The role of the IL23/IL17 pathway in Inflammatory Bowel Disease

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A thesis submitted in partial fulfilment of the requirements for degree of Doctor of Philosophy, Trinity Term 2011

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Abstract

The aetiology of IBD is unknown, but available evidence suggests that an aberrant immune response towards the commensal microbial flora is responsible for intestinal inflammation in genetically susceptible individuals. Studies from animal models of intestinal inflammation have greatly advanced our understanding of the immunological basis of IBD. However, translation of results from animal research into human studies is essential in order to improve treatment options and patient quality of life. In this thesis we present the successful introduction of translational studies on human tissue in our laboratory. In particular, we evaluated the role of the IL23/IL17 pathway in the human immune response and its role in IBD. IL23-driven inflammation has been primarily linked to its activity on Th-17 cells; however, work from our laboratory has identified a novel population of IL23-responsive ILC, which are responsible for innate colitis in mice. Here we have analyzed the role of IL23-responsive innate cells in IBD. Our results show increased expression of Th-17 signature genes amongst intestinal $CD3^-$ cells in patients with IBD. Furthermore, we observed a marked and selective increase in IL17 producing $CD56^-$ ILC in the inflamed intestine of patients with CD. ILC may contribute to intestinal inflammation through secretion of cytokines, such as IL17A and IL17F, and recruitment of other inflammatory cells, representing a novel tissue-specific target for the treatment of IBD. In addition, we present here our preliminary data on the characterization of human intestinal and systemic DC populations. In particular, we aimed to evaluate if in the context of the intestinal microenvironment DC develop specific regulatory features, as observed in murine $CD103^+$ DC. We show that human intestinal DC populations exhibit specific regulatory properties, such as expression of genes associated with TGF-β and RA activity. Furthermore, $CD103^+$ DC are present in the human gut and are characterized by tolerogenic markers. Remarkably, patients with IBD have reduced frequencies of intestinal $CD103^+$ DC, which display a more pro-inflammatory phenotype. Alteration in DC subset composition and functional activity may result in a distort balance between immune effector and regulatory responses, promoting the development of intestinal inflammation.

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List of abbreviations

ALDH	Aldehyde dehydrogenase
APC	Antigen presenting cells
AHR	Aryl hydrocarbon receptor
BCR	B-cell receptor
BM	Bone marrow
BMI	Body mass index
CD	Crohn's disease
cDC	Conventional DC
CIA	Collagen induced arthritis
CLR	C-type lectin receptors
CRC	Colorectal cancer
CRP	C-reactive protein
CRTH2	chemoattractant receptor–homologous molecule expressed on T_H2 lymphocytes
CTLA4	Cytotoxic T lymphocyte antigen 4
DC	Dendritic cells
DSS	Dextran sulphate sodium
EAE	Experimental autoimmune encephalomyelitis
ER	Endoplasmic reticulum
FACS	Fluorescence activated cell sorting
FAE	Follicle-associated epithelium
Flt3	FMS-like tyrosine kinase 3
Foxp3	Forkhead box P3
GALT	Gut associated lymphoid tissue
GATA3	GATA-binding protein 3
G-CSF	Granulocyte-colony-stimulating-factor
GITR	Glucocorticoid-induced TNF receptor–related protein
GM-CSF	Granulocyte-macrophage colony-stimulating-factor
GWAS	Genome-wide association scans
H&E	Haematoxylin and eosin
HLA	Human leukocyte antigen
IBD	Inflammatory bowel disease
ICAM1	Intercellular adhesion molecule-1
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IL7Rα	IL7 receptor α
ILC	Innate lymphoid cells
ILF	Isolated lymphoid follicles
iNKT	Invariant NKT
IPEX	Immunedysregulation polyendocrinopathy enteropathy X-linked syndrome
IRF	Interferon regulatory factor
iTreg	Induced Treg
JAK	Janus kinase
Lin	Lineage markers
LP	Lamina propria
LPMC	Lamina propria mononuclear cells

LPS	Lipopolysaccharide
LRR	Leucin-rich repeat
LT	Lymphotoxin
LTBP	Latent TGF beta binding protein
LTi	Lymphoid Tissue inducer
LTo	lymphoid tissue organizers
MAdCAM1	Mucosal-addressin cell adhesion molecule-1
MAIT	Mucosal associated invariant T
MALT	Mucosal associated lymphoid tissue
M-cells	Microfold-cells
MHC	Major histocompatibility complex
MLN	Mesenteric lymph nodes
MMP	Matrix metalloproteinases
MyD88	myeloid differentiation primary response gene (88)
NFAT	Nuclear factor of activated T-cells
NF-kB	nuclear factor kB
NK	Natural killer
NKR	NK cell receptors
NLR	NOD-like receptors
NOD	Nucleotide-binding oligomerization domain
nTreg	Naturally occurring Treg
PAMP	Pathogen-associated molecular patterns
PB	Peripheral blood
PBMC	Peripheral blood mononuclear cells
pDC	Plasmocytoid DC
PLAT	Tissue plasminogen activator
PP	Peyer's patches
PRR	Pattern recognition receptors
RA	Retinoic acid
RAR	RA receptor
RIG	Retinoic acid inducible gene
RLR	RIG -I-like receptors
RORγ-t	RAR-related orphan receptor gamma-t
RT	Room temperature
RXR	retinoid X receptor
qPCR	Quantitative real time polymerase chain reaction
SEM	Standard error of the mean
Smad	Mothers against decapentaplegic Drosophila homolog
SNPs	Single nucleotide polymorphisms
SOCS3	Suppressor of cytokine signalling 3
STAT	Signal transducer of activated T cells
T-bet	T-cell-specific T-box transcription factor
TCR	T-cell receptor
Th	T helper
TIR	Toll/IL1R
TGF	Transforming growth factor
TGU	Translational gastroenterology unit
TLR	Toll-like receptors
TNBS	Trinitrobenzenesulfonic acid
TNF	Tumour necrosis factor

TRANCE	TNF-related activation-induced cytokine
Treg	T regulatory cells
TREM-1	Triggering receptor expressed on myeloid cells 1
TRIF	TIR-domain-containing adapter-inducing interferon
TSLP	Thymic stromal lymphopoietin
UC	Ulcerative colitis
UPR	Unfolded protein response
VCAM1	Vascular adhesion molecule-1
WBC	White blood cells

Contents

Chapter 1- Introduction

Our immune system has evolved in order to protect us against invasion by infectious agents and its vital role in host survival and well-being is highlighted by the occurrence of severe opportunistic infections in immune-suppressed or immune-deficient individuals. On the other hand, the immune system faces the delicate task of discriminating between self or innocuous antigens and harmful pathogens in order to efficiently mount protective immune responses only against the latter, avoiding any unnecessary tissue damage for the host. When tolerance towards self or harmless antigens is compromised, aberrant immune reactions are responsible for the occurrence of pathologies, such as allergies, autoimmune diseases and chronic inflammatory disorders.

The players of the immune system are the leukocytes, which can be divided in myeloid cells and lymphoid cells. Myeloid cells are represented by granulocytes, monocytes, macrophages and dendritic cells (DC) and are the orchestrators of the innate immune response, our first line of defence against infection. Lymphoid cells include two main cell populations, B-cells and T-cells, which are responsible for the adaptive immune response, the ability of the immune system to specifically recognize infectious agents and to develop immunological memory, providing enhanced protection against re-infection. After activation B-cells differentiate into plasma cells and secrete antibodies. On the other hand, T-cells upon activation undergo differentiation into either cytotoxic T-cells, which are able to kill infected and transformed cells, or helper T-cells that assist the maturation and activation of other immune cells in different types of responses.

1.1 Organisation of the lymphoid tissue

The lymphoid tissue is organised in primary lymphoid structures, where lymphocytes are produced and undergo maturation -the bone marrow (BM) and the thymus- and secondary lymphoid structures, where naïve lymphocytes encounter their specific antigen presented by antigen presenting cells (APC) and differentiate into effector cells. The secondary lymphoid structures are represented by the peripheral lymph nodes, the spleen and the mucosal associated lymphoid tissue (MALT) [1].

The lymph nodes receive the lymph, an extracellular fluid that drains from the surrounding tissue through the afferent lymphatic vessel and contains antigens and APC. B-cells and T-cells are organised in specialized regions of the lymph node: the B-cell primary and, after antigen challenge, secondary follicles with germinal centres, and the paracortical T-cell zones, which also contain DC, the specialized APC of the immune system. Naïve lymphocytes continuously re-circulate from the blood to the lymph nodes. In presence of an infection, APC carrying infectious antigens migrate from the tissue to the lymph node, where they induce proliferation and differentiation of antigen-specific lymphocytes into effector cells, which can leave the lymph node through the efferent lymphatic vessel, re-enter the bloodstream and contribute to fight the infection.

By contrast, APC enter the spleen through the blood vessels and they encounter antigen-specific B-cells and T-cells in the specialized region of the white pulp, the B-cell corona and the periarteriolar lymphoid sheath.

The MALT is a specialized secondary lymphoid tissue that collects antigens from the mucosal surfaces of the body, the respiratory and gastrointestinal tracts and represents the largest lymphoid tissue in the body. The architecture of the gut associated lymphoid tissue (GALT) will be discussed in details below.

Finally, so called tertiary lymphoid structures can develop in the presence of chronic inflammation. They are characterized by an organisation in B-cell zones and T-cell areas, which resembles the architecture of secondary lymphoid organs. It is envisaged that tertiary lymphoid structure formation has a protective function at sites of pathogenic infections, but may be detrimental in perpetuating inflammation and tissue damage in the presence of autoimmunity [2].

1.2 Intestinal immunity

The gastrointestinal mucosa represents the largest surface of interaction with the external world in the human body, with an area of around 200 m^2 [3]. The gut lumen is continuously exposed to both food antigens introduced with the diet and bacterial antigens of the extremely rich and diversified resident microbial flora. In fact, it is calculated that around 10^{13}-10^{14} bacteria reside in the human intestine, with increasing distal concentration and the highest bacterial load in the terminal ileum and in the colon [4]. The intestinal commensal flora is necessary for many beneficial processes for the host, such as the metabolism of non-absorbed food components and the production of vitamins. On the other hand, the gut lumen may also represent the route of entry for pathogenic microorganisms that can lead to tissue damage. It is clear then that the intestinal immune system faces a very delicate task: on one hand tolerance must be maintained towards food and "good" bacterial antigens, but on the other hand a rapid and effective immune response needs to be mounted against pathogenic bacteria, which may enter the gut. This balance is achieved through the presence of a very efficient epithelial barrier and a complex and specialized immune system in the gut.

1.2.1 Anatomical organisation of the intestinal immune system

The GALT is composed by lymphocytes and other immune cells scattered through the intestinal mucosa and by organised lymphoid structures, such as Peyer's patches (PP) in the small intestine, isolated lymphoid follicles (ILF) in both large and small intestine and mesenteric lymph nodes (MLN) [5] (Figure 1.1).

PP have a dome-like structure and contain large B-cell follicles separated by T-cell zones. A specialized epithelium, the follicle associated epithelium, covers PP and is characterized by the presence of microfold (M)-cells that can uptake luminal antigens

and transfer them through the basal membrane into the dome of PP. Intact antigens are then processed by specialized APC, mainly DC, and presented to naïve T-cells, initiating the adaptive immune response. Primed T-cells migrate to the MLN in the afferent lymphatic vessels and from the MLN they enter the blood through the thoracic duct. Priming of naïve T-cells can also occur in the MLN by antigen loaded DC, that have received the antigen from M cells in PP or by direct antigen sampling through the intestinal epithelium and have migrated to the MLN [6]. From the systemic circulation primed T-cells can finally home back to the intestinal mucosa where they accumulate. Migration of lymphocytes to the gut is enabled by the expression of molecules, such as the α4β7 integrin, whose ligand mucosal-addressin cell adhesion molecule-1 (MAdCAM1) is highly expressed on intestinal endothelial cells and the chemokine receptor CCR9, which binds to CCL25 in the small intestine [7, 8].

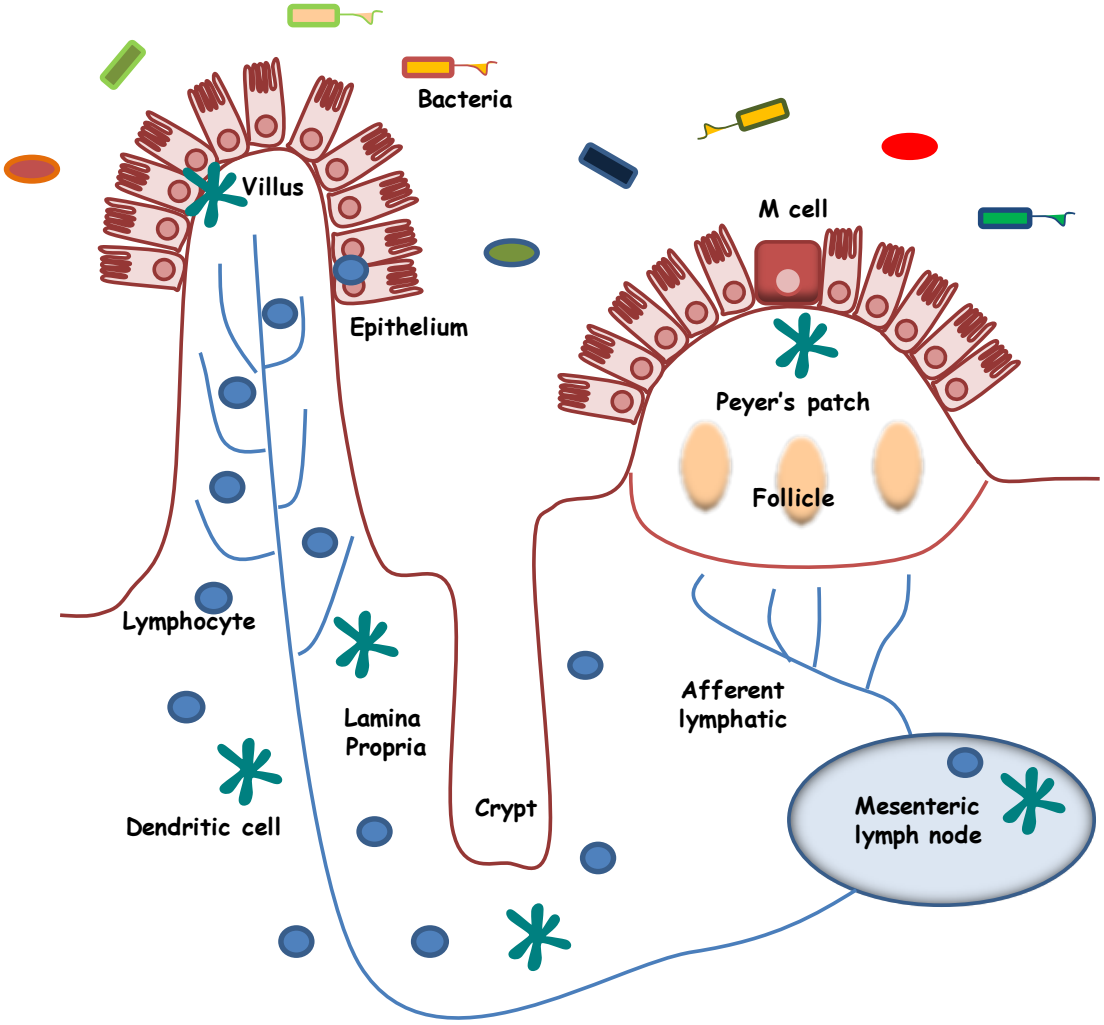


Figure 1.1 Anatomical organisation of the intestinal immune system

DC can receive the antigen from M-cells in PP or by direct sampling of the luminal content through the intestinal epithelium. Antigen-loaded DC migrate to the MLN through the afferent lymphatics where they can present the antigen to T-cells leading to T-cell activation. T-cell priming can also occur in the LP. Primed T-cells enter the systemic circulation through the thoracic duct and finally home back to the intestinal mucosa where they can accumulate.

Adapted from Mowat. Nature Reviews Immunology 3: 2003

1.2.2 The innate immune response

Mucous layer and intestinal epithelial barrier

The first physical barrier that intestinal bacteria and food antigens encounter on the mucosal surface is represented by the mucous layer that covers the intestinal epithelium. Animal studies have shown that this layer is thicker and more homogenous in the stomach and colon, while it appears to be less represented and organised in the small bowel [9]. Mucous is organised in an inner firm layer and an outer loose layer that are produced by polymerization of the gel-forming mucin MUC2, which is secreted by goblet cells and expands in the lumen due to its capacity to bind water. The resulting mucin net is firm and dense in the inner layer, which adheres to the epithelium and is not usually colonized by bacteria. Probably due to the proteolytic activity of both host and bacterial enzymes the outer mucous layer appears to be less organised and more permeable and it is inhabited by commensal bacteria that find important nutrients in the mucin glycans [10]. The importance of mucus in preventing bacterial break-through and intestinal inflammation has been highlighted by studies in $MUC2^{-/-}$ mice that develop colitis and have increased risk of colorectal cancer (CRC), associated with the presence of bacteria in direct contact with the intestinal epithelium [11, 12].

In contact with the inner layer of mucous layers it is the intestinal epithelium that forms the second line of defence to bacterial invasion [13]. The intestinal epithelium mainly consists of enterocytes and specialized epithelial cells, such as goblet cells and Paneth cells, with the latter only present at the base of the crypts in the small intestine. Integrity of the epithelial barrier is maintained by intercellular tight junctions and desmosomes that create an efficient physical barrier to bacterial invasion. Moreover, epithelial cells can secrete a number of bactericidal agents that include defensins (α-

defensins produced by Paneth cells and β-defensins produced by most epithelial cells) and C-type lectins, such as RegIII-γ [14-16]. Some of these agents are produced constitutively, while others are induced by the recognition of bacterial components by pattern recognition receptors (PRR) expressed both extra- and intra-cellularly in epithelial cells [17]. These antibacterial products are then trapped in the inner mucous layer where they contribute to limiting any bacterial-epithelial contact.

Phagocytes

Due to the huge numbers of commensal bacteria that are present in the gut lumen, it is still possible for some microbes to invade the mucous and epithelial barrier and enter the lamina propria (LP), the connective tissue that underlies the intestinal epithelium. In these circumstances, the first cells that intervene to limit bacterial spread are macrophages that are largely represented in the LP and often in close contact with the epithelium. Macrophages are professional phagocytes, that are capable of engulfing and killing bacteria trough mechanisms that involve acidification and production of reactive oxygen species, antimicrobial products (defensins and cationic proteins), and enzymes (lysozyme, hydrolases) [18]. They are classically activated by interferon (IFN)-γ stimulation, but alternative activation can be induced by T helper (Th)-2 cytokines, such as interleukin (IL)4 and IL13 [19-21]. Macrophages can also produce a range of inflammatory cytokines in response to bacterial recognition, initiating an inflammatory response. However, these cells show a more tolerant behaviour in the intestine compared to other body tissues. Intestinal macrophages show no or very low expression of most innate receptors, such as the lipopolysaccharide (LPS) receptor CD14, Fcγ, Fcα, Toll-like receptors (TLR)1-5 and triggering receptor expressed on myeloid cells 1 (TREM-1) [22, 23]. Recent data have also suggested that intestinal macrophages can induce the differentiation of T regulatory cells (Treg) in mice [24].

These mechanisms can contribute to the tolerogenic environment of the intestine, where, due to the large exposure to bacteria, a fine balance between inflammatory responses and regulation is particularly crucial. Human intestinal macrophages are characterized by the expression of the surface marker CD11b and low expression of CD11c and MHCII in the colon, while they express higher levels of MHCII and low levels of CD11b and CD11c in the small intestine [25, 26].

Neutrophils can also be recruited to the LP in response to bacterial invasion breaching to the LP. These are short-lived cells that extravasate from the blood vessels in response to different chemotactic and inflammatory molecules and die soon after phagocytosis.

Pattern recognition receptors

Sensing of microbial antigens by immune cells, such as macrophages and DC, but also non-immune cells, such as epithelial cells and fibroblasts, is mediated by PRR that recognize conserved microbial components or pathogen-associated molecular patterns (PAMP). PRR include trans-membrane TLR and C-type lectin receptors (CLR), as well as intracytoplasmic receptors such as retinoic acid inducible gene (RIG)-I-like receptors (RLR) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLR). TLR represent the best characterized family of PRR and they recognize PAMP localized extracellularly or in the intracellular vacuoles, such as lysosomes and endosomes [27]. Their structure is characterized by the presence of a leucin-rich repeat (LRR) region, a trans-membrane domain and an intracytoplasmic Toll/IL1R (TIR) domain. Different members of this family are activated by a number of microbial components, including lipoproteins, LPS, CpG-DNA, ds-RNA, ss-RNA and flagellin [28]. TLR activation can lead to myeloid differentiation primary response gene (88) (MyD88) or TIR-domain-containing adapter-inducing interferon-β

(TRIF) dependent intracellular pathways that culminate in the induction of inflammatory gene transcription.

RLR are activated by viral DNA and RNA and their expression is induced by type I IFN and viral infections. NLR are characterized by a C-terminal LRR region, a central nucleotide binding domain and an N-terminal CARD, pyrin or BIR domain [28]. The NLR family members NOD1 and NOD2 recognize different components of the bacterial peptidoglycan and induce nuclear factor kB (NF-kB) activation, while other NLR contribute to the formation of the inflammosome that leads to caspase-activation and IL1 secretion [29-31].

PRR stimulation, through the activation of intracellular cascades, leads to the induction of gene-transcription and to the production of inflammatory mediators that contribute to an effective immune response against pathogens. Furthermore, PRR ligation stimulates the expression of co-stimulatory molecules on DC and macrophages that are required for efficient antigen presentation and T-cell activation.

Natural killer cells

Natural killer (NK) cells are innate cells that originate in the BM from a common lymphoid progenitor. Distinct from B and T lymphocytes, NK cells do not undergo the genetic rearrangement necessary for the expression of antigen-specific receptors, but they express germline-encoded activating and inhibitory NK cell receptors (NKR) that mainly bind major histocompatibility complex (MHC) class I molecules. NK cells are activated when ligation of activating NKR prevails over inhibitory signals [32, 33]. Their effector function is mainly characterized by production of a wide range of cytokines and killing of target cells, such as virus-infected and transformed cells, through mechanisms involving granzyme, perforin and death receptor ligand expression. NK cells can also be activated directly by cytokine stimulation. In

particular, type I IFN, IL12 and IL18 stimulate the production of large amounts of IFN-γ by NK cells, while IL15 or IL2 together with IL12 have been shown to induce IL10 secretion [34].

Human NK cells are classically subdivided in two subsets based on the expression of the adhesion molecule CD56. $CD56^{dim}$ cells are the more represented subset in the blood and are characterized by a cytotoxic phenotype, while $CD56^{bright}$ are mainly found in secondary lymphoid organs and produce large amounts of IFN-γ. Recent studies have suggested that these two populations represent two stages of NK maturation, with the $CD56^{dim}$ representing the most differentiated cells [35].

LTi/LTi-like cells

Lymphoid Tissue inducer (LTi) cells are involved in the organogenesis of secondary lymphoid structures, such as lymph nodes and PP of the small intestine [36]. Murine LTi cells do not express lineage markers (Lin), they are $CD45^{+}$, c-kit^{+} and express the IL7 receptor α (IL7Rα or CD127) and lymphotoxin (LT)α1β2 [37]. Both $CD4^{+}$ and $CD4^{-}$ LTi cells have been identified in murine embryonic and adult tissues [38, 39]. Binding of LTα1β2 on the LTβ receptor on mesenchymal cells (lymphoid tissue organizers, LTo) induces expression of adhesion molecules, such as intercellular adhesion molecule-1 (ICAM1), vascular adhesion molecule-1 (VCAM1) and MAdCAM1 and secretion of the chemokines CCL19, CCL21 and CXCL13, which lead to LTi clustering, leukocyte recruitment and organisation of haematopoietic cells in secondary lymphoid structures [1]. Human LTi cells have been recently identified in foetal tissue as $Lin^{-}CD45^{int}CD127^{+}$c-$Kit^{+}$ cells, but distinct from their murine counterparts do not express CD4 [40]. Furthermore, both murine and human LTi cells are characterized by the expression of the transcription factor RAR-related orphan receptor gamma-t (RORγ-t). Mice deficient for RORγ have been shown to lack LTi

cells and do not develop lymph nodes and PP [41]. Similar findings are observed in mice lacking the transcription factors Id2 and Ikaros [42, 43]. Moreover tumour necrosis factor (TNF)-related activation-induced cytokine (TRANCE) and IL7-R are required for lymph node and PP development respectively [44, 45]. Both TRANCE and IL7 induce the expression of LTα1β2 on LTi cells and their differential requirement in lymph node and PP development might reflect their expression in the different microenvironments. Accordingly, $Lt\alpha^{-/-}$ mice, $Ltb^{-/-}$ and $Ltbr^{-/-}$ mice all present disrupted secondary lymphoid organ development [46-49].

Beside their role in development, LTi-like cells have been found in adult tissue and in particular in the intestine in both mice and men [40, 50]. Interestingly, recent data show that LTi and LTi-like populations respond to the pro-inflammatory cytokine IL23 and produce Th-17 signature cytokines, such as IL17A and IL22, suggesting they might play a role in both response to infections and autoimmunity (further discussed below) [40, 51].

Unconventional T-cells

These cells have a distinct developmental origin from conventional T-cells and are characterized by only having a limited antigen discrimination and specificity. They preferentially home to mucosal sites and take part in early immune responses against pathogens, suggesting they represent an evolutionary-conserved, ancient innate mechanism of protection [52].

i. γδ-T-cells

γδ-T-cells are non-conventional, innate-like T-cells that develop in the thymus in parallel to αβ-T-cells. They represent a minority of T-cells in blood, spleen and peripheral lymph nodes, but they are enriched in epithelial tissues, and particularly in the intestine. Variability of the γδ-T cell receptor (TCR) is generally more limited

than the corresponding αβ-TCR, and cells with homologous γδ-TCR seem to accumulate in specific tissues, representing invariant or semi-invariant TCR populations [53]. γδ-T-cells recognize non-peptide antigens expressed on stressed cells through ligation of the TCR, but also TLR (TLR1, TLR2 and dectin-1) and NKR, such as NKG2D [54-56]. γδ-T-cells can exert extremely different effector functions, depending on their intra-thymic developmental pathway and their peripheral activation. They can kill infected or tumour cells, through FAS-mediated mechanisms or the production of perforin and granzymes, and they can secrete a large range of cytokines, such as IFN-γ, TNF-α, IL17A, but also IL4, IL5, IL13, suppressive cytokines, such as IL10 and TGF-β, and growth factors [53, 57-61].

ii. iNKT-cells

Invariant NKT-cells (iNKT-cells) or type I NKT-cells develop in the thymus and are characterized by an invariant TCR, formed by the Vα24 chain (Vα14 in mice) associated with a limited range of Vβ chains [62, 63]. iNKT-cells recognize glycolipid antigens presented by the MHC-related molecule, CD1d [64]. In contrast to MHC class I and II molecules, CD1d is non polymorphic and can only present a restricted set of antigens. iNKT-cells represent less than 0.2% of total blood lymphocytes and they preferentially home to mucosal sites [65].

iii. MAIT-cells

Mucosal associated invariant T (MAIT)-cells also develop in the thymus and are characterized by the expression of a semi-invariant TCR, which results form the Vα7.2-Jα33 rearrangement associated Vβ2 and Vβ13 chains [63, 66]. MAIT-cells are $CD8^{+}$ or $CD8^{-}CD4^{-}$ and $CD161^{high}$, and are selected by the MHC-related non polymorphic molecule MR1 [67, 68]. The nature of the antigens presented by the MR1 molecule is currently unknown. MAIT-cells represent 1-4% of total blood T-

cells and they are enriched in the gut and lungs [66, 69].

Dendritic cells

DC are specialized APC that are responsible for the initiation of an adaptive immune response, representing the link between intestinal innate and adaptive immunity.

DC are classically subdivided into plasmocytoid DC (pDC) and conventional DC (cDC). pDC require an inflammatory or microbial stimulus to develop a typical DC form and function, they are characterized by the secretion of large amounts of type I IFN and take part in anti-viral responses. cDC already have DC form and function at steady state and they can be further classified on the base of their migratory behaviour and on the expression of different surface markers [70]. Migratory DC, such as Langerhans cells in the skin, are found in peripheral tissues and after sampling and processing antigens migrate to the regional lymph nodes through the afferent lymphatics. By contrast, lymphoid tissue-resident DC reside in secondary lymphoid organs, such as spleen and lymph nodes, where they contribute to antigen presentation and T-cell activation. On the basis of their maturation state, DC can be also divided into mature and immature DC. Immature DC mainly exert phagocytic activity in the periphery. Upon microbial recognition or inflammatory cytokine exposure, DC undergo maturation, which consists of increased expression of MHC and co-stimulatory molecules on their cell-membrane which optimize their APC function [71].

Intestinal DC constantly migrate from the LP and from the organised lymphoid structures of PP and ILF to the MLN through a CCR7 mediated mechanism, and there they present antigens to T-cells [72, 73]. Here they induce the expression on T-cells of gut homing receptors, such as CCR9 and α4β7 integrin, allowing the recirculation of primed T-cells to the intestine [73, 74]. Nevertheless, under homeostatic conditions,

intestinal DC have a characteristically tolerogenic phenotype and mainly induce Treg functions. This observation has been primarily linked to the production of anti-inflammatory cytokines by gut DC. In fact, small intestinal DC have been shown to constitutively express IL10 and transforming growth factor (TGF)-β and to induce the differentiation of IL10-producing Treg [75]. This regulatory activity is probably induced by micro-environmental factors, such as the presence of high concentrations of TGF-β, retinoic acid (RA) and thymic stromal lymphopoietin (TSLP) produced by intestinal epithelial cells (IEC) [76]. Recent studies in mice have led to the identification of different subsets of migratory and tissue-resident intestinal DC with regulatory and inflammatory properties.

i. $CD103^+$ DC

A specific subset of sentinel DC, characterized by the expression of the α_E integrin CD103, has been identified in the murine GALT, in PP, MLN and in both the small bowel and colon LP, where $CD103^+$ cells represent the majority of DC [77]. By contrast, $CD103^+$ DC are only found in very low frequencies in the spleen. The only known ligand for CD103 is E-cadherin, which is expressed on the baso-lateral membrane of IEC and may therefore be responsible for their accumulation in the gut. Studies conducted in our laboratory and others have shown that murine $CD103^+$ DC, compared to their $CD103^-$ counterparts, are strong inducers of Forkhead box P3 $(Foxp3)^+$ Treg differentiation [77-80]. This induction is dependent on the presence of RA, a metabolite of vitamin A, which can be metabolized by $CD103^+$ DC through their preferential expression of the RA converting enzymes, aldehyde dehydrogenase (ALDH)2. Moreover, $CD103^+$ DC express higher levels of *tgfb2* mRNA and enzymes involved in the conversion of latent TGF-β into its active form (tissue plasminogen activator, *plat*, and latent TGF beta binding protein 3, *ltbp3*). $CD103^+CX3CR1^-$ DC

have been shown to differentiate from the common-DC progenitor via FMS-like tyrosine kinase 3 (Flt3) activation, while $CD103^{-}CX3CR1^{-}$ DC derive from $Gr1^{hi}$ monocytes [81, 82].

ii. E-cadherin^{+} DC

We have recently identified a population of inflammatory E-cadherin^{+} DC, that accumulate in the colon and MLN in colitic mice [83]. E-cadherin^{+} DC have a pro-inflammatory phenotype, expressing high levels of many TLR, *Ccr6, Ccr2* and lower levels of expression of the regulatory genes *tgfb2, plat, Aldh1a2.* Moreover, they secrete high amounts of inflammatory cytokines and chemokines after stimulation. Adoptive transfer of BM-derived E-cadherin^{+} DC resulted in the exacerbation of T-cell mediated colitis, associated with increased frequencies of inflammatory Th-17 cells. Like $CD103^{-}CX3CR1^{+}$ DC, E-cadherin^{+} DC also derive from $Gr1^{high}$ inflammatory monocytes under granulocyte-macrophage colony-stimulating-factor (GM-CSF) stimulation, while TGF-β down-regulates E-cadherin expression on BM precursors. The *in vivo* relevance is revealed by an increase in E-cadherin^{+} DC in TGF-$\beta^{-/-}$ mice prior to signs of intestinal inflammation, suggesting that TGF-β can limit the accumulation of this inflammatory subset under homeostatic conditions.

iii. Human intestinal DC

The study of intestinal human DC has been hampered by the low availability of human tissue and the difficult isolation of these cells from the gut, which requires long enzymatic digestion protocols. Most studies on human DC have therefore relied on blood DC, which are more easily available but are mainly represented by immature DC and pDC. DC have been also generated in *vitro* from BM and umbilical cord blood $CD34^{+}$ haematopoietic precursors or $CD14^{+}$ monocytes. However, the *in vivo* relevance of these routes of DC generation remains controversial [84]. The literature

data on human intestinal DC is rendered more confused by the use of different markers in different studies, which have led to controversial results. Recently, a population of $CD11c^+$ human leukocyte antigen (HLA)-DR^+ cells that lack the markers for other cell lineages and show morphological and functional properties of DC, have been identified by flow cytometry in colon preparations of healthy human subjects. Immature DC, characterized by high endocytic activity and low expression of maturation markers, such as CD83, CD86, CD80 and CD25, were recovered directly after enzymatic digestion of the colonic tissue. Interestingly, $CD11c^+$HLA-DR^+ DC with a more mature phenotype were found to migrate out of the digested colonic tissue in the following 24 hours and, in accordance with their maturation state, showed the ability to induce allogeneic mixed leukocyte reactions [85].

