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Characterization of immune receptor-cognate ligands expression and signalling pathways in human intestinal myofibroblasts

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CHARACTERIZATION OF IMMUNE RECEPTOR-COGNATE LIGANDS EXPRESSION AND SIGNALLING PATHWAYS IN HUMAN INTESTINAL MYOFIBROBLASTS

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A thesis submitted for the degree of Doctor of Philosophy

University of Bath

Department of Pharmacy and Pharmacology

June 2004

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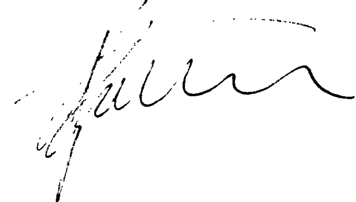

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Abstract

Inflammatory bowel disease is a chronic relapsing and remitting inflammatory disorder of the gastrointestinal system. Although its aetiology remains unknown, increasing evidence has outlined that immune mechanisms, including aberrant T-cell activation and abnormal cytokine production in the intestine may contribute to its pathogenesis. A role for intestinal myofibroblasts in chronic inflammation is gradually emerging even though the mechanisms by which they participate in this process are ill defined. In this study the role of myofibroblasts in expression of inflammatory mediators and their interactions with the adjacent colonic epithelial cells and T-lymphocytes was investigated.

Human intestinal myofibroblasts were found to constitutively express a variety of co-stimulatory molecules such as PD-L1, B7-RP-1and B7H3, whereas B7.1 and ICOS expression was induced by the combination of the pro-inflammatory cytokines TNF-α/IFN-γ. Expression of a cognate receptor, for the IFN-γ inducible chemokines, IP-10, Mig and I-TAC that are known to be produced by human intestinal epithelium, was demonstrated indirectly, by the ability of these chemokines to initiate various signalling events in myofibroblasts. Stimulation with all three chemokines induced phosphorylation of PKB, ERK1/2, p38 and p90RSK, whereas only I-TAC stimulation resulted in an increase in intracellular calcium. All signalling events exhibited a varying degree of sensitivity to the PI3K inhibitor LY294002, but were insensitive to the Gαi inhibitor pertussis toxin. The different kinetics of the signalling events in myofibroblasts compared to activated T-cells in addition to unresponsiveness to pertussis toxin, suggested that a receptor different to CXCR3 is expressed on myofiborblasts, which also induced actin polymerisation and stress fiber formation.

Taken together, the data presented here suggest a possible trimeric model consisting of intestinal myofibroblasts, T-cells and colonic epithelial cells that may have a role in modulating physiologic and pathologic mucosal inflammation.

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ABBREVIATIONS

aa	Amino acid
APS	Ammonium persulfate
APC	Antigen presenting cell
Arp 2/3	Actin related protein complex 2/3
BAD	Bcl-x_L/Bcl-2 associated death promoter
bFGF	Basic fibroblast growth factor
BSA	Bovine serum albumin
BTLA	B and T lymphocyte attenuator
cAMP	Cyclic adenosine monophosphate
CD	Crohn's Disease
CRIB	Cdc42 and Rac interactive binding
CTL	Cytotoxic lymphocyte
CTLA-4	Cytotoxic T-lymphocyte antigen 4
DAG	Diacylglycerol
DC	Dendritic cell
DMEM	Dulbecco's modified essential medium
DMSO	Dimethyl sulfoxide
DNA	Deozyribonucleic acid
DSS	Dextran sodium sulfate
DTT	Dithiothreitol

ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGTA	Ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N′*,*N′*-tetraacetic acid
ERK	Extracellular regulated kinase
FACS	Fluorescence-activated cell sorter
F-actin	Filamentous actin
FAE	Follicle-associated epithelium
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
GAG	Glycosaminoglycan
GAP	GTPase activating protein
GDI	Guanine nucleotide dissociation inhibitors
GDP	Guanosine 5'-diphosphate
GTP	Guanosine triphosphate
GEF	Guanine exchange factor
GI	Gastrointestinal
GPCR	G-protein coupled receptor
HBSS	Hank's balanced salt solution
HGF	Hepatocyte growth factor

HIV	Human immunodeficiency virus
HSC	Hepatic stellate cell
IBD	Inflammatory bowel disease
ICAM	Intracellular adhesion molecule
ICC	Interstitial cell of Cajal
ICOS	Inducible costimulator
IEC	Intestinal epithelial cells
IEL	Intraepithelial lymphocyte
Ig	Immunoglobulin
IP_3	Inositol 1,4,5-trisphosphate
ISEMF	Intestinal subepithelial myofibroblast
kDa	Kilo dalton
KGF	Keratinocyte growth factor
LIM	Lin-11, Isl-1, Mec-3 (Zn binding domain)
MAPK	Mitogen-activated protein kinase
MAPKK	MAPK kinase
MAPKKK	MAPKK kinase
mDia	Mammalian homolog of Diaphanous
MEK	MAPK/ERK kinase
MHC	Major histocompatibility complex
MLC	Myosin light chain

mRNA	Messenger ribonucleic acid
NFκB	Nuclear factor κB
NK	Natural killer cell
PAK	p21 activated kinase
PBL	Peripheral blood lymphocyte
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PD-1	Programmed death-1
PDGF	Platelet-derived growth factor
PDK	Phosphoinositide-dependent kinase
PD-L	Programmed death ligand
PH	Pleckstrin homology
PtdIns	Phosphatidylinositol
PtdIns4,5-P_2	Phosphatidylinositol 4,5 bi-phosphate
PtdIns3,4,5-P_3	Phosphatidylinositol 3,4,5 trisphosphate
PI	Phosphoinositide
PI3K	Phosphoinositide 3 kinase
PKA, B, C	Protein kinase A, B ,C
PLC	Phospholipase C
PTEN	Phosphatase and tensin homolog deleted on chromosome ten
PTK	Protein tyrosine kinase

PTX	Pertussis toxin
PX	Phox homology domain
Rho	Ras homology
ROCK	Rho-associated coiled-coil kinase
RTK	Receptor tyrosine kinase
SCF	Stem cell factor
SDS	Sodium dodecyl sulfate
SEB	Staphylococcal enterotoxin B
SHIP	SH2 domain-containing inositol-5-phosphatase
TBE	Tris, boric acid, EDTA buffer
TBS	Tris-buffered saline
TCR	T-cell receptor
TEMED	*N,N,N′,N′*-tetramethylethelenediamine
TGF-β	Transforming growth factor-β
Th	T helper
TNF	Tumor necrosis factor
TRITC	Tetramethylrhodamine isothiocyanate
UC	Ulcerative colitis
WASP	Wiskott-Aldrich syndrome protein
WAVE	WASP family Verprolin-homologous protein

Chapter 1

1 Introduction

1.1 The mucosal immune system

The mucosal immune system comprises those lymphoid elements associated with the internal mucosal surfaces of the body, namely the gastrointestinal tract (GI), the respiratory and the urogenital tract. The physiological roles of the mucosae are to absorb, digest and exchange nutrients. In addition to being a considerable size, most of the relevant organs employ a variety of anatomical strategies to increase their functional surface area. For example, the human GI tract alone comprises almost $400m^2$ surface, approximately 200 times that of the skin. The resulting large potential for antigenic stimulation is increased further by the fact that tissues such as the GI are exposed continuously to a vast array of diverse antigens. It is not surprising then that the lymphoid tissues associated with the mucosal surfaces comprise the largest compartment of the immune system, with more lymphocytes present there than in all parts of the immune system added together.

1.2 The gastrointestinal system

The gastrointestinal system is primarily involved in reducing food for absorption into the body. This process occurs in five main phases within defined regions of the gastrointestinal system: ingestion, fragmentation, digestion, absorption, and elimination of waste products. Ingestion and initial fragmentation of food occurs in the oral cavity. The next segments of the tract, the pharynx and oesophagus conduct food from the oral cavity to the stomach where fragmentation is completed and digestion initiated. This process reduces the stomach contents to a

semi-digested liquid called chyme and which is passed on through the pylorus, into the duodenum, the short, first part of the small intestine where it is neutralised partly by an alkaline secretion from the duodenal mucosa. The duodenal contents pass onwards along the small intestine where the process of digestion is completed and the main absorptive phase occurs. After the duodenum, the next segment of the small intestine, where the major part of absorption occurs, is called the jejunum; the rest of the small intestine is called the ileum, but there is no distinct junction between these parts of the tract. The unabsorbed liquid residue from the small intestine passes through a valve, the ileo-caceal valve, into the large intestine. In the large intestine, water is absorbed from the liquid residue, which becomes progressively more solid as it passes towards the anus. The first part of the intestine is called the caceum, from which projects a blind-ended sac, the appendix. The next part of the large intestine, the colon is divided anatomically into ascending, descending and sigmoid segments although histologically the segments are similar. Contractile activities in the final segment of the gastrointestinal tract, the rectum, eliminate the waste products by the process of defaecation via the anal canal.

Structure of the gastrointestinal tract

The structure of the gastrointestinal tract conforms to a general plan, which is clearly evident from the oesophagus to the anus. The tract is essentially a muscular tube lined by a mucous membrane. The arrangement of the major muscular component remains relatively constant throughout the tract whereas the mucosa shows marked variations in the different regions of the tract.

The gastrointestinal tract has four distinct functional layers:

1. The mucosa: the mucosa is the divided into three layers: an epithelial lining, a supporting connective tissue lamina propria and a thin smooth muscle layer, the muscularis mucosae, which produces local movements and folding of the mucosa.

2. The submucosa: this is a second connective tissue layer that supports the mucosa and contains the larger blood vessels, lymphatics and nerves.

3. The muscularis propria: this muscular wall is subdivided into two histological layers, a relatively thick inner layer of circular muscle and a thinner outer layer of longitudinal muscle. The action of these smooth muscle layers is the basis of peristaltic contraction.

4. The adventitia: this outer layer of connective tissue conducts the major vessels and nerves. Where the adventitia is exposed to the abdominal cavity, it is referred to as the serosa.

The intestine as a lymphoid organ

The best-studied organ of the mucosal immune system is the intestine, partly because of its essential role in host resistance to bacteria, viruses, and parasites and in the host's interaction with environmental antigens (e.g., food antigens).

The lymphoid tissue that forms the mucosal immune system in the intestine can be divided on anatomic, morphologic, and functional grounds into three major populations, Peyer's patches, lamina propria lymphoid cells and intraepithelial lymphocytes (Figure 1.1).

Peyer's patches: have an important role in the initiation of the mucosal immune response and are an important source of B and T lymphocytes that ultimately populate other regions of the intestinal mucosa. These lymphoid areas are

separated from the intestinal lumen by a single layer of columnar epithelial cells, known as the follicle-associated epithelium (FAE), which differs from the epithelium that covers the villus mucosa and is infiltrated by large numbers of B cells, T-cells, macrophages and dendritic cells. Draining the Peyer's patches via lymphatics are the mesenteric lymph nodes (Mowat and Viney, 1997).

Lamina propria lymphoid cells: The lamina propria is the layer of connective tissue between the epithelium and muscularis mucosa. It is made up of smooth muscle cells, fibroblasts, lymphatics and blood vessels, and makes up the villus core over which the absorptive epithelial cells migrate from the crypts to the villus tips. The most striking feature of adult human large and small intestinal lamina propria is the infiltrate of lymphoid cells. The large numbers of macrophages, dendritic cells and T-cells in the lamina propria make it likely that antigen crossing the epithelium may be processed and presented to lamina propria T-cells.

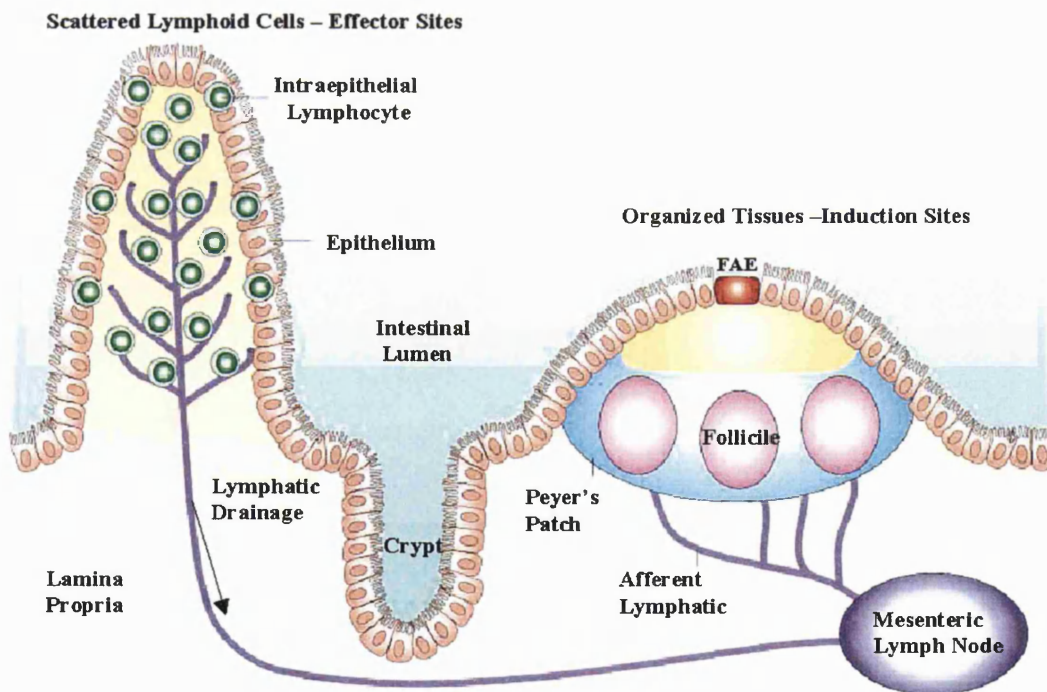

Figure 1.1 Schematic representation of the lymphoid elements of the intestinal mucosal immune system, acquired from (Mowat, 2003).

Intraepithelial lymphocytes: The intestinal mucosa also contains a specialized subset of lymphocytes that are interspersed within the epithelial layer, so called intraepithelial lymphocytes (IELs). The major physiologic functions of these cells are still uncertain, but their role in host defence as cytolytic cells and response to antigens presented on the surface of adjacent epithelial cells are being studied. It is possible that IELs and epithelial cells interact in ways that modify each other's functions (Hayday et al., 2001).

1.3 *Intestinal myofibroblasts*

Myofibroblasts are a unique group of cells that have a similar appearance and function regardless of their tissue of residence. These cells share characteristics of both fibroblasts and smooth muscle cells and have therefore been designated myofibroblasts.

Phenotypical characterisation of myofibroblasts

Immunohistochemical characterization of myofibroblasts is based on antibody reactions to two of the three filament systems of eukaryotic cells. These three systems are composed of:

- actin, a component of the microfilaments
- vimentin, desmin, lamin, or glial fibrillary acidic protein (GFAP), members of the intermediate filament system; and
- tubulins of the microtubules.

Myofibroblasts have not been characterized with regard to tubulins. Based on immunohistochemical staining of these filaments in a given tissue, a classification system has been proposed (Schmitt-Graff et al., 1994). Myofibroblasts that

express only vimentin are termed V-type myofibroblasts, those that express vimentin and desmin are called VD-type, those that express vimentin, α-smooth muscle actin, and desmin are called VAD-type, those that express vimentin and α-smooth muscle actin are called VA-type.

Origin of myofibroblasts

Several questions remain to be answered regarding myofibroblast origin. It is unclear whether myofibroblasts originate from progenitor stem cells or simply transdifferentiate from resident tissue fibroblasts or from tissue smooth muscle cells. Nevertheless two soluble factors have been shown to promote differentiation from embryonic stem cells: platelet-derived growth factor (PDGF) (Jobson et al., 1998) and stem cell factor (SCF) (Der-Silaphet et al., 1998). Myofibroblasts are also thought to represent an intermediate state between conventional fibroblasts and smooth muscle cells. Transforming growth factor-β (TGF-β) (Vaughan et al., 2000) and PDGF (Tang et al., 1996) appear to be the most important growth factors for the differentiation of fibroblasts to myofibroblasts (Figure 1.2.).

In some tissues, e.g., the liver, intestine the synoviocyte of the joint space and brain (astrocyte), myofibroblasts exist in two distinct morphological states: 1) the "activated" myofibroblast, as described above, and 2) the stellate-transformed myofibroblast, which is considered to be a transiently differentiated myofibroblast. *In vivo* activation, as signified by the development of α-SM actin positivity, may be separable from proliferation. Whereas many fibrogenic cytokines such as IL-1, tumor necrosis factor (TNF)-α, PDGF, fibroblast growth factor (FGF), and TGF-β have been incriminated in this process (Kovacs and DiPietro, 1994), TGF-β appears to be the most important cytokine causing the

development of α-SM actin staining and an activated phenotype capable of collagen secretion. It has been determined that the activation of the myofibroblast also requires the presence of matrix molecules, specifically, the ED-A (EIIIA) domain of fibronectin (Serini et al., 1998). Tissue injury gives rise to this specific ED-A domain splice variant of fibronectin. ED-A is the binding site for cell membranes and for other matrix molecules. Following activation of the myofibroblast, PDGF appears to be the factor primarily responsible for myofibroblast proliferation (Jobson et al., 1998).

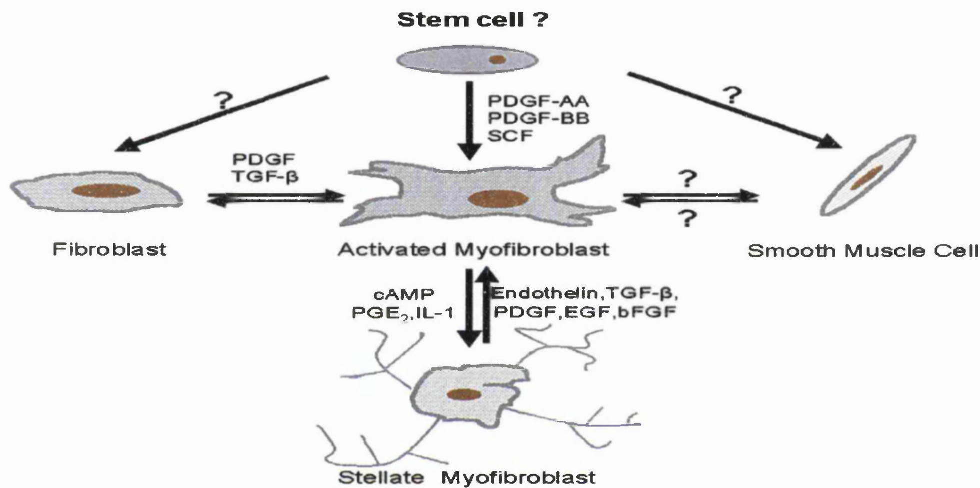

Figure 1.2 Proposed scheme depicting the origin, transdifferentiation, activation, and stellate transformation of myofibroblasts (adapted from (Powell et al., 1999)). Abbreviations used in the diagram: PDGF, platelet-derived growth factor; TGFβ, transforming growth factor β; SCF, stem cell factor; cAMP, cyclic adenosine monophosphate; PGE_2, prostaglandin E_2; IL-1, interleukin-1; EGF, epidermal growth factor; bFGF, basic fibroblast growth factor).

Subtypes of intestinal myofibroblasts

Intestinal myofibroblasts can be divided in two main types: 1) the interstitial cells of Cajal (ICC) and 2) intestinal subepithelial myofibroblasts (ISEMF). The ICC

are located in the submucosa and muscularis propria in association with the smooth muscle layers of the gut. The ISEMF are located in the lamina propria under the epithelial cells immediately subjacent to the basement membrane.

This study concentrates on ISEMF which have attracted the most interest between the two types of cells, primarily because an easy protocol for their isolation is available (Mahida et al., 1997). ISEMF initially were thought to exist as a sheath of fibroblasts, more dense in the region of crypts than at the surface of the colon or in the villi of the small intestine (Pascal et al., 1968), but were later shown to exist as a two-dimensional network that extends throughout the lamina propria of the gut (Joyce et al., 1987). ISEMF stain positive for vimentin and α-smooth muscle actin and negative (or weakly) for desmin (VA-type) (Valentich et al., 1997).

Function of intestinal myofibroblasts

The function of ISEMF is gradually being extended from merely structural components of the intestine to key mediators of various biological processes that occur in the intestine including, cell proliferation, differentiation, apoptosis, morphogenesis, tissue repair, inflammation and the immune response. These essential processes are initiated, maintained and terminated by local interactions between cells and myofibroblasts.

ISEMF are likely to be important in the regulation of intestinal epithelial cell proliferation (Fritsch et al., 1997), differentiation (Halttunen et al., 1996) and migration in response to minor injuries that occur in the intestine (McKaig et al., 1999), both because their location under the basement membrane is ideal for paracrine action and because myofibroblasts secrete the agents that thus far have

been shown to enhance epithelial cell migration in experimental disease states *in vivo* and in wound healing models *in vitro*. It has been proposed that myofibroblast–epithelium cross talk occurs via ECM proteins (Mahida et al., 1997), especially those making up the basement membrane. The latter contains discrete pores that would allow myofibroblast-derived secretory products reach the basal surface of epithelial cells.

Tissue repair is a complex, coordinated event, in which there is release of various lipid mediators such as eicosanoids, gases such as nitric oxide, cytokines such as TNFα , IL-1, IL-6, IL-2, and IL-15, and various growth factors. Many of these factors activate myofibroblasts, resulting in myofibroblast motility and the release of extracellular matrix (ECM) proteins and other growth factors. Remodelling of intestinal tissue is also an important response to gut injury. There is evidence that myofibroblasts take part in this process through the secretion of matrix metalloproteinases and other proteases (Daum et al., 1999), as well as secretion of TGF-α and KGF (Bajaj-Elliott et al., 1998).

Interestingly, even though myofibroblasts are not traditionally regarded as immune cells, they are capable of many immune functions such as secretion of cytokines, growth and differentiation factors, chemokines and expression of adhesion proteins (summarized in table 1.1). Many of the factors secreted by activated myofibroblasts, as well as their respective receptors, are up-regulated in the intestine in various disease states. Examples include prostaglandins via cyclooxygenase (COX)-2 activity (Kim et al., 1998), EGF, TGF-β, HGF, and KGF in small bowel injury (Dignass et al., 1996), in gastric ulcer models or disease (Hull et al., 1998), and in inflammatory bowel disease (Babyatsky et al.,

1996). Adhesion protein expression such as, ICAM-1, VCAM-1, and αβ integrins, by myofibroblasts has also been documented in response to inflammation (Pang et al., 1994). Finally the close proximity of ISEMF to lamina propria T-cells suggests a possible role for them in the growth and development of these cells (Roberts et al., 1997).

Inflammatory mediators secreted by ISEMF	**Receptors expressed by ISEMF**
Cytokines	
IL-1 (Valentich et al., 1997)	IL-1 R(Strong et al., 1998)
IL-6 (Pang et al., 1994)	IL-1Ra (Hinterleitner et al., 1996)
IL-10 (Pang et al., 1994)	TNF-α R(Hernandez-Munoz et al., 1997)
Growth factors	
TGF- β (McKaig et al., 1999)	TGF- β R (Graham et al., 1990)
EGF (Barnard et al., 1995)	PDGF R(Jobson et al., 1998)
KGF (Bajaj-Elliott et al., 1997)	FGF R(Jobson et al., 1998)
HGF (Goke et al., 1998)	EGF R (Jobson et al., 1998)
Chemokines	**Adhesion proteins**
ENA-78 (Casola et al., 1997)	ICAM-1 (Pang et al., 1994)
MIP-1 α (Casola et al., 1997)	VCAM-1 (Pang et al., 1994)
RANTES (Casola et al., 1997)	VLA-4 (Ebert and Roberts, 1996)
IL-8 (Furuta et al., 2000)	

EGF, epidermal growth factor; ENA-78, epithelial neutrophil-activating peptide 78; FGF, fibroblast growth factor; HGF, hepatocyte growth factor; ICAM-1, intracellular adhesion molecule-1; IL,interleukin; KGF, keratinocyte growth factor; MIP-1α macrophage protein 1α; PDGF, platelet derived growth factor; RANTES, regulated, upon activation, normal T cell expressed and secreted; TGF- β, transforming growth factor β; TNF-α, tumor necrosis factor-α; VCAM-1, vascular cell adhesion molecule-1; VLA-4.very late antigen 4

Table 1.1 Soluble factors and receptors important in inflammation expressed by intestinal sub-epithelial myofibroblasts.

1.4 Inflammation

Inflammation is the body's reaction to invasion by an infectious agent, antigen challenge or even just physical, chemical or traumatic damage. The main purpose of inflammation seems to be to bring fluid, proteins, and cells from the blood into the damaged tissues. Under normal conditions tissues lack most of the proteins and cells that are present in blood, since the majority of proteins are too large to cross the blood vessel endothelium. Thus there have to be mechanisms that allow cells and proteins to gain access to extravascular sites where and when they are needed if damage and infection has occurred. The discovery of the detailed processes of inflammation has revealed a close relationship between inflammation and the immune response. Many effector mechanisms capable of defending the body against such antigens and agents have developed and these can be mediated by soluble molecules or by cells. If infection occurs as a consequence of the tissue damage, the innate and, later, the adaptive immune systems are triggered to destroy the infectious agent.

The main features of the inflammatory response are: vasodilation, i.e. widening of the blood vessels to increase the blood flow to the infected area; increased vascular permeability, which allows diffusible components to enter the site; cellular infiltration by the directed movement of inflammatory cells through the walls of blood vessels into the site of injury; and activation of cells of the immune system as well as of complex enzymatic systems of blood plasma. Of course, the degree to which these occur is normally proportional to the severity of the injury and the extent of infection. The development of inflammatory reactions is controlled by cytokines, by products of the plasma enzyme systems, by lipid mediators (prostaglandins and leukotrienes) released from different cells, and by

vasoactive mediators released from mast cells, basophils and platelets. Fast-acting mediators, such as vasoactive amines, modulate the immediate response. Later, newly synthesized mediators such as leukotrienes are involved in the accumulation and activation of other cells. Once leukocytes have arrived at a site of inflammation, they also release mediators that control the later accumulation and activation of other cells.

In inflammatory reactions initiated by the immune system, the ultimate control is exerted by the antigen itself, in the same way as it controls the immune response itself. For this reason, the cellular accumulation at the site of chronic infection, or in autoimmune reactions (where the antigen cannot ultimately be eradicated), is quite different from that at sites where the antigenic stimulus is rapidly cleared.

The inflammatory process inevitably causes tissue damage and is accompanied by simultaneous attempts at healing and repair. The attempts at reconstruction may have different outcomes. If there is little tissue destruction then some organs may be able to regenerate their original structure. This involves removal of the destroyed tissue by phagocytosis with proliferation of capillary blood vessels and lymphatics into the lesion together with fibroblasts and collagen production (so-called granulation tissue), resulting in a dense fibrous scar.

Inflammatory bowel disease

Inflammatory Bowel Disease (IBD) in humans, mainly encompassing Crohn's disease (CD) and ulcerative Colitis (UC), is a complex immunological disorder. CD is a chronic inflammatory disorder of unknown aetiology. The small intestine is most commonly affected, but any part of the gut may be involved. The pathological features include thickening of the submucosa, ulceration, transmural

inflammation with granulomas and dense fibrosis. The lesions may be single or there may be multiple affected areas separated by normal bowel described as 'skip lesions'. UC is a chronic relapsing inflammatory disorder, which may have an acute fulminating presentation. It affects only the colon and rectum, sometimes confined to the latter. The lesion is essentially of the mucous membrane with loss of goblet cells associated with acute inflammatory cell infiltrate.

Our understanding of IBD has been greatly enhanced by research performed in human *in vitro* studies and in particular by *in vivo* studies using appropriate animal models. Such animal models allow both the examination of inflammatory processes (both early and late events) as well as the evaluation of new therapeutic modalities. Overall 63 models have been described, most within the last decade. These IBD animal models can be divided into 5 different categories: (1) antigen-induced colitis and colitis induced by microbials; (2) other inducible forms of colitis (chemical, immunological, and physical); (3) genetic colitis models (transgenic and knock-out models); (4) adoptive transfer models, and (5) spontaneous colitis models. In spite of the high overall number of models, none of them is the 'perfect' model and therefore numerous aspects of IBD are still under intensive investigation. Even though the aetiology is still unclear, the pathogenesis of these disorders is better understood, and it is increasingly clear that these diseases represent the outcome of three essential interactive cofactors: genetic host susceptibility, enteric microflora and mucosal immunity.

Genetic factors influencing IBD

Linkage studies have implicated several genomic regions as likely containing IBD susceptibility genes, with some observed uniquely in CD or UC, and others

common to both disorders (summarised in table 1.2). The best replicated linkage region, termed IBD1, on chromosome 16q contains the CD susceptibility gene, nucleotide-binding oligomerization domain 2 (NOD2). NOD2 was identified by searching public genomic libraries for genes encoding similar proteins to NOD1. NOD1 is an intracellular protein composed of a N-terminal caspase recruitment domain (CARD), a centrally located nucleotide binding domain (NBD), and a leucine rich repeat (LRR) domain at its C-terminus which could activate nuclear factor κB (NFκB) and also promote apoptosis (Inohara et al., 1999). NOD2 has one more CARD at its N-terminal than NOD1. It is expressed primarily in monocytes and following stimulation by bacterial lipopolysaccharide (LPS), activates NF-κB. So far, approximately one hundred sequence variants have been detected in NOD2 gene, most of which are rare mutations, located in LRR domain and are thought to initiate CD. The most likely mechanism by which the mutations in NOD2 contribute to the pathogenesis of IBD is either by raising the sensitivity of monocytes to bacterial pathogenic agents, resulting in overexpression of certain pro-inflammatory cytokines, or by causing deficiency of apoptosis, leading to monocyte accumulation in intestinal mucosa (Schreiber et al., 1998; Hugot et al., 2001; Ogura et al., 2001a; Ogura et al., 2001b; Inohara et al., 2001).

The significance of the colonic microflora in IBD

In human IBD, inflammation is present in parts of the gut containing the highest bacterial concentrations. Furthermore, increased mucosal absorption of viable bacteria and bacterial products is found in IBD. Serum and secreted antibodies are increased and mucosal T-lymphocytes that recognize luminal bacteria are present.

IBD locus designation	Chromosomal location	Diagnoses	Candidate genes within or near locus
IBD1	16q12	CD	NOD2 (Hugot et al., 1996)
IBD2	12q13	UC	VDR, IFN-γ (Satsangi et al., 1996)
IBD3	6p13	CD, UC	MHC I and II, TNF-α (Duerr et al., 1998)
IBD4	14q11	CD	TCR α/δ complex (Duerr et al., 2000)
IBD5	5q31–33	CD	IL-3, IL-4, IL-5, IL-13, CSF-2 (Rioux et al., 2000)
IBD6	19p13	CD, UC	ICAM-1,C3,TBXA2R, LTB4H (Rioux et al., 2000)
Other loci	1p36	CD, UC	TNF-R family, CASP9 (Cho et al., 1998)
Other loci	7q	CD, UC	MUC-3 (Satsangi et al., 1996)
Other loci	3p	CD, UC	HGFR, EGFR, GNAI2 (Satsangi et al., 1996)

VDR, vitamin D receptor; IFN, interferon; TCR, T-cell receptor; CSF, cerebrospinal fluid; TBXA2R, thromboxane A2 receptor; LTBH4H, leukotriene B4 hydroxylase; CASP, caspase; MUC3, mucin 3; GNAI2, inhibitory guanine nucleotide-binding protein

Table 1.2 IBD locus designation, chromosomal location, diagnoses and candidate genes. Table adapted with modifications from (Bonen and Cho, 2003).

However, there is evidence that the immune system reacts over-aggressively towards the normal luminal flora rather than the flora being altered in IBD (Swidsinski et al., 2002). Several approaches have been used in attempts to discover a specific microbial agent in the cause of IBD, but no specific micro-organism has been directly associated with the pathogenesis of IBD so far. Analysis of the luminal enteric flora, however has revealed differences in the composition of the flora compared to healthy controls (Schultsz et al., 1999). In CD, concentrations of *Bacteroides, Eubacteria* and *Peptostreptococcus* are increased (Krook et al., 1981; Van de Merwe et al., 1988), whereas *Bifidobacteria*

numbers are significantly reduced (Favier et al., 1997). Furthermore, in UC, concentrations of facultative anaerobic bacteria are increased (Campieri and Gionchetti, 2001).

Cell mediated immunity in IBD

The first study implicating classical immune mechanisms in IBD pathogenesis demonstrated the cytotoxic action of peripheral blood white blood cells derived from UC patients, against human fetal colon cells in vitro (Perlmann and Broberger, 1963). Since then various studies have provided evidence that all intestinal cell types are affected in IBD. Increasing evidence suggests that dysregulation of mucosal T-cells plays a key role in the pathogenesis of IBD (MacDonald et al., 2000). Studies in animal models of experimental colitis have also definitely confirmed abnormal immune responses to potential immunogenic stimuli (Sadlack et al., 1993; Mombaerts et al., 1993; Kuhn et al., 1993; Koh et al., 1999; Wirtz et al., 1999). However, the molecular mechanisms responsible for the initiation and maintenance of lymphocyte activation in the intestinal mucosa remains elusive.

Patients with CD and UC both overproduce macrophages, probably because of an increased demand of macrophages in the inflamed gut (Grip et al., 2003). Emerging evidence demonstrates that various cell types populating the mucosa have an active role in intestinal immunity and inflammation. Epithelial, endothelial, fibroblasts, and nerve cells display broad and previously unsuspected effector and regulatory functions, including immune-like functions, and interact intimately with lymphoid cells. Among the various immune-nonimmune cell interactions occurring in the gut, the functional communication existing between

epithelial cells and lymphocytes has been the most extensively studied (Campbell et al., 1999b). Studies showing that human intestinal epithelial cells produce cytokines that regulate the proliferation of intestinal lamina propria mononuclear cells, e.g IL-7 (Watanabe et al., 1995), and express functional cytokine receptors for several T- cell-derived cytokines such as IL-2R beta, IL-4R, IL-7R (Reinecker and Podolsky, 1995) have strengthened the concept of an exchange of regulatory signals between the epithelial and immune compartments of the mucosa.

Role of mesenchymal cells in IBD

A variety of other nonimmune cells actively participate in IBD pathogenesis, including "structural" cells such as fibroblasts, myofibroblasts, and muscle cells the so-called cells of mesenchymal origin. These cells produce a variety of extracellular matrix proteins, but this production is altered in IBD, as exemplified by the increased production of collagen observed in IBD patients (Lawrance et al., 2001). These alterations are associated with classical pathological features of IBD, such as the increased proliferation of muscle cell layers, formation of thickened bowel wall, and strictures. Intestinal smooth muscle cells and/or myofibroblasts have also been incriminated in the fibrotic process that characterizes CD. Myofibroblast proliferation is seen in response to inflammation in many tissues regardless of the aetiology of the insult. Excess myofibroblast proliferation persisting beyond the inflammatory insult, may be a risk factor leading to scarring and pathological remodelling of the tissue.

The role of the mucosal immune system in the pathogenesis of IBD is probably the most complex, as it involves various cellular components as well as a large variety of inflammatory mediators, of which the most important are probably cytokines, many of which are dysregulated in IBD.

Pro-inflammatory cyokines in IBD

Differing cytokine profiles have been identified for UC and CD. While it has been hypothesized that CD is a T-helper 1 (Th1) dominated immune reaction, there is also evidence that UC is characterized by T-helper 2 (Th2) domination. However, pro-inflammatory cytokines tend to be consistently elevated in IBD. IL-1, IL-6, and tumor necrosis factor-alpha (TNF-α) are found elevated in both inflammatory bowel conditions (Reinecker et al., 1993; Nikolaus et al., 1998). Several studies have suggested particular relevance of IFN-γ to CD, as indicated by the spontaneous release of IFN-γ and increased IFN-γ mRNA expression by lamina propria mononuclear cells and the presence of IFN-γ–secreting T-cells in actively inflamed mucosa (Fais et al., 1991; Breese et al., 1993).

Evidence from human and animal studies have highlighted the central role for TNF-α in the pathogenesis of IBD. These include the dramatic results from a clinical trial where by approximately 2/3 of the patients responded positively to a single infusion of the mouse/human chimeric monoclonal anti-TNF-α antibody infliximab (Targan et al., 1997) and the development of a Crohn's like phenotype in mice overexpressing TNF-α (Kontoyiannis et al., 1999). Infliximab was approved for clinical use in active CD in the USA in the autumn of 1998, and received a positive advice for the European Medicines Evaluation Agency in May 1999 and its success has resulted in various alternative attempts aimed at reducing TNF-α in IBD patients. Examples include the humanized monoclonal antibody CDP571, the human monoclonal antibody D2E7 (adalimumab), the anti-TNF human antibody Fab' fragment-polyethelene glycol (PEG) conjugate CDP870, and the small molecules thalidomide and CNI-1493 (MAP-kinase inhibitor).

Anti-inflammatory cytokines in IBD

In IBD significant changes also occur in the tissue expression of immunoregulatory cytokines. Contradicting results exist for the presence of IL-12 in IBD. Various immunohistological studies indicate that *in situ* IL-12 is overproduced by macrophages in CD, but not in UC (Monteleone et al., 1997; Parronchi et al., 1997) and macrophages that are isolated from the inflammatory lesions of patients with CD produce increased amounts of IL-12 *ex vivo* (Liu et al., 1999) , whereas macrophages that are isolated from patients with UC produce decreased amounts of IL-12, compared with those from normal tissues. The cytokine profile in UC patients provides more evidence of an exaggerated Th2 response, elevated IL-5 but no significant elevation of IFN-γ and other cytokines associated with an overactive Th1 response (Fuss et al., 1996), even though there is no evidence of increased amounts of IL-4, the definitive Th2 cytokine. Overall the pathogenesis of IBD is characterized by an imbalanced activation of Th1 and Th2-cytokines. This is exemplified by IL-10, an anti-inflammatory cytokine which down-regulates the production of Th1-derived cytokines. The relative deficiency of IL-10 in patients with UC may contribute to persistent inflammatory changes (Ishizuka et al., 2001). IL-10-deficient mice spontaneously develop intestinal inflammation characterized by discontinuous transmural lesions affecting the small and large intestines and by dysregulated production of pro-inflammatory cytokines, indicating that endogenous IL-10 is a central regulator of the mucosal immune response (Rennick and Fort, 2000).

Even though cytokines have been the most studied area in IBD, other soluble mediators like growth factors, eicosanoids, reactive oxygen and nitrogen

metabolites especially inducible nitric oxide synthase (iNOS), short chain fatty acids, are increasingly being recognized as having a role in IBD.

1.5 Chemokines

Chemokines constitute a superfamily of small (8-10 kDa) pro-inflammatory cytokines that are involved in a variety of immune and inflammatory responses, acting primarily as chemoattractants and activators of specific types of leukocytes. Four classes of chemokines have been defined depending on the number and arrangement of the conserved cysteine residues (C) at the N-terminal cysteine residues of the mature proteins. These four classes are: CC chemokines in which the first two conserved cysteine residues are adjacent, CXC chemokines which have one amino acid separating the first two conserved cysteine residues, C chemokines which lack two of the four conserved cysteine residues and finally CX3C chemokines in which three amino acids separate the two cysteines.

The human chemokine system comprises about 50 distinct chemokines (Table 1.3) and 20 chemokine receptors. The CXC chemokine genes, with a few exceptions, most notably Stromal Derived Factor 1 (SDF-1/CXCL12), which has been localized to human chromosome 10 (Shirozu et al., 1995) are all clustered on human chromosome 4 (Oppenheim et al., 1991). The majority of CC chemokines are clustered around chromosome 17 (Opdenakker and Van Damme, 1994).

Recently, the nomenclature of chemokines has been changed to a systematic system. The CC chemokines have been renamed CC chemokine ligand (CCL) 1, 2, 3, etc., and the CXC chemokines, (CXCL1), 2, etc (Murphy, 2002). In this study we use the old nomenclature but also provide the new name when introducing a new chemokine.

Official name	Commonly used synonyms	Gene locus
CXC(α) Chemokines		4q12-q13
CXCL1	GROα	4q12-q13
CXCL2	GROβ; MIP-2α	4q12-q13
CXCL3	GROγ; MIP-2β	4q12-q13
CXCL4	PF4	4q12-q13
CXCL5	ENA-78	4q12-q13
CXCL6	GCP-2	4q12-q13
CXCL7	NAP-2	4q12-q13
CXCL8	IL-8	4q21.21
CXCL9	Mig	4q21.21
CXCL10	IP-10	4q21.21
CXCL11	I-TAC	10q11.1
CXCL12	SDF-1α; SDF-1β; PBSF	4q21
CXCL13	BCA-1; BLC	5q31
CXCL14	BRAK	Unknokwn
CXCL15	Not applicable	17p13
CXCL16	Not applicable	
CC(β) Chemokines		17q11.2
CCL1	I-309	17q11.2
CCL2	MCP-1; MCAF	17q11.2
CCL3	MIP-1α; MIP-1αS; LD78α	17q11.2
Not applicable	LD78β, MIP-1aP	17q11.2
CCL4	MIP-1β	17q11.2
CCL5	RANTES	17q11.2
CCL7	MCP-3	17q11.2
CCL8	MCP-2	17q11.2
CCL11	Eotaxin	17q11.2
CCL13	MCP-4	17q11.2
CCL14	CC-1; HCC-1	17q11.2
CCL15	HCC-2; Lkn-1; MIP-5; MIP-1δ	17q11.2
CCL16	HCC-4; LEC; NCC-4; LMC	16q13
CCL17	TARC	17q11.2
CCL18	DC-CK-1; PARC; MIP-4	9p13
CCL19	MIP-3β; ELC; exodus-3	2q33-q37
CCL20	MIP-3α; LARC; exodus-1	9p13
CCL21	6Ckine; SLC; exodus-2	16q13
CCL22	MDC	17q11.2
CCL23	MPIF-1; MIP-3	7q11.23
CCL24	MPIF-2; eotaxin-2	19q13.2
CCL25	TECK	7q11.23
CCL26	Eotaxin-3; MIP-4α	9p13
CCL27	ESkine; CTACK	5p12
CCL28	MEC	
C(γ) Chemokines		1q23
XCL1	Lymphotactin α; SCM-1α	1q23
XCL2	Lymphotactin β; SCM-1β	
CX3C (δ) Chemokine		16q13
CX3CL1	Fractalkine	

Table 1.3 Human chemokines: systematic nomenclature, common names and chromosomal location. This table also highlights the clusters of chemokines that are typically found on chromosome 4 (CXC) and 17 (CC).

Chemokines can also be classified based on their pattern of expression. Homeostatic chemokines are expressed constitutively and participate in re-circulation of leukocytes between tissue and lymphatics and in the traffic of leukocytes within compartments of lymph nodes and thymus (Cyster, 1999). In contrast, inflammatory chemokines are induced by infection and other pro-inflammatory stimuli. This division however should not be regarded as absolute but rather as a rule with some exceptions (Figure 1.3). Constitutive expression of inducible chemokines is observed in neoplastic disorders (Haghnegahdar et al., 2000; Azenshtein et al., 2002). Moreover a number of molecules behave as both constitutive and inducible chemokines. For instance, Macrophage-Derived Chemokine (MDC/CCL22) was initially described as a chemokine constitutively expressed in certain cell types and lymphoid organs (Godiska et al., 1997). Subsequent work, prompted by the recognition that this molecule attracted preferentially polarized Th2 cells, has shown that MDC is expressed in a regulated way (Bonecchi et al., 1998a; Andrew et al., 1998).

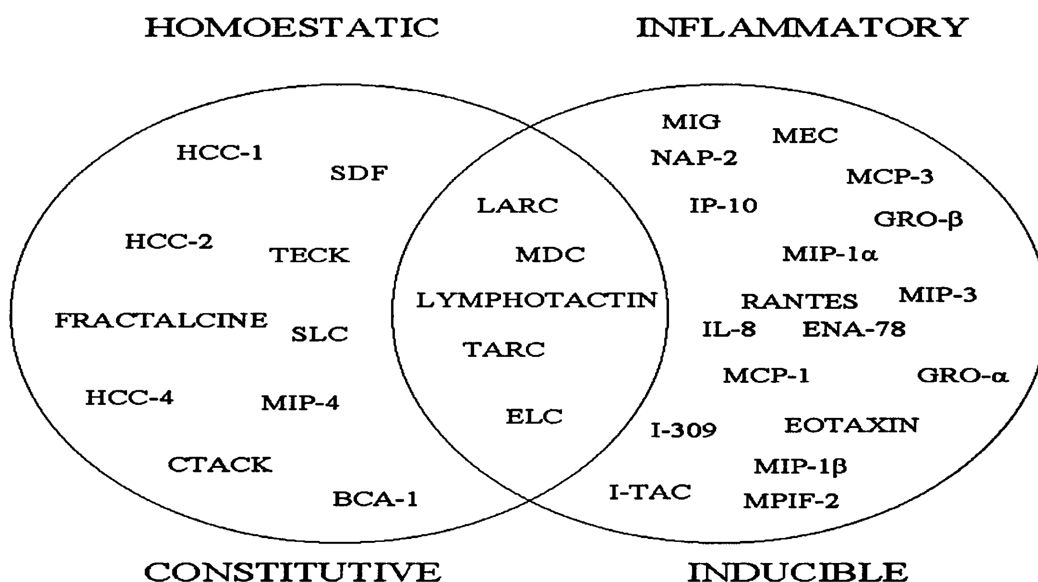

Figure 1.3 Chemokines can be classified as constitutive or inducible. This arrangement also determines their functions as regulating leukocyte trafficking or involvement in inflammatory processes.

ELR chemokines

The CXC chemokines can be further subdivided into those that contain the sequence glutamic acid–leucine–arginine (the ELR motif) near the N-terminal preceding the CXC sequence and those that do not. This motif dictates the angiogenic activity of those chemokines, with members containing the ELR motif (ELR^+) being potent promoters of angiogenesis (Strieter et al., 1995). In contrast, members that are induced by interferons and lack the ELR motif (ELR^-) are potent inhibitors of angiogenesis (Angiolillo et al., 1995; Sgadari et al., 1997). Most CXC chemokines are ELR^+, apart from the CXC3 ligands, IP-10, Mig, I-TAC and Platelet Factor 4 (PF-4/CXCL4). Although SDF-1 is another ELR^- CXC chemokine, its role in angiogenesis remains unclear (Arenberg et al., 1997; Zou et al., 1998).

Chemokine structure

Despite considerable differences in primary sequence, CXC and CC chemokines have a remarkably similar, three-dimensional structure. This structure is comprised of a short, NH_2-terminal region, a large core, which is stabilized by the disulfide bonds and hydrophobic interactions and characterized by three anti-parallel β-strands, and a COOH-terminal α-helix. The NH_2-terminal binding site is required for receptor signalling upon ligation, and the length and amino acid composition of the NH_2 terminus determines whether a chemokine will bind with high affinity to a receptor and whether binding has agonistic vs. antagonistic effects (Clark-Lewis et al., 1995). The second major site of interaction between a chemokine and its cognate receptor lies within the loop that follows the second

cysteine (Blanpain et al., 2001). The relative importance of each of these two contact regions to overall ligand affinity varies depending on the receptor examined and reflects synergy between several important contacts.

Chemokine receptors

The chemokine receptors (Table 1.4) are seven-transmembrane-spanning, G-protein-coupled receptors (GPCRs) and are classified based on the class of chemokines they bind, e.g., CC chemokine receptors bind CCLs, and CXC chemokine receptors bind CXCLs. Two highly promiscuous non-signalling (silent) receptors or binding proteins, DARC (Neote et al., 1994) and D6 (Nibbs et al., 1997), have also been identified, even though their function remains unclear.

CXC chemokine receptors	**Main ligands**	**Expression**
CXCR1	IL-8, GCP-2	N, M, EN
CXCR2	GROα-γ, ENA78, NAP-2	N, M, E, EN
CXCR3 -A	IP-10, MIG, I-TAC	T, B, SM
CXCR3 -B	IP-10, MIG, I-TAC, PF-4	T, B, SM
CXCR4	SDF1-α	Ubiquitous
CXCR5	BCA-1	B, T
CXCR6	CXCL16	T
CC chemokine receptors		
CCR1		
CCR2	RANTES,MIP1-α, MCP-2, 3	M, DC, T, N, E
CCR3	MCP-1, 2, 3, 4	M, DC, T, NK,
CCR4	Eotaxin, RANTES, MCP-4	FB, EN
CCR5	MDC, TARC	E, B, T, P, EP
CCR6	MIP1-α, β, RANTES	T, DC, B, P
CCR7	MIP3- α	T, DC, NK, M
CCR8	ELC, SLC	DC, T, B
CCR9	I-309	DC, T, B, NK
CCR10	TECK	T, M, NK, B, EN
C chemokine receptor	MEC, CTACK	T, EN, FB
XCR1	Lymphotactin α-β	T
CX3Cchemokine receptor		
CX3CR1	Fraktalkine	T, NK

N: neutrophils, M: monocytes, E: eosinophils, DC: dendritic cells
EN: endothelial, EP: epithelial, NK: natural killer cells, FB; fibroblasts

Table 1.4 Human chemokine receptors, their ligands and pattern of expression.

Chemokine receptors belong to Class A GPCRs, which are characterized by high homology with rhodopsin, the prototypical family member, although their structure has not been completely resolved yet. In addition to the transmembrane spanning domains, the receptor contains three intracellular loops, three extracellular loops, an N-terminal extracellular domain, and a C-terminal cytoplasmic tail. Typically, chemokine receptors are 340-370 amino acids in length with 25-80% amino acid identity. Common features include an acidic NH_2 terminus, a conserved 10-amino acid sequence in the second intracellular loop, a short basic third intracellular loop, and one cysteine in each of the four extracellular domains (Murphy et al., 2000). The interaction of chemokines with their receptors is characterized by considerable promiscuity. Most known receptors have been reported to interact with multiple ligands and most ligands interact with more than one receptor. For instance, all four Monocyte Chemotactic Proteins (MCPs) interact with CCR2, and at least MCP-2/CCL8, MCP-3/CCL7 and MCP-4/CCL13 also recognize CCR1 or CCR3. However, certain chemokines bind only one receptor and vice versa, such as the exclusive interactions of CXCR4 with SDF-1, CXCR5 with B-cell-attracting chemokine (BCA-1/CXCL13), CCR6 with Macrophage-Inflammatory Protein-3α (MIP-3α/CCL20), CCR9 with Thymus-Expressed ChemoKine (TECK/CCL25), CCR10 with Cutaneous T-cell-Attracting ChemoKine (CTACK/CCL27), and CXCR6 with CXCL16. Historically, the identification and characterization of chemokine-receptor–ligand specificities was based on agonist activity. Recently, it has become clear that the interplay of the receptors and ligands in physiological conditions is complicated by the presence of agonist and antagonist activities. The CXCR3 agonists have been reported to be antagonists of CCR3, (Loetscher et al.,

2001). Additionally the CCR1 agonist MCP-3 is an antagonist for CCR5 (Blanpain et al., 1999). A thorough characterization of the binding of all known chemokine ligands with all known receptors is likely to reveal additional examples of cross-reactivity between chemokine ligands and receptors.

Biological functions of chemokines

The role of chemokines initially was thought to be restricted to provide directional cues for the trafficking of leukocytes to sites of inflammation. The discoveries that chemokines can block human immunodeficiency virus (HIV) replication (Cocchi et al., 1995) and that their receptors have essential functions in fusion of HIV to target cells (Feng et al., 1996) propelled this field into the limelight, and raised expectations that chemokines might hold the key to understanding HIV-mediated pathogenesis. Although this promise has yet to be fulfilled, the increased interest in the field resulted in many findings that suggest chemokine functions are not limited to cellular recruitment and might be involved in a variety of biological functions including, T-cell activation (Taub et al., 1996), dendritic cell maturation (Sozzani et al., 1998), neutrophil degranulation (Meddows-Taylor et al., 1999), B cell antibody class switching (Bacsi et al., 1999), macrophage activation (Liu et al., 2000b) and gene transcription, proliferation and apoptosis (Thelen and Baggiolini, 2001).

Evidence for the roles chemokines and chemokine receptors might play in the pathogenesis of different acute or chronic inflammatory diseases is also rapidly increasing (Gerard and Rollins, 2001). Many studies in this field are focused on examining the response of animal models of acute inflammation to genetic elimination of the chemokine receptors (tables 1.5 and 1.6). Interestingly, the

deletion of receptors pivotal to basal trafficking and homing have striking phenotypes, whereas those that are inducible remain viable and healthy when maintained in a standard pathogen-free environment but are more susceptible to infection with large doses of pathogen than their wild type counterparts. The fact that only one chemokine receptor knockout has proved to be embryo lethal could imply that some compensation occurs for the loss of a given chemokine receptor, and is probably due to the redundancies of the ligands and receptors. Of all the chemokine knockouts (a selection of which is summarised in Table 1.7), MCP-1 deletion has the greatest effect (Gu et al., 1998; Izikson et al., 2000; Huang et al., 2001), an unexpected finding since its receptor CCR2 can be activated by all five MCPs.

Chemokines in the gastrointestinal system

Expression of IL-8/CXCL8, Epithelial Neutrophil Activating Peptide-78 (ENA-78/CXL5), MCP-1, eotaxin/CCL11, IP-10 and Fraktalkine/CX3CL1 has been detected in animal models as well as human disease condition of the gastrointestinal inflammation (Mazzucchelli et al., 1994; MacDermott et al., 1998; Uguccioni et al., 1999; Muehlhoefer et al., 2000; Hogan et al., 2001). TECK and its specific receptor CCR9, which is preferentially expressed on gut-homing intestinal memory T-cells (Zabel et al., 1999; Agace et al., 2000; Kunkel et al., 2000), are found up-regulated in small bowel but not colonic CD (Papadakis et al., 2001), suggesting that homing of T-cells to distinct gastrointestinal sites is differentially regulated in both inflammatory and basal conditions. Analysis of colitis induction in $CCR2^{-/-}$ or $CCR5^{-/-}$ mice showed significant protection from disease (Andres et al., 2000). Furthermore, in a rat model of chronic colitis, a CCR1 and CCR5 antagonist reduced cellular infiltration and inflammation (Ajuebor et al., 2001).

However, individuals carrying a mutated form of CCR5 (D32-CCR5) are equally susceptible to colitis (Martin et al., 2001), indicating that CCR5 is not necessary for development of disease.

Ablated gene	Phenotype
CCR1	Reduced NK-cell recruitment (Shang et al., 2000) Prevention of heart transplant rejection (Gao et al., 2000)
CCR2	Protection in EAE (Izikson et al., 2000) Reduced symptoms of atherosclerosis (Boring et al., 1998) Partial protection in DSS induced colitis (Andres et al., 2000) Increased airway allergic inflammation in response to Aspergillus (Blease et al., 2000) Reduced airway hypersensitivity following allergen challenge (Campbell et al., 1999a) Reduced Langerhans cell migration to draining lymph nodes (Sato et al., 2000) Increased severity of experimental glomerulonephritis (Bird et al., 2000) Inability to clear *Listeria* infections (Kurihara et al., 1997)
CCR3	Decreased eosinophils, increased airway hyper responsiveness (Humbles et al., 2002)
CCR4	No protection of airway inflammation in the OVA sensitization model, reduced fatality in LPS induced sepsis (Chvatchko et al., 2000) Reduced airway hyper responsiveness (Bishop and Lloyd, 2003)
CCR5	Reduced clearance of *Listeria* infections (Zhou et al., 1998) No protection in EAE (Tran et al., 2000) Resistance to DSS induced colitis (Andres et al., 2000)
CCR6	Reduced humoral immune response to oral antigens and increase in cells of select T lymphocyte populations within the mucosa (Cook et al., 2000)
CCR7	Altered secondary lymphoid organ structure (Forster et al., 1999) Reduced humoral immune responses and contact sensitivity (Saeki et al., 1999)
CCR8	Reduced Th2 responses (Chensue et al., 2001) Decreased eosinophilia in airway inflammation (Chung et al., 2003)
CCR9	Reduced pre B cells and reduction in T-cell receptor $\gamma\delta$ (+) gut intraepithelial lymphocytes (Wurbel et al., 2001)

Table 1.5 CC chemokine knock out mice. Highlighted in red are the knockouts and the studies with relevance to the gastrointestinal system.

Ablated gene	Phenotype
CXCR1	Reduced neutrophil recruitment (Gerard and Rollins, 2001)
CXCR2	Lymphadenopathy (Cacalano et al., 1994) Delayed wound healing (Devalaraja et al., 2000) Defective acute neutrophil accumulation (Hall et al., 2001) Decreased protection against Toxoplasma gondii infection (Del Rio et al., 2001) Reduce macrophage recruitment in atherosclerosis (Boisvert et al., 1998)
CXCR3	Long term protection in heart transplant (Hancock et al., 2000)
CXCR4	Embryo lethal (Nagasawa et al., 1996) Vasculature defects (Nagasawa et al., 1996; Tachibana et al., 1998)
CX3CR1	Defects in B-cell homing and lymph nodes (Forster et al., 1996)

Table 1.6 CXC and CX3C chemokine receptors knock out mice

Ablated gene	Phenotype
MCP-1	Development of resistance by the gastrointestinal nematode *Trichuris muris* (deSchoolmeester et al., 2003) Suppression of IFN-γ and up-regulation of TGF-β production in lamina propria (Gonnella et al., 2003) Disruption in the balance of Th1 and Th2 cytokines (DePaolo et al., 2003)
Eotaxin	Reduced eosinophil accumulation in the gastrointestinal tract (Hogan et al., 2001)
IP-10	Small bowel allografts are resistant to acute allograft rejection (Zhang et al., 2004)
BCA-1	Impaired development of Peyer's patches and mesenteric lymph nodes (Ansel et al., 2000)

Table 1.7 Chemokine knock out mice with relevance to the gastrointestinal tract.

CXCR3: expression and functions

Because this study will concentrate on CXCR3-mediated effects a detailed section on CXCR3 and its ligands is included in this introduction.

