Visualising intestinal inflammation and fibrosis using zirconium-89 labelled antibodies in a preclinical model of inflammatory bowel disease

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Abstract

Inflammatory bowel disease (IBD) is a chronic, relapsing and remitting inflammatory condition of the gastrointestinal tract. The diagnosis and monitoring of IBD is reliant on endoscopic techniques which are invasive and do not provide quantification. Molecular imaging approaches such as immuno-PET have superior sensitivity and provide quantitative information of the entire body. Immuno-PET combines the superior target selectivity provided by antibodies with the sensitivity of PET. This thesis outlines the development of two novel radiolabelled antibody tracers against intestinal inflammation in a preclinical model of IBD and one novel tracer against intestinal fibrosis in a model of chronic murine IBD. Symptom flare in IBD typically corresponds with an increased activation of innate immune pathways. We aimed to compare immuno-PET of the innate immune mediators IL-1β and CD11b against standard ^{18}F-FDG and MRI to detect colonic inflammation. For visualising intestinal inflammation, ^{89}Zr-α-IL-1β and ^{89}Zr-α-CD11b immuno-PET detected colonic inflammation, as did ^{18}F-FDG, and all PET tracers were more sensitive than MRI. While ^{18}F-FDG volumes of interest correlated with colitis severity and a strong trend was observed with ^{89}Zr-α-IL-1β, no correlation was observed for ^{89}Zr-α-CD11b or MRI. ^{89}Zr-α-IL-1β was distributed mainly to the gastrointestinal tract, while ^{89}Zr-α-CD11b was distributed in more tissue types. Intestinal fibrosis is one of the most common complications of IBD, with severe fibrosis leading to stricture and stenosis in approximately 30% of patients. Currently, intestinal fibrosis is diagnosed and monitored using endoscopies and MRI. Fibrosis is characterised by the excessive deposition of extracellular matrix (ECM) as a result of multiple periods of inflammation and subsequent healing, as seen in IBD and murine colitis. Matrix metalloproteinases (MMP) play a large role in fibrogenic pathways by regulating the

deposition of ECM during tissue renewal. MMP-9 is found to be elevated in fibrotic tissue resected from IBD patients and in preclinical models of intestinal fibrosis. We aimed to visualise intestinal fibrosis by targeting pro-MMP-9 using $f(ab')_2$ antibody fragments, radiolabelled with zirconium-89. This was the first immuno-PET study of fibrosis in any tissue in a preclinical or clinical setting. In our preclinical model of chronic IBD, ^{89}Zr-pro-MMP-9-$f(ab')_2$ successfully detected intestinal fibrosis in the absence of inflammation. Furthermore, immuno-PET and biodistribution studies indicated that the kidneys became fibrotic after multiple rounds of DSS. This was further confirmed by an increase in collagen and pro-MMP-9 levels in the kidneys, in the absence of elevated immune markers. As the mechanisms underlying fibrosis are similar across all organs, immuno-PET of pro-MMP-9 may be a valuable addition to the detection of fibrosis in all tissues. Additionally, development of these technologies for human subjects will provide a less invasive approach than endoscopy for diagnosing and monitoring IBD.

Declaration of originality

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

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Publications arising from this thesis

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Dmochowska N, Tieu W, Keller MD, Wardill HR, Mavrangelos C, Campaniello M, Takhar P, Hughes PA. Immuno-PET of Innate Immune Markers CD11b and IL-1beta Detects Inflammation in Murine Colitis. *Journal of Nuclear Medicine.* 2019;60:858-863.

Chapter 1 Introduction

This thesis investigates the pathogenesis of inflammation and fibrosis in inflammatory bowel disease (IBD) and how disease mediators can be targeted with radiolabelled antibodies to provide improved diagnostic imaging techniques. The mechanisms underpinning IBD and intestinal fibrosis have not been fully elucidated leading to a lack of effective therapeutics and management. Current diagnostic imaging modalities are invasive and lack specificity, selectivity and the option for quantification. Elucidation of IBD immune and profibrogenic mechanisms will provide targets for novel molecular imaging tracers. I investigated the use of zirconium-89 (^{89}Zr) labelled antibodies for the detection of intestinal inflammation and fibrosis in a preclinical model of IBD. This introduction will provide a broad overview of IBD, focusing on current diagnostic modalities and emerging radiolabelled antibody imaging techniques which will form the basis of this thesis.

1.1 Inflammatory bowel disease

IBD is a chronic idiopathic inflammatory condition of the gastrointestinal tract. The two main subtypes of IBD are ulcerative colitis (UC) and Crohn's disease (CD). IBD causes symptoms such as gastrointestinal bleeding, diarrhoea, severe pain and cramping, with more serious cases leading to weight loss and malnutrition. CD commonly occurs in the colon and lower small intestine, but can affect the entire gastrointestinal tract, leading to multiple separate areas of inflammation. Ulceration in CD is typically transmural, damaging all layers of the gut

and is commonly associated with fistulae, strictures and abscesses. In contrast, UC generally affects the sigmoid colon and rectum, forming continuous patches of inflammation which damage the innermost layers of the intestinal wall. Histologically, UC shows inflammatory changes limited to the mucosa and submucosa with cryptitis and crypt abscesses, in contrast to CD which presents with a thickened submucosa, transmural inflammation, fissuring ulceration and non-caseating granulomas (*1*). These conditions are debilitating to live with and are invisible to others.

The incidence of IBD is steadily increasing, with Western diets thought to be a driving cause (*2*). Asian countries have also seen an increased incidence of IBD, which correlates with an increase in the consumption of Western diets. For example, in South Korea, there was a 100% and 136% increase in UC and CD incidence from 1991-2000, respectively (*3*). The rates of IBD in Australia have been gradually increasing, with a prevalence of IBD 308.3/100,000 in 2005 (*4, 5*). Due to this, the economic burden of IBD is significantly increasing. In 2013 it was estimated that the total hospitalisation costs associated with IBD were close to $100 million per annum in Australia alone (*6*). Due to the severity and chronic nature of IBD, it often leads to losses in productivity - in 2012 productivity losses due to IBD were estimated to be over $380 million (*6*). On average IBD sufferers lose 7.2 days of work per annum, whereas CD sufferers can average up to 30 days loss (*7)*. Productivity at work can often be decreased due to flare of IBD and ill health in addition to higher rates of mental illnesses such as depression and anxiety (*8*). Although IBD largely affects people of work age, early age onset is common and can disrupt the sufferer’s education due to pain, frequent toileting and discrimination by teachers and peers. IBD affects many aspects of life and thus requires a

high quality of primary and multidisciplinary care in an attempt to improve quality of life. To prevent long term complications and preserve future quality of life, it is essential to diagnose IBD within the early stages.

1.2 Pathogenesis

The pathogenesis of IBD is believed to be multifactorial, with environmental, genetic and immune factors playing a role. IBD is thought to arise due to an uncontrolled immune activation to otherwise innocuous stimulation, such as commensal bacteria, initiating a chain of inflammatory events which damage the intestinal wall, allowing for the translocation of bacteria and bacterial products into the mucosa, further stimulating inflammation. Healthy gut epithelial cells form a tight barrier against the luminal contents, with Goblet cells forming IgA-rich mucus and Paneth cells secreting antimicrobial peptides such as defensins and TNF-α, which help to defend against pathogens and regulate commensal gut bacteria. Increased intestinal permeability is often observed in IBD patients (*9*). Dendritic cells sample the gut microenvironment and respond to pathogens and molecules secreted by the gut bacteria, initiating an immune response. This inflammatory activation disrupts the tight seal of the intestinal barrier, whilst dendritic cells continue to induce inflammation in the layers below the epithelial cells.

The innate immune system is our first line of defence against pathogens. The gastrointestinal tract is constantly being bombarded with stimuli whether pathogenic or innocuous. Innate immunity is non-specific and provides prompt responses to stimuli through a variety of cells including neutrophils, dendritic cells, monocytes, macrophages and natural killer cells.

Immunity at this level is largely driven by interactions with pattern recognition receptors, such as toll like receptors (TLRs), present on the cell surface and nucleotide-binding oligomerization domain-like (NOD) receptors in the cytoplasm (*10*). It has been suggested that innate immune responses and the expression and function of TLRs and NOD proteins are dysregulated in IBD. NOD2 mutations as seen in CD, may contribute towards disease pathogenesis through a dampened response to lipopolysaccharides (LPS) in the gut, decreasing the secretion of antibacterial agents, thus permitting pathogenic microbial invasion (*11*).

In contrast to innate immunity, adaptive immunity is highly specific, provides long-lasting immunity and takes days to produce pathogen-specific immune responses which are largely dependent on T cells. T helper type 1 (T_H1) cells are induced by IL-12 and secrete IFN-γ, in contrast to T_H2 cells which secrete IL-4, IL-5 and IL-13. T_H1 cells are crucial for the elimination of intracellular pathogens, whereas T_H2 cells are protective against parasites and mediate allergic responses and T_H17 cells defend against extracellular bacteria and fungi (*12*). Consequently, dysregulation of T cell immunity leading to abnormal T cell expansion and activation may lead to the onset of inflammation through the increased secretion of cytokines and chemokines which will further stimulate the innate and adaptive immune systems. Abnormal T_H1 responses, induced by mucosal IL-18 and IL-12, are thought to be involved with CD with macrophage-secreted IL-12 elevated in CD patients (*13, 14*). Mucosal T cells from CD patients also secrete higher levels of IL-2 and IFN-γ than UC T cells and healthy controls (*15, 16*). As such, CD is often characterised by abnormal T_H1 immune responses, in contrast to UC which is considered to be T_H2-mediated (*12*). T_H17 cells are induced by IL-6 and TGF-β and are characterised by their production of IL-17A, IL-17F, IL-21 and IL-22 (*17*). The expression of IL-

17A is found to be elevated in UC and CD mucosa, as well as being secreted in larger amounts in the lamina propria, relative to health controls. Regulatory T cells (T_{REG}) maintain gut homeostasis by supressing abnormal immune responses against innocuous stimuli such as commensal bacteria and dietary or self-antigens. T_{REG} cells are characterised by their expression of FOXP3 and secretion of the anti-inflammatory cytokines IL-10 and TGF-β. Proportions of T_{REG} cells have been found to be decreased in the peripheral blood of patients with active IBD, compared to quiescent IBD patients and controls (*18*). As such, the decrease in T_{REG} anti-inflammatory and subsequent increase in proinflammatory responses, contribute towards IBD pathogenesis. Genome-wide association studies (GWAS) have identified multiple IBD susceptibility genes, including NOD2, ATGL16L1 and IRGM, which are related to autophagy (*19*).

Recent evidence suggests that the microbiome plays a large role in IBD pathogenesis, which has led researchers to trial faecal microbiota transplantation (FMT) (*20*). FMTs have demonstrated some success in IBD treatment, increasing the chance of UC remission at 8-weeks post FMT. Multiple studies have analysed the microbiome in UC and CD in inflamed and noninflamed segments and found that there is a significantly decreased microbiome biodiversity when compared with healthy controls (*21*). In the healthy gut, Firmicutes and Bacteroidetes phyla predominate the microbiome. In contrast, there is a relative lack of Firmicutes and Bacteroidetes and an overgrowth of enterobacteria in CD and reduction in Clostridium spp and increased *Escherichia coli* in UC (*22*).

1.3 Clinical significance and management

Although there is no cure for IBD, the understanding of these debilitating diseases has significantly grown over the past decade. This has provided targets for more effective therapeutics that improve mucosal health, consequently inducing remission and decreasing the frequency and duration of inflammation. However, many of these therapeutics lack efficacy and often induce a myriad of side effects and as a result there remains a need to develop novel therapies. In some cases, patients develop tolerance to these drugs, leading to decreased efficacy, with a small subset of patients where all drugs fail (*23*). Current treatment of IBD in Australia involves the induction of remission by biologics, sulfasalazine, 5-aminosalicylates (5-ASAs) given orally, rectally or both (*24*).

Mild to moderate UC that does not respond to oral or rectal 5-ASAs may be attenuated with oral glucocorticoids or immunosuppressive agents (azathioprine or 6-mercaptopurine). For patients that continue to require or do not respond to glucocorticoids, biological agents such as monoclonal antibodies such as infliximab may induce remission. Once remission has been induced, oral and rectal 5-ASAs are effective for maintenance, particularly in distal colitis (*25*). When 5-ASAs are poorly tolerated or inefficient, thiopurines (azathioprine) can be used for maintenance (*26*). When pharmacological agents have been trailed with no success or if serious complications develop (e.g. perforation, toxic megacolon and uncontrollable bleeding), sections of gut may be surgically removed (*26*). Unlike CD, surgery for UC can be curative, with 5-20% of patients requiring the intervention; however, this can be associated with severe side effects such as short bowel syndrome or pouchitis, intestinal failure and the

possible requirement of enteral nutrition (*27*). Intestinal fibrosis is a common complication of IBD which occurs after repeated bouts of tissue injury, extracellular matrix (ECM) remodelling and tissue repair, resulting in ECM protein accumulation and scar formation, leading to the loss of function and anatomical abnormalities of the gastrointestinal tract. Complications of intestinal fibrosis include stricture, fistulae and abscesses, which typically require surgical repair and are the most common indication for surgery in CD. Although the development of intestinal fibrosis can be prevented by controlling intestinal inflammation, once fibrosis has developed, the control of inflammation cannot reverse the fibrosis. Apart from surgical intervention, there are no therapies to reverse the development of intestinal fibrosis, as such further research is required to elucidate fibrogenic pathways in IBD.

1.4 Diagnostic imaging

Currently, imaging of IBD is used to differentiate UC and CD in newly diagnosed patients, assess and monitor the progression of GI inflammation and extrasystemic side effects and to monitor the progression of already diagnosed patients (*28*). Diagnostic tools in IBD include colonoscopies and or endoscopies and can include barium x-ray, magnetic resonance imaging (MRI) and computed tomography (CT) and clinical investigations like histology and various blood tests for erythrocyte sedimentation rate, faecal lactoferrin and calproctectin which can indicate the level of inflammation. Scope investigations are largely invasive and uncomfortable due to a change in diet at least 3 days prior to the procedure (low residue diet), overnight fasting and bowel preparation by purging the colon of its contents for colonoscopies. Double contrast barium enemas can be utilised in IBD; however, CT and MRI are largely replacing this technique. Barium contrast studies may reveal signs such as:

pseudopolyps, filiform polyps, loss of haustra, strictures and ulcerations but may return unremarkable in some patients with mild UC (*29*). Barium enema findings for CD may include cobblestone appearance of gastrointestinal tract, fistulas, abscesses, strictures and separation of bowel loops (*29*). Barium enemas should be used with caution in patients with severe colitis as it may induce ileus with toxic megacolon. CT and MRI may reveal thickening of the bowel wall which is not specifically indicative of UC or CD and are less suitable than barium x-ray for detection of early colitis due to lower sensitivity (*29*). Additionally, ultrasound with Doppler may reveal thickening of the mucosal layer in active mild UC or transmural thickening in severe UC; however, neither of these signs are indicative of UC and may arise due to other causes. There has been a shift from traditional contrast radiography to CT, MRI, ultrasound and positron emission tomography (PET), with future applications focusing on PET-CT and magnetic resonance colonography. The diagnosis of IBD is largely achieved by endoscopies and colonoscopies; however, the evaluation of the small bowel appears to remain a challenge. The length and many overlapping loops of the small intestine limits the utility of endoscopies. CD and UC can be differentiated by colonoscopy, based on the focality and pattern of ulceration. Biopsies are routinely obtained during scope studies for confirmation of diagnosis through analysis of ulceration patterns and acute or chronic inflammation (*29*). The use of wireless capsule endoscopies has been increasing for assessment of the small bowel; however, it must be used with caution in patients with suspected strictures as the capsule may require surgical removal.

Currently, diagnostic imaging relies on the identification of anatomical signs for CD or UC. This provides minimal information on the degree of gastrointestinal inflammation present. Although colonoscopies are routinely performed for a range of conditions, including IBD, up

to 33% of patients experience at least 1 minor temporary gastrointestinal symptom as a result of the procedure (*30*). A relatively common adverse effect, perforation of the gastrointestinal tract due to mechanical manipulation of the scope occurs in up to 0.3% of patients (*31*). Haemorrhage may occur as a result of colonoscopy, but most commonly occurs in colonoscopy with polypectomy, compared with colonoscopies without polypectomy (*32*).

1.5 Molecular imaging and immuno-PET

Molecular imaging techniques are indispensable in the diagnosis and management of many conditions. They have the power to characterise, visualise and measure the activity of biological processes. In terms of gastrointestinal exploration and imaging, colonoscopies are generally used for investigations of the large intestine in addition to MRIs; however, colonoscopies are restricted to the mucosal surface. In the small bowel, MRIs and endoscopies are utilised; however, it can be difficult to observe inflammation in the small intestine using these methods. PET is a non-invasive functional imaging modality mostly used in the detection of cancer. This technique relies on the use of a radiolabelled probe attaching to its target and on radioactive decay, releasing a positron and subsequent gamma rays, which are detected by the scanner. Utilising this concept, it is possible to synthesize a radiolabelled antibody which will track to a desired target at high sensitivity and resolution, illustrating the areas of inflammation in 3D. The use of radiolabelled antibodies in PET is an attractive non-invasive imaging modality, which remains at the forefront of molecular imaging. The past forty years have seen the development of multiple radiolabelled antibodies for diagnostic and therapeutic purposes, particularly in the detection of tumours (*33*). Currently (^{18}F)-fluoro-2-deoxy-D-glucose (FDG) PET or radiolabelled leukocyte scintigraphy is utilised for IBD.

FDG-PET is used as a non-invasive imaging modality to illustrate aberrant glucose uptake, particularly in oncology. Inflamed or cancerous tissues often have higher glycolytic rates and tend to accumulate FDG, allowing for the visualisation of inflammation and tumours. Due to this, the specificity of this tracer can be lower in comparison to tracers with a specified target. Radiolabelled leukocyte scintigraphy also depends on a broad set of targets. This technique relies on the extraction of leukocytes from the patient’s blood which are radiolabelled. In contrast to other techniques such as FDG-PET, the synthesis of this tracer is much more time consuming and there is also the risk of cross contamination between patients and staff when working with blood products. Consequently, the currently available imaging tracers for IBD lack specificity and sensitivity, often targeting non-specific processes. Immuno-PET is a largely non-invasive technique, as such the risk of adverse effects is reduced. In addition to imaging, immuno-PET also possesses the ability to quantify molecular interactions. The amount of radiation exposed to a patient during the procedure is small, accordingly, the risk of follow-on effects is limited. Additionally, the beneficial results and improvement in patient management and treatment outcomes outweighs the risks of the test. There is also a small risk of allergic reaction to the tracer in some patients, as well as infection at the site of injection. Immuno-PET of IBD will provide functional and quantitative information by directly showing areas of inflammation, particularly in areas in and around current lesions or newly forming lesions, in addition to extramural manifestations and may reduce the need for biopsies.

Creating a tracer that is based on an antibody against a target fundamental to the development of IBD will increase the sensitivity and specificity of gastrointestinal inflammation imaging. This will provide clinicians and researchers with an increased

understanding into the patient's condition and treatment requirements, as well as differentiating between CD and UC, in addition to other conditions. This will play a key role in personalised medicine against IBD as patients can be imaged to evaluate the efficacy of their pharmacological therapies. In addition to this, this technique would greatly benefit drug development against IBD, with this technology being transferrable to other inflammatory conditions such as arthritis.

1.6 PET imaging with ^{89}Zr

The use of zirconium-89 (^{89}Zr) in radiolabelling of antibodies is relatively new, compared with other radionuclides such as technetium-99m. This radionuclide provides many advantages over other alternatives, such as a relatively long half-life of 78.4 hours and a relatively low average positron energy and good *in vivo* stability which are amongst characteristics which makes it suitable for immuno-PET with monoclonal antibodies (mAbs). ^{89}Zr decays by positron emission (23%) and electron capture (77%) to the stable isotope of ^{89}Y which also has suitable properties for immuno-PET (*34*). The half-life of ^{89}Zr is well-matched with the time required for mAbs to circulate and bind to their targets, resulting in optimal target-to-background ratios. The E_{max} for positron emission is 897 keV and E_{ave} of 396.9 keV could result in improved spatial resolution due to not having prompt photons with energies near 511 keV. Disadvantages of ^{89}Zr include high energy gamma emission of 908.97 keV which limit the radioactive dose which can be administered to patients and its limited availability. However, an increase in the commercial availability of ^{89}Zr will make this radionuclide widely available for research, with the possibility of developing novel clinical applications. The use and development of mAbs is rapidly expanding, with the US Food and Drugs Administration having approved over 30 for therapeutic use.

1.7 Production and purification of ^{89}Zr

^{89}Zr is typically formed by cyclotron bombardment of ^{89}Y foil, most often by $^{89}Y(p,n,)^{89}Zr$ or $^{89}Y(d,2n)^{89}Zr$ reactions (*35*). These reactions are preferred over other alternatives due to its higher yield of ^{89}Zr from ^{89}Y which is naturally abundant, and the reaction can occur with high purity using low level energy as yttrium only has 1 stable isotope (*36*). However, one of the largest issues associated with the use of ^{89}Zr is removing impurities and maintaining purity. Bombardment of ^{89}Y as mentioned above can form many impurities, one of which is the radiochemical impurity ^{88}Zr (~0.01%) which has a half-life of 83.4 days, in addition to ^{65}Zn, ^{48}V, ^{56}Co, and ^{156}Tb which may interfere with synthesis of the immunoconjugate (*37, 38*). This can be resolved by dissolving ^{89}Zr in hydrochloric acid and separating it from impurities by ion-exchange chromatography, particularly by hydroxamate resin purification (*37*). The final purified ^{89}Zr solution should be of high chemical and radiochemical purity with endotoxin concentrations below accepted levels (*39*). Using the methodology above, ^{89}Zr can be produced reliably with high purity (>99.99%) and high specific activity (470 – 1195 mCi/mmol) (*40*).

1.8 Conjugation of ^{89}Zr PET tracers with DFO

The combination of an intact mAb with ^{89}Zr allows for a relatively long-lived tracer which would be advantageous in transportation. Ideally, the compound which will be conjugated to ^{89}Zr should have a residency time similar to the half-life of the radionuclide. This allows for imaging to continue days post-injection, allowing for longer-term observations of treatment efficacy. ^{89}Zr is a residualizing isotope, meaning that free ^{89}Zr is internalised into cells, in contrast to other radionuclides such as ^{124}I, which remains internalised for a short period of

time before re-entering the blood stream (*39*). To prevent non-specific uptake, it is imperative that ^{89}Zr chelators are effective for the entire duration that contrast agents remain within the body. Previous ^{89}Zr chelators have lacked efficiency, allowing for cleavage of ^{89}Zr from the tracer. Multiple studies investigating ^{89}Zr biodistribution have shown that free ^{89}Zr accumulates within the bones and causes degradation of the bone in mice; however, bone uptake is not particularly observed in humans (*41, 42*). Zirconium is commonly conjugated to antibodies by desferrioxamine (DFO). There are two commonly used synthetic strategies used to achieve this: firstly, by a succinic acid linkage to the amine residue of DFO and an amine residue of the antibody and secondly, in a two-step process involving *p*-isothiocyanatobenzyl-desferrioxamine (*p*-NCS-Bz-DFO) (43, *44*). The latter being the preferred methodology due to its simplicity and commercial availability.

Synthesis of ^{89}Zr-DFO-Bz-NCS complexes can result in high immunoreactivity, radiochemical purity and structural integrity (*44*). Due to the relatively simple conjugation protocol, *p*-NCS-Bz-DFO allows for easier compliance to GMP guidelines as well as rapid and efficient preparation. *P*-NCS-Bz-DFO has been proven to be an efficient chelator of ^{89}Zr with no adverse uptake in normal organs or accumulation within the bone (*44*). *P*-NCS-Bz-DFO is currently being widely utilised in the development of zirconium tracers due to its superior chelating properties. In the past ten years there has been a significant amount of interest in the use of ^{89}Zr in radiopharmaceuticals, partly due to its

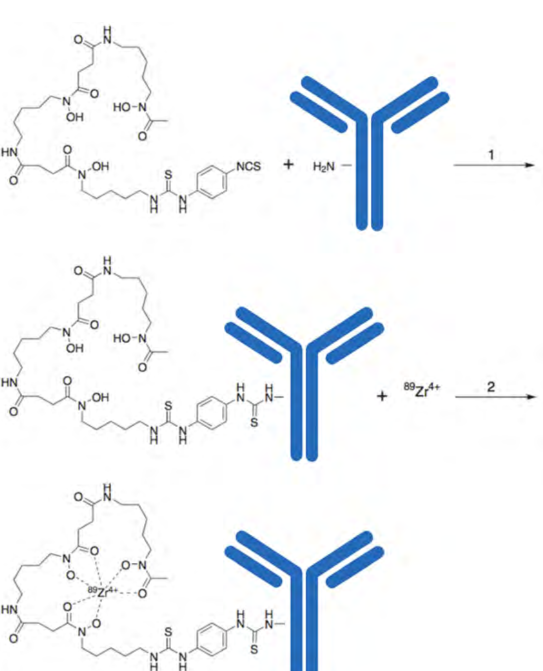

Figure 1 Schematic representation of p-*NCS-Bz-DFO* coupling with a monoclonal antibody (1) and labelling with 89-zirconium (2) (adapted from 45).

recent commercialization and increased availability. ^{89}Zr-mAb immuno-conjugates are already being utilised to provide high quality PET images in both clinical and preclinical settings. As mentioned above, *p*-NCS-Bz-DFO is the most suitable chelator at present, due to its superior chelating properties. A streamlined and more efficient coupling method has been developed in contrast to initial methodology (*45, 46*). Firstly, the chelator *p*-NCS-Bz-DFO attaches to the mAb by binding to a lysine-NH_2 group at pH 9.0 (step 1, figure 1). This product is purified by gel filtration prior the addition of ^{89}Zr-oxalic acid, from which the ^{89}Zr is complexed into the hexadentate ligand DFO (step 2, figure 1). The final product is purified by gel filtration. Another benefit of updated methodology is that there is no need to work under strict metal-free conditions, in contrast to other chelators such as DOTA and DTPA (*45*). Labelling of the *p*-NCS-Bz-DFO-mAb conjugate with ^{89}Zr results in >85% purity. The optimal purity of the final immuno-conjugate product should be >95% which can be confirmed by instant thin-layer chromatography (ITLC), high performance liquid chromatography (HPLC) and SDS-PAGE (*45*).

1.9 Antibody fragments

Antibodies have demonstrated their supreme utility as therapeutics through their highly selective and effective binding, to treat a range of diseases in immunology, cardiology and oncology (*47*). As such, the knowledge and availability of antibodies have significantly increased in recent decades. The long biological half-life of antibodies is ideal for therapeutic application but for use as imaging contrasts, intact antibodies have a long half-life and can require days to achieve good signal-to-noise ratios (see *47* for a thorough review on antibodies and engineered fragments). For *in vivo* imaging, this leads to increased radiation exposure.

Ideally imaging contrasts will not have any biological activity as this can alter the mechanisms of interest and may lead to side effects. Additionally, tracer targets should have an increased expression in disease and low expression in health. An antibody's binding properties and kinetics can be altered by engineering fragments through manipulations such as removing the fragment crystalizing (Fc) portion. This portion is not required for biological or therapeutic activity and its removal can improve target binding, signal-to-noise ratio and clearance. Retention of the fragment variable (Fv) portion is required for antigen binding. Furthermore, removal of the Fc portion prevents recycling through the neonatal Fc receptor pathway and complement, and effector cell-mediated immune interactions (*47*). These factors combined lead to rapid binding to target tissues, rapid blood clearance and improved contrast. Due to this, antibody fragments can be paired with isotopes that decay more rapidly, such as ^{18}F, reducing radiation exposure. The route of antibody clearance can be altered by the size, charge and hydrophobicity/hydrophilicity of the protein as well as any conjugated moieties (*47*). Use of antibody fragments and proteins less than approximately 60 kDa, promotes renal excretion, whereas larger fragments are cleared via the liver (*48*).

1.10 Conclusion

IBD is a debilitating and lifelong condition which is often invisible to others. Although the understanding of IBD pathogenesis has increased in recent decades, there is still a lack of effective pharmacological therapies and imaging modalities. Molecular imaging is emerging as the forefront of medical imaging. PET in combination with a pathology-specific tracer has a multitude of advantages over conventional diagnostic imaging modalities, including providing temporal and spatial visualisation of a molecular probe within a biological pathway *in vivo*, as

well as quantification of tracer uptake which may indicate severity. Personalised medicine is becoming more prominent in clinical settings, which will only increase in the future– molecular imaging and personalised medicine go hand in hand. Immuno-PET in general may allow clinicians to assess a patient's responsiveness to their treatment regime. This may increase the patient's prognosis, sparing the patient extra exams, time and decrease treatment costs. Early management due to immuno-PET and tailored treatment plans will also decrease the development of lesions, inflammation and fibrosis, prior to the requirement of invasive procedures such as a colectomy. Patient's drug dosing can be adjusted to achieve maximum therapeutic potential, whilst decreasing potential side effects. This technology also has the potential to quickly and non-invasively assess drug efficacy *in vivo*, while providing quantification into the biological pathway in question. Accordingly, I aimed to define the immune and epithelial barrier changes associated in DSS colitis and fibrosis. This will identify targets for molecular probes, which I will design and validate.

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Chapter 2 Gut barrier, tolerance and dietary emulsifiers: a review

2.1 Statement of authorship

Statement of Authorship

Title of Paper	Immune homeostasis and tolerance in the gastrointestinal tract: how do dietary emulsifiers interact with our gut?
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Overall percentage (%)	70		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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By signing the Statement of Authorship, each author certifies that:

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

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2.2 Introduction

The gastrointestinal tract has an array of innate and adaptive mechanisms for maintaining homeostasis by limiting immunogenicity against the host and by mounting the appropriate immune responses against pathogens. This can range from innate mechanisms such the antibacterial properties of the gastrointestinal mucus layer, to adaptive, anti-inflammatory activity by regulator T cells (T_{REG}). The intestinal epithelium and thick mucus layer are primary modes of defence against pathogens. The mucus itself is an active regulator of commensal bacterial colonisation and species diversity (*1*). The intestinal barrier primarily consists of epithelial cells in which tight junction complexes create a tight seal preventing the passage of the luminal contents into the mucosa. Intestinal biopsies from patients with CD, shows a decreased expression of the tight junction protein e-cadherin, impairing tight junction function (*2*). In addition to the physical intestinal barrier, Goblet cells, enterocytes, Paneth cells, neuroendocrine cells and M cells also contribute to the maintenance and homeostasis of the intestinal barrier (*3*). Accordingly, dysfunction of these cell types may contribute towards IBD pathology. Everything that we ingest has an interplay with our immune system, whether pathogenic or innocuous. This may be dietary components which are key for health, or more sinister stimuli such as pathogens like *Salmonella*. Regardless, our gastrointestinal immune system is being constantly bombarded with stimuli which will either be tolerated or deemed immunogenic by our gut. This fine balance between tolerance and immunogenicity is crucial in maintaining gut health and is implicated in many gastrointestinal disorders including inflammatory bowel disease (IBD). IBD encompasses both ulcerative colitis (UC) and

Crohn's disease (CD), which are characterised by chronic remitting and relapsing inflammation of the gastrointestinal tract (GIT), causing gastrointestinal ulcerations, bleeding, bowel disturbances and pain. The pathogenesis of IBD is not fully elucidated but is believed to be multifactorial in nature with host immunological, genetic, environmental and bacterial factors involved. Typical treatment of IBD involves the use of corticosteroids, immunosuppressants and biological agents such as anti-TNF-α agents. Tolerance of and side effects to pharmacological treatments is common, as such surgery is often required. Patients with IBD have been found to have decreased diversity of their microbiome (*4*). A decrease in bacteria populations which have anti-inflammatory properties may contribute to intestinal pathology. A decrease in short chain fatty acid-producing bacterium, such as *Faecalibacterium prausnitzii*, can contribute towards pathology by modulating epithelial cell growth and T_{REG} differentiation and expansion (*5*). Bacteria such as *Escherichia coli* can also modulate intestinal permeability, alter the microbiome composition and induce inflammation by modulating the expression of inflammatory genes (*6*). Modulation of the microbiome in active UC by faecal biota transfer has shown therapeutic promise (*7*). This review aims to assess the current understanding of tolerance and the maintenance of homeostasis in the gastrointestinal tract and how dietary emulsifiers can modulate this.

2.3 Modulation of intestinal homeostasis by dietary emulsifiers

The gastrointestinal tract is constantly exposed to a range of stimuli, which can be pathogenic or innocuous in nature. The gastrointestinal tract is home to a multitude of bacteria which can be beneficial to the host or can contribute towards pathologies. The disturbance of this fine balance between tolerance and pathogenicity of the gut microbiota can modulate the

host's mucosal immunity, possibly leading to inflammatory conditions such as IBD. A primary defence in the intestine is the layer of mucus which protects the epithelium from the microbiota (*8*). Consequently, disturbance of this mucus layer has the potential to potentiate inflammatory conditions of the gut. It has been hypothesised that compounds like dietary emulsifiers have the potential to disturb this mucus layer by altering its physicochemical properties, allowing for bacterial translocation into the lamina propria and subsequent inflammation of the gastrointestinal tract. Emulsifiers are widely used in the Western diet and may partially explain the rise of inflammatory conditions of the gut in Westernised countries. Emulsifiers are often added to foods such as mayonnaise, ice-cream and some processed meats to evenly distribute fats throughout the mixture, providing a more palatable texture and consistency. Two commonly used emulsifier carboxymethyl cellulose (CMC) and polysorbate-80 (P-80) work to stabilise emulsions in beverages and food. CMC increases viscosity by reducing the rate of sedimentation and P-80 is a non-ionic surfactant. It has been shown, that the administration of relatively low concentrations of the commonly used emulsifiers CMC and P-80 induced low-grade intestinal inflammation in WT hosts and potentiated robust colitis in IL10$^{-/-}$ and TLR5$^{-/-}$ mice (*9*). It is speculated that this occurred through a shift in microbiota which favoured pro-inflammatory interactions. Changes in microbiota can cause gastrointestinal inflammation or can occur as a result of inflammation, and therefore it can be difficult to discern the extent that a substance can alter an organisms' microbiota. Emulsifier-treated mice showed an average reduction of 50% in the distance between the epithelium and nearest bacteria (*9*). This microbiota encroachment was also associated with a reduced mucus thickness, with no change in Muc2 expression. Reduction in mucus thickness was not observed in germ-free mice, suggesting the CMC and P-80 act on the microbiota, rather than the mucus layer itself. Treatment with CMC or P-80 induced

significant alterations in microbiota composition in WT, $IL10^{-/-}$ and $TLR5^{-/-}$ mice. Faecal levels of proinflammatory flagellin and bioactive LPS were also increased in emulsifier-treated WT, $IL10^{-/-}$ and $TLR5^{-/-}$ mice. Additionally, it has been shown that P-80 can increase *Escherichia coli* translocation through CACO2-cl1 monolayers, M-cells and across human Peyer's patches (*10*).

More recent studies have supported the premise that the microbiota is an important target of CMC and P-80 (*11*). Using the *ex vivo* M-SHIME model it was shown that the human microbiota undergoes shifts in composition in response to CMC and P-80, increasing proinflammatory interactions (*11*). In mice with a pathobiont-free microbiota, there were no changes in microbiota composition in response to CMC and P-80. Additionally, M-SHIME suspensions from CMC and P-80-treated mice, when transferred into germ-free mice were able to produce low-grade inflammation and metabolic disease. A recent study has shown that acute exposure to CMC and P-80 is sufficient to alter intestinal mucus structure and transport properties (*12*). These changes result in the translocation of bacteria into the lamina propria, which may contribute towards the development of intestinal inflammation. CMC exposure induced a decrease in mucus pore size which correlates with significantly slower *Escherichia coli* speed and particle diffusion. Exposure to P-80 caused negligible changes to the mucus microstructure and particle diffusion but increased the movement speed of *Escherichia coli*. Both CMC and P-80 were found to alter mucus volume and thickness in rat intestinal tissue and mucus-producing cell cultures (*12*). These results suggest that dietary exposure to CMC may result in compaction of the intestinal mucus and the loss of some clumped material. In contrast, exposure to P-80 may alter microbial binding and transport in addition to altering the mucosal make-up through the loss of some mucosal components (*12*).

Although the consumption of emulsifiers has not been linked to directly causing gastrointestinal conditions, it has been shown to alter homeostatic conditions of the gut, perhaps permitting and potentiating the development of inflammation. The Westernised diet is often described as a risk factor for IBD. However, it must also be considered that it is common to consume multiple emulsifiers and dietary additives simultaneously, thus more work is required into the effects of multiple emulsifiers – are these effects additive or synergistic? Additionally, the concentration of dietary additives such as emulsifiers are not well reported or regulated as they are generally regarded as safe (GRAS) for consumption.

2.4 Innate lymphoid cells

Innate lymphoid cells (ILCs) are relatively recently discovered players in the field of mucosal immunology and inflammation. Although their functions are not entirely known, it has become apparent that they play a significant role in immune responses, particularly in chronic inflammation by mediating the transition from innate to adaptive immune responses and in tissue repair (*13, 14*). Although ILCs make up a small proportion of immune cells throughout the body, their strategic positioning in the epithelial layers, amongst others, enables them to be highly efficient in maintaining gastrointestinal homeostasis. Currently, three subtypes of ILCs are described, which are based on cytokine secretion, extracellular markers and transcription factors (table 1).

	Secrete	Transcription factors	Location	Function	Implicated in
ILC1	IFN-γ TNF-α	Tbet	Mucosal-associated lymph tissues (MALTs) Gut Skin Liver Tonsils Endometrium	Protective against bacteria, intracellular parasites	Autoimmune disorders
ILC2	IL-4, IL-5, IL-9, IL-13	GATA3	Fetal gut Small intestine Adipose tissue Tonsils Skin Lungs Peripheral blood	Protective against helminths and parasites	Allergy, asthma
ILC3	IL-22, IL-17, GM-CSF, TNF-α	RORγt	Lamina propria Spleen Tonsils Lungs Endometrium Skin	Protective against extracellular bacteria, fungi	Autoimmune disorders, IBD

Table 1. Innate lymphoid cell subtypes and characteristics (*13, 14*).

ILCs do not express T or B cell antigen receptors, nor do they express lymphoid differentiation lineage markers (*13*). ILCs are crucial for maintaining the fine balance between homeostasis and autoimmune inflammation, suggesting that they play a key role in autoimmune, allergic and inflammatory conditions, accordingly an understanding into their roles and functions may provide novel targets for much needed therapeutics. ILCs have been found to be crucial in the maintenance and repair of various tissues throughout the body, such as the intestinal

epithelium, which is crucial in the control of inflammation and homeostasis. The analysis of ILC populations in healthy control intestinal biopsies revealed that NKp44+ ILC3s are most dominant of the ILCs throughout the ileum, caecum and colon, with the lowest proportions in the ileum (*15*). ILC1s were most frequent in the ileum and ILC2s were in low proportions throughout the entire gastrointestinal tract. Proportions of NKp44+ ILC3s were significantly decreased in inflamed active UC and CD biopsies, relative to health. This reduction is observed in newly diagnosed and chronic IBD patients. Interestingly, this reduction correlated with the local endoscopic disease severity in both UC and CD patients. This reduction of NKp44+ ILC3s has severe consequences for maintaining epithelial barrier integrity and homeostasis, as discussed further below. This decrease in NKp44+ ILC3s correlated with an increase in ILC1 and ILC2 proportions in CD and UC at the time of diagnosis, respectively. However, in patients with chronic UC or CD both proportions of ILC1 and ILC2 were increased. Changes in ILC composition primarily occurred in inflamed tissue only and correlated with endoscopic damage scores. There were no changes to the proportions of ILCs in the noninflamed mucosa or peripheral blood of IBD patients, compared to healthy controls.

Group 1 innate lymphoid cells

Deriving from natural killer cell progenitors, T-bet$^+$ group 1 ILCs secrete IFN-γ and TNF-α in response to IL-12, IL-15 and IL-18 (*16, 17*). Although deriving from the same progenitor, group 1 ILCs differ from NK cells as they lack the cellular markers CD16, CD56 and CD94 (*16*). Currently, there are three distinct subsets of ILC1s, which includes natural killer cells (NK cells), intraepithelial CD127$^-$ CD103$^+$ ILC1 and CD127$^+$ ILC1 (*18*). CD127$^-$ CD103$^+$ ILC1 are cytotoxic and typically reside in the intestinal epithelium, in contrast to CD127$^+$ ILC1 which

are not cytotoxic and mostly reside in the lamina propria (*19*). In health, ILC1s are potent stimulators of mononuclear phagocytes and are key for immunity against intracellular pathogens such as *Toxoplasma gondii* through their inherent secretion of TNF-α and IFN-γ (*20*). Group 1 ILCs have been found to accumulate in the inflamed intestine of patients with CD and out of the three subsets, group 1 ILCs were the most frequent, suggesting a role for this subset in CD (*16*). RORγt^{+}T-bet^{dim} ILC3 cells can differentiate into IFN-γ-producing RORγt^{-} T-bet^{Hi} ILC1 cells when cultured with IL-2 and Il-12 (*16*). Previous studies have suggested that both human and mouse ILC3s can upregulate T-bet and downregulate RORγt (*16*). Additionally, the differentiation of ILC1 to ILC3 can be induced by IL-23 and is accelerated by IL-1β and retinoic acid (*18*). This is supported by an increase in RORγt and decreased levels of T-bet. Furthermore, retinoic acid has been shown to upregulate RORγt (*21*). This multi-directional differentiation from ILC1 to ILC3 and vice versa is mediated by a subset of dendritic cells (*16*). Intestinal resections from patients with CD were found to have a higher proportion of CD14^{+} dendritic cells which promote the differentiation of ILC3 to CD127^{+} ILC1 (*18*). Conversely, CD14^{-} dendritic cells can promote the reverse differentiation of CD127^{+} ILC1 to ILC3. This may explain the increased proportion of ILC1s in the inflamed intestine of CD patients. This coincides with a previous study which indicated that IFN-γ and IFN-γ producing cells, such as ILC1s, were significantly increased in the intestine of patients with active CD (*22*). Interestingly, fontolizumab, an anti-IFN-γ antibody was not found to be efficacious in treating CD (*23*). Experiments with DSS-treated human immune system mice indicate that proportions of CD127^{+} ILC1s increase as a result of acute inflammation, rather than as a side-effect of chronic intestinal inflammation (*16*). This is thought to occur through the inflammation-induced differentiation of NKp44^{+} ILC3s, which are a predominant subset in homeostatic conditions into ILC1 (*16*).

This differentiation is driven by IL-12 secreted by phagocytes which influx into the gastrointestinal tract during inflammation (*16, 24, 25*).