In accordance with the identification in mice of migratory $CD103^+$ DC, recent studies have identified $CD103^+$ DC in the human MLN. These cells share with their murine counterpart the ability to induce CCR9 expression on T-cells in an RA-dependent manner and to drive Treg differentiation [78, 86]. This successful translation of murine findings into human studies opens new possibilities for the development of therapeutic strategies.

1.2.3 The adaptive immune response

T-cells

T-cells are key players of the adaptive immune response and are characterized by the expression of an antigen-specific TCR complex. The TCR is composed of α and β chains or γ and δ chains, associated with CD3 molecules (γε and δε) ant the ζζ complex [87]. Most T-cells are characterized by a αβ-TCR and can be distinguished on the basis of their co-receptor expression in $CD8^+$ and in $CD4^+$ T-cells. $CD8^+$ T-cells recognize their specific peptides in association with self MHC class I molecules, which are widely expressed on nucleated cells. They recognize peptides mainly derived from intra-cytoplasmic pathogens, such as intracellular bacteria or virus, and they differentiate in cytotoxic effector cells, that are specialized in killing of target cells. On the contrary, $CD4^+$ T-cells recognize their specific antigen in association with MHC class II molecules, which are expressed on professional APC, mainly matured DC, macrophages and B-cells. However, thymic and IEC have also been shown to express MHC class II molecules and present to $CD4^+$ T-cells [88]. They recognize peptides that derive from both intracellular and extracellular bacteria, which have been sampled, processed and presented by APC, and they differentiate into different subsets of Th-cells in the presence of different stimuli, such as the presence of specific cytokines produced by innate cells in the microenvironment. These subsets differ largely in their cytokine production and function. The activation and differentiation of $CD4^+$ T-cells will be further discussed below.

B-cells

B-cells are lymphocytes that express the B-cell receptor (BCR) on their cell surface and when activated differentiate into plasma cells and produce antibodies that are soluble forms of the specific BCR. Similar to T-cells, B-cells can respond to a huge

variety of antigens, due to the high diversity of their specific BCR, obtained through V-(D)-J gene recombination during development, but also somatic hypermutation, which introduces point mutations in the variable regions of the BCR in mature B-cells [89, 90]. For activation B-cells require specific antigen recognition by the BCR together with co-stimulation by antigen-specific T-cells, which bind the antigen, associated with MHC class II molecules on the B-cells. T-cell independent activation of B-cells can also occur in response to some polysaccharide antigens and polymeric proteins [91, 92].

Naïve B-cells express immunoglobulin (Ig)M and IgD on their cell membrane, but upon maturation they undergo isotype switching, that leads to the secretion of functionally different antibodies, such as IgG, IgE and IgA, which differ on their heavy chain constant regions conferring specific effector functions [93].

B-cell responses in the intestine are characterized by the production of high amounts of IgA [94]. IgA dimers can be internalized by IEC through the polymeric Ig receptor expressed on their baso-lateral membrane and are secreted into the lumen as secretory IgA, where they can contribute to limiting bacterial and toxin attachment to the epithelial cells [95]. IgA producing cells mainly derive from the germinal centres of PP, but naïve B-cells can also be activated directly in the LP and the ILF [96-99]. Another subset of B-cells, deriving from peritoneal cells, is also found in the murine intestine, the B1-cells. The production of IgA from B1-cells is probably induced by antigen recognition, but differently from the production of IgA in PP, it does not require T-cell dependent activation [100, 101]. However, one study has suggested that B1-cells might not be present in the gut immune system in humans [102].

1.3 Lymphocyte development

All blood cells, including leukocytes, but also erythrocytes and platelets, originate in the BM from the same precursor, the pluripotent haematopoietic stem cell. The common myeloid progenitor and the common lymphoid progenitor originate from the pluripotent haematopoietic stem cell and will give rise to myeloid cells and to lymphoid cells, respectively. Further maturation of B-cells and T-cells occurs in specific environments and is driven by the presence of different growth factors or adhesion molecules expressed on specialized stromal cells. While B-cell development continues in the BM (at least until the stage of immature B-cells), T-lymphocytes develop in a dedicated lymphoid organ situated in the thorax, the thymus.

1.3.1 B-cell development

The different steps of maturation of B-cells in the BM are defined by the stage of rearrangement of the Ig gene regions [103]. The following populations can be distinguished: early pro-B-cells, that have rearranged the D-J regions or the heavy chain; late pro-B-cells, that have completed the V-D-J rearrangement of the heavy chain; large pre-B-cells, that express the pre-BCR (composed by the combination of the rearranged heavy chain with a surrogate light chain); small pre-B-cells, that have rearranged the V-J region of the light chain and immature B-cells, that express complete IgM on their cell-membrane. Only the latest stage of B-cell maturation is acquired in the periphery, whereas mature B-cells express both IgD and IgM.

1.3.2 T-cell development

As mentioned above, T-cells develop in the thymus, which is a lymphoid organ mainly composed of thymic epithelial cells, thymocytes (immature T-cells) and intrathymic DC [104].

Different steps of T-cell development in the thymus can also be distinguished based

on different rearrangement of the TCR [105, 106].

At earlier stages of differentiation thymocytes do not express the surface co-receptors, CD4 and CD8, and are therefore called double-negative cells. At this stage the rearrangement of the V-D-J gene regions of the β-chain occurs and if this rearrangement is productive, thymocytes express the pre-TCR, composed of a functional β-chain and a surrogate α-chain. This induces both CD4 and CD8 expression on the cell-surface in the double-positive cells, which rearrange the V-J regions of the α-chain leading to the expression of the complete TCR. At the double-positive stage, thymocytes will also encounter positive selection, where only T-cells that recognize self MHC molecules on cortical epithelial cells will survive [107]. Positive selection is associated with apoptosis of the majority of double-positive cells that do not recognize self MHC molecules. Surviving thymocytes will then down-regulate one of the co-receptors and become single-positive cells ($CD4^+$ if they recognize class II MHC and $CD8^+$ if they recognize class I MHC). In order to prevent the development of auto-reactive T-cells, thymocytes are also subject to a process of negative selection, which occurs at both the double-positive and single-positive stages and is associated with the apoptosis of cells that strongly recognize self-antigens [108].

1.4 Activation and differentiation of $CD4^+$ T-cells

$CD4^+$ T-cells are activated when they encounter their cognate-antigen in association with HLA class II molecules on APC, in particular mature DC. However, TCR triggering on its own is not sufficient for sustained T-cell activation and in fact it can induce T-cell anergy, deletion and tolerance in the absence of a second stimulatory signal. Co-stimulation is mainly mediated by binding of CD28 on T-cells with its ligands CD80 or CD86 on mature DC, which induces the secretion of IL2 by T-cells and promotes their proliferation and survival [109]. Other T-cell-DC interactions are also involved in this second step of T-cell activation, which results from a fine-tuned balance of stimulatory and inhibitory signals. The inhibitory molecule cytotoxic T lymphocyte antigen 4 (CTLA4) is induced on T-cells by CD28 triggering and competes with CD28 in binding CD80 and CD86 [110]. Other molecules involved in co-stimulation are CD40-L, OX40, RANKL/TRANCE, glucocorticoid-induced TNF receptor–related protein (GITR) and CD27 on T-cells and their counterparts CD40, OX40L, RANK, GITRL and CD70 on DC [111]. Once activated, $CD4^+$ T-cells can then differentiate into different subsets of effector cells, depending on different factors, such as type of antigen, strength of TCR triggering, subset of presenting DC and cytokine milieu [112] (Figure 1.2).

1.4.1 T helper-1

Th-1 cells play a major role in the adaptive immune response to intracellular bacteria and virus, but have also been implicated in the pathogenesis of immune disorders, such as diabetes and Crohn's disease (CD) [113-117]. They are characterized by the production of IFN-γ and LT-α, but also produce high amount of IL2, and can co-secrete TNF-α [112]. Th-1 cells induce cytotoxic T-cell responses and can stimulate IgG switching in B-cells, leading to the production of IgG2a and IgG3 antibodies that

mediate opsonisation and phagocytosis [118]. Th-1 cells are normally present in the intestine in the steady state and they have been shown to accumulate in the presence of inflammation.

Th-1 cells differentiate when TCR stimulation by the cognate antigen is associated with the presence of IL12 and IFN family cytokines, such as IFN-α, IFN-β and IFN-γ. IL12 is produced by innate cells, such as DC and macrophages in response to CD40 and TLR triggering and it acts on the IL12R complex, composed of the IL12Rβ1 and the IL12Rβ2 subunits [119]. IL12 and IFNs are able to induce the signal transducer of activated T-cells (STAT)-4 and STAT-1 respectively, which lead to the activation of the master transcription factor for Th-1 differentiation, T-cell-specific T-box transcription factor (T-bet) [120]. T-bet induces the expression of IFN-γ, through epigenetic remodelling of the *ifnγ* gene, which is responsible for a positive-feedback loop that amplifies Th-1 differentiation [121]. In addition, T-bet promotes IL12Rβ2 expression on T-cells, resulting in IL12 dependent expansion of Th-1 cells and further amplification of IFN-γ secretion [122]. Furthermore, STAT-4 can also directly activate the transcription of IFN-γ and IL12Rβ2 [123, 124]. Finally, IL12 and IL18 can both induce production of IFN-γ by differentiated Th-1 cells in a TCR-independent way, representing an innate mechanism of Th-1 activation [125, 126].

1.4.2 T helper-2

Th-2 cells play a major role in the protective immune response against extracellular parasites, such as helminths, but they are also involved in allergic reactions, such as eczema, hay fever or asthma [127, 128]. Their differentiation is driven by IL4, which through activation of STAT-6, induces the master transcription factor for Th-2 differentiation, GATA-binding protein 3 (GATA3) [129-132]. GATA3 activates the transcription of IL4, IL5 and IL13 that are cytokines characteristically produced by

Th-2 cells. Other transcription factors have also been shown to induce Th-2 differentiation, such as c-MAF, TCF-1 and nuclear factor of activated T-cells (NFAT) [133-137].

Th-2 cells can also induce isotype switching in B-cells leading to the production of IgE antibodies.

Th-2 cells are normally very rare in the intestine in health, but they can accumulate in the presence of intestinal inflammation.

1.4.3 T helper-17

Th-17 cells are a newly characterized population of T-cells that have been shown to contribute to the physiological immune response to extra-cellular bacteria and fungi especially at mucosal surfaces, such as the lung and intestine, through the induction of inflammatory and chemotactic molecules and antibacterial agents. In addition to their role in host defence, a number of studies have been produced that support a major pathogenic role for Th-17 cells in different forms of murine and human immune pathology.

Murine Th-17 cells differentiate in the presence of TGF-β together with inflammatory cytokines, such as IL6, but also IL21, which is induced in activated T-cells by IL6 in a STAT-3-dependent manner [138-143]. IL1 has also been reported to promote Th-17 cell development, with IL1RI-/- mice showing decreased Th-17 cell differentiation and reduced incidence of experimental autoimmune encephalomyelitis (EAE) [144]. Induction of the transcription factor RORγ-t drives the differentiation of Th-17 cells [143, 145]. However, other transcription factors contribute to Th-17 differentiation, such as RORα, Aryl hydrocarbon receptor (AHR), Interferon regulatory factor (IRF)4, STAT3, Runx and c-MAF [146-152]. The role of TGF-β in Th-17 differentiation has highlighted once more the pleiotropic properties of this cytokine, also known to exert

immune regulatory activity through the induction of Treg that are major players in dampening the immune response [153, 154]. However, recent evidence suggests that TGF-β independent differentiation of Th-17 cells might also occur. In fact, a combination of IL23, IL6 and IL1β has been shown to induce differentiation of Th-17 cells that express both T-bet and RORγ-t and have pathogenic activity in an EAE model [155].

Initial observations suggested that differentiation of human Th-17 cells did not require the presence of TGF-β [156, 157]. Other studies have subsequently shown that TGF-β together with a combination of inflammatory cytokines, such as IL1β, IL6, IL21 and IL23 is necessary for human Th-17 cell differentiation [158-160]. Even if not necessary for Th-17 differentiation, the IL12 related cytokine, IL23, is required for their maintenance, expansion and terminal commitment [161, 162], thus representing a tissue regulator of Th-17 phenotype and function.

Human Th-17 cells originate from $CD161^+$ precursors and are enriched in the $CD161^+$ population [163, 164]. CD161 is the human equivalent of the murine NK1.1 and is expressed on the majority of NK cells, NK-T cells and on a proportion of T cells. CD161 can bind to different ligands, such as different members of C-type lectin domain family 2, but its role on human Th-17 cells remain unknown [165]. They also express the chemokine receptor CCR6 [166] and are characterized by the production of IL17A, IL17F, IL26 and IL22, and can also produce IL21, TNF-α and IFN-γ. IL21 can induce further Th-17 differentiation creating a positive feedback loop.

Th-17 are present in the intestine in the absence of inflammation and their role in immune pathology will be further discussed below.

1.4.4 T regulatory cells

Naturally occurring Treg (nTreg) develop in the thymus after the recognition of high affinity self-peptide-MHC complex [167]. In addition to nTreg, it has been shown that induced Treg (iTreg) can develop in the periphery from naïve $CD4^+$ T cells in the presence of TCR stimulation and high levels of TGF-β [154]. TGF-β activation of mothers against decapentaplegic Drosophila homolog (Smad)3 and NFAT is responsible for the expression of the Treg master transcription factor Foxp3, which is necessary for the regulatory activity of both nTreg and iTreg [168, 169]. Foxp3 is a highly specific marker of murine Treg, while transient expression of Foxp3 has been observed in activated human T-cells generating some confusion in the definition of human Treg [170, 171]. Mutations in the *FOXP3* gene are responsible for a very rare and often fatal systemic autoimmune disease, the immunedysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX), highlighting the fundamental role of Treg in prevention of autoimmunity [172]. Treg are characterized by constitutively high expression of the IL2 receptor, CD25, and they also express CTLA4, GITR and OX40 [173].

Treg exert their immune suppressive properties on effector T-cells, B-cells and innate cells, through various and only partly understood mechanisms. These include modulation of APC function, T-cell inhibition by cell-cell contact and secretion of immunosuppressive cytokines, such as IL10 and TGF-β, induction of T-cell apoptosis by essential amino acid degradation and cAMP mediated mechanisms [174-178].

1.4.5 Plasticity of T-cell subsets

The original Th-1/Th-2 paradigm has been challenged not only by the identification of new lineage subsets, such as Treg and Th-17 cells, but also by the observation that T-cell subsets retain a certain degree of flexibility in their phenotype and function [179].

IL17/IFN-γ double positive Th-17 cells have been described in both mice and men [163, 180-183]. Th-17 cells can even loose the expression of their signature cytokine IL17 and become selective IFN-γ-producers [184]. Furthermore, polarized Th-17 cells can be converted in Th-2 cells in presence of IL4 *in vitro*[185]. Th-1 and Th-2 cells appear to be more stable, however, in some experimental settings differentiated Th-2 cells have been shown to start producing IFN-γ and Th-1 cells can be induced to produce IL13 [186, 187]. Similarly, $Foxp3^+$ Treg cells can be converted into IL17-producing cells in the presence of pro-inflammatory cytokines [188-191]. Accordingly with such flexibility in cytokine production, transcription factor expression is also not strictly lineage-specific. Foxp3 can be expressed together with RORγ-t, RORα, GATA3 or T-bet [147, 192-194]. AHR can induce Th-17 and or Treg differentiation, depending on the activating agonist [149, 195]. Similarly, IRF4 is involved in both Th-2 and Th-17 differentiation [196, 197]. All together, these observations support the presence of T-cell plasticity (Figure 1.2). However, the relevance *in vivo* and under physiologic conditions remains to be established.

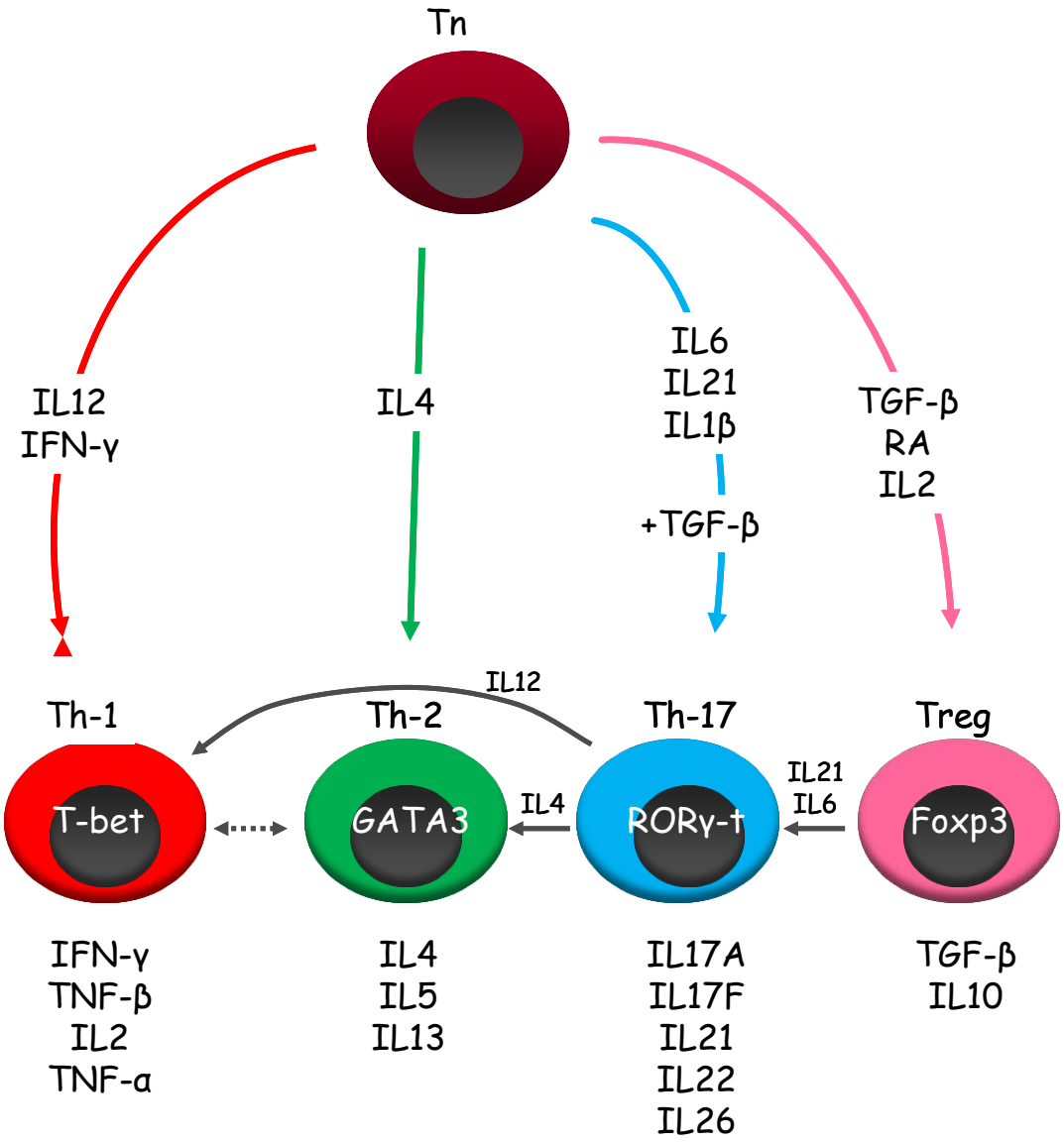


Figure 1.2 Differentiation of CD4 $^{+}$ T-cells

Th-cells differentiate from naïve precursors (Tn) in the presence of polarising cytokines. Each subset is characterized by the expression of specific transcription factors and by the secretion of signature cytokines that confer unique effector functions. However, the presence of plasticity of $CD4^{+}$ T-cell lineage differentiation has emerged from recent studies. Th-1 and Th-2 cells appear to have a more stable phenotype. Treg cells differentiate into IL17-producing cells in the presence of inflammatory cytokines, such as IL21 and IL6. Th-17 cells are particularly instable and can be converted into IFN-γ- and IL4-producing cells by stimulation with IL12 and IL4, respectively.

1.5 The IL23/IL17 pathway in autoimmunity and inflammation

1.5.1 A breakthrough: the discovery of IL23

Until recently, most human autoimmune disorders were considered to be driven by a Th-1 type of response, characterized by high levels of IFN-γ and TNF-α [198]. As discussed above, Th-1 cells differentiate from naïve T-cells in response to IL12, which is a heterodimeric type 1 cytokine mainly produced by activated macrophages, monocytes and DC [199]. IL12 comprises the IL12p40 subunit combined with the IL12p35 subunit and acts on the IL12 receptor, which is composed of the IL12Rβ1 and IL12Rβ2 chains and signals primarily through STAT4 activation. The recent discovery that IL12p40 can also combine with IL23p19 to form the closely related cytokine IL23 has led to reappraisal of the roles of IL12 and IL23 in a variety of inflammatory disorders [200]. In the seminal work by Cua *et al*, IL23p19$^{-/-}$ mice that specifically lack IL23, but not IL12, did not develop EAE, indicating that it is IL23 and not IL12 that drives inflammation in this model of multiple sclerosis [201]. Subsequent studies have demonstrated that IL23 is responsible for tissue-specific inflammation in various other models of immune diseases, such as rheumatoid arthritis, psoriasis and inflammatory bowel disease (IBD) and translational research has confirmed these findings in patients [181, 182, 202-208].

Like IL12, IL23 is produced by activated monocytes, macrophages, DC and endothelial cells in response to PRR binding (in particular dectin1, TLR2 and NOD2), prostaglandin E2 stimulation and CD40/CD40L interaction [209-212]. IL23 acts on the IL23 receptor, which is composed of the IL12Rβ1 (shared with the IL12 receptor), and the specific IL23R subunit [213]. The IL23R is widely expressed on immune cells, such as memory T-cells, NK cells, LTi-like cells and other innate lymphoid cells (ILC), myeloid cells and on non-immune cells [201, 213-215]. RORγ-t is

required for induction as well as maintenance of the IL23R [141, 216]. Low concentrations of TGF-β favour IL23R expression, whereas high concentrations have inhibitory effects [141]. IL23R is also upregulated by IL23, IL6 and IL21 in a STAT3-dependent manner and by prostaglandin E2 [141, 142, 217, 218]. Recently the microRNA Let-7f has been shown to inhibit IL23R expression in human $CD4^+$ memory T-cells [219]. Signalling through the IL23R involves the Janus kinase (JAK)/STAT pathway, with predominant activation of STAT3, however STAT1, STAT4 and STAT5 can also be activated [213]. After dimerization the STATs translocate to the nucleus, where they regulate gene transcription.

1.5.2 IL23 and Th-17 cells

IL23 driven inflammation has been primarily linked to its effect on Th-17 cells, which have been shown to play a pathogenic role in many autoimmune disorders [220]. As discussed above, IL23 is not strictly required for differentiation of Th-17 cells, as initially believed, but it appears to be necessary for the acquisition of Th-17 effector functions and for Th-17 accumulation [155, 161, 162]. It becomes clear that Th-17 represent a very heterogeneous population of cells that produce IL17A together with a range of other cytokines such as IL17F, IL21, IL22 and IL26, but also IFN-γ, TNF-α and IL6. Furthermore, they secrete CCL20, a ligand of the chemokine receptor CCR6, which in turn is preferentially expressed on the Th-17 cell surface, representing an autocrine loop that can contribute to Th-17 accumulation.

1.5.3 Th-17 cytokines

IL17A and IL17F

IL17A and IL17F are encoded by genes that lie in adjacent regions on human chromosome 6 and share the highest sequence homology (60%) amongst the other members of the IL17 family of cytokines. They are normally co-expressed by Th-17 cells and their expression appears to be dependent on the transcription factor STAT3 [221]. Both IL17A and IL17F production is also impaired in the absence of RORγ, whereas production of IL17F but not IL17A is independent of RORα [145, 147]. IL17A/IL17F heterodimers have been recently identified [222]. Interestingly, human Th-17 cells seem to produce mainly IL17F homodimers and IL17A/IL17F heterodimers *in vitro*, with only very low IL17A homodimers. IL17A and IL17F act on a heteromeric receptor composed of IL17RA and IL17RC subunits [223-225]. IL17F binds to IL17RA with lower affinity compared to IL17A, while both IL17F and IL17A show high affinity binding to IL17RC (murine IL17A does not bind IL17RC) [225]. On target cells, IL17A induces the secretion of inflammatory cytokines, such as IL6, TNF-α and IL1-β, chemokines, granulocyte-colony-stimulating-factor (G-CSF) and GM-CSF, which favour local accumulation of neutrophils and other inflammatory cells. Furthermore IL17A stimulates the production of matrix metalloproteinases (MMP) that are involved in tissue remodelling and tissue damage. Beside its inflammatory activity, IL17A contributes to the regulation and integrity of mucosal barrier functions through the production of antimicrobial peptides such as beta-defensins and S100 proteins [226]. Available evidence suggests that IL17F has overlapping biological functions with IL17A, such as induction of chemokines, GM-CSF, MMP and antimicrobial peptides [227-230]. Accordingly, $IL17RA^{-/-}$ mice develop *S. aureus* infections with a similar phenotype observed in IL17A/IL17F

double KO, but not in single KO, and IL17RA$^{-/-}$ mice are more susceptible to *K. pneumoniae* infection than IL17A$^{-/-}$ mice [231, 232]. These data confirm the presence of some redundancy in the functional activity of IL17A and IL17F in conferring protection against extra-cellular pathogens. Nevertheless, other studies have highlighted distinct and even opposite roles for IL17A and IL17F in inflammation [229, 231]. IL17A has been shown to play a dominant role in the development of arthritis, EAE and delayed type and contact hypersensitivity, with IL17F having only minor effects, if any in these models [231]. This finding may be explained by the stronger signalling and downstream gene-activation mediated by IL17A compared to IL17F [222, 229]. In the OVA-alum induced model of asthma, while IL17A contributes to the induction of Th-2 responses, IL17F has been shown to have Th-2 inhibitory effects. Moreover, IL17F appears to be pathogenic in dextran sulphate sodium (DSS)-induced colitis, whereas IL17A plays a protective role in this model of acute intestinal inflammation [229]. Considering the different affinity of IL17A and IL17F for IL17RA and IL17RC, a tissue-specific variability in the expression of the receptor complex subunits could be responsible for their differential activity. Interestingly, while IL17RA is expressed at high levels on haematopoietic cells, IL17RC is predominantly expressed on non-immune cells [233]. However, further studies are needed in order to elucidate overlapping and unique effects of IL17A and IL17F in different contexts.

IL21

IL21 is produced primarily by activated CD4^{+} T-cells and acts on a heteromeric receptor composed of the common γ-chain and the IL21Rα specific subunit, which is widely expressed on both immune cells (B-cells, T-cells and NK cells) and non-immune cells (epithelial cells, fibroblasts) [234, 235]. The IL21R complex signals via

the JAK/STAT pathway leading primarily to the activation of STAT1 and STAT3, and to weaker activation of STAT5 [236]. IL21 can stimulate proliferation and activation of $CD4^+$ and $CD8^+$ T-cells, inhibits Treg differentiation and increases resistance of target cells to Treg-mediated inhibition [234, 237-239]. As already discussed, recent studies have shown that IL21 together with TGF-β can induce the differentiation of Th-17 cells from naïve T-cells [141, 143, 160]. Since Th-17 cells in turn produce IL21, this represents an autocrine positive feedback loop that can contribute to the enhancement of Th-17 responses. Some studies have indicated that IL21 can enhance IFN-γ production and Th-1 responses [240, 241]. However, no difference in the production of IFN-γ was observed in *IL21r*$^{-/-}$ mice, which instead showed higher delayed hypersensitivity reactions associated with higher levels of IFN-γ, suggesting only partial effects of IL21 in shaping Th-1 and Th-2 responses [242, 243]. IL21 can also promote antibody production, IgG switching and plasma cell differentiation and expansion and activation of NK cells [234, 242, 244, 245]. Finally, on non immune cells, such as epithelial cells and fibroblasts, IL21 has been shown to induce the secretion of chemokines and the production of MMP [246, 247].

A role for IL21 has been suggested in the pathogenesis of many immune disorders, such as type 1 diabetes, atopic dermatitis, systemic sclerosis, systemic lupus erythematosus and IBD [244, 248-251]. In particular, IL21 has been shown to play a pathogenic role in chemically induced colitis, such as DSS- and trinitrobenzenesulfonic acid (TNBS)-induced colitis through the induction of Th-17 cell responses [252]. Furthermore, IL21 expression has been shown to be increased in the inflamed intestine in patients with IBD [241].

IL22

IL22 is a member of the IL10 cytokine family, together with IL26, and acts on the

heteromeric receptor that comprises the IL10R2 subunit, which is a component of the IL10R and ubiquitously expressed, and the specific IL22R1 subunit, which is only expressed on non-immune cells, such as epithelial cells and mesenchymal cells, particularly in skin, kidney and in the digestive and respiratory systems [253]. The highest levels of expression of the IL22R1 were found in the pancreas and the skin, where the IL22R1 is particularly expressed by keratinocytes [254]. Responsiveness to IL22 can be further induced during inflammation by exposure to IFN-γ and TNF-α [255, 256]. The IL22R complex signals through the JAK/STAT pathway leading to phosphorylation of STAT3. In humans the main producers of IL22 are activated T-cells, not only Th-17 cells, but also Th-1 and the recently described Th-22 subset, characterized by the production of IL22, but not IL17A nor IFN-γ [257, 258]. Furthermore, innate cells, such as γδ T-cells and LTi-like cells, have also been shown to produce IL22 [56, 259].

IL22 is involved in tissue defence, regeneration and healing, through the induction of antimicrobial agents (β-defensins and S100 proteins) and proteins involved in epithelial cell differentiation and cell mobility, as shown by gene expression arrays in keratinocytes [255]. IL22 has been shown to play a major role in bacterial intestinal and pulmonary infections. $IL22^{-/-}$ mice succumb to intestinal infection with *C. rodentium*, due to the lack of epithelial cell-derived RegIII antibacterial peptides [260]. Similarly, neutralization of IL22 has been shown to aggravate pulmonary infection with *K. pneumniae* [261]. Effects of IL22 on keratinocyte differentiation have been extensively studied and there is now strong evidence that IL22 plays a pathogenic role in different models of psoriasis, where it is responsible for the induction of acanthosis and hypogranulosis [203, 262]. These data are supported by findings in patients, which show high levels of IL22 in both skin lesions and

peripheral blood (PB) [254, 255]. Conversely, a protective role for IL22 has been shown in several models of liver inflammation, but the molecular mechanisms remain unclear [263, 264]. A protective role for IL22 has also been suggested in intestinal inflammation, but data are still controversial. In a model of Th-2 colitis, gene-delivery of IL22 to the colonic epithelium led to goblet cell restitution and amelioration of colitis [265, 266]. Furthermore IL22 has been shown to induce expression of LPS binding protein that could contribute to limiting systemic inflammatory responses to LPS in patients with CD [267].

IL26

IL26 was first identified in human T-cells, transformed with simian rhadinovirus herpesvirus saimiri as another member of the IL10 cytokine family, which does not have a homologue in mice [268]. IL26 is mainly produced by activated memory T-cells, with both Th-1 and Th-17 phenotypes [157, 269]. In addition, activated NK cells and $CD3^{-}CD56^{+}NKp44^{+}$ cells have also been shown to produce IL26 [259, 269]. The IL26 receptor complex is composed of the broadly-expressed IL10R2 subunit and IL20R1, which is expressed only on limited tissue types. Binding of the receptor leads to JAK/STAT activation and phosphorylation of STAT1 and STAT3 [270]. IL26 has been shown to target epithelial cells, such as intestinal cells and keratinocytes, where it induces secretion of IL10 and IL8 and over-expression of the adhesion molecule ICAM1 [271]. Furthermore, treatment of intestinal cell lines with IL26 was shown to inhibit cell proliferation, with an effect opposite to IL22, and induce expression of suppressor of cytokine signalling 3 (SOCS3) and pro-inflammatory cytokines, such as TNF-α and IL8. A role for IL26 in intestinal inflammation has been suggested by the observation of increased gene expression of IL26 in the inflamed *versus* the non-inflamed intestine of patients with CD [272].

1.5.4 IL23 and innate cells

Recent studies have shown that, besides its activity on Th-17 cells, IL23 can also act on cells of the innate immune system.

