# Complement Receptor Immunoglobulin (CRIg): expression and function in human phagocytes


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(B. Sc. with Hons)


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## Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint award of this degree.

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## Publications

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## Presentations

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**Awards During PhD**

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| | |
|---|---|
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| **2018** | Member of ASI Day of Immunology and Adelaide Immunology Retreat organising committee |

## Abbreviations

| | |
|---|---|
| 1,25D | 1,25-dihydroxyvitamin $D_3$; calcitriol |
| 25D | 25-hydroxy-vitamin $D_3$; calcifediol |
| 3H-TdR | methyl-3H Thymidine |
| $^{-/-}$ | Knock out |
| A20 | Tumor necrosis factor, alpha-induced protein 3 |
| Ab | Antibody |
| Akt | Protein kinase B |
| ANOVA | Analysis of variance |
| AP | Alternative pathway of complement |
| APS | Ammonium Persulfate |
| APC | Antigen presenting cells |
| Arp2/3 | Actin-related protein 2/3 complex |
| ARPC1B | Actin-related protein 2/3 complex subunit B |
| BAPTA-AM | 1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid (calcium chelator) |
| BCR | B cell receptor |
| bDMARDs | Biologic disease-modifying anti-rheumatic drugs |
| bp | Base pairs |
| BSA | Bovine serum albumin |
| $C_2$-type | Constant immunoglobulin domain |
| C3 | Complement component 3 |
| C3b | Complement component 3b |
| $Ca^{2+}$ | Calcium ions |
| CAIA | Collagen antibody induced arthritis |
| cAMP/cGMP | Cyclic adenosine monophosphate/cyclic guanosine monophosphate |
| CD | Cluster of differentiation |
| cDNA | Complementary DNA |
| CGD | Chronic granulomatous disease |
| CIA | Collagen induced arthritis |
| $cPLA_2$ | Cytosolic phospholipase A2 |
| $CO_2$ | Carbon dioxide |
| CR | Complement receptor |
| CR1 | Complement receptor 1; CD35 |
| CR2 | Complement receptor 2; CD21 |
| CR3 | Complement receptor 3; heterodimer consisting of CD18 and CD11b |
| CR4 | Complement receptor 4; heterodimer consisting of 2CD18 and CD11c |
| CRIg | Complement receptor immunoglobulin |
| CYP27B1 | Cytochrome P450 25-hydroxyvitamin D3-1alpha-hydroxylase |
| *d* | Density |
| DC | Dendritic cell |

| | |
|---|---|
| DMEM | Dulbecco's Modified Eagle Media |
| DMSO | Dimethylsulfoxide |
| DNA | Deoxyribonucleic acid |
| DPM | Disintegrations per minute |
| DSS | Dextran sulfate sodium |
| DTT | DL-Dithiothreitol |
| EAE | Experimental autoimmune encephalomyelitis |
| EDTA | Ethylenediaminetetraacetic acid |
| ELISA | Enzyme-linked immunosorbent assay |
| ERK1/ERK2 | Extracellular signal-regulated kinase 1/2 |
| FcγR | Fcγ receptor |
| FCS | Foetal calf serum |
| fDC | Follicular dendritic cell |
| FH | Complement factor H |
| FITC | Fluorescein isothiocyanate |
| fMLF | N-formyl-L-methionyl-L-leucyl-L-phenylalanine |
| fMLP | N-formyl-L-methionyl-L-leucyl-L-phenylalanine |
| fMLP-R | fMLP receptor |
| FMO | Fluorescence minus one |
| FSC | Forward scatter |
| GAPDH | Guanosin-adenosin-phosphate dehydrogenase |
| GM-CSF | Granulocyte/macrophage colony stimulating factor |
| HBSS | Hank's balanced salt solution |
| HBV | Hepatitis B virus |
| HCC | Hepatocellular carcinoma |
| HRP | Horseradish peroxidase |
| HuCRIg(L) | 'Long' variant of human complement receptor immunoglobulin |
| HuCRIg(S) | 'Short' variant of human complement receptor immunoglobulin |
| HOCl | Hypochlorous acid |
| iC3b | Inactive derivative of the complement component C3b |
| IFN-γ | Interferon gamma |
| Ig | Immunoglobulin |
| Ig-$C_2$ | Immunoglobulin constant domain |
| Ig-V | Immunoglobulin variable domain |
| IHC | Immunohistochemistry |
| JAK2 | Janus kinase 2 |
| JNK | c-jun amino terminal kinase |
| kDa | Kilodalton |
| KO | Knock out |
| LAD | Leukocyte adhesion deficiency |
| $LN_2$ | Liquid nitrogen |
| LPS | Lipopolysaccharide |
| LT | Lymphotoxin |
| LTA | Lipoteichoic acid |
| LTB4 | Leukotriene B4 |
| M1 | Classically activated macrophage |
| M2 | Alternatively activated macrophage |
| mAb | Monoclonal antibody |

| | |
|---|---|
| MAC | Membrane attack complex; also known as C5b-9 and the terminal complement complex |
| MAPK | Mitogen-activated protein kinase |
| M-CSF | Macrophage colony-stimulating factor |
| MDDC | Monocyte-derived dendritic cell |
| MDM | Monocyte-derived macrophage |
| MFI | Median fluorescence intensity |
| MHC | Major histocompatibility complex |
| MPO | Myeloperoxidase |
| Mφ | Macrophage |
| mRNA | Messenger ribonucleic acid |
| MS4A6D | Membrane-spanning 4-domains subfamily A member 6D |
| NF-κB | Nuclear factor-kappa B |
| NK cell | Natural killer cell |
| NLRP3 | NLR family pyrin domain containing 3 |
| OA | Osteoarthritis |
| p38 | p38 mitogen-activated protein kinase |
| PAGE | Polyacrylamide gel electrophoresis |
| PBMC | Peripheral blood mononuclear cells |
| PBS | Phosphate buffered saline |
| PCR | Polymerase chain reaction |
| pDC | Plasmacytoid dendritic cell |
| PE | Phycoerythrin |
| PEC | Peritoneal exudate cells |
| PHA | Phytohemagglutinin |
| PI3K | Phosphatidylinositol-3-OH kinase |
| PK | Proteinase K |
| PMA | Phorbol 12-myristate 13-acetate |
| PMN | Polymorphonuclear granulocyte |
| PKC | Protein kinase C |
| PRR | Pattern recognition receptor |
| PsA | Psoriatic arthritis |
| qRT-PCR | Quantitive RT-PCR |
| RA | Rheumatoid arthritis |
| RLU | Relative luminescence units |
| RNA | Ribonucleic acid |
| RNA-seq | RNA sequencing |
| ROS | Reactive oxygen species |
| RPMI | Roswell Park Memorial Institute medium |
| RT-PCR | Reverse-transcription PCR |
| SD | Standard deviation |
| SDS | Sodium dodecyl sulphate |
| SLE | Systemic lupus erythematosus |
| SSC | Side scatter |
| ST | Synovial tissue |
| STAT3 | Signal transducer and activator of transcription 3 |

| | |
|---|---|
| STIA | Serum-transfer induced arthritis |
| T1D | Type 1 diabetes |
| TAM | Tumour-associated macrophage |
| TBS | Tris-buffered saline |
| TBST | Tris-buffered saline supplemented with Tween-20 |
| TCR | T cell receptor |
| TGF-β | Transforming growth factor beta |
| $T_H1$ | T helper 1 cells |
| TLR | Toll-like receptor |
| TLR1/2 | Toll-like receptor 1 and 2 heterodimer |
| TNF-α | Tumour necrosis factor alpha |
| $T_{reg}$ | T regulatory cells |
| UV | Ultraviolet |
| VDR | Vitamin D receptor |
| VDRE | Vitamin D response element |
| V-type | Variable immunoglobulin domain |
| *VSIG4* | V-set and Immunoglobulin-domain-containing protein 4; the gene encoding complement receptor immunoglobulin |
| WT | Wild type |
| Z39Ig | Alternative name given to complement receptor immunoglobulin; often used interchangeably with CRIg and VSIG4 |

**Abstract**

The fifth human complement receptor, complement receptor immunoglobulin (CRIg; VSIG4; Z39Ig), was first documented in the year 2000. Since this initial discovery, CRIg has been described as a highly efficient phagocytosis promoting complement receptor, a player in the regulation of homeostasis, a regulator of the alternative pathway of complement, and also as a regulator of T cell activation. However, despite this broad range of attributed functions, CRIg remains to be the lesser known receptor of the complement receptor family; this is likely owing to the current lack of clarification as to its cellular expression in humans, mystery surrounding the mechanisms by which expression is controlled, a lack of commercially available antibodies, along with an inability to directly relate findings in murine models with human disease. Thus, before we can begin to investigate the role of CRIg in immune disease and how CRIg may potentially be used therapeutically, we must first come to understand the role of CRIg in the human state of health.

In this thesis, a comprehensive study into the immune cellular expression of CRIg in healthy human phagocytes will be presented as we attempt to unravel the uncertainties which cloud the field of CRIg biology. Currently, there are limited antibodies available which detect human and murine CRIg, and thus, the investigation presented herein will begin with the development and screening of multiple new, cross-reactive rat anti-murine CRIg monoclonal antibodies suitable for use in a range of methodologies, including Western blot, flow cytometry, and immunohistochemistry. These antibodies stand to be the first of their kind.

Next, we performed an in-depth investigation into the influence of cytokines/inflammatory mediators on CRIg expression by human monocyte-derived macrophages and dendritic cells. We observed a differential effect of cytokines/mediators on macrophage CRIg expression which was dependent on the developmental state of the cells, and demonstrated that those agents which induced an upregulation of CRIg protein expression also enhanced the ability of the cells to phagocytose the fungal pathogen, *Candida albicans*, identifying a control point through which these mediators act. In dendritic cells, we observed a similar influence of cytokines/mediators over CRIg expression, and found that cells stimulated to express high protein levels of CRIg negatively regulated the T cell proliferative response to phytohaemagglutinin and allogeneic stimulation, and reduced the cytokine production of T cells in response to allogeneic stimulation. This negative regulation could be inhibited by

the addition of an anti-CRIg antibody to the cultures, and thus identifies CRIg as a key effector molecule in dendritic cell-control over the T cell response.

The steroid hormone vitamin D has vitally important roles in many processes, including in the modulation of the immune response. With this in consideration, along with the growing global concern that is vitamin D deficiency, we next assessed the relationship between vitamin D and CRIg expression by human monocyte-derived macrophages. We observed that cells matured in the presence of the active form of vitamin D showed a marked increase in CRIg protein and mRNA expression, and that this increase correlated with the ability of the cells to phagocytose the pathogens *Candida albicans* and *Staphylococcus aureus.* Interestingly, treatment had no effect on the expression of the 'classical' complement receptors, CR3 or CR4, indicating that vitamin D promotes innate immune defence through promoting CRIg expression by macrophages.

Finally, in our endeavour to recharacterise CRIg in the state of human health, we demonstrate that CRIg expression in humans is not restricted to tissue-resident macrophages as previously described. For the first time, we present evidence of the expression and regulation of CRIg on the cell surface of human circulating phagocytes; monocytes and neutrophils. We additionally assessed this expression in comparison with cells from a case of immunodeficiency caused by a novel mutation in *ARPC1B*. We found that healthy human neutrophils express functional CRIg protein within intracellular stores, and upon stimulation with endogenous and exogenous inflammatory mediators, export this protein to the cell surface. This was in comparison with neutrophils which have an inability to polymerise microfilaments due to a mutation in *ARPC1B*, which failed to upregulate CRIg expression. Similarly, inhibition of the upstream regulator of Arp2/3, Rac-2 inhibited the ability of healthy neutrophils to upregulate surface CRIg protein, indicating that CRIg upregulation on the cell surface of neutrophils is a result of exocytosis.

Together, the findings to be presented herein represent a substantial increase in our knowledge of the biology of CRIg in healthy human systems, and with the information gained, it will form a backbone for future comprehensive studies into the role of CRIg in immunity and in inflammatory diseased states, such as in rheumatoid arthritis and type 1 diabetes.

# Chapter 1. Introduction

## 1.1. Introduction: The Cells of The Immune System

The human immune system can be divided into two main branches: the innate and the adaptive. The innate immune system consists of non-specific defence mechanisms, forming a first line of defence against invading pathogens, tissue damage, and cancer. It is rapidly initiated and includes physical barriers such as the skin, chemicals present in the blood such as enzymes, and immune cells which attack foreign cells/particles in the body, including neutrophils, monocytes and macrophages. These 'innate' cells express a broad range of pattern recognition receptors (PRRs) which non-specifically recognise foreign pathogens and are fully encoded in the germline genome (Kubelkova & Macela, 2019).

The adaptive immune response, although slower to establish, is more flexible than the innate, and is required to fight infections which evade the innate immune system (Paul, 2011). Comprised of antigen-specific defences which work to clear infections, the adaptive immune system is able to generate long term defences, many of which can be life-long for the host. This branch of immunity is composed of antibodies in circulation, cytokines in the blood and tissue, and the specialised cells of the adaptive immune system, T and B lymphocytes. These cells express highly specific, custom made receptors (T and B cell receptors; TCR and BCR respectively) which result from germline gene segment recombination, allowing the generation of millions of unique antigen receptors (Chaplin, 2010). As a result, the response of the adaptive immune system is highly specific to the inducing pathogen and can generate long lasting protection through the induction of immunological memory. This enables the immune system of the host to react faster, and more specifically to re-infection.

Both the adaptive and the innate branches of immunity are essential for full-functioning immunity, and interaction between the two systems is crucial (Clark & Kupper, 2005; Jain & Pasare, 2017). Without crosstalk between the innate and the adaptive immune systems, the adaptive is unable to be initiated. This is exemplified as defects within crucial genes of either the innate or adaptive immune systems are able to lead to primary immunodeficiencies (Rosenzweig & Holland, 2011).

## 1.2. Phagocytes

### 1.2.1. Neutrophils

Neutrophils are highly versatile cells considered to be of the innate arm of immunity and are the most abundant leukocyte in the circulation. These phagocytes are the first to arrive at sites of infection and injury, responsible for non-specifically clearing bacterial and fungal pathogens (Takashima & Yao, 2015). They are efficient phagocytes, able to internalise opsonised latex beads in less than 20 seconds (Segal et al., 1980). Neutrophils are vital for a fully functional immune system, exemplified by the predisposition of patients with neutrophil disorders such as leukocyte adhesion deficiency (LAD) or chronic granulomatous disease (CGD) to serious, life-threatening infections (Almarza Novoa et al., 2018; Amulic et al., 2012).

Neutrophils exert their anti-infective function through the key functional characteristics of: chemotaxis, the ability to rapidly migrate into sites of inflammation toward gradients of inflammatory molecules; phagocytosis, the ability to engulf and destroy invading pathogens; and degranulation, the ability to release stores of antimicrobial molecules to induce the killing of extracellular pathogens (Ferrante, 2005). They are also able to capture foreign particles through the formation of neutrophil extracellular traps (NETs) (Papayannopoulos, 2018). Neutrophils store a wide variety of anti-microbial products, enzymes and surface receptors within granules, ready to be exported out of the cell to the cellular membrane upon encountering certain stimuli (Ferrante, 2010; Lacy, 2006) (figure 1.1). These granules have been characterised by their constituents. Azurophilic granules (also known as primary granules) are characterised by the enzyme myeloperoxidase (MPO). Specific granules (or secondary granules) predominantly contain membrane bound proteins, while gelatinase granules (or tertiary granules) are identified by the enzyme gelatinase (Cowland & Borregaard, 2016). Mechanisms of the process of degranulation still remain somewhat mysterious, however, the importance of a range of molecules/pathways have been well established, such as calcium ions ($Ca^{2+}$) (Lacy, 2006), the mitogen activated protein kinase (MAPK) pathway (Pillinger et al., 1996), and the Rho family of small GTPases, including Rac1 and Rac2, which are regulators of actin polymerisation (McCormick et al., 2019).

Circulating quiescent neutrophils show limited antimicrobial activity, requiring prior stimulation to reach their maximum phagocytic capability (Yao et al., 2015). This process is known as ‘priming’, and induces the transport and fusion of the granule

membrane to the plasma membrane, resulting in the upregulation of cell surface receptors and signalling molecules contained within these granules (Borregaard et al., 1983; Uriarte et al., 2011). This primed state can be induced by inflammatory cytokines and microbial substances such as TNF and LPS (Aida & Pabst, 1990; Ferrante, 1992). Following granule and plasma membrane fusion, primed neutrophils express higher amounts of surface receptors than their inactivated counterparts (Paoliello-Paschoalato et al., 2015), and thus, are able to respond to pathogens and foreign particles more efficiently than inactivated cells. Among the receptors that are transported to the cell surface through priming, complement receptor (CR)3 is highly upregulated by exposure to TNF (Ferrante et al., 1993; Montecucco et al., 2008) and it is widely accepted that this increased expression of CR3 is responsible for the increased phagocytic function of primed neutrophils.

Activation of neutrophil Fc receptors and integrins on the cell surface induces the production of reactive oxygen species (ROS) at sites of infection (Belambri et al., 2018). Such activation initiates intracellular signalling pathways which lead to the assembly of the nicotinamide adenine dinucleotide phosphate (NAPDH) oxidase complex (Ferrante, 2005). This complex is a multicomponent enzyme system which is formed at the cell membrane and is responsible for the production of ROS (Nguyen et al., 2017). The NADPH oxidase-derived superoxide anion ($O_2^-$) acts as a precursor to hydrogen peroxide and other ROS, including the highly microbicidal hypochlorous acid (HOCl) (Winterbourn et al., 2016) . These molecules represent a major component of innate defence against bacterial and fungal pathogens, and are essential for microbial killing (Belambri et al., 2018; Nguyen et al., 2017).

While the view of neutrophils as cells of innate immunity is not incorrect, work in recent decades has revealed the importance of neutrophils in adaptive immunity (Rosales et al., 2017). Neutrophils can directly influence the function of DCs, delivering activation signals and antigens, thus enabling downstream DC-T cell crosstalk (Megiovanni et al., 2006). They can also directly influence lymphocyte function; chemokines produced by neutrophils (e.g. CCL2, CXCL9, CCL20) attract T helper 1 ($T_H1$) and 17 ($T_H17$) cells to sites of inflammation, and neutrophil-derived cytokines can activate T and B lymphocytes to promote proliferation (Li et al., 2019; Mantovani et al., 2011; Pelletier et al., 2010). Additionally, it has been published that neutrophils can act as antigen presenting cells following some form of activation (Lin

& Loré, 2017; Vono et al., 2017). While resting neutrophils express minimal levels of MHC class II molecules and are unable to induce T cell proliferation *in vitro*, neutrophils can acquire antigen presenting functions following activation. For instance, it was recently shown that following phagocytosis of IgG-opsonised red blood cells, neutrophil surface expression of the APC molecules MHC II, CD40, and CD80 is increased, and these cells can elicit antigen-specific T cell responses (Meinderts et al., 2019).

Whether distinct neutrophil subsets exist is a topic surrounded by debate. While different functional phenotypes of neutrophils have been consistently reported in cancer (Fridlender et al., 2009; Houghton, 2010), there remains a question of whether the proposed ‘anti-tumour’ N1 neutrophils are simply more active cells (Cowland & Borregaard, 2016; Piccard et al., 2012). Additionally, in the blood, high density- and low density- neutrophils have been reported, with functional studies suggesting that the former represents mature cells, while the latter represents immature neutrophil subsets (Scapini & Cassatella, 2014). Thus, while there is currently a push into research into lymphocytes—the traditional cell types we think of when we consider adaptive immunity—there remains a wealth of information to be uncovered revolving around these ‘unconventional’ cells of adaptive immunity.

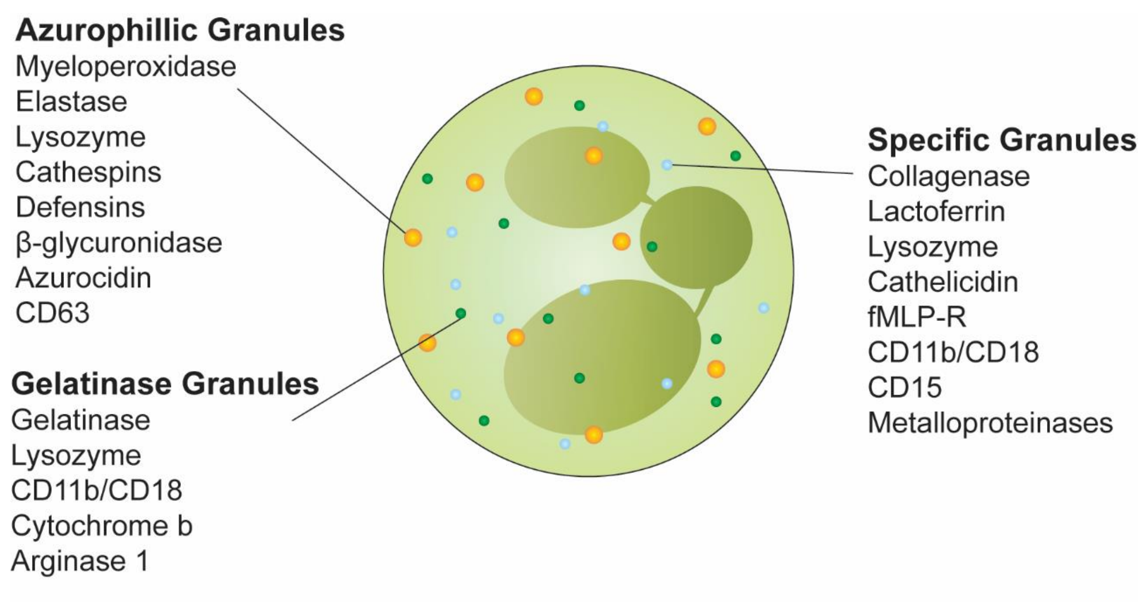


*Figure 1.1. Neutrophils store a wide range of enzymes, anti-microbial effectors, and membrane-bound proteins within three main types of granules; azurophilic, specific, and gelatinase (Cassatella et al., 2019).*

### 1.2.2. Monocytes

Comprising between 3-8% of all blood leukocytes, monocytes are important cells of myeloid lineage which have long been reported to have the main function of replenishing populations of macrophages and myeloid dendritic cells in the tissues (Yang et al., 2014). However, monocytes are also able contribute to other immune processes of both the innate and adaptive branches. They contribute to innate immunity where they can function as phagocytic cells (Kantari et al., 2008). They also interact with the adaptive immune system and function as antigen presenting cells (APCs), facilitating crosstalk between the two systems. Thus, monocytes are important for a healthy immune system, and patients in states of monocyte deficiency such as those suffering MonoMAC syndrome have high mortality rates (Calvo et al., 2012; Leon & Ardavin, 2008).

Studies in recent years have identified different subsets of monocytes with unique cytokine profiles, with different subsets responsible for different functions (Boyette et al., 2017). Traditionally, monocytes are divided into two groups determined by CD16 expression: classical monocytes ($CD14^{++}$ $CD16^{-}$), and non-classical ($CD14^{+}$ $CD16^{++}$) (Passlick et al., 1989). However, a third subset, 'intermediate' monocytes have more recently been included ($CD14^{++}$ $CD16^{+}$) (Ziegler-Heitbrock et al., 2010). These three subsets express distinct mRNA expression profiles, produce different cytokines, and show different levels of antigen processing and presentation capabilities (Wong et al., 2012).

### 1.2.3. Macrophages

Macrophages are highly heterogeneous cells primarily of haematopoietic origin. Arising from haematopoietic stem cells in the bone marrow, macrophage precursors are released into the bloodstream as monocytes (Murray & Wynn, 2011). All monocyte subsets have the potential to differentiate into macrophages, with differentiation beginning as monocytes migrate from the bloodstream into the tissue (Coillard & Segura, 2019). Within the tissue, macrophages play a major role with three major functions; phagocytosis, cytokine production, and antigen presentation. These properties enable macrophages, like DC, to act as a bridge and facilitate crosstalk between the innate and adaptive immune systems.

#### 1.2.3.1. Macrophage Subsets

As a highly heterogenous population of cells which serves different functions in different niches, macrophages can be divided into several subpopulations. The nomenclature of these populations has been based on their location within the body, along with their functional phenotype. Additionally, through the use of genetic fate-mapping techniques and single-cell RNA sequencing, different macrophage populations have been identified in adulthood based on their origins (Culemann et al., 2019), further complicating the way we characterise macrophages. Many populations of fixed tissue resident macrophages such as Kupffer cells in the liver, Hofbauer cells in the placenta, and synovial lining macrophages of the joints have been identified as of embryonic origin, and exist independently from monocyte-derived macrophages (Culemann et al., 2019; Epelman et al., 2014). These populations are self-renewing and play important roles in tissue homeostasis (Gentek et al., 2014).

The liver is a crucial organ which plays many roles in vertebrates including the production of metabolites, the regulation of immune responses, and the clearance of toxins from the circulation (Protzer et al., 2012). Connected to the bloodstream by the portal vein transporting draining blood from the intestines and the hepatic artery, the liver also has the pivotal role of clearing microbes and/or pathogens that cross the intestinal barrier or enter the bloodstream from peripheral sites (van Lookeren Campagne & Verschoor, 2018). The resident Kupffer cells in the liver sinusoids are the most abundant of resident macrophage populations in mammals and are the cell subset responsible for this role of the liver. While these are highly phagocytic cells with antigen presenting function (Crispe, 2011), Kupffer cells are promotors of tolerance in the healthy state (Thomson & Knolle, 2010), expressing low levels of MHC class II and co-stimulatory molecules, allowing them to contribute to liver-mediated immune tolerance (Horst et al., 2016; Ju et al., 2003; You et al., 2008).

#### 1.2.3.2. Macrophage Polarisation

The concept of macrophage activation and the existence of differing activation states was first proposed in 1962, where ‘classical activation’ was observed in cells in response to challenge by *Listeria monocytogenes* (Mackaness, 1962). These cells showed enhanced immunity upon secondary exposure, with the phenomenon later shown to be linked to T helper 1 (Th1) responses and interferon gamma (IFN-γ) production (Nathan, 1983). It has since been demonstrated that these macrophages

play a critical role in initiating and maintaining inflammation in response to lipopolysaccharide (LPS) and IFN-γ (Sica & Mantovani, 2012). ‘Alternative activation’ of macrophages was reported three decades later, with the T helper 2 (Th2) cytokines interleukin(IL)-4 and IL-13 shown to inhibit inflammatory cytokine production by macrophages (Doyle et al., 1994; Stein et al., 1992). Thus, the classical and alternative activation states of macrophages were termed ‘M1’, and ‘M2’, following the Th1 and Th2 nomenclature.

Although the effects of the cytokines IFN-γ, IL-4, and IL-13 on macrophage polarisation follow the idea of M1 and M2 states following Th1 and Th2 responses, since then, the effects of other cytokines or agents such as IL-10 and glucocorticoids have been shown to be contradictory to this idea. As a result, alternatively activated macrophages have been further characterised into three additional subsets; M2a, M2b, and M2c (Mantovani et al., 2004; Wang et al., 2019) (figure 1.2). Each of these three subsets have been documented to have distinct functions, and characteristic cytokine secretion profiles.

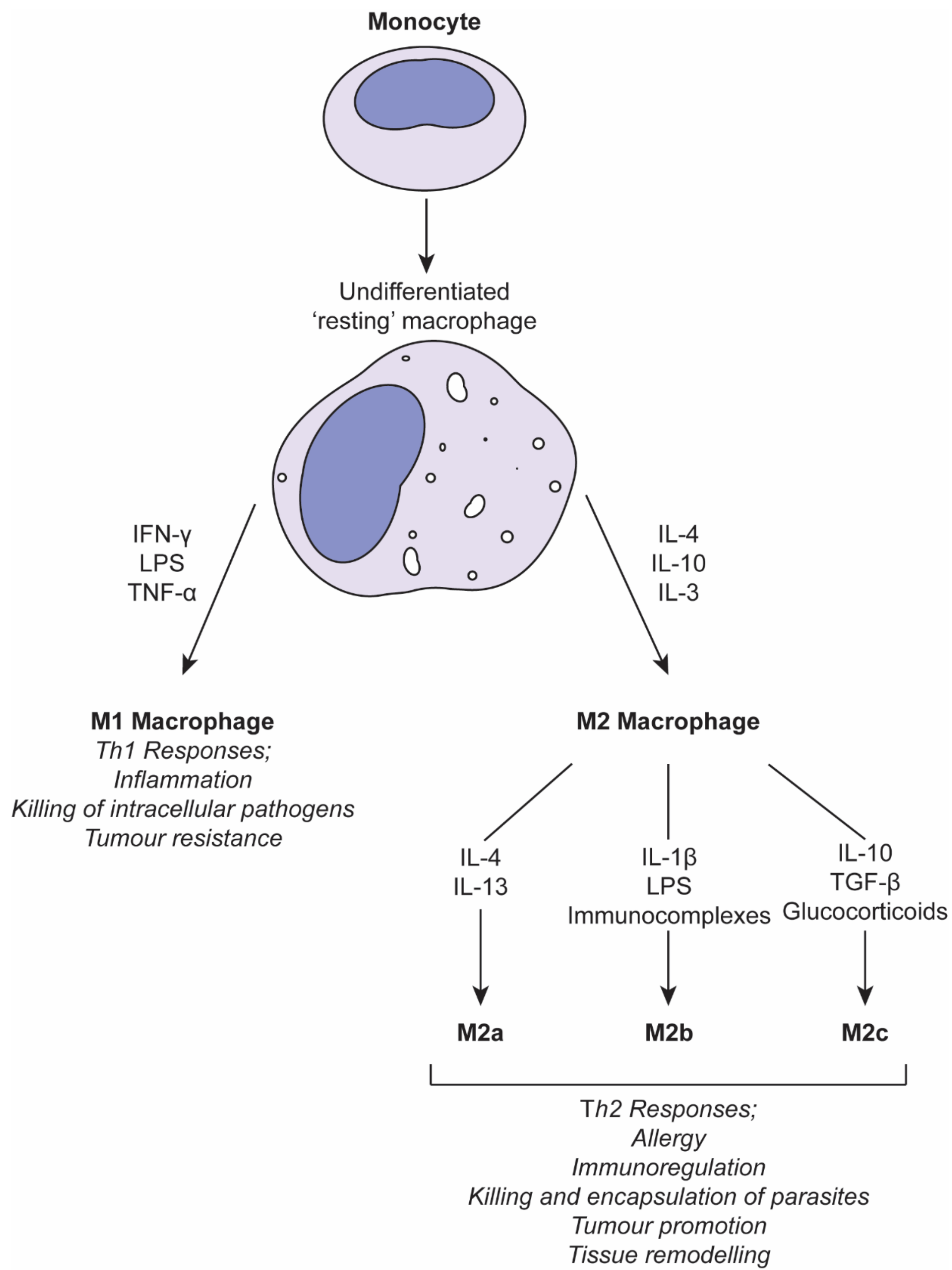


*Figure 1.2. Differential macrophage polarisation.* Following monocyte recruitment from the blood into tissues, environmental stimuli induce differentiation into macrophage subsets with distinct functional characteristics. Diagram adapted from Mantovani et al. (2004).

### 1.2.4. Dendritic Cells

Dendritic cells (DC) are large phagocytes and originate from several lineages. These include Langerhans cells, dermal DC, and DC in lymph node follicles and Peyer's patches. Follicular dendritic cells (FDC) are of mesenchymal origin (Aguzzi et al., 2014), while plasmacytoid dendritic cells (pDC) have long been thought to be of lymphoid origin, although this concept has been challenged (Ishikawa et al., 2007; Yang et al., 2005). DC can also be derived from classical and intermediate monocytes (termed monocyte derived DC; MDDC) under specific conditions. However, non-classical are not able into differentiate to MDDC (Boyette et al., 2017). Deemed as 'professional' APCs, DC were first discovered in 1973 (Steinman & Cohn, 1973, 1974), where they were initially received as an artefact. DCs were soon shown to express high levels of major histocompatibility complex (MHC) class I and II molecules, and function through their unrivalled ability to stimulate T cells (Nussenzweig et al., 1980). Although macrophages and B cells are also able to act as APCs, DC are unique in that they are the only cell able to activate naïve T cells (Howard et al., 2007). They can do so in different ways depending on phenotype; DCs can induce a tolerogenic response to certain antigens such as self-antigens and an immunogenic response to others (Takenaka & Quintana, 2017). As such, DC are the main players in facilitating immune crosstalk between the innate and adaptive systems, and are vital for the immune system's ability to distinguish between 'self' and 'non-self' (Geijtenbeek et al., 2004).

## 1.3. The Initiation of Inflammation

The inflammatory response is an event which can be triggered by tissue damage or infection and leads to the migration of leukocytes and inflammatory mediators to the location of damage. Initial infection or damage is recognised by local tissue-resident macrophages or mast cells through innate PRRs, which in turn produce further inflammatory mediators, including complement components, cytokines, and chemokines (Medzhitov, 2008). The first response to these mediators causes increased vascular permeability, allowing complement components and cells such as neutrophils, which continuously and randomly probe the vessel wall, to enter the area from the blood stream (Headland & Norling, 2015). On reaching the site of infection, infiltrating neutrophils become activated. This can occur through direct contact between invading pathogens and neutrophils, or through the response to inflammatory cytokines or mediators released by the resident cells of the tissue (Amulic et al., 2012). Neutrophils then act as previously

described, by releasing the toxic components of their granules (including ROS, enzymes such as proteinase 3), or through upregulating levels of functional surface receptors. This upregulation enhances their ability to phagocytose and kill invading pathogens (Ferrante, 2010).

## 1.4. The Complement System

The complement system is an intricate branch of innate immunity, forming a line of defence against pathogens in the blood and tissue fluids of the host (Merle, Church, et al., 2015). Comprising of more than 40 soluble and membrane-bound proteins, complement also functions in maintaining tissue homeostasis and influencing the adaptive immune response (Hovland et al., 2015). Complement activation can occur through three pathways: the alternative, the classical, and the lectin pathways (figure 1.3). These pathways differ by their activating stimuli and in the mechanisms of how their convertase enzymes are formed (Noris & Remuzzi, 2013). In normal physiological conditions, the alternative pathway (AP) is the dominant activation pathway (Merle, Church, et al., 2015). Constantly active at low levels, the AP is triggered by the spontaneous hydrolysis of C3 into its biologically active subunits C3a and C3b. Deposited C3b is then able to form the C3 and C5 convertases by forming complexes with complement factor Bb (the C3 convertase C3bBb, and the C5 convertase $(C3b)_2Bb$). The formed C3 convertase can then cleave additional C3 molecules, resulting in an amplification loop of complement activation. Along with this convertase action, C3b and its inactive derivative iC3b are also able to function as a potent opsonins owing to the conformational changes of the molecule which render binding sites accessible to phagocytosis-promoting receptors (Jongerius et al., 2010). Opsonisation is a critical mechanism of complement-mediated defence, by which foreign particles or apoptotic self-cells are ‘tagged’ for rapid recognition and subsequent phagocytosis by the phagocytes of the host. The lectin and the classical pathways differ from the AP, as they use an alternative C3 convertase, C4bC2b, and an alternative C5 convertase, C4bC2aC3b (figure 1.3). Although the activation mechanisms of the lectin and classical pathways differ to that of the AP, they are both able to trigger the downstream activation of the AP by the production of C3b molecules (Harboe et al., 2004).

Other effectors of the complement system include the anaphylatoxins, C3a and C5a. These function as proinflammatory molecules and are able to both attract and activate leukocytes through their corresponding receptors (C3aR, and C5aR) (Noris & Remuzzi, 2013). The final product of complement activation is the formation of the membrane

attack complex (MAC, C5b-9) in the membrane of the targeted cell, which can directly lyse targeted pathogens or self-cells by forming pores in the lipid bilayer. The MAC is a common product of all three complement activation pathways, and is formed though the association of the complement proteins C5b, C6, C7, C8, and C9 (Serna et al., 2016), a process which is irreversible.

As the complement system serves such a broad function in innate immunity and tissue homeostasis and is composed of such a large amount of inactive precursors requiring cleavage before activation can occur (Mathern & Heeger, 2015), fine-tuned regulation mechanisms are critical. Under-activation or uncontrolled activation of complement can contribute to or cause a wide range of complement associated disorders (Ricklin et al., 2016), and has been associated with autoimmune diseases such as rheumatoid arthritis (Holers & Banda, 2018) and systemic lupus erythematosus (Leffler et al., 2014).

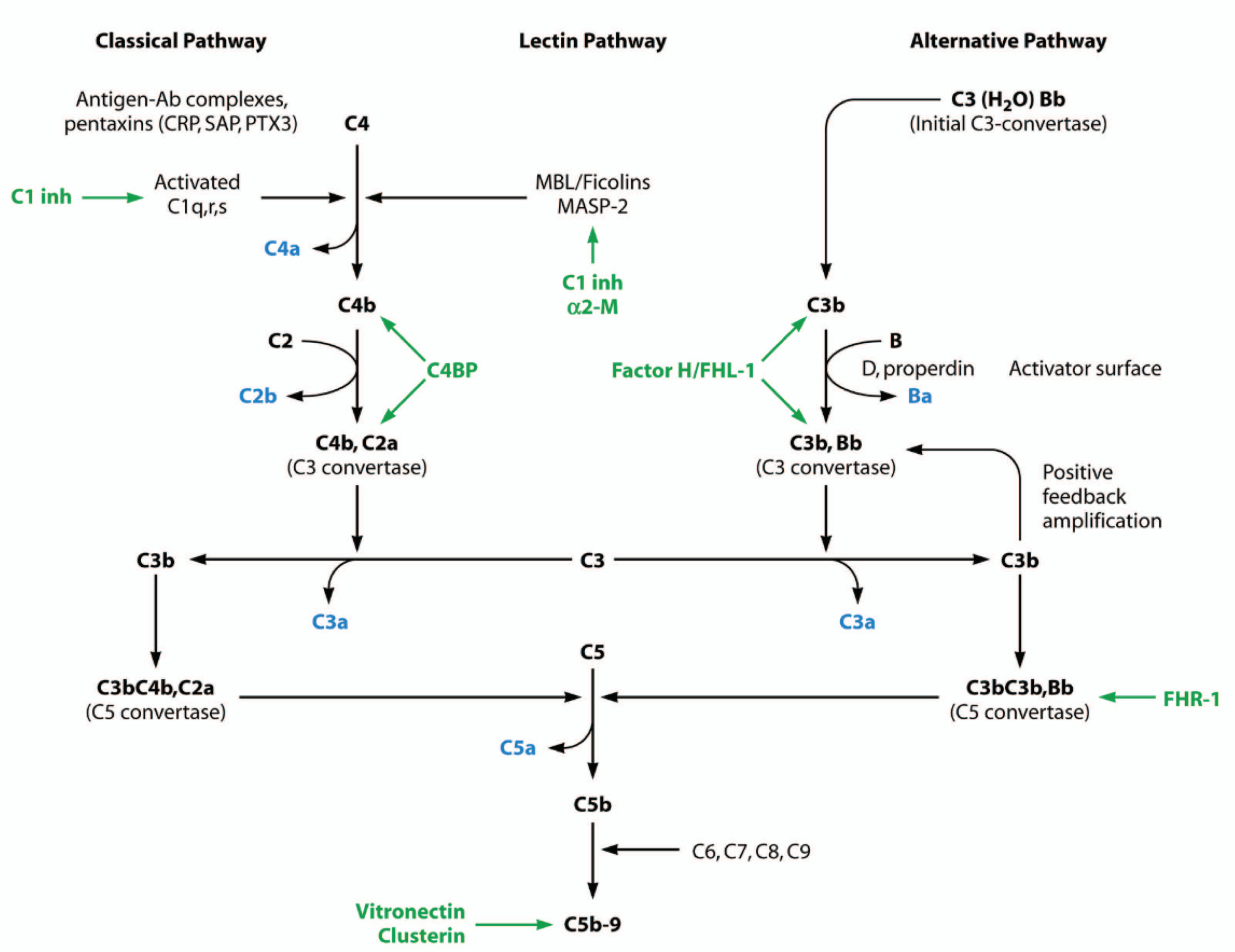


*Figure 1.3. Schematic representation of the activation of the complement cascade.* The fragments released into solution are indicated in blue. The key fluid-phase regulators are indicated in green. Ab, antibody; CRP, C-reactive protein; SAP, serum amyloid P component; PTX3, pentraxin 3; C1 inh, C1 inhibitor; α2-M, α2-macroglobulin; C4BP, C4b-binding protein; FHL-1, factor H-like protein-1; FHR-1, factor H-related molecule-1. Image adapted from Ram et al. (2010), reused with permission from the American Society for Microbiology (2020).

## 1.5. Phagocytosis

Phagocytosis is a critical process by which the cells of a host engulf other cells, fragments, invading microbial pathogens, and other foreign particles, and is closely related to endocytosis. Phagocytosis occurs in three main steps. Firstly, the opsonised foreign particle becomes attached through surface specific receptors on the phagocyte. Secondly, pseudopod extensions are formed by actin remodelling-dependent mechanisms around the attached particle where it is still exposed to the environment. Lastly, the complete evagination of the particle takes place to generate a phagosome, an outside-in compartment within the cell (van Kessel et al., 2014). Once inside of the cell, the phagosome interacts with recycling endosomes prior to fusing selectively with primary lysosomes to form a phagolysosome (Russell, 2011). Following fusion, the phagolysosome undergoes fusion with secondary vescicles. This pathway leads to changes in pH which progressively acidifies the inside of the phagolysosome, resulting in the destruction of its contents, and is accompanied by membrane recycling (Gordon, 2016). In phagocytic APCs, this process leads to the presentation of the resulting protein fragments on the cell surface major histocompatibility complex (MHC) class II molecules, or cross presentation on MHC class I molecules (Mantegazza et al., 2013).

## 1.6. Complement Receptors

The receptors of the complement system are widely distributed within mammals. They contribute in all three-complement activation pathways, and most are able to act as phagocytosis-promoting receptors (Holers 2014). Five main receptors for the C3 activation products are found in human cells originating from three different gene families: CR1 and CR2 from the short consensus repeat (SCR) modules, CR3 and CR4 from the β2-integrin family, and CRIg from the immunoglobulin superfamily (Merle, Church, et al., 2015) .

CR1, also known as CD35, is a glycoprotein expressed by/on the surface of erythrocytes, neutrophils, monocytes, B cells, and subsets of T cells. CR1 on erythrocytes plays a role in phagocytosis along with immune adhesion and clearing immune complexes by binding C3b (Nielsen et al., 1997). The receptor has also been shown to negatively regulate the complement cascade by inhibiting the C3 convertases. However, while it is able to take part in phagocytosis of immune-bound complexes in neutrophils and macrophages, CR1 binding to C3b-coated particles alone is not sufficient to induce phagocytosis. Instead, this binding assists the phagocyte in phagocytosing particles via

either CR3 or Fcγ receptor (FcγR)-mediated mechanisms (Fallman et al., 1993; Merle, Church, et al., 2015; Merle, Noe, et al., 2015).

CR2 (or CD21) is expressed by B cells, follicular dendritic cells and some epithelial cells (Zabel & Weis, 2001), where it functions through binding the C3 cleavage products iC3b, C3d and C3dg. On B cells, CR2 forms a component of the B cell co-receptor complex with molecules CD19 and Tapa-1, where it lowers the threshold of cell activation when the receptor recognises an antigen, while on follicular dendritic cells, CR2 assists in the uptake of opsonised immune complexes (Das et al., 2017). Additionally, like CR1, CR2 has the ability to act as the Epstein-Barr virus (EBV) receptor on B cells and nasopharyngeal epithelial cells (Du Clos & Mold, 2008; Ogembo et al., 2013).

CR3 and CR4 are heterodimers comprised of an alpha-subunit (CD11b or CD11c, respectively), which non-covalently associate with a common beta-subunit, CD18 (van Lookeren Campagne et al., 2007). Both CR3 and CR4 recognise iC3b, and play important roles in cell adhesion, leukocyte trafficking and migration, and phagocytosis. They are widely expressed, present on macrophages, neutrophils, monocytes, and follicular dendritic cells (Holers, 2014). While both receptors play important roles in complement-mediated phagocytosis, CR3 is also able to initiate phagocytosis in a complement-independent manner. This has been shown to contribute to the phagocytosis of *Candida albicans*, and is facilitated by a region on CR3 which acts as a PRR against β-glucan on the fungal cell wall (A. Small et al., 2018; Zheng et al., 2015).

## 1.7. Complement Receptor Immunoglobulin

Complement receptor immunoglobulin (CRIg), previously referred to as Z39Ig, is the most recently discovered of the complement receptor family, and currently remains the most mysterious. Initially documented in 2000, CRIg was first identified in a study assessing candidate genes involved in mental retardation (Langnaese et al., 2000). While this was found not to be the case, it was soon identified as a member of the immunoglobulin superfamily, and further shown to function through the recognition and binding of the complement component C3b, and its inactive derivative, iC3b (Helmy et al., 2006; Langnaese et al., 2000; Wiesmann et al., 2006). While CR3 and CR4 can also bind iC3b, CRIg is the only phagocytosis-promoting receptor expressed on macrophages that can bind active C3b. This ability enables CRIg to induce rapid phagocytosis as the first receptor able to recognise opsonised particles, and also contributes to the immunosuppressive characteristics of the protein. Although the first thorough study into

the biology of CRIg was published in 2006 (Helmy et al., 2006), many uncertainties revolving the expression and function of CRIg remain present in the current literature. These will be discussed further herein and constitute the rationale for the study detailed in this thesis.

## 1.8. CRIg in Health

### 1.8.1. Structural Properties of CRIg

The gene encoding CRIg is known as v-set and immunoglobulin containing protein 4 (*VSIG4*) and can produce a pre-mRNA transcript which can give rise to multiple variants when alternatively spliced (Langnaese et al. 2000). This gives the receptor the unique property in humans in that it is expressed as multiple variants: huCRIg(L), containing both a variable (V-type) and a constant ($C_2$-type) immunoglobulin domain, and a short variant, huCRIg(S), containing only the V-type domain (Helmy et al. 2006). The V-type domain is responsible for recognition of C3b and iC3b, while the role of the $C_2$-type domain currently remains unknown (Wiesmann, et al. 2006). Along with two documented proteins in humans, further alternative splicing of the gene can potentially produce four additional variants (figure 1.4, table 1.1) (data accessible at the National Center for Biotechnology Information (NCBI) Nucleotide database; reference sequences NM_001184831.1, NM_001184830.1, NM_001257403.1, XM_017029251.2). It is important to note that the sixth variant was added to this list in 2017, after the work to be presented in this thesis began. Currently, these remain as unreported translated proteins, and whether there are any functional differences between these different forms remains to be studied. Curiously, while these forms are able to be transcribed in humans, there has only been one transcript variant and corresponding protein reported to be present in mice (Helmy, et al. 2006) and this is a homolog to the human short variant. This suggests that only the V-type domain is required for protein function, as murine CRIg does not contain a $C_2$-type domain. On this note, it is also important to consider that the bulk of the experimental work currently published to date on the biological properties of CRIg has been gathered through the use of murine models. This suggests that most of the attributed characteristics of the receptor likely relate more closely to the human short variant.

Both huCRIg(L) and huCRIg(S) contain several extracellular *O*-glycosylation sites, and two intracellular phosphorylation sites; a cAMP/cGMP-dependent protein kinase phosphorylation site, and a protein kinase C (PKC) phosphorylation site (S-311 and T-333 respectively or S-217 and T-239) (Langnaese et al. 2000). CRIg variants 3, 4, 5, and

6 differ from the long and short forms as they incorporate either an extra intron, or an alternative splice site in exon 8 encoding the cytoplasmic domain (figure 1.4). These differences would give the translated proteins a truncated cytoplasmic region, leaving them with only one phosphorylation site, S-311, or S-217. Whether this variation results in translated proteins with functional differences remains to be studied.

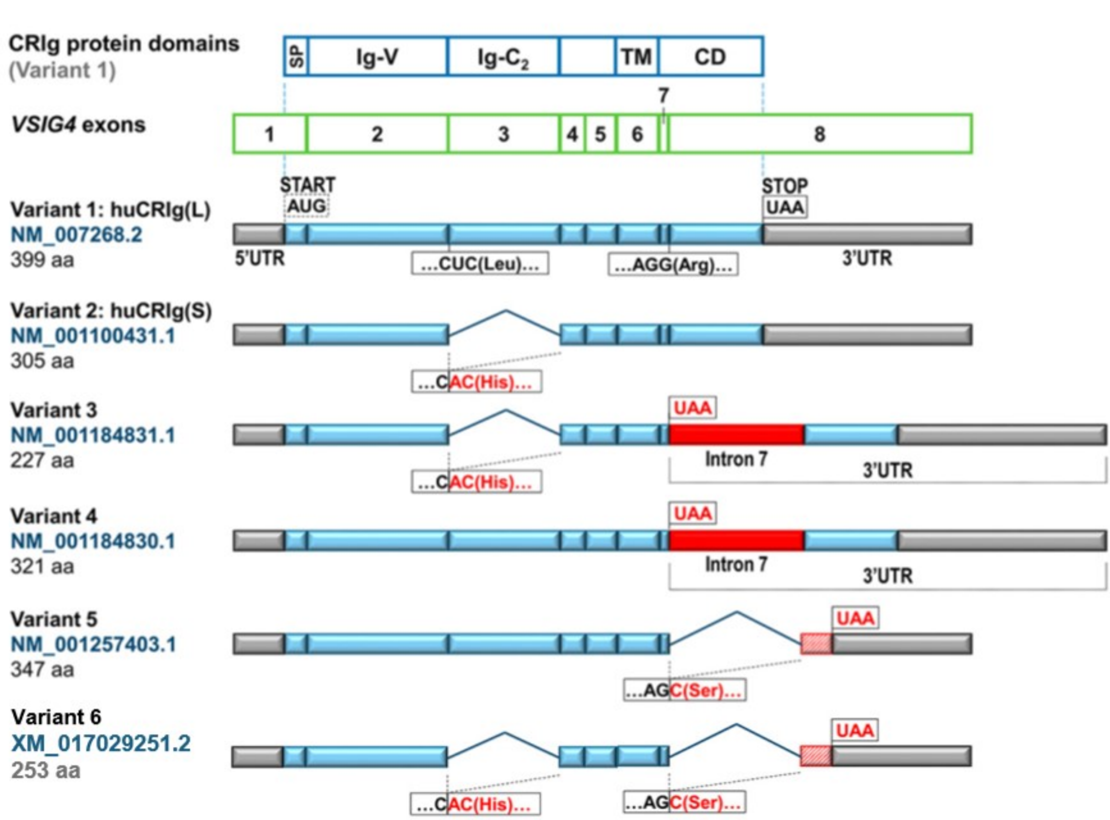


*Figure 1.4. Schematic diagram showing the CRIg protein domains aligned with the exons producing cDNA specific for the long form.* Below, the transcript variants are shown with exon arrangements aligned. The transcript structures are derived from the NCBI Reference Sequence Database (RefSeq) using the following mRNA accession numbers: NM_007268.2, NM_001100431.1, NM_001184831.1, NM_001184830.1, and NM_001257403.1. Adapted from Small et al. (2016).

*Table 1.1. Summary of the six predicted human alternatively spliced transcript products, their length in amino acids, and their predicted molecular weight in kilodaltons (kDa).*

| Transcript variant | No. of amino acids | Predicted molecular weight (kDa) |
|---|---|---|
| *Variant 1 – huCRIg(L)* | 399 | 44 |
| *Variant 2 – huCRIg(S)* | 305 | 33.9 |
| *Variant 3* | 227 | 25.4 |
| *Variant 4* | 321 | 35.6 |
| *Variant 5* | 347 | 38.2 |
| *Variant 6* | 253 | 28.08 |

### 1.8.2. Phagocytic Properties of CRIg

#### 1.8.2.1. CRIg vs. CR3/CR4

CRIg shares the function to bind iC3b to induce phagocytosis with CR3, the latter being the dominant phagocytic receptor in inflammatory macrophages. In resident quiescent cells, however, CR3 is retained in an inactive state, requiring activating stimuli to initiate phagocytosis. To activate, it is proposed that an 'inside-out' signal via an extracellular ligand and further activation of phosphatidylinositol-3-OH kinase (PI3K) is required (Means & Luster, 2010). Conversely, CRIg has been found to be the dominant phagocytic receptor expressed by resting tissue macrophages (Gorgani et al., 2008), and unlike CR3, it does not require this signal to activate, nor does it require dimerisation. In addition, CRIg has the added capability of recognising molecules opsonised with C3b (Helmy et al., 2006), the first C3 cleavage product of complement formed on the pathogen cell surface (Croize et al., 1993). Thus, when these cells first encounter an opsonised particle, CRIg is the first receptor able to initiate phagocytosis through its interaction with C3b/iC3b. Furthermore, when phagocytosis is initiated through CRIg, it has been suggested that the receptor recycles from internalising endosomes back to the cell surface, avoiding degradation, leaving the receptor able to initiate subsequent rounds of phagocytosis (Gorgani et al., 2008; Helmy et al., 2006). These together suggest that CRIg is more efficient at promoting phagocytosis than its β-integrin counterpart.

While the concept of a phagocytosis-promoting receptor with anti-inflammatory function is contradictory in essence, there are significant benefits to the existence of such a molecule, particularly for cells such as Kupffer cells. It takes the blood pool approximately twenty seconds to circulate throughout the entire vascular system (van Lookeren Campagne & Verschoor, 2018), and with the vitally important task of clearing circulating pathogens from the blood flow of the host, Kupffer cells need to act rapidly. Armed with both complement receptors and a wide array of PRRs, Kupffer cells have evolved to be one of the most effective phagocytic cellular subsets in the body (Tacke, 2017). Thus, high expression of CRIg, a phagocytic receptor which does not require activation or dimerisation to act, would be highly beneficial. Indeed, CRIg is expressed by murine Kupffer cells (Ikarashi et al., 2013) and has been shown to be crucial for the clearance of bacterial, parasitic and viral pathogens from the circulation, preventing dissemination throughout the body of the host (He et al., 2013; Helmy et al., 2006; Liu et al., 2019). Along with phagocytic clearance,

Kupffer cells have also been reported to ‘trap’ microbial pathogens extracellularly, thus removing them from the circulation while neutrophils assist in phagocytic removal (Gregory et al., 1996). While the lack of expression of the different isoforms of CRIg in mice makes *in vivo* experiments not possible, it is tempting to suggest that this may be a role for the truncated isoforms in humans which cannot signal. Furthermore, CRIg binds to the β-chain of C3b/iC3b (Wiesmann et al., 2006), while CR3 binds to the α-chain near the C345C domain of iC3b (Xu et al., 2017). This makes it entirely possible from a molecular perspective that both CRIg and CR3 can bind to the same molecule of iC3b at the same time, suggesting that the two may potentially work collaboratively in phagocytosis.

#### 1.8.2.2. CRIg as a Pattern Recognition Receptor?

Late in 2016, a novel function of CRIg as a pattern recognition receptor on liver Kupffer cells was reported, further likening CRIg to CR3 (Zeng et al., 2016). Utilising spinning-disk confocal intravital microscopy of liver sinusoids in murine C3 and CRIg KO models, the group described the ability of CRIg to selectively bind to wild-type (WT) *Staphylococcus aureus* in the absent of complement, and that this binding could be partially inhibited by the addition of either lipoteichoic acid (LTA) derived from *S. aureus* or by anti-LTA antibody *in vitro.* Further, the group report that mice infected with LTA-deficient *S. aureus* show significantly higher levels of bacterial dissemination from the liver, with increased bacterial burdens in the kidneys and blood. However, although an exciting report, these findings present a complete contradiction to the ‘classical’ findings of Helmy, et al (2006), which concluded that CRIg requires complement to induce phagocytosis. Furthermore, these experiments lack some consideration for alterations in pathogenesis that LTA-deficient *S. aureus* may have compared with WT.

These findings of Zeng and colleagues (2016) were complemented by a second study published in the same journal issue which provides further insight into a dual-track pathogen clearance mechanism (Broadley et al., 2016). This mechanism details CRIg expressed on liver Kupffer cells as the mediator of ‘slow clearance’ of opsonised bacteria, while alternative PRRs on the same cells mediate the ‘rapid’ clearance of unopsonised bacteria. This study potentially explains the discrepancies between the two above articles; through the use of double-knock out animal models, Broadley, et al (2016) show that in dual $C3^{-/-}$ $CRIg^{-/-}$ mice, opsonised bacteria are unable to be cleared from the circulation, while unopsonised bacteria are still able to be cleared at

normal levels. This is opposed to the data from Zeng et al. (2016), who use single knockout models and conclude that CRIg is still able to mediate phagocytosis in $C3^{-/-}$ mice. Thus, taking the findings of Broadley et al (2016) into consideration, the findings of Zeng et al. (2016) can be explained, as in C3 depleted conditions, all bacteria will be unopsonised, and thus, all bacteria will be able to be phagocytosed by alternative PRRs expressed on liver Kupffer cells aside from CRIg, as was shown to be the case by Broadley et al. (2016).

#### 1.8.2.3. Phagocytic Properties of CRIg in Human Cells

Prior to 2015, the bulk of studies investigating the role of CRIg in phagocytosis focus on murine models (Gorgani et al., 2008; He et al., 2013; Helmy et al., 2006), and whether the phagocytic function of CRIg is conserved in primary human macrophages remained essentially unstudied until recently. Irvine et al. (2016) investigated the phagocytic capability of CRIg expressing peritoneal macrophages in patient cases of liver cirrhosis and ascites. They reported a significant correlation between the numbers of $CRIg^{+}$ macrophages and disease severity, with patients with higher levels of $CRIg^{+}$ cells showing less severe symptoms. Interestingly, the group show that $CRIg^{+}$ macrophages can phagocytose unopsonised and heat inactivated serum-opsonised latex beads. This finding may potentially support the hypothesis proposed by Zeng et al (2016) that CRIg can act as a PRR to bacterial LTA, although this was not fully investigated in this particular study. Additionally, the findings of Irvine et al. (2016) may not necessarily be due to a CRIg-dependent, complement-independent mechanism, similarly to the conclusions drawn by Broadley et al. (2016). Alternatively, the observed results may be again explained by the action of scavenger receptors present on the cells. This would explain the contradictory findings when compared to Helmy et al. (2006) and suggests that this mechanism is conserved in both human and mice. However, an important limitation to note when attempting to interpret the results of Irvine and colleagues, is the absence of healthy human controls used in the study.

THP-1 cells are a human monocytic cell line originally derived from a childhood M5 subtype of acute monocytic leukaemia (Tsuchiya et al., 1980). These cells can be differentiated into macrophages, and as such, they have been widely used as an *in vitro* model for studying the biology of human macrophages. Using THP-1 derived macrophages, Kim et al. (2013) investigated the signalling cascade as a result of CRIg engagement and the ability of CRIg to phagocytose and kill the intracellular

bacteria *Listeria monocytogenes.* This study reported that upon CRIg engagement with either anti-CRIg antibody or with the complement component C3b, the bactericidal activity of the cells was enhanced, and this killing occurs within *L. monocytogenes*-containing phagosomes. Utilising yeast two-hybrid methodologies, the group also demonstrated that the cytoplasmic domain of CRIg interacts with chloride intracellular channel 3 (CLIC3), and further that this protein is essential for the CRIg-mediated killing to occur. Thus, CRIg is able to induce the phagocytosis and the killing of phagocytosed intracellular bacteria in human cells.

### 1.8.3. The Regulation of CRIg in Macrophages

The anti-inflammatory steroid dexamethasone has been shown to induce a significant increase in macrophage CRIg expression in human cells *in vitro* at both the mRNA and protein levels, including on the cell surface, while the inflammatory agent arachidonate causes the opposite effect (Gorgani et al., 2011). This depression of CRIg expression by arachidonate is dependent on activation of protein kinase C (PKC), while PI3K, the mitogen-activated protein (MAP) kinase p38, and the extracellular signal-regulated kinases 1 and 2 (ERK1/ERK2) are not involved (Gorgani et al., 2011; Ma et al., 2015). Furthermore, macrophages deficient in the PKC isozyme PKCα have been shown to express enhanced CRIg mRNA and total protein, exhibit enhanced phagocytosis of *Candida albicans*, and a decreased ability to produce the inflammatory cytokines IL-6 and TNF (Ma et al., 2015). Similarly to arachidonate, TNF has been shown to negatively regulate CRIg expression, and the addition of an anti-TNF monoclonal antibody (mAb) to macrophage cultures induced an increase in CRIg expression (Ma et al., 2015). This same study also demonstrated that macrophages induced to express high levels of CRIg protein produce significantly less TNFα and IL-6 in response to exposure to opsonised *C. albicans.*

Upon macrophage activation with either of the M1-inducing agents lipopolysaccharide (LPS) or interferon-γ (IFN-γ), CRIg is negatively regulated (Gorgani et al., 2008; Guo et al., 2010). Additionally, CRIg has been shown to be similarly downregulated by tissue resident macrophages in inflamed tissues at the time of flare up in cases of autoimmune myocarditis (Vogt et al., 2006) and experimental autoimmune uveoretinitis (Chen et al., 2010). In the case of uveoretinitis, CRIg expression within the inflamed tissue became undetectable at the peak of inflammation and was only once again detectable at levels comparable to healthy tissue during the recovery phase from the disease flare up. These findings

strengthen the association of CRIg with non-inflammatory macrophages and supports the hypothesis that CRIg may be involved in the resolution of inflammation rather than contributing to inflammation. As M1-polarised macrophages and activated macrophages in sites of inflammation either express low levels of CRIg or none at all, while conversely, macrophages expressing higher levels of CRIg produce lower levels of TNF upon encounter with opsonised pathogens, this suggests that in macrophages, there may be an autocrine feedback loop controlling CRIg expression that is dependent upon TNF (Ma et al., 2015).

While agents which regulate CRIg expression has been a key research focus, the role played by CRIg itself in influencing the function of the cells per se is a more obscure area of research. Recently, it has been demonstrated that CRIg can influence macrophage activation by inhibiting mitochondrial pyruvate metabolism (Li et al., 2017), and retains the cell in a non-activated state upon challenge with LPS by antagonising activation signals in the cells involving the stimulation of PI3K/Akt-STAT3 cascades, and preventing the generation of ROS by its mitochondria. Furthermore, it was shown that forced overexpression of CRIg resulted in suppression of M1 genes, further associating CRIg expression with the M2 macrophage phenotype. Supporting this, activation of macrophages in *Vsig4*$^{-/-}$ mice is enhanced and these cells express higher amounts of the pro-inflammatory factors pro-IL-1β, IFN-γ and TNF (Li et al., 2017). Further, mice deficient in CRIg expression are more susceptible to high fat diet-induced obesity. These animals exhibit increased liver damage and mortality as a result of murine hepatitis virus (MHV-3) infection, and the authors deduce that this is likely a result of enhanced macrophage activation (Li et al., 2017).

The NLRP3 inflammasome is a multimeric signalling complex of the innate immune system which has an important role in mediating caspase-1 activation and producing the active inflammatory cytokines IL-1β and IL-18 (Kelley et al., 2019). As the NLRP3 inflammasome can be activated by mitochondrial ROS, the same group of researchers as above assessed the relationship between CRIg and the NLRP3 inflammasome (Huang et al., 2019). Using *VSIG4*$^{-/-}$ KO mice, Huang and colleagues (2019) concluded that CRIg regulates expression of the NLRP3 inflammasome in isolated murine peritoneal exudate macrophages. While a significant portion of this study is largely based on correlation, the finding that the murine form of CRIg interacts with membrane-spanning 4-domains subfamily A member 6D (MS4A6D)

to form a signalling complex at the cell membrane is entirely novel. A combination of immunofluorescence and Western blot revealed colocalization between CRIg and MS4A6D on the cell membrane and that MS4A6D can then activate a JAK2-STAT3-A20 signalling cascade. This pathway negatively regulates NF-κB activation, thus preventing initiation of the immune response and the downstream transcription of inflammatory molecules such as *Nlrp3* and *Il-1β* (figure 1.5). Additionally, the group assessed the role of CRIg in two commonly used murine disease models, experimental autoimmune encephalomyelitis (EAE) and dextran sulfate sodium (DSS)-induced colitis. The group show that *VSIG4*$^{-/-}$ mice experience higher EAE disease severity compared to their WT counterparts, and show that this is as a result of increased production of the inflammatory cytokine IL-1β, which has previously been shown to be a contributor to the pathogenesis of EAE (Lin & Edelson, 2017). Conversely, they show higher disease severity in WT mice in the case of DSS-induced colitis. It is concluded that the increased activity of the NLRP3 inflammasome in VSIG4$^{-/-}$ mice contributes to exacerbation of disease.

NLRP3 inflammasome activation requires the activation of caspase-1, which cleaves proIL-1β and proIL-18, generating active IL-1β and IL-18. Interestingly, there is also a single caspase-1 cleavage site in the extracellular regions of both huCRIg(L) and huCRIg(S) at residue 266 and 172 respectively (Gasteiger et al., 2003; Gasteiger et al., 2005). Thus, it is tempting to speculate that upon activation of the inflammatory response, secreted caspase-1 in its active form may be able to cleave surface CRIg molecules, resulting in decreased surface expression on activated cells and a released soluble form of CRIg, which prevents CRIg from maintaining the inactive state of the cell. Indeed, the presence of soluble CRIg has been detected in human serum by ELISA and by proteomic techniques (Byun et al., 2017; Yuan et al., 2020). A soluble form of CRIg may be beneficial in the control of the alternative pathway of complement, however further research into the presence and the production of a bona fide soluble CRIg protein, by either caspase-1 cleavage or otherwise, remains to be undertaken.

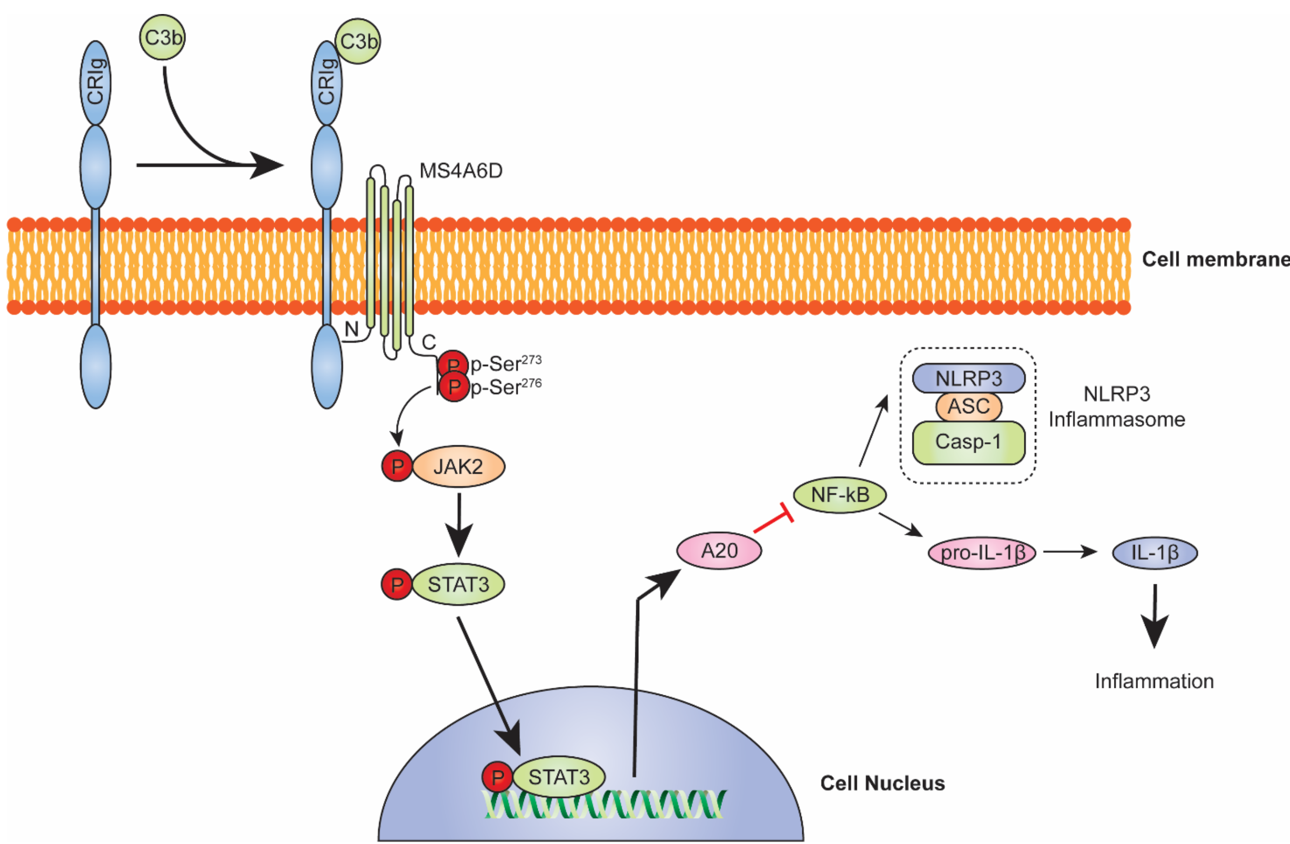


*Figure 1.5. Schematic diagram of the proposed model of the CRIg/MS4a6D signalling complex and its interaction with the NLRP3 inflammasome.* Liganding VSIG4 with complement C3b induces recruitment and formation of the VSIG4/MS4A6D surface inhibitory signalling complex. Both Ser232 and Ser235 phosphorylation at C-terminal of MS4A6D further triggers JAK2-STAT3-A20 cascades to inactivate NF-kB. Lastly, VSIG4 prevents *NLRP3* and *Il-1β* gene transcription, controlling NLRP3-mediated pathogenesis. Diagram adapted from Huang et al. (2019).

### 1.8.4. CRIg Expression by Dendritic Cells

While murine DC have been shown to express CRIg mRNA, both naïve and active DC from the spleen and blood were found to stain negatively for protein when assessed by flow cytometry (Vogt et al., 2006). Since this first initial study, numerous other studies have also detected CRIg mRNA in both human and murine DC (Li et al., 2011; Toivonen et al., 2016), however the detection of CRIg protein in these cells has not been reported. Thus, the functional role of CRIg in the adaptive immune response remains unclear.

### 1.8.5. CRIg and the Resolution of Inflammation

CRIg, having long been thought to contribute to the anti-inflammatory properties of tissue-resident macrophages, has homeostatic properties. Owing its ability to clear complement opsonised particles, and capacity to promote signal transduction with a minimal inflammatory response, CRIg has been hypothesised to clear low level infectious agents rapidly, preventing onset of a 'cytokine storm' (Gorgani et al., 2011). This most likely works in collaboration with the more inflammatory CR3, which is equipped to combat larger scale infections and initiate a full-blown inflammatory response.

*VSIG4* expression is upregulated in tissues during the resolution phase of inflammation. Through the use of a murine model of zymosan-induced peritonitis, Tani et al. (2014) show that *VSIG4* is upregulated in macrophages during the resolution phase. A further study by Wang and Tatakis (2017) show that *VSIG4* is significantly upregulated in human gingiva during the process of wound healing. Additionally, the numerous studies discussed above which utilised animal models of autoimmune inflammation support these findings and show that CRIg protein is downregulated during inflammation and replenished only in the recovery phase (Chen et al., 2010; Vogt et al., 2006). These, together with the finding that the resolution-promoting cytokine IL-10 (Siqueira Mietto et al., 2015; Sugimoto et al., 2016) has been shown to induce high expression of CRIg mRNA and protein in human monocyte-derived macrophages (Gorgani et al., 2011), while TGF-β1 induces a decrease, suggests that there is perhaps a unique cytokine network operating within the tissue during the recovery phase which controls CRIg expression .

### 1.8.6. CRIg and the Alternative Pathway of Complement

The crystal structure of CRIg in complex with C3b was published in 2006 (Wiesmann et al., 2006). This work revealed that while CRIg is bound to the β-chain of C3b, it is unable to form the C3 and C5 convertase complexes with complement factor Bb. This, in turn, prevents further cleavage of C3 and C5 to their active subunits, and as a result, leads to the inhibition of progression of the alternative pathway of complement (He et al., 2008). Thus, CRIg has been found to inhibit inflammation by inhibiting the activation of the alternative complement pathway – an amplifier of the inflammatory response. It's important to note, however, that while CRIg has been shown to inhibit the alternative pathway both *in vitro* and *in vivo*, CRIg does not inhibit either the classical or lectin pathways, as these use an alternative C3 convertase, C4bC2a (Katschke et al., 2007). CRIg is, however, able to inhibit the alternative pathway-driven amplification loop that is triggered by these pathways (Qiao et al., 2014).

Following the discovery of the complement-inhibitory function of CRIg, numerous studies utilising soluble CRIg fusion proteins as complement inhibitors have been performed in a variety of complement-associated disorders. Notably, Qiao et al. (2014) generated a soluble protein consisting of the functional domains of CRIg and the alternative pathway regulator factor H (FH), and demonstrated that this molecule (termed CRIg/FH) was highly effective at inhibiting complement activation and C3/C3b deposition. They went on to show that CRIg/FH was able to protect erythrocytes in patients with paroxysmal nocturnal haemoglobinuria from complement-mediated damage. Additionally, CRIg/FH was shown to be an efficacious therapeutic *in vivo*, with the group demonstrating that CRIg/FH is able to specifically target sites of ongoing complement activation to provide long-lasting inhibition of complement, thus providing a potent therapeutic effect in their rat model of mesangial proliferative glomerulonephritis. The therapeutic application of soluble CRIg fusion proteins have also been investigated in autoimmune conditions such as systemic lupus erythematosus (SLE) (Lieberman et al., 2015), experimental arthritis (Katschke et al., 2007), diabetes type 1 (Fu et al., 2012), and intestinal ischemia/reperfusion-induced injury (Chen et al., 2011) and these studies will be discussed in further detail below.

### 1.8.7. CRIg as a Regulator of T Cell Activation

In an attempt to identify novel members of the B7 protein family of immunoregulatory ligands, Vogt et al (2006) reported a novel function of CRIg. They documented that the murine ortholog of CRIg shares ~20% identity with known B7 protein family members, and thus, their study aimed to investigate whether CRIg can regulate T cell activation. They reported CRIg to be a potent inhibitor of T cell activation, inhibiting both IL-2 production and cell proliferation by arresting the cell cycle at the G0/G1 phase, and that this inhibition was as potent as that induced by protein cell death protein 1 (PD-1) ligand 1 (PD-L1). In addition, it was shown that the ability of CRIg to inhibit T cell proliferation was conserved across both mice and humans, suggesting that the short variant of CRIg plays the role in T cell regulation. The ligand/mechanism by which this regulation functions remains unknown, although as no receptor able to bind CRIg has been reported, it has been proposed that the inhibitory action of CRIg over T cell activation and proliferation may be a further manifestation of its ability to bind C3b and iC3b molecules (Zang & Allison, 2006). CR1 and CR3 are known to be expressed by T cells, and the addition of antibodies against these molecules to T cells has been reported to inhibit their proliferation (Wagner et al., 2006). Thus, it is possible that CR1/CR3 may bind to the same C3b/iC3b molecules at the same time as CRIg, hence triggering T cell regulation (figure 1.6).

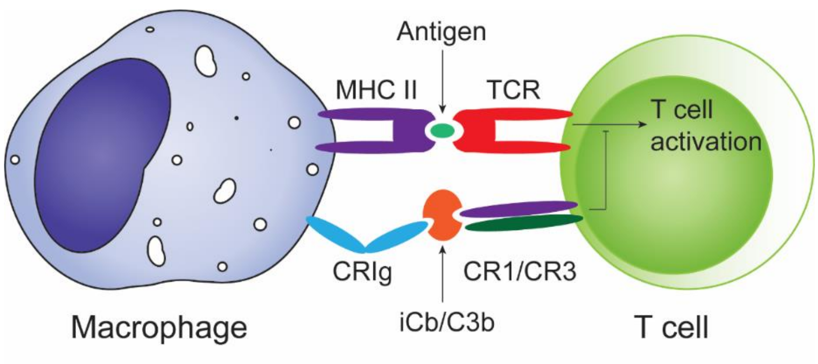


*Figure 1.6. CRIg acts as a negative regulator of T cell activation.* Proposed mechanism by which CRIg expressed by macrophages may be triggering the inhibition of T cell activation through binding to the same C3b or iC3b molecule as CR1/CR3 on T cells, which may in turn trigger an inhibiting signal to the T cell. Image is adapted from Zang and Allison (2006) .

In 2010, Xu et al. (2010) transfected mature, inflammatory human DC—which had been matured in the presence of TNF to induce an immunogenic phenotype—with human CRIg-recombinant adenoviral vectors, forcing expression of CRIg on these cells. These transfected DC were then used in mixed lymphocyte reactions with allogenic T cells and were shown to have the ability to suppress allogenic T cell proliferation, cytokine production, and activation marker expression compared with non-transfected and mock-transfected control cells. Thus, it can be concluded that the gain of CRIg protein was responsible for this observed shift in phenotype from immunogenic to 'tolerogenic'.

This work was expanded on by Jung et al. (2015), where they studied the contribution of CRIg in helper T cell activation and differentiation, and in turn, how CRIg contributes to isotype switching in B cells. Using CRIg KO mice, they show enhanced levels of isotype switching to IgG subclasses compared to WT. They further show that activated T cells co-cultured with macrophages from CRIg KO mice show significantly higher levels of cytokine production. This data supports the view that CRIg serves as a co-inhibitory molecule expressed by macrophages.

Yuan et al. (2017) sought to investigate the mechanisms by which CRIg regulates T cell responses. Stimulating isolated conventional T cells with anti CD3/CD28 antibodies to induce proliferation *in vitro*, the group showed that CRIg interferes with early T cell activation, while addition of CRIg-Ig late in the culture period (48 hours), was unable to suppress T cell proliferation or influence expression of the surface activation markers CD25 and CD69. Further, the ability of CRIg to regulate T cell activation was shown to be complement-independent, suggesting that this 'bridging' effect of CRIg binding C3b/iC3b at the same time as another receptor expressed on T cells (such as CR1 or CR3) is unlikely to be the mechanisms responsible for interacting with CRIg. The group also investigated whether CRIg directly binds to candidates of a panel of known co-inhibitory molecules expressed by T cells, CTLA-4, PD-1, VISTA, CD226, and TIGIT, and found that blocking these molecules had no effect on blocking the regulatory action of CRIg. To date, the ligand/receptor to which CRIg binds on T cells remains unknown.

## 1.9. CRIg in Disease

### 1.9.1. Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a chronic autoimmune disorder which targets the synovial tissue and manifests as articular damage and swelling (Small & Wechalekar,

2019). Affecting approximately 0.5-1% of the population in Western countries (Hunter et al., 2017), the disease is a major cause of disability and chronic pain in the adult population. Both genetic and environmental factors influence RA, with association with autoantibodies to various known proteins such as rheumatoid factor, and citrullinated proteins (Smolen et al., 2018).

As a regulatory molecule with established immunosuppressive function, CRIg and its relationship with RA have been investigated in several published studies (Katschke et al., 2007; Lee et al., 2006; Tanaka et al., 2008; Wen et al., 2017; Zheng et al., 2016; F. Zheng et al., 2014). However, in similar fashion to the articles discussed above, there are substantial conflicting results reported amongst these studies. The initial report by Lee et al. (2006) suggests that not only is CRIg expressed by macrophages in the synovial lining layer, but that CRIg actively contributes toward the pathogenesis of disease by induction of the secretion of IL-8 and matrix metalloproteinase 9 (MMP-9) through the activation of NF-κB. In the subsequent year, following the discovery that CRIg can act as an inhibitor of the alternative pathway of complement (Wiesmann et al., 2006), a soluble CRIg fusion protein fused with complement FH (CRIg-FH) was investigated in two mouse models of experimental arthritis; collagen induced arthritis (CIA) and collagen antibody induced arthritis (CAIA) (Katschke et al., 2007). The group reported that in both models, CRIg-FH, as a selective inhibitor of the alternative pathway of complement, worked to prevent disease onset and also alleviated symptoms of inflammation in established arthritis, thus providing support for the use of CRIg-FH as a potential therapeutic in human chronic inflammatory diseases.

The finding that this soluble form of CRIg can act therapeutically is of great interest; although significant advances in therapy have been made for RA patients in the last two decades with the development of TNF inhibitors and biologic disease-modifying anti-rheumatic drugs (bDMARDs) (Hopkins et al., 2016), up to 50% of patients continue to suffer from ongoing disease activity and joint damage. This indicates that there remains a strong need to develop further approaches to manage disease, and the work of Katschke et al. (2007) provides evidence for CRIg-FH to be further studied as a therapeutic. However, no clinical trials of the use of CRIg-FH have been registered to date, and this is likely owing to both the conflicting report by Lee et al. (2006) and due to the potential of a broad range of adverse side-effects of an

immunosuppressive agent such as CRIg causing broad inhibition of the alternative pathway of complement.

More recently, fluorescently labelled nanobodies targeting CRIg have been proposed as a tool to monitor arthritis progression and response to therapy (Zheng et al., 2019; Zheng et al., 2016). These nanobodies (NbV4m119) have been shown to accumulate in the joints of mice with serum-transfer induced arthritis (STIA) (a model of murine arthritis in which the animals recover following inflammation) in a manner that correlates with disease severity, and decreases in the recovery phase of disease (Zheng et al., 2016). Additionally, the nanobody accumulation was decreased in the joints of mice treated with dexamethasone compared with untreated mice. Thus, as dexamethasone is well documented as a potent driver of CRIg expression in macrophages (Gorgani et al., 2011; Tanaka et al., 2008), this raises the question of whether CRIg expression, per se, is correlated with disease severity, or whether the observed accumulation in the joints of mice with arthritis is rather a reflection of increased macrophage numbers within the joints. Further, as the accumulation of these anti-CRIg nanobodies within the joints in mice with STIA peaked at day 8—the time point at which these mice reach the most severe inflammation and begin to recover—it's possible that CRIg expression serves as the mediator of the beginning of the recovery phase.

The above articles provide strong support for the potential applications of CRIg protein, or CRIg-targeting agents in arthritis, however, there remains a need to fully elucidate the role of CRIg in disease, and there remain significant gaps in our knowledge of CRIg in human states. Human macrophages expressing the long form of CRIg on the cell surface are known to be present in the intimal lining layer of RA, normal, osteoarthritis (OA), and psoriatic arthritis (PsA) synovial tissue (Tanaka et al., 2008). These cells are CD16 and CD163 (a marker used for distinguishing M2 polarised macrophages) positive, suggesting they may potentially exert a regulatory function. Tanaka et al. (2008) observed that while numbers of CRIg$^+$ cells were found to remain consistent between early RA, established RA, OA, and PsA populations, the numbers of dual-positive CRIg$^+$CD11c$^+$ cells were significantly increased in both early and established RA, as was the ratio of CRIg$^+$CD11c$^+$ cells to CRIg$^+$ cells. However, aside from the finding that these cells are expanded in RA, the functional role of these cells remains to be elucidated.

### 1.9.2. CRIg in Other Autoimmune Disorders

RA, along with other autoimmune disorders such as systemic lupus erythematosus (SLE) have been associated with over activation of the alternative pathway of complement (Thurman & Holers, 2006). As such, soluble fusion proteins consisting of the extracellular domains of CRIg have been studied in several other autoimmune disease models. Lieberman et al. (2015) expanded on the potential therapeutic use initially proposed by Katschke et al. (2007), showing that their CRIg fusion protein diminished skin lesions, proteinuria and pyuria, and kidney damage in a mouse model of SLE. CRIg treated mice showed equal amounts of autoantibodies as their untreated counterparts. The group concluded that CRIg acts therapeutically through limiting the recruitment of effectors of tissue damage, rather than through preventing autoimmunity.

Chen et al. (2010) administered CRIg-Fc to mice with experimental autoimmune uveoretinitis and showed repressed retinal inflammation. They documented the role of the AP in disease pathogenesis and showed that it was the inhibition of this pathway that supressed disease. Along with this finding, they report lower production of the inflammatory cytokines TNF-α, IFN-γ, IL-17 and IL-6 produced by T cells of the mice treated with CRIg-Fc. Another interesting finding of this study was the pattern of CRIg expression in the retina. In healthy mice, $CRIg^{+}F4/80^{+}$ macrophages were detected, while at the peak of disease, CRIg could not be detected in the macrophages of the tissue nor in the infiltrating macrophages of the inflamed retina. However, as the disease severity decreased at 35 days post immunisation., $CRIg^{+}$ macrophages could one again be detected within the tissue. This finding provides further evidence that CRIg expression on macrophages may play a role in the resolution of inflammation, likely through its ability to regulate complement and the activation of T cells.

Together, these studies show that the immunosuppressive properties of CRIg make it a promising therapeutic. However, although promising in their results, these works use soluble CRIg fusion proteins to inhibit disease symptoms. As previously discussed, there have currently been no registered clinical trials for the use of CRIg-Fc as a therapeutic.

### 1.9.3. CRIg and Preeclampsia

Genome-wide transcriptional profiling has revealed that upregulated levels of CRIg mRNA in the peripheral blood is associated with the incidence of severe

preeclampsia (Textoris et al., 2013). Follow up specific quantitative PCR (qPCR) supported this finding. In agreement with this report, a second study reported that the transcriptional profiles of women suffering severe preeclampsia exhibit higher levels of CRIg mRNA than women with non-severe preeclampsia (Chaiworapongsa et al., 2013). This indicates that this pathogenic environment leads to an upregulation of CRIg production, suggesting that a measure of CRIg mRNA in the blood of women at risk of preeclampsia may serve as a diagnostic test to distinguish between the severe and non-severe forms.

### 1.9.4. CRIg and Cancer

Tumour associated macrophages (TAMs) are myeloid, tumour-promoting immune cells and represent a largely heterogeneous population (Liu & Cao, 2015). TAMs can facilitate tumour metastasis, promote tumour angiogenesis and can suppress the anti-tumour mechanisms of the host (Yang et al., 2018). With this range of immunosuppressive properties, TAMs exhibit a phenotype similar to the regulatory M2 polarised state and typically express CD68, CD163 (Jeong et al., 2019), and other negative co-stimulatory molecules of the B7 protein family.

The previously discussed documentation of CRIg as a B7 family-related protein (Vogt et al., 2006) has led to recent investigations into its potential role in various forms of cancer. Using double immunofluorescence staining, Liao et al. (2014) documented the presence of $CRIg^{+}CD68^{+}$ macrophages in lung cancer tissue sections. These $CRIg^{+}$ cells also expressed B7-H1 (PD-L1/CD274) and B7-H3 (CD276), two other members of the co-stimulatory B7 protein family members with known roles in the negative regulation of T cells. Further, the group demonstrated that mice deficient in CRIg expression developed larger tumours than wild type (Liao et al., 2014). Numerous studies since this initial publication have been published demonstrating the association of CRIg with other forms of cancer (table 1.2), and there is a general consensus that higher CRIg expression is associated with a poorer prognosis, fitting the known homeostasis-promoting function of the receptor and further likening CRIg to the B7 family of proteins. However, contrary to the concept of CRIg as pro-tumourigenic, Zhu et al. (2018) report that low expression of CRIg is associated with poor prognosis in hepatocellular carcinoma (HCC) patients with hepatitis B infection (HBV). Through the use of quantitative reverse-transcription polymerase chain reaction (qRT-PCR), they demonstrate poorer survival rates in patients with low CRIg expression in their tumours, compared with those with high

expression. Interestingly, this association is only observed in HCC patients with HBV infection and not those without. To complement this, analysis of *VSIG4* expression in the human hepatoma cell lines HepG2, Sk-hep-1, Huh7 and MHCC-97H revealed lower *VSIG4* expression than in healthy human liver cells. While the conclusions drawn by this study suggested that CRIg may play a role as a tumour suppressor gene specifically in HCC, further studies into the role of CRIg in this particular cancer are necessary to elucidate its role.

Along with tumour expression of CRIg, several reports of the presence of a soluble form of CRIg present in the serum of cancer patients have been published. In 2017, Byun et al. (2017) detected and measured plasma-soluble CRIg by enzyme-linked immunosorbent assay (ELISA), and found increased levels in patients with ovarian cancer compared with those with benign tumours. Three years later, Yuan et al. (2020) aimed to discover novel biomarkers for the diagnosis of lymphoma-associated haemophagocytic lymhohistiocytosis (L-HLH). Utilising quantitative mass spectrometry and ELISA, the group compared the serum proteome of lymphoma patients with L-HLH, and those without haemorphagocytic lymphohistiocytosis (HLH). The group detected a soluble form of CRIg (termed sVSIG4) in patient serum and found that its concentration was significantly increased in patients with L-HLH compared with non-HLH lymphoma patients. Additionally, when assessing healthy control serum and serum from patients with breast cancer, gastric cancer, and lung cancer, the group found that all of the median values of sVSIG4 concentration were undetectable, while the mean values were significantly lower than patients with non-HLH lymphoma and those with L-HLH. This suggests that increased sVSIG4 in the serum may be a specific marker for both non-HLH lymphoma and L-HLH. Further, the findings indicate that the presence of sVSIG4 in human serum is not associated with the healthy state.

Overall, these findings together liken CRIg with other B7 ligand family members in that expression is enhanced in many types of cancer, and that serum soluble protein can be detected in patients, and this concentration is correlated with disease prognosis (Buderath et al., 2019; Gu et al., 2018; Z. Zheng et al., 2014). CRIg in cancer likely contributes to the tumour-promoting function of TAMs through the suppression of T cell responses, and thus attests to the potential usefulness of measuring CRIg expression in cancer as a prognostic marker.

*Table 1.2. Summary of the cancers which have been associated with CRIg expression, the key findings of the individual studies, and their references.*

| Cancer | Finding | Reference |
|---|---|---|
| Lung cancer | Infiltration of CRIg$^{+}$ macrophages into tumour specimens by immunohistochemistry (IHC); mice deficient in CRIg develop smaller tumours than their wild-type counterparts. | (Liao et al., 2014) |
| Ovarian cancer | *VSIG4* expression enhanced in ovarian cancer tissue compared with benign tumours; soluble CRIg levels increased in cancer patients, specifically in advanced staged disease. | (Byun et al., 2017) |
| Glioma | CRIg protein enhanced in glioblastoma; IHC demonstrating CRIg upregulation in high-grade glioma tissue compared with healthy controls, and the association of higher CRIg expression levels with poor prognosis. | (Xu et al., 2015; Zhang et al., 2016) |
| Colorectal cancer | *VSIG4* overexpressed in colorectal cancer specimens with known disruptive mutations and linked to lower survival outcomes. | (Menyhart et al., 2019) |
| Hepatocellular carcinoma (HCC) | Downregulation of *VSIG4* expression in HCC associated with poor prognosis | (Zhu et al., 2018) |
| Multiple myeloma | Correlation of high *VSIG4* mRNA with poor prognosis | (Jimenez et al., 2017; Roh et al., 2017) |
| Prostate cancer | RNA-seq on tumour infiltrating cells revealed elder patients with prostate cancer express higher levels of *VSIG4* which is associated with higher levels of biochemical relapse. | (Bianchi-Frias et al., 2019) |
| Breast cancer | Increased expression of *VSIG4* in invasive breast adipose tissue and co-expression with *CD163*, *MARCO*, complement component 1, q subcomponent B-chain (*C1QB*), complement component 1, q subcomponent A-chain (*C1QA*). | (Sturtz et al., 2014) |
| Lymphoma | Soluble CRIg concentration is enhanced in patients with L-HLH compared with non-HLH | (Yuan et al., 2020) |

## 1.10. Of Mice and Men: Expression of CRIg in Human Versus Murine Tissues

CRIg has long been reported to be exclusively expressed by certain subsets of tissue resident macrophages (Helmy et al., 2006; van Lookeren Campagne & Orozco, 2018). While there is an overwhelming amount of published evidence that this is true in mice, whether CRIg is expressed by any other cell types in humans remains in need of elucidation. This is because the initial studies into the expression profiles of CRIg on various immune cell types were conducted using murine cells (Helmy et al., 2006; Vogt et al., 2006), and these results show striking differences to the expression profiles that are observed in human tissues. For instance, *VSIG4* mRNA is highly expressed in murine liver tissue but only in low levels in humans, while the opposite is seen in lung tissues (Langnaese et al., 2000; Thul et al., 2017; Uhlen et al., 2015). The function of CRIg in murine liver Kupffer cells has been investigated in great depth (Broadley et al., 2016; Zeng et al., 2016), however, as the expression pattern in the human liver is so different, this leads us to question the relevance of these studies to human biology. Furthermore, as there is only one documented transcript variant of *VSIG4* expressed in mice, while there are up to six in humans, the relevance of these and whether different tissues or cell types express differing isoforms of the protein require further clarification.

Following the initial identification of CRIg as a complement receptor, Kim et al. (2005) generated the first commercial anti-CRIg monoclonal antibody, and screened for CRIg protein in human immune cells, finding that human monocytes express CRIg protein. However, less than a year later, Helmy et al. (2006) published their own screening study using their own in-house generated monoclonal antibody. Their group reported monocytes to stain negative for CRIg, and since this study, there have been no other reports of expression nor function of CRIg on monocytes.

While this initial discrepancy was published more than a decade ago, conflicting reports of the expression patterns of CRIg in various cells and tissues have only continued to become more prominent throughout recent years. While investigating their protein of interest, Nagre et al. (2018) show that CRIg interacts with tripartite motif protein 72 (TRIM72) in both human and murine lungs. This study (Nagre et al., 2018) is a stark contradiction to the initial findings of Helmy, et al (2006), who report no expression of CRIg in the murine lung. Furthermore, Nagre et al. (2018) report murine CRIg to migrate at ~32 kDa by gel electrophoresis, while Helmy, et al (2006) reported it to migrate at ~45 kDa. It is of note that the groups use differing

antibodies against CRIg in their studies, and it is tempting to speculate that antibody choice may be a contributing factor to this disagreement. Together, these studies indicate that we cannot rely on murine models alone to reveal the functional role that CRIg may play in human health and disease, and that a substantial amount of fundamental research into the isoforms and expression CRIg is needed to be undertaken before we can begin to fully understand this receptor.

## 1.11. Rationale of This Thesis

In order to investigate the role of CRIg in immune disease and how CRIg may potentially be used therapeutically, we must first come to understand the role of CRIg in the human state of health. It is clear from the current state of the published literature revolving the biological function/s of CRIg that there is a need to re-characterise its expression in human phagocytic cells with consideration of the multiple protein forms which may be expressed, to re-assess the function of these proteins, and to better understand the differences between the role of this protein in mice compared with humans. Thus, this is the subject of this thesis.

## 1.12. Project Hypotheses, Aims, and Significance

We propose that CRIg is an anti-inflammatory, phagocytosis-promoting receptor, and that its expression is not restricted to tissue-fixed macrophages as previously believed. Thus, in this thesis, we aim to perform a comprehensive investigation into the CRIg mRNA and protein variants expressed by human populations of immune cells including macrophages, DCs, monocytes and neutrophils. This re-characterisation will include the generation of novel anti-CRIg monoclonal antibodies, and studies into the regulation over CRIg expression in human macrophages, and the relationship between cellular expression of CRIg and the 'classical' complement receptors, CR3 and CR4.

