An Immunological Perspective of the Mucosal Inflammation in Chronic Rhinosinusitis – Lymphoid Neo-organogenesis and Humoral Immunity

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by

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Declaration

I, Aden Ho Yin Lau, 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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Abbreviations

AEC	Airway Epithelial Cell	HPF	High power field
AERD	Aspirin-exacerbated respiratory disease	HPRT1	Hypoxanthine Phosphoribosyltransferase 1
AICDA/AID	Activation-induced cytidine deaminase	HRP	Horseradish peroxidase
AIM2	Absent in melanoma 2	IBD	Inflammatory bowel disease
ALI	Air-liquid interface	ICAM1	Intercellular Adhesion Molecule 1
ANA	Anti-nuclear autoantibodies	IFN-a/g	Interferon alpha/gamma
APC	Antigen presenting cells	IL	Interleukin
APRIL	A proliferation inducing ligand	IL-7R	Interleukin-7 Receptor
AR	Allergic rhinitis	LPS	Lipopolysacaride
ASA	Acetylsalicylic acid (Aspirin)	LTα	Lymphotoxin alpha
ASC	Apoptosis-associated speck-like protein containing a CARD	LTβ	Lymphotoxin beta
BAFF	B cell activating factor of the TNF family	LTβR	Lymphotoxin beta receptor
BCL6	B cell lymphoma 6	LTi	Lymphoid tissue inducer
BCR	B cell receptor	MHC	Major histocompatibility complex
BEGM	Bronchial epithelial growth medium	MPO	Myeloperoxidase
BPI	Bacterial permeability increasing protein	MRSA	Methicillin-resistant Staphylococcus aureus
CARD	Caspase activation and recruitment domain	MyD88	Myeloid differentiation primary response gene 88
CCL	C-C Motif Chemokine Ligand	NET	Neutrophilic extracellular trap
CD	Cluster of Differentiation	NF-κB	Nuclear factor kappa-light-chain -enhancer of activated B cells
CD40LG	CD40 ligand	NK cell	Natural killer cell
CENP B	Centromere protein B	NLR	NOD-like receptor
CNS	Central nervous system	NSAID	Non-steroid anti-inflammatory drug
COPD	Chronic obstructive pulmonary disease	OPG	Osteoprotegerin
CpG	C-phosphate-G	PAMP	Pathogen associated molecular pattern
CRS	Chronic rhinosinusitis	PBMC	Peripheral blood mononuclear cell
CRSsNP	CRS without nasal polyps	PD-1/ PDCD1	Programmed cell death 1
CRSwNP	CRS with nasal polyps	PDCD1LG2 /PD-L2	Programmed cell death 1 ligand 2
CT	Computed tomography	PDPN	Podoplanin
CXCL	C-X-C Motif Chemokine Ligand	PNAd	Peripheral node addressin
CXCR	C-X-C Motif Chemokine Receptor	PRR	Pattern recognition receptor
DAMP	Damage associated molecular pattern	pSS	Primary Sjögren's syndrome

DAPI	4',6-Diamidino-2-phenylindole dihydrochloride	PYHIN	Pyrin and HIN domain family
DC	Dendritic cell	qPCR	Quantitative polymeric chain reaction
DNase	Deoxyribonuclease	QS	Quorum sensing
dsDNA/RNA	double stranded DNA/RNA	RA	Rheumatoid Arthritis
DSS	Dextran sodium sulphate	RAGE	Receptor for advanced glycation end-product
ECP	Eosinophil cationic protein	RANK	Receptor Activator of Nuclear Factor Kappa B
ECRS	Eosinophilic CRS	RANKL	Receptor Activator of Nuclear Factor Kappa B Ligand
eDNA	Extracellular DNA	RORγT	RAR-related orphan receptor gamma T
ELISA	Enzyme-linked immunosorbent assay	ROS	Reactive oxygen species
EPOS	European position paper	SEA/B	Staphylococcal enterotoxin A/B
EPS	Exopolymeric substance	SELPLG	Selectin P ligand
ET	extracellular trap	SLE	Systemic lupus erythematosus
FDC	follicular dendritic cell	SLO	Secondary lymphoid organ
FESS	functional endoscopic sinus surgery	SS-A/B	Sjögren's syndrome A/B
FGF2	Fibroblast Growth Factor 2	TACI	transmembrane activator and calcium-modulating cyclophilin ligand interactor
FMT	Fecal microbiota transplantation	T_{fh}	T follicular helper
FOXP3	Forkhead Box P3	T_h	T helper
GA2LEN	Global Allergy and Asthma European Network project	TLO	Tertiary lymphoid organ
GC	Germinal centre	TMA	Tissue microarray
GPR183	G Protein-Coupled Receptor 183	TNF-α	Tumor necrosis factor alpha
HEV	High endothelial venule	TSLP	Thymic stromal lymphopoietin
HGF	Hepatocyte Growth Factor	TSLPR	Thymic stromal lymphopoietin receptor
HMGB1	High Mobility Group Box 1	VCAM1	Vascular Cell Adhesion Molecule 1
HNEC/NEC	Human nasal epithelial cell/nasal epithelial cell	VEGF-C	Vascular Endothelial Growth Factor C

Abstract

Chronic rhinosinusitis (CRS) is defined by the symptomatic inflammation of the nose and paranasal sinuses longer than 12 weeks. These symptoms include nasal discharge, nasal obstruction, facial pain and pressure, leading to a substantial impact on the quality of life of CRS patients. CRS can be phenotypically classified into either CRS without nasal polyps (CRSsNP) or CRS with nasal polyps (CRSwNP), based on the presence of endoscopically visualized nasal polyps in the middle meatus. Interestingly, ectopic accumulations of lymphoid cells are often observed within the nasal polyps of CRSwNP. This raises the question as to whether these aberrant lymphoid cell aggregates play a role in orchestrating the perpetual inflammation in CRS. Studies in the past have identified the increased amount of local class-switched antibodies in nasal polyps, but few studies have investigated the source of these immunoglobulins and utilized specific markers to study the presence of the organized lymphoid structures and their relation to disease severity in the context of CRS. This thesis investigates the significance of organized lymphoid neo-organogenesis in CRS pathogenesis and its effect on humoral immunity within both CRSsNP and CRSwNP patients.

Chapter 1: Introduction

1.1 Background

Definition of Chronic Rhinosinusitis

According to the most updated European Position Paper on Rhinosinusitis and Nasal Polyps (EPOS) published in 2012[1], Rhinosinusitis is defined as inflammation of the nose and the paranasal sinuses characterized by at least two symptoms, which comprise at least one from each of the two must-have categories of signs and symptoms. The first must-have sign/symptom category consists of nasal blockage/obstruction/congestion and nasal discharge (anterior/posterior nasal drip), ± facial pain/pressure, ± reduction or loss of smell. The second must-have sign/symptom category consists of endoscopic signs of polyps or mucopurulent discharge primarily from middle meatus or oedema/mucosal obstruction primarily in middle meatus, and CT changes showing mucosal changes within the ostiomeatal complex and/or sinuses. When these symptoms last for 12 weeks or more in adults, the disease is defined as Chronic Rhinosinusitis (CRS)[1, 2] and its definition remained unchanged in the latest EPOS update[1].

Prevalence of CRS

CRS is a highly impactful disease worldwide with high prevalence in the general population. Up to 10.5% of the Australian population is affected by CRS[3]. In the United States, a household survey was conducted in 2012 and the prevalence of CRS was found to be 16%[4]. The actual question regarding the prevalence of CRS was "In the last 12 months, has the patient had sinusitis diagnosed by a doctor or a health professional?"[4]. In the European populations, there were found to be substantial variations in the prevalence of CRS between countries[5]. In 2011, the Global Allergy and Asthma European Network project (GA2LEN) performed a multi-centre study which included 19 centres in 12 European countries. The study revealed that the self-reported doctor-diagnosed CRS ranges from 1.1% (Umea, Sweden) to 18.1% (Coimbra, Portugal) whereas the self-reported CRS by EPOS 2007 criteria ranges from 6.9% (Helsinki, Finland and Brandenburg, Germany) to 27.1% (Coimbra, Portugal)[5]. There was a statistically significant association between the self-reported doctor-diagnosed CRS and the self-reported CRS by EPOS criteria. These findings reflected, to a certain extent, that there is true geographical discrepancy in the prevalence of CRS across these cities. In Sao Paulo, the prevalence of CRS was found to be 5.51%, using a

complex two-stage cluster sampling plan and personal interviews which included EPOS criteria for defining CRS[6]. In South Korea, the Korean National Health and Nutrition Examination Survey 2009 has found the prevalence of CRS, which is defined as the presence of nasal obstruction and nasal discharge lasting more than 3 months along with endoscopic evidence such as nasal polyps, to be 6.95%[7]. A follow-up study published in 2016 separated the overall prevalence of CRS into symptom-based CRS and endoscopy-based CRS, and the prevalence was found to be 10.78% and 1.2% respectively. In China, the prevalence of CRS in 7 major cities is found to be 7.99% using the standardized questionnaire developed by GA2LEN[8]. Due to the need for surgery and frequent recurrence of the disease, it incurs billions of dollars of health care expenses per year[9].

Classification of CRS

CRS patients are phenotypically classified into CRS with nasal polyps (CRSwNP) or CRS without nasal polyps (CRSsNP). This is based on the presence of CRS with or without endoscopically visualized polyps in the middle meatus[1]. Despite the considerable overlap between CRSwNP and CRSsNP in terms of clinical presentation[10, 11], treatment options and treatment efficacy[12, 13], there are a variety of differences in their respective inflammatory profiles[14, 15] and treatment outcomes[16]. Although current knowledge of polyp development is very limited, the presence of polyps is useful for differentiating the two groups in many aspects. CRSwNP patients tend to have a more pronounced nasal obstruction and loss of smell whereas headache and postnasal drip is more common among CRSsNP patients [17]. At cellular and molecular levels, CRSwNP has a distinctive immune cell activation and cytokine expression profile in comparison with CRSsNP. These two CRS subgroups possess different clinical and morphological characteristics[18], and they have fundamental differences in terms of the type of inflammation, orchestrating immune activities, tissue remodelling, risk of recurrence and co-morbidities[18].

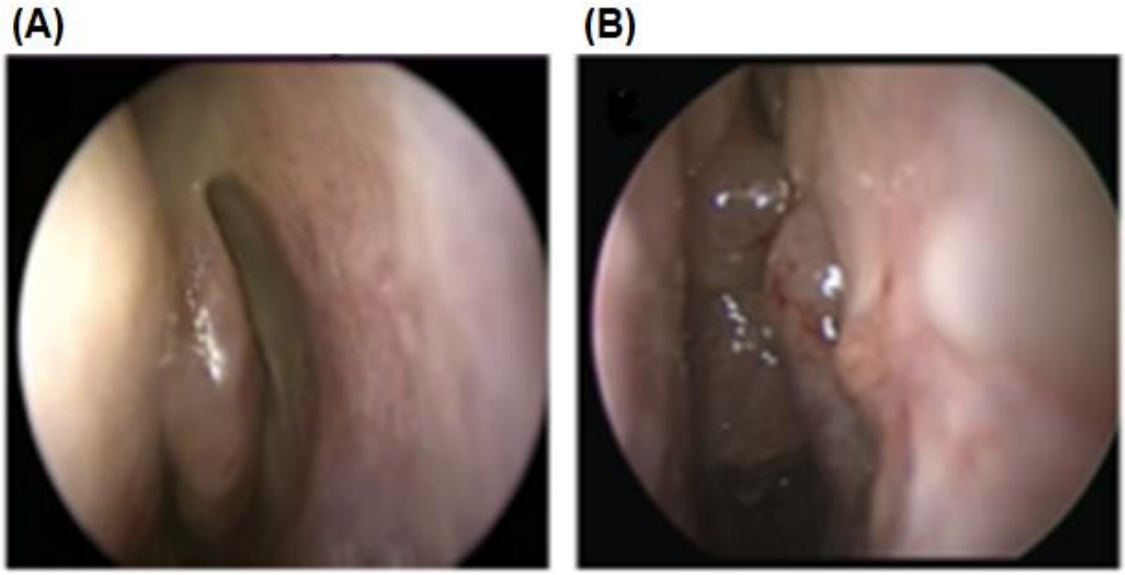

Figure 1.1 CRS phenotypes.

CRS can be phenotypically classified into CRS without nasal polyps (A) and CRS with nasal polyps (B) based on the bilateral absence or presence of endoscopically visualized nasal polyps in the middle meatus.

Current Treatment Options and disease recalcitrance

Once a patient is clinically diagnosed to have CRS, the initial therapy is medical with oral antibiotics and topical or oral steroids. When medical therapy fails, the patients might be offered endoscopic sinus surgery (ESS). The purpose of the surgery is to remove diseased tissue, which is blocking sinus drainage passages and possibly cultivating infection, in order to restore normal functions in the sinuses[19] (therefore, the previously used term functional endoscopic sinus surgery or FESS). ESS significantly improves the quality of life in most individuals[20] and has a very high frequency of application worldwide. However, a conservative approximation of 10% of CRS patients do not respond well to ESS and develop refractory disease[21]. In CRSwNP, post-operative endoscopic examinations have found the rate of polyp recurrence up to 18 months after ESS to be approximately 60-70% and the risk factors of polyp recurrence include previous ESS and worse pre-operative polyposis severity[22]. Furthermore, in cases with more severe disease or when the outcomes were assessed by objective measurements, the failure rate of ESS can rise to as high as 50%[23]. These CRS patients who do not respond to both medical treatments and surgical

interventions are defined as having recalcitrant CRS (rCRS). Revision surgery is frequently required for rCRS patients to provide mere symptomatic relief because there are no curative medical treatment options for these patients. This recurrent need for surgery in rCRS patients presents a substantial quality of life and economic burden. Therefore, new effective treatment options for rCRS are urgently needed.

Co-morbidities and risk factors of CRS

Asthma

According to the National Institute of Health Guidelines on Asthma in 2007[24], asthma is defined as the following: "Asthma is a chronic inflammatory disorder of the airways in which many cells and cellular elements play a role: in particular, mast cells, eosinophils, T lymphocytes, macrophages, neutrophils, and epithelial cells. In susceptible individuals, this inflammation causes recurrent episodes of wheezing, breathlessness, chest tightness, and coughing, particularly at night or in the early morning. These episodes are usually associated with widespread but variable airflow obstruction that is often reversible either spontaneously or with treatment. The inflammation also causes an associated increase in the existing bronchial hyperresponsiveness to a variety of stimuli. Reversibility of airflow limitation may be incomplete in some patients with asthma." The GA2LEN has revealed that asthma was strongly associated with CRS consistently in all European centres (overall OR: 3.48; 95% CI: 3.21–3.77; test for heterogeneity P = 0.001) irrespective of age, gender and smoking behaviour[25]. Up to 70% of both severe and mild-to-moderate asthma patients are positive of rhinosinusitis by CT scanning[26]. Furthermore, the CT score of sinus mucosa thickening is significantly higher in the severe asthma group compared to the mild-to-moderate asthma group[26]. The risk of co-morbid asthma is increased in T_{h2}-type CRSwNP in Caucasians[27], matching the usually T_{h2}-skewed and eosinophilic inflammatory profile in asthma[24]. Intriguingly, sputum eosinophilia is an independent predictor of worse lung functions in severe asthma[28] and medical or surgical treatments of CRS improve pulmonary functions of asthmatic patients[29]. These findings altogether suggest that asthma and CRS share common pathophysiology through T_{h2}-mediated immunity, which could be the connection between sinonasal, upper and lower airway in a united airway concept[30].

Aspirin-exacerbated respiratory disease (AERD)

Precipitation of asthma attacks after ingestion of aspirin (ASA) and non-steroid anti-inflammatory drugs (NSAID) is considered the hallmark of Aspirin-exacerbated respiratory

disease (AERD), which is a clinical syndrome characterized by CRS, nasal polyposis and asthma[31, 32]. The prevalence of AERD in adult asthma patients is 9 to 20%[33, 34], tested by oral ASA challenges. In the subset of adult asthmatic patients with associated sinusitis and nasal polyposis, the prevalence of AERD increases to 33% to 40%[35, 36]. AERD patients have a much higher rate of nasal polyposis, up to 80%, compared to 4% in the general population[37] and develop substantially more severe, long lasting and refractory CRS[36, 38]. Although the pathophysiological mechanisms of AERD have yet to be fully understood, it is well established that abnormal arachidonic acid metabolism is involved, leading to imbalanced cyclooxygenase (COX) and lipoxygenase pathways[36]. This anomaly leaves AERD patients with an overzealous lipoxygenase pathway which leads to an overproduction of leukotrienes upon ASA or NSAIDs intake, eventually setting off the precipitation of asthma attacks[36, 39]. There are multiple reports on elevated T_{h2} cytokines, including IL-4 and IL-13[40], in CRSwNP patients with AERD, especially those involved in eosinophilic infiltration[41], namely RANTES, eotaxin and eotaxin-2.

Allergic rhinitis (AR)

Allergic rhinitis (AR) is a heterogeneous disorder characterized by at least one of the following symptoms including sneezing, itching, nasal congestion and rhinorrhea[42]. AR is another manifestation of atopic syndrome, like asthma, associated with CRS[43, 44] and it often co-exists with asthma[45, 46, 47]. The number of eosinophils is also increased in the bronchial mucosa of AR patients and, remarkably, in asymptomatic atopic individuals[48], suggesting that aberrant immune activities precede the disease onset in atopic CRS patients. Furthermore, both AR and asthma feature a T_{h2}-type inflammation with increased IL-31 expression and elevated levels of serum eosinophils[49]. IL-31 is a T_{h2} cytokine expressed by activated T_{h2} cells and is important in the development of both atopic and non-atopic dermatitis[50]. About 80% of children with atopic dermatitis develop asthma or AR[51], implying a defective T_{h2}-mediated immunity with the potential involvement of IL-31 shared between asthma, AR, atopic dermatitis and possibly a subset of CRS. Such frequent co-occurrence and similar inflammatory profile of asthma and AR suggest that there is a systemic immunologic cross talk between the upper and lower airway[30]. In terms of the CRS subtypes, the clinical association profile differs between CRSsNP and CRSwNP. Like asthma and aspirin sensitivity, co-morbid AR is associated mainly with CRSwNP [18, 52, 53] whereas CRSsNP is often associated with lower airway disease[1].

Smoking

Smoking is a risk factor for CRS[5] and many chemicals in tobacco smoke have been shown to be ciliotoxic (i.e. having high toxicity to respiratory cilia)[54, 55, 56]. Tobacco smoke exposure promotes inflammation and connective tissue breakdown[57, 58]. Coherently, it is evident in the literature that active smokers and individuals exposed to second-hand smoke are more susceptible to CRS[59].

1.2 Pathophysiology of CRS

The one feature in common among all CRS subtypes is the chronic inflammation. However, the question as to the etiology underlying this chronic inflammation in an individual patient does not have a clear answer yet[60].

Persistent infections and the microbiome in CRS

Microbial infections have long been proposed to play a role in the pathogenesis of CRS[61, 62]. The concept of the disruption of symbiosis between commensal bacteria and humans, or dysbiosis, in the sinonasal mucosa might add clarity to how microbial infections are linked to CRS. Dysbiosis is defined as the change in the composition of residential commensal bacterial communities relative to those found in healthy individuals[63]. Dysbiosis in the gut is relatively well-studied and restoring the healthy microbiome has been proposed to be a therapeutic application in some gastrointestinal diseases[64]. Fecal microbiota transplantation (FMT) has shown excellent therapeutic efficacy in treating recurrent *Clostridium difficile* infection[64] and some initial success in treating inflammatory bowel disease[65]. In CRS, a literature review has shown that a large number of association studies have been done to demonstrate the different microbiomes between CRS patients and non-CRS controls[66], indicating a dysbiosis in a subset of CRS patients[67]. Highlighted bacterial species with higher colonization rate in CRS include *Staphylococcus aureus*[68, 69] and *Pseudomonas aeruginosa*[69]. Although the microbial communities are set to have a significant impact on CRS, the highly complex microbiological ecology, which includes interspecies and intraspecies communications as well as the potential influences from virus and fungi, renders the concept of a microbial etiology in CRS poorly understood[70].

Mucosal biofilms

One of the proposed mechanisms that support the pathogenic role of bacteria in CRS is by forming biofilms. Bacterial biofilm is a mode of living characterized by cell attachment to a surface and encapsulation in a self-produced matrix of exopolymeric substances (EPS)[71]. This EPS matrix

physically shields the bacteria from external stresses (e.g. antibiotics), provides a scaffold for adhesion to a surface and cohesion between bacterial cells, and sequesters enzymes and nutrients important for survival[71]. Once bacteria enter this communal mode of living, the biofilm matrix provides a platform for cell-to-cell communication, exchange of genetic materials and the recycling of lysed cell materials[72]. Bacterial biofilms are commonly perceived as an adaptation to resist host defences and antibiotics in the setting of CRS, and thus foster recalcitrant disease[73]. Despite a research interest in the development of anti-biofilm therapies in CRS, a definitive causal relationship between CRS and biofilm, and the role of bacterial biofilms in the initial establishment of CRS have not been identified[74]. The potential pro-inflammatory property of *S. aureus* biofilms and whether it is linked to the initiation of CRS at the nasal epithelium warrant further investigation.

While it is well known that bacteria in their biofilm form are much more persistent than their planktonic counterpart[75], how bacterial biofilms generate a pathological chronic inflammation is less clear. In a general event of an acute infection, an inflammatory response is induced at the site of infection to initiate innate immunity against the invading pathogens. The innate immune system then calls for the more sophisticated adaptive immunity, which is slower but more specific to the antigen, through the help of bridging mediators and cells. A successful immune response would clear the acute infection and the resolution of inflammation would begin within the next few hours due to the high turnover rate of immune cells[76]. However, when the immune system fails to effectively clear an infection, it leads to a persistent infection where the inflammatory response is constantly being active and, consequently, gives rise to pathogenic chronic inflammatory conditions. An example of persistent infection leading to chronic mucosal inflammation is *Helicobacter pylori* infection at the gastric epithelium[77]. Forming biofilms is one of the ways that microbes use to evade or block immune responses against them. Biofilms, combined with host susceptibilities or niches for infection, are associated with the transition from transient to persistent infections[71, 74]. Therefore, understanding the host-microbial interactions, especially those within the microenvironment of the infection, is crucial to tackling the infection-associated chronic inflammation. As previously mentioned, one intensively studied bacterium central to CRS pathogenesis is *Staphylococcus aureus*, which has been identified in 64% of CRSwNP patients compared with approximately 30% of CRSsNP patients or healthy individuals [78]. Evasive strategies to escape immunity by *S. aureus* include biofilm formation and intracellular invasion, which are extensively studied in CRS for their roles in helping the bacterium persist [79, 80]. Although the exact

mechanisms underlying the self-perpetuating inflammation in CRS remain to be defined, our research group has found that the AIM2 inflammasome, which is a cytosolic dsDNA sensor and produces an inflammatory response when activated[81], has consistently and significantly increased gene expression in CRSwNP patients and *S. aureus* biofilms [82]. Given the association between biofilms and AIM2, one potential source of immunogenic microbial DNA is extracellular DNA (eDNA) in biofilms. The term eDNA used here is specific for DNA that forms the majority of the biofilm physical structure and is functionally active in favour of the microbes. Therefore, eDNA is distinctive from any free DNA, host or foreign, that exists outside the cells. Since the first demonstration of DNase I treatment on *Pseudomonas aeruginosa* leading to the dissolution of its biofilms in 2002 [83], a wide range of bacteria have been subjected to DNase treatment and have shown the disruption of biofilms, especially at the early stage of biofilm development[72]. In addition, eDNA also exists in fungal biofilms and plays a central role in biofilm formation[84], meaning that eDNA is a well-conserved mechanism in biofilm biology. Although the importance of eDNA to the formation and integrity of biofilms has been undisputedly proven in many bacterial species, it is less clear as to how eDNA promotes biofilm formation and maintenance and, most importantly, how eDNA contributes to antibiotics resistance and pathogenic inflammation. Mulcahy et al. have shown that in *P. aeruginosa* eDNA acts as a cation chelator to maintain a cation gradient, which triggers antibiotic-resistance genes to defend against cationic host-derived anti-microbial peptides and antibiotics (e.g. aminoglycosides) by modifying cell surface lipopolysaccharides (LPS) in order to conceal the negative charge of the bacterial outer membrane[85]. Apart from its chelating property, eDNA is also a core component of the bacterial quorum-sensing (QS) system, which is the mechanism by which bacteria detect the surrounding bacterial population density in order to adapt to the environment by regulating the expression of relevant genes[86]. Targeting eDNA as a means to disrupt QS has emerged as a promising method to mitigate biofilm-mediated pathogenesis. *S. aureus* has decreased biofilm formation and increased anti-microbial susceptibility when treated with recombinant human DNase I[87]. A study by Bass et al has also shown that *P. aeruginosa lasI rhlI* mutant strain, which has significantly reduced eDNA, has reduced ability to stimulate the release of IL-8 and IL-1β when co-cultured with neutrophils compared to wild-type strain[88]. In a more recent study, inhibiting the target of RNAIII-activating protein (TRAP) QS systems[89] in *S. aureus* using Hamamelitannin (2',5'-di-*O*-galloyl-D-hamamelose; HAM) decreased the amount of eDNA present in the biofilm matrix and increased the susceptibility of *S. aureus* to vancomycin[90].

In view of these findings, decreasing the load of eDNA within the biofilm matrix (e.g. direct digestion by DNase or inhibiting QS to limit the release of eDNA) has a promising potential in treating biofilm-associated infection. This is particularly important in fighting biofilm-forming microbes that are resistant to antibiotics such as Methicillin-resistant *Staphylococcus aureus* (MRSA). In the context of CRS, it is of particular interest whether *S. aureus* eDNA has the similar pro-inflammatory effects to the *P. aeruginosa* eDNA in stimulating immune cells[88], especially eosinophils, given their prevalence and immunogenicity in CRS[91], particularly CRSwNP[92].