In particular unconventional, innate-like T-cell populations have been found to respond to IL23 stimulation and secrete Th-17 cytokines. A subpopulation of RORγ-t expressing γδ-T-cells has been shown to produce IL17A, IL22 and IL21 in response to IL23 in mice and to contribute to inflammation in EAE and CIA [56, 273-276]. Similarly, IL17 and IL22-producing γδ-T-cells have been described in the human peripheral blood, although in low frequencies [277]. In mice, iNKT-cells have also been shown to express IL23R and RORγ-t and to produce IL17A after IL23 stimulation or antigen recognition [278]. Similarly, human $CD56^{+}TCR\beta^{+}$ NKT cells also secrete IL17A after stimulation with anti-CD3 and IL23 [279]. MAIT-cells have also been found to express the IL23R and secrete IL17A after PMA/ionomycin stimulation [67]. This work suggests that MAIT cells represent the majority of previously described populations of IL-17A-producing $CD161^{high}CD8^{+}$T-cells [280, 281].

Takatori *et al* have shown that IL23 can also induce IL17 and IL22 production by ILC that share the phenotype of $CD3^{-}CD4^{+}$ LTi cells [51]. IL22-producing LTi-like populations have also been identified in adult mice and particularly in the MALT, such as intestine and tonsils, where they are thought to contribute to innate anti-microbial defence [282-285]. These cells are characterized by the expression of the NKR NKp46, but they are mainly $NK1.1^{-}$, do not show any cytotoxic activity and do not produce IFN-γ, therefore lacking conventional NK cell functions. Similar to Th-17 cells, LTi and related LTi-like cells are characterized by RORγ-t expression. Recent studies have shown that foetal and perinatal RORγ-t^{+} LTi cells express high levels of

c-kit and IL7Rα and can be $CD4^+$ or $CD4^-$. Conversely, adult RORγ-t^+ LTi-like cells are c-kit^{low}, IL7Rα^{low} and can be distinguished in $NKp46^+$ and $NKp46^-$ cells, which are mainly localized in the intestinal mucosa [286]. $NKp46^+$ LTi-like cells derive from NKR^-RORγ-t^+ precursors, while they are not related to conventional RORγ-t^- NK cells. Fate mapping experiments have shown that LTi-like cell differentiation involves progressive acquisition of NKR and loss of RORγ-t expression, which is regulated by tissue-specific microenvironmental factors. Development of LTi-like cells is influenced by the presence of the intestinal microflora, which has been shown to stabilise the expression of RORγ-t, probably through an IL7-mediated mechanism [287]. However, the microflora is not strictly necessary for LTi-like cell development since $NKp46^+$ cells are found in the intestine of germ-free mice [286]. A gradient of RORγ-t expression is associated with functional plasticity of LTi-like populations, with RORγ-t^+ NKR^+ LTi-like cells producing IL22 in response to IL23 and RORγ-t^- NKR^+ LTi-like cells responding to IL12 with IFN-γ production [287]. We have recently identified a population of IL23 responsive ILC characterized by a $Sca1^+Thy1^{high}cKit^-$ phenotype, which are responsible for intestinal inflammation in innate models of colitis, through secretion of IL17A and IL22 or IFN-γ depending on the experimental model (discussed in detail below) [214].

IL23-responsive ILC populations have also been identified in the human MALT, such as intestinal PP and tonsils. Cella and colleagues described $CD3^-CD56^+NKp44^+$ cells, termed NK22 that produce IL22, but not IL17 in response to IL23 [259]. $CD3^-$ $CD56^+NKp44^+$ cells also produce IL26 and express the transcription factor *RORC*. Although originally thought to represent a subset of NK cells, recent studies suggest that human $CD56^+$ LTi-like cells are developmentally and functionally related to LTi cells [288, 289]. Both human $CD56^+$ and $CD56^-CD127^+$ LTi-like cells can also

produce IL2, IL5 and IL13, in accordance with the description of Th-2-cytokine-producing ILC in mice [290-294]. Human LTi-like cells were found to express different members of the TLR family and are therefore able to sense and respond to microbial components. In particular association of IL2 or IL23 together with TLR2 stimulation is able to induce proliferation of LTi-like cells and IL22 production [291].

1.5.5 The IL23/IL17 axis in intestinal inflammation

Murine studies

Results from our group and others have shown that IL23 is the key cytokine driving intestinal inflammation in both T-cell independent and T-cell dependent murine models of colitis.

In an anti-CD40-induced innate model of acute colitis, T- and B-cell deficient $RAG^{-/-}$ mice treated with agonistic CD40 antibodies develop colitis and wasting disease associated with high serum levels of inflammatory cytokines [204]. Administration of monoclonal antibodies against the IL23 specific p19 subunit completely abrogates colitis in this model, while it does not affect wasting disease. Consistently, results in $RAG^{-/-}$ $IL23p19^{-/-}$ and $IL12p35^{-/-}$ mice that specifically lack IL23 and IL12 respectively, show that intestinal inflammation is highly dependent on IL23, while IL12 is necessary for the development of systemic disease.

In another model of innate colitis, $RAG^{-/-}$ mice are orally infected with the pathogenic bacterium *H. hepaticus* leading to coecal and colonic inflammation associated with marked systemic inflammatory responses [295]. Administration of anti-IL23 p19 antibodies is sufficient to completely abrogate colitis in these mice [181]. By contrast with the anti-CD40 model though, systemic disease is also attenuated by anti-IL23 p19 treatment, which may reflect the sequential activation of intestinal and systemic responses by the orally administered bacteria, compared to the systemic anti-CD40

stimulation.

Both *H. hepaticus* and anti-CD40 induced innate colitis are associated with high intestinal expression of Th-17 and Th-1 cytokines, such as IL17A and IFN-γ. In the *H. hepaticus*-driven model, blockade of IL17A or IFN-γ with specific antibodies is sufficient to significantly decrease intestinal and systemic inflammation indicating a functional role for both cytokines in this disease [214]. By contrast IL17A is dispensable for the development of anti-CD40 induced colitis and wasting disease, which appear to be dependent on other inflammatory cytokines such as IFN-γ and TNF-α.

Data from the T-cell independent models suggested the presence of an IL23 dependent innate source of IL17A in the murine intestine. Recently, we were able to identify a novel population of $Sca1^{+}Thy1^{high}c\text{-}kit^{-}$ ILC that is responsible for intestinal inflammation in both the anti-CD40 and *H. hepaticus* induced innate models. Strikingly, ILC express both RORγ-t and the Th-1 master regulator T-bet and respond to IL23 with IL17A, IL22 and IFN-γ production [214].

IL23 was also found to specifically drive intestinal inflammation in different T-cell dependent experimental models of colitis [181, 182, 205, 296]. In the well established T-cell transfer model, the adoptive transfer of $CD45RB^{high}$ naïve T-cells into immune deficient $RAG^{-/-}$ mice induces the development of colitis and systemic disease [297-299]. We have shown that $RAG^{-/-}$ IL23 $p19^{-/-}$ recipients do not develop intestinal inflammation after T-cell transfer, while colitis is not affected by specific deletion of IL12 in $RAG^{-/-}p35^{-/-}$ recipients. Interestingly, in this model the presence of either IL12 or IL23 is sufficient to induce systemic signs of disease [181]. Strikingly, the inflammatory activity of IL23 does not require IL17A production, since $IL17^{-/-}$ $CD45RB^{high}$ cells can induce colitis in $RAG^{-/-}$ mice [300]. Conversely, our data

demonstrate that in these settings IL23 exerts its pro-inflammatory effects restraining the IL10 and TGF-β dependent immune-regulatory activity of intestinal Treg [300]. Furthermore, recent work from our laboratory has shown that IL23 drives colitis, but not systemic disease, through direct effects on T-cells, inducing intestinal T-cell proliferation and accumulation and promoting the emergence of $IL17A^{+}IFN\text{-}\gamma^{+}$ double-positive T-cells [180].

Human studies

Available data from human studies strongly support a role for the IL23/IL17 pathway in IBD.

Some compelling evidence comes from results of genome-wide association scans (GWAS) that have identified IBD susceptibility single nucleotide polymorphisms (SNPs) in many genes involved in the IL23/IL17 axis, such as *IL23R, IL12b, STAT3, JAK2* and *CCR6* (further discussed below) [301-304].

Increased IL23 expression has been found in the inflamed intestine of patients with IBD and at higher levels in CD compared to ulcerative colitis (UC) [305-308]. Moreover, Th-17 cytokines are over-expressed in both colon and serum of patients. Increased mRNA expression of IL17A has been described in the mucosa of patients with both active UC and CD [308-310]. IL17A expression has been detected in both $CD3^{+}$ and $CD3^{-}CD68^{+}$ cells by immunofluorescence, suggesting the presence of both T and non-T sources of IL17A in the inflamed intestine [309]. In one study, preferential expression of IL17A was observed in $CD4^{+}$ T-cells isolated from the LP compared to PB of normal controls *ex vivo* and after TCR and IL23 stimulation [311]. These data confirm the compartmentalization of the IL23/IL17 axis previously described in mice. IL17F is also over-expressed in the inflamed compared to the uninflamed mucosa of patients with CD and higher levels of colonic IL17F are

observed in UC, even if there is no difference between inflamed and uninflamed tissue in the latter [312]. Similarly, IL26 is increased in the inflamed mucosa of patients with CD, but not UC, and RORγ-t^{+} cells expressing IL26 can be visualized by immunofluorescence analysis in active CD [272].

Recently, circulating and intestinal CD161^{+} Th-17 cells with an activated phenotype have been isolated from patients with CD [163, 164]. Interestingly, a high frequency of both IL17A^{+} and IL17A^{+}IFN-γ^{+} double-positive CD161^{+} Th-17 cells has been observed after *in vitro* expansion with anti-CD3 and anti-CD28 stimulation [163].

1.6 IBD: clinical features

IBD is a chronic inflammatory disorder of the gastrointestinal tract that encompasses CD, UC and indeterminate colitis (when overlapping features of CD and UC are observed). The prevalence of disease is around 1 in 1000 people in Europe, with ranges from 21.4 to 243/100000 people for UC and from 8.3 to 214/100000 for CD. Both prevalence and incidence rates seem to be higher in Northern Europe and North America, with a characteristic north-south and west-east gradient. Generally, IBD appears to be more frequent in westernized and industrialized countries, even if incidence appears to be rising in the last years even in southern and eastern countries, previously characterized by very low incidence of disease [313]. IBD is usually diagnosed in adolescents or young adults (mean age reported between 33.4 and 45 years in different studies) but it can still occur throughout life [314]. Differently from most classic autoimmune disorders, clear gender predominance is not observed.

IBD is characterized by alternating phases of clinical relapse and remission and both long standing UC and CD have been associated with increased risk of intestinal cancers. Symptoms are mainly represented by diarrhoea, abdominal pain and rectal bleeding. Nevertheless, extra-intestinal manifestations of the disease are also frequent, with possible involvement of joints (arthritis), skin (erythema nodosum and pyoderma gangrenosum), eyes (uveitis and episleritis) and kidneys (kidney stones) [315]. Patients with IBD also present increased risk of developing other chronic immune disorders, such as psoriasis, ankylosing spondilitis and primary sclerosing cholangitis [316].

1.6.1 Crohn's disease

In CD inflammation is typically discontinuous and can affect every part of the gastrointestinal tract, from the mouth to the anus. However, most frequently it is the

ileum and colon that are affected by the disease. Inflammation is characteristically transmural, with all different layers of the bowel wall (mucosa, submucosa, muscolaris and sierosa) involved in the inflammatory process. This can lead to the occurrence of complications, such as formation of strictures (narrowing of the bowel lumen) and fistulas (communications between intestinal loops, or with other pelvic organs, such as the bladder or the vagina, or opening on the skin surface) and abscesses [317]. Histologically, granulomas (aggregates of epithelioid cells, giant cells and lymphocytes) are pathognomonic of CD, but they are only found in 15-36% of colonoscopic biopsies [318]. CD is also characterised by the presence of patchy chronic inflammation and focal crypt irregularity.

Patients with CD are usually classified depending on the age of onset, localization and pattern of the disease (Montreal classification) [319].

Clinical symptoms can vary depending on different localizations and type of disease. They include cramping abdominal pain, diarrhoea, fever, anorexia, weight loss and growth retardation in children [317] (Table 1.1).

1.6.2 Ulcerative Colitis

Distinct from CD, UC involves only the colon and always starts in the rectum, from where it can spread proximally. Lesions are typically continuous, with inflammation being confined to the most superficial layer of the bowel wall, the mucosa. Microscopically, inflammation is diffuse and confined to the mucosa; other frequent findings are hyperaemia, mucin depletion, cryptitis, crypt abscess and superficial ulcers. Clinical symptoms can vary, but usually include diarrhoea, urgency, tenesmus and rectal bleeding, with abdominal pain being generally less severe than in CD (Table1.1) [320, 321].

Patients with UC are usually classified according to the extension of disease, which

can be limited to the rectum (proctitis), left-sided or can involve the majority of the colon (pancolitis) [320].

1.6.3 Therapeutic options

Therapeutic strategies for patients with IBD include interventions on life-style habits (such as stopping cigarette smoking in patients with CD) and medical and surgical treatments. The medical management includes anti-inflammatory drugs (such as different formulations of 5-aminosalicylic acid), corticosteroids, immunosuppressant agents (such as azathioprine, 6-mercaptopurine, cyclosporine and methotrexate) and biologic therapies (such as different anti-TNF compounds) [322]. Surgical treatment is required by majority of patients with CD (80%) in the course of their life for complications of the disease such as intestinal obstruction, fistulisation and abscess. However surgical treatment is not curative in patients with CD and endoscopic recurrence is observed in 65-90% of patients already one year after resection. Clinical recurrence in the absence of treatment occurs in 20-25% of patients per year [323]. Surgery is also required in UC patients with severely active disease or chronically active disease, not responding to medical treatment or intolerant to immunosuppressive therapy and in patients with dysplasia or cancer. Total colectomy is curative in patients with definite UC; nevertheless quality of life after surgery can be seriously affected by the frequent occurrence of pouchitis or pouch dysfunction in patients where an ileal J-pouch-anal anastomosis is performed, or by the presence of a permanent ileostomy [324].

	Ulcerative Colitis	Crohn's disease
Localization	Colon	All GI tract
Symptoms		
Diarrhoea	Common	Common
Rectal bleeding	Very common	Less common
Abdominal pain	Less severe	Common, often severe
Weight loss	Uncommon	Common
Fever	Uncommon	Common
Complications		
Perianal disease	Uncommon	Common
Endoscopy		
Distribution	Continuous	Discontinuous (skip lesions)
Rectum	Always involved	Often spared
Ileum	Not involved, unless backwash ileitis in pancolitis	Often involved
Histology		
Distribution	Mucosal, continuous	Transmural, patchy
Crypt abscesses	Common	Rare
Mucine depletion	Common when active	Absent
Granuloma	Absent	Diagnostic
Radiology		
Distribution	Continuous	Discontinuous
Symmetry	Symmetrical	Asymmetrical
Mucosa	Shallow ulcers	Deep ulcers
Strictures	Very rare	Common
Fistulae	Absent	Common

Table 1.1 Differences between UC and CD

1.7 IBD pathogenesis

The aetiology of IBD remains unknown. A role for both genetic and environmental etiologic factors in IBD pathogenesis is supported by the available evidence, suggesting that IBD represent a multifactorial disease.

Recent advance in clinical and basic science research suggests that an aberrant immune response towards the commensal intestinal bacterial flora is the pathogenic mechanism responsible for intestinal inflammation in genetically susceptible individuals. Here the evidence is presented that supports a role for both genetic and environmental factors and review the different immune pathways that have been implicated in the induction and perpetuation of chronic intestinal inflammation in patients with IBD.

1.7.1 Genetic factors

The involvement of genetic factors in the pathogenesis of IBD was first highlighted by epidemiological studies that have shown the presence of familial association of the disease, with families presenting multiple members affected by IBD [325]. In most cases family members with IBD share the same disease type, either CD or UC, but in 20% of multiply affected families different members present with either UC or CD. This observation suggests that some genetic factors might be specific for CD or UC, while others predispose to the development of either type of IBD. Epidemiological studies have also shown 50-75% concordance rates for CD in monozygotic twins, while concordance appears to be lower (around 20%) in patients with UC suggesting that genetic factors might play a stronger role in CD *versus* UC.

Linkage studies analyse the transmission of genetic markers in families with multiple affected members in order to identify genome regions that contain genes that predispose to disease [326]. These studies have led to the identification of nine risk

loci for IBD that have been denominated IBD1-9 [327]. However, a limitation of this approach is that the genetic regions identified are still very big and contain huge numbers of genes that could be responsible for the observed linkage. The underlying gene can be identified by fine mapping or association studies using a candidate gene approach, where candidate genes are selected on the base of the functional activity of the encoded protein. Nevertheless, undoubtedly the greatest advance in the identification of genetic risk variations in multifactorial diseases has been represented by the introduction of GWAS that scan the entire genome for multiple genetic variations [328]. In GWAS, several hundred thousand SNPs with an allele frequency greater than 5% and selected from the HapMap database in order to cover the whole genome, are genotyped on large populations of cases and controls leading to the identification of risk SNPs that are in linkage disequilibrium with the causal genetic variation.

GWAS conducted in patients with IBD have led to the identification of many susceptibility SNPs in different genes, mainly involved in innate immunity, autophagy and adaptive immunity [329]. *NOD2* was the first risk gene to be identified to confer increased risk to CD by two independent groups using different genetic approaches [330, 331]. Three uncommon SNPs in *NOD2* have all been associated with susceptibility to ileal CD with an odds ratio equal to 2.4 in heterozygote individuals and 17.1 in homozygotes or compound heterozygotes, representing the strongest association with IBD to date [329, 332]. Interestingly, this association has been largely replicated in populations of European ancestry, while no association has been found in Asian or African-American populations [333, 334]. A decade later, the functional role of *NOD2* mutations is still controversial, but available evidence suggests they represent loss of function mutations that lead to reduced NFkB

activation [335]. This inadequate response might result in reduced antibacterial agent production and pathogenic microbial invasion [336]. Other studies suggest that loss of function in NOD2 may result in lack of inhibition of TLR2 stimulation, leading to activation of inflammatory pathways and excessive Th-1 responses [337]. Furthermore, the NOD2 *3020ins*C variant has been shown to inhibit IL10 expression in human monocytes raising the possibility that NOD2 mutations may result in inadequate immune regulation [338].

CD has also been associated with mutations in *ATG16L1* and *IRGM* genes, which are involved in autophagy, a cellular process involving degradation of intracellular bacteria and cellular cytoplasmic material [339, 340]. Interestingly, recent studies have linked NOD2 with autophagy induction and have shown that both *NOD2* and *ATG16L1* variants induce altered autophagy, antigen presentation and intracellular bacterial handling in DC [341].

Results from genetic studies support the hypothesis that impaired immunosuppressive mechanisms may also be implicated in the pathogenesis of IBD. A polymorphism in the *IL10* gene has been associated to UC susceptibility in a recent GWAS [342]. Furthermore, autosomal recessive mutations in the genes encoding for IL10 and for the two subunits of the IL10R complex, *IL10RA* and *IL10RB*, have been identified in paediatric patients with severe, early onset CD [343, 344]. These findings indicate that a defective function of the regulatory cytokine IL10 plays a key role in the development of some forms of IBD. Notably, this hypothesis has long been suggested by studies with $IL10^{-/-}$ mice, which develop spontaneous colitis [345].

A role for the IL23/IL17 pathway in IBD has strongly been supported by results of GWAS that have identified susceptibility variants in different genes involved in this pathway. SNPs in the *IL23R* gene that encodes for the specific subunit of the IL23

receptor have been identified and largely replicated in independent cohorts of patients with IBD [301]. The strongest association was found with the uncommon non-synonimous coding variant Arg381Gln, which lies in the cytoplasmic domain of the IL23R and confers increased protection against IBD. Other SNPs in the intronic and intergenic regions of the *IL23R* gene have also been associated with increased susceptibility. To date, the functional role of the *IL23R* variants remains unknown. Interestingly *IL23R* polymorphisms have been associated with both CD and UC, suggesting that the IL23 axis might represent a shared inflammatory pathway in chronic intestinal inflammation. Furthermore variants in the *IL23R* gene have also been linked to other immune disorders, which can be associated with IBD, such as psoriasis and ankylosing spondilitis [346, 347].

IBD risk variants in other genes involved in the IL23/IL17 axis have also been identified, such as *STAT3* and *JAK2*, involved in the IL23 signal transduction, *IL12b*, encoding for the common subunit of IL12 and IL23, and *CCR6*, a chemokine receptor preferentially expressed on IL17 producing cells [302-304]. Furthermore, variants in *IL17A* and *IL17F* have also been reported to increase UC susceptibility and phenotype in Asian populations, but this has not been replicated in Caucasians [312, 348, 349] (Figure 1.3).

All together GWAS have greatly advanced our knowledge on the pathogenesis of IBD, identifying novel innate and adaptive pathways, which contribute to the development of chronic intestinal inflammation. However, it is estimated that the susceptibility genes identified by GWAS only account for a small fraction of the genetic contribution to IBD (only around 20% for CD) [302]. Moreover, the SNPs identified by GWAS are also very common in healthy individuals and have very low penetrance and effect sizes [350]. They do not represent the causal variants but point

to genomic regions that can contain variable number of genes and in some cases non-coding genomic regions. The actual causal variants can only be identified by deep sequencing and may affect protein structure or levels of expression. As highlighted by the case of NOD2 mutations, identified a decade ago and whose effects remain to date not completely understood, functional studies are needed to shed some light on the pathogenic mechanisms that predispose to IBD in individuals carrying genetic susceptibility.

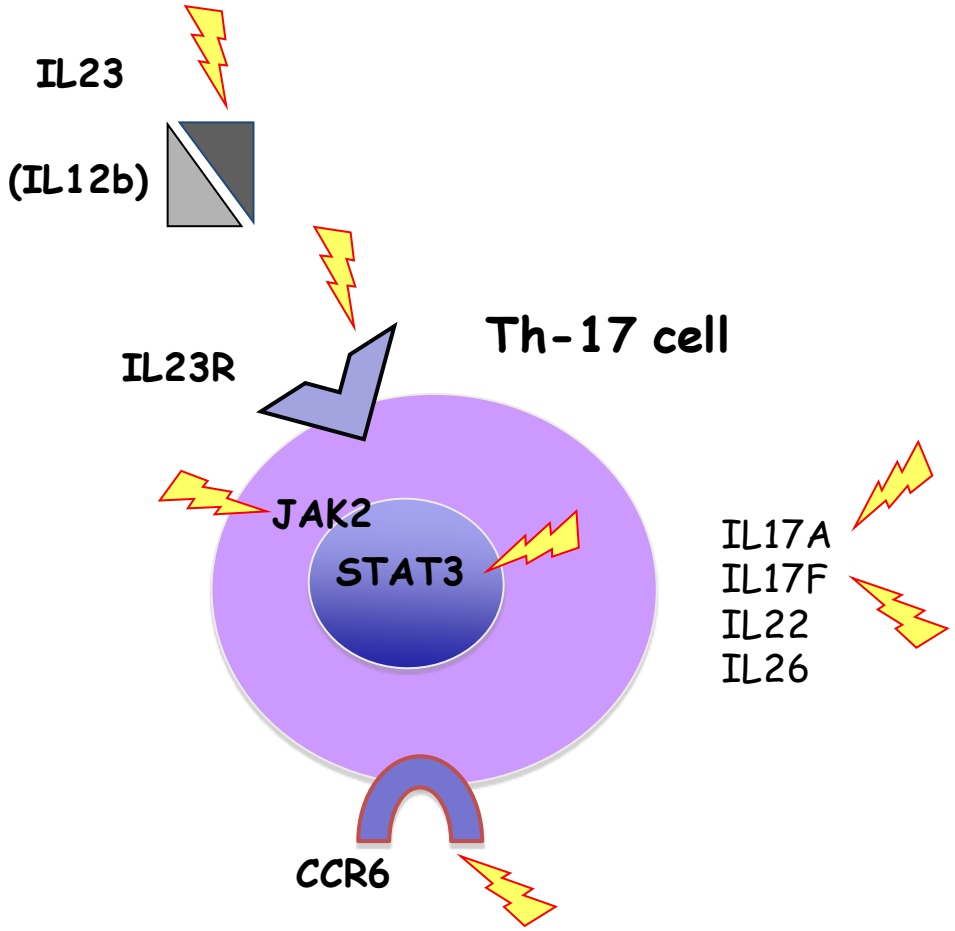


Figure 1.3 Multiple SNPs in genes involved in the IL23/IL17 pathway have been associated to IBD

Mutations in the gene encoding for the IL12b subunit of IL23, the *IL23R*, *JAK2*, *STAT3* and *CCR6* genes have been associated with IBD susceptibility. SNPs in the *IL17A* and *IL17F* genes have also been associated with UC susceptibility and phenotype in Asian populations.

1.7.2 Environmental factors

The epidemiological observation that concordance rates for both CD and UC in homozygotic twins do not reach 100% indicates on its own a substantial role for exposure to environmental factors in the development of IBD [325]. IBD is more frequent in the industrialized world and westernization is constantly accompanied by the emergence of IBD, first UC and then CD. The study of migrant populations that acquire the risk of the country of residence clearly shows that lifestyle changing and environmental factors are responsible for this geographical distribution of the disease [351, 352].

The most likely environmental factor to play a role in chronic intestinal inflammation is represented by the microbial flora that can shape the innate and adaptive intestinal immune response [353]. The strongest evidence that supports a role for the commensal flora in IBD comes from the observation that in many models of colitis, mice kept in germ-free environment do not develop intestinal inflammation [354-357]. A disrupted composition of the intestinal flora has been observed in a subset of patients with IBD, both CD and UC patients, which show reduction of the two bacterial *phyla* that represent the main components of a normal intestinal microflora, *Bacteroidetes* and *Firmicutes*, including *Lachnospiraceae* and *Faecalibacterium prausnitzii*, and a relative increase of *Proteobacteria* [358, 359]. It remains to date unclear if these changes represent a primary mechanism, induced by genetic predisposition, or may be secondary to inflammation and disease. Specific pathogens have also been implicated in the development of IBD, but evidence on their role is still controversial. *M. paratubercolosis*, which causes bovine enterocolitis with features that strongly resemble CD, has been isolated from CD patients, but later studies have led to conflicting and inconclusive results [360-362]. Adherent-invasive

species of *E. coli* have also been associated with CD, but it remains unclear if this represents a primary event or more probably it is secondary to mucosal damage [363]. Finally, the "cold-chain hypothesis" has correlated the increased incidence of IBD in the developed countries with the introduction of food refrigeration, which would allow growth of some bacterial species, such as *Yersinia Enterocolitica* and *pseudotubercolosis* that can cause enteropathies similar to CD [364].

Some hypotheses include a possible role for viral agents, such as measles infection or even measles vaccination, and reduced exposure to helminths in the westernized world [365]. In support of the latter, helminths are capable of inducing immune regulatory pathways and *Trichuris suis ova* have been shown to be effective as a therapeutic agent for both UC and CD in some recent clinical trials [366, 367].

However, the environmental factor that has been confirmed by the strongest evidence to have an effect on IBD risk and behaviour is represented by cigarette smoking. CD patients are more often smokers compared to control individuals and smokers bare an increased risk of clinical relapse, complications of disease and recurrence after surgery [368-371]. On the contrary, UC patients are more often non-smokers or ex-smokers, while smokers usually present milder disease activity and reduced need of immune suppressant and steroid use and hospitalization rates [368, 372, 373].

Other factors, such as appendicitis and appendectomy, use of oral contraceptives, vaccinations, diet and more generally increased hygiene have all been implicated in the higher frequency of IBD associated with economic development, but the pathogenic basis of this distribution remains to date controversial [374].

1.7.3 Immune effector pathways

Most studies conducted in the last thirty years have focused on the role of abnormal T-cell responses in the pathogenesis of IBD. Intestinal inflammation in CD has long been considered to be driven by a Th-1-like type of immune response, characterized by high levels of IL12 and Th-1 cytokines, such as IFN-γ and TNF-α in the LP. Conversely, UC has been rather associated with a non-conventional Th-2 response, characterized by increased expression of IL5 and IL13, but not IL4 in the inflamed colon [113, 375-378]. In contrast with a primary role for T-cell dysfunction in disease pathogenesis, the recent advances arisen from GWAS and immunological studies have moved the focus on early intestinal innate responses, such as autophagy and NLR activation, as central pathogenic pathways in IBD and particularly in CD. Closely related to autophagy and innate immunity, deregulation of the unfolded protein response (UPR) may also contribute to IBD. The UPR is induced by endoplasmic reticulum (ER) stress due to misfolded or unfolded protein accumulation in the ER [379]. Linkage and candidate gene studies have identified mutations in the *XBP1* gene, which is involved in the UPR, as risk factors for both UC and CD [380-383]. Consistently, specific deletion of Xbp1 in the intestinal epithelial cell compartment results in small intestinal inflammation and increased susceptibility to DSS colitis in mice [383]. Interestingly, alterations in NOD2, ATG16L1 or XBP1 activities have all been linked to Paneth cell dysfunction, which may represent a convergent pathogenic pathway affecting antimicrobial responses [379]. The newly identified IL23/IL17 pathway has also been shown to play a major role in the development of chronic intestinal inflammation in animal models and its pathogenic role in both UC and CD has been further supported by human studies. As discussed above, IL23 is a key cytokine in orchestrating the crosstalk between innate and

adaptive immunity and has a central role in driving early responses to microbes. All together, these observations support a role for a defective intestinal innate response in the development of chronic intestinal inflammation, in accordance with the immunodeficiency theory first postulated in the 1970s [384]. A defective early acute response would be unable to contain the overwhelming bacterial load present in the gut and lead to granulomatous reactions and secondary T-cell activation, characteristic of CD. Finally, the reduced activity of regulatory pathways may represent another mechanism contributing to the induction of chronic intestinal inflammation, as suggested by immunological studies and the recent identification of *IL10* mutations in patients with UC [342].

1.8 Aims

As presented in this chapter, huge advances in our understanding of IBD pathogenesis have arisen from studies conducted in animal models of intestinal inflammation and from genetic studies. Nevertheless, research projects in human tissues are necessary in order to translate these findings into effective changes in patient care and disease behaviour. Translational research projects in IBD have been hampered by an often inadequate communication between scientists and physicians, scarce availability of tissue from patients and adequate controls and technical experimental challenges. The recent establishment of the Translational Gastroenterology Unit (TGU) in Oxford that put together world leading scientists and clinicians in the field of IBD research and patient care has provided us with the ideal environment in order to overcome these limitations. In fact, through a very efficient collaboration, we have been able to collect both intestinal and blood samples from large numbers of patients with IBD and controls. We first aimed to optimize the techniques necessary for the isolation of cells from different tissues and for the characterization of human systemic and intestinal immune responses in order to achieve reliable and informative tools four our experiments (discussed in Chapter 3). We then wanted to elucidate the contribution of different immune pathways to chronic intestinal inflammation in patients with IBD. In light of our previous findings in the murine models of colitis and the results of the human GWAS, our interest particularly focused on the role of the newly described IL23/IL17 pathway in mucosal and systemic immunity. We evaluated the contribution of adaptive and innate IL23 responsive cells to initiation and propagation of chronic intestinal inflammation in patients with IBD. We especially aimed to investigate if IL23 responsive ILC are present in the human gut and if they play a role in IBD, as suggested by our recent work in the innate models of colitis (discussed in Chapter 4)

[214]. In addition, we wanted to characterize the phenotype and function of human DC subsets in both peripheral and intestinal immune responses and to evaluate if an altered composition of the DC pull or a dysfunction of specific DC subsets might contribute to shaping the aberrant intestinal immune-response in patients with IBD (discussed in Chapter 5).

Chapter 2- Materials and methods

2.1 Study subjects

All patients and controls were recruited from the gastroenterology unit and the colorectal surgery department at the John Radcliffe Hospital and the Churchill Hospital in Oxford, UK. The diagnosis of IBD was confirmed by established clinical, radiological, endoscopic and histological criteria. Blood samples, gut specimens and MLN were obtained from patients with UC and CD undergoing surgery for severe disease, chronically active disease or complications of disease. Further blood samples were collected from patients with IBD attending the outpatient clinic. Blood samples and gut specimens from macroscopically healthy areas were collected from CRC patients as non-inflammatory controls. Blood samples were also collected from healthy volunteers. Blood samples and gut specimens were obtained from patients with diverticulitis as inflammatory controls.

Colonic and ileal biopsies were collected from patients with IBD undergoing colonoscopy for clinical relapse, surveillance or assessment of disease extent and from healthy controls undergoing colonoscopy for chronic abdominal pain or CRC screening.

2.2 Ethics

Ethical approval was obtained from the Oxfordshire Research Ethics Committee (REC reference number 07/Q1605/35) and informed written consent was given by all study participants.

2.3 Clinical information

The relevant clinical information was collected by the physician or surgeon who obtained the informed consent and was stored in the patient clinical notes. A non identifiable number was given to each sample.

2.4 Sample collection

After obtaining informed consent, blood was drawn before surgery and before intravenous administration of general anaesthetics or at the time of clinical assessment in the IBD clinic. Blood was collected into three 5 ml EDTA-coated tubes and kept in ice before processing it. Surgical specimens of inflamed colon and terminal ileum and MLN of patients with IBD, inflamed colon of patients with diverticulitis and unaffected colon and terminal ileum of patients with CRC were collected after macroscopic examination by a pathologist and before tissues were fixed. All samples were kept in RPMI in ice until processing.