Thus, the following aims have been constructed:

1. To re-characterise CRIg expression in human immune cells, including the circulating phagocytes of the immune system, monocytes and neutrophils.
2. To develop monoclonal antibodies for human and mouse forms of CRIg.
3. To examine CRIg expression and its regulation in human monocyte-derived macrophages, and to examine its relationship to the expression of the 'classical' complement receptors, CR3 and CR4.

4. To investigate the expression and regulation of CRIg isoforms in human monocyte-derived DC by cytokines at the mRNA and protein levels.
5. To investigate the inflammatory mediator network that regulate the expression of CRIg in human macrophages.

By meeting these aims, the findings from this investigation will represent a substantial increase in our knowledge of the biology of CRIg in healthy human phagocytic cells, and with the information gained, it will form a backbone for future comprehensive studies into the role of CRIg in inflammatory disease states, such as in rheumatoid arthritis and type 1 diabetes.

## 1.13. Publication: 'Complement Receptor Immunoglobulin: a control point in infection and immunity, inflammation and cancer'

### 1.13.1. Introduction and Contextual Statement

This section of this introductory chapter presents a comprehensive review of the literature, which includes the structure of human vs. murine CRIg, the various immune functions attributed to the protein, and the expression profiles of CRIg on human immune cell types, current up until April 2016. The review summarises the established roles of CRIg in infection and immunity, inflammation, and cancer, and also highlights importance of future foundational studies in the area of CRIg biology before in-depth studies into specific disease models can take place.

This chapter presents the first published manuscript included in this thesis. The following invited review article entitled *'Complement receptor immunoglobulin: a control point in infection and immunity, inflammation and cancer'*, by **Annabelle Small**, Marwah Al-Baghdadi, Alex Quach, Charles S. Hii & Antonio Ferrante was published in the peer reviewed journal, Swiss Medical Weekly, in April 2016 (doi:10.4414/smw.2016.14301).

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# Statement of Authorship

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## Principal Author

| | | | |
|---|---|---|---|
| Name of Principal Author (Candidate) | Annabelle Small | | |
| Contribution to the Paper | Critically reviewed the literature, collated data, drafted manuscript. | | |
| Overall percentage (%) | 70% | | |
| Certification: | This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper. | | |
| Signature | | Date | 7/3/2020 |

## Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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| Signature | | Date | 03/03/2020 |

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|---|---|---|---|
| Contribution to the Paper | CS assisted in the drafting of the manuscript + drawing Fig 2 + legend | | |
| Signature | | Date | 5/3/20 |

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# Complement receptor immunoglobulin: a control point in infection and immunity, inflammation and cancer

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## Summary

The B7 family-related protein, V-set and Ig domain (VSIG4) / Z39Ig / complement receptor immunoglobulin (CRIg), is a new player in the regulation of immunity to infection and inflammation. The unique features of this receptor as compared with classical complement receptors, CR3 and CR4, have heralded the emergence of new concepts in the regulation of innate and adaptive immunity. Its selective expression in tissue macrophages and dendritic cells has been considered of importance in host defence and in maintaining tolerance against self-antigens. Although a major receptor for phagocytosis of complement opsonised bacteria, its array of emerging functions which incorporates the immune suppressive and anti-inflammatory action of the receptor have now been realised. Accumulating evidence from mouse experimental models indicates a potential role for CRIg in protection against bacterial infection and inflammatory diseases, such as rheumatoid arthritis, type 1 diabetes and systemic lupus erythematosus, and also in promotion of tumour growth. CRIg expression can be considered as a control point in these diseases, through which inflammatory mediators, including cytokines, act. The ability of CRIg to suppress cytotoxic T cell proliferation and function may underlie its promotion of cancer growth. Thus, the unique properties of this receptor open up new avenues for understanding of the pathways that regulate inflammation during infection, autoimmunity and cancer with the potential for new drug targets to be identified. While some complement receptors may be differently expressed in mice and humans, as well as displaying different properties, mouse CRIg has a structure and function similar to the human receptor, suggesting that extrapolation to human diseases is appropriate. Furthermore, there is emerging evidence in human conditions that CRIg may be a valuable biomarker in infection and immunity, inflammatory conditions and cancer prognosis.

***Key words:*** *CRIg/VSIG4/Z39Ig; macrophages; dendritic cells; infection and immunity; cytokines; alternative complement pathway, complement receptors; inflammatory diseases; CRIg-Fc fusion protein; cancer*

**Abbreviations**

APC antigen presenting cell

CR complement receptor

CRIg complement receptor immunoglobulin

HBV hepatitis B virus

IL interleukin

MAP mitogen activated protein

PI3 phosphatidylinositol 3

PKC protein kinase C

TNF tumour necrosis factor

VSIG4 B7 family-related protein V-set and Ig domain

Z39Ig Protein with immunoglobulin domains derived from expressed sequence tag #Z39624

## Introduction

Complement plays an important role in the opsonisation of circulating pathogens, facilitating the phagocytosis and removal of these pathogens by phagocytes. Fragments of complement are recognised by four complement C3 fragment receptors, CR1 (CD35), CR2 (CD21), CR3 (CD11b/CD18), and CR4 (CD11c/CD18), with an additional receptor, complement receptor immunoglobulin (CRIg), being added to this list in 2006 (fig. 1; [1, 2]). The major developments surrounding CRIg are summarised in table 1. Although CRIg (originally named Z39Ig) was first described in 2000 [3], its biological properties were not evident until van Lookeren Campagne and colleagues published their extensive work on its prime role as a complement receptor promoting phagocytosis of bacteria *in vivo* and *in vitro* [4]. Working with mice, this group not only continued to provide supportive evidence of its importance in defence against infection and promoting phagocytosis but also discovered its property of uniquely interacting with components of the alternative complement pathway and inhibiting its activation [2, 5]. This led to the development of a CRIg-Fc fusion protein, which demonstrated anti-inflammatory activity in several murine models of inflammatory diseases [6–10]. In a parallel publication, Vogt et al. [6] demonstrated the immunosuppressive activity of CRIg by using a VSIG4(CRIg)-Fc fusion protein *in vitro* and also when injected into mice. Interestingly, human dendritic cells ex-

pressing CRIg were found to suppress T cell proliferation, expression of activation markers (CD25 and CD69) and production of helper T cell (Th1) cytokines [11]. This suggests that CRIg+ dendritic cells may promote tolerance

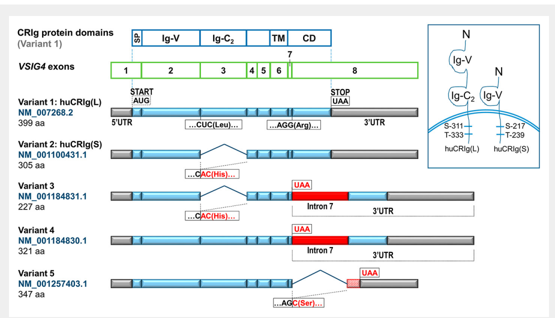


**Figure 1**

Schematic representation of the CRIg protein domains aligned with the five *VSIG4* splice variant transcript structures. The transcripts structures are derived from the NCBI Reference Sequence Database (RefSeq) using the following mRNA accession numbers: NM_007268.2, NM_001100431.1, NM_001184831.1, NM_001184830.1, and NM_001257403.1, as noted in the figure. The structure of the longest CRIg isoform (variant 1, huCRIg(L)) is used as the reference, with the protein domains denoted as: SP, signal peptide; Ig-V, immunoglobulin domain V-type; Ig-$C_2$, immunoglobulin domain V-type; TM, transmembrane region, and CD, cytoplasmic domain. The relative *VSIG4* exon structure reveals that SP, Ig-V, Ig-$C_2$, and TM are encoded specifically by exons 1, 2, 3, and 6, respectively, whereas CD is encoded by exons 7 and 8. The short form (variant 2, huCRIg(S)) differs from the long form by the exclusion of exon 3, the Ig-$C_2$ domain, and a change at codon 138 from CUC (Leucine) to CAC (Histidine). In this isoform, the overall size of the CRIg protein is reduced from 399 to 305 amino acids (aa). Variant 3 features this identical alteration, in addition to the inclusion of intron 7 which creates a stop codon (UAA) adjacent the last codon of exon 7, causing the majority of the CD to be missing (227 aa). Variant 4 features the same loss of the CD as variant 3, but retains all other domains (321 aa). Variant 5 retains the same domains as variants 1 and 4 (exon 1 to 7 inclusive), but is unique from the other four variants in that a portion of exon 8 is excluded, which creates a change at codon 321 (exon 7-8 junction) from AGG (Arginine) to AGC (Serine), followed by a 'frameshift' that results in a stop codon generated 26 aa downstream (347 aa). Insert: shows a schematic representation of the long and short forms of CRIg, expressed in macrophages. The extracellular portion of CRIg contains an Ig-V and/or Ig-$C_2$ domain(s). Putative phosphorylation sites for cAMP/cGMP-dependent protein kinase (S-311 or S-217) and protein kinase C (T-333 or T-239) have been proposed to be present in the cytoplasmic domain of CRIg.

and immunosuppression. This may possibly explain recent findings that CRIg+ human macrophages may have a role in regulating malignancy [12–15].

Inflammatory mediators exert their effects on macrophages by regulating CRIg expression. Accordingly, cytokines have been shown to alter CRIg expression on human macrophages [16, 17]. Work along this line should be expanded to gain a better understanding of how inflammatory mediator networks operate in infection and immunity, chronic inflammatory diseases and cancer. Since CRIg and the classical complement receptors, CR3 and CR4, which also bind complement opsonised bacteria, are expressed concomitantly on macrophages, the relative commonalities and differences when engaged need to be appreciated. Finally, the mechanisms of CRIg-mediated protection against infection and inflammatory conditions as well as presumed susceptibility to cancer are becoming research topics of intense interest.

## CRIg structure

CRIg, a member of the transmembrane protein of the type 1 immunoglobulin (Ig) superfamily, is encoded by the *VSIG4* gene located in the pericentromeric region of the human X chromosome. The gene, first documented as "Z39Ig" by Langnaese et al. [3], contains eight exons and has a length of 18.3 kb. In humans, the product, CRIg, referred to as the long form (huCRIg(L) [4], contains both a constant (C2-type) and a variable (V-type) immunoglobulin domain (fig. 2). In addition, a short form, huCRIg(S), was also identified. The huCRIg(S) contains only the V-type immunoglobulin domain, with no C2-type [4]. The two different forms of CRIg arise from alternative splicing of the *VSIG4* gene, which also has the potential to give rise to a total of five different variants (fig. 2). Only one form of CRIg, containing a single IgV-type domain, is expressed in murine macrophages and the data show that the V-type domain of the CRIg protein is essential for its ability to bind complement components and to promote phagocytosis, whereas the significance of the C2-type domain remains uncertain. The intracellular portion of huCRIg(L) harbours two potential phosphorylation sites, a cAMP/cGMP-dependent protein kinase phosphorylation site at S-311, and a protein kinase C phosphorylation site at T-333 [3]. Both S and T

**Table 1:** Key developments in the field of complement receptor immunoglobulin (CRIg).

| Development | References |
|---|---|
| Identification of Z39Ig – immunoglobulin superfamily member, gene localisation in the pericentriomere region of human X chromosome. | [3] |
| Z39Ig/CRIg expressed predominantly in fixed tissue macrophages. | [4] |
| CRIg promotes clearance of bacteria and viruses in experimental models; a key role for CRIg+ Kupffer cells. | [4, 19] |
| CRIg is a complement receptor which promotes phagocytosis of bacteria by macrophages. Unique properties of the receptor described. | [4, 20, 21] |
| CRIg uniquely interacts with components of the alternative complement activation pathway; is an inhibitor of the alternative pathway. | [2, 5] |
| VSIG4 induces T cell immunosuppression; CRIg+ dendritic cells and tolerogenic responses; negatively regulates T cell-dependent immunoglobulin isotype switching in the mouse and human immune system. | [6, 11, 24] |
| Development of CRIg-Fc fusion protein; protects against experimental arthritis, systemic lupus erythematosus, type 1 diabetes and other diseases. | [6–10, 27] |
| Adoptive transfer of CRIg+ macrophages protects against immune-mediated liver injury in a mouse model. | [29] |
| CRIg expression in tumour- associated macrophage; poor prognosis; promotes tumour growth (mouse and human immune system). | [12–15] |
| Regulation of CRIg expression in human macrophages by inflammatory mediators (including cytokines) and anti-inflammatory agents. | [16, 17, 33] |
| CRIg expression in macrophages in human tissues, large intestine, synovial tissue, liver. | [12–15, 26, 28, 30, 33, 36] |
| CRIg as a biomarker in human conditions: preeclampsia, chronic hepatitis B virus infection, heart failure, cancer | [12–15, 33–36] |

residues are present on the intracellular domain of huCRIg(S) at residues 217 and 239 (fig. 1). The significance of these sites is currently unknown but could provide a means to regulate CRIg function. Surprisingly, the reported sizes of the protein (50kDa and 45kDa for the long and short forms, respectively) [4] do not agree with the estimated protein sizes. This suggests that some post-translational modifications, such as glycosylation, have occurred [18].

## CRIg promotes phagocytosis and antimicrobial action of macrophages

Although there has been limited publication of data showing that CRIg plays a role in protection against infection, the results are quite convincing. Using CRIg$^{-/-}$ mice, Helmy et al. [4] showed that CRIg was important in the clearance of the intracellular bacterium *Listeria monocytogenes* by liver resident/fixed macrophages (Kupffer cells), and preventing dissemination of the bacteria to other organs. This protection provided by CRIg was evident by a reduction in numbers of bacteria and an increase in mouse survival. Similar results were obtained when the extracellular pathogen *Staphylococcus aureus* was examined, leading to the conclusion that CRIg is required for the rapid clearance of

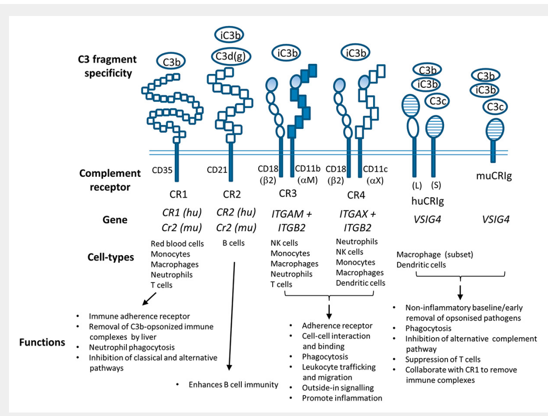


**Figure 2**

Characteristics, function and expression of complement receptors
The structural domains of the five known types of complement receptors are depicted, together with their specificity of C3 fragments, the genes encoding them, their distribution amongst the different leucocyte types and their known functions. CR1, CR2 and CRIg are single transmembrane proteins with extracellular portions, transmembrane domains and cytoplasmic tails whereas CR3 and CR4 are transmembrane heterodimers of a common β2 integrin (CD18) chain and an α integrin chain, αM (CD11b) or αX (CD11c). Murine CR1 and CR2 are derived from the same gene by alternative splicing whereas the human counterparts are encoded by 2 different genes. CR1 contains thirty short consensus repeats (SCR) and CR2 has fifteen SCR. CD18 contains four repeats and a Von Willebrand factor type A domain (lightly shaded oblong shape). The α integrins contain, within their extracellular portions, seven FG-GAP repeats (rectangles) and a Von Willebrand factor type A domain. Two human CRIg isoforms, huCRIg(L) for the long form and huCRIg(S) for short, have been described. Both isoforms contain an N-terminal ligand binding domain that belongs to the IgV-type of immunoglobulin domains (horizontal stripes). The long form of CRIg also contains a membrane proximal domain that is an IgC-type immunoglobulin domain. The function of this domain is unclear. The murine form, similar to CRIg(S), contains only the IgV-type of immunoglobulin domain but the cytoplasmic tail is shorter than that of huCRIg(S). The IgV domains are believed to be responsible for binding C3 fragments.

both intracellular and extracellular C3-opsonised bacteria. More recently, the anti-infective actions of CRIg have been demonstrated with adenoviruses in mice [19]. The work showed that CRIg-mediated viral clearance by liver Kupffer cells was significantly reduced in CRIg$^{-/-}$ mice compared with wild type mice.

CRIg functions as a complement receptor on macrophages, promoting phagocytosis by binding to C3b and iC3b-coated particles [4]. CRIg with bound C3b is internalised into Kupffer cells and becomes localised in a pool of constitutively recycling membranes. At the initial stages of phagosome formation, CRIg was actively recruited from recycling endosomes to the sites of C3b-coated particle ingestion. Then, prior to the fusion of the phagosome and lysosome to avoid degradation, CRIg is likely recycled from the phagosome to the endosome for use in further phagocytic events, ensuring a readily available source of CRIg on the cell surface and thereby enabling a faster rate of phagocytosis when bacteria are encountered [1].

CRIg differs from CR3 not only in its expression (selectively in macrophages and dendritic cells) but also in the mechanisms of phagocytosis and induction of immune responses. Whereas CRIg binds to both C3b and iC3b, CR3 binds only iC3b ([1, 4], table 2). In murine peritoneal macrophages, Gorgani et al. [20] reported that, whereas efficient binding of opsonised particles to CR3 required divalent cations ($Mg^{2+}$ and $Ca^{2+}$), binding to CRIg did not require these ions. CRIg-mediated binding also did not require integrin activation. Gorgani et al. [20] further investigated the relative contribution of CRIg and CR3 to complement-mediated phagocytosis, finding that CRIg enhanced phagocytosis only in resident macrophages, while CR3 acted as the dominant complement receptor in infiltrating activated macrophages at sites of inflammation. It was demonstrated that CRIg expression is capable of increasing the rate of phagocytosis in resident peritoneal macrophages, and is absent from inflammatory macrophages, suggesting that CRIg has a function in immune clearance and tissue homeostasis, and that initiating CRIg-mediated phagocytosis avoids the inflammatory cytokine cascade that is associated with phagocytosis via CR3 [4, 16]. Consistent with this idea, CRIg has been shown to play a role in clearing pathogens during early infection such as in the liver sinusoidal lumen through which invading pathogens have to pass [4]. Furthermore, this receptor may participate in the baseline removal of C3-opsonised apoptotic cells and cell debris without involving CR3 [1, 2], the engagement of which leads to a systemic inflammatory response. CRIg may not work alone in this function. CR1 is known to recognise C3b without internalisation. In this manner, CR1 would remove immune complexes from the circulation, thus preventing pathological deposition. The role for CRIg may be to mediate removal of potentially pathological agents through internalisation. Thus, CR1 and CRIg may have important roles in maintenance of homeostasis, providing baseline clearance of pathogens [1, 2].

Apart from promoting phagocytosis of opsonised bacteria, CRIg may also affect their killing. It has been reported in the human macrophage cell line THP1 and the J774 mouse macrophage cell line that CRIg binds chloride intracellular channel 3 (CLIC3), an intracellular chloride channel protein

that is required for the clearance of *Listeria monocytogenes* [21]. Thus, CRIg was shown to co-localise with CLIC3 on the membranes of *Listeria monocytogenes*-containing vacuoles in the mouse macrophage cell line. Using an anti-CRIg antibody that enhanced macrophage-mediated killing of intracellular *Listeria monocytogenes*, it was demonstrated that this killing-enhancing effect of anti-CRIg antibody required CLIC3 as the effect was abolished in macrophages from CLIC3$^{-/-}$ mice. The mechanism probably involved a CRIg-mediated increase in Cl$^-$ concentration and a decrease in pH in the vacuoles. This contrasts with CR3 in the killing of phagocytosed microorganisms such as *Salmonella* serovar Typhimurium by human phagocytes in that this receptor is solely needed for the phagocytic step whereas the killing mechanism depends on a Toll-like receptor 4-mediated activation of NADPH oxidase [22].

Since the majority of the above data have been generated from mouse macrophages and experimental mouse models, care needs to be exercised when translating the findings to human diseases. There are major differences in complement receptors between mouse and humans; for example, CR1 has a very limited expression profile in the mouse. In terms of CRIg, mouse macrophages express one form that also contains the IgV domain ([4]; fig 1 and 2, table 2). Studies with human macrophages and phagocytosis have been limited to monocyte-derived macrophages in culture which express CRIg. The levels of this receptor in these macrophages correlated with the degree of phagocytosis of complement-opsonised *Candida albicans* [17]. The comparison between mouse and human CRIg expressing macrophages are shown in table 2.

## Immunosuppressive and anti-inflammatory functions

CRIg is able to bind the alternative pathway complement components C3b and iC3b, which functions to bind foreign particles for phagocytosis. A study by Wiesmann et al. [5] investigated whether the binding of CRIg to C3b inhibits the inflammatory convertase activation of the alternative complement pathway. They successfully solved the crystal structure of CRIg bound to C3b, which indicated that unlike most C3b binding molecules, CRIg predominantly binds to the β-chain (rather than the α-chain). It was demonstrated that CRIg inhibited both the C3 and C5 convertases of the alternative but not the classical pathway. This inhibition is related to the ability of CRIg to bind C3b, whose function in both convertases is to recruit C3 and C5, thereby enabling their cleavage by factor Bb, the catalytic subunit of the convertases. Further studies with C5 revealed that when CRIg was bound to C3b, it prevented C5 from interacting with C3b. As a consequence, C5 could not be cleaved by the C5 convertase (C3bBb3b) to C5a and C5b [2]. Selectivity for alternative pathway convertases can be explained on the basis that CRIg does not bind C4b and hence cannot compete effectively with the classical pathway C5 convertase (C4b2a3b) for C5 binding [2].

Katschke et al. [7] demonstrated, through the generation of a soluble CRIg fusion protein (CRIg-Fc), that it was possible to reverse inflammation and bone loss in two different experimental models of arthritis. By inhibiting the alternative complement pathway with the fusion protein, the study demonstrated that the alternative pathway of complement is essential for disease induction and progression. This fusion protein was further investigated in a recent study in which it was administered to lupus-prone MRL/lpr mice [8]. The group reported decreased skin and kidney inflammation, and decreased proteinuria and pyuria in mice administered CRIg-Fc fusion protein. The protein also protected against other conditions known to involve complement. Chen et al. [9] demonstrated protection against experimental ischaemia/reperfusion injury in a mouse model. Administration of the CRIg-Fc protein prevented local intestinal and remote lung damage, which was associated with decreased complement deposition. In experimental autoimmune uveoretinitis, Chen et al. [10] showed that retinal inflammation was suppressed by treatment with the CRIg-Fc protein, which was also associated with reduced deposition of C3b and factor B in the tissues.

Vogt et al. [6] further investigated the immune function of CRIg. It is known that T cell activation by antigen presenting cells (APCs) is both positively and negatively regulated by the B7 family of proteins that are found on the surface of the APC [23]. Through screening, it was found that a mouse copy DNA sequence that appeared to be derived from messenger RNA encoding the CRIg protein, when translated was 20% identical to the amino acid sequences of known B7 family members. It was therefore hypothesised that CRIg, being related to the B7 protein family, would show some degree of regulation over T cells. They showed CRIg to be a strong negative regulator of murine and human T cell proliferation and interleukin-2 (IL-2) production, though the mechanisms of this regulation remain uncertain. It was further found that CRIg was only expressed on the surface of resting tissue macro-

**Table 2:** Comparison between mouse and human complement receptor immunoglobulin (CRIg) on macrophage phagocytosis.

| Function | Experimental approach | Human (L) | Human (S) | Murine | References |
|---|---|---|---|---|---|
| Recognition of C3 fragments | Formation of rosettes following incubation of CRIg+ cells with complement-opsonised sheep erythrocytes | Yes | Not determined | Yes | [4] |
| Binding to C3b and iC3b | Binding of huCRIg (L)-Fc, huCRIg(S)-Fc or muCRIg-Fc to C3b and iC3b | Yes | Yes | Yes | [4] |
| Phagocytosis | Phagocytosis of complement-opsonised particles | (Yes; but isoform not determined) | | Yes | [16, 17, 20] |
| Clearance of *Listeria monocytogenes* and *Staphylococcus aureus* | Assessment of bacterial load in the blood, spleen and lung of mice | Not determined | Not determined | Yes | [4] |
| Inhibition of the alternative complement pathway | Cleavage of C3 and C5 by their respective convertases | Yes (but isoform not stated) | | Yes | [5, 7] |

phages, and not on the surface of macrophages activated by lipopolysaccharide. This fact indicates that CRIg may have an important role in maintaining T cell unresponsiveness in healthy tissues. The regulatory role of CRIg on antibody production has also been demonstrated [24]. The work conducted in mice showed that CRIg on macrophages negatively regulates T cell-dependent immunoglobulin isotype switching through an action on T cell activation and differentiation.

Expression of CRIg in human dendritic cells was first reported by Ahn et al. [25]. While the immunosuppressive effects of CRIg-Fc fusion protein have been demonstrated by its direct interaction with T cells, Xu et al. [11] transfected human dendritic cells with CRIg such that they constitutively expressed CRIg and showed that these dendritic cells inhibited proliferation of allogenic T-cells, and decreased expression of activation markers and pro-inflammatory cytokines production from these cells. These suggest that CRIg expression on dendritic cells has anti-inflammatory outcomes; plays a role in tissue homeostasis and host defence, and suggest a potential function in suppressing effector T cells.

It has been reported with respect to inflammatory diseases that CRIg expression and levels of CRIg$^+$ macrophages relate to the intensity of the inflammatory reaction. For example, Tanaka et al. [26] showed that CRIg$^+$ macrophages were present in the large intestine of mice and this expression decreased during inflammatory colitis. Fu et al. [27], using the nonobese diabetic mouse model of type 1 diabetes, found that CRIg$^+$ macrophages were associated with diabetes resistance. Mice given injections of CRIg-Fc fusion protein had lower incidence of diabetes. CRIg expression has been found in macrophages infiltrating tissue in other inflammatory conditions, including in atherosclerosis where receptor expression was associated with foamy macrophages in human carotid atherosclerotic plaques [28]. The role of CRIg$^+$ macrophages in protection against inflammation is best seen from the results of Jung et al. [29], who demonstrated the protective role of these macrophages in a model of immune-mediated liver injury. Mice lacking CRIg showed increased liver pathology and poor survival rates, associated with increased antigen-induced responses by liver T and natural killer T cells. Interestingly the effect of lack of CRIg in these mice could be overcome by adoptive transfer of CRIg$^+$ Kupffer cells.

Since at least one of the human CRIg receptors, the S form, is structurally similar to the murine CRIg in that they contain only the IgV-type domain (fig. 1 and fig. 2), we can tentatively conclude that these results from experimental models of human diseases are relevant to the human immune system and inflammatory diseases, but this obviously requires examination. The presence of CRIg$^+$ macrophages in synovial tissue has been reported in rheumatoid arthritis [28, 30] and in experimental arthritis in mice [31]. In one study the CRIg+ macrophages in the synovial tissue were defined as CRIg$^+$CR4$^+$ and CRIg$^+$CR4$^-$. Interestingly, the former predominated in rheumatoid arthritis compared with osteoarthritis. Although data were not presented, CR3 was expressed in a similar manner to CR4 [30]. It is tempting to speculate that the CRIg$^+$CR4$^-$ subpopulation may be playing a protective role in this disease and that manipulating the number of this subpopulation may be potential new avenues to treat rheumatoid arthritis [29].

## CRIg$^+$ macrophages and cancer

Recently, it has been shown that macrophages infiltrating lung tissue in patients with non-small-cell lung cancer express high level of CRIg [12]. The authors of this article highlighted that CRIg downregulated CD4$^+$ and CD8$^+$ T-cell proliferation and cytokine production. This work was further extended into a mouse model of Lewis lung carcinoma in which the effect of CRIg deficiency on tumour growth was examined. The result showed that CRIg$^{-/-}$ mice had significantly smaller tumours than wild type mice [12]. The role of CRIg in cancer pathogenesis is likely to be of relevance across different cancers. Sturtz et al. [13] conducted a gene microarray study in breast cancer patients and showed that tumour-adjacent tissue had >5 fold increase in CRIg expression compared with distant tissue. Investigation extended to CRIg expression in glioma using tissue microarray. The result of this study showed that CRIg expression in glioma patients is higher than control. Indeed, CRIg upregulation correlated with poor prognosis in this type of cancer [14]. Similarly, by using gene microarray from lymphoma patients, CRIg was found to be one of the most upregulated genes in T cell / histiocyte-rich large B-cell lymphoma [15]. Cancer has been shown to be associated with decreased production of interferon-γ by T cells in the tumour environment [32]. Previous studies have shown that this cytokine causes a decrease in CRIg expression in human macrophages, *in vitro* and *in vivo* [17, 33], and is conducive to the increased CRIg expression in tumour-associated macrophages.

The outcome of immune responses following the engagement of the T cell antigen receptor to the peptide of the antigen expressed on the major histocompatibility complex of the APC is dependent on the costimulatory and coinhibitory signals between the CD28 receptor family on the T cells and B7 family on the APC. These costimulatory and coinhibitory signals may be exploited by tumours for immune evasion. CRIg, a member of the B7 family which is a coinhibitory molecule, being increased in tumour associated macrophages is likely to prevent T-cell mediated tumour destruction [34].

## Regulation of CRIg expression in macrophages

Since experimental disease models have shown that CRIg promotes anti-infective and anti-inflammatory events, there is a need to understand whether inflammatory mediators regulate CRIg expression on macrophages. The major evidence for inflammatory networks regulating CRIg expression and associated phagocytosis comes from the work of Gorgani et al. [17]. They demonstrated that the inflammatory mediator and cell activator, arachidonate, caused a marked decrease in CRIg expression in human macrophages, both at the mRNA and cell surface protein expression level [17]. The action of cytokines on this expression in human monocyte-derived macrophages is interesting. The results showed that tumour necrosis factor (TNF),

interferon-γ, IL-4 and transforming growth factor-β1 decreased CRIg expression but the immunosuppressive cytokine IL-10 caused a marked increase in expression. These changes in CRIg expression correlated with the amount of phagocytosis of *Candida albicans* [17]. Further studies in human monocyte-derived macrophages also demonstrated that IL-1β and IL-6 caused a decrease in expression, but were not as potent as TNF [16]. Interestingly, Guo et al. [33] found that interferon-γ not only decreased expression of CRIg on human macrophages *in vitro* but also played a role in decreasing expression in liver macrophages of patients with chronic hepatitis B virus (HBV) infection. A reduced CRIg expression was associated with an increase in plasma HBV load and increased serum alanine aminotransferase levels. This finding supports the infection protective actions of CRIg as well as the anti-inflammatory characteristics.

The findings reported by Gorgani et al. [17] and Ma et al. [16] collectively suggest that mediators of inflammation, including cytokines, may control CRIg expression at two levels: firstly by regulating the development of monocytes into CRIg positive macrophages and secondly on mature macrophages *per se*. The results suggest that exogenously and endogenously generated mediators not only regulate tissue/resident macrophage function by modulating CRIg expression but also act on infiltrating monocytes to control their development into CRIg$^+$ macrophages. This is supported by the findings of Vogt et al. [6] and Gorgani et al. [20] in mice.

While arachidonate is a powerful down-regulator of CRIg expression in human macrophages, the steroidal anti-inflammatory agent, dexamethasone, caused a marked upregulation of CRIg expression [16, 17]. Evidence suggested that these agents regulate CRIg expression via protein kinase Cα (PKCα) [16, 17]. The arachidonate action on CRIg expression was found to be independent of the cyclooxygenase and lipoxygenase pathways, and did not involve the mitogen-activated protein kinases p38 and ERK1/ERK2, as well as independent of PI3 kinase but dependent on PKC activation [17]. The increase induced by dexamethasone can also be accounted for by an action on PKCα, namely an inhibitory effect [16, 17]. It is interesting that an anti-inflammatory agent has the ability to upregulate a macrophage receptor which has both phagocytic and anti-inflammatory functions. This suggests that some of the anti-inflammatory properties of dexamethasone may be, in part, mediated by causing changes in CRIg expression. Ma et al. [16] made the further observation that TNF production by human macrophages may autoregulate the full expression of CRIg. Thus the addition of anti-TNF neutralising antibodies increased CRIg expression in cultured human macrophages. Since CRIg$^+$ macrophages are found in synovial tissue of rheumatoid arthritis patients, it is tempting to speculate that the anti-TNF therapy may be protective via this mechanism.

## CRIg as a potential disease biomarker

Once we understand better the relationship between CRIg expression and disease progression in inflammatory disorders, it is possible that CRIg expression may be a helpful biomarker to ascertain diagnosis or disease progression and outcomes. The work of Tanaka et al. [30] suggests that there may be benefits for gauging inflammation in rheumatoid arthritis and this is supported by the findings in experimental mouse arthritis [31]. The most convincing example to date comes from the work on preeclampsia, a leading cause of neonatal and maternal morbidity and death [35]. Although there are biomarkers which distinguish between normal pregnancy and preeclampsia, these markers do not distinguish between the non-severe versus the severe form. Using a microarray approach, the upregulated *VSIG4* gene was found to be a marker for the severe form [35]. Others have found that CRIg was differentially expressed between right ventricular and left ventricular dysfunction in human heart failure and suggested this as a biomarker [36]. Furthermore it is evident that CRIg expression in tumour-associated macrophages can be a potential biomarker of prognostic value in cancer patients [12–15]. In chronic HBV infection the levels may be useful to gauge viral load and liver damage [33].

## Concluding remarks

The exciting properties of CRIg, first revealed in animal models just over a decade ago, appear not to have escalated into a search for its role in health and disease, which is often associated with findings of this type. The information available, however, places the role of the receptor, from potentially being a major player in protection against infection and chronic inflammatory disease to the other end of the spectrum of increasing susceptibility to cancer. Clearly there is justification to pursue more actively work that will culminate in a greater understanding of (i) the regulatory inflammatory mediator network which controls the expression of the receptor on both macrophages and dendritic cells, (ii) the consequences of complement opsonised bacteria engaging CRIg versus CR3/CR4 on macrophages, (iii) the translation of the experimental models findings to the clinical diseases. The therapeutic potential in chronic inflammatory diseases has already been realised through the generation of CRIg-Fc fusion protein but the many effects of this protein may warrant other approaches of altering the actual expression of CRIg on macrophages at inflammatory sites. Finally, it remains now for "experiments of nature" to teach us what role CRIg and CRIg+ macrophages / dendritic cells play in human diseases, by identifying genetic mutations in the *VSIG4* gene and clinical presentation associated with CRIg deficiency.

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## References

1 van Lookeren Campagne M, Wiesmann C, Brown EJ. Macrophage complement receptors and pathogen clearance. Cell Microbiol. 2007;9(9):2095–102.

2 He JQ, Wiesmann C, van Lookeren Campagne M. A role of macrophage complement receptor CRIg in immune clearance and inflammation. Mol Immunol. 2008;45(16):4041–7.

3 Langnaese K, Colleaux L, Kloos DU, Fontes M, Wieacker P. Cloning of Z39Ig, a novel gene with immunoglobulin-like domains located on human chromosome X. Biochim Biophys Acta. 2000;1492(2-3):522–5.

4 Helmy KY, Katschke KJ, Jr., Gorgani NN, Kljavin NM, Elliott JM, Diehl L, et al. CRIg: a macrophage complement receptor required for phagocytosis of circulating pathogens. Cell. 2006;124(5):915–27.

5 Wiesmann C, Katschke KJ, Yin J, Helmy KY, Steffek M, Fairbrother WJ, et al. Structure of C3b in complex with CRIg gives insights into regulation of complement activation. Nature. 2006;444(7116):217–20.

6 Vogt L, Schmitz N, Kurrer MO, Bauer M, Hinton HI, Behnke S, et al. VSIG4, a B7 family-related protein, is a negative regulator of T cell activation. J Clin Invest. 2006;116(10):2817–26.

7 Katschke KJ, Jr., Helmy KY, Steffek M, Xi H, Yin J, Lee WP, et al. A novel inhibitor of the alternative pathway of complement reverses inflammation and bone destruction in experimental arthritis. J Exp Med. 2007;204(6):1319–25.

8 Lieberman LA, Mizui M, Nalbandian A, Bosse R, Crispin JC, Tsokos GC. Complement receptor of the immunoglobulin superfamily reduces murine lupus nephritis and cutaneous disease. Clin Immunol. 2015;160(2):286–91.

9 Chen J, Crispin JC, Dalle Lucca J, Tsokos GC. A novel inhibitor of the alternative pathway of complement attenuates intestinal ischemia/reperfusion-induced injury. J Surg Res. 2011;167(2):e131–6.

10 Chen M, Muckersie E, Luo C, Forrester JV, Xu H. Inhibition of the alternative pathway of complement activation reduces inflammation in experimental autoimmune uveoretinitis. Eur J Immunol. 2010;40(10):2870–81.

11 Xu S, Sun Z, Li L, Liu J, He J, Song D, et al. Induction of T cells suppression by dendritic cells transfected with VSIG4 recombinant adenovirus. Immunol Lett. 2010;128(1):46–50.

12 Liao Y, Guo S, Chen Y, Cao D, Xu H, Yang C, et al. VSIG4 expression on macrophages facilitates lung cancer development. Laboratory investigation; a journal of technical methods and pathology. 2014;94(7):706–15.

13 Sturtz LA, Deyarmin B, van Laar R, Yarina W, Shriver CD, Ellsworth RE. Gene expression differences in adipose tissue associated with breast tumorigenesis. Adipocyte. 2014;3(2):107–14.

14 Xu T, Jiang Y, Yan Y, Wang HX, Lu CY, Xu HC, et al. VSIG4 is highly expressed and correlated with poor prognosis of high-grade glioma patients. Am J Transl Res. 2015;7(6):1172–80.

15 Van Loo P, Tousseyn T, Vanhentenrijk V, Dierickx D, Malecka A, Vanden Bempt I, et al. T-cell/histiocyte-rich large B-cell lymphoma shows transcriptional features suggestive of a tolerogenic host immune response. Haematologica. 2010;95(3):440–8.

16 Ma Y, Usuwanthim K, Munawara U, Quach A, Gorgani NN, Abbott CA, et al. Protein kinase calpha regulates the expression of complement receptor Ig in human monocyte-derived macrophages. J Immunol. 2015;194(6):2855–61.

17 Gorgani NN, Thathaisong U, Mukaro VR, Poungpair O, Tirimacco A, Hii CS, et al. Regulation of CRIg expression and phagocytosis in human macrophages by arachidonate, dexamethasone, and cytokines. Am J Pathol. 2011;179(3):1310–8.

18 Prabakaran S, Lippens G, Steen H, Gunawardena J. Post-translational modification: nature's escape from genetic imprisonment and the basis for dynamic information encoding. Wiley Interdiscip Rev Syst Biol Med. 2012;4(6):565–83.

19 He JQ, Katschke KJ, Jr., Gribling P, Suto E, Lee WP, Diehl L, et al. CRIg mediates early Kupffer cell responses to adenovirus. J Leukoc Biol. 2013;93(2):301–6.

20 Gorgani NN, He JQ, Katschke KJ, Jr., Helmy KY, Xi H, Steffek M, et al. Complement receptor of the Ig superfamily enhances complement-mediated phagocytosis in a subpopulation of tissue resident macrophages. J Immunol. 2008;181(11):7902–8.

21 Kim KH, Choi BK, Song KM, Cha KW, Kim YH, Lee H, et al. CRIg signals induce anti-intracellular bacterial phagosome activity in a chloride intracellular channel 3-dependent manner. Eur J Immunol. 2013;43(3):667–78.

22 van Bruggen R, Zweers D, van Diepen A, van Dissel JT, Roos D, Verhoeven AJ, et al. Complement receptor 3 and Toll-like receptor 4 act sequentially in uptake and intracellular killing of unopsonized Salmonella enterica serovar Typhimurium by human neutrophils. Infect Immun. 2007;75(6):2655–60.

23 Zang X, Allison JP. To be or not to be B7. J Clin Invest. 2006;116(10):2590–3.

24 Jung K, Seo SK, Choi I. Endogenous VSIG4 negatively regulates the helper T cell-mediated antibody response. Immunol Lett. 2015;165(2):78–83.

25 Ahn JH, Lee Y, Jeon C, Lee SJ, Lee BH, Choi KD, et al. Identification of the genes differentially expressed in human dendritic cell subsets by cDNA subtraction and microarray analysis. Blood. 2002;100(5):1742–54.

26 Tanaka M, Nagai T, Usami M, Hasui K, Takao S, Matsuyama T. Phenotypic and functional profiles of CRIg (Z39Ig)-expressing macrophages in the large intestine. Innate immunity. 2011;18(2):258–67.

27 Fu W, Wojtkiewicz G, Weissleder R, Benoist C, Mathis D. Early window of diabetes determinism in NOD mice, dependent on the complement receptor CRIg, identified by noninvasive imaging. Nat Immunol. 2012;13(4):361–8.

28 Lee MY, Kim WJ, Kang YJ, Jung YM, Kang YM, Suk K, et al. Z39Ig is expressed on macrophages and may mediate inflammatory reactions in arthritis and atherosclerosis. J Leukoc Biol. 2006;80(4):922–8.

29 Jung K, Kang M, Park C, Hyun Choi Y, Jeon Y, Park SH, et al. Protective role of V-set and immunoglobulin domain-containing 4 expressed on kupffer cells during immune-mediated liver injury by inducing tolerance of liver T- and natural killer T-cells. Hepatology. 2012;56(5):1838–48.

30 Tanaka M, Nagai T, Tsuneyoshi Y, Sunahara N, Matsuda T, Nakamura T, et al. Expansion of a unique macrophage subset in rheumatoid arthritis synovial lining layer. Clin Exp Immunol. 2008;154(1):38–47.

31 Zheng F, Put S, Bouwens L, Lahoutte T, Matthys P, Muyldermans S, et al. Molecular imaging with macrophage CRIg-targeting nanobodies for early and preclinical diagnosis in a mouse model of rheumatoid arthritis. Journal of nuclear medicine: official publication, Society of Nuclear Medicine. 2014;55(5):824–9.

32 Hodge G, Barnawi J, Jurisevic C, Moffat D, Holmes M, Reynolds PN, et al. Lung cancer is associated with decreased expression of perforin, granzyme B and interferon (IFN)-gamma by infiltrating lung tissue T cells, natural killer (NK) T-like and NK cells. Clin Exp Immunol. 2014;178(1):79–85.

33 Guo S, Yang C, Mei F, Wu S, Luo N, Fei L, et al. Down-regulation of Z39Ig on macrophages by IFN-gamma in patients with chronic HBV infection. Clin Immunol. 2010;136(2):282–91.

34 Zang X, Allison JP. The B7 family and cancer therapy: costimulation and coinhibition. Clinical cancer research: an official journal of the American Association for Cancer Research. 2007;13(18 Pt 1):5271–9.

35 Textoris J, Ivorra D, Ben Amara A, Sabatier F, Menard JP, Heckenroth H, et al. Evaluation of current and new biomarkers in severe preeclampsia: a microarray approach reveals the VSIG4 gene as a potential blood biomarker. PloS one. 2013;8(12):e82638.

36 di Salvo TG, Yang KC, Brittain E, Absi T, Maltais S, Hemnes A. Right ventricular myocardial biomarkers in human heart failure. J Card Fail. 2015;21(5):398–411.

## Figures (large format)

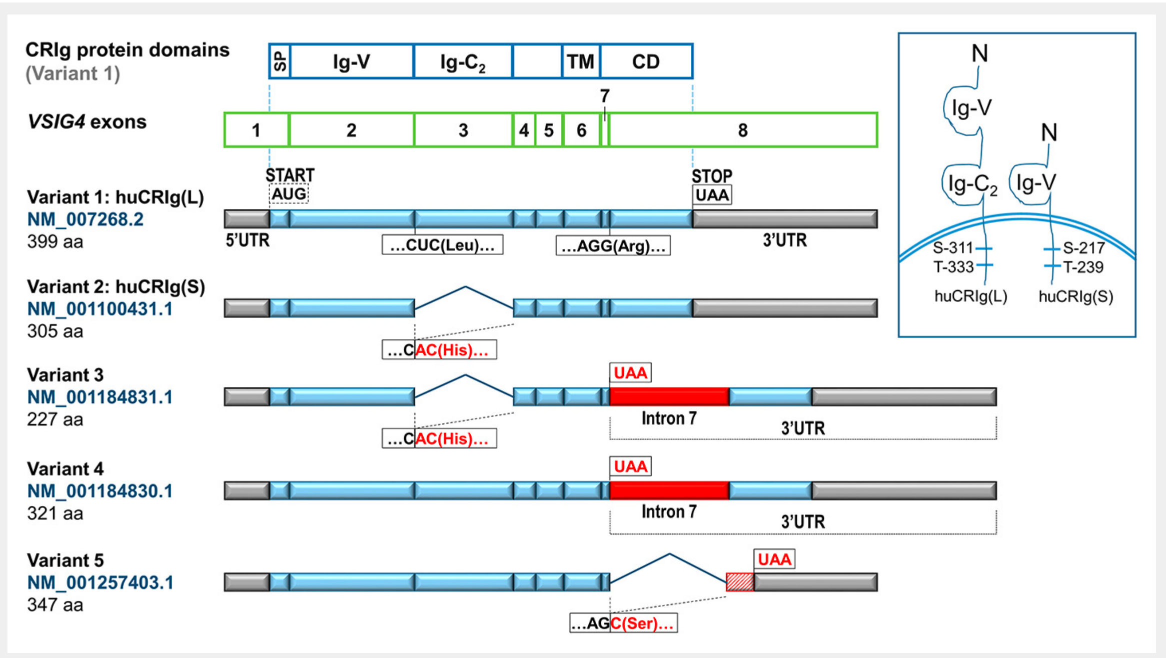


**Figure 1**

Schematic representation of the CRIg protein domains aligned with the five *VSIG4* splice variant transcript structures. The transcripts structures are derived from the NCBI Reference Sequence Database (RefSeq) using the following mRNA accession numbers: NM_007268.2, NM_001100431.1, NM_001184831.1, NM_001184830.1, and NM_001257403.1, as noted in the figure. The structure of the longest CRIg isoform (variant 1, huCRIg(L)) is used as the reference, with the protein domains denoted as: SP, signal peptide; Ig-V, immunoglobulin domain V-type; Ig-$C_2$, immunoglobulin domain V-type; TM, transmembrane region, and CD, cytoplasmic domain. The relative *VSIG4* exon structure reveals that SP, Ig-V, Ig-$C_2$, and TM are encoded specifically by exons 1, 2, 3, and 6, respectively, whereas CD is encoded by exons 7 and 8. The short form (variant 2, huCRIg(S)) differs from the long form by the exclusion of exon 3, the Ig-$C_2$ domain, and a change at codon 138 from CUC (Leucine) to CAC (Histidine). In this isoform, the overall size of the CRIg protein is reduced from 399 to 305 amino acids (aa). Variant 3 features this identical alteration, in addition to the inclusion of intron 7 which creates a stop codon (UAA) adjacent the last codon of exon 7, causing the majority of the CD to be missing (227 aa). Variant 4 features the same loss of the CD as variant 3, but retains all other domains (321 aa). Variant 5 retains the same domains as variants 1 and 4 (exon 1 to 7 inclusive), but is unique from the other four variants in that a portion of exon 8 is excluded, which creates a change at codon 321 (exon 7-8 junction) from AGG (Arginine) to AGC (Serine), followed by a 'frameshift' that results in a stop codon generated 26 aa downstream (347 aa). Insert: shows a schematic representation of the long and short forms of CRIg, expressed in macrophages. The extracellular portion of CRIg contains an Ig-V and/or Ig-$C_2$ domain(s). Putative phosphorylation sites for cAMP/cGMP-dependent protein kinase (S-311 or S-217) and protein kinase C (T-333 or T-239) have been proposed to be present in the cytoplasmic domain of CRIg.

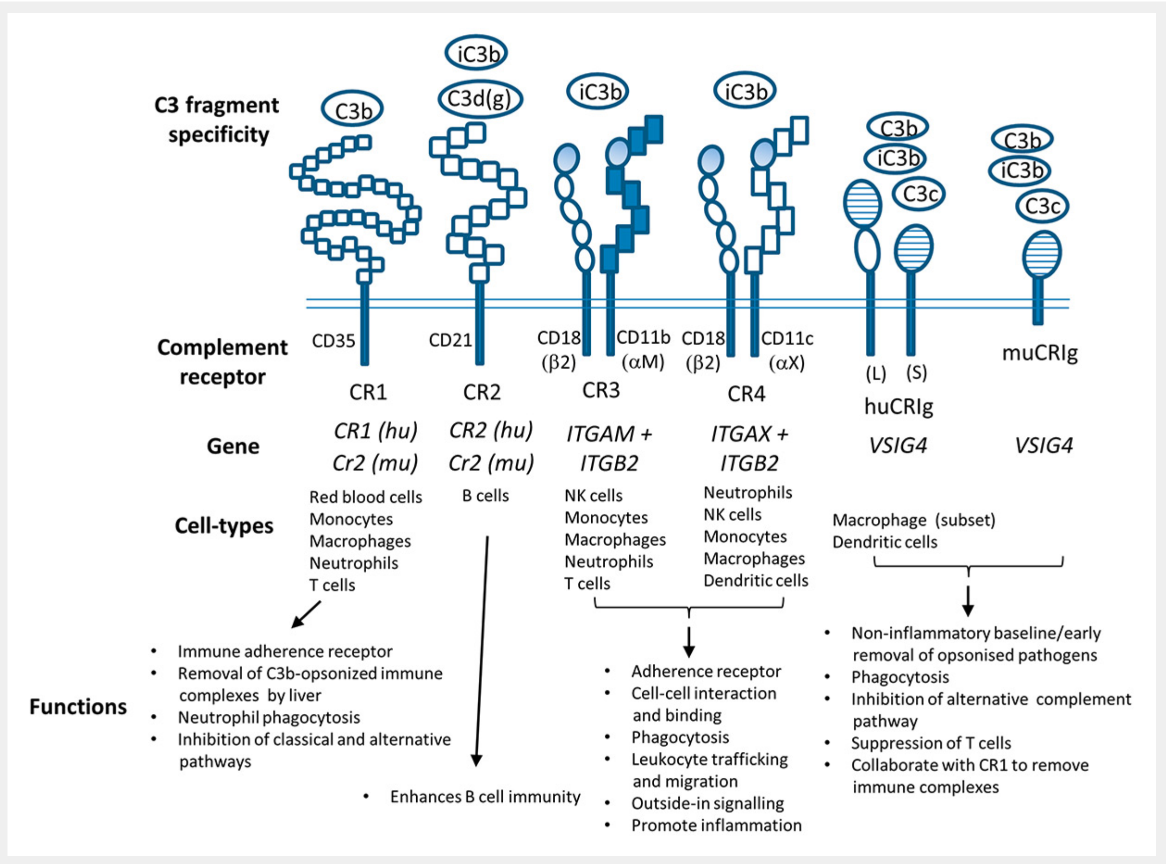


**Figure 2**

Characteristics, function and expression of complement receptors The structural domains of the five known types of complement receptors are depicted, together with their specificity of C3 fragments, the genes encoding them, their distribution amongst the different leucocyte types and their known functions. CR1, CR2 and CRIg are single transmembrane proteins with extracellular portions, transmembrane domains and cytoplasmic tails whereas CR3 and CR4 are transmembrane heterodimers of a common β2 integrin (CD18) chain and an α integrin chain, αM (CD11b) or αX (CD11c). Murine CR1 and CR2 are derived from the same gene by alternative splicing whereas the human counterparts are encoded by 2 different genes. CR1 contains thirty short consensus repeats (SCR) and CR2 has fifteen SCR. CD18 contains four repeats and a Von Willebrand factor type A domain (lightly shaded oblong shape). The α integrins contain, within their extracellular portions, seven FG-GAP repeats (rectangles) and a Von Willebrand factor type A domain. Two human CRIg isoforms, huCRIg(L) for the long form and huCRIg(S) for short, have been described. Both isoforms contain an N-terminal ligand binding domain that belongs to the IgV-type of immunoglobulin domains (horizontal stripes). The long form of CRIg also contains a membrane proximal domain that is an IgC-type immunoglobulin domain. The function of this domain is unclear. The murine form, similar to CRIg(S), contains only the IgV-type of immunoglobulin domain but the cytoplasmic tail is shorter than that of huCRIg(S). The IgV domains are believed to be responsible for binding C3 fragments.

# Chapter 2. Materials and Methods

## 2.1. Ethical Considerations

All research included in this thesis was performed in strict accordance with the ethical standards outlined in The National Statement on Ethical Conduct in Human research (2007), and the Australian Code of Practice for Care and Use of Animals for Scientific Purposes ($8^{th}$ edition, 2013) and South Australian Animal Welfare Act 1985.

Ethics approval was sought and obtained from the Women's and Children's Health Network (WCHN) Human Research Ethics Committee, the WCHN Animal Ethics Committee, and Research Governance Committee;

- HREC/15/WCHN/21
- REC/2165/04/2021
- AE1023/10/2021

## 2.2. Materials

### 2.2.1. Tissue Culture Media

Roswell Park Memorial Institute (RPMI) 1640 tissue culture medium, Dulbecco's Modified Eagle Media (DMEM), foetal calf serum (FCS) and L-glutamine were purchased from SAFC Biosciences (Lenexa, Kansas, USA). Penicillin, streptomycin and Hank's balanced salt solution (HBSS) were obtained from Sigma-Aldrich (St. Louis, MO). Dulbecco's Modified Eagle Medium (DMEM) was purchased from ThermoFisher Scientific (Waltham, MA).

### 2.2.2. Gradients/Cell Separation Media

Ficoll-Paque Plus was purchased from GE Healthcare (Uppsala, Sweden; Little Chalfont, UK), and Percoll PLUS was purchased from Sigma-Aldrich (St. Louis, MO).

### 2.2.3. Cytokines

Recombinant cytokines, (LT)-α (TNF-β), GM-CSF, M-CSF, IL-1β, IL-6, IL-4, TNF-α, IL-13, IFN-γ and IL-10 were purchased from ProSpec-Tany Technogene (Rehovot, Israel), and TGF-β1 was purchased from R&D Systems (Minneapolis, Minnesota, USA).

### 2.2.4. Antibodies

A mouse monoclonal antibody that recognizes the IgV domain of human CRIg (clone 3C9) was kindly provided by Dr. van Lookeren Campagne (Genentech, San Francisco, California, USA). Mouse monoclonal antibodies against CRIg/Z39Ig

(clone 6H8), and CD11b (clone M1/70) were purchased from Santa Cruz Biotechnology (Dallas, Texas, USA). Rabbit polyclonal anti-VSIG4 was purchased from Proteintech (Manchester, UK). The anti- CD11c monoclonal antibody and mouse IgG were purchased from Abcam (Cambridge, UK). Mouse $IgG_1$ isotype phycoerythrin-conjugated antibodies were purchased from eBioscience (San Diego, Ca). Horse-radish peroxidase (HRP)-conjugated rabbit anti-mouse IgG was purchased from Dako, Agilent Technologies (Denmark). A further list of all antibodies used in flow cytometric assays is shown in table 2.2.

### 2.2.5. General Reagents and Biochemicals

Sodium hydroxide, chloroform, and isopropanol were purchased from Ajax Chemicals (Auburn, New South Wales, Australia). Dimethyl sulphoxide (DMSO), absolute ethanol and paraformaldehyde were obtained from Merck (Kilsyth, Victoria, Australia). Bovine Serum Albumin (BSA) was purchased from Bovogen Biologicals (Essendon, Victoria, Australia). Trizma base, trypan blue, β-mercaptoethanol, and ethylenediaminetetraacetic acid (EDTA) were from Sigma-Aldrich. HEPES, Ponceau S, TEMED, DTT, Glycine, Folin and Ciocalteau's phenol reagent were purchased from Sigma-Aldrich. Polyacrylamide was purchased from Bio-Rad (Hercules. CA).

### 2.2.6. Protease and Phosphotase Inhibitors

Benzamidine, leupeptin, pepstatin A, phenylmethylsulfonyl fluoride (PMSF), and Sigma 104 (Phosphotase substrate) were purchased from Sigma-Aldrich and aprotinin from Calbiochem (Merck, Darmstadt, Germany).

### 2.2.7. Cell stimulating agents

Recombinant tumour necrosis factor (TNF) was purchased from ProSpec-Tany Technogene (Rehovot, Israel). Dexamethasone, phorbol myristate acetate (PMA), N-Formyl-Met-Leu-Phe (fMLP), and lipopolysaccharide (LPS) from *Escherichia coli* O127:B8 were purchased from Sigma Aldrich (St. Louis, MO). LTB4 was purchased from Cayman Chemical.

1α,25-dihydroxyvitamin D3 (1,25D) and 25-dihydroxyvitamin D3 (25D) were purchased from Sigma-Aldrich. Stock solutions of 1,25D and 25D were prepared to $10^{-3}$ M in 95% ethanol and stored at -80 °C. The synthetic bacterial lipopeptide Pam3CSK4 was purchased from Invivogen, with stock preparation at 1 mg/mL in endotoxin-free water and storage at -20 °C.

### 2.2.8. Other inhibitors and chelators

The p38 inhibitors SB202190 and SB203580 were purchased from SelleckChem (Houston, TX). The phosphoinositide 3-kinase (PI3K) inhibitor Wortmannin, cytochalasin B, the calcium chelator 1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid (BAPTA-AM), and the Rab27a inhibitor Nexinhib20 were purchased from Sigma-Aldrich (St. Louis, MO). The PKC inhibitor GF109203X was purchased from Biomol Research Laboratories (Plymouth Meeting, PA). Rac-1 inhibitor NSC 23766 and the rac family inhibitor EHT 1864 were purchased from TOCRIS (Bristol, UK).

## 2.3. Purification of Human Monocytes from Whole Blood

Human peripheral blood mononuclear cells (PBMC) were purified from whole blood from healthy volunteers by centrifuging over a medium of Ficoll-Paque PLUS (GE Healthcare) at 600 × *g* for 35 min. After centrifugation, the band containing peripheral blood mononuclear cells (PBMCs) was harvested, and plasma was harvested from above the band. The PBMCs were washed three times with complete media by repeated centrifugation (3 × 5 minutes, 600 × *g*). The cells were then layered over a 46% Percoll gradient (GE Healthcare) and centrifuged for 35 minutes at 600 × *g* with no brake. The single resulting band containing monocytes was harvested and washed a further 3 times (3 × 5 minutes, 600 × *g*). Cells were then counted using a haemocytometer and the viability of the leukocytes as judged by their ability to exclude trypan blue was routinely >99%. Cells were then resuspended in complete media, and $2 \times 10^7$ cells were transferred into a single 22.1 $cm^2$ culture dish. Dishes were then incubated for 1 hour in a 37 °C, 5% $CO_2$, high humidity incubator. Following incubation, the non-adherent cells were carefully removed, leaving purified, adherent monocytes in the dish.

## 2.4. Purification of Human Monocytes from Buffy Coats

Monocytes were isolated from blood buffy coats of healthy donors (Australian Red Cross Blood Service, Adelaide, South Australia). Briefly, 50 mL buffy coats were diluted 1:3 with sterile 1 × PBS by centrifugation on Ficoll-Paque PLUS (GE Healthcare) medium for 35 minutes at 600 × *g*, with no brake. After centrifugation, the leukocytes resolved into two discrete bands with red blood cells at the bottom of the tube. PBMCs consisting of monocytes and lymphocytes were obtained from the top band, and plasma was harvested from above the top band. The upper band was gently aspirated and washed three times with complete media by repeated centrifugation (3 × 5 minutes, 600 × *g*) and re-suspension of the cells. Cells were counted with a haemocytometer and viability of the leukocytes as judged

by their ability to exclude trypan blue was >98%. Cells were then resuspended in 28 mL fresh complete media, and 7 mL of cell suspension were transferred into each 150 mm culture dish. Dishes were then left to incubate for 1 hour in a 37°C, 5% $CO_2$, high humidity incubator. Following incubation, the non-adherent cells were carefully removed, leaving purified, adherent monocytes in the dish.

### 2.5. Isolation of Human Neutrophils from Whole Blood

Healthy human donor neutrophils were isolated from peripheral blood by the rapid single-step technique (Ferrante & Thong, 1982). Briefly, blood was layered onto Hypaque-Ficoll, $d = 1.114$ and centrifuged at 600 × g for 35 minutes with no brake. After centrifugation, the leukocytes resolved into two discrete bands; an upper PBMC-containing band and the neutrophil-containing band below. Neutrophils were carefully aspirated and washed with complete media (600 × g, 5 minutes). Cells were then counted using a haemocytometer and viability judged by their ability to exclude typan blue. Cell preparations were routinely >99% viable and >98% pure.

### 2.6. Preparation of Human Monocyte-Derived Macrophages (MDM)

PBMCs at $2 \times 10^7$ cells in 4 mL of complete media were added to 6 cm tissue culture petri dishes and incubated at 37 ° C in a high humidity, 5% $CO_2$ atmosphere for one hour. After incubation, the non-adherent cells were removed by gentle pipetting, and 4 mL of fresh complete media was added to each plate. The dishes were incubated for 3 days before being used for RNA extraction, or 5 days for protein analysis. Media was changed every second day.

### 2.7. Preparation of Human Monocyte-Derived Dendritic Cells (MDDC)

PBMCs at $2 \times 10^7$ cells in 4 mL of complete media were added to 6 cm tissue culture petri dishes and incubated at 37 ° C in a high humidity, 5% $CO_2$ atmosphere for one hour. The non-adherent cells were removed by gentle pipetting, and 4 mL of fresh complete media was added to each dish. The cells in the dishes were then supplemented with GM-CSF at a final concentration of 50 ng/ml and IL-4 at a concentration of 20 ng/ml, and the cells were incubated at 37 ºC under an atmosphere of 5% $CO_2$/air and high humidity over a period of 5 days (for harvesting RNA) or for 7 days (for harvesting protein).

### 2.8. Cryopreservation of Cells

For cryopreservation studies, freshly counted cells of interest were cryopreserved at $1 \times 10^7$ cells/mL in freezing media containing 90% heat-inactivated FCS and 10% DMSO. Cells were incubated in a 'Mr. Frosty' Freezing Container (Thermo-Fisher Scientific, Scoresby,

Australia) overnight in a -80 °C freezer, before being transferred into liquid nitrogen ($LN_2$) for storage. Prior to use, vials were removed from $LN_2$ and thawed rapidly at 37 °C before washing in RPMI-1640. Cells were then re-counted, and viability assessed by the trypan blue-exclusion method prior to application.

## 2.9. DC-T Cell Co-cultures

Autologous DC and T cell co-cultures were set up using MDDCs as prepared above, with autologous T cells purified from the remaining lymphocyte fraction following the centrifugation of PBMC over 46% iso-osmotic Percoll gradient. T cells were enriched from the lymphocyte fractions by subjecting the cell preparation through two cycles of nylon wool (Polysciences Inc.,Warrington, PA) columns. T cell preparations were routinely >95% pure and >99% viable as determined by FACS analysis (judged by CD45 and CD3 expression) and trypan blue dye exclusion assay respectively, and T cells were cryopreserved in $LN_2$ as above until use. MDDCs were added to 96-well round-bottom plates at $1 \times 10^4$ cells/well and stimulated with 50 ng/mL dexamethasone for 24 hours and washed. The cryopreserved T cells were thawed and added to the autologous MDDC ($2 \times 10^5$ T cells/well). Phytohemagglutinin (PHA) was used as a stimulus in the appropriate wells (0.5 μg/well) (Remel Inc., San Diego, CA), with or without either anti-CRIg (clone 6H8) antibody or isotype control. The mixed lymphocyte reactions were then cultured at 37 °C in an atmosphere of 95% air and 5% $CO_2$ for 72 hours. Following incubation, cells were pulsed with 1 μCi methyl-3H Thymidine (3H-TdR) (PerkinElmer, Waltham, MA) 6 hours prior to harvest. Incorporation of 3H-TdR was then measured as disintegrations per minute (DPM) using a Wallac 1409 liquid scintillation beta counter (Wallac, Turklo, Finland).

In the case of experiments where allogenic T cells were used, allogeneic T cells were isolated from fresh or cryopreserved PBMCs using the EasySep™ Human T Cell Isolation Kit (Stem Cell Technologies, Vancouver, Canada), and added to allogeneic DCs as the stimulus in a DC:T cell ratio of 1:10 as $2 \times 10^5$ total cells/well, with or without anti-CRIg antibody or isotype control. DCs were untreated or dexamethasone treated DC at $2 \times 10^4$ cells/well in 96-well round-bottom plates. Cells were cultured at 37 °C in an atmosphere of 95% air and 5% $CO_2$ for 120 hours and pulsed with 3H-TdR 6 hours prior to harvest. At harvest, culture supernatants were harvested and stored at -80 °C for later quantification of cytokines, followed by measurement of the remaining cells for 3H-TdR incorporation.

## 2.10.Measurement of Cytokines

Cytokines in the culture supernatants were quantitated using BD™ Cytometric Bead Array kits for IFN-γ, TNF-α, IL-13, TGF-β1, IL-4, and IL-10 (BD Biosciences). The

manufacturer's protocols were adapted for assay in 96-well v-bottom plates, and data was acquired using a BD FACS Canto I with an attached BD™ High Throughput Sampler, and analysis with FCAP Array v3 software (BD Biosciences).

## 2.11. Analysis of CRIg Expression at the mRNA Level

### 2.11.1. Isolation of Total RNA

RNA was extracted from cells by using the TRIzol reagent (Life Technologies) according to the manufacturer's instructions. Briefly, adherent cells were detached from culture plate by gentle scraping using a 'rubber policeman' and pelleted by centrifuging for 5 minutes at 600 × *g*. Non-adherent cells were collected and pelleted as above. The supernatant was discarded, and cell pellet resuspended in 500 µL TRIzol reagent. To the samples, 100 µL of chloroform was added, and tubes was shaken vigorously by hand for 15 seconds. The samples were incubated at room temperature for 2-3 minutes, and then the tubes were centrifuged at 12,000 × *g* for 15 minutes at 4 °C. The resulting clear, aqueous phase was harvested and transferred into a new 1.5 mL tube. To these collected fractions, 250 µL of 100% isopropanol was added and all samples were incubated for 10 minutes at room temperature, before centrifuging again at 12,000 × *g* for 10 minutes. The supernatant was removed, and the pellet was washed with 500 µL 75% ethanol (7500 × *g* for 5 minutes). The supernatant was discarded, and the pellet air-dried before being resuspended in 30 µL RNAse-free water. The RNA sample was heated to 50 °C for 15 minutes before quality and purity analysis using a NanoDrop. RNA was harvested from macrophages at day three of culture, from DCs at day five of culture, or immediately after separation from monocytes and neutrophils.

### 2.11.2. Generation of cDNA

Following RNA quantification on the NanoDrop, a BioRad iScript™ cDNA synthesis kit was used to generate cDNA, following the manufacturer's instructions (Bio-Rad Laboratories). Briefly, approximately 300 ng of total RNA, 4 µL of 5 × Reaction Mix, 1 µL of iScript, and water up to 20 µL total volumes. The mixture was heated at 25 °C for 5 minutes, 42 °C for 30 minutes, and 85 °C for 5 minutes and then stored at -20 °C for further use in PCR.

### 2.11.3. Primer Design

Primers used for detection and amplification of the *VSIG4* transcript variants, *CR1*, total *VSIG4*, *GAPDH*, *ITGAM* (CD11b), and *ITGAX* (CD11c) are as previously

published (Munawara et al., 2017). A full list of the primer sequences and combinations are detailed in table 2.2.

### 2.11.4. Standard PCR

Forward and reverse primers (0.1 µM) were added to a reaction mixture containing 1 × AmpliTaq Gold® 360 Master Mix. Approximately 300 ng of cDNA was then added to each reaction. The PCR was then run using a BioRad MyCycler (Bio-Rad Laboratories) at the following conditions: 95 °C for 5 minutes, followed by 40 cycles of 95 °C for 30 seconds, 60 °C for 30 seconds, 72 °C for 30 seconds, finishing with 72 °C for 5 minutes. The PCR products were then kept at room temperature for either analysis by gel electrophoresis or purification.

### 2.11.5. Agarose Gel Electrophoresis of PCR Products

To 5 µL of each PCR product, 1.5 µL of loading buffer was added before being loaded onto a 2% agarose gel containing 1.5 µL GelRed to stain the DNA, and electrophoresed at 70 V in 1 × SB buffer. To determine product size, 1kb Plus DNA Ladder (Invitrogen) was loaded onto each gel. DNA in the gel was visualised using a BioRad UV transilluminator in a Chemidoc XRS+ imaging system, and the results were analysed using Image Lab Software version 3.0 (BioRad).

### 2.11.6. Quantitative Real Time PCR (SYBR Green)

Expression levels of *VSIG4* (CRIg), *CR1*, *ITGAM* (CD11b), and *ITGAX* (CD11c) were quantitively assessed using the primer pairs outlined in table 2.1. *GAPDH* mRNA was quantitated and utilised for data normalisation. Each reaction was performed in triplicate and had a final volume of 20 µL containing 100 nM of each primer, 1 µL of cDNA, and iQ SYBR Green Supermix (Bio-Rad Laboratories). Reactions were assayed in an iQ5 Real Time Detection System with iQ5 Optical System v2.1 software (Bio-Rad Laboratories), with thermal cycling performed with an initial denaturation at 95 °C for 5 minutes, followed by 40 cycles of 95 °C for 30 seconds, 60 °C for 30 seconds, and 72 °C for 30 seconds.

*Table 2.1. Full list of primer sequences used in this study. Direction is indicated (F-forward; R-reverse), and in the case of CRIg transcript-specific primers, pairings are indicated along with the transcript each pair is specific for.*

| Gene/Primer | Sequence (5' to 3') | Pairing | Transcript No. |
|---|---|---|---|
| *VSIG4 (transcripts)* | | | |
| F1 | TTTGTGGTCAAAGACTCCTCAAAGC | F1 and R1 | **1** |
| F2 | TGTCCAGAAACACTCCTCAAAGCT | F2 and R1 | **2** |
| R1 | TGGCATGTGCCCTGGCT | F2 and R2 | **3** |
| R2 | GAGAGACTTTCTTACCTGGCTGCTT | F1 and R2 | **4** |
| R3 | GACACTTTGGGCTGGCTGCT | F1 and R3 | **5** |
| | | F2 and R3 | **6** |
| *CR1* | | | |
| F | CCCTTTGGAAAAGCAGTAAA | | |
| R | TCAACTTGGCAAACAGAAAA | | |
| *VSIG4 (total)* | | | |
| F | ACACTTATGGCCGTCCCAT | | |
| R | TGTACCAGCCACTTCACCAA | | |
| *GAPDH* | | | |
| F | GAGTCAACGGATTTGGTCGT | | |
| R | GACAAGCTTCCCGTTCTCAGCCT | | |
| *ITGAM* (CD11b) | | | |
| F | CCTGGTGTTCTTGGTGCCC | | |
| R | TCCTTGGTGTGGCACGTACTC | | |
| *ITGAX* (CD11c) | | | |
| F | CCGATTGTTCCATGCCTCAT | | |
| R | AACCCCAATTGCATAGCGG | | |

## 2.12. Analysis of CRIg Expression by Flow Cytometry

All protein analysis by flow cytometry was performed in 5 mL round bottom polystyrene FACS tubes (BD, Franklin Lakes, NJ). Cells (between 1-2.5 × $10^5$ depending on the assay) were aliquoted into FACS tubes and blocked for non-specific antibody binding for 10 minutes on ice with 100 µg human IgG (Kiovig, Baxter, Old Toongabbie, NSW, Australia). Cells were then stained for 20 minutes with the appropriate antibody cocktail in the dark on ice. Complete lists of antibodies and the cocktails used are outlined in table 2.2. Cells were washed in 2 mL of PBS supplemented with 5% FCS at 500 × *g* for 5 minutes prior to analysis. In cases where cells were not immediately acquired, cells were fixed in 4% formaldehyde and stored at 4 °C.

In cases where secondary antibodies were required, cells were washed as above prior to incubation with the appropriate secondary antibody for 20 minutes in the dark on ice. Then, cells were washed again prior to analysis.

For experiments requiring intracellular staining, surface antigens were stained as above before fixation and permeabilization with the BD Fixation/Permeabilization kit (BD Biosciences) as per the manufacturer's instructions. Intracellular antigens were immunostained in the presence of BD Perm/Wash™ Buffer to maintain permeabilization for 20 minutes in the dark on ice. Following staining, cells were washed twice in 2 mL of BD Perm/Wash™ Buffer prior to analysis.

Data acquisition was carried out using a BD FACS Canto I and data analysed using FlowJo 10.1 software (FlowJo, LLC, Ashland, Oregon). All gates were set using fluorescence-minus-one controls (FMO), and fluorescence intensities determined by subtracting isotype control and unstained control fluorescence values

*Table 2.2. Full list of antibodies used in this study for flow cytometric purposes, their conjugates, concentrations, sources, and clone numbers. In the case of polyclonal antibodies, the commercial product number is listed.*

| Antibody | Conjugate | Concentration | Source | Clone/product number |
|---|---|---|---|---|
| **Anti-human primary antibodies** | | | | |
| CD45 | APC-H7 | 100 μg/ mL | BD Biosciences | 2D1 |
| CD14 | FITC | 20 μL per test | BD Biosciences | M5E2 |
| CD3 | PE-Cy5 | 20 μL per test | BD Biosciences | UCHT1 |
| CD20 | APC | 40 μg/mL | BD Biosciences | L27 |
| Z39Ig (CRIg) | PE | 50 μg/mL | Santa Cruz | 6H8 |
| CD11b | PE | 500 μg/mL | BD Biosciences | M1/70 |
| CD11c | FITC | 5 μL per test | BD Biosciences | B-ly6 |
| CRIg | Unconjugated | 1 mg/mL | Genentech | 3C9 |
| CRIg | Unconjugated | 1 mg/mL | Generated in house | 14B11 |
| **Anti-mouse primary antibodies** | | | | |
| B220 | APC | 200 μg/mL | BD Biosciences | RA3-6B2 |
| CD11b | PE | 500 μg/mL | BD Biosciences | M1/70 |
| CRIg | PE | 1 mg/mL | Genentech | 14G6 |
| CRIg | Unconjugated | 1 mg/mL | Generated in house | 14B11 |
| **Isotype controls** | | | | |
| Mouse IgG1 κ | PE | 50 μg/mL | BD Biosciences | MOPC-21 |
| Mouse IgG1 κ | FITC | 50 μg/mL | BD Biosciences | MOPC-21 |
| Mouse IgG1 κ | Unconjugated | 500 μg/mL | BD Biosciences | MOPC-21 |
| Rat IgG2a κ | Unconjugated | 500 μg/mL | BD Biosciences | R35-95 |
| **Secondary antibodies** | | | | |
| Goat anti-mouse | PE | 400 μg/mL | Santa Cruz | sc-3738 |
| Goat anti-rat | FITC | 500 μg/mL | BD Biosciences | 554016 |

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## 2.13. Analysis of CRIg Expression by Western Blot

### 2.13.1. Sample Preparation

Cells in suspension were counted with a haemocytometer, and centrifuged at 600 × *g* for 5 min at 4 °C. After aspirating the supernatant, the cell pellet was resuspended and washed in 1 × PBS, before being resuspended in Lysis buffer at 30 µL per 1 × $10^6$ cells. For adherent cells, the culture supernatant was removed, and Lysis buffer was added directly to the cells with detachment assisted with a cell scraper. Lysis preparations were transferred to 1.5 mL screw top tubes, sealed and incubated under ice with rocking for 30 min. These tubes were centrifuged at 3,500 × *g* for 5 min at 4 °C and the supernatants (cell lysates) were harvested and stored at -20 °C until analysis.

### 2.13.2. Protein Quantitation

#### 2.13.2.1. Qubit Protein Assay

Concentration of the protein lysates were determined using the Qubit® Protein Assay kit according to the manufacturer's instructions (Invitrogen). Briefly, 1 µL of each cell lysate was mixed with 199 µL of Qubit® Protein Reagent diluted 1:200 with Qubit® Protein Assay Buffer in 0.5 mL tubes. Samples were then incubated for 15 minutes at room temperature, before fluorometric analysis on the Qubit® 3.0 (Invitrogen). Measurements were compared with standards supplied with the kit.

#### 2.13.2.2. Lowry's Protein Assay

The protein content of the cell lysates was quantitated by the method described by Lowry (Lowry et al., 1951). BSA protein standards (0, 3.125, 6.25, 12.5, 25 and 50 µg) were prepared for each assay by serially diluting 1% BSA (1 mg/ml in PBS) with $H_2O$, while samples of cell lysates were diluted 1:10 for quantitation. To each 50 µL preparation of standards and diluted protein lysate sample, 150 µL of Lowry's solution was added. All samples were incubated for 20 minutes at room temperature. Following incubation, 15 µL of 50% Folin and Ciocalteau's Phenol Reagent (diluted in water) was added. Following 20 minutes of incubation at room temperature, 180 µL of each standard and sample was transferred into a 96 well flat-bottomed plate (Nunc, Roskilde, Denmark) and the optical density at 540 nm was measured using a Dynatech MR 5000 plate reader (Dynatech Laboratories, Alexandria, VA). A standard curve was generated from the protein standards, enabling the concentration of protein in each sample to be determined.

#### 2.13.3. Western Blot

Following protein quantification, lysates were diluted at 2:1 in 3 x Laemmli buffer supplemented with 10% β-mercaptoethanol. Samples were boiled at 100 °C for 5 minutes, and 50-100 µg of total protein was subjected to 10% SDS-PAGE at 175 V for approximately 1 hour. The separated proteins were electrophoretically transferred to nitrocellulose using the TransBlot®-Turbo™ Transfer System (Bio-Rad), as per the manufacturer's instructions. To monitor the extent of protein transfer, the membrane was stained with 0.1 % Ponceau stain (in 5 % acetic acid). The membrane was blocked using either 5 % skim milk in 1 × TBST, or 3 % BSA in 1 × TBST for phospho-proteins. After blocking, the membrane was incubated with primary antibody diluted in the blocking agent for either an hour at room temperature or overnight at 4 °C. The membrane was washed in blocking solution (3 × 5 minutes), and then incubated in HRP-conjugated secondary antibody for either an hour room temperature or overnight at 4°C. Immunoreactive material present on the membrane was detected by enhanced chemiluminescence according to the manufacturer's instructions (Western Lightning chemiluminescence, Perkin Elmer, Waltham, MA). The protein bands on the membrane were then visualised by a ChemiDoc XRS+ Imaging System, and quantitated using ImageLab™ Software, Version 3.0 (Bio-Rad Laboratories, Hercules, CA).

## 2.14. Chemiluminescence Assay

Luminol-dependent chemiluminescence assay was performed as previously described (Kumaratilake & Ferrante, 1992). Briefly, $1x10^6$ neutrophils were added to 125 µg lucigenin (bis-N-methylacridinium nitrate, Sigma Aldrich) in 500 µL HBSS prior to the addition of the respiratory burst-inducing agent. Oxidative burst was measured as chemiluminescence using an LB 953 Autolumat Plus luminometer (Berthold Technologies), and peak fluorescence recorded. Data is expressed as mean relative luminescence units (RLU).

## 2.15. Phagocytosis Assays

### 2.15.1. Flow Cytometric Phagocytosis Assay

Neutrophil and macrophage phagocytosis was measured using *Staphylococcus aureus* pHrodo™ Red Bioparticles™ (Invitrogen) as previously described (Annabelle Small et al., 2018). Briefly, $1 \times 10^6$ cultured macrophages or isolated neutrophils in HBSS were combined with 8% human AB serum, and 80 µg pHrodo™ Red *S. aureus* Bioparticles™ (Invitrogen), in a final volume of 400 µL in 5 mL polystyrene round bottom tubes with caps BD, Franklin Lakes, NJ). These were

gassed with 5% $CO_2$/air and capped before incubation at 37 °C for 1 hour for macrophages, or 15 minutes for neutrophils. To stop the reactions, 2 mL of ice-cold HBSS was added to each tube and cells were washed (500 × *g*, 5 minutes). Then, samples were analysed using a BD FACSCanto I flow cytometer, and resulting data analysed using FlowJo 10.1 software (FlowJo LLC) to determine bioparticle uptake by changes in median fluorescence intensity in the PE channel.

### 2.15.2. Phagocytosis Assay by Microscopy Analysis

This phagocytosis assay was performed as described previously (Gorgani et al., 2011; Munawara et al., 2017). Briefly, $1 \times 10^5$ *C. albicans* yeast particles were combined with $5 \times 10^4$ macrophages in a final volume of 0.5 mL HBSS. Complement-containing human AB serum was added to a final concentration of 10%. The cells were incubated for 1 hour at 37 °C on a rocking platform. Following incubation, unphagocytosed yeast particles were removed by differential centrifugation at 175 × *g* for 5 minutes, and the remaining macrophages in the pellet were cytocentrifuged onto a microscope slide and stained with Giemsa. The particles in phagocytic vacuoles were enumerated. Phagocytosis was scored as both the number of macrophages that had engulfed >4 fungi as well as the number of fungi engulfed per cell.

## 2.16. Generation of anti-murine CRIg Hybridomas and Culture Supernatant

### 2.16.1. Peptide selection

A 168 amino acid peptide, corresponding to N-terminal amino acids 20-187 of murine Vsig4 was selected as immunogen;

HPTLKTPESVTGTWKGDVKIQCIYDPLRGYRQVLVKWLVRHGSDSVTIFLR
DSTGDHIQQAKYRGRLKVSHKVPGDVSLQINTLQMDDRNHYTCEVTWQT
PDGNQVIRDKIIELRVRKYNPPRINTEAPTTLHSSLEATTIMSSTSDLTTNGT
GKLEETIAGSGRNLP

Amino acids 20-137 have 79.67% homology to the region of human CRIg encoded by *VSIG4* exon 2 (Basic local alignment search tool (Altschul et al., 1990).

### 2.16.2. Rat immunisation and hybridoma generation

Rats were immunised with the recombinant protein of the sequence above generated through the *E. coli* expression system and hybridomas were generated through a contract with GenScript (GenScript; Piscataway, NJ). Customised antibody specificity was assessed using ELISA followed by Western blot.

### 2.16.3. Hybridoma Cell Culture

Rat hybridoma cell lines received from GenScript (Piscataway, NJ) were maintained at a concentration < $1 \times 10^6$ cells/mL in DMEM supplemented with 1% FCS, 100 U/mL penicillin, and 0.1 mg/mL streptomycin in a 37 °C, 5% $CO_2$, high humidity incubator. The cell lines were routinely tested for mycoplasma by PCR.

### 2.16.4. Hybridoma Supernatant Harvesting and Concentration

Twice a week, all cells were removed from the culture flask and centrifuged at 500 × *g* for 5 minutes. Supernatant was removed and retained while the pellet was resuspended in 5 mL of residual supernatant. Resuspended cells were returned to the culture flask and topped up to 50 mL with fresh DMEM supplemented with 1% FCS, 100 U/mL penicillin, and 0.1 mg/mL streptomycin, and returned to culture in a 37 °C, 5% $CO_2$, high humidity incubator. The harvested hybridoma supernatant was filter sterilised using a 0.2 µm filter and supplemented with 0.05% sodium azide. Supernatant was stored at 4 °C for short term storage and at -20 °C for long term storage.

### 2.16.5. Antibody Purification

Generated monoclonal antibodies were purified from culture supernatant using anti-rat IgG conjugated agarose beads (Abcam, Cambridge, UK). Briefly, to 100 µL of culture supernatant, 50 µL of anti-rat conjugated agarose slurry was added. Tubes were incubated at 4 ° C on a rocking platform for 1 hour before they were centrifuged at 200 × *g* for 1 minute. The supernatant was discarded, and agarose conjugates were washed twice in Tris buffered saline (TBS; 50 mM Tris pH 7.5, 150 mM NaCl) before a final wash in 0.5 M Tris, pH 6.8. Isolated antibody was then eluted from agarose complexes by incubation for 10 minutes with glycine (pH 2.6) at 1:1 with frequent agitation prior to centrifugation at 200 × *g* for 1 minute. The eluate was harvested and neutralised by adding an equal volume of Tris (pH 8.0). The elution steps were repeated, and eluates pooled. Antibody concentration was determined using the Qubit® protein assay as per 2.10.2. and purification protocol validated using Western blot as per 2.10.4.

## 2.17. Immunohistochemistry

Paraffin embedded murine paw or human synovial tissue sections were prepared for histologic analysis using standard protocols (Wang et al., 2016). Briefly, paraffin embedded tissue serial sections were deparaffinised by incubating in xylene for 10 minutes, followed

by a further 10 minutes in xylene, 5 minutes in 100% ethanol, and 5 minutes in 95% ethanol. Slides were then washed twice in MilliQ water (2 × 5 minutes) followed by a five-minute wash in PBS. Antigen retrieval was performed by covering the entirety of the section in Proteinase K (PK) at a dilution of 1/50 and incubating at 37 °C for 30 minutes in a high humidity atmosphere. Alternately, antigen retrieval was performed using antigen retrieval solution (1mM EDTA/10mM Tris pH9) heated to 95-100 °C. Following antigen retrieval, slides were washed three times in PBS (3 × 5 minutes) before endogenous peroxidase (EP) blocking in 0.3% hydrogen peroxide in sodium azide. Following three washes in PBS (3 × 5 minutes), non-specific antibody binding was blocked using 20% donkey serum diluted in PBS (Jackson) for 30 minutes at room temperature. Sections were subsequently stained with rat anti-murine CRIg (clone 14B11, neat), followed by rabbit anti-rat secondary antibody (Dako, P0450, 1:100) and horseradish peroxidase-conjugated swine anti-rabbit tertiary antibody (Dako, P0399, 1:100). Staining was visualised using aminoethyl carbazole (AEC) (10-minute incubation at room temperature in the dark). Slides were then counterstained with haematoxylin before mounting in Aquamount (ThermoFisher, Waltham, MA). Slides were analysed and images taken using an Olympus BX51 and Olympus AnalySIS Life Science Starter software (Shinjuku, Tokyo, Japan).

## 2.18. Isolation of Murine Peritoneal Exudate Cells

Peritoneal macrophages were harvested from male swiss white laboratory mice using peritoneal washouts after the mice were euthanised by $CO_2$ asphyxiation. Briefly, the peritoneal cavity was injected with 3 mL of RPMI-1640 medium and gently massaged, before the cell-containing fluid was withdrawn. Harvested cells were washed in complete cell culture media consisting of RPMI-1640, 10% heat-inactivated FCS, penicillin/streptomycin, and L-glutamine, prior to processing for flow cytometric analysis.

## 2.19. Isolation of Murine Synovial Cells

Synovial cells were isolated using the published method as described by Misharin et al. (2014). Briefly, from routinely culled healthy BALB/c male mice, legs were removed, skinned and ankles cut 3 mm above the heel. Bone marrow was flushed with HBSS and toes disarticulated by pulling with forceps. Tibiotalar joints were opened by posterior access, exposing the synovial lining. Feet were then incubated in digestion buffer comprising of 2 mg/mL dispase II, 2 mg/mL collagenase D and 1 mg/mL DNase I in HBSS for 1 hour at 37 °C on a rocking platform. Released mononuclear cells were purified by density gradient centrifugation (Ficoll-Paque PLUS, GE Healthcare), and macrophages were isolated by adherence to 6 cm plastic culture dishes for 1 hour at 37 °C and non-adherent cells removed

by gentle washing in complete culture media. Enriched macrophages were removed from the plates by a combination of gentle pipetting and scraping with a ‘rubber policeman’ cell scraper and subjected to flow cytometric analysis as described above.

### 2.20. Statistical Analysis

Statistical significance was calculated using GraphPad Prism 8.0.0 (GraphPad Software, Inc., La Jolla, CA, USA). To compare the control response to multiple groups, a two-way ANOVA or one-way ANOVA followed by Dunnett’s Multiple Comparison test was performed. A paired or unpaired two-tailed Student’s *t*-test was used to compare the means of two groups with matched or unmatched responses, respectively. A value of $p < 0.05$ was considered significant.

## 2.21. Publication: 'Facilitating THP-1 macrophage studies by differentiating and investigating cell functions in polystyrene test tubes'

### 2.21.1. Introduction and Contextual Statement

This section of the chapter presents the second published manuscript to be included within this thesis, which outlines the basic method for differentiating the THP-1 human monocytic cell line in polystyrene test tubes suitable for downstream usage in flow cytometry, thus avoiding the necessity of detachment processes which may lead to poor cell yield. The article also includes the methods utilised for the flow cytometric phagocytosis assay and surface immunostaining protocols which were utilised in other chapters of this thesis.

The following methods article entitled *'Facilitating THP-1 macrophage studies by differentiating and investigating cell functions in polystyrene test tubes'*, by **Annabelle Small**, Nikki Lansdown, Marwah Al-Baghdadi, Alex Quach, and Antonio Ferrante was published in the peer reviewed journal, The Journal of Immunological Methods, in October 2018 (doi: 10.1016/j.jim.2018.06.019).

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# Statement of Authorship

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## Principal Author

| Name of Principal Author (Candidate) | Annabelle Small | | |
|---|---|---|---|
| Contribution to the Paper | AS conducted the experiments, interpreted data, compiled the figures and wrote the initial draft of the manuscript. | | |
| Overall percentage (%) | 60% | | |
| Certification: | This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper. | | |
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## Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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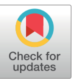
Research paper

# Facilitating THP-1 macrophage studies by differentiating and investigating cell functions in polystyrene test tubes



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ABSTRACT

Macrophage cell lines are a useful model to explore the properties of primary macrophages. However, a major limitation in the use of these cells is that when they are differentiated, they become adherent and hence present with the same limitation as natural macrophages. The cells need to be detached and are often subjected to detachment techniques such as detachment buffers containing proteolytic enzymes or scraping with a rubber 'policeman'. These steps are time-consuming, reduce cell yields as well as cell viability and function. We have therefore investigated the possibility of differentiating the human macrophage THP-1 cell line in polystyrene FACS tubes to enable cells to be directly used for investigations by flow cytometry. Here we demonstrate that when the human macrophage cell line THP-1 are cultured in FACS tubes with phorbol myristate acetate added, they undergo differentiation into macrophages, assessed morphologically and by autofluorescence expression, in a similar manner to those cultured in tissue culture dishes. The cells can be readily washed and adjusted in concentration by centrifugation in the same tubes and can be directly tested for expression of cell surface markers and function by flow cytometry. This avoids the use of either detachment reagents or physical cell scraping. Consequently, we showed that the tube culture method results in increased cell yield and viability compared to those subjected to detachment procedures. The tube method generated functional macrophages which expressed the complement receptors, CR3 and CR4, and effectively phagocytosed complement opsonised *Staphylococcus aureus* via these receptors.

## 1. Introduction

THP-1 cells are a human monocytic cell line derived from a childhood M5 subtype of acute monocytic leukaemia (Tsuchiya et al., 1980). Since their establishment, these cells have been extensively used as a model to study monocyte and macrophage function (Bosshart and Heinzelmann, 2016). However, like primary macrophages, THP-1 differentiated cells are highly adherent (Lund et al., 2016), a characteristic which hinders the ability to use the cells in investigations. In order to overcome this issue, multiple techniques to detach these cells have been established, including the use of buffers containing trypsin, commercial buffers such as Accutase, EDTA containing buffers, and physically scraping cells using rubber cell scrapers (Chen et al., 2015). However, these buffers can be expensive, and the use of trypsin has been known to cleave cell surface proteins (Zhang et al., 2012), resulting in changes to cell function. In addition, cell scraping and the use of EDTA buffers often result in low cell yields with low viability due to damage to the cell structure (Van Veldhoven and Bell, 1988). Thus, there remains a need for new approaches to handling adherent cell types such as THP-1 derived macrophages.

Here, we describe a method for differentiating functional THP-1 macrophages using 5 mL polystyrene FACS tubes; a method which is cheaper, provides higher cell yields, and higher cell viability compared to the classical culture dishes.

## 2. Materials and methods

### 2.1. Reagents and antibodies

RPMI 1640 tissue culture medium, foetal calf serum (FCS) and L-glutamine were purchased from SAFC Biosciences (Lenexa, KS). Phorbol myristate acetate (PMA) was purchased from Sigma Aldrich (St. Louis, MO). PE-conjugated CD11b (clone 2LPM19c) and FITC-conjugated CD11c (clone KB90) antibodies were purchased from Dako

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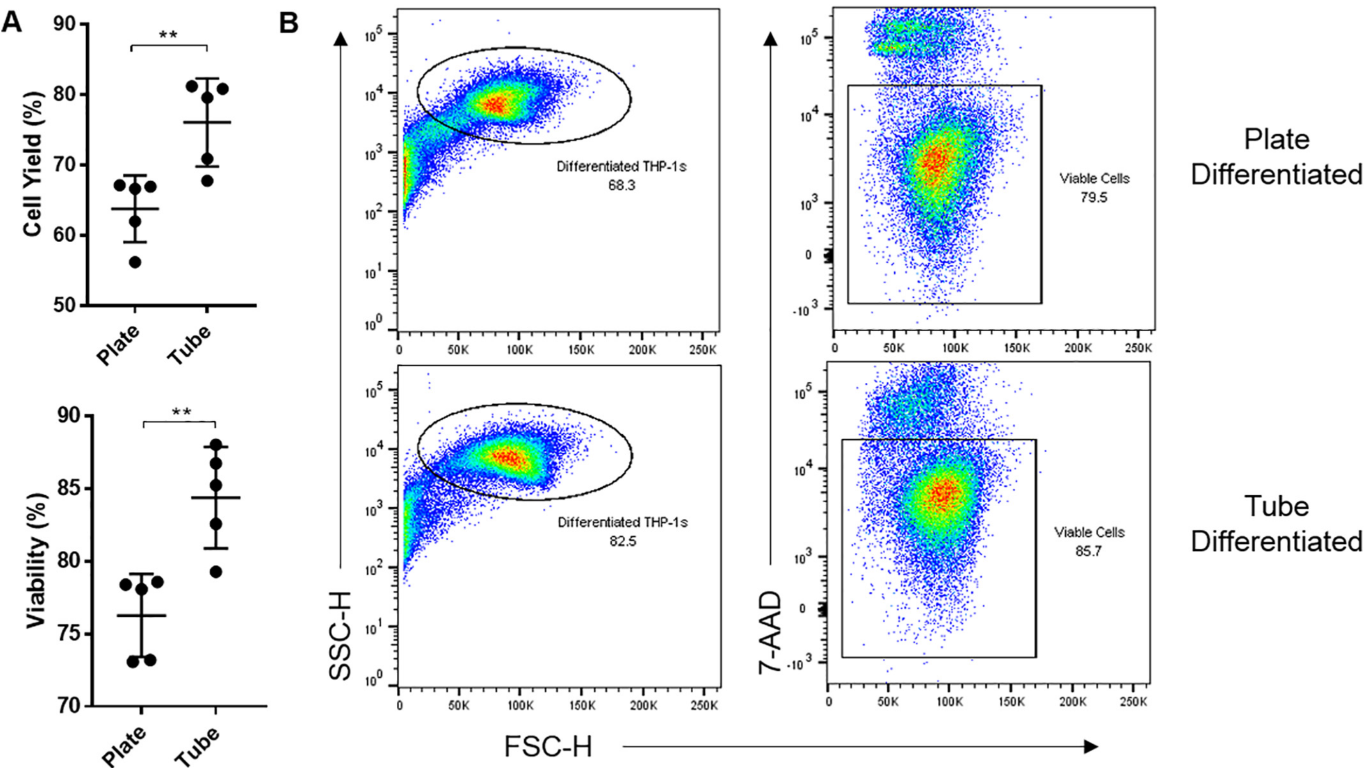


**Fig. 1.** Differentiating THP-1s in polystyrene FACS tubes yields higher cell numbers and higher viability than those differentiated in tissue culture plates (A). (B) Gating strategy for differentiated cells is based on side vs. forward scatter (left panel) and viable cells are gated based on their ability to exclude 7-AAD (right panel). Data are presented as percentage viable cells of the gated differentiated population ± SD, and is representative of five experiments. $^{**}p < .01$, unpaired student's *t*-test.