CXCR3 is a seven-transmembrane receptor, which is highly induced by T-cell activation. CXCR3 expression can be detected on IL-2-activated T-cells, on allogeneically activated T-cells, and on thymocytes during lymphopoiesis (Loetscher et al., 1996; Loetscher et al., 1998; Qin et al., 1998; Ebert and McColl, 2001; Romagnani et al., 2001b). Although exogenous IL-2 can enhance CXCR3 expression on T-cells in culture, CXCR3 is also expressed on 35–40% of normal blood T-cells (Loetscher et al., 1998; Qin et al., 1998; Mohan et al., 2002). CXC3 was first identified in incomplete form in 1995 on a genomic clone isolated by polymerase chain reaction (PCR)-based homology hybridization. At the same time, a full-length cDNA was independently isolated from an IL-2-activated T-cell library (Loetscher et al., 1996). The gene was named GPR9 and was originally mapped incorrectly to human chromosome 8p11.2-12 (Marchese et al., 1995) and later mapped correctly to Xq13 (Loetscher et al., 1998). The open reading frame is interrupted by one intron in the region encoding the N-terminal segment and the predictive polypeptide is 368 aa in length with a molecular mass of ~ 40 kDa. Other lymphocytes expressing CXCR3 are NK cells, and a small subset of normal circulating B cells, (Trentin et al., 1999; Inngjerdingen et al., 2001).

Furthermore CXCR3 has also been detected in eosinophils (Jinquan et al., 2000), endothelial (Salcedo et al., 2000; Romagnani et al., 2001a), pericytes (Bonacchi et al., 2001), and microglia (Biber et al., 2001; Rappert et al., 2002). CXCR3 has been detected preferentially on Th1 cell lines and clones in vitro (Sallusto et al., 1998; Bonecchi et al., 1998a; Bonecchi et al., 1998b; Sallusto et al., 1999). Blood T-cells positive for CXCR3 are mostly CD45RO^{+} memory cells, which express high levels of β_1 integrins (Qin et al., 1998). Additionally, several studies have shown that CXCR3 plays an important role in the pathophysiology of Th1-type

diseases such as autoimmune disorders and viral infections (Balashov et al., 1999; Liu et al., 2000a; Reinhart et al., 2002) and has therefore been suggested as a marker of Th1 cells, even though CXCR3 did not discriminate between Th1 and Th2-dominated responses *in vivo* (Annunziato et al., 1999).

CXCR3 knockout mice have been tested in allograft rejection models. Like most knockouts of inducible chemokine receptors, CXCR3$^{-/-}$ mice retain a normal phenotype when unchallenged, but showed profound resistance to development of acute allograft rejection and CXCR3-deficient allograft recipients treated with cyclosporin A maintained their allografts permanently and without evidence of chronic rejection (Hancock et al., 2000). Similar results were observed in pancreatic islet allograft CXCR3-deficient recipients (Baker et al., 2003).

CXCR3 agonists

CXCR3 binds three inflammatory/inducible, ELR$^-$ CXC chemokines: **M**onokine **i**nduced by human interferon-γ (Mig/CXCL9), **I**nterferon-inducible **10**-kDa **P**rotein (IP-10/CXCL10) and, **I**nterferon-inducible **T**-cell **α** **C**hemoattractant (I-TAC/CXCL11) (Loetscher et al., 1996; Cole et al., 1998), all of which chemoattract and induce calcium flux in activated T-cells and CXCR3-transfected cells.

IP-10

IP-10 is expressed constitutively at low levels in thymic, splenic, and lymph node stroma (Gattass et al., 1994) but its expression can be highly induced by IFN-α, β and γ and LPS in a variety of cell types, including endothelial cells, keratinocytes, fibroblasts, mesangial cells, astrocytes, monocytes, and neutrophils (Luster and

Ravetch, 1987). It has been demonstrated to be highly expressed in many Th1-type inflammatory diseases, including skin diseases (Gottlieb et al., 1988; Flier et al., 1999; Flier et al., 2001), atherosclerosis (Mach et al., 1999), multiple sclerosis (Sorensen et al., 1999; Balashov et al., 1999), allograft rejection (Melter et al., 2001; Zhao et al., 2002) and others. Studies with inhibitory antibodies and IP-10-deficient mice have revealed that IP-10 plays an important role in the recruitment of effector T-cells into inflammatory tissues (Hancock et al., 2001; Dufour et al., 2002; Zhang et al., 2002).

Mig

Mig was originally identified by differential screening of a cDNA library prepared from lymphokine-activated macrophages (Farber, 1990). Mig expression can be induced on monocytes and macrophages, hepatocytes, fibroblasts, keratinocytes, and endothelial cells in response to IFN-γ (Farber, 1990; Farber J. M., 1993; Farber, 1993; Amichay et al., 1996). In mice, systemic administration of IFN-γ and infection with protozoa or virus was associated with induction of the Mig gene in a variety of tissues, including liver, spleen, heart, and lung (Amichay et al., 1996). A comparison of chemokine protein sequences shows that human Mig is related to IP-10, with the chemokines sharing 37% amino acid identity. The genes for human Mig and IP-10 were found to be adjacent on chromosome 4q21, suggesting a close evolutionary relationship (Lee and Farber, 1996).

I-TAC

I-TAC was initially identified by screening a cDNA library from primary human astrocytes stimulated with various cytokines and is ~ 40% identical at the amino

acid level to IP-10 and Mig. Expression of human I-TAC has been detected in cultured primary monocytes, cultured foetal astrocytes, microglial cell line (Cole et al., 1998), astrocytoma cells (Rani et al., 1996), atheroma-associated cells (Mach et al., 1999), bronchial epithelial cells (Sauty et al., 1999), neutrophils (Gasperini et al., 1999) and keratinocytes (Tensen et al., 1999). Moderate expression has also been detected in human central nervous system (Luo et al., 1998), pancreas, lung, thymus and spleen tissues (Cole et al., 1998). In addition, I-TAC was recently shown to be up-regulated in IFN-γ-stimulated human endothelial cells, suggesting a role for this chemokine in T lymphocyte recruitment to sites of inflammation (Mazanet et al., 2000). I-TAC appears to have the highest affinity for CXCR3 with a dissociation constant (K_d) $\approx$ 3nM (Clark-Lewis et al., 2003).

Despite the redundancy in the chemokine system, there is evidence to support the notion that IP-10, Mig and I-TAC exert different biological activities. Although freshly isolated T-cells respond to I-TAC, curiously they are relatively less responsive to IP-10 or Mig (Rabin et al., 1999; Mohan et al., 2002). Recent studies have shown that the CXCR3 ligands exhibit unique temporal and spatial expression patterns suggesting that they have non-redundant functions *in vivo* (Sorensen et al., 1999; Flier et al., 2001; Agostini et al., 2001). Moreover a CXCR3-specific mAb named 1C6 has been reported to block human IP-10, but not human Mig, binding to CXCR3 (Qin et al., 1998). Finally it has been demonstrated that I-TAC binds allotopically with IP-10 and Mig to the active conformation of CXCR3 (Cox et al., 2001).

Additional ligands for CXCR3

It has been suggested that leukocyte recruitment during inflammatory responses may be tightly regulated by chemokines acting as agonists at some receptors and antagonists at others. With respect to CXCR3 and CCR3, it has been suggested that eotaxin could act as a natural antagonist of CXCR3 by blocking IP-10-mediated receptor activation *in vitro* (Weng et al., 1998). This was subsequently disputed by a study, which demonstrated, reciprocally, that the CXCR3 ligands could antagonize CCR3 function (Loetscher et al., 2001). CXCR3 ligands inhibit CCR3 responses not only to eotaxin but also to eotaxin-2/CCL24 and eotaxin-3/CCL26. Additionally, although the three eotaxins exhibit low structural homology, they were all similarly inhibited by the CXCR3 ligand. Finally mouse Secondary Lymphoid-tissue Chemokine (SLC/CCL21) which binds to CCR7 has been reported to induce calcium flux through mouse CXCR3 (Soto et al., 1998) but this was not observed with human SLC with either human or the highly homologous (87% aa identity) mouse CXCR3 (Jenh et al., 1999). However a recent study has demonstrated that human SLC is a functional ligand for endogenously expressed CXCR3 in human adult microglia. In absence of CCR7 expression, SLC induced chemotaxis of human microglia with efficiency similar to Mig and IP-10 (Dijkstra et al., 2004). The fact that SLC did not show any effects in CXCR3-transfected HEK293 cells, suggests that CXCR3 signalling depends on the cellular background in which the receptor is expressed.

Other receptors that bind IP-10, Mig and I-TAC

An alternative functional high-affinity receptor for IP-10 but not Mig or I-TAC, has been found recently to be expressed on epithelial and endothelial cells

(Soejima and Rollins, 2001), however, cloning and more detailed analysis will be necessary to determine the function of this putative alternative receptor in mediating the actions of IP-10 *in vivo.* Several studies have also pointed out that IP-10 and PF-4 share a great number of activities, such as inhibition of chemotaxis and proliferation of endothelial cells or inhibition of hematopoiesis (Aronica et al., 1995; Luster et al., 1995; Strieter et al., 1995). This large overlap of biological activities was finally explained with the discovery of an alternatively spliced variant of CXCR3, termed CXCR3-B on endothelial cells (Lasagni et al., 2003) which binds PF4 as well as the other CXCR3 ligands. IP-10, like many chemokines, also binds to cell surface glycosaminoglycans (GAGs) (Luster et al., 1995). GAGs are polysaccharides with a high negative charge due to sulfate and carboxyl groups and are usually attached to core proteins to form proteoglycans. Chemokines are largely basic molecules that exhibit electrostatic interactions with GAGs, especially heparin and heparan sulfate. This heparan sulfate-binding site for IP-10 can also bind PF-4. While our understanding of the biological activities of the CXCR3 ligands has increased, relatively little is known about the importance of their interaction with GAGs. It has been postulated that GAGs on cells bearing the chemokine receptors facilitate chemokine binding to their high affinity receptor by sequestering chemokines, raising their effective concentration and, thus, their probability of encountering the receptor (Hoogewerf et al., 1997). The role of GAGs on endothelial cells and in the extracellular matrix might be important for retaining chemokines close to their site of secretion (Tanaka et al., 1993). A summary of all the ligands and receptors related to CXCR3 are presented in table 1.8.

Receptor	Expression	Function
CXCR3	T-cells, natural killer cells, B cells, pericytes, microglia, eosinophils, endothelial cells	T-cell migration, possible in Th1 type diseases and allograft rejection
CXCR3-B	Endothelial cells	Inhibits endothelial cell proliferation
IP-10 Receptor	Endothelial cells, epithelial cells	Unknown
Ligand	**Expression**	**Function**
IP-10	Induced by IFN-α, β and γ in endothelial cells, keratinocytes, fibroblasts, astrocytes, monocytes, neutrophils	T-cell migration, angiostatic, inhibits endothelial cell proliferation
Mig	Induced by IFN- γ in monocytes, macrophages, hepatocytes, fibroblasts, keratinocytes and endothelial cells	T-cell migration, angiostatic, promotes vascular pericyte proliferation
I-TAC	Induced by IFN- γ in monocytes, astrocytes, microglial, neutrophils, keratinocytes and epithelial cells	T-cell migration, angiostatic, promotes vascular pericyte proliferation
PF-4	Secreted by activated platelets	Inhibits endothelial cell proliferation

Table 1.8 CXCR3, related receptors and their ligands.

Role for CXCR3 and its ligands in IBD

Consistent with the involvement of Th1 cells in the pathogenesis of CD, expression of CXCR3 has been reported on T-cells infiltrating the inflamed gastrointestinal submucosa of patients (Yuan et al., 2001). However its expression was also largely found on lymphocytes isolated from patients with ulcerative

colitis (Qin et al., 1998) which appears to be a Th2 disease. Another study using two different Th1 mediated models of IBD, demonstrated increased amounts of Mig in mice deficient in IL-10, whereas in the RAG knock out model, IP-10 and Mig production was enhanced (Scheerens et al., 2001). Furthermore, expression of IP-10 and CXCR3 has been found to be up-regulated in the epithelium in the dextran sulfate sodium (DSS) animal model of colitis (Sasaki et al., 2002). Neutralization of IP-10 protected the mice from epithelial ulceration by promoting crypt cell survival without any evidence of altered immune cell infiltration, indicating a possible role for IP-10 as a new therapeutic target for IBD by controlling the dynamics of epithelial homeostasis.

1.6 Signalling pathways associated with chemokines

Phosphoinositide metabolism

Phosphoinositides (PIs) are minor lipid components of biological membranes, which have emerged as essential regulators of a variety of cellular processes, both on the plasma membrane and on several intracellular organelles. The versatility of these lipids stems from their ability to function either as substrates for the generation of second messengers, as membrane anchoring sites for cytosolic proteins or as regulators of the actin cytoskeleton. This allows them to interact with proteins and to orchestrate the spatio-temporal organization of key signalling pathways.

Phosphatidylinositol (PtdIns), the basic building block for the intracellular inositol lipids in eukaryotic cells, consists of D-*myo*-inositol-1-phosphate (Ins1P) linked via its phosphate group to diacylglycerol (DAG). This molecule is the target of a

number of lipid kinases that phosphorylate the inositol ring on positions D3, D4, and D5 in different combinations. The 2 and 6 positions in these lipids are not known to be esterified with phosphate. PtdIns and its phosphorylated derivatives are collectively referred to as phosphoinositides. Eight PI species have been documented in eukaryotic cells (Figure 1.4).

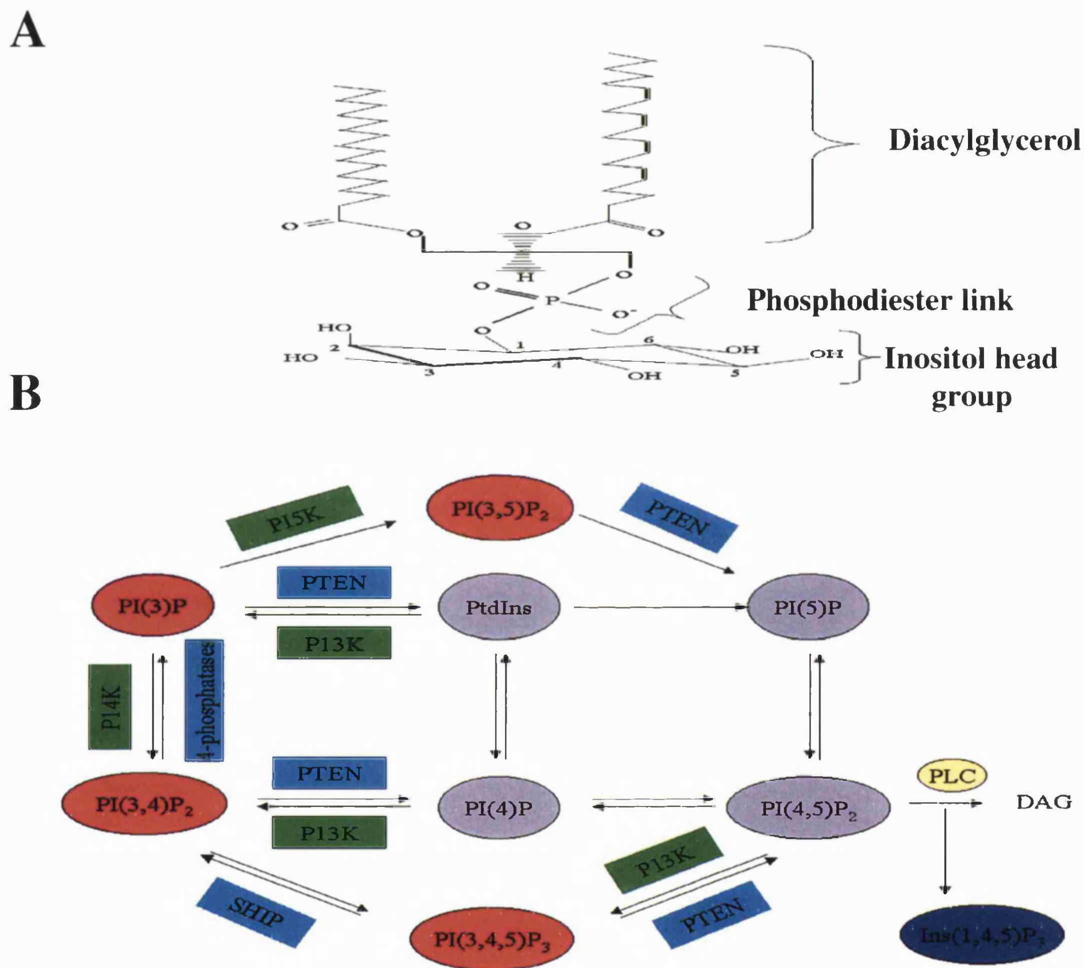

Figure 1.4 Chemical structure of PtdIns (A) and summary of phosphosinositide lipid metabolism (B). Only kinases specifically involved in 3-PI metabolism are included. Blue boxes represent phosphatases, green boxes represent kinases that catalyse phosphorylation of the lipid substrates and 3-PIs are represented in red. Abbreviations used in the diagram: SHIP, Src homology domain-containing inositol 5'-phosphatase; PTEN, phosphatase and tensin homologue deleted on chromosome TEN; PLC, phospholipase C; PI5K, phosphoinositide 5 kinase; DAG, diacylglycerol.

Phospholipase C

Eleven distinct isoforms of PI-specific phospholipase C (PLC), which are grouped into four subfamilies (β, γ, δ and ε), have been identified in mammals. These proteins catalyze the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PtdIns4,5-P_2) to inositol 1,4,5-trisphosphate (Ins1,4,5-P_3) and DAG in response to the activation of various cell surface receptors. These messengers then promote the activation of protein kinase C (PKC) and the release of Ca^{2+} from intracellular stores, respectively. All PLC isoforms apart from a highly conserved catalytic core, also contain various combinations of regulatory domains. The latter target the different PLC isoforms to the vicinity of their substrate through protein-protein or protein-lipid interactions. For instance the β, γ, and δ isoforms all contain an NH_2-terminal pleckstrin homology domain (PH), a module that is present in many signalling proteins and that binds to various PIs (Rameh and Cantley, 1999). The presence of distinct regulatory domains in PLC isoforms renders them susceptible to different modes of activation. Given that the partners that interact with these regulatory domains of PLC isoforms are generated or eliminated in specific regions of the cell in response to changes in receptor status, the activation and deactivation of each PLC isoform is a highly regulated process.

Phosphoinositide 3-kinase

Phosphoinositide 3-kinase (PI3K) is an enzyme that participates in many cellular processes and whose activity has been linked to cell growth and transformation, differentiation, motility, insulin action, and cell survival to name a few. Thus it is not surprising that considerable effort has gone into understanding the mechanisms by which PI3K mediates these responses. PI3K compromises a

family of signalling enzymes that catalyse the phosphorylation of the D3 position of the inositol ring of PIs. PI3K initiate signalling cascades by generating three distinct membrane inositol lipids, PtdIns-3-P, PtdIns-3,4-P_2, and PtdIns-3,4,5-P_3 (referred from now on as 3-PIs). Approximately 5% of cellular PI is phosphorylated at the 4-position, and another 5% is phosphorylated at both the 4 and 5 positions . However, less than 0.25% of the total inositol-containing lipids are phosphorylated at the 3-position, consistent with the idea that these lipids exert specific regulatory functions inside the cell, as opposed to a structural function. Resting mammalian cells contain significant levels of PtdIns3-P, but hardly any of the other 3-PIs. Whereas the overall levels of PtdIns3-P do not seem to increase upon cellular stimulation, the levels of the other 3-PIs can rise sharply, although they probably never match the levels of PtdIns4,5-P_2 or PtdIns4-P. PI3K lipid products are not substrates for the PI-specific PLC enzymes. Instead, 3-PIs are metabolised by kinases and phosphatases that act on the inositol ring (summarized in Figure 1.4).

PI3K isoforms

Based on structural characteristics, regulatory mechanisms and their selective in vitro substrate specificity, PI3Ks can be grouped into three classes: class I, class II and class III (summarized in Figure 1.5).

Class I PI3K

The prototypical PI3Ks are the class I PI3Ks which are heterodimers of approximately 200 kDa, composed of a 110–120 kea catalytic subunit and a 50–100 kDa adaptor subunit and are able to phosphorylate, PtdIns, PtdIns 4-P and PtdIns 4,5-P_2 *in vitro*. The preferred in vivo substrate for class I PI3Ks, however,

seems to be PtdIns 4,5-P_2 (Stephens et al., 1991; Hawkins et al., 1992). Depending on the adaptor proteins involved in this process, class I PI3Ks can be segregated into two groups: those able to associate with p85 will be directed to phosphorylated tyrosine motifs (class IA), while PI3Kγ interacts with trimeric G proteins and the p101 protein (class IB).

Class	Catalytic Subunit	Adaptor Subunit	Lipid Substrate
I A	p110 α,β,δ	p85 α,β p55 α,γ p50	PtdIns, PtdIns-4P, PtdIns 4,5 P_2
B	p110γ	p101	PtdIns, PtdIns-4P, PtdIns 4,5 P_2
II	$C2_{\alpha}$, $C2_{\beta}C2\gamma$	clathrin	PtdIns, PtdIns-4P
III	Vps34p	p150	PtdIns

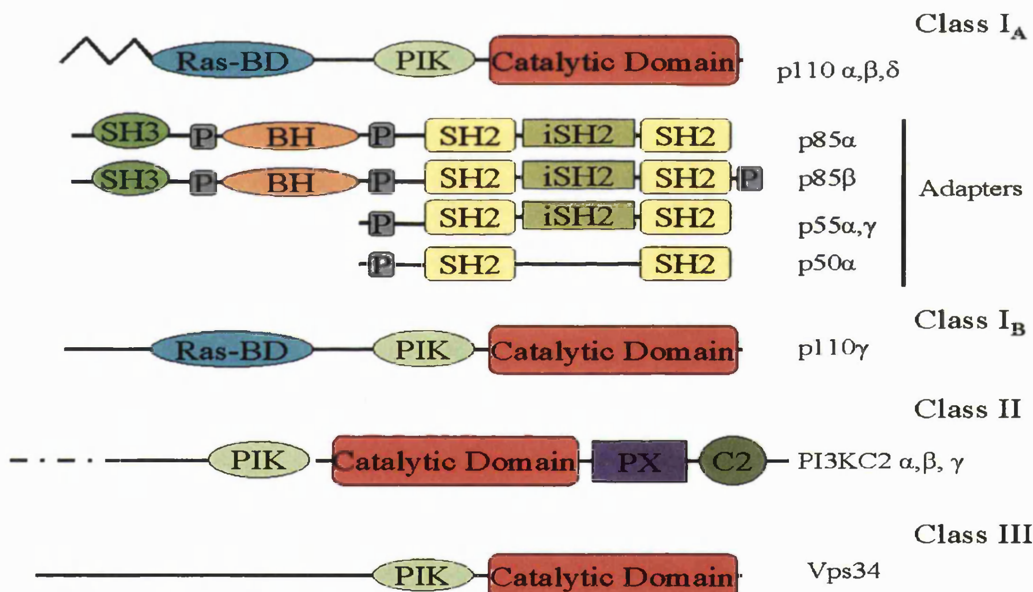

Figure 1.5 Classes, subunits, lipid substrates of mammalian PI3Ks and structural characteristics of the different PI3K isoforms (adapted from (Curnock et al., 2002). PI3Ks have been divided into three classes, based on primary structure, substrate specificity and regulatory mechanisms; class 1 is further subdivided according to the associated adapter subunit. The protein domains are as follows: BH, breakpoint-cluster region; C2, C2 domain; P, proline-rich motif; PIK, phosphatidylinositol kinase domain; PX, phox homology domain; Ras-BD; Ras-binding domain; SH2, src-homology domain 2; SH3, src-homology domain; iSH2, inter SH2 domain.

Class IA PI3Ks are tightly and constitutively associated with a 50–85 kDa regulatory subunit, of which p85 is the prototype. The p85 is composed, starting from the N terminus, of a Src homology 3 (SH3) domain, a breakpoint-cluster-region homology (BH) domain flanked by two proline-rich regions and two C-terminal SH2 domains spaced by an inter-SH2 region (Otsu et al., 1991; Skolnik et al., 1991; Hiles et al., 1992). The latter mediates tight binding of p85 to the catalytic subunit. The subsequent cloning of PI3Kγ (Stoyanov et al., 1995) identified a protein with similarities to the class IA PI3K, but without an N-terminal p85-binding site. Instead, PI3Kγ was found associated with a novel 101 kDa protein without any functional homology to known proteins, and was proposed to be essential in conferring Gβγ sensitivity to p110γ (Stephens et al., 1997). Others have reported that Gβγ-dependent p110γ activation in absence of p101 occurs *in vitro* reconstitution assays with permeabilized neutrophils (Kular et al., 1997; Leopoldt et al., 1998).

Class II PI3K

The drosophila PI 3-kinase PI3K68D/cpk has been identified as the first member of a new class of PI3Ks (MacDougall et al., 1995), They are distinguished from other PI3K isoforms by the presence of two tandem domains at their carboxyl terminus that. The first is termed a phox homology (PX) domain and the second a C2 domain which are known to act as a specific PI-binding modules in other signalling molecules. Class II isoforms are characterized PtdIns and PtdIns4-P specificity in vitro, although PI3-K-C2α was claimed to phosphorylate PtdIns4,5-P_2 as well (Domin et al., 1997). Of the PI3K-II α, β and γ families, drosophila PI3K68D/cpk resembles most the mammalian PI3K-IIβ and shares with it a

ubiquitous expression pattern (Brown et al., 1997). Class II PI3Ks display homologies with class I enzymes, but have different N-termini and do not associate with p85. The C2 domain of PI3K-IIs, although related to the one of synaptotagmin, is Ca^{2+}-insensitive due to the lack of conserved Asp residues necessary for Ca^{2+}-binding (Sutton et al., 1995). Indeed it has been shown to bind weakly to phospholipids in a Ca^{2+}-independent fashion (MacDougall et al., 1995) and to be essential for catalytic activity of the enzyme (Misawa et al., 1998). The observation that drosophila PI3K68D/cpk can be phosphorylated on Tyr suggests that protein tyrosine kinases and protein–protein interactions could mediate the activation of PI3K-IIs (Molz et al., 1996) .

Class III PI3K

Class III PI3Ks are homologues of *S. cerevisiae* Vps34p (vacuolar protein sorting mutant, and phosphorylate exclusively PtdIns (Schu et al., 1993). Based on observations in yeast Vps34, the mammalian homologue of this class III PI3K is considered to be the principle mediator of vesicle transport from the Trans-Golgi network (TGN) to lysosomes, the organelle in mammalian cells that is functionally equivalent to the yeast vacuole. In support of this, transport of newly synthesized lysosomal enzymes from the TGN is inhibited by PI3K inhibition (Brown et al., 1995).

Lipid phosphatases

To ensure that activation of this pathway is appropriately suppressed/terminated, there are three major lipid phosphatases involved in negatively regulating the levels of 3-PIs: the ubiquitously expressed 54-kDa tumor suppressor PTEN

(Phosphatase and TENsin homologue deleted on chromosome TEN) which hydrolyzes PtdIns3,4,5-P_3 to PtdIns4,5-P_2, and the 145-kDa hematopoietic-restricted SHIP (Src homology domain 2 (SH2)-containing inositol 5'-phosphatase, also known as SHIP1), as well as the more widely expressed 150-kDa SHIP2, which breaks it down to PtdIns3,4-P_2. PTEN will de-phosphorylate several phosphoinositide signalling molecules in vitro, specifically removing phosphate from the D-3 position of the inositol ring in each case (Maehama and Dixon, 1998; Myers et al., 1998) . However, PItdIns3,4,5-P_3 and PItdIns3,4-P_2 are the most efficient substrates for PTEN in vitro, although it will also de-phosphorylate PI3-P and the soluble head group of PItdIns3,4,5-P_3, inositol 1,3,4,5-tetrakisphosphate(Ins(1,3,4,5)P_4). SHIP 1 and 2 are capable of hydrolysing PtdIns3,4,5-P_3 at position 5 of the inositol ring to produce PItdIns3,4-P_2 (Damen et al., 1996; Lioubin et al., 1996). SHIP1 is also capable of de-phosphorylating Ins(1,3,4,5)P_3, whereas SHIP2 is not (Wisniewski et al., 1999). The D-3 position of the inositol phospholipid must be phosphorylated before SHIP can de-phosphorylate the D-5 position (Damen et al., 1996), suggesting that SHIP acts sequentially with PI3K in the inositol phospholipid pathway.

Targeting PI3K isoforms

The specific PI3K inhibitors, wortmannin (Wymann et al., 1996) and LY294002 (Vlahos et al., 1994), have been invaluable tools for elucidating the roles of these enzymes in signal transduction pathways in various cellular responses *in vitro*. However, it has been difficult to study the function of this enzyme family *in vivo*. Moreover, neither of these inhibitors exhibit any degree of selectivity for

individual PI3K isoforms, even though PI3K-C2α has been reported as displaying reduced sensitivity to wortmannin (Domin et al., 1997).

The recent development of genetic approaches based on gene-manipulated mouse systems has provided a breakthrough in elucidating the *in vivo* role of the individual PI3K enzymes. To date, each of the class I PI3K catalytic subunits has been inactivated by gene targeting, as have the p85α and p85β regulatory subunits (summarised in table 1.9).

Ablated gene	Phenotype
p85α/p55α/p50 α	Perinatal lethal (Fruman et al., 1999), Impaired B-cell development and activation (Suzuki et al., 1999), Over production of IL-2 from DCs and enhanced Th1 responses to *Leishmania major* infection (Fukao et al., 2002a)
p85β	Selective loss of gastrointestinal mast cells and impaired responses to intestinal nematodes (Fukao et al., 2002b), No immune phenotype reported (Ueki et al., 2002)
p110α	Embryonic lethal (Bi et al., 1999)
p110β	Embryonic lethal (Bi et al., 2002)
p110δ	Impaired T and B antigen receptor signaling, impaired B cell development and activation (Clayton et al., 2002; Jou et al., 2002; Okkenhaug et al., 2002)
p110γ	Impaired T cell development , activation, chemotaxis and inflammatory responses, improved heart function, decreased oxidative burst and thromobembolism (Hirsch et al., 2000; Li et al., 2000; Sasaki et al., 2000; Hirsch et al., 2001; Laffargue et al., 2002; Crackower et al., 2002)

Table 1.9 Immune phenotypes of genetically targeted PI3K isoforms (adapted from (Ward and Finan, 2003).

Despite the advantages of this approach, the results obtained are incomplete because the p110α and β knockouts are embryonic lethal, and need to be interpreted with caution due to the complex regulation of p110 by p85. Targeting p85α interferes with recruitment of p110 to tyrosine-phosphorylated receptor complexes, while expression of each of the class I_A catalytic subunits is reduced, supporting a role for p85 in protecting p110 from proteolysis. In addition it is thought that loss of one member of the family might be compensated for by another isoform.

Lipid products as mediators of PI3K downstream signalling

PI levels are exquisitely regulated within cells and constitute important spatial and temporal signals coordinating a wide range of cellular processes. The effects of 3-PIs are mediated primarily by direct interaction with a large number of downstream effector proteins (summarized in Figure 1.6), and these protein-lipid interactions involve numerous, specific lipid-binding modules, including the PH, FYVE and PX domains.

The pleckstrin homology (PH) domain was first identified in 1993 as a 100–120-residue stretch of amino-acid-sequence similarity that occurs twice in pleckstrin and is found in numerous proteins involved in cellular signalling. It was originally proposed that PH domains, like SH2 and SH3 domains, might be involved in protein–protein interactions in cellular signalling (Haslam et al., 1993). Subsequent work has shown that many PH domains direct membrane targeting of their host proteins, but by binding to PIs rather than proteins in cellular membranes. They are the only domains known to exhibit PtdIns3,4,5-P_3-binding

properties in molecules such as protein kinase B (PKB) (James et al., 1996), Bruton's tyrosine kinase (Btk) (Salim et al., 1996), the general receptor for phosphoinositides-1 (Grp1) (Klarlund et al., 1997), and the dual adaptor for phosphotyrosine and 3-phosphoinositides-1 (DAPP1) (Dowler et al., 1999). PtdIns3,4-P_2 has also several well-known targets, which all contain PH domains. Several of the PH domains that are recruited by PtdIns3,4,5-P_3 also recognize PtdIns3,4-P_2. These include the PH domains from PKB and DAPP1, but not those from Btk or Grp1 (which are PtdIns3,4,5-P_3-specific) (Kavran et al., 1998). Only one PH domain, from TAPP1 (tandem PH domain containing protein 1), has been reported to bind exclusively to PtdIns3,4-P_2, and evidence has been presented to suggest that PtdIns3,4-P_2 is the target of this PH domain *in vivo* (Kimber et al., 2002).

Of all physiological PIs, PtdIns(3)P is the one that has the largest number of known specific binding partners, being recognized specifically by most FYVE domains and PX domains, of which there are 30 and 42, respectively, in the human proteome. The FYVE domain contains approximately 60-70 amino acids, and is named for the four proteins in which it was first identified: **F**ab1p, **Y**OTB, **V**ac1p, and **E**EA1 (Stenmark et al., 2002). Since their initial description, it has been clear that more than just the FYVE domain is required for efficient targeting of proteins to PtdIns3-P-containing membranes *in vivo*, simply because PtdIns3-P is not very abundant, and that head group binding, while specific, is relatively weak (Dumas et al., 2001). FYVE finger proteins regulate distinct trafficking steps such as membrane fusion, receptor sorting, membrane invagination, and the endocytic pathway, consistent with the localisation of PtdIns3-P on both endosomes and vacuoles (Gillooly et al., 2000). PX domains were pointed out in

1996 as a 130-amino acid homology region in two components of the phagocyte NADPH oxidase complex (p40 ^{phox} and p47 ^{phox}) as well as many other proteins with diverse functions (Ponting, 1996). PX domains are now recognized as PtdIns3-P binding modules, although the PX domains from PI3K C2α has been reported to bind PtdIns4,5-P_2 (Song et al., 2001).

The potential of some PH domains to specifically bind PItdIns3,4P_2 and PtdIns3,4,5-P_3 correlates with in vivo data defining the same PH domain-containing proteins as PI3K effectors. For example, PI3K activity leads to multiple phosphorylations of p70^{S6K}, which is involved in G1 cell cycle transition and proliferation (Chung et al., 1994; Alessi et al., 1998). p70^{S6K} can also associate with and is activated by the Rho family G proteins Rac and Cdc42 (Chou and Blenis, 1996; Welch et al., 1998) which are again under the control of PI3K. The list of PI3K targets further includes the atypical PKCs ε, ζ, η, λ (Toker and Cantley, 1997), and PLC γ (Falasca et al., 1998; Bae et al., 1998).

Protein Kinase B

Although the serine/threonine protein kinase PKB was not the first PI3K effector discovered, intense interest in this field has led to what is arguably the best understood mechanism of activation and function of any PtdIns3,4,5-P_3 target. PKB was identified as a 57kDa serine/threonine kinase with high homology to protein kinases A and C, and was therefore termed PKB. PKB is cytosolic in unstimulated cells, and is activated trough membrane localization (Andjelkovic et al., 1997) and Ser/Thr phosphorylation (Andjelkovic et al., 1996; Alessi et al., 1996). Both events are dependent on PI3K: the PH domain of PKB promotes

translocation to the plasma membrane by binding to PtdIns3,4-P_2 (Franke et al., 1997), and phosphorylation at Thr^{308} and Ser^{473} requires phosphoinositide-dependent kinases (PDKs) 1 and 2 respectively. Activity of PDK1 is specifically controlled by interaction of PtdIns3,4,5-P_3, and PtdIns3,4-P_2 with its PH domain (Stokoe et al., 1997; Stephens et al., 1998). Apart from phosphorylating PKB on Thr^{308}, PDK1 phosphorylates members of the AGC subfamily members on the equivalent residues such as on PKC isoforms (Dutil et al., 1998), p70-S6K (Alessi et al., 1998), PKA (Cheng et al., 1998) and others.

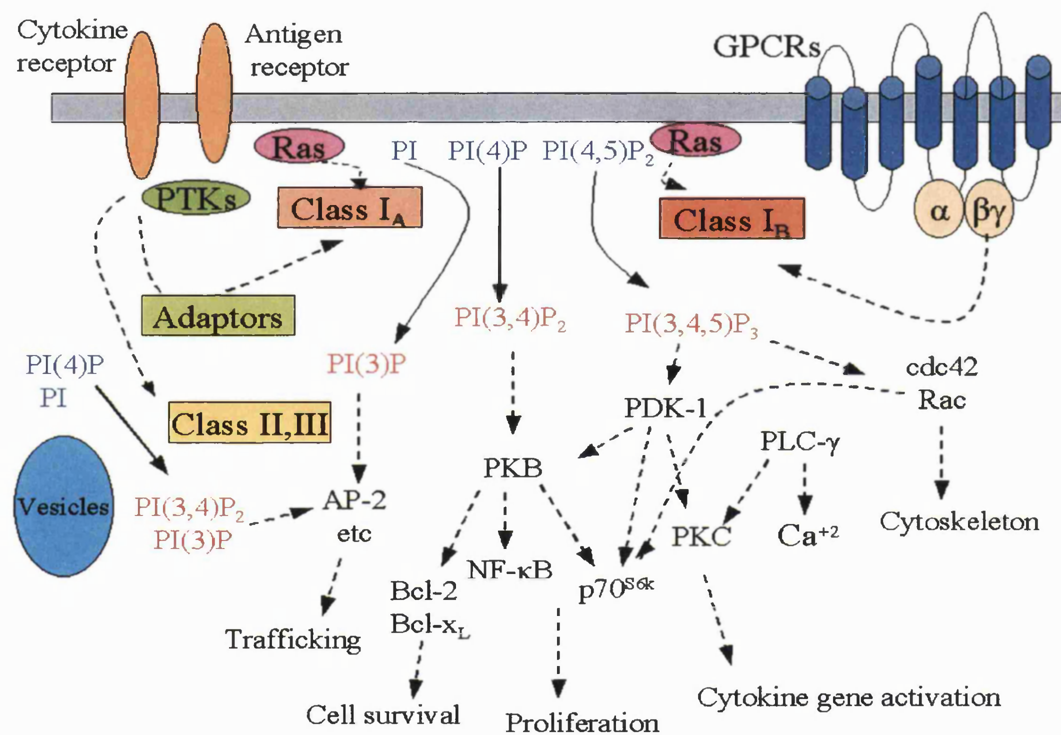

Figure 1.6 Overview of PI3K and phosphoinositide signalling. Proteins containing PH domains (PKB, PDK1, and PLC-γ) are present downstream of PI(3,4)P_2 and PI(3,4,5)P_3. Various target proteins function downstream of these molecules. Proteins containing FYVE and PX domains function upon binding to PI(3)P and/or PI(3,4)P_2. Activation mechanisms of class II and class III PI3Ks are largely unknown. Abbreviations: AP-2, adaptor-related protein complex 2; PKB, protein kinase B; PKC, protein kinase C; PDK-1, phosphoinositide dependent kinase-1; PLC-γ, phospholipase Cγ ; PTKs, protein tyrosine kinases.

PKB has been implicated in many biological processes including intermediary metabolism, protein synthesis, and anti-apoptotic signalling. Once activated, PKB leaves the plasma membrane to phosphorylate intracellular substrates. Consistent with this, translocation of PKB to the nucleus has been reported (Andjelkovic et al., 1997) and this undoubtedly links PKB to phosphorylation of transcription factors such as c-AMP-responsive element-binding protein (CREB), forkhead transcription factors, and NF-κB (Kandel and Hay, 1999). PKB phosphorylates and activates endothelial nitric oxide synthase (eNOS) leading to sustained production of NO by endothelial cells, which has been implicated in gene regulation and angiogenesis (Snyder and Jaffrey, 1999; Fulton et al., 1999). The glycogen synthase kinase 3 (GSK3) is also phosphorylated and inactivated by PKB leading to an increase in glycogen synthesis (Cross et al., 1995).

One of the major functions of PKB is as a cell survival factor, and a number of proteins have been shown to mediate its anti-apoptotic function. The pro-apoptotic Bcl-2 family member BAD is phosphorylated and inactivated by PKB leading to protection from apoptosis (Datta et al., 1997). However it is unlikely that this represents the major anti-apoptotic mechanism by which PKB, as Bad is not ubiquitously expressed. A cysteine protease, caspase-9, as well as forkhead transcription factors such as FKHR, FKHRL1 and AFX also induce apoptosis, an event that is inhibited by PKB mediated phosphorylation of these proteins (Cardone et al., 1998; Brunet et al., 1999a). Another anti-apoptotic action of PKB may operate via the transcription factor NF-kB. When bound to its cytosolic inhibitor, IκB, NF-kB is sequestered in the cytoplasm. PKB has been reported to associate with and activate IκB kinases (IKKs), which are known to phosphorylate and degrade IκB. This results in translocation of NF-κB to the nucleus where it

activates the transcription of anti-apoptotic members of the inhibitor-of-apoptosis proteins (IAP) such as c-IAP-1 and c-IAP-2 (Kane et al., 1999; Wang et al., 1999). The PI3K/PKB pathway is summarized in figure 1.7.

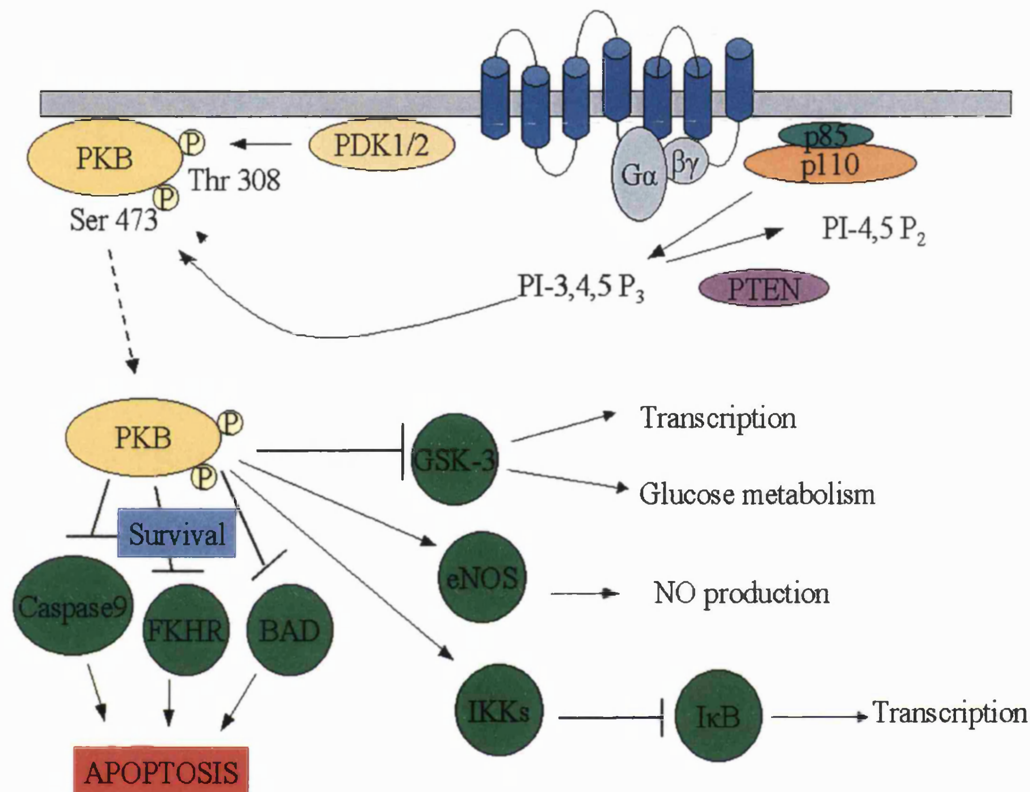

Figure 1.7 The PI3K/PKB pathway. Activation of PI3K results in the local accumulation of PtdIns3,4,5-P_3 at the plasma membrane. Newly synthesized PtdIns3,4,5-P_3 recruits both PDK-1 and PKB to the plasma membrane where the combination of lipid binding and phosphorylation by PDK-1 serves to activate PKB. Once activated PKB has profound effects on cell function leading to gene transcription and cell survival. Abbreviations used in the diagram: FKHR, Forkhead transcription factors; BAD, **B**cl-2/Bcl-X_L-**a**ntagonist, causing cell **d**eath; eNOS, endothelial nitric oxide synthase; GSK-3, glycogen synthase kinase-3; IKKs, IκB kinases.

PI3K activation by chemokines

The first evidence for the involvement of PI3K in chemokine-stimulated cell migration was the demonstration that the chemotaxis and polarization of T-cells induced by RANTES is inhibited by the PI3K inhibitor wortmannin (Turner et al., 1995b). Subsequent studies by several groups showed that other chemokines, such as MIP-3, MCP-1, IL-8 and SDF-1 stimulate wortmannin-sensitive chemotaxis of eosinophils, THP-1 monocytic cells, neutrophils and T–cells respectively (Knall et al., 1997; Turner et al., 1998; Sotsios et al., 1999; Sullivan et al., 1999). Moreover SDF-1 and certain SDF-1 peptide analogues stimulate the transient accumulation of PtdIns3,4,5-P_3 in leukaemic T-cell lines and peripheral blood-derived T-cells (Sotsios et al., 1999). Given that chemokine receptors are G protein coupled, one might predict an involvement of the $G\beta\gamma$-dependent PI3K in mediating PtdIns3,4,5-P_3 accumulation. Indeed, the accumulation of PtdIns3,4,5-P_3 stimulated by SDF-1 and MCP-1 can be completely inhibited by pre-treatment with pertussis toxin, strongly indicating that 3'-phosphoinositide lipid accumulation occurs via the Gi protein-coupled PI3K (Turner et al., 1998; Sotsios et al., 1999). However, in PI3K$\gamma^{-/-}$ mice, neutrophils are still capable of migrating to several chemoattractants (Hirsch et al., 2000; Li et al., 2000; Sasaki et al., 2000), suggesting that other PI3K isoforms are also activated. *In vitro* assays of immunoprecipitated p85 subunits of PI3K indicate that the p85/p110 heterodimer is activated by SDF-1 and RANTES in T cells (Turner et al., 1995a; Sotsios et al., 1999) and by MCP-1 in THP-1 cells (Turner et al., 1998). The study with MCP-1 revealed that PtdIns3,4,5-P_3 accumulation in THP-1 cells is wortmannin resistant, yet entirely pertussis toxin sensitive, suggesting the involvement of PI3K-C2α, which is thought to exhibit reduced sensitivity to wortmanin.

Calcium (Ca^{+2}) signalling

Originally thought to be a structural element for bone and teeth formation, Ca^{2+} is now known to be involved in many intracellular signalling processes (e.g. gene transcription, proliferation, apoptosis, migration and muscle contraction) and its intracellular mobilisation is initiated by a wide variety of receptors.

The process of Ca^{2+} signalling involves regulated changes in the concentration of Ca^{2+} in the cytoplasm from 100 nM in resting conditions to roughly 1000 nM upon activation. This is achieved by an extensive molecular repertoire of signalling components, which comprise the Ca^{2+} signalling toolkit (Figure 1.8). In most cell types, the major internal Ca^{2+} stores are the endoplasmic reticulum (ER) or the sarcoplasmic reticulum (SR). Release of Ca^{2+} from these stores is attained via multimeric ligand gated ion channels: IP_3 receptors (IP_3Rs) and ryanodine receptors (RRs). The latter is gated by Ca^{2+} itself (also known as calcium-induced calcium release (CICR), but can also be operated by an endogenous ligand named cyclic ADP ribose (cADPR) (Galione and Churchill, 2000). There are currently three IP3Rs and three RRs (Berridge et al., 2003). A further internal store operated by nicotinic acid adenine dinucleotide phosphate (NAADP) has been recently identified (Genazzani and Billington, 2002).

Additionally, Ca^{2+} is able to enter the cell externally via various entry channels: voltage-operated channels (VOCs) are plasma membrane ion channels that are activated by membrane depolarisation; receptor-operated channels (ROCs) open in response to the binding of an extracellular ligand; second-messenger-operated channels (SMOCs) open in response to the binding of intracellular second messengers such as DAG, cyclic nucleotides or arachiodonic acid; and store-

operated channels (SOCs) open in response to the depletion of internal stores of Ca^{2+}.

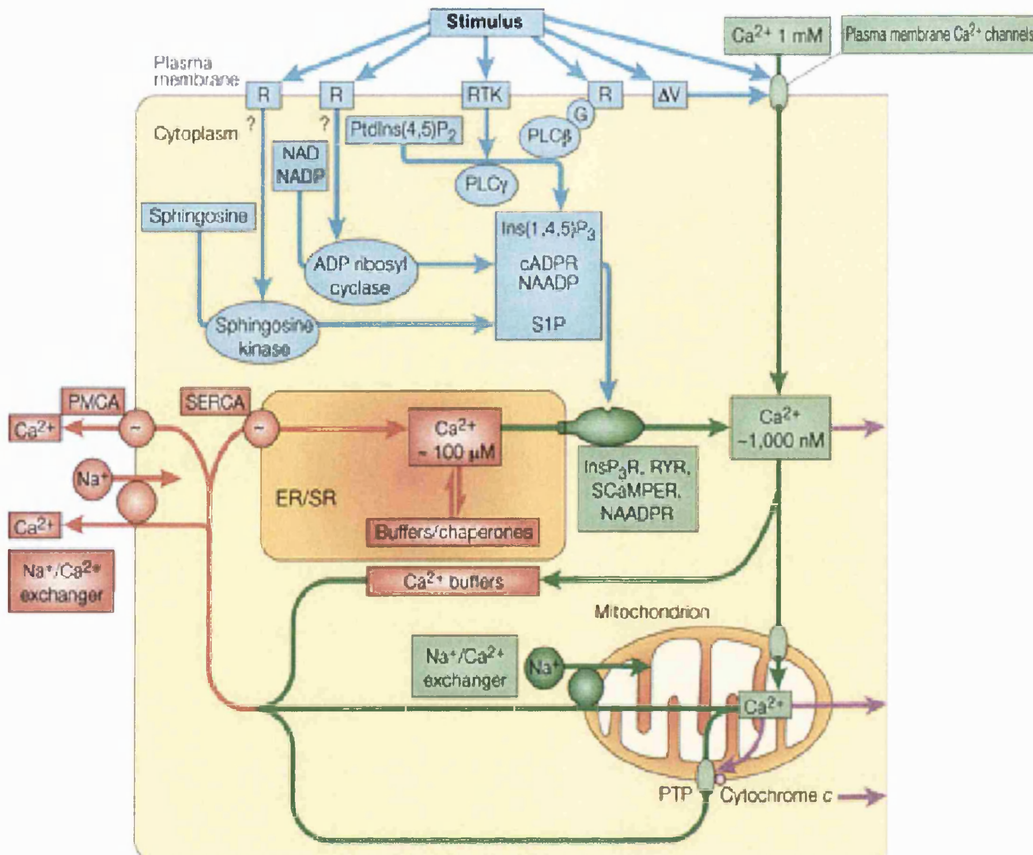

Figure 1.8 An overview of the pathways involved in calcium mobilisation, Acquired from (Berridge et al., 2000). Calcium mobilising signals are shown in blue. Influx mechanisms are in green, with efflux pathways highlighted in red. Abbreviations: cADPR, cyclic adenosine diphosphate ribose; ER, endoplasmic reticulum; G, G-protein; InsP3R, inositol (1,4,5)-trisphosphate receptor; NAD, nicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide phosphate; NAADP, nicotinic acid adenine dinucleotide phosphate; PLC, phospholipase C; PMCA, plasma membrane calcium ATPase; PTP, permeability transition pore; R, receptor; RTK, receptor tyrosine kinase; RYR, ryanodine receptor; S1P, sphingosine 1-phosphate; SCaMPER, sphingolipid calcium release-mediating protein of the ER; SERCA, sarco(endo) plasmic reticulum calcium ATPase; SR, sarcoplasmic reticulum.

For a cell to be able to maintain viability, the calcium influx must be balanced by calcium efflux. Four different mechanisms are responsible: plasma membrane calcium ATPase (PMCA), sodium/calcium exchanger (NCX), sarco (endo) plasmic reticulum calcium ATPase (SERCA), and the mitochondrial uniporter. PMCA and SERCA have high affinities but limited capacities meaning they can respond to modest increases in calcium levels and set basal calcium levels. The remaining two calcium transporters, NCX and the mitochondrial uniporter, have much higher capacities and can limit the calcium transient over a wider range (i.e. optimal mitochondrial calcium accumulation occurs when calcium is in the μM range but do accumulate calcium even when presented with modest nM global calcium changes). The PMCA couples ATP hydrolysis to the transport of calcium from cytosolic to extracellular spaces. NCX (plasma membrane) exchanges three moles of sodium for one mole of calcium, either inward or outward, depending on the ionic gradients across the membrane. The mitochondrial uniporter transports calcium from the cytosol into the mitochondrial matrix and is located on the inner mitochondrial membrane. SERCA is located on SR and ER membranes and couples ATP hydrolysis to the transport of calcium from the cytosol to the lumenal space (for reviews (Berridge et al., 2000; Berridge et al., 2003)). Rises in cytosolic calcium levels are decoded by various intracellular calcium binding proteins coupling the calcium flux to a biochemical and cellular response, with calmodulin being the most abundant and well known of these calcium sensors.

Calmodulin binding to calcium leads to its conformational change and activation of the serine-threonine phosphatase calcineurin, allowing this protein to then activate various transcription factors and subsequent transcription of various genes, such as IL-2.

Chemokines and calcium signalling

The activity of chemokine receptors is most commonly assayed by measuring changes in intracellular Ca^{2+} levels following application of agonists, although it does not define which G protein family has transduced the chemokine message from receptor to effector. For instance in cultured cerebellar Purkinje cells, CCR8-induced Ca^{2+} fluxes were not affected by PTX treatment of the cells indicating the lack of involvement of Gi-coupled pathways (Gillard et al., 2002). In the same system, activation of CCR3 by the specific ligand eotaxin induces calcium transients, but the calcium release is completely inhibited by pre-treatment of the cells with PTX (Gillard et al., 2002).

Increases in intracellular Ca^{2+} levels do not necessarily prove activation of IP3Rs. An alternative explanation would be the chemokine-mediated opening of channels in the outer cell membrane allowing Ca^{2+} flux into the cell. Indeed it has been observed that the rise in intracellular Ca^{2+} levels following activation of CCR1 expressed in HEK-293 cells is completely inhibited by the PLC inhibitor U73122, indicating that the Ca^{2+} is released from intracellular pools. However, it was noticed that in the absence of extracellular Ca^{2+}, the intracellular levels induced by CCR1-mediated events were considerably reduced (Nardelli et al., 1999). This implies that CCR1 is able to promote the influx of Ca^{2+} into a cell by activating Ca^{2+} channels as well as by promoting the release of Ca^{2+} from IP3-sensitive intracellular pools. Another interesting observation from calcium studies with chemokines is that chemokine receptor stimulation can have biological effects in the absence of measurable calcium mobilization. SDF-1 was unable to stimulate increases in $[Ca^{2+}]_i$ in Jurkat cells, although these cells still elicited a chemotactic response to SDF-1 (Turner et al., 1995b; Sotsios et al., 1999).

The Mitogen-Activated Protein Kinase pathways

Mitogen-activated protein kinase (MAPK) pathways are conserved in all eukaryote organisms and are common participants in signal transduction pathways from the cell membrane to the nucleus. These kinases regulate directly or indirectly a number of transcription factors that control a very large number of important genes that are responsible for such fundamental cellular processes as proliferation, differentiation, survival and apoptosis. The mammalian MAP kinase family includes: 7 members of the **ERK** family, 4 isoforms of **p38** MAP kinase and 10 or more splice variants of the **JNK/SAPK family** (c-Jun N-terminal /stress activated protein kinases).

Mammalian MAPK pathways can be activated by various receptor families, such as tyrosine (Tyr), serine/threonine (Ser/Thr) kinase receptors, cytokine receptors or GPCRs, including chemokine receptors. All the known MAP kinases can be categorized by the sequence of the canonical dual phosphorylation site Thr-Xaa-Tyr (TXY) in a regulatory loop between kinase subdomains VII and VIII (Tanoue and Nishida, 2003). The ERK group members have the Thr-Glu-Tyr (TEY) dual phosphorylation motif; all the p38 group kinases have the Thr-Gly-Tyr (TGY) and JNK/SAPK group has a Thr-Pro-Tyr (TPY) motif. All MAPK pathways feature three-tiered central 'core signalling modules' (Figure 1.9), consisting of three kinases: a MAPK kinase kinase (MAPKKK) that activates a MAPK kinase (MAPKK) by Ser/Thr phosphorylation, which in turn activates a MAPK. MAPKs are activated by concomitant Thr/Tyr phosphorylation within a conserved motif in the activation loop of the kinase domain.

The Extracellular Regulated Kinase pathway

The ERKs are a very heterogeneous group within the MAPKs. They include the ERK1/2, ERK3/4 and ERK5/BMK (Zhou et al., 1995) subfamilies, as well as the newly discovered ERK7 (Abe et al., 2001) and ERK8 (Abe et al., 2002). ERK1, ERK2 and ERK5, participate in signal transduction pathways that originate from the cell surface receptors.

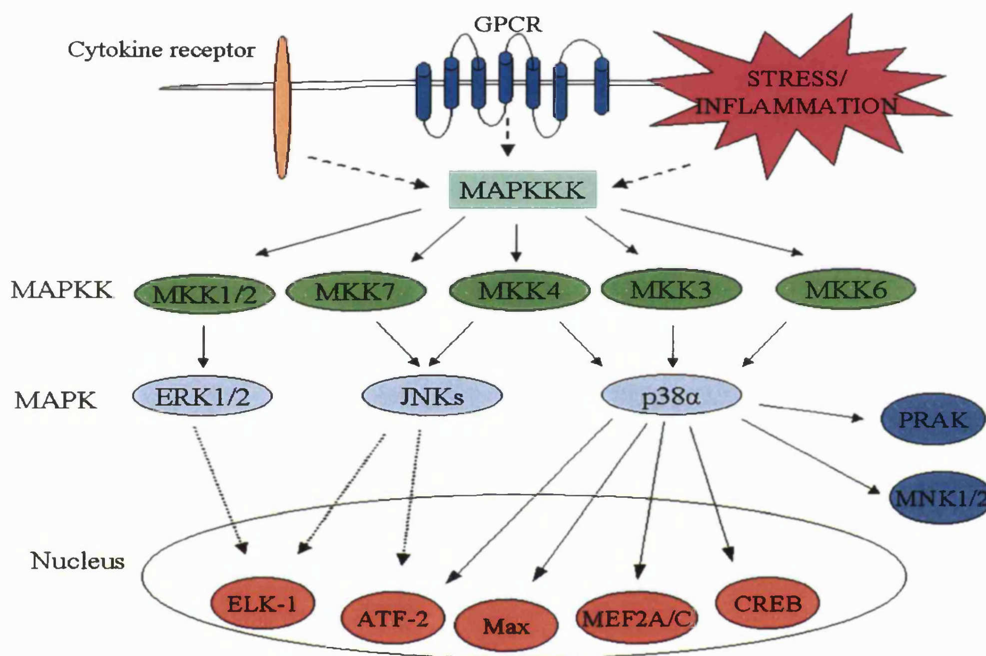

Figure 1.9 Overview of the mitogen-activated protein kinase (MAPK) core signalling module. Divergent inputs feed into a core MAPK-kinase-kinase (MAPKKK) →MAPK-kinase (MAPKK)→ MAPK pathways. MAPKs, in turn, coordinate activation of gene transcription factors or further downstream targets. Abbreviations:ATF-2, activating transcription factor-2; CREB, cAMP response element-binding protein; Elk-1, Ets-like gene-1; Max, Myc-associated factor X; MEF2A/C, myocyte enhancer factor-2A/-2C; MNK1/2, MAPK-interacting kinase-1/-2; PRAK, p38-regulated/activated kinase.

