The Role of Membrane Tumour Necrosis Factor Alpha in the Function and Efficacy of Anti-Tumour Necrosis Factor Antibodies in Inflammatory Bowel Disease.

Iona Maree Bell

May 2017

'Submitted in partial fulfilment of the requirements of the Degree of Doctor of Philosophy'

Barts and The London School of Medicine and Dentistry

Queen Mary University of London

Statement of originality

I, Iona Maree Bell 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.

I attest that I have exercised reasonable care to ensure that the work is original, and does not to the best of my knowledge break any UK law, infringe any third party's copyright or other Intellectual Property Right, or contain any confidential material.

I accept that the College has the right to use plagiarism detection software to check the electronic version of the thesis.

I confirm that this thesis has not been previously submitted for the award of a degree by this or any other university.

Signature: Iona Maree Bell

Date: 31/05/2017

Details of collaboration:

I declare that the work presented in this thesis is my own with the exception of Chapters 6 and 7, in which some of the experiments were performed in collaboration with Paolo Biancheri and Paolo Giuffrida respectively.

Abstract

Tumour Necrosis Factor Alpha (TNFα) is central to the immunopathogenesis of inflammatory bowel disease. It is initially expressed on the cell surface as a trimer, membrane TNFα, and cleaved from the cell surface by TNFα Converting Enzyme (TACE) to release a soluble trimeric form of the cytokine (sTNFα). Infliximab, an anti-TNFα antibody revolutionised the treatment of IBD. However infliximab is ineffective in up to a third and nearly half of patients lose response to infliximab over time. As yet the mechanisms of action of infliximab are unclear, although the importance of mTNFα is emerging.

The aim of the study was to establish whether pharmacological and endogenous TACE inhibitors change the expression of mTNFα with a reciprocal change in sTNFα and other pro-inflammatory cytokines. Additionally possible mechanisms of action of anti-TNFα antibodies were tested.

The expression of mTNFα and sTNFα were measured with FACS and ELISA respectively in vitro and ex vivo in controls and IBD patients. The functional effects of infliximab and etanercept were explored using a TNFα transfected CHO cell line and peripheral blood and lamina propria mononuclear cells. Changes in receptor tyrosine kinase (RTK) phosphorylation as a result of TNFα neutralisation in IBD explants were determined using a RTK phosphoarray.

A significant reduction in sTNFα and an increase in mTNFα were seen with some TACE inhibitors, without any change in other pro-inflammatory cytokines. Apoptosis was not seen with anti-TNFα antibodies in either the TNFα

expressing cell line or in PBMCs. The study showed for the first time that infliximab reduces phosphorylation of RTKs such as EGFR, FGFR, Eph as well as those involved in T cell receptor signalling such as ZAP-70 and Lck.

Acknowledgements

I am sincerely grateful to my supervisor, Professor Tom MacDonald, for his enthusiastic encouragement and guidance on this momentous journey. He has been an endless and valuable source of advice and expertise. I would also like to thank Dr Andy Stagg, my second supervisor, who provided positive, helpful feedback, throughout my PhD.

Life in the lab has been an enjoyable and rewarding experience, primarily because of the kind, entertaining and helpful group of scientists I have met and become friends with. Thank you Aneta, Renata, Nadja and Anna for your all your help. A special thanks to Paolo Biancheri who willingly discussed and made wise suggestions on how to proceed, and when to stop for coffee! As well as for the collaboration on apoptosis. I am also indebted to Paolo Giuffrida for the collaborative work on tyrosine kinases which led to such interesting results.

I made a great friend through learning about flow cytometry and how to make tiramisu- Francesca, you made coming to the lab something to look forward to even when the experiments were all going wrong!

I am considerably lucky to be surrounded by a supportive family who have made this possible. My parents have always encouraged me and shown me all that can be achieved with determination and passion, and given me all the love and support I needed to finish this thesis. My sister Corrie, who has the gift of knowing just how to help me back on course however stormy the winds, with a lot of laughter too.

My parents- in- law have willingly helped at a moment's notice, encouraged me to carry on, and shown such interest in my work.

My children have been fantastic throughout it all, with impressive suggestions on planning and writing up, and cheering me on in the final stages.

A special thanks to my amazing husband, Haythem, who has believed in me from the start.

I dedicate this thesis to my wonderful daughters:

***Sohaila, Maaria, Leila, Elia and Omaya**,*

For the sunshine you each bring into my life

Table of contents

List of figures

List of tables

List of abbreviations

ADAM17	A disintegrin and metalloproteinase
ADCC	Antibody Dependent Cytoxicity
AJ	Adherens junctions
ATGI6LI	Autophagy gene 16 Like 1
APC	Antigen presenting cells
CARD	Caspase activation and recruitment domain
CD	Crohn's disease
CDAI	Crohn's disease activity index
CDC	Complement Dependent Cytotoxicity
CHO	Chinese hamster ovary
CRP	C-Reactive Protein
CSF	Colony stimulating factor
DMSO	Dimethyl sulfoxide
DC	Dendritic cell
DSS	Dextran sulphate sodium
EDTA	ethylenediaminetetraacetic acid
Eph	Erythropoietin producing hepatocellular
Eph R	Erythropoietin producing hepatocellular Receptor
EGFR	Epidernal growth factor receptor

ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence activated cell sorting
FAE	Follicle associated epithelium
FGFR	Fibroblast growth factor receptors
FLT3	A receptor tyrosine kinase required for dendritic cell development
FSC	Forward scatter
GALT	Gastrointestinal associated lymphoid tissue
GC	Germinal centres
GDF-1	Growth differentiation factor-1
GI	Gastrointestinal
GM-CSF	Granulocyte macrophage colony-stimulating factor
HBSS	Hanks's balanced salt solution
HER	Heregulin is one of a family of ligands with tumour initiating potential
HGFR	Hepatocyte growth factor receptor
HLA	Human leukocyte antigen
HRP	Horseradish peroxidase
IBD	Inflammatory bowel disease
IE	Intestinal epithelium
IFN	Interferon

IFX	Infliximab
Ig	Immunoglobulin
IGF-IR	Insulin–like growth factor -1 receptor
IL	Interleukin
Ins R	Insulin R
ITAM	Immunoreceptor tyrosine-based activation motif
JAK	Janus Kinase
LP	Lamina propria
LPMCs	Lamina propria mononuclear cells
LPS	Lipopolysaccharide
M	Microfold
MAP	Mitogen-activated protein
MAPK	Mitogen-activated protein kinase
M-CSFR	Macrophage colony stimulation factor receptor
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase
MLN	Mesenteric lymph node
MyD88	Myeloid differentiation factor 88
NADPH	Nicotinamide adenine dinucleotide phosphate-oxidase

NFKB	Nuclear factor kappa B
NK	Natural killer
NLR	Nod-like receptor
NO	Nitric oxide
NOD	Nucleotide-binding oligomerisation domain
PAMP	Pathogen associated molecular pattern
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGFR	Platelet derived growth factor
PLC	Phospholipase C
PMA	Phorbol 12-myristate 13-acetate
PP	Peyer's patches
PRR	Pattern recognition receptor
PSA	Polyssacharide A
RA	Retinoic acid
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute-1640 medium
RTK	Receptor tyrosine kinase

SCID	Severe combined immunodeficiency
SED	Subepithelium dome
SFB	Segmented filamentous bacteria
SSC	Side scatter
STAT	Signal transducer and activator of transcription
TACE	Tumour Necrosis Factor Alpha converting enzyme
TAM	Tumour-associated macrophage
TED	Trans epithelial dendrites
TGF	Transforming growth factor
Th1	T helper 1
Th2	T helper 2
Th17	T helper 17
TJ	Tight junctions
TIMP	Tissue inhibitors of metalloproteinases
TLRs	Toll-like receptors
TNF	Tumour necrosis factor
TNFRI	Tumour Necrosis Factor Receptor I
TNFRII	Tumour Necrosis Factor Receptor II
Tregs	T regulatory cells

TSLP	Thymic stromal lymphopoietin
UC	Ulcerative colitis
VEGFR	Vascular endothelial growth factor receptor

1 General Introduction

1.1 The Intestinal Immune System

1.1.1 The Gut Mucosal Immune System

The gastrointestinal tract is the largest immune organ in the body and is responsible for absorbing nutrients as well as maintaining the integrity of the mucosal barrier to potentially harmful antigens and organisms. However, it is also important that tolerance is induced to both innocuous antigens to prevent potentially harmful and inappropriate inflammation as well as tolerance to commensal microorganisms that are required for digestive function (Mowat, 2003). The cross talk of cells of the innate and adaptive immune system with the microbiome, in combination with the specialised properties of the cells in the intestine, all contribute to immune homeostasis.

In the classic paradigm of mucosal immunity, responses are divided into inductive and effector sites against antigens from the gut lumen. Briefly, the inductive sites are the Peyer's patches (PP), where luminal antigens are sampled by M cells and dendritic cells, and presented to naïve T and B cells. The sensitised cells migrate to the mesenteric lymph nodes (MLN), traverse the thoracic duct to enter the blood stream where they home to the intestinal lamina propria using specific adhesion molecules and chemokines.

The glycocalyx forms the first physical barrier to the luminal contents of ingested nutrients and the microbiome. It is composed of mucus produced by goblet cells and anti-microbial peptides released by Paneth cells. The thick consistency of mucus traps microorganisms preventing their invasion. The

intestinal epithelium (IE), beneath the glycocalyx is a single layer of cells that originate from multipotent stem cells located within the intestinal crypt. The epithelial cells have a number of adhesive properties to maintain the barrier, such as cadherins, desmosomes and tight junctions (Garcia-Hernandez et al., 2017). The epithelium absorbs nutrients and can produce cytokines and chemokines, which help maintain the balance between tolerance and inflammation. The selective permeability of the IE is achieved via the transcellular and paracellular pathways. The transcellular pathway operates via specialised transporter mechanisms located on the apical and basolateral surfaces (Kiela and Ghishan, 2009). The paracellular pathways are associated with the transport in the intercellular space between adjacent epithelial cells. It is composed of tight junctions (TJs) and adherens junctions (AJs), which form the apical junctional complex. Adherens junctions together with desmosomes are responsible for forming strong, adhesive, intercellular bonds (Baum and Georgiou, 2011). Permeability, on the other hand is determined by the TJs. Four integral transmembrane proteins have been identified; occludin (Furuse et al., 1993), junctional adhesion molecule (JAM) (Martìn-Padura et al., 1998), claudins (Furuse et al., 1998) and tricellulin (Ikenouchi et al., 2005). The intracellular domains are anchored to the peri-junctional actomysin ring via the interaction with an intracellular protein such as zonular occludens (ZO) proteins. The extra-cellular component of the protein forms the barrier.

The intestinal epithelium is composed of various cell types with specialised functions, namely enterocytes which are primarily involved in the absorption of nutrients; goblet cells, which contain trefoil peptide factors, important in intestinal repair (Taupin and Podolsky, 2003). Paneth cells, found at the bottom

of crypts, secrete antimicrobial peptides which regulate the gut microbiota as well as growth factors involved in maintenance of neighbouring stem cells. The epithelium also contains tuft cells whose function has previously been unclear, but has recently been shown to initiate type 2 responses to parasites by secreting IL-25 (Gerbe et al., 2016). IL-25 activates tissue resident group 2 innate lymphoid cells (ILC2s) to secrete IL-13 which acts on the crypt progenitor cells to promote differentiation of tuft and goblet cells (von Moltke et al., 2016). Finally, enteroendocrine cells sense nutrients (Gribble and Reimann, 2016).

Microfold (M) cells located within the epithelium play a vital role in the follicular associated epithelium (FAE) of Peyer's patches. Peyer's patches are organised into 3 regions namely the FAE, which consists of a single cell layer of epithelial cells adjacent to the intestinal lumen; the subepithelium dome (SED) underlying the FAE; and B cell follicles and T cells areas (Victora et al., 2012). Ten per cent of the epithelial cells are M cells (Kanaya et al., 2012). M cells are specialised phagocytic cells in the FAE, which capture luminal antigens and present them to the immune cells in the dome. M cells differ from other epithelial cells in that the glycocalyx overlying them is thinner making it easier for them to phagocytose antigens. They also have an irregular brush border and smaller microvilli. They phagocytose and transcytose antigen across the epithelium where the antigen enters intraepithelial pockets beneath the M cell basolateral membrane which contains lymphocytes and mononuclear phagocytes.

Mononuclear phagocytes are composed of dendritic cells and macrophages. There is a substantial overlap in the surface phenotype of DCs and macrophages

and differentiating between them can be difficult. For example CD11c and MHC class II are expressed by both macrophages and DCs. However the expression of CD64 and Flt3 can identify cells as macrophages, as opposed to CD64-, Flt- cells which are DCs (Bain et al., 2013).

Macrophages are found in the lamina propria throughout the entire GI tract and are located predominantly close to the epithelium. They capture and destroy material breaching the intestinal barrier due to their location and their phagocytic and bactericidal properties (Smythies et al., 2005). Trans epithelial dendrites (TEDs) extend their processes into the lumen to capture antigen; although these cells were initially thought to be dendritic cells due to their expression of CD11c and MHC class II, it is now known that the trans epithelial dendrites originate from CXCR1+ macrophages (Niess et al., 2005). In the steady state intestinal macrophages are hypo responsive to cytokine and Toll-like Receptor (TLR) activation. CD103+ dendritic cells in the lamina propria promote Foxp3+ regulatory T cells in the presence of retinoic acid and TGFβ, whereas CD103- dendritic cells drive T cells to produce pro-inflammatory cytokines such as IL-6 and TNFα (Ng et al., 2010).

One of the cytokines produced by epithelial cells is thymic stromal lymphopoietin (TSLP) which exerts its biological activities by binding to IL-7 receptor IL7Rα and the specific TSLP receptor resulting in the phosphorylation of signal transducer and activator of transcription (STAT)5 (Takai et al., 2012). TSLP promotes the development of tolerogenic dendritic cells in the lamina propria (Iliev et al., 2009) which induce the differentiation of naïve T cells into

Foxp3+ Tregs and block the development of Th1 and Th17 cells (Rescigno and Iliev, 2009).

Naive T cells are presented with microbial antigens either in the Peyer's patches or in the MLN by antigen-loaded dendritic cells which have migrated to the MLN via the afferent lymphatics resulting in the priming and expansion of effector T cells. The primed T cells then exit the MLN via the efferent lymphatics, return to the circulation via the thoracic duct and home to the gut lamina propria.

The trafficking of T cells to the appropriate organs is a highly regulated process mediated by members of the selectin, integrin and chemokine protein family. Selectins expressed on the endothelial surface and selectin ligands on the circulating leucocyte bind loosely, tethering the leukocyte to the endothelium. Chemokines activate integrins, which then form a tight bond to their specific adhesion molecules, which enables the extravasation of the leukocyte across the endothelium.

Different combinations of chemokines and integrins are found in different parts of the body to ensure that leukocytes are recruited to the areas needed. Cells expressing CCR9 and $\alpha4\beta7$ generated in the PP migrate back to the intestinal lamina propria. CCR9 binds to the ligand CCL25 that is preferentially secreted by the small intestinal epithelium (Bekker et al., 2015).

The integrin $\alpha4\beta7$ binds mucosal addressin cell adhesion molecule-1 (MAdCAM-1). The expression of CCR9 and $\alpha4\beta7$ is induced by dendritic cells in Gut Associated Lymphoid Tissue (GALT). Iwata et al., (2004) showed that

this was mediated by retinoic acid (RA), a metabolite of vitamin A. Vitamin A deficient mice have few T cells in the small intestinal lamina propria (Iwata et al., 2004). Retinoic acid also induces gut-homing IL-10 producing regulatory T cells (Bakdash et al., 2015). DCs in GALT express RALDH, the key enzyme in converting retinol into retinoic acid, while DCs at other sites do not. Retinoic acid binds to cognate nuclear receptors which act as transcription factors to drive $\alpha 4\beta 7$ and CCR9 gene expression.

The epithelium also contains a large number of lymphocytes, mostly CD8+ T cells and is enriched in gamma delta T cells. These cells are thought to be able to kill virally infected epithelial cells. Space precludes a detailed discussion of these cells because there is very little evidence they play a role in IBD.

1.1.2 The Effector Site of Mucosal Immunity- The Lamina Propria

The lamina propria is the effector site of mucosal immune responses. After interaction with APCs, T cells receive signals via their T cell receptors (TCRs), co-stimulatory molecules and a complex network of cytokine signals and undergo activation and differentiation from naïve T cells into effector CD4+ T cells. One of the most important signalling pathways, which transduce cytokines, is the JAK-STAT pathway (Soldevila et al., 2004). The pathway consists of the Janus kinase (JAK) proteins JAK1, JAK2, and TYK2 and signal transducer and activator of transcription (STAT) proteins one to six. JAK activation occurs following ligand binding to a cell surface receptor, which brings two JAKS into close proximity. This results in phosphorylation of tyrosine residues on STATS, which are latent transcription factors located in the cytoplasm. The activated STAT enters the nucleus and activates or represses transcription of target genes.

This provides a mechanism to translate an extracellular signal into a transcriptional response. The suppressors of cytokine signalling (SOCS) proteins are inhibitors of STAT proteins by interacting with Janus kinases to prevent STAT activation. (Nicholson et al., 1999).

The main types of effector T cell lineages are Th1, Th2, Th17 and Tregs and their differentiation is related to particular cytokines and transcription factors.

1.1.2.1 Th1 cell development

Dendritic cells secrete IL-12 on exposure to pathogens, which binds to the IL-12 receptor on the naïve T cell, upregulates the Th1 transcription factor T- box expressed in T cells (T-bet), and down-regulates GATA3, the transcription factor for Th2 cells. IL-12 activates STAT4 signalling giving rise to the transcription and secretion of IFNγ. In addition Th1 cells secrete TNFα and IL-2 to regulate cell-mediated immunity, such as to intracellular pathogens. T-bet expression is central in the balance between susceptibility to infection and autoimmunity. T-bet deficient mice are resistant to autoimmune diseases such as diabetes and colitis, but are more susceptible to mycobacterial and viral infections (Powell et al., 2010).

1.1.2.2 Th2 cell development

Following CD4+ activation through the TCR, the activation of STAT6 by IL-4 leads to the transcription of GATA-3 which results in the release of IL-4, IL-5, IL-10 and IL-13 which promote B cell function. IL-4/ STAT6 signalling in PP leads to class switching to IgA. TGF-beta is also an Ig switch factor (Seo et al., 2009).

1.1.2.3 Th17 cell development

Th17 cells require TGFβ, IL-21, and IL-6 (rather than IL-17) (Bettelli et al., 2006). Th17 cells produce IL-17 and IL-22 providing defence against extracellular pathogens. They express a specific transcription factor ROR gamma t.

1.1.2.4 T regulatory cell development

T regulatory cells are a population of T cells responsible for suppressing the proliferation of effector T cells and the production of Th1 and Th2 cytokines. T reg cells normally constitute 1-10 % of the T cell population. Natural T regs are thymus derived and are characterized by their expression of CD4, CD25 and have high expression of the transcription factor Foxp3. They are suppressed by both cytokines (ie IL-10) and contact dependent pathways (CTLA-4). Inducible Tregs are the CD4 subset, which become regulatory when they see antigen in the presence of TGFβ. They are also FoxP3+ and suppress by making TGFβ. Recent studies have shown that numbers of functional Tregs in the gut in IBD are the same as in normal gut, the high expression of smad7 in effector cells makes them resistant to TGFβ made by Tregs (Fantini et al., 2009).

1.1.3 The Intestinal Microbiome

The intestinal microbiome consists of up to 10^{13} microorganisms maximally concentrated in the colon (MacDonald et al., 2011b). These are made up of just a few main phyla, with the two most dominant being Bacteroidetes and Fermicutes, and at lower numbers of the phyla Actinobacteria, Proteobacteria, Fusobacteria and Verrocomicrobia (Eckburg et al., 2005). The proportions of

these are dynamic within an individual and vary between individuals depending on the type of birth, diet, age and antibiotic use. The microbiome consists of transient and permanent species depending on their ability to colonise the gut. Permanent microbiota have adapted to establish a mutual relationship with the host and do not usually survive as free-living organisms. These are true commensals. Transient microbiota on the other hand do not have a mutualistic relationship with the host but survive despite host defences. The effect can be innocuous, pathogenic or beneficial.

Commensal bacteria are beneficial to the host by competing for space and nutrients within the lumen and thereby preventing other pathogenic bacteria increasing in numbers that could be harmful to the individual. The microbiome also provides nutritional benefits to the host such as synthesizing vitamins (Kau AL et al., 2011) and ferment fibre to produce short chain fatty acids, such as butyrate, which is a major energy source for colonocytes, and stimulates colonic blood flow and electrolyte exchange (Bhat and Kapila, 2017). Butyrate is also an inhibitor of histone deacetylation which affects transcription of pro- and anti-inflammatory genes (MacDonald et al., 2011b). However it is becoming increasingly clear that their presence is also vital in developing the gut immune system. Indeed germ free mice have small Peyer's patches, and have very low numbers of T cells and plasma cells in their gut, which increases following introduction of microbes (Hooper et al., 2001).

The intestinal microbiota influences mucosa and T-cell responses. T-helper type I (Th1) and Th2 cells control intracellular microorganisms and helminths respectively. Th17 cells protect the host from bacterial and fungal infections

(Ivanov et al., 2008). Ivanov et al., (2008) initially showed the direct association between the presence of a single microbe, the segmented filamentous bacterium (SFB), in the small intestine of mice and the accumulation of Th17 cells in the small intestine lamina propria, and their ability to mount effective Th17 responses. Germ free mice have significantly impaired mucosal and systemic Th17 responses. Mice treated with antibiotics also show a reduction in Th17 cells in the SI LP. The reduction in Th17 cells was inversely proportional to T reg cell numbers in the SI LP (Ivanov et al., 2009).

SBF are different from other gut commensals in that they adhere to the ileal epithelium (Atarashi et al, 2015). Other bacterial species, which are able to adhere to the epithelium, are also able to induce Th17 cell responses. These include E.coli 0157 and Citrobacter rodentium (Atarashi et al., 2015). However mutant forms of E.coli 0157 deficient in a protein required for epithelial adhesion, called intimin, are unable to adhere to the IE and fail to induce Th17 responses (Atarashi et al., 2015).

Recent findings by Sano et al., (2015) demonstrate that the induction of Th17 cells by SFB is due to the expression of a protein called serum amyloid A (SAA) protein 1 and 2 on epithelial cells in the ileum.

The induction of T reg cells is associated with the non-pathogenic Clostridium clusters XVIa and IV (Atarashi et al., 2011). Treg cells of mice colonised with these clusters also contain a large number of Helios- Foxp3+ cells suggesting that the Clostridium IV clusters may induce colonic iTreg conversion. Additionally these cells also express high levels of CTLA-4 on their surface and make IL-10 (Atarashi et al., 2011). Round and Mazmanian, (2010) showed that

polysaccharide A (PSA), an immunomodulatory molecule produced by Bacteroides fragilis increases expression of IL-10 and requires TLR2 signalling. PSA has over the years been shown to have many apparently conflicting functions in the gut and its real role has not yet been discovered.

1.1.4 Pattern Recognition Receptors (PRRs)

PRRs recognise conserved structures of bacteria and viruses, called pathogen associated molecular pattern (PAMP)s, and alert the host to a potential infection. PRRs include transmembrane Toll-like receptors (TLR) and intracytoplasmic receptors such as NOD receptors. There are 10 TLRs and their distribution varies throughout the gut. TLR5 is basically expressed in the colonic epithelium and recognises invasive flagellated bacteria, whilst TLR2 and TLR4 are found predominantly in the colonic crypts. TLR3 is expressed in mature enterocytes in the small and large bowel (Elia et al., 2015). Alternatively NOD1 and -2, also known as CARD 4 and -15 are found in the cytosol of epithelial cells. Following injury or ulceration, PRRs are exposed to PAMPs such as lipopolysaccharide (LPS). Recognition of LPS by TLR4 and its co-factors CD14 and MD2 triggers signalling through MYD88 and results in NFκB and MAPK activation. Mice lacking either TLR2, TLR4, TLR9, or Myd88 are highly susceptible to DSS colitis (Rakoff-Nahoum et al., 2004). TLR4-deficient mice have decreased proliferation following DSS injury than wild-type mice. TLR4 expression is reduced in the IEC and LPMCs in IBD (Abreu, 2010). Activation through TLR4 expression leads to increased TNFα production, as well as phagocytosis and translocation of bacteria. TLR2 and 4 are expressed at low levels by IECs in the normal colon and TLR3 in the small intestine and normal colon.

1.2 Inflammatory Bowel Disease

Inflammatory bowel disease consists of 2 main conditions, Crohn's disease (CD) and Ulcerative Colitis (UC), which are chronic relapsing and remitting diseases affecting 261,000 and 115,000 people in the UK respectively (NICE 2013). The aetiology is incompletely understood, but appears to be a complex combination of genetic and environmental interactions which result in immune dysfunction. Although there are similarities between UC and CD they are in fact different diseases. The challenge that they present is the increasing incidence worldwide (Molodecky et al., 2012) and the limitations of current treatments.

UC and CD are both relatively recent diseases whose incidence has increased dramatically since the 1950s. Both are more common in Northern Europe and North America (Bernstein et al., 2006b) (Bernstein et al., 2006a) although the incidence is increasing in Asia (Thia et al., 2008) and Eastern Europe (Ng et al., 2015) . It has also been traditionally more common in Caucasians, and genetic mutations in NOD 2, which are found in 15 % of CD individuals, are only found in Caucasians. However the incidence in Caucasians is also environmental, as the incidence in 2nd generation Asians in the UK is greater than resident Caucasians (Probert and Brown, 2008). The age of onset is different for UC and CD. UC develops throughout life whereas CD has two thirds of cases diagnosed in late adolescence and early adulthood. The age range of onset appears to be changing with increasing presentations in childhood as well as in elderly patients (Nguyen et al., 2017). There is a slight female preponderance in CD, which is not seen in UC. Smoking is a risk factor for CD (Calkins, 1989) as well as being associated with earlier recurrence following surgery and poorer response to

medical treatment. Smoking in UC however, is protective (Ponder and Long, 2013) . Appendectomy before the age of 20 is also strongly protective against UC (Baron et al., 2005)

1.2.1 Clinical and Histological Features of IBD

The main symptoms of Crohn's Disease are diarrhoea, abdominal pain and weight loss. Unexplained anaemia and growth failure in children may also be the only presenting features. The symptoms are often non-specific and chronic although acute abdominal pain raising the possibility of acute appendicitis can also be due to CD. Ulcerative colitis also presents with diarrhoea and abdominal pain, although bleeding per rectum is a more predominant feature than in CD, likely due to the different parts of the gut affected.

Crohn's Disease can affect any part of the intestine from the mouth to the anus, often discontinuously, although it more commonly affects the terminal ileum. Ulcerative colitis initially affects the rectum and can extend proximally to involve the whole colon. Both UC and CD are associated with extra intestinal features in up to 50% of patients. These include arthralgia, uveitis, pyoderma gangrenosum (Weizman et al., 2014) as well as other life-threatening conditions such as venous thrombo-embolism and primary sclerosing cholangitis. Extra intestinal manifestations are generally more common in active disease with the exception of ankylosing spondylitis and uveitis. CD is also associated with fistulas between loops of bowel or external and internal surfaces as well as abscesses (Yamamoto and Watanabe, 2014).

Diagnosis is based on symptoms, examination, blood tests suggesting inflammation, and imaging. The gold standard is endoscopic and

histopathological evidence of inflammation. Biopsies favouring a diagnosis of CD show the presence of granulomas, show patchy lesions, a normal mucosal surface and predominantly involvement of the cecum and ileum (Feakins, 2014). In UC on the other hand, the inflammation and ulceration is limited to the mucosa, there is lymphocytic but a mainly granulocytic infiltration and crypt abscesses. There is a loss of goblet cells from the epithelium and extensive epithelial cell damage (DeRoche et al., 2014). Although CD and UC are 2 separate diseases, the overlap in their presenting symptoms and areas of the bowel affected can make diagnosis difficult. In fact in some patients the diagnosis is changed (Prenzel and Uhlig, 2009).

In CD, inflammation extends from the mucosa into the deeper muscle layers. In addition to inflammation, fibrosis is also seen in areas of previous inflammation and characterised by an excessive deposition of extracellular matrix proteins (Biancheri et al., 2015). Although the tissue can heal without the development of fibrosis, chronic inflammation and the repeated healing required appears to be associated with the development of fibrosis (Rieder et al., 2017).

In UC the rectum is always affected and the disease can extend proximally to involve the whole of the colon. Twenty per cent of patients with UC have pan colitis which is associated with an increased risk of developing colon cancer (Munkholm, 2003).

1.2.2 Management of IBD

Management is both medical and surgical, although the need for surgery has reduced since the introduction of anti-TNF antibodies (Vogelaar et al., 2009). Treatment varies depending on the site and severity of disease and whether the treatment is to induce or maintain remission. The majority of drugs are used for both CD and UC although their indications vary slightly. Thus an accurate diagnosis is vital prior to treatment.

Steroids are used in the acute phase in mild disease in CD in order to induce remission. Enteral therapy is used instead of steroids in children, however its efficacy in adults is limited, possibly due to poor compliance (Penagini et al., 2016). In ulcerative colitis steroids may be required to induce remission in the acute phase. Mesalazines, either topically for left sided disease, or orally for more extensive disease, are the mainstay of treatment in UC. In both CD and UC more powerful immunosuppressants such as azathioprine, 6 mercaptopurine or less frequently methotrexate, are introduced if steroids or aminosalicylates are insufficient to induce remission, or if relapse occurs after cessation of steroid therapy (Timmer et al., 2016).

In a proportion of patients with IBD in whom azathioprine is ineffective anti-TNF treatments are possible. Those with clinical features of a poor prognosis such as severe endoscopic lesions, age less than 40 years, ileal or ileocolonic location and perianal disease at time of diagnosis, should be considered for anti-TNF early (Jauregui-Amezaga et al., 2015). Anti-TNF treatment will be considered in a separate section.

Surgical intervention for CD is considered in individuals presenting with bowel obstruction, localised peritonitis thought to be due to appendicitis (Gomollón et al., 2017) (Yarur et al., 2014), and in individuals with strictures amenable to surgery. Surgery plays an important role in the complications of CD such as strictures, fistulae and abscesses. There is no medical therapy shown to be effective in fibrosis, although endoscopic dilatation can be used if the strictures are short. Anti-TNF antibodies are used to help close fistulae and are effective in up to 60% of patients (Allocca et al., 2017) .

In UC, fewer individuals have surgery than in CD, (Singh et al., 2015). This may however occur at first presentation with severe colitis, or in those patients in whom medical therapy is not an option. It is thought that surgical intervention is reducing with the use of biologics (Fu et al., 2014).

1.2.3 Genetics of IBD

The incidence of CD and UC is increased in first-degree relatives compared to the general population, and the incidence of CD particularly, is increased amongst monozygotic twins compared to dizygotic twins (38% concordance compared to 2% respectively); whereas in UC the concordance rates are 15% and 8% respectively (Halfvarson, 2011). However the absence of simple Mendelian inheritance suggests that multiple gene loci are responsible and that the pathophysiology is multifactorial. In 2001, Hugot et al., (2001) and Ogura et al., (2001) discovered that mutations in the gene encoding NOD (nucleotide oligomerization domain) 2, also known as caspase activation and recruitment domain (CARD) 15 was associated with CD in 15% of patients. NOD2 consists of two amino-terminal caspase recruitment domains (CARDs), a centrally

located domain, and multiple leucine-rich repeats (LRRs) at its carboxy-terminal. Three different mutations were found in NOD2, all located near the leucine rich repeat (LRR) region (Hugot et al., 2001). Nucleotide oligomerization domain is expressed in gut epithelial cells, antigen presenting cells (macrophages and dendritic cells) and Paneth cells (Strober et al., 2014). NOD2 acts as an intracellular receptor for the peptidoglycan components of gram-positive bacteria and the mutations result in loss of function (van Heel et al., 2005). Activation leads to the activation of NFκB and mitogen-activated protein (MAP) kinase pathways and increases the production of pro-inflammatory cytokines such as TNFα and IL-1β. The mutations are only found in Caucasians with CD and are associated with an increased incidence of ileal and fibrotic disease (Radford-Smith and Pandeya, 2006). Individuals homozygous for a single NOD2 mutation or compound heterozygotes have a 20-40 fold increase in the risk of developing CD. However it is important to emphasise that many healthy individuals carry the same NOD2 mutations. NOD1, although structurally similar to NOD2 does not play a major role in disease susceptibility (Zouali et al., 2003).

Since the advent of genome wide association studies, 163 different IBD loci containing gene variants either over-expressed or under expressed in IBD have been identified. The genes cluster into groups with different functions. NOD2 for example or the autophagy genes, ATG16L1 and IRGM relate to bacterial handling (Parkes et al., 2007). The IBD3 region (6p21.1-23) is an area that encompasses the human leukocyte antigen (HLA) genes and is associated with UC and CD (Muro et al., 2014). This region also encodes TNFα and the TNFα receptor. A polymorphism in the TNFα receptor was found to be associated with

infliximab response in Japanese patients (Matsukura et al., 2008). The Immunochip study led to the validation of a total of 163 genetic loci containing susceptibility to IBD (Jostins et al., 2012). IBD loci are shown in Table 1.

	Cellular innate immunity	Immune mediated	Other
CD	NOD2	IL2RA	GCKR
	IRGM	IL18RAP	THADA
	ATG16	IL27	SP140
	LRRK2	PTPN22	PRDX5
		CCR6	ZPF36L1
		ERAP2	ZIMZ1
		CCL2/CCL7	CPEB4
		TNFSF11	FADS1
		BACH2	TAGAP
		ITLN1	DENNDIB
		TAGAP	DNMT3A
		VAMP3	MUC1/ SCAMP3

	Immune mediated	Other	HLA	TH17
IBD	IL10	CRMD3	DRB103	STAT3J
	CARD9	RTEL1		L23R
	MST1	PTGER4		IL12B
	REL	KIF21B		AK2
	ICOSLG	NKX2-3		FUT2
	IL1R2	CREM		TYK2
	YDJC	CDKAL1		
	PRDM1	STAT3		
	TNFSF15	ZNF365		
	SMAD3	PSMG1		
	PTPN2			
	TNFRSF6B			

	Immune mediated	Other	Epithelial barrier
UC	IL8RA / IL8RB	OTUD3 / PLA2G2E	ECM1
	IL2 / IL21	PIM3	HNF-4A
	IFNY / IL26	DAP	CDH1
	IL7R	CAPN10	LAMB1
	TNFRSF9	JAK2	GNA12
	TNFRSF14		
	IRF5		
	LSP1		
	FCGR2A		

Table 1.1 Inflammatory bowel disease (IBD) loci, represented by lead gene name, according to pathway. (Ek et al, 2014)

1.2.4 The Microbiome in IBD

There have been huge advances recently in the understanding of the relationship between the gut microbiome and IBD (especially CD) with the advent of next generation sequencing. Although the association between IBD and microbes has been suspected for many years due to clinical observations. Firstly the majority of lesions are seen in the areas of the bowel in which the microflora are most abundant, such as the terminal ileum and colon. The use of metronidazole, an antibiotic, is an effective treatment of peri-anal CD (Rutgeerts et al., 2004) Crohn's disease has been successfully treated if the area of inflamed bowel is

diverted, and lesions return when the faecal stream is reintroduced into the bowel (Flanagan et al., 2012), (Sauk et al., 2014). Bacteria, which are associated with the mucosa such as Bacteroides fragilis, have been found in increased numbers in the biofilm of IBD patients compared to controls (Swidsinski et al., 2005). Dubinsky et al., (2006) showed that raised immune responses to microbial antigens were associated with a more aggressive phenotype in paediatric CD. There is still controversy as to the pathogenesis of the microbiota and IBD. The proposed theories are discussed below.

1.2.4.1 Functional changes in commensal bacteria

Some strains of commensal bacteria are able to adhere to and invade intestinal epithelial cells such as a more virulent strain called AIEC (adherent/ invasive E. coli). AIEC showed the ability to survive and replicate within macrophages in ileal CD (Baumgart et al., 2007) and increased numbers of E. coli are associated with reduced time before relapse (Lopez-Siles et al., 2014) and active disease (Mylonaki et al., 2005). The receptor, which enables ileal colonisation in CD by AIEC, was identified by (Barnich et al., 2007) as CEACAM6. In addition its expression is activated by TNFα and IFNγ.

1.2.4.2 Commensal Microbiota Dysbiosis

The microbiome in IBD patients is less diverse than normal patients. Species which are reduced in IBD are clostridium species and Faecalibacterium praunitzii (Sokol et al., 2009), (Joossens et al., 2011). Low numbers of F prausnitzii are associated with post- resection relapse in CD, which is likely related to its purported ability to stimulate secretion of IL-10 by dendritic cells

(Wu et al., 2017). Nonetheless there are many who consider that the changes in the microbiota seen in IBD are secondary to inflammation, and that to imply that a dysbiosis is responsible for immune mediated diseases is far fetched.

1.2.4.3 Defective containment of commensal bacteria

NOD2, which is predominantly expressed in Paneth cells, senses muramyl dipeptide (MDP) in the cytoplasm and triggers downstream signalling pathways such as NFκB thus initiating antimicrobial activities. NOD2/CARD15 polymorphisms in CD prevent the effective binding of bacterial peptidoglycan and reduce the production of antimicrobial α-defensins and subsequent defective clearance of invasive bacteria (Wehkamp et al., 2005).

1.2.4.4 Defective host immunoregulation

IBD patients have aggressive T cell responses to commensal bacteria and fail to develop tolerance to oral antigens (Sartor and Muehlbauer, 2007). The NOD2/ CARD15 mutation reduces effective clearance of bacteria. Finally IBD in IL-2 null mice and IL-10 null mice depends on the presence of a normal gut flora. When the mice are placed in a germ-free environment they do not develop colitis but when they are exposed to either a single species of bacteria or a mixed bacterial colonisation they develop colitis very quickly (Podolsky, 1997). In 2007 two autophagy related genes, immune-related GTPase family M protein (IRGM) and autophagy-related 16-like 1 (ATG16L1), were identified and associated with CD (Hampe et al., 2007), (Massey and Parkes, 2007). These are variants that control autophagy, the breakdown of bacteria by the autophagosome. Saitoh et al., (2008) showed that in mice knocked out for

ATG16L, a protein necessary for autophagic recruitment, TLR and TNF were stimulated leading to abnormal inflammasome activity in innate immune cells.

1.2.5 Adaptive Immunity in IBD

In CD there is an excessive Th1 response. CD4+ cells release large amounts of IFNγ, and TNFα, and Th1 inducing cytokines such as IL-12 and IL-18 are also increased. Rovedatti et al., (2009) showed that there is an increase in the number of Th17 and Th1/Th17 cells in IBD. In health, lamina propria T cells would normally undergo apoptosis in the absence of the peptides that activated them in PP, but in IBD, the presence of luminal peptides and survival cytokines such as IL-2, IL-7 and IL-15 allow the cells to persist and continue to secrete cytokines (MacDonald et al., 2011a). The large amounts of TNFα and IFNγ induce macrophages to fuse and become epithelioid giant cells and form granulomata. Resident myofibroblasts activated by pro-inflammatory cytokines produce matrix metalloproteinase (MMP)-3 that causes local mucosal damage and ulceration (Pender et al., 1997), (Salmela et al., 2002). MMP3 is increased in the fistulous tracts of patients with CD (Scharl and Rogler, 2014) possibly due to increased IL-17A expression (Biancheri et al., 2013). The defective barrier allows microbes to infiltrate and exposes T cells and APC in the lamina propria to an increased amount of antigen that perpetuates the inflammatory cycle. Regulatory T cells have been shown to be vital in inhibiting inflammation in animal models of IBD and an inflammatory enteropathy similar to CD occurs following mutations in FOXP3 gene and results in no Tregs (Bennett et al., 2001).

1.3 TNFα and IBD

The most important advance in IBD has been related to TNFα. Tumour Necrosis Factor α is a key cytokine in inflammation. It was initially found to be increased in the serum of children with an acute episode of UC and colonic CD (Murch et al., 1991). Following this TNFα secreting cells were increased in inflamed mucosa of CD and UC children (Breese et al., 1994). It was a step change in IBD therapy when (van Dullemen et al., 1995) showed that a single infusion of an anti-TNFα human/mouse chimeric monoclonal antibody (later called infliximab) led to the normalisation of the CDAI scores and mucosal healing in 8 out of 10 Crohn's patients. The clinical response to a single dose of infliximab was still evident 12 weeks after the infusion (Targan et al., 1997). Infliximab is effective in fistula healing (Yang et al., 2015). It was later shown to be effective in UC as well (Armuzzi et al., 2004).

1.3.1 TNFα production

TNFα is produced mainly by macrophages but also by CD4+ T cells, NK cells, neutrophils and eosinophils. TNFα can regulate enterocyte growth and alter the permeability of the epithelial barrier (Feng et al., 2013). TNFα also stimulates the production of MMPs and other tissue remodelling enzymes in the intestinal mesenchymal cells that are central to regulating epithelial cell function (Salmela et al., 2002).

Tumour Necrosis Factor alpha exists in two forms, initially as a trimer on the cell membrane called membrane TNFα, and after cleavage from the cell surface

by the matrix metalloproteinase TNFα converting enzyme (TACE/ ADAM17) into a secreted trimeric molecule, soluble TNFα (sTNFα) (Black et al., 1997). Soluble TNFα is produced by activated macrophages and lymphocytes and can bind to TNFRI and TNFRII. TNFRI and TNFRII are expressed on almost all nucleated cells.

TACE also cleaves other transmembrane cytokines from the cell surface such as epidermal growth factor receptor ligands, TGFα, L-selectin, and IL-6 signalling pathways (Sharma et al., 2014). In addition, TACE deficient mice die at birth (Black et al., 1997). Mice with barely detectable levels of ADAM17 (due to generation of a new exon within the ADAM17 gene) show enhanced susceptibility in inflammatory bowel disease models as well as showing severe skin inflammation (Chalaris et al., 2010). The role of TACE in humans however may be less important as a TACE knockout patient has been described and leads a relatively normal life despite recurrent inflammation of the skin and bowel (Blaydon et al., 2011) .

The natural inhibitor of TACE is TIMP-3, which also inhibits matrix metalloproteinases and has been shown to reduce pro-inflammatory cytokine transcripts in IBD biopsies (Monteleone et al., 2012). Virtually all cells in the body can express TACE, but it is barely detectable on the surface of most cells, suggesting that the expression of TACE on the cell surface is regulated. Erk-mediated phosphorylation has been shown to translocate TACE to the cell surface (Soond et al., 2005) and iRhom2 is also needed for surface expression (Adrain et al., 2012).

Membrane TNFα is constitutively expressed on Natural Killer cells but not on resting T, B cells or monocytes (Caron et al., 1999) but is expressed on T cells, macrophages and monocytes following activation (Horiuchi et al., 2010). Membrane TNFα can bind to both receptors but generally has a greater affinity for TNFRII. Binding of TNFRI results in activation of the caspase dependent apoptosis, whereas the binding of TNFRII signals TRAF2 and NFκB induction, which has anti-apoptotic effects (Atreya et al., 2011). Both these effects occur locally due to the cell-to-cell interaction. In addition to acting as a ligand, mTNFα can also bring about changes in the cell expressing mTNFα via reverse signalling (Horiuchi et al., 2010). The cytoplasmic tail of the transmembrane TNFα protein can trigger intracellular signals such as activating MAP kinase increasing TGFβ production (Pallai et al., 2016).

TNFα and particularly mTNFα play a major role in defending the host against bacterial, viral and parasitic infections. Various studies using transgenic mice expressing only mTNFα have shown the involvement of TNFα in macrophage migration and granuloma formation following *Mycobacterium tuberculosis* infection. Membrane TNFα was shown to be sufficient to allow cell-cell signalling and control of mycobacterial infection in TNF KO mice and mTNFα mice (Fremond, 2005).

1.3.2 Anti-TNFα Agents

Infliximab is a human/mouse chimeric monoclonal antibody, which is effective in UC and CD. Other TNFα antibodies have been developed which differ in their structure. Adalimumab is a recombinant whole antibody and certolizumab

pegol is a PEGylated Fab' fragment of a humanised monoclonal antibody that binds and neutralises human TNFα and both are effective in IBD (Nesbitt et al., 2007). Etanercept is engineered from TNFRII coupled to the Fc receptor of IgG, and is not licensed for use in IBD due to its poor efficacy shown in a double blind trial (Sandborn et al., 2001). Although it is still not clear how anti-TNFα antibodies work in IBD it is helpful to consider their different structures to help understand their mechanisms of action.

1.3.3 Proposed mechanisms of action of Anti-TNF Antibodies

Originally TNFα was thought to be a key cytokine because when produced by macrophages it fed back and induced secretion of other inflammatory mediators such as IL-8 and diminished expression of adhesion molecules E-selectin and ICAM-1 (Feldman et al., 1998). Neutralising soluble TNFα in activated PBMC blocked this feedback loop. However this is clearly not the whole picture. The duration of the response to a single dose exceeds the half-life of the antibody. In addition, etanercept, which neutralises sTNFα is ineffective in the treatment of Crohn's disease (Sandborn et al., 2001). Infliximab reduces the pro-inflammatory cytokine IL-1β in PBMCs stimulated with LPS (Nesbitt et al., 2007). Other pro-inflammatory cytokines such as IL-6 and IL-8 are reduced in the serum of CD patients following treatment with infliximab (Suzuki et al., 2015).