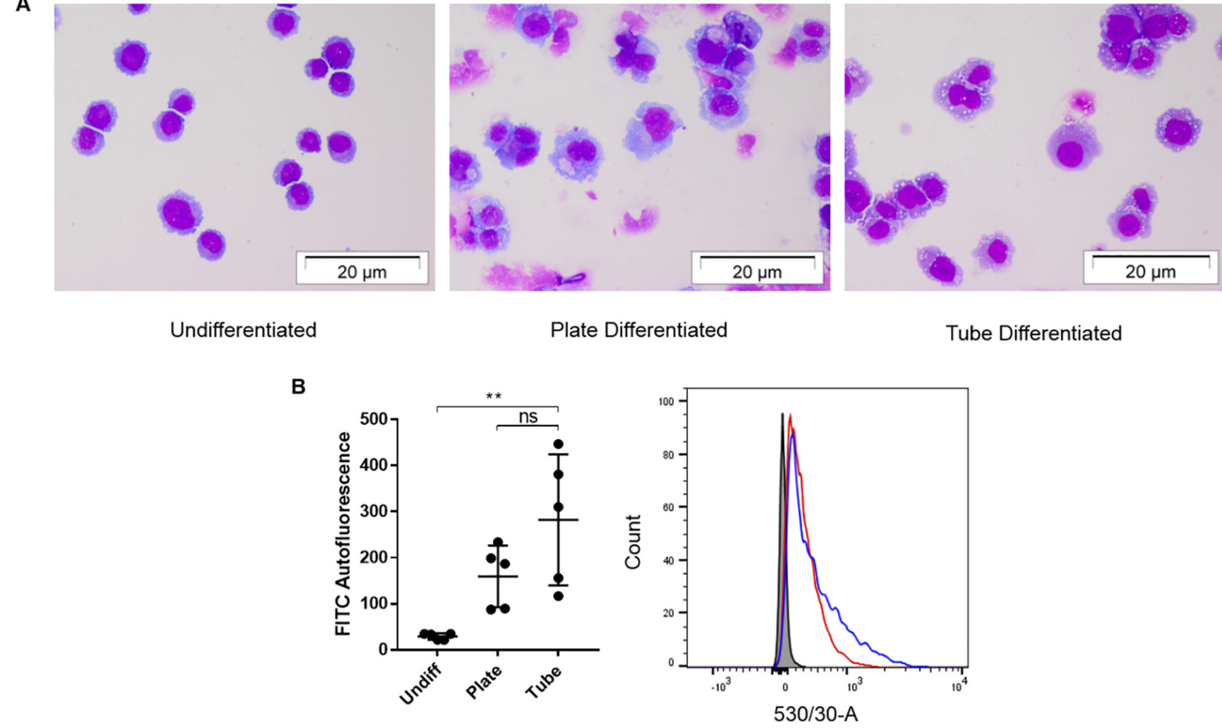


**Fig. 2.** Differentiated THP-1 macrophages produced in either FACS tubes or tissue culture dishes show similar morphological changes and equal amounts of autofluorescence. (A) Giemsa stained smears comparing undifferentiated THP-1s (left panel), with pDMs (centre panel) and tDMs (right panel). (B) Autofluorescence of undifferentiated THP-1s, pDMs, and tDMs detected by blue (488 nm) excited green fluorescence (530 nm, 30 nm bandwidth), with representative autofluorescence histogram shown (bottom right). Undifferentiated cells show close to no fluorescence (shaded), compared with increased levels observed in pDMs (red), and tDMs (blue). Data are presented as median fluorescent intensity of gated macrophages ± SD, and is representative of five experiments, ns = not significant, $^{**}p < .01$, Dunnett's multiple comparison test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

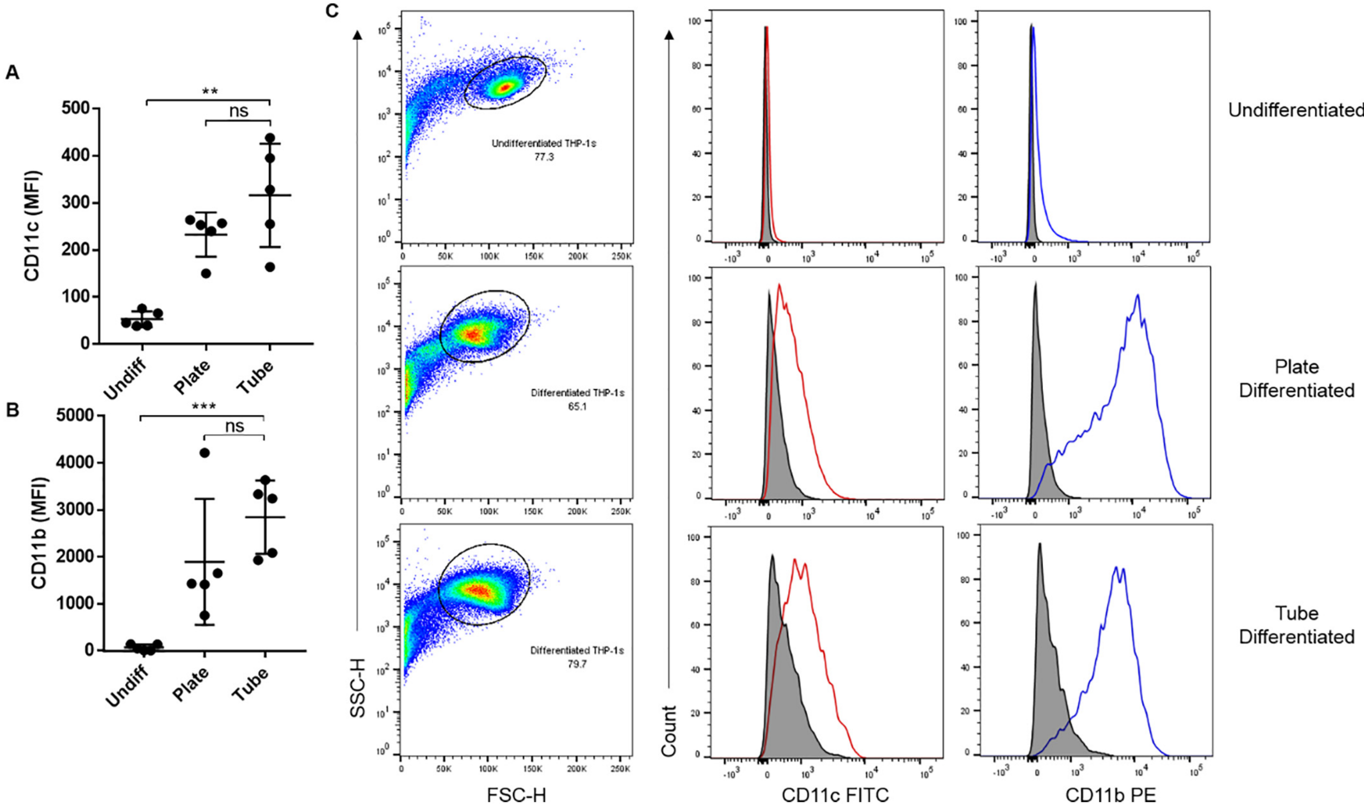


**Fig. 3.** Expression of CD11c and CD11b by THP-1 derived macrophages differentiated in FACS tubes. tDMs show similar amounts of expression of CD11c (A) and CD11b (B) as pDMs. Gating strategy of THP- 1 cells is shown (C), with representative histograms of CD11c (red) and CD11b (blue) expression, overlayed over unstained control cells (shaded). Data are presented as median fluorescent intensity of CD11c (FITC) or CD11b (PE) staining ± SD, and are representative of five experiments, ns = not significant, $^{**}p < .01$, $^{***}p < .001$, Dunnett's multiple comparison test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Victoria, Australia). 7-AAD viability dye was purchased from BD Biosciences (San Jose, CA; material number 559925).

### 2.2. Cell lines

THP-1 cell line was purchased from the ECACC (Salisbury, UK; catalog number: 88081201). Cells were maintained in culture in RPMI +10% FCS, supplemented with 100 U/mL penicillin, and 0.1 mg/mL streptomycin below $1 \times 10^6$ cells/mL in a 37 °C, 5% $CO_2$, high humidity incubator. The cell line was routinely tested for mycoplasma by PCR (Uphoff and Drexler, 2011).

### 2.3. Giemsa stain

A total of $1 \times 10^5$ cells in 100 µL of HBSS with 10% FCS were cytospun onto Superfrost® microscope slides (Thermo Scientific, Waltham, MA) using the Cytospin 3 centrifuge (Shandon Scientific, Cheshire, UK). The slides were retrieved and allowed to airdry for 10 min. The slides were then Giemsa stained using the UniCel DxH 800 Cellular Analysis System (Beckman Coulter, Brea, CA) and mounted with a coverslip. The cells were visualised under an Olympus BX51 microscope at 1000× magnification and images taken using analySIS LS Starter 3.1 (Olympus, Tokyo, Japan).

### 2.4. Flow cytometry

Following differentiation, cell culture media was removed, and cells were blocked directly in culture FACS tubes with 100 µg (10 µL) human IgG (Kiovig, Baxter, Old Toongabbie, NSW, Australia) for 10 min on ice, followed by a 20 min incubation in the dark and on ice, with the appropriate fluorochrome-conjugated anti-human antibodies (either 0.5 µg anti-CD11b FITC or 0.5 µg anti-CD11b PE). For plate differentiated THP-1 cells, $1 \times 10^6$ cells were added per tube. FACS Wash solution (2 mL) was added to each tube before centrifugation at 500 ×*g* for 5 min and the supernatant decanted. Following repeat of the wash step, the cells were analysed on a BD FACSCanto I flow cytometer. A minimum of 50,000 events were acquired. The data was analysed using FlowJo 10.1 (FlowJo, LLC, Ashland, Oregon).

### 2.5. Phagocytosis assay

The pHrodo™ Red *S. aureus* Bioparticles™ were purchased from ThermoFisher (Waltham, MA; catalog number A10010). Phagocytosis assay was performed according to the manufacturer's instructions. Briefly, $1 \times 10^6$ differentiated THP-1 macrophages were combined with HBSS, 10% *S. aureus* bioparticles, and 8% human AB serum to a final volume of 400 µL. Tubes were briefly gassed with 5% $CO_2$/air before incubation at 37 °C for 1 h. Following washing, results were then analysed using a BD FACSCanto flow cytometer, and processed using FlowJo 10.1 software (FlowJo, LLC, Ashland, Oregon).

### 2.6. Statistics

Unpaired comparisons were analysed using the two-tailed Student's *t*-test and multiple comparison were performed using Dunnett's test, with $p < .05$ considered significant. All statistical analyses were performed using GraphPad Prism 7 software (Graphpad Software Inc., San Diego, CA).

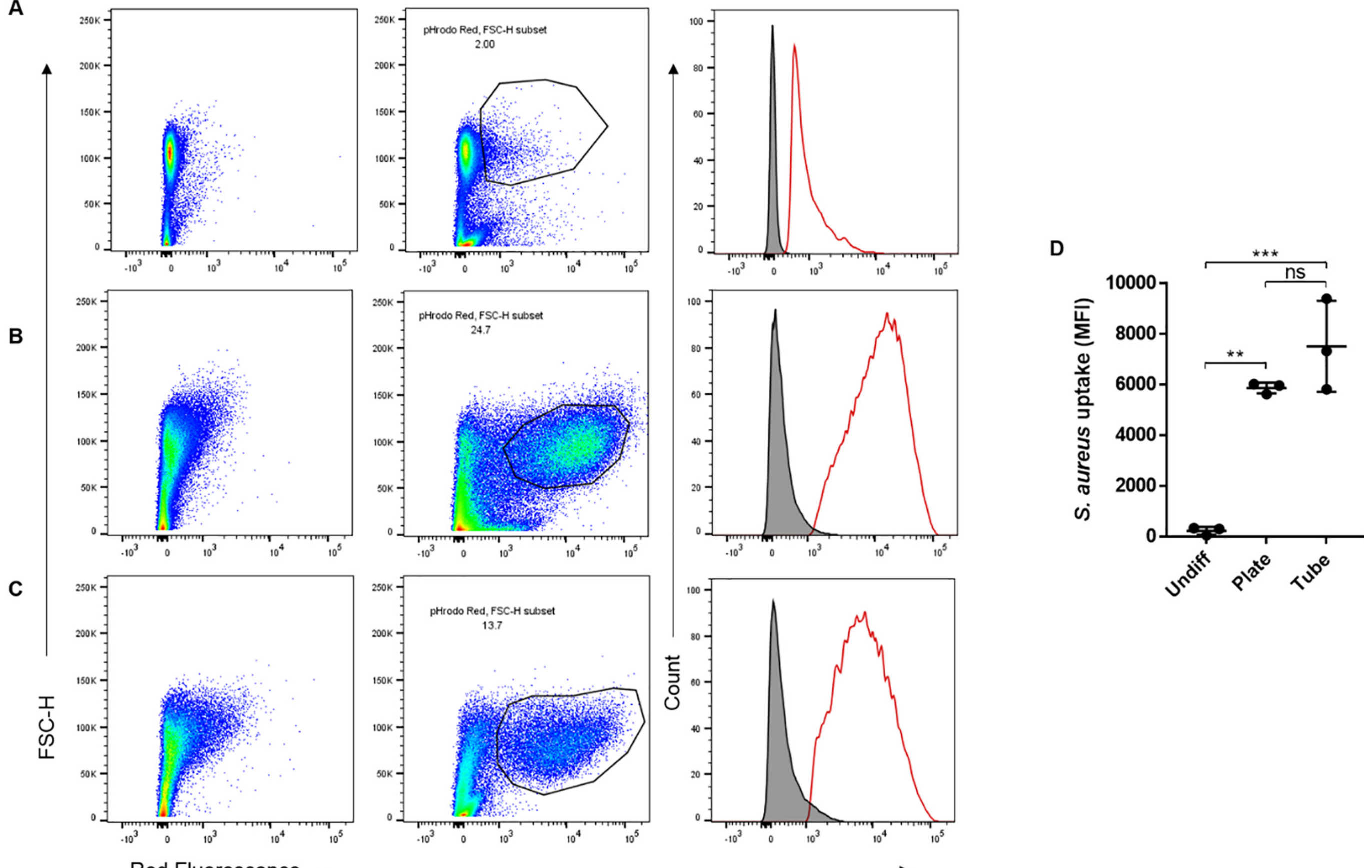


**Fig. 4.** Phagocytic activity of THP-1 macrophages differentiated in either FACS tubes or tissue culture dishes. tDMs efficiently phagocytose *S. aureus* bioparticles. (A) Undifferentiated THP-1s. Left panel shows THP-1 cells alone, with THP-1s and *S. aureus* in centre panel. Representative histogram comparing phagocytic THP-1s (red) vs. THP-1s alone (shaded) is shown to the right. (B) Differentiated tDMs. Left panel shows tDMs alone, with tDMS and *S. aureus* in centre panel. Representative histogram comparing phagocytic tDMs (red) vs. tDMs alone (shaded) is shown to the right. (C) Differentiated pDMs. Left panel shows pDMs alone, with pDMS and *S. aureus* in centre panel. Representative histogram comparing phagocytic pDMs (red) vs. pDMs alone (shaded) is shown to the right. (D) Both tDMs and pDMs show similar levels of phagocytic capabilities. Data are expressed as MFI ± SD, and are representative of triplicate experiments, ns = not significant, $^{**}p < .01$, $^{***}p < .001$, Dunnett's multiple comparison test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

## 3. Results

Cells were differentiated in sterile 5 mL polystyrene FACS tubes with caps (BD, Franklin Lakes, NJ) by adding 1 mL of cell suspension at $1 \times 10^6$ cells/mL per tube. PMA was added to the tubes at 50 ng/mL, and cells were incubated at 37 °C for three days. On the fourth day, cells were washed and allowed to rest in PMA-free media for a minimum of 24 h before analysis. Cells were detached from the inner surface of the tube by pipetting with culture fluid. For plate-differentiated THP-1 cells, $2 \times 10^6$ cells were seeded into each culture dish (6 mm in diameter), and PMA added at 50 ng/mL. Cells were incubated for three days at 37 °C. On the fourth day, cells were washed, non-adherent cells were removed, and cells allowed to rest in PMA-free media for a minimum of 24 h before analysis. Cells were detached prior to analysis by pipetting followed by gentle cell scraping.

Cell yields resulting from THP-1 differentiation in plates (pDMs) and differentiation in tubes (tDMs) were compared by flow cytometry. Cells were determined as differentiated by morphology and gated by forward vs side scatter (Fig. 1B). Cell yields were calculated as a percentage of differentiated cells out of 50,000 total events (Fig. 1A). Higher cell yields were consistently obtained from tDM preparations as compared with pDMs. A 7-AAD fluorometric assay was used to assess the viability of pDMs vs tDMs, with viable cells judged by their ability to exclude the dye (Fig. 1A, right panels of B). Significantly higher viability was found for cells cultured in FACS tubes. Thus, by adopting the method of differentiating THP-1 cells in FACS tubes, macrophages of higher yield and viability can be prepared.

In order to assess whether THP-1 cells were in fact differentiating in the FACS tubes, both cell morphology and differentiation marker expression were assessed and compared with those produced by pDMs. The morphology of undifferentiated THP-1 s was compared with pDMs and tDMs by Giemsa stain (Fig. 2A). A similar increase in cell/cytoplasm size consistent with macrophage differentiation (Aldo et al., 2013) was observed in pDMs and tDMs.

Autofluorescence is a key marker for macrophage differentiation (Daigneault et al., 2010), and was assessed by flow cytometry by blue (488 nm)-excited green fluorescence (530 nm, 30 nm bandwidth) (Fig. 2B). Both pDMs and tDMs show significantly increased amounts of autofluorescence after 4-day maturation periods compared with undifferentiated controls, with no significant levels of difference between the two groups, suggesting that macrophage differentiation has occurred.

CD11b and CD11c are molecules of the integrin family and represent the α-chain of complement receptor 3 (CR3) and complement receptor 4 (CR4) respectively when dimerised with the β-subunit CD18. CD11b and CD11c are reported as lowly expressed or absent from undifferentiated THP-1 s and are highly upregulated upon differentiation (Chanput et al., 2014; Daigneault et al., 2010; Mittar et al., 2011). Expression of these molecules was assessed on the surface of undifferentiated THP-1s (Fig. 3) and compared with expression by pDMs

and tDMs. CD11c and CD11b were substantially increased in both cell populations, and no significant difference between the two groups was observed. These findings, together with cell morphology and autofluorescence, shows that tDMs differentiate as efficiently as pDMs.

To assess whether tDMS are functional, the cells were assessed for phagocytic activity using commercially available labelled *S. aureus* bioparticles. These bacteria are non-fluorescent outside of the cell but highly fluorescent once taken into phagosomes. We were able to perform the entire assay within the same tubes used for differentiation. The results showed that undifferentiated THP-1 s were poorly phagocytic (Fig. 4A). However, both tDMs were highly phagocytic, similar to plate differentiated cells (Fig. 4B-D).

## 4. Discussion

The data demonstrates that the key limitations and challenges associated with culturing and experimenting with THP-1 macrophages due to their adherence properties can be overcome by differentiating and conducting experiments in polystyrene FACS tubes. This approach not only facilitates the handling of the cells in procedures such as washing, but also leads to greater cell recovery with increased numbers of viable cells compared to those cultured on tissue culture dishes. Centrifugation in these tubes enables the cells to be pelleted for further treatment and functional studies without requiring harvesting of the adhered cells as is the normal practice.

Examination of the resulting THP-1 macrophages demonstrates that those differentiated in FACS tubes differentiate to cells morphologically similar to those derived in tissue culture dishes. This is supported by the high degree of autofluorescence which they display as differentiated macrophages. Levels of autofluorescence as judged by flow cytometry were similar between the cells from the different culturing techniques, indicating that differentiation was effective in the FACS tubes.

The functionality of the THP-1 macrophages cultured in FACS tubes was assessed using two key biomarkers and this data showed that cells derived from FACs tubes are optimal for use in functional studies. Thus, while the undifferentiated THP-1 cells showed low expression of the complement receptors CR3 and CR4, the expression was markedly increased in the differentiated cells. This is supported by the phagocytosis assays which tested their ability to phagocytose complement opsonised *S. aureus*. Thus, this was low in undifferentiated cells, and high in the THP-1 macrophages.

In some experiments the tube-prepared cells showed greater experimental variability, although the difference between the plate and tube procedures was not significant. Because this difference was not always observed, it is likely that this is due to experimental variation that may be resolved by generating more data points.

Differentiating cells directly in the polystyrene FACs tubes has clear advantages. Firstly, and most importantly, this method avoids the problem of having to detach adhered cells, hence preventing loss of cell viability. Using both detachment buffers containing proteolytic enzymes and or the anaesthetic lidocaine with EDTA in tissue culture dishes attached macrophages may also have sublethal effects, compromising the experimentation with these cells. Using a cell scraper to dislodge the macrophages does not overcome these issues because of cell damage.

## Conflicts of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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## References

Aldo, P.B., Craveiro, V., Guller, S., Mor, G., 2013. Effect of culture conditions on the phenotype of THP-1 monocyte cell line. Am J. Reprod. Immunol. 70 (1), 80–86. http://dx.doi.org/10.1111/aji.12129.

Bosshart, H., Heinzelmann, M., 2016. THP-1 cells as a model for human monocytes. Ann. Transl. Med. 4 (21), 438. http://dx.doi.org/10.21037/atm.2016.08.53.

Chanput, W., Mes, J.J., Wichers, H.J., 2014. THP-1 cell line: An in vitro cell model for immune modulation approach. Int. Immunopharmacol. 23 (1), 37–45. http://dx.doi.org/10.1016/j.intimp.2014.08.002.

Chen, S., So, E.C., Strome, S.E., Zhang, X., 2015. Impact of detachment methods on M2 macrophage phenotype and function. J. Immunol. Methods 426, 56–61. http://dx.doi.org/10.1016/j.jim.2015.08.001.

Daigneault, M., Preston, J.A., Marriott, H.M., Whyte, M.K.B., Dockrell, D.H., 2010. The identification of markers of macrophage differentiation in PMA-stimulated THP-1 cells and monocyte-derived macrophages. PLoS One 5 (1), e8668. http://dx.doi.org/10.1371/journal.pone.0008668.

Lund, M.E., To, J, O'Brien, B.A., Donnelly, S., 2016. The choice of phorbol 12-myristate 13-acetate differentiation protocol influences the response of THP-1 macrophages to a pro-inflammatory stimulus. J. Immunol. Methods 430, 64–70. http://dx.doi.org/10.1016/j.jim.2016.01.012.

Mittar, D., Paramban, R., McIntyre, C., 2011. Flow Cytometry and High-Content Imaging to Identify Markers of Monocyte-Macrophage Differentiation. BD Biosciences.

Tsuchiya, S., Yamabe, M., Yamaguchi, Y., Kobayashi, Y., Konno, T., Tada, K., 1980. Establishment and characterization of a human acute monocytic leukemia cell line (THP-1). Int. J. Cancer 26 (2), 171–176.

Uphoff, C.C., Drexler, H.G., 2011. Detecting mycoplasma contamination in cell cultures by polymerase chain reaction. Methods Mol. Biol. 731, 93–103. http://dx.doi.org/10.1007/978-1-61779-080-5_8.

Van Veldhoven, P.P., Bell, R.M., 1988. Effect of harvesting methods, growth conditions and growth phase on diacylglycerol levels in cultured human adherent cells. Biochim. Biophys. Acta 959 (2), 185–196.

Zhang, B., Shan, H., Li, D., Li, Z.R., Zhu, K.S., Jiang, Z.B., Huang, M.S., 2012. Different methods of detaching adherent cells significantly affect the detection of TRAIL receptors. Tumori 98 (6), 800–803. http://dx.doi.org/10.1700/1217.13506.

# Chapter 3. Development and characterisation of cross-reactive monoclonal antibodies to human and murine complement receptor immunoglobulin

## 3.1. Introduction and Contextual Statement

Currently available on the market, is a limited selection of monoclonal anti-CRIg monoclonal antibodies. The two key publications which document anti-CRIg antibody generation were published by Kim et al. (2005) and (Helmy et al., 2006). Between these articles, multiple striking differences in their findings and conclusions exist, making it difficult to form a foundation of knowledge of CRIg biology on which to build upon. In this chapter, we present experimental comparisons between the two most commonly used anti-CRIg antibodies currently commercially available, followed by the documentation of the generation and characterisation of novel, cross-reactive anti-CRIg antibodies.

This chapter is presented in the format of a submitted publication, and incorporates the **supplementary findings** of '*Functional expression of CRIg/VSIG4 on neutrophils and monocytes under activating conditions involving PKC, p38, $Ca^{2+}$ and cytoskeleton*', by **Annabelle Small**, Trishni Putty, Khalida Perveen, Nikita Patel, Asmitabahen Patel, Muhammad Y. Gulam, Patrick Quinn, Helen Weedon, Anak A.S.S.K. Dharmapatni, Mihir D. Wechalekar, Charles S. Hii, Alex Quach, & Antonio Ferrante, which at the time of submission of this thesis, is currently under consideration for publication (November 2020).

## 3.2. Statement of Authorship

# Statement of Authorship

| | |
|---|---|
| Title of Paper | 'Activated neutrophils express functional Complement Receptor Immunoglobulin involving p38 and cytoskeleton', AND 'Development and characterization of cross-reactive monoclonal antibodies to human and murine complement receptor immunoglobulin (CRIg)' |
| Publication Status | ☐ Published ☐ Accepted for Publication ☒ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style |
| Publication Details | Unpublished works presented in manuscript style, submitted together and currently under consideration for publication |

### Principal Author

| | |
|---|---|
| Name of Principal Author (Candidate) | Annabelle Small |
| Contribution to the Paper | AS was principally responsible for running the project, including the design, generation and characterisation of the monoclonal antibodies. This included comprehensively reading and assessing published data in the field, organising laboratory facilities, designing experiments, and coordinating staff assisting with experiments and data interpretation. AS performed the majority of the experiments, collated the data, interpreted data, produced the first draft of the manuscript, and wrote the manuscript. |
| Overall percentage (%) | 70% |
| Certification: | This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper. |
| Signature | Date 14/8/20 |

### Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

| | |
|---|---|
| Name of Co-Author | Professor Antonio Ferrante |
| Contribution to the Paper | AF proposed and designed the experiments, interpreted data, supervised all aspects of the study, was responsible for the clinical/laboratory work up of the patient with ARPC1B deficiency, initiated and contributed to the characterisation of the monoclonal antibodies, wrote the manuscript and acted as corresponding author. |
| Signature | Date 14/08/20 |

| | |
|---|---|
| Name of Co-Author | Trishni Putty |
| Contribution to the Paper | TP contributed to the experiments, the clinical/laboratory work up of the patient with ARPC1B deficiency, data interpretation, and was involved with the critical reading and writing of the manuscript. |
| Signature | Date 14/08/20 |

Please cut and paste additional co-author panels here as required.

| Name of Co-Author | Nikita Patel | | |
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| Contribution to the Paper | MYG was involved in the initial establishment of the project and contributed to some experimental runs and data interpretation. | | |
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| Name of Co-Author | Dr. Patrick Quinn | | |
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| Contribution to the Paper | PQ was responsible for the clinical and laboratory work up of the patient with ARPC1B deficiency and contributed to the critical reading and writing of the manuscript. | | |
| Signature | | Date | 20/08/2020 |

| Name of Co-Author | Khalida Perveen | | |
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| Contribution to the Paper | KP contributed to some experimental runs, data interpretation, the characterisation of the monoclonal antibodies, and in the critical reading and writing of the manuscript. | | |
| Signature | | Date | 14/8/2020 |

| Name of Co-Author | Helen Weedon | | |
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| Signature | | Date | 19-8-20 |

| Name of Co-Author | Dr. Kencana Dharmapatni | | |
|---|---|---|---|
| Contribution to the Paper | KP was responsible for the acquisition of the murine synovial tissue and assisted in the characterisation of the monoclonal antibodies and critical reading of the manuscript. | | |
| Signature | | Date | 19/08/2020 |

| Name of Co-Author | Associate Professor Charles Hii | | |
|---|---|---|---|
| Contribution to the Paper | CH was involved in designing aspects of the project including signalling pathways and monoclonal antibody generation. CH was also involved in data interpretation and in the critical reading and writing of the manuscript. | | |
| Signature | | Date | 14/8/20 |

| Name of Co-Author | Dr. Alex Quach | | |
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| Contribution to the Paper | AQ was responsible for the clinical/laboratory work up of the patient with ARPC1B deficiency, assisted in data interpretation, compiling of the figures, statistics, and in the critical reading and writing of the manuscript. | | |
| Signature | | Date | 14.8.20 |

| Name of Co-Author | Associate Professor Mihir Wechalekar | | |
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| Contribution to the Paper | MW was responsible for the acquisition of the human synovial tissue samples, and assisted in the characterisation of the monoclonal antibodies and critical reading of the manuscript. | | |
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| Name of Co-Author | Asmitabahen Patel | | |
|---|---|---|---|
| Contribution to the Paper | AP contributed to some experimental runs and in data interpretation. | | |
| Signature | | Date | 19.8.20 |

# Supplementary Materials for

## Functional expression of CRIg/VSIg4 on neutrophils and monocytes under activating conditions involving PKC, p38, $Ca^{2+}$ and cytoskeleton

Annabelle G Small, Trishni Putty, Khalida Perveen, Nikita Patel, Asmitabahen Patel, Muhammad Y Gulam, Patrick Quinn, Helen Weedon, Anak A. S. S. K. Dharmapatni, Mihir D. Wechalekar, Charles S. Hii, Alex Quach and Antonio Ferrante*

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**This file includes:**

**Part 1. Development and characterization of cross-reactive monoclonal antibodies to mouse and human CRIg**

Table S1. Summary of anti- human and anti-mouse CRIg antibodies currently commercially available, their uses, and cell types they detect CRIg

Fig. S1. Differing reactivity of two widely used clones of anti-human CRIg monoclonal antibody was observed in Western blotting

Table S2. Reactivity of hybridoma culture supernatant from clones 1A3, 2D6, 4H8, 8E12, and 14B11, to murine CRIg by ELISA

Fig. S2. Diagram showing the, theoretical, six human CRIg isoforms and their relationship to the selected immunogen for monoclonal antibody production

Fig. S3. Specificity of the anti-CRIg monoclonal antibody clone 14B11 was demonstrated by competitive binding against recombinant CRIg peptide in Western blotting of human macrophages

Fig. S4. The generated monoclonal antibodies stain CRIg on the surface of human MDMs and murine PECs

Fig. S5. Hybridoma clone 14B11 detects CRIg on both human and murine synovial tissue macrophages

**Part 2. Other Supplementary Material**

Fig. S6. CRIg expression by human monocyte-derived macrophages

Table S3. Summary of the inflammatory mediators used in this study and the known neutrophil compartments from which they induce degranulation, as well as signalling pathways involved in cytoskeleton rearrangements and the granule release

# Part 1. Development and characterization of cross-reactive monoclonal antibodies to mouse and human CRIg

## Introduction

The fifth human complement receptor, complement receptor immunoglobulin (CRIg), was discovered two decades ago (1-3). Since this initial documentation, CRIg has been largely regarded as an efficient promotor of phagocytosis expressed selectively by tissue resident macrophages (2) with a critical role in mediating blood clearance by liver Kupffer cells (2, 4, 5). However, over the last two decades, other studies have attributed a broad range of additional functions to the receptor. CRIg has been reported to function as a promoter of inflammation (6), a potent inhibitor of the alternative pathway of complement (7, 8), a pattern recognition receptor (PRR) (9), and an inhibitor of macrophage activation (10, 11). While investigations into the root causes of the differences and contradictions present between these studies have not been undertaken, it is likely they stem down to two main causes. Firstly, there are fundamental differences between CRIg biology in murine compared with human systems; in mice, one 'short' splice variant is expressed, while in humans, up to six different isoforms can be produced (2, 12). Secondly, there remains a lack of currently available monoclonal antibodies raised against human and particularly the murine form of CRIg in today's commercial market.

Currently available is a modest selection of antibodies available from either commercial or other sources (Table S1). However, previously published studies using these antibodies have made strikingly different conclusions with regard to the pattern of CRIg expression by human cells (Table S1). Of note, in 2005, human monocytes were reported to express high levels of CRIg on the cell surface (3), while later in 2006, monocytes were reported negative (2). This blatant disagreement in findings has remained undiscussed in today's literature, even though the implications of this are not benign. CRIg expression on the surface of monocytes—the macrophage-replenishing phagocytic cells of the circulation—may have many implications in a broad range of biological processes, particularly with respect to the application of CRIg blocking antibodies as potential therapeutics to disorders such as cancer where expression is elevated (13-16). This is not the only discrepancy in the reports of CRIg expression on differing cellular populations. In humans, CRIg is highly expressed by alveolar macrophages, while in mice, expression in the lung is debated (2, 17-19). Similarly, in the liver, CRIg is highly

expressed at the protein level in murine tissues, but in humans, protein is conspicuously absent (20).

These substantial discrepancies in the reports of CRIg cellular expression in human systems compared with mice indicate that there remains a need in today's market for an antibody capable of detecting all human protein forms of CRIg. Thus, in order to address this need, we sought to generate new anti-CRIg monoclonal antibodies, suitable for use in a range of techniques. Using a portion of murine CRIg as an immunogen, we describe the successful generation of two new, cross-reactive rat anti-murine CRIg monoclonal antibodies which detect CRIg on the surface of healthy human monocytes, supporting the findings of Kim, et al (2005) (3). Additionally, we demonstrate that these antibodies are suitable for use in a range of applications, including flow cytometry, Western blot, and immunohistochemistry on both human and murine tissues.

**Table S1. Summary of anti- human and anti-mouse CRIg antibodies currently commercially available, their uses, and cell types they detect CRIg.**

| Species | Antibody | Reported applications | Reported Positive Expression | Reported Negative Expression | References |
|---|---|---|---|---|---|
| **Human** | Genentech mouse anti-human clone 3C9 | Western blot, flow cytometry | Monocyte-derived macrophages | Granulocytes, NK cells, B cells, T cells, monocytes, THP-1 cells | **2, 21** |
| | Santa Cruz mouse anti human clone 6H8 | Western blot, flow cytometry, ELISA, paraffin immuno-histochemistry | Macrophages, Monocytes, DC, THP-1 cells | Granulocytes, NK cells | **3, 12, 21** |
| | Invitrogen mouse anti-human clone JAV4 | Flow cytometry | Kupffer cells, monocyte-derived macrophages | N/A | **22** |
| | Proteintech rabbit anti-human polyclonal | Western blot, ELISA | N/A | N/A | **-** |
| | Aviva Systems Biology polyclonal rabbit anti-human | Western blot, immunofluorescence | Alveolar macrophages | N/A | **17** |
| | Abcam polyclonal rabbit anti-human (ab56037) | Western blot, ELISA, tissue microarray-immunohistochemistry | Primary human melanoma tissues | N/A | **23** |
| | Invitrogen mouse anti-human clone 3 | ELISA | N/A | N/A | **-** |
| **Mouse** | Genentech rat anti-mouse clone 14G6 | Flow cytometry, Western blot | Kupffer cells, peritoneal macrophages | Monocytes, B cells, T cells, granulocytes, NK cells | **2** |
| | Aviva Systems Biology polyclonal rabbit anti-human | Western blot, immunofluorescence | Alveolar macrophages | N/A | **17** |
| | Invitrogen rat anti-mouse clone NLA14 | Flow cytometry, not suitable for blocking CRIg-ligand interaction | Peritoneal macrophages | N/A | **-** |

## Methods

### Ethics statement

Procurement of human blood and the conduction of experimental procedures were approved by the Women's and Children's Health Network (WCHN) Human Ethics Committee and the Southern Adelaide Clinical Human Research Ethics Committee, in accordance to the National Statement on Ethical Conduct in Human Research (2007, updated 2018) (National Health and Medical Research Council Act 1992). Peripheral blood was donated by healthy donors who had given informed consent. Scavenged murine tissue and peritoneal exudate cells were procured from the University of Adelaide animal house. All murine experimental procedures were approved by the WCHN Animal Ethics Committee and work conducted in accordance to the *Australian code for the care and use of animals for scientific purposes.* In all cases scavenger tissue and peritoneal cells were used.

### Immunogen and hybridoma generation

A 168 amino acid peptide, corresponding to N-terminal amino acids 20-187 of murine Vsig4, was selected as immunogen;
HPTLKTPESVTGTWKGDVKIQCIYDPLRGYRQVLVKWLVRHGSDSVTIFLRDSTGDH
IQQAKYRGRLKVSHKVPGDVSLQINTLQMDDRNHYTCEVTWQTPDGNQVIRDKIIEL
RVRKYNPPRINTEAPTTLHSSLEATTIMSSTSDLTTNGTGKLEETIAGSGRNLP

Amino acids 20-137 have 80% homology to the region of human CRIg encoded by *VSIG4* exon 2.

The peptide was synthesized and used to immunise rats for the generation hybridomas by GenScript. The list of generated cell lines/clones are shown in Table S2, where antibody clone reactivity to murine CRIg was determined by ELISA. Western blotting was used to assess custom antibody specificity in-house.

### Hybridoma cell culture and supernatant harvesting

*Cell culture maintenance.* Rat hybridoma cell lines were maintained at a concentration below $1 \times 10^6$ cells/mL in DMEM supplemented with 1% FCS, 100 U/mL penicillin, and 0.1 mg/mL streptomycin in a 37 °C, 5% $CO_2$, high humidity incubator. The cell lines were routinely tested for mycoplasma by PCR.

*Supernatant harvesting and concentration.* Biweekly, all hybridoma cells were transferred from their flask into tubes and centrifuged at 500 × *g* for 5 minutes. Supernatant was retained while the pellet was resuspended in 5 mL of residual supernatant. Resuspended cells were returned to the flask and topped up to 50 mL with fresh DMEM supplemented with 1% FCS, 100 U/mL penicillin, and 0.1 mg/mL streptomycin, and returned to culture. The harvested hybridoma supernatant was filter sterilised through 0.2 µm and supplemented with 0.02% sodium azide. Supernatant was stored at 4 °C or -20 °C for short- or long-term storage, respectively.

**Antibody Purification**

Monoclonal antibodies were crudely purified from the hybridoma culture supernatant using anti-rat IgG conjugated agarose beads (Abcam). Briefly, 50 µL of anti-rat conjugated agarose slurry was added to 100 µL of culture supernatant. Tubes were incubated at 4 °C on a rocking platform for 1 h before they were centrifuged at 200 × *g* for 1 min. The pelleted agarose conjugates were washed twice in Tris-buffered saline (TBS; 50 mM Tris at pH 7.5, 150 mM NaCl) before a final wash in 0.5 M Tris at pH 6.8. Antibodies were eluted from agarose complexes by incubation for 10 min with glycine at pH 2.6 at 1:1 with frequent agitation prior to centrifugation at 200 × *g* for 1 min. Eluate was harvested and neutralised by adding an equal volume of Tris at pH 8.0. The elution was repeated once more, and the product pooled with the initial eluate. Antibody concentration was determined using the Qubit® protein assay previously described[12], and purification protocol validated by Western blot.

**Flow cytometry**

Flow cytometric measurement of CRIg expression was determined on monocyte-derived macrophages or murine synovial tissue macrophages, essentially as described in the manuscript methods, but with unconjugated anti-human CRIg primary antibodies: either 100 µL neat hybridoma supernatant containing rat anti-CRIg; 0.2 µg of purified rat anti-CRIg clone 14B11; 0.2 µg of clone 6H8; or matched isotype control. Following incubation and washing, goat anti-rat IgG FITC (sc-2011, Santa Cruz Biotechnology) was added for a further 20 min. The cells were washed twice in PBS-FCS, then acquired and analysed.

**Western blot**

Western blotting were performed on macrophages detached from culture dishes through gentle scraping with a rubber cell scraper, essentially as described in the manuscript methods, with

staining using other primary anti-human antibodies: mouse anti-human CRIg clone 3C9 at 1:3000 and/or clone 6H8 at 1:1000, (provided by Dr Menno van Lookeren Campagne, Genentech Inc. CA) and purified rat anti-human CRIg clone 14B11 at 1:1000 in blocking solution overnight at 4 °C. Secondary antibody staining and immunoreactive material detection on nitrocellulose membrane followed as per the manuscript methods. Blots were stripped using Re-Blot Plus Mild Solution (Millipore) and re-probed with mouse anti-human GAPDH (G8795, Sigma-Aldrich) at 1:20,000 and analysed as above.

**Immunohistochemistry**

Paraffin embedded murine paw or synovial tissue sections were prepared for histologic analysis using standard protocols. Briefly, sections were dewaxed with xylene followed by ethanol prior to antigen retrieval with proteinase K (1:50), or antigen retrieval solution (1 mM EDTA/10 mM Tris at pH 9), heated to 95-100 °C. Slides were washed before endogenous peroxidase (EP) blocking (0.1% sodium azide in Tris PBS/1% $H_2O_2$), with further blocking in 20% donkey serum (Jackson ImmunoResearch). Sections were subsequently stained with rat anti-murine CRIg (clone 14B11 hybridoma supernatant, neat), followed by rabbit anti-rat secondary antibody (Dako, P0450) and horseradish peroxidase-conjugated swine anti-rabbit tertiary antibody (Dako, P0399). Slides were then counterstained with haematoxylin.

**Isolation of murine peritoneal exudate cells**

Peritoneal macrophages were harvested from male swiss white laboratory mice using peritoneal washouts after the mice were euthanised by $CO_2$ asphyxiation. Briefly, the peritoneal cavity was injected with 3 mL of RPMI-1640 medium and gently massaged, before the cell-containing fluid was withdrawn. Harvested cells were washed in complete cell culture media consisting of RPMI-1640, 10% heat-inactivated FCS, penicillin/streptomycin, and L-glutamine, prior to processing for flow cytometric analysis.

**Isolation of murine synovial tissue macrophages**

Synovial cells were isolated as described by Misharin, et al (2014) (24). Briefly, legs were removed, skinned and ankles cut 3 mm above the heel. Bone marrow was flushed with HBSS and toes disarticulated by pulling with forceps. Tibiotalar joints were opened by posterior access, exposing the synovial lining. Feet were then incubated in digestion buffer comprising of 2 mg/mL dispase II, 2 mg/mL collagenase D and 1 mg/mL DNase I in HBSS for 1 h at 37 °C on a rocking platform. Released mononuclear cells were purified by density gradient

centrifugation on Ficoll-Paque PLUS (GE Healthcare), and macrophages were isolated by adherence to 6 cm plastic culture dishes for 1 h at 37 °C. Macrophages were removed from the plates and subjected to flow cytometric analysis.

## Results

### Assessment of commercially available antibodies

The two most commonly referenced antibody clones for the detection of CRIg in human studies are mouse anti-human clone 6H8 (Santa Cruz Biotechnology) (3, 25, 26) and mouse anti-human clone 3C9 (Genentech) (2, 21). Thus, we began our study by comparing the staining patterns of these antibodies by Western blot analysis. Using the same monocyte-derived macrophage total protein lysate, we probed the same blot with either clone 6H8, clone 3C9, or a combination of both (with primary antibody stripping steps between each stain) (Fig. S1). Strikingly, we observed distinctly different staining patterns of each antibody, suggesting that while both antibodies were raised against human CRIg, by Western blot analysis, the antibodies detect differing proteins. Following this finding, we assessed the staining pattern of a combination of both antibodies on the same blot, and found that when used together, the antibody combination detects up to six proteins in MDM whole lysates.

### Anti-CRIg antibody generation

In humans, up to six isoforms of CRIg as a result of alternative splicing exist (data accessible at NCBI Nucleotide database, reference sequences NM_007268.3, NM_001100431.2, NM_001184831.1, NM_001184830.1, NM_001257403.1, XM_017029251.2). These have been shown to be expressed by human MDM at the mRNA level (21). Therefore, the finding that the commercially available monoclonal anti-CRIg antibodies detect different proteins to one another suggests that these are potentially recognising different splice variants. As a result, there remains a need for improved anti-human CRIg antibodies on the market which are able to detect all forms of CRIg expressed by human cells, and thus, we sought to generate our own monoclonal antibodies for this purpose.

In order to minimise both time and cost, we elected to raise our antibody against a peptide sequence of high homology between human and mice, with the expectation that the produced antibody may be cross-reactive. Taking the variability of the six potential human forms of CRIg into consideration, we aimed to select a peptide sequence which is present in the extracellular domain of all six proteins, allowing for the generation of an antibody suitable

for the staining of all human CRIg variant isoforms on the cell surface. As a result, we selected a 168 amino acid region of murine CRIg which is ~80% conserved with human CRIg (Fig. S2) to use as immunogen. Monoclonal antibodies against the peptide were raised in rat (GenScript) and of the twenty generated hybridoma clones, five were selected progress to the next stage of antibody screening. Culture supernatants from duplicate preparations of each of the five clones (giving a total of ten supernatants) were screened for their ability to bind the peptide immunogen by ELISA (Table S2). Of these clones, 14B11 and 4H8 were selected as the better performing clones for Western blot (not shown) and flow cytometric (Fig. S4) applications respectively.

**Monoclonal antibody screening**

We first assessed the ability of our generated antibody to detect CRIg protein in human MDM total lysates. Anti-CRIg clone 14B11 detected a strong band migrating at ~50 kDa (consistent with the previously reported size of CRIg protein (2)), but unfortunately did not detect additional protein bands (Fig. S3, left panel). Performing the antibody probing step in the presence of recombinant CRIg peptide (2:1) completely prevented detection of this protein (Fig. S3, right panel), suggesting that 14B11 specifically recognises human CRIg protein by Western blot.

Next, we assessed the ability of our antibodies to perform in flow cytometry. Unstimulated human monocytes were differentiated into macrophages for 5 days as previously described (21) before being subject to flow cytometric analysis. All three tested hybridoma supernatants (clones 4H8, 1A3, and 2D6) positively detected CRIg protein on the cell surface and stained comparably well with the commercial clone 6H8 (Fig. S4B). Additionally, we sought to reproduce the observed upregulation in CRIg surface expression induced by dexamethasone treatment as described by Gorgani, et al. (2011) (27). Using our antibody clone 4H8, we observed a 2-4-fold increase in surface protein as a result of treatment with 50 ng/mL dexamethasone (Fig. S4C). Further, we assessed the ability of clone 4H8 to detect CRIg on the surface of murine peritoneal exudate cells (PEC). Consistent with previous reports, our antibody successfully detected two populations of F4/80$^{+}$ peritoneal macrophages based on expression of CRIg (25, 28).

CRIg protein is expressed by tissue resident macrophages in both human and murine synovial tissues (6, 29, 30). To assess the suitability of our antibody for the application of immunohistochemistry, we stained serial sections of human healthy control and early RA synovial tissue (ST) with hybridoma clone 14B11. Using standardised protocols as previously

described (31), we observed positive staining in the synovial lining of healthy control tissue (Fig. S5A, left panel), with increased staining in early RA tissue (Fig. S5A, right panel), consistent with previous reports (6).

We next investigated the ability of 14B11 to stain murine synovial cells by both flow cytometry and immunohistochemistry. Of the dissociated synovial cells from healthy BALB/c mice of macrophage morphology, ~8% stained positive for CD11b alone, while ~30% stained dual positive for both CD11b and CRIg, consistent with the observed population proportions reported in the peritoneal cavity by Gorgani, et al[28]. Next, we stained intact ST from the paws of mice with collagen antibody-induced arthritis (CAIA). We observed cells of macrophage-like morphology staining highly positive for CRIg in both the synovial lining and the sublining layers. Together, these data demonstrate that our generated anti-CRIg antibody clone 14B11 specifically detects CRIg protein in both human and murine ST resident macrophages by immunohistochemistry and detects CRIg on the surface of dissociated murine synovial cells.

## Discussion

Although documented almost two decades ago, CRIg currently remains the most elusive member of the complement receptor family, and this likely stems to the lack of available commercial monoclonal antibodies. Now, adding to the growing uncertainties in the field of CRIg biology, is the finding that the commonly used monoclonal antibodies are actually detecting distinctly different proteins in human macrophages.

The finding that each of the monoclonal antibody clones 3C9 and 6H8 appear to recognise three proteins by Western blot of different sizes is perhaps not surprising, and can potentially be explained by the existence of the six alternatively spliced protein products (12). Anti-CRIg clone 6H8 was generated in 2005 by Kim, et al (3), one year before the documentation of alternative splice variants of CRIg. Thus, during the steps of antibody generation, this antibody may have been unknowingly selected to recognise certain protein isoforms of CRIg, while the antibody clone 3C9, generated in 2006, was specifically raised to the full-length isoform of CRIg. As shown in Fig. S2, three of the CRIg proteins contain identical 'long' form extracellular portions (exon 3), while the other three contain the 'short' extracellular portions. Thus, in the initial generation of the antibodies, if one clone (potentially 3C9) recognises the 'long' CRIg proteins (isoforms 1, 4, and 5) while the other recognises the

'short' CRIg proteins (isoforms 2, 3, and 6), then, the different protein detection patterns can be explained.

The data presented here highlights the importance of inclusive consideration of the numerous CRIg protein isoforms resulting from alternative splicing. The finding that the currently available antibodies raised against CRIg indeed recognise proteins of different molecular weights present in human MDM total protein lysates by Western blot suggests that what we currently know about CRIg expression in humans may not be fully reflective of the 'true' story, and that further, in depth studies into the full expression profile of the CRIg isoforms are required. While the functional differences which may exist between the CRIg protein isoforms currently remain unknown, the short form of CRIg has a higher affinity for complement C3 fragments than the long form (7), and the isoform pairs 3 and 4, and 5 and 6 have differing intracellular portions compared to the 'traditional' long and short forms, suggesting that these isoforms may signal differently. Specifically, forms 3 and 4 do not contain any intracellular phosphorylation sites, and the cytoplasmic domains are lacking. Thus, it stands to reason that these forms of CRIg may serve to capture opsonised pathogens or particles from the extracellular environment, while avoiding initiating an inflammatory response within the cell. The phenomenon of extracellular capture of pathogens has been known to occur for decades in liver Kupffer cells (which express high levels of CRIg protein in mice), where the phagocytes of the circulation, the neutrophil, assist in clearing extracellularly trapped pathogens (32). Alternatively, the existence of soluble CRIg has recently been described (13, 33). It would be interesting to isolate serum CRIg protein and confirm whether this soluble form of CRIg may correspond to the shorter CRIg protein isoforms which lack cytoplasmic protein portions.

Here, we present two novel, cross-reactive rat anti-murine CRIg monoclonal antibody clones, 14B11 and 4H8, which together are suitable for the applications of Western blot, flow cytometry, and immunohistochemistry on human and murine tissues.

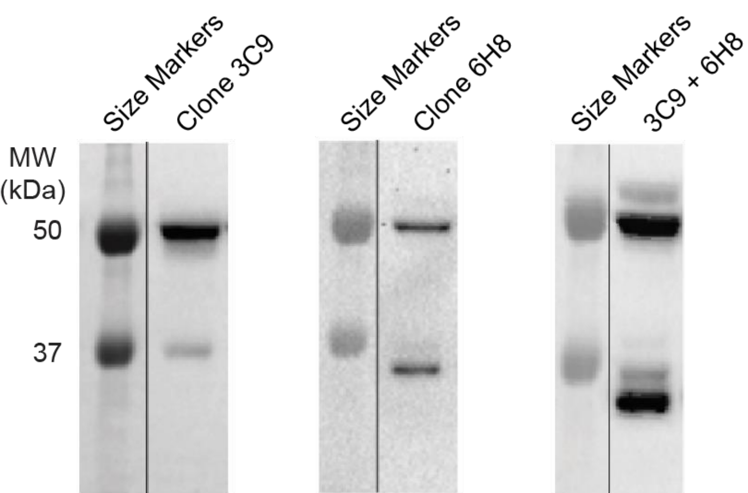


**Fig. S1. Differing reactivity of two widely used clones of anti-human CRIg monoclonal antibody was observed in Western blotting.** The mouse anti-human Z39Ig clone 6H8 (Santa Cruz Biotechnology) and mouse anti-human CRIg clone 3C9 (Genentech), were applied to human macrophage lysates that had undergone SDS-PAGE and transfer to nitrocellulose membranes.

**Table S2. Reactivity of hybridoma culture supernatant from clones 1A3, 2D6, 4H8, 8E12, and 14B11, to murine CRIg by ELISA.**

| Cell Line | Supernatant Dilution | | | | | | Neg Control | Titer |
|---|---|---|---|---|---|---|---|---|
| | 1:10 | 1:30 | 1:90 | 1:270 | 1:810 | 1:2430 | | |
| 1A3-1 | 2.896 | 2.733 | 2.433 | 1.869 | 1.060 | 0.475 | 0.082 | 1:2430 |
| 1A3-2 | 2.811 | 2.687 | 2.389 | 1.775 | 1.017 | 0.477 | 0.082 | 1:2430 |
| 2D6-1 | 1.047 | 0.791 | 0.462 | 0.236 | 0.121 | 0.084 | 0.082 | 1:270 |
| 2D6-2 | 1.033 | 0.735 | 0.447 | 0.202 | 0.119 | 0.099 | 0.082 | 1:270 |
| 4H8-1 | 2.775 | 2.428 | 1.871 | 1.233 | 0.627 | 0.271 | 0.082 | 1:2430 |
| 4H8-2 | 2.544 | 2.099 | 1.434 | 0.843 | 0.402 | 0.182 | 0.082 | 1:2430 |
| 8E12-1 | 3.399 | 3.376 | 3.372 | 3.192 | 2.695 | 1.785 | 0.082 | >1:2430 |
| 8E12-2 | 3.426 | 3.306 | 3.277 | 3.017 | 2.285 | 1.358 | 0.082 | >1:2430 |
| 14B11-1 | 3.100 | 3.047 | 2.895 | 2.239 | 1.329 | 0.593 | 0.082 | >1:2430 |
| 14B11-2 | 3.051 | 2.994 | 2.783 | 2.167 | 1.321 | 0.616 | 0.082 | >1:2430 |

Indirect ELISA was performed by GenScript to test various dilutions of parental hybridoma culture supernatant reactivity (from duplicate preparations per cell line) to full length murine CRIg protein coated at 1 μg/mL in PBS (at pH 7.4), at 100 μL per well of a 96-well plate. Peroxidase AffiniPure goat anti-rat IgG, Fcγ fragment specific (Jackson ImmunoResearch) was used as secondary antibody, with absorbance measured spectrophotometry at 450 nm. The optical densities ($OD_{450}$) are presented. The titer was determined as the highest dilution with signal/negative ≥ 2.1, whilst the $OD_{450}$ in the negative control is the average of two technical replicates.

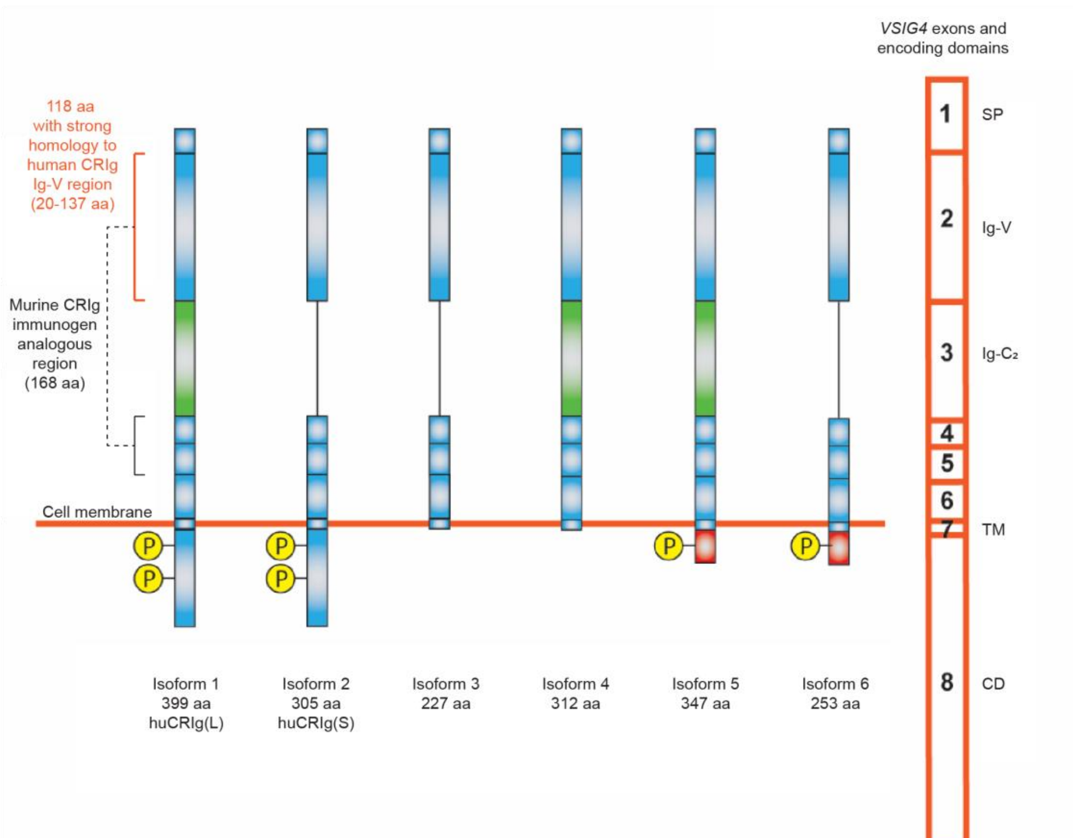


**Fig. S2. Diagram showing the, theoretical, six human CRIg isoforms and their relationship to the selected immunogen for monoclonal antibody production.** The isoforms resulting from alternative splicing were deduced from Genbank mRNA sequence identifiers and NM_007268.3, NM_001100431.2, NM_001184831.1, NM_001184830.1, NM_001257403.1, XM_017029251.2 (adapted from Small, et al (2016) (34)). The immunogen, amino acids 20-187 of murine CRIg (Genbank RefSeq NP_808457.1), has an initial 118 amino acid sequence that has significant homology (80%) to amino acids 20-137 of human CRIg (NP_009199.1), the extracellular immunoglobulin-variable region encoded by *VSIG4* exon 2. All of the six predicted CRIg isoforms harbour this sequence. The remainder of the immunogen sequence has some homology to the regions encoded by *VSIG4* exons 4-5. The abbreviated domain name definitions are as follows: SP, signal peptide; Ig-V, immunoglobulin variable region; Ig-C, immunoglobulin constant region; TM, transmembrane domain; CD, cytoplasmic domain, P, phosphorylation sites.

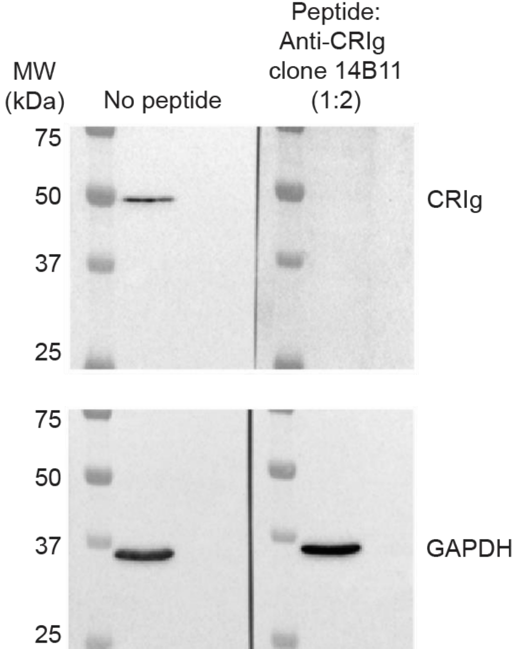


**Fig. S3. Specificity of the anti-CRIg monoclonal antibody clone 14B11 was demonstrated by competitive binding against recombinant CRIg peptide in Western blotting of human macrophages.** Anti-CRIg clone 14B11 was incubated with separate nitrocellulose membranes with identical human MDM lysate, in the absence or presence of recombinant CRIg peptide. One-part peptide nullified detection of CRIg by 2-parts monoclonal antibody.

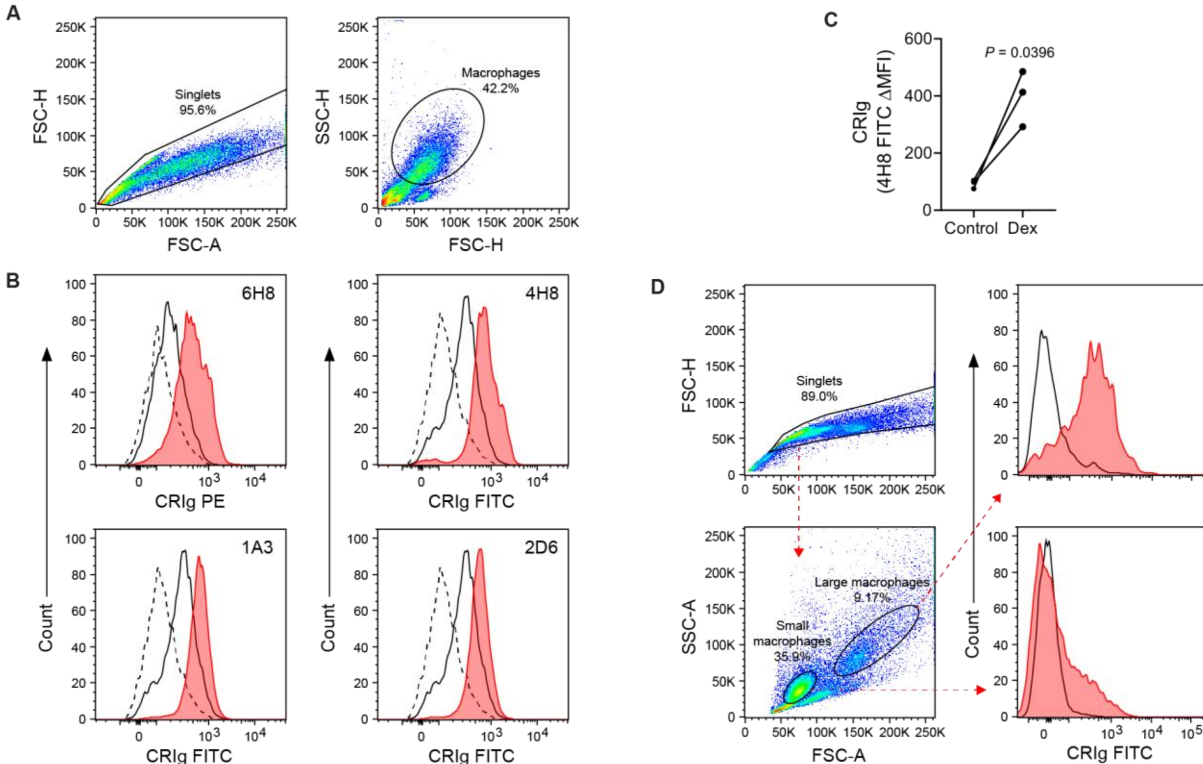


**Fig. S4. The generated monoclonal antibodies stain CRIg on the surface of human MDMs and murine PECs.** (**A**) MDM gating strategy based on size and complexity. (**B**) Representative histogram overlays demonstrating MDM staining with anti-CRIg clone 6H8 (top left) compared with hybridoma supernatant clones 4H8 (top right), 1A3 (bottom left) and 2D6 (bottom right). Dashed line: unstained; solid black: isotype control, shaded: anti-CRIg staining. (**C**) CRIg expression of untreated MDM compared with MDMs differentiated in the presence of 50 ng/mL dexamethasone, stained with purified anti-CRIg hybridoma clone 4H8. Δ median fluorescence intensity (MFI) for CRIg staining minus isotype control is shown from three individual experiments. Data was analyzed by the paired two-tailed Student's *t*-test. (**D**) Gating strategy of murine peritoneal exudate myeloid cells is shown, along with representative histogram demonstrating surface staining using purified rat anti-CRIg monoclonal antibody clone 4H8.

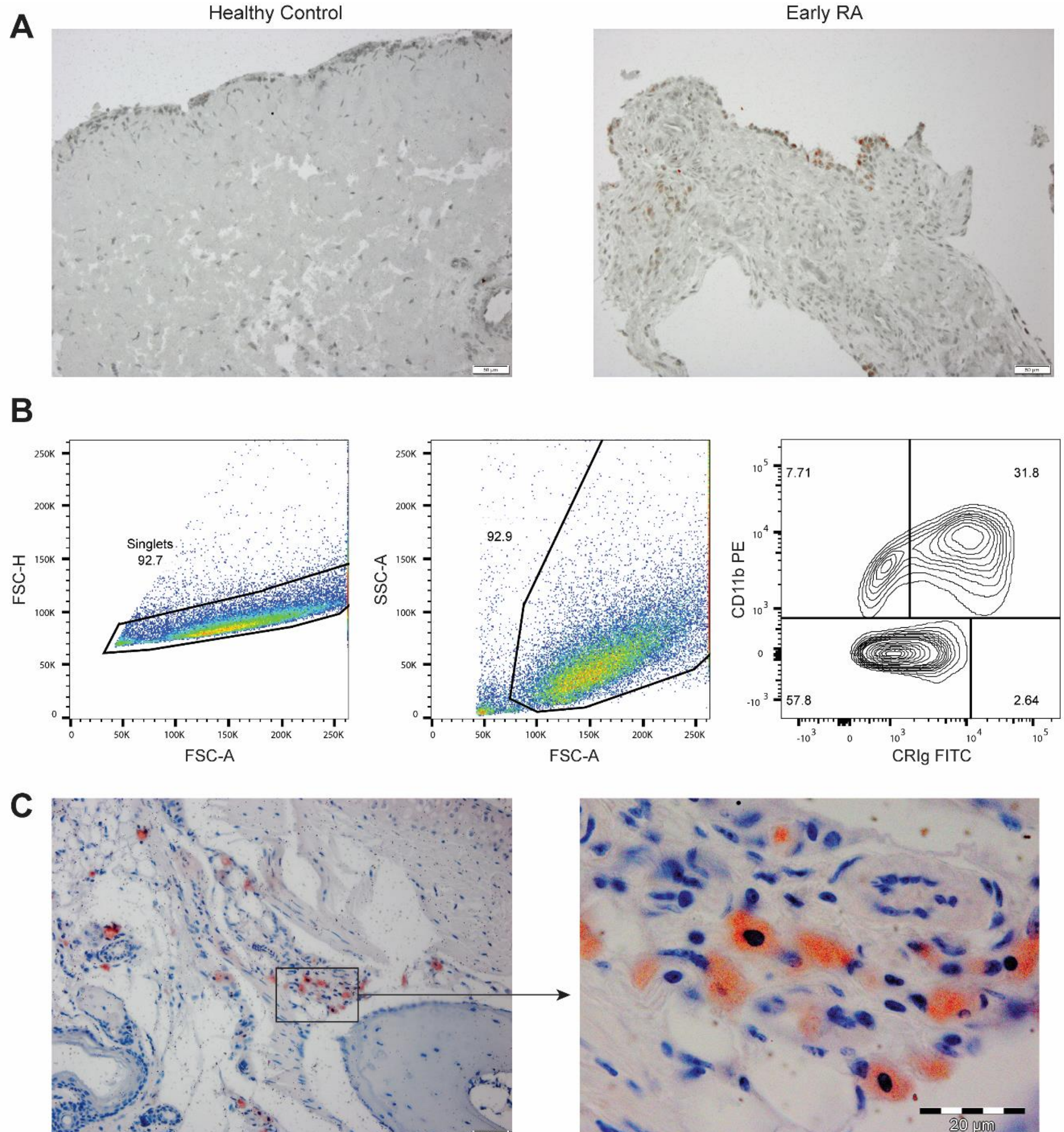


**Fig. S5. Hybridoma clone 14B11 detects CRIg on both human and murine synovial tissue macrophages.** (**A**) Healthy (left panel) and early rheumatoid arthritis (right panel) synovial tissue sections were stained with neat dilution of 14B11 anti-CRIg, followed by rabbit anti-rat secondary antibody, followed by swine anti-rabbit-HRP. Positively stained cells appear red. (**B**) Flow cytometric analysis of murine mononuclear cells. Non-lymphocyte mononuclear cells as judged by size and complexity were gated. Following singlet gating by FSC-A vs. FSC-H (left panel), cells were gated based on CRIg and CD11b expression. (**C**) Healthy murine synovial tissue stained with neat dilution of anti-CRIg clone 14B11, followed by rabbit anti-rat secondary antibody, followed by swine anti-rabbit-HRP. Positively stained cells appear red.

**References**


1 Langnaese, K., Colleaux, L., Kloos, D.U., Fontes, M. & Wieacker, P., Cloning of Z39Ig, a novel gene with immunoglobulin-like domains located on human chromosome X. *Biochimica et biophysica acta* **1492**, 522-525 (2000).

2 Helmy, K.Y., Katschke Jr, K.J., Gorgani, N.N., Kljavin, N.M., Elliott, J.M., Diehl, L., Scales, S.J., Ghilardi, N., van Lookeren Campagne, M., CRIg: a macrophage complement receptor required for phagocytosis of circulating pathogens. *Cell* **124**, 915-927, doi:10.1016/j.cell.2005.12.039 (2006).

3 Kim, J.K., Choi, E.M., Shin, H.I., Kim, C.H., Hwang, S.H., Kim, S.M., Kwon, B.S., Characterization of monoclonal antibody specific to the Z39Ig protein, a member of immunoglobulin superfamily. *Immunol Lett* **99**, 153-161, doi:10.1016/j.imlet.2005.02.012 (2005).

4 van Lookeren Campagne, M. & Verschoor, A. Pathogen clearance and immune adherence “revisited”: Immuno-regulatory roles for CRIg. *Seminars in Immunology* **37**, 4-11, doi:https://doi.org/10.1016/j.smim.2018.02.007 (2018).

5 Liu, G., Fu, Y., Yosri, M., Chen, Y., Sun, P., Xu, J., Zhang, M., Sun, D., Strickland, A.B., Mackey, Z.B., Shi, M., CRIg plays an essential role in intravascular clearance of bloodborne parasites by interacting with complement. *Proc Natl Acad Sci U S A*, doi:10.1073/pnas.1913443116 (2019).

6 Lee, M.Y., Kim, W.J., Kang, Y.J., Jung, Y.M., Kang, Y.M., Suk, K., Park, J.E., Choi, E.M., Choi, B.K., Kwon, B.S., Lee, W.H., Z39Ig is expressed on macrophages and may mediate inflammatory reactions in arthritis and atherosclerosis. *Journal of leukocyte biology* **80**, 922-928, doi:10.1189/jlb.0306160 (2006).

7 Wiesmann, C. Katschke, K.J., Yin, J., Helmy, K.Y., Steffek, M., Fairbrother, W.J., McCallum, S.A., Embuscado, L., Deforge, L., Hass, P.E., van Lookeren Campagne, M., Structure of C3b in complex with CRIg gives insights into regulation of complement activation. *Nature* **444**, 217-220, doi:10.1038/nature05263 (2006).

8 Katschke, K.J., Jr., Helmy, K.Y., Steffek, M., Xi, H., Yin, J., Lee, W.P., Gribling, P., Barck, K.H., Carano, R.A.D., Taylor, R.E., Rangell, L., Diehl, L., Hass, P.E., Wiesmann, C., van Lookeren Campagne, M., A novel inhibitor of the alternative pathway of complement reverses inflammation and bone destruction in experimental arthritis. *The Journal of experimental medicine* **204**, 1319-1325, doi:10.1084/jem.20070432 (2007).

9 Zeng, Z., Surewaard, B.G.J., Wong, C.H.Y., Geoghegan, J.A., Jenne, C.N., Kubes, P., CRIg Functions as a Macrophage Pattern Recognition Receptor to Directly Bind and Capture Blood-Borne Gram-Positive Bacteria. *Cell host & microbe* **20**, 99-106, doi:10.1016/j.chom.2016.06.002 (2016).

10 Li, J., Guo, S., Huang, X., Yang, C., Feng, Z., Yan, W., Ning, Q., Zheng, L., Chen, Y., Wu, Y., VSIG4 inhibits proinflammatory macrophage activation by reprogramming mitochondrial pyruvate metabolism. *Nature Communications* **8**, 1322, doi:10.1038/s41467-017-01327-4 (2017).

11 Huang, X., Feng, Z., Jiang, Y., Li, J., Xiang, Q., Guo, S., Yang, C., Fei, L., Guo, G., Zheng, L., Wu, Y., Chen, Y., VSIG4 mediates transcriptional inhibition of Nlrp3 and Il-1beta in macrophages. *Sci Adv* **5**, eaau7426, doi:10.1126/sciadv.aau7426 (2019).

12 Munawara, U., Perveen, K., Small, A.G., Putty, T., Quach, A., Gorgani, N. N., Hii, C.S., Abbott, C.A., Ferrante, A., Human Dendritic Cells Express the Complement Receptor Immunoglobulin Which Regulates T Cell Responses. *Frontiers in Immunology* **10**, doi:10.3389/fimmu.2019.02892 (2019).

13 Byun, J.M., Jeong, D.H., Choi, I.H., Lee, D.S., Kang, M.S., Jung, K.O., Jeon, Y.K., Kim, Y.N., Jung, E.J., Lee, K.B., Sung, M.S., Kim, K.T., The Significance of VSIG4 Expression in Ovarian Cancer. *Int J Gynecol Cancer* **27**, 872-878, doi:10.1097/igc.0000000000000979 (2017).

14 Zhang, X. H., Qian, Y., Li, Z., Zhang, N. N. & Xie, Y. J. Let-7g-5p inhibits epithelial-mesenchymal transition consistent with reduction of glioma stem cell phenotypes by targeting VSIG4 in glioblastoma. *Oncol Rep* **36**, 2967-2975, doi:10.3892/or.2016.5098 (2016).

15 Xu, T., Jiang, Y., Yan, Y., Wang, H., Lu, C., Xu, H., Li, W., Fu, D., Lu, Y., Chen, J., VSIG4 is highly expressed and correlated with poor prognosis of high-grade glioma patients. *Am J Transl Res* **7**, 1172-1180 (2015).

16 Menyhart, O., Kakisaka, T., Pongor, L.S., Uetake, H., Goel, A., Győrffy, B., Uncovering Potential Therapeutic Targets in Colorectal Cancer by Deciphering Mutational Status and Expression of Druggable Oncogenes. *Cancers (Basel)* **11**, doi:10.3390/cancers11070983 (2019).

17 Nagre, N., Cong, X., Terrazas, C., Pepper, I., Schreiber, J.M., Fu, H., Sill, J.M., Christman, J.W., Satoskar, A.R., Zhao, X., Inhibition of Macrophage Complement Receptor CRIg by TRIM72 Polarizes Innate Immunity of the Lung. *Am J Respir Cell Mol Biol* **58**, 756-766, doi:10.1165/rcmb.2017-0236OC (2018).

18 Vogt, L., Schmitz, N., Kurrer, M.O., Bauer, M., Hinton, H.I., Behnke, S., Gatto, D., Sebbel, P., Beerli, R.R., Sonderegger, I., Kopf, M., Saudan, P., Bachmann, M.F., VSIG4, a B7 family-related protein, is a negative regulator of T cell activation. *J Clin Invest* **116**, 2817-2826, doi:10.1172/jci25673 (2006).

19 Yuan, X., Yang, B.-H., Dong, Y., Yamamura, A. & Fu, W. CRIg, a tissue-resident macrophage specific immune checkpoint molecule, promotes immunological tolerance in NOD mice, via a dual role in effector and regulatory T cells. *eLife* **6**, e29540, doi:10.7554/eLife.29540 (2017).

20 Uhlén, M., Fagerberg L., Hallström, B.M., Lindskog, C., Oksvold, P., Mardinoglu, A, Sivertsson, Å., Kampf, C., Sjöstedt, E., Asplund, A., Olsson, I., Edlund, K., Lundberg, E., Navani, S., Szigyarto, C.A.K., Odeberg, J., Djureinovic, D., Takanen, J.O., Hober, S., Alm, T., Edqvist, P.H., Berling, H., Tegel, H., Mulder, J., Rockberg, J., Nilsson, P., Schwenk, J.M., Hamsten, M., von Feilitzen, K., Forsberg, M., Persson, L., Johansson, F., Zwahlen, M., von Heijne, G., Nielsen J., and Pontén, F., Proteomics. Tissue-based map of the human proteome. *Science* **347**, 1260419, doi:10.1126/science.1260419 (2015).

21 Munawara, U., Small, A.G., Quach, A., Gorgani, N.N., Abbott, C.A., Ferrante, A., Cytokines regulate complement receptor immunoglobulin expression and phagocytosis of Candida albicans in human macrophages: A control point in anti-microbial immunity. *Scientific Reports* **7**, 4050, doi:10.1038/s41598-017-04325-0 (2017).

22 Martrus, G. Goebels, H., Langeneckert, A.E., Kah, J., Flomm, F., Ziegler, A.E., Niehrs, A., Löbl, S.M., Russu, K., Hess, L.U., Salzberger, W., Poch, T., Nashan, B., Schramm, C., Oldhafer, K.J., Dandri, M., Koch, M., Lunemann, S., Altfeld, M., CD49a Expression Identifies a Subset of Intrahepatic Macrophages in Humans. *Frontiers in Immunology* **10**, doi:10.3389/fimmu.2019.01247 (2019).

23 Scott, K.L., Nogueira, C., Heffernan, T.P., van Doorn, R., Dhakal, S., Hanna, J.A., Min, C., Jaskelioff, M., Xiao, Y., Wu, C.J., Cameron, L.A., Perry, S.R., Zeid, R., Feinberg, T., Kim, M., Vande Woude, G., Granter, S.R., Bosenberg, M., Chu, G.C., DePinho, R.A., Rimm, D., L., Chin, L., Proinvastion Metastasis Drivers in Early-Stage Melanoma Are Oncogenes. *Cell Press* **20**, 92-103, doi: https://doi.org/10.1016/j.ccr.2011.05.025 (2011)

24 Misharin, A.V., Cuda, C.M., Saber, R., Turner, J.D., Gierut, A.K., Haines 3rd, G.K., Berdnikovs, S., Filer, A., Clark, A.R., Buckley, C.D., Mutlu, G.M., Budinger, G.R.S., Perlman, H., Nonclassical Ly6C− Monocytes Drive the Development of Inflammatory

Arthritis in Mice. *Cell Reports* **9**, 591-604, doi:https://doi.org/10.1016/j.celrep.2014.09.032 (2014).

25 Irvine, K.M., Banh, X., Gadd, V.L., Kojcik, K.K., Ariffin, J.K., Jose, S., Lukowski, S., Baillie, G.J., Sweet, M.J., Powell, E.E., CRIg-expressing peritoneal macrophages are associated with disease severity in patients with cirrhosis and ascites. *JCI insight* **1**, e86914, doi:10.1172/jci.insight.86914 (2016).

25 Ma, Y., Usuwanthim, K., Munawara, U., Quach, A., Gorgani, N.N., Abbott, C.A., Hii, C.S., Ferrante, A., Protein Kinase Cα Regulates the Expression of Complement Receptor Ig in Human Monocyte–Derived Macrophages. *The Journal of Immunology* **194**, 2855 (2015).

27 Gorgani, N.N., Thathaisong, U., Mukaro, V.R.S., Poungpair, O., Tirimacco, A., Hii, C.S.T., Ferrante, A., Regulation of CRIg expression and phagocytosis in human macrophages by arachidonate, dexamethasone, and cytokines. *The American journal of pathology* **179**, 1310-1318, doi:10.1016/j.ajpath.2011.05.021 (2011).

28 Gorgani, N.N., He, J.Q., Katschke Jr., K.J., Helmy, K.J., Xi, H., Steffek, M., Hass, P.E., van Lookeren Campagne, M., Complement receptor of the Ig superfamily enhances complement-mediated phagocytosis in a subpopulation of tissue resident macrophages. *Journal of immunology (Baltimore, Md. : 1950)* **181**, 7902-7908 (2008).

29 Zheng, F., Put, S., Bouwens, L., Lahoutte, T., Matthys, P., Muyldermans, S., De Baetselier, P., Devoogdt, N., Raes, G., Schoonooghe, S., Molecular imaging with macrophage CRIg-targeting nanobodies for early and preclinical diagnosis in a mouse model of rheumatoid arthritis. *Journal of nuclear medicine : official publication, Society of Nuclear Medicine* **55**, 824-829, doi:10.2967/jnumed.113.130617 (2014).

30 Tanaka, M., Nagai, T., Tsuneyoshi, Y., Sunahara, N., Matsuda, T., Nakamura, T., Tsuyama, S., Hasui, K., FitzGerald, O., Matsuyama, T., Expansion of a unique macrophage subset in rheumatoid arthritis synovial lining layer. *Clinical and experimental immunology* **154**, 38-47, doi:10.1111/j.1365-2249.2008.03733.x (2008).

31 Smith, M.D., Weedon, H., Papangelis, V., Walker, J., Roberts-Thomson, P.J., Ahern, M.J., Apoptosis in the rheumatoid arthritis synovial membrane: modulation by disease-modifying anti-rheumatic drug treatment. *Rheumatology* **49**, 862-875, doi:10.1093/rheumatology/kep467 (2010).

32 Gregory, S. H., Sagnimeni, A. J. & Wing, E. J. Bacteria in the bloodstream are trapped in the liver and killed by immigrating neutrophils. *Journal of immunology (Baltimore, Md. : 1950)* **157**, 2514-2520 (1996).

33 Yuan, S., Wang, Y., Luo, H., Jiang, Z., Qiao, B., Jiang, Y., Hu, Y., Cheng, Y., Chen, X., Weihua, G., Huang, Y., Zhao, W., Luo, D., Liu, B., Su, H., Zhou, J., Song, S., Serum soluble VSIG4 as a surrogate marker for the diagnosis of lymphoma-associated hemophagocytic lymphohistiocytosis. *British Journal of Haematology* **n/a**, doi:10.1111/bjh.16299 (2020).

34 Small, A. G., Al-Baghdadi, M., Quach, A., Hii, C. & Ferrante, A. Complement receptor immunoglobulin: a control point in infection and immunity, inflammation and cancer. *Swiss medical weekly* **146**, w14301, doi:10.4414/smw.2016.14301 (2016).

## Part 2. Other Supplementary Material

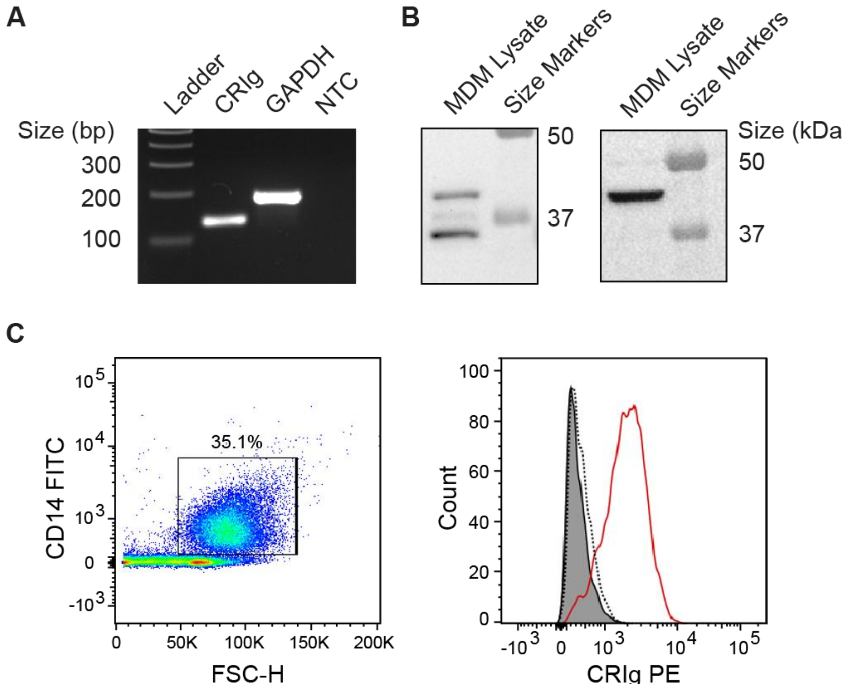


**Fig. S6. CRIg expression by human monocyte-derived macrophages**. (**A**) Gel of CRIg and *GAPDH* cDNA amplicons from monocyte RNA. NTC, no template control. (**B**) Western blot of macrophage protein lysate, stained with anti-CRIg monoclonal antibody, clone 6H8 (left), or clone 14B11 (right panel). (**C**) CRIg expression on the surface of MDM gated by $CD14^+$ staining. The histogram overlay shows unstained (shaded), isotype (dashed line), and CRIg clone 6H8 PE staining (red line).

**Table S3. Summary of the inflammatory mediators used in this study and the known neutrophil compartments from which they induce degranulation, as well as signalling pathways involved in cytoskeleton rearrangements and the granule release.**

| | | Azurophilic Granules | Specific Granules | Gelatinase Granules | Secretory Vescicles | References |
|---|---|---|---|---|---|---|
| Stimulants | TNF | - | - | + | + | [1] |
| | fMLP | - | - | + | + | [1-3] |
| | PMA | - | + | + | - | [2,4-7] |
| | LPS | - | + | + | low | [8,9] |
| | LTB4 | - | + | + | + | [10-13] |
| | fMLP + Cyt B | + | + | *ND* | *ND* | [14-17] |
| Cytoskeleton related proteins | Rac1 | + | + | *ND* | *ND* | [18,19] |
| | Rac2 human | + | - | *ND* | *ND* | [20] |
| | Rab27a | + | + | + | low | [5] |
| | ARPC1B | *+ | *ND* | *ND* | *ND* | [21] |

+, indicates that the stimulant induces degranulation, -, indicates that the stimulant does not induce degranulation, ND; indicates role is not defined.

*, Deficiency in this molecule results in enhanced granule release in human neutrophils.

**Fig. S6 and Table S3 References**


1 McLeish, K.R., Merchant, M.L., Creed, T.M., Tandon, S., Barati, M.T., Uriarte, S.M., Ward, R.A., Frontline Science: Tumor necrosis factor-α stimulation and priming of human neutrophil granule exocytosis. *Journal of leukocyte biology* **102**, 19-29, doi:10.1189/jlb.3HI0716-293RR (2017).

2 Videm, V. & Strand, E., Changes in Neutrophil Surface-Receptor Expression After Stimulation with FMLP, Endotoxin, Interleukin-8 and Activated Complement Compared to Degranulation. *Scandinavian journal of immunology* **59**, 25-33, doi:10.1111/j.0300-9475.2004.01351.x (2004).

3 Kjeldsen, L., Sengelov, H., Lollike, K., Nielsen, M. H., Borregaard, N., Isolation and characterization of gelatinase granules from human neutrophils. *Blood* **83**, 1640-1649 (1994).

4 Parker, H., Albrett, A.M., Kettle, A.J., Winterbourn, C.C., Myeloperoxidase associated with neutrophil extracellular traps is active and mediates bacterial killing in the presence of hydrogen peroxide. *Journal of leukocyte biology* **91**, 369-376, doi:10.1189/jlb.0711387 (2012).

5 Herrero-Turrión, M. J., Calafat, J., Janssen, H., Fukuda, M., Mollinedo, F., Rab27a Regulates Exocytosis of Tertiary and Specific Granules in Human Neutrophils. *The Journal of Immunology* **181**, 3793, doi:10.4049/jimmunol.181.6.3793 (2008).

6 Tseng, A., Kim, K., Li, J., Cho, J., Myeloperoxidase Negatively Regulates Neutrophil-Endothelial Cell Interactions by Impairing αMβ2 Integrin Function in Sterile Inflammation. *Frontiers in medicine* **5**, 134-134, doi:10.3389/fmed.2018.00134 (2018).

7 Mollinedo, F., Calafat, J., Janssen, H., Martín-Martín, B., Canchado, J., Nabokina, S.M., Gajate, C., Combinatorial SNARE complexes modulate the secretion of cytoplasmic granules in human neutrophils. *Journal of immunology (Baltimore, Md. : 1950)* **177**, 2831-2841, doi:10.4049/jimmunol.177.5.2831 (2006).

8 Naegelen, I., Beaume, N., Plançon, S., Schenten, V., Tschirhart, E.J., Bréchard, S., Regulation of Neutrophil Degranulation and Cytokine Secretion: A Novel Model Approach Based on Linear Fitting. *J Immunol Res* **2015**, 817038, doi:10.1155/2015/817038 (2015).

9 Almkvist, J., Fäldt, J., Dahlgren, C., Leffler, H., Karlsson, A., Lipopolysaccharide-Induced Gelatinase Granule Mobilization Primes Neutrophils for Activation by Galectin-3 and Formylmethionyl-Leu-Phe. *Infection and immunity* **69**, 832 (2001).

10 Smith, R.J., Iden, S.S., Bowman, B.J., Activation of the human neutrophil secretory process with 5(S),12(R)-dihydroxy-6, 14-cis-8,10-trans-eicosatetraenoic acid. *Inflammation* **8**, 365-384, doi:10.1007/BF00918213 (1984).

11 O'Dwyer, C.A., O'Brien, M.E., Wormwald, M.R., White, M.M., Banville, N., Hurley, K., McCarthy, C., McElvaney, N.G., Reeves, E.P., The BLT1 Inhibitory Function of alpha-1 Antitrypsin Augmentation Therapy Disrupts Leukotriene B4 Neutrophil Signaling. *Journal of immunology (Baltimore, Md. : 1950)* **195**, 3628-3641, doi:10.4049/jimmunol.1500038 (2015).

12 Cassatella, M. A. *The neutrophil: an emerging regulator of inflammatory and immune response*. Vol. 83 (Karger Medical and Scientific Publishers, 2003).

13 Di Gennaro, A., Kenne, E., Wan, M., Soehnlein, O., Lindbom, L., Haeggström, J.Z., Leukotriene B4-induced changes in vascular permeability are mediated by neutrophil release of heparin-binding protein (HBP/CAP37/azurocidin). *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **23**, 1750-1757, doi:10.1096/fj.08-121277 (2009).

14 Bignold, L. P. & Ferrante, A. Effects of cytochalasin B, N-formyl peptide and plasma on polarisation, zeiosis (blebbing) and degranulation of polymorphonuclear leukocytes in suspension. *Cell Biology International Reports* **12**, 195-203, doi:10.1016/0309-1651(88)90096-3 (1988).

15 Smith, R. J., Wierenga, W., Iden, S. S. Characteristics ofN-formyl-methionyl-leucyl-phenylalanine as an inducer of lysosomal enzyme release from human neutrophils. *Inflammation* **4**, 73-88, doi:10.1007/BF00914105 (1980).

16 Yurewicz, E. C. & Zimmerman, M. Cytochalasin B-dependent release of azurophil granule enzymes from human polymorphonuclear leukocytes. *Inflammation* **2**, 259-264, doi:10.1007/bf00921005 (1977).

17 Ferrante, A., Nandoskar, M., Walz, A., Goh, D. H. B., Kowanko, I. C. Effects of Tumour Necrosis Factor Alpha and Interleukin-1 Alpha and Beta on Human Neutrophil Migration, Respiratory Burst and Degranulation. *International Archives of Allergy and Immunology* **86**, 82-91, doi:10.1159/000234610 (1988).

18 Mitchell, T., Lo, A., Logan, M. R., Lacy, P., Eitzen, G., Primary granule exocytosis in human neutrophils is regulated by Rac-dependent actin remodeling. *Am J Physiol Cell Physiol* **295**, C1354-C1365, doi:10.1152/ajpcell.00239.2008 (2008).

19 Chi, X., Wang, S., Huang, Y., Stamnes, M., Chen, J.-L., Roles of rho GTPases in intracellular transport and cellular transformation. *Int J Mol Sci* **14**, 7089-7108, doi:10.3390/ijms14047089 (2013).

20 Ambruso, D.R., Knall, C., Abell, A.N., Panepinto, J., Kurkchubasche, A., Thurman, G., Gonzalez-Aller, C., Hiester, A., deBoer, M., Harbeck, R.J., Oyer, R., Johnson, G.L., Roos, D., Human neutrophil immunodeficiency syndrome is associated with an inhibitory Rac2 mutation. *Proceedings of the National Academy of Sciences of the United States of America* **97**, 4654-4659, doi:10.1073/pnas.080074897 (2000).

21 Kuijpers, T.W., Tool, A.T.J., van der Bijl, I., de Boer, M., van Houdt, M., de Cuyper, I.M., Roos, D., van Alphen, F., van Leeuwen, K., Cambridge, E.L., Arends, M.J., Dougan, G., Clare, S., Ramirez-Solis, R., Pals, S.T., Adams, D.J., Meijer, A.B., van der Berg, T.K., Combined immunodeficiency with severe inflammation and allergy caused by ARPC1B deficiency. J. Allergy Clin. Immunol., 2017. 140. p. 273-277.

# Chapter 4. Expression of CRIg by Human MDM and Modulation by Inflammatory Mediators

## 4.1 Introduction and Contextual Statement

This chapter presents the third published manuscript included in this thesis. The work presented within focused on investigating the inflammatory mediator network which regulates CRIg expression by healthy human MDMs at both the developmental stages and following maturation. Importantly, this work demonstrates that cytokines that are considered inflammatory such as TNF and IFN-γ down regulate CRIg expression at both the mRNA and protein levels, while anti-inflammatory agents such as dexamethasone and IL-10 induce the opposite. These modulations in CRIg expression were independent of expression levels of CR3 and CR4. Additionally, the regulation of CRIg was found to correlate with the ability of MDMs to phagocytose the fungal pathogen *Candida albicans.*

The following paper entitled *'Cytokines regulate complement receptor immunoglobulin expression and phagocytosis of Candida albicans in human macrophages: A control point in anti-microbial immunity'*, by Usma Munawara, **Annabelle G. Small**, Alex Quach, Nick N. Gorgani, Catherine A. Abbott & Antonio Ferrante was published in the peer reviewed journal, Scientific Reports, in June 2017 (7: 4050, DOI:10.1038/s41598-017-04325-0). Supplementary information follows the paper.