Innate Immunity in CRS

The epithelial barrier

The sinonasal mucosa is in direct contact with the environment, meaning the exposure to inhaled allergens and irritants, and the colonization of both commensal and pathogenic organisms. Mucociliary clearance and apical junctional complexes (AJCs) between sinonasal epithelial cells serve as a mechanical barrier that protects the host from the environment. Goblet cells and submucosal glands produce respiratory mucus to trap foreign materials and remove them from the sinuses and nasal cavity. Both genetic and acquired defects in mucociliary flow have been linked to the disease process of CRS[93, 94, 95]. A disrupted tight and adherence junction, and thus a disrupted mucosal barrier, is associated with CRSwNP as demonstrated by significantly altered levels of adhesion molecules of AJCs[96, 97, 98]. Functional studies using an in-vitro air-liquid interface (ALI) model have shown that the epithelial barrier in CRSwNP is more permeable [98, 99], suggesting an increased susceptibility to infection in these patients with a defective epithelial barrier. An innate immune response is triggered through pattern recognition receptors (PRRs) when the epithelial barrier is breached[15].

Pattern recognition receptors

Sinonasal epithelial cells are one of the cell types at the mucosal tissues that express PRRs[100, 101], which recognize pathogen associated molecular patterns (PAMPs) from microbes. Some studies have suggested that Toll-like receptors (TLRs), which are the best-characterized PRRs, have potential defects that might contribute to the pathogenesis of CRS[102, 103, 104, 105]. TLRs are expressed by many cell types including epithelial cells, fibroblasts, endothelial cells and immune cells such as dendritic cells, macrophages, B cells and T cells[106]. TLR 1-9 are relatively well understood and have known agonists. TLR stimulation by their corresponding agonists is the strongest in TLR 3, 6 & 9 in primary nasal epithelial cells in vitro[101]. Each TLR has a distinctive

apical-basal polarization pattern in human airway epithelial cels[107], indicating that airway epithelial cells have a significant role to play in regulating the innate immune response.

TLR3 is expressed in the endosome of myeloid dendritic cells and macrophages whereas epithelial cells and fibroblasts can express TLR3 both intracellularly and on the cell surface[108]. TLR3 is mainly expressed on the luminal and basal mucosal surface of airway epithelial cells[107]. TLR3 recognizes double-stranded RNA (dsRNA), which is a PAMP associated with viral infection because it is produced by most viruses during replication[109]. When activated by poly(I:C), a synthetic dsRNA analogue, TLR3 transduces the signal through a MyD88-dependent signalling pathway to induce inflammatory cytokine production such as IL-6 and TNF-α[109]. MyD88 is an adaptor protein that is shared by all known TLRs[109]. TLR3 stimulation by poly(I:C) in human tracheal epithelial cells resulted in a strong induction of type I and type III interferons as well as pro-inflammatory cytokine IL-8[107].

TLR6 is expressed on the cell surface and it recognizes diacyl lipopeptide when coupled with TLR2[110]. The expression of TLR2 and TLR6 on airway epithelial cells have a predominant basolateral distribution[107]. TLR6 can also heterodimerize with TLR4 and recognizes oxidized low-density lipoprotein (LDL) and amyloid-beta to produce sterile inflammation that is thought to be important for atherosclerosis and Alzheimer's disease[110].

As for TLR9, it is expressed by various immune cells including dendritic cells, macrophages, natural killer cells (NK cells) and other antigen presenting cells[111]. In human airway epithelial cells, TLR9 has a moderate surface expression with a clear luminal polarization[107]. TLR9 recognizes the unmethylated CpG motifs, which are relatively common in the genomes of most bacteria and DNA viruses[112]. CpG motifs are generally suppressed and methylated in vertebrate genomes[112]. Nevertheless, a recent study has implicated that TLR9 may also recognize the CpG regions of host DNA exposed during apoptosis in autoimmune diseases where there is delayed clearance of apoptotic cells[113]. When activated in a MyD88-dependent manner, inflammatory cytokines such as IL-6, IL-12 and TNF-α, and type I interferon cytokines such as IFN-α are induced[114]. In the respiratory system, TLR9 activation reduces T_{h2}-dependent allergic inflammation by inducing a T_{h1} response[115]. *E. coli* DNA, but not human DNA, stimulates TLR9 expressed on human large airways epithelial cells cultivated as air-liquid interface cultures to induce the dose-dependent release of IL-6, IL-8 and β-defensin 2 via the NF-κB pathway in a MyD88-dependent manner[116]. TLR9 also plays a role in a variety of autoimmune diseases[117] but

it appears to have protective effects in some autoimmune diseases while having detrimental effects in the others. In a murine model of systemic lupus erythematosus (SLE), TLR9 has regulatory effects on the development of lupus nephritis as shown by the loss of TLR9 exacerbating the autoimmune disease[118]. On the contrary, TLR9 activation has pro-inflammatory effects in erythema nodosum leprosum (ENL) as demonstrated by the TLR9 antagonist inhibiting the secretion of pro-inflammatory cytokines by ENL PBMCs[119]. In CRS, TLR9 at the protein level is decreased in CRSwNP compared to non-CRS controls[105] and this finding is consistent with the predominant T_{h2} phenotype in CRSwNP.

Nasal epithelial cells constitutively express and secrete a variety of host defensive molecules, with the levels of these molecules altered upon PRR stimulation[120, 121, 122]. Lowered expression of some defensive molecules, such as lactoferrin, psoriasin and calprotectin is associated with CRS[122, 123, 124], but the underlying mechanisms are unclear. The reduced levels of anti-microbial molecules are likely to reflect the decreased number of submucosal glands, which is further supported by our histological observation of decimated glands particularly in CRSwNP. Besides these host defense molecules, sinonasal epithelial cells are also capable of secreting chemokines and cytokines to attract and activate innate lymphoid cells, respectively, in response to PRR stimulations[120, 125, 126], especially the epithelial cells of nasal polyps[127].

The innate immune system plays a vital role in immune surveillance and coordinating the inflammatory response as a first-line defence, especially at the barriers that are constantly exposed to the external environment such as the airway epithelium, which is able to respond to a wide range of pro-inflammatory stimuli from both host and microbes by releasing various cytokines (e.g. IL-5, IL-6, IL-8, etc.) [128]. Defective regulations of cytokines or mediators at the epithelium have critical effects in chronic inflammatory diseases, with examples including the airway epithelium in chronic obstructive pulmonary disease (COPD) and asthma [129], the intestinal epithelium in inflammatory bowel disease (IBD)[130] and the salivary gland epithelium in primary Sjögren's Syndrome (pSS) [131]. There is emerging evidence for the importance of epithelial innate immunity in inflammatory diseases [132, 133, 134] and TLR expression levels in epithelial cells are associated with inflammatory cytokine profiles [131]. In the settings of the canonical pathway, the signal is transduced from TLRs to the nucleus by the NFκb pathway, which regulates the gene expression of certain important downstream inflammatory mediators including inflammasomes and pro-inflammatory cytokine IL-1β [135]. The inflammasome, which is a member of PRRs found in the cytoplasm, has

become a popular research area for inflammation and innate immunity studies since its discovery in 2002 [136]. In general, inflammasomes consist of a pattern recognition domain (e.g. Nod-like receptors in the NLR family or PYHIN in AIM2) and a caspase activation and recruitment domain (CARD) or an adaptor protein ASC that binds to CARD. The pattern recognition domain of each inflammasome is specific for a pro-inflammatory danger or damage signal (e.g. AIM2 detects cytosolic dsDNA[81]). The CARD domain cleaves caspase-1 into its mature form upon activation. Active caspase-1 in turn cleaves IL-1β into its active form and then the active IL-1β is secreted as a pro-inflammatory cytokine (figure 1). It is speculated that defects among these components of the inflammatory machinery in epithelial cells such as the NFκb pathway are contributing factors to chronic inflammation[137]. The inflammasome protein itself can also be defective as shown by the study where AIM2 activation is achieved by the overexpression of AIM2 and inflammasome apparatus in otherwise inflammasome-deficient cells[138]. In non-canonical activation of AIM2, type I IFN is required and intracellular stimuli such as intracellular bacterial DNA from *Francisella novicida* has been shown to stimulate AIM2-mediated release of inflammatory cytokines IL-1β and IL-18[139]. AIM2 recognizes DNA in a sequence-independent manner but the DNA strands must be at least 80 bp long[140]. Activation of AIM2 results in pyroptosis[141], which is a pro-inflammatory type of programmed cell death that leads to the release of damage associated molecular patterns (DAMPs), such as DNA, ATP, heat-shock proteins[142], as well as IL-1β and IL-18[143]. AIM2 is involved in the severe inflammation driven by microbial insults and autoimmunity[144]. In terms of bacterial infection, there is a range of bacterial species that can be recognized by AIM2 in immune cells[144]. Examples include AIM2-deficient macrophage in mice releasing reduced levels of IL-1β and IL-18 against infection by *Listeria monocytogenes*, *Streptococcus pneumoniae* and *Francisella tularenesis*[145, 146, 147]. AIM2-deficient THP-1 human monocytic cell lines have also shown reduced release of IL-1β against some bacterial infections such as *Porphyromonas gingivalis* and *Mycobacterium tuberculosis*[148, 149]. Interestingly, AIM2 plays a significant role in a murine model of acute central nervous system (CNS) infection by *S. aureus*[150]. Also, an increased colonization of *S. aureus* in CRSwNP[78] has been shown to correlate with the increased AIM2 expression in CRSwNP and biofilm-positive patients[82] but further investigations of their interactions in the context of CRS are needed. In the settings of inflammation derived from autoimmunity, AIM2 also plays a critical role in promoting chronic inflammatory conditions. Host DNA is not present in the cytosol under normal circumstances because it is normally sequestered in the nucleus or

mitochondria. However, individuals with genetic predispositions may have aberrant accumulation of host DNA in the cytosol caused by impaired degradation of DNA[151] or excess uptake of extracellular DNA derived from neighbouring dying cells. This accumulated host DNA in the cytosol can serve as an endogenous danger signal, which can set off IL-1β release by activating AIM2 as in the AIM2-dependent pathogenesis of psoriasis[152]. In brief, given that AIM2 plays a pivotal role in driving inflammation derived from various origins and its expression being elevated in CRSwNP[82] as mentioned above, it is speculated that AIM2 promotes severe inflammation in CRS. Nevertheless, it is not clear as to how AIM2 is activated and whether host-derived DNA or microbial DNA (or both) activates AIM2 in the context of its pathogenicity in CRS.

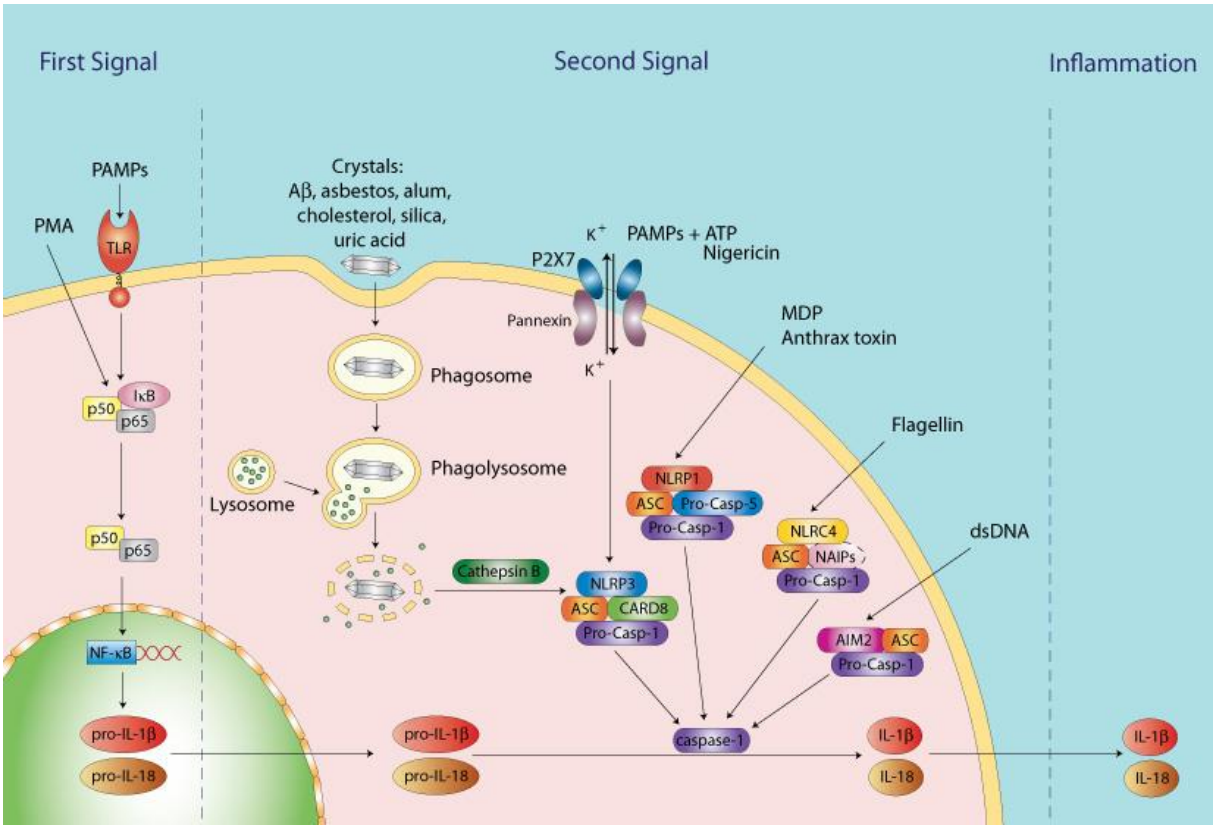

Figure 1.2 Inflammasome activation pathways in general

Inflammasome activation requires a first signal of TLR ligation and an inflammasome-specific second signal. The activation leads to the cleavage of caspase-1 and subsequently cleavage and secretion of pro-inflammatory cytokine IL-1β and IL-18. Image adopted from Inflammasome Review (2012), Invivogen, http://www.invivogen.com/review-inflammasome (accessed on 25 Mar, 18)

Innate lymphoid cells (ILCs)

ILCs are the subjects of a rapidly expanding field of study in the realm of immunology and play an important role in autoimmune and inflammatory diseases[153]. ILCs can be categorized into group 1 to 3, each carrying a unique set of functions that ochestrate the type of immune response generated and the transition from innate to adaptive immunity[154]. Here we focus on group 2 and 3 ILCs due to their great relevance in CRS and the formation of lymphoid organs respectively. Group 2 ILC population comprises ILC2s, which can produce IL-4, IL-5 and IL-13[153], and are a potent driver of type II inflammation. Studies have shown that ILC2s play a decisive role in T_{H2} cell activities, in terms of both memory[155] and effector functions[156]. ILC2s

are activated by IL-25, IL-33 and TSLP[157, 158], all of which can be produced by epithelial cells[159, 160], giving ILC2s an extra layer of importance given the emphasis on epithelium-mediated inflammation in this thesis. In CRS, our group has found that ILC2s are enriched in nasal polyps[161] and these ILC2s are responsible for producing T_{h2} cytokines[162]. Given the importance of T_{h2}-type inflammation and its cytokines involved in CRSwNP, ILC2s are likely to contribute significantly to the pathogenesis of CRSwNP. Nonetheless, their precise role in the context of CRS and what therapeutic implications they can bring on remain to be elucidated.

Group 3 ILC consists of ILC3s and lymphoid tissue inducer (LTi) cells. ILC3s are a robust source of IL-17/IL-22[163] whereas LTi cells are crucial to the initation of lymphoid organ formation[164, 165]. Currently, there is very limited evidence in the literature on the direct involvement of group 3 ILCs in CRS pathogenesis. However, given our research focus on lymphoid neo-organogenesis in CRS, LTi cells could be a relevant research target and their roles in CRS warrant further investigation. In murine lymph node and Peyer's patch development, LTi cells were proven to be essential by knocking out RORγt[166], which is indispensible for fetal LTi cell ($CD4^+CD3^-IL\text{-}7R\alpha^+$ cell) functions. Another mouse model knocking out RORγ, the only different isoform of the same protein, yielded similar detrimental effects[167]. During fetus development, LTi cells are attracted to mesenchymal lymphoid tissue organizer (LTo) cells in the lymph node and Peyer's patch anlagens through ICAM-1 and VCAM-1 expressed by both parties[168]. $LT\alpha_1\beta_2$-expressing LTi cells then engage LTβR-expressing mesenchymal cells to induce lymph nodes and Peyer's patches development[168, 169]. In the setting of lymphoid neo-organogenesis, the involvement and necessity of LTi cells are still controversial[164, 165]. RORγt-expressing LTi cells feature the production of IL-17[170, 171], a characteristic property of group 3 ILCs. This phenotype implicates an ancestral relationship between $ROR\gamma t^+$ LTi cells and IL-17 producing $CD4^+$ T_{h17} cells[172]. In the intestine, RORγt+ LTi cells are clustered in the cryptopatches between crypts of the intestinal lamina propria, where they govern the formation of isolated lymphoid follicles and produce IL-22[172], which is a critical cytokine for the immunological activation of epithelial cells. This raises an interesting question as to whether the same interactions between LTi cells and epithelial cells take place at the respiratory tracts or sinuses. Adoptive transfer of adult LTi cells into $CXCR5^{-/-}$ mice induced the formation of intestinal lymphoid tissues[170]. However, some studies suggest that lymphoid tissue formation can be induced independently of LTi cells in response to infections[173, 174]. It is proposed that LTi cells

are substitutable by other immune cells in post-natal lymphoid tissue formation[164, 165, 175] and this will be further discussed in later sections.

Immune cells and extracellular traps

Apart from the sensing of pathogenic patterns through the likes of TLRs and inflammasomes, it is also important to understand the effector cells in the innate immunity that directly give rise to the pathogenic inflammation and other symptoms in chronic inflammatory conditions. In the context of CRS, eosinophils have been highlighted in past studies as they are correlated with disease severity[176], recurrence rate[177, 178] and clinically distinct subsets of CRS[179]. In western countries, CRSwNP has a strong association with eosinophilic inflammation[180], as represented by the eosinophilic markers eotaxin and eosinophil cationic protein (ECP)[17]. In contrast, CRSwNP in Asian countries is predominantly associated with neutrophilic inflammation but CRSwNP with eosinophilic infiltration has increased over the past few years[180]. T_{h2} cytokines such as IL-5 and eotaxin are essential in tissue eosinophilia[180, 181] whereas neutrophilic infiltration is primarily associated with T_{h1} cytokines such as IFN-γ[182]. The T_{h1}/neutrophilic profile versus T_{h2}/eosinophilic profile exemplifies the interplay between innate and adaptive immunity, which will be discussed in more details in the next section. The way that innate immunity and adaptive immunity influence each other to give rise to a particular phenotype in CRS, such as CRSsNP versus CRSwNP and neutrophilic versus eosinophilic inflammation, is not well understood thus warrants further investigation.

In the past decade, there is an emerging interest in a new concept of immune cells releasing extracellular traps (ETs) to immobilize and kill microorganisms. ETs were reported for the first time in 2004 by Brinkmann et al where neutrophils released these web-like structures when encountering bacteria[183]. Due to the bactericidal effects of ETs, their composition and how they exert their functions have become a hot spot in the field of biomedical research. As for the structure of ETs, their backbone comprises DNA and histones binding a wide range of antibacterial molecules, including elastase, cathepsin G, bacterial permeability increasing protein (BPI) and myeloperoxidase (MPO) [183, 184, 185]. Some of the antimicrobial properties of ETs are attributed to the reactive oxygen species (ROS) produced by the myeloperoxidase (MPO) on neutrophil ETs (NETs) with activated immune cells at the inflammation site as the source of hydrogen peroxide[186]. There are two modes of releasing ETs from an immune cell – with or without triggering a special form of programmed cell death called ETosis[185], which features vacuolization and nuclear

chromatin decondensation with disintegration of the nuclear membrane[187]. In a non-ETosis scenario, neutrophilic vesicles containing ETs can travel across the cell membrane through budding to the extracellular space and then rupture to release their content[188]. In addition to chromosomal DNA, mitochondrial DNA can also be utilized to form ETs and be ejected by neutrophils[189] and eosinophils[190] but the mechanisms behind this peculiar way of ET formation are not clear. *S. aureus* has been reported to induce a rapid NET release without neutrophil lysis and the NET exhibited bactericidal effects[188]. After a prolonged exposure to pathogens, the nuclear envelope of neutrophils ruptured and the cells enter ETosis[188], which is distinctive from other programmed cell death like apoptosis and does not require the activity of caspases[187]. The cell membrane of granulocytes undergoing ETosis erupt and release DNA mixed with granular content into the extracellular milieu[185]. Other cell types capable of releasing ETs include mast cells[191], monocytes/macrophages[192] and, notably, eosinophils[193]. Eosinophil ETosis (EETosis) can be triggered by a variety of stimulating factors including phorbol myristate acetate (PMA), cytokines with a platelet activating factor and, remarkably, immunoglobulins (IgG and IgA)[194, 195]. EETosis mainly occurs in inflamed tissues[196] and EETs are thought to be involved in some chronic inflammatory diseases[197] such as atopic dermatitis[198] and allergies like allergic asthma[199].

The role of EETs in the characteristic eosinophilic inflammation in CRSwNP has been implicated by The Japan Epidemiological Survey of Refractory Eosinophilic Chronic Rhinosinusitis Study (JESREC Study). This multi-centre retrospective study, classified CRSwNP into eosinophilic CRS (ECRS) and non-ECRS in order to differentiate their refractory phenotypes[178]. Mucosal eosinophilia, which is defined as 70 eosinophils per high power field or higher[178], significantly correlated with higher recurrence rates of polyps after surgery. ECRS with more severe mucosal eosinophilia was significantly associated with higher rates of recurrence. There are pronounced clinical differences between ECRS and non-ECRS[200], particularly the highly viscous, glue-like mucous in ECRS. The massive eosinophilic infiltrates also account for the thick secretion observed in other persistent eosinophilic diseases including eosinophilic otitis media and allergic fungal sinusitis[201, 202]. Excessive amount of DNA was visualized in the eosinophilic mucin from an ECRS patient, where the DNA was confirmed to be EETs by immunostaining, indicating that EETs, in addition to mucin glycoproteins, are a major contributor to the high viscosity of the mucous in ECRS[195].

1.3 Adaptive Immunity in CRS

T cells and T_h type immune response

The adaptive immune system comprises different B cell and T cell subsets, which are equipped with different abilities and coordinate with each other in order to generate an appropriate type of immune response corresponding to the type of pathogenic challenge. The T helper cell, in particular, is a critical determinant of the type of mucosal inflammation in regard to neutrophilic versus eosinophilic predominance[73]. In CRS, the T_h cell profiles are mostly T_{h1}, T_{h2} and T_{h17}[14, 17], depending on the polyp versus non-polyp differentiation and ethnicity. A Spanish cohort of 18 CRSwNP patients had shown activated T cell infiltration expressing both IFN-γ and IL-5 simultaneously, representing a mixed T_{h1}/T_{h2} profile. This finding also suggests that the type of T_h cell response is not predetermined and is likely to be influenced by the local immunological micro-environment. In agreement with this study, a later study by Lam et al has demonstrated that T_{h17} is the default T_h phenotype in healthy nasal mucosa whereas T_h cells derived from nasal polyps express both T_{h2} and T_{h17} cytokines. By comparing the cellular and mediator profile of 10 CRSwNP patients and 13 CRSsNP patients in Belgium[17], T_{h2} cytokine IL-5 was highly upregulated in CRSwNP patients but not in CRSsNP patients or healthy controls whereas T_{h1} mediator IFN-γ was upregulated in CRSsNP patients only. The T_{h2}-dominant response in CRSwNP is consistent with the fact that *S. aureus* is prevalent in CRSwNP and a major driver of T_{h2} response [203, 204]. TSLP, another cytokine favouring a T_{h2}-type immune response, was also found to be elevated in CRSwNP[205]. A more recent study has shown that a subset of T_{h2} effector cells that express receptors for T_{h2} cytokines IL-25 and IL-33 were only present in CRSwNP but not in non-CRS controls[206]. These cells can respond to IL-25 and IL-33 to give an elevated IL-5 and IL-13 production, therefore augmenting the T_{h2}-response specifically in CRSwNP[206]. When these findings appear to suggest that CRSwNP and CRSsNP are mediated by T_{h2} and T_{h1} response respectively in a clear-cut fashion, eotaxin and eosinophil cationic protein (ECP), which are involved in eosinophil chemotaxis and degranulation respectively, are upregulated in both CRSwNP and CRSsNP patients compared to healthy control with the degree of upregulation being much higher in CRSwNP [17]. Since eosinophils are activated by T_{h2} cytokines [207] thus represent a T_{h2} response, it is speculated that a subset of CRSwNP and CRSsNP patients are at different progressive stages of CRS where the immune response shifts from type I to type II as a consequence of eosinophilic infiltration during nasal polyp development. In our study, we have found that tissue eosinophilia in CRSwNP patients

(n = 75) is more than twice as prevalent as in CRSsNP patients (n=59)[208], supporting the notion that inflammation in CRSwNP is predominantly eosinophilic and T_{h2}-skewed. Nevertheless, a T_{h1}/T_{h17} cytokine profile and neutrophil predominance is observed in Southern-Chinese CRSwNP patients in contrast to the T_{h2} predominance in Belgian CRSwNP patients as shown in the study by Zhang *et al* [14], suggesting that the association between T_{h2} and nasal polyposis may not be causative and complications such as ethnicity and geographical distribution need to be taken into account.

Infections are also one of the contributing factors to the T_{h1}/T_{h2} polarization[73]. Superantigens can skew the cytokine profile towards a T_{h2} response as in *S. aureus* enterotoxins in CRSwNP[27]. The previously discussed *S. aureus* biofilms could also favour a T_{h2} response independent of the superantigen effect[209]. In addition, microbes might manipulate the adaptive immunity to produce a mal-adaptive T_{h2}-skewed immune response in order to reduce innate immunity at the epithelium and consequently facilitate infection in CRS [105, 210]. To summarize, the T_h endotypes in CRS are determined by a variety of highly complex and continuous interactions between host factors (e.g. predisposition to lymphoid neo-organogenesis and ethnicity) and environmental factors (e.g. superantigens and biofilms). The question as to why T_{h2} response is associated with nasal polyposis in the western population is currently unclear and warrants further investigation.