Group 2 innate lymphoid cells

ILC2s are highly responsive to epithelial cell secreted cytokines such as IL-25 and IL-33 which are released following breaking of the intestinal barrier (*26*). They are capable of secreting type 2 cytokines such as IL-5, IL-13, IL-4 and IL-9 and originate from common lymphoid progenitors via common helper innate lymphoid cell progenitors, like ILC3s. These cells are dependent on GATA-3 for their development and function and are crucial for immune responses to helminths through their stimulation of eosinophilia and development of goblet cell hyperplasia (*26*). In health, ILC2s can produce factors required for epithelial barrier repair such as amphiregulin (*27*). They are predominantly located in non-lymphoid tissues such as those in the gastrointestinal tract, lungs and skin (*26*). ILC2s exist in very small proportions in the human colon and accordingly aren't speculated to play a large role in gastrointestinal inflammation, particularly if not due to parasites (*28*). Interestingly, some intestinal biopsies from patients with CD have been found to have increased numbers of ILC2s, with no change found in UC (*21, 29*). They have also been found to be protective in murine DSS colitis, suggesting that they may be involved in maintenance of the intestinal epithelial barrier (*30*). Recently it has been shown that T-bet regulates intestinal ILC2 functions (*31*). To date, T-bet has been understood to be critical for the function of ILC1s and NCR^{+} ILC3s and the depletion of T-bet prevents the development of these subsets. Additionally, the depletion of T-bet leads to dysregulated ILC3 responses, causing microbiota-dependent colitis. Accordingly, in the innate immune system, T-bet is considered to inhibit pathogenic immune responses. The selective depletion of T-bet in ILCs causes increased activity and expansion of ILC2s

which is beneficial for mucosal immunity, particularly in the defence against *Trichinella spiralis* and inflammatory colitis (*31*). This T-bet-dependent regulation of intestinal ILCs potentially occurs through IL-7 receptor signalling. This mechanism is crucial for maintaining the fine balance between pathogenic and protective immune responses, which may play a role in IBD. T-bet transcriptionally represses the *Il7ra* gene in ILCs. In a T-bet deficient environment, increased IL-7Rα expression in ILCs causes increased activation of STAT-5 on stimulation with IL-7 (*31*). This can occur in normal GATA-3 levels, further suggesting that T-bet acts directly as a transcriptional repressor of IL-7Rα. It has been previously shown that increased IL-7 sensitivity, independent of retinoic acid signalling is associated with higher ILC2 proliferation and accumulation (*32*). Adding to this, it has been found that T-cell plasticity and lineage stability can be influenced by interactions between the retinoic acid receptor-α and T-bet (*31*). STAT-5 stimulation may stabilise the expression of GATA-3 in ILCs, increasing the proportion of ILC2s, which is observed in T-bet deficient animals. It is suspected that human ILC3s also comprise of multiple subtypes, but their similarity to murine subset is yet to be determined.

Group 3 innate lymphoid cells

Group 3 innate lymphoid cells are characterised by their expression of RORγt and the secretion of IL-17A, IL-17F, IL-22 and GM-CSF (*33*). Multiple subsets of ILC3s exist in both humans and mice and are defined by their localisation in different tissues and transcriptional, developmental and functional differences (*1, 34*). ILC3s are constitutively present throughout the murine and human gastrointestinal tract, however their densities throughout these organs differs.

They are the most abundant ILC subset at intestinal mucosal surfaces, such as the ileum and colon (*16, 28*). Distribution of ILC subsets is dictated by IL-7 expression as IL-7 stimulation promotes RORγt expression, which in turn promotes ILC3 proliferation (*19*). In mice, there are at least 2 ILC3 subsets: the lymphoid tissue inducer (LTi)-like ILC3 and NCR^+ILC3 cells. LTi-like ILC3s are characterised by the expression of c-kit, CCR6 and their localisation within lymphoid tissues. Conversely, NCR^+ ILC3 lack LTi markers such as CCR6 and instead express NKp46, the transcription factor T-bet and reside in the lamina propria of the small intestine. In health, ILC3s are extremely responsive to different environmental stimuli in various tissues including cytokine and cell surface receptor dependant mediated mechanisms to maintain homeostasis of the gut immune system. Crucial to IBD, ILC3s maintain gut barrier function and integrity, whilst balancing immunogenic and tolerance mechanisms against the microbiota. When immunogenic bacterial antigens have been sensed, ILC3s are stimulated by IL-23 or IL-1β secreted by $CX3CR1^+$ mononuclear cells and dendritic cells (*19*). This stimulates the ILC3s to secrete their main effector cytokines IL-22, IL-17 and GM-CSF.

Recent studies suggest that LTi-like ILC3 tend to be polarised towards maintaining homeostatic functions and regulating adaptive immunity, in contrast to NCR^+ILC3s which are involved in inflammatory processes through the T-bet-dependent secretion of IFN-γ. NCR^+ILC3s predominantly reside within the small intestine and are seldom present in lymph tissues. This is maintained through their expression of CXCR6, which retains the cells within the intestinal lamina propria (*35*). In contrast, LTi-like ILC3 reside within the lymph tissue, which includes Peyer's patches and mesenteric lymph nodes and aggregate around cells of the adaptive immune system such as $CD4^+$ T cells and B cells (*36*). ILC3 are a main source of

IL-22 during homeostasis. Both NCR^+ILC3 and LTi-like ILC3 can produce IL-22 when stimulated by myeloid cell-derived signals such as IL-1β (*37*).

In mice, T_H17 cells are also a source of IL-22, but this does not translate to humans where T_H22 and T_H1 subsets produce majority of IL-22. IL-22 is in the IL-10 family of cytokines which also includes, IL-10, IL-19, IL-20, IL-24, IL-26, IL-28 and IL-29 (*38*). IL-22 is crucial for mucosal host defence against extracellular pathogens such as bacteria and also for tissue repair and acts on only non-hematopoietic stromal cells such as epithelial cells (*39*). IL-22 target tissues include the small intestine, colon, liver, lung, kidney, skin and pancreas (*40*). Dependent on location, IL-22 secretion can be pathogenic due to its pro-inflammatory properties. This is amplified when IL-22 is secreted with other proinflammatory cytokines such as IL-17, which can lead to abnormal epithelial proliferation and differentiation as seen with many inflammatory conditions (*39*). To prevent this, IL-22 and IL-17 secretion are highly and independently regulated. Both IL-22 and IL-17 regulation is dependent on RORγt and STAT3 expression but independent regulation of IL-22 comes from other factors such as c-Maf (*39*). ILC3 secretion of IL-22 is regulated by the aryl hydrocarbon receptor (Ahr), which stimulated by endogenous and xenobiotic ligands (*41*).

In the absence of ILC3s, epithelial maintenance is decreased, leading to increased pathology and severe damage to the intestinal crypts (*42*). Loss of ILC3-secreted IL-22 leads to the translocation of commensal bacteria into the peripheral organs, which allows bacteria to reside closely within the epithelium or within lymphoid tissues (*43*). This suggests that IL-22 is crucial for the maintenance of the intestinal barrier. IL-22 producing ILC3 within cryptopatches can maintain epithelial barrier homeostasis through the maintenance of crypt

stem cells which can differentiate to intestinal epithelial cells and other epithelial and enteroendocrine cell subsets (*42*). To ensure and maintain a closely sealed barrier, crypt stem cells continuously proliferate and replenish all intestinal epithelial cells within days. It has been found that the restoration of proliferating crypt cells and epithelial phosphorylation of Stat3 after tissue damage occurs, is dependent on $Thy1^+$ cells (*42*). In response to tissue damage, $Thy1^+$ cells are essential for the generation of IL-22 and GM-CSF, working to restore intestinal homeostasis.

2.5 Innate lymphoid cell migration

It has been shown that ILCs migrate into the intestine using CCR9 and $\alpha_4\beta_7$ and that ILC1s and ILC3s in both the small intestine and colon highly express $\alpha_4\beta_7$ (*44*). All ILC subsets require CCR9 to migrate into the small intestine, whereas $\alpha_4\beta_7$ serves as a general gut migration marker. Interestingly, 30-50% of ILC1s and ILC3s within the mesenteric lymph nodes (MLN) express CCR9 and $\alpha_4\beta_7$, but these markers are not required for migration into the MLN, suggesting that the MLN are a site for the generation of ILCs, but not an endpoint (*44*). CCR7 is highly expressed on ILC1s and ILC3s in the MLN and may play a role in the bidirectional migration between the MLN and intestine (*44*). The accumulation of homing receptor deficient ILCs in the spleen suggests that migration into the spleen occurs passively without specific homing receptors. Furthermore, the migration of ILC1s and ILC3s but not ILC2s is regulated by mucosal dendritic cells and retinoic acid (*44*). Retinoic acid secreted by dendritic cells within gut-lymphoid tissues can induce upregulation of gut homing receptors on ILC1 and ILC3, thus causing migration between organs (*44*). It has been shown that retinoic acid plays a role in the upregulation of both CCR9 and $\alpha_4\beta_7$ on ILC1 and ILC3 (*44*). However, it is

still not known whether retinoic acid regulates all ILC subsets in the same manner. It has been observed that ILC3s are the most sensitive to retinoic acid-induced homing receptor switching from CCR7 to CCR9 and $\alpha_4\beta_7$, as with ILC1s, but ILC2s did not respond at all (*44*). Interestingly, regardless of organ, all ILC2s were found to express CCR9, including in the spleen (*45*) and intestinal ILC2s do not highly express $\alpha_4\beta_7$ (*44*). Upregulation of gut homing receptors appears to occur in the periphery for ILC1 and ILC3, in contrast to ILC2 which occurs in the bone marrow. Vitamin A levels can alter ILC distribution, causing abhorrent mucosal immunology through a decreased secretion of anti-microbial peptides and IL-22 (*44*). However, ILC migration mechanisms are not fully elucidated so it is likely that non-retinoic acid-based pathways may be involved.

2.6 Regulatory T cells

Regulatory T cells (T_{REG}) powerfully modulate mucosal immunity throughout the gastrointestinal tract by exerting anti-inflammatory effects. They are crucial for restraining immune responses against self and innocuous antigens, whilst permitting antipathogen and antitumor activity (*46*). To operate effectively, T_{REG} cells must interact with both the adaptive and innate immune systems, which depends on their proximity to their targets and their ability to migrate to specific tissues. Loss of these cells can promote intestinal inflammation. T_{REG} cells are constitutive within secondary lymphoid tissues and can be also found within most non-lymphoid tissues, even during homeostatic conditions. T_{REG} cells can secrete suppressive messengers such as TGF-β, IL-10 and IL-35. Expansion of T_{REG} cells from $CD4^+$ T cells is induced by IL-2 and TGF-β (*47*). Forkhead box P3 (FOXP3) expression of $CD4^+$ cells is characteristic of T_{REG} cells (*46*). Stimulation of T cell receptors on T_{REG} cells in secondary

lymphoid organs can promote the homing of T_{REG} cells (*48*), however little is understood about T_{REG} priming and migration to the gut. T_{REG} cells can migrate to other tissues by various homing and chemokine receptors (See *46* for a thorough review). Currently it is thought that a large proportion of peripheral T_{REG} (p T_{REG}) cells differentiate in the mesenteric lymph nodes, where they upregulate gut-homing receptors such as $\alpha_4\beta_7$ and CCR9 (*49*). Both thymus-derived T_{REG} cells (tT_{REG}) and pT_{REG} are present in the gastrointestinal tract, but it is currently thought that pT_{REG}s are responsible for tolerance to microbial and dietary factors (*50*). Furthermore, pT_{REG}s have been found to prevent aberrant T_H1 immunity to food antigens (*51*).

T_{REG} cells have been implicated in the onset and development of UC and CD (*52*). Evidence suggests that an altered balance between anti-inflammatory T_{REG} cells and proinflammatory effector cells in the intestinal microenvironment can contribute to the pathogenesis of IBD (*53*). This allows the mucosal immune system to mount a response against the host. T_{REG} subset phenotype and function in inflamed gastrointestinal tissue of patients with IBD are drastically different from T_{REG} cells obtained from peripheral lymphoid tissue of healthy controls (*54*). Patients with IBD have decreased peripheral T_{REG} cells but increased peripheral T_H17 cells. Also, mRNA expression of FOXP3, IL-17A, IL-1β and IL-6 are elevated in the mucosa of patients with IBD. Interestingly, it has recently been shown that patients with IBD with active endoscopic disease, the intestine has increased proportions of CD4+ T cells, T_{REG} and $T_{central\ memory}$ cells, compared with healthy controls and endoscopic inactive IBD (*55*). Proportions intestinal of CD8+ and CD103+ T cells were decreased in active IBD. The composition of T cell subsets in patients with active endoscopic disease was maintained between the initial and follow up visit, suggesting that this composition maintains consistency over time. T lymphocyte composition returned to the levels of healthy controls when IBD

patients were in endoscopic inactive disease. These proportions of lymphocyte subsets also correlates with endoscopic disease activity. There was no variation in lymphocyte subsets between the ileum and colon, suggesting that it is more so based on disease and not restricted to one location (*55*). Patients with CD that had a higher baseline proportion of T_{REG}s were associated with disease with more strictures, penetrations and or abdominal surgeries. Interestingly, CD patients with a lower baseline of T_{REG} proportions usually experienced milder colitis and treatment with steroids was the strongest treatment used. It is difficult to explain the purpose of this increase in T_{REG} populations in active IBD; however, this expansion may be in an effort to dampen inflammation.

2.7 T-helper cell subset 17

T-helper cell 17 (T_H17) is subset of effector memory T cells characterised by the secretion of IL-17 that have been implicated in the pathogenesis of CD and UC (*56-58*). T_H17 cells are distinct from T_H1 and T_H2 cells and are crucial for the clearance of pathogens and homeostasis of the intestinal mucosa. Loss of this subset can permit the translocation of bacteria into the intestine, causing chronic inflammation. It has been shown that T_H17 cells which induce T_H1 cells are critical for the pathogenesis of colitis (*59*). This occurs primarily through the secretion of IFN-γ by T_H1-like cells which derive from T_H17 precursors. T-bet is required for the transition from T_H17 precursor cells to T_H1-like cells (*60, 61*). T_H17 cells secrete IL-17A, IL-17F, IL-21, IL-22, IL-26, IL-8, IL-10, TNF-α, CXCL8, CCL20 and GM-CSF (*62*). IL-17A and IL-17F are crucial for the defence against extracellular pathogens. T_{REG} cells are the anti-inflammatory counterparts of T_H17 cells and are crucial for maintaining the balance between tolerance and immunogenicity. Interestingly, secukinumab, a monoclonal antibody against IL-17A, failed in

the treatment of IBD, suggesting that IBD is not a typical T_H17-mediated autoimmune disorder (*63*). Binding of IL-17 to its receptor stimulates the recruitment of ACT1, subsequently activating TNF receptor-associated factor 6, activating NF-κB and MAPK pathways (*64*). This leads to the recruitment, activation and migration of granulocytes, but also stimulates epithelial cells, endothelial cells and fibroblasts. Macrophages can also be stimulated to produce proinflammatory mediators such as IL-6, TNF-α and IL-1β (*64, 65*). Differentiation of T_H17 cells is induced by cytokines such as IL-1β, IL-6 and IL-21. The T_{REG}- T_H17 balance can be modulated by environmental cues such as all-trans retinoic acid which promotes T_{REG} differentiation and inhibits T_H17 differentiation (*66*). Studies in germ-free mice have shown a decreased capacity for differentiation between T_H17 and T_{REG} cells, suggesting that the microbiota can modulate these cell populations (*67, 68*). The Clositridia species have been found to be a prominent regulator of intestinal homeostasis and T cell plasticity. Transplantation of human or murine faecal *Clostridium* mixture induces strong polarisation towards T_{REG} cells (*68, 69*). It has been shown that the epithelial adhesion of microbes such as *Citrobacter rodentium* causes the induction of T_H17 cells by segmented filamentous bacteria (SFB) and enterohemorrhagic *Escherichia coli* (EHEC) (*70*). In contrast, adhesion-defective SFB and EHEC were not able to trigger these T_H17 responses. 20 different bacterial strains were isolated from faecal samples of a patient with UC were able to cause the induction of T_H17 cells in the mouse colon and bind to the epithelium (*70*). However, the authors suggest that only 3 of these strains are found to be significantly elevated in the microbiomes of CD and UC patients. Consequently, it is believed that the intestinal immune system evokes T_H17 responses through the recognition of microbes themselves, rather than microbial secretions/components or metabolites. Adhesion of microbes to the epithelium

correlated with an increase in intestinal IgA+ cells, thus T_H17 and IgA+ cells may play a role in the control of epithelial cell-adhering microbes (*70*).

2.8 Mechanisms in the maintenance of intestinal homeostasis and tolerance

Recently it was found that the transcriptional repressor Hypermethylated in cancer 1 (HIC1) was involved in the retinoic acid-dependant regulation of intestinal homeostasis by ILCs (*71*). Additionally, HIC1 has also been found to regulate intestinal T-cell function during inflammation and homeostasis (*72*). Mice that were fed a diet deficient of vitamin A lacked HIC1-positive cells in the intestine (*72*). These cells were found to over-secrete IL-17A, which led to a failure to mount an intestinal immune response. The expression of HIC1 on ILCs occurs in a vitamin A-dependant manner. Without HIC1, proportions of IL-22-producing RORγt$^+$T-bet$^+$ ILC3s decreased, leading to the increased risk of *Citrobacter rodentium* infection. There were no HIC1-dependant changes to the proportions of ILC2s and CD4$^+$ ILC3s (lymphoid tissue inducing-cells, LTi). Additionally, a loss of HIC1 did not disturb the development of ILC progenitor cells in the bone marrow, suggesting that HIC1 regulates ILC function and development in the periphery. The authors hypothesised that retinoic acid induces the expression of HIC1, promoting cellular quiescence in the intestine, through Myc-dependant mechanisms. Tolerance of *Citrobacter rodentium* is mediated by IL-22, ILC3s being one of the largest sources, consequently, HIC1 is crucial for immunity to *Citrobacter rodentium* (*71*). In addition to this, it has been shown that retinoic acid receptor-α (RARα) signalling is essential in maintaining intestinal homeostasis and in the development of the intestinal immune system (*73*). RARα signalling in the intestinal epithelia influences the cellular composition of the intestinal barrier which plays a large role in intestinal immune homeostasis and defence against bacterial infections (*73*). A RARα underdeveloped immune system also has difficulty

in clearing *Citrobacter rodentium* infection. ILC3 are sensitive to modulation by dietary stimuli (*1*). Their ability to react to dietary stimuli suggests that ILC3 have a role in host tolerance of food-derived antigens. It has been shown that commensal bacteria can regulate vitamin A metabolism and storage, which is crucial for the maintenance of intestinal epithelial cells (*74*). As briefly discussed previously, retinoic acid can modulate transcriptional programs in a concentration-dependant manner, which can encourage protective or pathogenic immune responses in the gastrointestinal tract. Recent studies have shown that Clostridia species can modulate gut levels of retinoic acid by supressing intestinal epithelial cell expression of retinol dehydrogenase 7 (*Rdh7*), which catalyses the oxidation of retinol to retinaldehyde. This mechanism is key for the maintenance of tolerance, whilst preventing bacterial colonisation of the gut by enteric pathogens. Partial depletion of the gut bacteria by oral antibiotic administration in mice caused a significant increase in *Rdh7*, compared with untreated control mice but did not reach the same level as germ-free mice (*74*). This correlated with an increased concentration of retinoic acid and decreased retinol in the intestine, compared to control mice. Vancomycin-induced dysbiosis in mice caused a significant increase in the expression of *Rdh7*, which correlated with significantly higher amounts of gut retinoic acid. This data suggests that symbiotic bacterial colonies supress *Rdh7* expression and retinoic acid secretion in the gut, whereas dysbiotic microbiomes do not (*74*).

In addition to vitamin A, dietary metabolites such as vitamin D and aryl hydrocarbon receptor ligands can modulate ILC3 populations and intestinal tolerance (*1*). Vitamin D deficiency has been indicated as a risk factor for inflammatory bowel diseases, with a correlation between vitamin D levels and disease activity (*75*). When ILC3 were stimulated with IL-23 and IL-1β,

vitamin D receptors were upregulated (*76*). Stimulation of gut ILC3 with vitamin D3 caused a downregulation of the IL-23 pathway, leading to suppressed IL-22, IL-17F and GM-CSF, but upregulated genes involved in the IL-1β pathway as well as IL-6 and IL-8. GM-CSF is crucial for tolerance to dietary antigens by supporting the tolerogenic activity of intestinal mononuclear phagocyte populations which can modulate intestinal T_{REG} populations (*77*) Vitamin D3-induced changes in ILC3 subsets did not alter cell proliferation, viability or phenotype. Aryl hydrocarbon receptors (Ahr) are known to interact with dietary and endogenous bacterial metabolites and xenobiotic environmental toxins (*78*). Ahr are essential for the maintenance and function of ILC3 and the secretion of IL-22 (*41*). It has been shown that IL-22-dependant signalling between ILC3 and epithelial cells can modulate the composition of the microbiota in mice, altering tolerance to pathogenic and commensal species (*43*). ILC3-secreted IL-22 is also key for restricting bacterial translocation and in the defence against opportunistic pathogens (*19*). Mice that are deficient in IL-22 or ILC3s showed decreased barrier integrity, which decreases their capacity to prevent the translocation of commensal bacteria. Under a deficit of IL-2, ILC3s can inhibit commensal-specific T cells through the MHCII receptor (*79*). Due to their expression of B cell activating factors such as CD40L, BAFF and APRIL, ILC3s are able to promote B cell-mediated defence against commensals (*80*). Furthermore, through lymphotoxin-dependent pathways ILC3s are able to stimulate the secretion of T cell-dependent and independent IgA and IgG antibodies (*80*). These IgA and IgG antibodies contribute towards maintaining intestinal homeostasis by modulating the size, composition and anatomical containment of the commensal microbiota (*81*). The modulation of ILCs by toll like receptors (TLRs) is still largely unknown. However, it has been shown that human LTi-like ILCs can be stimulated by TLR2 ligands to express IL-5, IL-13 and IL-22 in a NK-κB-dependent manner (*82*). The IL-23 receptor has been found as a susceptibility allele in IBD

and as such is suspected to be involved in the pathogenesis (*83*). IL-23-driven intestinal pathology was initially thought to be due to T_H17 cells, but it is now understood that ILCs also play a role (*84*). Stimulation of colonic leukocytes with IL-23 induced an increase in the production of IL-17 and IFN-γ, which was solely secreted by Thy1+ SCA-1+ RORγt+ IL-23R+ ILCs (*83*). This subtype of ILCs is found to be increased in the inflamed colon.

Ahr $^{-/-}$ mice were found to have increased IL-17 secretion by $CD4^+$ T cells in the small intestine, enhancing T_H17 responses (*41*). The authors suggest that Ahr is required to suppress T_H17 responses under homeostatic conditions in the gut. This increase of IL-17 by $CD4^+$ T cells in *Ahr* $^{-/-}$ mice seems to arise from an increase in RORγt expression. Microbiota such as segmented filamentous bacteria can promote T_H17 cell differentiation through the induction of RORγt expression in $CD4^+$ T cells (*85*). *Ahr* $^{-/-}$ mice were found to have a significant increase in faecal segmented filamentous bacteria, which correlates with previous studies showing that segmented filamentous bacteria can induce T_H17 responses in the gut (*86*). This suggests that expansion of gut segmented filamentous bacteria in *Ahr* $^{-/-}$ mice plays a role in increased T_H17 responses. It has also been shown that a depletion of ILCs leads to an increase in segmented filamentous bacteria in *Rag* $^{-/-}$ mice, suggesting that ILCs play role in the maintenance of levels of commensal bacteria during homeostasis (*41*). Mice deficient in *Ahr* also spontaneously developed intestinal pathology, showing signs of thickening and fibrosis of the intestine, without displaying weight loss or diarrhoea. These studies suggest that the Ahr-IL-22 axis in ILC3s works to maintain intestinal homeostasis through the suppression of T_H17 responses (*41*).

In addition to this, Ahr signalling has also been shown to modulate ILC2 function (*87*). Out of the ILC subsets, Ahr is most highly expressed on ILC2s and controls the accessibility of chromatin at the *Ahr* locus by positive feedback. Ahr stimulation supresses the transcription factor Gfi1-mediated expression of IL-33 receptor ST2 on ILC2. This also decreases secretion of the ILC2 effector molecules IL-5, IL-13 and amphiregulin. Removal of Ahr increases the capacity for anti-helminth immunity in the gut, but pharmacological or genetic stimulation of Ahr suppresses ILC2 function but enhances ILC3 immunity against *Citrobacter rodentium* infection. This suggests that through modulation of the Ahr pathway, the host is able to regulate the gut ILC2-ILC3 balance to mount the appropriate immune response against various pathogens (*87*).

2.9 Conclusion

Everything that we ingest has the potential to modulate our mucosal immunity and intestinal barrier. Additives such as emulsifiers and thickeners are commonly used in an array of foods and have been shown to contribute towards intestinal pathology. It is crucial to build our understanding of how dietary elements such as emulsifiers and other food additives affect our intestinal tolerance and microbiome and how they may contribute towards disease. The mechanisms underpinning IBD are highly complex and interwoven, but our understanding of these debilitating diseases continues to improve. Understanding of these mechanisms is crucial for the development of novel therapeutics to improve patient care. New therapies must be developed as current therapies lack efficacy and may decrease in efficacy over time and frequently cause debilitating side effects. It is evident that the interplay between the intestinal immune system and the microbiota can contribute towards pathology. Future

therapies should be personalised based on an individual's aberrant inflammatory responses or microbiota and may even include dietary interventions.

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Chapter 3 Dietary emulsifier exacerbates murine colitis

3.1 Statement of authorship

Statement of Authorship

Title of Paper	Dietary emulsifier exacerbates chemically induced murine colitis
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)	Nicole Dmochowska		
Contribution to the Paper	Conceptualized the study and interpreted the data Acquired data Contributed toward methodology Curated and analysed data Drafted the manuscript		
Overall percentage (%)	70		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	**24/10/2019**

Co-Author Contributions

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

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

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

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

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3.2 Introduction

The gastrointestinal tract has an array of innate and adaptive defences and tolerogenic mechanisms to regulate and maintain the fine balance and control of gastrointestinal homeostasis and mucosal immunity. Dietary emulsifiers are detergent-like compounds added to foods to evenly distribute fats throughout the mixture creating a more palatable texture and consistency. The use of emulsifiers is particularly high in Westernised diets and can be found in foods such as mayonnaise, ice-cream and even some processed meats. Carboxymethyl cellulose (CMC) is a commonly used emulsifier which stabilises emulsions in beverages and foods by reducing the rate of sedimentation. It has been shown that dietary emulsifiers can disturb the mucus layer of the gut by altering its physicochemical properties. This allows bacterial translocation into the lamina propria, promoting inflammation (*1*).

It has previously been demonstrated that emulsifier-treated mice show increased intestinal bacterial encroachment which correlated with reduced mucus thickness but did not change mucin 2 (MUC2) expression, which is required for maintenance of the insoluble mucus membrane (*2*). The administration of relatively low concentrations (1% w/v) of CMC incudes low-grade intestinal inflammation in WT hosts and promotes robust colitis in IL-10$^{-/-}$ and TLR5$^{-/-}$ mice (*2*). Faecal levels of proinflammatory flagellin and bioactive LPS were also increased in emulsifier-treated WT, IL-10$^{-/-}$ and TLR5 $^{-/-}$ mice. Additionally, emulsifiers altered host-microbiome interactions resulting in polarisation of the microbiota that favours mucolytic and

pro-inflammatory activity, promoting intestinal inflammation and leading to the manifestation of colitis.

Although the consumption of dietary emulsifiers has not directly been shown to cause inflammatory conditions of the gastrointestinal tract, it has been demonstrated that they have the potential to alter homeostatic conditions of the gut, promoting the development of inflammation. The effects of emulsifier treatment on metabolism and microbiome have been well characterised, however, changes to the mucosal immune profile have not been determined. Innate lymphoid cells (ILCs) are crucial for the maintenance of intestinal homeostasis and the mucosal barrier by responding to effector cytokines in the immediate microenvironment, as well as exogenous or endogenous danger patterns (*3*). Group 3 ILCs predominate the gastrointestinal tract and are dependent on RORγT expression and secrete IL-17, IL-22 and colony stimulating factor 2. Although considered to be innate, ILCs can produce and secrete cytokines that are typically regarded as CD4+ T_H cells (*3*). $T_{REGULATORY}$ cells (T_{REG}), typified by their expression of forkhead box P3 (FOXP3) and secretion of suppressive IL-10 and TGF-β, are crucial for the maintenance of immune tolerance in the gastrointestinal tract through their secretion of regulatory cytokines, modulation of antigen-presenting cell functions and the alteration in cellular metabolism (*4*). Furthermore, CD4+ T cells can migrate into effector sites of the gut epithelium during inflammation through the expression of $\alpha_4\beta_7$ integrin and mucosal addressin cell adhesion molecular-1 (*5*). We aimed to analyse the CMC-induced changes in innate and adaptive immune regulation and intestinal barrier properties in WT healthy control and DSS colitis mice.

3.3 Methods

Mouse treatments

Colitis was induced in group-housed male SPF mice with 2% dextran sodium sulfate (DSS) in drinking water, as previously described (*6*). Emulsifier treated mice received daily oral gavage of 100 µL 1% carboxymethylcellulose sodium (CMC) for 21 days. The DSS emulsifier group completed a course of DSS colitis to coincide with the completion of CMC administration. Healthy control mice received no treatments. See figure 1 for a summary of mouse treatments.

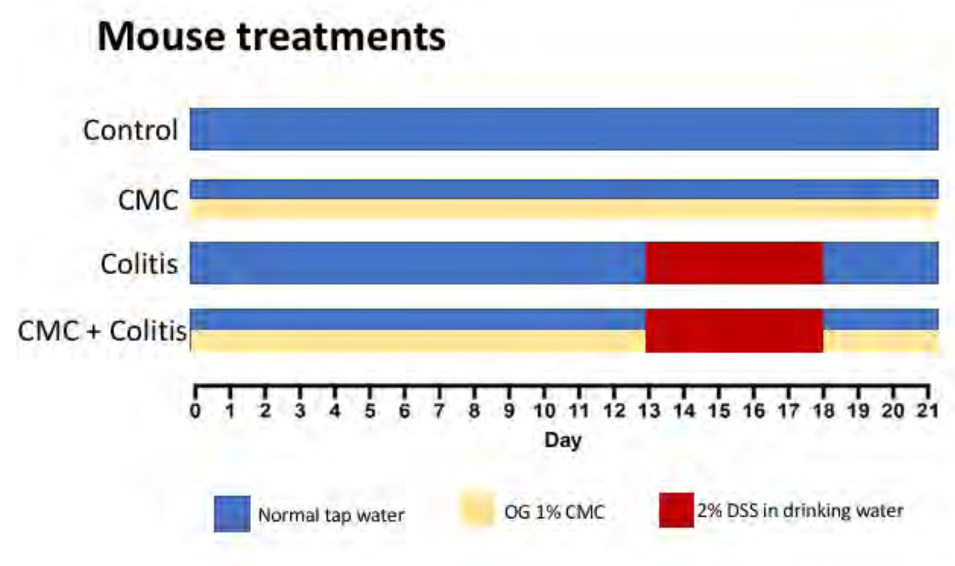

Figure 1. Summary of mouse treatments.

Colon histopathological analysis

Following euthanasia, a 1 cm section of distal colon was dissected and stored in 4% buffered formalin at 4°C prior to embedding in paraffin. 5 µm thick sections were stained with hematoxylin and eosin (H&E) using standard protocols and 20x images were scored from inflammatory and epithelial changes as previously described (*7*).

Using Chamber studies

Segments of distal colon were mounted into 0.1 cm^2 aperture slides in 5 mL baths with oxygenated glucose-fortified Ringers solution for analysis by Ussing chamber as previously described (*6, 8*).

PCR

mRNA extracted from segments of distal colon was analysed for the expression of sodium-potassium adenosine triphosphate (Na-K-ATPase) and sodium-potassium-chloride cotransporter (NKCC1), relative to the housekeeper EEF2 by quantitative RT-PCR as previously described (*7*).

Flow cytometry

Colon lamina propria mononuclear cells (LPMCs) were isolated enzymatically as previously described (*6, 7*). Splenocytes and mesenteric lymph node (MLN) cells were isolated by flushing with PBS, followed by filtration and centrifugation.

Prior to staining for innate lymphoid cells, single cell LPMC suspensions were cultured with 10 nm/ml IL-23 and GolgiPlug, overnight. 0.5 x 10^6 F_c blocked LPMC, MLN cells or splenocytes were stained with the extracellular anti-mouse monoclonal antibodies (BD Biosciences) for T cells CD45-BV605 (30-F11), CD4-BUV737 (GK-1.5), CD326-BV510 (G8.8), CD25-PE-Cy7 (PC61), CD49d-AF647 (R1-2), B7-BB515 (FIB504), CD29-PE (HM β 1-1), CD103-BV786 (M290) and for ILC3s CD45-BV605 (30-F11), CD326-BV510 (G8.8), Ly-6G/Ly-6C-BV510 (RB6-8C5), CD11b-BUV737 (M1/70), CD3-BUV737 (145-2C11), B220-BUV737 (RA36B2), TER-119-BV650 (TER-

119), NK-1.1-BUV395 (PK136) and FCεRIa-PE-Cy7 (MAR-1, BioLegend) (lineage negative: CD326, Ly6G/Ly-6C, CD11b, CD3, B220, FCεRIa, TER119). Cells were stained with intracellular anti-mouse monoclonal antibodies following fix/permeation (BD Biosciences): FOXP3-PECF594 (MF23) for T_{REG} and IFN-γ-AF488 (XMG 1.2), IL-17A-AF647 (TC11-18H10), RORγT-PE (Q31-378), T-bet-BV786 (04-46) and GATA3-BV421 (L50-823) for ILC3, following standard protocols. 50,000 events per test were analysed using a FACS-Fortessa (BD Biosciences) and proportions of live (FVS700, BD Biosciences) singlets were determined using FlowJo as described previously (*6, 7*).

MLN and bone marrow cell stimulations

Bone marrow cells were isolated by flushing of the femur with PBS, followed by filtration and centrifugation. MLN cells and splenocytes were plated at 1 x 10^5 cells per 200 µL in complete media in 96-well U-bottom plates. MLNs were cultured in the presence of CD3/CD28 beads (1:4, cell:bead) for 96 hours. Bone marrow cells were cultured in the presence of 1µg/mL lipopolysaccharide (LPS). Cultures were centrifuged, the supernatant was collected and stored at -80°C until multiplex immunoassay analysis.

Innate and adaptive cytokine concentrations

Colonic proteins were extracted from a 1 cm segment of distal colon as previously described (*6, 7)*. A Milliplex bead-based multiplex immunoassay was used to quantify the colonic concentrations of G-CSF, GM-CSF, IFN-γ, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12p40, IL-12p70, IL-13, IL-15, IL-17, IP-10, KC, MCP-1, MIP-1a, MIP-1b, MIP-2, RANTES and TNF-α, as per the manufacturer’s instructions. LPS stimulated bone marrow supernatants were assessed for the innate cytokines IL-1β, IL-6, IL-10, CXCL2, CXCL10, CCL2, CCL3 and TNF-

α, as per the manufacturer's instructions. CD3/CD28 stimulated MLN cells were assessed for the adaptive cytokines IFN-γ, IL-10, IL-13, IL-17, IL-2, IL-4, IL-5 and TNF-α, as per the manufacturer's instructions. Plates were read and analysed using a Luminex MAGPIC with xPONENT software. Concentrations were normalised to total protein content as determined by a BCA assay, as previously described (*6, 7*). Limits of detection were CXCL10 (48.0 pg/ml), CCL2 (17.1 pg/ml), CCL3 (10.0 pg/ml), CXCL2 (16.7 pg/ml), G-CSF (72.2 pg/ml), GM-CSF (14.8 pg/ml), IFN-γ (35.2 pg/ml), IL-1a (32.8 pg/ml), IL-1β (18.2 pg/ml), IL-2 (8.3 pg/ml), IL-4 (7.5 pg/ml), IL-5 (8.5 pg/ml), IL-6 (21.0 pg/ml), IL-7 (26.2 pg/ml), IL-9 (439.0 pg/ml), IL-10 (23.1 pg/ml), IL-12p40 (192.6 pg/ml), IL-12p70 (11.4 pg/ml), IL-13 (61.4 pg/ml), IL-15 (154.2 pg/ml), IL-17 (16.5 pg/ml), CXCL10 (26.9 pg/ml), CXCL1 (26.0 pg/ml), CCL2 (14.7 pg/ml), CCL3 (17.2 pg/ml), CCL4 (113.9 pg/ml), CCL5 (73.8 pg/ml) and TNF-α (44.4 pg/ml).

Statistical analysis

All data has been expressed as mean ± SEM in all cases. The significance of the results was determined by one-way ANOVA with a Bonferroni post-hoc. Differences where $P<0.05$ were deemed statistically significant. Multiple comparisons were only made for groups of interest: control vs emulsifier, control vs DSS colitis, DSS colitis vs emulsifier and DSS colitis and emulsifier vs DSS colitis.

3.4 Results

Macro and microscopic inflammatory changes

CMC treatment increased DSS induced colitis weight loss, at end point (P<0.001), with no change in microscopic damage score (P>0.05) (figure 2). DSS colitis induced significant weight loss at end point (P<0.001), colon contraction (P<0.001) and histological microscopic damage score (P<0.001), relative to controls. Administration of CMC had no significant effect on colon length or weight loss, (P>0.05), but induced histological microscopic damage, compared to healthy controls (P<0.05).

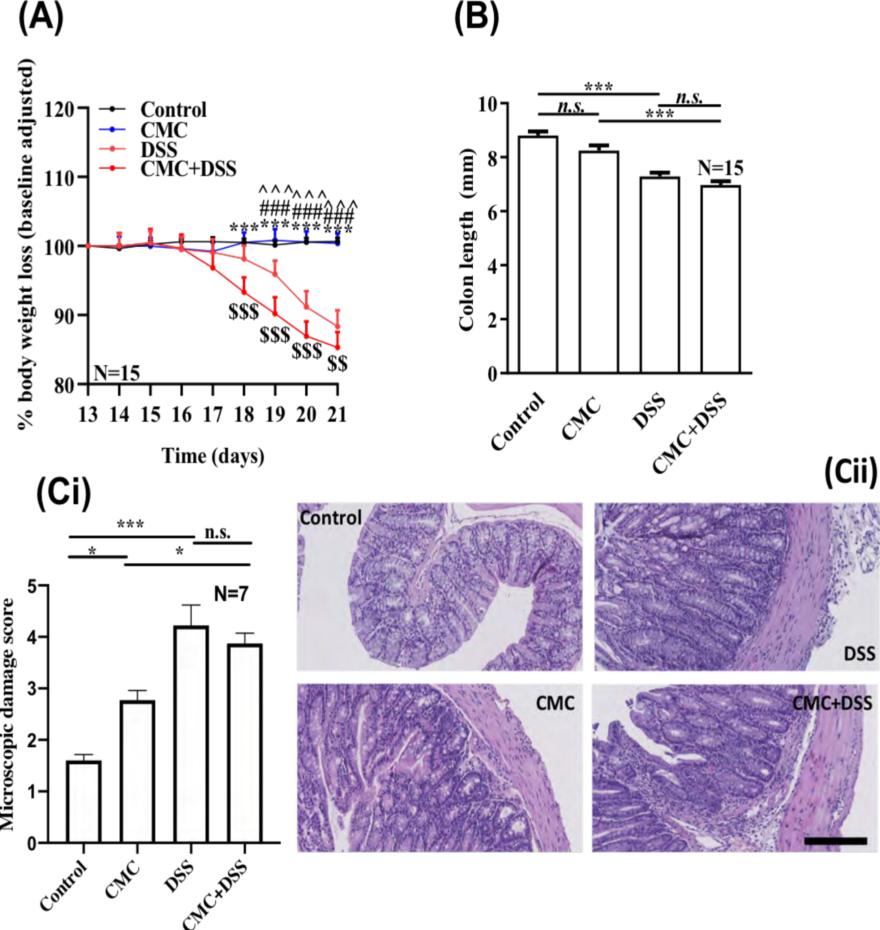

Figure 2. CMC treatment exacerbates DSS colitis. A) CMC treatment worsens DSS colitis weight loss (N=15/group). B) Colon length is decreased in DSS colitis, which did not change with CMC treatment (N=15/group). Ci) CMC treatment increases colonic microscopic damage scores, but not to the same extent as DSS colitis (N=6/group). Cii) Representative images of colon histology. # is CMC vs DSS, * is CMC vs CMC+DSS, $ is DSS vs CMC+DSS. *P0.05, **P<0.01, ***P<0.001. Scale bar = 200 μm.

Epithelial barrier properties

DSS colitis induced a significant decrease in TEER and short circuit current, relative to controls (P<0.01) (figure 3). This effect on TEER was abolished in the presence of CMC (P<0.01), with no change in short circuit current (P>0.05). DSS colitis significantly decreased forskolin (P<0.001) and carbachol (P<0.05) induced epithelial short circuit changes, relative to health. DSS colitis induced a decrease in the colonic expression of Na-K-ATPase, which was abolished in the presence of CMC (P<0.01). CMC treatment had no effect on TEER, baseline short circuit current or secretagogue-evoked changes to short circuit current, Na-K-ATPase or NKCC1 expression, relative to controls (P>0.05).