Complement-dependent cytotoxicity (CDC) of Jurkat cells and CHO cells bearing mTNFα is seen with infliximab but not etanercept (Mitoma et al, 2008, Arora et al, 2009) possibly due to the lack of the CH1 domain, which contains

the C1q binding site in etanercept. However Kaymakcalan et al (2009) was unable to show CDC by infliximab in activated human PBMCs.

Antibody-dependent cytotoxicity (ADCC) occurs when the Fc portion of IgG1 binds to Fc receptors on NK cells resulting in granzyme B and perforin release and ultimately target cell lysis. ADCC was seen using an mTNFα expressing cell line incubated with infliximab but not etanercept (Arora et al., 2009), (Nesbitt et al., 2007). The concept of reverse signalling on binding to mTNF leading to apoptosis has also been shown.

Apoptosis, also known as programmed cell death, is an important process in the regulation of the immune system. A balance of pro- and anti- apoptotic factors exist and these can be set in motion in 2 ways; via the intrinsic cell death pathway or the extrinsic pathway. P53 is a regulatory molecule found in the cytoplasm which detects cell stress and DNA damage. If this is detected, p53 translocates to the nucleus to initiate transcription of pro-apoptotic factors. The extrinsic pathway can be activated via various means such as the death receptor ligand interacting with FAS, TNF and TNFR1 interaction.

Lugering et al., (2001) were the first to show apoptosis of peripheral blood monocytes following infliximab infusions in patients with CD. Apoptosis was then shown to occur in T cells in the lamina propria 24 hours after infliximab infusion as well as in vitro using an activated Jurkat cell line cultured with infliximab (Ten Hove et al 2002). Van den Brande et al., (2003) compared infliximab and etanercept and showed that both neutralise TNFα, however only infliximab induced apoptosis. Increased apoptosis was also shown in mucosal biopsies 10 weeks following infliximab and was caspase dependent (Di Sabatino

et al., 2004). Waetzig et al., (2005) showed apoptosis occurs via reverse signalling through mTNFα.

A final non-immunological explanation for the success of infliximab is that activated fibroblasts in IBD express mTNFα and infliximab binding induces inside out signalling and a large increase in their production of TIMP-1, the endogenous MMP inhibitor (Di Sabatino et al., 2007).

Anti-TNFα antibodies have allowed patients with IBD to achieve more than just symptomatic relief. Infliximab has been shown to induce mucosal-healing, and reduced the number of hospitalisations and surgery (Rutgeerts et al., 2004). The ACT I and ACT II trials showed that infliximab was also effective in steroid refractory ulcerative colitis (Rutgeerts et al., 2005). However only 50% of patients respond to infliximab and those that do respond do not all continue to do so. In addition to this, the side effects of anti-TNFα antibodies, principally infections, are significant. This indicates that better pharmaceutical options are still needed.

2 Aims

1. To establish whether pharmacological and endogenous TACE inhibitors reduce the production of sTNFα and whether other pro-inflammatory cytokines would or would not be affected.
2. To elucidate which cells express mTNFα in PBMCs and LPMCs and whether pharmacological and endogenous TACE inhibitors increase mTNFα expression.
3. To elucidate whether infliximab reduces pro-inflammatory cytokines or causes apoptosis and the role of mTNFα in these activities.
4. To determine if the addition of TNFα neutralising agents to mucosal biopsies from patients with IBD ex vivo has any effect on RTKs.
5. To establish whether the addition of infliximab to mucosal biopsies influences the production of other cytokines and chemokines.

3 Regulation of Soluble Tumour Necrosis Factor Production by Tumour Necrosis Factor Alpha Converting Enzyme (TACE) Inhibitors

3.1 Introduction

Tumour Necrosis Factor α is a key cytokine in inflammation. It was initially found to be increased in the serum of children with an acute episode of UC and colonic CD (Murch et al., 1991). Breese et al., then showed that TNFα secreting cells were increased in inflamed mucosa of CD and UC children (Breese et al., 1994). It was a step change in IBD therapy when Targan et al. (1997) showed that neutralising TNFα reduced inflammation in CD, and later in UC by (Armuzzi et al., 2004). Since then various anti-TNFα antibodies have been developed and are the mainstay of treatment for steroid-dependent CD and UC.

Tumour Necrosis Factor α is a unique cytokine because it exists as a trimer in 2 forms. Firstly on the cell membrane (mTNFα) and as a secreted molecule known as soluble TNFα (sTNFα). Both forms have been shown to be responsible for driving inflammation (Perrier et al., 2013). Soluble TNFα preferentially binds to TNFα receptor I whereas mTNFα preferentially binds to TNFRII (Rauert et al., 2010) .

Soluble TNFα is formed when membrane TNFα is cleaved from the cell surface by the TNFα converting enzyme (TACE) (Black et al., 1997). Virtually all cells in the body can express TACE but it is barely detectable on the surface of most cells, suggesting that the expression of TACE on the cell surface is regulated.

Erk-mediated phosphorylation has been shown to translocate TACE to the cell surface (Soond et al., 2005) and iRhom2 is also needed for surface expression (Adrain et al., 2012).

Tumour necrosis factor α converting enzyme also known as A Disintigrin and Metalloproteinase-17 (ADAM 17) is responsible for a wide range of physiological processes in addition to TNFα release. These include processing adhesion proteins (L-selectin (Le Gall et al., 2009) and ICAM-1), cytokine receptors (IL-6R (Chalaris et al., 2011) and TNFR) and epidermal growth factor-receptor ligand (Dey et al., 2010). In addition, TACE deficient mice die at birth (Black et al., 1997). Mice with barely detectable levels of ADAM17 (due to generation of a new exon within the ADAM17 gene) show enhanced susceptibility to inflammatory bowel disease models as well as severe skin inflammation (Chalaris et al., 2010). The role of TACE in humans however may be less important as a TACE knockout patient has been described and leads a relatively normal life despite recurrent inflammation of the skin and bowel (Blaydon et al., 2011) .

The natural inhibitor of TACE is tissue inhibitor of metalloproteinase (TIMP)-3 (Amour et al., 2000), (Black et al., 1997). TIMP-3 is constitutively expressed in the intestinal epithelial cells in normal and inflamed human gut (Fréour et al., 2009). TIMP-3 deficiency is associated with increased hepatic inflammation and TIMP-3 knockout mice develop severe colitis when exposed to 2,3,4 trinitri-benzene-sulfonic acid (TNBS) compared to TIMP-3 transgenic mice which are resistant to TNBS colitis (Monteleone et al., 2012). TACE inhibition has been shown to increase TNFα mediated hyper permeability of the intestinal barrier

(Fréour et al., 2009). In IBD (Monteleone et al., 2012) showed that TIMP-3 is down regulated in inflamed CD but up regulated when Smad7 (an inhibitor of TGF-β1 signalling) was knocked down. (Cesaro et al., 2009) found that increased TACE expression in mucosal biopsies was associated with inflammation in CD and TIMP-3 expression was reduced.

There has been an interest in the therapeutic benefits of TACE inhibitors to reduce TNFα production. Animal models have shown improved inflammation in arthritis and an iatrogenic inhibitor of TACE, compound 11p, was also shown to result in an improvement in inflammation in DSS induced colitis (Sharma et al., 2014). However the role of TACE inhibitors in IBD is still unclear.

3.2 Aims

To elucidate whether sTNFα production by LPMCs, PBMCs and biopsies would decrease with the addition of TACE inhibitors and whether other pro-inflammatory cytokines would or would not be affected. Accordingly the following experiments were carried out.

1. Soluble TNFα production was measured by activated PBMCs and LPMCs with the TACE inhibitor Marimastat in healthy controls.
2. Soluble TNFα production was measured in supernatants of IBD colonic biopsies incubated with the endogenous TACE inhibitor TIMP-3.
3. IL-1β, IL-6 and IL-8 were measured in supernatants of IBD colonic biopsies incubated with the endogenous TACE inhibitor TIMP-3.
4. Soluble TNFα and other pro-inflammatory cytokine production was measured with a specific TACE inhibitor in activated PBMCs, and in the supernatants of biopsies cultured overnight.

3.3 Materials and Methods

3.3.1 Patients and Sample Selection

Colonic samples were taken from patients undergoing colonoscopy or colonic resections after obtaining consent. Specimens from intestinal resections were obtained from CD and UC patients requiring surgery or healthy control specimens of patients without CD or UC requiring surgery for cancer. Colonic biopsies were also taken from patients with diarrhoea without any endoscopic macroscopic inflammation who had functional diarrhoea at the end of their diagnostic work-up. Inflamed mucosal samples were from patients with active symptoms from their IBD and macroscopic evidence of inflammation. Patients were consented to take part in research which had been approved by the City and East London research ethics committee (10/H0704/74).

3.3.2 Isolation of Peripheral Blood Mononuclear Cell (PBMC)

Healthy individuals and patients with a confirmed diagnosis of CD or UC gave their consent to donate blood for the study. The blood was collected in heparinised tubes and then diluted with phosphate buffered saline (PBS Invitrogen, Paisley, UK) in a 1:1 ratio and layered onto an equal volume of Ficoll (Amersham Pharmacia Biotech, Little Chalfont, UK) for centrifugation at 150g for 20 minutes at 21°C. The mononuclear cell layer was removed and washed with PBS and centrifuged again at 150g for 5 minutes at 10°C. The cell pellet was isolated and then resuspended. The cells were counted using a haemocytometer with trypan blue to measure cell viability.

3.3.3 Isolation of Lamina Propria Mononuclear Cells (LPMC) from Surgical Resections

Specimens from intestinal resections were obtained following consent of CD and UC patients requiring surgery. Control specimens were obtained following consent of patients without CD or UC requiring surgery for cancer. The specimens were taken immediately to the histopathology department for macroscopic examination. Full thickness pieces of gut in length from 3-7cm were then removed from the specimen by the histopathologist and placed in ready to use Roswell Park Memorial Institute-1640 medium (RPMI)(Invitrogen) on ice and transported to the lab. The mucosa was removed from the specimens with scissors and incubated in calcium- and magnesium-free Hank's balanced salt solution (HBSS) with 1mM ethylene tetra-acetic acid (EDTA)(Sigma-Aldrich, Gillingham, Dorset, UK) for 30 minutes at 37°C with agitation in order to remove the mucus and epithelial cells. The tissue was then cut into 3-4mm pieces with a scalpel and digested in 1mg/ml collagenase (Sigma-Aldrich, Gillingham, UK) and DNAase I (10U/ml; Roche, Mannheim, Germany) with agitation for 60 minutes at 37°C. The suspension was then passed through a 100μm cell strainer and washed in RPMI (Sigma-Aldrich, Gillingham, UK). The pellet was resuspended in 20ml of PBS and the LPMCs purified using Ficoll density gradient (centrifuged at 150g for 30 minutes at 20°C). The cell pellet was resuspended in RPMI and the cells counted using trypan blue.

3.3.4 PBMC cell activation

Following PBMC isolation, PBMCs were activated using LPS (Sigma-Aldrich, Pool, UK), anti CD3/ anti CD28 antibody or PMA/ Ionomycin. One millilitre

medium containing 1 million PBMCs was placed in a well of a 24 well plate in the following conditions; medium, LPS (100ng/ml; Sigma-Aldrich, Pool, UK), anti CD3/28 antibody (1μg/ml; eBioscience, San Diego, CA), LPS and Marimastat (10μM; Sigma-Aldrich, Pool, UK) and anti CD3/28 antibody and Marimastat (10μM) for 24 and 48 hours. A dose response experiment was later carried out with 0.1μM, 1μM and 10μM concentrations of Marimastat and LPS (100ng/ml; Sigma-Aldrich, Pool, UK). One millilitre of 1 million PBMCs were also placed in a 24 well plate in the following conditions; DMSO, PMA (50ng/ml Sigma-Aldrich, Pool, UK)/ Ionomycin (500ng/ml) for 4 hours, or PMA / Ionomycin and GW280264X, a gift from Prof Stefan Rose-John (10μM). The supernatant was stored at -80°C for further analysis by ELISA.

3.3.5 Organ Culture of Intestinal Mucosal Biopsies

Uninflamed and inflamed colonic biopsies obtained during colonoscopy were cultured (one biopsy per well) in 24 well plates (VWR International, Lutterworth, UK) in 300μl of serum free HL-1 medium (Cambrex BioScience, Wokingham, UK) for 24 and 48 hours at 37°C, 5% CO. TACE inhibitors (Marimastat 10μM, TIMP-3 100ng/ml (R and D Systems, Abingdon, UK) and GW280264X 10μM) were added. The supernatant was collected and stored at -80°C and cytokines measured by ELISA.

3.3.6 Measurement of Cytokine Production by ELISA

Ninety-six well microplates (VWR International, Lutterworth, UK) were coated with anti-TNF-α (480μg/ml; R and D Systems, Abingdon, UK), anti-IL-6 (360μg/ml; R and D Systems, Abingdon, UK), anti-IL-1β (480μg/ml; R and D

Systems, Abingdon, UK) or anti-IL-8 (720μg/ml; R and D Systems, Abingdon, UK) overnight at room temperature. The plates were then washed 3 times using ELISA wash solution (PBS 0.05% Tween (Sigma-Aldrich, Gillingham, UK)) and then blocked with PBS 0.1% bovine serum albumin (BSA) (TNFα, IL-6, IL-1β) or PBS 0.01% BSA (IL-8) for 1 hour at 37°C. Following another 3 washes, the plates were incubated with serially diluted recombinant cytokine standards and diluted samples (1:2 for TNFα, 1:10 for IL-6, 1:5 for IL-1β, 1:50 for IL-8). After 2 hours the plates were washed and then incubated with a biotinylated anti-TNF-α (4g/ml), anti-IL-6 (9μg/ml), anti-IL-1β (4μg/ml) and anti-IL-8 (3.6μg/ml) for 1 hour. The plates were then washed and incubated with Streptavidin HRP (1 in 200) for 20 minutes before washing, and substrate solution was added. Finally without washing, 50μl of stop solution (H2SO4) was added in order to measure the optical density of each well using a microplate reader at 450nm.

3.4 Results

3.4.1 Soluble TNFα production is reduced by Marimastat in both activated PBMCs and LPMCs

Soluble TNFα levels were undetectable in non-activated PBMCs of healthy controls as expected. Soluble TNFα increased when PBMCs were activated with LPS to 1574pg/ml and 2422pg/ml in the 2 healthy donors after 24 hours. This increase is greater in one individual when PBMCs are incubated for 48 hours (2949pg/ml) but less in the other individual (901pg/ml). When Marimastat, was co-incubated with LPS for 24 or 48 hours the concentration of sTNFα was markedly reduced (Figure 4.1).

Soluble TNFα also increased when PBMCs were activated with anti CD3/anti CD28 antibody for 24 hours (977.30pg/ml and 3975.30pg/ml) (Figure 3.1). Soluble TNFα is further increased when the PBMCs are activated for 48 hours (2696.50pg/ml and 10477.80pg/ml respectively). Marimastat again markedly reduced sTNFα production (Figure 3.1).

3.4.2 The concentration of sTNFα is reduced in a dose dependent manner by Marimastat

PBMCs from one healthy individual were activated with either LPS or anti CD3/28 antibody and then increasing doses of Marimastat (0.1μM, 1μM and 10μM) were added. Inhibition of sTNFα was in a dose dependent manner. Inhibition was much less in activated T cells than LPS activated monocytes (Figure 3.2).

Similar findings were seen when LPMCs were activated with anti CD3/28 and co-incubated with Marimastat (Figure 3.3). LPS was not used to activate LPMCs because it is well known that resident macrophages in normal colon are refractory to LPS (Nakata et al., 2006). Very little sTNFα is detected in LPMCs from normal colonic mucosa; a mean of 32.58pg/ml (with a range between 0 and 124 pg/ml). Activation with anti-CD3/28 increases sTNFα to a mean of 1389.48 (range between 174.60 pg/ml and 2724.60 pg/ml). Co-incubation with Marimastat reduced sTNFα to a mean of 573.18 (range between 365.50 pg/ml and 1224.60 pg/ml).

3.4.3 Tissue Inhibitor of Metalloproteinase (TIMP)-3 significantly reduces sTNFα in IBD biopsies.

The natural inhibitor of TACE is TIMP-3, which also inhibits MMPs and has been shown to reduce pro-inflammatory cytokines expressed on CD LPMCs, as well as cytokine RNA in CD biopsies treated with recombinant TIMP-3 (Monteleone et al., 2012). Further experiments were carried out adding TIMP-3 to mucosal biopsies from four CD and three UC patients with active disease (Figures 3.4). There was a statistically significant (P=0.0241) reduction in sTNFα concentration in the supernatant of inflamed mucosal biopsies incubated with TIMP-3 when a paired t test was used. The reduction seen in CD biopsies alone was also significant (P=0.0437). The UC biopsies reduced from a mean of 120.52 pg/ml in HL-1 to 49.30 pg/ml.

3.4.4 Tissue Inhibitor of Metalloproteinase (TIMP)-3 did not reduce IL-1β, IL-8 or IL-6 in IBD biopsies.

The pro-inflammatory cytokines IL-6, IL-1β and IL-8 in the supernatant of cultured biopsies from UC and CD patients were then determined using ELISA (Figure 3.4). There was a small reduction in IBD biopsies in the mean IL-1β, IL-8 and IL-6 concentration. IL-1β reduced from 244 pg/ml (range of 0 to 845) in medium to 92.70 pg/ml (range of 18.12 to 403) with TIMP-3; IL-8 reduced from 44709.38 pg/ml (range of 35886 to 48266) to 35,985.33 pg/ml (range of 75 to 58826), and IL-6 reduced from 9899.54 pg/ml (range 1787.41 to 35,886) to 7,029.43 pg/ml (range of 624.44 to 19976).

3.4.5 The highly specific TACE inhibitor GW280264X reduces sTNFα in activated PBMCs.

We next tested a highly specific ADAM17 inhibitor, GW280264X, a gift from Prof Stefan Rose-John. PBMCs from healthy controls were activated with PMA/Ionomycin (Figure 3.5). There was a significant increase in sTNFα with activation ($p=0.012$) and in addition co-incubation with the selective TACE inhibitor, significantly reduced secretion of TNFα ($p=0.019$).

Lamina propria mononuclear cells isolated from surgical resections of one UC and one CD individual were activated with anti CD3/28 antibody. There was an increase in sTNFα (1964.22 pg/ml from 97.56 pg/ml in the individual with CD and 1515.33 pg/ml from 159.78 pg/ml in the UC individual) (Figure 3.6).

3.4.6 The highly specific TACE inhibitor GW280264X significantly reduces sTNFα in IBD.

There was a statistically significant reduction in sTNFα (P=0.0033) in IBD biopsies (six UC patients and four CD patients). When a paired t test was applied to the UC biopsies alone there was also a significant reduction in sTNFα (P=0.037); sTNFα reduced from a mean of 221.05 pg/ml (a range of 156.27 to 320.93) to a mean of 109.74 pg/ml (range of 25.1 to 220.9). A reduction was also seen ex vivo in inflamed CD biopsies from a mean sTNFα concentration of 145.77 pg/ml (128.18 to 157.56) to a mean of 101.52 pg/ml (range of 56.44 to 134.55) with 10μM GW280264X. The reduction however was not statistically significant.

There was no change in the concentration of IL-1β, IL-8 or IL-6 in UC or CD biopsies.

3.5 Figures

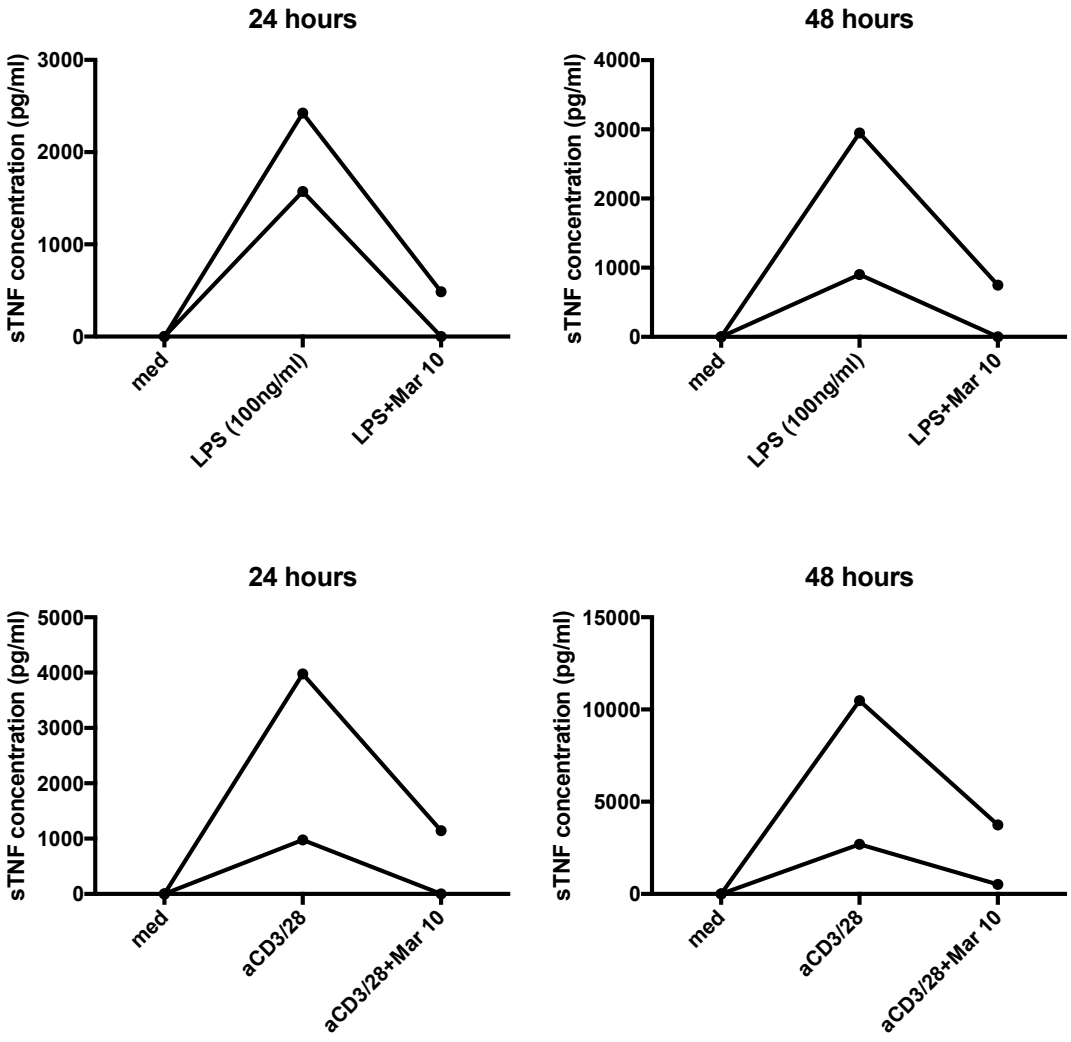


Figure 3-1 Soluble TNFα increases with activation by LPS and anti CD3/28 and reduces with Marimastat 10μM. PBMCs from 2 control donors were incubated for 24 or 48 hours with medium, anti CD3/28 antibody or anti-CD3/28 antibody with 10μM Marimastat or LPS with or without Marimastat. There was an increase in sTNFα with activation of both LPS and anti CD3/28 antibody, which decreased with Marimastat.

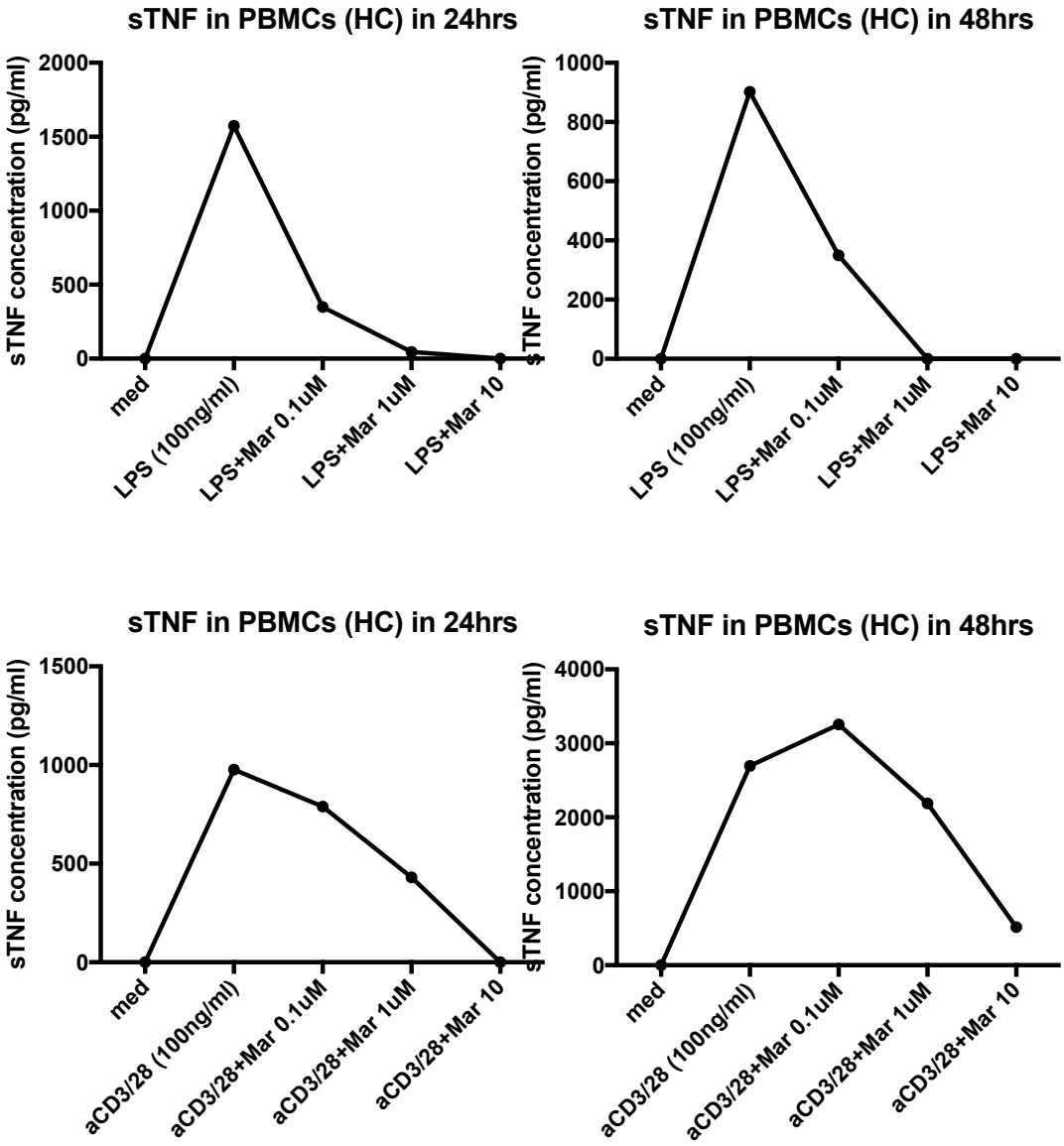


Figure 3-2 Soluble TNFα increases with activation by LPS and anti CD3/28 and reduced with Marimastat 10μM in a dose dependent manner. PBMCs from one healthy donor was incubated for 24 or 48 hours with medium, anti CD3/28 antibody or anti-CD3/28 antibody with 0.1μM, 1μM and 10μM Marimastat or LPS with or without 0.1μM, 1μM and 10μM Marimastat. There was an increase in sTNFα in the supernatant with activation of both LPS and anti CD3/28 antibody which decreased with Marimastat.

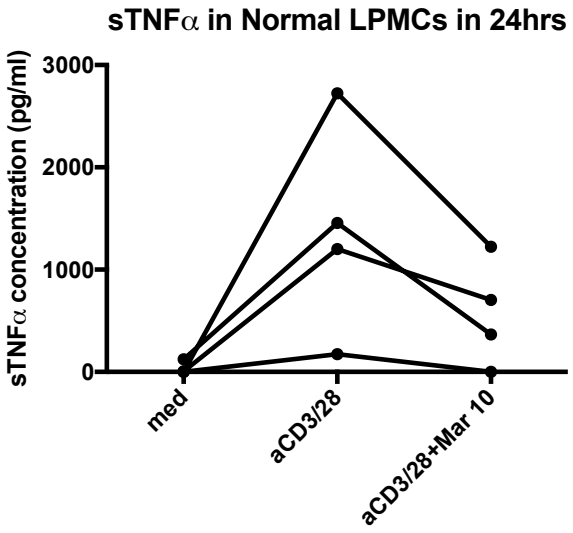


Figure 3-3 Soluble TNFα increased in LPMCs isolated from four healthy individuals following activation with 24 hours anti CD3/28 antibody (1μl/ml) and was reduced with 10μM Marimastat.

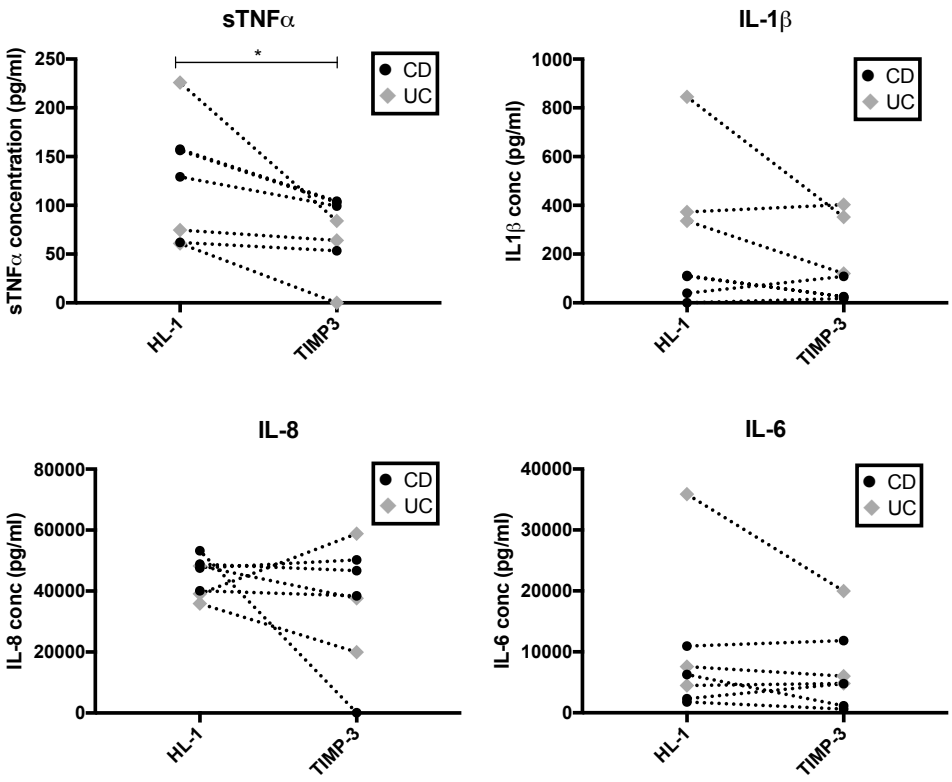


Figure 3-4 TIMP-3 significantly reduces sTNFα in IBD biopsies. Biopsies from 4 CD and 3 UC patients were incubated in HL-1 medium with or without TIMP-3 (100ng/ml) for 24 hours. The concentrations of sTNFα, IL-1β, IL-6 and IL-8 in the supernatant were measured by ELISA. *=P<0.05

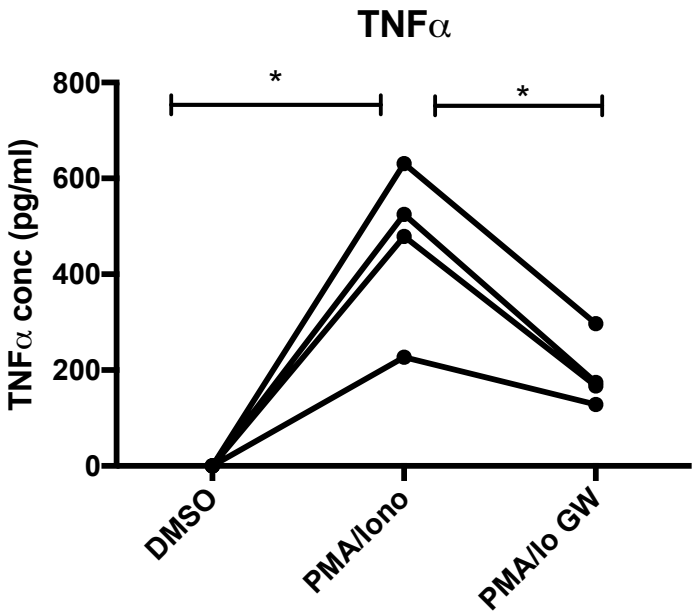


Figure 3-5. Soluble TNFα significantly decreases with co-incubation of GW280264X. Peripheral blood mononuclear cells were incubated for 4 hours in DMSO, PMA/Ionomycin and PMA/I and 10μM GW280264X. *=P<0.05

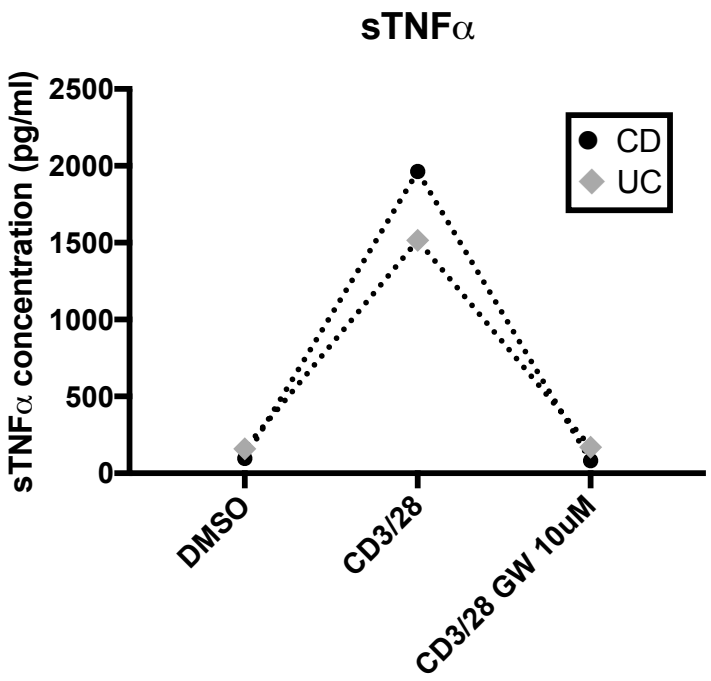


Figure 3-6. The TACE inhibitor GW280264X reduces sTNFα in activated IBD LPMCs of incubated for 24 hours. LPMCs were incubated with DMSO, anti CD3/28 antibody (1µl/ml) and anti CD3/28 antibody and GW280264X in one CD individual and one UC individual. The sTNFα concentration was measured in the supernatant by ELISA.

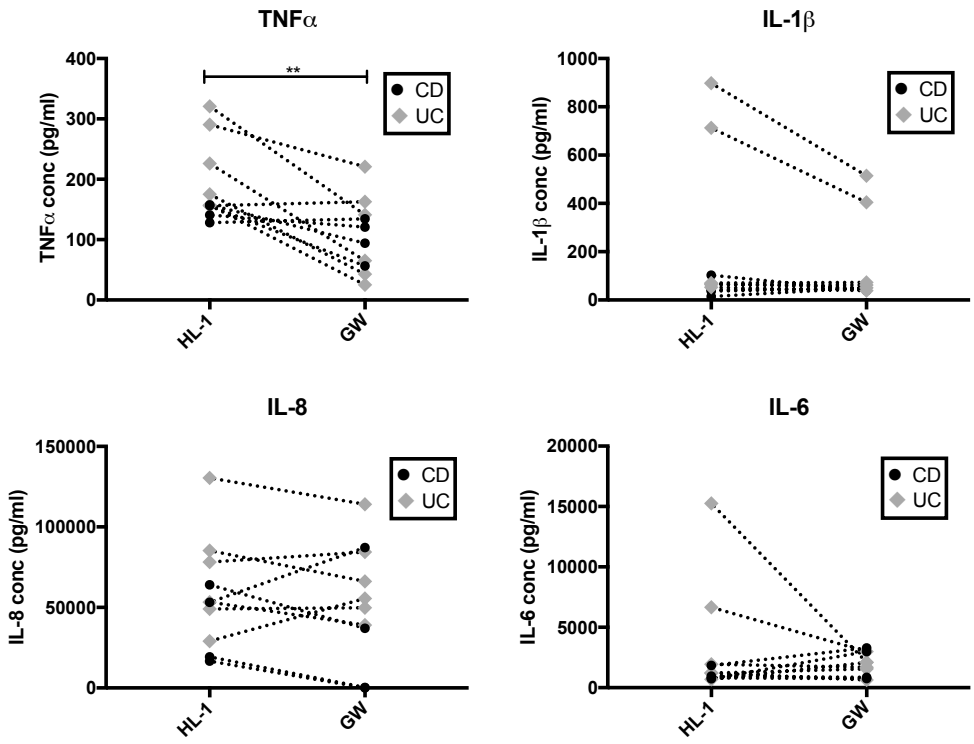


Figure 3-7 The TACE inhibitor GW280264X significantly reduces sTNFα in IBD biopsies. Inflamed biopsies from 6 UC patients and 4 CD patients were incubated in HL-1 medium or GW280264X (10μM) for 24 hours. The concentration of sTNFα, IL-1β, IL-6 and IL-8 in the supernatant was measured by ELISA. **=P<0.005

3.6 Discussion

I aimed to establish whether pharmacological and endogenous TACE inhibitors reduce soluble TNFα and identify any changes in other pro-inflammatory cytokines. Preliminary studies with a broad spectrum MMP inhibitor, Marimastat (Tsuji et al., 2002), showed a reduction in sTNFα secretion with 10μM Marimastat. In fact a dose of 0.1μM was sufficient to reduce sTNFα production (Figure 3.2). Although Marimastat has not been tested in the gut previously, Tsuji et al showed that LPS-induced sTNFα in the plasma was reduced in mice following oral administration of Marimastat (Tsuji et al., 2002).

The naturally occurring TACE inhibitor, TIMP 3 was then examined ex vivo. Consistent with the findings of Marimastat, a significant reduction in sTNFα was seen in the supernatant of organ culture medium following 24 hours incubation with TIMP-3. Previous studies have looked at endogenous TIMP-3 expression in IBD, finding that its expression is reduced in active CD (Cesaro et al., 2009), but upregulated in quiescent CD and reduced in DSS induced colitis in mice (Sharma et al., 2014). Monteleone et al. (2012) and Sharma et al. (2014) found similar effects on sTNFα following the administration of TIMP-3 or compound 11p, a pharmacological TACE inhibitor.

However their findings differ in the changes seen with other pro-inflammatory cytokines. Both studies showed a reduction in pro-inflammatory cytokines. The variation in these results could possibly be due to the patchy nature of inflammation in CD and greater numbers of samples are required to minimise this variability. It could also be due to the different experiments used.

(Monteleone et al., 2012) measured pro-inflammatory cytokines by post translational transcripts. Tumour necrosis factor is a master cytokine, which results in downstream release of pro-inflammatory cytokines; it is possible therefore that 24 hours is too short a time period to detect these changes. Carrying out an ELISA following 48 hours incubation may reveal a reduction in cytokines. Sharma et al., (2014) used a specific TACE inhibitor as opposed to TIMP-3 and experiments were in mice not human gut. Monteleone et al., (2012) showed the reduction in pro-inflammatory cytokines using flow cytometry of cell expression on LPMCs which again is likely to show changes earlier than in the supernatant. The concentration of TIMP-3 used was the same as Monteleone et al., (2012) (100ng/ml) but it may be helpful to use higher concentrations to see if the results are different.The reduction in sTNFα was not significant in UC, although the numbers are small.

A specific TACE inhibitor was given by Professor Rose-John and tested in similar experiments to see if changes found were consistent and significant. The reduction in sTNFα in PBMCs was significant with GW280264X. The activation with PMA/ Ionomycin did not result in sTNFα concentrations comparable to activation with LPS or anti CD3/28 however. The changes seen ex vivo with GW280264X were similar to TIMP-3, showing a reduction in sTNFα. Interestingly the reduction was significant in UC biopsies but not CD biopsies (the opposite finding to TIMP-3). This again may be due to the patchy nature of inflammation. They may also suggest a difference in TACE inhibition between UC and CD individuals.

It is not clear from these results whether the reduction in sTNFα is anti-inflammatory or pro-inflammatory. Fréour et al., (2009) showed increased intestinal permeability with TIMP-3 and postulated that this was due to the associated reduction in TNFR shedding. These results support previous findings that TACE inhibitors reduce sTNFα.

Having examined the effects of TACE inhibitors on sTNFα and other pro-inflammatory cytokines, the next step was to elucidate whether TACE inhibitors induce a reciprocal change in mTNFα expression.

4 Regulation of Membrane Tumour Necrosis Factor Alpha by Tumour Necrosis Factor Alpha Converting Enzyme Inhibitors

4.1 Introduction

Tumour necrosis Factor α is a key cytokine, which drives disease inflammation in inflammatory bowel disease. It is initially expressed on the cell surface as a trimer on the cell membrane. Membrane TNFα is constitutively expressed on Natural Killer cells but not on resting T, B cells or monocytes (Caron et al., 1999), but is expressed on T cells, macrophages and monocytes following activation (Horiuchi et al., 2010).

Membrane TNFα binds to TNFRI but has a greater affinity for TNFRII. Binding of TNFRI results in activation of caspase dependent apoptosis, whereas the binding of TNFRII signals TRAF2 and NFKappaB induction, which has anti-apoptotic effects (Atreya et al., 2011). Both these effects occur locally due to the cell-to-cell interaction. In addition to acting as a ligand, mTNFα can also bring about changes in the cell expressing mTNFα via reverse signalling (Horiuchi et al., 2010). The cytoplasmic tail of the transmembrane TNFα protein can trigger intracellular signals such as activating MAPkinase increasing TGFβ production (Pallai et al., 2016).

The importance of mTNFα is crucial to resolve colonic inflammation induced in immunodeficient mice (Perrier et al., 2013) . This was shown by comparing the improvement in colitis between mice treated with XENP1595, which neutralises

sTNFα alone, a monoclonal anti-TNFα antibody that neutralises both mTNFα and sTNFα and an isotype. Improvement was only seen when mTNFα was neutralised. The role of mTNFα in IBD is also supported by the different efficacies of two anti-TNFα antibodies. Infliximab a monoclonal anti-TNFα antibody is effective in IBD, whereas etanercept is not (Sandborn et al., 2001). Both anti-TNFα antibodies neutralise sTNFα with similar binding, but etanercept binds weakly to mTNFα (Nesbitt et al., 2007) and (Vos et al., 2011). (Atreya and Neurath, 2016) also showed that response to infliximab was better in patients with an increased number of mTNFα cells in the mucosa.

The 26 kilodalton (kd) mTNFα is cleaved into a 17kd fragment from the cell surface by the adamalysin TNFα Converting Enzyme (TACE) (Black et al., 1997). Cesaro et al., (2009) showed that TACE mRNA is strongly expressed in biopsies from CD patients, but TIMP-3 the natural inhibitor of TACE is poorly expressed. Increased TACE expression at mRNA level was also shown in DSS induced colitis by Sharma et al., (2014), which was associated with a suppression in TIMP-3. Treatment with compound 11p, a selective TACE inhibitor, ameliorated the colitis and restored pro-inflammatory cytokines to normal levels.

Monteleone et al., (2012) showed mTNFα expression significantly increased on CD LPMCs when incubated with TIMP-3 for 36 hours and significantly reduced expression of IL-6, IFNγ and IL-17A.

4.2 Aims

To elucidate which cells express mTNFα in PBMCs and LPMCs and whether TACE inhibitors increase mTNFα expression the following experiments were carried out.

1. PBMCs were activated with LPS, anti-CD3/28 antibody and PMA/ Ionomycin and analysed using flow cytometry for mTNFα expression.
2. Activated PBMCs and LPMCs were incubated with different TACE inhibitors and the change in mTNFα expression measured by flow cytometry.
3. LPMCs from UC and CD patients were incubated with TACE inhibitors and the change in mTNFα expression measured by flow cytometry.

4.3 Materials and Methods

4.3.1 Isolation of Peripheral Blood Mononuclear Cell (PBMC)

Healthy individuals and patients with a confirmed diagnosis of CD or UC gave their consent to donate blood for the study. The blood was collected in heparinised tubes and then washed with phosphate buffered saline (PBS Invitrogen, Paisley, UK) in a 1:1 ratio and layered onto an equal volume of Ficoll (Amersham Pharmacia Biotech, Little Chalfont, UK) for centrifugation at 150g for 20 minutes at 21°C. The mononuclear cell layer was removed and washed with PBS and centrifuged again at 150g for 5 minutes at 10°C. The cell pellet was isolated and then resuspended. The cells were counted using a haemocytometer with trypan blue to measure cell viability.