B cells

Apart from the differential T helper cell profiles between CRSwNP and CRSsNP, there are also increased B cell activities in CRSwNP compared to CRSsNP. The number of $CD138^+$ plasma cells was significantly increased in CRSwNP compared to CRSsNP and non-CRS control[17]. The expression of B cell-activating factor of the TNF family (BAFF), a potent regulator of class-switch recombination and immunoglobulin production, is also elevated in CRSwNP[211] and this could be a contributing factor to the increase in plasma cell number. More $CD19^+$ cells were observed in CRSwNP compared to CRSsNP and control but not CRSsNP versus control[212], indicating that there are germinal centre reactions and B cell maturation specifically to the CRSwNP phenotype. Coherently, there were increased local, but not systemic, class-switched immunoglobulins, including IgG, IgA and IgE in CRSwNP versus CRSsNP[212]. The immunoglobulin overproduction in CRS might be related to bacteria since 71% of CRSwNP and 57% of CRSsNP patients possess bacteria-specific IgE as compared to 10% in allergic rhinitis[213]. The association between bacteria and the increased humoral immune response in CRSwNP is further supported by the fact that

specific IgE antibodies against staphylococcal superantigens SEA and SEB have been detected in nasal polyps[214]. It is proposed that the overproduction of local IgE and IgA contributes to the inflammation in nasal polyps by activating mast cells and eosinophils[215]. There were signs of inducible, follicle-like lymphoid structures in the airway mucosa of nasal polyps[216, 217, 218] and these lymphoid structures within the nasal polyps were later further characterized and confirmed, as tertiary lymphoid organs (TLOs), in our study[208].

1.4 Epithelial cells mediating Inflammation

Given the increasing evidence for epithelial cells actively participating in immune surveillance and inflammation[219], there is a rising interest in studying the innate immune mechanisms in mucosal surfaces and how they interact with pathogens and the "epimmunome", that is all the factors in epithelial cells associated with immunology, in the initiation of inflammatory disorders. As for innate immunity in epithelial cells, the inflammasome is an important member of the epimmunome when it comes to inflammation. Inflammasome is expressed by many epithelial cell types such as intestinal, skin and airway epithelial cells and epithelial inflammasome mediates inflammation in multiple inflammatory disease models[220]. Strong induction of AIM2 has been observed in the lesions of psoriasis and atopic dermatitis[152, 221], suggesting that AIM2 can induce severe inflammation at the epithelial barrier. Given that nasal epithelial cells can strongly express AIM2 inflammasome, which is a crucial factor for biofilm and polyp status in CRS[82], the role of AIM2 produced by nasal epithelial cells in promoting the pathogenic inflammation in CRS became one of our research interests. Apart from inflammasome in the innate immunity arsenal, airway epithelial cells have also been shown to express TLRs with a clear apical-basolateral differentiation in each individual TLR[107], demonstrating that epithelial cells possess the innate immune machinery which is well adapted to the exposure to pathogens of various types in the airway lumen. In functional terms, the agonists of TLR1/2, TLR3, TLR6 and TLR9 have the ability to stimulate primary human nasal epithelial cells to produce either IL-6 or IL-8[101], showing that the innate immune machinery of airway epithelial cells is actively involved in mediating an inflammatory response. The versatility of the airway epithelium in innate immunity (Figure 1.3) has made them important inflammatory mediators[132]. Furthermore, airway epithelial cells (AECs) can also regulate the adaptive immunity by producing T_{h1} or T_{h2} cytokines. AECs can produce α-defensins [132], which serve as a chemoattractant of naïve $CD4^+$ T cells, $CD8^+$ T cells and immature DCs [222]. α-defensins also induce the release of IFN-γ from T cells[223], favouring a T_{h1}

response. AECs can also promote T_{h2} type inflammation by producing TSLP[159], which is induced by TLR3 or T_{h2} cytokines stimulation. TSLP-stimulated myeloid DC (mDC) induce the differentiation of naïve $CD4^+$ T cells into T_{h2} cells that produce IL-4, IL-13 and TNF-α but not IL-10 and IFN-γ[224, 225]. Furthermore, TSLP is also one of the activators of cytokine-primed eosinophils to eject EETs[226] and this is remarkable given the prominence of eosinophilic inflammation in CRSwNP. These immune-mediating properties coupled with being the physical barrier in direct contact with the lumen, nasal epithelial cells have become a promising target to study the initiation of inflammation in CRS. Given that AIM2 can detect bacterial DNA and the extracellular DNA (eDNA) within bacterial biofilms are theoretically in direct contact with nasal epithelial cells, it is hypothesized that eDNA, particularly when produced by *S. aureus*, is a potent inflammation driver via AIM2 and responsible for increased disease severity in CRSwNP. Nonetheless, AIM2-deficient mice are more susceptible to dextran sodium sulphate (DSS)-induced colitis, implicating that AIM2 has a regulatory role in an inflammatory response[227]. This study has also shown that AIM2 exerts its regulatory effect by controlling the growth of opportunistic bacteria in the gut[227], implying that an increase in AIM2 might favour microbes that are more resistant to inflammatory responses such as biofilm-forming *S. aureus*. One condition has to be met before the hypothetical AIM2 activation by eDNA can happen – bacterial DNA has to be internalized into the corresponding inflammation-inducing cells in order to activate AIM2 in the cytosol. One mechanism that is central to the recognition of bacterial CpG-DNA and involves DNA internalization is the pathway controlled by two main proteins - high mobility group box 1 (HMGB1) and receptor for advanced glycation end-products (RAGE). The members of HMGB protein family bind DNA at the minor groove[228] and then exert their regulatory functions by increasing the affinity of the bound DNA to certain proteins, mainly transcription factors[229]. HMGB1, one of the damage associated molecular patterns, is secreted in response to pro-inflammatory stimuli[230]. Secreted HMGB1 binds to extracellular DNA in a structure-dependent (i.e. unmethylated CpG-DNA) rather than sequence-dependent manner[229]. The engagement of HMGB1-DNA complexes on RAGE expressed on the membrane of cells leads to the induction of IFN-α production in human peripheral blood mononuclear cells (PBMCs)[231]. AECs also can express HMGB1 and RAGE[232], hence the treatment with CpG-DNA results in IL-1β secretion in human bronchial epithelial cells[232], presumably through HMGB1-RAGE interactions. HMGB-1 complexes with DNA and thus facilitates its internalization[231] through endocytosis[233] but whether RAGE is essential in the process is still in question[231, 233]. The

internalization of HMGB1-DNA complexes results in the activation of AIM2 in the cytosol, triggering pyroptosis in macrophages[233] and eventually setting off an inflammatory response as discussed previously. Since airway epithelial cells also possess the necessary HMGB1-RAGE-TLR9-AIM2 machinery, it is hypothesized that bacterial CpG-DNA derived from an infection can initiate inflammation at the nasal epithelium through the activation of AIM2.

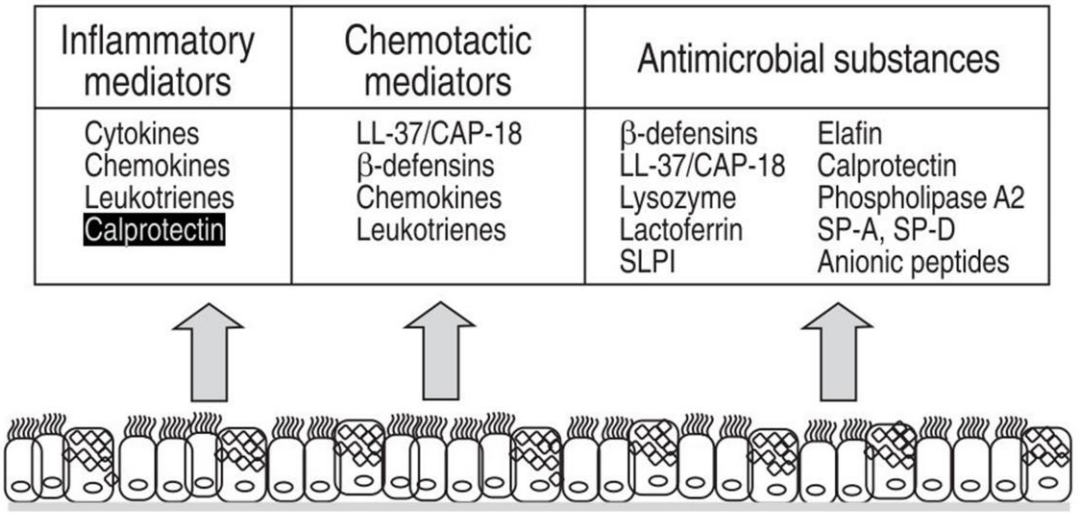

Figure 1.3 The repertoire of the innate immune mediators produced by AECs[132]

1.5 Secondary lymphoid organs and tertiary lymphoid organs

Overview of lymphocytes and lymphoid organs

Lymphocytes are formed in primary lymphoid organs, which consist of the thymus and the bone marrow. After the creation at the bone marrow, the maturation of B cells also takes place in the bone marrow whereas T cells travel to the thymus where they become mature. Some data suggests that Peyer's patches and other gut-associated lymphoid tissues (GALTs) may also be involved in B cells maturation[234]. Lymphocytes precursors undergo V(D)J-recombination, which randomly determines the receptor gene segments an individual lymphocyte will express, and thus giving rise to the complementarity determing regions (CDR) of the receptor (i.e. the determinant of the specificity for an antigen). For T maturation, immature T cells undergo a positive selection process in which T-cells progenitors not interacting with major histocompatibility complexes

(MHC)-self-antigen complex will undergo apoptosis and be deleted from the selection[235]. MHC molecules are expressed on all nucleated cells and they function as a marker of "self" identity and present antigens to lymphocytes[234]. T-cells that bind to MHC-self-antigen complex with high affinity are also deleted to avoid autoimmunity, leaving only T-cell progenitors binding MHC molecules with low affinity to develop further. Next, these cells passing the positive selection undergo negative selection in which T-cells reacting with tissue-specific antigens presented by dendritic cells, macrophages and thymic epithelial cells on MHC molecules are removed to prevent auto-reactive T cells from developing[235]. As for B cell maturation, it starts in the bone marrow where immature B cells expressing B cell receptors (BCRs) with no or weak binding affinity to self antigen can become transitional B cells and travel to the spleen[236]. Splenic transitional B cells enter a second checkpoint for autoreactivity screening where only B cells with low binding affinity to self-antigens are allowed to migrate to germinal centres and differentiate into memory B cells or short-lived plasma cells with the support of T helper cells in the follicles[236]. Mature lymphocytes enter circulation and are activated when presented with the antigens they are specific for in secondary lymphoid organs (SLOs) such as lymph nodes and mucosa-associated lymphoid tissues (MALTs).

SLOs provide a highly organized interface for antigen presentation to lymphocytes in order to generate an efficient immune response upon encountering a challenge by foreign antigens. SLOs develop in pre-determined locations with drainage from peripheral tissues. Entry of lymphocytes into SLOs is controlled by high endothelial venules (HEVs), which are blood vessels specialized in naïve lymphocytes binding and trans-endothelial trafficking into SLOs by expressing adhesion molecules and C-C motif chemokine ligand 21 (CCL21, a.k.a. secondary lymphoid tissue chemokine)[237]. There are very precise cell compartmentalizations within a SLO, orchestrated by chemokine-based homing of cells (i.e. cell migration towards a central spot) [237], that ultimately lead to the highly organized anatomy of SLOs. B cell homing is achieved by CXCR5-expressing B cells being attracted to CXCL13 expressed by B cell zone resident stromal cells whereas T cell homing is achieved by C-C motif chemokine receptor 7 (CCR7)-expressing T cells being attracted to T cell zone stromal cells expressing CCL21 and CCL19 (also called Epstein-Barr virus-induced molecule 1 ligand chemokine). Dendritic cells from the infected peripheral tissues translocate to the nearby draining SLOs, carrying the antigens derived from the infectious agent[238]. These mature DCs in SLOs become antigen-presenting DCs and interact with T cells in the T cell zone. T cells

specific for the presented antigens will be activated. Some activated T_h cells migrate to the boundary between the B and T cell areas or differentiate into follicular T helper cells (T_{fh}) that move to germinal centres[239]. These T_{fh} cells provide support to B cells in T-dependent antibody responses[239]. Other activated T cells exit SLOs and travel to the inflamed site as effector cells[237].

Tertiary Lymphoid Organs

The highly specialized and organized structures in SLOs can be induced in peripheral tissues, in the form of tertiary lymphoid organs (TLOs), when there is a persistent source of antigen and a constant need for extravasation of leukocytes as in zones of infection, transplant rejection and autoimmunity [165]. The immunological functions of TLOs are very similar to those of SLOs. It has become well understood that when inflammation becomes a chronic condition, lymphocytic infiltrates increase gradually, and local plasma cells expand in numbers [165]. While the functions of TLOs are meant to provide protective immunity as shown in peripheral lymphoid organ-knockout disease models[240, 241], TLOs can also have detrimental effects when the enhanced immune response fails to clear the infection or targets self-antigens (or grafted tissues). There are a surging number of studies reporting the identification of TLOs in various chronic inflammatory or autoimmune disease models [164, 165, 242], including diseases that involve mucosal inflammation such as COPD [243], IBD [244] and Sjögren's syndrome [245].

In SLO formation, $LT\alpha_1\beta_2$-expressing lymphoid tissue-inducer cells (LTi) bind LTβR that is expressed on stromal cells to instruct them to develop into FDCs and HEVs[246, 247]. In the context of TLO formation, the instructive signal, that is $LT\alpha_1\beta_2$-LTβR binding, for lymphoid tissue neogenesis remains essential but the cell types carrying this signal are likely to be dendritic cells [248, 249], which instruct naïve B cells to acquire LTi-like properties and interact with stromal cells [250]. Lymphocytes are also thought to be actively involved in the process of TLO formation[251, 252]. Subsequently, TLO construction is orchestrated by the chemokines (e.g. CXCL13 for B cells, CCL19/CCL21 for T cells) produced in accordance to stromal organization [241, 253]. Recent studies have shown that T_{h17} cells constitute a part of this process as well[254, 255] and thus the T_{h17} pathway has emerged as an important target to study in the initiation of TLO development. For instance, a model of pulmonary inflammation induced by LPS has shown that IL-17 is a key mediator for the formation of inducible bronchus-associated lymphoid tissue (iBALT)[256]. Furthermore, T_{h17} cells inhibitor IL-27 has been shown to prevent TLO development in inflammatory arthritis by controlling CD4 T cell response[257]. Interestingly, IL-17 is dispensable in the formation of iBALT

in response to replication-deficient poxvirus modified vaccinia virus ankara[258], implying that other cytokines belonging to the T_{h17} pathway can substitute IL-17 in inducing TLO formation. Recent studies have shown that other T_{h17} cytokines, namely IL-21, IL-22 and IL-23, also play a key role in TLO formation[259, 260]. However, there is yet to be a unifying theory encompassing the mechanisms of TLO formation and the variable degrees of structural organization of the infiltrates in different individuals. Although TLOs are induced in response to chronic inflammatory stimuli and thus can form theoretically in any inflamed non-lymphoid tissue, some tissues may have a higher propensity to develop TLOs than others[261]. Our data has shown that primary nasal epithelial cells can strongly express LTβ, BAFF and, intriguingly, IL-7Rα. When IL-7Rα heterodimerizes with the common cytokine receptor γ-chain (γc) or thymic stromal lymphopoietin receptor (TSLPR), the heterodimer recognizes IL-7 or TSLP respectively. The IL-7/IL-7R ligation enhances the expression of $LT\alpha_1\beta_2$ in LTi cells, which are the main source of $LT\alpha_1\beta_2$ signals for lymph node and Peyer's patch development[262]. Similar to the requirement of $LT\alpha_1\beta_2$, TLO development resembles that of SLO development as it also requires IL-7/IL-7R engagement[263]. IL-7R, but not IL-7, has significantly increased expression in CRSwNP patients compared to CRSsNP patients and is clustered with genes that are important for TLO development[208]. Therefore, it is inferred that epithelial cells can be a source of $LT\alpha_1\beta_2$ to initiate TLO development, as in $LT\alpha_1\beta_2$ -expressing LTi cells initiating SLO development, upon encountering foreign antigens. The ability of epithelial cells to produce these initiation signals might explain the propensity of TLO formation within nasal polyps.

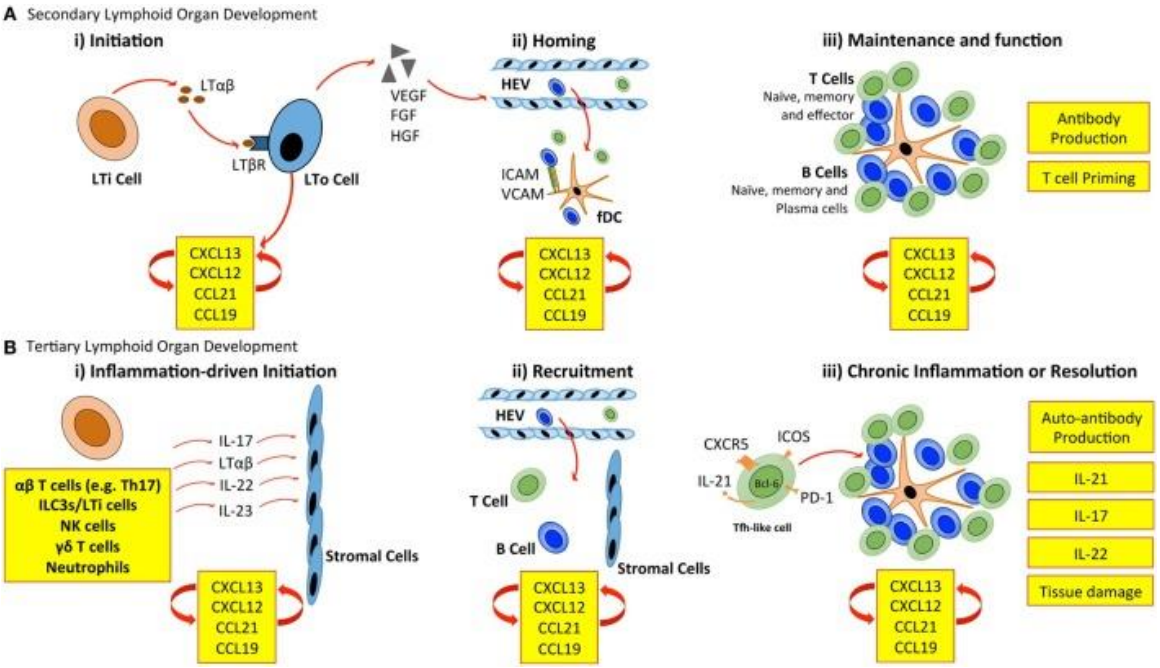

Figure 1.4 Comparison of the signaling for SLO and TLO development[175].

SLO initiation requires Lti cells to instruct LTo cells, through LTαβ-LTβR binding, to secrete chemokines for the contruction and organization of SLO. In TLO development, the initiation signal, LTαβ-LTβR binding, remains essential but LTi and LTo are replaced by relavant immune cells and peripheral tissue stromal cells respectively.

Identification of TLO

Neyt *et al.* proposed a set of criteria that can be used to define and identify TLOs [165] as follows:

(i) the organized infiltrate contains anatomically distinct yet adjacent T and B cell compartments;

(ii) the T cell area contains an extensive network of fibroblastic reticular cells (FRCs);

(iii) PNAd+ high endothelial venules (HEVs) are present;

(iv) evidence of B cell class switching and GC reactions in the B cell follicles;

(v) AID enzyme, which is necessary for ongoing class-switching, is present;

(vi) FDCs are present.

It appears that organized structures partly fulfilling these criteria can still function as a TLO [165]. TLOs can be identified, using immunohistochemistry, by demonstrating the clustering of B cells and FDCs within germinal centres and the presence of T cells and HEVs around the same follicles.

These combine to give the presence of necessary cell types and the high levels of organization between them in TLOs as required in the aforementioned criteria. This technique has been illustrated in other studies that successfully identified TLOs [264, 265].

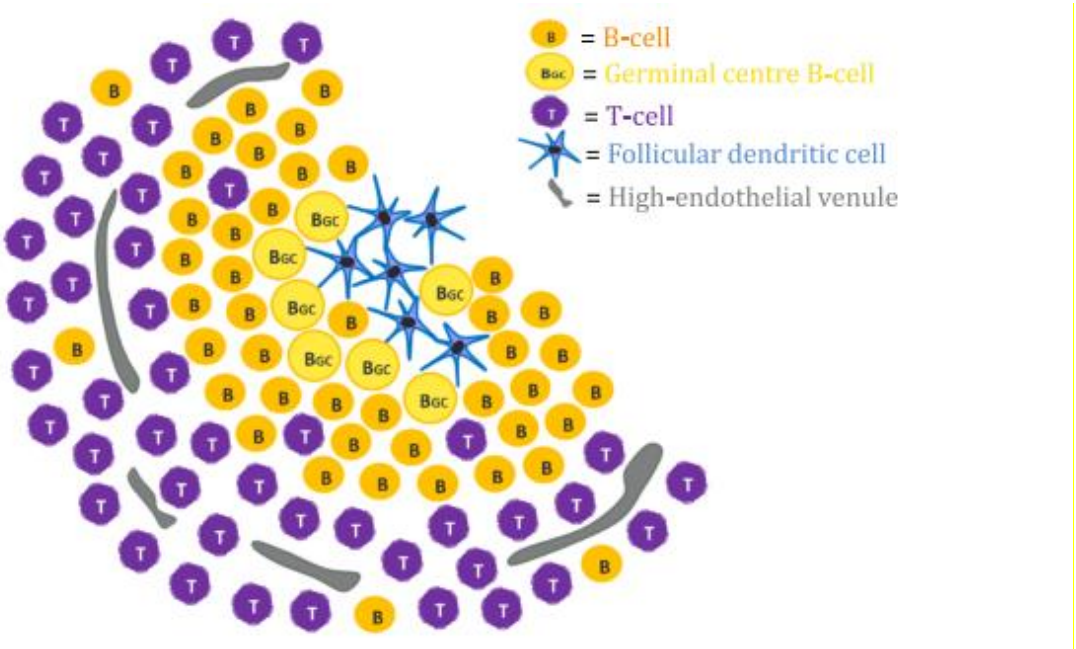

Figure 1.5 The structure of a TLO[264]

There are B cell and T cell zones in TLO with HEVs sitting at the outskirt of the boundary of B & T cell zones. An active GC is located at the centre of a TLO and it consists of FDCs and GC-B cells. Unlike SLO, TLO is a very dynamic structure and, hence, some TLOs may not possess all of these features but still retain some TLO functions.

TLOs in inflammation

The significance of identifying TLOs is that they are closely related to the immunopathology in inflammatory diseases and autoimmunity. In a model of dextran sodium sulphate (DSS)-induced colitis in RORγT-negative mice (i.e. lacking programmed lymphoid tissues, LTi and T_{h17} cells) [266], the immunity is mostly reliant on the generation of tertiary lymphoid tissues and the mice developed severe intestinal inflammation along with significantly more tissue damage upon DSS-introduced inflammation in the gut. This can be prevented by LTβR immunoglobulin treatment that blocks the LTβR receptor, indicating that TLOs are responsible for the severe inflammation observed in this disease model. Furthermore, the disease can also be

prevented by saturation of Fc receptors through intravenous immunoglobulin treatment, meaning that humoral immunity is crucial to the TLO-related immunopathology and this is coherent to the plasma cell expansion in TLOs. In a study that looked into immunologic memory in TLOs, memory B cells can be generated and maintained by TLOs independently of SLOs[267]. Since memory B cells are capable of responding to polyclonal stimuli such as CpG-DNA in the absence of BCR[268] and thus differentiating into antibody-secreting cells in response to inflammatory signals, TLOs can sustain local antibody production through the memory B cells generated by them. In view of these findings, TLOs form in response to chronic insults (e.g. persistence infection, autoimmunity, graft rejection etc) and then promote severe local ongoing inflammation, which leads to further tissue damage, via GC activities and humoral immune response. The even more disrupted epithelial barrier becomes more susceptible to further infection[269], causing the local inflammation to intensify and thus maintenance of TLOs. Therefore, it is hypothesized that a persistent source of inflammation, such as infections by biofilm-forming *S. aureus*, can set off a chronic inflammation that is maintained and aggravated by TLOs. A vicious circle of severe chronic inflammation is ultimately formed between the source of persistent inflammation, TLO formation, the inflammation-promoting TLO activities and the breached infection-prone epithelial barrier. The immunopathological role of TLOs has also been proposed in autoimmune diseases such as multiple sclerosis[270], primary Sjögren's syndrome [271], systemic lupus erythematosus (SLE) [272] and rheumatoid arthritis (RA) [273]. In regard to autoimmunity, TLOs have a hypothetical role in facilitating a local breakdown of self-tolerance [271, 272, 273] due to intensive cell death and processed self-antigen presentation in the microenvironment of inflamed nasal mucosal tissues in CRS.

Potential clinical implications of TLOs

Given the contributions of TLOs to the pathogenic chronic inflammation in chronic infections and autoimmune diseases, TLOs have become an important biomarker for severe chronic inflammation and this has profound implications in the clinical value of TLOs. In rheumatic autoimmune diseases, TLOs have been extensively studied due to the increasing recognition of the importance of TLOs in the pathogenesis of these diseases[274]. In rheumatoid arthritis, frontline anti-TNF biological therapies have a lower response in patients with TLOs in the synovial tissues compared to their TLO-negative counterparts[275], demonstrating the prognostic values of TLOs. Interestingly, the phase III clinical trial of anti-IL-17 biologics Secukinumab has shown some

success in RA patients with inadequate response to anti-TNFα agents (clinical trial identifier: NCT01377012, information from the ClinicalTrials.gov website, accurate as of April 2017) and this could be related to the significant role T_{h17} cytokines play in TLO formation and maintenance. Furthermore, there are some promising results in animal models of arthritis[276] and Sjögren's syndrome[277], where targeting LTβ using a LTβR-Ig fusion protein has led to the retardation of TLO development and disease amelioration. Nonetheless, translation of the beneficial effects of TLO-specific therapies from animal models to clinical application presents to be a great challenge. The human LTβ blocker failed to clinically replicate the therapeutic efficacy in rheumatoid arthritis or Sjögren's syndrome [278]. It is speculated that the success in animal models is attributed to intervening TLO development at an early stage. However, patients receiving LTβ-targeted treatment would have relatively mature TLOs and thus the blockage of LTβ at this stage may have substantially diminished effects. Given the importance of B cells in TLO-mediated immunopathology, B cell depletion biologics might have therapeutic efficacy against relevant chronic inflammatory conditions. There are a number of ongoing or completed phase II or phase III clinical trials investigating the efficacy of drugs targeting BAFF in TLO-related autoimmune diseases like SLE and SS[164, 274]. T_{h17} cytokines IL-17 and IL-21 are also hotspots for clinical trials of biologics against rheumatic autoimmune diseases[164, 274]. Results of these clinical trials might shed light on the future of targeting TLO mediators as a novel therapeutic strategy. However, proper stratification based on TLO positivity is desperately needed in clinical trials since the prevalence of TLOs is low in these diseases (30-40% in RA[279] and 27% in CRSwNP[208]).