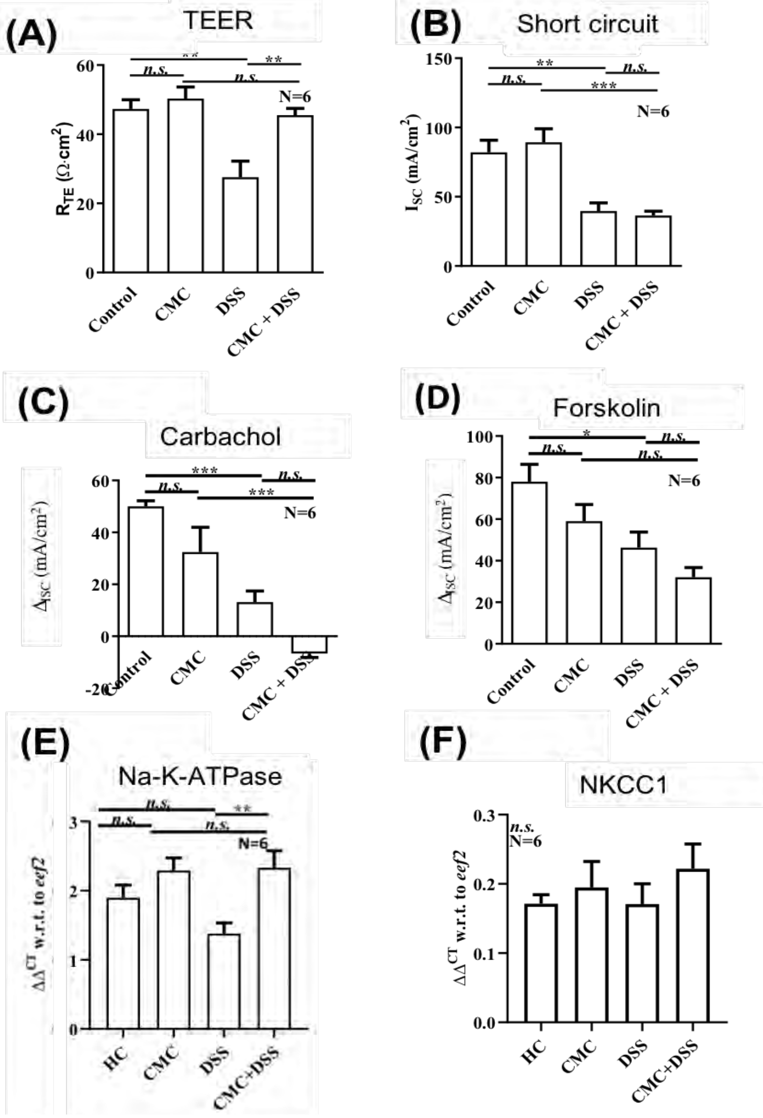

Figure 3. Epithelial responsiveness. A) Baseline TEER (N=6/group). B) Baseline short circuit current (N=6/group). C) Carbachol-evoked changes in short circuit current (N=6/group). D) Forskolin-evoked changes in short circuit current (N=6/group). E) Colonic expression of Na-K-ATPase (N=6/group). F) Colonic expression of NKCC1 (N=6/group). *P<0.05, **P<0.01, ***P<0.001.

Innate and adaptive mediators

DSS colitis induced increased secretion of colonic CCL2, CCL5 and CXCL10 (P<0.05, P<0.05, P<0.01), which were unchanged in the presence of CMC (P>0.05) (figure 4). However, CXCL1 was significantly increased in DSS colitis in the presence of CMC (P<0.01). CMC treatment did not induced changes in colonic chemokine secretion, relative to controls (P>0.05).

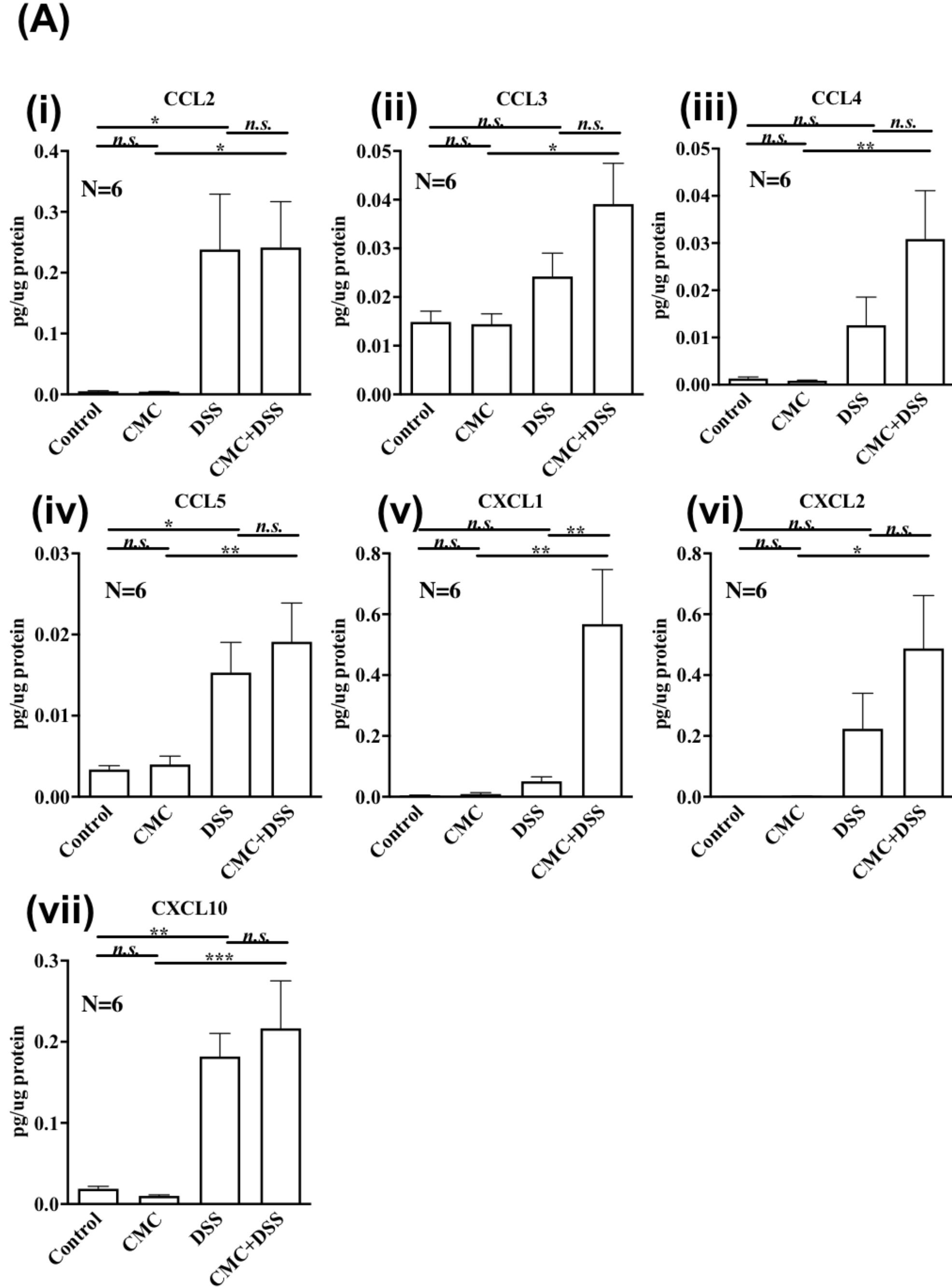

Figure 4. Colonic concentrations of innate chemokines. N=6/group. *P<0.05, **P<0.01, ***P<0.001.

DSS colitis induced significant increases in colonic IL-1α (P<0.05), which was unchanged with CMC treatment (P>0.05) (figure 5). DSS colitis when treated with CMC resulted in significant increases in IL-1β (P<0.01), IL-6 (P<0.01), IL-12p40 (P<0.05), IL-12p70 (P<0.05), G-CSF (P<0.001) and GM-CSF (P<0.05). CMC treatment increased colonic secretion of IL-12p40 (P<0.001), with no changes in other innate cytokines, relative to controls (P>0.05).DSS colitis in the presence of CMC increased colonic secretion of IL-13 (P<0.001) but did not alter the secretion of adaptive cytokines in controls (P>0.05) (figure 6).

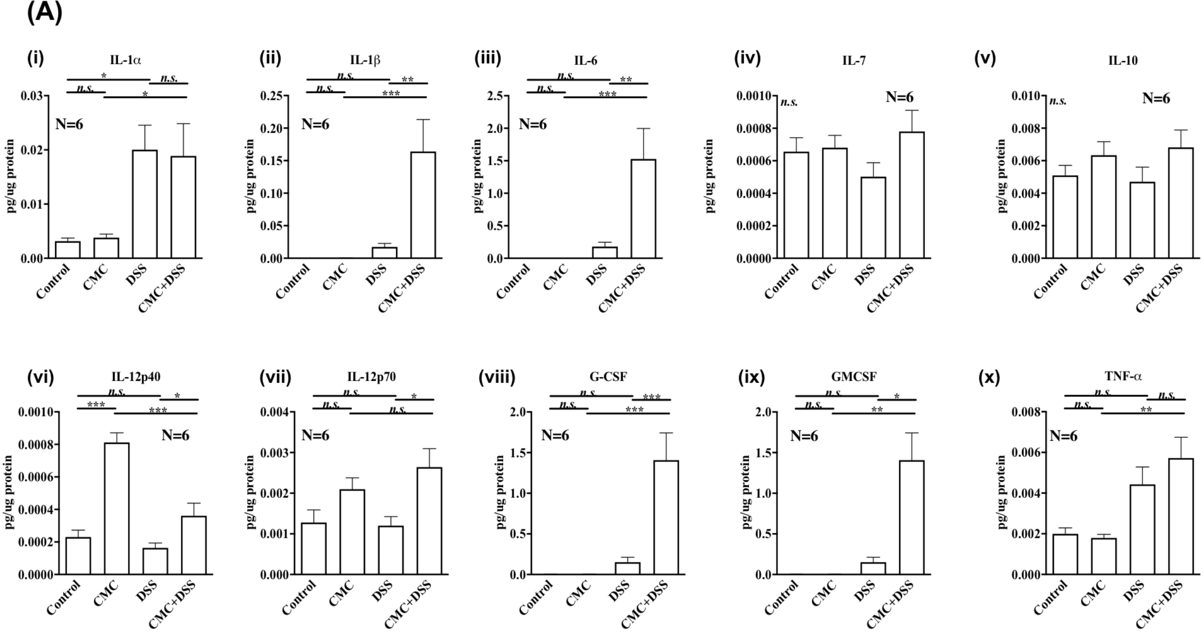

Figure 5. Colonic concentrations of innate cytokines. N=6/group. *P<0.05, **P<0.01, ***P<0.001.

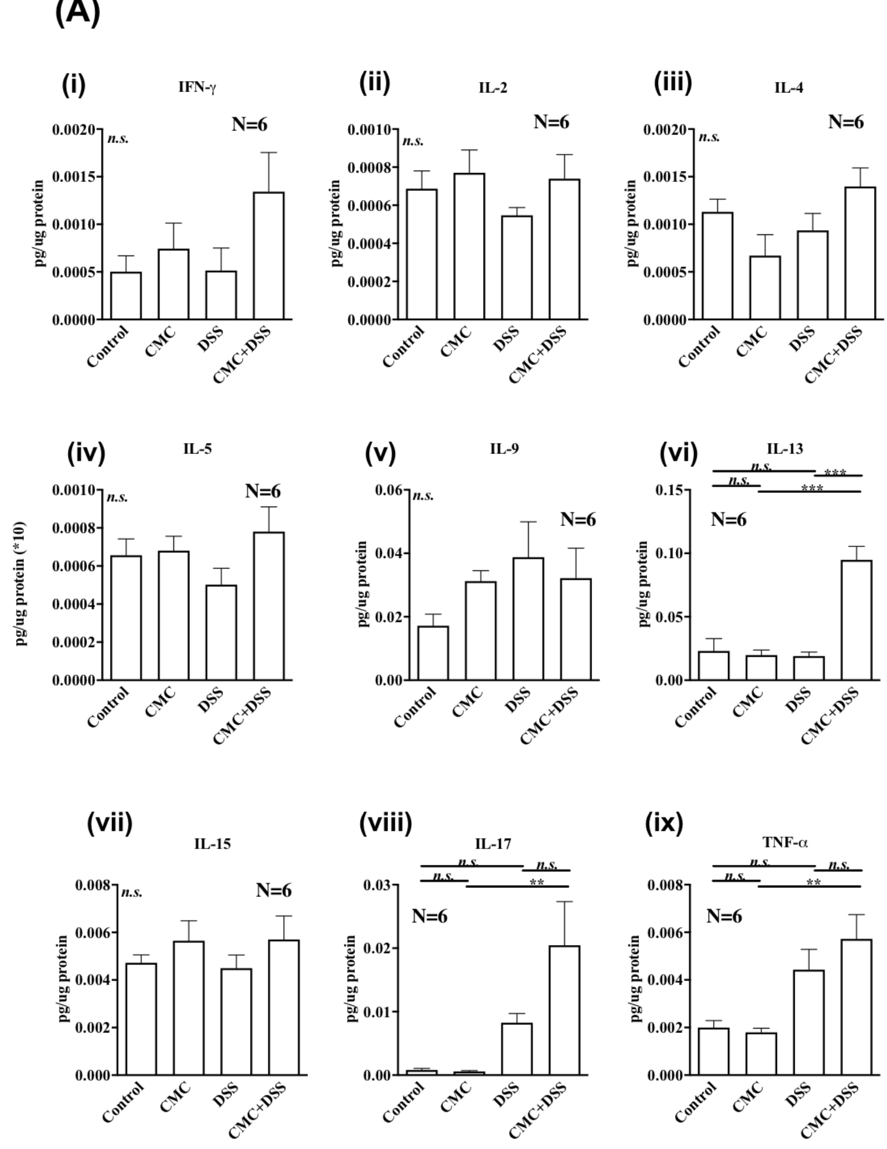

Figure 6. Colonic concentrations of adaptive cytokines. N=6/group. *P<0.05, **P<0.01, ***P<0.001.

CD3/CD28 stimulated MLN cell cytokine secretion

DSS colitis induced CD3/CD28 stimulated MLN cells to secrete increased IFN-γ (P<0.01), IL-2 (P<0.05), IL-5 (P<0.01), IL-10 (P<0.001) and IL-13 (P<0.05), relative to controls (figure 7). CMC-treated DSS colitis resulted in the increased secretion of IL-2 (P<0.05), IL-4 (P<0.05), IL-17 (P<0.001) and TNF-α (P<0.01) from CD3/CD28 stimulated MLN cells, compared to DSS colitis. CMC treatment did not alter the cytokine secretion of CD3/CD28 stimulated MLN cells (P>0.05).

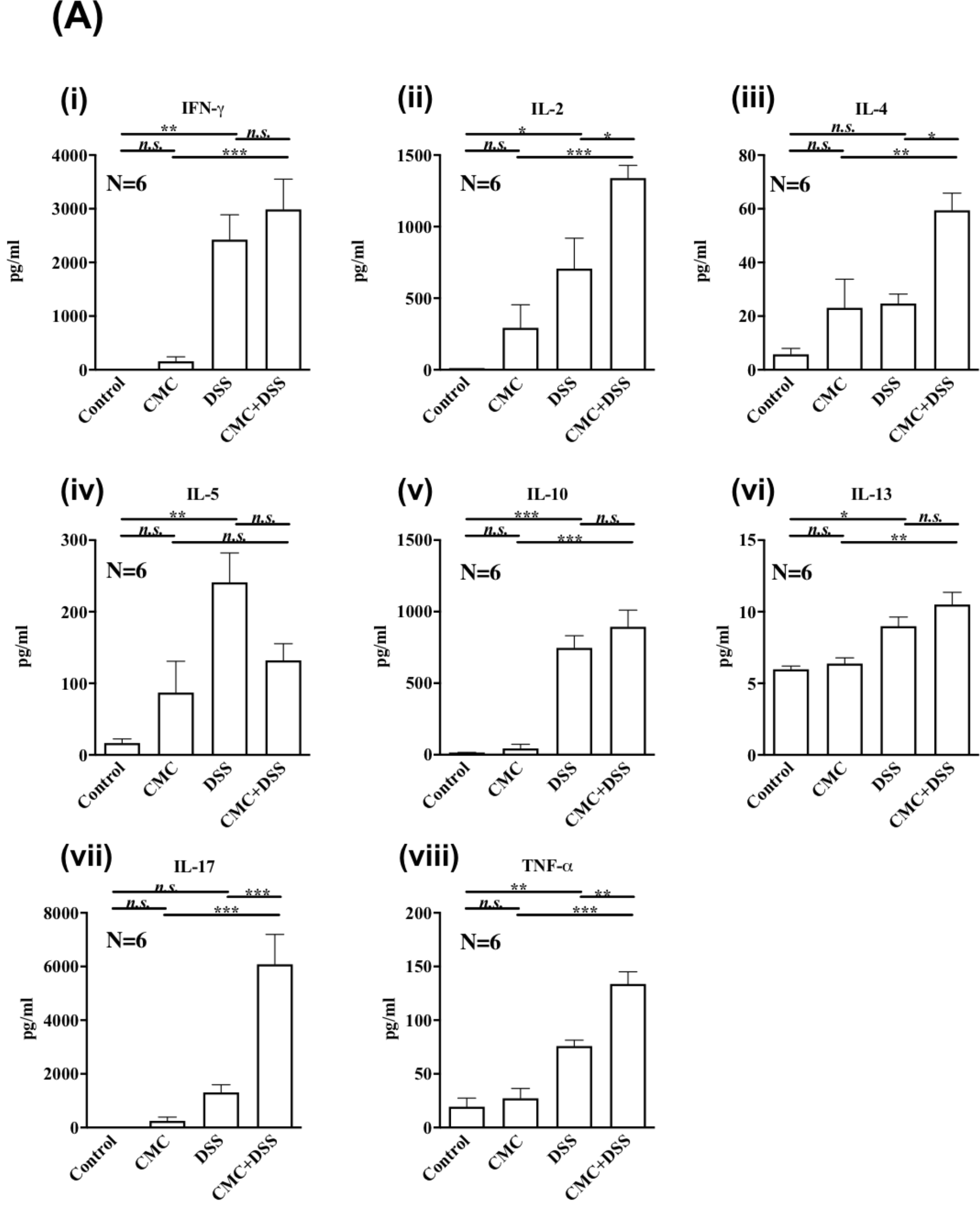

Figure 7. CD3/CD28 stimulated MLN cytokine secretion. N=6/group. *P<0.05, **P<0.01, ***P<0.001.

LPS stimulated bone marrow cell cytokine and chemokine secretion

DSS colitis induced LPS-stimulated bone marrow cells to secrete increased IL-1β (P<0.01), IL-10 (P<0.001), CCL3 (P<0.001), CXCL2 (P<0.05) and CXCL10 (P<0.001), relative to controls (figure 8). CMC-treated DSS colitis resulted in the decreased LPS stimulated bone marrow secretion of IL-1β (P<0.05), IL-10 (P<0.001) and CXCL10 (P<0.001), but increased CCL3 (P<0.001), CXCL2 (P<0.05) and TNF-α (P<0.05), relative to DSS colitis. CMC-treatment decreased the secretion of IL-1β (P<0.05), IL-6 (P<0.01), CCL3 (P<0.001) and CXCL2 (P<0.05) from LPS stimulated bone marrow cells, relative to controls .

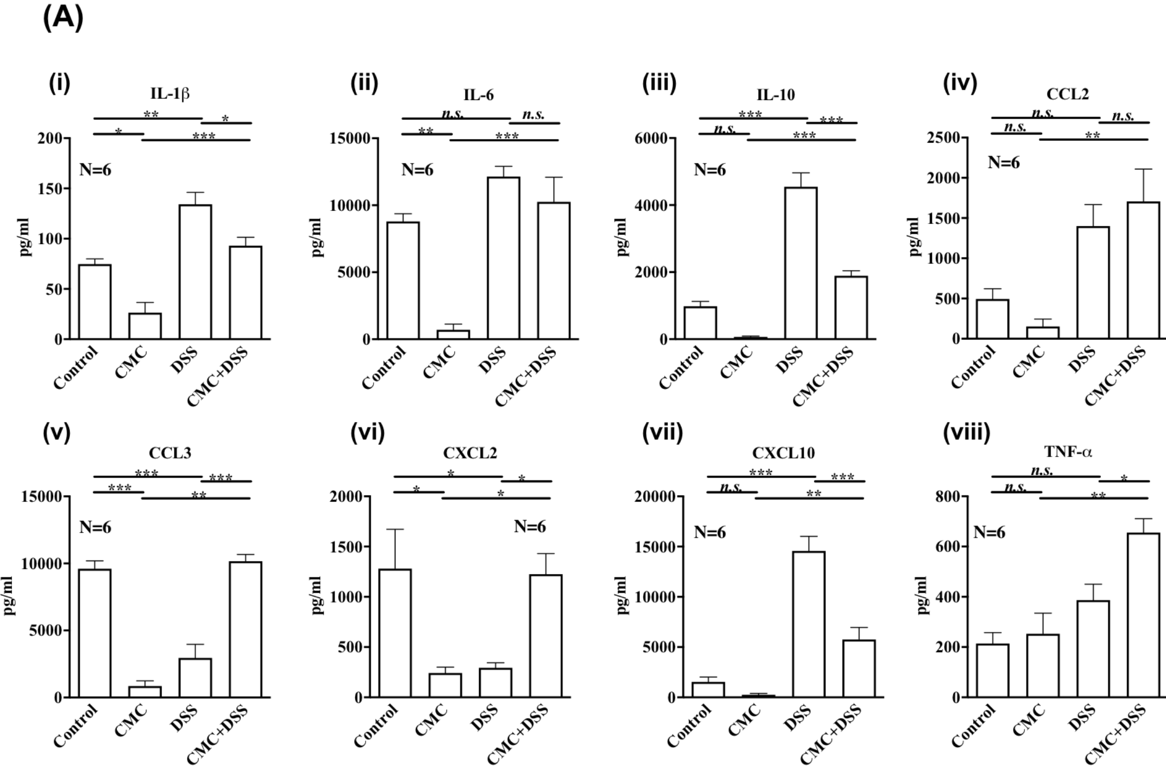

Figure 8. LPS stimulated bone marrow cytokine secretion. N=6/group. *P<0.05, **P<0.01, ***P<0.001.

Cells

DSS colitis increased proportions of colonic T_{REG} cells (P<0.001), which was abolished when treated with CMC (P<0.001). CMC treatment did not induce changes in T_{REG} populations in controls (P>0.05). DSS colitis did not alter the percentage of CD4+ T helper cells expressing the integrins $\alpha_4\beta_7$, $\alpha_4\beta_1$ or $\alpha_E\beta_7$, when compared to controls (P>0.05); however, expression of $\alpha_4\beta_7$ was increased when treated with CMC (P<0.05). CMC treatment induced increased expression of $\alpha_4\beta_7$ (P<0.05), $\alpha_4\beta_1$ (P<0.01) and $\alpha_E\beta_7$ (P<0.001), relative to controls. DSS colitis decreased colonic proportions of ILC3s, relative to controls (P<0.001), which was abolished in the presence of CMC (P<0.01). CMC treatment decreased colonic ILC3 proportions, relative to controls (P<0.001).

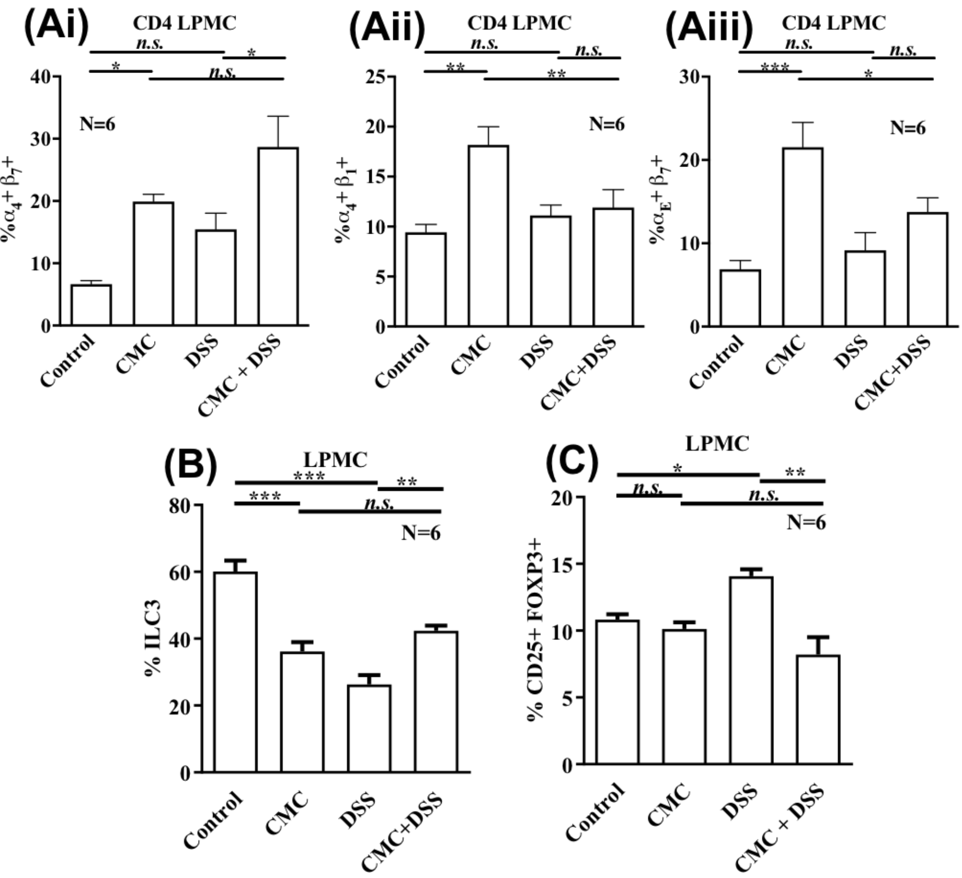

Figure 9. Proportions of CD4+ LPMCs expressing integrin $\alpha_4\beta_7$ (Ai), $\alpha_4\beta_1$ (Aii) or $\alpha_E\beta_7$ (Aiii). B) Proportions of colonic ILC3s. C) Colonic proportions of T_{REG} cells. N=6/group. *P<0.05, **P<0.01, ***P<0.001.

3.5 Discussion

We aimed to elucidate the immune and epithelial barrier changes induced by CMC administration in DSS colitis and healthy controls. Our findings demonstrate that CMC administration alone is not sufficient to induce profound innate or adaptive immune activation or changes to intestinal epithelial permeability. Treatment of WT mice with CMC prior to and during DSS colitis induces a more severe and prolonged disease course, with amplified innate and adaptive immune responses, without changes to intestinal epithelial permeability. This suggests that dietary additives such as CMC may have the potential to provide an environment which promotes intestinal inflammation in human IBD, but not necessarily initiate inflammation in health. Our results support previous studies examining the effects of CMC to the microbiota and intestine, contributing to intestinal inflammation (*1,2*).

CMC treatment induces a more severe DSS colitis course

Despite no robust induction of innate or adaptive immune mechanisms, mice pre-treated with CMC lost more body weight at a faster rate during DSS colitis compared to DSS alone. Just after 3 days of treatment with DSS, CMC-pre-treated mice showed a steep decline in percentage original body weight, which persisted until the completion of the treatment course. Typically, we observe these changes and other symptoms of colitis 3-4 days later in mice treated with DSS only (*6*). This suggests that CMC increases the susceptibility of DSS colitis in mice, supporting previous studies which showed CMC-induced degradation of the intestinal mucus barrier promotes of inflammation.

CMC administration does not alter epithelial barrier properties in control or DSS colitis mice

Although CMC can alter properties of the intestinal mucus layer, leading to increased exposure of the intestinal contents such as the microbiota to the intestine, it is not known how this alters epithelial barrier function. Our findings demonstrate that CMC administration in control or DSS colitis mice does not alter the secretory capacity of the colonic epithelium, including the transcellular movement of Cl^-. Baseline ion transport and secretagogue-provoked Cl^- secretion were not altered by CMC treatment, despite an induction in innate and adaptive immune responses in CMC-treated DSS group relative to DSS colitis alone. However, DSS colitis alone reduced baseline I_{SC} and carbachol-provoked change in I_{SC} relative to health, indicating that DSS treatment induces changes in intestinal epithelial barrier function. These results suggest that DSS colitis leads to a saturated reduction in epithelial barrier permeability, restricting deficits in epithelial Cl^- secretion which may be induced by CMC administration in DSS despite an increase in immune activation. Carbachol-provoked changes in I_{SC} are largely due to activity by Na-K-ATPase and NKCC1. Epithelial sodium channels (ENaC), sodium-hydrogen antiporter 3 (NHE), Na-K-ATPase and NKCC have been shown to be downregulated in colonic biopsies from active IBD patients and in acute TNBS colitis (*9-11*). However, the effect we observed is unlikely to be caused by ENaC and NHE as they were blocked by amiloride in our study. Interestingly, the changes in carbachol-provoked Cl^- secretion were not due to changes in the expression of Na-K-ATPase or NKCC in our study. In fact, administration of CMC in DSS colitis preserved the expression of Na-K-ATPase, relative to DSS colitis alone. Further investigations into the mechanisms of CMC-induced epithelial barrier function in DSS colitis are required as increased Cl^- secretion induces diarrhoea, which is a common symptom of IBD.

Induction of innate and adaptive immune mechanisms in CMC-treated DSS colitis

Our results indicate induction of innate and adaptive immune responses in CMC-treated DSS colitis, relative to DSS colitis alone. This was characterised by striking increases in the secretion of colonic IL-1β and IL-6. Increased secretion of IL-1β and IL-6 may be due to the amplified attraction of macrophages via CXCL1. Interestingly, IL-6 secretion can be increased in response to microbial PAMPs, suggesting an increased translocation of the microbiota or microbial secreted products into the lamina propria (*12*). Colonic IL-13 secretion was also significantly increased, as DSS colitis classically produces a T_H2-like immune response (*13*). LPS stimulated bone marrow cell secretion also revealed polarisation of innate immune responses through the increased secretion of CCL3, CXCL2 and TNF-α in CMC-treated DSS colitis, relative to DSS colitis. This suggests that immature bone marrow cells in CMC-treated DSS colitis are more primed to attract innate immune cells, despite not migrating from the bone marrow microenvironment. Interestingly, CD3/CD28 stimulated MLN cells show enhanced T_H1, T_H2 and T_H17 immune activation through the increased secretion of IL-2, IL-4 and IL-17 in CMC-treated DSS colitis, relative to DSS colitis. DSS colitis has previously been described as a T_H1-T_H17-mediated acute inflammatory response which shifts to a predominant T_H2-mediated inflammatory response in chronic disease, which may explain the mixed T_H responses that we observed (*13*). Alternatively, these mixed immune responses may be due to asynchronous stimulation from CMC-induced breaks in the mucosal barrier, subsequent bacterial translocation into the lamina propria and DSS colitis. CMC-treatment abolished the increase in T_{REG} cells that was seen in DSS colitis perhaps due to the gradual exposure of luminal antigens due to CMC-induced break in mucus barrier.

There was also a greater induction of T helper cells in CMC-treated DSS colitis, shown by the increased migration of $\alpha_4\beta_7$+ T helper cells to the colon. Interestingly, there was also an increase in T helper cell migration to the colon as a result of CMC-treatment, which was not accompanied by an increase in adaptive cytokine secretion. The increase in gut-homing T helper cells in response to CMC treatment in controls may indicate early adaptive immune activation brought on by retinoic acid secretion by activated dendritic cells (*14*). This may occur due to CMC-induced alterations to the mucosal barrier, as shown in previous studies (*1*).

ILC3 populations were significantly decreased in the lamina propria in CMC-treated mice, relative to controls, suggesting a loss of tolerance and dysregulation of intestinal homeostasis. ILC3s are crucial for the maintenance of intestinal homeostasis and oral tolerance, particularly through their secretion of IL-22 which acts on non-hematopoietic cells, including intestinal epithelial cells to maintain crypt stem cells, promote the differentiation of mucus-producing goblet cells as well as stimulating Paneth cells to secrete antimicrobial peptides (*15*). Loss of beneficial ILC3 functions as well as dysregulation of ILC3 mechanisms have been reported in murine and human IBD, which results in an inflammatory phenotype, exacerbating disease (*16*). Interestingly, the loss of ILC3s in the lamina propria was slightly conserved in CMC-treated DSS colitis when compared to DSS colitis alone.

In summary, our results indicate that the commonly used dietary emulsifier CMC exacerbates DSS colitis but does not induce significant immune activation or intestinal damage in healthy controls. This suggests that dietary additives such as CMC may have the potential to provide an environment which promotes intestinal inflammation in human IBD, but not necessarily

initiate inflammation in health. Our results support previous studies examining the effects of CMC to the microbiota and intestine, contributing to intestinal inflammation (*1, 2*). A limitation of this study may be that although Bonferroni corrections were applied within each test, due to the number of tests performed in this study, it may be prudent to adjust the P-value cut off to <0.001 (***) to account for the overall risk of statistical error when comparing between groups. Furthermore, concentrations of emulsifiers used in food products are not reported, therefore a limitation of this study may be the emulsifier dosing amount and timing used which may or may not represent typical human consumption.

In recent decades there has been a steady increase in the consumption of the Westernised diet and food additives such as emulsifiers, many of which were not strenuously tested when approved for use and were given GRAS status. As multiple food additives are frequently and concurrently consumed, further research is required as to whether food additives such as CMC have an additive or synergistic effect. Furthermore, human clinical trials on dietary emulsifiers and other food additives are required to fully understand their physiological effects and impacts on the microbiome.

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Chapter 4 Advances in imaging specific mediators of inflammatory bowel disease: a review

4.1 Statement of authorship

Statement of Authorship

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Overall percentage (%)	80%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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Co-Author Contributions

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

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

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4.2 Abstract

Inflammatory bowel disease (IBD) is characterised by chronic remitting and relapsing inflammation of the lower gastrointestinal tract. The aetiology underlying IBD remains unknown, but it is thought to involve a hypersensitive immune response to environmental antigens, including the microbiota. Diagnosis and monitoring of IBD is heavily reliant on endoscopy, which is invasive and does not provide information regarding specific mediators. This review describes recent developments in imaging of IBD with a focus on positron emission tomography (PET) and single-photon emission computed tomography (SPECT) of inflammatory mediators, and how these developments may be applied to the microbiota.

4.3 Introduction

Inflammatory bowel disease (IBD) is an inflammatory disorder of the gastrointestinal (GI) tract that impacts substantially on quality of life. The causes of inflammation in IBD remain unknown but are thought to involve a hypersensitive immune response to the intestinal microbiota. IBD is commonly associated with dysbiosis, but it remains unknown whether dysbiosis is a cause or consequence of inflammation. Diagnosing and monitoring IBD is heavily reliant on endoscopy, which is an invasive technique that requires bowel preparation and typically, also anaesthesia.

Although generally well tolerated, endoscopy noticeably impacts on patient quality of life and does not provide any direct information regarding the role that specific mediators contribute toward inflammation. Therefore, new technologies are required that are sensitive enough to grade disease severity, are non- or only minimally invasive, and can be optimised for the detection of specific mediators. Recent developments in positron emission tomography (PET), particularly with the use of antibody-conjugated tracers, have demonstrated success in cancers, but have only recently been adapted to IBD. This review summarises the current advances of these technologies in IBD, how they have been used to detect specific mediators of inflammation, and their potential to image microbiota.

4.4 Inflammatory bowel disease

IBD is a collection of debilitating idiopathic diseases characterised by chronic inflammation of the lower gastrointestinal tract that have a remitting and relapsing disease course. The cause(s) of IBD remain unknown; however, they are thought to involve aberrant immune responses to environmental stimuli in people with a complex genetic predisposition. The global prevalence of IBD is estimated to be 0.3% and is widely regarded as increasing (*1*). The relatively recent increase in the incidence of IBD is perplexing and highlights the likely importance of yet-to-be-determined environmental factors in its aetiology. IBD is typically diagnosed in adolescence and early adulthood; however, it can also occur in the very young paediatric population, and approximately 25% of paediatric cases present prior to reaching 10 years of age (*2,3*). It is currently unclear whether the mechanisms underlying IBD differ between the very young paediatric and late adolescent populations. However, it is probable

that yet-to-be-characterised genetic factors feature more prominently in the very young, as the length of time of exposure to environmental factors is unlikely to be long enough to sensitize the developing immune system. IBD is a chronic disease in paediatric and adult populations; however, the paediatric population is particularly vulnerable to the consequences of IBD, including impacts on growth and development, bone health, and psychosocial function and development (*2*).

There are two major subtypes of IBD: Crohn's disease (CD) and ulcerative colitis (UC), which can typically be distinguished by pathological and histological differences. Inflammation in UC is typically restricted to the mucosal layer of the colon and progresses in a contiguous manner. CD differs in that it is characterised by transmural skip lesions that can occur anywhere in the GI tract but are typically located in the ileum with and without involvement of the colon. The initiating factors and inflammatory aspects are likely to differ between CD and UC given the distinctions between their pathology but are only poorly understood relative to what is known regarding how inflammation is perpetuated. This increased understanding has led to the relatively recent development of biologic drugs targeting specific immune mechanisms, most prominently the cytokines TNF-α, interleukin (IL)-12 and -23, and integrins involved in the migration of immune cells into the GI tract, including $\alpha_4\beta_7$ (*4*). These treatments and other nonspecific immune suppressants can be beneficial in IBD; however, treatment gaps remain due to intolerance, incomplete efficacy, and side effects of current therapies, leading to a disease course characterised by variable periods of remission and relapse. Prognostic indicators of relapse in IBD are only poor, and therefore patients are required to undertake constant surveillance, typically via endoscopy. Severe persistent or relapsing symptoms also lead to fibrosis and stricture in >30% of CD and approximately 5% of UC patients (*5*).

Intestinal fibrosis and stricture occurs as a result of excessive deposition of extracellular matrix proteins that accumulate following tissue remodeling and lead to serious complications, including obstructions (6). No drugs are currently clinically available to treat fibrosis and stricture, and therefore these cases often lead to surgery, which can be curative for UC (withstanding complications), but is not curative for CD (*7*). This high rate of surgery highlights the ongoing need for a deeper understanding of the mechanisms involved in initiating and perpetuating inflammation in IBD.

4.5 Imaging IBD: current approaches

Treatments for IBD depend on clinical severity, which is typically assessed by endoscopy. The gastrointestinal mucosa is directly imaged by endoscopy for assessment of disease stage and monitoring, and when forceps are attached, which allows for the collection of biopsy material for analysis of pathology and mechanistic studies. Endoscopy can differentiate between UC and CD in the majority of cases (*8*). However, endoscopic approaches have limitations, as the quality of results is operator-dependent, and it may not be sensitive enough for assessment of clinical severity in some cases. Furthermore, endoscopic imaging is restricted to the superficial mucosal layers of the intestine and therefore provides no information regarding inflammatory damage to the deeper layers of the intestinal wall, the degree of muscle thickness, or the diameter of the lumen. Finally, a major limitation of endoscopy is the difficulty in reaching the small intestine, due to the distance needed to be covered and the complexities of intestinal anatomy with its multiple loops and folds. These limitations are particularly relevant to the paediatric population, who are sensitive to perforation of the intestinal wall and risks associated with sedation (*9*).

A number of novel imaging technologies are emerging that complement or may even replace endoscopy as the gold standard for clinical diagnosis, as recently reviewed in detail (*10,11*). Chromoendography involves the application of dyes onto the mucosa, improving endoscopic characterization of lesions and neoplasia, and has the potential to correlate with histological damage, although the latter remains controversial (*12*). Confocal laser endomicroscopy and endocytoscopy combine high-resolution microscopic imaging with endoscopy, and again, are primarily used to detect colonic dysplasia and neoplasia (*13*). Wireless capsule technologies may circumvent the limitations that endoscopy has in accessing difficult-to-reach areas of the small intestine. While these capsules provide high-resolution imaging, they have similar limitations to standard endoscopy regarding assessment of inflammatory damage to the deeper layers of the colon wall and may also require surgery for removal when stricture occurs (*14,15*).

Less invasive imaging technologies include barium X-rays, ultrasound, magnetic resonance imaging (MRI), computed tomography (CT), PET, and single photon emission computed tomography (SPECT). Barium X-rays provide excellent visualisation of the bowel and can reveal thickening of the bowel wall, but are not recommended in patients with severe inflammation due to the risk of complications such as toxic megacolon, and while they have been traditionally used to assess stricture location and severity, this is largely being replaced by CT and MRI (*2*). Ultrasound, MRI, CT, PET, and SPECT are minimally invasive relative to endoscopy and offer the additional benefits of visualizing planes through the colon wall and extraintestinal manifestations. Bowel ultrasound has some attractions as a non-invasive diagnostic tool for IBD, including relative inexpensiveness, high accuracy in CD, and ease of use.

It has recently been used to reveal ulcer location and severity in adult CD, and ultrasound and MRI are currently the preferred tools for assessment of paediatric perianal abscesses and fistulas (*16,17,18*). However, ultrasound is highly operator-dependent and its accuracy is sensitive to anatomical location, as it is reduced for detecting ulcers in the jejunum and rectum (*17,18*). MRI is currently the imaging technique of choice for follow-up of CD patients due to its lack of ionising radiation. Notably, MRI can differentiate between normal and pathological sections of the bowel by analysis of changes in bowel wall thickness (*19*). Recent developments with MRI and CT include using them to assess fibrosis and stricture. Gadolinium-contrasted MRI has recently been shown to reliably predict severe fibrosis in CD and, interestingly, also revealed that areas of fibrosis often coexist with inflammation within the same intestinal segment (*20*). A recent comprehensive review of the use of PET/SPECT for IBD diagnosis, prognosis, and follow-up indicated that 2-deoxy-2-(^{18}F)fluoro-D-glucose (^{18}F-FDG) coupled with CT has the highest accuracy for detecting inflammation in IBD (*11*). ^{18}F-FDG is a radiolabelled glucose analogue that detects tissue glucose metabolism and has been the tracer of choice for PET studies for decades; however, radiolabelled leukocytes are also typically used to assess GI inflammation. Indeed, SPECT of technetium-99m (^{99m}Tc)-leukocytes was shown nearly two decades ago to accurately assess the extent and severity of disease for people with severe UC, correlating with endoscopic and histological findings (*21*). However, limitations with radiolabelled leukocytes exist, including a relatively extensive protocol required for working up the cells, risks of cross-contamination, and importantly, the nonspecific nature of the tracer. Only limited information exists regarding the use of PET, SPECT, or CT for paediatric IBD, although the combination of ^{18}F-FDG PET/CT has been suggested to be the most reliable due to its improved anatomical mapping (*22*). Importantly, ^{18}F-FDG dosing and imaging protocols are yet to be standardised for PET of paediatric patients, highlighting the need for more studies in this area.

Nonetheless, the potential benefits of PET/CT in the paediatric IBD population were highlighted in the first published case, where scans in a 12-year-old child revealed diffuse heterologous uptake of ^{18}F-FDG in the gut wall, particularly in the stomach (*23*). CT confirmed that the uptake was focal, and endoscopy revealed ulceration of the gastric wall, with an ensuing diagnosis of gastric and intestinal CD. It is important to note that while these technological advances are welcome for IBD management, none of them currently target specific mediators. This highlights the need for the development of new imaging technologies that are highly sensitive, quantitative, and able to provide longitudinal data in real time, but are also only minimally invasive.