Termed MAPK/ERK kinase (MEK1 and MEK2), these dual specificity kinases are the immediate upstream activating kinases for ERK1 and ERK2 (Crews et al.,

1992). The MAPK kinase kinase for this module, which is the most extensively studied, Raf, has been shown to activate MEK1 and/or MEK2 (Kyriakis et al., 1992; Dent et al., 1992). Only the knockout of ERK1 has been described (Pages et al., 1999). ERK1 deficient mice are viable and appear normal and with a modest defect in T-cell development, suggesting that ERK1 is dispensable and that the second isoform, ERK2, can compensate for the loss of ERK1. A similar and more marked defect is present in transgenic mice expressing dominant-negative MEK1 in thymocytes (Alberola-Ila et al., 1995).

The p38 pathway

Four isoforms of p38 have been identified: p38 (also called p38α), p38β, p38γ (also called ERK6), and p38δ. p38α and p38β are expressed in almost all tissues and are particularly abundant in brain and heart (Jiang et al., 1996). In contrast, p38γ and p38δ show very selective tissue distribution, with p38γ predominantly expressed in skeletal muscle and p38δ enriched in lung, kidney, testis, pancreas, and small intestine. In the past few years, intensive study has been done regarding the activation of p38a in many systems, whereas there is not much information concerning activation of the other isoforms. Growth factors, GPCR agonists, heat shock, cell stretching, and ischemia have all been found to be able to trigger the activation of this pathway (Kumar et al., 2003). Like all MAP kinases, p38 is activated by dual kinases, the MAPKKs. MKK6 can activate all four p38 isoforms, whereas MKK3 activation of both the p38 and JNK pathways, which may be the reason why p38 and JNK are often co-activated. However specific activation of p38 and JNK has been observed, implying that there is specific activation of the p38 pathway at this level. The biological consequences of p38

activation are very diverse, varying from production of pro-inflammatory cytokines, induction of enzymes like cyclo-oxyenase 2 (COX-2), induction of adherent proteins such as VCAM-1 and many other inflammatory related molecules (Kumar et al., 2003).

ERK, p38 and chemokines

Several reports have shown that ERK and/or p38 is involved in chemotaxis induced by serum, lysophosphatidylcholine, and chemokines in leukocytes and smooth muscle cells (Jing et al., 2000; Ayala et al., 2000; Stupack et al., 2000). p38 is also involved in chemotaxis induced by MCP-1 in THP-1 cells (Ashida et al., 2001), contradicting previous results, which showed that ERK but not p38 is responsible for MCP-1-mediated chemotaxis (Yen et al., 1997). On the other hand, IL-8-mediated chemotaxis is both ERK and p38 independent (Knall et al., 1997). It has also been demonstrated that GROα in parental melanoma cells enhances Ras, MEKK1, MEK3/6, p38, but not ERK activity (Wang and Richmond, 2001), whilst the pro-apoptotic signal SDF1 sends through CXCR4 in $CD4^+$ T cell are p38 phosphorylation-dependent (Vlahakis et al., 2002).

1.7 *Actin and the cytoskeleton*

The cytoskeleton is a cellular network of structural, adaptor and signalling molecules that regulates most cellular functions including those related to the immune response, such as migration, extravasation, antigen recognition, activation and phagocytosis. Cytoskeletal genes represent 2.8% of the human genome, and they form a part of complex and finely regulated polymer networks, including microfilaments, microtubules and intermediate filaments.

Microfilaments, composed of filamentous (polymerised) actin (F-actin), are mainly utilized in eukaryotic cells to drive locomotion by the extension of pseudopods. Depending on their morphology and the cellular context, pseudopods, are called lamellipods, leading lamellae, or ruffles. Many different processes depend on cell locomotion, including morphogenetic movements during embryonic development, movement of neurites during development and remodelling of the nervous system, chemotactic movements of immune cells, and fibroblast migration during wound healing.

The high rates of actin polymerisation or depolymerisation, are regulated by many capping, nucleator and adaptor proteins which allow fast growth and deconstruction of microfilament-based structures (Pollard and Borisy, 2003). The Arp2/3 complex, an abundant assembly of seven subunits (Machesky et al., 1994), which is comprised of two actin-related proteins (Arp2 and Arp3) with five novel proteins: p40 (ARPC1), p35 (ARPC2), p19 (ARPC3), p18 (ARPC4), and p14 (ARPC5) is integral in this process. At the leading edge of motile cells, the entire network of actin filaments is a branched array with Arp2/3 complex localized to the branch sites (Svitkina and Borisy, 1999). The best studied Arp2/3 complex activators are members of the Wiskott-Aldrich syndrome protein (WASP) and WASP family Verprolin-homologous proteins (WAVE) (Machesky and Insall, 1998; Miki et al., 1998), which are regulated by Rho-type small GTPases.

Rho GTPases

Ras homology (Rho) family GTPases, are small (20-30 kDa) GTP-binding proteins of the Ras superfamily. The prototype Rho family members are RhoA,

(Rac1), and Cdc42. Their distinct biological were first demonstrated in microinjection experiments in fibroblasts, in which the introduction of Rho induced the formation of actin stress fibers and focal contacts, whereas the introduction of Rac, in a distinct pathway, led to membrane ruffling and formation of lamellipodia. Cdc42 induced a third signal transduction pathway, producing finger-like structures known as filopodia, which contain bundles of F-actin (Ridley and Hall, 1992; Ridley et al., 1992; Nobes and Hall, 1995). These proteins function as binary switches by cycling between the active GTP-bound state and the inactive GDP-bound state. In the GTP-bound form, these proteins bind to and activate a variety of downstream effector proteins including kinases, actin-binding proteins, and lipid-modifying enzymes. The guanine nucleotide binding cycle of Rho family GTPase is controlled by: guanine nucleotide exchange factors (GEFs) which promote the transition from the inactive GDP-bound state to the active GTP-bound conformation; GTPase-activating proteins (GAPs) that stimulate the inactivation; and guanine nucleotide dissociation inhibitors (GDIs) act to lock the GTPase in either the active or inactive state.

Rac-1 and Cdc42

By regulating F-actin, Cdc42 and Rac exert a profound effect on cell shape, polarity, migration, cell:cell and cell:matrix adhesion, protein traffic, and cytokinesis. Rac and Cdc42 are required at the front of the cell to regulate actin polymerisation and membrane protrusion. For efficient cell migration, this activity would be expected to be spatially restricted as demonstrated for Rac which can be visualized in migrating fibroblasts with the highest concentrations at the leading edge (Kraynov et al., 2000).

Over 30 target proteins have been identified that interact with either Cdc42 or Rac, or both, specifically in their GTP-bound forms (reviewed in (Bishop and Hall, 2000)). Many, though not all, of these contain a recognizable motif, the Cdc42 and Rac interactive binding (CRIB) motif, as part of their Rac/Cdc42 binding domain (RBD) (Burbelo et al., 1995). Cdc42 binds to WASP or N-WASP, which are primary effectors mediating filopodia formation, whereas Rac activates WAVE. Interestingly, while the WASPs contain a recognizable CRIB motif, the WAVEs do not. Instead, their linkage to small GTPases is provided by an adaptor protein, insulin receptor substrate p53 (IRSp53) (Miki et al., 2000). WASP binding to Cdc42 and PtdIns3,4-P_2 (via a PH domain), opens its normally masked and auto-inhibited C-terminal domain that binds Arp2/3, thus regulating the position of newly assembled actin filaments (Prehoda et al., 2000). Another downstream effector of Rac that has been implicated in cytoskeletal rearrangements and membrane ruffling are the p21 activated kinases (PAKs). PAKs are serine/threonine protein kinases that associate with Cdc42, and usually also with Rac, via a conventional CRIB motif. PAKS have been implicated in MAP kinase signalling pathways, apoptosis, and cytoskeletal regulation (Knaus and Bokoch, 1998).

Rho A

RhoA-induced stress fiber formation is associated with focal adhesion assembly and cell contractility and is responsible for cell body contraction and rear end retraction (Ridley and Hall, 1992). RhoA activation induces changes in the actin cytoskeleton through a large number of downstream targets, of which the best characterised are the Rho-activated kinases, the Rho-associated coiled-coil forming protein kinases (ROCK). Rho and p160 ROCK have been shown to be

essential for rear cell detachment in single migrating leukocytes (Alblas et al., 2001). p160 ROCK can phosphorylate and activate LIM kinase (named from the Lin-11, Isl-1 and Mec-3 genes) (LIMK), which in turn phosphorylates and inactivates cofilin, leading to stabilization of actin filaments within actin:myosin filament bundles (Maekawa et al., 1999). p160ROCK also inhibits by phosphorylation the myosin binding subunit of myosin light chain (MLC) phosphatase (Kawano et al., 1999) thereby regulating actin–myosin contraction formation of actin stress fibers (Ridley, 2001). Another important downstream target of Rho is the mammalian ortholog of *Drosophila* Diaphanous (mDia), mDia belongs to the formin-homology containing family of proteins, which have been linked to actin filament assembly in both *Drosophila* and yeast (Pruyne et al., 2002; Sagot et al., 2002).

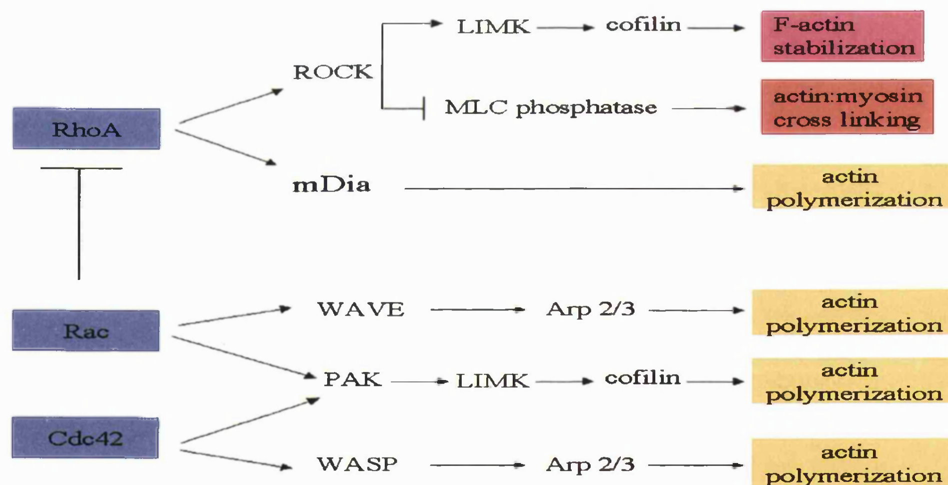

Figure 1.10 Rho-GTPase pathways in actin filament organization. Rho promotes contractile actin:myosin filament assembly through two effectors, mDia and p160ROCK. Rac and Cdc42 both regulate actin polymerisation through WASP/WAVE or through PAK kinases. Abbreviations: Arp2/3, actin related proteins 2/3 complex; mDia, mammalian ortholog Diaphanous; MLC, myosin light chain; LIMK, LIM kinase, PAK, p21 activated proteins; WASP, Wiskott-Aldrich syndrome proteins; WAVE, WASP family Verprolin-homologous proteins.

Rho activity at the front of a migrating cell is incompatible with membrane protrusion and hence mechanisms must inhibit its activity at the leading edge. One way this might occur is through Rac. Expression of activated Rac has been shown to inhibit Rho function in many cell types, (Sander et al., 1999). A summary of the pathways of the Rho GTPases regulating actin organization is shown in figure 1.10.

1.8 *Antigen presentation and the CD28/B7 model of co-stimulation*

T lymphocytes play a key role in immunity by distinguishing self from nonself peptide antigens and regulating both the cellular and humoral arms of the immune system. To avoid damage to the host, these immune responses must be tightly regulated. Molecular recognition is performed by the antigen receptor of T lymphocytes, the T- cell receptor (TCR). The TCR is limited to scanning 9-12 amino acid long peptides bound in the groove of major histocompatibility complex (MHC) class I or class II glycoprotein heterodimers (Germain, 1994), or glycolipids bound to the MHC-like molecule CD1d (Beckman et al., 1994). MHC class I molecules, which present peptides form endogenous sources to $CD8^+$ T-cells, are expressed on the majority of nucleated cells. In contrast, MHC class II molecules, which present peptides from exogenous sources to $CD4^+$ T cells, are found primarily on the surface of specialised antigen presenting cells (APCs) due to the tightly controlled expression of the class II transactivator (CIITA), which is essential for MHC class II transcription (Chang et al., 1994).

The three classical MHC class I molecules (HLA-A, HLA-B and HLA-C) play essential roles in the detection and elimination of virus-infected cells, tumor cells and transplanted allogeneic cells. The non-classical MHC class I molecules

(HLA-E, HLA-F and HLA-G) have specialized immune regulatory functions (Braud et al., 1999). HLA-E functions predominantly as an inhibitor of NK-cell functions, whereas HLA-G inhibits both T- and NK-cell functions, including the transendothelial migration of human NK cells (Dorling et al., 2000).

MHC class II genes encode the polymorphic HLA-DR, HLA-DQ and HLA–DP proteins, which are expressed as αβ heterodimers on the cell surface. MHC class II molecules play a central role in the initiation of the cellular and humoral immune responses, but they have also been implicated as contributing factors for a variety of autoimmune disorders, and they play an important role in transplant rejection. Constitutive expression of MHC class II proteins is confined to APCs, which include dendritic cells, macrophages, B lymphocytes and thymic epithelial cells. On most other cell types, expression of MHC class II molecules can be induced in an environment rich in inflammatory cytokines of which IFN-γ is the most potent (Giacomini et al., 1988).

T-cell anergy and co-stimulation

Acquired, antigen-specific unresponsiveness is the most important mechanism by which T-cell responses to antigen are regulated *in vivo.* The term *unresponsiveness* was coined by Bretscher and Cohn in 1970 to describe 'the immunological state of an animal to which antigen has been administered and which cannot subsequently respond to that antigen but can respond to other non cross-reacting foreign antigens' (Bretscher and Cohn, 1970).

Engagement of the TCR with MHC-peptide complexes may elicit four distinct functional outcomes: no response (ignorance), productive T-cell activation, induction of unresponsiveness to subsequent antigen, or activation-induced cell

death (peripheral deletion). T-cell responses need therefore to be tightly regulated and are subject to finely tuned control mechanisms. One such mechanism is the requirement for a co-stimulatory signal (Signal 2) provided by soluble factors or cell-surface molecules on APCs, in order to produce full T-cell activation. T-cell clones fail to proliferate in the absence of co-stimulatory signals and become refractory to further activation (Jenkins and Schwartz, 1987).

The finding that T-cell inactivation, termed T-cell anergy, was a direct consequence of regulated IL-2 production (DeSilva et al., 1991) led to the search for a master co-stimulatory signal that targeted the IL-2 pathway. This search resulted in the identification of the CD28/B7 pathway as a prominent co-stimulatory pathway for T-cells (Harding et al., 1992). Although additional co-stimulatory pathways have since been identified, including the CD40 ligand CD154/CD40, CD2/CD58, LFA-1 (CD18)/ICAM-1 (CD54), and others, the CD28 CD28/B7 pathway remains the most potent and well characterized.

Receptors of the CD28 family

CD28

CD28 is expressed on virtually 100% of murine T-cells, all human $CD4^+$cells and about 50% of human $CD8^+$ T cells. Although CD 28 is expressed constitutively, its levels increase after T cell activation (Turka et al., 1990). CD28 is also highly expressed on developing thymocytes (Gross et al., 1992) although its role in thymocytes is not well understood. Signalling through CD28 is required for optimal IL-2 production, IL-2 receptor expression and cell cycle progression (Jenkins et al., 1991). CD28 also regulates cell survival by induction of the anti-apoptotic protein Bcl-X_L and activation of PKB (Parry et al., 1997).

CTLA-4

Cytotoxic T lymphocyte antigen 4 (CTLA-4) is expressed on the surface of activated $CD8^+$ and $CD4^+$ T cells. However, unlike CD28, CTLA-4 is not expressed on the surface of resting T cells. Moreover, CTLA-4 expression is only 2-3% of the levels of CD28 (Linsley et al., 1992). In contrast to CD28, CTLA-4 delivers a negative signal to the activated T cell, opposing CD28-mediated co-stimulation (Walunas et al., 1994). Mice deficient in CTLA-4 have been shown to exhibit profound lymphoproliferative defects that are characterized by polyclonal T-cell activation and a high frequency of cells expressing activation and/or memory T cell antigens (Tivol et al., 1995).

ICOS

Inducible co-stimulator (ICOS) was first identified in a screen for unique molecules expressed on human peripheral blood T-cells following activation. ICOS enhances all basic T-cell responses to foreign antigen and like CTLA-4 it has to be induced on the T cell surface (Hutloff et al., 1999). ICOS-mediated co-stimulation does not induce IL-2 production but increases secretion of IL-4, IL-5, IL-10, INF-γ and TNF-α suggesting that ICOS functions primarily to induce T-cell effector function (Yoshinaga et al., 1999).

PD-1

Programmed Death 1 (PD-1) is unique among the CD28 family members in that it is widely expressed on hematopoietic-derived tissues. It is constitutively expressed on a subset of $CD4^-$ $CD8^-$ thymocytes, immature B cells and some peripheral T-cells, and is expressed on T-cells, B cells, monocytes and myeloid

cells following activation (Ishida et al., 1992). PD-1 like CTLA-4, appears to mediate an inhibitory signal. The exact role of PD-1 is just beginning to be elucidated. It has been reported that PD-1 ligation inhibits IFN-γ, IL-10, and IL-2 secretion (Freeman et al., 2000), but unlike CTLA-4 , PD-1 can influence positive and negative thymocyte selection (Nishimura et al., 2000) .

BTLA

The B and T lymphocyte attenuator (BTLA), is the most recently discovered Ig superfamily member (Watanabe et al., 2003). BTLA engagement results in down-regulation of T-cell activation, and mice deficient in BTLA show increased incidence and severity of autoimmune disorders. Table 1.10 summarizes the ligands for the CD28 family receptors.

Ligand	Alternative name	Receptor(s)	Expression
B7-1	CD80	CD28, CTLA-4	Induced on DC, T, B (Hathcock et al., 1994)
B7-2	CD86	CD28, CTLA-4	DC, monocytes (Chang et al., 1995)
PD-L1	B7 H1	PD-1	DC, induced on monocytes (Dong et al., 1999)
PD-L2	B7 DC	PD-1	DC, monocytes (Tseng et al., 2001)
B7 RP-1	B7h, GL-50	ICOS	B, DC, up-regulated on monocytes by IFN-γ (Aicher et al., 2000)
B7 H3	B7 RP-2	?	DC (Chapoval et al., 2001)
B7 H4	B7S1, B7x	BTLA	DC, B, macrophages (Prasad et al., 2003)

Table 1.10 B7 ligands for the CD28 family receptors, alternative names and expression in the immune system. Abbreviations: B7 RP-1, and 2, B7 related proteins 1 and 2; BTLA, B and T lymphocyte attenuator CTLA-4, cytotoxic T lymphocyte antigen 4; DC, dendritic cells; ICOS, inducible co-stimulator; PD-1, programmed death 1; PD-L1 and 2, programmed death ligands 1 and 2.

Co-stimulation in the intestine

The notion that T-cells primed in the gut play an important role in regulating mucosal immune responses has been long suggested. Normal intestinal epithelial cells (IECs) can process and present antigen to T-cells, including $CD8^+$ regulatory T-cells, which may control the inflammation seen in the intestine (Allez et al., 2002). However, the absence of conventional co-stimulatory molecules on normal intestinal epithelium would suggest that antigen presented by IECs would result in anergy. Lack of co-stimulatory molecule expression could be a way to control mucosal immune responses in the gastrointestinal tract, where exposure to dietary, viral, and bacterial antigens is constant.

In UC, however, B7.2 is expressed (Nakazawa et al., 1999), while PD-L1 can be induced by IFN-γ on a colonic epithelial cell line (Dong et al., 1999), underscoring, at least in this disease, the potential contribution of the IEC to mucosal T-cell responses. Moreover, regulatory T-cells were demonstrated to inhibit inflammation through ICOS-B7RP-1 engagement (Akbari et al., 2002), suggesting that regulation of co-stimulation by IECs (and other APCs in the mucosa) may have a beneficial therapeutic effect in patients with inflammatory responses characteristic of IBD.

1.9 *Aims of the study*

During the past decade, several experimental approaches have stressed the functional importance of the mesenchymal cell compartment in the intestine. The permissive and instructive actions of myofibroblasts on gastrointestinal epithelial cells has been demonstrated and underlined by the observation that these cells

secrete important growth factors whose receptors are found on the epithelial cell surface, exemplified by the expression of HGF and KGF which regulate the epithelial cell behaviour. These studies have highlighted the central role of intestinal epithelial cells in regulating the mucosal immune system and its response. It is now well established that these cells are capable of performing various immunological functions, such as expression of class I and II MHC antigens, presentation of antigens to lymphocytes, expression of adhesive molecules, and production of cytokines. These functions allow them to interact with other cells of the immune system in order to induce an efficient inflammatory response.

The role of myofibroblasts has been slightly overlooked, even though there is growing evidence that these mesenchymal derived cells present in the gut lamina propria interact with various other cell types, among which are immune cells, indicating their involvement in the inflammatory cascade. The aim of this thesis is therefore to investigate the participation of myofibroblasts in a trimeric model consisting of epithelial cells, T lymphocytes and myofibroblasts. More specifically this project will investigate two main areas.

The first is to determine a possible role for myofibroblasts in the activation process of T-cells. This will be done by investigating the possibility of co-stimulatory molecules expression on myofibroblasts, as has already been demonstrated for intestinal epithelial cells.

The second is to establish a possible cross-talk pathway between myofibroblasts and epithelial cells. Colonic epithelial cells are known to secrete members of the chemokine superfamily, which are strong chemoattractants for T-cells. Possible

expression of the cognate chemokine receptors on intestinal myofibroblasts would therefore substantially influence the immune response during intestinal inflammation.

Chapter 2

2 Methods and materials

2.1 *Methods*

Cell Culture

All cells were cultured at 37° C in a humidified atmosphere of air supplemented with 5% CO_2. For long term storage cells were frozen under liquid nitrogen. Cells were pelleted (400g, 5 min), resuspended at 10^7 cells/ml of freeze medium (90% FBS / 10% DMSO) and aliquoted in cryovials. Vials were then gradually cooled in vapour phase of liquid nitrogen overnight and tubes were stored in liquid nitrogen tanks. For resuscitation of cells from liquid nitrogen, cells were rapidly defrosted at 37°C in a water bath, washed twice in fresh medium, resuspended in complete medium and returned to culture. Cells from one cryovial were seeded into 175cm^2 tissue culture flasks in 50 ml of medium.

Mucosal Tissue

Fresh, histologically normal, colonic mucosal samples, were obtained from human intestinal specimens resected at operation. Normal colonic mucosal samples were obtained >5 cm from the tumor, from multiple colonic biopsies in patients who underwent colonoscopy at the Royal United Hospital, Bath. Biopsies were immediately placed in transport medium, Hanks balanced salts solution (HBSS) pH 7.3, supplemented with antibiotics (penicillin 100 U/ml, streptomycin 100 μg/ml, gentamicin 50 μg/ml, and fungizone 2.5 μg/ml), transferred to the laboratory and gently washed 3 times for 15 minutes in HBSS and cut into small pieces of about 1 cm.

Isolation of intestinal myofibroblasts

Intestinal myofibroblasts were isolated from these mucosal samples as previously described (Mahida et al., 1997). Biopsy specimens were treated with 1mM DTT for 15 minutes and washed again 3 times in HBSS. They were then incubated for 30 minutes at 37° in the presence of 1mM EDTA, pH 8.0 for 3 times. At the end of the EDTA treatment the mucosal samples were completely denuded of epithelial cells and were subsequently cultured in RPMI medium supplemented with antibiotics. During culture numerous cells appeared both in suspension and adhered to the culture flask. The cells in suspension were removed every 24h-72h culture period and the denuded mucosal tissue was maintained in culture for up to 4 weeks till myofibroblasts appeared attached to the bottom of the culture flask. Tissue specimens were then removed, and intestinal myofibroblasts were cultured in DMEM medium supplemented with penicillin (10 u/ml), streptomycin (10 μg/ml), fungizone 0.5 μg/ml, 1% (v/v) non-essential amino acids and 10% (v/v) foetal bovine serum (referred to as complete medium). Cells were passaged when fully confluent in a 1:2 to 1:3 split ratio.

Intestinal myofibroblasts were routinely cultured in 500 cm^2 tissue culture flasks in complete DMEM medium. The medium was changed every 3 days. To subculture confluent monolayers, the medium was removed and the cells were washed twice with PBS (without Ca^{2+} and Mg^{2+}). Cells were then treated once with a 20 ml Trypsin-EDTA mixture of 0.05% (w/v) trypsin and 0.02% (w/v) EDTA. The cells were then incubated for approximately 5 minutes at 37°C until the cells had detached from the flask. Adding 50ml of complete medium inhibited the action of Trypsin-EDTA and the cell suspension was centrifuged at 400g for 5

minutes. The cell pellet was resuspended in complete medium and cell counting and viability were checked in a Neubauer haemocytometer after mixing with Trypan Blue. Dead cells stained blue, due to the uptake of Trypan Blue. Cell viability was always greater than 95%. Cells were counted and then seeded at 2-3 x 10^4/ml of DMEM complete medium, into 500 cm^2 tissue culture flasks for further culture, or into 6 well plates or smaller tissue culture flasks for experimental protocols. Cells were used between passage number 6-12, since they have been reported to acquire an altered phenotype at higher passage numbers.

18 Co cells

The 18Co colon adenocarcinoma cell line is human fibroblast cell line that was isolated from a primary tumour in a 2.5month year old Black female (ATCC). They are well characterised with features that match the myofibroblast phenotype (Valentich et al., 1997). 18Co cells were provided by Dr Don Powell (University of Texas, Department of Internal Medicine and Physiology, Texas, USA) and cultured in MEM medium supplemented with penicillin (10 u/ml), streptomycin (10 μg/ml), and 10% (v/v) foetal bovine serum. Cells were passaged when fully confluent and used between passage numbers 6-12 since they have been reported to acquire an altered phenotype at higher passage numbers. (Valentich et al., 1997)

Peripheral blood mononuclear cell isolation

Blood form healthy donors was taken aseptically in 50ml syringes containing heparin 1U/ml of blood, via 19-gauge 'butterfly' needles. The blood was diluted 1:1 with RPMI 1640 culture medium and 35 ml aliquots of the mix were carefully

layered on to 15ml Lymphoprep™ in 50 ml transparent centrifuge tubes and centrifuged brake-free for 30 min at 400g. The monocyte/lymphocyte (PBMCs) band was carefully removed, washed three times in RPMI (400g, 10min, 20° C), and counted under a x200 microscope with a Neubauer haemocytometer.

Peripheral blood derived T-blast preparation

PBMCs obtained from centrifugation with Lymphoprep™ as described above, were re-suspended at 1 x 10^6 cells /ml in RPMI 1640 with 10% (v/v) FBS, 50 U/ml penicillin and 50μg/ml streptomycin. They were then incubated for 72h with 1 μg/ml Staphylococcal Enterotoxin B. After 72h and every 48 for 10-15 days, the T lymphoblasts were supplemented with 20ng/ml hr IL-2. T-cells were maintained at 0.5-1.5 x 10^6 cells /ml. Prior to use, the cells were washed of IL-2 and deprived for 18 hours to allow accumulation in the G_0 phase of the cell cycle so that they represented a more homogenous population with respect to IL-2 receptor expression and signalling potential (Cantrell et al., 1989).

Chinese Hamster Ovary cells

CHO cells stably transfected with B7.1, were cultured in Ham's F12 medium supplemented with, 2mM glutamine, penicillin (10 u/ml), streptomycin (10 μg/ml), and 10% (v/v) foetal bovine serum.

Jurkat cells

Jurkat cells were cultured in RPMI 1640 medium supplemented with, penicillin (10 u/ml), streptomycin (10 μg/ml), and 10% (v/v) foetal bovine serum.

Experimental protocol

Unless otherwise stated, primary intestinal myofibroblasts or 18 Co cells were grown until confluent. Prior to experiments, monolayers were washed and cultured in DMEM without serum for 24 hours. Growth-arrested cultures were washed twice with PBS, treated with fresh serum free medium and stimulated with the appropriate doses of either drugs, cytokines or vehicle controls for the times described in the results section.

Peripheral blood derived T-cells were washed in RPMI 1640 without serum three times and left in a water bath at 37°C for 60 minutes prior to stimulation. Supernatants were collected, centrifuged to remove cellular debris and stored at -70°C until assayed. Total RNA and cellular proteins were extracted as described below.

Polymerase chain reaction

Kleppe and colleagues first described the polymerase chain reaction, a technique used to amplify virtually any DNA segment that lies between two regions of known sequence, in 1979. PCR was applied to amplify DNA encoding for chemokine receptors and members of the B7 family. The cDNA was generated by reverse transcription from mRNA isolated from cells and PCR enabled visualisation of DNA segment bands in UV illuminated ethidium bromide gels. With this qualitative process, the extent of constitutive transcription of chemokine receptor or B7 family members was assessed, as well as responses to various stimuli.

Sample mRNA extraction

Sample mRNA was extracted from primary intestinal myofibroblasts, 18 Co cells and T-lymphoblasts at various time points. Initially total cellular RNA was isolated from the cells using RNAzol B according to the manufacturers instructions. For the adherent cells, monolayers were lysed directly in the culture dish by the addition of 1ml RNAzol B per well. The lysate was homogenised with a sterile cell scraper and transferred to sterile eppendorf tubes by pipette. For the suspension cells 1 x 10^6 cells were quickly centrifuged at 13000 rpm, the supernatant was discarded and the cells were homogenized in 400 μl RNAzol B. 100 μl of chloroform per 1 ml of homogenate was then added, the samples shaken vigorously for 15 seconds and then cooled on ice for 15 minutes. The samples were then centrifuged at 14.000 rpm for 15 minutes at 4°. This results in the formation of two phases: a lower blue phenol-chloroform phase and the RNA containing upper aqueous phase. The aqueous phase was then transferred to a clean eppendorf tube and an equal volume of isopropanol was added. The samples were then cooled on ice for 15 minutes and centrifuged again at 14.000 rpm for 15 minutes at 4°. RNA will then form a precipitate at the bottom of the tube. The supernatants were removed and the RNA pellet washed in 1ml 70% ethanol. The pellets were then dried and re-dissolved in RNase free water.

RNA was quantified, using a deuterium lamp spectrophotometer, Gene Quant II RNA/DNA calculator, (Pharmacia, UK). RNA concentration is measured by the absorbance of 1 μl of RNA sample diluted in 500 μl of water at 260 nm. The amount of RNA (in μg) present in each sample was calculated by the following formula:

A_{260} x dilution factor (500) x40 x volume of remaining RNA solution in ml (0.048)

ODs were also read at 280 nm and 230 nm to assess the purity of RNA. A value of less than 2 for the OD_{260} : OD_{280} ratio indicated protein contamination. A low OD_{260} : OD_{230} ratio indicated guanidine contamination. 1 µg of total RNA was dissolved in 8 µl of nuclease free water and was treated with 1 µl DNase I for possible DNA contamination. DNase I was then inactivated by the addition of 1µl EDTA and heating at 65° for 10 minutes.

Reverse Transcription (RT) step

The resulting solution was then mixed with 2µl oligo (dT) cellulose and denatured at 70° for 10 minutes. The reverse transcription mixtures were made up in 0.2 ml PCR tubes and contained 4 µl reverse transcription buffer, 1µl DTT, 1µl DNTP's, 1µl RNAsin and 1µl of reverse transcriptase per sample and together with the 12 µl mRNA sample gave a final volume of 20 µl per PCR tube. The final concetration in 20 µl of the constituents was: 1µM of pd $(T)_{12-18}$, 0.5mM from each of the deoxynucleoside triphosphates dATP, dCTP, dGTP and dTTP, 10 U/µl Superscript II RNase H^- Reverse Transcriptase, and 1 U/µl RNAsin, a non-competitive ribonuclease inhibitor.

The tubes were placed in a Perkin Elmer Gene Amp 2400 thermocycler (Warrington, UK) and followed a reverse transcription program of: 42° C for 60 min, 94° C for 2 min and 4° C thereafter. The RT products were either used immediately, or briefly stored at -80° C.

PCR step

A forward and reverse primer was obtained for all the genes of interest, using Primer 3 design software, which is available on the Internet. The DNA sequence that was amplified by PCR was selected in a way that the primers were spanning at least one intron, which would result in the generation of a larger intron-containing PCR product in the case of DNA contamination. Each RT template from an experimental sample, apart from the genes of interest, was also tested for a positive control, the house keeping gene β-actin and a negative control of original RNA before the RT step, to check for DNA contamination. Each PCR reaction was carried out in 0.2 ml thin PCR tubes in 25 μl total volume containing 0.05 μg cDNA template, 15.9 μl of nuclease-free water, and the following final concentrations of constituents (in appropriate volumes to give a final volume of 25 μl per tube): 200 μM of each of the 4 deoxynucleoside triphosphates dATP, dCTP, dGTP and dTTP, 500nM of the forward primer, 500 nM of the reverse primer, 1.25 Unit of Expand ™ High Fidelity enzyme mix which comprised of 2 DNA polymerase enzymes: Taq and a proofreading polymerase and 1x the Expand ™ PCR buffer with Mg^{2+}. The polymerase enzymes and PCR buffers were used according to 'Expand ™ High Fidelity PCR System' manufacturer's specifications, and the theromocycler was given the following programme: 30 sec 94° C, 30 sec 60° C, 30 sec 72° C repeated for 30 cycles and two holds, one at the beginning of the programme for 5min/94° C and one at the end for 7 min/72° C, prior to cooling to 4° C thereafter. The temperatures set were optimal for the phases of the PCR cycle: 94° C for denaturation of the template, 60° C for annealing of the primers to the open DNA strand and 72° C for optimum DNA synthesis by the heat stable polymerase enzymes. Cycle composition was

determined by manufacturer's instructions and by GC content of primers (annealing temperature). The PCR products were either loaded immediately on an agarose gel, or briefly stored at 4° C.

Detection of PCR products

A 2% agarose gel was made in TBE (10 mM Tris base, 10 mM Boric acid, 2 mM EDTA, pH 8.0), boiled and cooled with the addition of 1 μg/ml ethidium bromide. 5 μl from each tube containing the PCR end products were coloured with 5 μl of Blue Juice (15% (w/v) Ficoll 400, 0.25% (w/v) bromo-phenol blue in water). A 100 base pair ladder comprising of 15 blunt end fragments (100-1500 base pair scale) was also coloured with Blue Juice. Samples and ladder were loaded onto the agarose-ethidium bromide gel and run by gel electrophoresis with 100V/0.1 mA current (BioRad Instruments). The resulting bands were visualised with a UV illuminator and photographed with a Polaroid camera and film or using the Gene Quant imaging software program

Sequence and design of primers

The sequence of the primers used are summarized in the following table:

Gene	Forward primer	Reverse primer
β-actin	CATCACCATTGGCAATGAGC	ATACTCCTGCTTGCTGATCC
B7.1	CTTACCACCTTGCTTCTGTG	AGGATCACAATGGAGAGGTT
B7.2	CTTACCACCTTGCTTCTGTG	CCCATAGTGCTGTCACAAAT
PD-L1	GGTCATCCCAGAACTACCTC	ACGGAAGATGAATGTCAGTG
B7 RP-1	AGAACAGCTCCTTGGAAAAC	TCACATTGGAGTTGCGAGTT
B7-H3	AGCAGGGCTTGTTTGATGTG	TGATCTTTCTCCAGCACACG
ICOS	ACAAACACCCTCTTGCAACC	TCCAGCTTTGAAGCATCTCC
CD28	ATCCCTTCACAAAGGACTGG	GGTGTTTCCCTTTCACATGG
CTLA-4	TCACTATCCAAGGACTGAGG	TAGACCCCTGTTGTAAGAGG
CXR3	GCCAATACAACTTCCCACAG	TGACCCCTACAAAGGCATAG

Parameters that were taken into account when designing the primers were: the length of the primer, the melting temperature, the specificity and the G/C content of the primers. One of the most critical parameters in primer design is the melting temperature (T_m). Both of the oligonucleotide primers should be designed such that they have similar melting temperatures. If primers are mismatched in terms of T_m, amplification will be less efficient or may not work at all since the primer with the higher T_m will miss-prime at lower temperatures and the primer with the lower T_m may not work at higher temperatures. The melting temperatures of oligos are most accurately calculated using thermodynamic calculations with the formula:

$$\mathbf{T_m^{primer} = \Delta H\ [\Delta S + \mathit{R}\ ln\ (c/4)] - 273.15°C + 16.6\ log_{10}\ [K^+]}$$

where H is the enthalpy and S is the entropy for helix formation, *R* is the molar gas constant and c is the concentration of primer. This is most easily accomplished using any of a number of primer design software packages on the market. Fortunately, a good working approximation of this value (generally valid for oligos in the 18–24 base range) can be calculated using the formula:

$\mathbf{T_m = 2(A+T) + 4(G+C)}$, which is known as the Wallace formula.

Using the above formula and keeping the length and the G/C content of the primers constant, at 20 bases and 50% respectively, the resulting primers had T_m of 60° C. Specificity was ensured by putting the primers through the NCBI blast search facility, and primers that amplified additional sequences of DNA to the gene of interest were redesigned.

Cell Lysis and sample preparation for SDS-PAGE

Monolayers of primary intestinal myofibroblasts were trypsinized, washed three times in DMEM and re-suspended at the desired concentration in 500 μl DMEM/ 20 mM HEPES and placed in a water bath at 37° C. After appropriate treatments and stimulations, reactions were terminated by rapid-pulse cell pelleting and supernatant aspiration, followed by lysis with the addition of 50 μl/ point of freshly prepared ice cold lysis buffer (137 mM NaCl, 20 mM Tris pH 7.5, 10 mM NaF, 1mM EDTA, 1% w/v IGEPAL CA-630, 10% w/v glycerol, 1mM sodium orthovanadate, 1μg/ml leupeptin, 1 μg/ml pepstatin A, 1 μg/ml aprotinin and 1 mM phenyl methyl sulphonyl fluoride. The samples were rotated at 4° C for ten minutes before removal of the nuclear lysate debris by centrifugation for 15 min at 14000 rpm / 4° C in a microfuge. Supernatants were removed to a clean tube and used immediately or stored at -20° C.

Protein assay

Total protein per lysate was estimated using the Bio-Rad *DC* Protein Assay. This assay is based on the Bradford dye-binding procedure. Known concentrations of bovine serum albumin (BSA) diluted in lysis buffer were used as a standard curve. 5 μl of sample or standard were placed in a 96-well plate with 25 μl of working reagent A' (20 μl reagent S into 1 ml reagent A), plus 200 μl of Bio-Rad reagent B, provided in the kit. After 15', the plate was read at 595 nm on a Dynatech MR5000 platereader. The protein concentrations were calculated by linear regression from the standard curve and, if significantly variable, the lysate volumes were adjusted using lysis buffer, thus ensuring equal concentrations of protein in each sample.

Total protein preparation

Protein samples were resolved essentially as described by Laemmli (1970), according to protocols established in our laboratory. Proteins to be analysed from total cell lysates were solubilised by boiling for 5 minutes in SDS-PAGE sample buffer consisting of 4% (w/v) SDS, 20% (w/v) glycerol, 125 mM Tris, 10% (v/v) 2-mercaptoethanol and coloured appropriately with bromophenol blue.

Western Blot Analysis

Proteins were separated by SDS-PAGE using the Bio-Rad Mini Protean III. Minigels of the appropriate percentage were prepared as described below. The resolving gel was poured into the gel equipment and overlaid with Milli-Q water. Polymerisation took 20 – 30 minutes, after which the water was aspirated off, the stacking gel was poured and a 10 or 15 lane comb inserted. Polymerisation took 20 minutes, the comb was removed and the wells washed thoroughly with Milli-Q water APS and TEMED were added immediately prior to casting the gels. The wells were then filled with 1X SDS-PAGE running buffer consisting of 25mM Tris, 192 mM glycine and 0.1% w/v SDS.

20 μl of each sample was then loaded into the wells in parallel with molecular weight markers and the gels run at 80 V through the stacking gel, followed by 150 V through the resolving gel, until the bromophenol blue reached the bottom of the gel. Gels were then placed in transfer buffer. The acrylamide gels were prepared as presented in the table below:

	Resolving gel (20 ml)					Stacking gel (12 ml)	
Final % gel	5%	7.5%	10%	12%	14%		5.0%
dH_20 (ml)	11.31	9.63	7.97	6.64	5.31		6.72
Resolving gel buffer pH(8.8)	5.0	5.0	5.0	5.0	5.0	Stacking gel buffer (pH 6.8)	3.0
Bis-Acryl (30%) (ml)	3.33	5.00	6.67	8.00	9.33		2.0
10% APS	150μl	150 μl	150 μl	150 μl	150 μl		150 μl
TEMED	15 μl	15 μl	15 μl	15 μl	15 μl		15 μl

Resolving gel – 5 ml is sufficient for 1 mini gel

Stacking gel – 1.5 ml is sufficient for 1 mini gel

Semi-dry transfer of proteins to nitrocellulose

The graphite electrodes of the semi-dry transfer apparatus (Pharmacia-Biotech Multiphor II) were dampened with semi-dry transfer buffer, followed by placing a sandwich of 4 pieces of 3MM Whatmann paper (the same size as the gel), one piece of nitrocellulose membrane, the gel and another 4 pieces of 3MM paper, all soaked in transfer buffer. Each layer was rolled gently to expel air bubbles. The transfer was run for 60 minutes at 0.8 mA/cm^2 of membrane. The membrane was then stained with Ponceau S to check for transfer and even loading of the samples and to determine the location of the molecular weight markers. The stain was removed by washing the membrane in distilled water for 2 minutes, followed by a 10 minute wash in Tris buffered saline (TBS).

Blocking and Developing

The non-specific protein binding was blocked by incubation of the membrane with the appropriate blocking buffer at room temperature for 60 minutes on a rocking platform. After a10 minute wash in TBS, the membrane was incubated with the primary antibody diluted in a 1:5 dilution of fresh blocking buffer for 2h – overnight (usually 3h). Membranes were washed 1X with TBS, 3X with TBSN (TBS with 0.005% (v/v) Tween 20), 1X with TBS for ~10 minutes each wash. The membrane was incubated for 1 – 2 hours with the appropriate secondary antibody diluted in TBSN, followed by extensive washing as described above. Antibody dilutions ranged from 1:1000 to 1:2000 for primary antibodies and 1:7000 to 1:2000 for secondary antibodies An extra TBS wash for 10 minutes was done before adding 5 ml of Enhanced Chemiluminescent (ECL) reagent for 1 minute. The membrane was exposed to X-ray film for a few seconds up to 30 minutes and the film was developed using an RGII Fuji X-ray film developer.

Membrane stripping

Where appropriate, blots were stripped of bound protein and re-probed with a different primary antibody. After the ECL procedure described above, the membrane would be washed twice in TBS for 10 minutes, placed in 50 ml of stripping buffer (100 mM 2-mercaptoethanol, 2% (w/v) SDS, 62,5 mM Tris pH 6.7) in a sealed sandwich box and incubated for 1 hour at 55°C. After extensive washing in at least three changes of TBSN and one wash in TBS, the membrane would be re-blocked for 1 hour in blocking buffer. A different primary antibody could then be applied to the membrane for further protein detection.

Immunoprecipitation and *In Vitro* Lipid Kinase Assay

Cells were aliquoted at 1x 10^7 per point and lysed as described above in Cell Lysis. The lysates were pre-cleared by adding 20µl of a 50% v/v suspension of either protein-A or protein-G sepharose beads (depending on the antibody to be used) and rotated for 15-30 minutes at 4°C. The beads were spun down at 12000g for 1 minute and the supernatant removed to a clean tube. The protein G sepharose beads from Sigma were provided in methanol and were therefore washed three times with 1 ml of lysis buffer and then re-suspended as a 50% suspension in lysis buffer. 500µl aliquots were stored at 4°C until required. Protein A sepharose is provided in powder which swells up to 4x its weight. Therefore 100 mg of protein A sepharose are re-hydrated by the addition of 1ml ice cold lysis buffer and kept on ice for 30 minutes with regular mixing. The beads are then treated like the protein G beads to give the 50% suspension.

The appropriate antibody was then added to the pre-cleared extract, briefly vortexed and rotated for 1-2h at 4°C. Then 30 µl of the 50 % slurry of the corresponding protein A or G sepharose beads was added for another 1-2h rotation at 4° C.

After completing the immunoprecipitation incubation, the beads were pulse pelleted, and the supernatant was removed. Thereafter, the immunoprecipitates were washed 3 times with 1% IGEPAL CA-630 in PBS, three times with 5mM lithium chloride (in 100 mM Tris, 0.25 mM EDTA pH 7.4), and twice with lipid kinase buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA) spinning at 10000g between each wash. After the final wash, surplus kinase buffer was removed using a Hamilton syringe.

Each of the samples was resuspended in 50μl of lipid kinase buffer. 70μl of the lipid substrate mixture (50 μl of lipid kinase buffer, 10 μl PtdIns and 10 μl 100 mM $MgCl_2$ or $CaCl_2$ was added to the immunoprecipitates. The reaction was initiated by the addition of 5 μCi of [γ-^{32}P]-ATP (S.A. 3000Ci/mmol, 0.5mCi/ml, 18.5MBq) and 100μM ATP. The samples were incubated in a 25°C water bath for 15' and the reaction quenched using 20μl 1M HCl and 160μl 1:1 chloroform:methanol. The samples were spun for 10 at 10000g to separate the phases. 50 μl of the lower chloroform layer was removed and separated by thin layer chromatography TLC, as described previously (Ward et al., 1992).

Laned silica gel 60 plates were pre-treated with 1% sodium oxalate in water and allowed to dry. The extracted phospholipid samples were loaded onto the plates and placed in a pre-equilibrated solvent tank containing chloroform: methanol: water: ammonium hydroxide (60: 47: 11: 2) and lined with filter paper to ensure adequate vapour equilibration. The samples were allowed to run till the solvent front had reached the end of the plate. Thereafter, the plate was air dried and the samples were visualised by exposure to iodine vapour, to confirm even extraction of substrate lipids between individual samples, and finally exposed to a film for 1-12 hours at -70°C. The film was developed using an RGII Fuji X-ray film processor.

FACS Analysis

Cells were trypsinized and centrifuged at 1500 rpm for 5 minutes, washed 3 times in PBS, and re-suspended at 5×10^6 cells/ml in PBS/20% FBS. 90 μl of this suspension was added to polypropylene FACS tubes with 10 μl of the appropriate antibody or isotype control and shaken at 4° C for 30 min. All antibodies were

used at a final concentration of 1 μg/ml. To remove unbound antibody, cells were washed three times in 4 ml of PBS. Following this, they were re-suspended again in 90 μl PBS/20% FBS with 10 μl goat anti-mouse polyvalent (anti-IgM, IgG, IgA) secondary antibody conjugated to FITC. After 30 min shaking at 4° C the cells were washed with PBS and analysed immediately or fixed in 4% parafolmadehyde/ 1% glutaraldehyde in PBS at 4° C. All FACS analyses were performed on a Beckton Dickinson FACS Vantage using a 200 mW 488 argon laser with light being channelled by an FL-1 filter (520nm ± 20) and an FL-2 filter (580 nm ± 20). Cell quest software was used for subsequent analysis and WinMDI software for presentation.

Calcium fluorimetry

Fura-2 is a UV light–excitable, ratiometric Ca^{2+} indicator that has become the dye of choice for ratio-imaging microscopy, in which it is more practical to change excitation wavelengths than emission wavelengths. Upon binding Ca^{2+}, fura-2 exhibits an absorption shift that can be observed by scanning the excitation spectrum between 300 and 400 nm, while monitoring the emission at ~510 nm.

The sodium and potassium salts of fura-2 are cell-impermeant probes that can be delivered into cells by microinjection. In addition, these salts are useful as standards for calibrating Ca^{2+} measurements. Unlike the salt forms, the acetoxymethyl (AM) esters of fura-2 can passively diffuse across cell membranes, avoiding the use of invasive loading techniques. Once inside the cell, these esters are cleaved by intracellular esterases to yield cell-impermeant fluorescent indicators.

Loading cells with fura-2/AM

A 10 mM stock solution of the ester probe in anhydrous dimethylsulfoxide (DMSO) was prepared and divided into appropriately sized aliquots that can be stored at –20°C. This procedure curtails the spontaneous ester hydrolysis that can occur in moist environments. Before loading, the DMSO stock solution was diluted in 'calcium buffer' (140 mM NaCl, 5 mM KCl, 1mM $MgCl_2$, 25mM HEPES) to a final concentration of 5μM. The non-ionic and non-denaturing detergent Pluronic F-127 was added to help disperse the indicator in the loading medium.

Cells were grown on 22 mm glass cover slips. When subconfluent, the cells were washed twice in 'calcium buffer' and incubated at 37°C for 30 minutes in the dark with fura-2, and then washed again three times with fresh 'calcium buffer'. It is important that the loading medium is free of amino acids or buffers containing primary or secondary amines because aliphatic amines may cleave the AM esters and prevent loading.

$[Ca^{2+}]_i$ measurements

The coverslip was positioned on a Axiovert S100 inverted epifluorescence microscope from Zeiss, (Oberkochen, Germany) which is connected to an ultrapix camera with Kodak KAF1400 chip (Eastman Kodak), and a personal computer with Merlin Imaging software (Olympus America, New York, NY, USA). The cells were covered with 500 μl of 'calcium buffer' in which 1mM $CaCl_2$ had been added to adjust external calcium concentration.

A population of approximately 30 cells was selected by adjusting the field of view. Using a X40 oil immersion objective, the fluorescence changes were monitored over 5 minutes for a basal intracellular calcium measurement. After addition of the agonist, the response was monitored for at least 10 minutes and detected using dual excitation wavelengths of 340 nm and 380 nm and a single emission of 510 nm provided by a dual excitation/single emission spectromaster from Perkin Elmer Life Science (Boston, MA, USA).

Indicators that show an excitation or emission spectral shift upon ion binding, like fura-2 can be calibrated using a ratio of the fluorescence intensities measured at two different wavelengths, resulting in the cancellation of artifactual variations in the fluorescence signal that might otherwise be misinterpreted as changes in ion concentration. Calibration procedures consisted of recording fluorescence signals corresponding to a series of precisely manipulated ion concentrations.

Chelation of all free calcium ions with 10 mM EGTA at pH 8.5 was used to produce the minimum fluorescence signal (R_{min}) equivalent to less than 10 nM $[Ca^{2+}]_i$. Maximum fluorescence signal (R_{max}) was produced by treating the cells with 5mM ionomycin. Sample $[Ca^{2+}]_i$ was determined using Merlin software based on the formula:

$$[Ca^{2+}]_i = (R-R_{min})/(R-R_{max}) \times K_d$$

where R is the measured sample fluorescence ratio and K_d the dissociation constant for the fura- Ca^{++} complex (224 nM).

Cell staining for immunofluorescence microscopy

Cells were grown on 22 mm glass cover slips in 6 well plates. When subconfluent, the cells were serum starved for 24 hours and then washed twice in PBS, treated with fresh serum free medium placed in a water bath at 37° C and stimulated with the appropriate doses of drugs, chemokines or vehicle controls for various times.

Stimulations were terminated by aspirating the medium and fixing the cells for 10 minutes in either 4% w/v parafolmadehyde, 1% glutaraldehyde in PBS, or in ice cold methanol depending on the antibody that would be used. The coverslips were then placed in a petri dish, rinsed with PBS and permeabilised with 0.2% v/v Triton X100 at room temperature for 5 minutes. After washing 3 times with PBS, the cells were covered with blocking buffer (1% BSA in PBS) for 30 minutes at 37°C to minimize non-specific adsorption of the antibodies to the coverslip.

The blocking buffer was removed by holding each coverslip on its edge with forceps and draining it onto a sheet of fiber-free paper. Primary antibodies were diluted to 1-10 μg/ml in blocking buffer (optimal concentration depended on several variables, such as the affinity of the antibody and the abundance of the antigen). 100 μl of the primary antibody solution was placed on each coverslip and distributed evenly, by covering each coverslip with a small piece of parafilm. The antibody was left on for 1 hour at room temperature after which it was removed by aspiration. The coverslips were again washed three times in PBS, for 5 minutes each time.

The coverslips were then incubated with secondary antibodies conjugated to a fluorochrome; e.g. anti-mouse IgG- FITC, depending on the donor species of the

primary antibody and the desired fluorochrome. The procedure for applying the secondary antibody was exactly the same as for the primary antibody.

Mounting coverslips and preparation for microscopy

Mounting media is prepared by adding 6 gr of glycerol and 2.4 gr of Mowiol to 6 ml of water and 12 ml 0.2 M Tris buffer pH 8.5 and mixed on a rocker for at least 3 hours. The mixture is then left to settle for 2 hours followed by incubation for 10 minutes at 50° C. Non soluble material is pelleted by centrifugation for 15 minutes at 5000g, the supernatant is collected and 0.1 % (v/v) DABCO is added to the solution as an anti-bleaching agent. The mounting media is aliquoted and kept at -20° C.

After the final washes each coverslip was inverted onto a slide containing 20 μl of mounting media and the excess mounting media was removed with fiber-free paper. The coverslips were placed in and allowed to dry overnight.

Imaging of the cells was performed using an epifluorescence imaging system comprised of an Olympus IX70 inverted epifluorescence microscope (Olympus America, New York, NY, USA), an Ultrapix camera with Kodak KAF1400 chip (Eastman Kodak)(6.7 x 6.7 μm physical pixels, giving 67 nm per image pixel with a 100x oil immersion objective), and a personal computer with Fluoview imaging software (Olympus America).

