Type 1 Interferon signalling in human intestinal T cells

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Statement of Originality

I, Edward Mark Giles, confirm that the research included within this thesis is my own work or that where it has been carried out in collaboration with, or supported by others, that this is duly acknowledged below and my contribution indicated. Previously published material is also acknowledged below.

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Ed Giles
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Details of collaboration and publications:
Work in chapter 4 (section 4.4.5) on peripheral T cells was assisted by James Lung, a student in the laboratory. Work in chapters 4 and 5 (Sections 4.5.7.4 and 5.4.1) on mRNA expression of Interferon Stimulated Genes was assisted by Mohini Pathak, also a student in the laboratory. Some primers used in those sections were a kind gift of Dr Raj Lahiri and Professor Graham Foster. The 'Phosflow' protocol, described in detail in chapters 2 and 3, was used in a separate work, published recently: Lahiri R, Derwa Y, Bashir Z, Giles E, Torrance HD, Owen HC, O'Dwyer MJ, O'Brien A, Stagg AJ, Bhattacharya S, Foster GR, Alazawi W; Systemic Inflammatory Response Syndrome After Major Abdominal Surgery Predicted by Eary Upregulation of TLR4 and TLR5. Ann Surg. 2015 May 27. [Epub ahead of print]

Abstract

Intestinal immune homeostasis depends on the appropriate balance of pro-inflammatory and regulatory T cell activities which are themselves influenced by local mucosal signals. Dysregulation of these pathways leads to inflammatory bowel disease (IBD). Type 1 Interferon (T1IFN; IFNα/β), signalling via the phosphorylation of Signal Transduction and Activation of Transcription (STAT)1, is critical for innate protection against viruses but also has diverse effects on the immune system. They are potential local regulators of intestinal T cells. In mice, endogenous T1IFN ameliorates colitis by promoting regulatory T cell function. T1IFN has been used to treat IBD patients with limited success, but little is known about T1IFN in the human intestine. The aim of this work was test the hypothesis that T cells in the human intestine are modulated by intestinal factors such as T1IFN and that these responses are altered in IBD.

A 'Phosflow' technique was developed to measure activation of key signalling molecules such as STATs at the single cell level. Responsiveness of human intestinal T cells to T1IFN was confirmed and increased expression of phosphorylated (p) STAT1 observed in CD4 T cells from IBD colonic tissue, irrespective of T1IFN stimulation. Increased pSTAT1 was associated with reduced expression of the STAT1 regulator *SOCS1.* Phosphorylation of STAT3 and STAT5 was not altered in IBD. Constitutive STAT1 phosphorylation was due in part to exposure to IFNβ. A potential role for T1IFN was supported by increased expression of Interferon Stimulated Genes (ISGs) by intestinal CD4 T cells in IBD and by the detection of endogenous IFNβ in fresh frozen sections of human colon. Addition of neutralising anti-IFNβ antibody to intestinal biopsy cultures led to an enhanced pro-inflammatory T cell cytokine profile and, in cells from healthy tissue, fewer $IL10^{+}$ T cells. These data suggest that in health the endogenous T1IFN-STAT1 pathway supports a regulatory cytokine profile in human CD4+ intestinal T cells. There is evidence for dysregulation of this pathway in IBD, but the functional significance of this is less clear.

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Abbreviations

APC	Antigen Presenting Cell
CD	Crohn's Disease
CD(x)	Cluster of Differentiation (x)
CM	Conditioned Medium
DC	Dendritic Cell
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic Acid
dsRNA	Double-stranded RNA
DSS	Dextran Sulphate Sodium
EAE	Experimental Autoimmune Encephalitis
ERK	Extracellular signal-regulated kinase
FoxP3	Forkhead box P3
GALT	Gut-associated Lymphoid Tissue
GWAS	Genome Wide Association Study
IBD	Inflammatory Bowel Disease
IFN	Interferon
IFNAR	Interferon Alph/Beta Receptor
Ig(x)	Immunoglobulin (x)
IL-(x)	Interleukine – (x)
IRG	Interferon Regulated Gene
ISG	Interferon Stimulated Gene
JAK	Janus Kinase
LP	Lamina Propria
LPMC	Lamina Propria Mononuclear Cell
LPS	Lipopolysaccharide
MHC	Major Histocompatibility Complex
MLN	Mesenteric Lymph Node

MS	Multiple Sclerosis
NOD	Nucleotide-binding oligomerization domain-containing protein
PBMC	Peripheral Blood Mononuclear Cell
PP	Peyer's Patches
PRR	Pattern Recognition Receptor
RA	Retinoic Acid
RNA	Ribonucleic Acid
RORγt	RAR (Retinoic Acid Receptor)-related Orphan Receptor, isoform 2
SD	Standard Deviation
SFB	Segmented Filamentous Bacteria
SOCS	Suppressor of Cytokine Signalling
STAT	Signal of Transducer and Activator of Transcription
T1IFN	Type 1 Interferon
T-bet	T-box Expressed in T cells
TCR	T cell Receptor
TFH	Follicular B Helper T cell
TGF	Transforming Growth Factor
TH(X)	T helper subtype (x)
TLR	Toll-Like Receptor
TNF	Tumour Necrosis Factor
TNFα	Tumour Necrosis Factor α
Treg	Regulatory T cell
UC	Ulcerative Colitis

Chapter 1

1 Introduction

1.1 Chapter Overview

In this overview there will be a brief summary of the major topics covered in the introduction, providing the context for the thesis, which will be explored in greater detail in the corresponding sections.

1.1.1 Background

The human intestine contains the majority of immune cells in the body, and is in direct association with a vast amount of foreign antigen. The various component layers of the mucosal immune system all work in concert to facilitate nutrition while sampling antigen and maintaining homeostasis. Intestinal T cells are important in maintaining mucosal integrity, and their function can be affected by various mechanisms. It is increasingly recognized that even after priming in organized lymphoid tissue and returning to the mucosa as effector memory cells, intestinal T cells retain the capacity to alter their phenotype in response to environmental cues. This is sometimes referred to as T cell plasticity. This may allow a more dynamic response to pathogenic or other challenges from the intestine. New techniques in the laboratory allow for a greater understanding of T cell function, and do not rely on traditional measurements of cytokine production potential alone.

There are potentially multiple mechanisms by which T cell function can be modulated, but the role of Type 1 Interferon (T1IFN) signalling in T cells has been shown to be important in chronic viral infection with implications for other forms of chronic inflammation. Indeed, many mouse models show that T1IFN maintains signalling proteins in T cells at a level so they are primed to produce a pro-

regulatory response. This may be a direct effect, but is complicated by the ubiquitous nature of the T1IFN receptor (IFNAR), and its complex effects on all parts of the mucosal immune system.

1.1.2 IBD

The Inflammatory Bowel Diseases (IBD), comprised primarily of Crohn's Disease (CD) and Ulcerative Colitis, are thought to be caused by inappropriate immune reactivity to components of the commensal microbiota driving chronic intestinal inflammation (Kaser et al., 2010). Genome wide association studies (GWASs) have identified over 90 genetic loci/genes that confer IBD susceptibility (Jostins et al., 2012; Lees et al., 2011), but these alone do not explain preponderance to develop disease. Many of the candidate genes, however, are involved in different aspects of the mucosal immune system thought to be pertinent to IBD pathogenesis. Precise immunological mechanisms are not fully defined but encompass dysregulated recognition of bacterial components by cells of the innate immune system with 'downstream' effects on the balance of pro-inflammatory and regulatory components of the adaptive immune response (Duchmann et al., 1995; Peyrin-Biroulet et al., 2008).

Both CD and UC are often viewed as T cell mediated diseases. GWAS data and mouse models of IBD have suggested the importance of T cells and their cytokine products in the pathogenesis and perpetuation of disease. *Ex vivo* analysis of human IBD patient samples have supported this and treatments are used which target various T cell pathways with mixed success. Greater understanding of the plasticity of T cell response to inflammation in the intestine, as well as differences in T cell phenotype in patient samples, may provide insights into new therapies or improved targeting of current drugs.

As described below, this thesis focuses on T1IFN as a particular factor important in the control of T cell plasticity. T1IFN has been used as a treatment in IBD in the

past with limited success. Greater understanding of its ability to promote immune homeostasis via T cell responsiveness may provide understanding of these mechanisms in individuals in health and disease.

1.1.3 “Plasticity” of mucosal T cells

While the pathogenesis of IBD is largely unknown, it is thought that the adaptive immune system is the main driver of chronic intestinal inflammation (Neurath, 2014b; Shale et al., 2013). $CD4^+$ 'helper' T cells are critical in this inflammation. These have traditionally been characterized by their phenotype in association with IBD and many other inflammatory conditions (Shale et al., 2013). The interaction between the antigen presenting cell (APC) that presents the cognate antigen to a naïve T cell, including the cytokine milieu in which it occurs, determines the phenotype of T helper cell differentiation (ie TH1, TH2 etc). Recently, the traditional labeling of helper T cells has been questioned and a more plastic system is being explored (O'Shea and Paul, 2010). Much of the previous research into the T cell phenotype of intestinal inflammation has used the “TH” model of the adaptive immune system, based on cytokine production potential. Modern scientific instruments may allow a more dynamic approach to investigating T cell responses, including at mucosal surfaces.

As the mucosal immune system is constantly interacting with an extraordinarily large amount of antigen, predominantly from the microbiota, T cells being able to respond more flexibly to this challenge would be of great benefit to the host.

1.1.4 Signal Transduction and Activators of Transcription (STATs) as mediators of T cell response

Signalling pathways in T cells are extremely complex and interact at multiple levels. However, of principal importance in the differentiation of T cell phenotype and cytokine production are the STAT family of proteins. There are 7 members of the

STAT family, which all have distinct functions that are context and cell type dependent. All the STATs are responsive to T1IFN (Darnell et al., 1994; Gough et al., 2012). Many members of the STAT family have been implicated in different ways in the immunological processes underlying IBD, but the studies in humans have generally looked at inflamed whole tissue specimens which may miss significant cell specific effects.

A new technique that allows measurement of phosphorylated or activated STAT proteins at a single cell level, Phosflow, may therefore allow a better understanding of the responsiveness of mucosal T cells to their environment.

1.1.5 Type I Interferon as a co-ordinator of T cell response

There are a very large number of signals that the immune system uses to respond such a complex environment. In order to better understand T cell responses in a dynamic manner, it is useful to probe the signalling machinery by response to known cytokines. Type I Interferon (T1IFN), while traditionally associated with restriction of viral replication, is increasingly recognized for its role in the immune system (Hertzog and Williams, 2013). This includes directly or indirectly priming T cell responses (Le Bon et al., 2006; Le Bon et al., 2001). Its constitutive role in the gut is recently been shown to have a regulatory role in mouse models of intestinal inflammation (Kole et al., 2013; Lee et al., 2012). The role of T1IFN in the human gut is less well understood; however T1IFN has been used as a treatment for both forms of IBD (Gasché et al., 1995; Mannon et al., 2011; Moschen et al., 2008).

1.1.6 Summary

The data presented in this thesis explores new techniques for assessing T cell responsiveness in the human intestine. In particular, it attempts to assess the role of T cell responses to environmental signals, such as T1IFN, as potential modulators of gut inflammation or regulation. Finally, the work in this thesis explores the

differences in T cell response to T1IFN between humans in health, and those with IBD. This should give a clearer understanding of the role of T cells in this complex condition.

The first chapter is divided into 4 further sections. First, IBD is described in greater detail, with a focus on the current understanding of disease pathogenesis and the inflammatory response. Next, general mucosal immunology is briefly summarized, with a focus on the role of intestinal T cells in humans. This then leads on to a more in depth description on intestinal T cells in IBD. Finally, the role of T1IFN in the mucosal immune system is explored, with a particular focus on its role in human intestinal T cells and IBD.

1.2 Inflammatory Bowel Disease

1.2.1 Background

Inflammatory Bowel Disease is an increasingly common disease of chronic relapsing intestinal inflammation (Henderson et al., 2012). The two predominant forms are Crohn's Disease and Ulcerative Colitis, and both may present with symptoms of pain, diarrhoea, per rectal bleeding and significant weight loss. IBD is believed to be caused by an inappropriate immune response to the commensal microbiota, in the context of a complex interplay between genetic factors and environmental factors that are not well understood (reviewed by Baumgart and Carding 2007).

1.2.2 Clinical Phenotypes

1.2.2.1 Crohn's Disease

Crohn's Disease can manifest as inflammation that can affect all layers of the intestine (transmural). It can affect any part of the Gastro-Intestinal (GI) tract, from the mouth to the anus, and there can be isolated areas of inflammation separated by apparently normal areas (reviewed by Baumgart and Sandborn, 2012). One of the major difficulties in better understanding IBD pathogenesis is the vast heterogeneity in the condition. CD in particular has such wide variation that the new Paris criteria for the categorisation of paediatric and adult CD defines 3 different groups of ages of onset; 5 different combinations of sites of disease; 3 different types of disease activity; as well as extra-intestinal and perianal features, and effect on growth – and any combination of these features can occur (Levine et al., 2011).

These different phenotypes may represent the endpoint of different and poorly understood immunological mechanisms.

The most common area of the GI tract affect by CD is the terminal ileum (45%), then the colon (32%) and both in 18.6% (Thia et al., 2010). Paediatric patients have a more severe phenotype (Van Limbergen et al., 2008) and the disease progresses over time, with surgery necessary in 50% of patients within 5 years of diagnosis (Vernier-Massouille et al., 2008). Histological features include inflammatory infiltrates in all layer of the intestine (mucosa, submucosa and serosa), with non-caseating granulomas in any location in up to 50% of patients (reviewed by Baumgart and Sandborn, 2012).

1.2.2.2 Ulcerative colitis

Inflammation in UC is confined to the mucosal layer, and is almost always present in the rectum. The disease then extends proximally along the colon in a continuous manner, and may include just the rectum or as much as the entire colon (pancolitis; reviewed by (Ford et al., 2013). Histological features include leukocyte infliltration in the LP, with distorted mucosal architecture (reviewed by Ordás et al., 2012). Again, paediatric patients have a more severe phenotype, with a much greater incidence of pan-colitis (Van Limbergen et al., 2008).

1.2.2.3 IBD Unclassified

A small percentage of patients with IBD have features of both CD and UC and are termed IBDU (Levine et al., 2013). Over time, these patients may progress to a final diagnosis of either CD or UC, or remain unclassified (Levine et al., 2013).

Crohn's Disease	Ulcerative Colitis
Pan-intestinal disease (any area mouth to anus can be affected)	Colon only
Diarrhoea++, Weight loss++, Blood per rectum+, Abdominal pain++	Blood per rectum+++, Diarrhoea++, Abdominal pain+, Weight loss+
Skip lesions	Continuous inflammation from rectum
Transmural inflammation	Inflammation usually confined to mucosa
Granulomas may be present	No granulomas
Fistulae can occur	Fistulae very rare
Strictures occur	Strictures unusual

Table 1.1 *Distinguishing features between Crohn's disease and Ulcerative Colitis*

1.2.2.4 Extraintestinal Features

All forms of IBD can present or be complicated by extraintestinal manifestations in approximately 25% of cases. Involvement can include almost any organs system but most commonly include skin, eye, liver, biliary system and joints (reviewed by Das, 1999). Progression of the extraintestinal disease usually, but not always, follows the disease course of the bowel (reviewed by Das, 1999).

1.2.3 Current (and future) therapy in IBD

IBD therapy differs in CD and UC, and is dependent on many patient factors, some of which have been described above. The aim of management for both conditions, however, is the same – that of clinical remission. This means that patients should be able to lead a symptom-free existence. More recently, there has been a move towards deeper remission, termed mucosal healing. This term describes aiming to return the intestinal mucosa to normal both macroscopically and histologically, on the evidence that this improves long term outcomes for patients (reviewed in Neurath, 2014b).

1.2.3.1 Crohn's disease

The mainstay of therapy to induce remission in CD is corticosteroids. These are thought to work by various mechanisms, including inhibition of several inflammatory pathways: suppressing interleukin transcription, stabilising the NFκβ complex, suppression of arachidonic acid metabolism and stimulation of apoptosis of lamina propria lymphocytes (Mowat et al., 2011). An alternative to steroid therapy is exclusive enteral nutrition. This is where patients consume only a diet of a nutritionally complete feed, and exclude all other nutrition, for a period of some weeks, and is commonly used in paediatric patients in Europe. This has similar efficacy to corticosteroids, without the many side effects (Ruemmele et al., 2014). The mechanism of action of enteral nutrition remains unknown.

After induction of remission, many patients with CD do not require further ongoing therapy. However, should the disease be refractory, the therapy can be escalated. The treatment paradigm is complex, but second line agents are immunomodulators, principally azathioprine. Azathioprine induces T cell apoptosis by altering Rac1 signalling (Tiede et al., 2003).

Third line therapy is usually a biological agent targeting the pro-inflammatory cytokine Tumour Necrosis Factor α (TNFα) (Hanauer et al., 2002; Sandborn et al., 2007). Other therapies include cyclosporin, a calcineurin inhibitor, preventing clonal expansion of T cell subsets (Mowat et al., 2011).

Surgery remains an important therapy in CD, particularly for isolated small bowel disease, or in stricturing and penetrating disease (Ruemmele et al., 2014).

Newer therapies include other biological agents targeting lymphocyte homing markers (see section 1.3.5) and other (non-TNF) cytokines, some of which will be discussed later.

1.2.3.2 Ulcerative colitis

The first line therapy for mild UC is an agent containing 5 amino-salicylic acid (5-ASAs). These work as anti-inflammatory agents locally in the mucosal epithelium by a variety of mechanisms to inhibit release of lipid mediators, suppress inflammatory cells and their cytokine production, and inhibit reactive oxygen species (reviewed in Mowat et al., 2011). They are also used, less frequently, in mild CD, particularly with disease affecting the colon.

Induction of remission of more significant UC is with corticosteroids. Unlike in CD, exclusive enteral nutrition is not effective.

Second and indeed third line therapy in UC is similar to CD, although surgery is considered more frequently in UC, as colectomy may be considered a curative operation (Mehta et al., 2013).

Newer therapies for UC include a small molecule inhibitor, tofacitinib, which selective inhibits Jak3, in the Jak-STAT pathway (Sandborn et al., 2011a). This has also been trialed in CD, as many of the other newer CD treatments are tested in UC.

Faecal transplantation has been attempted in UC and to a lesser extent in CD with variable success, with more studies no doubt to follow (Colman and Rubin, 2014).

Treatment of IBDU varies according to clinical phenotype, but broadly is similar to treatment in UC. A summary of the common IBD therapies is shown in table 1.2.

	Crohn's Disease	Ulcerative Colitis
5-ASA	Occasionally used, especially for mild colitis induction of remission	Very frequently used, induction and maintenance, sole agent in mild disease
Exclusive Enteral Nutrition	Effective, 1st line in paediatrics for induction of remission	Ineffective
Corticosteroids	Effective for induction of remission	Effective for induction of remission
Azathioprine and other immunomolators	Effective in maintenance of remission for some	Effective in maintenance of remission for some
Monoclonal anti-TNFα antibodies	Effective in induction and maintenance of remission, variable duration	Effective in induction and maintenance of remission (only recently used for maintenance)

Table 1.2 *Table comparing common current therapies used in CD and UC*

1.2.4 Pathogenesis of IBD (see schematic figure 1.2)

1.2.4.1 Genetic Predisposition to IBD

Twin studies have shown concordance in monozygotic twins for CD is between 20-50%, and in dizygotic twins is 10% (Halme et al., 2006). In UC the heritability is less, with monozygotic twins having concordance of only 16%, and dizygotic 4% (Halme et al., 2006).

The first gene identified with disease-associated variants in CD patients was nucleotide oligomerization domain (NOD)-2 (Hugot et al., 2001). One of three polymorphisms is present in approximately 40% of Western CD patients (Cuthbert et al., 2002). NOD2 is an intracellular receptor for muramyl dipeptide (MDP), which is a component of bacterial peptidoglycan, although can also be triggered by viral infection (reviewed in Shaw et al., 2011). Signalling via NOD2 leads to activation of NF-κB and production of pro-inflammatory cytokines (Girardin et al., 2003; Inohara

et al., 2003). While this shows the NOD2 pathway to be associated with pro-inflammatory effects, the main variants found in CD, namely amino-acid substitutions Arg702Trp and Gly908Arg, and the frameshift mutation FS1007insC, are loss of function, and yet apparently contribute to increased gut inflammation. The variant of NOD2 FS1007insC, associated with a severe CD phenotype, strongly suppresses transcription of pro-regulatory IL-10 in from mononuclear cells in patients with CD (Noguchi et al., 2009). It has also been shown that a microRNA (microRNA-29) is induced by NOD2 activation, which controls colitis associated with a TH17 cytokine signature (Brain et al., 2013).

MDPs, signalling via NOD pathways and NFκB, lead to the production of T1IFN (Leber et al., 2008; Park et al., 2007). It is conceivable therefore that this pathway of T1IFN induction may be important in the pathogenesis of IBD. Viral infection with T1IFN production, involving activation of the NOD1/2 pathway, augments mortality to *E. coli* sepsis (Kim et al., 2011).

GWAS have identified 163 genetic loci implicated in risk for IBD (Jostins et al., 2012). Of these, 53 are specific to either CD or UC, but the majority is common to both diseases (Jostins et al., 2012). The magnitude of risk associated with any polymorphism is very small, but may provide an insight into pathways involved in the pathogenesis of IBD (reviewed by Graham and Xavier, 2013).

For example, both CD and UC are associated with genes associated with the development and function of TH17 cells, including *IL12R*, *IL12B* (encoding IL-12/23p40), *JAK2* and *STAT3* (Anderson et al., 2011), and the *IFNAR* gene, which encodes IFNAR, the receptor for all T1IFNs, is itself a susceptibility loci for IBD (Jostins et al., 2012). Despite the large number of known loci, these only account for 13.6% of CD cases and 7.5% of UC (Jostins et al., 2012). This, in addition to the concordance in monozygotic twins being less than 100% demonstrates the importance of environmental factors in pathogenesis. Additionally, it has been proposed that epigenetics, or heritable non-coding DNA modifications that influence

gene expression, could bridge the “heritability-gap” between known disease variants and environmental factors (reviewed by Ventham et al., 2013).

Finally, a mouse model incorporating one of known susceptibility gene variants, *Atg16L1* (a gene involved in the autophagy pathway), has shown that the mutation alone causes no intestinal damage, but this mouse is particularly sensitive to viral infection (Cadwell et al., 2010). This suggests that genetic predisposition to IBD may require further triggers, including viral infection (hence environmental T1IFN), before development of overt disease.

1.2.4.2 Environmental factors

Tobacco smoking is the most well identified environmental factor, and is associated with increasing severity of CD (Seksik et al., 2009) and is protective against UC (Mahid et al., 2006). Other environmental factors are thought to be related to the “western lifestyle”, including sanitation, diet, antibiotics, urbanization, microbial exposure and pollution (reviewed by Ng et al., 2013). High incidence areas vary according to geography, and include North America, Northern Europe and Australia (Ng et al., 2013).

IBD is thought to be due to an aberrant response of the host immune system to the commensal microbiota (reviewed by Baumgart and Carding, 2007). Therefore the microbiota itself is postulated to be a significant environmental factor in the pathogenesis of IBD.

The first line of cellular defence against intestinal pathogens is the epithelial barrier, described in section 1.3.2. Increased intestinal permeability is associated with both CD and UC, which suggests an increase in exposure to the intestinal microbiota (reviewed by Baumgart and Sandborn, 2012; Ordás et al., 2012).

Patients with IBD are found to have reduced diversity of their microbiota (reviewed by Sartor, 2008). An increase in adherent invasive *E. coli* (AIEC) has been found in ileal CD (Boudeau et al., 1999; Darfeuille-Michaud et al., 2004). A reduction in the

numbers of *Faecalibacterium prasunitzii* (*F. prau*) has been associated with increased relapse of post-operative CD (Sokol et al., 2008). However, an increase in *F. prau* has been identified in paediatric CD patients at diagnosis (Hansen et al., 2012). Much less is known about the resident viruses of the intestine, or virome, compared to bacterial populations. However, recently there has been a study showing that the virome in IBD patients appears to differ from healthy controls (Norman et al., 2015).

There is a strong association between acute GI infection and either the onset or exacerbation of IBD (García Rodríguez et al., 2006; Gradel et al., 2009; Porter et al., 2008). Given that the majority of GI infections are viral, it is plausible that in a genetically susceptible host, the immunological response to a viral infection may be inappropriate or otherwise lead to chronic inflammation.

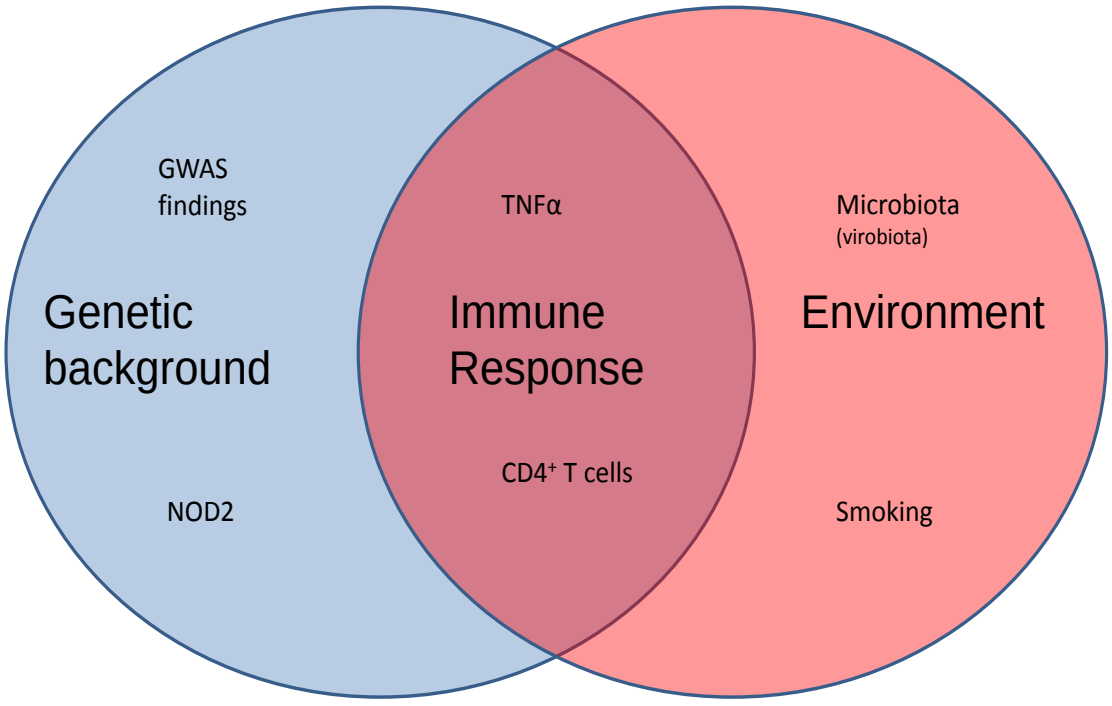

Figure 1.1 Schematic representation of the pathogenesis of IBD. *IBD is considered to be an abnormal immune response triggered by environmental factors in a genetically susceptible host. This schematic lists some of the key factors identified in this process.*

1.2.5 Inflammatory response in IBD

Currently, the most clinically relevant pro-inflammatory cytokine in IBD is TNFα, which is produced by Lamina Propria Mononuclear Cells (including T cells) in significantly higher quantities than healthy controls (MacDonald et al., 1990; Reinecker et al., 1993). This is also true for IL-6 and IL-1β (MacDonald et al., 1990; Reinecker et al., 1993). Monoclonal antibodies to TNFα such as infliximab and adalimumab are effective therapies in CD and UC (reviewed by Akobeng and Zachos, 2004; Lawson et al., 2006), as discussed in section 1.2.3. However, the soluble TNF receptor-Fc fusion protein etanercept is ineffective in CD (Sandborn et al., 2001). While both etanercept and infliximab neutralise soluble TNFα, only infliximab binds surface TNFα on activated T cells to induce apoptosis (ten Hove et al., 2002; Van den Brande et al., 2003). This indicates that neutralization of soluble TNFα is insufficient to resolve intestinal inflammation, which may require the removal of cells which produce pro-inflammatory cytokines.

1.2.6 IBD as a (T cell) immunological disease

There is various circumstantial evidence that inflammation in both UC and CD is perpetuated by intestinal T cells. For example, histological specimens of affected areas of intestine show a predominance of lamina propria $CD4^+$ T cells. Immunomodulatory therapy that is effective in IBD appears to selectively target T cells (see above section 1.2.3). Patients with Human Immunodeficiency Virus (HIV), with a loss of $CD4^+$ T cells, have shown clinical improvement with co-existent CD (Pospai et al., 1998). Certainly many of the mouse models of colitis are dependent on T cell driven inflammation.

However, in order to fully understand the importance of T cells in human disease, it is important to understand their place in a complex mucosal immune system. It is therefore worth summarizing current understanding of mucosal immunology, with a particular focus on T cells, before returning to IBD as a T cell-mediated disease.

1.3 Intestinal Immunity

At birth, the small intestine already has a length of approximately 300cm (Weaver et al., 1991), necessary to facilitate sufficient nutrient absorption for growth. The human intestine is exposed to vast amounts of antigen in the form of food components and commensal micro-organisms. The existence of intestinal pathogens that do not need to penetrate host tissues to cause disease mandates active sampling of luminal contents. However multiple immune mechanisms act to limit responsiveness to commensal organisms and other non-pathogenic antigens, while maintaining the ability to detect and clear pathogens. This balance is termed immune homeostasis and it maintains the healthy intestine in an active immune state termed physiological inflammation. There are multiple components of the mucosal immune system that all interact in order to maintain this delicate balance.

1.3.1 The microbiota

The intestine contains a vast number of bacteria, fungi, parasites and viruses that are known as the microbiota. In the human, the number exceeds 100 trillion, of which most are bacteria and largely found in the colon (reviewed in Kamada et al., 2013). The complex interaction with the host is largely beneficial as bacteria can degrade otherwise indigestible material releasing nutrients available for absorption (reviewed by Bäckhed et al., 2005). Commensal bacteria, that is those in a symbiotic relationship with the host, also compete with more pathogenic strains to protect the host from severe infection (reviewed by Hooper and Macpherson, 2010). The microbiota varies widely between species and within individuals, and this variation provides great complexity in the interactions of the mucosal immune system.

For some time, the microbiota has been known to interact with the immune system, including effects on $CD4^+$ T cells (Saparov et al., 1999). For example, particular species of the microbiota of the *Clostridia* species have been associated with

induction of regulatory T cells and protection from colitis in mice (Atarashi et al., 2013).

Much less is known or understood about the virobiota. There are a large number of both eukaryotic viruses and bacteriophages in the intestine at any given time (Duerkop and Hooper, 2013). It is not yet clear which of these are stable members of the “virobiota” or represent a more varied population over time compared to the bacterial species. It is estimated that the average human has between 8 to 12 chronic viral infections at any one time (Virgin et al., 2009).

1.3.2 Barrier Defences

The first line of interaction between the microbiota and the host is a physical barrier. The intestinal epithelium is a single cell layer covered by mucous which protects the body from the intestinal lumen but allows the mucosal immune system to sample antigen. This is while also allowing nutrients to freely pass through all its layers.

1.3.2.1 Mucous Layer

There are two layers to the mucous of the murine colon, and one layer in the small intestine. The inner colonic layer, adjacent to the epithelium, is considered sterile. The outer layer, as with the small intestinal mucous, is penetrated by bacteria (Ermund et al., 2013; Johansson et al., 2008). Mucous is predominantly formed by the protein MUC2. MUC2 is produced by goblet cells to form its viscous structure through disulphide bonds (reviewed in Johansson et al., 2011). There are also anti-bacterial factors within the mucous which act to protect the host. The defensins HBD-1 and HBD-4, the cathelicidin LL37 and multiple lysozymes have all been found within human mucous (Antoni et al., 2013). Emulsifiers, which interrupt the

mucous barrier, have been shown to enhance bacterial translocation across the intestinal epithelium (Roberts et al., 2010).

1.3.2.2 Intestinal Epithelium

The intestinal epithelium is a single continuous layer of cells separated from the lamina propria (LP) by its basement membrane. There are four differentiated cell types within this layer. Enterocytes are the most common, which are columnar cells with a brush border lined with microvilli. The microvilli, combined with the long physical length of the human small intestine and its villus projections, maximize the surface area for absorption. Goblet cells, as described above, produce mucins to form the mucous layer (Johansson et al., 2013). Paneth cells produce the majority of the anti-microbial peptides, including α-defensins, while most epithelial cells produce β-defensins (reviewed by Hooper and Macpherson, 2010). The rarest cell type are the enteroendocrine cells which produce a variety of hormones (Rehfeld, 1998).

All of these cells are differentiated from intestinal stem cells located in the crypt, which provide a continuous replacement of cells as they are sloughed off during intestinal transit (reviewed by Humphries and Wright, 2008). Cells are kept in close association (while allowing some paracellular transport) by tight junction proteins such as claudins (reviewed by Abreu, 2010). In mice, the tight junction protein ZO-1 has its expression enhanced in bacterial infection (Gibson et al., 2008), demonstrating that even the physical barrier of the gut can respond to pathogens. Recently, IFNβ (a cytokine discussed in detail in the thesis) has been shown to enhance epithelial barrier function in mice by a pathway inhibited by *E. coli* infection (Long et al., 2014).

In specialized areas of the intestine, overlying gut-associated lymphoid (GALT) tissue, the epithelium contains microfold cells (M cells). These cells lack both

microvilli and mucous protection and therefore allow preferential antigen uptake (reviewed by Mowat, 2003).

1.3.2.3 Secretory IgA and other products

A large quantity of IgA (approximately 3 grams per day) is secreted into the intestinal lumen (Hooper, 2009). This IgA is produced in the LP by terminally differentiated B cells, termed plasma cells. This IgA forms large polymers of its dimeric form and is transported across the epithelium (reviewed by Macpherson et al., 2008). Luminal IgA limits bacterial adherence to the epithelium (Suzuki et al., 2004). Production of IgA by plasma cells will be further discussed in section 1.3.2.3.

Other antimicrobial products, such as defensins, cathelicidins and C-type lectins, are secreted into the lumen can kill bacteria directly via enzymatic digestion (reviewed by Hooper and Macpherson, 2010). In murine models, the presence of bacteria in the intestinal lumen is necessary for the production of some of these products as well the mucous protein MUC-2 (Frantz et al., 2012; Rakoff-Nahoum et al., 2004). This demonstrates the close relationship between the microbiota and the development of the mucosal immune system at many levels.

1.3.3 Innate Immune Defence

The first line of the cellular immune system is the innate system. The nature of the intestinal barrier ensures that microbes and their products cross into the LP to encounter the innate system. In the intestine, many bacterial pathogens utilize the transcytosis of M cells to directly invade the LP (reviewed by Mowat, 2003). Due to the common entry of bacteria into the LP, the mucosal immune system needs to be able to rapidly clear them without always prompting a large inflammatory response. Indeed, when mice were genetically altered to impair phagocytic killing, they developed large abscesses filled with commensal bacteria (Shiloh et al., 1999). The immune cells that are responsible for this response initially are primarily

macrophages, although neutrophils are rapidly recruited to the intestine when there is an inflammatory response (reviewed in Kinnebrew and Pamer, 2012). Finally, there are a variety of antigen presenting cells, in particular dendritic cells (DC), that co-ordinate the sampling of microbial products and orchestrate response from the adaptive immune system.

1.3.3.1 Intestinal Macrophages

In health, human intestinal macrophages can phagocytose potentially pathogenic bacteria without a pro-inflammatory cytokine response (Smythies et al., 2005). Intestinal macrophages, unlike other populations, appear to be derived continuously from circulating monocytes, as has been recently demonstrated in mice (Bain et al., 2014). This suggests that local factors, such as those derived from the microbiota, may determine the differentiation fate of these cells. The non-responsiveness of these cells is associated with the low or absent expression of many pattern recognition receptors (Schenk et al., 2005; Smythies et al., 2005). Intestinal macrophages are also non-responsive to poly I:C (a synthetic dsRNA molecule), despite their expression TLR3 (Smythies et al., 2010) A major receptor for this ligand, This appears to be due to a reduction in some aspects of the signalling machinery, including NF-κB and/or MyD88 and TRIF (Smythies et al., 2010).

In murine models, a population of intestinal macrophages is also capable of antigen presentation, and can sample antigens across the epithelium directly from the intestinal lumen (Rescigno et al., 2001; Rivollier et al., 2012; Schulz et al., 2009). However, these cells have a poor ability to stimulate naïve T cells, and do not generally migrate to draining lymph nodes (Rivollier et al., 2012; Schulz et al., 2009).

1.3.3.2 Intestinal Neutrophils

While absent in the steady-state, neutrophils are rapidly recruited to the mucosa in inflammation by chemokines including CXL1 and CXCL2. These are secreted by various cell types in response to detection of microbial products via TLRs or NOD receptors (reviewed by Kinnebrew and Pamer, 2012). In mice, this chemokine expression and subsequent neutrophil recruitment, can be enhanced by both IL-17 and TNFα (Griffin et al., 2012).

1.3.4 Antigen Sampling and Antigen Presenting Cells

The linking of the innate and adaptive immune system in the gut occurs via antigen presenting cells (APCs). This allows pathogens that have evolved virulence factors to avoid the innate system to be detected and cleared. In addition, harmless antigens from food particles or commensal organisms need to be detected without a pro-inflammatory response. While some antigen sampling occurs by macrophages, much of this is done by a variety of subtypes of dendritic cells (Ng et al., 2010). In order to maximize the chance of an APC, such as a dendritic cell (DC), presenting their cognate antigen, naïve T cells migrate between the bloodstream and the secondary lymphoid tissues (reviewed by Agace, 2006).

1.3.4.1 Dendritic Cells

After sampling antigen in the LP, including in close association with M cells (see section 1.3.2.2), DCs migrate to the T cell zones of draining lymph nodes (reviewed by Alvarez et al., 2008). During this CCR7-dependant migration, the DCs mature in order to interact efficiently with naïve T cells and promote T cell activation (reviewed by Sharpe and Freeman, 2002). DCs interact with naïve $CD4^+$ and $CD8^+$ T cells which express TCRs specific for the presented antigen. The cytokine milieu during the interaction between DCs and T cells has traditionally thought to determine the

phenotype of the proliferating T cells (reviewed in Banchereau and Steinman, 1998). It is this T cell phenotype that will discussed in section 1.3.6.3.

In the mouse, after ingestion of the commensal bacteria *E. cloacae*, DCs containing the bacteria can be detected in Peyer's patches (PPs), but notin mesenteric lymph nodes (Macpherson and Uhr, 2004), suggesting that PPs are the primary route for sampled commensal bacterial antigen.

1.3.5 Lymphocyte migration and "gut homing"

In order to exert their effect in the lamina propria, the T cells must re-circulate to the intestine via the systemic circulation (see figure 1.2). This re-circulation occurs via the "gut-homing" integrin pair α4β7, which interacts with the mucosal addressin cellular adhesion molecule (MAdCAM)-1, expressed on endothelial cells in murine and human small intestinal and colonic mucosa (Agace, 2006; Briskin et al., 1997). The chemokine receptor CCR9 can also be induced to mediate homing to the mouse small intestine by the ligand CCL25 (Kunkel et al., 2000). This homing has been demonstrated in the mouse, and there is much circumstantial evidence to support the same processes in humans (Feagan et al., 2005; Jaensson et al., 2008; Johansen et al., 2005).

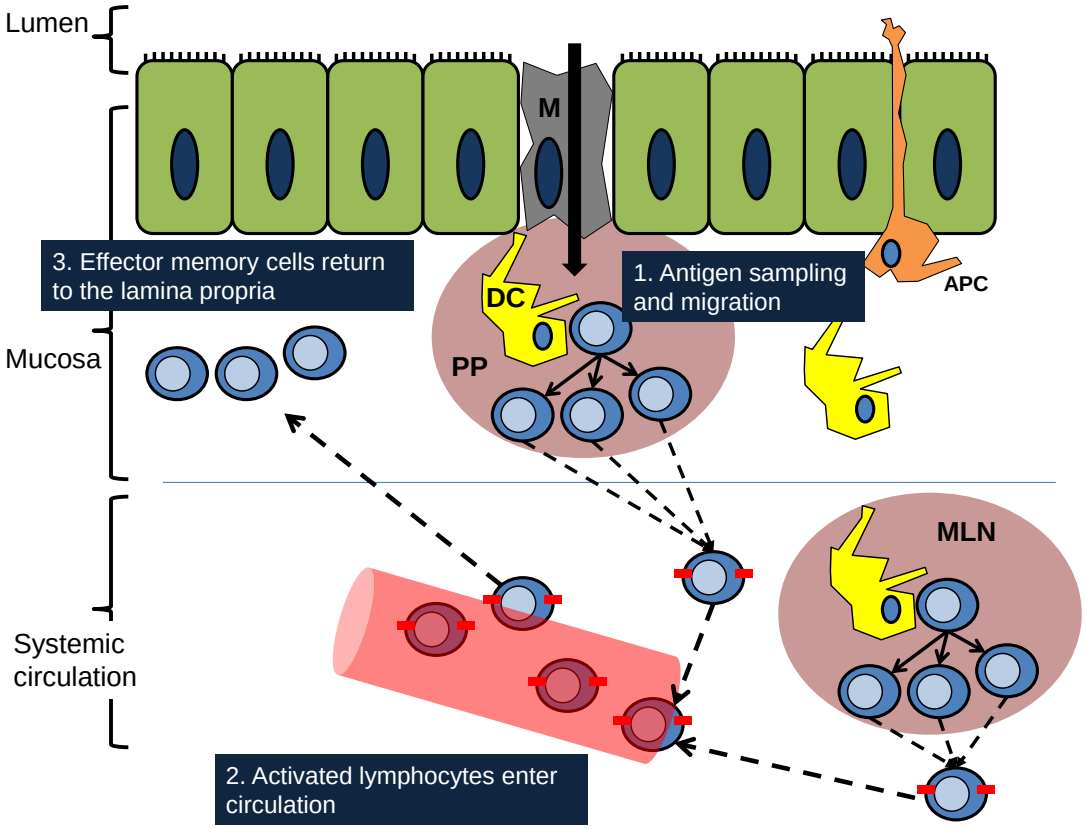

Figure 1.2 Overview of T cell responses in the intestine. *Antigen is sampled from the gut lumen by various mechanisms, including via M cells and directly by antigen presenting cells (APCs). 1 The APCs migrate to organised lymphoid tissue such as Peyer's patches or Mesenteric lymph nodes and present Ag which leads to proliferation of the appropriate T cell and imprinting of gut-homing capability. 2 The T cells recirculate via the systemic circulation and using these markers (3) move back into the lamina propria to exert their effect. However, the effector memory T cells may still be responsive to the local environment, as described in later sections. PP Peyer's Patch. DC Dendritic cell. MLN. Mesenteric Lymph Node. APC Antigen Presenting Cell. M Microfold "M" Cell.*

1.3.6 Lymphocyte Subsets in the Intestine

The predominant T cell subset in the human intestine are $CD4^+$ effector memory T cells (Sathaliyawala et al., 2013), with the next largest the $CD8^+$ effector memory cells. $CD4^+$ central memory cells are more often found in the lymphoid tissues, as are naïve $CD8^+$ T cells (Sathaliyawala et al., 2013).

Intracellular pathogens, such as Salmonella, are cleared by macrophages when they receive signals from T cells of a TH1 phenotype. TH2-polarised T cells effectively prime B cell responses to respond to parasitic infections. Recently

identified TH17 cells can be protective against bacterial, viral or fungal infection (Khader et al., 2009a).

1.3.6.1 B cells

Bacteria specific IgA can be produced in PPs by B cells without T cell help (Macpherson et al., 2000), at least in mice, although this lacks the affinity of IgA produced with T cell help. The interaction via the B cell receptor (BCR) results in the formation of a germinal centre. Here, B cells class switch from IgM to IgA and undergo somatic hypermutation with a range of mutations in the variable region of their antibody. The mutants with expression of antibody with greater affinity are positively selected (reviewed by Pabst, 2012).

These activated B cells differentiate into plasma cells and enter the circulation. They home back to the intestine (by mechanisms similar to those described for T cells, in in section 1.3.5), and continue to produce large quantities of sIgA (reviewed by Hadis et al., 2011).

1.3.6.2 Cytotoxic T cells

Antigens are presented by DCs to naïve $CD8^+$ T cells during the process of "cross presentation" (reviewed by Joffre et al., 2012). IL-12 induces the differentiation of cytotoxic T lymphocytes (CTLs). Their function includes induction of apoptosis of virally infected cells expressing their cognate peptide-MHC class I target (reviewed by Kaech and Cui, 2012). CTLs express T-bet and can produce IFNγ and TNFα (Intlekofer et al., 2005).

1.3.6.3 Helper T cell phenotype

The differentiation of $CD4^+$ T cells into functionally distinct subsets occurs in the organised lymphoid tissue. The phenotype of intestinal T cells is determined at least partly by the cytokine environment at the time of antigen presentation (reviewed in

Banchereau and Steinman, 1998). This environment provides cytokine signals to the T cell, mediated via Signal Transducer and Activator of Transcription (STAT) proteins (which will be discussed in much greater detail in section 1.3.9), and leads to expression of lineage specific "master regulator" transcription factors (Darnell, 1997). These phenotypes have been well demonstrated in mouse models, and to a lesser extent in humans, as described below (and see table 1.3).

TH Phenotype	**TH1**	**TH2**	**TH17**	**Treg**
Signature Cytokine(s) produced	IFNγ, TNFα	IL-4, IL-5, IL-13	IL-17A, IL-17F, IL-22	IL-10
Induction cytokine(s)	IL-12	IL-4	TGF-β, IL-21/IL-6	RA, TGFβ
Transcription Factor/ "Master Regulator"	T-bet	GATA-3	RORγt	FoxP3
STAT proteins	STAT4, STAT1	STAT6	STAT3	STAT5
Function	Pro-inflammatory, anti-viral, anti-intracellular bacteria	Pro-allergic responses, anti-parasite	Anti-extracellular bacteria and fungi	Pro-regulatory, inhibit other TH cell functions

Table 1.3 Simplified characterisation of classic Helper T cell phenotypes. *$CD4^+$ T cells are characterized by their phenotype, which is imprinted depending on the conditions under which they proliferate in organized lymphoid tissue, as previously described. Many of these characteristics are less clear than described in the table, and some of the complexity is described in the following sections.*

1.3.6.3.1 TH1 cells

$CD4^+$ T cells primed in conditions with high levels of IL-12 and/or Type 1 Interferon (T1IFN) have been shown to differentiate into T helper 1 (TH1) cells (Chua et al., 1994; Presky et al., 1996). These cells express the transcription factor T box expressed in T cells (T-Bet) and produce the signature cytokines Interferon γ (IFNγ) and Tumour Necrosis Factor (TNF) α (Afkarian et al., 2002; Macatonia et al., 1995). IFNγ is important in activating intestinal macrophages by various mechanisms, in

order to facilitate clearing of intracellular pathogens (reviewed by Boehm et al., 1997).

1.3.6.3.2 TH2 Cells

In order to differentiate into a TH2 phenotype, naïve T cells must be primed in an environment with IL-4, which signals via STAT6, and then express the master regulator GATA-3 (Swain et al., 1990; Zheng and Flavell, 1997). IL-2 signalling via STAT5 is also important (Cote-Sierra et al., 2004; Zhu et al., 2010), although dispensable in mice (reviewed by Rochman et al., 2009). TH2 cells produce IL-4, IL-5 and IL-13 (Swain et al., 1990; Zheng and Flavell, 1997). They are involved in defence against parasites via IL-4-dependent B cell class switching to IgE (reviewed by Paul and Zhu, 2010). TH2 cells recruit eosinophils and mast cells via IL-5 and IL-9, respectively (reviewed by Paul and Zhu, 2010).

1.3.6.3.3 TH17 Cells

Murine TH17 cells are induced by a combination of TGFβ and IL-6, via STAT3, to express the transcription factor RORγt and produce IL-17A and IL-17F (Ivanov et al., 2006; Yang et al., 2008a; Zhou et al., 2007).

When cultured with only IL-6 and TGF-β, TH17 cells are non-inflammatory, and secrete IL-10 as well as IL-17A (McGeachy et al., 2007). Further stimulation with IL-23 pushes the TH17 away from regulation, with loss of IL-10 production (McGeachy et al., 2009). This is an example of functional plasticity of a T helper phenotype (see section 1.3.7 below).

TH17 cells have a reciprocal relationship with Treg cells. IL-2 suppresses TH17 cells, while enhancing Treg induction (Laurence et al., 2007; Setoguchi et al., 2005). This is via a balance of signals between STAT3 and STAT5 (Yang et al., 2011).

Human TH17 cells are not produced simply by activation of naïve T cells in the presence of IL-6 and TGF-β (Acosta-Rodriguez et al., 2007; van Beelen et al., 2007; Wilson et al., 2007) but instead require IL-21 instead of IL-6 (Yang et al., 2008c). Human TH17 cells do produce IL17A and IL-17F, as well as IL-22 and GM-CSF (Annunziato et al., 2007; Wilson et al., 2007). These IL-17 isoforms recruit neutrophils via chemokine induction and other methods (reviewed by Witowski et al., 2004), providing defence against extracellular bacterial and fungal infections.

1.3.6.3.4 Regulatory T Cells

Regulatory T cells (Tregs) are classically described by the transcription factor Forkhead Winged helix P3 (FoxP3), have high expression of CD25 and produce IL-10 (Andersson et al., 2008; Fontenot et al., 2003; Hori et al., 2003). As there are FoxP3- as well as FoxP3+ Tregs, they are perhaps better defined by their function; Tregs suppress T cell proliferation and cytokine production by both contact dependent and independent mechanisms. Contact-dependent mechanisms include expression of Cytotoxic T Lymphocyte associated antigen 4 (CTLA-4) which down regulates the co-stimulatory molecules CD80 and CD86 on APCs (Takahashi et al., 2000). Contact independent mechanisms include IL-10 production (reviewed by Barnes and Powrie, 2009).

Murine FoxP3$^+$ Tregs are either natural occurring (nTregs) or induced Tregs (iTregs). nTregs develop during T cell differentiation in the thymus and are thought to express TCRs with self antigen specificity. iTregs develop from naïve CD4$^+$ T cells in the periphery, which potentially allows other non-self antigens to be subject to Treg responses. iTregs are differentiated under the influence of conditions including retinoic acid (RA) and TGF β (Andersson et al., 2008; Chen et al., 2003; Coombes et al., 2007; Fontenot et al., 2003; Mucida et al., 2007). It has been proposed that RA does not induce Tregs directly, but rather inhibits cytokines such as IFNγ which would usually suppress Treg induction (Hill et al., 2008).

In experimental autoimmune encephalitis (EAE), a mouse model of multiple sclerosis, FoxP3$^+$ T cells were not sufficient to suppress effector T cells, while they could suppress naïve T cells (Korn et al., 2007), with similar results *ex vivo* from patients with Multiple Sclerosis (MS) (Viglietta et al., 2004). This suggests that in inflammation, FoxP3$^+$ Tregs may not always be sufficient to control effector T cell responses.

There are also subtypes of FoxP3$^-$ inducible regulatory T cells. Type 1 regulatory cells (TR1) have been identified in mice and humans which have low proliferation, high IL-10 expression, and low expression of IFNγ and IL-2 (Groux et al., 1997). Recently, the transcription factor FOXA1 has been shown to identify a population of FOXP3$^-$ T cells that are regulatory and are induced by IFNβ (Liu et al., 2014).

In humans, the identification of Tregs is more difficult, due to the transient expression of FOXP3 by activated CD4$^+$ T cells (Allan et al., 2007; Gavin et al., 2007; Merlo et al., 2008), and this does not associate with suppressive function. However, patients with mutations of the *FOXP3* gene, have the fatal autoimmune condition of Immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome (Bennett et al., 2001; Wildin et al., 2001). This demonstrates the importance of *FOXP3* to maintain immune homeostasis in humans, including in the intestine.

A schematic of the different type of helper T cell differentiation is shown in figure 1.3.

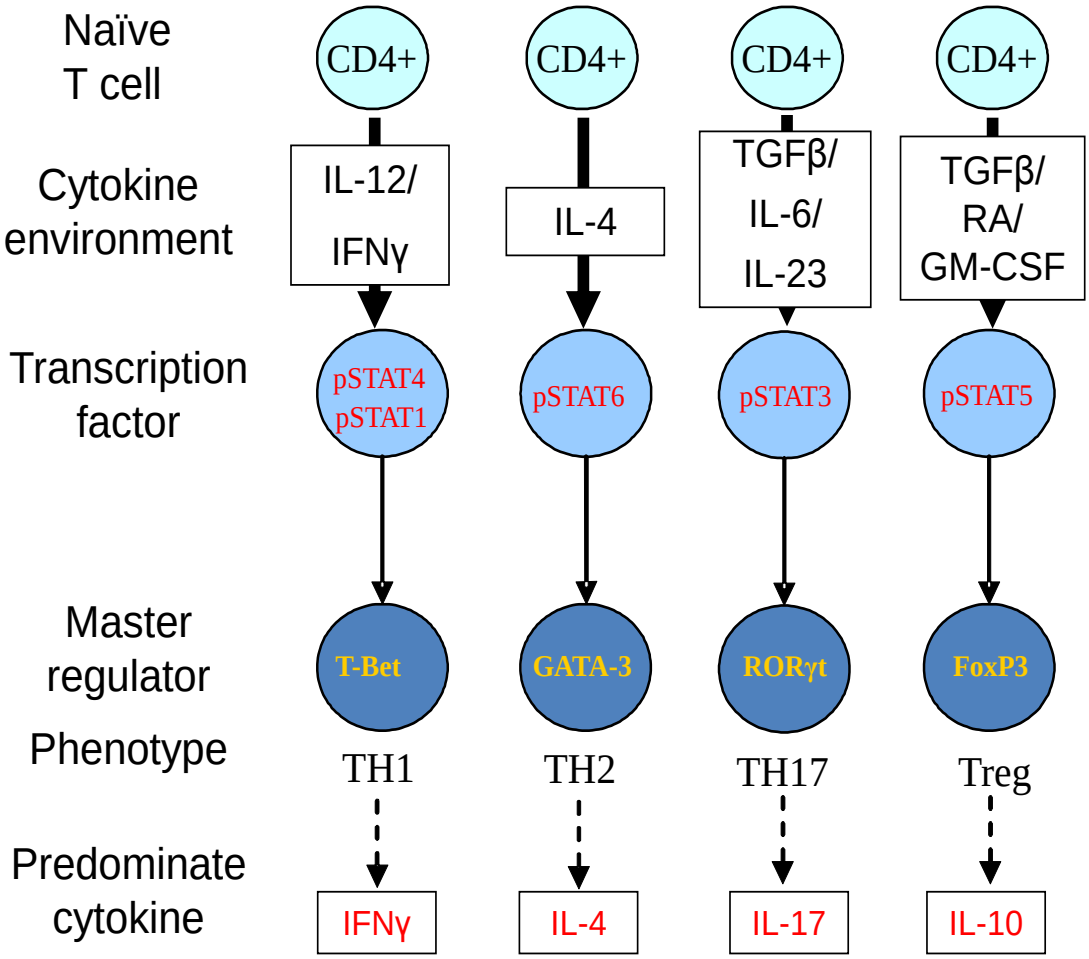

Figure 1.3 A simplified overview of T cell differentiation. *Naïve CD4 T cells receive their cognate antigen in the gut in mesenteric lymph nodes, Peyer's patches or other lymphoid aggregates and depending on the cytokine milieu differentiate into different phenotypes which produce different predominant cytokines*

1.3.6.4 Intestinal $CD4^+$ T cells

In humans, the largest subtype of effector T cells in the healthy human intestine is TH1, with the potential to produce IFNγ (Rovedatti et al., 2009; Van Damme et al., 2001). The proportion depends largely on the method of detection (eg method of *ex vivo* stimulation), but along with IL-2, IFNγ is generally the most abundant cytokine measured from lamina propria T cells (Rovedatti et al., 2009; Van Damme et al., 2001). After intestinal infections, TH1 cells can persist in the mucous of mice for extended periods (Hand et al., 2012).

The role of TH17 cells in the intestine is less clear, and may be either pro- or anti-inflammatory (Monteleone et al., 2012). The microbiota may influence the induction of the TH17 phenotype. Mice with segmented filamentous bacteria (SFB) in their intestine have higher levels of TH17 cells in the intestine than those without (Ivanov et al., 2009). This greater TH17 cell presence provides improved protection against the pathogen *Citrobacter rodentium* (Ivanov et al., 2009). This protective effect, however, must be balanced against the evidence of IL-17 involvement in the pathogenesis of colitis and IBD (discussed below).

Given the ability of many intestinal effector T cells to produce IFNγ (Barnes and Powrie, 2009; Rovedatti et al., 2009), it is perhaps surprising that the gut is not in a constant state of severe chronic inflammation. Multiple mouse models have demonstrated the capacity of intestinal Tregs to maintain homeostasis in the intestine (reviewed by Barnes and Powrie, 2009). The mechanisms of their function in the gut will be briefly described.

IL-10 is a key anti-inflammatory cytokine in the gut (Berg et al., 1996; Gazzinelli et al., 1996), and is produced by intestinal Tregs (reviewed in Saraiva and O'Garra, 2010), both $FoxP3^+$ and $FoxP3^-$ (Maynard et al., 2007). IL-10 signalling via STAT3, has been shown to be necessary in murine intestinal ($FoxP3^+$) Tregs to control TH17-driven colitis (Chaudhry et al., 2011).

There is some controversy on the relative importance of induced versus naturally occurring Tregs in the murine intestine. It has been suggested that it is the thymus-derived T regs that are most important in mediating tolerance to commensal bacteria in the colon (Cebula et al., 2013), but this is in contrast to other published work (Furusawa et al., 2013; Lathrop et al., 2011).

1.3.7 “Plasticity” of mucosal T cells

The classic model of helper T cell priming in gut associated lymphoid tissue (GALT) into fully committed cells expressing a particular phenotype (TH1, 2, etc) has been recently challenged. This is the concept of immune “plasticity”; differentiated $CD4^+$ T cells remaining responsive to their environment (O'Shea and Paul, 2010), possibly orchestrated by epigenetic modifications (Wei et al., 2009). T Helper phenotype in experimental models requires sustained cytokine exposure, and therefore may not be representative of most human *in vivo* environments (Curtis et al., 2010). Most human $CD4^+$ T cells have been shown to retain both memory and flexibility of gene expression (Messi et al., 2003). Secondary encounters with antigen or other factors in the gut may affect the survival, retention and proliferation of effector T cells (see schematic figure 1.4).

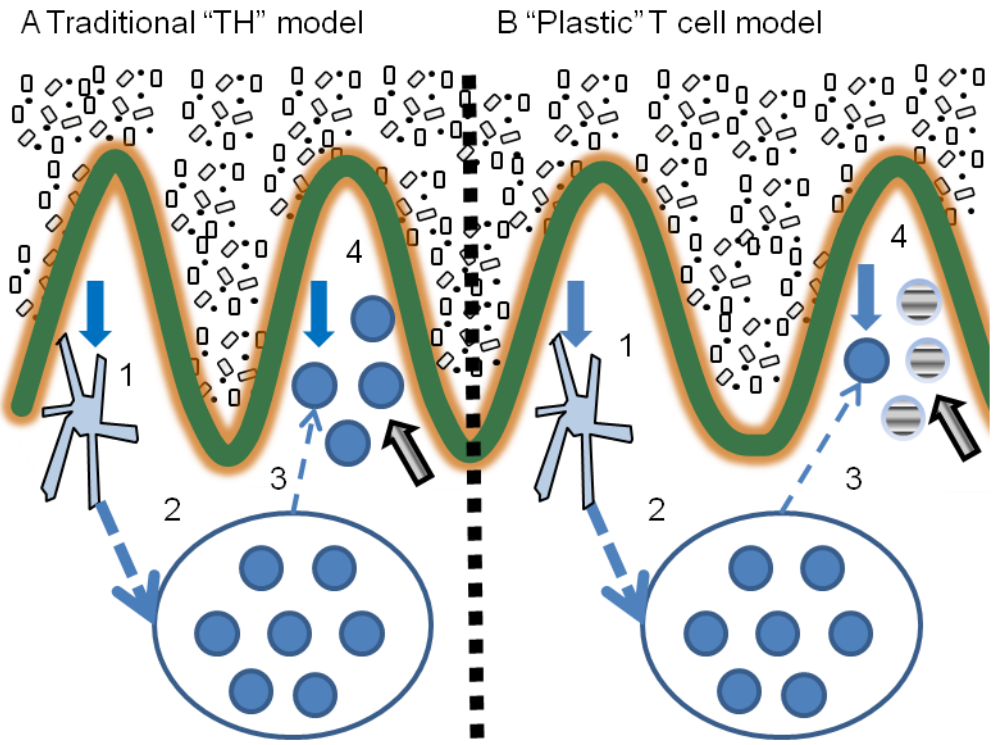

Figure 1.4 Intestinal resident memory T cells may remain responsive to environmental triggers. *1. Antigens are processed by professional APCs under different conditions. 2. These conditions are reflected in the phenotype that T cells are primed with when the APC moves to organised lymphoid tissue. 3. T cells then home back to the gut via the circulation to exert their effect. 4. In the traditional model on the left (A), these resident memory T cells retain their phenotype despite differing signals in the gut, represented by the stiped arrow. In the more recently*

recognised plastic model (B), the T cells in the intestine remain responsive to environmental triggers, potentially altering the phenotype of cells, making them more or less inflammatory or regulatory.

1.3.7.1 Intestinal Tregs

It has been shown that antigen-specific memory T cell expansion and differentiation can occur directly in the lamina propria in mice, via $CD70^+$ APCs (Laouar et al., 2005), suggesting that local factors can influence final effector function. Again in the mouse, it has been shown that local factors, particularly IL-10 production from intestinal macrophages, drive Treg education and expansion in the lamina propria (Hadis et al., 2011).

Even in mice, the Treg phenotype is considered unstable (Feng et al., 2011; Oldenhove et al., 2009; Tsuji et al., 2009; Wei et al., 2009). Fate-mapping experiments show loss of FoxP3 expression from T cells and conversion into TH17 cells that contribute to arthritis, and $IL17^+FoxP3^+CD4^+$ cells have been detected in the synovium of patients with RA (Komatsu et al., 2014). The commensal flora, in particular segmented filamentous bacteria (SFB) have been shown to alter antigen responsiveness in intestinal T cells, at least in part due to the an inflammatory environment modifying the TCR signalling cascade (Chappert et al., 2013). Intestinal $FoxP3^+$ T cells in Peyer's patches have been shown to convert to IgA enhancing TFH cells (Tsuji et al., 2009). Environmental factors can also act to stabilize T cell function. For example, IL-10 can act on Tregs to maintain FoxP3 expression and suppress T cell mediated colitis in mice (Hadis et al., 2011; Murai et al., 2009).

1.3.7.1.1 Gut homing versus local factors

The relationship between T cell phenotype, particularly Tregs, and intestinal "homing" is complex and age-related. The majority of human Tregs at birth express α4β7, but this switches to CCR4 in adults and appears to correlate with the change from naïve to memory Treg phenotype between 1 and 3 years of age (Grindebacke et al., 2009). The induction of oral tolerance in mice via Tregs has been shown to require the gut homing markers CCR9 and β7, but can be restored by exogenous IL-10 provision in *ccr9* knockout mice (Cassani et al., 2011). This suggests that factors in the gut, including IL-10, are capable of influencing the functional determination of regulating T cells in the lamina propria.

1.3.7.2 Intestinal TH17 cells

TH17 cells have been extensively studied due to their apparently changeable phenotype. *In vivo*, TH17 cells are commonly found to produce IFNγ, the TH1 signature cytokine (Chen et al., 2007; Wilson et al., 2007). Indeed, fate mapping experiments suggest that TH17 cells produce IL-17 in response to their microenvironment but can lose this capacity (Kurschus et al., 2010), and can induce IgA production as TFH cells (Hirota et al., 2013), as well as produce IFNγ and other cytokines (Hirota et al., 2011). Intestinal Th17 cells in mice have been shown to express the IL-10 receptor, and with blockade of this receptor increase numbers of $IL17A^{+}IFN\gamma^{+}$ cells (Huber et al., 2011). Indeed, different microbial triggers appear to alter the cytokine potential of mucosal TH17 cells (Zielinski et al., 2012).

It is likely that the balance of signals in the intestine affects the phenotype of local T cells. TGFβ in the environment is produced by Tregs and, in the presence of the pro-inflammatory IL-6, may lead to T cell differentiation to a TH17 phenotype in both mice and humans (Koenen et al., 2008; Yang et al., 2008c). This plasticity of response may allow the intestine to change between regulation or inflammation

depending on the balance of signals in the local environment (Strober and Fuss, 2011).

1.3.7.3 Intestinal TH1 and TH2 cells

Even the canonical TH1 transcription factor, T-bet, can be ambiguous, as its expression in Tregs has been shown to be necessary to suppress TH1 driven inflammation (Koch et al., 2009). Also, $FoxP3^{+}IFN\gamma^{+}$ cells were shown to retain suppressive ability in a T cell model of colitis (Feng et al., 2011). The cytokine milieu drives differing phenotypes of Tregs required for suppression of inflammation (Hall et al., 2012)(Hall et al., 2012)(Hall et al., 2012)(Hall et al., 2012)(Hall et al., 2012). $T\text{-}bet^{+}FoxP3^{+}$ T cells, so called TH1-Tregs, poorly upregulate IL-12R in response to STAT1 mediated IFNγ signalling (Koch et al., 2012). Exposure to the combination of T1IFN andT2IFN (IFNγ) can drive TH2 cells to become “TH2+1” cells, producing IL-4 and IFNγ (Hegazy et al., 2010). While some homeostatic mechanisms are in place to prevent too much instability in T cell phenotype (Burzyn et al., 2013), it is increasingly clear that, particularly in the gut, that the traditional terminology of describing functional T cell phenotypes is becoming inadequate.

1.3.8 Dynamic measurement of T cell response

Traditionally, mucosal $CD4^{+}$ T cells, like their peripheral counterparts, have been characterized as a T helper phenotype based on their cytokine production potential. This is an over-simplification of T cell responsiveness *in vivo*. Furthermore, at a single cell level, cytokine production is routinely measured in the context of *in vitro* stimulation. Such stimulation commonly uses agents such as phorbol-12-myristate-13-acetate (PMA) and Ionomycin, to activate the cells and this is far from physiological. PMA mimics the effect of diacylglycerol (DAG), in that it binds to and activates protein kinase C (PKC), which then leads to downstream cytokine

production in T cells (Galron et al., 1994). Ionomycin synergises with PMA, as it mimics inositol 1,4,5 triphosphate (IP3), and so increases intracellular calcium ions and activates protein kinase C (Chatila et al., 1989). These mechanisms avoid the need for cell surface signalling in classical T cell engagement, and will thus activate all T cells to produce cytokines which then may be detected. This is then a method of detection of the cytokine production *potential* of these cells.

There are other methods of T cell activation, some which will be discussed in detail in chapter 2 and the results section. However, one method to measure more directly the T cells ability to respond to its environment in a more dynamic way, is by measuring levels of phosphorylation of important signalling molecules within these cells (Krutzik et al., 2004). This was traditionally performed by Western Blot of the phosphorylated protein. However, Western blotting does not allow for simultaneous measurement of cell surface or other parameters, and is less amenable to quantification (see table).

Phosflow is a relatively new technology that combines the use of phospho-specific antibodies with flow cytometry (Krutzik et al., 2004) enabling signalling pathways to be analysed at the single cell level. It is this technique which will be assessed for its suitability for analysis of intestinal T cells in chapter 3. Technical details will be discussed further in that chapter and within the materials and methods section (chapter 2).