1.6 Potential autoimmunity in CRS

In general terms, self-antigens are inert to our immune system in a healthy individual owing to negative selection during lymphocyte maturation. Autoimmunity arises when lymphocytes start recognizing self-antigens and generate an immune response against them. It is still largely unclear why and how autoimmunity occurs but there are emerging hypothetical explanations such as defects in genes that are involved in the negative selection process like genes governing the selection checkpoints of BCR and T cell receptor (TCR)(e.g. autoimmune regulator, AIRE)[280]. Apart from defective negative selection genes, some infections could also trigger autoimmunity by means of molecular mimicry of self-antigen or superantigen[281]. In terms of molecular mimicry of self-antigen, when the structure of microbial peptides is sufficiently similar to that of self-peptides,

lymphocytes that are activated by those microbial peptides can also be activated by self-peptides, leading to autoimmunity[282]. Another mechanism by which autoimmunity can be caused by molecular mimicry is a process referred to as epitope spreading, which is defined as the diversification of epitope specificity from the dominant epitope on the immunogenic protein to other sub-dominant epitopes on the same protein[283]. These sub-dominant epitopes could be structurally similar to the epitopes of self-proteins, causing autoimmunity. As for superantigens, they are defined as a family of microbial proteins that have the ability to activate a massive number of T cells in a non-specific fashion so as to camouflage the microbes from the adaptive immunity in an infection[284]. Superantigens can activate up to 25% of an individual's T cells[285] whereas only less than 0.01% of the T cell repertoire would be activated via conventional MHC-restricted antigen processing[284]. The massive pool of activated T cells created by superantigen could include self-reactive T cells, resulting in autoimmunity. Studies have found that CRSwNP is associated with the loss of tolerance in different forms like allergy and asthma. It has been shown that CRSwNP patients have increased local production of IgE against *S. aureus* enterotoxins[217]. IgE is a class of antibody that is closely related to allergic reactions due to its ability to sensitize basophils and mast cells by binding the receptors of these cells when activated by allergens[286]. Furthermore, there are signs of autoimmunity, which represents the loss of self-tolerance, being linked with CRSwNP. It has been shown that BAFF is upregulated in CRSwNP patients compared to CRSsNP patients and healthy controls[211]. BAFF plays a central role in class-switching and immunoglobulin production[287] and the overexpression of BAFF in transgenic mice leads to major manifestations of autoimmunity[288] such as sialadenitis that is reminiscent of pSS[289]. Our research associates have identified that sicca symptoms (i.e. dryness in the eyes and mouth, typical symptoms of pSS) are associated with CRS and asthma in an epidemiological study[290], suggesting that sicca symptoms, possibly pSS, share common pathogenic mechanisms governing inflammatory diseases of the upper and lower respiratory tract. In a recent study that looked at a cohort of SLE patients, the presence of *S. aureus* in the nose was shown to be associated with increased production of anti-nuclear autoantibodies (ANAs)[291], which are a major manifestation of autoimmunity and are used as diagnostic markers of autoimmune diseases (e.g. anti-dsDNA for SLE, anti-Ro/La for pSS), and disease severity of SLE[292]. This suggests that microbial infection could be a trigger for autoimmunity or self-perpetuating inflammation. Since *S. aureus* can cause persistent infection at the sinuses and is closely associated with CRS pathogenesis, it is possible that *S. aureus* or other

persistent infections drive an ongoing inflammation which sets off a secondary autoimmunity in a subset of CRS patients. In studies that are relevant to autoimmunity specifically in CRS, CRSwNP patients were found to possess higher levels of class-switched immunoglobulins (IgG, IgE and IgA)[211, 212]. Furthermore, the levels of anti-dsDNA ANAs IgG and IgA, normalized to total IgG and IgA respectively, are elevated within the polyps compared to inferior turbinate mucosal tissues from CRSwNP, CRSsNP and non-CRS control[291]. These results altogether suggest that on top of the hyperimmunoglobulinemia observed in nasal polyps, the immune activities in the polyps, possibly from TLOs, specifically produce more ANAs, particularly anti-dsDNA. Tan *et al.* hypothesized that the chronic inflammation in the local mucosal microenvironment is facilitated by the expansion of autoreactive B-cell clones that helped perpetuating inflammation[291]. In the context of the higher incidence of ANA generation in CRSwNP, their hypothesis strikingly resonates with our TLO theory where germinal centres at the site of local inflammation may produce autoreactive B cells due to the release of pro-inflammatory signals from intensive cell death, processed self-antigen presentation during apoptosis or pyroptosis and the enhanced humoral immune response in the TLO. It is known that TLOs can be the local production sites of autoantibodies[271, 293] and these autoantibodies are derived from the autoreactive B cells generated by the TLOs[175]. Examples include anti-citrullinated protein (anti-CCP) antibodies in rheumatoid arthritis[273], anti-Sjögren's syndrome antigen A (anti-SS-A) and anti-Sjögren's syndrome antigen B (anti-SS-B) in Sjögren's syndrome[293], anti-thyroglobulin and anti-thryoperoxidase antibodies in Hashimoto's thyroiditis, and anti-acetylcholine receptor antibodies in Myasthenia Gravis[294]. A recent study has looked at the therapeutic potential of stopping TLOs from generating ANAs by blocking IL-22[259], which is a cytokine necessary for the differential expression of CXCL12 in epithelial cells and CXCL13 in fibroblastic stromal cells and thus crucial to B cell recruitment and organization in TLO formation. The blockade of IL-22 has led to the impairment of TLO formation and, consequently, the reverse of autoantibody production[259], showing that TLOs can indeed produce ANAs and are a promising therapeutic target particularly in autoimmune diseases and possibly in a subset of CRS patients too. The theory where TLOs can induce autoimmunity would also explain why epithelial inflammatory diseases, as in the likes of pSS and asthma, are common co-morbidities in CRSwNP, given that epithelial cells are actively involved in mediating immune responses, both innate immunity and adaptive immunity[295], and defects in these epithelial immune-mediating pathways would ultimately contribute to the loss of tolerance through TLOs.

Chapter 2: Characterization of tertiary lymphoid organs in chronic rhinosinusitis

Statement of Authorship

Title of Paper	Tertiary lymphoid organs in recalcitrant chronic rhinosinusitis
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
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Principal Author

Name of Principal Author (Candidate)	Aden Lau
Contribution to the Paper	Preparation of samples Experiments conducted Data analysis and manuscript writing
Overall percentage (%)	100
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 25/3/18

Co-Author Contributions

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

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

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2.1 Abstract

Background: Chronic Rhinosinusitis (CRS) is a persistent inflammatory condition of the sinus epithelium. Tertiary lymphoid organs (TLOs) have been found in severe chronic inflammatory diseases. However, their presence in CRS has not been demonstrated.

Objective: To investigate the prevalence and the potential role of TLOs in CRS.

Methodology: Sinonasal tissue along with detailed information on disease severity and clinical history was obtained from CRS patients with nasal polyps (CRSwNP), CRS without nasal polyps (CRSsNP) and non-CRS controls. mRNA was analysed using a microfluidic qPCR array, generating an expression profile of 29 TLO-related genes. Relative expression was determined by normalizing to 2 housekeeping genes and to non-CRS controls. Haematoxylin & Eosin stained tissue sections from 158 patients were analysed for the presence of TLO-like lymphoid aggregates and representative samples from the 3 groups were analysed by immunohistochemistry for the presence of TLOs.

Results: TLOs were observed in 28/75 CRSwNP (37%), 6/59 CRSsNP (10%), and in 0/24 control patients. TLOs were exclusive to CRS patients compared to non-CRS controls ($p = 0.002$) and were 5.2-times more prevalent in CRSwNP than in CRSsNP patients ($p = 0.003$). 17 TLO-related genes had increased relative expression in CRSwNP patients ($p<0.05$). TLOs were associated with disease recalcitrance ($p = 0.01$), as defined by the number of previous operations, and with tissue eosinophilia ($p = 0.003$).

Conclusions: Our data indicates that recalcitrant CRSwNP patients demonstrate massive inflammation with TLO formation in association with tissue eosinophilia. Analysis of matched gene expression and histopathology data suggests that CRS patients could be at different stages of TLO development.

2.2 Introduction

Chronic Rhinosinusitis (CRS) is a common chronic inflammatory condition of the paranasal sinus mucosa, affecting approximately 10.5% of western populations [3, 4]. CRS poses a heavy economic burden to the health care system owing to its high prevalence and need for surgery [9]. Moreover, at least 10% of CRS patients do not respond to medical and surgical treatment regimens and develop refractory disease [21]. CRS patients can be phenotypically classified according to the presence or absence of nasal polyps [1]. CRS patients with polyps (CRSwNP) tend to have higher disease severity and risk of recurrence compared to patients without polyps (CRSsNP) [18]. The self-perpetuating inflammation in CRSwNP is closely associated with persistent infections such as intracellular and mucosal biofilm-associated *Staphylococcus aureus* infections [79, 80], but the underlying mechanisms remain unclear.

In order to generate an efficient immune response upon encountering antigen, the immune system has evolved to develop secondary lymphoid organs (SLOs) (e.g. Spleen, lymph nodes) present at specific anatomical localizations. SLOs allow intensive screening of pathogens in circulation or tissues and provide a highly organized interface for antigen presentation to lymphocytes. This kind of highly efficient and organized lymph node-like structure can also be induced, in the form of tertiary lymphoid organs (TLOs), when there is a persistent source of antigen and a constant need for extravasation of leukocytes as in zones of infection, transplant rejection and autoimmunity [165]. TLOs are thought to play an important role in severe chronic inflammation and have been found in chronic obstructive pulmonary disease (COPD) [243], inflammatory bowel disease (IBD) [244] and Sjögren's syndrome [245]. It has become well understood that when inflammation becomes a chronic condition, lymphocytic infiltrates increase gradually and local plasma cells expand in numbers [165]. Given that massive lymphocytic infiltration can be observed in CRSwNP patients and TLOs are commonly found in different severe chronic inflammatory conditions, it is hypothesized that TLOs are present in CRS.

The purpose of this study is to determine whether TLO formation occurs in the sinonasal tissues of CRS patients using current histopathological criteria [165] as shown in Box 1. Using matched mRNA expression and immunohistochemistry from CRSwNP, CRSsNP and control patients, we determined the expression of genes known to be involved in TLO formation and maintenance. The

Box 1. Definition and identification criteria of TLOs

(i) The organized infiltrate contains anatomically distinct yet adjacent T and B cell compartments;

(ii) The T cell area contains an extensive network of fibroblastic reticular cells (FRCs);

(iii) Peripheral node addressin PNAd+ high endothelial venules (HEVs) are present;

(iv) Evidence of B cell class switching and germinal centre (GC) reactions in the B cell follicles;

(v) Activation-induced cytidine deaminase (AICDA), which is necessary for ongoing class-switching, is present;

(vi) Follicular dendritic cells (FDCs) are present.

relationship of TLO formation to clinical data such as disease severity scores and tissue eosinophilia was also evaluated.

2.3 Methods

Patients and Samples

This study was approved by the Human Research Ethics Committee of The Queen Elizabeth Hospital, Adelaide, Australia (reference number: HREC/15/TQEH/132). Biopsies were obtained from the ethmoid sinuses from patients undergoing endoscopic sinus surgery for CRS at the Department of Otolaryngology, Head and Neck Surgery, The Queen Elizabeth Hospital, Adelaide, Australia. Control patients were patients undergoing endoscopic skull base procedures without clinical or radiological evidence of sinus disease. CRS patients fulfilled the diagnostic criteria set out in the recent position papers by the American Academy of Otolaryngology and Head and Neck Surgery and the European Position Statement (EPOS) on CRS [296, 297]. Patients with CRS were further sub-classified according the absence (CRSsNP) or presence (CRSwNP) of visible polyps present within the middle meatus on nasal endoscopy as defined by EPOS guidelines [296, 297]. Nasal polyp tissues were obtained from CRSwNP patients and mucosal tissues were obtained from CRSsNP and control patients. Tissues for RNA extraction were placed in RNAlater (Sigma, St. Louis, USA) for 24 hours at 4˚C and then stored at -80˚C before total RNA preparation. Samples for immunohistochemistry were washed twice in 1x PBS and fixed in 10% neutral buffered formalin, followed by paraffin-embedding.

Histology

Paraffin-embedded tissue samples were cut in 4µm thickness, stained with Haematoxylin & Eosin (H&E) and scanned using digital whole-slide imaging (WSI) technology (NanoZoomer, Hamamatsu). Tissue eosinophilia was determined by averaging the number of eosinophils per High Power Field (HPF) (0.035 mm^3) from at least 6 HPF's/slide as specified by Ramezanpour et al [298].

Immunofluorescence

Tissue handling and processing were performed as described by Cantero et al [299]. In short, formalin-fixed paraffin-embedded tissue blocks were sectioned in 4µm thickness. Slides were de-paraffinized and rehydrated. Antigen retrieval was induced at 100˚C for 10 minutes in 10mM sodium citrate buffer, pH 6. Slides were stained with various combinations of primary antibodies against the following human antigens using overnight incubation at 4˚C: mouse mAb L26 to CD20 at 1:10 dilution (Abcam, Cambridge, UK); rabbit mAb EP3093 to CD21 at 1:50 dilution (Abcam, Cambridge, UK); rat MECA-79 to PNAd at 1:10 dilution (Biolegend, California, USA); mouse mAb PS1 to CD3 at 1:5 dilution (Abcam, Cambridge, UK). Control primary antibodies matching the species and antibody isotypes respectively with irrelevant binding specificity are the following: mouse IgG2a ICIGG2A to a synthetic hapten (Abcam, Cambridge, UK); rabbit serum IgG I5006 (Sigma, St. Louis, USA); rat IgM RTK2118 to trinitrophenol (Biolegend, California, USA); mouse IgG1 MG100 isotype control (Invitrogen, Massachusetts, USA). The following fluorescent dye-conjugated secondary antibodies were used at 1:200 dilutions: Alexa Fluor 488-conjugated polyclonal anti-mouse IgG (Jackson ImmunoResearch Laboratories, Pennsylvania, USA); Cy3-conjugated polyclonal anti-rat IgM (Jackson ImmunoResearch Laboratories, Pennsylvania, USA); Alexa Fluor 647-conjugated polyclonal anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Pennsylvania, USA). Cell nuclei were visualised with DAPI (4',6-Diamidino-2-phenylindole dihydrochloride, Sigma, St. Louis, USA). Slides were incubated with Sudan Black solution (10mg/mL in ethanol, Sigma, St. Louis, USA) for 5 minutes to reduce auto-fluorescence. Slides were analysed with a Zeiss LSM700 confocal microscope.

Quantitative Real-time PCR

Total RNA was prepared with TRIzol (Invitrogen, Massachusetts, USA) according to the manufacturer's instructions; total RNA with RNA quality index 6.5 or above only, as determined by Experion RNA Analysis kit (Bio-Rad, California, USA), was allowed to proceed to cDNA synthesis; cDNA was synthesized from total RNA using Quantitect Reverse Transcription kit (Qiagen, Hilden, Germany). Biomark HD System (Fluidigm, California, USA) was used with Taqman chemistry (Applied Biosystems, California, USA) for all genes as instructed by the manufacturer's protocol to perform qRT-PCR analysis. All cDNA was pre-amplified for 14 cycles before performing 35 cycles of 48x48 Dynamic Array Integrated Fluidic Circuit.

Statistical analysis

Gene relative expression was determined by the delta-delta Ct method according to Vandesompele 2002 [300]. Ct values were normalised to endogenous control genes, B-actin and HPRT1, present on each PCR plate, and results for CRSsNP and CRSwNP patients were normalised to non-CRS controls Analysis was performed using R version 3.2.1 and the R library MCMC.qPCR [301]. Full details, including the unique assay ID, for all proprietary primers used are listed on Table 2.2 in the supplementary data.

Multivariate correlations between gene expression data was analysed by hierarchical clustering, using R library pvclust [302]. The dendrogram was constructed from the Pearson correlation matrix of the log(expression) values (i.e. normalized to the endogenous control genes), using a distance measure of (1-|correlation|) and the complete linkage method, with subsequent bootstrapping to identify significant gene expression clusters.

Associations between TLO and patient subgroups or clinical data within CRS patients were analysed by Monte Carlo simulated exact tests, by either exact logistic regression (R library elrm [303] or other standard appropriate statistical tests from the R library coin[304]. All analyses within CRS patients were stratified by CRS subgroup.

2.4 Results

Patient demographical data

181 patients in total were included in this study. 68 were females and 111 were males (2 without data), aged 18 to 83 years old. The patients include 29 non-CRS controls, 69 CRSsNP patients and 83 CRSwNP patients. Summary of demographic information is listed in table 2.1.

Table 2.1 Summary of patient demographics

	Control	CRSsNP	CRSwNP
Average Age	53.92	51.30	54.41
Sex (Male)	13/24 (54.17%)	35/59 (59.32%)	50/74 (67.57%)
TLO	0/24 (0%)	6/59 (10.2%)	28/75 (37.3%)
Asthma	1/24 (4.2%)	18/59 (30.5%)	42/75 (56.0%)
Aspirin Sensitivity	0/24 (0%)	1/59 (1.7%)	10/75 (13.3%)
Average Previous. OP	0	0.88	1.77
Revision Surgery	0/24(0%)	27/59(45.8%)	49/75(65.3%)
Eosinophilia	3/24 (12.5%)	22/59(37.29%)	61/74(82.43%)
Oral Steroid*	3/24(12.5%)	2/59(3.4%)	6/75(8%)
Antibiotics*	0/24(0%)	16/44(36.4%)	9/56(16.1%)
Group Total	24	59	75

*3 weeks prior to operation

Relative expression of TLO-related genes in CRSwNP patients

Data were derived from 2 PCR assays. Assay#1 had 21 CRSwNP patients, 17 CRSsNP patients and 7 controls. Assay#2 had 26 CRSwNP patients, 11 CRSsNP patients and 9 controls. 11 CRSwNP patients, 10 CRSsNP patients and 6 controls were common to both assays. In the CRSwNP patient group, there was a significant increase in the mRNA expression of 17 genes including AICDA (fold-change = 8.79, 95% CI = (2.32, 32.45), p-value = 0.002), CCR7 (5.16, CI = (2.77, 8.41), p<0.001), CD274 (2.18, CI = (1.58, 3.15), p<0.001), CD40LG (3.94, CI = (2.78,

5.52), p<0.001), CXCL13 (4.12, CI = (0.63, 14.51), p=0.032), CXCR5 (11.57, CI = (4.81, 29.82), p<0.001), GPR183 (2.69, CI = (1.93, 3.92), p<0.001), ICAM1 (2.21, CI = (1.17, 4.32), p=0.024), IL-7R (3.22, CI = (2.33, 4.53), p<0.001), LTα (1.9, CI = (1.20, 3.03), p=0.002), LTβ (5.55, CI = (3.53, 8.74), p<0.001), PDCD1LG2 (2.18, CI = (1.58, 3.10), p<0.001), PDPN (1.54, CI = (1.15, 2.23), p=0.014), SELPLG (4.43, CI = (2.48, 7.78), p<0.001), RANKL (4.09, CI = (2.16, 7.67), p<0.001), BAFF (2.32, CI = (1.47, 3.89), p=0.002), VCAM1 (2.44, CI = (1.56, 3.81), p<0.001) and a significant reduction in mRNA expression of 2 genes including RANK (0.31, CI = (0.15, 0.59), p<0.001) and VEGF-C (0.61, CI = (0.44, 0.84), p=0.008). A comparison of the fold-changes between CRSwNP and CRSsNP for all genes with differential relative expression (p<0.05) in at least one of the two groups is shown in Figure 2.1. Relative expression details of all genes are provided in Table 2.2.

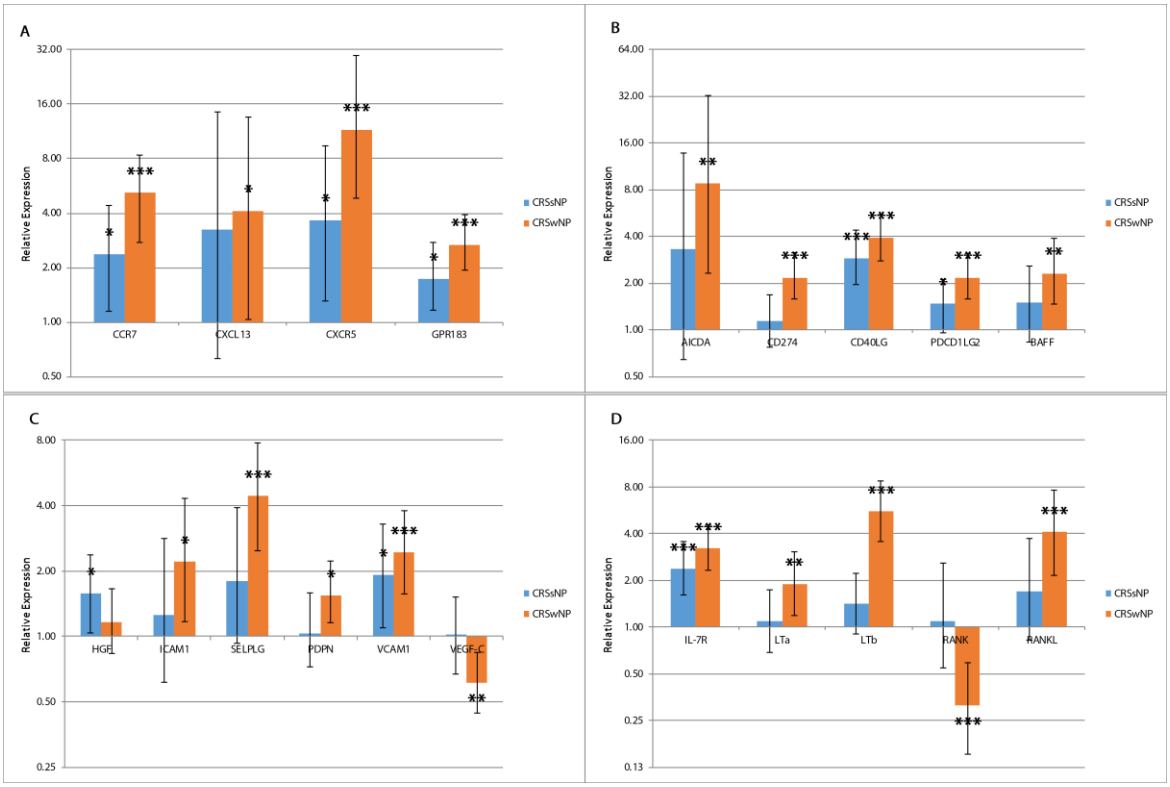

Figure 2.1 Relative expression of differentially expressed TLO-related genes in CRSsNP and CRSwNP tissue

Genes are grouped according to function into lymphocyte homing and compartmentalization (A), GC reaction and T-cell regulation (B), lymphangiogenesis and white blood cell recruitment (C), and lymph node development (D). Y-axes are plotted in log-2 scale. Error bars indicate the 95% CIs. *P ≤ .05 **P < .01, and ***P < .001

Hierarchical clustering of TLO-related gene expression

Hierarchical clustering of TLO-related gene expression (Figure 2.2) identified three clusters of genes which reached statistical significance, with 7, 2 and 7 genes in cluster 1, 2 and 3 respectively. Positively correlated genes within the same cluster were collectively either increased or decreased in the same patient. All genes in these clusters are positively correlated within the same cluster, except TNFRSF11A (RANK), which is negatively correlated with the rest of the genes in cluster 1. Change in expression of each gene in all 3 clusters is statistically significant ($p<0.05$) in the CRSwNP group, except PDCD1 ($p=0.12$).

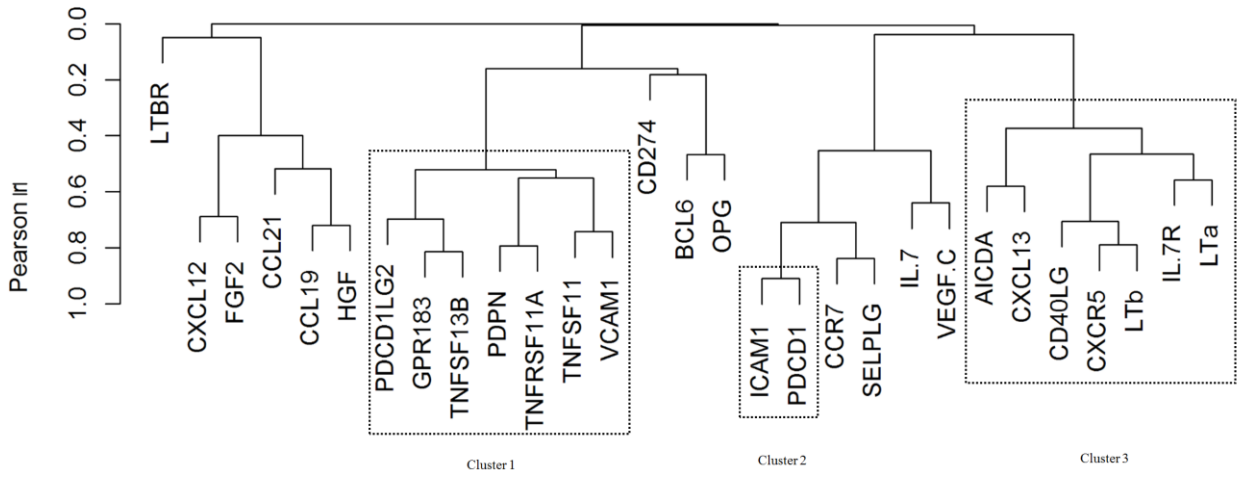

Figure 2.2 Hierarchical clustering of TLO genes based on expression

Cluster dendrogram for correlations of the expression of TLO-related genes. Each dotted-line represents a cluster of genes that has statistically significant Pearson correlation ($P < .05$).