2.2 *Materials*

Material	Source
[γ-^{32}P]ATP (3000 Ci/mmol)	Amersham Biosciences, Little Chalfont (UK)
2-Mercaptoethanol	Sigma, Poole (UK)
4G10, monoclonal Ab	Upstate Biotechnology, (USA)
Absolute Ethanol	Fisher Scientific (UK)
Acrylamide/bis acrylamide	Bio-Rad (UK)
Actin α-smooth muscle, monoclonal antibody	Sigma, Poole (UK)
Adenosine triphosphate	Sigma, Poole (UK), stock 100 mM dissolved in 100 mM Tris pH 7.4
Agarose	Sigma, Poole (UK)
Akt polyclonal antibody	New England Biolabs, MA (USA)
Ammonium hydroxide	Sigma, Poole (UK)
Ammonium persulphate	BDH, Poole (UK)
Bovine serum albumin (BSA)	Sigma, Poole (UK)
Bromophenol blue	BDH, Poole (UK)
Calcium Chloride	Sigma, Poole (UK)
CD80 monoclonal antibody FITC conjugate	BD Biosciences, CA (USA)
Cell culture plastics	Nunc, (UK)
Chloroform	Fisher Scientific (UK)
Deoxynucleoside triphosphate: dATP, dCTP, DGTP and dTTP	Roche, Basel, Switzerland
Desmin, monoclonal antibody	Sigma, Poole (UK)
Dimethyl sulphoxide (DMSO)	Sigma, Poole (UK)
Dithiothreitol (DTT)	Sigma, Poole (UK)
DNAase I	Invitrogen Ltd, Paisley, (UK)
DNA 100 base pair ladder (100-1500 bp scale)	Invitrogen Ltd, Paisley, (UK
Dulbeccos' modified essential medium	Invitrogen Ltd, Paisley, (UK)
Endothelin	Sigma, Poole (UK)
Enhanced chemiluminescence detection kit for Western blotting (ECL)	Amersham Biosciences, Little Chalfont (UK)
Ethanol	Fisher Scientific (UK)
Ethidium bromide	Sigma, Poole (UK)
Ethylenediaminetetraacetic acid (EDTA)	Sigma, Poole (UK)
Ethyleneglucol –bis (β- amino-ethylether)-N,N,N',N' tetraacetic acid (EGTA)	Sigma, Poole (UK)

Expand Polymerase	Roche, Basel, Switzerland
Filter paper	Whatman (UK)
Foetal bovine serum (FBS)	Invitrogen Ltd, Paisley, (UK)
Formaldehyde	BDH, Poole (UK)
Fura-2 acetoxylmethyl ester (Fura-2AM)	Calbiochem (UK)
Glacial acetic acid	Amersham Biosciences, Little Chalfont (UK)
Glass slide covers	BDH, Poole (UK)
Glutaraldehyde	Sigma, Poole (UK)
Glycerol	Sigma, Poole (UK)
Glycine	Sigma, Poole (UK)
Goat anti-mouse IgG-FITC conjugated	DAKO, Denmark
Goat anti-mouse immunoglobulins peroxidase conjugate	DAKO, Denmark
Goat anti-rabbit immunoglobulins peroxidase conjugate	DAKO, Denmark
Hank's balanced salt solution	Invitrogen Ltd, Paisley, (UK)
Hepes (1M liquid)	Invitrogen Ltd, Paisley, (UK)
Hydrochloric acid	BDH, Poole (UK)
ICOS polyclonal antibody	Alexis Corporation, CA (USA)
IFN-γ: human recombinant; specific activity > 2×10^7 U/mg	Boehringer Mannheim, Germany
IGEPAL CA-630	Sigma, Poole (UK)
IL-1α: human recombinant	Gift from Glaxo (Greenford, UK); diluted in sterile PBS + 0.25% (w/v) BSA
IL-2: human recombinant	PeproTech EC Ltd, London (UK)
IL-4: human recombinant	PeproTech EC Ltd, London (UK)
IL-10: human recombinant	PeproTech EC Ltd, London (UK)
IL-13: human recombinant	PeproTech EC Ltd, London (UK)
IP-10: human recombinant	PeproTech EC Ltd, London (UK)
I-TAC: human recombinant	PeproTech EC Ltd, London (UK)
Lantruncilin B	Calbiochem, Nottingham (UK)
Lymphoprep ™	Nycomed, Birmingham (UK)
Methanol	Fisher Scientific (UK)
MIG: human recombinant	PeproTech EC Ltd, London (UK)
Molecular weight markers	Bio-Rad (UK)
Minimum Essential Medium (MEM)	Gibco BRL, Paisley, UK
Mouse anti-hCXCR3 FITC conjugate	R&D Systems, Abingdon (UK)
Mouse anti-rabbit immunoglobulins peroxidase conjugate	DAKO, Denmark

Mouse IgG1 isotype	Sigma, Poole (UK)
Mouse IgG2α isotype	Sigma, Poole (UK)
Mowiol® 4-88	Calbiochem, Nottingham (UK)
Myosin monoclonal antibody	Sigma, Poole (UK)
Nitrocellulose blotting membrane 0.45μM	BDH, Poole (UK)
Non Essential amino acids 10x	Sigma, Poole (UK)
Nuclease free water	Promega, WI (USA)
p38 MAPK polyclonal antibody	New England Biolabs, MA (USA)
p42 MAPK monoclonal antibody	New England Biolabs, MA (USA)
p44/42 MAPK polyclonal antibody	New England Biolabs, MA (USA)
p85 α, monoclonal Ab	Dr. D. Cantrell, University of Dundee (UK)
Parafolmadehyde	Sigma, Poole (UK)
PCR filter tips	Greiner BioOne (UK)
PCR tubes	Anachem, Luton (UK)
Penicillin	Sigma, Poole (UK)
Petussis Toxin	Calbiochem (UK)
Pepstatin	Sigma, Poole (UK)
Phalloidin-TRITC	Sigma, Poole (UK); stock 0.3 mM in PBS stored at -20° C
Phosphate buffered saline	Invitrogen Ltd, Paisley, (UK)
Phosphatidylinositol	Sigma, Poole (UK)
Phospho Akt (Ser 473) polyclonal antibody	New England Biolabs, MA (USA)
Phospho Akt (Thr 308) polyclonal antibody	New England Biolabs, MA (USA)
Phosphotyrosine antibody (4G10)	Upstate technology, NY (USA)
Phospho p38 MAP kinase (Thr180/Tyr182) polyclonal antibody	New England Biolabs, MA (USA)
Phospho p44/42 MAP Kinase (Thr 202/Tyr 204) polyclonal antibody	New England Biolabs, MA (USA)
Phospho p90RSK (Thr 573) antibody	New England Biolabs, MA (USA)
PI3K C2α polyclonal antibody	Dr J Domin, Imperial college, London (UK)
PI3K C2β monoclonal antibody	BDH, Poole (UK)
PMSF	Sigma, Poole (UK)
Polaroid film (type 55)	Sigma, Poole (UK)
Poly d(T) 12-18	Pharmacia (UK)
Polypropylene FACS tubes	Beckton & Dickinson (USA)
Ponceau S	Sigma, Poole (UK)
Potassium oxalate	Sigma, Poole (UK)

Primers for PCR	MWG, Ebersberg, Germany
Propan-1-ol	Fisher Scientific (UK)
Propan-2-ol	Fisher Scientific (UK)
Propidium iodide	Sigma, Poole (UK)
Protein A beads	Sigma, Poole (UK)
Protein G beads	Sigma, Poole (UK)
RNase A	Sigma, Poole (UK)
RNAsin-,non competitive ribonuclease inhibitor	Promega, WI (USA)
RPMI 1640 cell culture medium	Gibco BRL, Paisley (UK)
RNAzol B	Tel Test, Texas, (USA)
Silica gel 60 Thin Layer Chromatography laned plates	Whatman (UK)
Sodium azide	Sigma, Poole (UK)
Sodium chloride	Sigma, Poole (UK)
Sodium dodecyl sulfate (SDS)	Sigma, Poole (UK)
Sodium fluoride	Sigma, Poole (UK)
Sodium hydroxide	Sigma, Poole (UK)
Sodium molybdate	Sigma, Poole (UK)
Sodium nitrite	Sigma, Poole (UK)
Sodium orthovanadate	Sigma, Poole (UK)
Staphylococcal Enterotoxin B	Sigma, Poole (UK); stock 1mg/ml in 0.1% BSA stored at -20 °C
Superscript	Invitrogen Ltd, Paisley, (UK)
TEMED	Sigma, Poole (UK)
Tissue culture reagents	Invitrogen Ltd, Paisley, (UK)
TNF-α: human recombinant; specific activity 6×10^7 U/mg	Gift from Bayer (Slough, UK); diluted in sterile PBS + 0.1% (w/v) BSA
Triton X-100	Sigma, Poole (UK)
Trizma base	Sigma, Poole (UK)
Trypan Blue	Invitrogen Ltd, Paisley, (UK)
Tween-20	Sigma, Poole (UK)
Vimentin monoclonal antibody	Sigma, Poole (UK)
Wortmannin	Sigma, Poole (UK)
X-OMAT film	Amersham Biosciences, Little Chalfont (UK)
Y27632	Calbiochem, Nottingham (UK)

Chapter 3

3 Results I

Profile of B7/CD28 family members expression in intestinal myofibroblasts

3.1 Background

In the intestine, T-cell activation, mediated by the interaction of T-cells with MHC-peptide complexes and B7 co-stimulatory molecules on antigen-presenting cells, is an essential event in the pathogenesis of IBD (Powrie et al., 1994). This second effect is most potently effected by ligation of CD28 on T cells via the B7 molecules, CD80 and CD86 (Guinan et al., 1994), on APCs. APC's of the human gut are heterogeneous, including both macrophages, a variety of dendritic cells and B cells. They are found both in gut-associated lymphoid tissue and in the mucosal lamina propria, especially beneath the surface epithelium. Moreover, recent studies (Hogaboam et al., 1996; Roberts et al., 1997), have established a role for mesenchymal cells in T-cell activation in the gut.

Intestinal myofibroblasts are considered to have an important role in intestinal fibrosis. This is based primarily on the role of similar cells in other systems such as hepatic stellate cells (HSC), the equivalent of myofibroblasts in the liver. HSCs play a key role in the development of liver fibrosis and are the major producers of extracellular matrix in the liver after undergoing an activation process that results in a phenotypic change from retinoid-storing quiescent cells to activated HSCs with a myofibroblast phenotype (Friedman, 2000). Migration of HSCs is believed to be critical for the accumulation of HSCs at the site of injury. It has been suggested that chemokines induce the migration of HSCs to the site of injury and

attract a leukocytic infiltrate to the site of injury (Marra et al., 1998; Marra et al., 1999). Therefore, chemokines may be part of a cytokine network within the liver that regulates the interaction of resident and non-resident cells during the hepatic wound-healing response. This hypothesis is supported by the fact that HSCs express the molecular machinery to interact with infiltrating leukocytes such as ICAM, VCAM, and CD40 and are able to present antigen and to stimulate the proliferation of allogenic lymphocytes.

To investigate the possibility of a cross-talk between myofibroblasts and cells of the immune system in the gut to achieve a concerted cellular response during the intestinal wound healing process, expression of the B7 co-stimulatory molecules on primary intestinal myofibroblasts as well as the relevant 18 Co cell line (Valentich et al., 1997) at the mRNA and protein level was studied.

3.2 *Results*

Phenotypical characterisation of isolated cells

In order to ensure that the cells isolated following the procedure described in Materials and Methods, have the myofibroblast phenotype, cells were grown on coverslips fixed with methanol and then stained for α-smooth muscle actin, vimentin and desmin (Figure 3.1).

Cells isolated from biopsy specimens, stain positive for α-smooth muscle actin and vimentin but not for desmin. Negative controls were performed by using non-specific mouse IgG instead of the specific antibodies.

To further demonstrate that the cells are indeed myofibroblasts, cells were lysed in order to isolate protein for western blot analysis of α-smooth muscle actin and

vimentin (Figure 3.2). This experiment compared to immunocytochemistry has the advantage of providing additional information for the identity of the protein detected, by means of its molecular weight. Both proteins correspond to the predicted molecular weight for α-smooth muscle actin and vimentin.

Induction of B7.1

It is commonly reported that B7.1 is inducible in other cell lines by stimulation with a variety of pro-inflammatory cytokines, tumour promoters and other mitogens. The pro-inflammatory cytokines TNF-α and IFN-γ were chosen in an attempt to induce B7.1 in intestinal myofibroblasts as it has been previously demonstrated that these cytokines induce expression of B7.1 on murine fibroblasts (Pechhold et al., 1997). Due to the limitation of the numbers of myofibroblasts available, the concentrations of the cytokines used in the following experiments were chosen to be within physiological limits and are routinely used in studies of the gastrointestinal system (Weaver et al., 2001).

B7.1 mRNA induction in intestinal myofibroblasts

Primary intestinal myofibroblasts and 18 Co cells were stimulated with TNF-α (100 ng/ml) or IFN-γ (300 u/ml) or in combination and mRNA was isolated for PCR analysis for B7.1. The time course for this study was elected to be up to 48 hours since B7.1 expression is usually detectable only at 24 hours and peaks at around 48-72 hours following stimulation in a variety of systems (Coyle and Gutierrez-Ramos, 2001). The combination of the two cytokines caused B7.1 induction in both primary intestinal myofibroblasts (Figure 3.3 A) and the 18Co cell line (Figure 3.3 B). The induction in the primary cells appeared quicker, starting at 6 hours after stimulation and was sustained untill the end of the time

course, whereas B7.1 induction was detected in 18 Co cells only 12 hours after stimulation. In both cases the induction appeared to be sustained with gradually increasing amounts of B7.1 mRNA being produced at the later time points.

Neither cytokine alone, over the same time frame of 48 hours, was able to induce B7.1 expression in either primary intestinal myofibroblasts (Figure 3.4A) or in the 18 Co cell line (Figure 3.4 B). Stably transfected CHO cells expressing B7.1 were used as a positive control for these experiments to verify that the PCR products were valid (Figures 3.3 and 3.4).

Cell surface expression of B7.1 in intestinal myofibroblasts

In light of TNF-α/IFN-γ inducing ICOS mRNA in both primary intestinal myofibroblasts, FACS analysis was employed to assess whether the same stimulation also caused induction of B7.1 cell surface expression. Primary intestinal myofibroblasts and 18 Co cells were stimulated over a 72 hour time course with TNF-α (100 ng/ml) or IFN-γ (300 u/ml) or a combination of both and subsequently prepared for FACS (Figures 3.5-3.6).

B7.1 cell surface was detected only 72 hours after stimulation in both cell types. This lags behind the induction of B7.1 mRNA, possibly in order to allow for protein translation and translocation to the membrane.

Cell surface expression was slightly higher in the primary cells (Figure 3.5), compared to the 18 Co cells (Figure 3.6). The two cytokines were also used individually in order to confirm the PCR findings that both TNF-α and IFN-γ on their own are not able to induce B7.1 expression in our system. In these

experiments stably transfected CHO cells expressing B7.1 were again used as a positive control.

B7.2 mRNA in intestinal myofibroblasts

After the successful induction of B7.1 in both primary intestinal myofibroblasts and 18Co cells, B7.2 inducibility in these cells was investigated. Primary intestinal myofibroblasts were again stimulated over a 48hour time course with TNF-α (100 ng/ml) or IFN-γ (300 u/ml) (Figure 3.8) or a combination of both (Figure 3.7) and mRNA was isolated for PCR analysis for B7.2. This combination of cytokines proved unable to induce B7.2 expression in either primary intestinal myofibroblasts or 18 Co cells (Figure 3.7).

The two cytokines when used on their own, over the same period of 48 hours were also unable to induce B7.2 expression in the two cell types (Figure 3.8). In these experiments stably transfected CHO cells expressing B7.2 were used as a positive control.

Expression of other B7 family members in intestinal myofibroblasts

Assessment of the more recently described members of the B7 family in our system was also performed. Primary intestinal myofibroblasts and 18 Co cells were stimulated over 48 hours with TNF-α (100 ng/ml) and IFN-γ (300 u/ml) and mRNA was isolated for PCR analysis for various B7 family members (Figure 3.9). Both primary intestinal myofibroblasts and 18 Co cells demonstrated a constitutive expression of all the novel B7 family members they were tested for, namely PD-L1, B7 RP-1 and B7 H3. The cytokines that were used to stimulate the

cells did not appear to have any effect in the expression of these proteins (Figure 3.9).

Expression of CD28 and CTLA-4 in intestinal myofibroblasts

After completing the studies of B7 family member expression, we also wanted to determine the expression of their cognate receptors in our system. Even though these receptors are thought to be T-cell specific, there are some studies suggesting that their expression is not restricted to the lymphoid cell lineage. CTLA-4 has been found to be expressed on placental fibroblasts (Kaufman et al., 1999). Moreover various pro-inflammatory cytokines induced the expression of COLA-4 and COD on normal human muscle cells (Niagara et al., 1999).

Primary intestinal myofibroblasts and 18 Co cells were stimulated over a 48hour time course with TNF-α (100 ng/ml) or IFN-γ (300 u/ml) or a combination of both and mRNA was isolated for PCR analysis for CD28 and CTLA-4.

As expected both cell types did not express CD28 and CTLA-4 and the cytokines were also unable to induce their expression (Figure 3.10). Peripheral blood derived activated T-cells, which are known to express both CD28 and CTLA-4, was used as a positive control to verify validity of PCR products.

Expression of ICOS in intestinal myofibroblasts

Our studies then concentrated on the most recently discovered receptor of the CD28 family, inducible co-stimulator (ICOS). Primary intestinal myofibroblasts were stimulated over a 48hour time course with 100 ng/ml TNF-α or 300 units/ml IFN-γ or a combination of both and mRNA was isolated for PCR analysis for ICOS. The combination of TNF-α /IFN-γ surprisingly proved able to induce the

expression of ICOS in primary intestinal myofibroblasts. Induction was visible at 6 hours after stimulation and peaked at 48 hours (Figure 3.11 A). The two cytokines when applied on their own, over the same time frame of 48 hours were unable to induce ICOS expression in primary intestinal myofibroblasts (Figure 3.12 A).

In contrast to primary cells, the combination of TNF-α/IFN-γ was unable to induce ICOS expression in the 18 Co cell line (Figure 3.11 B). The two cytokines on their own were also not capable of inducing ICOS expression in these cells (Figure 3.12B).

Regulation of ICOS expression in primary intestinal myofibroblasts by anti-inflammatory cytokines

Having established that the pro-inflammatory cytokines TNF-α/IFN-γ can induce ICOS mRNA in primary intestinal myofibroblasts, the next aim was to assess any possible regulatory role for other cytokines relevant to gastro-intestinal biology. Primary intestinal myofibroblasts were pre-treated with IL-4 or IL-10 (both at 30ng/m for 1 hour), prior to stimulation with TNF-α/IFN-γ for up to 48 hours.

The two cytokines used had very different effects on ICOS expression. Pre-treatment with IL-10 completely inhibited the induction of ICOS mRNA (Figure 3.13 B). Pre-treatment with IL-4 on the other hand, promoted early induction of ICOS mRNA at 1hour, following the addition of TNF-α/IFN-γ, but the response was more transient as no ICOS mRNA could be detected beyond the 2 hour time point (Figure 3.13 A).

The cells were also stimulated with IL-4 and IL-10 for 1 hour to verify the synergistic effect of the anti with the pro-inflammatory cytokines. Both IL-4 and

IL-10 failed to induce any detectable levels of ICOS mRNA in primary intestinal myofibroblasts (Figure 3.13 C and D).

Protein expression of ICOS in primary intestinal myofibroblasts

The unexpected finding that ICOS mRNA could be induced in primary intestinal myofibroblasts, prompted us to assess ICOS protein expression in these cells. Unfortunately, the only available antibody for ICOS at the time of these experiments recognizes an intracellular epitope of ICOS. Moreover according to the manufacturer it is not suitable for FACS analysis. Therefore possible positive results do not necessarily correlate with surface expression of this molecule.

Activated T-cells were first tested for ICOS expression as a positive control for the antibody (Figure 3.14A). Peripheral blood mononuclear cells were isolated as described in Materials and Methods, stimulated with SEB and left in culture for up to 12 days. Samples of these cells were collected at days 0 (day of isolation), 3, 5,7,10,12 post isolation and whole cell lysates were isolated for Western blot analysis. No ICOS protein could be detected in the freshly isolated cells. However activation of the cells by SEB proved to induce ICOS expression at days 3 and 5 following activation. The identified protein has a molecular weight of approximately 55kDa. The molecular weight for ICOS is 23kDa, but was initially recognized as a 55kDa homodimeric glycosylated protein when purified and cloned from a T cell line cDNA library (Hutloff et al., 1999).

Primary intestinal myofibroblasts cells were stimulated with TNF-α (100 ng/ml) or IFN-γ (300 u/ml) or a combination of both and protein was isolated for analysis of ICOS (Figure 3.14 B). This combination of cytokines was able to induce ICOS expression in a pattern very similar to the one observed for the mRNA induction.

ICOS protein was first detected 6 hours after the addition of the cytokines, peaked at 24 hours and was still visible 48 hours after stimulation.

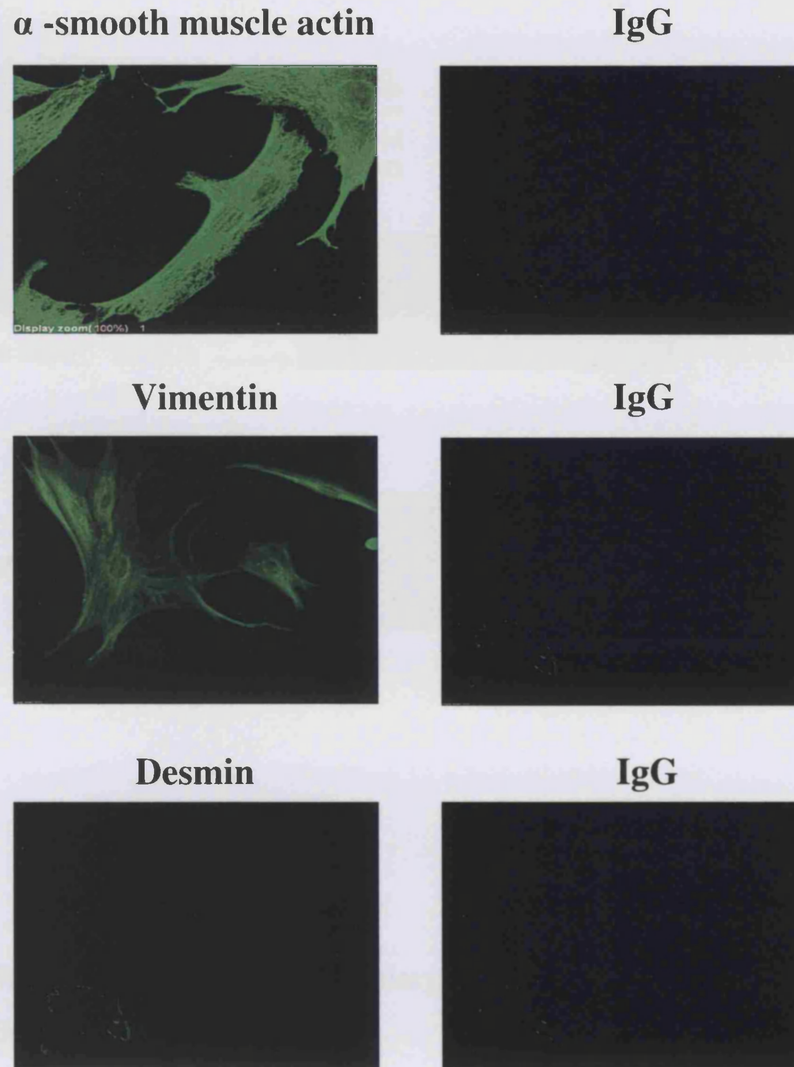

Figure 3.1 Immunofluorescence staining of primary human intestinal myofibroblasts for α-smooth muscle actin, vimentin and desmin.

$2x10^5$ primary intestinal myofibroblasts were plated on coverslips in 35mm plates and cultured for 48 hours. Cell were then fixed in methanol and labelled with monoclonal mouse antibodies against α-smooth muscle actin (A), vimentin (B), desmin (C) or mouse IgG as an isotype control (D-F) for 1 hour to a final concentration of 20 μg/ml for all antibodies. After three washes, the coverslips were incubated for 30-45 min with FITC-labelled goat-anti-mouse IgG secondary antibody and visualized under a confocal microscope as described in Methods and and Materials. Results are from single experiments representative of 6 replicate experiments.

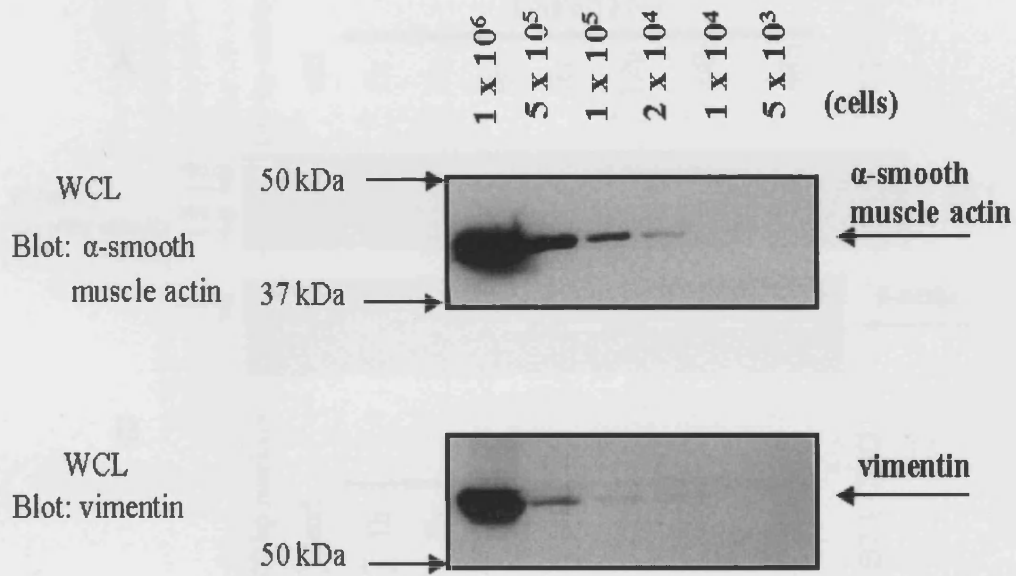

Figure 3.2 Western blot analysis of primary human intestinal myofibroblasts for α-smooth muscle actin and vimentin.

Indicated numbers of unstimulated primary intestinal myofibroblasts were lysed in lysis buffer as described in Methods and Materials and then probed with specific mouse monoclonal antibodies against α-smooth muscle actin (upper panel) or vimentin (lower panel) at a final concentration of 0.5 μg/ml for both antibodies. Results are from single experiments representative of 3 replicate experiments.

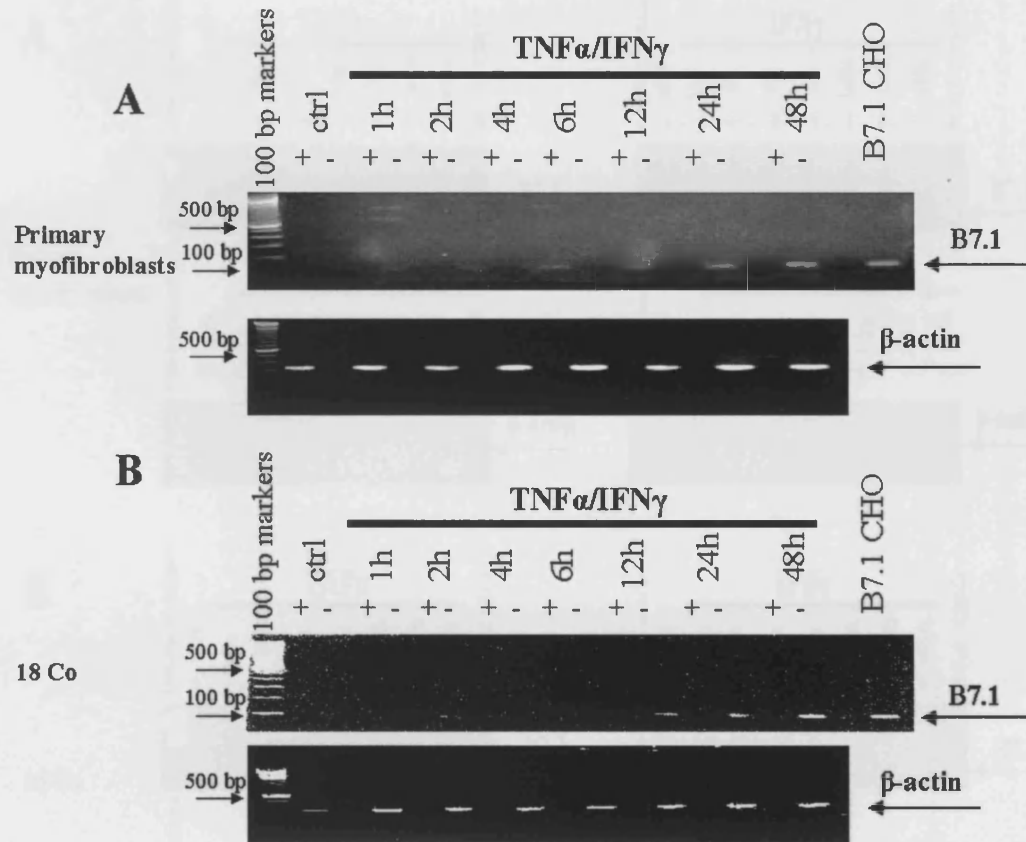

Figure 3.3 The combination of TNFα and IFN-γ induces expression of B7.1mRNA in primary human intestinal myofibroblasts and 18 Co cells.

1x10^6 primary intestinal myofibroblasts (A), or 18Co cells (B) were left unstimulated (ctrl) or stimulated with 100ng/ml TNF-α and 300 units/ml IFN-γ for indicated times and then lysed in 400 μl RNAzol. 1x10^6 CHO cells, stably transfected with B7.1 were also lysed in 400 μl RNAzol as a positive control for B7.1 expression. RT PCR was then performed on the lysates as described in Methods and Materials using primers for B7.1 and β-actin as a housekeeping gene to verify equal amounts of mRNA and equal amplification. (+) and (-) refers to RT positive and negative samples respectively. Results are from single experiments representative of 3 replicate experiments.

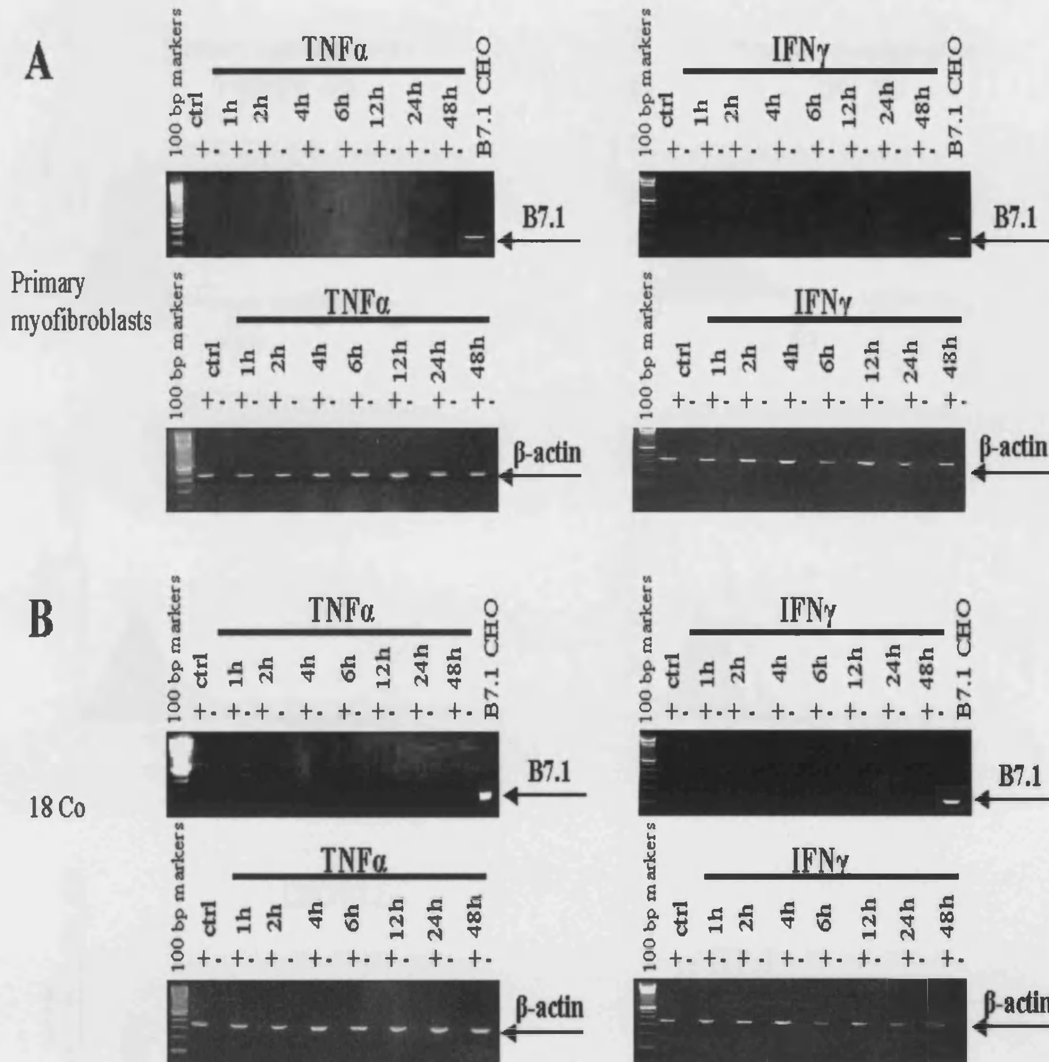

Figure 3.4 TNFα and IFN-γ fail to induce expression of B7.1mRNA in primary human intestinal myofibroblasts and 18 Co cells.

1x10^6 primary intestinal myofibroblasts (A) or 18Co cells (B) were lysed in 400 μl RNAzol (ctrl) or stimulated with 100ng/ml TNF-α (left panels) or 300 units/ml IFN-γ (right panels) for indicated time points and lysed in RNAzol. 1x10 ^{6} CHO cells, stably transfected with B7.1 were also lysed in 400 μl RNAzol as a positive control for B7.1 expression. RT PCR was performed on the lysates as described in Methods and Materials using primers for B7. (+) and (-) refers to RT positive and negative samples respectively. Results are from single experiments representative of 3 replicate experiments.

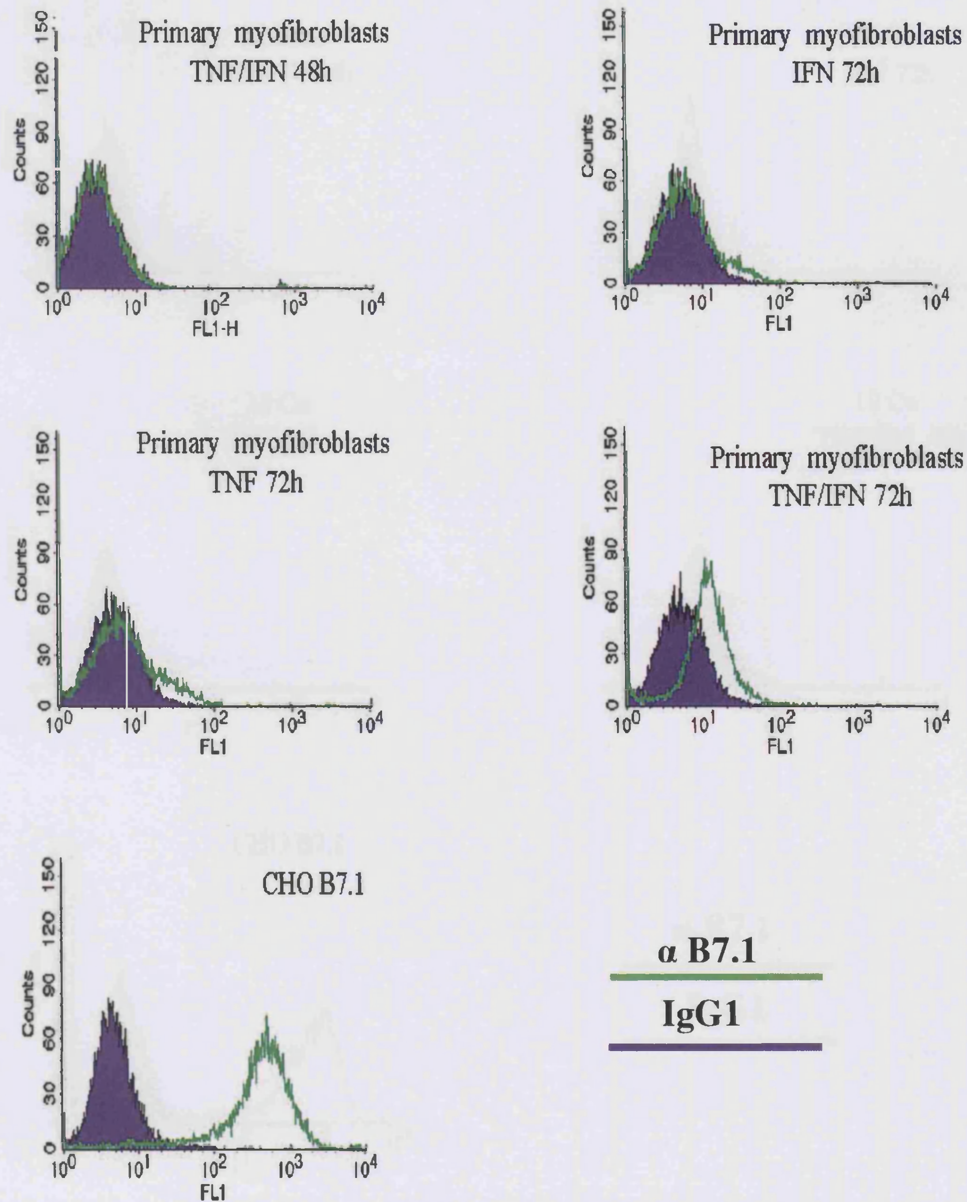

Figure 3.5 B7.1 surface expression on primary intestinal myofibroblasts.

1x10^6 cells / tube primary intestinal myofibroblasts were stimulated with 100ng/ml TNF-α or 300 units/ml IFN-γ or a combination of both for indicated time points and then stained with FITC conjugated anti-B7.1 (green histograms) or isotype matched mouse IgG1 (purple filled histograms), as described in Methods and Materials.1x10^6 CHO cells stably transfected with B7.1 were used as a positive control for B7.1 staining.

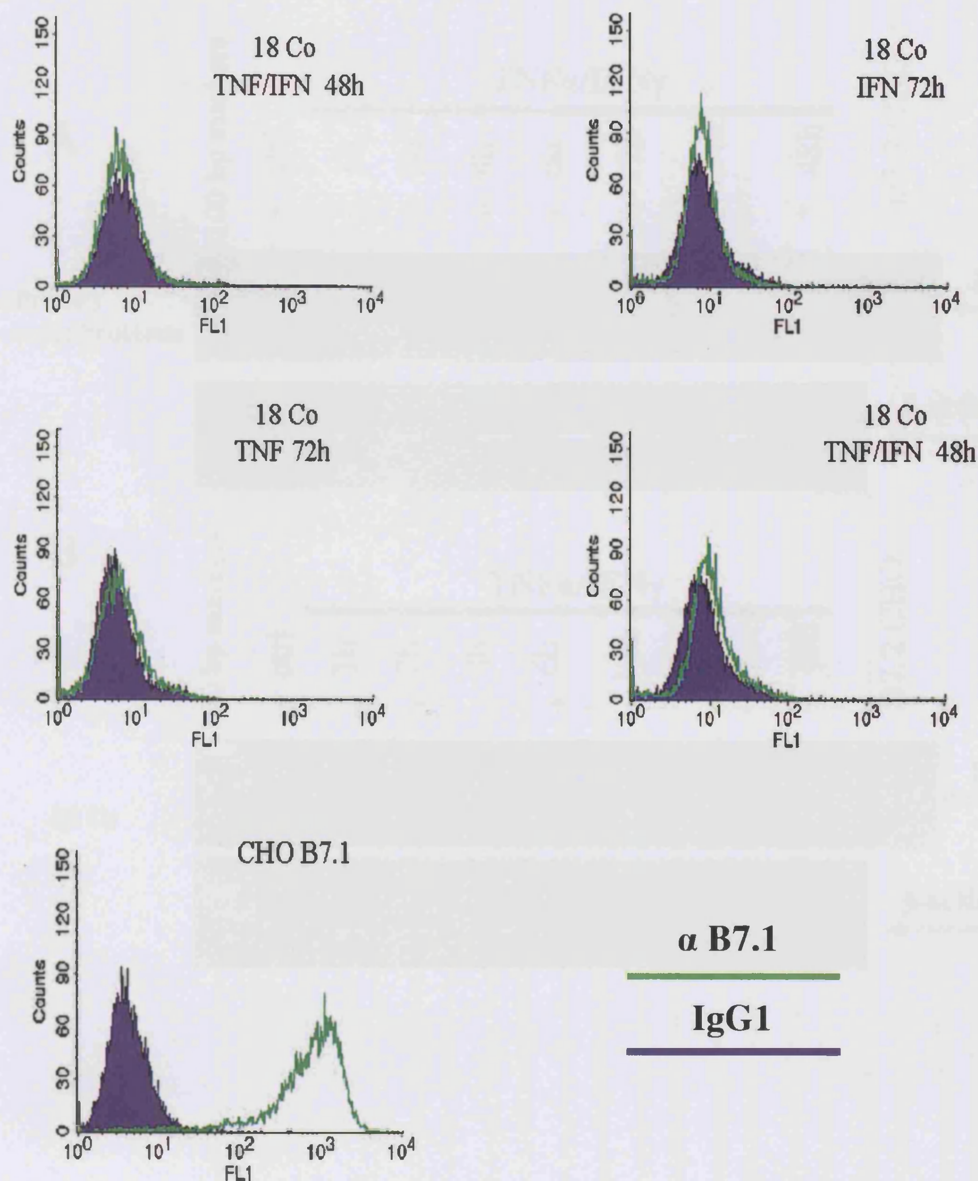

Figure 3.6 B7.1 surface expression on 18 Co cells.

1×10^6 cells / tube 18 Co cells were stimulated with 100ng/ml TNF-α or 300 units/ml IFN-γ or a combination of both for indicated time points and then stained with FITC conjugated anti-B7.1 (green histograms) or isotype matched mouse IgG1 (purple filled histograms), as described in Methods and Materials. 1×10^6 CHO cells stably transfected with B7.1 were used as a positive control for B7.1 staining.

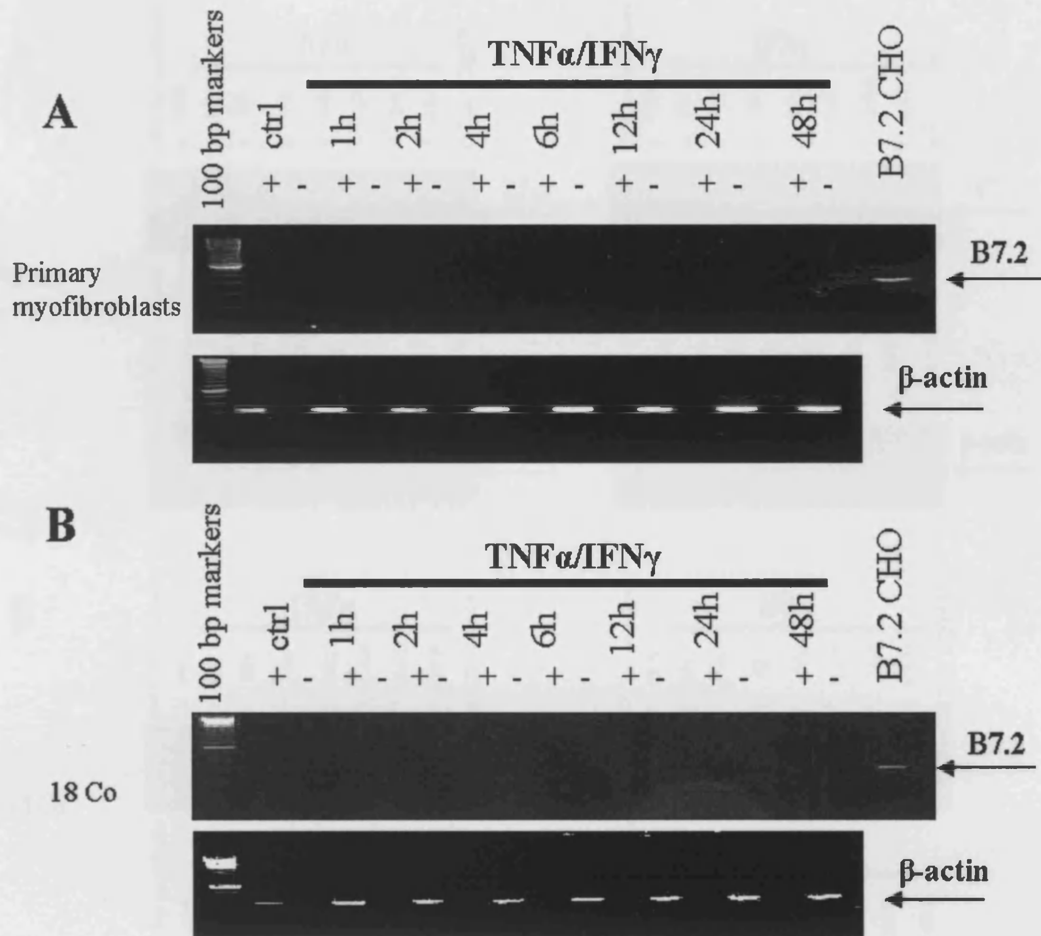

Figure 3.7 The combination of TNFα and IFN-γ fails to induce expression of B7.2 mRNA in primary human intestinal myofibroblasts and 18 Co cells.

1x10^6 primary intestinal myofibroblasts (A), or 18Co cells (B) were lysed in 400 μl RNAzol (ctrl) or stimulated with 100ng/ml TNF-α and 300 units/ml IFN-γ for indicated times and then lysed in RNAzol. 1x10^6 CHO cells, stably transfected with B7.2 were also lysed in 400 μl RNAzol as a positive control for B7.2 expression. RT PCR was then performed on the lysates as described in Methods and Materials using primers for B7.2 (+) and (-) refers to RT positive and negative samples respectively. Results are from single experiments representative of 3 replicate experiments.

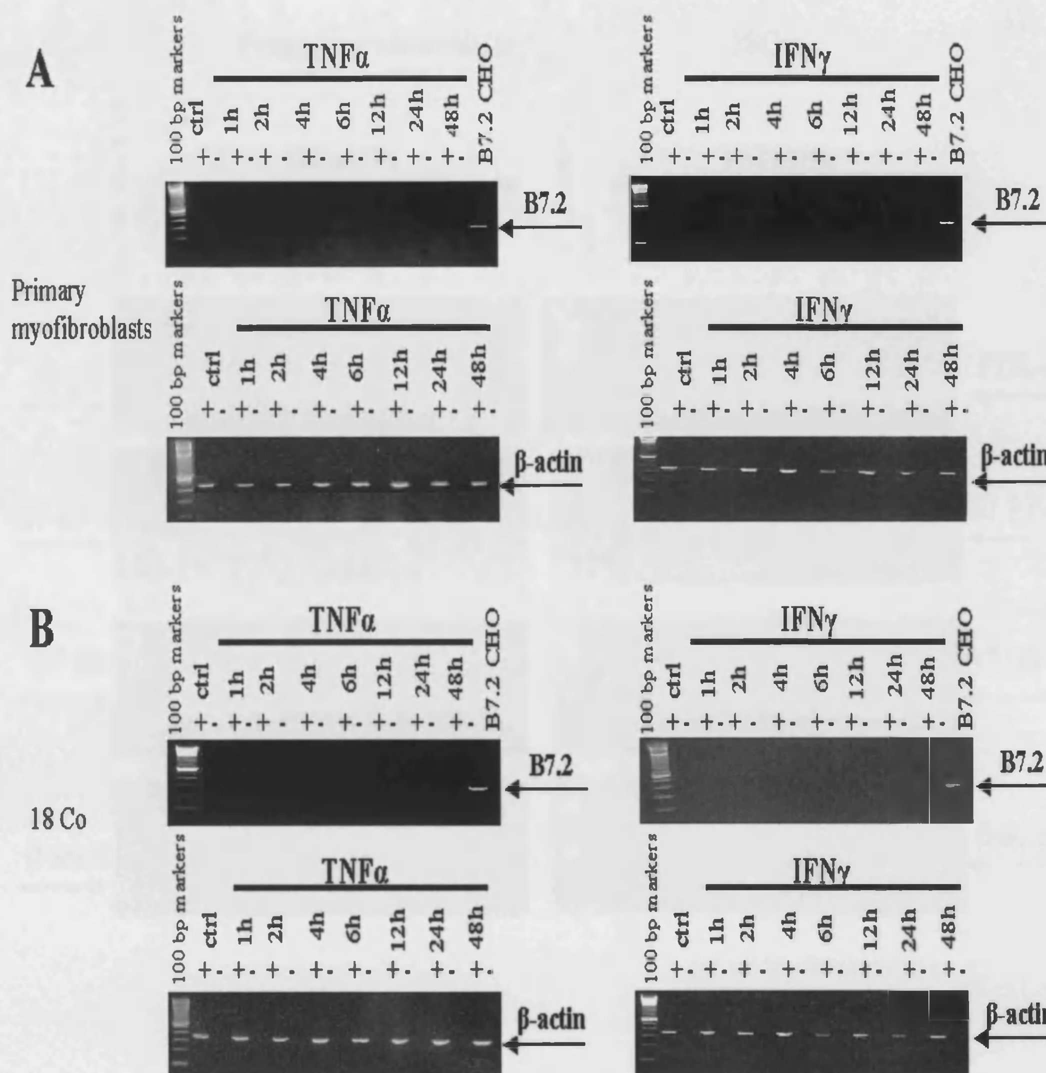

Figure 3.8 PCR analysis of B7.2 expression in primary human intestinal myofibroblasts and 18 Co cells.

1x10^6 primary intestinal myofibroblasts (A) or 18Co cells (B) were lysed in 400 μl RNAzol (ctrl) or stimulated with 100ng/ml TNF-α (left panels) or 300 units/ml IFN-γ (right panels) for indicated time points and lysed in RNAzol. 1x10 ^6 CHO cells, stably transfected with B7.2 were also lysed in 400 μl RNAzol as a positive control for B7.2 expression. RT PCR was performed on the lysates as described in Methods and Materials using primers for B7.2 (+) and (-) refers to RT positive and negative samples respectively. Results are from single experiments representative of 3 replicate experiments

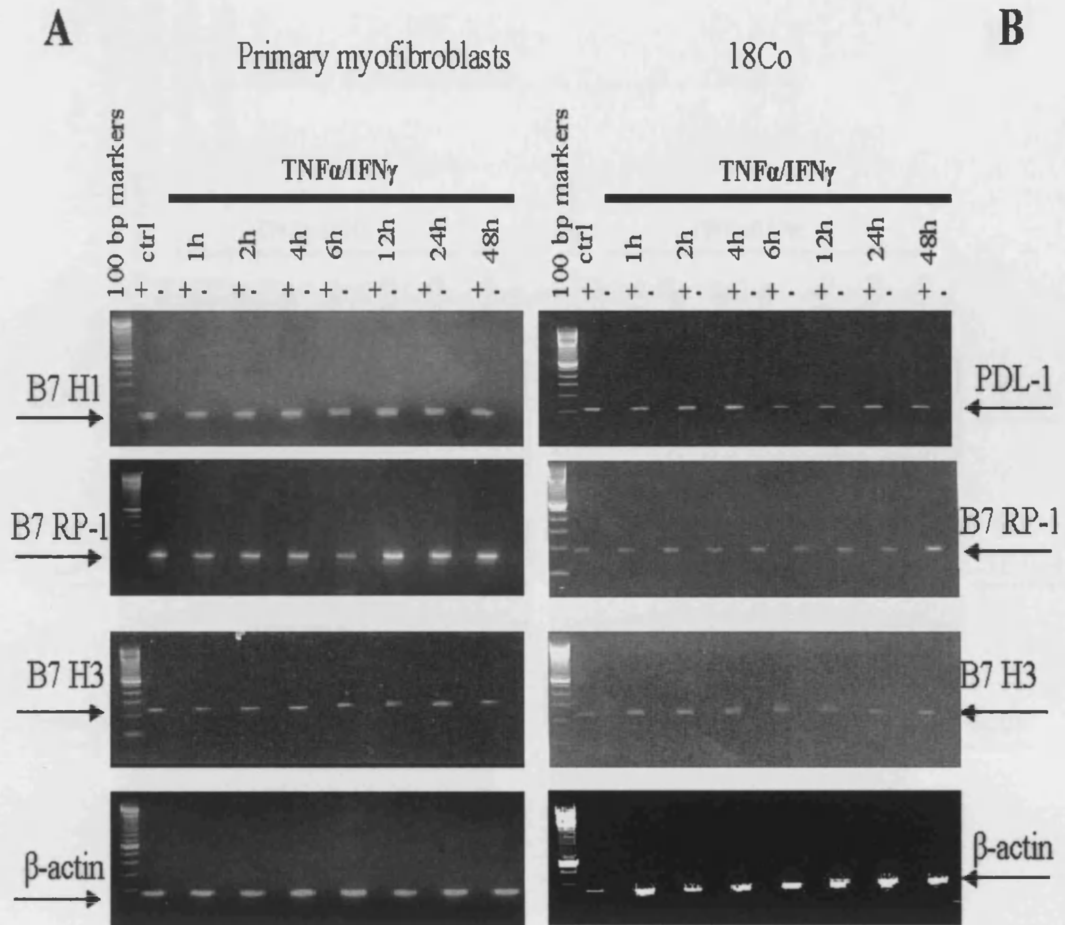

Figure 3.9 Primary human intestinal myofibroblasts and 18Co cells constitutively express mRNA for PD-L1, B7 RP-1 and B7 H3.

$1x10^6$ primary intestinal myofibroblasts (A) or 18 Co cells (B) were lysed in 400 µl RNAzol (ctrl) or stimulated with 100ng/ml TNF-α and 300 units/ml IFN-γ for indicated time points and lysed in RNAzol. RT PCR was performed on the lysates as described in Methods and Materials using primers for PD-L1, B7 RP-1, B7 H3. (+) and (-) refers to RT positive and negative samples respectively. Results are from single experiments representative of 3 replicate experiments.

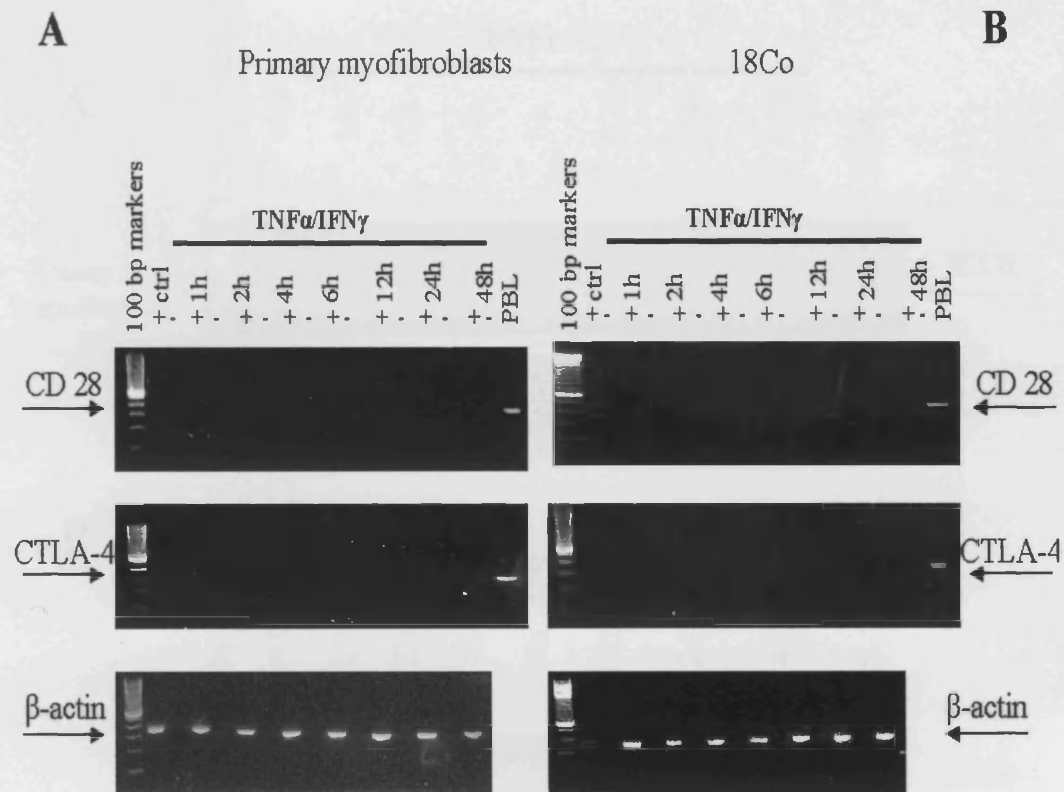

Figure 3.10 PCR analysis of CD28 and CTLA-4 expression in primary human intestinal myofibroblasts and 18 Co cells

1x10^6 primary intestinal myofibroblasts (A) or 18 Co cells (B) were lysed in 400 μl RNAzol (ctrl) or stimulated with 100ng/ml TNF-α and 300 units/ml IFN-γ for indicated time points and lysed in RNAzol. 1x10 ^6 12 days old SEB activated peripheral blood derived T cells (PBL) were also lysed in 400 μl RNAzol as a positive control for CD28 and CTLA-4 expression. RT PCR was performed on the lysates as described in Methods and Materials using primers for CD28, CTLA-4. (+) and (-) refers to RT positive and negative samples respectively. Results are from single experiments representative of 3 replicate experiments.

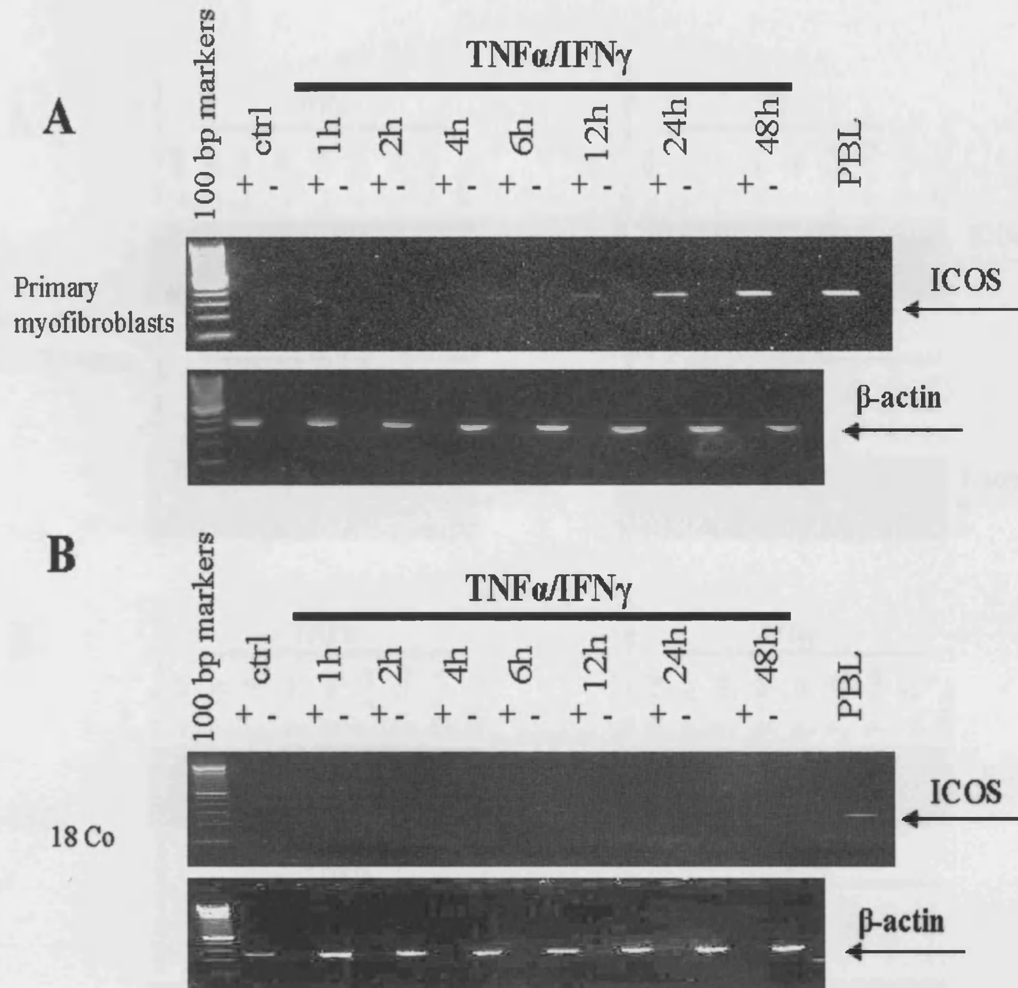

Figure 3.11 The combination of TNFα and IFN-γ induces expression of ICOS mRNA in primary human intestinal myofibroblasts but not in 18Co cells.

$1x10^6$ primary intestinal myofibroblasts (A) or 18 Co cells (B) were lysed in 400 μl RNAzol (ctrl) or stimulated with 100ng/ml TNF-α and 300 units/ml IFN-γ for indicated time points and lysed in RNAzol. $1x10^6$ 12 days old SEB activated peripheral blood derived T cells (PBL) were also lysed in 400 μl RNAzol as a positive control for ICOS expression. RT PCR was performed on the lysates as described in Methods and Materials using primers for ICOS. (+) and (-) refers to RT positive and negative samples respectively. Results are from single experiments representative of 3 replicate experiments.