Western blot	**Flow cytometry**
Average of population of cells	Single Cell Analysis
Homogeneous sample	Heterogeneous sample
One parameter	Multi-parameter
Large number of cells	Small numbers of cells
Time consuming for large subsets	Scalable
Antibodies highly selective	Antibody must be validated

Table 1.4 *Comparison of phospho-specific flow cytometry*

and Western blot. Adapted from (Krutzik et al., 2004).

1.3.9 T cell signalling – the Jak/STAT pathway

There a many different signalling pathways in T cells which interact, and it is not currently possible to dynamically assess all of these pathways simultaneously (Gough et al., 2008). The Jak-STAT pathway is known to be crucial of T cell differentiation and expression of major pro- and anti-inflammatory cytokines (see figure 1.3 above) (reviewed in Levy and Darnell, 2002; O'Shea et al., 2011). For this reason this study focuses on STATs as the main molecules of interest for both the analysis of T cell responsiveness and also their role in human intestinal T cells in health and IBD (chapters 3 and 4, respectively). Indeed, STAT proteins and their regulators, the Suppressor of Cytokine Signalling (SOCS) proteins, have been associated with murine models as well as human IBD (see sections 1.4.6 and chapter 4).

There are 7 known STAT molecules; STATs 1, 2, 3, 4, 5A, 5B and 6. These all share a common method of activation. Cytokines bind to specific receptors on T cells (and other cell types) leading to downstream Janus Kinase (Jak) phosphorylation which leads to binding and phosphorylation of STAT proteins which then dimerise and move to the nucleus where they directly and indirectly effect transcription (see figure 1.5). Each STAT molecule may form homo- or hetero-dimers and may be phosphorylated at different amino acid residues and these and other factors alter their function.

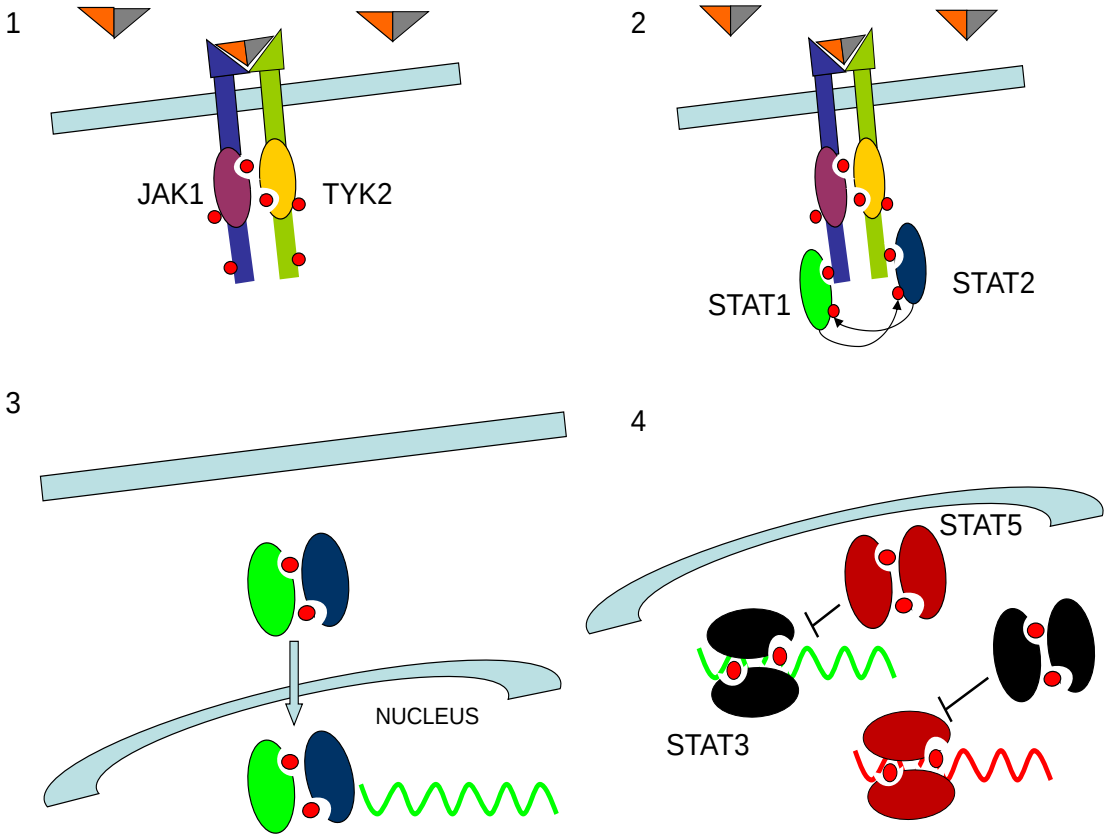

Figure 1.5 Representation of JAK/STAT signalling*. Panel 1 shows cytokines or other factors binding to receptors which dimerise, leading to phosphorylation (red circles) of Janus Kinase (Jak) proteins. 2. This leads to binding and phosphorylation of STAT proteins which themselves (3) dimerise and move to the nucleus where they can either promote or repress the transcription of a large number of genes. 4. Different STATs can interact/compete for binding sites which can lead to opposing transcriptional outcomes (eg STAT3 and STAT5 at the IL-17 promoter region)*

The STATs have a variety of functions in many cell types and are critical in helper T cell differentiation and function. These functions can be mediated directly by modifying permissive and repressive epigenetic events on target genes and indirectly by the control of expression additional transcription factors which function as master regulators of cell fate. (O'Shea et al., 2011; Thieu et al., 2008). The following paragraphs will briefly describe the STAT proteins known major functions in T cells. Many of these functions have been well described in mouse models, but where human data is known it is highlighted.

1.3.9.1 STAT1

STAT1 was originally discovered in mice as a target of T1IFN signalling (Levy and Darnell, 2002), and was shown to be critical in antiviral and antibacterial responses. After activation by T1IFN of the Type I Interferon receptor (IFNAR), Jak1 and Tyk2 are phosphorylated. This leads to the downstream phosphorylation of STAT proteins. Canonically, phosphorylated (p) STAT1 forms heterodimers with pSTAT2 (see figure 1.6 below), although it can also form pSTAT1 homodimers or form combinations with other STAT molecules (reviewed in Ivashkiv and Donlin, 2014).

STAT1 has also been shown to be activated by Type II Interferon (T2IFN or IFNγ) signalling. After stimulation with IFNγ, signalling via the IFNγ receptor (IFNGR), Jak1 and Jak2 are phosphorylated. This leads to the formation of pSTAT1 homodimers (González-Navajas et al., 2012). Apart from T1 and T2IFN, STAT1 has been shown to also be activated by IFN λ and IL-27 (Maródi et al., 2012). Nevertheless, both T1 and T2IFN signalling are dependent on STAT1 (Meraz et al., 1996).

STAT1 is an important component of apoptotic signalling, where it functions largely by sensitising cells to other pro-apoptotic signals, and also contributes to non-apoptotic cell death pathways (Kim and Lee, 2007). The pro-apoptotic effects of STAT1 activation are only partially dependant on IFNγ, and may be related to T1IFN responses (Kumar et al., 1997).

In mouse T cells, STAT1 has been shown to be necessary for induction of T-bet and other components of TH1 differentiation (Afkarian et al., 2002). However, there are species differences between mice and humans in the signalling of TH1 differentiation. Specifically, T1IFN signals via murine STAT2 (in conjunction with STAT1) and this is responsible for a selective loss of STAT4 signalling, required for TH1 development (Farrar et al., 2000). In humans, T1IFN signalling involving STATs 1, 2 and 4 leads to TH1 polarisation, but this does not occur in mice. So

while in mice STAT1 is necessary for TH1 differentiation, this is not via T1IFN signalling, thought to be due to the species differences in STAT2 (see below). In mice, however, Stat1 has also been shown to be essential for development of Tregs (Nishibori et al., 2004).

Stat1 deficient mice are phenotypically normal, although they have no response to T1 or T2IFN, and are therefore susceptible to viral infection. The mice do respond to other factors including IL-10, so (at least in early studies) the loss of STAT1 appears to be relatively specific to the IFN (types 1 and 2) response (Durbin et al., 1996; Meraz et al., 1996). In the mouse model of Multiple Sclerosis (EAE), Tregs from *Stat1* KO mice are unable to suppress TH17 mediated inflammation (Nishibori et al., 2004).

In humans, STAT1 loss-of-function mutations have been associated with defective immunity to bacteria, mycobacteria and viruses (Averbuch et al., 2011; Dupuis et al., 2001; Dupuis et al., 2003). In contrast, a STAT1 gain-of-function mutation is one cause of chronic mucocutaneous candidiasis disease, and in this condition there is impaired IL-17 production in T cells (Liu et al., 2011). The defective IL-17 production is thought to be due to increased STAT1 signalling at the expense of STAT3 (van de Veerdonk et al., 2011), which is required for IL-17 production/TH17 differentiation – see below in section on STAT3. This is an example of the importance of the balance of expression of different members of the STAT family for a given T cell phenotype.

Indeed, in viral infection models in mice, STAT1 expression is decreased in virus-specific CD8+ T cells, and the alteration in STAT protein balance appears to change the effect of T1IFN signalling from anti-proliferative to a pro-proliferative signal, leading to increased viral clearance (Gil et al., 2012; Gil et al., 2006).

In summary, STAT1 is primary involved in T1 and T2IFN responses in various cell types. The effects are pleiotropic, and can differ between species, particular between mice and humans.

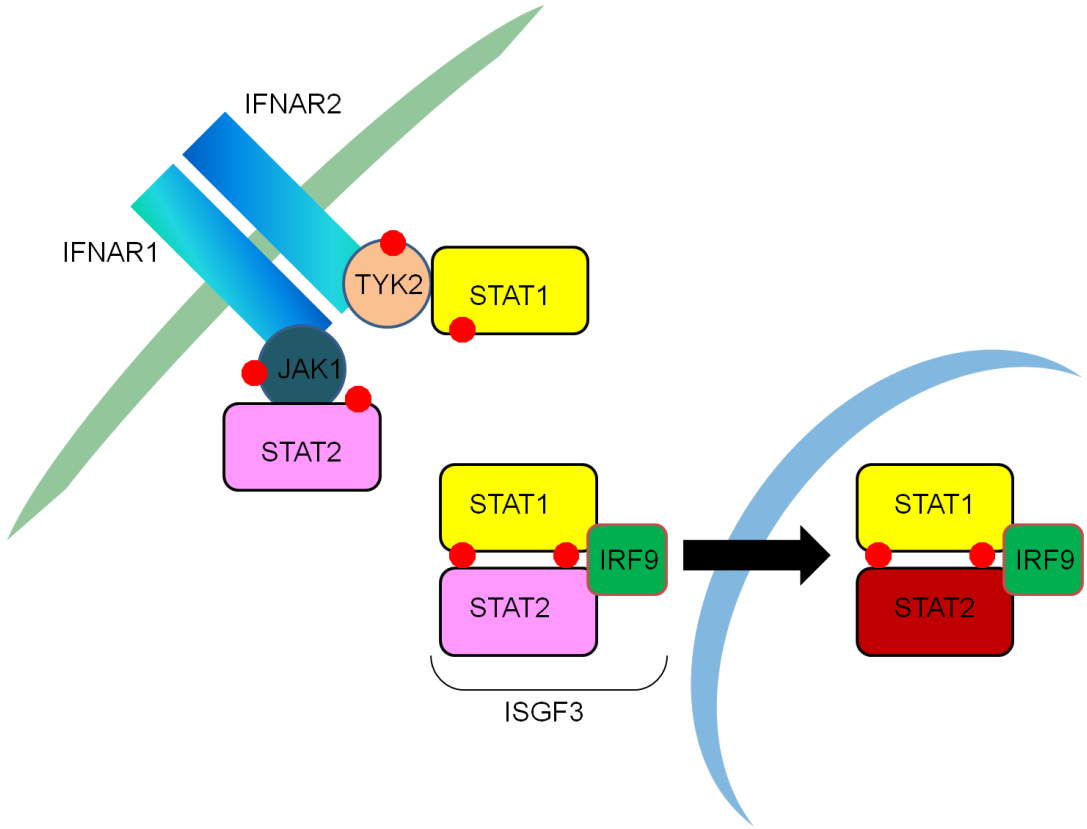

Figure 1.6 Canonical T1IFN-STAT1/2 signalling. *T1IFN signals via the ubiquitous T1IFN receptor, IFNAR, which dimerises its two components IFNAR1 and IFNAR2 on signalling. This leads to phosphorylation (red circles) of the Janus Kinases Jak1 and Tyk2. This in turn leads to phosphorylation of STAT1 and STAT2. Phosphorylated STAT1 and STAT2 then dimerise and form a complex with Interferon Regulatory Factor 9 (IRF9), called Interferon Stimulated Gene Factor 3 (ISGF3). This in turn enters the nucleus and has multiple effects on gene transcription.*

1.3.9.2 STAT2

Like STAT2, STAT1 was also originally discovered as a target for IFN in mice, and these two form a heterodimer on Type I IFN stimulation to activate IFN-inducible genes (Trinchieri, 2010). Unlike STAT1, STAT2 does not form homodimers; nuclear import depends on heterodimerization with STAT1 (Chen and Khurana Hershey, 2007). In human T cells Type I IFN signalling via STAT2 recruits and activates STAT4 thereby inducing TH1 differentiation. Due to species differences in the *STAT2* gene this does not occur in mice, as mentioned above. Specifically, it was

thought a minisatellite insertion in the murine *Stat2* gene renders mouse T cells unable to activate Stat4 in response to T1IFN (Farrar et al., 2000). However, subsequent work suggested that the response to T1IFN may be more similar between species when STAT1 (as opposed to STAT4) signalling predominates (Nguyen et al., 2002). It therefore remains unclear exactly the nature of the differences in STAT signalling between mice and humans and the importance of the STAT2 gene differences.

1.3.9.3 STAT3

STAT3 in murine T cells is activated by IL-6, and in combination with TGFβ leads to increased expression of the retinoid orphan receptor γt (RORγt), the master regulator for TH17 cells (Zhou et al., 2007). Humans with dominant-negative mutations in the *STAT3* gene develop Job's (Hyper IgE) Syndrome, and are deficient in TH17 cells (Minegishi et al., 2007). STAT3 activation by IL-10 has been shown to be essential for the development and function of murine Treg cells. Disruption to this IL-10-STAT3 pathway specifically in Tregs affected the mouse's ability to control inflammatory TH17 responses in murine colitis (Chaudhry et al., 2009). Massive parallel sequencing with chromatin immunoprecipitation (ChIP-Seq) has demonstrated many targets of *Stat3* in mouse T cells, including genes involved in Th17 differentiation (eg *Il-17a, Il-17f, Rorc*), cell activation, proliferation and survival (eg *Bcl2, Ier3, Fos, Jun* and *Fosl2*) (Durant et al., 2010).

In humans, *STAT3* mutations lead to increased infections, particularly Staphylococcus and Candida, due to decreased TH17 cell numbers and therefore decreased neutrophil recruitment (Minegishi et al., 2009).Genetic variants associated with the STAT3 gene have been identified in GWAS studies of patients to confer increased risk to both CD and UC.

1.3.9.4 STAT4

STAT4 is required in mice for differentiation into TH1 CD4 T cells (Jacobson et al., 1995) in response to stimulation with IL-12 and induces expression of the master regulator T-Bet (Thieu et al., 2008). STAT4, in conjunction with T-Bet, is required for the expression of *Ifng*, and induces the receptors IL-18Rα and IL-12β2 (Thieu et al., 2008; Zhu et al., 2010). Alongside IL-12, T1IFN can also induce production of IFNγ in murine T cells by STAT4 (as well as it's classic signalling by STAT1 and 2) (Freudenberg et al., 2002). As mentioned above, in human T cells STAT4 is bound and activated by STAT2 after T1IFN signalling to induce TH1 differentiation (Farrar et al., 2000).

1.3.9.5 STAT5A and STAT5B

The two isoforms of STAT5 perform similar functions and it is not clear whether their differences relate simply to cell type localisation. Therefore they will be discussed here as a single entity. In humans, a STAT5b mutation has been associated with growth hormone insensitivity and also abnormal T cell responses (Kofoed et al., 2003). In particular, in these patients there is a decrease in regulatory T cell number and function (Cohen et al., 2006). More generally, STAT5 is critical in the mouse in both B and T cell development (reviewed in Farrar and Harris, 2011). Interleukin-2 signals via STAT5 in T cells to induce proliferation following T cell receptor (TCR) cross-linking (Moriggl et al., 1999b). STAT5 is required for the development of murine $FoxP3^+$ regulatory T cells, with T cell specific deletion of STAT5 preventing Treg development, and STAT5 shown to directly bind to the *FoxP3* gene promotor (Burchill et al., 2007). Again in mice, activating STAT5 skews $CD4^+$ T cell differentiation towards a regulatory type, as well as a TH2 phenotype, and away from both TH1 and TH17 (Vogtenhuber et al., 2010; Yang et al., 2011). However, other studies have shown a requirement of IL-2/Jak3 mediated STAT5 activation for

optimal IFNγ secretion, indicating an importance in TH1 lineage commitment (Liao et al., 2011; Murphy, 2008; Shi et al., 2008). It has been shown that activation of STAT5 can occur in association with CD69, a poorly understood marker of T cell activation, and Jak3, to abrogate TH17 differentiation (Martín et al., 2010). In mice, the shift between TH17 and Treg differentiation appears to depend on a balance between STAT3 and STAT5 signalling, due at least in part to competitive binding at the same sites on, for example, the *foxp3* and *Il-17* loci (Decker et al., 2005; Yang et al., 2011). This is another example of the importance of the balance of STAT signalling in determining T cell phenotype.

1.3.9.6 STAT6

STAT6 is activated by IL-4, and is the main determinant of TH2 differentiation, leading to increased transcription of IL-4, IL-13 and the transcription factor GATA-3 (Zheng and Flavell, 1997). In mice, hyperimmunoglobulinaemia E and eosinophilia associated with *FoxP3* deficiency can be reversed with additional STAT6 deficiency (Lin et al., 2005). STAT6, like the other STAT molecules, binds to many transcription sites, and it more commonly acts as a repressor rather than initiator of transcription, commonly antagonistic to the role of STAT4 (Wei et al., 2010) and so TH1 differentiation (Tamachi et al., 2009).

1.3.10 Regulation of STAT proteins

1.3.10.1 Stimulation of STAT Signalling by Type I IFN

Not only are T cell intracellular signalling pathways complex, and STAT pathways are only one important type, the large number of cytokines, chemokines, growth factors and other extracellular molecules that can trigger these pathways make their analysis extremely challenging. While different cytokines are known to trigger

phosphorylation of different STATs (as partially described above), type I interferon (IFN) is known to activate all STATs to varying degrees (Darnell et al., 1994; Gough et al., 2012). This makes type I interferon an excellent candidate for probing STAT responses in different cell populations, including T cells.

There are 5 subtypes of type I IFN in humans; IFNα (with at least 13 isoforms), IFNε, IFNκ, IFNω and IFNβ (Platanias, 2005). All of these subtypes signal through a ubiquitously expressed receptor composed of IFNAR1 and IFNAR2 chains. The IFNAR signal via the Janus family kinases Tyk2 and Jak1, which bind STAT1 and STAT2 which then form a heterodimer and in the nucleus lead to expression of the Type I IFN-inducible genes (Li et al., 1996). In response to the Type II IFN, IFNγ, the IFNAR can also signal via Jak1 and Jak2 followed by STAT1 homodimers which then bind to IFNγ-induced genes (Decker et al., 2005). Apart from STAT1 and STAT2, the other STAT proteins are directly and indirectly involved in IFN signalling. The mechanisms of non-canonical STAT signalling (eg STAT3, 4, 5 and 6) in response to T1IFN are not completely understood, but appear to require Jak/Tyk phosphorylation similar to classic STAT1/STAT2 dimerization (reviewed in Ivashkiv and Donlin, 2014). STAT3 acts as a negative regulator of T1IFN responses in mice (Wang et al., 2011). Signalling via STAT4 in the absence of STAT1 directly induces production of IFNγ in murine T cells (Freudenberg et al., 2002), and in humans also occurs but in conjunction with STAT1 (Farrar et al., 2000).

Historically, T1IFN was extremely difficult to directly measure and thought only to be present after viral infection. However, constitutive T1IFN (in this case, IFNβ) can be detected in healthy (uninfected) mouse tissue (Hamilton et al., 1996). This IFNβ is necessary for priming cells to be responsive to further cytokine signals (Gough et al., 2010; Taniguchi and Takaoka, 2001). It has been hypothesized that this responsiveness is altered through changing the ratios of STAT proteins available for phosphorylation, and thus their downstream effects (Gough et al., 2012). This

makes the T1IFN-STAT pathway an attractive one for measuring T cell responsiveness.

1.3.10.2 The Suppressors of Cytokine Signalling (SOCS) proteins

Regulation of STAT signalling can occur at all levels of their function, but a major determinant is the action of Suppressors of Cytokine Signalling (SOCS) proteins. SOCS proteins interact with cytokine receptors or the Jaks to prevent activation of STATs, and play a key role in $CD4^+$ T cell differentiation and plasticity (Elliott and Johnston, 2004; Knosp and Johnston, 2012). There are eight members of the SOCS family (SOCS1 to SOCS7 and CIS) that are all induced by STAT activation and down-regulate the Jak-STAT pathway in a negative feedback loop.

SOCS1 is a key inhibitor (Fenner et al., 2006) of IFN signalling and blocks STAT1 activation (Alexander et al., 1999; Starr et al., 1998). SOCS3 can also contribute to regulation of TH1 differentiation by preventing STAT4 activation (Seki et al., 2003). However, SOCS3 also limits Treg differentiation (Kinjyo et al., 2006), and promotes a TH17 phenotype (Kleinsteuber et al., 2012). TH2 differentiation appears to be controlled via SOCS1 and SOCS5 (Egwuagu et al., 2002; Seki et al., 2002). SOCS3 regulation of STAT3 and STAT4 can also promote commitment to a TH2 lineage (Stumhofer et al., 2007; Tanaka et al., 2008). SOCS3 inhibition of STAT3 inhibits TH17 polarisation (Horino et al., 2008). It has been suggested that TGFβ can inhibit SOCS3 expression, and this may explain how TGFß enhances TH17 differentiation (Qin et al., 2009). The roles of the other members of the SOCS family are varied and complex, but also less well explored than SOCS1 and SOCS3, and are beyond the scope of this investigation, but briefly mentioned in figure 1.7.

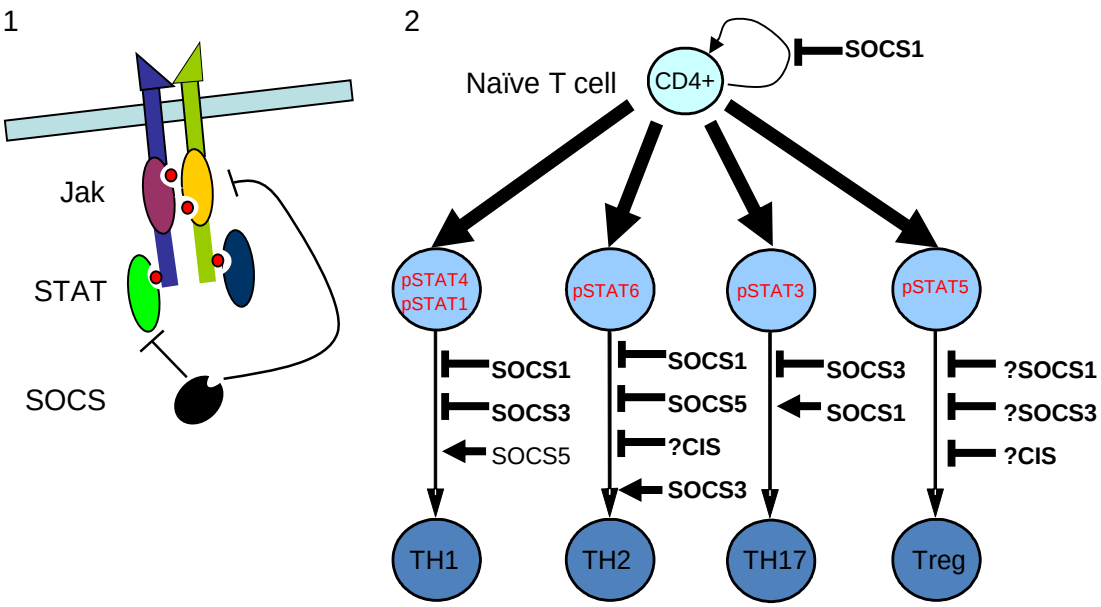

Figure 1.7 SOCS proteins in T cell differentiation. *1. SOCS proteins inhibit the Jak-STAT pathway by various mechanisms, including blocking STAT recruitment and binding to Jaks and inhibiting their kinase activity. 2. Potential roles for SOCS in the differentiation of naïve CD4$^+$ T cells.*

1.4 T cells in IBD

The main mediators of intestinal damage in IBD are generally considered to be mucosal T cells, and these are the most common targets for therapy (reviewed in Neurath, 2014b; Shale et al., 2013). Most T cells in the gut mucosa are found in the lamina propria, and are effector memory T cells (Sallusto et al., 1999). Crohn's Disease (CD) and Ulcerative Colitis (UC) have been associated with different immunological signatures, particularly in regard to CD4$^+$ T cells (Brown and Mayer, 2007). A schematic view of IBD as a T cell mediated disease, as either an excess of pro-inflammatory effector cells, or a deficiency of regulatory T cells, is displayed in figure 1.8.

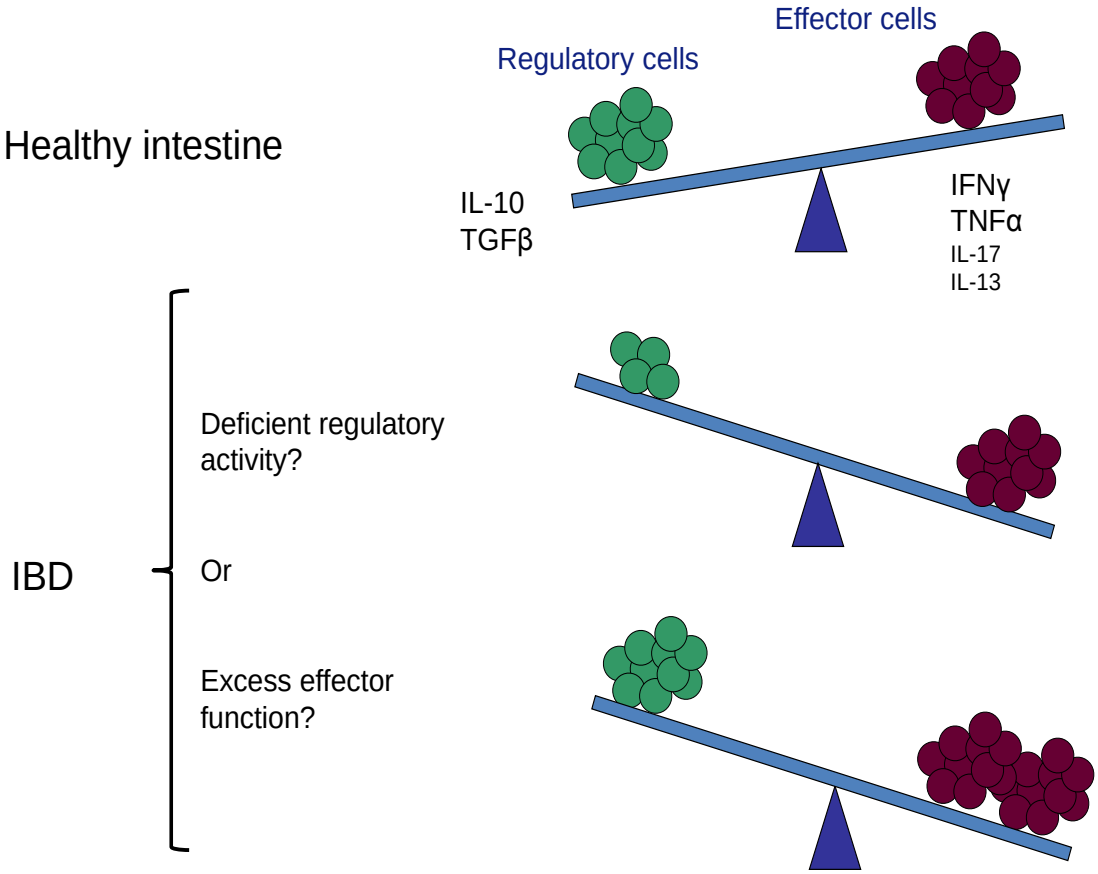

***Figure 1.8 Schematic representation of the traditional models of T cells in IBD**. T cell homeostasis in the intestine is dependent on a balance of regulatory T cells, producing IL-10 and TGFβ, and effector T cells, producing IFNγ and TNFα. The precise phenotype and cytokine profile of CD4+ T cells is described in detail in the text, but traditionally IBD is seen as either an excess effector T cell function, or failure of regulatory T cells. However, as mentioned, the phenotype of T cells is less fixed than these concepts allow, suggesting this model is an over-simplification.*

1.4.1 Crohn's disease as a "TH1" disease

Traditionally, CD has been viewed as a TH1-mediated disease (Monteleone et al., 1997). Inflammation in CD is associated with high levels of IFNγ production by LP T cells (Fuss et al., 1996), and APCs in CD patients also produce more IL-12 compared with UC patients and healthy controls (Fuss et al., 2006; Hart et al., 2005; Neurath et al., 2002). There is also increased $Tbet^{+}$ T cells in inflamed LP of CD patients compared with UC and controls (Neurath et al., 2002). However, monoclonal antibodies to IFNγ are ineffective in CD patients, suggesting that

transient reduction in IFNγ alone is not sufficient to halt intestinal inflammation (Reinisch et al., 2010; Reinisch et al., 2006).

1.4.2 UC as a "TH2" disease

UC has been shown to have a modified T helper 2 (TH2) signature, with increased IL-5 and IL-13 production, but decreased IL-4 (Fuss et al., 2004; Fuss et al., 1996). However, $CD4^+$ T cells in both UC and CD have elevated IFNγ and IL-17A production compared with controls (Rovedatti et al., 2009). The overall gene expression in CD and UC inflamed mucosa is remarkably similar, with a rare exception of increased IL-23 expression in UC relative to CD (Granlund et al., 2013).

1.4.3 CD as a "TH1/TH17" disease

With the discovery of the T helper 17 (TH17) lineage, CD is now seen as a disease in which both TH1 and TH17 cells are major driving forces (Brand, 2009), with TH17 cells producing cytokines associated with tissue damage in IBD (reviewed in Neurath, 2014a). Increased IL-17 producing T cells have been found in both CD and UC compared with controls (Fujino et al., 2003; Kleinschek et al., 2009; Rovedatti et al., 2009). More IL-17-producing T cells were detected in mesenteric lymph nodes of CD patients compared to both UC and controls (Sakuraba et al., 2009), and more IL-17 has been found by ELISA after stimulation of intestinal T cells in CD patients compared with controls (Wilke et al., 2011). In this study, levels of IL-17 were negatively correlated with (pro-regulatory) IL-10.

However, monoclonal antibodies to IL-17A are ineffective in CD, and are associated with increased susceptibility to infections (Hueber et al., 2012). This is consistent with the proposal that IL-17 can be both pro- and anti-inflammatory in the gut (Monteleone et al., 2012). There are six members of the IL-17 family (IL17A-F), with similar structures and functions, although IL17A and F are considered the most

similar (Khader et al., 2009b; Songhet et al., 2010). However, in mouse models of colitis, *Il17a* knockout mice have more severe inflammation (O'Connor et al., 2009; Yang et al., 2008b), but *Il17f* knockout mice have attenuated colitis (Yang et al., 2008b). Monoclonal antibodies to IL-6 (IL-6 is associated with production of a pro-inflammatory TH17 phenotype) were not shown to be effective in treating active CD (Ito et al., 2004). These studies suggest that while IL-17 may be important in the inflammation of IBD, its role is still not fully understood.

1.4.4 IBD as a defective Treg disease

It has been suggested that inflammation in IBD may be the result of a failure of Treg populations to restrain effector T cells (Mayne and Williams, 2013). However, identifying Tregs with the human mucosa is difficult, due to the transient expression of FoxP3 in activated T cells (see section 1.3.6.3.2). Also, $CD25^{hi}$ $FoxP3^{+}$ T cells are increased in the intestine of UC and CD patients compared to controls (Makita et al., 2004; Maul et al., 2005; Uhlig et al., 2006). The Tregs in the intestine can suppress proliferation of autologous peripheral blood T cells (Kelsen et al., 2005; Makita et al., 2004; Maul et al., 2005), so their increased presence in IBD suggests the effector T cell population is somehow refractory to suppressive signals. For example, effector T cells in inflamed IBD mucosa express SMAD7, which makes them resistant to TGF-β mediated suppression by Tregs (Fantini et al., 2009).

Recombinant IL-10, another anti-inflammatory cytokine produced by Tregs, has been shown to be ineffective in the treatment of CD (Buruiana et al., 2010). However, Ova-specific Tregs have been used as an autologous treatment for refractory Crohn's disease with limited success (Desreumaux et al., 2012). The suppressive effects of the Tregs in this study were felt to be largely due to increased T cell production of IL-10 in the gut, but perhaps in conjunction with cell contact suppression mechanisms.

So while it is clear that T cells play an important regulatory role in the intestine, including IL-10 production, their place in the pathogenesis and therapy of IBD is not yet certain.

1.4.5 The role of plasticity in the IBD T cell subsets

The early studies in Crohn's disease T cell phenotype demonstrated increased IFNγ mRNA from Northern Blot analysis (Breese et al., 1993; Fais et al., 1991). However, flow cytometry analysis of lamina propria T cells from CD has shown a decrease in the proportion of IFNγ-producing cells (Van Damme et al., 2001). In both CD and UC, a mixed cell population containing a high proportion of T cells was shown by ELISPOT to have fewer cells producing IL-4 than in the control group (Karttunnen et al., 1994). It has also been suggested that cytokine patterns in CD change with time and disease progression, from a TH2 to a TH1 dominated pattern (Desreumaux et al., 1997; Holland et al., 2008), although other studies show an early TH1 predominance (Kugathasan et al., 2007; Zorzi et al., 2013), with a later progression to a TH1/TH17 profile (Zorzi et al., 2013). A study of paediatric patients with IBD showed a varied pattern of increased TH1 and or TH17 cytokine transcripts in CD and UC (Verdier et al., 2012). Given the varied clinical phenotype of CD it is difficult to compare these studies as they sample different patient populations in different ways.

Recently, there have been increasing examples of studies demonstrating intestinal T cells producing cytokines that vary from their traditional T helper phenotype. TH17 cells are found in increased numbers in CD lesions, and can express IFNγ as well as IL-17 more readily than the same cell population in healthy control (Kleinschek et al., 2009). Both intestinal TH17 and TH1/17 cells have been found to be increased in patients with IBD compared to controls (Rovedatti et al., 2009)

In CD patients, a proportion of $CD4^+FoxP3^+$ T cells have the capacity to produce IL-17A, and these cells were not seen in UC or healthy controls (Hovhannisyan et al., 2011). These cells could suppress proliferation of peripheral blood T cells, express IL-17A and RORγt, as well as lFNγ and Tbet (Annunziato et al., 2007; Hovhannisyan et al., 2011). These cells are not seen in the peripheral circulation, suggesting that there are factors in the intestinal mucosa influencing the phenotype of the effector T cell pool. Separately, a similar population of $CD4^+FoxP3^+IL\text{-}17^+$ T cells was identified in patients with UC and were thought to be associated with carcinogenesis (Kryczek et al., 2011).

Analysis of gene expression from whole biopsies showed increased TH17-related gene expression in IBD, particularly in UC, but these changes was more significant in colonic than ileal CD, suggesting a relationship between site of inflammation of T cell phenotype (Bogaert et al., 2010). Another study of inflamed whole biopsy specimens showed increased expression in TH1 and TH17 related genes compared with controls, with no difference between UC and CD (Granlund et al., 2013). Of note in this study, there were minimal differences in gene expression between inactive IBD samples and healthy controls.

It has been shown that T cell cytokine production in response to stimulation differs with increasing age, with little TH2 cytokine production in children under 2 years of age, and varying widely between individuals (Smart and Kemp, 2001). This may confound any findings in T cell phenotype within IBD patients, particularly if the control groups are not matched closely. This is often the case when surgical resection specimens are used, and these individuals usually have colorectal cancer are substantially older than the IBD cohort.

1.4.6 STAT and SOCS proteins in models of colitis and in IBD

Due to their importance in cell signalling and T cell differentiation, STAT proteins have been previously studied in the context of IBD. In mice, adoptive transfer of T cells with transgenic mice with constitutively activated STAT4 to a SCID model leads to a chronic colitis (Wirtz et al., 1999). Also, knockout of *Stat4* abolished colitis in a *Stat6/T-bet* knockout mouse model as the result of increased Treg development (Xu et al., 2011). Also in the T cell transfer model of colitis, it has also been shown that STAT3 is required for the development of disease, as T cells from *Stat3* KO mice did not cause colitis (Durant et al., 2010). STAT3 also appears to be necessary for the regulatory function of intestinal T cells, as conditional knockout of *Stat3* in $FoxP3^+$ cells in mice led to an early fatal colitis (Chaudhry et al., 2009).

Perhaps unsurprisingly, STAT3 responses in colitis are context and cell-type dependent. In murine intestinal epithelial cells, STAT3 has been shown to be protective in models of colitis (Pickert et al., 2009). Separately, STAT3 signalling in these epithelial cells has been shown to be necessary for IL-10 production, which is itself protective against murine colitis (Pickert et al., 2009).

SOCS1, the main regulator of STAT1 and a suppressor of other STATs, particularly in T cells, has been shown to be necessary to prevent colitis by inhibiting a STAT1 signalling pathway (Horino et al., 2008). SOCS3 appears to negatively regulate intestinal inflammation by inhibiting the STAT3 pathway (Suzuki et al., 2001).

In humans, levels of STAT1 have been found to be increased in whole biopsies by Western Blotting (WB) from inflamed tissue of Ulcerative Colitis (UC) patients compared to Crohn's disease (CD) patients but both higher compared with controls (Schreiber et al., 2002). STAT3 levels have been found increased $CD4^+$ T cells in CD compared to controls as measured by WB, after multiple *ex vivo* stimulations (Lovato et al., 2003). STAT3 expression in T cells has been found to be increased in both CD and UC patient colonic specimens, after CD2/28 stimulation and separation

by magnetic beads (Mudter et al., 2005). In a study of paediatric UC, levels of STAT4, as well as GATA-3 and IL-4, were increased from mucosal specimens (Ohtani et al., 2010). In one study, high levels of SOCS3 protein (the main STAT3 inhibitor) were found in healthy control and CD patient biopsies compared to UC patients (Schreiber et al., 2002), although a different analysis of CD patients showed increased SOCS3 expression in intestinal samples compared with healthy controls (Lovato et al., 2003). More recently, SOCS3 mRNA expression levels from whole biopsies of inflamed areas of colon from CD and UC patients were found to be increased compared to healthy controls (León et al., 2009).

These studies have, in many cases, used whole tissue samples or heterogenous cell populations and therefore may miss cell type specific changes. For example, exposure of human dendritic cells to IL-10 induces a tolerogenic phenotype which appears to be dependent on STAT3 in the DCs (Saito et al., 2011). In contrast, in T cells STAT3 signalling skews away from a regulatory function (Yang et al., 2011).

All of the studies in the above paragraph use samples from inflamed areas of intestine (or do not state degree of inflammation) in IBD samples and therefore may miss changes in the non-inflamed areas. There is some evidence of an altered cytokine environment in areas of unaffected bowel in CD and UC patients. Increased IL-1β, IL-6 and TNFα have been detected from non-inflamed (whole) tissue samples (Akazawa et al., 2002; Reimund et al., 1996). Altered signalling pathways in non-inflamed patient tissues may indicate events relevant to immune dysregulation that could become ‘swamped’ by a dominant inflammatory signature in tissue which is actively inflamed.

As mentioned in section 1.2.4.1, IBD GWAS studies have shown that STAT3, JAK2 and STAT4 are all loci associated with increased risk for CD and/or UC (Glas et al., 2010; Jostins et al., 2012; Lees et al., 2011).

As discussed in section 1.2.3, new agents have been developed to target the JAK/STAT pathway in IBD. Toficitanib (CP-690,550), a putatively selective JAK1/JAK3 inhibitor has been used in Phase II trials in both CD and UC, with statistically significant benefit for patients with UC but not CD (Sandborn et al., 2011a; Sandborn et al., 2011b). In murine T cells, this new drug has been shown to have downstream effects on STAT1, STAT3, STAT5 and STAT6 signalling (Ghoreschi et al., 2011). This underlies the importance of further understanding these pathways to improve treatment of IBD patients.

1.5 T1IFN in mucosal immune regulation

The viral “Interferon” was first identified in the 1950s (ISAACS and LINDENMANN, 1957). T1IFN is essential for anti-viral defence in mice and humans (Theofilopoulos et al., 2005). This was initially demonstrated using neutralising anti-IFNα/β antiserum which increased the pathogenicity of many different viruses (Gresser et al., 1976a; Gresser et al., 1976b). The lesser known immunological, and predominantly anti-inflammatory, effects of T1IFN have nevertheless been known for nearly 30 years (Ling et al., 1985). T1IFN, as a group of cytokines in many contexts, can be considered a bridge between the innate and adaptive immune systems (Mangan and Fung, 2012).

There are many subtypes of Type I Interferon (IFN) recognized; 14 IFNα subtypes, as well as a single IFNβ, ε, κ,τ, δ and limitin (reviewed in Hertzog and Williams, 2013). These many T1IFNs are coded by multiple genes but they are expressed in a co-ordinated fashion, and it has been suggested that IFNβ is the master regulator of this network (Gough et al., 2012). T1IFNs are typically induced by activation of a pattern recognition receptor (PRR) (reviewed in Cavlar et al., 2012). All T1IFNs signal via the T1IFN receptor comprising IFNAR1 and IFNAR2 components. The

differing responses of different T1IFN isoforms may be due to a mixture of conserved and subtype-specific ligand binding sites modulating downstream signalling (Thomas et al., 2011). That is, the different T1IFN subtypes bind to the IFNAR receptor in slightly different ways, which may affect the downstream signalling pathways. These pathways are various but include the canonical JAK/STAT pathway (reviewed in Noppert et al., 2007; Platanias, 2005). Pre-associated with the IFNAR are the Janus Kinases Tyk2 (with IFNAR1) and Jak1 (with IFNAR2) (Domanski et al., 1997; Yan et al., 1996). In most cells STAT1, STAT2, STAT3 and STAT5 are phosphorylated after T1IFN binding but in lymphocytes so are STAT4 and STAT6 (Cho et al., 1996; Fasler-Kan et al., 1998; Matikainen et al., 1999). Recently, it has been shown that IFNβ can signal independently of INFAR2 and the Jak/STAT signalling machinery (de Weerd, 2013). Regardless of the mechanism of signalling, these pathways all lead to the expression of a very large number of IFN-stimulated genes (ISGs)(Samarajiwa et al., 2009).

1.5.1 The cellular and anatomical source of T1IFN

T1IFNs can be produced from almost every cell type (reviewed in González-Navajas et al., 2012). An early murine study suggested that macrophages are the main source of T1IFN (Vogel and Fertsch, 1984). It was then shown that, in humans, IFNα mRNA could be detected in the tissue of various organs (although the gut was not tested in this study) and peripheral blood of humans, but that IFNβ required viral stimulation to be detectable by western blot (Tovey et al., 1987). In mice, IFN-producing cells (IPCs) were identified as plasmacytoid-like immature APCs from various organs, including the lung (Asselin-Paturel et al., 2001). Plasmacytoid dentritic cells (pDCs) were identified as the main producers of T1IFN

in human blood (Siegal et al., 1999). However, in the duodenum at least, several different APC populations have been shown to produce small amounts of IFNα in equal quantity (Ráki et al., 2013).

IFN reporter mice, which are genetically altered to express a product alongside T1IFN that is easily detectable (eg green fluorescent protein - GFP) have enabled the detection of T1IFN more accurately, and have also shown its production from a variety of sources in viral infections. For example, LCMV infection has been shown to promote IFNα production from pDCs and not conventional DCs (cDCs) or macrophages in the spleen or other organs (Jung et al., 2008). However, in Newcastle disease viral respiratory infection, the IFNα production was from alveolar macrophages and cDCs, although in systemic infection the IFNα was produced from pDCs (Kumagai et al., 2007). So-called inflammatory monocytes ($Ly6C^{hi}CD11b^{+}CD11c^{-}B220^{-}$) can also produce T1IFN in mice in response to MCMV and vaccinia (Barbalat et al., 2009).

From reciprocal mouse chimera experiments, which enable determination whether cells have a haematopoeitc origin, it has been suggested that IFNβ production from respiratory epithelial cells is more important than from immune cells in protection against viral infection (Shornick et al., 2008). Subsequently, epithelial cells have been shown to be strong producers of T1IFN in the murine respiratory tract in bacterial infection (Parker et al., 2011).

However, in a different mouse model of viral intestinal infection, the primary source of IFNα and IFNβ production in Peyer's Patches (PPs) was cDCs (and, to a lesser extent, pDCs) with no epithelial contribution (Johansson et al., 2007). It is unclear where the main source of T1IFN in the gut is in the absence of infection. In the steady state, murine gut stromal cells have also been shown to produce T1IFN (Tezuka et al., 2011). In the human intestine, epithelial cells have been shown to

produce large quantities of IFNβ (Watanabe et al., 2010). pDCs, often considered that archetypal T1IFN-producing cell, were found to be rare in the human duodenal mucosa, with IFNα produced in low levels by a variety of cell types in active celiac disease (Ráki et al., 2013). However, it remains unclear whether this is the predominant source in health or only after infection or with inflammation. Interestingly, in mice, endogenous T1IFN appears to be driven by signalling from commensal microbiota (Yamamoto et al., 2012).

There has been recent huge interest in the microbiome and its importance in diseases from obesity to inflammatory bowel disease. However, there has been relatively little research into the viral component of the intestinal contents in health or IBD. However, there is a “virome” and it is reasonable to suppose that the interactions with the immune system are important in health as well as disease (Duerkop and Hooper, 2013). This virome will, along with the bacteria in the intestine, provide signals to the innate and adaptive immune systems of the intestine. Recently, it has been shown that the virome in IBD appears to differ in patients with IBD to controls (Norman et al., 2015), and this may have significant implications in the understanding of viral responses, including T1IFN, in this patient group.

1.5.2 Triggers of T1IFN production

IFNs are induced mainly via the family of PRRs called the Toll-Like Receptors (TLRs), and are susceptible to bacterial as well as viral induction (Monroe et al., 2010). TLRs 2,3,4,7,8 and 9 (both cell membrane and intracellular) have all been shown to induce T1IFN (reviewed in Monroe et al., 2010)). The best characterized of these is LPS binding of TLR4 which induces T1IFN in multiple cell types via TIR-domain-containing adapter-inducing interferon-β (TRIF) (Fitzgerald et al., 2003;

Toshchakov et al., 2002). In the gut, viral (but not bacterial) ligands signalling via TLR2 on “inflammatory monocytes” lead to a T1IFN response (Barbalat et al., 2009). This selective responsiveness to viral and not bacterial antigens may be a method the gut uses to prevent a pro-inflammatory response to the large number of commensal bacteria, while maintaining effector response to viral infection. In contrast, a different transduction protein, TNF receptor associated factor (TRAF)3 is important in downstream signalling from TLRs for the T1IFN-mediated induction of anti-inflammatory IL-10. This use of different adaptor proteins is a possible mechanism by which signalling through the same TLR may trigger pro- or anti-inflammatory responses in different circumstances (Häcker et al., 2006; Tseng et al., 2010). Non-TLR bacterial induction of T1IFN has also been shown by various cytosolic receptors (Kawai and Akira, 2009). Notably, *Mycobacterium tuberculosis* induced IFNα/β production via NOD2 and Interferon Regulatory Factor 5 (IRF 5) in murine macrophages (Pandey et al., 2009). As mentioned in section 1.2.4.1, NOD2 variants are strongly associated with Crohn’s disease., (Hugot et al., 2001). This suggests that bacterial products may trigger T1IFN responses via NOD2 in the human colon, which may be dysregulated in patients with NOD2 variants.

It was initially thought that T1IFN was only produced in response to (viral) infection, but there is now increasing evidence of its constitutive production. Indeed, in mice T1IFN is constitutively produced in many tissues, including the gut (Chirdo et al., 2005; Gresser and Belardelli, 2002; Lienenklaus et al., 2009; Munakata et al., 2008).

T1IFN has also been shown to be produced in response to DNA degradation products from dying cells or aberrant nucleic acid metabolism (Okabe et al., 2005). An early study demonstrated the presence of endogenous T1IFN as an inhibitor of TNFα and LPS effects on murine bone-marrow derived macrophages (Hamilton et

al., 1996). More recently, in mice, DCs were found to be significant constitutive producers of T1IFN (Chirdo et al., 2005).

Differing expressions of various parts of interferon signalling machinery have been shown to be important in the stochastic production of IFNβ (Zhao et al., 2012). It has been hypothesised that constitutive T1IFN is responsible for maintaining presence of signalling intermediaries, such as STAT proteins, required for its own, as well as many other, cytokine responses (Gough et al., 2012; Hertzog and Williams, 2013). Indeed inconsistent expression of IFNβ by immune cells in response to stimuli (Zhao et al., 2012) has been suggested to underlie the necessity for constitutive T1IFN production to maintain the cellular machinery necessary for its own production in response to pathogen (Hertzog and Williams, 2013).

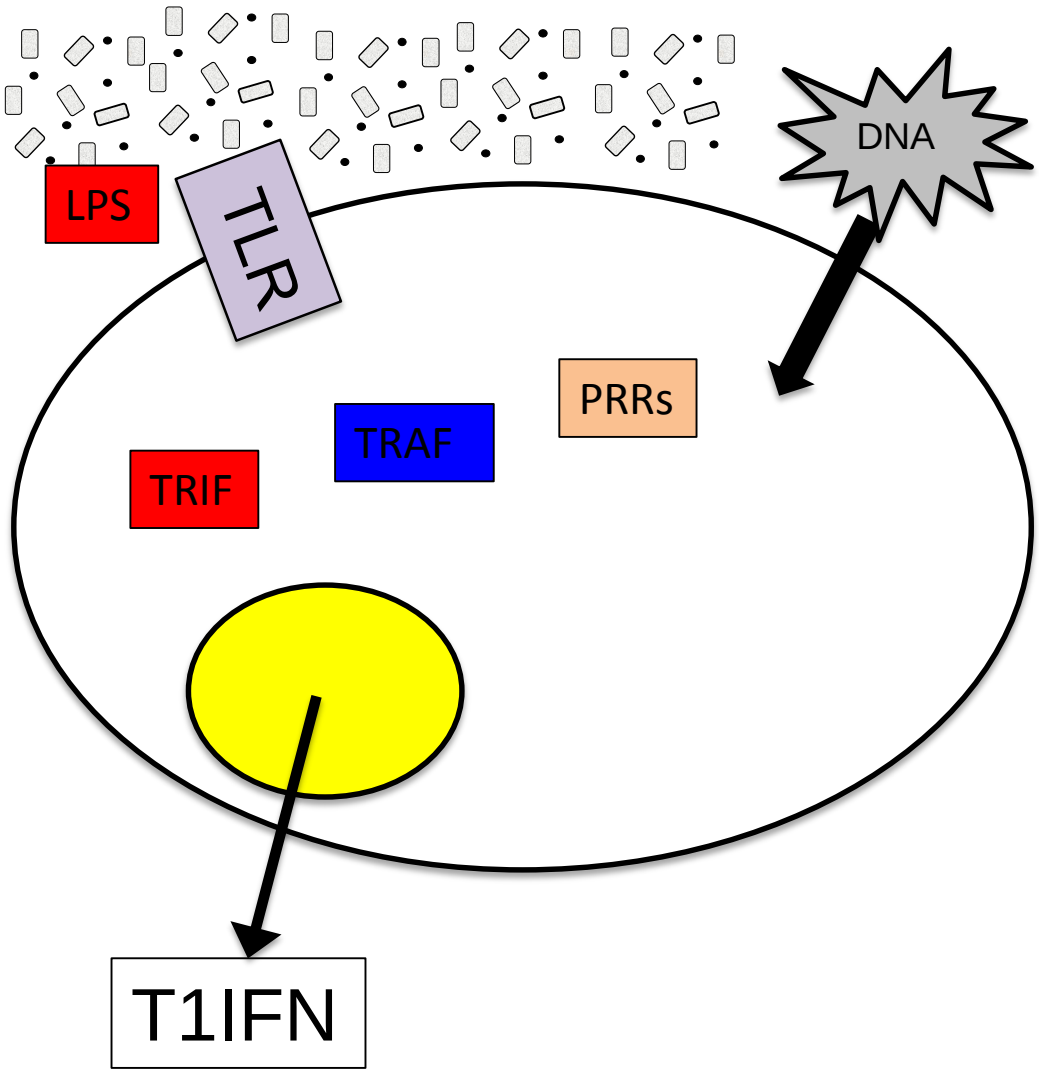

Figure 1.9 Cellular triggers of T1IFN production in the human intestine. *Microbial products, including LPS, signal largely via TLRs (including TLRs 2,3,4,7,8 and 9) which then use complex signalling machinery to promote T1IFN production. Depending on the cellular pathways, this may lead to a more pro-inflammatory effect (eg involving TRIF) or regulatory (TRAF). Intracellular Pattern Recognition Receptors (PRR), such as NOD2, may also lead to T1IFN secretion, after detection of DNA breakdown products, from microbial or host cells. The cellular source of T1IFN in the human gut is not known; all cells are capable of producing T1IFN, but epithelial cells, stromal cells, conventional and plasmacytoid DCs have all been implicated as primary producers in different circumstances.*

1.5.3 T1IFN in innate immunity

T1IFN has been shown to have contradictory roles in the innate immune response. IFNβ has been shown to be necessary for the LPS shock response *in vivo* from knockout mice experiments (Karaghiosoff et al., 2003). Mice without the T1IFN

receptor are also resistant to *Listeria monocytogenes* infection, and produce increased IL-12p70 and TNFα, but not IFNγ (Auerbuch et al., 2004) with no change in their adaptive response to re-challenge. In support of this result, addition of T1IFN increased susceptibility to the same infection (O'Connell et al., 2004). T1IFN also appears to increase susceptibility to *Mycobacterium tuberculosis* in mice (Stanley et al., 2007), and Poly I:C (a potent inducer of T1IFN) exacerbated *M. tuberculosis* infection in mice dependent on monocyte/macrophages (with no effect of T cell function) (Antonelli et al., 2010). IFNAR knockout mouse have also been shown to be resistant to *Chlamydia murinarum*, due to decreased apoptosis of macrophages (Qiu et al., 2008).

In contrast, IFNAR knockout mice have decreased survival following infection with *Streptococcus pneumonia* or *Escherichia coli* (Mancuso et al., 2007). In *S .pneumonia* lung infection, T1IFN signalling is active in the murine lung, particularly in epithelial cells, neutrophils and cDCs, and contributes to protection from disease (Parker et al., 2011).

T1IFN has also been shown to inhibit inflammation by suppressing inflammasome activity. Specifically, in murine BMDMs under various conditions, T1IFN inhibits IL-1β production in a STAT1-dependant manner, and increases IL-10 production (Chang et al., 2007; Guarda et al., 2011). T1IFN has also been shown to be important in reducing mortality from influenza A infection in mice, through enhanced IL-10 production (Arimori et al., 2013). However, in a separate mouse model of influenza, T1IFN has been shown to increase IL-1β release (Henry et al., 2007).

In the murine intestine, there is some evidence that T1IFN has a role in maintaining mucosal homeostasis. IFNAR knockout mice have been shown to be more susceptible to dextran sulfate sodium (DSS) colitis (McFarland et al., 2011). Also,

T1IFN induced by TLR9 signalling has been shown to abrogate colitis in mouse models (Katakura et al., 2005). Specifically, IFNβ, induced by TLR9 signalling in $CD11c^{hi}$ DCs, reduces colitis in the DSS model (Abe et al., 2007).. However, local delivery of IFNβ by a transgenic *Lactobacillus acidophilus* that constitutively expresses IFNβ led to worsening of DSS colitis (McFarland et al., 2011). A possible explanation of this apparently contradictory finding was that the *L. acidophilus* caused a decreased expression of IFNAR1 by CD103+ DCs in the murine Peyer's patches and this may lead to an inability to protect against the DSS-induced colitis via endogenous T1IFN. The TLR7 agonist, Imiquimod, promoted T1IFN expression from the intestinal tract and ameliorated DSS colitis (Sainathan et al., 2012).

Commensal *Escherichia coli* increased IFNα mRNA and was protective against induced epithelial apoptosis, as was exogenous IFNα (Mirpuri et al., 2010). DNA products of the probiotic combination VSL-3 were shown to protect against DSS colitis via TLR9 (often involved in T1IFN production), although this was thought to be via a systemic effect of the TLR9 agonists (Rachmilewitz et al., 2004).

In contrast, two strains of *Lactobacillus acidophilus*, but not other probiotic strains, have been shown to induce IFNβ in murine bone marrow derived DCs (BMDCs) via TLR2, associated with downstream induction of pro-inflammatory IL-12 that could be inhibited by anti- IFNβ (Weiss et al., 2010b). In related work a strain of *Bifidobacterium bifidum* was shown to block the above effect of *L. acidophilus* and reduce Th1 skewing by abrogating the IFNβ induction (Weiss et al., 2010a).

More recently, a commensal small intestinal bacteria has been shown to trigger IFNβ production from murine BMDCs (via TLR3) and this is protective against DSS colitis (Kawashima et al., 2013)(Kawashima et al., 2013)(Kawashima et al., 2013)(Kawashima et al., 2013)(Kawashima et al., 2013)(Kawashima et al., 2013). Furthermore, antibiotic treated mice have shown decreased T1IFN response (from intestinal macrophages), and pathogen clearance in viral infections (Abt et al., 2012).

These studies show that the complex interplay between microbiota may result in varying effects on the downstream T1IFN pathway. Furthermore, in response to various microbial products, T1IFN is usually (but not always) anti-inflammatory in the mouse gut mucosa, and multiple TLRs are involved in this innate response.

1.5.4 T1IFN in adaptive immunity

While T1IFN is more traditionally associated with the innate immune system, due to both its rapid production and source, there is increasing interest in its downstream effect on the adaptive immune response. There is evidence of a direct of T1IFN on T cells that this is dependent on IFNAR (Le Bon et al., 2006). More specifically, T1IFN can enhance both CD4 and CD8 responses, when given at or after immunization (Le Bon et al., 2006; Proietti et al., 2002). TLR9, commonly associated with immune-sensing on innate immune cells (see section 1.3.3), has also been found to be expressed by $CD3^+$ cells in the murine gastric mucosa, where it is associated with increased T1IFN mRNA production and an anti-inflammatory cytokine profile (Otani et al., 2012)

There has been relatively less work on the effect of T1IFN on B cells than on T cells. However, T1IFN has been shown to enhance antibody responses and isotype switching in mice (Tezuka et al., 2011). Interestingly, in the absence of B cells or IgA, the murine intestinal epithelium upregulates T1IFN related genes, and similar gene expression profiles were seen in duodenal biopsies from humans with CVID and also those infected with HIV (Shulzhenko et al., 2011). These are different examples of murine and human deficiencies in the adaptive immune system, where a related increase in T1IFN effects may be a marker of the innate immune system attempting to compensate for the defect in the adaptive system.

Recent work in chronic viral infection models have shown that in contrast to its critical importance in controlling early viral infection, endogenous T1IFN can contribute to the persistence of chronic viral infection, dependant on both $CD4^+$ T cells and IL-10 production (Teijaro et al., 2013; Wilson et al., 2013). This lends weight to the important immunomodulatory role that T1IFN plays in T cells, particularly in chronic infection and perhaps in chronic inflammatory disorders (Odorizzi and Wherry, 2013).

1.5.4.1 The effects of T1IFNs on CD4 T cell subsets

1.5.4.1.1 Regulatory T cells

In contrast to the regulatory effects on dendritic cells, TLR9 knockout mice have a reduction in Treg cell induction and an increase in IFNγ production (Hall et al., 2008). This suggests the DNA products from the microbiota may have a regulatory effect. Indeed, in a T cell transfer model of colitis, regulatory effects of bacterial DNA on T cells appeared to be via CD11c DCs. But even direct treatment of murine T cells *in vitro* with IFNβ prior to transfer abrogated colitis (Hofmann et al., 2010). Using CpG-ODN a (potent T1IFN inducer) in SCID mice showed induction of regulatory T cells and protection from colitis via both TGF-β and IFNα/β signalling (Bleich et al., 2009). In a murine tumour model, Tregs and their IL-10 production were dependent on IFNAR and STAT1 signalling, and limited TH17 inflammation (Stewart et al., 2013).

Intestinal $CD11c^{lo}$ DCs in mice have been shown to have increased capacity for IFNα production compared with peripheral DCs, and these gut DCs are susceptible to CpG activation and induce regulatory T cells and suppress effector T cell expansion (Bilsborough et al., 2003). IFNAR knockout mice had reduced Tregs in

Peyers patches and were more susceptible to DSS colitis with upregulation of many pro-inflammatory cytokines (McFarland et al., 2011). In a more recent study, it was shown that in a mouse model of colitis, constitutive IFNβ signalling in the intestine was necessary to maintain FoxP3 expression in T cells and suppress inflammation (Lee et al., 2012). However, exogenous IFNα has been shown to impair the suppressive effects of Tregs (Bacher et al., 2013), and IFNβ can inhibit Treg function in mice to allow chronic viral infection clearance (Srivastava et al., 2014). There are various possibilities for these contradictory results, including a difference in endogenous versus exogenous effects of T1IFN, a difference between IFNα and IFNβ subtypes, or even a concentration dependant outcome.

In human PBMCs treated under TH1 polarising conditions (anti-CD3 antibody in presence of IL-12), IFNβ had no effect on IFNγ or IL-17 production from $CD4^+$ T cells, but did increase IL-10 production (Axtell et al., 2010). IFNα has been shown to induce the regulatory Tr1 cells in addition to IL-10 production (Levings et al., 2001). In LPS-stimulated human PBMCs and T cells lines, T1IFN has been shown to promote IL-10 production and inhibit IL-12 production (Wang et al., 2000).

Studies in MS and EAE suggest TIFN promotes Treg frequency and function ((Namdar et al., 2010; Pace et al., 2010), but the opposite effect has been shown (Golding et al., 2010). These suggest a differing response depending on timing and possibly subtype of T1IFN used. Patients with MS treated with IFNβ also have higher FoxP3 mRNA than untreated patients or controls in $CD4^+CD25^+$ T cells (Vandenbark et al., 2009), as well as increased $CD4^+CD25^+$ cell numbers and improved suppressive function (de Andrés et al., 2007). This is perhaps the most direct evidence of a pro-regulatory effect of T1IFN on T cells in humans *in vivo* although it cannot be determine whether T1IFN effects regulatory T cells directly in this context.

1.5.4.1.2 TH17 cells

In a mouse model of multiple sclerosis (EAE), T1IFN has been shown to reduce IL-17 production by T cells and restrain inflammation (Guo et al., 2008). However, in a more recent paper, IFNβ was shown to exacerbate EAE induced by TH17 cells, despite reducing IL-17 production. IFNβ was effective in improving EAE driven by TH1 cells, and this was associated with an increase in the amount of IL-10 production from isolated splenic T cells. and these effects were dependent on IFNγ (Axtell et al., 2010). Indeed, the immunosuppressive effects of T1IFN in EAE have been shown to be dependent on IFNγ, and that without IFNγ signalling, T1IFN has a pro-inflammatory effect (Naves et al., 2013).

In human PBMCs, both TH17 differentiation and IL-17 production were suppressed by IFNα (Moschen et al., 2008). In patients with relapsing-remitting MS (RRMS), non-responders to IFNβ have higher serum IL-17F than responders (Axtell et al., 2010). It has been suggested that IFNβ may worsen diseases (including some patients with MS) who have an underlying TH17 driven inflammation, but may help to contain TH1 driven disease (Axtell et al., 2011).

1.5.4.1.3 TH1 cells

In early studies, *in vitro* T1IFN was shown to induce TH1 differentiation in humans via STAT4 activation, by increasing responsiveness to IL-12 (Rogge et al., 1998; Shibuya et al., 2003). Systemic IFNβ treatment in mice increase TH1 responses and decreases TH2 responses (Yoo et al., 2010). In addition, in LCMV infection models, T1IFN induces IFNγ production from $CD8^+$ T cells independently of IL-12 (Cousens et al., 1999). In a similar model, IFNα was shown to be required for IFNγ production, and this suggested that T1IFN signalling via STAT1 was regulatory, but via STAT4 promoted TH1 polarisation (Nguyen et al., 2002). It has subsequently been shown that T1IFN is not sufficient to sustain a TH1 response alone (Ramos et al., 2007).

In contrast to some of the above effects, T1IFN has also been shown to skew away from a TH1 phenotype. A hypervirulent strain of *Myocobacterium tuberculosis* was associated with increased IFNα mRNA in murine lungs, with reduced TH1 cytokine transcripts; and intra-nasal IFNα/β increased the bacterial load, with these differences not seen in SCID mice (Manca et al., 2001). It has been shown in a mouse viral (LCMV) infection model, STAT1-dependant signalling of T1IFN reduces T cell production of IFNγ, but STAT1-independent signalling increases IFNγ (Nguyen et al., 2000). This suggests that T1IFN, produced in response to bacterial or viral infection, can provide an anti-inflammatory role via the adaptive immune system and is dependent on STAT1.

1.5.4.1.4 TH2 cells

IFNα has been shown to alter TH2 cytokine production in human PBMCs by downregulating IL-13 production but increasing IL-4 (Kaser et al., 1998), as well as reversing Th2 commitment via suppression of GATA3 (Huber et al., 2010). T1IFN has been shown to inhibit STAT6 activation in human monocytes, possibly due to induction of SOCS1, and this prevented induction of IL-4-inducible genes (Dickensheets et al., 1999). This could have downstream effects on T cell differentiation by reducing the development of TH2 cells .

1.5.4.1.5 Summary of T1IFN effects on T Helper phenotype (see figure 1.10)

Overall, it is clear that T1IFN has multiple effects on the properties of CD4 T cells. It is likely that these effects are context specific, but appear to be generally pro-regulatory, including in the (less frequent) human studies. This is perhaps not surprising as T1IFN has been used for many years as a treatment of MS, considered to be a T-cell driven disease.