4.6 Imaging inflammatory mechanisms in IBD: which targets to choose?

4.6.1 Immune targets

Inflammation is central to IBD; however, much remains to be understood regarding the causes of inflammation in humans. It is currently thought that inflammation in IBD is driven by a loss of immune tolerance to autologous proteins in the colon wall and foreign antigens in the lumen, including the microbiota. This initiates a cascade of cytokines and chemokines which cause an influx of immune cells, as comprehensively reviewed elsewhere (*24,25*). Immune cells are attracted to sites of inflammation by chemokines and also by upregulated expression of “gut-homing” integrins on the cell surface, including $\alpha_4\beta_7$ (*26*). Subsequent inflammatory lesions and damage to the epithelial wall and deeper layers of intestinal tissue result in the production of damage-related mediators during active disease, which progresses

toward mediators related to tissue-remodeling and wound healing as disease improves toward remission, but also toward mediators associated with fibrosis after repeated or extended periods of inflammation. The immunological basis for the differences in pathophysiology of UC and CD are not clear, and while it is suggested that CD is driven by a T_H1/T_H17-type immune response and UC is mediated by a T_H2-type immune response (↑ IL-5, IL-13) that is atypical as IL-4 is not involved, much of the evidence for this generalization results from animal models. Furthermore, while there is evidence supporting a genetic predisposition, large-cohort IBD human studies indicate this is clearly complicated and contains loci that coexist in both CD and UC, but also other loci that are not shared (*27,28*). Environmental factors are thought to contribute to IBD; however, evidence for or against these are heavily biased toward animal studies and are yet to be definitively determined in humans. The best evidence supporting an aggravating role for specific immune mediators in IBD is the success of several biologic medications targeting the cytokines TNF-α and IL-12/23 and the gut-homing integrin $\alpha_4\beta_7$. This success is tempered by the lack of efficacy of therapies directed against IL-17, IFN-γ, and IL-13, amongst others (*29*). However, as failure of biologics in the clinic can be related more to toxicity and side effects than to a lack of involvement of their target, tracers directed to these cytokines may still prove to be valuable for imaging. With this in mind, new immune modulators are currently undergoing clinical trials, including those targeting specific cytokines such as IL-6, inhibitors of intracellular pathways including Janus kinases (JAK), and mediators related to tissue remodeling and fibrosis, such as matrix metalloproteases (MMP) (*29*).

Recent advances in two-photon laser scanning microscopy have enabled *in vivo* imaging of deep visceral tissues and immune cells in particular, indicative of the potential for this

technique to provide preclinical evidence prior to relatively expensive immuno-PET studies. Two-photon laser scanning microscopy was instrumental in identifying goblet cell-associated passage (GAPs) as the major mechanism for delivering luminal antigens to dendritic cells in the small intestine in health (*30*). Interestingly, GAPs only appear in the colon following the disruption of microbe-sensing pathways, highlighting a potential role in IBD (*31*). We have recently applied two-photon laser scanning microscopy to investigate immune cell migration in the trinitro benzene sulphonic acid (TNBS) model of colitis, demonstrating *in vivo* that not only are immune cells in very close apposition to nerves within the colon wall, but also that myeloid cell infiltration is increased in TNBS colitis (*32*). This technique can be easily combined with reporter mice and promises to increase the understanding of the mechanisms involved in immune cell migration from blood vessels into the lower GI tract in IBD.

4.6.2 Microbiota

Recent improvements in high-throughput "omics" technology have also revealed the involvement of the microbiome in IBD. The general consensus indicates that active IBD is characterised by an overall loss of diversity coupled with a dysbiotic phenotype primarily depicted by elevated Proteobacteria and decreased Firmicutes, although inconsistencies between studies remain (*33,34,35*). Functionally, the major classes of bacteria affected relate to the production of short-chain fatty acids (e.g., *Faecalibacerium prausnitzii*), bacteria with mucolytic activity (e.g., *Ruminococcous torques*), and sulfate-reducing species including *Desulfovibrio* (*36,37,38*). While this dysbiotic composition may contribute to disease progression through dysregulated epithelial barrier function and aberrant immune

signalling, it is currently contentious whether dysbiosis itself causes IBD or simply reflects the disease course (*39*).

Uncovering the compositional shifts in the microbiome has undoubtedly increased our understanding of its role in disease initiation, progression, and relapse. However, techniques such as 16S pyrosequencing are inherently limited by the variations that exist in aspects of their methodology, including inconsistent or incomplete reference libraries, the unknown impact of batched analyses, non-standardised statistical analyses, and an inability to adequately appreciate the heterogeneity in the microbiome (*40*). This is further confounded by the fact that the annotation is based on putative association of the 16S rRNA gene, with a taxon defined as an operational taxonomic unit (OTU) (*41,42*). Although whole-genome shotgun sequencing has enhanced our ability to define species taxa with more accuracy, it remains an expensive alternative to 16S and requires significantly greater data analysis (*40*). As such, conclusions regarding the functional implications of compositional shifts remain difficult to draw, relying on metagenomic inference using phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt). Nonetheless, changes in microbiota composition offer the intriguing potential to image microbiota as markers of disease severity in IBD.

4.7 PET and SPECT of specific mediators in IBD

PET of radioisotope-labelled antibodies is currently being used in multiple clinical trials in cancer to tailor personalised therapies based on specifically targeted diagnostic tests (*43,44*). However, immuno-PET of IBD is currently limited to preclinical studies. When deciding which

inflammatory mediators to image in IBD, it is firstly important to understand which immune processes are likely to be involved in the model of choice, and secondly to understand what the information will provide regarding the initiating, perpetuating, and resolving phases of colitis.

4.8 Imaging colitis in animal models

Animal models of colitis have significantly improved our understanding of how inflammation develops and perpetuates, highlighting roles for both the innate and adaptive arms of the immune response. There are currently numerous animal models of IBD, including colitis that develops due to primary genetic defects, adoptive transfer of activated immune cells, or is chemically induced (*45*). Importantly, most of these models progress directly from acute through to fulminant colitis and therefore are not useful for investigating pathways involved in the resolution of inflammation and tissue remodeling. Nuclear imaging of colitis has so far relied upon two models of chemically induced colitis: the TNBS model and the dextran sulfate sodium (DSS) model. TNBS is administered by enema and inflammation is therefore restricted to the colon. TNBS is a hapten; it is not directly toxic, but instead binds to autologous proteins and luminal microbiota, turning them immunogenic. The colitis that develops is transmural, involves an IL-12-driven T_H1/T_H17-type response, and is therefore considered to model aspects of inflammation associated with CD (*46*). DSS differs as it is directly toxic to epithelial cells and, when administered by the oral route in drinking water, results in a colitis that takes longer to develop than TNBS. DSS colitis is considered to model aspects of inflammation associated with UC as the colitis results from direct damage to the epithelial layer and is not as deep as that which occurs with the TNBS model. While animal models are useful for

emphasizing the importance of specific mediators and mechanisms, it is important to note that findings from animal models can be difficult to translate to human IBD. This is common with most chronic diseases that have a remitting and relapsing course due to the involvement of multiple and overlapping pathways that are intertwined with disease stage as inflammation progresses and regresses, highlighting the requirement for extensive validation in human subjects.

4.9 PET and SPECT of immune mediators in colitis

Cell-secreted immune mediators are generally classified as chemokines or cytokines, with chemokines mediating cell attraction and cytokines mediating immune responses. Table 1 provides an overview of PET/SPECT studies of immune mediators in human IBD and animal models of colitis. The first study investigating whether radiolabelled antibodies are efficacious for imaging colitis was disappointing, as uptake of ^{111}In-labelled IgG was substantially lower than ^{111}In-labelled leukocytes, the gold standard at the time, and also the newly developed ^{111}In-labelled liposomes in TNBS-colitic rabbits (*47*). While imaging of liposomes and leukocytes enabled grading of inflammation, the contrast provided from IgG labelling was not sufficient for this to occur. This result was not surprising given the large abundance of IgG in circulating blood and its relative non-selectivity for colitis. Interleukin (IL)-8, more recently renamed CXCL8, is a chemokine produced by innate immune cells, but also cells in other tissues, including epithelial cells and endothelial cells. IL-8 is primarily involved in attracting neutrophils to inflammatory sites, but also attracts other granulocytes, including mast cells. Colonic inflammation in TNBS-treated rabbits was detected more readily with ^{99m}Tc-labelled IL-8 than ^{99m}Tc-labelled granulocytes (*48*). Furthermore, the severity of inflammation was also

able to be graded with ^{99m}Tc-IL-8, but not with ^{99m}Tc-granulocytes. More recently, IL-8 was labelled with ^{99m}Tc for SPECT studies and was found to have higher sensitivity for detecting inflammation than endoscopy in a large clinical study of people with IBD, although specificity was lower than for endoscopy (*49*).

Table 1. Summary of PET/SPECT of human IBD and animal models of colitis. - Not suitable for colitis imaging, + satisfactory image quality, ++ excellent image quality.

Target	Tracers	Species	Model	Outcome	Reference
Leukocytes	^{99m}Tc-HMPAO-leukocytes	Humans	UC	+	(21)
CXCL8	^{99m}Tc-CXCL8	Humans	CD and UC	+	(49)
β_7	^{64}Cu-FIB504.64-Fab	Mice	DSS	+	(53)
$\alpha_4\beta_7$	^{64}Cu-DATK32			+	
β_7	^{64}Cu-FIB504.64-Fab	Mice	DSS	+	(54)
β_7	^{64}Cu-FIB504.64-F(ab')$_2$ (fragments)			++	
CD4	^{89}Zr-GK1.5 cys-diabody	Mice	DSS	++	(55)
TNF-α	^{99m}Tc-Infliximab	Rats	TNBS	+	(52)
IgG	^{111}In-IgG			-	
Leukocytes	^{111}In-WBC	Rabbits	TNBS	++	(47)
Liposomes	^{111}In-liposomes			+	
IL-8	^{99m}Tc-HYNIC-IL-8	Rabbits	TNBS	++	(48)
Granulocytes	^{99m}Tc-HMPAO-Granulocytes			+	

Abbreviations: UC: ulcerative colitis. CD: Crohn's disease. DSS: dextran sulphate sodium. TNBS: tri-nitro benzene sulphonic acid. WBC: white blood cell. Tc: technetium.

TNF-α is a cytokine that is secreted by a range of immune cells from both the innate and adaptive arms of the immune response and is produced in large amounts in many autoimmune diseases, including IBD. Neutralising TNF-α with antibodies, for example infliximab, has proven success in treating IBD (*50,51*). In preclinical studies, ^{99m}Tc-labelled infliximab was able to discriminate between moderate and severe colonic inflammation in TNBS-treated rats (*52*). However, enthusiasm for infliximab as a tracer in preclinical studies was hampered by high background uptake in healthy control rats. This study is of importance as it not only revealed TNF-α mediated inflammation, but it also demonstrated target specificity of a clinically useful drug for IBD. This is also particularly significant given the increased development of biologics, particularly antibody-based therapies, but also the relatively recent expansion of personalised medicine and theragnostic approaches toward treating disease.

4.10 Imaging immune cells in colitis

The DSS colitis model is the most commonly used model to image immune cells in colitis. Table 1 provides an overview of PET/SPECT studies of immune cells in human IBD and animal models of colitis. The integrins α_4 and β_7 form a heterodimer ($\alpha_4\beta_7$) which specifically directs the migration, or homing, of immune cells to the gastrointestinal tract via interactions with its ligand, the addressin MAdDCAM-1 (*26*). Targeting this pathway has proved successful in IBD with the recent development of vedolizumab, a monoclonal antibody that blocks $\alpha_4\beta_7$ (56). Uptake of ^{64}Cu-labelled β_7 antibodies is increased in DSS-colitic mice relative to healthy controls and also to ^{64}Cu-labelled IgG isotype negative control, indicating the potential for β_7 as a tracer for human IBD (*53*). However, high levels of background were also observed in nontarget organs including the small intestine and stomach, prompting the development of antibody fragments. In the same model, uptake of ^{64}Cu-labelled β_7 antibody fragments was superior to both whole ^{64}Cu-labelled β_7 antibodies and ^{64}Cu-labelled $\alpha_4\beta_7$, suggesting that antibody fragments may provide enhanced signalling of inflammation in IBD compared to whole antibodies (*54*). The concept of increased sensitivity with small antibodies has recently been validated in cancer biology and in inflammation. ^{18}F-labelled antibody fragments directed against the antigen-presenting complex major histocompatibility complex (MHC)II were much more sensitive than standard ^{18}F-FDG in detecting xenoplanted tumours, even detecting tumours when growth was neither palpable nor visible (*57*). Furthermore, ^{18}F-labelled antibody fragments directed against CD11b detected complete Freund's adjuvant (CFA)-induced inflammation in the mouse paw much earlier than standard ^{18}F-FDG (*57*). Most recently, ^{89}Zr-labelled antibody fragments against the T_{HELPER} marker CD4 were observed to

have higher uptake in the colon of DSS mice compared to healthy controls (*55*). However, uptake was also increased in the spleen and lymph nodes, highlighting the intrinsic difficulties in imaging immune cells during inflammation, as they generally migrate to and from inflammatory sites and lymph nodes, incorporating blood vessels and lymphatics

4.11 Imaging the microbiota in colitis

As our understanding of microbiota–host interactions grows, it is necessary to move beyond current genomics technology and compositional descriptions. This remains a daunting task when imaging specific microbiota in the gut, as it is limited by the diversity of tissue types that need to be maintained, which is of particular importance when considering the sensitivity of the mucous layer to standard fixation methods and the heterogeneity within gut microenvironments. As such, it is critical that a robust protocol including mucous-preserving sample preparation, image segmentation, and quantitative analysis tools be developed. A framework satisfying these criteria was recently developed by Earle et al. (*58*), where high-resolution quantification of the spatial organisation of the gut microbiome revealed that changes in the proximity of microbes to the epithelium are sufficient to induce increased expression of key inflammatory markers, despite negligible shifts in microbiota composition. Most recently, chemical imaging techniques exploiting a blinking surface enhanced Raman spectroscopy (SERS) have been used to visualise bacteria *in vitro* with exquisite resolution, and have demonstrated the ability to differentiate between the chemical signatures of different bacteria (*59*). MRI, CT, fluorescence/bioluminescence imaging, and PET have also been applied to investigate the activity of bacteria *in vivo*; however, these typically only provide indirect information about bacterial activity by inference from immune function (*60,61*).

Recent advances exploiting the bacterial uptake of carbohydrates have proven successful for the selective *in vivo* imaging of bacteria independently of host factors and secondary pathologies (*61,62*). Furthermore, specific bacteria have also been labelled with iron oxide nanoparticles for MRI to track bacteria longitudinally during infection (*60*). These developments highlight the potential to image specific bacteria during inflammation but are yet to be applied to IBD.

4.12 Limitations of radiolabelled antibodies

Despite the evidence outlined above supporting the potential use of radiolabelled antibodies or immune mediators for *in vivo* diagnostic imaging, several issues remain that currently limit enthusiasm for widespread use. The primary limitation is economy-based, as specialised equipment is required for the manufacture and detection of radiolabels. These costs are high, but may be mitigated by improved clinical outcomes, including early detection, and the potential development of personalised medicines to improve clinical responses and reduce side effects. Scientific challenges also exist, particularly with relation to the relatively long half-life of antibodies and the time required for them to move through the circulatory system to the site of inflammation and clear after studies have been performed. These issues relate to the increased risk of cancer due to radiation exposure, particularly as IBD patients already have a higher risk of colon cancer. However, PET is considered safe and the effective dose of whole-body ^{18}F-FDG was approximately 7 mSv, much less that the 16 mSv used for a routine abdominal–pelvic CT with contrast (*63,64*). Notable efforts are underway to reduce circulation time by using pretargeted methods, as explained in more detail below. The relatively large structure of antibodies may lead to difficulties in penetration of the site of

inflammation, although this may be mitigated by altering the structure of the antibody, also explained in more detail below. A limitation also relates to the single mode of PET detection, as only a single mediator can be imaged. This is problematic in terms of detailed studies of immune cell subsets, as they are typically identified by flow cytometry techniques requiring multiple targets. Furthermore, mediators must be accessible to antibodies and are therefore restricted to soluble cytokines and chemokines or markers located on the outer surface of the cell membrane. While intracellular cytoplasmic cytokines and nuclear transcription factors such as FOXP3 (T_{REG}) and ROR-γ (T_H17) are increasingly being used to characterise immune cell subsets, antibody penetration inside immune cells typically requires cell permeabilization, which is not possible *in vivo*.

4.13 Future directions of imaging IBD

Remarkable progress has been achieved in recent years toward understanding the mechanisms underlying IBD. This has resulted in a number of specific biologics for treatment, but they are yet to be applied to diagnosis or surveillance. The complex immunological mechanisms of IBD indicate that there are multiple markers that could potentially be used for diagnostic imaging and drug bioavailability. Imaging specific sites of inflammation should be achievable relatively easily; however, determining markers that differentiate between IBD and other gastrointestinal diseases, such as cancer, is more problematic and requires extensive preclinical validation.

Intact antibodies are increasingly being used as therapeutics; however, their utility as imaging agents is reduced by their extended half-life, as the duration of time required to reach a sufficient signal-to-noise ratio is long.

Furthermore, extended half-lives may also lead to other harmful biological activity and side effects, potentially altering the biological function being imaged; however, in theory, this risk is minor as concentrations are typically much lower for imaging than for treatment. Recent developments in antibody engineering include a shift from whole IgG antibodies to fragments, enhancing their physical properties and leading to enhanced efficacy as extensively reviewed in (*65*). Enzymatic cleavage or restructuring of the antibody attenuates problems associated with half-life by decreasing clearance times. Furthermore, decreasing the amount of time required to reach a suitable signal also permits the use of short-lived isotopes, reducing radiation exposure.

Pretargeting strategies have also emerged as a means of improving efficacy whilst reducing radiation dose; for example, by using a novel biorthogonal inverse electron demand Diels–Alder reaction between a ^{64}Cu-labelled radioligand and a site-specific immunoconjugate (*66*). This occurs in four steps: (a) injection of the modified antibody, (b) accumulation of the antibody at its target site and clearance from the blood, (c) injection of the radioligand 24 h later, and (d) the *in vivo* click ligation between the immunoconjugate and radioligand and subsequent excretion of excess radioligand. The target was able to be delineated four hours post-injection of the radioligand in tumour tissue, with image contrast improved over time and an ideal signal-to-noise ratio at 24 h. This pretargeting approach and similar approaches may expedite the clinical translation of future immunoconjugates for imaging.

A tantalizing approach is the potential to image different bacterial species; however, this is again currently limited by the lack of knowledge of functional roles for specific microbiota in IBD and the limited ability of PET to simultaneously label multiple targets. Additionally, it may be useful to perform simultaneous PET-MR which would combine the strengths and

weaknesses of each technique. MRI biomarkers of intestinal inflammation can include anatomical signs but also intestinal motility indices, small bowel water content and whole gut transit time (*67*). Cine sequence MRI can be used to identify bowel segments with abnormal motility, such as increased or decreased peristaltic activity which may indicate early signs of inflammation such as in CD (*67*). Furthermore, imaging of microbiota in general currently requires models where the bacteria can be isolated, labelled, and readministered. Future developments including the ability to use multiple labels, but also advances in the identification of bacterial species of interest and anaerobic culturing of microbiota, would be a welcome addition to preclinical models of IBD in the first instance before clinical studies are warranted. Furthermore, the potential to exploit factors that are selectively produced by bacteria and differentiate between active and quiescent states may provide intriguing insights. Bacterial toxins may be the ideal sensor as they are typically only secreted during disease, are readily identifiable by antibodies, and are relatively easily attached to various detection technologies. However, biosensing technology remains limited in its ability to provide insight into the spatial organisation of the microbiome, and as such, the ideal microbial analysis tool remains elusive and current investigations must combine genomic, visual, and activity-based measures.

4.14 Summary

Imaging has a critical role in the diagnosis and management of IBD patients, where endoscopic studies are currently the gold standard. However, there is a clear need for new technologies that are less invasive than endoscopy, but more penetrative at both the local level within the intestinal wall and more broadly along the entire intestinal length. These factors are most relevant for paediatrics IBD, where risks of adverse outcomes are increased, and also in small intestinal inflammation and fibrosis, which is generally not accessible by endoscopy.

Animal studies indicate that PET and SPECT of immune cells and/or mediators are potentially useful for IBD management and diagnosis. The potential targets for PET/SPECT of immune mediators and cells in active IBD are numerous; however, options for imaging of microbiota are currently limited by the lack of understanding of the role that specific microbiota contribute toward IBD. Translation of these technologies into human studies in IBD is currently non-existent, yet applicability in other diseases such as cancer is encouraging. Imaging of currently available biologics should be relatively achievable in the short term, while longer-term goals include the development of tracers with deep tissue penetration and short half-lives. Concurrent research into the immune mechanisms underlying IBD should deliver targets that offer insight into disease mechanisms, for example in paediatrics IBD, and can differentiate between diseases and/or disease states, for example between active inflammation and fibrosis in UC and CD. In conclusion, the future for the development of imaging techniques in IBD is bright, and further refinement will clearly lead to benefits in the diagnosis and management of IBD patients.

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Chapter 5 Immuno-PET of innate immune markers CD11b and IL-1β detect inflammation in murine colitis.

5.1 Statement of authorship

Statement of Authorship

Title of Paper	Immuno-PET of innate immune markers CD11b and IL-1β detect inflammation in murine colitis
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Name of Principal Author (Candidate)	Nicole Dmochowska
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Overall percentage (%)	70
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
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By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
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5.2 Abstract

Inflammatory bowel disease (IBD) is a chronic relapsing and remitting inflammatory disease of the gastrointestinal tract. The diagnosis and monitoring of IBD are reliant on endoscopy, which is invasive and does not provide information on specific mediators. Symptom flare in IBD is associated with increased activation of innate immune pathways. Immuno-PET approaches have previously demonstrated the ability to detect colitis; however, a direct comparison of antibodies targeted to innate immune mediators and cells has not been done. We aimed to compare immuno-PET of antibodies to IL-1β and CD11b against standard ^{18}F-FDG and MRI approaches to detect colonic inflammation. Methods: Colonic concentrations of IL-1β and myeloperoxidase were determined by ELISA, and colonic infiltration by CD11b-positive CD3-negative innate immune cells was determined by flow cytometry and compared between healthy and dextran sodium sulphate–treated colitic mice. PET of ^{89}Zr-α-IL-1β, ^{89}Zr-α-CD11b, and ^{18}F-FDG was compared by volume-of-interest analysis and with MRI by region-of-interest analysis. Imaging results were confirmed by *ex vivo* biodistribution analysis. Results: Colonic inflammation was associated with impaired colonic epithelial barrier permeability, increased colonic IL-1β and myeloperoxidase concentrations, and increased CD11b-positive CD3-negative innate immune cell infiltration into the colon. ^{89}Zr-α-IL-1β and ^{89}Zr-α-CD11b immuno-PET detected colonic inflammation, as did ^{18}F-FDG, and all PET tracers were more sensitive than MRI.

Although ^{18}F-FDG volumes of interest correlated with colitis severity and a strong trend was observed with ^{89}Zr-α-IL-1β, no correlation was observed for ^{89}Zr-α-CD11b or MRI. ^{89}Zr-α-IL-1β was distributed mainly to the gastrointestinal tract, whereas ^{89}Zr-α-CD11b was distributed to more tissue types. Conclusion: Immuno-PET using antibodies directed to innate immune markers detected colonic inflammation, with ^{89}Zr-α-IL-1β providing a more tissue-specific signal than ^{89}Zr-α-CD11b. Development of these technologies for human subjects will potentially provide a less invasive approach than endoscopy for diagnosing and monitoring IBD.

5.3 Introduction

Inflammatory bowel disease (IBD) is a collection of chronic idiopathic inflammatory diseases of the gastrointestinal tract, including Crohn disease and ulcerative colitis (*1*). IBD substantially affects quality of life, and although treatable, significant gaps remain because of incomplete efficacy, lack of tolerance, and side effects, leading to variable periods of remission and relapse (*2*). Patients with IBD require constant surveillance because prognostic indicators of symptom flare remain poor and patients with long-standing disease have an increased risk of developing colon cancer (*2,3*). The diagnosis and management of IBD are currently heavily reliant on endoscopy, which is generally well tolerated but is invasive, is restricted to imaging of the superficial mucosal surface, cannot easily reach all areas of the small intestine, and does not provide immediate information on specific mediators or mechanisms.

Therefore, new technologies are required for imaging IBD that are less invasive than endoscopy and can provide information on specific pathologic processes in real time. Clinical studies support the use of PET, SPECT, and MRI for detecting inflammation in the gastrointestinal tract, satisfying the criteria for reduced invasiveness and real-time information (*4,5*). However, current clinical PET, SPECT, and MRI approaches in IBD are restricted to using radiolabelled autologous leukocytes and ^{18}F-FDG in PET and SPECT and using gadolinium contrast in MRI, none of which provides information on specific mediators. This lack of detail is of increasing importance because of the increase in use of target-specific biologics, particularly antibodies, and the emergence of personalised medicine.

Monoclonal antibodies have exquisite selectivity for their target, and their applicability to immuno-PET has been appreciated for decades, particularly in the cancer field (*6–8*). However, the use of immuno-PET in IBD is currently limited to preclinical studies, in which T-cell infiltration into the colon of colitic animals has been demonstrated either directly by imaging the T$_{HELPER}$ cell marker CD4 (*9*) or indirectly by targeting the gut-homing integrin α4β7 (*10*). In contrast, immuno-PET of immune mediators is yet to be reported; however, direct labelling of the neutrophil-attracting chemokine CXCL8 (also known as IL-8) (*11,12*) and antibody-directed imaging of TNF-α (*13*) have shown some efficacy for SPECT in both colitic animals and human IBD.

Activation of the innate immune system is intimately linked to inflammation in IBD, most obviously illustrated by the clinical utility of detection of the neutrophil-secreted mediators calprotectin and lactoferrin in stool (*3*). IL-1β is an inducible cytokine that has a key role in

innate immune responses and is predominantly secreted by neutrophils, monocytes, macrophages, and dendritic cells, whereas CD11b is a pan-myeloid innate immune marker predominantly expressed on these same cell types. Here, we aimed to compare the ability of PET of ^{89}Zr-conjugated antibodies against IL-1β and CD11b with the ability of standard (^{18}F-FDG) PET and MRI to detect inflammation in colitic mice.

5.4 Materials and methods

All experiments were approved by the Animal Ethics Committee of the South Australian Health and Medical Research Institute (SAHMRI) and the University of Adelaide.

Mice

Male C57BL/6jSah mice aged 10–14 week (20–30 g) were bred and group-housed in a specific pathogen-free environment at SAHMRI. Animals had *ad libitum* access to food and water. Experiments were conducted with only male mice to eliminate the potential confounding effects of the oestrous cycle. Mice were humanely euthanised via CO_2 inhalation and cervical dislocation to remove tissues.

DSS Colitis Model

Colitis was induced by replacing normal drinking water with 2% (w/v) dextran sodium sulphate (DSS) (molecular mass, 40–50 kDa; Alfa Aesar) for 5 d, followed by an additional 3 d of normal drinking water. Mice were assessed daily for signs of acute colitis, including a decrease in body weight and diarrhoea. Healthy mice were age and weight matched. Colon length was measured on excised colons dissected free from surrounding tissue from the tip of the anus to the distal end of the cecum.

Ussing Chamber Studies

Distal colon segments were mounted into 0.1 cm^2 aperture sliders bathed in 5 mL baths with oxygenated glucose-fortified Ringers solution for Ussing chamber analysis (Physiologic Instruments) as previously described (*14*). Briefly, voltage-clamped tissues were equilibrated for 20 minutes before baseline transepithelial electrical resistance (V cm^2) and short circuit current (mA/cm^2) were averaged over 10 minutes.

Myeloperoxidase Activity

Myeloperoxidase activity in the distal colon was determined as previously described (*15,16*). Briefly, phosphate-buffered saline–washed tissue segments were homogenised in 0.5% hexadecyl-trimethylammonium bromide (Sigma-Aldrich) and reacted with *o*-dianisidine hydrochloride (Sigma-Aldrich). Absorbance at 450 nm was averaged over 5 minutes at 30-s intervals.

Myeloperoxidase activity is expressed as units/mg of tissue, with 1 unit of myeloperoxidase activity defined as the amount of myeloperoxidase required to degrade 1 mmol of peroxide per minute at 25°C.

IL-1β ELISA

Proteins were extracted from the distal colon as previously described (*14,15*). Briefly, colon segments were homogenised for 5 minutes in cell extraction buffer (ThermoFisher Scientific) supplemented with protease inhibitor cocktail (Sigma-Aldrich), 1 mM phenyl-methanesulfonyl fluoride (Sigma-Aldrich), and 50 mM ethylenediaminetetraacetic acid at 1 mL/50 mg of tissue. The lysate was incubated (1 h, 4°C, with rotation), sonicated (2 minutes), and centrifuged (14,000 rpm, 15 minutes, 4°C). Supernatant was collected and stored at -80°C until analysis. IL-1β concentrations were determined by OptEIA IL-1β ELISA (BD Biosciences) as previously described (*14,15*). The limit of sensitivity was less than 4 pg/mL. Concentrations were normalised to total protein concentration as determined by a bicinchoninic acid assay (Abcam) as previously described (*15*).

Flow Cytometry

Colon lamina propria mononuclear cells (LPMCs) were isolated enzymatically, essentially as previously described (*14,15*). Briefly, the colon was excised free of fat and blood vessels, flushed with cold phosphate-buffered saline, roughly chopped, and incubated in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffered Hanks balanced salt solution

supplemented with 1 mM ethylenediaminetetraacetic and 1 mM dithiothreitol (20 minutes, 37°C, gentle shaking). Tissue segments were filtered (100 mM), and the remaining tissue was minced and incubated (20 minutes, 37°C, gentle shaking) in complete RPMI 1640 (Gibco) supplemented with foetal calf serum, GlutaMAX (Life Technologies Corp.), penicillin/streptomycin, collagenase D (1 mg/mL), DNAse1 (0.5 mg/mL), and dispase (3 mg/mL) (all from Sigma-Aldrich) before being strained (40 mM) and resuspended over a 40/80 Percoll gradient and the interphase LPMC layer collected and pelleted for LPMCs. Viability was determined (trypan blue), and only preparations with at least 80% viability were investigated further. F_C-blocked LPMCs ($0.5 \cdot 10^6$) were stained with the antimouse monoclonal antibodies CD45-BV605 (30-F11), CD3-BUV395 (145-2C11), and CD11b-APC- Cy7 (M1/70) (all from BD Biosciences). Per test, 20,000 events were analysed using a FACS-Fortessa (BD Biosciences), and proportions of live (FVS700; BD Biosciences) singlets were determined using FlowJo (Tree Star), as described previously (*14,15*).

Antibody–Chelator Conjugation and Radiolabelling

^{89}Zr-oxalate was produced via proton irradiation of ^{89}Y target on a PETtrace 880 cyclotron (GE Healthcare) and purified on Aleco (Comecer) as previously described (*17*). Monoclonal α-CD11b (M1/ 70) and α-IL-1β (B122) (BD Biosciences) were conjugated to ^{89}Zr with NCS-Bz-DFO (Macrocyclics) linkage as previously described (*18,19*). Competitive blocking assays determined the specificity of ^{89}Zr-conjugated antibodies, whereby $0.5 \cdot 10^6$ healthy mouse splenocytes were incubated with 1.25 mg of ^{89}Zr-labelled α-IL-1β or α-CD11b for 20 minutes at 37°C, in the presence or absence of 50 mg of unlabelled α-IL-1β or α-CD11b added for 20 minutes beforehand.

Cells were pelleted and counted using a Hidex γ-counter for accumulation of ^{89}Zr. All counts were background- and decay-corrected. ^{18}F-FDG (phosphate buffer) was produced by the Molecular Imaging and Therapy Research Unit of SAHMRI on a FASTLab synthesiser (GE Healthcare).

***Ex Vivo* Biodistribution Studies**

Immediately after post-imaging culling, organs of interest were dissected, weighed, and counted for ^{89}Zr accumulation as outlined above for blocking experiments.

Statistical Analysis

Data are expressed as mean 6 SEM in all cases. The significance of results was determined by unpaired t testing except for weight loss data, for which a 2-way ANOVA with Bonferroni post hoc testing was used. The strength and significance of correlations were determined by Pearson analysis. Differences with a P value of less than 0.05 were considered statistically significant.

MRI-PET

A 3 to 5 MBq quantity of ^{89}Zr-α-IL-1β (37.5 mg) and ^{89}Zr-α-CD11b (85 mg) in 100–200 mL of saline was administered intravenously (tail vein) 24 h before imaging, and a 10 to 15 MBq

quantity of ^{18}F-FDG in 100–200 mL of saline was administered intravenously 30 minutes before imaging. Some mice underwent MRI first. The mice were anesthetised with isoflurane (2%), and a T2-weighted rapid- acquisition-with-refocused-echoes (RARE) spin-echo sequence was acquired with a benchtop MRI system (Bruker Icon 1T; Bruker Physik GmbH) (repetition time, 84 ms; echo time, 4,314 ms; number of excitations, 1; RARE factor, 8; field of view, 80x80 mm; slice thickness, 2 mm; scan time, 2 minutes). ITK snap (www.itksnap.org) was used for consequent segmentation of the scans (*20*). PET experiments were performed over 15 minutes using a submillimeter-resolution (0.7 mm) Albira PET-SPECT small-animal scanner (Bruker Biospin GmbH). Manually drawn volumes of interest in the area of interest were applied to analyze average and total radiotracer accumulation using the PMOD imaging suite (PMOD Technologies).

5.5 Results

DSS Colitis Association with Body Weight Loss, Colon Shortening, and Impaired Epithelial Permeability

Body weight was reduced in the DSS-treated group 4 d after treatment began (P<0.01, Figure 1A) and was further reduced by about 10% 8 d after treatment began (P<0.001, Figure 1A). By day 8, DSS-treated mice also had approximately 15% shorter colons (P<0.001, Figure 1B), an approximately 35% reduction in epithelial resistance (P<0.01, Figure 1C), and an approximately 60% reduction in short-circuit current (P<0.01, Figure 1C), indicative of colonic inflammation and damage to the epithelial barrier.

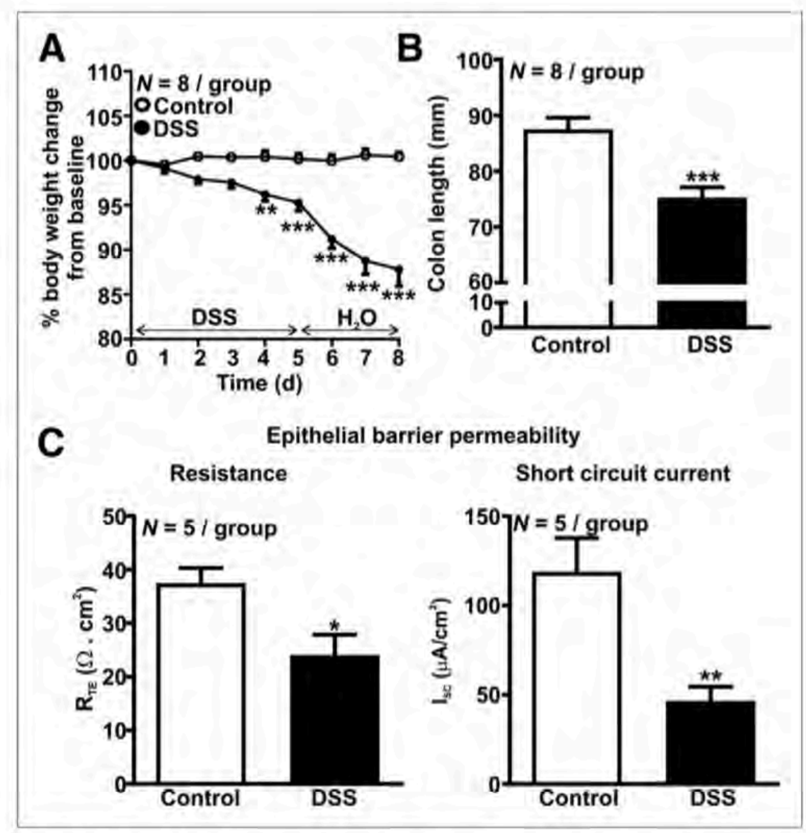

FIGURE 1. Characterization of DSS colitis. (A) Loss of body weight. (B) Shortening of colon length. (C) Impaired epithelial barrier transepithelial electric resistance (left) and short-circuit current (right). $^{*}P < 0.05$. $^{**}P < 0.01$. $^{***}P < 0.001$. R_{TE} = transepithelial electrical resistance.

Increase in Colonic Innate Immune Infiltration in DSS Colitis

DSS-treated mice had an approximately 7.5-fold increase in colonic myeloperoxidase activity (P<0.001, Figure 2A) and an approximately 2.5-fold increase in colonic IL-1β concentration (P<0.01, Figure 2B), indicating that innate immune mediators are increased in colitic mice. The proportion of CD11b colonic LPMCs was increased about 6-fold in DSS-treated mice (P <0.001, Figure 2C), indicative of an infiltration by innate myeloid cells, including neutrophils, monocytes, macrophages, and dendritic cells.

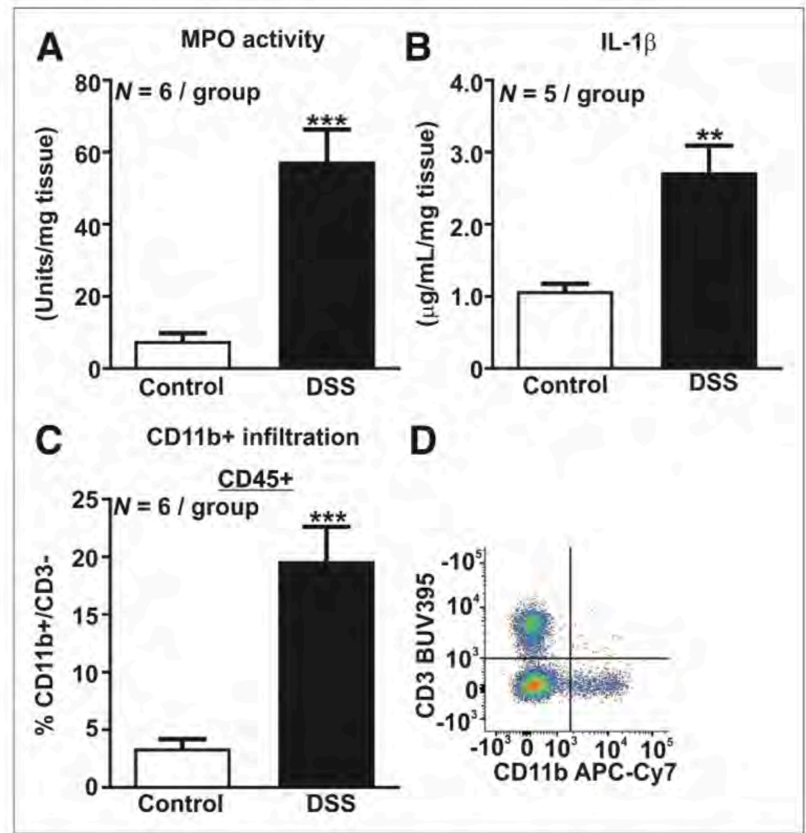

FIGURE 2. Activation of innate immune system by DSS colitis. (A) Increase in colonic myeloperoxidase activity. (B) Increase in IL-1β concentrations. (C) Increase in CD11b-positive CD3-negative innate immune cell infiltration. (D) Gating strategy. **$P < 0.01$. ***$P < 0.001$.

Immuno-PET Detection of Colonic Innate Immune Activation in Colitis

^{89}Zr was successfully conjugated to α-IL-1β and α-CD11b as demonstrated by outcompeting binding in the presence of excess unlabelled antibody (^{89}Zr-α-IL-1β: 8,135 ± 1,510 counts per minutes (cpm) hot vs. 3,026 ± 337 cpm cold, P<0.05, n =3; ^{89}Zr-α-CD11b: 61,535 ± 8,355 cpm hot vs. 34,811 ± 4,081 cpm cold, n=3, P<0.05). PET imaging revealed that in DSS colitic mice, distal colonic uptake of ^{89}Zr-α-IL-1β was increased about 3-fold (P<0.001, Figure 3A, Supplemental Videos 1 and 2; supplemental materials are available at

http://jnm.snmjournals.org), 89Zr-α-CD11b was increased about 5-fold (P<0.05, Figure 3B, Supplemental Videos 3 and 4), and 18F-FDG was increased about 3.5-fold (P<0.01, Figure 3C, Supplemental Videos 5 and 6). A robust positive correlation (R^2 = 0.70, P<0.05) was observed between colonic uptake of 18F-FDG and percentage body weight loss (Figure 3C), with a strong trend toward a similar effect observed for 89Zr-α-IL-1β (R^2 =0.54, P=0.09, Figure 3A) but not for 89Zr-α-CD11b (Figure 3B).

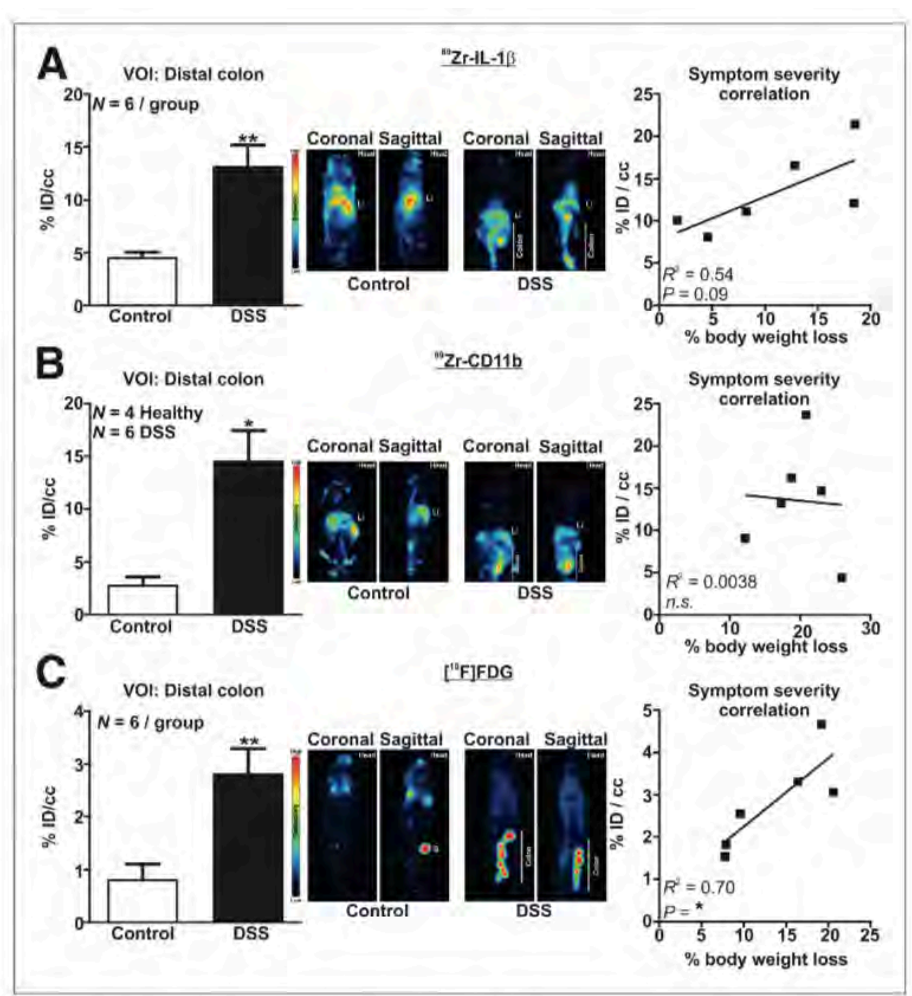

FIGURE 3. α-IL-1β, α-CD11b, and 18F-FDG PET detection of colonic inflammation. Shown are volume-of-interest analysis with representative PET image (coronal and sagittal) of control and DSS mice (left) and correlation of percentage body weight loss with volume of interest (right) for 89Zr-α-1 L-1β (A), 89Zr-α-CD11b (B), and 18F-FDG (C). Li = liver; B = bladder; ID = injected dose; n.s. = not statistically significant; VOI = volume of interest. *P < 0.05. **P < 0.01.