GC-like structures are identified in sinus tissue from CRSwNP patients

H&E stained sections of 24 controls, 59 CRSsNP and 75 CRSwNP patients were examined for the presence of TLO-like structures and number of eosinophils. A TLO-like structure was defined as an organised lymphoid structure present in the sinonasal mucosa as exemplified in Figure 2.3A. These were observed in 6 out of 59 CRSsNP (10%) and in 28 out of 75 CRSwNP patients (37%) but in none of the 24 control patients (Table 2.3). TLO-like structures are exclusive to CRS patients ($p = 0.002$, Fisher's exact) and are more frequent in CRSwNP compared to CRSsNP (OR 5.2, 95% CI = 1.9, 16.7, $p = 0.003$). To confirm that these structures were indeed TLOs, we undertook an immunohistochemical analysis on tissue sections from representative patient groups that had suspected TLO-like structures (group 1: 5 CRSwNP, 1 CRSsNP) and those that did not (group 2: 2 CRSwNP, 2 CRSsNP and 3 non-CRS controls). In our immunofluorescence analysis, only CRSwNP patients in group 1 possessed structures where FDCs were clustered with B cells to give a clear meshwork that resembled an active GC (Figure 2.3B). HEVs were observed in the T cell zone surrounding a putative GC (Figure 2.3C), matching the anatomical feature of a TLO.

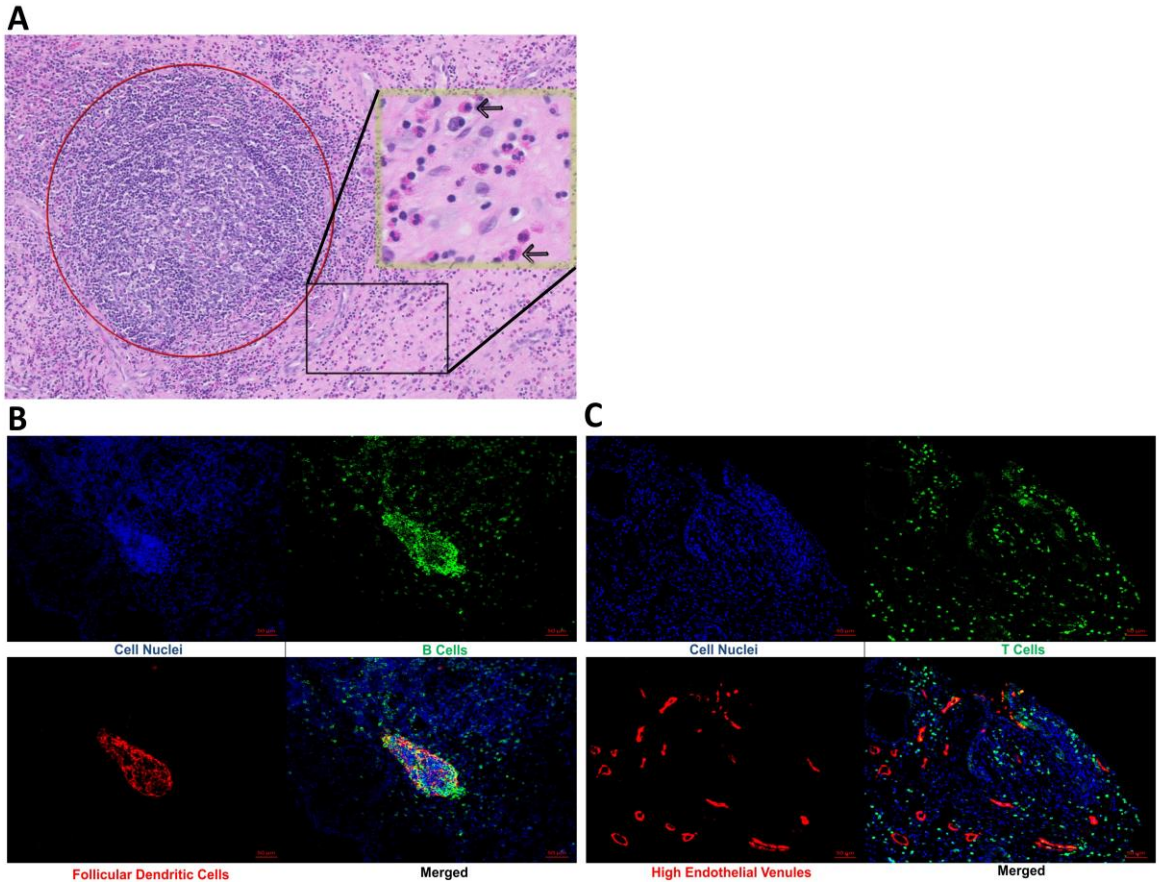

Figure 2.3 Representative images of TLOs in patients with CRSwNP

A) Hematoxylin and eosin staining showing a TLO (red circle) and tissue eosinophilia (black arrows in inlet). B, A highly organized TLO containing follicular dendritic cells (red) and B cells (green). C, Abundant high endothelial venules (red) and T-cell infiltration (green). Cell nuclei stained by 49-6-diamidino-2-phenylindole, dihydrochloride (blue).

Associations of TLOs with clinical data, demographic factors and histological characteristics in CRS patients

All analyses within CRS patients were stratified by patient subgroup. There was no association between the presence of TLO and age ($p = 0.70$), gender ($p = 1.0$), asthma ($p = 0.37$), or aspirin sensitivity ($p = 0.72$). However, TLOs were positively associated with tissue eosinophilia (OR 6.0, 95% CI 1.5, 48.0, $p = 0.003$, exact logistic regression, stratified by CRS subgroup, Table 2.4). TLOs were also positively associated with the number of previous operations ($p = 0.010$, exact Wilcoxon-Mann-Whitney test, stratified by CRS subgroup, Table 2.5).

2.5 Discussion

Our data strongly support the notion that TLOs are present within polyp tissue in a subset of CRS patients. This is evidenced by our immunofluorescence analysis showing (1) that FDCs and HEVs were found exclusively in nasal polyp tissues in CRS patients but not in any of the control patient tissues; (2) that HEVs were found in the outer-follicle, corresponding to an important anatomical feature of a TLO [264]; and (3) by our gene expression analysis, showing an upregulation in CRS patients of a number of genes that are essential in TLO formation and maintenance. Nonetheless, not all CRS patients had observable TLOs and in those who did, the degree of structural organization varied from one patient to another. Similarly, TLOs or TLO-like structures reported in other disease models have variable compliance of the proposed TLO definition (Box 1) [165]. We speculate that a spectrum of structural organization of TLOs exists within CRS patients. Whether or not the extent of structural organisation associates with disease severity is unclear and warrants further investigation.

There are profound implications of the presence of TLOs in CRS. In the context of infections, TLOs function as inductive sites for local protective immunity and disintegrate once the infection is cleared [240, 241]. When the immune system is unable to clear the infection or there are frequent re-infections, TLOs generate ongoing and often pathological humoral immune responses associated with significant tissue damage [241, 266, 267]. Epithelial damage in turn facilitates bacterial infection and TLO formation, creating a self-perpetuating cycle of disease [259, 269]. Our finding of an association between TLO formation and a higher number of previous surgeries supports the notion that TLOs are associated with recalcitrant or more severe CRS. Further research will be needed to understand whether TLO formation in CRS patients negatively impacts on disease progression and severity. Deleterious effects of TLO formation are typically encountered in autoimmunity and transplant rejection. This is exemplified in Sjogren's syndrome [271], rheumatoid arthritis [273], autoimmune diabetes [305] and transplant rejections of different organs [306, 307, 308]. In auto-immune diseases, TLOs can be the local production sites of anti-nuclear autoantibodies (ANAs) [271, 293]. Interestingly, there is some evidence of autoantibody production, particularly ANAs, within the polyps of CRSwNP patients [291]. The association between local and systemic ANAs, TLO formation and CRS warrants further investigation.

The presence of TLOs in CRSwNP patients was found to be positively associated with tissue eosinophilia and eosinophils were observed both within TLOs and in the surrounding micro-environment. The significance of this finding is not clear since, apart from infiltration of eosinophils as part of a general influx of reactive immune cells in the context of chronic inflammation and TLO formation, a specific association of TLOs and eosinophilia has not previously been reported. It is well known that CRSwNP patients often have a predominant Th2-type of inflammation with increased expression of Th2 cytokines and tissue eosinophilia [161, 309]. It is also known that the airway micro-environment intrinsically favours the generation of Th2 types of responses. This is supported by the finding that T cell priming by intranasal delivery of a Th1-inducing antigen promotes Th2-dominated responses with eosinophilia, rather than the expected Th1 responses [310]. In a follow-up study by Constant et al, it was shown that these Th2 responses are critically dependent on the presence and activation of resident tissue $CD11c^+$ Dendritic Cells (DCs) [311]. DCs were furthermore shown to be essential for the organization and maintenance of inducible bronchus-associated lymphoid tissue (iBALT), a form of TLO induced in the lungs after influenza virus infection [241]. In view of these findings, it can be hypothesized that our finding of a specific association of TLO formation and tissue eosinophilia might be dependent on the presence and activation of specific DC subsets. Further research will be needed to define the role of eosinophils in the context of TLO structure and function and potential common immune pathways of TLO-formation and tissue eosinophilia in the context of CRS.

In our gene expression analysis, many genes that are critical to lymph node development were upregulated in CRSwNP patients, especially LTα, LTβ, IL-7R and RANKL. These results indicate that the TLO formation we observe here shares a number of the signalling pathways of lymph node development as reported [312] . Nevertheless, the inducing agents, instructive signals and cell types involved in TLO formation remain undefined. For instance, the involvement of LTi in TLO formation is still controversial [165]. Gevaert et al suggested that organized lymphoid structures exist in nasal polyps of CRS patients with locally produced IgE against *Staphylococcus aureus* enterotoxins and increased eosinophilic inflammation [217]. Given the association between TLOs and eosinophilia as described in our study, and the notion that *S. aureus* infections and biofilms are associated with worse prognosis and disease recalcitrance in CRS [209], it is likely that microbial antigens contribute to the formation of TLOs in CRS. Various infectious agents, such as

Helicobacter pylori [313], *Influenza A* [240] and *Epstein-Barr virus* [314], have been shown to be associated with lymphoid neogenesis, however, a specific role for *S. aureus* in the induction of TLO formation has not yet been reported.

We next examined correlations between relative gene expression to identify co-regulated networks relevant to TLO organogenesis [315]. Our analysis generated 3 gene clusters, 2 of which were large enough (gene clusters 1 and 3, 7 genes each) to allow further assessment. Gene cluster 1 consists of GPR183, PD-L2, PDPN, RANK, RANKL, BAFF and VCAM-1. These genes are mainly associated with committing stromal cells to lymph node development (PDPN, RANK, RANKL and VCAM-1) and functions related to T-cell-dependant humoral immune responses (GPR183, VCAM-1 and arguably PD-L2). Two genes are more ambiguous in terms of fitting to these functions. First, BAFF enhances B cell survival and boosts humoral immune responses presumably in a general fashion, rather than being dedicated to T-cell-dependant humoral immune responses [316]. The fact that BAFF also fell within this gene cluster may point to the involvement of type I IFN as in other inflammatory diseases [317, 318]. Second, PD-L2 is expressed on professional APCs and has anti-proliferative effects on $CD4^+$ T cells when bound to PD-1 [319]. Although it appears to be an immunosuppressive gene as PD-L2-deficient mice suffer from loss of tolerance [319], a more recent study had found that PD-L2 also inhibits $CD4^+$ $FOXP3^+$ T regulatory cell (T_{reg}) homing to lymph nodes [320].

Gene cluster 3 consists of AICDA, CD40LG, CXCL13, CXCR5, IL-7R, LTα and LTβ. These genes are all strongly linked to B cell follicle development, homeostasis and functions and give rise to GC reactions and class-switched antibody production. $LT\alpha_1\beta_2$ is a crucial instructive signal for lymphoid organogenesis by activating two NF-κB pathways [321], which in turn activate the transcription of important TLO-related genes such as VCAM-1, CXCL13 and BAFF. Mice lacking LTα or LTβ have disrupted FDC-B-cell networks and thus underdeveloped GCs due to the loss of $LT\alpha_1\beta_2$ [322, 323]. $LT\alpha_1\beta_2$-expression is enhanced by IL-7-IL-7R signalling [324], which is essential for lymphoid follicle formation as a result. IL-7R is expressed on germinal centre B cells only when they are activated [325]. IL-7R is also expressed in late follicular T helper cells (T_{fh}) [326], which, through the expression of CD40LG [239], play a central role in the selection of GC B cells that then become plasma cells and produce class-switched antibodies [327]. Mice lacking CD40LG or CD40

have impaired class-switching and GC formation [328, 329]. Hence, the fact that IL-7R was found elevated in our gene expression analysis justifies the follicle-related properties of these genes. Another core gene for affinity maturation and class-switching, AICDA [330], also belongs to this gene cluster. The importance of AICDA to TLO-related pathology has been exemplified in chronic inflammatory diseases as in infections [331] and autoimmune diseases [273, 332]. CXCL13 is expressed by FDCs to attract B cells that express CXCR5, the receptor for CXCL13, providing the basis for B cell homing. Furthermore, CXCL13-CXCR5 interaction promotes $LT\alpha_1\beta_2$ expression on B cells to give a positive feedback loop, reinforcing B cell follicle formation and homeostasis [333]. Based on these associations, we propose that CRS patients with high levels of cluster 3 genes are likely to have more mature forms of TLOs (i.e. at the structurally organized end of the TLO status spectrum) and, therefore, more likely to have recalcitrant disease after surgery.

2.6 Conclusion

The presence of TLOs in CRS patients has been demonstrated by immunofluorescence and gene expression studies. Analysis of a cohort of 158 patients with matching histology and clinical data has shown that TLOs are positively associated with tissue eosinophilia and the number of previous operations. The gene expression profile suggests that CRS patients could be at different progressive stages of the disease, which are reflected by the TLO status. Our data suggests that TLOs may have prognostic value in predicting the chance of disease relapse. Targeting the triggers and molecular pathways that govern TLO formation and maintenance may help alleviate the ongoing severe inflammation in recalcitrant CRS.

2.7 Supplementary data

Table 2.2 Details of all primers used in the microfluidic qPCR assay

Assay ID	Gene Symbol	Gene Name
Hs01060665_g1	ACTB	beta actin
Hs02800695_m1	HPRT1	hypoxanthine phosphoribosyltransferase 1
Hs00989654_g1	CCL21	chemokine (C-C motif) ligand 21
Hs00171149_m1	CCL19	chemokine (C-C motif) ligand 19
Hs01013469_m1	CCR7	chemokine (C-C motif) receptor 7
Hs01099203_m1	VEGFC	vascular endothelial growth factor C
Hs00266645_m1	FGF2	fibroblast growth factor 2 (basic)
Hs00300159_m1	HGF	hepatocyte growth factor (hepapoietin A; scatter factor)
Hs00174202_m1	IL7	interleukin 7
Hs00233682_m1	IL7R	interleukin 7 receptor
Hs00921372_m1	TNFRSF11A	tumor necrosis factor receptor superfamily member 11a; RANK
Hs00243522_m1	TNFSF11	tumor necrosis factor (ligand) superfamily member 11; RANKL
Hs03676656_mH	CXCL12	chemokine (C-X-C motif) ligand 12
Hs00757930_m1	CXCL13	chemokine (C-X-C motif) ligand 13
Hs01003372_m1	VCAM1	vascular cell adhesion molecule 1
Hs00164932_m1	ICAM1	intercellular adhesion molecule 1
Hs00366766_m1	PDPN	podoplanin
Hs00757808_m1	AICDA	activation-induced cytidine deaminase
Hs00153368_m1	BCL6	B-cell CLL/lymphoma 6
Hs00163934_m1	CD40LG	CD40 ligand
Hs04276253_m1	SELPLG	selectin P ligand
Hs00270639_s1	GPR183	G protein-coupled receptor 183; EBI2
Hs01125301_m1	CD274	CD274 molecule; PD-L1
Hs01057777_m1	PDCD1LG2	programmed cell death 1 ligand 2; PD-L2

Assay ID	Gene Symbol	Gene Name
Hs01550088_m1	PDCD1	programmed cell death 1; PD-1
Hs00198106_m1	TNFSF13B	tumor necrosis factor (ligand) superfamily member 13b; BAFF
Hs00242739_m1	LTB	lymphotoxin beta
Hs04188773_g1	LTA	lymphotoxin alpha
Hs00540548_s1	CXCR5	chemokine (C-X-C motif) receptor 5

2.8 Citations

This publication has been cited by:

- Sokoya M, Ramakrishnan VR, Frank DN, Rahkola J, Getz A, Kofonow JM, Nguyen Q, Janoff EN. Expression of immunoglobulin D is increased in chronic rhinosinusitis. Annals of Allergy, Asthma & Immunology. 2017 Oct 1;119(4):317-23.

- Song J, Wang H, Zhang YN, Cao PP, Liao B, Wang ZZ, Shi LL, Yao Y, Zhai GT, Wang ZC, Liu LM. Ectopic lymphoid tissues support local immunoglobulin production in patients with chronic rhinosinusitis with nasal polyps. Journal of Allergy and Clinical Immunology. 2017 Nov 3

Table 2.3 Relative expression of genes tested in the qPCR assay

	CRSsNP				CRSwNP			
Gene	**Rel. Expr**	**Lower CI**	**Upper CI**	**p-val**	**Rel. Expr**	**Lower CI**	**Upper CI**	**p-val**
HPRT1, B-actin	1				1			
AICDA	3.30	0.65	13.76	0.114	8.79	2.32	32.45	0.002
BCL6	0.90	0.55	1.56	0.670	0.87	0.53	1.37	0.524
CCL19	1.52	0.63	4.11	0.362	1.44	0.60	3.17	0.398
CCL21	1.25	0.53	3.45	0.614	0.77	0.36	1.71	0.522
CCR7	2.37	1.15	4.40	0.018	5.16	2.77	8.41	<0.001
CD274	1.15	0.77	1.70	0.524	2.18	1.58	3.15	<0.001
CD40LG	2.89	1.97	4.38	<0.001	3.94	2.78	5.52	<0.001
CXCL12	1.44	0.78	2.92	0.26	0.73	0.41	1.22	0.27
CXCL13	3.24	0.63	14.51	0.140	4.12	1.03	13.60	0.032
CXCR5	3.66	1.32	9.43	0.012	11.57	4.81	29.82	<0.001
FGF2	1.28	0.90	1.96	0.218	0.78	0.56	1.10	0.174
GPR183	1.73	1.17	2.77	0.018	2.69	1.93	3.92	<0.001
HGF	1.58	1.04	2.36	0.022	1.17	0.84	1.65	0.372
ICAM1	1.25	0.61	2.83	0.568	2.21	1.17	4.32	0.024
IL-7	0.98	0.65	1.44	0.894	0.85	0.58	1.19	0.398
IL-7R	2.38	1.60	3.58	<0.001	3.22	2.33	4.53	<0.001
LTα	1.09	0.68	1.73	0.74	1.90	1.20	3.03	0.002
LTβ	1.41	0.91	2.22	0.15	5.55	3.53	8.74	<0.001
LTBR	0.76	0.48	1.19	0.25	0.79	0.50	1.21	0.296
OPG	0.93	0.52	1.72	0.82	1.40	0.83	2.34	0.19
PDCD1	1.36	0.53	3.58	0.520	1.91	0.87	4.07	0.124
PDCD1LG2	1.47	0.97	2.12	0.046	2.18	1.58	3.10	<0.001
PDPN	1.03	0.72	1.58	0.880	1.54	1.15	2.23	0.014
SELPLG	1.80	0.93	3.90	0.104	4.43	2.48	7.78	<0.001

	CRSsNP				CRSwNP			
Gene	**Rel. Expr**	**Lower CI**	**Upper CI**	**p-val**	**Rel. Expr**	**Lower CI**	**Upper CI**	**p-val**
TNFRSF11A	1.09	0.54	2.57	0.838	0.31	0.15	0.59	<0.001
TNFSF11	1.69	0.83	3.74	0.164	4.09	2.16	7.67	<0.001
TNFSF13B	1.50	0.84	2.59	0.172	2.32	1.47	3.89	0.002
VCAM1	1.93	1.10	3.28	0.022	2.44	1.56	3.81	<0.001
VEGF-C	1.02	0.67	1.51	0.916	0.61	0.44	0.84	0.008

Table 2.4 Prevalence of TLO by patient group

Diagnosis	TLO		Total
	Neg	Pos	
Control	24	0 (0%)	24
CRSsNP	53	6 (10%)	59
CRSwNP	47	28 (37%)	75
Total	124	34	158

Table 2.5 Association of TLO with eosinophilia stratified by diagnosis

Diagnosis	Eosinophilia	TLO*		N
		Neg	Pos	
CRSsNP	Neg	36	1 (3%)	37
	Pos	17	5 (23%)	22
CRSwNP	Neg	11	2 (15%)	13
	Pos	35	26 (43%)	61

Table 2.6 Association of TLO with the number of previous operations.

Diagnosis	TLO: Median (IQR) Operations*	
	Neg	Pos
CRSsNP	0 (1)	2.5 (2.5)
CRSwNP	1 (2)	1 (3.5)

Chapter 3: The Nasal Epithelium Mediates Tertiary Lymphoid Organ Formation in Chronic Rhinosinusitis

Statement of Authorship

Title of Paper	The Nasal Epithelium Mediates Tertiary Lymphoid Organ Formation in Chronic Rhinosinusitis
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☑ Unpublished and Unsubmitted work written in manuscript style
Publication Details	

Principal Author

Name of Principal Author (Candidate)	Aden Lau
Contribution to the Paper	Preparation of samples Experiments conducted Data analysis and manuscript writing
Overall percentage (%)	100
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 25/3/18

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 to 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	Maureen Rischmueller		
Contribution to the Paper	Project supervision Manuscript editing		
Signature		Date	23 March 2018

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 to 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	Susan Lester		
Contribution to the Paper	Project supervision Data analysis Manuscript editing		
Signature		Date	23 March 2018

Name of Co-Author	Sarah Vreugde		
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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.

Name of Co-Author	Takayoshi Suzuki		
Contribution to the Paper	Data analysis		
Signature		Date	03/23/2018

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	Masanobu Suzuki		
Contribution to the Paper	Data analysis		
Signature		Date	23/3/18

3.1 Abstract

Background: Chronic Rhinosinusitis (CRS) is characterized by persistent inflammation of the sinonasal mucosa. Recalcitrant CRS patients frequently present with organized ectopic accumulations of lymphoid cells within nasal mucosa and polyps termed Tertiary Lymphoid Organs (TLOs). The factors that drive TLO formation in CRS are unknown.

Objective: To investigate the role of the nasal epithelium in mediating TLO formation in CRS.

Methodology: Primary human nasal epithelial cells (HNECs) from 3 donors were treated with TLR 1-9 agonists for 48 hours and the mRNA expression of interleukin-7 receptor (IL-7R), lymphotoxin beta (LTB) and B-cell activating factor (BAFF) were measured by qPCR. Sinonasal tissue was obtained from 5 non-CRS controls, 9 CRS without nasal polyps (CRSsNP) and 14 CRS with nasal polyps (CRSwNP) patients and prepared into a tissue microarray (TMA) block followed by immunofluorescence analysis of IL-7R, LTB and BAFF.

Results: TLR3 agonists (poly I:C) induced a significant upregulation of BAFF (fold-change=36.39, 95% CIs=7.46, 144.02), IL-7R (fold-change=95.46, 95% CIs=13.29, 620.06) and LTB (fold-change=37.44, 95% CIs=6.05, 253.76) after 48 hours of challenge of HNECs. LTB expression of submucosal cells was significantly increased in CRSsNP (p=0.032) and CRSwNP (p=0.005) compared to non-CRS control. IL-7R expression of submucosal cells was significantly increased in CRSwNP (p=0.034) compared to non-CRS control. Submucosal IL-7R expression levels inversely correlated with the number of eosinophils ($p_{\text{linear trend}}$=0.004).

Conclusion: The elevated expression levels of IL-7R, LTB and BAFF in HNECs in response to TLR3 agonists and increased influx of LTB and IL-7R expressing cells within the submucosa in CRSwNP patients imply that the nasal epithelium may contribute to immune cell infiltration involved in TLO formation.

Key Words: tertiary lymphoid organs, nasal polyps, nasal epithelial cells, IL-7R, LTB

3.2 Introduction

Chronic Rhinosinusitis (CRS) is a common chronic inflammatory condition of the paranasal sinus mucosa, affecting approximately 10.5% of populations in the US and Australia [3, 4]. CRS accounts for massive healthcare costs due to its high prevalence and need for surgery[9] and has a strong impact on the quality of life of patients. Furthermore, a conservative estimation of 10% of CRS patients do not respond to medical treatment and surgery, and develop refractory disease[21]. CRS patients can be phenotypically classified according to the presence or absence of nasal polyps into CRS with nasal polyps (CRSwNP) and CRS without nasal polyps (CRSsNP)[1]. CRSwNP patients tend to have more severe disease and a higher risk of recurrence compared to CRSsNP patients[18]. We have recently found that almost 40% of CRSwNP patients present with organized ectopic accumulations of lymphoid cells within their nasal mucosa and polyps termed Tertiary Lymphoid Organs (TLOs). TLOs in the context of CRS were found in association with increased disease severity and with tissue eosinophilia[334]. TLOs are organized lymph node-like structures formed in response to persistent antigenic stimuli (e.g. chronic infection). TLOs possess the functions of a secondary lymphoid organ including the trafficking of antigen-carrying professional antigen-presenting cells and the extravasation of circulating T and B cells, hence enhancing the generation of local T-cell dependent humoral immunity.