*Disclaimer: Significant portions of the results presented in this manuscript were generated by Dr. Usma Munawara throughout the completion of her Ph.D. studies and submitted for the award of her degree.*

**Article Metrics (as of November 2020)**

*Journal Impact Factor: 4.122*

*Citations: 7*

# Statement of Authorship

| | |
|---|---|
| Title of Paper | Cytokines regulate complement receptor immunoglobulin expression and phagocytosis of Candida albicans in human macrophages: A control point in anti-microbial immunity |
| Publication Status | [X] Published [ ] Accepted for Publication [ ] Submitted for Publication [ ] Unpublished and Unsubmitted work written in manuscript style |
| Publication Details | Munawara, U., Small, A. G., Quach, A., Gorgani, N. N., Abbott, C. A., & Ferrante, A. (2017). Cytokines regulate complement receptor immunoglobulin expression and phagocytosis of Candida albicans in human macrophages: A control point in anti-microbial immunity. Scientific Reports, 7(1), 4050. doi:10.1038/s41598-017-04325-0 |

## Principal Author

| | | | |
|---|---|---|---|
| Name of Principal Author (Candidate) | Annabelle Small | | |
| Contribution to the Paper | AS conducted the experiments, performed data analysis, was involved in data interpretation, and assisted in the writing and critical reading of the manuscript. | | |
| Overall percentage (%) | 30% | | |
| Certification: | This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. | | |
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## Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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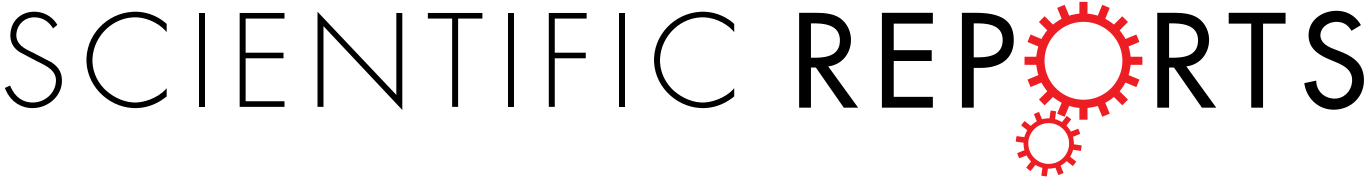
| Signature | | Date | 03/03/2020 |



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# Cytokines regulate complement receptor immunoglobulin expression and phagocytosis of *Candida albicans* in human macrophages: A control point in anti-microbial immunity

Usma Munawara[1,2], Annabelle G. Small[2], Alex Quach[2], Nick N. Gorgani[2,3,5], Catherine A. Abbott[1] & Antonio Ferrante[2,4]

**Complement Receptor Immunoglobulin (CRIg), selectively expressed by macrophages, plays an important role in innate immunity by promoting phagocytosis of bacteria. Thus modulation of CRIg on macrophages by cytokines can be an important mechanism by which cytokines regulate anti-microbial immunity. The effects of the cytokines, tumor necrosis factor, transforming growth factor-β1, interferon-γ, interleukin (IL)-4, IL-13, IL-10, IL-1β, IL-6, lymphotoxin-α, macrophage-colony stimulating factor (M-CSF) and GM-CSF on CRIg expression were examined in human macrophages. We demonstrated that cytokines regulated the CRIg expression on macrophages during their development from monocytes in culture at the transcriptional level using qPCR and protein by Western blotting. Both CRIg spliced forms (Long and Short), were similarly regulated by cytokines. Direct addition of cytokines to matured CRIg+ macrophages also changed CRIg mRNA expression, suggesting that cytokines control macrophage function via CRIg, at two checkpoints. Interestingly the classical complement receptors, CR3 and CR4 were differentially regulated by cytokines. The changes in CRIg but not CR3/CR4 mRNA expression correlated with ability to phagocytose *Candida albicans* by macrophages. These findings suggest that CRIg is likely to be a control point in infection and immunity through which cytokines can mediate their effects, and is differentially regulated from CR3 and CR4 by cytokines.**

Members of complement, Toll-like and scavenger receptors as well as C-type lectins are amongst the groups of receptors that initially recognize opsonised-pathogen or pathogen-associated molecular patterns. In the last decade, the B7 family-related protein V-set and Ig domain-containing 4 (VSIG4) (Z39Ig)[1–3], was found to be an important complement (CRIg) receptor[4]. This receptor differs structurally and functionally from the classical complement receptors, CR3 and CR4. CRIg is expressed selectively by macrophages and is involved in the rapid phagocytosis of complement (C3b/iC3b)-opsonised pathogens[5]. The presence of CRIg on Kupffer cell surfaces results in the rapid uptake of circulating *Listeria monocytogenes* and *Staphylococcus aureus*, thereby limiting bacterial dissemination and pathogenesis[4]. CRIg$^{-/-}$ mice infected with these bacteria exhibited exaggerated levels of inflammatory cytokines, and died earlier than wild type mice. More recently the uniqueness of this receptor in the

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clearance of bacteria by Kupffer cells was evident by showing that it promoted bacterial clearance by a dual track system in a complement dependent manner[6] and clearance of gram-positive bacteria via non-complement ligands[6,7]. While the function of CRIg in immunity to infection appears well established, there is little known about the modulation of expression of CRIg by inflammatory mediators generated during infection and inflammation.

It has been previously reported that monocytes in culture begin to express CRIg as they differentiate into macrophages[4,8]. The development of $CRIg^+$ macrophages was found to be up- (IL-10) or down (IFN-$\gamma$, IL-4 and TGF-$\beta$1) regulated by cytokines based on CRIg mRNA levels. The purpose of our investigation was to extend the work to include other important cytokines generated during infection and inflammation: IL-13, IL-1$\beta$, IL-6, lymphotoxin-$\alpha$, M-CSF and GM-CSF, examining whether this relates to changes in CRIg protein expression level to enable us to evaluate the effects on the two spliced forms of CRIg, as well as assessing effects on mature macrophages. Since CRIg is likely to co-exist with CR3 and CR4 on these macrophages, comparisons were made with the expression of these receptors. Finally the cytokine-induced modulation of expression of these complement receptors was examined in the context of their anti-microbial action against complement opsonised *Candida albicans*.

## Results

We have previously shown that cultured human monocytes displayed maximal increase in CRIg mRNA expression on day 3 of culture and protein on day 5–7[8]. We confirmed these results in preliminary experiments (data not presented). Thus experiments were designed around these time points for examining the effects of cytokines on macrophage CRIg expression. Cytokines known to regulate macrophage function and which are produced in inflammatory sites were evaluated for their effects on CRIg expression.

**Effect of cytokines on the development of $CRIg^+$ macrophages.** Monocytes were cultured in the presence of either the Th1 cytokines, LT-$\alpha$ and IFN-$\gamma$ or the Th2 cytokines IL-4 and IL-13 and then examined for levels of CRIg mRNA after 3 days by qPCR and protein at day 7 by Western blot analysis using anti-CRIg antibody. In the presence of LT-$\alpha$ there was an increase in CRIg mRNA and marked increase in CRIg protein (Fig. 1a and b). In contrast, IFN-$\gamma$ caused a substantial decrease in CRIg mRNA and protein expression (Fig. 1c and d). These effects were seen over a concentration range of 5–40 ng/ml for LT-$\alpha$ and 10–40 ng/ml for IFN-$\gamma$. The Th2 cytokines, IL-4 and IL-13 both markedly inhibited the expression of CRIg at the mRNA and protein levels (Fig. 2). The effects occurred in a concentration range of 1–40 ng/ml for IL-4 and 5–40 ng/ml for IL-13. Western blot analysis enabled us to distinguish between the two different forms of CRIg, the long (L) and short (S) forms (Fig. S1). The data in Figs 1(b,d,e) and 2(b,d,e) showed that the two forms were similarly regulated by the cytokines. It is also evident that CRIg(L) is the more prominent form in these macrophages, even after treatment with cytokines.

TNF, IL-1$\beta$ and IL-6 are cytokines referred to as pyrogenic and pro-inflammatory cytokines which predominate during infection and inflammation, associated with chronic inflammatory diseases. Because of the importance of CRIg in phagocytosis and regulation of inflammation, their effects on CRIg expression in cultured macrophages were examined. Treatment of monocytes with TNF caused a marked reduction of CRIg mRNA and protein in the maturing macrophages (Fig. 3a and b). This reduced expression occurred in a concentration dependent manner. In relation to CRIg protein expression, TNF caused approximately 80% reduction. IL-1$\beta$, and in particular IL-6 increased CRIg expression in macrophages (Fig. 3c–f). CRIg protein expression analysed by Western blotting demonstrated that expression of both forms, L and S, were altered in a similar manner in cells cultured in the presence of these cytokines (Fig. 3b,d,f,g). In order to gain more physiological meaningful information in regards to cytokine profiles and CRIg expression on macrophages, mixtures of cytokines that are up-regulated in bacterial infections and chronic inflammatory conditions such as rheumatoid arthritis were examined, namely IL-1$\beta$, IL-6 and TNF. When monocytes were cultured in the presence of this mixture of cytokines, there was a resultant increase in expression of CRIg during their development i.e. the down regulation induced by TNF was overcome by having IL-1$\beta$ and IL-6 present (Fig. 3h).

TGF-$\beta$1 and IL-10 share a number of properties and have been shown to regulate and depress inflammation. Their effects on macrophage function have been reported. We have now examined their effects on CRIg mRNA and protein expression. Culturing monocytes with TGF-$\beta$1 led to a concentration (2–15 ng/ml) related decrease in CRIg mRNA expression with almost complete suppression of CRIg protein expression (Fig. 4a and b). In contrast IL-10 caused a marked increase in CRIg expression in macrophages (Fig. 4c and d). When we compared this with the effects of dexamethasone it was evident that IL-10 was as effective as dexamethasone in increasing CRIg expression (Fig. 4e and f). This was seen at both the mRNA and protein level. Although the effects of dexamethasone on total CRIg cellular protein was not previously studied[8] it is evident from the Western blot analysis that the steroid increased the cellular expression of both CRIg(L) and CRIg(S) forms (Fig. 4f). Both forms of CRIg were similarly regulated by TGF-$\beta$1 and IL-10 (Fig. 4b and d).

We extended our studies to another set of cytokines which are involved in controlling macrophage function, M-CSF and GM-CSF. When monocytes were cultured in the presence of these cytokines, both caused an increase in CRIg mRNA and protein expression in the macrophage population (Fig. 5). Both of these cytokines caused a marked increase in expression, comparable to that induced by IL-10. The expression of both CRIg L and S forms was increased by M-CSF and GM-CSF (Fig. 5b and d).

**The effect of cytokines on CRIg expression on mature macrophages (MDM).** In the previous section we have presented data which resulted from examining the effects of cytokines on the development of $CRIg^+$ macrophages, monocyte-derived macrophages (MDM). While this forms one stage of understanding of how mediators control CRIg expression in macrophages in particular during inflammation and monocytes infiltration into tissues, it does not reveal whether mature macrophages present already expressing CRIg can be modulated by

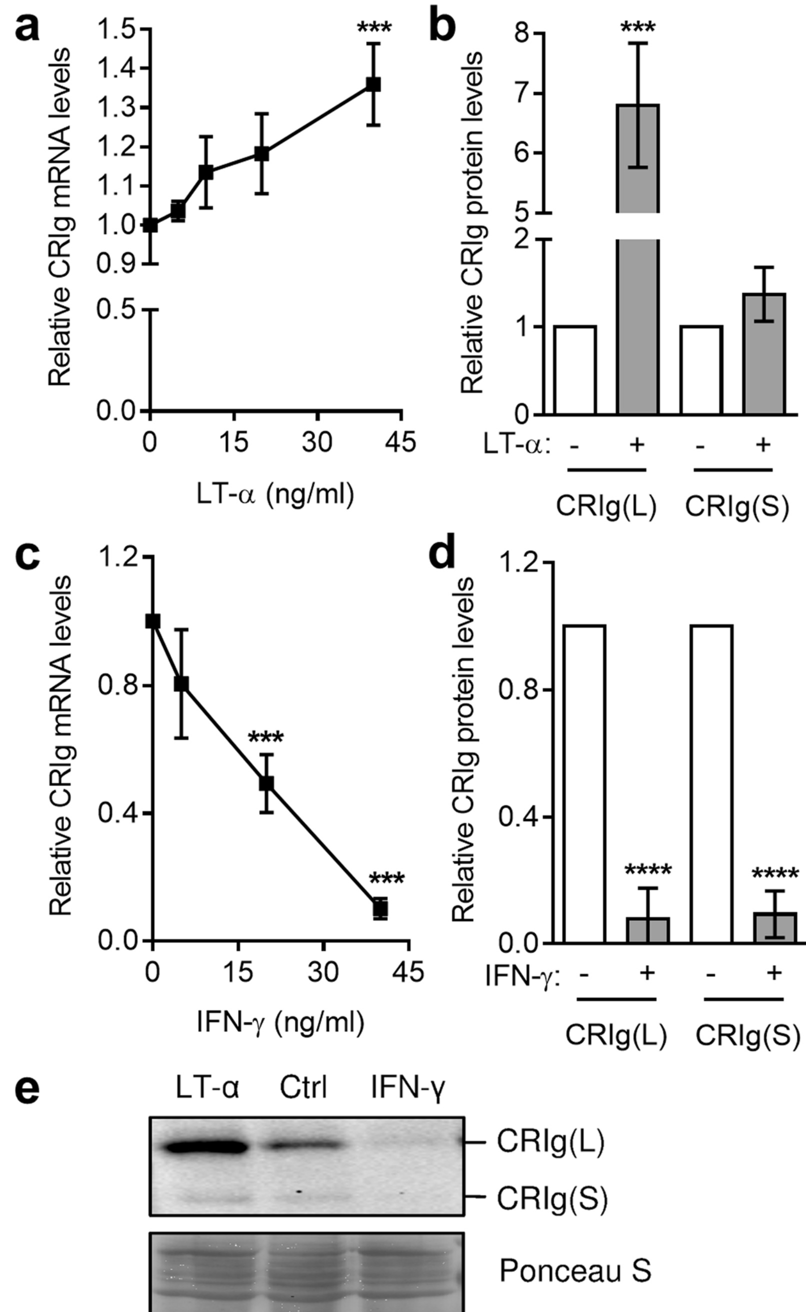


**Figure 1.** The development of CRIg$^+$ macrophages is differentially modulated by LT-α and IFN-γ. Monocytes were cultured in the presence of 0, 5, 10, 20, and 40 ng/ml LT-α (**a**) or 0, 10, 20, and 40 ng/ml IFN-γ (**c**) for 3 days and then CRIg mRNA expression measured. Data are expressed as fold-change over GAPDH-normalised CRIg mRNA in the absence of cytokine set as 1. For CRIg protein expression monocytes were treated with 40 ng/ml LT-α (**b**,**e**) or IFN-γ (**d**,**e**) for 7 days and then the CRIg protein levels measured. Note both the Long and Short forms of CRIg are expressed. (**e**) A representative Western blot of total protein lysates is shown with Ponceau S staining showing consistency of protein load. Data are expressed as fold-difference in CRIg band intensity as determined by densitometry with CRIg expression in the absence of cytokine set as 1. Data are presented as means ± SD of three experiments each conducted with cells from three different individuals. ***$p < 0.001$, ****$p < 0.0001$.

cytokines. Thus a second stage for regulating inflammation is for cytokines to act on already mature macrophages, such as MDM.

MDM expressing CRIg were generated from monocytes in culture in the absence of cytokines. The MDM were then examined to see what effects cytokines had on expression of CRIg mRNA expression. The macrophages were treated with the cytokines for 24 h and then examined for levels of CRIg mRNA. Treatment with 5–40 ng/ml of LT-α caused an increase in CRIg mRNA (Fig. 6a). In comparison, another Th1 cytokine IFN-γ caused a

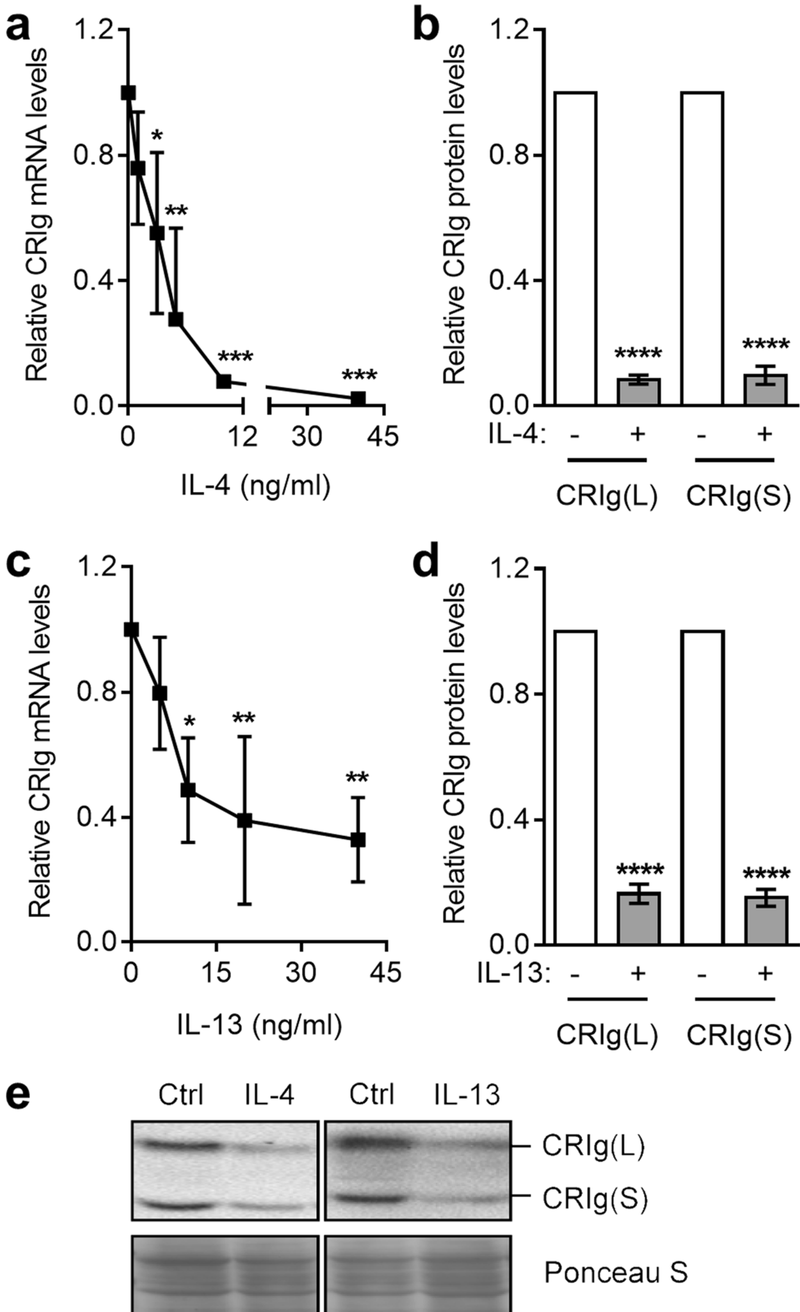


**Figure 2.** IL-4 and IL-13 down-regulate the development of $CRIg^{+}$. Monocytes were cultured in the presence of 0, 1, 3, 5, 10, and 40 ng/ml IL-4 (**a**) or 0, 5, 10, 20, and 40 ng/ml IL-13 (**c**) and CRIg mRNA expression was measured by qPCR. For CRIg protein expression the monocytes were treated with 40 ng/ml (**b**) IL-4 or IL-13 (**d**). (**e**) Representative Western blots of CRIg levels (IL-4 and IL-13 treatments were analysed on separate blots). Data are presented as means ± SD of three experiments, each conducted with cells from different individuals. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

marked decrease in CRIg mRNA expression over a concentration range of 5–40 ng/ml reaching a decrease of approximately 60% at 40 ng/ml (Fig. 6b). TNF is also considered a Th1 lymphocyte cytokine. Under these same conditions TNF caused a substantial decrease in CRIg mRNA expression, compared to IL-1β and IL-6, both of which, had little effect on CRIg expression[9]. IL-4 down regulated CRIg mRNA expression in MDM over a concentration range of 1–40 ng/ml, with a 60% reduction at 10 ng/ml (Fig. 6c). Decreased expression could be detected as low as 1–3 ng/ml concentrations of IL-4. IL-13 caused a reduction in expression of CRIg mRNA over a concentration range of 5–40 ng/ml (Fig. 6d).

The regulatory cytokine TGF-β1 caused a substantial decrease in CRIg mRNA over a concentration range of 2–15 ng/ml (Fig. 6e) and similarly for IL-10 over a concentration range of 5–40 ng/ml (Fig. 6f). In contrast treatment with dexamethasone increased CRIg expression (Fig. 6g). The colony stimulating factors differed in

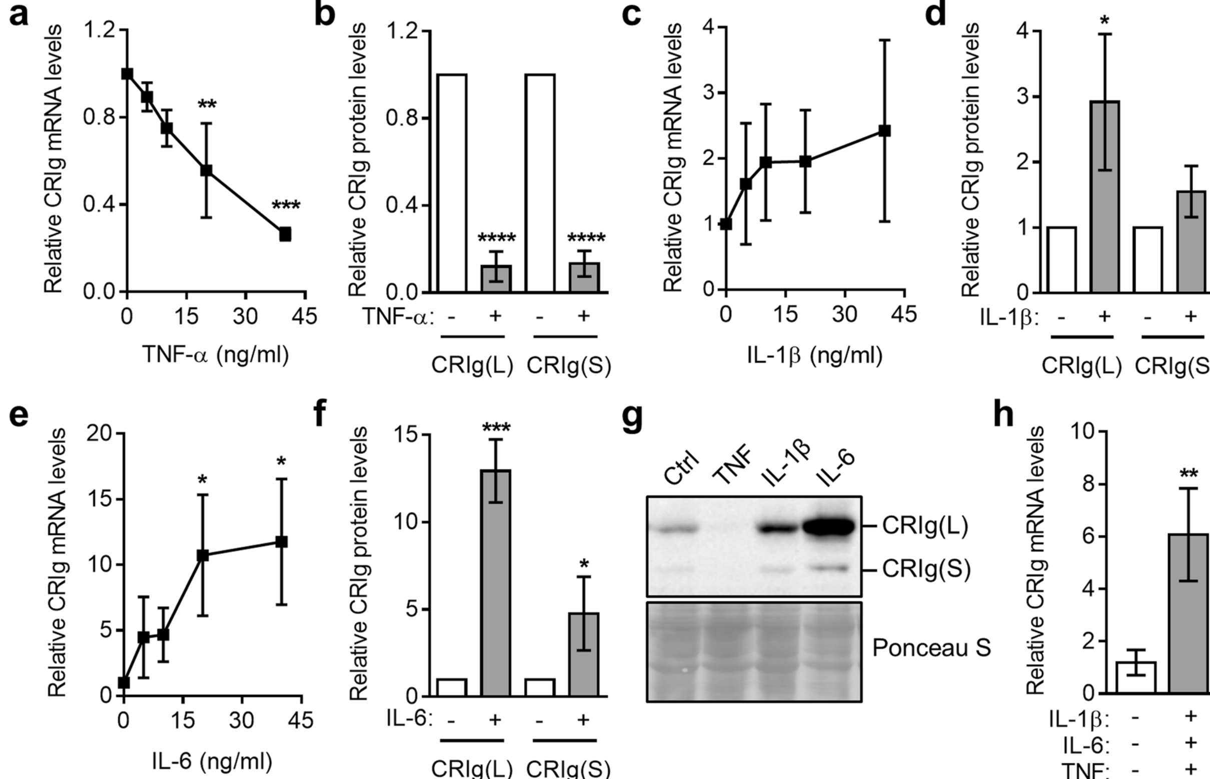


**Figure 3.** The pyrogenic cytokines, TNF, IL-1β and IL-6 differentially regulate CRIg$^{+}$ macrophage development. Monocytes were cultured in the presence of 0, 5, 10, 20, 40 ng/ml TNF (**a**), IL-1β (**c**) or IL-6 (**e**) and CRIg mRNA expression measured by qPCR. For CRIg protein expression monocytes were treated with 40 ng/ml TNF (**b**), IL-1β (**d**) or IL-6 (**f**). (**e**) A representative Western blot of CRIg levels and total protein in lysates is shown. (**h**) shows the effect of combined addition of 40 ng/ml of TNF, IL-1β and IL-6 to the development of CRIg$^{+}$ MDM. Data are presented as means ± SD of three experiments, each conducted with cells from different individuals. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

their effects on MDM CRIg expression. While GM-CSF down regulated expression, M-CSF caused an increase in expression (Fig. 6h and i).

**Effect of cytokines on CR3 and CR4 expression in macrophages.** To gain a greater understanding of the consequences of cytokine-induced modulation of CRIg expression it is important to assess these changes relative to those induced in other functional receptors. Particularly important in this context is the expression of the classical complement receptors, CR3 and CR4, which also promote the phagocytosis of iC3b-opsonized particle[5,10]. Thus the relative expression of these receptors may be a critical determinant of the severity of the inflammatory reaction. It was therefore considered important to understand whether CR3 and CR4 were also regulated by these cytokines and the type of changes the cytokines induced.

The effect of cytokines on the development of CR3$^{+}$/CR4$^{+}$ macrophages from monocytes, as well as their direct effect on MDM was examined. In differentiating macrophages, the cytokines influenced the final expression of these complement receptors. In the majority of cases the increase or decrease in CD11b and CD11c mRNA caused by the cytokines were similar for CR3 and CR4 expression (Fig. 7a). The results demonstrated a clear decrease in CR3 and CR4 expression caused by TNF, IL-6, M-CSF and GM-CSF (Fig. 7a). In contrast several cytokines, while having no effect on CR3, increased expression of CR4 (Fig. 7a).

To examine the direct effects of cytokines on mature macrophages, the MDM were treated with cytokines and after 24 h the cells were examined for expression of CD11b and CD11c mRNA. The data showed that several of the cytokines had very little effect or decreased expression of these receptors (Fig. 7b). However IL-13, TNF and IL-10 caused an increase in CR3 and CR4 expression. Both M-CSF and GM-CSF reduced expression of these receptors (Fig. 7b). In contrast to the effects on developing macrophages (Fig. 7a), dexamethasone increased CR3 and CR4 expression in MDM (Fig. 7b).

**Effects of cytokines on macrophages phagocytosis of *C. albicans*.** To examine whether the effects of cytokines on CRIg expression in MDM corresponded to functional changes, we examined phagocytosis. In these experiments the MDM were treated with the cytokines for 24 h and were then challenged with *C. albicans* which had been opsonised with complement-containing human AB group serum. It has been established that *C. albicans* activates complement via the alternative pathway and that we see no phagocytosis when serum is heat inactivated[11,12]. The data presented in Fig. 8 show that cytokine treatment of MDM altered their capacity

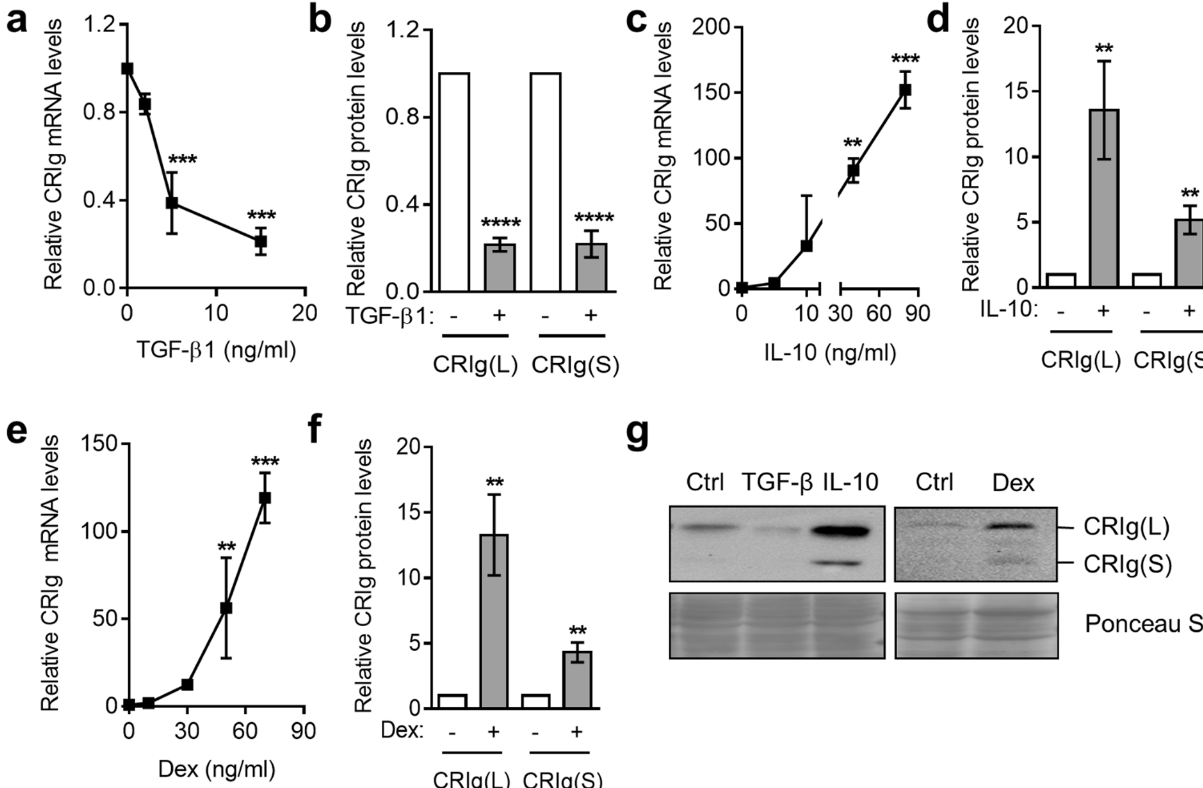


**Figure 4.** The effect of regulatory cytokines TGF-β1 and IL-10 on CRIg$^+$ macrophage development. Monocytes were cultured in the presence of 0, 2, 5, 15 ng/ml TGF-β1 (**a**) or 0, 5, 10, 40, 80 ng/ml IL-10 (**c**) and CRIg mRNA expression was measured by qPCR. For CRIg protein expression monocytes were treated with 15 ng/ml TGF-β1 (**b**) or 40 ng/ml IL-10 (**d**). (**e**) Monocytes were cultured in the presence of dexamethasone and the CRIg mRNA determined. (**f**) For protein expression monocytes were treated with 30 ng/ml dexamethasone. (**g**) Representative blots are shown (dexamethasone treatment was analysed on a separate blot from TGF-β1 and IL-10). Data are presented as means ± SD of three experiments, each conducted with cells from different individuals. **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

to phagocytose *C. albicans*. While LT-α and M-CSF caused an increase in phagocytosis, all the other cytokines caused a decrease in fungal phagocytosis by the macrophages. This paralleled the effects of the cytokines on CRIg expression but not in the expression of CR3 and CR4. Similarly, dexamethasone which upregulated CRIg expression, increased the rate of phagocytosis of fungi by MDM (Fig. 8).

To gain further confidence in this correlation we examined CRIg protein expression by western blot analysis in the MDM which had been treated with cytokines for 24 h. The data presented in Supplementary Fig. S2 demonstrated that total CRIg protein expression, unlike the expression of CRIg mRNA, did not correlate with phagocytic activity of the cell, although the effects of some cytokines were consistent with mRNA levels. There was also no correlation with CD11b and CD11c protein expression. Because it has been previously reported that there are five different transcripts of CRIg, it is tempting to speculate that this may explain the discrepancy of the effects of cytokines seen at the mRNA and protein level. When we examined whether these transcripts were present in MDM, five were detected when the cells were stimulated with dexamethasone (Fig. S3). The antisera used only detected the L and S forms. As further antibodies to the different forms become available, this question will need to be revisited.

## Discussion

The data demonstrate that cytokines regulate the development of CRIg$^+$ macrophages from monocytes, supporting and extending previous observations[8] and the view that CRIg expression may be a control point in infection and immunity, through which cytokines control macrophage function. These cytokines could be divided into the group which promoted the development of CRIg$^+$ macrophages, LT-α, IL-1β, IL-6, IL-10, GM-CSF, M-CSF and those which depressed this development, IFN-γ, TNF, TGF-β1, IL-4 and IL-13 (Table 1). This data not only identifies for the first time the cytokine patterns which regulate CRIg expression in macrophages but also reveal new and unexpected properties for some of these cytokines, which may have implications in the understanding of mechanisms of immunity to infection and in inflammation.

Since a major and primary role of CRIg is to promote phagocytosis of bacteria[4–7] our findings that cytokines can significantly alter the expression of CRIg suggest that the effects of these intercellular signalling molecules in infection and inflammation may occur via changes in CRIg expression. The data show that modulation of CRIg

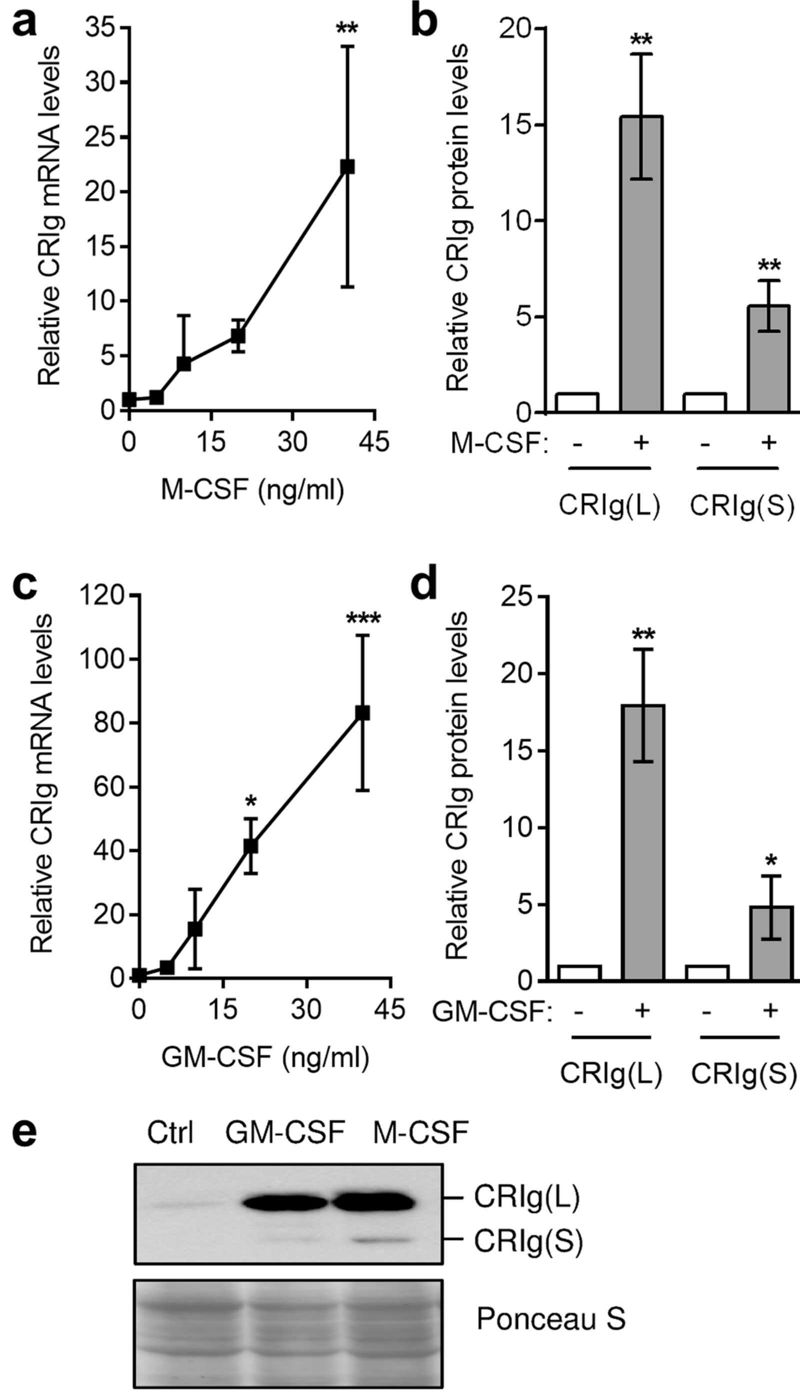


**Figure 5.** M-CSF and GM-CSF promote the development of CRIg$^+$ macrophages. Monocytes were cultured in the presence of 0, 5, 10, 20, 40 ng/ml M-CSF (**a**) or GM-CSF (**c**). Then CRIg mRNA expression measured. For CRIg protein expression monocytes were treated with 40 ng/ml M-CSF (**b**) or GM-CSF (**d**). (**e**) A representative Western blot. Data are presented as means ± SD of three experiments, each conducted with cells from different individuals, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

expression by cytokines is at a pre-transcriptional level and eventually emanates into corresponding changes in CRIg protein expression. Thus the effects of cytokines on CRIg protein expression by Western blot correlate with the changes seen at the mRNA level. The findings significantly extend the previous observation which only examined a restricted number of cytokines and which mainly assessed effects at CRIg mRNA level[8].

While the effects of cytokines were found at concentrations that might be measured in septic patients they are untypically high for many inflammatory conditions such as rheumatoid arthritis (RA), general viral or bacterial infections). Potential technical reasons why higher than normal (*in vivo*) cytokine concentrations were required in these assays include protein absorption to the tubes. Although it is evident that in biological fluids even during

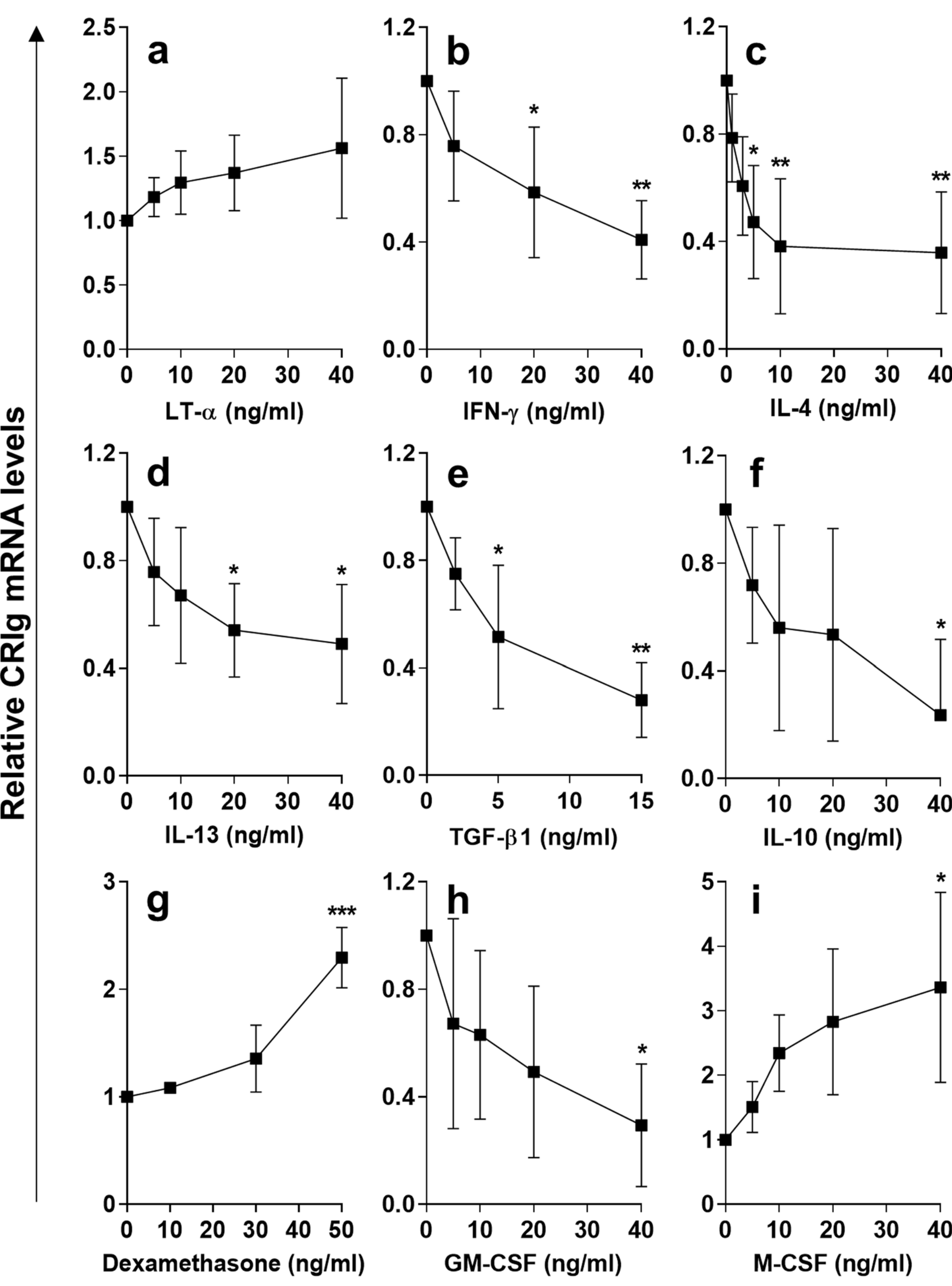


**Figure 6.** Effects of cytokines on CRIg expression in matured macrophages (MDM). In these studies MDM were prepared by culturing human monocytes for 3 days. MDM from 3 day cultures were treated with LT-α (**a**) (0, 5, 10, 20 and 40 ng/ml) or IFN-γ (**b**) (0, 5, 20 and 40 ng/ml) or IL-4 (**c**) (0, 1, 3, 5, 10 and 40 ng/ml) or IL-13 (**d**) (0, 5, 10, 20 and 40 ng/ml), TGF-β1 (**e**) (0, 2, 5 and 15 ng/ml) or (**f**) IL-10 (0, 5, 10, 20 and 40 ng/ml) or M-CSF/GM-CSF (**g**,**h**) (0, 5, 10, 20 and 40 ng/ml) or dexamethasone (**i**) (0, 10, 30 and 50 ng/ml) for 24 h and then CRIg mRNA levels relative to GAPDH mRNA were assessed by qPCR. Data are expressed as fold-change over GAPDH-normalised CRIg mRNA in the absence of cytokine set as 1. Data are presented as means ± SD of three experiments, each conducted with cells from different individuals, *p < 0.05, **p < 0.01, ***p < 0.001.

inflammation that pg/ml and not ng/ml levels are found, in some fluids even levels up to 500ng/ml have been reported[13]. Other factors include, from our experience e.g. with TNF that detection in inflammatory fluids may not be indicative of the absolute cytokine levels as these are bound by tissue receptors especially as these seem to increase during an infection[14]. There may also be other serum/fluid factors which may cause measurement errors

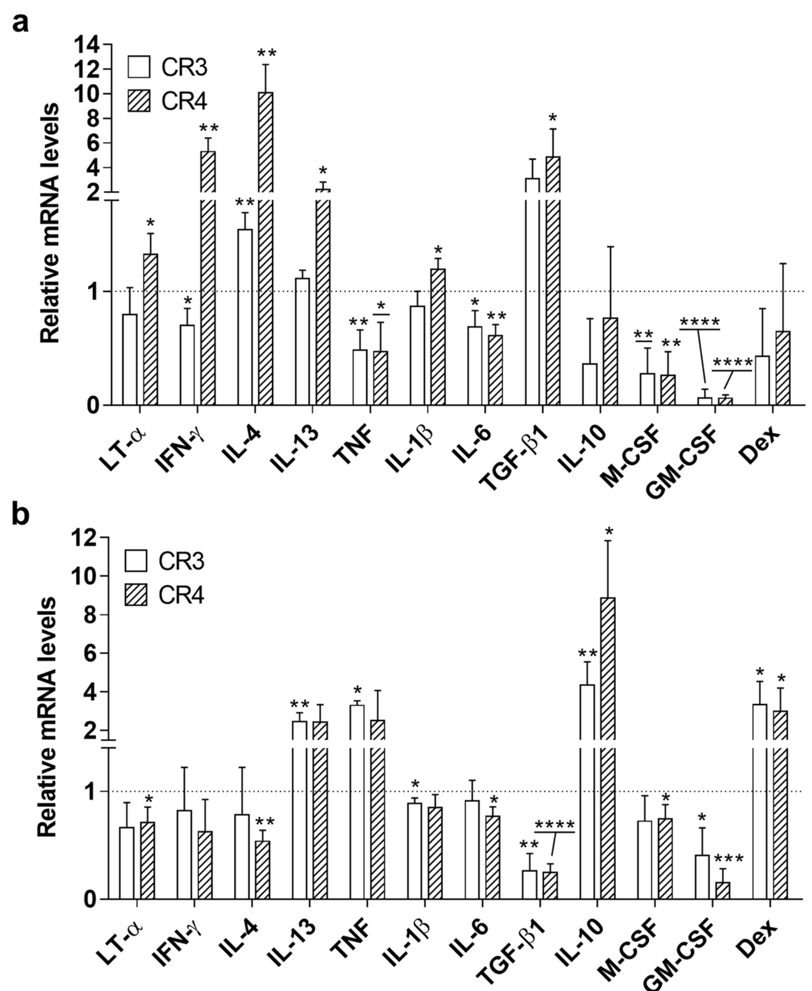


**Figure 7.** The effect of cytokines on the development of CR3$^+$ and CR4$^+$ macrophages and in MDM. (**a**) Monocytes were treated with 40 ng/ml LT-α, IFN-γ, IL-4, IL-13, IL-1β, IL-6, IL-10, M-CSF, GM-CSF or dexamethasone, 20 ng/ml TNF, 15 ng/ml TGF-β1. (**b**) Monocytes were cultured for 3 days for maturation into macrophages. The MDM were then incubated for 24 h with 40 ng/ml of the cytokines, LT-α, IFN-γ, IL-4, IL-1β, IL-6, IL-10, IL-13, M-CSF, GM-CSF, 20 ng/ml TNF, 15 ng/ml TGF-β1 or Dexamethasone (50 ng/ml). The level of CD11b and CD11c mRNA was measured using qPCR. Data are expressed as fold-change over GAPDH-normalized CD11b and CD11c mRNA in the absence of cytokine set as 1. Data are presented as means ± SD of three experiments, each conducted with cells from different individuals, *$p < 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

as well as cytokine decay, which we have experienced for biological fluids. It must also be appreciated that we are using recombinant cytokines, lacking glycosylation, and these may give different activities to the natural forms. Our use of concentrations between 2.5–40 ng/ml has followed other reports examining the effects of cytokines on macrophages. Perhaps one approach to resolving this issue is to look at levels produced by cells in culture following stimulation. Published data show that blood leukocytes stimulated with mitogens, bacteria and parasites produce ng/ml quantities of cytokines[15–17]. In conditions of severe pneumonia in patients serum levels of TNF and IL-6 reach ng quantities[18].

The immuno-suppressive cytokine IL-10 caused a substantial increase in CRIg protein expression. In comparison another regulatory cytokine, TGF-β1, which shares properties with IL-10, profoundly decreased CRIg protein expression in developing macrophages. Our findings not only confirmed these results but also demonstrated a corresponding effect on CRIg protein expression. The two cytokines may thus form a regulation for CRIg expression in M2 macrophages in the killing of parasites[19–21]. While the pyrogenic cytokines, TNF, IL-1β and IL-6 share many biological activities, the effects on the development of CRIg$^+$ macrophages differed. TNF caused a decrease and IL-1β and IL-6 increased CRIg expression. These changes were seen at both the mRNA and CRIg protein expression. Thus TNF versus IL-1β/IL-6 are likely to regulate CRIg expression in macrophages developing into M1 type[19–21]. Exposure to dexamethasone is likely to promote M2c macrophage development[20,21] with high CRIg expression (Table 1).

IFN-γ, IL-4, IL-10 and TGF-β1 altered CRIg expression, with both forms being affected. By measuring CRIg protein by Western blotting, the fate of both spliced forms of the receptor could be followed. The present studies

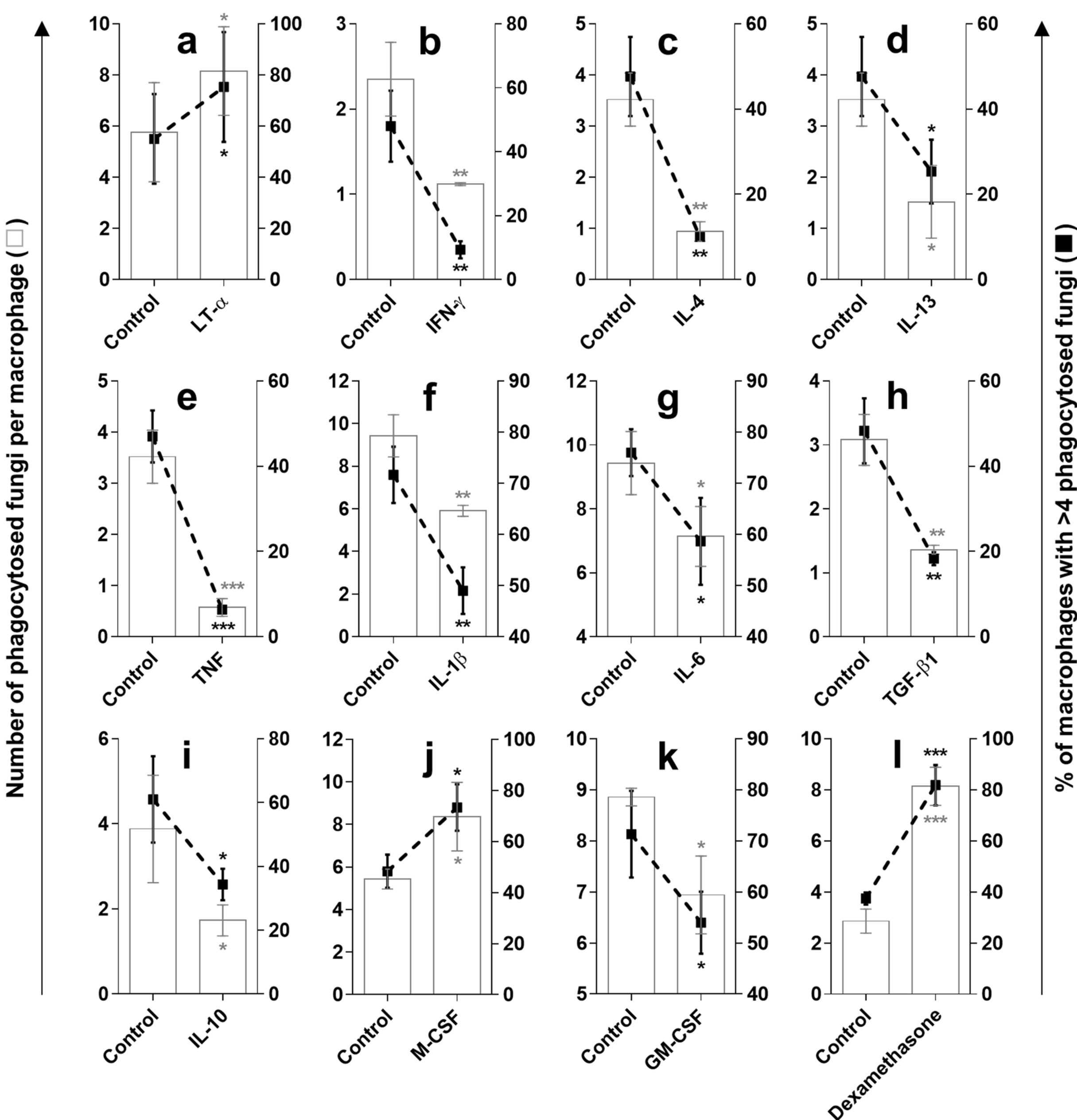


**Figure 8.** Effects of cytokines on the phagocytosis of *C. albicans* by MDM. MDM were prepared by culturing human monocytes for 5 days. The MDM were treated with 40 ng/ml of LT-α (**a**), IFN-γ (**b**), IL-4 (**c**), IL-13 (**d**), or 20 ng/ml TNF (**e**), or 40 ng/ml IL-1β (**f**), IL-6 (**g**), or 15 ng/ml TGF-β1 (**h**) or 40 ng/ml IL-10 (**i**), M-CSF/GM-CSF (**j**,**k**) or 50 ng/ml dexamethasone (**l**) for 24 h and examined for their ability to phagocytose complement opsonised *C. albicans*. Phagocytosis was scored as both the number of macrophages that had engulfed more than >4 fungi (line graph) and the number of fungi engulfed per cell (bar graph). Data are presented as means ± SD of three experiments, each conducted with cells from different individuals, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

revealed that the CRIg(L) and CRIg(S) were similarly, down- or up-, regulated by these cytokines. While both forms are found in human macrophages, murine macrophages possess only the latter[4]. Thus the finding that cytokines regulate the CRIg (S) form is also relevant to the murine models of infection and immunity and inflammation, since this is the form found in mouse macrophages.

Cytokine networks play an important role in regulating inflammation and those tested in our present study act on the macrophage, a cell which is central to infection and immunity, including immunity to *C. albicans*[22]. Cytokines are known for their differences in either promoting disease or protecting against these diseases. It is tempting to speculate that CRIg may be one of the control points in infection and immunity through which cytokines and other intercellular acting inflammatory mediators act. Indirect support for this view can be

| Cytokine | During Macrophage Development | | | Expression in MDM | | |
|---|---|---|---|---|---|---|
| | CR3/CD11b | CR4/CD11c | CRIg | CR3/CD11b | CR4/CD11c | CRIg |
| LT-α | ↓ | ↑ | ↑ | ↓ | ↓ | ↑ |
| IFN-γ | ↓ | ↑ | ↓ | ↓ | ↓ | ↓ |
| IL-4 | ↑ | ↑ | ↓ | ↓ | ↓ | ↓ |
| IL-13 | ↓ | ↑ | ↓ | ↑ | ↑ | ↓ |
| IL-10 | ↓ | ↓ | ↑ | ↑ | ↑ | ↓ |
| TGF-β1 | ↑ | ↑ | ↓ | ↓ | ↓ | ↓ |
| TNF | ↓ | ↓ | ↓ | ↑ | ↑ | ↓ |
| IL-1β | ↓ | ↑ | ↑ | ↓ | ↓ | ↓ |
| IL-6 | ↓ | ↓ | ↑ | ↓ | ↓ | ↓ |
| M-CSF | ↓ | ↓ | ↑ | ↓ | ↓ | ↑ |
| GM-CSF | ↓ | ↓ | ↑ | ↓ | ↓ | ↓ |
| Dexamethasone | ↓ | ↓ | ↑ | ↓ | ↓ | ↑ |

**Table 1.** Effect of cytokines on CR3/CD11b, CR4/CD11c and CRIg mRNA expression in macrophages. The ↑ and ↓ arrows represents an increase and a decrease in receptor expression.

derived from the findings that CRIg$^+$ macrophages disappear from inflammatory sites and with the intensity of inflammation[23].

It was interesting to find that both of the Th2 cytokines, IL-4 and IL-13 caused a decrease in expression of CRIg at the mRNA and protein level in maturing macrophages. This may be a mechanism by which macrophages promote pathogenesis induced by helminths such as schistosomes and other Th2 mediated inflammation such as that seen in allergy[21]. The observation could be given consideration in future research.

Often cytokines have been examined singly for their effects as this enables the contribution that the respective cytokine may have on cellular function. But to gain more physiological meaningful perspective we need to also understand the impact of cells interacting with the different cytokines simultaneously which may more closely mimic the *in vivo* inflammatory environment. To illustrate this we subjected monocytes during their development of CRIg$^+$ macrophages to a combination of IL-1β, IL-6 and TNF. The depressive effects on CRIg$^+$ macrophage development induced by TNF could be overcome by concomitant addition of IL-1β and IL-6. While the results suggest that during infection and inflammation the fluids generated are likely to increase the express ion of CRIg on macrophages, it is important to appreciate that the levels of these relative to each other will vary significant at different times of the inflammatory reaction, and may hence dictate the final outcome.

Here we have highlighted that cytokines not only affected the development of CRIg$^+$ macrophages but also regulated the expression of this receptor on mature macrophages, indicative of events in tissues. However most of the cytokines caused a down-regulation of CRIg mRNA. Only LT-α and M-CSF induced an up regulation, similar to the anti-inflammatory agent dexamethasone. The findings indicate that mature macrophages are amenable to cytokine-induced modulation of CRIg (Table 1). This then becomes a second control point in inflammation through which cytokines may have their influence once the macrophages are matured and localized in tissues. The ability of LT-α and M-CSF to increase CRIg expression both during development and directly on mature macrophages is interesting. We have previously demonstrated that TNF caused these effects via activation of PKCα and those macrophages treated with anti-TNF antibody showed increased expression of CRIg[9]. It is therefore tempting to speculate that one important action of anti-TNF therapy is to prevent the loss of CRIg expression induced by TNF in RA and thereby improve phagocytic uptake of microbial pathogen, a possible reason as to why patients on anti-TNF therapy do not experience the expected wider increase in susceptibility to infection.

Cytokines which altered CRIg expression in macrophages, also caused changes to the expression of CR3 and CR4. It is evident from these results that some cytokines had opposite effects on these three receptor types (Table 1). The receptors, apart from performing similar functions, display other differing key functional properties. Thus their differential expression caused by cytokines will have an impact in the final response precipitated during microbial interaction. While IL-4 and TGF-β1 promoted the development of CR3 expressing macrophages, the development of CR4 expressing macrophages was promoted by the rest. Thus although CR3 may be decreased on macrophages subjected to LT-α, IFN-γ, IL-13 and IL-1β their phagocytic function is likely to be retained through the up regulation of CR4 by these cytokines. In comparison to this scenario, IL-4 and TGF-β1 promote the development of macrophages with increased expression of both CR3 and CR4; increasing the potential phagocytic capability of the macrophage. Although these are *in vitro* models, consideration should be given to these mimicking the monocyte invasion of tissue and their development into macrophages to interact with complement opsonised microbial pathogens, such as *Candida*[24]. Macrophage development towards cells with lower phagocytic activity may occur when the same cytokines cause a decrease in expression of both CR3 and CR4. Cytokines which gave rise to this decrease were TNF, IL-10 and IL-6.

Although CR1 (CD35), is a complement control protein (CCP) module containing molecule, is present on the surface of macrophages, its role may not be to directly enhance phagocytosis of complement opsonized pathogens as opposed to the roles for CR3, CR4 and CRIg. In contrast, CR1 enhances clearance of soluble immune complexes via Fc receptors. CR1 on erythrocytes plays a major role in the clearance of soluble immune complexes, by transporting them to the liver and spleen, where they are cleared by macrophages. The binding of C3b-coated targets to phagocyte CR1 is not sufficient to trigger phagocytosis, but C3b–CR1 interaction enhances

the FcγR-mediated phagocytosis of targets bearing both IgG and C3b[25–27]. When we examined the expression of CR1, it was evident that the cytokines did not alter the expression of this receptor (Fig. S2).

Examination of effects of cytokines on mature macrophages, MDM, demonstrated a different pattern of alteration in CR3 and CR4 mRNA (Table 1). The ability of cytokines to regulate these receptors provides a second check point for regulating macrophage function in infection and immunity, depending on the infection type and cytokines generated. The cytokines IFN-γ, TNF, IL-6, M-CSF and GM-CSF decreased the development of $CR3^+$ macrophages. But this did not necessarily correspond to a similar effect on mature macrophages (Table 1). The findings show that CR3 and CR4 expression may be differentially regulated by some cytokines. Since Kupffer cells not only express CRIg but also CR3 and CR4[28], the findings are also relevant to this tissue fixed macrophage. But further studies are required to ascertain whether this differential expression of CRIg versus CR3/CR4 induced by cytokines is also relevant to Kupffer cells.

Because most cytokines examined caused a decrease in CRIg expression on mature macrophages, it is inevitable that those monocytes which respond to infection in tissues and develop into macrophages will be susceptible to the action of these cytokines and this may be a reason why CRIg expressing macrophages are low at inflammatory sites and infection foci[23]. Previously we found that IFN-γ decreases the development of $CRIg^+$ macrophages and caused reduced phagocytosis of complement opsonised *C. albicans*[8]. The present study demonstrated that IL-4 caused a decrease in the expression of CRIg mRNA and reduction in the phagocytosis of *C. albicans*. We have previously reported that IL-4 caused a decrease in the phagocytosis and killing of complement-opsonised *Plasmodium falciparum* infected red blood cells by macrophages[29]. The changes in CRIg mRNA levels in MDM correlated with their altered rates of phagocytosis of complement opsonised *C. albicans*. Complement deposition on this fungi results from activation of complement via the alternative pathway[11, 12]. Complement opsonisation is required to see the effects of changes in CRIg expression[9]. Thus innate immunity may function through components of microbial pathogens stimulating human lymphocytes to produce LT-α[30]. As previously demonstrated by Helmy *et al.*[4], once phagocytosis has been initiated by liver macrophages (Kupffer cells), CRIg expression is dramatically reduced. Our results indicate that this is most likely due to the release of cytokines, in particular TNF which decreases CRIg expression[9].

Although we are emphasising a potentially important function for CRIg in the phagocytosis of fungi, the study has not been designed to conclusively prove this. Approaches such as blocking the receptor and or the other complement receptors would need to be under taken to establish their role in this function. Furthermore our results revealed that the MDM expression of CRIg protein neither correlated with expression of CRIg mRNA nor phagocytic activity. The most appropriate explanation for this discrepancy is that this anti-CRIg antibody only reveals the changes in the L and S forms. We identified five transcripts of CRIg in MDM and it is possible that changes in the expression of other forms may account for changes in rates of phagocytosis.

While our studies have focussed on phagocytosis of fungi, the importance of CRIg in phagocytosis of bacteria has been highlighted. Apart from implications in infections, our results suggest that cytokines may work through alterations in CRIg expression to modulate the inflammatory response in chronic inflammatory diseases such as RA. The pro-inflammatory, Th1 cytokine IFN-γ, in contrast to LT-α, causes a marked decrease in CRIg expression, in line with their reported effects in the pathogenesis of RA. IFN-γ is present in RA patients' synovium and synovial fluid[31]. CD4 T cells in RA patients contribute to the pathogenesis by producing IFN-γ[32, 33]. Another Th1 cytokine, TNF, caused a decrease in CRIg expression. TNF is a major mediator of joint inflammation and bone destruction in inflammatory arthritis and several studies have measured large amounts of TNF in synovial fluid of patients with RA, psoriatic arthritis and in children with juvenile idiopathic arthritis[34–37] TNF targeting biological drugs proved effective in the treatment of RA patients[38]. The role of LT-α, a close homolog of TNF[39], found in synovial tissue of diseased joints, is not well defined[40]. In psoriatic arthritis patients, anti TNF-α monoclonal antibodies have been developed for neutralization of TNF and etanercept for LT-α[41]. Psoriatic arthritis patients undergoing etanercept treatment showed significantly increased serum levels of LT-α after 3 and 6 months which returned to baseline levels after 12 months[41]. These findings are conducive with our data showing that LT-α up regulated the expression of CRIg in macrophages. The difference between LT-α and TNF which act on the same receptor is not surprising as previously we have found that the two cytokines have some distinct biological effects on phagocytes[42]. For example in terms of mediating articular cartilage damage, LT-α plays a protective role compared to the destructive role of TNF[42].

The immuno-suppressive cytokine IL-10 caused a substantial increase in CRIg mRNA and corresponding CRIg protein. This is consistent with its protective and anti-inflammatory effects observed in several murine arthritis models and its praised therapeutic potential in this disease[43, 44]. Another regulatory cytokine, TGF-β1 which shares properties with IL-10, however, plays a major role in the progression of RA and several studies reported that TGF- β1 has been detected in the synovial tissue of patients with RA[45, 46]. Our findings show that TGF-β1 which has regulatory effects on macrophages profoundly decreases CRIg mRNA[8] and protein expression in macrophages and suggest that this may be a mechanism in the pathogenesis of RA. Although IL-1β is expressed in RA[47], its role in inflammation has been controversial. Injection of recombinant IL-6 into the joint cavity reduced cartilage destruction in experimental arthritis[48]. Some studies reported that increases in serum IL-6 levels are associated with clinical improvements[49]. IL-6 reduces TNF production[50, 51] which may explain its protective role in joint pathology. Our findings are in line with its protective effects by increasing CRIg expression.

It was interesting to find that the Th2 cytokines, IL-4 and IL-13 both caused a decrease in expression of CRIg at both the mRNA and protein level. It has been reported that there is an association of IL-4 gene 70 bp VNTR and MTHFRC677T polymorphism in the development of RA[52]. Furthermore, it has been suggested that IL-4 and its receptor could play a role in the pathogenesis of RA[53]. Similarly, IL-13 is also identified as a risk locus for psoriatic arthritis investigated in a number of studies[54].

Because of the critical functions played by CRIg in infection and immunity and inflammation, our results suggest that cytokines have the potential to modify inflammation and resistance to microbial pathogens by

modulating this receptor, hence identifying a mechanism by which cytokines regulate defence against infection and inflammation[55]. The research extended to show that cytokines could regulate the expression of CRIg on mature macrophages to provide a second control point by which cytokines could modify macrophage microbial killing, inflammation and immune responsiveness. Other classes of inflammatory mediators are likely to also regulate CRIg expression, as we previously found with arachidonate[8]. The importance of CRIg in Kupffer cell-mediated phagocytosis of bacteria has been demonstrated[4,6,7] and it is likely that CRIg expression in these cells is also regulated by cytokines during infection and inflammation[56]. While the complexity of the CRIg system and its varied roles in infection and immunity is becoming appreciated[25], we have now provided further evidence of its importance in host defence and understanding the mechanisms regulating macrophages in immunity to infection.

## Methods

**Cytokines and cell culture reagents.** Recombinant granulocyte-macrophage colony stimulating factor (GM-CSF), macrophage (M)-CSF, interleukin (IL)-1β, IL-6, IL-4, IL-10, IL-13, interferon (IFN)-γ, lymphotoxin (LT)-α, tumor necrosis factor (TNF), M-CSF and GM-CSF were purchased from ProSpec-Tany Technogene (Rehovot, Israel), transforming growth factor (TGF)-β1 from R&D Systems (Minneapolis, MN), and dexamethasone was purchased from Sigma-Aldrich (St. Louis, MO). A mouse monoclonal antibody (clone 3C9) that recognizes the IgV domain of human CRIg was kindly provided by Dr. Menno van Lookeren Campagne (Genentech, San Francisco, CA). RPMI 1640 tissue culture medium, foetal calf serum (FCS) and L-glutamine were purchased from SAFC Biosciences (Lenexa, KS).

**Ethics statement.** Venous blood was collected from healthy adult volunteers under guidelines and approval of the Women's and Children's Health Network Human Research Ethics Committee. Written informed consent was obtained from all participants.

**Purification and culture of monocytes.** Peripheral blood mononuclear cells (PBMC) were prepared by centrifugation of blood on Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden). The interface layer containing PBMC was harvested and cells were washed in RPMI-1640 medium supplemented with 2 mmol/L L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% foetal calf serum, pH 7.4 (RPMI-FCS). Cell viability was determined by the trypan blue-exclusion method. Monocytes were purified from the PBMC by density gradient centrifugation, as described previously[9]. Briefly, PBMC were layered onto a 46% iso-osmotic Percoll gradient (GE Healthcare, Uppsala, Sweden) and centrifuged at 600 × g for 30 min at room temperature. The monocytes-containing layer was harvested. Monocytes were >90% pure as judged by staining with anti-human CD14-FITC (BD Pharmingen, San Jose, CA) and analysing on a BD FACSCanto (BD Biosciences, San Diego, CA). Monocytes were cultured in RPMI-FCS in humidified air containing 5% $CO_2$ at 37 °C at $10^6$ cells/ml under the influence of cytokines or dexamethasone. Cells were harvested after either 3 days (for CRIg mRNA analysis) or 7 days (for CRIg protein analysis) culture by gentle scrapping with a 'rubber policeman'.

**Quantitative PCR.** RNA was converted to cDNA using an iScript cDNA synthesis kit (Bio-Rad). QPCR was conducted using primers for human CD11b (Forward: CCTGGTGTTCTTGGTGCCC; Reverse: TCCTTGGTGTGGCACGTACTC), CD11c (F: CCGATTGTTCCATGCCTCAT; R: AACCCCAATTGCATAGCGG), and CRIg (F: ACACTTATGGCCGTCCCAT; R: TGTACCAGCCACTTCACCAA) with GAPDH (F: GAGTCAACGGATTTGGTCGT; R: GACAAGCTTCCCGTTCTCAGCCT) as the reference gene[8,9]. Assayed in triplicate, each reaction contained 100 nM of each primer, 1 μl of cDNA, and iQ SYBR Green Supermix (Bio-Rad Laboratories) in a 20 μl final volume. Thermal cycling was performed with an initial denaturation at 95 °C for 5 min, followed by 40 cycles of 95 °C for 30 sec, 60 °C for 30 sec and 72 °C for 30 sec, using an iQ5 Real Time Detection System with iQ5 Optical System v2.1 software (Bio-Rad Laboratories). Expression data was normalised to GAPDH transcript levels.

**Western blotting.** Macrophages were harvested after 7 days, washed, and resuspended in 100 μl of lysis buffer containing 20 mmol/L HEPES, pH 7.4, 0.5% Nonidet P-40 (v/v), 100 mmol/L NaCl, 1 mmol/L EDTA, 2 mmol/L $Na_3VO_4$, 2 mmol/L dithiothreitol, 1 mmol/L PMSF, and 10 μg/ml of each protease inhibitor (Benzamidine, leupeptin, pepstatin A and phenylmethylsulfonyl fluoride (PMSF) purchased from Sigma-Aldrich and aprotinin from Calbiochem (Merck, Darmstadt, Germany)[57]. Protein was quantitated by the Lowry method, prior to the addition of Laemmli buffer. Samples were boiled at 100 °C for 5 min and 60 μg of each were subjected to 12% SDS-PAGE at 175 V for approximately 1 h using the Mini-PROTEAN 3 system (Bio-Rad Laboratories, Hercules, CA). The samples were electrophoretically transferred to nitrocellulose membrane (Pierce Biotechnology, Thermo Fisher Scientific, Rockford, IL) at 100 V for 1 h. To monitor the extent of protein transfer, the membrane was stained with 0.1% Ponceau S (in 5% acetic acid). After blocking, the membrane was incubated with mouse anti-human CRIg (3C9) at 1:20000 in blocking solution overnight at 4 °C. Following washing in blocking solution (3 × 10 min), the membrane was incubated with secondary HRP-conjugated rabbit anti-mouse IgG (Dako, Glostrup, Denmark) at 1:2000 in blocking solution for 1 h at room temperature. Immunoreactive material was detected by enhanced chemiluminescence according to the manufacturer's instructions (Western Lightning Chemiluminescence, Perkin Elmer, Waltham, MA). The protein bands on the membranes were visualised by a ChemiDoc XRS+ Imaging System and quantitated using Image LabTM Software, Version 3.0 (Bio-Rad Laboratories, Hercules, CA).

**Phagocytosis assay.** The phagocytosis assay was performed essentially as described previously[8]. Twenty four hours post treatment of MDM with cytokine treatment, the cells were washed and detached with detachment buffer. Then $1 \times 10^5$ *C. albicans* yeast particles were added to $5 \times 10^4$ MDM in a final volume of 0.5 ml HBSS.

Complement-containing human AB serum was added to a final concentration of 10%. The cells were incubated for 15 min at 37 °C on a rocking platform. Unphagocytosed yeast particles were removed by differential centrifugation at 175 × g for 5 min and then the MDM in the pellet were resuspended and cytocentrifuged onto a microscope slide and stained with Giemsa. The number of particles in phagocytic vacuoles was then determined[8]. Phagocytosis was scored as both the number of macrophages that had engulfed >4 fungi (line graph) as well as the number of fungi engulfed per cell (bar graph).

**Statistical analysis.** Unpaired comparison were analysed using the two-tailed Student's t-test and multiple comparison were performed using Dunnett's test, with $p < 0.05$ considered significant.

## References


1. Langnaese, K., Colleaux, L., Kloos, D. U., Fontes, M. & Wieacker, P. Cloning of Z39Ig, a novel gene with immunoglobulin-like domains located on human chromosome X. *Biochimica et biophysica acta* **1492**, 522–525 (2000).
2. Walker, M. G. Z39Ig is co-expressed with activated macrophage genes. *Biochimica et biophysica acta* **1574**, 387–390 (2002).
3. Kim, J. K. *et al.* Characterization of monoclonal antibody specific to the Z39Ig protein, a member of immunoglobulin superfamily. *Immunology letters* **99**, 153–161, doi:10.1016/j.imlet.2005.02.012 (2005).
4. Helmy, K. Y. *et al.* CRIg: a macrophage complement receptor required for phagocytosis of circulating pathogens. *Cell* **124**, 915–927, doi:10.1016/j.cell.2005.12.039 (2006).
5. Gorgani, N. N. *et al.* Complement receptor of the Ig superfamily enhances complement-mediated phagocytosis in a subpopulation of tissue resident macrophages. *J Immunol* **181**, 7902–7908, doi:10.4049/jimmunol.181.11.7902 (2008).
6. Zeng, Z. *et al.* CRIg Functions as a Macrophage Pattern Recognition Receptor to Directly Bind and Capture Blood-Borne Gram-Positive Bacteria. *Cell host & microbe* **20**, 99–106, doi:10.1016/j.chom.2016.06.002 (2016).
7. Broadley, S. P. *et al.* Dual-Track Clearance of Circulating Bacteria Balances Rapid Restoration of Blood Sterility with Induction of Adaptive Immunity. *Cell host & microbe* **20**, 36–48, doi:10.1016/j.chom.2016.05.023 (2016).
8. Gorgani, N. N. *et al.* Regulation of CRIg expression and phagocytosis in human macrophages by arachidonate, dexamethasone, and cytokines. *Am J Pathol* **179**, 1310–1318, doi:10.1016/j.ajpath.2011.05.021 (2011).
9. Ma, Y. *et al.* Protein kinase calpha regulates the expression of complement receptor Ig in human monocyte-derived macrophages. *J Immunol* **194**, 2855–2861, doi:10.4049/jimmunol.1303477 (2015).
10. van Lookeren Campagne, M., Wiesmann, C. & Brown, E. J. Macrophage complement receptors and pathogen clearance. *Cellular microbiology* **9**, 2095–2102, doi:10.1111/j.1462-5822.2007.00981.x (2007).
11. Thong, Y. H. & Ferrante, A. Alternative pathway of complement activation by Candida albicans. *Australian and New Zealand journal of medicine* **8**, 620–622 (1978).
12. Kozel, T. R. Activation of the complement system by pathogenic fungi. *Clinical microbiology reviews* **9**, 34–46 (1996).
13. Gonzales, J. R. *et al.* Concentration of interleukin-1beta and neutrophil elastase activity in gingival crevicular fluid during experimental gingivitis. *Journal of clinical periodontology* **28**, 544–549 (2001).
14. Chan, R. M.-C. Mechanisms of pathophysiologic processes in a murine model of cerebral malaria (Plasmodium berghei Anka), University of Adelaide (1994).
15. Staugas, R. E., Harvey, D. P., Ferrante, A., Nandoskar, M. & Allison, A. C. Induction of tumor necrosis factor (TNF) and interleukin-1 (IL-1) by Pseudomonas aeruginosa and exotoxin A-induced suppression of lymphoproliferation and TNF, lymphotoxin, gamma interferon, and IL-1 production in human leukocytes. *Infection and immunity* **60**, 3162–3168 (1992).
16. Groeneveld, A. B., Bossink, A. W., van Mierlo, G. J. & Hack, C. E. Circulating inflammatory mediators in patients with fever: predicting bloodstream infection. *Clinical and diagnostic laboratory immunology* **8**, 1189–1195, doi:10.1128/CDLI.8.6.1189-1195.2001 (2001).
17. de Jager, W., te Velthuis, H., Prakken, B. J., Kuis, W. & Rijkers, G. T. Simultaneous detection of 15 human cytokines in a single sample of stimulated peripheral blood mononuclear cells. *Clinical and diagnostic laboratory immunology* **10**, 133–139 (2003).
18. Matsuda, K., Tsutsumi, H., Okamoto, Y. & Chiba, C. Development of interleukin 6 and tumor necrosis factor alpha activity in nasopharyngeal secretions of infants and children during infection with respiratory syncytial virus. *Clinical and diagnostic laboratory immunology* **2**, 322–324 (1995).
19. Mantovani, A., Garlanda, C. & Locati, M. Macrophage diversity and polarization in atherosclerosis: a question of balance. *Arteriosclerosis, thrombosis, and vascular biology* **29**, 1419–1423, doi:10.1161/ATVBAHA.108.180497 (2009).
20. Mantovani, A., Sica, A. & Locati, M. New vistas on macrophage differentiation and activation. *European journal of immunology* **37**, 14–16, doi:10.1002/eji.200636910 (2007).
21. Martinez, F. O. & Gordon, S. The M1 and M2 paradigm of macrophage activation: time for reassessment. *F1000prime reports* **6**, 13, doi:10.12703/P6-13 (2014).
22. Erwig, L. P. & Gow, N. A. Interactions of fungal pathogens with phagocytes. *Nature reviews. Microbiology* **14**, 163–176, doi:10.1038/nrmicro.2015.21 (2016).
23. Vogt, L. *et al.* VSIG4, a B7 family-related protein, is a negative regulator of T cell activation. *J Clin Invest* **116**, 2817–2826, doi:10.1172/JCI25673 (2006).
24. Ngo, L. Y. *et al.* Inflammatory monocytes mediate early and organ-specific innate defense during systemic candidiasis. *The Journal of infectious diseases* **209**, 109–119, doi:10.1093/infdis/jit413 (2014).
25. Small, A. G., Al-Baghdadi, M., Quach, A., Hii, C. & Ferrante, A. Complement receptor immunoglobulin: a control point in infection and immunity, inflammation and cancer. *Swiss medical weekly* **146**, w14301, doi:10.4414/smw.2016.14301 (2016).
26. Merle, N. S., Church, S. E., Fremeaux-Bacchi, V. & Roumenina, L. T. Complement System Part I - Molecular Mechanisms of Activation and Regulation. *Frontiers in immunology* **6**, 262, doi:10.3389/fimmu.2015.00262 (2015).
27. Merle, N. S., Noe, R., Halbwachs-Mecarelli, L., Fremeaux-Bacchi, V. & Roumenina, L. T. Complement System Part II: Role in Immunity. *Frontiers in immunology* **6**, 257, doi:10.3389/fimmu.2015.00257 (2015).
28. Hinglais, N., Kazatchkine, M. D., Mandet, C., Appay, M. D. & Bariety, J. Human liver Kupffer cells express CR1, CR3, and CR4 complement receptor antigens. An immunohistochemical study. *Laboratory investigation; a journal of technical methods and pathology* **61**, 509–514 (1989).
29. Kumaratilake, L. M. & Ferrante, A. IL-4 inhibits macrophage-mediated killing of Plasmodium falciparum *in vitro*. A possible parasite-immune evasion mechanism. *J Immunol* **149**, 194–199 (1992).
30. Ferrante, A. *et al.* Production of tumor necrosis factors alpha and beta by human mononuclear leukocytes stimulated with mitogens, bacteria, and malarial parasites. *Infection and immunity* **58**, 3996–4003 (1990).
31. Baccala, R., Kono, D. H. & Theofilopoulos, A. N. Interferons as pathogenic effectors in autoimmunity. *Immunol Rev* **204**, 9–26, doi:10.1111/j.0105-2896.2005.00252.x (2005).
32. Rodeghero, R. *et al.* Location of CD4+ T cell priming regulates the differentiation of Th1 and Th17 cells and their contribution to arthritis. *J Immunol* **190**, 5423–5435, doi:10.4049/jimmunol.1203045 (2013).

33. Park, J. K. *et al*. CD70-expressing CD4 T cells produce IFN-gamma and IL-17 in rheumatoid arthritis. *Rheumatology* **53**, 1896–1900, doi:10.1093/rheumatology/keu171 (2014).
34. Feldmann, M., Brennan, F. M., Elliott, M., Katsikis, P. & Maini, R. N. TNF alpha as a therapeutic target in rheumatoid arthritis. *Circulatory shock* **43**, 179–184 (1994).
35. Redlich, K. *et al*. Osteoclasts are essential for TNF-alpha-mediated joint destruction. *J Clin Invest* **110**, 1419–1427, doi:10.1172/JCI15582 (2002).
36. Ritchlin, C. T., Haas-Smith, S. A., Li, P., Hicks, D. G. & Schwarz, E. M. Mechanisms of TNF-alpha- and RANKL-mediated osteoclastogenesis and bone resorption in psoriatic arthritis. *J Clin Invest* **111**, 821–831, doi:10.1172/JCI16069 (2003).
37. Scardapane, A., Breda, L., Lucantoni, M. & Chiarelli, F. TNF-alpha Polymorphisms in Juvenile Idiopathic Arthritis: Which Potential Clinical Implications? *International journal of rheumatology* **2012**, 756291, doi:10.1155/2012/756291 (2012).
38. Geiler, J., Buch, M. & McDermott, M. F. Anti-TNF treatment in rheumatoid arthritis. *Current pharmaceutical design* **17**, 3141–3154 (2011).
39. Schneider, K., Potter, K. G. & Ware, C. F. Lymphotoxin and LIGHT signaling pathways and target genes. *Immunol Rev* **202**, 49–66, doi:10.1111/j.0105-2896.2004.00206.x (2004).
40. Grom, A. A. *et al*. Patterns of expression of tumor necrosis factor alpha, tumor necrosis factor beta, and their receptors in synovia of patients with juvenile rheumatoid arthritis and juvenile spondylarthropathy. *Arthritis and rheumatism* **39**, 1703–1710 (1996).
41. Murdaca, G., Colombo, B. M., Contini, P. & Puppo, F. Determination of lymphotoxin-alpha levels in patients with psoriatic arthritis undergoing etanercept treatment. *Journal of interferon & cytokine research: the official journal of the International Society for Interferon and Cytokine Research* **32**, 277–279, doi:10.1089/jir.2011.0120 (2012).
42. Kowanko, I. C., Bates, E. J. & Ferrante, A. Tumour necrosis factor-beta modulates human neutrophil-mediated cartilage damage. *Scandinavian journal of immunology* **28**, 591–598 (1988).
43. Finnegan, A. *et al*. Collagen-induced arthritis is exacerbated in IL-10-deficient mice. *Arthritis research & therapy* **5**, R18–24 (2003).
44. Fellowes, R. *et al*. Amelioration of established collagen induced arthritis by systemic IL-10 gene delivery. *Gene therapy* **7**, 967–977, doi:10.1038/sj.gt.3301165 (2000).
45. Taketazu, F. *et al*. Enhanced expression of transforming growth factor-beta s and transforming growth factor-beta type II receptor in the synovial tissues of patients with rheumatoid arthritis. *Laboratory investigation; a journal of technical methods and pathology* **70**, 620–630 (1994).
46. Lafyatis, R. *et al*. Transforming growth factor-beta production by synovial tissues from rheumatoid patients and streptococcal cell wall arthritic rats. Studies on secretion by synovial fibroblast-like cells and immunohistologic localization. *J Immunol* **143**, 1142–1148 (1989).
47. McInnes, I. B. & Schett, G. The pathogenesis of rheumatoid arthritis. *The New England journal of medicine* **365**, 2205–2219, doi:10.1056/NEJMra1004965 (2011).
48. van de Loo, F. A., Kuiper, S., van Enckevort, F. H., Arntz, O. J. & van den Berg, W. B. Interleukin-6 reduces cartilage destruction during experimental arthritis. A study in interleukin-6-deficient mice. *Am J Pathol* **151**, 177–191 (1997).
49. de Benedetti, F. *et al*. Correlation of serum interleukin-6 levels with joint involvement and thrombocytosis in systemic juvenile rheumatoid arthritis. *Arthritis and rheumatism* **34**, 1158–1163 (1991).
50. Aderka, D., Le, J. M. & Vilcek, J. IL-6 inhibits lipopolysaccharide-induced tumor necrosis factor production in cultured human monocytes, U937 cells, and in mice. *J Immunol* **143**, 3517–3523 (1989).
51. de Hooge, A. S. *et al*. Male IL-6 gene knock out mice developed more advanced osteoarthritis upon aging. *Osteoarthritis and cartilage* **13**, 66–73, doi:10.1016/j.joca.2004.09.011 (2005).
52. Inanir, A., Yigit, S., Tekcan, A., Tural, S. & Kismali, G. IL-4 and MTHFR gene polymorphism in rheumatoid arthritis and their effects. *Immunology letters* **152**, 104–108, doi:10.1016/j.imlet.2013.05.004 (2013).
53. Prots, I. *et al*. Association of the IL4R single-nucleotide polymorphism I50V with rapidly erosive rheumatoid arthritis. *Arthritis and rheumatism* **54**, 1491–1500, doi:10.1002/art.21832 (2006).
54. Bowes, J. *et al*. Evidence to support IL-13 as a risk locus for psoriatic arthritis but not psoriasis vulgaris. *Annals of the rheumatic diseases* **70**, 1016–1019, doi:10.1136/ard.2010.143123 (2011).
55. Schett, G., Elewaut, D., McInnes, I. B., Dayer, J. M. & Neurath, M. F. How cytokine networks fuel inflammation: Toward a cytokine-based disease taxonomy. *Nature medicine* **19**, 822–824, doi:10.1038/nm.3260 (2013).
56. Guo, S. *et al*. Down-regulation of Z39Ig on macrophages by IFN-gamma in patients with chronic HBV infection. *Clinical immunology* **136**, 282–291, doi:10.1016/j.clim.2010.03.007 (2010).
57. Hii, C. S. *et al*. Characterization of the MEK5-ERK5 module in human neutrophils and its relationship to ERK1/ERK2 in the chemotactic response. *The Journal of biological chemistry* **279**, 49825–49834, doi:10.1074/jbc.M406892200 (2004).

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## Author Contributions

Conceived and designed the experiments: U.M., C.A.A. and A.F. Execution of experiments: U.M., A.S., A.Q. Data analysis: U.M., A.S., A.Q. Data interpretation: U.M., A.S., A.Q., C.A.A., N.N.G. and A.F. Wrote and or critical reading of manuscript: U.M., A.S., A.Q., N.N.G., C.A.A. and A.F.

## Additional Information

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**Competing Interests:** The authors declare that they have no competing interests.

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## Supplementary Methods

*Primers for CRIg transcript variants and CR1*

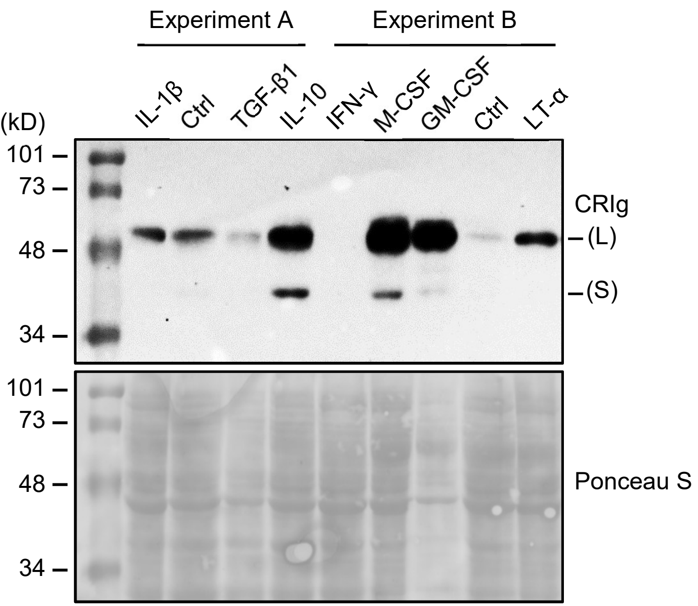
Reverse-transcriptase PCR to detect five CRIg (*VSIG4*) transcript variants and SYBR Green qPCR for CR1 were performed using the primers and pairings listed in Table S1. The different CRIg variants are distinguished by their NCBI RefSeq Accession numbers. Each *VSIG4* primer was designed to anneal across either exons 3-4 or 7-8.

**Supplementary Table S1.** PCR primer sequences specific for CRIg transcript variants and CR1.

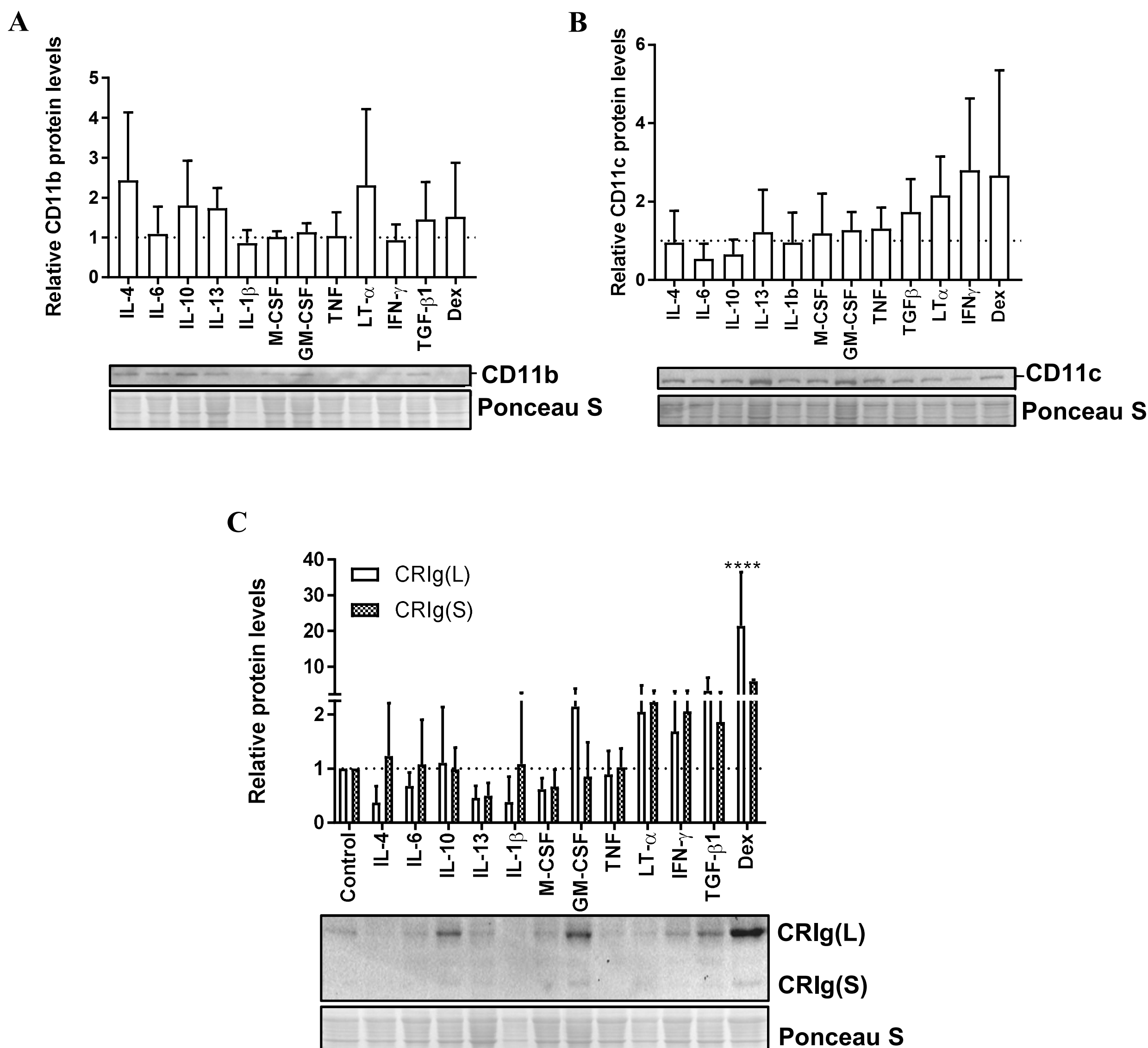
| Gene/ Primer | Sequence (5' to 3') | Pairing | NCBI RefSeq | Transcript No. |
|---|---|---|---|---|
| ***VSIG4*** | | | | |
| **F1** | TTTGTGGTCAAAGACTCCTCAAAGC | F1 and R1 | NM_007268.2 | 1 |
| **F2** | TGTCCAGAAACACTCCTCAAAGCT | F2 and R1 | NM_001100431.1 | 2 |
| **R1** | TGGCATGTGCCCTGGCT | F2 and R2 | NM_001184831.1 | 3 |
| **R2** | GAGAGACTTTCTTACCTGGCTGCTT | F1 and R2 | NM_001184830.1 | 4 |
| **R3** | GACACTTTGGGCTGGCTGCT | F1 and R3 | NM_001257403.1 | 5 |
| ***CR1*** | | | | |
| **F** | CCCTTTGGAAAAGCAGTAAA | | | |
| **R** | TCAACTTGGCAAACAGAAAA | | | |

*VSIG4* primer F1 paired with R1 are specific for transcript variant 1, F2 with R1 for transcript variant 2, F2 with R2 for transcript variant 3, F1 with R2 for transcript variant 4, and F1 with R3 for transcript variant 5. All *VSIG4* primer pairings generate an amplicon of 292 bp in length. The primers for CR1 are from Anand et al (2014)[1] and expected to generate a 193 bp amplicon.

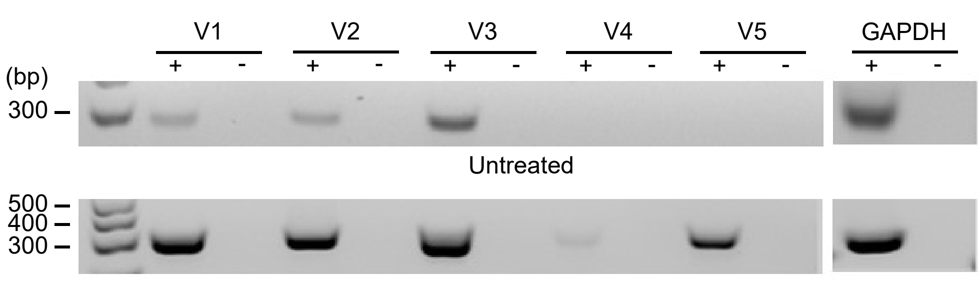
**Supplementary Figures**



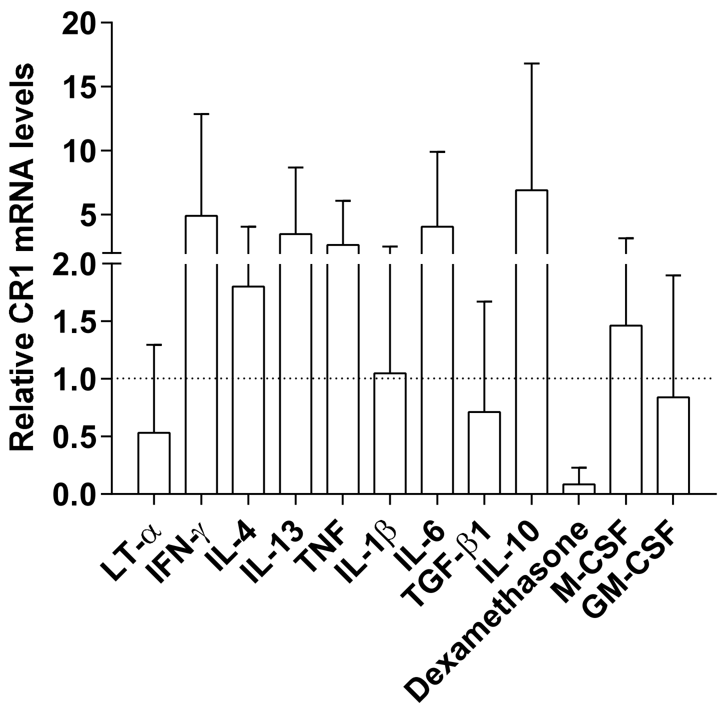

**Supplementary Figure S1. Representative Western blot demonstrating molecular sizing of CRIg isoforms in cytokine-induced development of macrophages.** The blot presents lysates from monocytes cultured in the presence of 40 ng/ml LT-α, IFN-γ, IL-1β, IL-10, M-CSF, GM-CSF, or 15 ng/ml TGF-β1 in separate individuals (experiment A and B) that were examined by staining with CRIg 3C9 monoclonal antibodies. The corresponding Ponceau S staining shows the consistency of protein load. Low Range Prestained SDS-PAGE Standards (Bio-Rad Laboratories) were used for determining the long (L) and short (S) forms of CRIg with ladder band sizes indicated in kilodaltons (kD).