4.3.2 Isolation of Lamina Propria Mononuclear Cells (LPMC) from Surgical Resections

Specimens from intestinal resections were obtained following consent of CD and UC patients requiring surgery. Control specimens were obtained following consent of patients without CD or UC requiring surgery for cancer. The specimens were taken immediately to the histopathology department for macroscopic examination. Full mural thickness sections varying in length from 3-7cm were then removed from the specimen by the histopathologist and placed in ready to use Roswell Park Memorial Institute-1640 medium (RPMI)(Invitrogen) on ice and transported to the lab. The mucosa was removed from the specimens with scissors and incubated in calcium- and magnesium-free Hank's balanced salt solution (HBSS) (Life Technologies, Paisley, UK) with

1mM ethylene tetra-acetic acid (EDTA)(Sigma-Aldrich, Gillingham, UK) for 30 minutes at 37°C with agitation in order to remove the mucus and epithelial cells. The tissue was then cut into 3-4mm pieces with a scalpel and incubated in 1mg/ml collagenase (Sigma-Aldrich, Gillingham, UK) and DNAase I (10U/ml; Roche, Mannheim, Germany) with agitation for 60 minutes at 37°C to break down intercellular connective tissues. The suspension was then passed through a 100μm cell strainer and washed in RPMI (Sigma-Aldrich, Gillingham, UK). The pellet was resuspended in 20ml of PBS (Invitrogen, Paisley, UK) and the LPMCs purified using Ficoll density gradient (centrifuged at 150g for 30 minutes at 20°C. The cell pellet was resuspended in RPMI and the cells counted using trypan blue.

4.3.3 Separation of CD14+ and CD14- cells by magnetic beads.

MACS (Miltenyi Biotec, Bergisch Gladbach, Germany) was also used to separate CD14+ and CD14- PBMCs which could then be activated with LPS or anti CD3/28 antibody respectively. The MACS separator uses magnetically labeled microbeads with CD14+ antibody, which bind to CD14+ PBMCs. The cell suspension is loaded onto a MACS column, which is placed in a magnetic field. The CD14+ cells are retained in the column, whilst the CD14- cells pass through and collected. When the magnetic field is removed from the column the CD14+ cells also pass through and can be collected separately. Following PBMC isolation, PBMCs were centrifuged at 300g for 10 minutes at 4 °C and resuspended in 80μl of PBS (Invitrogen, Paisley, UK) per 10^7 cells. Cells were passed through a strainer to remove clumps and 20μl of CD14+ microbeads added. Cells were agitated and then refrigerated for 10 mins. Cells were then

washed in PBS (Invitrogen, Paisley, UK) and resuspended in up to 500μl of PBS before being passed through the MACS separator.

4.3.4 PBMC and LPMC cell activation

Following PBMC and LPMC isolation, 1 million PBMCs or LPMCs were activated using LPS (Sigma-Aldrich, Gillingham, UK), anti CD3/ anti CD28 antibody (eBioscience, San Diego, CA) or PMA/ Ionomycin (Sigma-Aldrich, Gillingham, UK). One millilitre of 1 million PBMCs were placed in a well of a 24 well plate in the following conditions; medium, LPS (100ng/ml; Sigma-Aldrich, Gillingham, UK), anti CD3/28 antibody (1μg/ml; eBioscience, SanDiego, CA), LPS and Marimastat (10μM) and anti CD3/28 antibody and Marimastat (10μM) for 24 and 48 hours. One millilitre of 1 million PBMCs were also placed in a 24 well plate in the following conditions; DMSO, PMA (50ng/ml; Sigma-Aldrich, Gillingham, UK)/ Ionomycin (500ng/ml) for 4 hours, or PMA / Ionomycin and GW280264X, a gift from Prof Stefan Rose-John (10μM). After 4 hours the supernatant was stored at -80°C for analysis by ELISA whilst the cells were washed and prepared for FACS analysis.

4.3.5 Staining for Flow Cytometry

LPMCs and PBMCs from each of the conditions following activation were washed in 1 ml of FACS buffer and resuspended in 100μl of FACS buffer (PBS + 2mM EDTA + 2% BSA). Cells were then incubated on ice with 20μl of FACS blocking buffer (Human Fc Receptor Binding Inhibitor) (eBioscience; Hatfield, England) to reduce non-specific binding via Fc receptors. The cells were then incubated with FITC bound mTNFα antibody or its isotype control (R and D

systems, Abingdon, UK), PeCy7 bound TNFα antibody or its isotype control (BioLegend, London, UK), Pacific Blue anti-CD14 antibody (BD Bioscience, Oxford, UK) and PE anti-CD3 antibody (BioLegend, London, UK) for 20-30 minutes at 37°C in the dark. Cells were then washed three times in FACS buffer and analysed using LSRII flow cytometry (BD Bioscience, Oxford, UK). Isotype controls were purchased from the same manufacturer as the antibodies. Data was analysed using FlowJo software (Tree Star Inc, OR, USA).

4.3.6 Gating Strategy

Lymphocytes were gated using forward scatter and side scatter properties. Doublets were then excluded using forward scatter and side scatter width. CD3+ or CD14+cells were then identified by PE and Pacific Blue fluorescence respectively. The FITC isotype and mTNF FITC fluorescence enabled the final gating to quantify mTNF+ CD3+ and mTNF+ CD14+ cells.

4.4 Results

4.4.1 Membrane TNFα expression significantly increases in PBMCs activated with anti CD3/28 antibody

PBMCs were activated with anti CD3/28 for 48 hours and analysed by flow cytometry. Initially an antibody for intracellular TNFα detection was used as the vendor claimed that it was also effective in detecting membrane TNFα. However another antibody specific for membrane TNFα was also purchased and compared to optimise the detection of mTNFα expression (Figure 4.1). There was a significant increase in mTNFα expression (P=0.019) with anti CD3/28 activation using the membrane specific antibody (Figure 4.2). There was no difference in mTNFα expression on PBMCs incubated with medium by the two antibodies (a mean of 1.6 for iTNFα antibody and a mean of 0.8 for the mTNFα antibody), but there was an increased detection in mTNFα by the latter in PBMCs incubated with anti-CD3/ anti-CD28 antibody (mean of 3.6 for iTNFα and a mean of 9.7 for mTNFα) (Figure 4.2).

4.4.2 mTNFα expression was greater with a PE bound CD3+ antibody than MACS separated CD14- cells.

In order to measure the mTNFα expression in monocytes and T cells two different methods were carried out and then compared. PBMCs were separated into CD14+ and CD14- cells. The former were activated with LPS and mTNFα expression measured by FACS (Figure 4.3). CD14- cells were activated with anti CD3/ anti CD28 and mTNFα expression measured by FACS (Figure 4.4). The mTNFα expression was also measured in similar experiments in which

PBMCs were not separated but anti CD3 or antiCD14 antibodies used to quantify the cell populations. There was a higher detection of CD3+cells when a PE bound CD3+ antibody was used than when the CD14- cells separated by magnetic beads. There was no difference between the two methods in the detection of CD14+ mTNFα cells (Figure 4.5).

4.4.3 Marimastat does not increase the mTNFα expression on activated PBMCs.

Membrane TNFα is significantly increased on PBMCs activated with anti CD3/28 antibody but the expression of mTNFα is not increased further with Marimastat. PBMCs do not show an increase in mTNFα with LPS activation or with LPS and Marimastat (Figure 4.6).

4.4.4 Marimastat increases mTNFα in UC but not in CD LPMCs.

LPMCs from inflamed colon were incubated in medium or activated with anti CD3/28 antibody (1μg/ml) and Marimastat (10μM) and CD3+mTNFα cells calculated by FACS. In CD mTNFα increased with anti CD3/28 from 1.1% to 20.4% but reduced with Marimastat to 1.45%. In UC mTNFα increased with activation from 2.72% to 11.1% and further increased with Marimastat to 23.5% (Figure 4.7).

4.4.5 The highly specific TACE inhibitor GW280264X significantly increases mTNFα expression in CD3+ PBMCs.

There was a statistically significant (P=0.01) increase in mTNFα expression between activated lymphocytes with GW280264X compared to no GW280264X in CD3+ PBMCs. The mean fluorescence intensity was also statistically significant (P=0.0045) (Figures 4.8 and 4.9).

4.5 Figures

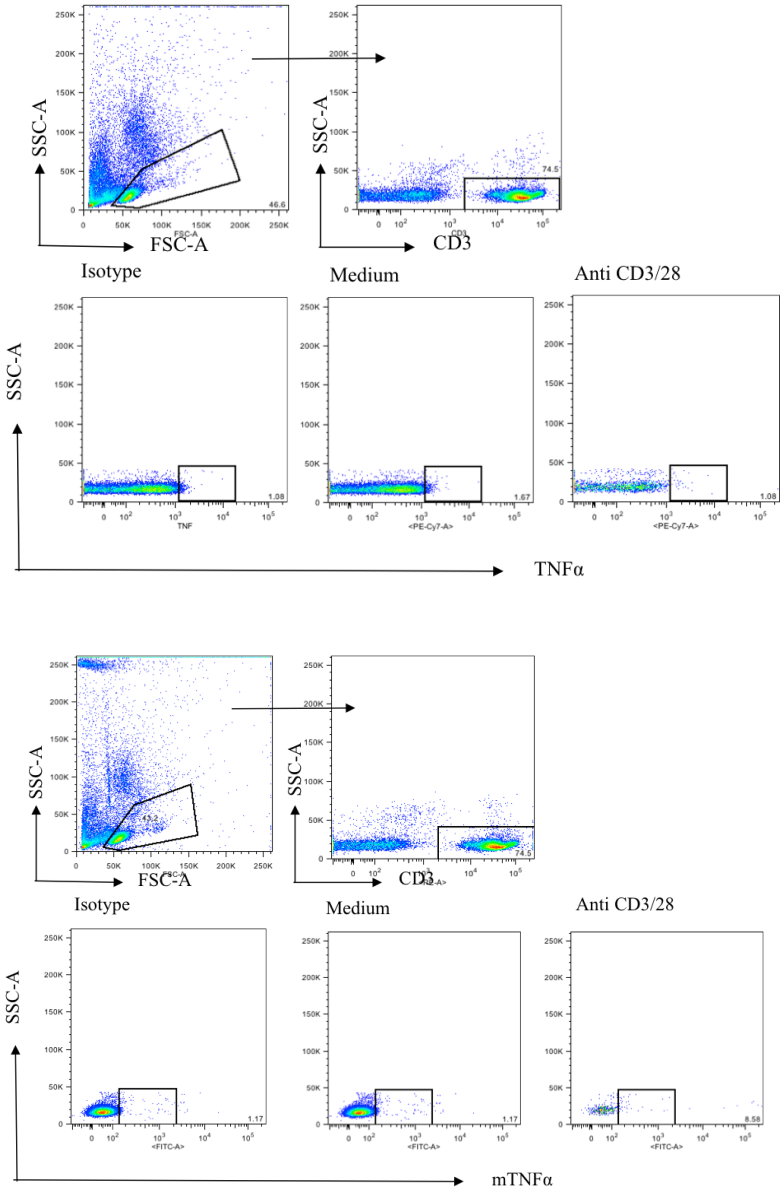


Figure 4-1. PBMCs from healthy donors were incubated in medium or anti CD3/28 antibody for 48 hours. The mTNFα expression was measured by flow cytometry comparing two antibodies, one for TNFα (intracellular or membrane) and the other specifically for membrane TNFα. The upper panel is the non specific antibody and mTNFα detected was 1.08% of total cell count. The lower panel is the specific mTNFα and 8.50% cells expressed mTNFα. Representative plots of 3 experiments.

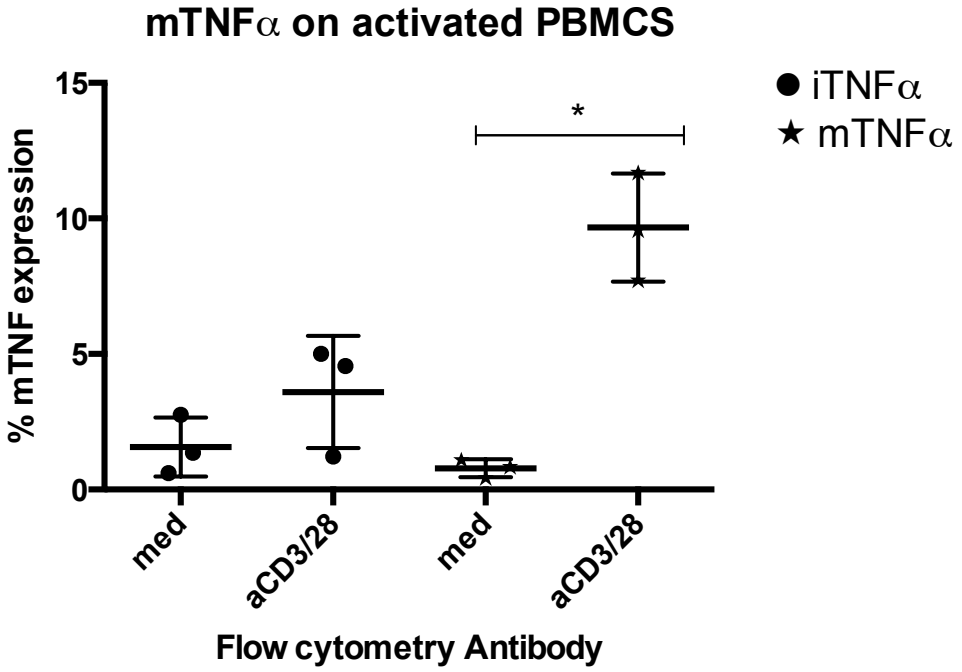


Figure 4-2 . mTNFα expression increased significantly when detected with mTNFα antibody. PBMCs from 3 healthy donors activated with anti CD3/28 for 48 hours and stained with either non specific TNFα (iTNFα) antibody or mTNFα antibody for FACS analysis. Figure shows the mean and standard deviation of 3 experiments. The mTNFα antibody detected an increased percentage of mTNFα on the activated lymphocytes, which was significant (P<0.5) using a paired t test.

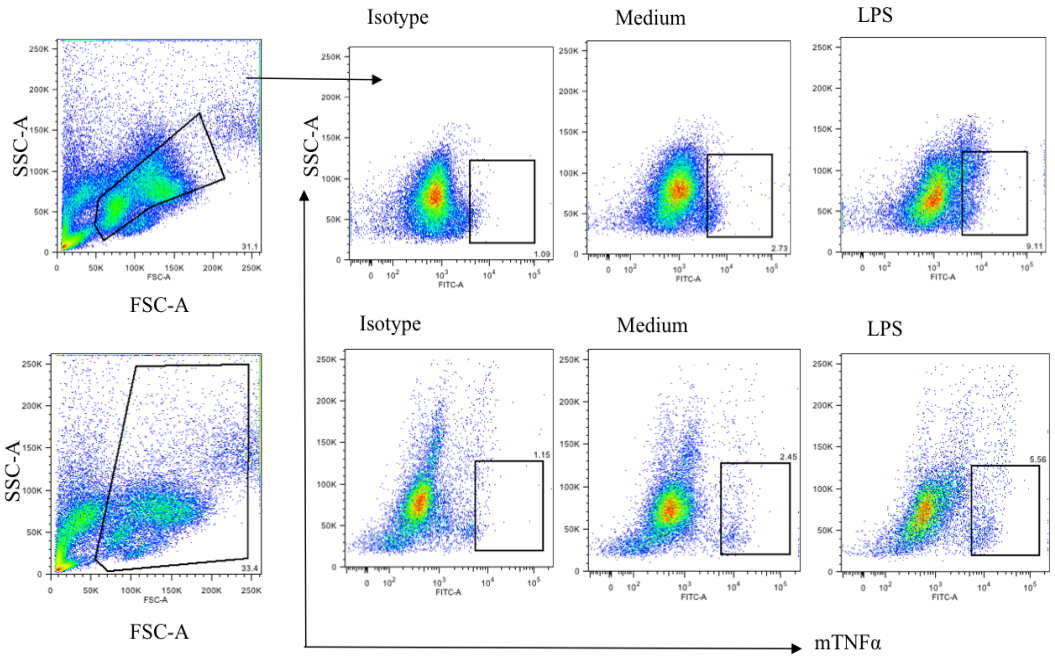


Figure 4-3. PBMCs from healthy controls were separated into CD14+ and CD14-ve cells by magnetic beads. CD14+ve cells were incubated in medium or LPS (100ng/ml) for 24 hours and the cells analysed for mTNFα expression by flow cytometry. Membrane TNFα expression increased with LPS from 2.73% to 9.11% in the first experiment (upper panel) and 2.45 to 5.56% in the second experiment (lower panel).

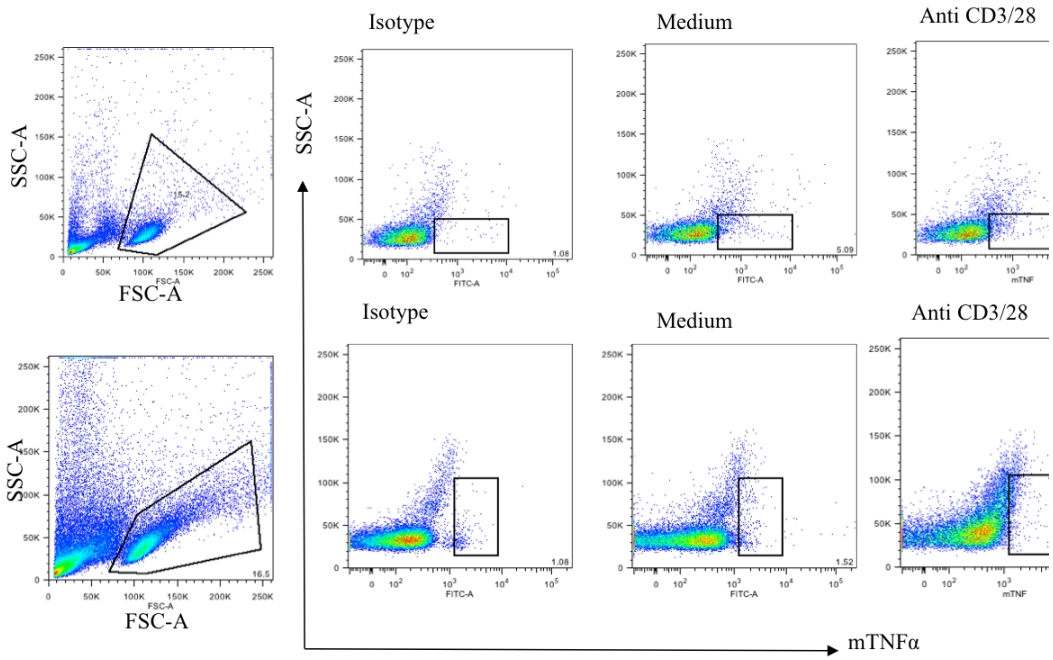


Figure 4-4 . PBMCs from healthy controls were separated into CD14+ and CD14-ve cells by magnetic beads. CD14-ve cells were incubated in medium and anti CD3/28 antibody (1µg/ml) for 48 hours and the cells analysed for mTNF expression with flow cytometry. Membrane TNFα expression increased with anti CD3/28 5.09% to 5.2% in the first experiment and 1.52 to 3.64% in the second experiment.

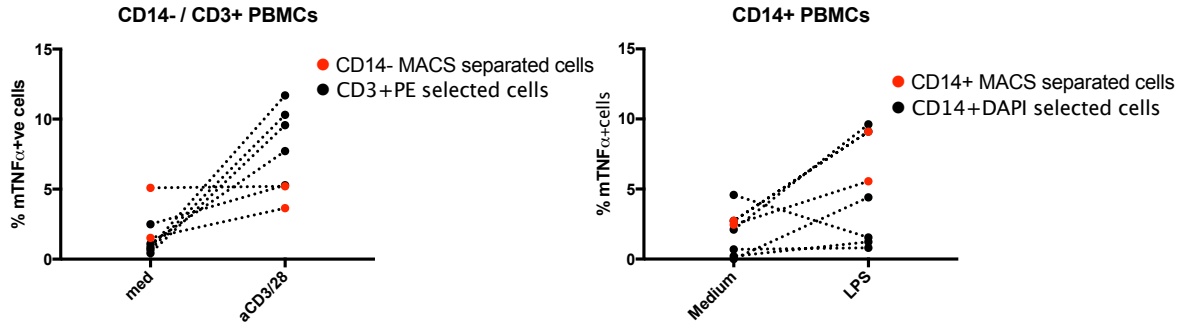


Figure 4-5. CD3+PE staining detected more mTNFα cells than MACS selection of CD14- cells. PBMCs were separated into CD14+ and CD14- populations and then stimulated before FACS analysis of mTNFα+ expression. In addition PBMCs were stimulated without prior MACS separation CD3+PE and CD14+Pacific Blue used to detect the different cell populations. The result between the two were compared. Results from 6 donors.

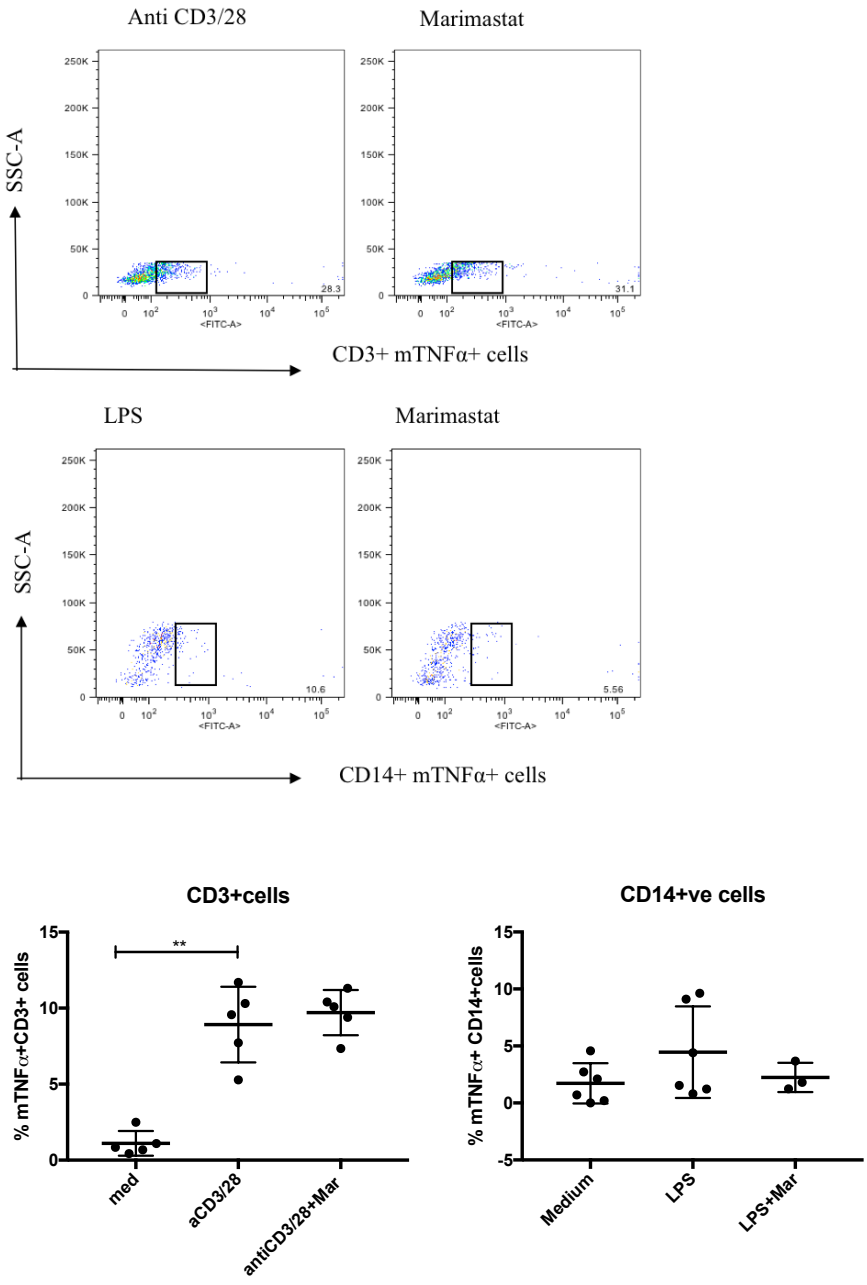


Figure 4-6. Membrane TNFα is significantly increased with anti CD3/28 antibody. PBMCs were incubated in medium or activated with anti CD3/28 antibody (1µg/ml) or LPS (100ng/ml) and Marimastat (10µM) added. The FACS plot shows CD3+mTNFα cells and CD14+mTNFα cells calculated by FACS. Marimastat does not increase mTNFα following activation with anti CD3/28 or LPS. Graph showing the mean % of mTNFα and the standard deviation where n=6 in CD3+ cells and n=5 in CD14+ cells. **=P<0.005

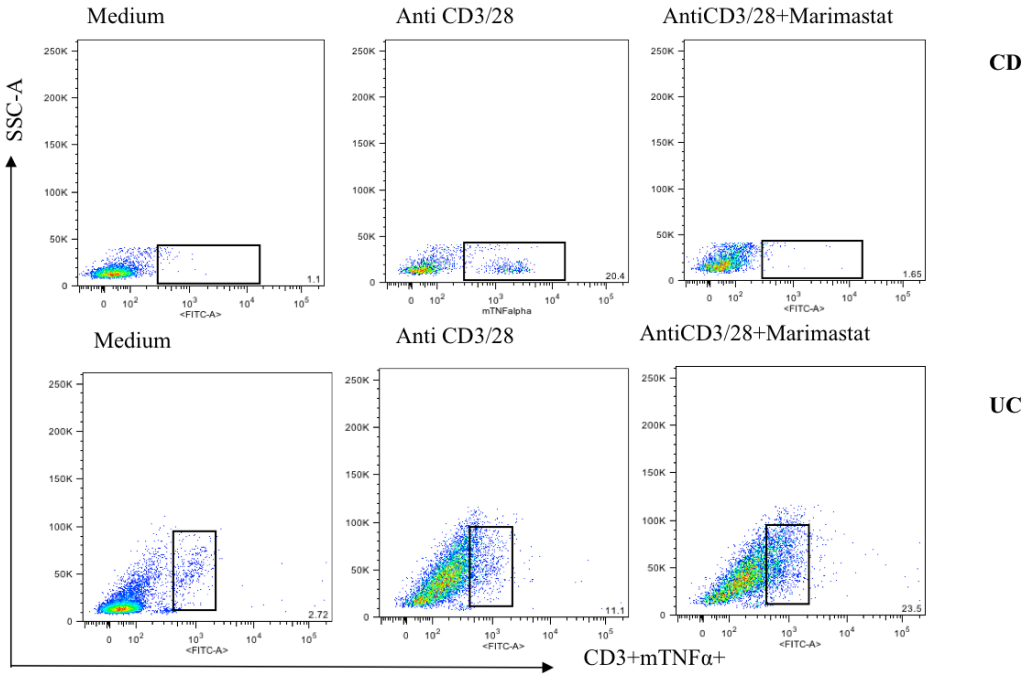


Figure 4-7. Marimastat increases mTNFα in UC but not in CD. Plots show one experiment with UC and one with CD. LPMCs from inflamed colon were incubated in medium or activated with anti CD3/28 antibody (1μg/ml) and Marimastat (10μM) and CD3+mTNFα cells calculated by FACS. In CD mTNFα increased with anti CD3/28 from 1.1 to 20.4% but reduced with Marimastat to 1.45%. In UC mTNFα increased with activation from 2.72% to 11.1% and further increased with Marimastat to 23.5%.

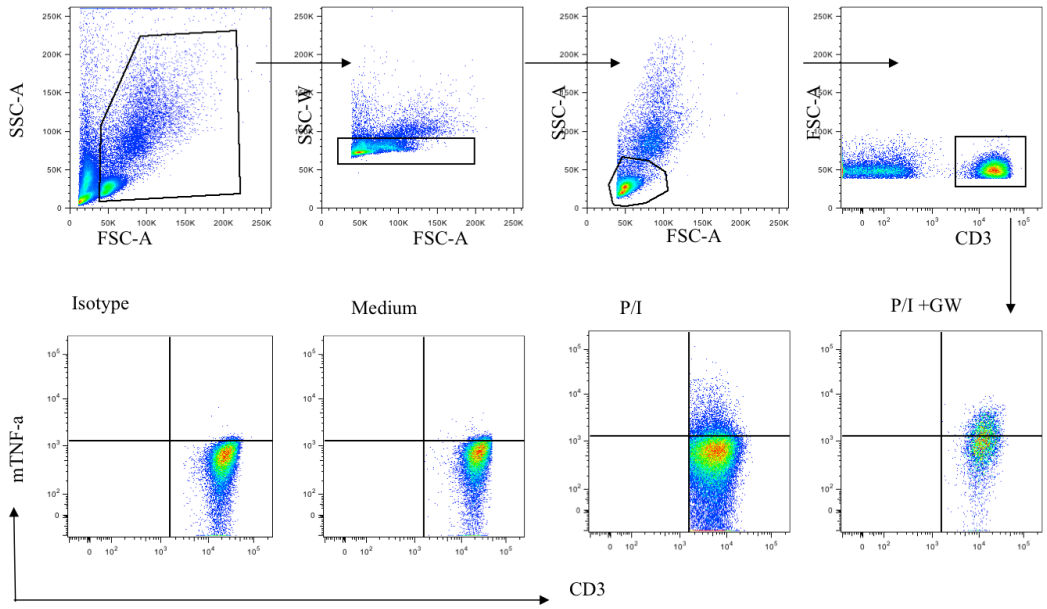


Figure 4-8. The highly specific TACE inhibitor GW280264X significantly increases mTNFα expression in CD3+ cells. A representative plot of PBMCs cultured in medium, P/I or P/I with GW280264X (10μM) for 4 hours and stained for CD3+ and mTNF+ cells.

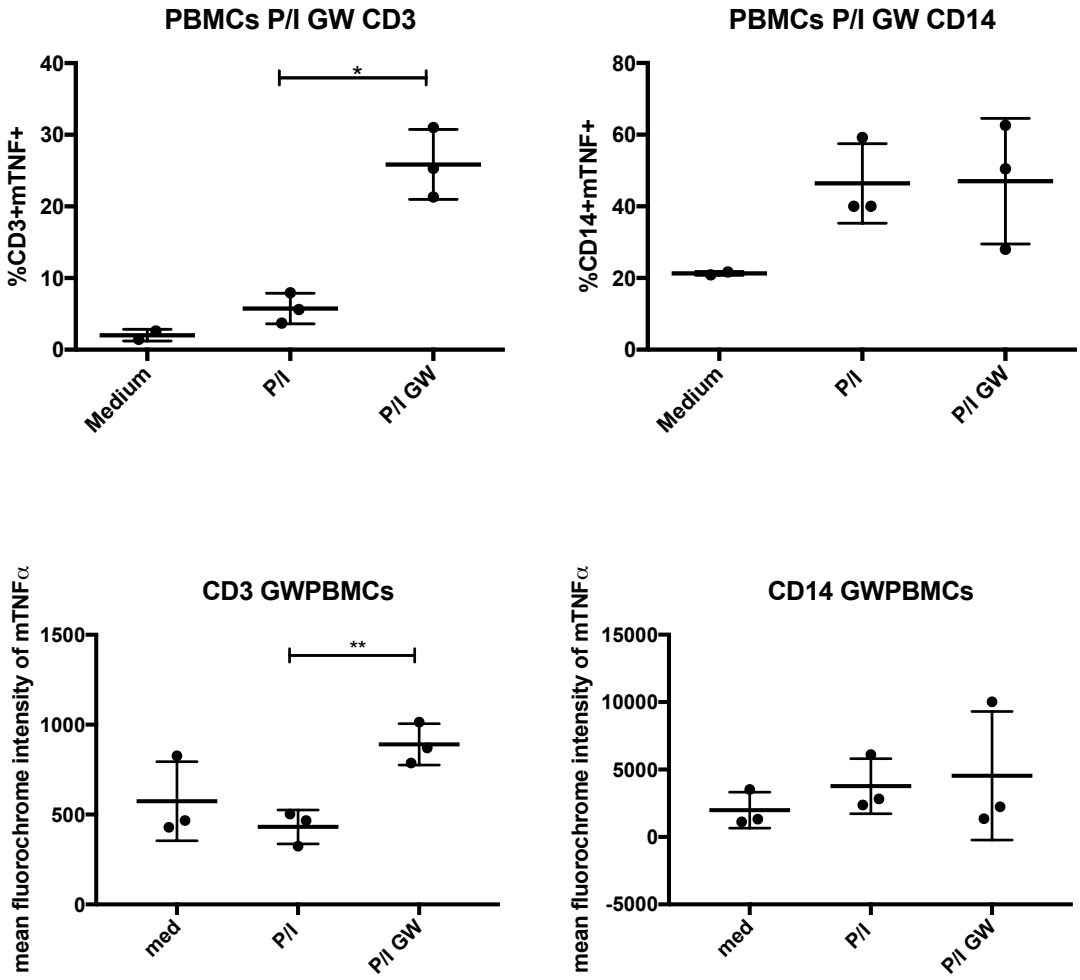


Figure 4-9 The highly specific TACE inhibitor GW280264X significantly increases mTNFα expression in CD3+ cells. PBMCs were cultured in medium, P/I or P/I with GW280264X (10μM) for 4 hours. There was a statistically significant (P=0.01) increase in mTNFα expression between activated lymphocytes with GW280264X compared to no GW280264X in CD3+cells. The mean fluorescence intensity was also statistically significant (P=0.0045). Error bars indicate the standard deviation.

4.6 Discussion

The aim was to establish which cells express mTNFα and whether pharmacological TACE inhibitors increase the expression of mTNFα. Membrane TNFα expression was not detected in resting CD3+ or CD14+ control PBMCs or LPMCs. This is consistent with findings by Caron et al., (1999) in which resting T cells did not express mTNFα. The expression of mTNFα increased significantly in CD3+ PBMCs with anti CD3/28 antibody activation but not with P/I activation. Alternatively mTNFα expression was increased on CD14+ PBMCs with P/I activation but not LPS. It is known that LPS can cause a silencing of mTNFα through reverse signalling (Eissner et al., 2000), it is possible that these differences are due to the small number.

The first TACE inhibitor tested in this study was the broad spectrum MMP inhibitor Marimastat (Tsuji et al., 2002). There was no further increase in mTNFα expression following activation with Marimastat in PBMCs activated with LPS or anti CD3/28 antibody. Tsuji et al., (2002) show Marimastat reduces plasma sTNFα in mice in LPS induced sTNFα production, however mTNFα was not measured. In this study Marimastat increases mTNFα expression in one experiment with UC LPMCs. Similarly Monteleone et al., (2012) show mTNFα expression increases in CD LPMCs with the endogenous TACE inhibitor TIMP3. Membrane TNFα was measured by flow cytometry using infliximab to bind to mTNFα and a secondary antibody bound to a fluorochrome to detect

mTNFα. Comparative experiments were not done in this study between the specific mTNFα antibody and infliximab.

The selective TACE inhibitor GW280264X statistically increased mTNFα in CD3+ activated PBMCs. The differences in the results with the two TACE inhibitors tested are likely due to their selectivity. Marimastat was originally designed as a MMP-9 inhibitor but at the concentrations used in this study, the selectivity for TACE is poor. GW280264X alternatively is a highly selective TACE inhibitor as demonstrated by the significant increase in mTNFα expression found.

Having shown how sTNFα and mTNFα expression change with stimulation and TACE inhibition, the next step was to elucidate possible mechanisms of action of infliximab and whether increasing mTNFα expression enhances these changes.

5 Functional Effects of Anti-Tumour Necrosis Factor Alpha Antibodies

5.1 Introduction

Anti-TNFα agents are used in patients with CD and UC who continue to have active disease despite being treated with steroids or azathioprine. Infliximab was the first anti-TNFα antibody to be licensed for use in CD and is effective in inducing remission in about half of patients with CD and about a third of patients with UC (Hanauer, 2002) (Rutgeerts et al., 2005). Other anti-TNF agents have since been developed, namely adalimumab, etanercept, certolizumab and golimumab, however etanercept which showed comparable efficacy to infliximab in patients with rheumatoid arthritis, is not licenced for use in CD due to less convincing efficacy (Sandborn et al., 2001).

Hanauer (2002) showed that infliximab was more effective in maintaining remission when 8 weekly infusions are given rather than a single infusion, however a loss of response still occurs in up to one third of patients. The mechanism by which infliximab induces remission is not clear but proposed mechanisms have included neutralising TNFα and reducing the production of pro-inflammatory cytokines downstream, inducing direct or indirect apoptosis of T cells or macrophages bearing mTNFα, regulatory macrophages, and complement mediated lysis of these cells (Mizutani et al., 2013, ten Hove et al., 2002a, Mitoma et al., 2008). Many studies have used the different efficacies of etanercept and infliximab to support whether the mechanism of action studied is

likely to play a significant role in IBD. These mechanisms are discussed in more detail below.

Infliximab is a human/mouse chimeric monoclonal antibody and etanercept is engineered from TNFRII coupled to the Fc receptor of IgG. Originally TNFα was thought to be a key cytokine because when produced by macrophages it fed back and induced secretion of IL-1, IL-6, IL-8 and GM-CSF (Butler et al., 1995). Neutralising soluble TNFα blocked this feedback loop (Butler et al, 1995). Infliximab reduces the pro-inflammatory cytokine IL-1β in PBMCs stimulated with LPS (Nesbitt et al., 2007). Other pro-inflammatory cytokines such as IL-6 and IL-8 are reduced in the serum of CD patients following treatment with infliximab (Mizutani et al., 2013).

Lügering et al., (2001) were the first to show apoptosis of peripheral blood monocytes following infliximab infusions in patients with CD. Apoptosis was then shown to occur in T cells in the lamina propria 24 hours after infliximab infusion as well as in vitro using an activated Jurkat cell line cultured with infliximab (ten Hove et al., 2002b). Van den Brande et al., (2003) compared infliximab and etanercept and showed that both neutralise TNFα, however only infliximab induced apoptosis. Increased apoptosis was also shown in mucosal biopsies 10 weeks following infliximab and was caspase dependent (Di Sabatino et al., 2004). Waetzig et al., (2005) showed apoptosis occurs via reverse signalling through mTNFα.

Complement-dependent cytotoxicity (CDC) of Jurkat cells and CHO cells bearing mTNFα is seen with infliximab but not etanercept (Mitoma et al., 2008, Arora et al., 2009) possibly due to the lack of the CH1 domain by etanercept. However Kaymakcalan et al., (2009) was unable to show CDC by infliximab in activated human PBMCs.

Antibody-dependent cytotoxicity (ADCC) occurs when the Fc portion of IgG1 binds to Fc receptors on NK cells resulting in granzyme B and perforin release and ultimately target cell lysis. ADCC was seen using an mTNFα expressing cell line incubated with infliximab but not etanercept (Arora et al., 2009). Nesbitt et al., (2007) however showed ADCC using infliximab and etanercept in a TNFα expressing cell line, although the level of ADCC seen with etanercept was much less than infliximab. Mitoma et al., (2008) however showed similar levels of ADCC with etanercept as with infliximab in a mTNFα expressing Jurkat cell line. It has also been suggested that infliximab binds to mTNFα but etanercept does not (Vos et al., 2011).

5.2 Aims

To elucidate whether infliximab reduces pro-inflammatory cytokines, causes apoptosis and the role of mTNFα in these activities. Accordingly the following experiments were carried out

1. Pro-inflammatory cytokines were measured by ELISA in the supernatants of inflamed IBD biopsies incubated with infliximab.
2. Apoptosis was measured in an mTNFα expressing cell line incubated with IgG1 or infliximab.
3. Apoptosis was measured in PBMCs and LPMCs activated with anti CD3/28 and infliximab in cells from healthy controls and IBD patients. This work was carried out in collaboration with Paolo Biancheri.
4. Apoptosis was measured in PBMCs activated with PMA/ Ionomycin and co-incubated with the TACE inhibitor GW280264X (a gift from Professor Rose-John) before the addition of infliximab or IgG1.

5.3 Materials and Methods

5.3.1 Organ Culture of Intestinal Mucosal Biopsies

Uninflamed and inflamed colonic biopsies obtained during colonoscopy were cultured (one biopsy per well) in 24 well plates (VWR International, Lutterworth, UK) in 300μl of serum free HL-1 medium (Cambrex BioScience, Wokingham, UK) and infliximab (10μg/ml; Remicade; Schering-Plough, Welwyn Garden City, UK) and IgG1 (10μg/ml) for 24 hours at 37°C, 5% CO. The supernatant was collected and stored at -80°C and cytokines measured by ELISA.

5.3.2 Measurement of Cytokine Production by ELISA

Ninety-six well microplates (VWR International, Lutterworth, UK) were coated with anti-IL-6 (360μg/ml; R and D Systems, Abingdon, UK), anti-IL-1β (480μg/ml; R and D Systems, Abingdon, UK) or anti-IL-8 (720μg/ml; R and D Systems, Abingdon, UK) overnight at room temperature. The plates were then washed 3 times using ELISA wash solution (PBS 0.05% Tween (Sigma-Aldrich, Gillingham, UK)) and then blocked with PBS 0.1% bovine serum albumin (BSA) (IL-6, IL-1β) or PBS 0.01% BSA (IL-8) for 1 hour at 37°C. Following another 3 washes, the plates were incubated with serially diluted recombinant cytokine standards and diluted samples (1:10 for IL-6, 1:5 for IL-1β, 1:50 for IL-8). After 2 hours the plates were washed and then incubated with a biotinylated anti-IL-6 (9μg/ml), anti-IL-1β (4μg/ml) and anti-IL-8 (3.6μg/ml) for 1 hour. The

plates were then washed and incubated with Streptavidin HRP (1 in 200) for 20 minutes before washing and substrate solution was added. Finally without washing 50μl of stop solution (H2SO4) was added in order to measure the optical density of each well using a microplate reader at 450nm.

5.3.3 Culture of Chinese Hamster Ovary (CHO) cell lines

A CHO cell line transfected with membrane TNFα and a CHO cell line with an empty vector (VH2, Hinxton, Cambridge) were cultured in tissue culture flasks with F12 GIBCO Nutrient mixture with L Glutamine (Invitrogen, Paisley, UK) with 1% penicillin/ streptomycin, 10% FBS and 400μg/ml Hygromycin B added. Cells were passaged two or three times a week by removing the medium, rinsing the cell layer with sterile PBS and adding 3 mls Accutase (PAA Laboratories, Pasching, Austria) and placing in an incubator for 20 minutes. Ten ml of complete medium was then added to deactivate the Accutase (PAA Laboratories, Pasching, Austria) and 1/6 of the total volume of cells added to a new flask with fresh medium. The remaining cells were washed with PBS and centrifuged at 150g for 5 minutes at 10°C and the cell pellet resuspended before cells were counted with trypan blue to measure cell viability.

5.3.4 Isolation of Peripheral Blood Mononuclear Cell (PBMC)

Healthy individuals and patients with a confirmed diagnosis of CD or UC gave their consent to donate blood for the study. The blood was collected in heparinised tubes and then washed with phosphate buffered saline (PBS Invitrogen, Paisley, UK) in a 1:1 ratio and layered onto an equal volume of

Ficoll (Amersham Pharmacia Biotech, Little Chalfont, UK) for centrifugation at 150g for 20 minutes at 21°C. The mononuclear cell layer was removed and washed with PBS and centrifuged again at 150g for 5 minutes at 10°C. The cell pellet was isolated and then resuspended. The cells were counted using a haemocytometer with trypan blue to measure cell viability.

5.3.5 Isolation of Lamina Propria Mononuclear Cells (LPMC) from Surgical Resections

Specimens from intestinal resections were obtained following consent of CD and UC patients requiring surgery. Control specimens were obtained following consent of patients without CD or UC requiring surgery for cancer. The specimens were taken immediately to the histopathology department for macroscopic examination. Full thickness pieces of gut in length from 3-7cm were then removed from the specimen by the histopathologist and placed in ready to use Roswell Park Memorial Institute-1640 medium (RPMI)(Invitrogen, Paisley, UK) on ice and transported to the lab. The mucosa was removed from the specimens with scissors and incubated in calcium- and magnesium-free Hank's balanced salt solution (HBSS) with 1mM ethylene tetra-acetic acid (EDTA)(Sigma-Aldrich, Gillingham, UK) for 30 minutes at 37°C with agitation in order to remove the mucus and epithelial cells. The tissue was then cut into 3-4mm pieces with a scalpel and digested in 1mg/ml collagenase (Sigma-Aldrich, Gillingham, UK) and DNAase I (10U/ml; Roche, Mannheim, Germany) with agitation for 60 minutes at 37°C. The suspension was then passed through a 100μm cell strainer and washed in RPMI (Sigma-Aldrich, Gillingham, UK). The

pellet was resuspended in 20ml of PBS and the LPMCs purified using Ficoll density gradient (centrifuged at 150g for 30 minutes at 20°C). The cell pellet was resuspended in RPMI and the cells counted using trypan blue.