Biodistribution of ^{89}Zr-α-IL-1β and ^{89}Zr-α-CD11b

Ex vivo analysis indicated that uptake of ^{89}Zr-α-IL-1β and ^{89}Zr-α-CD11b was increased throughout the gastrointestinal tract in DSS-treated mice relative to control mice but also in some extragastrointestinal sites. Specifically, uptake of ^{89}Zr-α-IL-1β was increased about 3-fold in the colon (P <0.05), 5-fold in the cecum (P<0.01), 1.5-fold in the small intestine (P<0.05), and 1.75-fold in the stomach (P<0.05), whereas uptake in the spleen was also increased about 1.75-fold (P<0.05) (Figure 4A). Uptake was similar between DSS-treated and control mice in all other tissues investigated. Uptake of ^{89}Zr-α-CD11b was more discrete in the gastrointestinal tract, with increases observed only in the colon (26-fold, P<0.05) and small intestine (20-fold, P<0.05) and a trend toward an increase in the cecum (21-fold, P=0.083) (Figure 4B). However, increased uptake of ^{89}Zr-α-CD11b was observed in more sites outside the gastrointestinal tract than ^{89}Zr-α-IL-1β, including approximately 6-fold in the spleen (P<0.05) but also approximately 7-fold in the liver (P<0.05) and 8-fold in the bone marrow (P<0.05). Notably, trends toward increased uptake of ^{89}Zr-α-CD11b were observed across most other tissues, including the cecum, tail, and stomach; however, uptake was generally more variable for ^{89}Zr-α-CD11b than for ^{89}Zr-α-IL-1β, limiting the significance of these observations.

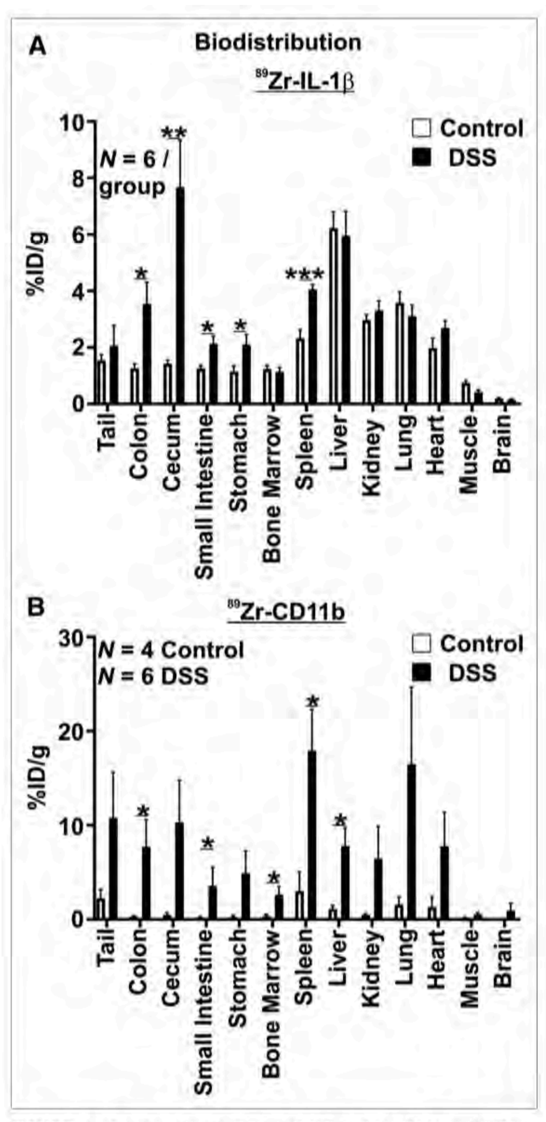

FIGURE 4. Ex vivo biodistribution of ^{89}Zr-α-1 L-1β (A) and ^{89}Zr-α-CD11b (B). Decay-corrected values for uptake are displayed. *$P < 0.05$. **$P < 0.01$. ***$P < 0.001$. ID = injected dose.

MRI Detection of Colonic Inflammation in Colitic Mice

MRI analysis of colonic inflammation revealed an approximately 2-fold increase in the T2 signal intensity ratio in DSS colitis relative to control (P<0.01, Figure 5, Supplemental Videos 7 and 8), but this analysis did not correlate with percentage body weight loss (Figure 5).

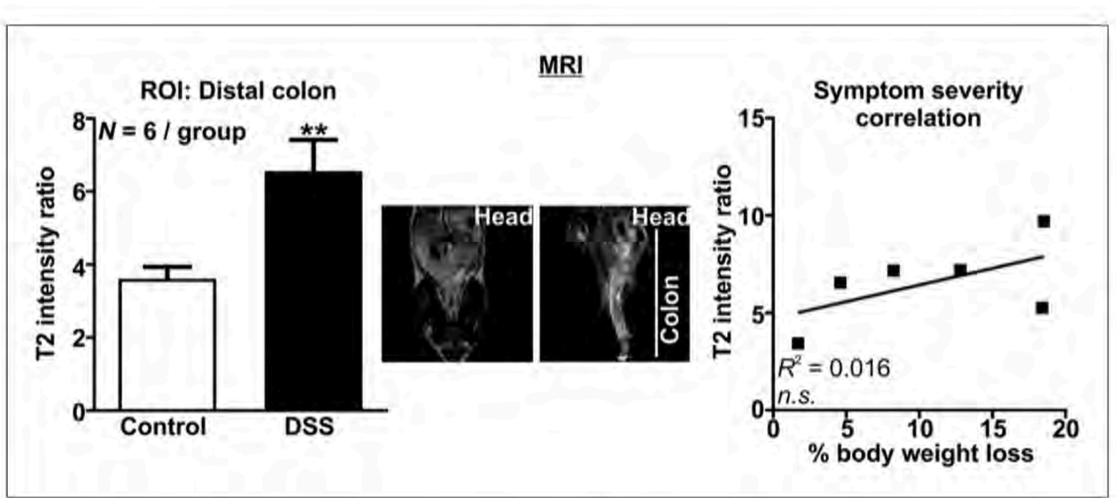

FIGURE 5. MRI detection of colonic inflammation. Region-of-interest analysis with representative MR image (coronal) of control and DSS mice (left) and correlation of percentage body weight loss with region-of-interest MRI analysis (right). $^{**}P < 0.01$. n.s. = not statistically significant; ROI = region of interest.

5.6 Discussion

We demonstrate for the first time, to our knowledge, that immuno-PET directed against innate immune cells and mediators is applicable for detection of colitis. The sensitivity of ^{89}Zr-labelled α-IL-1β and α-CD11b immuno-PET for detecting colonic inflammation was similar to that of ^{18}F-FDG, and all PET approaches were more sensitive than MRI. Furthermore, uptake of ^{89}Zr-α-IL-1β was restricted to the gastrointestinal tract and spleen, whereas ^{89}Zr-α-CD11b was distributed over a broader range of tissue types. Our comparison demonstrates the strong potential that immuno-PET of innate immune mediators has for diagnosing and monitoring IBD.

Activation of the innate immune system is a hallmark of inflammation in IBD both in humans and in animal models (*3,21*). DSS is directly toxic to epithelial cells, and we confirm that epithelial damage in DSS colitis is associated with increased colonic infiltration of CD11b-expressing cells and increased colonic concentrations of innate immune cell–secreted IL-1β (*22*), both of which we were reliably able to detect by PET of ^{89}Zr-conjugated monoclonal antibodies. Previous immuno-PET studies have identified increased infiltration of T cells in DSS colitis, either by directly labelling of the THELPER marker CD4 or by labelling of the gut-homing integrin complex α4β7, which is upregulated on T cells in IBD (*9,10,23*). However, we believe our study to be the first to describe the potential that radiolabelled antibodies against innate immune markers have for PET in IBD diagnosis and monitoring.

We observed a striking difference in the biodistribution of ^{89}Zr-α-IL-1β and ^{89}Zr-α-CD11b, with ^{89}Zr-α-IL-1β being restricted to the gastrointestinal tract and spleen whereas ^{89}Zr-α-CD11b was observed in a broad range of tissues. Furthermore, ^{89}Zr-α-IL-1β also correlated with colitis severity whereas ^{89}Zr-α-CD11b did not. These findings are likely to relate to immunologic processes underlying innate immune activation in colitis. Innate immune cells migrate from induction sites in lymphatic tissue through the bloodstream into sites of inflammation, where they mature and secrete cytokines, including IL-1β, before clearing from tissue by the lymphatics or dying (*24*). CD11b is expressed on innate cell subsets that promote inflammation, such as M1 macrophages and most neutrophil subsets, but also on innate immune cells that promote tolerance, wound healing, and immune suppression, including M2 macrophages, dendritic cells, and CXCL4-expressing neutrophil subsets (*25,26*). These cells express CD11b at all stages of maturity, and imaging CD11b is therefore likely to detect sites of inflammation but also migration between tissues and lymph nodes, including the bone marrow and, particularly in mice, the spleen. Furthermore, whereas CD11b expression is high on myeloid innate immune cells, it is also expressed on other immune cells, including T- and B-cell subsets, which may contribute toward the signal we observed in our imaging (*27*). The expression of individual markers on multiple cell types is common; for example, CD4 is expressed on macrophages in addition to T_{HELPER} cells, and complex flow cytometry using multiple markers is typically required to conclusively characterise individual immune cell subsets. For IBD, this is particularly relevant to the ever expanding T_{HELPER} cell subsets and innate lymphoid cells, which rely on staining of cytoplasmic cytokines or nuclear transcription factors for their characterisation (*28*). Definitively identifying these subsets for *in vivo* imaging is difficult in live cells because antibodies are typically too large to passively enter cells.

In contrast, IL-1β has 2 favourable characteristics for immuno-PET. First, it is inducible rather than constitutive and therefore is secreted only by activated cells, which, in colitis, are predominately located at sites of inflammation. Second, proteolytic processing of IL-1β into its mature form typically occurs within the cell, and it is secreted as a mature functional protein (*29*). This is somewhat an anomaly for cytokines but is beneficial for immuno-PET because only mature IL-1β is imaged. Our results support further investigation into the use of imaging inducible and intracellularly processed immune mediators for detecting inflammation in IBD. Furthermore, immuno-PET using radiolabels with long half-lives, such as ^{89}Zr, enables longitudinal imaging, which would provide valuable information on the temporal nature of immune responses in IBD.

SPECT is a cost-effective technique for nuclear imaging but generally has reduced resolution relative to PET. Nonetheless, SPECT imaging of the neutrophil-attracting chemokine CXCL8, and CD4, has also shown potential in preclinical and human studies of IBD (*11,12,30*). Furthermore, whereas SPECT imaging of the TNF-α–neutralising antibody infliximab also demonstrated some efficacy in preclinical colitis, it was hindered by high background, the cause of which was likely its chimeric mouse–human properties (*13*). The most readily available alternative markers for immuno-PET imaging of IBD include antibodies that are currently used for treatment, including variations targeting TNF-α, α4β7 (vedolizumab), $\alpha 4\beta_1$ (natalizumab), and IL-12/23 (ustekinumab) (*31*). However, markers do not have to be limited to the immune system. Epithelial damage and increased epithelial barrier permeability are hallmarks of IBD and colitis, as we demonstrate in our model (*32,33*). The permeability of the epithelial monolayer to luminal contents is regulated by a large family of tight-junction proteins, many of which are reduced in degraded tissue in the inflamed intestine, impeding

their use as markers. Conversely, expression of some of these proteins promotes increased permeability and is increased during IBD, such as the pore-forming claudin-2 (*34,35*). Other potential markers include those associated with tissue remodeling associated with the resolution of inflammation (*36*). Matrix metalloproteases are involved in tissue remodeling and, interestingly, are thought to play a key role in promoting fibrosis. Although an ant body against matrix metalloprotease 9 was ultimately withdrawn from clinical trials on IBD, interest in this target remains and it may have more promise as a marker of fibrosis (*37*).

The clinical utility of immuno-PET for IBD is currently limited by several issues. Primarily, antibodies are large molecules with molecular weights of about 150 kDa. This large size prolongs their half-life and reduces excretion, which is beneficial when used as treatments but is challenging for imaging because tissue penetration is reduced and excretion prolonged. Issues related to tissue penetration may be somewhat diminished in IBD because vascular permeability is increased in inflammation, aiding immune cell migration into inflammatory sites (*38*). However, increased leukocyte extravasation is unlikely to drastically reduce the time taken for systemically administered antibodies to reach inflammatory sites in IBD, and the current clinical paradigm would require dosing and imaging to occur at separate visits over several days. This requirement results in increased exposure to radiolabels, potentially increasing the risk of side effects. Promising alternatives to intact antibodies that potentially limit these concerns are in development (*39*). Notably, engineered small antibody fragments against anti-CD4 and β7 have already shown promise in preclinical colitis (*9,10*). A different strategy uses a 2-step process for pretargeting. The initial step comprises administration of

an antibody modified to incorporate a radiolabel capture site, which is allowed enough time to localize to its target. A few days later, the radiolabel is injected and binds to the antibody. This strategy has shown promise in cancer (*40*) but has yet to be applied to IBD and may prove difficult because the harsh protease-rich environment in inflammation may rapidly degrade targets.

5.7 Conclusion

We have demonstrated that radiolabelled antibodies against innate immune cells and mediators are suitable for immuno-PET of colitis. Our results provide further evidence of the clinical utility of immuno-PET for IBD diagnosis and monitoring. Development of these techniques would be of particular benefit to patients who are difficult to scope, including the paediatric population and those that have inflamed or fibrotic regions of the small intestine.

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Chapter 6 Mechanisms underpinning intestinal fibrosis in IBD and current and future diagnostic imaging: a review

6.1 Statement of authorship

Statement of Authorship

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Principal Author

Name of Principal Author (Candidate)	Nicole Dmochowska		
Contribution to the Paper	Wrote, drafted and proofread the manuscript		
Overall percentage (%)	70		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	28/10/2019

Co-Author Contributions

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

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

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Contribution to the Paper	Wrote, drafted and proofread the manuscript		
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6.2 Introduction

Intestinal fibrosis is a major common complication of IBD and occurs after extended or recurrent periods of intestinal damage and healing, leading to an excessive deposition of extracellular matrix (ECM). The excessive deposition of ECM is driven by an imbalance between matrix metalloproteinases (MMPs) and tissue inhibitors of matrix metalloproteinases (TIMPs) during periods of tissue repair and the activation of ECM-producing cells (*1*). This increased deposition of ECM and lack of normal tissue restructuring can lead to loss of function of the gastrointestinal tract. Complications of fibrostenotic disease such as fistulae, strictures and abscesses are the main indications for surgery in Crohn's disease (CD). Within 5 years of being diagnosed with CD, 49% of patients are diagnosed with stricturing disease or penetrative complications, this increases to 53%-70% at 10 years post-diagnosis (*2, 3*). The risk of intestinal fibrosis is lower in ulcerative colitis (UC) with 5% of patients developing strictures during the course of their disease (*4*). In patients with CD this leads to thickening of all layers of the gut from the mucosa and muscularis mucosa to the muscularis propria (*5*). This is associated with an infiltration of dense bands of collagen and collections of smooth muscle cells as well as an expansion of the muscularis mucosa (*6*). Thickening of the muscularis mucosae and the increased deposition of collagen in the colon leads to shortening and increased rigidity of the colon (*7*).

In CD, the degree of fibroblast accumulation correlates with the expression of ECM mediators and fibrotic cytokines (*8*). Intestinal fibrotic changes are associated with an increase in fibronectin, tenascin C and total collagen which is predominantly collagen III and V (*9*). Patients with UC or CD can also display fibro-muscular obliteration of the submucosa which usually occurs with thickening of the muscularis propria. Although the formation of intestinal fibrosis can be decreased by inhibiting inflammatory pathways, in conditions such as IBD, regulation of the intestinal inflammatory pathways can be difficult as therapies such as commonly used anti-TNF-α monoclonal antibodies can decrease in efficacy over time. Currently, apart from surgical intervention, there are no therapies to reverse the development of intestinal fibrosis, as such an increased understanding into the mechanisms underpinning intestinal fibrosis is urgently required.

6.3 Clinical impact and significance

The presence of internal penetrating disease in patients with CD is usually accompanied with strictures, with fistulae being highly predictive of the presence of associated strictures (*10*). As a result, it is often thought that strictures precede the development of fistulae and internal penetrating disease. Acute obstruction due to strictures can be removed by surgical resection, but in CD reoccurrence is possible. The formation of strictures seems to correlate with the location of inflammation with strictures most commonly occurring in the terminal ileum or ileocecal area. Currently there are no reliable clinical markers or predictors of intestinal fibrosis. Endoscopic, clinical and environmental risk factors have been identified, but most biomarkers only identified a complex course of CD which is not specific for fibrostenotic disease.

Risk factors for patients currently include under 40 years of age at time of diagnosis, perianal disease and early requirement of treatment with steroids (*11, 12*). The prediction and prevention of intestinal fibrosis and stricturing is crucial for the management of IBD. Studies have attempted to identify serologic and genetic markers that are associated with formation of strictures. Nucleotide-binding oligomerisation domain-containing protein 2 (NOD2) and MMP-3 were genes linked with fibrostenotic disease in CD (*13*). In terms of serological markers, although TIMP-1 is increased in CD mucosa in areas of stricturing disease, there is no correlation between serum TIMP-1 and the fibrostenotic phenotype (*14, 15*). Fibronectin, an ECM protein, is decreased in CD patients relative to controls but is increased in the plasma of CD patients with stricturing disease (*16, 17*). Serum basic fibroblast growth factor (bFGF) activates wound healing and is increased in CD and correlates with thickness of the bowel wall and fibrostenotic disease (*18, 19*).

6.4 Current treatments

The prevention of fibrostenotic disease relies on the effective management and treatment of periods of inflammation and relapse in IBD. There are a few pharmacological therapies available which can slow the rate of fibrosis progression, but there are no therapies to reverse the progression of fibrosis, thus more effective therapies are needed. Immunosuppressive therapies can slow or delay the progression of fibrosis, but surgery and endoscopic balloon dilation still remains the most common therapies for strictures in CD patients (*20*). Patients should be prescribed immunosuppressants such as anti-tumour necrosis factor (TNF) agents to reduce gut wall oedema, which may reduce bowel wall thickness and decrease obstructive symptoms (*20*). The reoccurrence rate after strictureplasty was found to be 23%, 10% of

patients had recurrence requiring reoperation at the previous site of strictureplasty, which may suggest that strictures can be reversible (*21*). Additionally, it has been found that there is no significant difference between recurrence rates following strictureplasty versus resection (*21*).

6.5 Diagnosis of intestinal fibrosis and imaging techniques

Intestinal fibrosis is a common IBD complication, however, patients initially present to the clinic due to an inflammatory phenotype, in the absence of complications such as fistulae or strictures (*5*). Currently, no validated and reliable diagnostic tools exist to detect intestinal fibrosis with a high specificity and sensitivity. Diagnosis of fibrotic strictures is particularly difficult in the setting of CD as inflammation often coexists. Differentiating between inflammation and fibrosis remains the largest challenge in antifibrotic therapies and diagnosis. Additionally, it may be beneficial to differentiate between established fibrosis and active disease (fibrogenesis). Cross-sectional imaging modalities such as magnetic resonance imaging (MRI) and computed tomography (CT) are currently the gold standard in differentiating intestinal inflammation from intestinal fibrosis. This relies on differing anatomical features. For example, on MRI intestinal inflammation in CD is associated with increased T2 hyperintensity, ulcerations and mucosal enhancement, in contrast to intestinal fibrosis which presents as abnormalities in contrast uptake; however, diffusion weighted MRI and magnetisation MRI have also shown potential for the quantification of fibrosis (*13*). In addition, magnetic resonance elastography, MRI-defined motility, T2* mapping and intravoxel incoherent motion DWI may also prove useful for the imaging of fibrosis. Ultrasound provides a non-invasive and radiation-free option to visualise fibrostenotic disease in some cases.

Due to the many overlapping loops of the bowel and the depth of some segments, ultrasound cannot be used to visualise the entire gastrointestinal tract. The terminal ileum is well visualised with ultrasound, in contrast to the proximal ileum and jejunum which usually cannot be visualised. Patient body habitus may also impede ultrasound sensitivity. The diagnostic accuracy of ultrasound is also highly operator-dependant, when compared to MRI or CT. Ultrasound strain elastography is commonly used in fibrostenotic disease and can be used to estimate the stiffness of the tissue, which often correlates with fibrosis histopathological grade (*22*). Shear wave velocity ultrasound (SWE) is another technique used to estimate tissue stiffness through pulse waves generated by the ultrasound probe, in contrast to strain elastography which utilises direct pressure from the probe. In rat models SWE can successfully differentiate between fibrotic and acutely inflamed bowel (*23*). Contrast-enhanced ultrasound enhances the signal from blood cells and vessels, where fibrotic bowel will demonstrate a decreased blood flow and volume. A recent study has identified strain-photoacoustic imaging as a potential modality for characterizing intestinal fibrosis (*24*). Increased haemoglobin and collagen content in fibrotic intestinal strictures were identified using spectroscopic photoacoustic imaging (*25, 26*). Parallel photoacoustic ultrasound is capable of visualising reduced blood content in intestinal strictures during the compression of the abdominal walls (*24*).

Molecular imaging is at the forefront of medical imaging, providing non-invasive, quantitative technologies to visualise disease pathology at a cellular and subcellular level. This type of imaging will allow for the early detection, disease staging, assessment of disease activity and response to treatments. Molecular probes comprise of small molecules, peptides and antibodies that bind to specific mediators/targets involved in the disease pathology of interest.

PET and single-photon emission computed tomography (SPECT) can be highly sensitive, detecting targets within picomolar concentrations and can provide quantification of the target (*27*). However, PET and SPECT also have lower spatial resolution and require radioactive probe production, thus making these options less suitable than non-ionising modalities for long term assessment. In contrast to MRI which has a much higher resolution, no ionising radiation but much smaller range of biological contrasts are available. As such, PET and MRI can be combined to achieve highly sensitive target quantification with high resolution anatomical detail. As fibrogenic pathways share similarities between various fibrotic diseases, probes designed for one organ may also detect fibrosis in others. Currently, there are over 25 molecular probes available for imaging of fibrosis and fibrosis-associated pathways such as vascular leak and immune activation (see *27* for a thorough review). The ideal molecular probe for any disease should target a highly expressed molecular process which is unique to that disease and is in low levels or not present in areas not pathologically involved. Additionally, ideal probes will produce low background signals, which is achieved through low non-specific binding, rapid elimination and low binding to non-target areas. For probes designed for disease staging, uptake should increase linearly with an increase in disease. Somatostatin receptors become upregulated on fibroblasts during fibrosis (*28*). Octresoscan is an FDA-approved somatostatin peptide mimic, radiolabelled with ^{111}In. It can successfully detect idiopathic pulmonary fibrosis, systemic sclerosis, sarcoidosis and idiopathic interstitial pneumonia. Somatostatin analogues such as ^{68}Ga-DOTATATE (NETSPOT) can also detect idiopathic pulmonary fibrosis (*29*). However, proinflammatory M1 macrophages also express type 2 somatostatin receptors, thus may interfere with the true fibrosis signal (*30*). The deposition of ECM is one of the primary targets for the imaging of fibrosis and type 1 collagen is the most abundant of the collagens in the body (*31*).

A peptide-based gadolinium probe (EP-3533) against type 1 collagen can successfully detect myocardial injury after infarction and liver and pulmonary fibrosis in preclinical models using MRI (*32, 33*). To date, no studies have attempted to use type 1 collagen probes to visualise intestinal fibrosis, although type 1 collagen deposition is significantly elevated in the gut during fibrosis. CM-101 is a more stable gadolinium probe, targeting type 1 collagen which has improved pharmacokinetic properties, compared with EP-3533 and has been used to detect liver fibrosis in rodents on MRI (*34*). CM-101 binding is increased in fibrotic animals and correlates well with hydroxyproline content. The same peptide probe used in CM-101 has also been optimised for PET of pulmonary fibrosis (*35*). Elastin has also previously been the target of molecular imaging probes for fibrosis and has been used to image atherosclerosis (*36*). However, elastin may not be a suitable target for intestinal fibrosis as it is often found to be decreased in the fibrotic gut (*37*). Immuno-PET relies on the use of radiolabelled antibodies or antibody fragments which will circulate throughout the body before binding to its target. The utility of this technique has been demonstrated in preclinical studies, imaging bowel inflammation in murine models of IBD, but also clinically in the imaging of various cancers (*38-40*).

6.6 Models of intestinal fibrosis

Advances in this field can be restricted by accessibility to human biopsies and samples, as such, a number of well-characterised animal models of intestinal fibrosis have been developed. These models can be categorised into their modes of induction: spontaneous, genetically engineered, chemical, immune, bacteria, radiation-induced and post-operative (*41, 42*).

6.6.1. Chemically induced models

Trinitrobenzene sulfonic acid

Chemically induced models develop some of the immunological and histopathological features seen in human IBD. These models rely on repeated rounds of chemically induced gastrointestinal inflammation, followed by periods of healing and remission. However, many factors can influence the development of colitis in these models, therefore careful protocol standardisation is required to ensure translational value. Trinitrobenzene sulfonic acid (TNBS) is one of the most common compounds used to induce colitis in mice. Colitis is induced in susceptible strains by rectal administration of TNBS in ethanol. Ethanol is required to break the intestinal barrier, allowing TNBS to access the gut wall and haptenize gut proteins, initiating an immune response to colonic or microbiota-derived proteins, inducing an expansion and activation of CD4+ T cells. Consequently, the TNBS model of colitis is extensively used to investigate the role of CD4+ T cells in gut inflammation. The duration and intensity of TNBS colitis is dependent on multiple factors including, the dose of TNBS, concentration of ethanol, strain of mouse, genetic make-up of mice, time between treatments and the presence of certain T-cell activating microbiota. Typically, TNBS colitis results in T_H1, T_H2 and T_H17 type responses (*43*). Histopathologically, in our experience, TNBS colitis results in much harsher intestinal damage. TNBS colitis classically induces transmural infiltration of macrophages, neutrophils and lymphocytes, loss of crypt architecture, blunted villi, thickening of the colon muscle wall and loss of goblet cells (*44*). Some of the immunological and histopathological features of TNBS colitis resemble CD and as such, is often used to study aspects of this disease.

Dextran Sodium Sulfate

Dextran sodium sulfate (DSS) is dissolved in water and administered orally to induce self-limiting colitis characterised by body weight loss, diarrhoea and bloody stools (*45*). DSS induces loss of epithelial cells, infiltration by neutrophils and ulcer formation, closely resembling some features of flare of UC (*45*). The colitogenic mechanism of DSS is not fully understood, but it thought to be directly toxic to the intestinal epithelial cells of the basal crypts. Breaking the intestinal barrier results in the translocation of luminal antigens into the mucosa and submucosa, causing a rapid and penetrating inflammatory response. As such, repeated DSS treatment can result in the development of intestinal fibrosis. Recent studies suggest that medium-chain fatty acids are required for the induction of DSS colitis and that the extent and severity of colitis is significantly impacted by various microbiota (*46, 47*). Additionally, short-chain fatty acids have been found to be protective in DSS colitis and have been identified as a possible therapeutic for IBD (*48*). DSS colitis incudes a T_H2-like inflammatory response, leading to an increase of TNF-α, IL-6, IL-17 and KC in acute colitis and an increase in IL-4, IL-10 and decrease in TNF-α, IL-6, IL-17 and KC in chronic inflammation (*43*). Severe combined immunodeficiency (SCID) and $Rag^{-/-}$ mice can develop severe DSS colitis, suggesting that colitis can be induced without activation of the adaptive immune system (*49, 50*). As such, the DSS model of colitis is often used to study innate immune mechanisms in colitis. Recently, it has been shown that fibrogenesis in chronic DSS is not influenced by the neutralisation of T_{REG} cells and the depletion of IL-13, IFN-γ and IL-17F (*51*). Inhibition of IL-17A slightly increased colonic collagen deposition without inducing any colonic or systemic inflammation. This may suggest that although increased in chronic DSS, T_H1, T_H2, T_H17 cytokines and T_{REG} cells are not crucial for the chronicity of inflammation and the development of fibrosis (*51*).

Peptidoglycan-polysaccharides

Microbial models of intestinal fibrosis are becoming of greater interest as IBD GWAS studies continue to identify genes responsible for innate immunity, mucosal barrier integrity and bacterial sensing (*52*). Additionally, the microbiome of IBD patients differs from healthy controls. A number of models attempt to alter the microbiome by utilising specific microbiota or microbial components to induce inflammation and intestinal fibrosis. Transmural injection of peptidoglycan-polysaccharides (PG-PS) into the distal colon, cecum or small bowel of genetically susceptible rats results in transmural enterocolitis (*53, 54*). Intramural injection of PG-PS results in an acute phase of inflammation 3 days after injection, followed by a spontaneous reactivation of colitis characterised by immune infiltration by macrophages, neutrophils and lymphocytes into the submucosa and muscularis propria at 3-4 weeks (*54*). Transmural administration of PG-PS into the gastrointestinal tract induces colonic thickening, adhesions, increased colonic permeability and increased myeloperoxidase activity, compared with controls. Granulomatous areas of intestine have an increased expression of collagen-α1 (COL1A1), TGF-β1 and IL-6 (*55*). The areas surrounding the granulomas are fibrotic in nature and have an increased proportion of mesenchymal cells, particularly myofibroblasts. This technique is time-consuming, technically difficult and is limited by its poor reproducibility. Similar to the PG-PS model, intramural injection of rat faecal suspension into the colon bowel wall in rats can also induce intestinal fibrosis (*56*).

Chronic *Salmonella* typhimurium infection

As mentioned in this review, chronic infection with *Salmonella* typhimurium in antibiotic mice can cause colitis and subsequent fibrosis (*57, 58*). Mice pre-treated with streptomycin 24 hours prior to oral administration of *S.* typhimurium leads to transmural inflammation and

mucosal ulcerations, accompanied by increased expression of TGF-β1, connective tissue growth factor, insulin-like growth factor 1 as well as an increased deposition of collagen in the caecal mucosa, submucosa and muscularis mucosa (*58*). Areas of fibrosis also had elevated levels of fibroblasts and myofibroblasts. In this study, fibrosis is evident by 7 days post-infection, peaking at 21 days and persisting until 70 days. This model is easy to use and highly reproducible, as such is used extensively to research intestinal fibrosis.

Chronic adherent-invasive *Escherichia coli* infection

Chronic infection with adherent-invasive *Escherichia coli* (AIEC) leads to chronic inflammation and intestinal fibrosis (*59*). Typically, AIEC dysbiosis is associated with human CD, however their role in CD immunopathology is unclear, due to a lack of suitable experimental models. Mice pre-treated with streptomycin receive oral administration of AIEC, inducing a T_H1 and T_H17-like immune response, resulting in increased levels of colonic IL-17, TNF-α, MCP-1, TGF-β1, connective tissue growth factor, insulin-like growth factor and ECM deposition. Increased ECM deposition was visible as early as 7 days post-infection and was evident in the muscularis mucosa and lamina propria. Fibrosis persisted in the caecal wall of these mice 63 days post-infection. Expression of *Col1a2* and *Col3a1* mRNA was increased in the caeca of AIEC-infected mice, compared with controls. The above changes in cytokines, growth factors and profibrotic mediators shows many similarities with the profiles seen in CD.

6.6.2 Immune-mediated models

T cell adoptive transfer

It is known that T cell abnormalities may indirectly modulate mesenchymal cells to exert profibrotic effects. Accordingly, T cell adoptive transfer may provide an immune-mediated model of intestinal fibrosis. Transfer of CD4+ CD45RBhigh T cells into b-17 SCID mice results in a lethal wasting disease with severe infiltration of mononuclear cells into the colon, and elevated IFN-γ mRNA (*60-62*). In contrast, transfer of CD4+ CD45RBlow or co-transfer of both T cell subsets did not result in colitis (*61*). Transfer of CD4+CD45RBhigh T cells resulted in diffuse inflammation from the caecum to the rectum, which was restricted to the lamina propria, but transmural in a few mice. This colitis resulted in mucin depletion, epithelial hyperplasia and mucosal thickening. The changes to the large intestine mirror those seen in UC and CD patients.

6.6.3 Genetic models

TGF-β1 induction

TGF-β1 is a crucial mediator of fibrosis and mesenchymal cell proliferation, as such induction of the TGF-β1 gene in mice can lead to intestinal fibrosis (*63*). Transfection of spontaneously biologically active TGF-β1 recombinant gene via an adenovirus vector leads to increased TGF-β1 levels in the colon, relative to uninfected mice. TGF-β1 gene transfer induces body weight loss, distension and hardening of the colon, as well as accumulation of faecal matter in the dilated region, indicative of severe fibrosis. Acute inflammation, mucosal ulceration and transmural immune infiltration was visible over the first 3 days post-infection, with inflammation and tissue injury resolving by day 14 post-infection. ECM deposition was seen in the submucosa of transfected mice and the serosa and subserosal region showed thickening in addition to increased cellularity. 14-28 days post-infection, more than 50% of

transfected mice developed colonic dilation and subsequent obstruction. These changes were associated with a thickening of the muscularis mucosa and muscularis externa. Masson's trichome and Verhoeff's elastic staining showed that the presence of fibrosis was not uniform throughout the colon but appeared as focal points, affecting regions 0.5 to 2 cm in length in the distal colon. In some instances, circumferential fibrosis was observed and, in some samples, only part of the tissue cross-section was fibrosed. Despite severe mucosal inflammation in the first week post-infection in transfected mice, at 14-28 days the colonic mucosa appeared similar to uninfected controls. However, the submucosa continued to show signs of fibrosis such as the accumulation of fibroblasts, granulocytes and monocytes and a significant deposition of collagen in the external muscle layers. Increased staining of α-smooth muscle actin (α-SMA) was observed in the heavily fibrotic serosal and subserosal areas of colon of transfected mice, but not in controls. Weak desmin staining within these sections confirmed that these α-SMA positive cells were in fact myofibroblasts and not smooth muscle cells. The TGF-β1 transfection model mimics many features of CD-induced fibrosis, especially the development of bowel obstructions. This model may be useful in the elucidation of mechanisms leading to intestinal fibrosis in CD.

IL-10 deficiency

IL-10 is a well characterised immunoregulatory and anti-inflammatory cytokine and genetic deficiency of the IL-10 receptor leads to severe IBD (*64*). As such, IL-10$^{-/-}$ mice show aberrant cytokine production and T_H1-like responses, consequently leading to intestinal inflammation and fibrosis (*65*). In 3-week old IL-10$^{-/-}$ mice, inflammatory changes initially occur in the caecum, ascending and transverse colon. As time progressed, lesions appeared in the remaining distal colon and rectum, with some mice developing inflammation in the small intestine.

These changes seem to be driven by uncontrolled cytokine secretion by activated macrophages and CD4+ T_H1-like T cells. T_H1-like inflammatory mechanisms were reinforced by anti-IFN-γ antibody attenuation of intestinal inflammation in IL-$10^{-/-}$ mice. Treatments of infant mice with IL-10 also attenuated intestinal inflammation but did not alter disease progression. By 3 months of age, IL-$10^{-/-}$ mice developed multifocal lesions throughout the large intestine, including the caecum and rectum. The large intestine developed prominent epithelial hyperplasia, transmural inflammation predominantly of lymphocytes, plasma cells, macrophages and neutrophils and occasionally crypt abscesses were also present, signs consistent with human CD. Mice also showed signs of fibrosis in the absence of fissures, fistulae and ileal inflammation and as a result the authors suggest that IL-$10^{-/-}$ cannot be viewed as a true model of CD.

Macrophage chemoattractant protein overexpression (MCP-1)

Macrophage chemoattractant protein (MCP-1) is increased throughout the gastrointestinal tract in IBD. Increased levels of MCP-1 drives intestinal inflammation, however the exact role of MCP-1 in intestinal fibrosis is not known. Transfection of the MCP-1 gene significantly increased collagen, TGF-β1 and TIMP-1 production in the distal colon (*66*). Transmural collagen deposition was associated with an infiltration of T cells, macrophages and vimentin+ cells. Collagen type I was most predominantly deposited in the muscularis mucosa and muscularis propria, whereas collagen type III in the submucosa and myenteric plexus. Overexpression of MCP-1 was unable to induce overproduced collagen in immunodeficient Rag$2^{-/-}$ mice. Overexpression of MCP-1 peaked on day 3 post-infection but persisted to day 42 following administration of the recombinant gene. Collagen deposition was observed in the sub mucosa 3 days post-infection and the muscularis propria at later time points. MCP-1

overexpressing mice also had a significant increase in total collagen levels, relative to controls. Immunological assessment saw an increase in α-SMA+ fibroblasts within the muscularis propria. MCP-1 overexpressing mice showed a significant increase in colonic TGF-β1, TIMP-1 and MMP-3 levels, relative to controls. This suggests that MCP-1 overexpression can be used a model for intestinal fibrosis and that MCP-1 may be a novel therapeutic target against intestinal fibrosis.

TNF-like cytokine 1A overexpression

Tumour necrosis factor-like cytokine (TL1A, TNFSF15) is involved in IBD pathogenesis, modulating the location and severity of intestinal inflammation and fibrosis (*67*). TL1A is overexpressed in the inflamed gut mucosa and regions of fibrostenosing disease in CD. Overexpression of TL1A in mice leads to spontaneous ileitis, proximal colitis and fibrosis. It has been demonstrated that the inflammatory effects driven by overexpression of TL1A can be ameliorated in the absence of resident microbiota. Reconstitution of germ-free Tl1a transgenic mice with specific pathogen free (SPF) mouse but not human microbiota resulted in increased fibroblast activation and collagen deposition. Fibroblast activation and migration was reduced in germ-free mice compared to native conditions, suggesting that intestinal fibrosis can be modulated by the gut microbiota. WT mice showed no change in intestinal inflammation or collagen deposition when colonised with SPF or human flora. Tl1a transgenic mice colonised with SPF flora showed increased collagen deposition in the inflamed ileum and noninflamed caecum. Interestingly, Tl1a transgenic mice colonised with human flora showed no change in ileal or caecal collagen deposition or inflammation. This data suggests that Tl1a-mediated intestinal inflammation requires the intestinal microbiota or microbial secreted products found in SPF mouse microbiota but not in human microbiota.

Fibroblasts express toll-like receptors and therefore can be activated by bacterial products (*68*). In the cecum of SPF-colonised mice which showed signs of fibrosis such as increased collagen deposition and fibroblasts activation, several microbes were found to correlate with fibrosis. The mucin-degrading bacteria *M.* schaedleri was found to correlate with fibrosis in the caecum, versus the decreased abundance of fibrosis in the colon or small intestine, in addition to *Ruminococcus* which are increased in CD and are associated with stricturing disease in paediatric CD (*69*). Additionally, *Anaeroplasma* was also increased in the fibrosed caecum of SPF-colonised mice. In contrast, members of *Streptococcus* and *Lactobacillus* correlated with fibrosis in the ileum. This is the first study to implicate the intestinal microbiome in intestinal fibrosis, suggesting that TL1A pathways and the microbiome may provide novel therapeutic targets against fibrostenosing CD.

6.6.4 Spontaneous models

SAMP mouse strain

The SAMP1/YitFc (SAMP) mouse strain develops spontaneous chronic ileitis and subsequent ileal fibrosis and is a relatively new model of intestinal fibrosis. Although the exact pathogenetic mechanisms of this model are unknown, SAMP mice show T_H1/T_H2 immune activation, with T_H1 responses predominating the acute phase, with a switch to T_H2 in the chronic phase (*70*). This model highly mimics CD in regard to the disease location, histologic features, extra-intestinal manifestations and response to common therapeutics. SAMP mice show signs of intestinal inflammation as early as 4 weeks of age, with an expansion of secretory epithelial cells such as goblet and Paneth cells, ileal hyperplasia of the muscularis propria, increased collagen deposition and stricture formation (*71*). Ileal mRNA expression of

COL1A1, COL3A, insulin-like growth factor-1 and connective tissue growth factor are all significantly increased in SAMP mice, relative to controls. Interestingly, neutralisation of IL-33 mitigates ileitis and ileal fibrosis in SAMP mice when compared with vehicle controls (*72*). This model may allow for the consistent and reliable examination of fibrogenic pathways in CD at various timepoints.

6.6.5 Human intestinal organoids

Current *in vitro* models lack the ability to replicate the intricate architecture of the intestine - intestinal organoids develop as 3D structures which resemble the intestine and are also comprised of a variety of specialised cell types, normally found in the gut. Intestinal organoids can be developed using human intestinal stem cells, thus provide a higher physiological resemblance to human disease, offering a more practical model for high-throughput screening (*73*). Generated from normal human pluripotent stem cells, intestinal organoids proceed through normal embryonic intestinal development and contain epithelium and mesenchyme, including myofibroblasts and as such is a feasible *in vitro* human model of intestinal fibrosis (*74).* Stimulation of human intestinal organoids with TGF-β1 results in upregulation of fibrogenic genes, including COL1A1, FN1, ACTA2, MYLK and MKL1 and a marked induction of α-SMA (*75*). Organoids can also be generated from the gastrointestinal tracts of mice, in particular the organoid-based epithelial to mesenchymal transition model may also be used as a novel model of intestinal fibrosis (*76*). Cells that contribute to fibrosis in CD can originate from intestinal epithelial cells via epithelial-to-mesenchymal transition (EMT) or from endothelial cells via endothelial-to-mesenchymal transition (*77, 78*). Recently, an EMT-inducting transcription factor, ZNF281 is induced in HT29 cells when treated with inflammatory agents, cultured uninflamed colonic biopsies from patients with CD and in DSS-treated mice (*79*).

Expression of ZNF281 is found to correlate with disease severity in CD and UC and inhibition reduces proinflammatory and profibrogenic factors such as TIMP-1, fibronectin, α-SMA, IL-1β, IL-17 and IL-23. As with human intestinal organoids, mouse-derived intestinal organoids also respond to TGF-β1 in a concentration and time-dependent manner, causing disruption and death of organoids (*76*). Co-culture of intestinal epithelial organoids with macrophages significantly improves epithelial to mesenchymal transition, compared to organoids stimulated with TGF-β1 without co-culture. The authors suggest that this may be due to the increased concentration of TNF-α secreted by the LPS-activated macrophages.