**Supplementary Figure S2. Effects of cytokines on CRIg/CD11b/CD11c expression in matured macrophages (MDM).** In these studies, MDM were prepared by culturing human monocytes for 7 days. MDM from 7 day cultures were treated with 40 ng/ml LT-α, IFN-γ, IL-4, IL-13, IL-1β, IL-6, IL-10, M-CSF, GM-CSF or dexamethasone, 20 ng/ml TNF, or 15 ng/ml TGF-β1 for 24 h and then (**a**) CD11b, (**b**) CD11c, (**c**) CRIg protein levels relative to Ponceau S loading control were assessed by Western blot. Data are normalised against untreated control cells and expressed as means ± SD of three experiments, each conducted with cells from different individuals.




**Supplementary Figure S3. CRIg transcript variants in macrophages.** Agarose gel electrophoresis was used to visualise CRIg transcript variant amplicons generated from the cDNA of untreated macrophages (top row) and macrophages cultured for 3 days with 50 ng/mL dexamethasone (bottom row). The primers used are as shown in Table S1. Lanes labelled V1, V2, V3, V4 and V5 represent CRIg transcript variants 1, 2, 3, 4, and 5 respectively, with (+) indicating PCR with macrophage cDNA and (-) indicating PCR with no template. Amplification of GAPDH was used as an internal control. A 1kb Plus DNA Ladder (Invitrogen) was used to verify the size of the amplicons, with ladder band sizes indicated in base pairs (bp). Results are representative of three experiments.




**Supplementary Figure S4. Effects of cytokines on the development of CR1+ macrophages.** Monocytes were treated with 40 ng/ml LT-α, IFN-γ, IL-4, IL-13, IL-1β, IL-6, IL-10, M-CSF, GM-CSF or dexamethasone, 20 ng/ml TNF, 15 ng/ml TGF-β1, then CR1 mRNA expression measured. Data are normalised against untreated control cells and expressed as means ± SD of three experiments, each conducted with cells from different individuals.

## Supplementary References

1 Anand, D., Kumar, U., Kanjilal, M., Kaur, S. & Das, N. Leucocyte complement receptor 1 (CR1/CD35) transcript and its correlation with the clinical disease activity in rheumatoid arthritis patients. *Clinical and experimental immunology* **176**, 327-335, doi:10.1111/cei.12274 (2014).

# Chapter 5. Expression of CRIg by human dendritic cells

## 5.1. Introduction and Contextual Statement

As discussed in earlier chapters, CRIg is a potent negative regulator of T cell activation, proliferation, and cytokine production (Vogt et al., 2006). This function is retained by both recombinant, soluble forms of the protein (Yuan et al., 2017) and membrane-bound protein when induced on the cell surface (Xu et al., 2010). However, whether unaltered, naturally expressed CRIg exerts this function, and whether human DC are able to express protein naturally without transfection remains to be studied.

In this chapter, we expand upon the groundwork presented in earlier chapters and deepen our investigations into the expression of CRIg on human immune cell types in states of health. We demonstrate the expression and regulatory function of CRIg expressed by human MDDC, and show that MDDC stimulated to express high levels of CRIg on their surface through treatment with dexamethasone significantly inhibit both phytohemagglutinin (PHA)-induced and alloantigen-induced T cell proliferation responses.

The following paper entitled *'Human Dendritic Cells Express the Complement Receptor Immunoglobulin Which Regulates T Cell Responses'*, by Usma Munawara, Khalida Perveen, **Annabelle G. Small**, Trishni Putty, Alex Quach, Nick N. Gorgani, Charles S. Hii, Catherine A. Abbott & Antonio Ferrante was published in the peer reviewed journal, Frontiers in Immunology, in December 2019 (7: 4050, DOI: 10.3389/fimmu.2019.02892). Supplementary information follows the paper.

*Disclaimer: Significant portions of the results presented in this manuscript were generated by Dr. Usma Munawara throughout the completion of her Ph.D. studies and submitted for the award of her degree.*

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| Name of Principal Author (Candidate) | Annabelle Small | | |
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| Contribution to the Paper | AS assisted with the data presentation, the writing of the manuscript and the conducting of experiments | | |
| Overall percentage (%) | 10% | | |
| Certification: | This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. | | |
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## Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

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|---|---|---|---|
| Contribution to the Paper | UM performed the majority of the experiments, was involved in the planning of the experiments, collating the data and the writing of the paper. | | |
| Signature | | Date | 12/3/20 |

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| Contribution to the Paper | KP was responsible for the allogeneic-induced T cell proliferation and cytokine production experiments, as well as the Treg measurements. | | |
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# Human Dendritic Cells Express the Complement Receptor Immunoglobulin Which Regulates T Cell Responses

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The B7 family-related protein V-set and Ig containing 4 (VSIG4), also known as Z39Ig and Complement Immunoglobulin Receptor (CRIg), is the most recent of the complement receptors to be identified, with substantially distinct properties from the classical complement receptors. The receptor displays both phagocytosis–promoting and anti-inflammatory properties. The receptor has been reported to be exclusively expressed in macrophages. We now present evidence, that CRIg is also expressed in human monocyte-derived dendritic cells (MDDC), including on the cell surface, implicating its role in adaptive immunity. Three CRIg transcripts were detected and by Western blotting analysis both the known Long (L) and Short (S) forms were prominent but we also identified another form running between these two. Cytokines regulated the expression of CRIg on dendritic cells, leading to its up- or down regulation. Furthermore, the steroid dexamethasone markedly upregulated CRIg expression, and in co-culture experiments, the dexamethasone conditioned dendritic cells caused significant inhibition of the phytohemagglutinin-induced and alloantigen-induced T cell proliferation responses. In the alloantigen-induced response the production of IFNγ, TNF-α, IL-13, IL-4, and TGF-β1, were also significantly reduced in cultures with dexamethasone-treated DCs. Under these conditions dexamethasone conditioned DCs did not increase the percentage of regulatory T cells (Treg). Interestingly, this suppression could be overcome by the addition of an anti-CRIg monoclonal antibody to the cultures. Thus, CRIg expression may be a control point in dendritic cell function through which drugs and inflammatory mediators may exert their tolerogenic- or immunogenic-promoting effects on dendritic cells.

**Keywords: dendritic cells, complement receptor immunoglobulin (CRIg), dexamethasone, cytokines, T cells, immunosuppression**

## INTRODUCTION

The Complement Receptor Immunoglobulin (CRIg), unlike other complement receptors, is expressed selectively in macrophages (1). The receptor plays a key role in the phagocytosis and clearance of bacteria in a complement dependent (1–3) and complement independent manner (4). But in addition, it has been reported to inhibit T cell responses. Vogt et al. (5) demonstrated that CRIg-Ig fusion protein inhibited the anti-CD3 or anti-CD3/28 antibody(s) induced mouse and human T cell proliferation and IL-2 production *in vitro*. When this fusion protein was injected into mice, there was a reduction in the numbers of antigen-induced $CD8^+$ T cells and a reduction in the IFN-γ producing population. In these mice the Th-dependent IgG antibody response was reduced. CRIg expression in macrophages has been suggested to regulate the T cell response (6, 7). We demonstrate that human DC express CRIg mRNA and protein, including cell surface expression and that expression could be modulated by cytokines. Furthermore, dexamethasone was found to cause upregulation of CRIg expression on DC which inhibited the mitogen- and alloantigen-induced T cell response. This highlights an additional mechanism involved in the regulation of the adaptive immune response.

## METHODS

### Cytokines and Cell Culture Reagents

Recombinant human cytokines used for DC treatments were as follows: LT-α (TNF-β), GM-CSF, M-CSF, IL-1β, IL-6, IL-4, TNF-α, IL-13, IFN-γ, IL-10 (ProSpec-Tany Technogene, Rehovot, Israel) and TGF-β1 (R&D Systems, Minneapolis, Minnesota, USA) were used in culture within a final concentration range of 5–80 ng/ml. Dexamethasone was used at a final concentration in culture at 30 ng/ml (Sigma-Aldrich, St. Louis, MO). All cell culture experiments utilized RPMI 1640 tissue culture medium, heat-inactivated (56°C/20 min) fetal calf serum (FCS), penicillin/streptomycin and L-glutamine (SAFC Biosciences, Lenexa, KS).

### Antibodies

Anti-human protein antibodies used in this study were as follows: mouse monoclonal anti-CRIg clone 3C9 (kindly provided by Dr. van Lookeren Campagne, Genentech, San Francisco, CA), phycoerythrin (PE)-conjugated and unconjugated anti-CRIg clone 6H8 (Santa Cruz Biotechnology, Dallas, TX, USA), fluorescein isothiocyanate (FITC)-conjugated anti-DC-SIGN/CD209 clone 120507 (R&D Systems). Isotype controls used were as follows: PE-conjugated and unconjugated mouse $IgG_1$ (eBioscience, San Diego, CA), and FITC-conjugated mouse $IgG_{2b}$ (R&D Systems). The secondary antibody used for Western blotting was horse-radish peroxidase (HRP)-conjugated rabbit anti-mouse IgG (Dako, Glostrup, Denmark).

### Preparation of Dendritic Cells

The study was approved by the CYWHS Human Ethics Committee (approval number REC 2165/4/2011). Dendritic cells were prepared from peripheral blood of healthy donors, who had given informed consent, utilizing previously described methods (8, 9). Blood was layered onto Ficoll® Paque PLUS (GE Healthcare, Uppsala, Sweden), $d = 1.077$, and centrifuged at 400 × $g$ for 30 min. Firstly blood monocytes were prepared as described previously (9). The peripheral blood mononuclear cell (PBMC) layer was harvested and washed in RPMI-1640 medium with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% heat-inactivated FCS. Then, the PBMCs were layered onto 46% iso-osmotic Percoll® gradient (GE Healthcare, Uppsala, Sweden) and centrifuged at 600 × $g$ for 30 min. After centrifugation the lymphocytes were pelleted, and the upper monocyte-containing interphase layer was harvested and washed, with preparations routinely being of >98% viability and >90% purity by Giemsa. For all studies unless otherwise stated, monocytes were seeded at $1 \times 10^6$ cells per 60 × 15 mm culture dish pre-treated with autologous plasma and left to adhere at 37°C for 1 h. Any contaminating non-adherent cells were removed, and the adherent monocytes cultured with RPMI-1640 medium with L-glutamine, penicillin, streptomycin, FCS, 50 ng/ml GM-CSF, and 20 ng/ml IL-4 at 37°C in an atmosphere of 95% air and 5% $CO_2$ over 5 days for differentiation into DCs. The DCs were harvested by gentle pipetting and washed prior to use in experiments.

### DC-T Cell Co-cultures

Autologous DC and T cell co-cultures were setup using DCs as prepared above, with autologous T cells purified from the remaining lymphocyte fraction following the centrifugation of PBMC over 46% iso-osmotic Percoll® gradient. The T-cells were purified by subjecting the lymphocyte fraction through two cycles of nylon wool (Polysciences Inc., Warrington, PA) columns using an established protocol (10). The T-cell preparation was of >95% purity and >99% viability as determined by FACS analysis and trypan blue dye exclusion assay, respectively. The T cells were cryopreserved in liquid nitrogen until use (11). The DCs were added to 96-well round-bottom plate (Nunc) at $1 \times 10^4$ cells/well and treated with dexamethasone for 24 h and washed. The cryopreserved T cells were thawed and added to the autologous DC ($2 \times 10^5$ T-cells/well). PHA was used as a stimulus in the appropriate wells (0.5 μg/well) (Remel Inc., San Diego, CA), with or without either anti-CRIg (clone 6H8) antibody or isotype control. The cells were cultured at 37°C in an atmosphere of 95% air and 5% $CO_2$ for 72 h. Cells were pulsed with 1 μCi methyl-$^3$H Thymidine ($^3$H-TdR) (PerkinElmer, Waltham, MA) 6 h prior to harvest. $^3$H-TdR incorporation was measured as disintegrations per minute (DPM) in a Wallac 1409 liquid scintillation beta counter (Wallac, Turklo, Finland).

For allogeneic DC-T cell cultures, instead of autologous T cells, allogeneic T cells were isolated from fresh or cryopreserved PBMCs using the EasySep™ Human T Cell Isolation Kit (Stem Cell Technologies, Vancouver, Canada), and added to allogeneic DCs as the stimulus in a DC:T cell ratio of 1:10 as $2 \times 10^5$ total cells/well, with or without anti-CRIg antibody or isotype control. DCs were untreated or dexamethasone treated DC at $2 \times 10^4$ cells/well in 96-well round-bottom plates. Cells were cultured at 37°C in an atmosphere of 95% air and 5% $CO_2$ for 120 h and pulsed with $^3$H-TdR 6 h prior to harvest. At harvest,

culture supernatants were harvested and stored at −80°C for later quantification of cytokines, followed by measurement of the remaining cells for $^{3}$H-TdR incorporation. Cytokines in the culture supernatants were quantitated with BD™ Cytometric Bead Array kits for IFN-γ, TNF-α, IL-13, TGF-β1, IL-4, and IL-10 (BD Biosciences) following adaptation of the manufacturer's protocols for assay in 96-well v-bottom plates, with acquisition on a BD FACSCanto with an attached BD™ High Throughput Sampler (HTS), and analysis with FCAP Array v3 software (BD Biosciences).

In similar culture setups, we examined the T cells for the presence of Treg cells in the alloreactive stimulation as above. After 7 days of culture the cells were harvested and the levels of $CD4^{+}CD25^{+}$ $CD127^{lo}Foxp3^{+}$ cells measured by flow cytometry. Anti-human CD4-FITC, CD25-PE-Cy7, CD127-Alexa Fluor 647, Foxp3-PE, and corresponding isotype controls were from BD Biosciences. Cell surfaces were stained with appropriate antibodies for 20 min at room temperature (RT), washed once with PBS supplemented with 0.1% FCS, and incubation for 60 min at RT in Fixation/Permeabilization buffer (eBioscience). Following washing with Permeabilization buffer (eBioscience) and blocking with mouse IgG for 10 min at RT, intracellular staining of Foxp3 was performed in Permeabilization buffer with 30 min incubation at RT with the appropriate antibody or isotype control. Acquisition was performed on a BD FACSCanto and data analyzed using FlowJo v10.1 (FlowJo, LLC, Ashland, Oregon). The gating strategy to identify $Foxp3^{+}$ Tregs is described in **Supplementary Figure 3**.

## Measurement of CRIg by RT-PCR and qPCR

For determination of total CRIg mRNA levels and isoform transcript detection, RNA was isolated using a RNeasy® Plus kit (Qiagen, Venlo, Limburg, Netherlands) according to the manufacturer's instructions, and treated with DNase I (DNA-free Kit, Ambion, Life Technologies, Mulgrave, Vic, Australia) to remove any genomic DNA contamination. The quantity of RNA was assessed on a NanoDrop™ (Thermo Fisher Scientific, MA, USA), and converted to cDNA using the iScript™ cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA).

Reverse transcriptase (RT)-PCR for CRIg isoform transcripts was conducted as previously described (12), using primers for isoform 1 (F1: TTTGTGGTCAAAGACTCCTCAAAGC; and R1: TGGCATGTGCCCTGGCT), isoform 2 (F2: TGTCCAGA AACACTCCTCAAAGCT; and R1), isoform 3 (F2; and R2: GAGAGACTTTCTTACCTGGCTGCTT), isoform 4 (F1 and R2), and isoform 5 (F1; and R3: GACACTTTGGGCTGGC TGCT). GAPDH primer sequences were used as previously described (12) (F: GAGTCAACGGATTTGGTCGT; R: GACA AGCTTCCCGTTCTCAGCCT). Separate reactions were set up for each isoform, containing 100 nM of each primer (pairing as described above), 1 μl of cDNA, and AmpliTaq Gold® 360 Master Mix (Applied Biosystems) in a 25 μl final volume. PCR reactions were performed with an initial denaturation at 95°C for 7 min, followed by 35 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 60 s, and a final extension at 72°C for 7 min, using a SimpliAmp™ Thermal Cycler (Applied Biosystems). The RT-PCR products were visualized following electrophoresis on a 2% GelRed-stained agarose gel (Biotium) along with a 1 kb Plus DNA Ladder (Invitrogen).

qPCR for total CRIg mRNA expression was conducted as previously described (12) using the primer pair detecting all five known isoforms of CRIg (F: ACACTTATGGCCGTCCCAT; R: TGTACCAGCCACTTCACCAA) with the GAPDH primer pair described above for expression data normalization. Each reaction had a final volume of 20 μl containing 100 nM of each primer, 1 μl of cDNA, and iQ SYBR Green Supermix (Bio-Rad Laboratories). Triplicate reactions were assayed in an iQ5 Real Time Detection System with iQ5 Optical System v2.1 software (Bio-Rad Laboratories), with thermal cycling performed with an initial denaturation at 95°C for 5 min, followed by 40 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s.

## Measurement of CRIg Cell-Surface Expression

The expression of CRIg on the cell surface of DCs was measured by flow cytometry. At the conclusion of treatment, $1.5 \times 10^{5}$ harvested DCs had Fc receptors on their surface blocked with ice-cold PBS supplemented with 0.5% (w/v) BSA, 10 mg/ml Intragam P, and 5% (v/v) human AB serum for 30 min. PE-conjugated anti-CRIg (clone 6H8) or isotype control antibodies, along with FITC-conjugated anti-CD209 antibodies were incubated with the DCs in a final staining volume of 50 μl for 30 min. The cells were washed in PBS with 0.5% (w/v) BSA, and following centrifugation (600 × *g* for 5 min), the cells were then fixed in PBS containing 1% (v/v) formaldehyde. A minimum of 20,000 events were acquired from the stained DC samples on a BD FACSCanto (BD Biosciences, CA, USA), with data analysis performed with FlowJo 10.1 (FlowJo, LLC, Ashland, Oregon). Doublets were excluded by gating with SSC-A vs. SSC-H. Trypan blue was used to determine cell viability (>95%) following harvest and prior to flow cytometric staining. Using 7-aminoactinomycin D (7-AAD), we were able to demonstrate specific DC viability in a set of replication experiments (**Supplementary Figure 1**) where similar results of enhanced CRIg expression by dexamethasone treatment was found.

## Western Blotting for CRIg Isoforms

Western blot for CRIg expression in DCs was performed using methods previously described (13). DCs harvested from each culture were lysed in 100 μl of 20 mM HEPES, pH 7.4, with 0.5% (v/v) Nonidet P-40, 100 mM NaCl, 1 mM EDTA, 2 mM $Na_3VO_4$, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride and 10 μg/ml leupeptin, aprotonin, pepstatin A, and benzamidine, for 2 h at 4°C with constant mixing. These samples were centrifuged at 12,000 × *g* for 5 min to obtain lysates (supernatants), and the protein content quantitated by Lowry assay, prior to the addition of Laemmli buffer supplemented with 3% β-mercaptoethanol. The lysates were boiled at 100°C for 5 min and 60 μg of protein loaded and electrophoresed on 12% SDS polyacrylamide gels, followed by transfer of protein onto nitrocellulose membrane (Pierce Biotechnology, Thermo Fisher Scientific, Rockford, IL). The membrane was stained with 0.1% Ponceau S (in 5% acetic

acid) to ascertain protein loading equality. The amounts of the CRIg L, S and I isoforms were detected using monoclonal mouse anti-human CRIg clone 3C9 and HRP-conjugated rabbit anti-mouse IgG. The immune complexes on the membranes were visualized by enhanced chemiluminescence on a ChemiDoc XRS+ Imaging System and quantitated using Image Lab™ software version 3.0 (Bio-Rad Laboratories, Hercules, CA).

## Statistical Analysis

Statistical significance was calculated using GraphPad Prism 7.0 (GraphPad Software, Inc., La Jolla, CA, USA), with testing as follows: two-way ANOVA with *post-hoc* Bonferroni's Multiple Comparison testing for relative CRIg protein isoforms; Student *t*-testing for relative CRIg surface expression and dexamethasone-modulated CRIg mRNA expression; One-way ANOVA with *post-hoc* Dunnett's Multiple Comparison testing for cytokine dose-dependent CRIg mRNA expression; and One-way ANOVA with *post-hoc* Bonferroni's Multiple Comparison testing for $^3$H-TdR incorporation between DC-T cell co-culture treatments. Statistical significance was defined as $P < 0.05$.

# RESULTS

## Expression of CRIg on Human DC

Human monocyte derived dendritic cells (MDDC) were generated in culture by treating monocytes with IL-4 and GM-CSF. The MDDC expressed CRIg mRNA by RT-PCR (**Figure 1A**) and CRIg protein on their surface by flow cytometry analysis (**Figure 1B**). Examination of transcripts showed that at least three isoforms of CRIg were present (**Figure 1A**). Furthermore, by Western blot analysis we identified the expression of the prominent long (L) and short (S) isoforms, as previously described in human macrophages (1) and an additional intermediate form migrating between the L and S isoforms (**Figure 1C**).

## Dexamethasone Increases CRIg Expression in DC Leading to Immunosuppression

Previously, we have demonstrated that the anti-inflammatory steroid dexamethasone is a strong enhancer of CRIg expression in human macrophages (12, 14, 15). In addition, it has been reported that DC generated under the influence of dexamethasone have a tolerogenic functional phenotype (16). It was therefore of interest to determine whether dexamethasone alters the expression of CRIg on DC. The MDDC were treated with varying concentrations of dexamethasone for 24 h, washed and CRIg expression measured. Dexamethasone caused an increase (3-fold) in CRIg mRNA levels (**Figure 1D**). This was reflected in an increase in CRIg protein measured by Western blotting (**Figure 1C**). Examination of the Western blots also revealed that dexamethasone caused an increase in the levels of all 3 isoforms of CRIg on DC (**Figure 1C**). The changes induced by dexamethasone were also evident in expression of CRIg on the surface of DC (**Figure 1E**), which has implications for the function of DC as antigen presenting cells and adaptive immunity.

To assess the functional consequences of increasing CRIg expression, we examined whether DC which had been treated with dexamethasone, expressing increased amounts of cell surface CRIg, were immunosuppressive in cell co-culture studies. Mononuclear leukocytes (MNL) from single individuals were separated into T cells and monocytes. The T cells were cryopreserved and the monocytes were treated with GM-CSF and IL-4 to allow development into DC. Then, the T cells were thawed and reconstituted with DC which had been pre-treated with either diluent or dexamethasone. The cells were stimulated with phytohemagglutinin (PHA) and proliferation was measured by a radiometric assay. The data showed that T cells cultured in the presence of dexamethasone conditioned DC were significantly depressed in proliferation (**Figure 2A**). Further studies examined the importance of surface expressed CRIg in the immunosuppression by adding anti-CRIg monoclonal antibody to the cultures (clone 6H8, Santa Cruz Biotechnology, Dallas, TX). The results showed that the suppression by dexamethasone conditioned DC could be completely prevented by the antibody (**Figure 2A**). The normalized data has been presented in **Supplementary Figure 3**.

In the second set of experiments, the effects of dexamethasone were assessed in an allogeneic T cell stimulation culture model. Monocyte derived DC were treated with dexamethasone, washed and added to allogeneic T cells. After 5 days of culture the culture fluids removed and the cells were replenished with fresh medium containing $^3$HTdR and harvested after 6 h of further incubation. The amount of radioactivity incorporated was determined and proliferation quantitated. The dexamethasone treated DCs caused a significant decrease in the allogeneic proliferative response (**Figure 2B**). When anti-CRIg monoclonal antibody was added to the cultures the effect was essentially abolished, suggesting that CRIg played a role in the immunosuppression. When we examined the cytokines IFNγ, TNF-α and IL-4, IL-13, IL-10, and TGF-β1 in the supernatants from these cultures, we observed that production of all these cytokines was significantly reduced in the presence of dexamethasone treated DCs except for IL-10 (**Figures 2C–H**), and that the addition of anti-CRIg monoclonal antibody prevented this suppression in cytokine production (**Figures 2C–G**). Examination of the lymphocyte population for Treg cells demonstrated that based on the expression of CD127, CD25, and FoxP3 expression there was no increase but if anything a decrease in this subset (**Figure 2I**). The normalized data has been presented in **Supplementary Figure 3**.

## Th1 and Th2 Cytokines Alter the Expression of CRIg

In all of the following studies, we used CRIg$^+$ DC that had been derived from monocytes cultured in the presence of GM-CSF and IL-4. No dexamethasone treatment was conducted. Our study examined the effects of four cytokines, IFN-γ and LT-α representing Th1 cell and IL-4 and IL-13 representing Th2 cell products on DC CRIg expression. When CRIg$^+$ DC were treated with LT-α for 24 h, the cells showed a concentration dependent decrease in CRIg mRNA over a concentration range of 5–40 ng/ml (**Figure 3A**). This effect was supported by the finding

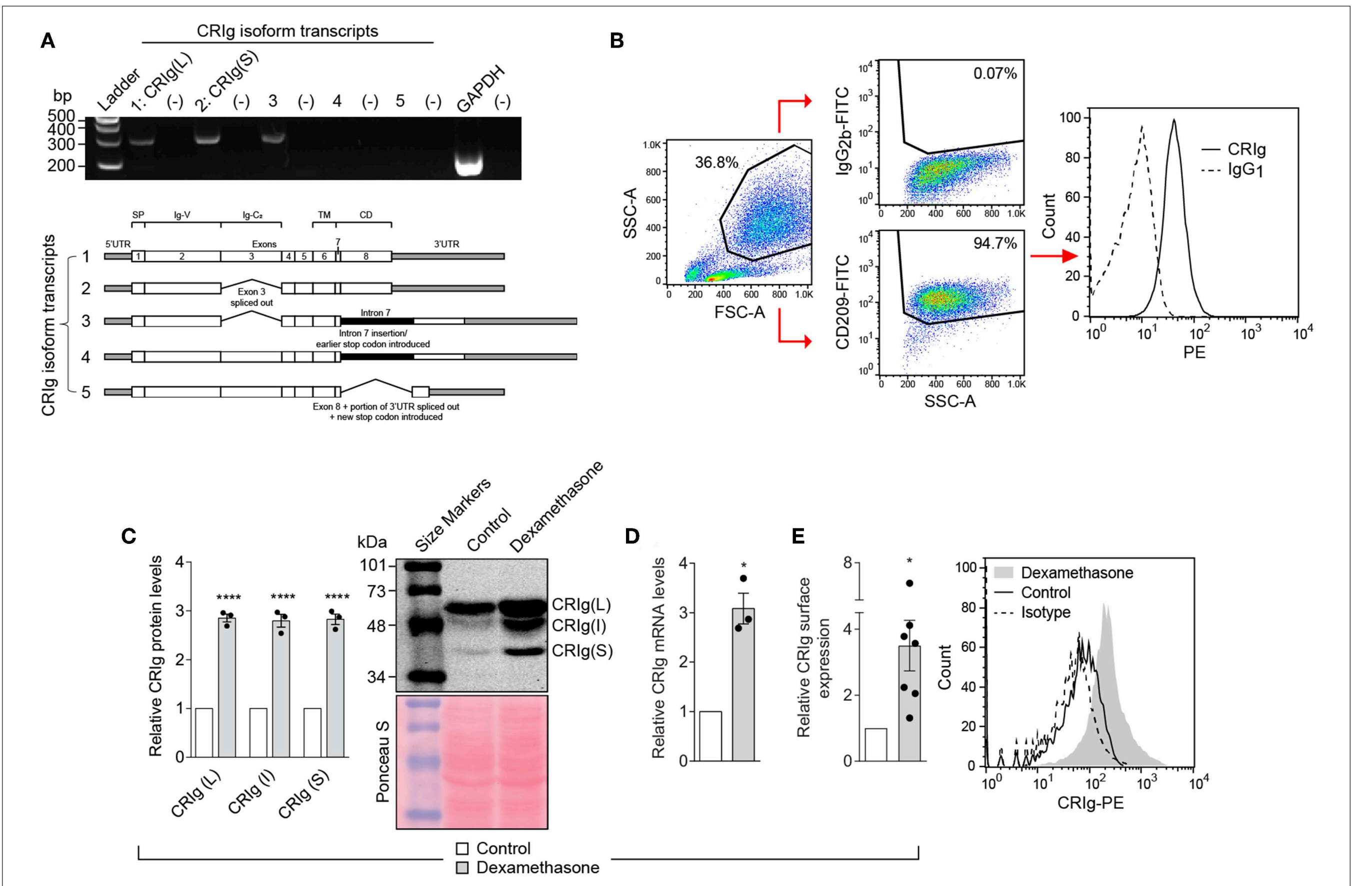


**FIGURE 1 |** CRIg expression in MDDC and effects of dexamethasone. **(A)** CRIg isoform transcripts detected in DC. Agarose gel electrophoresis (2%) was used to visualize transcript variant amplicons generated from the cDNA of HMDC. Lanes are labeled 1, 2, 3, 4, and 5 representing each CRIg isoform with (–) to the right of each isoform lane indicating the respective no template controls. GAPDH was a positive control. Gels are representative of three experiments. **(B)** Gating strategy for CRIg expression on DC by flow cytometry. $CD209^+$ cells were gated before assessing anti-CRIg antibody (6H8-PE) staining; representative histogram shown. **(C)** CRIg isoform expression by Western blot using anti-CRIg monoclonal antibody (clone 3C9). A representative blot of total protein stained with Ponceau S shows consistency of protein loading. Band intensity for each isoform (L, I, and S) over protein load was determined by densitometry. Data are expressed as fold-change over control DC ($n = 3$). **(D)** Relative CRIg mRNA expression as detected by qPCR, normalized to GAPDH, expressed as fold-change over control DCs ($n = 3$). **(E)** Relative CRIg cell-surface expression by flow cytometry. Data are expressed as fold-change in CRIg-PE (6H8) mean fluorescence intensity minus isotype control (IgG1) of treated over control DCs ($n = 7$); representative histogram shown. Data are presented as means ± SEM of experiments conducted with cells from different individuals. Significance levels are indicated by asterisks: $^*P < 0.05$, $^{****}P < 0.0001$.

that LT-α caused a significant decrease in total CRIg protein measured by Western blotting (**Figure 3B**), with a concomitant decrease of the L and S as well as the intermediate isoforms. A corresponding effect on cell surface expression was observed (**Figure 3C**). Treatment with 5–40 ng/ml of IFN-γ showed a similar decrease in CRIg mRNA, total protein and cell surface expression as seen with LT-α (**Figures 3D–F**).

The Th2 cytokines, IL-4, and IL-13 caused an even more profound decrease in CRIg expression in the DC (**Figure 4**). The cytokines caused a decrease in CRIg mRNA expression over a concentration range of 5–40 ng/ml. A similar decrease was observed when total CRIg protein was measured by Western blot (**Figures 4B,E**). Expression of all three CRIg isoforms was decreased by treatment with either IL-4 or IL-13. However, this decrease was not reflected in a reduced expression of cell surface CRIg (**Figures 4C,F**).

## The Regulatory and Immunosuppressive Cytokines Increase CRIg Expression

The cytokine TGF-β1 regulates inflammation and IL-10 has immunosuppressive activity (17–19). Their action could in part be through the regulation of CRIg on DC. We found that MDDC treated with IL-10 for 24 h showed a significant increase in CRIg mRNA expression in a concentration dependent manner (**Figure 5A**). Examination by Western blot showed a corresponding increase in CRIg protein expression (**Figure 5B**). However, this increase was not as evident in cell surface CRIg expression (**Figure 5C**). TGF-β1 also increased CRIg expression of mRNA, total CRIg protein and cell-surface expression on DC (**Figures 5D–F**). Examination of cell lysates subjected to Western blots showed that both TGF-β1 and IL-10 caused an increase in the levels of the L and S isoforms as well as the intermediate form on DC (**Figures 5B,E**).

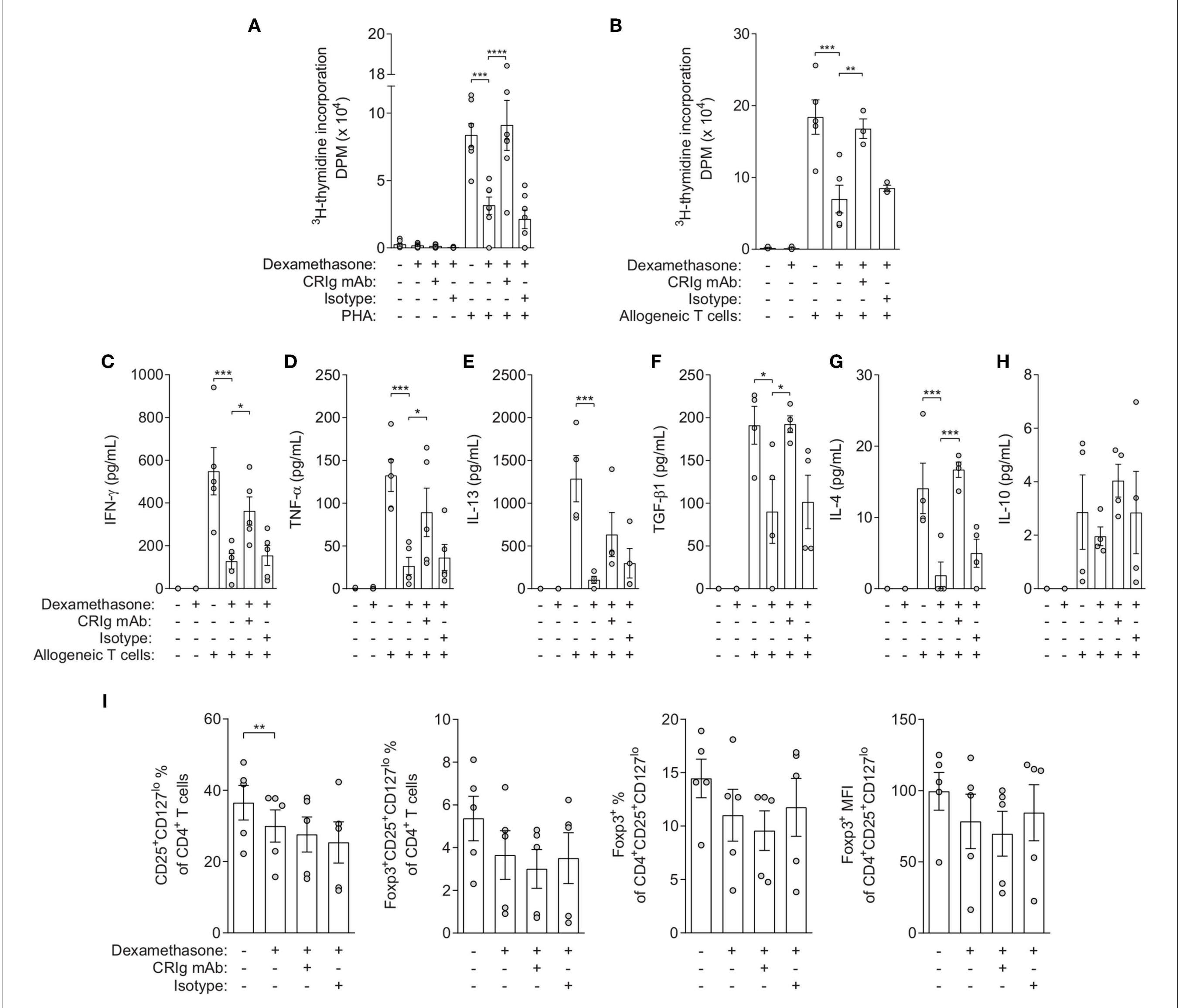


**FIGURE 2 |** Effects of dexamethasone conditioned DCs and anti-CRIg monoclonal antibody on PHA- and allogeneic-induced T cell proliferation, cytokine production and generation of iTreg. **(A)** $^{3}$H-TdR incorporation (DPM) in autologous DC-T cell co-culture in the presence/absence of dexamethasone, anti-CRIg 6H8 or isotype control antibodies, and PHA. Data are presented as means ± SEM of 6 experiments conducted with cells from different individuals. **(B)** $^{3}$H-TdR incorporation (DPM) in allogeneic DC-T cell co-culture in the presence/absence of dexamethasone treated DCs, anti-CRIg 6H8 antibody or isotype control antibodies. Data are presented as mean ± SEM of 5 experiments. **(C–H)** Effects on cytokine production in allogeneic DC-T cell cultures. Data are presented as mean ± SEM of 4–5 experiments. **(I)** Effects on generation of iTreg in allogeneic DC-T cell cultures. Data are presented as mean ± SEM of 4 experiments. Note that normalized data for the above cytokine production and Treg generation is presented in **Supplementary Figure 3**. Significance levels are indicated by asterisks: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

## Effects of Pyrogenic Cytokines on the Expression of CRIg in DC

The cytokines TNF-α, IL-1β, and IL-6 are pyrogenic cytokines (20, 21) which have direct effects on monocytic cells, including the modulation of macrophage and DC differentiation (22–25) or macrophage function and cell death (26, 27). Since these are produced during the innate phase of the inflammatory response, they may influence the adaptive immune response through their effects on DC. It was therefore of interest to examine this group of cytokines on CRIg expression in DC. Cells treated for 24 h with either TNF-α, IL-1β, or IL-6 showed a significant decrease in CRIg mRNA expression, in a concentration dependent manner (**Figure 6**). This effect was reflected in the total CRIg protein expression decreased by the cytokines. However, there was no corresponding decrease in cell surface expression, apart from TNF-α (**Figure 6**). It was also evident that both TNF-α and IL-1β caused a decrease in all three isoforms of CRIg, shown by Western blot analyses but the S form was not significantly decreased by the IL-6 treatment (**Figures 6B,E,H**).

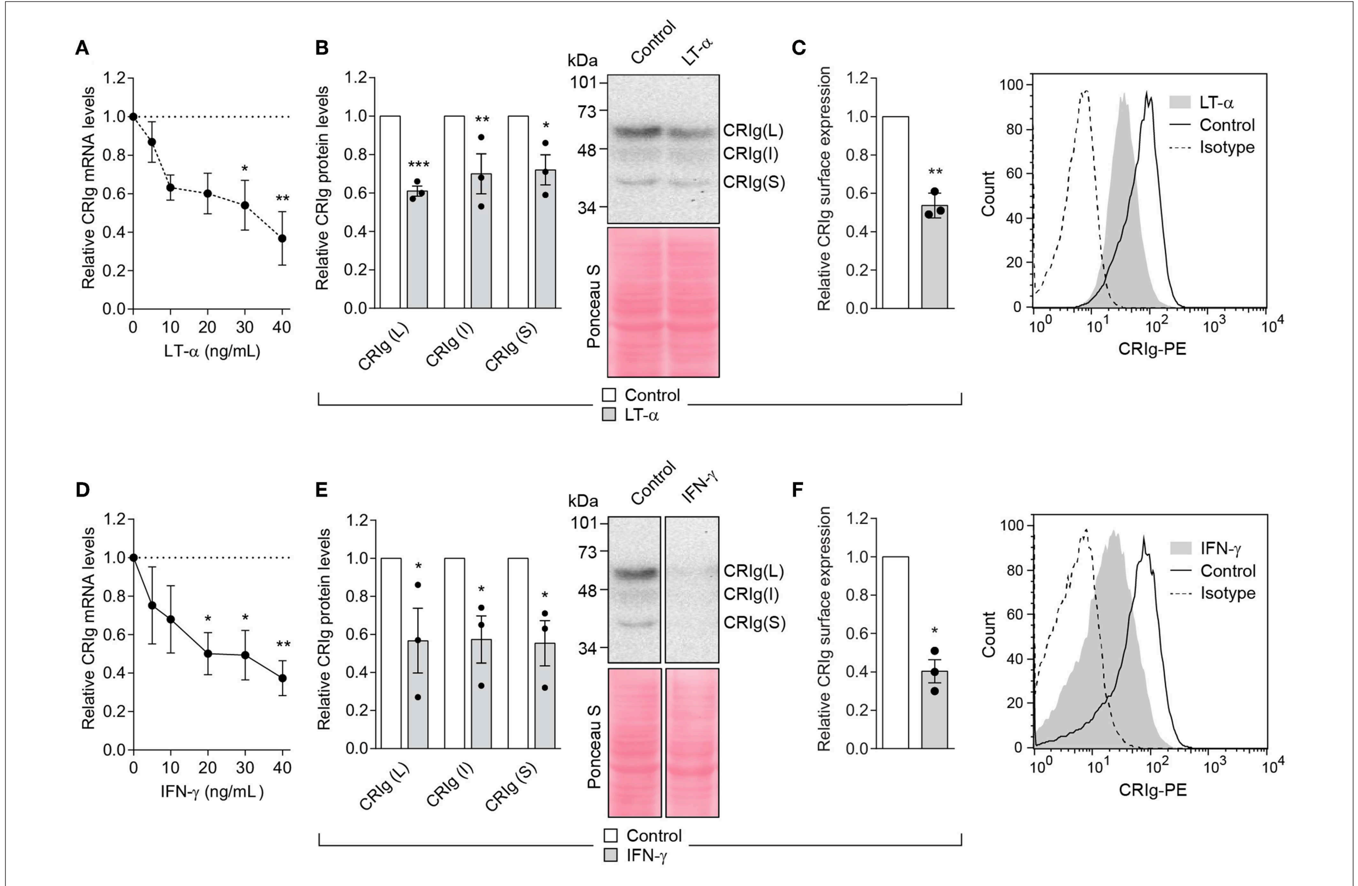


**FIGURE 3 |** LT-α and IFN-γ decrease CRIg expression in DC. **(A,D)** DC were treated with varying concentrations of cytokines and the levels of CRIg mRNA determined after 24 h of culture. **(B,E)** In other experiments, CRIg isoform proteins were assessed by Western blot after cells were treated with 40 ng/ml of each of the cytokines. **(C,F)** The effects on cell surface expression of CRIg was examined after a similar treatment. Data are presented as means ± SEM of three experiments, each conducted with cells from different individuals. The blot image **(E)** was spliced to exclude intervening lanes that represent other treatments and the complete un-spliced blot can be found in **Supplementary Figure 2**. Significance levels are indicated by asterisks: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

## Effect of Colony Stimulating Factors, M-CSF, and GM-CSF

Both M-CSF and GM-CSF have been reported to alter DC differentiation and/or function (22, 28). Examination of the effects of M-CSF and GM-CSF on the expression of CRIg in DC showed that cells treated with M-CSF display a marked increase in CRIg mRNA expression (**Figure 7A**). The increase paralleled the increase seen in total CRIg protein assayed by Western blot. The cytokine caused several-fold increase in the levels of CRIg protein expression (**Figure 7B**). Similar increases in CRIg expression of mRNA and total protein (**Figures 7D,E**) occurred in the presence of GM-CSF. However, we found that neither of these cytokines caused any changes in expression of cell surface CRIg (**Figures 7C,F**). As with other cytokines, all three isoforms of CRIg were concomitantly increased by the CSFs (**Figures 7B,E**).

## DISCUSSION

The data provide evidence that CRIg is expressed by human monocyte derived dendritic cells (MDDC). Expression is observed at the mRNA, protein and cell surface level. The level of expression may dictate whether the cell promotes T cell responsiveness or unresponsiveness. Thus, increasing the surface expression of CRIg by treating with dexamethasone rendered the DC not capable of supporting the T cell response to PHA or alloantigen stimulation. Evidence that the dexamethasone-conditioned DC work through CRIg is provided by the finding that addition of an anti-CRIg monoclonal antibody to the cultures prevents the immunosuppression in both culture models. It has already been suggested that CRIg participates in adaptive immunity (5, 6, 29, 30). While Xu et al. (29) did not find CRIg expression in human MDDC, when these cells were transfected with the CRIg gene (representing the L form), the protein was expressed. The induced expression of CRIg in the transfected DC led to an immunosuppressed response or tolerance (29). This supports our data that CRIg expression regulates immune responsiveness. The inability to show expression in the non-transfected cells may be due to the fact that Xu et al. (29) treated the cells with TNF-α toward the end of their maturation phase. Our results show that TNF-α causes the down regulation of CRIg expression. While

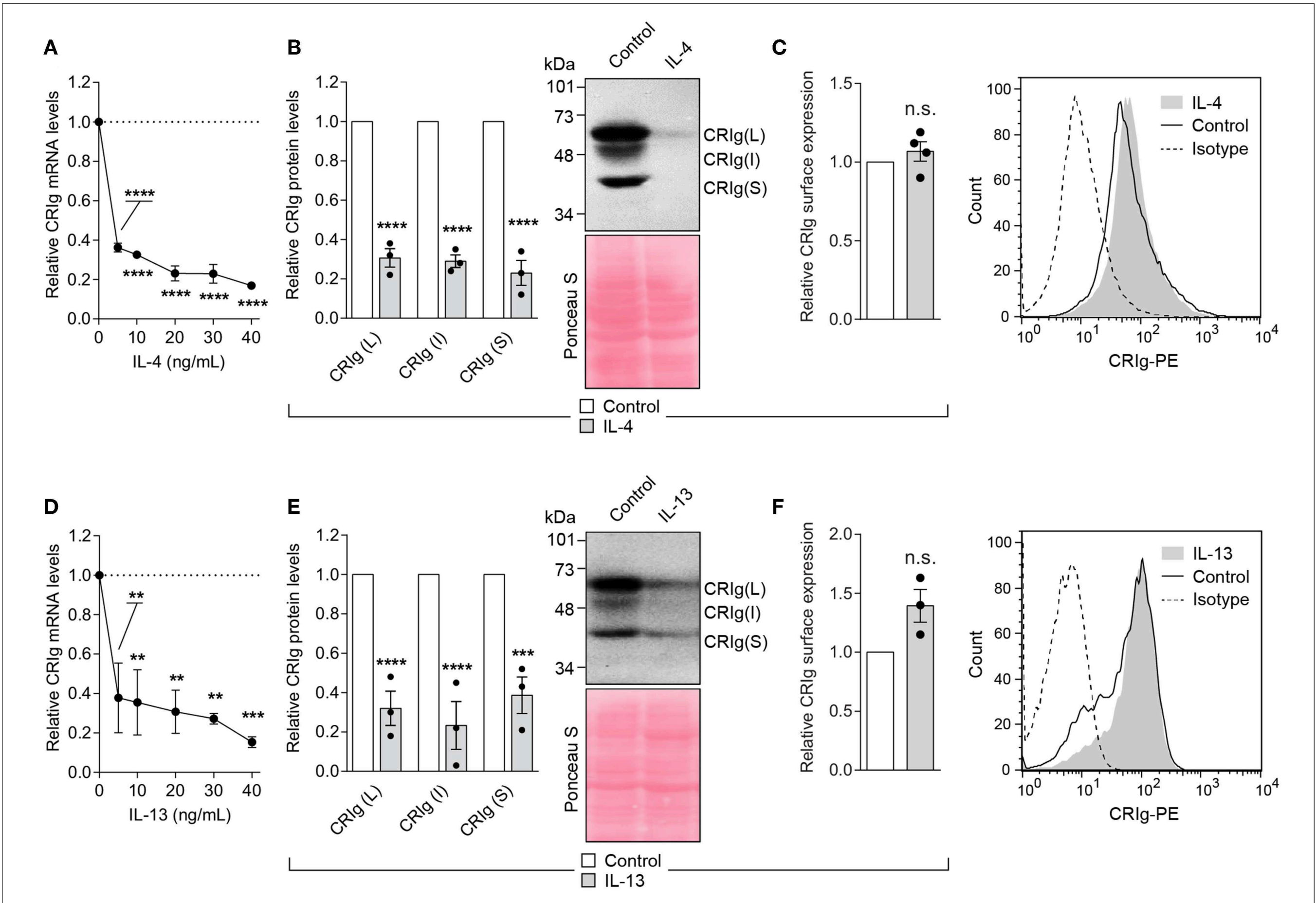


**FIGURE 4 |** IL-4 and IL-13 down regulate CRIg expression in DC. **(A,D)** DC were treated with a dose range of the cytokines for 24 h and then examined for CRIg mRNA expression. **(B,E)** The levels of total CRIg isoform proteins measured by Western blot in DC treated with 20 ng/ml of cytokines. **(C,F)** Similarly treated DC were examined for surface expression of CRIg by flow cytometry. Data are presented as means ± SEM of at 3–4 experiments, each conducted with cells from a different individual. Significance levels are indicated by asterisks: $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, whilst n.s. indicates non-significance.

further studies need to be undertaken with DCs from different tissues, transcriptomic data indicate that CRIg is likely to be expressed in tissue DCs (**Supplementary Figure 4**). It is evident that expression ranges from medium to high in different DC types but expression can be as high as in macrophages. Of interest, although in limited studies, Tanaka et al. (31) described surface expression CRIg$^+$ dendritic-like cells in the synovial tissue from rheumatoid arthritis, osteoarthritis and psoriatic arthritis patients.

Examination of the culture fluids in our DC-T allogeneic cell cultures for cytokine production supported the immunosuppressive effects of dexamethasone treated DCs, acting via CRIg expression. The dexamethasone-conditioned DCs-T cell cultures produced significantly less Th1 cytokines, IFN-γ, and TNF-α, as well as reduced Th2 cytokines, IL-4, and IL-13. In addition, the production of regulatory cytokine TGF-β1 was also reduced. With respect to all of these cytokines, the addition of anti-CRIg monoclonal antibody prevents the decrease in cytokine production. This indicates that the major effect precipitating the immunosuppression is the increased CRIg expression on the DCs. In mouse T cell cultures, Yuan et al. (7) showed that CRIg-Ig fusion protein suppressed the phosphorylation of signaling molecules, such as ZAP-70, ERK1/2, and Akt, thus acting early in the T cell activation response. Such inhibition was likely to cause the reported CRIg-Ig-mediated suppression of mTORC1 activation, thereby promoting inducible (i)Treg generation (32). Furthermore, the immunosuppressive effects of CRIg are unlikely to result from changes in proportions of Treg cells, since these were not increased but in fact decreased in these cultures, consistent with decreased production of the regulatory cytokine TGF-β1. In contrast, in mice CRIg-Ig fusion protein promoted the differentiation of Treg cells and the stabilization of Foxp3, although this was less evident when CRIg expressing macrophages were used (7). Whether these differences are due to macrophages vs. DCs or mouse vs. human leukocytes, as well as other factors, remain to be identified.

Expression on the cell surface indicates that CRIg will have the ability to mediate the tolerogenic properties of DC. Inevitably the level of CRIg expression on these cells may be a

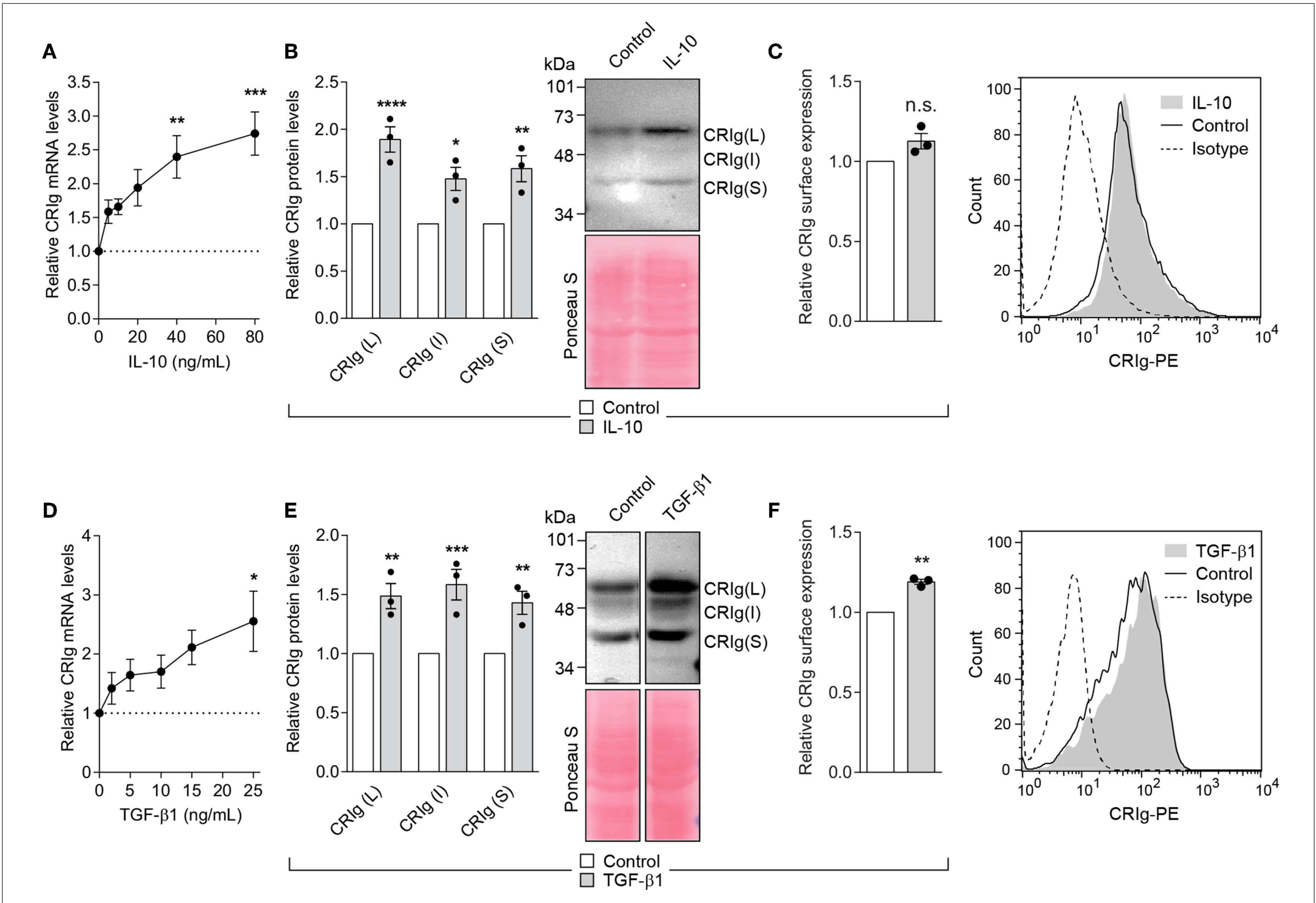


**FIGURE 5 |** IL-10 and TGF-β1 increase CRIg expression in DC. **(A,D)** DC were treated with varying concentrations of the cytokines for 24 h and then examined for CRIg mRNA expression. DC were treated with either 40 ng/ml of IL-10 or 25 ng/ml of TGF-β1 and CRIg isoform protein expression in DC lysates **(B,E)** or CRIg expression on the cell surface **(C,F)** were measured. Data are presented as means ± SEM of three experiments, each conducted with cells from different individuals. The blot image in **(E)** was spliced to exclude intervening lanes that represent other treatments and the complete un-spliced blot can be found in **Supplementary Figure 2**. Significance levels are indicated by asterisks: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$, whilst n.s. indicates non-significance.

determining factor as to the potency of a resultant immunogenic or tolerogenic response the T cells may express, as the DC transform from providing an immunostimulatory signal to a tolerogenic signal following expression of CRIg (29). Thus, the composition of cytokine milieu at tissue sites is likely to be important in determining the role played by DC in the adaptive immune response, to which CRIg contributes. Our data demonstrate that cytokines significantly modulate the expression of CRIg in MDDC. CRIg expression on DC was increased by TGF-β1, IL-10, M-CSF, and GM-CSF. In comparison, LT-α, IFN-γ, IL-4, IL-13, TNF-α, IL-1β, and IL-6 decreased expression. In this manner, the cytokines could participate in tolerogenic vs. immunogenic responses, respectively through their ability to alter expression of CRIg on DC. However, it is not clear as to why DC treated with some cytokines did not show a corresponding alteration in expression at the cell surface. This may be an assay time related effect. But the ability of a cytokine to increase the intracellular CRIg levels may operate collaboratively with another cytokine to increase release to the cell surface, an area for future investigation. It is therefore tempting to speculate that inflammatory mediators may regulate expression at the transcriptional, translation and release to the cell surface. The regulatory effects of cytokines on CRIg expression has also been demonstrated for macrophages (12, 33). **Supplementary Table 1** depicts the effects of cytokines on CRIg expression in MDM and MDDC. While most of the cytokines had similar effects on both cell types, LT-α, GM-CSF and the regulatory cytokines, IL-10 and TGF-β1, had the opposite effects on the two cell types.

The finding that IL-10 and TGF-β1 cause an increase in CRIg expression on DC is of interest and importance in adaptive immunity and immune responsiveness. Tolerogenic DC can be generated by immunosuppressive cytokines including IL-10, TGF-β1 (34–37), and immunomodulatory drugs, such as dexamethasone (38). Since tolerogenic DC are being considered as a therapeutic strategy in transplantation (39) and autoimmune inflammatory diseases (40, 41), these findings are likely to be helpful in developing tolerogenic DC for this purpose. IL-10

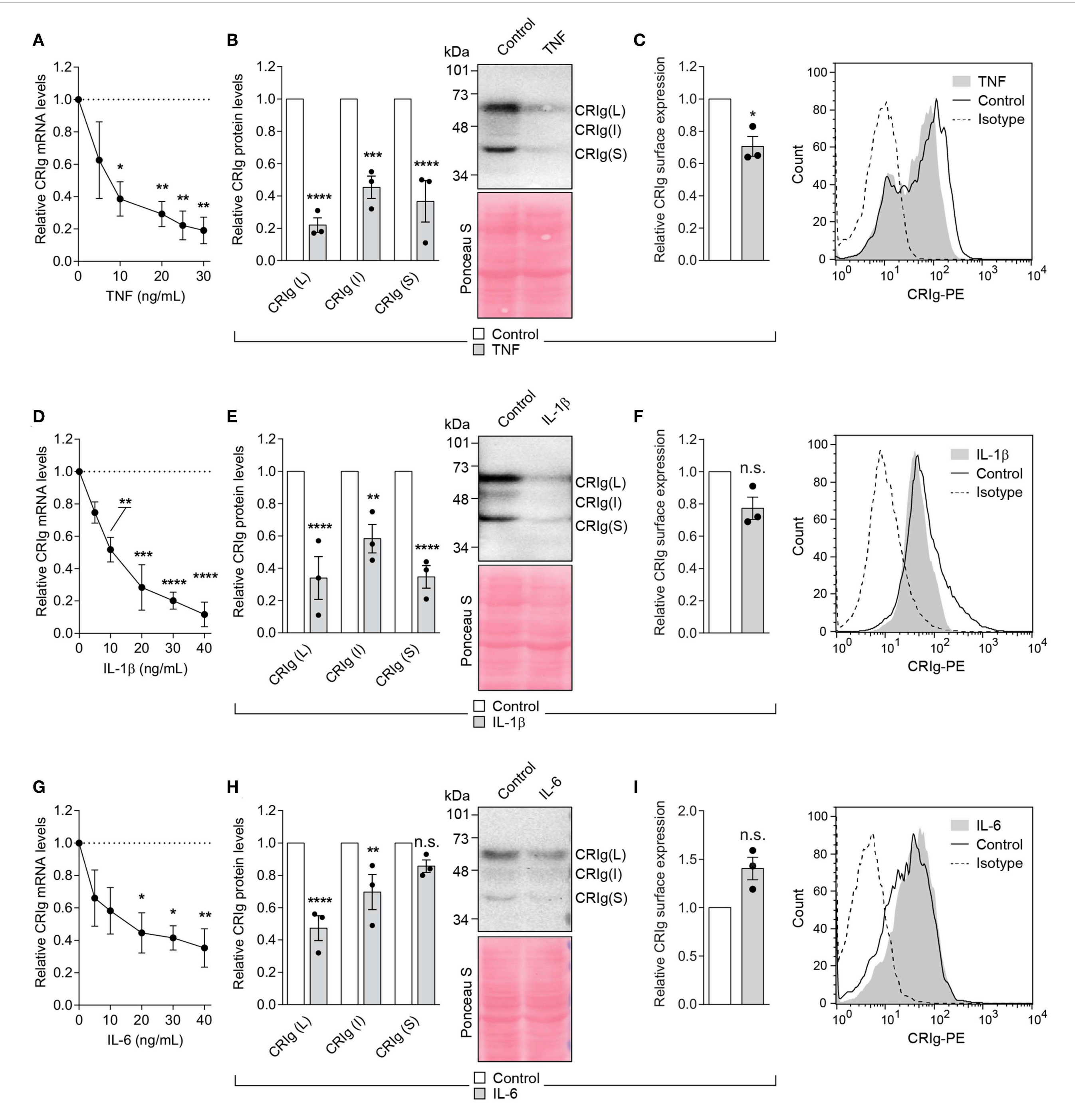


**FIGURE 6** | Effect of TNF-α, IL-1β, and IL-10 on CRIg expression in DC. **(A,D,G)** DC were treated with varying concentrations of the cytokines for 24 h and then examined for CRIg mRNA expression. For examination of CRIg isoform protein expression **(B,E,H)** and cell surface expression **(C,F,I)**, the cells were treated with 40 ng/ml of cytokine. Data are presented as means ± SEM of three experiments, each conducted with cells from different individuals. Significance levels are indicated by asterisks: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$, whilst n.s. indicates non-significance.

caused a substantial increase in CRIg mRNA and corresponding CRIg protein in human DCs. Dexamethasone treated DCs generates tolerogenic DCs that have reduced alloantigenic capacity, higher IL-10 secretion and inhibit Th2 differentiation of naïve $CD4^+$ T cells in latex-allergic patients (42).

Our findings of CRIg being expressed by DC have important implications in autoimmunity, chronic inflammation and cancer (43). CRIg expression has been associated with decreased T cell and B cell responses (5, 44). The importance of CRIg in protecting against autoimmune inflammation has been

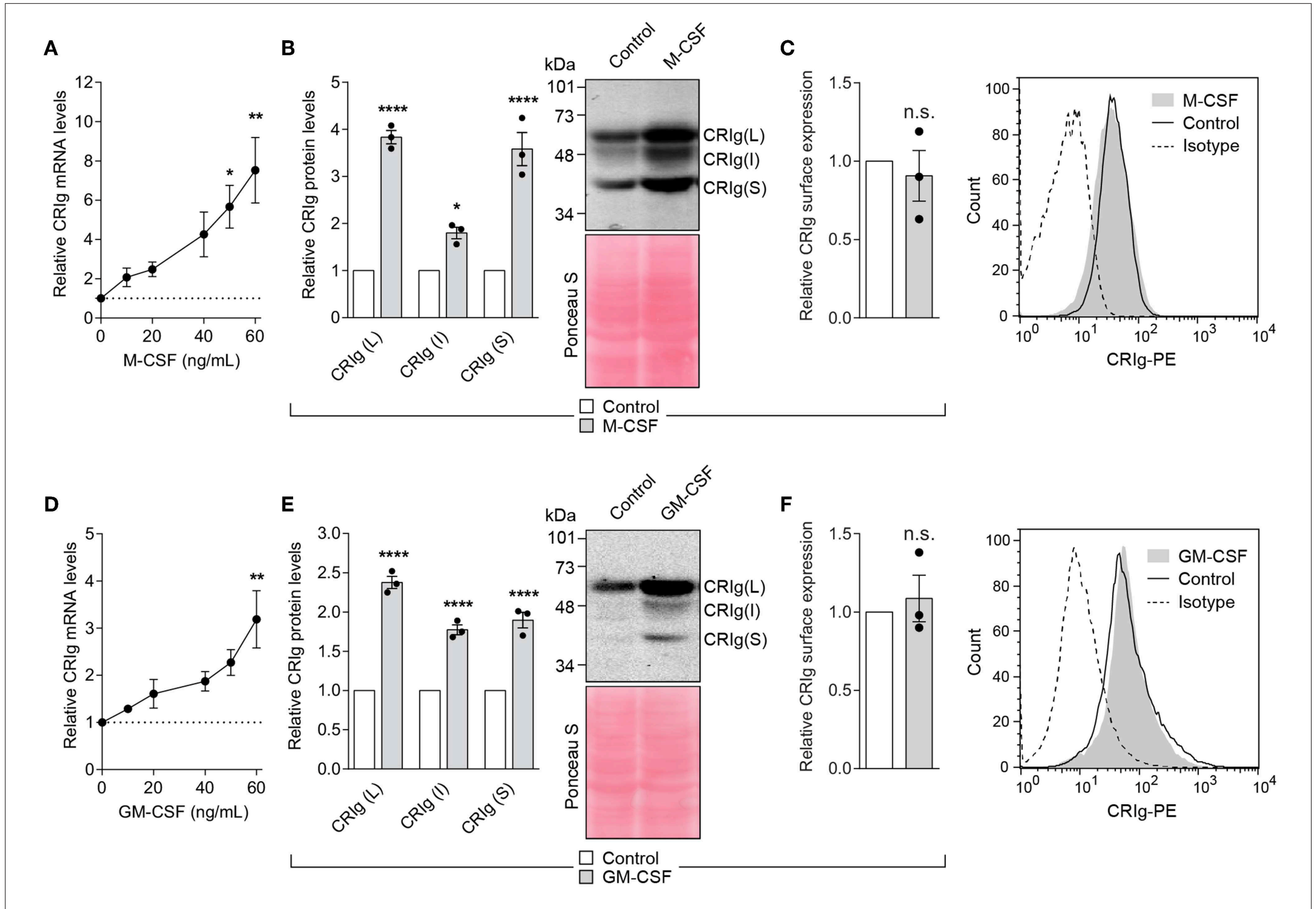


**FIGURE 7** | M-CSF and GM-CSF increase CRIg in DC. **(A,D)** DC were treated with varying concentrations of the CSF and the CRIg mRNA expression determined. Changes in CRIg isoform proteins **(B,E)** and cell surface **(C,F)** CRIg expression on DC treated with 40 ng/ml of each CSF are shown. Data are presented as means ± SEM of three experiments, each conducted with cells from different individuals. Significance levels are indicated by asterisks: $^*P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$, whilst n.s. indicates non-significance.

demonstrated in experimental models of inflammatory arthritis (45), renal tubulointestitial injury (46), lupus nephritis (47), immune-mediated liver injury (48), type 1 diabetes (7, 30), and inflammatory bowel disease (49). In addition, the levels of CRIg expression in macrophages has been associated with disease severity in rheumatoid arthritis (31, 50) and patients with cirrhosis and ascites (51). In cancer, the level of CRIg expression by tumor associated macrophages has been shown to be a prognostic marker for tumors metastasizing, with high expression being prognostic for poor outcome (52–54). This also raises the potential for CRIg being a check point in the development of metastatic cancer and hence a drug target.

Our results demonstrate that three transcripts of CRIg are expressed in human MDDC. By Western blot analysis, we were able to identify the L and S isoforms along with an additional form not previously described and designated as the intermediate or I form. This most likely corresponds to the third transcript detected by PCR. However, because of lack of appropriate monoclonal antibodies specific for the different CRIg isoforms, we were not able to relate these to the changes seen in the Western blots. Nevertheless, it is evident that the I isoform is less prominent than the L and S isoforms. While the function of the I form remains unknown, it is tempting to speculate that since some of the extracellular domain is the same as the short form, its interaction with ligands should be the same. But because the I form has absence of intracellular phosphorylation sites as a result of alternative splicing, it is questionable that this form would be able to signal.

The ability of cytokines and dexamethasone to regulate the expression of CRIg was evident at the mRNA level and this correlated with protein expression, suggesting that inflammatory mediators and the immunosuppressive drug act at the pre-transcriptional level. It has been postulated by us that dexamethasone acts via the glucocorticoid receptor to downregulate CRIg expression as well as acting via the inhibition of PKCα activation and increasing CRIg expression in this manner (15). While the mechanisms of $CRIg^+$ antigen presenting cell-induced immunosuppression remain to be elucidated, it has recently been demonstrated that engaging this receptor in macrophages reprograms the mitochondrial pyruvate

metabolism and inhibits their activation (55). Here we have not only shown the expression of CRIg on DC, but that increased expression can lead to suppression of T lymphocyte proliferation. This provides important support for its role in protection against autoimmune inflammatory diseases and poor prognosis in metastasizing cancer (43). Furthermore, the findings expand our knowledge on CRIg and the regulation of the adaptive immune response, from that of its elegant role in clearance of pathogens and regulation of the alternative complement pathway activation (56, 57).

## DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

## ETHICS STATEMENT

This study was carried out in accordance with the recommendations of The Women's and Children's Hospital Network Human Ethics Committee with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the WCHN Human Ethics Committee, approval number REC 2165/4/2011.

## AUTHOR CONTRIBUTIONS

UM performed the majority of the experiments and was involved in planning the experiments, collating the data, and writing the paper. KP was responsible for the allogeneic-induced T cell proliferation and cytokine production, as well as Treg measurements. AS assisted with the data presentation, writing of the manuscript, and conducting some experiments. TP conducted the cytokine measurements. AQ assisted with the collating of data, statistical analysis, and writing of the manuscript. NG critically read the manuscript and contributed its writing. CH was involved with reviewing of the data and writing of the manuscript. CA assisted with the planning of the research, supervised the work, and wrote the manuscript. AF initiated the study, supervised the research, reviewed the data, and wrote the manuscript.


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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.02892/full#supplementary-material

## REFERENCES


1. Helmy KY, Katschke KJ Jr., Gorgani NN, Kljavin NM, Elliott JM, Diehl L, et al. CRIg: a macrophage complement receptor required for phagocytosis of circulating pathogens. *Cell.* (2006) 124:915–27. doi: 10.1016/j.cell.2005.12.039
2. Gorgani NN, He JQ, Katschke KJ Jr., Helmy KY, Xi H, Steffek M, et al. Complement receptor of the Ig superfamily enhances complement-mediated phagocytosis in a subpopulation of tissue resident macrophages. *J Immunol.* (2008) 181:7902–8. doi: 10.4049/jimmunol.181.11.7902
3. Broadley SP, Plaumann A, Coletti R, Lehmann C, Wanisch A, Seidlmeier A, et al. Dual-track clearance of circulating bacteria balances rapid restoration of blood sterility with induction of adaptive immunity. *Cell Host Microbe.* (2016) 20:36–48. doi: 10.1016/j.chom.2016.05.023
4. Zeng Z, Surewaard BG, Wong CH, Geoghegan JA, Jenne CN, Kubes P. CRIg functions as a macrophage pattern recognition receptor to directly bind and capture blood-borne gram-positive bacteria. *Cell Host Microbe.* (2016) 20:99–106. doi: 10.1016/j.chom.2016.06.002
5. Vogt L, Schmitz N, Kurrer MO, Bauer M, Hinton HI, Behnke S, et al. VSIG4, a B7 family-related protein, is a negative regulator of T cell activation. *J Clin Invest.* (2006) 116:2817–26. doi: 10.1172/JCI25673
6. Zang X, Allison JP. To be or not to be B7. *J Clin Invest.* (2006) 116:2590–3. doi: 10.1172/JCI30103
7. Yuan X, Yang BH, Dong Y, Yamamura A, Fu W. CRIg, a tissue-resident macrophage specific immune checkpoint molecule, promotes immunological tolerance in NOD mice, via a dual role in effector and regulatory T cells. *Elife.* (2017) 6:E28. doi: 10.7554/eLife.29540.028
8. Sallusto F, Lanzavecchia A. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. *J Exp Med.* (1994) 179:1109–18. doi: 10.1084/jem.179.4.1109
9. Marantos C, Mukaro V, Ferrante J, Hii C, Ferrante A. Inhibition of the lipopolysaccharide-induced stimulation of the members of the MAPK family in human monocytes/macrophages by 4-hydroxynonenal, a product of oxidized omega-6 fatty acids. *Am J Pathol.* (2008) 173:1057–66. doi: 10.2353/ajpath.2008.071150
10. Harb H, Irvine J, Amarasekera M, Hii CS, Kesper DA, Ma Y, et al. The role of PKCzeta in cord blood T-cell maturation towards Th1 cytokine profile and its epigenetic regulation by fish oil. *Biosci Rep.* (2017) 37:BSR20160485. doi: 10.1042/BSR20160485
11. Jones AP, D'Vaz N, Meldrum S, Palmer DJ, Zhang G, Prescott SL. 25-Hydroxyvitamin D3 status is associated with developing adaptive and innate immune responses in the first 6 months of life. *Clin Exp Allergy.* (2015) 45:220–31. doi: 10.1111/cea.12449
12. Munawara U, Small AG, Quach A, Gorgani NN, Abbott CA, Ferrante A. Cytokines regulate complement receptor immunoglobulin expression and phagocytosis of Candida albicans in human macrophages: a control point in anti-microbial immunity. *Sci Rep.* (2017) 7:4050. doi: 10.1038/s41598-017-04325-0
13. Hii CS, Huang ZH, Bilney A, Costabile M, Murray AW, Rathjen DA, et al. Stimulation of p38 phosphorylation and activity by arachidonic acid in HeLa cells, HL60 promyelocytic leukemic cells, and human neutrophils. Evidence for cell type-specific activation of mitogen-activated protein kinases. *J Biol Chem.* (1998) 273:19277–82. doi: 10.1074/jbc.273.30.19277
14. Gorgani NN, Thathaisong U, Mukaro VR, Poungpair O, Tirimacco A, Hii CS, et al. Regulation of CRIg expression and phagocytosis in human

macrophages by arachidonate, dexamethasone, and cytokines. *Am J Pathol.* (2011) 179:1310–8. doi: 10.1016/j.ajpath.2011.05.021

15. Ma Y, Usuwanthim K, Munawara U, Quach A, Gorgani NN, Abbott CA, et al. Protein kinase calpha regulates the expression of complement receptor Ig in human monocyte-derived macrophages. *J Immunol.* (2015) 194:2855–61. doi: 10.4049/jimmunol.1303477
16. Spiering R, Wagenaar-Hilbers J, Huijgen V, van der Zee R, van Kooten PJ, van Eden W, et al. Membrane-bound metallothionein 1 of murine dendritic cells promotes the expansion of regulatory T cells *in vitro*. *Toxicol Sci.* (2014) 138:69–75. doi: 10.1093/toxsci/kft268
17. Palomares O, Martin-Fontecha M, Lauener R, Traidl-Hoffmann C, Cavkaytar O, Akdis M, et al. Regulatory T cells and immune regulation of allergic diseases: roles of IL-10 and TGF-beta. *Genes Immun.* (2014) 15:511–20. doi: 10.1038/gene.2014.45
18. Comi M, Amodio G, Gregori S. Interleukin-10-producing DC-10 is a unique tool to promote tolerance via antigen-specific T regulatory type 1 cells. *Front Immunol.* (2018) 9:682. doi: 10.3389/fimmu.2018.00682
19. Liu M, Li S, Li MO. TGF-beta control of adaptive immune tolerance: a break from Treg cells. *Bioessays*. (2018) 40:e1800063. doi: 10.1002/bies.201800063
20. Dinarello CA. Infection, fever, and exogenous and endogenous pyrogens: some concepts have changed. *J Endotoxin Res.* (2004) 10:201–22. doi: 10.1179/096805104225006129
21. Fumagalli R, Bellani G, Perri A. Which drugs for the control of fever in critical patients. *Curr Drug Targets.* (2009) 10:881–6. doi: 10.2174/138945009789108828
22. Chomarat P, Banchereau J, Davoust J, Palucka AK. IL-6 switches the differentiation of monocytes from dendritic cells to macrophages. *Nat Immunol.* (2000) 1:510–4. doi: 10.1038/82763
23. Chomarat P, Dantin C, Bennett L, Banchereau J, Palucka AK. TNF skews monocyte differentiation from macrophages to dendritic cells. *J Immunol.* (2003) 171:2262–9. doi: 10.4049/jimmunol.171.5.2262
24. Fernando MR, Reyes JL, Iannuzzi J, Leung G, McKay DM. The pro-inflammatory cytokine, interleukin-6, enhances the polarization of alternatively activated macrophages. *PLoS ONE.* (2014) 9:e94188. doi: 10.1371/journal.pone.0094188
25. Hu S, Marshall C, Darby J, Wei W, Lyons AB, Korner H. Absence of tumor necrosis factor supports alternative activation of macrophages in the liver after infection with *Leishmania* major. *Front Immunol.* (2018) 9:1. doi: 10.3389/fimmu.2018.00001
26. Pilli M, Arko-Mensah J, Ponpuak M, Roberts E, Master S, Mandell MA, et al. TBK-1 promotes autophagy-mediated antimicrobial defense by controlling autophagosome maturation. *Immunity.* (2012) 37:223–34. doi: 10.1016/j.immuni.2012.04.015
27. He X, Qian Y, Li Z, Fan EK, Li Y, Wu L, et al. TLR4-upregulated IL-1beta and IL-1RI promote alveolar macrophage pyroptosis and lung inflammation through an autocrine mechanism. *Sci Rep.* (2016) 6:31663. doi: 10.1038/srep31663
28. Sun L, Rautela J, Delconte RB, Souza-Fonseca-Guimaraes F, Carrington EM, Schenk RL, et al. GM-CSF quantity has a selective effect on granulocytic vs. monocytic myeloid development and function. *Front Immunol.* (2018) 9:1922. doi: 10.3389/fimmu.2018.01922
29. Xu S, Sun Z, Li L, Liu J, He J, Song D, et al. Induction of T cells suppression by dendritic cells transfected with VSIG4 recombinant adenovirus. *Immunol Lett.* (2010) 128:46–50. doi: 10.1016/j.imlet.2009.11.003
30. Fu W, Wojtkiewicz G, Weissleder R, Benoist C, Mathis D. Early window of diabetes determinism in NOD mice, dependent on the complement receptor CRIg, identified by noninvasive imaging. *Nat Immunol.* (2012) 13:361–8. doi: 10.1038/ni.2233
31. Tanaka M, Nagai T, Tsuneyoshi Y, Sunahara N, Matsuda T, Nakamura T, et al. Expansion of a unique macrophage subset in rheumatoid arthritis synovial lining layer. *Clin Exp Immunol.* (2008) 154:38–47. doi: 10.1111/j.1365-2249.2008.03733.x
32. Chapman NM, Chi H. mTOR signaling, Tregs and immune modulation. *Immunotherapy.* (2014) 6:1295–311. doi: 10.2217/imt.14.84
33. Guo S, Yang C, Mei F, Wu S, Luo N, Fei L, et al. Down-regulation of Z39Ig on macrophages by IFN-gamma in patients with chronic HBV infection. *Clin Immunol.* (2010) 136:282–91. doi: 10.1016/j.clim.2010.03.007
34. Geissmann F, Revy P, Regnault A, Lepelletier Y, Dy M, Brousse N, et al. TGF-beta 1 prevents the noncognate maturation of human dendritic Langerhans cells. *J Immunol.* (1999) 162:4567–75.
35. Steinbrink K, Graulich E, Kubsch S, Knop J, Enk AH. CD4(+) and CD8(+) anergic T cells induced by interleukin-10-treated human dendritic cells display antigen-specific suppressor activity. *Blood.* (2002) 99:2468–76. doi: 10.1182/blood.V99.7.2468
36. Torres-Aguilar H, Sanchez-Torres C, Jara LJ, Blank M, Shoenfeld Y. IL-10/TGF-beta-treated dendritic cells, pulsed with insulin, specifically reduce the response to insulin of $CD4^+$ effector/memory T cells from type 1 diabetic individuals. *J Clin Immunol.* (2010) 30:659–68. doi: 10.1007/s10875-010-9430-5
37. Tai N, Yasuda H, Xiang Y, Zhang L, Rodriguez-Pinto D, Yokono K, et al. IL-10-conditioned dendritic cells prevent autoimmune diabetes in NOD and humanized HLA-DQ8/RIP-B7.1 mice. *Clin Immunol.* (2011) 139:336–49. doi: 10.1016/j.clim.2011.03.003
38. Unger WW, Laban S, Kleijwegt FS, van der Slik AR, Roep BO. Induction of Treg by monocyte-derived DC modulated by vitamin D3 or dexamethasone: differential role for PD-L1. *Eur J Immunol.* (2009) 39:3147–59. doi: 10.1002/eji.200839103
39. Marin E, Cuturi MC, Moreau A. Tolerogenic dendritic cells in solid organ transplantation: where do we stand? *Front Immunol.* (2018) 9:274. doi: 10.3389/fimmu.2018.00274
40. Morelli AE, Thomson AW. Tolerogenic dendritic cells and the quest for transplant tolerance. *Nat Rev Immunol.* (2007) 7:610–21. doi: 10.1038/nri2132
41. Thomas R. Dendritic cells and the promise of antigen-specific therapy in rheumatoid arthritis. *Arthritis Res Ther.* (2013) 15:204. doi: 10.1186/ar4130
42. Escobar A, Aguirre A, Guzman MA, Gonzalez R, Catalan D, Acuna-Castillo C, et al. Tolerogenic dendritic cells derived from donors with natural rubber latex allergy modulate allergen-specific T-cell responses and IgE production. *PLoS ONE.* (2014) 9:e85930. doi: 10.1371/journal.pone.0085930
43. Small AG, Al-Baghdadi M, Quach A, Hii C, Ferrante A. Complement receptor immunoglobulin: a control point in infection and immunity, inflammation and cancer. *Swiss Med Wkly.* (2016) 146:w14301. doi: 10.4414/smw.2016.14301
44. Jung K, Seo SK, Choi I. Endogenous VSIG4 negatively regulates the helper T cell-mediated antibody response. *Immunol Lett.* (2015) 165:78–83. doi: 10.1016/j.imlet.2015.04.004
45. Katschke KJ Jr., Helmy KY, Steffek M, Xi H, Yin J, Lee WP, et al. A novel inhibitor of the alternative pathway of complement reverses inflammation and bone destruction in experimental arthritis. *J Exp Med.* (2007) 204:1319–25. doi: 10.1084/jem.20070432
46. Li Y, Wang YQ, Wang DH, Hou WP, Zhang Y, Li M, et al. Costimulatory molecule VSIG4 exclusively expressed on macrophages alleviates renal tubulointerstitial injury in VSIG4 KO mice. *J Nephrol.* (2014) 27:29–36. doi: 10.1007/s40620-013-0022-3
47. Lieberman LA, Mizui M, Nalbandian A, Bosse R, Crispin JC, Tsokos GC. Complement receptor of the immunoglobulin superfamily reduces murine lupus nephritis and cutaneous disease. *Clin Immunol.* (2015) 160:286–91. doi: 10.1016/j.clim.2015.05.006
48. Jung K, Kang M, Park C, Hyun Choi Y, Jeon Y, Park SH, et al. Protective role of V-set and immunoglobulin domain-containing 4 expressed on kupffer cells during immune-mediated liver injury by inducing tolerance of liver T- and natural killer T-cells. *Hepatology.* (2012) 56:1838–48. doi: 10.1002/hep.25906
49. Tanaka M, Nagai T, Usami M, Hasui K, Takao S, Matsuyama T. Phenotypic and functional profiles of CRIg (Z39Ig)-expressing macrophages in the large intestine. *Innate Immun.* (2012) 18:258–67. doi: 10.1177/1753425911400641
50. Lee MY, Kim WJ, Kang YJ, Jung YM, Kang YM, Suk K, et al. Z39Ig is expressed on macrophages and may mediate inflammatory reactions in arthritis and atherosclerosis. *J Leukoc Biol.* (2006) 80:922–8. doi: 10.1189/jlb.0306160
51. Irvine KM, Banh X, Gadd VL, Wojcik KK, Ariffin JK, Jose S, et al. CRIg-expressing peritoneal macrophages are associated with disease severity

in patients with cirrhosis and ascites. *JCI Insight.* (2016) 1:e86914. doi: 10.1172/jci.insight.86914
52. Liao Y, Guo S, Chen Y, Cao D, Xu H, Yang C, et al. VSIG4 expression on macrophages facilitates lung cancer development. *Lab Invest.* (2014) 94:706–15. doi: 10.1038/labinvest.2014.73
53. Xu T, Jiang Y, Yan Y, Wang H, Lu C, Xu H, et al. VSIG4 is highly expressed and correlated with poor prognosis of high-grade glioma patients. *Am J Transl Res.* (2015) 7:1172–80.
54. Byun JM, Jeong DH, Choi IH, Lee DS, Kang MS, Jung KO, et al. The significance of VSIG4 expression in ovarian cancer. *Int J Gynecol Cancer.* (2017) 27:872–8. doi: 10.1097/IGC.0000000000000979
55. Li J, Diao B, Guo S, Huang X, Yang C, Feng Z, et al. VSIG4 inhibits proinflammatory macrophage activation by reprogramming mitochondrial pyruvate metabolism. *Nat Commun.* (2017) 8:1322. doi: 10.1038/s41467-017-01327-4
56. Wiesmann C, Katschke KJ, Yin J, Helmy KY, Steffek M, Fairbrother WJ, et al. Structure of C3b in complex with CRIggives insights into regulation of complement activation. *Nature.* (2006) 444:217–20. doi: 10.1038/nature05263
57. van Lookeren Campagne M, Verschoor A. Pathogen clearance and immune adherence "revisited": immuno-regulatory roles for CRIg. *Semin Immunol.* (2018) 37:4–11. doi: 10.1016/j.smim.2018.02.007

**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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*Supplementary Material*

# Human Dendritic Cells Express the Complement Receptor Immunoglobulin Which Regulates T Cell Responses

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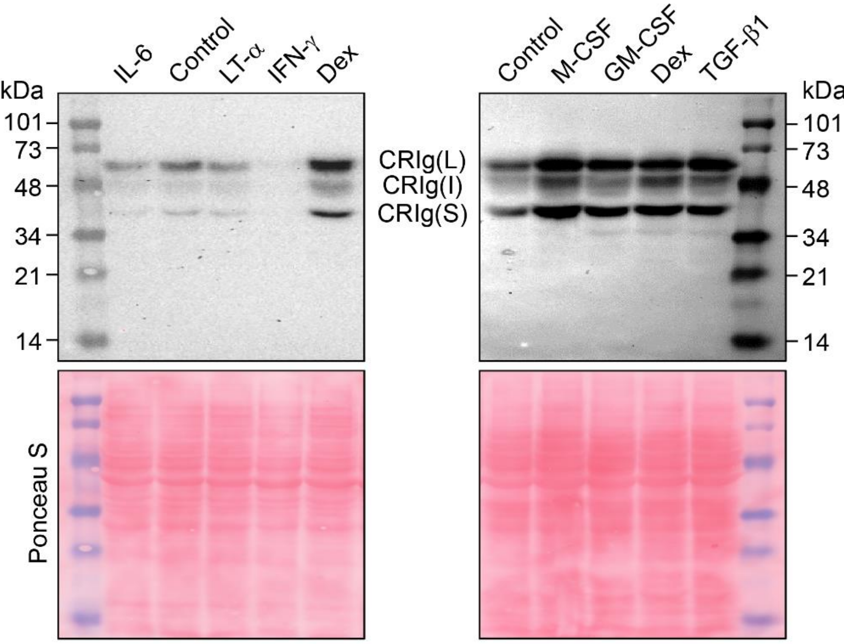
**Supplementary Table 1.** The arrows indicate the effects of treating MDM and MDDC for 24 hours with the indicated cytokine/agent. MDM data from Munawara et al (2017).

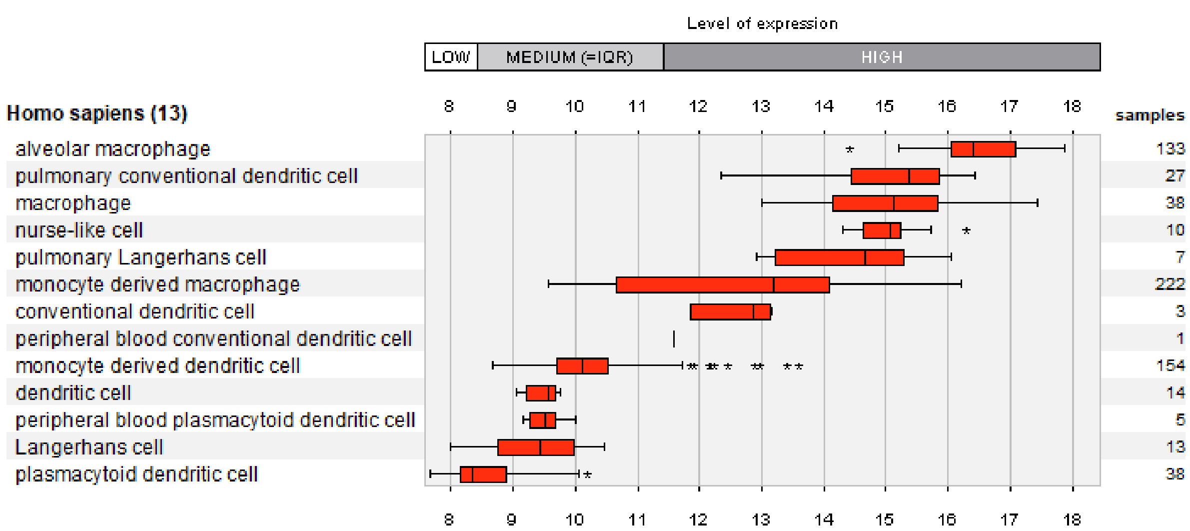
| Cytokine | MDM | DC |
|---|---|---|
| LTα | ↑ | ↓ |
| IFN-γ | ↓ | ↓ |
| IL-4 | ↓ | ↓ |
| IL-13 | ↓ | ↓ |
| IL-10 | ↓ | ↑ |
| TGF-β1 | ↓ | ↑ |
| TNF-α | ↓ | ↓ |
| IL-13 | ↓ | ↓ |
| IL-6 | ↓ | ↓ |
| M-CSF | ↑ | ↑ |
| GM-CSF | ↓ | ↑ |
| Dexamethasone | ↑ | ↑ |

**Supplementary Figure 1.** Gating strategies for determining CRIg expression on the DC surface and Foxp3$^{+}$ regulatory T cells in allogeneic DC-T cell co-cultures. **(A)** Representative plots show DC gating by capture of the high FSC/SSC population, followed by doublet exclusion by SSC-A vs SSC-H, and then exclusion of 7-aminoactinomycin D (7-AAD)$^{+}$ cells (non-viable). Representative histogram overlays are also shown of isotype control and CRIg-PE staining in control and 24 h dexamethasone-treated viable DCs. The relative fold-increase in viable DC surface CRIg expression with dexamethasone treatment was shown to be significant from experiments of DCs from four individual donors. *, $P < 0.05$ by two-tailed t-testing. **(B)** Representative plots show Treg gating by capture of FSC and SSC singlets, followed by CD4$^{+}$ T cell gating, and resolution of the CD25$^{+}$CD127$^{lo}$ Treg cell population. An isotype control (IgG1-PE) was used to adjudicate Foxp3$^{+}$ Treg cells.




**Supplementary Figure 2.** Complete Western blots of CRIg isoforms in cytokine-treated dendritic cells from which spliced blots presented in Figure 3 and 5 were obtained. The left blot was the source of the spliced IFN-γ and corresponding control blot in Figure 3, whilst the right blot was the source of the spliced TGF-β1 and corresponding control blot in Figure 5. Each blot presents lysates from DC cultured in the presence of the indicated cytokines in an individual, that were examined by staining with CRIg 3C9 monoclonal antibodies. The corresponding Ponceau S staining shows the consistency of protein load. Low Range Prestained SDS-PAGE Standards (Bio-Rad Laboratories) were used for determining the long (L), intermediate (I) and short (S) forms of CRIg with ladder band sizes indicated in kilodaltons (kDa).

**Supplementary Figure 3.** Cytokine production and Treg cell populations in allogeneic DC-T cell-anti-CRIg reactions relative to the absence of dexamethasone treatment. **(A)** Relative IFN-γ, TNF-α, IL-13, IL-10, IL-4, and TGF-β1 production. **(B)** Relative $CD25^{+}CD127^{lo}$ and $Foxp3^{+}CD25^{+}CD127^{lo}$ Treg percentages of $CD4^{+}$ T cells, and $Foxp3^{+}$ percentages and MFI of $CD25^{+}CD127^{lo}$ Treg cells. Significance levels are indicated by asterisks: *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$, ****, $P < 0.0001$.




**Supplementary Figure 4.** *VISG4* expression levels in various human macrophages and dendritic cells. Data were extracted and compiled from public gene expression data repositories via Genevestigator V7.3.1 (Hruz et al. 2008). The expression levels are normalized by Genevestigator and presented as boxplots with the mean and interquartile range (IQR), and whiskers denoting 1.5 IQR from the lower and upper quartile, with asterisks indicating outliers. The sample sizes are indicated on each row of data.

## Supplementary References


Munawara, U., Small, A. G., Quach, A., Gorgani, N. N., Abbott, C. A., Ferrante, A. (2007) Cytokines regulate complement receptor immunoglobulin expression and phagocytosis of Candida albicans in human macrophages: A control point in anti-microbial immunity. *Sci Rep* 22;7(1):4050. doi: 10.1038/s41598-017-04325-0.

Hruz, T., Laule, O., Szabo, G., Wessendorp, F., Bleuler, S., Oertle, L., et al. (2008). Genevestigator v3: a reference expression database for the meta-analysis of transcriptomes. *Adv Bioinformatics* 2008, 420747. doi: 10.1155/2008/420747.

# Chapter 6. Complement receptor immunoglobulin in human macrophage innate immunity

### 6.1. Introduction and Contextual Statement

In the currently published literature and as presented in chapter 4, CRIg is an important phagocytosis-promoting receptor which contributes to the innate immune function of macrophages, and its expression is heavily inducible by the synthetic steroid dexamethasone. This increase of expression manifests functionally as an increase in cellular phagocytic capability. In light of this, we next sought to investigate whether naturally occurring steroid hormones exert a similar form of control over CRIg and macrophage immune function.

The naturally occurring steroid hormone vitamin D is an important molecule which has extensive roles in a multitude of biological processes such as calcium metabolism and in innate immunity. In this chapter, we document the existence of a vitamin D-primed innate host defence mechanism in macrophages which promotes enhanced removal of bacterial and fungal pathogens and is dependent on CRIg. Results from investigation into the effects of the active form of vitamin D (1,25-dihydroxyvitamin D3) on developing and pre-matured MDM expression of CRIg and phagocytic function are presented, and these findings are supplemented by the assessment of the effects of treatment with the inactive precursor 25-hydroxyvitamin D3 in combination with the toll like receptor 2 agonist, triacylated lipopeptide, Pam3CSK4, which promotes the conversion of 25-hydroxyvitamin D3 to 1,25D.