After obtaining informed consent, up to ten biopsies from each individual were collected during endoscopic sessions by an experienced endoscopist from the colon and ileum of IBD patients and non-inflammatory controls. Biopsies were kept in complete media in ice until processing. Occasionally, the tissue was kept overnight at 4°C in complete media before processing.

2.5 Histology

2.5.1 Haematoxilin and Eosin staining

Intestinal tissue was fixed in a 10% formaldehyde saline solution and 4.0 μm sections were prepared using a microtome and stained with haematoxylin and eosin (H&E). Images were captured using a CoolScope microscope (Nikon).

2.5.2 Immunofluorescence

Three-colour immunofluorescence was used to determine CD127 and CD3CD20 expression together with DAPI nuclear counterstaining in acetone-fixed, frozen tissue from tonsils and formalin-fixed, sucrose-embedded intestinal tissue. Blocking of endogenous peroxidase activity was achieved with 0.13% sodium azide and 3% H2O2 and tissue was incubated with 10% normal donkey serum to avoid non-specific binding. Tissue was stained with monoclonal mouse anti-human CD127 antibodies (clone R34-34, Dendritics, Lyon, FR) (1:20). Polyclonal donkey anti-mouse IgG (Abcam, Cambridge, UK) were used as secondary antibodies (1:100) and signal was amplified with FITC-tyramide (Perkin Elmer Lifesciences, Cambridge, UK) (1:300). Another round of staining was carried out with monoclonal rabbit anti-human CD20 (clone EP459Y, Abcam, Cambridge, UK) (1:150) and polyclonal rabbit anti-human CD3 (Dako, Ely, UK) (1:150). HRP-conjugated polyclonal donkey anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA, USA) (1:100) were used as secondary antibodies and signal amplification was performed with Cy5-tyramide (1:300).

The same protocol was used to determine CD103 expression and DC LAMP expression together with DAPI nuclear counterstaining in PFA-fixed, frozen intestinal tissue. Tissue was stained with monoclonal anti-human CD103 antibodies (clone MCA708, AbDSerotec, Oxford, UK) (1:100) and DC LAMP (clone 104G4, Dendritics, Lyon, FR) (1:100).

2.6 Cell isolation

2.6.1 Isolation of LPMC from surgical specimens of large and small bowel

LP mononuclear cells (LPMC) were isolated using a modified version of the protocol described by *Bull* and *Bookman* [385]. The gut specimen was washed multiple times in HBSS. Peritoneal tissue was dissected and the specimen was incubated in HBSS and 1 mM DTT at room temperature (RT) for 15 minutes, shaking, to dissolve the mucous layer. Supernatant was aspirated and the specimen was washed in HBSS. The mucosa was then dissected along the *muscularis mucosae* and cut in pieces <25 mm^2. Fragments were transferred in a vented Erlenmeyer flask and stirred in HBSS and 0.75 mM EDTA in the incubator at 37°C, for 45 minutes to detach the epithelial crypts. Supernatant was aspirated and the incubation was repeated for a total of three times or until the supernatant looked clear and no epithelial cells were observed under the microscope. The tissue was then washed three times in HBSS in order to remove any EDTA, which is known to inhibit collagenase activity, and digested overnight in RPMI/10%FCS and 0.1 mg/ml collagenase D solution (Roche Diagnostics, Burgess Hill, UK), with a stirrer in the incubator. In some experiments 0.16 mg/ml collagenase V solution was used (Sigma-Aldrich, Dorset, UK). After 12-18 hours, the supernatant was harvested, filtered through a nylon mesh and washed three times in HBSS. Cells were then resuspended in a 40% Percoll solution, layered on a 100/60/40/30 Percoll gradient and centrifuged for 30 minutes at 1500 rpm at 4°C with break off. LPMC were collected at the 40%-60% interface and washed in HBSS. All solutions used were supplemented with antibiotics (Penicillin/Streptomycin, Gentamicin 40 μg/ml and Amphotericin B 0.025 μg/ml).

2.6.2 Isolation of LPMC from endoscopic biopsies of large and small bowel

Biopsies were incubated in HBSS and 1 mM DTT at RT for 15 minutes, shaking, to dissolve the mucous layer. After three washes in HBSS, biopsies were incubated in RPMI/5%FCS/1.5 mg/ml collagenase A (Roche Diagnostics, Burgess Hill, UK) and 0.01 mg/ml DNase (Qiagen, Crawley, UK) solution in gentleMACS C tubes (Miltenyi Biotec, Bisley, UK). In some experiments collagenase D (Roche Diagnostics, Burgess Hill, UK) was used (1.5 mg/ml). After a short shaking step with the gentleMACS dissociator (Miltenyi Biotec, Bisley, UK), biopsies were incubated for one hour shaking at 37°C. After a second shaking in the gentleMACS dissociator, the tissue appeared completely digested. The supernatant was harvested and washed three times in HBSS. Cells were then resuspended in a 40% Percoll solution, layered on a 100/60/40/30 Percoll gradient and centrifuged for 30 minutes at 1500 rpm at 4°C with break off. LPMC were collected at the 40%-60% interface and washed in HBSS. All solutions used were supplemented with antibiotics (Penicillin/Streptomycin, Gentamicin 40 μg/ml and Amphotericin B 0.025 μg/ml).

2.6.3 Cell isolation from blood

PB was diluted in an equal volume of PBS and centrifuged over a Ficoll-Hypaque layer at 2000 rpm at RT for 20 minutes. PB mononuclear cells (PBMC) were collected at the Ficoll-dilute plasma interface and washed in PBS. PBMC were resuspended in PBS and stored overnight at 4°C.

2.6.4 Cell isolation from MLN

Fat was removed and MLN were cut into 1-2 mm pieces using a razor blade. Pieces were digested in RPMI/10% FCS/15mM HEPES and 0.75mg/ml collagenase VIII (Sigma-Aldrich, Dorset, UK) or collagenase D (Roche Diagnostics, Burgess Hill, UK)

in the shaking incubator at 150-200rpm at 37°C for 40 minutes. The supernatant was harvested and filtered through a cell strainer. The remaining undigested tissue was also squished through a 70μm or 100 μm cell strainer with a 2 ml syringe piston and added to the pooled digested mixture. Cells were washed and resuspended in PBS.

2.7 Cell sorting

2.7.1 Magnetic cell sorting

$CD3^+$ and $CD3^-$ cells were sorted by CD3 positive selection using the CD3 MicroBeads (Miltenyi Biotec, Bisley, UK) according to the producer protocol. Shortly, cells were incubated with microbeads conjugated to monoclonal human anti-CD3 antibodies for 15 minutes at 4°C. Cells were washed and passed through a MACS column in a magnetic field (Miltenyi Biotec, Bisley, UK). The unlabeled $CD3^-$ cells passed through the column and were recovered. After removing the column from the magnet, the $CD3^+$ labelled fraction was flushed out and collected.

2.7.2 Fluorescence Activated Cell Sorting (FACS)

$CD3^+$, $CD3^-$, CD3-$CD19^-$, Lin(CD3, CD19, CD14, $CD16)^-CD45^+CD56^-$, $Lin^-CD45^+CD56^+$, $Lin^-CD45^+CD127^+$ and $Lin^-CD45^+CD127^-$ cells were sorted by FACS in the experiments presented in Chapter 4. $LIN(CD3, CD14, CD16, CD20, CD56)^-$ $HLA\text{-}DR^+CD11c^{high}$, $LIN^-HLA\text{-}DR^+CD11c^{high}CD103^+$ and $LIN^-HLA\text{-}DR^+CD11c^{high}CD103^-$ DC were sorted by FACS in the experiments presented in Chapter 5. Before staining, cells were incubated in 2% normal rat or mouse serum for 30 minutes at 4°C in order to block the Fc-receptor and avoid non specific antibody binding. Cells were then incubated for 30 minutes at 4°C in the dark with specific combinations of conjugated antibodies in different experiments and then washed and resuspended in PBS. The antibodies used in the different sorts are shown in Table 2.1. In some experiments secondary streptavidin reagents were added for 15 minutes 4°C in the dark. Cells were then washed twice and resuspended in PBS.

Cells were sorted using Dako Cytomaiton MoFlo or FACS Aria depending on the experiments. The different gating strategies will be shown in the relevant sections.

2.8 Flow cytometry

2.8.1 Flow cytometry antibodies

Antibodies used for flow cytometry are shown in Table 2.1. Stained cells were acquired on a FACS Sort, FACS Calibur, Dako Cyan or LSRII. Analysis was performed using FlowJo software (TreeStar Inc.).

2.8.2 Surface staining

Cells were incubated in 2% normal rat or mouse serum for 30 minutes at 4°C before staining to avoid aspecific binding. After washing, cells were incubated with the appropriate antibody mix for 20 minutes at 4°C. When biotin conjugated antibodies were used, cells were washed and incubated with streptavidin reagents for 15 minutes at 4°C in the dark. Cells were then washed twice in PBS and fixed in 2% paraformaldehyde PBS solution.

2.8.3 Intracellular staining

For cytokine intracellular staining cells were stimulated with PMA (100 ng/ml) (Sigma Aldrich, Dorset, UK) and ionomycin (1 μg/ml) (Sigma Aldrich, Dorset, UK) in the presence of Brefeldin A (3 μg/ml) (eBioscience, Hatfield, United Kingdom) for 4 hours before staining. After centrifugation for 5 minutes at 1500 rpm, supernatant was aspirated and cells were washed once in PBS. Foxp3 staining buffers (eBioscience, Hatfield, United Kingdom) were used for cell fixation and permeabilization. After surface staining, cells were incubated in fixation/permeabilization buffer at 4°C overnight or at RT for 20 minutes in the dark. Cells were then incubated in permeabilization buffer with 2% rat or mouse serum for 30 minutes at 4°C in the dark. The appropriate antibodies for intracellular staining were then added and cells were incubated for other 30 minutes at 4°C in the dark.

Cells were washed twice with permeabilization buffer and resuspended in PBS for acquisition.

Specificity	Clone	Source	Fluorochrom	Dilution
Surface				
CD3	HIT3a	Mouse	Biotin, PE	1:100
CD3	UCHT1	Mouse	FITC, APC,	1:100
CD4	OKT4	Mouse	APC, PB	1:100, 1:50
CD8a	HIT8a	Mouse	FITC	1:100
γδ-TCR	B1.1	Mouse	FITC	1:50
CD19	HIB19	Mouse	Biotin	1:100
CD19	HIB19	Mouse	APC/Cy7	1:100
CD14	61D3	Mouse	Biotin	1:50
CD16	3G8	Mouse	Biotin	1:50
CD45	HI30	Mouse	PE/Cy7	1:100
CD56	HCD56	Mouse	PERCP	1:50
CD127	eBioRDR5	Mouse	PB	1:5
LIN CD3 CD16 CD19 CD20 CD14 CD56	 SK7 3G8 SJ25C1 L27 MΦP9 NCAM16.2	 Mouse Mouse Mouse Mouse Mouse Mouse	FITC	1:100
HLA-DR	L243	Mouse	PERCP	1:100
CD11c	SHCL3	Mouse	APC	1:100
CD103	LF61	Mouse	PE	1:100
NKp44	P44-8	Mouse	APC	1:50
CCR6	11A9	Mouse	PE	1:500
β8	37E1	Mouse	APC	1:50
Intracellular				
IFN-γ	4S.B3	Mouse	FITC/APC	1:20
IL17A	BL168	Mouse	PE	1:10
IL17A	eBio64DEC1	Mouse	PE	1:10

Table 2.1 Antibodies used for FACS analysis and sorting

2.9 Preparation of samples for cytokine analysis

2.9.1 Preparation of intestinal tissue

Two small fragments of intestinal mucosa (around 2 mm in size) were snap-frozen in liquid nitrogen and stored at -80°C for protein and RNA analysis. Frozen tissue was homogenized in a FastPrep^{TM} 24 homogenizer (MP, Biomedicals, Cambridge, UK). For protein analysis, 600 µl of PBS supplemented with protease-inhibitor was added to the tissue fragment in a lysing Matrix tube (MP, Biomedicals, Cambridge, UK). For RNA extraction, 700 µl of RLT buffer supplemented with β-mercaptoethanol were added to the sample. The fragment was then homogenized at 6.5 M/s for 40 seconds. This step was repeated three times or until the fragment looked completely homogenized. The sample was centrifuged at >13000 rpm for 5 minutes. Supernatant was collected and centrifugation repeated for other 5 minutes. Supernatant was collected, aliquoted in 100 µl aliquots and stored at -80°C for protein analysis. For RNA analysis, supernatant was loaded on a Qiagen RNAeasy mini column (Qiagen, Crawley, UK) and RNA was extracted as described below.

2.9.2 RNA extraction and cDNA synthesis

RNA was extracted using the RNAeasy mini or micro kit (Quiagen, Crawley, UK), according to the manufacturer protocol. A DNase incubation step was performed in all cases to avoid DNA contamination. RNA concentration was measured by NanoDrop (Thermo Scientific, Wilmington, USA). RNA was stored at -80°C. cDNA was synthesized using the Superscript III reverse transcriptase and Oligo DT primers (Invitrogen, Paisley, UK) as per manufacturer protocol and was diluted up to 1 in 5 in Milli-Q water. cDNA was stored at -20°C.

2.10 Analysis of cytokine expression

2.10.1 qPCR

SyberGreen based quantitative real time polymerase chain reaction (qPCR) was performed using Quantitect primer assays (Qiagen, Crawley, UK) and Platinum SYBR Green qPCR Supermix (Invitrogen, Paisley, UK). TaqMan® Gene Expression Assays were used in some experiments (Applied Biosystems, Warrington, UK) (Table 2.2). cDNA samples were assayed in triplicate using the Chromo4 detection system (GMI, Ramsey, MN, USA), and gene expression levels for each individual sample were normalized to β-actin. Mean relative gene expression was determined and expressed as $2^{-\Delta CT}$ ($\Delta CT=CT_{gene}-CT_{\beta\text{-actin}}$) x10000, unless otherwise specified.

2.10.2 Protein analysis by bead assays

Protein concentration in intestinal tissue homogenates, serum and supernatants were assessed using the FlowCytomix human Basic Kit in combination with the human simple kits (Bender Medsystem, Vienna, Austria) according to the manufacturer instructions. Samples were acquired on FACS Calibur and data analysis was performed using the Flowcytomix Pro 2.2 software (Bender Medsystem, Vienna, Austria). For analysis of total TGF-β, samples were activated in the presence of HCl. Latent TGF-β concentration was calculated subtracting total TGF-β concentration and active TGF-β concentration measured without acidification with HCl.

Gene	Entrez Gene ID	Transcript	Reporter Dye	Provider
IL22	50616	NM_020525	SYBR Green	Qiagen
IL17A	3605	NM_002190	SYBR Green	Qiagen
IL17F	112744	NM_052872	SYBR Green	Qiagen
IL21	59067	NM_021803	SYBR Green	Qiagen
IL23R	149233	NM_144701	SYBR Green	Qiagen
RORC	6097	NM_005060	SYBR Green	Qiagen
AHR	196	NM_001621	SYBR Green	Qiagen
IFNG	3458	NM_000619	SYBR Green	Qiagen
IL13	3596	NM_002188	SYBR Green	Qiagen
LTA	4049	NM_000595	SYBR Green	Qiagen
LTB	4050	NM_002341	SYBR Green	Qiagen
TNF	7124	NM_000594	SYBR Green	Qiagen
LTBP1	4052	NM_000627	SYBR Green	Qiagen
PLAT	5327	NM_033011	SYBR Green	Qiagen
SMAD7	4092	NM_005904	SYBR Green	Qiagen
ITGB8	3696	NM_002214	SYBR Green	Qiagen
TGFB2	7042	NM_003238	SYBR Green	Qiagen
ALDH1A2	8854	NM_003888	SYBR Green	Qiagen
TBX21	30009	NM_013351	SYBR Green	Qiagen
IL1B	3553	NM_000576	SYBR Green	Qiagen
ACTB	60	NM_001101	SYBR Green	Qiagen
ACTB	60	NM_001101	Taqman/FAM	Applied Biosystem
IL23A	51561	NM_016584	Taqman/FAM	Applied Biosystem
IL26	55801	NM_018402	Taqman/FAM	Applied Biosystem
CCR6	1235	NM_031409	Taqman/FAM	Applied Biosystem

Table 2.2 Primers used for qPCR

2.11 Cell cultures

Cells were cultured in RPMI with 10% FCS, antibiotics (Penicillin/Streptomycin, Gentamicin 40 μg/ml and Amphotericin B 0.025 μg/ml) and L-Glutamine with or without recombinant human IL23 or IL12 (R&D Systems Europe, Abingdon, UK) at 10 ng/ml concentration.

2.12 Statistics

The nonparametric, two-tailed Mann-Whitney test was performed in Prism (Graphpad Software, SD, USA) in all cases. Mean +/- standard error of the mean (SEM) is represented on bar charts. Differences were considered statistically significant when $p<0.05$.

Chapter 3- Optimization of cell isolation protocols and characterization of human intestinal and systemic immune cells in patients with IBD and controls

3.1 Introduction

The immunological basis of IBD was supported by results of pivotal studies conducted in the 1950s-60s. The initial observation that antibodies against colonic epithelial cells were found in high titres in children and in a proportion of adults with UC, first suggested that this may represent an autoimmune disorder [386]. In 1965, Williams observed that patients with CD presented delayed hypersensitivity reactions to tuberculin in the Mantoux skin test, compared to normal controls [387]. In the same years, it was shown that lymphocytes isolated from the blood of CD patients exhibited impaired responsiveness to mitogens *in vitro* [388]. These findings indicated the presence of some systemic depression of T-cell function in patients with CD. Subsequent research efforts aimed to characterize the immune response in patients with IBD, but most initial studies focused on PB immune cells due to their easier accessibility. However, only minimal variations, if any, were observed in systemic immune responses in patients with IBD compared to healthy individuals [389-392]. It became soon evident that local mucosal immune responses needed to be investigated in order to advance the understanding of the immunological basis of IBD. An attempt to investigate the intestinal immune system *in situ* was performed using immunofluorescence techniques, but these had the intrinsic limitation of allowing only semi quantitative analysis [391]. The introduction of intestinal cell isolation methods in the late ‘70s marked a real cornerstone in the development of human IBD research and they still represent invaluable tools for immunologists with an interest in

the intestinal immune system. Both enzymatic and mechanical techniques were investigated and variations of the original protocols are still in use today [385, 393]. In 1977 Bull and Bookman established an enzymatic digestion protocol that allows the isolation and purification of LPMC from the human intestine [385]. Some variations of the original protocol have been proposed in more recent years by different groups, but the main steps are maintained [394-396]. These include epithelial cell dissociation by EDTA incubation, collagenase digestion of the mucosal layer and purification of lymphocytes on a Fycoll-Hypaque or percoll gradient. Mechanical digestion protocols are of interest as avoiding enzymatic digestion steps might better to preserve cell-surface protein expression and cell function. Nevertheless, results from both original studies and more recent work seem to favour enzymatic *versus* mechanical digestion protocols in terms of cell yields, cell viability and cell function [394, 397].

In this project we have introduced and successfully established isolation protocols of human cells from surgical and biopsy intestinal samples and MLN in our laboratory. This has required optimization of methods, which will be discussed at the beginning of this chapter. The successful isolation of immune cells from blood and intestinal samples from IBD patients and controls has then allowed us to phenotype both systemic and mucosal immune responses in the presence and absence of intestinal inflammation and has opened door to subsequent functional analysis. Here some phenotypic characterization of lymphocytes and myeloid cells from PB, intestine and MLN is presented.

3.2 Results

3.2.1 Samples collected

From June 2007 until December 2010, we have been able to collect 120 blood samples, 97 colonic surgical specimens, 30 ileal surgical specimens, 26 MLN and endoscopic biopsies from 18 subjects (Table 3.1).

Blood samples were collected from subjects from all groups undergoing surgery. In addition, further blood samples were obtained from patients with IBD attending the outpatient clinic and from healthy volunteers. Intestinal specimens have been collected from patients with IBD, both UC and CD, and from uninflamed controls (CRC patients) and inflamed controls (patients with diverticulitis). MLN were mainly from patients with CD, due to the associated mesenteric lymphoadenopathy in this condition, which allows macroscopic identification of MLN by surgeons and pathologists. Isolation of MLN from control patients was limited by the small dimensions of MLN in the absence of intestinal inflammation and by the absolute necessity for the pathologist to keep MLN from CRC patients to perform histological analysis for cancer staging. In the last months of this project, we also collected endoscopic biopsies (up to 10 per patient) from colon or ileum of 18 patients undergoing endoscopy for IBD or for CRC screening.

On average, more than 3 surgical intestinal samples and blood samples per month were collected and processed in the last three years.

Diagnosis	**Blood samples**	**Colon specimens**	**Ileal specimens**	**MLN**	**Colon biopsies**	**Ileal biopsies**

Crohn's disease	45	29	26	21	4	2
Ulcerative Colitis	22	18	-	3	6	-
Colorectal cancer	43	47	4	1	-	
Diverticulitis	2	3	-	1	-	-
Healthy	8	-	-	-	6	-
Total	120	97	30	26	16	2

Table 3.1 Samples collected from June 2007 until December 2010

3.2.2 Patient clinical information

Blood test results at time of inclusion were only available from a proportion of subjects undergoing surgery. In particular we have analysed haemoglobin levels, white blood cell, lymphocyte and platelet counts, and C-reactive protein (CRP). Platelets were significantly higher in patients with CD compared to CRC and a trend was also observed in patients with UC ($p=0.06$). A trend to an increase was also observed for CRP levels in patients with CD compared to controls ($p=0.08$) (Figure 3.1, A).

Body mass index (BMI) was calculated in some IBD patients and controls and we observed significantly lower BMI in patients with CD and UC compared to CRC patients (Figure 3.1, B).

3.2.3 Macroscopic and microscopic assessment of intestinal inflammation

All surgical specimens from IBD patients included in our analysis presented macroscopic signs of intestinal inflammation, such as presence of ulcers, wall thickening, and hyperaemia.

Absence and presence of inflammation were confirmed by microscopic examination after staining with H&E in intestinal tissue from surgical specimens of CRC and IBD patients in some experiments (Figure 3.2, A-F). Transmural mononuclear inflammation, mainly represented by lymphocytes and plasma cells, was observed in patients with CD and typical granulomas could be identified in the ileal mucosa in one patient (Figure 3.2, C-D). Inflammation limited to the mucosa, associated to goblet cell depletion and crypt abscesses were common findings in the inflamed colon of UC patients (Figure 3.2, E-F).

Endoscopic biopsies from IBD patients were collected from macroscopically inflamed areas of colon and ileum, while control biopsies were taken from macroscopically normal tissue.

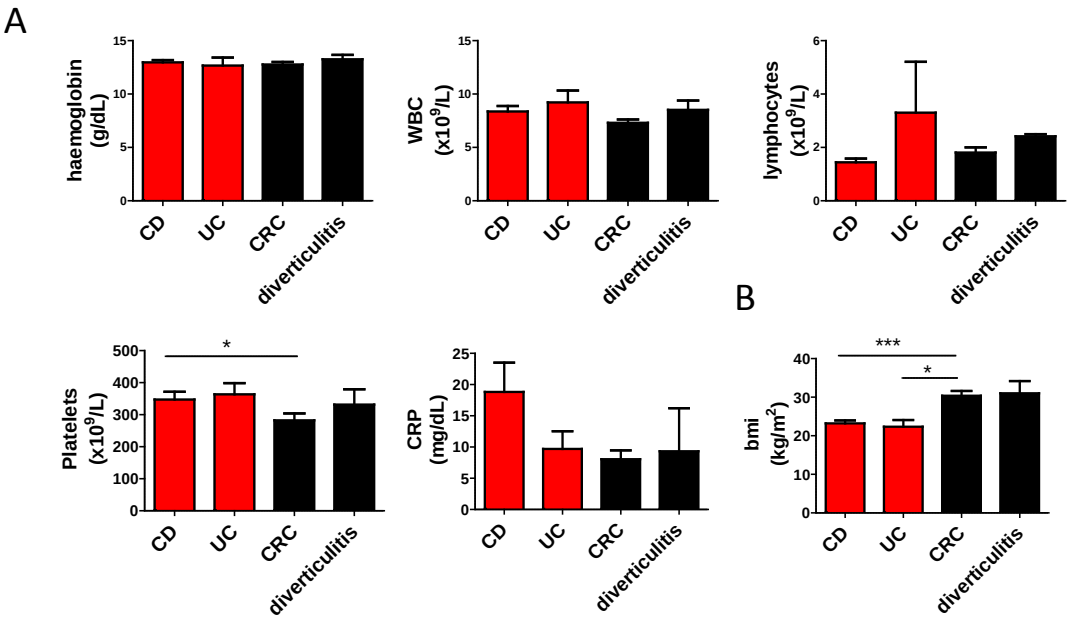


Figure 3.1 Clinical parameters from patients undergoing surgery

(A) Blood test results at time of surgery for haemoglobin (CD n=28, UC n=11, CRC n=33 and diverticulitis n=4), white blood cells (WBC) (CD n=27, UC n=11, CRC n=33 and diverticulitis n=4), lymphocytes (CD n=26, UC n=5, CRC n=21 and diverticulitis n=3), platelets (CD n=28, UC n=8, CRC n=30 and diverticulitis n=4) and CRP (CD n=28, UC n=10, CRC n=24 and diverticulitis n=3). (B) BMI at time of surgery (CD n=27, UC n=3, CRC n=19 and diverticulitis n=4). Bars represent mean +/- SEM. *p<0.05, ***p<0.0001

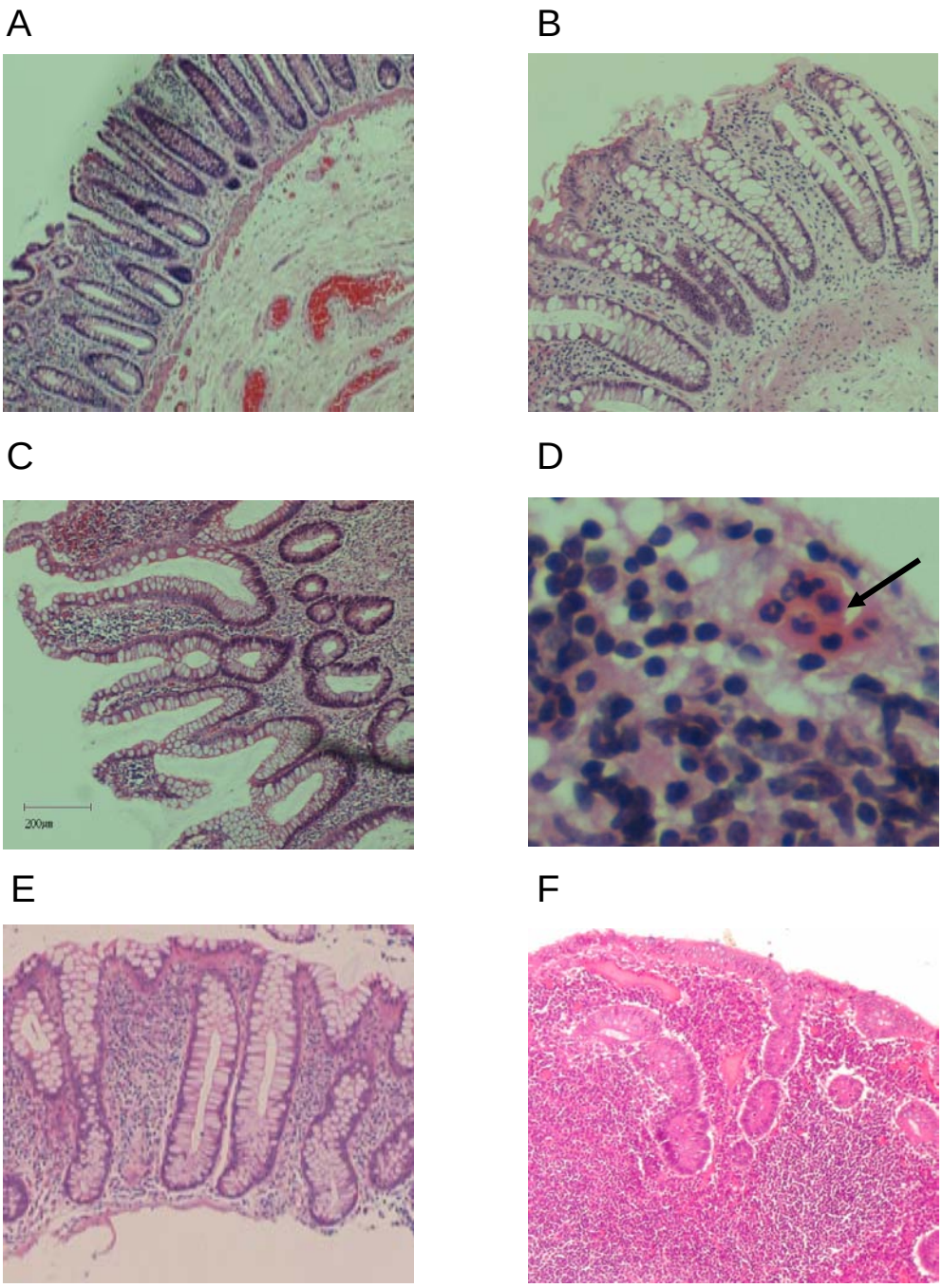


Figure 3.2 Microscopic examination of intestinal tissue from controls and IBD patients

Intestinal tissue was fixed in a 10% formaldehyde saline solution and 4 μm sections were cut with a microtome and stained with H&E. Images were captured using a CoolScope microscope (Nikon). (A-B) Non-inflamed colonic mucosa from unaffected areas of colon in patients with CRC. (C) Mononuclear cell infiltrate in the ileal LP and submucosa in a patient with CD. (D) Granuloma (arrow) and inflammatory infiltrate in the ileal mucosa from a patient with CD. (E-F) Mucosal inflammatory infiltrate and crypt distortion in the colonic mucosa of patients with UC.

3.2.4 Optimization of cell isolation protocols

LPMC isolation from intestinal surgical specimens

In initial experiments, we optimized the LPMC isolation protocol first described by Bull and Bookman in 1977 [385]. As per original protocol, intestinal mucosa is dissected along the fibrous connective tissue and incubated for 15 minutes in DTT solution at RT to remove any adherent mucous. This step is followed by 90 minute incubation in 0.75 mM EDTA solution at 37ºC in order to detach the epithelial layer. Authors state that this step is repeated until no epithelial crypt can be observed in the medium under phase microscopy. However, at microscopic observation of the supernatants, we found that after repeated EDTA washes increasingly higher numbers of rounded lymphocyte-looking cells were present in the supernatant, alongside with lower numbers of epithelial cells. This observation suggested that LP lymphocytes might be released in the medium with repeated EDTA washes, leading to lower final cell yields. In order to characterize the cells released after incubation with EDTA, we performed three 45 minute EDTA washes and surface-stained the cell suspensions from each wash with a fluorescently labelled anti-CD3 antibody and analysed by FACS. We observed that cell suspensions from second and third EDTA washes included increasingly higher frequencies of $CD3^+$ cells (Figure 3.3, A). Even if intraepithelial lymphocytes can account for the presence of some $CD3^+$ cells in the medium, we decided to limit these washes to three 45 minutes incubations, in order to reduce the risk of losing high numbers of LP lymphocytes in the supernatants. Any subsequent epithelial contamination of our cell-suspension would be excluded by performing a percoll gradient after the collagenase digestion step.

We were also able to test different preparations of *Clostridium Histolyticum* collagenase for overnight digestion of the intestinal mucosa isolated from surgical

specimens. Initially, we used collagenase type V (Sigma-Aldrich, Dorset, UK) and we then moved to collagenase type D (Roche Diagnostics, Burgess Hill, UK), as suggested by other researchers in the field of human IBD and in order to achieve consistency with previous published data (personal communications by I. Monteleone, University of Rome "Tor Vergata", Rome, Italy and A. J. Stagg, Queen Mary University of London, London, UK). Collagenase type D maintains high collagenase activity while bearing very low tryptic activity, therefore being particularly indicated when functionality and integrity of cell-surface proteins are crucial. We were able to compare collagenase V (0.16 mg/ml) and collagenase D (0.1 mg/ml) in the digestion of a colonic specimen from a control patient and observed no major differences in size of LPMC isolated, in frequency of $CD3^-$ cells, $CD3^+$ T-cells and $CD4^+$ T-cells. However, some reduction in frequency of $LIN^-HLA\text{-}DR^+CD11c^{high}$ cDC was observed when collagenase D was used (Figure 3.3, B, C).

Collagenase D appeared to be more effective than collagenase V in obtaining complete tissue digestion and led to the isolation of more than twice the numbers of LPMC/gram of tissue (Figure 3.3, D). Therefore, collagenase D was used for mucosal digestion from surgical specimens in most experiments.

LPMC isolation from intestinal endoscopic biopsies

For digestion of endoscopic biopsies, we combined collagenase enzymatic digestion with mechanical digestion by gentleMACS dissociator (Miltenyi Biotec, Bisley, UK). In initial experiments, we compared two different preparations of *Clostridium Histolyticum* collagenase: collagenase D, which we used for mucosal digestion from surgical specimens, and collagenase A (Roche Diagnostics, Burgess Hill, UK). Collagenase A is particularly indicated when cell yields are the main limiting factor

and therefore we thought it might be preferable in cell isolation from very small tissue samples, such as endoscopic biopsies.