5.3.6 PBMC cell activation

Following PBMC isolation, PBMCs were activated using anti CD3 / anti CD28 antibody (eBioscience, SanDiego,CA) or PMA/ Ionomycin. One millilitre medium containing 1 million PBMCs was placed in a well of a 24 well plate in the following conditions; medium, anti CD3/28 antibody (1μg/ml; eBioscience, SanDiego, CA), anti CD3/28 antibody (1μg/ml) and infliximab (10μg/ml; Remicade, Scering-Plough, Welwyn Garden City, UK) and anti CD3/28 antibody (1μg/ml) and IgG1 (10μg/ml) for 24 hours. One millilitre of 1 million PBMCs were also placed in a 24 well plate in the following conditions; PMA (50 ng/ml Sigma-Aldrich, Gillingham, UK)/ Ionomycin (500 ng/ml) for 4 hours, or PMA/ Ionomycin and GW280264X, a gift from Prof Stefan Rose-John (10μM) and either IgG1 (100μg/ml) or infliximab (100 μg/ml; Remicade, Schering-Plough, Welwyn Garden City, UK).

5.3.7 Flow Cytometric detection of membrane TNFα

CHO cells were aspirated and washed in 1 ml of FACS buffer and resuspended in 100μl FACS buffer (PBS, 2mM EDTA, 2% BSA). Cells were then incubated with 5μl fluorescin conjugated membrane TNF antibody (R and D, Abingdon, UK), or its matched isotype, for 20-30 minutes at 37°C in the dark. Cells were

then washed three times in ice-cold FACS buffer and analysed by flow cytometry using LSRII (BD Bioscience, Oxford, England).

5.3.8 Measurement of Apoptosis using Annexin V

Annexins are a family of calcium dependent phospho-lipid binding proteins that preferentially bind phosphatidylserine (PS). PS is normally located on the inner layer of the plasma membrane, however when apoptosis is initiated, PS is translocated to the outer layer of the phospholipid bilayer marking cells for phagocytosis. Fluorescently labelled Annexin V binds to the PS on the cell surface. In the early stages of apoptosis viability dyes such as propidium iodide are excluded by the plasma membrane. Thus cells in early apoptosis are positive for Annexin V but negative for PI. Whereas cells in late apoptosis, in which the cell membrane has lost its integrity, stain positively for Annexin V and PI.

PBMCs were washed once in PBS and then once with binding buffer. PBMCs were then resuspended in 250µl of binding buffer and 10µl of Annexin V (eBioscience, San Diego, CA) and incubated for 15 minutes at room temperature. Cells were then washed in binding buffer and resuspended in 200µl of binding buffer (eBioscience,San Diego, CA) and 5µl of PI (eBioscience, SanDiego, CA) added. Cells were put on ice in the dark until analysis by flow cytometry. Data was analysed using FlowJo software (Tree Star Inc, OR, USA).

5.4 Results

5.4.1 Infliximab and etanercept do not reduce the production of IL-1β, IL-6 or IL-8 in inflamed IBD biopsies cultured ex vivo.

Inflamed biopsies from four CD and 1 UC patient were incubated for 24 hours with infliximab (10μg/ml), etanercept (10μg/ml) or IgG1 (10μg/ml). There was a wide range of IL-1β, IL-6 and IL-8 produced which did not change with the addition of infliximab or etanercept (Figure 5.1).

5.4.2 Surface TNFα on CHO cells

CHO cells were cultured and passaged two to three times per week. At passage cells that were removed were washed and mTNFα expression measured using a FITC conjugated anti-mTNFα antibody (Figure 5.2). The mean from 3 experiments was 74.3% (range of 55-91%) (Figure 5.3). There was no staining of control CHO cells.

5.4.3 Apoptosis is not increased in the mTNFα transfected CHO cell line cultured with infliximab.

There was no difference in apoptosis in CHO cells incubated for 24 hours with infliximab (10μg/ml) compared to IgG1 (10μg/ml). Apoptosis was measured using Annexin V and PI (Figure 5.4). These results were similar in CHO cells, which did not express mTNFα (results not shown).

5.4.4 Apoptosis does not increase in CD14-ve PBMCs incubated with infliximab compared to IgG1 or etanercept.

Figure 5.5 shows two experiments in which CD14-ve PBMCs activated with anti CD3/28 antibody for 48 hours are then incubated with IgG1 (10μg/ml), infliximab (10μg/ml) and etanercept (10μg/ml). Apoptosis with infliximab is greater in one experiment only but no different in the second experiment.

5.4.5 Apoptosis in unstimulated CD3+LPMCs in inflamed CD was not increased.

Figure 5.6 shows one experiment in which inflamed CD LPMCs were isolated and incubated with infliximab or IgG1 for 24 hours. There was no difference in apoptotic cells although this was only one experiment.

5.4.5 The TACE inhibitor GW280264X does not increase the apoptosis in PBMCs incubated with infliximab.

PBMCs from healthy controls were incubated for 48 hours with anti-CD3/ anti CD28 (1μg/ml) antibody with and without GW280264X (10μM). Infliximab (100μg/ml) or IgG1 (100μg/ml) was then added for 24 hours. A higher concentration was used as this was the concentration used by Nesbitt et al (2007). The cells were then stained with Annexin V and Propidium Iodide for apoptosis and analysed by flow cytometry. Greater apoptosis was detected with infliximab and GW280264X, however an increase in apoptosis was also detected with IgG1 and GW280264X (Figure 6.7).

5.5 Figures

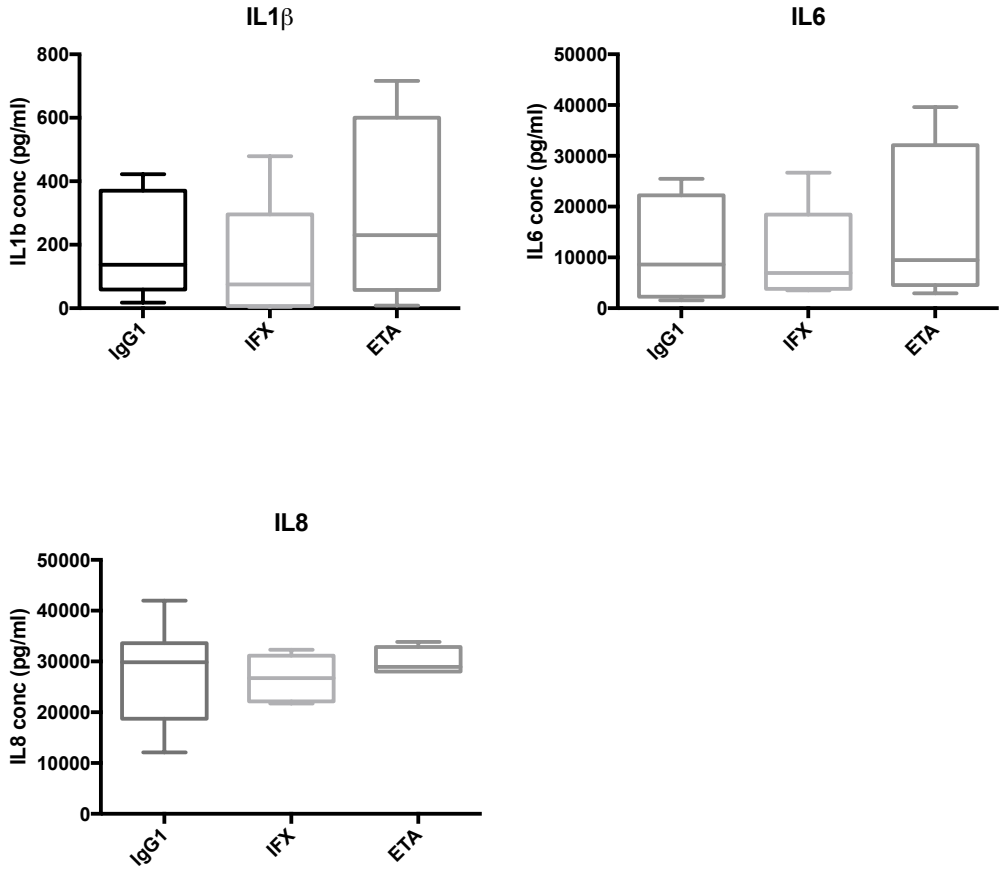


Figure 5-1. Infliximab and etanercept did not reduce IL-1β, IL-6 or IL-8 in inflamed IBD biopsies. Inflamed biopsies from four CD and 1 UC patient were incubated for 24 hours with infliximab (10 μg/ml), etanercept (10μg/ml) or IgG1 (10μg/ml). The concentration of IL-1β, IL-6 and IL-8 was measured by ELISA. The box plots represent the mean concentration of 5 patients for each cytokine and the the standard deviation.

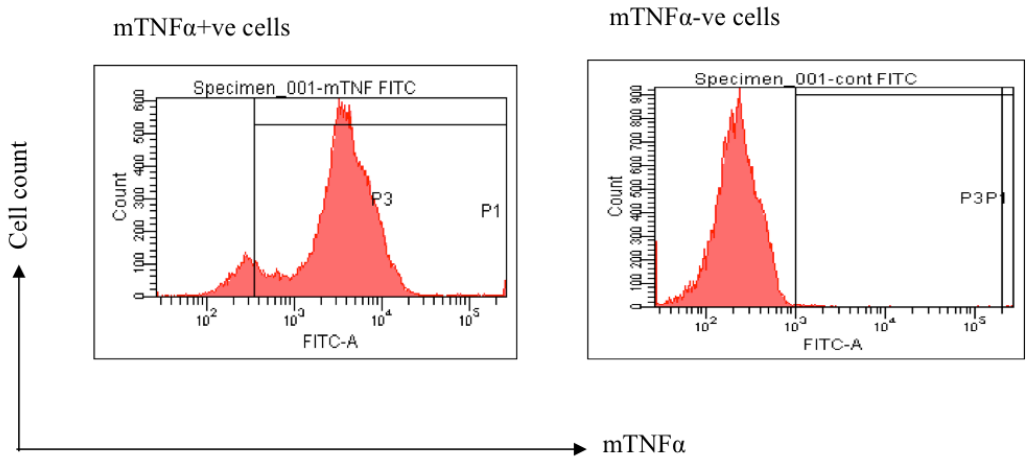


Figure 5-2. A representative FACS plot showing mTNFα expression in the mTNFα transfected CHO cells compared to the mock transfected CHO cells.

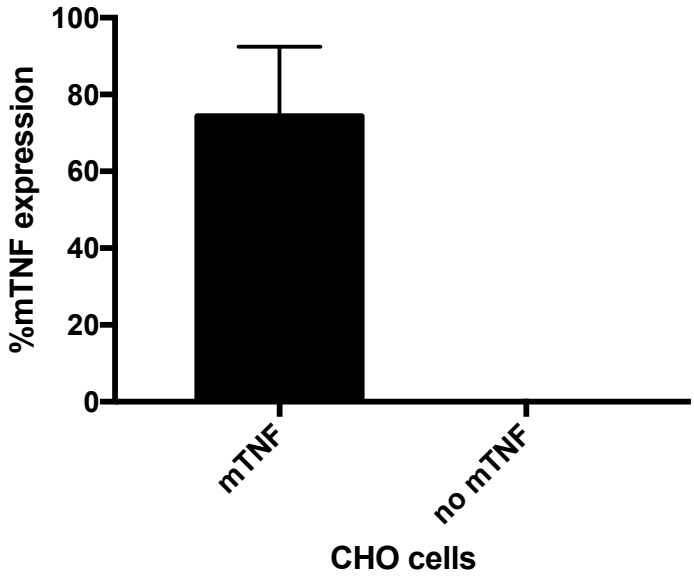


Figure 5-3. CHO cells transfected with mTNFα showed a mean mTNFα expression of 74.3% and the standard deviation. CHO cells transfected with mTNFα were analysed 3 times by FACS to assess mTNFα expression compared to the control CHO cells that were not transfected.

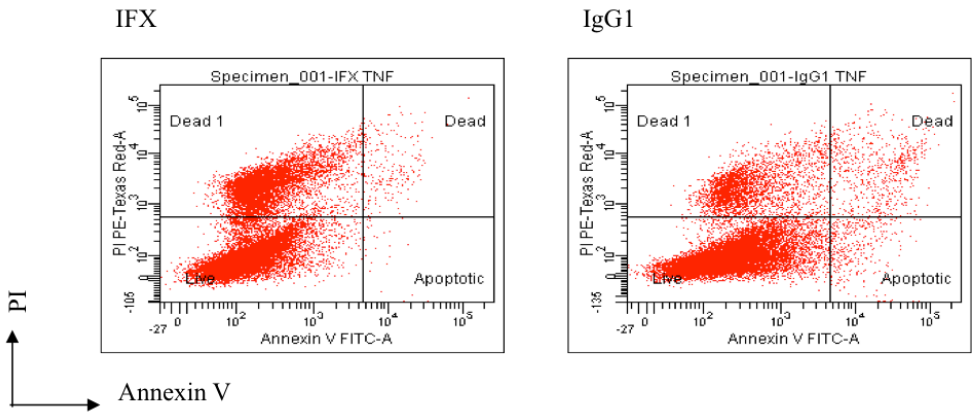


Figure 5-4. Apoptosis is not increased in mTNFα transfected CHO cells with infliximab compared to IgG1. A representative FACS showing CHO cells after 24 hour incubation with infliximab (10µg/ml) or IgG1 (10µg/ml) and the percentage apoptotic cells calculated by flow cytometry using Annexin V and propidium iodide. Apoptotic cells were 0.1% with infliximab and 1.9% with IgG1.

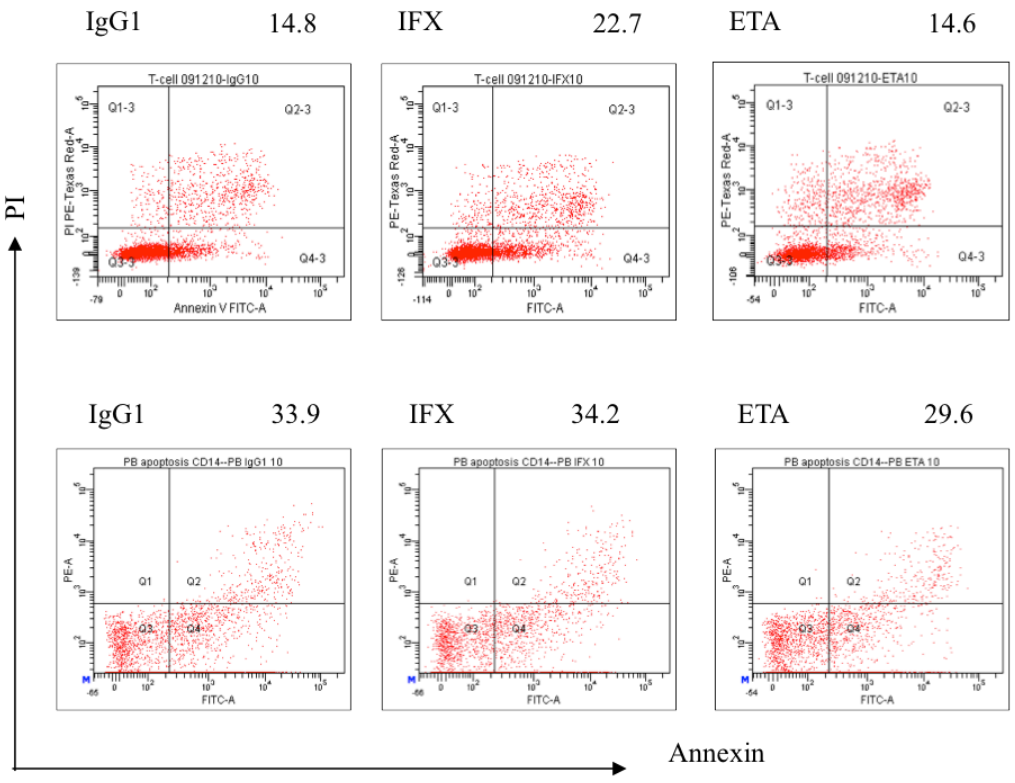


Figure 5-5. Apoptosis is not increased in CD14-ve activated PBMCs incubated with infliximab compared to IgG1 or etanercept. Two experiments in which CD14-ve PBMCs activated with anti CD3/28 antibody for 48 hours and then incubated with IgG1 (10µg/ml), infliximab (10µg/ml) and etanercept (10µg/ml). Apoptosis (Annexin V +ve/ PI-ve cells) was quantified by flow cytometry. The percentage of apoptotic cells are shown above each FACS plot; IgG1 14.8% and 33.9%; infliximab 22.7% and 34.2%; etanercept 14.6% and 29.6%.

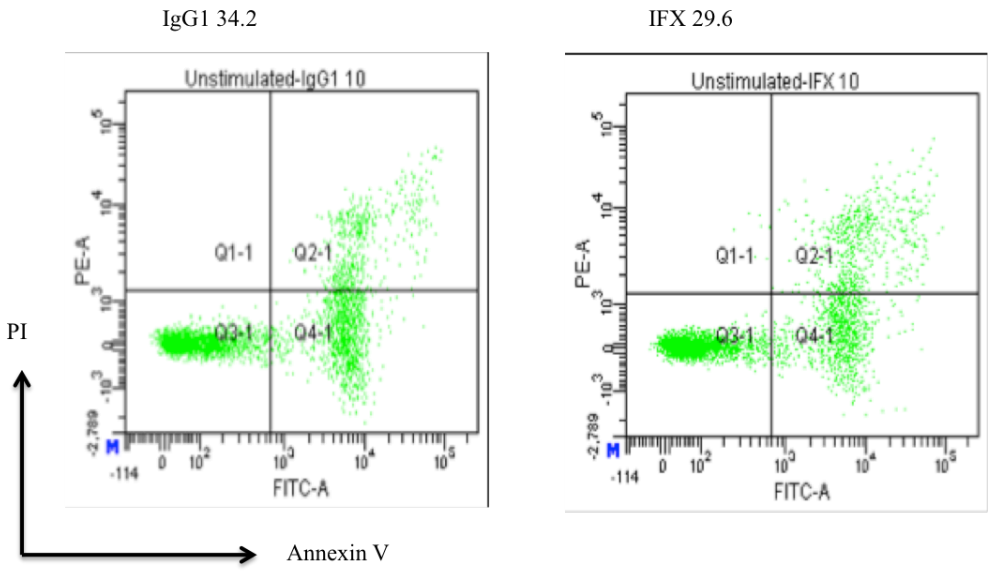


Figure 5-6. There was no increase in apoptosis in unstimulated CD3+ LPMCs of inflamed CD with infliximab compared to IgG1. LPMCs were isolated and incubated with infliximab (10μg/ml) or IgG1 (10μg/ml) for 24 hours before analysis with Annexin V, PI and an anti CD3 antibody to detect CD3+ cells.

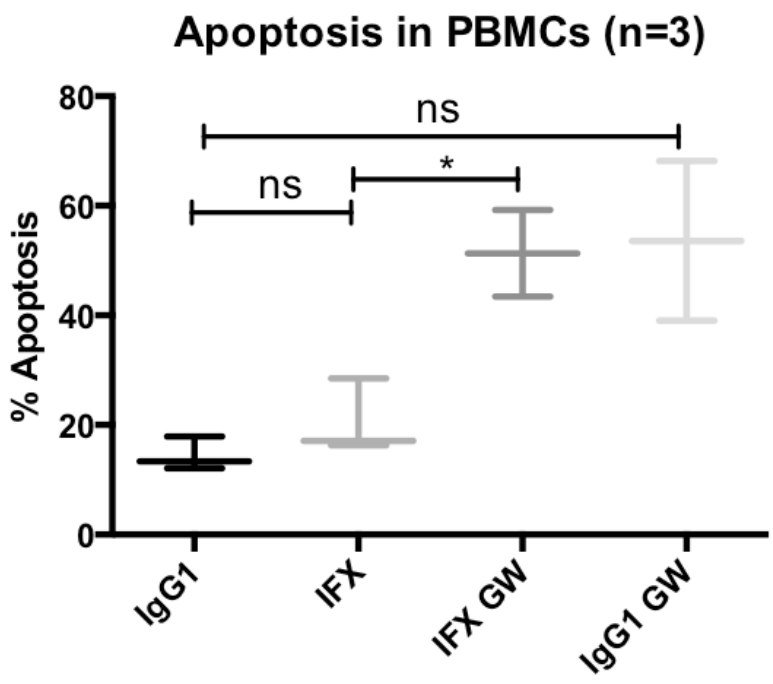


Figure 5-7. GW280264X does not increase apoptosis in PBMCs incubated with infliximab. PBMCs from healthy controls were incubated for 48 hours with anti-CD3/ anti CD28 (1μg/ml) antibody with and without GW280264X 10μM. Infliximab (100μg/ml) or IgG1 (100μg/ml) was then added for 24 hours before staining cells with Annexin V and Propidium Iodide and analysed by flow cytometry . Figure shows the mean of 3 experiments and the standard deviation. A significant difference was seen between IFX and IFX +GW280264X however there was also an increase seen in GW280264X +IgG1. Therefore this difference is not due to IFX. *=P<0.05

5.6 Discussion

The aim of this chapter was firstly to elucidate whether infliximab reduces pro-inflammatory cytokines. No change in the concentration of IL-1β, IL-8 or IL-6 was detected ex-vivo. This is inconsistent with other studies. Nesbitt et al., (2007) showed a reduction in IL-1β in PBMCs following incubation with LPS although PBMCs were initially incubated with infliximab or IgG1 before the addition of LPS. Petito et al., (2016) recently showed a reduction in IL-6 and IL-8 in the supernatant of colonic biopsies incubated with infliximab.

The second aim was to establish the contentious issue of apoptosis with infliximab. Apoptosis was not seen in the mTNFα transfected CHO cell line in this study. Preliminary experiments showed that the CHO cell line expressed mTNFα and CHO cell lines are recognised for their robust nature in vector expression (Meleady et al., 2008). Ten Hove et al., (2002a) showed apoptosis in a Jurkat cell line with infliximab.

Apoptosis was not seen in this study in PBMCs or LPMCs with infliximab or etanercept. Aghnolt et al., (2001) did not show apoptosis in colonic cultures from IBD patients. However apoptosis has been shown in PBMCs and LPMCs with infliximab (Nesbitt et al., 2007, Van den Brande., 2004, Di Sabatino et al., 2004) using Annexin V/PI to measure apoptosis. More recently Eder et al., (2013) failed to show apoptosis in uninflamed biopsies following infliximab or adalimumab using immunohistochemical analysis. Inflamed biopsies were not

sampled. (Atreya et al., 2011) proposed a possible explanation for the detection of apoptosis when they showed apoptosis in TNFRII expressing CD4+ lamina propria T cells following incubation with mTNFα expressing CD14+ cells. However in this study, there was no apoptosis detected when PBMCs activated with P/I were incubated with the TACE inhibitor GW280264X and infliximab. Although the expression of mTNFα on CD14+ cells had not increased, which may have been an explanation for failing to show apoptosis. It is acknowledged that the sample size is small.

This study did not support two of the proposed mechanisms of action of infliximab in IBD. Vossenkaemper et al., (2014) showed increased phosphoprotein expression in IBD using phosphoarrays. The next step was to elucidate whether infliximab reduced inflammation in IBD by their effect on RTK signalling pathways.

6 The Effect of Anti-Tumour Necrosis Factor Antibodies on Receptor Tyrosine Kinase Phosphorylation

6.1 Introduction

Infliximab is effective in reducing inflammation in IBD and increasing the time between relapses (Van Dulleman et al., 1995), (Targan et al., 1997), (Rutgeerts et al., 1999). However the mechanisms of action are still not clear. It has been suggested that it induces T cell apoptosis in the inflamed gut (ten Hove et al., 2002), (Van den Brande et al., 2003), kills cells expressing mTNFα by ADCC (Nesbitt et al., 2007), or kills them by complement activation (Mitoma et al., 2008), (Arora et al., 2009). It has also been suggested that infliximab induces regulatory macrophages (Vos et al., 2011). Initially it was considered that TNFα was a master cytokine and when made by activated macrophages fed back and induced the production of other inflammatory molecules such as IL-6 or IL-1-β. (Butler et al., 1994). If this is indeed the case then it would be expected that infliximab would inhibit many of the downstream signalling pathways associated with cytokine driven inflammation.

Receptor tyrosine kinases (RTK) are a family of receptors that signal primarily through phosphorylation events leading to a cascade of signalling pathways downstream which control cell signalling, the cell cycle, migration as well as cell proliferation and differentiation. Receptor tyrosine kinases (RTK) include Eph proteins, EGFR, PDGFR. The RTK families, which have been shown to be involved in the gut, are discussed in more detail below.

The Erythropoietin producing hepatocellular (Eph) receptor tyrosine kinases are the largest family of RTK. Eph proteins are expressed on almost all cells and are involved in wound healing and maintenance of intestinal cell populations. Eph proteins are divided into two subgroups –A and B. With few exceptions Eph A receptors bind to Ephrin A ligands and Eph B receptors to Ephrin B ligands. Eph A and B are similar in structure; extracellularly they have a ligand binding domain and a cys- rich domain (encompassing Sushi and Epidermal Growth Factor domain) and 2 fibronectin bound domains. Intracellularly a transmembrane region, a tyrosine kinase domain, the sterile alpha motif and the PDZ (postsynaptic density protein 95, discs large 1, and zonula occludens 1) domain. The interaction between Eph receptors and Ephrin ligands result in phosphorylation of tyrosine residues located between the transmembrane and tyrosine kinase domains.

Ephrin ligands are unusual for RTK ligands because they are membrane bound rather than soluble. Ephrins can be divided into 2 two subsets; named A and B, based on their molecular structure; A-Ephrins are bound to the cell membrane by a glycosyl-phosphatidylinositol (GPI) anchor; B-Ephrins are transmembrane proteins (Pasquale., 1997). The binding of the Eph proteins and the Ephrin ligands direct collaborative cell movements by cell-cell signalling (Drescher., 2002). Eph-Ephrin signalling is vital embryologicaly in axonal and vascular development (Flanagan and Vanderhaeghen, 1998), (Gale and Yancopoulos, 1999). In the intestine Eph-Ephrins interactions organise the position of cells within the intestinal epithelial crypts (Batlle et al., 2002) and cell migration up the crypts by changing the expression of Eph receptors (Poliakov et al., 2004). (Holmberg et al., 2006) showed that Eph B receptors on progenitor cells

coordinate migration and proliferation in the stem cell niche. Thus they are likely to play an important role in the regenerative capacities in the gut following damage. In IBD the damage leads to massive increases in epithelial renewal as the gut tries to cover over ulcers with new epithelium. Interestingly Hafner et al., (2005) showed an up-regulation of Eph B2 receptor expression in both lesional and peri-lesional epithelial cells of IBD patients.

Epidermal Growth Factor Receptors (EGFR) are cell surface growth receptors widely expressed in the intestinal tract on the apical and basolateral surface of villus enterocytes (Avissar et al., 2000). When EGF binds to EGFR the receptor dimerises resulting in the autophosphorylation of the EGFR RTKs which triggers various signalling pathways downstream. The signalling pathways involved include Ras/MAPK, phospholipase C/protein kinase C, and STATS signal pathways (Diegelmann et al., 2012). The signalling pathways play a vital role in cellular proliferation and differentiation that help in intestinal development and mucosa repair (Wee et al., 2015). EGFR expression is present in 60-80% of sporadic colorectal cancer (CRC) (Ciardiello and Tortora, 2003) and EGFR inhibitors appear to be effective in the treatment of CRC. There is also an increase in EGFR expression in IBD associated cancer (Svrcek et al., 2007).

The fibroblast growth factor receptors (FGFRs) are a family of tyrosine kinases, which consist of four members FGFR1, FGFR2, FGFR3 and FGFR4 which are very similar structurally (Touat et al., 2015). The extracellular component consists of two or three immunoglobulin-like structures which are required for FGF binding, an acidic region and a heparin-binding region (Brooks et al.,

2012). Phosphorylated tyrosine kinase sites on the transmembrane region act as docking sites for various adaptor proteins. The proteins are then phosphorylated by the activated FGFR and stimulate four possible signalling pathways downstream; PLC, JAK, PI3K and GRB2. The result is a regulation of cell proliferation, differentiation, migration and survival (Beenken and Mohammadi, 2009), (Ornitz, 2000) and plays a role in embryonic development and wound healing (Oladipupo et al., 2014). The association between FGFRs' dysregulation and tumorigenesis has led to the development of a large number of FGFR inhibitors in cancer treatment (Turner et al., 2010).

Platelet derived growth factor (PDGF) is a major growth factor which plays a central role in the repair process. PDGF transduces signals by binding to PDGF receptor- of which there are two- PDGFR-α and PDGFR-β which are tyrosine kinase receptors. They are structurally similar with 5 Ig-like domains extracellularly, two of which form the binding site for PDGF. In the intracellular region, up to eleven tyrosine kinase residues are located. These sites are autophosphorylated on binding of PDGF and trigger a range of signaling cascades such as ERL MAPK. Defects in PDGFR signaling has been associated with various cancers (Medves and Demoulin, 2012).

The mitogen-activated protein kinase (MAPK) pathways are important in controlling inflammation, cell differentiation, cell growth and malignancy. There are three major constituents; the extracellular signal-regulated kinases (ERKs) such as p42/p44, the c-Jun N-terminal kinases, and the p38 MAPKinases. Apart from ERK 3 and 4 and NLK, full activation of MAPKinases requires dual simultaneous phosphorylation of threonine and tyrosine within a Threo-X-Tyro

motif where X is a specific amino acid. The amino acid varies between MAPKinases and confers specificity for the upstream kinase. Phosphorylation results in a conformational change in the protein exposing the kinase active site and enable substrate binding (Chen et al., 2001). Activation of the signaling cascade can be triggered by a wide range of stimuli, in general ERKs are activated by mitogens and differentiation signals whilst Jun and MAPK are activated by cellular stresses (Riemann et al., 2015). TNF can activate all three groups (including hormones, growth factors, PAMPS, cytokines and environmental stresses and cellular stress particularly by TNFα).

Increased expression of ERK 1 and 2, p38 and MAPK are seen in IBD patients compared to controls (Waetzig et al., 2002). Levels of activated p38 MAPK were found to be raised in the inflamed mucosa of patients with IBD (Docena et al., 2010) and inhibitors of MAPK reduce phosphorylated MAPK in biopsies cultured ex vivo from patients with IBD (Docena et al., 2010). Hommes et al., (2002) showed that a Jnk and p38 MAPK inhibitor given for 12 days reduced MAPK phosphorylation when tested in vitro on colonic biopsies. After 4 weeks, 67% showed a clinical response and a significant reduction in CDAI.

The Jak/ Signaling transducer and activation of transcription (STAT) 3 pathway is used by cytokines such as IL-6 (De Simone et al., 2015) and IL-10 (Lejeune et al., 2002). On binding of cytokines to its membrane receptor a conformational change occurs in associated JAK molecules resulting in autophosphorylation and a second phosphorylation on the cytokine receptor. Subsequently docking sites for STAT are exposed which are also phosphorylated and translocate to the nucleus regulating transcription of various genes (O'Shea et al., 2004). STAT 5

is involved in the development of different T cell subsets, specifically T regs (Yao and Shao., 2007). The role of STAT 3 in IBD is well documented (Kaur et al., 2016), (Zundler and Neurath, 2016), (Jauregui-Amezaga et al., 2015), and its expression is increased in both UC and CD lamina propria T cells (Mudter et al., 2005). In addition a JAK1, 3 and 5 inhibitor (Tofacitinib) shows efficacy in UC (Sandborn et al., 2012) although has not been shown to be more effective than placebo in CD (Panés et al., 2015).

T cell subsets are important in IBD. How T cells are activated and differentiate into different subsets is of interest. ZAP-70 is a cytoplasmic tyrosine kinase mainly expressed in T cells and mediates T cell receptor (TCR) signaling. Following TCR engagement, Lck associated to CD4 is brought into proximity with the TCR enabling the phosphorylation of the ITAM motifs situated on the TCRζ chain. After ITAM binding, ZAP-70 is phosphorylated by Lck, activating ZAP-70 (Wang et al., 2010). ZAP-70 activation triggers a number of signalling cascades resulting in rearrangement of the T cell cytoskeleton, IL-2 synthesis and release and T cell proliferation (Boubek et al., 1996). Loss of function of expression of ZAP-70 in humans leads to SCID in which CD4+ T cells are nonfunctional and CD8+ T cells are absent (Elder et al., 1994). Bouzid et al recently showed an association between a ZAP-70 polymorphism and CD in a Tunisian population (Bouzid et al., 2013). A heterozygous mutation in ZAP-70 was identified in a Chinese patient who developed early onset and recurrent infections as well as IBD amongst other autoimmune clinical manifestations (Liu et al., 2017).

The different efficacies of infliximab and etanercept in IBD have been discussed in chapter 5. The reasons for this difference is not yet understood and research is still needed to explain why etanercept, an anti-TNFα antibody is ineffective in CD whilst infliximab is effective.

6.2 Aims

To use phosphoarrays to determine if the addition of TNFα neutralising agents to mucosal biopsies from patients with IBD ex vivo has any effect on RTKs. Accordingly the following experiments were carried out.

1. The phosphorylation events in inflamed IBD biopsies incubated in infliximab were measured. Samples were initially snap frozen and stored at -80°C prior to cell lysis and phosphoarray.
2. The phosphorylation changes in inflamed biopsies of UC and CD individuals incubated in etanercept were then measured and compared to infliximab.

6.3 Materials and Methods

6.3.1 Organ Culture of Intestinal Mucosal Biopsies

Inflamed colonic biopsies obtained during colonoscopy were cultured (one biopsy per well) in 24 well plates (VWR International, Lutterworth, UK) in 300μl of serum free HL-1 medium (Cambrex BioScience, Wokingham, UK) for 24 hours at 37°C, in 5% CO_2. At the start of the culture infliximab (5μg/ml; Remicade®, Schering-Plough, Welwyn Garden City, UK), etanercept (5μg/ml ; Enbrel®, Wyeth, Maidenhead, UK) or IgG1 were added. The supernatant was collected after 24 hrs and stored at -80°C before undergoing cytokine array analysis. The biopsy was snap-frozen and stored at -80°C prior to cell lysis and phosphoarray.

6.3.2 Phosphoarray

Pathscan RTK signalling arrays (Cell Signalling Technology, Danvers, MA) were used to determine the phosphorylation status of receptor tyrosine kinases. One hundred microlitres RIPA buffer (containing 10μl/ml protease inhibiter and 10μl/ml phosphatase inhibiter) was added to each biopsy on ice. The biopsy was then sonicated for 2-3 seconds three times before centrifugation for 10 minutes at 12000g at 4°C. The supernatant was aspirated and the protein concentration calculated using the Bradford assay. The supernatants were stored at -80°C.

The PathScan RTK Signalling Antibody Array kit is a slide based antibody array based upon the sandwich immunoassay principle. It allows for the simultaneous detection of 28 receptor tyrosine kinases and 11 signalling nodes phosphorylated

at tyrosine or other residues. The manufacturer precoats the slide, with capture antibodies spotted in duplicate onto nitro-cellulose glass slides. A multi-well gasket is fixed onto the slide and 150µl of blocking buffer is introduced into each well. The slide is placed on a rocker at room temperature for 15 minutes. The blocking buffer is then decanted from each well and 150µl of sample lysate containing 75µg of protein is introduced into the well and incubated on a rocker at room temperature for two hours. The wells are then washed four times with array wash buffer for five minutes each before 150µl of Array Detection Antibody Cocktail is introduced and incubated for one hour. The slide is washed again four times and 150µl of streptavidin HRP is introduced into each well. The slide is washed and then removed from the gasket. Lumiglow and peroxide were then pipetted onto the slide, which was then exposed to X-ray film, and the pixel intensity quantified using Image J.

6.4 Statistical Analysis

Data was analysed using GraphPad Prism (GraphPad Software, San Diego, CA) software. Student's paired t-test was used to compare two values from the same individual. P values of less than 0.05 were considered statistically significant.

6.5 Results

6.5.1 The phosphorylation levels detected in inflamed CD and UC biopsies were high.

Inflamed biopsies from five CD patients and four UC patients were incubated with IgG1 or infliximab for 24 hrs. The pixel intensities of healthy controls were studied in Vossenkaemper et al., (2014) in our group and shown to be low compared to IBD. Figure 6.1 shows the array after exposure to X ray film and the RTK map identifying the different RTKs on the array. The pixel intensity of inflamed CD and UC biopsies showed high phosphoprotein levels in the majority of phosphoproteins. Figures 6.2 and 6.3 show each of the biopsies' phosphoprotein levels for CD and UC respectively as represented by a heatmap. There is some variation between the samples as well as between the different phosphoproteins from the same biopsy. The range varied from 0.01 (the value in TrkA and TrkB in CD2, CD3 and CD4) up to 78.4 (the value of Src in UC1).

6.5.2 The phosphoprotein levels were higher in the UC individuals compared to the CD individuals.

The mean pixel intensity in the CD biopsies and the UC biopsies for each receptor tyrosine kinase was greatest for the EGFR, FGFR, Insulin R and Stat families (19.36 in FGFR4 up to 39.04 in EGFR) (Figures 6.2 and 6.3,6.4 to 6.7). The mean pixel intensity was low in the NGFR and HGFR families (2.79 in TrkA and up to 10.67 in Ron/ MST1R) (Figure 6.2), in fact in 3 of the individuals the pixel intensity was the same as the background in these families (CD2, CD3 and CD4 in Figure 6.2).

6.5.3 There was a global reduction in phosphoprotein levels with infliximab in CD and UC biopsies.

Figure 6.1 shows a representative slide of a CD biopsy with infliximab and IgG1 and the array map for identification of RTKs. The relative pixel intensity is reduced in all phosphoproteins in each CD individual (Figure 6.2) and three out of four UC individuals (Figure 6.3).

6.5.4 PDGFR, c-Kit, M-CSFR/CSF and FLT3 phosphoproteins in the PDGFR family were reduced significantly with infliximab.

Four RTK were measured in the PDGFR family, which were c-Kit, FLT3, PDGFR and M-CSFR. The mean change in pixel intensity of all five CD and four UC individuals reduced in all four RTK (Figure 6.4). The reduction was significant in c-Kit ($p=0.001$), PDGFR ($p=0.026$), M-CSFR/ CSF (0.022) and FLT3 ($p=0.008$) (Figure 6.8).

6.5.5 Infliximab significantly reduced the phosphoproteins Eph A2, Eph A3, Eph B1 and Eph B3 in IBD.

Six RTKs from the Ephrin family were measured; Eph A1, Eph A2, Eph A3, Eph B1, Eph B3 and Eph B4. The mean pixel intensity in the phosphoproteins in the biopsies incubated in IgG1 and infliximab showed a reduction in all members in CD and in UC (Figure 6.4). The reduction in IBD biopsies was

significant in Eph A2 (p=0.002); Eph A3 (p= 0.02), Eph B1 (p=0.004), and Eph B3 (p=0.007) (Figure 6.9).

6.5.6 Infliximab significantly reduced the phosphoproteins EGFR and HER2.

Three phosphoproteins from the EGFR family were measured which were EGFR/ Erb1, HER2/ Erb2 and HER 3/ Erb3 and infliximab reduced the levels of each (Figure 6.5). The mean pixel intensity in the 5 CD biopsies was reduced from 39.04 to 27.70 and in the UC biopsies from 44.7 to 42.8. Together there was a significant reduction with infliximab in inflamed IBD biopsies in EGFR (p=0.02) and HER 2 (p=0.02) (Figure 6.10). The reduction in HER 3 was not significant.

6.5.7 Infliximab significantly reduced the phosphoproteins FGFR3 and FGFR4.

Three phosphoproteins from the FGFR family were measured, namely FGFR1, FGFR3 and FGFR4. Infliximab significantly reduced the levels in each phosphoprotein (Figure 6.5). FGFR3 reduced from 27.40 to 17.13 (p=0.016) and FGFR4 reduced from 19.40 to 10.20 (p=0.021) (Figure 6.11). The pixel intensity for each phosphoprotein in IgG1 and infliximab can be seen in Figure 6.11 showing the reduction in all but one individual with infliximab.

6.5.8 Infliximab significantly reduced Axl, Tyro3, Tie2 and VEGFR phosphoproteins.

The RTK measured in the Axl family were Axl and Tyro3. There was a significant reduction in Axl, Tyro and Tie2 and VEGFR when a paired t test is carried out on all IBD biopsies where p=0.001 (Axl), p=0.004 (Tyro3), p=0.001 (Tie2) and p=0.003 (VEGFR) (Figure 6.6 and 6.12).

6.5.9 Infliximab significantly reduced phosphoproteins p44/MAPK, S6 Ribosomal protein and c-Abl in inflamed IBD biopsies.

Infliximab reduced phosphorylation of p44/MAPK and s6 ribosomal protein and c-abl which is significant in the IBD biopsies in p44/MAPK (p=0.015), S6 Ribosomal protein (p=0.026) and c-abl (p=0.024) (Figure 6.7 and 6.13).

6.5.10 Infliximab significantly reduces phosphoproteins IRS-1 and Zap-70 in CD biopsies.

Although the reduction in phosphoprotein intensity in IBD biopsies together is not significant, when a paired t test is applied to the CD biopsies alone the reduction is significant where p=0.007 in Zap-70 and p=0.017 in IRS-1 (Figure 6.13 and 6.14).

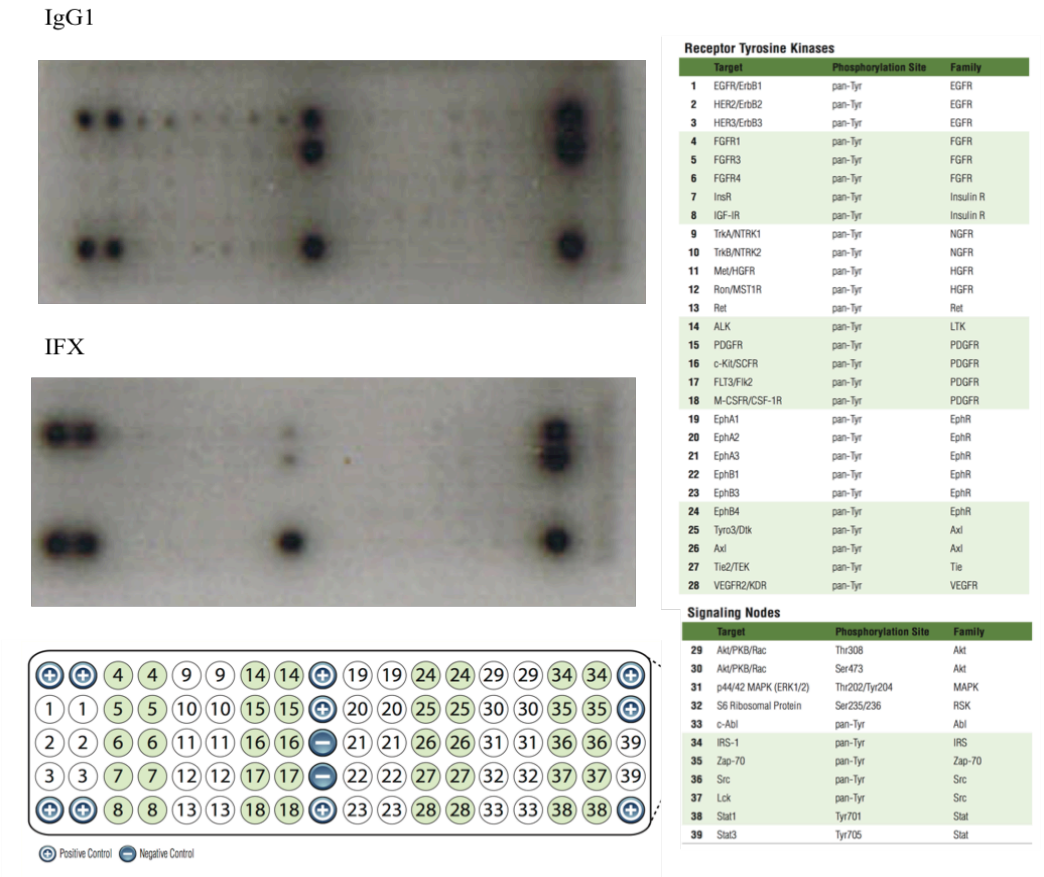
6.5.11 Infliximab significantly reduced phosphoproteins Akt/PKB/Rac on Ser 473 in inflamed IBD biopsies.

There was a significant reduction in Ser473 in inflamed IBD biopsies (p=0.007) (Figure 6.7 and 6.15). In the CD biopsies alone however there was a significant reduction in Src (p=0.02), Lck (p=0.015) and Sat 1 (p=0.016) (Figure 6.16).

6.5.12 Etanercept reduces PDGFR, c-Kit FLT3 and CSFR phosphorylation in inflamed IBD biopsies

The phosphorylation intensities were high in the IBD biopsies with IgG1, seen in the heatmap showing the changes in all four of the inflamed IBD biopsies (3 UC, 1 CD) (Figure 6.17) There was a reduction in receptors in the PDGFR family, including PDGFR, c-Kit, FLT3 and CSFR, although the reduction is not significant. There was no change in the phosphorylation of the other RTKs tested with etanercept (Figure 6.18 and 6.21).