TLOs are associated with severe chronic inflammation[242, 335], and have been found in the context of autoimmunity, chronic infection, graft rejection and cancer[164, 165, 242]. The formation process of TLOs is complex and not clearly understood and may involve both intrinsic (e.g. autoimmunity) and extrinsic (e.g. chronic infection) factors. Previous studies on lymphoid neo-organogenesis have shown that some cell types (e.g. dendritic cells [248, 249]and B cells [250]) and signalling pathways (e.g. $LT\alpha_1\beta_2$-LTβR[250]) are involved in TLO formation. However, current knowledge on TLO initiation, the specific cell types involved and its relation to disease progression in the context of chronic infections is very limited. Furthermore, current research in TLOs has been focused on immune cells and a potential role of epithelial cells in this process has not yet been established[259]. In oral squamous cell carcinoma, TLOs are typically located in the peritumoral stroma in close proximity to the epithelial layer[264]. Given the increasingly recognized roles of airway epithelial cells in mediating inflammation[336] and interacting with both innate and adaptive immunity[337], we hypothesized that epithelial cells are involved in TLO formation. This study investigates key factors of TLO development in ex vivo cell cultures of primary HNECs and in the context of CRS.

We focused on IL-7R[263, 338], LTα/LTβ[312, 339] and BAFF[340] for their well described functions in the process of TLO development and maintenance.

3.3 Methods

Patients and samples

The study was approved by The Queen Elizabeth Hospital Human Research Ethics Committee, Adelaide, Australia (reference number: HREC/15/TQEH/132). Informed consent was obtained from all donors of cells and biopsies. Control patients were patients undergoing endoscopic skull base procedures without clinical or radiological evidence of sinus disease. Biopsies were obtained from the ethmoid sinus mucosa from patients undergoing endoscopic sinus surgery for CRS at the Department of Otolaryngology, Head and Neck Surgery, The Queen Elizabeth Hospital, Adelaide, Australia. CRS patients fulfilled the diagnostic criteria set out in the position papers by the American Academy of Otolaryngology and Head and Neck Surgery and the European Position Statement (EPOS) on CRS[296, 297]. Patients with CRS were further sub-classified according the absence (CRSsNP) or presence (CRSwNP) of visible polyps present within the middle meatus on nasal endoscopy as defined by EPOS guidelines[296, 297]. Nasal polyp tissues were obtained from CRSwNP patients and mucosal tissues were obtained from CRSsNP and control patients. Samples for immunohistochemistry were washed twice in 1x PBS and fixed in 10% neutral buffered formalin, followed by paraffin-embedding.

Cell culture

Culturing of primary human nasal epithelial cells (HNECs) was done as described by C. Cooksley *et al*[101]. Briefly, the inferior turbinate was brushed using sterile nasal brushes and extracted cells were suspended in Bronchial Epithelial Growth Medium (BEGM, CC-3170, Lonza, Walkersville, MD). The cell suspension was depleted of monocytes using anti-CD68 (Dako, Glostrup, Denmark) coated culture dishes. HNECs were expanded in routine cell culture conditions (37 °C, humidified, 5% CO2) in collagen-coated flasks (Thermo Fisher, Waltham, MA). Cells were seeded onto a 24-well plate (2 cm^2 per well), pre-coated overnight with 50 μg/mL human type IV collagen (C7521, Sigma-Aldrich, Missouri, US), at passage 1 with a cell density of 50,000 cells per well. Once cells reached 80% confluence, TLR 1-9 agonists (tlrl-kit1hw, Invivogen, California, USA) treatments were applied for 48 hours under routine cell culture conditions. BEGM without any TLR agonist

was used as a no treatment control. Working concentrations of all TLR agonists are listed in Table 3.3 in the supplementary data. For immunofluorescence, primary HNECs were seeded onto Falcon 8-chamber slides (BD, New Jersey, USA), pre-coated with collagen as described above, at $8x10^4$ cells/chamber, grown to 80% confluence, then treated with the TLR 3 agonist poly (I:C) for 48 hours. BEGM only was used as a no treatment control. Cells were fixed with 2.5% formalin in PBS after treatment.

Quantitative PCR

RNA was isolated from HNECs using RNeasy Mini Kit (Qiagen, Hilden, Germany). cDNA was synthesized from RNA using Quantitect Reverse Transcription kit (Qiagen, Hilden, Germany). Taqman chemistry (Applied Biosystems, California, USA) was used with Biorad CFX96 Thermocycler (Bio-rad Laboratories, California, USA) for all genes as instructed by the manufacturer's protocol to perform qRT-PCR analysis to perform 45 cycles of cDNA amplification. Details of the Taqman probes used are listed in Supplementary Table 3.4 in the supplementary data.

Histology

Paraffin-embedded tissue samples were cut in 4µm thickness, stained with Haematoxylin & Eosin (H&E) and scanned using digital whole-slide imaging (WSI) technology (NanoZoomer, Hamamatsu). Tissue eosinophilia was determined by averaging the number of eosinophils per High Power Field (HPF) (0.035 mm^3) from at least 6 HPF's/slide as specified by Bassiouni et al[341]. The eosinophilia scores were determined by the average number of eosinophils per HPF as the following scale: 0=0-1.0; 1= 1.1-10.0, 2=10.1-20.0, 3= >20.0. The presence of TLOs was determined by examining the H&E-stained slides for the presence of lymphoid infiltrations as described by Lau et al[334].

Immunohistochemistry / Immunofluorescence

Tissue handling and processing were performed as described by Cantero et al [299]. In short, tissue cores (2mm) were prepared from formalin-fixed paraffin-embedded tissue blocks and embedded into the tissue microarray (TMA) block using TMA Master (3DHistech, Budapest, Hungary). These tissue cores were sectioned in 5µm thickness. Antigen retrieval was done at 100ºC for 20 minutes in pH6 sodium citrate solution. For immunofluorescence, LTB antibody (HPA048884,

Sigma-Aldrich, Missouri, USA) was used in 1:10 dilution, followed by Alexa Fluor-647 anti-rabbit Ab (711-606-152, Jackson ImmunoResearch Laboratory, Pennsylvania, USA) in 1:800 dilution. BAFF antibody (ALX-804-131-C100, Enzo Life Sciences, New York, USA) was used in 1:100 dilution, followed by Cy3-conjugated anti-Rat Ab (112-165-075, Jackson ImmunoResearch Laboratory, Pennsylvania, USA) in 1:800 dilution. All primary incubations were overnight at 4ºC and secondary incubations were 1 hour at room temperature. For immunohistochemistry, IL-7R antibody (I8409, Sigma-Aldrich, Missouri, USA) was used in 1:20 dilution and the incubation conditions are overnight at 4ºC, followed by using VECTORSTAIN universal quick HRP kit (Vector Laboratories, California, USA) and the incubation conditions of all reagents are in compliance with the manufacturer's manual. Each primary antibody has a negative staining control, which was incubated with non-specific antibodies raised from the corresponding host under the same incubation conditions. Immunofluorescence images were captured using Olympus FV3000 confocal laser scanning microscope (Olympus, Tokyo, Japan). Immunohistochemistry images were taken using NanoZoomer-XR Digital slide scanner C12000-01 (Hamamatsu Photonics, Hamamatsu, Japan) under high resolution (40x object magnification power). For immunohistochemistry, protein expression levels were derived from the number of positive cells divided by the representative area set at 0.3 mm^2. For immunofluorescence, protein expression levels were derived from the percentage of positively stained cells over the total number of cells, as determined by cell nuclei stained by 4',6-diamidino-2-phenylindole (DAPI), in a representative area. Image analyses were carried out by independent investigators in a double-blinded manner.

Statistical analysis

Statistical analysis for qRT-PCR was performed using R v 3.2.1[342]. Relative expression of qRT-PCR data was analysed using the MCMC.qpcr library[301]. Briefly, Ct values were normalised to the β-actin gene (ΔCt), and then normalised to the no treatment control group (ΔΔCt), and exponentiated to report relative expression values for each treatment group. Image data analysis was performed using R v3.4.2 and the coin library v1.2-2[343]. p-values were derived by permutation (N = 10000) to approximate exact tests, and analysis within CRS patients were stratified by CRSsNP/CRSwNP status.

3.4 Results

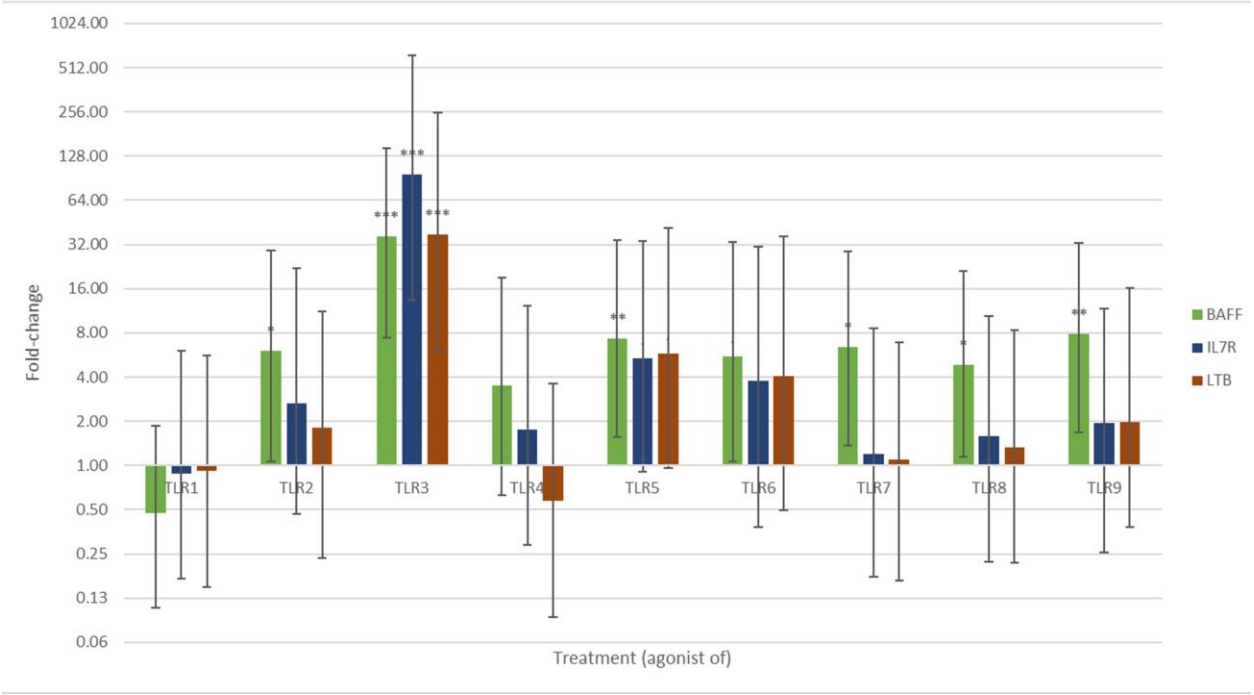
Patient demographics

30 patients in total were recruited to this study. 18 were male and 12 were female, aged 19 to 83. These patients included 6 non-CRS controls, 9 CRSsNP and 15 CRSwNP patients. 0 non-CRS control, 1 CRSsNP patient and 8 CRSwNP patients were TLO-positive. Demographic information is summarised in Table 3.2 in the supplementary data.

Nasal epithelial cells express TLO genes in response to TLR agonists

Epithelial cells were used from 3 non-CRS donors (2 females,1 male, aged 28-59). All cell donors were non-asthmatic and non-smokers. Gene expression was normalized to the housekeeping gene β-actin and to the no treatment control. TLR3 agonists (poly (I:C)) induced a significant upregulation of BAFF (fold-change=36.39, 95% CIs=7.46, 144.02), IL-7R (fold-change=95.46, 95% CIs=13.29, 620.06) and LTB (fold-change=37.44, 95% CIs=6.05, 253.76) after 48 hours of challenge (Figure 3.1A). BAFF was also upregulated in response to TLR2 (fold-change=6.00, 95% CIs=1.07, 29.32), TLR5 (fold-change=7.30, 95% CIs=1.56, 34.03), TLR7 (fold-change=6.41, 95% CIs=1.38, 28.59), TLR8 (fold-change=4.84, 95%CIs=1.14, 20.91) and TLR9 agonists (fold-change=7.86, 95% CIs=1.67, 32.97). Protein expression of IL-7R, LTB and BAFF induced by poly I:C treatment in NECs was demonstrated by immunofluorescence (Figure 3.1B).

A



B

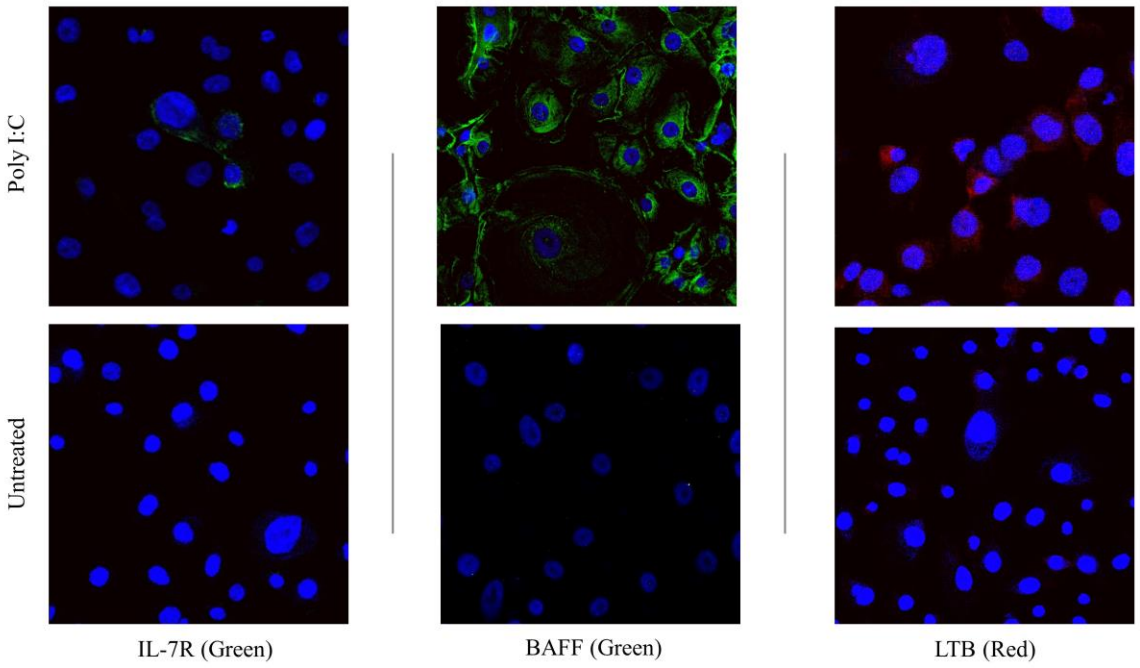


Figure 3.1 Expression of TLO-related genes/proteins in primary nasal epithelial cells in response to TLR agonists treatment.

A) The mRNA expression of IL-7R, LTB and BAFF in response to TLR 1-9 agonist treatment for 48 hours, normalized to the housekeeping gene and to the no-treatment control. *p<0.05, **p<0.01, ***p<0.001 B) Poly (I:C) induces IL-7R, BAFF and LTB protein expression as shown by immunofluorescence.

Expression of TLO genes by immunohistochemical analysis

The p-values in this section were derived from Kruskal-Wallis tests unless otherwise specified. IL-7R was expressed in epithelium and submucosal immune cells (Figure 3.2) and was significantly higher in submucosal cells in CRSwNP compared to control (p=0.034) (Figure 3.3A). IL-7R was lower in asthmatic CRS patients versus non-asthmatic CRS patients, however, this was not statistically significant (p=0.076) (Figure 3.3D). IL-7R protein expression in submucosal cells in CRS inversely correlated with tissue eosinophilia score (p=0.012) by linear-by-linear association test (Figure 3.3F). LTB was also expressed in the epithelium with stronger expression in immune cells within the submucosa (Figure 3.4). Submucosal LTB expression was significantly increased in both CRSsNP and CRSwNP patients compared to non-CRS controls ($p_{overall}$=0.013) (Figure 3.3B). Epithelial IL-7R and BAFF expression was variable between different patient tissues and appeared to be higher in CRSwNP but the difference did not reach statistical significance (IL-7R, p=0.201, BAFF, p=0.07) (Figure 3.5). There was no significant difference in BAFF expression by diagnosis in submucosal cells. There was no significant difference in the epithelial or submucosal expression of IL-7R, LTB and BAFF by TLO phenotype in CRS patients. Details of expression levels of IL-7R, LTB and BAFF in epithelial cells and submucosal cells are listed in Table 3.1.

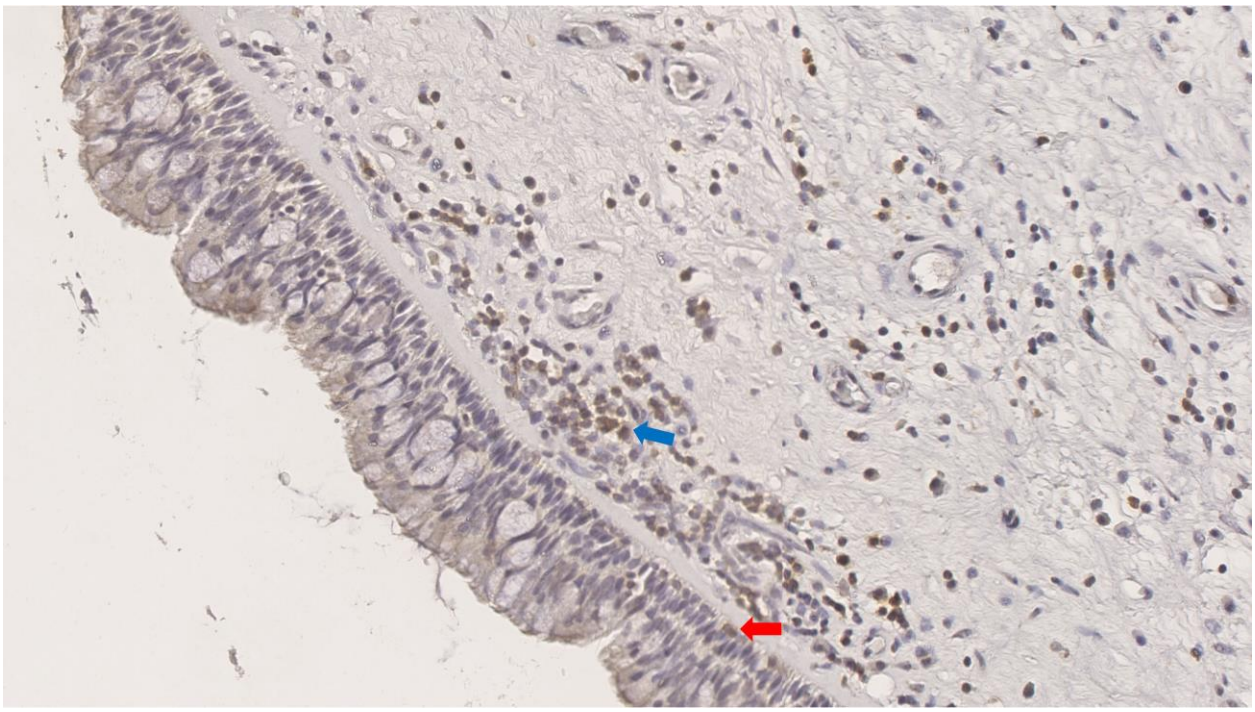
Clinical/demographical associations

IL-7R, LTB or BAFF expression neither differs by gender by Kruskal-Wallis test nor correlates with age or the number of previous operations by linear regression.

Table 3.1 Expression of IL-7R, LTB and BAFF in epithelial cells and submucosal cells.

Epithelial cells					Submucosal cells				
IL-7R					**IL-7R**				
Diagnosis	**Mean expression (cells/mm^2)**	**SD**	**P-val**	**N**	**Diagnosis**	**Mean expression (cells/mm^2)**	**SD**	**P-val**	**N**
Control	**110.2**	**140.0**		**4**	**Control**	**90.8**	**74.3**		**4**
CRSsNP	**242.1**	**250.6**	**0.481**	**8**	**CRSsNP**	**485.2**	**494.9**	**0.126**	**8**
CRSwNP	**270.8**	**243.8**	**0.202**	**14**	**CRSwNP**	**432.2**	**655.9**	**0.034***	**14**
Total				**26**	**Total**				**26**
LTB					**LTB**				
Row Labels	**Mean expression (cell count)**	**SD**	**P-val**	**N**	**Diagnosis**	**Mean expression (cell count)**	**SD**	**P-val**	**N**
Control	**28.6**	**18.2**		**6**	**Control**	**4.4**	**7.5**		**6**
CRSsNP	**29.4**	**11.2**	**0.831**	**9**	**CRSsNP**	**36.8**	**29.5**	**0.032***	**9**
CRSwNP	**32.9**	**14.9**	**0.904**	**15**	**CRSwNP**	**58.0**	**26.3**	**0.005***	**15**
Total				**30**	**Total**				**30**
BAFF					**BAFF**				
Diagnosis	**Mean expression (cell count)**	**SD**	**P-val**	**N**	**Diagnosis**	**Mean expression (cell count)**	**SD**	**P-val**	**N**
Control	**1.5**	**1.3**		**6**	**Control**	**17.9**	**23.4**		**6**
CRSsNP	**6.0**	**7.7**	**0.172**	**9**	**CRSsNP**	**16.9**	**13.9**	**0.806**	**9**
CRSwNP	**7.9**	**8.6**	**0.077**	**15**	**CRSwNP**	**25.0**	**14.8**	**0.360**	**15**
Total				**30**	**Total**				**30**

(A)



(B)

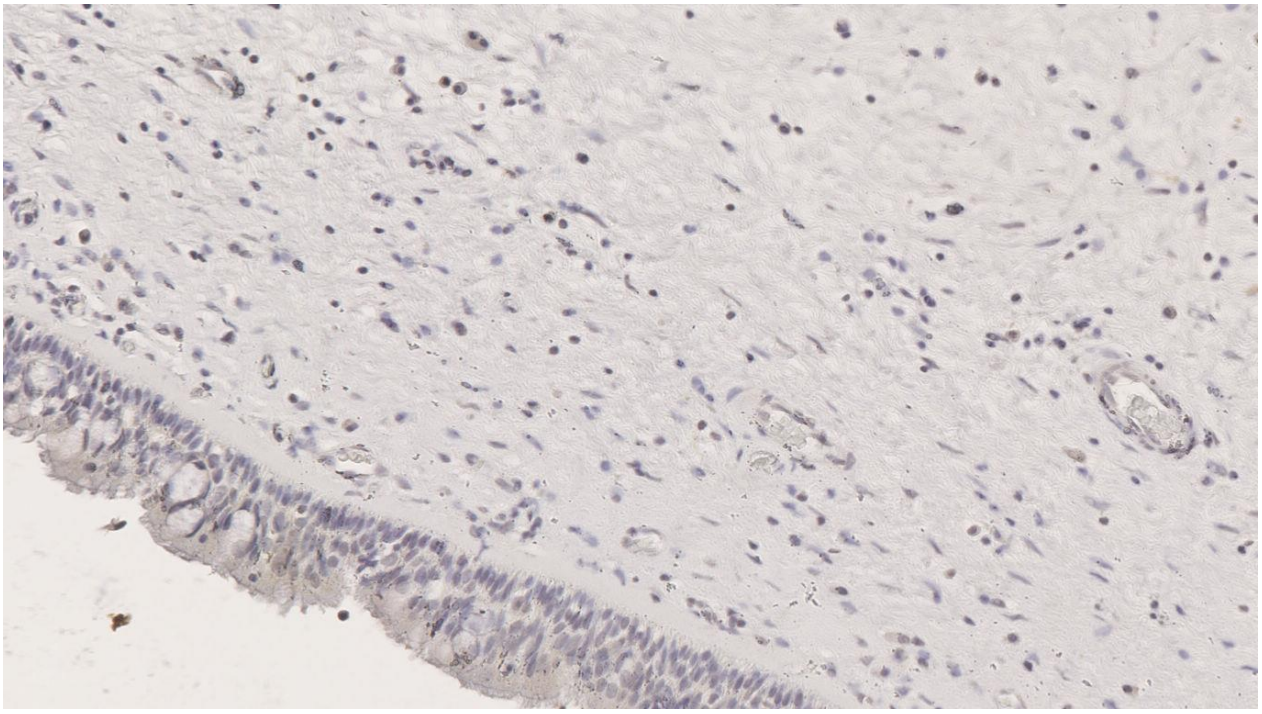


Figure 3.2 Representative images of IL-7R expression in a CRSwNP patient.