Human intestinal organoids are ideal for testing responses to anti-fibrotic drugs, as such the authors assessed whether TGF-β1-mediated fibrogenic responses could be reversed by treatment with spironolactone, which is used as an anti-fibrotic agent in heart patients (*75*). Spironolactone suppressed TGF-β1-stimulated induction of COL1A1, FN1, ACTA2, MYLK and α-SMA protein expression. Accordingly, human intestinal organoids are highly effective for testing anti-fibrotic drugs and provide a more relevant disease state than *in vivo* animal models, as such they should be utilised when studying fibrogenic pathways and antifibrotic therapeutics.

6.7 Cellular mediators of fibrosis

Fibrosis occurs due to the excessive deposition of ECM by activated mesenchymal cells, predominantly myofibroblasts (*13*). Fibroblasts are essential for the synthesis of various ECM proteins, the main type being collagen. Matrix-secreting cells can be stimulated by a variety of signals including pathogen-associated molecular patterns and para- and autocrine signals (*80*).

Both innate and adaptive immune responses can induce activation and differentiation of fibroblasts. Persistent, low-grade inflammation with multiple cycles of acute damage and wound healing leads to fibrosis. Consequently, the prevention of cyclic periods of tissue inflammation is a certain way to prevent the development of fibrosis. However, this may be difficult when the aetiology of the underlying inflammatory mechanism is unknown or poorly understood, such as in IBD.

Stellate cells are a subset of mesenchymal cell precursors which can contribute towards fibrosis as they differentiate into myofibroblasts (*81*). Stellate cells from IBD tissues produce collagen sooner and in greater levels, when compared to cells from healthy controls and also differentiate *in vitro* into myofibroblasts at a higher rate than controls (*82*). This suggests that stellate cells can be primed *in vivo* by inflammatory mediators, contributing towards a fibrogenic phenotype in IBD. Fibroblast expansion and activation occurs in response to inflammatory signals in the gastrointestinal tract. Fibroblast cell culture studies suggest altered responses to profibrogenic cytokines in IBD (*83*). Fibroblasts from UC and CD patients replicate faster than those from healthy controls, with no difference between CD and UC. Fibroblast collagen secretion, regardless of subtype, was increased IBD, relative to health. Additionally, platelet derived growth factor (PDGF), bFGF and TGF-β1-induced fibroblast secretion of collagen is increased from IBD fibroblasts. This activated subset of fibroblasts in both UC and CD are present in the tissue, regardless of the presence of intestinal fibrosis or inflammation. Fibroblasts can migrate along the intestine via concentration gradients of chemical signals involved in intestinal inflammation. Molecules which can induce chemotaxis include fibronectin, PDGF, IGF-I, EGF and TGF-β (when in the presence of fibronectin) (*84-86*).

TNF and IFN-γ have been found to repress fibroblast chemotaxis *in vitro*, possibly leading to the retention of fibroblasts in areas of inflammation.

In the first stage of tissue repair, platelets are activated to induce coagulation, in turn releasing growth factors such as TGF-β1, which stimulates fibroblast-mediated ECM synthesis, in addition to platelet-derived growth factor which is a potent proinflammatory cell chemoattractant (*87*). As such, dysfunction within the coagulation cascade can lead to fibrosis (*88*). Damaged endothelial and epithelial cells also release a myriad of chemoattractant factors which can recruit inflammatory monocytes and neutrophils to sites of tissue damage. The persistent presence of neutrophils and macrophages can lead to the secretion of reactive oxygen and nitrogen compounds which can damage the surrounding tissues, leading to the formation of scars.

6.8 Immune regulation of fibrosis

The innate immune system is the first line of defence in the gastrointestinal tract, providing rapid and non-specific protection against pathogens. Dysfunction or loss of innate immune regulation leading to dysregulated immune responses has been implicated in the pathogenesis of IBD. Innate immunity is largely driven through the interaction of immune cell pattern-recognition receptors (PRR) with pathogen associated molecular patterns (PAMP), which are pathogen-specific and crucial for their survival. PRRs are expressed on predominantly macrophages and dendritic cells and can identify a range of pathogens including bacteria, viruses, parasites, fungi and protozoa. Activation of PRR via the recognition of PAMPs leads to proinflammatory activation, particularly stimulation of phagocytic cells such as macrophages, neutrophils and dendritic cells, leading to the secretion of

proinflammatory cytokines such as TNF-α, IL-1β and IL-6 and chemokines such as CCL5. Furthermore, dysfunction of PRRs such as toll-like receptors (TLR) and NOD-like receptors (NLR) have been found to be implicated in the pathogenesis of IBD.

In contrast, the adaptive immune system provides a highly powerful and pathogen specific response which takes days to develop. Adaptive immunity is reliant on the recognition of specific antigens by T or B cells, resulting in the activation of T_H lymphocytes (T_H1, T_H2, T_H17 and T_H22 cells). Cells such as macrophages and dendritic cells bridge innate and adaptive immunity through the presentation of antigens to T cells via major histocompatibility complexes (MHC) in lymphoid tissue. Activated T cells proliferate and differentiate into memory or effector T cells which migrate towards the site of inflammation via the circulatory system. The migration of T cells is mediated by adhesion molecules such as integrins and selectins found on the cell surface. In IBD inflammation, T cells are directed to the lamina propria via expression of gut homing integrin $\alpha_4\beta_7$ and the mucosal addressin-cell adhesion molecule 1 (MAdCAM-1). T_H1 immune responses are predominantly against intracellular pathogens such as bacterial, viruses and fungi, leading to the secretion of IFN-γ, IL-12 and TNF-α, via activation of STAT1 (*89*). In contrast, T_H2 responses are targeted against extracellular pathogens through the secretion of IL-4, IL-5, IL-13, IL-21 and IL-25, which leads to the activation of STAT6 and GATA-3 (*89*). Classically, CD has been considered to be a T_H1-driven disease, whilst UC was T_H2-mediated, however recent studies show overlap with multiple subsets of T lymphocytes (*90, 91*). Additionally, T_H17 pathways are activated by IL-6, IL-23 and TGF-β1 via STAT3 in the absence of IL-4 and IL-12 and leads to the increased

secretion of IL-22, IL-13, TNF-α and fibroblast growth factor. Accordingly, T_H17 lymphocytes have been implicated in the pathogenies of intestinal fibrosis.

Innate immune regulation of fibrosis

Innate immune cells such as monocytes, neutrophils and mast cells produce proinflammatory and profibrotic mediators such as interleukins, tumour necrosis factor, TGF-β1 and PDGF, promoting myofibroblast differentiation. As such, innate immune pathways contribute towards the cellular activation of fibrosis. Monocyte-derived cells such as macrophages and dendritic cells can regulate activated myofibroblasts and their progenitors by acting directly on the matrix (*92*). Activated M1 macrophages are induced by IFN-γ, TNF-α or bacterial productions which act via MyD88 and NF-κB which lead to further tissue damage and increased myofibroblast resistance to apoptosis. M2 macrophages are key inducers of wound healing and have been implicated in the development of fibrosis. They are produced following the secretion of IL-4 or IL-13 through STAT6 activation. It has been shown that M2 cells compete with T_H2 cells and fibroblasts for L-arginine and L-proline which are crucial for regulating cell growth and collagen synthesis, respectively (*93*). M2 cells are also powerful inducers of anti-inflammatory effects by T_{REG} cells, leading to the suppression of fibrosis. As such, M2 macrophages, through multiple mechanisms, contribute towards the inhibition of ECM synthesis by myofibroblasts. Additionally, M2 macrophages produce profibrotic factors such as TGF-β1, connective tissue growth factor, PDGF, fibrosis growth factor and insulin-like growth factor (*94*).

During epithelial injury, neutrophils are recruited to exterminate invading pathogens but to also remove tissue debris. Prolonged neutrophil activation can exacerbate tissue damage and promote the activation of myofibroblasts through the secretion of fibrotic cytokines, chemokines and reactive oxygen and nitrogen species (*94*). Neutrophils are also one of the main sources of MMP-9 during intestinal inflammation which is degranulated upon stimulation (*95*). Furthermore, proline-glycine-proline is a product of collagen breakdown by MMPs and propyl endopeptidase and has been found to be a chemoattractant for neutrophil (*96*). Recently this has been suggested as a mechanism for MMP-induced neutrophil infiltration in IBD. Neutralisation of proline-glycine-proline leads to reduced neutrophil infiltration and subsequent intestinal inflammation (*97*). Mast cells and eosinophils can promote fibrosis through their secretion of TGF-β1 and IL-13 and activation of proinflammatory lymphocytes.

Adaptive immune regulation of fibrosis

The T_H17-secreted cytokine IL-17a has been implicated in the pathogenesis of fibrosis, particularly in lungs, heart and liver (*98, 99*). IL-17a secretion induces amplified neutrophil recruitment, leading to tissue damage and tissue fibrosis. Additionally, IL-17a directly induces the expression of MMP-1 in human cardiac fibroblasts, suggesting that IL-17a regulates fibrosis by inducing inflammatory responses and regulating the downstream activation of fibroblasts (*93*). T_H2 cytokines such as IL-4, IL-5 and IL-13 have all been shown to be involved in profibrogenic mechanisms (*100, 101*). IL-13 is crucial for fibrotic tissue remodelling and has been implicated in lung, skin, gut and liver fibrosis and stimulates the production of TGF-β1 (*102-105*). IL-13-induced fibrosis is regulated by the abundance of IL-13Ra1 signalling receptor and IL-13Ra2 decoy receptor expressed on cells such as myofibroblasts (*106, 107*). Low or

absent IL-13 binding to the decoy receptor exacerbates fibrosis (*108*). T_H1 immune activation such as IFN-γ, can have antifibrotic activity, partially by antagonising TGF-β1 (109). IFN-γ also directly dampens fibroblasts proliferation, TGF-β1-induced expression of procollagen I and procollagen III and collagen deposition by myofibroblasts (*93, 110*). Additionally, IFN-γ can also prevent T_H2-cytokine-induced differentiation of peripheral blood monocytes into fibrocytes, of which are believed to participate in fibrogenic pathways (*93, 111*).

6.9 Profibrotic mediators of fibrosis

The gastrointestinal microbiota and microbial-secreted products, as well as growth factors and cytokines secreted from immune and non-immune cells promote the differentiation and activation of mesenchymal cells which drives intestinal fibrosis (*13*). TGF-β1 is undeniably the key inducer of fibrosis and has been shown to have both anti-inflammatory and profibrotic effects. Increased TGF-β1 secretion correlates with the development of liver, lung, kidney, skin and cardiac fibrosis and inhibition of this signalling pathway reduces the progression of fibrosis in experimental models (*93*). TGF-β1 can directly induce the differentiation and activation of fibroblasts in collagen-secreting myofibroblasts. It has been suggested that the source of TGF-β1 secretion can dictate its activity (*112*). TGF-β1 secreted from macrophages generally causes wound-healing leading to profibrotic activity and secretion from T_{REG} cells leads to anti-inflammatory and antifibrotic affects (*112*).

Interestingly, mouse models do not develop fibrosis in absence of microbiota (*41*). It has been shown that persistent infection with *Salmonella enterica* Serovar Typhimurium can induce protease expression during intestinal fibrosis (*113*). Mice develop severe and persistent

intestinal fibrosis in response to chronic *S.* Typhimurium infections, closely resembling the phenotype of human disease. MMPs, serine proteases, cysteine proteases and protease inhibitors were among the proteins which had differential regulation in fibrosis. Of the 96 significantly regulated proteases and inhibitors, 56 were upregulated and 40 were downregulated in fibrotic caecal tissue, when compared to control ceca from mock-infected mice. Some of the most strongly upregulated proteases in fibrotic caecal tissue included MMP-3, MMP-7, MMP-8, MMP-10 and MMP-13. Expression of MMP-2 remained unchanged during infection. The epithelial barrier-promoting metalloprotease meprin-β (Mep1b) and the glandular kallikreins Klk1b5, Klk1b8 and Klk1b22 and tryptase-γ (Tpsg1) were amongst some of the proteins which were significantly downregulated in fibrotic tissue. It was also found that *S.* Typhimurium infection can directly stimulate protease expression in macrophages and epithelial cells but not in fibroblasts. Several cathepsins (Ctsc, Ctsd, Ctsk), protease inhibitors Serpina3m and stefinA1 (Stfa1) and TIMP-1 were upregulated in *S.* Typhimurium-induced intestinal fibrosis, overall showing upregulated expression of MMPs and impairment of serine protease expression. The epithelium of noninflamed controls also stained positive for MMP-3 and MMP-8, in contrast to the epithelia of CD and UC patients, where MMP-3 and MMP-8-positive inflammatory cells were only identified in the lamina propria. Additionally, MMP-7, MMP-10 and granzyme K were not detected in control tissue, but a small subset of UC and CD crypt epithelial cells stained strongly for MMP-7. Epithelial and inflammatory cells from IBD patients stained positive for MMP-10 and granzyme K. Staining of cathepsin C, cathepsin D and cystatin A was decreased in the lamina propria of healthy control tissue, when compared to tissue from both UC and CD patients. Overall, there were no changes in protease staining between UC and CD intestinal tissue, which were both significantly changed when compared to noninflamed controls. Additionally, protease changes that were observed in

intestinal mouse tissues were largely reflected in human IBD tissues. This study suggests that the specific inhibition of some proteases could be a novel target in the treatment of chronic inflammation and subsequent fibrosis in IBD.

TGF-β is highly produced in CD and has been shown to drive pro-fibrogenic action in CD by stimulating TIMP-1 and reducing MMP-12 activity. In contrast, TGF-β neutralising is also upregulated in segments of fibrostenotic bowel, stimulating the production of collagen, TIMP-1, MMP-3 and MMP-12 (*114*). Mucosal and submucosal biopsies from IBD patients have higher collagen content, including type VI collagen, compared to control tissue (*115*). In tissues from CD patients with fibrostenotic disease, levels of IL-36A were significantly increased and correlated with high numbers of activated fibroblasts that were positive for α-SMA. IL-36R stimulation of mouse and human fibroblasts leads to upregulation of genes required for tissue remodelling and fibrosis, as well as an increased expression of collagen VI. $Il1rl2^{-/-}$ mice and mice administered antibodies against IL-36R developed less severe DSS and TNBS colitis and fibrosis. Administration of IL-36R neutralising antibodies significantly reduced established fibrosis in mice with chronic intestinal inflammation. As such, antagonising or blocking IL-36R signalling may be beneficial for the prevention and treatment of intestinal fibrosis in IBD.

6.10 Antifibrotic mediators of fibrosis

IFN-γ and IL-12 are two of the main mediators which work to supress fibrosis (*116*). IFN-γ directly inhibits fibroblast proliferation as well as the TGF-β1 dependent expression of procollagens I and III and collagen in activated myofibroblasts (*110*). Additionally, IFN-γ

interferes with fibroblast migration by modulating the cellular distribution of fibronectin and the cytoskeleton (*117*). Despite showing therapeutic potential, clinical studies investigating IFN-γ in idiopathic pulmonary fibrosis have been discouraging (*118*). IL-12 also induces antifibrotic mechanisms through its stimulation of natural killer and T_H1 cells and subsequent IFN-γ secretion.

The induction of autophagy in $CX3CR1^+$ mononuclear cells limits intestinal fibrosis through modulation of the IL-23/IL-22 axis (*119*). MTOR inhibition by rapamycin or mTOR deletion in $CX3CR1^+$ mononuclear phagocytes inhibits the expression of IL-23 and is accompanied by reduced intestinal production of IL-22 which improves TNBS-induced fibrosis in mice. Decreased IL-23 expression results in increased autophagy activity. Suppression of the autophagy gene Atg7 results in the increased expression of IL-23 and the subsequent expression of IL-22 and increased intestinal fibrosis. This induction of IL-22 and intestinal fibrosis can occur in $RAG^{-/-}$ mice and depletion of innate lymphoid cells attenuates fibrogenesis, suggesting that this process is independent of T and B cells. IL-22 stimulates the differentiation of fibroblasts to myofibroblasts. Neutralisation of IL-22 or IL-23 suppresses fibrogenesis. This study shows that altered mTOR/autophagy in $CX3CR1^+$ mononuclear phagocytes stimulates the IL-23/IL-22 axis, causing a disproportionate fibrotic response. This suggests that this signalling cascade may provide novel targets for the treatment of fibrosis in CD.

6.11 Conclusion

In contrast to other organs such as the lungs and liver, intestinal fibrosis is understudied, despite being one of the most common complications of IBD. Dysregulated and uncontrolled periods of inflammation drive the development of fibrosis and the promotion of fibroblast activation and alteration of the normal ECM. Although fibrogenic mechanisms are conserved between various organs, fibrosis in the setting of IBD is complicated by the myriad of inflammatory mechanisms underpinning gastrointestinal inflammation. Although fibrosis can be prevented by reducing inflammatory processes, there are no current pharmacological therapies to reverse already established fibrosis. Treatment of this complex complication will require a multifactorial approach, consisting of anti-inflammatory, anti-fibrotic and epigenetic elements. The emergence of more clinically relevant models such as human intestinal organoids will allow for a more accurate replication of fibrotic diseases. In addition to poor pharmacological treatments, diagnostic imaging of fibrosis of any organ, but particularly the gastrointestinal tract is lacking and requires attention. Molecular imaging and immuno-PET may provide the sensitivity and quantification of the entire gastrointestinal tract that is required for improved patient care and outcomes.

6.12 Literature cited

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Chapter 7 ^{89}Zr-pro-MMP-9-F(ab')$_2$ detects colitis induced intestinal and kidney fibrosis

7.1 Statement of authorship

Statement of Authorship

Title of Paper	^{89}Zr-pro-MMP-9 F(ab')$_2$ detects colitis induced intestinal and kidney fibrosis.
Publication Status	Published; Accepted for Publication; X Submitted for Publication; Unpublished and Unsubmitted work written in manuscript style
Publication Details	

Principal Author

Name of Principal Author (Candidate)	Nicole Dmochowska		
Contribution to the Paper	Conceptualized the study and interpreted the data Acquired data Contributed toward methodology Curated and analysed data Drafted the manuscript		
Overall percentage (%)	70		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	24/10/2019

Co-Author Contributions

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

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

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

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

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7.2 Abstract

Intestinal fibrosis is a common complication of inflammatory bowel disease (IBD) but remains difficult to detect and treat. Matrix metalloproteases (MMPs) and tissue inhibitors of matrix metalloproteases (TIMPs) have key roles in fibrosis and are therefore potential targets for detection of fibrosis by immuno-PET, however it remains unclear whether they differ between inflamed and fibrotic states. Here we aimed to determine whether immuno-PET of $F(ab')_2$ antibody fragments targeting MMPs can detect DSS colitis induced colonic fibrosis. **Methods:** Mice were administered 2% DSS treated water for 1 cycle (inflamed) or 3 cycles (fibrotic) or were untreated (control). Colonic and kidney collagen, innate cytokine, MMPs, TIMP-1 and faecal MPO concentrations were analysed by multiplex / ELISA. α-pro-MMP-9 $F(ab')_2$ fragments were engineered, conjugated to ^{89}Zr and administered i.v. prior to PET imaging. Ex-vivo analysis was determined by Cherenkov imaging (IVIS spectrum) and biodistribution by γ-counts. **Results:** Colonic innate cytokine concentrations were increased in inflamed mice but not fibrotic mice. Colonic collagen concentrations were increased in fibrotic mice. MMPs were increased in inflamed mice, but only pro-MMP-9 remained increased in fibrotic mice. ^{89}Zr-pro-MMP-9 $F(ab')_2$ uptake was increased in the intestine but also in the kidney of fibrotic mice, where collagen and pro-MMP-9 concentrations were increased. **Conclusion:** ^{89}Zr-pro-MMP-9 $F(ab')_2$ detects colitis induced intestinal fibrosis and associated kidney fibrosis. This is the first immuno-PET study of fibrosis and highlights the utility of combining antibody specificity with PET sensitivity for detecting disease targets in tissues that are distant to the disease origin.

7.3 Introduction

Inflammatory bowel disease (IBD), incorporating Crohn's disease and ulcerative colitis, is characterised by remitting and relapsing inflammation of the lower gastrointestinal (GI) tract (*1*). Intestinal fibrosis is one of the most common IBD related complications, with severe fibrosis leading to stricture and stenosis in ~30% of IBD patients (*2-4*). Intestinal fibrosis is not currently treatable and can become life threatening, leaving endoscopic mechanical manipulation or surgical resection as the only options (*5, 6*). Importantly, no validated diagnostic tools or biomarkers currently exist for detecting or staging intestinal fibrosis (*5, 6*).

Fibrosis is characterised by the excessive deposition of extracellular matrix (ECM) proteins including collagen by persistently activated fibroblasts (*7-9*). Fibrosis typically occurs in a progressive manner following repeated episodes of inflammatory damage and remission, or healing, such as occurs in IBD and in the dextran sodium sulphate (DSS) preclinical model of intestinal fibrosis (*8, 10, 11*). Matrix metalloproteinases (MMPs) regulate fibrosis by degrading the ECM that is normally laid down as the tissue renews (*7, 12*). MMP function is tightly regulated by tissue inhibitors of matrix metalloproteinases (TIMPs), which inhibit MMP activity in a 1:1 ratio. MMP-2, -3, -8 and -9, and TIMP-1 are altered to varying extents in fibrotic tissue resected from IBD patients and in preclinical models of intestinal fibrosis (*10, 11, 13-15*).

However, MMPs and TIMPs are also increased in inflamed intestinal tissue and it remains unclear how their expression is altered in inflamed relative to fibrotic tissue.

Diagnostic tools are ideally sensitive, quantitative and selective for their pathology, non-invasive to the patient, provide information rapidly and do not influence the disease process (*5, 16*). Stricture can be detected by endoscopy, however endoscopy is limited to imaging only the mucosal surface and not the deeper layers of the colon wall where fibrosis develops (*17*). Molecular imaging approaches including positron emission tomography (PET) have superior sensitivity and provide quantitative information (*16, 18*). Immuno-PET combines the superior target selectivity provided by antibodies with the sensitivity of PET and we, amongst others, have previously demonstrated that immuno-PET strategies effectively detect intestinal inflammation (*16, 19-21*). However, the use of intact antibodies as molecular imaging probes is challenging as their relatively high molecular weight results in long half-lives, potentially translating into increased patient exposure to ionising radiation, and it may also impede the penetration of fibrotic tissue (*22*). Engineering $F(ab')_2$ antibody fragments from intact antibodies by cleaving the effector F_C region retains the target specificity of the antibody whilst reducing its molecular weight, thereby increasing excretion and promoting tissue penetration.

Here we aimed to undertake the first immuno-PET study of fibrosis in any tissue, preclinical or clinical, to determine whether immuno-PET of $F(ab')_2$ antibody fragments targeting MMPs can detect DSS colitis induced colonic fibrosis.

7.4 Materials and methods

All experiments were approved by the Animal Ethics Committee of the South Australian Health and Medical Research Institute (SAHMRI) and The University of Adelaide.

Mice

Male C57BL/6jSah mice aged 10-14 weeks (20-30g) were bred and group housed in a specific pathogen-free environment at the South Australian Health and Medical Research Institute (SAHMRI). Animals had *ad libitum* access to food and water. Experiments were conducted with only male mice to eliminate the potential confounding effects of the oestrous cycle. Mice were humanely euthanised via CO_2 inhalation and cervical dislocation to remove tissues.

DSS Colitis Model

Colonic inflammation was induced by the addition of 2% (w/v) dextran sodium sulphate (DSS) (molecular mass 40–50 kDa, Alfa Aesar, Lancashire, United Kingdom) for 5 days followed by an additional 3 days of normal drinking water for inflamed mice (d8 inflamed) (*20*), while fibrosis was induced by repeating 3 cycles of 5 days DSS treatment followed by 9 days of normal drinking water. Mice were assessed daily for signs of colitis including body weight and diarrhoea. Healthy mice were age and weight matched. Colon length was measured from the tip of the anus to the distal end of the colon.

Hydroxyproline assay

Hydroxyproline concentrations were determined essentially as described in the manufacturer's protocol (Abcam, Cambridge, UK). Briefly, ~1cm sections of distal colon or kidney were excised free of surrounding fat and blood vessels and homogenised in 100μL dH_2O / 10mg tissue before being hydrolysed in 10N NaOH (Sigma, Sydney, Australia) for 1 hr. at 120°C, then neutralised with 10N HCl (Chem Supply, Gillman, Australia) and the supernatant collected after centrifugation. Samples and standards were evaporated on a 96 well flat bottom plate, oxidised (Chloramine-T for 20 minutes), and absorbance measured at 560nm after 4-(Dimethylamino)benzaldehyde incubation for 45 minutes at 65°C. Hydroxyproline concentrations were determined based on their relationship with standards of known concentration and normalised to tissue weight.

Histology

1cm sections of distal colon were excised with surrounding fat, stored in 4% formalin at 4°C and mounted in paraffin blocks. 10x4 μm sections of formalin fixed (4°C overnight) paraffin embedded sections were stained with Masson's Trichome and processed for image capture (Nanozoomer (Hamamatsu, Japan)).

Innate cytokine and matrix metalloprotease concentrations

Proteins were extracted from a 1cm section of distal colon or kidney as previously described (20, 23-25). Concentrations of the innate cytokines IL-1α, IL-1β, IL-6, IL-10 and TNF-α (Merck Millipore, Massachusetts, USA) and matrix metalloproteases (MMP) MMP-2, MMP-3, MMP-8 and Pro-MMP9 (Merck Millipore) were determined by multiplex assay, while TIMP-1 concentrations were determined by ELISA (R&D systems, Minnesota, USA). Kidney pro-

MMP-9 concentrations were determined by ELISA (R&D systems). Concentrations were normalised to total protein concentration as determined by a BCA assay (Abcam, UK) as previously described (*20, 24, 25*).

MPO ELISA

Luminal faecal pellets were removed and homogenised for MPO processing and supernatants collected as previously described (*20, 23*). MPO concentrations were determined by ELISA essentially as per manufacturer's instructions (ThermoFisher Scientific, Waltham, MA, USA), with MPO concentrations determined based on a relationship to standards of known concentration and results standardised to faecal wet weight. The limit of sensitivity was <14ng/ml.

F(ab')$_2$ generation, conjugation and radiolabelling

Rat α-mouse pro-MMP-9 IgG_{2A} (R&D Systems Clone # 116134) F(ab')$_2$ fragments were generated by digesting on an immobilised pepsin agarose resin for 4 hours. at 37°C, followed by purification on a protein A column essentially as per manufacturer's instructions (ThermoFisher Scientific). Intact and fragmented antibodies were collected and analysed by SEC-HPLC (LC-20, Shimadzu, Kyoto, Japan) with an AdvanceBio SEC 300 column (7.8x300 mm, 2.7µm, Agilent Technologies, Foster City, USA) under isocratic conditions, with peaks detected at 280nM. Mass was calculated from a 15-600kDa protein standard mix (Sigma). ^{89}Zirconium (^{89}Zr) production and antibody radiolabelling and conjugation was performed essentially as previously described (*20*). Briefly, ^{89}Zr-oxalate was produced via proton irradiation of an ^{89}Y target on a PETtrace 880 cyclotron (GE Healthcare, Illinois, USA) and purified on the ALECO solid target processing system (Comecer, Italy).

NCS-Bz-DFO (Macrocyclics, Texas, USA) was conjugated to α-pro-MMP-9 $F(ab')_2$ fragments and radiolabelled with ^{89}Zr. Purity was confirmed by SEC-HPLC as described above. Specificity was determined by shift assay (SEC-HPLC) following incubation of recombinant pro-MMP-9 (R&D systems, 5mg/mL) in the presence or absence of α-pro-MMP9 $F(ab')_2$ in 1:10 ratio for 2hours. at room temperature.

MRI-PET

1-2 MBq of ^{89}Zr-α-pro-MMP-9 $F(ab')_2$ (71µg) in 100-200µL of normal saline was administered intravenously (tail vein) 18 hours. prior to imaging and PET scans performed 18 hours later over 30 minutes using a submillimeteric-resolution (0.7mm) (Albira PET-SPECT small animal scanner, Bruker Biospin GmbH) as previously described (*20*). Manually drawn volumes of interest (VOI) were analysed as previously described (*20*).

***Ex vivo* analysis**

Organs were dissected immediately after PET imaging, weighed and intestinal ^{89}Zr accumulation counted by Cherenkov luminescence imaging performed with an optimal imager (IVIS Lumina, Perkin Elmer USA) equipped with a CCD camera (exposure time 600sec., binning factor 8, field of view 23cm, in open filter mode), with counts background corrected. ^{89}Zr biodistribution was determined in organs using a Hidex γ-counter (Hidex, Finland) with counts background and decay-corrected as previously described (*20*).

Statistical Analysis

Data are expressed as mean±SEM in all cases. The significance of results were determined by unpaired *t* test or one-way ANOVA with Tukey's post-hoc test. Differences with $P<0.05$ were considered statistically significant.

7.5 Results

Repeated cycles of DSS induces colonic fibrosis

Colon length was reduced in d8 inflamed mice, however colonic shortening did not occur in fibrotic mice to the same extent as inflamed mice (Figure 1a). Collagen deposition was increased in the colons of fibrotic mice relative to inflamed mice and controls as indicated by hydroxyproline content and Masson's trichrome staining (Figure 1B).

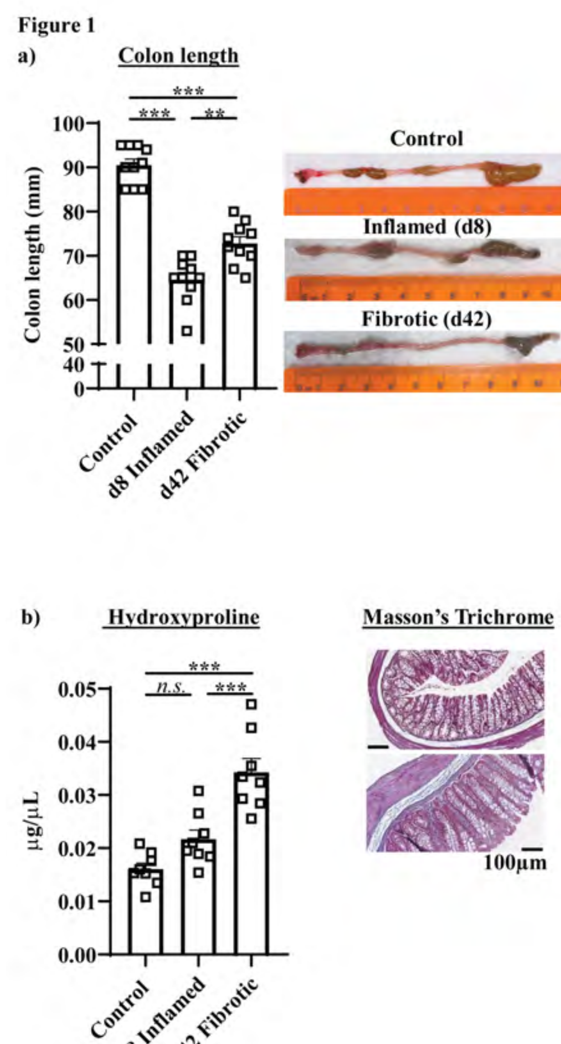

Figure 1. Colonic collagen content increases in inflamed (1 DSS cycle) and fibrotic (3 DSS cycles) mice. (A) Colon length did not shorten to the same extent in fibrotic mice as in inflamed mice. (B) Repeated DSS cycles increased the colonic collagen content as indicated by hydroxyproline concentration (left) and Masson's trichrome staining (right) **P<0.01, ***P<0.001.

Innate cytokines are not altered in fibrotic mice

The colonic concentrations of innate cytokines IL-1α (Figure 2a), IL-1β (Figure 2b), IL-6 (Figure 2c) and TNF-α (Figure 2d), and faecal MPO (Figure 2f) were substantially increased in inflamed mice relative to controls. However, the colonic concentrations of IL-1α, IL-1β, IL-6 and TNF-α and faecal MPO did not differ between controls and fibrotic mice. IL-10 intestinal concentrations did not differ between d8 inflamed, d42 fibrotic and control mice (Figure 2e). These results indicate that the key innate immune mediators of intestinal inflammation are not altered in the fibrotic colon.

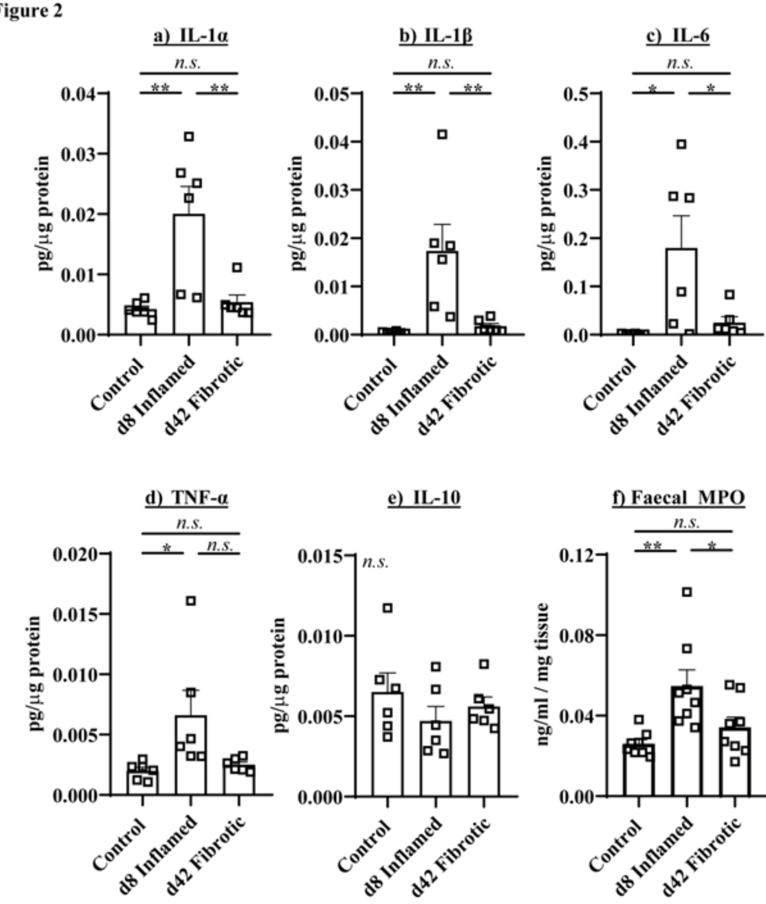

Figure 2. Innate immune mediators are not altered in colonic fibrosis. A) Colonic concentrations of A) IL-1α, B) IL-1β, C) IL-6 and D) TNF-α were increased in inflamed but not fibrotic colons, as are F) faecal MPO concentrations, while E) IL-10 concentrations were not altered in inflamed or fibrotic mice. *P<0.05, **P<0.01.

Pro-MMP9 remains increased in the fibrotic colon.

The colonic concentrations of the MMP members MMP-2 (Figure 3a), MMP-3 (Figure 3b), MMP-8 (Figure 3c), Pro-MMP-9 (Figure 3d) and TIMP-1 (Figure 3e) were substantially increased in inflamed mice relative to control mice. However, while the colonic concentrations of MMP-3, MMP-8 and TIMP-1 were higher in fibrotic mice than controls, they were substantially reduced compared to inflamed mice. MMP-2 concentrations did not differ in d42 fibrotic mice relative to control mice. Importantly, only pro-MMP-9 concentrations remained as high in fibrotic colons as in inflamed colons.

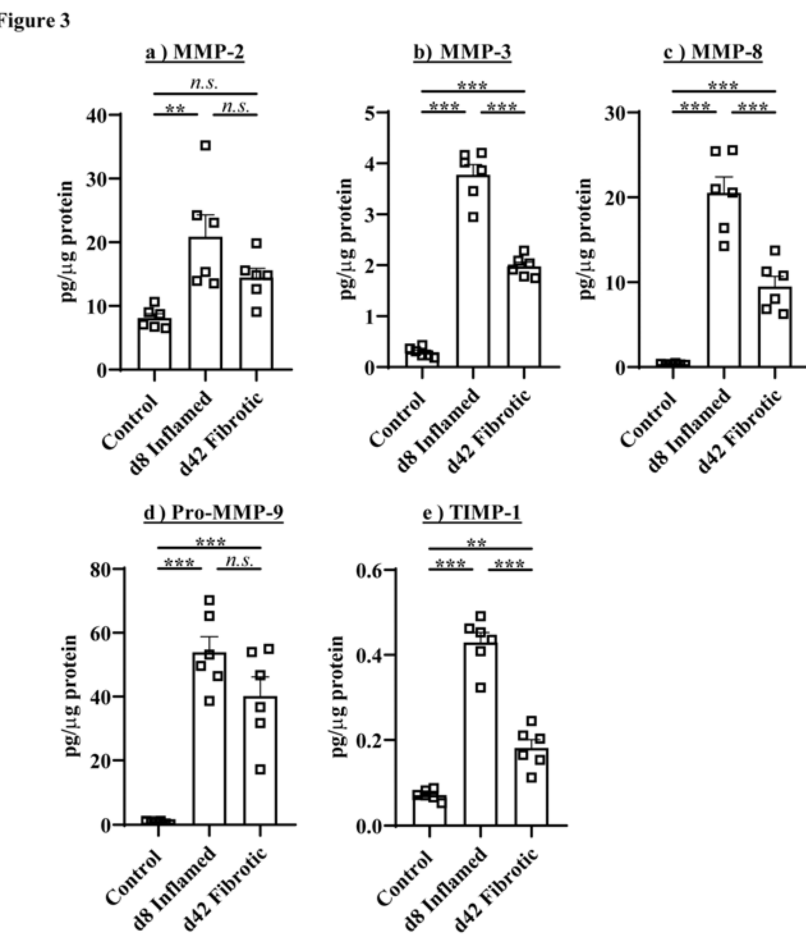

Figure 3. Pro-MMP-9 concentrations remain increased in fibrosis. (A) MMP-2, (B) MMP-3, (C) MMP-8, (D) pro-MMP-9 and (E) TIMP-1 were increased in inflamed and fibrotic mice, but only pro-MMP-9 remained as high in fibrotic colons as in inflamed colons. *P<0.05, **P<0.01, ***P<0.001.

^{89}Zr-Pro-MMP-9 F(ab')$_2$ immuno-PET Detects Intestinal fibrosis

α-pro-MMP9 F(ab')$_2$ fragments had a retention time of 10.24 minutes. and a molecular weight of 103kDa, while the intact pro-MMP-9 antibody had a retention time of 9.82 minutes and a molecular weight of 138kDa (Figure 4a). The specificity of our tracer was confirmed by the reduction in pro-MMP-9 signal in samples co-incubated with α-pro-MMP9 F(ab')$_2$ (Figure 4b). PET imaging revealed intestinal uptake of ^{89}Zr-Pro-MMP-9 F(ab')$_2$ was increased in the colon (Figure 5a), but, unexpectedly, also in the kidney (Figure 5b) relative to controls (See online resource video 1 (control) and 2 (fibrotic)). These findings were confirmed by *ex-vivo* analysis that demonstrated that ^{89}Zr-α-pro-MMP-9 F(ab')$_2$ uptake was increased in the large intestine and kidney in d42 fibrotic mice relative to controls by analysis of Cherenkov luminescence (Figure 6a) and γ-counts (Figure 6b). ^{89}Zr-α-pro-MMP-9 F(ab')$_2$ uptake was not observed in other gastrointestinal sites including the small intestine and caecum but was increased the spleen and liver (Figure 6b). Uptake was similar between DSS treated and control mice in all other tissues investigated.

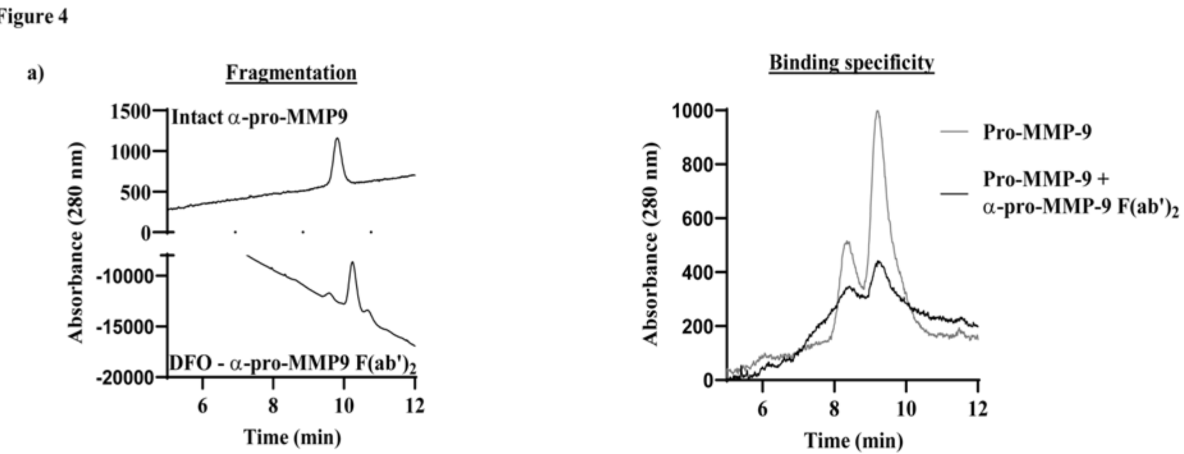

Figure 4. A) α-pro-MMP-9 F(ab')$_2$ fragments had a longer retention time than intact antibody and B) specifically bound pro-MMP-9.

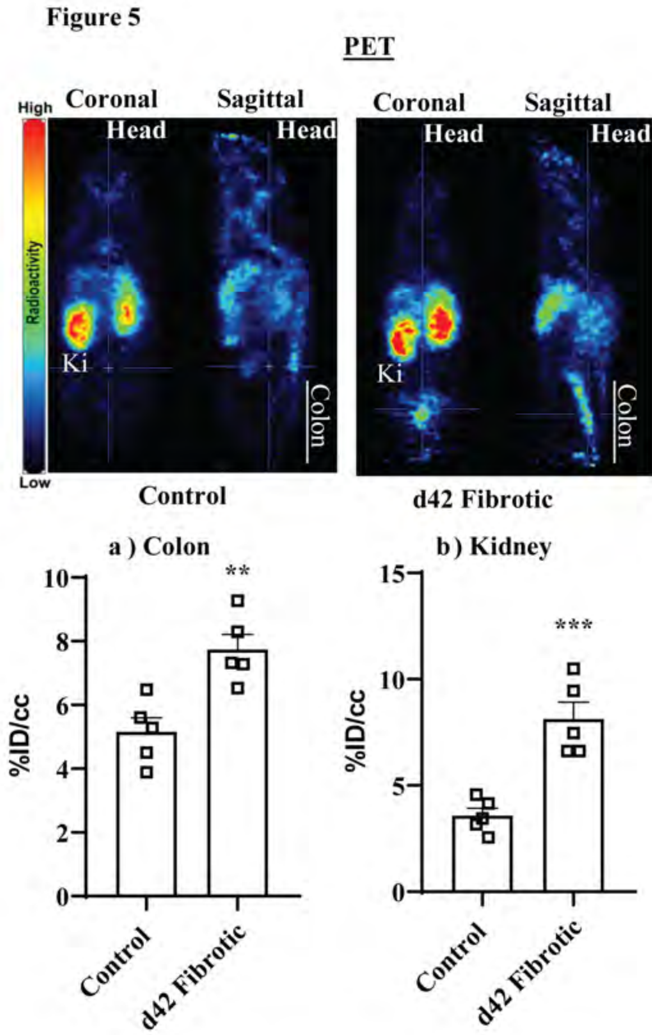

Figure 5. ^{89}Zr-α-pro-MMP-9 F(ab')$_2$ PET detection of A) colonic and B) kidney fibrosis. Volume of interest (VOI) analysis with representative PET image (coronal and sagittal) of control and fibrotic mice. Ki=kidney. **P <0.01, ***P <0.001.