By FACS analysis there was no difference in the expression of surface markers, such as LIN, CD45 and CD11c, after collagenase A or collagenase D digestion. Only some reduction in HLA-DR expression could be observed with collagenase A (Figure 3.4). However, collagenase A was strikingly more efficient than collagenase D in digesting mucosal tissue. In fact, we observed complete digestion of biopsy specimens after only one hour incubation with collagenase A, while undigested tissue was always detected when collagenase D was used, resulting in lower cell yields. Therefore, collagenase A was used for LPMC isolation from biopsies in most experiments.

Cell isolation from MLN

For the enzymatic digestion of human MLN we initially used collagenase type VIII (Sigma-Aldrich, Dorset, UK), according to the protocol used in our laboratory to isolate leukocytes from murine MLN. However, in some experiments we noticed that cells isolated from MLN with collagenase VIII completely lacked the expression of the chemokine receptor CCR6, which was instead expressed by a proportion of both intestinal $CD3^+$ and $CD3^-$ cells isolated from ileum and colon using collagenase D. To address this discrepancy, we performed collagenase D (0.75 mg/ml) and collagenase VIII (0.75 mg/ml) digestion on two different halves of the same human MLN. No difference was observed in cell yields between the two preparations. However, collagenase VIII digestion resulted once again in the complete lack of CCR6 expression on all cells, while the use of collagenase D preserved CCR6 expression on a proportion of both $CD3^+$ and $CD3^-$ cells (Figure 3.5). This result highlights the high variability of different collagenase preparations in maintaining the integrity of cell-surface proteins. In all subsequent experiments, we used collagenase D in order to

have maximum consistency between intestinal and MLN digestion protocols and to be able to compare intestinal and MLN cell surface marker expression.

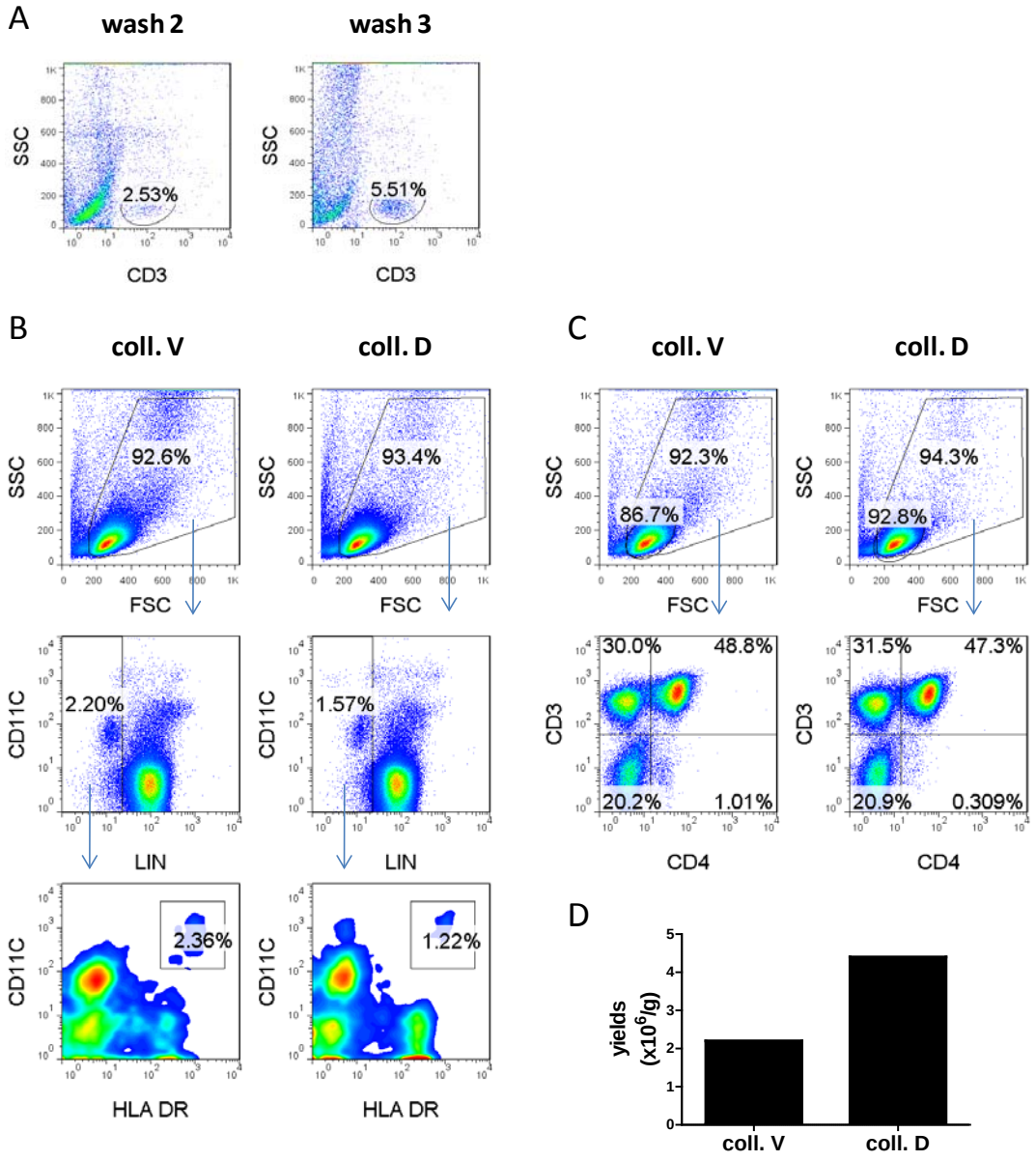


Figure 3.3 Optimization of LPMC isolation protocol from surgical specimens

(A) CD3 staining of cells released after the second and third washes with EDTA. (B) Staining of $LIN^-HLA\text{-}DR^+CD11c^{high}$ cDC using collagenase V and collagenase D. Cells were gated on the live cells on the FSC/SSC plot, on the LIN^- and on the $HLA\text{-}DR^+CD11c^{high}$ populations. (C) T-cell staining using collagenase V and collagenase D. Cells were gated on the live cells on the FSC/SSC plot and on the $CD3^+CD4^+$, $CD3^+CD4^-$, $CD3^-CD4^+$, $CD3^-CD4^-$ populations. (D) Cell yields using collagenase V and collagenase D overnight digestion in one experiment.

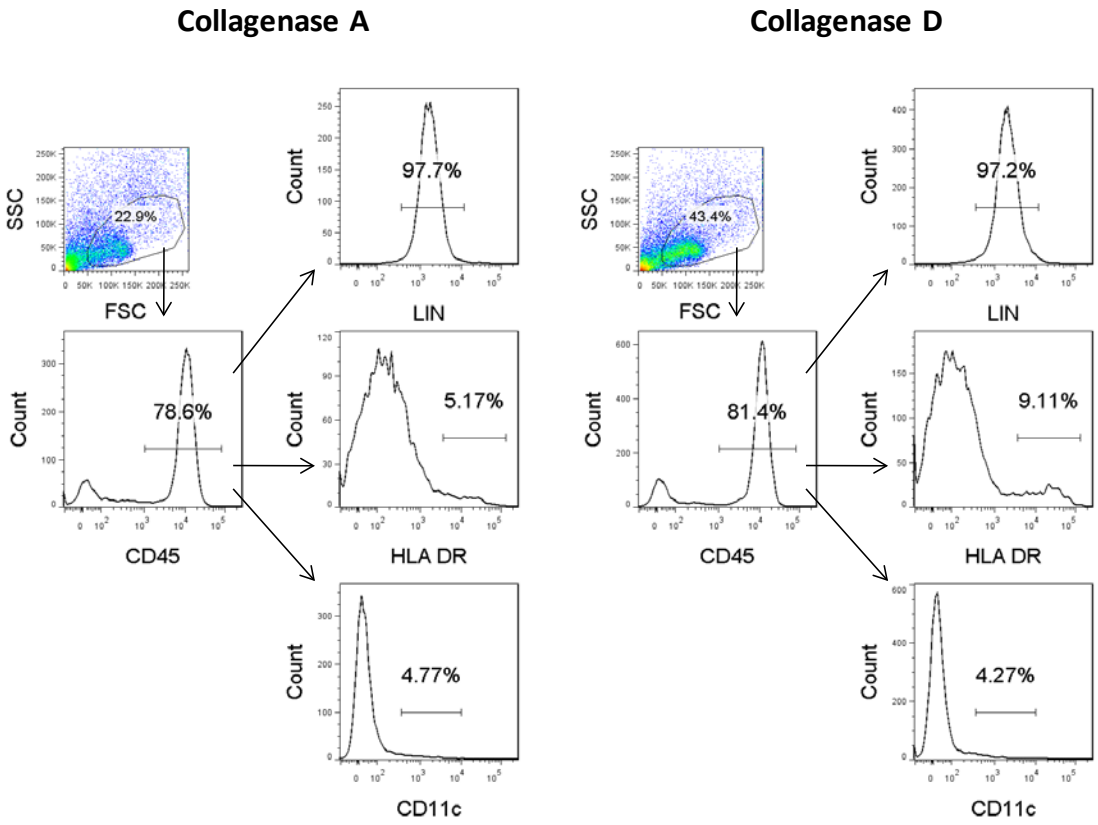


Figure 3.4 Optimization of LPMC isolation from intestinal endoscopic biopsies

LPMC were isolated from colonic endoscopic biopsies combining mechanical and enzymatic digestion using collagenase A (1.5 mg/ml) or collagenase D (1.5 mg/ml) and were stained for CD45, LIN, HLA-DR and CD11c. Cells are gated on the FSC/SSC plot and on the $CD45^{+}$ population.

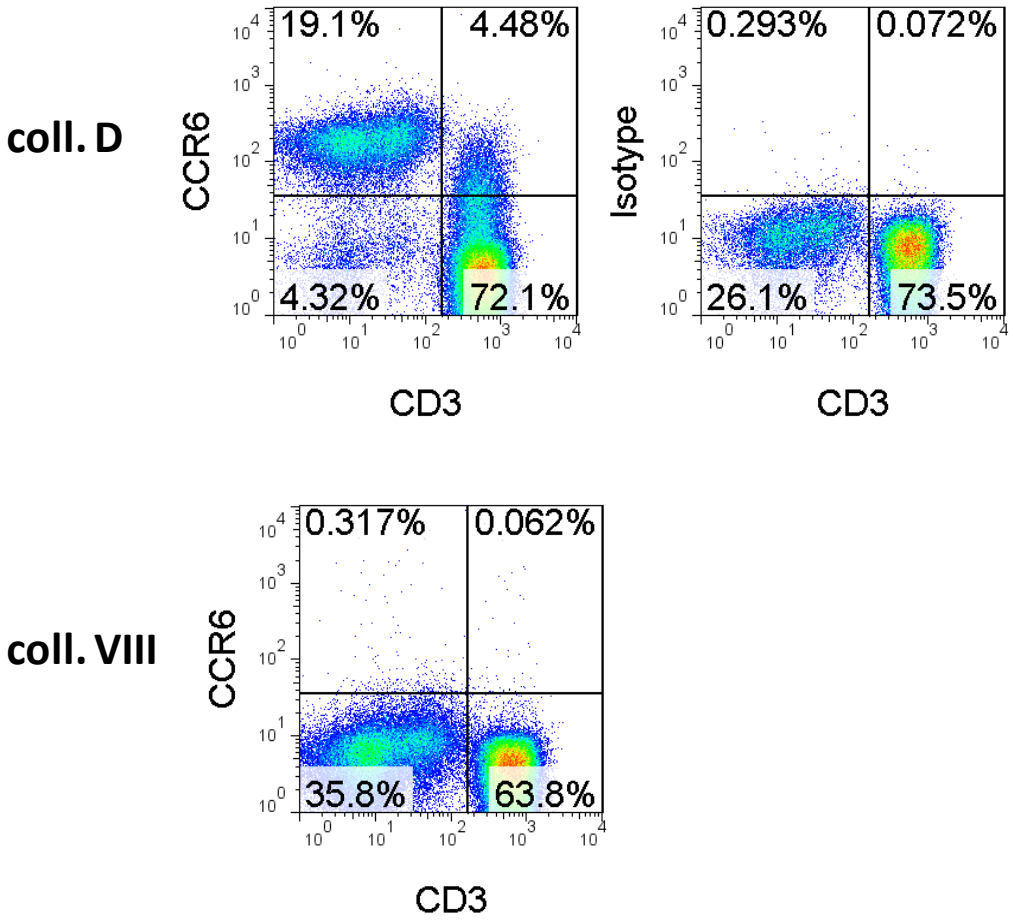


Figure 3.5 Optimization of cell isolation from MLN

Cells were isolated from two halves of the same MLN using either collagenase D (0.75 mg/ml) or collagenase VIII (0.75 mg/ml) enzymatic digestion and were stained for CCR6 and CD3. Gates are set using the appropriate isotype control.

3.2.5 Characterization of PBMC in patients with IBD and controls

In order to characterize the systemic immune response in patients with IBD and un-inflamed controls, we evaluated the frequency of T-cells and T-cell subsets, NK cells, B-cells, granulocytes and DC in the PB from healthy donors, CRC patients, CD patients, UC patients and IBD patients (CD and UC patients pulled together).

T-cells

For T-cell analysis, cells were gated on the lymphocytic cell population on the FSC/SSC scatter (Figure 3.6, A). Lymphocyte frequency in total PBMC did not differ between patients and controls and they represented 48% of PBMC (range=7.6-90.0). No significant difference was observed in frequency of $CD3^+$ T-cells amongst lymphocytes and in the relative frequency of $CD4^+$, $CD8^+$ and $\gamma\delta^+$ T-cells amongst $CD3^+$ cells between patients and controls. $CD3^+$ T-cells represented 67% of lymphocytes (range=13.8-93.5). $CD4^+$ and $CD8^+$ T-cells accounted for 68% (range=22.4-94.8) and 24% (range=3.0-68.0) of $CD3^+$ respectively, with only 4% of $CD3^+$ cells expressing the γδ TCR (range=0.3-16.2) (Figure 3.6, A-B).

Non-T lymphocytes

We analysed the frequency of NK cells and B-cells amongst the $CD3^-$ lymphocytes in patients and controls.

Significantly higher frequency of $CD56^+$ NK cells was observed amongst $CD3^-$ cells in patients with CRC (mean=55.7, range=44.7-65.7) compared to healthy controls and this difference was due to increased frequency of $CD56^{dim}$ cells amongst $CD3^-$ in CRC patients (mean=50.0, range=41.3-60.0) (Figure 3.7, A-B). $CD56^{dim}$ cells are typically cytotoxic and represent the major subset in the blood, while $CD56^{bright}$ are mainly found in secondary lymphoid organs and secrete large amounts of IFN-γ. No difference was observed in the frequency of $CD56^+$ NK cells between patients with

either UC or CD and healthy controls, where they accounted for 28% of $CD3^-$ lymphocytes (range=1.2-69.2) with majority of NK cells represented by $CD56^{dim}$ cells.

Similar frequencies of $CD19^+$ B-cells were observed in patient and control groups, where they represented 17% of $CD3^-$ lymphocytes (range=2.3-73.4) (Figure 3.7, C-D).

Granulocytes

Granulocytes were defined on the base of their size and granularity and gated on the FSC/SSC scatter (Figure 3.8, A). Granulocyte frequency did not differ between groups of patients and controls and they represented 12% of PBMC (range=1.6-26.8) (Figure 3.8, B).

Dendritic cells

We analysed the frequency of blood DC, defined as $LIN^-HLA\text{-}DR^+$ cells. Significantly higher frequency of $LIN^-HLA\text{-}DR^+$ DC was found in patients with CD (mean=2, range=0.3-4.6) and patients with IBD (mean=2, range=0.3-8.0) compared to healthy controls (mean=1, range=0.4-2.2), but not to CRC patients (mean=2, range=0.4-9). cDC, defined as $LIN^-HLA\text{-}DR^+CD11c^{high}$ cells represented 0.4% of PBMC in CD patients, UC patients and healthy controls (range=0.0-1.0). Higher frequency of cDC was observed in CRC patients (mean=1.1%, range=0.2-5.6) and this difference was statistically significant when compared to patients with UC (mean=0.3, range=0.0-0.7) (Figure 3.8, C-D). However, more samples will need to be analysed to confirm these preliminary data.

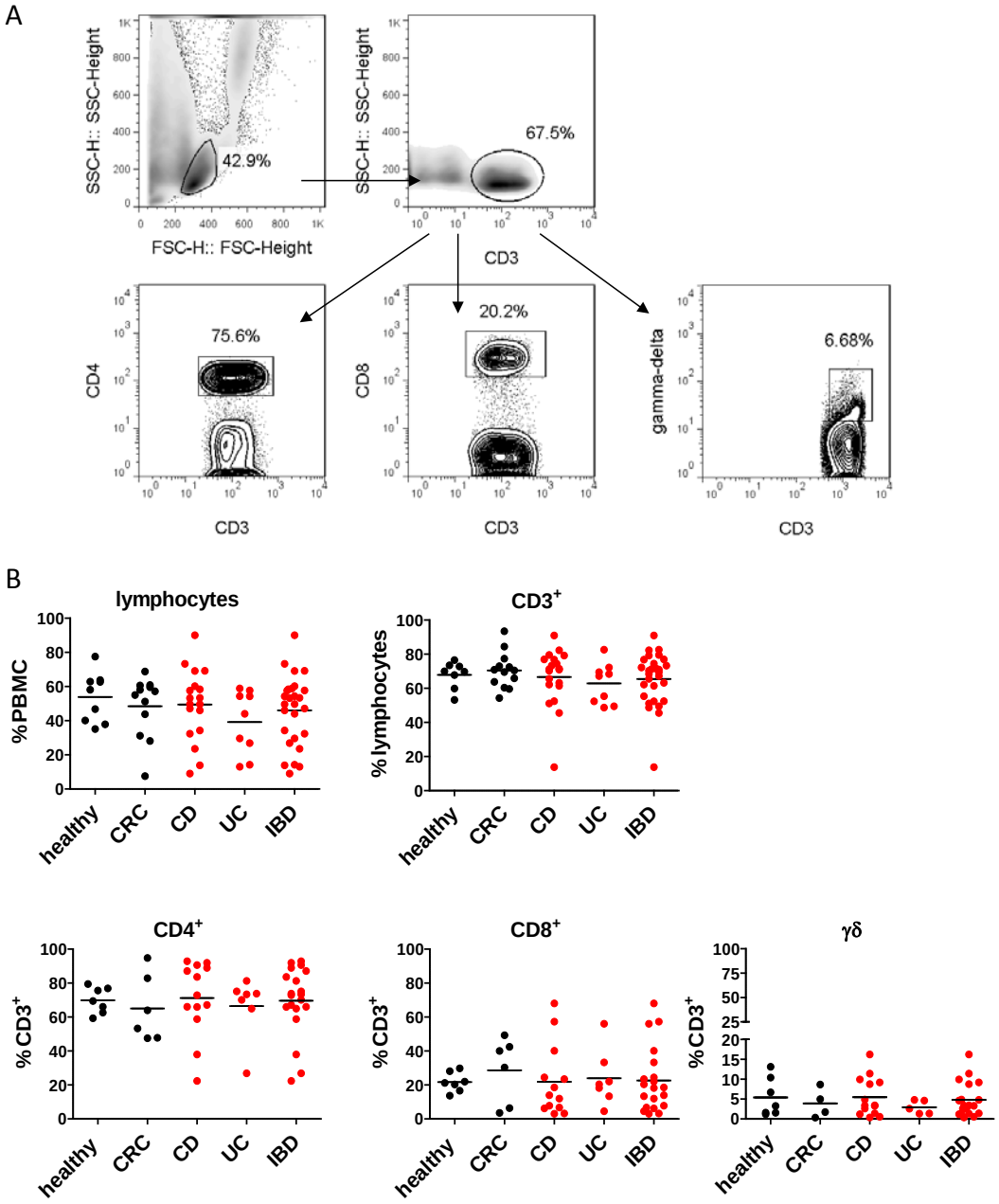


Figure 3.6 Characterization of PB T-cells

(A) Representative staining of $CD3^+$, $CD4^+$, $CD8^+$ and $\gamma\delta^+$ T-cells in PBMC. Cells were gated on the lymphocytic gate, on the $CD3^+$ population and on the $CD4^+$, $CD8^+$ or $\gamma\delta^+$ cells as shown in the FACS plots. (B) Frequency of lymphocytes in total PBMC, $CD3^+$ in lymphocytes, and $CD4^+$, $CD8^+$ and $\gamma\delta^+$ in $CD3^+$ T-cells isolated from the blood of healthy volunteers, CRC, CD, UC and IBD (CD+UC) patients, using the gates shown in (A).

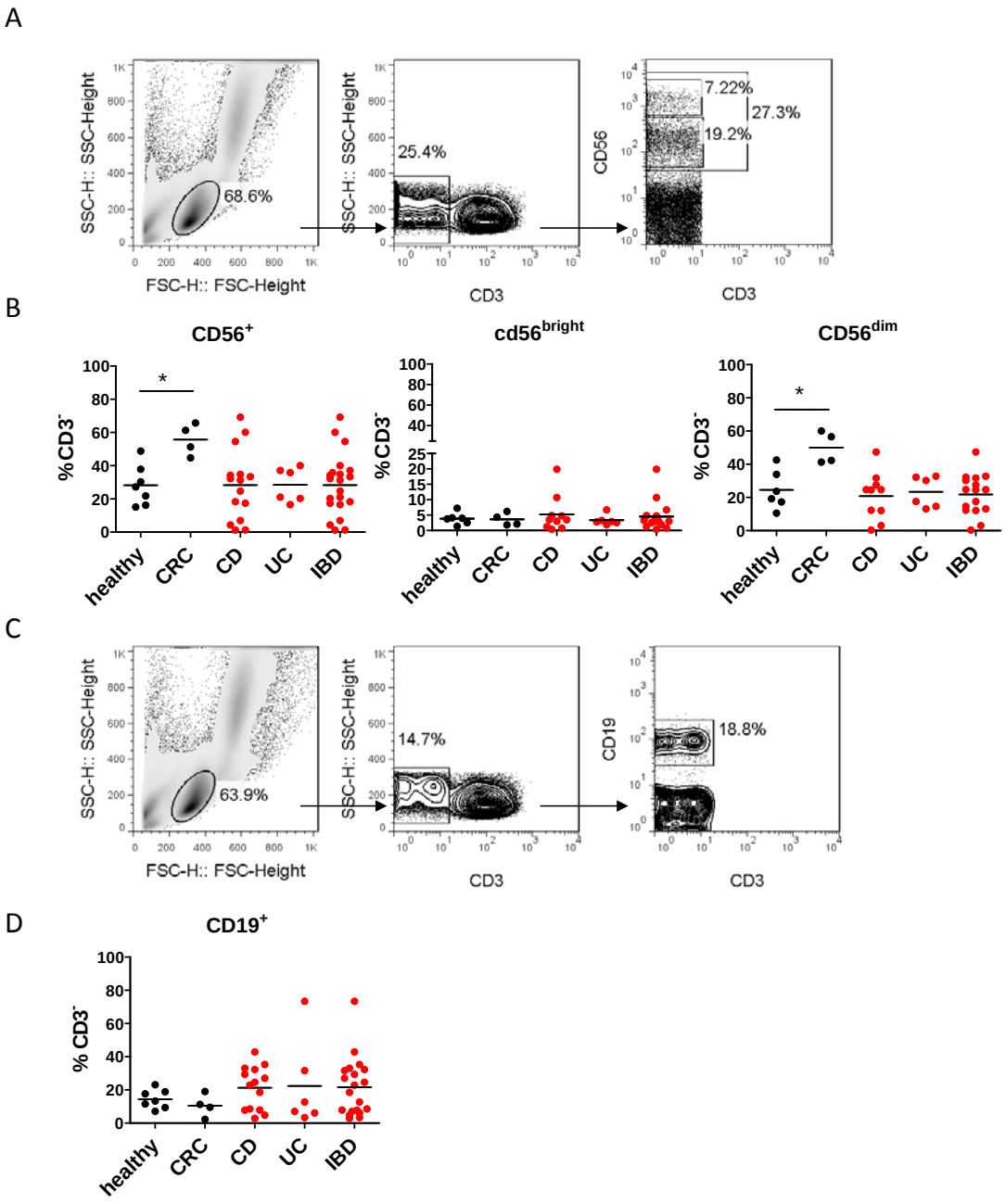


Figure 3.7 Characterization of PB NK and B-cells

(A) Representative staining of NK cells in PBMC. Cells were gated on the lymphocytic gate, on the $CD3^-$ population and on the $CD56^+$, $CD56^{bright}$ or $CD56^{dim}$ cells as shown in the FACS plots. (B) Frequency of $CD56^+$, $CD56^{bright}$ or $CD56^{dim}$ NK cells in the $CD3^-$ cells from healthy volunteers, CRC, CD, UC and IBD (CD+UC) patients, using the gates shown in (A). (C) Representative staining of B-cells in PBMC. Cells were gated on the lymphocytic gate, on the $CD3^-$ population and on the $CD19^+$ cells as shown in the FACS plots. (D) Frequency of $CD19^+$ B-cells in the $CD3^-$ cells from healthy volunteers, CRC, CD, UC and IBD (CD+UC) patients, using the gates shown in (C). *$p<0.05$

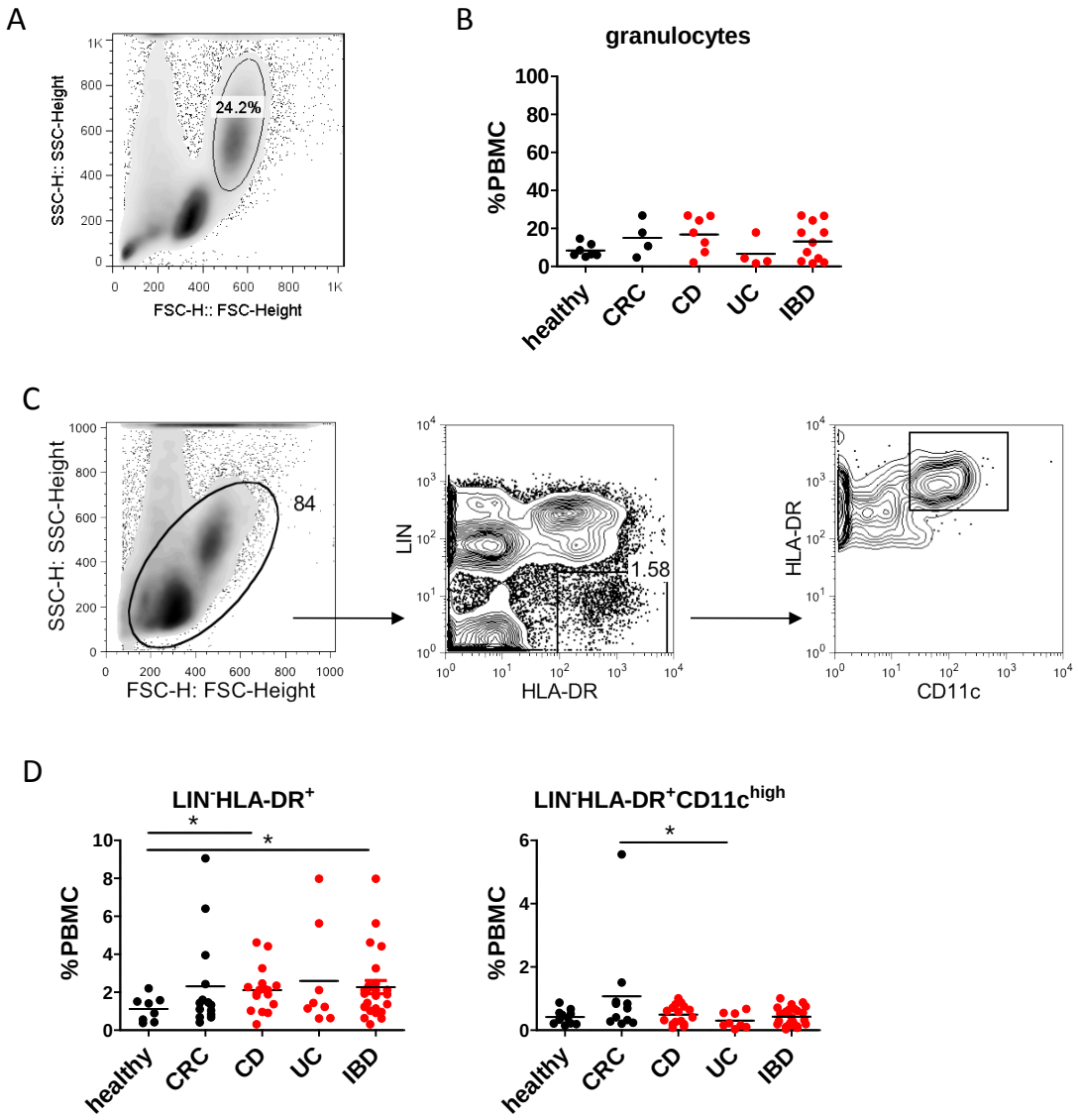


Figure 3.8 Characterization of PB granulocytes and DC

(A) Representative gating strategy for granulocytes on the FSC/SSC plot. (B) Frequency of granulocytes in PBMC from healthy volunteers, CRC, CD, UC and IBD (CD+UC) patients, using the gate shown in (A). (C) Representative staining of DC and cDC in PBMC. Cells were gated on the live cells on the FSC/SSC plot, on the $LIN^-HLA\text{-}DR^+$ population (DC) and on the $CD11c^{high}$ cells (cDC) as shown in the FACS plots. (D) Frequency of $LIN^-HLA\text{-}DR^+$ DC and $LIN^-HLA\text{-}DR^+CD11c^{high}$ cDC amongst PBMC from healthy volunteers, CRC, CD, UC and IBD (CD+UC) patients, using the gates shown in (C). *$p<0.05$

3.2.6 Characterization of LPMC in patients with IBD and controls

To characterize the intestinal immune response, we analysed the frequency of T-cells and T-cell subsets, NK cells, B-cells and DC in LPMC isolated from uninflamed colon of CRC patients, from inflamed colon of CD, UC and IBD patients (UC+CD patients), from control ileum of CRC patients or normal controls and from inflamed ileum of CD patients.

T-cells

Cells were gated on the FSC/SSC scatter on the lymphocytic population, which represented 48% of total LPMC with no significant difference between inflamed and uninflamed tissues (range=3.3-92.8). Only a trend to a higher frequency of lymphocytes was observed in control ileum *versus* inflamed ileum of CD patients (p=0.08). We did not observe any difference in the frequency of $CD3^+$ T-cells in the colon or ileum of IBD patients and uninflamed controls and they represented 69% of lymphocytes (range=27.7-90.5). Similarly, no difference was found in the relative frequency of $CD4^+$ and $CD8^+$ between patients and controls and they represented 56% (range=22.6-76.4) and 31% (range=8.1-58.2) of $CD3^+$ cells, respectively. Only 4% of $CD3^+$ cells expressed the γδ TCR in colon and ileum of IBD patients (range=0.4-13.7), while $\gamma\delta^+$ T-cells were 12% and 15% of $CD3^+$ cells in the colon of two CRC patients (Figure 3.9, A-B).

Non-T lymphocytes

$CD56^+$ NK cells represented 14% of $CD3^-$ lymphocytes (range=2.3-33.3) and no significant difference was observed in inflamed and uninflamed colon and ileum from IBD patients and controls (Figure 3.10, A-B). CD56 staining of LPMC did not clearly distinguish between $CD56^{bright}$ and $CD56^{dim}$ cells in most samples in our hands; therefore we could not perform subpopulation analysis.

Interestingly, significantly higher frequency of $CD19^+$ B-cells was observed amongst $CD3^-$ lymphocytes in the colon of patients with CD (mean=54.72, range=49.0-60.5) and IBD patients (mean=53, range=45.1-60.5) *versus* the uninflamed colon of CRC patients (mean=26%, range=11.1-53.7) (Figure 3.10, C-D).

Dendritic cells

We evaluated the frequency of intestinal DC and cDC, defined as $LIN^-HLA\text{-}DR^+$ and $LIN^-HLA\text{-}DR^+CD11c^{high}$ cells, respectively. This analysis showed that LP DC represent 4% (range=0.6-28.5) and cDC 0.5% (range=0.0-3.1) of LPMC. No significant difference was observed between patients and controls in colon or ileum (Figure 3.11, A-B).