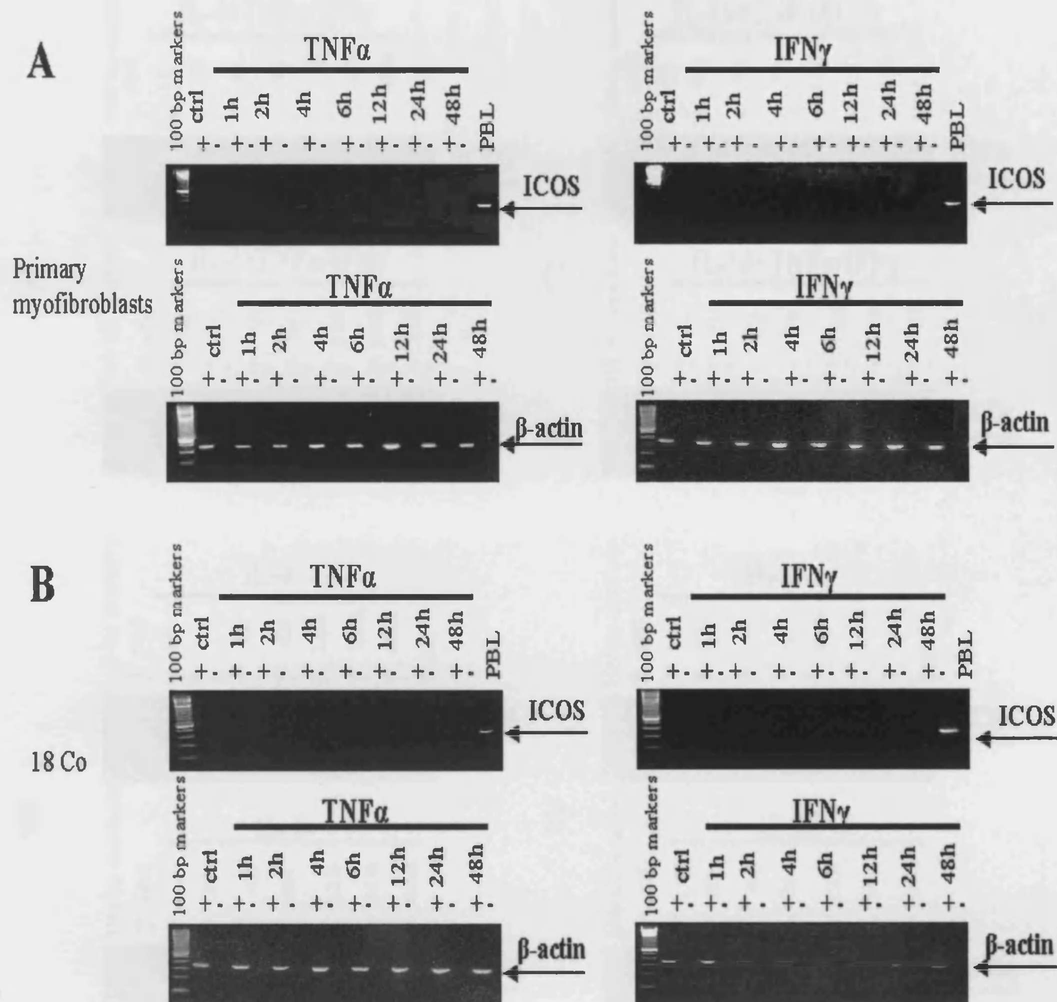

Figure 3.12 TNFα and IFN-γ are unable to induce expression of ICOS mRNA in primary human intestinal myofibroblasts and 18Co cells.

$1x10^6$ primary intestinal myofibroblasts (A) or 18Co cells (B) were lysed in 400 µl RNAzol (ctrl) or stimulated with 100ng/ml TNF-α (left panels) or 300 units/ml IFN-γ (right panels) for indicated time points and lysed in RNAzol. $1x10^6$ 12 days old SEB activated peripheral blood derived T cells (PBL) were also lysed in 400 µl RNAzol as a positive control for ICOS expression. RT PCR was performed on the lysates as described in Methods and Materials using primers for ICOS. (+) and (-) refers to RT positive and negative samples respectively. Results are from single experiments representative of 3 replicate experiments.

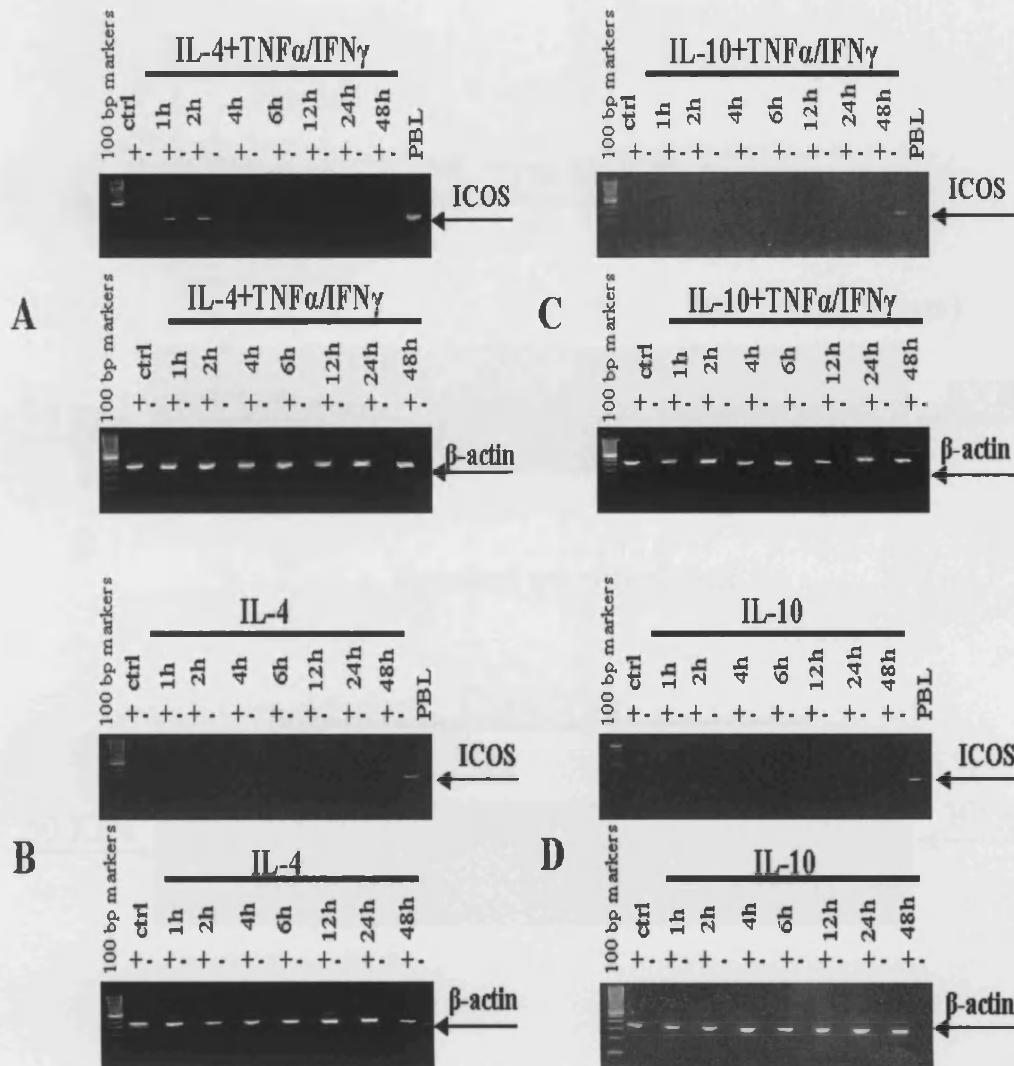

Figure 3.13 Regulation of of ICOS expression mRNA expression in primary human intestinal myofibroblasts by IL-4 and IL-10.

1x10^6 primary intestinal myofibroblasts were lysed in 400 µl RNAzol (ctrl) or pre-treated with IL-4 (100 ng/ml) (A) or IL-10 (100 ng/ml) (C) for 1 hour and then stimulated with 100ng/ml TNF-α and 300 units/ml IFN-γ for indicated time points or treated with IL-4 (B) or IL-10 (D) for indicated time points and then lysed in RNAzol. 1x10 ^6 12 days old SEB activated peripheral blood derived T cells (PBL) were also lysed in 400 µl RNAzol as a positive control for ICOS expression. RT PCR was performed on the lysates as described in Methods and Materials using primers for ICOS. (+) and (-) refers to RT positive and negative samples respectively. Results are from single experiments representative of 3 replicate experiments.

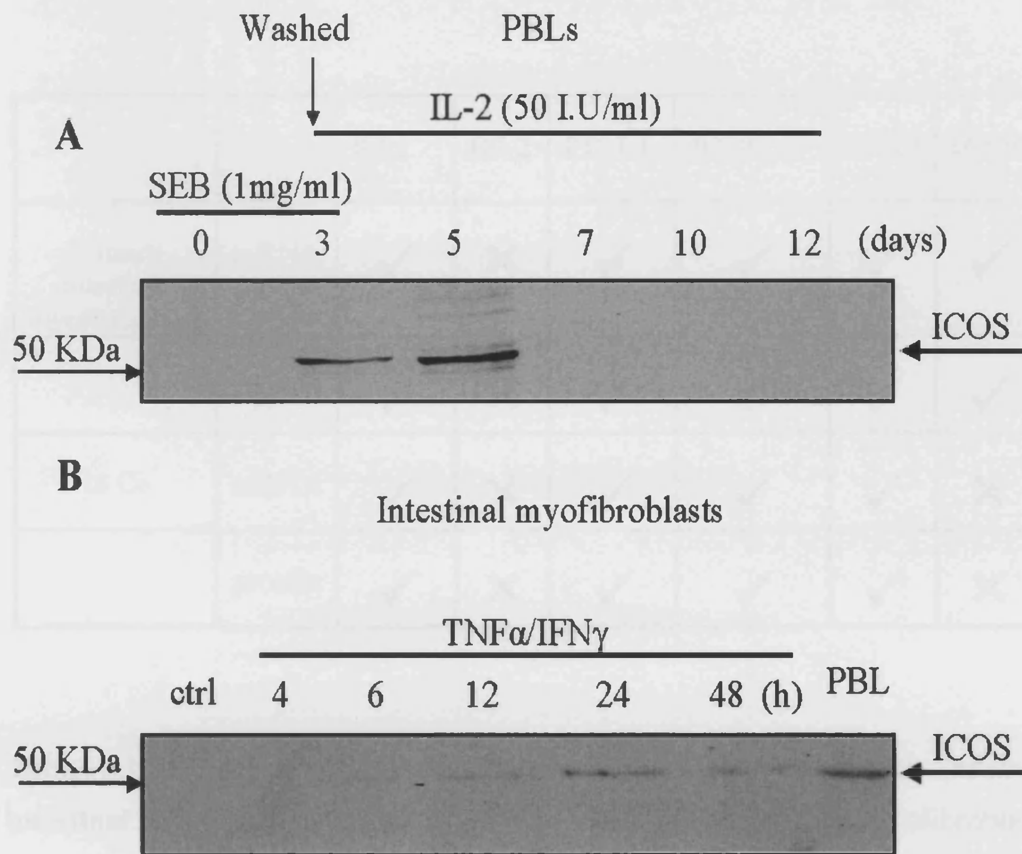

Figure 3.14 Protein expression of ICOS in peripheral blood derived activated T-cells and primary intestinal myofibroblasts.

A. $1x10^6$ peripheral blood mononuclear cells were lysed in lysis buffer (ctrl) or stimulated with SEB for 72 hours. Cells were then washed and maintained in 50 I.U/ml IL-2 and lysed at indicated time points. The resulting lysates were probed with a specific goat polyclonal antibody against ICOS at a final concentration of 0.1 μg/ml. **B**. $0.2x10^6$ primary intestinal myofibroblasts were plated in 35mm plates and cultured till confluence. Cells were then lysed (ctrl) or stimulated with 100ng/ml TNF-α and 300 units/ml IFN-γ for indicated times and then lysed in lysis buffer as described in Methods and Materials and then probed with a specific goat polyclonal antibody against ICOS at a final concentration of 0.1 μg/ml. Results are from single experiments representative of 3 replicate experiments.

3.3 Summary of findings

		B7.1	**B7.2**	**PD-L1**	**B7 RP-1**	**B7H3**	**ICOS**
Primary intestinal myofibroblasts	mRNA	✓	✗	✓	✓	✓	✓
	protein	✓	✗	✓	✓	✓	✓
18 Co	mRNA	✓	✗	✓	✓	✓	✗
	protein	✓	✗	✓	✓	✓	✗

Table 3.1 B7 and CD28 family members expression on human primary intestinal myofibroblasts and in 18 Co cells. Human primary myofibroblasts and 18 Co cells, express most of the B7 family members at the mRNA level and at least B7.1 on the cell surface. Primary myofibroblasts also express at both mRNA and protein level ICOS.

3.4 Discussion

Aberrant T-lymphocyte responses are implicated in a wide range of gastrointestinal disorders including IBD, celiac disease and autoimmune hepatitis. Numerous studies over the past decade have shown that stimulation through the antigen-specific TCR is not sufficient for full activation of a T-cell. Specifically, the induction of stimulatory T-cell responses requires two independent signals from the APC. As outlined, one is via TCR interaction with the MHC-peptide complex, while the other is via interaction of a co-stimulatory molecule on the

APC with its counter-ligand on the surface of the T-cell. Data from various experimental systems suggest that delivery of the first signal in the absence of the second results in T-cell anergy. This model highlights co-stimulatory molecules as potentially important therapeutic targets to intervene in autoimmune diseases.

Progression from early to chronic gut inflammation is accompanied by multiple structural abnormalities in the affected bowel segments, where persistent infiltration by immune cells is associated with qualitative and quantitative changes of local non-immune cells. Therefore, it is reasonable to assume that the cellular and molecular mechanisms underlying IBD vary during the course of the disease. In particular, it is possible that non-classic immune cells (such as epithelial, endothelial, and mesenchymal cells) become increasingly more important in sustaining chronic inflammation. This notion, yet to be explored in IBD, has already gained wide acceptance in other chronic diseases (Smith et al., 1997). A chronic intestinal inflammatory condition in which non-immune cells are recognized to play a key pathogenic role is celiac disease (Schuppan, 2000), where activated endothelial cells and fibroblasts are the prime source of tissue transglutaminases that selectively deaminate gliadin peptides, generating new epitopes recognized by mucosal T-cells that proliferate, produce cytokines, and perpetuate inflammation (Molberg et al., 1998). The fact that fibroblasts also play an active role in inflammation has finally been appreciated, and now fibroblasts are recognized as sentinel cells that not only modulate inflammation but actually regulate the switch from acute resolving to chronic persistent inflammation (Buckley et al., 2001).

In this report it has been demonstrated using flow cytometry and RT-PCR amplification that primary intestinal myofibroblasts and the relevant 18Co cell line express co-stimulatory molecules such as B7.1, PD-L1, B7-RP-1 and B7H3.

B7.1 and B7.2 expression in intestinal myofibroblasts

B7.1 expression was observed as a response to the pro-inflammatory cytokines TNFα and IFNγ. On the other hand the combination of theses cytokines failed to induce expression of B7.2 in both cell types B7.1 expression in intestinal myofibroblasts is in accordance with a previous study of regulated expression of B7.1 but not B7.2 in murine fibroblasts by the same cytokines (Pechhold et al., 1997).

To appreciate the implications of this finding, one needs to consider the potential role of intestinal myofibroblasts as important APCs in mucosal immune responses. In the intestinal mucosa, large numbers of lymphocytes normally reside in close physical proximity to a rich network of sub-epithelial and lamina propria fibroblasts and myofibroblasts. Preliminary evidence indicates that human intestinal fibroblasts can bind T-cells, modulate mucosal T-cell proliferation and apoptosis, and synthesize extracellular matrix capable of adhering T-cells (Fiocchi, 1997). It is more than likely that the net outcome of antigen presentation events associated with intestinal myofibroblasts are complex and occur via a variety of molecules. A previous study has already proposed that ICAM-1 is such a molecule, responsible for mediating mucosal fibroblasts-T-cell cross-talk (Musso et al., 1999).

Whether intestinal myofibroblasts can stimulate naïve $CD4^+$ T cells or CD28-dependent T-cells in the intestinal mucosa remains a matter for speculation. It is

conceivable however, that the delivery of 'signal 1' in the absence of 'signal 2' via B7 will result in the induction of tolerance in a subset of $CD4^{+}$ T-cells in the intestinal mucosa. Under physiological conditions, the net response (or lack of observed response) represents a balance between the stimulation of T-cells with suppressive activity versus those with a potential role in inflammation. With antigen exposure consistently high, the net result (i.e. suppressive versus inflammatory T-cell activity) is likely to depend on the underlying degree of inflammation and the state of activation of intestinal myofibroblasts. Due to reciprocal interactions between local fibroblasts and T-cells contribute to persistence of inflammation; blocking these interactions could limit T-cell accumulation and, eventually, down-regulate inflammation. It should also be noted that under the same conditions that intestinal myofibroblasts express B7.1, intestinal epithelial cells express B7.2, suggesting a supplementary role between the two cell types for regulating co-stimulatory events in the intestine

PD-L1, B7-RP-1 and B7H3 expression in intestinal myofibroblasts

Data presented here, also demonstrate, that intestinal myofibroblasts express B7 - H1, PD-L1 and B7-H3, the more recently identified co-stimulatory molecules of the B7 family, at the mRNA level. Unfortunately the lack of commercially available antibodies for these molecules at the time of this study, did not allow confirmation of their expression at the protein level.

These molecules are expressed mainly on professional APCs and T-cells. Although their precise function(s) and relationship with their cognate receptors CD28 and CTLA-4 are still not completely understood, there is universal

agreement that these B7 molecules provide critical co-stimulatory signals to T-cells and play an essential role in normal and pathologic immune reactions.

What might be the role of PD-L1 in intestinal myofibroblasts? PD-L1 expression has been detected not only in lymphoid organs, but also in non-lymphoid organs, such as heart, lung, placenta, kidney, and liver (Dong et al., 1999; Freeman et al., 2000). PD-L1 expression is also found in the majority of human cancers and leads to increased apoptosis of activated T-cells (Dong et al., 2002), raising the possibility that some tumours may use PD-L1 to inhibit an anti-tumour immune response. It has also been proposed that PD-L1 may be involved in setting thresholds for activation in the peripheral immune system (Freeman et al., 2000). In the intestine, a balance must be met between tolerance and the ability to rapidly induce memory responses. This involves the setting and maintaining of finely tuned thresholds and it seems likely that PD-L1 could have an important role in this process.

In humans, cell surface expression of B7-RP1 has been described on B cells, dendritic cells, monocytes/macrophages, T-cells and endothelial cells (Carreno and Collins, 2002). B7-RP1 mRNA expression has been detected in a variety of lymphoid and non-lymphoid organs (Liu et al., 2001), but the functional significance of B7-RP1 on non-lymphoid cells has remained unclear. Current models propose that B7-RP1–ICOS interactions play a more prominent role in the co-stimulation of effector or memory T-cell responses (Hutloff et al., 1999; Coyle et al., 2000), whereas CD28 co-stimulates primary T-cell functions. It is tempting therefore to speculate that B7-RP1 on intestinal myofibroblasts may augment the production of Th1 and Th2 cytokines by interaction with $CD4^+$ effector/memory

T-cells, thus augmenting antigen-specific immune responses in inflammatory conditions. Another possibility is that activated T-cells expressing ICOS may encounter and interact with B7-RP1 expressed on intestinal myofibroblasts, resulting in maintenance of activation in intestine in the presence of continued antigen exposure.

The possible role of B7-H3 on intestinal myofibroblasts is more difficult to assess. B7-H3 is expressed in multiple organs in humans and mouse and in several human tumour cell lines, and binds to an unknown receptor expressed on activated $CD4^+$ and $CD8^+$ T cells (Chapoval et al., 2001). This receptor is distinct from CD28, CTLA-4, PD-1 and ICOS, the receptors known to bind to the other B7 family proteins. Human B7-H3 augments TCR-mediated T-cell proliferation, IFN-γ production and generation of cytotoxic T lymphocytes (CTL) *in vitro*, indicating that B7-H3 may have positive regulatory functions in CTL responses. It seems likely that expression by intestinal myofibroblasts of different combinations of molecules mediating intestinal myofibroblast-T-cell interactions, such as B7.1, B7-H1, PD-L1 and B7-H3 may be important in shaping immune responses under different conditions. For instance in normal conditions, human gut mucosa is infiltrated with a large number of mononuclear cells reflecting the fact that the human intestine is continuously subjected to a massive stimulation by luminal antigens. This state of "physiological" inflammation is a tightly controlled phenomenon, as several mucosal cells interact to generate and maintain an appropriate local immune response. Lamina propria T-cells play the dual role of providing protection against pathogens, dietary and enteric florae antigens while limiting this potentially damaging immune response. This daunting task of adaptive immunity is complemented by that of other cells (macrophages,

eosinophils, mast cells, and natural killer cells) mediating innate immunity. To preserve an anti-inflammatory environment, lamina propria T-cells must put in motion complementary mechanisms to concomitantly provide active immunity and tolerance. To maintain this balance indispensable to gut homeostasis lamina propria T-cells effector functions must be turned on and off, which is accomplished through signals mediating cell activation, growth, differentiation, survival and death. In other words, health is the result of homeostasis between death and proliferation and the co-stimulatory signals provided by intestinal myofibroblasts might be crucial in regulating this process.

Induced expression of ICOS in primary intestinal myofibroblasts

The most unexpected finding of our study was the fact that primary human intestinal myofibroblasts were able to express ICOS at both the mRNA and protein level. This finding was not confirmed in the 18Co cells. Even though this result was confirmed for five different patients, it will be necessary to study fibroblasts from multiple additional subjects without colonic disease and from patients with adenomas, adenocarcinomas, and other colonic diseases to define the range of colonic fibroblast responsiveness to cytokine stimulation and the temporal relationship of ICOS expression to the stage of colon carcinogenesis. It may be that the distinct phenotypic attributes of an individual's colonic fibroblasts, such as relative sensitivity to cytokine stimulation, can have a protective role or contribute to the pathogenesis of large bowel disease.

However, one obvious possibility arising from ICOS expression in these cells is that myofibroblasts act as pluripotent stem cells capable of expressing a variety of molecules at low levels. Our RT-PCR results suggest that CD28 and CTLA-4

were not present in those cells, excluding the possibility of contamination of myofibroblasts with mucosal T-cells, while ICOS was detected both in myofibroblasts and in lymphocytes. Therefore, intestinal myofibroblasts express specifically ICOS but not CD28 or CTLA-4 under cytokine stimulation.

Assuming that the function of this molecule on myofibroblasts is similar to that of their T-cell counterparts, the induced expression of ICOS in intestinal myofibroblasts may be a critical parameter in determining the state of activation of these cells. Recent studies have shown that ICOS regulates both Th1 and Th2 responses up-regulating the levels of IFN-γ and IL-2 as well as IL-4 and IL-10 *in vivo* (Khayyamian et al., 2002), and therefore may also have a role in determining the cytokine milieu in the intestine. IL-4 in particular is known to be important in the formation of germinal centres in Peyer's patches (Vajdy et al., 1995).

Indirect evidence for the role of ICOS in intestinal myofibroblasts comes from a study in a well-validated Th1-mediated mouse colitis model, which found that expression of ICOS and B7-RP1, was increased during experimental colonic inflammation (Totsuka et al., 2003). Furthermore, anti-ICOS mAb could both prevent and reverse established inflammation, suggesting a potential therapeutic application in human Th1-mediated intestinal inflammatory conditions such as CD. On the other hand, blockade of B7RP- 1 did not have a protective effect, indicating that the interaction between these co-stimulatory molecules was not critical to the development of colitis. The resulting phenotype was attributed to the fact that anti-ICOS mAb induced apoptosis of activated T-cells. ICOS expression in intestinal myofibroblasts, raises the possibility of a similar mechanism for apoptosis in myofibroblasts which could provide a means to

control myofibroblast proliferation and therefore limit the damage of the tissue observed in CD.

Chapter 4

4 Results II

CXC3 ligand-mediated signalling events in human primary intestinal myofibroblasts

4.1 Background

The first suggestion that chemokines contribute to the pathogenesis of IBD came from a series of clinical studies published nearly a decade ago, in which rectal biopsies from patients with active ulcerative colitis or Crohn's disease were found to produce high levels of the chemokine IL-8 (Izzo et al., 1993; Raab et al., 1993). Subsequent studies have also implicated other chemokines such as RANTES (Mazzucchelli et al., 1996), MCP-1(Grimm et al., 1996) and MCP-3 (Wedemeyer et al., 1999).

One major source of chemokines in the gut is the intestinal epithelium. Stimulation of human intestinal epithelial cells with TNFα, IL-1 or infection with enteroinvasive bacteria such as *Salmonella*, causes the increased expression and secretion of a number of chemokines with pro-inflammatory functions. Thus, stimulated epithelial cells express and secrete relatively high levels of IL-8, GROα, GROβ, GROγ, and ENA-78 (Eckmann et al., 1993; Yang et al., 1997). These cytokines belong to the C-X-C family of chemokines and are characterized by their ability to chemoattract and activate polymorphonuclear leukocytes. Activated epithelial cells also secrete, albeit at lower levels, a range of C-C chemokines, including MCP-1, MIP-1β, MIP-1α, RANTES, MDC and MIP-3α (Jung et al., 1995; Berin et al., 2001; Izadpanah et al., 2001) which variably can act as chemoattractants of monocytes/macrophages, eosinophils, and

subpopulations of T-cells. More recently the T-cell chemoattractants IP-10, I-TAC and Mig have also been shown to be secreted by intestinal epithelial cells (Dwinell et al., 2001). Interestingly the receptor for these chemokines, CXCR3, has been found to be expressed on HSC, the equivalent of myofibroblasts in the liver (Bonacchi et al., 2001). The close proximity of intestinal myofibroblasts to intestinal epithelial cells, being separated only by the basement membrane, makes it reasonable to propose that the CXCR3 ligands may play a role in mediating cross talk between these two cell types.

In order to explore this possibility the effect of the CXCR3 ligands on various biochemical responses was investigated, using activated T-cells, an established CXCR3 expressing cell type, as a positive control for these experiments.

4.2 Results

Expression of CXCR3 in primary intestinal myofibroblasts

To determine if intestinal myofibroblasts express mRNA transcripts for CXCR3, total RNA was isolated from cells derived from two different biopsy specimens, and was analysed for expression of CXCR3 transcripts by RT-PCR. As shown in Fig. 4.1, cells from both biopsies constitutively expressed CXCR3 mRNA. This result was consistent for the cells that were isolated from all the biopsy specimens used throughout this project. Peripheral blood derived, SEB activated T-cells 12 days old, were used as a positive control for this experiment.

To determine if this constitutive expression of mRNA CXCR3 was paralleled by expression of CXCR3 on the cell surface, cells were stained with an anti-CXCR3 antibody and examined by flow cytometry (Figure 4.1, lower panel). No

detectable levels of CXCR3 were observed on the cell membrane of any of the different patient derived cells tested, as opposed to the high levels of CXCR3 expression detected on activated T-cells, which were used as a positive control for this experiment.

Effect of endothelin-1 in $[Ca^{2+}]_i$ in primary intestinal myofibroblasts

The disparity between the presence of CXCR3 mRNA and lack of detectable protein expression in primary human intestinal myofibroblasts, leaves several possibilities regarding the presence or not of that receptor on those cells. One possibility is that the receptor is expressed on the cell surface, but below sensitivity limits of the antibody used in this study. Another possibility is that the receptor is post-translationally modified. In order to verify that CXCR3 is indeed not expressed on primary intestinal myofibroblasts we tested the ability of the known CXCR3 ligands to generate various biochemical signals in these cells.

The process of cellular Ca^{2+} signalling involves regulated changes in the intracellular cytosolic concentration of Ca^{2+} ($[Ca^{2+}]_i$) and is known to regulate a broad range of secondary signals and functional responses. Calcium mobilization was the first and one of the best characterized responses to chemokine stimulation (Loetscher et al., 1994; Baggiolini et al., 1997), and was thus chosen as the first functional response to the CXCR3 ligands in our system.

However, we first examined the ability of human primary intestinal myofibroblasts to mobilize Ca^{2+}, using endothelin-1, which has recently been shown to mobilize calcium in a relevant cell line (Kernochan et al., 2002).

Cultured intestinal myofibroblasts grown on glass coverslips, were loaded with 1 μM fura-2 AM for 30 minutes, at room temperature, before the coverslip was mounted in a chamber and the cells stimulated at 37°C with endothelin-1 at 100 nM. Live images of the cells were obtained using digital fluorescence imaging microscopy. Endothelin-1 transiently increased $[Ca^{2+}]_i$, in most but not all cells (Figure 4.2). In all subsequent experiments, 100 nM endothelin-1 was used to ensure responsiveness of the cells.

Differential effect of the CXCR3 ligands in $[Ca^{2+}]_i$ in intestinal myofibroblasts

Having established the ability of intestinal myofibroblasts to elicit calcium responses, the effect of IP-10, I-TAC and Mig in $[Ca^{2+}]_i$ was next investigated. Intestinal myofibroblasts were prepared for digital fluorescence imaging microscopy as described above. Cells were temperature-equilibrated to 37°C for five minutes, during which the basal fluorescence spectrum was observed. Chemokines were added to the chamber at concentrations between 10 and 100 nM, and the fluorescence was monitored. Endothelin-1 at 100 nM was used at the end of each experiment to verify that the cells would respond to a calcium-mobilizing stimuli. Intestinal myofibroblasts did not respond to IP-10 (Figure 4.3) or Mig (Figure 4.5). However, it was found that intestinal myofibroblasts showed a rapid and transient increase in intracellular Ca^{2+} following stimulation with I-TAC (Figure 4.4). All of these cells showed a positive control response to endothelin-1, indicating that the cells are indeed non-responsive to IP-10 and Mig. It should be noted that two factors render the interpretation of the results of these experiments difficult. First, in most cases there were several Ca^{2+} oscillations

before and after the addition of the chemokines or endothelin-1, resulting in high basal levels of intracellular Ca^{2+}. Secondly, these cells almost constantly undergo shape changes due to spontaneous contraction and expansion, thus limiting the accuracy of the technique since a specific area, indicating the space of a cell, must be outlined.

CXCR3 ligands and PKB phosphorylation

The data obtained from the calcium assays, further complicated the possibilities regarding the presence or absence of CXCR3 in primary human myofibroblasts. These cells could be expressing a receptor that responds only to I-TAC, like the receptor that has been reported to respond only to IP-10 but not the other CXCR3 ligands (Soejima and Rollins, 2001).It was therefore imperative to compare any further biochemical responses to the CXCR3 ligands in the myofibroblasts, with responses in activated T cells which are known to express CXCR3 (Kim and Broxmeyer, 1999).

The serine/threonine kinase PKB has been shown to be critical for cell survival, proliferation, and gene expression. The products of PI3K activity are absolutely required for phosphorylation at Thr-308 and Ser-473 and consequent activation of PKB, a reaction catalyzed by PDK-1, which phosphorylates Thr-308, and a second unidentified kinase that targets PKB Ser-473 (Alessi et al., 1997). Thus, phosphorylation of PKB is a facile readout for activation of the PI3K pathway. Most chemokines are known to phosphorylate PKB and therefore this phosphorylation event was examined as a possible downstream target of IP-10, Mig and I-TAC in our system.

Primary human intestinal myofibroblasts were stimulated over a short time course of up to 20-minutes with IP-10, I-TAC or Mig at 10 nM, since this concentration was sufficient to mobilize calcium in response to I-TAC. Protein was then isolated for western blot analysis. Whole cell lysates were run on SDS-polyacrylamide gels and probed with a specific rabbit antibody against the phosphorylated (activated) PKB phospho ^{473} PKB. Membranes were then stripped and re-probed for the respective un-phosphorylated isoform of PKB (Figure 4.6 A, B, C).

Activation of PKB, for all three ligands is sustained, with a peak of phosphorylation occurring five minutes after stimulation but remaining above basal levels at the end of the time course.

Using similar experimental protocols, SEB activated peripheral blood-derived T-cells that had been maintained in IL-2 for 12 days were stimulated with IP-10, I-TAC or Mig over a 20-minute time course. Protein was isolated for western blot analysis and membranes were probed for phospho ^{473} PKB (Figure 4.6 D, E, F). This was used as a positive control since it has recently been shown that all CXCR3 ligands result in a robust PKB phosphorylation in activated T-cells (Smit et al., 2003). Indeed the chemokines again induced a transient phosphorylation of PKB but with very different kinetics compared to the one observed in myofibroblasts. Phosphorylation levels were very high for the first 2 minutes of the time course, but were not detectable 5 minutes after the addition of the chemokines.

As mentioned earlier, phosphorylation at S473 partially activates PKB, whereas full activation of PKB requires phosphorylation on a second site, T308 (Alessi et

al., 1996). In order to determine if PKB was phosphorylated on this second site, intestinal myofibroblasts and activated T-cells were again stimulated with the CXCR3 ligands over a 20-minute time course, protein was isolated for western blot analysis and membranes were probed for the activated phospho ^{308} PKB (Figure 4.7).

The phosphorylation patterns for T308 were identical to the ones observed for S473, with activation of PKB being sustained throughout the time course in the myofibroblasts (Figure 4.7 A, B and C), and returned to basal levels after 2 minutes in T cells (Figure 4.7 D, E, F).

PKB phosphorylation in the presence of PI3K inhibition

PKB is one of the most well known downstream targets of PI3K. To confirm this intestinal myofibroblasts and blood-derived T-cells were treated with the PI3K inhibitor LY294002 at 10 μM or a vehicle control for 30 minutes and then stimulated with IP-10, I-TAC or Mig for up to 20-minutes. As expected PKB phosphorylation is PI3K dependent in both intestinal myofibroblasts (Figure 4.8) and activated T-cells (Figure 4.9) as demonstrated by its complete inhibition by the PI3K inhibitor. It should be noted however, that phosphorylation of PKB does not necessarily indicate activation of this kinase. In order to examine PKB activation, one would have to investigate downstream targets of PKB such as GSK-3 or FKHR. Those experiments were carried out but did not result in detection of any phosphorylation of these proteins in intestinal myofibroblasts. This could be because the amounts of protein in our samples are very low (approximately 3 μg/sample) due to the primary nature of the cells and the limitation in the numbers that can be realistically obtained from biopsy tissues.

Sensitivity of various PI3K isoforms to LY294002

The availability of a PI3K inhibitor such as LY294002 has contributed greatly to our understanding of the biological role of PI3K and its effector proteins. However, the issue of the selectivity of LY294002 for individual PI3K isoforms is highly controversial. The generally accepted view is that LY294002 inhibits all PI3K with an IC_{50} in the 1-50 μM range (Foster et al., 2003). Use of this inhibitor can therefore implicate a PI3K activity in a cellular process of interest, but is not suitable for dissecting the involvement of individual PI3K isoforms. In order to verify the selectivity of LY294002 on different PI3K isoforms, whole cell lysates from unstimulated Jurkat cells, which are known to exhibit a high basal PI3K activity (Freeburn et al., 2002), were immunoprecipitated with antibodies against various PI3K isoforms. The isoforms chosen for investigation were limited by the lack of reliable commercially available antibodies for the other PI3K isoforms. The resulting immunoprecipitates were treated with various concentrations of the inhibitor and assayed for PI3K activity using an *in vitro* lipid kinase (Figure 4.10). Both class IA PI3K isoforms examined, p85α and p110δ, were very sensitive to LY294002 and almost completely inhibited at 10μM (Figure 410 A and B). In contrast both class II isoforms, C2α and C2β, proved very resistant to LY294002, even at concentrations well beyond the range which is normally used, namely > 30μM (Figure 4.10 C and D).

PI3K isoform expression in intestinal myofibroblasts

In order to investigate the involvement of specific PI3K isoforms in chemokine induced signalling in intestinal myofibroblasts and activated T cells, it was essential to establish which isoforms were expressed in these cells. Different cell

numbers were lysed in order to isolate protein for western blot analysis of p85α, p110 δ and the two class II PI3K isoforms, PI3K-C2α and PI3K-C2β. All four isoforms examined were found to be expressed in both intestinal myofibroblasts (Figure 4.11) and T-cells (Figure 4.12).

Chemokine induced PI3K isoform activation

The limitation of relying on PKB phosphorylation experiments is that it does not provide any information about the specific isoforms involved in those processes. The only valid method for examining specific PI3K isoform activation, is by employing *in vitro* lipid kinase assays, to assess catalytic activity within isoform-selective immunoprecipitates. One disadvantage of this method is the large number of cells required ($1x10^7$ cells per point). Due to the difficulty of obtaining sufficient number of primary human intestinal myofibroblasts, these studies were confined to only one of the three CXCR3 ligands, IP-10. In addition, the activity of the PI3K isoforms in these established assays, is not studied in its natural environment, i.e., inside the cell and therefore possible activity might not reflect events in a living cell.

With antibodies suitable for immunoprecipitation available for the PI3K isoforms found to be expressed in intestinal myofibroblasts, the cells were stimulated with IP-10 at 10 nM over a short time of thirty minutes and then immunoprecipitated with antibodies recognizing p85α, p110δ, PI3K-C2α or PI3K-C2β (Figure 4.13). Buffers containing Ca^{+2} were used for all isoforms except PI3K-C2β since it has been demonstrated that an increase in Ca^{2+} would have a negative effect on the production of $PI(3,4)P_2$ by PI3K-C2β (Arcaro et al., 1998).

In contrast to p85α and p110δ, which did not appear to be activated above basal levels, both PI3K-C2α and PI3K-C2β phosphorylated the exogenous substrate PtdIns. The kinetics for the activation of the two isoforms was slightly different, with C2α activation being observed two minutes after stimulation and returning to basal levels within the end of the time course applied, whereas C2β activation was faster and sustained throughout the stimulation period.

Having demonstrated that only class II PI3K isoforms are detectably activated by IP-10 in intestinal myofibroblasts, a similar range of experiments were performed in T-cells to determine PI3K isoform activity in this system. SEB activated peripheral blood derived T-cells, maintained in IL-2 for 12 days were stimulated as previously with IP-10 at 10 nM over a short time course of 30 minutes. Protein was isolated and an *in vitro* lipid kinase assay was performed using specific PI3K isoform immunoprecipitates (Figure 4.14).

The results were very different compared to those obtained with the myofibroblasts. In T-cells, both class I PI3K isoforms examined were found to respond to IP-10, with activity peaking in both cases five minutes after stimulation. However the activity of C2α and C2β was not altered by the chemokine.

It should be noted though that the two different cell types appear to have high basal levels of activity for the isoforms that appear unresponsive, which could explain why no activation is observed in those experiments.

IP-10 stimulates the recruitment of PI3K-C2α and PI3K-C2β to phosphotyrosine complexes in intestinal myofibroblasts

Reports of tyrosine phosphorylation of PI3K have mainly concerned class I PI3Ks. Tyrosine phosphorylation of the p85 subunit has been shown to occur in many different systems, such as in response to platelet-derived growth factor (Kaplan et al., 1987), insulin (Hayashi et al., 1993), B cell antigen receptor ligation (Gold et al., 1992), and interleukin-2 (Karnitz et al., 1994). The only reports of tyrosine phosphorylation of class II PI3K has been in Drosophila (Molz et al., 1996), and in EGF-stimulated A431 cells (Arcaro et al., 2000). The same study demonstrated tyrosine phosphorylation of PI3KC2β in PDGF-stimulated fibroblasts. The observation that these proteins were phosphorylated on tyrosines could imply that protein tyrosine kinases and protein–protein interactions could mediate the activation of class II PI3Ks. Since activation of both of the class II PI3Ks in response to IP-10 had been demonstrated in this study, the possibility that these proteins were tyrosine phosphorylated was investigated.

Intestinal myofibroblasts were treated with IP-10 at a concentration of 10 nM for up to thirty minutes. The lysates generated were immnunoprecipitated with an anti-phospotyrosine specific antibody (4G10) and the recovered immune complexes were collected on protein G-sepharose beads for one hour at 4°C and finally was analysed by Western blotting with antibodies against PI3K-C2α or PI3K-C2β (Figure 4.15).

Both class II PI3K enzymes were found to be tyrosine phosphorylated. Phosphorylation for C2α occurred between one and ten minutes after stimulation while maximal phosphorylation for C2β was observed five minutes after the

addition of the chemokine but was still above basal levels at the end of the time course. This pattern of activation corresponds with the results obtained from the *in vitro* lipid kinase assays.

CXC3 ligand-induced phosphorylation of ERK 1/2

Having established that IP-10, I-TAC and Mig activate the PI3K pathway, regulation of the MAPK pathway was next investigated. Activation of the MAPK pathway has been demonstrated to occur in response to many cytokines and is involved in the activation of transcription factors required for various cell functions (Chang and Karin, 2001).

Intestinal myofibroblasts were stimulated over a time course of up to 20-minutes with IP-10, I-TAC or Mig at a concentration of 10 nM and then protein was isolated for Western blot analysis with a specific rabbit antibody against the phosphorylated MAP kinases phospho ^{202,204} ERK 1/2.

Basal levels of constitutive phosphorylation of ERK were detected in intestinal myofibroblasts. However, all three chemokines were able to phosphorylate ERK transiently between two and ten minutes following stimulation above the basal levels observed (Figure 4.16 A, B and C).

Similarly SEB activated T-cells, were stimulated over a 20-minute time course and analysed for ERK 1/2 phosphorylation. In contrast to the intestinal myofibroblasts, no basal levels of phospho-ERK 1/2 was found in this system. Nevertheless, stimulation in these cells, resulted in phosphorylation of ERK 1/2 which like phosphorylation of PKB was much more rapid and transient compared

to intestinal myofibroblasts. Phosphorylation was maximal two minutes following stimulation and was not detectable after 10 minutes (Figure 4.16 D, E and F).

CXC3 ligand-induced p90RSK phosphorylation

Having demonstrated that IP-10, I-TAC and Mig phosphorylate ERK 1/2 in intestinal myofibroblasts and activated T-celis, a downstream target of ERK was selected to correlate phosphorylation of ERK with its activity. It is well known that p90RSK lies downstream of ERK (Richards et al., 2001), and it has been suggested that when activated, both ERK 1/2 and p90RSK are translocated to the nucleus (Pierrat et al., 1998). Cells were stimulated with IP-10, I-TAC or Mig and protein was isolated for western blot analysis as previously, probing for phosphorylated form p90RSk, namely phospho ^{380}p90RSK (Figure 4.17).

In primary human intestinal myofibroblasts, activation of p90RSK is delayed and more transient compared to the activation of ERK 1/2, with phosphorylation observed only between 5 and 10 minutes after the addition of the chemokines (Figure 4.17 A, B and C). These delayed kinetics should be expected since p90RSK lies downstream of ERK 1/2.

In activated T-cells, all three chemokines also induce transient phosphorylation of p90RSK. This event is also delayed compared to ERK 1/2 phosphorylation in this system, with optimal phosphorylation occurring 2 minutes post-ligand stimulation and then declining rapidly (Figure 4.17 D, E and F).

CXC3 ligands and p38 phosphorylation in primary intestinal myofibroblasts

Mig has been shown to be able to activate the p38 pathway human melanoma cells (Robledo et al., 2001). Having previously shown that the chemokines are

able to induce phosphorylation of one MAPK pathway in our systems, their ability to phosphorylate the p38 pathway was also investigated.

Intestinal myofibroblasts were stimulated with IP-10, I-TAC or Mig at 10 nM and the protein lysates were probed with a specific rabbit polyclonal antibody against the phosphorylated MAP kinase phospho $^{180/182}$ p38 (Figure 4.18). Phosphorylation of p38 was found to be sustained with a peak of activation between 2 and 5 minutes following stimulation but still quite prominent at 10 minutes, and in the case of Mig (Figure 4.18 C), phosphorylation levels were above basal even at the end of the time course. This discrepancy most likely reflects the heterogeneity and the individuality within a population of primary cells rather than any differences between the three different ligands.

The same experiment was performed with SEB activated T-cells but no detectable levels of phosphorylated p38 were observed. This may not necessarily imply that p38 is not phosphorylated in those cells in response to the CXCR3 ligands but might simply reflect the low levels of phosphorylated p38 beyond the sensitivity levels of the antibody.

CXCR3 ligand-induced ERK phosphorylation in the presence of PI3K inhibition

The involvement of PI3K in the activation of the ERK in chemokine signalling appears to be highly dependent on the chemokine and cell type involved. Indeed it has been shown that ERK phosphorylation can be both dependent (Sotsios et al., 1999) or independent (Bonacchi et al., 2001) of PI3K activation. To assess the role of PI3K signalling pathway in ERK phosphorylation, cells were pre-treated

with either the PI3K inhibitor LY294002, or a vehicle control for thirty minutes before adding the chemokines. Protein was isolated for Western blot analysis and membranes were probed for activated phospho $^{202/204}$ ERK (Figures 4.19-4.20).

The results obtained from these experiments are not very consistent for human primary intestinal myofibroblasts. The data presented here show that in the presence of LY294002, ERK1/2 phosphorylation appears to be modestly inhibited in IP-10 and Mig stimulated cells, whereas I-TAC appears to be the most sensitive to LY294002 treatment (Figure 4.19 B). However, interpretation of these results is difficult due to the discrepancies of the effect of LY294002 on basal levels of phosphorylation of ERK 1/2. The PI3K inhibitor markedly reduced the basal phosphorylation levels of ERK 1/2 in I-TAC stimulated cells, something not observed in the experiments with the other 2 ligands. The only explanation we can give for these apparent contradictions is donor variability in conjunction with the nature of primary cells renders them very susceptible to the handling procedures employed, resulting in varying responses. Overall though, it seems likely that there is some involvement of PI3K in the activation the ERK pathway.

Results obtained from SEB activated T-cells are more uniform than the results from intestinal myofibroblasts. LY294002 appeared to partially inhibit ERK 1/2 phosphorylation in IP-10, I-TAC or Mig stimulated cells (Figure 4.20). ERK phosphorylation was not completely abolished indicating that PI3K kinase activity is involved in the activation of the ERK pathway in this system.

p90RSK phosphorylation in the presence of PI3K inhibition

Little is known about the signalling mechanisms involving p90RSK in response to chemokine stimulation. However it is known that p90RSK is phosphorylated and

activated by the PI3K target PDK-1 (3-phosphoinositide-dependent protein kinase-1) (Jensen et al., 1999). To examine the involvement of PI3K in chemokine-mediated p90RSK activation, cells were pre-treated with LY294002, prior to stimulation over a short time course of up to twenty minutes with IP-10, I-TAC or Mig at 10 nM and then protein was isolated for western blot analysis (Figures 4.21-4.22).

Treatment with LY294002 in human myofibroblasts partially inhibited p90RSK phosphorylation. Consistent with the previous finding for ERK 1/2 phosphorylation, this event was more significant for I-TAC stimulated cells where LY294002 had a more profound effect on basal phosphorylation levels (Figure 4.21 B). Therefore, PI3K appears to be a critical upstream kinase responsible for activation of p90RSK in the IP-10 / I-TAC/ Mig signal transduction pathway in human primary intestinal myofibroblasts. The effect of LY294002 in p90RSK phosphorylation in activated T cells was very similar to the effect of the inhibitor in ERK 1/2 phosphorylation (Figure 4.22). This event was partially inhibited suggesting a possible role for PI3K in this event in this system as well.

p38 phosphorylation in the presence of PI3K inhibition

Having previously shown that p38 could be phosphorylated by the CXCR3 ligands in our system, the role of PI3K in activating this pathway was investigated. Previous studies have indicated that activation of p38, is PI3K dependent (Rane et al., 1997; Madrid et al., 2001). In addition, a new mechanism for PKB-mediated nuclear factor- kappaB (NF-κB) activation has been proposed by demonstrating that PKB utilizes IκB kinase (IKK) and p38 to stimulate the transactivation potential of the RelA/p65 subunit of NF-κB (Madrid et al., 2001) .

Intestinal myofibroblasts were incubated with IP-10, I-TAC or Mig at 10nM up to 20 minutes, in the presence or absence of LY294002 pre-treatment at 10 μM for 30 minutes (Figure 4.23). Treatment of the cells with the PI3K inhibitor markedly reduces the chemokine-induced phosphorylation of p38. Moreover it should be noted that the cells appear to have higher basal levels of phosphorylation when treated with LY294402. This would suggest that the cells are stressed, since p38 is known to be a stress signal and could explain the inconsistencies observed earlier in respect of ERK 1/2 phosphorylation when LY294002 was used.

Effect of pertussis toxin in chemokine signalling in intestinal myofibroblasts

The major G protein attributed to be coupled to chemokine receptor is $G\alpha_i$ because in the hands of most investigators pertussis toxin inhibits the biological activities induced by chemokines. However, pertussis toxin -sensitive G proteins also include $G\alpha_{01}$, and $G\alpha_{02}$. In addition to this it has been observed that while pertussis toxin completely inhibits IP-10, and the C chemokine lymphotactin-induced calcium mobilisation in NK cells, anti-$G\alpha_q$ also inhibits these responses, suggesting that pertussis toxin-insensitive G proteins might also be involved in those processes (Maghazachi et al., 1997). Recently it has been found that CXCR3 signalling in activated T-cells is completely inhibited by the use of pertussis toxin (Smit et al., 2003). On the other hand the newly identified alternative splice variant of CXCR3, CXCR3-B, appears to exert its biological activities by coupling to G proteins other than $G\alpha_i$ (Lasagni et al., 2003). Finally the as yet unidentified receptor that binds only IP-10 is probably coupled to Gαi, as demonstrated by sensitivity to pertussis toxin (Soejima and Rollins, 2001).

In light of the knowledge that pertussis toxin can have opposing effects on CXCR3 ligand-mediated events, it was decided to examine the effect of this inhibitor in IP-10 induced signalling in both human intestinal myofibroblasts and activated T-cells.

Cells were therefore treated with IP-10 at 10nM for up to 20 minutes in the presence or absence of pre-treatment with pertussis toxin at 100 ng/ml for 16 hours and analysed by western blot analysis with specific antibodies against the active forms of ERK 1/2, PKB and p90RSK. In the myofibroblasts, phosphorylation of all proteins was found to be insensitive to pertussis toxin (Figure 4.24). Moreover, p90RSK phsosphorylation in cells treated with pertussis toxin was sustained compared to the chemokine only induced signals, with phosphorylation levels still increasing at the end of the time course (Figure 4.24 C). These observations contrasted the results in the activated T-cells. Pertussis toxin completely abrogated chemokine induced ERK phosphorylation but was unable to block phorbol 12-myristate 13-acetate (PMA)-induced activation which is known to activate several proteins independently of G-protein mechanisms (Volpi et al., 1985) and was therefore used as a positive control (Figure 4.25 A). Consistent with this, pertussis toxin completely blocked ERK 1/2 downstream target p90RSK activation (Figure 4.25 B). Finally, phosphorylation of PKB was also found to be pertussis toxin sensitive (Figure 4.25 C), confirming the involvement of $G\alpha i$ in CXCR3 signalling events in activated T-cells.

Differences in potency of the CXCR3 ligands in intestinal myofibroblast signalling

There have been contradicting reports concerning the relative potency of IP-10, I-TAC and MIG in receptor binding, calcium flux, and chemotaxis (Cole et al., 1998; Stanford and Issekutz, 2003). However, I-TAC appears to be more potent and efficacious than either IP-10 or Mig in its ability to mobilize intracellular calcium and as a chemotactic factor in activated T-cells (rank order potency I-TAC > MIG ≈ IP-10 (Cole et al., 1998). Studies comparing the ability of these chemokines to initiate intracellular signals are lacking.

Having established that all three CXCR3 ligands are capable of activating various signalling pathways in intestinal myofibroblasts and activated T-cells, the potency of these chemokines to activate those pathways was compared in both of our systems. Cells were stimulated over time course of up to 10 minutes, within which maximal phosphorylation of ERK 1/2 and PKB had previously been demonstrated for both cell types, with IP-10, I-TAC or MIG at 10 nM and then protein was isolated for western blot analysis.

In intestinal myofibroblasts, no differences could be detected in the magnitude of the signals elicited by those 3 ligands, suggesting that their efficacy for the signalling events examined is similar in these cells (Figure 4.26). On the other hand, in activated T-cells. I-TAC appeared to confirm the calcium and chemotaxis studies (Cole et al., 1998) as the most potent of the 3 CXCR3 ligands. Phosphorylation of both ERK 1/2 and PKB by I-TAC was induced to a greater magnitude than those observed with IP-10 and Mig, which were very similar between them (Figure 4.27).

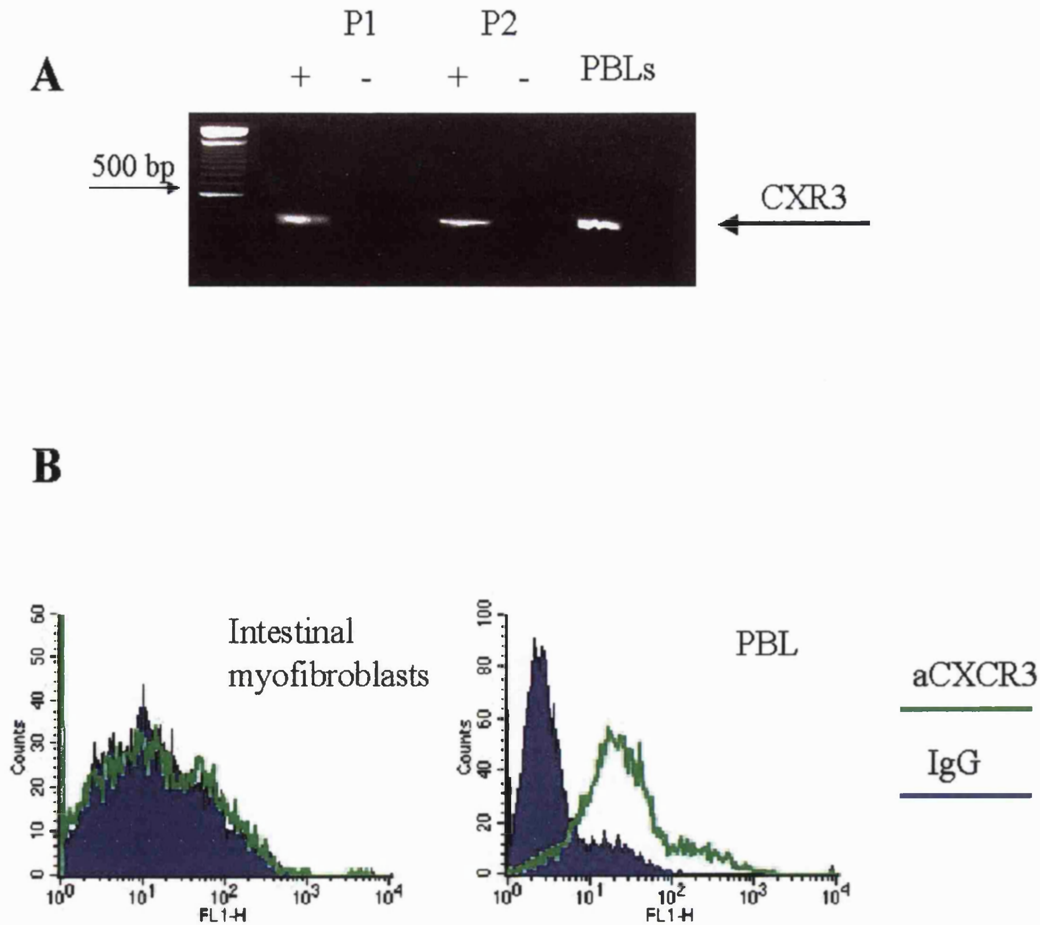

Figure 4.1 mRNA and cell surface expression of CXCR3 in primary intestinal myofibroblasts.

A. PCR analysis of CXCR3 mRNA expression of intestinal myofibroblasts isolated from two different patients (P1, P2). (+) and (-) refers to reverse transcriptase (RT) positive/negative samples respectively. Activated peripheral blood derived T cells (PBLs) were used as a positive control (upper panel). **B.** Fluorescence Activated Cell Sorting (FACS) analysis of CXCR3 expression on intestinal myofibroblasts, again using activated peripheral blood derived T-cells (PBLs) (lower panel). Cells were stained with a specific mouse monoclonal fluorescein isothiocyanate (FITC) conjugated anti-CXCR3 antibody (green line) at a concentration of 1μg/ml or with FITC conjugated mouse IgG at the same concentration (purple area), as an isotype matched control. All three results are from single experiments representative of 3 replicate experiments.

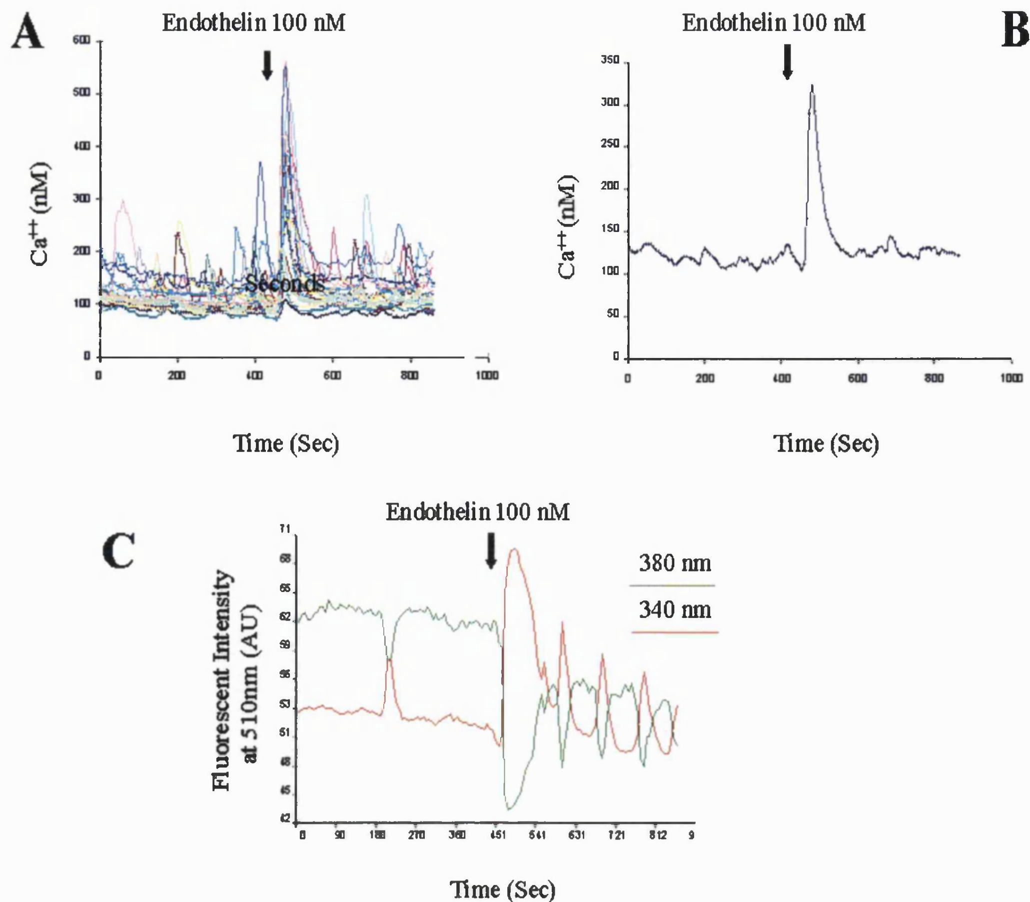

Figure 4.2 Effect of endothelin-1 on calcium mobilization in human primary intestinal myofibroblasts.