6.6 Figures



Receptor Tyrosine Kinases

	Target	Phosphorylation Site	Family
1	EGFR/ErbB1	pan-Tyr	EGFR
2	HER2/ErbB2	pan-Tyr	EGFR
3	HER3/ErbB3	pan-Tyr	EGFR
4	FGFR1	pan-Tyr	FGFR
5	FGFR3	pan-Tyr	FGFR
6	FGFR4	pan-Tyr	FGFR
7	InsR	pan-Tyr	Insulin R
8	IGF-IR	pan-Tyr	Insulin R
9	TrkA/NTRK1	pan-Tyr	NGFR
10	TrkB/NTRK2	pan-Tyr	NGFR
11	Met/HGFR	pan-Tyr	HGFR
12	Ron/MST1R	pan-Tyr	HGFR
13	Ret	pan-Tyr	Ret
14	ALK	pan-Tyr	LTK
15	PDGFR	pan-Tyr	PDGFR
16	c-Kit/SCFR	pan-Tyr	PDGFR
17	FLT3/Flk2	pan-Tyr	PDGFR
18	M-CSFR/CSF-1R	pan-Tyr	PDGFR
19	EphA1	pan-Tyr	EphR
20	EphA2	pan-Tyr	EphR
21	EphA3	pan-Tyr	EphR
22	EphB1	pan-Tyr	EphR
23	EphB3	pan-Tyr	EphR
24	EphB4	pan-Tyr	EphR
25	Tyro3/Dtk	pan-Tyr	Axl
26	Axl	pan-Tyr	Axl
27	Tie2/TEK	pan-Tyr	Tie
28	VEGFR2/KDR	pan-Tyr	VEGFR

Signaling Nodes

	Target	Phosphorylation Site	Family
29	Akt/PKB/Rac	Thr308	Akt
30	Akt/PKB/Rac	Ser473	Akt
31	p44/42 MAPK (ERK1/2)	Thr202/Tyr204	MAPK
32	S6 Ribosomal Protein	Ser235/236	RSK
33	c-Abl	pan-Tyr	Abl
34	IRS-1	pan-Tyr	IRS
35	Zap-70	pan-Tyr	Zap-70
36	Src	pan-Tyr	Src
37	Lck	pan-Tyr	Src
38	Stat1	Tyr701	Stat
39	Stat3	Tyr705	Stat

Figure 6-1 Phosphoarray showing a sample chemiluminescent film of IgG1 and infliximab and the array map with the RTKs and signaling nodes listed. IBD biopsies were incubated for 24 hours with IgG1 (5µg/ml) or infliximab (5µg/ml) and the biopsies then lysed and analysed for phosphorylation intensity with a phosphoarray.

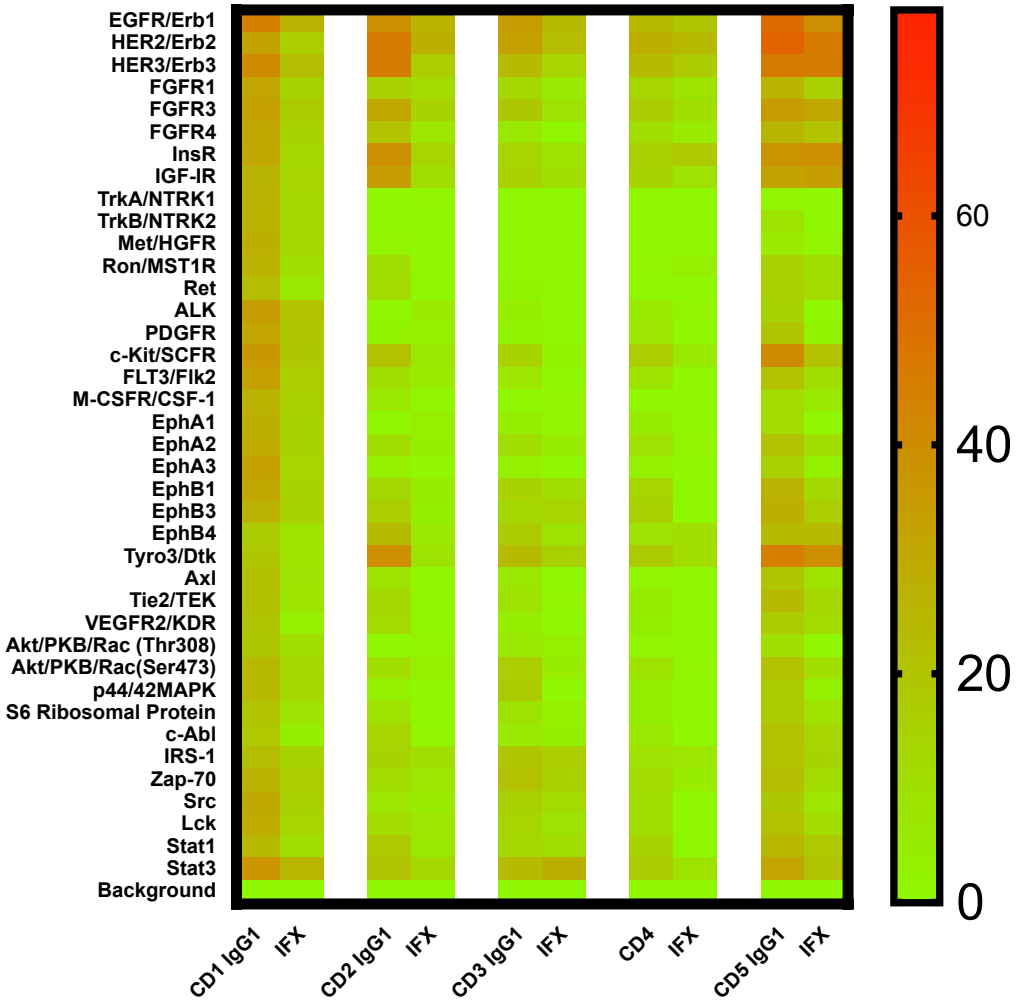


Figure 6-2. Infliximab reduces the phosphorylation in all RTKs measured in CD biopsies. A heatmap representing the mean pixel intensity measured in the inflamed biopsies of five CD individuals following incubation for 24 hours with infliximab (5µg/ml) or its control IgG1 (5µg/ml).

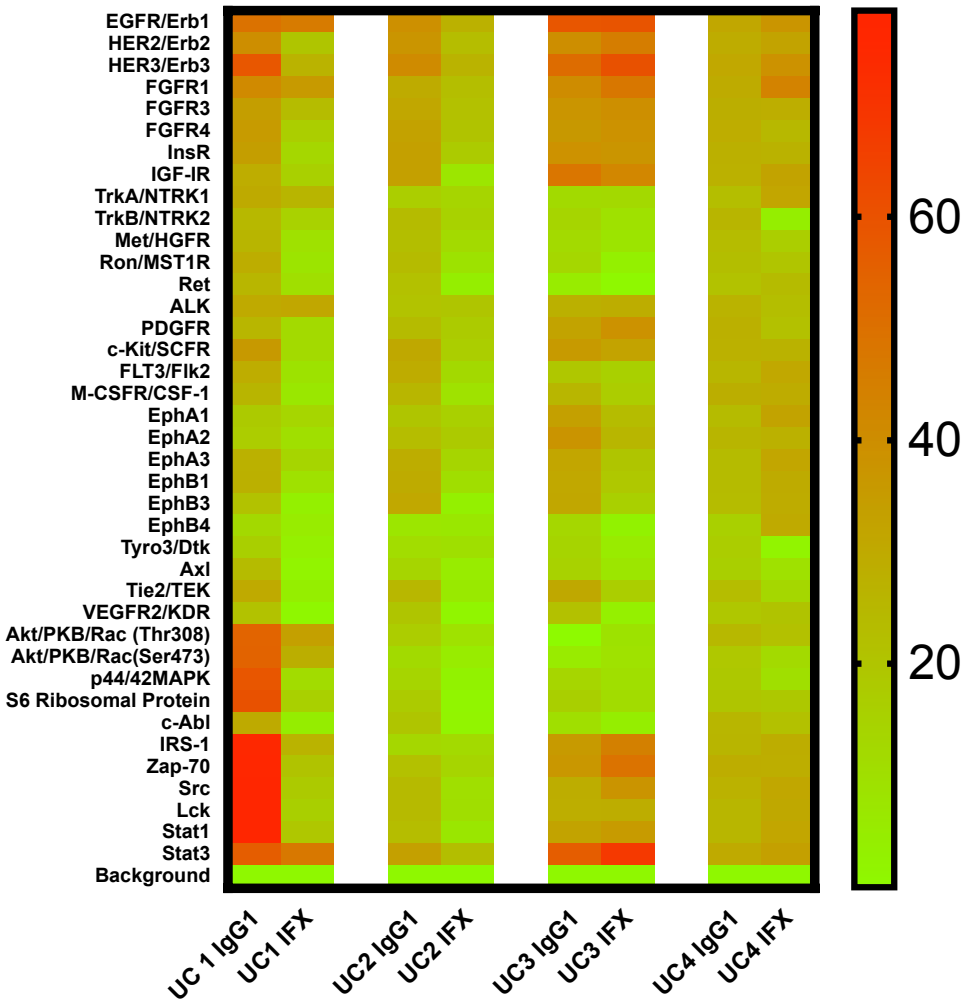


Figure 6-3. Infliximab reduces the phosphorylation in all RTKs measured in UC biopsies. A heatmap representing the mean pixel intensity measured in the inflamed biopsies of four UC individuals following incubation for 24 hours with infliximab (5µg/ml) or its control IgG1 (5µg/ml).

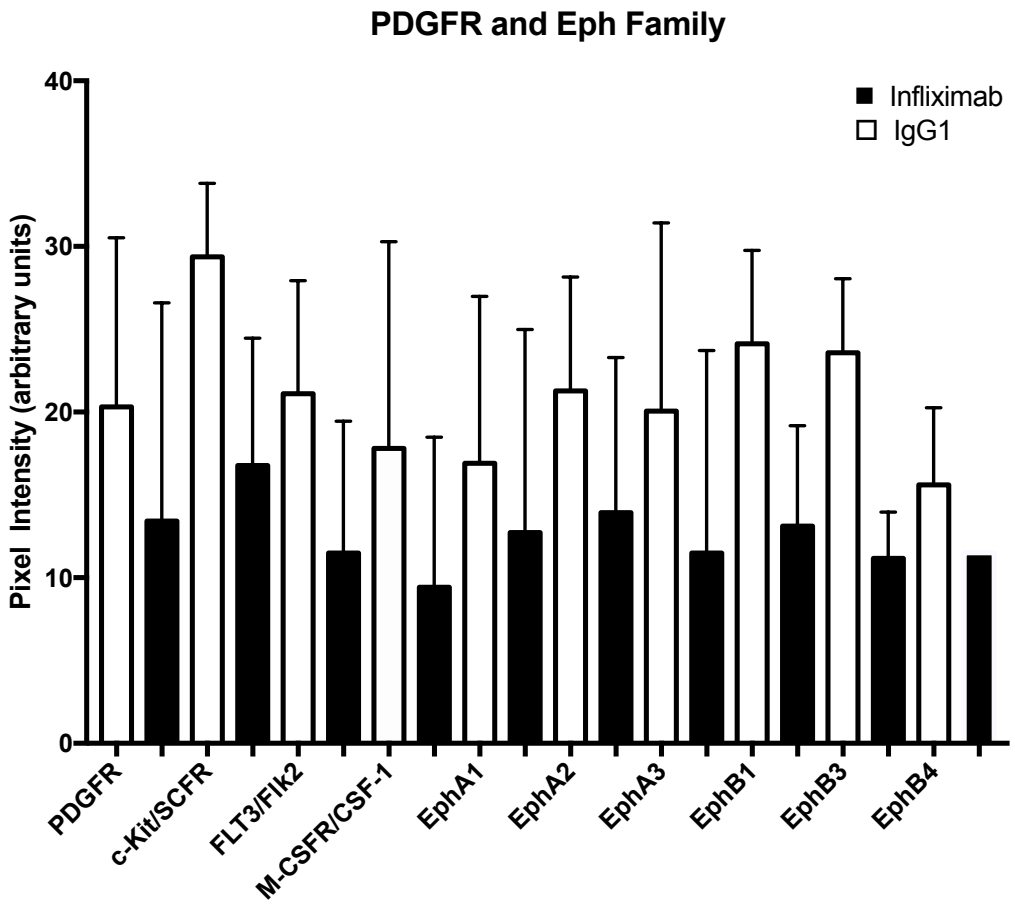


Figure 6-4 Infliximab reduces phosphorylation in IBD explants in PDGFR and Eph family. Mean pixel intensity and the standard deviation from explants of 5 CD and 4 UC patients incubated for 24 hours with Infliximab (5μg/ml) and IgG1 (5μg/ml).

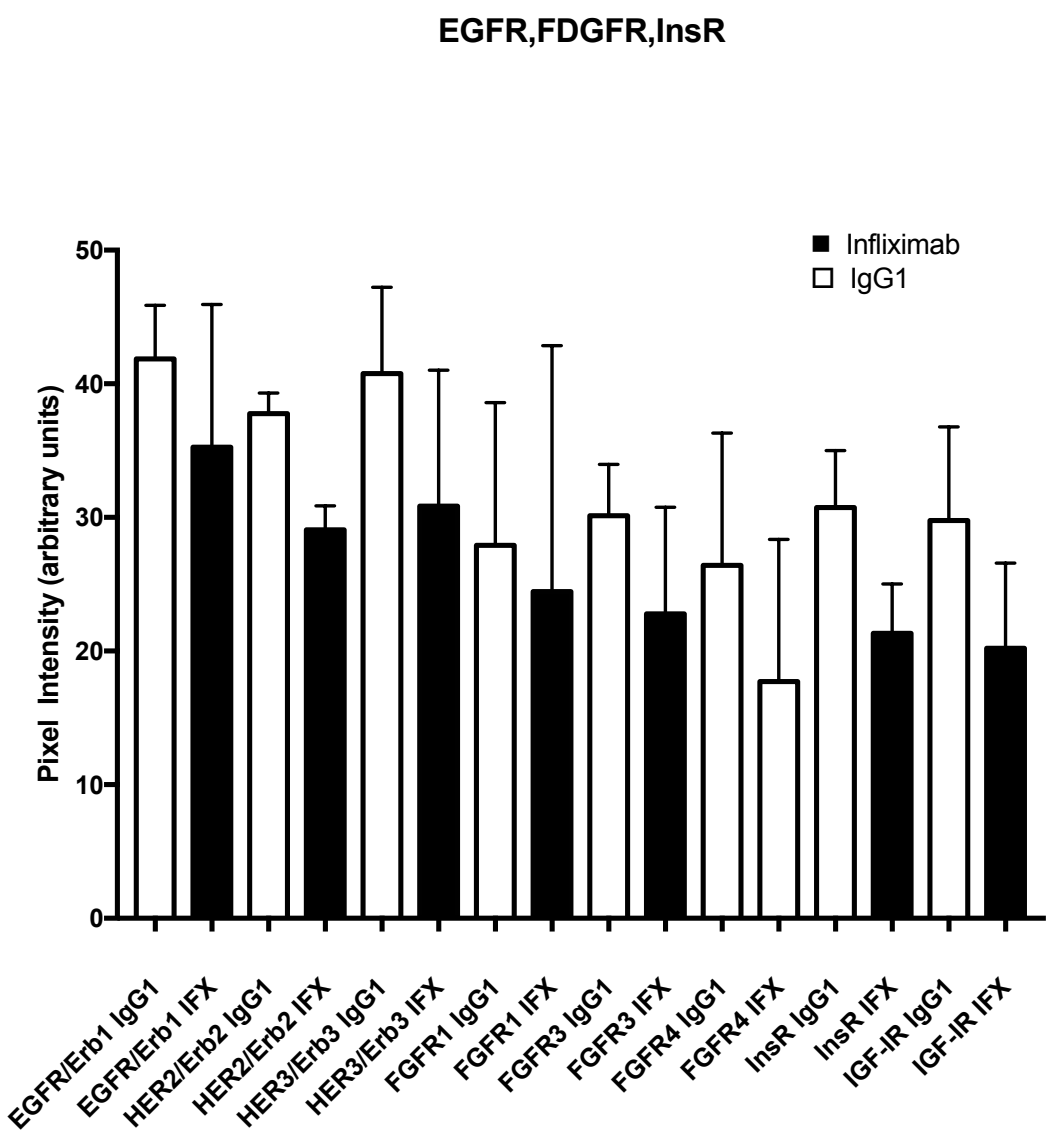


Figure 6-5 Infliximab reduces phosphorylation in IBD explants in EGFR anf FGFR family. Mean pixel intensity and the standard deviation for 18 receptor tyrosine kinases from explants of 5 CD and 4 UC patients incubated for 24 hours with Infliximab (5μg/ml) and IgG1 (5μg/ml).

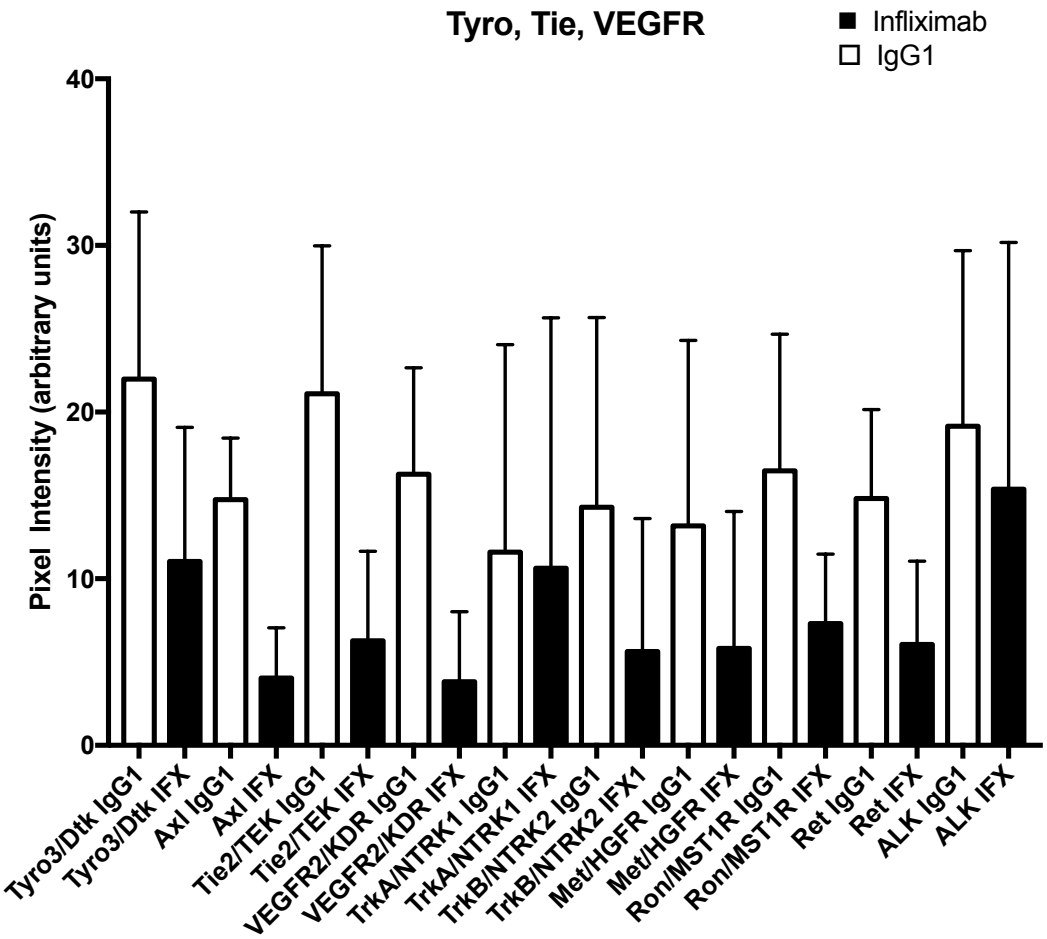


Figure 6-6. Infliximab reduces phosphorylation in IBD explants in RTKs. Mean pixel intensity and the standard deviation for receptor tyrosine kinases from explants of 5 CD and 4 UC patients incubated for 24 hours Infliximab (5μg/ml) or IgG1 (5μg/ml).

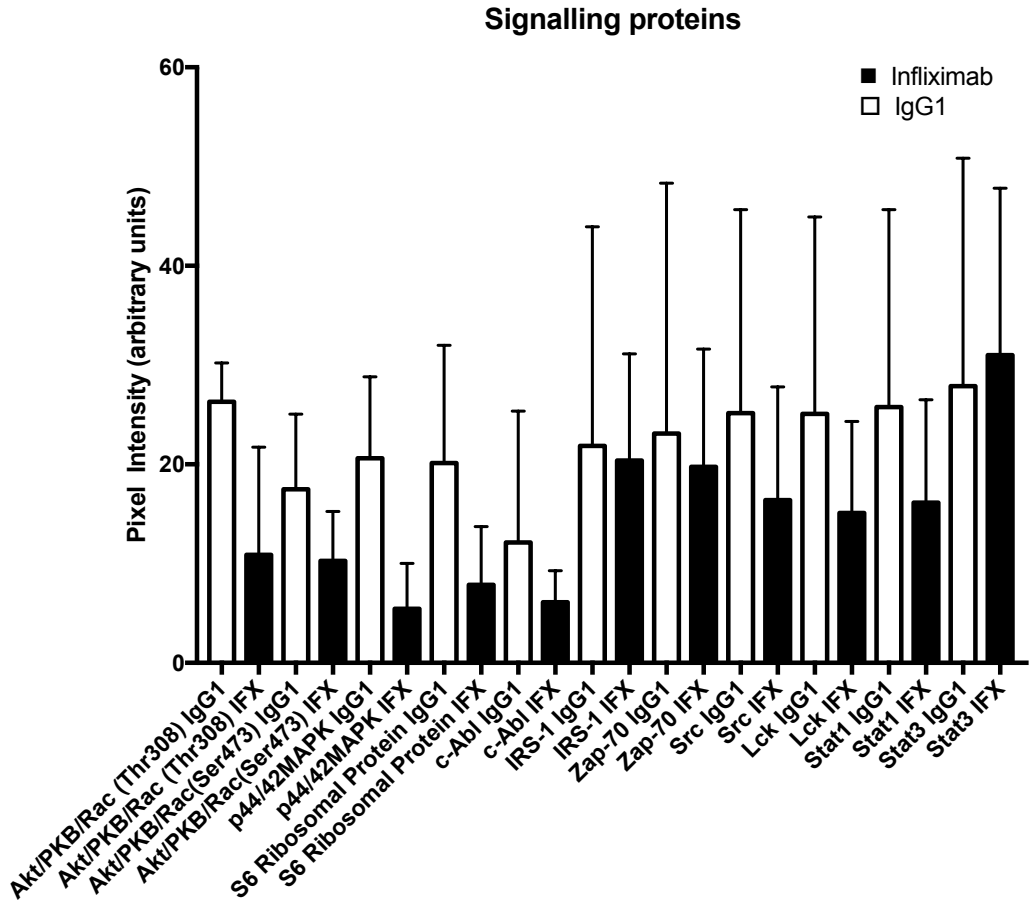


Figure 6-7. Mean pixel intensity and the standard deviation of RTKs and signalling nodes from explants of 5 CD and 4 UC patients incubated for 24 hours with Infliximab (5µg/ml) or IgG1 (5µg/ml).

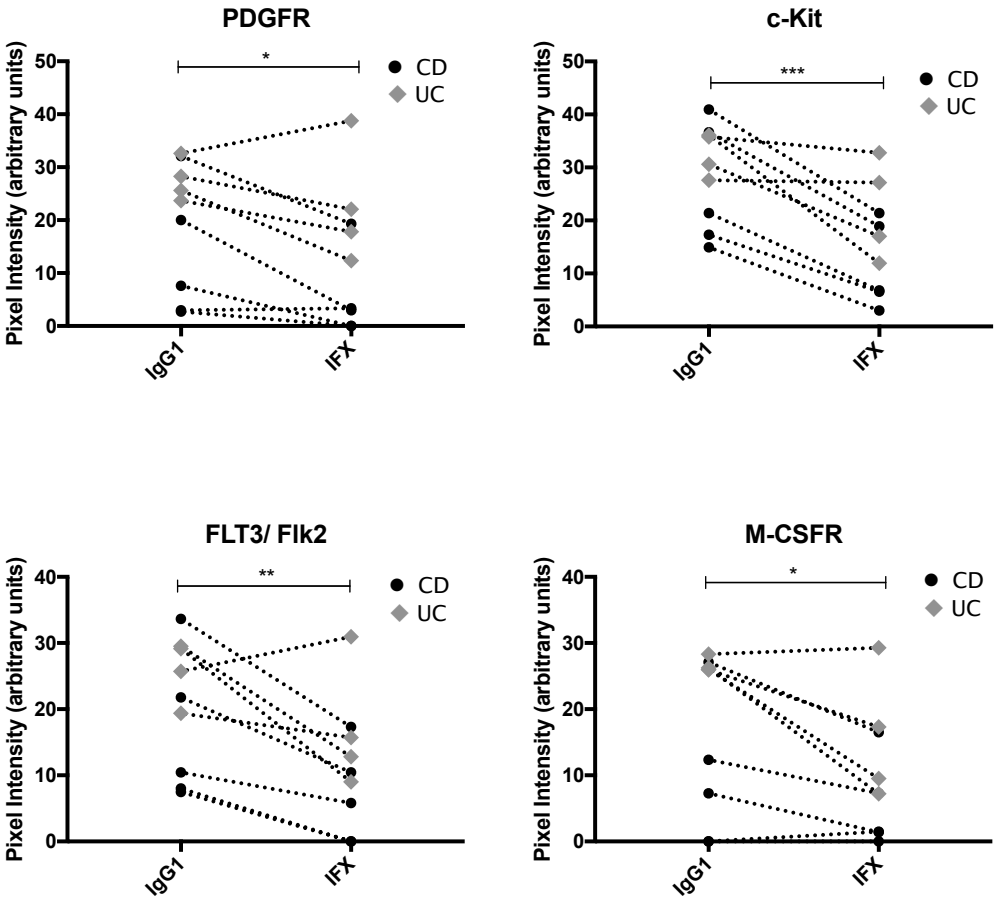


Figure 6-8. All phosphoproteins in the PDGFR are reduced significantly with infliximab. Five CD and 4 UC biopsies were incubated for 24 hours with infliximab (5μg/ml) or IgG1 (5μg/ml) and the change in phosphoprotein expression measured by calculating the pixel intensity. * = p<0.05, ** = p<0.005.

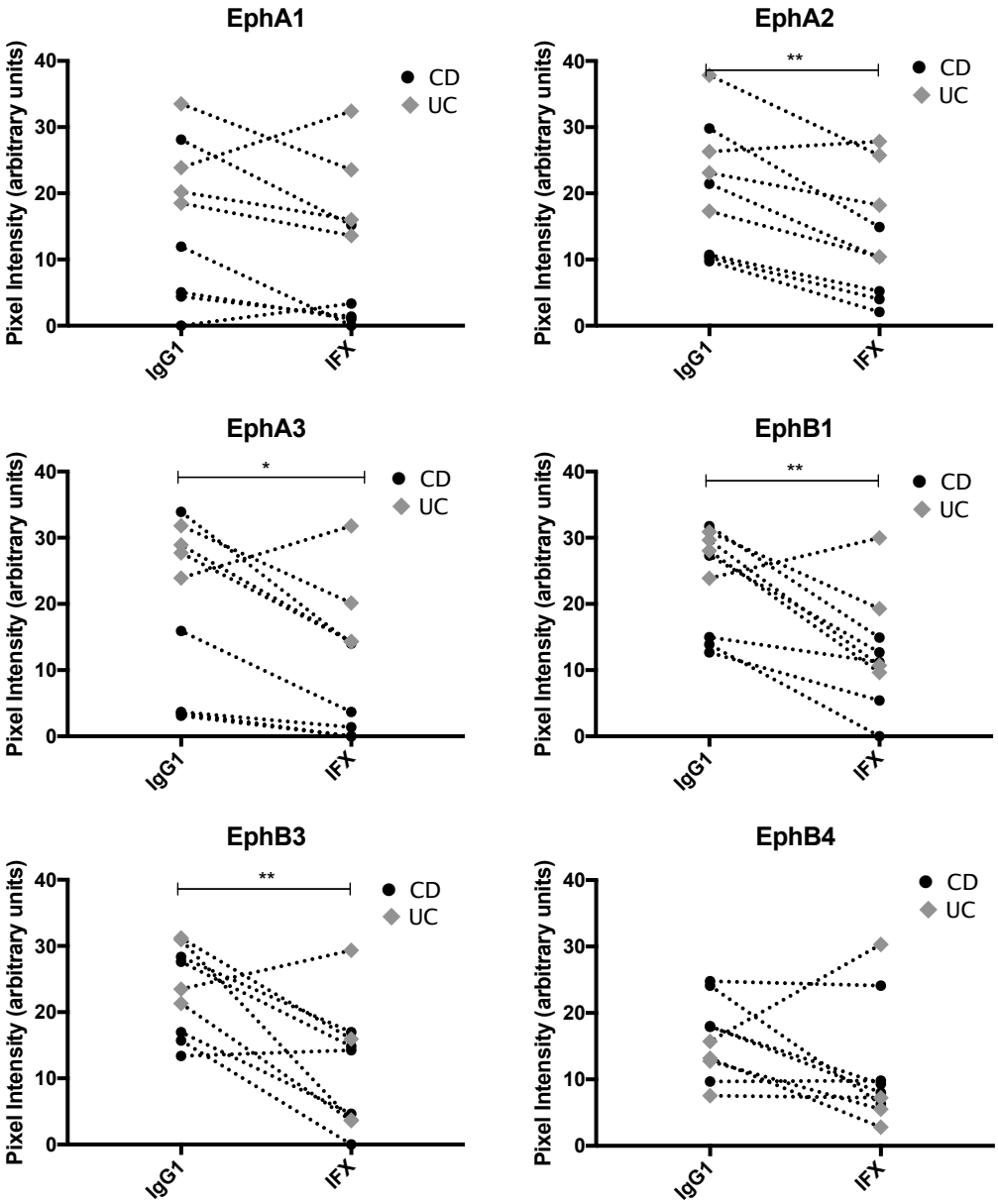


Figure 6-9 EphA2, EphA3, EphB1 and Eph B3 in the Eph family are significantly reduced in IBD biopsies with infliximab. Five CD and four UC biopsies were incubated for 24 hours with Infliximab (5µg/ml) or IgG1 (5µg/ml) and the phosphoprotein expression measured by calculating the pixel intensity. * = p<0.05, ** = p<0.005.

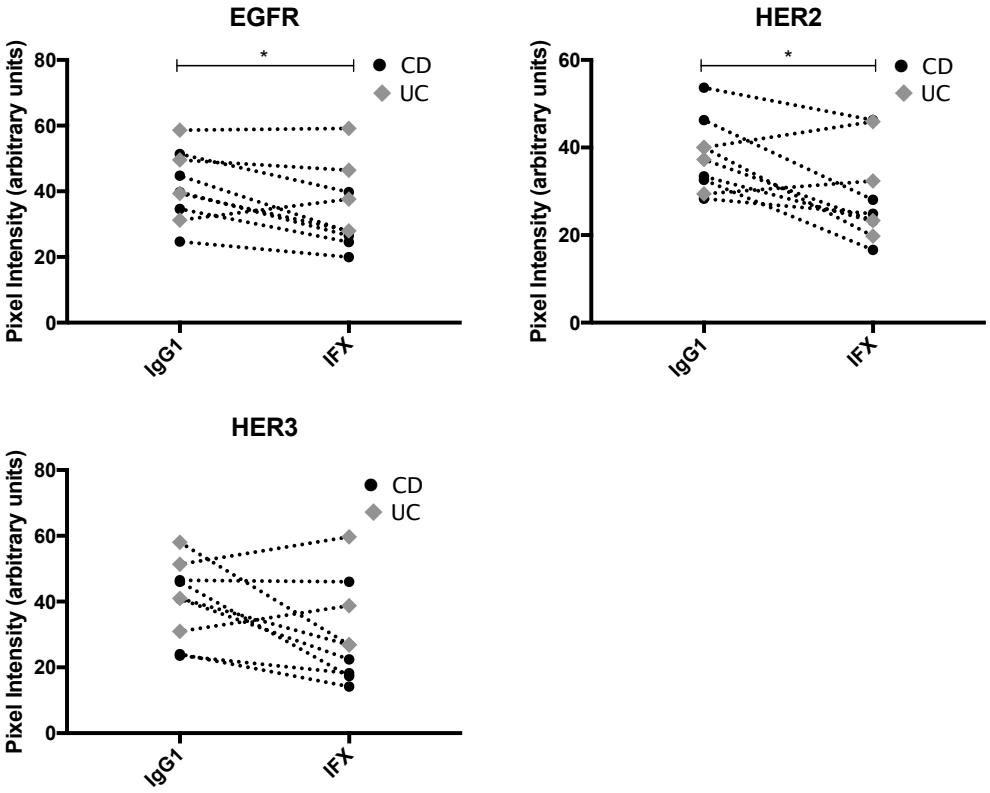


Figure 6-10. Infliximab reduced the phosphoprotein levels of EGFR and HER 2 in the EGFR family significantly in IBD biopsies. Five CD and four UC inflamed biopsies were incubated for 24 hours with infliximab (5µg/ml) or IgG1 (5µg/ml) and the phosphoprotein expression measured by calculating the pixel intensity. * = p<0.05, ** = p<0.005.

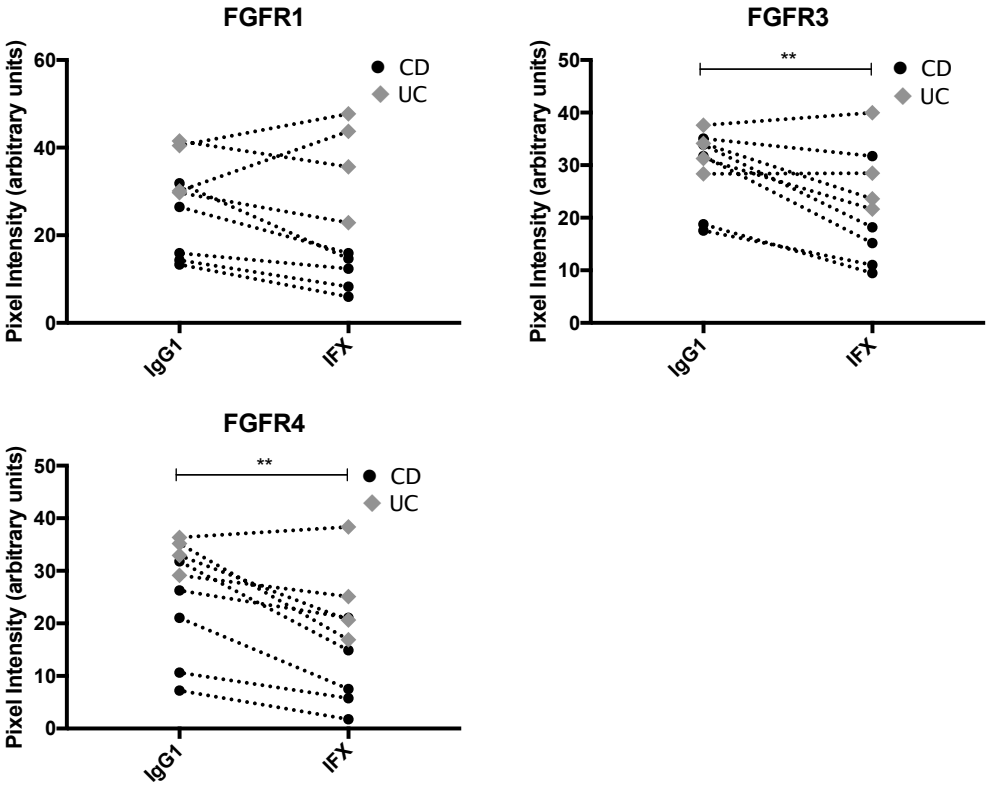


Figure 6-11. Infliximab reduced the phosphoprotein levels of FGFR3 and FGFR4 significantly in inflamed IBD biopsies. Five CD explants and four UC were incubated for 24 hours with infliximab (5µg/ml) or IgG1 (5µg/ml) and the phosphoprotein expression measured by calculating the pixel intensity. These phophoproteins are from the FGFR family. * = p<0.05, ** = p<0.005.

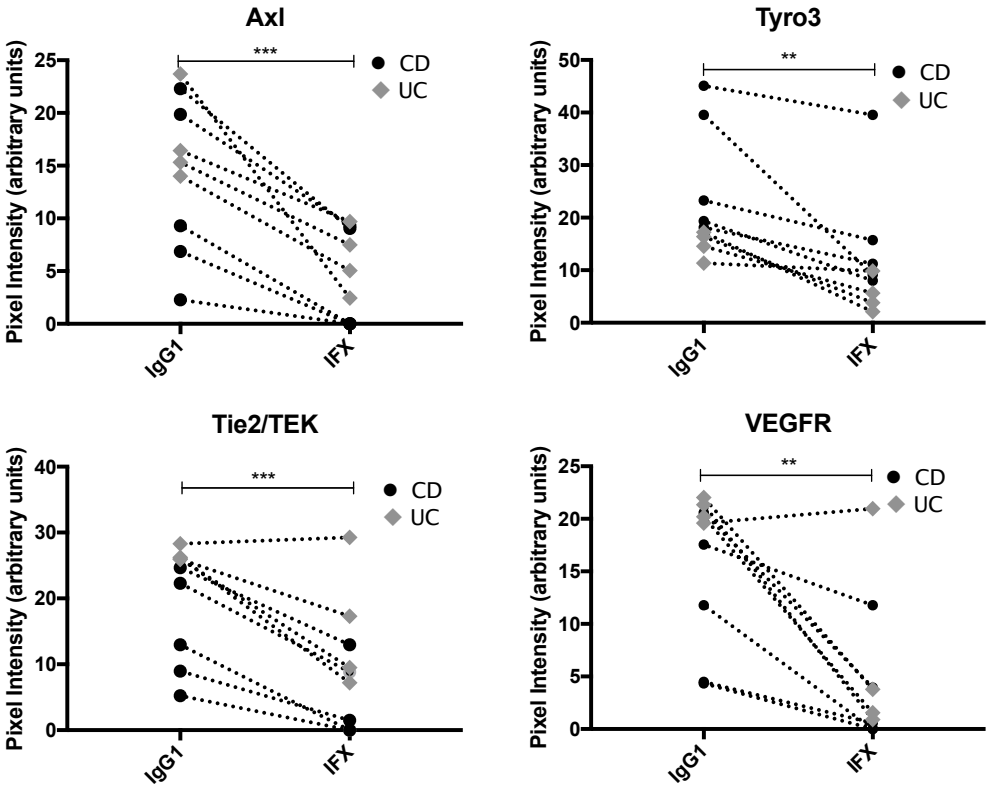


Figure 6-12. Infliximab significantly reduces Axl, Tyro3, Tie2/TEK and VEGFR in inflamed IBD biopsies. Five CD and four UC explants were incubated for 24 hours with Infliximab (5µg/ml) or IgG1 (5µg/ml) and the phosphoprotein expression measured by calculating the pixel intensity. * = p<0.05, ** = p<0.005, *** = p<0.0005.

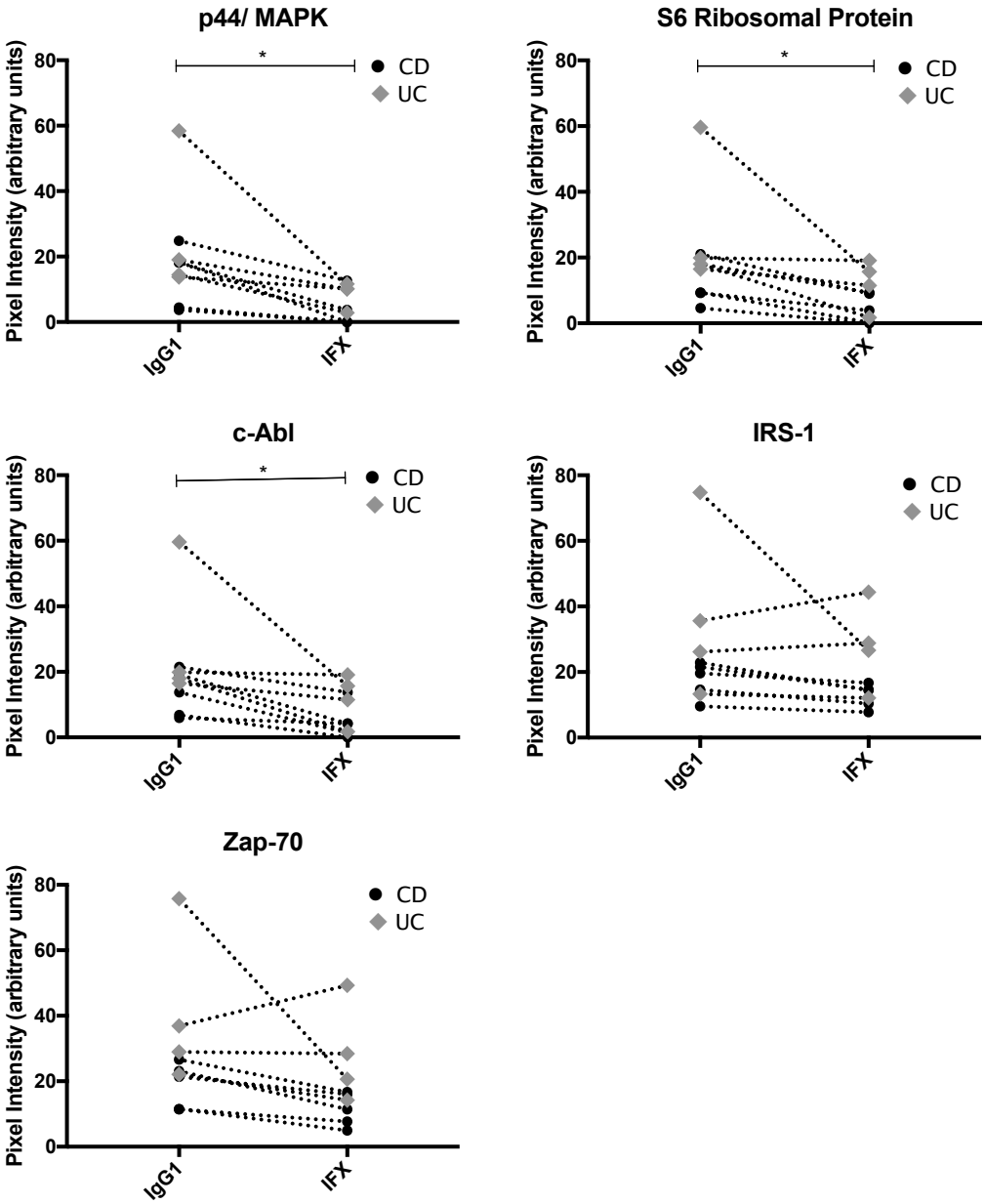


Figure 6-13. Infliximab significantly reduces the phosphoproteins p44/ MAPK, S6 Ribosomal Protein and c-Abl in inflamed IBD biopsies. Five CD and four UC biopsies were incubated for 24 hours with Infliximab (5µg/ml) or IgG1 (5µg/ml) and the phosphoprotein expression measured by calculating the pixel intensity. * = $p<0.05$.

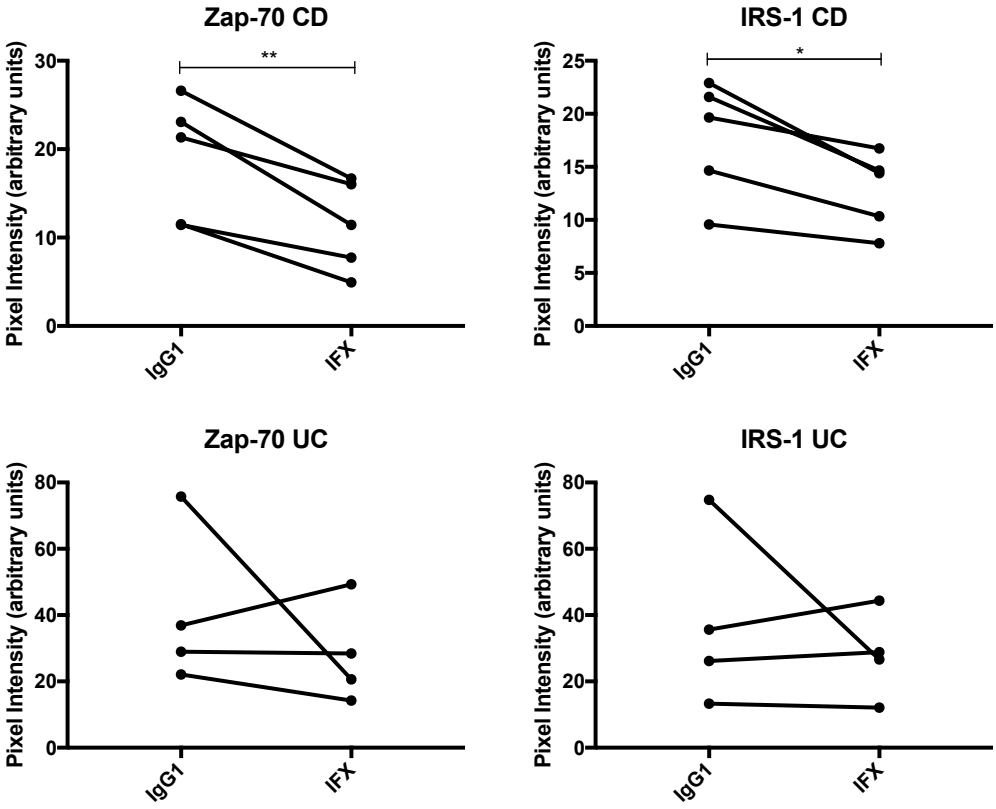


Figure 6-14. Infliximab significantly reduces the phosphoproteins IRS-1 and Zap-70 in inflamed CD biopsies. Five CD explants and four UC explants were incubated for 24 hours with Infliximab (5µg/ml) or IgG1 (5µg/ml) and the phosphoprotein expression measured by calculating the pixel intensity. * = p<0.05, ** = p<0.005.