Repeated DSS cycles induce kidney fibrosis

Hydroxyproline (Figure 7a) and pro-MMP-9 (Figure 7b) concentrations were increased in the kidney of fibrotic mice relative to inflamed mice and controls.

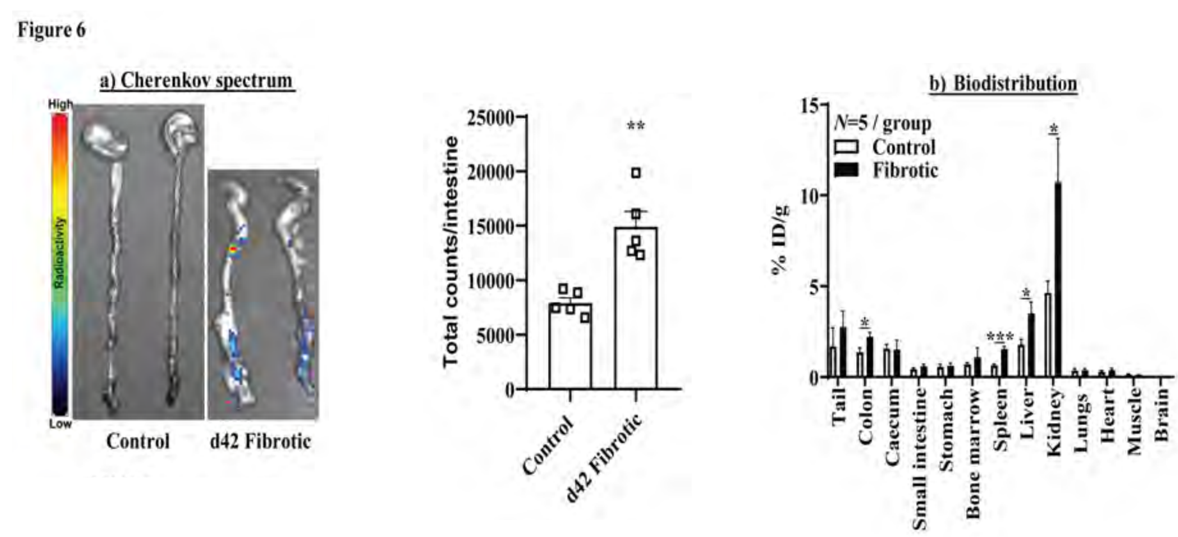

Figure 6. *Ex vivo* A) Cherenkov luminescence analysis and B) biodistribution of ^{89}Zr-α-Pro-MMP-9 F(ab')$_2$. *P<0.05, **P<0.01, ***P<0.001.

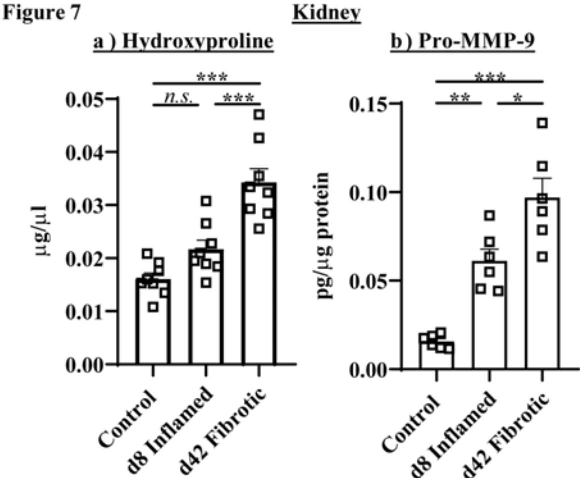

Figure 7. Repeated DSS cycles increased A) hydroxyproline and B) pro-MMP-9 concentrations in the kidney *P<0.05, **P<0.01, ***P<0.001.

7.6 Discussion

Fibrosis is a common and serious complication of IBD but remains difficult to detect and treat. We conducted the first immuno-PET study of fibrosis to demonstrate that ^{89}Zr labelled $F(ab')_2$ antibody fragments directed against pro-MMP-9 reliably detects colonic fibrosis in the absence of inflammation. We demonstrate that the pro-MMP-9 signal in the colon co-exists with a strong signal in the kidney, which we also reveal becomes fibrotic in this model. These findings highlight the utility of immuno-PET for detecting changes in specific mediators of fibrosis in the organ of interest and also in organs that are distant from the initially affected site.

We compared MMP, TIMP and innate immune cytokine concentrations in inflamed and fibrotic intestines and demonstrate that pro-MMP-9 selectively remains as high in fibrotic tissue as in inflamed tissue. Our findings of increased MMP-2, -3, -9 and TIMP-1 in fibrotic intestinal tissue confirm previous findings in preclinical models of intestinal fibrosis *(10, 11, 26*). However MMP and TIMP expression in fibrotic intestinal resections from IBD patients remains controversial; MMP-2, -3, -9 and TIMP-1 are reportedly increased to a similar extent in fibrotic and inflamed intestinal tissue relative to tissue from healthy subjects (*14, 15*), while others observed that TIMP-1 is increased but MMP-3 is reduced in intestinal fibrosis relative to nearby unstrictured tissue (*13*). Furthermore, MMPs and TIMPs are typically also upregulated in inflamed regions of IBD patients, and some have suggested that serum MMP concentrations may be suitable as biomarkers of intestinal inflammation (*14, 27-31*). These observations highlight the difficulty in identifying unique markers of fibrosis as inflammation

and fibrosis can co-exist (*6*), highlighting the importance of comparing inflamed and fibrotic tissue. We found that of all the MMPs and innate cytokines investigated, only pro-MMP-9 remained as high in fibrotic tissue as in inflamed tissue indicating that pro-MMP-9 concentrations remain high in the absence of inflammation. Our findings related to MPO are particularly important as MPO is closely related to calprotectin in humans and faecal calprotectin content is a clinically useful marker of intestinal inflammation. Therefore, our study demonstrates that high pro-MMP-9 is a biomarker of intestinal fibrosis in the absence of inflammation and would potentially be of use in IBD patients experiencing symptoms of fibrosis that are concomitant with low faecal calprotectin levels. The focus of this study was to image and compare intestinal fibrosis with healthy, non-fibrotic mice, as such we did not perform imaging experiments on d8 inflamed mice. As such, this may limit some of the conclusions that can be drawn from this study.

There are no validated diagnostic tests for intestinal fibrosis, highlighting a major unmet need as early intervention is prevented. Furthermore, there are no endorsed endpoints for clinical trials of urgently needed therapeutics. Endoscopy has long been the backbone tool for diagnosis of intestinal disease, but is only useful for detecting fibrosis in its later stages when stricture and stenosis manifest, and the jejunum and proximal ileum are normally difficult to access by endoscopy (*17, 32*). Additionally, there is no accepted histopathological scoring system for grading the severity of intestinal fibrosis in biopsies obtained from endoscopy. Recent developments in MRI, CT and intestinal ultrasound have provided some promise for imaging intestinal fibrosis (*5*), however their sensitivity is typically lower than PET and generating quantifiable data can be difficult (*18*). Additionally, these technologies typically focus on discrete body regions and therefore potentially miss concurrent disease in other organs, as we observed in the kidney in the current study. Only one study to date has assessed

PET/SPECT approaches for detecting fibrosis in IBD patients, demonstrating that PET combined with MR enterography could differentiate fibrotic strictures from inflammation while PET/CT did not perform as well (*17, 33*). However, the metabolic marker ^{18}F-FDG was used as the probe in this study and discriminating the changes in metabolism that are induced in fibrosis from those that are related to inflammation involved changes is likely to be difficult. Advances in the molecular imaging of fibrosis have been made in other tissues, and clinical/preclinical studies have now identified useful probes targeting the stomatostatin receptor, ECM constituents including collagen and fibrin, cathepsins and integrins involved in TGF-β activation (*18*). However, these are yet to be applied to intestinal fibrosis in clinical or preclinical studies, and, to the best of our knowledge, our study is the first immuno-PET based approach to identify fibrosis in any tissue.

Immuno-PET directed against immune cells or mediators has demonstrated efficacy for detecting colonic inflammation in preclinical models (*16, 19-21*). We recently demonstrated that targeting inflammation induced immune mediators such as IL-1β results in a substantially more localised bio-distribution than targeting immune cells, presumably as while immune cells migrate to inflamed sites they are also widely distributed throughout the body (*20*). These findings prompted our examination of inducible mediators of fibrosis as opposed to markers on the surface of fibroblasts or constitutively secreted mediators such as TGF-β1. Here, using the same model of colonic inflammation as our previous study, we confirmed that IL-1β is the most favourable innate immune cytokine for detecting intestinal inflammation as it had the highest fold increase. While there was also a substantial increase in colonic IL-6 concentrations during inflammation, targeting IL-6 for imaging is complicated by its constitutive secretion from a range of non-immune cell types including muscle, its relatively high serum concentration, and its known variability in human disease states (*34*).

Enthusiasm for developing antibody-based probes for PET imaging has been tempered by concerns related to the use of ionising radiation, which are amplified by the high molecular weight of antibodies, and the potential for the probe to interfere with the disease process (*5*). Our use of engineered antibody fragments to detect fibrosis extends from studies employing antibody fragments against the T_{HELPER} immune cell surface marker CD4 and the gut homing immune cell integrin β_7 to detect colonic inflammation in preclinical models (*19, 21*). $F(ab')_2$ antibody fragments have a substantially reduced size compared to intact antibodies which promotes tissue penetration and increases clearance, thereby reducing patient exposure to ionising radiation (*16, 22*). Furthermore, $F(ab')_2$ antibody fragments retain target specificity and removal of the effector F_C region effectively renders the antibody inert as F_C mediated activation of innate immune cells and the complement cascade is eliminated. $F(ab')_2$ fragments may theoretically influence the disease process by neutralising their target, however this effect is improbable as the antibody dose for their use as probes for immuno-PET is much lower than when they are used as conventional drugs (*18*). The dose of ionising radiation we used in our current study was similar to that used in these previous studies and much lower than the dose we previously used with conjugated intact antibodies to detect colonic inflammation (*20*), reducing exposure to radiation (*19, 21*). Furthermore, it should be noted that the ionising radiation dose for whole body ^{18}F-FDG PET scans is much lower than typically used for abdominal pelvic CT scans, but much more information regarding distant organs can be obtained (*16, 35, 36*). Importantly, immuno-PET offers the potential for both early detection and theranostic treatment which are critically needed for treatment in the intestine and other fibrotic tissues.

We observed that the increased pro-MMP-9 signal in the fibrotic colon occurred concurrently with increased signal in the kidney, which we demonstrate for the first time develops fibrosis in this model. IBD is frequently associated with extra-intestinal manifestations. Nephritis and nephrolithiasis, or stone formation, is the most frequently experienced renal diseases in IBD patients, and is particularly prevalent in those with long standing disease (*37*). MMP-9 expression is increased in nephritis and nephrolithiasis has been linked to MMP-9 polymorphisms, however in relation to IBD it remains difficult to determine whether these diseases result from immune mechanisms that are shared or distinct to those in the intestine, or whether they result from drug toxicity (*37, 38*). Kidney injury and increased inflammatory mediator content has previously been demonstrated in DSS induced colitis (*39*). However, our study is the first to investigate the kidney in a model of intestinal fibrosis. The mechanisms underlying colitis induced kidney fibrosis that we observed warrant further study.

7.7 Conclusion

We performed the first immuno-PET study in any organ, clinical or preclinical, and demonstrated that pro-MMP-9 $F(ab')_2$ fragments detected inflammation induced intestinal fibrosis after inflammation had resolved. Furthermore, we also established that the kidney becomes fibrotic in this model, underscoring the benefit of PET for detecting disease in organs that are distant from the original disease site and are therefore potentially ignored. As the mechanisms underlying fibrosis are broadly similar across all organs, and ~45% of all natural deaths in the western world can be attributed to fibroproliferative disease (*8*), we propose that immuno-PET of pro-MMP-9 antibody fragments are a valuable addition to the detection of fibrosis in all tissues.

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Chapter 8 Discussion

8.1 Overview

IBD is a chronic, debilitating disease that currently lacks efficient and long-term therapeutics and sensitive, quantitative diagnostic imaging that can visualise the entire gastrointestinal tract. I analysed the underlying mechanisms of DSS colitis and how dietary emulsifiers can modulate these mechanisms, this study provided targets for immuno-PET of gastrointestinal inflammation. I designed and validated two immuno-PET tracers against innate immune mediators and compared tracer efficacy between targeting immune cells and inducible cytokines, in a preclinical model of acute IBD. Furthermore, I designed and validated an antibody fragment immuno-PET tracer against intestinal fibrosis in a preclinical model of chronic IBD, which to our knowledge is the first immuno-PET study of fibrosis in any tissue.

8.2 Dietary emulsifiers exacerbate DSS colitis

To be able to image certain disease processes, there must be an understanding of the underlying mechanisms. As such, I elucidated the inflammatory mechanisms involved in murine DSS colitis but also how the dietary emulsifier CMC affects gastrointestinal homeostasis, as the increased consumption of a Westernised diet has been linked with the increasing incidence of IBD. In the past decade, there has been an increased awareness of healthy eating habits and reducing our consumption of processed foods. This has been followed by a number of studies examining the effects of dietary emulsifiers such as CMC or P80 on the gastrointestinal tract. Our results support the current literature, suggesting that CMC can disrupt gastrointestinal homeostasis and exacerbate murine chemically induced colitis.

8.3 Immuno-PET of innate immune markers

Following the identification of tracer targets, I visualised and compared immuno-PET directed against a key innate immune cytokine IL-1β and cell marker CD11b. My results showed that immuno-PET targeting cell secreted immune mediators provided a higher colonic uptake with decreased uptake in non-target organs, in contrast to targeting immune cells. These findings correlate to the underlying immunologic mechanisms involved in colitis. When activated, innate immune cells migrate from induction sites in the lymphatic vessels through the bloodstream to sites of inflammation, where they mature and secrete cytokine such as IL-1β, before clearing from the area. Innate cells such as macrophages, dendritic cells and neutrophils express CD11b, regardless of their maturity or state of activation and thus imaging of CD11b will highlight areas of inflammation but also show migration throughout the blood and lymph, which may help explain the uptake distribution that we saw. In contrast, IL-1β is inducible and is only secreted by activated cells located in inflammatory loci. Additionally, proteolytic manipulation of IL-1β into its mature form occurs within the cell and it is secreted in its functional form. My results also supported the current literature in the use of FDG-PET for the detection of inflammatory lesions in colitis, however, as previously mentioned, FDG uptake is based on the tissue metabolism of glucose. As such, the specificity, sensitivity and clinical utility of PET in the setting of IBD can be improved through the direct targeting of inflammatory markers. Additionally, in some cases it has been found that FDG-PET is unable to differentiate between pathologic findings and adjacent regular intestinal segments (*1*). It is also unclear in which context FDG-PET in IBD would prove clinically useful and provide a change in management.

Imaging modalities are ideally sensitive, quantitative and selective for their pathology and are non-invasive to the patient, in contrast to the current gold standard of endoscopies which are

highly invasive and require thorough bowel preparation. Molecular imaging techniques such as immuno-PET provide superior sensitivity in comparison to traditional methods such as MRI or CT through the combination of the high target selectivity of antibodies with the sensitivity of PET. Typically, PET images are combined with CT or MR to provide anatomical mapping. Immuno-PET provides whole body imaging of disease processes, which is useful for IBD as patients often experience extra-intestinal manifestations such as arthritis. Additionally, in contrast to endoscopic techniques, immuno-PET can detect inflammation below the mucosal surface and the deeper layers of the colon. Our study supports further investigation into the use of targeting inducible, intracellularly processed immune mediators for immuno-PET of intestinal inflammation.

8.4 Immuno-PET of intestinal fibrosis

Intestinal fibrosis is one of the most common complications of IBD and lacks an efficient imaging modality. As such, I developed a murine model of fibrosis that is stimulated by chronic chemically induced colitis. I assessed the changes in fibrotic mediators such as TIMP, MMPs and immune cytokines and chemokines and found that pro-MMP-9 was suitable for immuno-PET due to its persistent induction, in the absence of inflammation. As expected, innate and adaptive immune activation decreased in chronic DSS colitis, in contrast to acute DSS colitis. Concurrently, there was an increase in markers such as MMP-3, MMP-8, pro-MMP-9 and TIMP-1, in chronic DSS, however only pro-MMP-9 remained as high in fibrotic colons as in inflamed colons.

The use of intact IgG antibodies as imaging probes leads to an increased half-life and retention in non-target organs, potentially leading to an increase in the exposure of ionising radiation. Radiation exposure from PET tracers is much less than that of an abdominal CT study. As such,

if the patient receives improved care, prognosis and quality of life as a result of the PET study, the radiation dose can be justified. The Fc portion of intact antibodies can be cleaved to form a $F(ab')_2$ fragment which results in decreased molecular weight and non-specific binding, whilst retaining the target specificity of the antibody. The use of antibody fragments decreases the antibody half-life as well as its circulation time and binding to non-target organs, increasing excretion and promoting tissue penetration. Consequently, the patient's radiation dose is reduced. Targeting intestinal fibrosis using anti-pro-MMP-9 antibody fragments provided visualisation of fibrotic segments of the colon. This study suggests that targeting MMPs may be suitable for the visualisation of intestinal fibrosis.

It is imperative to select tracer targets that will not influence the mechanism or disease pathway that is being imaged – this may lead to decreased accuracy. Targets like TGF-β, although critical for fibrogenesis would not be suitable immuno-PET targets as they are constitutively secreted and involved in many other disease mechanisms and may influence the very mechanisms that are going to be visualised. However, this effect is typically minimalised as the dose used for imaging is much less than what is used therapeutically. Furthermore, proteins such as collagen, although increased in fibrotic tissue would not be suitable for immuno-PET targeting due to a high basal expression throughout the body in health. Interestingly, our results showed for the first time that chronic DSS colitis leads to fibrosis of the kidneys in addition to the large intestine. Interestingly, I observed mediators of fibrosis such as collagen and pro-MMP-9 were also elevated in the kidneys in the absence of inflammation, indicative of fibrosis. As this is the first description of fibrotic kidneys in this model, the mechanisms underlying these effects require further study. However, patients with IBD have been shown to develop kidney pathologies such as nephritis or nephrolithiasis,

particularly in longstanding disease. To our knowledge, this is the first study to demonstrate immuno-PET of fibrosis in any tissue in a preclinical or clinical setting.

8.5 Discussion of combined results

Elucidation of immune mechanisms underlying DSS colitis and emulsifier-induced exacerbation of colitis showed strong innate immune activation, particularly a large increase in colonic IL-1β and CD11b+ immune cells, suggesting possible targets for visualisation of intestinal inflammation with immuno-PET. Consequently, radiolabelling of monoclonal antibodies against IL-1β and CD11b provided visualisation of gastrointestinal inflammation in acute DSS colitis. Targeting of IL-1β proved to be superior due to its localised secretion in areas of inflammation, in contrast to targeting CD11b+ immune cells which migrate throughout the body. This study suggests that future immuno-PET studies of inflammatory changes should ideally target inducible cytokines or mediators to achieve the best visualisation of disease changes. For the fibrosis immuno-PET study this prompted us to target inducible mediators such as MMPs when detecting intestinal fibrosis, in contrast to cells such as fibroblasts.

Chronic administration of DSS colitis resulted in the induction of MMPs, particularly pro-MMP-9 which was persistently elevated in the absence of inflammation. Targeting of pro-MMP-9 provided visualisation of intestinal fibrosis in chronic DSS colitis but also showed uptake in the kidneys which was shown to be due to fibrotic changes and not tracer excretion. As expected, $F(ab')_2$ fragment tracer resulted in an altered biodistribution, showing decreased uptake in nontarget areas, in contrast to our innate immune marker study which utilised intact antibodies. The use of antibodies of less than 60 kDa favours the renal route of excretion, in contrast to larger proteins which are hepatically cleared. The results from the intestinal fibrosis immuno-PET study highlighted the superior use of immuno-PET for inflammatory

diseases, as pathological changes can be detected in organs other than the original disease site, which may be potentially missed when using conventional techniques such as colonoscopies. Further optimisation of anti-IL-1β and anti-pro-MMP-9 F(ab')2 immuno-PET is required which may lead to clinical trials.

8.6 Future studies

The use of humanised immune system (HIS) mice may bridge the gap between murine models of colitis and clinical trials, improving bench-to-bedside translation. HIS mice provide a more immunologically relevant mouse model of IBD, relying on the implantation of human hematopoietic stem cells or peripheral blood mononuclear cells (PBMC) into immuno-deficient NOD/scid-IL2rγ$^{-/-}$ or NSG mice. Recently a novel model of chemically induced IBD in human PBMC NSG mice has been described (*2*). Briefly, these mice were implanted with human PBMCs from UC patients and were later sensitised via dermal application, and then intrarectally challenged. Histopathological assessment showed mice developed signs intestinal inflammation as seen in IBD such as oedema, fibrosis and the infiltration of T cells into the lamina propria. The development of these murine models may offer a more immunologically relevant model for the preclinical testing of immuno-PET tracers. Furthermore, although there have been previous studies using repeated doses of ^{89}Zr tracers in humans the radiation burden to patients must always considered. Pretargeting imaging approaches can mitigate some of the disadvantages associated with conventional antibody imaging such as further decreasing radiation doses. This involves injection of the antibody, uptake period (days), followed by injection of a clearing agent which will remove excess antibody tags and then injection of the radioactive tag which takes minutes to hours to bind to the previously injected antibody agent. This technique removes the dilemma of having to match long half-life antibodies with long half-life radioisotopes, as such, shorter-lived isotopes

such as fluorine-18 (110 minutes), carbon-11 (20 minutes) and gallium-68 (70 minutes) can be used. However, the logistics of these tracers may be challenging as the radiopharmacy unit must be in close proximity to the clinic where the imaging will be performed. Additionally, PET cameras have a greater sensitivity and higher spatial and temporal resolution than SPECT cameras, therefore offering high quality images at lower radiation doses.

8.7 Conclusion

IBD is a debilitating chronic condition that currently lacks effective therapeutics and non-invasive imaging modalities. Unravelling the pathogenesis of IBD does not only provide novel therapeutic targets, but also targets for immuno-PET. Molecular imaging is a rapidly expanding field at the forefront of diagnostic imaging, which may satisfy the unmet need of highly sensitive, quantitative and non-invasive imaging of IBD. We have shown that immuno-PET of innate immune markers, particularly inducible cytokines, can be used to successfully visualise intestinal inflammation in a preclinical model of IBD and for the first time, that targeting pro-MMP-9 allows for the visualisation of intestinal fibrosis in a preclinical model of chronic IBD. Further research into these techniques is required as they show clinical potential.

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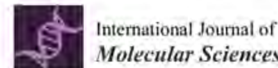
Chapter 9 Appendix of publications arising from this thesis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Review

Advances in Imaging Specific Mediators of Inflammatory Bowel Disease

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Abstract: Inflammatory bowel disease (IBD) is characterized by chronic remitting and relapsing inflammation of the lower gastrointestinal tract. The etiology underlying IBD remains unknown, but it is thought to involve a hypersensitive immune response to environmental antigens, including the microbiota. Diagnosis and monitoring of IBD is heavily reliant on endoscopy, which is invasive and does not provide information regarding specific mediators. This review describes recent developments in imaging of IBD with a focus on positron emission tomography (PET) and single-photon emission computed tomography (SPECT) of inflammatory mediators, and how these developments may be applied to the microbiota.

Keywords: Inflammatory Bowel Disease (IBD), colitis; PET; SPECT; microbiota; cytokine; chemokine; inflammation

1. Introduction

Inflammatory bowel disease (IBD) is an inflammatory disorder of the gastrointestinal (GI) tract that impacts substantially on quality of life. The causes of inflammation in IBD remain unknown, but are thought to involve a hypersensitive immune response to the intestinal microbiota. IBD is commonly associated with dysbiosis, but it remains unknown whether dysbiosis is a cause or consequence of inflammation. Diagnosing and monitoring IBD is heavily reliant on endoscopy, which is an invasive technique that requires bowel preparation and typically also anesthesia. Although generally well tolerated, endoscopy noticeably impacts on patient quality of life and does not provide any direct information regarding the role that specific mediators contribute toward inflammation. Therefore, new technologies are required that are sensitive enough to grade disease severity, are non- or only minimally invasive, and can be optimized for the detection of specific mediators. Recent developments in positron emission tomography (PET), particularly with the use of antibody-conjugated tracers, have demonstrated success in cancers, but have only recently been adapted to IBD. This review summarizes the current advances of these technologies in IBD, how they have been used to detect specific mediators of inflammation, and their potential to image microbiota.

2. Inflammatory Bowel Disease

IBD is a collection of debilitating idiopathic diseases characterized by chronic inflammation of the lower gastrointestinal tract that have a remitting and relapsing disease course. The cause(s) of IBD remain unknown; however, they are thought to involve aberrant immune responses to environmental stimuli in people with a complex genetic predisposition. The global prevalence of IBD is estimated to be 0.3% and is widely regarded as increasing [1]. The relatively recent increase in the incidence of IBD is perplexing and highlights the likely importance of yet-to-be-determined environmental factors

in its etiology. IBD is typically diagnosed in adolescence and early adulthood; however, it can also occur in the very young pediatric population, and approximately 25% of pediatric cases present prior to reaching 10 years of age [2,3]. It is currently unclear whether the mechanisms underlying IBD differ between the very young pediatric and late adolescent populations. However, it is probable that yet-to-be-characterized genetic factors feature more prominently in the very young, as the length of time of exposure to environmental factors is unlikely to be long enough to sensitize the developing immune system. IBD is a chronic disease in pediatric and adult populations; however, the pediatric population is particularly vulnerable to the consequences of IBD, including impacts on growth and development, bone health, and psychosocial function and development [2].

There are two major subtypes of IBD: Crohn's disease (CD) and ulcerative colitis (UC), which can typically be distinguished by pathological and histological differences. Inflammation in UC is typically restricted to the mucosal layer of the colon and progresses in a contiguous manner. CD differs in that it is characterized by transmural skip lesions that can occur anywhere in the GI tract but are typically located in the ileum with and without involvement of the colon. The initiating factors and inflammatory aspects are likely to differ between CD and UC given the distinctions between their pathology, but are only poorly understood relative to what is known regarding how inflammation is perpetuated. This increased understanding has led to the relatively recent development of biologic drugs targeting specific immune mechanisms, most prominently the cytokines TNF-α, interleukin (IL)-12 and -23, and integrins involved in the migration of immune cells into the GI tract, including $\alpha_4\beta_7$ [4]. These treatments and other nonspecific immune suppressants can be beneficial in IBD; however, treatment gaps remain due to intolerance, incomplete efficacy, and side effects of current therapies, leading to a disease course characterized by variable periods of remission and relapse. Prognostic indicators of relapse in IBD are only poor, and therefore patients are required to undertake constant surveillance, typically via endoscopy. Severe persistent or relapsing symptoms also lead to fibrosis and stricture in >30% of CD and approximately 5% of UC patients [5]. Intestinal fibrosis and stricture occurs as a result of excessive deposition of extracellular matrix proteins that accumulate following tissue remodeling and lead to serious complications, including obstructions [6]. No drugs are currently clinically available to treat fibrosis and stricture, and therefore these cases often lead to surgery, which can be curative for UC (withstanding complications), but is not curative for CD [7]. This high rate of surgery highlights the ongoing need for a deeper understanding of the mechanisms involved in initiating and perpetuating inflammation in IBD.

Imaging IBD: Current Approaches

Treatments for IBD depend on clinical severity, which is typically assessed by endoscopy. The gastrointestinal mucosa is directly imaged by endoscopy for assessment of disease stage and monitoring, and when forceps are attached, which allows for the collection of biopsy material for analysis of pathology and mechanistic studies. Endoscopy can differentiate between UC and CD in the majority of cases [8]. However, endoscopic approaches have limitations, as the quality of results is operator-dependent and it may not be sensitive enough for assessment of clinical severity in some cases. Furthermore, endoscopic imaging is restricted to the superficial mucosal layers of the intestine and therefore provides no information regarding inflammatory damage to the deeper layers of the intestinal wall, the degree of muscle thickness, or the diameter of the lumen. Finally, a major limitation of endoscopy is the difficulty in reaching the small intestine, due to the distance needed to be covered and the complexities of intestinal anatomy with its multiple loops and folds. These limitations are particularly relevant to the pediatric population, who are sensitive to perforation of the intestinal wall and risks associated with sedation [9].

A number of novel imaging technologies are emerging that complement or may even replace endoscopy as the gold standard for clinical diagnosis, as recently reviewed in detail [10,11]. Chromoendography involves the application of dyes onto the mucosa, improving endoscopic characterization of lesions and neoplasia, and has the potential to correlate with histological damage,

although the latter remains controversial [12]. Confocal laser endomicroscopy and endocytoscopy combine high-resolution microscopic imaging with endoscopy, and again, are primarily used to detect colonic dysplasia and neoplasia [13]. Wireless capsule technologies may circumvent the limitations that endoscopy has in accessing difficult-to-reach areas of the small intestine. While these capsules provide high-resolution imaging, they have similar limitations to standard endoscopy regarding assessment of inflammatory damage to the deeper layers of the colon wall and may also require surgery for removal when stricture occurs [14,15].

Less invasive imaging technologies include barium X-rays, ultrasound, magnetic resonance imaging (MRI), computed tomography (CT), PET, and single photon emission computed tomography (SPECT). Barium X-rays provide excellent visualization of the bowel and can reveal thickening of the bowel wall, but are not recommended in patients with severe inflammation due to the risk of complications such as toxic megacolon, and while they have been traditionally used to assess stricture location and severity, this is largely being replaced by CT and MRI [2]. Ultrasound, MRI, CT, PET, and SPECT are minimally invasive relative to endoscopy and offer the additional benefits of visualizing planes through the colon wall and extraintestinal manifestations. Bowel ultrasound has some attractions as a noninvasive diagnostic tool for IBD, including relative inexpensiveness, high accuracy in CD, and ease of use. It has recently been used to reveal ulcer location and severity in adult CD, and ultrasound and MRI are currently the preferred tools for assessment of pediatric perianal abscesses and fistulas [16–18]. However, ultrasound is highly operator-dependent and its accuracy is sensitive to anatomical location, as it is reduced for detecting ulcers in the jejunum and rectum [17,18]. MRI is currently the imaging technique of choice for follow-up of CD patients due to its lack of ionizing radiation. Notably, MRI can differentiate between normal and pathological sections of the bowel by analysis of changes in bowel wall thickness [19]. Recent developments with MRI and CT include using them to assess fibrosis and stricture. Gadolinium-contrasted MRI has recently been shown to reliably predict severe fibrosis in CD and, interestingly, also revealed that areas of fibrosis often coexist with inflammation within the same intestinal segment [20]. A recent comprehensive review of the use of PET/SPECT for IBD diagnosis, prognosis, and follow-up indicated that 2-deoxy-2-[^{18}F]fluoro-D-glucose (^{18}F-FDG) coupled with CT has the highest accuracy for detecting inflammation in IBD [11]. ^{18}F-FDG is a radiolabeled glucose analogue that detects tissue glucose metabolism and has been the tracer of choice for PET studies for decades; however, radiolabeled leukocytes are also typically used to assess GI inflammation. Indeed, SPECT of technetium-99m (^{99m}Tc)-leukocytes was shown nearly two decades ago to accurately assess the extent and severity of disease for people with severe UC, correlating with endoscopic and histological findings [21]. However, limitations with radiolabeled leukocytes exist, including a relatively extensive protocol required for working up the cells, risks of cross-contamination, and importantly, the nonspecific nature of the tracer. Only limited information exists regarding the use of PET, SPECT, or CT for pediatric IBD, although the combination of ^{18}F-FDG PET/CT has been suggested to be the most reliable due to its improved anatomical mapping [22]. Importantly, ^{18}F-FDG dosing and imaging protocols are yet to be standardized for PET of pediatric patients, highlighting the need for more studies in this area. Nonetheless, the potential benefits of PET/CT in the pediatric IBD population were highlighted in the first published case, where scans in a 12-year-old child revealed diffuse heterologous uptake of ^{18}F-FDG in the gut wall, particularly in the stomach [23]. CT confirmed that the uptake was focal, and endoscopy revealed ulceration of the gastric wall, with an ensuing diagnosis of gastric and intestinal CD. It is important to note that while these technological advances are welcome for IBD management, none of them currently target specific mediators. This highlights the need for the development of new imaging technologies that are highly sensitive, quantitative, and able to provide longitudinal data in real time, but are also only minimally invasive.

3. Imaging Inflammatory Mechanisms in IBD: Which Targets to Choose?

3.1. *Immune Targets*

Inflammation is central to IBD; however, much remains to be understood regarding the causes of inflammation in humans. It is currently thought that inflammation in IBD is driven by a loss of immune tolerance to autologous proteins in the colon wall and foreign antigens in the lumen, including the microbiota. This initiates a cascade of cytokines and chemokines which cause an influx of immune cells, as comprehensively reviewed elsewhere [24,25]. Immune cells are attracted to sites of inflammation by chemokines and also by upregulated expression of "gut-homing" integrins on the cell surface, including $\alpha_4\beta_7$ [26]. Subsequent inflammatory lesions and damage to the epithelial wall and deeper layers of intestinal tissue result in the production of damage-related mediators during active disease, which progresses toward mediators related to tissue-remodeling and wound healing as disease improves toward remission, but also toward mediators associated with fibrosis after repeated or extended periods of inflammation. The immunological basis for the differences in pathophysiology of UC and CD are not clear, and while it is suggested that CD is driven by a T_H1/T_H17-type immune response and UC is mediated by a T_H2-type immune response (↑ IL-5, IL-13) that is atypical as IL-4 is not involved, much of the evidence for this generalization results from animal models. Furthermore, while there is evidence supporting a genetic predisposition, large-cohort IBD human studies indicate this is clearly complicated and contains loci that coexist in both CD and UC, but also other loci that are not shared [27,28]. Environmental factors are thought to contribute to IBD; however, evidence for or against these are heavily biased toward animal studies and are yet to be definitively determined in humans. The best evidence supporting an aggravating role for specific immune mediators in IBD is the success of several biologic medications targeting the cytokines TNF-α and IL-12/23 and the gut-homing integrin $\alpha_4\beta_7$. This success is tempered by the lack of efficacy of therapies directed against IL-17, IFN-γ, and IL-13, amongst others [29]. However, as failure of biologics in the clinic can be related more to toxicity and side effects than to a lack of involvement of their target, tracers directed to these cytokines may still prove to be valuable for imaging. With this in mind, new immune modulators are currently undergoing clinical trials, including those targeting specific cytokines such as IL-6, inhibitors of intracellular pathways including Janus kinases (JAK), and mediators related to tissue remodeling and fibrosis, such as matrix metalloproteases (MMP) [29].

Recent advances in two-photon laser scanning microscopy have enabled in vivo imaging of deep visceral tissues and immune cells in particular, indicative of the potential for this technique to provide preclinical evidence prior to relatively expensive immuno-PET studies. Two-photon laser scanning microscopy was instrumental in identifying goblet cell-associated passage (GAPs) as the major mechanism for delivering luminal antigens to dendritic cells in the small intestine in health [30]. Interestingly, GAPs only appear in the colon following the disruption of microbe-sensing pathways, highlighting a potential role in IBD [31]. We have recently applied two-photon laser scanning microscopy to investigate immune cell migration in the trinitro benzene sulphonic acid (TNBS) model of colitis, demonstrating in vivo that not only are immune cells in very close apposition to nerves within the colon wall, but also that myeloid cell infiltration is increased in TNBS colitis [32]. This technique can be easily combined with reporter mice and promises to increase the understanding of the mechanisms involved in immune cell migration from blood vessels into the lower GI tract in IBD.

3.2. *Microbiota*

Recent improvements in high-throughput "omics" technology have also revealed the involvement of the microbiome in IBD. The general consensus indicates that active IBD is characterized by an overall loss of diversity coupled with a dysbiotic phenotype primarily depicted by elevated Proteobacteria and decreased Firmicutes, although inconsistencies between studies remain [33–35]. Functionally, the major classes of bacteria affected relate to the production of short-chain fatty acids (e.g., *Faecalibacerium*

prausnitzii), bacteria with mucolytic activity (e.g., *Ruminococcous torques*), and sulfate-reducing species including *Desulfovibrio* [36–38]. While this dysbiotic composition may contribute to disease progression through dysregulated epithelial barrier function and aberrant immune signaling, it is currently contentious whether dysbiosis itself causes IBD or simply reflects the disease course [39].

Uncovering the compositional shifts in the microbiome has undoubtedly increased our understanding of its role in disease initiation, progression, and relapse. However, techniques such as 16S pyrosequencing are inherently limited by the variations that exist in aspects of their methodology, including inconsistent or incomplete reference libraries, the unknown impact of batched analyses, nonstandardized statistical analyses, and an inability to adequately appreciate the heterogeneity in the microbiome [40]. This is further confounded by the fact that the annotation is based on putative association of the 16S rRNA gene, with a taxon defined as an operational taxonomic unit (OTU) [41,42]. Although whole-genome shotgun sequencing has enhanced our ability to define species taxa with more accuracy, it remains an expensive alternative to 16S and requires significantly greater data analysis [40]. As such, conclusions regarding the functional implications of compositional shifts remain difficult to draw, relying on metagenomic inference using phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt). Nonetheless, changes in microbiota composition offer the intriguing potential to image microbiota as markers of disease severity in IBD.

4. PET and SPECT of Specific Mediators in IBD

PET of radioisotope-labelled antibodies is currently being used in multiple clinical trials in cancer to tailor personalized therapies based on specifically targeted diagnostic tests [43,44]. However, immune-PET of IBD is currently limited to preclinical studies. When deciding which inflammatory mediators to image in IBD, it is firstly important to understand which immune processes are likely to be involved in the model of choice, and secondly to understand what the information will provide regarding the initiating, perpetuating, and resolving phases of colitis.

4.1. Imaging Colitis in Animal Models

Animal models of colitis have significantly improved our understanding of how inflammation develops and perpetuates, highlighting roles for both the innate and adaptive arms of the immune response. There are currently numerous animal models of IBD, including colitis that develops due to primary genetic defects, adoptive transfer of activated immune cells, or is chemically induced [45]. Importantly, most of these models progress directly from acute through to fulminant colitis and therefore are not useful for investigating pathways involved in the resolution of inflammation and tissue remodeling. Nuclear imaging of colitis has so far relied upon two models of chemically induced colitis: the TNBS model and the dextran sulfate sodium (DSS) model. TNBS is administered by enema and inflammation is therefore restricted to the colon. TNBS is a hapten; it is not directly toxic, but instead binds to autologous proteins and luminal microbiota, turning them immunogenic. The colitis that develops is transmural, involves an IL-12-driven T_H1/T_H17-type response, and is therefore considered to model aspects of inflammation associated with CD [46]. DSS differs as it is directly toxic to epithelial cells and, when administered by the oral route in drinking water, results in a colitis that takes longer to develop than TNBS. DSS colitis is considered to model aspects of inflammation associated with UC as the colitis results from direct damage to the epithelial layer and is not as deep as that which occurs with the TNBS model. While animal models are useful for emphasizing the importance of specific mediators and mechanisms, it is important to note that findings from animal models can be difficult to translate to human IBD. This is common with most chronic diseases that have a remitting and relapsing course due to the involvement of multiple and overlapping pathways that are intertwined with disease stage as inflammation progresses and regresses, highlighting the requirement for extensive validation in human subjects.

4.1.1. PET and SPECT of Immune Mediators in Colitis

Cell-secreted immune mediators are generally classified as chemokines or cytokines, with chemokines mediating cell attraction and cytokines mediating immune responses. Table 1 provides an overview of PET/SPECT studies of immune mediators in human IBD and animal models of colitis. The first study investigating whether radiolabeled antibodies are efficacious for imaging colitis was disappointing, as uptake of ^{111}In-labelled IgG was substantially lower than ^{111}In-labelled leukocytes, the gold standard at the time, and also the newly developed ^{111}In-labelled liposomes in TNBS-colitic rabbits [47]. While imaging of liposomes and leukocytes enabled grading of inflammation, the contrast provided from IgG labelling was not sufficient for this to occur. This result was not surprising given the large abundance of IgG in circulating blood and its relative nonselectivity for colitis. Interleukin (IL)-8, more recently renamed CXCL8, is a chemokine produced by innate immune cells, but also cells in other tissues, including epithelial cells and endothelial cells. IL-8 is primarily involved in attracting neutrophils to inflammatory sites, but also attracts other granulocytes, including mast cells. Colonic inflammation in TNBS-treated rabbits was detected more readily with ^{99m}Tc-labelled IL-8 than ^{99m}Tc-labelled granulocytes [48]. Furthermore, the severity of inflammation was also able to be graded with ^{99m}Tc-IL-8, but not with ^{99m}Tc-granulocytes. More recently, IL-8 was labelled with ^{99m}Tc for SPECT studies and was found to have higher sensitivity for detecting inflammation than endoscopy in a large clinical study of people with IBD, although specificity was lower than for endoscopy [49]. TNF-α is a cytokine that is secreted by a range of immune cells from both the innate and adaptive arms of the immune response and is produced in large amounts in many autoimmune diseases, including IBD. Neutralizing TNF-α with antibodies, for example infliximab, has proven success in treating IBD [50,51]. In preclinical studies, ^{99m}Tc-labelled infliximab was able to discriminate between moderate and severe colonic inflammation in TNBS-treated rats [52]. However, enthusiasm for infliximab as a tracer in preclinical studies was hampered by high background uptake in healthy control rats. This study is of importance as it not only revealed TNF-α mediated inflammation, but it also demonstrated target specificity of a clinically useful drug for IBD. This is also particularly significant given the increased development of biologics, particularly antibody-based therapies, but also the relatively recent expansion of personalized medicine and theragnostic approaches toward treating disease.

Table 1. Summary of PET/SPECT of human IBD and animal models of colitis. - Not suitable for colitis imaging, + satisfactory image quality, ++ excellent image quality.