This chapter is presented in the form of a submitted manuscript by **Annabelle Small**, Sarah Harvey, Jaspreet Kaur, Trishni Putty, Alex Quach, Usma Munawara, Andrew McPhee, Charles S. Hii, & Antonio Ferrante, entitled *'The 'sunshine' vitamin D upregulates the macrophage complement receptor immunoglobulin in innate immunity to microbial pathogens'*. At the time of the revision of this thesis, this publication was accepted for publication in Nature Communications Biology ($12^{th}$ February 2021) as manuscript COMMSBIO-20-0217A.

# Statement of Authorship

| Title of Paper | The sunshine vitamin D upregulates the macrophage complement receptor immunoglobulin in innate immunity to microbial pathogens |
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| Publication Details | Unpublished work presented in manuscript style, submitted and currently under consideration for publication. |

## Principal Author

| Name of Principal Author (Candidate) | Annabelle Small | | |
|---|---|---|---|
| Contribution to the Paper | AS designed the experiments, carried out the experiments, interpreted data, and wrote the manuscript. | | |
| Overall percentage (%) | 55% | | |
| Certification: | This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper. | | |
| Signature | | Date | 7/3/2020 |

## Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

| Name of Co-Author | Professor Antonio Ferrante | | |
|---|---|---|---|
| Contribution to the Paper | AF designed the experiments, interpreted data, supervised and initiated the project, wrote the manuscript, and acted as corresponding author. | | |
| Signature | | Date | 03/03/2020 |

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|---|---|---|---|
| Contribution to the Paper | SH designed and carried out the experiments. | | |
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| Contribution to the Paper | TP carried out the experiments and assisted in data interpretation. |
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| Signature | Date 12/3/20 |

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| Contribution to the Paper | AQ was involved in collating data, statistical analysis, data interpretation and writing of the manuscript. |
| Signature | Date 19.3.20 |

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|---|---|
| Contribution to the Paper | AMcP assisted with the cord blood cell study and critical reading of the manuscript. |
| Signature | Date 10/3/20 |

| Name of Co-Author | Associate Professor Charles Hii |
|---|---|
| Contribution to the Paper | CH was incolved in collating data, statistical analysis, data interpretation and writing of the manuscript |
| Signature | Date 5/3/20 |

# The 'sunshine' vitamin D upregulates the macrophage complement receptor immunoglobulin in innate immunity to microbial pathogens

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**Vitamin D deficiency remains a global concern[1]. Known as the 'sunshine' vitamin, the secosteroid is converted through a multistep process to the hormonally active 1,25-dihydroxyvitamin $D_3$ (1,25D), the final step of which can occur in macrophages.[2] Here we demonstrate a role for vitamin D in innate immunity to infection, that expression of the complement receptor immunoglobulin (CRIg), which plays an important role in innate anti-microbial host defence[3,4,5], is upregulated by 1,25D in human macrophages. Monocytes cultured in the presence of 1,25D differentiated into macrophages that display increased CRIg mRNA, protein and cell surface expression but not in expression of the classical complement receptors, CR3 and CR4. Under these conditions, the macrophages show increased phagocytosis of complement opsonised *Staphylococcus aureus* and *Candida albicans*. Treating macrophages *per se* with 1,25D for 24h causes an increase in CRIg expression. Interestingly, while treating macrophages with 25-hydroxyvitamin $D_3$ does not increase CRIg expression, when added together with the toll like receptor 2 agonist, triacylated lipopeptide, Pam3CSK4, which promotes the conversion of 25-hydroxyvitamin $D_3$ to 1,25D[6], leads to an increase in CRIg expression. These findings suggest that macrophages harbour a vitamin D-primed innate host defence mechanism against bacterial and fungal pathogens, involving the upregulation of CRIg.**

Vitamin D is generated in humans by a two-step process. Firstly, the ultraviolet light band B (UVB) converts the cholesterol precursor 7-dehydrocholesterol to pre-vitamin D in the epidermis[7]. The second step involves the isomerisation to vitamin $D_3$ (or cholecalciferol) in a thermo-sensitive, non-catalytic reaction[7]. Vitamin $D_3$ is an inactive precursor which is bioactivated by the liver to form 25-hydroxy–vitamin $D_3$ (25D). This is the main form of vitamin D present in the circulation and the form measured to determine 'vitamin D status' in an individual[8]. To form the biologically active metabolite, 1,25-dihydroxyvitamin $D_3$

(1,25D), 25D requires hydroxylation by the enzyme CYP27B1, or 25-hydroxyvitamin $D_3$ 1-α-hydroxylase. This is an intracellular process which occurs predominantly within the proximal and distal tubules of the kidneys but also extrarenally in activated macrophages[9]. Here we show that human macrophages differentiated from monocytes in the presence of 1,25D for 3 days, display increased CRIg mRNA expression (Fig 1a, b). This effect is seen in a concentration dependent manner over 0.5 – 200 nM (Fig 1a, b). The increase induced by 1,25D on CRIg mRNA expression is seen in cultures initiated with either peripheral blood mononuclear cells (PBMC) (Fig 1a) or purified monocytes (Fig 1b). Because CRIg plays an important role in innate immunity, it was of interest to examine its expression in cord blood macrophages. CRIg is expressed to a similar degree in macrophages from adult and cord blood and is similarly upregulated by the presence of 1,25D (Fig 1c).

Further studies with purified monocytes show that the increase in CRIg expression is evident at the protein level and is reflected in an increase in the predominant isoform, the long (L) as well as the less prominent short (S) forms, revealed by Western blot analysis using a mouse anti-human CRIg monoclonal antibody (clone 3C9, Genentech, CA)[3] (Fig 1d). Flow cytometry analyses of cell surface CRIg expression using the same monoclonal antibody show that macrophages derived from monocytes treated with 100 nM of 1,25D display significant increases in surface expression of CRIg, compared with vehicle-treated control cells, suggesting that the increase in CRIg expression is likely to have an impact on cell function (Fig 1e). While the finding that CRIg expression is modulated at the mRNA level by 1,25D suggests that regulation may occur at the transcriptional level, possibly through a direct genomic effect of 1,25D, this is more likely to be an indirect action or non-genomic action, since the presence of a vitamin D receptor (VDR) binding site has not been predicted in the promoter regions of the *VSIG4* gene.

As CRIg is not the only phagocytosis-promoting complement receptor expressed by macrophages[10], we next assessed the levels of the β-integrin complement receptors 3 and 4 (CR3 and CR4, respectively) in macrophages differentiated from monocytes in the presence of 1,25D, by measuring the levels of the α-subunits CD11b (CR3) and CD11c (CR4) expression. There is no increase in CD11b mRNA. While there is a decrease in CD11c mRNA expression in these macrophages (Fig 2a), this is not reflected in changes in either of these receptors at the protein level, revealed by Western blot analysis (Fig 2b), and in their cell surface expression, compared with untreated controls (Fig 2c). With the finding that 1,25D upregulates CRIg, but not CR3 and CR4 in macrophages, we investigated whether the phagocytic capabilities of the cells were altered by the 1,25D treatment. Using commercially available *Staphylococcus aureus* bioparticles which fluoresce once within the phagosomes of the macrophage[11], we found that phagocytosis is significantly increased in 1,25D-treated cells, compared to untreated control cells (Fig 2d). Using a second assay involving addition of heat-killed *Candida albicans* and analysis of cells under a microscope, phagocytosis is significantly higher in macrophages generated in the presence of 1,25D (Fig 2e), with more particles engulfed per individual macrophage and more cells engulfing $\geq$ 4 particles. As the process of phagocytosis in both of these assays is promoted by complement and the other phagocytosis-promoting complement receptors CR3 and CR4 were essentially not influenced by 1,25D treatment, it can be concluded that the upregulation of phagocytic activity is most likely a direct result of the increase in CRIg expression on these cells. Interestingly, vitamin D or 1,25D has been associated with the promotion of M2 macrophage polarisation, a cell which is less inflammatory but has higher phagocytic activity than M1 macrophages[12-14]. Additionally, CRIg is an important phagocytosis-promoting receptor able to mediate capture of bacterial, fungal, and parasitic pathogens[15], with increased phagocytic rates compared with CR3[3,16,17].

Monocyte-derived macrophages have a lifespan ranging from weeks to years in the tissues[18]. As a result, these cells can potentially be exposed to a range of homeostatic or inflammatory conditions. As their local microenvironment fluctuates, macrophages are able to display a high level of phenotypic plasticity reflecting this environment. Because of this, we sought to investigate whether adding 1,25D directly to the macrophages also causes a change in CRIg expression. Macrophages were prepared by incubating monocytes in culture for 5 days. These were then treated with 100 nM of 1,25D for 24 hours. The macrophages show an increase in expression of CRIg mRNA (Fig. 3a) and protein (Fig 3b).

We surmise that macrophages with an active cytochrome P450 25-hydroxyvitamin D3-1alpha-hydroxylase (CYP27B1) and ability to convert the inactive 25D to 1,25D would show increased expression of CRIg, possibly through an autocrine or paracrine mechanism (Fig 4a). The TLR1/2 agonist Pam3CSK4, is known to increase the expression of CYP27B1 in macrophages[6]. Using a combination of 25D and Pam3CSK4, we investigated whether treatment with these agents for 24h causes an increase in CRIg expression. While treating macrophages with either 50 ng/mL Pam3CSK4 or 100 nM 25D independently has no significant effect, combined addition of these to cells causes an increase in CRIg mRNA and protein expression, particularly the long form (Fig. 4b, c, d). These results indicate that 1,25D produced by macrophages following engagement of TLR1/2[6] is able to act in an autocrine or intracrine manner to enhance CRIg expression.

Emerging interest on the non-classical biological effects of vitamin D has recently been highlighted[19], which includes an ability to regulate innate immune responses. Thus, 1,25D has been reported to increase the production of anti-microbial peptides e.g. cathelicidin and β-defensin 2, and stimulate phagocytosis in macrophages[20]. Recently, the secosteroid has been shown to be required for IL-22 production by type 3 innate lymphoid cells and in defence against *Citrobacter rodentium* infection[21]. In macrophages, vitamin D is known to be

required for defence against the intracellular pathogen *Mycobacterium tuberculosis*[2,22]. Macrophages express both the vitamin D receptor (VDR) and CYP27B1[9], the latter enabling the generation of 1,25D[23]. VDR and CYP27B1 expression is upregulated by engaging TLR1/2 by triacylated lipoproteins on the microbial surface[2,24]. Another important piece of this immunobiology of the vitamin D 'jigsaw' puzzle shown by the present results is the upregulation of CRIg expression through the stimulation of TLR1/2 in the presence of 25D, providing evidence for a global role in anti-infective innate immunity. The results also make prominent the point that while CRIg is readily modulated, CR3 and CR4 are essentially not affected by 1,25D. It has been reported that cytokines and inflammatory mediators as well as the steroid drug dexamethasone display this differential effect on these receptors[25-27]. Our findings reveal an important mechanism in innate anti-microbial activity of macrophages, influenced by vitamin D. This study furthermore supports the importance of vitamin D sufficiency for a functional innate immune response.

## References


1 van Schoor, N. & Lips, P. Global Overview of Vitamin D Status. *Endocrinology and metabolism clinics of North America* **46**, 845-870, doi:10.1016/j.ecl.2017.07.002 (2017).

2 Bloom BR, Modlin RL. Mechanisms of Defense against Intracellular Pathogens Mediated by Human Macrophages. *Microbiol Spectr.* 2016 Jun;4(3). doi: 10.1128/microbiolspec.MCHD-0006-2015.

3 Helmy, K. Y. *et al.* CRIg: a macrophage complement receptor required for phagocytosis of circulating pathogens. *Cell* **124**, 915-927, doi:10.1016/j.cell.2005.12.039 (2006).

4 Zeng, Z. et al. CRIg Functions as a Macrophage Pattern Recognition Receptor to Directly Bind and Capture Blood-Borne Gram-Positive Bacteria. *Cell Host & Microbe* **20** (2016).

5 Broadley, S.P., et al. Dual-Track Clearance of Circulating Bacteria Balances Rapid Restoration of Blood Sterility with Induction of Adaptive Immunity. *Cell Host & Microbe* **20** (2016).

6 Zavala K, Gottlieb CA, Teles RM, Adams JS, Hewison M, Modlin RL, Liu PT. Intrinsic activation of the vitamin D antimicrobial pathway by M. leprae infection is inhibited by type I IFN. PLoS Negl Trop Dis. 2018 Oct 9;12(10):e0006815. doi: 10.1371/journal.pntd.0006815.

7 Bikle, D. D. Vitamin D Metabolism, Mechanism of Action, and Clinical Applications. *Chemistry & biology* **21**, 319-329, doi:10.1016/j.chembiol.2013.12.016 (2014).

8 Harrison SR, Li D, Jeffery LE, Raza K, Hewison M. Vitamin D, Autoimmune Disease and Rheumatoid Arthritis. *Calcif Tissue Int.* 2019 Jul 8. doi: 10.1007/s00223-019-00577-2.6

9 Cantorna, M.T., Rogers, C.J., Arora, J. Aligning the Paradoxical Role of Vitamin D in Gastrointestinal Immunity. *Trends in Endocrinology & Metabolism*, **30**, No. 7 (2019)

10 Sun, D. et al. Fungal dissemination is limited by liver macrophage filtration of the blood. *Nat Commun* 10, 4566, (2019).

11 Small, A., Lansdown, N., Al-Baghdadi, M., Quach, A. & Ferrante, A. Facilitating THP-1 macrophage studies by differentiating and investigating cell functions in polystyrene test tubes. *Journal of Immunological Methods*, doi:https://doi.org/10.1016/j.jim.2018.06.019 (2018).

12 Gunasekar, P. *et al.* Vitamin D and macrophage polarization in epicardial adipose tissue of atherosclerotic swine. *PLOS ONE* **13**, e0199411, doi:10.1371/journal.pone.0199411 (2018).

13 Zhang, X., Zhou, M., Guo, Y., Song, Z. & Liu, B. 1,25-Dihydroxyvitamin $D_3$ Promotes High Glucose-Induced M1 Macrophage Switching to M2 via the VDR-PPARγ Signaling Pathway. *BioMed research international* **2015**, 157834-157834, doi:10.1155/2015/157834 (2015).

14 Dionne, S., Duchatelier, C. F. & Seidman, E. G. The influence of vitamin D on M1 and M2 macrophages in patients with Crohn's disease. *Innate immunity* **23**, 557-565, doi:10.1177/1753425917721965 (2017).

15 Liu, G. et al. CRIg plays an essential role in intravascular clearance of blood borne parasites by interacting with complement. *Proc Natl Acad Sci U S A*, (2019).

16 Gorgani, N. N. *et al.* Complement receptor of the Ig superfamily enhances complement-mediated phagocytosis in a subpopulation of tissue resident macrophages. *Journal of immunology (Baltimore, Md. : 1950)* **181**, 7902-7908 (2008).

17 Small, A. G., Al-Baghdadi, M., Quach, A., Hii, C. & Ferrante, A. Complement receptor immunoglobulin: a control point in infection and immunity, inflammation and cancer. *Swiss medical weekly* **146**, w14301, doi:10.4414/smw.2016.14301 (2016).

18 Hoeffel G, Ginhoux F. Fetal monocytes and the origins of tissue-resident macrophages. *Cell Immunol*. 2018 Aug;330:5-15. doi: 10.1016/j.cellimm.2018.01.001. Epub 2018 Jan 12.

19 Hii, C. S. & Ferrante, A. The Non-Genomic Actions of Vitamin D. *Nutrients* 8, 135, doi:10.3390/nu8030135 (2016).

20 Hart, P.H. Sun-immune connection. *Nat Rev Immunol.* **19**, 661, doi:10.1038/s41577-019-0216-6 (2019).

21 Lin, Y.D., Arora, J., Diehl, K., Bora, S.A., Cantorna, M.T. Vitamin D is required for ILC3 derived IL-22 and protection from Citrobacter rodentium infection. Front. Immunol. **10**, 1. (2019)

22 Liu, P. T. *et al.* Toll-like receptor triggering of a vitamin D-mediated human antimicrobial response. *Science* **311**, 1770-1773, doi:10.1126/science.1123933 (2006).

23 Booth, D. R. *et al.* Cistromic and genetic evidence that the vitamin D receptor mediates susceptibility to latitude-dependent autoimmune diseases. *Genes and Immunity* **17**, 213-219, doi:10.1038/gene.2016.12 (2016).

24 Meyer V, Saccone DS, Tugizimana F, Asani FF, Jeffery TJ, Bornman L. Methylation of the Vitamin D Receptor (VDR) Gene, Together with Genetic Variation, Race, and Environment Influence the Signaling Efficacy of the Toll-Like Receptor 2/1-VDR Pathway. *Front Immunol.* 2017 Sep 11;8:1048. doi: 10.3389/fimmu.2017.01048. eCollection 2017.

25 Gorgani, N. N. *et al.* Regulation of CRIg expression and phagocytosis in human macrophages by arachidonate, dexamethasone, and cytokines. *The American journal of pathology* **179**, 1310-1318, doi:10.1016/j.ajpath.2011.05.021 (2011).

26 Munawara, U. *et al.* Cytokines regulate complement receptor immunoglobulin expression and phagocytosis of Candida albicans in human macrophages: A control point in anti-microbial immunity. *Scientific Reports* **7**, 4050, doi:10.1038/s41598-017-04325-0 (2017).

27 Ma, Y. *et al.* Protein Kinase Cα Regulates the Expression of Complement Receptor Ig in Human Monocyte–Derived Macrophages. *The Journal of Immunology* **194**, 2855 (2015).

## Data availability

The data supporting this study are available within the paper and Supplementary Information. Any additional data relating to the study are available from the corresponding author on reasonable request.

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**Contributions.** AGS, SH and AF designed the experiments. AGS, SH, TP, JP and UM carried out the experiments. AMcP assisted with the cord blood cell study and critical reading of the manuscript. AGS, AQ, CSH and AF were involved in collating data, statistical analyses, data interpretation and writing of the manuscript. AF initiated and supervised the project, and AGS and AF were responsible for drafting the manuscript.

**Competing Interest.** The authors declare that there is no conflict of interest regarding the publication of this manuscript.

**Correspondence and request for materials** should be addressed to AF

## Methods

**Materials.** *Human blood specimens*. The procurement of human blood and all experimental procedures were approved by the Human Research Ethics Committee of the Women's and Children's Health Network (WCHN), Adelaide, South Australia, in accordance to The National Statement on Ethical Conduct in Human Research (2007, updated 2018) (National Health and Medical Research Council Act 1992). Venous blood was collected from healthy adult volunteers by venipuncture with their informed consent, under approval number HREC/15/WCHN/21.

*Antibodies*. The mouse monoclonal antibody (clone 3C9, for flow cytometry, 0.2 μg; for Western blotting, 1:3000) that recognizes the IgV domain of human CRIg was kindly provided by Dr. Menno van Lookeren Campagne (Genentech, San Francisco, CA). The rabbit recombinant monoclonal anti-CD11b antibody (ab133357, clone EPR1344, 1:1,000), and mouse IgG1 isotype control antibody (ab37355) were purchased from Abcam. The mouse monoclonal anti-CD11c antibody (clone N-19, 1:1,000) and goat PE-conjugated anti-mouse IgG antibody were purchased from Santa Cruz Biotechnology. The mouse monoclonal anti-GAPDH (clone 71.1, 1:20,000) was obtained from Sigma-Aldrich. The polyclonal HRP-conjugated rabbit anti-mouse (P0260), anti-goat (P0449), and goat anti-rabbit (P0448) immunoglobulin antibodies (1:2000) were obtained from Dako.

*Reagents*. Roswell Park Memorial Institute (RPMI) 1640 tissue culture medium, Hank's Buffered Saline Solution (HBSS), foetal calf serum (FCS), L-glutamine, penicillin and streptomycin were purchased from SAFC Biosciences. Dithiothreitol (DTT), benzamidine, leupeptin, pepstatin A, phenylmethylsulfonyl fluoride (PMSF), 1α,25-dihydroxyvitamin D3 (1,25D) and 25-dihydroxyvitamin D3 (25D) were purchased from Sigma-Aldrich. Stock solutions of 1,25D and 25D were prepared to $10^{-3}$ M in 95% ethanol and stored at -80 °C. Pam3CSK4 was purchased from Invivogen, with stock preparation at 1 mg $ml^{-1}$ in endotoxin-free water and storage at -20 °C. Aprotinin was purchased from Merck.

**Cell preparation and culture.** Peripheral blood mononuclear cells (PBMC) or cord blood mononuclear cells were prepared by density gradient centrifugation of blood on Ficoll-Paque PLUS (GE Healthcare). The interface layer containing PBMC was harvested and cells were washed in RPMI 1640 medium. Monocytes were purified from the MC following seeding of the latter at $2 \times 10^7$ per autologous plasma-coated 6 cm culture dish (TPP) and incubation at

37 °C, 5% $CO_2$/air, in a high humidity incubator for 2 hours. Non-adherent cells were removed by three gentle washes resulting in > 90% monocytes purity, and each dish replenished with 4 mL of RPMI 1640 supplemented with 2 mmol $L^{-1}$ L-glutamine, 100 U $ml^{-1}$ penicillin, 100 µg $ml^{-1}$ streptomycin and 10% FCS, pH 7.4. Experiments either utilized total PBMC or purified monocytes. Cells were stimulated with either, 1,25D, 25D, Pam3CSK4, or diluent and cultured for the duration specified in the Results section. Cells were harvested after either 3 days (for CRIg mRNA analysis) or 5 days (for CRIg protein analysis or phagocytosis assays) culture by gentle scraping with a 'rubber policeman'.

**Quantitative PCR assays.** The quantitative PCR (qPCR) assays were performed as previously described[26]. In brief, total RNA was extracted from harvested cells using TRIzol reagent (Invitrogen). cDNA was prepared using iScript cDNA synthesis kit (Bio-Rad). qPCR analysis was performed using iTaq™ Universal SYBR® Green Supermix (Bio-Rad) with the following conditions: initial denaturation for 5 min at 95 °C followed by 40 cycles at 95 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s using an iQ5 Real Time Detection System with iQ5 Optical System v2.1 software (Bio-Rad). Data were normalized to expression of a control gene GAPDH for each experiment. The primer pairs used were for human CRIg (Forward: 5'-ACACTTATGGCCGTCCCAT-3'; Reverse: 5'-TGTACCAGCCACTTCACCAA-3'), CD11b (F: 5′-CCTGGTGTTCTTGGTGCCC-3′ and R: 5′-TCCTTGGTGTGGCACGTACTC-3′) CD11c (F: 5′-CCGATTGTTCCATGCCTCAT-3′; R: 5′-AACCCCAATTGCATAGCGG-3′), and GAPDH (F: 5′-GAGTCAACGGATTTGGTCGT-3′; R: 5′-GACAAGCTTCCCGTTCTCAGCCT-3′).

**Phagocytosis assays.** *Staphylococcus aureus bioparticle uptake quantitation by flow cytometry*[11]. Briefly, 1 × $10^6$ macrophages in HBSS with 8% human AB serum, were incubated with 80 µg pHrodo™ Red *S. aureus* Bioparticles™ (Invitrogen), in a final volume of 400 µL in 12 x 75 mm round bottom tubes. These were gassed with 5% $CO_2$/air and capped, with incubation at 37 °C for 1 hour. Following washing in HBSS, samples were acquired using a BD FACSCanto I flow cytometer, with analysis using FlowJo 10.1 software (FlowJo LLC) to determine bioparticle uptake by changes in median fluorescence intensity in the PE channel.

*Candida albicans particle uptake quantitation by microscopy*. This phagocytosis assay was performed essentially as described previously[25,26]. Briefly, 1 × $10^5$ *C. albicans* yeast particles were added to 5 × $10^4$ macrophages in a final volume of 0.5 ml HBSS. Complement-

containing human AB serum was added to a final concentration of 10%. The cells were incubated for 15 min at 37 °C on a rocking platform. Following removal of unphagocytosed yeast particles by differential centrifugation at 175 × *g* for 5 min, the remaining macrophages in the pellet were cytocentrifuged onto a microscope slide and stained with Giemsa. The particles in phagocytic vacuoles were enumerated, with phagocytosis was scored as both the number of macrophages that had engulfed >4 fungi as well as the number of fungi engulfed per cell.

**Cell surface CRIg expression determination.** Macrophage surface CRIg expression was determined by flow cytometry[25]. Briefly, harvested cells were incubated in 12 × 75 mm round bottom tubes on ice with 100 µg purified human IgG (Kiovig, Baxter) for 15 min. This was followed by addition of 0.2 µg of either anti-human CRIg or mouse IgG1 isotype control antibodies, with further incubation for 20 min. Cells were washed with 2 mL PBS with centrifugation at 500 × g for 5 min. Goat anti-mouse IgG PE secondary antibody was then added, with continued incubation in the dark on ice for 20 min. Following washing twice more, the cells were acquired (50,000 event minimum) on a BD FACSCanto I with data analysed using FlowJo 10.1.

**Western blotting assays.** Protein analysis in harvested macrophages was performed using Western blot essentially as previously described[26]. Lysates were generated from macrophages in each culture dish with 100 µL of buffer containing 20 mmol $L^{-1}$ HEPES, pH 7.4, 0.5% Nonidet P-40 (v/v), 100 mmol $L^{-1}$ NaCl, 1 mmol $L^{-1}$ EDTA, 2 mmol $L^{-1}$ $Na_3VO_4$, 2 mmol $L^{-1}$ DTT, 1 mmol $L^{-1}$ PMSF and 1 µg $mL^{-1}$ of each protease inhibitor, benzamidine, leupeptin, and pepstatin A. Total protein in the soluble fractions were quantitated using the Qubit™ Protein Assay Kit on a Qubit 3.0 (Invitrogen), prior to the addition of Laemmli buffer. Samples were boiled at 100 °C for 5 min and 60 µg of protein were subjected to 10% SDS-PAGE at 170 V for approximately 1 hour, using the Mini-PROTEAN 3 system (Bio-Rad). The samples were transferred onto nitrocellulose membrane using the Trans-Blot® Turbo™ Transfer System (Bio-Rad). The extent of protein transfer was ascertained using 0.1% Ponceau S membrane staining. After blocking in TBST with 5% skim milk (blocking solution), the membrane was incubated with either mouse anti-human CRIg, rabbit anti-human CD11b, or mouse anti-human CD11c antibodies in blocking solution overnight at 4 °C. The membrane was washed in blocking solution (3 × 5 min) and then incubated with the appropriate secondary HRP-conjugated antibody (anti-mouse, anti-rabbit, or anti-goat IgG) in

blocking solution for 1 hour at room temperature. Immunoreactive material was detected using the Western Lightning Plus-ECL Enhanced Chemiluminescence Substrate (PerkinElmer), with protein bands visualized on a ChemiDoc™ XRS+ Imager and quantitated using Image Lab™ Software, Version 3.0 (Bio-Rad). For GAPDH determination, stained membranes were subjected to antibody stripping using ReBlot Plus Mild Solution (Millipore) and incubated with mouse anti-human GAPDH antibody, followed by the staining and visualization steps as described above.

**Statistical analysis.** Graphpad Prism 8.0 (Graphpad Software) was used for statistical analysis. Mean differences were compared using t-tests (for comparisons of two groups) or one-way ANOVA followed by multiple-comparison tests (for comparisons of three of more groups). *P* values <0.05 were considered to be statistically significant.

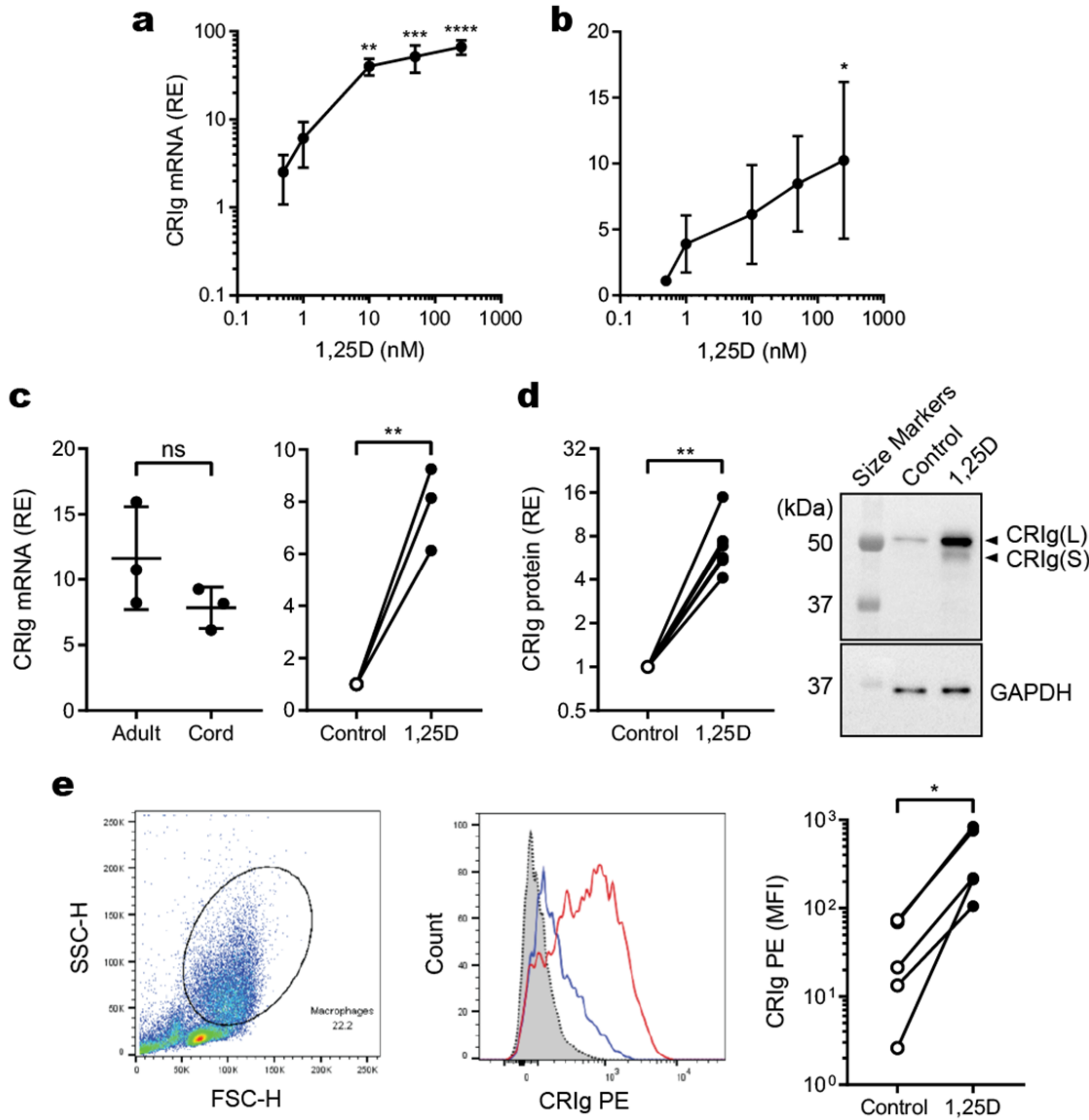



**Fig. 1. CRIg is upregulated in human macrophages by 1,25D.** PBMC or purified monocytes from the blood of healthy human adult donors were cultured in the presence or absence of 1,25D. The cells were harvested to determine levels of CRIg mRNA on day 3 of culture, and CRIg protein on day 5 of culture. Relative expression (RE) of mRNA or protein was measured against GAPDH. **a,** CRIg mRNA expression in PBMC cultured with varying concentration of 1,25D. **b,** CRIg mRNA expression in macrophages derived from monocytes cultured with varying concentrations of 1,25D. **c,** CRIg mRNA expression in macrophages derived from cord blood monocytes compared with macrophages from adult cells (left panel), and comparison of macrophages cultured for 3 days in the presence or absence of 100 nM 1,25D (right panel). **d,** CRIg protein in macrophages derived from monocytes cultured in the presence or absence of 100 nM 1,25D.

Western blot data are presented as fold-difference in CRIg band intensity normalized against GAPDH (loading control) with 6 experimental runs each with cells from a different individual. Representative Western blot of CRIg expression (top panel) and GAPDH re-probe (bottom panel) are shown. **e,** Macrophages derived from monocytes cultured in the presence or absence of 100 nM 1,25D were analyzed for cell surface CRIg expression by flow cytometry. *Left panel*, Gating strategy based on size and granularity; *Centre panel*, representative histogram overlay of CRIg expression: secondary antibody control is shown in dotted black, unstimulated macrophage CRIg fluorescence is shown in blue, and 1,25D stimulated macrophage CRIg fluorescence is shown in red; *Right panel*, Δ median fluorescence intensity (MFI), for CRIg staining minus isotype control, is shown for control and 1,25D treated cells from 5 individual experiments. **a-b,** Data are presented as mean ± s.d. of 3 experiments each with cells from a different individual. $P$ values were calculated using one-way ANOVA followed by Dunnett's multiple comparison test. **c-e,** Data are analyzed by the paired, two-tailed Student's *t*-test. Statistical significance of 1,25D treated versus controls are represented as *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

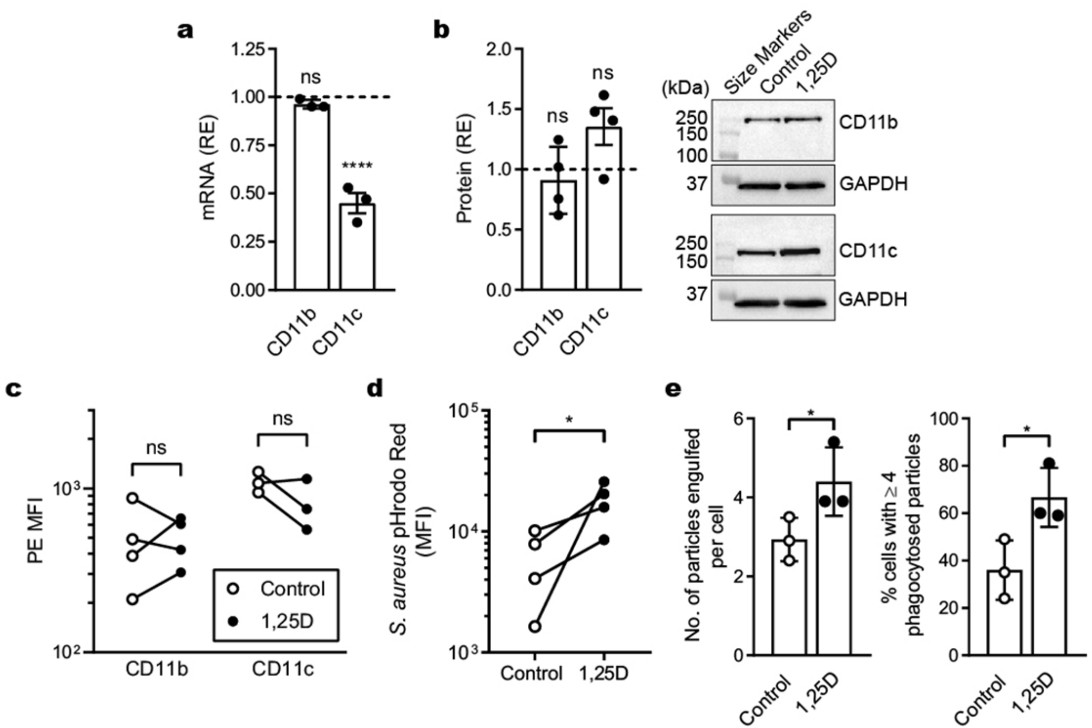



**Fig. 2. Effect of 1,25D on macrophage CR3 and CR4 expression and phagocytosis.** Monocytes were cultured for either 3 days (for mRNA expression) or 5 days (for protein expression) with 1,25D and examined for complement receptor expression. Relative expression (RE) of mRNA or protein was measured against GAPDH. **a,** Macrophages were examined for CD11b and CD11c mRNA expression. Data are expressed as fold-change compared with untreated control from 3 experiments each conducted with cells from a different individual. **b,** Macrophages were examined for CD11c and CD11b protein expression by Western blotting, normalized against GAPDH from 4 experiments each with cells from a different individual. Representative Western blots are shown. **c,** Macrophages were analyzed for CD11b and CD11c surface expression by flow cytometry. The PE MFI values are shown of 4 (CD11b) and 3 (CD11c) experiments, each conducted with cells from a different individual. **d,** Phagocytosis of *S. aureus* bioparticles by macrophages as measured by the pH-sensitive pHrodo™ Red dye. Data are expressed as MFI, each conducted with cells from a different individual. **e,** Phagocytosis of opsonized *C. albicans* by macrophages derived from monocytes cultured in either the presence or absence of 100 nM 1,25D for 5 days, is expressed as the number of engulfed particles per cell (left graph) and the percentage of cells with 4 or more phagocytosed particles (right graph). Data are presented as mean ± s.d. of 3 experiments each with cells from a different individual. **a-b,** Data are presented as mean ±

s.d. $P$ values were calculated using one-way ANOVA followed by Dunnett's multiple comparison test. **c-e,** $P$ values were calculated using paired two-tailed (c) or one-tailed (d-e) Student's t-test. Significance of differences between 1,25D versus control, *$P$ <0.05, ****$P$ <0.0001, ns = not significant.

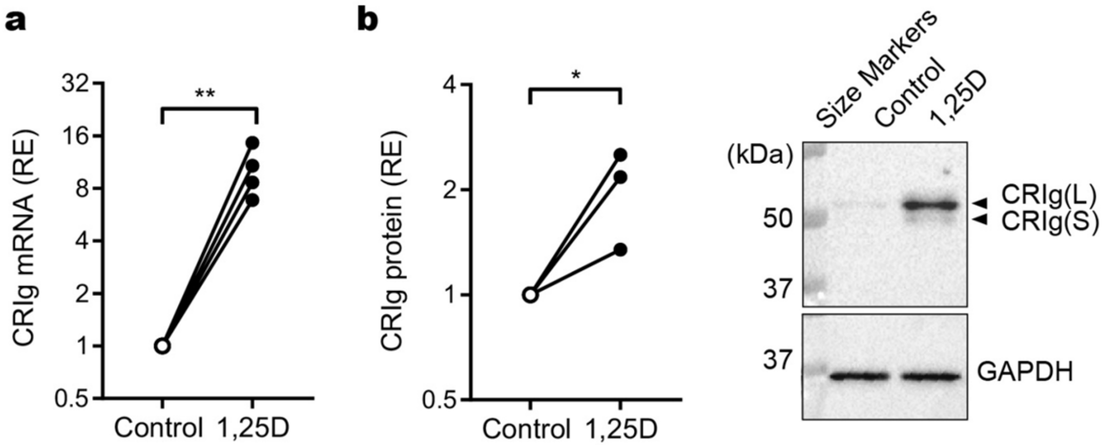



**Fig. 3. Effects of treating the macrophages directly with 1,25D on CRIg expression. a,** Macrophages matured after three days of monocyte culture, were treated for a further 24 h with 100 nM of 1,25D or diluent and then the CRIg mRNA levels measured by qPCR. Data are expressed as CRIg relative to GAPDH from 4 experiments, each conducted with cells from a different individual. **b,** Macrophages differentiated from culturing monocyte for 5 days culture, were treated as described above. The CRIg expression was measured by Western blot in three experiments, each conducted with cells from different individuals. A representative Western blot is shown of CRIg and GAPDH staining of the same blot. **a-b,** Relative expression (RE) of mRNA or protein was measured against GAPDH. *P* values were calculated by paired, one-tailed student's t-test. Significance of differences between 1,25D versus control, *$P$ <0.05; **$P$<0.01

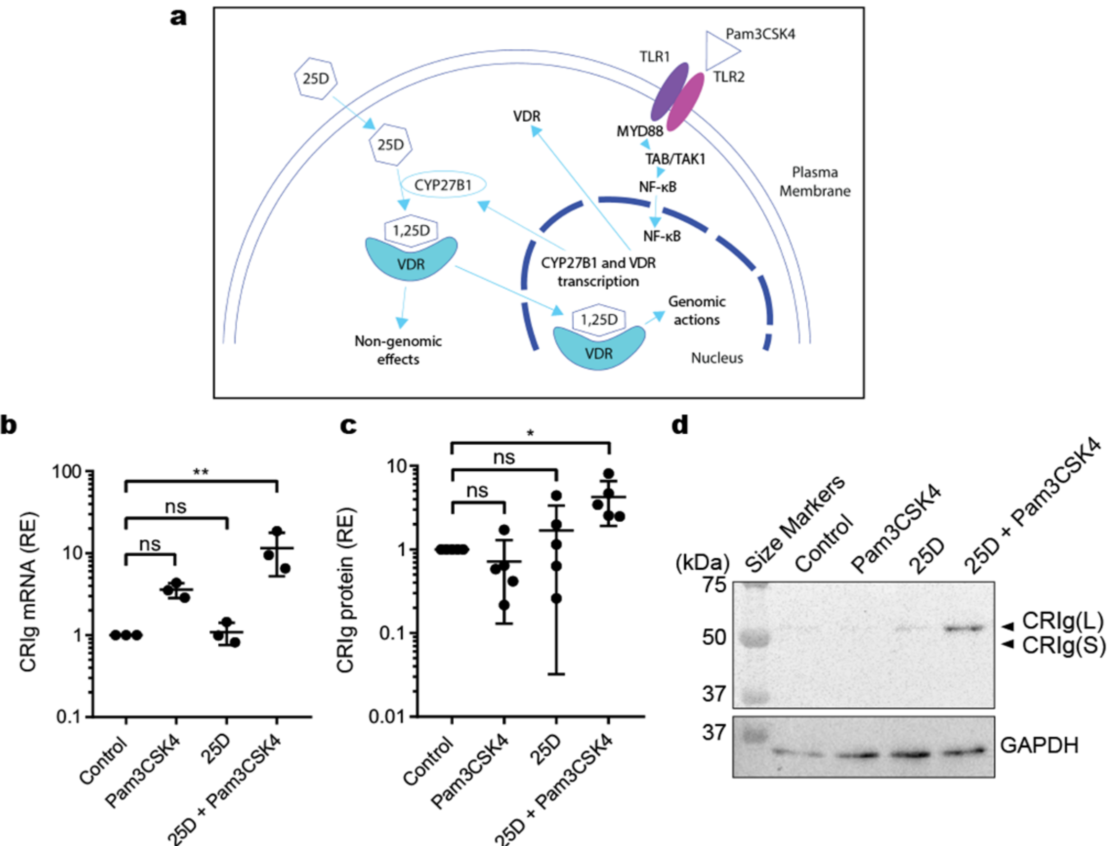
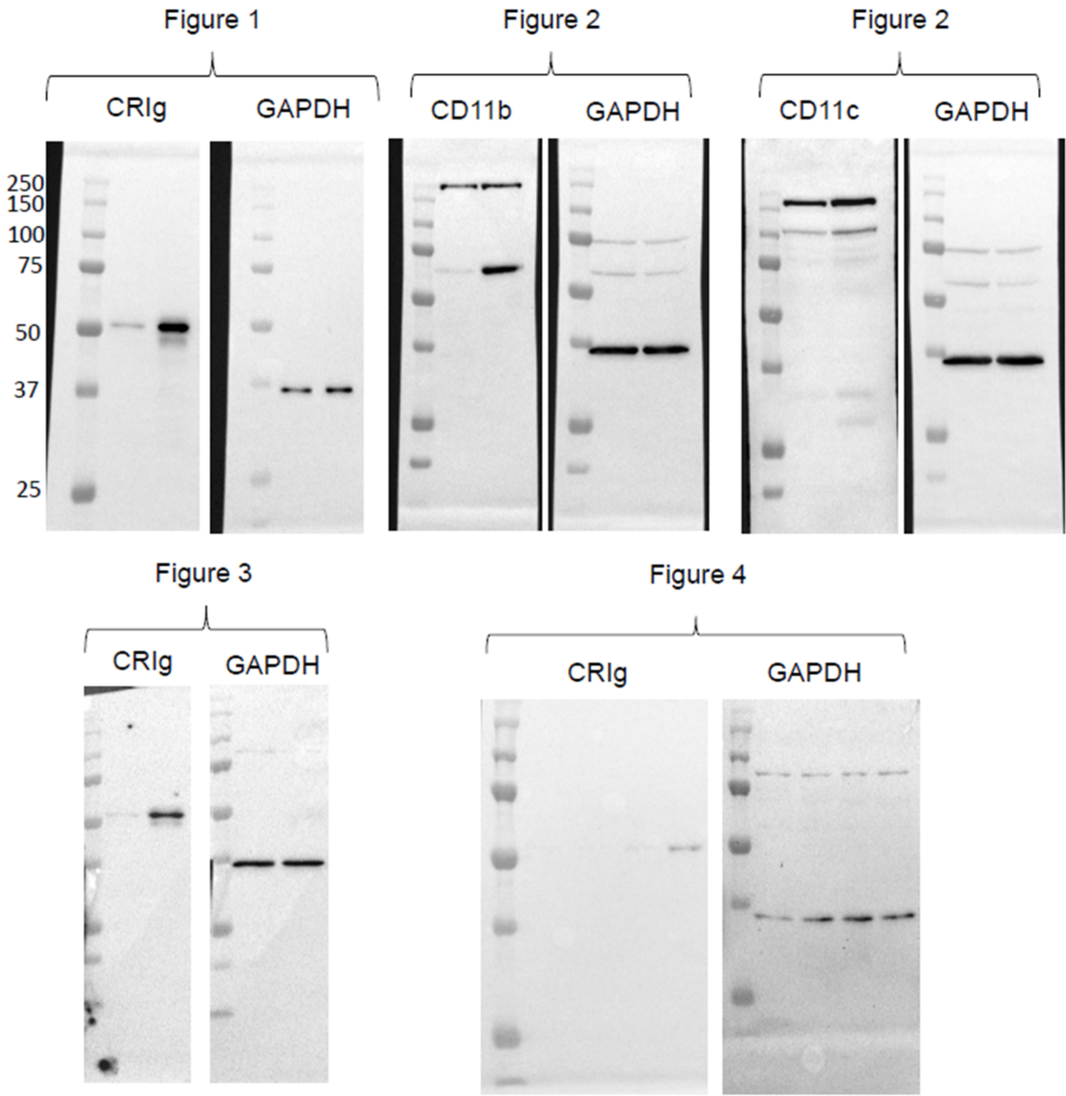



**Fig. 4. Vitamin $D_3$ promotes CRIg expression in macrophages treated with the TLR1/2 agonist Pam3CSK4. a,** Schematic diagram showing engagement of TLR1/2 inducing enhanced expression of CYP27B1 which then converts 25D to 1,25D **b,** Macrophages matured after three days of monocyte culture, were treated for a further 24 h with either 50 ng/mL Pam3CSK4, 100 nM 25D or a combination of both or neither and the levels of CRIg mRNA determined. The levels were expressed relative to GAPDH mRNA (RE). Data are expressed as individual values and as means ± s.d. of three experiments. **c,** Macrophages matured after five days of monocyte culture, were treated as described above. CRIg expression was measured by Western blot relative to GAPDH expression. Data are expressed as means ± s.d. of 5 experiments. **d,** A representative Western blot. *P* values were calculated using one-way ANOVA followed by Dunnett's multiple comparison test. Significance of differences between the different treatments are shown, **P* <0.05, ***P* <0.01, ns = not significant.




**Extended Data Fig. 1. Full length Western blots from which cropped blots in the manuscript originated.** CRIg, CD11b, CD11c and the corresponding GAPDH blots with lanes in their entirety are presented. Precision Plus Protein™ Standards (Bio-Rad) were used for determining the molecular weights of proteins in kilodaltons (kDa).

# Chapter 7. Expression by Neutrophils and Monocytes

### 7.1. Contextual Statement

As discussed in earlier chapters, CRIg has long been known to be exclusively expressed by particular subsets of tissue resident macrophages such as Kupffer cells (Fu et al., 2012; Helmy et al., 2006; Yuan et al., 2017). While in 2005, monocytes were reported to express CRIg protein (Kim et al., 2005), since this initial documentation, monocytes have been consistently reported negative (Helmy et al., 2006; Tanaka et al., 2008; Vogt et al., 2006). Similarly, while neutrophils have been reported to express CRIg mRNA, no protein expression of CRIg by these cells has been reported to date (Vogt et al., 2006). In this chapter, we sought to address these inconsistencies in the published reports of CRIg expression and investigated the presence and dynamics of CRIg expression in human circulatory phagocytes—monocytes and neutrophils.

This chapter is presented in the format of a submitted publication, and incorporates the **main findings** of '*Functional expression of CRIg/VSIg4 on neutrophils and monocytes under activating conditions involving PKC, p38 and cytoskeleton*', by **Annabelle Small**, Trishni Putty, Khalida Perveen, Nikita Patel, Asmitabahen Patel, Muhammad Y. Gulam, Patrick Quinn, Helen Weedon, Anak A.S.S.K. Dharmapatni, Mihir D. Wechalekar, Charles S. Hii, Alex Quach, & Antonio Ferrante, which at the time of submission of this thesis, is currently under consideration for publication (November 2020).

*Note that all references to 'supplementary materials figures and tables (termed 'Fig S1-6, and table S1-3) refer to the figures and tables presented in chapter 3 of this thesis.*

# Statement of Authorship

| Title of Paper | 'Activated neutrophils express functional Complement Receptor Immunoglobulin involving p38 and cytoskeleton', AND 'Development and characterization of cross-reactive monoclonal antibodies to human and murine complement receptor immunoglobulin (CRIg)' |
|---|---|
| Publication Status | ☐ Published ☐ Accepted for Publication ☒ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style |
| Publication Details | Unpublished works presented in manuscript style, submitted together and currently under consideration for publication |

## Principal Author

| Name of Principal Author (Candidate) | Annabelle Small | | |
|---|---|---|---|
| Contribution to the Paper | AS was principally responsible for running the project, including the design, generation and characterisation of the monoclonal antibodies. This included comprehensively reading and assessing published data in the field, organising laboratory facilities, designing experiments, and coordinating staff assisting with experiments and data interpretation. AS performed the majority of the experiments, collated the data, interpreted data, produced the first draft of the manuscript, and wrote the manuscript. | | |
| Overall percentage (%) | 70% | | |
| Certification: | This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper. | | |
| Signature | | Date | 14/8/20 |

## Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

| Name of Co-Author | Professor Antonio Ferrante | | |
|---|---|---|---|
| Contribution to the Paper | AF proposed and designed the experiments, interpreted data, supervised all aspects of the study, was responsible for the clinical/laboratory work up of the patient with ARPC1B deficiency, initiated and contributed to the characterisation of the monoclonal antibodies, wrote the manuscript and acted as corresponding author. | | |
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| Name of Co-Author | Trishni Putty | | |
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| Contribution to the Paper | TP contributed to the experiments, the clinical/laboratory work up of the patient with ARPC1B deficiency, data interpretation, and was involved with the critical reading and writing of the manuscript. | | |
| Signature | | Date | 14/08/20 |

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| Name of Co-Author | Nikita Patel |
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| Contribution to the Paper | HW was involved in the characterisation of the monoclonal antibodies and critical reading and writing of the manuscript. |
| Signature | Date 19-8-20 |

| Name of Co-Author | Dr. Kencana Dharmapatni |
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| Contribution to the Paper | KP was responsible for the acquisition of the murine synovial tissue and assisted in the characterisation of the monoclonal antibo s n critical reading of the manuscript. |
| Signature | Date 19/08/2020 |

| Name of Co-Author | Associate Professor Charles Hii | | |
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| Contribution to the Paper | CH was involved in designing aspects of the project including signalling pathways and monoclonal antibody generation. CH was also involved in data interpretation and in the critical reading and writing of the manuscript. | | |
| Signature | | Date | 14/8/20 |

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| Contribution to the Paper | AQ was responsible for the clinical/laboratory work up of the patient with ARPC1B deficiency, assisted in data interpretation, compiling of the figures, statistics, and in the critical reading and writing of the manuscript. | | |
| Signature | | Date | 14.8.20 |

| Name of Co-Author | Associate Professor Mihir Wechalekar | | |
|---|---|---|---|
| Contribution to the Paper | MW was responsible for the acquisition of the human synovial tissue samples, and assisted in the characterisation of the monoclonal antibodies and critical reading of the manuscript. | | |
| Signature | | Date | 21.8.2020 |

| Name of Co-Author | Asmitabahen Patel | | |
|---|---|---|---|
| Contribution to the Paper | AP contributed to some experimental runs and in data interpretation. | | |
| Signature | | Date | 19.8.20 |

**Research Article**

# Functional expression of CRIg/VSIg4 on neutrophils and monocytes under activating conditions involving PKC, p38, $Ca^{2+}$ and cytoskeleton

**One sentence summary:** While it has been reported that only macrophages express Complement Receptor Immunoglobulin (CRIg), we present evidence that activated neutrophils become armed with the surface expression of this phagocytosis-promoting receptor, involving PKC, p38 and ARPC1B, Rac2 and Rab27a.

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## ABSTRACT

The most recently identified phagocytosis-promoting complement receptor, Complement Receptor Immunoglobulin (CRIg), is exclusively expressed in macrophages, playing an important role in immunity to bacterial, viral, fungal and parasitic infections. Here we report that human neutrophils also express functional cell surface CRIg. Protein analysis by western blot reveals two isoforms. Cell surface expression is evident after activation with inflammatory mediators, such as tumour necrosis factor and bacterial lipopolysaccharide. Activation-induced surface expression on neutrophils requires p38 MAP kinase and protein kinase C, as well as intracellular calcium. Neutrophils which are defective in actin microfilament reorganisation due to a mutation in *ARPC1B* or inibition of its upstream regulator, Rac2 lose their ability to upregulate CRIg. Inhibition of another small GTPase, Rab27a, prevents the increase in CRIg expression, suggesting a requirement for the actin cytoskeleton and exocytosis. Engagement of CRIg on neutrophils with an anti-CRIg monoclonal antibody elicits the release of superoxide. In contrast, CRIg is highly expressed on monocytes and cellular activation causes a decrease in this expression. The data demonstrate the expression of functional CRIg on circulating phagocytes, regulated by inflammatory mediators. The findings imply that CRIg expression is more widely distributed in different leukocytes types and bring a new perspective on mechanisms of the body's anti-microbial defence system involving this receptor.

## INTRODUCTION

CRIg or V-set and immunoglobulin domain–containing 4 (VSIG4) has distinct structural and biological properties from the classical complement receptors, CR3 and CR4 (1, 2). Its role in innate immunity is exemplified by its ability to promote the rapid clearance of blood-borne bacteria in both a complement-dependent and independent pattern recognition receptor manner (3, 4). The restriction of CRIg expression to macrophages, and in particular to subpopulations

of fixed tissue mouse phagocytes such as Kupffer cells and resident peritoneal macrophages, has been the basis for formulating our concepts to date on the role of this receptor in infection and immunity and inflammation (5). Here, we have explored the possibility that CRIg may also be expressed by the phagocytes of the circulation, neutrophils and monocytes. We demonstrated the surface expression of CRIg on human neutrophils under cell-activating conditions, and naturally by monocytes under non-activating conditions. In addition, the upregulation of surface CRIg expression on neutrophils requires PKC, p38 MAP kinase, intracellular calcium, ARPC1B and the small GTP-binding proteins, Rac and Rab27a.

## RESULTS

### CRIg expression in neutrophils

Here, we report that human neutrophils also express CRIg (Fig. 1). Neutrophils purified from peripheral blood of healthy blood donors demonstrate expression of CRIg mRNA by RT-PCR (Fig. 1a, left), and protein by Western blot (Fig. 1a, right panel) using a rat anti-CRIg monoclonal antibody which we generated for this study (clone 14B11, Table S2, Fig. S1-5). The antibody was validated and shown to react with both mouse and human CRIg in ELISA assay, Western blot, flow cytometry and immunohistochemistry. Neutrophil lysates show the presence of two isoforms of CRIg migrating at ~45 kDa and ~40 kDa (Fig. 1a, right), consistent with the previously reported sizes of the CRIg protein splice variants in macrophages and dendritic cells (1, 6, 7). However, it has been demonstrated recently that human macrophages can express at least five isoforms of CRIg (8). Examination of neutrophil surface expression shows that minimal levels of CRIg is expressed compared to intracellular expression, revealed by flow cytometry using anti-CRIg monoclonal antibody (Santa Cruz Biotechnology, clone 6H8) (Fig. 1b).

**Activated neutrophils express functional cell surface CRIg**

Importantly, in further investigations with neutrophils, we find that activating neutrophils with either exogenous or endogenous inflammatory mediators leads to a significant increase in expression of cell surface CRIg (Fig. 1). Stimulation of purified neutrophils with either the tripeptide, f-met-leu-phe (fMLF), bacterial lipopolysaccharide (LPS), phorbol myristate acetate (PMA), or leukotriene (LT)$B_4$ causes a rapid increase in expression of CRIg in a concentration dependent manner (Fig.1c-g).

Previous studies have shown that TNF primes neutrophils for increased anti-microbial function, and that the mechanisms involved include an increase in cell surface receptor expression (9). Thus, we examined whether TNF modulates the expression of CRIg. The results showed that TNF causes a rapid increase in CRIg surface expression in a concentration and time dependent manner (Fig. 2a). When we compare the effects of TNF on intracellular protein, it was apparent that TNF did not cause any change in intracellular CRIg levels by Western blot analysis (Fig. 2b). This suggests that TNF promotes an increase in CRIg surface expression through the translocation of protein to the cell surface without synthesis of new CRIg protein. Additionally, adding either TNF or PMA to whole blood induces the cell surface CRIg expression on neutrophils (Fig. 2d). In contrast, we observed no expression of CRIg on murine neutrophils, including following TNF stimulation of murine whole blood (Fig 2e).

Next, to determine whether CRIg expressed on the surface of human neutrophils is functional, we treated the TNF-primed neutrophils with an anti-CRIg monoclonal antibody which has been previously shown to elicit a signalling response through CRIg in THP-1 macrophages (clone 6H8, Santa Cruz Biotechnology) (6), and measured the respiratory burst response using the lucigenin-dependent chemiluminescence assay, a measure of superoxide production. Addition of the anti-CRIg antibody causes a significant increase in respiratory burst or superoxide production compared to the non-TNF treated cells (Fig. 2c). These results show

that CRIg not only promotes the phagocytosis of bacteria, but also elicits a corresponding NADPH oxidase respiratory burst following engagement, inducing bactericidal reactive oxygen intermediates by neutrophils.

The ability of neutrophils to express CRIg under the influence of inflammatory mediators as shown in our studies is likely to have implications as to how we view the role of neutrophils in defence against bacteria and viruses. While the mouse reticuloendothelial system or Kupffer cells of the liver show high expression of CRIg (1), this is not the case in humans where proteomic studies have failed to show expression of CRIg (10). It is therefore tempting to speculate from our results that the infiltrating neutrophils and monocytes at infection sites are key players in controlling infection, particularly as the bacterial components and endogenously generated mediators upregulate CRIg expression in neutrophils. We have previously demonstrated that TNF primes neutrophils to kill bacteria and parasites in a complement-dependent manner (9, 11). The ability of this cytokine to increase surface expression of CRIg is likely also to play a role in the mechanisms involved in cytokine-induced priming of these cells. Unlike CR3 which binds iC3b, CRIg binds both C3b and iC3b fragments of complement (1, 12). Therefore, CRIg engages the complement-opsonised bacteria before CR3 as the latter receptor also needs to be activated through an 'inside-out' signal before these receptors can act (5), and thus, CRIg is most likely to be responsible for the rapid initial phagocytosis of microbial pathogens early in infection (12).

**Role of PKC and p38 MAP kinase**

Since agents such as PMA cause an increase in CRIg surface expression in neutrophils (Fig. 1e, g), this indicates that PKC activation is involved in the upregulation of CRIg on the surface as PKC is a receptor for PMA (13). Thus, treating the neutrophils with the PKC pharmacological inhibitor, GF109203X, prevents the expression of CRIg on the surface of

neutrophils, induced by PMA (Fig. 3a). This induction of CRIg expression via activation of PKC contrasts with the established role of PKC activation in the downregulation of CRIg expression in human macrophages (14, 15). In comparison, the inhibitor has no effect on the TNF-induced expression of this complement receptor in neutrophils (Fig. 3b). However, this is not surprising as TNF does not activate PKC in neutrophils (16), consistent with a lack of phosphoinositide generation, indicating the lack of phospholipase C activation (17). These findings therefore suggest that CRIg expression in neutrophils and macrophages is regulated through differing mechanisms when signalling via the TNF receptors.

The TNF-induced response in neutrophils is dependent on the activation of p38 MAP kinase, demonstrated by the ability of the p38 inhibitors SB203580 and SB202190 to abrogate this response (Fig. 3c, d). Other MAP kinases such as ERK1/2 and JNK are unlikely to be involved since TNF does not activate these in neutrophils (18). Further investigations show that the TNF effect is not dependent on phosphoinositide 3-kinase (PI3K) activation as it is not inhibited by wortmannin (Fig. 3e).

**Role of $Ca^{2+}$, Rac, Rab27a and ARPC1B**

The above observations (Fig. 2, 3) imply that CRIg is stored intracellularly and mobilized to the cell surface during stimulation, presumably via exocytosis. Neutrophil degranulation via exocytosis is known to require an increase in intracellular $Ca^{2+}$ (19) and involves actin remodelling and microtubule assembly (20). We therefore investigated whether the increase in cell-surface CRIg expression requires intracellular $Ca^{2+}$ and the function of the actin cytoskeleton. When neutrophils are treated with the cell permeable $Ca^{2+}$-selective chelator BAPTA, the increase in cell-surface expression of CD11b induced by fMLF is significantly reduced (Fig. 4a). While there similarly appears to be a reduction in cell-surface CRIg expression, this is not significant compared with cells treated with fMLF alone (*P*=0.1839).

However, this is likely a result of the increased baseline expression of CRIg on the surface of neutrophils treated with BAPTA alone. Thus, we only observe a significant increase in CRIg expression on neutrophils stimulated with fMLF in the absence of the inhibitor, indicating a requirement of intracellular $Ca^{2+}$ in mediating the upregulation of CRIg cell surface expression.

Since fMLF and TNF do not cause the degranulation of azurophilic and specific granules (Table S3), it is unlikely that CRIg is localised in these compartments. Similarly, the finding that PMA does not cause release from azurophilic granules supports our view that CRIg is not present in these granules (Table S3). Cytochalasin B inhibits actin network formation and it has been used widely in neutrophil degranulation studies. Addition of cytochalasin B to neutrophils promotes the fMLF-induced release from azurophilic (primary) and specific (secondary) granules (21) (Table S3). While cytochalasin B promotes the fMLF-induced expression of CD11b, there is no effect on CRIg expression (Fig 4b). This suggests that CRIg is not associated with the specific and azurophilic granules but is present in a store which is insensitive to cytochalasin B, potentially in gelatinase granules. This is supported by the finding that LPS treatment mobilizes gelatinase granules but only a minor fraction of specific granules (22) and was found to increase expression of CRIg in neutrophils. Further support comes from findings that both TNF and fMLF mobilise release from gelatinase granules and secretory vehicles (23) (Table S3).

To further investigate the role of the actin cytoskeleton, we examined the effects of blocking the function of rac, a small GTPase that regulates actin polymerisation and neutrophil function, including migration, superoxide production (24, 25) and degranulation (24). Three highly homologous forms of rac proteins (rac1, rac2 and rac3), are expressed in mammalian cells. Of these, rac1 and rac2 are expressed in neutrophils, and there is no evidence for the presence of rac3 in myeloid cells (26). Human neutrophils express predominantly rac2 (~97%) and a small amount of rac1 (27). We found that treating neutrophils with the rac1 inhibitor,

NSC 23766, doesn't alter either CRIg or CD11b expression in response to fMLF (Fig. 4c). Additionally, EHT 1864, a rac inhibitor with selectivity for rac1 and rac2 over rac3 (28), suppresses the stimulatory effect of fMLF and TNF on CRIg and CD11b expression (Fig. 4d). While the rac1/2 inhibitors have been reported to possess "identical" non-rac-related actions in platelets (29), the differing actions between the two pharmacological inhibitors suggest that upregulation of surface CRIg and CD11b expression in response to fMLF and TNF is unlikely to involve a non-rac-related mechanism of action. While our results imply that rac2 is involved, data from human neutrophils with the rac2 (D57N) mutation (24) show that the loss of rac2 function is associated with a selective loss of azurophilic granule release but did not affect release from specific granules (24). Thus, we infer from our findings that rac2 may promote release from gelatinase granules.

To support a role for rac2 and actin filament reorganisation in the induction of CRIg surface expression, we utilised neutrophils from a patient deficient in the expression of actin related protein 2/3 complex subunit 1B (ARPC1B), also known as Arc-p41, the only ARPC1 isoform expressed in haematopoietic cells (30, 31). Arp2/3 is a downstream effector of rac2 (32) and is required for actin filament branching, with total loss of function being embryonic lethal (33). We questioned whether neutrophils from an ARPC1B deficient individual with impaired actin polymerisation function (3) are able to upregulate CRIg surface expression after stimulation (Fig. 4e). Our data demonstrate that ARPC1B deficient neutrophils, while showing normal basal expression of CRIg, CD11b, and CD11c, did not display an increase in expression of these receptors in response to TNF and fMLF (Fig. 4e). This finding supports a role for ARPC1B and rac2 in regulating CRIg expression in neutrophils. Importantly, neutrophils deficient in ARPC1B fail to upregulate their CRIg expression, yet show an increase in release from azurophilic granules (31), further supporting that CRIg is not contained within these granules.

Membrane-membrane docking and fusion during exocytosis requires intracellular $Ca^{2+}$, as well as other effectors, including the small GTPase, rab27a (34). We therefore investigated whether Rab27a is required for the surface expression of CRIg. Nexinhib20, a specific Rab27a inhibitor, causes inhibition of CRIg expression on the cell surface (Fig. 4f). Interestingly, Rab27a regulates exocytosis of gelatinase and specific granules but not of azurophilic granules (35). However, when using fMLF in combination with cytochalasin B, degranulation from azurophilic granules was regulated by Rab27a (34). Indeed, Rab27a is largely absent from CD25-enriched secretory vesicles (35). This, together with the finding that secretory vescicles can be released by fMLF when intracellular $Ca^{2+}$ is chelated with BAPTA (21), leads us to conclude that CRIg is likely stored within the gelatinase granules.

**Monocytes express CRIg**

Further, we found that monocytes also express CRIg mRNA and protein (Fig. 5a). These cells express the 45 kDa isoform which is also expressed in monocyte-derived macrophages (Fig. S6). In comparison to neutrophils, resting monocytes express high levels of CRIg on the cell surface (Fig. 5b, c). Intracellular CRIg expression is also shown by flow cytometry and is compared with surface expression (Fig. 5b). This difference between cell surface expression on monocytes and neutrophils is also evident in whole blood assays (Fig 5c). In addition, the data reveal the absence of CRIg in T cells, B cells and NK cells (Fig. 5c). Previous studies have reported that monocytes do not express CRIg (1). It is most likely that this is due to loss of CRIg from the cell surface during cell isolation from the blood followed by further cell manipulation during culture. Accordingly, we demonstrate that CRIg expression on the monocyte cell surface decreases significantly following the isolation of the mononuclear cells (PBMC) from human blood (Fig. 5d). Note that cryopreservation of the monocytes (PBMC) and subsequent thawing leads to a significant loss of the receptor on cell surface. This contrasts

with expression of the classical complement receptors, CR3 and CR4 which may in fact be increased following cell separation and cryopreservation (Fig. 5d). Finally, In contrast to neutrophils, activation of whole blood monocytes with PMA leads to a significant decrease in CRIg expression on the cell surface (Fig. 5e). This is similar to the previously reported studies with macrophages treated with PMA (14). This difference between neutrophils and monocytes/macrophages suggests the existence of another regulatory role for CRIg in defence against bacterial infection in the blood stream and in tissues.

**Discussion**

The data demonstrate that human blood neutrophils and monocytes express CRIg mRNA and protein. While CRIg was abundantly expressed on the cell surface of monocytes, neutrophils required activation with inflammatory mediators to demonstrate functional cell surface expression. It is thus intriguing that this expression has not been reported previously. This can potentially be explained in part by our observations that the commonly used anti-human CRIg monoclonal antibody clones 6H8 and 3C9 detect different isoforms of CRIg to each other when used to examine the same macrophage protein sample (Fig. S1). Human MDM treated with dexamethasone express at least five transcript variants of CRIg (8). Thus, this difference in antibody reactivity can possibly be explained by the existence of multiple protein variants being expressed by MDM which are differentially detected by the two monoclonal antibodies. While studies to confirm which specific CRIg variants are being expressed by monocytes and neutrophils remain to be conducted, it is plausible that neutrophils and monocytes are expressing different protein variants compared to macrophages. This view is supported by our Western blot results, which detected a protein migrating at ~50 kDa in MDM total cell lysates using anti-CRIg clone 3C9 (Fig. S1), while the largest protein observed in neutrophils and monocytes using anti-CRIg clone 6H8 migrates around 45 kDa (Fig. 1a, 5a), suggesting that

the larger protein expressed by MDM is not expressed in circulating phagocytes. This view that there is an isoform of CRIg that is exclusively expressed by MDM which is detected by the commonly used antibody clone 3C9 and not 6H8, potentially explains why for the last two decades, CRIg has been known to be exclusively expressed by macrophages. Additionally, CRIg expression by neutrophils may have been missed as in our study, we found that surface expression of CRIg is not significantly detectable compared with isotype control fluorescence values unless the neutrophils have been stimulated with inflammatory mediators or agonists. The initial study which assessed CRIg expression by cells in the circulation and reported them negative was conducted using flow cytometry to assess unstimulated, healthy control blood samples (1). Similarly with monocytes, we demonstrated that CRIg is lost from the cell surface upon cell isolation and cryopreservation, and this loss could explain why monocyte expression has been previously missed.

Further studies into the mechanisms of the agonist-induced CRIg expression on the neutrophil surface demonstrated a role for PKC, p38 MAP kinase, ARPC1B, Rac 2, and Rab27a and by deduction, mobilisation from gelatinase granules (depicted in Fig. 6). The increase in CRIg surface expression induced by PMA, a known direct activator of PKC, was inhibited by the pharmacological inhibitor, GF 109203X. In comparison, the effects of TNF, which does not activate PKC, were not inhibited, but the response to TNF was dependent on p38, shown by inhibition by two of the p38 MAP kinase inhibitors, SB203580 and SB202190. The correlation of CRIg upregulation with the upregulation of CD11b, known to be present in neutrophil specific and azurophilic granules, suggests that CRIg could be stored in the same compartments as CD11b. Furthermore, as ARPC1B-deficient neutrophils exhibit enhanced secretion of azurophilic granules (31) and our findings showed that these deficient cells were unable to upregulate either of CD11b or CRIg in response to TNF and fMLF, this indicates that CRIg is not stored in azurophilic granules. Inhibition of Rab27a successfully inhibited surface

upregulation of CRIg in response to fMLF stimulation. Rab27a is known to be responsible for regulating the docking and fusion with the plasma membrane during exocytosis of both gelatinase and specific granule but not azurophilic granules (35). This, considered in conjunction with the fact that LPS predominantly induces the release of gelatinase rather than specific granules, and that LPS enhances the surface expression of CRIg, suggests it is unlikely that CRIg is stored in specific granules. Furthermore, in the absence of $Ca^{2+}$ ions, neutrophils are still able to release secretory vesicles in response to fMLF (21). Thus, our data demonstrating that neutrophils are unable to upregulate CRIg in response to fMLF in the presence of the $Ca^{2+}$ chelator BAPTA-AM, would argue against CRIg being stored in the secretory vesicles. Evidence strongly suggests that CRIg is stored in the gelatinase granules of human neutrophils.

A key observation emanating from the data is that CRIg expression is regulated differently in neutrophils compared to macrophages and monocytes. While TNF, PMA and LPS depress expression in monocytes and macrophages (8, 14), these increase expression in neutrophils. Indeed, PKC activation in macrophages depresses CRIg expression, but increases expression in neutrophils. Similarly, the activation of p38 is not required for macrophage CRIg expression (14).

CRIg is a promoter of phagocytosis, and as it binds to the complement component C3b which is a precursor to iC3b, it is able to act faster than the other complement receptors, CR3 and CR4 (1, 12). Thus, for neutrophils, the ability to upregulate a receptor with such efficient phagocytic capability would be highly beneficial. Particularly, in cases of low-grade infection where a fully-fledged immune response is not required, CRIg may be upregulated on the surface of these cells, where it then promotes pathogen clearance without inducing the 'cytokine storm' which is characteristic of engagement through CR3 (36). Clearance of low levels of opsonised particles from the blood by Kupffer cells typically does not induce an

inflammatory response (37), and as neutrophils are known to assist Kupffer cells in the removal of extracellularly captured particles from the blood (32), this could be a possible function for CRIg expressed by neutrophils. Alternatively, CRIg may function as a cell-activating molecule. We observed that engaging neutrophils through CRIg induced an oxidative burst response, indicating that CRIg is able to induce inflammatory signalling in neutrophils. Other studies have shown that in macrophages, CRIg engagement leads to secretion of IL-8 and matrix metalloproteinase 9.

Aside from novelty, the finding that circulating phagocytes express CRIg has broad ramifications in terms of the potential use of CRIg as a therapeutic agent or target. Similar to other B7 family ligands, CRIg expression levels have been shown to be elevated in multiple types of cancers (12, 14, 38). As a result, it has been suggested that CRIg may be a potential target for inhibiting antibodies (similar in action to blocking antibodies against the checkpoint molecules PD-1 and PD-L1). However, if CRIg is naturally expressed in the blood as well as the tissues and is an important player in neutrophil-mediated clearance of opsonised pathogens, then blocking CRIg may severely immunocompromise patients. Additionally, the role that CRIg plays in the function of monocytes remains to be studied. Therefore, the properties of CRIg as identified here will be vitally important properties to consider in future studies into the therapeutic efficacy of CRIg-targeting agents.

In summary, the study presented here establishes that neutrophils express functional CRIg on the cell surface when activated by exogenous and endogenous inflammatory mediators. The work also elaborates on the mechanisms involved in this expression with particular emphasis on PKC, p38 MAP kinase, rac2, and Rab27a as well as ARPC1B, suggesting the importance of the cytoskeleton and exocytosis, most likely from gelatinase granules (Fig. 6). This together with the identification of expression on monocytes, places an important perspective on the mechanisms of the inflammatory reaction in infection and

immunity. Thus, it is evident that tissue infiltrating blood phagocytes are armed with CRIg to participate in phagocytosis in bacterial and viral infection. In the unstimulated state, CRIg is abundantly present on the monocyte surface, but little or no expression is present on neutrophils (Fig. 7). Therefore, the monocyte may be the first line of anti-microbial immunity that involves this receptor. Following infection, inflammatory mediators are generated leading to the loss of CRIg expression on monocytes but an increase in expression on the neutrophil surface, enabling this cell to efficiently participate in CRIg mediated phagocytosis of bacteria. Both monocytes and neutrophils will migrate to the infection foci in tissues but by this stage of the inflammatory reaction the neutrophil will arrive at the inflammatory site armed with CRIg. It is also known that the inflammatory environment down regulates the CRIg expressed by tissue fixed macrophages. Hence, CRIg expression on neutrophils under the influence of inflammatory mediators makes this phagocyte the dominant phagocytosis promoting cell at the infection site.

## Methods

### Ethical statement

The procurement of human blood and all experimental procedures were approved by the Human Research Ethics Committee of the Women's and Children's Health Network (WCHN), Adelaide, South Australia, and the Southern Adelaide Clinical Human Research Ethics Committee in accordance to The National Statement on Ethical Conduct in Human Research (2007, updated 2018; National Health and Medical Research Council Act 1992). Venous blood was collected from healthy adult volunteers by venipuncture with their informed consent. Results from studies on ARCIB deficient neutrophils were obtained as part of the clinical laboratory assessment of the patient and informed consent was obtain from the parents to publish the results.

All murine experimental procedures were approved by the WCHN Animal Ethics Committee and work conducted in accordance to the *Australian code for the care and use of animals for scientific purposes.*

### Reagents

RPMI 1640 tissue culture medium, foetal calf serum (FCS) and L-glutamine were purchased from SAFC Biosciences. Recombinant human tumour necrosis factor (TNF) was purchased from ProSpec-Tany Technogene. Phorbol myristate acetate (PMA), lipopolysaccharide (LPS) from *Escherichia coli* O127:B8, N-Formyl-Met-Leu-Phe (fMLF), wortmannin, cytochalasin B, BAPTA-AM (calcium chelator), and Nexinhib20 (Rab27 inhibitor) were purchased from Sigma-Aldrich. LTB4 was purchased from Cayman Chemical. SB202190 and SB203580 were purchased from SelleckChem. The PKC inhibitor GF109203X was purchased from Biomol Research Laboratories. Rac-1 inhibitor, NSC 23766, and the Rac-1/Rac-2 inhibitor, EHT 1864, were purchased from TOCRIS.

**Antibodies**

FITC-conjugated goat anti-rat antibody and, unlabelled and PE-conjugated mouse anti-human CRIg monoclonal antibody (clone 6H8, 1:200 for Western blotting), were purchased from Santa Cruz Biotechnology. Fluorochrome-conjugated antibodies to CD45 (APC-H7; clone 2D1), CD3 (PE-Cy5; clone UCHT1), CD20 (APC; clone L27), CD14 (FITC; clone M5E2), CD11b (PE; clone D12), CD11c (FITC, clone B-ly6), and mouse IgG1-κ isotype control (PE; clone MOPC-21), were purchased from BD Biosciences. The rabbit anti-ARPC1B antibody (HPA004832, 1:1000) and mouse monoclonal anti-GAPDH (clone 71.1, 1:20,000) were obtained from Sigma-Aldrich. The polyclonal HRP-conjugated rabbit anti-mouse (P0260), anti-goat (P0449), anti-rat (P0450), and goat anti-rabbit (P0448) immunoglobulin antibodies (1:2000) were obtained from Dako. Custom made rat monoclonal antibody raised to the variable domain of human CRIg (clone 14B11, 1:25 for Western blotting), was produced through a contract with GenScript (for further information regarding antibody generation, see supplementary manuscript).

**Purification of leukocytes**

Neutrophils and peripheral blood mononuclear cells (PBMC) were isolated from the peripheral blood of healthy human donors using Hypaque-Ficoll as previously described (39. 40). Briefly, blood was layered onto Hypaque-Ficoll, d = 1.114 and centrifuged at 600 × *g* for 35 minutes with no brake. After centrifugation, the leukocytes resolved into two discrete bands: an upper PBMC-containing band and the neutrophil-containing band below. Neutrophils were gently aspirated from the lower band, and PBMCs from the upper band, and both fractions were washed with complete media (600 × *g*, 5 minutes). Cells were then counted using a haemocytometer and viability judged by their ability to exclude trypan blue. Cell preparations were routinely >99% viable and >98% pure. For cryopreservation studies, freshly isolated PBMCs were cryopreserved in freezing media containing 90% heat-inactivated FCS and 10%

DMSO. Cells were incubated in a 'Mr. Frosty' Freezing Container (Thermo-Fisher Scientific) overnight in a -80 °C freezer, before being transferred into liquid nitrogen for storage. Prior to use, cryopreserved PBMCs were removed from storage and thawed rapidly at 37 °C before washing in RPMI-1640. Cells were counted and viability assessed by the trypan blue-exclusion method prior to application.

**Reverse transcription PCR assays**

In brief, total RNA was extracted from monocyte-derived macrophages at day three of culture or immediately after separation from monocytes and neutrophils using TRIzol reagent (Invitrogen). cDNA was prepared using 300 ng RNA using iScript™ cDNA synthesis kit (Bio-Rad). RT-PCR analysis was performed using AmpliTaq Gold® 360 Master Mix (Applied Biosystems) with the following conditions: initial denaturation for 5 min at 95°C followed by 40 cycles at 95°C for 30 s, 60°C for 30 s and 72°C for 30 s using a Bio-Rad MyCycler. The primer pairs used were for human CRIg (Forward: 5'-ACACTTATGGCCGTCCCAT-3'; Reverse: 5'-TGTACCAGCCACTTCACCAA-3') and GAPDH (8). The PCR products were visualized by 2% agarose gel electrophoresis alongside 1 kb Plus DNA ladder (Invitrogen).

**Sanger sequencing**

Genomic DNA was isolated from heparinised blood using the Flexigene DNA kit (Qiagen). *ARPC1B* gene exon 2 and the flanking intronic regions were amplified using AmpliTaq Gold® 360 Master Mix (Applied Biosystems) with 0.1 µM of each M13-tagged primer (Forward: GCTGCCCCTCTAAACTGAGG; Reverse: AACTTTAACCCAGGAGGCCC), and 25–50 ng DNA in a 25 µL final PCR volume. Thermal cycling conditions were an initial denaturation of 95 °C for 10 min, followed by 35 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 60 s; with a final extension at 72 °C for 7 min. The PCR products were purified using Illustra ExoProStar1-Step (GE HealthCare) and sequenced using BigDye Terminator v3.1 on an ABI

3730 DNA Analyzer (Applied Biosystems). Mutation detection was performed by alignment with reference sequence LRG_1188 using Mutation Surveyor v4.0.11 (SoftGenetics).

**Western Blot**

Cell pellets were resuspended in 100 µL of lysis buffer containing 20 mM HEPES, pH 7.4, 0.5% Nonidet P-40 (v/v), 100 mM NaCl, 1 mM EDTA, 2 mM $Na_3VO_4$, 2 mM dithiothreitol, 1 mM PMSF and 1 µg/mL of each protease inhibitor (benzamidine, leupeptin, pepstatin A, and phenylmethylsulfonyl fluoride (PMSF), purchased from Sigma-Aldrich, and aprotinin purchased from Calbiochem. Total protein in the soluble fractions were quantitated using the Qubit™ Protein Assay Kit on a Qubit 3.0 (Invitrogen), prior to the addition of Laemmli buffer. Samples were boiled at 100°C for 5 min and 60 µg of each were subjected to 10% SDS-PAGE at 170 V for approximately 1 hour, using the Mini-PROTEAN 3 system (Bio-Rad), and then transferred to nitrocellulose membranes using the Trans-Blot® Turbo™ Transfer System (Bio-Rad). Protein transfer was examined by 0.1% Ponceau S staining. After blocking in TBST with 5% skim milk (blocking solution) for 1 h, the membrane was incubated primary antibodies in blocking solution for 1 h at room temperature or overnight at 4 °C. The membrane was washed 3 x 5 min in blocking solution and then incubated with appropriate secondary HRP-conjugated antibodies in blocking solution for 1 h at room temperature. Immunoreactive material was detected using the Western Lightning Plus-ECL Enhanced Chemiluminescence Substrate (PerkinElmer), with protein bands visualized on a ChemiDoc™ XRS+ Imager and quantitated using Image Lab™ Software, Version 3.0 (Bio-Rad).

**Flow cytometry**

Cell surface expression of CRIg, CD45, CD11b, CD11c and CD14 were analysed by flow cytometry as previously described (7, 15). Briefly, $2.5 \times 10^5$ cells were incubated in 12 x 75 mm round bottom tubes on ice with 100 µg purified human IgG (Kiovig, Baxter) for 15 min.

This was followed by addition of the appropriate fluorochrome-conjugated anti-human primary antibodies, with further incubation for 20 min. Cells were washed with 2 mL PBS with 5% heat-inactivated FCS with centrifugation at 500 × *g* for 5 min. The cells were acquired (50,000 event minimum) on a BD FACSCanto I with data analysed using FlowJo 10.1 (FlowJo, LLC).

**Chemiluminescence assay**

Luminol-dependent chemiluminescence assay was performed as previously described (39). Briefly, 1 x $10^6$ neutrophils were added to 125 µg lucigenin (bis-N-methylacridinium nitrate, Sigma-Aldrich) in 500 µL HBSS. Chemiluminescence was measured using a LB 953 Autolumat Plus luminometer (Berthold Technologies), and peak fluorescence recorded. Data is expressed at mean relative luminescence units (RLU).

**Statistical Analysis**

GraphPad Prism 8.0 (GraphPad Software) was used for statistical analysis. Mean differences were compared using t-tests (for comparisons of two groups) or one-way ANOVA followed by multiple-comparison tests (for comparisons of three of more groups). $P$ values $< 0.05$ were considered to be statistically significant.

**References**

1 Helmy, K.Y., Katschke Jr, K.J., Gorgani, N.N., Kljavin, N.M., Elliott, J.M., Diehl, L., Scales, S.J., Ghilardi, N., van Lookeren Campagne, M., CRIg: A Macrophage Complement Receptor Required for Phagocytosis of Circulating Pathogens. *Cell*, 2006. **124**(5): p. 915-927.

2 Small, A.G. Al-Baghdadi, M., Quach, A., Hii, S., Ferrante, A., Complement receptor immunoglobulin: a control point in infection and immunity, inflammation and cancer. *Swiss Med Wkly*, 2016. **146**: p. w14301.

3 Broadley, S.P., Plaumann, A., Coletti, R., Lehmann, C., Wanisch, A., Seidlmeier, A., Esser, K., Luo, S., Rämer, P.C., Massberg, S., Busch, D.H., van Lookeren Campagne, M., Verschoor, A., Dual-Track Clearance of Circulating Bacteria Balances Rapid Restoration of Blood Sterility with Induction of Adaptive Immunity. *Cell Host Microbe*, 2016. **20**(1): p. 36-48.

4 Zeng, Z., Surewaard, B.G.J., Wong, C.H.Y., Geoghegan, J.A., Jenne, C.N., Kubes, P., CRIg Functions as a Macrophage Pattern Recognition Receptor to Directly Bind and Capture Blood-Borne Gram-Positive Bacteria. *Cell Host Microbe*, 2016. **20**(1): p. 99-106.

5 van Lookeren Campagne, M. and L.D. Orozco, Chapter 29 - CRIg, in *The Complement FactsBook (Second Edition)*, S. Barnum and T. Schein, Editors. 2018, Academic Press. p. 309-316.

6 Kim, J.K., Choi, E.M., Shin, H.I., Kim, C.H., Hwang, S.H., Kim, S.M., Kwon, B.S., Characterization of monoclonal antibody specific to the Z39Ig protein, a member of immunoglobulin superfamily. *Immunol Lett*, 2005. **99**(2): p. 153-61.

7 Munawara, U., Perveen, K., Small, A.G., Putty, T., Quach, A., Gorgani, N. N., Hii, C.S., Abbott, C.A., Ferrante, A., Human Dendritic Cells Express the Complement

Receptor Immunoglobulin Which Regulates T Cell Responses. *Front Immunol*, 2019. **10**, doi:10.3389/fimmu.2019.02892 (2019).

8 Munawara, U., Small, A.G., Quach, A., Gorgani, N.N., Abbott, C.A., Ferrante, A., Cytokines regulate complement receptor immunoglobulin expression and phagocytosis of Candida albicans in human macrophages: A control point in anti-microbial immunity. *Scientific Reports*, 2017. **7**(1): p. 4050.

9 Ferrante, A., Martin, A.J., Bates, E.J., Goh, D.H., Harvey, D.P., Parsons, S., Rathjen, D.A., Guss, G., Dayer, J.M., Killing of Staphylococcus aureus by tumor necrosis factor-alpha-activated neutrophils. The role of serum opsonins, integrin receptors, respiratory burst, and degranulation. *J Immunol*, 1993. **151**(9): p. 4821-8.

10 Uhlén, M., Fagerberg L., Hallström, B.M., Lindskog, C., Oksvold, P., Mardinoglu, A, Sivertsson, Å., Kampf, C., Sjöstedt, E., Asplund, A., Olsson, I., Edlund, K., Lundberg, E., Navani, S., Szigyarto, C.A.K., Odeberg, J., Djureinovic, D., Takanen, J.O., Hober, S., Alm, T., Edqvist, P.H., Berling, H., Tegel, H., Mulder, J., Rockberg, J., Nilsson, P., Schwenk, J.M., Hamsten, M., von Feilitzen, K., Forsberg, M., Persson, L., Johansson, F., Zwahlen, M., von Heijne, G., Nielsen J., and Pontén, F., Proteomics. Tissue-based map of the human proteome. *Science*. 2015. 347, 1260419, doi:10.1126/science.1260419.

11 Kumaratilake, L.M., Rathjen, D.A., Mack, P., Widmer, F., Prasertsiriroj, Ferrante, A., A synthetic tumor necrosis factor-alpha agonist peptide enhances human polymorphonuclear leukocyte-mediated killing of Plasmodium falciparum in vitro and suppresses Plasmodium chabaudi infection in mice. *Journal of Clinical Investigation*, 1995. **95**(5): p. 2315-2323.

12 Gorgani, N.N., He, J.Q., Katschke Jr., K.J., Helmy, K.J., Xi, H., Steffek, M., Hass, P.E., van Lookeren Campagne, M., Complement receptor of the Ig superfamily enhances

complement-mediated phagocytosis in a subpopulation of tissue resident macrophages. *Journal of immunology*, 2008. **181**, 7902-7908.

13 Boneh, A., S. Mandla, and H.S. Tenenhouse, Phorbol myristate acetate activates protein kinase C, stimulates the phosphorylation of endogenous proteins and inhibits phosphate transport in mouse renal tubules. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, 1989. **1012**(3): p. 308-316.

14 Gorgani, N.N., Thathaisong, U., Mukaro, V.R.S., Poungpair, O., Tirimacco, A., Hii, C.S.T., Ferrante, A., Regulation of CRIg expression and phagocytosis in human macrophages by arachidonate, dexamethasone, and cytokines. *Am J Pathol*, 2011. **179**(3): p. 1310-8.

15 Ma, Y., Usuwanthim, K., Munawara, U., Quach, A., Gorgani, N.N., Abbott, C.A., Hii, C.S., Ferrante, A., Protein Kinase Cα Regulates the Expression of Complement Receptor Ig in Human Monocyte–Derived Macrophages. *The Journal of Immunology*, 2015. **194**(6): p. 2855.

16 Berkow, R.L., Dodson, M.R. Biochemical mechanisms involved in the priming of neutrophils by tumor necrosis factor. *J Leukoc Biol*. 1988. **44**(5):345-52.

17 Laudanna, C., Miron, S., Berton, G., Rossi, F. Tumor necrosis factor-alpha/cachectin activates the O2(-)-generating system of human neutrophils independently of the hydrolysis of phosphoinositides and the release of arachidonic acid. *Biochem Biophys Res Commun*. 1990. **166**(1):308-15.

18 Mukaro, V. R., Quach, A., Gahan, M.E., Boog, B., Huang, Z.H., Gao, X., Haddad, C., Mahalingam, S., Hii, C.S., Ferrante, A., Small tumor necrosis factor receptor biologics inhibit the tumor necrosis factor-p38 signalling axis and inflammation. *Nat Commun*, 2018. **9**, 1365, doi:10.1038/s41467-018-03640-y.

19 Hann, J., Bueb, J.L., Tolle, F., Bréchard, S. Calcium signaling and regulation of neutrophil functions: Still a long way to go. *J Leukoc Biol*. 2020. **107**(2):285-297.

20 Sheshachalam, A., Srivastava, N., Mitchell, T., Lacy, P., Eitzen, G., Granule Protein Processing and Regulated Secretion in Neutrophils. *Frontiers in Immunology*, 2014. **5**(448).

21 Niessen, H.W.M., Kuijpers, T.W., Roos, D., Verhoeven, A.J., Release of azurophilic granule contents in fMLP-stimulated neutrophils requires two activation signals, one of which is a rise in cytosolic free Ca2+. *Cellular Signalling*, 1991. **3**(6): p. 625-633.

22 Almkvist, J., Fäldt, J., Dahlgren, C., Leffler, H., Karlsson, A., Lipopolysaccharide-Induced Gelatinase Granule Mobilization Primes Neutrophils for Activation by Galectin-3 and Formylmethionyl-Leu-Phe. *Infection and Immunity*, 2001. **69**(2): p. 832.

23 Potera, R.M., Jensen, M.J., Hilkin, B.H., South, G.K., Hook, J.S., Gross, G.A., Moreland, J.G., Neutrophil azurophilic granule exocytosis is primed by TNF-α and partially regulated by NADPH oxidase. *Innate Immun,* 2016. **22**, 635-646

24 Ambruso, D.R., Knall, C., Abell, A.N., Panepinto, J., Kurkchubasche, A., Thurman, G., Gonzalez-Aller, C., Hiester, A., deBoer, M., Harbeck, R.J., Oyer, R., Johnson, G.L., Roos, D., Human neutrophil immunodeficiency syndrome is associated with an inhibitory Rac2 mutation. *Proc Natl Acad Sci U S A*. 2000. **97**(9):4654-9.

25 Williams, D.A., Tao, W., Yang, F., Kim, C., Gu, Y., Mansfield, P., Levine, J.E., Petryniak, B., Derrow, C.W., Harris, C., Jia, B., Zheng, Y., Ambruso, D.R., Lowe, J.B., Atkinson, S.J., Dinauer, M.C., Boxer, L., Dominant negative mutation of the hematopoietic-specific Rho GTPase, Rac2, is associated with a human phagocyte immunodeficiency. Blood. 2000. 96(5):1646-1654.

26 Bokoch, G. M. Regulation of innate immunity by Rho GTPases. *Trends Cell Biol*. 2005. **15** (3), 163-171.

27 Heyworth, P.G., Bohl, B.P., Bokoch, G.M., Curnutte, J.T. Rac translocates independently of the neutrophil NADPH oxidase components p47phox and p67phox. Evidence for its interaction with flavocytochrome b558. *J Biol Chem*. 1994. **269**(49):30749-30752.

28 Shutes, A., Onesto, C., Picard, V., Leblond, B., Schweighoffer, F., Der. C.J. Specificity and mechanism of action of EHT 1864, a novel small molecule inhibitor of Rac family small GTPases. *J Biol Chem*. 2007. **282**(49):35666-35678.

29 Dütting, S., Heidenreich, J., Cherpokova, D., Amin, E., Zhang, S.C., Ahmadian, M.R., Brakebusch, C., Nieswandt, B. Critical off-target effects of the widely used Rac1 inhibitors NSC23766 and EHT1864 in mouse platelets. *J Thromb Haemost*. 2015. **13**(5):827-838.

30 Quinn, P., Pearson, C., Cheung, A., King, J., Chan, D., Marchand, B., Rudaks, D., Quach, A., Ferrante, A., Gold, M., *P84:* Combined Immunodeficiency Due to Deficiency of Actinrelated Protein Complex 1b (ARPC1B). *Internal Medicine Journal*, 2017. **47**(S5): p. 29-30.

31 Kuijpers, T.W., Tool, A.T.J., van der Bijl, I., de Boer, M., van Houdt, M., de Cuyper, I.M., Roos, D., van Alphen, F., van Leeuwen, K., Cambridge, E.L., Arends, M.J., Dougan, G., Clare, S., Ramirez-Solis, R., Pals, S.T., Adams, D.J., Meijer, A.B., van der Berg, T.K., Combined immunodeficiency with severe inflammation and allergy caused by *ARPC1B* deficiency. *J. Allergy Clin. Immunol.*, 2017. **140**. p. 273-277.

32 Sun, C. X., Magalhaes, M. A. O., and Glogauer, M. Rac1 and Rac2 differentially regulate actin free barbed end formation downstream of the fMLP receptor. *J. Cell Biol.* 2007. **179**(2): 239–245.