0.2 x 10^6 primary human intestinal myofibroblasts were plated on coverslips in 35mm plates and cultured for 48 hours. Prior to experimentation, cells were washed and kept in physiological salt solution (PSS) and loaded with Fura-2 as described in Methods and Materials. Cells were stimulated with endothelin-1 (100nM) whilst alternately excited with light at 340 and 380 nm. Fura –2 fluoresence emission intensity was measured at 510nm and transformed to intracellular-free calcium concentrations, based on calibration of the cells with 30 μM ionomycin and 10 mM EGTA, for individual cells (A) or for the average of the cells in view (B). A representative graph of emission intensity at 340 nm and 380 is presented for a single cell(C). Results are from single experiments representative of 3 replicate experiments.

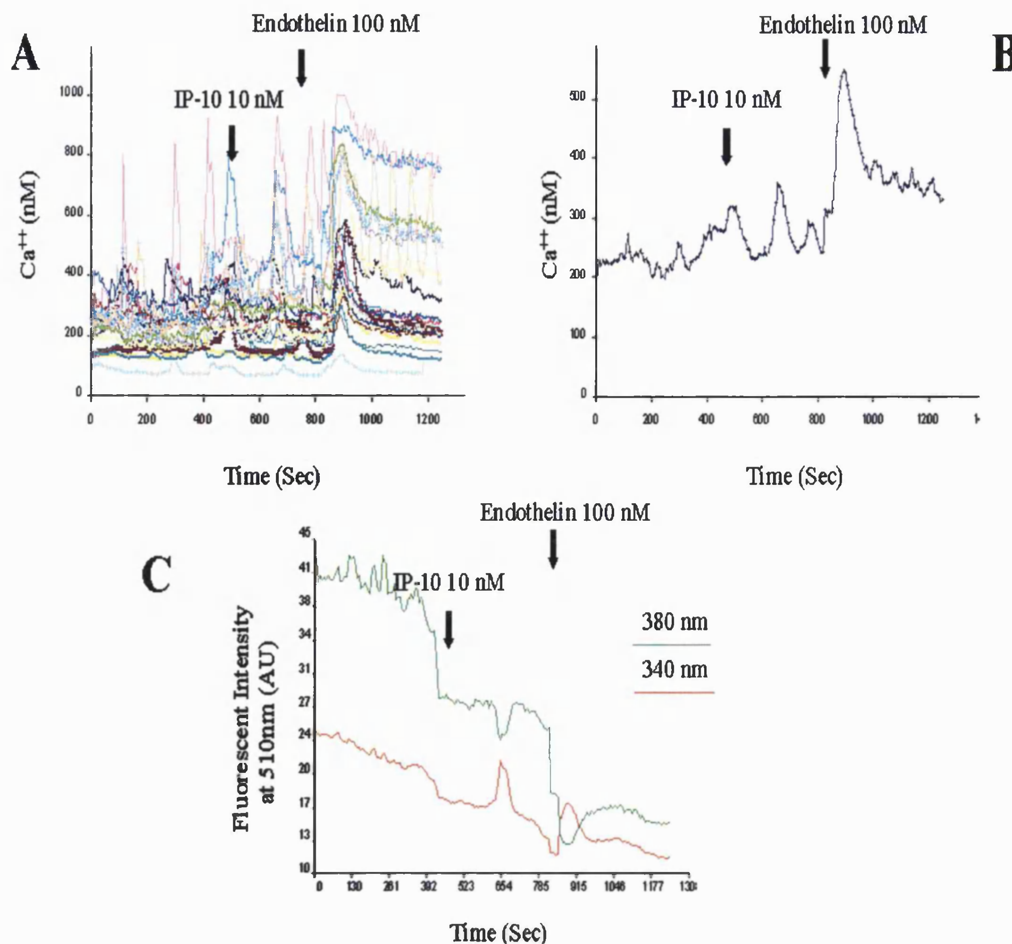

Figure 4.3 Effect of IP-10 on calcium mobilization in human primary intestinal myofibroblasts.

0.2 x 10^6 primary human intestinal myofibroblasts were plated on coverslips in 35mm plates and cultured for 48 hours. Prior to experimentation, cells were washed and kept in physiological salt solution (PSS) and loaded with Fura-2 as described in Methods and Materials. Cells were stimulated with IP-10 (10 nM) and then endothellin-1 (100 nM) whilst alternately excited with light at 340 and 380 nm. Fura –2 fluoresence emission intensity was measured at 510nm and transformed to intracellular-free calcium concentrations, based on calibration of the cells with 30 μM ionomycin and 10 mM EGTA, for individual cells (A) or for the average of the cells in view (B). A representative graph of emission intensity at 340 nm and 380 is presented for a single cell(C). Results are from single experiments representative of 3 replicate experiments.

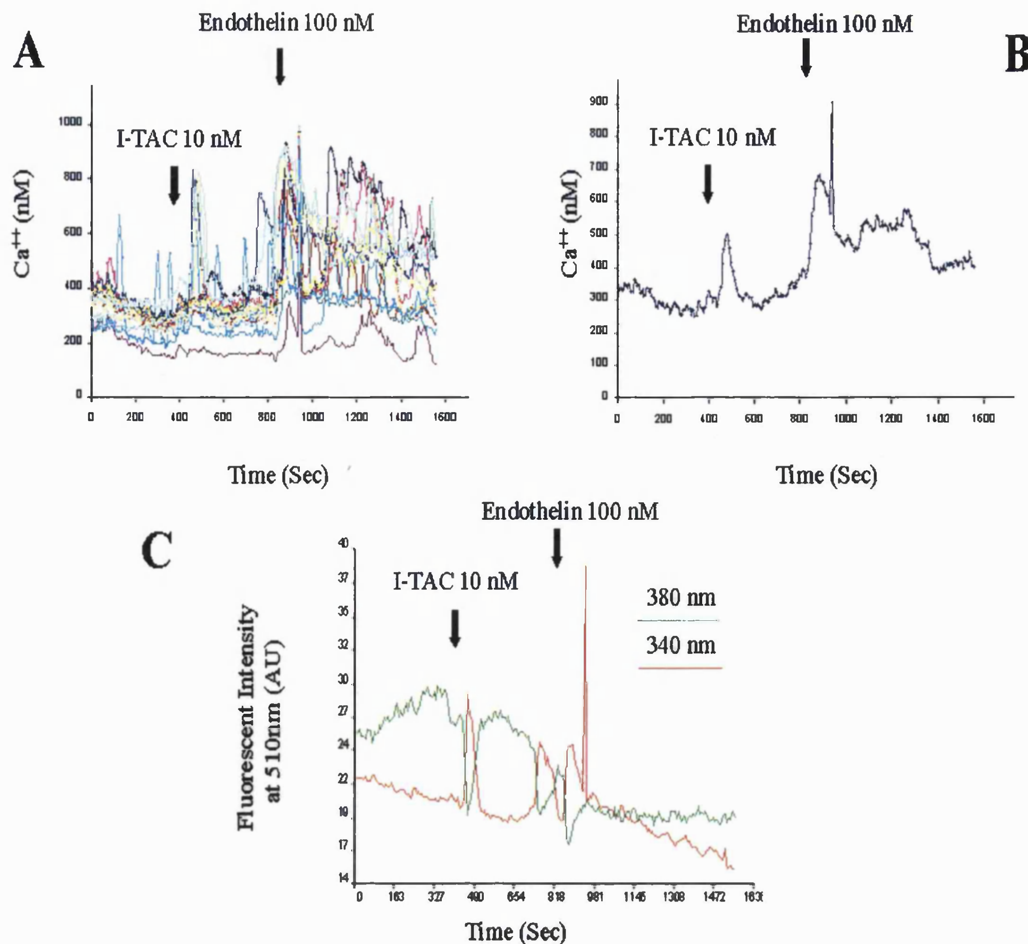

Figure 4.4 Effect of I-TAC on calcium mobilization in human primary intestinal myofibroblasts.

0.2 x 10^6 primary human intestinal myofibroblasts were plated on coverslips in 35mm plates and cultured for 48 hours. Prior to experimentation, cells were washed and kept in physiological salt solution (PSS) and loaded with Fura-2 as described in Methods and Materials. Cells were stimulated with I-TAC (10 nM) and then endothellin-1 (100 nM) whilst alternately excited with light at 340 and 380 nm. Fura –2 fluoresence emission intensity was measured at 510nm and transformed to intracellular-free calcium concentrations based on calibration of the cells with 30 μM ionomycin and 10 mM EGTA, for individual cells (A) or for the average of the cells in view (B). A representative graph of emission intensity at 340 nm and 380 is presented for a single cell(C). Results are from single experiments representative of 3 replicate experiments.

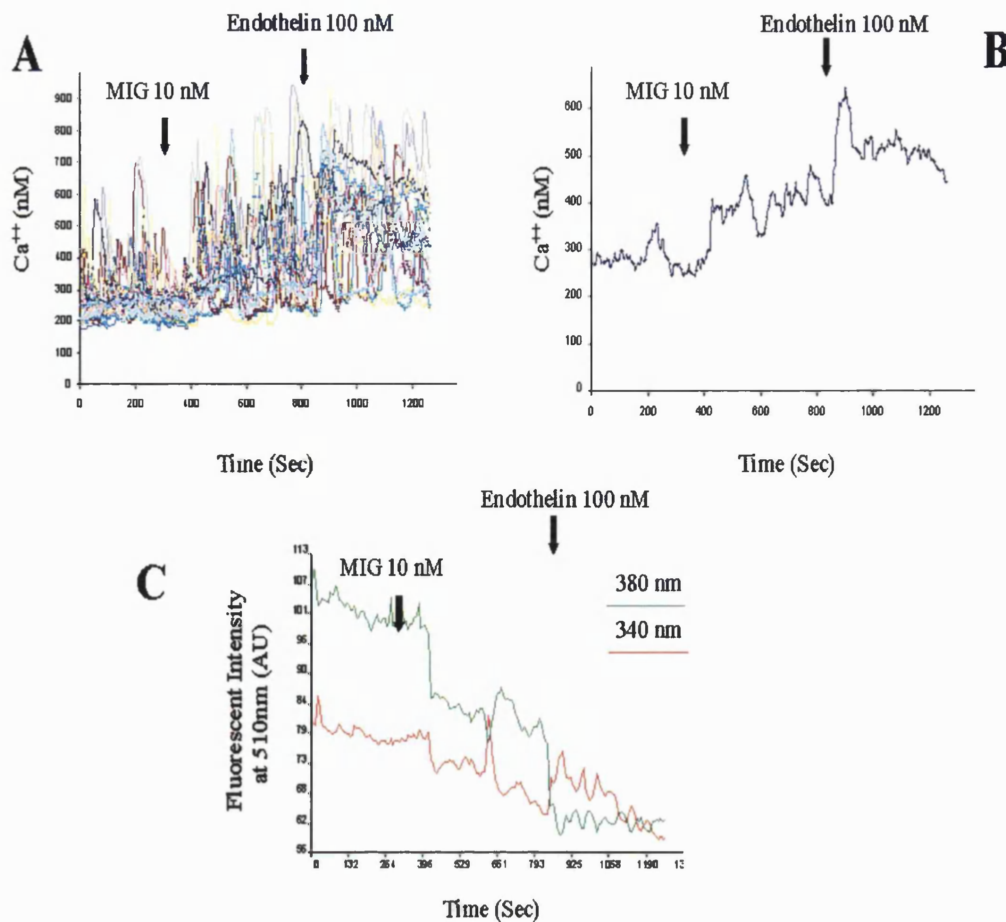

Figure 4.5 Effect of Mig on calcium mobilization in human primary intestinal myofibroblasts.

0.2 x 10^6 primary human intestinal myofibroblasts were plated on coverslips in 35mm plates and cultured for 48 hours. Prior to experimentation, cells were washed and kept in physiological salt solution (PSS) and loaded with Fura-2 as described in Methods and Materials. Cells were stimulated with Mig (10 nM) and then endothellin-1 (100 nM) whilst alternately excited with light at 340 and 380 nm. Fura –2 fluoresence emission intensity was measured at 510nm and transformed to intracellular-free calcium concentrations, based on calibration of the cells with 30 µM ionomycin and 10 mM EGTA, for individual cells (A) or for the average of the cells in view (B). A representative graph of emission intensity at 340 nm and 380 is presented for a single cell(C). Results are from single experiments representative of 3 replicate experiments.

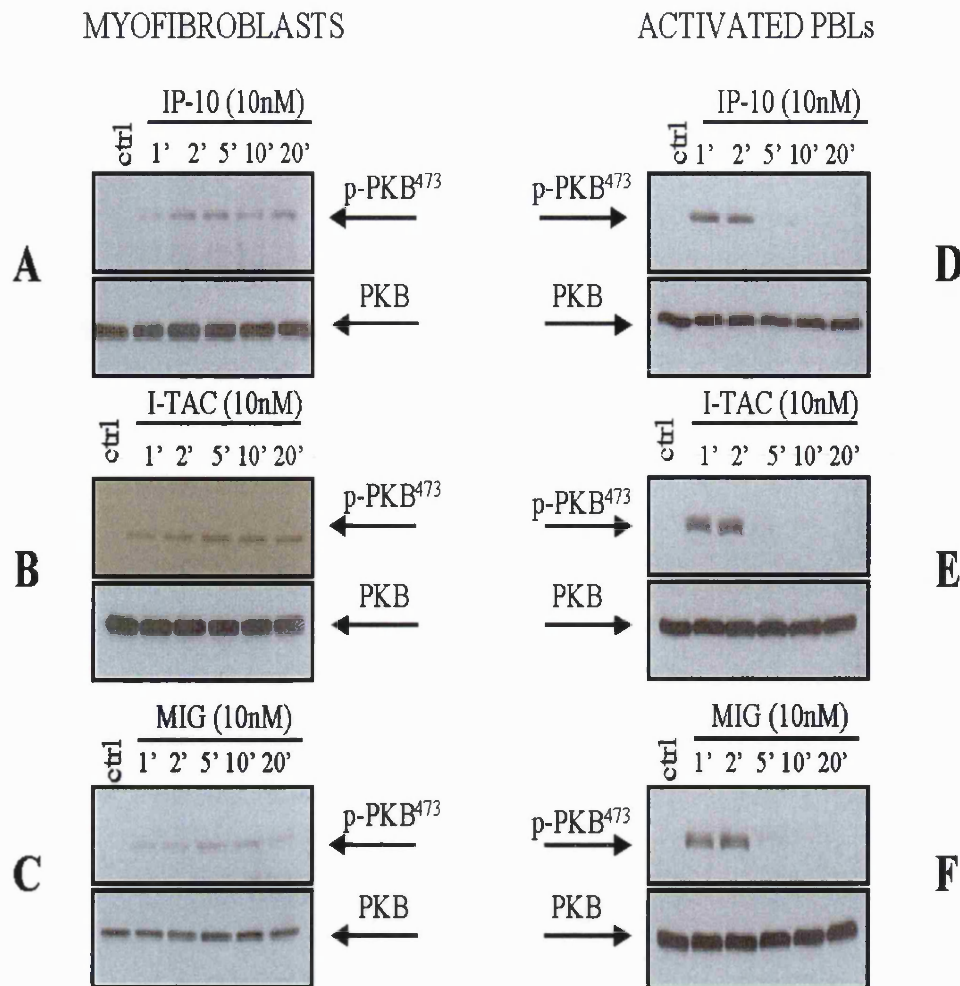

Figure 4.6 Effect of CXCR3 ligands in PKB^{473} phosphorylation in human primary intestinal myofibroblasts and peripheral blood derived activated T-cells.

1 x 10^6 intestinal myofibroblasts (left panels) or 1 x 10^6 12 days old SEB activated peripheral blood derived T cells (right panels) were lysed in 50 μl lysis buffer (ctrl) or stimulated with IP-10 (A, D) or I-TAC (B,E) or Mig (C, F all at 10 nM) for indicated time points, lysed in lysis buffer as described in Methods and Materials and then probed with a specific rabbit polyclonal antibody against $phospho^{473}$ PKB diluted 1:1000 from stock (upper panels). Membranes were then stripped and re-probed with an antibody against the non-phosphorylated protein to show equal loading (lower panels). Blots are from single experiments but are representative of at least two others.

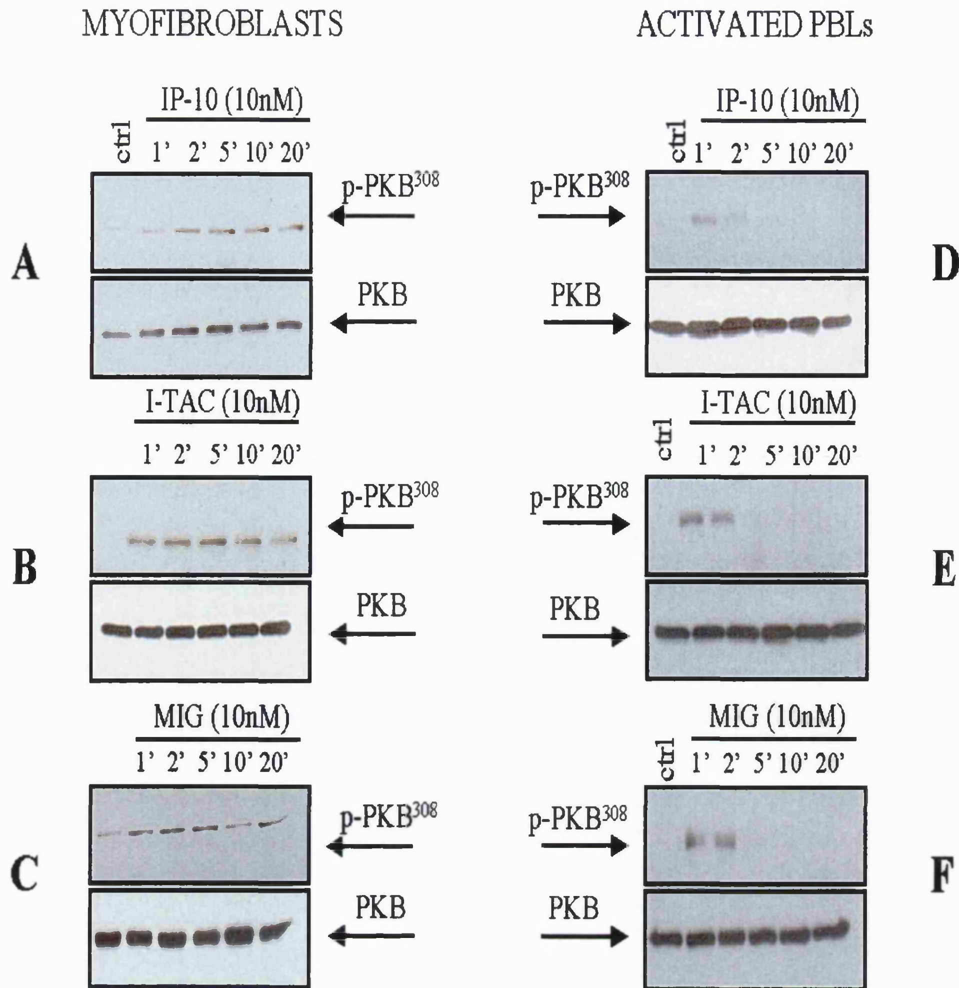

Figure 4.7 Effect of CXCR3 ligands in PKB^{308} phosphorylation in human primary intestinal myofibroblasts and peripheral blood derived activated T cells.

1 x 10^6 intestinal myofibroblasts (left panels) or 1 x 10^6 12 days old SEB activated peripheral blood derived T cells (right panels) were lysed in 50 μl lysis buffer (ctrl) or stimulated with IP-10 (A, D) or I-TAC (B,E) or Mig (C, F all at 10 nM) for indicated time points, lysed in lysis buffer as described in Methods and Materials and then probed with a specific rabbit polyclonal antibody against phospho^{308} PKB diluted 1:1000 from stock (upper panels). Membranes were then stripped and re-probed with an antibody against the non-phosphorylated protein to show equal loading (lower panels). Blots are from single experiments but are representative of at least two others.

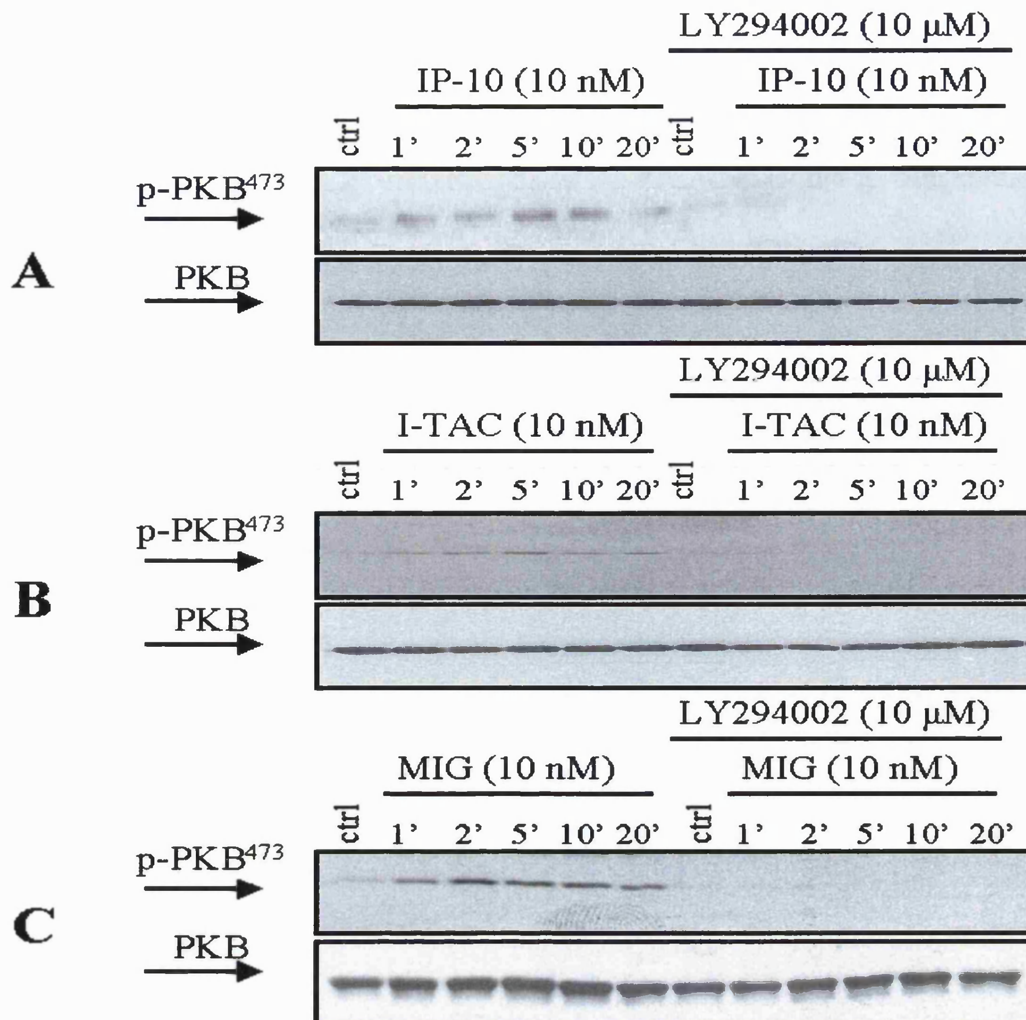

Figure 4.8 Phosphorylation of PKB in primary intestinal myofibroblasts is abrogated by PI3K inhibition.

1 x 10^6 intestinal myofibroblasts were lysed in 50 µl lysis buffer (ctrl) or stimulated with IP-10 (A) or I-TAC (B) or Mig (C, all at 10 nM) in the presence or absence of pre-treatment with the PI3K inhibitor LY294002 (10µM for 30 minutes) for indicated time points, lysed in lysis buffer as described in Methods and Materials and then probed with a specific rabbit polyclonal antibody against phospho^{473} PKB diluted 1:1000 from stock (upper panels). Membranes were then stripped and re-probed with an antibody against the non-phosphorylated protein to show equal loading (lower panels). Blots are from single experiments but are representative of at least two others.

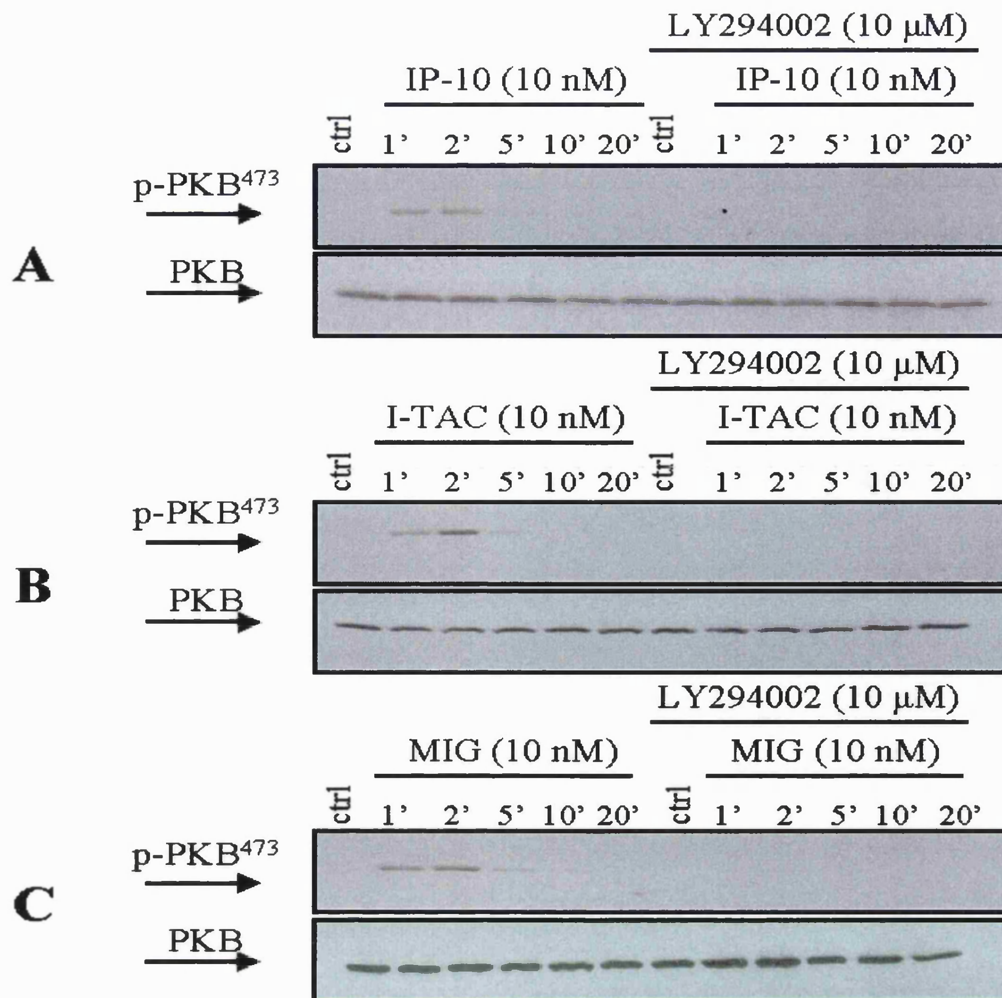

Figure 4.9 Phosphorylation of PKB in peripheral blood derived activated T cells is abrogated by PI3K inhibition.

1 x 10^6 12 days old SEB activated peripheral blood derived T cells were lysed in 50 μl lysis buffer (ctrl) or stimulated with IP-10 (A) or I-TAC (B) or Mig (C, all at 10 nM) in the presence or absence of pre-treatment with the PI3K inhibitor LY294002 (10μM for 30 minutes) for indicated time points, lysed in lysis buffer as described in Methods and Materials and then probed with a specific rabbit polyclonal antibody against $phospho^{473}$ PKB diluted 1:1000 from stock (upper panels). Membranes were then stripped and re-probed with an antibody against the non-phosphorylated protein to show equal loading (lower panels). Blots are from single experiments but are representative of at two experiments.

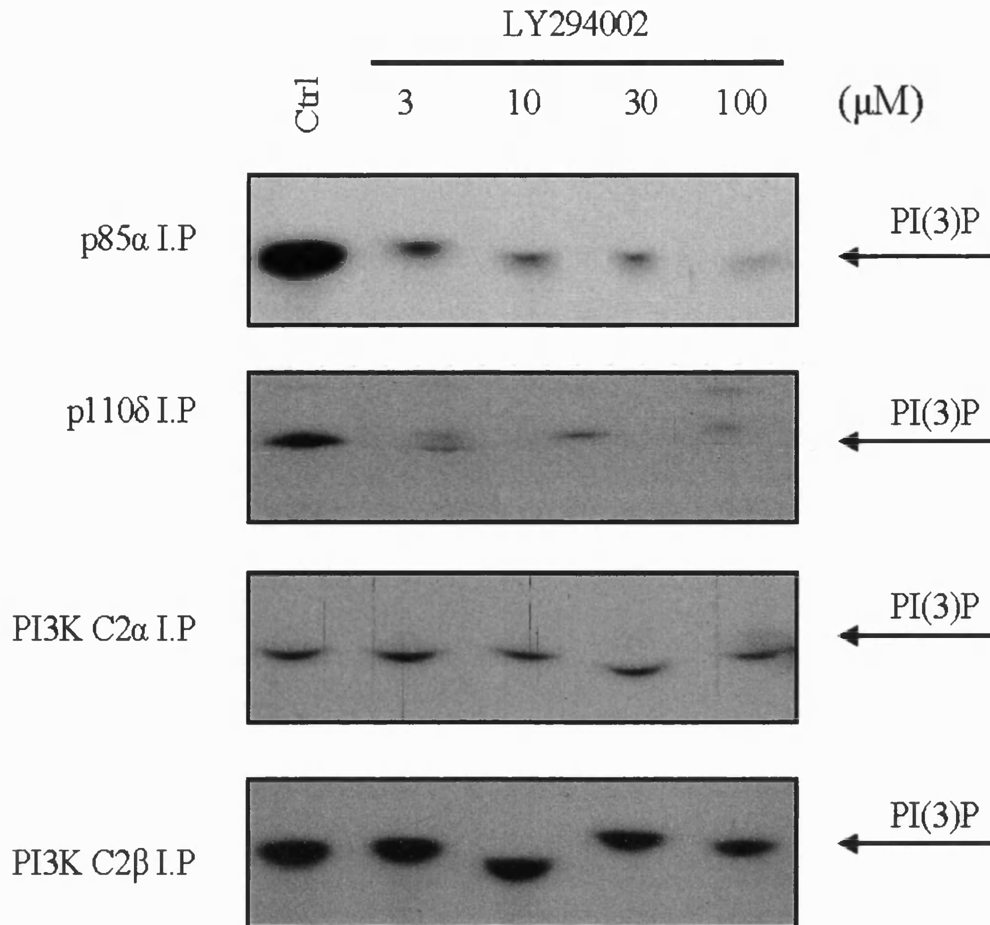

Figure 4.10 Sensitivity of various PI3K isoforms to the PI3K inhibitor LY294002.

1 x 10^7 Jurkat cells were lysed in 500 μl lysis buffer and then immunoprecipitated with an antibody against p85α (A), p110δ (B), PI3K C2α (C) or PI3K C2β (D). Lysates were then incubated with a vehicle control (ctrl) or with indicated concentrations of the PI3K inhibitor LY294002. An *in vitro* lipid kinase assay was then performed on the lysates as described in Methods and Materials, using PtdIns as a substrate. Results are from single experiments but are representative of three experiments.

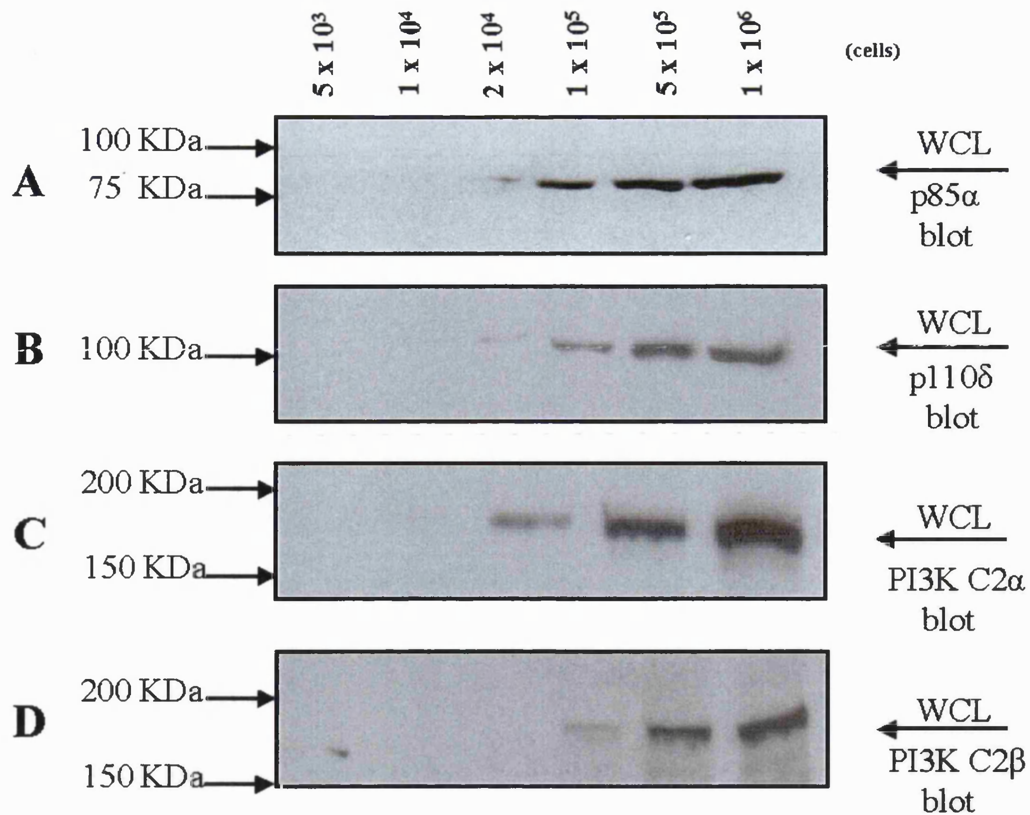

Figure 4.11 Western blot analysis of primary human intestinal myofibroblasts for various PI3K isoforms.

Primary human intestinal myofibroblasts were lysed in lysis buffer at indicated numbers as described in Methods and Materials and then probed with specific rabbit polyclonal antibodies against p85α (A), p110δ (B), PI3K-C2α (C) and PI3K-C2β (D). All antibodies were used at a final concentration of 0.1 μg/ml. Results are from single experiments representative of 2 replicate experiments.

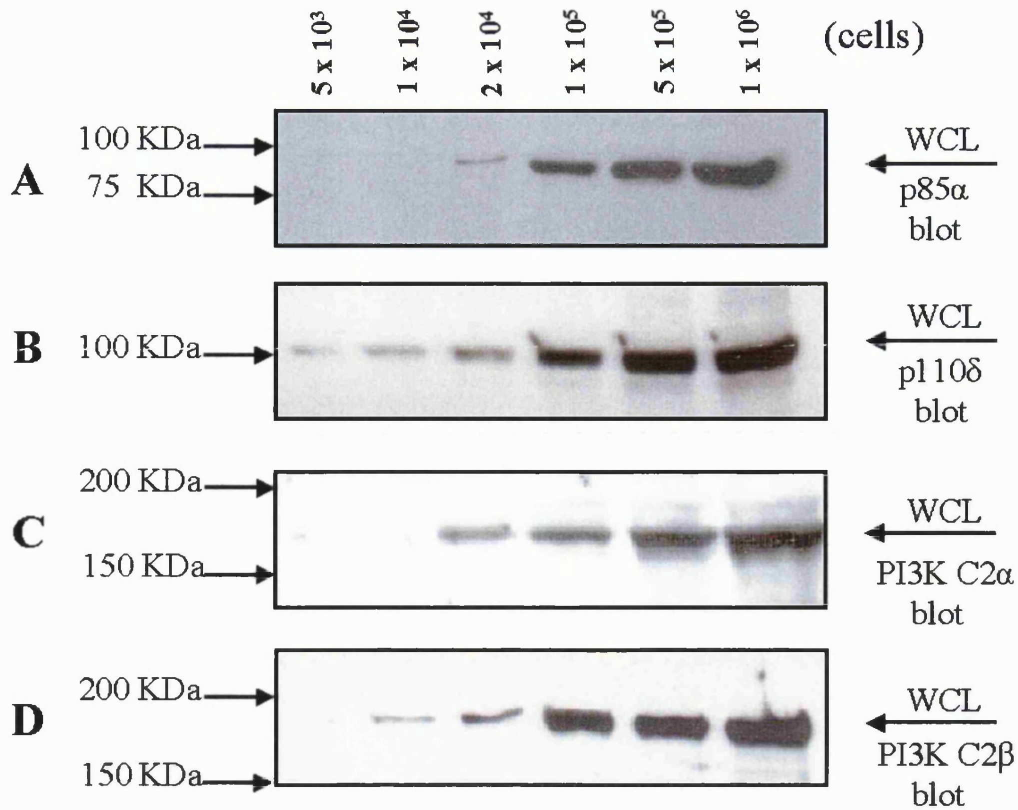

Figure 4.12 Western blot analysis of peripheral blood derived activated T-cells for various PI3K isoforms.

12 days old SEB activated peripheral blood derived T cells were lysed in lysis buffer at indicated numbers as described in Methods and Materials and then probed with specific rabbit polyclonal antibodies against p85α (A), p110δ (B), PI3K C2α (C) and PI3K C2β (D). All antibodies were used at a final concentration of 0.1 μg/ml. Results are from single experiments representative of 2 replicate experiments.

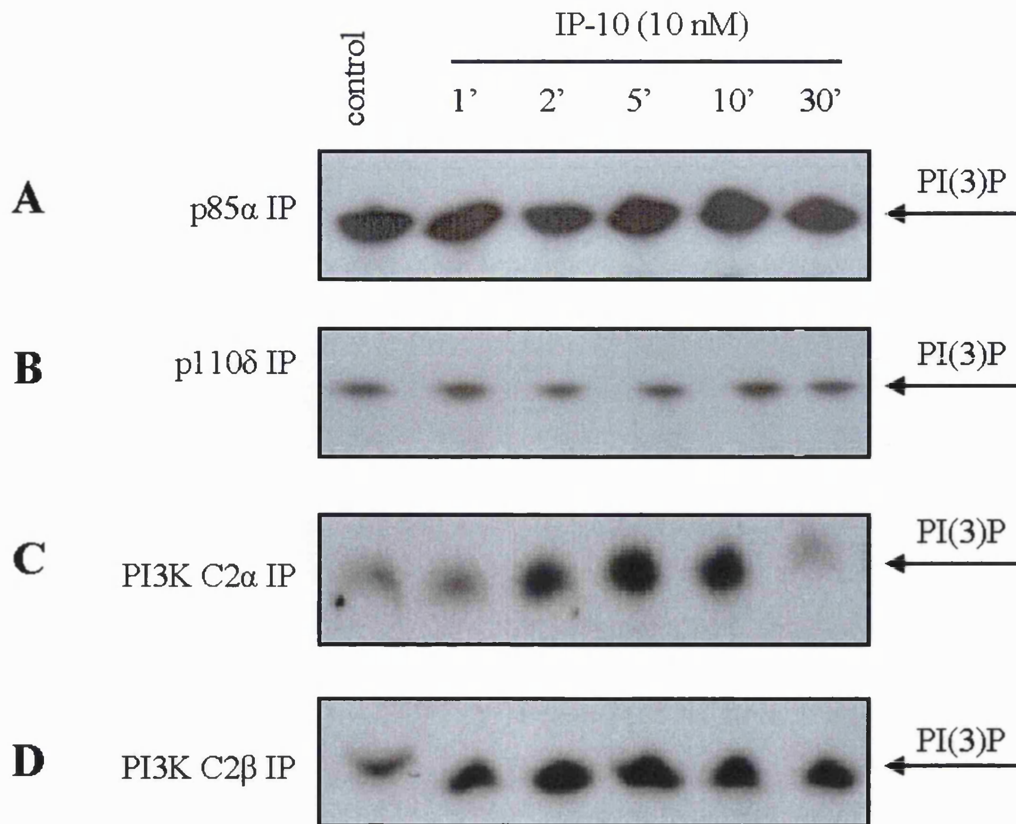

Figure 4.13 IP-10 activates class II but not class I PI3K isoforms in primary human intestinal myofibroblasts.

1 x 10^7 intestinal myofibroblasts were lysed in 500 μl lysis buffer (ctrl) or stimulated with IP-10 (10 nM) for indicated time points, lysed in lysis buffer and then immunoprecipitated with an antibody against p85α (A), p110δ (B), PI3K C2α (C) or PI3K C2β (D). An *in vitro* lipid kinase assay was then performed on the lysates as described in Methods and Materials. Results are from single experiments but are representative of two experiments.

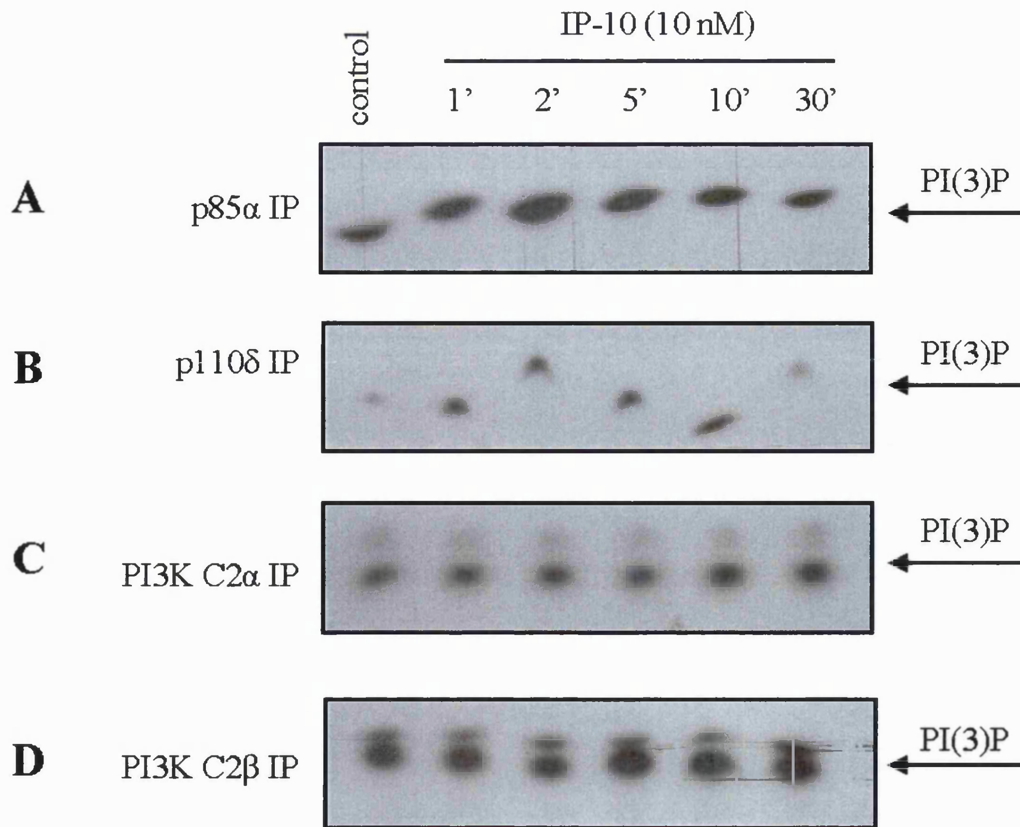

Figure 4.14 IP-10 activates class I but not class II PI3K isoforms in peripheral blood derived activated T-cells.

1 x 10^7 12 days old SEB activated peripheral blood derived T-cells were lysed in 500 μl lysis buffer (ctrl) or stimulated with IP-10 (10 nM) for indicated time points, lysed in lysis buffer and then immunoprecipitated with an antibody against p85α (A), p110δ (B), PI3K C2α (C) or PI3K C2β (D). An *in vitro* lipid kinase assay was then performed on the lysates as described in Methods and Materials. Results are from single experiments but are representative of two experiments.

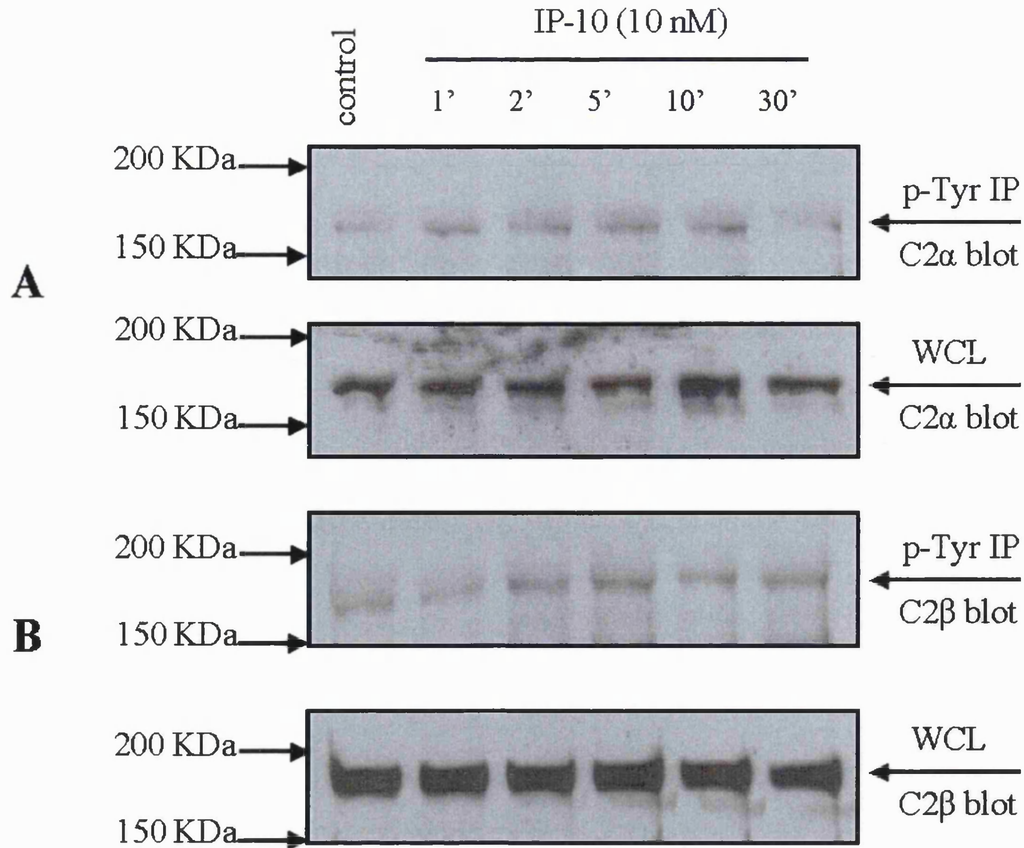

Figure 4.15 PI3K-C2α and PI3K-C2β are tyrosine phosphorylated in intestinal myofibroblasts upon chemokine ligation.

1 x 10^7 intestinal myofibroblasts were lysed in 500 μl lysis buffer (ctrl) or stimulated with IP-10 (10 nM) for indicated time points, lysed in lysis buffer, immunoprecipitated with the murine anti-phospho tyr monoclonal antibody 4G10, and then probed with specific rabbit polyclonal antibodies against PI3K-C2α (A) or PI3K-C2β (B). Both antibodies were used at a final concentration of 0.1 μg/ml. Whole cell lysates from each sample were also probed for isoform expression to verify equal loading (lower panels). Blots are from single experiments but are representative of two experiments

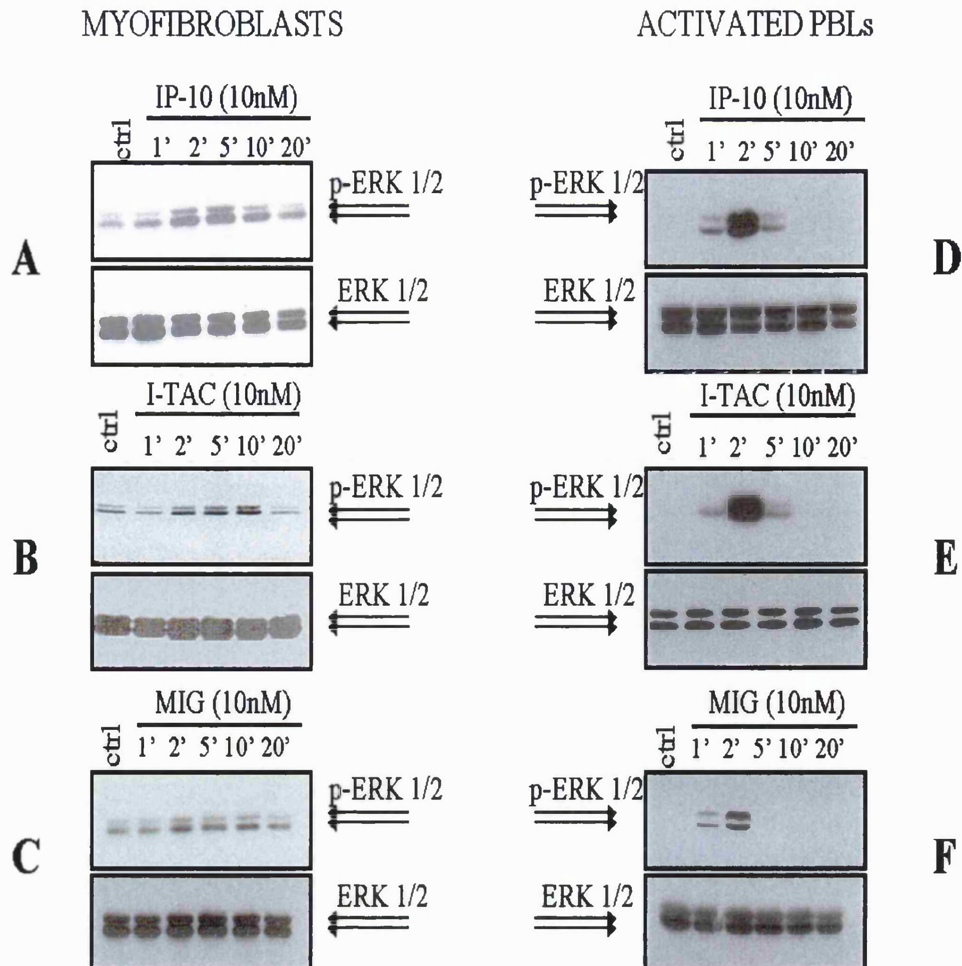

Figure 4.16 Effect of CXCR3 ligands on ERK1/2phosphorylation in human primary intestinal myofibroblasts and peripheral blood derived activated T-cells.

1 x 10^6 intestinal myofibroblasts (left panels) or 1 x 10^6 12 days old SEB activated peripheral blood derived T-cells (right panels) were lysed in 50 μl lysis buffer (ctrl) or stimulated with IP-10 (A, D) or I-TAC (B, E) or Mig (C, F all at 10 nM) for indicated time points, lysed in lysis buffer as described in Methods and Materials and then probed with a specific rabbit polyclonal antibody against phospho$^{202/204}$ ERK diluted 1:1000 from stock (upper panels). Membranes were then stripped and re-probed with an antibody against the non-phosphorylated protein to show equal loading (lower panels). Blots are from single experiments but are representative of at least two others.

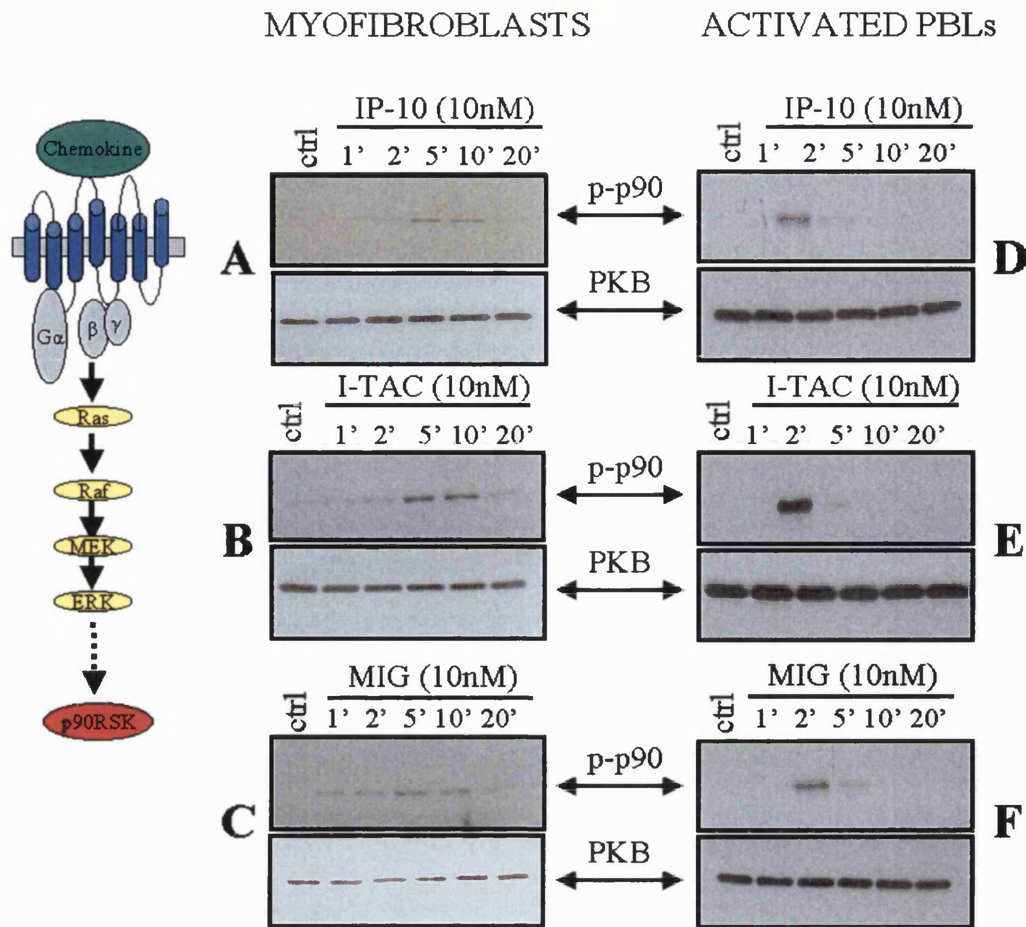

Figure 4.17 Effect of CXCR3 ligands on p90RSK phosphorylation in human primary intestinal myofibroblasts and peripheral blood derived activated T cells.

1 x 10^6 intestinal myofibroblasts (left panels) or 1 x 10^6 12 days old SEB activated peripheral blood derived T cells (right panels) were lysed in 50 µl lysis buffer (ctrl) or stimulated with IP-10 (A, D) or I-TAC (B, E) or Mig (C, F all at 10 nM) for indicated time points, lysed in lysis buffer as described in Methods and Materials and then probed with a specific rabbit polyclonal antibody against phospho^{380} p90RSK diluted 1:1000 from stock (upper panels). Membranes were then stripped and re-probed with an antibody against a non-phosphorylated protein to show equal loading (lower panels). Blots are from single experiments but are representative of at least two others. A diagram of the ERK pathway is included on the left, to indicate which part of the cascade is monitored with this experiment.

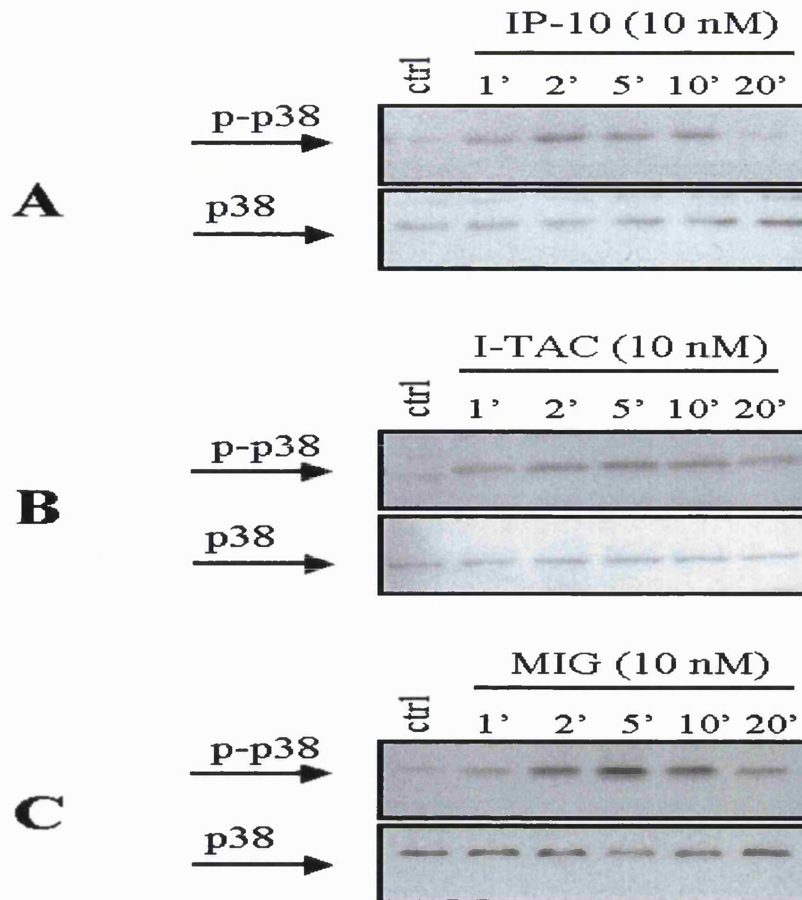

Figure 4.18 Effect of CXCR3 ligands on p38 phosphorylation in human primary intestinal myofibroblasts.