Target	Tracers	Species	Model	Outcome	Reference
Leukocytes	^{99m}Tc-HMPAO-leukocytes	Humans	UC	+	[21]
CXCL8	^{99m}Tc-CXCL8	Humans	CD and UC	+	[49]
β_7	^{64}Cu-FIB504.64-Fab	Mice	DSS	+	[53]
$\alpha_4\beta_7$	^{64}Cu-DATK32			+	
β_7	^{64}Cu-FIB504.64-Fab	Mice	DSS	+	[54]
β_7	^{64}Cu-FIB504.64-F(ab′)$_2$ (fragments)			++	
CD4	^{89}Zr-GK1.5 cys-diabody	Mice	DSS	++	[55]
TNF-α	^{99m}Tc-Infliximab	Rats	TNBS	+	[52]
IgG	^{111}In-IgG			-	
Leukocytes	^{111}In-WBC	Rabbits	TNBS	++	[47]
Liposomes	^{111}In-liposomes			+	
IL-8	^{99m}Tc-HYNIC-IL-8	Rabbits	TNBS	++	[48]
Granulocytes	^{99m}Tc-HMPAO-Granulocytes			+	

Abbreviations: UC: ulcerative colitis. CD: Crohn's disease. DSS: dextran sulphate sodium. TNBS: tri-nitro benzene sulphonic acid. WBC: white blood cell. Tc: technetium.

4.1.2. Imaging Immune Cells in Colitis

The DSS colitis model is the most commonly used model to image immune cells in colitis. Table 1 provides an overview of PET/SPECT studies of immune cells in human IBD and animal models of

colitis. The integrins α_4 and β_7 form a heterodimer ($\alpha_4\beta_7$) which specifically directs the migration, or homing, of immune cells to the gastrointestinal tract via interactions with its ligand, the addressin MAdDCAM-1 [26]. Targeting this pathway has proved successful in IBD with the recent development of vedolizumab, a monoclonal antibody that blocks $\alpha_4\beta_7$ [56]. Uptake of ^{64}Cu-labelled β_7 antibodies is increased in DSS-colitic mice relative to healthy controls and also to ^{64}Cu-labelled IgG isotype negative control, indicating the potential for β_7 as a tracer for human IBD [53]. However, high levels of background were also observed in nontarget organs including the small intestine and stomach, prompting the development of antibody fragments. In the same model, uptake of ^{64}Cu-labelled β_7 antibody fragments was superior to both whole ^{64}Cu-labelled β_7 antibodies and ^{64}Cu-labelled $\alpha_4\beta_7$, suggesting that antibody fragments may provide enhanced signaling of inflammation in IBD compared to whole antibodies [54]. The concept of increased sensitivity with small antibodies has recently been validated in cancer biology and in inflammation. ^{18}F-labelled antibody fragments directed against the antigen-presenting complex major histocompatibility complex (MHC)II were much more sensitive than standard ^{18}F-FDG in detecting xenoplanted tumors, even detecting tumors when growth was neither palpable nor visible [57]. Furthermore, ^{18}F-labelled antibody fragments directed against CD11b detected complete Freund's adjuvant (CFA)-induced inflammation in the mouse paw much earlier than standard ^{18}F-FDG [57]. Most recently, ^{89}Zr-labelled antibody fragments against the T_{HELPER} marker CD4 were observed to have higher uptake in the colon of DSS mice compared to healthy controls [55]. However, uptake was also increased in the spleen and lymph nodes, highlighting the intrinsic difficulties in imaging immune cells during inflammation, as they generally migrate to and from inflammatory sites and lymph nodes, incorporating blood vessels and lymphatics.

4.2. Imaging the Microbiota in Colitis

As our understanding of microbiota–host interactions grows, it is necessary to move beyond current genomics technology and compositional descriptions. This remains a daunting task when imaging specific microbiota in the gut, as it is limited by the diversity of tissue types that need to be maintained, which is of particular importance when considering the sensitivity of the mucous layer to standard fixation methods and the heterogeneity within gut microenvironments. As such, it is critical that a robust protocol including mucous-preserving sample preparation, image segmentation, and quantitative analysis tools be developed. A framework satisfying these criteria was recently developed by Earle et al. [58], where high-resolution quantification of the spatial organization of the gut microbiome revealed that changes in the proximity of microbes to the epithelium are sufficient to induce increased expression of key inflammatory markers, despite negligible shifts in microbiota composition. Most recently, chemical imaging techniques exploiting a blinking surface enhanced Raman spectroscopy (SERS) have been used to visualize bacteria in vitro with exquisite resolution, and have demonstrated the ability to differentiate between the chemical signatures of different bacteria [59]. MRI, CT, fluorescence/bioluminescence imaging, and PET have also been applied to investigate the activity of bacteria in vivo; however, these typically only provide indirect information about bacterial activity by inference from immune function [60,61]. Recent advances exploiting the bacterial uptake of carbohydrates have proven successful for the selective in vivo imaging of bacteria independently of host factors and secondary pathologies [61,62]. Furthermore, specific bacteria have also been labelled with iron oxide nanoparticles for MRI to track bacteria longitudinally during infection [60]. These developments highlight the potential to image specific bacteria during inflammation, but are yet to be applied to IBD.

5. Limitations of Radiolabeled Antibodies

Despite the evidence outlined above supporting the potential use of radiolabeled antibodies or immune mediators for in vivo diagnostic imaging, several issues remain that currently limit enthusiasm for widespread use. The primary limitation is economy-based, as specialized equipment is required for the manufacture and detection of radiolabels. These costs are high, but may be mitigated by improved

clinical outcomes, including early detection, and the potential development of personalized medicines to improve clinical responses and reduce side effects. Scientific challenges also exist, particularly with relation to the relatively long half-life of antibodies and the time required for them to move through the circulatory system to the site of inflammation and clear after studies have been performed. These issues relate to the increased risk of cancer due to radiation exposure, particularly as IBD patients already have a higher risk of colon cancer. However, PET is considered safe and the effective dose of whole-body ^{18}F-FDG was approximately 7 mSv, much less that the 16 mSv used for a routine abdominal–pelvic CT with contrast [63,64]. Notable efforts are underway to reduce circulation time by using pretargeted methods, as explained in more detail below. The relatively large structure of antibodies may lead to difficulties in penetration of the site of inflammation, although this may be mitigated by altering the structure of the antibody, also explained in more detail below. A limitation also relates to the single mode of PET detection, as only a single mediator can be imaged. This is problematic in terms of detailed studies of immune cell subsets, as they are typically identified by flow cytometry techniques requiring multiple targets. Furthermore, mediators must be accessible to antibodies and are therefore restricted to soluble cytokines and chemokines or markers located on the outer surface of the cell membrane. While intracellular cytoplasmic cytokines and nuclear transcription factors such as FOXP3 (T_{REG}) and ROR-γ (T_H17) are increasingly being used to characterize immune cell subsets, antibody penetration inside immune cells typically requires cell permeabilization, which is not possible in vivo.

6. Future Directions for Imaging IBD

Remarkable progress has been achieved in recent years toward understanding the mechanisms underlying IBD. This has resulted in a number of specific biologics for treatment, but they are yet to be applied to diagnosis or surveillance. The complex immunological mechanisms of IBD indicate that there are multiple markers that could potentially be used for diagnostic imaging and drug bioavailability. Imaging specific sites of inflammation should be achievable relatively easily; however, determining markers that differentiate between IBD and other gastrointestinal diseases, such as cancer, is more problematic and requires extensive preclinical validation.

Intact antibodies are increasingly being used as therapeutics; however, their utility as imaging agents is reduced by their extended half-life, as the duration of time required to reach a sufficient signal-to-noise ratio is long. Furthermore, extended half-lives may also lead to other harmful biological activity and side effects, potentially altering the biological function being imaged; however, in theory, this risk is minor as concentrations are much typically much lower for imaging than for treatment. Recent developments in antibody engineering include a shift from whole IgG antibodies to fragments, enhancing their physical properties and leading to enhanced efficacy as extensively reviewed in [65]. Enzymatic cleavage or restructuring of the antibody attenuates problems associated with half-life by decreasing clearance times. Furthermore, decreasing the amount of time required to reach a suitable signal also permits the use of short-lived isotopes, reducing radiation exposure.

Pretargeting strategies have also emerged as a means of improving efficacy whilst reducing radiation dose; for example, by using a novel biorthogonal inverse electron demand Diels–Alder reaction between a ^{64}Cu-labelled radioligand and a site-specific immunoconjugate [66]. This occurs in four steps: (a) injection of the modified antibody, (b) accumulation of the antibody at its target site and clearance from the blood, (c) injection of the radioligand 24 h later, and (d) the in vivo click ligation between the immunoconjugate and radioligand and subsequent excretion of excess radioligand. The target was able to be delineated four hours post-injection of the radioligand in tumor tissue, with image contrast improved over time and an ideal signal-to-noise ratio at 24 h. This pretargeting approach and similar approaches may expedite the clinical translation of future immunoconjugates for imaging.

A tantalizing approach is the potential to image different bacterial species; however, this is again currently limited by the lack of knowledge of functional roles for specific microbiota in IBD and the

limited ability of PET to simultaneously label multiple targets. Furthermore, imaging of microbiota in general currently requires models where the bacteria can be isolated, labelled, and readministered. Future developments including the ability to use multiple labels, but also advances in the identification of bacterial species of interest and anaerobic culturing of microbiota, would be a welcome addition to preclinical models of IBD in the first instance before clinical studies are warranted. Furthermore, the potential to exploit factors that are selectively produced by bacteria and differentiate between active and quiescent states may provide intriguing insights. Bacterial toxins may be the ideal sensor as they are typically only secreted during disease, are readily identifiable by antibodies, and are relatively easily attached to various detection technologies. However, biosensing technology remains limited in its ability to provide insight into the spatial organization of the microbiome, and as such, the ideal microbial analysis tool remains elusive and current investigations must combine genomic, visual, and activity-based measures.

7. Summary

Imaging has a critical role in the diagnosis and management of IBD patients, where endoscopic studies are currently the gold standard. However, there is a clear need for new technologies that are less invasive than endoscopy, but more penetrative at both the local level within the intestinal wall and more broadly along the entire intestinal length. These factors are most relevant for pediatric IBD, where risks of adverse outcomes are increased, and also in small intestinal inflammation and fibrosis, which is generally not accessible by endoscopy. Animal studies indicate that PET and SPECT of immune cells and/or mediators are potentially useful for IBD management and diagnosis. The potential targets for PET/SPECT of immune mediators and cells in active IBD are numerous; however, options for imaging of microbiota are currently limited by the lack of understanding of the role that specific microbiota contribute toward IBD. Translation of these technologies into human studies in IBD is currently nonexistent, yet applicability in other diseases such as cancer is encouraging. Imaging of currently available biologics should be relatively achievable in the short term, while longer-term goals include the development of tracers with deep tissue penetration and short half-lives. Concurrent research into the immune mechanisms underlying IBD should deliver targets that offer insight into disease mechanisms, for example in pediatric IBD, and can differentiate between diseases and/or disease states, for example between active inflammation and fibrosis in UC and CD. In conclusion, the future for the development of imaging techniques in IBD is bright, and further refinement will clearly lead to benefits in the diagnosis and management of IBD patients.

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Abbreviations

^{64}Cu	Copper-64
^{18}F	Fluorine-18
^{89}Zr	Zirconium-89
^{99m}Tc	Technetium-99m
^{111}In	Indium-111
CD	Crohn's disease
CD4	Cluster of differentiation 4
CD11b	Cluster of differentiation 11b
CFA	Complete Freund's adjuvant
CT	Computed tomography
CXCL8	C-X-C motif chemokine ligand 8; interleukin 8

DSS	Dextran sodium sulfate
GAP	Goblet cell-associated passage
GI	Gastrointestinal
GIT	Gastrointestinal tract
Fab	Fragment antigen-binding
HMPAO	Hexamethylpropyleneamine oxime
HYNIC	Hydrazinonicotinic acid
IBD	Inflammatory bowel disease
IgG	Immunoglobulin G
IFN	Interferon
IL	Interleukin
JAK	Janus kinase
MAdCAM-1	Mucosal vascular addressin cell adhesion molecule 1
MHCII	Major histocompatibility complex II
MMP	Matrix metalloproteinase
MRI	Magnetic resonance imaging
mSv	Milliseiverts
OTU	Operational taxonomic unit
PET	Positron emission tomography
rRNA	Ribosomal ribonucleic acid
SERS	Surface enhanced Raman spectroscopy
SPECT	Single photon emission computed tomography
TNBS	Trinitro benzene sulfonic acid
TNF-α	Tumor necrosis factor alpha
UC	Ulcerative colitis
VHH	Variable domain of heavy-chain antibodies
WBC	White blood cell

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FEATURED BASIC SCIENCE ARTICLE

Immuno-PET of Innate Immune Markers CD11b and IL-1β Detects Inflammation in Murine Colitis

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Inflammatory bowel disease (IBD) is a chronic relapsing and remitting inflammatory disease of the gastrointestinal tract. The diagnosis and monitoring of IBD are reliant on endoscopy, which is invasive and does not provide information on specific mediators. Symptom flare in IBD is associated with increased activation of innate immune pathways. Immuno-PET approaches have previously demonstrated the ability to detect colitis; however, a direct comparison of antibodies targeted to innate immune mediators and cells has not been done. We aimed to compare immuno-PET of antibodies to IL-1β and CD11b against standard ^{18}F-FDG and MRI approaches to detect colonic inflammation. **Methods:** Colonic concentrations of IL-1β and myeloperoxidase were determined by ELISA, and colonic infiltration by CD11b-positive CD3-negative innate immune cells was determined by flow cytometry and compared between healthy and dextran sodium sulphate–treated colitic mice. PET of ^{89}Zr-lα-IL-1β, ^{89}Zr-α-CD11b, and ^{18}F-FDG was compared by volume-of-interest analysis and with MRI by region-of-interest analysis. Imaging results were confirmed by ex vivo biodistribution analysis. **Results:** Colonic inflammation was associated with impaired colonic epithelial barrier permeability, increased colonic IL-1β and myeloperoxidase concentrations, and increased CD11b-positive CD3-negative innate immune cell infiltration into the colon. ^{89}Zr-α-IL-1β and ^{89}Zr-α-CD11b immuno-PET detected colonic inflammation, as did ^{18}F-FDG, and all PET tracers were more sensitive than MRI. Although ^{18}F-FDG volumes of interest correlated with colitis severity and a strong trend was observed with ^{89}Zr-α-IL-1β, no correlation was observed for ^{89}Zr-α-CD11b or MRI. ^{89}Zr-α-IL-1β was distributed mainly to the gastrointestinal tract, whereas ^{89}Zr-α-CD11b was distributed to more tissue types. **Conclusion:** Immuno-PET using antibodies directed to innate immune markers detected colonic inflammation, with ^{89}Zr-α-IL-1β providing a more tissue-specific signal than ^{89}Zr-α-CD11b. Development of these technologies for human subjects will potentially provide a less invasive approach than endoscopy for diagnosing and monitoring IBD.

Key Words: immuno-PET; innate immune system; colitis; inflammatory bowel disease

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Inflammatory bowel disease (IBD) is a collection of chronic idiopathic inflammatory diseases of the gastrointestinal tract, including Crohn disease and ulcerative colitis (*1*). IBD substantially affects quality of life, and although treatable, significant gaps remain because of incomplete efficacy, lack of tolerance, and side effects, leading to variable periods of remission and relapse (*2*). Patients with IBD require constant surveillance because prognostic indicators of symptom flare remain poor and patients with long-standing disease have an increased risk of developing colon cancer (*2,3*). The diagnosis and management of IBD are currently heavily reliant on endoscopy, which is generally well tolerated but is invasive, is restricted to imaging of the superficial mucosal surface, cannot easily reach all areas of the small intestine, and does not provide immediate information on specific mediators or mechanisms. Therefore, new technologies are required for imaging IBD that are less invasive than endoscopy and can provide information on specific pathologic processes in real time.

Clinical studies support the use of PET, SPECT, and MRI for detecting inflammation in the gastrointestinal tract, satisfying the criteria for reduced invasiveness and real-time information (*4,5*). However, current clinical PET, SPECT, and MRI approaches in IBD are restricted to using radiolabeled autologous leukocytes and ^{18}F-FDG in PET and SPECT and using gadolinium contrast in MRI, none of which provides information on specific mediators. This lack of detail is of increasing importance because of the increase in use of target-specific biologics, particularly antibodies, and the emergence of personalized medicine.

Monoclonal antibodies have exquisite selectivity for their target, and their applicability to immuno-PET has been appreciated for decades, particularly in the cancer field (*6–8*). However, the use of immuno-PET in IBD is currently limited to preclinical studies, in which T-cell infiltration into the colon of colitic animals has been demonstrated either directly by imaging the T_{HELPER} cell marker CD4 (*9*) or indirectly by targeting the gut-homing integrin $\alpha_4\beta_7$ (*10*). In contrast, immuno-PET of immune mediators is yet to be reported; however, direct labeling of the neutrophil-attracting

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chemokine CXCL8 (also known as IL-8) (*11,12*) and antibody-directed imaging of TNF-α (*13*) have shown some efficacy for SPECT in both colitic animals and human IBD.

Activation of the innate immune system is intimately linked to inflammation in IBD, most obviously illustrated by the clinical utility of detection of the neutrophil-secreted mediators calprotectin and lactoferrin in stool (*3*). IL-1β is an inducible cytokine that has a key role in innate immune responses and is predominantly secreted by neutrophils, monocytes, macrophages, and dendritic cells, whereas CD11b is a pan-myeloid innate immune marker predominantly expressed on these same cell types. Here, we aimed to compare the ability of PET of ^{89}Zr-conjugated antibodies against IL-1β and CD11b with the ability of standard (^{18}F-FDG) PET and MRI to detect inflammation in colitic mice.

MATERIALS AND METHODS

All experiments were approved by the Animal Ethics Committee of the South Australian Health and Medical Research Institute (SAHMRI) and the University of Adelaide.

Mice

Male C57BL/6jSah mice aged 10–14 wk (20–30 g) were bred and group-housed in a specific pathogen-free environment at SAHMRI. Animals had ad libitum access to food and water. Experiments were conducted with only male mice to eliminate the potential confounding effects of the estrous cycle. Mice were humanely euthanized via CO_2 inhalation and cervical dislocation to remove tissues.

DSS Colitis Model

Colitis was induced by replacing normal drinking water with 2% (w/v) dextran sodium sulphate (DSS) (molecular mass, 40–50 kDa; Alfa Aesar) for 5 d, followed by an additional 3 d of normal drinking water. Mice were assessed daily for signs of acute colitis, including a decrease in body weight and diarrhea. Healthy mice were age- and weight-matched. Colon length was measured on excised colons dissected free from surrounding tissue from the tip of the anus to the distal end of the cecum.

Ussing Chamber Studies

Distal colon segments were mounted into 0.1 cm^2 aperture sliders bathed in 5-mL baths with oxygenated glucose-fortified Ringers solution for Ussing chamber analysis (Physiologic Instruments) as previously described (*14*). Briefly, voltage-clamped tissues were equilibrated for 20 min before baseline transepithelial electrical resistance (Ω·cm^2) and short circuit current (μA/cm^2) were averaged over 10 min.

Myeloperoxidase Activity

Myeloperoxidase activity in the distal colon was determined as previously described (*15,16*). Briefly, phosphate-buffered saline–washed tissue segments were homogenized in 0.5% hexadecyl-trimethylammonium bromide (Sigma-Aldrich) and reacted with *o*-dianisidine hydrochloride (Sigma-Aldrich). Absorbance at 450 nm was averaged over 5 min at 30-s intervals. Myeloperoxidase activity is expressed as units/mg of tissue, with 1 unit of myeloperoxidase activity defined as the amount of myeloperoxidase required to degrade 1 μmol of peroxide per minute at 25°C.

IL-1β ELISA

Proteins were extracted from the distal colon as previously described (*14,15*). Briefly, colon segments were homogenized for 5 min in cell extraction buffer (ThermoFisher Scientific) supplemented with protease inhibitor cocktail (Sigma-Aldrich), 1 mM phenyl-methanesulfonyl fluoride (Sigma-Aldrich), and 50 μM ethylenediaminetetraacetic acid at 1 mL/50 mg of tissue. The lysate was incubated (1 h, 4°C, with rotation), sonicated (2 min), and centrifuged (14,000 rpm, 15 min, 4°C). Supernatant was collected and stored at −80°C until analysis. IL-1β concentrations were determined by OptEIA IL-1β ELISA (BD Biosciences) as previously described (*14,15*). The limit of sensitivity was less than 4 pg/mL. Concentrations were normalized to total protein concentration as determined by a bicinchoninic acid assay (Abcam) as previously described (*15*).

Flow Cytometry

Colon lamina propria mononuclear cells (LPMCs) were isolated enzymatically, essentially as previously described (*14,15*). Briefly, the colon was excised free of fat and blood vessels, flushed with cold phosphate-buffered saline, roughly chopped, and incubated in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffered Hanks balanced salt solution supplemented with 1 mM ethylenediaminetetraacetic and 1 mM dithiothreitol (20 min, 37°C, gentle shaking). Tissue segments were filtered (100 μM), and the remaining tissue was minced and incubated (20 min, 37°C, gentle shaking) in complete RPMI 1640 (Gibco) supplemented with fetal calf serum, GlutaMAX (Life Technologies Corp.), penicillin/streptomycin, collagenase D (1 mg/mL), DNAse1 (0.5 mg/mL), and dispase (3 mg/mL) (all from Sigma-Aldrich) before being strained (40 μM) and resuspended over a 40/80 Percoll gradient and the interphase LPMC layer collected and pelleted for LPMCs. Viability was determined (trypan blue), and only preparations with at least 80% viability were investigated further. F_c-blocked LPMCs (0.5 × 10^6) were stained with the antimouse monoclonal antibodies CD45-BV605 (30-F11), CD3-BUV395 (145-2C11), and CD11b-APC-Cy7 (M1/70) (all from BD Biosciences). Per test, 20,000 events were analyzed using a FACS-Fortessa (BD Biosciences), and proportions of live (FVS700; BD Biosciences) singlets were determined using FlowJo (Tree Star), as described previously (*14,15*).

Antibody–Chelator Conjugation and Radiolabeling

^{89}Zr-oxalate was produced via proton irradiation of ^{89}Y target on a PETtrace 880 cyclotron (GE Healthcare) and purified on Alceo (Comecer) as previously described (*17*). Monoclonal α-CD11b (M1/70) and α-IL-1β (B122) (BD Biosciences) were conjugated to ^{89}Zr with NCS-Bz-DFO (Macrocyclics) linkage as previously described (*18,19*). Competitive blocking assays determined the specificity of ^{89}Zr-conjugated antibodies, whereby 0.5 × 10^6 healthy mouse splenocytes were incubated with 1.25 μg of ^{89}Zr-labeled α-IL-1β or α-CD11b for 20 min at 37°C, in the presence or absence of 50 μg of unlabeled α-IL-1β or α-CD11b added for 20 min beforehand. Cells were pelleted and counted using a Hidex γ-counter for accumulation of ^{89}Zr. All counts were background- and decay-corrected. ^{18}F-FDG (phosphate buffer) was produced by the Molecular Imaging and Therapy Research Unit of SAHMRI on a FASTLab synthesizer (GE Healthcare).

MRI-PET

A 3 to 5 MBq quantity of ^{89}Zr-α-IL-1β (37.5 μg) and ^{89}Zr-α-CD11b (85 μg) in 100–200 μL of saline was administered intravenously (tail vein) 24 h before imaging, and a 10 to 15 MBq quantity of ^{18}F-FDG in 100–200 μL of saline was administered intravenously 30 min before imaging. Some mice underwent MRI first. The mice were anesthetized with isoflurane (2%), and a T2-weighted rapid-acquisition-with-refocused-echoes (RARE) spin-echo sequence was acquired with a benchtop MRI system (Bruker Icon 1T; Bruker Physik GmbH) (repetition time, 84 ms; echo time, 4,314 ms; number of excitations, 1; RARE factor, 8; field of view, 80 × 80 mm; slice thickness, 2 mm; scan time, <2 min). ITK snap (www.itksnap.org) was used for consequent segmentation of the scans (*20*). PET experiments were performed over 15 min using a submillimeter-resolution (0.7 mm) Albira PET-SPECT small-animal scanner (Bruker Biospin GmbH). Manually drawn volumes of interest in the area of interest were applied to analyze average and total radiotracer accumulation using the PMOD imaging suite (PMOD Technologies).

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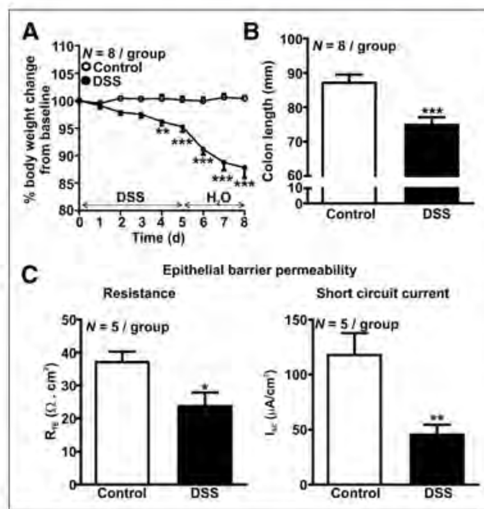

FIGURE 1. Characterization of DSS colitis. (A) Loss of body weight. (B) Shortening of colon length. (C) Impaired epithelial barrier transepithelial electric resistance (left) and short-circuit current (right). *$P < 0.05$. **$P < 0.01$. ***$P < 0.001$. R_{TE} = transepithelial electrical resistance.

Ex Vivo Biodistribution Studies

Immediately after postimaging culling, organs of interest were dissected, weighed, and counted for ^{89}Zr accumulation as outlined above for blocking experiments.

Statistical Analysis

Data are expressed as mean ± SEM in all cases. The significance of results was determined by unpaired t testing except for weight loss data, for which a 2-way ANOVA with Bonferroni post hoc testing was used. The strength and significance of correlations were determined by Pearson analysis. Differences with a P value of less than 0.05 were considered statistically significant.

RESULTS

DSS Colitis Association with Body Weight Loss, Colon Shortening, and Impaired Epithelial Permeability

Body weight was reduced in the DSS-treated group 4 d after treatment began ($P < 0.01$, Fig. 1A) and was further reduced by about 10% 8 d after treatment began ($P < 0.001$, Fig. 1A). By day 8, DSS-treated mice also had approximately 15% shorter colons ($P < 0.001$, Fig. 1B), an approximately 35% reduction in epithelial resistance ($P < 0.01$, Fig. 1C), and an approximately 60% reduction in short-circuit current ($P < 0.01$, Fig. 1C), indicative of colonic inflammation and damage to the epithelial barrier.

Increase in Colonic Innate Immune Infiltration in DSS Colitis

DSS-treated mice had an approximately 7.5-fold increase in colonic myeloperoxidase activity ($P < 0.001$, Fig. 2A) and an approximately 2.5-fold increase in colonic IL-1β concentration ($P < 0.01$, Fig. 2B), indicating that innate immune mediators are increased in colitic mice. The proportion of CD11b colonic LPMCs was increased about 6-fold in DSS-treated mice ($P < 0.001$, Fig. 2C), indicative of an infiltration by innate myeloid cells, including neutrophils, monocytes, macrophages, and dendritic cells.

Immuno-PET Detection of Colonic Innate Immune Activation in Colitis

^{89}Zr was successfully conjugated to α-IL-1β and α-CD11b as demonstrated by outcompeting binding in the presence of excess unlabeled antibody (^{89}Zr-α-IL-1β: 8,135 ± 1,510 counts per min [cpm] hot vs. 3,026 ± 337 cpm cold, $P < 0.05$, $n = 3$; ^{89}Zr-α-CD11b: 61,535 ± 8,355 cpm hot vs. 34,811 ± 4,081 cpm cold, $n = 3$, $P < 0.05$). PET imaging revealed that in DSS colitic mice, distal colonic uptake of ^{89}Zr-α-IL-1β was increased about 3-fold ($P < 0.001$, Fig. 3A, Supplemental Videos 1 and 2; supplemental materials are available at http://jnm.snmjournals.org), ^{89}Zr-α-CD11b was increased about 5-fold ($P < 0.05$, Fig. 3B, Supplemental Videos 3 and 4), and ^{18}F-FDG was increased about 3.5-fold ($P < 0.01$, Fig. 3C, Supplemental Videos 5 and 6). A robust positive correlation ($R^2 = 0.70$, $P < 0.05$) was observed between colonic uptake of ^{18}F-FDG and percentage body weight loss (Fig. 3C), with a strong trend toward a similar effect observed for ^{89}Zr-α-IL-1β ($R^2 = 0.54$, $P = 0.09$, Fig. 3A) but not for ^{89}Zr-α-CD11b (Fig. 3B).

Biodistribution of ^{89}Zr-α-IL-1β and ^{89}Zr-α-CD11b

Ex vivo analysis indicated that uptake of ^{89}Zr-α-IL-1β and ^{89}Zr-α-CD11b was increased throughout the gastrointestinal tract in DSS-treated mice relative to control mice but also in some extragastrointestinal sites. Specifically, uptake of ^{89}Zr-α-IL-1β was increased about 3-fold in the colon ($P < 0.05$), 5-fold in the cecum ($P < 0.01$), 1.5-fold in the small intestine ($P < 0.05$), and 1.75-fold in the stomach ($P < 0.05$), whereas uptake in the spleen was also increased about 1.75-fold ($P < 0.05$) (Fig. 4A). Uptake was similar between DSS-treated and control mice in all other tissues investigated. Uptake of ^{89}Zr-α-CD11b was more discrete in the gastrointestinal tract, with increases observed only in the colon (~26-fold, $P < 0.05$) and small intestine (~20-fold,

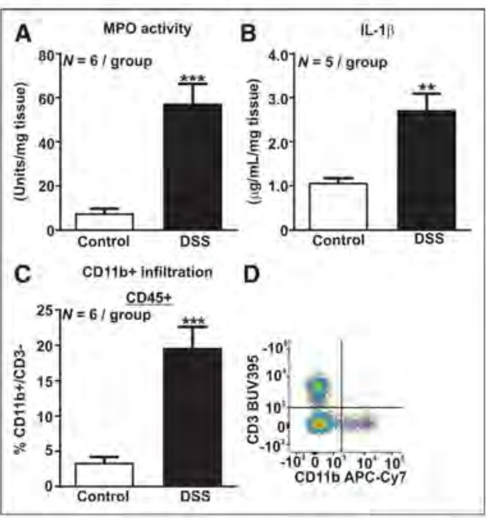

FIGURE 2. Activation of innate immune system by DSS colitis. (A) Increase in colonic myeloperoxidase activity. (B) Increase in IL-1β concentrations. (C) Increase in CD11b-positive CD3-negative innate immune cell infiltration. (D) Gating strategy. **$P < 0.01$. ***$P < 0.001$.

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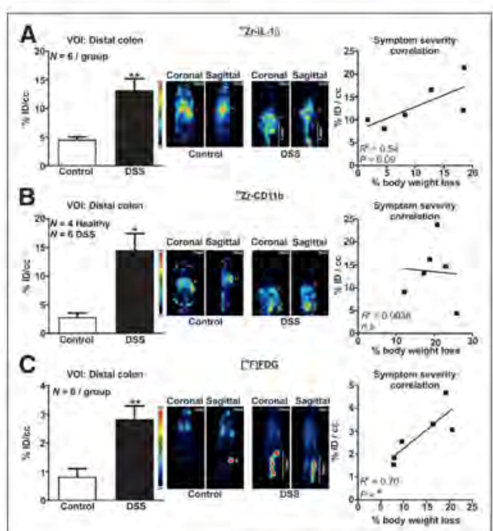

FIGURE 3. α-IL-1β, α-CD11b, and ^{18}F-FDG PET detection of colonic inflammation. Shown are volume-of-interest analysis with representative PET image (coronal and sagittal) of control and DSS mice (left) and correlation of percentage body weight loss with volume of interest (right) for ^{89}Zr-α-1 L-1β (A), ^{89}Zr-α-CD11b (B), and ^{18}F-FDG (C). Li = liver; B = bladder; ID = injected dose; n.s. = not statistically significant; VOI = volume of interest. *$P < 0.05$. **$P < 0.01$.

$P < 0.05$) and a trend toward an increase in the cecum (~21-fold, $P = 0.083$) (Fig. 4B). However, increased uptake of ^{89}Zr-α-CD11b was observed in more sites outside the gastrointestinal tract than ^{89}Zr-α-IL-1β, including approximately 6-fold in the spleen ($P < 0.05$) but also approximately 7-fold in the liver ($P < 0.05$) and 8-fold in the bone marrow ($P < 0.05$). Notably, trends toward increased uptake of ^{89}Zr-α-CD11b were observed across most other tissues, including the cecum, tail, and stomach; however, uptake was generally more variable for ^{89}Zr-α-CD11b than for ^{89}Zr-α-IL-1β, limiting the significance of these observations.

MRI Detection of Colonic Inflammation in Colitic Mice

MRI analysis of colonic inflammation revealed an approximately 2-fold increase in the T_2 signal intensity ratio in DSS colitis relative to control ($P < 0.01$, Fig. 5, Supplemental Videos 7 and 8), but this analysis did not correlate with percentage body weight loss (Fig. 5).

DISCUSSION

We demonstrate for the first time, to our knowledge, that immuno-PET directed against innate immune cells and mediators is applicable for detection of colitis. The sensitivity of ^{89}Zr-labeled α-IL-1β and α-CD11b immuno-PET for detecting colonic inflammation was similar to that of ^{18}F-FDG, and all PET approaches were more sensitive than MRI. Furthermore, uptake of ^{89}Zr-α-IL-1β was restricted to the gastrointestinal tract and spleen, whereas ^{89}Zr-α-CD11b was distributed over a broader range of tissue types. Our comparison demonstrates the strong potential that immuno-PET of innate immune mediators has for diagnosing and monitoring IBD.

Activation of the innate immune system is a hallmark of inflammation in IBD both in humans and in animal models (*3,21*). DSS is directly toxic to epithelial cells, and we confirm that epithelial damage in DSS colitis is associated with increased colonic infiltration of CD11b-expressing cells and increased colonic concentrations of innate immune cell–secreted IL-1β (*22*), both of which we were reliably able to detect by PET of ^{89}Zr-conjugated monoclonal antibodies. Previous immuno-PET studies have identified increased infiltration of T cells in DSS colitis, either by directly labeling of the T_{HELPER} marker CD4 or by labeling of the gut-homing integrin

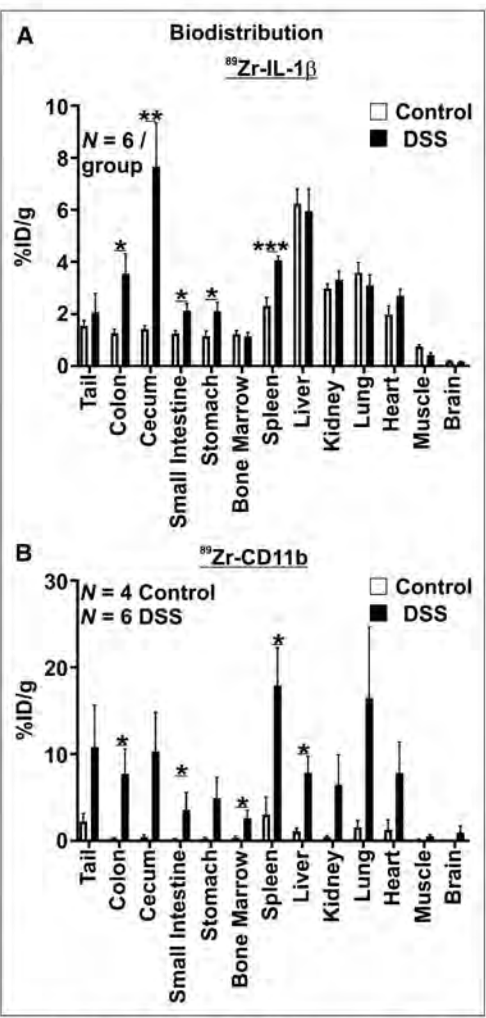

FIGURE 4. Ex vivo biodistribution of ^{89}Zr-α-1 L-1β (A) and ^{89}Zr-α-CD11b (B). Decay-corrected values for uptake are displayed. *$P < 0.05$. **$P < 0.01$. ***$P < 0.001$. ID = injected dose.

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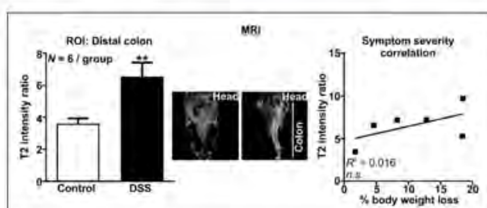

FIGURE 5. MRI detection of colonic inflammation. Region-of-interest analysis with representative MR image (coronal) of control and DSS mice (left) and correlation of percentage body weight loss with region-of-interest MRI analysis (right). $^{**}P < 0.01$. n.s. = not statistically significant; ROI = region of interest.

complex $\alpha_4\beta_7$, which is upregulated on T cells in IBD (*9,10,23*). However, we believe our study to be the first to describe the potential that radiolabeled antibodies against innate immune markers have for PET in IBD diagnosis and monitoring.

We observed a striking difference in the biodistribution of ^{89}Zr-α-IL-1β and ^{89}Zr-α-CD11b, with ^{89}Zr-α-IL-1β being restricted to the gastrointestinal tract and spleen whereas ^{89}Zr-α-CD11b was observed in a broad range of tissues. Furthermore, ^{89}Zr-α-IL-1β also correlated with colitis severity whereas ^{89}Zr-α-CD11b did not. These findings are likely to relate to immunologic processes underlying innate immune activation in colitis. Innate immune cells migrate from induction sites in lymphatic tissue through the bloodstream into sites of inflammation, where they mature and secrete cytokines, including IL-1β, before clearing from tissue by the lymphatics or dying (*24*). CD11b is expressed on innate cell subsets that promote inflammation, such as M1 macrophages and most neutrophil subsets, but also on innate immune cells that promote tolerance, wound healing, and immune suppression, including M2 macrophages, dendritic cells, and CXCL4-expressing neutrophil subsets (*25,26*). These cells express CD11b at all stages of maturity, and imaging CD11b is therefore likely to detect sites of inflammation but also migration between tissues and lymph nodes, including the bone marrow and, particularly in mice, the spleen. Furthermore, whereas CD11b expression is high on myeloid innate immune cells, it is also expressed on other immune cells, including T- and B-cell subsets, which may contribute toward the signal we observed in our imaging (*27*). The expression of individual markers on multiple cell types is common; for example, CD4 is expressed on macrophages in addition to T_{HELPER} cells, and complex flow cytometry using multiple markers is typically required to conclusively characterize individual immune cell subsets. For IBD, this is particularly relevant to the ever-expanding T_{HELPER} cell subsets and innate lymphoid cells, which rely on staining of cytoplasmic cytokines or nuclear transcription factors for their characterization (*28*). Definitively identifying these subsets for in vivo imaging is difficult in live cells because antibodies are typically too large to passively enter cells.

In contrast, IL-1β has 2 favorable characteristics for immuno-PET. First, it is inducible rather than constitutive and therefore is secreted only by activated cells, which, in colitis, are predominately located at sites of inflammation. Second, proteolytic processing of IL-1β into its mature form typically occurs within the cell, and it is secreted as a mature functional protein (*29*). This is somewhat an anomaly for cytokines but is beneficial for immuno-PET because only mature IL-1β is imaged. Our results support further investigation into the use of imaging inducible and intracellularly processed immune mediators for detecting inflammation in IBD. Furthermore, immuno-PET using radiolabels with long half-lives, such as ^{89}Zr, enables longitudinal imaging, which would provide valuable information on the temporal nature of immune responses in IBD.

SPECT is a cost-effective technique for nuclear imaging but generally has reduced resolution relative to PET. Nonetheless, SPECT imaging of the neutrophil-attracting chemokine CXCL8, and CD4, has also shown potential in preclinical and human studies of IBD (*11,12,30*). Furthermore, whereas SPECT imaging of the TNF-α–neutralizing antibody infliximab also demonstrated some efficacy in preclinical colitis, it was hindered by high background, the cause of which was likely its chimeric mouse–human properties (*13*). The most readily available alternative markers for immuno-PET imaging of IBD include antibodies that are currently used for treatment, including variations targeting TNF-α, $\alpha_4\beta_7$ (vedolizumab), $\alpha_4\beta_1$ (natalizumab), and IL-12/23 (ustekinumab) (*31*). However, markers do not have to be limited to the immune system. Epithelial damage and increased epithelial barrier permeability are hallmarks of IBD and colitis, as we demonstrate in our model (*32,33*). The permeability of the epithelial monolayer to luminal contents is regulated by a large family of tight-junction proteins, many of which are reduced in degraded tissue in the inflamed intestine, impeding their use as markers. Conversely, expression of some of these proteins promotes increased permeability and is increased during IBD, such as the pore-forming claudin-2 (*34,35*). Other potential markers include those associated with tissue remodeling associated with the resolution of inflammation (*36*). Matrix metalloproteases are involved in tissue remodeling and, interestingly, are thought to play a key role in promoting fibrosis. Although an antibody against matrix metalloprotease 9 was ultimately withdrawn from clinical trials on IBD, interest in this target remains and it may have more promise as a marker of fibrosis (*37*).

The clinical utility of immuno-PET for IBD is currently limited by several issues. Primarily, antibodies are large molecules with molecular weights of about 150 kDa. This large size prolongs their half-life and reduces excretion, which is beneficial when used as treatments but is challenging for imaging because tissue penetration is reduced and excretion prolonged. Issues related to tissue penetration may be somewhat diminished in IBD because vascular permeability is increased in inflammation, aiding immune cell migration into inflammatory sites (*38*). However, increased leukocyte extravasation is unlikely to drastically reduce the time taken for systemically administered antibodies to reach inflammatory sites in IBD, and the current clinical paradigm would require dosing and imaging to occur at separate visits over several days. This requirement results in increased exposure to radiolabels, potentially increasing the risk of side effects. Promising alternatives to intact antibodies that potentially limit these concerns are in development (*39*). Notably, engineered small antibody fragments against anti-CD4 and β_7 have already shown promise in preclinical colitis (*9,10*). A different strategy uses a 2-step process for pretargeting. The initial step comprises administration of an antibody modified to incorporate a radiolabel capture site, which is allowed enough time to localize to its target. A few days later, the radiolabel is injected and binds to the antibody. This strategy has shown promise in cancer (*40*) but has yet to be applied to IBD and may prove difficult because the harsh protease-rich environment in inflammation may rapidly degrade targets.

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CONCLUSION

We have demonstrated that radiolabeled antibodies against innate immune cells and mediators are suitable for immuno-PET of colitis. Our results provide further evidence of the clinical utility of immuno-PET for IBD diagnosis and monitoring. Development of these techniques would be of particular benefit to patients who are difficult to scope, including the pediatric population and those that have inflamed or fibrotic regions of the small intestine.

DISCLOSURE

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Immuno-PET of Innate Immune Markers CD11b and IL-1β Detects Inflammation in Murine Colitis

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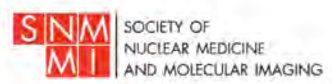

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