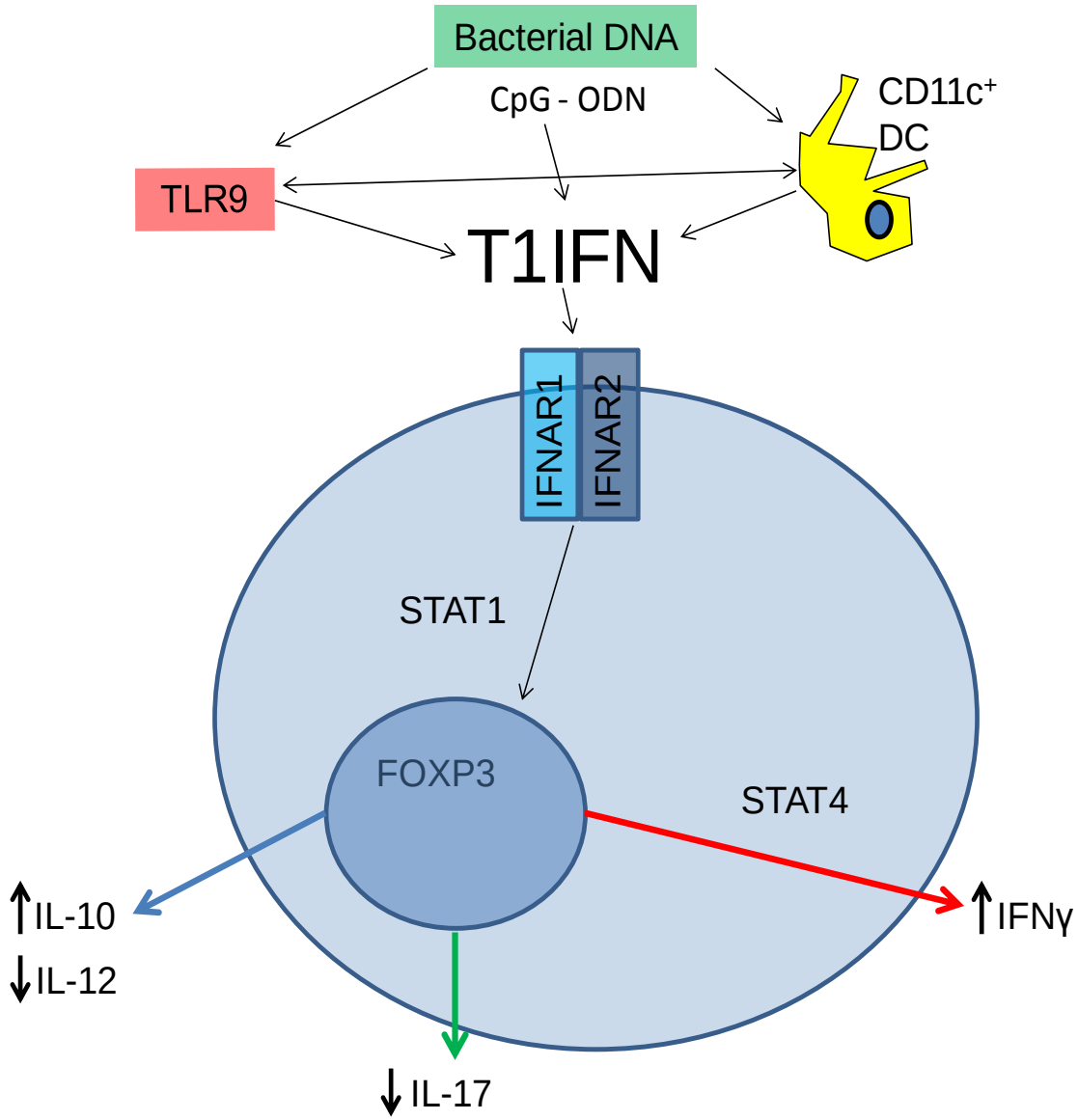

Figure 1.10 Summary of effects of T1IFN on T cell phenotype. *Bacterial products and other environmental triggers induce T1IFN, often requiring innate immune cells such as CD11c+ DCs and pattern recognition receptors such as TLR9. T1IFN signals via the IFNAR and STAT1, and tends to promote a pro-regulatory phenotype, with increased FoxP3 expression, and increased IL-10 production. IL-17 production is generally suppressed although this is of uncertain significance. Under different circumstances, however, T1IFN can signal via STAT4 and increase IFNγ production. The different effects of T1IFN on T cells are likely context specific and explored more in this and other chapters of the thesis.*

1.5.4.2 T cell survival

As well as its multiple effects on T cell phenotype, T1IFN has other global effects on T cell fate. T1IFN has been shown to sensitise murine lymphocytes to apoptosis, increasing susceptibility to *Listeria monocytogenes* infection (Carrero et al., 2004;

O'Connell et al., 2004). The susceptibility to infection was associated with apoptotic lymphocytes releasing IL-10 which may dampen the innate response to infection. In human *in vitro* experiments, IFNα with anti-CD3 stimulation increased apoptosis of T cells (Kaser et al., 1999). Also, it has been shown that while early in T cell activation T1IFN protects against apoptosis (Marrack 1999), it also enhances components of the apoptotic pathway to trigger cell death at the end of the immune response (Dondi et al., 2004). It may be that T1IFN-driven T cell apoptosis is mediated via STAT1, as this has been shown to be downregulated in proliferating CD8 T cells (Gil et al., 2006; Stark et al., 1998). STAT1 has also been shown to be necessary for the anti-proliferative effect of T1IFN (Bromberg et al., 1996; Tanabe et al., 2005).

It has been hypothesized that this anti-proliferative pathway may explain differences in effect of T1IFN in resting and activated T cells (Tough, 2012). That is, T1IFN effect on activated T cells is protective against apoptosis, mediated by STAT1, but with STAT1 signalling promoting apoptosis in resting T cells. This is supported by T1IFN increasing apoptosis of resting T cells in STAT1 knockout mice, but has no effect on apoptosis in wild-type mice (Tanabe et al., 2005). More specifically, T1IFN can activate both STAT1 and STAT3; these share great sequence similarity and similar structure (Qing and Stark, 2004). Nevertheless, *in vivo*, their activation can lead to opposing downstream effects (reviewed in Regis et al., 2008)). T1IFN is anti-apoptotic in resting mouse T cells when it signals via STAT3, but it is pro-apoptotic when it signals via STAT1 (Tanabe et al., 2005).

However, using another *in vitro* human model, IFNβ (from fibroblasts) has been shown to rescue activated T cells from apoptosis (Pilling et al., 1999). Indeed, in a very early mouse model of LCMV infection, when IFNAR knockout was restricted to the T cells alone, CD4 and CD8 expansion and survival were significantly reduced (Merigan et al., 1977). Overall this suggests that T1IFN can have pro- or anti-

apoptotic effects depending on both timing and context and that this is not yet fully understood.

In another example of T1IFN pro-survival effect, IFNα can enhance IL-2 production from human central memory T cells that then produce IFNγ (Davis et al., 2008). However, earlier human studies showed that high dose IFNα reduced IL-2 expression and IL-2 production from $CD4^+$ T cells, as well as decreasing CD3 and CD28 expression over time, and was associated with reduced proliferation (Zella et al., 2000). Prior exposure to IFNα, however, in a mouse model, has been shown to increase subsequent T cell proliferation (Feng et al., 2005). Again, this suggests that timing and dose of T1IFN exposure may be critical with regards to its effect on T cell function.

There is some evidence that T cells respond differently to T1IFN dependant on their level of activation, with reduced induction of ISGs after activation (Dondi et al., 2003). Also, expression of IL-2R as well as cell proliferation and survival following activation are further enhanced in response to IFNα (Hervas-Stubbs et al., 2010; Matikainen et al., 1999). In contrast, T cells that are treated with IFNα prior to CD3/CD28 stimulation have reduced IL-2 and IL-2R expression (Zella et al., 2000). All of this is further evidence of the conflicting potential of T1IFN to effect T cell survival by various methods.

This differential effect of T1IFN dependent on different signalling pathway may be related to the environment that the T cell has been exposed to, and may itself be affected by exposure to T1IFN. This “priming” of T cells by T1IFN may also be related to T cell migration and adhesion (Avraamides et al., 2007). Specifically, T1IFN has been shown to upregulate CD69, a marker of early T cell activation (Feng et al., 2005; Sun et al., 1998), which has also been proposed to promote retention of T cells in organized lymphoid tissue (Masopust and Schenkel, 2013), and is highly expressed in human intestinal T cells (Sathaliyawala et al., 2013). Indeed, CD69 has been found to be required for the tolerogenic effect of T1IFN in

mice (Radulovic et al., 2012). It is therefore conceivable that constitutive T1IFN, via CD69, has a role in retaining T cells in the organized lymphoid tissue of the gut, and promotes a pro-regulatory T cell response to commensal antigens.

1.5.5 T1IFN in human inflammatory diseases

Much of the continuing interest in T1IFN and its immunological effects are related to its success as a therapeutic agent in treating MS. As mentioned above, there is increasing evidence of different underlying immunological processes involved in patients who are responders to T1IFN compared with those who are non-responders (Axtell et al., 2010). But apart from the TH1/TH17 dichotomy, overall T1IFN (in MS) appears to decrease pro-inflammatory cytokine production via increasing IL-10 production, including from CD4+ T cells (Byrnes et al., 2002; Dikopoulos et al., 2005; Ersoy et al., 2005; Levings et al., 2001).

Psoriasis is a T-cell mediated inflammatory disease of the skin. In psoriasis, pDCs are recruited to the skin and produce large amounts of T1IFN (Nestle et al., 2005). In Systemic Lupus Erythematosus (SLE), elevated levels of T1IFN have been detected in patient serum, as well as increased ISGs in PBMCs (Baechler et al., 2003; Bennett et al., 2003; Hua et al., 2006). In paediatric patients with SLE, T1IFN has been shown to induce dendritic cell differentiation and drive inflammation (Blanco et al., 2001). T1IFN antibodies, which are associated with some autoimmune syndromes, often precede an increase in susceptibility to infections (Cheng and Anderson, 2012; Maródi et al., 2012).

In human mycobacterial infections, T1IFN has been shown to reduce IFNγ responses by macrophages, and there appears to be an inverse correlation between expression of IFNβ and expression of IFNγ in the lesions of patients with human *Mycobacterium leprae* infection.

With regards to mucosal diseases, T1IFN was used as a treatment for patients with IBD. There were individual trials of the use of IFNα and IFNβ in both UC and Crohn's disease with proven efficacy (Madsen et al., 2001; Musch et al., 2007; Nikolaus et al., 2003; Pena Rossi et al., 2009), but in a meta-analysis with other studies they were shown to have no clear benefit in UC (Seow et al., 2008). T1IFN was generally less used in Crohn's patients than UC due to questionable efficacy and significant side effects.

In contrast, for many years it has been recognized that in rare instances patients receiving T1IFN therapy for other reasons have developed or had exacerbations of IBD (Mitoro et al., 1993; Samson et al., 2009; Schott et al., 2007; Sprenger et al., 2005; Watanabe et al., 2011). This suggests that while T1IFN may be beneficial in selected patients, it also has the potential to trigger intestinal inflammation. This supports the idea that T1IFN can have either pro- or anti-inflammatory effects in the intestine under different circumstances.

It has been shown in UC patients who improved with IFNβ treatment, lamina propria T cell production of IL-13 was reduced, and non-responders had higher production of IL-17 (in peripheral and LPMC T cells) and IL-6 (in peripheral macrophages) (Mannon et al., 2011). In a separate study, UC patients showed increased IL-17 expression in whole colon biopsies, and this was reduced after IFNα treatment (Moschen et al., 2008).

As T1IFN is no longer used for the treatment of IBD, it will be difficult to assess in greater detail whether these immunological effects can help guide therapeutic interventions in the future. However, regardless of whether they have received IFN treatment, IBD patients have been found to have an increase in pDCs in the intestine compared to controls, but a decreased production of IFNα on CpG stimulation of peripheral blood pDCs (Baumgart et al., 2011). This could support the

idea of recruitment of pDCs to the intestine to provide T1IFN to the local environment, but does not address what the consequences of this would be.

Patients have also been known to develop coeliac disease while receiving T1IFN as a therapy for another condition. It has been suggested that this may be due to upregulation of HLA-DQ2 and DQ-8 (the MHC class II molecules on APCs that are able to bind gliadin peptides), increasing the likelihood of activation of effector T cells, and the subsequent intestinal damage (González-Navajas et al., 2012). IFNα has also been shown to promote a TH1 phenotype in intestinal T cells in patients with coeliac disease.(Di Sabatino et al., 2007).

On a final note, a new TLR9 agonist (and thus T1IFN inducer) DIMS0150 has been shown in a phase 1 clinical trial to offer benefit to patients with active UC, in terms of restoring sensitivity to glucocorticoids (Musch et al., 2013).

1.5.6 Summary of T1IFN in intestinal immunity

It is now recognized that T1IFN has a role in immune regulation beyond traditional anti-viral effects. These effects, particularly in the intestine, are varied but often pro-regulatory in nature. While critical for the clearing of acute viral infection, it now appears that T1IFN can perpetuate inflammation in chronic viral infections (Odorizzi and Wherry, 2013). Constitutive T1IFN in the intestine has at least the potential to be pro-regulatory in its effects on the innate immune system, while also decreasing pro-inflammatory T cells and support regulatory T cell function.

1.6 Summary

IBD is a complex disease with a partially understood pathogenesis that appears to be related to an inappropriate immunological response to the commensal microbiota. T cells are a significant component of the immune response in IBD and considered to be a major driver of persistent inflammation in the intestine. The

definition of T cell phenotypes based on cytokine production potential alone do not take into account for the ability of intestinal T cells to respond dynamically to their local environment. The Jak-STAT pathway is a major determinant of T cell signalling and abnormalities in this pathway occur in IBD. Measuring the activation of the Jak-STAT pathway may give a more nuanced picture of T cells in the gut.

Type 1 Interferon, which signals via the Jak-STAT pathway, has been used as a treatment in IBD, but with limited success. Mouse models of colitis suggest that constitutive T1IFN may be important in maintaining intestinal homeostasis via T cells. Therefore T1IFN signalling in intestinal T cells provides an interesting target to better understand an important mechanism of mucosal immune response.

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1.7 Overall Hypothesis and Aims

The objective of this study is to test the hypotheses that

(i) human intestinal T cells remain responsive to local mucosal signals, including Type I Interferon, and

(ii) these responses differ between health and disease and can be delineated by analysis of phosphorylation in key signalling pathways.

AIMS

The specific aims of this study were:

1. Develop a Phosflow technique for assessment of signalling pathways and responsiveness to external stimuli in human intestinal T cells.

2. Use this technique to compare the responsiveness of human intestinal T cells to signals including T1IFN in health and IBD

3. Assess the functional role of T1IFN in modifying the function of intestinal T cells in health and IBD.

2 Materials and Methods

2.1 Intestinal tissue and blood collection

2.1.1 Ethics

All tissue and blood donors gave informed written consent and the study was approved by the local ethics committee (refs 05/Q0405/71, 08/H0702/33, 11/LO/1543 and P/01/023).

2.1.2 Patients and controls

Controls consisted of patients (adults and children) with macroscopically and histologically normal intestine that had been referred with symptoms of abdominal pain, rectal bleeding or change in bowel habit. Patient samples were collected from patients with confirmed IBD (Crohn's disease, Ulcerative Colitis or IBDU) or coeliac disease. Patient details (demographics, treatments etc) are described in individual sections as appropriate, and in detail in appendix II. It should be noted that many of the patients and controls in this thesis were paediatric patients or young adults.

2.1.3 Intestinal tissue

Up to 8 colonic or ileal biopsies were obtained at colonoscopy from patients with inflammatory bowel disease (IBD) or from control subjects. For duodenal biopsies, up to 2 biopsies were obtained. Intestinal mucosa was also derived from surgically-resected tissue from IBD patients and from macroscopically unaffected areas of patients with colon cancer. The diagnosis for each patient was made using clinical

parameters, radiographic studies and histological criteria. Tissue was collected in ice cold base medium (RPMI1640 Dutch Modification; Sigma-Aldrich).

Patient details are summarized in Appendix 2.

2.1.4 Blood

Blood was obtained by venepuncture from patients or volunteer donors in 10 mL sodium heparin tubes (Becton Dickinson).

2.1.5 Cell extraction

Cells of interest were extracted by two methods, one involving a collagenase digestion process, and the other involving cell egress from intestinal tissue, termed "walk-out". Both are described below. All tissue handling occurred in sterile tissue culture hoods. Reagants used are described as below and common reagants are listed in detail in Appendix 1.

2.1.5.1 Lamina propria cells (LPCs) by collagenase digestion

Surgical resection specimens were cut into biopsy-sized pieces prior to tissue processing. LPCs were extracted from intestinal biopsies by enzymatic digestion (Bell et al., 2001; Hart et al., 2005). Colonic or ileal biopsies were incubated in T25 tissue culture flasks in 1 mM dithiothreitol (DTT; Sigma-Aldrich) in 25 mL calcium and magnesium free Hanks Balanced Salt Solution (HBSS, Sigma-Aldrich) for 20 minutes at room temperature to remove mucus. DTT-HBSS was removed using a 3 mL Pasteur pipette and replaced with 10 mL fresh HBSS. The flask was agitated and then removed as for DTT-HBSS. 25 mL ethylenediaminetetraacetic acid (EDTA, 1% w/v, pH7, Sigma-Aldrich) in HBSS was added to the T25 flask. This was incubated at 37 °C under agitation for 30 minutes; the EDTA-HBSS was removed and replaced with 10 mL fresh HBSS. The HBSS was removed following agitation

and replaced by a further 10 mL of fresh HBSS. The EDTA and wash steps were repeated once, to remove the epithelium from the biopsies.

The biopsies were removed and placed in a fresh T25 flask to which 10 mL of pre-heated collagenase solution (see reagents, appendix 1) was added. This was incubated for an hour at 37 °C under agitation, with violent shaking at 10 minute intervals thereafter until the medium appeared turbid. The contents of the flask were poured through a 10 µm cell strainer (Becton Dickinson) to remove undigested material. LPMCs were collected in a 50 mL tube (Becton Dickinson) and were centrifuged at 400 x g for 5 minutes. The supernatant was carefully aspirated and the cell pellet resuspended in Complete Medium and transferred to a 5 mL FACS tube (Becton Dickinson).

2.1.5.2 'Walk out' cells (see schematic figure 2.1)

Cells were also isolated by the "walk-out" technique, using the known property of LPMC migration from tissue into culture medium (Bell et al., 2001; Mahida et al., 1997). Biopsies were treated with DTT and EDTA as described above in section 2.2.1. The denuded biopsies were then cultured in Complete Medium with gentamicin (see reagents section, appendix 1). Cultures were established in 24 well plates (Becton Dickinson) at one biopsy in 0.5 mL of medium per well and placed in a humidified incubator for 48 hours at 37 ºC with 5% CO_2 in air. The biopsies were then discarded; the complete medium was collected in a 5mL FACS tube (Becton Dickinson) and centrifuged at 400 x g for 5 minutes to collect the egressed cells. The supernatant was also collected. This biopsy conditioned medium (BCM) was stored at - 80 °C for future use.

A comparison of the collagenase method and the walk-out method after flow cytometry analysis is shown in figure 2.2.

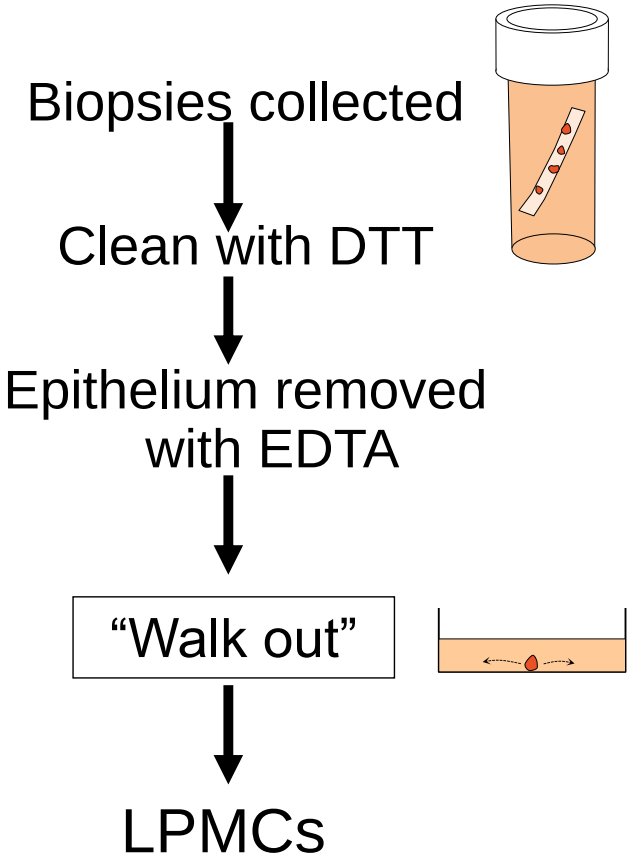

Figure 2.1 “Walk out” method of collection of LPMCs*. Biopsies or small pieces of cut surgical resection tissue were cleaned to remove faeces, the epithelium removed, and then placed for 48 hours in complete medium to allow LPMCs to egress. The biopsy (or equivalent sized piece) was removed and cells were collected in the medium for further analysis.*

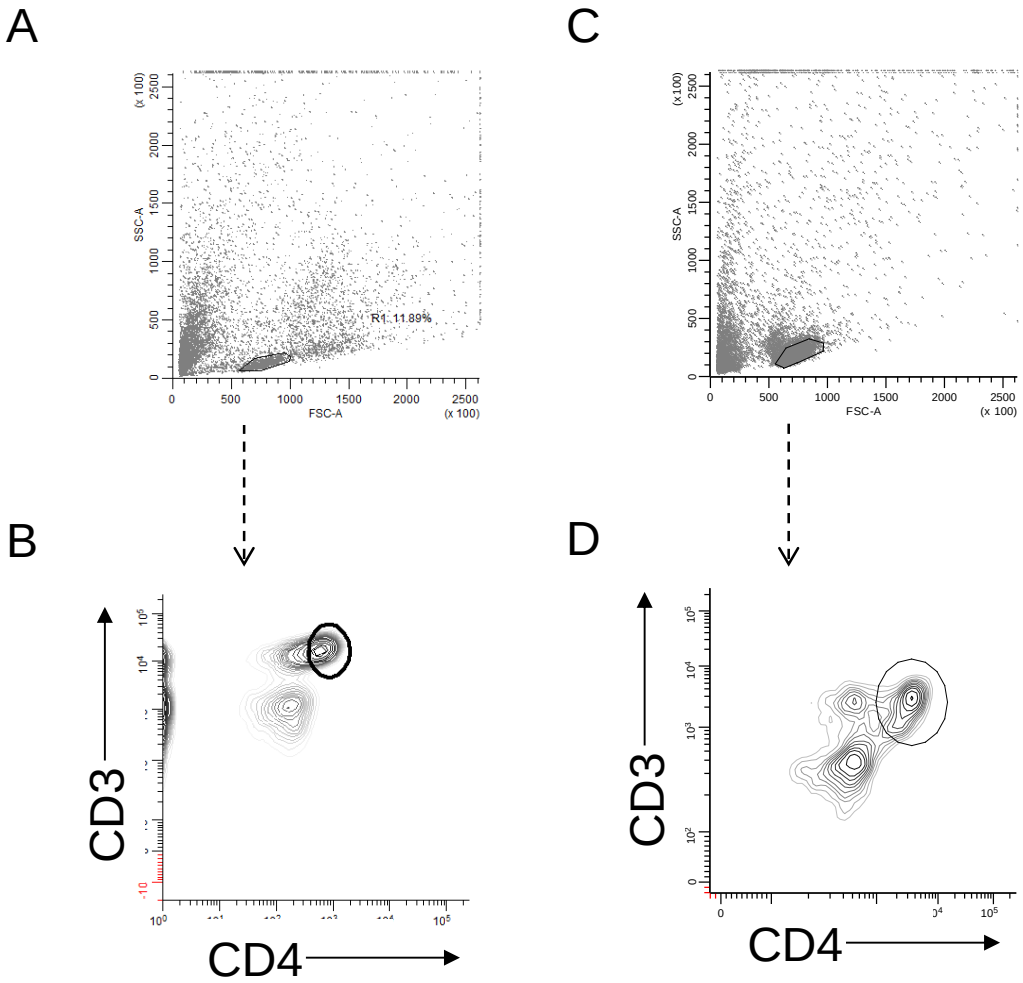

Figure 2.2 Comparison of Collagenase and Walk-out LPMCs. *Cells were processed by the collagenase method (A+B) or the walk-out method (C+D) and lymphocytes were gated by size scatter and labelled for CD3 and CD4 antibodies. Representative of 5 representative experiments.*

In some experiments walk-out cultures had differing additional reagents or additives depending on the experimental design. Specifically, cultures were added with the following agents.

2.1.5.2.1 T1IFN

IFNβ (Peprotech) at 1000IU/ml or with an equal volume as the diluent Phosphate Buffer Saline (PBS) as a control. This dose was chosen as it was commonly used in the laboratory on site that works with T1IFN regularly to generate a robust

response, and is also commonly used in the literature (for example, (McFarland et al., 2011)

2.1.5.2.2 Anti-IFNβ

Human Anti-IFNβ antibody (Biolegend) was added at 1 µg per mL or the same concentration of mouse IgG1 (Biolegend) as an isotype-matched control. This antibody was selected as it was felt to be the best available monoclonal antibody, and the polyclonal anti-IFNAR antibody was not successful in trial experiments. The dose was based on the technical data sheet.

2.1.5.2.3 Conditioned medium

50% v/v biopsy conditioned medium collected as described above, diluted in complete medium with gentamicin; control cultures were in complete medium with gentamicin alone.

2.1.5.3 Peripheral blood mononuclear cells (PBMCs)

10 mL Peripheral blood was diluted 2:1 with RPMI1640 Dutch Modification (Sigma-Aldrich) base medium, then layered over 15 mL Ficoll-Paque PLUS (GE Healthcare) in 50 mL falcon tubes (ie 30 mL of RPMI/blood mixed layered over 15 mL Ficoll) using a Pasteur pipette and centrifuged at 650 x g for 20 minutes with the brake at its lowest setting or deactivated. PBMCs were collected from the interface between Ficoll and serum with a Pasteur pipette, pooled into 15 mL Falcon tubes (Becton Dickinson) and centrifuged at 650 x g for 10 minutes. Supernatant was discarded and pellets were resuspended in the residual volume, pooled into a 5 mL FACS tube (Becton Dickinson) and washed twice into complete medium by centrifugation at 400 x g for 5 minutes.

2.1.5.4 Cell counts

Cell counts were performed by mixing 50 µL cell suspensions with 50 µL Trypan Blue Solution (0.4%, Invitrogen) and 150 µL RPMI-Dutch modification. A small sample of this mixture was applied to a haemocytometer (Neubauer) and cells counted within a 4 x 4 grid. An average count was taken from at least 2 grids and this number was converted to the cell concentration by the following formula:

Cell concentration (per mL) = Average number of cells in one grid x 5 x 10^4

2.2 Purification of cell populations

2.2.1 T cell isolation by miniMACS

T cells were positively selected from LPMCs after collagenase extraction on the basis of CD3 expression using magnetic bead separation according to the manufacturer's protocol (Miltenyi Biotec). Briefly, the cells were resuspended in 2 mL MiniMACS buffer (see reagents section, appendix 1). They were labeled with 15 µL anti-CD3-PE antibody (Biolegend) and incubated on ice for 15 minutes and then washed by resupsension in 5mL of MiniMACS buffer, centrifugation for 5 min at 400 g and discarding of supernatant. The cells were resuspended in 100 µL MiniMACS buffer and incubated with 15 µL anti-PE microbeads (Miltenyi Biotec) for 10 minutes on ice. The cells were again washed by the above method and the pellet resuspended in 0.5 mL MiniMACS buffer.

A pre-chilled MS column was placed into the specialised magnet and attached to a metal stand. The column was prepared by pipetting 0.5 mL MiniMACS buffer directly onto it and allowing the buffer to elute. The LPMCs were pipetted onto the column. The column was 'washed' by adding an additional 0.5 mL of MiniMACS buffer three times allowing the liquid to enter the column between each addition thereby eluting non labelled CD3- LPMCs. $CD3^+$ cells were eluted by removing the

column from the magnet, adding 1 mL MiniMACS buffer and forcing the buffer through the column with the plunger provided.

At all times where possible during the above separation the cells were kept on ice to minimise T cell activation. Purity of the recovered $CD3^+$ T cell population was verified by flow cytometry (Section 2.4) and was routinely >90 %.

2.2.2 Isolation of Intestinal or Peripheral T cells by flow sorting

Lamina Propria Cells (LPCs), collected by walk-out technique, or PBMCs, were washed by addition of excess FACS buffer, centrifugation at 400 x g for 5 min and discarding of supernatant. The cells were then resuspended into 100 µL filter sterilised (0.2 micron) modified FACS buffer (lacking sodium azide) and incubated with relevant monoclonal antibodies for 15 minutes on ice protected from light. Details of antibodies used are in Tables 2.1 and 2.2). Cell populations within LPCs and PBMCs were isolated by flow-sorting using a FACS Aria cytometer (Becton Dickinson). For the studies described in this thesis, four different cell populations were obtained by cell sorting. These populations and the gating strategy used to identify and select them are indicated in Table 2.1). The process of labeling the cells for flow sorting is described in the flow cytometry chapter below (section 2.4.3). In order to sort only viable cells, the agent 7-amino-actinomycin D (7-AAD; Biolegend) was used, as is also described in section 2.4.3. An example sort strategy for PBMCs is shown in figure 2.3 and for LPMCs in figure 2.4.

T cell subset	Gating strategy for sorting	Experiments
Total T cells (LPMCs)	$CD3^+7AAD^-$	RNA extraction for PCR (section 2.5.1)
Naïve helper T cells (PBMCs)	$CD3^+CD4^+CD45RA^+$	Phosflow (section 2.4.4)
"Gut-homing" memory T cells (PBMCs)	$CD3^+CD4^+CD45RA^-\beta7^+$	Phosflow (section 2.4.4)
"Non-gut-homing" memory T cells	$CD3^+CD4^+CD45RA^-\beta7^-$	Phosflow (section 2.4.4)

(PBMCs)		

Table 2.1 Gating strategies used to identify T cell subsets for purification by flow cytometry sorting. *All groups were identified by discrete positive/negative staining with the exception of β7, where an isotype control was used to determine gate position.*

The discrete nature of staining with antibodies to CD3, CD4 and CD45RA allowed the relevant cell populations to be identified without the routine use of isotype control staining, thereby maximizing the number of cells that could be separated. The exception was staining with anti-β7 where isotype control labelling was required to enable expressing and non-expressing cells to be discriminated. Cells were sorted at the lowest speed possible to minimise sort-associated cell damage, and were collected in sterile 1.5 mL TubeOne Microcentrifuge Tubes (Star Lab) containing 0.5 mL complete medium. Technical assistance for cell sorting was provided by Gary Warnes, Flow Cytometry Core Facility, Blizard Institute.

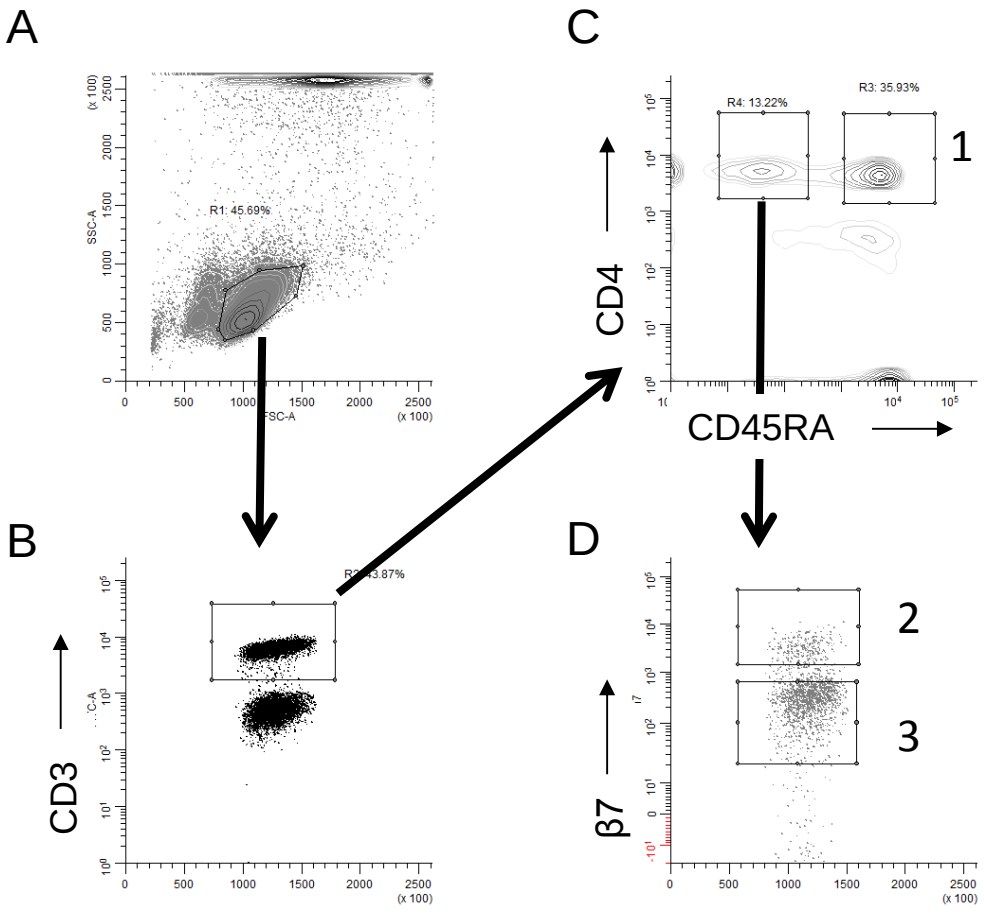

Figure 2.3 Example sort strategy for PBMCs. *PBMCs were isolated as described. In order to acquire the appropriate cell populations for subsequent Phosflow, the cells were sorted using the following strategy. A. Lymphocytes were isolated by size scatter. B. CD3+ events were gated. C Of CD3+, CD45RA gating was used and CD3+CD4+CD45+ cells were collected (1). D CD3+CD4+CD45RA- cells were gated, and sorted based on β7+ (2) or β7- (3). Thus, 3 populations were isolated. Representative of >20 experiments.*

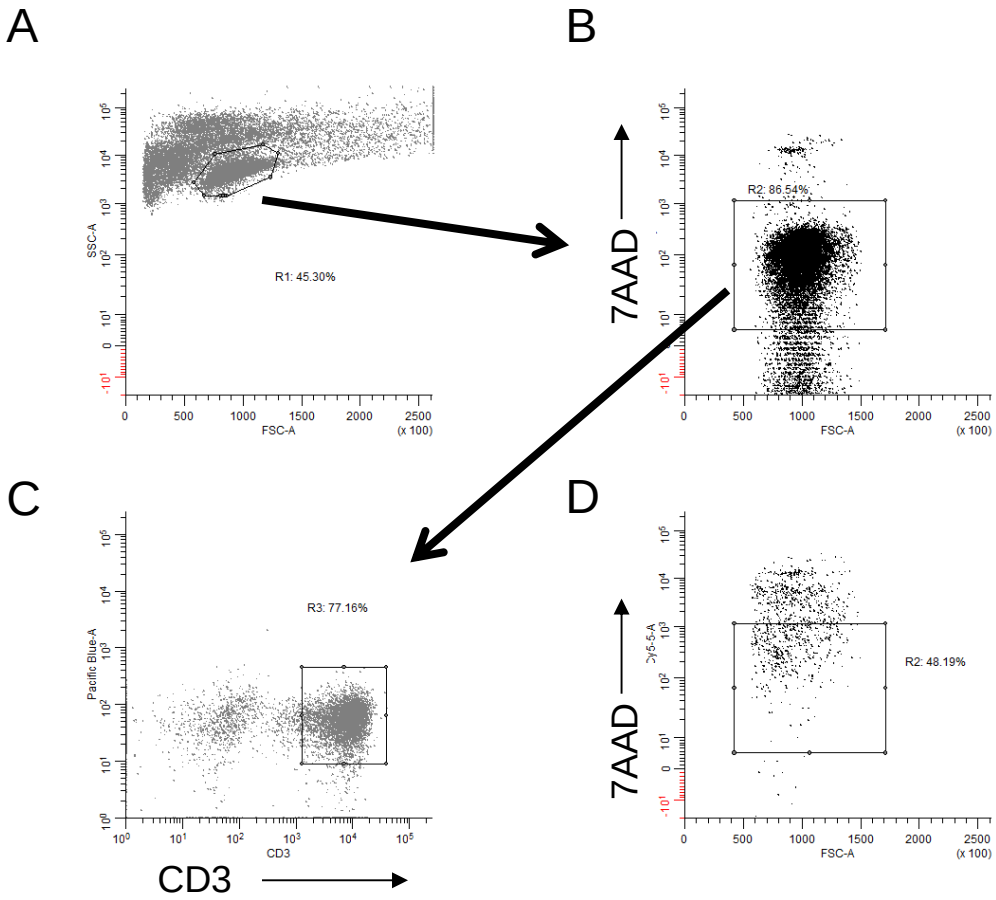

Figure 2.4 Example sorting strategy of viable intestinal T cells. *LPMCs were isolated by the walk out method as described. Cells were acquired by the following strategy. A. Lymphocytes were gated on size scatter. B. Viable cells were gated as 7AAD-. C. CD3+ cells were gated and acquired. D. An example of cells treated at 60°C for 15 minutes to give a positive control for 7AAD. Representative of >20 experiments.*

2.2.3 Stimulation of T cells for cytokine analysis

LPMCs were cultured in 200 µL complete medium in 96 well plates (Beckton Dickinson), and Low Endotoxin Azide Free (LEAF) anti-CD3 and anti-CD28 antibodies (Biolegend) were added each at a final concentration of 1 µg/mL. The

plates were put in a humidified incubator at 37 °C with 5% CO_2 in air for 20 hours. After 20 hours the cells were collected into 5mL FACS tubes, and the Golgi-blocking agent monensin (VWR) was added at 3 µM final concentration and the tube contents mixed thoroughly. The FACS tubes were replaced in the incubator for 4 hours. Cells were then collected and processed by flow cytometry after centrifugation and supernatants were stored at -80 °C.

2.3 Flow cytometry

2.3.1 Monoclonal antibodies

Antigen	Fluorochrome	Clone	Isotype	Supplier
CD4	APC	RPA-T4	Mouse IgG1	Biolegend
CD4	FITC	RPA-T4	Mouse IgG1	Biolegend
CD8	FITC	RPA-T8	Mouse IgG1	BioLegend
CD8	PerCP/Cy5.5	SK-1	Mouse IgG1	BD Biosciences
CD3	Pacific Blue™	UCHT1	Mouse IgG1	BioLegend
CD45RA	PE-Cy7	HI100	Mouse IgG2b	Biolegend
CD69	PE	FN50	Mouse IgG1	Biolegend
Beta7	PE	FIB504	Rat IgG2a	Biolegend
T-bet	PerCP-Cy5.5	4B10	Mouse IgG1	eBioscience
RORγt	PE	AFKJS-9	Rat IgG2a	eBioscience
FoxP3	Alexafluor 647	206D	Mouse IgG1	BioLegend
IL-17	PE	BL168	Mouse IgG1	Biolegend
IL-10	AF647	JES3-9D7	Rat IgG1	Biolegend
TNFα	PE	MAb11	Mouse IgG1	Biolegend
IFNγ	AF647	B27	Mouse IgG1	BD Biosciences
pSTAT1 (pY701)	Alexafluor 647	4a	Mouse IgG2A	BD Biosciences
Stat1 (N-Terminus)	PE	1/Stat1	Mouse IgG1	BD Biosciences
pSTAT3 (pY705)	Alexafluor 647	4/P–STAT3	Mouse IgG2A	BD Biosciences

pSTAT3 (pY705)	PerCP-Cy5.5	4/P-STAT3	Mouse IgG2A	BD Biosciences
pSTAT4 (pY693)	Alexafluor 647	38/p-STAT4	Mouse IgG2B	BD Biosciences
pSTAT5 (pY694)	Alexafluor 647	47/Stat5(pY 694)	Mouse IgG1	BD Biosciences
pSTAT6 (pY641)	Alexafluor 647	18/p-STAT6	Mouse IgG1	BD Biosciences
pErk1/2 (PT202/PY 204)	Alexafluor 647	20A	Mouse IgG1	BD Biosciences

Table 2.2 Antibodies used for flow cytometry

Isotype-matched control antibodies were obtained from the appropriate manufacturer, and were appropriate for intracellular staining as necessary.

2.3.2 Antibody labelling

Cells were transferred into 5 mL FACS tubes (Becton Dickinson) and washed by centrifugation (400 x g, 5 minutes) in cold FACS buffer. Supernatant was removed by inversion, and monoclonal antibodies were added to the residual volume (approximately 100 µl) containing the cell pellet (typically 5 µl antibody per test). Cells were vortexed and incubated on ice for 15 minutes and washed twice by centrifugation (400 x g, 5 minutes) and supernatant removed by inversion.

For detection of intracellular antigens (excluding phospho-antigens), cells were fixed following extracellular labelling by addition of 100 µl Leucoperm A solution (AbD Serotec), vortexed and then incubated in the dark for 15 minutes at room temperature. Cells were then washed by the centrifugation (400 x g, 5 minutes) with ice cold FACS buffer and the supernatant removed entirely by inversion of the tube onto a paper towel in order to remove the residual liquid. Cells were permeabilised with 100 µl Leucoperm B solution (AbD Serotec) in the presence of relevant monoclonal antibodies (5 µl per test). The tubes were vortexed and incubated at room temperature in the dark for 30 minutes. Cells were then washed in cold FACS

buffer by centrifugation (400 x g, 5 minutes) and fixed in paraformaldehyde (1 % w/v).

Viability was assessed in some unfixed cell populations by the addition of 3 µl 7-amino-actinomycin D (7-AAD; Biolegend) in 300 µl FACS buffer. Samples were vortexed, incubated for 5 minutes at room temperature and then analysed by flow cytometry (see below). 7-AAD enters cells with a damaged cell membrane (i.e. dead cells) and complexes with DNA, but is restricted to the exterior of cells with a viable membrane (i.e. live cells), providing a clear distinction between live and dead cells upon data analysis.

2.3.3 Flow cytometry

Labelled cells were acquired on a Canto II or LSR II (Becton Dickinson) using CellQuest software (Becton Dickinson). Single fluorophore compensation controls were generated by labelling compensation beads (Becton Dickinson) with individual antibodies tagged with dyes used in a particular experiment. The compensation beads contain one population of beads that non-specifically binds to mouse IgG, generating a positively stained population, and a second population of beads which do not bind to mouse IgG and so create a negatively stained population. In situations where antibodies not of mouse origin were used (e.g. anti-β7 which is a rat IgG2a antibody) compensation beads were unsuitable. As an alternative, PBMCs labelled with only this antibody were used as the compensation control. In the case of 7-AAD staining, 1 x 10^6 PBMCs were cultured at 60 °C in PBS for 5 minutes to generate a population of dead cells, placed on ice and then combined with 1 x 10^6 untreated viable PBMCs in PBS at room temperature. The combined cell population contained equal amounts of live and dead cells, and so when stained with 7-AAD immediately prior to flow cytometry provided a single fluorophore compensation control for 7-AAD.

At least 10,000 events were acquired for each sample when possible. Data were exported and analysed using WinList 7.0 or 7.1 software (Verity). Compensation was applied prior to data analysis using the single fluorophore controls described above. Positive staining was determined by comparison with populations within samples stained with matched isotype control antibodies. Alternatively, for some labellings (eg anti-CD3) where the distinction between stained and unstained cells was very clear, isotype-matched control were unnecessary. The level of staining was quantified as the mean fluorescence intensity (MFI), and geometric mean was used throughout so that the mean values were not distorted by the high levels of fluorescence of positively stained populations.

2.3.4 Phosflow

A technique for measuring intracellular phospho-antigens in single cells was developed and optimized as part of this study and is described in detail in Chapter 3. A brief outline of the optimized technique is presented below. The initial protocol was based on a T cell activation kit provided by BD Biosciences. Many of the basic flow cytometry techniques and equipment are used as described in the routine flow cytometry section above.

2.3.4.1 Stimulation with Type I Interferon and fixation

PBMCs or LPMCs were resuspended in 100 or 200 µL complete medium with gentamicin in 5 mL FACS tubes and either left unstimulated or stimulated with 40000 IU/mL IFNα2A or glycosylated IFNβ1 (Peprotech), for 15 minutes in a 37 °C waterbath. The α2A isoform and the dose were chosen initially as recommended by the manufacturers protocol but it should be noted that this dose (40000 IU/mL) is extremely high and used primarily to provide a positive control. The β isoform was also used later as it was the isoform that could be neutralised by an appropriate antibody (see section 2.1.5.2.2). The cells were then fixed by addition of an equal

volume (100 or 200 µL) of 4 % w/v paraformaldehyde (PFA) (to give a final concentration 2% PFA) for 10 minutes in a 37 °C water bath. PBMCs were then transferred to 1.5 mL Eppendorf tubes (Sigma-Aldrich) and stored at -80°C. These were then thawed before flow sorting to purity of different T cell subsets (see above, section 2.3.2). Both sorted PBMCs or LPMCs directly were permeabilised for antibody staining with phospho-antibodies. It was thought that freezing the sorted PBMCs would be possible, given that the cells were previously fixed, but this was confirmed with cells processed by the Phosflow protocol either with or without a freeze/thaw cycle (see figure 2.5).

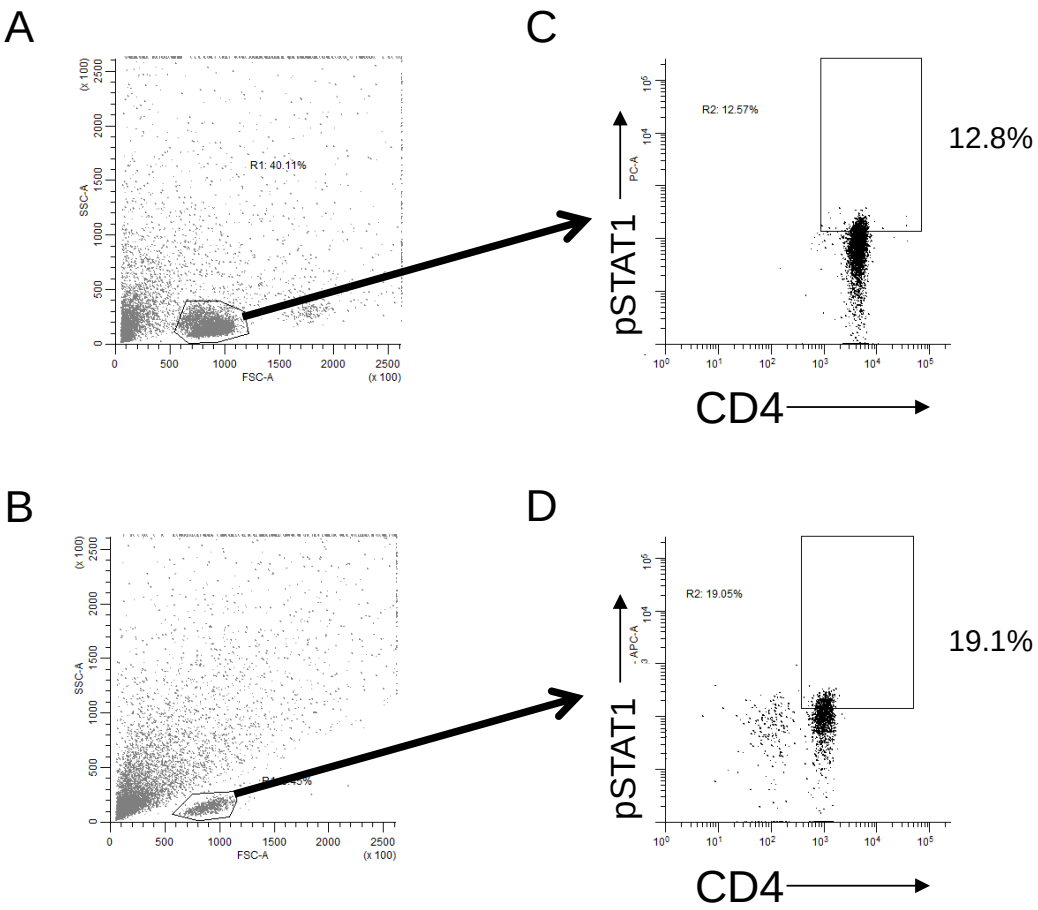

Figure 2.5 Comparison of fresh and frozen sorted PBMCs for Phosflow. *PBMCs were isolated (section 2.1.5.3) and then stimulated with T1IFN (section 2.3.4.1) for 15 minutes before fixation. Cells were then sorted according to table 2.1 and then processed directly by the Phosflow protocol (A+C) or frozen at -80 and then thawed before processing (B+D). Sorted CD3+CD4+β7- cells are shown. As seen, fresh samples maintain CD4 expression but only a small amount of this expression is lost after freeze/thaw cycles (D). pSTAT1 staining remains viable,*

gating based on strict isotype controls. Representative of 3 experiments with fresh samples and >10 experiments for freeze/thaw.

2.3.4.2 Permeabilisation and Phospho-antibody staining.

The fixed cells were washed by centrifugation in PBS and then incubated in 1 mL of 70% cold methanol (VWR, USA) on ice for 30 minutes for permeabilisation. The cells were washed twice in FACS buffer and then resuspended in 100 µL FACS buffer for simultaneous cell surface and intracellular antibody labeling. Cells were labeled as appropriate and incubated for 30-60 minutes at room temperature. After one further wash with FACS buffer the cells were resuspended in 300 µL of FACS buffer and analysed by flow cytometry.

Multicolour flow cytometry was performed on a FACS Canto II or an LSRII (Becton Dickinson) as described. Viable cells were identified on the basis of their forward and light scatter, unless otherwise specified in the results section (see below).

2.3.4.3 Viability testing during phosflow

Staining with the fixation compatible Live/Dead Staining kit (Invitrogen, USA) was used to assess cell viability. Cells isolated using walk out or collagenase technique were suspended in 100µL of PBS and 1µL of the LIVE/DEAD® reactive dye added. Following incubation in the dark for 30 minutes at room temperature, the cells were then washed in PBS by centrifugation and then fixed in 1% PFA. A positive control was provided by cells that had been killed by incubation at 60°C for 15 minutes.

Data was analysed using WinList 3D 7.1 (Verity Software House). Compensation was performed using single colour controls using antibody labeled compensation beads (Beckton Dickinson).

2.4 Quantitative real-time PCR

2.4.1 RNA extraction

The first step in the PCR process is extraction of RNA from cell populations of interest. Purified T cells were obtained from collagenase digests of biopsy tissues and from walk-out cultures by magnetic separation (Section 2.3.1) and FACS sorting (Section 2.3.2) respectively. T cells were washed in PBS by centrifugation (400 x g, 5 minutes), then supernatant was removed entirely by careful pipetting. RNA extraction was performed using the RNeasy Micro Kit (Qiagen) as per manufacturer's instructions. In brief, samples with low numbers of cells ($<1 \times 10^5$) were lysed with 75 µl RLT buffer by vortexing for 1 minute. Alternatively, samples with ($>1 \times 10^5$ cells) were lysed in 350 µl RLT buffer by vortexing for 1 minute. RNA extraction was either performed immediately after this point, or alternatively the cell lysate was stored at -80 ºC and thawed at a later date in a water bath at 37 ºC before continuing RNA extraction. One volume of 70 % ethanol (i.e. either 75 or 350 µl) was added to the cell lysate and mixed by repeated pipetting. The entire volume was then transferred onto an RNeasy MinElute spin column placed in a 2 mL collection tube and centrifuged (8,000 x g, 15 seconds). Flow-through liquid in the collection tube was discarded and the collection tube re-used. RNA trapped on the column membrane was washed by the addition of 700 µl RWI buffer, and centrifugation (8,000 x g, 15 seconds). The RNeasy MinElute spin column was then placed in a fresh 2 mL collection tube and 500 µl RPE buffer added, followed by centrifugation (8,000 x g, 15 seconds) to wash the spin column membrane. The flow-through liquid was then discarded and 2 mL collection tube re-used. Then 500 µl of 80 % ethanol was added to the column to wash the membrane, followed by centrifugation (8,000 x g, 2 minutes). The spin column was then placed in a fresh 2 mL collection tube and centrifuged at full speed for 5 minutes with the lid of the spin column open to completely remove all ethanol from the membrane. Finally, the spin

column was transferred to a 1.5 mL collection tube, 14 µl of RNase-free water added directly to the membrane and then spin column was centrifuged at full speed for 1 minute to elute the RNA.

Purified RNA was used either immediately for reverse transcription to cDNA (see below) or stored at -20 °C for processing at a later date.

2.4.2 Reverse transcription

Reverse transcription is the process of converting the extracted RNA into cDNA, suitbable for PCR. Reverse transcription was performed using the QuantiTect Reverse Transcription Kit (Qiagen) as per manufacturer's instructions. In brief, the entire RNA sample (approximately 12 µl) was incubated with 2 µl gDNA Wipeout Buffer for 2 minutes at 42 °C to degrade genomic DNA. The 14 µl gDNA elimination reaction was then mixed with Quantiscript Reverse Transcriptase (1 µl), Quantitect RT Buffer (4 µl) and RT primer mix (1 µl) and incubated at 42 °C for 15 minutes allowing reverse transcription of all RNA molecules present into cDNA. The sample was then incubated at 95 °C for 3 minutes to inactivate Quantiscript Reverse Transcriptase. The cDNA sample was then either used for real-time PCR or stored at -20 °C until required.

2.4.3 Real-time PCR with SYBR Green

SYBR technology is based on the ability of the fluorescent SYBR probes ability to bind to all double-stranded DNA in a sample. During PCR, the target dsDNA should be amplified, which binds the SYBR dye. This results in an increase in fluorescence intensity proportionate to the amount of PCR product.

Quantitative real-time PCR was performed using QuantiFast SYBR Green PCR Kit (Qiagen) as per manufacturer's instruction. In brief, master mixes containing 2x QuantiFast SYBR Green PCR Master Mix (12.5 µl), specific forward and reverse

primers (primers listed in Table 2.3). Primers were added for a final concentration of 1 µM. This entailed the addition of 2.5 µl each of forward and reverse primers or 2.5 µl total with custom primers. Primers used were either custom made from Qiagen, or were designed in house. In all cases, primers were exon spanning so as to prevent amplification of contaminating genomic DNA versions of the target gene.

Sufficient RNase-free water was finally added to bring the volume up to 24 µl for each amplification. This master mix was then pipetted into the wells of a 96 well PCR plate (24 µl/well; Star Lab). cDNA (1 µl) was added to the top of relevant wells, then the plate was covered with Advanced Polyolefin StarSeal (Star Lab) and briefly centrifuged to draw the cDNA droplet into the master mix at the base of the well. The plate was then run on a 7500 Real-Time PCR System (Applied Biosystems). The program used for all amplifications is displayed in Table 2.4.

Target	Forward primer	Reverse primer	Company	Cat. number
GAPDH	TGCACCACCAACT GCTTAGC	GCATGGACTGTGG TCATGAG	Invitrogen	N/A
RPL30			Qiagen	QT00056651
MxA	GTTGGAGGCACTG TCAGGAGTTGC	CTACCTCTGAAGC ATCCGAAATCTC	Sigma	N/A
25 0AS	GTGCGCTCAGCTT CGTACTGAGTTC	CTGCAGGTCGGT GGACTCCTCGATG	Sigma	N/A
SOCS1			Qiagen	QT00202475
SOCS3			Qiagen	QT02488983

Table 2.3: Primers used in real-time PCR

	Time	Temperature
1. Activation step	5 min	95 °C
2. Denaturation	10 sec	95 °C
3. Annealing / extension	33 sec	60 °C

Table 2.4: PCR programme

Data was collected at step 3, and steps 2 to 3 were cycled 40 times. Following amplification, a threshold was placed automatically by the software across the

beginning of the log-linear phase of amplification and the cycle number at which the individual amplifications crossed this threshold (Ct value) was collected. Expression of the target gene (e.g. *MxA*) was normalized to the reference gene (*GAPDH)* using the $2^{-\Delta Ct}$ method (Pfaffl, 2001) where ΔCt is the difference in threshold cycle number for target and reference gene. An example of an amplification plot is displayed in Figure 2.6. All amplifications were run in the presence of a "no template control" (NTC) containing water instead of cDNA to ensure that any amplification was not the result of contamination of the reagents.

Purity of amplified product and the presence of primer dimers were determined by running a melt curve analysis with every experiment. This step involves incremental increases in temperature and analysis of fluorescence at each increase. SYBR green is fluorescent in the presence of double stranded DNA, and the dissociation of double stranded DNA therefore leads to a loss of this fluorescence. The temperature at which double stranded DNA dissociates or 'melts' results from the size of the molecule, and the composition of GC and AT base pairs. If the amplified PCR product is pure, all the DNA molecules will melt at the same temperature, leading to a large loss of fluorescence at a specific temperature. However, where multiple products have been non-specifically generated, SYBR green fluorescence is lost at a range of temperatures associated with the distinct melting points of these various products. Primer dimers also produce a characteristic melt curve profile with a lower melting temperature than the desired product. Therefore, if amplification is detected but only primer dimers are observed by melt curve analysis, this may be taken as a negative result. An example of melt curve analysis for *SOCS1* and *SOCS3* amplification is also displayed in Figure 2.6.

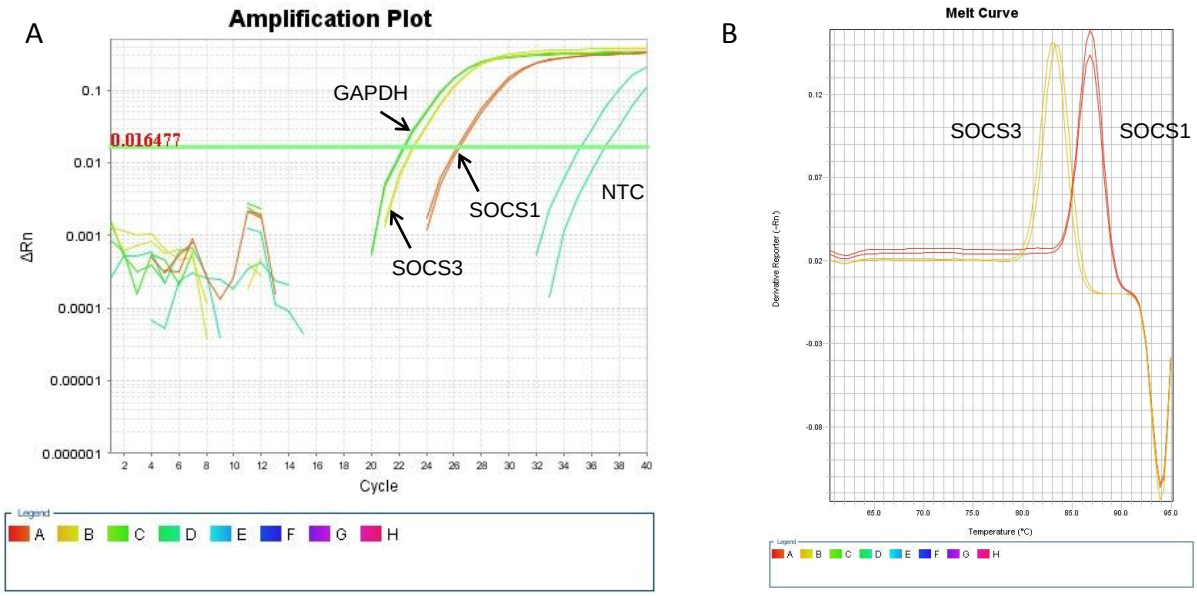

Figure 2.6 Example of real time PCR amplification. A *Real time PCR with SYBR green was performed using primers specific for SOCS1, SOCS3 and GAPDH . Amplification was also performed with SOCS1 and SOCS3 primers either in absence of cDNA (no template control; NTC) or with a sample in which reverse transcriptase was omitted from the reverse transcription step (i.e. no cDNA present; -RT). A threshold was placed at the beginning of the log-linear phase of amplification, with the cycle number at which the fluorescence crosses this line as the Ct value.* ***B*** *Purity of the amplified produce was verified by melt curve analysis. For the SOCS1 and SOCS3 amplified products there is a single discrete change in fluorescence at a specific temperature indicating a pure product. All experiments are performed as duplicates as shown, with the mean value taken for analysis.*

2.5 Multiplex ELISA

Enzyme-Linked immunosorbent assays (ELISAs) are routinely used tests to measure antigens (eg proteins) by using specific antibodies alongside a linked enzymatic process of colour change. Multiplex ELISA is a method where multiple different substrates, in this case cytokines, can be measured simultaneously. The technology used here involved measuring the ELISAs using a flow cytometry detector to measure the colour changes. This is an efficient way of measuring multiple antigens of interest from within a small sample.

Samples were thawed at room temperature. Analysis was performed using the Human Basic Kit PE FlowCytomix™ (eBioscience), with the cytokines TNFα, IL-10, IFNγ, IL-17 and IFNα (all eBioscience). All samples, mixtures and standards were prepared in 5 mL FACS tubes. Standards were provided for each cytokine and dilutions prepared as per manufacturer's instructions. The Bead Mixtures, used to

detect the various cytokines were prepared as per instructions, with volumes depending on the number of analytes performed (between 50-100 samples per experiment), diluted with the Reagant Dilution Buffer (RDB). For example, for 96 tests to be run simultaneously on all 5 cytokines, 150 mL of each Bead Set was mixed with 2250 mL of RDB (total 3000 mL). A PE-Conjugate mixture was also made according to similar conditions. Again, with 5 cytokines measured with 96 samples, 300 mL of each cytokine PE-conjugate was mixed with 4500 mL RDB (total 6000 mL).

To perform the ELISA, tubes were prepared as in table 2.5.

	Standard Dilutions	Blank Tubes	Assays
Standard Mixture Dilutions	25 µL	-	-
Assay Buffer	-	25 µL	-
Sample	-	-	25 µL
Bead Mixture	25 µL	25 µL	25 µL
PE-Conjugate	50 µL	50 µL	50 µL
Total	100 µL	100 µL	100 µL

Table 2.5 *Sample preparation quantities for multiplex ELISA*

All tubes were mixed well, and incubated for 2 hours at room temperature in the dark. All tubes were then washed twice by centrifugation at 200 x g for 5 minutes in 1 mL of Assay Buffer.

Analysis was performed using a Canto II Flow Cytometer (BD Bioscience), set up as per FlowCytomix™ instructions. Data was acquired from the flow cytometer and then analysed by FlowCytomixPro™ (eBioscience) software, provided with the kit.

2.6 Immunohistochemistry

Colonic biopsies were received from patients and snap frozen in liquid nitrogen. Slides were prepared by the pathology service at the Royal London Hospital (Barts Health, UK), and then stored at -80°C or used immediately for immunohistochemistry.

Slides were first left out at room temperature for 15 minutes. Then they were fixed with addition of 1mL of PFA 4% to each slide, followed by a wash with an excess of PBS for 5 minutes. Excess liquid was removed before each stage by gentle agitation of the slide. 1mL blocking serum (5% BSA, 0.1% Tween in PBS) was added and left for 1 hour at room temperature, followed by addition of primary antibodies. The antibodies used were as described in the results chapter (section 4.4.7.3) and as shown in table 2.6

Target	Primary Antibody	Secondary Antibody
T cells	Anti-CD3, rabbit polyclonal (Dako)	AF488 donkey anti-rabbit IgG (Invitrogen)
IFNβ	Anti-Human IFNβ, Clone MMHB-3, (PBL Interferon Source)	AF555 goat anti-mouse IgG (Invitrogen)
IFNAR	Anti-Human IFNAR, Clone MMHAR-1, (PBL Interferon Source)	AF555 goat anti-mouse IgG (Invitrogen)
Isotype control		AF555 goat anti-mouse IgG (Invitrogen, USA)

Table 2.6 *Primary and Secondary antibodies used in IHC.*

Primary antibodies were left at room temperature for 1 hour, followed by three washes with PBS for 5 minutes each. Then the secondary antibodies were applied: AF555 goat anti-mouse IgG (Invitrogen, USA), and/or AF488 donkey anti-rabbit IgG (Invitrogen, USA). The slides were then washed again three times in PBS for 5 minutes. Finally, the slides were air dried by agitation, and then 1 drop of mounting medium with DAPI (Vectorshield, Vector Laboratories, USA), was added to each slide, and the slide was mounted carefully to minimise air bubbles. The slides were left at 4°C overnight before analysis.

Analysis was performed with a Research Systems Microscope BX61 (Olympus), with digital image capture at 100x (standard) or 200x (high power) using the software system SmartCapture v3.0 (Digital Scientific, UK). Analysis of samples

was taken with autofocus, autocalibration and all 5 colour filters (DAPI, FITC, Cyan, Cy3 and Cy5) applied. Data was exported as TIFF files with all filters used for all images (ie allowing detection of autofluorescence).

2.7 Statistics

Statistical analyses were performed using Sigmaplot 11.0 (Systat Software, London, UK). Comparisons between two groups of normally distributed data were analysed using t-tests, paired where appropriate. Datasets containing more than two groups were compared by analysis of variance (ANOVA) with pair-wise comparisons using the Tukey test to correct for multiple comparisons. Non-normally distributed data were log10 transformed and then analysed by ANOVA and Tukey test where indicated in the text. The significance of correlations for non-normally distributed data was assessed by Spearman's Rank. Outcomes in which p values were less than 0.05 were regarded as statistically significant.

Chapter 3

3 Using Phosflow to analyse signalling pathways in intestinal T cells at the single cell level

3.1 Chapter Overview

Intestinal $CD4^+$ T cells are considered the main drivers of intestinal inflammation in IBD. These cells are traditionally characterised by assigning them to one of a limited set of functional subtypes based on their profile of cytokine production (eg TH1,TH2 etc). This phenotype is often determined by *ex vivo* stimulation of cytokine production, which may not reflect the *in vivo* responsiveness of the T cell population. In addition, these cells are functionally plastic, and not fixed to one pattern of cytokine production, therefore the “TH” subtype may not fully represent the dynamic nature of T cell function in the gut.

Local signals impact on key signalling pathways within intestinal T cells to modulate their function. It has not previously been possible to measure the influence of such environmental factors on T cell function at a single cell level. Flow cytometry is a commonly used tool in immunology to determine cellular properties (such as protein expression) at an individual cell level. A recently developed flow cytometry technique, called Phosflow, enables analysis of the phosphorylated forms of signalling proteins, thereby enabling detection of the “on” (active) and “off” (inactive) signalling pathways within cells. This potentially provides a tool for a more dynamic

assessment of the functional state of intestinal T cells. The aim of the work presented in this chapter is to develop Phosflow for the analysis of T cells in the human intestine.

This work focuses on the activation of Signal Transduction and Activator of Transcription (STAT) proteins, which are a crucial part of the signalling machinery in all cell types, including T cells. In particular, they are involved in differentiation of naive T cells into different T helper phenotypes, and the production of their hallmark cytokines. These and other roles for STAT proteins are incompletely understood, but all members of the STAT family are responsive to stimulation with Type I Interferon (T1IFN). The STAT family have also all been linked to IBD and/or its murine models. It may be that subtle effects on these pathways have significant effects on downstream T cell effector functions, which may influence a pathological phenotype.

An existing basic protocol for Phosflow analysis, provided by BD Biosciences, was modified for use in conjunction with small samples of intestinal T cells isolated from IBD patients and healthy controls. Using this protocol, it was possible to detect, in human gut T cells, constitutive expression of phosphorylated (p) STAT1, pSTAT 3 and pSTAT 5, which was significantly upregulated by brief stimulation with T1IFN. Expression of pSTAT4 was not detected under either unstimulated or T1IFN-stimulated conditions. The failure to detect pSTAT4 despite extensive modification of the protocol may indicate an unresolved technical issue with this antibody. Other phospho-antigens were detected, including pSTAT6 and pErk1/2, and required different agents to upregulate phosphorylation.

Using the multicolour parameters of flow cytometry it was possible to characterise the activated T cells simultaneously with other intracellular and cell surface markers. In particular, it was shown that it was predominantly memory T cells that expressed phospho-antigens (pAg). It was also shown that the intestinal T cells expressed high

levels of the activation marker CD69, and that the $CD69^+$ T cells were more responsive in pSTAT1.

Based on these observations it was concluded that Phosflow can be used to assess phosphorylation of signalling molecules, including STAT proteins, in human intestinal T cells. This provides a new tool for assessing dynamic aspects of immune function at this site.

3.2 Introduction

3.2.1 Background

Intestinal $CD4^+$ T cells are considered the main drivers of persistent intestinal inflammation (reviewed in Brand, 2009). Traditionally, these T cells have been characterized by their cytokine production potential. Specifically, TH1/TH17 cells are considered drivers of inflammation in Crohn's disease, whereas a modified TH2 response is considered important in UC (Neurath, 2014a). Tregs are also considered vital in maintaining intestinal homeostasis (Hadis et al., 2011). However, these simplified categories of T cells, as well as a likely over-simplification, also do not reflect the T cell's ongoing ability to respond to its environment.