1 x 10^6 intestinal myofibroblasts were lysed in 50 μl lysis buffer (ctrl) or stimulated with IP-10 (A) or I-TAC (B) or Mig (C, all at 10 nM) for indicated time points and then lysed in lysis buffer as described in Methods and Materials and then probed with a specific rabbit polyclonal antibody against phospho$^{180/182}$p38 diluted 1:1000 from stock (upper panels). Membranes were then stripped and re-probed with an antibody against the non-phosphorylated protein to show equal loading (lower panels). Blots are from single experiments but are representative of at least two others.

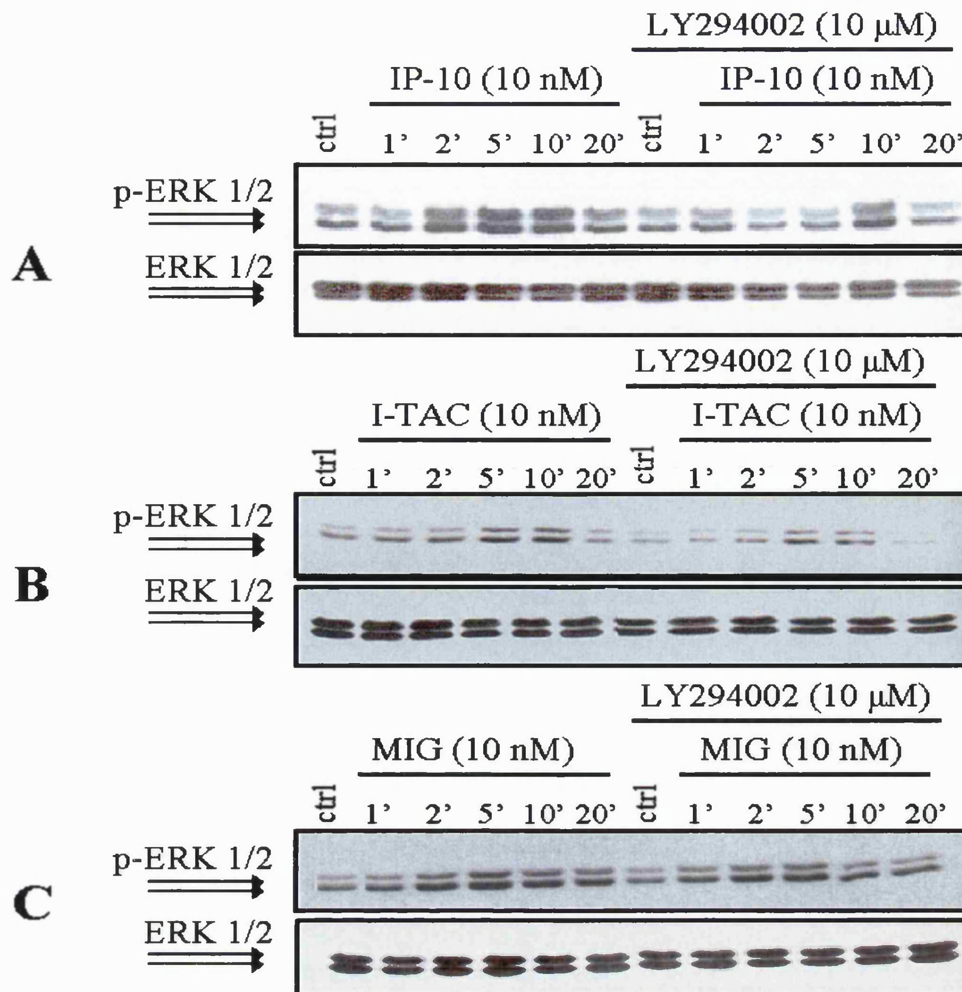

Figure 4.19 Phosphorylation of ERK 1/2 in human primary intestinal myofibroblasts is moderately affected by PI3K inhibition.

1 x 10^6 intestinal myofibroblasts were lysed in 50 μl lysis buffer (ctrl) or stimulated with IP-10 (A) or I-TAC (B) or Mig (C, all at 10 nM) in the presence or absence of pre-treatment with the PI3K inhibitor LY294002 (10μM for 30 minutes) for indicated time points, lysed in lysis buffer as described in Methods and Materials and then probed with a specific rabbit polyclonal antibody against phospho$^{202/204}$ ERK 1/2 diluted 1:1000 from stock (upper panels). Membranes were then stripped and re-probed with an antibody against the non-phosphorylated protein to show equal loading (lower panels). Blots are from single experiments but are representative of at least two others.

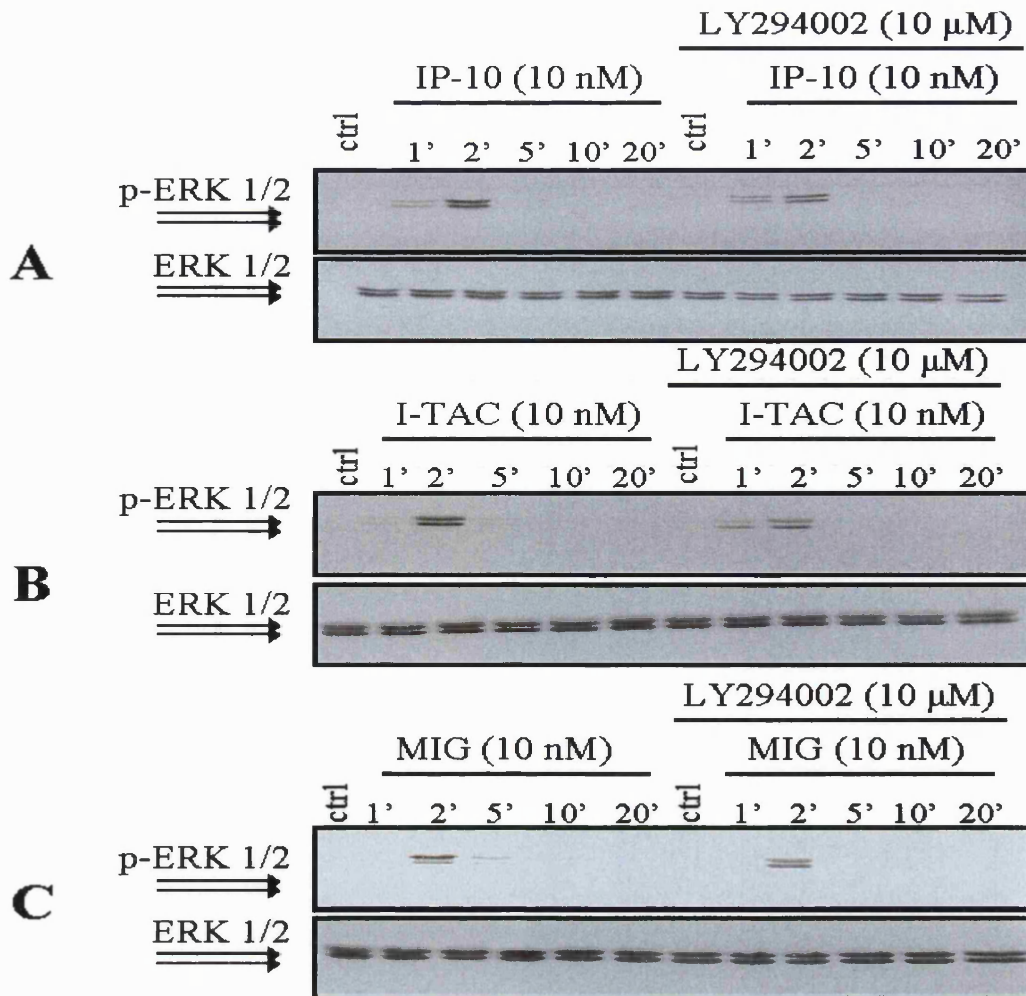

Figure 4.20 Phosphorylation of ERK 1/2 in peripheral blood derived activated T-cells is abrogated by PI3K inhibition.

1 x 10^6 12 days old SEB activated peripheral blood derived T-cells were lysed in 50 μl lysis buffer (ctrl) or stimulated with IP-10 (A) or I-TAC (B) or Mig (C, all at 10 nM) in the presence or absence of pre-treatment with the PI3K inhibitor LY294002 (10μM for 30 minutes) for indicated time points, lysed in lysis buffer as described in Methods and Materials and then probed with a specific rabbit polyclonal antibody against phospho$^{202/204}$ ERK diluted 1:1000 from stock (upper panels). Membranes were then stripped and re-probed with an antibody against the non-phosphorylated protein to show equal loading (lower panels). Blots are from single experiments but are representative of at two experiments.

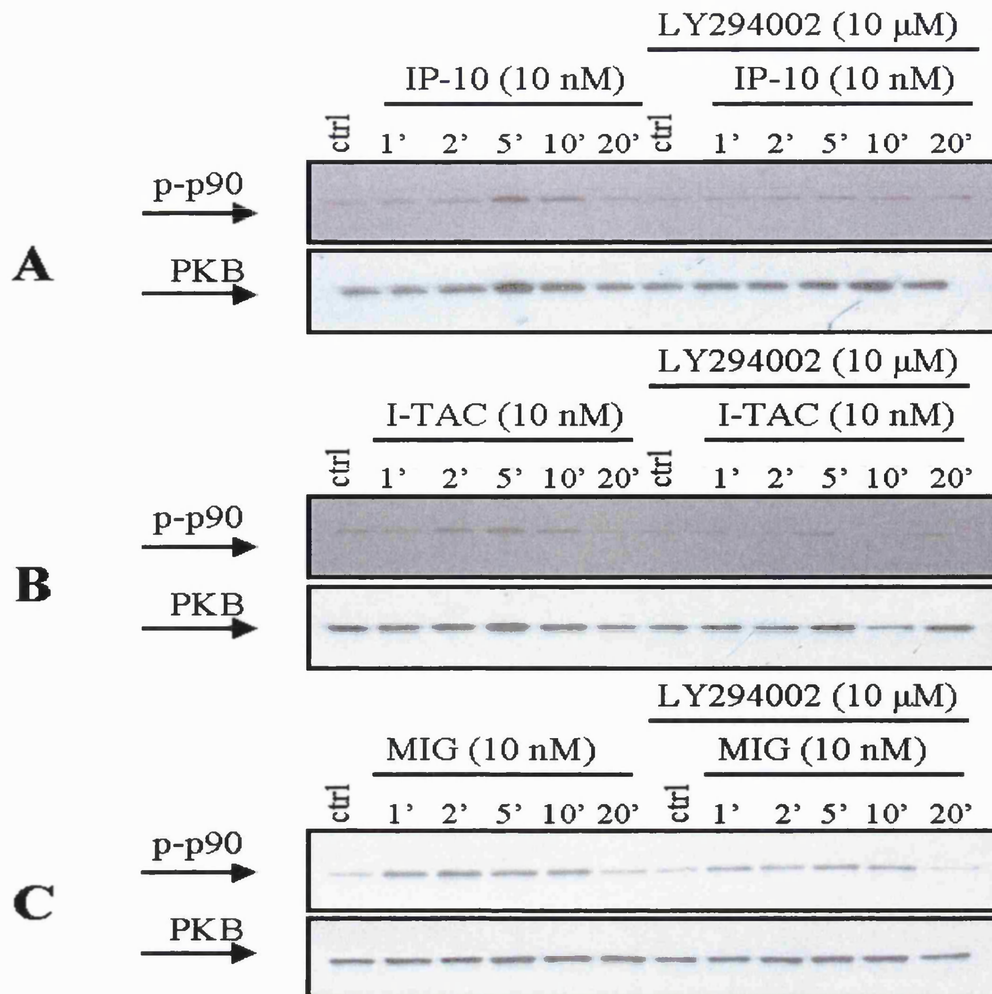

Figure 4.21 Phosphorylation of p90RSK in human primary intestinal myofibroblasts is moderately affected by PI3K inhibition.

1 x 10^6 intestinal myofibroblasts were lysed in 50 μl lysis buffer (ctrl) or stimulated with IP-10 (A) or I-TAC (B) or Mig (C, all at 10 nM) in the presence or absence of pre-treatment with the PI3K inhibitor LY294002 (10μM for 30 minutes) for indicated time points, lysed in lysis buffer as described in Methods and Materials and then probed with a specific rabbit polyclonal antibody against phospho^{380} p90RSK diluted 1:1000 from stock (upper panels). Membranes were then stripped and re-probed with an antibody against a non-phosphorylated protein to show equal loading (lower panels). Blots are from single experiments but are representative of at least two others.

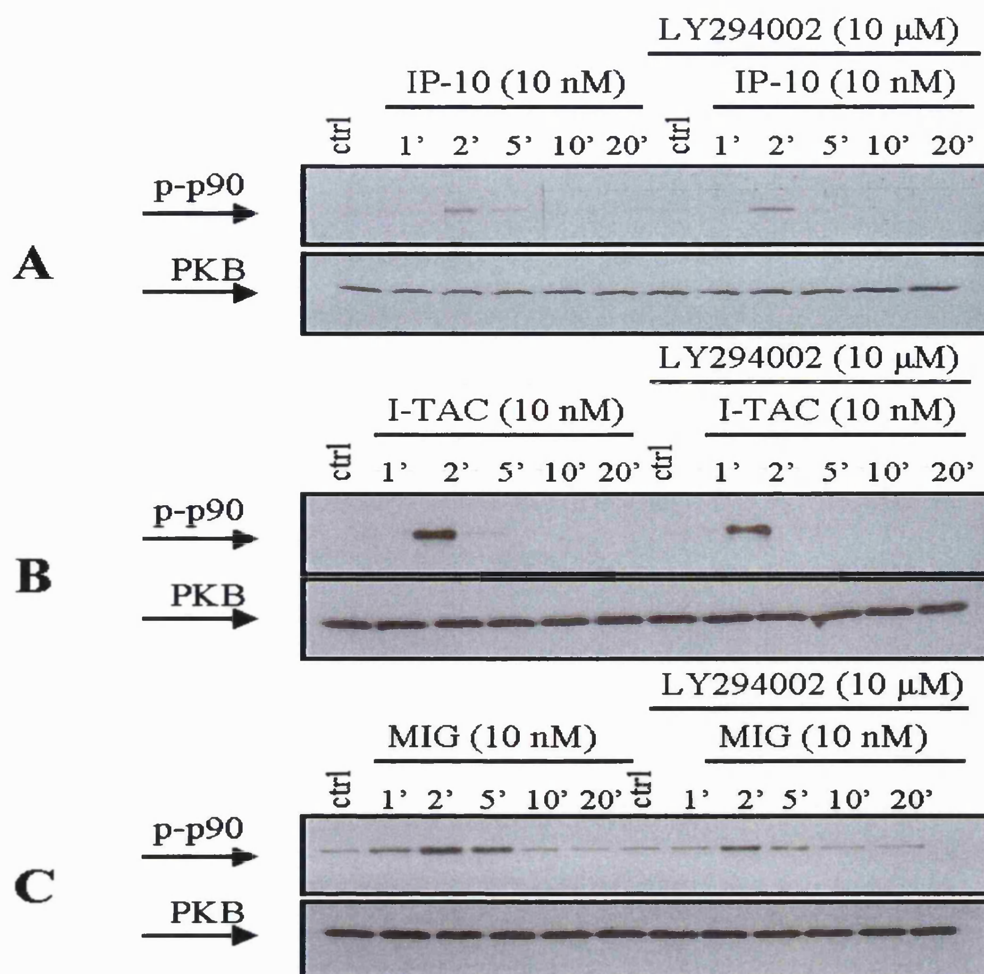

Figure 4.22 Phosphorylation of p90RSK in peripheral blood derived activated T-cells is abrogated by PI3K inhibition.

1 x 10^6 12 days old SEB activated peripheral blood derived T-cells were lysed in 50 μl lysis buffer (ctrl) or stimulated with IP-10 (A) or I-TAC (B) or Mig (C, all at 10 nM) in the presence or absence of pre-treatment with the PI3K inhibitor LY294002 (10μM for 30 minutes) for indicated time points, lysed in lysis buffer as described in Methods and Materials and then probed with a specific rabbit polyclonal antibody against phospho^{380} p90RSK diluted 1:1000 from stock (upper panels). Membranes were then stripped and re-probed with an antibody against a non-phosphorylated protein to show equal loading (lower panels). Blots are from single experiments but are representative of at two experiments.

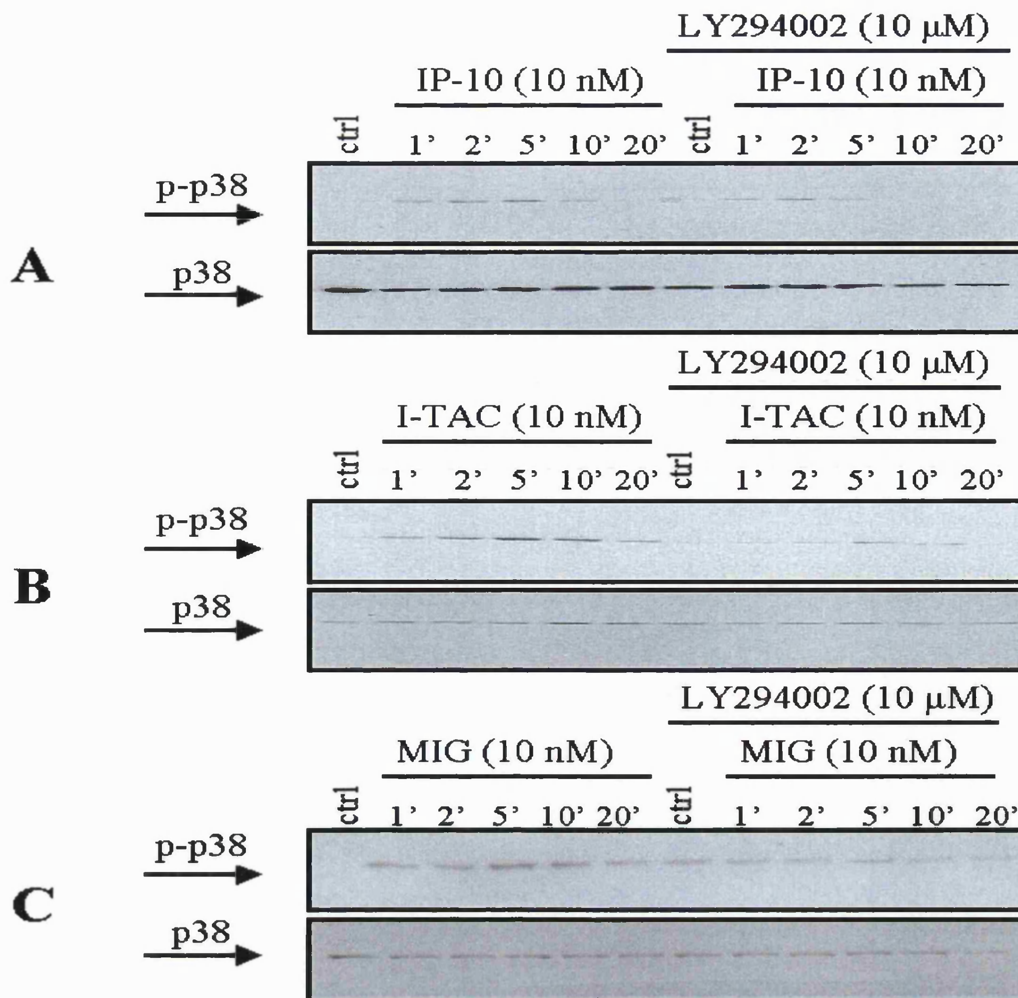

Figure 4.23 Activation of p38 in human primary intestinal myofibroblasts is moderately affected by PI3K inhibition.

1 x 10^6 intestinal myofibroblasts were lysed in 50 μl lysis buffer (ctrl) or stimulated with IP-10 (A) or I-TAC (B) or Mig (C, all at 10 nM) in the presence or absence of pre-treatment with the PI3K inhibitor LY294002 (10μM for 30 minutes) for indicated time points, lysed in lysis buffer as described in Methods and Materials and then probed with a specific rabbit polyclonal antibody against phospho$^{180/182}$ p38 diluted 1:1000 from stock (upper panels). Membranes were then stripped and re-probed with an antibody against a non-phosphorylated protein to show equal loading (lower panels). Blots are from single experiments but are representative of at least two others.

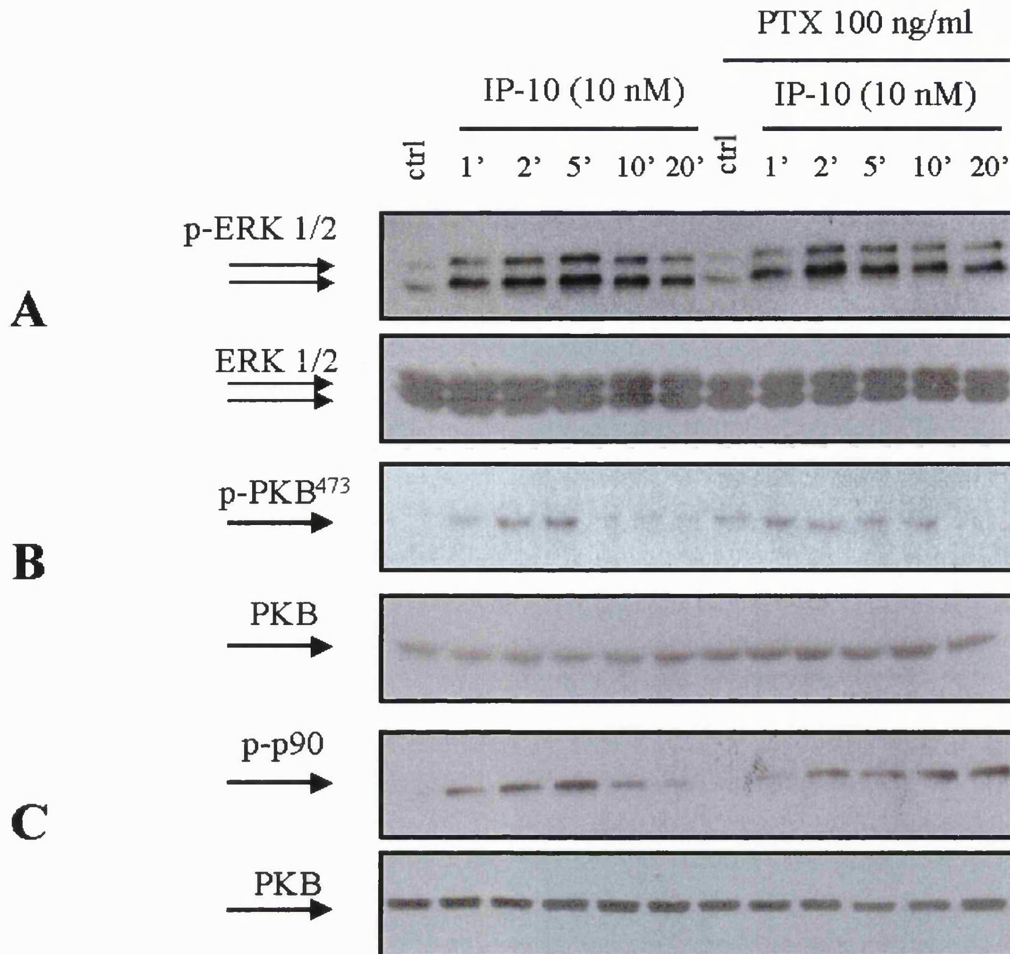

Figure 4.24 IP-10 signalling in primary human intestinal myofibroblasts is not affected by pertussis toxin.

0.2 x 10^6 primary intestinal myofibroblasts were plated on coverslips in 35mm plates, cultured till confluence and then starved for 24 hours. Cells were then lysed in 150 μl lysis buffer (ctrl) or stimulated with IP-10 in the presence or absence of pre-treatment with the Gai inhibitor pertussis toxin (100 ng/ml for 16 hours) for indicated time points and then lysed in lysis buffer as described in Methods and Materials and then probed with specific antibodies against phospho $^{202/204}$ ERK (A) or phospho ^{473} PKB (B) or phospho ^{380} p90RSK (C). Membranes were then stripped and re-probed with an antibody against a non-phosphorylated protein to show equal loading (lower panels). Blots are from single experiments but are representative of at least two others.

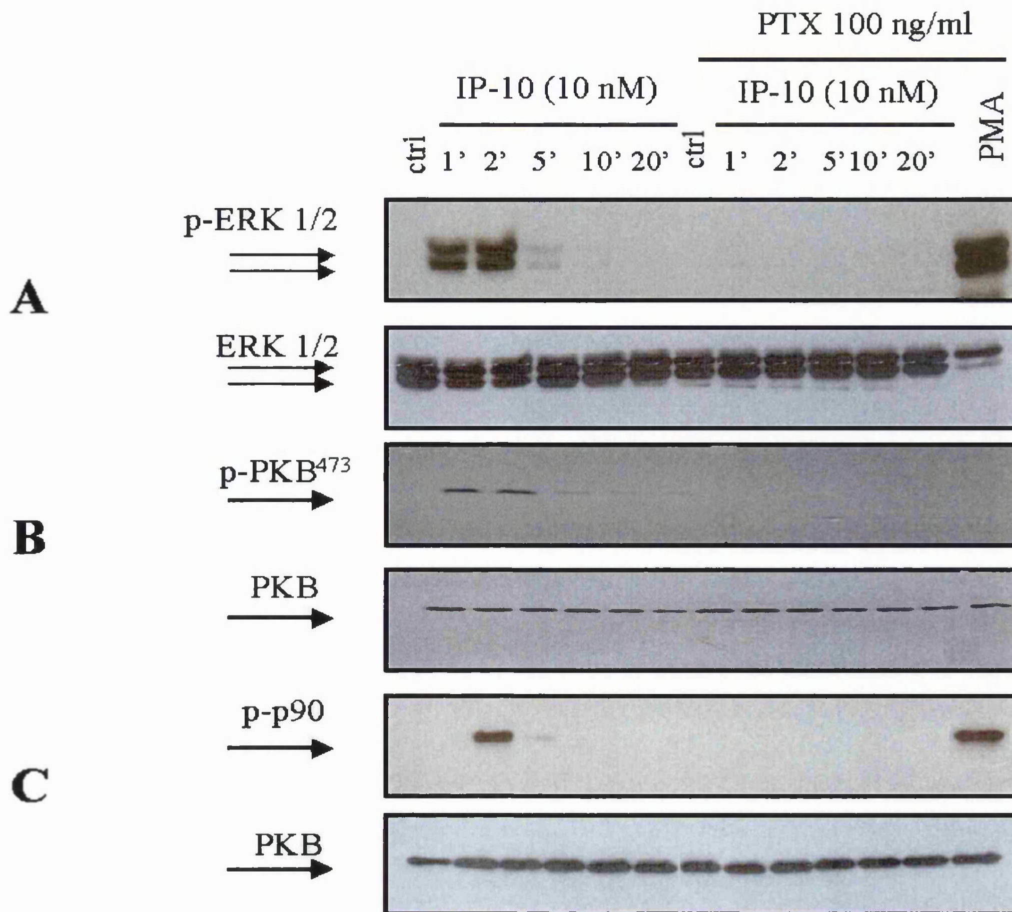

Figure 4.25 IP-10 signalling in peripheral blood derived activated T-cells is abrogated by pertussis toxin.

1 x 10^6 12 days old SEB activated peripheral blood derived T-cells were lysed in 50 μl lysis buffer (ctrl) or stimulated with IP-10 all at 10 nM) in the presence or absence of pre-treatment with the Ga_i inhibitor pertussis toxin (100 ng/ml for 16 hours) for indicated time points, lysed in lysis buffer as described in Methods and Materials and then probed with specific rabbit polyclonal antibodies against phospho $^{202/204}$ ERK (A) or phospho ^{473} PKB (B) or phospho ^{380} p90RSK (C). Membranes were then stripped and re-probed with an antibody against the non-phosphorylated protein to show equal loading (lower panels). Blots are from single experiments but are representative of two experiments.

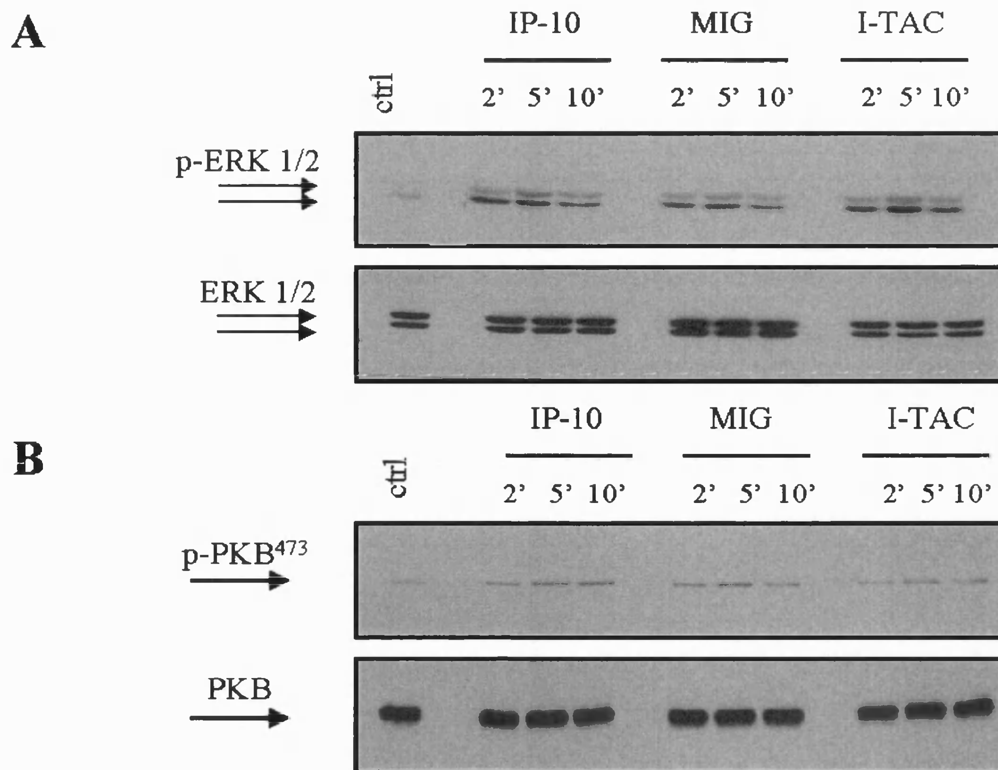

Figure 4.26 Comparison of the 3 CXC3 ligands in human primary intestinal myofibroblasts signalling.

1 x 10^6 intestinal myofibroblasts were lysed in 50 μl lysis buffer (ctrl) or stimulated with IP-10 or I-TAC or Mig (all at 10 nM) for indicated time points, lysed in lysis buffer as described in Methods and Materials and then probed with specific rabbit polyclonal antibodies against phospho$^{202/204}$ ERK (A) or.phosphp^{473} PKB (B). Membranes were then stripped and re-probed with antibodies against non-phosphorylated proteins to show equal loading (lower panels). Blots are from single experiments but are representative of two experiments.

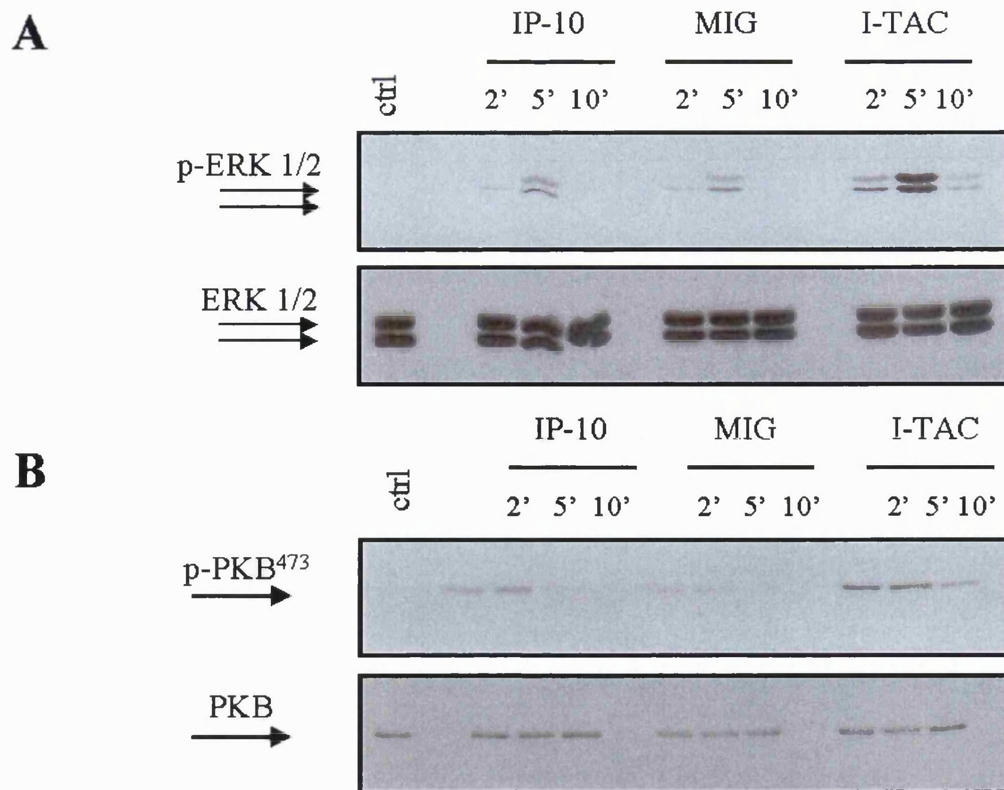

Figure 4.27 Comparison of the 3 CXC3 ligands in peripheral blood derived activated T-cells signalling.

1 x 10^6 12 days old SEB activated peripheral blood derived T-cells were lysed in 50 μl lysis buffer (ctrl) or stimulated with IP-10 or I-TAC or Mig (all at 10 nM) for indicated time points, lysed in lysis buffer as described in Methods and Materials and then probed with specific rabbit polyclonal antibodies against phospho$^{202/204}$ ERK (A) or.phosphp^{473} PKB (B). Membranes were then stripped and re-probed with antibodies against non-phosphorylated proteins to show equal loading (lower panels). Blots are from single experiments but are representative of two experiments.

4.3 Summary of findings

- CXCR3 is expressed at least at the mRNA level on primary human intestinal myofibroblasts
- I-TAC, but not IP-10 or Mig mediate calcium mobilisation in primary human intestinal myofibroblasts
- IP-10, I-TAC and Mig induce a time dependent phosphorylation of PKB at both phosphorylation sites in primary human intestinal myofibroblasts
- Activation of PKB is PI3K dependent
- Class II PI3Ks are activated and tyrosine phosphorylated in human intestinal myofibroblasts in response to IP-10 but not in peripheral blood-derived activated T-cells
- IP-10, I-TAC and Mig induce a time dependent phosphorylation of ERK 1/2 and p38 in primary human intestinal myofibroblasts, which is partially dependent on PI3K
- IP-10, I-TAC and Mig also induce a time dependent phosphorylation of p90RSK in primary human intestinal myofibroblasts again partially dependent on PI3K
- Phosphorylation events in primary intestinal myofibroblasts are Gαi independent in contrast to peripheral blood-derived activated T-cells
- The potency of IP-10, I-TAC and Mig in these phosphorylation events are similar in primary intestinal myofibroblasts, whereas in peripheral blood-derived activated T-cells I-TAC is the most potent

4.4 Discussion

Even though chemokines were first identified by their ability to act as chemoattractants for specific leukocyte subsets, leukocytes are not the only cell types that respond to chemokines. For example, although the CXC chemokine IP-10 was initially characterized as a chemoattractant for T lymphocytes (Taub et al., 1993), it also has anti-angiogenic activities that appear to be mediated by its direct effects on endothelial cells (Luster et al., 1995; Angiolillo et al., 1995; Strieter et al., 1995). Because some endothelial cells express no detectable CXCR3, it was suggested that a different receptor was responsible for these effects. This problem was solved with the identification of the alternative splice variant of CXCR3, CXCR3-B (Lasagni et al., 2003). In intestinal myofibroblasts we were unable to detect any surface expression of CXCR3, even though these cells expressed the message for this receptor. The disparity between the presence of CXCR3 mRNA and lack of detectable protein expression in primary human intestinal myofibroblasts, leaves several possibilities regarding the presence or not of that receptor on those cells. One possibility is that the receptor is expressed on the cell surface, but below sensitivity limits of the antibody used in this study. Another possibility is that the receptor is post-translationally modified in these cells, which might alter the recognition site of the antibody. Chemokine receptors are known to undergo a variety of post-translational modifications. For instance, phosphorylation of specific intracellular serine residues in the C-terminal region of chemokine receptors is essential for their signal transduction function. In some chemokine receptors, extracellular regions are also known to be post-translationally modified. Human chemokine receptors CCR2b, CCR5, CX_3CR1, and CXCR4 are reported to be sulfated and/or glycosylated at their N-terminal

extracellular domains (Farzan et al., 1999; Preobrazhensky et al., 2000; Farzan et al., 2002; Fong et al., 2002). On the other hand, one last possibility could be that CXCR3 was expressed at the mRNA level but the protein was either not translated at all or degraded post-translationally.

Calcium mobilization in intestinal myofibroblasts

In order to rule out the possibility that this receptor was indeed not present in our system, calcium mobilization of the cells to the CXCR3 ligands was investigated.

The nature of these cells however quickly revealed a limitation in this approach. In our hands intestinal myofibroblasts show spontaneous transient increases in $[Ca^{2+}]_i$. The Ca^{2+} oscillations were not synchronous among the cells and the frequency varied from cell to cell. This made the effect of the agonist difficult to determine. Unfortunately there are no calcium studies in primary intestinal myofibroblasts to compare this finding. However, in intact tissues of the gastrointestinal tract, fluorescent calcium imaging has already been employed to monitor calcium changes during spontaneous (and evoked) activities over large areas of the smooth muscle network (Stevens et al., 1999b; Stevens et al., 1999a; Stevens et al., 2000; Hennig et al., 2002). These studies demonstrated that contractions of gastrointestinal smooth muscle are produced by robust rises in $[Ca^{2+}]_i$ initiated by slow waves or Ca^{2+} influxes through calcium channels, and intestinal myofibroblasts seem to comply with this pattern.

Despite the limitations of this technique, it was clear that the cells do not respond to IP-10 and Mig. The only CXCR3 ligand that resulted in a response was I-TAC, which stimulated a rapid and transient elevation in $[Ca^{2+}]_i$, similar though lower in magnitude to the response seen previously with endothelin-1.

These observations further perplexed the possibilities regarding expression or not of CXCR3 on intestinal myofibroblasts. In CXCR3 expressing activated T-cells IP-10, MIG and I-TAC induce transient mobilization of intracellular calcium (Cole et al., 2001). Moreover, stimulation of cultured glial cells (Biber et al., 2002), or primary podocytes (Huber et al., 2002), with chemokine ligands for CXCR3 also induced intracellular calcium transients. On the other hand, ligation of CXCR3-B by any of its ligands does not result in calcium mobilization (Lasagni et al., 2003). However, a receptor that mobilizes calcium only in response to I-TAC has not been described yet. There are several potential explanations for these results. First, I-TAC-induced calcium mobilization might be due to a functionally wild-type CXCR3 expressed at very low levels in intestinal myofibroblasts. The fact that only I-TAC is able to mobilize calcium could be explained by the fact that it is known to be considerably more potent than the other two CXCR3 ligands (Cole et al., 2001). Second, a novel receptor that binds only I-TAC is responsible for the transient calcium increase. Such a receptor could either be the product of a post-translational modification of the mRNA of CXCR3, which is present in intestinal myofibroblasts, resulting in a new receptor capable of binding only I-TAC, or a receptor completely unrelated to CXCR3. A receptor like that has already been demonstrated to exist for IP-10 although its identity has yet to be revealed (Soejima and Rollins, 2001). The last possibility is that I-TAC could be binding to a glycosaminoglycan (GAGs), since it is known that these molecules bind various chemokines (Hoogewerf et al., 1997; Ali et al., 2000). However, this is highly unlikely to be the receptor we have identified since binding of chemokines to GAGs has never been shown to result in signal transduction.

PKB phosphorylation in intestinal myofibroblasts

In order to further examine the nature of this receptor, activation of the PI3K pathway was assessed as it has been shown to be activated by CXCR3 ligands in a similar cell type in the liver (Bonacchi et al., 2001). Indeed, the downstream target of PI3K, PKB, was found to be phosphorylated at both possible sites upon stimulation of the cells by I-TAC. Unexpectedly however, IP-10 and MIG stimulation also resulted in PKB phosphorylation. Moreover phosphorylation of PKB in intestinal myofibroblasts was much more sustained compared to phosphorylation events observed in activated T-cells. The dependence of this phosphorylation event by PI3K was confirmed by its complete inhibition by the PI3K inhibitor LY294002.

A possible molecular mechanism to explain the different kinetics of PKB phosphorylation could be the differential expression of various phosphatases in the different cell types. For example, the PTEN gene product dephosphorylates tyrosine and serine/threonine residues and exhibits phosphatase activities with both protein and lipid substrates (Cantley and Neel, 1999; Maehama and Dixon, 1999). The major substrate of PTEN is PtdIns3,4,5-P_3 a product of PI3K (Myers et al., 1998). The loss of PTEN function increases the concentration of PtdIns3,4,5-P_3, which in turn leads to PKB hyperactivation, which suggests that the tumor-suppressor function of PTEN is exerted through the negative regulation of the PI3K/PKB cell survival pathway (Di Cristofano and Pandolfi, 2000). Although the protein phosphatase activity of PTEN is not considered to be as important as its lipid phosphatase activity for tumor suppression, the PTEN function as protein phosphatase has been implicated in the inhibition of cell migration and invasion

via dephosphorylation of focal adhesion kinase (FAK), a molecule critical in the regulation of integrin signalling (Tamura et al., 1998). Another phosphatase that has recently been implicated in the regulation of PtdIns3,4,5-P_3, and thus PKB phosphorylation, is SHIP-1 (Freeburn et al., 2002). It would therefore be interesting to determine whether these proteins are expressed in intestinal myofibroblasts.

The robustness and duration of the activation of a given signalling pathway has far reaching biological consequences. For example, in T-cells, persistent activation of PKB by SDF through CXCR4 is thought to occur because SDF and CXCR4 are involved in homeostasis rather than inflammation; sustained activation could protect $CXCR4^+$ cells from undergoing apoptosis, a process that is critical for the activation of T-cells (Tilton et al., 2000). In contrast, CXCR3 ligands are up-regulated in the intestine in inflammatory conditions (Dwinell et al., 2001) . Sustained PKB activation by these chemokines could therefore not just regulate and guide the migration of CXCR3-bearing myofibroblasts but also have a protective role in apoptosis in case of local high expression of the CXCR3 ligands. Disturbance of such a balance might contribute to the presence of irregular numbers of myofibroblasts in certain inflammatory diseases like CD and UC.

The finding that intestinal myofibroblasts respond to the CXCR3 chemokines suggests that they might also regulate the number of T-cells migrating into the vicinity of the epithelium. IP-10, Mig, and I-TAC are thought to play an important role in the pathogenesis of intestinal inflammatory responses, particularly those driven by Th1-type responses by chemoattracting CXCR3-expressing

activated/memory T-cells (Luster, 2001). Indeed it is known that virtually all IELs and LPLs express CXCR3 (Agace et al., 2000). Binding of the chemokines on the surface of myofibroblasts would decrease the concentration of the chemokines available to CXCR3$^+$ T-cells. Interestingly at lower concentrations of I-TAC, PKB phosphorylation has been demonstrated to be sustained in T-cells and thought to have an anti-apoptotic role (Smit et al., 2003).

PI3K isoforms involved in CXC3 ligand-mediated signalling events

Although LY294002 has been used extensively to study the physiologic role of class I PI3Ks in various cellular responses (including chemotaxis), contradictory results have been obtained regarding its ability to inhibit class II isoforms. For example, LY284002 has been shown to inhibit all PI3K isoforms with an IC_{50} in the 1-50 μM range (Foster et al., 2003), whereas another study demonstrated inhibition of PI3K C2 α only at concentrations of the compound where it is known to inhibit other signalling enzymes, including PtdIns 4-kinases (PI4Ks) (Domin et al., 1997). In our hands both class II PI3K isoforms appear to be insensitive to LY294002, suggesting that PKB phosphorylation is not dependent on class II PI3K isoforms.

Nevertheless, class II PI3Ks are known to be activated by chemokines (Turner et al., 1998) . This notion was confirmed in intestinal myofibroblasts in which both class II isoforms are activated by IP-10, but not in T-cells. The apparent discrepancy could be due to differences in signalling pathways employed by different cell types. This is emphasized by the fact that class I P13K do not appear to be activated in our *in vitro* lipid kinase assays in intestinal myofibroblasts. However, the possibility that CXCR3-mediated signalling in human T-cells is

coordinated in a different way involving class II PI3K should not be excluded. It is more than likely, that high activity basal levels in the lipid kinase assays are a consequence of the experimental procedures, resulting in masking activation of some of these molecules in both systems. Indeed, the high levels of p85 and p110δ observed in the intestinal myofibroblasts, would lead someone to expect these cells to exhibit high basal levels of PKB phosphorylation, which is not the case. Moreover, if class I isoforms were not activated in this system it would be impossible to explain the chemokine induced and LY294002-dependent PKB phosphorylation.

Following phospho-tyrosine immunoprecipitation, we have found that class II PI3K activity is clearly increased in cells exposed to IP-10 albeit with different kinetics for the two isoforms. This difference suggests either differential compartmentalization of the class II PI3K isozymes or a difference in their mechanisms of regulation. Evidence for the former exists from reports that demonstrate that PI3K-C2α is concentrated in trans-Golgi network and is present in clathrin-coated pits (Domin et al., 2000), whereas PI3K-C2β was found in the nuclei of rat liver cells (Sindic et al., 2001).

Coupling of receptors to the class I p85/p110 PI3K is known to require interaction of src homology 2 (SH2) domains within the p85 regulatory subunit with specific phosphotyrosine-containing binding motifs (pYXXM; where pY represents phosphotyrosine) located in several growth factor receptors or adaptor molecules such as the insulin receptor substrate-1 (IRS-1). The mechanism by which the G protein-coupled CXCR3 could couple to class I PI3K is unclear, since there is no recognized binding motif for the p85 SH2 domains contained within the CXCR3

sequence. G-protein coupled receptors activate the p85/p110 isoform, by tyrosine kinase–regulated pathways (Ptasznik et al., 1996). It has also been proposed that activation of the p85/110 isoform may be mediated by binding of an adapter to the receptor and could be independent of hetero-trimeric G proteins (Luttrell et al., 1999). CXCR3 signalling has been demonstrated to activate the tyrosine kinase Src in hepatic stellate cells (Bonacchi et al., 2001) providing a possible mechanism for class I activation in intestinal myofibroblasts.

The possible mechanisms that allow coupling of class II PI3K to receptors, are less clear. Binding of Ca^{2+} or phospholipid to the C2 domains can play a role in regulating protein function (Rizo and Sudhof, 1998), making this a potential candidate to explain chemokine regulation of class II PI 3-kinases. Indeed, there is evidence that this domain is involved in regulating the activity of class II PI 3-kinases because deletion of this domain in PI3K-C2β increases catalytic activity (Arcaro et al., 1998). However, it appears unlikely that a similar mechanism operates in this case since IP-10 stimulation does not result in calcium mobilization in intestinal myofibroblasts. Another possibility is by direct interaction of Gβγ subunits with class II PI3K, or indirectly via protein tyrosine kinases (PTK(s)). The latter has been proven downstream of the epidermal growth factor receptor (EGFR) and is supported in this study by the fact that IP-10 stimulates the recruitment of PI3K-C2α and PI3K-C2β to phosphotyrosine complexes in intestinal myofibroblasts.

Characterization of PI3K-C2β has established that its N-terminus is responsible for the interaction with the activated EGFR. This sequence lacks phosphotyrosine binding motifs; instead it has 3 proline-rich regions that have the potential to bind

SH3 containing adaptor molecules. The adaptor Growth binding protein 2 (Grb2) has been proposed as the link between C2β and EGFR. This protein consists of a single, phosphotyrosine binding SH2 domain flanked by two polyproline binding SH3 domains (Lowenstein et al., 1992). Recruitment of Grb2 to the EGFR following ligand addition has been described extensively, and its interaction is dependent upon 2 phosphotyrosine residues (Rozakis-Adcock et al., 1993). Grb2 therefore couples pTyr-X-Asn motifs, recognized selectively by the SH2 domain, to signalling pathways that are recruited by the SH3 domains, and promote cell proliferation, growth, and survival. A variation on this theme is provided by mammalian docking proteins, such as Src homologue and collagen homologue (Shc), fibroblast growth factor substrate 2 (FRS2), and IRS-1 family members. These proteins all possess a phosphotyrosine-binding (PTB) domain that binds phosphorylated NPXY motifs on activated RTKs, and are phosphorylated on tyrosine on recruitment to the receptor. Their phosphorylation creates binding sites for the SH2 domains of cytoplasmic signalling proteins, including Grb2, and thereby potentiates the activation of specific biochemical pathways that stimulate growth and survival. The assembly of such large multi docking/adaptor protein complexes and the effectors that are bound to them may provide a mechanism for generation of signal diversity.

The hypothesis that different mechanisms are employed for the activation of different PI3K isoforms could be a reflection of the fact that different PI3K enzymes fulfil specific biological roles.

MAPK activation in intestinal myofibroblasts

In intestinal myofibroblasts and in activated primary human T-cells, IP-10, I-TAC and Mig induce activation of the p44/p42 MAPK- as well as phosphorylation of the ERK downstream target, p90RSK. However, considerable differences exist between the two cell types. Similar to PKB, phosphorylation of ERK and p90 in intestinal myofibroblasts is delayed and more sustained compared to the phosphorylation events in the T-cells. It should be noted that the efficacy of chemokines is subject to myofibroblast and T-cell donor variability but the results are very similar in terms of kinetics.

The duration and amplitude of MAP kinase activation represents the balance between the activating signal and inactivation mechanisms. Both are influenced by negative feedback triggered by the activating signal upstream of the MAP kinase. Sustained activation has been shown to induce nuclear translocation of ERK-1 and 2 (Lenormand et al., 1998; Brunet et al., 1999b).

Perhaps the most well defined signalling pathway from the cell membrane to ERK1 and ERK2 is that used by RTKs. Stimulation of these receptors by the appropriate ligand results in an increase in receptor catalytic activity and subsequent autophosphorylation on tyrosine residues. Phosphorylation of these receptors results in the formation of multi-protein complexes whose organization dictates further downstream signalling events. Quite often one of these functions is the activation of the monomeric G protein, Ras. This is achieved by the recruitment of adaptor proteins, such as Shc and Grb2, to the receptor through interactions between their SH2 domains and phosphotyrosine residues. The

guanine nucleotide exchange factor (GEF) Son of Sevenless (Sos) then becomes engaged with the complex and induces Ras to exchange GDP for GTP. GTP-ligated Ras is capable of directly interacting with a number of effectors, including Raf isoforms, of which the best characterized is Raf-1. Signalling to ERKs by GPCRs also involves modulation of Raf activity; however, the mechanisms employed by these receptors are widely varied. The existence of multiple classes of G proteins, the ability of some receptors to activate more than one class of G protein, and cell type-specific mechanisms contribute to the diversity. CXCR3-ligand induced activation of ERK is thought to be Gαi-dependent (Smit et al., 2003). In a proposed model for this type of ERK activation, the βγ subunits stimulate a Src family kinase activity in a PI3K γ dependent manner (Lopez-Ilasaca et al., 1997). Src may then phosphorylate a tyrosine kinase receptor, PYK2, or FAK, to create SH2 domain binding motifs (Dikic et al., 1996; Della Rocca et al., 1999). Then, analogous to the signalling mechanism used by receptor tyrosine kinases described above, a Shc-, Grb2-, and Sos-containing complex is formed at the membrane to activate Ras and, in turn, Raf-1. ERK activation in cell types where PI3K 110γ expression is low, as is probably the case for intestinal myofibroblasts, may be dependent on alternative means to activate Src or PYK2 (Dikic et al., 1996). This PI3K-ERK cross talk is supported by the fact that LY294002 seems to have an effect on ERK phosphorylation in both cell types used in this study. p90 RSK phosphorylation appears to be more sensitive to the PI3K inhibitor probably reflecting the involvement of PDK-1 in this event.

Another MAPK, the p38 pathway was found to be phosphorylated in intestinal myofibroblasts in response to the CXC3 ligands. The stimulation of the p38 family by GPCRs has been well documented, but their mechanism of activation is

far from being fully understood. A few studies have shown that βγ dimers activate p38 (Yamauchi et al., 1997), and two PTKs, Bruton's tyrosine kinase (Btk) (Bence et al., 1997) and Src (Nagao et al., 1998), have also been implicated in this mechanism. Another possible mechanism is through the small GTP binding proteins Rac and Cdc42 through their activation of p21-activated kinase which have been implicated as upstream regulators of p38 in transformed cells (Zhang et al., 1995; Bagrodia et al., 1995). Furthermore, MAPKK-3, MAPKK-4, and MAPKK-6 have been shown to phosphorylate and activate p38 (Derijard et al., 1995; Raingeaud et al., 1996). It is presently unclear whether there is a role for PI 3K in regulation of the p38 MAPK pathway. Studies in neutrophils demonstrate either partially dependent (Krump et al., 1997) or entirely PI 3K-independent p38 MAP kinase activation (Knall et al., 1997). From our results it appears that in intestinal myofibroblasts, CXCR3 ligand-mediated activation of p38 MAP kinases requires PI3K activity.

MAPK are believed to play a crucial role in many aspects of immune mediated inflammatory responses. The p44 and p42 ERK1/2 mediate responses mainly to mitogenic stimuli, whereas p38 mediate responses to cellular stress. However, very few studies of activation of the MAPK pathways exist for intestinal myofibroblasts. A possible role for these kinases emerges from a study in hepatic myofibroblasts. Whereas p38 MAP kinase mediates PDGF-BB-stimulated migration in hepatic myofibroblasts, ERKs mediate PDGF-induced proliferation, but not migration (Tangkijvanich et al., 2002). A similar role for the CXCR3 ligands and the importance of these pathways in our system would require further investigation.

Is CXCR3 responsible for the signals in intestinal myofibroblasts?

CXCR3-mediated signalling has been found to be sensitive to pertussis toxin in all studies to date. Pertussis toxin is a potent inhibitor of all three characterized Gi subunits, and blocks most chemokine-mediated chemotactic responses in leukocytes. However in intestinal myofibroblasts, pertussis toxin appeared to have no effect on IP-10-mediated signalling (Figure 4.24). Pertussis toxin-insensitive heterotrimeric G proteins include members of the G_{12} and Gq families and one Gi family member, i.e. Gz which is predominantly expressed in neurons, platelets (Casey et al., 1990).

A potentially important biochemical property of at least two pertussis toxin-resistant G-proteins is their ability to be phosphorylated. Gz and G_{12} have been shown to be excellent *in vitro* substrates for PKC (Kozasa and Gilman, 1996). Members of all three major subtypes of PKC (i.e. classical, calcium-independent and atypical) are able to phosphorylate these two G-proteins. While the biological significance of this phosphorylation is unclear, phosphorylation of both Gz and G_{12} blocks their interaction with $G\beta\gamma$ (Fields and Casey, 1995; Kozasa and Gilman, 1996), suggesting that this phosphorylation is a regulatory mechanism for amplifying signalling through these $G\alpha$ subunits by preventing subunit reassociation. This mechanism could explain the sustained signalling observed in intestinal myofibroblasts compared to the T-cells.

In addition to the identification of the pertussis toxin resistant G-proteins, many studies have also characterized the specific signalling pathways in which each participates. Signalling experiments through Gq have demonstrated that it can directly stimulate PLC-β isoenzymes in *in vitro* assays (Taylor et al., 1991). There

is evidence to suggest that this might link Gq with the MAPK pathway (Buhl et al., 1995) even though other reports have proposed a more direct involvement.

Since G_{12} -coupled receptors appear to also activate Gq family members, it has been difficult to selectively study the cellular signalling processes regulated by receptor-mediated activation of G_{12}. G-proteins of the G_{12} family, e.g. G_{12} and G_{13}, have been demonstrated to be involved in the induction of the platelet shape change. This is mainly based on the finding that in Gq-deficient platelets in which thromboxane A_2 (TXA_2) receptors only couple to G_{12} and G_{13}, a rapid shape change is observed when upon receptor stimulation. This effect appears to be mediated by the Rho/Rho kinase pathway (Klages et al., 1999). This is further supported by the fact that G_{12} proteins have also been linked to the regulation of the actin cytoskeleton. Mutationally activated G_{12} proteins induce actin polymerization and focal adhesion when transfected into Swiss 3T3 cells (Buhl et al., 1995).

In chemokine signalling, the opposite biological activities mediated by CXCR3-A and CXCR3-B are thought to reflect coupling of the two receptors to different signal transduction pathways. In contrast to CXCR3-A transfectants, in which pertussis toxin treatment inhibits proliferation, pertussis toxin had no effect on the proliferation and survival of CXCR3-B transfectants (Lasagni et al., 2003). Multifunctional coupling is common to many GPCRs and has been previously described for the putative IP-10 receptor (Soejima and Rollins, 2001). Together, the lack of calcium influx in response to the CXCR3 ligands (except for I-TAC), and pertsussis toxin insensitivity suggest the coupling of the receptor present in intestinal myofibroblasts to other than Gi types of G proteins. This receptor could

either be CXCR3, or a modified CXCR3, or finally a completely different receptor. Because intestinal myofibroblasts express mRNA for CXCR3, we favour the possibility that the signalling events are mediated by a CXCR3 type receptor, through a mechanism summarized in Figure 4.28.