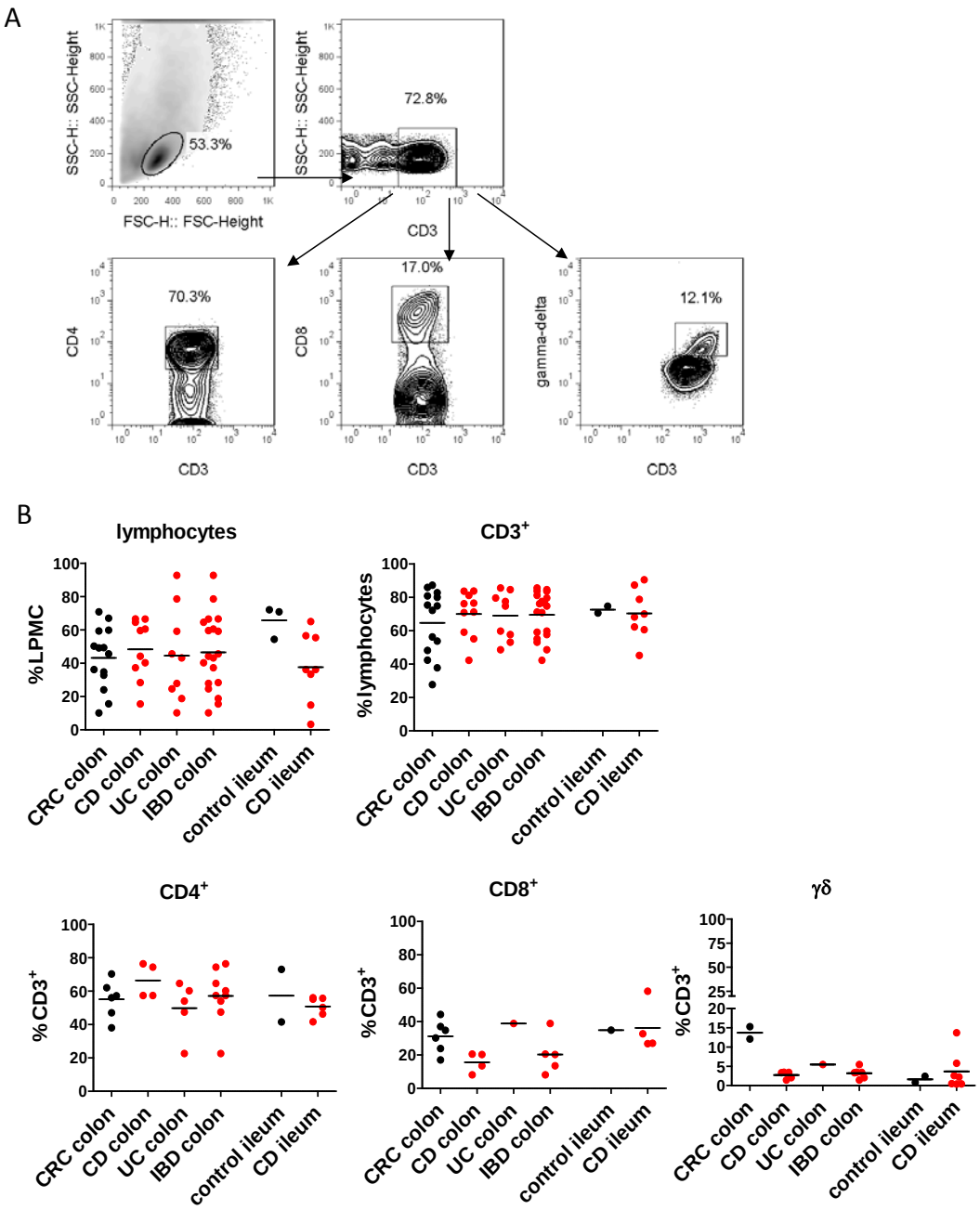


Figure 3.9 Characterization of LP T-cells

(A) Representative staining of $CD3^+$, $CD4^+$, $CD8^+$ and $\gamma\delta^+$ T-cells in LPMC. Cells were gated on the lymphocytic gate, on the $CD3^+$ population and on the $CD4^+$, $CD8^+$ and $\gamma\delta^+$ cells as shown in the FACS plots. (B) Frequency of lymphocytes in total LPMC, $CD3^+$ in lymphocytes, $CD4^+$, $CD8^+$ and $\gamma\delta^+$ in $CD3^+$ T-cells in the colon of CRC, CD, UC and IBD (CD+UC) patients and in the ileum of controls and CD patients, using the gates shown in (A).

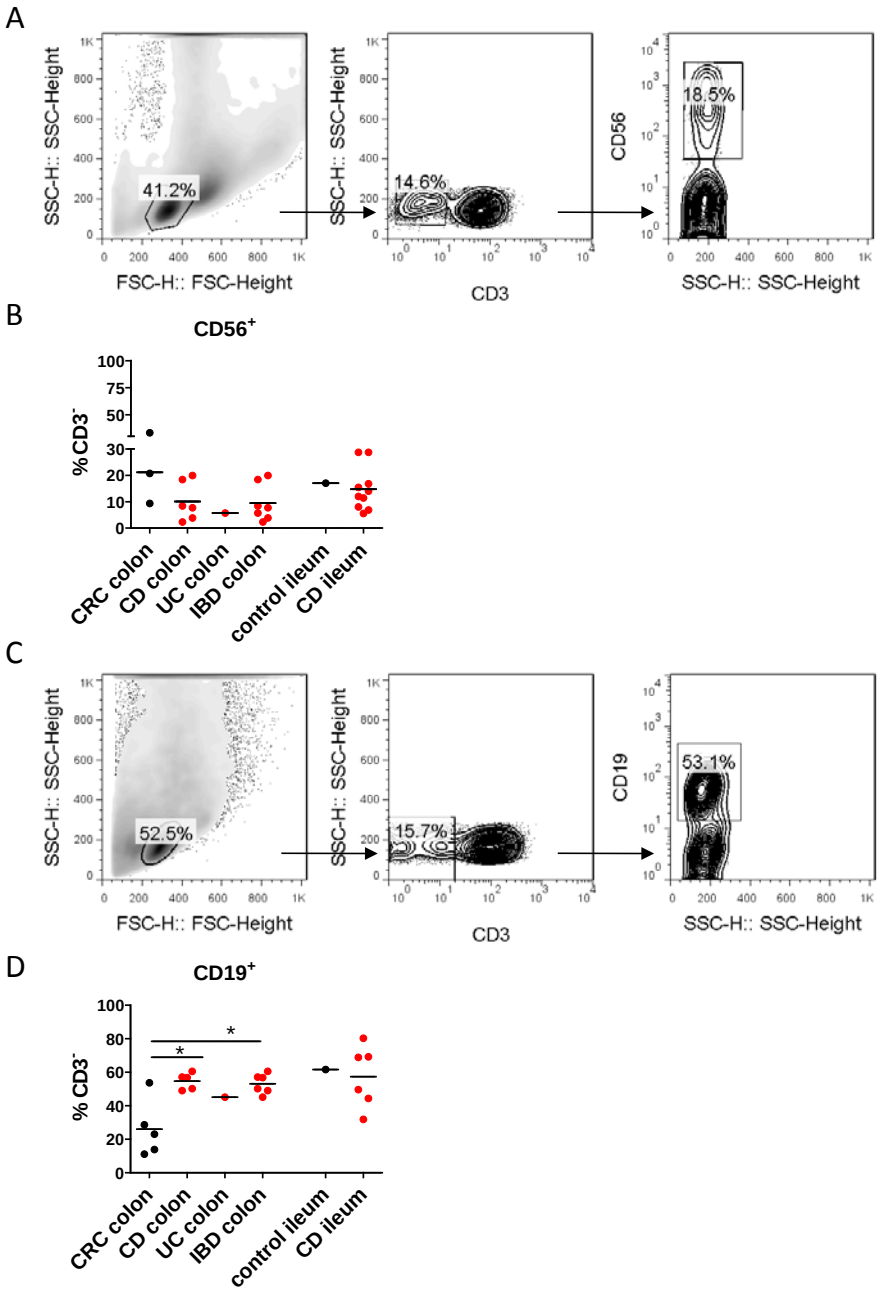


Figure 3.10 Characterization of LP B-cells and NK cells

(A) Representative staining of NK cells in LPMC. Cells were gated on the lymphocytic gate, on the $CD3^-$ population and on the $CD56^+$ cells as shown in the FACS plots. (B) Frequency of $CD56^+$ NK cells in the $CD3^-$ cells in LPMC isolated from the colon of CRC, CD, UC and IBD (CD+UC) patients and from the ileum of controls and CD patients, using the gates shown in (A). (C) Representative staining of B-cells in LPMC. Cells were gated on the lymphocytic gate, on the $CD3^-$ population and on the $CD19^+$ cells as shown in the FACS plots. (D) Frequency of $CD19^+$ B-cells in the $CD3^-$ cells in the colon of CRC, CD, UC and IBD (CD+UC) patients and in the ileum of controls and CD patients, using the gates shown in (C). *$p<0.05$

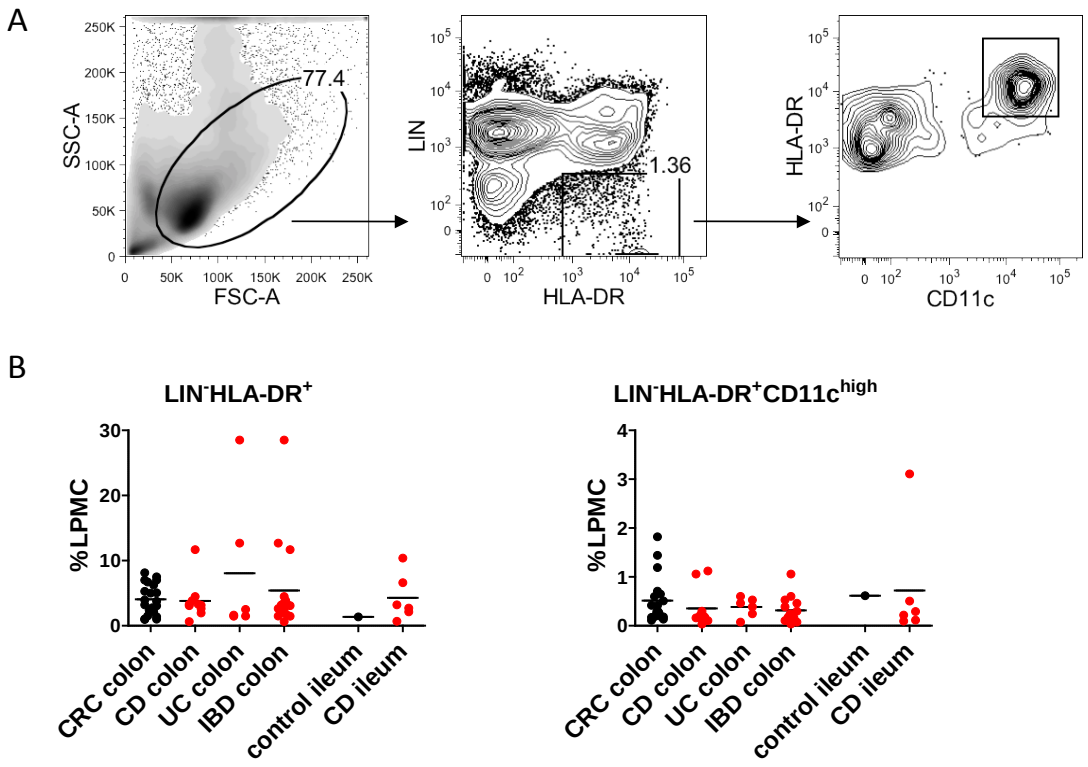


Figure 3.11 Characterization of LP DC

(A) Representative staining of DC and cDC in LPMC. Cells were gated on the live cells, on the LIN^- population, on the $HLA\text{-}DR^+$ cells (DC) and on the $CD11c^{high}$ cells (cDC) as shown in the FACS plots. (B) Frequency of $LIN^-HLA\text{-}DR^+$ DC and frequency of the $LIN^-HLA\text{-}DR^+CD11c^{high}$ cDC in LPMC in the colon of CRC, CD, UC and IBD (CD+UC) patients and in the ileum of controls and CD patients, using the gates shown in (A).

3.2.7 Characterization of MLN mononuclear cells in patients with IBD

We were able to evaluate T-cell, B-cell, NK and DC phenotype in haematopoietic cells isolated from MLN of patients with CD.

T-cells

Lymphocytes were gated on the FSC/SSC scatter and represented 68% of total cells (range=15.0-90.5). Of these, 64% were represented by $CD3^+$ T-cells (range=29.0-78.4). Amongst T-cells, 65% (range=35.9-81.3) were $CD4^+$, 20% (range=10.1-32.1) were $CD8^+$ and 3% (range=0.6-4.5) were $\gamma\delta^+$ T-cells (Figure 3.12, A-B).

Non-T lymphocytes

In MLN of CD patients, $CD19^+$ B-cells and $CD56^+$ NK cells represented 44% (range=4.9-62.1) and 5% (range=0.6-10.3) of $CD3^-$ lymphocytes, respectively (Figure 3.12, C-D). Similarly to what observed in LPMC staining, a clear distinction between $CD56^{dim}$ and CD56 ^{bright} NK cells was not observed in cells isolated from MLN.

Dendritic cells

$LIN^-HLA\text{-}DR^+$ DC and $LIN^-HLA\text{-}DR^+CD11c^{high}$ cDC represented 1.5% (range=0.3-5.5) and 0.1% (range=0.0-0.3) of cells in MLN from CD patients, respectively (Figure 3.13, A-B).

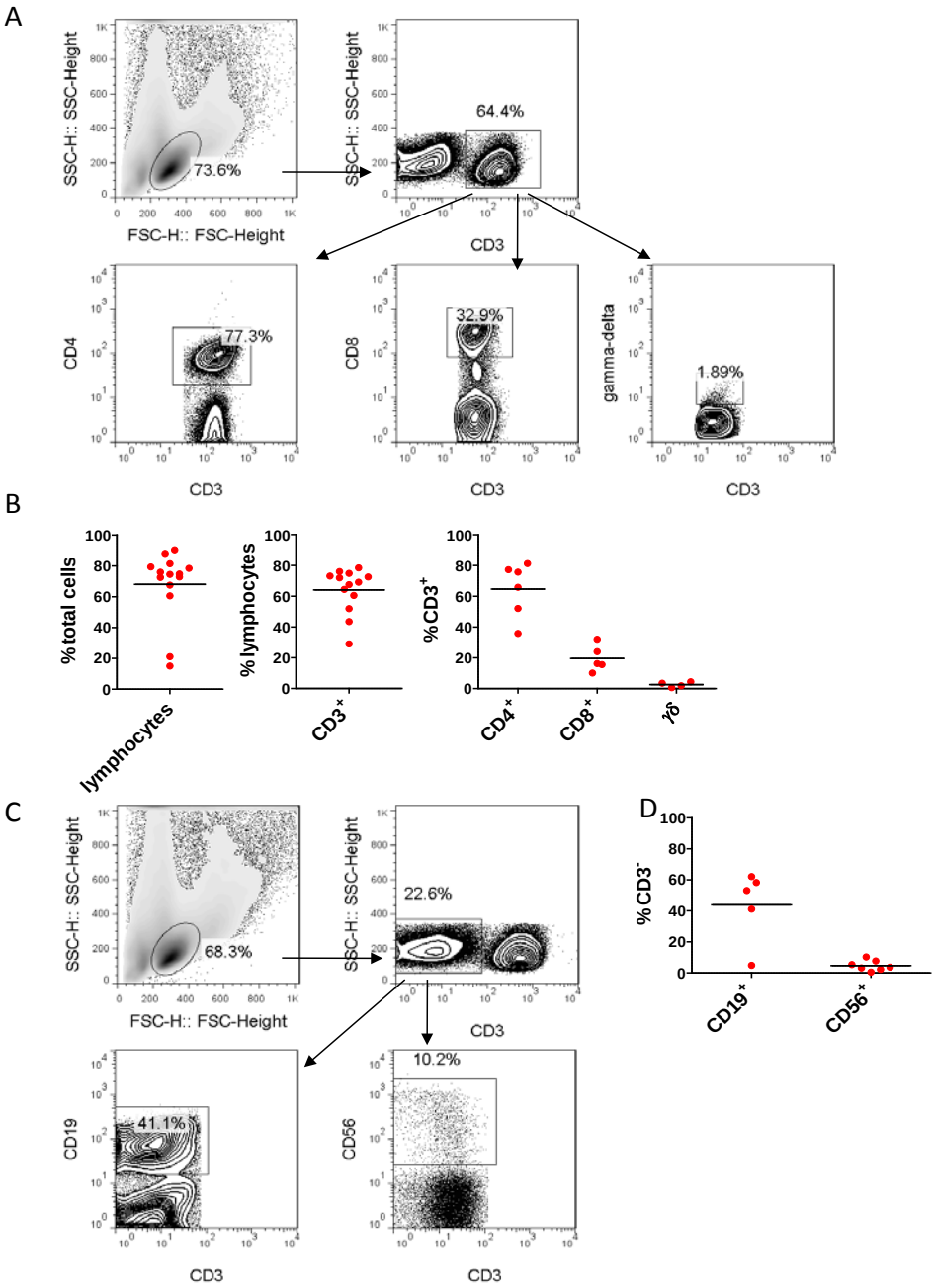


Figure 3.12 Characterization of T-cells, B-cells and NK cells in MLN in CD

(A) Representative staining of $CD3^+$, $CD4^+$, $CD8^+$ and $\gamma\delta^+$ T-cells in MLN. Cells were gated on the lymphocytic gate, on the $CD3^+$ population and on the $CD4^+$, $CD8^+$ or $\gamma\delta^+$ cells as shown in the FACS plots. (B) Frequency of lymphocytes in total cells, $CD3^+$ in lymphocytes, $CD4^+$, $CD8^+$ and $\gamma\delta^+$ in $CD3^+$ T-cells in MLN from CD patients, using the gates shown in (A). (C) Representative staining of B-cells and NK cells in MLN. Cells were gated on the lymphocytic gate, on the $CD3^-$ population and on the $CD56^+$ (NK cells) or the $CD19^+$ (B-cells) cells. (D) Frequency of B-cells and NK cells in the $CD3^-$ cells in MLN from CD patients, using the gates shown in (C).

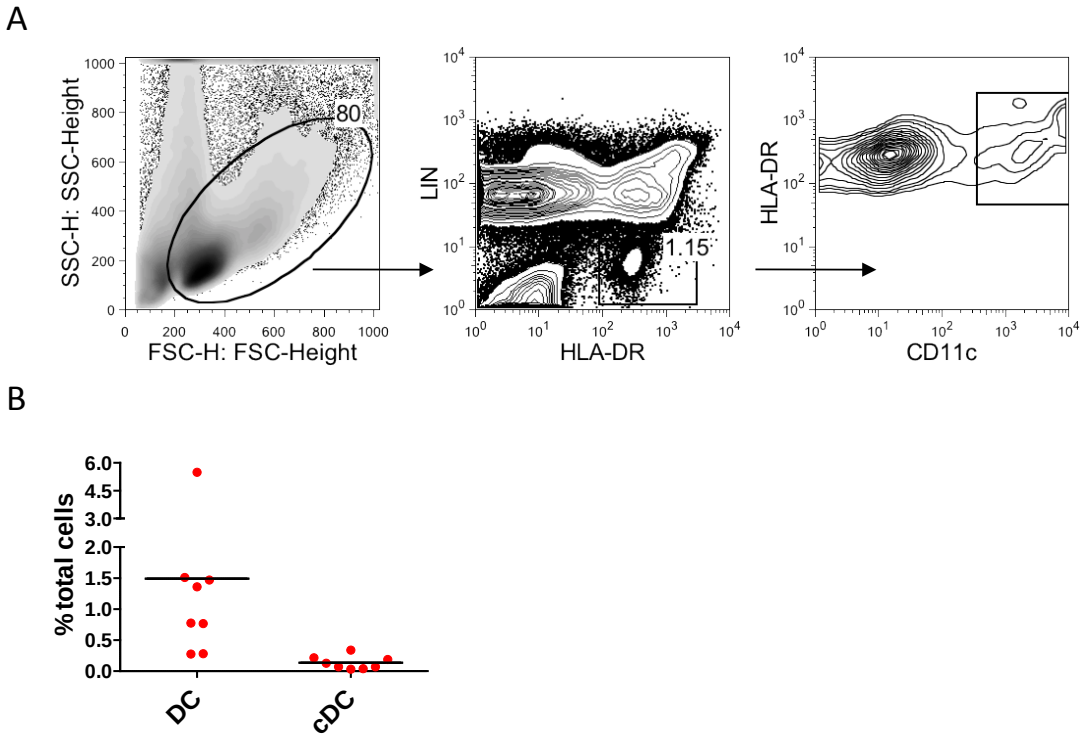


Figure 3.13 Characterization of MLN DC

(A) Representative staining of DC and cDC in MLN. Cells were gated on the live cells, on the LIN^- population, on the $HLA\text{-}DR^+$ (DC) and on the $CD11c^{high}$ (cDC) cells as shown in the FACS plots. (B) Frequency of DC and cDC in MLN from CD patients, using the gates shown in (A).

3.3 Conclusion

In this chapter, we have shown the successful isolation and characterization of human cells from blood samples, surgical intestinal specimens, MLN and endoscopic biopsies from IBD patients and controls. Isolation of mononuclear cells from human intestinal tissue and MLN has required protocol optimization, which particularly highlighted the importance of selecting the right collagenase preparation for each tissue in order to obtain satisfactory cell yields, while preserving surface marker integrity.

Our phenotyping of systemic and intestinal immune cells shows no difference in the frequency of T-cells and $CD4^+$, $CD8^+$ and $\gamma\delta^+$ T-cell subsets between patients and controls. These findings are in accordance with previous reports from the literature [392, 398, 399].

Amongst non-T lymphocytes, we found increased frequency of $CD56^+$ NK cells in the systemic immune response in patients with CRC and similar findings were reported earlier by Gibson and Jewell [400]. As previously shown, $CD56^{dim}$ NK cells were the major population in the periphery [35]. Conversely, it has been shown that tissue and intra-tumoural NK cells are mainly represented by $CD56^{bright}$ NK cells [401]. The increased peripheral frequency of NK cells in CRC patients compared to healthy controls highlights once more the intrinsic limitation of recruiting patients with cancer as non-inflammatory controls, especially when studying specific cell-populations, which might be altered in patients with cancer, as it is the case for NK cells. B-cell frequency did not differ between patients and controls in PB, but B-cell accumulation was observed in the inflamed colon of patients with CD. Increased B-cell and plasma-cell infiltrates in the inflamed intestine of IBD patients have been reported by early studies [402, 403]. This observation suggests that a local B-cell response could

contribute to intestinal inflammation in IBD, even if the precise mechanism remains unknown.

In our analysis of human DC populations, we used the definition proposed by Bell *et al*, who first isolated and characterized $LIN^{-}HLA\text{-}DR^{+}$ DC from the human colon and showed that they can stimulate allogeneic mixed leukocyte reactions and exert endocytic activity. In accordance with their initial observations, we have found similar frequencies of intestinal DC in IBD patients *versus* controls [85]. However, available evidence suggests that in IBD intestinal DC present a more activated phenotype, over-express TLRs and secrete inflammatory cytokines [404]. Interestingly, we observed an increase in circulating DC in patients with IBD, with no difference in cDC, suggesting this might be due to an increase in pDC.

We have already discussed the limitation of collecting MLN only from patients with IBD, which has not allowed us to compare MLN draining inflamed tissue with MLN draining uninflamed tissue. Nevertheless, our data show that T-cell subset frequency is conserved in blood, intestine and MLN from IBD patients, with only lower frequency of $CD4^{+}$ T-cells found in the colon in our cohort. On the contrary, we found higher frequency of B-cells and DC in MLN and intestinal cells compared to circulating cells, while NK cells are more represented in the blood.

Recruitment of patients undergoing intestinal surgery was very efficient for patients with IBD and for CRC patients as non-inflammatory controls. On the other hand, this approach obviously does not allow the study of intestinal tissue from healthy subjects and even the enrolment of inflammatory controls appeared to be quite challenging in our study. We were only able to collect three intestinal samples from patients with diverticulitis that were not included in our phenotypic analysis due to the very low numbers. Most patients with diverticulitis or other inflammatory intestinal conditions

are in fact successfully managed with medical treatment and colectomy is only rarely required as an emergency procedure, which makes it difficult to organise sample collection and perform laboratory experiments. However, the inclusion of inflammatory controls in human studies on IBD is of appreciable importance in order to differentiate between IBD specific and non-specific pathways involved in intestinal inflammation. In this respect, our recent optimization of a successful mechanical and enzymatic technique that allows isolation of cells from endoscopic biopsies appears particularly promising for future research. In fact, this approach opens access not only to much larger numbers of IBD patients with different spectrum of disease activity, but also to healthy controls, undergoing endoscopy for CRC screening or chronic abdominal pain for instance, and to a bigger range of inflammatory controls, such as patients with diverticulitis, infectious or ischemic colitis and microscopic colitis that do not require surgical intervention. Isolation of cells from surgical specimens is particularly useful for more extensive functional analysis, but correlation of phenotype with disease parameters requires large numbers of biopsy material. Initial studies will really benefit of cell isolation from surgical specimens to perform a wider and more flexible range of experiments. Once a specific cell-type or pathway of interest is identified, isolation of cells from endoscopic biopsies will be an important tool to evaluate big cohorts of patients and controls. Studies on isolated cells need to be combined with *in situ* analysis, which can be performed by immunofluorescent staining of biopsy material.

Chapter 4- The role of the IL23/IL17 pathway in human systemic and intestinal immune responses and its contribution to IBD

4.1 Introduction

IL23 plays a pivotal role in the development of chronic intestinal inflammation in experimental murine models of colitis [181, 205, 296, 300]. Compartmentalization of the IL23/IL17 pathway has been observed in these models with IL23 being the key cytokine driving intestinal inflammation while systemic disease is dependent on IL12 [204]. Results from human studies have converged with the identification in patients with IBD of multiple susceptibility SNPs in many genes encoding for proteins involved in the IL23/IL17 pathway including *IL23R*, *IL12B*, *STAT3*, *JAK2* and *CCR6* [301-304]. In addition, Th-17 signature cytokines are elevated in the intestine and serum of patients with IBD [309, 405, 406] and Th-17 cells with an activated phenotype are present in the colon and blood of patients with CD [163, 164]. IL23 has been shown to sustain Th-17 responses [201] but also acts on newly identified ILC populations to induce IL17 and IL22 production [51]. Recent findings from our laboratory have demonstrated that IL23-responsive ILC mediate innate colitis through an IL17- and IFN-γ-dependent mechanism indicating an important functional role for ILC in the intestinal inflammatory response [214]. IL23-responsive ILC populations have also been identified in human intestine and tonsils. Cella and colleagues have described $CD3^{-}CD56^{+}NKp44^{+}$ cells that are present in the human MALT and produce IL22, but not IL17 in response to IL23 [259]. It is notable that they also express the transcription factor *RORC*, typically expressed by Th-17 cells. Recent studies suggest that these cells belong to the same lineage as LTi cells [40, 288, 289]. LTi are

involved in the organogenesis of secondary lymphoid organs through TNF-α and LT-β-mediated induction of the adhesion molecules ICAM1, VCAM1 and MAdCAM1 on mesenchymal cells. Interestingly, murine LTi are known to produce IL22 and IL17 in response to IL23 [51]. Both $CD127^{+}CD56^{-}$ and $CD56^{+}$ ILC can be isolated from human adult tonsils and share the expression of NKp44, NKp46, CD161, c-Kit and *RORC*. *In vitro* expanded $CD56^{-}CD127^{+}$ and $CD56^{+}CD127^{+}$ cells showed a similar cytokine profile. However, clonal analysis revealed that although some clones express IL17, the frequency of IL22-producing clones is higher [288].

The role of human innate lymphoid sources of IL17 and IL22 in the human intestine and how they may contribute to IBD has not been investigated.

In this chapter, we have analysed the role of the IL23/IL17 pathway in the human intestinal and systemic immune response and its contribution to chronic intestinal inflammation in patients with IBD. We aimed to evaluate if in humans, as in mice, this axis is compartmentalized with preferential expression in the gut and if there is an over-expression in the presence of chronic intestinal inflammation in patients with IBD. Moreover, we wanted to investigate if innate sources of IL17 are found in the human intestine and whether they contribute to intestinal inflammation in IBD.

4.2 Results

4.2.1 The IL23/IL17 pathway is compartmentalized

Intestinal cells respond to IL23

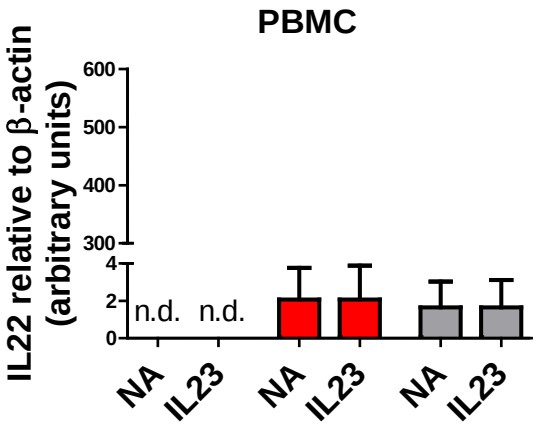
In order to assess the responsiveness of intestinal and blood cells to IL23 stimulation, we isolated PBMC and LPMC from control and UC subjects and cultured them overnight in the presence and absence of IL23. *IL22* and *IL17A* m-RNA expression was then analysed by qPCR as a readout of IL23 activity on blood and intestinal cells. *IL17A* expression was very low or undetectable in all cell culture conditions and no induction was observed after IL23 stimulation (data not shown). In initial experiments *IL22* was not detectable in PBMC from one control individual with or without IL23 stimulation. Detectable transcript levels of *IL22* were found in PBMC from some UC patients, but we did not observe any induction after culture in the presence of IL23 (Figure 4.1, A). Interestingly, *IL22* was expressed in LPMC from both controls and UC patients and significantly induced after IL23 stimulation (statistical significance was achieved when results from controls and UC patients were pooled together) (Figure 4.1, B). These observations suggest the presence of compartmentalization of IL23 responsiveness to the gut.

Th-17 genes are expressed in both intestinal T and non-T cells in the absence of inflammation

We next wanted to evaluate the contribution of innate and adaptive sources of Th-17 signature cytokines in the human systemic and intestinal immune response in the absence of inflammation. We sorted $CD3^+$ and $CD3^-$ cells from PB and LP of control individuals and analysed *ex vivo* expression of Th-17 genes. We found preferential expression of the Th-17 cytokine genes *IL17A, IL17F, IL22, IL26* and *IL21* in $CD3^+$ cells isolated from the colon compared to the blood, in the absence of any

pathological changes. Similarly *IL23R* and the *AHR* transcription factor genes were also expressed at higher levels in LP compared to PB $CD3^+$ cells (Figure 4.2). However, compartmentalization of Th-17 gene expression was not restricted to T-cells as we also found increased expression of *IL17F*, *IL22*, *IL26*, *IL23R*, *RORC* and *AHR* in LP $CD3^-$ cells with undetectable or very low expression amongst PB $CD3^-$ cells. A trend to a higher expression in intestinal $CD3^-$ cells was also found for *IL17A* (p=0.07) (Figure 4.3). Expression levels of *IL17A*, *IL17F* and *IL26* were significantly lower in LP $CD3^-$ than in LP $CD3^+$ cells. This suggests that either many cells express lower levels of these cytokines or only a minor population expresses these genes at high levels in the intestinal $CD3^-$ cell compartment in uninflamed controls. These results confirm our hypothesis of a specific role for the IL23 axis in the intestinal immune response and show that both T and non-T cells expressing Th-17 related genes are present in the human intestine.

A



B

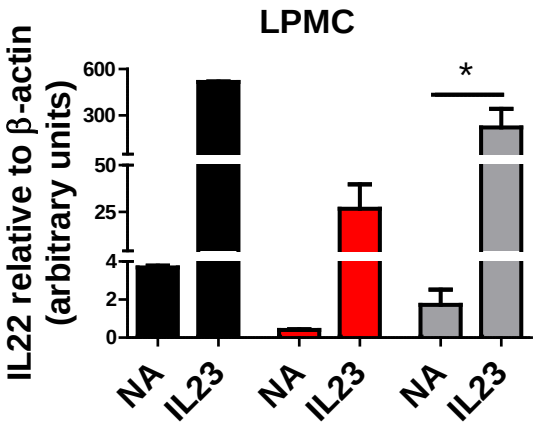


Figure 4.1 IL23 activity is compartmentalized to the gut.