(A) The red arrow indicates IL-7R expressing epithelial cells and the blue arrow indicates IL-7R expressing submucosal cells. (B) Negative stain control

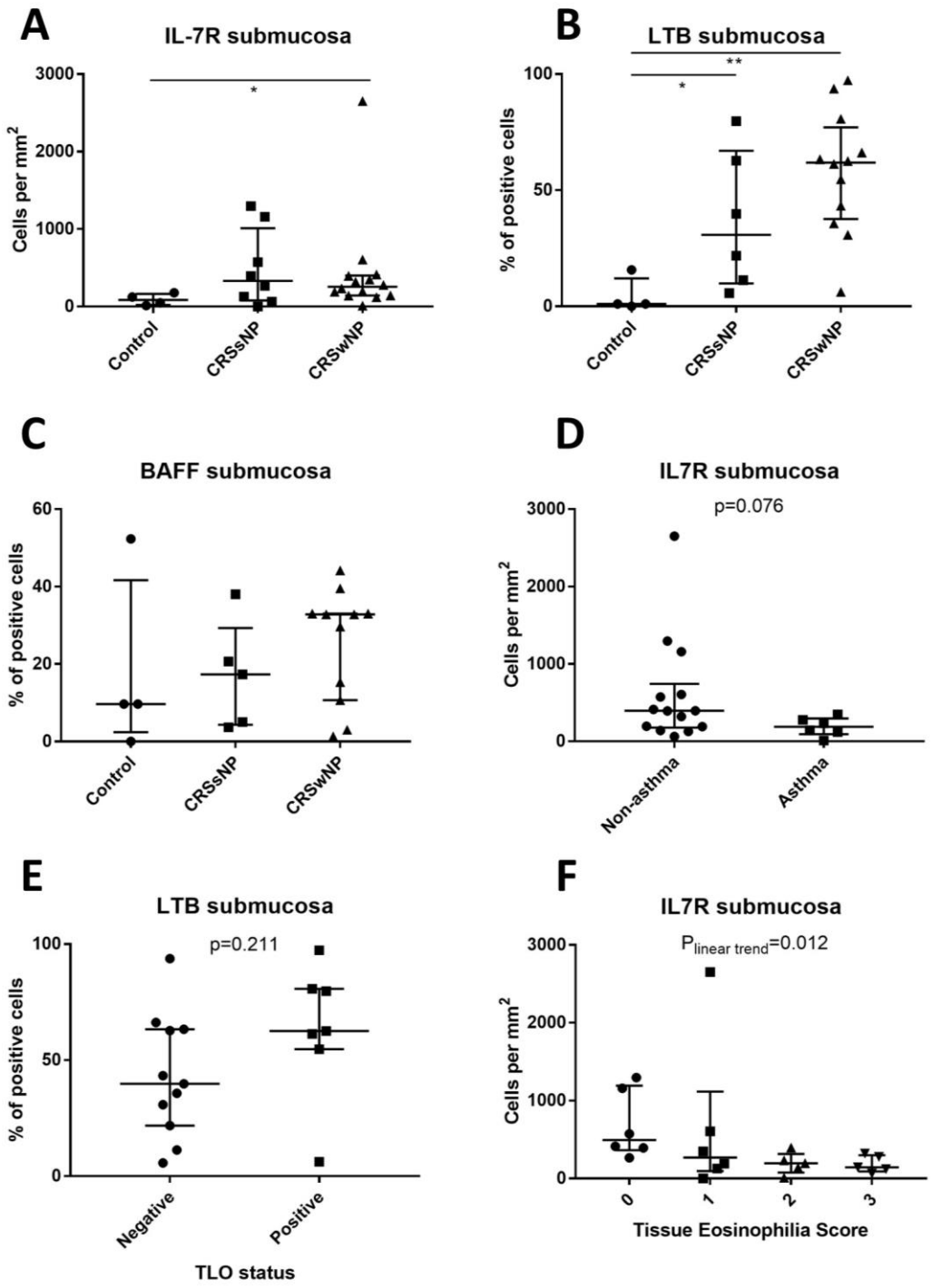


Figure 3.3 Protein expression in submucosal cells.

A-C) IL-7R, LTB and BAFF by patient groups respectively. D) Submucosal IL-7R expression in relation to asthma E) LTB expression in CRS patients by TLO status. F) Submucosal IL-7R expression in relation with tissue eosinophilia score *p<0.05, **p<0.01, ***P<0.001

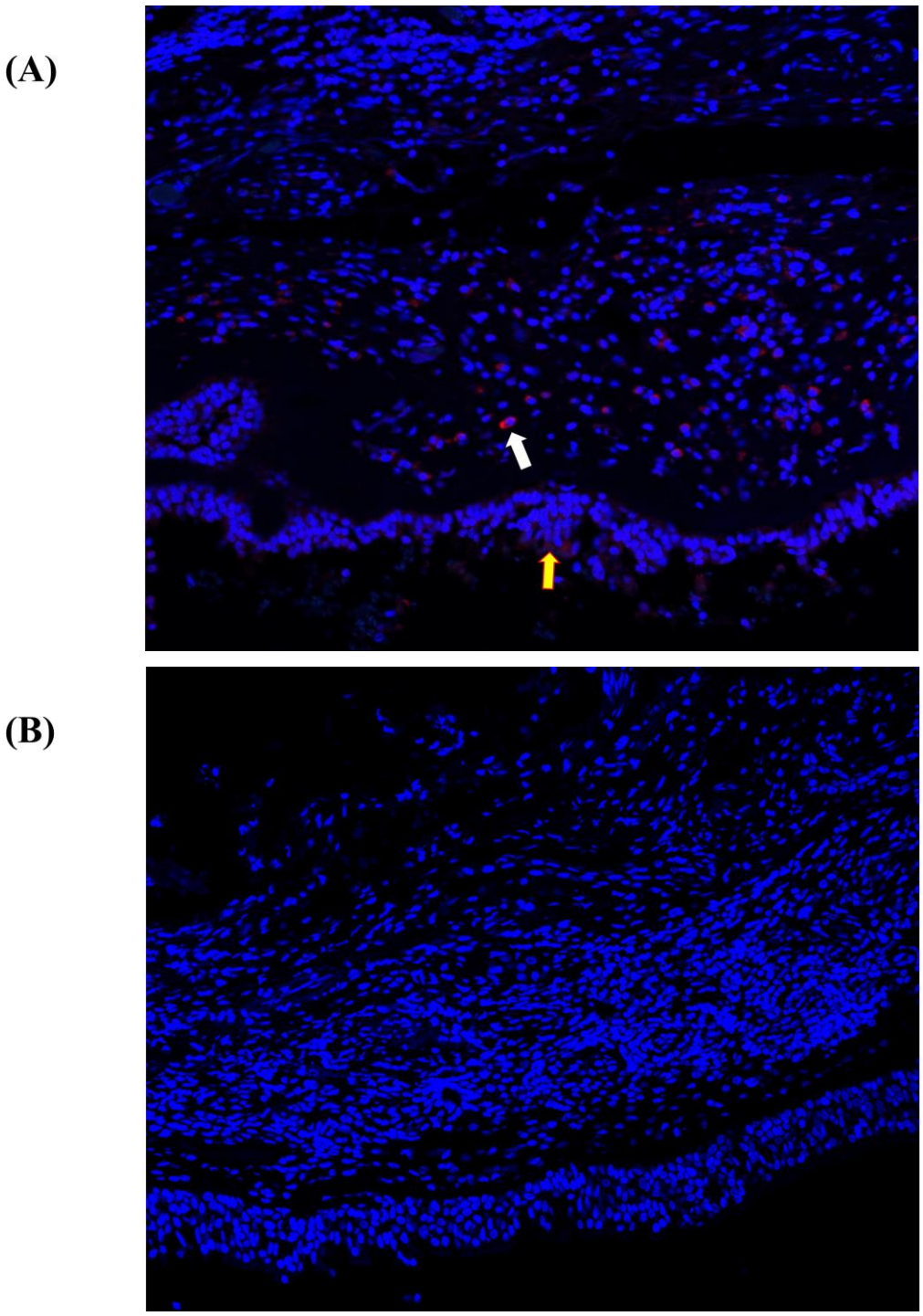


Figure 3.4 A representative image of LTB expression in a CRSwNP patient.

(A) The yellow arrow indicates LTB expressing epithelial cells and the white arrow indicates LTB expressing submucosal cells. (B) Negative stain control.

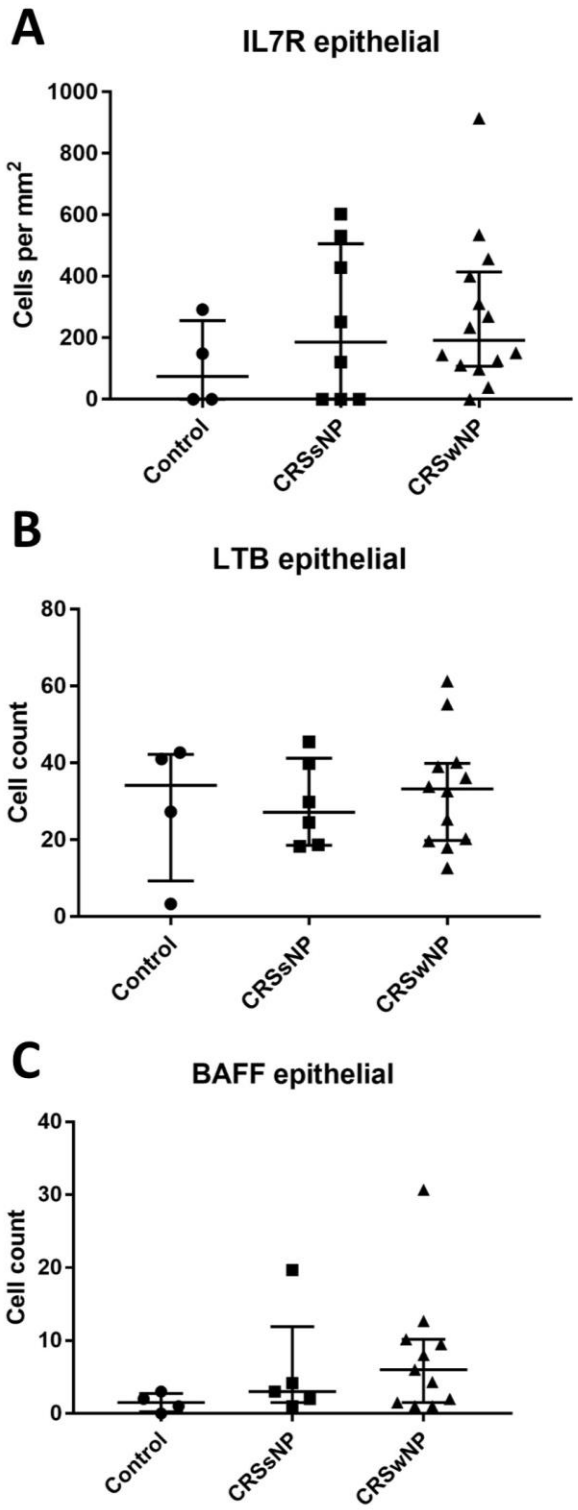


Figure 3.5 Protein expression of IL-7R (A), LTB (B) and BAFF (C) in nasal epithelium by patient groups.

3.5 Discussion

TLOs have been a hot-spot for research in recent years due to their contributions to chronic inflammation, especially in autoimmune diseases[164, 165, 175]. We have recently demonstrated the presence of TLOs in nasal polyps and its associations with tissue eosinophilia and disease recalcitrance represented by the number of previous operations[334]. It has been shown that nasal polyp TLOs locally produced IgG, IgA and IgE but not IgM[344]. This indicates the existence of an antigen-specific B-cell response that might drive chronic inflammation in these patients, supporting the hypothetic role of TLOs in promoting pathogenic inflammation in CRS. Our data provides evidence supporting the notion that nasal epithelial cells might be involved in TLO development. Our gene expression analysis has shown that HNECs strongly express genes important for TLO induction, namely IL-7R, LTB and BAFF, in response to Poly (I:C) for 48 hours. This finding suggests that HNECs are equipped with some of the genes that are required for TLO induction with activation occurring upon prolonged TLR3 activation. The TLR3 agonist Poly (I:C) is a synthetic analogue of dsRNA and mimics viral infection and replication. The TLR3 protein is constitutively expressed at high levels in epithelial cells [345] and HNECs have been shown to respond vigorously to TLR3 agonists[101] . Whether RNA viruses, such as Rhinovirus or influenza virus which are frequently found in mucosal washes of CRS patients [346], play a role in TLO initiation in the context of CRS warrants further investigation. Interestingly, influenza virus lung infections have been shown to induce TLO formation[241].

There has been a rising interest in epithelial cells for their ability to mediate immune responses[219]. Epithelial cells are well recognized as an integral part of the innate immunity[125, 128, 336, 347]. Their role in mediating adaptive immunity has recently been identified [219, 337], especially in the context of B-cell responses[295] which are particularly relevant to TLO functions. Using immunofluorescence, our study confirmed the expression of LTB, IL-7R and BAFF within the epithelial layer of the sinonasal mucosa. However, whilst the epithelium-specific expression of BAFF and IL-7R appeared increased in CRSwNP compared to control in some patients, these differences did not reach statistical significance in this study population. A larger study involving more samples is needed to fully elucidate the differential expression patterns of BAFF and IL-7R within the epithelium of CRS patients and controls.

Apart from the epithelium-specific expression, LTB and IL-7R expression was also identified in cells within the submucosa in close proximity to the epithelium and was significantly increased in

CRS patient tissues compared to controls. Interestingly, in the context of oral mucosal cancers, TLOs are typically found close to the tumor margin within the lymphocyte-rich subepithelial areas[264]. Given the crucial functions of IL7-IL-7R and $LT\alpha_1\beta_2$-LTBR engagement in early lymphoid organ development and the increased expression of these genes both within the epithelium upon stimulation with TLR agonists and in immune cells located in the immediate vicinity of the epithelium, these findings suggest that the epithelium plays a central role in the TLO induction/maintenance process. In view of our findings of significantly increased expression of these proteins within the submucosa rather than in the epithelium layer itself indicates that HNECs could play a role in attracting immune cells expressing TLO initiation signals rather than being the major source of these signals.

The inverse correlation of submucosal IL-7R expression with tissue eosinophilia is intriguing and its mechanism and significance unclear. IL-7R has a critical role in immune cell development and homeostasis, and mutations in this gene are associated with the pathogenesis of severe combined immunodeficiency (SCID). IL-7R (alias CD127) is a receptor for both IL-7 and for thymic stromal-derived lymphopoietin (TSLP) and has a role in T- and B-cell development and homeostasis (via IL-7) and allergic responses and Th2 inflammation (via TSLP)[348]. The regulation of IL-7R expression is complex and it has been reported that IL-7 can downregulate IL-7R protein expression and induces the shedding of IL-7R from CD8+ T cells[349]. Also, the presence of a persistent antigen can suppress IL-7R expression and this correlates with T cell exhaustion[350] and immune dysfunction. IL-7R is expressed on eosinophils and IL-7R Single Nucleotide Polymorphisms have been shown to confer susceptibility to mite-sensitized asthma[351]. Further research is needed to shed light on IL-7R protein expression and its relation to IL-7 and TSLP protein levels, tissue eosinophilia and immune dysfunction in the context of CRS.

3.6 Conclusion

Our study has indicated the capability of nasal epithelial cells to play a potential role in TLO development. Further comprehensive studies are needed to investigate the triggers and molecular and cellular pathways of TLO formation in the context of CRS.

3.7 Supplementary data

Table 3.2 Summary of patient demographics

	Control	CRSsNP	CRSwNP
Number	6	9	15
Age (IQR)	51 (47 - 56)	48 (26 - 56)	57 (43 - 62)
Male/Female	1/5	6/3	11/4
Asthma/non-asthma	1/5	3/6	6/9
TLO/non-TLO	0/6	1/8	8/7
Average previous ESS	NA	0.67	0.8

Table 3.3 Working Concentration of TLR 1-9 agonist treatment

Agonist of	Agonist name	Working concentration
TLR 1	Pam3CSK4	1 μg/ml
TLR 2	HKLM	10^8 cells/ml
TLR 3	Poly(I:C) High molecular weight *	10 μg/ml
TLR 3	Poly(I:C) Low molecular weight *	10 μg/ml
TLR 4	LPS	1 μg/mL
TLR 5	Flagellin	1 μg/ml
TLR 6	FSL-1	1 μg/ml
TLR 7	Imiquimod	1 μg/ml
TLR 8	ssRNA40	1 μg/ml
TLR 9	ODN2006	5 μM

* All results in this study from Poly(I:C) HMW and LMW were very close. The reported numbers are the average of the two.

Table 3.4 Details of Taqman probes

AssayID	Gene
Hs99999903_m1	β-actin (housekeeping gene)
Hs00233682_m1	IL-7R
Hs00242739	LTB
Hs00198106_m1	BAFF

Chapter 4: Reduced Serum Immunoglobulin G Levels in Chronic Rhinosinusitis Patients presenting with Tertiary Lymphoid Organs

Statement of Authorship

Title of Paper	Reduced Serum Immunoglobulin G Levels in Chronic Rhinosinusitis Patients presenting with Tertiary Lymphoid Organs
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☑ Unpublished and Unsubmitted w ork w ritten in manuscript style
Publication Details	

Principal Author

Name of Principal Author (Candidate)	Aden Lau
Contribution to the Paper	Preparation of samples Experiments conducted Data analysis and manuscript writing
Overall percentage (%)	100
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 25/3/18

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 to 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	Maureen Rischmueller		
Contribution to the Paper	Project supervision Manuscript editing		
Signature		Date	23 March 2018

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 to 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	Susan Lester		
Contribution to the Paper	Project supervision Data analysis Manuscript editing		
Signature		Date	23 March 2018

Name of Co-Author	Sarah Vreugde		
Contribution to the Paper	Project supervision Manuscript editing		
Signature		Date	22/3/18

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	Karen Patterson		
Contribution to the Paper	Experimental design. Conduct experiment Interpretation of results		
Signature		Date	24 March 2018

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	Dimitra Beroukas		
Contribution to the Paper	Experiment conducted		
		Date	23.3.18.

4.1 Abstract

Background: Organized ectopic accumulations of lymphoid cells, termed Tertiary Lymphoid Organs (TLOs), are frequently found in Chronic Rhinosinusitis with nasal polyps (CRSwNP) patients. TLOs are formed in response to persistent antigen stimulation and are thought to serve mainly local humoral immune responses. Matched systemic and local humoral immunity and the presence of Anti-Nuclear Antibodies (ANAs) in relation to TLO status have not been studied in the context of CRS.

Objective: To investigate the relationship of TLO status with serum and tissue immunoglobulin G (IgG) levels, and the presence of ANAs in CRS.

Methods: Sinonasal tissue and serum was obtained from 12 non-CRS controls, 18 CRS without nasal polyp (CRSsNP) patients and 34 CRSwNP patients. Haematoxylin & Eosin (H&E)-stained tissue sections were analysed for the presence of TLOs. Tissue lysates and sera were analysed for IgG levels by ELISA. The presence of ANAs against 12 autoantigens in sera and tissue lysates was determined by immunoblot assays.

Results: TLO+ CRSwNP patients had significantly reduced serum IgG levels compared to TLO- CRSwNP patients ($p=0.044$). Nasal polyp IgG levels were significantly increased compared to CRSsNP ($p=0.0005$) and non-CRS control mucosa ($p<0.0001$). Nasal polyp IgG levels did not differ by TLO status ($p=0.46$). No systemic ANAs were detected in any subjects tested.

Conclusion: CRSwNP patients with TLOs have lower serum IgG levels than their TLO-negative counterparts. No detectable ANAs were found in any subjects tested.

4.2 Introduction

Chronic Rhinosinusitis (CRS) is characterized by the persistent inflammation of the paranasal sinus mucosa. CRS is phenotypically classified into CRS with nasal polyps (CRSwNP) or CRS without nasal polyps (CRSsNP) based on the endoscopic evidence of the presence of nasal polyps in the middle meatus[1]. These two sub-classes of CRS possess different clinical and morphological characteristics with CRSsNP frequently presenting with a Th1-type inflammation and CRSwNP often having a predominant Th2-type of inflammation with increased expression of Th2 cytokines (such as IL-5 and IL-13), tissue eosinophilia and goblet cell hyperplasia [1, 18, 73, 352].

Our recent research highlights the presence of organized ectopic accumulations of lymphoid cells, termed tertiary lymphoid organs (TLOs), in up to 28% of CRSwNP patients and

the associations of TLOs with recalcitrant CRS and tissue eosinophilia[334]. TLOs form in tissues affected by severe chronic inflammation and can be protective (e.g. in the context of cancer) or detrimental to the disease process (e.g. in transplant rejection and autoimmune diseases) [164, 165, 175, 242]. We have shown that the expression of many genes related to chronic inflammation and TLOs is increased in nasal polyps of CRSwNP patients compared to the nasal mucosa of CRSsNP patients and control subjects. These genes include key players of TLO formation and function such as lymphotoxin alpha (LTA) and lymphotoxin beta (LTB) and genes critical for immunoglobulin class switching such as Activation Induced Cytidine Deaminase (AICDA) and B-cell Activating Factor of the TNF Family (BAFF) [334]. BAFF, a B-cell survival factor, is a therapeutic target in systemic autoimmune diseases[353], which are associated with both TLOs and anti-nuclear autoantibodies (ANAs).

Multiple reports have shown that there is an increased local class-switched immunoglobulin production in nasal polyps[78, 212, 216, 217], indicating an increased antigen-specific humoral immune response within nasal polyps[354]. In contrast, whereas up to 17.9% of CRS patients show humoral immune dysfunction with low serum IgG levels (reviewed in Berger et al[355]), average serum IgG levels were found to be similar in CRS versus non-CRS patients in different studies[350, 356]. Furthermore, Tan *et al* had demonstrated increased levels of anti-dsDNA IgG and IgA within nasal polyps of CRSwNP patients, indicating the presence of local ANAs within nasal polyp tissue[291]. Anti-dsDNA is an ANA specificity that is characteristic of a subset of patients with Systemic Lupus Erythematosus (SLE)[357]. Since TLOs can be the local production site of ANAs[271, 293], we hypothesised that an autoimmune component contributes to the persistent inflammation in CRSwNP patients with TLOs. Although TLOs have been identified in nasal polyps and found to be associated with CRS disease recalcitrance [334] and increased levels of local immunoglobulins[344], the link between TLOs, local and systemic IgG levels and ANAs has yet to be determined. In this study, we used matched serum and nasal tissue samples from CRS patients and non-CRS controls and assessed IgG and ANA levels in relation to the TLO status and disease severity scores.

4.3 Methods

Patients and samples

This study was approved by the Human Research Ethics Committee of The Queen Elizabeth Hospital, Adelaide, Australia (reference number: HREC/15/TQEH/132). Biopsies were obtained

from the ethmoid sinuses from patients undergoing endoscopic sinus surgery for CRS at the Department of Otolaryngology, Head and Neck Surgery, The Queen Elizabeth Hospital, Adelaide, Australia. Control patients were patients undergoing endoscopic skull base procedures without clinical or radiological evidence of sinus disease. CRS patients fulfilled the diagnostic criteria set out in the recent position papers by the American Academy of Otolaryngology and Head and Neck Surgery and the European Position Statement (EPOS) on CRS [296, 297]. Patients with CRS were further sub-classified according to the absence (CRSsNP) or presence (CRSwNP) of visible polyps present within the middle meatus on nasal endoscopy as defined by EPOS guidelines [296, 297]. Nasal polyp and non-polyp mucosa tissues were obtained from CRSwNP patients and mucosal tissues were obtained from CRSsNP and control patients.

Preparation of protein extract from tissues

Freshly obtained nasal polyps and turbinate tissues were snap-frozen and stored at -80^{o}C until needed. Tissues were thawed on ice. Approximately 100mg of tissue was suspended in 1mL of T-per tissue protein extraction buffer (product no. 78510, Thermo Fisher Scientific, MA, US) containing 1% v/v HALT protease inhibitor cocktail (product no. 87786, Thermo Fisher Scientific, MA, US). The samples were then homogenized with Qiagen Tissuelyser (product no. 85220, Qiagen, Hilden, Germany) at 30 hertz for 20 minutes. The homogenized suspensions were centrifuged at 17,000g for 10 minutes at 4^{O}C and the supernatants were stored at -80 ^{O}C until analysis. The protein concentrations of tissue extracts were determined using the Pierce BCA Protein Assay Kit (product no. 23225, Thermo Fisher Scientific, MA, US).

Measurement of serum and tissue IgG levels by ELISA

IgG levels were measured by using the total IgG ELISA kit (catalogue no. 88-50550-88, Thermo Fisher Scientific, MA, USA) as per the instructions by the manufacturer. The detection range of this ELISA is 1.5-100 ng/mL. Tissue lysate samples were diluted to 1 μg of total protein per mL and serum samples were diluted 100,000 folds. Hence, all tissue lysate IgG levels are reported in ng of IgG per μg of total protein.

Detection of autoantibodies

The presence of autoantibodies against 12 ANA-associated autoantigens (nRNP, Sm, SS-A (SS-A native and Ro-52), SS-B, Scl-70, Jo-1, CENP B, dsDNA, Nucleosomes, Histones and ribosomal P-Protein) were tested by Euroimmun ANA Profile I Immunoblot kits (catalogue no. DL 1590-6401-8G, Euroimmun, Lübeck, Germany). The tests were performed as per the manufacturer's

manual. For serum samples the standard protocol was used. For tissue lysate samples, 400 µL was used per sample and the primary incubation step was extended to 3 hours instead of 30 minutes. The blots were evaluated by the EUROLineScan (Euroimmun, Lübeck, Germany) for scanning and analysis. Background cut-off value was ≤10 intensity unit. 3 serum samples known to possess ANAs were used as positive controls.

Histology

Paraffin-embedded tissue samples were cut in 4µm thickness, stained with Haematoxylin & Eosin (H&E) and scanned using digital whole-slide imaging (WSI) technology (NanoZoomer, Hamamatsu). Tissue eosinophilia was determined by averaging the number of eosinophils per High Power Field (HPF) (0.035 mm^3) from at least 6 HPF's/slide as specified by Ramezanpour et al [298]. TLO status was determined by examining H&E-stained histology as described by Lau et al[334].

Statistical analysis

Gamma (log link) regression (coefficients were exponentiated and have interpretation as the ratio of IgG levels, relative to the base (reference level)) was performed using Stata v15.1 (StataCorp LLC, Texas, USA). Krusal Wallis test with Benjamini-Hochberg post-hoc correction was performed using Graphpad Prism v7.03 (Graphpad Software, California, USA).

4.4 Results

Subjects

64 patients were involved in this study including 12 non-CRS controls, 18 CRSsNP patients and 34 CRSwNP patients. These patients were aged between 23 and 76 years old with 27 females and 37 males. 0/12 non-CRS control, 1/18 CRSsNP and 9/34 CRSwNP patients were positive for TLOs. Demographical and clinical information of all patients is listed in Table 4.4 in the supplementary data.

Serum IgG levels are decreased in TLO-positive CRS patients

Within CRSwNP patients, the presence of TLOs was correlated with significantly lower serum IgG levels adjusted for gender ($p_{adjusted}$=0.044) (Figure 4.1A). There was no significant difference in serum IgG levels when comparing CRS versus control ($p_{adjusted}$=0.28) or CRSsNP versus CRSwNP ($p_{adjusted}$=0.27) (Figure 4.1B). Serum IgG analysis is summarized in Table 4.1. Within CRS patients, there were no correlations between serum IgG and asthma (p = 0.23), eosinophilia score (p_{linear} = 0.34) nor number of previous operations (p_{linear} = 0.73) (data not shown).