3.2.2 Phosphorylation of signalling pathways

At a single cell level, cytokine production is routinely measured in the context of *in vitro* stimulation in order to enable detection. For example, T cells are commonly stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin, and then require a golgi-blocking agent such as monensin in order to detect cytokine production at a single cell level. This *in vitro* chemical restimulation results in production of cytokines that reflect the history of the cell's cytokine potential and not its current state of responsiveness. Rather than measuring the potential of intestinal T cells to produce cytokines, this thesis was interested in exploring the ability of T

cells to dynamically respond to their environment. One way of measuring ongoing responsiveness of T cells in the intestine is to measure levels of phosphorylation of important signalling molecules within these cells.

Protein phosphorylation of involved molecules is a key component of signalling pathways. Kinases which phosphorylate specific residues in target proteins, together with phosphatase enzymes that remove phosphate groups, control the “on” and “off” states of signalling molecules. Thus, phosphorylation state identifies the activation state of a signalling molecule and the activity of its associated pathway(s) (reviewed in Suni and Maino, 2011).

Recently, technology has been developed that combines the use of phospho-specific antibodies with flow cytometry (Krutzik et al., 2004) enabling signalling pathways to be analysed at the single cell level. This technique, known as Phosflow, has been used to detect differences of signalling molecules (such as phospho-AKT) in peripheral T cells of patients with rheumatoid arthritis, a relapsing and remitting inflammatory condition of the joints (Galligan et al., 2009). While optimised for use in human blood samples, this technique had not been applied to human intestinal immune cells. It is a potential method of detecting ongoing T cell responsiveness, as well as constituently activated pathways within cells. Detecting the status of the intestinal T cells in this dynamic way would potentially allow a much more nuanced approach to defining how this cell type contributes to and restrains inflammation in the gut.

3.2.3 T cell signalling molecules – the STAT family

Signalling pathways are diverse, complex and interact (Gough et al., 2008). The Jak-STAT pathway is known to be crucial in T cell differentiation and expression of major pro- and anti-inflammatory cytokines (reviewed in Levy and Darnell, 2002; O'Shea et al., 2011). Given the large number of possible phospho-antigens that

could be measured by Phosflow, the STAT family is a reasonable early target for further analysis.

There are 7 known STAT molecules; STATs 1, 2, 3, 4, 5A, 5B and 6. These all share a common method of activation. The function of the STATs as well as their regulation is described in detail in chapter 1, section 1.3.9. Figure 3.1 recaps current understanding of STAT signalling in T cells.

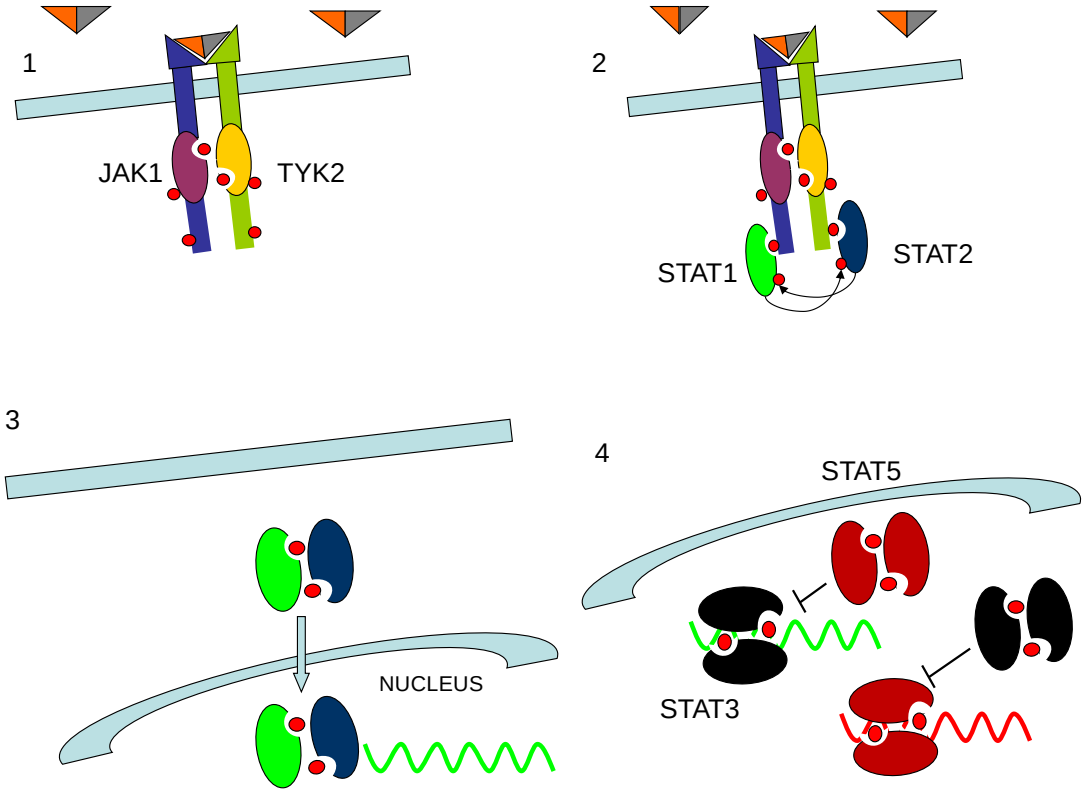

Figure 3.1 Representation of JAK/STAT signalling*.The examples (panels 1-3) show canonical T1IFN signalling with JAK1/TYK2 and STAT1/STAT2 heterodimers. Panel 1 Cytokines or other factors bind to receptors which dimerise, leading to the phosphorylation (red circles) of Janus Kinase (Jak) proteins. 2. This causes binding and phosphorylation of STAT proteins which themselves (3) dimerise and move to the nucleus where they can either promote or repress the transcription of a large number of genes. (4) While STATs may either promote or repress gene transcription, different STATs may also competitively bind at the same site on DNA sequences but with opposing effect, as exemplified by STAT3 and STAT5 binding at the IL-17 locus (Yang et al., 2011)*

3.2.4 Stimulation of STAT Signalling by Type I IFN

Not only are T cell intracellular signalling pathways complex, and STAT pathways are only one important group, the large number of cytokines, chemokines, growth factors and other extracellular molecules that can trigger these pathways make their analysis extremely challenging. While different cytokines are known to trigger phosphorylation of different STATs (as described in chapter 1), type I interferon (IFN) is known to activate all STATs to varying degrees (Darnell et al., 1994; Gough et al., 2012). This makes type I interferon an excellent candidate for probing STAT responses in different cell populations, including T cells.

There are 5 subtypes of type I IFN in humans; IFNα (with at least 13 isoforms), IFNε, IFNκ, IFNω and IFNβ (Platanias, 2005). All of these signal through a ubiquitously expressed receptor composed of IFNAR1 and IFNAR2 chains. The IFNAR signal via the Janus family kinases Tyk2 and Jak1, which bind STAT1 and STAT2 which then form a heterodimer and in the nucleus lead to expression of the Type I IFN-inducible genes (see figure 3.1 and (Li et al., 1996).

In response to the Type II IFN, IFNγ, the IFNAR can also signal via JAK1 and JAK2 followed by STAT1 homodimers which then bind to IFNγ-induced genes (Decker et al., 2005). Apart from STAT1 and STAT2, the other STAT proteins are directly and indirectly involved in IFN signalling. STAT3 acts as a negative regulator of type I IFN responses in mice (Wang et al., 2011). Signalling via STAT4 in the absence of STAT1 directly induces production of IFNγ in murine T cells (Freudenberg et al., 2002), and in humans this IFNγ production occurs with STAT4 and STAT1 combined (Farrar et al., 2000). This demonstrates an important species difference in T cell signalling that may contribute to a significant change in cytokine production which could potentially change the pro- or anti-inflammatory milieu of the gut. These issues are all discussed in greater detail in Chapter 1.

3.2.5 Using Phosflow to measure pSTATs in intestinal T cells

In concept, it was therefore desirable to use this new flow cytometry technique to measure activation of a key signalling pathway in this important cell type in the human gut. However, technical challenges were anticipated. First, cell numbers from this form of cell extraction and often small, and it was not clear whether the technique would be robust with small numbers of cells. Also, the extraction of intestinal T cells to be able to be processed by flow cytometry was thought to potentially affect the expression and/or detection of pAg. Finally, it was not clear that the preparation of the cells (including permeabilisation) would be compatible with cells extracted from the intestine.

3.2.6 Summary

Intestinal T cells are traditionally measured by their cytokine production potential. Phosflow, a new technique of intracellular flow cytometry, allows a more dynamic assessment of T cell responsiveness. While this technique has been used successful in human peripheral blood analysis, it has not yet been used in intestinal T cells. STAT proteins are key members of a a complex signalling cascade that have been show to be necessary for differentiation and function of T helper cell phenotypes and are important in murine and human intestinal and other inflammatory diseases. Therefore optimising Phosflow to measure the differing activation states of the STAT family of proteins in intestinal T cells would allow a much greater understanding of this critical mediator of gut inflammation.

3.3 Aims

1. Develop Phosflow methodology for analysis of T cell signalling pathways, particularly STAT proteins, in intestinal tissue.

2. Use optimised Phosflow to determine constitutive activity within key signalling pathways in intestinal cells and the responsiveness of these pathways to stimulation, particularly with T1IFN.

3.4 Results

3.4.1 Optimisation and selection of Phosflow reagants

To establish the technique of Phosflow and obtain proof of concept for its use in gut tissue, it was first important to show the feasibility of the technique in the laboratory. Using the BD Biosciences kit, expression of phospho-Erk1/2 (pErk) was initially assessed in $CD3^{+}CD4^{+}$ blood T cells following stimulation of whole blood with Phorbol myrsitate acetate (PMA) (figure 3.2). This was chosen as the first phospho-antigen (pAg) as it was recommended by the manufacturer, and was known to respond to PMA, which is a stimulus well known to activate T cells, and often used in the laboratory.

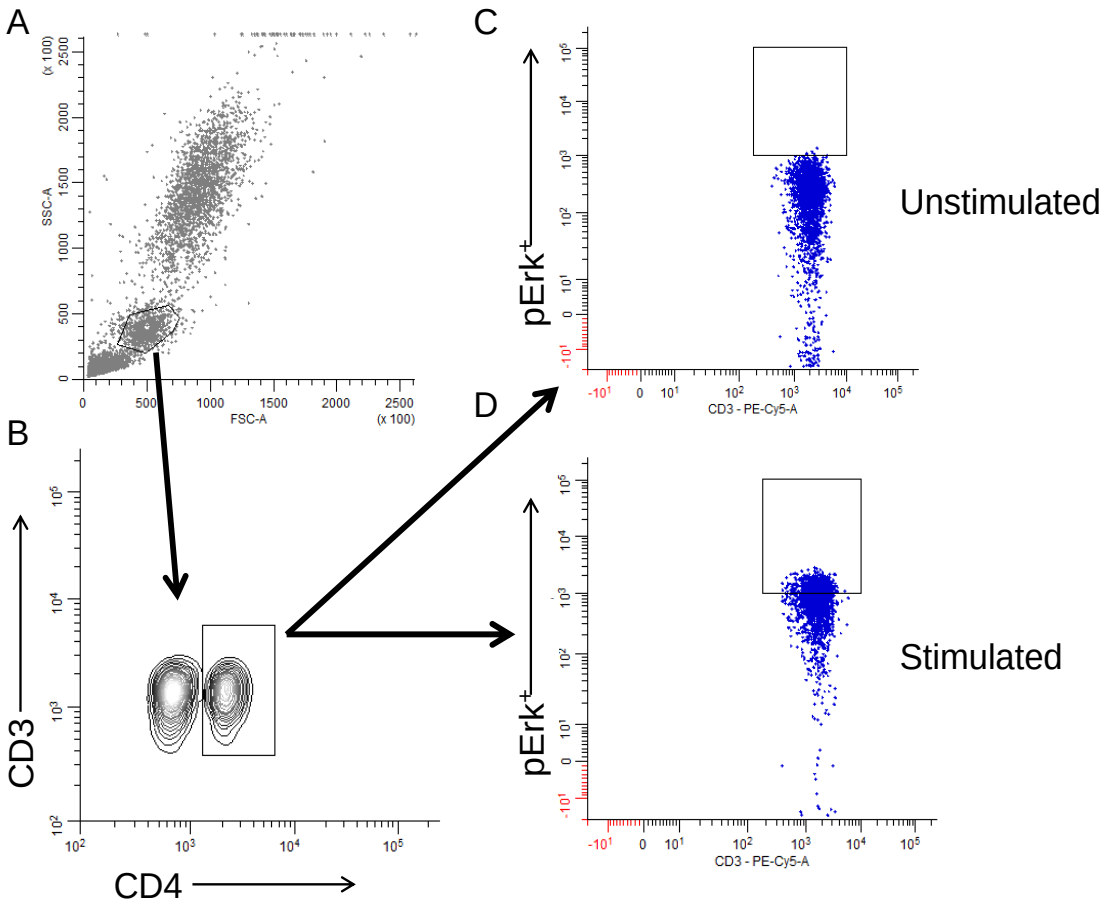

Figure 3.2 pErk is detectable after PMA stimulation in peripheral blood T helper cells. *Flow cytometry data from whole blood samples showing pErk1/2 staining.* ***A*** *lymphocyte gating on light scatter;* ***B*** *cell surface staining with CD3 and CD4 antibodies.* ***C*** *and* ***D*** *pErk1/2 signal in unstimulated (C: 0.1% gated), and stimulated cells (D: 29.1% gated)*

The technique was then applied to cells isolated from blood (PBMCs). While the BD kit was effective for whole blood using the manufacturer's protocol, there were number of potential difficulties in directly applying the protocol to analysis of mononuclear cells (PMBCs). For example, the fixation agent supplied in the BD kit was specifically designed for whole blood, and therefore contained a red blood cell lysis agent(s), unnecessary for PBMC processing. To address these issues, changes were made to a number of aspects of the protocol including the use of alternative fixation and permeablisation reagents, which are described below.

3.4.1.1 Fixation

The fixation agent provided was a BD Lyse/Fix buffer, and this was exchanged for Paraformaldehyde (PFA) 2% w/v (see chapter 2), based on previous published studies using PBMCs (Haas et al., 2008; Montag and Lotze, 2006a; Montag and Lotze, 2006b). It was not appropriate to be using an agent designed for lysing red blood cells in the intestine, and the 2% PFA appeared to adequately fix all cells, although no formal comparison was made.

3.4.1.2 Permeabilisation.

As this is a technique for measuring intracellular signalling molecules, it is necessary to permeabilise the cells before staining with antibodies. The BD protocols suggest different permeabilisation agents depending on the phospho-antigen of choice; reflecting the high degree of sensitivity of the targets to this stage of the process. Permeabilisation of the cells also can affect cell surface staining, and there is a need to obtain a compromise between maintaining cell surface staining whilst maximising intracellular signal (Krutzik et al., 2004; Suni and Maino, 2011). Detergent based permeabilisation, although widely used in flow cytometry does not appear to allow detection of phospho-antigens. In addition to proprietary reagents, methanol at a concentration of 70-75% has been demonstrated to allow detection of pAg while not compromising cell surface markers (Montag and Lotze, 2006b). In order to determine whether this would be effective, 70% methanol and BD perm buffer III (the proprietary agent) were directly compared (figure 3.3). While surface and intracellular staining was at a marginally higher intensity with the BD product, the final result was comparable. Therefore, 70% methanol was used for future studies.

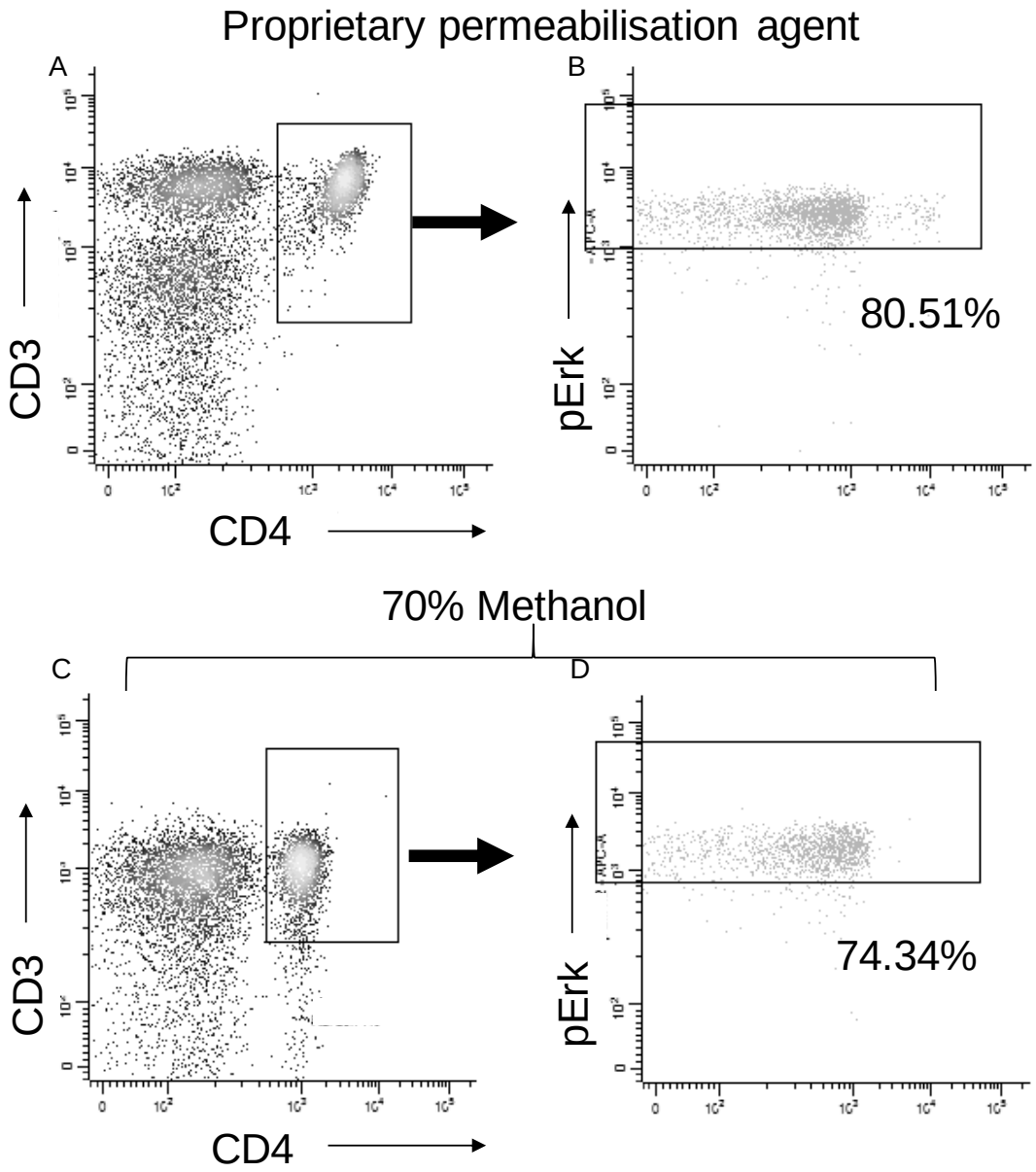

Figure 3.3 70% Methanol effectively permeabilises T cells for Phosflow. *Comparison of BD Perm Buffer III (A and B) with 70% Methanol (C and D) as permeabilisation agent in staining protocol for whole blood.* ***A*** *CD3 and CD4 staining with BD Perm Buffer III* ***B*** *pErk1/2 staining after stimulation with PMA (80.51%, gating based on unstimulated).* ***C*** *CD3 and CD4 staining with 70% methanol.* ***D*** *pErk1/2 as per figure B (74.34%, gating based on unstimulated). Representative of 3 independent experiments.*

3.4.2 Measurement of STAT phosphorylation

After detection of pErk was found to be feasible in blood after stimulation with PMA, it was next attempted to detect pSTAT1 with and without stimulation with T1IFN in blood, as pSTAT1 was one of the main signalling molecules of interest. It was

shown that, in response to 15 minute T1IFN stimulation, high levels of pSTAT1 could be detected in $CD4^+$ T cells from peripheral blood (figure 3.4 A and B).

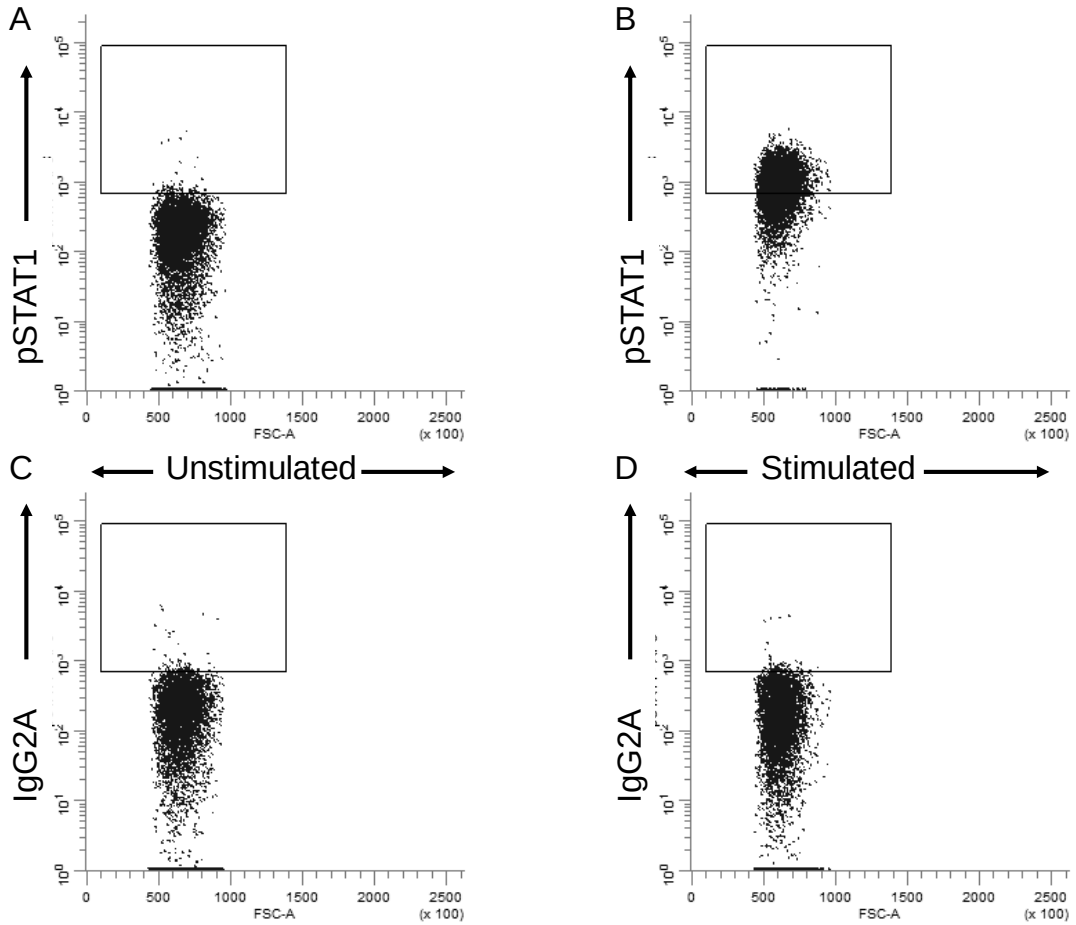

Figure 3.4 pSTAT1 is detectable after stimulation in peripheral blood T cells. *Whole blood from a healthy control was taken and processed according to the Phosflow protocol. $CD4^+CD3^+$ lymphocytes were gated and shown in all panels.* ***A*** *Negligible $pSTAT1^+$ events are detected constitutively, but were after 15 minutes T1IFN stimulation (**B**).* ***C*** *and* ***D*** *Isotype controls. Plots are representative of >10 experiments*

3.4.3 Detecting constitutive pSTAT levels using strict isotype controls

Staining of pAntigens, including pSTATs, in the BD kit was compared between stimulated and unstimulated control cells. In order to detect constitutive pSTAT levels in unstimulated cells from the human intestine, staining with anti-pSTAT antibodies was compared with staining with isotype-matched control antibodies. As staining with Phosflow antibodies provides an increase signal detected in the

population of cells, rather than a discrete positive population, it was necessary to gate strictly and consistently across experiments. Therefore all Phosflow experiments were gated strictly with isotypes to achieve consistency in detecting low levels of constitutive staining. Stimulation with T1IFN, which consistently increased specific pSTAT1 staining, was used as a positive control in all Phosflow experiments. The specificity of this labelling was confirmed because isotype control staining did not change with stimulation (example figures 3.4 C and D). This rigorous approach to isotype controls allowed detection of relatively low levels of constitutive staining.

3.4.4 Phosflow in intestinal T cells

Biopsies were collected and LPMCs were extracted using collagenase digestion as described in chapter 2, and the Phosflow protocol was performed. This experimental approach was used several times but in each case gave poor cell surface staining and no staining of pAg, except a low level of pErk1/2 staining after stimulation with PMA (data not shown). It was considered likely that this poor outcome was secondary to the effects of enzyme treatment on the intestinal T cells. Therefore, it was recognised that a cell isolation method that did not involve an enzymatic process may be more successful.

A well-recognised technique for isolation of LPMCs is known as the walk-out technique (described in detail in chapter 2, section 2.2.2)(Bell et al., 2001; Mahida et al., 1997). In brief, intestinal biopsies are placed in culture medium and lamina propria cells migrate out of the biopsy tissue into the surrounding medium, from where they are collected. It was decided to perform the Phosflow protocol using cells isolated using this process. Indeed, extracting LPMCs from biopsies or surgical specimens using the walk-out technique enabled successful detection of pSTAT1 in both unstimulated and stimulated cells by Phosflow (figure 3.5).

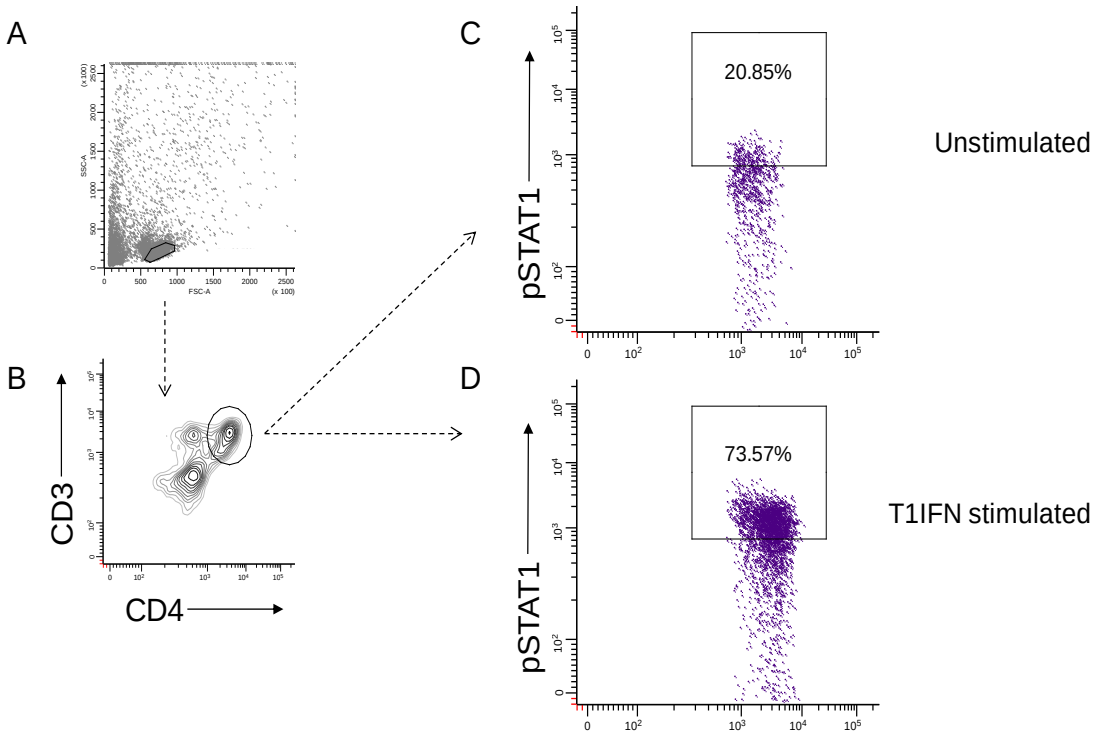

Figure 3.5"Walk-out" CD4$^+$ intestinal T cells show constitutive expression of pSTAT1 which is increased following.T1IFN stimulation. A *and* ***B*** *demonstrate the gating strategy used to identify CD4$^+$ T cells and are representative of all (>70) experiments.* ***C*** *and* ***D*** *pSTAT1 staining of CD4$^+$ T cells. Positivity was determined by strict gating on the isotype control.* ***C*** *unstimulated and* ***D*** *stimulated for 15 minutes with T1IFN.*

3.4.5 The vast majority of walk-out lymphocytes are viable

As the lymphocytes collected by the walk-out technique have spent 24-48 hours in complete medium, some cell death may be anticipated. It is therefore conceivable that it is dead cells that contribute to higher pAg signal, either via activation (ie phosphorylation) of various cell death pathways or because of increased auto-fluorescence or non-specific antibody binding. Figure 3.6 demonstrates that the lymphocytes gated on cell morphology are >99% viable by LIVE/DEAD® staining (Invitrogen, USA, chapter 2 section 2.4.4.3 for details of methodology). It is reasonable to suppose that some of the events outside of the lymphocyte gate on FSC/SSC plot are likely to be lymphocytes that have died either before or during cell extraction and processing. However, these data confirm high viability within the

population gated in all experiments for pAg analysis making it highly unlikely that artefacts related to cell death contribute to the staining observed.

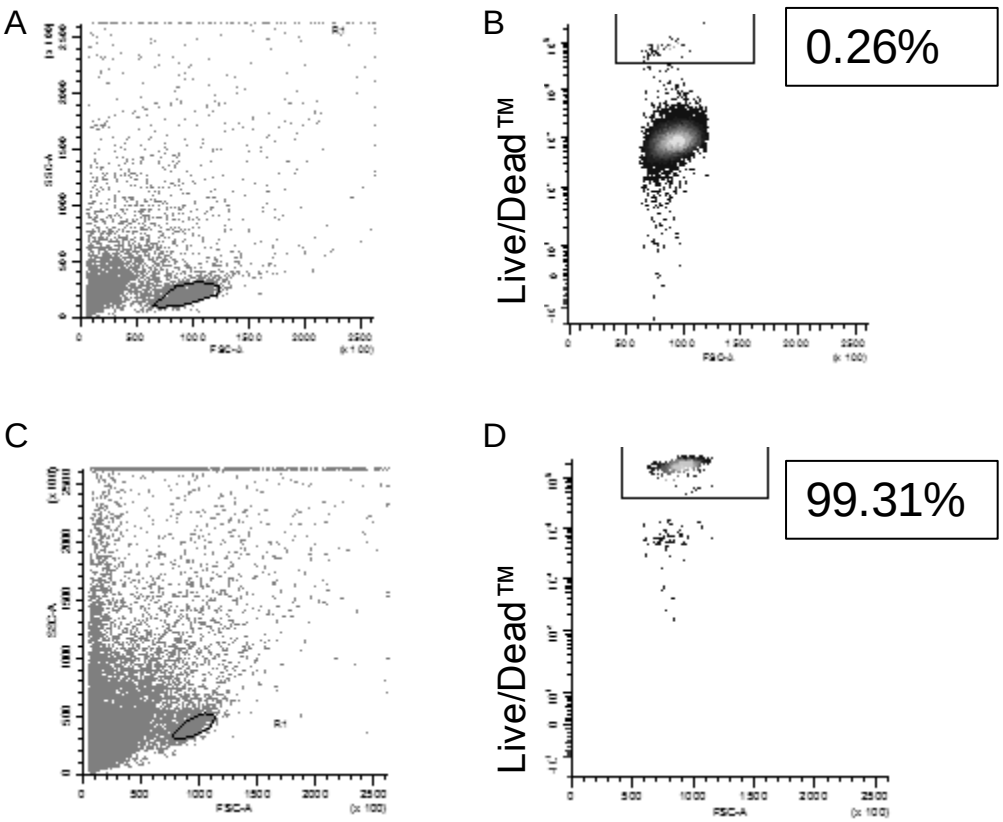

Figure 3.6 The vast majority of walk out cells are viable. *Intestinal cells were obtained by walk-out and immediately processed through the standard Phosflow protocol (**A** and **B**) or killed (15min at 60°C) prior to Phosflow analysis (**C** and **D**) **A** and **C** gating of lymphocytes; **B** and **D** staining of cells in the lymphocyte gate with "*LIVE/DEAD®*" staining. Representative of 2 independent experiments*

3.4.6 Multiple pSTATs can be detected in CD4$^+$ intestinal T cells with and without stimulation with T1IFN

As all members of the STAT family are potentially important in T cell responsiveness and IBD pathogenesis, it was envisaged to examine all members using the Phosflow protocol. However, no antibody specific for pSTAT2 available was available to us for use in this project. It was also not possible to obtain any staining with the available anti-pSTAT4 antibody using whole blood, PBMC or intestinal cell samples despite various modifications to the staining protocol. These modifications included using different stimulus agents (including IL-12), obtaining

different antibody samples (in case there was a faulty lot), and liaising closely with the manufacturer's representatives (BD Bioscience). However, it was not attempted to detected pSTAT4 using a different protocol or manufacturer.

Unlike anti-pSTAT4, specific staining with anti-pSTAT3 and anti-pSTAT5 was obtained following 15 min stimulation with T1IFN (see figures 3.7 and 3.8). Staining with anti-pSTAT6 was detectable after IL-4 stimulation (figure 3.9) but not with T1IFN stimulation (data not shown).

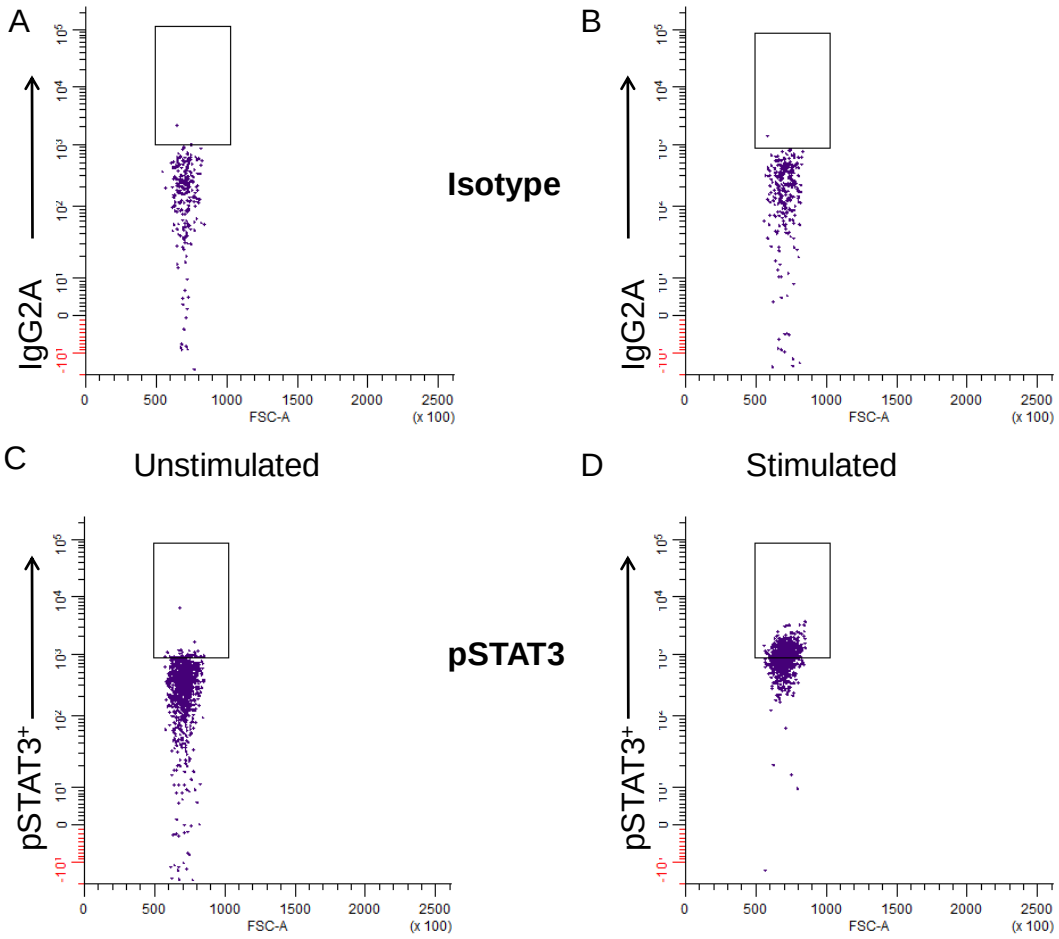

Figure 3.7 pSTAT3 can be detected in intestinal CD4$^+$ T cells with and without T1IFN stimulation. *LPMCs were isolated by the walk-out method. All panels are gated for CD3$^+$CD4$^+$ lymphocytes.* ***A*** *and* ***B*** *isotype controls,* ***C*** *and* ***D*** *Staining with anti-pSTAT3. A and C are unstimulated cells, B and D cells were stimulated with T1IFN. A and B <0.5% events are positive. C 2.08%. D 48.17%. Representative of >30 experiments.*

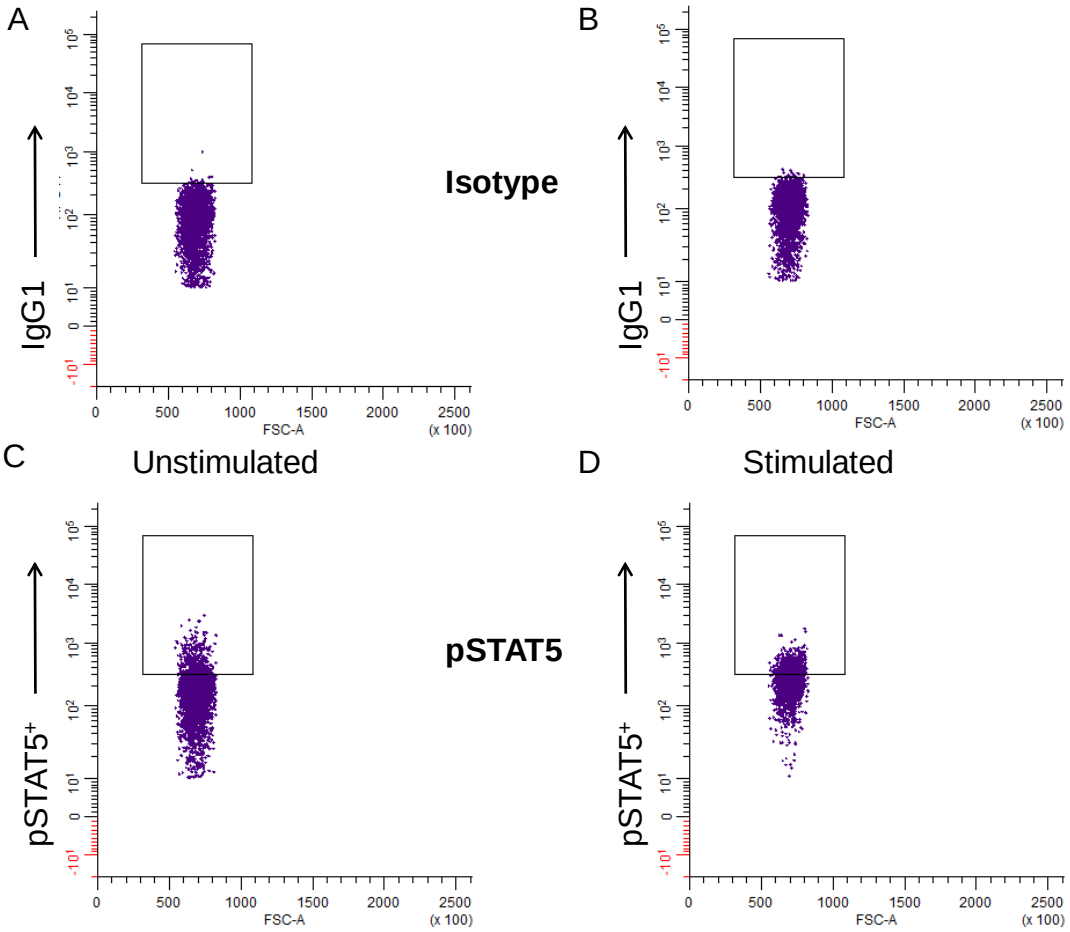

Figure 3.8 pSTAT5 can be detected in intestinal CD4$^+$ T cells with and without T1IFN stimulation. *LPMCs were isolated by the walk-out method. All panels are gated for CD3$^+$CD4$^+$ lymphocytes.* ***A*** *and* ***B*** *isotype controls,* ***C*** *and* ***D*** *staining for pSTAT5. A and C are unstimulated cells, B and D cells were stimulated with T1IFN. A and B <0.5% events are positive. C 8.58%. D 28.71%. Representative of >30 experiments.*

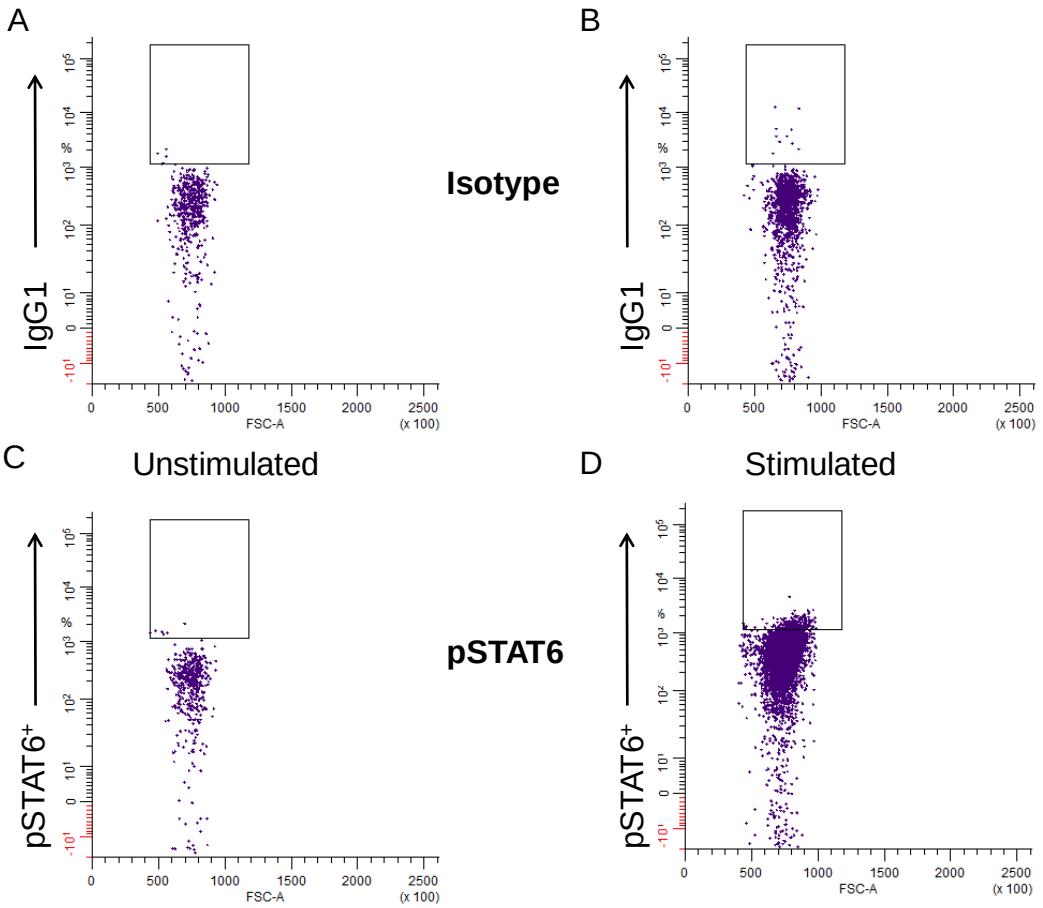

Figure 3.9 pSTAT6 can be detected in intestinal T cells with IL-4 stimulation. *LPMCs were isolated by the walk-out method. All panels show events gated for $CD3^+CD4^+$ lymphocytes.* ***A*** *and* ***B*** *isotype controls, with* ***C*** *and* ***D*** *staining for pSTAT6. A and C are unstimulated cells, B and D cells have been stimulated with IL-4. A, B and C <0.5% events are positive. D 5.83%. Representative of 2 experiments.*

In addition to T1IFN stimulated STAT phophorylation, a small frequency of cells were specifically stained with antibodies to pSTAT1, pSTAT3, and pSTAT5 in the absence of stimulation, thereby enabling the quantification of 'constitutive' levels of STAT phosphorylation (seen in figures 3.5, 3.7 and 3.8). Quantitative analysis of this data is presented in chapter 4.

3.4.7 Characterisation of pSTAT$^+$ intestinal CD4$^+$ T cells

3.4.7.1 Responsiveness of memory versus naïve intestinal T cells

In order to better characterise the CD4$^+$ T cells that were expressing pAgs, cells were labelled in combination with anti-CD45RA, a surface marker of naïve T cells. It is known that the majority of lamina propria T cells are memory T cells, but there are a small proportion of naive cells present. Indeed, in the experiments conducted for this thesis, approximately 90% of the CD4$^+$ T cells isolated by walk out culture were CD45RA$^-$, consistent with the predominance of effector/memory cells in the colonic lamina propria ((Mahida et al., 1997) and data not shown). A small proportion of CD4$^+$ T cells were CD45RA$^+$ and these cells may or may not represent cells derived from organised lymphoid tissue randomly sampled within the intestinal biopsies. While pSTAT1 could be detected in naïve and memory CD4$^+$ T cell populations both before and following T1IFN stimulation, the frequency of pSTAT1$^+$ cells was significantly higher in the memory cell population after stimulation (figure 3.10). There was no difference between the frequency of constitutive pSTAT1$^+$ expression in memory compared to naïve CD4$^+$ T cells (n=10, p=0.43, paired t test, data not shown).

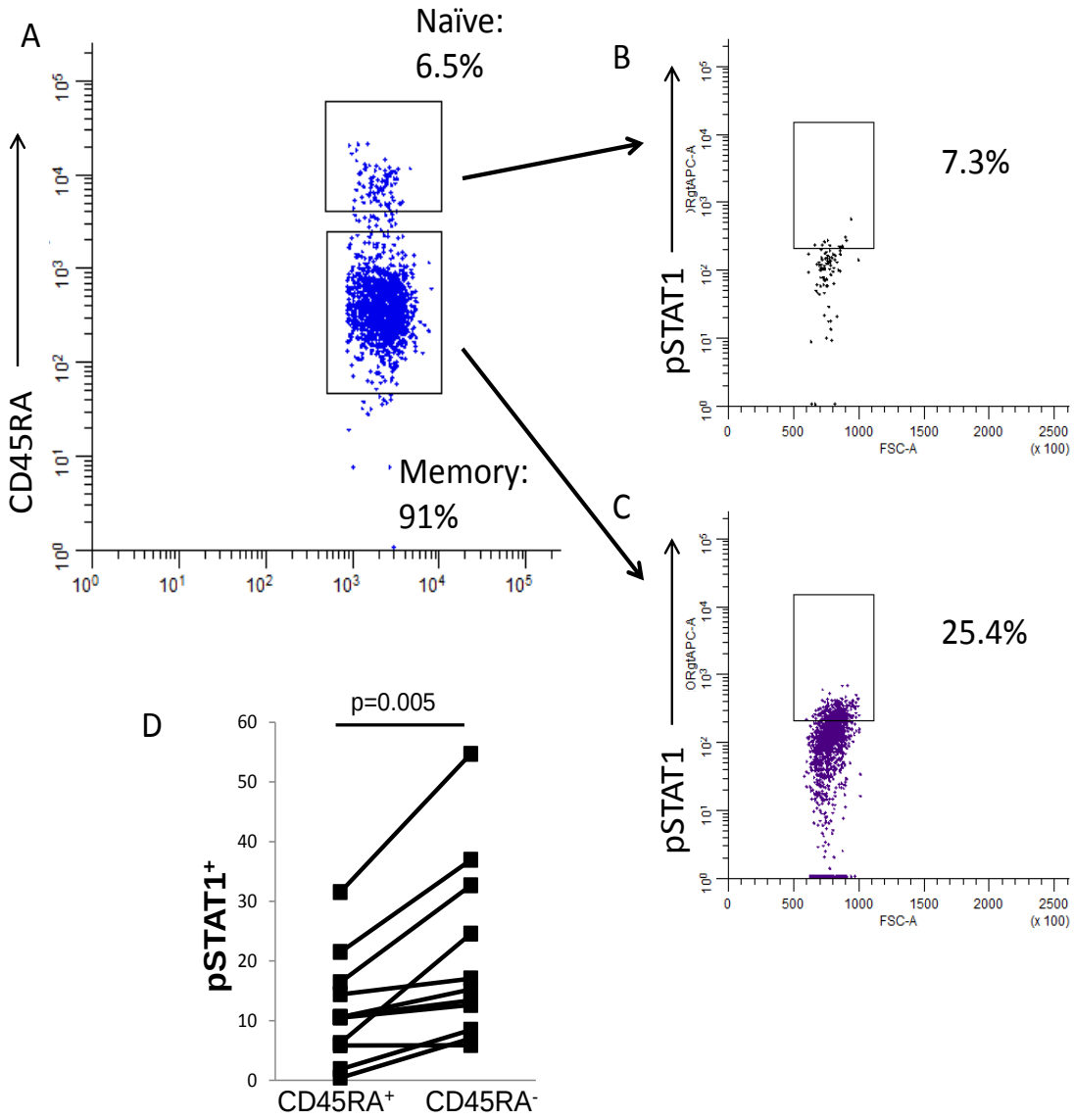

Figure 3.10 Most intestinal CD4$^+$ T cells are memory cells and these are responsive to T1IFN. A *Example plot of CD45RA expression on gated CD4$^+$ intestinal T cells isolated by walk out.* ***B*** *CD45RA$^+$ (naive) or* ***C*** *CD45RA$^-$ (memory) CD4$^+$ T cells after Type I IFN stimulation. Representative of 10 experiments.* ***D*** *Combined data from all (n=10) experiments showing percentage of pSTAT1+ intestinal T cells after T1IFN stimulation in naïve (CD45RA$^+$)* and memory (CD45RA$^-$) populations.

The percentages of pSTAT3$^+$ T cells were too low even after T1IFN to determine a consistent difference between memory and naïve phenotypes, although there was higher MFI of pSTAT3 after T1IFN in the CD45RA$^-$ cells, consistent with the pSTAT1 data (n=10, p=0.019, data not shown). There was no significant difference

between the percentages of pSTAT5$^+$ naïve or memory intestinal T cells after T1IFN stimulation (n=10, p=0.109, data not shown).

3.4.7.2 Measurement of T cell activation by CD69 expression

Expression of the early T cell activation marker CD69 indicated that a large proportion of the cells (CD45RA$^-$ only) were activated (n=16, range 20-80%, median 57%). This upregulation of CD69 may have occurred during cell isolation, or may represent *in vivo* activation status. While both CD69$^+$ and CD69$^-$ cells were responsive to T1IFN, CD69$^+$ cells showed a greater responsiveness with regards to percentage of pSTAT1$^+$ cells (figure 3.11). Due to relatively low cell numbers, it was not possible to analyse CD69 expression in conjunction with pSTAT3 and/or pSTAT5, and therefore not possible to comment on the relationship between CD69 expression and other pSTATs.

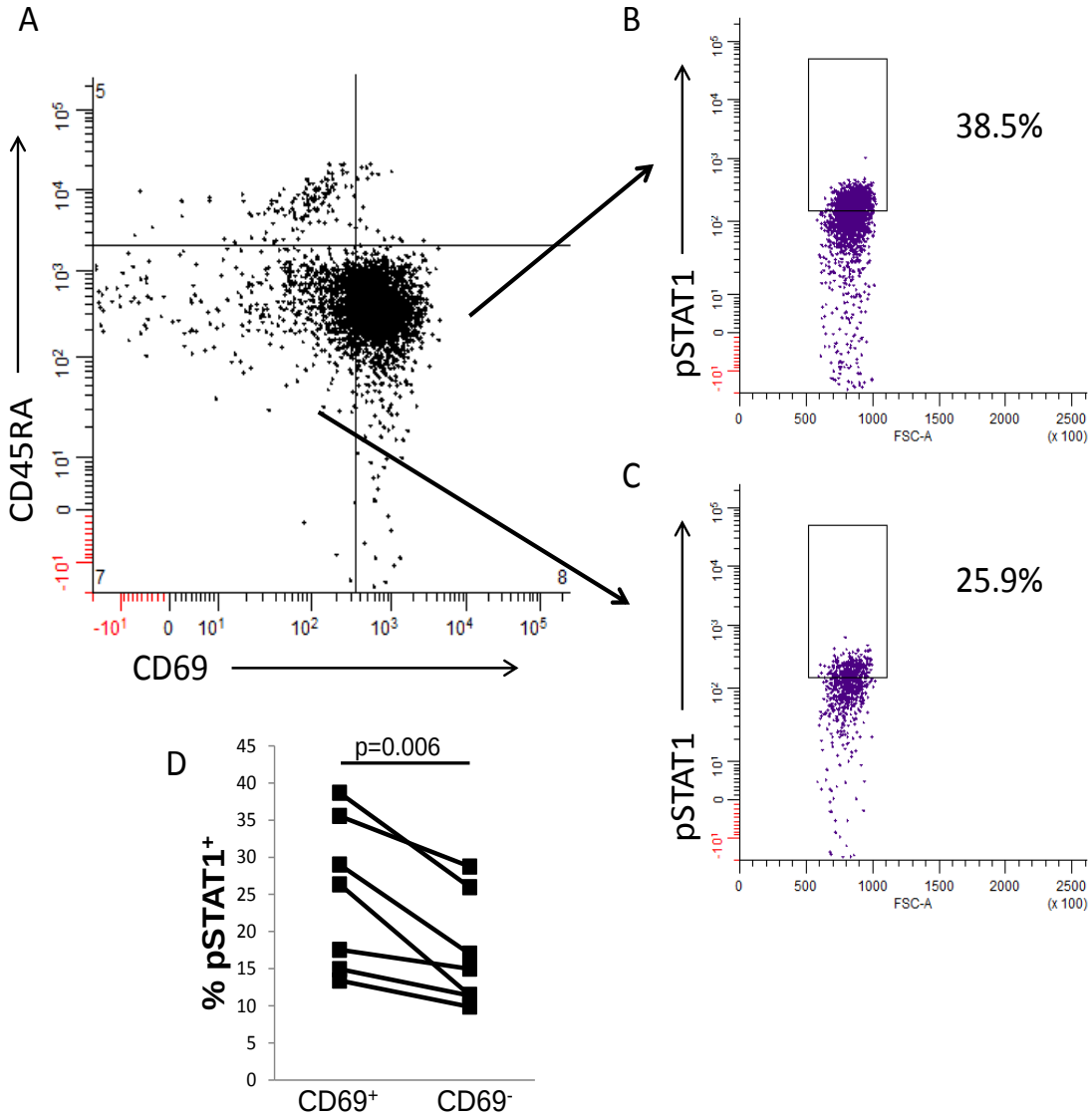

Figure 3.11 CD69 is expressed by memory intestinal CD4⁺ T cells and is associated with increased pSTAT1 expression. ***A*** *Expression of CD69 and CD45RA by CD4⁺ intestinal T cells. Examples of pSTAT1⁺ expression, after T1IFN stimulation, by CD45RA⁻CD69⁺ cells (**B**) and CD45RA⁻CD69⁻ cells (**C**). Representative of 7 experiments.* ***D*** *Combined data from all experiments showing percentage of pSTAT1⁺ intestinal CD4⁺ memory T cells after T1IFN stimulation in CD69⁺ and CD69⁻* populations.

3.4.7.3 TCR signalling in intestinal T cells by phosphor-Erk1/2 expression

Finally, pErk, which is directly downstream in the signalling pathway of T cell receptor (TCR) activation, had initially been measured in whole blood and LPMCs using the Phosflow technique, using PMA stimulation. It was of interest to know

whether the presence of STAT activation was related to signalling through the TCR, which may be demonstrated by pErk1/2 upregulation. There was no detectable pErk constitutively or after Type I IFN stimulation (n=5, data not shown).

3.5 Discussion

This chapter shows the feasibility of using Phosflow as a technique for measuring activation of signalling pathways in intestinal T cells. While this analysis appears unsuitable for cells isolated by collagenase digestion, it was effective when LPMCs were isolated by the walk-out technique. By this method, Phosflow enabled detection of both constitutive pSTAT expression and the induction of pSTAT expression in response to brief T1IFN stimulation. Expression of pSTAT1, pSTAT3, pSTAT5 and pSTAT6 was detectable using this approach. pSTAT4 was not detected under any experimental conditions evaluated; antibody to pSTAT2 was not available. Expression of pSTATs was a property of viable memory $CD4^+$ T cells and was increased after T1IFN, particularly in cells showing evidence of recent activation by CD69 expression. TCR activation did not appear to be linked to pSTAT expression as assessed by detection of pErk.

3.5.1 Optimisation and selection of Phosflow reagants

It was possible to modify the Phosflow (BD Biosciences) protocol to use different fixation and permeabilisation agents, and there was literature to support these alterations (Haas et al., 2008; Montag and Lotze, 2006a; Montag and Lotze, 2006b). In particular, 2% w/v PFA was used for cell fixation and 70% v/v methanol was used as a permeabilisation agent. It is worth noting that the requirement for permeabilisation with methanol, which is considered a harsh agent, may modify cell surface expression of epitopes (Chow et al., 2005; Suni and Maino, 2011), and this was reflected in what was technically feasible in the later experiments of this and subsequent chapters.

3.5.2 Measurement of STAT phosphorylation

3.5.2.1 Detecting constitutive phospho-antigen (pAg) levels and response to T1IFN stimulation

As discussed in the introduction, it was considered of greatest interest to measure STAT family protein activation within the T cells. The manufacturer's protocol is largely dependent on phosphorylation measurements after high dose stimulation over a very short period of time, as indeed is successful (figure 3.2). However, it was always the intention to measure constitutive signal in the intestinal T cells if possible, as this may give an indication of the baseline levels of responsiveness of these cells *in vivo*. Indeed, constitutive levels of STAT phosphorylation were detected in PBMCs of patients with rheumatoid arthritis using this technique (Galligan et al., 2009). However, that publication used Mean Fluorescence Intensity (MFI) as a measure of increased phosphorylation, comparing MFI of several antibodies to different pAgs with several measures of clinical activity. Indeed, the Galligan paper did not take into account isotype binding which must bring into question the specificity of their findings. Given the nature of *ex vivo* manipulation of human cells, it was felt important in this work to detect pAg with antibodies paired with their appropriate isotype control (figure 3.4). In this chapter's work there was no detectable constitutive pSTAT1 detected in PBMCs above isotype control in our laboratory.

3.5.2.2 Different pSTATs can be detected with and without T1IFN stimulation

It was extremely useful to be able to detect pSTATs 1, 3 and 5 with the same stimulus. This would potentially enable multiple STATs to be analysed in parallel using a single sample thereby maximising the information that could be obtained from the limited cell numbers available. Therefore, using multi-colour flow cytometry, it was possible to characterise relatively small numbers of cells in detail at a single

cell level, as well as the varying responses of different STATs to a single stimulant. Other pAgs were also detected, particularly pErk1/2, but also pSTAT6 and pMAPK38, although these latter antigens were not studied in detail. It would be presumably possible to detect many other pAgs using this technique. However, it was disappointing not to be able to detect pSTAT4, both as its importance in TH1 commitment (Jacobson et al., 1995) and in IBD and models of colitis (Glas et al., 2010; O'Malley et al., 2008; Ohtani et al., 2010; Wirtz et al., 1999). This failure of detection was despite multiple attempts at optimisation, including using different stimulants for a positive control, changing the cell isolation conditions and contacting the manufacturers for suggestions. It is possible that an experiment using different time-points may have detected pSTAT4, as it may be phosphorylated later than STAT1 in response to T1IFN. Given the longstanding association of Crohn's disease in particular as a "TH1 disease", it would have been of great interest to study this more dynamically in the human gut, and perhaps a different product would have been more successful.

3.5.3 Phosflow in LPMCs

No staining with anti-pSTAT antibodies was detectable, with or without *ex vivo* stimulation, when intestinal cells were isolated by standard collagenase digestion (see chapter 2, section 2.2.1). Notably, staining for surface antigens was also poor under these conditions using the adapted Phosflow protocol. It is likely that treatment with collagenase (and possibly other agents in this process), particularly in conjunction with 'harsh' methanol fixation, has a detrimental effect on the cell-surface and intracellular epitopes being analysed. Both of these treatments have previously been noted to impact upon antibody staining in flow cytometry (Chow et al., 2005; Wu et al., 2010). This impact may be related to either the ability of the T cells to respond dynamically to environmental triggers, or the ability of the antibodies to detect the response, or some combination of the two. Regardless, it

may be that with a “rest period” after isolation, such as placing the extracted cells in culture for 24 hours, the cells may more amenable to analysis with Phosflow. Of note, one of the first papers to use Phosflow in rheumatoid arthritis patients blood samples did not use the technique on synovial fluid where collagenase was used to isolate the T cells (Galligan et al., 2009).

Fortunately, using the walk-out technique of cell isolation (see chapter 2), both constitutive pSTAT signal, as well as responsiveness to T1IFN stimulation, were detectable for STATs 1, 3 and 5. In this protocol cells are isolated after 24-48 hours in culture medium, and there is therefore potential for the cells to become activated by the process or undergo other phenotypic or functional changes during the culture period. This, 'constitutive' expression of pSTATs may reflect a response stimuli present during this culture period. In addition, simply the act of taking a biopsy or surgical resection irrespective of the means of subsequent cell isolation could have and unanticipated effects on the isolated cells. Therefore, caution must be exercised in extrapolating directly from observations made in these *in vitro* cultures to the properties of cells *in situ*. Nonetheless, the data in this chapter demonstrate that the walk out procedure generates a population of viable human cells in which signalling activity can be explored using Phosflow (figure 3.5).

These results demonstrate the feasibility of detecting phospho-antigens in mucosal T cells. This technique is likely to be applicable to other cells types within the intestine, although given the poor staining achieved using collagenase cells it would currently be limited to cells types that can be obtained by the walk-out method. However, many lymphoid and myeloid lineage cells can be detected in this way (Mahida et al., 1997), so it is likely that at least these cell types could be probed.

3.5.4 Characterisation of pSTAT$^+$ intestinal CD4$^+$ T cells

It was consistent with previous observations that the majority of isolated T cells were of a memory phenotype and it was this population that appeared the most

responsive to T1IFN as determined by the greater frequency of pSTAT1^{+} cells following stimulation (figure 3.10). There was no clear difference in constitutive pSTAT1 levels between naïve and memory cells. The increased responsiveness is perhaps not surprising in that memory T cells from peripheral blood have been shown to be more responsive to other signals (such as TCR or CD3 activation) than naïve cells (Kersh et al., 2003; Sanders et al., 1989). The few CD45RA^{+} T cells could be any of a number of possibilities. These include naive cells from blood contamination within the biopsy samples, inadvertent sampling of organised lymphoid tissue, revertant CD45RA^{+} memory T cells or naïve T cells that have circulated through the lamina propria. Regardless of cause, the proportions of naïve T cells found were similar to a recent article published using an abbreviated collagenase method of cell isolation (Sathaliyawala et al., 2013).

A common method of assessing T cell activation is by measuring the surface marker CD69. The process of biopsy sampling, denuding the biopsy of epithelium, and the contact of the LPMCs on plastic could all conceivably lead to activation of all cell types. As the walk-out isolation process is dependent on the active movement of cells out of the biopsy, it was thought that these cells would be activated during this process, and thus might have expressed high levels of CD69. Interestingly, the CD69 expression was only seen in the CD45RA^{-} cells (figure 3.11). This is concordant with murine models, where CD69 expression has been found at high levels in lamina propria T cells, predominantly in the memory (CD45RB^{low}) population (Aranda et al., 1997), and a recent paper describing human T cell subsets (Sathaliyawala et al., 2013). Interestingly, in the human T cell study (which used collagenase) the CD69 expression levels were similar to the results in figure 3.11, suggesting that it may not be the walk-out process that drives this expression or perhaps that it is not T cell activation that is driving the CD69 expression.

It has been shown in mice that CD69 expression is essential to block $CD4^+$ T cell egress from Peyer's patches (Schulz et al., 2014), and this effect can be dependent on T1IFN signalling (Shiow et al., 2006). In a T cell transfer model of murine colitis, $CD4^+$ T cells from CD69 knockout mice showed reduced regulatory T cell capacity, and exacerbated colitis (Radulovic et al., 2012). It was also shown in this study that the CD69 expression was dependant on the presence of the IFNAR (Radulovic et al., 2012). These studies, in combination with the data from this chapter, suggests that CD69 expression in intestinal $CD4^+$ T cells is associated with T1IFN signalling, including upregulation of pSTAT1, and that this may have downstream effects on both T cell retention within the gut, and T cell function.

The T1IFN stimulation did not elicit any detectable phospho-Erk1/2, nor was this antigen detectable in intestinal T cells constitutively. This suggests that TCR signalling is not a major component of the ongoing responsiveness detectable in this cell population using Phosflow, although an absence of pErk cannot rule out TCR transduction.

3.5.5 Summary

This chapter showed the optimisation of a relatively new technique within flow cytometry for use with intestinal T cells. The major technical challenge of this was balancing the use of harsh fixation and permeabilisation agents required for detections of phospho-antigens with the maintenance of expression of cell surface antigens for cell identification in the context of human tissue with relatively small cell numbers. In particular, pSTAT1, 3 and 5, but not pSTAT4, could be detected. The pSTAT4 antigen was not detected in any experiments, with or without stimulation with various stimulants. The reason that pSTAT4 could not be detected is unclear but is presumed to be due to technical issue with this antibody.

Successful detection of pSTATs required isolation of intestinal cells by the walk out method; collagenase digestion of tissue was not suitable. Using this approach phosphorylated STATs were detectable in a small proportion of cells without stimulation and were markedly upregulated following brief stimulation with T1IFN. $CD45RA^-$ memory/effector intestinal CD4+ T cells were more responsive to T1IFN than CD45RA+ naive cells and within the former population, cells that expressed CD69 were more responsive than those that did not.

These observations provided a strong platform for using Phosflow to investigate the role of STAT signalling in intestinal T cells in both healthy humans and patients with IBD. These experiments are presented in chapter 4.

Chapter 4

4 STAT signalling in human peripheral and intestinal T cells in health and Inflammatory Bowel Disease

4.1 Chapter Overview

Control of T cell reactivity within the intestinal mucosa is poorly understood. T cells are increasing recognised to be functionally plastic and respond dynamically to signals in their local environment. However, these properties may not be adequately captured in a traditional measurement of cytokine profiles. Analysis of activity within key intracellular signalling pathways, as determined by measuring phosphorylation of proteins (such as Signal Transduction and Activator of Transcriptions (STATs)) may offer a more dynamic picture. As shown in Chapter 3, Phosflow enables these events to be resolved at the single cell level. In IBD, T cells are considered the main drivers of inflammation, with CD considered a TH1/TH17 disease, and UC a modified TH2 disease. It was hypothesised that Phosflow would enable the dynamic properties of intestinal T cells to be explored by defining activity in key signalling pathways. This would extend our understanding of these cells in health and IBD.

To test this hypothesis LPMCS were isolated and analysed using flow cytometry for pSTATs as well as traditional transcription factors, with and without stimulation with Type I Interferon (T1IFN). Expression of SOCS, the main regulators of STAT activation, were analysed by qRT-PCR after mRNA was extracted from purified intestinal T cells.