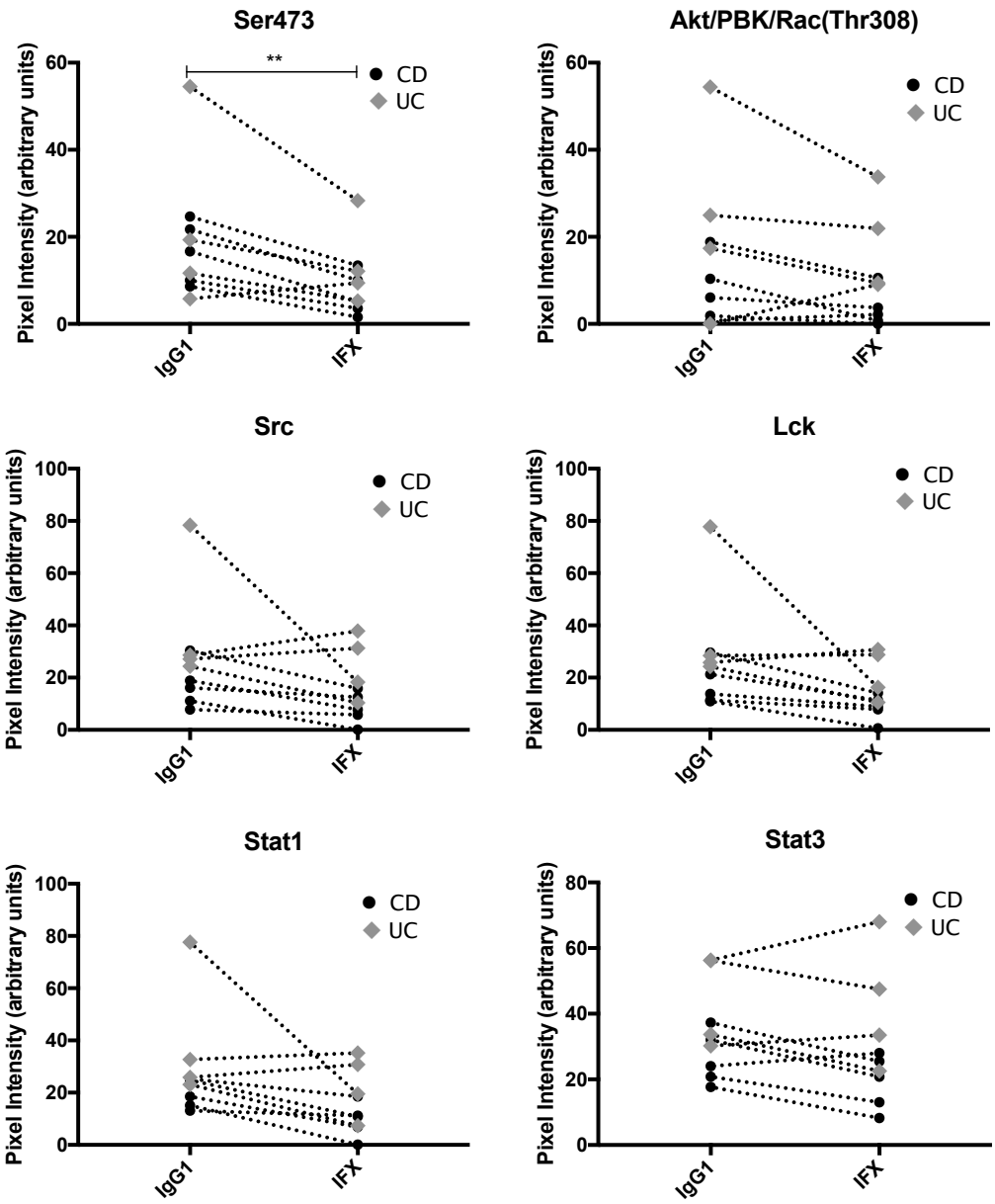


Figure 6-15. Infliximab significantly reduces the phosphoproteins Ser473 in inflamed IBD biopsies. Five CD and four UC biopsies were incubated for 24 hours with Infliximab (5µg/ml) or IgG1 (5µg/ml) and the phosphoprotein expression measured by calculating the pixel intensity. **= p<0.005.

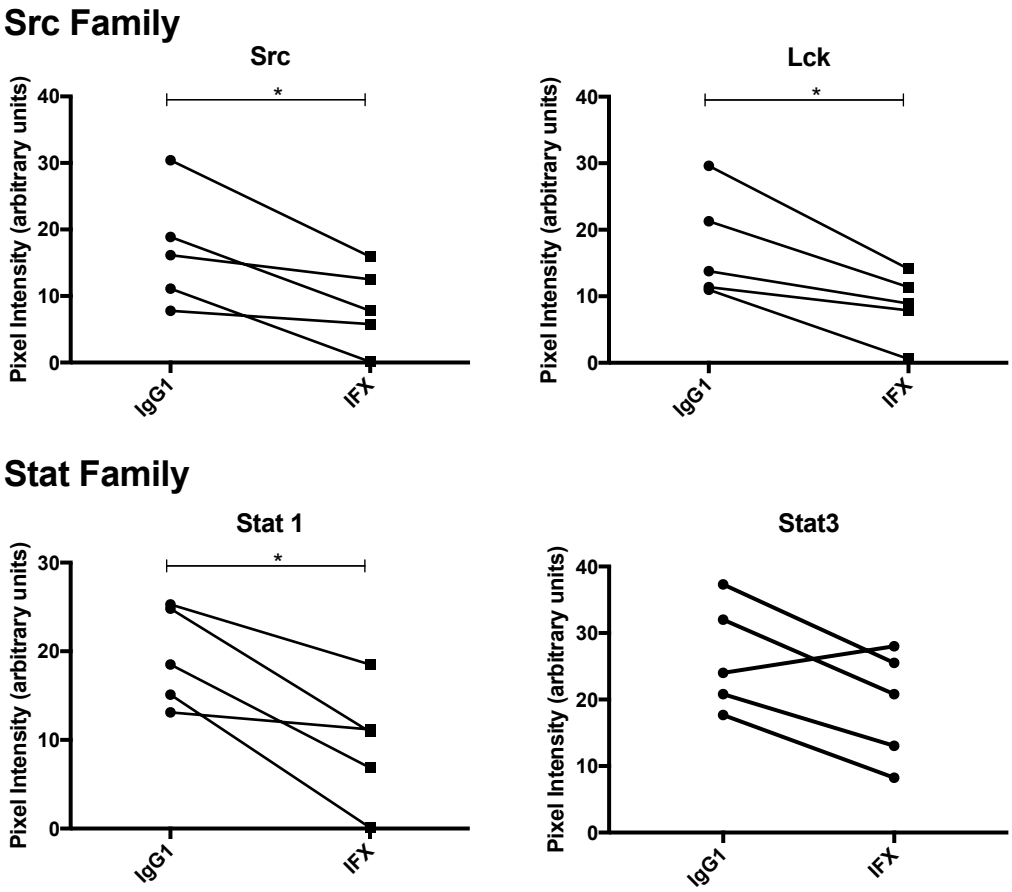


Figure 6-16. Infliximab significantly reduced Src, Lck and Stat 1. Five CD explants were incubated for 24 hours with Infliximab (5μg/ml) or IgG1 (5μg/ml) and the phosphoprotein expression measured by calculating the pixel intensity. * = $p<0.05$.

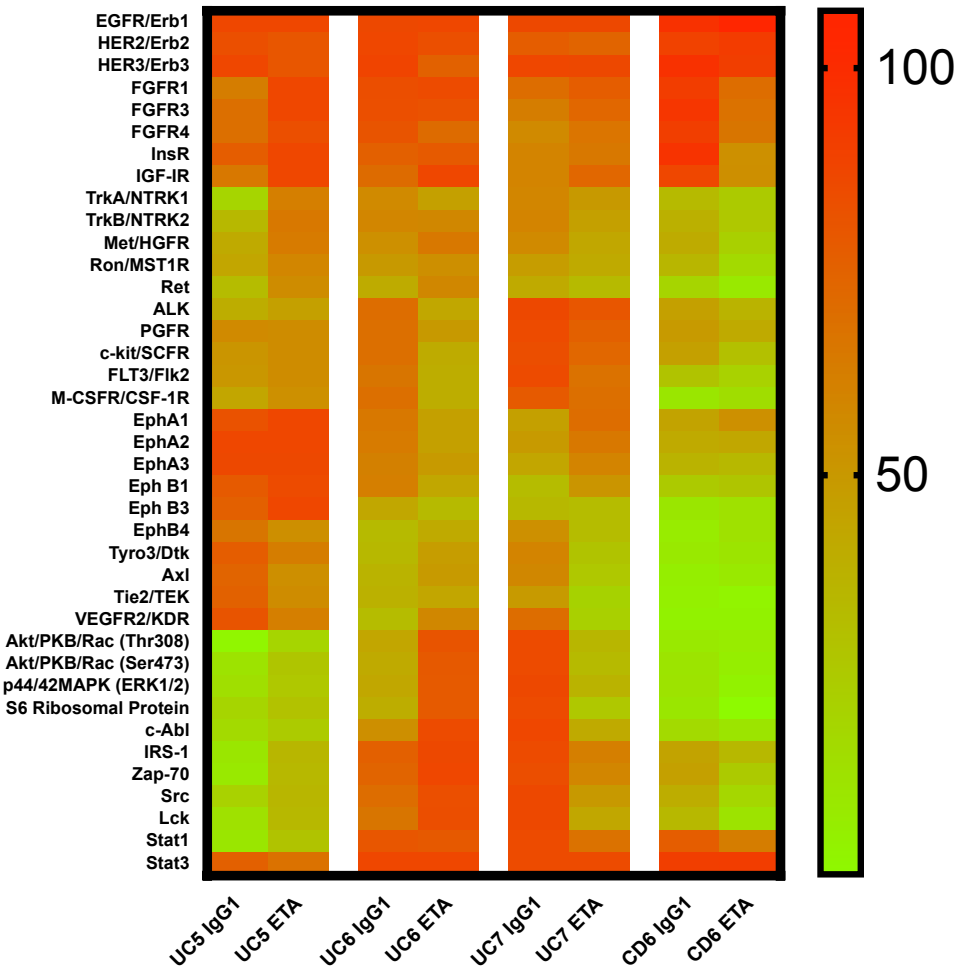


Figure 6-17. Etanercept does not reduce the phosphorylation intensity of IBD biopsies. Etanercept (5µg/ml) or IgG1 (5µg/ml) were added to inflamed IBD biopsies (3 UC and 1CD) for 24 hours. The phosphorylation intensity of RTKs was measured by phospoarray.

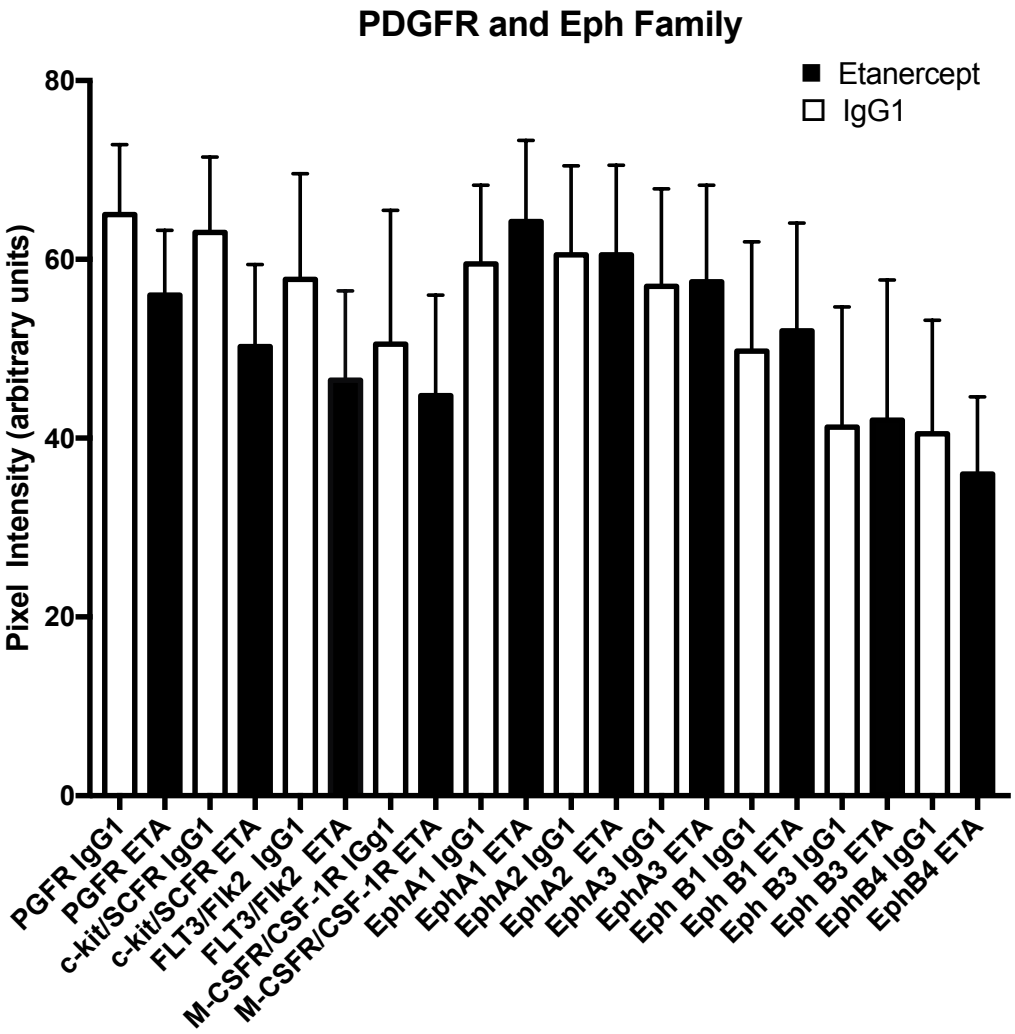


Figure 6-18. Etanercept does not reduce phosphorylation in IBD explants in PDGFR and Eph Family
Mean pixel intensity and standard deviation for receptor tyrosine kinases from explants of 1 CD and 3 UC patients incubated for 24 hours with Etanercept (5µg/ml) or IgG1 (5µg/ml).

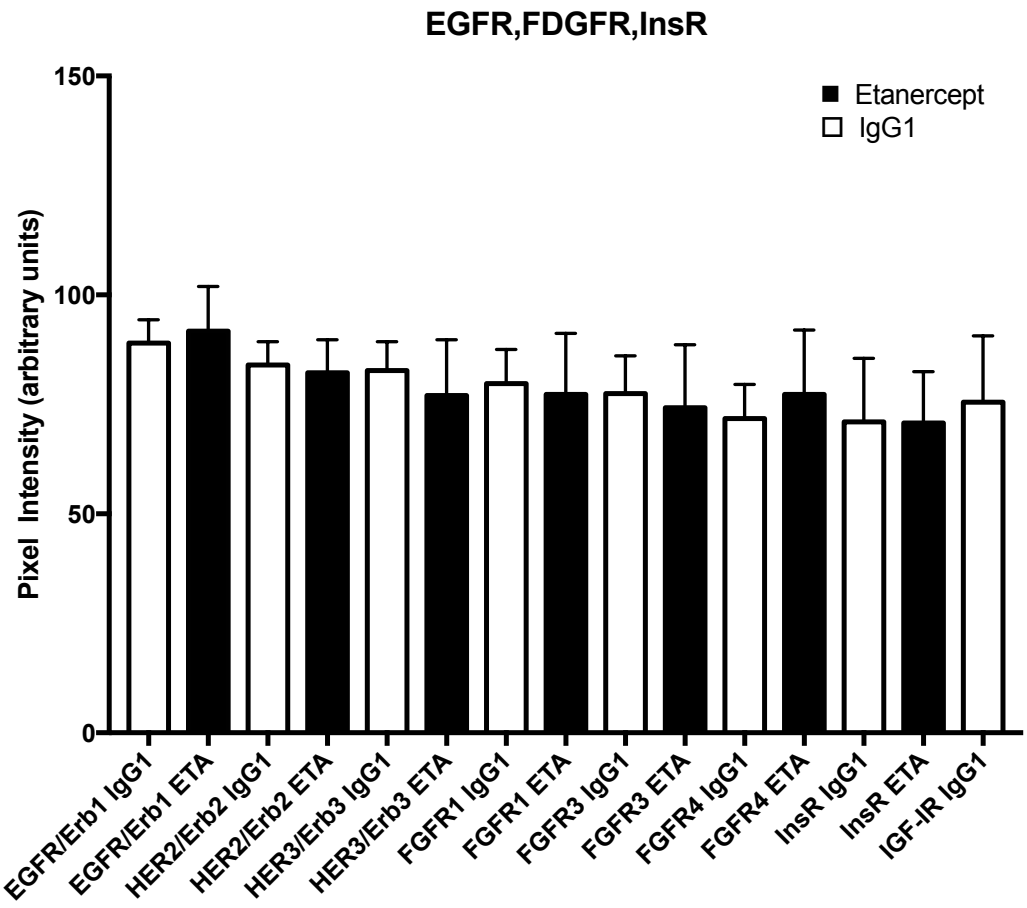


Figure 6-19. Etanercept does not reduce phosphorylation in IBD explants in EGFR and FGFR family
Mean pixel intensity and standard deviation for receptor tyrosine kinases from explants of 1 CD and 3 UC patients incubated for 24 hours with Etanercept (5µg/ml) or IgG1 (5µg/ml).

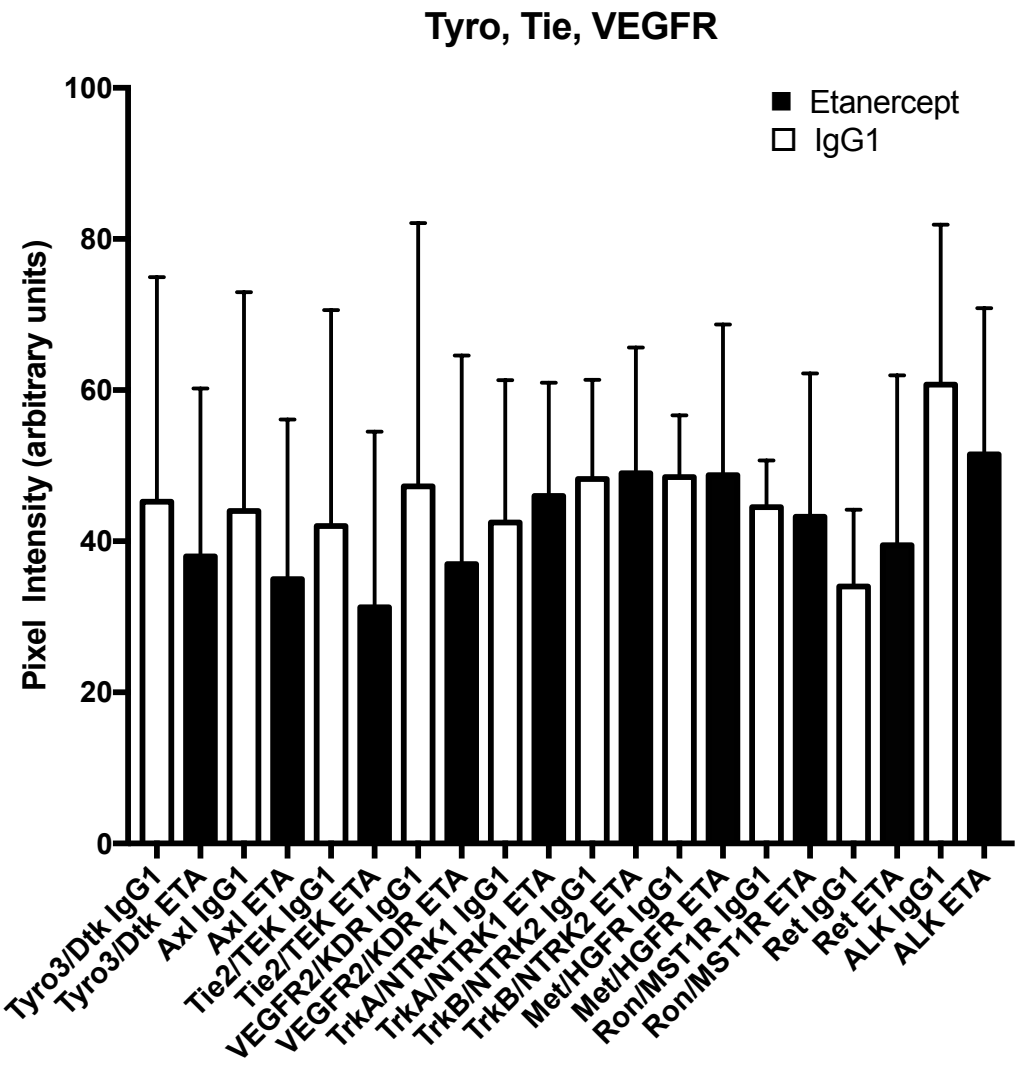


Figure 6-20 Etanercept does not reduce phosphorylation in IBD explants in RTKs. Mean pixel intensity and standard deviation for 18 receptor tyrosine kinases from explants of 1 CD and 3 UC patients incubated for 24 hours with Etanercept (5µg/ml) or IgG1 (5µg/ml).

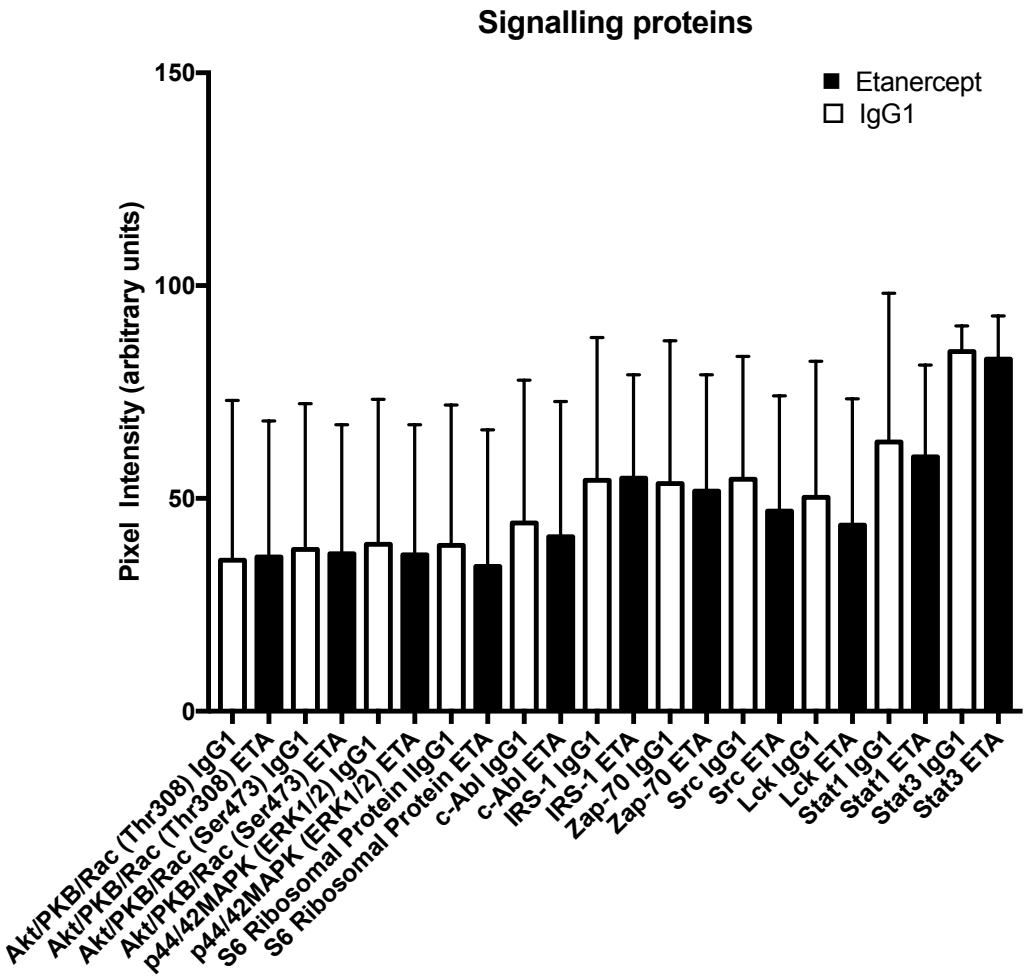


Figure 6-21 Etanercept does not reduce phosphorylation in IBD explants in signalling proteins. Mean pixel intensity and standard deviation for 18 receptor tyrosine kinases from explants of 1 CD and 3 UC patients incubated for 24 hours with Etanercept (5µg/ml) or IgG1 (5µg/ml).

6.7 Discussion

Vossenkämper et al., (2014) showed increased phosphorylation in CD and UC inflamed biopsies compared to controls. UC biopsies also showed a greater relative phosphorylation intensity than CD biopsies (Vossenkämper et al., 2014). This is understandable as there is a great deal of repair and regeneration required in IBD as a result of mucosal inflammation.

The RTKs involved in repair and wound healing are the EGFR, FGFR and PDGFR families. Whilst previous observations have included activity of RTKs in IBD these results show for the first time that infliximab significantly reduces the phorphorylation activity.

MAPK is known to be activated by TNF and increased expression of ERK 1 and 2 (also called p44/MAPK) and p38 MAPK was shown in IBD by Western blot and immunohistochemistry of IBD biopsies (Waetzig et al., 2002). Waetzig et al., (2002) also showed a reduction in ERK 1 and 2 (p44/MAPK) expression following glucocorticoid treatment.

ERK 1 and 2 levels have not been measured in IBD with infliximab, however Faurschou et al., (2008) showed a reduction in ERK 1/2 expression in keratinocytes following UVB damage. A reduction in Akt Thre and Akt Ser-473 was also shown similar to this study.

PDGF is a major growth factor which plays a central role in the repair process. PDGFR was significantly reduced in IBD biopsies (P=0.026) after 24 hours. Kumagai et al., (2001) showed increased expression of PDGFR by in situ

hybridization and immunohistochemistry on surgical IBD resections in active inflammation as well as areas of fibrosis adjacent to the ulcer base. The changes with infliximab have not been shown previously.

CSFR-1 RNA transcripts have been shown to be increased in IBD when measured by real time PCR (Franze et al., 2016). Franze et al., (2016) also used immunohistochemistry to show up-regulation of this receptor in epithelial and lamina propria mononuclear cells. This is consistent with an increased phosphorylation intensity seen by Vossenkaemper et al., (2014). However the effect of infliximab has not been looked into previously.

This study shows high phosphorylation of EGFR in inflamed colon. This was also shown by Hardbower et al., (2017) when they looked specifically at EGFR phosphorylation in macrophages. There was increased phosphorylation compared to inactive colon in IBD.

The role of Eph receptors in the intestinal crypt has been shown recently by Jurek et al., (2016) and previously by Holmberg et al., (2006). Cooperation between Eph A and B recptors is required for mitogenesis in the stem cell niche (Jurek et al., 2016). Hafner et al., (2005) showed increased expression of Eph B2 in CD biopsies. Eph B2 was not included in the phosphoarray used however Eph A2, EphB1 and EphB3 were shown to be raised in IBD and reduced with infliximab. It is not possible to say whether the reduction in Eph receptor phosphorylation is due to an improvement in healing by infliximab and therefore a down regulation of the proliferation of progenitor cells or whether infliximab directly effects Eph phosphorylation. No other studies have looked at the effect of infliximab on Eph receptors.

ZAP-70 was shown to be significantly reduced by infliximab in the CD samples but not in the UC samples. Bouzid et al., (2013) showed a single nucleotide polymorphism rs13420683 was associated with CD but not UC in a Tunisian population supporting the importance of T cell signaling in CD.

It is interesting that the phosphorylation intensity is not reduced with etanercept. The phosphorylation intensity is high showing that the biopsies were taken from inflamed areas. Patients also were anti-TNF naïve. Sandborn et al., (2001) showed that etanercept was ineffective in CD in a pilot study. This was supported more recently with the lack of improvement seen in ankylosing spondylitis- associated intestinal inflammation following treatment with etanercept compared to infliximab or adalimumab (Chitul et al., 2017). Etanercept does not bind to mTNFα with the same avidity as infliximab (Nesbitt et al., 2007), (Shealy et al., 2010), but binding to sTNFα is similar to infliximab (Nesbitt et al., 2007). This study does not indicate how infliximab reduces the phosphorylation of RTKs, but it is possible that this is via mTNFα rather than sTNFα. However another possible explanation for the lack of effect by etanercept is related to the matrix metalloproteinases (MMPs). Biancheri et al., (2015) showed that etanercept is extremely senstitive to breakdown by MMP 3 and 12 which are in excess in the inflamed mucosa in IBD. The findings indicate the possibility that RTKs may be significant signaling pathways of clinical benefit in the treatment of IBD.

Having shown a significant change in RTKs using phosphoarrays, the next step was to use a cytokine array to elucidate changes in a large number of cytokines ex vivo by infliximab.

7 The Effect of Anti-Tumour Necrosis Factor Antibodies on Cytokine Production in IBD biopsies

7.1 Introduction

Crohn's disease has characteristics of a Th1 type immune response with increased concentration of IFNγ and IL-12 (Breese et al., 1994), (Monteleone et al., 1997) and overexpression of Th1 transcription factors such as Stat-1 and T-bet (Neurath et al., 2002). UC however displays Th2 characteristics, increased production of IL-5, although not IL-13 as originally thought (Biancheri et al., 2014). Both CD and UC have increased numbers of Th17 cells which have the potential to secrete IL-17A, IL-17F, IL-21, IL-22 and IL-26 (Rovedatti et al., 2009), (Chen and Sundrud, 2016).

Tumour Necrosis Factor alpha (TNFα) is abundant in both CD and UC (Breese et al., 1994). The most successful anti-cytokine treatment for IBD in the last 20 years has been anti-TNFα antibodies. Infliximab is effective in the treatment of inflammatory and fistulising CD as well as in UC (Targan et al., 1997), (Sands, 2001). However infliximab is not effective in almost half of patients and with others the response wanes over time (Rutgeerts et al., 2009). Understanding the downstream effect on cytokines by neutralising TNFα may help to identify alternative targets.

The mechanisms of action of anti-TNFα antibodies are not fully understood. TNFα is thought to act as a master cytokine released by macrophages, which feeds back to the cell and leads to increased secretion of other pro-inflammatory cytokines. Neutralisation of mTNFα has been shown to result in a reduction in pro-inflammatory cytokines such as IL-6, IL-1β and IL-8 (Butler et al., 1995, (Nesbitt et al., 2007). (Mitoma et al., 2005) showed the induction of IL-10 in a mTNFα expressing Jurkat cell line following treatment with infliximab but not etanercept.

7.2 Aims

To establish whether the addition of infliximab to mucosal biopsies influences the production of other cytokines and chemokines, the following experiments were carried out

1. Inflamed biopsies of CD and UC patients were incubated in the presence of anti-TNF antibodies and the supernatants analysed using a cytokine array panel.
2. The results between infliximab and etanercept were compared.

7.3 Materials and Methods

7.3.1 Organ Culture of Intestinal Mucosal Biopsies

Inflamed colonic biopsies obtained during colonoscopy were cultured (one biopsy per well) in 24 well plates (VWR International, Lutterworth, UK) in 300ul of serum free HL-1 medium (Cambrex BioScience, Wokingham, UK) for 24 at 37°C, in 5% CO_2. At the start of the culture infliximab (Remicade®, Schering-Plough, Welwyn Garden City, UK; 5μg/ml), etanercept (Enbrel®, Wyeth, Maidenhead, UK; 5μg/ml) or IgG1 were added. The supernatant was collected after 24 hrs and stored at -80°C before undergoing cytokine array analysis.

7.3.2 Cytokine Array Analysis

A human cytokine array panel A (R and D Systems, Abingdon, UK) was used to determine changes in 38 cytokines and chemokines simultaneously in the supernatants. Nitrocellulose membranes were precoated with capture antibodies in duplicate. The membrane is then placed in a multi-well dish containing 1ml of blocking buffer for 1 hour at room temperature on a rocking platform. Array buffers containing a buffered protein base provided by the manufacturer are added to 250μl of supernatant to make a total of 1ml and 15μl of detection antibody cocktail added, mixed and incubated for 1 hour at room temperature. The buffer is then aspirated from the wells and the sample/ antibody mixtures added onto the nitrocellulose membranes. The membranes are incubated overnight on a rocking platform at 4°C. The membranes were then removed and placed into plastic containers and rinsed with 20ml wash buffer before being

washed for 10 minutes three times. Strepdavidin HRP (1 in 200) was then added to each well with the membranes and incubated for 30 minutes. The membranes were washed as before and placed on a plastic sheet. One ml chemilumescent mix was added and the membranes exposed on X-ray film and pixel intensity quantified using Image J.

7.3.3 Statistical Analysis

Data was analysed using GraphPad Prism (GraphPad Software, San Diego, CA) software. Student's paired t-test was used to compare two values from the same individual. P values of less than 0.05 were considered statistically significant.

7.4 Results

7.4.1 There is a high expression of pro-inflammatory cytokines in IBD biopsies

Figure 7.1 shows the expression of cytokines without treatment and the heatmap in Figure 7.2 shows the high expression of proinflammatory cytokines in biopsies cultures with IgG1. The cytokines which have the highest expression are G-CSF, CXCL1, sICAM-1, IL-1ra, IL-6, IL-8, IL-16, IL-17, IL-27, MIF, Serpin E and CCL5 (Figures 7.1 and 7.2).

7.4.2 Infliximab reduces C5/5a, CD40 ligand, IL-1ra, IL-2, IL-4, IL-8, IL-16, MIF, CCL5, IL-32a, CXCL10, CXCL11, CXCL12, TNFα and sTREM1.

There was a reduction in a large number of cytokines although the change was not significant. The fold change seen with infliximab was calculated by dividing the mean pixel intensity with IgG1 by the mean pixel intensity with infliximab. Table 7.1 shows six cytokines reduced by 2 to 5 fold namely C5/C5a (2.2), IL-10 (3.5), CCL5 (2.7), CXCL12 (3.2), TNFα (4) and sTREM1 (2.2)(2 CD and 1 UC patient) (Figure 7.3).

7.4.3 Etanercept reduces CD40 ligand, IL-6, IL-8, IL-17, IL-27, CCL3, CCL5 and TNFα.

Etanercept reduced the mean cytokines in the two CD patients tested. Although there was no change in the fold change seen. (Figure 7.4).

7.5 Figures

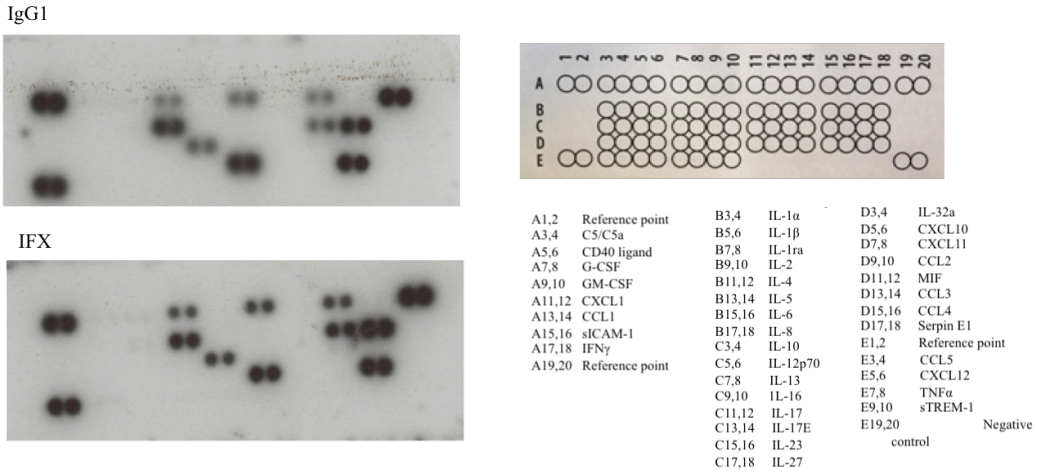


Figure 7-1. Infliximab and etanercept reduced most of the cytokines tested. Etanercept reduced G-CSF, IL-6 and IL-8 which were unaffected by infliximab in this patient. Membranes from cytokine array testing cytokine array from supernatants of CD biopsies incubated for 24 hours with IgG1 (5µg/ml), infliximab (5µg/ml) and etanercept (5µg/ml).

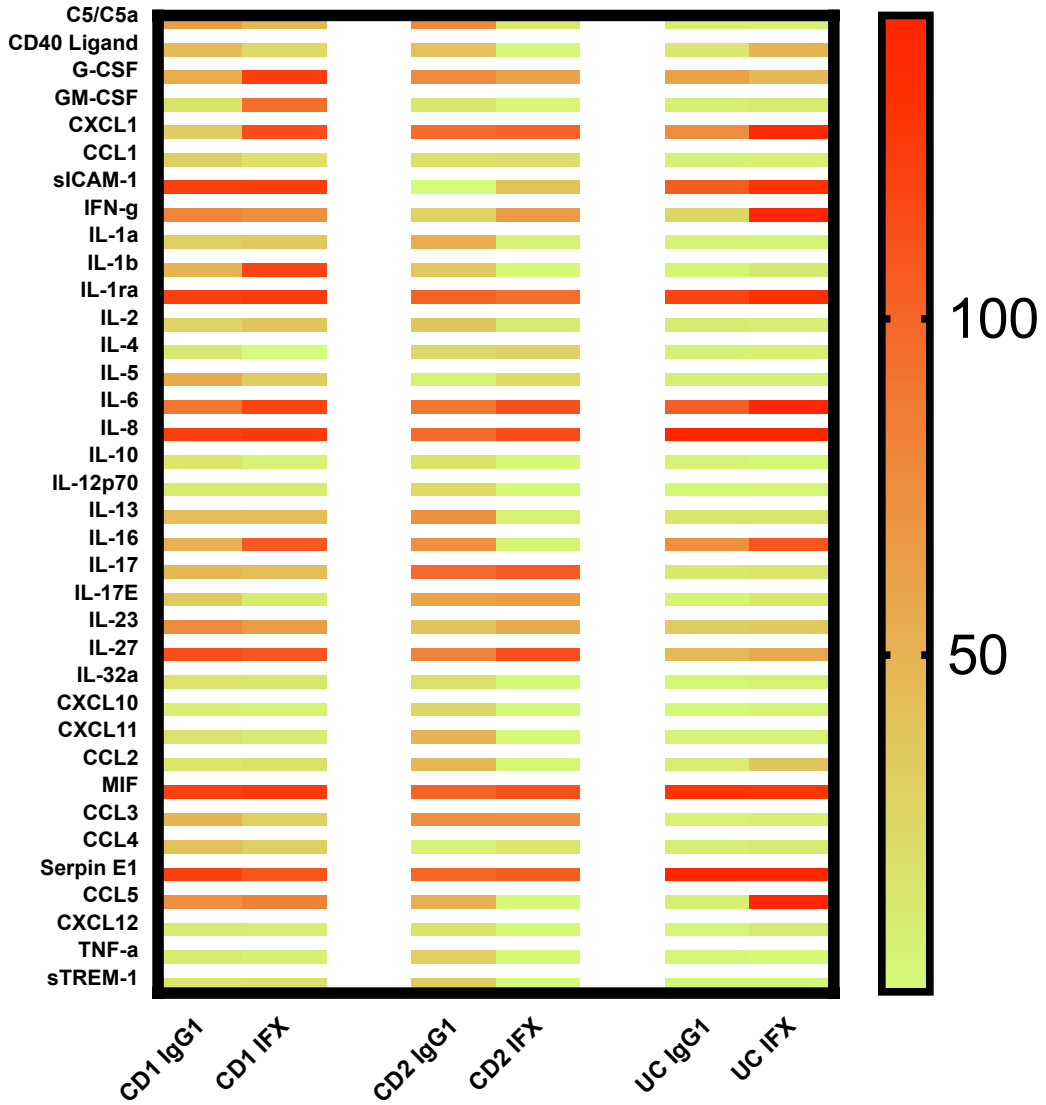


Figure 7-2There is a high expression of pro-inflammatory cytokines in the controls which show a small reduction with infliximab. A heatmap showing inflamed biopsies from 2 CD patients and 1UC patient incubated with IgG1 (5µg/ml) and infliximab (5µg/ml) for 24 hours.

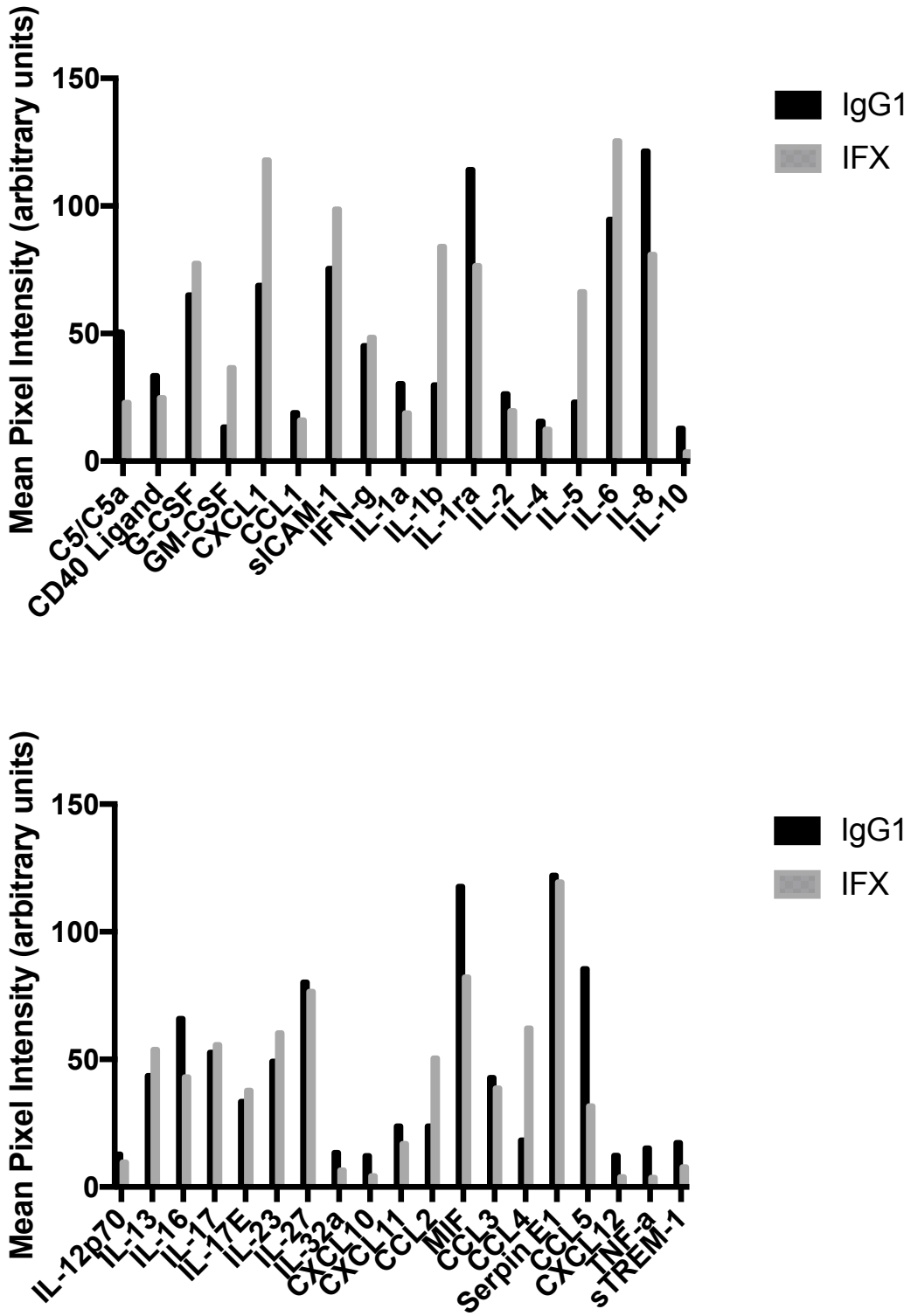


Figure 7-3. Infliximab reduces C5/5a, CD40 ligand, IL-1ra, IL-2, IL-4, IL-8, IL-16, MIF, CCL5, IL-32a, CXCL10, CXCL11, CXCL12 TNFα and sTREM1. Mean change in cytokine concentration in the supernatants of inflamed IBD biopsies (2 CD and 1 UC patient). Cytokine expression was measured by pixel intensity calculation on a cytokine array on the supernatants of biopsies incubated for 24 hours with infliximab (5µg/ml) or IgG1 (5µg/ml).