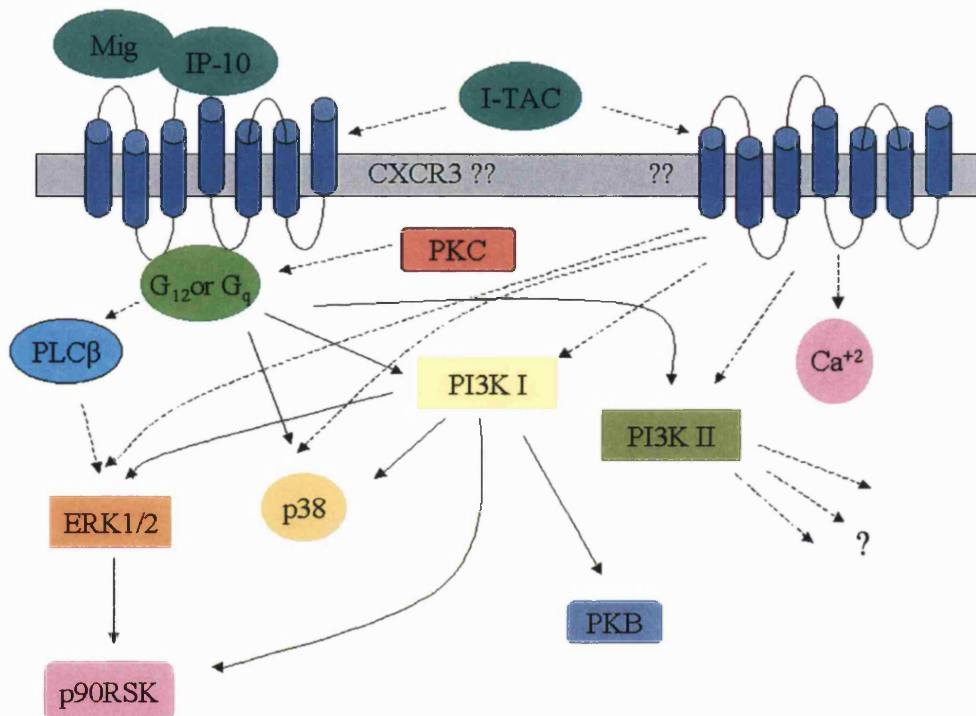

Figure 4.28 Model of signalling pathways induced by IP-10, I-TAC and Mig in human intestinal myofibroblasts. The solid lines represent pathways, which have been demonstrated in this section to be involved in IP-10, I-TAC and Mig mediated responses. Dotted lines provide potential pathways that are known to occur in different systems as outlined in the discussion section. IP-10 and Mig bind and signal through the same receptor, possibly CXCR3. I-TAC could be operating via the same receptor, a different receptor or both.

Chapter 5

5 Results III

CXCR3 ligand-mediated effects on actin polymerisation in primary intestinal myofibroblasts

5.1 Background

Amongst the most impressive effects of chemokines on leukocytes are the morphological changes of the cells: the cytoskeleton is rearranged and the cell becomes polarized. An early event in the leukocyte polarization induced by chemoattractants is a change in filamentous actin (F-actin) distribution from a radial symmetry around the cell to its concentration at a particular region, resulting in the switch from a spherical to a polarized shape. Then integrin-mediated focal adhesions are formed, and the cell binds and detaches from the substrate in a coordinated manner with extension and retraction of pseudopods to execute the directional migration (Bokoch, 1995; Ward et al., 1998).

Cell migration is not restricted to leukocytes but involves almost every cell type. For example, fibroblast adhesion and migration, albeit relatively slow compared to T-cells (Niggemann et al., 1997), are of critical importance to tissue homeostasis, wound healing, fibro-proliferation and tumour growth, and are therefore tightly controlled. In most cases, this dynamic process requires coordinated changes in the temporal and spatial organization of the actin cytoskeleton which are differentially modulated by diverse extracellular stimuli (Lauffenburger and Horwitz, 1996; Horwitz and Newsome, 1999; Ridley et al., 2003).

There are however some exceptions. PDGF-BB, one of strongest chemoattractants, stimulates migration of hepatic myofibroblasts through alterations in the actin cytoskeleton (Tangkijvanich et al., 2002). On the other hand, PDGF drives vascular smooth muscle cell (VSMC) motility without detectable effect on actin cytoskeleton (Abedi et al., 1995). These observations suggest that chemoattractant-induced movement might occur in actin reorganization –relevant or irrelevant fashions.

Based on this evidence, in order to determine whether the receptors that respond to IP-10, I-TAC and MIG on human primary intestinal myofibroblasts are functional, we examined these cells for actin re-organization in response to the CXC3 ligands.

5.2 Results

CXCR3 ligands induce F-actin polymerisation in intestinal myofibroblasts

Stellate-transformed myofibroblasts become activated in response to many fibrogenic cytokines like IL-1, TNF-α, PDGF, fibroblast growth factor (FGF), and TGF- β (Kovacs and DiPietro, 1994), typified by re-organization of actin into stress fibers. It has also been demonstrated, that chemokine ligand–receptor interactions trigger intracellular actin polymerisation in leukocytes (Burger et al., 1999) a process which is pre-requisite for cell motility and migration.

In light of this knowledge, the effect of the CXCR3 ligands on actin polymerisation was assessed. Intestinal myofibroblasts were treated with IP-10, I-TAC or MIG over a short time period of up to 60 minutes fixed, permeabilized

and finally stained with TRITC conjugated phalloidin and images were analysed on a confocal microscope.

Consistent with the findings in leukocytes, IP-10, I-TAC and MIG induced a transient increase in intracellular F-actin as indicated by the brighter red staining of the stimulated cells compared to cells treated with a vehicle control (Ctrl) (Figures 5.1-5.3). This increase was accompanied by a dramatic induction of stress fiber formation in intestinal myofibroblasts and a redistribution of F-actin to the cell periphery and polarization to the leading edge, resulting in the appearance of nuclear sparing. F-actin redistribution was observed as early as 2 min after addition of all three chemokines and peaked at thirty minutes following stimulation, before receding to basal conditions.

Effect of latruncillin B and Y27632 on chemokine-induced F-actin polymerisation in intestinal myofibroblasts

Latrunculins are novel marine compounds isolated from a Red Sea sponge that alter cell shape, disrupt microfilament organization and microfilament-mediated processes. They also inhibit polymerisation of actin by binding to monomeric G-actin in a 1:1 ratio (Spector et al., 1989; Yarmola et al., 2000). Latrunculins are frequently used to establish the effects of F-actin disassembly on particular physiological functions.

Intestinal myofibroblasts were pre-treated with various concentrations of latruncilin B for one hour before the addition of the chemokines at 10 nM for thirty minutes. The cells were then fixed, permeabilized and stained with TRITC conjugated phalloidin that recognizes polymerised actin (Figure 5.4).

Latruncilin B at concentrations as low as 1 μM not only completely inhibited chemokine induced actin polymerisation but also completely blocked the basal level of actin polymerisation observed in these cells.

RhoA, a small, monomeric G-protein, is a member of the Rho subfamily of the Ras family of G-proteins and its effector Rho dependent-kinase (ROCK) has been identified as upstream components of a major pathway involved in actin cytoskeleton-linked morphological changes. A relatively specific Rho kinase inhibitor, Y27632, developed by Narumiya and colleagues (Uehata et al., 1997) is a particularly useful reagent for identifying mechanisms mediated by Rho kinases. Rho works as a molecular switch for the induction of stress fibers in cultured cells (Ridley and Hall, 1992) and ROCK is a Rho effector in this process (Amano et al., 1997).

The possibility that Y27632 could inhibit Rho-induced formation of these structures in cultured cells was therefore tested. Intestinal myofibroblasts were pre-treated with various concentrations of Y27632 for one hour at various concentrations before the treatment with the chemokines for an additional thirty minutes at 10 nM (Figure 5.5). Treatment of cells with Y27632 results in a very different morphology of the cells compared to the one seen with latrunculin B. In this case, at concentrations of 10μM and above, Y27632 abolished stress fibers induced by all three chemokines as well as the fibers observed basally. However, in contrast to latrunculin B, it failed to inhibit actin polymerisation as staining of the cells is still very visible even at the highest concentrations of this inhibitor.

Effect of pertussis toxin on chemokine induced F-actin re-organization in intestinal myofibroblasts

As mentioned earlier both pertussis toxin-sensitive and pertussis toxin-insensitive signalling through chemokine receptors in lymphocytes have been reported. The previous finding that the CXCR3 ligands induced signals were all insensitive to pertussis toxin suggests that the receptor is coupled in intestinal myofibroblasts to G proteins other than $G_{\alpha i}$. However there have been reports of systems which display both pertussis toxin sensitive and in-sensitive chemotactic responses (Shibata et al., 2002; Jimenez-Sainz et al., 2003), suggesting that chemokines could elicit their various biological functions through distinct G-proteins.In view of this the effect of pertussis toxin on chemokine induced actin polymerisation and stress fiber formation was investigated.

Intestinal myofibroblasts were subjected to staining for F-actin following incubation with pertussis toxin for 16 hours at 100 ng/ml and treatment with the chemokines for thirty minutes at 10 nM (Figure 5.6). In agreement with previous findings pertussis toxin had no visible effect on either actin polymerisation or stress fiber induction.

Effect of PI3K inhibition on chemokine induced F-actin re-organization in intestinal myofibroblasts

The mechanisms through which Rho GTPases exert their effects are not completely elucidated, although a great number of effectors have been described (Hall, 1998). The understanding of the connection between PI3K and Rho GTPases remains fragmentary. For instance, PI3K apparently does not activate Rac in T lymphocytes (Reif and Cantrell, 1998). Nevertheless, PI3K inhibitors abolish polarization and chemotaxis (Turner et al., 1995b). Two distinct pathways

for F-actin polymerisation during chemoattractant-stimulated pseudopd extension, regulated by Rho family proteins, have also been proposed to exist in human neutrophils (Chodniewicz and Zhelev, 2003a). One of them is dependent on PI3K whereas the other is independent. Therefore, it is feasible that GTPase-induced effects could be mediated by multi-molecular complexes and not by linear pathways of biochemical cascades.

The involvement of PI3K on chemokine-induced F-actin re-organization in intestinal myofibroblasts was therefore explored given that we have showed that this pathway is activated in this system. Cells were pre-treated for thirty minutes with the PI3K inhibitor LY294002 at various concentrations before adding the chemokines for another thirty minutes at 10 nM. Cells were subsequently fixed, permeabilized and stained with TRITC conjugated phalloidin for polymerised actin and images were visualised on a confocal microscope (Figure 5.7).

Both actin polymerisation and stress fiber formation were both found to be PI3K independent, as LY294002 even at concentrations higher than the one normally used to inhibit PI3K appeared to have no effect on these processes.

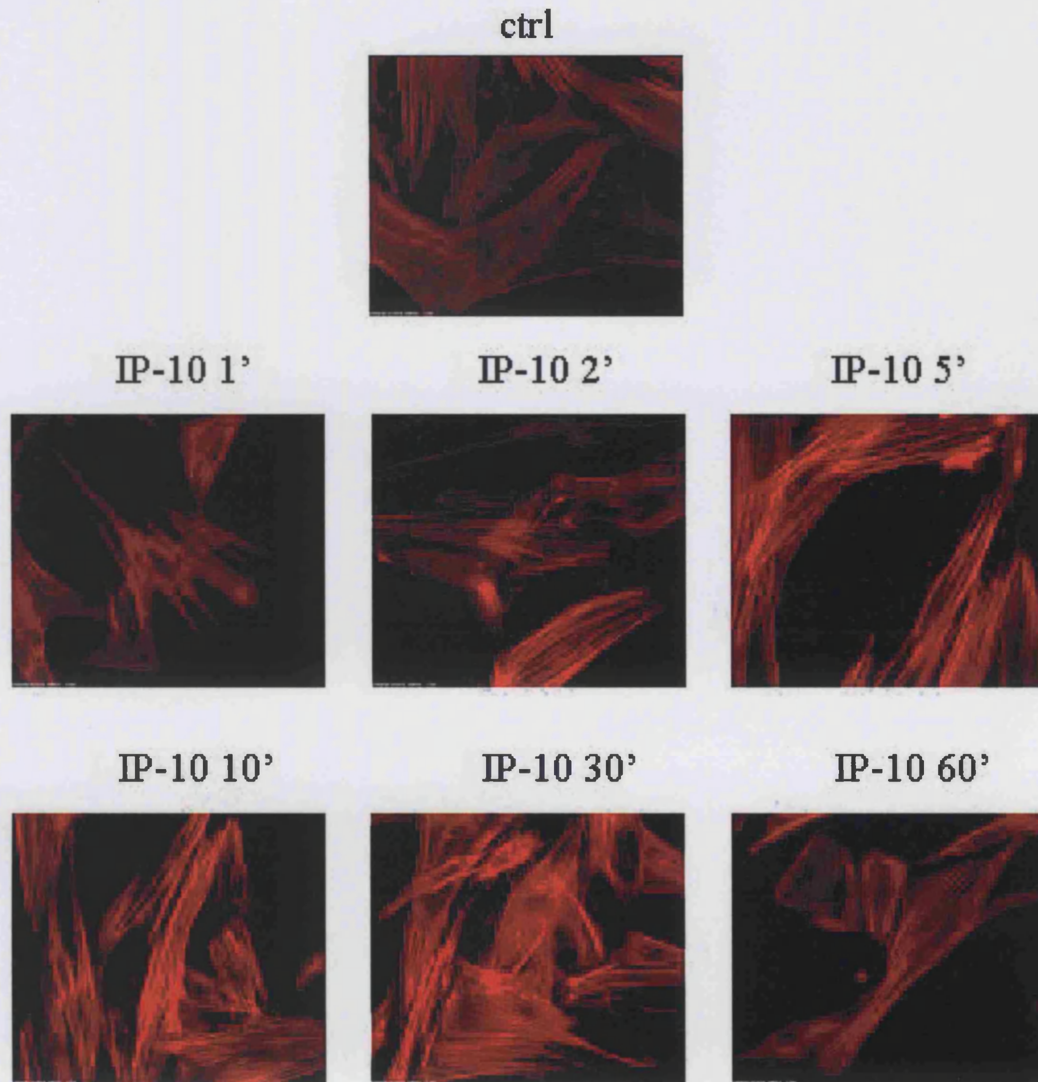

Figure 5.1 IP-10 induces F-actin polymerisation and re-organization in intestinal myofibroblasts.

0.2 x 10^6 primary intestinal myofibroblasts were plated on coverslips in 35mm plates and cultured for 48 hours. Cells were then fixed in 1ml parafolmadehyde /glutaraldeyhde (ctrl) or stimulated with IP-10 (10 nM) for indicated time points, fixed and then stained as described in Methods and Materials with a phalloidin-TRITC conjugated antibody, at a final concentration of 0.1 μg/ml, that recognizes polymerised actin. Results are from single experiments representative of 3 replicate experiments.

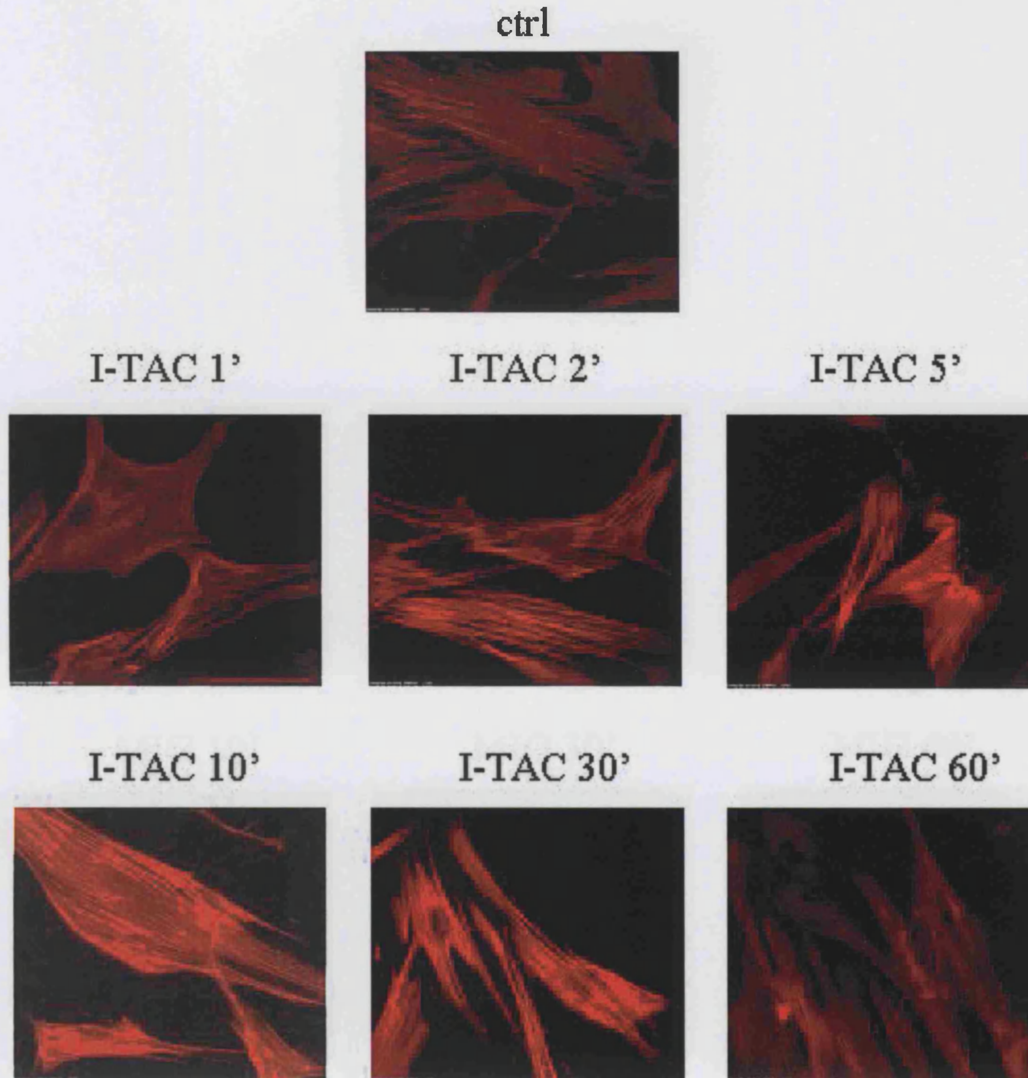

Figure 5.2 I-TAC induces F-actin polymerisation and re-organization in intestinal myofibroblasts.

0.2 x 10^6 primary intestinal myofibroblasts were plated on coverslips in 35mm plates and cultured for 48 hours. Cells were then fixed in 1ml parafolmadehyde /glutaraldeyhde (ctrl) or stimulated with I-TAC (10 nM) for indicated time points, fixed and then stained as described in Methods and Materials with a phalloidin-TRITC conjugated antibody, at a final concentration of 0.1 μg/ml, that recognizes polymerised actin. Results are from single experiments representative of 3 replicate experiments.

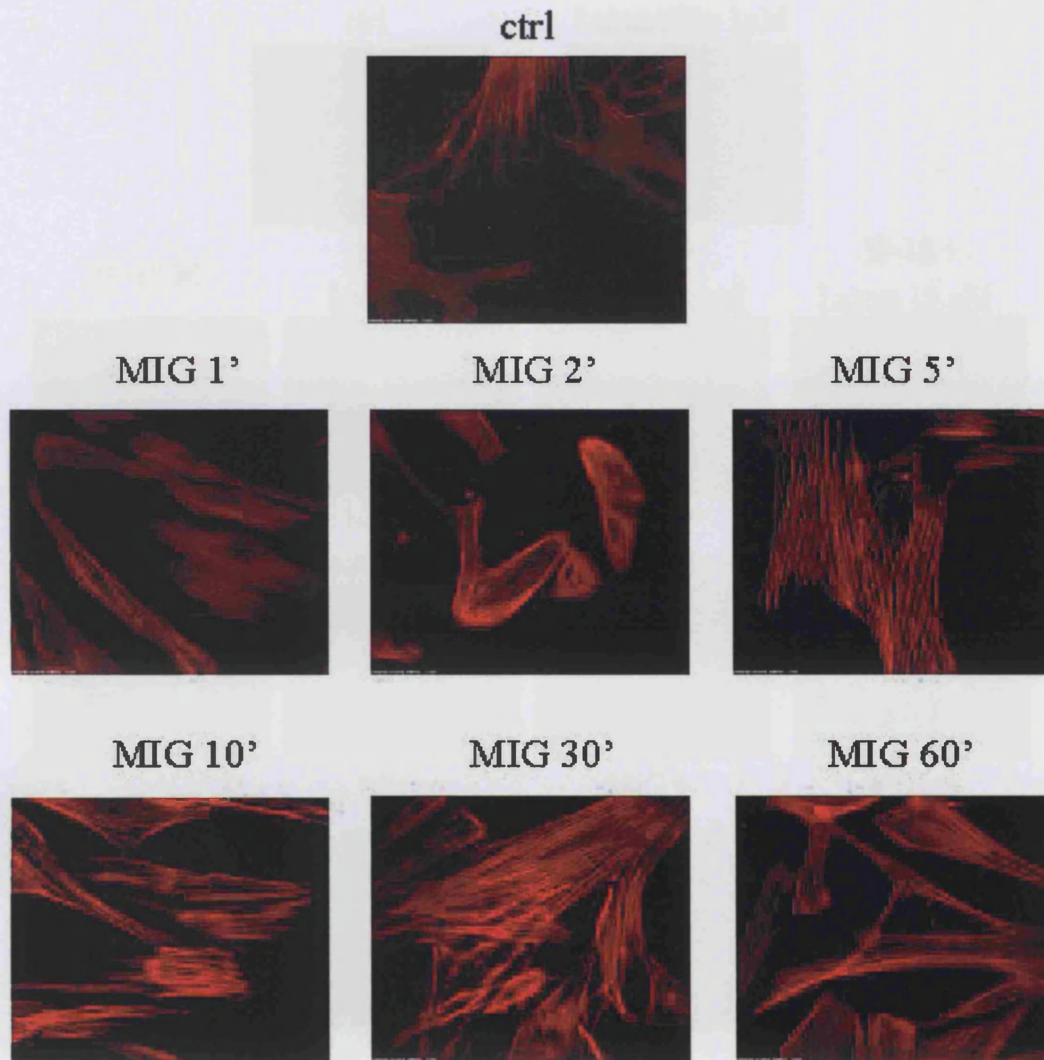

Figure 5.3 Mig induces F-actin polymerisation and re-organization in intestinal myofibroblasts.

0.2 x 10^6 primary intestinal myofibroblasts were plated on coverslips in 35mm plates and cultured for 48 hours. Cells were then fixed in 1ml parafolmadehyde /glutaraldeyhde (ctrl) or stimulated with Mig (10 nM) for indicated time points, fixed and then stained as described in Methods and Materials with a phalloidin-TRITC conjugated antibody, at a final concentration of 0.1 μg/ml, that recognizes polymerised actin. Results are from single experiments representative of 3 replicate experiments.

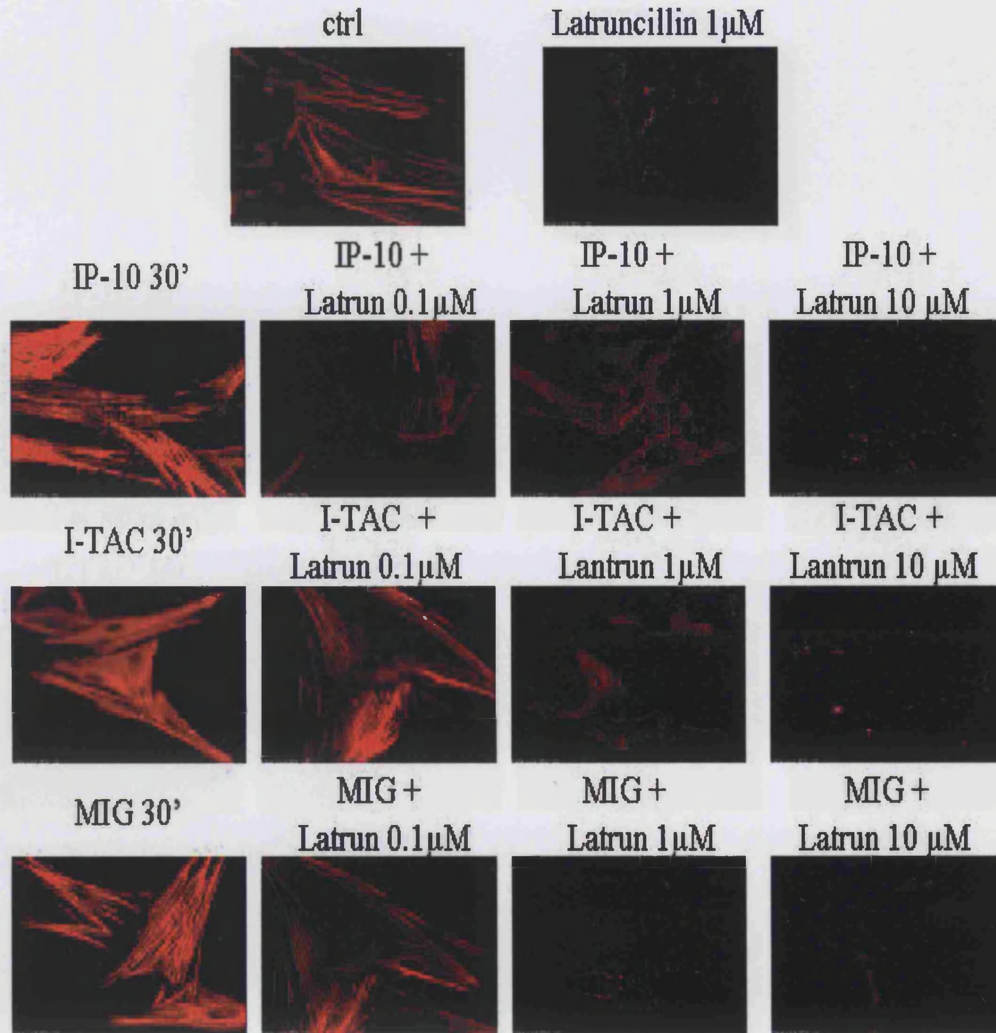

Figure 5.4 Effect of Latruncillin B on chemokine induced F-actin re-organization in intestinal myofibroblasts.

0.2 x 10^6 primary intestinal myofibroblasts were plated on coverslips in 35mm plates and cultured for 48 hours. Cells were then fixed in 1ml parafolmadehyde/ glutaraldeyhde (ctrl) or stimulated with IP-10, I-TAC or MIG (all at 10 nM) 10 in the presence or absence of pre-treatment with the actin polymerisation inhibitor Latruncillin B (0.1-10 nM for 30 minutes) and then fixed. Cells were stained as described in Methods and Materials with a phalloidin- TRITC conjugated antibody, at a final concentration of 0.1 μg/ml. that recognizes polymerised actin. Results are from single experiments representative of 3 replicate experiments.

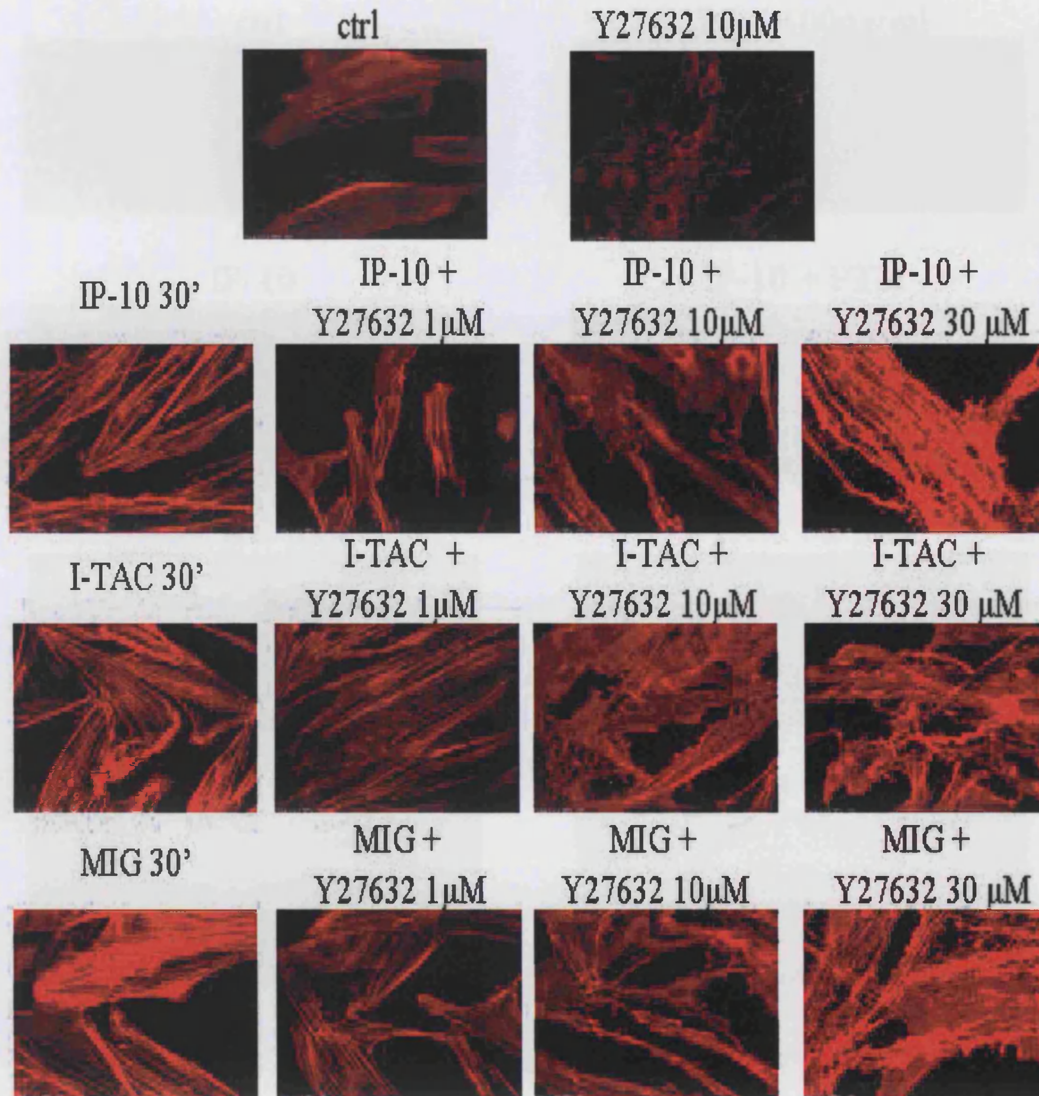

Figure 5.5 Effect of Y27632 on chemokine induced F-actin re-organization in intestinal myofibroblasts.

0.2 x 10^6 primary intestinal myofibroblasts were plated on coverslips in 35mm plates and cultured for 48 hours. Cells were then fixed in 1ml parafolmadehyde/ glutaraldeyhde (ctrl) or stimulated with IP-10, I-TAC or MIG (all at 10 nM) in the in the presence or absence of pre-treatment with the Rho kinase inhibitor Y27632 (1-30 nM for 30 minutes) and then fixed. Cells were stained as described in Methods and Materials with a phalloidin- TRITC conjugated antibody, at a final concentration of 0.1 μg/ml. that recognizes polymerised actin. Results are from single experiments representative of 3 replicate experiments.

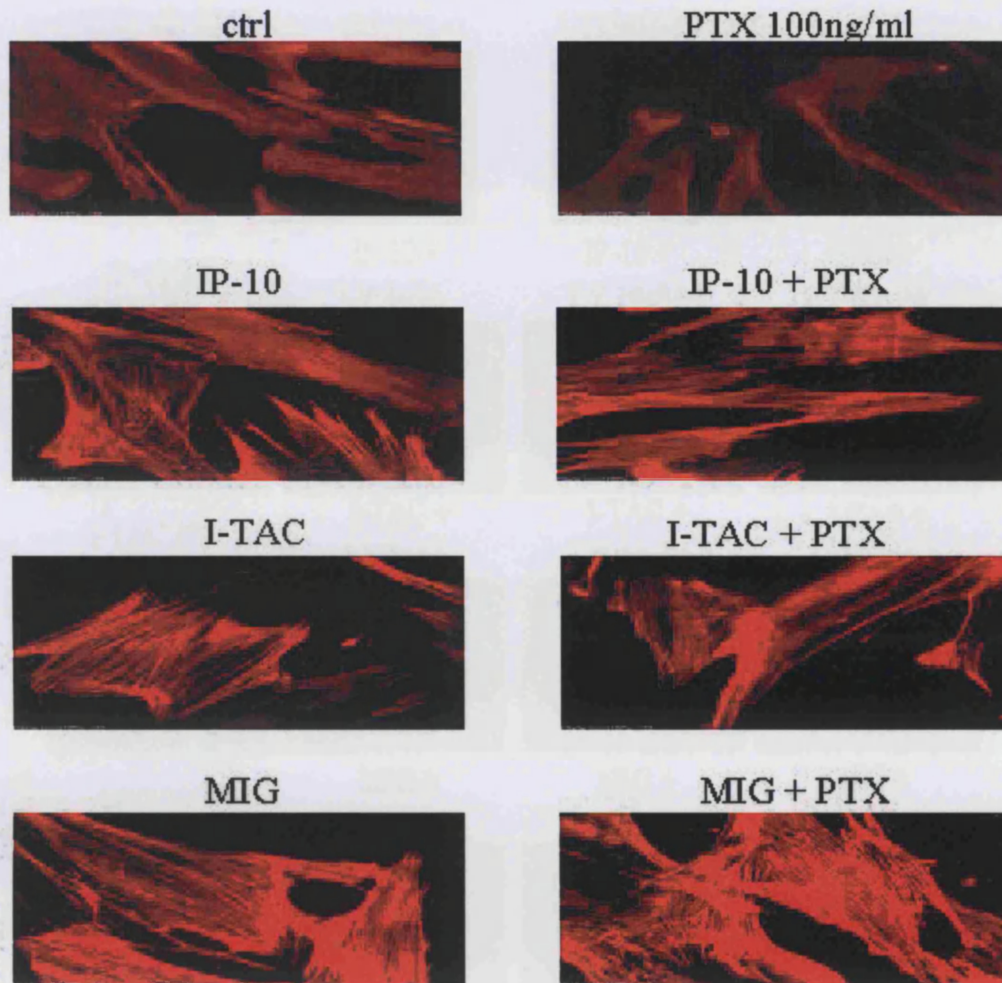

Figure 5.6 Effect of pertussis toxin on chemokine induced F-actin re-organization in intestinal myofibroblasts.

0.2 x 10^6 primary intestinal myofibroblasts were plated on coverslips in 35mm plates and cultured for 48 hours. Cells were then fixed in 1ml parafolmadehyde/ glutaraldeyhde (ctrl) or stimulated with IP-10, I-TAC or MIG (all at 10 nM) 10 in the presence or absence of pre-treatment with the $G\alpha_i$ inhibitor Pertussis Toxin (100 ng/ml for 16 hours) and then fixed. Cells were stained as described in Methods and Materials with a phalloidin- TRITC conjugated antibody, at a final concentration of 0.1 μg/ml. that recognizes polymerised actin. Results are from single experiments representative of 3 replicate experiments.

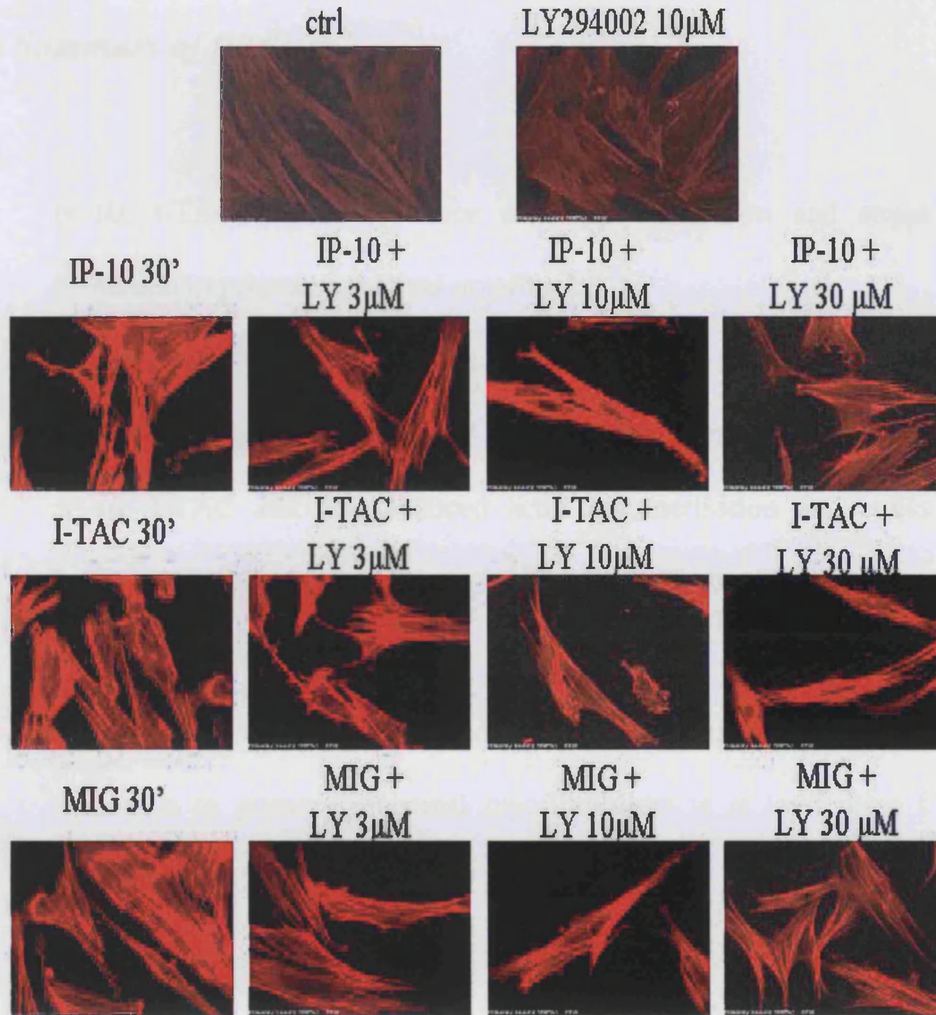

Figure 5.7 Effect of LY294002 on chemokine induced F-actin re-organization in intestinal myofibroblasts.

0.2 x 10^6 primary intestinal myofibroblasts were plated on coverslips in 35mm plates and cultured for 48 hours. Cells were then fixed in 1ml parafolmadehyde/ glutaraldeyhde (ctrl) or stimulated with IP-10, I-TAC or MIG (all at 10 nM) 10 in the presence or absence of pre-treatment with the PI3K kinase inhibitor LY294002 (1-10 nM for 30 minutes) and then fixed. Cells were stained as described in Methods and Materials with a phalloidin- TRITC conjugated antibody, at a final concentration of 0.1 μg/ml. that recognizes polymerised actin. Results are from single experiments representative of 3 replicate experiments.

5.3 Summary of findings

- IP-10, I-TAC and Mig induce actin polymerisation and stress fiber formation in primary intestinal myofibroblasts
- Chemokine induced stress fiber formation, but not actin polymerisation in primary intestinal myofibroblasts is ROCK dependent
- IP-10, I-TAC and Mig induced actin polymerisation and stress fiber formation in primary intestinal myofibroblasts is not facilitated by a Gαi dependent mechanism
- IP-10, I-TAC and Mig induced actin polymerisation and stress fiber formation in primary intestinal myofibroblasts is at least class I PI3K independent.

5.4 Discussion

Regardless of the identity of the receptor, it appears to be functional as demonstrated by the signaling events presented in the previous chapter. Moreover, all three CXCR3 ligands were able to promote actin polymerization and enhance formation of stress fibers in intestinal myofibroblasts. Transforming growth factor β1 (TGF-β1), is a cytokine known to have a similar effect on these cells (Simmons et al., 2002). TGF-β1 is a well-established mediator of wound healing and fibrosis in a number of organs, including skin, lungs, and the liver. In the intestine, however, increased expression of TGF-β1 accompanies ulcerative colitis UC, which generally is not associated with fibrosis, and CD, where fibrosis is a

common complication (Babyatsky et al., 1996). The sequence of cellular events that underlie fibrosis in the intestine is not well defined due, in part, to the complexity of mesenchymal cell subtypes. Subepithelial myofibroblasts and enteric smooth muscle cells both are smooth muscle actin positive, making it difficult to trace activated myofibroblasts during intestinal inflammation (Pucilowska et al., 2000). Nonetheless, our findings indicate that IP-10, I-TAC and Mig could profoundly influence the phenotype of intestinal myofibroblasts.

This finding is somewhat expected since chemokines are known to play a major role in re-organization of the actin cytoskeleton during cell motility (Sanchez-Madrid and del Pozo, 1999). The best studied effects of chemokines in morphological changes are in leucocytes, where binding of the chemokine to the receptor results in cytoskeleton rearrangement, integrin-mediated focal adhesions are formed, and the cell binds and detaches from the substrate in a coordinated manner with extension and retraction of pseudopods to execute the directional migration (Ward et al., 1998). An early event in this leukocyte polarization induced by chemoattractants is a change in filamentous F-actin distribution from a radial symmetry around the cell to its concentration at a particular region, resulting in the switch from a spherical to a polarized shape (Coates et al., 1992).

Fibroblasts are much less characterized in terms of migratory responses. In fact there is only one report about intestinal myofibroblast migration, which demonstrated that the growth factors PDGF-AB, insulin growth factor (IGF), EGF and TGF-β1 stimulate the migration of these cells (Leeb et al., 2002). The identification of further physiologically relevant migration inducing factors is still required to elucidate the network of interactions and the complex mechanisms

involved in intestinal wound healing or fibrosis. The ability of the CXCR3 ligands to induce actin polymerisation in this system suggests a possible role for them in this process.

ROCK in chemokine induced actin polymerisation in intestinal myofibroblasts

Within a cell, actin polymerisation is tightly regulated by a host of actin-associated proteins. As actin is involved in diverse cellular phenomena and signalling pathways, identifying the biochemical steps that lead to force generation has been difficult. Nevertheless it is now well established that the dynamics of actin cytoskeleton are closely regulated by the activation of members of the Rho GTPase family, including RhoA and Rac1, and their activities control cell migration and adhesion. Rho in particular has been associated with stress fiber formation (Ridley and Hall, 1992) and cell contractility (Burridge and Chrzanowska-Wodnicka, 1996). Rho acts on downstream effectors to exert the above actions. Several proteins have been isolated as putative Rho effectors on the basis of their selective interaction with the GTP-bound form of Rho. These include the ROCK family comprised of p160ROCK (ROCK-I) (Ishizaki et al., 1996) and ROKα/Rho-kinase/ROCK-II (Leung et al., 1995), protein kinase N (PKN) (Amano et al., 1996), citron kinase (Madaule et al., 1998) and mDia 1 and mDia 2 (Watanabe et al., 1997; Alberts et al., 1998). Among them, the ROCK family of kinases has been shown to be involved in Rho-induced formation of actin stress fibers and focal adhesions (Leung et al., 1996; Amano et al., 1997). The synthesis of a specific inhibitor of the ROCK family of kinases, Y27632 (Uehata et al., 1997) has proven to be an important tool in assessing the role of these kinases in mediating changes in the actin cytoskeleton. Indeed, in agreement

with the previously established importance of Rho for actin stress fiber formation pre-treatment of intestinal myofibroblasts with 1μM Y27632 abolishes chemokine induced stress fiber formation resulting in a phenotype very similar to the one observed in unstimulated cells. At higher concentrations this inhibitor resulted in increased polymerised actin as indicated by the intensity of the fluorescence. However no stress fibers are visible and actin appears to be concentrated in speckles in areas around the nucleus. The physiological importance of this result is unclear, but is probably not related to ROCK inhibition since at concentrations higher than 5 μM, Y27632 is known to inhibit other kinases (Davies et al., 2000).

PI3K in chemokine induced actin polymerisation in intestinal myofibroblasts

Regulation of actin polymerisation, downstream from GPCRs, is thought to depend on PI3K activation and PtdIns3,4,5-P_3 production, and involves the activation of PKB and the GTPases Cdc42 and Rac2 (Pollard et al., 2000). Cdc42 and Rac2 form complexes with the WASP family proteins and the Arp2/3 complex to promote the formation of free barbed ends which in turn initiate cytoskeletal actin polymerisation in the lamella region. This mechanism of actin polymerisation provides a useful framework for the understanding of the signalling of actin dynamics in the living cell during motility; however, it is far from complete. Recently an alternative mechanism, which is PI3K-independent, for actin polymerisation in human neutrophils has been proposed (Chodniewicz and Zhelev, 2003b). Our findings are in agreement with the latter mechanism since LY294002 did not appear to have any effect on IP-10, I-TAC or Mig-induced actin polymerisation. Further investigation is required to determine involvement of these proteins in actin polymerisation in intestinal myofibroblasts.

Moreover, since LY294002 does not inhibit class II PI3K, a possible role for PI3K C2α and or PI3KC2β in actin polymerisation and stress fiber formation should not be excluded.

Effect of pertussis toxin in chemokine induced actin polymerisation in intestinal myofibroblasts

A plethora of studies examining various cellular responses have revealed that agonist activation of heterotrimeric G protein-linked receptors can result in signalling to the small G-protein Rho. An intriguing question that remains to be answered is how GPCRs signal to and activate Rho. Both the nature of the G protein subunits that mediate this response and the molecular mechanisms involved are under intensive study. Even though chemokine receptors, which are Gai coupled receptors, are known to be able to activate Rho the majority of GPCR-induced, Rho-mediated effects on the cytoskeleton are pertussis toxin insensitive (Sah et al., 2000). Consistent with the signalling events, actin polymerisation and stress fiber formation were found to be insensitive in intestinal myofibroblasts.

Most of the GPCRs agonists shown to activate Rho were thought to be coupled to Gq-mediated signalling pathways. However there is evidence to suggest that G proteins of the pertussis toxin-insensitive $G_{12/13}$ family also are able to control Rho-dependent stress fiber formation. Microinjection of either $G\alpha_{12}$ or $G\alpha_{13}$ into fibroblasts resulted in stress fiber formation (Buhl et al., 1995). Stimulation of $G_{12/13}$-dependent MLC phosphorylation and platelet shape change by thromboxane A_2 receptors are known to be dependent on both Rho and Rho kinase (Klages et al., 1999). Because coupling to Gq leads to activation of PKC, it is likely that this

kinase might regulate Rho function. PKC also has been shown to phosphorylate $G\alpha_{12}$ and $G\alpha_{13}$ (Offermanns et al., 1996) providing the possibility of an additional level of Gq regulation of Rho signalling. Stress fiber formation signalling events downstream of the putative receptor(s) for IP-10, I-TAC and Mig are summarized in Figure 5.8.

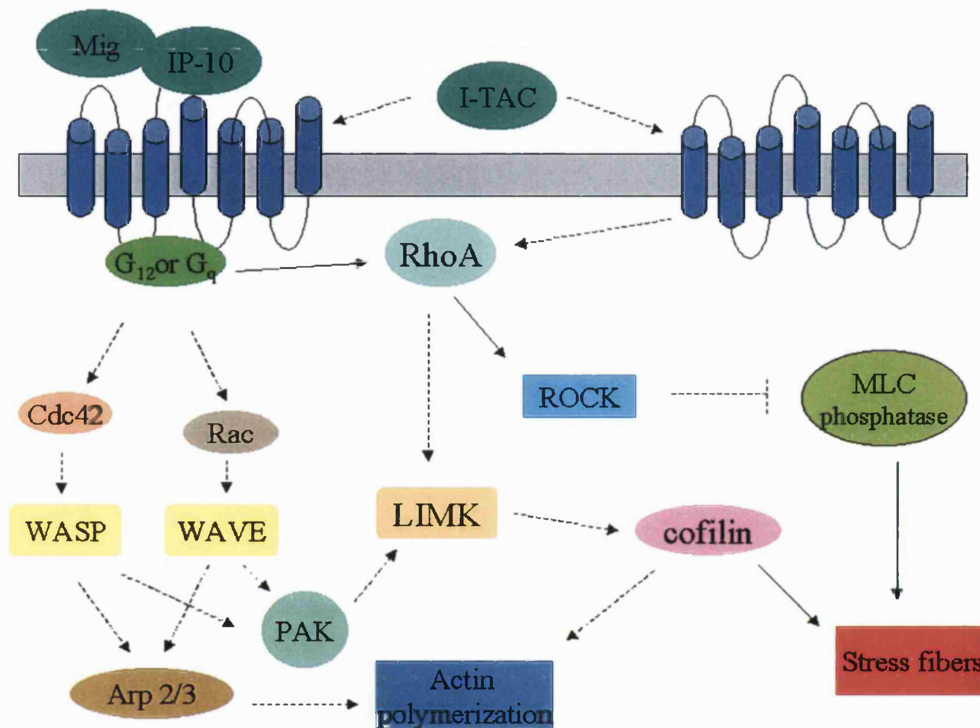

Figure 5.8 Proposed model of signalling pathways induced by IP-10, I-TAC and Mig in human intestinal myofibroblasts leading to actin polymerisation and stress fiber formation. The solid lines represent pathways, which have been demonstrated in this section to be involved in IP-10, I-TAC and Mig mediated stress fiber formation. Dotted lines provide additional potential pathways for stress fiber formation and actin polymerisation. Abbreviations: Arp2/3, actin related proteins 2/3 complex; MLC, myosin light chain; LIMK, LIM kinase, PAK, p21 activated proteins; RhoA Ras homology A; WASP, Wiskott-Aldrich syndrome proteins; WAVE, WASP family Verprolin-homologous proteins.

Chapter 6

6 Overall discussion and future directions

6.1 *Discussion*

Intestinal inflammation has traditionally been viewed as a process in which effector immune cells are the key mediators while the other mucosal cell types have been regarded as passive bystander targets. Progress in understanding the process of intestinal inflammation has led to a much broader and more integrated picture of the various mucosal components, a picture in which cytokines, chemokines, growth factors and adhesion molecules act as functional mediators. The existence of specialized communication pathways between non-immune and immune cells is now well documented, in which abnormal epithelial cell mediated T-cell activation during inflammation has attracted the most attention. Data presented here suggest that intestinal myofibroblasts may act in a similar way, since they are capable of expressing various co-stimulatory molecules. Interestingly, B7.1 expression in intestinal myofibroblasts occurs under the same conditions that favour B7.2 expression by intestinal epithelial cells, implying a complementary role for these two cell types in regulating activation of T-cells in the inflamed gut.

The importance of mesenchymal cells in morphogenetic processes and in the maintenance of the tissue integrity in the gut is now well established and underlined by the observation that these cells express important growth factors whose receptors are found on the epithelial cell surface. This study has provided evidence to support the notion that this cross-talk between these cells might be extended in order to encompass more interactions, such as chemokine-chemokine receptor expressed on the surface of the cells, adding to the emerging evidence

that all cell types populating the mucosa have an active role in intestinal immunity and inflammation. A receptor that binds the chemokines secreted by intestinal epithelial cells seems to be expressed on intestinal myofibroblasts, and this receptor was shown to activate various signalling pathways in these cells and markedly alters their phenotype. Myofibroblasts appear to be an integral part of a trimeric model consisting of intestinal myofibroblasts, T-cells and colonic epithelial cells that may have a role in modulating physiologic and pathologic mucosal inflammation.

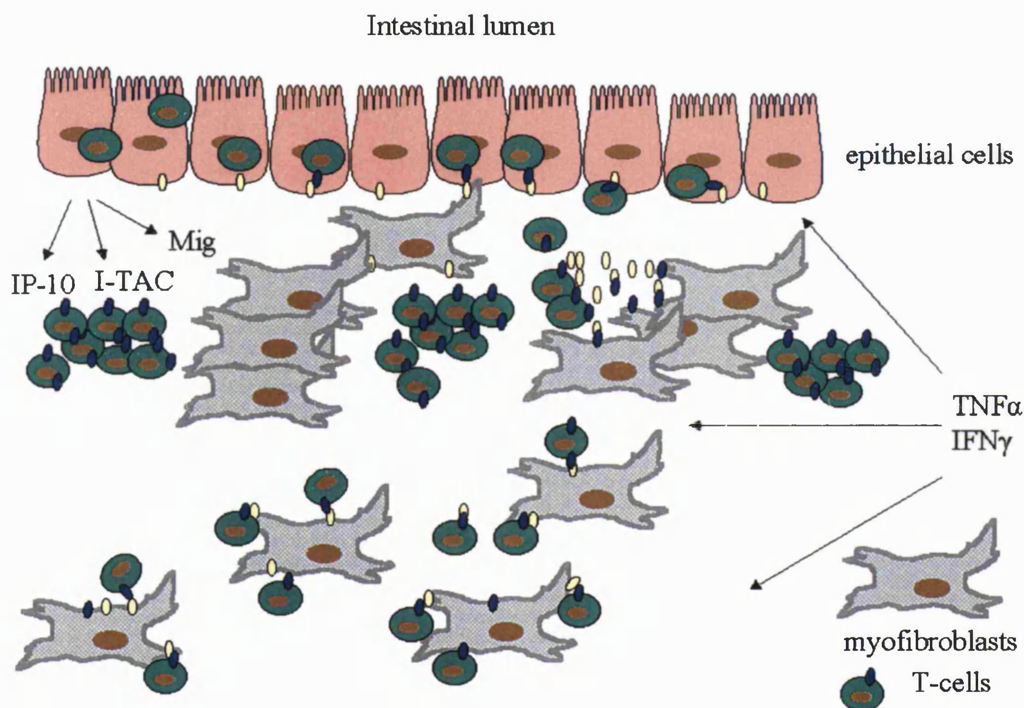

Figure 6.1 Trimeric model of interactions between myofibroblasts, epithelial cells and T-lymphocytes in the intestine. Pro-inflammatory cytokines induce expression of co-stimulatory molecules (represented in yellow) on intestinal myofibroblasts and colonic epithelial cells that interact with receptors of the CD28 family (represented in blue) on the surface of gut homing T-cells. IP-10, I-TAC and Mig produced by the intestinal epithelium chemoattarcts T-cells expressing CXCR3 which have to compete with myofibroblasts for chemokine binding.

An impairment of the integrity of the mucosal epithelial barrier is commonly observed in the course of various intestinal disorders including IBD, celiac disease and intestinal infections. If the wound is deep, the sub-epithelial tissues that contain interstitial substance, blood vessels, nerves, and fibroblasts must also be reconstituted. Wound healing is an interactive process that involves soluble mediators, extracellular matrix components, resident cells and infiltrating leukocyte subtypes, which participate differentially in the classically defined three phases of wound healing: inflammation, tissue formation, and tissue remodelling. Understanding the network of wound healing requires a profound analysis of all soluble mediators and adhesion factors involved in the recruitment and trafficking of the different cell types during the inflammatory reaction. The presence of a receptor on intestinal myofibroblasts, which binds T-cell chemoattractants is likely to have important implications in the cellular milieu in the lamina propria. Various studies have demonstrated the important role of chemokines for the accompanying inflammatory reaction as well as for repair processes during wound healing. However, the importance of chemokines during pathological wound-healing conditions has not been investigated and needs particular attention. It appears conceivable that chemokines could be exploited therapeutically, as major adjuvants to stimulate wound healing provided that the timely and spatially different expression patterns, as detected in physiological wound healing, are considered adequately. Therefore, the orchestrated processes of wound healing in the gastrointestinal tract with respect to treatment would certainly require a highly complex and sophisticated approach and should target chemokines as important traffic lights for migration of resident and inflammatory cells as well as essential regulators of repair mechanisms. Based on evidence in this study, chemokines

agonists and/or antagonists targeted against the CXCR3 ligands and their receptors could provide potential therapeutic targets in the treatment of gastrointestinal diseases.

6.2 *Future directions*

- The lack of commercially available antibodies at the time of this study against the various B7 family members limited the investigation of the expression of these molecules in intestinal myofibroblasts to the mRNA level. Since then antibodies have become available and should allow detection at the protein level and most importantly confirm cell surface expression of these molecules.
- The functionality of the B7 family members in intestinal myofibroblasts has not been tested. This can be achieved by stimulating T-cells that express the receptors for the B7 molecules by co-sedimenting them with activated intestinal myofibroblasts, and assessing the effect it has in downstream biochemical events in those cells.
- Assessment of the role of ICOS expression would be of great interest. ICOS appears to co-stimulate distinct effector functions in different immune responses, depending on factors such as the nature of the antigen encountered and localization and chronicity of the immune response. For example in the severe combined immunodeficiency transfer colitis model, ICOS expression is strongly associated with IFN-γ and IL-2 production. It would therefore be very interesting to determine whether intestinal

myofibroblasts expressing ICOS are capable of producing the same or different pattern of cytokines.

- Of equal importance would be to identify the signalling pathways downstream of ICOS in intestinal myofibroblasts. The ICOS cytoplasmic tail contains a YMFM motif that binds the p85 subunit of PI3K analogous to the YMNM motif of CD28. It will be essential therefore to examine in detail the activation of the PI3K/PKB3 pathway as well as activation of the various MAPK signalling pathways in response to ICOS ligation.
- A question that remains unanswered is the identity of the receptor responsible for eliciting the signaling events in intestinal myofibroblasts. Advances in small interfering RNA (SiRNA) directed against CXCR3 should all allow to verify if this is the receptor or not. Furthermore, the use of blocking peptides against the various G-protein subunits could determine the nature of the G-proteins that couple to this receptor. Finally the use of inhibitors like PD98059 (MEK inhibitor), RO320432 (PKC inhibitor) will help further elucidate the signalling events in intestinal myofibroblasts.
- Further analysis is required to resolve the exact involvement of the different PI3K isoforms in the different signaling events. SiRNA could again be a useful tool to overcome the absence of PI3K isoform specific inhibitors.
- Studies in other systems suggest that other chemokines and chemokine receptors are likely to be expressed in intestinal myofibroblasts. Moreover, it would be imperative to determine expression of chemokine receptors and their ligands from patients with IBD versus normal controls. Signal

transduction events downstream of those receptors can be determined and compared to our knowledge of chemokine signalling in other cell types. Regulation of expression of these receptors by pro-inflammatory cytokines should also be addressed.

- Even though, actin polymerisation is required it is not sufficient for cell migration. Additional chemotactic experiments need to be carried out in order to establish a role for the CXCR3 ligands in the motility of intestinal myofibroblasts.
- The effect of the chemokines on production of various ECM proteins by intestinal myofibroblasts including type IV collagen and β1- and γ1-laminin and fibronectin should be examined to determine a possible role for these proteins in intestinal fibrosis.

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