In the absence of T1IFN stimulation, $pSTAT1^+$ $CD4^+$ T cells were more frequently detected from non-inflamed mucosa of IBD patients compared with similar tissue from controls. In paired IBD samples, constitutive pSTAT1 was more frequently detected in $CD4^+$ T cells from non-inflamed than inflamed areas. T1IFN stimulation increased $pSTAT1^+$ frequency irrespective of the source of intestinal T cells but the IBD-related differences were maintained. The frequency of $pSTAT1^+$ peripheral blood T cells did not differ between IBD patients and controls, indicating that changes in this signalling pathway were specific for the intestine. The frequency of T cells expressing the TH1 transcription factor T-Bet was increased in samples from IBD patients compared with controls. However, expression of pSTAT1 was not

restricted to T-bet$^+$ cells indicating that it is not simply a marker of TH1 cells in this context.

There was a trend towards reduced expression of SOCS1 mRNA, the main regulator of STAT1, in T cells from non-inflamed IBD mucosa. Neutralisation of IFNβ, considered the most important T1IFN subtype, reduced frequency of pSTAT1$^+$ and total STAT1$^+$ cells, suggesting that endogenous T1IFN is an important driver of STAT1 signalling in intestinal CD4$^+$ T cells.

It was therefore determined to detect evidence of T1IFN in the human intestine. IFNβ could be identified by immunohistochemistry (IHC) in fresh frozen sections of colonic lamina propria from both control subjects and IBD patients. To our knowledge this is the first time T1IFN has been identified directly in the human intestine. In addition, expression of mRNA of two classic Interferon Stimulating Genes (ISGs), MxA and 2'5' OAS, were also detected from intestinal T cells, further evidence of endogenous T1IFN activity.

Previous published studies showing altered STAT expression in IBD mucosal tissue have examined only inflamed gut, and usually have assessed whole biopsies or tissue specimens, not specific cell types. These differences in approach may explain some differences between the results presented in this chapter and previously published findings. The increased pSTAT1 reported here does not simply reflect a TH1 phenotype, but instead may reflect ongoing responsiveness to the intestinal environment. IFNβ has been demonstrated, for the first time, in the human lamina propria in both control and IBD samples, where it would exert its constitutive properties. The plasticity of T cell response, especially from signalling pathways involving IFNβ-STAT1, may be important in controlling inflammatory responses in the human gut.

4.2 Introduction

4.2.1 Background

The Inflammatory Bowel Diseases (IBD), comprised primarily of Crohn's Disease (CD) and Ulcerative Colitis, are thought to be caused by inappropriate immune reactivity to components of the commensal microbiota, driving chronic intestinal inflammation (Kaser et al., 2010). There are several aspects of the mucosal immune system, including mucosal barrier function and innate anti-microbial immunity, which have shown to be defective in patients with IBD and in animal models of intestinal inflammation, as discussed in Chapter 1. However, intestinal T cells are ultimately the main drivers of persistent inflammation (Brand, 2009) and also the target of most current therapies in IBD (Neurath, 2014b). Crohn's disease is often regarded as a TH1/TH17 disease (reviewed in Brand, 2009), and UC a modified TH2 disease (Fuss et al., 2004). These terms, however, may become less relevant as new techniques to assess T cell phenotype become available.

Given the increasing understanding in the plasticity of the T cell phenotype from animal and *ex vivo* models (reviewed in Hirahara et al., 2013), it is reasonable to assume that T cells in the human mucosa also maintain a degree of flexibility that traditional analysis may not have appreciated. Having established the ability to measure activation of key signalling pathways in intestinal T cells, as described in Chapter 3, it was now hoped to use this technique to further understand the responsiveness of T cells in the human gut in health and IBD.

4.2.2 "Plasticity" of mucosal T cells

The classic model of helper T cell priming in gut associated lymphoid tissue (GALT) into fully committed cells expressing a particular phenotype (TH1, 2, etc) has been recently challenged (see figure 4.1). This is the concept of immune "plasticity";

differentiated $CD4^+$ T cells remain responsive to their environment (reviewed in O'Shea and Paul, 2010). It has been shown that antigen specific memory T cell expansion and functional commitment can occur directly in the lamina propria in mice dependant on $CD70^+$ APCs (Laouar et al., 2005), demonstrating that local factors can influence final effector function. Again in the mouse, it has been shown that local cytokines, particularly IL-10 produced by intestinal macrophages, can drive Treg education and expansion in the lamina propria (Hadis et al., 2011). The induction of oral tolerance in mice by Tregs has been shown to require CCR9 and β7, which enable trafficking to the intestinal mucosa where the cells receive a critical IL-10 signal. In *ccr9* knockout mice, oral tolerance can be restored by provision of exogenous IL-10 (Cassani et al., 2011). That is, the normal "education" step of T cell proliferation and differentiation, which requires gut homing after T cell priming in organised lymphoid tissue, can be achieved by local factors. This suggests that environmental factors, including IL-10, can be responsible for the functional determination of T cells in the lamina propria.

Within $CD4^+$ cells, TH17 cells are perhaps the best example of T cells with a modifiable phenotype, as discussed in chapter 1. It is likely that the balance of signals in the intestine affects the phenotype of local T cells. This plasticity would allow the intestine to be flexible in either maintaining an either regulatory or inflammatory adaptive response depending on the balance of signals in the local environment (Strober and Fuss, 2011).

4.2.3 T cells in IBD

Most T cells in the gut mucosa are found in the lamina propria, and are effector memory T cells (Sallusto et al., 1999). Traditionally, CD has been viewed as a TH1-mediated disease (Monteleone et al., 1997). UC has been shown to have a modified T helper 2 (TH2) signature, with increased IL-5 and IL-13 production, but decreased IL-4 (Fuss et al., 2004; Fuss et al., 1996). More recently, with the discovery of the T

helper 17 (TH17) lineage, CD is now seen as a disease in which both TH1 and TH17 cells are major driving forces (Brand, 2009).

4.2.3.1 Heterogeneity of IBD T cell phenotype

While the TH1/TH2/TH17 labels for the IBDs are commonly used, they are based on studies using relatively old techniques of analysis, often with at least partially conflicting results. These studies were described in chapter 1, section 1.4. Given the varied clinical phenotype of IBD, it is difficult to compare these papers as they sample different patient populations, and it is possible that this alone could explain the different findings.

The technical approach of Phosflow does give an opportunity to measure responsiveness of human intestinal T cells. Given the strong relationship between STAT proteins and T cell phenotype, as discussed in both chapter 1 and chapter 3, exploring these pathways in intestinal T cells would give a more refined approach understanding the pathogenesis of IBD. Previous studies had looked at STAT proteins and molecules closely related to them in IBD patients, but these had generally not been with specific regard to T cells.

4.2.4 STAT and SOCS proteins in models of colitis and in IBD

The Jak-STAT pathway is known to be crucial in T cell differentiation and expression of major pro- and anti-inflammatory cytokines (see introduction, figure 1.3). For this reason the STATs were chosen in this thesis as the main molecules of interest for both the analysis of T cell responsiveness and also their role in human intestinal T cells in health and IBD. Indeed, STAT proteins and their regulators, the Suppressor of Cytokine Signalling (SOCS) proteins, have been associated with murine models as well as human IBD.

In humans, the numerous studies showing changes in STAT and SOCS expression in both CD and UC (see introduction, section 1.4.6) predominately show an increase in STAT3, but also STAT1 and STAT4. There is some conflicting data, due to multiple variations in the studies. In most cases the samples are from inflamed areas of intestine, and whole tissue biopsies, which may miss cell-type specific changes. It was therefore hypothesised that by using Phosflow to study T cells at a single cell level one would more fully understand the role of STAT proteins in intestinal T cells in IBD.

Previous studies have shown persistent immunological differences between non-inflamed IBD mucosa and controls, as well as between inflamed and non-inflamed areas (Akazawa et al., 2002; Andus et al., 1997; León et al., 2009; Matsuda et al., 2009; Reimund et al., 1996). While it appears self evident (as well as from the literature), that the immunological mechanisms that underly the intestinal inflammation of UC and CD must differ, it is not clear whether the regulatory mechanisms differ between these two patient groups. Given that T cells from non-inflamed areas of IBD mucosa may be considered to be (on balance) functionally pro-regulatory, for the purposes of this thesis IBD samples (CD and UC) are generally analysed together. This was also to allow a technique which is very sensitive, such as Phosflow, to be able to detect subtle differences that may not be seen in the setting of overt inflammation. That is, altered signalling pathways in non-inflamed patient tissues may indicate events relevant to immune dysregulation that could become ‘swamped’ by a dominant inflammatory signature in tissue which is actively inflamed..

STAT signalling can be initiated by numerous cytokines and other factors. T1IFN can activate all members of the STAT family (Darnell 1994, Gough 2012). In combination with its known role in murine intestinal homeostasis via T cell response, T1IFN is an excellent candidate for exploring STAT-mediated human intestinal T

cell responsiveness. It was for this reason that T1IFN was used as the primary stimulus for exploring STAT responses in this chapter and the previous one.

4.2.5 Summary

STAT proteins have been previously shown to be of potential significance in the pathogenesis of IBD. New IBD therapies, such as tofacitinib, the putatively selective Jak3 inhibitor, have effects on STAT activation. T cell function is plastic, and new techniques, such as Phosflow, allow a more nuanced approach to understanding responsiveness of cells to their environment. Given this, it was proposed to study the activated STAT proteins in health and IBD. The focus was specifically on STATs 1, 3 and 5, as they are implicated in T cell function (in TH1, TH17 and Treg pathways respectively). As shown in Chapter 3, phosphorylation of these STATs can be measured in intestinal T cells using Phosflow. All three are phosphorylated following stimulation with T1IFN, allowing responsiveness to a relevant environmental mediator to be assessed. It was thought that measuring these pathways in T cells from non-inflamed areas of mucosa as well as inflamed tissue would give insights into subtle changes of T cell phenotype at the interface of health and disease.

4.3 Aims

(i) To identify IBD associated differences in activation of STATs (1, 3 and 5) in intestinal T cells.

(ii) To compare STAT signalling in T cells isolated from inflamed areas of IBD mucosa with those from macroscopically and histologically non-inflamed areas.

(iii) To explore the relationship between activated STAT signalling pathways and T cell populations defined by more classical markers.

(iv) To explore potential mechanisms of STAT activation in intestinal T cells in health and IBD.

4.4 Results

4.4.1 pSTAT1 expression by $CD4^+$ T cells in non-inflamed and inflamed IBD intestinal mucosa compared with controls samples

Expression of pSTAT1 was detectable in *ex vivo* walk-out lamina propria T cells with and without stimulation with T1IFN (figure 4.1). The percentage of pSTAT1 positive T cells was variable and low under unstimulated conditions (constitutive expression) but was significantly increased after 15 min stimulation with T1IFN (n= 35, $p<0.001$, paired t test). This data is not directly shown but constitutes the differences between figures 4.1A and 4.1B.

Constitutive pSTAT1 expression was significantly more frequent in $CD4^+$ T cells from non-inflamed biopsies of patients with IBD than in similar cells from control tissue (figure 4.1A). There was also a trend towards a higher frequency of pSTAT1+ cells amongst $CD4^+$ T cells from inflamed IBD tissue than from controls but this difference did not reach statistical significance ($p=0.05$). The expression of pSTAT1 by $CD4^+$ T cell from non-inflamed tissues was also significantly more frequent than that of cells from paired inflamed samples (figure 4.1A). Although the frequency of pSTAT1+ cells was significantly increased in $CD4^+$ T cells from all three types of samples following T1IFN stimulation, the relatively high expression by cells from non-inflamed IBD tissue was maintained (figure 4.1B).

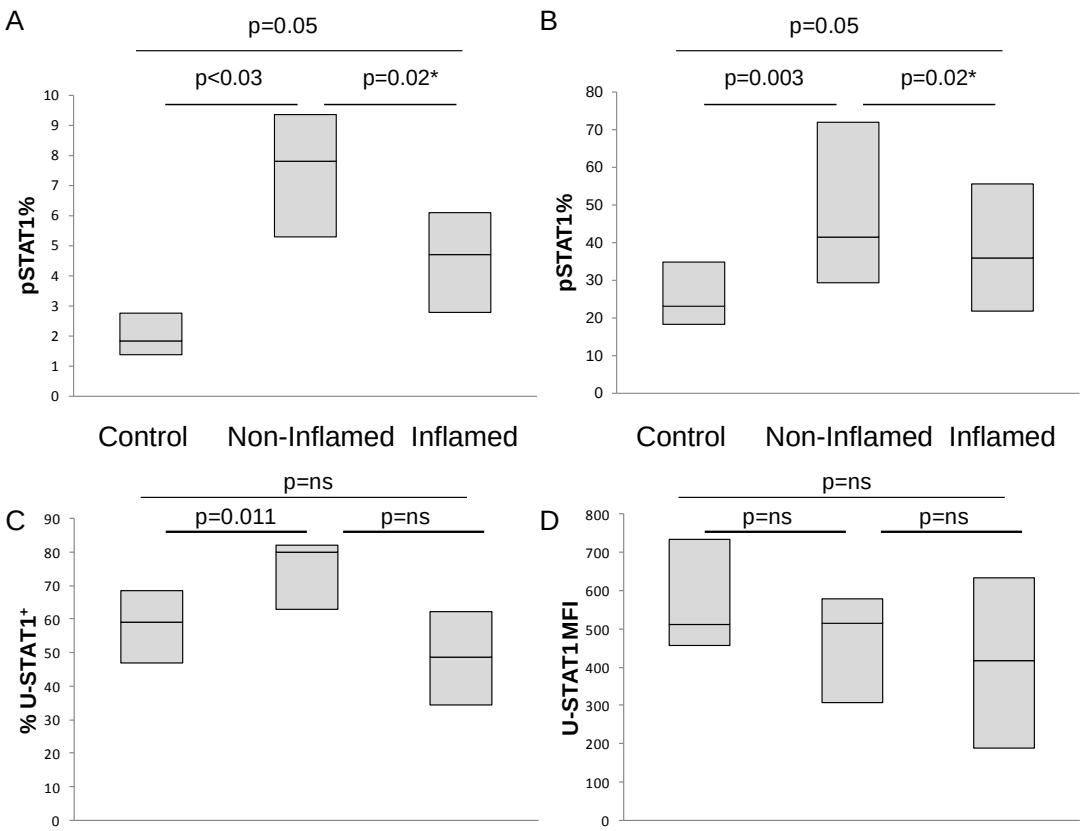

Figure 4.1 Expression of pSTAT1 is more frequent in Intestinal T cells from non-inflamed areas in IBD patients. *Intestinal biopsies of controls or IBD patients (normal macro/microscopically – Non-Inflamed or Inflamed), were processed by the walk out method as described in chapter 2.* ***A,B*** *Percentage of pSTAT1$^+$ CD4$^+$ T cells in healthy (n=16) and IBD (n=24) patients intestinal walk-out cells in both unstimulated (**A**) and after 15 minute stimulation with T1IFN (**B**).* ***C*** *Total STAT1 expression (phosphorylated and non-phosphorylated; U-STAT1$^+$) as a percentage of intestinal CD4+ in unstimulated samples and* ***D*** *U-STAT1 Mean Fluorescence Intensity (MFI) levels from the same samples. Boxes indicate 1st and 3rd quartiles, with the middle line the median. p values obtained by ANOVA * indicates significance only in paired samples from the same patients.*

4.4.1.1 Total (unphosphorylated) STAT1

The increases in pSTAT1 may have reflected an increase in total STAT1 levels, rather than a selective increase in the fraction of STAT1 molecules that are phosphorylated. While activated STAT1 was detected in few cells and expression different significantly between the different sample groups, most cells were U-STAT$^+$ and there were no significant differences in expression between samples. (figure 4.1 C and D). Since the frequency of U-STAT1$^+$ T cells was very high in all samples,

since all T cells would be expected to express some STAT1, U-STAT expression was also compared using the Mean Fluorescence Intensity (MFI, figure 4.1D). Although statistically non-significant, there was a trend towards an increased percentage of U-STAT1$^+$ CD4$^+$ T cells in non-inflamed IBD samples compared to controls mirroring the pSTAT1 data, but this was not reflected in the mean fluorescence intensity. All pSTAT1$^+$ cells showed high U-STAT1 MFI, and were U-STAT1$^+$, as may be expected, given that U-STAT1 antibodies should bind to STAT1 regardless of its phosphorylation status (data not shown). Overall, these data suggest the differences in pSTAT1 expression are related, at least in part, to the available pool of STAT1 molecules.

4.4.1.2 pSTAT1 expression by intestinal CD4$^+$ T cells from Ulcerative Colitis and Crohn's Disease patients

When samples from UC (n=12) and CD (n=12) patients were considered separately, the differences between control tissue and non-inflamed patient tissue were generally maintained for both patient groups, although did not always retain statistical significance. In non-inflamed biopsies from UC patients, the constitutive frequency of pSTAT1$^+$ CD4$^+$ intestinal T cells trended to increase compared to healthy control cells ($p=0.08$, ANOVA) and this difference was significant after 15 min stimulation with T1IFN ($p=0.017$, ANOVA). In CD, the same differences were observed in unstimulated ($p=0.08$, ANOVA) and stimulated ($p=0.011$, ANOVA) cells. In paired samples from CD patients, pSTAT1$^+$ T cells tended to being more frequent in CD4$^+$ T cells from non-inflamed biopsies than from inflamed tissue in unstimulated cells ($p=0.064$, Signed Rank test) but this difference was not statistically significant after T1IFN stimulation ($p=0.168$, paired t test). In the UC patient samples, there was the same trend for $p=0.12$ for constitutive expression ($p=0.12$, Signed Rank test), which was just significant after T1IFN stimulation

(p=0.049, Signed rank test). These results imply that there is not a difference between Crohn's and UC with regards to the STAT1 activation in intestinal $CD4^+$ T cells.

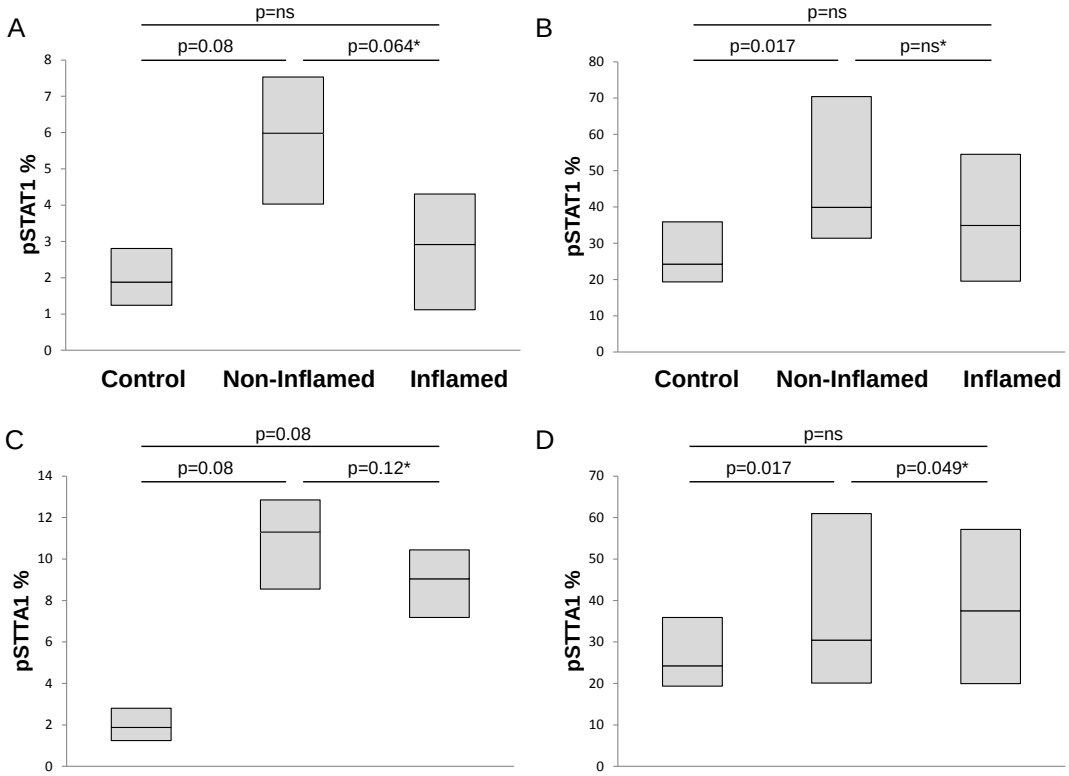

Figure 4.2 Expression of pSTAT1 is more frequent in Intestinal T cells from non-inflamed areas in UC and Crohn's disease patients. *Intestinal biopsies of controls or IBD patients (normal macro/microscopically – Non-Inflamed or Inflamed), were processed by the walk out method as described in chapter 2.* ***A,B*** *Percentage of $pSTAT1^+$ $CD4^+$ T cells in healthy (n=16) and UC (n=12) patients intestinal walk-out cells in both unstimulated (**A**) and after 15 minute stimulation with T1IFN (**B**).* ***C,D*** *Percentage of $pSTAT1^+$ $CD4^+$ T cells in healthy (n=16) and Crohn's (n=12) patients intestinal walk-out cells in both unstimulated (**C**) and after 15 minute stimulation with T1IFN (**D**). Boxes indicate 1st and 3rd quartiles, with the middle line the median. p values obtained by ANOVA * indicates significance only in paired samples from the same patients.*

4.4.1.3 $pSTAT1^+$ Intestinal $CD4^+$ T cells in Coeliac disease

To determine whether increased pSTAT1 expression by intestinal $CD4^+$ T cells was specific for IBD, a similar analysis was performed on samples obtained from patients with coeliac disease. The coeliac biopsies may be considered inflamed samples and samples were also taken from age-matched controls (see patient

details, appendix II). It should be noted that cells from this analysis were obtained from walk-out cultures of duodenal biopsies. There were no differences seen between control samples and coeliac samples with regard to frequency of $pSTAT1^+$ $CD4^+$ T cells, either constitutively or after T1IFN stimulation (figure 4.3). There was no equivalent to the non-inflamed biopsies as inactive coeliac disease patients are not routinely biopsied and it is impossible to macroscopically determine which areas of small bowel are affected or unaffected by active coeliac disease.

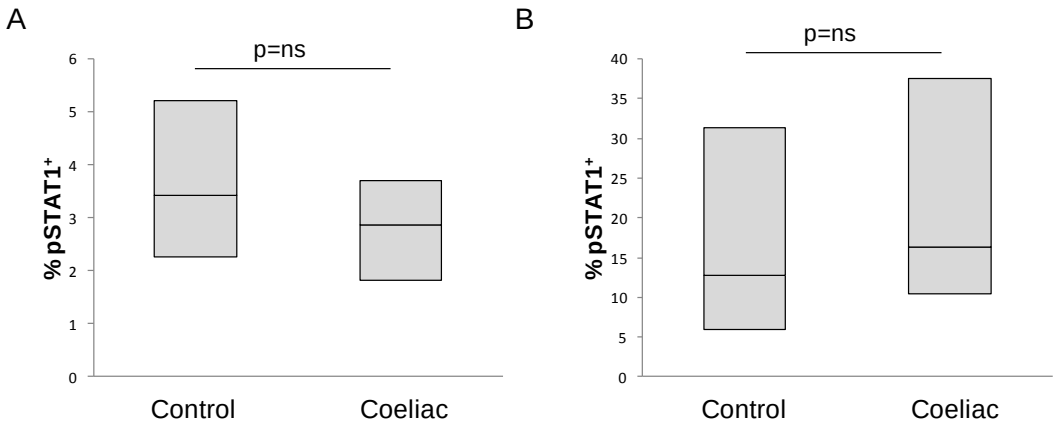

Figure 4.3 $pSTAT1^+$ intestinal T cells are not different in frequency between health and active coeliac disease. *Intestinal T cells were isolated by walk-out as previously described. Samples were taken from patients at diagnosis of coeliac disease (n=9) or age-matched controls (n=7). $CD4^+$ T cells were gated and the pSTAT1+ percentages are shown in constitutive (A) and T1IFN stimulated (B) samples.*

4.4.2 Expression of pSTAT3 and pSTAT5 in intestinal $CD4^+$ T cells in IBD and controls

Given the IBD related differences in pSTAT1 expression, and the literature suggesting that other STATs (particularly STAT3) may be involved in the pathogenesis and persistence of IBD, it was also attempted to look for changes in other pSTATs. In particular, it was of interest as to whether all measured pSTATs would be increased, or whether the increase in the frequency of pSTAT1+ cells was a specific finding.

Both pSTAT3 and pSTAT5 could be detected in intestinal CD4+ T cells (see figure 4.4). Overall, the frequency of T cells that expressed $pSTAT3^+$ was low in all types

of sample. Fewer than 3% of T cells were $pSTAT3^+$ in unstimulated cultures and although stimulation with T1IFN significantly increased expression of pSTAT3 (paired t test, $p<0.001$, data not directly shown), the magnitude of this effect was small. In contrast, pSTAT5 was universally detectable in unstimulated samples and the proportion of $pSTAT5^+$ T cells significantly increased, as expected, followingT1IFN stimulation.

When comparing between constitutive pSTAT expression, in all tissue types (control, non-inflamed and inflamed), $pSTAT5^+$ T cells were more frequent than $pSTAT3^+$ ($p=0.001$, ANOVA on ranks, data not directly shown). In control and inflamed tissue, pSTAT5+ T cells were also more frequent than $pSTAT1^+$ T cells ($p=0.001$, ANOVA on ranks, data not directly shown), but this was not the case in T cells from non-inflamed samples. This reflects the increased frequency of $pSTAT1^+$ cells from the non-inflamed group as shown in figure 4.1.

In contrast to the pSTAT1 data, the frequency of intestinal $CD4^+$ T cells expressing pSTAT3 or pSTAT5 did not differ between samples (control, non-inflamed IBD and inflamed IBD) in either unstimulated or stimulated cells (figure 4.4). However, there was a trend for an increase in constitutive $pSTAT3^+$ T cells in both non-inflamed and inflamed samples. No differences were observed between cells from coeliac disease patients and healthy controls with regards to $pSTAT3^+$ or $pSTAT5^+$ T cells (data not shown).

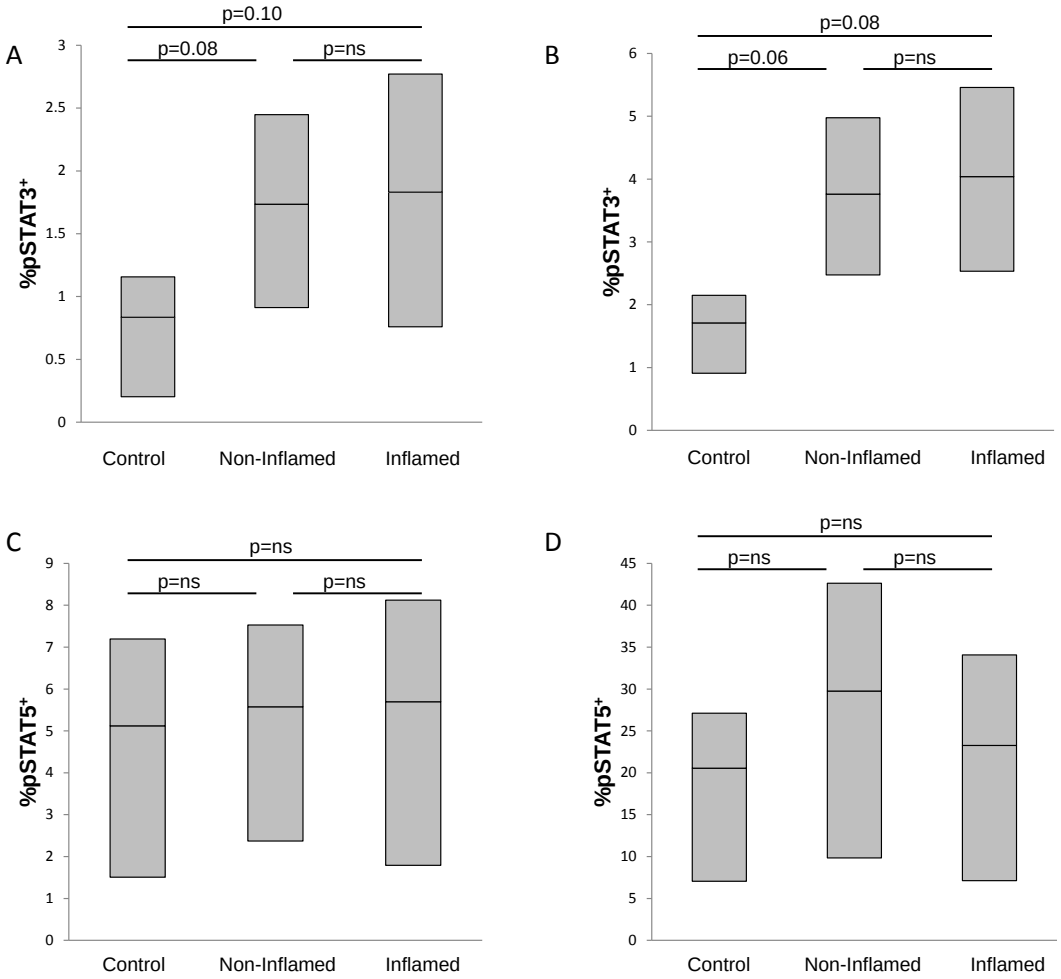

Figure 4.4 pSTAT3 and pSTAT5 expression by intestinal CD4+ T cells is not altered in IBD. *LPMCs were isolated by walk out and intestinal CD4$^+$ T cells are gated. pSTAT3 (A+B) and pSTAT5 (C+D) expression in CD4$^+$ walk-out cells were measure (n=16 control, n=24 IBD). No significant differences are seen between control and IBD or within IBD groups in unstimulated (A+C) or stimulated (T1IFN, B+D) cells. Boxes indicated quartiles with median at middle line.* Statistical analysis is by ANOVA.

4.4.3 pSTAT1$^+$ intestinal CD4$^+$ T cells as a measure of T-bet$^+$ "TH1" cells

The so-called master regulators of T cell transcription are essential factors in determining the function of the cells. It has been shown that expression of T-bet, the TH1 transcription factor, by intestinal CD4$^+$ T cells is increased in CD compared with equivalent cells from healthy controls (Matsuoka et al., 2004). As STAT1 is activated in T cells as they differentiate into the TH1 subset (Zhu et al., 2010), it was thought that the increased proportion of pSTAT1$^+$ intestinal T cells detected in IBD

patient samples may be a reflection of an increase proportion of TH1 cells in the lamina propria of these patients.

Overall expression of T-bet, the TH1 factor, and FoxP3, for Tregs, is shown in figures 4.3 A and B respectively. There is increased expression in T-bet in T cells from both inflamed and non-inflamed IBD mucosa, (figure 4.5A). Expression of FoxP3 did not different significantly between the three groups, but there was a trend towards an increase in the IBD samples compared with controls (p=0.10, ANOVA). Expression of RORγt, the TH17 master regulator, was detected in very few cells, making detection difficult and quantitation unreliable; no significant differences were found between groups (data not shown).

There was no clear relationship within experiments between expression of any 'master regulator' transcription factor and expression of pSTAT1, 3 or 5 (example in figure 4.5). This suggests that the presence of pSTAT1 in A CD4 T cells is not simply a marker of TH1 phenotype. The proportion of pSTAT1+ cells that co-expressed T-bet is shown for each sample examined in figure 4.5E. It is evident that there is no clear relationship between expression of these two markers supporting the idea that the presence of pSTAT1 in intestinal $CD4^+$ cells does not simply reflect a "TH1" phenotype.

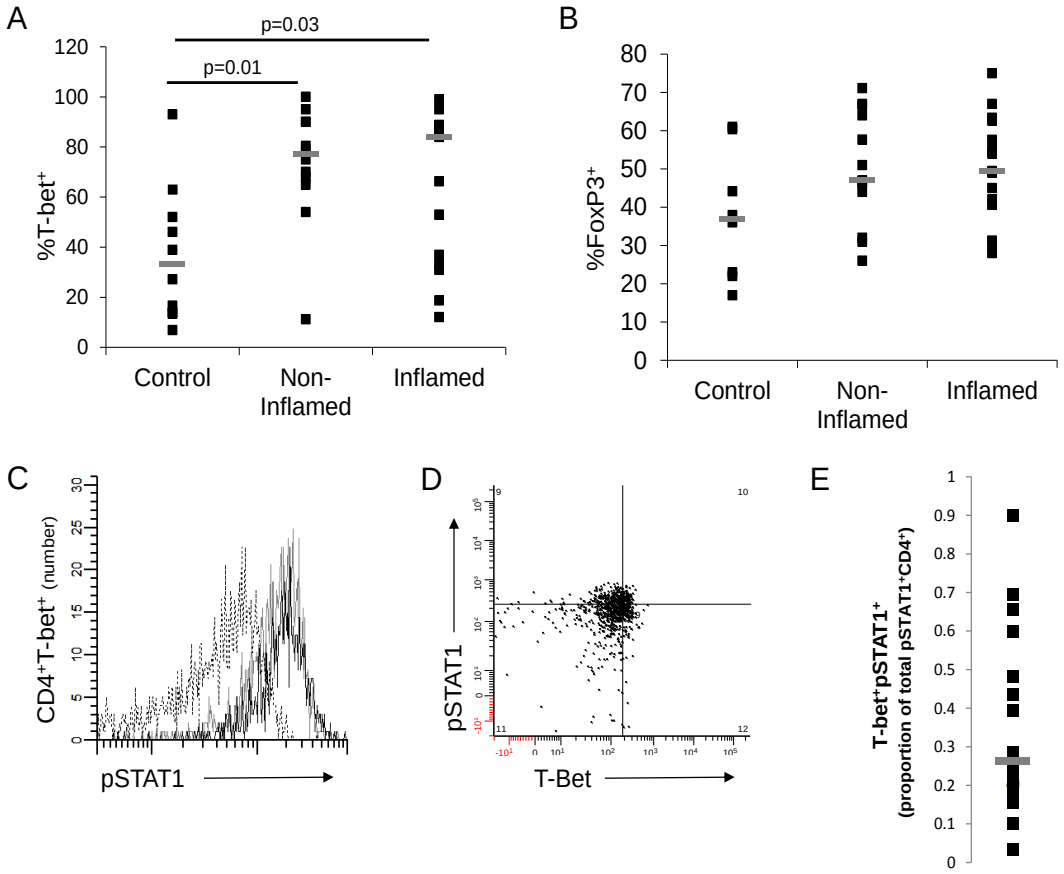

Figure 4.5 pSTAT1 expression is not restricted to T-bet$^+$ TH1 cells. *Expression of transcription factors T-Bet (A, n=9 control, n=12 IBD) and FoxP3 (B, n=7 control, n=9 IBD) as a percentage of all walk-out CD4$^+$ T cells. C Representative FACS plot of pSTAT1 expression in T-bet$^+$ (black line) and T-bet$^-$ (grey line) walk-out CD4$^+$ T cells (dotted line isotype). D 2D representative FACS plot of CD4$^+$ T cells showing co-expression of T-bet and pSTAT1. E Proportion of all T-Bet$^+$CD4$^+$ T cells that are also pSTAT1$^+$ (n=21). Grey bars indicate median values,*

4.4.4 CD69 expression as a measure of activation of Intestinal CD4$^+$ T cells in IBD and controls

Given the possibility of activation of the LPMCs during the walk-out process, it was thought that increased pSTAT1 expression, or indeed other pSTAT signatures, may relate to T cell activation. CD69 is a marker of early T cell activation known to be present on intestinal T cells (De Maria et al., 1993). CD69 expression in the CD4$^+$ T cells can be detected in cells labelled using the Phosflow protocol, as described in chapter 3, section 3.4.4. Results from IBD samples compared with controls are shown in figure 4.6. There were no statistically significant differences in CD69 expression in CD4$^+$ intestinal T cells between sample groups, although there may

be a trend towards decreased expression in cells from inflamed IBD tissue. As shown in chapter 3, there was increased pSTAT1$^+$ expression in CD69$^+$ intestinal T cells compared to CD69$^-$ cells (figure 3.11).

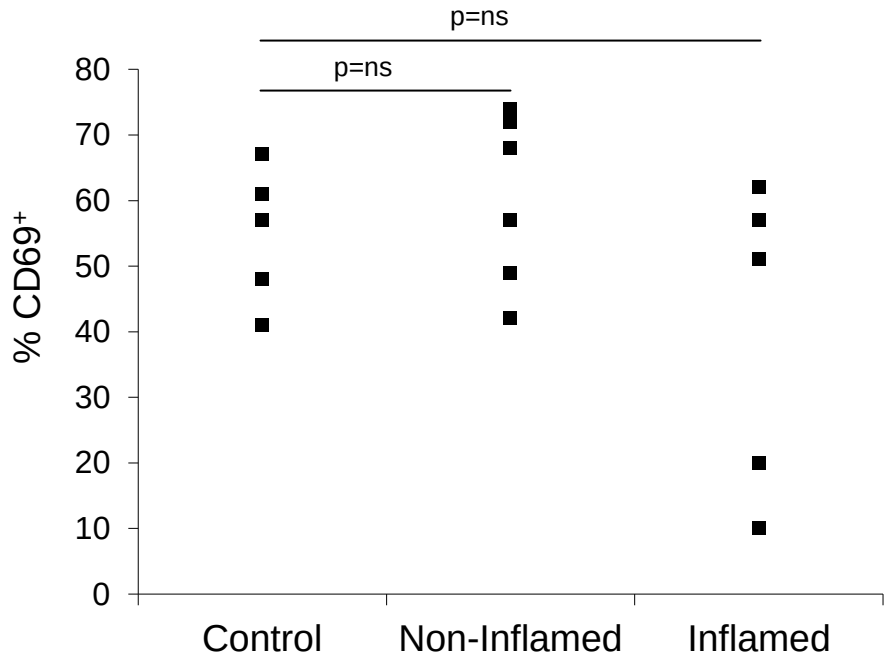

Figure 4.6 CD69 is expressed by many CD4$^+$ intestinal T cells from both IBD patients and controls *LPMCs were isolated by the walk out method. The percentage of CD4$^+$ T cells expressing CD69 is shown in healthy controls (n=5) and IBD patients (Non-inflamed, n=6, and inflamed, n=5). Statistical analysis was performed by ANOVA.*

4.4.5 STAT signalling in peripheral blood T cells

Given the known changes in pSTAT1 in intestinal T cells, it was considered whether these changes would be reflected in peripheral T cells. This would be critical in determining whether any effect on the STAT signalling in T cells was imprinted at the time of T cell "priming" or was rather a local effect related to the intestinal environment.

4.4.5.1 "Gut-primed" T cells in PBMCs in health and IBD

Blood was taken from IBD patients and controls. PBMCs were isolated and stimulated or not with 40000U/mL T1IFN. Specific cell populations (as described

below) sorted by flow cytometry, before the pSTAT expression was analysed using the Phosflow protocol. In order to determine which T cells were likely primed in the gut, the gut homing integrin β7 was used to differentiate T cell subsets. PBMCs were sorted into either "gut-primed" memory/effector ($CD3^+CD4^+CD45RA^-\beta7^+$) T cells, or non-gut effector ($CD3^+CD4^+CD45RA^-\beta7^-$), or naive ($CD3^+CD4^+CD45RA^+$) T cells. Fewer $\beta7^+$ cells were found across both control and IBD samples (p=0.0006, when all samples considered, data not shown), showing that the "gut primed" T cells were the minority population in this analysis. There were no differences seen between controls and IBD in cell numbers in any of these T cell subsets (see figure 4.7). No differences were detected when the groups were analysed by proportion of total T cells either (data not shown).

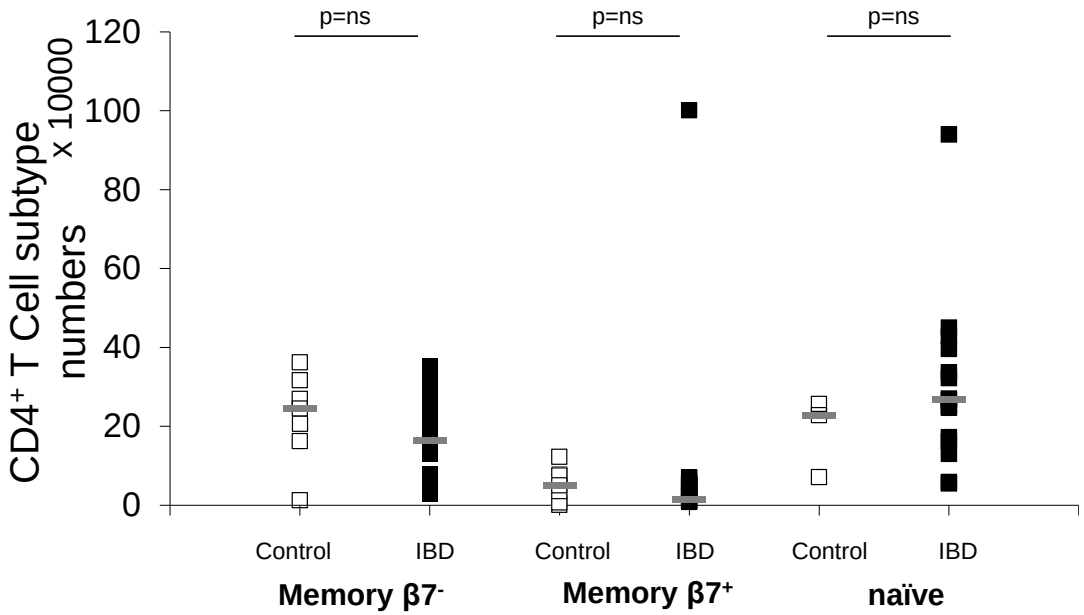

Figure 4.7 "Gut-primed" memory T cells are a minority population, with no significant differences between health and IBD. *PBMCs were isolated from IBD patients (black boxes, n=18) and age-matched controls (white boxes, n=8). Cells were sorted by flow cytometry as follows.* ***β7-****:* $CD3^+CD4^+CD45RA^-\beta7^-$*;* ***β7+****:* $CD3^+CD4^+CD45RA^-\beta7^+$*;* ***naive****:* $CD3^+CD4^+CD45RA^+$*. Statistical analysis by Student's t test.*

4.4.5.2 Responsiveness of PBMCs as measured by phosflow

In the absence of stimulation, expression of pSTAT1 and pSTAT3 was not detectable in any of the populations of peripheral $CD4^+$ T cells examined, ($\beta7^+$ effector/memory, $\beta7^-$ effector/memory or naive cells) from either IBD patients or controls (data not shown). Expression of constitutive pSTAT5 was detectable in a small proportion (0-9%) of T cells in the different populations examined but no differences were detected, either between the different T cell subsets or between health and disease (data not shown).

4.4.5.3 PBMC pSTAT responses to T1IFN in health and IBD

After stimulation of PBMCs with T1IFN, pSTAT1, 3 and 5 could all be detected. While there were no significant differences in pSTAT expression between $\beta7^-$ and $\beta7^+$ effector cells, there was a strong trend towards a lower frequency of pSTAT1 after T1IFN stimulation in the naive T cells when IBD and control samples were combined ($p=0.05$, ANOVA, data not directly shown). There was no difference in the T1IFN stimulated expression of pSTAT1 between IBD samples and age-matched controls in any blood T cell population analysed (figure 4.8)

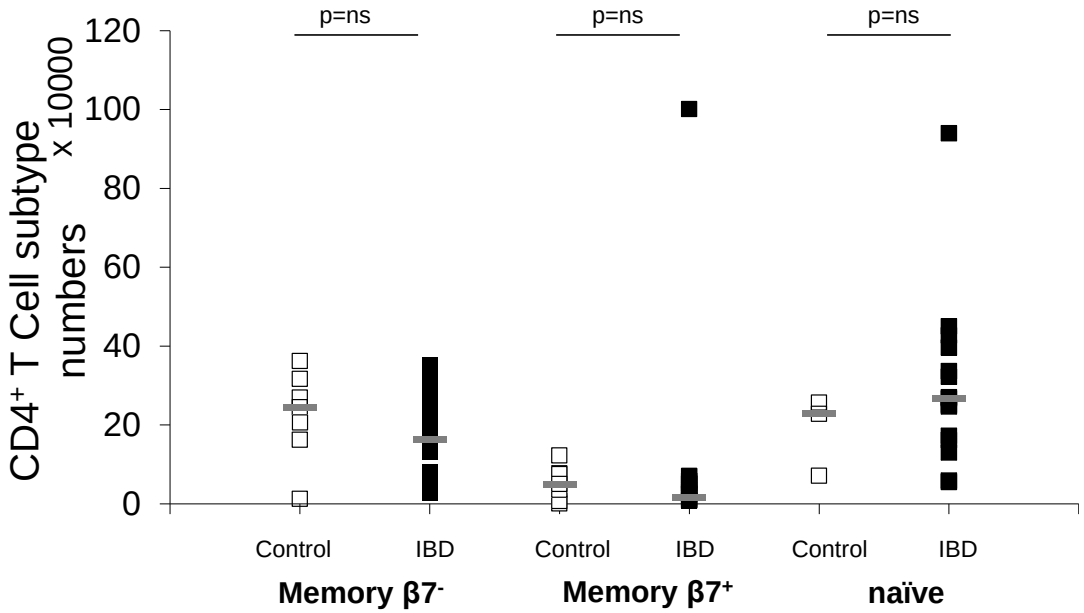

Figure 4.8 pSTAT1 response to T1IFN show no difference in IBD in peripheral T cell subsets*. PBMCs were isolated from IBD patients (n=18) and age matched controls (8). Cells were analysed for pSTAT1 expression after 15 minute stimulation with T1IFN, gating on the following T cell subsets, $CD3^+CD4^+CD45RA^-\beta7^-$ ($\beta7^-$, n=8 controls, n=17 IBD); $CD3^+CD4^+CD45RA^-\beta7^+$ ($\beta7^+$, n=8 controls, n=17 IBD) or $CD3^+CD4^+CD45^+$ (naive, n=5 controls, n=13 IBD). Statistical analyses were performed using Student's t tests.*

4.4.5.4 pSTAT3 and pSTAT5 in peripheral T cell subsets

pSTAT3 was detectable at between 0-25% (median 1.24) in T cells in all subsets after T1IFN stimulation. There were no significant differences seen between T cell subsets or patient groups (data not shown). pSTAT5 was by a high proportion of cells after stimulation (generally 50-60%)., Again, there was no significant difference between groups, although a trend towards decreased pSTAT5 in the naive cells and increased signal in the $\beta7^+$ cells (p=0.11, ANOVA, data not shown).

4.4.6 Mechanisms underlying STAT1 activation in mucosal T cells

4.4.6.1 Tissue culture medium conditioned by IBD biopsies

Given the variety of mechanisms by which STAT1 can be activated, there were many pathways that could potentially be involved in intestinal T cells to account for the IBD-related differences in pSTAT1 expression. In broad terms, one possibility would be that it could have been due to the different cytokine environment that the cells were exposed to during the walk-out process. Alternatively, it may have been inherent in the phenotype of the intestinal T cells. In other words, were there extrinsic factors in the (IBD) mucosa activating the STAT1 pathway, or were there intrinsic factors in the (IBD) intestinal $CD4^+$ T cells that made them more responsive in this pathway.

In order to test whether there were IBD biopsy tissue release factors which could induce pSTAT1 expression during *in vitro* culture, intestinal biopsies from healthy controls were cultured for 24-48 hours in supernatant (conditioned medium) from IBD tissue (n=4) previously shown to contain $CD4^+$ T cells with increased pSTAT1 expression. Overall there was no consistent effect of conditioned medium on the frequency of $pSTAT1^+CD4^+$ T cells in the control biopsies, although there was a trend for an increase in $pSTAT1^+$ frequency in unstimulated cells (figure 4.9).

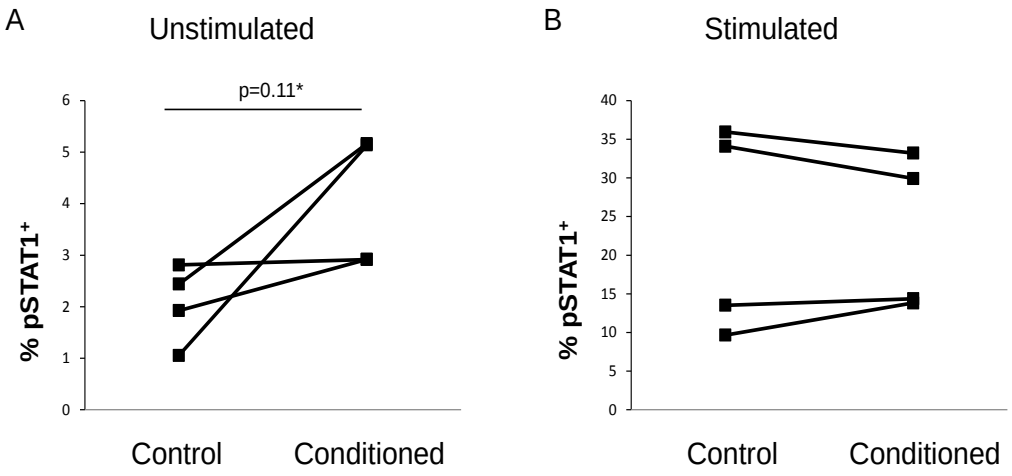

Figure 4.9 Frequency of pSTAT1 expression by control intestinal T cells is not increased by medium conditioned by IBD biopsies. *Control patient biopsies were isolated by the walk-out process as usual (control, n=4) or in culture medium supplemented with supernatant from IBD biopsies known to contain T cells with a high frequency of pSTAT1$^+$ expression (Conditioned). The frequency of pSTAT1$^+$ intestinal CD4$^+$ T cells is shown.* ***A*** *without exogenous T1IFN and* ***B*** *After T1IFN stimulation. *paired t test*

4.4.6.2 Suppressors of Cytokine Signalling (SOCS) Expression

It was then attempted to assess some of the intrinsic T cell factors that may affect responsiveness to STAT activation.

There are several cellular mechanisms underlying the control of STAT signalling. The Suppressors of Cytokine Signalling (SOCS) family of proteins is the most widely described, and thought to be the most important regulators of STAT phosphorylation (Alexander 1999). Pathways are complex and interact, but SOCS1 and SOCS3 are the major regulators of STAT1 activity (reviewed in Palmer and Restifo, 2009). In order to determine whether differential expression of *SOCS* genes could contribute to the differences in pSTAT1 expression, mRNA extracted was extracted from magnetically separated CD3$^+$ sorted intestinal T cells, converted to cDNA and expression of *SOCS1* and *SOCS3* quantified by qRT-PCR in comparison to the housekeeping gene *GAPDH* (see section 2.4). For these experiments, T cells were isolated from LPMC that were obtained by immediate collagenase digestion of tissue, thereby enabling levels of *SOCS* expression to be determined prior to any *in vitro* culture (see chapter 2, section 2.1.5.1).

There were no significant differences seen in either *SOCS1* or *SOCS3* expression between the groups, but there was a trend for a decrease in *SOCS1* expression in intestinal T cells from non-inflamed IBD samples (figure 4.10).

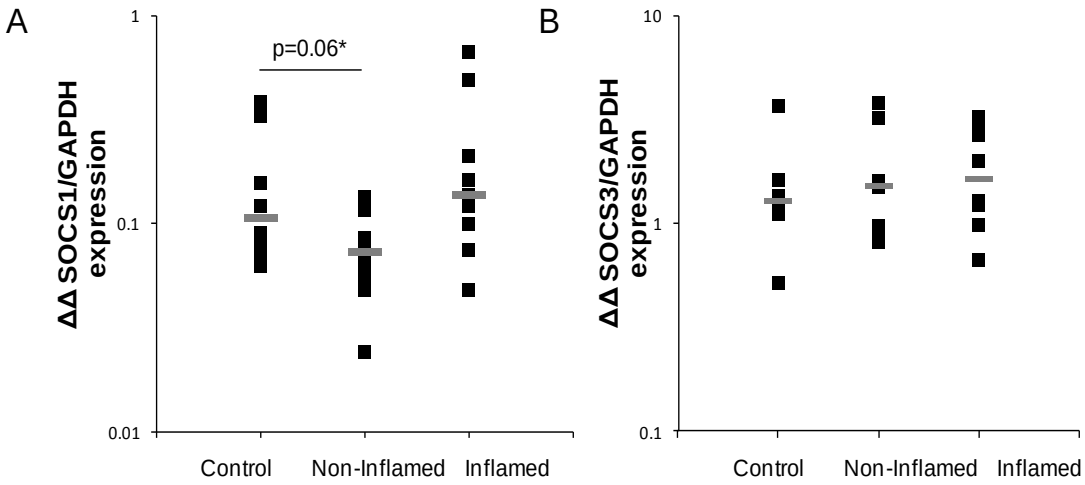

Figure 4.10 SOCS1 expression may be reduced in intestinal T cells from non-inflamed IBD tissue. *Relative expression of SOCS1 (A) and SOCS3 (B) mRNA compared to GAPDH housekeeping gene in $CD3^+$ LPMCs. Cells were purified by magnetic bead selection after collagenase processing, RNA was extracted, converted to cDNA, and SOCS1 and SOCS3 expression was determined by q-RT-PCR as described. n=8 controls, n=9 IBD samples. Grey bars indicate median values. *ANOVA*

4.4.6.3 Functional significance of altered pSTAT1 signalling

Altered pSTAT1 levels in lamina propria $CD4^+$ T cells of IBD patients would have downstream consequences on cytokine production. This, however, is difficult to measure directly as traditional methods of detecting cytokines are not compatible with the Phosflow protocol. The stimulation of T cells required to detect T cell cytokine production using flow cytometry would also affect pSTAT levels, therefore not allowing a simultaneous comparison. Attempts to detect cytokine levels without stimulation, using only Golgi-blocking agents, were unsuccessful (data not shown). It was therefore attempted to selectively block the STAT1 pathway, so the downstream effects of activation of this pathway could be measured by more traditional methods (for example, by cytokine production of the T cells). This was hoped to inform the functional significance of the high pSTAT1 levels in non-inflamed IBD samples. Therefore pSTAT1 blockade was tested using the known antagonist, Fludarabine (Frank, 1999).

4.4.6.3.1 Fludarabine as a selective STAT1 inhibitor

There are no known specific and selective inhibitors of STAT1 activation. However, Fludarabine, a cancer drug which interferes with DNA replication, has also been shown to be a STAT1-specific inhibitor (Frank et al., 1999). That is, it inhibits phosphorylation of STAT1 and reduces mRNA transcripts of STAT1 and not other STAT proteins.

Fludarabine was added to the walk out process in order to completely or partially neutralise STAT1 phosphorylation. Unfortunately there appeared to be no significant, specific or reproducible effect of the fludarabine treatment of pSTAT1 levels (figure 4.11). Given this may have been due to the half-life of the drug, it was also added during the short (15 minute) stimulation period. While this did slightly reduce the response of pSTAT1, it had the same effect on pSTAT3 and 5, suggesting a non-specific effect (data not shown). Unfortunately, there were no other commercially available pSTAT1 inhibitors at the time of the experiments.

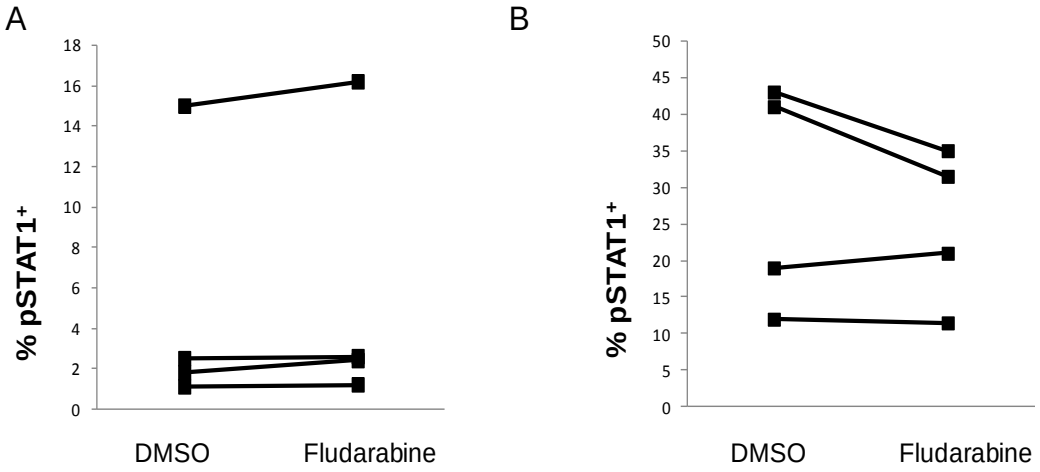

Figure 4.11 Fludarabine does not selectively inhibit pSTAT1 in intestinal CD4+ T cells. *Fludarabine was added or not (DMSO vehicle control) to the cells during the walk-out period and then processed as per the Phosflow protocol (n=4). pSTAT1+ expression in gated CD4+ intestinal T cells are shown without T1IFN (A) and with brief stimulation (B)*

Given the inability to selectively inhibit STAT1 activation, different mechanisms of exploring the functional significance of the activation of this pathway were explored.

In particular, T1IFN was considered a likely candidate to be driving STAT1 activation, and this is discussed in detail below.

4.4.7 Endogenous T1IFN as a promoter of STAT activation in intestinal T cells

There are only a small number of cytokines that are known to signal via STAT1 (reviewed in Stark and Darnell, 2012). Type I and Type II Interferon (T1IFN and T2IFN) are prototypical inducers of signalling via STAT1 (Gough et al., 2010). However, the more recently recognised Type III IFN (T3IFN), as well as IL-28 (Dumoutier et al., 2004) and IL-26 (Hör et al., 2004), can also induce phosphorylation of STAT1.

4.4.7.1 The effect of IFNβ on STAT1 expression and phosphorylation

The data early in this chapter demonstrated an increased frequency of pSTAT1 expression in intestinal T cells from non-inflamed IBD mucosa, both with and without T1IFN stimulation (figure 4.1). It was felt likely that this pSTAT1 was driven constitutionally by T1IFN but there were other candidates, including T2IFN (reviewed by Stark and Darnell, 2012). Therefore experiments were conducted to test whether STAT1 activation was predominantly driven by T1IFN and in particular by IFNβ.

In order to test whether STAT1 activation in the intestinal T cells was driven by endogenous IFNβ, intestinal biopsies were “walked-out” in the presence of neutralising anti-IFNβ antibody or its isotype-matched control. It was decided to use anti-IFNβ for several reasons. Firstly, it was attempted to use anti-IFNAR neutralising antibodies but the antibodies available were polyclonal and the isotype controls were found to be not suitable. Secondly, of the T1IFN agents, there was available a suitable monoclonal antibody. Finally, the literature appeared to suggest

that IFNβ was the coordinator of T1IFN responses and also was the isoform detected in the human skin (Gough et al., 2012; Teles et al., 2013). The cells were then processed as per the Phosflow protocol described in detail in chapters 2 and 3. The results are shown in figure 4.12, and showed a significant reduction in pSTAT1 expression in cells across all sample types, cultured in the presence of anti-IFNβ (figure 4.12A). When analysed separately (healthy controls, non-inflamed IBD or inflamed IBD samples), no individual group reached statistical significance although samples from healthy controls showed a strong trend (p=0.051) Interestingly, statistical significance was increased if the inflamed samples were excluded (not shown) suggesting that non-IFNβ–dependent effects in these inflamed samples may partially obscure effects in the overall dataset which are attributable to IFNβ. Similarly, levels of total STAT1 (U-STAT1) was also measured (figure 4.12B) and when samples from all patient groups were combined in the analysis, IFNβ neutralisation led to decreased U-STAT1 expression by a small but highly statistically significant degree (p=0.008).

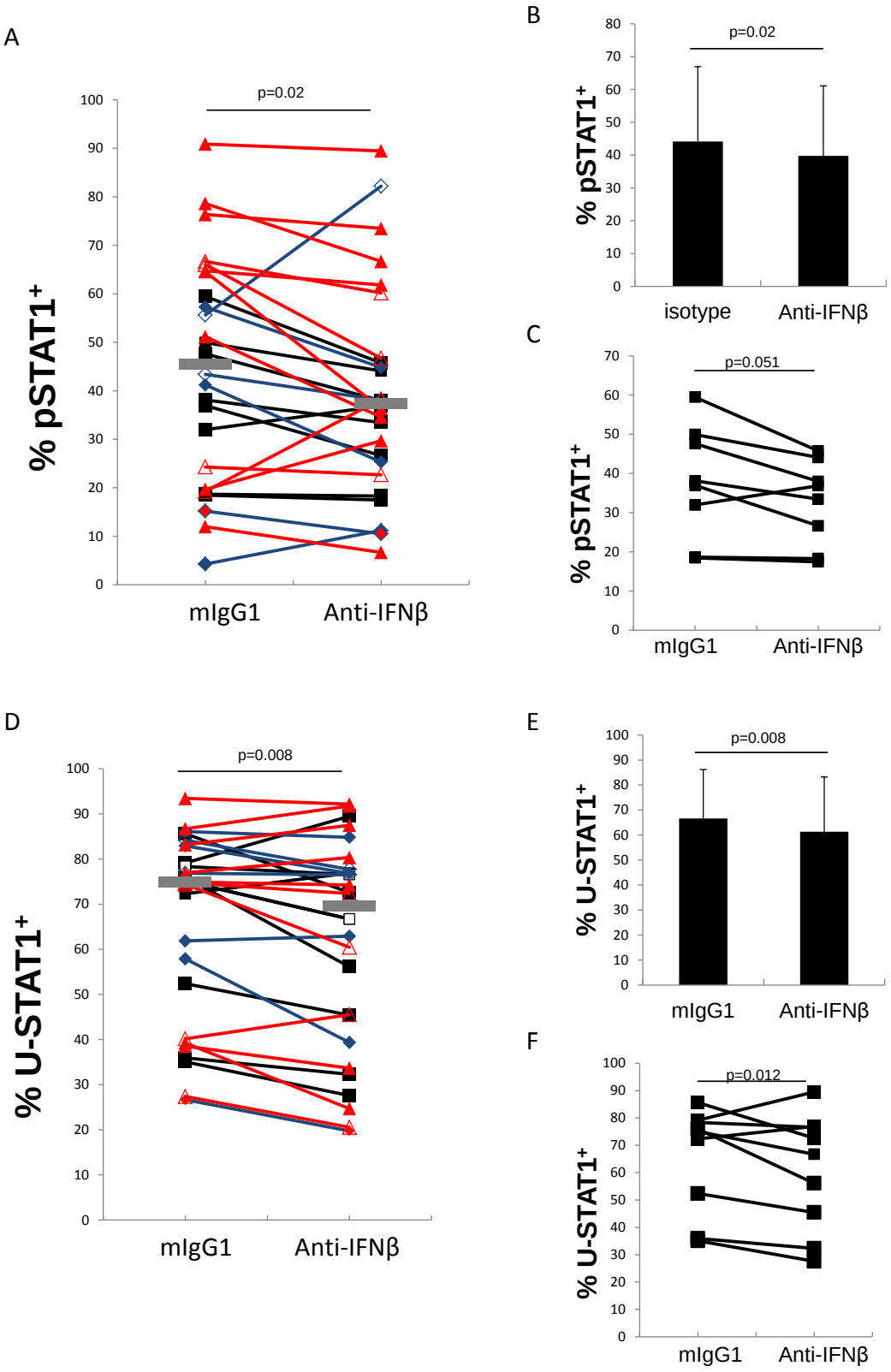

Figure 4.12 Both pSTAT1 and U-STAT1 expression are reduced in intestinal CD4⁺ T cells in the presence of neutralising anti-IFNβ. *LPMCs were walked-out as previously described in the presence of neutralising anti-IFNβ antibody or isotype-matched control antibody (mIgG1). Cells were then harvested after 48 hours and analysed by flow cytometry. A pSTAT1+ frequency in CD4+ intestinal T cells from all sample types B Summary of data in A, showing mean and standard*

deviation C Control samples only from A shown. D Total STAT1 (U-STAT1) frequency in intestinal CD4$^+$ T cells in all sample types. E Summary of data from D, showing mean and standard deviation. F Control samples only from D. All results are gated on light scatter and CD4$^+$. Black boxes and lines: paired control samples, n=8, Blue diamonds and lines: paired non-inflamed IBD samples, n=7 (filled blue diamonds Crohn's, empty UC). Red triangles and lines: inflamed samples, n=11 (filled red triangles Crohn's, empty UC). Grey lines: median values. Statistical analysis by signed rank in A, B, C and by paired t test in D,E and F.

4.4.7.2 The effect of Type II IFN neutralisation on STAT1 activation

In order to further explore the hypothesis that the phosphorylation of STAT1 the intestinal T cells was driven primarily by T1IFN/IFNβ, the effect of neutralising the other main inducer of STAT1 phosphorylation, IFNγ, was examined. Although there was a significant (p=0.03, n=3) reduction in total STAT1 and a trend towards decreased expression of the Th1 associated transcription factor T-bet (data not shown), the frequency of pSTAT1$^+$ intestinal CD4+ T cells was not affected by IFNγ neutralisation during walk-out (n=3, figure 4.13). There were no attempts to neutralise the other known activators of STAT1, namely Type III IFN, IL-26 or IL-28 (Dumoutier et al., 2004; Gough et al., 2010; Hör et al., 2004), and so any contribution of these cytokines remains unknown.

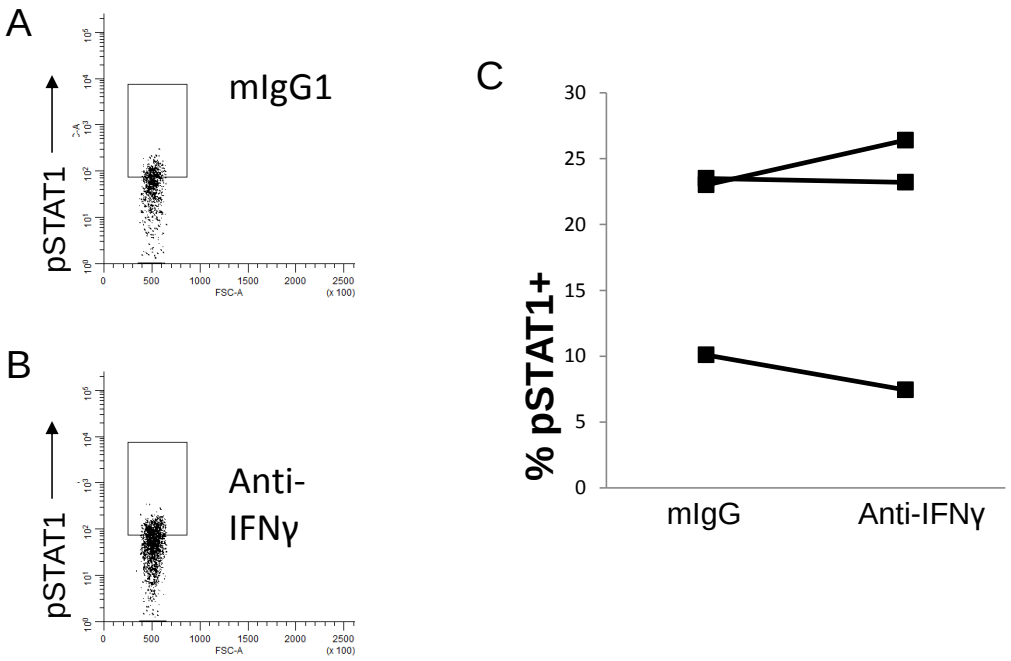

Figure 4.13 Neutralisation with Anti-IFNγ has no effect on pSTAT1 expression on intestinal CD4+ T cells*. LPMCs were walked-out as previously described in the presence of neutralising anti-IFNγ antibody or isotype-matched control antibody (mIgG1). Cells were then harvested after 48 hours and analysed by flow cytometry. A and B are example flow cytometry plots. Gating was on $CD3^{+}CD4^{+}$ lymphocytes C Combined data of 3 experiments.*

4.4.8 Direct evidence of constitutive T1IFN in the human intestine

The preceding data suggests an important role of endogenous IFNβ in regulating intestinal T cells in health. However, it is not known whether IFNβ (or indeed any other T1IFN subtype) can be directly detected in the human intestine in health or disease. In order to detect the *ex vivo* presence of T1IFN, sections were stained using antibodies by Immunohistochemistry (IHC). To optimise antigen detection, the authors of a recent publication were contacted (Teles et al., 2013). This publication showed direct evidence of both IFNβ in human skin for the first time, by IHC. Staining of IFNβ control colon tissue with anti-IFNβ, but not an isotype-matched control antibody, was detected (figure 4.14) suggesting the presence of endogenous IFNβ.