Relative mRNA expression of *IL22* in PBMC from one control subject (black bars), UC patients (red bars, n=4) and control and UC individuals pooled together (grey bars, n=5) after overnight culture in complete media with no addition (NA) and in the presence of IL23 (10 ng/ml). (B) *IL22* expression in LPMC from control subjects (black bars, n=2), UC patients (red bars, n=3) and control and UC individuals pooled together (grey bars, n=5) after overnight culture with no addition (NA) and in the presence of IL23 (10 ng/ml). Bars represent mean +/- SEM. *p<0.05

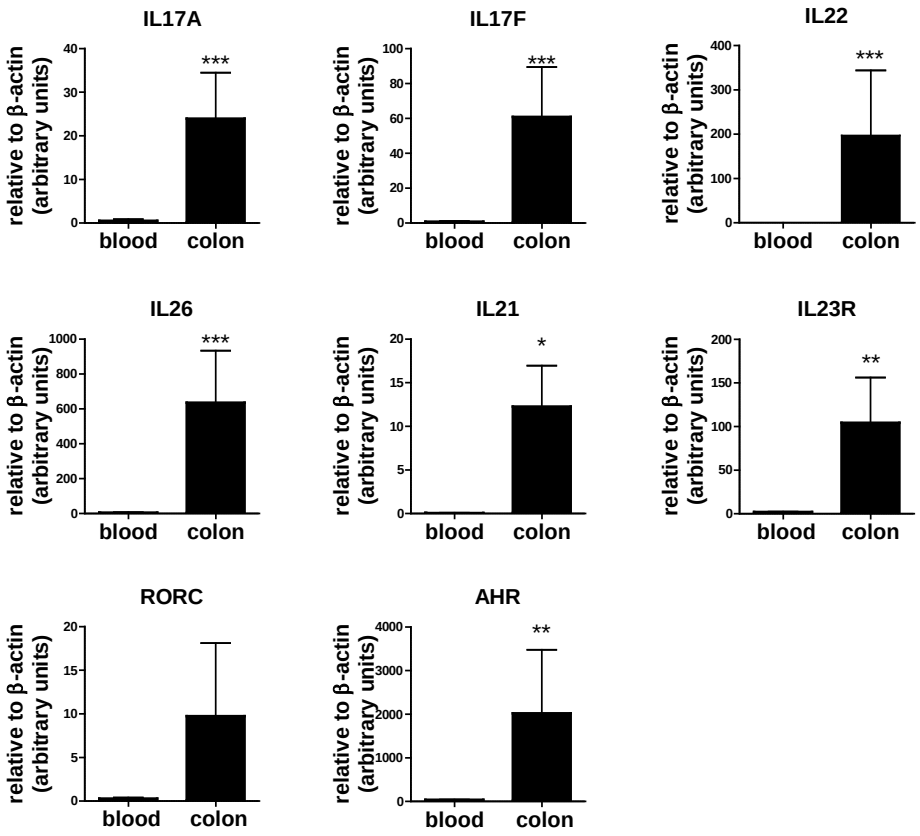


Figure 4.2 Th-17 genes are expressed in intestinal T-cells

Relative mRNA expression of Th-17 signature genes in $CD3^+$ cells isolated from blood (n=7) and colon (n=9) of control patients *ex vivo*. Bars represent mean +/- SEM. *p<0.05; **p<0.01; ***p<0.001

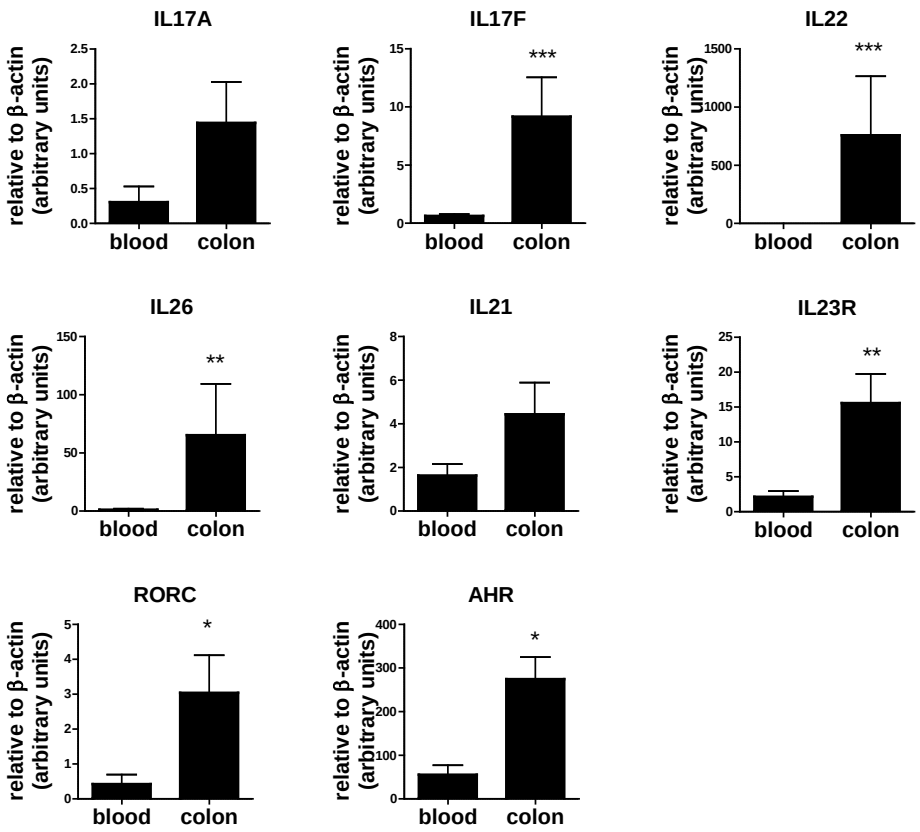


Figure 4.3 Th-17 genes are expressed in intestinal non-T cells

Relative mRNA expression of Th-17 signature genes in $CD3^-$ cells isolated from blood (n=7) and colon (n=10) of control patients *ex vivo*. Bars represent mean +/- SEM. *p<0.05; **p<0.01; ***p<0.001

4.2.2 The IL23/IL17 pathway is over-expressed in IBD

To evaluate if the IL23/IL17 pathway contributes to chronic intestinal inflammation in IBD, we compared the expression of Th-17 signature genes in the inflamed intestinal mucosa from patients with CD and UC and in the uninflamed mucosa from control individuals. No significant difference was observed between patients and controls in the expression of the Th-17 transcription factors *RORC* and *AHR* and in the expression of *IL23A* and *IL23R*. However, mucosal expression of *IL17A* and *IL22* was significantly increased in the inflamed intestine in UC and CD. Significantly higher expression of *IL17F* was also observed in IBD patients, when patients with CD and UC were pooled together. Interestingly, *IL26* was only increased in the inflamed colon of patients with UC, while no difference was observed between CD patients and controls (Figure 4.4, A). We also looked at protein expression of IL23 and IL17A in the intestinal mucosa from some patients with IBD and control. We found no difference in IL23, as observed at mRNA level. An increase in protein levels of IL17A was found in some intestinal samples from IBD patients; however the difference in IL17A protein expression between patients and controls did not reach statistical significance (Figure 4.4, B).

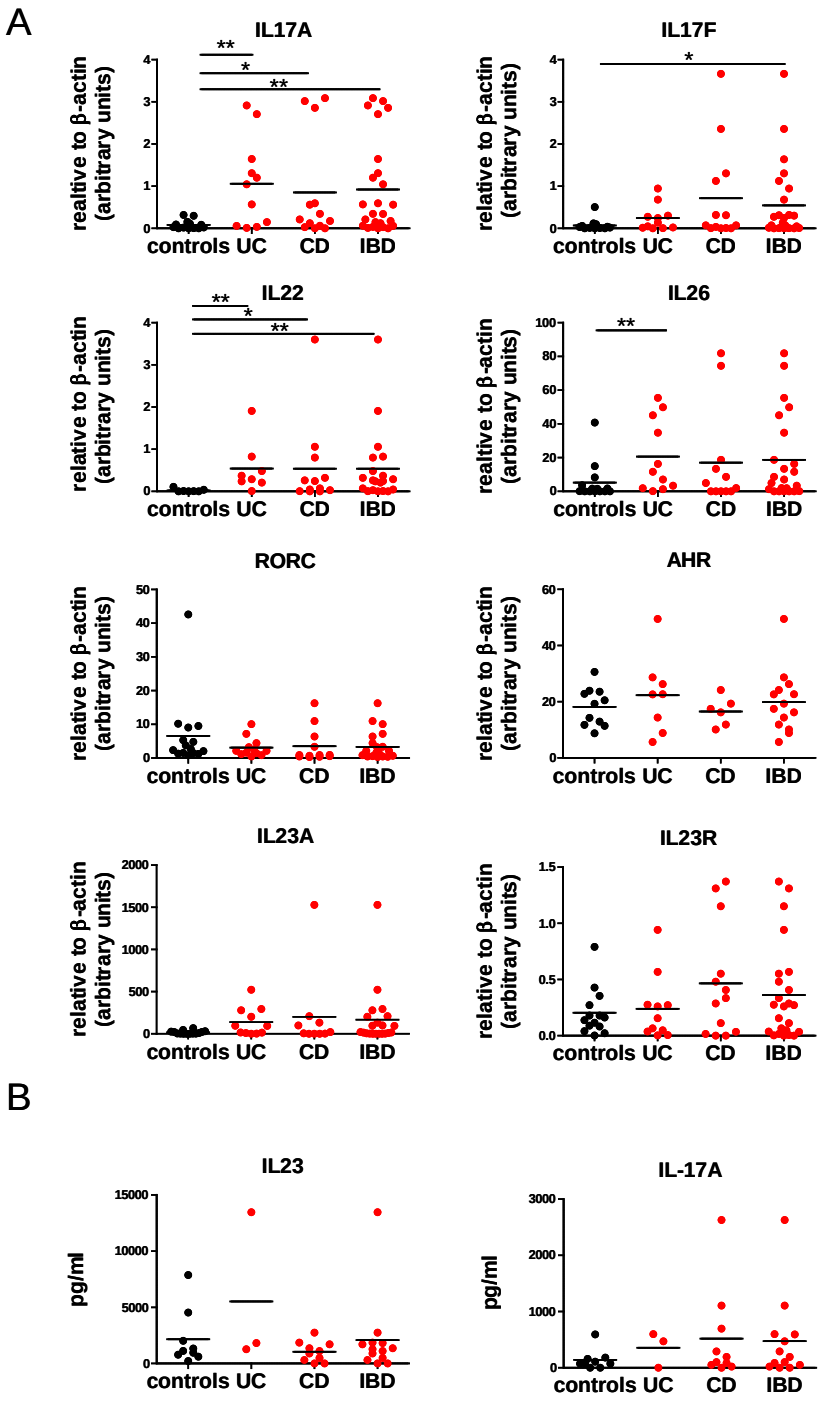


Figure 4.4 The IL23/IL17 pathway is over-expressed in IBD

(A) Relative mRNA expression of Th-17 signature genes in the intestinal mucosa from controls, UC, CD and IBD (UC+CD) patients. (B) Protein expression of IL23 and IL17A in intestinal mucosa homogenates from controls, UC, CD and IBD (UC+CD) patients. *p<0.05; **p<0.01

4.2.3 IL23-dependent non-T sources of IL17 contribute to intestinal inflammation in IBD

To evaluate the contribution of T and non-T sources of Th-17 cytokines to IBD, we analysed the expression of Th-17 genes in sorted LP $CD3^+$ and $CD3^-$ cells isolated from the intestine of patients with IBD *versus* controls.

No significant differences were observed in the expression of *IL22*, *IL17A*, *IL17F*, *IL26*, *RORC* and *IL23R* in LP $CD3^+$ cells between patients and controls. These data suggest that there is no increase in expression of Th-17 genes in T-cells in IBD (Figure 4.5, A). Conversely, significantly higher expression of *IL17A* and *IL17F* was observed in LP $CD3^-$ cells isolated from patients with IBD compared to controls. A trend to an increase was also observed in the expression of *IL26* ($p=0.07$) and *IL23R* ($p=0.06$), while no significant difference was found in *IL22* and *RORC* (Figure 4.5, B). These data suggest that innate sources of IL17A and IL17F might contribute to intestinal inflammation in IBD.

To determine if intestinal non-T cells are responsive to IL23 and whether the innate response is altered in IBD, sorted LP $CD3^-$ cells isolated from the colon of control subjects and IBD patients and from the inflamed ileum of patients with CD were cultured overnight with or without IL23, and Th-17 gene expression was evaluated. *IL22* was induced by IL23 stimulation in the non-T cells isolated from control colons, confirming the presence of IL23 responsive innate cells in the human intestine. No significant induction of *IL22* was observed after IL23 stimulation in non-T cells isolated from the colon or ileum of patients with IBD. Interestingly, the expression of *IL17A* in non-T cells after IL23 stimulation was significantly higher in cells isolated from the inflamed colon, but not the ileum, of patients with IBD compared to controls (Figure 4.6). All together, these data suggest that an IL23-inducible source of IL17 is

present in the $CD3^-$ compartment in the inflamed colon of patients with IBD, but not in controls.

The same experiments were performed with sorted $CD3^-$ and $CD3^-CD19^-$ cells isolated from the MLN of patients with IBD. Significant *IL22* induction was observed after IL23 stimulation in $CD3^-$ cells from MLN, confirming the presence of IL23 responsive innate cells in the MLN. No difference was found in *IL17A* expression in $CD3^-$ cells cultured with or without IL23. Interestingly, higher transcript levels of Th-17 cytokines were detected after IL23 stimulation in sorted $CD3^-CD19^-$ cells from MLN of patients with IBD (Figure 4.7, A). Similarly, higher expression of *IL22* and *IL17F* was observed in $CD3^-CD19^-$ cells from IBD colons (Figure 4.7, B). This observation suggests that exclusion of B-cells from the $CD3^-$ compartment results in the enrichment for the IL23 responsive innate source of Th-17 cytokines, supporting the idea that only a minority of cells are responding.

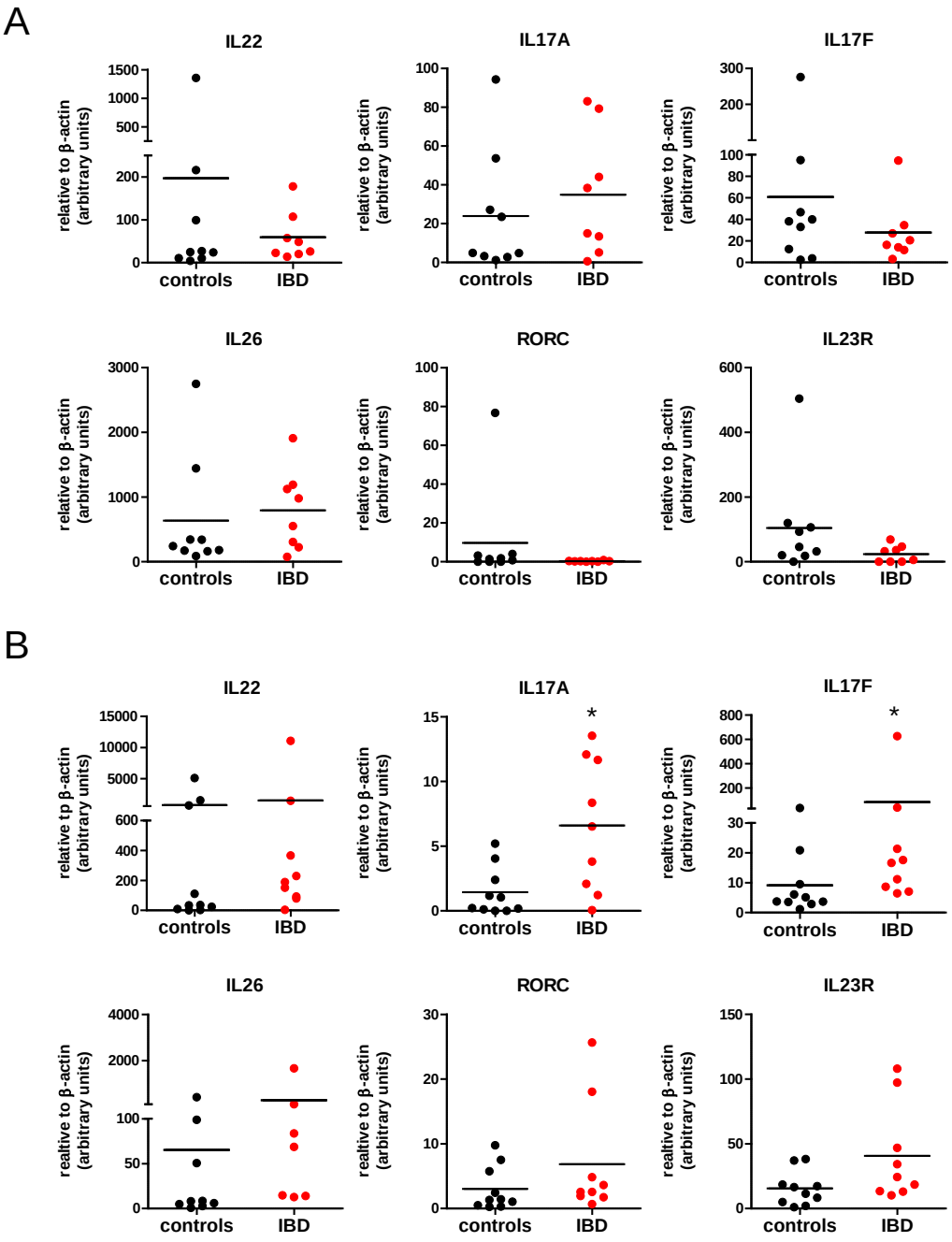


Figure 4.5 IL17 is over-expressed in intestinal non-T cells in IBD

(A) Relative mRNA expression of Th-17 signature genes in sorted intestinal $CD3^+$ cells from controls and IBD (UC+CD) patients. (B) Relative mRNA expression of Th-17 signature genes in sorted intestinal $CD3^-$ cells from controls and IBD (UC+CD) patients. *$p<0.05$

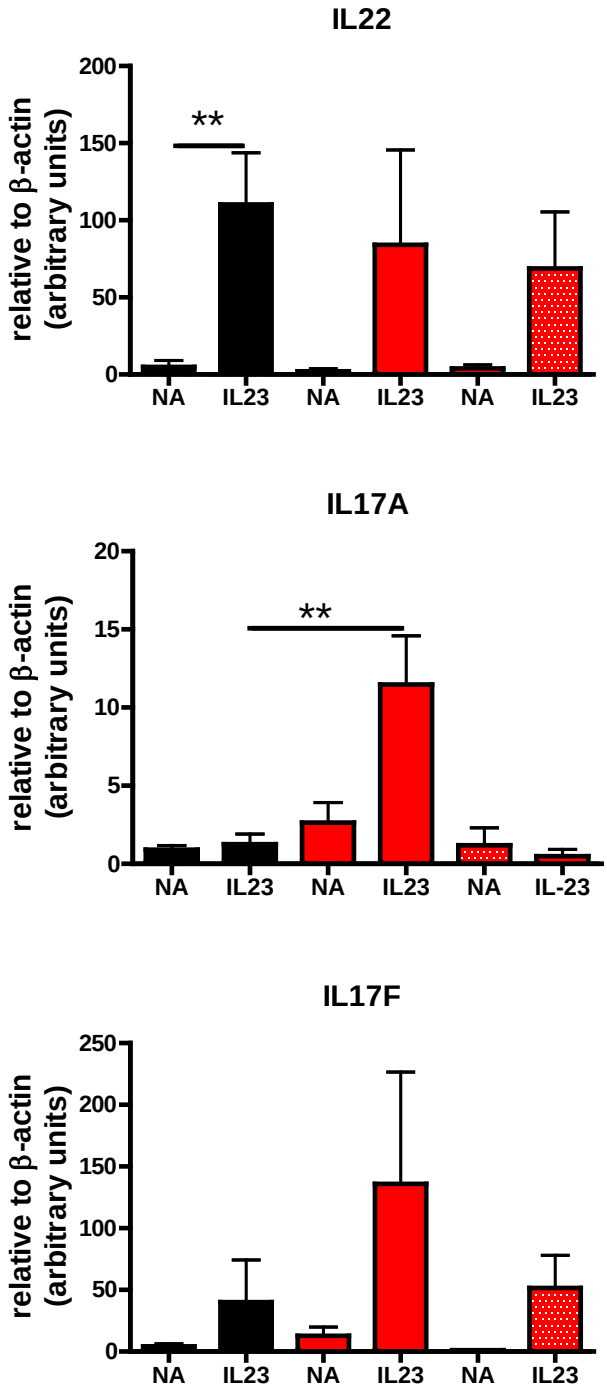


Figure 4.6 IL23 induces IL17A in colonic non-T cells from IBD patients

Relative mRNA expression of *IL22*, *IL17A* and *IL17F* in $CD3^-$ cells from the colon of controls (black bars, n=9) and IBD patients (red bars, n=4) and from the ileum of CD patients (red dotted bars, n=3) after overnight culture in complete media with no addition (NA) and in the presence of IL23 (10 ng/ml). In some experiments B-cells have been excluded ($CD3^-CD19^-$ cells). Bars represent mean +/- SEM. **p<0.01

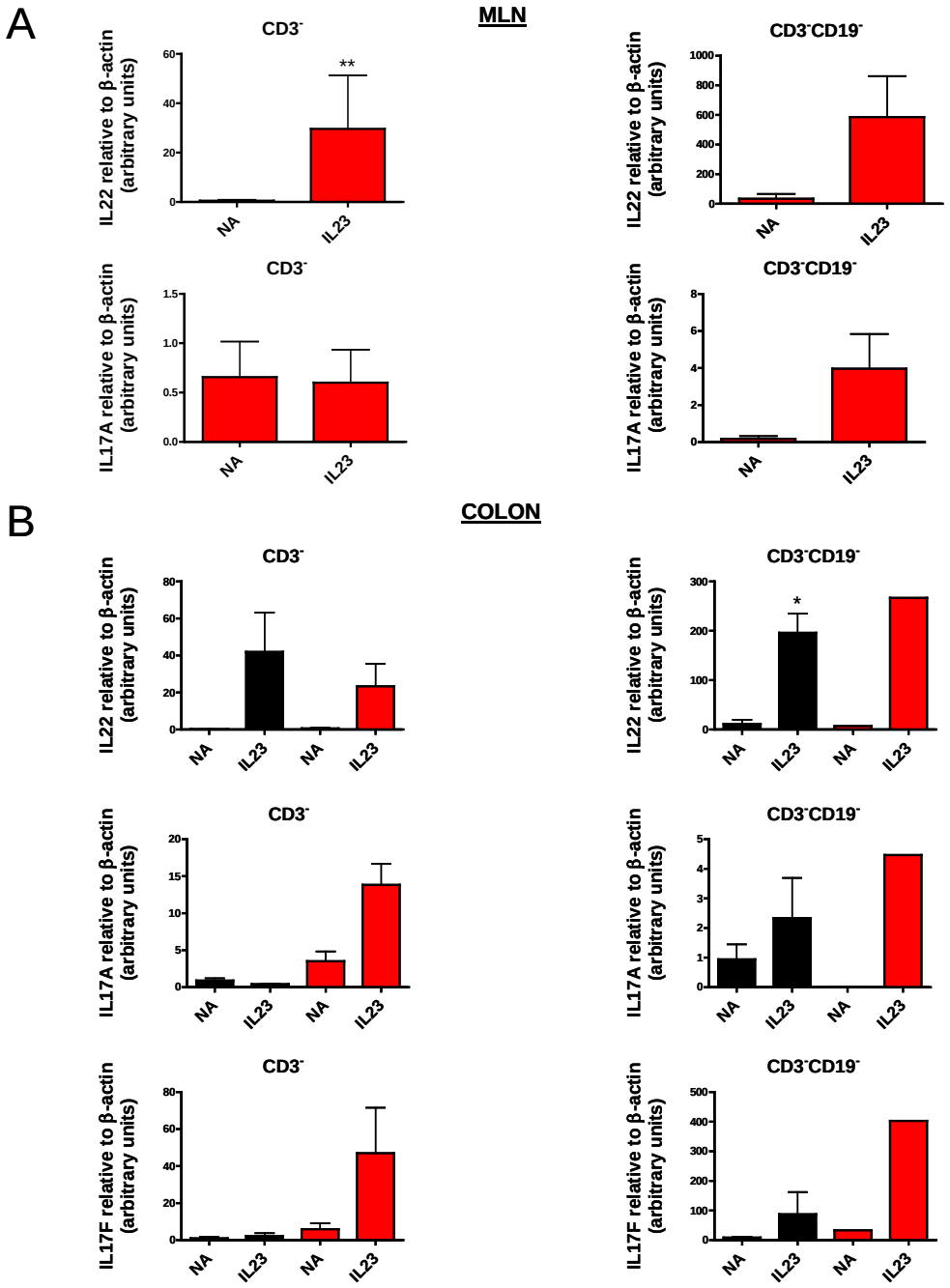


Figure 4.7 non-T, non-B cells respond to IL23 in MLN and colon from IBD patients

(A) Relative mRNA expression of *IL22* and *IL17A* in $CD3^-$ (n=5) and $CD3^-CD19^-$ (n=3) cells from MLN of IBD patients after overnight culture in complete media with no addition (NA) and in the presence of IL23 (10 ng/ml). (B) Relative mRNA expression of *IL22*, *IL17A* and *IL17F* in $CD3^-$ from the colon of controls (black bars, n=5) and IBD patients (red bars, n=3) and $CD3^-CD19^-$ cells from the colon of controls (black bars, n=4) and IBD patients (red bars, n=1) after overnight culture in complete media with no addition (NA) and in the presence of IL23 (10 ng/ml). Bars represent mean +/- SEM. *p<0.05; **p<0.01

4.2.4 T-cell contamination is not responsible for Th-17 cytokine expression and IL23 responsiveness amongst CD3$^-$ populations

One possible explanation for these results is that contaminating T-cells may be responsible for cytokine expression and IL23 responsiveness amongst CD3$^-$ populations isolated from the inflamed intestine and MLN of patients with IBD. However, the highest purity of sorted CD3$^-$ and CD3$^-$CD19$^-$ cells was obtained through FACS sorting in most experiments. Furthermore, when negative selection of CD3$^-$ cells was performed using anti-CD3 magnetic beads, FACS staining of CD3$^-$ enriched cells showed purity greater than 95% (Figure 4.8).

We also excluded the possibility that T-cells that have down-regulated CD3 upon activation might be contaminating the CD3$^-$ compartment in patients with IBD. TCR down-regulation has been shown to be mediated through retention and degradation of the internalized TCR [407]. If intestinal T-cells down-regulate TCR expression secondary to intestinal inflammation in patients with IBD, we would expect to see a population of T-cells expressing intermediate levels of CD3 (CD3^{dim}) in LPMC isolated from these patients. However, FACS analysis of LPMC from IBD patients show two clearly distinct populations of CD3$^+$ and CD3$^-$ cells in the absence of CD3^{dim} cells (Figure 4.9, A). Furthermore, T-cells that have down-modulated their surface-CD3 after activation are still expected to express CD3 at the intracellular level. To address this issue, we have performed intracellular staining for CD3 in LPMC from the colon and ileum of one patient with CD and we show that, while surface-CD3$^+$ cells co-express intracellular CD3 as expected, no intracellular expression of CD3 is observed in surface-CD3$^-$ cells (Figure 4.9, B). Finally we did not observe any significant difference in the mRNA expression of *CD3ε* and the constant region of the TCRβ (*TRBC2)* in intestinal sorted CD3$^-$ cells isolated from

IBD patients *versus* controls (Figure 4.9, C). Together these data indicate that contamination with T-cells does not explain cytokine production and IL23 responsiveness amongst $CD3^-$ populations in IBD.

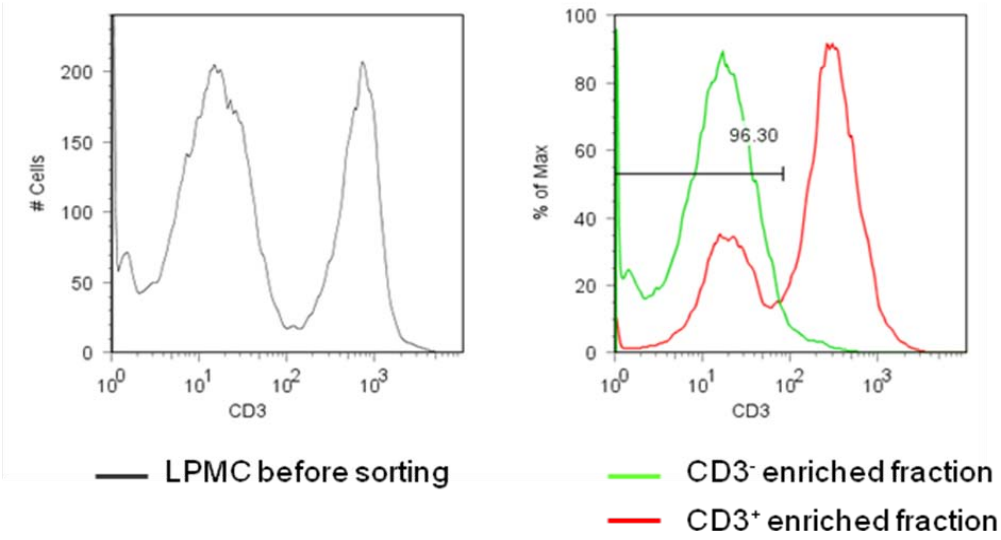


Figure 4.8 Purity of intestinal CD3$^-$ cells after MACS sorting

Representative FACS staining for CD3 in all LPMC (left panel) and in the CD3$^+$ and CD3$^-$ enriched fractions after MACS sorting.

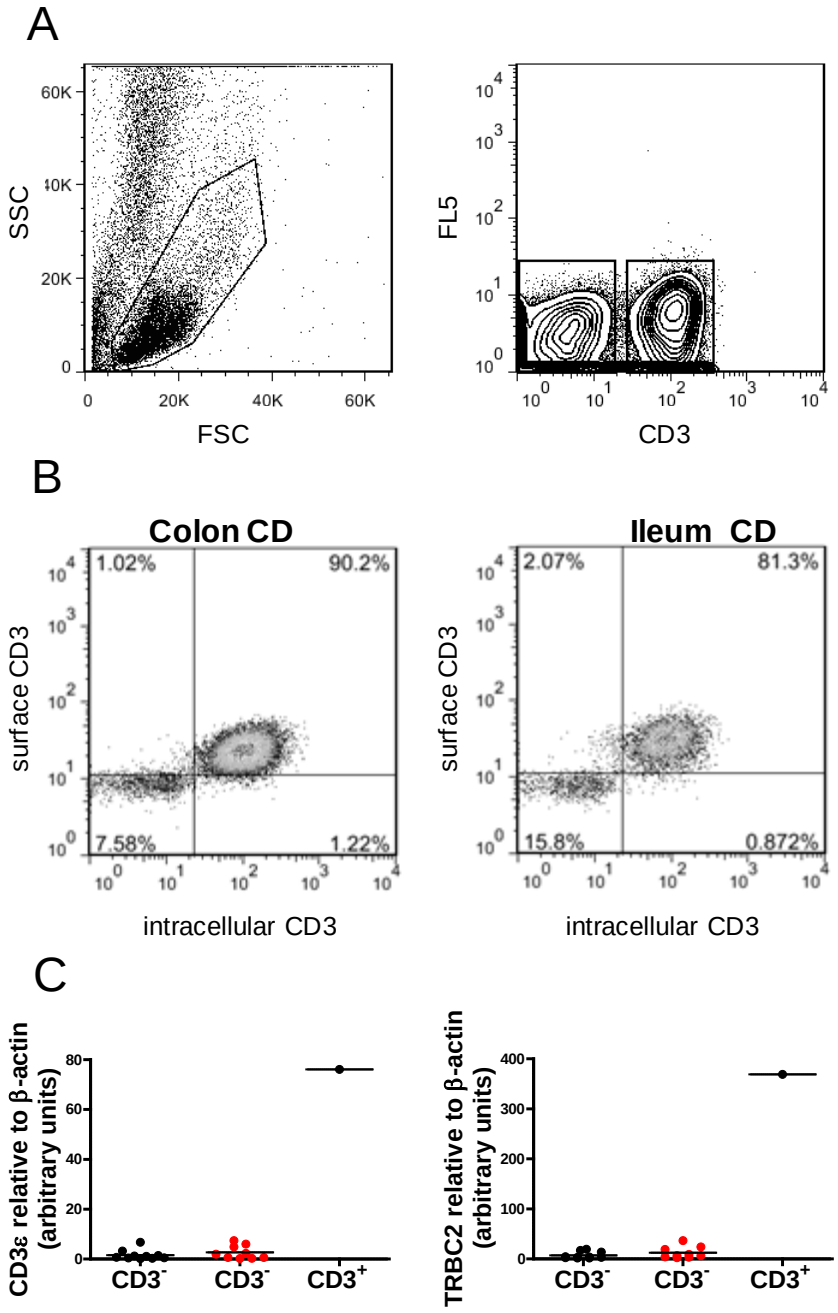


Figure 4.9 Intestinal CD3$^+$ do not down-regulate CD3 in IBD patients

(A) Representative surface staining for CD3 on LPMC isolated from the inflamed ileum of one CD patient. Cells were gated as shown on the FSC/SSC plot (left). (B) Staining for surface and intracellular CD3 of LPMC isolated from the inflamed colon and ileum of one patient with CD. Different fluorochrome-conjugated antibodies were used for surface and intracellular staining. The UCHT1 antibody clone, which stains both the extracellular and intracellular CD3ε, was used in this experiment. Cells are gated on the lymphocytic gate on the FSC/SSC plot. (C) mRNA expression of *CD3ε* and *TRBC2* in sorted CD3$^-$ cells isolated from the intestine of controls (black dots) and IBD patients (red dots) and in sorted CD3$^+$ cells isolated from the intestine of one control individual.