No change with infliximab			2-5 fold reduction with infliximab
CD40 ligand	IL-6	CXCL10	C5/C5a
G-csf	IL-8	CXCL11	IL-10
GM-CSF	IL12-p70	CCL2	CCL5
CCL1	IL-13	MIF	CXCL12
SICAM-1	IL-16	CCL3	TNFα
IFN g	IL-17	CCL4	sTREM1
IL-1a	IL-17E	Serpin E1	
IL-2	IL-23		
IL-4	IL-27		
IL-5	IL-32a		

Table 7.1. The fold change in cytokine intensity with infliximab and IgG1 in the supernatants of IBD biopsies incubated for 24 hours.

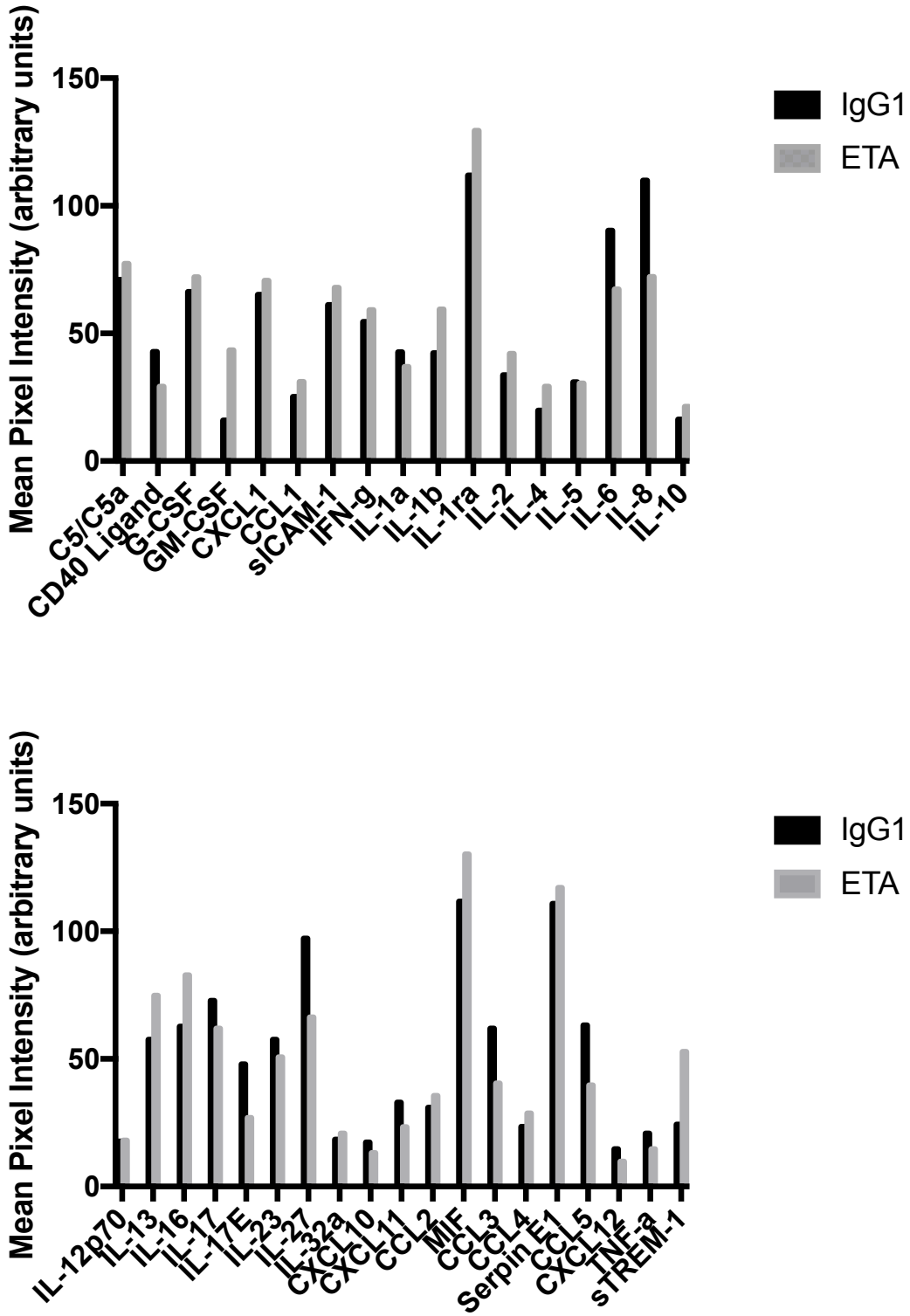


Figure 7-4 Etanercept reduces CD40ligand, IL-6, IL-8, IL-17, IL-27, CCL3, CCL5 and TNFα. Mean chemokine expression in the supernatants of 2 CD patients in which biopsies incubated in etanercept (5µg/ml) or IgG1 (5µg/ml) for 24 hours.

7.6 Discussion

The development, growth, activation and function of the innate and adaptive immune response is controlled largely by cytokines. A fine balance exists to maintain tissue integrity. In IBD, in which the immune system is dysregulated infliximab is effective in achieving mucosal healing (Corica and Romano, 2017). It was hypothesized that infliximab would affect the concentrations of a wide range of cytokines and chemokines. By using a cytokine array, the role of new cytokines might be discovered.

The cytokines CCL5 (RANTES), MIF, IL-8 and IL 1ra reduced with infliximab in IBD biopsies. Increased concentrations of CCL5, MIF, IL-8 and IL1ra have previously been associated with IBD (McCormack et al., 2001), (Ansari et al., 2006), (Singh et al., 2016), (Neurath, 2014), (Daryani et al., 2015). This is the first study to show a reduction in the CCL5 and MIF with infliximab in IBD. Etanercept did not reduce MIF but there was a reduction in CCL5. The reduction seen with infliximab is not significant. One possibility for this is the numbers are very small (2 CD and 1 UC patient). The other possibility is that the time frame was too short. Vossenkämper et al., (2014) used a similar cytokine array to test a CD3 antibody otelixumab. Biopsies were incubated for 48 hours before the supernatants tested for cytokines. In the previous chapter, a significant reduction was seen in a wide range of RTKs which are involved in the regulation of cytokines and therefore it is reasonable to expect a change in cytokines. Incubating the IBD biopsies for 48 hours and increasing the number of samples would therefore be an important next step.

8 General Discussion

Tumour Necrosis Factor alpha is central to the immunopathogenesis of inflammatory bowel disease. It is initially expressed on the cell surface as a trimer and cleaved from the cell surface by TNFα Converting Enzyme (TACE) to release the soluble trimeric form of the cytokine. The aim of this study was to establish whether pharmacological and endogenous TACE inhibitors increase membrane TNFα with reciprocal reduction in soluble TNFα and identify any changes in other pro-inflammatory cytokines. Atreya et al., (2014) have shown an association between mTNFα expression on macrophages in the gut and the clinical response to adalimumab, another anti-TNF antibody used to treat IBD. In animal models, neutralisation of mTNFα is also important in improving colitis (Perrier et al., 2013).

The TACE inhibitors tested were a broad spectrum MMP inhibitor called Marimastat which has been reported to inhibit TACE, (Tsuji et al., 2002), the endogenous inhibitor TIMP-3 (Monteleone et al., 2012), and a specific inhibitor, GW280264X, which was a gift from Prof Stefan Rose-John. All TACE inhibitors showed a reduction in sTNFα by activated PBMCs and by explants of IBD mucosa cultured ex vivo. An increase in mTNFα was also seen in PBMCs activated with anti CD3/28 antibody in the presence of GW280264X. With the exception of Monteleone et al., (2012), who showed an increase in mTNFα expression on LPMCs from patients with IBD cultured with TIMP-3, most of the

studies with TACE inhibitors in IBD focus on sTNFα and not mTNFα. This study therefore adds to the work by Monteleone et al., (2012) in showing the effect of TACE inhibitors on mTNFα specifically. There was no change in the production of other pro-inflammatory cytokines by inflamed biopsies ex vivo, namely IL-1β, IL-6 and IL-8. This study does not indicate if TACE inhibitors have a pro- or anti-inflammatory effect or any other downstream effect as a result of increasing mTNFα expression. It also suggests that reducing TNF-alpha production has no effects on other cytokines. Although some studies have shown a reduction in pro-inflammatory cytokines with TACE inhibitors (Monteleone et al., 2012), (Sharma et al., 2014), Fréour et al., (2009) showed an increase in gut epithelial permeability when cultured with TIMP-3. A TACE knockout patient suffers from recurrent mild gut inflammation but the etiology of this inflammation is not known (Blaydon et al., 2011). A shortcoming of this study is that the number of samples was quite small and in cases where trends did not reach significance, increasing the number of samples studied would possibly show significant results.

Infliximab, an anti-TNFα antibody, has revolutionised the treatment of IBD (Kuek et al., 2007). However up to half of patients fail to respond and up to a third of patients lose response over time. The mechanisms of action are also incompletely understood. There is therefore a need to understand the mechanisms by which infliximab induces remission to help develop further treatments for IBD. Two of the suggested mechanisms of action were tested in this study, namely neutralisation of TNFα and subsequent reduction in other pro-inflammatory cytokines, and apoptosis.

The concentration of IL-1β, IL-8 or IL-6 did not change in inflamed IBD biopsies incubated with infliximab ex-vivo. Taken together with the fact that TACE inhibitors also did not reduce the production of other cytokines, it appears that in the gut, the paradigm originally thought to explain the mode of action of infliximab does not apply. Specifically it was suggested that TNFα was a master cytokine which when made by macrophages fed back and increased the production of other pro-inflammatory cytokines (Ogata and Hibi, 2003). The implication of these results is that it supports the use of other anti-cytokine therapies in patients who become infliximab unresponsive, such as anti-IL-6 or anti-IL-6R.

The second aim was to establish the contentious issue of apoptosis induction when mTNFα binds infliximab. This study did not show apoptosis either in a TNFα transfected cell line which expressed high levels of mTNFα or using activated PBMCs. Some studies have shown that infliximab induces apoptosis on Jurkat T cells and T cells from IBD mucosa (Lügering et al., 2001), (Van den Brande et al., 2003), (Di Sabatino et al., 2012). Other studies have failed to replicate these observations (Agnholt and Kaltoft, 2001), (Biancheri-unpublished observations). Apoptosis however is only one mechanism that has been suggested to explain the therapeutic benefits of infliximab. Others include the induction of regulator macrophages (Levin et al., 2016) and breakdown of the antibody in inflamed gut by matrix metalloproteinases (Biancheri et al., 2015). In addition etanercept does not cause apoptosis (Nesbitt et al., 2007) and is not clinically effective in CD, and likewise the anti-TNF antibody certolizumab is effective in treating CD but also does not cause apoptosis (Nesbitt et al., 2007). Some caution however must be given to the fact that etanercept is not effective

in patients because (Biancheri et al., 2015) showed that etanercept is exquisitely sensitive to proteolytic degradation and loss of function. The capacity to induce apoptosis is therefore insufficient to explain the therapeutic benefit of infliximab.

The cytoplasmic tail of the transmembrane TNFα protein can trigger intracellular signals such as MAPkinase increasing TGFβ production (Pallai et al., 2016). MAPkinases are a member of receptor tyrosine kinases, which are important in controlling inflammation and cell regeneration. (Waetzig et al., 2002) showed increased expression of ERK 1 and 2 and Docena et al., (2010) showed increased activated p38 MAPK in the inflamed mucosa of patients with IBD. Vossenkämper et al. (2014) showed increased phosphorylation in a large number of RTKs including EGFR, FGFR and PDGFR in CD and UC inflamed biopsies compared to controls. There is a great deal of repair and regeneration required in IBD and infliximab has been shown to achieve mucosal healing (Corica and Romano, 2017) .

The next aim was to elucidate the effect of infliximab on receptor tyrosine kinase phosphorylation in biopsies from IBD patients cultured ex vivo. The RTKs involved in repair and wound healing are the EGFR, FGFR and PDGFR families. Whilst previous observations have included activity of RTKs in IBD these results show for the first time that infliximab significantly reduces their phorphorylation activity. ERK 1 and 2 levels have not been measured in IBD after infliximab therapy, however Faurschou et al., (2008) showed a reduction in ERK 1 and 2 expression following infliximab treatment in keratinocytes

following UVB damage. A reduction in Akt Thr308 and Akt Ser-473 was also seen here.

It is not possible to say whether the reduction in phosphorylation is due to an improvement in healing by infliximab and therefore a down-regulation of the proliferation of progenitor cells or whether infliximab directly effects RTK phosphorylation. Related to this question is also that is is not clear the role for mTNFα in the down-regulation of RTK phosphorylation.

The TCR signalling proteins, ZAP-70 and Lck, are also reduced significantly in CD biopsies. This observation is of interest because Lck phosphorylates CD3 zeta allowing the recruitment of Zap70, which is then again phosphorylated by Lck to initiate T cell signalling. This suggests that infliximab is having a direct effect on T cell signalling, a key event in the pathogenesis of CD.

The significance of the effect of infliximab on RTK phosphorylation is accentuated by the lack of effect of etanercept which was not effective in CD (Levin et al., 2016).

Again however one must be cautious in over-emphasising these results because Biancheri et al., (2015) showed that etanercept loses its ability to neutralise TNFα when cleaved by MMPs. Repeating these experiments with effective anti-TNF antibodies such as certolizumab would be helpful in understanding the significance of the down-regulation of RTK phosphorylation.

The final chapter aimed to identify any previously unidentified cytokines related to the clinical efficacy of infliximab. The cytokines CCL5 (RANTES), MIF, IL-

8 and IL-1ra were reduced by infliximab. The reduction is not significant although it is acknowledged that the number of samples was very small. Increased concentrations of CCL5 (Zhou et al., 2009), MIF (Tamaki et al., 2006), IL-8 and IL-1ra have previously been associated with IBD (Marlow et al., 2013). This is the first study to show a reduction in CCL5 and MIF with infliximab in IBD.

9 Future Work

The reduction in phosphorylation of RTKs by infliximab is the main positive outcome of this work. It would be interesting to carry out the same experiments for 48 or 72 hours to see if the reduction in RTK phosphorylation is sustained or reduced further. Whilst MAPkinase inhibitors have been shown to be effective in reducing inflammation in IBD (Docena et al., 2010), (Coskun et al., 2011) it would be helpful to test the therapeutic benefit of some of the other RTK inhibitors such as sunitinib (Shin et al., 2010) already in use for colonic cancer in IBD.

Further experiments with TCR ZAP70 and Lck would also be interesting in CD patients. Quantifying changes in intracellular expression of ZAP70 and Lck in IBD patients with infliximab in LPMCs with flow cytometry or western blot could support the findings in reduced phosphorylation seen with infliximab in this study.

One of the questions, which remain unanswered, is whether TACE inhibitors are pro- or anti-inflammatory. The phosphoarray could be used to measure downstream signalling in IBD biopsies which have been incubated with the specific TACE inhibitor GW280264X.

10 References

ABREU, M. T. 2010. Toll-like receptor signalling in the intestinal epithelium: how bacterial recognition shapes intestinal function. *Nat Rev Immunol,* 10**,** 131-44.

ADRAIN, C., ZETTL, M., CHRISTOVA, Y., TAYLOR, N. & FREEMAN, M. 2012. Tumor necrosis factor signaling requires iRhom2 to promote trafficking and activation of TACE. *Science,* 335**,** 225-8.

ALLOCCA, M., BONIFACIO, C., FIORINO, G., SPINELLI, A., FURFARO, F., BALZARINI, L., BONOVAS, S. & DANESE, S. 2017. Efficacy of tumour necrosis factor antagonists in stricturing Crohn's disease: A tertiary center real-life experience. *Dig Liver Dis*.

AMOUR, A., KNIGHT, C. G., WEBSTER, A., SLOCOMBE, P. M., STEPHENS, P. E., KNÄUPER, V., DOCHERTY, A. J. & MURPHY, G. 2000. The in vitro activity of ADAM-10 is inhibited by TIMP-1 and TIMP-3. *FEBS Lett,* 473**,** 275-9.

ANSARI, N., ABDULLA, J., ZAYYANI, N., BRAHMI, U., TAHA, S. & SATIR, A. A. 2006. Comparison of RANTES expression in Crohn's disease and ulcerative colitis: an aid in the differential diagnosis? *J Clin Pathol,* 59**,** 1066-72.

ARMUZZI, A., DE PASCALIS, B., LUPASCU, A., FEDELI, P., LEO, D., MENTELLA, M. C., VINCENTI, F., MELINA, D., GASBARRINI, G., POLA, P. & GASBARRINI, A. 2004. Infliximab in the treatment of steroid-dependent ulcerative colitis. *Eur Rev Med Pharmacol Sci,* 8**,** 231-3.

ARORA, T., PADAKI, R., LIU, L., HAMBURGER, A. E., ELLISON, A. R., STEVENS, S. R., LOUIE, J. S. & KOHNO, T. 2009. Differences in binding and effector functions between classes of TNF antagonists. *Cytokine,* 45**,** 124-31.

ATARASHI, K., TANOUE, T., ANDO, M., KAMADA, N., NAGANO, Y., NARUSHIMA, S., SUDA, W., IMAOKA, A., SETOYAMA, H., NAGAMORI, T., ISHIKAWA, E., SHIMA, T., HARA, T., KADO, S., JINNOHARA, T., OHNO, H., KONDO, T., TOYOOKA, K., WATANABE, E., YOKOYAMA, S., TOKORO, S., MORI, H., NOGUCHI, Y., MORITA, H., IVANOV, I. I., SUGIYAMA, T., NUÑEZ, G., CAMP, J. G., HATTORI, M., UMESAKI, Y. & HONDA, K. 2015. Th17 Cell Induction by Adhesion of Microbes to Intestinal Epithelial Cells. *Cell,* 163**,** 367-80.

ATARASHI, K., TANOUE, T., SHIMA, T., IMAOKA, A., KUWAHARA, T., MOMOSE, Y., CHENG, G., YAMASAKI, S., SAITO, T., OHBA, Y., TANIGUCHI, T., TAKEDA, K., HORI, S., IVANOV, I. I., UMESAKI,

Y., ITOH, K. & HONDA, K. 2011. Induction of colonic regulatory T cells by indigenous Clostridium species. *Science,* 331**,** 337-41.

ATREYA, R., NEUMANN, H., NEUFERT, C., WALDNER, M. J., BILLMEIER, U., ZOPF, Y., WILLMA, M., APP, C., MÜNSTER, T., KESSLER, H., MAAS, S., GEBHARDT, B., HEIMKE-BRINCK, R., REUTER, E., DÖRJE, F., RAU, T. T., UTER, W., WANG, T. D., KIESSLICH, R., VIETH, M., HANNAPPEL, E. & NEURATH, M. F. 2014. In vivo imaging using fluorescent antibodies to tumor necrosis factor predicts therapeutic response in Crohn's disease. *Nat Med,* 20**,** 313-8.

ATREYA, R. & NEURATH, M. F. 2016. Predicting Therapeutic Response by in vivo Molecular Imaging in Inflammatory Bowel Diseases. *Dig Dis,* 34**,** 552-7.

ATREYA, R., ZIMMER, M., BARTSCH, B., WALDNER, M. J., ATREYA, I., NEUMANN, H., HILDNER, K., HOFFMAN, A., KIESSLICH, R., RINK, A. D., RAU, T. T., ROSE-JOHN, S., KESSLER, H., SCHMIDT, J. & NEURATH, M. F. 2011. Antibodies against tumor necrosis factor (TNF) induce T-cell apoptosis in patients with inflammatory bowel diseases via TNF receptor 2 and intestinal $CD14^{+}$ macrophages. *Gastroenterology,* 141**,** 2026-38.

AVISSAR, N. E., WANG, H. T., MILLER, J. H., IANNOLI, P. & SAX, H. C. 2000. Epidermal growth factor receptor is increased in rabbit intestinal brush border membrane after small bowel resection. *Dig Dis Sci,* 45**,** 1145-52.

BAIN, C. C., SCOTT, C. L., URONEN-HANSSON, H., GUDJONSSON, S., JANSSON, O., GRIP, O., GUILLIAMS, M., MALISSEN, B., AGACE, W. W. & MOWAT, A. M. 2013. Resident and pro-inflammatory macrophages in the colon represent alternative context-dependent fates of the same Ly6Chi monocyte precursors. *Mucosal Immunol,* 6**,** 498-510.

BAKDASH, G., VOGELPOEL, L. T., VAN CAPEL, T. M., KAPSENBERG, M. L. & DE JONG, E. C. 2015. Retinoic acid primes human dendritic cells to induce gut-homing, IL-10-producing regulatory T cells. *Mucosal Immunol,* 8**,** 265-78.

BARNICH, N., CARVALHO, F. A., GLASSER, A. L., DARCHA, C., JANTSCHEFF, P., ALLEZ, M., PEETERS, H., BOMMELAER, G., DESREUMAUX, P., COLOMBEL, J. F. & DARFEUILLE-MICHAUD, A. 2007. CEACAM6 acts as a receptor for adherent-invasive E. coli, supporting ileal mucosa colonization in Crohn disease. *J Clin Invest,* 117**,** 1566-74.

BARON, S., TURCK, D., LEPLAT, C., MERLE, V., GOWER-ROUSSEAU, C., MARTI, R., YZET, T., LEREBOURS, E., DUPAS, J. L., DEBEUGNY, S., SALOMEZ, J. L., CORTOT, A. & COLOMBEL, J. F. 2005. Environmental risk factors in paediatric inflammatory bowel diseases: a population based case control study. *Gut,* 54**,** 357-63.

BATLLE, E., HENDERSON, J. T., BEGHTEL, H., VAN DEN BORN, M. M., SANCHO, E., HULS, G., MEELDIJK, J., ROBERTSON, J., VAN DE

WETERING, M., PAWSON, T. & CLEVERS, H. 2002. Beta-catenin and TCF mediate cell positioning in the intestinal epithelium by controlling the expression of EphB/ephrinB. *Cell,* 111**,** 251-63.

BAUM, B. & GEORGIOU, M. 2011. Dynamics of adherens junctions in epithelial establishment, maintenance, and remodeling. *J Cell Biol,* 192**,** 907-17.

BAUMGART, M., DOGAN, B., RISHNIW, M., WEITZMAN, G., BOSWORTH, B., YANTISS, R., ORSI, R. H., WIEDMANN, M., MCDONOUGH, P., KIM, S. G., BERG, D., SCHUKKEN, Y., SCHERL, E. & SIMPSON, K. W. 2007. Culture independent analysis of ileal mucosa reveals a selective increase in invasive Escherichia coli of novel phylogeny relative to depletion of Clostridiales in Crohn's disease involving the ileum. *ISME J,* 1**,** 403-18.

BEENKEN, A. & MOHAMMADI, M. 2009. The FGF family: biology, pathophysiology and therapy. *Nat Rev Drug Discov,* 8**,** 235-53.

BEKKER, P., EBSWORTH, K., WALTERS, M. J., BERAHOVICH, R. D., ERTL, L. S., CHARVAT, T. T., PUNNA, S., POWERS, J. P., CAMPBELL, J. J., SULLIVAN, T. J., JAEN, J. C. & SCHALL, T. J. 2015. CCR9 Antagonists in the Treatment of Ulcerative Colitis. *Mediators Inflamm,* 2015**,** 628340.

BENNETT, C. L., CHRISTIE, J., RAMSDELL, F., BRUNKOW, M. E., FERGUSON, P. J., WHITESELL, L., KELLY, T. E., SAULSBURY, F. T., CHANCE, P. F. & OCHS, H. D. 2001. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nat Genet,* 27**,** 20-1.

BERNSTEIN, C. N., RAWSTHORNE, P., CHEANG, M. & BLANCHARD, J. F. 2006a. A population-based case control study of potential risk factors for IBD. *Am J Gastroenterol,* 101**,** 993-1002.

BERNSTEIN, C. N., WAJDA, A., SVENSON, L. W., MACKENZIE, A., KOEHOORN, M., JACKSON, M., FEDORAK, R., ISRAEL, D. & BLANCHARD, J. F. 2006b. The epidemiology of inflammatory bowel disease in Canada: a population-based study. *Am J Gastroenterol,* 101**,** 1559-68.

BETTELLI, E., CARRIER, Y., GAO, W., KORN, T., STROM, T. B., OUKKA, M., WEINER, H. L. & KUCHROO, V. K. 2006. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature,* 441**,** 235-8.

BHAT, M. I. & KAPILA, R. 2017. Dietary metabolites derived from gut microbiota: critical modulators of epigenetic changes in mammals. *Nutr Rev,* 75**,** 374-389.

BIANCHERI, P., BREZSKI, R. J., DI SABATINO, A., GREENPLATE, A. R., SORING, K. L., CORAZZA, G. R., KOK, K. B., ROVEDATTI, L., VOSSENKÄMPER, A., AHMAD, N., SNOEK, S. A., VERMEIRE, S., RUTGEERTS, P., JORDAN, R. E. & MACDONALD, T. T. 2015. Proteolytic cleavage and loss of function of biologic agents that

neutralize tumor necrosis factor in the mucosa of patients with inflammatory bowel disease. *Gastroenterology,* 149**,** 1564-1574.e3.

BIANCHERI, P., DI SABATINO, A., AMMOSCATO, F., FACCIOTTI, F., CAPRIOLI, F., CURCIARELLO, R., HOQUE, S. S., GHANBARI, A., JOE-NJOKU, I., GIUFFRIDA, P., ROVEDATTI, L., GEGINAT, J., CORAZZA, G. R. & MACDONALD, T. T. 2014. Absence of a role for interleukin-13 in inflammatory bowel disease. *Eur J Immunol,* 44**,** 370-85.

BIANCHERI, P., PENDER, S. L., AMMOSCATO, F., GIUFFRIDA, P., SAMPIETRO, G., ARDIZZONE, S., GHANBARI, A., CURCIARELLO, R., PASINI, A., MONTELEONE, G., CORAZZA, G. R., MACDONALD, T. T. & DI SABATINO, A. 2013. The role of interleukin 17 in Crohn's disease-associated intestinal fibrosis. *Fibrogenesis Tissue Repair,* 6**,** 13.

BLACK, R. A., RAUCH, C. T., KOZLOSKY, C. J., PESCHON, J. J., SLACK, J. L., WOLFSON, M. F., CASTNER, B. J., STOCKING, K. L., REDDY, P., SRINIVASAN, S., NELSON, N., BOIANI, N., SCHOOLEY, K. A., GERHART, M., DAVIS, R., FITZNER, J. N., JOHNSON, R. S., PAXTON, R. J., MARCH, C. J. & CERRETTI, D. P. 1997. A metalloproteinase disintegrin that releases tumour-necrosis factor-alpha from cells. *Nature,* 385**,** 729-33.

BLAYDON, D. C., BIANCHERI, P., DI, W. L., PLAGNOL, V., CABRAL, R. M., BROOKE, M. A., VAN HEEL, D. A., RUSCHENDORF, F., TOYNBEE, M., WALNE, A., O'TOOLE, E. A., MARTIN, J. E., LINDLEY, K., VULLIAMY, T., ABRAMS, D. J., MACDONALD, T. T., HARPER, J. I. & KELSELL, D. P. 2011. Inflammatory skin and bowel disease linked to ADAM17 deletion. *N Engl J Med,* 365**,** 1502-8.

BREESE, E. J., MICHIE, C. A., NICHOLLS, S. W., MURCH, S. H., WILLIAMS, C. B., DOMIZIO, P., WALKER-SMITH, J. A. & MACDONALD, T. T. 1994. Tumor necrosis factor alpha-producing cells in the intestinal mucosa of children with inflammatory bowel disease. *Gastroenterology,* 106**,** 1455-66.

BROOKS, A. N., KILGOUR, E. & SMITH, P. D. 2012. Molecular pathways: fibroblast growth factor signaling: a new therapeutic opportunity in cancer. *Clin Cancer Res,* 18**,** 1855-62.

BUTLER, D. M., MAINI, R. N., FELDMANN, M. & BRENNAN, F. M. 1995. Modulation of proinflammatory cytokine release in rheumatoid synovial membrane cell cultures. Comparison of monoclonal anti TNF-alpha antibody with the interleukin-1 receptor antagonist. *Eur Cytokine Netw,* 6**,** 225-30.

CALKINS, B. M. 1989. A meta-analysis of the role of smoking in inflammatory bowel disease. *Dig Dis Sci,* 34**,** 1841-54.

CARON, G., DELNESTE, Y., AUBRY, J. P., MAGISTRELLI, G., HERBAULT, N., BLAECKE, A., MEAGER, A., BONNEFOY, J. Y. & JEANNIN, P. 1999. Human NK cells constitutively express membrane

TNF-alpha (mTNFalpha) and present mTNFalpha-dependent cytotoxic activity. *Eur J Immunol,* 29**,** 3588-95.

CESARO, A., ABAKAR-MAHAMAT, A., BREST, P., LASSALLE, S., SELVA, E., FILIPPI, J., HÉBUTERNE, X., HUGOT, J. P., DOGLIO, A., GALLAND, F., NAQUET, P., VOURET-CRAVIARI, V., MOGRABI, B. & HOFMAN, P. M. 2009. Differential expression and regulation of ADAM17 and TIMP3 in acute inflamed intestinal epithelia. *Am J Physiol Gastrointest Liver Physiol,* 296**,** G1332-43.

CHALARIS, A., ADAM, N., SINA, C., ROSENSTIEL, P., LEHMANN-KOCH, J., SCHIRMACHER, P., HARTMANN, D., CICHY, J., GAVRILOVA, O., SCHREIBER, S., JOSTOCK, T., MATTHEWS, V., HÄSLER, R., BECKER, C., NEURATH, M. F., REISS, K., SAFTIG, P., SCHELLER, J. & ROSE-JOHN, S. 2010. Critical role of the disintegrin metalloprotease ADAM17 for intestinal inflammation and regeneration in mice. *J Exp Med,* 207**,** 1617-24.

CHALARIS, A., GARBERS, C., RABE, B., ROSE-JOHN, S. & SCHELLER, J. 2011. The soluble Interleukin 6 receptor: generation and role in inflammation and cancer. *Eur J Cell Biol,* 90**,** 484-94.

CHEN, M. L. & SUNDRUD, M. S. 2016. Cytokine Networks and T-Cell Subsets in Inflammatory Bowel Diseases. *Inflamm Bowel Dis,* 22**,** 1157-67.

CHEN, Z., GIBSON, T. B., ROBINSON, F., SILVESTRO, L., PEARSON, G., XU, B., WRIGHT, A., VANDERBILT, C. & COBB, M. H. 2001. MAP kinases. *Chem Rev,* 101**,** 2449-76.

CIARDIELLO, F. & TORTORA, G. 2003. Epidermal growth factor receptor (EGFR) as a target in cancer therapy: understanding the role of receptor expression and other molecular determinants that could influence the response to anti-EGFR drugs. *Eur J Cancer,* 39**,** 1348-54.

CORICA, D. & ROMANO, C. 2017. Biological Therapy in Pediatric Inflammatory Bowel Disease: A Systematic Review. *J Clin Gastroenterol,* 51**,** 100-110.

COSKUN, M., OLSEN, J., SEIDELIN, J. B. & NIELSEN, O. H. 2011. MAP kinases in inflammatory bowel disease. *Clin Chim Acta,* 412**,** 513-20.

DARYANI, N. E., SADR, M., MOOSSAVI, S., SHAHKARAMI, S., SOLTANI, S., FARHADI, E. & REZAEI, N. 2015. Significance of IL-1RA Polymorphism in Iranian Patients with Inflammatory Bowel Disease. *Dig Dis Sci,* 60**,** 1389-95.

DE SIMONE, V., FRANZÈ, E., RONCHETTI, G., COLANTONI, A., FANTINI, M. C., DI FUSCO, D., SICA, G. S., SILERI, P., MACDONALD, T. T., PALLONE, F., MONTELEONE, G. & STOLFI, C. 2015. Th17-type cytokines, IL-6 and TNF-α synergistically activate STAT3 and NF-kB to promote colorectal cancer cell growth. *Oncogene,* 34**,** 3493-503.

DEROCHE, T. C., XIAO, S. Y. & LIU, X. 2014. Histological evaluation in ulcerative colitis. *Gastroenterol Rep (Oxf),* 2**,** 178-92.

DEY, M., BALDYS, A., SUMTER, D. B., GÖOZ, P., LUTTRELL, L. M., RAYMOND, J. R. & GÖOZ, M. 2010. Bradykinin decreases podocyte permeability through ADAM17-dependent epidermal growth factor receptor activation and zonula occludens-1 rearrangement. *J Pharmacol Exp Ther,* 334**,** 775-83.

DI SABATINO, A., CICCOCIOPPO, R., CINQUE, B., MILLIMAGGI, D., MORERA, R., RICEVUTI, L., CIFONE, M. G. & CORAZZA, G. R. 2004. Defective mucosal T cell death is sustainably reverted by infliximab in a caspase dependent pathway in Crohn's disease. *Gut,* 53**,** 70-7.

DI SABATINO, A., PENDER, S. L., JACKSON, C. L., PROTHERO, J. D., GORDON, J. N., PICARIELLO, L., ROVEDATTI, L., DOCENA, G., MONTELEONE, G., RAMPTON, D. S., TONELLI, F., CORAZZA, G. R. & MACDONALD, T. T. 2007. Functional modulation of Crohn's disease myofibroblasts by anti-tumor necrosis factor antibodies. *Gastroenterology,* 133**,** 137-49.

DIEGELMANN, J., OLSZAK, T., GÖKE, B., BLUMBERG, R. S. & BRAND, S. 2012. A novel role for interleukin-27 (IL-27) as mediator of intestinal epithelial barrier protection mediated via differential signal transducer and activator of transcription (STAT) protein signaling and induction of antibacterial and anti-inflammatory proteins. *J Biol Chem,* 287**,** 286-98.

DOCENA, G., ROVEDATTI, L., KRUIDENIER, L., FANNING, A., LEAKEY, N. A., KNOWLES, C. H., LEE, K., SHANAHAN, F., NALLY, K., MCLEAN, P. G., DI SABATINO, A. & MACDONALD, T. T. 2010. Down-regulation of p38 mitogen-activated protein kinase activation and proinflammatory cytokine production by mitogen-activated protein kinase inhibitors in inflammatory bowel disease. *Clin Exp Immunol,* 162**,** 108-15.

DRESCHER, U. 2002. Eph family functions from an evolutionary perspective. *Curr Opin Genet Dev,* 12**,** 397-402.

DUBINSKY, M. C., LIN, Y. C., DUTRIDGE, D., PICORNELL, Y., LANDERS, C. J., FARRIOR, S., WROBEL, I., QUIROS, A., VASILIAUSKAS, E. A., GRILL, B., ISRAEL, D., BAHAR, R., CHRISTIE, D., WAHBEH, G., SILBER, G., DALLAZADEH, S., SHAH, P., THOMAS, D., KELTS, D., HERSHBERG, R. M., ELSON, C. O., TARGAN, S. R., TAYLOR, K. D., ROTTER, J. I., YANG, H. & ALLIANCE, W. R. P. I. R. 2006. Serum immune responses predict rapid disease progression among children with Crohn's disease: immune responses predict disease progression. *Am J Gastroenterol,* 101**,** 360-7.

ECKBURG, P. B., BIK, E. M., BERNSTEIN, C. N., PURDOM, E., DETHLEFSEN, L., SARGENT, M., GILL, S. R., NELSON, K. E. & RELMAN, D. A. 2005. Diversity of the human intestinal microbial flora. *Science,* 308**,** 1635-8.

EDER, P., LYKOWSKA-SZUBER, L., KRELA-KAZMIERCZAK, I., STAWCZYK-EDER, K., ZABEL, M. & LINKE, K. 2013. The influence of infliximab and adalimumab on the expression of apoptosis-related

proteins in lamina propria mononuclear cells and enterocytes in Crohn's disease - an immunohistochemical study. *J Crohns Colitis,* 7**,** 706-16.

EISSNER, G., KIRCHNER, S., LINDNER, H., KOLCH, W., JANOSCH, P., GRELL, M., SCHEURICH, P., ANDREESEN, R. & HOLLER, E. 2000. Reverse signaling through transmembrane TNF confers resistance to lipopolysaccharide in human monocytes and macrophages. *J Immunol,* 164**,** 6193-8.

ELDER, M. E., LIN, D., CLEVER, J., CHAN, A. C., HOPE, T. J., WEISS, A. & PARSLOW, T. G. 1994. Human severe combined immunodeficiency due to a defect in ZAP-70, a T cell tyrosine kinase. *Science,* 264**,** 1596-9.

ELIA, P. P., TOLENTINO, Y. F., BERNARDAZZI, C. & DE SOUZA, H. S. 2015. The role of innate immunity receptors in the pathogenesis of inflammatory bowel disease. *Mediators Inflamm,* 2015**,** 936193.

FANTINI, M. C., RIZZO, A., FINA, D., CARUSO, R., SARRA, M., STOLFI, C., BECKER, C., MACDONALD, T. T., PALLONE, F., NEURATH, M. F. & MONTELEONE, G. 2009. Smad7 controls resistance of colitogenic T cells to regulatory T cell-mediated suppression. *Gastroenterology,* 136**,** 1308-16, e1-3.

FAURSCHOU, A., GNIADECKI, R & WULF, H.C. 2008. Infliximab inhibits DNA repair in ultraviolet B-irradiated premalignant keratinocytes. predicts utility in other diseases. *Experimental Dermatology, 17(11), pp933-8*.

FEAKINS, R. M. 2014. Ulcerative colitis or Crohn's disease? Pitfalls and problems. *Histopathology,* 64**,** 317-35.

FELDMAN, M., TAYLOR, P., PALEOLOG, E., BRENNAN, F. M. & MAINI, R. N. 1998. Anti-TNF alpha therapy is useful in rheumatoid arthritis and Crohn's disease: analysis of the mechanism of action predicts utility in other diseases. *Transplant Proc,* 30**,** 4126-7.

FENG, Y., NI, L. & WANG, Q. 2013. Administration of cathepsin B inhibitor CA-074Me reduces inflammation and apoptosis in polymyositis. *J Dermatol Sci,* 72**,** 158-67.

FLANAGAN, J. G. & VANDERHAEGHEN, P. 1998. The ephrins and Eph receptors in neural development. *Annu Rev Neurosci,* 21**,** 309-45.

FLANAGAN, P. K., CAMPBELL, B. J. & RHODES, J. M. 2012. Lessons from diversion studies and antibacterial interventions. *Dig Dis,* 30**,** 347-50.

FRÉOUR, T., JARRY, A., BACH-NGOHOU, K., DEJOIE, T., BOU-HANNA, C., DENIS, M. G., MOSNIER, J. F., LABOISSE, C. L. & MASSON, D. 2009. TACE inhibition amplifies TNF-alpha-mediated colonic epithelial barrier disruption. *Int J Mol Med,* 23**,** 41-8.

FU, Y. T., HONG, T., ROUND, A. & BRESSLER, B. 2014. Impact of medical therapy on patients with Crohn's disease requiring surgical resection. *World J Gastroenterol,* 20**,** 11808-14.

FURUSE, M., FUJITA, K., HIIRAGI, T., FUJIMOTO, K. & TSUKITA, S. 1998. Claudin-1 and -2: novel integral membrane proteins localizing at

tight junctions with no sequence similarity to occludin. *J Cell Biol,* 141**,** 1539-50.

FURUSE, M., HIRASE, T., ITOH, M., NAGAFUCHI, A., YONEMURA, S. & TSUKITA, S. 1993. Occludin: a novel integral membrane protein localizing at tight junctions. *J Cell Biol,* 123**,** 1777-88.

GALE, N. W. & YANCOPOULOS, G. D. 1999. Growth factors acting via endothelial cell-specific receptor tyrosine kinases: VEGFs, angiopoietins, and ephrins in vascular development. *Genes Dev,* 13**,** 1055-66.

GARCIA-HERNANDEZ, V., QUIROS, M. & NUSRAT, A. 2017. Intestinal epithelial claudins: expression and regulation in homeostasis and inflammation. *Ann N Y Acad Sci*.

GERBE, F., SIDOT, E., SMYTH, D. J., OHMOTO, M., MATSUMOTO, I., DARDALHON, V., CESSES, P., GARNIER, L., POUZOLLES, M., BRULIN, B., BRUSCHI, M., HARCUS, Y., ZIMMERMANN, V. S., TAYLOR, N., MAIZELS, R. M. & JAY, P. 2016. Intestinal epithelial tuft cells initiate type 2 mucosal immunity to helminth parasites. *Nature,* 529**,** 226-30.

GOMOLLÓN, F., DIGNASS, A., ANNESE, V., TILG, H., VAN ASSCHE, G., LINDSAY, J. O., PEYRIN-BIROULET, L., CULLEN, G. J., DAPERNO, M., KUCHARZIK, T., RIEDER, F., ALMER, S., ARMUZZI, A., HARBORD, M., LANGHORST, J., SANS, M., CHOWERS, Y., FIORINO, G., JUILLERAT, P., MANTZARIS, G. J., RIZZELLO, F., VAVRICKA, S., GIONCHETTI, P. & ECCO 2017. 3rd European Evidence-based Consensus on the Diagnosis and Management of Crohn's Disease 2016: Part 1: Diagnosis and Medical Management. *J Crohns Colitis,* 11**,** 3-25.

GRIBBLE, F. M. & REIMANN, F. 2016. Enteroendocrine Cells: Chemosensors in the Intestinal Epithelium. *Annu Rev Physiol,* 78**,** 277-99.

HAFNER, C., MEYER, S., LANGMANN, T., SCHMITZ, G., BATAILLE, F., HAGEN, I., BECKER, B., ROESCH, A., ROGLER, G., LANDTHALER, M. & VOGT, T. 2005. Ephrin-B2 is differentially expressed in the intestinal epithelium in Crohn's disease and contributes to accelerated epithelial wound healing in vitro. *World J Gastroenterol,* 11**,** 4024-31.

HALFVARSON, J. 2011. Genetics in twins with Crohn's disease: less pronounced than previously believed? *Inflamm Bowel Dis,* 17**,** 6-12.

HAMPE, J., FRANKE, A., ROSENSTIEL, P., TILL, A., TEUBER, M., HUSE, K., ALBRECHT, M., MAYR, G., DE LA VEGA, F. M., BRIGGS, J., GÜNTHER, S., PRESCOTT, N. J., ONNIE, C. M., HÄSLER, R., SIPOS, B., FÖLSCH, U. R., LENGAUER, T., PLATZER, M., MATHEW, C. G., KRAWCZAK, M. & SCHREIBER, S. 2007. A genome-wide association scan of nonsynonymous SNPs identifies a susceptibility variant for Crohn disease in ATG16L1. *Nat Genet,* 39**,** 207-11.

HANAUER, S. B. 2002. New steroids for IBD: progress report. *Gut,* 51**,** 182-3.

HARDBOWER, D.M., COBURN, L.A., ASIM, M., SINGH, K., SIERRA, J.C., BARRY, D.P., GOBERT, A.P., PIAZUELO, M.B., WASHINGTON, M.K., WILSON, K.T. 2017. Infliximab EGFR-mediated macrophage activation promotes colitis-associated tumorigenesis. *Oncogene, 36(27):3807-19.*

HOLMBERG, J., GENANDER, M., HALFORD, M. M., ANNERÉN, C., SONDELL, M., CHUMLEY, M. J., SILVANY, R. E., HENKEMEYER, M. & FRISÉN, J. 2006. EphB receptors coordinate migration and proliferation in the intestinal stem cell niche. *Cell,* 125**,** 1151-63.

HOMMES, D., VAN DEN BLINK, B., PLASSE, T., BARTELSMAN, J., XU, C., MACPHERSON, B., TYTGAT, G., PEPPELENBOSCH, M. & VAN DEVENTER, S. 2002. Inhibition of stress-activated MAP kinases induces clinical improvement in moderate to severe Crohn's disease. *Gastroenterology,* 122**,** 7-14.

HOOPER, L. V., WONG, M. H., THELIN, A., HANSSON, L., FALK, P. G. & GORDON, J. I. 2001. Molecular analysis of commensal host-microbial relationships in the intestine. *Science,* 291**,** 881-4.

HORIUCHI, T., MITOMA, H., HARASHIMA, S., TSUKAMOTO, H. & SHIMODA, T. 2010. Transmembrane TNF-alpha: structure, function and interaction with anti-TNF agents. *Rheumatology (Oxford),* 49**,** 1215-28.

HUGOT, J. P., CHAMAILLARD, M., ZOUALI, H., LESAGE, S., CÉZARD, J. P., BELAICHE, J., ALMER, S., TYSK, C., O'MORAIN, C. A., GASSULL, M., BINDER, V., FINKEL, Y., CORTOT, A., MODIGLIANI, R., LAURENT-PUIG, P., GOWER-ROUSSEAU, C., MACRY, J., COLOMBEL, J. F., SAHBATOU, M. & THOMAS, G. 2001. Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. *Nature,* 411**,** 599-603.