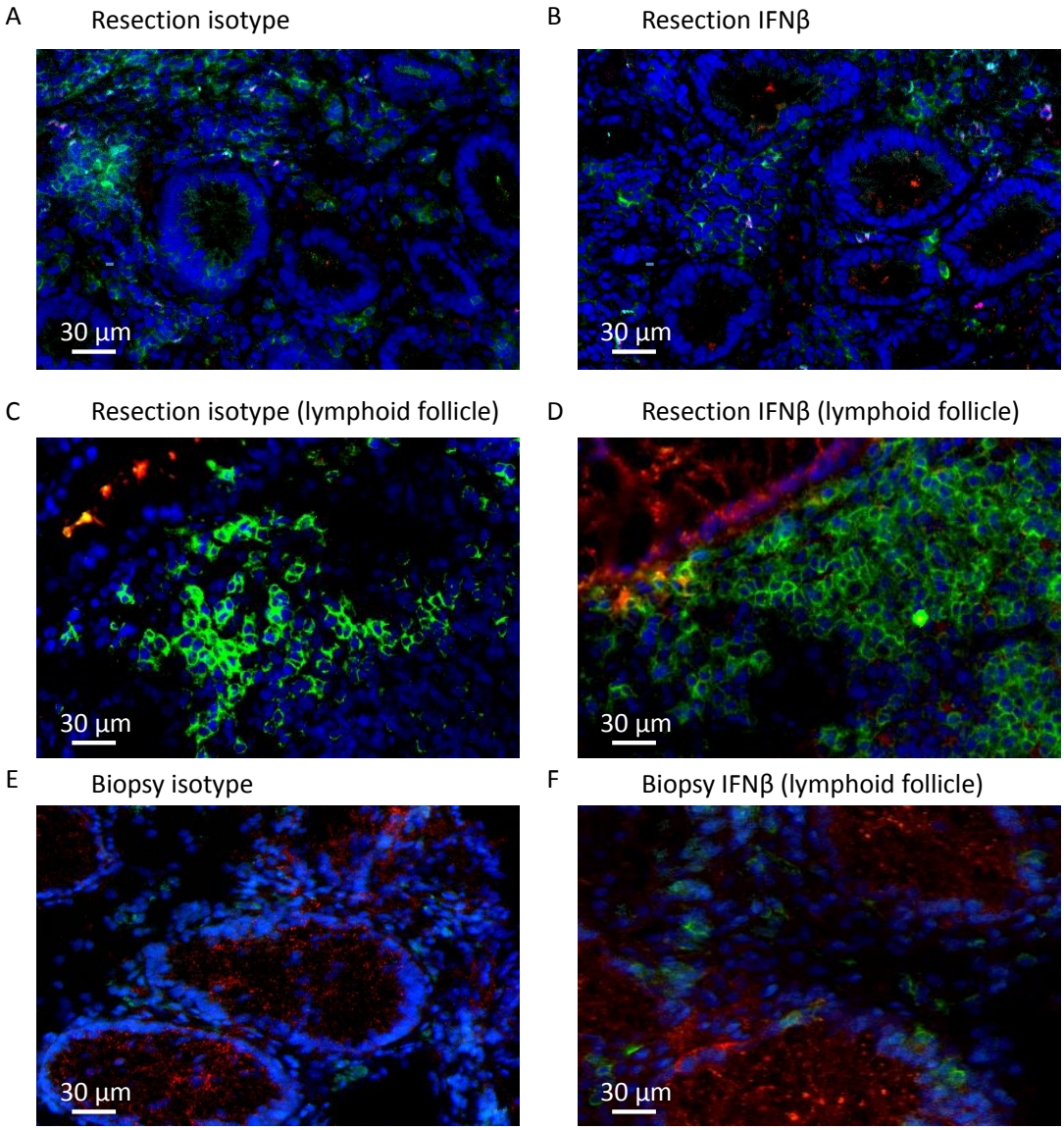

Figure 4.14. IFNβ is detectable in control colonic human lamina propria and is closely associated with T cells. *Fresh frozen sections prepared from control colonic resection or biopsy tissue and prepared for immunohistochemistry. All panels show CD3 labelling in green (AF488). A, C and E: isotype control staining in red (AF555) (red). B, D and F: IFNβ staining in as red (AF555). C and D include a colonic lymphoid follicle. A-D are from surgical specimens, E-F from biopsy specimens. A-D show colonic lamina propria, with a lymphoid follicle evident in C & D. All sections are from control donors. Representative of 5 independent experiments.*

These sections demonstrate the presence of IFNβ in the lamina propria, with only very occasional co-staining with intestinal T cells. It is of note that in all samples there is significant "red" (AF555) signal within the crypts (represented largely as circular structures that contain the colonic epithelial monolayer and lumen). While it

is conceivable that some of this AF555 signal represents specific staining of IFNβ, it is more likely that this is non-specific staining. Indeed, unlike staining in the lamina propria, there was some staining with mouse IgG as well as anti- IFNβ in the crypts, although this was not consistently observed. Also, there was occasional co-staining of CD3 (AF488 – green) with IFNβ or mIgG, combining to yellow, in the crypts. It is highly unlikely that $CD3^+$ cells would be present in the middle of crypts (ie inside the intestinal lumen), supporting that this is non-specific staining.

It is also of note that there is very rare co-staining of IFNβ and CD3 (combined yellow) in the lamina propria. This suggests that while T cells may be one possible source of IFNβ, they are unlikely to be the primary producers of IFNβ in the human colon.

IFNβ was also detectable in samples from IBD patients. There were no obvious qualitative differences between these and control samplers, or between inflamed and non-inflamed samples, although the sample size was small (figure 4.15).

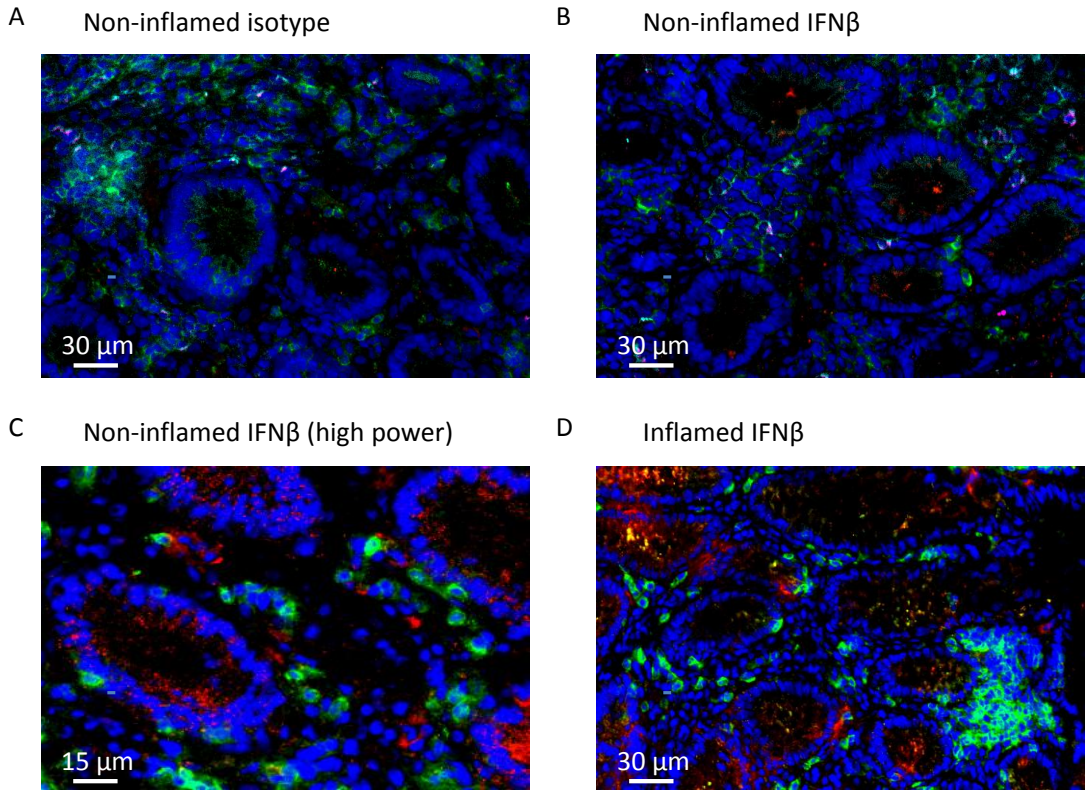

Figure 4.15 IFNβ was detectable in the lamina propria of both non-inflamed and inflamed IBD colon. *Fresh frozen sections prepared from colonic resection*

tissue and prepared for immunohistochemistry as described. All panels show CD3 labelling in green(AF488). . A: isotype control staining (mIgG1 – AF555, red) from non-inflamed IBD sample. B, C and D: IFNβ staining in red (AF555) B Non-inflamed sample (same sample as A) C High-power non-inflamed IBD sample. D Inflamed IBD sample. Representative of 3 independent experiments for both inflamed and non-inflamed samples.

4.4.8.1 Detection of constitutive Interferon Stimulated Gene Signatures in intestinal T cells and their response to T1IFN stimulation in vitro.

In order to look for further evidence of endogenous T1IFN, a more traditional method of detection of interferon signature was used - the measurement of the mRNA expression of Interferon Stimulated Genes (ISGs). ISGs are a large family of genes whose expression is increased by stimulation with T1IFN (Hertzog et al., 2011). Detection of the presence of ISGs without exogenous T1IFN has been shown as evidence of constitutive T1IFN signalling (Cho and Kelsall, 2014).

Cells were isolated from the intestine, viable T cells sorted to purity by flow cytometry, and the RNA was extracted and reversed transcribed. Expression of two Interferon Stimulated Genes (ISGs) were then measured using qRT-PCR. The two ISGs selected, were MxA and 2’5’ OAS, which are commonly used in T1IFN research (Coelho et al., 2007; Wang et al., 2013). The cells were isolated by walk out with or without the addition of T1IFN, enabling the quantitation of ISGs at “baseline”, giving an approximation of constitutive T1IFN signalling.

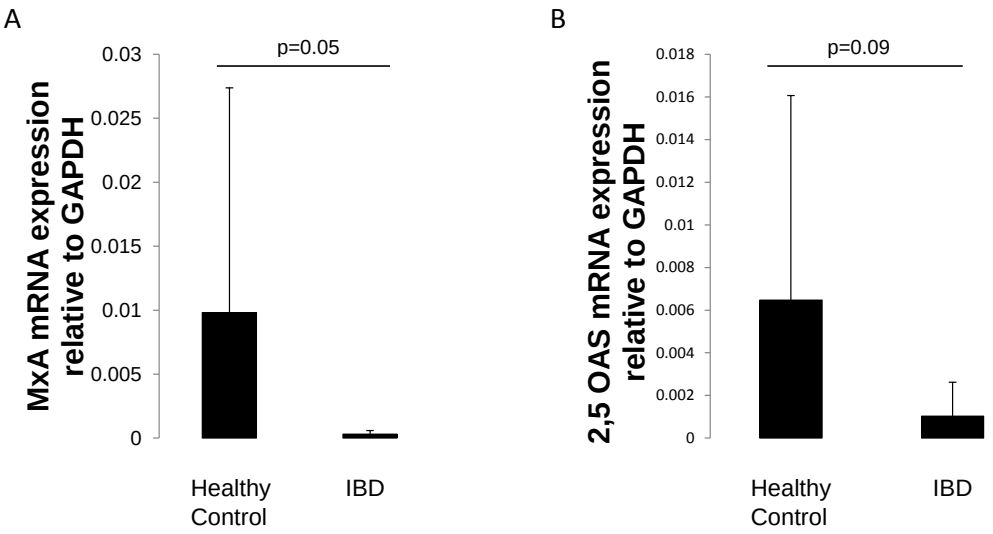

Figure 4.16 Intestinal T cells have a trend to higher baseline ISG expression in controls compared to IBD patients. *Cells were walked-out and T cells were sorted by flow cytometry prior to RNA extraction. A and B mean (+standard deviation) MxA (A) and 2,5 OAS (B) mRNA expression compared to GAPDH housekeeping gene (n=10) healthy controls, solid squares, n=12 IBD samples, white squares). Statistical analysis by Signed Rank tests.*

These results (figure 4.16) confirm the 'footprint' of constitutive T1IFN signalling but the presence of ISGs in unstimulated *ex vivo* intestinal T cells. Of note, there was a strong trend in both MxA and 2'5' OAS mRNA for increased expression in healthy control samples compared to (non-inflamed) IBD samples. This suggests that healthy intestinal T cells are either exposed to more endogenous IFNβ signalling than those in IBD or that intestinal T cells in IBD are relatively refractory to similar signals.

4.5 Discussion

The work described in this chapter demonstrated an increased frequency of pSTAT1$^+$ intestinal CD4$^+$ T cells in IBD (figure 4.1). Frequency of pSTAT1$^+$ T cells was highest in cells obtained from histologically normal areas of mucosa from IBD patients. Expression of pSTAT1 was increased by stimulation with T1IFN *in vitro* in all groups but remained highest in cells from non-inflamed IBD tissue. pSTAT1 did not associate with T-bet expression suggesting that pSTAT1 is not a marker of TH1 differentiation in intestinal CD4$^+$ T cells. Increased expression of pSTAT1 by

intestinal CD4 T cells appeared to be specific for this STAT and for cells in this compartment, in that expression of pSTAT3 and pSTAT5 did not differ in IBD and expression of pSTAT1 by blood $CD4^+$ T cells was similar in IBD patients and controls.

There was no strong evidence that IBD tissue released factors that increased STAT1 phosphorylation in intestinal T cells. There was a trend for reduced expression of *SOCS1* in T cells from non-inflamed IBD tissue which may suggest increase sensitivity to STAT1 activation signals in these cells. There was evidence that IFNβ was at least partially responsible for STAT1 signalling in intestinal T cells. The presence of endogenous IFNβ was detected in the human colon by IHC and also be the presence of ISG expression in intestinal T cells.

4.5.1 $pSTAT1^+$ $CD4^+$ T cells are more frequent in non-inflamed IBD mucosa compared to inflamed and healthy gut

The first significant finding in this chapter was the specific increase in frequency of pSTAT1 expression in $CD4^+$ intestinal T cells from non-inflamed areas of mucosa of IBD patients. Given that the cell isolation period occurs over 48 hours, it seems possible that the pSTAT1 signal, prior to exogenous T1IFN, represented an effect of the cell isolation method, rather than a true representation of the *in vivo* state of the T cells. Also, earlier experiments (discussed in chapter 3, section 3.4.4), using collagenase for extraction of lamina propria cells, did not show constitutive pSTAT expression. However, T cells isolated by collagenase did not respond to *in vitro* T1IFN stimulation either, as measured by Phosflow, so these results must be interpreted with caution. It should also be noted that the experiments using supernatants of IBD samples to attempt to induce pSTAT1 expression in control samples were not completely successful (figure 4.9), suggesting that the walk-out method does not universally induce increased pSTAT expression. Whatever the

driver of the pSTAT1 expression in the T cells, the differences between patient and control groups remain, and warrant further investigation.

The increased pSTAT1 frequency from T cells of IBD patients was consistent with some of the previous literature. Using Western blots of extracts from whole biopsies, a relatively early study (Schreiber et al., 2002) showed increased STAT1 in inflamed tissue from UC and CD patients compared to controls, which reduced after steroid treatment in the UC patients. However, in this paper pSTAT1 was identified on neutrophils and not on T cells by immunofluorescence (IF). The variance from this chapter could be explained by various differences in experimental approach. Firstly, there is a lower sensitivity of IF compared with Phosflow. Secondly, the omission from the Schreiber paper of non-inflamed biopsies, where T cell expression of pSTAT1 was more frequent, may have missed the more significant finding. Finally, the different techniques involved in sample preparation could have profound effects on activation of STAT1.

Similarly to the Schreiber paper, our differences in STAT1 signalling were common to both UC and CD. The findings were analysed as separate patient groups and remained statistically significant when comparing control samples with non-inflamed CD or UC samples. This suggests that the difference between health and non-inflamed IBD mucosa is either related to a final common pathway of inflammation in the gut, or that (given that the pSTAT1 was most frequent in non-inflamed areas), the changes may reflect the adaptive immune system's attempt to maintain intestinal homeostasis (ie as a pro-regulatory pathway). It is likely that the differences between non-inflamed and inflamed samples did not reach statistical significance only due to lack of sample numbers, as there were strong trends to replicate the pSTAT1 differences found when IBD samples were combined.

In the largest study of STAT signalling in IBD, multiple STATs were examined in IBD using flow cytometry in $CD4^+$ LPMCs (a population enriched for, but not exclusively, $CD4^+$ T cells), STAT1 was increased, but not significantly, and pSTAT1 was shown

in the lamina propria and epithelium by IF (Mudter et al., 2005). These findings are consistent with this chapter's results, although it is unclear whether the Mudter study samples were from inflamed or non-inflamed sites. If they were, as is common, from inflamed sites, it may explain why the flow cytometry results were not significant. However, in an older paper, resections from CD and UCs showed no increase in pSTAT1 in whole specimens, compared to infectious colitis and controls (Suzuki 2001). However, these were whole tissue specimens and not T cell specific, cells were prepared differently, and the controls may not have been as well matched. It should also be noted that the large majority of samples in these experiments were from paediatric patients (or controls), or young adults. These patients would likely have fewer co-morbidities, be earlier in the course of their disease, and be on less immunomodulation therapy than those from adult populations.

4.5.1.1 Phosphorylation of pSTAT1 is greatest in non-inflamed IBD tissue.

Strikingly, pSTAT1 phosphorylation was significantly greater in $CD4^+$ T cells from non-inflamed tissue from IBD patients than in cells from either healthy control tissue or inflamed IBD mucosa. Tissue was regarded as non-inflamed if it was macroscopically and histologically normal in appearance. The biopsies used in these studies categorised histologically on the basis of a blinded evaluation, by a consultant histopathologist, of adjacent biopsies taken for clinical purposes (see Chapter 2).

The vast majority of the research in the mucosal immunology of human IBD has focussed on samples from macroscopically inflamed tissue. In this context, underlying immunological abnormalities may be masked by the predominant inflammatory profile. Conversely, altered immunological pathways in non-inflamed tissue may be free of this confounding issue and inform about underlying immune dysregulation. It is therefore unclear as to whether many of the published

immunological changes in Crohn's or UC occur in non-inflamed intestine. This may explain differences found here compared with the literature. Once inflammation is established, it is reasonable to assume that it is more difficult to detect subtle abnormalities in, for example, T cell signalling, that could either predispose to or protect from pathology.

The prospect of the increased pSTAT1 frequency in intestinal T cells being a pro-regulatory signal is superficially counter-intuitive. STAT1 is traditionally seen as part of a TH1 differentiation signalling pathway (see chapter 1 and (Afkarian et al., 2002). However, in murine EAE, STAT1 is necessary for the function of $CD4^+CD25^+$ regulatory T cells and supports a regulatory phenotype (Nishibori et al., 2004). As will be discussed in further detail in chapter 5, T1IFN signalling via STAT1 in T cells can inhibit IFNγ secretion (Nguyen et al., 2000). It was therefore our hypothesis that this increase STAT1 signalling was pro-regulatory, thus contributing to the prevention of inflammation in these areas of intestine.

4.5.1.2 Differences in STAT1 phosphorylation do not fully correlate with total levels of STAT1

Unphosphorylated STAT1 (U-STAT1, or total STAT1) was measured simultaneously with pSTAT1 and was detectable in the majority of T cells. There was an increase in U-STAT1 in non-inflamed IBD samples compared to controls but no other groups differed (figure 4.1 C). Given that in certain samples U-STAT1 expression was close to 100%, the U-STAT1 mean fluorescence intensity (MFI) was also compared. It should be noted that MFI was also analysed in all Phosflow experiments and differences were consistent with expression measured as percentage $pSTAT^+$ (data not shown). Levels of U-STAT1 as assessed by MFI did not differ significantly between samples from control or IBD (inflamed or non-inflamed) samples. The general lack of increase in U-STAT1, particularly from inflamed tissue, is in contrast to the previous IBD literature on STAT1 (Mudter et al.,

2005; Schreiber et al., 2002) described earlier, but the differences, as with pSTAT1, could be explained by the use of the walk-out method for cell isolation, or other experimental differences, such as different cell populations. For example, the Schreiber study uses whole tissue sections, and the Mudter paper uses a lamina propria cell population enriched for $CD4^+$ cells, not a specific $CD4^+$ T cell population identified by flow cytometry. Another possibility for the difference in findings is that during walk-out culture, T cells are exposed to factors that induce phosphorylation of the ubiquitously expressed STAT1 and that this pathway is more active in cultures if IBD tissue. It would be expected that this signal would itself lead to increased STAT1 expression as there is a known positive feedback loop in the activation of STAT1 (Gil et al., 2006). Thus, overall this chapter's results are consistent with persistent activation of the STAT1 signalling pathway leading to upregulation of total STAT1.

4.5.1.3 Phosphorylation of STAT1 is not increased in intestinal T cells from coeliac disease patients

To assess whether an increased frequency of $pSTAT1^+CD4^+$ T cells is a feature of other types of intestinal inflammation, cells from duodenal biopsies taken from coeliac disease patients were also examined. In contrast to the findings in IBD, there was no consistent increase in pSTAT1 frequency in the intestinal $CD4^+$ T cells from active coeliac disease patients (fig 4.3). This observation is surprising since pSTAT1 has been detected in duodenal samples by various techniques in published work on celiac disease (Di Sabatino et al., 2007; Mazzarella et al., 2003). Again, however, these experiments were done on whole tissue samples, not isolated T cells, and it is possible that in these samples pSTAT1 is present in other cells types. For example, in whole tissue specimens the pSTAT1 could reflect changes in the epithelium, or indeed any other cell type present. However, in this chapter's work the numbers of samples taken were quite small, so it is also possible that any

differences were missed. Finally, the control group in Mazzarella's paper were different from in our group, in that they had duodenal inflammation (in these results the controls were all histologically normal), which also could explain different findings. Overall, considering this chapter's results in T cell pSTAT1, one could infer that the pSTAT1 changes in IBD are disease specific. However, another possibility is that the differences are related to the site of the gut, and it is conceivable that the function of T cells in the duodenum would be different than those in the distal bowel. That is, the importance of increased STAT1 signalling may be context as well as cell-type specific (Stark and Darnell, 2012).

4.5.2 Expression of pSTAT3 and pSTAT5 by intestinal $CD4^+$ T cells does not differ between control and IBD samples

One of the more surprising findings from this chapter's experiments was the lack of increase in pSTAT3 in the intestinal T cells from IBD patients. This appears to be in contrast to many published studies showing increased pSTAT3 or STAT3 in the intestine in IBD, more than any other STAT protein (Carey et al., 2008; Lovato et al., 2003; Mudter et al., 2005; Schreiber et al., 2002; Suzuki et al., 2001). Once again this earlier data often comes from whole inflamed biopsies (Carey et al., 2008; Lovato et al., 2003; Schreiber et al., 2002; Suzuki et al., 2001). The Mudter study, however, did investigate $CD4^+$ enriched LPMCs; and STAT3 and pSTAT3 were both increased in frequency, in both Crohn's and UC patients compared to controls, albeit from presumably inflamed samples. The results for pSTAT3 in this chapter do show a strong trend for increase in pSTAT3 in IBD, but the overall frequencies were very low. It is unclear why the frequencies were so low, particularly after stimulation with T1IFN. It may be related to the medium used in which the cells are isolated. It is known that aromatic hydrocarbons in certain tissue culture media promote TH17 differentiation, which is STAT3 dependent (Veldhoen et al., 2009; Yang et al., 2011). The medium used in the laboratory (RPMI, Dutch modification, see appendix

I) contains only very low levels of aromatic hydrocarbons, which would then explain detection of low levels of IL-17 and related proteins. This would also explain the generally very low levels of RORγt expression seen in the chapter, and generally TH17 cytokines in our laboratory. It is also possible that the low pSTAT3 expression is related to the higher pSTAT1 frequency, as these two STAT proteins have been thought to interfere with transcription and/or phosphorylation of each other (reviewed in Stephanou and Latchman, 2005).

In contrast, pSTAT5 was detected at both constitutive levels and after T1IFN stimulation. There were no differences in the frequency of $pSTAT5^+$ T cells between control and IBD samples, and this is consistent with the literature (Lovato et al., 2003; Suzuki et al., 2001), despite the differing methodologies. IL-2 signalling via STAT5 is an important survival signal for T cells (Moriggl et al., 1999a) so it is unsurprising to find it ubiquitous in the T cell population at a constitutive level.

4.5.3 Expression of pSTAT1 is not confined to intestinal T cells with a TH1 phenotype.

T-bet expression was increased in the IBD samples compared with control samples (figure 4.5), consistent with previous studies (Matsuoka et al., 2004; Neurath et al., 2002). Interestingly the frequency of $T\text{-}bet^+CD4^+$T cells was similarly increased in both non-inflamed and inflamed IBD tissue (figure 4.5). However, as the frequency of $T\text{-}bet^+CD4^+$ T cells was high in both IBD sample groups, it may be that they could not be increased further with inflammation, at least by this detection method.

More importantly in this section, it was demonstrated that pSTAT1 expression did not strongly associate with T-bet expression (fig 4.5C). This suggests that the increased pSTAT1 levels from IBD samples do not simply reflect an increased TH1 cell phenotype.

4.5.3.1 FoxP3 expression in intestinal $CD4^+$ T cells

No differences were observed in the expression of FoxP3 (4.5B). It has been reported that there is an increase in Treg cells from inflamed compared to non-inflamed mucosa in IBD patients (Maul et al., 2005). However, this paper reported analysis of only a small number of patients, and used a different form of cell isolation and analytical approach to those used in this thesis. While undoubtedly FoxP3 expression is crucial in the regulation of autoimmunity, its expression in humans may also be related to cell activation, and is therefore not a robust marker of human regulatory T cells (Allan et al., 2007; Wang et al., 2007).

The frequency of $FoxP3^+$ intestinal $CD4^+$ T cells in this chapter did show a trend to being lower in control samples (fig 4.5B). The number of samples was quite small, due to technical aspects of measuring FoxP3 simultaneously with pSTATs in samples with relatively low cell numbers. While it is possible that with increased sample numbers a statistically significant increase in IBD samples may have been seen, there appeared to be no difference between inflamed and non-inflamed IBD sample groups. This is a different pattern than the increased $pSTAT1^+$ frequency.

4.5.3.2 CD69 expression in IBD samples

Given the known association with CD69, a marker or T cell activation (Testi et al., 1989), and expression of pSTAT1 (see chapter 3, figure 3.11), the expression of CD69 in intestinal $CD4^+$ T cells may have been expected to follow the same pattern in IBD as that of pSTAT1. As discussed in chapter 3, the high CD69 expression was thought to be possibly related to activation during cell egress in the walk-out process. However, as was also mentioned in chapter 3, a recent study on human intestinal T cells showed high CD69 expression when cells were isolated by collagenase (Sathaliyawala et al., 2013), suggesting that the CD69 expression measured may reflect an *in vivo* process.

The pattern of CD69 expression, however, did not mirror that of pSTAT1 expression in the different patient groups (figure 4.6). There was a non-significant trend for a reduction in CD69 expression in T cells from inflamed areas. As one would expect more, rather than less, T cell activation in areas of inflammation, this result is perhaps surprising. It may be that the relative loss of CD69 expression (and perhaps pSTAT1 expression) represents a degree of T cell exhaustion in this group. Certainly there was more cell death seen in the inflamed samples after walk-out compared to either non-inflamed or controls, as measured by light scatter and/or viability dye staining (data not shown). Overall, as the pattern of CD69 and pSTAT1 expression differs, this suggests that there must be some differences in the processes that drive their expression in IBD.

4.5.4 STAT signalling in peripheral blood T cells

In order to determine whether the differences in STAT1 signalling in intestinal T cells were specific to the mucosal environment, similar experiments were designed to look at the peripheral T cell population. If, during the priming of intestinal T cells, there was some imprinting of a disease or regulatory phenotype via STAT1 reactivity, then it was conceivable that this would be detectable in the peripheral blood. To detect T cells that have been primed in the gut, memory T cells with the integrin marker β7 were chosen as these could be considered “gut-homing” T cells (Stefanich et al., 2011). After several experiments, it was clear that the harsh permeabilisation with methanol used in Phosflow was not compatible with maintaining β7 expression at detectable levels (data not shown). Therefore the PBMCs were flow sorted to purity according to β7 expression and memory phenotype as described in Chapter 2, and then pSTAT1 (or other pSTATs) expression was determined by flow cytometry.

There were no difference seen between T cell subsets as measured between control samples and IBD. The only significant difference seen in the peripheral blood was that naive T cells were less responsive than memory cells (either $\beta7^+$ or $\beta7^-$) to T1IFN, in the increase in any pSTAT. It is known that memory T cells have increased responsiveness to antigen and other stimulation compared to naive T cells (reviewed in Berard and Tough, 2002). However, this difference appears to depend on the signalling pathway, with increased pSTAT1 in memory T cells compared to naive with T1IFN stimulation, but decreased pSTAT1 with IFNγ stimulation (Marino et al., 2006). This is consistent with the results in this chapter, where the stimulus was T1IFN.

Unlike the intestinal T cells, there was no constitutive expression of pSTAT1 detectable in the PBMCs (data not shown). This may be due to the isolation methods, but may also reflect the more active cytokine milieu that intestinal T cells experience, whereas in the peripheral blood there is minimal ongoing pathway activation. pSTAT1 was detected in peripheral $CD4^+$ T cells after T1IFN stimulation, but there was no significant difference between IBD samples and controls, in any of the T cell groups. This would suggest that the differences in pSTAT1 frequency in intestinal T cells are specific to the environment that they are in, and perhaps reflect the plasticity of phenotype that is increasingly recognised in the literature.

4.5.5 Mechanisms underlying STAT1 activation in mucosal T cells

Given that the changes in pSTAT1 in particular were not mirrored in the peripheral blood T cells, it was felt important to return to the intestine to more fully understand the implications and possible cause of the altered STAT signalling.

4.5.5.1 Factors released by IBD intesinal tissue do not reliably induce phosphorylation of STAT 1 in intestinal T cells

Exactly what factor(s) were driving the increased constitutive pSTAT1 in the $CD4^+$ T cells from non-inflamed IBD samples was unclear. Attempts to determine whether the differences observed were due to factors released from the biopsy samples from experiments with known high pSTAT1 levels in $CD4^+$ T cells were unable to reliably induce high pSTAT1 levels in control samples, although there was a trend in unstimulated samples (fig 4.9). Unfortunately the number of cells available to conduct this experiment was limited which made repetition difficult. It is certainly possible that conducted differently, or with increased sample size, this experiment may have shown a significant effect.

4.5.5.2 Suppressors of Cytokine Signalling (SOCS) Expression

The signalling pathway responsible for STAT1 activation is complex. SOCS1 is the main member of this group to regulate STAT1, and does so by various mechanisms. These include direct binding to Jak and inhibiting Jak-STAT signalling (Horino et al., 2008). The expression of *SOCS1* did show a strong trend for reduction in T cells from non-inflamed areas although this does not reach statistical significance. The cells used in these experiments were purified by magnetic bead separation, as described in chapter 2. This only lead to purities of about 80-90% $CD3^+$ cells. Higher levels of purity of isolation of $CD3^+CD4^+$ cells may reveal significant difference in SOCS expression. In addition, the SOCS1 expression measured was mRNA, and protein levels may differ if measured directly.

SOCS1 expression in T cells has been shown in mouse models to ameliorate DSS colitis by inhibiting IFNγ/STAT1 signalling (Horino et al., 2008). Specifically, mice with a conditional knockout of *socs1* in T cells developed more severe colitis than

controls. In this chapter, there was some suggestion of decreased SOCS1 in T cells from non-inflamed areas. pSTAT1 levels were shown to be increased in the *socs1* KO mice in the Horino study mentioned above, but this was from whole tissue, and in association with IFNγ production. This suggests those cells had a predominantly TH1 phenotype, so this effect may have been a different pathway and/or cell type than is involved in this thesis' human data.

SOCS1 has various other properties in T cells, including roles in apoptosis, proliferation and activation (Kimura et al., 2004; Tanaka et al., 2008; Yu et al., 2008), so it is also possible that these pathways could also be significant in maintaining SOCS1 levels in the human gut. Overall it is difficult to comment of the importance of the trend to decrease SOCS1 expression in non-inflamed IBD samples, but it is consistent with the increase $pSTAT1^{+}$ frequency shown in figure 4.1.

Of note, while no differences were seen in *SOCS3* expression, the levels were extremely high when compared to the *GAPDH* housekeeping gene. This would correlate with the very low levels of pSTAT3, and the poor response to T1IFN stimulation in this group, as SOCS3 is the main regulator of STAT3 (Croker et al., 2003; Kinjyo et al., 2006; Yu et al., 2003). Indeed, just as STAT3 has been shown to promote intestinal inflammation in humans (Carey et al., 2008; Lovato et al., 2003; Mudter et al., 2005), SOCS3 has been shown to be protective (Suzuki et al., 2001). This rise in SOCS3 could not be attributed to the walk-out process, as the cells were extracted using the collagenase digestion methodology (see chapter 2, section 2.1.5.1). It is possible that the 2-3 hours of collagenase processing in RPMI-containing medium (or any other technical factor) would be sufficient to alter expression of SOCS mRNA, but it is also possible that the SOCS levels measured are representative of *in vivo* properties (or at least mRNA expression) of human intestinal T cells. However, as mentioned, the purity of the T cell population was not 100%, and this could have influenced data obtained for both SOCS1 and SOCS3.

4.5.6 The functional significance of altered pSTAT1 signalling

In order to determine the significance of the increased pSTAT1 frequency in T cells from non-inflamed areas, it was attempted to neutralise the STAT1 activation. Fludarabine, a chemotherapeutic agent, has been described as a selective STAT1 inhibitor (Frank 1999). Unfortunately, in the model used in this chapter, fludarabine did not appear to inhibit phosphorylation of STAT1, either constitutively or in its response to T1IFN stimulation (fig 4.11). This may be due to the complex interplay of cytokines in the walk out medium, and/or temporal effects of the drug versus STAT1 activation. When fludarabine was used in an attempt to inhibit STAT1 phosphorylation induced by T1IFN stimulation, it did have an inhibitory effect but this was not selective for STAT1. It may be that this measurement was not sensitive enough to detect the small differences in the effect on STAT1 compared with other STATs. It is possible that with more experiments conducted at different time-points, including a more robust positive control, a more specific effect of Fludarabine may have been shown. It may also be possible that fludarabine is not as selective an inhibitor as originally thought. Recently, toficitanib, the putative specific Jak3 inhibitor, used as a novel treatment for IBD (Rosengren et al., 2012), was found to have a broader range of inhibitory activities against other members of the Jak family (Ghoreschi et al., 2011).

Given the failure to robustly manipulate STAT using either conditioned media or pharmacological inhibitors, it was felt that investigating other components of the STAT1 signalling pathway, particularly T1IFN, would be more successful. To begin, it was therefore felt important to test whether the STAT1 signalling in the intestinal T cells was related to T1IFN.

4.5.7 The relationship between constitutive pSTAT1 and IFNβ

4.5.7.1 T1IFN signals via STAT1 and possibly other pathways in intestinal T cells

Somewhat surprisingly there was only a small (although statistically significant) reduction in phosphorylated STAT1 in the intestinal T cells isolated from all patient groups with anti-IFNβ antibody present (figure 4.12). In the subgroup analysis, the only near significant difference was in the cells from healthy control samples. It was particularly in the inflamed samples where there was no effect of IFNβ neutralisation on pSTAT1 expression. As mentioned previously, the inflamed condition would presumably contain much higher levels of cytokines and other factors and this may make the T cells more refractory to a measurable effect by the manipulation of low levels of tonic signalling. That is, there may be other factors driving STAT1 activation, which are of greater relative significance than endogenous IFNβ. It is less clear why the IFNβ neutralisation did not significantly affect the STAT1 phosphorylation in $CD4^+$ T cells from non-inflamed IBD tissue samples. It is unlikely that IFNγ is the major driver of STAT1 activation in this context as neutralisation of this cytokine had no effect on phosphorylation of any of the STATs measured.

Across all sample types, there are multiple possible reasons for the lack of a more substantial absolute reduction in pSTAT1 levels. It may be that the effect of the IFNβ antibody on STAT1 signalling occurs primarily in the early stages of the walk-out process, and that this is overcome by the time of measurement. It may be that the other signals (eg T2IFN) on STAT1 have a more significant effect, although the experiments using a IFNγ antibody did not show any effect on pSTAT, but did (as with T1IFN, discussed below) decrease U-STAT1. There could also be a role for other cytokines that signal via STAT1, including other T1IFNs, such as the various IFNα subtypes.

There was an overall reduction in the levels of total (U)-STAT1 in all sample types (control and all IBD, figure 4.12) in the context of IFNβ neutralisation. This suggests a prolonged effect on STAT1 signalling leading to reduced transcription of STAT1, although other pathways may be involved. Similar to observations on the phosphorylated form of STAT1, it was in the inflamed samples that were there was no consistent effect anti-IFNβ antibody on U-STAT1 expression. Again, similar possible mechanisms to those described above may explain this observation in inflamed tissue.

4.5.7.2 Type II IFN neutralisation has less effect on STAT1 activation than T1IFN neutralisation

There were too few experiments using anti-IFNγ antibodies to draw many conclusions about the role of IFNγ. However, given there was no effect seen on the number of $pSTAT1^{+}$ cells it is likely that IFNγ is not the main constitutive driver of STAT1 activation of the intestinal T cells. In has been shown that alternative activation pathways of STAT1, rather than the traditional tyrosine phosphorylation, are of importance in modulating IFNγ responses in T cells (Bancerek et al.; Begitt et al., 2014). This may explain the lack of pSTAT1 effect of IFNγ neutralisation experiments, while there retained a significant decrease in U-STAT1 expression (see figure 4.12).

4.5.7.3 Detection of endogenous T1IFN in the human intestine

The final experiments in this chapter dealt with attempting to determine whether T1IFN (specifically IFNβ) was present in the human intestine. Two approaches were used; a novel use of Immunohistochemistry and a more traditional technique of quantitative real-time PCR of mRNA of classical Interferon Stimulated Genes (ISGs).

4.5.7.3.1 Endogenous IFNβ in the human intestine as detected by Immunohistochemistry

To our knowledge, T1IFN had never been shown directly in the human intestine before. IFNβ in particular is very difficult to detect directly for a variety of reasons including that heterophilic serum proteins non-specifically bind and interfere with ELISA assays (Redondo et al., 1999). T1IFN has also been traditionally extremely difficult to detect directly in tissue, but a recent publication demonstrated IFNβ directly in human skin by IHC on fresh frozen samples (Teles et al., 2013). After direct contact with the authors of this paper, a protocol was developed which enabled detection of IFNβ in fresh frozen sections of human gut tissue. IFNβ could only be seen in what appeared relatively low quantity, perhaps mostly associated with the gut epithelium (although non-specific staining makes this assertion difficult to be certain about). There was only rare co-staining with T cells, and this would be consistent with a low level of tonic signalling. The lack of sufficiently sensitive quantitation methods as well as the small number of experiments conducted meant that it was not possible to determine with confidence if there were differences between controls and IBD specimens.

It is interesting to speculate on the source of this endogenous T1IFN. The literature surrounding this was described in more detail in the introduction (see section 1.5.1), but briefly both epithelial and immune cells (including pDCs discussed below) have been implicated. It has been shown that with stimulation, human epithelial cells can produce large amounts of IFNβ (Watanabe et al., 2010), and perhaps these results support this from the location of most of the IFNβ staining by IHC. It is interesting to note that in an early paper showing that IFNβ required viral stimulation to be detected in humans, the intestine was not one of the organs tested (Tovey et al., 1987). Recent data would support endogenous IFNβ in the human skin (Teles et al., 2013), and the IHC data (figures 4.14 and 4.15) would suggest the presence of

endogenous IFNβ in the human intestine, although it is unclear whether this expression is dependent on any environmental signals.

4.5.7.4 Endogenous Interferon Stimulated Gene Signatures in intestinal T cells are decreased in IBD patients

Given the difficulties of direct detection of T1IFN, it is common to look for its footprint, such as through the Interferon Stimulated Genes (ISGs). ISGs are a large group of genes whose protein product transcription is significantly enhanced by T1IFN signalling (Hertzog et al., 2011). ISGs are routinely used to evaluate response to T1IFN (Coelho et al., 2007; Wang et al., 2013). ISG expression is dependent on signalling via the IFNAR (Cho and Kelsall, 2014), therefore detection of ISGs at baseline does suggest tonic IFNAR signalling.

Using primers for two ISGs (MxA and 2'5'OAS) validated in our institute (a kind gift from Dr Raj Lahiri and Professor Graham Foster), the 'footprint' of T1IFN signalling could be inferred by the detection of specific mRNA product in T cells studied directly *ex vivo*. MxA and 2'5' OAS are thought to be two of the few ISGs that are stable in different cytokine environments (Touzot et al., 2014). The T cells were sorted to high purity by flow cytometry to separate them from other cell types which may also be T1IFN responsive.

Indeed, not only were these ISGs detectable in purified intestinal T cells, but they were found at higher levels in healthy control samples than in IBD samples, although this didn't quite reach statistical significance for both genes (figure 4.16). Only non-inflamed IBD samples were used, to support the findings from previous experiments showing higher pSTAT1 frequency in non-inflamed IBD samples (figure 4.1). In previous work from 30 years ago, 2'5' OAS response to poly I:C (a potent T1IFN inducer) was measured in PBMCs from active IBD patients and found to be no different to controls (Stalnikowicz et al., 1985). This is consistent with the lack of

differences detected in the peripheral T cell subset data discussed earlier (see section 4.5.4), but does not preclude differences in the gut.

Previous published work has shown higher levels of plasmacytoid DCs in the lamina propria from IBD patient samples, which have been shown to be predominant producers of T1IFN (Baumgart et al., 2011). This would initially seem to be in contrast with our ISG data. However, the pDCs in that study were from inflamed IBD tissue, which may explain any difference. Perhaps more interestingly, the pDCs also showed decreased T1IFN secretion after CpG stimulation compared to control pDCs. In a separate murine study, increased pDCs appear to be protective against TNBS colitis, where they mediate signals from the microbiota to support Treg production of IL-10 (Dasgupta et al., 2014). Importantly, however, in this study the effects on Tregs were not dependent on T1IFN. Other human work has shown that pDCs are only rarely found in the (small) intestine (Ráki et al., 2013). Further work would need to be done to establish likely sources of endogenous human intestinal T1IFN.

Overall the IHC and PCR data show strong evidence for the presence of endogenous IFNβ in the human intestine, and it's interaction with intestinal T cells.

4.6 Conclusion

Phosflow is an effective tool for measuring intestinal T cell responsiveness in health and disease. $pSTAT1^{+}CD4^{+}$ intestinal T cells are increased in IBD compared with controls, particularly from non-inflamed areas of intestine. These differences are across both Crohn's disease and Ulcerative colitis, and do not reflect a measure of TH1 phenotype. This suggests that this may reflect an increase in pro-regulatory signals in the T cells maintaining homeostasis in these unaffected areas.

Differences between the data in this thesis and previously published reports with regard to STAT expression are likely explicable by the differences in experimental approach, the focus on T cells specifically (as opposed to whole tissue samples),

and the interest in non-inflamed samples. This chapter shows a more nuanced picture that reflects the complex T cell pathways that exist, where the same factor (eg STAT1) may be pro- or anti- inflammatory, depending on context (Nguyen et al., 2000; Odorizzi and Wherry, 2013).

The mechanisms that underlie these STAT changes in remain to be fully defined, but expression of SOCS1, a major regulator of STAT1 may contribute as there were indications that its expression is in T cells from non-inflamed IBD samples. The pSTAT1 differences were not replicated in peripheral blood samples, suggesting something specific to the intestinal environment, affecting the responsiveness of intestinal T cells.

Endogenous IFNβ was shown to be important in STAT1 signalling in the intestinal T cells. In addition, IFNβ was shown directly in the human intestine to our knowledge of the first time.

Overall, these results suggest that factor(s), including IFNβ, in the intestinal environment can influence memory T cells after they have homed back to the gut. These effects may have different functional consequences depending on different variables, but those affecting pSTAT1 in T cells may be pro-regulatory. T1IFN has been used as a treatment in IBD with varying success (Nikolaus et al., 2003; Seow et al., 2008), and has been shown to promote Tregs in mouse models (Kole et al., 2013; Lee et al., 2012). Given the close relationship of T1IFN to STAT1, and the known response of these cells to IFNβ from this chapter, it was considered that the next area of investigation would be human intestinal T cell responses to T1IFN, which will be the basis of the final chapter of results.

Chapter 5

5 The modulation of intestinal T cell function by Type 1 Interferon

5.1 Chapter overview

Constitutively produced T1IFN has been shown in mouse models to promote the development and function of intestinal T_{regs} and ameloriate colitis. T1IFN has also been used as a treatment for both UC and Crohn's disease. Data in the previous chapters showed that pSTAT1, activated by T1IFN, was increased in intestinal T cells from non-inflamed areas of IBD patients compared with both inflamed IBD and healthy control samples.

Experiments in the previous chapter also showed that neutralisation of IFNβ reduced the frequency of $pSTAT1^+$ and $U\text{-}STAT1^+$ intestinal $CD4^+$ T cells. This suggests strongly that IFNβ-STAT1 signalling is important in modulating T cell responses in the human intestine. Indeed, endogenous IFNβ and its 'footprint' could be detected in samples from patients and healthy controls. It was therefore hypothesised that endogenous IFNβ may have pro-regulatory effects on human intestinal T cells.

While the pSTAT1 data had implied the T cells responsiveness to T1IFN, it was first planned to demonstrate more directly that the intestinal T cells could respond ex vivo to T1IFN. Indeed, Interferon Stimulated Gene (ISG) expression increased in intestinal T cells exposed to IFNβ. Moreover, the samples from non-inflamed tissue of IBD patients appeared to be more responsive the T1IFN *in vitro*.

In order to further explore the functional consequences of IFNβ signalling, a model system using predominantly cell and supernatant isolation with the presence or absence of IFNβ-neutralising antibody was used. In this, the $CD4^+$ intestinal T cells of healthy controls had a more “inflammatory” phenotype (ie an increased frequency of IFNγ-producing T cells with a decreased frequency of IL-10-producing T cells,) when isolated by 'walk-out' in the presence of neutralising anti-IFNβ antibody. In contrast, addition of neutralising anti-IFNβ to cultures of IBD tissues, lead to a non-selective increase in the production of nearly all cytokines measured (IFNγ, TNFα and IL17).

In summary, endogenous T1IFN does appear to have a regulatory effect on human intestinal T cells in health. This effect is disrupted in IBD, where the IBD lamina propria T cells appear to be more responsive to T1IFN.

5.2 Introduction

5.2.1 Background

Given the increased pSTAT1 levels in intestinal T cells from non-inflamed areas of IBD patients intestine in chapter 4 (figure 4.1), it was hypothesised that this could be a reflection of either a "pre-disease" state of these T cells, or, in contrast, an increase in regulatory signalling preventing overt inflammation in these regions.

Evidence already acquired from earlier experiments showed that the intestinal T cells were responding *ex vivo* to T1IFN (IFNα2a and IFNβ were the stimuli used in chapter 4), and that IFNβ was shown to be at least partially responsible for STAT1 activation in intestinal T cells (chapter 4, figure 4.12). This in combination with recent murine research suggesting a role for T1IFN in maintaining T_{reg} function in the gut (Kole et al., 2013; Lee et al., 2012), led to the hypothesis that T1IFN was acting as a pro-regulatory cytokine in the human intestine via $CD4^+$ T cells.

This is also in the context of recent research suggesting a role for T1IFN, acting via T cells, in the persistence of chronic viral infections, which may mimic chronic inflammatory disorders such as IBD (Odorizzi and Wherry, 2013; Teijaro et al., 2013; Wilson et al., 2013). This effect is in contrast to T1IFN's canonical role in clearing acute/early viral infection, where T1IFN has a pro-inflammatory effect (Cousens et al., 1999). These contrasting observations suggest a complex interaction of the T1IFN pathway in the adaptive immunity of chronic inflammation, which has been suggested to be dependent on different signalling pathways (Gough et al., 2010; Nguyen et al., 2002).

5.2.2 T1IFN in adaptive immunity

5.2.2.1 T1IFN effects on T cell differentiation and function (figure 5.1)

5.2.2.1.1 T1IFN and TH1 cells

T1IFN was originally shown to increase T_H1 differentiation in T cells in humans dependent on STAT4 (Rogge et al., 1998; Shibuya et al., 2003), although T1IFN without other cytokines (eg IL-12) is not sufficient to sustain T-bet expression (Ramos et al., 2007). However, in contrast to this, in murine *Mycobacterium tuberculosis* T1IFN was negatively associated with T_H1 cytokine signatures (Manca et al., 2001). Later work in murine LCMV models has confirmed that T1IFN signalling predominantly through STAT4 activation promotes IFNγ production, but that signalling via STAT1 reduces IFNγ production and is pro-regulatory (Nguyen et al., 2000; Nguyen et al., 2002). Therefore T1IFN can either support or reduce T_H1 cell polarisation, depending on the engagement of different signalling pathways.

5.2.2.1.2 T1IFN and regulatory T cells

Recently, various studies have shown that T1IFN, largely via effects on the innate immune system, supports the induction and function of murine T_{regs} (Bilsborough et al., 2003; Bleich et al., 2009; Hall et al., 2008; Hofmann et al., 2010; McFarland et al., 2011). This effect has also been shown in human PBMCs (Axtell et al., 2010; Levings et al., 2001; Wang et al., 2000). Indeed, patients treated with IFNβ for Multiple Sclerosis have increased FoxP3 expression after therapy, (Vandenbark et al., 2009). In mice, the effect of T1IFN on T_{reg} IL-10 production has been shown to be at least partially a consequence of signally directly through the IFNAR1 receptor on T cells and STAT1 (Stewart et al., 2013), consistent with the known differential signalling pathways (see above, section 5.2.2.1.1).

5.2.2.2 T1IFN and TH17 cells

The effects of T1IFN on IL-17 production or T_H17 cells are conflicting in the literature. In general, mouse models and human PBMC studies suggest that T1IFN generally suppresses IL-17 production (Guo et al., 2008; Moschen et al., 2008). However, it has also been suggested that IFNβ treatment in patients with Multiple Sclerosis (MS) is successful in those patients with T_H1 driven disease (and indeed a mouse model of EAE driven by T_H1 cells) but worsens disease in patients with T_H17 driven inflammation (and its corresponding mouse model) (Axtell et al., 2011; Axtell et al., 2013).

The negative effect of T1IFN on T cell IL-17 production would be consistent with the increase in IL-10 production that T1IFN has been shown to induce in T cells (see above, section 5.2.2.1.2). This is because there is a strong negative relationship between T cell IL-10 production and IL-17 production, which is dependent on the balance of different STAT signalling (Chaudhry et al., 2009; Chaudhry et al., 2011; Gu et al., 2008).

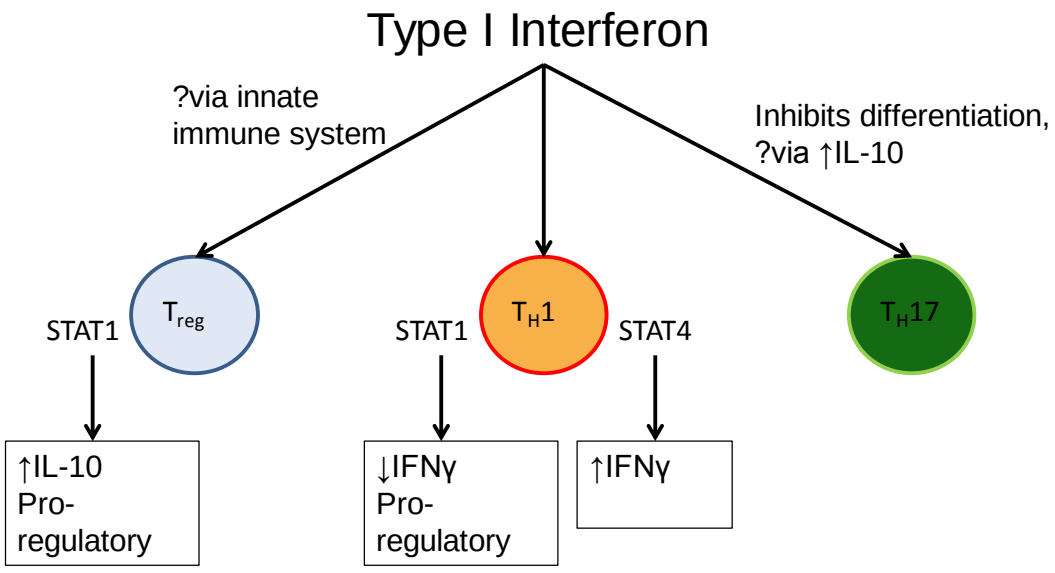

Figure 5.1 Schematic representation of known T1IFN effects on T cell differentiation and function. *T1IFN has been shown to have direct and indirect effects on T cell phenotype/function. In general, it is thought that T1IFN has a positive effect on T_{regs}, via the innate immune system. T1IFN effects on T_H1 cells appear to depend on whether signalling is via STAT1 or STAT3. T1IFN has been shown to have a negative effect on T_H17 cell differentiation, largely via increasing IL-10 production.*

5.2.3 T1IFN in human inflammatory disorders

There are multiple inflammatory conditions where T1IFN has been implicated in the pathogenesis, including psoriasis (Nestle et al., 2005) and SLE (Blanco et al., 2001; Hua et al., 2006). In these conditions, increased levels of T1IFN are associated with pathology, and therefore T1IFN signalling has been a considered a target for treatments (Petri et al., 2013). This is in contrast with Multiple Sclerosis and some chronic viral conditions, where T1IFN is given exogenously as a treatment with significant success (Hauser et al., 2014; Oliver et al., 2011). This conflicting use of pharmacological targeting of T1IFN in human inflammation suggests that this is a pathway that is finely balanced to maintain homeostasis in different anatomical locations and genetic pre-dispositions. Disruption in either direction may lead to chronic pathology.

The most pertinent knowledge of the role of T1IFN in human intestinal T cells comes from experience with patients with IBD. In IBD, systemic T1IFN has been used as a treatment with variable success in both Crohn's disease and UC (Musch et al., 2005; Pena Rossi et al., 2009), but eventually felt to be ineffective (Seow et al., 2008). Alongside this, there have been multiple case reports of people developing IBD when treated with T1IFN for other conditions (Mitoro et al., 1993; Schott et al., 2007; Watanabe et al., 2011). This again implies a complex relationship of T1IFN signalling in the mucosa.

Specifically in regard to T cell effects, in UC patients who were treated with T1IFN, those individuals who were non-responders to T1IFN were shown to have higher IL-17 production from peripheral and intestinal T cells (Mannon et al., 2011). A separate study in UC showed that IFNα treatment caused a reduction of IL-17 production from whole colon biopsies (Moschen et al., 2008). This would be consistent with successful treatment being associated with an increase in IL-10 T

cell production, and the corresponding IL-17 changes demonstrated. However, the effects on other intestinal T cell properties or cytokine production (including IL-10), have not been investigated in humans.

Despite its previous use in IBD and current use in many other viral and inflammatory disorders, the mechanisms of the action of T1IFN remain poorly understood, particularly in the intestine.

5.2.4 Summary

Given the presence of a constitutive pSTAT1 signal shown in earlier experiments on intestinal T cells (figure 4.1), it was hypothesised that there could be endogenous T1IFN in the lamina propria that was "priming" these memory T cells to respond in a pro-regulatory fashion. There is considerable research from mouse models and *ex vivo* peripheral blood work to support this. This endogenous IFNβ was demonstrated in chapter 4 (figure 4.15). Therefore, it was postulated, that by blocking this constitutive T1IFN with a neutralising antibody, it should be possible to make more pro-inflammatory, or less regulatory, T cells.

The increased number of $pSTAT1^+$ T cells from non-inflamed areas of colon, compared to inflamed, could also be an indication of a relatively increased pro-regulatory signal, maintaining mucosal integrity in some areas of the gut when it may otherwise be lost. This would be indirect evidence of a functional plasticity to these memory T cells, potentially being more responsive to maintain mucosal integrity in an IBD environment.

5.3 Aims

To determine whether:

(i) intestinal T cells are responsive to T1IFN signalling, and if this differs between health and IBD.

(ii) endogenous T1IFN has a pro-regulatory effect on human intestinal T cells in health

(iii) the effects of endogenous and exogenous T1IFN on intestinal T cells differ between health and IBD, including between non-inflamed and inflamed areas of bowel in IBD

5.4 Results

5.4.1 T cell Responsiveness to IFNβ in health & IBD

The experiments described in the first part of this chapter on the role of T1IFN in intestinal T cells focussed on measuring the responsiveness of the cells to exogenous T1IFN. This could provide further evidence to support the theory that intestinal T cells maintain ability to respond to local environmental factors.

5.4.1.1 Induction of Interferon Stimulated Gene Signatures in intestinal T cells by exogenous IFNβ

Cells were isolated from the intestine, viable T cells were then sorted to purity by flow cytometry, and the RNA was extracted and reversed transcribed. Expression of two Interferon Stimulated Genes (ISGs) were then measured using qRT-PCR. The two ISGs selected were MxA and 2'5' OAS, which are commonly used in T1IFN research (Coelho et al., 2007; Wang et al., 2013). The cells were isolated by walk out with or without the addition of T1IFN, enabling the quantitation of ISGs in response to T1IFN stimulation. As in previous experiments, IFNβ was used, at a concentration of 1000 IU/mL for 24 hours. This is a standard concentration used in many previous studies assessing the effects of T1IFN on ISG expression, but notably much lower than used in the Phosflow experiments in chapter 3 and the first part of chapter 4 (40 000 IU/mL).

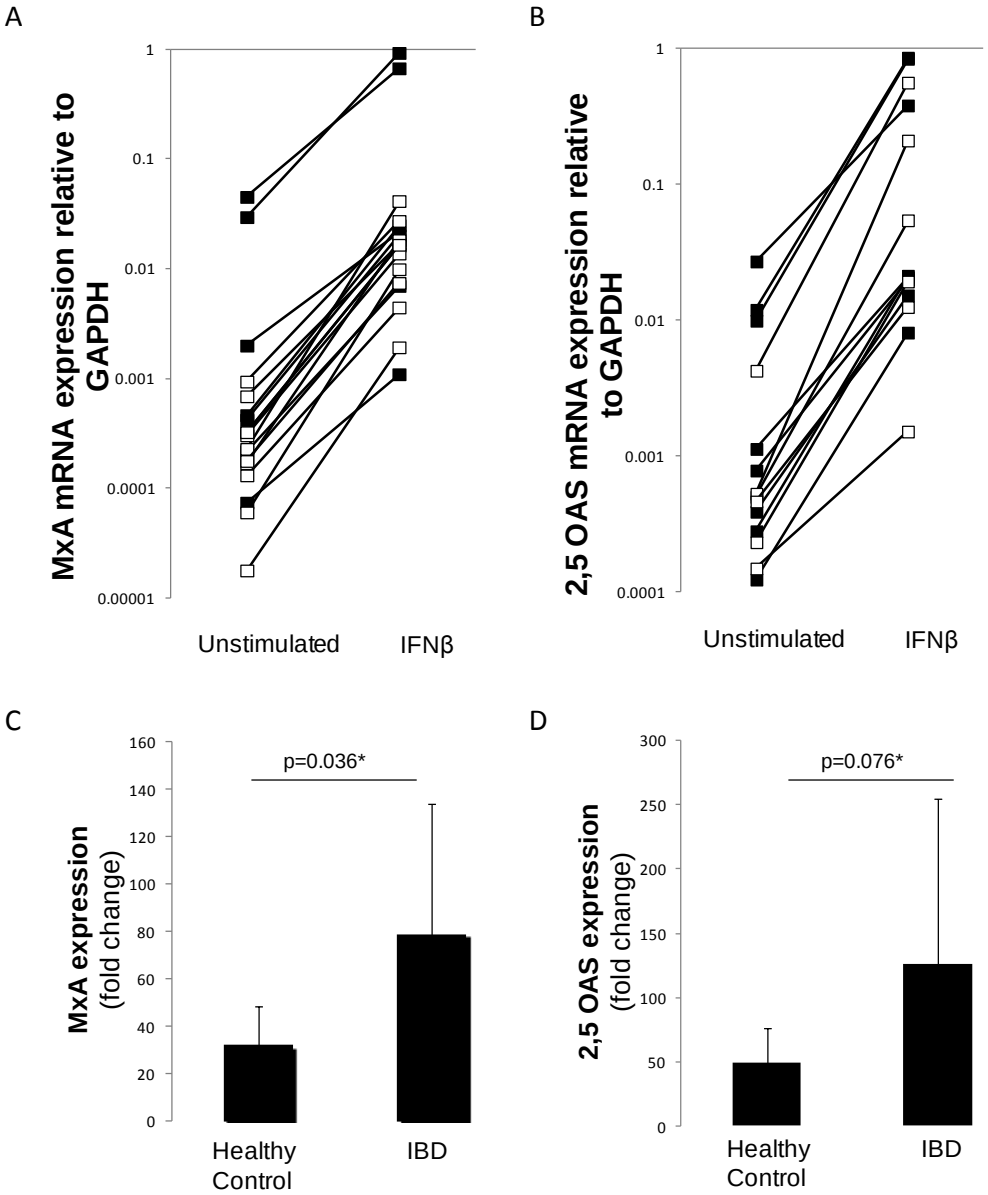

Figure 5.2 Intestinal T cells from IBD patients are more responsive to T1IFN stimulation than controls. *Cells were walked-out in the presence or absence (unstimulated) of 1000U/mL IFNβ and T cells were sorted by flow cytometry prior to RNA extraction. A and B qPCR data for unstimulated and T1IFN-stimulated expression of MxA (A) and 2,5 OAS (B) relative to expression of the housekeeping' gene GAPDH (n=10 healthy controls, solid squares, n=12 IBD samples, white squares) C and D Fold change in PCR data from A and B in MxA expression (C), and 2,5 OAS expression (D), comparing T cells isolated with and without exogenous IFNβ (1000U/mL) over 24 hours.*

These data confirm that the MxA and 2,5 OAS ISG response is inducible in human colonic $CD4^+$ T cells. Interestingly, the $CD4^+$ T cells isolated from IBD patients were more responsive to exogenous IFNβ than those from controls with regard to ISG

induction, as demonstrated by their fold change in relative MxA expression with a similar trend for 2,5 OAS (figure 5.2, panels C and D).

5.4.2 The effect of exogenous and endogenous IFNβ on intestinal T cell function

5.4.2.1 Experimental approach to explore T1IFN signalling effects on T cell cytokine production

In order to explore the effect of T1IFN on intestinal T cells, a functional outcome of the effect of T1IFN signalling was required. Given the relatively small samples available, it was considered that measuring the cytokine output of the intestinal T cells would be the method best suited to this outcome.

Lamina propria mononuclear cells (LPMCs) were isolated using the walk-out method as in previous chapters. Added to the medium in which the denuded intestinal specimens were placed was either a neutralising antibody to IFNβ or the isotype-matched control (see figure 5.3). The possibility of blocking all T1FN signalling by using antibodies to its receptor (anti-IFNAR antibodies) was also considered but at the time of the experiments there were no reliable monoclonal antibodies available. Furthermore, although experiments were attempted using a polyclonal anti-IFNAR antibody, selection of an adequate control was problematic (data not shown). Therefore experiments exclusively utilised monoclonal anti-IFNβ antibody. Using this approach it was possible to demonstrate at least partial neutralisation of T1IFN signalling, as demonstrated by reduced induction of STAT1 phosphorylation (see section 4.12 and data not shown). IFNβ is considered the co-ordinator of many T1IFN effects (Gough et al., 2012), and has been the most studied in mouse models of colitis (Katakura et al., 2005; Kawashima et al., 2013; McFarland et al., 2011), and was the T1IFN subtype detected by IHC in chapter 4.

Therefore it seemed reasonable to focus on IFNβ in the further study of the effects of T1IFN in the human gut.

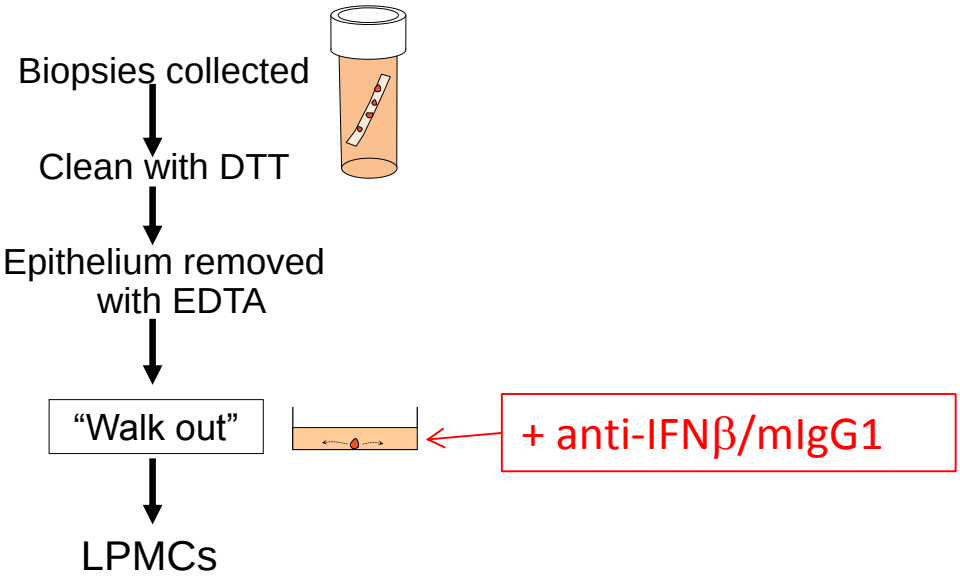

Figure 5.3 Neutralising endogenous IFNβ in intestinal LPMCs. *Schematic demonstrating the process of* ex vivo *manipulation of endogenous T1IFN in intestinal LPMCs. Biopsies or small pieces of tissue were cleaned to remove faeces, the epithelium removed, and then placed for 48 hours in complete medium to allow LPMCs to egress. During this process, neutralising anti-IFNβ antibody or isotype control was added. The biopsy was removed and cells were collected for further analysis. See Chapter 2 for more details.*

5.4.2.1.1.1 Optimisation of intracellular staining for T cell cytokine detection

It was determined to measure T cell cytokine output to determine the effect of IFNβ neutralisation. This was done initially by measuring IFNγ, IL-10, TNFα and IL-17 by intracellular staining and flow cytometry. These cytokines were chosen due to their known importance in IBD pathology (reviewed by Neurath, 2014a). In order to measure the individual cytokine production of T cells it is necessary to both stimulate the cells and to block secretion of cytokines out of the cells. Traditionally, a combination of phorbol 12-myristate 13-acetate (PMA) and ionomycin as stimulus agents with Monensin as a golgi-blocking agent is used. The combination of PMA and Ionomycin activates protein kinase C and mobilises calcium to promote cytokine production from any genes that are, at the time of stimulation, in an "open" configuration to be transcribed. It was felt that such a powerful stimulus would be

likely to override the effects of potentially low endogenous concentrations of T1IFN in the micro-environment. In the murine intestine, concentrations of IFNβ are at very low levels (Kole et al., 2013), and it is not unreasonable to assume that endogenous IFNβ would also only be present at very low concentrations in the human gut, at least in the absence of viral infection. Therefore a more subtle form of T cell activation was preferred.

As an alternative method, an anti-CD3 antibody was employed, which mimics TCR engagement. This allows a much broader response than would be seen in an antigen-specific manner, while retaining more physiological downstream signalling events. This system was considered to be more likely to be impacted by neutralisation of the endogenous T1IFN.