33 Kahr, W.H.A., Pluthero, F.G., Elkadri, A., Warner, N., Drobac, M., Chen, C.H., Lo, R.W., Li, L., Li, R., Li, Q., Thoeni, C., Pan, J., Leung, G., Lara-Corrales, I., Murchie,

R., Cutz, E., Laxer, R.M., Upton, J., Roifman, C.M., Yeung, R.S.M., Brumell, J.H., Muise, A.M., Loss of the Arp2/3 complex component ARPC1B causes platelet abnormalities and predisposes to inflammatory disease. *Nat. Commun.* 2017. **8**: p. 14816.

34 Catz SD. Regulation of vesicular trafficking and leukocyte function by Rab27 GTPases and their effectors. *J Leukoc Biol*. 2013. **94**(4): p. 613–622.

35 Herrero-Turrión, M. J., Calafat, J., Janssen, H., Fukuda, M. & Mollinedo, F. Rab27a Regulates Exocytosis of Tertiary and Specific Granules in Human Neutrophils. *The Journal of Immunology*, 2008. **181**, 3793.

36 Wolf, D., Anto-Michel, N., Blankenbach, H., Wiedemann, A., Buscher, K., Hohmann, J. D., Lim, B., Bäuml, M., Marki, A., Mauler, M., Duerschmied, D., Fan, Z., Winkels, H., Sidler, D., Diehl, P., Zajonc, D.M., Hilgendorf, I., Stachon, P., Marchini, T., Willecke, F., Schell, M., Sommer, B., von zur Muhlen, C., Reinöhl, J., Gerhardt, T., Plow, E.F., Yakubenko, V., Libby, P., Bode, C., Ley, K., Peter, K., Zirlik, A., A ligand-specific blockade of the integrin Mac-1 selectively targets pathologic inflammation while maintaining protective host-defense. *Nature Communications,* 2018, **9** (1), 525-525.

37 Crawford, J. M., Bioulac-Sage, P., & Hytiroglou, P. 1 - Structure, Function, and Responses to Injury. In A. D. Burt, L. D. Ferrell, & S. G. Hübscher (Eds.), *Macsween's Pathology of the Liver (Seventh Edition)* (pp. 1-87), 2018. Elsevier.

38 Liao, Y., Guo, S., Chen, Y., Cao, D., Xu, H., Yang, C., Fei, L., Ni, B., Ruan, Z., VSIG4 expression on macrophages facilitates lung cancer development. *Lab Invest,* 2014, **94** (7), 706-715. doi:10.1038/labinvest.2014.73

39 Ferrante, A., Robinson, B.S., Singh, H., Jersmann, H.P.A., Ferrante, J.V., Huang, Z.H., Trout, N.A., Pitt, M.J., Rathjen, D.A., Easton, C.J., Poulos, A., Prager, R.H., Lee, F.S.,

Hii, C.S.T., A novel beta-oxa polyunsaturated fatty acid downregulates the activation of the IkappaB kinase/nuclear factor kappaB pathway, inhibits expression of endothelial cell adhesion molecules, and depresses inflammation. *Circ Res*, 2006. **99**(1): p. 34-41.

40 Kumaratilake, L.M., Ferrante, A., Jaeger, T., Rzepczyk, C.M., Effects of cytokines, complement, and antibody on the neutrophil respiratory burst and phagocytic response to Plasmodium falciparum merozoites. *Infection and Immunity*, 1992. **60**(9): p. 3731-3738.

41 Benard V, Bohl BP, Bokoch GM. Characterization of rac and cdc42 activation in chemoattractant-stimulated human neutrophils using a novel assay for active GTPases. J Biol Chem. 1999 May 7;274(19):13198-204. doi: 10.1074/jbc.274.19.13198.

**Data availability**

The data supporting this study are available within the paper and Supplementary Information. Any additional data relating to the study are available from the corresponding author on reasonable request.

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**Author contributions**

AGS and AF were responsible for the initial draft of the MS. AF proposed and supervised the study and together with AGS planned the experiments. In the main the experiments were carried out by AGS with a contribution by TP, KP, NP, AP and MYG. PQ, AQ, TP and AF were responsible for the clinical and laboratory work up of the patient with ARPC1B deficiency. AGS, CSH, AF, KP, MDW, HW, and AASSKD were responsible for initiating, designing/production and characterization of the monoclonal antibodies. All authors contributed to critical reading and writing of the manuscript.

**Competing interests**

The authors declare no competing interests.

**Supplementary materials**

Supplementary document: Supplemental data tables 1-3, and figures 1-6

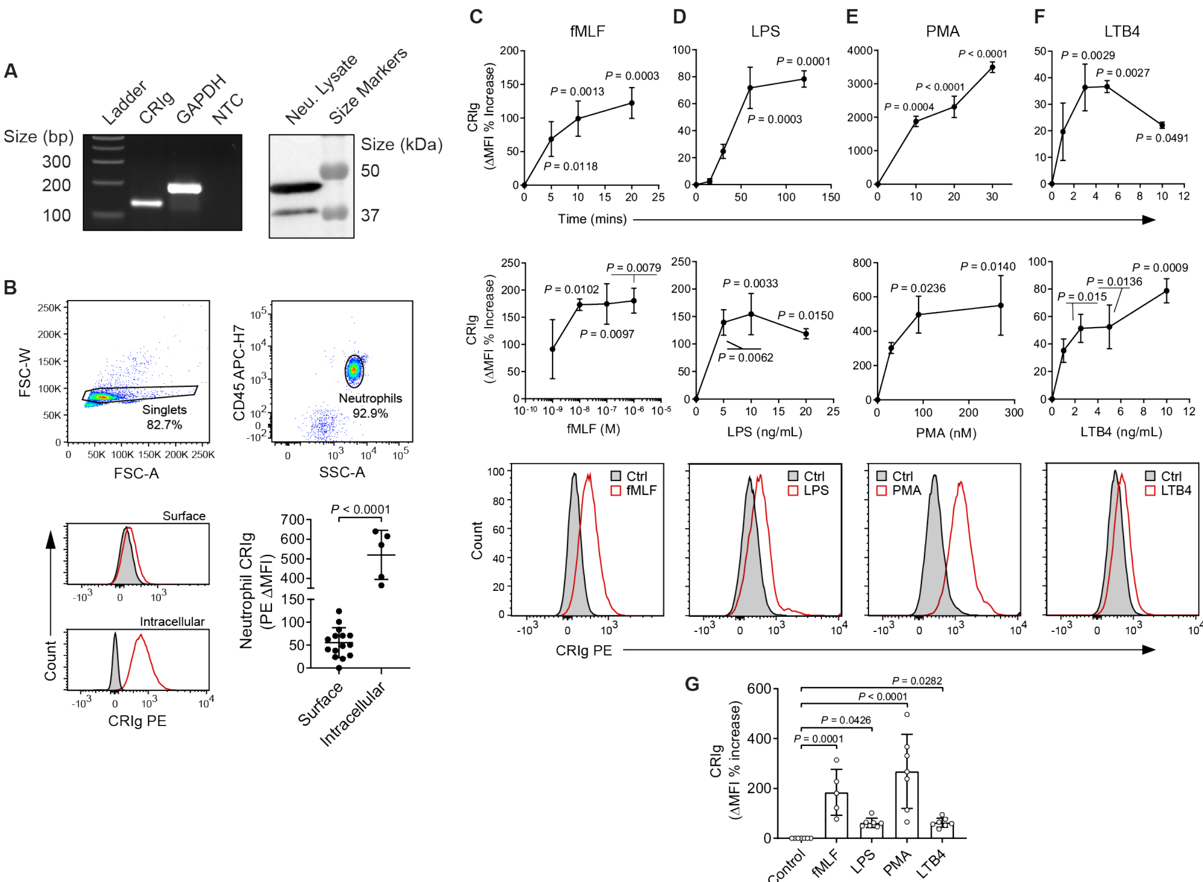

**Fig. 1. Expression of CRIg by human neutrophils.** (**A**) Representative gel of CRIg (*VSIG4*) and *GAPDH* cDNA amplicons from neutrophil RNA (left) and Western blot of neutrophil lysate stained using anti-CRIg clone 14B11 monoclonal antibody (right). NTC, no template control. (**B**) Surface and intracellular CRIg expression in neutrophils, including gating strategy with histogram overlays of PE anti-CRIg clone 6H8 (red line) and isotype control (black line with grey shading) staining. Bottom graph shows MFI of CRIg PE minus isotype control (ΔMFI) for surface ($n$ = 15) and intracellular ($n$ = 5) staining. Measurements were taken from distinct specimens and graphs show mean ± SD. Two-tailed, unpaired $t$-test with Welch's correction compared with surface expression. One-way analysis of variance (ANOVA) with Tukey's post-test compared between all sample types. (**C** to **F**) Graphs of CRIg expression change on the surface of neutrophils over time-courses (upper panels) and concentration ranges (middle panels), are shown along with representative histograms (lower panels) of responses to fMLF, LPS, PMA, and LTB4. Expression was assessed as described above with ΔMFI as a

percentage increase over control treatment. (**G**) Summary of the stimulation of neutrophil CRIg expression by fMLF, LPS, PMA and LTB4 at optimal times (20, 60, 20, and 5 min, respectively) and concentrations ($10^{-6}$ M, 10 µg/ml, 90 nM and 5 ng/ml, respectively). Measurements were taken from distinct specimens and graphs show mean ± SD of at least three distinct experiments. **C** to **F**, One-way ANOVA with Dunnett's post-test compared with expression at the start of the time-course or in the absence of added inflammatory mediator. **G**, Kruskal-Wallis test with uncorrected Dunn's post-test compared with control treatment.

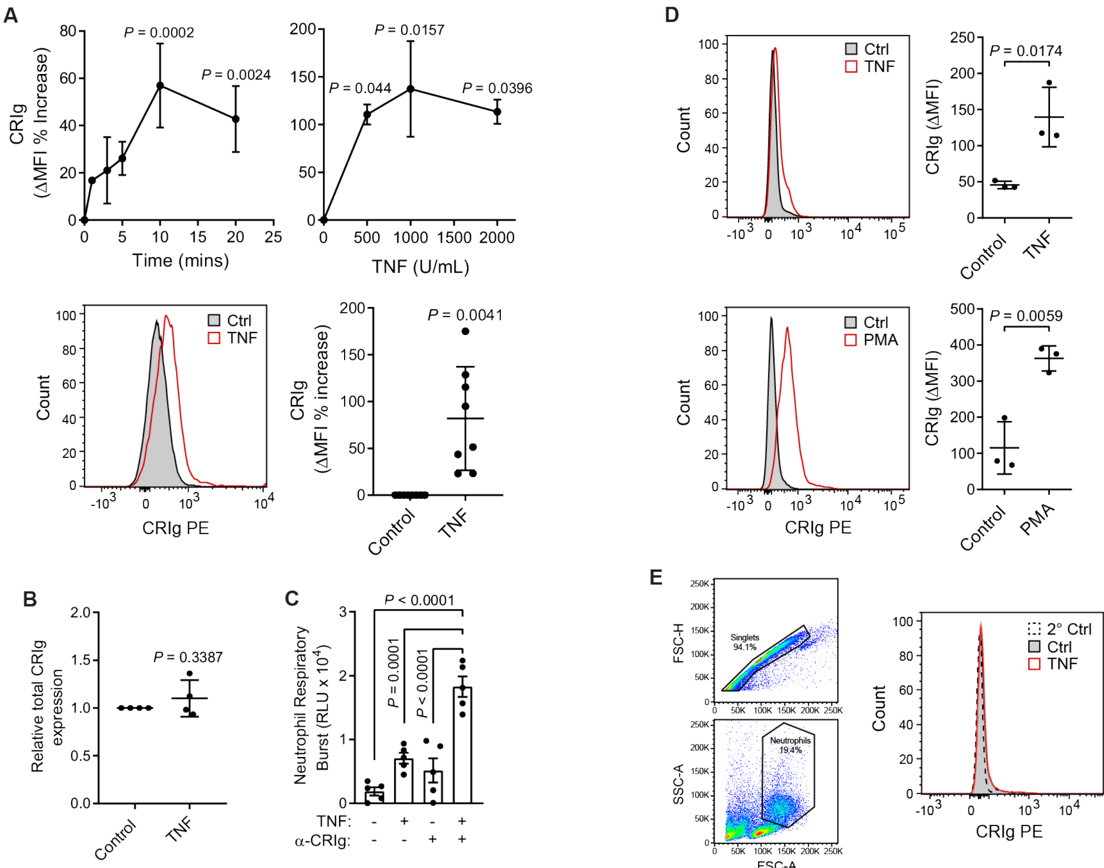


**Fig. 2. TNF induces CRIg expression on neutrophils.** (**A**) Shows CRIg expression changes on the surface of neutrophils over time-courses and TNF concentration ranges, are shown along with representative histograms (lower panels) of responses to TNF. Expression was assessed as described in Fig. 1 with ΔMFI as a percentage increase over control treatment. Summary of the stimulation of neutrophil CRIg expression by TNF at optimal time of 20 min and concentration of $10^3$ U/ml. (**B**) Total CRIg protein expression of unstimulated or TNF stimulated neutrophils as assessed by Western blot, using anti-CRIg clone 14B11, with CRIg protein normalised against GAPDH loading control. (**C**) Neutrophils were treated for 20 min with $10^3$ U/mL TNF with or without challenge with 4 µg/mL anti-CRIg mAb (6H8), and the respiratory burst measured by lucigenin-induced chemiluminescence (RLU, relative luminescence units). (**D**) Neutrophil surface CRIg expression in whole blood treated with $10^4$ U/mL TNF or 90 nM PMA for 20 min. (**E**) The effects of TNF on mouse neutrophil CRIg expression in whole blood assay. Measurements were taken from distinct specimens and graphs

show mean ± SD of at least three distinct experiments. **A**, One-way ANOVA with Dunnett's post-test compared with expression at the start of the time-course or in the absence of added inflammatory mediator. **B, D,** Two-tailed, unpaired *t*-test compared with control expression; **C**, One-way ANOVA with Tukey's post-test compared between all treatments.

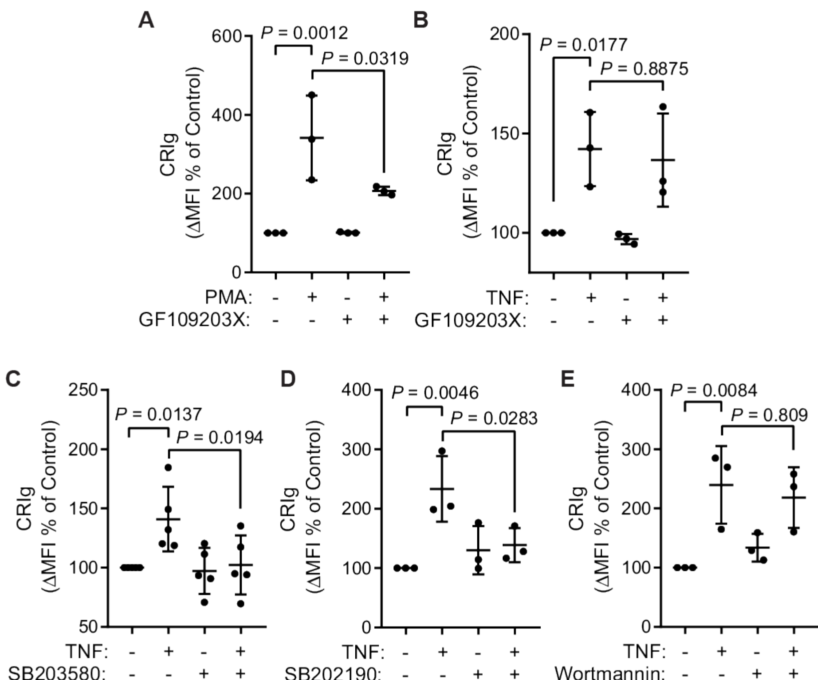


**Fig. 3. The role of PKC and p38 MAP kinase**. (**A**, **B**) Surface CRIg expression induced by PMA or TNF measured on neutrophils pre-treated for 10 min with 500 nM of the PKC inhibitor, GF109203X. (**C, D**) Effects of p38 inhibitors. Surface CRIg induced by TNF on neutrophils pre-treated for 30 min with either 10 μM SB203580 or 20 μM SB202190. (**E**) Effects of PI3 kinase inhibitor, wortmannin. Surface CRIg induced by TNF on neutrophils pre-treated for 10 min with 100 nM wortmannin. Expression was assessed as described in Fig. 1 with either ΔMFI *per se* or as a percentage increase over control treatment. Measurements were taken from distinct specimens and graphs show mean ± SD of at least three distinct experiments, Sidak's post-test compared between TNF/PMA treatment and control or treatment in the presence of inhibitor.

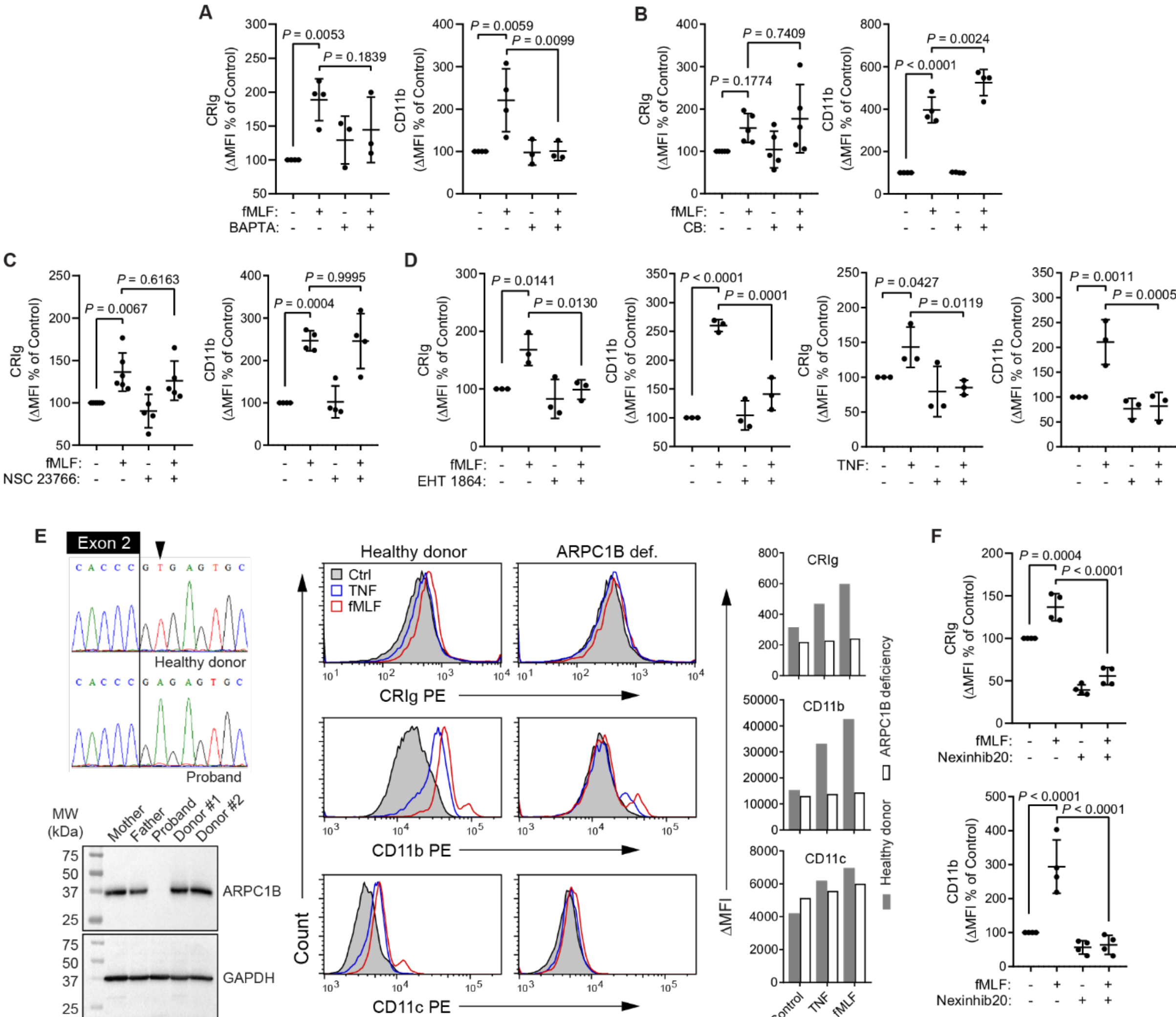


**Fig. 4. Expression of CRIg on the neutrophil surface is dependent on intracellular calcium, Rac, ARPC1b and Rab27a.** (**A** to **D**), Surface CRIg and CD11b expression was measured on neutrophils challenged with $10^{-6}$ M fMLF, following pre-treatment with 25 µM of $Ca^{2+}$ chelator, BAPTA-AM for 30 min, 10 µg/mL of the actin polymerization inhibitor, Cytochalasin B (CB) for 10 min, 50 µM of the Rac-1 inhibitor, NSC 23766 for 1 h, or 100 µM of the Rac-1/Rac-2 inhibitor, EHT 1864, for 30 min. The effect of EHT 1864 was also examined on TNF ($10^3$ U/mL)-induced CRIg and CD11b expression. (**E**) Sequence electropherograms of the end of *ARPC1B* exon 2 and flanking intron in a healthy donor and a patient with a splice-site substitution (LRG_1188t1:c.64+2T>A). The resulting deficiency in ARPC1B is shown in the Western blot showing absence of the protein in neutrophil lysate

compared to parental and other healthy donor neutrophils. Flow cytometric histograms present a lack of up-regulation of cell surface CRIg, CD11b and CD11c in response to TNF is shown in ARPC1B-deficient neutrophils. Graphs show mean ΔMFI of two experiments. (**F**) Surface CRIg and CD11b expression was measured on neutrophils challenged with $10^{-6}$ M fMLF, following pre-treatment with 20 μM of Rab27a inhibitor, Nexinhib20, for 1 h. Expression was assessed as described in Fig. 1 with either ΔMFI itself or as a percentage increase over control treatment. Measurements were taken from distinct specimens and graphs show mean ± SD of at least three distinct experiments. One-way ANOVA with Sidak's post-test compared between fMLF/TNF treatment and control or fMLF/TNF in the presence of inhibitor.

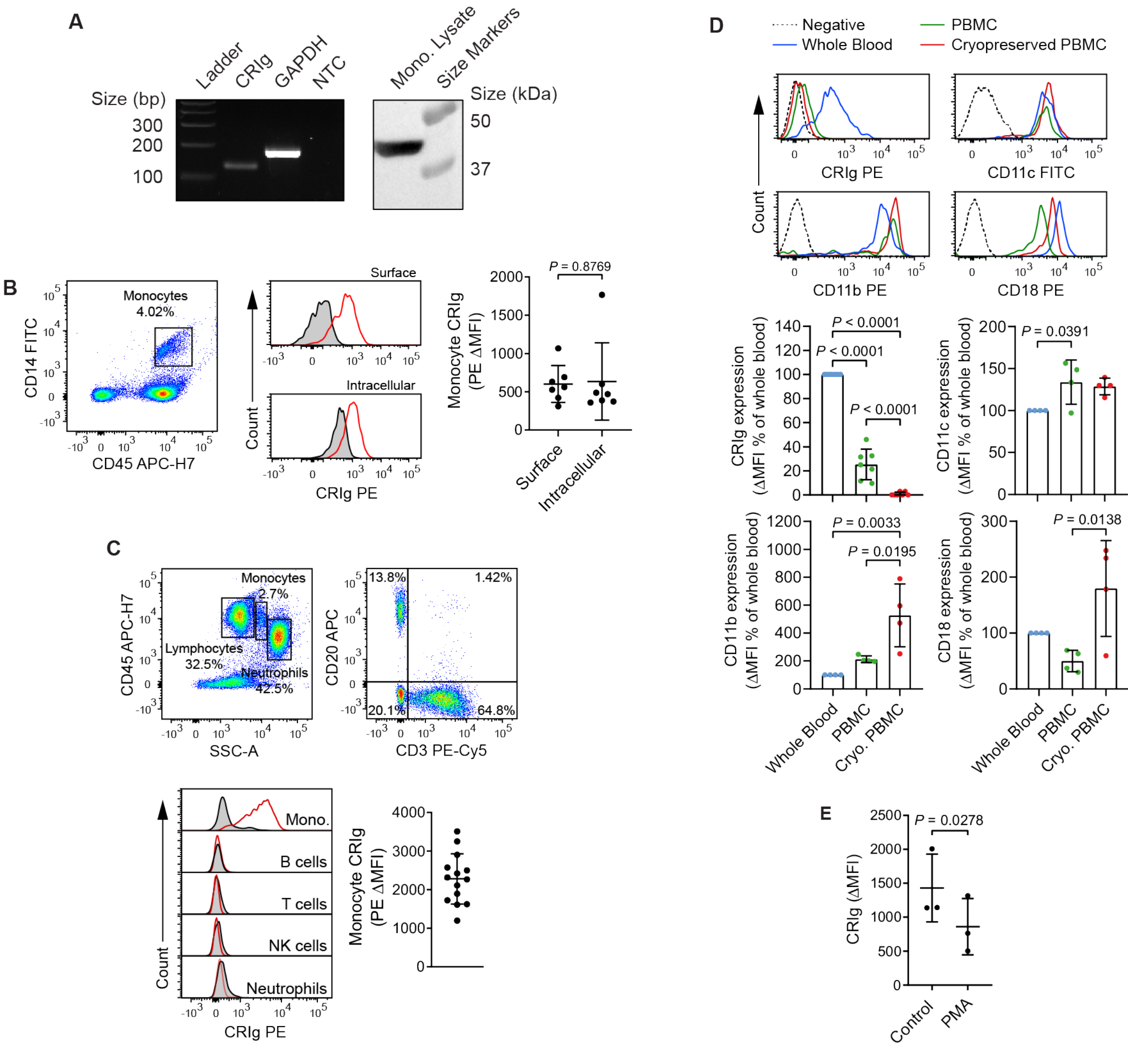

**Fig. 5. Monocytes express CRIg mRNA and protein.** (**A**) Representative gel of CRIg and GAPDH cDNA amplicons from monocyte RNA (left) and Western blot of monocyte lysate stained using anti-CRIg clone 14B11 monoclonal antibody (right). (**B**) Surface and intracellular expression of CRIg in the monocyte fraction of PBMC and; (**C**) CRIg expression on the surface of whole blood leukocyte subpopulations, with gating strategy and histogram overlays of PE anti-CRIg clone 6H8 (red line) and isotype control (black line with grey shading) staining. The values for samples from 14 different individuals. (**D**) Comparison of monocyte surface CRIg ($n$ = 7), CD11b ($n$ = 4), CD11c ($n$ = 4) and CD18 ($n$ = 4) expression in whole blood, against freshly isolated and cryopreserved PBMC from the same specimens, with representative histograms and graphs showing ΔMFI as a percentage of that observed in whole blood. (**E**)

Shows the effects of PMA treatment of whole blood on monocyte CRIg surface expression. Measurements were taken from distinct specimens and graphs show mean ± SD. **B and E**, Two-tailed, unpaired *t*-test with Welch's correction compared with surface expression. **D**, One-way analysis of variance (ANOVA) with Tukey's post-test compared between all sample types.

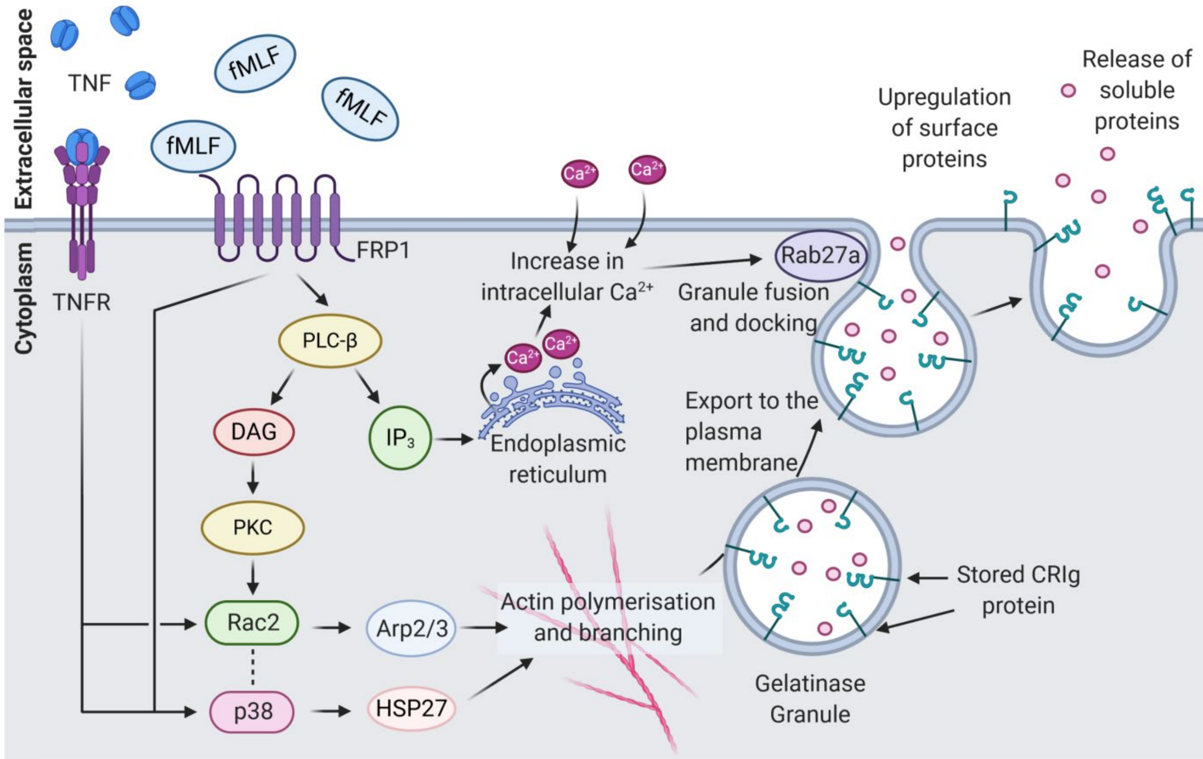


**Fig. 6. *Diagrammatic representation of data in Fig 3 and 4, and Table S3 showing the intracellular events leading to the expression of CRIg on the neutrophil surface following activation.** Inflammatory mediators act via p38, PKC, Rac2/Arp2/3 (41) increases in intracellular calcium and Rab27a to promote exocytosis and release of CRIg to the surface. Whereas Rac/Arp2/3 and PKC are likely to act via actin reorganisation, calcium and rab27a are likely to regulate granule-plasma membrane docking and fusion. As argued, CRIg is most likely stored in the gelatinase granules. *Image created with BioRender.

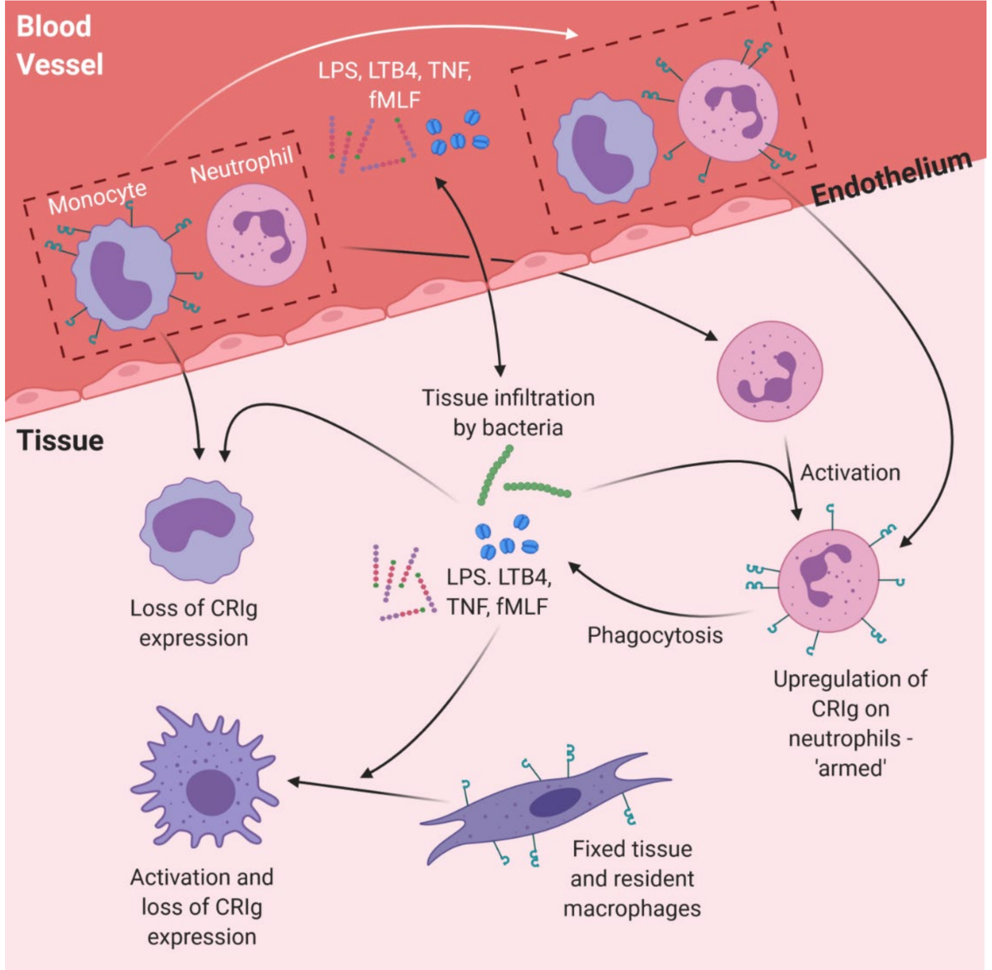


**Fig. 7.** *CRIg is expressed on monocytes and activated neutrophils, altering our understanding of the dynamics of the inflammatory reaction in response to infection. Under physiological conditions, the circulating monocytes show high CRIg expression and neutrophils show very low expression. Thus, infiltration of microbial pathogens to the blood stream are most likely phagocytosed by monocytes, but as exogenous and endogenous inflammatory mediators are generated, the neutrophil will show increased expression, while conversely, expression in monocytes is significantly reduced, leading to the neutrophil being the major phagocytic cell. Neutrophils are rapidly deployed to sites of infection in the tissues by which stage the CRIg on tissue macrophages has been downregulated. The neutrophils continue to express CRIg and deal with residual bacteria. The monocytes under the influence of the inflammatory environment lose their expression. *Image created with BioRender.

# Chapter 8. Discussion

## 8.1. Introductory Remark

The investigation that has been presented in this thesis initially sought out to fully recharacterise the expression of CRIg in human cells, with the overall goal of unravelling the uncertainties present in the field of CRIg biology at the time of commencement. Here, we have provided the preliminary assessment of the functionality of multiple new anti-murine CRIg monoclonal antibodies, and have substantially built upon our existing knowledge of the expression of CRIg in human macrophages and the network of mediators which influence this expression. We have also demonstrated that human circulating monocytes express CRIg mRNA, which is reflected by protein expression on the cell surface. Finally, and possibly most importantly, the data presented in this thesis have shown for the first time, functional CRIg expressed by human MDDC and neutrophils, identifying novel roles for CRIg in the regulation of T cell responses, and in infection and immunity.

## 8.2. Generation of Anti-CRIg Monoclonal Antibodies

Our lab has successfully generated two monoclonal antibodies reactive to murine and human CRIg which are suitable for use in Western blot, immunohistochemistry, and flow cytometry. The generation of our new anti-murine CRIg monoclonal antibody clones has several benefits. Firstly, at the time of the compiling of this thesis, there is a fundamental lack of anti-murine CRIg monoclonal antibodies commercially available; specifically, there are no monoclonal anti-murine CRIg antibodies available that have been validated for use in immunohistochemistry and Western blot. Secondly, there are no commercial monoclonal antibodies that are capable of recognising both murine and human forms of CRIg. The work presented here remedies these issues, and the generated antibodies stand to be the first of their kind.

When designing and screening our antibodies, we were particularly interested in whether our new clones would be suitable for use in functional assays. As such, the ability of our antibodies to block or elicit a signal through CRIg was an important consideration throughout the design process. As shown in chapter 3 figure 2 (Fig. S2), we selected a raising peptide from the extracellular region of CRIg which was central to the V-type domain of the protein. As the V-type domain is the portion of the protein responsible for binding C3b and iC3b (Wiesmann et al., 2006), we expect that while the experiments to confirm this were unable to be performed in the timeframe of the completion of this thesis and are yet to be conducted, that our clones will perform functionally. At the time of the writing of this thesis, there are no anti-murine CRIg monoclonal antibodies commercially available which have been validated for functional usage. Such antibodies would have the potential to be

investigated as therapeutics in complement-driven inflammatory disorders (such as RA), or also as checkpoint inhibitors in cancer, as CRIg has been shown to be upregulated on tumour cells (Liao et al., 2014).

The use of a raising peptide with identical sequence to the V-type region of murine CRIg was also beneficial as this portion is 80% conserved with human, and accordingly, we have demonstrated that our antibody clones were indeed cross-reactive with human CRIg. The clones successfully recognised human CRIg protein by immunohistochemistry, flow cytometry, and Western blot. While the specific isoforms of human CRIg that our generated antibodies recognise and whether using combinations of our antibodies will allow us to detect all of the human protein variants remains to be assessed, our preliminary data presented here show that clone 14B11 detects at least one protein in human monocytes, MDMs, and two forms in human neutrophils. Thus, while our antibodies are suitable for the uses outlined in this thesis, it is evident that further monoclonal antibody development is required for us to be able to detect all CRIg protein variants simultaneously.

### 8.3. CRIg Expression and Role in Phagocytosis in Human MDMs

CRIg expressed by macrophages is a rapid promotor of phagocytosis of both bacterial and fungal pathogens (Helmy et al., 2006) (further discussed in 8.8). We have shown that CRIg expression on human MDMs can be positively modulated by the synthetic anti-inflammatory steroid dexamethasone, and also by the naturally occurring steroid hormone 1,25D. While dexamethasone and 1,25D can engage immune cells via differing mechanisms, these agents both induce an upregulation of CRIg protein on the cell surface which is directly reflected by an increase in cellular phagocytic capability. This increase in phagocytic capability is a hallmark feature of the M2 macrophage phenotype (Yao et al., 2019). Remarkably, we observed that treatment with dexamethasone, 1,25D, and other cytokines which induced a significant change in CRIg expression did not affect the expression of CR3 and CR4, while in the case of 1,25D, we found a decrease in expression of CD11c (CR4) mRNA levels. The 'traditional' complement receptor CR3 has previously been considered to be the dominant receptor responsible for the phagocytosis of opsonised bacteria by monocytes, MDM, and neutrophils, while CR4 plays a role in internalisation of opsonised bacteria by MDM (Lukácsi et al., 2017). However, the presented data in this thesis indicate that inflammatory mediators induce an increase in the ability of MDM to internalise opsonised particles, and they do this through modulating expression of CRIg and not CR3 or CR4. Thus, it is tempting to speculate that CRIg is the crucial molecule on the cell surface of human MDM that is

responsible for bacterial and fungal phagocytosis, and not CR3 or CR4 as previously thought.

### 8.4. CRIg Expression in Human MDDCs

The work presented in chapter 5 demonstrated CRIg expression on human MDDC for the first time, and that cells expressing high levels of CRIg significantly suppress the T cell proliferative response to both PHA and alloantigen stimulation. This suppression was able to be inhibited by the addition of the anti-CRIg monoclonal antibody clone 6H8 (Santa Cruz) to the reactions. However, an interesting aspect of the study was that surface expression of CRIg did not necessarily correlate with total protein levels when assessed by Western blot. For instance, when DC samples were stimulated with IL-10, we observed a significant increase of CRIg total protein, while no modulation was observed when assessing via flow cytometry. Similarly, we observed significant decreases in total protein when the MDDC were stimulated with IL-4 and IL-13 which were not reflected by surface expression. However, when taken together with the data presented in chapter 3 which clearly demonstrate that the monoclonal anti-CRIg clones 3C9 and 6H8 detect differing proteins to each other, the discrepancy may be explained as 3C9 was used for all Western blot work, while 6H8 was used for flow cytometry. As our quantitative PCRs used primers that would detect total CRIg expression levels (i.e., all six transcript variants), it is plausible that while the CRIg variants detected by 3C9 are being significantly modulated, the variants detected by 6H8 are not. Thus, it would be interesting to assess levels of CRIg on the surface of DC with either our generated anti-CRIg monoclonal antibodies, or a combination cocktail of both 3C9 and 6H8 to see if these better correlate with our Western blot findings. Additionally, using our CRIg variant-specific primers, it may also be beneficial to quantitatively assess which specific isoforms are being modulated at the mRNA level by each cytokine. Experiments such as these would enable us to relate the immunosuppressive function of CRIg-expressing cells with the specific CRIg variants and may potentially reveal functional differences between the proteins.

We found that IL-10, GM-CSF, and TGF-β1 induce upregulated levels of CRIg protein in MDDC. This is important, as these cytokines are known to induce a 'tolerogenic' phenotype in DC, and the fact that we can suppress this tolerogenic phenotype using a blocking anti-CRIg antibody suggests that CRIg may be the molecule responsible for this alteration in phenotype. Interestingly, while TGF-β1 induced CRIg upregulation in MDDC, we observed a significant decrease in MDM expression of CRIg at the same concentration of cytokine. Conversely, lymphotoxin-α (LT-α) induced a significant increase in MDM CRIg expression,

while it decreased expression in MDDC. This indicates that while IL-10 and GM-CSF positively regulate CRIg in both MDM and MDDC, TGF-β1 and LT-α have differential effects, indicating that there may be differing signalling pathways being activated by the cytokines across the two cell types, further supporting the notion that CRIg plays differing functional roles between the cell types.

### 8.5. CRIg Expression in Human Neutrophils

Unexpectedly, we found that human neutrophils and monocytes from both whole blood and isolated preparations express CRIg mRNA and protein. While CRIg can be readily detected on the cell surface of monocytes in whole blood and in isolated PBMC fractions, we unexpectedly found that CRIg is only able to be detected on the neutrophil cell surface following stimulation with inflammatory mediators. A major question which arises from these findings is; *how has CRIg expression by these circulating phagocytes been missed until now?* The answer that can be concluded by the presented results has two aspects. Firstly, as shown in chapter 3, figure 1 (Fig. S1), we observed that the commonly used anti-human CRIg monoclonal antibody clones 6H8 and 3C9 detect different proteins to each other when used to examine the same macrophage protein sample. As demonstrated in chapter 4, human MDM which have been stimulated with dexamethasone during culture express at least five transcript variants of CRIg. Thus, this difference in antibody reactivity can be explained by the existence of multiple protein variants being expressed by MDMs which are differentially detected by the two monoclonal antibodies. While studies to confirm which specific CRIg variants are being expressed by monocytes and neutrophils were not conducted in the work presented here, it is plausible that these cells are expressing different protein variants compared with macrophages. Indeed, by Western blot, we detect protein migrating at ~50 kDa in MDM and DC total cell lysates, while the largest protein observed in neutrophils and monocytes migrates around 45 kDa, suggesting that the larger protein expressed by MDM and DC is not expressed in circulating phagocytes. This hypothesis—that there is an isoform of CRIg that is exclusively expressed by MDM and DC—potentially explains why for the last two decades, CRIg has been known to be exclusively expressed by macrophages. Secondly, CRIg expression by neutrophils may have been missed as in our study, we see that surface expression of CRIg is not significantly detectable compared with isotype control fluorescence values unless the neutrophils have been stimulated with inflammatory mediators prior to assessment. This is supported by the fact that the initial study which assessed CRIg expression by cells in the circulation and reported them negative was conducted using flow cytometry to assess unstimulated, healthy control blood samples (Helmy et al., 2006). Similarly with monocytes, we demonstrated that CRIg is lost from the

cell surface upon cell isolation and cryopreservation, and this loss could explain why expression on monocytes was not evident.

The finding that human neutrophils from healthy individuals express CRIg protein and can be stimulated to express the protein on the cell surface is novel. However, prior to the undertaking of this study and in the work presented in chapter 4, it has been demonstrated that CRIg expression by human macrophages can be positively induced by anti-inflammatory mediators such as dexamethasone and IL-10 (Gorgani et al., 2011). Thus, the finding that the expression of CRIg on the cell surface of neutrophils is only significantly detectable following stimulation of the cells by agonists and inflammatory agents such as PMA and TNF was surprising. This contrasts with macrophages, which have been shown, in this thesis and in other studies, to downregulate CRIg following exposure to inflammatory conditions or to inflammatory stimulants such as TNF (Chen et al., 2010; Ma et al., 2015; Vogt et al., 2006). This difference is likely to be a result of a difference in the mechanism of regulation between the two cell types. In the case of macrophages, we observed that CRIg expression was negatively regulated by TNF at the mRNA level and this decrease was reflected by lower protein expression (a culture period of three days was required to detect mRNA modulation, while a culture period of 5 days was required to observe a difference in protein levels). However, in neutrophils—terminally differentiated, short-lived effector cells which need to respond to environmental factors rapidly—we see that TNF stimulation has an immediate effect on surface CRIg expression, with expression levels peaking after 20 minutes of stimulation. Further, we do not observe an alteration in total protein levels of CRIg in TNF-stimulated cells compared with unstimulated controls. This indicates that in neutrophils, the upregulation of CRIg on the cell surface following stimulation with TNF is a result of rapid translocation of existing protein to the plasma membrane and that CRIg expression by these cells is controlled by a different mechanism to that that has previously been observed in macrophages (figure 8.2). The correlation of CRIg upregulation with upregulation of CD11b—a surface receptor known to be present in neutrophil specific and azurophilic granules—suggests that CRIg is stored in one of the same compartments as CD11b. Additionally, as ARPC1B-deficient neutrophils exhibit enhanced secretion of azurophilic granules (Kuijpers et al., 2017) and our work presented in chapter 7 showed that these deficient cells were unable to upregulate both CD11b and CRIg in response to TNF and fMLP, this indicates that CRIg is not stored within azurophilic granules. Furthermore, inhibition of Rab27a successfully inhibited surface upregulation of CRIg in response to fMLP stimulation. Rab27a is known to be responsible for regulating the docking and fusion

with the plasma membrane during exocytosis of both gelatinase and specific granules, and not azurophilic (Herrero-Turrión et al., 2008). This, together with the fact that LPS predominantly induces the release of gelatinase granules rather than specific suggests it is unlikely CRIg is stored within specific granules. Lastly, in the absence of $Ca^{2+}$ ions, neutrophils are still able to release secretory vescicles in response to fMLP (Niessen et al., 1991). As demonstrated in chapter 7, neutrophils were unable to upregulate CRIg in response to fMLP in the presence of the $Ca^{2+}$ chelator BAPTA-AM, indicating that CRIg is not stored in the secretory vescicles. Thus, our work strongly suggests that CRIg is stored within the gelatinase granules of human neutrophils.

CRIg is a rapid promoter of phagocytosis, and as it binds to the complement component C3b which is produced sequentially prior to iC3b, it is able to act faster than the other complement receptors, CR3 and CR4 (Gorgani et al., 2008; Helmy et al., 2006). Thus, for neutrophils, the ability to upregulate a receptor with such efficient phagocytic capability would be highly beneficial. Particularly, in cases of low-grade infection where a fully-fledged immune response is not required, CRIg may be upregulated on the surface of these cells, where it then clears pathogens without promoting the 'cytokine storm' which is characteristic of engagement through CR3 (Wolf et al., 2018). Clearance of low levels of opsonised particles from the blood by Kupffer cells typically does not induce an inflammatory response (Crawford et al., 2018), and as neutrophils are known to assist Kupffer cells with the removal of extracellularly captured particles from the blood (Gregory et al., 2002; Gregory et al., 1996), this could be a possible function for CRIg expressed by neutrophils. Alternatively, CRIg may function as a cell-activating molecule. We observed that engaging neutrophils through CRIg induced an oxidative burst response, indicating that CRIg is able to induce inflammatory signalling in neutrophils. Other studies have shown that in macrophages, CRIg engagement leads to secretion of IL-8 and matrix metalloproteinase 9. Thus, future research which this work may provide the basis for is whether CRIg operates cooperatively with CR3 and CR4 by inducing their activation on the neutrophil cell surface through providing an activating signal.

Aside from novelty, the finding that circulating phagocytes express CRIg has broad ramifications in terms of the potential use of CRIg as a therapeutic agent or target. Similarly to other B7 family ligands, CRIg expression levels have been shown to be elevated in many types of cancer (Byun et al., 2017; Liao et al., 2014; Xu et al., 2015). As a result, it has been suggested that CRIg may be a potential target for inhibiting antibodies (similar in action to blocking antibody against the checkpoint molecules PD-1 and PD-L1). However, if CRIg is

naturally expressed in the blood as well as the tissues and is an important player in neutrophil-mediated clearance of opsonised pathogens, then blocking CRIg may severely immunocompromise patients. Additionally, the role that CRIg plays in the function of monocytes remains to be studied. Therefore, the properties of CRIg as identified here will be vitally important properties to consider in future studies into the therapeutic efficacy of CRIg-targeting agents.

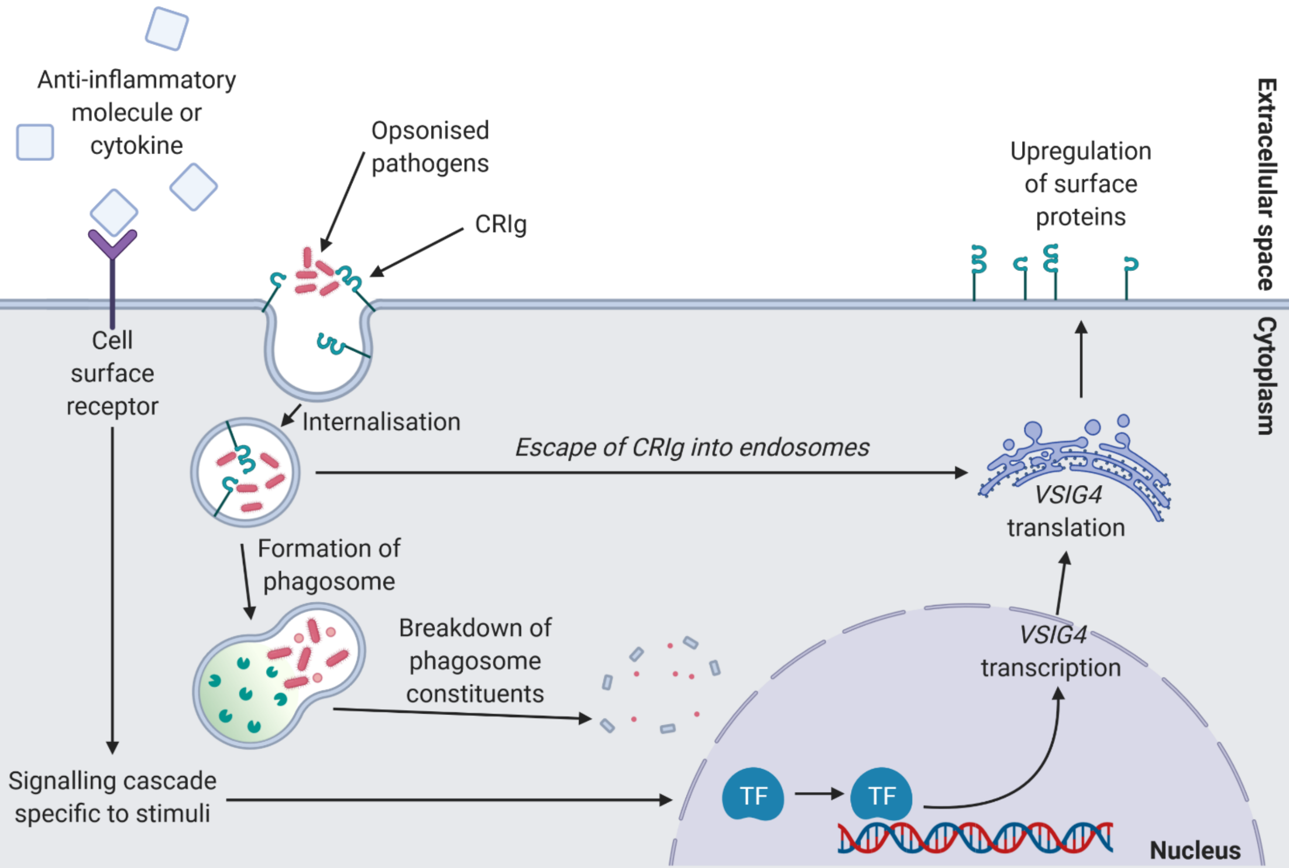


*Figure 8.1. *Schematic diagram demonstrating the mechanisms of regulation of surface CRIg protein in macrophages.* CRIg upregulation is controlled by two mechanisms; first, the recycling pool of receptors contained in recycling endosomes, and secondly, the generation of new protein as a result of increased transcription upon cellular stimulation with specific mediators such as IL-10, 1,25D, and dexamethasone. TF- transcription factor. *Image generated with BioRender.

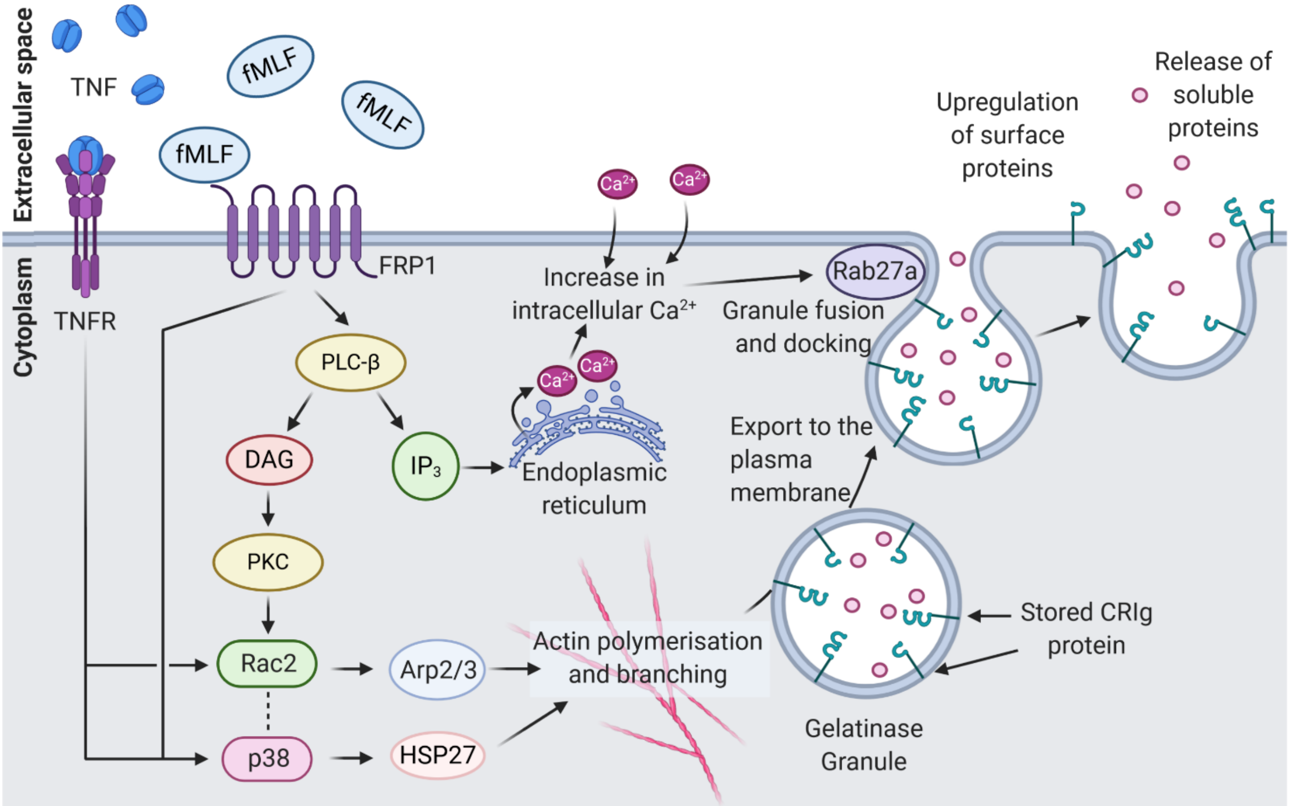


*Figure 8.2. *Schematic diagram demonstrating the mechanism of upregulation of surface CRIg protein in neutrophils.* In resting cells, CRIg is stored in internal granules (possibly the gelatinase granules). Upon exposure to inflammatory mediators such as TNF (PKC independent) or fMLP (either PKC dependent or independent), a signalling cascade as a result of activation of p38 is initiated, and granule exocytosis takes place, translocating stored protein to the cell surface. This process is dependent on actin polymerisation and branching, and requires membrane docking which is dependent on rab27a (image duplicated from chapter 7 for ease of reading, *generated with BioRender).

## 8.6. The Transcript Variants of CRIg

As presented in this thesis, we have successfully detected mRNA transcripts of three human CRIg isoforms in unstimulated MDM and MDDC, and all five in dexamethasone stimulated cells (the existence of the sixth transcript variant of CRIg was not known at the time of conducting this work). While the transcript variants present in neutrophils were not assessed in this thesis due to the poor quality and low RNA yields from neutrophils, it would be interesting to assess how these compare with what we see in macrophages, and whether neutrophils are expressing 'unique' isoforms. Additionally, as the existence of a soluble form of human CRIg has been reported since the commencement of this work (Yuan et al., 2020), it would be interesting to examine whether this soluble form represents one, or multiple, of the shorter CRIg variants, such as variants 3 and 4 (chapter 3, figure 2; Fig. S2), as these proteins have truncated intracellular domains. Alternatively, as there is a single caspase-1 cleavage site in the extracellular regions of both huCRIg(L) and huCRIg(S) at residue 266 and 172 respectively (Gasteiger et al., 2003; Gasteiger et al., 2005), it would be interesting to assess whether soluble CRIg is produced by protein being cleaved from the cell surface upon inflammation and cell activation. This could perhaps explain how CRIg is being lost from monocytes upon isolation and cryopreservation.

## 8.7. Limitations, Future Directions and Final Conclusions

A major limitation restricting our ability to investigate the numerous protein variants of human CRIg is the fact that due to the high homology of the extracellular domains of the proteins, antibodies specific to each of the protein variants are not able to be designed. The only way antibodies could be used to distinguish between each of the six variants would be to use a combination of antibodies: one that is able to distinguish variants with the short versus the long extracellular domains, and a second which is able to distinguish between the three possible intracellular domains, and thus would not be able to be used to examine live cells. While our lab has an interest in this line of experimentation, it was unable to be performed in timeframe of this thesis. Additionally, while our findings suggest that CRIg in resting neutrophils is stored within granules, the next natural step in continuing this work would be to confirm the specific CRIg-containing compartment through co-localisation experiments with markers for each of the individual granules and vesicles. This is of interest to our lab and will be a focus of future study.

Overall, the presented findings have revealed further complexities in the field of CRIg biology and have further distanced what we know in mice from what we know in humans. We have identified CRIg as a major control point molecule in the innate immune response

of human macrophages, and have demonstrated the important role of CRIg on MDDC where it exerts control over the T cell response. Finally, we have identified multiple cell types present in the healthy human periphery which express CRIg, providing the basis for undertaking future works to address how this expression may or may not be associated or modulated with diseased states such as in rheumatoid arthritis (where we would expect to see an increase in active, inflammatory neutrophils (Grayson et al., 2016)), but also in certain types of cancer where CRIg has been found to be increased (Yuan et al., 2020). Thus, this may lead to the development of a blood test to gauge neutrophil activation in the case of inflammatory disorders.

## 8.8. Publication: 'The Role of Phagocytes in Immunity to *Candida albicans*'

### 8.8.1. Introduction and Contextual Statement

This section of the final discussion chapter presents a comprehensive review in the form of a published book chapter into the role of phagocytes in immunity against the fungal pathogen, *Candida albicans*. This article discusses the mechanisms of phagocytosis and killing of *C. albicans* by human phagocytes, particularly neutrophils and macrophages, with a focus on the complement-dependent and -independent receptors involved in this process. Importantly, the known role of CRIg in immunity against *C. albicans* is summarised, and primary data demonstrating the kinetics of complement-dependent vs. complement-independent phagocytosis is shown. We also discuss cytokine priming in immunity against *C. albicans*, along with primary and secondary immunodeficiencies associated with susceptibility to fungal infection.

This chapter presents the final published manuscript included in this thesis. The following book chapter entitled 'The Role of Phagocytes in Immunity to *Candida albicans*', by **Annabelle Small**, Jovanka R. King, Deborah A. Rathjen & Antonio Ferrante was published in the peer reviewed, IntechOpen open access book entitled *Candida albicans*, edited by Doblin Sandai in May 2018 (doi: 10.5772/intechopen.80683).

**Article Metrics (as of November 2020)**

*Altmetrics score: 1*

*Citations: 1*

# Statement of Authorship

| Title of Paper | The Role of Phagocytes in Immunity to Candida albicans |
|---|---|
| Publication Status | [X] Published [ ] Accepted for Publication [ ] Submitted for Publication [ ] Unpublished and Unsubmitted work written in manuscript style |
| Publication Details | Small, AG, King, JR, Rathjen, DA, & Ferrante, A (2018), 'The role of phagocytes in immunity to Candida albicans.' IntechOpen, DOI: 10.5772/intechopen.80683 |

## Principal Author

| Name of Principal Author (Candidate) | Annabelle Small | | |
|---|---|---|---|
| Contribution to the Paper | AS conducted original experiments included in the article, complied figures, critically reviewed the literature and assisted in critical reading and writing of the manuscript. | | |
| Overall percentage (%) | 50% | | |
| Certification: | This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper. | | |
| Signature | | Date | 3/3/2020 |

## Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

| Name of Co-Author | Professor Antonio Ferrante | | |
|---|---|---|---|
| Contribution to the Paper | AF initiated the work, acted as corresponding author, and wrote the manuscript. | | |
| Signature | | Date | 03/03/2020 |

| Name of Co-Author | Dr. Jovanka King | | |
|---|---|---|---|
| Contribution to the Paper | JK compiled data, critically reviewed the literature, wrote and critically read the manuscript. | | |
| Signature | | Date | 5/3/2020 |

Please cut and paste additional co-author panels here as required.

| Name of Co-Author | Dr. Deborah Rathjen | | |
|---|---|---|---|
| Contribution to the Paper | DA was involved in conducting the murine experiments, interrogation of data, and critically read the manuscript. | | |
| Signature | | Date | 16 November 2020 |

Chapter 5

# The Role of Phagocytes in Immunity to *Candida albicans*

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Deborah A. Rathjen and Antonio Ferrante

Additional information is available at the end of the chapter

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**Abstract**

Body clearance of fungi such as *Candida albicans* involves phagocytosis by fixed tissue macrophages as well as infiltrating monocytes and neutrophils. Through phagocytosis, the fungi are confined and killed by the oxidative and non-oxidative anti-microbial systems. These include oxygen derived reactive species, generated from the activation of the NADPH oxidase complex and granule constituents. These same mechanisms are responsible for the damage to hyphal forms of *C. albicans*. Complement promotes phagocytosis, through their interaction with a series of complement receptors including the recently described complement receptor immunoglobulin. However, it is also evident that under other conditions, the killing of yeast and hyphal forms can occur in a complement-independent manner. Phagocytosis and killing of *Candida* is enhanced by the cytokine network, such as tumour necrosis factor and interferon gamma. Patients with primary immunodeficiency diseases who have phagocytic deficiencies, such as those with defects in the NADPH oxidase complex are predisposed to fungal infections, providing evidence for the critical role of phagocytes in anti-fungal immunity. Secondary immunodeficiencies can arise as a result of treatment with anti-cancer or other immunosuppressive drugs. These agents may also predispose patients to fungal infections due to their ability to compromise the anti-microbial activity of phagocytes.

**Keywords:** *Candida albicans*, macrophages, neutrophils, complement, innate immunity, phagocytosis, fungal killing mechanisms, cytokines, trained immunity, immunodeficiency, immunopharmacology

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## 1. Introduction

*C. albicans* is considered to be the most common fungus causing both skin and disseminated disease, particularly in immunodeficient and immunocompromised patients. Phagocytes, particularly neutrophils, play an important role in clearing candidal infections. The importance of neutrophils in immunity to *C. albicans* is clearly evident from the increased rate of infection seen in patients with severe neutropenia [1].

In neutrophils, the major response associated with phagocytosis of microbial pathogens is the oxygen-dependent respiratory burst and the generation of reactive oxygen species (ROS). Several decades ago it became evident that neutrophils displayed a unique respiratory burst in the absence of mitochondria, where the generation of ATP comes mainly from glycolysis (reviewed in [2]). It also became apparent that the majority of the oxygen consumed was converted to superoxide ($O\cdot_2^-$) which is then converted to further oxygen intermediates, including singlet oxygen and $H_2O_2$. The enzyme which catalyses the conversion of $O_2$ to $O\cdot_2^-$ is assembled in the phagocytic vacuole membrane, facilitating its release into the bacteria or fungus-containing vacuole. In neutrophils, the release of the azurophilic granule content simultaneously into the phagocytic vacuole leads to the generation of HOCl, a highly potent anti-microbial agent, as a result of the action of myeloperoxidase on $H_2O_2$ in the presence of chloride ions. In addition, ingestion of microbial pathogens and their confinement to the vacuolar environment may restrict the supply of essential nutrients necessary for growth.

The NADPH oxidase complex is responsible for the respiratory burst and consists of a number of different proteins which assemble in the neutrophil vacuole membrane following cell stimulation. This is typically initiated during phagocytosis of bacteria and fungi [3]. The complex consists of the oxidase-specific phox proteins $gp91^{phox}$, $p22^{phox}$, $p40^{phox}$, $p47^{phox}$, $p67^{phox}$ and the small GTPases, Rac1 and Rac2. Cell activation leads to the assembly of these components in the membrane and the initiation of enzymatic activity.

The non-oxidative microbicidal system complements the respiratory burst. Components of the azurophilic granules in neutrophils have been shown to have anti-microbial activity. These include defensins, serprocidins and bactericidal/permeability increasing protein (BPI). Defensins are cationic peptides with broad spectrum antimicrobial activity [2]. The seroprodins, elastase, azurocidin and cathepsin G have antimicrobial activity independent of their enzymatic activity [2].

As with neutrophils, most bacteria and fungi are confined and killed within phagosomes by macrophages [4], involving a variety of agents such as toxic metabolites, peptides and enzymes. These may act either alone or synergistically. In addition, macrophages can produce ROS which have anti-microbial action but unlike monocytes, macrophages lack MPO. Most striking is the marked heterogeneity of macrophages enabling these leukocytes to perform functions relevant to specific tissues in which they are located.

The extrusion of neutrophil extracellular traps (NETs) is also considered to be a defence mechanism against microbial pathogens. NETs are structures composed of DNA as well as anti-microbial substances, elastase, calprotectin and MPO [5]. NETs not only trap the microbial pathogens, but also kill them. Interestingly, it has been reported that the formation of NETs requires the presence of ROS [6].

Effective recognition of microbial pathogens by neutrophils and macrophages requires receptors which bind peptides deposited on bacterial and fungal surfaces which have been generated through the activation of complement, namely C3b and iC3b. Receptors recognising iC3b include CR3 (CD18/CD11b) and CR4 (CD18/CD11c), which are present on both neutrophils and macrophages. Recently, another complement receptor type, complement immunoglobulin receptor (CRIg), expressed only by a subpopulation of macrophages has been described, which binds both iC3b and C3b (reviewed in [7]). It has been shown that this receptor plays an important role in clearance of bacteria from the circulation by liver Kupffer cells [8] and may also be a pattern recognition receptor, facilitating clearance of bacteria in the absence of complement [9].

Antibody bound to microbial pathogens also promotes phagocytosis through the Fcγ receptors, FcγRI (CD64), FcγRIIA (CD32) and FcγRIIIB (CD16), all of which engage the Fc domain of Immunoglobulin G (IgG). The FcαRI which binds the Fc domain of Immunoglobulin A (IgA) also promotes microbial phagocytosis and killing [2].

Apart from the integrins and FcγRs, neutrophils and macrophages express a range of pattern recognition receptors (PPR) which recognise conserved microbial pathogen structures, such as lipoteichoic acid, β-glucans and lipopolysaccharide. Families of PPRs include those found in serum (pentraxins, collectins, complement), those which are membrane bound (classic C-type lectins, non-classic C-type lectin leucine-rich proteins, scavenger receptors) and those which are located intracellularly (NODs, interferon induced proteins).

## 2. Complement dependent and independent phagocytosis of *C. albicans*

Despite the importance of complement-independent mechanisms for host anti-candidal immunity, it is evident that complement is required for optimal resistance to fungal infection [10–12]. It was also evident in these studies that complement could be activated by *C. albicans* by the alternative pathway. Activation of complement leads to the generation of chemotactic peptides and C5a, which attracts neutrophils to the site of candidal infection [13, 14]. Thong and Ferrante [11], in their studies on the generation of chemotaxis promoting factors by serum treated with *C. albicans*, showed that this activity was totally dependent on heat-labile factors and activation of complement via the alternative pathway. Chemotaxis of neutrophils towards fungus-treated serum was abolished when the serum was either heated at 56°C for 20 min or was C2 deficient (where the alternative but not the classical pathway can be activated). The subsequent step, phagocytosis, was also highly dependent on heat labile opsonins [12]. However, while the chemotactic response was totally dependent on serum complement, the heat labile opsonins only acted to enhance other phagocytosis-promoting mechanisms. Thus, significant phagocytosis was still observed in the presence of heat-inactivated serum. In both of these studies on chemotaxis and phagocytosis-promoting activity of serum, it was shown that these principles applied to a wide-range of clinical isolates of *C. albicans* from patients and both including Serotypes A and B [11, 12].

Zymosan A is a yeast cell wall glucan and, like *C. albicans* derived β(1,3) (1,6)-glucan, is an agonist to TLR2 and Dectin-1 [15]. Using commercially available labelled zymosan A bioparticles

which are non-fluorescent outside of the cell and fluoresce once taken into acidic phagosomes, we showed that neutrophils require opsonising conditions to phagocytose particles efficiently (**Figure 1**). This supports the findings of [16], and demonstrates that like monocytes and macrophages, neutrophils require complement for the rapid phagocytosis of yeast particles. Interestingly, the complement dependency of phagocytosis diminished at incubation times of 45–60 min, where complement-independent mechanisms of phagocytosis become more prominent (**Figure 1C**).

The classical complement pathway is likely to be activated by mannan-specific antibodies found in human serum [19] whereas the lectin pathway is activated by the binding of mannose-binding lectin to mannan on the cell wall of the fungus [20]. However, it has also been shown that *C. albicans* can bind the complement regulatory protein, C4b-binding protein (C4BP), thereby inactivating C4b and hence preventing complement activation on the yeast surface. As a result, the microbial pathogen will evade complement activation via the classical and lectin pathways, but the alternative pathway remains operative, generating chemotactic factors and opsonins. Furthermore, *C. albicans* has the ability to regulate the alternative pathway and factors H and FHL-1 [21]. The binding of these regulators is seen with both the cellular and hyphal forms of *C. albicans* [22].

The unique complement receptor CRIg is a member of the transmembrane protein of the type 1 immunoglobulin superfamily, encoded by *VSIG4*. Although two spliced forms of CRIg have been described, a long (L) and short (S) form [8], we have recently identified five forms based on expression of transcripts and western blot analysis [23]. The receptor is expressed selectively by a subpopulation of macrophages, probably of the M2 type, and is abundant in fixed tissue macrophages such as liver Kupffer cells and resident peritoneal macrophages [24, 25]. Unlike CR3 and CR4 which require prior activation, CRIg is naturally active and its activity is controlled by its recycling pattern from the endoplasmic reticulum [8]. Our studies have demonstrated that cytokines alter CRIg expression in human macrophages and this was associated with a corresponding change in ability of neutrophils to phagocytose *C. albicans* in a complement-dependent manner [23, 26, 27].

While CR3 and FcRγ mediate phagocytosis of complement and antibody opsonised *C. albicans*, in the absence of these opsonins, adherence and phagocytosis by neutrophils and macrophages is promoted by C-Type Lectin Receptors (CLRs), in particular Dectin-1 [28–31]. The targets for Dectin-1 are β-1,3 glucan polymers, major components of the fungal cell wall. In *C. albicans* hyphae, this polymer is masked and appears to be different in the yeast form [32].

Cells of the phagocytic system are able to recognise *C. albicans* through multiple classes of receptors [33]. These include pattern recognition receptors (PRRs) such as Toll-like receptor (TLRs) 4 and 2 [34, 35], and CLRs such as Dectin-1 and the mannose receptor [36]. While these receptors are able to induce phagocytosis independently of complement, efficiency of uptake in both macrophages and neutrophils can be significantly increased when *C. albicans* is opsonised [16]. Under these conditions CR3 present on phagocytes is able to recognise iC3b deposited on the fungal cell surface and promote phagocytosis. In macrophages, this process is also able to occur through CRIg [27]. Agents such as dexamethasone that promote the upregulation of CRIg protein expression are also able to induce increased levels of phagocytosis of *C albicans* [23], suggesting that CRIg rather than CR3 plays an important role in the phagocytosis of *C. albicans* in macrophages.

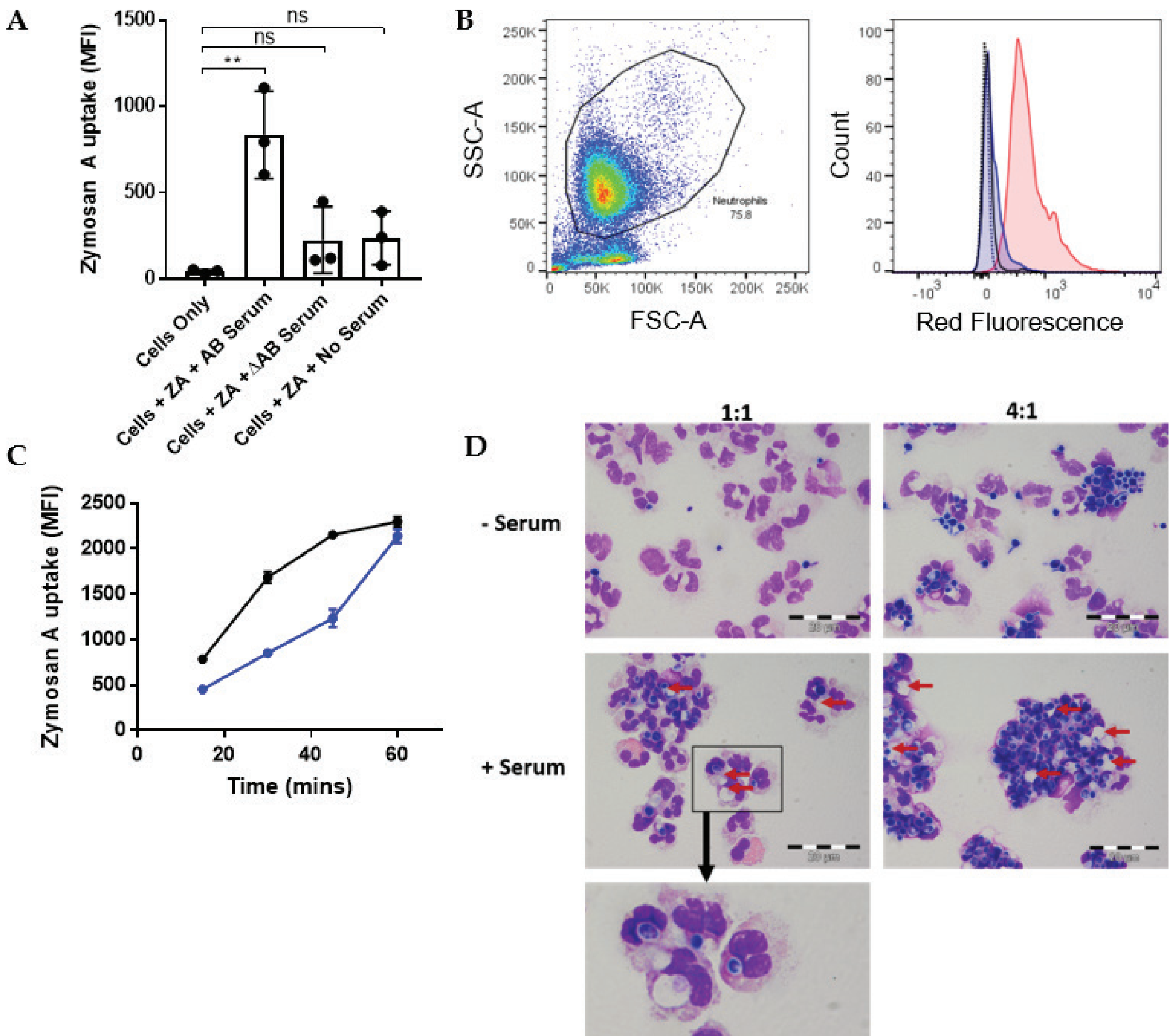


**Figure 1.** Complement-dependent and -independent phagocytosis of zymosan A bioparticles by human neutrophils. (A) Phagocytosis of *C. albicans* under the different treatment conditions indicated in the x-axis. Results are the mean ± SD of three experiments. (B) Representative histogram/gating strategy for these experimental runs. In these experiments the reaction was terminated at 30 min. Neutrophils only are shown by the dashed line, no serum in black, heat-inactivated serum shown in blue, and native AB serum shown in red. (C) Phagocytosis kinetics over a 60 min incubation period in the presence or absence of serum. Results are presented as mean ± SD of triplicate reactions. Neutrophils were prepared from human peripheral blood from healthy volunteers, using the high density gradient method [17]. Phagocytosis was assayed using pHrodo™Red Zymosan A bioparticles (ThermoFisher, Walter MA, Cat no. P35364) as described previously [18]. Human AB serum was prepared from peripheral blood of healthy volunteers. The serum was shown to have normal levels of complement activity using the CH50 assay. AB serum heated at 56°C for 20 min was confirmed to lack complement activity. The cell samples were analysed using a FACSCanto I flow cytometer (BD). The work was approved by the Human Research Ethics Committee of the Women's and Children's Hospital Network, Adelaide. Statistical analyses were carried out by ANOVA followed by Dunnet's post hoc test. (D) Photomicrographs of the interaction of *C. albicans* with neutrophils in the presence or absence of serum at a 1:1 and 1:4 neutrophil:fungal ratio. Red arrows indicate phagocytic vacuoles following the digestion of the yeast or non-degraded yeast particles (following 30 min of incubation).

Neutrophils recognise *C. albicans* through the PRRs TLR2, TLR4 and Dectin-1, and also under opsonising conditions through FcγR and CR3 [37]. Similar to macrophage phagocytosis of *C. albicans*, uptake of isolated fungal zymosan A is more efficient in opsonising conditions, with phagocytosis after a 15-min incubation time being three times higher in reactions with complement compared to no serum and heat-inactivated serum controls (**Figure 1**).

*C. albicans* is able to exist in multiple forms, as a single-celled budding yeast or in pseudohyphal or hyphal filamentous forms [38]. While in its unicellular form, the fungus can be tolerated as a commensal organism by the oral or vaginal epithelium. However, when it converts to its hyphal form, the fungus displays pathogenic properties. The host is able to discriminate against the potential danger [37] through MAPK-based recognition in the epithelial cells [39], which leads to mitogen-activated protein kinase phosphatase 1 (MKP1) and c-Fos activation. Neutrophils also play a role in this protection through TLR4-mediated recognition [40].

## 3. Trained macrophage immunity in anti-fungal immunity

Trained immunity (TI) refers to the ability of innate immune cells to exhibit 'memory' and prevent reinfection of previously encountered invading pathogens [41]. Termed by Netea and colleagues [42], TI induces a state of enhanced antimicrobial action in cells of the innate immune system, particularly monocytes and macrophages, which is distinct from both typical innate immunity and the memory of the adaptive immune system. Alternatively, TI refers to the enhanced response to reinfection against the initial invading microorganisms and cross-protection against different pathogens. Although the concept of TI is relatively new, the phenomenon of protection afforded by previous infection in a manner distinct from adaptive immunity has long been known, particularly in plant and insect systems [43, 44].

TI has been shown to have a role in infection and immunity against *C albicans.* Bistoni et al. [45] demonstrated that not only did injection with a non-pathogenic strain of *C. albicans* induce protection against reinfection, but also cross-protected against the other pathogens *Candida tropicalis* and *Staphylococcus aureus*. This protection was determined to be macrophage-dependent, as transfer of adherent splenic cells from mice administered with the non-pathogenic strain conferred protection to the recipient mice. Two decades later, Quintin et al. [46] expanded on this concept, demonstrating that mice injected with low doses of *C. albicans* showed increased survival rates when administered lethal infection loads, and increased proinflammatory cytokine production upon secondary exposure. This protection was also shown in mice deficient in T and B cells and not in mice lacking *CCR2*, indicating that similar to the results of Bistoni et al. [45], the observed protection was monocyte-dependent. It was also shown that training of monocytes could be induced through purified β-glucan, a polysaccharide that makes up the cell wall of selected bacteria and fungi [47]. The group further investigated the mechanisms behind this protection by analysis of the genome-wide binding pattern of the methylation marks on histone 3 lysine 4 (H3K4me3) and on histone 3 lysine 27 (H3K27me3), and concluded that protection was controlled at the epigenetic level through H3K4me3 in known genes involved in innate immunity. Furthermore, mRNA levels of TNF and IL-6 were higher in trained monocytes compared with non-trained control cells.

While other molecules such as fungal chitin have also been shown to induce TI [48], β-glucan remains the most well-studied molecule with respect to *C. albicans*, which has been shown to induce TI in both human and murine systems [46, 49, 50]. Along with its antimicrobial priming, β-glucan-induced TI has also been investigated in anti-tumour immunity [51].

## 4. Killing of *C. albicans* by neutrophils and macrophages

Ferrante [52] demonstrated that killing of yeast forms of *C. albicans* and *Candida glabrata* was associated with release of the ROS, superoxide, and constituents of azurophilic granules and specific granules. During this interaction the generation of HOCl occurred, another potent anti-fungal agent. The importance of ROS was demonstrated by the finding that inhibitors of superoxide and $H_2O_2$ decreased intracellular killing of *C. albicans* [53]. Further proof of the role of ROS generation in the killing of *C. albicans* came from the demonstration that neutrophils and macrophages from patients with chronic granulomatous disease (CGD) (who have defective NADPH oxidase activity), were unable to effectively kill the fungi [54]. However, whether ROS *per se* are responsible for the killing of *C. albicans* remains to be established [55]. The reaction of $H_2O_2$ with MPO, in the presence of chloride ions, forms a very potent antimicrobial system. We have previously demonstrated that opsonised *C. albicans* induces the release of both $H_2O_2$ and MPO, thereby establishing an anti-candidal environment [52]. The importance of MPO in the killing of *C. albicans* is supported by the finding that neutrophils and monocytes from MPO deficient patients failed to kill *C. albicans* [56, 57].

*In vivo* the absence of MPO in macrophages may be overcome by the cells incorporating MPO released by neutrophils at infection sites. Thus, resident peritoneal mouse macrophages in the presence of recombinant MPO caused an increase in intracellular killing of *C. albicans* [58]. However, it is noteworthy that using mouse models of X-linked CGD and MPO deficiency, susceptibility was most evident in the former, suggesting that ROS are the major mediators of candidicidal activity [59]. In comparison, the neutrophil-mediated damage to *C. albicans* pseudohyphae was found to be mediated by the oxidative burst and MPO [60]. Interestingly, this neutrophil-mediated damage occurred in the absence of serum complement.

Two distinct mechanisms for human neutrophil-mediated killing have been documented, depending on the state of fungal opsonisation. Using *in vitro* fungicidal assays, Gazendam et al. [61] showed that killing of un-opsonised *C. albicans* was dependent on CR3 and phosphatidylinositol-3-kinase (PI3K) signalling, but was independent of NADPH oxidase activation. However, the killing of antibody opsonised *C. albicans* by neutrophils was dependent on Fcγ receptors and protein kinase C (PKC) in addition to NADPH.

## 5. Intracellular signalling required for killing of *C. albicans*

Approximately two decades ago it was demonstrated that human neutrophil-mediated killing of *C. albicans*, in a complement-dependent manner, required the activation of the extracellular signal-regulated protein kinase cascade [62]. More recently it has been reported that PKCδ activation downstream of the receptors Dectin-1 and Mac-1 is important in the neutrophil-mediated resistance to *C. albicans* and fungi-induced ROS generation [63]. In contrast, while PKCδ deficiency in macrophages prevented the stimulation of production of ROS induced by *C. albicans*, this did not affect the killing of the fungus. It has been demonstrated that BTK and

Vav1 are Dectin-1 interacting proteins [64]. These were found to be recruited to phagocytic cups containing yeast or hyphae of *C. albicans*, at the less mature stage of phagosome development. These contribute to the Dectin-1 dependent phagocytosis of *C. albicans*.

In comparison, Gazendam et al. [61] demonstrated that neutrophils display two different mechanisms in the killing of *C. albicans* by evaluating patients with Dectin-1 deficiency, CARD9 deficiency or NADPH deficiency. One of these mechanisms was CR3, PI3K and CARD9 dependent, but independent of ROS generation. The other was selectively dependent on Fcγ, PKC and ROS generation. Both of these candidicidal pathways required Syk tyrosine activation but were independent of Dectin-1.

## 6. Neutrophil extracellular traps in immunity to *C. albicans*

*C. albicans* has been shown to induce NET extrusion in phagocytes, particularly in neutrophils. While the formation of this structure is considered as part of cell death or NETosis [65], Byrd et al. [66] reported that the rapid extrusion of NETs in response to *C. albicans* occurs in the absence of cell death. However, others have demonstrated that the yeast forms of *C. albicans* stimulated NETs through autophagy and ROS generation in the early stage of the interaction (first 15 min) [67]. However, with the hyphal forms, NET formation occurred via autophagy and not ROS generation. In the longer term (4 h), only the hyphae stimulated NETs. Interestingly, they found less killing of yeast forms by NETs compared to the high level of damage to the hyphae forms. Other strategic functions of extracellular protrusions of neutrophils have been demonstrated for *Plasmodium falciparum*. Here, the neutrophils were observed to 'throw out' protrusions which penetrated the parasitophorous vacuole containing the intraerythrocytic stage of the parasite and withdrawing the parasite without damaging the erythrocyte [68].

## 7. Cytokine priming in phagocyte-mediated killing of *C. albicans*

Over three decades ago it became evident that neutrophil responses to microbial pathogens could be significantly increased if the cells were pre-sensitised with products released by activated lymphocytes and macrophages [69], a process dependent on the presence of TNF [70, 71]. The importance of cytokine priming in killing of *C. albicans* by neutrophils was also observed [72]. Thus, neutrophil mediated killing of *C. albicans* and a related fungus, *Candida glabrata* was significantly increased if the phagocytes had been pre-treated with either TNF or GM-CSF [52, 73]. The TNF treatment also increased the candida-induced release of ROS and MPO, consistent with the increased anti-fungal activity induced by the cytokines [52]. The mechanism by which TNF primes neutrophils for increased killing of *C. albicans* has not been studied. However, these mechanisms can be inferred from studies with other microbial pathogens. Kowanko et al. [74] demonstrated that the TNF-induced effects responsible for increased microbial killing could be mediated by both oxygen-dependent and oxygen-independent mechanisms, with respect to killing of opsonised *S. aureus* and *Plasmodium falciparum* infected erythrocytes, respectively. Furthermore, studies with the pathogenic soil amoeba,

*N. fowleri* have shown that the TNF-enhanced killing requires a functional $H_2O_2$-MPO-halide system [75]. The priming of neutrophils by TNF is reflected by an increase in expression of CR3 and CR4 on the surface of these cells. The enhanced killing of *S. aureus* was dependent on these receptors, given that this was not seen upon the addition of anti-CD11b and -CD11c monoclonal antibodies [76].

The use of TNF to enhance immunity against various microbial infections has not been considered appropriate because of the highly toxic and tissue damaging effects of TNF. In an effort to harness the anti-infective properties of TNF and exclude some of its tissue damaging properties, we synthesised short peptides representative of the TNF sequence [77]. One of these elevenmer peptides, $TNF_{70–80}$, was found to activate neutrophils and macrophages to increase microbial killing both *in vitro* and *in vivo* [77–81].

Our studies with *C. albicans* demonstrated that $TNF_{70–80}$ also protected against infections with this fungus (**Tables 1** and **2**). In the first set of experiments, the effect of administering either TNF or $TNF_{70–80}$ to mice infected with *C. albicans* was examined. The recovery of fungi from

| Treatment | No. mice/group | Log CFU/g kidney (M ± SD) |
|---|---|---|
| PBS | 23 | 7.3 ± 0.6 |
| Amphotericin B | 15 | 2.7 ± 2.4*** |
| TNF (0.1 mg/kg) | 29 | 5.6 ± 1.2*** |
| $TNF_{70–90}$ (4 mg/kg) | 9 | 5.75 ± 1.7** |

Eight week old Balb/c mice were challenged with $5 \times 10^5$ CFU *C. albicans* intravenously. Treatment of mice commenced 24 h prior to infection, and continued with daily administration until 2 days post-infection. Mice were sacrificed on day 2 and kidney preparations plated on Sabouraud agar. The degree of infection was determined by enumeration of the number of organisms in the kidney at the time of euthanisation (**p < 0.01, ***p < 0.001, 1-way ANOVA, SNK test). The research received approval from the Women's and Children's Hospital Animal Ethics Committee.

**Table 1.** The effect of TNF and $TNF_{70–80}$ on ***C. albicans*** infection in mice.

| Treatment | Route | Dose (mg/kg) | Survivors 10 days post-infection |
|---|---|---|---|
| Vehicle control | IP | — | 8 |
| Cyclophosphamide | PO | 30 | 2* |
| $TNF_{70–80}$ + cyclophosphamide | IP | 100 | 7ns |
| $TNF_{70–80}$ + cyclophosphamide | IP | 10 | 4ns |
| $TNF_{70–80}$ + cyclophosphamide | IP | 1 | 4ns |
| $TNF_{70–90}$ + cyclophosphamide | IP | 0.1 | 2* |
| Azimexone + cyclophosphamide | IP | 100 | 6ns |

Balb/c mice (10/group) were treated with 3 doses of oral (OP) cyclophosphamide (30 mg/kg) and infected with *C. albicans* as described in **Table 1**. Mice were also treated with three doses of $TNF_{70–80}$ at the schedule described in **Table 1**. Azimexone (used as a positive control) was administered intraperitoneally (IP) (n = 10 mice, *p < 0.05, ns: not significant, one-sided Fisher's exact test). The research received approval from by Women's and Children's Hospital Animal Ethics Committee.

**Table 2.** Effect of $TNF_{70–80}$ on ***C. albicans*** infection in immunocompromised mice.

the kidneys of these mice was significantly lower than in non-treated control mice (**Table 1**). In the second experimental set-up, mice treated with cyclophosphamide became highly susceptible to *C. albicans* with the survival of mice dropping from 80 to 20%, 10 days after infection. If the mice had been treated with $TNF_{70-80}$, survival was increased with 70% survival observed at the highest dose (**Table 2**).

Cytokines also influence the ability of macrophages to phagocytose and kill fungi. Human monocyte-derived macrophages (MDMs) treated with interferon gamma showed increased ability to phagocytose and kill yeast forms of *C. albicans* [82]. The cytokine treated cells showed a corresponding increase in ROS production when challenged with the fungus. This effect of interferon gamma was evident with non-opsonised *C. albicans* and was independent of CR3. These effects of interferon gamma were reproduced with mouse peritoneal macrophages [83]. M-colony stimulating factor has also been shown to increase macrophage phagocytosis and killing of *C. albicans* yeast forms and cause damage to hyphae [84].

From the described studies, it is evident that when considering killing of microbial pathogens including *C. albicans*, this needs to be interpreted in terms of the cytokine milieu generated during the infection. It is evident from other published work that several cytokines regulate phagocyte-mediated microbial killing properties, including interferon gamma, lymphotoxin and interleukin-1 [71, 85].

## 8. Primary immunodeficiency diseases associated with susceptibility to fungal infection

Primary immunodeficiency diseases (PID) are a heterogeneous group of inborn errors of immunity. Affected individuals develop severe, unusual or recurrent infections, and may also develop features of immune dysregulation with autoimmune manifestations. There are currently over 320 described molecular genetic causes of PID, which can be categorised according to presenting phenotypic features [86]. The International Union of Immunological Sciences (IUIS) classify PID into the following disease categories: immunodeficiencies affecting cellular and humoral immunity, combined immunodeficiencies (CID) with associated or syndromic features, predominantly antibody deficiencies, diseases of immune dysregulation, congenital defects of phagocyte number, function or both, defects in intrinsic and innate immunity, auto-inflammatory disorders, complement deficiencies and phenocopies of PID [86].

Intact immunological processes and pathways are required to mount an effective immune response against fungi, incorporating both innate and adaptive components [87]. Several immune cells and immunological mediators such as cytokines are of critical importance to maintenance of anti-fungal immunity. These include phagocytes, dendritic cells, T cells (particularly T helper 1 (TH1) and T helper 17 (TH17) cells) [87]. The importance of these effectors is evidenced by patients with PID affecting cellular or phagocytic immunity developing severe, invasive or recurrent fungal infections [1].

Primary phagocytic disorders result from mutations in genes encoding key proteins that are essential for normal phagocytic development and function. These disorders may be classified

according to whether phagocyte number, function or both are affected, and by the presence or absence of associated syndromic features [86]. These disorders and their underlying, causative genetic abnormality are summarised in **Table 3**.

| **Congenital defects of phagocytic number, function or both** | | | |
|---|---|---|---|
| **Associated with syndromic features** | | **Not associated with syndromic features** | |
| **Disorder** | **Gene(s)** | **Disorder** | **Gene(s)** |
| Shwachman-Diamond syndrome | *SBDS*, *DNAJC21* | Elastase deficiency (SCN1) | *ELANE* |
| G6PC3 deficiency (SCN4) | *G6PC3* | Kostmann disease (HAX1 deficiency; SCN3) | *HAX1* |
| Glycogen storage disease type 1b | *G6PT1* | GFI1 deficiency (SCN2) | *GFI1* |
| Cohen syndrome | *COH1* | X-linked neutropaenia/myelodysplasia WAS GOF | *WAS* |
| Barth syndrome (3-methylglutaconic aciduria type II) | *TAZ* | G-CSF receptor deficiency | *CSF3R* |
| Clericuzio syndrome (poikiloderma with neutropaenia) | *C16ORF57 (USB1)* | Neutropaenia with combined immune deficiency | *MKL1* |
| VPS45 deficiency (SCN5) | *VPS45* | | |
| P14/LAMTOR2 deficiency | *LAMTOR2* | | |
| JAGN1 deficiency | *JAGN1* | | |
| 3-methylglutaconic aciduria | *CLPB* | | |
| SMARCD2 deficiency | *SMARCD2* | | |
| WDR1 deficiency | *WDR1* | | |
| HYOU1 deficiency | *HYOU1* | | |
| **Congenital defects of phagocytic function** | | | |
| **Associated with syndromic features** | | **Not associated with syndromic features** | |
| **Disorder** | **Gene(s)** | **Disorder** | **Gene(s)** |
| Cystic fibrosis | *CFTR* | Chronic granulomatous disease | *CYBB, NCF1, CYBA, NCF4, NCF2* |
| Papillon-Lefevre syndrome | *CTSC* | Rac2 deficiency | *RAC2* |
| Localised juvenile periodontitis | *FPR1* | G6PD deficiency Class 1 | *G6PD* |
| Leukocyte adhesion deficiency (LAD) 1 | *ITGB2* | GATA2 deficiency (MonoMac syndrome) | *GATA2* |
| Leukocyte adhesion deficiency (LAD) 2 | *SLC35C1* | Specific granule deficiency | *C/EBPE* |
| Leukocyte adhesion deficiency (LAD) 3 | *FERMT3* | Pulmonary alveolar proteinosis | *CSF2RA*, *CSF2RB* |

Adapted from [86].
SCN = severe congenital neutropaenia, WAS = Wiskott-Aldrich Syndrome, GOF = gain of function.