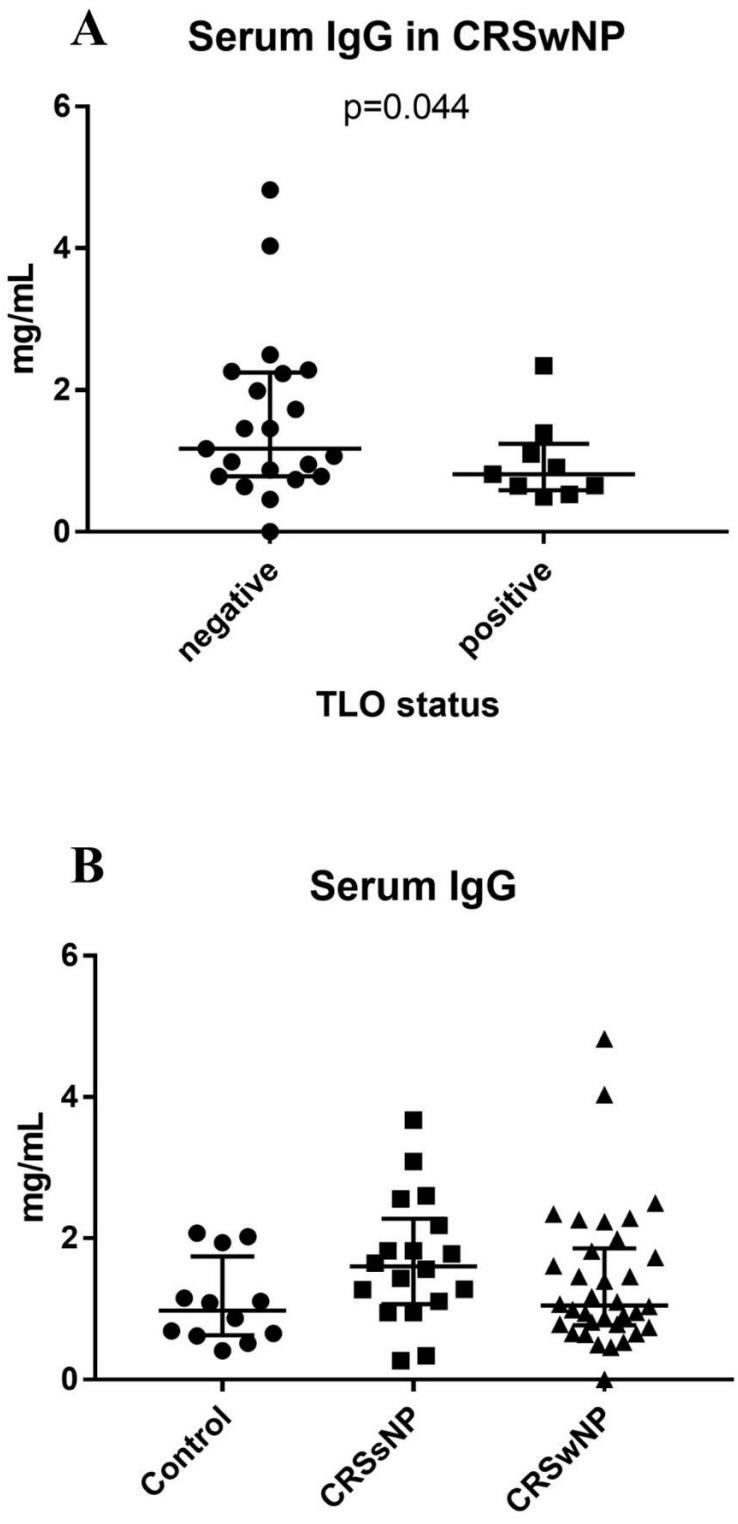


Figure 4.1 Serum IgG levels within CRSwNP patients

Serum IgG levels by TLO (A) and patient groups (B).

Table 4.1 Serum IgG analysis

(A) Serum IgG levels (mg/mL) by patient groups:

Group	N	Median	IQR
Control	12	0.980	0.911
CRSsNP	18	1.604	1.078
CRSwNP:TLO neg	20	1.315	1.422
CRSwNP:TLO pos	9	0.815	0.449
Total	59	1.152	1.209

(B) Serum IgG regression analysis estimates adjusted for gender:

Group	IgG Ratio	Lower CI	Upper CI	p-value
Controls	1 (base)			
CRSsNP	1.37	0.91	2.08	0.13
CRSwNP:TLO neg	1.33	0.86	2.04	0.20
CRSwNP:TLO pos	0.81	0.50	1.32	0.40
Females	1 (base)			
Males	1.30	0.95	1.78	0.10

(C) Serum IgG Helmert contrasts:

Helmert Contrast	Unadjusted			Adjusted for Gender		
	df	chi2	p-val	df	chi2	p-val
Controls vs CRS	1	3.21	0.073	1	1.18	0.28
CRSsNP vs CRSwNP	1	1.03	0.31	1	1.23	0.27
CRSwNP: TLO neg vs CRSwNP: TLO pos	1	4.39	0.036	1	4.04	0.044

Local IgG levels are elevated in CRSwNP patients versus CRSsNP patients and non-CRS controls

IgG levels in CRSwNP mucosal tissue were significantly higher than in non-CRS control ($p_{adjusted}$=0.021) (Figure 4.2A). Mucosal IgG levels in CRSwNP were also higher than CRSsNP but this did not reach statistical significance ($p_{adjusted}$=0.060) (Figure 4.2A). Nasal polyp IgG levels were significantly increased compared to CRSsNP (p=0.0005) and non-CRS control mucosa

($p<0.0001$) (Figure 4.2A). Within CRSwNP, nasal polyp IgG levels appeared to be higher than that of mucosa, but this did not reach statistical significance ($p=0.059$) (Figure 4.2A). Nasal polyp IgG levels did not differ by TLO status ($p=0.46$) (Figure 4.2B). Tissue IgG levels in CRSwNP and mucosal IgG analysis is summarized in Table 4.2 and Tables 4.3 respectively.

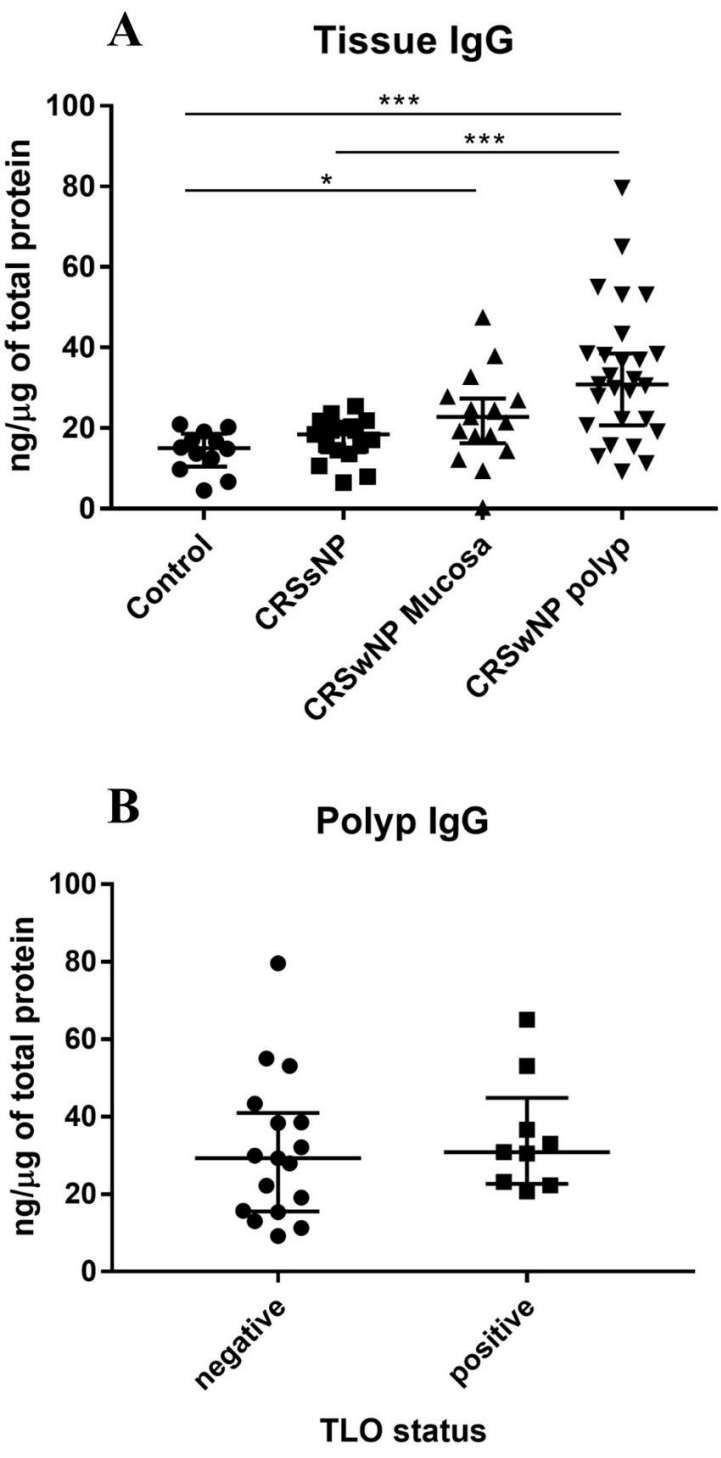


Figure 4.2 Tissue IgG levels

Tissue IgG levels by patient groups and tissue type (A); IgG levels in nasal polyps by TLO status (B). *p<0.05, **p<0.01, ***p<0.001

Table 4.2 Tissue IgG level in CRSwNP

(A) Polyp versus mucosa IgG levels (left) and polyp IgG levels by TLO status (right):

Group	N	Median	IQR	Group	N	Median	IQR
Mucosa:CRSwNP	17	22.75	8.79	Polyp:TLO neg	17	29.3	22.9
Polyp:CRSwNP	27	30.85	16.98	Polyp:TLO pos	8	31.9	18.5
Total	44	53.60	25.77	Total	25	30.5	17.8

(B) Polyp IgG regression analysis estimates:

Unadjusted (n = 25)				
Group	IgG Ratio	Lower CI	Upper CI	p-value
CRSwNP:TLO neg	1 (base)			
CRSwNP:TLO pos	1.16	0.78	1.74	0.46

Table 4.3 Mucosal IgG analysis

(A) Mucosal IgG (ng/μg of total protein) by group:

Group	N	Median	IQR
Control	12	15.0	6.9
CRSsNP	17	18.4	5.9
CRSwNP	17	22.8	8.8
Total	46	18.3	8.2

(B) Mucosa IgG regression analysis estimates:

Unadjusted (n = 44)					Adjusted for Gender (n = 44)				
Group	IgG Ratio	Lower CI	Upper CI	p-value	Group	IgG Ratio	Lower CI	Upper CI	p-value
Control	1 (base)				Controls	1 (base)			
CRSsNP	1.20	0.94	1.55	0.15	CRSsNP	1.19	0.91	1.54	0.20
CRSwNP	1.57	1.15	2.15	0.004	CRSwNP	1.55	1.15	2.10	0.004
					Females	1 (base)			
					Males	1.04	0.83	1.30	0.74

(C) Mucosa IgG Helmert contrasts:

Helmert Contrast	Unadjusted			Adjusted for Gender		
	df	chi2	p-val	df	chi2	p-val
Controls vs CRS	1	6.43	0.011	1	5.99	0.014
CRSsNP vs CRSwNP	1	3.69	0.055	1	3.53	0.060
Joint	2	8.24	0.016	2	8.24	0.016

Assessing the presence of autoantibodies against 12 autoantigens by immunoblot

The presence of antibodies to 12 autoantigens was tested in serum and tissue. All 3 positive controls were positive for at least one autoantibody. No autoantibodies were detected in the sera of any of the 64 patients. Autoantibodies were detected in the tissue lysates of 0/8 TLO-positive nasal polyps.

4.5 Discussion

This is the first study demonstrating reduced serum IgG levels in CRSwNP patients presenting with TLOs compared to CRSwNP patients without TLOs. The significance of this finding, and in particular the relationship of low serum IgG with TLOs is unclear and warrants further investigation. Whilst it is thought that the contribution of TLOs to adaptive immunity serves mainly local immune responses to a persistent antigen, previous studies have shown positive correlations between serum IgG levels and Germinal Centre (GC)-positive immune infiltrations in the context of Sjögren's Syndrome[358]. Furthermore, studies in mice have shown inducible bronchus-associated lymphoid tissue (iBALT), a form of TLO induced in the lungs after influenza virus infection, contributes significantly to systemic humoral immunity in a process that is dependent on dendritic cell function[359]. In contrast to our finding of a reduction of serum IgG levels, these studies indicate increased serum IgG levels in relation to TLO formation. Reduced circulating total IgG levels have been associated with recurrent respiratory tract infections[360]. In a recent study involving more than 1600 samples, serum IgG levels, in a dose-dependent manner, were significantly and independently related to Chronic Obstructive Pulmonary Disease (COPD) exacerbations and hospitalizations[361]. A number of studies have shown humoral immune dysfunction with low serum IgG levels in 9-17.9% of CRS patients (reviewed in Berger et al[355]). In a systematic review and meta-analysis involving >1400 CRS patients, immunoglobulin deficiencies were identified in 12.7% of patients with recurrent CRS (defined as CRS patients not controlled by appropriate conservative management for 4 months) and in 22.8% of patients with difficult-to-treat CRS (defined as non-controllable rhinosinusitis despite successful sinus surgery and appropriate conservative management for at least 1 year)[362]. In contrast, less than 1% of the general population are estimated to have immunoglobulin deficiencies[363, 364]. In view of these findings of a greater than 10-fold higher prevalence of immunoglobulin deficiencies in patients with recurrent CRS, our finding of an increased prevalence of TLOs in recalcitrant CRS patients[334] and the association of TLO positivity with reduced serum IgG levels, it is tempting to speculate that TLO+ CRS patients might represent a distinct CRS endotype characterised by low serum IgG levels. The exact nature and cause of reduced IgG levels and whether, apart from IgG, also other immunoglobulins or IgG subtypes are involved is unknown and warrants further investigation. Interestingly, specific pathogens have been shown to ablate B cell responses by suppressing the expression of transmembrane activator and calcium-modulating cyclophilin ligand interactor (TACI) and/or B

cell-activating factor of the tumor necrosis factor family-receptor (BAFF-R) on B cells[365]. Together with BAFF and A Proliferation-Inducing Ligand (APRIL), these "BAFF system molecules" play critical roles in antibody responses to microbial infections. The downregulation of TACI and/or BAFF-R by specific pathogens is regarded as a potent virulence mechanism rendering the immune response to these pathogens ineffective and resulting in reduced systemic immunoglobulin levels[366]. Further studies are required to elucidate the identity of the microbiome in associated with TLO formation in CRSwNP patients and how this relates to markers of B-cell specific immune activation and function.

The finding of reduced systemic IgG levels is important since for symptomatic immunoglobulin deficiencies, early antibiotic treatment and preventive intravenous or subcutaneous immunoglobulin substitution therapy could help alleviate the chronic inflammation and infection[367, 368]. Restoring normal serum IgG has shown to be beneficial, improving lung function and reducing acute exacerbations for patients who present with asthma or COPD and are found to have underlying antibody deficiency [369].

In terms of local IgG levels, nasal polyps contained elevated IgG compared to the mucosa of CRSsNP patients and non-CRS controls as reported by other groups[291, 370]. Despite the previous report of elevated IgG levels in nasal polyps with TLOs compared their TLO-negative counterpart[344], there was no significant difference in nasal polyp IgG levels by TLO status in our study population. It is clear that various immune cells, including antibody-secreting plasma cells and other B cells, are enriched in nasal polyps[161, 356, 370, 371], indicating that germinal centres in TLOs are not the sole contributors to the IgG levels. Perhaps the selection of more mature TLOs (or high grade TLO as described by Song et al[344]) is needed to demonstrate TLO-driven IgG production versus a plasma-cell-rich nasal polyp without TLOs.

Tan et al. used ELISA assays to show elevated local anti-dsDNA IgG and IgA levels in nasal polyps compared to CRSsNP and control mucosa, which, when detected in serum, are markers for autoimmune diseases such as SLE[291, 357]. To test the hypothesis that TLOs in the context of CRS are associated with increased production of auto-antibodies, we tested the presence of systemic and local IgG against 12 autoantigens, using immunoblot assays, which are routinely used in the standard diagnostics of auto-immune diseases [372]. There were no significant ANA levels detected in any of the 64 tested sera, indicating that none of the CRS patients tested had evidence of auto-immunity. In addition, no ANAs were detected in any of the TLO-positive nasal polyp lysate

samples. Since the levels of IgG in tissue lysates are much lower than that of sera as shown in our ELISA results, the ANA levels in our tissue lysates might not fall under the detection range of the immunoblot assay. There are flaws in detecting ANAs using ELISA[373, 374] and validations are needed before the its results can be properly interpreted. Interpretations of anti-dsDNA ELISA results in previous studies were based on simple variation in absorbances, which were not validated against positive and negative samples. On the contrary, the immunoblot assay has a good agreement with gold-standard technique for the detection of ANAs[375, 376] and can test multiple ANAs per run. Therefore, we chose the immunoblot assay in our study and the choice of methods must be carefully considered in future ANA research.

4.6 Conclusion

In conclusion, this study has demonstrated that CRSwNP patients with TLOs have lower serum IgG levels than their TLO-negative counterparts. Future studies are required to determine the significance and pathophysiology of this finding.

4.7 Supplementary data

Table 4.4 Summary of patient demographics

	Control	**CRSsNP**	**CRSwNP**
Number	12	18	34
Age (IQR)	56 (41 - 61)	58.5 (48 - 66)	57 (45 - 67)
Male/Female	3/9	12/6	22/12
Asthma/non-asthma	0/12	3/15	14/20
TLO/non-TLO	0/12	1/16	9/21
Average previous ESS	NA	1.11	1.38

Chapter 5: Discussion

CRS is a multifactorial disease characterized by the persistent inflammation of the sinonasal mucosa with a high degree of heterogeneity between patients. According to the widely accepted criteria proposed by the European Position Statement (EPOS), CRS is classified into CRSsNP and CRSwNP based on the absence or presence of endoscopically visualised nasal polyps in the middle meatus[1]. This classification has been widely adopted and useful in the clinical setting as it proposes treatment strategies adapted to these CRS phenotypes[73]. CRSwNP is characterised by eosinophilic inflammation and T_{h2-} type immune responses [73], and is usually responsive to steroid treatment as manifested by reduced eosinophil activation[377] and reduced polyp size[378, 379]. In contrast, CRSsNP is frequently characterized by a Th1-type of inflammation and is usually treated with topical steroid[1]. However, more advanced methods of CRS patient classification have emerged recently, such as the Japanese Epidemiological Survey of Refractory Eosinophilic Chronic Rhinosinusitis (JESREC) study[178] which sub-divided CRSwNP patients by the degree of eosinophilic inflammation. These different classification systems reflect the high levels of heterogeneity in CRS phenotypes and perhaps the classification of CRS sub-types based solely on nasal polyps is an over simplification. For example, it does not take into account the wide spectrum of immunological disease profiles, such as the involvement of the ectopic aggregation of lymphoid cells[217] and different immunoglobulin isotypes[216, 370], underlying the persistent inflammation in CRS. Organized structures of lymphoid cells called TLOs have been found in many severe chronic inflammatory conditions including autoimmune diseases, chronic infection, organ transplant rejection and cancer[164, 165, 242]. We hypothesized that TLOs are present in CRS and contribute to its persistent inflammation. To investigate this, we examined tissue from CRSsNP and CRSwNP for the expression of genes related to TLO formation and functions, and the presence of histopathological structures that resemble TLOs.

Our results showed that many genes important for TLO formation and maintenance were upregulated in CRS, mainly in CRSwNP. Hierarchical clustering analysis of the expression pattern of these TLO-related genes showed a cluster of genes that are crucial for TLO formation and GC reactions tended to co-express in the same patients, indicating that TLO-mediated pathology drives the inflammation in a distinct subset of CRS patients. We also confirmed that the aberrant lymphoid cell aggregates found in the nasal polyps were in fact TLOs by demonstrating the presence of FDCs, HEVs and B/T cell zones, and the lymphoid-follicle-specific spatial organization between them.

We examined the histology of 158 patients and found that TLOs were present in 37% of CRSwNP patients, 10% of CRSsNP patients and none of non-CRS controls, demonstrating that TLOs were exclusive to CRS and were more prevalent in CRSwNP versus CRSsNP. Correlation of clinical and demographical information with the presence of TLOs was then evaluated. We showed that tissue eosinophilia and the number of previous operations were associated with TLOs, suggesting that TLOs contribute to severe inflammation and the development of recalcitrant disease in CRSwNP. The discovery of the presence of TLOs in CRS adds to the understanding of the pathophysiology of refractory CRS. The discovery may pave the way for the discovery and development of new non-steroid treatments for recalcitrant CRS such as biologics blocking TLO pathways[380].

With the knowledge of the significance of TLOs in CRS, we next investigated the role of the nasal epithelium in mediating TLOs and its interactions with other aspects of CRS immunology. Airway epithelial cells substantially contribute to T_{h2}-type responses[162], making them highly relevant in driving the chronic inflammation in CRSwNP[336]. Our study found an increased expression of three genes that are crucial for TLO formation, namely IL-7R, LTB and BAFF, in primary HNECs in response to TLR 3 agonist (poly (I:C)) treatment by both qPCR and immunofluorescence, demonstrating that HNECs are capable of facilitating TLO development and this supports the hypothetical role of HNECs in TLO formation. We next examined the expression of these three proteins in the epithelium and the adjacent submucosal cells using patient-derived tissue microarrays. Our results showed that submucosal LTB and IL-7R were upregulated in CRS patients compared to non-CRS controls. Given the importance of IL7-IL-7R and $LT\alpha_1\beta_2$-LTBR engagement in early lymphoid organ development, the increased expression of IL-7R and LTB in the immune cells in close proximity to the nasal epithelium and in the epithelial cells upon stimulation by TLR agonists suggests that the epithelium is central to the TLO formation/maintenance process. Previous studies had demonstrated the ability of epithelial cells to recruit B cells to the subepithelial area[381, 382] and directly mediate immunoglobulin class-switching[295]. Our findings of elevated expression of LTB and IL-7R in the submucosa rather than in the epithelium layer itself also supports the notion that the epithelium could facilitate TLO formation by attracting immune cells carrying TLO initiation signals, such as LTi cells, to the submucosa.

Intriguingly, there were inverse correlations of submucosal IL-7R expression with both tissue eosinophilia and asthma, but the mechanism and significance of this finding is unclear. Apart from its importance in hematopoietic cell development[383, 384, 385], IL-7R is expressed by many immune cell types, especially during activation in the peripheral tissue including DCs[386], monocytes[387], $CD4^+$ T cells[386], LTi cells[170] and eosinophils[351], and its immune functions and regulation are complex[338]. The IL-7 signalling is required in a defined time-frame during early Peyer's patch development[388, 389]. Given the resemblance of the process of SLO and TLO neo-organogenesis, strong submucosal expression of IL-7R could represent an early stage in TLO development and inflammatory stage that is distinctive from the well-developed eosinophilic/asthmatic inflammation.

Few studies have investigated the local and systemic immunity in matching samples in the context of CRS, and their relationships with the presence of TLOs. Our study is the first to demonstrate lowered serum IgG levels in CRS patients with TLOs compared to CRS patients without TLOs. Traditionally, TLOs are thought to have enhancing effects on humoral immunity mainly on a local level. Some studies have shown positive correlations of serum IgG levels and TLOs as in Sjögren's Syndrome[358] and a murine model of influenza virus-induced TLOs[241]. The discordant relationship of TLOs and reduced serum IgG in the context of CRS warrants further investigations. Interestingly, low immunoglobulin levels are not uncommon in respiratory diseases. Reduced IgG2 and IgA levels were observed in recurrent respiratory tract infections[390], and lower serum IgG levels were associated with worse disease outcomes in COPD[361]. Furthermore, there was more than a 10-fold increase of the prevalence of immunoglobulin deficiencies in recurrent CRS patients than in the general population[362, 363, 364]. It is speculated that immunoglobulin deficiency is associated with a distinct CRS endotype that is represented by CRS patients with TLOs. Most importantly, these findings may have profound therapeutic implications as preventive treatments have been shown to help alleviate the chronic inflammation and infection in symptomatic immunoglobulin deficiencies[367, 368].

Increased levels of anti-dsDNA IgG and IgA, which are ANAs routinely used in the diagnosis of SLE[357], have previously been demonstrated in nasal polyps compared to CRSsNP and non-CRS control mucosa[291]. TLOs are a hallmark of severe inflammation in the context of auto-immune diseases and can be the local production site of ANAs[271, 293]. Therefore, we hypothesized that TLOs contribute to the perpetuating inflammation in CRSwNP by the local generation of ANAs.

However, our study did not provide a definitive answer to this question potentially because of the sub-detectable levels of immunoglobulins by immunoblot assays in our tissue lysate samples and the compatibility issue of tissue lysate samples with immunoblot assays, which are only guaranteed to work with serum or plasma samples. In the sera, there were no detectable ANAs in any of the tested subjects. These results indicate that TLO positivity in CRS is not associated with auto-immunity. Further research is needed to determine the specificity of the local immunoglobulins within nasal polyps, as this may account for the increased disease severity in CRSwNP patients with TLOs.

Chapter 6: Conclusion

In conclusion, this PhD thesis has advanced our understanding of the immunology in CRS. Firstly, we have identified the ectopic lymphoid structures termed TLOs in the nasal polyps of CRSwNP patients and found the positive correlation of the presence of TLOs with severe inflammation and recalcitrant disease in CRS. We have also identified the increased expression of genes important for TLO formation and maintenance in HNECs and immune cells in the subepithelial area, indicating that the nasal epithelium layer plays a critical role in mediating TLO formation in CRS. We also demonstrated that TLOs are correlated with lowered serum IgG levels in CRSwNP patients. Although the significance of this finding is unclear, it showed, for the first time, that CRS pathology could have a systemic influence on the immune system through TLOs. We hope that ongoing research in CRS immunology will ultimately yield a better understanding of the complex patho-etiology of CRS and provide directions in the development of targeted therapeutic strategies.

Appendices

Appendix 1: Publication

Lau A, Lester S, Moraitis S, Ou J, Psaltis AJ, McColl S, et al. Tertiary lymphoid organs in recalcitrant chronic rhinosinusitis. The Journal of allergy and clinical immunology. 2017 Apr;139(4):1371-3.e6. DOI: 10.1016/j.jaci.2016.08.052.

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