The initial experiments used soluble anti-CD3 antibodies to stimulate the cells after collection from walk-out. Cells were washed into fresh complete medium, counted, and $2x10^5$ cells were stimulated with anti-CD3 antibodies for 24 hours, with monensin added for the last 4 hours before analysis by flow cytometry (see chapter 2, section 2.2.3). Despite the expectation that APCs within the mixed cell population in the walk outs would provide sufficient co-stimulatory signals for T cell activation, cytokine production induced by anti-CD3 alone was poor (data not shown). Therefore a combination of anti-CD3 and anti-CD28 (to enhance co-stimulation) antibodies were used in subsequent experiments. Figure 5.4 shows an example of cytokine levels detected with different stimulants. Good cytokine production was demonstrable under conditions anti-CD3/28 stimulation although the frequency of cytokine-producing cells was fewer that that observed stimulation with PMA/Ionomycin.

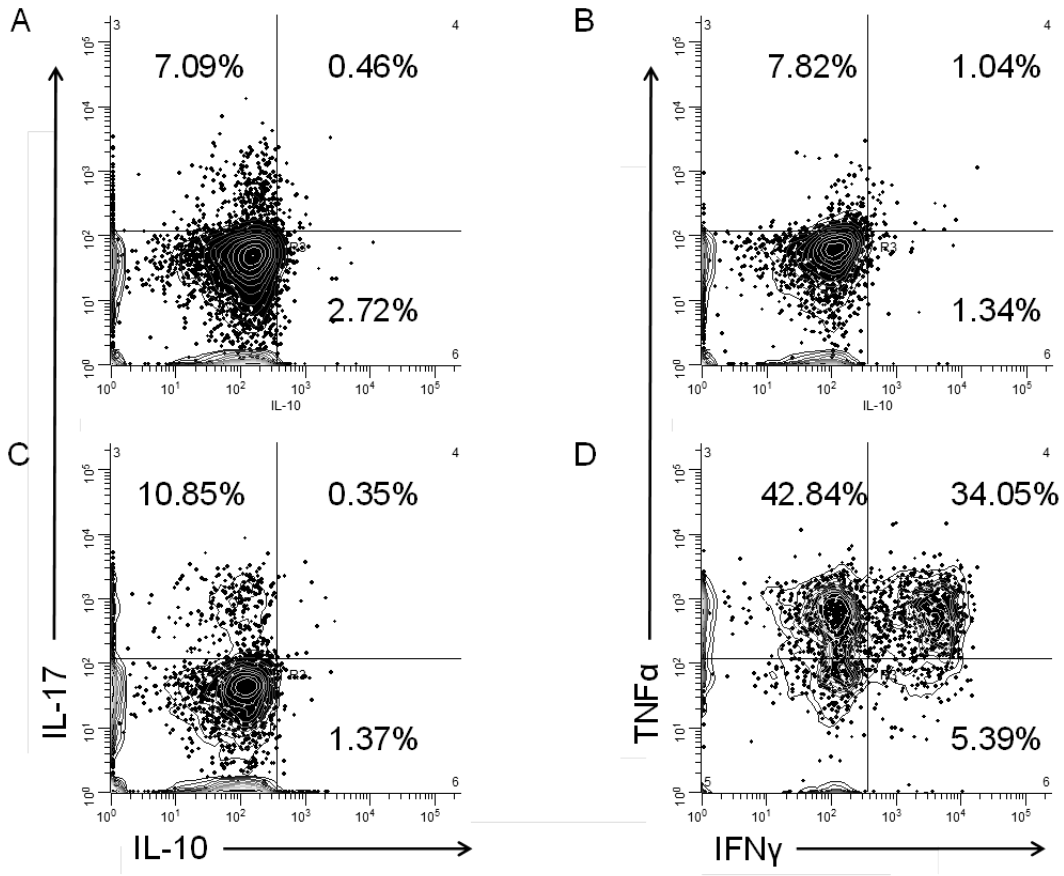

Figure 5.4 CD3/CD28 stimulation induces detectable cytokine production by colonic CD4$^+$ T cells. *Walk-out cells were resuspended in complete medium and stimulated for 24 hours with anti-CD3 and anti-CD28 antibodies (A+B) or left for 20 hours then stimulated with PMA, Ionomycin (C+D). All were then treated with monensin and intracellular cytokines as shown were measured by flow cytometry. Cells were gated on CD4$^+$ T cells. Representative of 3 independent experiments.*

Although the intention was predominantly to determine CD4$^+$ T cell responses, both CD8$^+$ and CD4$^+$ were measured (figure 5.5). In almost all experiments, more CD4$^+$ T cells had detectable cytokine production than CD8+ T cells (see detailed results later in chapter).

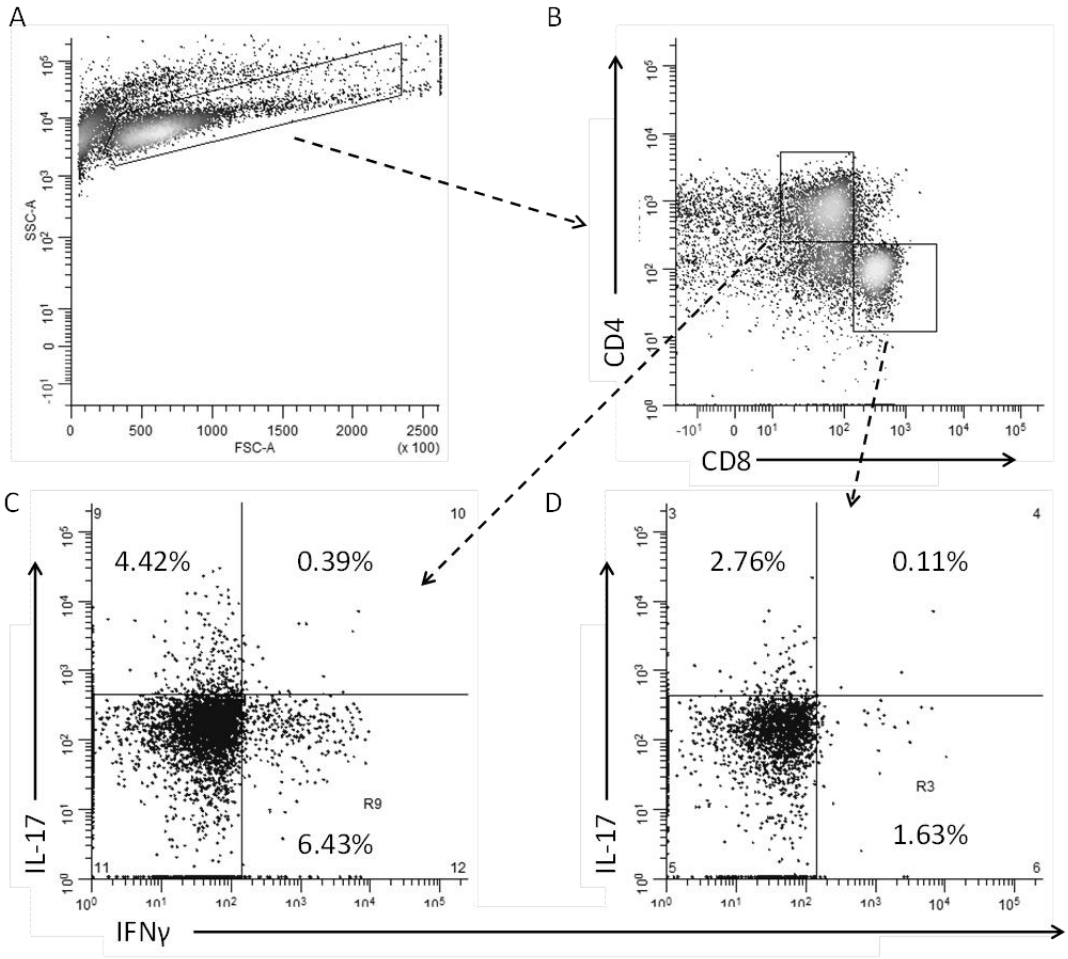

Figure 5.5 More CD4⁺ T cells than CD8⁺ colonic T cells produce cytokine in these conditions. *LPMCs were isolated and stimulated as described. Briefly, cells were stimulated with anti-CD3/CD28 antibodies for 24 hours and intracellular cytokines were measured by flow cytometry. Cells were gated on scatter (A) and either CD4 or CD8 (B). IFNγ and IL-17 results are shown but the pattern was similar for TNFα and IL-10 Representative of >20 experiments.*

5.4.2.1.1.2 Multiplex ELISA to measure the effect of IFNβ neutralisation on cytokine production

During the experiments to measure the intracellular cytokines by flow cytometry, the supernatants of the cell cultures were collected and stored at -80°C. The LPMCs from the walk-out preparation contain a range of different cell types as characterised by the original study showing the use of this cell isolation technique (Mahida et al., 1997). However, following T cell stimulation it is reasonable to assume that the predominant source of cytokines would be the T cells themselves. Even in the absence of stimulation, the production of IFNγ and IL-17 would be predominantly

from T cells (O'Shea and Murray, 2008; Schoenborn and Wilson, 2007). It is less clear which cells would be producing TNFα and IL-10 but measurement of these four cytokines in the supernatants of the walk-outs with and without IFNβ neutralisation would provide further evidence of the role of endogenous T1IFN in the human intestine.

5.4.2.1.1.3 Overall experimental approach in exploring the effects of IFNβ

To summarise, intestinal biopsies were cultured (walked-out) in the presence or absence of neutralising anti-IFNβ, excess IFNβ, or the appropriate control. Furthermore, cells cultured under these conditions were stimulated with anti-CD3/anti-CD28 antibodies and cytokines produced were detected by intracellular flow cytometry. Supernatant was also collected at different time-points in order to measure cytokine release by multiplex ELISA. This is summarised in the schematic figure 5.6

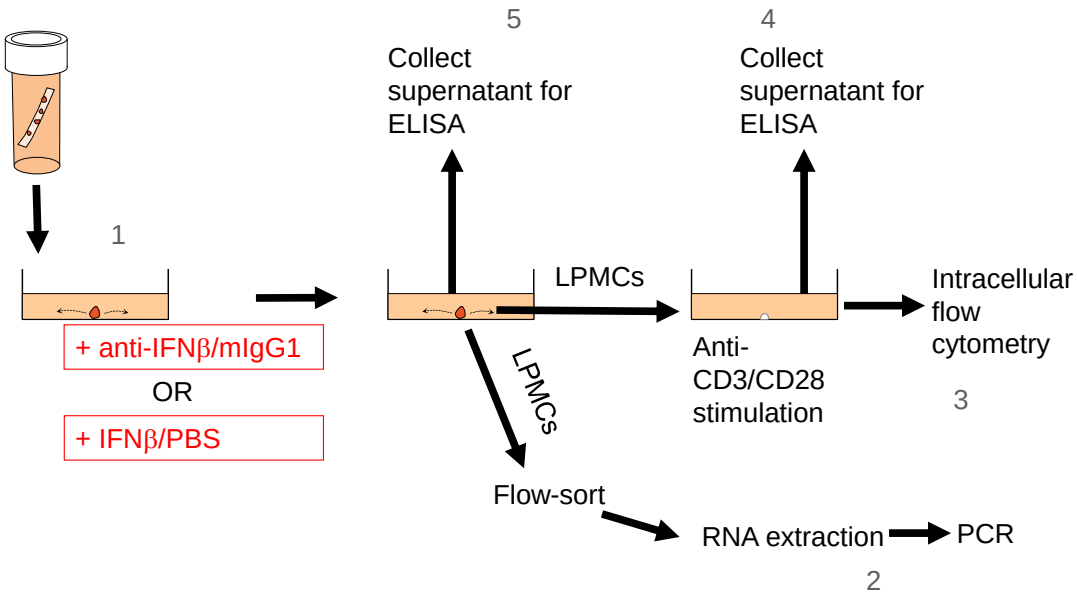

Figure 5.6 Schematic model of overall experimental approach. *1. Walk-out cultures were set up as previously described in chapter 2, but with either neutralising anti-IFNβ antibody or isotype-matched control, or excess 1000 IU IFNβ or PBS for 24 hours. 2. LPMCs were recovered from the walk-out and sorted to purity for RNA extraction and PCR experiments.3. Alternatively, LPMCs were recultured in the presence of anti-CD3/CD28 antibodies for T cell stimulation and cells were processed for intracellular flow cytometry. Supernatants were collected*

for multiplex ELISA analysis both after T cell stimulation (4) and from whole biopsy cultures (5). Details of experimental techniques are in chapter 2.

5.4.2.2 The Effect of endogenous TIFN signalling on intestinal T cells in health

Once conditions had been optimised (section 5.4.2.1), multiple samples were taken to look first at the effect of neutralisation of IFNβ in walk-out samples from healthy control samples.

5.4.2.2.1 The effect of IFNβ neutralisation on cytokine production by $CD4^+$ T cells from healthy individuals measured by intracellular flow cytometry

Figure 5.7 shows cytokine production by $CD4^+$ intestinal T cells from eight healthy donors, with or without IFNβ neutralisation.

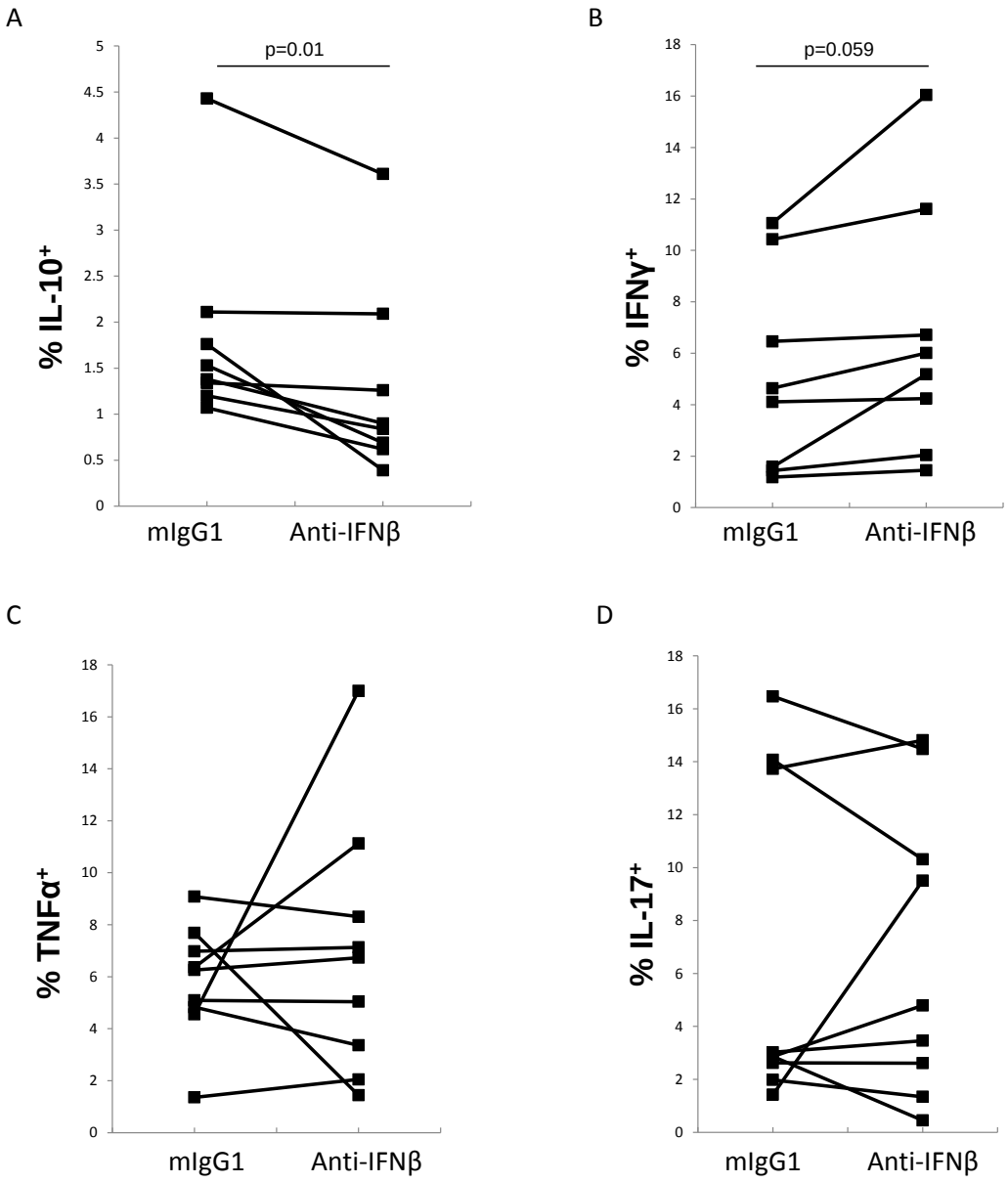

Figure 5.7 IFNβ promotes IL-10 production from intestinal CD4$^+$ T cells in healthy controls. *LPMCs were isolated and stimulated as described. Briefly, cells were walk-out in the presence of anti-IFNβ or isotype control (mIgG1). LPMCs were then collected and stimulated with CD3/CD28 antibodies for 24 hours. Intracellular cytokines were measured by flow cytometry as follows: A IL-10, B IFNγ C TNFα and D IL-17. Cells were gated on scatter and CD4$^+$ (see example figure 5.4). Statistical analysis was performed by paired t tests.*

The frequency IL-10-producing CD4+ T cells was reduced in the presence of anti-IFNβ suggesting that constitutive production of this cytokine enhances IL-10 production (figure 5.7A). The effect on IFNγ production did not reach statistical significance but there was a trend for the presence of anti-IFNβ antibody to increase the frequency of IFNγ-producing T cells (figure 5.7B). This lends weight to the

hypothesis of a pro-regulatory effect of T1IFN in the healthy intestine dependent on T cells. There were no consistent effects seen on the frequency of cells producing either TNFα or IL-17 (figure 5.7C and D).

Intracellular cytokine production by $CD8^+$ intestinal T cells was simultaneously measured (figure 5.8). All measured cytokines were less frequently detected in the $CD8^+$ cells compared with the $CD4^+$ cells. There were no statistically significant effects of anti-IFNβ on cytokine production by $CD8^+$ T cells, with only a trend for reduction in frequency of IL-10 producing cells.

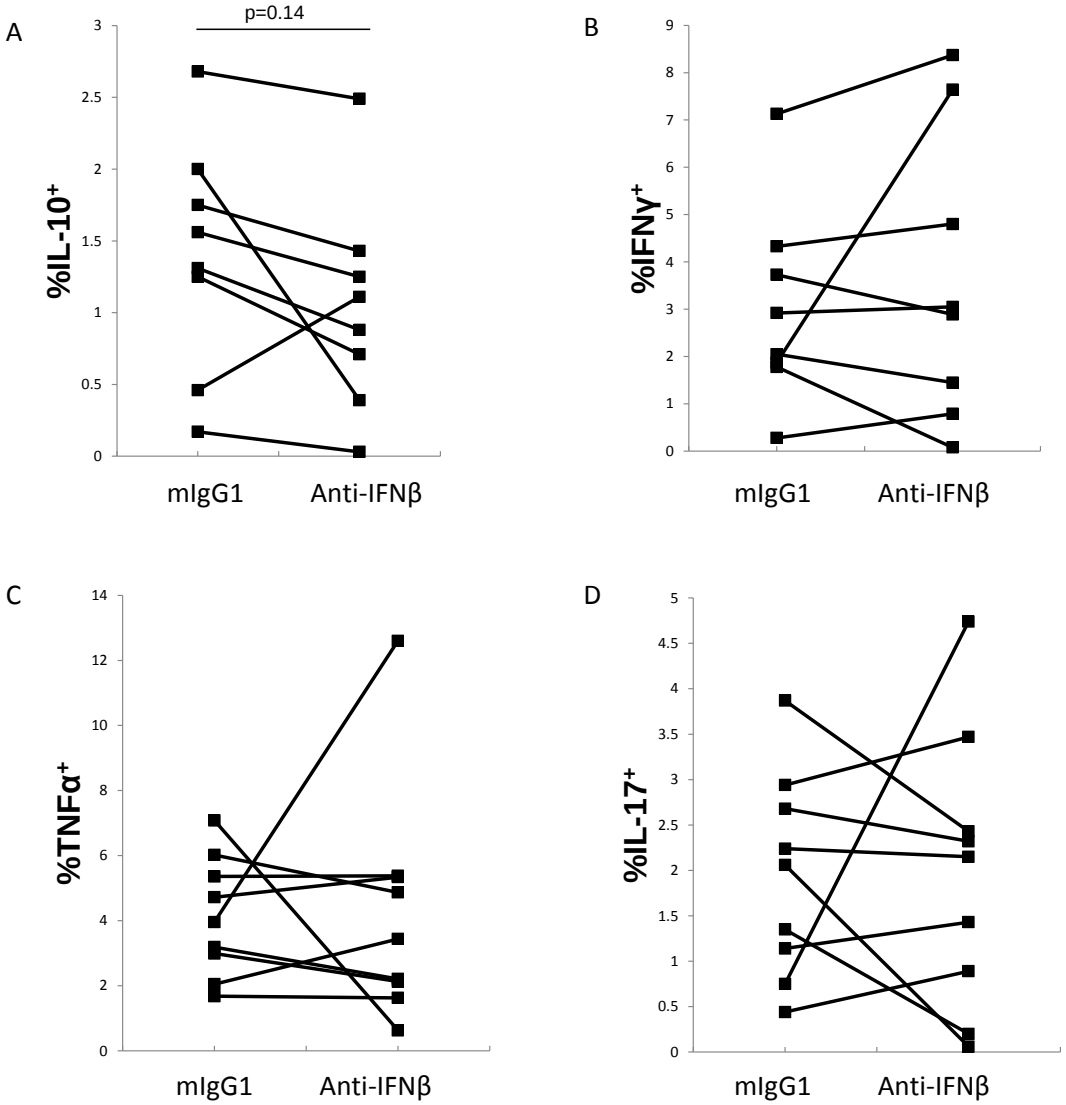

Figure 5.8 IFNβ neutralisation has no effect on cytokine production from $CD8^+$ intestinal T cells from healthy controls. *LPMCs were isolated from walk-out cultures in the presence of anti-IFNβ antibody or isotype control (mIgG1). Cells were resuspended and activated with anti-CD3/28 antibodies for 24 hours and intracellular cytokines were measured using flow cytometry as above. Cells gated*

on light scatter and CD8 expression (see example in figure 5.4). Statistical analysis was performed by paired t tests.

5.4.2.2.2 The effect of IFNβ neutralisation on cytokine production from intestinal biopsies of healthy individuals, after T cell activation, as measured by multiplex ELISA

In contrast to the equivalent intracellular cytokine results shown above, analysis of the supernatants from the cultures of stimulated T cells by ELISA (after 24 hours of anti-CD3/28 antibody activation), showed no significant effect of anti-IFNβ antibody, although the number of experiments was quite small (n=8, figure 5.9). The number of experiments possible was limited by the number of cell obtained from walk-out cultures from control tissue. However, there was a trend towards an increase in IFNγ production in cultures in which IFNβ was neutralised, consistent with the flow cytometry results.

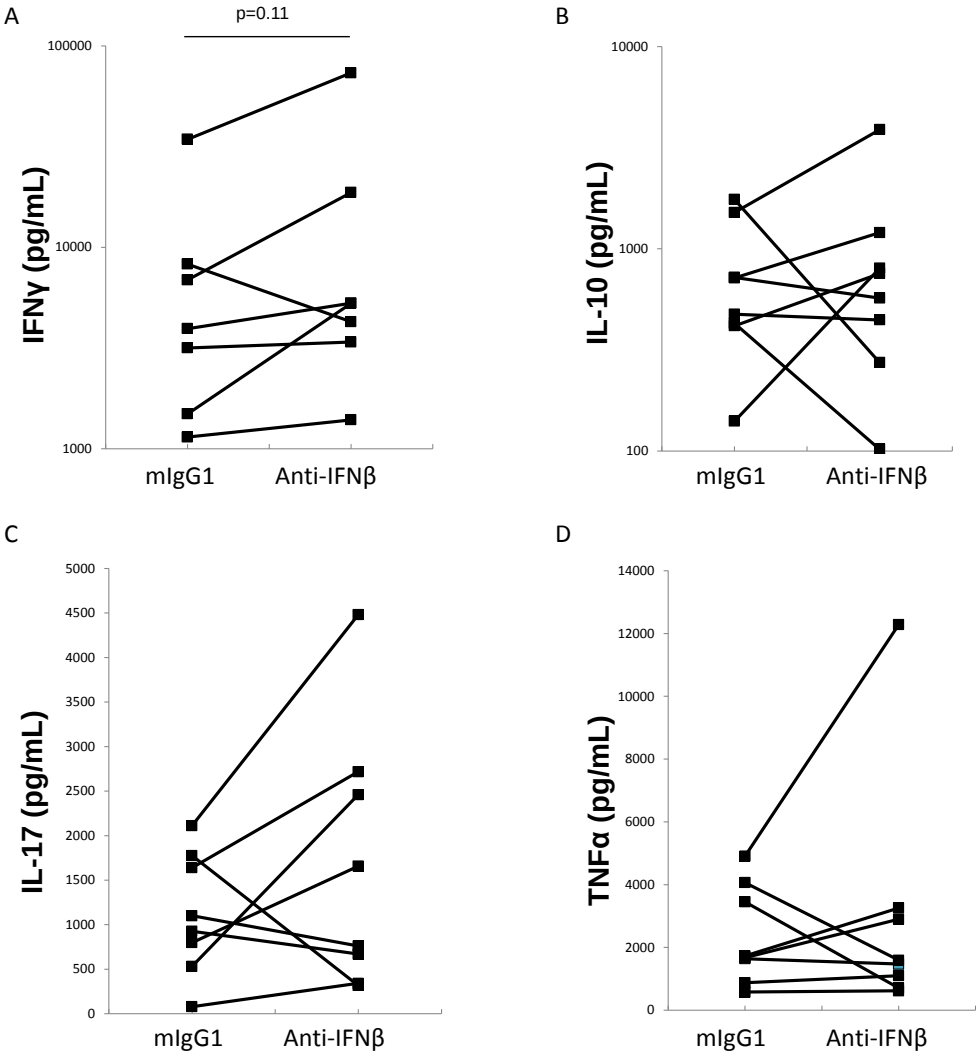

Figure 5.9 No differences detected in supernatant cytokines from healthy controls with IFNβ neutralisation after T cell activation, measured by ELISA. *Walk-outs were prepared in the presence of neutralising anti-IFNβ antibody or isotype control (mIgG1). After 48 hours, the cells were harvested and stimulated with anti-CD3/CD28 for a further 24 hours and the supernatant was collected, frozen, and later analysed by multiplex ELISA for IFNγ, IL-10, TNFα and IL-17 (n=8). Statistical analysis was performed by paired t tests.*

5.4.2.2.3 The effect of IFNβ neutralisation on cytokine production from whole intestinal biopsies from healthy individuals

Cytokines were measured from the stored supernatants of the walk-out experiments as described in section 5.4.2.1.1.2. This was done using multiplex ELISA,

measuring quantities of IFNγ, IL-10, TNFα and IL-17. It was also attempted to measure IFNα, but this was not detected in any supernatant.

Figure 5.10 demonstrates detection of a variety of cytokines in the supernatants, even in the absence of T cell activation, or indeed any other *in vitro* stimulation. The cytokines detected in the supernatants would have been produced from a mixed cell population, and not exclusively T cells. It was felt likely that IFNγ would be largely T cell produced (Schoenborn and Wilson, 2007), as (at least in the gut) would IL-17 (O'Shea et al., 2008). Therefore measuring the effect of IFNβ neutralisation on their production by this method would be supportive of the endogenous effect of T1IFN on T cell function.

Both IL-10 and TNFα are produced by a variety of cell types in the intestine, but are important cytokines in the current understanding of IBD pathogenesis (Neurath, 2014a), therefore it was felt that detecting effects of endogenous T1IFN on their production would be of interest, even if not directly attributable to a T cell effect. Also, the hypothesis proposed a role of T1IFN in the regulatory function of T cells, and IL-10 is one of the main regulatory cytokines (Saraiva and O'Garra, 2010). In addition, TNFα is the main target of biological therapies in patients with IBD (Neurath, 2014b).

Given the number of cells collected from individual walk-out experiments varied (see sections 2.1.5.4 and 5.4.2.5), it was important to control for the number of cells in each culture in determining the amount of cytokine produced. Therefore, the raw value produced by the multiplex ELISA was adjusted according to the relative number of cells between the isotype control and the anti-IFNβ antibody containing culture (see section 2.5). In brief, the raw cytokine concentration for the anti-IFNβ result was multiplied by the ratio of cell numbers in that condition compared to the isotype. Adjustment was not necessary in cultures after anti-CD3/CD28 stimulation, as the same number of LPMCs ($2x10^5$) were placed in each well.

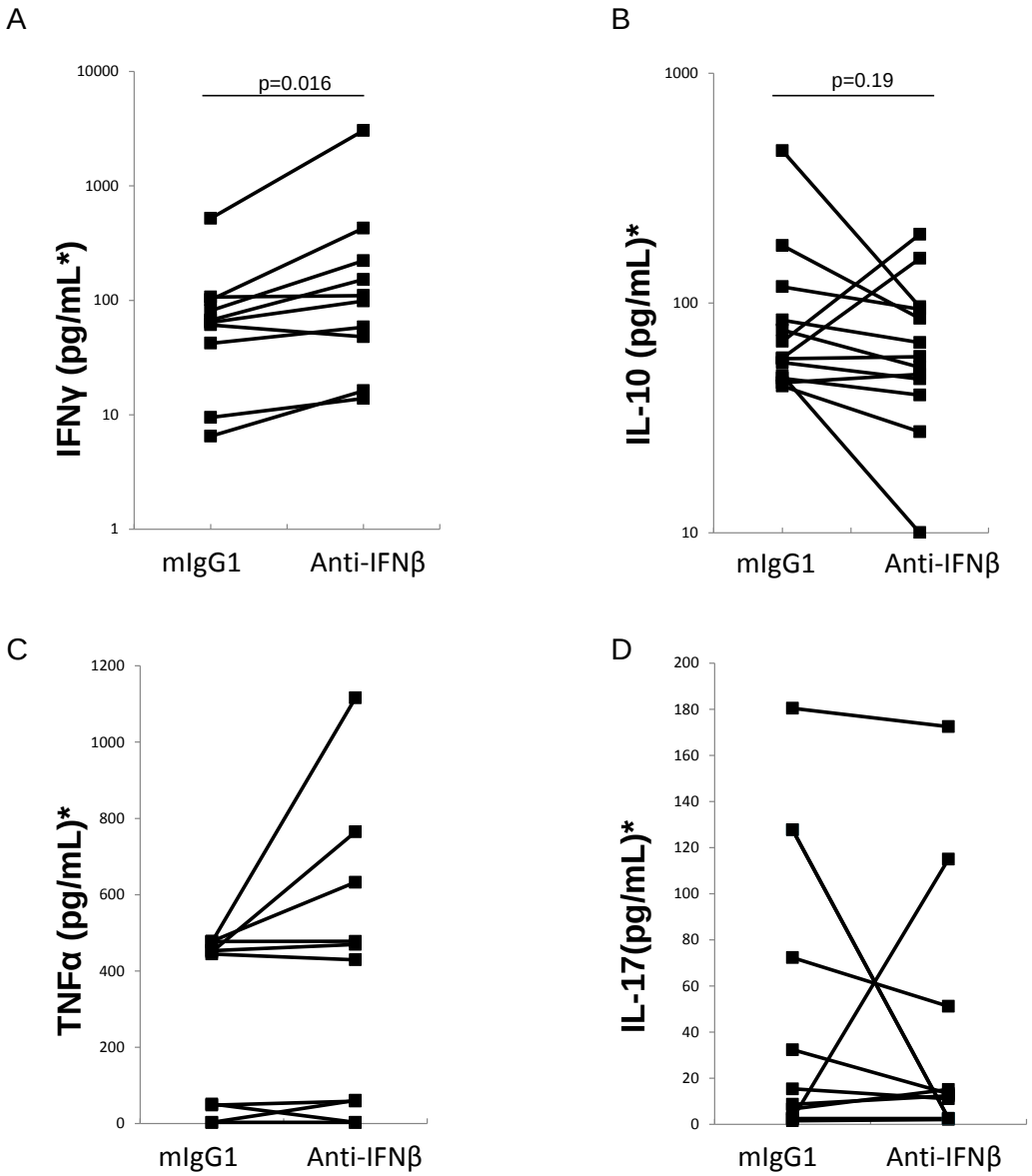

Figure 5.10 IFNβ neutralisation increases IFNγ concentrations in supernatants from control intestine. *Biopsy specimens were placed in complete medium as previously described, in the presence of neutralising anti-IFNβ antibody or isotype control (mIgG1). After 48 hours, the supernatant was collected and IFNγ (A), IL-10 (B), TNFα (C), IL-17 (D), and IFNα and were measured by multiplex ELISA(n=11). IFNα results are not shown as there was little or no detectable cytokine in any sample. *concentrations were corrected according to the ratio of number of cells in the samples, as described in chapter 2 and chapter 5, section 5.4.2.1.1.2. Statistical analysis was performed by paired t tests.*

The IL-10 production did not change significantly after IFNβ neutralisation, although there was a trend towards reduction. However, to allow for variation in overall cytokine production between individual experiments, a ratio of IFNγ:IL-10 was

calculated and this ratio did differ with a significantly higher value for cells isolated in the presence of anti- IFNβ antibody (signed rank test, p=0.014).

There were no consistent effects on TNFα production, nor IL-17, although the IL-17 concentrations in particular were generally very low.

5.4.2.2.4 The effect of IFNβ neutralisation on intestinal T cell phenotype measured by "master regulators" of transcription

As well as measuring cytokine production as a functional consequence of IFNβ neutralisation, it was possible to detect transcription factors that are associated with particular T cell phenotypes by intracellular staining in flow cytometry. This could be done simultaneously with the intracellular cytokine staining, although cell numbers were often a limiting technical factor. There was no effect seen on the percentage of T cells expressing FoxP3 (the master regulator associated with regulatory T cells) after IFNβ neutralisation compared with isotype control (mean 30% in both groups, n=4). The transcription factor associated with T_H1 cells, T-box expressed in T cells (T-bet), was also not affected by IFNβ neutralisation, with the mean percentage of cells expressing T-bet being 27% in the presence of anti-IFNβ and 26% in the presence of the isotype-matched control antibody (n=7). RORγt, the transcription factor associated with T_H17 cells, was only detected in a small proportion of T cells in either condition (1.5%-4.5%) with no differences found with anti-IFNβ (n=7).

5.4.2.2.5 The effect of IFNβ neutralisation on T cell recovery and viability

It was also considered whether endogenous T1IFN may be having other effects on intestinal T cells, as well as affecting cytokine production. In particular, whether the presence or absence of endogenous T1IFN may affect T cell survival. It was noted that with exogenous T1IFN added to the culture medium (see section 5.4.2.3 below), cell recovery from the walk-out experiments was increased. The mean yield

of total cells (LPMCs) was $1.65x10^4$ (+/- 0.73 SD) cells per biopsy with no IFNβand increased to $3.05x10^4$ (+/- 2.20 SD) cells per biopsy in cultures containing additional 1000 IU/mL IFNβ (n=8, p=0.04, paired t test). However, with use of the anti-IFNβ antibody, the cell yield change did not differ significantly between cultures with mouse IgG1 antibody (mean $3.6x10^4$ +/- 1.78 SD cells per biopsy) and cultures with anti-IFNβ (mean $3.2x10^4$ +/- 1.47 SD cells per biopsy) (p=0.10, n=35, paired t test). Unfortunately, there was no quantification of the effect of IFNβ either the total or $CD4^+$ T cells, so it is unclear whether there may or may not be a cell-type specific effect.

From the literature, T1IFN is known to have effects on cell survival (Dondi et al., 2004; Gil et al., 2006; Stark et al., 1998), which, over a relatively short time period, may not be detected by cell numbers. Therefore the viability of the cells during the walk-out process was also assessed. Viability was demonstrated using a fixable viability stain on the cells (see materials and methods chapter). In fact, cell viability within the lymphocyte gate was always >98% (n=8) with no differences between biopsies exposed to anti- IFNβ antibody or to isotype-matched control antibody.

5.4.2.3 Effect of exogenous IFNβ on intestinal T cells in health

As well as measuring the effects of endogenous T1IFN in the walk-out biopsy cultures, it was of interest to known how responsive to exogenous T1IFN the walk-out cultures were, in particular the $CD4^+$ T cells. Due to low cell numbers, not as many conditions were analysed, as many of the cells recovered were used for mRNA extraction in the previously described T cell responsiveness by expression of ISGs (see section 5.4.1 above).

5.4.2.3.1 The effect of exogenous IFNβ on cytokine production from supernatants after T cell stimulation of walk-out cells from healthy individuals

LPMCs isolated from these cultures produced high levels of cytokines following stimulation with anti-CD3/28 antibodies, irrespective of the addition of IFNβ to the biopsy culture (figure 5.11). Production of IL-17 was significantly lower by cells obtained from IFNβ-supplemented cultures. There were no significant differences in the production of IFNγ, IL-10, and TNFα between cells derived from IFNβ-supplemented and control cultures although there was a trend towards reduced production of IL-10 which mirrored the effect in production by the biopsy itself. The enhancement of IFNγ production that was observed in IFNβ supplemented whole biopsy culture (see figure 5.12A below) was not reflected in production by anti-CD3/28 activated cells derived from these cultures. However, production of IFNγ in particular was often very high in these antibody stimulated cultures it is possible that maximal production was achieved even in the absence of prior exposure to IFNβ.

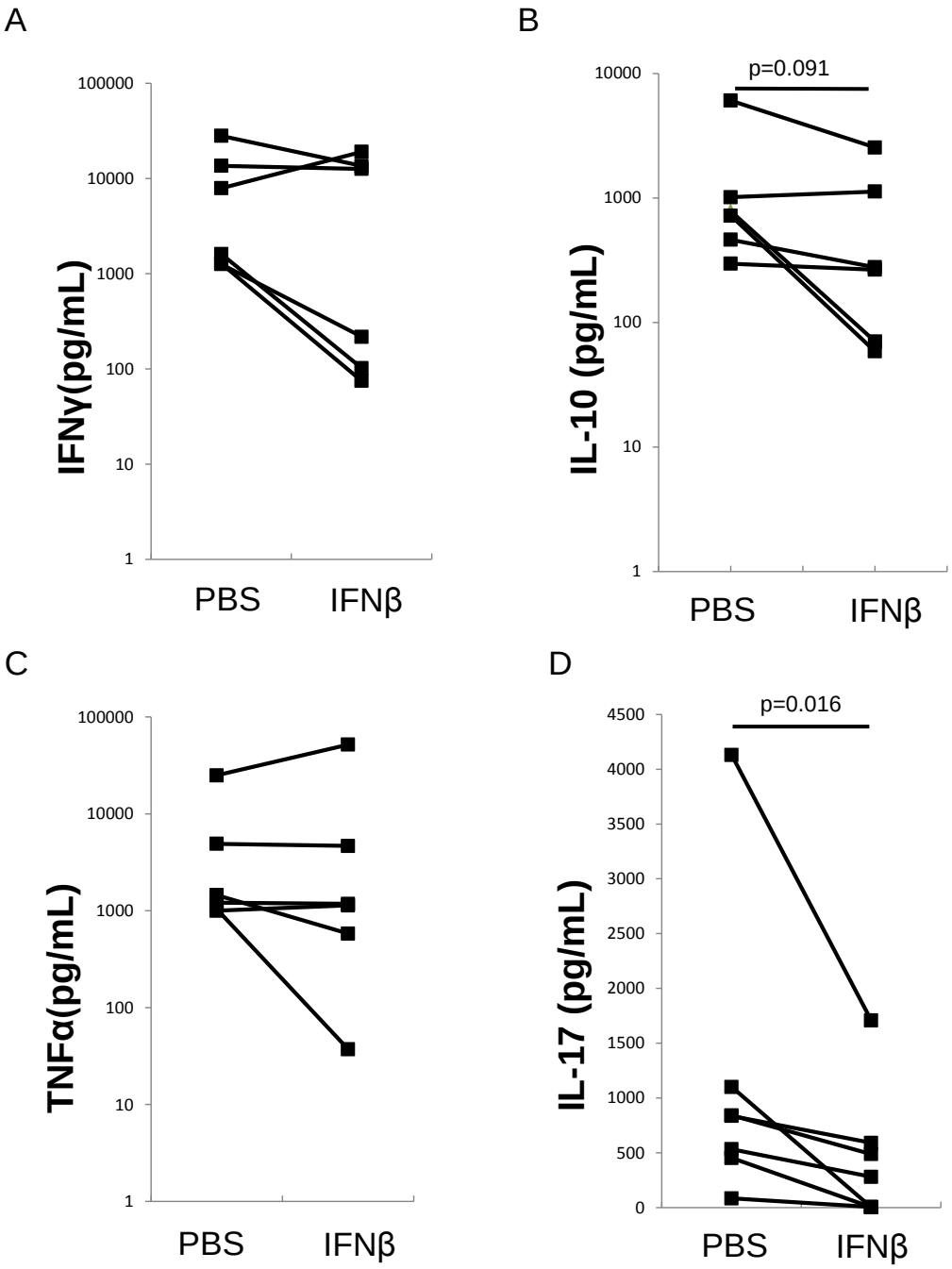

Figure 5.11 Addition of IFNβ to biopsies from healthy donors alters cytokine production by walk-out LPMCs after T cell activation *Cells were walked-out in the presence or absence (PBS) of IFNβ (1000U/mL) for 48 hours. The cells were collected and stimulated for 24 hours with anti-CD3/CD28 antibodies as previously described. The supernatant was collected and IFNγ, IL-10, TNFα and IL-17 were measured by multiplex ELISA . A IFNγ B IL-10 C TNFα and D IL-17 (n=6). Statistical analysis was performed by paired t tests.*

5.4.2.3.2 The effect of exogenous IFNβ on cytokine production of whole intestinal biopsies from healthy individuals

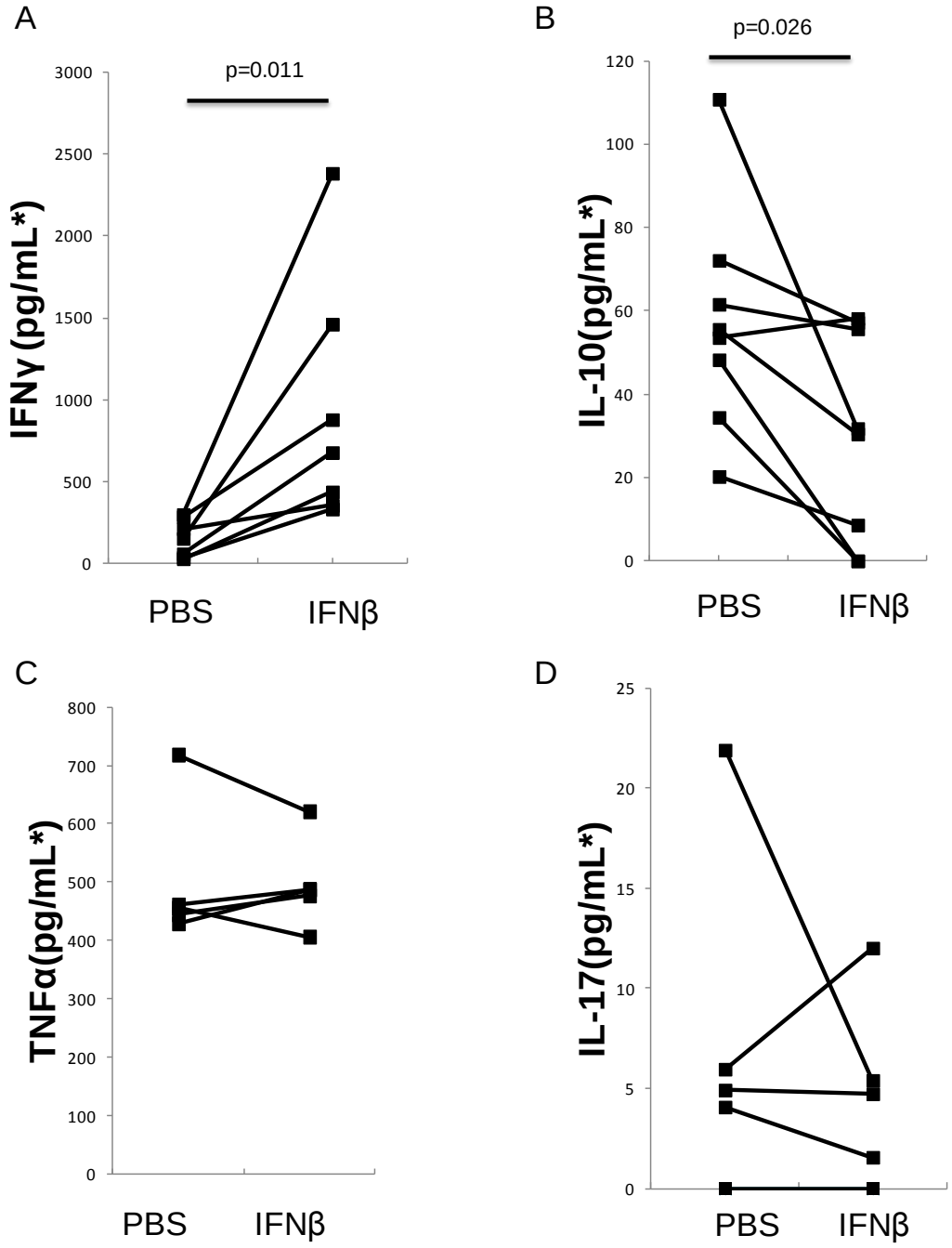

Figure 5.12 Exogenous IFNβ alters cytokine production in biopsy organ culture from control subjects *Intestinal biopsies were cultured in the presence or absence (PBS) of IFNβ (1000U/mL). After 48 hours, the supernatant was collected and IFNγ, IL- 10, TNFα and IL-17 were measured by multiplex ELISA as previously described. (A) IFNγ; (B) IL-10; C TNFα; and D IL-17 (n=8) Note: Levels of IL-17 were very low and several samples contained no detectable L-17 irrespective of the presence of IFNβ to the limits of the detection of the cytokine by the multiplex kit*

*(see chapter 2, section 2.5). *concentrations were corrected according to the ratio of number of cells in the samples, as described in chapter 2 and chapter 5, section 5.4.2.1.1.2.*

Figure 5.12 shows the effect on cytokine production of the addition of T1IFN cultures of colonic biopsies from healthy tissue. The striking effect was the increase in IFNγ production in the presence of added T1IFN, with a reduction in IL-10. This was surprising as these are similar findings to the results with the use of antibody neutralisation of endogenous IFNβ. No significant effect was seen with other cytokines measured, although concentrations were generally low.

5.4.2.4 The effect of IFNβ on intestinal T cell function in IBD

5.4.2.4.1 The effect of IFNβ neutralisation on cytokine production by T cells from Non-Inflamed IBD intestine, measured by intracellular flow cytometry

In chapter 4, it was demonstrated that pSTAT1, both with and without exogenous T1IFN stimulation, was more frequently detected in the intestinal $CD4^+$ T cells from non-inflamed areas of IBD patients compared to both inflamed areas and control samples. It was hypothesised that this may be a reflection of increased responsiveness to signalling from endogenous T1IFN. It was therefore of interest whether this group was also more sensitive to manipulation with neutralisation of endogenous IFNβ. It may have been expected, given the higher frequency of constitutive pSTAT1 in intestinal T cells from non-inflamed IBD samples, that there would be a greater effect in neutralisation of IFNβ on T cell cytokine production. This is particularly as the pSTAT1 activation has been shown to be at least partially driven by endogenous IFNβ (see chapter 4, figure 4.12).

Data showing the effect of IFNβ neutralisation on $CD4^+$ T cells cytokine production from the non-inflamed IBD samples, as measured by intracellular flow cytometry, are shown in figure 5.13. Similar to the T cells from control samples, there were significantly more IFNγ-producing T cells in these samples after IFNβ neutralisation (figure 5.13B). However, there was not an associated decrease in IL-10-producing T cells. In fact, the trend was for more IL-10-producers. Also, there were increased numbers of T cells producing both TNFα and IL-17, an effect that was not seen from the healthy control samples. So, in contrast to the healthy controls, where endogenous IFNβ appears to helping to maintain a regulatory T cell phenotype, in these non-inflamed samples the endogenous IFNβ appears to be restraining a broad range of both pro- and anti-inflammatory cytokine production.

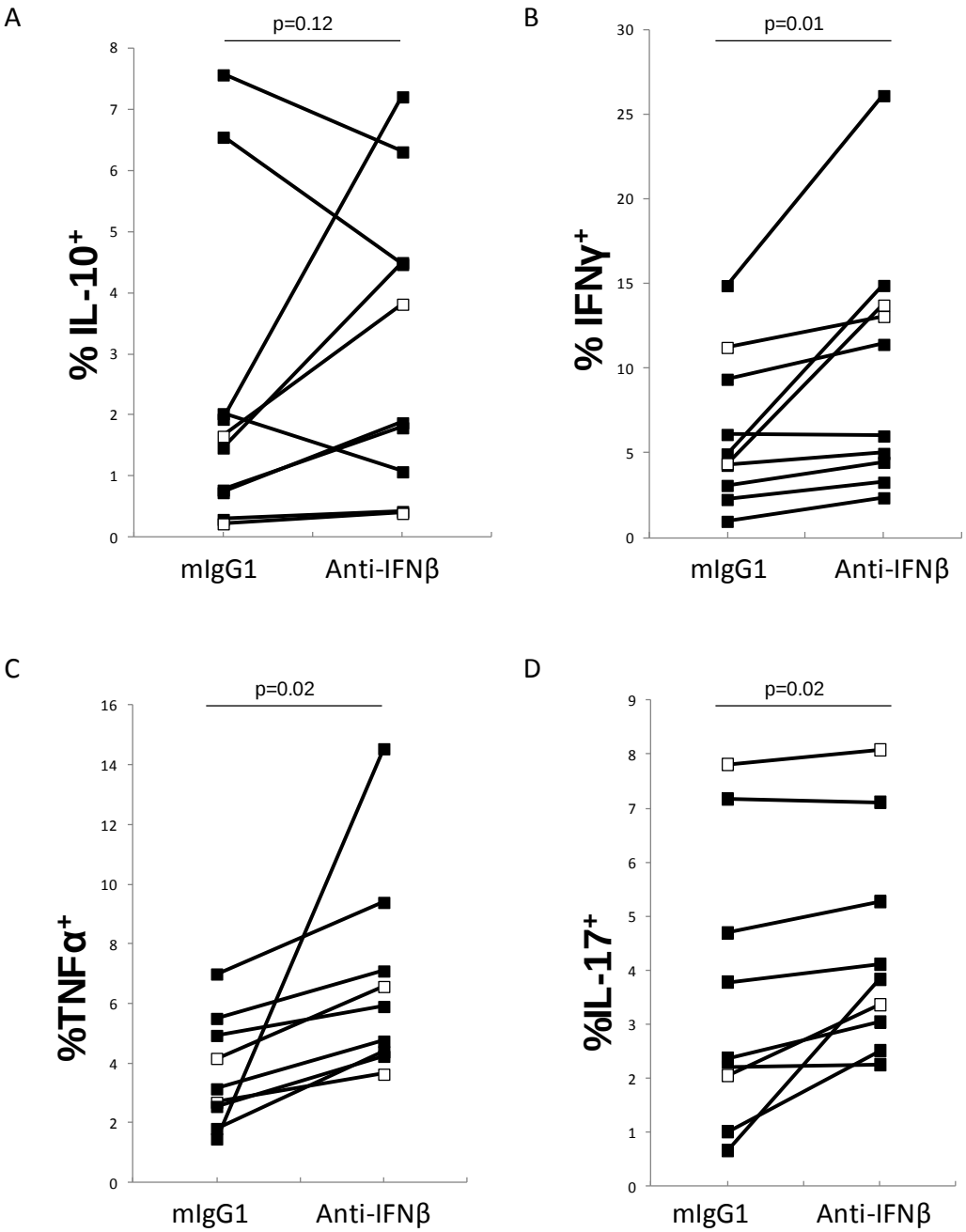

Figure 5.13 IFNβ neutralisation increases production of multiple cytokines by intestinal CD4+ T cells from non-inflamed IBD samples*. LPMCs were isolated by walk-out in the presence of anti-IFNβ antibody or isotype-matched control (mIgG1) for 48 hours. Cells were harvested and T cells activated with CD3/CD28 antibodies for 24 hours. Intracellular cytokines were measured by flow cytometry. Results shown are for cells gated on the basis of light scatter and CD4 expression. Black boxes: Crohn's disease, n=8; white boxes: UC, n=2. Statistical analysis was performed by paired t tests.*

As observed with T cells from the healthy control samples, $CD8^+$ T cells from non-inflamed IBD tissue had overall a reduced frequency of cytokine producing cells.

The only significant difference that remained between the samples that were exposed to the anti-IFNβ antibody and isotype control was that the frequency of IFNγ-producing cells was increased, as in the $CD4^+$ cells (figure 5.14B).

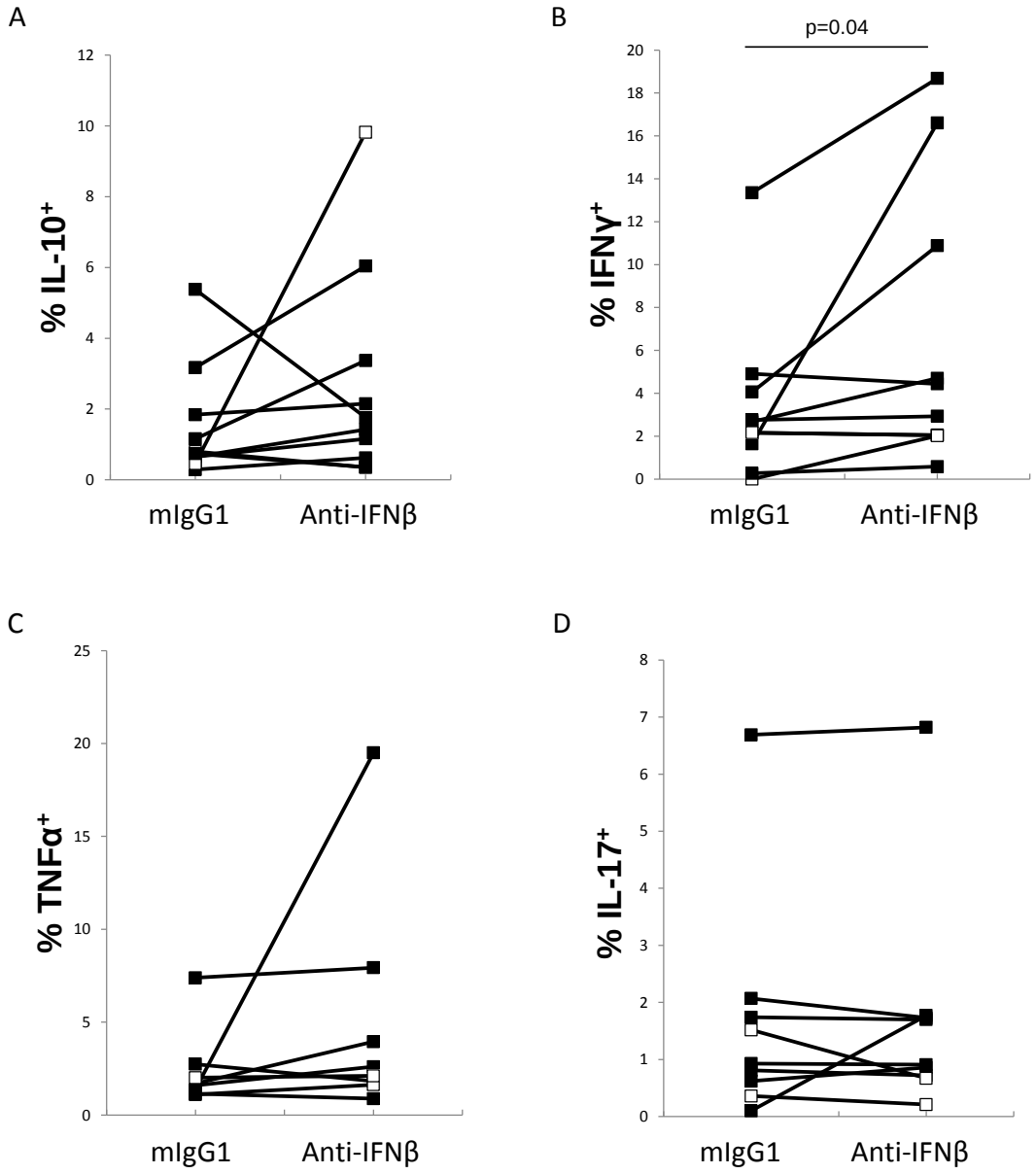

Figure 5.14 IFNβ neutralisation increased the frequency of IFNγ-producing $CD8^+$ intestinal T cells from non-inflamed IBD samples*. Experiments performed as per earlier figures; LPMCs were isolated by walk-out in the presence of neutralising anti-IFNβ antibody or isotype-matched control (mIgG1) for 48 hours. Cells were harvested and T cells activated with anti-CD3/CD28 antibodies for 24 hours. Intracellular cytokines were measured by flow cytometry. Results shown for cells gated on the basis of light scatter and CD8 expression. Black boxes: Crohn's disease; white boxes: UC. Statistical analysis was performed by paired t tests.*

5.4.2.4.2 The effect of IFNβ neutralisation on cytokine production from whole intestinal biopsies of Non-Inflamed IBD samples

When the absolute quantity of cytokine released from non-inflamed IBD biopsy supernatants was measured by multiplex ELISA (figure 5.15), no consistent effect of anti-IFNβ was seen. This finding contrasts with the intracellular cytokine staining data obtained with antibody-stimulated $CD4^+$ T cells described above (figure 5.13) However, it should be borne in mind that cytokines produced by non-T cells may contribute to what is measured in biopsy supernatants and that, unlike the intracellular cytokine analysis, these cultures were not subject to stimulation with anti-CD3/28 antibodies. Unfortunately, there were insufficient samples to analyse the cytokines by ELISA after anti-CD3/CD28 stimulation.

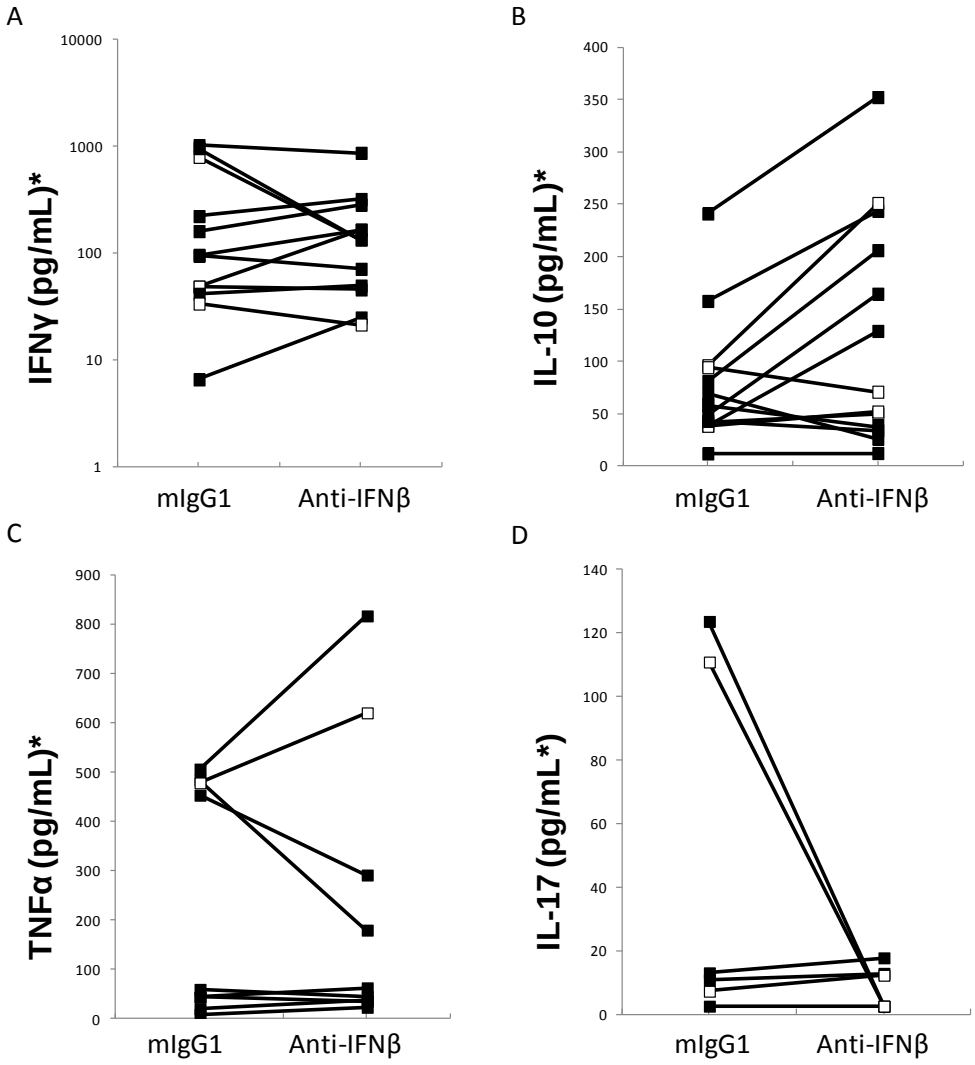

Figure 5.15 IFNβ neutralisation has no effect on spontaneous cytokine release from biopsies obtained from non-Inflamed IBD mucosa. *Supernatants were collected from biopsies cultured with neutralising anti-IFNβ antibody or isotype control (mIgG1) as previously described. Cytokines were measured by multiplex ELISA as follows: IFNγ (A), IL-10 (B), TNFα (C) and IL-17 (D). n=12. Black boxes: Crohn's disease; white boxes: UC. *concentrations were corrected according to the ratio of number of cells in the samples, as described in chapter 2 and chapter 5, section 5.4.2.1.1.2.*

5.4.2.4.3 The effect of IFNβ neutralisation on the expression of master regulators of transcription by intestinal CD4+ T cells from non-inflamed IBD mucosa

As with the healthy control specimens, it was possible to simultaneously measure the proportion of T cells expressing FoxP3, T-bet and RORγt transcription factors (associated with T_{regs}, T_H1 and T_H17 cells respectively) in CD4 T cells obtained from non-inflamed IBD biopsies in the presence or absence of neutralising anti-IFNβ. Similar to $CD4^+$ T cells from control tissue, there was no consistent effect of the presence of anti-IFNβ antibody on the expression of the transcription factors tested. The percentage of $CD4^+$ intestinal T cells expressing FoxP3 was mean 50% (n=3), with no change in cells cultured IFNβ antibody. This was a trend for higher expression than in healthy controls (mean 30%, see section 5.4.2.2.4 above). The percentage of intestinal T cells expressing T-bet was mean 63% (n=5) with no significant change with IFNβ antibody (mean 70%, p=0.40). T-bet expression in intestinal T cells appeared to be more common in non-inflamed IBD samples compared with controls (mean 27%, section 5.4.2.2.4, but this did not reach statistical significance with these small numbers of samples (ANOVA, p=0.11), although this was shown in chapter 4 (figure 4.5). RORγt expression was only rarely detected (0.8% to 2.4% of $CD4^+$ T cells) with no differences detected between cells cultured with or without IFNβ neutralisation and or between cells from non-inflamed IBD or control tissue.

5.4.2.4.4 The effect of IFNβ neutralisation on cytokine production by T cells from Inflamed IBD intestine

Given the large amounts of cytokine produced by T cells from inflamed IBD mucosa, it was felt unlikely that (potentially only partial) neutralisation of IFNβ in cultures of inflamed biopsies from IBD patients would have a significant measurable effect.

Indeed, no effect of neutralising anti- IFNβ was observed for either $CD4^+$ or $CD8^+$ intestinal T cells from inflamed IBD samples on any of the measure cytokines (figures 5.16 and 5.17 respectively).