IKENOUCHI, J., FURUSE, M., FURUSE, K., SASAKI, H. & TSUKITA, S. 2005. Tricellulin constitutes a novel barrier at tricellular contacts of epithelial cells. *J Cell Biol,* 171**,** 939-45.

ILIEV, I. D., SPADONI, I., MILETI, E., MATTEOLI, G., SONZOGNI, A., SAMPIETRO, G. M., FOSCHI, D., CAPRIOLI, F., VIALE, G. & RESCIGNO, M. 2009. Human intestinal epithelial cells promote the differentiation of tolerogenic dendritic cells. *Gut,* 58**,** 1481-9.

IVANOV, I. I., ATARASHI, K., MANEL, N., BRODIE, E. L., SHIMA, T., KARAOZ, U., WEI, D., GOLDFARB, K. C., SANTEE, C. A., LYNCH, S. V., TANOUE, T., IMAOKA, A., ITOH, K., TAKEDA, K., UMESAKI, Y., HONDA, K. & LITTMAN, D. R. 2009. Induction of intestinal Th17 cells by segmented filamentous bacteria. *Cell,* 139**,** 485-98.

IVANOV, I. I., FRUTOS, R. E. L., MANEL, N., YOSHINAGA, K., RIFKIN, D. B., SARTOR, R. B., FINLAY, B. B. & LITTMAN, D. R. 2008. Specific microbiota direct the differentiation of IL-17-producing T-helper cells in the mucosa of the small intestine. *Cell Host Microbe,* 4**,** 337-49.

IWATA, M., HIRAKIYAMA, A., ESHIMA, Y., KAGECHIKA, H., KATO, C. & SONG, S. Y. 2004. Retinoic acid imprints gut-homing specificity on T cells. *Immunity,* 21**,** 527-38.

JAUREGUI-AMEZAGA, A., RIMOLA, J., ORDÁS, I., RODRÍGUEZ, S., RAMÍREZ-MORROS, A., GALLEGO, M., MASAMUNT, M. C., LLACH, J., GONZÁLEZ-SUÁREZ, B., RICART, E. & PANÉS, J. 2015. Value of endoscopy and MRI for predicting intestinal surgery in patients with Crohn's disease in the era of biologics. *Gut,* 64**,** 1397-402.

JOOSSENS, M., HUYS, G., CNOCKAERT, M., DE PRETER, V., VERBEKE, K., RUTGEERTS, P., VANDAMME, P. & VERMEIRE, S. 2011. Dysbiosis of the faecal microbiota in patients with Crohn's disease and their unaffected relatives. *Gut,* 60**,** 631-7.

JOSTINS, L., RIPKE, S., WEERSMA, R. K., DUERR, R. H., MCGOVERN, D. P., HUI, K. Y., LEE, J. C., SCHUMM, L. P., SHARMA, Y., ANDERSON, C. A., ESSERS, J., MITROVIC, M., NING, K., CLEYNEN, I., THEATRE, E., SPAIN, S. L., RAYCHAUDHURI, S., GOYETTE, P., WEI, Z., ABRAHAM, C., ACHKAR, J. P., AHMAD, T., AMININEJAD, L., ANANTHAKRISHNAN, A. N., ANDERSEN, V., ANDREWS, J. M., BAIDOO, L., BALSCHUN, T., BAMPTON, P. A., BITTON, A., BOUCHER, G., BRAND, S., BÜNING, C., COHAIN, A., CICHON, S., D'AMATO, M., DE JONG, D., DEVANEY, K. L., DUBINSKY, M., EDWARDS, C., ELLINGHAUS, D., FERGUSON, L. R., FRANCHIMONT, D., FRANSEN, K., GEARRY, R., GEORGES, M., GIEGER, C., GLAS, J., HARITUNIANS, T., HART, A., HAWKEY, C., HEDL, M., HU, X., KARLSEN, T. H., KUPCINSKAS, L., KUGATHASAN, S., LATIANO, A., LAUKENS, D., LAWRANCE, I. C., LEES, C. W., LOUIS, E., MAHY, G., MANSFIELD, J., MORGAN, A. R., MOWAT, C., NEWMAN, W., PALMIERI, O., PONSIOEN, C. Y., POTOCNIK, U., PRESCOTT, N. J., REGUEIRO, M., ROTTER, J. I., RUSSELL, R. K., SANDERSON, J. D., SANS, M., SATSANGI, J., SCHREIBER, S., SIMMS, L. A., SVENTORAITYTE, J., TARGAN, S. R., TAYLOR, K. D., TREMELLING, M., VERSPAGET, H. W., DE VOS, M., WIJMENGA, C., WILSON, D. C., WINKELMANN, J., XAVIER, R. J., ZEISSIG, S., ZHANG, B., ZHANG, C. K., ZHAO, H., SILVERBERG, M. S., ANNESE, V., HAKONARSON, H., BRANT, S. R., RADFORD-SMITH, G., MATHEW, C. G., RIOUX, J. D., SCHADT, E. E., et al. 2012. Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease. *Nature,* 491**,** 119-24.

KANAYA, T., HASE, K., TAKAHASHI, D., FUKUDA, S., HOSHINO, K., SASAKI, I., HEMMI, H., KNOOP, K. A., KUMAR, N., SATO, M., KATSUNO, T., YOKOSUKA, O., TOYOOKA, K., NAKAI, K., SAKAMOTO, A., KITAHARA, Y., JINNOHARA, T., MCSORLEY, S. J., KAISHO, T., WILLIAMS, I. R. & OHNO, H. 2012. The Ets transcription factor Spi-B is essential for the differentiation of intestinal microfold cells. *Nat Immunol,* 13**,** 729-36.

KAU AL, AHERN PP, GRIFFIN NW, GOODMAN AL & JI., G. 2011. Human nutrition, the gut microbiome and the immune system. Nature.

KAUR, M., PANIKKATH, D., YAN, X., LIU, Z., BEREL, D., LI, D., VASILIAUSKAS, E. A., IPPOLITI, A., DUBINSKY, M., SHIH, D. Q., MELMED, G. Y., HARITUNIANS, T., FLESHNER, P., TARGAN, S. R. & MCGOVERN, D. P. 2016. Perianal Crohn's Disease is Associated with Distal Colonic Disease, Stricturing Disease Behavior, IBD-Associated Serologies and Genetic Variation in the JAK-STAT Pathway. *Inflamm Bowel Dis,* 22**,** 862-9.

KAYMAKCALAN, Z., SAKORAFAS, P., BOSE, S., SCESNEY, S., XIONG, L., HANZATIAN, D. K., SALFELD, J. & SASSO, E. H. 2009. Comparisons of affinities, avidities, and complement activation of adalimumab, infliximab, and etanercept in binding to soluble and membrane tumor necrosis factor. *Clin Immunol,* 131**,** 308-16.

KIELA, P. R. & GHISHAN, F. K. 2009. Ion transport in the intestine. *Curr Opin Gastroenterol,* 25**,** 87-91.

KUMAGAI, S., OHTANI, H., NAGAI, T., FUNA, K., HIWATASHI, N. O., SHIMOSEGAWA & NAGURA, H. 2001. Platelet-derived growth factor and its receptors are expressed in areas of both active inflammation and active fibrosis in inflammatory bowel disease. *Tohoku J Exp Med,* 195**,** 21-33.

LE GALL, S. M., BOBÉ, P., REISS, K., HORIUCHI, K., NIU, X. D., LUNDELL, D., GIBB, D. R., CONRAD, D., SAFTIG, P. & BLOBEL, C. P. 2009. ADAMs 10 and 17 represent differentially regulated components of a general shedding machinery for membrane proteins such as transforming growth factor alpha, L-selectin, and tumor necrosis factor alpha. *Mol Biol Cell,* 20**,** 1785-94.

LEJEUNE, D., DUMOUTIER, L., CONSTANTINESCU, S., KRUIJER, W., SCHURINGA, J. J. & RENAULD, J. C. 2002. Interleukin-22 (IL-22) activates the JAK/STAT, ERK, JNK, and p38 MAP kinase pathways in a rat hepatoma cell line. Pathways that are shared with and distinct from IL-10. *J Biol Chem,* 277**,** 33676-82.

LIU, Q., WANG, Y. P., ZHAO, Q., CHEN, X. M., XUE, X. H., ZHOU, L. N., DING, Y., TANG, X. M., ZHAO, X. D. & ZHANG, Z. Y. 2017. Novel compound heterozygous mutations in ZAP70 in a Chinese patient with leaky severe combined immunodeficiency disorder. *Immunogenetics,* 69**,** 199-209.

LOPEZ-SILES, M., MARTINEZ-MEDINA, M., BUSQUETS, D., SABAT-MIR, M., DUNCAN, S. H., FLINT, H. J., ALDEGUER, X. & GARCIA-GIL, L. J. 2014. Mucosa-associated Faecalibacterium prausnitzii and Escherichia coli co-abundance can distinguish Irritable Bowel Syndrome and Inflammatory Bowel Disease phenotypes. *Int J Med Microbiol,* 304**,** 464-75.

LÜGERING, A., SCHMIDT, M., LÜGERING, N., PAUELS, H. G., DOMSCHKE, W. & KUCHARZIK, T. 2001. Infliximab induces apoptosis in monocytes from patients with chronic active Crohn's disease by using a caspase-dependent pathway. *Gastroenterology,* 121**,** 1145-57.

MACDONALD, T. T., BELL, I. & MONTELEONE, G. 2011a. The opposing roles of IL-21 and TGFβ1 in chronic inflammatory bowel disease. *Biochem Soc Trans,* 39**,** 1061-6.

MACDONALD, T. T., MONTELEONE, I., FANTINI, M. C. & MONTELEONE, G. 2011b. Regulation of homeostasis and inflammation in the intestine. *Gastroenterology,* 140**,** 1768-75.

MARTÌN-PADURA, I., LOSTAGLIO, S., SCHNEEMANN, M., WILLIAMS, L., ROMANO, M., FRUSCELLA, P., PANZERI, C., STOPPACCIARO, A., RUCO, L., VILLA, A., SIMMONS, D. & DEJANA, E. 1998. Junctional adhesion molecule, a novel member of the immunoglobulin superfamily that distributes at intercellular junctions and modulates monocyte transmigration. *J Cell Biol,* 142**,** 117-27.

MASSEY, D. C. & PARKES, M. 2007. Genome-wide association scanning highlights two autophagy genes, ATG16L1 and IRGM, as being significantly associated with Crohn's disease. *Autophagy,* 3**,** 649-51.

MATSUKURA, H., IKEDA, S., YOSHIMURA, N., TAKAZOE, M. & MURAMATSU, M. 2008. Genetic polymorphisms of tumour necrosis factor receptor superfamily 1A and 1B affect responses to infliximab in Japanese patients with Crohn's disease. *Aliment Pharmacol Ther,* 27**,** 765-70.

MCCORMACK, G., MORIARTY, D., O'DONOGHUE, D. P., MCCORMICK, P. A., SHEAHAN, K. & BAIRD, A. W. 2001. Tissue cytokine and chemokine expression in inflammatory bowel disease. *Inflamm Res,* 50**,** 491-5.

MEDVES, S. & DEMOULIN, J. B. 2012. Tyrosine kinase gene fusions in cancer: translating mechanisms into targeted therapies. *J Cell Mol Med,* 16**,** 237-48.

MELEADY, P., HENRY, M., GAMMELL, P., DOOLAN, P., SINACORE, M., MELVILLE, M., FRANCULLO, L., LEONARD, M., CHARLEBOIS, T. & CLYNES, M. 2008. Proteomic profiling of CHO cells with enhanced rhBMP-2 productivity following co-expression of PACEsol. *Proteomics,* 8**,** 2611-24.

MITOMA, H., HORIUCHI, T., HATTA, N., TSUKAMOTO, H., HARASHIMA, S., KIKUCHI, Y., OTSUKA, J., OKAMURA, S., FUJITA, S. & HARADA, M. 2005. Infliximab induces potent anti-inflammatory responses by outside-to-inside signals through transmembrane TNF-alpha. *Gastroenterology,* 128**,** 376-92.

MITOMA, H., HORIUCHI, T., TSUKAMOTO, H., TAMIMOTO, Y., KIMOTO, Y., UCHINO, A., TO, K., HARASHIMA, S., HATTA, N. & HARADA, M. 2008. Mechanisms for cytotoxic effects of anti-tumor necrosis factor agents on transmembrane tumor necrosis factor alpha-expressing cells: comparison among infliximab, etanercept, and adalimumab. *Arthritis Rheum,* 58**,** 1248-57.

MIZUTANI, H., ISHIHARA, Y., IZAWA, A., FUJIHARA, Y., KOBAYASHI, S., GOTOU, H., OKABE, E., TAKEDA, H., OZAWA, Y., KAMIYA, Y., KAMEI, H., KIKUCHI, T., YAMAMOTO, G., MITANI, A.,

NISHIHARA, T. & NOGUCHI, T. 2013. Lipopolysaccharide of Aggregatibacter actinomycetemcomitans up-regulates inflammatory cytokines, prostaglandin E2 synthesis and osteoclast formation in interleukin-1 receptor antagonist-deficient mice. *J Periodontal Res,* 48**,** 748-56.

MOLODECKY, N. A., SOON, I. S., RABI, D. M., GHALI, W. A., FERRIS, M., CHERNOFF, G., BENCHIMOL, E. I., PANACCIONE, R., GHOSH, S., BARKEMA, H. W. & KAPLAN, G. G. 2012. Increasing incidence and prevalence of the inflammatory bowel diseases with time, based on systematic review. *Gastroenterology,* 142**,** 46-54.e42; quiz e30.

MONTELEONE, G., BIANCONE, L., MARASCO, R., MORRONE, G., MARASCO, O., LUZZA, F. & PALLONE, F. 1997. Interleukin 12 is expressed and actively released by Crohn's disease intestinal lamina propria mononuclear cells. *Gastroenterology,* 112**,** 1169-78.

MONTELEONE, I., FEDERICI, M., SARRA, M., FRANZÈ, E., CASAGRANDE, V., ZORZI, F., CAVALERA, M., RIZZO, A., LAURO, R., PALLONE, F., MACDONALD, T. T. & MONTELEONE, G. 2012. Tissue inhibitor of metalloproteinase-3 regulates inflammation in human and mouse intestine. *Gastroenterology,* 143**,** 1277-87.e1-4.

MOWAT, A. M. 2003. Anatomical basis of tolerance and immunity to intestinal antigens. *Nat Rev Immunol,* 3**,** 331-41.

MUDTER, J., WEIGMANN, B., BARTSCH, B., KIESSLICH, R., STRAND, D., GALLE, P. R., LEHR, H. A., SCHMIDT, J. & NEURATH, M. F. 2005. Activation pattern of signal transducers and activators of transcription (STAT) factors in inflammatory bowel diseases. *Am J Gastroenterol,* 100**,** 64-72.

MUNKHOLM, P. 2003. Review article: the incidence and prevalence of colorectal cancer in inflammatory bowel disease. *Aliment Pharmacol Ther,* 18 Suppl 2**,** 1-5.

MURCH, S. H., LAMKIN, V. A., SAVAGE, M. O., WALKER-SMITH, J. A. & MACDONALD, T. T. 1991. Serum concentrations of tumour necrosis factor alpha in childhood chronic inflammatory bowel disease. *Gut,* 32**,** 913-7.

MURO, M., LÓPEZ-HERNÁNDEZ, R. & MROWIEC, A. 2014. Immunogenetic biomarkers in inflammatory bowel diseases: role of the IBD3 region. *World J Gastroenterol,* 20**,** 15037-48.

MYLONAKI, M., RAYMENT, N. B., RAMPTON, D. S., HUDSPITH, B. N. & BROSTOFF, J. 2005. Molecular characterization of rectal mucosa-associated bacterial flora in inflammatory bowel disease. *Inflamm Bowel Dis,* 11**,** 481-7.

NAKATA, K., INAGAWA, H., NISHIZAWA, T., KOHCHI, C. & SOMA, G. I. 2006. Specific messenger RNA expression for signal transduction molecules by lipopolysaccharide in intestinal macrophages. *Clin Exp Immunol,* 143**,** 484-93.

NESBITT, A., FOSSATI, G., BERGIN, M., STEPHENS, P., STEPHENS, S., FOULKES, R., BROWN, D., ROBINSON, M. & BOURNE, T. 2007. Mechanism of action of certolizumab pegol (CDP870): in vitro comparison with other anti-tumor necrosis factor alpha agents. *Inflamm Bowel Dis,* 13**,** 1323-32.

NEURATH, M. F. 2014. Cytokines in inflammatory bowel disease. *Nat Rev Immunol,* 14**,** 329-42.

NEURATH, M. F., WEIGMANN, B., FINOTTO, S., GLICKMAN, J., NIEUWENHUIS, E., IIJIMA, H., MIZOGUCHI, A., MIZOGUCHI, E., MUDTER, J., GALLE, P. R., BHAN, A., AUTSCHBACH, F., SULLIVAN, B. M., SZABO, S. J., GLIMCHER, L. H. & BLUMBERG, R. S. 2002. The transcription factor T-bet regulates mucosal T cell activation in experimental colitis and Crohn's disease. *J Exp Med,* 195**,** 1129-43.

NG, S. C., KAMM, M. A., STAGG, A. J. & KNIGHT, S. C. 2010. Intestinal dendritic cells: their role in bacterial recognition, lymphocyte homing, and intestinal inflammation. *Inflamm Bowel Dis,* 16**,** 1787-807.

NG, S. C., TANG, W., LEONG, R. W., CHEN, M., KO, Y., STUDD, C., NIEWIADOMSKI, O., BELL, S., KAMM, M. A., DE SILVA, H. J., KASTURIRATNE, A., SENANAYAKE, Y. U., OOI, C. J., LING, K. L., ONG, D., GOH, K. L., HILMI, I., OUYANG, Q., WANG, Y. F., HU, P., ZHU, Z., ZENG, Z., WU, K., WANG, X., XIA, B., LI, J., PISESPONGSA, P., MANATSATHIT, S., ANIWAN, S., SIMADIBRATA, M., ABDULLAH, M., TSANG, S. W., WONG, T. C., HUI, A. J., CHOW, C. M., YU, H. H., LI, M. F., NG, K. K., CHING, J., WU, J. C., CHAN, F. K., SUNG, J. J. & GROUP, A.-P. C. S. A. C. E. S. A. 2015. Environmental risk factors in inflammatory bowel disease: a population-based case-control study in Asia-Pacific. *Gut,* 64**,** 1063-71.

NGUYEN, G. C., BERNSTEIN, C. N. & BENCHIMOL, E. I. 2017. Risk of Surgery and Mortality in Elderly-onset Inflammatory Bowel Disease: A Population-based Cohort Study. *Inflamm Bowel Dis,* 23**,** 218-223.

NICHOLSON, S. E., WILLSON, T. A., FARLEY, A., STARR, R., ZHANG, J. G., BACA, M., ALEXANDER, W. S., METCALF, D., HILTON, D. J. & NICOLA, N. A. 1999. Mutational analyses of the SOCS proteins suggest a dual domain requirement but distinct mechanisms for inhibition of LIF and IL-6 signal transduction. *EMBO J,* 18**,** 375-85.

NIESS, J. H., BRAND, S., GU, X., LANDSMAN, L., JUNG, S., MCCORMICK, B. A., VYAS, J. M., BOES, M., PLOEGH, H. L., FOX, J. G., LITTMAN, D. R. & REINECKER, H. C. 2005. CX3CR1-mediated dendritic cell access to the intestinal lumen and bacterial clearance. *Science,* 307**,** 254-8.

OGURA, Y., BONEN, D. K., INOHARA, N., NICOLAE, D. L., CHEN, F. F., RAMOS, R., BRITTON, H., MORAN, T., KARALIUSKAS, R., DUERR, R. H., ACHKAR, J. P., BRANT, S. R., BAYLESS, T. M., KIRSCHNER, B. S., HANAUER, S. B., NUÑEZ, G. & CHO, J. H. 2001. A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. *Nature,* 411**,** 603-6.

OLADIPUPO, S. S., SMITH, C., SANTEFORD, A., PARK, C., SENE, A., WILEY, L. A., OSEI-OWUSU, P., HSU, J., ZAPATA, N., LIU, F., NAKAMURA, R., LAVINE, K. J., BLUMER, K. J., CHOI, K., APTE, R. S. & ORNITZ, D. M. 2014. Endothelial cell FGF signaling is required for injury response but not for vascular homeostasis. *Proc Natl Acad Sci U S A,* 111**,** 13379-84.

ORNITZ, D. M. 2000. FGFs, heparan sulfate and FGFRs: complex interactions essential for development. *Bioessays,* 22**,** 108-12.

PALLAI, A., KISS, B., VEREB, G., ARMAKA, M., KOLLIAS, G., SZEKANECZ, Z. & SZONDY, Z. 2016. Transmembrane TNF-α Reverse Signaling Inhibits Lipopolysaccharide-Induced Proinflammatory Cytokine Formation in Macrophages by Inducing TGF-β: Therapeutic Implications. *J Immunol,* 196**,** 1146-57.

PANÉS, J., SU, C., BUSHMAKIN, A. G., CAPPELLERI, J. C., MAMOLO, C. & HEALEY, P. 2015. Randomized trial of tofacitinib in active ulcerative colitis: analysis of efficacy based on patient-reported outcomes. *BMC Gastroenterol,* 15**,** 14.

PARKES, M., BARRETT, J. C., PRESCOTT, N. J., TREMELLING, M., ANDERSON, C. A., FISHER, S. A., ROBERTS, R. G., NIMMO, E. R., CUMMINGS, F. R., SOARS, D., DRUMMOND, H., LEES, C. W., KHAWAJA, S. A., BAGNALL, R., BURKE, D. A., TODHUNTER, C. E., AHMAD, T., ONNIE, C. M., MCARDLE, W., STRACHAN, D., BETHEL, G., BRYAN, C., LEWIS, C. M., DELOUKAS, P., FORBES, A., SANDERSON, J., JEWELL, D. P., SATSANGI, J., MANSFIELD, J. C., CARDON, L., MATHEW, C. G. & CONSORTIUM, W. T. C. C. 2007. Sequence variants in the autophagy gene IRGM and multiple other replicating loci contribute to Crohn's disease susceptibility. *Nat Genet,* 39**,** 830-2.

PASQUALE, E. B. 1997. The Eph family of receptors. *Curr Opin Cell Biol,* 9**,** 608-15.

PENAGINI, F., DILILLO, D., BORSANI, B., COCOCCIONI, L., GALLI, E., BEDOGNI, G., ZUIN, G. & ZUCCOTTI, G. V. 2016. Nutrition in Pediatric Inflammatory Bowel Disease: From Etiology to Treatment. A Systematic Review. *Nutrients,* 8.

PENDER, S. L., TICKLE, S. P., DOCHERTY, A. J., HOWIE, D., WATHEN, N. C. & MACDONALD, T. T. 1997. A major role for matrix metalloproteinases in T cell injury in the gut. *J Immunol,* 158**,** 1582-90.

PERRIER, C., DE HERTOGH, G., CREMER, J., VERMEIRE, S., RUTGEERTS, P., VAN ASSCHE, G., SZYMKOWSKI, D. E. & CEUPPENS, J. L. 2013. Neutralization of membrane TNF, but not soluble TNF, is crucial for the treatment of experimental colitis. *Inflamm Bowel Dis,* 19**,** 246-53.

PETITO, V., LOPETUSO, L. R., ARENA, V., STIGLIANO, E., BONINSEGNA, A., BIBBÒ, S., POSCIA, A., ALFIERI, S., ROSA, F., AMATO, A., CAMMAROTA, G., PAPA, A., SGAMBATO, A., GASBARRINI, A. & SCALDAFERRI, F. 2016. Direct effect of

infliximab on intestinal mucosa sustains mucosal healing: exploring new mechanisms of action. *Dig Liver Dis,* 48**,** 391-8.

PODOLSKY, D. K. 1997. Healing the epithelium: solving the problem from two sides. *J Gastroenterol,* 32**,** 122-6.

POLIAKOV, A., COTRINA, M. & WILKINSON, D. G. 2004. Diverse roles of eph receptors and ephrins in the regulation of cell migration and tissue assembly. *Dev Cell,* 7**,** 465-80.

PONDER, A. & LONG, M. D. 2013. A clinical review of recent findings in the epidemiology of inflammatory bowel disease. *Clin Epidemiol,* 5**,** 237-47.

POWELL, N., CANAVAN, J. B., MACDONALD, T. T. & LORD, G. M. 2010. Transcriptional regulation of the mucosal immune system mediated by T-bet. *Mucosal Immunol,* 3**,** 567-77.

PRENZEL, F. & UHLIG, H. H. 2009. Frequency of indeterminate colitis in children and adults with IBD - a metaanalysis. *J Crohns Colitis,* 3**,** 277-81.

PROBERT, C. S. & BROWN, M. 2008. Are there any ethnic groups that are more likely to develop IBD? *Inflamm Bowel Dis,* 14 Suppl 2**,** S24-5.

RADFORD-SMITH, G. & PANDEYA, N. 2006. Associations between NOD2/CARD15 genotype and phenotype in Crohn's disease--Are we there yet? *World J Gastroenterol,* 12**,** 7097-103.

RAKOFF-NAHOUM, S., PAGLINO, J., ESLAMI-VARZANEH, F., EDBERG, S. & MEDZHITOV, R. 2004. Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. *Cell,* 118**,** 229-41.

RAUERT, H., WICOVSKY, A., MÜLLER, N., SIEGMUND, D., SPINDLER, V., WASCHKE, J., KNEITZ, C. & WAJANT, H. 2010. Membrane tumor necrosis factor (TNF) induces p100 processing via TNF receptor-2 (TNFR2). *J Biol Chem,* 285**,** 7394-404.

RESCIGNO, M. & ILIEV, I. D. 2009. Interleukin-23: linking mesenteric lymph node dendritic cells with Th1 immunity in Crohn's disease. *Gastroenterology,* 137**,** 1566-70.

RIEDER, F., FIOCCHI, C. & ROGLER, G. 2017. Mechanisms, Management, and Treatment of Fibrosis in Patients With Inflammatory Bowel Diseases. *Gastroenterology,* 152**,** 340-350.e6.

RIEMANN, A., IHLING, A., THOMAS, J., SCHNEIDER, B., THEWS, O. & GEKLE, M. 2015. Acidic environment activates inflammatory programs in fibroblasts via a cAMP-MAPK pathway. *Biochim Biophys Acta,* 1853**,** 299-307.

ROUND, J. L. & MAZMANIAN, S. K. 2010. Inducible Foxp3+ regulatory T-cell development by a commensal bacterium of the intestinal microbiota. *Proc Natl Acad Sci U S A,* 107**,** 12204-9.

ROVEDATTI, L., KUDO, T., BIANCHERI, P., SARRA, M., KNOWLES, C. H., RAMPTON, D. S., CORAZZA, G. R., MONTELEONE, G., DI SABATINO, A. & MACDONALD, T. T. 2009. Differential regulation

of interleukin 17 and interferon gamma production in inflammatory bowel disease. *Gut,* 58**,** 1629-36.

RUTGEERTS, P., SANDBORN, W. J., FEAGAN, B. G., REINISCH, W., OLSON, A., JOHANNS, J., TRAVERS, S., RACHMILEWITZ, D., HANAUER, S. B., LICHTENSTEIN, G. R., DE VILLIERS, W. J., PRESENT, D., SANDS, B. E. & COLOMBEL, J. F. 2005. Infliximab for induction and maintenance therapy for ulcerative colitis. *N Engl J Med,* 353**,** 2462-76.

RUTGEERTS, P., VAN ASSCHE, G. & VERMEIRE, S. 2004. Optimizing anti-TNF treatment in inflammatory bowel disease. *Gastroenterology,* 126**,** 1593-610.

RUTGEERTS, P., VERMEIRE, S. & VAN ASSCHE, G. 2009. Biological therapies for inflammatory bowel diseases. *Gastroenterology,* 136**,** 1182-97.

SAITOH, T., FUJITA, N., JANG, M. H., UEMATSU, S., YANG, B. G., SATOH, T., OMORI, H., NODA, T., YAMAMOTO, N., KOMATSU, M., TANAKA, K., KAWAI, T., TSUJIMURA, T., TAKEUCHI, O., YOSHIMORI, T. & AKIRA, S. 2008. Loss of the autophagy protein Atg16L1 enhances endotoxin-induced IL-1beta production. *Nature,* 456**,** 264-8.

SALMELA, M. T., MACDONALD, T. T., BLACK, D., IRVINE, B., ZHUMA, T., SAARIALHO-KERE, U. & PENDER, S. L. 2002. Upregulation of matrix metalloproteinases in a model of T cell mediated tissue injury in the gut: analysis by gene array and in situ hybridisation. *Gut,* 51**,** 540-7.

SANDBORN, W. J., HANAUER, S. B., KATZ, S., SAFDI, M., WOLF, D. G., BAERG, R. D., TREMAINE, W. J., JOHNSON, T., DIEHL, N. N. & ZINSMEISTER, A. R. 2001. Etanercept for active Crohn's disease: a randomized, double-blind, placebo-controlled trial. *Gastroenterology,* 121**,** 1088-94.

SANDBORN, W. J., VAN ASSCHE, G., REINISCH, W., COLOMBEL, J. F., D'HAENS, G., WOLF, D. C., KRON, M., TIGHE, M. B., LAZAR, A. & THAKKAR, R. B. 2012. Adalimumab induces and maintains clinical remission in patients with moderate-to-severe ulcerative colitis. *Gastroenterology,* 142**,** 257-65.e1-3.

SANDS, B. E. 2001. Biological therapies for ulcerative colitis. *Acta Gastroenterol Belg,* 64**,** 205-9.

SANO, T., HUANG, W., HALL, J. A., YANG, Y., CHEN, A., GAVZY, S. J., LEE, J. Y., ZIEL, J. W., MIRALDI, E. R., DOMINGOS, A. I., BONNEAU, R. & LITTMAN, D. R. 2015. An IL-23R/IL-22 Circuit Regulates Epithelial Serum Amyloid A to Promote Local Effector Th17 Responses. *Cell,* 163**,** 381-93.

SARTOR, R. B. & MUEHLBAUER, M. 2007. Microbial host interactions in IBD: implications for pathogenesis and therapy. *Curr Gastroenterol Rep,* 9**,** 497-507.

SAUK, J., NGUYEN, D., YAJNIK, V., KHALILI, H., KONIJETI, G., HODIN, R., BORDEIANOU, L., SHELLITO, P., SYLLA, P., KORZENIK, J., FRIEDMAN, S. & ANANTHAKRISHNAN, A. N. 2014. Natural history of perianal Crohn's disease after fecal diversion. *Inflamm Bowel Dis,* 20**,** 2260-5.

SCHARL, M. & ROGLER, G. 2014. Pathophysiology of fistula formation in Crohn's disease. *World J Gastrointest Pathophysiol,* 5**,** 205-12.

SEO, G. Y., YOUN, J. & KIM, P. H. 2009. IL-21 ensures TGF-beta 1-induced IgA isotype expression in mouse Peyer's patches. *J Leukoc Biol,* 85**,** 744-50.

SHARMA, M., MOHAPATRA, J., WAGH, A., PATEL, H. M., PANDEY, D., KADAM, S., ARGADE, A., DESHPANDE, S. S., SHAH, G. B., CHATTERJEE, A. & JAIN, M. R. 2014. Involvement of TACE in colon inflammation: a novel mechanism of regulation via SIRT-1 activation. *Cytokine,* 66**,** 30-9.

SHIN, H. W., CHO, C. H., KIM, T. Y. & PARK, J. W. 2010. Sunitinib deregulates tumor adaptation to hypoxia by inhibiting HIF-1alpha synthesis in HT-29 colon cancer cells. *Biochem Biophys Res Commun,* 398**,** 205-11.

SINGH, S., AL-DARMAKI, A., FROLKIS, A. D., SEOW, C. H., LEUNG, Y., NOVAK, K. L., GHOSH, S., EKSTEEN, B., PANACCIONE, R. & KAPLAN, G. G. 2015. Postoperative Mortality Among Patients With Inflammatory Bowel Diseases: A Systematic Review and Meta-analysis of Population-Based Studies. *Gastroenterology,* 149**,** 928-37.

SINGH, U. P., SINGH, N. P., MURPHY, E. A., PRICE, R. L., FAYAD, R., NAGARKATTI, M. & NAGARKATTI, P. S. 2016. Chemokine and cytokine levels in inflammatory bowel disease patients. *Cytokine,* 77**,** 44-9.

SMYTHIES, L. E., SELLERS, M., CLEMENTS, R. H., MOSTELLER-BARNUM, M., MENG, G., BENJAMIN, W. H., ORENSTEIN, J. M. & SMITH, P. D. 2005. Human intestinal macrophages display profound inflammatory anergy despite avid phagocytic and bacteriocidal activity. *J Clin Invest,* 115**,** 66-75.

SOKOL, H., SEKSIK, P., FURET, J. P., FIRMESSE, O., NION-LARMURIER, I., BEAUGERIE, L., COSNES, J., CORTHIER, G., MARTEAU, P. & DORÉ, J. 2009. Low counts of Faecalibacterium prausnitzii in colitis microbiota. *Inflamm Bowel Dis,* 15**,** 1183-9.

SOLDEVILA, G., LICONA, I., SALGADO, A., RAMÍREZ, M., CHÁVEZ, R. & GARCÍA-ZEPEDA, E. 2004. Impaired chemokine-induced migration during T-cell development in the absence of Jak 3. *Immunology,* 112**,** 191-200.

SOOND, S. M., EVERSON, B., RICHES, D. W. & MURPHY, G. 2005. ERK-mediated phosphorylation of Thr735 in TNFalpha-converting enzyme and its potential role in TACE protein trafficking. *J Cell Sci,* 118**,** 2371-80.

STROBER, W., ASANO, N., FUSS, I., KITANI, A. & WATANABE, T. 2014. Cellular and molecular mechanisms underlying NOD2 risk-associated polymorphisms in Crohn's disease. *Immunol Rev,* 260**,** 249-60.

SUZUKI, Y., MATSUI, T., ITO, H., ASHIDA, T., NAKAMURA, S., MOTOYA, S., MATSUMOTO, T., SATO, N., OZAKI, K., WATANABE, M. & HIBI, T. 2015. Circulating Interleukin 6 and Albumin, and Infliximab Levels Are Good Predictors of Recovering Efficacy After Dose Escalation Infliximab Therapy in Patients with Loss of Response to Treatment for Crohn's Disease: A Prospective Clinical Trial. *Inflamm Bowel Dis,* 21**,** 2114-22.

SWIDSINSKI, A., WEBER, J., LOENING-BAUCKE, V., HALE, L. P. & LOCHS, H. 2005. Spatial organization and composition of the mucosal flora in patients with inflammatory bowel disease. *J Clin Microbiol,* 43**,** 3380-9.

TAKAI, A., MARUSAWA, H., MINAKI, Y., WATANABE, T., NAKASE, H., KINOSHITA, K., TSUJIMOTO, G. & CHIBA, T. 2012. Targeting activation-induced cytidine deaminase prevents colon cancer development despite persistent colonic inflammation. *Oncogene,* 31**,** 1733-42.

TARGAN, S. R., HANAUER, S. B., VAN DEVENTER, S. J., MAYER, L., PRESENT, D. H., BRAAKMAN, T., DEWOODY, K. L., SCHAIBLE, T. F. & RUTGEERTS, P. J. 1997. A short-term study of chimeric monoclonal antibody cA2 to tumor necrosis factor alpha for Crohn's disease. Crohn's Disease cA2 Study Group. *N Engl J Med,* 337**,** 1029-35.

TAUPIN, D. & PODOLSKY, D. K. 2003. Trefoil factors: initiators of mucosal healing. *Nat Rev Mol Cell Biol,* 4**,** 721-32.

TEN HOVE, T., CORBAZ, A., AMITAI, H., ALONI, S., BELZER, I., GRABER, P., DRILLENBURG, P., VAN DEVENTER, S. J., CHVATCHKO, Y. & TE VELDE, A. A. 2001. Blockade of endogenous IL-18 ameliorates TNBS-induced colitis by decreasing local TNF-alpha production in mice. *Gastroenterology,* 121**,** 1372-9.

TEN HOVE, T., VAN DEN BLINK, B., PRONK, I., DRILLENBURG, P., PEPPELENBOSCH, M. P. & VAN DEVENTER, S. J. 2002a. Dichotomal role of inhibition of p38 MAPK with SB 203580 in experimental colitis. *Gut,* 50**,** 507-12.

TEN HOVE, T., VAN MONTFRANS, C., PEPPELENBOSCH, M. P. & VAN DEVENTER, S. J. 2002b. Infliximab treatment induces apoptosis of lamina propria T lymphocytes in Crohn's disease. *Gut,* 50**,** 206-11.

THIA, K. T., LOFTUS, E. V., SANDBORN, W. J. & YANG, S. K. 2008. An update on the epidemiology of inflammatory bowel disease in Asia. *Am J Gastroenterol,* 103**,** 3167-82.

TIMMER, A., PATTON, P. H., CHANDE, N., MCDONALD, J. W. & MACDONALD, J. K. 2016. Azathioprine and 6-mercaptopurine for maintenance of remission in ulcerative colitis. *Cochrane Database Syst Rev***,** CD000478.

TOUAT, M., ILEANA, E., POSTEL-VINAY, S., ANDRÉ, F. & SORIA, J. C. 2015. Targeting FGFR Signaling in Cancer. *Clin Cancer Res,* 21**,** 2684-94.

TSUJI, F., OKI, K., OKAHARA, A., SUHARA, H., YAMANOUCHI, T., SASANO, M., MITA, S. & HORIUCHI, M. 2002. Differential effects between marimastat, a TNF-alpha converting enzyme inhibitor, and anti-TNF-alpha antibody on murine models for sepsis and arthritis. *Cytokine,* 17**,** 294-300.

TURNER, N., PEARSON, A., SHARPE, R., LAMBROS, M., GEYER, F., LOPEZ-GARCIA, M. A., NATRAJAN, R., MARCHIO, C., IORNS, E., MACKAY, A., GILLETT, C., GRIGORIADIS, A., TUTT, A., REIS-FILHO, J. S. & ASHWORTH, A. 2010. FGFR1 amplification drives endocrine therapy resistance and is a therapeutic target in breast cancer. *Cancer Res,* 70**,** 2085-94.

VAN DEN BRANDE, J. M., BRAAT, H., VAN DEN BRINK, G. R., VERSTEEG, H. H., BAUER, C. A., HOEDEMAEKER, I., VAN MONTFRANS, C., HOMMES, D. W., PEPPELENBOSCH, M. P. & VAN DEVENTER, S. J. 2003. Infliximab but not etanercept induces apoptosis in lamina propria T-lymphocytes from patients with Crohn's disease. *Gastroenterology,* 124**,** 1774-85.

VAN DULLEMEN, H. M., VAN DEVENTER, S. J., HOMMES, D. W., BIJL, H. A., JANSEN, J., TYTGAT, G. N. & WOODY, J. 1995. Treatment of Crohn's disease with anti-tumor necrosis factor chimeric monoclonal antibody (cA2). *Gastroenterology,* 109**,** 129-35.

VAN HEEL, D. A., GHOSH, S., HUNT, K. A., MATHEW, C. G., FORBES, A., JEWELL, D. P. & PLAYFORD, R. J. 2005. Synergy between TLR9 and NOD2 innate immune responses is lost in genetic Crohn's disease. *Gut,* 54**,** 1553-7.

VICTORA, G. D., DOMINGUEZ-SOLA, D., HOLMES, A. B., DEROUBAIX, S., DALLA-FAVERA, R. & NUSSENZWEIG, M. C. 2012. Identification of human germinal center light and dark zone cells and their relationship to human B-cell lymphomas. *Blood,* 120**,** 2240-8.

VOGELAAR, L., SPIJKER, A. V. & VAN DER WOUDE, C. J. 2009. The impact of biologics on health-related quality of life in patients with inflammatory bowel disease. *Clin Exp Gastroenterol,* 2**,** 101-9.

VON MOLTKE, J., JI, M., LIANG, H. E. & LOCKSLEY, R. M. 2016. Tuft-cell-derived IL-25 regulates an intestinal ILC2-epithelial response circuit. *Nature,* 529**,** 221-5.

VOS, A. C., WILDENBERG, M. E., DUIJVESTEIN, M., VERHAAR, A. P., VAN DEN BRINK, G. R. & HOMMES, D. W. 2011. Anti-tumor necrosis factor-α antibodies induce regulatory macrophages in an Fc region-dependent manner. *Gastroenterology,* 140**,** 221-30.

VOSSENKÄMPER, A., HUNDSRUCKER, C., PAGE, K., VAN MAURIK, A., SANDERS, T. J., STAGG, A. J., DAS, L. & MACDONALD, T. T. 2014. A CD3-specific antibody reduces cytokine production and alters

phosphoprotein profiles in intestinal tissues from patients with inflammatory bowel disease. *Gastroenterology,* 147**,** 172-83.

WAETZIG, G. H., ROSENSTIEL, P., ARLT, A., TILL, A., BRÄUTIGAM, K., SCHÄFER, H., ROSE-JOHN, S., SEEGERT, D. & SCHREIBER, S. 2005. Soluble tumor necrosis factor (TNF) receptor-1 induces apoptosis via reverse TNF signaling and autocrine transforming growth factor-beta1. *FASEB J,* 19**,** 91-3.

WAETZIG, G. H., SEEGERT, D., ROSENSTIEL, P., NIKOLAUS, S. & SCHREIBER, S. 2002. p38 mitogen-activated protein kinase is activated and linked to TNF-alpha signaling in inflammatory bowel disease. *J Immunol,* 168**,** 5342-51.

WANG, H., KADLECEK, T. A., AU-YEUNG, B. B., GOODFELLOW, H. E., HSU, L. Y., FREEDMAN, T. S. & WEISS, A. 2010. ZAP-70: an essential kinase in T-cell signaling. *Cold Spring Harb Perspect Biol,* 2**,** a002279.

WEE, P., SHI, H., JIANG, J., WANG, Y. & WANG, Z. 2015. EGF stimulates the activation of EGF receptors and the selective activation of major signaling pathways during mitosis. *Cell Signal,* 27**,** 638-51.

WEHKAMP, J., FELLERMANN, K. & STANGE, E. F. 2005. Human defensins in Crohn's disease. *Chem Immunol Allergy,* 86**,** 42-54.

WEIZMAN, A., HUANG, B., BEREL, D., TARGAN, S. R., DUBINSKY, M., FLESHNER, P., IPPOLITI, A., KAUR, M., PANIKKATH, D., BRANT, S., OIKONOMOU, I., DUERR, R., RIOUX, J., SILVERBERG, M., ROTTER, J. I., VASILIAUSKAS, E., HARITUNIANS, T., SHIH, D., LI, D., MELMED, G. Y. & MCGOVERN, D. P. 2014. Clinical, serologic, and genetic factors associated with pyoderma gangrenosum and erythema nodosum in inflammatory bowel disease patients. *Inflamm Bowel Dis,* 20**,** 525-33.

WU, C. H., HUANG, T. C. & LIN, B. F. 2017. Folate deficiency affects dendritic cell function and subsequent T helper cell differentiation. *J Nutr Biochem,* 41**,** 65-72.

YAMAMOTO, T. & WATANABE, T. 2014. Surgery for luminal Crohn's disease. *World J Gastroenterol,* 20**,** 78-90.

YANG, B. L., CHEN, Y. G., GU, Y. F., CHEN, H. J., SUN, G. D., ZHU, P. & SHAO, W. J. 2015. Long-term outcome of infliximab combined with surgery for perianal fistulizing Crohn's disease. *World J Gastroenterol,* 21**,** 2475-82.

YAO, D. K. & SHAO, J. Y. 2007. Flexibility of single microvilli on live neutrophils and lymphocytes. *Phys Rev E Stat Nonlin Soft Matter Phys,* 76**,** 021907.

YARUR, A. J., MANDALIA, A. B., DAUER, R. M., CZUL, F., DESHPANDE, A. R., KERMAN, D. H., ABREU, M. T. & SUSSMAN, D. A. 2014. Predictive factors for clinically actionable computed tomography findings in inflammatory bowel disease patients seen in the emergency

department with acute gastrointestinal symptoms. *J Crohns Colitis,* 8**,** 504-12.

ZOUALI, H., LESAGE, S., MERLIN, F., CÉZARD, J. P., COLOMBEL, J. F., BELAICHE, J., ALMER, S., TYSK, C., O'MORAIN, C., GASSULL, M., CHRISTENSEN, S., FINKEL, Y., MODIGLIANI, R., GOWER-ROUSSEAU, C., MACRY, J., CHAMAILLARD, M., THOMAS, G., HUGOT, J. P., GROUP, E. & GROUP, E. 2003. CARD4/NOD1 is not involved in inflammatory bowel disease. *Gut,* 52**,** 71-4.

ZUNDLER, S. & NEURATH, M. F. 2016. Integrating Immunologic Signaling Networks: The JAK/STAT Pathway in Colitis and Colitis-Associated Cancer. *Vaccines (Basel),* 4.