**Table 3.** Primary immunodeficiency diseases affecting phagocytic number and/or function.

Of the described primary immunodeficiency diseases of phagocytic number or function, recurrent or invasive candidal disease has been reported in cases of chronic granulomatous disease and myeloperoxidase deficiency [1] and GATA2 deficiency [88]. Candidosis is reported but tends to be less common in leukocyte adhesion deficiency and congenital neutropaenic syndromes [1].

Chronic granulomatous disease (CGD) occurs as a result of defects in components of the NADPH oxidase system, resulting in defective neutrophil oxidative burst and susceptibility to a narrow range of organisms, particularly those which are catalase-producing. As well as the predisposition to infection, patients with CGD develop a hyperinflammatory response and granuloma formation [89]. X-linked CGD occurs due to mutations in the *CYBB* gene which encodes the NADPH oxidase complex component gp91$^{phox}$ [86]. Autosomal recessive forms of CGD are less common, and occur due to mutations in the *NCF1*, *CYBA*, *NCF4* or *NCF2* genes, which encode for other components of the complex, namely p47$^{phox}$, p22$^{phox}$, p40$^{phox}$ and p67$^{phox}$, respectively [86, 89].

Candidosis is well described in CGD patients, with candidal species implicated in episodes of meningitis, fungaemia, suppurative adenitis, pneumonia, subcutaneous abscesses and liver abscess reported in a cohort of 368 patients with CGD [90]. Although the majority of these infections were expected to be due to underlying, impaired phagocytic function, additional factors such as steroid use likely increase the risk of invasive candidiasis. Candidal oesophagitis, keratitis and disseminated infection (particularly affecting young infants) have also been described, however mucocutaneous candidiasis is uncommon in CGD patients [1].

Patients with gp40$^{phox}$ mutations have been noted to have a distinct clinical phenotype as compared with those with other forms of CGD, with a milder clinical course and lower frequency of invasive fungal infection [91]. There is no impairment in the ability of the neutrophils of affected patients to kill candida, suggesting residual NADPH oxidase activity and a potential gp40$^{phox}$-independent process for reactive oxygen species production. Furthermore, monocyte and monocyte-derived macrophage NADPH oxidase generation appears to occur independently of gp40$^{phox}$ [91]. In patients with CGD, a correlation has been shown between residual production of reactive oxygen intermediates (ROI) and improved long-term survival [92]. The specific mutation in NADPH oxidase predicts the amount of residual production of ROI [92].

CGD may be conservatively managed with antibiotic and antifungal prophylaxis, along with adjunctive therapies including subcutaneous interferon therapy. CGD is curable by haematopoietic stem cell transplantation (HSCT), and trials are underway to evaluate the role of gene therapy as an alternative definitive management strategy [93].

MPO deficiency is autosomal recessive with variable penetrance, may be complete or partial, and has an estimated incidence of between 1:2000 and 1:4000 individuals [94]. Most individuals are clinically asymptomatic, although severe infections are reported in around 5% of those affected. MPO-deficient phagocytes have an impaired capacity to kill *C. albicans*, as evidenced by severe infection in MPO-deficient mice.

*GATA2* encodes a zinc finger transcription factor which is critical for haematopoetic cell development [95]. Mutations in this gene give rise to a syndrome also known as 'MonoMac', which

refers to the monocytopaenia and predisposition to mycobacterial infection which are characteristic of this condition [95, 96]. In addition, affected patients have other haematological anomalies including thrombocytopaenia and neutropaenia, predisposition to haematological malignancy and severe mycobacterial, fungal and human papilloma viral infections [88, 96]. In a recent study of 79 French and Belgian patients with *GATA2* mutations, 16 patients were reported to have had 18 episodes of fungal infection, 5 of which were candidoses [88]. Eight of the 18 infections were associated with chemotherapy or HSCT. The neutrophils from some *GATA2* deficient patients were noted to have reduced granularity [97]. When stimulated with PHA (phytohaemagglutinin), patient PBMCs (peripheral blood mononuclear cells) demonstrated reduced lymphocyte proliferative and cytokine production capacity, which normalised after addition of monocytes [96], highlighting the important role of these cells in eliciting an effective immune response.

In addition to the critical role of phagocytes in anti-fungal immunity, defects in other immune cells and immunologic pathways also give rise to susceptibility to infection with candida and other fungi. A range of single-gene inborn errors of immunity resulting in severe or recurrent superficial or invasive candidiasis have been described [86, 98]. Cell-mediated immunity is essential for anti-fungal immunity. This is evidenced by the predisposition to severe fungal infection in infants with severe combined immunodeficiency (SCID), a life-threatening condition manifested by low, absent or severely dysfunctional T cells [86]. Other forms of combined immunodeficiency, for example, those due to deficiencies in CD25, NEMO/IKBG, DOCK8, TCR-α, ORAI1, MST1/STK4, MHC Class II, along with *IKBA* gain of function mutations and idiopathic CD4+ T cell lymphopaenia are associated with chronic mucocutaneous candidiasis (CMC) [98]. In addition, CMC is a feature of several PID with syndromic features, including STAT3 deficiency (autosomal dominant hyper-immunoglobulin E syndrome), APECED (autoimmune polyendocrinopathy-candidiasis-ectodermal dysplasia), also known as APS-1 (autoimmune polyglandular syndrome type 1) which occurs due to mutations in the *AIRE* gene), and deficiencies of IL12Rβ, IL-12p40 and CARD9 [98, 99]. The importance of the TH17 pathway and IL-17 signalling in anti-candidal immunity has become apparent [100, 101], with severe CMC described in patients with deficiencies of IL-17RA, IL-17F, RORC and *STAT1* gain of function mutations [98, 102]. In particular, AIRE has been demonstrated to have a key role in anti-candidal immunity, as evidenced by its role in fungal synapse formation which is required for initial macrophage recognition of fungal hyphae [103]. AIRE, along with Dectin-1, Dectin-2, Syk and CARD9 are required for formation of the fungal synapse upon stimulation of macrophage-like THP-1 cells after stimulation with *C. albicans* [103].

## 9. Secondary immunodeficiency diseases associated with disorders of phagocyte number or function

Immunosuppression is a well-described risk factor for infection with candida and other fungal species [98]. Corticosteroids are commonly used in the management of a range of inflammatory and malignant conditions, and use of these agents is a known risk factor for fungal infection [104]. The precise mechanisms by which corticosteroids lead to impaired anti-candidal

immunity remain unclear, and this is likely multifactorial [105]. In terms of phagocytic cell function, corticosteroids appear to alter leukocyte differentiation programs. They induce monocytes and macrophages to adopt an anti-inflammatory phenotype. This is modulated by the cytokine environment (including increased IL-10 expression on macrophages), increased apoptotic activity and induction of transcription of anti-inflammatory genes which impact upon chemotaxis, phagocytosis and resistance to oxidative stress [105]. However, despite these observations it has been recently shown that dexamethasone increases the expression of CRIg on human MDMs but not CR3 or CR4, and that this increase was associated with an increase in phagocytosis of complement opsonised *C. albicans* [23, 26, 27].

Cancer patients are at an increased risk of systemic candidiasis, and *C. albicans* is reported to be one of the most common causes of sepsis in this patient group [104]. This predisposition to fungal infection is multifactorial, and may be due to a secondary immunodeficiency caused by the underlying malignancy itself, or due to the effects of chemotherapeutic agents. Chemotherapeutic drugs may induce neutropaenia or affect neutrophil function, thereby impairing anti-candidal immunity. Neutrophil function may be impaired as a result of reduced trafficking, chemotaxis or phagocytic activity. For example, chemotherapeutics targeting microtubule structures likely impair cytoskeletal processes and actin polymerisation, thereby reducing neutrophil chemotaxis and phagocytosis. Chemotherapeutic agents can also interfere in NETosis, which is important for antimicrobial activity. Some drugs may also induce monocytopaenia and impaired monocytic function, further increasing the risk of candidal infection [104].

Patients with liver disease are at an increased risk of fungal infection. Those with cirrhosis have been found to have reduced complement levels and impaired monocyte activation and neutrophil mobilisation [106]. Patients with liver disease are at risk for infectious peritonitis, and *C. albicans* and *C. neoformans* were amongst the main species isolated in these cases. Renal disease is also a risk factor for invasive fungal disease [104]. Neonatal candidal sepsis has been reported in association with jaundice [107]. Interestingly, unconjugated bilirubin in hyperbilirubinemia has also been linked to reduced phagocytic cell function; phagocytosis and killing of fungi [108, 109]. Burns patients are at increased risk of fungal infection owing to a breached skin barrier and use of antimicrobial agents, with candidal infection in particular being associated with increased morbidity and mortality in these patients [106]. In addition to these disease states, other physical factors, alone or in combination, such as the use of intravenous catheters and mechanical ventilation also increase the risk of invasive fungal disease [98, 104].

Finally, it is also evident that anti-fungal drugs *per se* can compromise immunity [109–111]. Several of the imidazoles were found to inhibit neutrophil functions, chemotaxis, phagocytosis and microbial killing of bacteria and candida [110].

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## Conflicts of interest

Authors AGS and JRK declare no conflicts of interest. Authors DAR and AF declare that they are inventors on patent relating to $TNF_{70-80}$ technology.

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## References

[1] Antachopoulos C, Walsh TJ, Roilides E. Fungal infections in primary immunodeficiencies. European Journal of Pediatrics. 2007;**166**(11):1099-1117. DOI: 10.1007/s00431-007-0527-7

[2] Ferrante A. In: Kaufmann S, Steward M, editors. Phagocytes Part 2: Neutrophils. 10th ed. London: Hodder Arnold; 2005. pp. 35-54

[3] Quinn MT, Gauss KA. Structure and regulation of the neutrophil respiratory burst oxidase: Comparison with nonphagocyte oxidases. Journal of Leukocyte Biology. 2004;**76**(4):760-781. DOI: 10.1189/jlb.0404216

[4] Brown GD, Gordon S. In: Kaufmann S, Steward M, editors. Phagocytes Part 1: Macrophages. 10th ed. London: Hodder Arnold; 2005. pp. 21-34

[5] Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, et al. Neutrophil extracellular traps kill bacteria. Science. 2004;**303**(5663):1532-1535. DOI: 10.1126/science.1092385

[6] Fuchs TA, Abed U, Goosmann C, Hurwitz R, Schulze I, Wahn V, et al. Novel cell death program leads to neutrophil extracellular traps. The Journal of Cell Biology. 2007;**176**(2):231-241. DOI: 10.1083/jcb.200606027

[7] Small AG, Al-Baghdadi M, Quach A, Hii C, Ferrante A. Complement receptor immunoglobulin: A control point in infection and immunity, inflammation and cancer. Swiss Medical Weekly. 2016;**146**:w14301. DOI: 10.4414/smw.2016.14301

[8] Helmy KY, Katschke KJ Jr, Gorgani NN, Kljavin NM, Elliott JM, Diehl L, et al. CRIg: A macrophage complement receptor required for phagocytosis of circulating pathogens. Cell. 2006;**124**(5):915-927. DOI: 10.1016/j.cell.2005.12.039

[9] Zeng Z, Surewaard BG, Wong CH, Geoghegan JA, Jenne CN, Kubes P. CRIg functions as a macrophage pattern recognition receptor to directly bind and capture blood-borne gram-positive bacteria. Cell Host & Microbe. 2016;**20**(1):99-106. DOI: 10.1016/j.chom.2016.06.002

[10] Ray TL, Wuepper KD. Activation of the alternative (properdin) pathway of complement by *Candida albicans* and related species. The Journal of Investigative Dermatology. 1976;**67**(6):700-703

[11] Thong YH, Ferrante A. Alternative pathway of complement activation by *Candida albicans*. Australian and New Zealand Journal of Medicine. 1978;**8**(6):620-622

[12] Thong YH, Ness D, Ferrante A. Effect of bilirubin on the fungicidal capacity of human neutrophils. Sabouraudia. 1979;**17**(2):125-129

[13] Hurley DL, Fauci AS. Disseminated candidiasis. I. An experimental model in the Guinea pig. The Journal of Infectious Diseases. 1975;**131**(5):516-527

[14] Maibach HI, Kligman AM. The biology of experimental human cutaneous moniliasis (albicans). Archives of Dermatology. 1962;**85**(2):233-257. DOI: 10.1001/archderm.1962.01590020073009

[15] Dillon S, Agrawal S, Banerjee K, Letterio J, Denning TL, Oswald-Richter K, et al. Yeast zymosan, a stimulus for TLR2 and dectin-1, induces regulatory antigen-presenting cells and immunological tolerance. The Journal of Clinical Investigation. 2006;**116**(4):916-928. DOI: 10.1172/JCI27203

[16] Marodi L, Korchak HM, Johnston RB Jr. Mechanisms of host defense against Candida species. I. Phagocytosis by monocytes and monocyte-derived macrophages. Journal of Immunology. 1991;**146**(8):2783-2789

[17] Quach A, Ferrante A. The application of dextran sedimentation as an initial step in neutrophil purification promotes their stimulation, due to the presence of monocytes. Journal of Immunology Research. 2017;**2017**:10. DOI: 10.1155/2017/1254792

[18] Small A, Lansdown N, Al-Baghdadi M, Quach A, Ferrante A. Facilitating THP-1 macrophage studies by differentiating and investigating cell functions in polystyrene test tubes. Journal of Immunological Methods. 2018;**461**:73-77. DOI: 10.1016/j.jim.2018.06.019

[19] Zhang MX, Kozel TR. Mannan-specific immunoglobulin G antibodies in normal human serum accelerate binding of C3 to *Candida albicans* via the alternative complement pathway. Infection and Immunity. 1998;**66**(10):4845-4850

[20] Kozel TR. Activation of the complement system by pathogenic fungi. Clinical Microbiology Reviews. 1996;**9**(1):34-46

[21] Meri T, Hartmann A, Lenk D, Eck R, Wurzner R, Hellwage J, et al. The yeast *Candida albicans* binds complement regulators factor H and FHL-1. Infection and Immunity. 2002;**70**(9):5185-5192

[22] Meri T, Blom AM, Hartmann A, Lenk D, Meri S, Zipfel PF. The hyphal and yeast forms of *Candida albicans* bind the complement regulator C4b-binding protein. Infection and Immunity. 2004;**72**(11):6633-6641. DOI: 10.1128/iai.72.11.6633-6641.2004

[23] Munawara U, Small AG, Quach A, Gorgani NN, Abbott CA, Ferrante A. Cytokines regulate complement receptor immunoglobulin expression and phagocytosis of *Candida albicans* in human macrophages: A control point in anti-microbial immunity. Scientific Reports. 2017;**7**(1):4050. DOI: 10.1038/s41598-017-04325-0

[24] Gorgani NN, He JQ, Katschke KJ Jr, Helmy KY, Xi H, Steffek M, et al. Complement receptor of the Ig superfamily enhances complement-mediated phagocytosis in a subpopulation of tissue resident macrophages. Journal of Immunology. 2008;**181**(11):7902-7908

[25] Irvine KM, Banh X, Gadd VL, Wojcik KK, Ariffin JK, Jose S, et al. CRIg-expressing peritoneal macrophages are associated with disease severity in patients with cirrhosis and ascites. JCI Insight. 2016;**1**(8):e86914. DOI: 10.1172/jci.insight.86914

[26] Gorgani NN, Thathaisong U, Mukaro VR, Poungpair O, Tirimacco A, Hii CS, et al. Regulation of CRIg expression and phagocytosis in human macrophages by arachidonate, dexamethasone, and cytokines. The American Journal of Pathology. 2011;**179**(3):1310-1318. DOI: 10.1016/j.ajpath.2011.05.021

[27] Ma Y, Usuwanthim K, Munawara U, Quach A, Gorgani NN, Abbott CA, et al. Protein kinase Cα regulates the expression of complement receptor Ig in human monocyte–derived macrophages. The Journal of Immunology. 2015;**194**(6):2855

[28] Herre J, Marshall AS, Caron E, Edwards AD, Williams DL, Schweighoffer E, et al. Dectin-1 uses novel mechanisms for yeast phagocytosis in macrophages. Blood. 2004;**104**(13):4038-4045. DOI: 10.1182/blood-2004-03-1140

[29] Herre J, Willment JA, Gordon S, Brown GD. The role of Dectin-1 in antifungal immunity. Critical Reviews in Immunology. 2004;**24**(3):193-203

[30] Miramon P, Kasper L, Hube B. Thriving within the host: Candida spp. interactions with phagocytic cells. Medical Microbiology and Immunology. 2013;**202**(3):183-195. DOI: 10.1007/s00430-013-0288-z

[31] Naglik JR. Candida immunity. New Journal of Science. 2014;**2014**:27. DOI: 10.1155/2014/390241

[32] Lowman DW, Greene RR, Bearden DW, Kruppa MD, Pottier M, Monteiro MA, et al. Novel structural features in *Candida albicans* hyphal glucan provide a basis for differential innate immune recognition of hyphae versus yeast. The Journal of Biological Chemistry. 2014;**289**(6):3432-3443. DOI: 10.1074/jbc.M113.529131

[33] Cheng SC, Joosten LA, Kullberg BJ, Netea MG. Interplay between *Candida albicans* and the mammalian innate host defense. Infection and Immunity. 2012;**80**(4):1304-1313. DOI: 10.1128/iai.06146-11

[34] Jouault T, Ibata-Ombetta S, Takeuchi O, Trinel PA, Sacchetti P, Lefebvre P, et al. *Candida albicans* phospholipomannan is sensed through toll-like receptors. The Journal of Infectious Diseases. 2003;**188**(1):165-172. DOI: 10.1086/375784

[35] Tada H, Nemoto E, Shimauchi H, Watanabe T, Mikami T, Matsumoto T, et al. Saccharomyces cerevisiae- and *Candida albicans*-derived mannan induced production of tumor necrosis factor alpha by human monocytes in a CD14- and Toll-like receptor 4-dependent manner. Microbiology and Immunology. 2002;**46**(7):503-512

[36] Netea MG, Gow NAR, Munro CA, Bates S, Collins C, Ferwerda G, et al. Immune sensing of *Candida albicans* requires cooperative recognition of mannans and glucans by lectin and Toll-like receptors. Journal of Clinical Investigation. 2006;**116**(6):1642-1650. DOI: 10.1172/JCI27114

[37] Miramón P, Dunker C, Windecker H, Bohovych IM, Brown AJP, Kurzai O, et al. Cellular responses of *Candida albicans* to phagocytosis and the extracellular activities of neutrophils are critical to counteract carbohydrate starvation, oxidative and nitrosative stress. PLoS One. 2012;**7**(12):e52850. DOI: 10.1371/journal.pone.0052850

[38] Sudbery PE. Growth of *Candida albicans* hyphae. Nature Reviews Microbiology. 2011; **9**:737. DOI: 10.1038/nrmicro2636

[39] Moyes DL, Runglall M, Murciano C, Shen C, Nayar D, Thavaraj S, et al. A biphasic innate immune MAPK response discriminates between the yeast and hyphal forms of *Candida albicans* in epithelial cells. Cell Host & Microbe. 2010;**8**(3):225-235. DOI: 10.1016/j.chom.2010.08.002

[40] Weindl G, Naglik JR, Kaesler S, Biedermann T, Hube B, Korting HC, et al. Human epithelial cells establish direct antifungal defense through TLR4-mediated signaling. The Journal of Clinical Investigation. 2007;**117**(12):3664-3672. DOI: 10.1172/jci28115

[41] Cassone A. The case for an expanded concept of trained immunity. MBio. 2018;**9**(3). DOI: 10.1128/mBio.e00570-18

[42] Netea M, Quintin J, van der Meer JW. Trained immunity: A memory for innate host defense. Cell Host & Microbe. 2011;**9**(5):355-361. DOI: 10.1016/j.chom.2011.04.006

[43] Durrant WE, Dong X. Systemic acquired resistance. Annual Review of Phytopathology. 2004;**42**:185-209. DOI: 10.1146/annurev.phyto.42.040803.140421

[44] Pham LN, Dionne MS, Shirasu-Hiza M, Schneider DS. A specific primed immune response in Drosophila is dependent on phagocytes. PLoS Pathogens. 2007;**3**(3):e26. DOI: 10.1371/journal.ppat.0030026

[45] Bistoni F, Vecchiarelli A, Cenci E, Puccetti P, Marconi P, Cassone A. Evidence for macrophage-mediated protection against lethal *Candida albicans* infection. Infection and Immunity. 1986;**51**(2):668-674

[46] Quintin J, Saeed S, Martens JHA, Giamarellos-Bourboulis EJ, Ifrim DC, Logie C, et al. *Candida albicans* infection affords protection against reinfection via functional reprogramming of monocytes. Cell Host & Microbe. 2012;**12**(2):223-232. DOI: 10.1016/j.chom.2012.06.006

[47] Akramiene D, Kondrotas A, Didziapetriene J, Kevelaitis E. Effects of beta-glucans on the immune system. Medicina (Kaunas, Lithuania). 2007;**43**(8):597-606

[48] Rizzetto L, Ifrim DC, Moretti S, Tocci N, Cheng S-C, Quintin J, et al. Fungal chitin induces trained immunity in human monocytes during cross-talk of the host with *Saccharomyces cerevisiae*. The Journal of Biological Chemistry. 2016;**291**(15):7961-7972. DOI: 10.1074/jbc.M115.699645

[49] Garcia-Valtanen P, Guzman-Genuino RM, Williams DL, Hayball JD, Diener KR. Evaluation of trained immunity by beta-1, 3 (d)-glucan on murine monocytes in vitro and duration of response in vivo. Immunology and Cell Biology. 2017;**95**(7):601-610. DOI: 10.1038/icb.2017.13

[50] Ifrim DC, Quintin J, Meerstein-Kessel L, Plantinga TS, Joosten LA, van der Meer JW, et al. Defective trained immunity in patients with STAT-1-dependent chronic mucocutaneaous candidiasis. Clinical and Experimental Immunology. 2015;**181**(3):434-440. DOI: 10.1111/cei.12642

[51] Alexander MP, Fiering SN, Ostroff GR, Cramer RA, Mullins DW. Beta-glucan-induced trained innate immunity mediates antitumor efficacy in the mouse lung. The Journal of Immunology. 2016;**196**(1 Supplement):142.124

[52] Ferrante A. Tumor necrosis factor alpha potentiates neutrophil antimicrobial activity: Increased fungicidal activity against Torulopsis glabrata and *Candida albicans* and associated increases in oxygen radical production and lysosomal enzyme release. Infection and Immunity. 1989b;**57**(7):2115-2122

[53] Thompson HL, Wilton JM. Interaction and intracellular killing of *Candida albicans* blastospores by human polymorphonuclear leucocytes, monocytes and monocyte-derived macrophages in aerobic and anaerobic conditions. Clinical and Experimental Immunology. 1992;**87**(2):316-321

[54] Leijh PCJ, van den Barselaar MT, van Furth R. Kinetics of phagocytosis and intracellular killing of *Candida albicans* by human granulocytes and monocytes. Infection and Immunity. 1977;**17**(2):313-318

[55] Vázquez-Torres A, Balish E. Macrophages in resistance to candidiasis. Microbiology and Molecular Biology Reviews. 1997;**61**(2):170-192

[56] Lehrer RI. The fungicidal mechanisms of human monocytes. I. Evidence for myeloperoxidase-linked and myeloperoxidase-independent candidacidal mechanisms. The Journal of Clinical Investigation. 1975;**55**(2):338-346. DOI: 10.1172/JCI107937

[57] Lehrer RI, Cline MJ. Leukocyte myeloperoxidase deficiency and disseminated candidiasis: The role of myeloperoxidase in resistance to Candida infection. The Journal of Clinical Investigation. 1969;**48**(8):1478-1488. DOI: 10.1172/JCI106114

[58] Lefkowitz SS, Gelderman MP, Lefkowitz DL, Moguilevsky N, Bollen A. Phagocytosis and intracellular killing of *Candida albicans* by macrophages exposed to myeloperoxidase. The Journal of Infectious Diseases. 1996;**173**(5):1202-1207

[59] Aratani Y, Kura F, Watanabe H, Akagawa H, Takano Y, Suzuki K, et al. Relative contributions of myeloperoxidase and NADPH-oxidase to the early host defense against

pulmonary infections with *Candida albicans* and *Aspergillus fumigatus*. Medical Mycology. 2002;**40**(6):557-563

[60] Diamond RD, Krzesicki R, Jao W. Damage to pseudohyphal forms of *Candida albicans* by neutrophils in the absence of serum in vitro. The Journal of Clinical Investigation. 1978;**61**(2):349-359. DOI: 10.1172/jci108945

[61] Gazendam RP, van Hamme JL, Tool ATJ, van Houdt M, Verkuijlen PJJH, Herbst M, et al. Two independent killing mechanisms of *Candida albicans* by human neutrophils: Evidence from innate immunity defects. Blood. 2014;**124**(4):590

[62] Hii CST, Stacey K, Moghaddami N, Murray AW, Ferrante A. Role of the extracellular signal-regulated protein kinase Cascade in human neutrophil killing of *Staphylococcus aureus* and *Candida albicans* and in migration. Infection and Immunity. 1999;**67**(3):1297-1302

[63] Li X, Cullere X, Nishi H, Saggu G, Durand E, Mansour MK, et al. PKC-delta activation in neutrophils promotes fungal clearance. Journal of Leukocyte Biology. 2016;**100**(3):581-588. DOI: 10.1189/jlb.4A0915-405R

[64] Strijbis K, Tafesse FG, Fairn GD, Witte MD, Dougan SK, Watson N, et al. Bruton's tyrosine kinase (BTK) and Vav1 contribute to Dectin1-dependent phagocytosis of *Candida albicans* in macrophages. PLoS Pathogens. 2013;**9**(6):e1003446. DOI: 10.1371/journal.ppat.1003446

[65] Steinberg BE, Grinstein S. Unconventional roles of the NADPH oxidase: Signaling, ion homeostasis, and cell death. Science's STKE. 2007;**2007**(379):pe11. DOI: 10.1126/stke.3792007pe11

[66] Byrd AS, O'Brien XM, Johnson CM, Lavigne LM, Reichner JS. An extracellular matrix-based mechanism of rapid neutrophil extracellular trap formation in response to *Candida albicans*. Journal of Immunology. 2013;**190**(8):4136-4148. DOI: 10.4049/jimmunol.1202671

[67] Kenno S, Perito S, Mosci P, Vecchiarelli A, Monari C. Autophagy and reactive oxygen species are involved in neutrophil extracellular traps release induced by *C. albicans* morphotypes. Frontiers in Microbiology. 2016;**7**:879

[68] Kumaratilake LM, Ferrante A, Kumaratilake JS, Allison AC. Extraction of intraerythrocytic malarial parasites by phagocytic cells. Parasitology Today. 1994;**10**(5):193-196. DOI: 10.1016/0169-4758(94)90029-9

[69] Ferrante A, Mocatta TJ. Human neutrophils require activation by mononuclear leucocyte conditioned medium to kill the pathogenic free-living amoeba, *Naegleria fowleri*. Clinical and Experimental Immunology. 1984;**56**(3):559-566

[70] Ferrante A. Augmentation of the neutrophil response to *Naegleria fowleri* by tumor necrosis factor alpha. Infection and Immunity. 1989a;**57**(10):3110-3115

[71] Ferrante A. In: Coffey RG, editor. Activation of Neutrophils by Interleukins-1 and -2 and Tumour Necrosis Factors. New York, USA: Marcel Dekker Inc; 1992. pp. 417-436

[72] Murphy JW, Wu-Hsieh BA, Singer-Vermes LM, Ferrante A, Moser S, Russo M, et al. Cytokines in the host response to mycotic agents. Journal of Medical and Veterinary Mycology. 1994;**32**(Suppl 1):203-210

[73] Kowanko IC, Ferrante A, Harvey DP, Carman KL. Granulocyte-macrophage colony-stimulating factor augments neutrophil killing of *Torulopsis glabrata* and stimulates neutrophil respiratory burst and degranulation. Clinical and Experimental Immunology. 1991;**83**(2):225-230

[74] Kowanko IC, Ferrante A, Clemente G, Kumaratilake LM. Tumor necrosis factor primes neutrophils to kill *Staphylococcus aureus* by an oxygen-dependent mechanism and *Plasmodium falciparum* by an oxygen-independent mechanism. Infection and Immunity. 1996;**64**(8):3435-3437

[75] Ferrante A, Hill NL, Abell TJ, Pruul H. Role of myeloperoxidase in the killing of *Naegleria fowleri* by lymphokine-altered human neutrophils. Infection and Immunity. 1987;**55**(5):1047-1050

[76] Ferrante A, Martin AJ, Bates EJ, Goh DH, Harvey DP, Parsons D, et al. Killing of *Staphylococcus aureus* by tumor necrosis factor-alpha-activated neutrophils. The role of serum opsonins, integrin receptors, respiratory burst, and degranulation. Journal of Immunology. 1993;**151**(9):4821-4828

[77] Rathjen DA, Ferrante A, Aston R. Differential effects of small tumour necrosis factor-alpha peptides on tumour cell cytotoxicity, neutrophil activation and endothelial cell procoagulant activity. Immunology. 1993;**80**(2):293-299

[78] Kumaratilake LM, Rathjen DA, Mack P, Widmer F, Prasertsiriroj V, Ferrante A. A synthetic tumor necrosis factor-alpha agonist peptide enhances human polymorphonuclear leukocyte-mediated killing of *Plasmodium falciparum* in vitro and suppresses *Plasmodium chabaudi* infection in mice. Journal of Clinical Investigation. 1995;**95**(5):2315-2323

[79] Britton WJ, Meadows N, Rathjen DA, Roach DR, Briscoe H. A tumor necrosis factor mimetic peptide activates a murine macrophage cell line to inhibit mycobacterial growth in a nitric oxide-dependent fashion. Infection and Immunity. 1998;**66**(5):2122-2127

[80] Roach DR, Briscoe H, Baumgart K, Rathjen DA, Britton WJ. Tumor necrosis factor (TNF) and a TNF-mimetic peptide modulate the granulomatous response to *Mycobacterium bovis* BCG infection in vivo. Infection and Immunity. 1999;**67**(10):5473-5476

[81] Mukaro VR et al. Small tumor necrosis factor receptor biologics inhibit the tumor necrosis factor-p38 signalling axis and inflammation. Nature Communications. 2018;**9**(1):1365

[82] Maródi L, Schreiber S, Anderson DC, MacDermott RP, Korchak HM, Johnston RB. Enhancement of macrophage candidacidal activity by interferon-gamma. Increased phagocytosis, killing, and calcium signal mediated by a decreased number of mannose receptors. Journal of Clinical Investigation. 1993;**91**(6):2596-2601

[83] Brummer E, Morrison CJ, Stevens DA. Recombinant and natural gamma-interferon activation of macrophages in vitro: Different dose requirements for induction of killing activity against phagocytizable and nonphagocytizable fungi. Infection and Immunity. 1985;**49**(3):724-730

[84] Roilides E, Lyman CA, Sein T, Gonzalez C, Walsh TJ. Antifungal activity of splenic, liver and pulmonary macrophages against *Candida albicans* and effects of macrophage colony-stimulating factor. Medical Mycology. 2000;**38**(2):161-168

[85] Kumaratilake LM, Ferrante A. IL-4 inhibits macrophage-mediated killing of *Plasmodium falciparum* in vitro. A possible parasite-immune evasion mechanism. Journal of Immunology. 1992;**149**(1):194-199

[86] Bousfiha A, Jeddane L, Picard C, Ailal F, Bobby Gaspar H, Al-Herz W, et al. The 2017 IUIS phenotypic classification for primary immunodeficiencies. Journal of Clinical Immunology. 2018;**38**(1):129-143. DOI: 10.1007/s10875-017-0465-8

[87] Antachopoulos C. Invasive fungal infections in congenital immunodeficiencies. Clinical Microbiology and Infection. 2010;**16**(9):1335-1342. DOI: 10.1111/j.1469-0691.2010.03289.x

[88] Donadieu J, Lamant M, Fieschi C, Sicre de Fontbrune F, Caye A, Ouachee M, et al. Natural history of GATA2 deficiency in a survey of 79 French and Belgian patients. Haematologica. 2018;**103**(8):1278-1287

[89] Rider NL, Jameson MB, Creech CB. Chronic granulomatous disease: Epidemiology, pathophysiology, and genetic basis of disease. Journal of the Pediatric Infectious Diseases Society. 2018;**7**(suppl_1):S2-S5. DOI: 10.1093/jpids/piy008

[90] Winkelstein JA, Marino MC, Johnston RB, Boyle J, Curnutte J, Gallin JI, et al. Chronic granulomatous disease. Report on a national registry of 368 patients. Medicine (Baltimore). 2000;**79**(3):155-169

[91] van de Geer A, Nieto-Patlan A, Kuhns DB, Tool AT, Arias AA, Bouaziz M, et al. Inherited p40phox deficiency differs from classic chronic granulomatous disease. The Journal of Clinical Investigation. 2018;**128**(9):3957-3975. DOI: 10.1172/jci97116

[92] Kuhns DB, Alvord WG, Heller T, Feld JJ, Pike KM, Marciano BE, et al. Residual NADPH oxidase and survival in chronic granulomatous disease. The New England Journal of Medicine. 2010;**363**(27):2600-2610. DOI: 10.1056/NEJMoa1007097

[93] Keller MD, Notarangelo LD, Malech HL. Future of care for patients with chronic granulomatous disease: Gene therapy and targeted molecular medicine. Journal of the Pediatric Infectious Diseases Society. 2018;**7**(suppl_1):S40-S44. DOI: 10.1093/jpids/piy011

[94] Milligan KL, Mann D, Rump A, Anderson VL, Hsu AP, Kuhns DB, et al. Complete myeloperoxidase deficiency: Beware the "false-positive" dihydrorhodamine oxidation. The Journal of Pediatrics. 2016;**176**:204-206. DOI: 10.1016/j.jpeds.2016.05.047

[95] Spinner MA, Sanchez LA, Hsu AP, Shaw PA, Zerbe CS, Calvo KR, et al. GATA2 deficiency: A protean disorder of hematopoiesis, lymphatics, and immunity. Blood. 2014;**123**(6):809-821. DOI: 10.1182/blood-2013-07-515528

[96] Vinh DC, Patel SY, Uzel G, Anderson VL, Freeman AF, Olivier KN, et al. Autosomal dominant and sporadic monocytopenia with susceptibility to mycobacteria, fungi, papillomaviruses, and myelodysplasia. Blood. 2010;**115**(8):1519-1529

[97] Li J, Vinh DC, Casanova J-L, Puel A. Inborn errors of immunity underlying fungal diseases in otherwise healthy individuals. Current Opinion in Microbiology. 2017;**40**:46-57. DOI: 10.1016/j.mib.2017.10.016

[98] Lanternier F, Cypowyj S, Picard C, Bustamante J, Lortholary O, Casanova JL, et al. Primary immunodeficiencies underlying fungal infections. Current Opinion in Pediatrics. 2013;**25**(6):736-747. DOI: 10.1097/mop.0000000000000031

[99] Alves de Medeiros AK, Lodewick E, Bogaert DJ, Haerynck F, Van Daele S, Lambrecht B, et al. Chronic and invasive fungal infections in a family with CARD9 deficiency. Journal of Clinical Immunology. 2016;**36**(3):204-209. DOI: 10.1007/s10875-016-0255-8

[100] Conti HR, Bruno VM, Childs EE, Daugherty S, Hunter JP, Mengesha BG, et al. IL-17 receptor signaling in oral epithelial cells is critical for protection against oropharyngeal candidiasis. Cell Host & Microbe. 2016;**20**(5):606-617. DOI: 10.1016/j.chom.2016.10.001

[101] Patel DD, Kuchroo VK. Th17 cell pathway in human immunity: Lessons from genetics and therapeutic interventions. Immunity. 2015;**43**(6):1040-1051. DOI: 10.1016/j.immuni.2015.12.003

[102] Okada S, Markle JG, Deenick EK, Mele F, Averbuch D, Lagos M, et al. Impairment of immunity to *Candida* and *Mycobacterium* in humans with bi-allelic *RORC* mutations. Science. 2015;**349**(6248):606

[103] Albuquerque JATD, Banerjee PP, Castoldi A, Ma R, Zurro NB, Ynoue LH, et al. The role of AIRE in the immunity against *Candida albicans* in a model of human macrophages. Frontiers in Immunology. 2018;**9**(567). DOI: 10.3389/fimmu.2018.00567

[104] Teoh F, Pavelka N. How chemotherapy increases the risk of systemic candidiasis in cancer patients: Current paradigm and future directions. Pathogens. 2016;**5**(1). DOI: 10.3390/pathogens5010006

[105] Coutinho AE, Chapman KE. The anti-inflammatory and immunosuppressive effects of glucocorticoids, recent developments and mechanistic insights. Molecular and Cellular Endocrinology. 2011;**335**(1):2-13

[106] Pilmis B, Puel A, Lortholary O, Lanternier F. New clinical phenotypes of fungal infections in special hosts. Clinical Microbiology and Infection. 2016;**22**(8):681-687. DOI: 10.1016/j.cmi.2016.05.016

[107] Celebi S, Hacimustafaoglu M, Koksal N, Ozkan H, Cetinkaya M, Ener B. Neonatal candidiasis: Results of an 8 year study. Pediatrics International. 2012;**54**(3):341-349. DOI: 10.1111/j.1442-200X.2012.03574.x

[108] Thong YH, Ferrante A, Ness D. Neutrophil phagocytic and bactericidal dysfunction induced by bilirubin. Australian Paediatric Journal. 1977;**13**(4):287-289

[109] Ferrante A, Thong YH. Amphotericin B-induced immunosuppression in tumor-bearing mice. International Journal of Immunopharmacology. 1979;**1**(4):299-301

[110] Rowan-Kelly B, Ferrante A, Thong YH. Modification of polymorphonuclear leucocyte function by imidazoles. International Journal of Immunopharmacology. 1984;**6**(4):389-393. DOI: 10.1016/0192-0561(84)90059-6

[111] Thong YH, Ferrante A, Secker LK. Suppression of delayed-type hypersensitivity in tumour-bearing mice by treatment with miconazole. Immunology Letters. 1980;**2**(2):119-121. DOI: 10.1016/0165-2478(80)90062-0

# References

Aguzzi, A., Kranich, J., & Krautler, N. J. (2014). Follicular dendritic cells: origin, phenotype, and function in health and disease. *Trends Immunol, 35*(3), 105-113. doi:10.1016/j.it.2013.11.001

Aida, Y., & Pabst, M. J. (1990). Priming of neutrophils by lipopolysaccharide for enhanced release of superoxide. Requirement for plasma but not for tumor necrosis factor-alpha. *J Immunol, 145*(9), 3017-3025.

Almarza Novoa, E., Kasbekar, S., Thrasher, A. J., Kohn, D. B., Sevilla, J., Nguyen, T., . . . Bueren, J. A. (2018). Leukocyte adhesion deficiency-I: A comprehensive review of all published cases. *The Journal of Allergy and Clinical Immunology: In Practice, 6*(4), 1418-1420.e1410. doi:10.1016/j.jaip.2017.12.008

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology, 215*(3), 403-410. doi:https://doi.org/10.1016/S0022-2836(05)80360-2

Amulic, B., Cazalet, C., Hayes, G. L., Metzler, K. D., & Zychlinsky, A. (2012). Neutrophil Function: From Mechanisms to Disease. *Annu Rev Immunol, 30*(1), 459-489. doi:10.1146/annurev-immunol-020711-074942

Belambri, S. A., Rolas, L., Raad, H., Hurtado-Nedelec, M., Dang, P. M., & El-Benna, J. (2018). NADPH oxidase activation in neutrophils: Role of the phosphorylation of its subunits. *Eur J Clin Invest, 48 Suppl 2*, e12951. doi:10.1111/eci.12951

Bianchi-Frias, D., Damodarasamy, M., Hernandez, S. A., Gil da Costa, R. M., Vakar-Lopez, F., Coleman, I. M., . . . Nelson, P. S. (2019). The Aged Microenvironment Influences the Tumorigenic Potential of Malignant Prostate Epithelial Cells. *Mol Cancer Res, 17*(1), 321-331. doi:10.1158/1541-7786.Mcr-18-0522

Borregaard, N., Heiple, J. M., Simons, E. R., & Clark, R. A. (1983). Subcellular localization of the b-cytochrome component of the human neutrophil microbicidal oxidase: translocation during activation. *J Cell Biol, 97*(1), 52-61.

Boyette, L. B., Macedo, C., Hadi, K., Elinoff, B. D., Walters, J. T., Ramaswami, B., . . . Metes, D. M. (2017). Phenotype, function, and differentiation potential of human monocyte subsets. *PLOS ONE, 12*(4), e0176460. doi:10.1371/journal.pone.0176460

Broadley, S. P., Plaumann, A., Coletti, R., Lehmann, C., Wanisch, A., Seidlmeier, A., . . . Verschoor, A. (2016). Dual-Track Clearance of Circulating Bacteria Balances Rapid Restoration of Blood Sterility with Induction of Adaptive Immunity. *Cell Host Microbe, 20*(1), 36-48. doi:10.1016/j.chom.2016.05.023

Buderath, P., Schwich, E., Jensen, C., Horn, P. A., Kimmig, R., Kasimir-Bauer, S., & Rebmann, V. (2019). Soluble Programmed Death Receptor Ligands sPD-L1 and sPD-L2 as Liquid Biopsy Markers for Prognosis and Platinum Response in Epithelial Ovarian Cancer. *Frontiers in Oncology, 9*(1015). doi:10.3389/fonc.2019.01015

Byun, J. M., Jeong, D. H., Choi, I. H., Lee, D. S., Kang, M. S., Jung, K. O., . . . Kim, K. T. (2017). The Significance of VSIG4 Expression in Ovarian Cancer. *Int J Gynecol Cancer, 27*(5), 872-878. doi:10.1097/igc.0000000000000979

Calvo, K. R., Hickstein, D. D., & Holland, S. M. (2012). MonoMAC and GATA2 deficiency: overlapping clinical and pathological features with aplastic anemia and idiopathic CD4+ lymphocytopenia. Reply to Haematologica 2012;97(4):058669. *Haematologica, 97*(4), e12-e13. doi:10.3324/haematol.2012.063396

Cassatella, M. A., Östberg, N. K., Tamassia, N., & Soehnlein, O. (2019). Biological Roles of Neutrophil-Derived Granule Proteins and Cytokines. *Trends Immunol, 40*(7), 648-664. doi:10.1016/j.it.2019.05.003

Chaiworapongsa, T., Romero, R., Whitten, A., Tarca, A. L., Bhatti, G., Draghici, S., . . . Hassan, S. S. (2013). Differences and similarities in the transcriptional profile of peripheral whole blood in early and late-onset preeclampsia: insights into the molecular basis of the phenotype of preeclampsiaa. *J Perinat Med, 41*(5), 485-504. doi:10.1515/jpm-2013-0082

Chaplin, D. D. (2010). Overview of the immune response. *The Journal of allergy and clinical immunology, 125*(2 Suppl 2), S3-S23. doi:10.1016/j.jaci.2009.12.980

Chen, J., Crispín, J. C., Dalle Lucca, J., & Tsokos, G. C. (2011). A Novel Inhibitor of the Alternative Pathway of Complement Attenuates Intestinal Ischemia/Reperfusion-Induced Injury. *Journal of Surgical Research, 167*(2), e131-e136. doi:https://doi.org/10.1016/j.jss.2009.05.041

Chen, M., Muckersie, E., Luo, C., Forrester, J. V., & Xu, H. (2010). Inhibition of the alternative pathway of complement activation reduces inflammation in experimental autoimmune uveoretinitis. *European Journal of Immunology, 40*(10), 2870-2881. doi:10.1002/eji.201040323

Clark, R., & Kupper, T. (2005). Old Meets New: The Interaction Between Innate and Adaptive Immunity. *Journal of Investigative Dermatology, 125*(4), 629-637. doi:http://dx.doi.org/10.1111/j.0022-202X.2005.23856.x

Coillard, A., & Segura, E. (2019). In vivo Differentiation of Human Monocytes. *Frontiers in Immunology, 10*(1907). doi:10.3389/fimmu.2019.01907

Cowland, J. B., & Borregaard, N. (2016). Granulopoiesis and granules of human neutrophils. *Immunol Rev, 273*(1), 11-28. doi:10.1111/imr.12440

Crawford, J. M., Bioulac-Sage, P., & Hytiroglou, P. (2018). 1 - Structure, Function, and Responses to Injury. In A. D. Burt, L. D. Ferrell, & S. G. Hübscher (Eds.), *Macsween's Pathology of the Liver (Seventh Edition)* (pp. 1-87): Elsevier.

Crispe, I. N. (2011). Liver antigen-presenting cells. *Journal of hepatology, 54*(2), 357-365. doi:10.1016/j.jhep.2010.10.005

Croize, J., Arvieux, J., Berche, P., & Colomb, M. G. (1993). Activation of the human complement alternative pathway by Listeria monocytogenes: evidence for direct binding and proteolysis of the C3 component on bacteria. *Infect Immun, 61*(12), 5134.

Culemann, S., Gruneboom, A., Nicolas-Avila, J. A., Weidner, D., Lammle, K. F., Rothe, T., . . . Kronke, G. (2019). Locally renewing resident synovial macrophages provide a protective barrier for the joint. *Nature, 572*(7771), 670-675. doi:10.1038/s41586-019-1471-1

Das, A., Heesters, B. A., Bialas, A., O'Flynn, J., Rifkin, I. R., Ochando, J., . . . Carroll, M. C. (2017). Follicular Dendritic Cell Activation by TLR Ligands Promotes Autoreactive B Cell Responses. *Immunity, 46*(1), 106-119. doi:https://doi.org/10.1016/j.immuni.2016.12.014

Doyle, A. G., Herbein, G., Montaner, L. J., Minty, A. J., Caput, D., Ferrara, P., & Gordon, S. (1994). Interleukin-13 alters the activation state of murine macrophages in vitro: comparison with interleukin-4 and interferon-gamma. *Eur J Immunol, 24*(6), 1441-1445. doi:10.1002/eji.1830240630

Du Clos, T. W., & Mold, C. (2008). 20 - Complement and complement deficiencies A2 - Rich, Robert R. In T. A. Fleisher, W. T. Shearer, H. W. Schroeder, A. J. Frew, & C. M. Weyand (Eds.), *Clinical Immunology (Third Edition)* (pp. 305-325). Edinburgh: Mosby.

Epelman, S., Lavine, Kory J., & Randolph, Gwendalyn J. (2014). Origin and Functions of Tissue Macrophages. *Immunity, 41*(1), 21-35. doi:10.1016/j.immuni.2014.06.013

Fallman, M., Andersson, R., & Andersson, T. (1993). Signaling properties of CR3 (CD11b/CD18) and CR1 (CD35) in relation to phagocytosis of complement-opsonized particles. *J Immunol, 151*(1), 330-338.

Ferrante, A. (1992). Activation of neutrophils by interleukins-1 and -2 and tumor necrosis factors. *Immunol Ser, 57*, 417-436.

Ferrante, A. (2005). *Neutrophils* (S. Kaufmann, and Steward, M. Ed. 10 ed.). London: Hodder Arnold.

Ferrante, A. (2010). Phagocytes Part 2: Neutrophils *Topley & Wilson's Microbiology and Microbial Infections*.

Ferrante, A., Martin, A. J., Bates, E. J., Goh, D. H., Harvey, D. P., Parsons, D., . . . Dayer, J. M. (1993). Killing of Staphylococcus aureus by tumor necrosis factor-alpha-activated neutrophils. The role of serum opsonins, integrin receptors, respiratory burst, and degranulation. *J Immunol, 151*(9), 4821-4828.

Ferrante, A., & Thong, Y. H. (1982). Separation of mononuclear and polymorphonuclear leucocytes from human blood by the one-step Hypaque-Ficoll method is dependent on blood column height. *J Immunol Methods, 48*(1), 81-85. doi:10.1016/0022-1759(82)90212-5

Fridlender, Z. G., Sun, J., Kim, S., Kapoor, V., Cheng, G., Ling, L., . . . Albelda, S. M. (2009). Polarization of Tumor-Associated Neutrophil Phenotype by TGF-β: "N1" versus "N2" TAN. *Cancer Cell, 16*(3), 183-194. doi:https://doi.org/10.1016/j.ccr.2009.06.017

Fu, W., Wojtkiewicz, G., Weissleder, R., Benoist, C., & Mathis, D. (2012). Early window of diabetes determinism in NOD mice, dependent on the complement receptor CRIg, identified by noninvasive imaging. *Nature immunology, 13*(4), 361-368. doi:10.1038/ni.2233

Gasteiger, E., Gattiker, A., Hoogland, C., Ivanyi, I., Appel, R. D., & Bairoch, A. (2003). ExPASy: The proteomics server for in-depth protein knowledge and analysis. *Nucleic Acids Res, 31*(13), 3784-3788.

Gasteiger, E., Hoogland, C., Gattiker, A., Duvaud, S. e., Wilkins, M. R., Appel, R. D., & Bairoch, A. (2005). Protein Identification and Analysis Tools on the ExPASy Server. In J. M. Walker (Ed.), *The Proteomics Protocols Handbook* (pp. 571-607). Totowa, NJ: Humana Press.

Geijtenbeek, T. B., van Vliet, S. J., Engering, A., t Hart, B. A., & van Kooyk, Y. (2004). Self- and nonself-recognition by C-type lectins on dendritic cells. *Annu Rev Immunol, 22*, 33-54. doi:10.1146/annurev.immunol.22.012703.104558

Gentek, R., Molawi, K., & Sieweke, M. H. (2014). Tissue macrophage identity and self-renewal. *Immunol Rev, 262*(1), 56-73. doi:10.1111/imr.12224

Gordon, S. (2016). Phagocytosis: An Immunobiologic Process. *Immunity, 44*(3), 463-475. doi:10.1016/j.immuni.2016.02.026

Gorgani, N. N., He, J. Q., Katschke, K. J., Jr., Helmy, K. Y., Xi, H., Steffek, M., . . . van Lookeren Campagne, M. (2008). Complement receptor of the Ig superfamily enhances complement-mediated phagocytosis in a subpopulation of tissue resident macrophages. *J Immunol, 181*(11), 7902-7908.

Gorgani, N. N., Thathaisong, U., Mukaro, V. R., Poungpair, O., Tirimacco, A., Hii, C. S., & Ferrante, A. (2011). Regulation of CRIg expression and phagocytosis in human macrophages by arachidonate, dexamethasone, and cytokines. *Am J Pathol, 179*(3), 1310-1318. doi:10.1016/j.ajpath.2011.05.021

Grayson, P. C., Schauer, C., Herrmann, M., & Kaplan, M. J. (2016). Review: Neutrophils as Invigorated Targets in Rheumatic Diseases. *Arthritis & rheumatology (Hoboken, N.J.), 68*(9), 2071-2082. doi:10.1002/art.39745

Gregory, S. H., Cousens, L. P., van Rooijen, N., Döpp, E. A., Carlos, T. M., & Wing, E. J. (2002). Complementary adhesion molecules promote neutrophil-Kupffer cell interaction and the elimination of bacteria taken up by the liver. *J Immunol, 168*(1), 308-315. doi:10.4049/jimmunol.168.1.308

Gregory, S. H., Sagnimeni, A. J., & Wing, E. J. (1996). Bacteria in the bloodstream are trapped in the liver and killed by immigrating neutrophils. *J Immunol, 157*(6), 2514-2520.

Gu, D., Ao, X., Yang, Y., Chen, Z., & Xu, X. (2018). Soluble immune checkpoints in cancer: production, function and biological significance. *Journal for ImmunoTherapy of Cancer, 6*(1), 132. doi:10.1186/s40425-018-0449-0

Guo, S., Yang, C., Mei, F., Wu, S., Luo, N., Fei, L., . . . Wu, Y. (2010). Down-regulation of Z39Ig on macrophages by IFN-gamma in patients with chronic HBV infection. *Clin Immunol, 136*(2), 282-291. doi:10.1016/j.clim.2010.03.007

Harboe, M., Ulvund, G., Vien, L., Fung, M., & Mollnes, T. E. (2004). The quantitative role of alternative pathway amplification in classical pathway induced terminal complement activation. *Clin Exp Immunol, 138*(3), 439-446. doi:10.1111/j.1365-2249.2004.02627.x

He, J. Q., Katschke, K. J., Jr., Gribling, P., Suto, E., Lee, W. P., Diehl, L., . . . van Lookeren Campagne, M. (2013). CRIg mediates early Kupffer cell responses to adenovirus. *J Leukoc Biol, 93*(2), 301-306. doi:10.1189/jlb.0612311

He, J. Q., Wiesmann, C., & van Lookeren Campagne, M. (2008). A role of macrophage complement receptor CRIg in immune clearance and inflammation. *Mol Immunol, 45*(16), 4041-4047. doi:10.1016/j.molimm.2008.07.011

Headland, S. E., & Norling, L. V. (2015). The resolution of inflammation: Principles and challenges. *Seminars in Immunology, 27*(3), 149-160. doi:https://doi.org/10.1016/j.smim.2015.03.014

Helmy, K. Y., Katschke, K. J., Jr., Gorgani, N. N., Kljavin, N. M., Elliott, J. M., Diehl, L., . . . van Lookeren Campagne, M. (2006). CRIg: a macrophage complement receptor required for phagocytosis of circulating pathogens. *Cell, 124*(5), 915-927. doi:10.1016/j.cell.2005.12.039

Herrero-Turrión, M. J., Calafat, J., Janssen, H., Fukuda, M., & Mollinedo, F. (2008). Rab27a Regulates Exocytosis of Tertiary and Specific Granules in Human Neutrophils. *The Journal of Immunology, 181*(6), 3793. doi:10.4049/jimmunol.181.6.3793

Holers, V. M. (2014). Complement and Its Receptors: New Insights into Human Disease. *Annual Review of Immunology, 32*(1), 433-459. doi:10.1146/annurev-immunol-032713-120154

Holers, V. M., & Banda, N. K. (2018). Complement in the Initiation and Evolution of Rheumatoid Arthritis. *Frontiers in Immunology, 9*, 1057-1057. doi:10.3389/fimmu.2018.01057

Hopkins, A. M., Proudman, S. M., Vitry, A. I., Sorich, M. J., Cleland, L. G., & Wiese, M. D. (2016). Ten years of publicly funded biological disease-modifying antirheumatic drugs in Australia. *Med J Aust, 204*(2), 64-68.

Horst, A. K., Neumann, K., Diehl, L., & Tiegs, G. (2016). Modulation of liver tolerance by conventional and nonconventional antigen-presenting cells and regulatory immune cells. *Cellular & molecular immunology, 13*(3), 277-292. doi:10.1038/cmi.2015.112

Houghton, A. M. (2010). The paradox of tumor-associated neutrophils: Fueling tumor growth with cytotoxic substances. *Cell Cycle, 9*(9), 1732-1737. doi:10.4161/cc.9.9.11297

Hovland, A., Jonasson, L., Garred, P., Yndestad, A., Aukrust, P., Lappegard, K. T., . . . Mollnes, T. E. (2015). The complement system and toll-like receptors as integrated players in the pathophysiology of atherosclerosis. *Atherosclerosis, 241*(2), 480-494. doi:10.1016/j.atherosclerosis.2015.05.038

Howard, C. J., Charleston, B., Stephens, S. A., Sopp, P., & Hope, J. C. (2007). The role of dendritic cells in shaping the immune response. *Animal Health Research Reviews, 5*(2), 191-195. doi:10.1079/AHR200468

Huang, X., Feng, Z., Jiang, Y., Li, J., Xiang, Q., Guo, S., . . . Chen, Y. (2019). VSIG4 mediates transcriptional inhibition of Nlrp3 and Il-1beta in macrophages. *Sci Adv, 5*(1), eaau7426. doi:10.1126/sciadv.aau7426

Hunter, T. M., Boytsov, N. N., Zhang, X., Schroeder, K., Michaud, K., & Araujo, A. B. (2017). Prevalence of rheumatoid arthritis in the United States adult population in healthcare claims databases, 2004-2014. *Rheumatol Int, 37*(9), 1551-1557. doi:10.1007/s00296-017-3726-1

Ikarashi, M., Nakashima, H., Kinoshita, M., Sato, A., Nakashima, M., Miyazaki, H., . . . Seki, S. (2013). Distinct development and functions of resident and recruited liver Kupffer cells/macrophages. *J Leukoc Biol, 94*(6), 1325-1336. doi:10.1189/jlb.0313144

Irvine, K. M., Banh, X., Gadd, V. L., Wojcik, K. K., Ariffin, J. K., Jose, S., . . . Powell, E. E. (2016). CRIg-expressing peritoneal macrophages are associated with disease severity in patients with cirrhosis and ascites. *JCI Insight, 1*(8), e86914. doi:10.1172/jci.insight.86914

Ishikawa, F., Niiro, H., Iino, T., Yoshida, S., Saito, N., Onohara, S., . . . Akashi, K. (2007). The developmental program of human dendritic cells is operated independently of conventional myeloid and lymphoid pathways. *Blood, 110*(10), 3591-3660. doi:10.1182/blood-2007-02-071613

Jain, A., & Pasare, C. (2017). Innate Control of Adaptive Immunity: Beyond the Three-Signal Paradigm. *The Journal of Immunology, 198*(10), 3791. doi:10.4049/jimmunol.1602000

Jeong, H., Hwang, I., Kang, S. H., Shin, H. C., & Kwon, S. Y. (2019). Tumor-Associated Macrophages as Potential Prognostic Biomarkers of Invasive Breast Cancer. *J Breast Cancer, 22*(1), 38-51. doi:10.4048/jbc.2019.22.e5

Jimenez, C., Jara-Acevedo, M., Corchete, L. A., Castillo, D., Ordonez, G. R., Sarasquete, M. E., . . . Garcia-Sanz, R. (2017). A Next-Generation Sequencing Strategy for Evaluating the Most Common Genetic Abnormalities in Multiple Myeloma. *J Mol Diagn, 19*(1), 99-106. doi:10.1016/j.jmoldx.2016.08.004

Jongerius, I., Puister, M., Wu, J., Ruyken, M., van Strijp, J. A., & Rooijakkers, S. H. (2010). Staphylococcal complement inhibitor modulates phagocyte responses by dimerization of convertases. *J Immunol, 184*(1), 420-425. doi:10.4049/jimmunol.0902865

Ju, C., McCoy, J. P., Chung, C. J., Graf, M. L., & Pohl, L. R. (2003). Tolerogenic role of Kupffer cells in allergic reactions. *Chem Res Toxicol, 16*(12), 1514-1519. doi:10.1021/tx0341761

Jung, K., Seo, S.-K., & Choi, I. (2015). Endogenous VSIG4 negatively regulates the helper T cell-mediated antibody response. *Immunol Lett, 165*(2), 78-83. doi:https://doi.org/10.1016/j.imlet.2015.04.004

Kantari, C., Pederzoli-Ribeil, M., & Witko-Sarsat, V. (2008). The role of neutrophils and monocytes in innate immunity. *Contrib Microbiol, 15*, 118-146. doi:10.1159/000136335

Katschke, K. J., Jr., Helmy, K. Y., Steffek, M., Xi, H., Yin, J., Lee, W. P., . . . van Lookeren Campagne, M. (2007). A novel inhibitor of the alternative pathway of complement reverses inflammation and bone destruction in experimental arthritis. *The Journal of Experimental Medicine, 204*(6), 1319-1325. doi:10.1084/jem.20070432

Kelley, N., Jeltema, D., Duan, Y., & He, Y. (2019). The NLRP3 Inflammasome: An Overview of Mechanisms of Activation and Regulation. *International journal of molecular sciences, 20*(13), 3328. doi:10.3390/ijms20133328

Kim, J. K., Choi, E. M., Shin, H. I., Kim, C. H., Hwang, S. H., Kim, S. M., & Kwon, B. S. (2005). Characterization of monoclonal antibody specific to the Z39Ig protein, a member of immunoglobulin superfamily. *Immunol Lett, 99*(2), 153-161. doi:10.1016/j.imlet.2005.02.012

Kim, K. H., Choi, B. K., Song, K. M., Cha, K. W., Kim, Y. H., Lee, H., . . . Kwon, B. S. (2013). CRIg signals induce anti-intracellular bacterial phagosome activity in a chloride intracellular channel 3-dependent manner. *Eur J Immunol, 43*(3), 667-678. doi:10.1002/eji.201242997

Kubelkova, K., & Macela, A. (2019). Innate Immune Recognition: An Issue More Complex Than Expected. *Frontiers in cellular and infection microbiology, 9*(241). doi:10.3389/fcimb.2019.00241

Kuijpers, T. W., Tool, A. T. J., van der Bijl, I., de Boer, M., van Houdt, M., de Cuyper, I. M., . . . van den Berg, T. K. (2017). Combined immunodeficiency with severe inflammation and allergy caused by <em>ARPC1B</em> deficiency. *Journal of Allergy and Clinical Immunology, 140*(1), 273-277.e210. doi:10.1016/j.jaci.2016.09.061

Kumaratilake, L. M., & Ferrante, A. (1992). IL-4 inhibits macrophage-mediated killing of Plasmodium falciparum in vitro. A possible parasite-immune evasion mechanism. *J Immunol, 149*(1), 194-199.

Lacy, P. (2006). Mechanisms of degranulation in neutrophils. *Allergy, asthma, and clinical immunology : official journal of the Canadian Society of Allergy and Clinical Immunology, 2*(3), 98-108. doi:10.1186/1710-1492-2-3-98

Langnaese, K., Colleaux, L., Kloos, D. U., Fontes, M., & Wieacker, P. (2000). Cloning of Z39Ig, a novel gene with immunoglobulin-like domains located on human chromosome X. *Biochim Biophys Acta, 1492*(2-3), 522-525.

Lee, M. Y., Kim, W. J., Kang, Y. J., Jung, Y. M., Kang, Y. M., Suk, K., . . . Lee, W. H. (2006). Z39Ig is expressed on macrophages and may mediate inflammatory reactions in arthritis and atherosclerosis. *J Leukoc Biol, 80*(4), 922-928. doi:10.1189/jlb.0306160

Leffler, J., Bengtsson, A. A., & Blom, A. M. (2014). The complement system in systemic lupus erythematosus: an update. *Annals of the Rheumatic Diseases, 73*(9), 1601. doi:10.1136/annrheumdis-2014-205287

Leon, B., & Ardavin, C. (2008). Monocyte-derived dendritic cells in innate and adaptive immunity. *Immunol Cell Biol, 86*(4), 320-324. doi:10.1038/icb.2008.14

Li, J., Diao, B., Guo, S., Huang, X., Yang, C., Feng, Z., . . . Wu, Y. (2017). VSIG4 inhibits proinflammatory macrophage activation by reprogramming mitochondrial pyruvate metabolism. *Nature Communications, 8*(1), 1322. doi:10.1038/s41467-017-01327-4

Li, K., Fazekasova, H., Wang, N., Sagoo, P., Peng, Q., Khamri, W., . . . Zhou, W. (2011). Expression of complement components, receptors and regulators by human dendritic cells. *Molecular Immunology, 48*(9-10), 1121-1127. doi:10.1016/j.molimm.2011.02.003

Li, Y., Wang, W., Yang, F., Xu, Y., Feng, C., & Zhao, Y. (2019). The regulatory roles of neutrophils in adaptive immunity. *Cell Communication and Signaling, 17*(1), 147. doi:10.1186/s12964-019-0471-y

Liao, Y., Guo, S., Chen, Y., Cao, D., Xu, H., Yang, C., . . . Ruan, Z. (2014). VSIG4 expression on macrophages facilitates lung cancer development. *Lab Invest, 94*(7), 706-715. doi:10.1038/labinvest.2014.73

Lieberman, L. A., Mizui, M., Nalbandian, A., Bosse, R., Crispin, J. C., & Tsokos, G. C. (2015). Complement receptor of the immunoglobulin superfamily reduces murine lupus nephritis and cutaneous disease. *Clin Immunol, 160*(2), 286-291. doi:10.1016/j.clim.2015.05.006

Lin, A., & Loré, K. (2017). Granulocytes: New Members of the Antigen-Presenting Cell Family. *Frontiers in Immunology, 8*, 1781-1781. doi:10.3389/fimmu.2017.01781

Lin, C. C., & Edelson, B. T. (2017). New Insights into the Role of IL-1beta in Experimental Autoimmune Encephalomyelitis and Multiple Sclerosis. *J Immunol, 198*(12), 4553-4560. doi:10.4049/jimmunol.1700263

Liu, G., Fu, Y., Yosri, M., Chen, Y., Sun, P., Xu, J., . . . Shi, M. (2019). CRIg plays an essential role in intravascular clearance of bloodborne parasites by interacting with complement. *Proc Natl Acad Sci U S A*. doi:10.1073/pnas.1913443116

Liu, Y., & Cao, X. (2015). The origin and function of tumor-associated macrophages. *Cellular & molecular immunology, 12*(1), 1-4. doi:10.1038/cmi.2014.83

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *J Biol Chem, 193*(1), 265-275.

Lukácsi, S., Nagy-Baló, Z., Erdei, A., Sándor, N., & Bajtay, Z. (2017). The role of CR3 (CD11b/CD18) and CR4 (CD11c/CD18) in complement-mediated phagocytosis and podosome formation by human phagocytes. *Immunology Letters, 189*, 64-72. doi:https://doi.org/10.1016/j.imlet.2017.05.014

Ma, Y., Usuwanthim, K., Munawara, U., Quach, A., Gorgani, N. N., Abbott, C. A., . . . Ferrante, A. (2015). Protein Kinase Cα Regulates the Expression of Complement Receptor Ig in Human Monocyte–Derived Macrophages. *The Journal of Immunology, 194*(6), 2855.

Mackaness, G. B. (1962). Cellular resistance to infection. *J Exp Med, 116*, 381-406.

Mantegazza, A. R., Magalhaes, J. G., Amigorena, S., & Marks, M. S. (2013). Presentation of phagocytosed antigens by MHC class I and II. *Traffic (Copenhagen, Denmark), 14*(2), 135-152. doi:10.1111/tra.12026

Mantovani, A., Cassatella, M. A., Costantini, C., & Jaillon, S. (2011). Neutrophils in the activation and regulation of innate and adaptive immunity. *Nature Reviews Immunology, 11*(8), 519-531. doi:10.1038/nri3024

Mantovani, A., Sica, A., Sozzani, S., Allavena, P., Vecchi, A., & Locati, M. (2004). The chemokine system in diverse forms of macrophage activation and polarization. *Trends Immunol, 25*(12), 677-686. doi:10.1016/j.it.2004.09.015

Mathern, D. R., & Heeger, P. S. (2015). Molecules Great and Small: The Complement System. *Clin J Am Soc Nephrol, 10*(9), 1636-1650. doi:10.2215/cjn.06230614

McCormick, B., Chu, J. Y., & Vermeren, S. (2019). Cross-talk between Rho GTPases and PI3K in the neutrophil. *Small GTPases, 10*(3), 187-195. doi:10.1080/21541248.2017.1304855

Means, T. K., & Luster, A. D. (2010). Integrins limit the Toll. *Nat Immunol, 11*(8), 691-693. doi:10.1038/ni0810-691

Medzhitov, R. (2008). Origin and physiological roles of inflammation. *Nature, 454*(7203), 428-435.

Megiovanni, A. M., Sanchez, F., Robledo-Sarmiento, M., Morel, C., Gluckman, J. C., & Boudaly, S. (2006). Polymorphonuclear neutrophils deliver activation signals and antigenic molecules to dendritic cells: a new link between leukocytes upstream of T lymphocytes. *J Leukoc Biol, 79*(5), 977-988. doi:10.1189/jlb.0905526

Meinderts, S. M., Baker, G., van Wijk, S., Beuger, B. M., Geissler, J., Jansen, M. H., . . . van Bruggen, R. (2019). Neutrophils acquire antigen-presenting cell features after phagocytosis of IgG-opsonized erythrocytes. *Blood Advances, 3*(11), 1761-1773. doi:10.1182/bloodadvances.2018028753

Menyhart, O., Kakisaka, T., Pongor, L. S., Uetake, H., Goel, A., & Gyorffy, B. (2019). Uncovering Potential Therapeutic Targets in Colorectal Cancer by Deciphering Mutational Status and Expression of Druggable Oncogenes. *Cancers (Basel), 11*(7). doi:10.3390/cancers11070983

Merle, N. S., Church, S. E., Fremeaux-Bacchi, V., & Roumenina, L. T. (2015). Complement System Part I – Molecular Mechanisms of Activation and Regulation. *Frontiers in Immunology, 6*(262). doi:10.3389/fimmu.2015.00262

Merle, N. S., Noe, R., Halbwachs-Mecarelli, L., Fremeaux-Bacchi, V., & Roumenina, L. T. (2015). Complement System Part II: Role in Immunity. *Frontiers in Immunology, 6*(257). doi:10.3389/fimmu.2015.00257

Misharin, Alexander V., Cuda, Carla M., Saber, R., Turner, Jason D., Gierut, Angelica K., Haines, G. K., . . . Perlman, H. (2014). Nonclassical Ly6C− Monocytes Drive the Development of Inflammatory Arthritis in Mice. *Cell Reports, 9*(2), 591-604. doi:https://doi.org/10.1016/j.celrep.2014.09.032

Montecucco, F., Steffens, S., Burger, F., Da Costa, A., Bianchi, G., Bertolotto, M., . . . Ottonello, L. (2008). Tumor necrosis factor-alpha (TNF-alpha) induces integrin CD11b/CD18 (Mac-1) up-regulation and migration to the CC chemokine CCL3 (MIP-1alpha) on human neutrophils through defined signalling pathways. *Cell Signal, 20*(3), 557-568. doi:10.1016/j.cellsig.2007.11.008

Munawara, U., Small, A. G., Quach, A., Gorgani, N. N., Abbott, C. A., & Ferrante, A. (2017). Cytokines regulate complement receptor immunoglobulin expression and phagocytosis of Candida albicans in human macrophages: A control point in anti-

microbial immunity. *Scientific Reports, 7*(1), 4050. doi:10.1038/s41598-017-04325-0

Murray, P. J., & Wynn, T. A. (2011). Protective and pathogenic functions of macrophage subsets. *Nature reviews. Immunology, 11*(11), 723-737. doi:10.1038/nri3073

Nagre, N., Cong, X., Terrazas, C., Pepper, I., Schreiber, J. M., Fu, H., . . . Zhao, X. (2018). Inhibition of Macrophage Complement Receptor CRIg by TRIM72 Polarizes Innate Immunity of the Lung. *Am J Respir Cell Mol Biol, 58*(6), 756-766. doi:10.1165/rcmb.2017-0236OC

Nathan, C. F. M., H.W.; Wiebe, M.E.; Rubin, B.Y. (1983). Identification of interferon-gamma as the lymphokine that activates human macrophage oxidative metabolism and antimicrobial activity. *J Exp Med, 158*(3), 670-689.

Nguyen, G. T., Green, E. R., & Mecsas, J. (2017). Neutrophils to the ROScue: Mechanisms of NADPH Oxidase Activation and Bacterial Resistance. *Frontiers in cellular and infection microbiology, 7*, 373-373. doi:10.3389/fcimb.2017.00373

Nielsen, C. H., Matthiesen, S. H., Lyng, I., & Leslie, R. G. (1997). The role of complement receptor type 1 (CR1, CD35) in determining the cellular distribution of opsonized immune complexes between whole blood cells: kinetic analysis of the buffering capacity of erythrocytes. *Immunology, 90*(1), 129-137.

Niessen, H. W. M., Kuijpers, T. W., Roos, D., & Verhoeven, A. J. (1991). Release of azurophilic granule contents in fMLP-stimulated neutrophils requires two activation signals, one of which is a rise in cytosolic free Ca2+. *Cellular Signalling, 3*(6), 625-633. doi:https://doi.org/10.1016/0898-6568(91)90039-W

Noris, M., & Remuzzi, G. (2013). Overview of complement activation and regulation. *Seminars in nephrology, 33*(6), 479-492. doi:10.1016/j.semnephrol.2013.08.001

Nussenzweig, M. C., Steinman, R. M., Gutchinov, B., & Cohn, Z. A. (1980). Dendritic cells are accessory cells for the development of anti-trinitrophenyl cytotoxic T lymphocytes. *J Exp Med, 152*(4), 1070-1084.

Ogembo, J. G., Kannan, L., Ghiran, I., Nicholson-Weller, A., Finberg, R., Tsokos, G. C., & Fingeroth, J. D. (2013). Human Complement Receptor Type 1/CD35 is an Epstein-Barr Virus Receptor. *Cell reports, 3*(2), 371-385. doi:10.1016/j.celrep.2013.01.023

Paoliello-Paschoalato, A. B., Marchi, L. F., de Andrade, M. F., Kabeya, L. M., Donadi, E. A., & Lucisano-Valim, Y. M. (2015). Fcγ and Complement Receptors and Complement Proteins in Neutrophil Activation in Rheumatoid Arthritis: Contribution to Pathogenesis and Progression and Modulation by Natural Products.

*Evidence-based Complementary and Alternative Medicine : eCAM, 2015*, 429878. doi:10.1155/2015/429878
Papayannopoulos, V. (2018). Neutrophil extracellular traps in immunity and disease. *Nature Reviews Immunology, 18*(2), 134-147. doi:10.1038/nri.2017.105
Passlick, B., Flieger, D., & Ziegler-Heitbrock, H. W. (1989). Identification and characterization of a novel monocyte subpopulation in human peripheral blood. *Blood, 74*(7), 2527-2534.
Paul, William E. (2011). Bridging Innate and Adaptive Immunity. *Cell, 147*(6), 1212-1215. doi:10.1016/j.cell.2011.11.036
Pelletier, M., Maggi, L., Micheletti, A., Lazzeri, E., Tamassia, N., Costantini, C., . . . Cassatella, M. A. (2010). Evidence for a cross-talk between human neutrophils and Th17 cells. *Blood, 115*(2), 335-343. doi:10.1182/blood-2009-04-216085
Piccard, H., Muschel, R. J., & Opdenakker, G. (2012). On the dual roles and polarized phenotypes of neutrophils in tumor development and progression. *Critical Reviews in Oncology/Hematology, 82*(3), 296-309. doi:https://doi.org/10.1016/j.critrevonc.2011.06.004
Pillinger, M. H., Feoktistov, A. S., Capodici, C., Solitar, B., Levy, J., Oei, T. T., & Philips, M. R. (1996). Mitogen-activated protein kinase in neutrophils and enucleate neutrophil cytoplasts: evidence for regulation of cell-cell adhesion. *J Biol Chem, 271*(20), 12049-12056. doi:10.1074/jbc.271.20.12049
Protzer, U., Maini, M. K., & Knolle, P. A. (2012). Living in the liver: hepatic infections. *Nature Reviews Immunology, 12*, 201. doi:10.1038/nri3169
Qiao, Q., Teng, X., Wang, N., Lu, R., Guo, L., Zhang, X., . . . Hu, W. (2014). A novel CRIg-targeted complement inhibitor protects cells from complement damage. *Faseb j, 28*(11), 4986-4999. doi:10.1096/fj.14-258046
Ram, S., Lewis, L. A., & Rice, P. A. (2010). Infections of people with complement deficiencies and patients who have undergone splenectomy. *Clin Microbiol Rev, 23*(4), 740-780. doi:10.1128/CMR.00048-09
Ricklin, D., Reis, E. S., & Lambris, J. D. (2016). Complement in disease: a defence system turning offensive. *Nat Rev Nephrol, 12*(7), 383-401. doi:10.1038/nrneph.2016.70
Roh, J., Jeon, Y., Lee, A. N., Lee, S. M., Kim, Y., Sung, C. O., . . . Park, C. S. (2017). The immune checkpoint molecule V-set Ig domain-containing 4 is an independent prognostic factor for multiple myeloma. *Oncotarget, 8*(35), 58122-58132. doi:10.18632/oncotarget.19468

Rosales, C., Lowell, C. A., Schnoor, M., & Uribe-Querol, E. (2017). Neutrophils: Their Role in Innate and Adaptive Immunity 2017. *Journal of Immunology Research, 2017*, 9748345-9748345. doi:10.1155/2017/9748345

Rosenzweig, S. D., & Holland, S. M. (2011). Recent Insights into the Pathobiology of Innate Immune Deficiencies. *Current allergy and asthma reports, 11*(5), 369-377. doi:10.1007/s11882-011-0212-9

Russell, D. G. (2011). Mycobacterium tuberculosis and the intimate discourse of a chronic infection. *Immunological Reviews, 240*(1), 252-268. doi:10.1111/j.1600-065X.2010.00984.x

Scapini, P., & Cassatella, M. A. (2014). Social networking of human neutrophils within the immune system. *Blood, 124*(5), 710-719. doi:10.1182/blood-2014-03-453217

Segal, A. W., Dorling, J., & Coade, S. (1980). Kinetics of fusion of the cytoplasmic granules with phagocytic vacuoles in human polymorphonuclear leukocytes. Biochemical and morphological studies. *J Cell Biol, 85*(1), 42-59. doi:10.1083/jcb.85.1.42

Serna, M., Giles, J. L., Morgan, B. P., & Bubeck, D. (2016). Structural basis of complement membrane attack complex formation. *Nat Commun, 7*, 10587. doi:10.1038/ncomms10587

Sica, A., & Mantovani, A. (2012). Macrophage plasticity and polarization: in vivo veritas. *The Journal of Clinical Investigation, 122*(3), 787-795. doi:10.1172/JCI59643

Siqueira Mietto, B., Kroner, A., Girolami, E. I., Santos-Nogueira, E., Zhang, J., & David, S. (2015). Role of IL-10 in Resolution of Inflammation and Functional Recovery after Peripheral Nerve Injury. *J Neurosci, 35*(50), 16431-16442. doi:10.1523/jneurosci.2119-15.2015

Small, A., King, J. R., Rathjen, D. A., & Ferrante, A. (2018). The Role of Phagocytes in Immunity to Candida albicans *Candida Albicans*: IntechOpen.

Small, A., Lansdown, N., Al-Baghdadi, M., Quach, A., & Ferrante, A. (2018). Facilitating THP-1 macrophage studies by differentiating and investigating cell functions in polystyrene test tubes. *Journal of Immunological Methods*. doi:https://doi.org/10.1016/j.jim.2018.06.019

Small, A., & Wechalekar, M. D. (2019). Synovial biopsies in inflammatory arthritis: precision medicine in rheumatoid arthritis. *Expert Rev Mol Diagn*. doi:10.1080/14737159.2020.1707671

Smolen, J. S., Aletaha, D., Barton, A., Burmester, G. R., Emery, P., Firestein, G. S., . . . Yamamoto, K. (2018). Rheumatoid arthritis. *Nat Rev Dis Primers, 4*, 18001. doi:10.1038/nrdp.2018.1

Stein, M., Keshav, S., Harris, N., & Gordon, S. (1992). Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation. *J Exp Med, 176*(1), 287-292.

Steinman, R. M., & Cohn, Z. A. (1973). Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. *J Exp Med, 137*(5), 1142-1162.

Steinman, R. M., & Cohn, Z. A. (1974). Identification of a novel cell type in peripheral lymphoid organs of mice. II. Functional properties in vitro. *J Exp Med, 139*(2), 380-397.

Sturtz, L. A., Deyarmin, B., van Laar, R., Yarina, W., Shriver, C. D., & Ellsworth, R. E. (2014). Gene expression differences in adipose tissue associated with breast tumorigenesis. *Adipocyte, 3*(2), 107-114. doi:10.4161/adip.28250

Sugimoto, M. A., Sousa, L. P., Pinho, V., Perretti, M., & Teixeira, M. M. (2016). Resolution of Inflammation: What Controls Its Onset? *Frontiers in Immunology, 7*, 160-160. doi:10.3389/fimmu.2016.00160

Tacke, F. (2017). Targeting hepatic macrophages to treat liver diseases. *J Hepatol, 66*(6), 1300-1312. doi:10.1016/j.jhep.2017.02.026

Takashima, A., & Yao, Y. (2015). Neutrophil plasticity: acquisition of phenotype and functionality of antigen-presenting cell. *J Leukoc Biol, 98*(4), 489-496. doi:10.1189/jlb.1MR1014-502R

Takenaka, M. C., & Quintana, F. J. (2017). Tolerogenic dendritic cells. *Semin Immunopathol, 39*(2), 113-120. doi:10.1007/s00281-016-0587-8

Tanaka, M., Nagai, T., Tsuneyoshi, Y., Sunahara, N., Matsuda, T., Nakamura, T., . . . Matsuyama, T. (2008). Expansion of a unique macrophage subset in rheumatoid arthritis synovial lining layer. *Clin Exp Immunol, 154*(1), 38-47. doi:10.1111/j.1365-2249.2008.03733.x

Tani, Y., Isobe, Y., Imoto, Y., Segi-Nishida, E., Sugimoto, Y., Arai, H., & Arita, M. (2014). Eosinophils control the resolution of inflammation and draining lymph node hypertrophy through the proresolving mediators and CXCL13 pathway in mice. *The FASEB Journal, 28*(9), 4036-4043. doi:10.1096/fj.14-251132

Textoris, J., Ivorra, D., Ben Amara, A., Sabatier, F., Menard, J. P., Heckenroth, H., . . . Mege, J. L. (2013). Evaluation of current and new biomarkers in severe

preeclampsia: a microarray approach reveals the VSIG4 gene as a potential blood biomarker. *PLOS ONE, 8*(12), e82638. doi:10.1371/journal.pone.0082638

Thomson, A. W., & Knolle, P. A. (2010). Antigen-presenting cell function in the tolerogenic liver environment. *Nature Reviews Immunology, 10*, 753. doi:10.1038/nri2858

Thul, P. J., Akesson, L., Wiking, M., Mahdessian, D., Geladaki, A., Ait Blal, H., . . . Lundberg, E. (2017). A subcellular map of the human proteome. *Science, 356*(6340). doi:10.1126/science.aal3321

Thurman, J. M., & Holers, V. M. (2006). The central role of the alternative complement pathway in human disease. *J Immunol, 176*(3), 1305-1310.

Toivonen, R., Kong, L., Rasool, O., Lund, R. J., Lahesmaa, R., & Hanninen, A. (2016). Activation of Plasmacytoid Dendritic Cells in Colon-Draining Lymph Nodes during Citrobacter rodentium Infection Involves Pathogen-Sensing and Inflammatory Pathways Distinct from Conventional Dendritic Cells. *J Immunol, 196*(11), 4750-4759. doi:10.4049/jimmunol.1600235

Tsuchiya, S., Yamabe, M., Yamaguchi, Y., Kobayashi, Y., Konno, T., & Tada, K. (1980). Establishment and characterization of a human acute monocytic leukemia cell line (THP-1). *International Journal of Cancer, 26*(2), 171-176. doi:10.1002/ijc.2910260208

Uhlen, M., Fagerberg, L., Hallstrom, B. M., Lindskog, C., Oksvold, P., Mardinoglu, A., . . . Ponten, F. (2015). Proteomics. Tissue-based map of the human proteome. *Science, 347*(6220), 1260419. doi:10.1126/science.1260419

Uriarte, S. M., Rane, M. J., Luerman, G. C., Barati, M. T., Ward, R. A., Nauseef, W. M., & McLeish, K. R. (2011). Granule Exocytosis Contributes to Priming and Activation of the Human Neutrophil Respiratory Burst. *J Immunol, 187*(1), 391-400. doi:10.4049/jimmunol.1003112

van Kessel, K. P. M., Bestebroer, J., & van Strijp, J. A. G. (2014). Neutrophil-Mediated Phagocytosis of Staphylococcus aureus. *Frontiers in Immunology, 5*, 467. doi:10.3389/fimmu.2014.00467

van Lookeren Campagne, M., & Orozco, L. D. (2018). Chapter 29 CRIg *The Complement FactsBook* (pp. 309-316).

van Lookeren Campagne, M., & Verschoor, A. (2018). Pathogen clearance and immune adherence “revisited”: Immuno-regulatory roles for CRIg. *Seminars in Immunology, 37*, 4-11. doi:https://doi.org/10.1016/j.smim.2018.02.007

van Lookeren Campagne, M., Wiesmann, C., & Brown, E. J. (2007). Macrophage complement receptors and pathogen clearance. *Cell Microbiol, 9*(9), 2095-2102. doi:10.1111/j.1462-5822.2007.00981.x

Vogt, L., Schmitz, N., Kurrer, M. O., Bauer, M., Hinton, H. I., Behnke, S., . . . Bachmann, M. F. (2006). VSIG4, a B7 family-related protein, is a negative regulator of T cell activation. *J Clin Invest, 116*(10), 2817-2826. doi:10.1172/jci25673

Vono, M., Lin, A., Norrby-Teglund, A., Koup, R. A., Liang, F., & Loré, K. (2017). Neutrophils acquire the capacity for antigen presentation to memory CD4(+) T cells in vitro and ex vivo. *Blood, 129*(14), 1991-2001. doi:10.1182/blood-2016-10-744441

Wagner, C., Ochmann, C., Schoels, M., Giese, T., Stegmaier, S., Richter, R., . . . Hansch, G. M. (2006). The complement receptor 1, CR1 (CD35), mediates inhibitory signals in human T-lymphocytes. *Mol Immunol, 43*(6), 643-651. doi:10.1016/j.molimm.2005.04.006

Wang, J. J., Macardle, C., Weedon, H., Beroukas, D., & Banovic, T. (2016). Mucosal-associated invariant T cells are reduced and functionally immature in the peripheral blood of primary Sjögren's syndrome patients. *European Journal of Immunology, 46*(10), 2444-2453. doi:10.1002/eji.201646300

Wang, L.-x., Zhang, S.-x., Wu, H.-j., Rong, X.-l., & Guo, J. (2019). M2b macrophage polarization and its roles in diseases. *J Leukoc Biol, 106*(2), 345-358. doi:10.1002/JLB.3RU1018-378RR

Wang, Y., & Tatakis, D. N. (2017). Human gingiva transcriptome during wound healing. *J Clin Periodontol, 44*(4), 394-402. doi:10.1111/jcpe.12669

Wen, Y., Ouyang, Z., Schoonooghe, S., Luo, S., De Baetselier, P., Lu, W., . . . Zheng, F. (2017). Structural evaluation of a nanobody targeting complement receptor Vsig4 and its cross reactivity. *Immunobiology, 222*(6), 807-813. doi:10.1016/j.imbio.2016.11.008

Wiesmann, C., Katschke, K. J., Yin, J., Helmy, K. Y., Steffek, M., Fairbrother, W. J., . . . van Lookeren Campagne, M. (2006). Structure of C3b in complex with CRIg gives insights into regulation of complement activation. *Nature, 444*(7116), 217-220. doi:10.1038/nature05263

Winterbourn, C. C., Kettle, A. J., & Hampton, M. B. (2016). Reactive Oxygen Species and Neutrophil Function. *Annu Rev Biochem, 85*, 765-792. doi:10.1146/annurev-biochem-060815-014442

Wolf, D., Anto-Michel, N., Blankenbach, H., Wiedemann, A., Buscher, K., Hohmann, J. D., . . . Zirlik, A. (2018). A ligand-specific blockade of the integrin Mac-1 selectively targets pathologic inflammation while maintaining protective host-defense. *Nature Communications, 9*(1), 525-525. doi:10.1038/s41467-018-02896-8

Wong, K. L., Yeap, W. H., Tai, J. J. Y., Ong, S. M., Dang, T. M., & Wong, S. C. (2012). The three human monocyte subsets: implications for health and disease. *Immunologic Research, 53*(1), 41-57. doi:10.1007/s12026-012-8297-3

Xu, S., Sun, Z., Li, L., Liu, J., He, J., Song, D., . . . Wu, X. (2010). Induction of T cells suppression by dendritic cells transfected with VSIG4 recombinant adenovirus. *Immunol Lett, 128*(1), 46-50. doi:10.1016/j.imlet.2009.11.003

Xu, S., Wang, J., Wang, J.-H., & Springer, T. A. (2017). Distinct recognition of complement iC3b by integrins α(X)β(2) and α(M)β(2). *Proceedings of the National Academy of Sciences of the United States of America, 114*(13), 3403-3408. doi:10.1073/pnas.1620881114

Xu, T., Jiang, Y., Yan, Y., Wang, H., Lu, C., Xu, H., . . . Chen, J. (2015). VSIG4 is highly expressed and correlated with poor prognosis of high-grade glioma patients. *Am J Transl Res, 7*(6), 1172-1180.

Yang, G. X., Lian, Z. X., Kikuchi, K., Moritoki, Y., Ansari, A. A., Liu, Y. J., . . . Gershwin, M. E. (2005). Plasmacytoid dendritic cells of different origins have distinct characteristics and function: studies of lymphoid progenitors versus myeloid progenitors. *J Immunol, 175*(11), 7281-7287. doi:10.4049/jimmunol.175.11.7281

Yang, J., Zhang, L., Yu, C., Yang, X.-F., & Wang, H. (2014). Monocyte and macrophage differentiation: circulation inflammatory monocyte as biomarker for inflammatory diseases. *Biomarker Research, 2*, 1-1. doi:10.1186/2050-7771-2-1

Yang, M., McKay, D., Pollard, J. W., & Lewis, C. E. (2018). Diverse Functions of Macrophages in Different Tumor Microenvironments. *Cancer Res, 78*(19), 5492-5503. doi:10.1158/0008-5472.Can-18-1367

Yao, Y., Matsushima, H., Ohtola, J. A., Geng, S., Lu, R., & Takashima, A. (2015). Neutrophil priming occurs in a sequential manner and can be visualized in living animals by monitoring IL-1beta promoter activation. *J Immunol, 194*(3), 1211-1224. doi:10.4049/jimmunol.1402018

Yao, Y., Xu, X.-H., & Jin, L. (2019). Macrophage Polarization in Physiological and Pathological Pregnancy. *Frontiers in Immunology, 10*, 792-792. doi:10.3389/fimmu.2019.00792

You, Q., Cheng, L., Kedl, R. M., & Ju, C. (2008). Mechanism of T cell tolerance induction by murine hepatic Kupffer cells. *Hepatology, 48*(3), 978-990. doi:10.1002/hep.22395

Yuan, S., Wang, Y., Luo, H., Jiang, Z., Qiao, B., Jiang, Y., . . . Song, S. (2020). Serum soluble VSIG4 as a surrogate marker for the diagnosis of lymphoma-associated hemophagocytic lymphohistiocytosis. *British Journal of Haematology, n/a*(n/a). doi:10.1111/bjh.16299

Yuan, X., Yang, B.-H., Dong, Y., Yamamura, A., & Fu, W. (2017). CRIg, a tissue-resident macrophage specific immune checkpoint molecule, promotes immunological tolerance in NOD mice, via a dual role in effector and regulatory T cells. *eLife, 6*, e29540. doi:10.7554/eLife.29540

Zabel, M. D., & Weis, J. H. (2001). Cell-specific regulation of the CD21 gene. *International Immunopharmacology, 1*(3), 483-493. doi:https://doi.org/10.1016/S1567-5769(00)00046-1

Zang, X., & Allison, J. P. (2006). To be or not to be B7. *J Clin Invest, 116*(10), 2590-2593. doi:10.1172/jci30103

Zeng, Z., Surewaard, B. G., Wong, C. H., Geoghegan, J. A., Jenne, C. N., & Kubes, P. (2016). CRIg Functions as a Macrophage Pattern Recognition Receptor to Directly Bind and Capture Blood-Borne Gram-Positive Bacteria. *Cell Host Microbe, 20*(1), 99-106. doi:10.1016/j.chom.2016.06.002

Zhang, X. H., Qian, Y., Li, Z., Zhang, N. N., & Xie, Y. J. (2016). Let-7g-5p inhibits epithelial-mesenchymal transition consistent with reduction of glioma stem cell phenotypes by targeting VSIG4 in glioblastoma. *Oncol Rep, 36*(5), 2967-2975. doi:10.3892/or.2016.5098

Zheng, F., Luo, S., Ouyang, Z., Zhou, J., Mo, H., Schoonooghe, S., . . . Wen, Y. (2019). NIRF-Molecular Imaging with Synovial Macrophages-Targeting Vsig4 Nanobody for Disease Monitoring in a Mouse Model of Arthritis. *International journal of molecular sciences, 20*(13), 3347. doi:10.3390/ijms20133347

Zheng, F., Perlman, H., Matthys, P., Wen, Y., Lahoutte, T., Muyldermans, S., . . . Raes, G. (2016). Specificity Evaluation and Disease Monitoring in Arthritis Imaging with Complement Receptor of the Ig superfamily targeting Nanobodies. *Scientific Reports, 6*, 35966. doi:10.1038/srep35966

Zheng, F., Put, S., Bouwens, L., Lahoutte, T., Matthys, P., Muyldermans, S., . . . Schoonooghe, S. (2014). Molecular imaging with macrophage CRIg-targeting

nanobodies for early and preclinical diagnosis in a mouse model of rheumatoid arthritis. *J Nucl Med, 55*(5), 824-829. doi:10.2967/jnumed.113.130617

Zheng, N.-X., Wang, Y., Hu, D.-D., Yan, L., & Jiang, Y.-Y. (2015). The role of pattern recognition receptors in the innate recognition of Candida albicans. *Virulence, 6*(4), 347-361. doi:10.1080/21505594.2015.1014270

Zheng, Z., Bu, Z., Liu, X., Zhang, L., Li, Z., Wu, A., . . . Ji, J. (2014). Level of circulating PD-L1 expression in patients with advanced gastric cancer and its clinical implications. *Chinese journal of cancer research = Chung-kuo yen cheng yen chiu, 26*(1), 104-111. doi:10.3978/j.issn.1000-9604.2014.02.08

Zhu, S., Tan, W., Li, W., Zhou, R., Wu, X., Chen, X., . . . Chen, Y. (2018). Low expression of VSIG4 is associated with poor prognosis in hepatocellular carcinoma patients with hepatitis B infection. *Cancer management and research, 10*, 3697-3705. doi:10.2147/CMAR.S165822

Ziegler-Heitbrock, L., Ancuta, P., Crowe, S., Dalod, M., Grau, V., Hart, D. N., . . . Lutz, M. B. (2010). Nomenclature of monocytes and dendritic cells in blood. *Blood, 116*(16), e74.