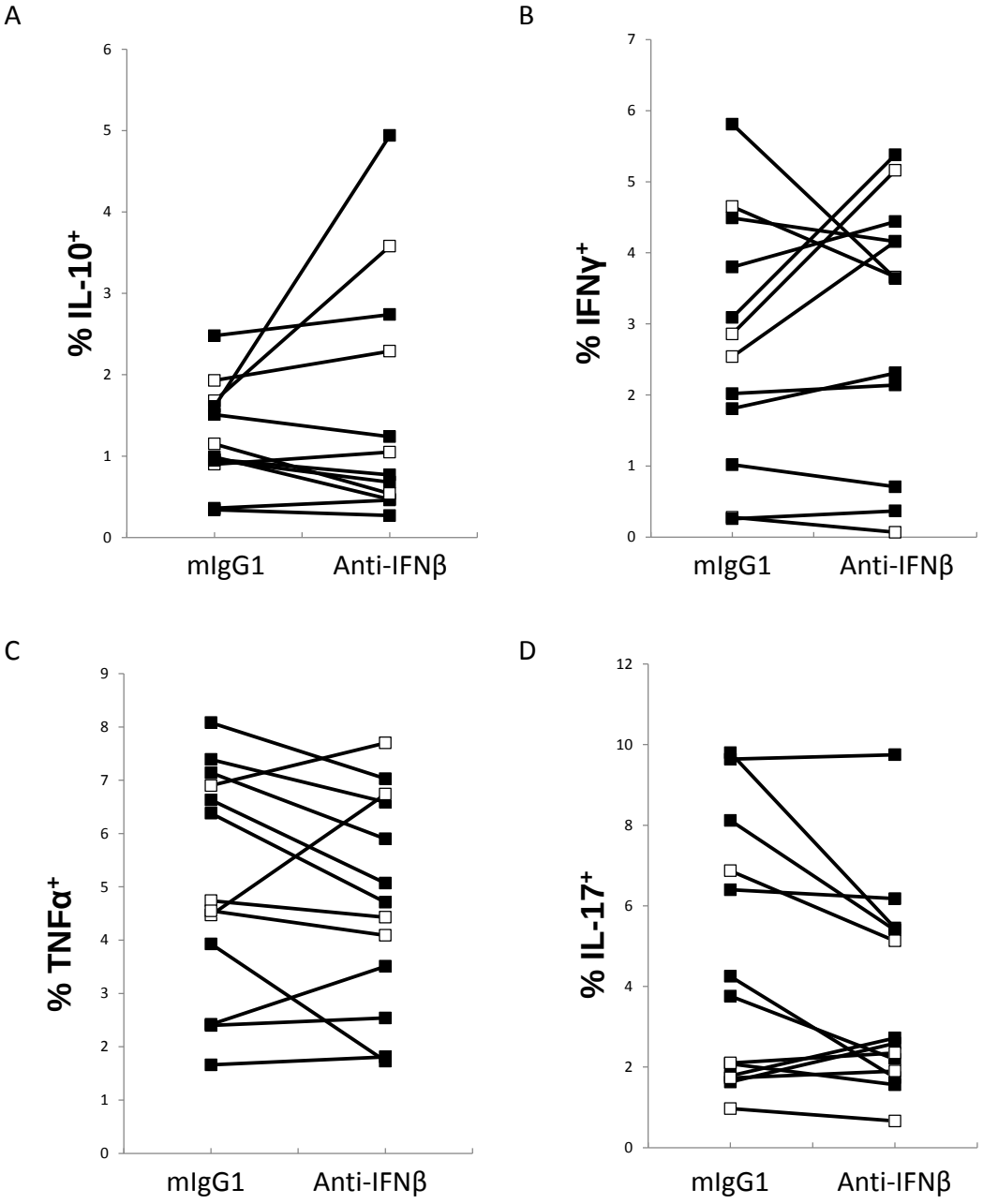

Figure 5.16 IFNβ neutralisation has no consistent effect on $CD4^+$ T cell cytokine production from inflamed IBD samples. *LPMCs were walked-out in the presence of neutralising anti-IFNβ antibody or isotype-matched control (mIgG) for 48 hours. Cells were harvested and T cells activated with anti-CD3/CD28 antibodies for 24 hours. Intracellular cytokines were measured by flow cytometry as displayed. Results shown are for cells gated on the basis of light scatter and CD4 expression. Black boxes: Crohn's disease samples, n=9; white boxes: UC samples, n=3.*

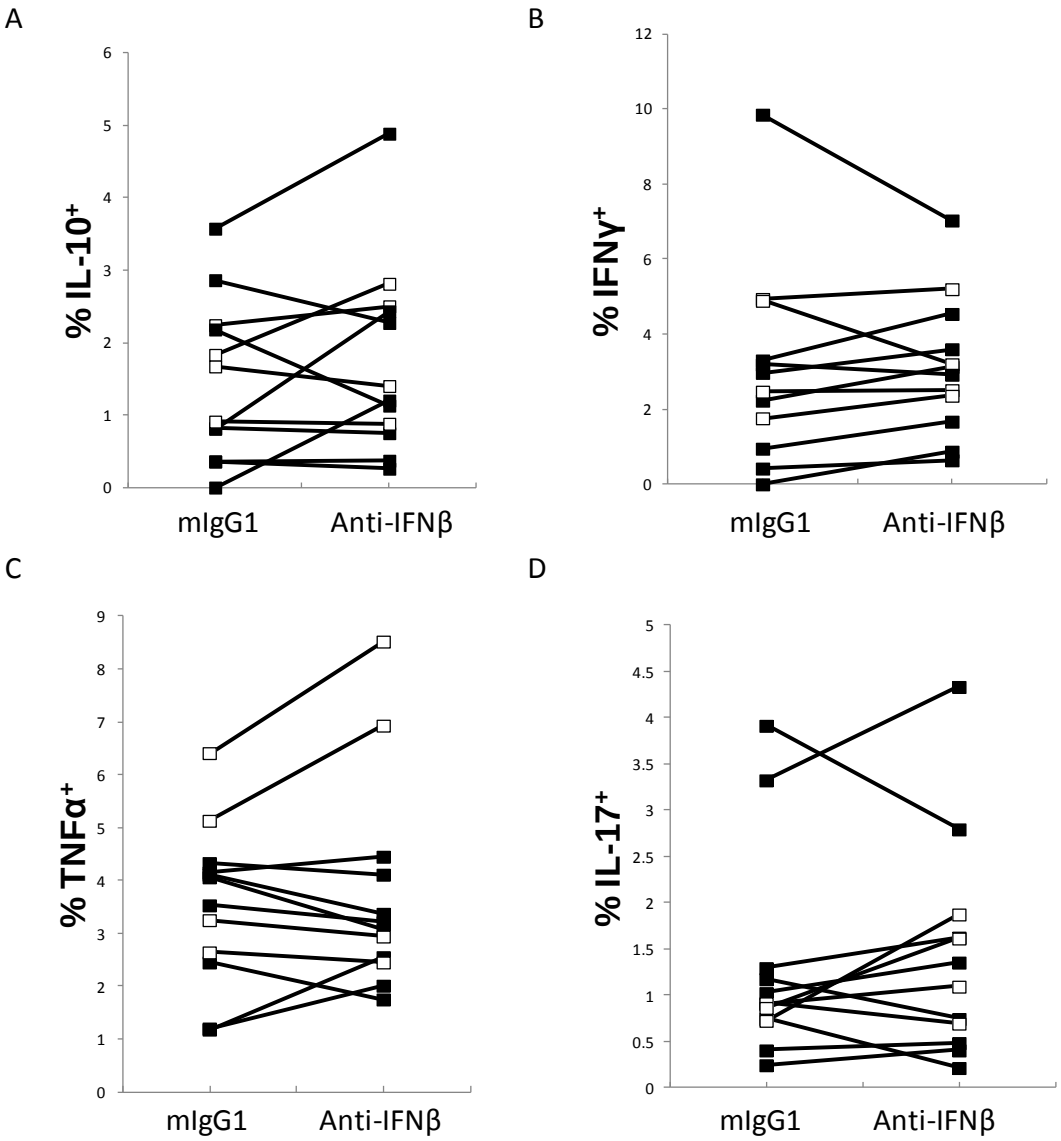

Figure 5.17 IFNβ neutralisation has no consistent effect on CD8+ T cell cytokine production from inflamed IBD samples. *LPMCs were walked-out in the presence of IFNβ antibody or isotype control for 48 hours. Cells were harvested and T cells activated with anti-CD3/CD28 antibodies for 24 hours. Intracellular cytokines were measured by flow cytometry as displayed. Results were gated on scatter and CD8+. Black boxes: Crohn's samples; white boxes: UC samples.*

There were no experiments performed by multiplex ELISA on the effects of IFNβ neutralisation on supernatants from samples of inflamed IBD mucosa.

5.4.2.4.5 The effect of IFNβ neutralisation on master regulators of transcription in intestinal T cells from inflamed IBD mucosa

Again, the percentage of $CD4^+$ T cells expressing FoxP3, T-bet and RORγt were measured and there were no differences between cells obtained from cultures with IFNβ neutralisation compared with control cultures. FoxP3 was expressed by a mean of 45% of $CD4^+$ T cells (n=5). This was a proportion similar to that observed with similar cells from non-inflamed samples (mean 50%; see above section 5.4.2.4.3). There were similar trends for differences between inflamed IBD tissue and healthy control tissue as were observed with non-inflamed IBD tissue and control samples (ie, more frequent FoxP3 and T-bet expression), but again these did not reach statistical significance in this small series.

5.4.2.4.6 Summary of effects of IFNβ on intestinal T cells in non-inflamed and inflamed IBD samples

The above section (5.4.2.4) shows the data on the effects of IFNβ neutralisation on whole biopsy and ($CD4^+$) T cell cytokine production in samples from IBD patients. In general, the presence of anti-IFNβ antibody did not show any consistent effect, with the main exception being in the changes in cytokine profile in $CD4^+$ T cells from non-inflamed IBD samples (figure 5.13).

Regarding exogenous IFNβ, unfortunately there were insufficient IBD supernatants stored to comment on these effects in cells from either inflamed or non-inflamed IBD samples. In addition, ELISA experiments were also not performed in all conditions, particularly from biopsies from inflamed samples. This was as the focus of the experiments was a on the non-inflamed samples, particularly to correlate with the differences found in pSTAT1 expression from these cells in chapter 4 (figure 4.1). These issues will be discussed further below and in the final chapter.

5.5 Discussion

This chapter showed the increased responsiveness of intestinal T cells to exogenous IFNβ by increased ISG expression. It also showed the effects of IFNβ neutralisation on intestinal T cells, suggesting a role for endogenous IFNβ in the human intestine promoting a regulatory T cell cytokine profile. The role for endogenous IFNβ in IBD samples is less clear, but appears to restrain a broad range of cytokine production in intestinal $CD4^+$ T cells.

5.5.1 T cell Responsiveness to T1IFN

After stimulation with exogenous IFNβ, the ISGs were highly upregulated (figure 5.2), confirming, in intestinal T cells, the relationship between T1IFN exposure and expression of these genes. The IBD samples, however, were significantly more responsive by this measure. This correlates with the increased pSTAT1 signal found in chapter 4 (figure 4.1). However, both these results are perhaps surprising given that it has been shown that more activated T cells have reduced responsiveness to T1IFN by measurement of ISG expression (Dondi et al., 2003). The expression of TLR9, a PRR strongly associated with induction of T1IFN, has been shown to be increased in peripheral B cells from patients with IBD compared to healthy controls (Berkowitz et al., 2013). However, in this study the B cells were less responsive to ODN-CpG (in producing T1IFN), than healthy controls. This implies a complex picture of responsiveness related to the T1IFN pathway in patients with IBD. As noted in chapter 4, section 4.5.4, differences in $CD4^+$ T cell responsiveness in the intestine are not necessarily reflected in the peripheral blood. The data from this chapter supports a picture in which intestinal T cells in IBD are more responsive to T1IFN, but this does not necessarily have the same pro-regulatory consequence found in health (discussed below).

5.5.2 The effect of T1IFN on intestinal T cell cytokine production in health

5.5.2.1 Endogenous T1IFN has pro-regulatory effects on $CD4^+$ intestinal T cells in health

As hypothesised, neutralisation of endogenous T1IFN did change elements of the phenotype of the intestinal T cells from control samples *in vitro*, particularly with regard to cytokine production. Both intracellular cytokine analysis of $CD4^+$ T cells and ELISA of cytokines releases into the supernatant of cultured biopsies showed that IFNβ in the human intestine has a predominately pro-regulatory effect on T cells in the healthy human intestine and therefore may contribute to the maintenance of homeostasis. This is evidenced by a reduced frequency of IL-10-producing $CD4^+$ T cells when IFNβ was neutralised with a coincident strong trend for an increased frequency of IFNγ producing cells (figure 5.7), combined with an increase in the amount of IFNγ detectable in the supernatants of biopsies cultured with anti-IFNβ in association with a trend towards reduction in IL-10 secretion (figure 5.10).

These ELISA data showed only a trend towards reduced IL10 production in the presence of anti-IFNβ. It is likely that there would be many cells present with the biopsy tissue that could contribute, in addition to T cells, to the production of IL-10 detected in the culture supernatants; the effects of endogenous IFNβ on these other cell types is unknown but likely to be complex. IFNγ is primarily produced by T cells (O'Shea and Murray, 2008), so is a more sensitive marker of the effect of T1IFN on this cell type in the context of a mixed cell population. The effects of IFNβ neutralisation on IFNγ production, however, also did not reach significance by intracellular staining of $CD4^+$ T cells. This may be because the percentage of IFNγ-producing cells was already very high, and therefore difficult to further increase. In addition, the significant effect of anti-IFNβ on secretion of IFNγ as measured by multiplex ELISA may be reconciled with the intracellular cytokine data if it reflects an

increased production on a per cell basis rather than an increase in the frequency of cells committed to IFNγ production. However this was not seen after the T cells were activated with anti-CD3/CD28 antibodies (figure 5.9). Once again, it is possible that the IFNγ production was already so high under conditions of strong TCR and co-stimulatory signalling that it was difficult to increase, or it may be related to the kinetics of cytokine release and the possibilty that this experiment missed the critical time point at which modulation of cytokine production was evident.

The numbers of cells shown to produce cytokine were generally low in the intracellular staining experiments. It must be noted that the activation was produced by anti-CD3/CD28 antibody stimulation, not the more traditionally used PMA/Ionomycin combination. This may explain differences with other publications in intracellular cytokine measurements.

As discussed in the results section, the anti-CD3/CD28 method of T cell activation was chosen as it was felt more likely to be amenable to modulation. The effect of presumably low levels of T1IFN constitutively setting a “tonic” signal to T cells, priming them to a particular cytokine response may well not be detectable when PMA/Ionomycin directly activate transcription without cell surface receptor interaction. It has been shown that lamina propria T cells are relatively hyporesponsive to CD3 activation (De Maria et al., 1993; Ebert, 2006). This responsiveness could be restored by “resting” of tissue in culture medium for 24 hours (De Maria et al., 1993). It is unclear whether isolated cells via the walk-out method would restore this anti-CD3 “responsiveness” (ie that the walk-out period would be equivalent to the ‘rest’ in the previously published study). The relatively low number of cytokine producing cells in response to anti-CD3 antibody in this chapter would suggest otherwise.

The presence of anti-IFNβ antibody did effect TNFα and IL-17 T cell production, but in an inconsistent way with no overall significant change. This is in contrast to the increase in the production of TNFα seen in the murine IFNαβR KO mice, where

T1IFN was shown to support Treg function in colitis (Kole et al., 2013; Lee et al., 2012). There are many possible reasons for this difference, but include that targeting the receptor may more completely block the effects of IFNβ, and will also impact on signalling by other T1IFNs, which haven't been explored in these human experiments. The murine studies also provide conflicting data: one suggests that the effects of T1IFN on T cells are direct (Lee et al., 2012), but another shows the requirement for APCs (Kole et al., 2013). The need for APCs to mediate the tolerogenic potential of T1IFN on T cells is supported by other work where bacterial CpG motifs promote regulatory T cell functions (Hofmann et al., 2010). In this chapter, it is impossible to comment on whether the IFNβ effects on T cells observed in the current study are direct or indirect, although there is certainly a suggestion that the effects of IFNβ neutralisation are not restricted to T cells alone.

5.5.2.2 Additional effects on intestinal T cells of endogenous T1IFN

5.5.2.2.1 The effect of IFNβ neutralisation on the master regulators of T cell transcription

In contrast to published murine studies the IFNβ neutralisation experiments reported here found no evidence for an effect of IFNβ on FoxP3 expression, or indeed on expression of T-bet or RORγt expression, the master regulators of T_{regs}, T_H1 and T_H17 cells respectively. However, FoxP3 expression in humans can be induced with activation of the cell (Wang et al., 2007) and is therefore not necessary a reliable correlate of regulatory phenotype in T cells from cultured human biopsies. This is particularly so because the walk-out of LPMCs may be associated with significant T cell activation, as indicated by high CD69 expression on the T cells (see chapter 3, figure 3.11). It is perhaps more surprising that there was no change in the T-bet expression with anti-IFNβ, given the increase in IFNγ production. It is unclear why

this would be, but perhaps the T-bet expression is stable in the gut memory T cells and the effects on IFNγ are via different mechanisms. Also, the frequency of T cells expressing T-bet was generally high even in the absence of anti-IFNβ and, unlike IFNγ production, may not be readily increased further by neutralisation of IFNβ.

Previous work on human PBMCs showed that under T_H1 polarising conditions, IFNβ increased IL-10 production by $CD4^+$ T cells (Axtell et al., 2010), and after LPS stimulation, T1IFN increased T cell IL-10 production and inhibited IL-12 (Wang et al., 2000). The findings presented in this thesis are consisted with this earlier work since human intestinal T cells from walk-out cultures express high levels of T-bet (figure 4.5) suggesting they are very "T_H1-like", and they would have been exposed to LPS during isolation (Austin et al., 2005). Therefore it is consistent that the IFNβ neutralisation would reduce IL-10 production as was seen (figure 5.7).

There were consistently fewer $CD8^+$ T cells producing cytokines than $CD4^+$ cells under the conditions used in these studies. This could be for a large variety of reasons, but the $CD4^+$ cells were of primary interest as they are considered particularly important in the regulation of gut inflammation (Shale et al., 2013). The effect of IFNβ neutralisation on the function of CD8+ cells was also less marked, with the only statistically significant effect seen being an increase in number of IFNγ-producing $CD8^+$ cells from non-inflamed IBD samples (figure 5.14). This suggests that whatever effect endogenous IFNβ has on intestinal T cells, it is mediated via $CD4^+$ more than $CD8^+$ cells

This constitutive or tonic IFNβ signalling in health would likely be important in maintaining levels of signalling molecules in the gut T cells, priming them for an appropriate response to a, for example, viral stimulus. This priming has been suggested in mouse models to be critical in maintaining homeostasis (Hertzog and Williams, 2013; Zhao et al., 2012). The pathways of T cell responsiveness to T1IFN, including via STAT activation, will be discussed later in this chapter.

5.5.2.2.2 The effect of IFNβ neutralisation of T cell survival and recovery from tissue

Given the pleotropic effects of T1IFN, other possible effects of neutralising IFNβ on intestinal T cells were considered. Total cell numbers collected from cell samples did not differ in the presence of anti-IFNβ, although there was a trend towards decreased yield, consistent with a (small) effect on cell survival and/or migration of the cells from the biopsy tissue. However, there was no significant difference in the viability of cells cultured with or without the antibody as assessed by the Live/Dead™ staining (always >98% viability). It was, of course, not possible to assess the viability of the cells that did not egress from the biopsy. When T1IFN (1000 IU/mL for 24 hours) was added to the walk-out culture, there was a significant increase in the numbers of cells recovered. This suggests that exogenous T1IFN has the capacity to increase T cell survival and/or migration.

The known effects of T1IFN on T cell survival are complex, but appear to favour an increase in apoptosis with addition of T1IFN in mouse models (Carrero et al., 2004; O'Connell et al., 2004). This is in contrast to the findings from this chapter. However, in human PBMCs, when the T cells are activated, T1IFN appears to be protective against apoptosis (Kaser et al., 1999). These differences are likely to be due to timing and context of the T1IFN exposure. In resting (mouse) T cells different effects on T1IFN on cell survival appear to be reflect the involvement of different signalling machinery with signalling via STAT1 supporting apoptosis, but signalling via STAT3 being anti-apoptotic (Tanabe et al., 2005). These effects on cell survival are of interest given the theory that (failure of) apoptosis may play a significant role in the pathogenesis of IBD (Boirivant et al., 1999), particularly because in this thesis STAT1 signalling was increased in non-inflamed IBD samples (Fig 4.1), but STAT3 was increased in inflamed IBD samples (Fig 4.4), and STAT3 has been shown to

have increased expression in intestinal T cells from other studies (Lovato et al., 2003; Mudter et al., 2005; Suzuki et al., 2001).

Collectively these observations may suggest a role for T1IFN driving T cell apoptosis to support intestinal homeostasis. This could be disrupted in an inflammatory environment, in which increased activation of T cells would switch T1IFN signalling from predominately STAT1 mediated to predominately STAT3 mediated with a resulting switch to increased cell survival. Increased survival of pathogenic CD4+ T cells could help perpetuate T cell driven inflammation.

T1IFN has also been shown to enhance survival of human memory T cells, via enhanced production of IL-2, which then produce IFNγ (Davis et al., 2008). However, again there are conflicting reports: in another study high dose IFNα reduced IL-2 secretion by human $CD4^+$ T cells (Zella et al., 2000). Again these different outcomes are likely to be related to timing, dose and context of T1IFN exposure, with reduced responsiveness (to T1IFN) after T cell activation (Dondi et al., 2003).

It is more difficult to comment on the effect of T1IFN on T cell migration, as the process of egress from denuded biopsies into culture medium may not be comparable to functional T cell migration. However, it is known that PBMCs from IFNβ-treated MS patients had reduced $CD4^+$ T cell migration in an *ex vivo* transwell assay (Dressel et al., 2007). In contrast, T1IFNs have also been shown to enhance T cell chemotaxis (Avraamides et al., 2007). T1IFN has been shown to upregulate CD69 (Feng et al., 2005; Sun et al., 1998), which has also been proposed to promote retention of T cells in organized lymphoid tissue (Masopust and Schenkel, 2013). CD69 is highly expressed by human intestinal T cells as reported here in Chapter 4 and also in the literature (Sathaliyawala et al., 2013). However, the high levels of CD69 expression from the human intestinal T cells, and the proposed related ability to be retained in, for example, Peyer's Patches or MLNs, may not be related to cell recovery from intestinal biopsy samples (which are lamina propria

cells). CD69 has also been found to be required for the tolerogenic effect of T1IFN in mice, in that Tregs from CD69 knockout mice do not retain the ability to ameliorate colitis (Radulovic et al., 2012). The CD69 expression shown in the walk-out samples was associated with pSTAT1 (see chapter 4, figure 4.6), and this suggests a possible link between T1IFN signalling, CD69 expression and tolerogenic $CD4^+$ T cells.

Overall, it is possible that the effect of IFNβ neutralisation and/or additional IFNβ on cell recovery from biopsies could have almost any combination of positive and negative effects on both cell survival and migration. It would require a large set of separate experiments to address the likelihood of any factor predominating, and was beyond the scope of this thesis.

5.5.2.3 The effects of exogenous T1IFN on mucosal T cell phenotype

The experiments showing the effect of additional IFNβ added to the walk-out samples from controls showed unexpected effects on cytokine production. Given that in cultures of healthy tissue addition of anti-IFNβ antibody lead to a more pro-inflammatory cytokine profile, it was expected that additional T1IFN would have the opposite effect and reduce the production of inflammatory cytokines. However, in whole biopsy cultures, IFNγ was *increased* by the addition of 1000U/mL of IFNβ to biopsy cultures for 48 hours, and IL-10 was decreased (figure 5.11). This was a very similar pattern to that observed with neutralisation of IFNβ. The fact that the main effect observed was on IFNγ production suggests this is a predominately T cell driven effect. It may be due to the increased survival of intestinal T cells, or other effects, as demonstrated by enhanced cell recovery with additional IFNβ (see section 5.4.2.2.5). While the cytokine concentrations are corrected for cell number *per se*, better cell survival may be reflected in enhanced cytokine release by the cells present in the walk out cultures.

Following stimulation with anti-CD3/CD28 antibodies, the effects of prior exposure to added IFNβ on IFNγ production were no longer observed. There was also no significant effect on IL-10 production, although there remained a trend towards reduction (figure 5.10). Once again, the high concentrations of IFNγ induced by anti-CD3/CD28 stimulation may have represented maximum production and have precluded detection of enhancing effect of additional IFNβ. The subtle effects on IL-10 production may suggest a contribution of other cell types, although it must be stressed that levels of all cytokines examined were low in the absence of anti-CD3/CD28 activation. In contrast to the lack of effect on IFNγ and IL-10, production of IL-17 in response to anti-CD3/CD28 was significantly reduced in cell previously exposed to the exogenous IFNβ. This observation is consistent with the literature on the effect of T1IFN on T_H17 cells (Guo et al., 2008; Moschen et al., 2008).

These results observed with additional T1IFN at first seem contradictory to the IFNβ neutralisation experiments, but in fact are not necessarily inconsistent. It is likely that there are real differences between the effects of low concentration of endogenous T1IFN which provide important tonic signals and the effects of exogenous T1IFN added at a concentration of 1000U/mL. This concentration, which, although standard for published works involving IFNβ, is unlikely to be physiological.

Having said this, the apparent pro-inflammatory effect on cytokine production of exogenous IFNβ is consistent with the early literature on the effects of T1IFN. The early work analysing the effect of T1IFN on T cell differentiation in humans showed STAT4-dependent T_H1 cell enhancement (Rogge et al., 1998; Shibuya et al., 2003). It therefore may be that different dominant signalling pathways can lead to very different functional outcomes of T1IFN signalling. It has indeed been previously shown in mice that T1IFN signalling via STAT1 was regulatory, but via STAT4 pro-T_H1 phenotype (Nguyen et al., 2002). This model of T1IFN signalling using different

STAT molecules for different functional outcomes will be discussed further in the section on IBD below.

5.5.3 T1IFN effects on cytokine production from intestinal T cells in Inflammatory Bowel Disease

5.5.3.1 Endogenous IFNβ restrains general cytokine production from T cells in non-inflamed IBD mucosa

While the effects of IFN neutralisation on T cells from healthy tissue had a distinct pattern which implicated endogenous IFNβ in the promotion of IL-10 production but inhibition of IFNγ, the results from the IBD patient samples were less clear. The IBD samples were a very heterogeneous group. This heterogeneity was in a number of different parameters including most obviously the grouping together of Crohn's disease and UC patients together. The primary hypothesis of this chapter, however, was that T1IFN has a pro-regulatory role in maintaining homeostasis in health and it is perhaps unsurprising that these pro-regulatory effects are disrupted in the context of inflammatory disease. It was, nevertheless, of interest to examine the effect of T1IFN particularly in the non-inflamed areas of IBD intestine. Specifically, we wished to determine whether the non-inflamed samples would show a “pre-inflammatory” phenotype, and be similar to inflamed IBD samples, or a more “pro-regulatory” phenotype, that might be involved in locally restraining inflammation of the context of disease

The effect of IFNβ neutralisation in these cultures of non-inflamed tissue did not map definitively to either pattern. Instead, the data suggest that endogenous IFNβ non-selectively restrains T cell production of a broad range of cytokines. With the exception of IL-10, production of all cytokines tested was significantly increased when endogenous IFNβ was neutralised as measured by the frequency of cytokine producing cells in intracellular flow cytometry experiments (figure 5.13). The

increased frequency of T cells producing IFNγ, TNFα, and IL17 is consistent with published effects of T1IFN on intestinal T cells assessed by mouse models (Kole et al., 2013; Lee et al., 2012). However, none of these effects were seen in the ELISA experiments. This is difficult to explain, but may be due to kinetics, or the greater contribution of non ($CD4^+$) T cells to cytokine production in IBD tissue compared to healthy controls. This is particularly relevant, as the only multiplex ELISA data obtained for the IBD samples was from whole biopsy supernatants rather than cultures of activated T cells. Of note, the cytokine release measured by ELISA was broadly equivalent in the cultures of control and non-inflamed IBD samples. This is as expected given the non-inflamed IBD samples are from macroscopically and histologically uninflamed areas, but it does not help explain the differences in response to IFNβ neutralisation in the intracellular flow cytometry data.

5.5.3.2 Endogenous T1IFN has little effect on cytokine production from T cells in inflamed IBD mucosa

In samples from inflamed IBD mucosa, there was no significant effect of IFNβ neutralisation on the percentage of T cells producing any of the measured cytokines. This was perhaps not surprising, as the large quantities of various cytokines and other factors produced from inflamed IBD mucosa would have subjected the T cells to a strong inflammatory milieu, and this would conceivably leave them refractory to what would be relatively low levels of T1IFN signalling. Thus any manipulation of endogenous T1IFN, such as neutralisation of IFNβ, would not have any demonstrable effect.

What was more unexpected was the finding of relatively low frequencies of pro-inflammatory cytokine-producing T cells found from the inflamed IBD samples. Indeed, there were lower percentages of IFNγ-producing T cells from the inflamed samples than the non-inflamed samples ($p=0.01$, signed rank test). It is very unlikely

that this is a true reflection of the cytokine production potential of these cells *in vivo*. What is more probable is that during the process of cell isolation, the T cells became refractory to further stimulation/activation, even with anti-CD3/CD28 antibodies. This would be consistent with the lack of effect seen by IFNβ neutralisation, and may also be an explanation for the higher levels of $pSTAT1^{+}$ T cells from non-inflamed IBD samples compared to inflamed (chapter 4, figure 4.1). It is also of note that, unsurprisingly, inflamed samples yield higher cell number than non-inflamed, which themselves yield very similar numbers to control sample (data not shown). There are many possible reasons why T cells from the inflamed samples may become hypo-responsive. These include the relatively higher numbers of LPMCs in each well which may lead to secondary effects on the T cells, even though the cell numbers were counted and activated in a fixed quantity. This does have implications for some of the results from the inflamed IBD samples isolated by this technique, especially when assessing T cell "responsiveness" or plasticity. That is why, in many experiments only samples from non-inflamed areas were chosen as the IBD samples, and there was less emphasis on exploring the effects T1IFN signalling on T cells from inflamed samples.

5.5.3.3 Summary of the possible effects of T1IFN on intestinal T cells in IBD

Focusing on the non-inflamed sites, it is still not clear what the functional consequences of the cytokine changes in the IBD patients would be, given that the anti-IFNβ antibody increased the percentage of cytokine producing cells in all cytokines measured, both pro- and anti-inflammatory. This result may be due to both the relatively small number and the heterogeneity of the samples. The non-inflamed IBD samples were a mix of samples from Crohn's disease and Ulcerative Colitis, with varying disease duration and treatment exposure. Even though these samples were predominantly from children and young adults, there is still a wide

variety of disease phenotype, duration and treatment (see appendix II). It may be with a larger sample size and/or a more homogeneous patient population, a more nuanced picture of the consequence of disordered T1IFN-STAT1 signalling in intestinal T cells in IBD could be obtained.

Results from chapter 4 suggest that there may be differences in the T1IFN-STAT signalling pathway in intestinal $CD4^+$ T cells in health and IBD. The concept of different STAT signalling downstream of T1IFN determining functional outcome is attractive and supported by previous published work, including increased STAT4 in human IBD samples (Mudter et al., 2005; Ohtani et al., 2010). This increase in STAT4 however is potentially at odds with the data presented in chapter 4 on increased pSTAT1 from T cells isolated from non-inflamed IBD samples (chapter 4, figure 4.1). However, the data on STAT4 is from inflamed areas of IBD patients, whereas the STAT1 data in this thesis is from non-inflamed area. It is therefore possible that the STAT1 signalling is regulatory, although this was not shown clearly in this chapter. It is also possible that for some reason in IBD the T1IFN-STAT1 pathway in T cells is not regulatory, as indeed is suggested by the cytokine data presented here.

A potential model for T1IFN signalling is shown in schematic form below (figure 5.18). In health, higher expression of IFNβ (as shown by ISGs in chapter 4, figure 4.16), primes intestinal CD4 T cells to signal using STAT1/STAT2 homodimers. This canonical T1IFN signalling is pro-apoptotic and promotes IL-10 production. In contrast, in IBD, higher basal IFNβ primes more responsive intestinal $CD4^+$ T cells to signal using STAT4 and STAT3, which leads to anti-apoptotic signals and increased IFNγ production.

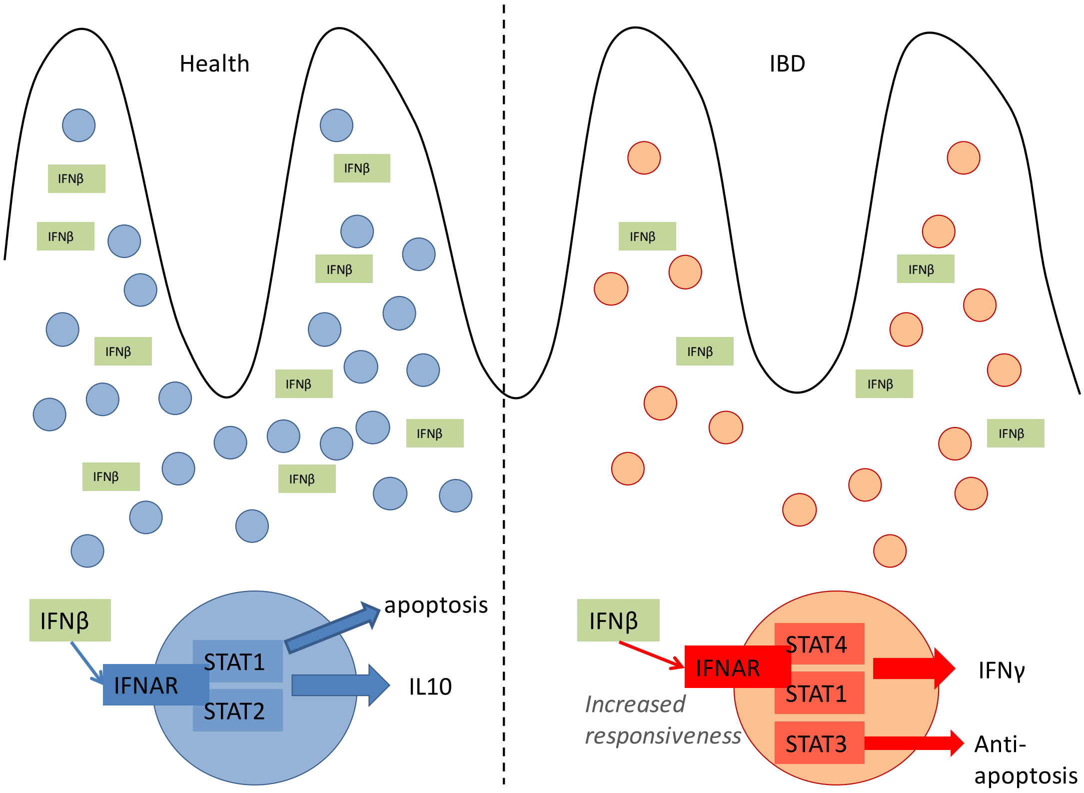

Figure 5.18 Schematic representation of constitutive IFNβ signalling in intestinal T cells. *IFNβ, of uncertain and likely mixed cellular sources, signals via the IFNAR in intestinal T cells. In health, this signalling is predominantly via STAT1, canonically associated with STAT2. This signalling increases the regulatory phenotype of the T cell, in particular enhancing IL-10 production, and also supports apoptotic pathways. In contrast, in IBD, signalling via STAT4 leads to an increase IFNγ production, and signalling via STAT3 promotes T cell survival.*

5.5.4 Conclusion

In summary these findings support the hypothesis that endogenous production of IFNβ in the healthy human intestine provides signals to $CD4^+$ T cells (directly and/or indirectly) which prime them to a more regulatory phenotype. In IBD patients, the relationship between T cells and T1IFN is altered: in IBD intestinal T cells are more responsive to IFNβ *ex vivo* than in controls. Furthermore, in IBD, endogenous IFNβ does not have a distinct pro-regulatory role, but rather restrains a broad range of cytokine production. The possible significance of these findings, in conjunction with the previous chapters, will be discussed in the final discussion.

Chapter 6

6 Final Discussion

6.1 Introduction

This chapter will briefly summarise the results obtained in this study, and discuss the possible significance that these have in the context of what is already known in the field. There will follow a brief discussion of some areas of potential future research, and the possible clinical relevance of the increased understanding of this area that this thesis brings.

6.2 Summary of results

The hypothesis that lead to the work detailed in the results chapters presented in this thesis was that T cells in the intestine remained responsive to environmental factors, in particular T1IFN, and that the responses may differ in health and IBD. This was explored initially using a relatively new method of analysis, Phosflow, which enables detection of phosphorylated (activated) signalling molecules at the single cell level.

Chapter 3 described the optimisation of this new technique for use with cells obtained from human gut tissue, and demonstrated the ability to detect low levels of constitutively activated STAT proteins, namely STAT1, 3 and 5, in intestinal $CD4^+$ T cells. Furthermore, the cells were responsive to further *ex vivo* T1IFN stimulation. Induction of STAT1 phosphorylation in response to T1IFN stimulation was greater in memory $CD4^+$ intestinal T cells and in those expressing the activation marker CD69 but did not appear to be related to signalling through the TCR. The use of Phosflow allowed the measurement of the ongoing responsiveness of memory $CD4^+$ T cells at a single cell level.

The following chapter compared these signalling molecules in intestinal T cells in health and IBD, and showed an increase in the frequency of pSTAT1$^+$ CD4$^+$ T cells from IBD patients, particularly from non-inflamed areas of intestine. There was a trend toward reduced expression of SOCS1, the main regulator of STAT1, in intestinal T cells, consistent with these findings. T1IFN stimulation also induced STAT1 phosphorylation in peripheral blood T cells but this response did not differ between cells from IBD patients and controls. The response was also equivalent in β7$^+$ memory T cells (that represent a population of cells primed in the intestinal lymphoid tissue) and β7$^-$ memory T cells. These findings support the concept that STAT phosphorylation in intestinal T cells is influenced by local tissue factors, possibly including T1IFN.

The data presented in the final results chapter explored T1IFN, which is dependent on STAT1 signalling, as an environmental factor in the gut and particularly its constitutive effect on intestinal T cells. To our knowledge, IFNβ was shown for the first time directly in fresh frozen sections of human colon tissue. It was demonstrated that, in health, constitutive IFNβ supports pro-regulatory functions of intestinal T cells, either by direct or indirect mechanisms. In IBD, this role is disrupted, and overall the CD4$^+$ T cells appear to be more responsive to exogenous T1IFN.

6.3 Significance of Thesis Results

6.3.1 Functional significance of increased intestinal T cell responsiveness in IBD

The increased responsiveness to T1IFN in intestinal T cells from IBD patients was shown by increased ISG expression (chapter 5, figure 5.2) and also by the increased frequency of pSTAT1$^+$ cells following *ex vivo* T1IFN stimulation (chapter

4, figure 4.1). The functional consequences of these cells being more responsive in IBD are unclear. In health, endogenous T1IFN appeared to promote a pro-regulatory T cell, particularly in terms of cytokine production. However, in IBD, this was not the case. There was also a trend for lower expression of ISGs in intestinal T cells in IBD prior to *ex vivo* stimulation, suggesting that there is more constitutive T1IFN tonic signalling in health.

A lower expression of ISG would initially appear to be inconsistent with the higher percentage of $pSTAT1^+$ cells found prior to *ex vivo* stimulation in chapter 4. There are, however, various possible explanations for this discrepancy. One possibility would be that consistent higher exposure to T1IFN would increase ISG expression, but would also increase regulators of T1IFN signalling, such as SOCS1, and this would constrain both constitutive and stimulated STAT1 activation. This is indeed the pattern of SOCS1 expression shown in chapter 4 (figure 4.10), with a strong trend towards higher expression in healthy intestinal T cells. That is, persistent low level T1IFN signalling in the gut in health leads to increased expression of SOCS1, and perhaps other negative regulators of T1IFN signalling, which would help constrain intestinal T cell responses. An example of this is in a mouse HIV model, where giving exogenous T1IFN increases ISG expression, which by day 7 return to baseline, despite ongoing T1IFN treatment, suggesting homeostatic mechanisms can limit responsiveness (Sandler et al., 2014).

In IBD, there appears to be a failure to induce pathways that would normally limit responses to T1IFN as part of the normal feedback response to an endogenous cytokine. This would mean an increased sensitivity to signals that induce STAT1 phosphorylation and thereby an altered signalling ‘landscape’ within the intestinal T cells (see figure 1).

This model, however, does not readily explain the different effect on cytokine production of neutralising constitutive T1IFN in cultures of healthy and IBD biopsies It may be that in the altered signalling landscape proposed above that different

signalling mechanisms predominate in the response to T1IFN. Indeed, it has been demonstrated that differing pathways of T1IFN signalling can have opposite outcomes, in that T1IFN by STAT1 is pro-regulatory in murine T cells, but by STAT4 is pro-inflammatory (Nguyen et al., 2002). Alternatively, additional mechanisms such as epigenetic changes to key loci or possibly the induction of specific microRNAs might qualitatively altered the nature of the response induced.

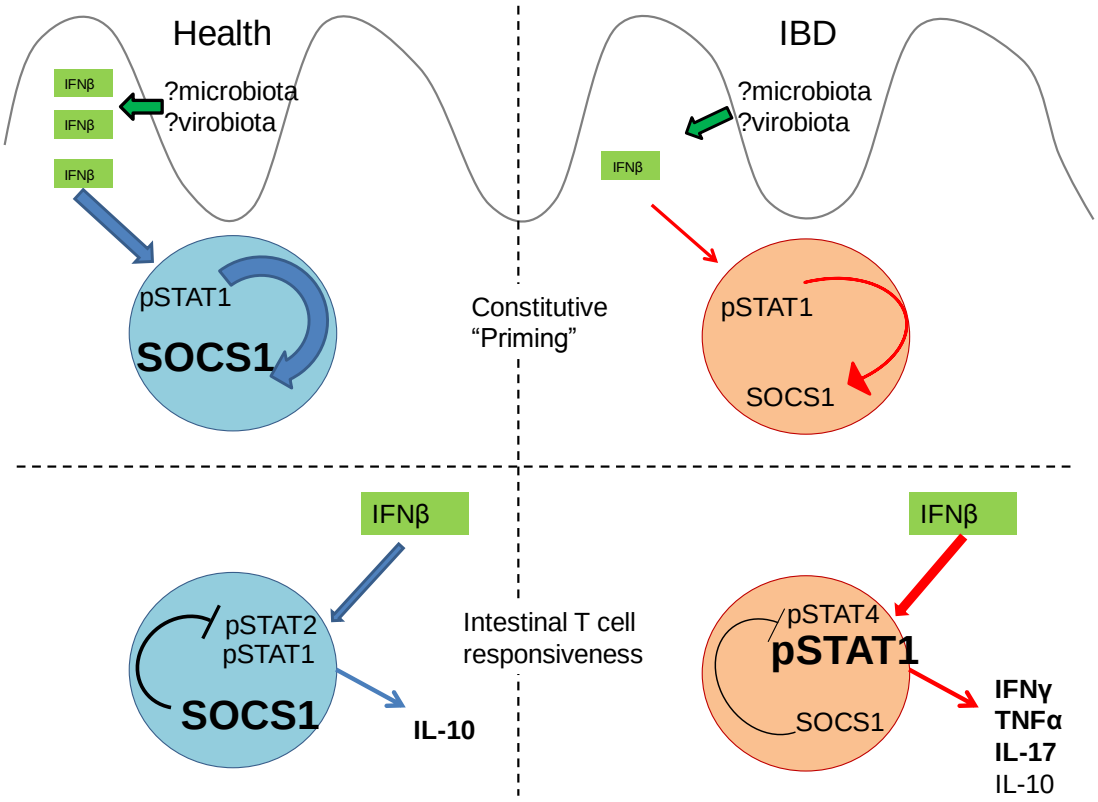

Figure 6.1 Suggested model for T1IFN priming of intestinal T cells in the human intestine. *On the left, in health, environmental factors such as bacteria or viruses in the gut promote T1IFN (especially IFNβ) expression. Tonic T1IFN signalling increases SOCS1 expression. Later, T1IFN (and/or potentially other factors) trigger a regulatory response from the T cell. On the right, in IBD patients, there may be less constitutive T1IFN signalling and therefore lower SOCS1 expression. This may alter the signalling pathways that are predominant in the T cells, driving a more pro-inflammatory phenotype.*

6.3.2 Endogenous versus exogenous T1IFN

6.3.2.1 Endogenous T1IFN

It is important throughout this discussion of the significance of the thesis results to distinguish between the role of constitutive T1IFN signalling and the effects of exogenous T1IFN. It was proposed in the early 1980s that T1IFN was produced constitutively due to the ongoing low grade exposure of the mucosa to pathogens and the requirement for continuous tissue remodelling. This was based on the observation that uninfected tissue preparations had antiviral activity similar to that of IFN (Bocci, 1980). The difficulties of measuring T1IFN directly have previously made it difficult to demonstrate the presence of the cytokine in the intestine. Recent murine work has confirmed that T1IFN is only present at extremely low levels in the colon (Kole et al., 2013).

The work in this thesis has shown, for the first time, the presence of IFNβ directly in the human colon by antibody staining and immunofluorescence analysis (chapter 4, figures 4.14 and 4.15). In addition, the ISG expression detected in freshly isolated intestinal T cells (figures 4.16 and 5.2) is likely dependant on low levels of endogenous T1IFN produced in the gut (Yan and Chen, 2012). This supports the results that the trend towards lower ISG expression in IBD samples compared to controls (figure 4.16) suggests lower constitutive T1IFN signalling in intestinal T cells as suggested in figure 6.1 above. The significance of this lower tonic signalling is suggested by evidence from MS patients, where MxA expression in whole blood was lower in patients who were relapsing than those in long term remission (van der Voort et al., 2010). This suggests endogenous T1IFN signalling can have a regulatory role in a systemic disease, and may be a parallel with the lower ISG expression in the IBD patient intestinal T cells.

6.3.2.2 Exogenous T1IFN

The discussion in the previous paragraphs (section 6.3.1) is regarding the effects of endogenous T1IFN. However, the effect of exogenous T1IFN on intestinal T cells does not appear to mirror its constitutive properties, as both neutralisation of IFNβ and addition of exogenous IFNβ appear to promote an inflammatory T cell cytokine response (figures 5.11 and 5.12). However, these two observations should not necessarily be seen as contradictory. They may represent effects at opposite ends of a concentration spectrum. The exogenous T1IFN used in this thesis was at supra-physiological doses of IFNβ (either 1000 IU/mL or 40000 IU/mL), whereas the endogenous IFNβ would be at much lower concentrations. But perhaps more importantly, the literature consistently demonstrates the importance of timing in the effects of T1IFN (Beilharz et al., 2007; Davidson et al., 2014; McNab et al., 2015). For example, low level exogenous T1IFN has been shown to be protective against viral infection where high dose had no effect (Beilharz et al., 2007). In an influenza infection model that a combination of host factors, timing/dose of T1IFN and viral factors are all important in determining mortality and morbidity in mice (Davidson et al., 2014)(Davidson et al., 2014)(Davidson et al., 2014)(Davidson et al., 2014)(Davidson et al., 2014). It is these concentration and timing dependent effects that may help explain the varied clinical response in the intestine to therapeutic T1IFN.

6.3.3 The role of STAT1 in endogenous T1IFN signalling

Endogenous T1IFN has been shown to maintain STAT1 expression in murine T cells (Gough et al., 2010). Interestingly, while T1IFN can signal via all of the members of the STAT family, endogenous T1IFN is thought to signal via STATs 1, 2 and 6, but not STAT4 (Mangan and Fung, 2012). It is unfortunate that in this work we were unable to evaluate the role of STAT4 in the T1IFN signalling of intestinal $CD4^+$ T cells in health and disease because we were unable to obtain staining with

the available reagent. The ability to have analysed STAT4 activation in intestinal T cells would have contributed substantially to the understanding of the importance of T cell signalling pathways and their functional outcomes. This is because STAT4 is known as a canonical signalling molecule in TH1 cell differentiation (Rogge et al., 1998), is important in determining the functional outcome of T1IFN signalling (Nguyen et al., 2002), and has been demonstrated to be of importance in previous work in the investigation of IBD (Mudter et al., 2005).

There is, however, work from mice and other human diseases that suggests how the T1IFN-STAT1 pathway may regulate the adaptive immune response. For example, it has been suggested that in multiple sclerosis, endogenous IFNβ is regulatory in its effect by directly decreasing TH17 cell responses (Tao et al., 2014). Indeed, it seems clear that, at least in EAE, endogenous T1IFN modulates TH1/TH17-mediated autoimmunity in a way that is different to exogenous T1IFN administered as a therapy (Kalinke and Prinz, 2012). Specifically, the level of steady-state T1IFN signalling by endogenous T1IFN, acting via STAT1, is critical in determining the response of cells of the adaptive immune system to further T1IFN exposure (Mangan and Fung, 2012). That is, in EAE, analogous to the model proposed for IBD above (figure 6.1), a dysregulated adaptive system, primed by low activity of T1FN, may be more likely to produce a pro-inflammatory response to a (viral) stimulus.

This does, however, suggest that low level endogenous T1IFN may prime the signalling molecules of intestinal T cells to maintain ISG expression which would in turn allow the cells to respond appropriately to other triggers of T1IFN, such as viral infection. In virus-specific $CD8^+$ T cells, STAT1 expression is decreased and as a result of the altered STAT protein balance, the consequence of T1IFN signalling shifts from anti-proliferative to a pro-proliferative effect on effector T cells. The result is increased viral clearance (Gil 2006 and Gil 2012).

The opposite effect of T1IFN appears to be the case in Tregs, related to higher STAT1 and lower SOCS1 expression, where T1IFN signalling has an anti-inflammatory effect, and leads to inefficient viral clearance (Srivastava et al., 2014). Indeed, T1IFN has been shown to increase Tregs number and suppress TH17-driven inflammation via STAT1 (Stewart et al., 2013). This enhancement of Treg number or function and suppression of (TH17) inflammation, dependent on STAT1, is consistent with the data in chapter 5. That is, the IFNβ neutralisation led to a more "pro-inflammatory" intestinal $CD4^+$ T cell cytokine profile. The effect on IL-17 production is this work were more complex, and are described and discussed below.

While there was no significant effect of IFNβ neutralisation on intestinal IL-17 production in either healthy controls or IBD, the addition of exogenous T1IFN to organ cultures did suppress IL-17 production by tissue from healthy controls (figure 5.11). Unfortunately there were insufficient IBD samples available to comment on this effect in disease. In humans, STAT1 gain of function mutations lead to decreased IL-17 production, as the result of an increase in T1IFN signalling. The functional consequence of these mutations is recurrent severe candida infections (Liu et al., 2011; van de Veerdonk et al., 2011). Interestingly, homozygous mutations in the CARD9 gene are also associated with severe candida infections and this gene has also been identified as a locus associated with increased risk of developing IBD (Maródi et al., 2012). Given that the phenotype of STAT1 and CARD9 mutations show some similarities, it does raise the possibility that STAT1 signalling may also be implicated in gut inflammation. While the STAT1 mutations in humans do not appear to lead to a phenotype consistent with IBD, it must be remembered that these are gain-of-function mutations, which, the results of this thesis would suggest would drive a more regulatory T cell response in the gut.

6.4 Possible further research

6.4.1 The source of intestinal T1IFN

As described in chapter 1 (section 1.5.1), the source of T1IFN in the human intestine is not known, with varied and partially conflicting data in the literature. Unfortunately the work presented here was unable to clarify this further. IFNβ staining by IF suggested that T cells were not the likely main source of IFNβ, and perhaps epithelial cells would be more likely, based on the proximity of staining (figure 4.15), although the evidence for this is not strong. The murine literature has reported an important role for various other cell types in the production of T1IFN. The cell types implicated include plasmacytoid DC (pDCs), but the contribution of pDCs in the context of human mucosal inflammation is yet to be fully established. It has been shown that RNA from commensal (but not pathogenic) bacteria promote TLR3-dependent IFNβ production from intestinal conventional (cDCs) (Kawashima et al., 2013), which highlights the importance of the microbiota (and possibly virobiota) in this process. The interaction between the host micro/virobiota, epithelium/stromal cells, innate immune system and adaptive cells in the context of T1IFN production remains an area with much to be explored.

6.4.2 The role of other components of the T1IFN signalling pathway

Due to the nature of this research on human tissue, and the limited number cells available, the ability to comprehensively explore signalling pathways was limited; some pragmatic choices had to be made with regard to which elements of the T1IFN pathway were most likely to be of importance in intestinal T cells. Therefore the data largely focuses on IFNβ signalling via STAT1. STAT3 and STAT5 were studied to a lesser extent. Doses of neutralising antibody and exogenous stimuli were rarely varied, and were often supra-physiological. This is clearly an over-

simplification of the variety of T1IFN signalling, but was necessary in order to make experiments feasible.

The relative contribution of IFNβ compared with other T1IFNs (such as the IFNα subtypes or IFNλ) may be an important consideration, as well as the contribution of other components of the downstream signalling pathways, including the IFNAR receptor. In macaques with SIV infection, both the blockade of the IFNAR receptor and the addition of exogenous T1IFN (IFNα2a) worsened the outcomes following infection (Sandler et al., 2014). Recently, it has been shown that IFNβ can signal independently of both IFNAR2 subunit and the Jak/STAT signalling machinery (de Weerd, 2013). This is a possible mechanism by which T1IFN signalling could be pro-inflammatory, as opposed to the canonical T1IFN-STAT1 pathway, which is traditionally (and appears in this work) to support regulatory T cell function.

It is likely therefore that host factors, such as expression of IFNAR receptor components and their downstream proteins, and T1IFN dose/timing (including endogenous/exogenous sources), in combination with other unknown environmental factors, determine the outcome of T1IFN signalling in the human intestine.

6.4.3 Role of the innate immune system in regulating T cell responses to T1IFN

While there appears to be consensus in murine models that T1IFN signalling in intestinal T cells is important in constraining inflammation, it is controversial as to whether this is a direct effect on the T cells, or an effect via the innate immune system. There is some evidence that T1IFN affects intestinal $CD4^+$ T cells directly (Lee et al., 2012; Radulovic et al., 2012). However, other studies suggest the need for dendritic cells (both pDCs and cDCs), particularly in the development and function of intestinal Tregs (Bilsborough et al., 2003; Bleich et al., 2009; Hofmann et al., 2010; Kole et al., 2013). The effects of T1IFN depend upon the cytokine milieu and vice versa (Touzot et al., 2014).

The experiments reported in this thesis utilised mixed cell populations including LPMCs and therefore cannot comment directly on the relative importance of direct action of T1IFN on intestinal $CD4^+$ T cells versus indirect effects via other cell populations present. While the induction of pSTAT1 expression in T cells after stimulation with T1IFN for only 15 minute (chapter 3, figure 3.4 and others) is suggestive of a direct action at least at high concentrations of exogenous T1IFN, it is not clear how important this direct effect is functionally in the human intestinal environment.

6.4.4 Role of the microbiota/virobiota

There has been rapidly increasing interest and understanding about the role of the microbiota in mucosal immunology in recent years, although the understanding of the virobiota is still in its infancy (Duerkop and Hooper, 2013). T1IFN is generated in the gut not only in response to viral triggers, but also in response to bacteria and bacterial components. Indeed, systemic antiviral immune responses have been shown to be dependent on the microbiota (Ichinohe et al., 2011; Kuss et al., 2011). In addition, T1IFN, regulated by the microbiota, contributes to the priming of the inflammatory and immune response in the gut (Abt et al., 2012; Touzot et al., 2014). Moreover, viral infection, by stimulating T1IFN production, is thought to sensitise the immune system to bacterial infection (McNab et al., 2015).

It would be of great interest to further understand the role of the known dysregulated microbiota, and recently virobiota (Norman et al., 2015), in IBD and to explore the contribution of T1IFN to these effects. There are many groups studying this field, but T1IFN signalling may provide an important mechanism in understanding the inflammatory response to the environment.

6.5 Clinical relevance of T1IFN signalling in the human intestine

The complex findings of the role of T1IFN signalling in the gut are consistent with the overall disappointing clinical experience of the use of pharmacological T1IFN in IBD (Madsen et al., 2001; Nikolaus et al., 2003; Pena Rossi et al., 2009; Seow et al., 2008). In addition, as discussed in chapter 1, when used in treatment of other conditions, T1IFN has led to the development of IBD (Mitoro et al., 1993; Samson et al., 2009; Schott et al., 2007; Sprenger et al., 2005; Watanabe et al., 2011). The results in chapter 5 suggest that in IBD, intestinal T cells are primed to be more pro-regulatory by constitutive T1IFN, but unfortunately there were insufficient samples to show the effect of additional T1IFN in disease. In healthy controls, additional IFNβ increased IFNγ and decreased IL-17 production (figure 5.11).

The mucosa of patients with IBD has been known for many years to produce large quantities of the canonical TH1 cytokine IFNγ (MacDonald et al., 1990). Given, the known effect of exogenous T1IFN on T cell IFNγ production, and the findings in this thesis, it is plausible that T1IFN could promote the development or severity of Crohn's disease or UC by enhancing IFNγ production.

The role of IL-17 in IBD is still unclear, but there is little doubt that the IL-23/IL-17 axis is involved (Brand, 2009; Neurath, 2014a). There is much literature, discussed in detail in chapter 1, which supports the role of T1IFN increasing T cell IL-10 secretion and reducing IL-17 secretion from T cells (also reviewed in (Axtell et al., 2013). Endogenous IL-10 in the human gut has been suggested to have a role in Crohn's disease patients by suppression of TH17 driven inflammation (Wilke et al., 2011). This thesis would support a theory that constitutive T1IFN signalling may support endogenous IL-10 production. Trials of monoclonal antibodies to IL-17 as treatments for IBD have had negative results (Hueber et al., 2012; Reinisch et al., 2010). However, as discussed in Chapter 1, the role of IL-17 and TH17 cells in the human gut and IBD have yet to be fully understood, and may be pro- or anti-

inflammatory, and this may also vary between individuals (Monteleone et al., 2012). This an impact of T1IFN on IL-17 may be predicted to have different outcomes in different individuals and may provide a partial explanation as to why pharmacological T1IFN had a variable, and ultimately disappointing, effect in the treatment of IBD.

As the understanding of the dynamic nature of T1IFN signalling slowly increases, the approach to therapeutics may change. In particular, given the role of low dose endogenous T1IFN in regulating the immune response, it has been suggested that low dose more frequent T1IFN treatment may give better results in treating immunological dysfunction in humans (Schreiber and Piehler, 2015). This approach would be strongly supported by the data in this thesis, which would suggest that supporting tonic T1IFN signalling in health may help prevent a pro-inflammatory response to later environmental insults.

6.6 Final Summary

This thesis has explored the role of T1IFN signalling in human intestinal T cells. This was done initially by utilising a relatively new technology, Phosflow, which has allowed single cell analysis of the activation of STAT molecules in $CD4^+$ lamina propria T cells. This technique has allowed a greater understanding of the plasticity of the T responses in the intestine, and supports a more nuanced approach to understanding of $CD4^+$ T cells beyond traditional T helper phenotypes.

In particular, this thesis has demonstrated an increased responsiveness in intestinal $CD4^+$ T cells to T1IFN in IBD, with regards to increased frequency of $pSTAT1^+$ cells, and also increased upregulation of ISGs. Endogenous IFNβ has been detected for the first time directly in the human gut. The functional significance of this constitutive T1IFN-STAT1 pathway in human intestinal T cells appears to support a regulatory cytokine profile in health. This is consistent with the literature in the mouse, and in

other human diseases and their mouse models. The pathway appears to be dysregulated in IBD, but the functional significance of this is less clear. However, a greater understanding of the role of T1IFN as a critical regulator in the gut provides some potential explanation for previous therapeutic failures in IBD, and also supports novel approaches for supporting intestinal homeostasis and treating or preventing gut inflammation in the future.

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Appendix 1

Reagents

The majority of commonly used reagents are listed below. These as well other less commonly used reagents are referred to in the text. Reagents were stored at room temperature, -20 °C or -80 °C as appropriate; frozen aliquots were discarded following the second freeze/thaw cycle to minimize degradation.

General reagents

Complete medium: RPMI1640 Dutch Modification supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin (all Sigma-Aldrich) and 10% heat-treated (56 °C for 30 minutes) foetal calf serum (FCS; PAA).

Complete medium with gentamicin: Complete medium with 100 µg/mL Gentamicin (Sigma-Aldrich) in addition.

FACS buffer: Phosphate-buffered saline solution (PBS) containing 2% FCS, 1 mM ethylenediaminetetraacetic acid (EDTA) and 0.2% (w/v) sodium azide.

EDTA: 0.93g EDTA disodium salt dihydrate (Sigma-Aldrich) in 50ml distilled water (dH_2O). EDTA solution adjusted to pH 7.4 using NaOH. Sterile filtered. Stored 4^oC.

This produces 50mM stock. Stock is diluted 1 in 50 with HBSS prior to use → 1mM working concentration.

MiniMACS buffer: PBS with Bovine Serum Albumin (BSA, 0.5 %, w/v) and EDTA (2 mM)

Tissue Processing Reagents

Dithiothreitol (***DTT):*** 0.39g DTT (Sigma-Aldrich) in 25ml dH_2O for 100mM stock. Stored at -20^oC.

Collagenase stock: 500mg collagenase D (Roche) in 50ml serum-free RPMI-HEPES (Sigma-Aldrich) to make 10mg ml^{-1} stock. Sterile filtered. Stored -20^oC.

***DNase I*:** 100mg DNase I (Roche) in 10ml dH_2O to make 10mg ml^{-1} stock. Sterile filtered. Stored -20^oC.

Collagenase solution: 0.3 mL FCS (PAA) + 1.5 mL collagenase stock (Roche) + 30 μL DNase I (Roche) in 15 mL total volume with warm RPMI-HEPES (Sigma-Aldrich)

Interferons

Recombinant Human IFN-γ, Peprotech

Recombinant Human IFN-β, Peprotech

Recombinant Human IFN-α, Peprotech

IFN/STAT neutralization agents

Low Endotoxin Azide Free (LEAF) Purified anti-human IFN-β, clone IFNb/A1, isotype mIgG1, Biolegend

LEAF Purified anti-human IFN-γ, clone MD-1, Biolegend

LEAF Purified Mouse IgG1, clone MOPC-21, Biolegend

Fludarabine – 2-Fluoroadenine-9-β-D-arabinofuanoside, Sigma-Aldrich

T cell stimulation agents

LEAF Purified anti-human CD3, clone OKT-3, Biolegend

LEAF™ Purified anti-human CD28, clone CD28.2 Biolegend

Multiplex ELISA

Human Basic Kit FlowCytomix, eBioscience

eBioscience FlowCytomix Simplex Kit, Human IL-17A, TNF-α, IFN-α, IL-10, IFN-γ

Immunohistochemistry

Blocking serum: 5% BSA in 0.1% Tween (Biorad) in PBS

DAPI Vectorshield (Vector Laboratories)

Appendix 2

Patient details

Abbreviations
IBS: Irritable Bowel Syndrome; FHx: Family history of; FAP: Familial Adenomatous Polyposis; 5asa: 5-aminosalicylic acid (eg mesalazine); aza: azathioprine; pred: prednisolone; anti-TNF: anti-TNFα monoclonal antibody (eg infliximab); PR: per rectal; GOR: gastro-oesopheal reflux; PPI: Proton Pump Inhibitor; MTX: methotrexate

Chapter 3

Section 3.4.4 Phosflow in intestinal T cells

Sample type	n	Median age (range)	Diagnosis
Control	1	13	Constipation

Section 3.4.5 The vast majority of walk-out lymphocytes are viable

Sample type	n	Median age (range)	Diagnosis
Control	2	13.5 (13-14)	IBS

Section 3.4.6 Multiple pSTATs can be detected in $CD4^+$ intestinal T cells with and without stimulation with T1IFN

pSTAT3

Sample type	n	Median age (range)	Diagnosis
Control	1	7	FHx FAP

pSTAT5

Sample type	n	Median age (range)	Diagnosis
Control	1	7	FHx FAP (same as above)

pSTAT6

Sample type	n	Median age (range)	Diagnosis
Control	1	13	IBS

Section 3.4.7 Characterisation of $pSTAT^+$ intestinal $CD4^+$ T cells

Responsiveness of memory versus naïve intestinal T cells

Sample type	n	Median age (range)	Diagnosis	Medications
Control	5	13 (9-16)	IBS 3, FHx FAP 2	nil
IBD	5	13 (9-16)	CD 3, UC 2	5asa 3, pred 2, aza 2

Measurement of T cell activation by CD69 expression

Sample type	n	Median age (range)	Diagnosis	Medications
Control	5	15 (8-16)	IBS 3, FHx FAP 2	nil
IBD	2	15 (8-16)	CD 2	pred 1, aza 1

Chapter 4

Sections 4.4.1 and 4.4.2

pSTAT1, 3 and 5 and total STAT1 experiments

Sample type	n	Median age (range)	Diagnosis	Medications
Controls	16	12 (3-16)	IBS 7, constipation 4, abdo pain 1, PR bleeding 2, FHx FAP 2	nil
UC	12	14 (6-17)	4 new, 3 patients with IBDU	nil 4, 5asa 8, pred 4, aza 6
Crohns	12	13 (7-17)	7 new diagnosis	nil 7, 5ASA 6, pred 2, aza 5, anti-TNF 4

Section 4.4.1

pSTAT1 in Coeliac patients

Sample type	n	Median age (range)	Diagnosis	Medications
Controls	7	9 (1-12)	PR bleeding ?IBD, eosinophilic oesophagitis, GOR, IBS (2), feeding difficulties, post-infectious diarrhoea	PPI 3
Coeliac	9	9 (3-13)	New coeliac	nil

Section 4.4.3

pSTAT1 and relationship with Transcription factors

T-bet

Sample type	n	Median age (range)	Diagnosis	Medications
Control	9	14 (6-16)	IBS 4, constipation 2, abdo pain 1, FHx FAP 2	nil
IBD	12	14 (6-17)	CD 6, UC 6	nil 3, 5asa 3, pred 1, aza 1, anti-TNF 1

FoxP3

Sample type	n	Median age (range)	Diagnosis	Medications
Control	7	14 (6-16)	IBS 4, constipation 2, abdo pain 1	Nil
IBD	9	15 (9-17)	CD 5, UC 4	Nil 3, 5asa 4, pred 1, aza 1

Section 4.4.4

CD69 as a measure of intestinal T cell activation

Sample type	n	Median age (range)	Diagnosis	Medications
Control	5	12 (7-15)	2 FHx FAP, 1 IBS, 1 abdo pain, 1 constipation	Nil
IBD	5 (3 paired samples)	13.5 (10-16)	4 CD, 1 UC	Nil 2, 5asa 3, pred 1, aza 2, anti-TNF 1

Section 4.4.5

STAT signalling in Peripheral Blood T cell subsets

Sample type	N	Median age (range)	Diagnosis	Medications
Control	8	15(7-25)	volunteers (n=4), IBS=2, PR bleeding=2	nil
IBD	18	12.5 (6-21)	CD 10, UC 8, 9 active, 9 in remission	nil 5, 5asa not recorded, aza 7, mtx 1, anti-TNF 3

Section 4.4.6.1

Conditioned medium experiments

Sample type	n	Median age (range)	Diagnosis
Control	4	14 (12-17)	polyposis 1, FHx FAP 1, IBS 2

Section 4.4.6.2

SOCS1 and SOCS3 expression

Sample type	n	Median age (range)	Diagnosis	Medications
Control	8	12(7-15)	IBS =4, 1= diarrhoea, 1 FHx polyposis, 2=PR bleeding	nil
IBD	9 (3 paired samples)	14(11-16)	4 CD, 2 UC	nil 1, 5asa 3, aza 4, anti-TNF 1

Section 4.4.6.3.1

Fludarabine as a STAT1 inhibitor

Sample type	N	Median age (range)	Diagnosis
control	4	13 (11-15)	IBS 2, polyposis 1, PR bleeding 1

Section 4.4.7.1

IFNβ neutralisation pSTAT1/U-STAT1

Sample type	N	Median age (range)	Diagnosis	Medications
Control	8	13 (4-14)	IBS=3, constipation=2, abdo pain=3	nil
IBD	15 (4 paired samples)	12.5 (8-15)	CD 12, UC 3	Nil 6, 5ASA 4, pred 3, aza 5, mtx 1

Section 4.4.7.3

Immunohistochemistry

Sample type	N	Median age (range)	Diagnosis	Medications
Control	5	14 (11-62)	IBS 3, colon cancer 2	Nil 3 unknown 2
IBD	3 paired	14 (12-16)	CD 2 UC 1	Unknown 3

Section 4.4.7.4

mRNA expression of ISGs

Sample type	N	Median age (range)	Diagnosis	Medications
Control	10	11.5 (8-16)	IBS 4, polyp 1, abdo pain 2, diarrhoea 3	nil
IBD (all NI)	12	13.5 (6-16)	CD 9, UC 3	Nil 5, 5ASA 5, pred 2, aza 4, mtx 1,anti-TNF 2

Chapter 5

Section 5.4.1

mRNA expression of ISG

Sample type	N	Median age (range)	Diagnosis	Medications
Control	10	11.5 (8-16)	IBS 4, polyp 1, abdo pain 2, diarrhoea 3	nil
IBD (all NI)	12	13.5 (6-16)	CD 9, UC 3	Nil 5, 5ASA 5, pred 2, aza 4, mtx 1,anti-TNF 2

Sections 5.4.2.2.1 and 5.4.2.2.2

IFNβ neutralisation in health controls – flow cytometry and multiplex ELISA T cells

Sample type	N	Median age (range)	Diagnosis	Medications
Control	8	13 (4-14)	IBS 3, constipation 2, abdo pain 3	nil

Section 5.4.2.2.3

IFNβ neutralisation in health controls – multiplex ELISA - whole biopsy

Sample type	N	Median age	Diagnosis	Medications

		(range)		
Control	11	13.5 (4-16)	IBS 5, constipation 2, abdo pain 3, PR bleeding 1	nil

Section 5.4.2.3.1

Exogenous IFNβ in healthy controls – multiplex ELISA from T cells

Sample type	n	Median age (range)	Diagnosis	Medications
Control	6	12 (8-16)	IBS 3, abdo pain 2, diarrhoea 1	nil

Section 5.4.2.3.2

Exogenous IFNβ in healthy controls – multiplex ELISA from whole biopsies

Sample type	n	Median age (range)	Diagnosis	Medications
Control	8	11.5 (8-16)	IBS 4, abdo pain 2, diarrhoea 2	Nil

Section 5.4.2.4.1 and 5.4.2.4.2

IFNβ neutralisation in NI IBD – flow cytometry and multiplex ELISA T cells

Sample type	n	Median age (range)	Diagnosis	Medications
IBD (all NI)	10	14 (6-16)	CD 8, UC 2	Nil 4, 5ASA 4, pred 2, aza 3, mtx 1,anti-TNF 2

Section 5.4.2.4.3

IFNβ neutralisation in NI IBD –multiplex ELISA whole biopsy

Sample type	n	Median age (range)	Diagnosis	Medications
IBD (all NI)	12	13.5 (6-16)	CD 9, UC 3	Nil 5, 5ASA 5, pred 2, aza 4, mtx 1,anti-TNF 2

Section 5.4.2.4.4

IFNβ neutralisation in I IBD – flow cytometry

Sample type	n	Median age (range)	Diagnosis	Medications
IBD (all I)	12	12 (6-16)	CD 9, UC 3	Nil 6, 5ASA 4, pred 2, aza 4, anti-TNF 1