4.2.5 ILC are a source of IL17 in the inflamed intestine

In humans $CD3^-CD127^+$ ILC can be further subdivided based on expression of the NK marker CD56 [288]. To further characterize the intestinal IL23-responsive non-T source of Th-17 cytokines in the inflamed intestine of patients with IBD, we sorted $Lin^-CD45^+CD56^+$ cells and $Lin^-CD45^+CD56^-$ cells from the ileum and colon of patients with CD and cultured them with IL23. *IL22* and *IL26* were induced by IL23 in the $CD56^+$ population. By contrast, *IL17A* and *IL17F* were preferentially expressed in the $Lin^-CD45^+CD56^-$ cells (Figure 4.10, A-B). We observed no difference in the expression of the transcription factor genes *AHR* and *RORC* and of the *IL23R* between the two cell populations and no induction was found after IL23 stimulation. Expression of *IFNG* was also equally expressed amongst ileal and colonic $Lin^-CD45^+CD56^+$ and $Lin^-CD45^+CD56^-$ cells from patients with CD. It has recently been shown that both human $CD56^+$ and $CD56^-$ ILC can also produce IL5 and IL13, in accordance with the description of Th-2-cytokine-producing ILC in mice (so called nuocytes and natural helper cells) [291-293]. We observed similar levels of expression for *IL13* in ileal and colonic $Lin^-CD45^+CD56^+$ and $Lin^-CD45^+CD56^-$ cells, while *IL5* was not detected in most cultures. Other LTi-related genes, such as *LTA*, *LTB* and *TNF*, were also equally expressed in both populations in the colon and ileum and no induction was observed after culture in the presence of IL23 (Figure 4.11).

In one experiment $Lin^-CD45^+CD127^+$ and $Lin^-CD45^+CD127^-$ cells were sorted from the colon of a patient with CD, regardless of the expression of CD56 in order to obtain sufficient cell numbers, and cytokine expression was analysed *ex vivo*. In accordance with an LTi-like phenotype, $CD127^+$ ILC expressed higher transcript levels of *LTA*, *LTB* and *TNF*. Interestingly, *IL17A* expression was also higher in $CD127^+$ ILC (Figure 4.12, A-B). However more experiments are required to confirm this result.

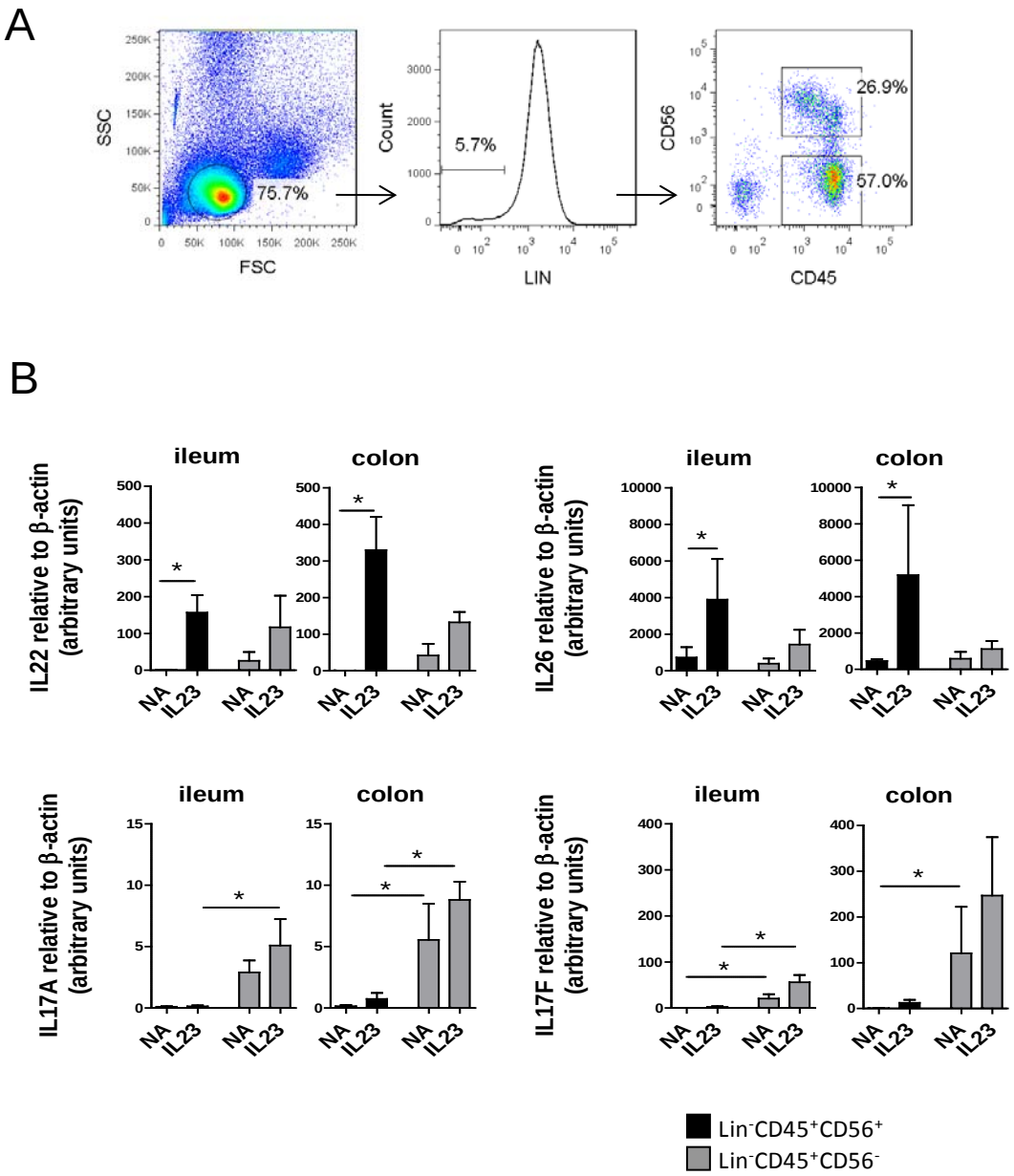


Figure 4.10 Lin$^-$CD45$^+$CD56$^-$ cells are a source of IL17 in IBD

(A) Representative gating strategy for Lin$^-$CD45$^+$CD56$^+$ and Lin$^-$CD45$^+$CD56$^-$ cell FACS sorting from LPMC from the ileum of a patient with CD. (B) mRNA expression of *IL22*, *IL26*, *IL17A*, *IL17F*, in Lin$^-$CD45$^+$CD56$^+$ (black bars) and Lin$^-$CD45$^+$CD56$^-$ (grey bars) cells from the ileum (n=4) and colon (n=4) of patients with CD after overnight stimulation in complete media with no addition (NA) and in the presence of IL23 (10 ng/ml). Bars represent mean +/- SEM. *p=0.029

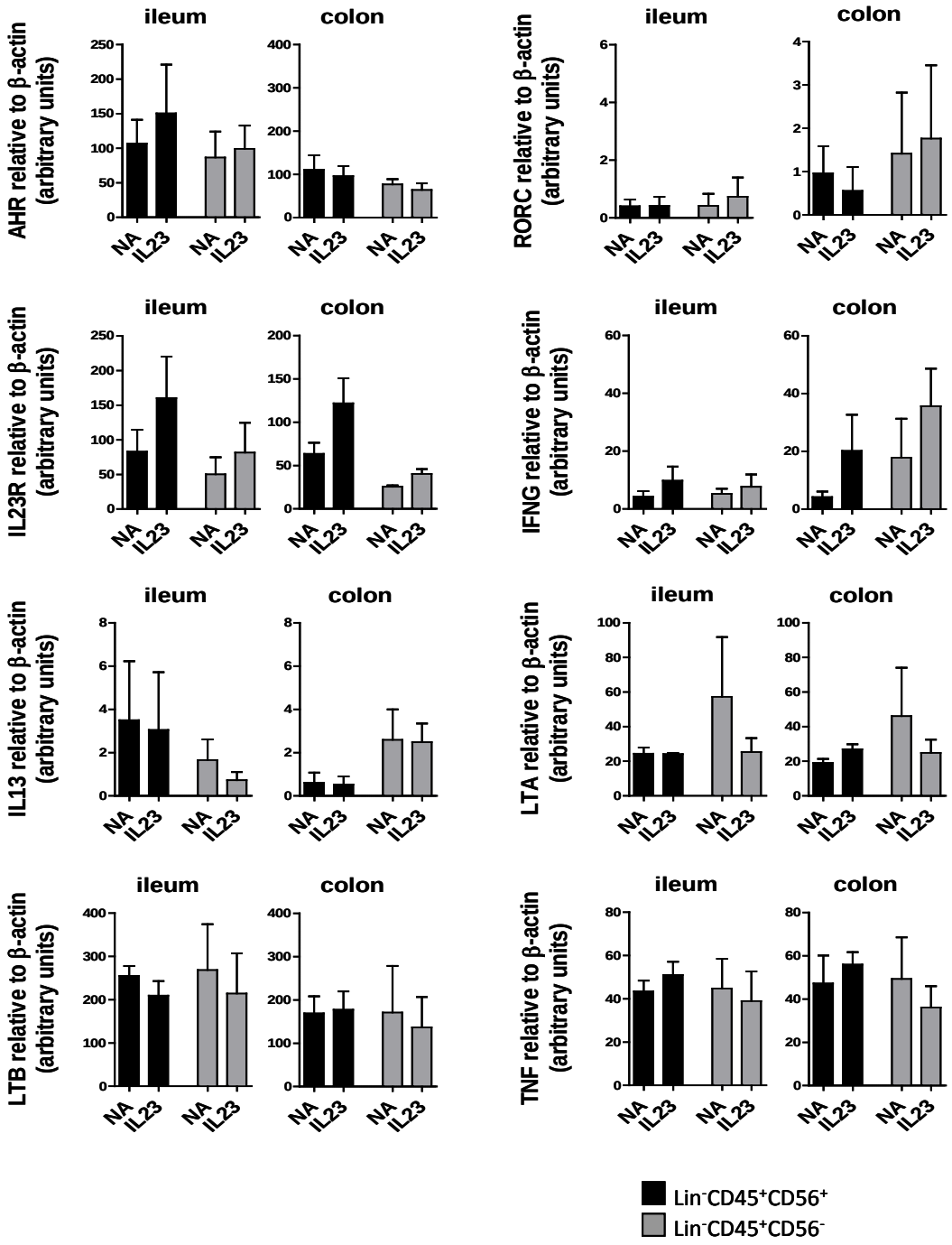


Figure 4.11 Gene expression profiles of Lin$^-$CD45$^+$CD56$^+$ and Lin$^-$CD45$^+$CD56$^-$ cells

mRNA expression of *AHR*, *RORC*, *IL23R*, *IFNG*, *IL13*, *LTA*, *LTB* and *TNF* in Lin$^-$CD45$^+$CD56$^+$ (black bars) and Lin$^-$CD45$^+$CD56$^-$ (grey bars) cells from the ileum (n=3) and colon (n=3) of patients with CD after overnight stimulation in complete media with no addition (NA) and in the presence of IL23 (10 ng/ml). Bars represent mean +/- SEM.

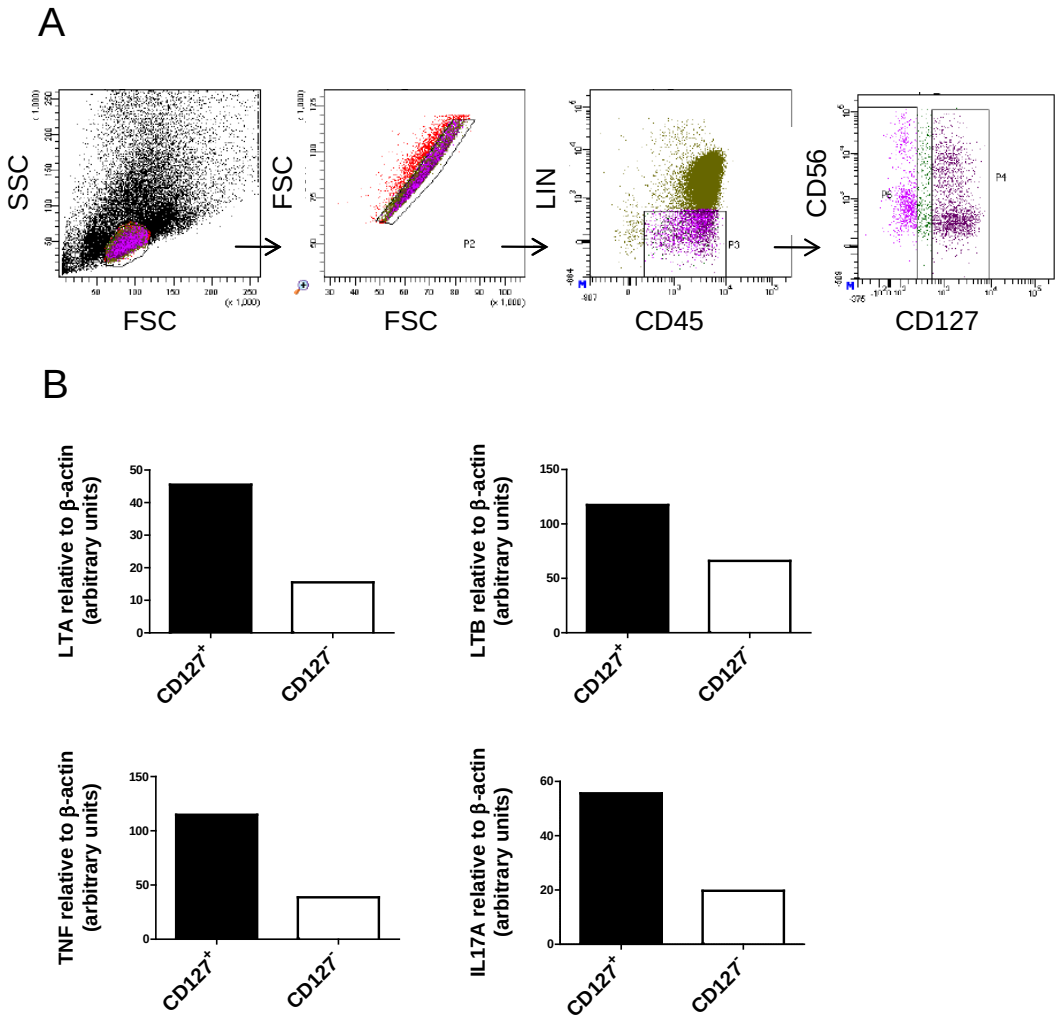


Figure 4.12 CD127$^+$ ILC share features of LTi-like cells and express IL17

(A) Representative gating strategy for Lin$^-$CD45$^+$CD127$^+$ and Lin$^-$CD45$^+$CD127$^-$ cell FACS sorting from LPMC from the colon of a patient with CD. (B) mRNA expression of *LTA*, *LTB*, *TNF* and *IL17A* in CD127$^+$ and CD127$^-$ ILC from the colon of a patient with CD.

4.2.6 CD56$^-$ ILC accumulate in CD

We next wanted to evaluate if ILC populations contribute to intestinal inflammation in IBD. Analysis of the frequency of intestinal Lin$^-$CD45$^+$CD127$^+$CD56$^-$ cells and Lin$^-$CD45$^+$CD127$^+$CD56$^+$ (from now on called CD56$^-$ and CD56$^+$ ILC) in patients with CD, UC and controls showed that while both populations were present at similar frequencies in the uninflamed colon and ileum of control individuals, there was a marked increase specifically in the CD56$^-$ ILC in the inflamed ileum and colon of CD patients. Interestingly, no difference in the frequency of CD56$^-$ and CD56$^+$ ILC was observed in the colon of UC patients *versus* controls, indicating that accumulation of CD56$^-$ ILC might be a specific feature of CD (Figure 4.13, A-B). Moreover, analysis of the phenotype of IL17A- and IFN-γ-producing cells in the Lin$^-$ compartment in the ileum of CD patients by intracellular FACS staining showed these were primarily CD127$^+$CD56$^-$ (Figure 4.14). All together, these data indicate that CD56$^-$ ILC are accumulating in the inflamed intestine in CD and might contribute to intestinal inflammation through the production of inflammatory cytokines, such as IL17A, IL17F and IFN-γ.

Only a small percentage of CD56$^-$ ILC that accumulate in CD express NKp44 suggesting that they represent a distinct population from IL22 producing NK22 cells, which express NKp44 and represent one fourth of CD56$^+$ ILC (Figure 4.15, A). Interestingly, we found that both intestinal CD56$^-$ and CD56$^+$ ILC also express the chemokine receptor CCR6 (Figure 4.15, B). CCR6 might be involved in the recruitment of ILC to the inflamed intestinal tissue that over-expresses CCR6 ligands such as CCL20 and β-defensins [408-410]. A CCR6-CCL20 mediated mechanism could also determine the topographical organization of the inflammatory infiltrate. In fact, CCL20 has been shown to be mainly expressed on the follicle-associated

epithelium (FAE) and might induce clustering of ILC in secondary and tertiary organised lymphoid structures, rather than scattered localization in the LP [408, 411]. In order to study intestinal ILC *in situ* we have performed immunohistochemistry staining of $CD3^{-}CD20^{-}CD127^{+}$ cells in tissue sections. ILC could be identified in MALT in acetone-fixed, frozen sections from human tonsils (Figure 4.16, A). Staining of formalin-fixed intestinal tissue has proven to be more challenging and it is still work in progress. Nevertheless, we were able to clearly identify $CD3^{-}CD20^{-}$ $CD127^{+}$ cells in the inflamed colon of a patient with CD (Figure 4.16, B).

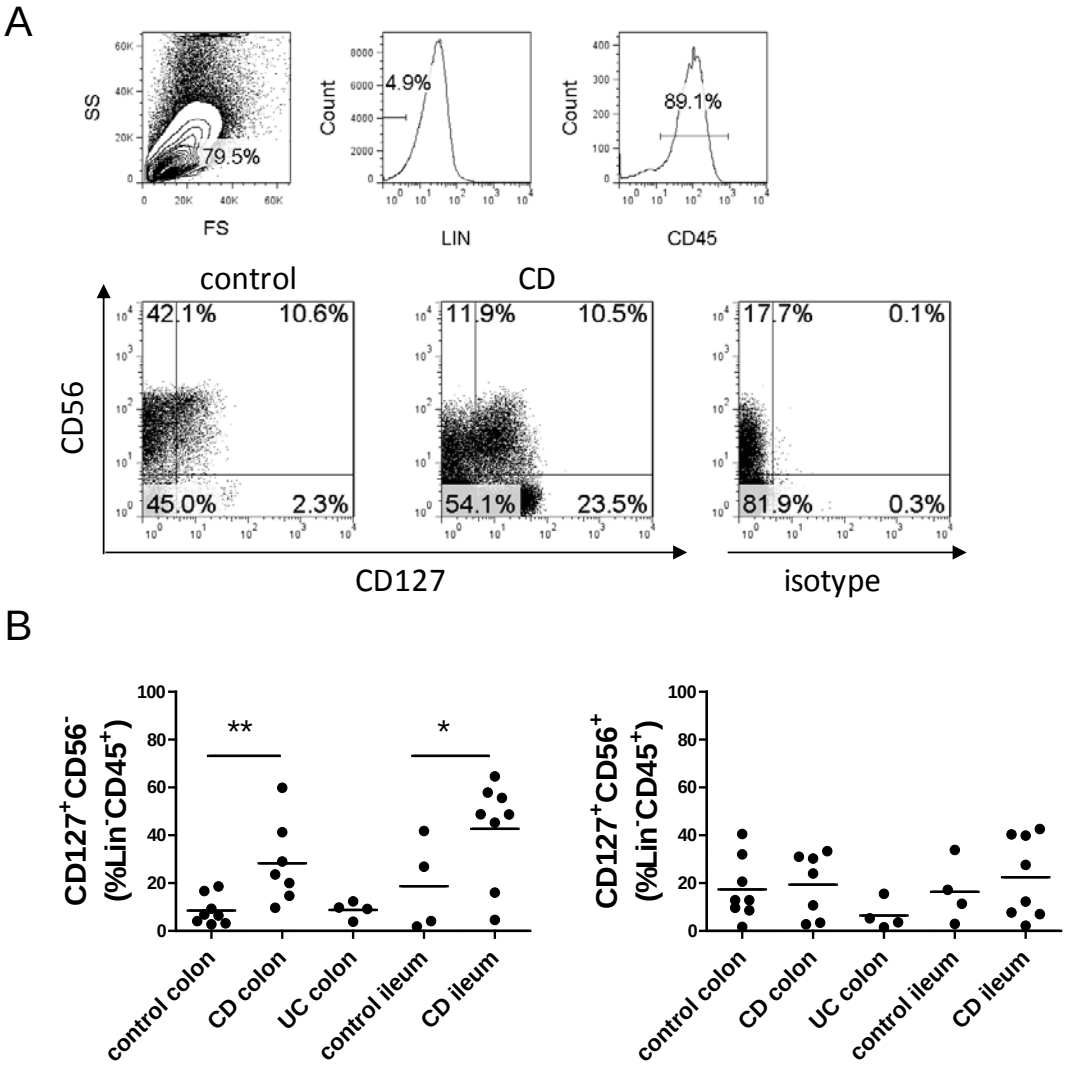


Figure 4.13 CD56$^-$ ILC accumulate in the intestine in CD

(A) Representative staining of CD127$^+$CD56$^-$ and CD127$^+$CD56$^+$ ILC from control and CD intestine. LPMC are gated on the lymphocytic gate in the FSC/SSC plot, the Lin$^-$ and CD45$^+$ population. (B) Percentage of CD127$^+$CD56$^-$ and CD127$^+$CD56$^+$ ILC in the Lin$^-$CD45$^+$ population, using the gates shown in A, in the colon of control, CD and UC patients and in the ileum of control and CD patients.

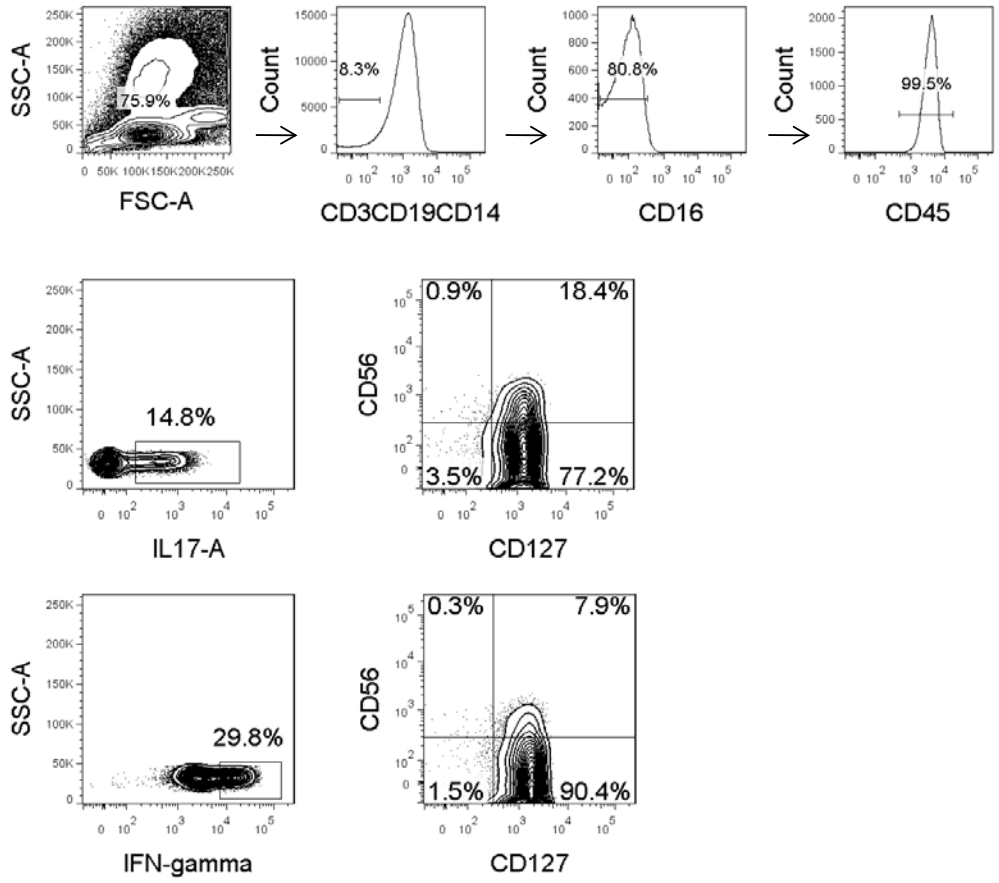


Figure 4.14 CD56$^-$ ILC produce inflammatory cytokines in CD

Intracellular staining for IL17A and IFN-γ in the LPMC isolated from the ileum of a patient with CD (representative of two experiments). LPMC were also stained for CD56 and CD127 and gated on the lymphocytic gate in the FSC/SSC plot, on CD3$^-$ CD19$^-$CD14$^-$, CD16$^-$ and CD45$^+$ population.

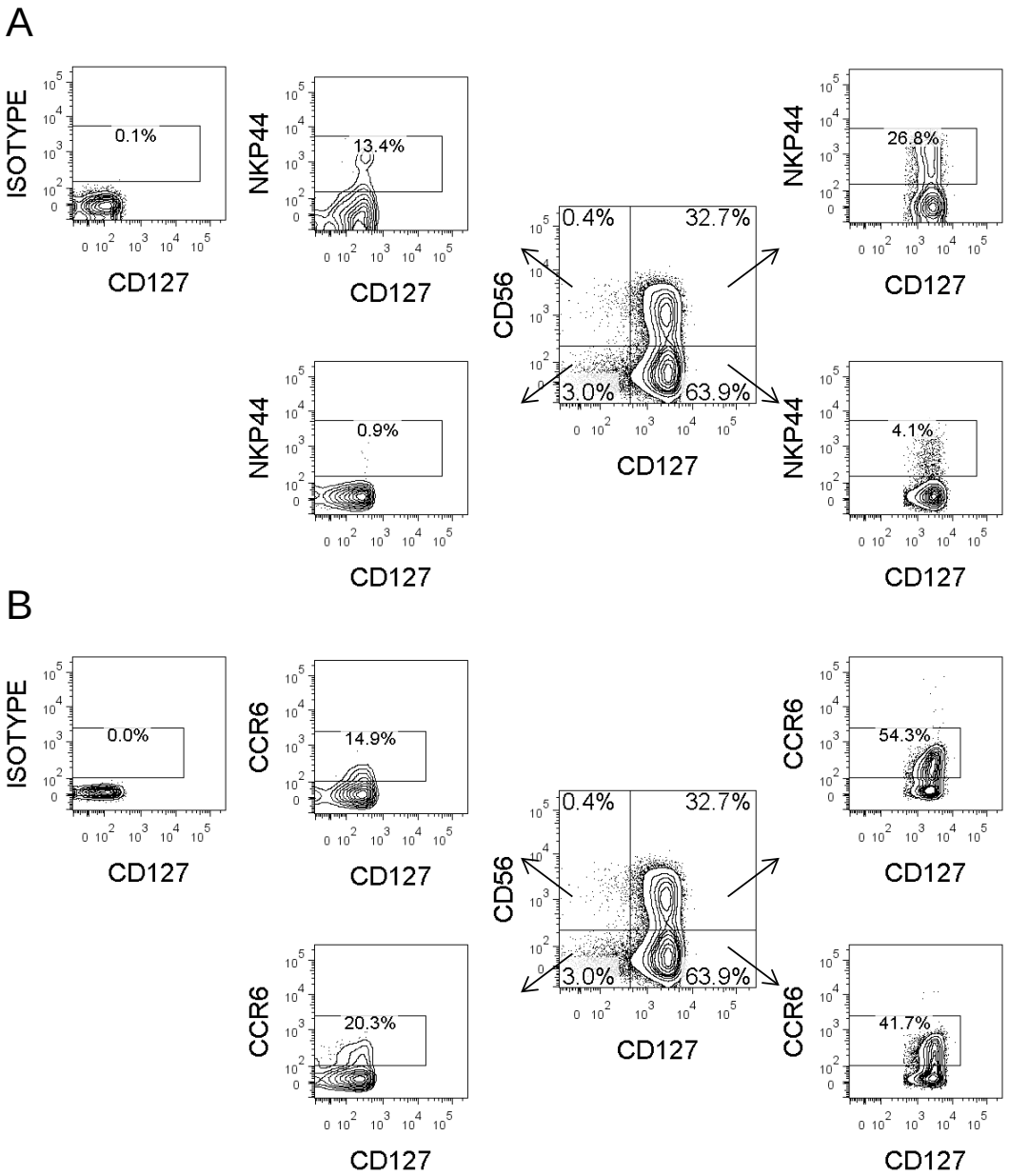
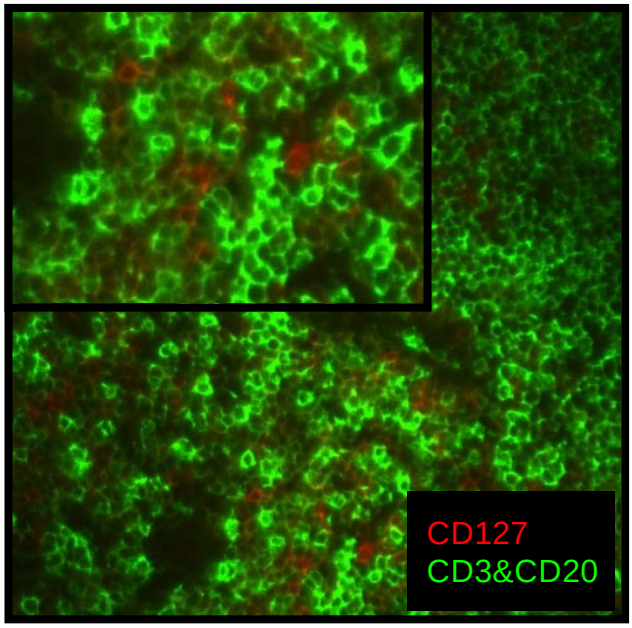


Figure 4.15 Phenotype of intestinal CD56$^-$ and CD56$^+$ ILC

(A) FACS staining for NKp44 in CD56$^-$ and CD56$^+$ ILC in the ileum of one CD patient. (B) FACS staining for CCR6 in CD56$^-$ and CD56$^+$ ILC in the ileum of one CD patient. Cells are gated on the lymphocytic gate in the FSC/SSC plot, the Lin$^-$ and CD45$^+$ population.

A



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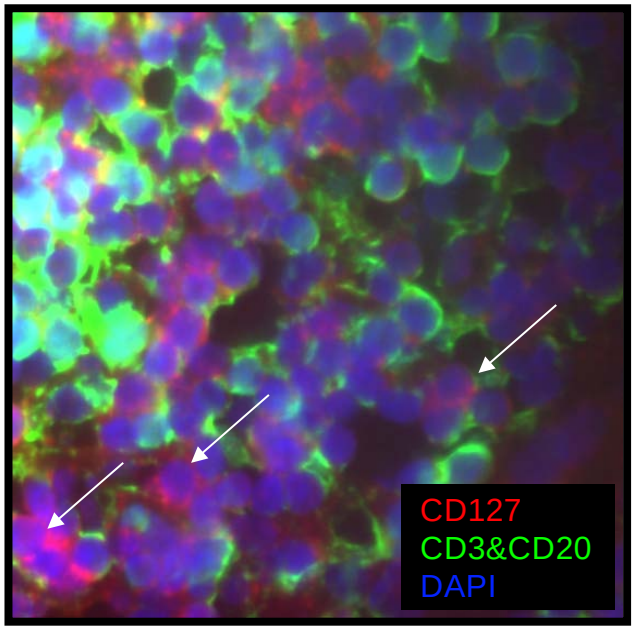

Figure 4.16 $CD3^-CD20^-CD127^+$ ILC are found in human tonsils and in the inflamed colon in CD

(A) Immunofluorescent staining of $CD3^-CD20^-CD127^+$ ILC in acetone-fixed, frozen tissue from a tonsil. Two-colour immunofluorescence was used to determine CD127 expression (FITC, green fluorescence) and CD3CD20 expression (Cy5-tyramide, red fluorescence). (B) Immunofluorescent staining of $CD3^-CD20^-CD127^+$ ILC in formalin-fixed, sucrose-embedded tissue from the colon of a CD patient. Three-colour immunofluorescence was used to determine CD127 expression (Cy5-tyramide, red fluorescence), CD3CD20 expression (FITC, green fluorescence), nuclear counterstaining (DAPI, blue fluorescence). Examples of $CD3^-CD20^-CD127^+$ ILC are shown by arrows. Experiment performed by Holm Uhlig and Anna-Lena Schaupp.

4.3 Conclusion

In this chapter we presented our data on the role of the IL23/IL17 axis in the human intestinal and systemic immune response and in chronic intestinal inflammation in patients with IBD.

First, we show here that in human immune responses the IL23/IL17 axis appears to be tissue-compartmentalized. While blood cells are unresponsive to IL23 *in vitro*, intestinal cells can respond to IL23 stimulation. Furthermore, Th-17 signature genes, such as genes encoding for Th-17 cytokines, transcription factors and the IL23R are preferentially expressed in intestinal cells *versus* blood cells, where only low or undetectable transcript levels are found. These data are in accordance with our previous work in mice that indicated the presence of compartmentalization of the IL23/IL17 pathway [204].

In line with our results showing preferential expression of Th-17 genes in intestinal T-cells, Kobayashi *et al*. described higher expression of IL17A in LP $CD4^+$ cells compared to the PB $CD4^+$ population [311]. Interestingly, in our study compartmentalization of Th-17 gene expression was also observed in non-T-cells as we found increased expression of *IL22*, *IL17F*, *IL26*, *RORC*, *AHR* and *IL23R* in LP $CD3^-$ cells with very low expression levels amongst PB $CD3^-$ cells. These findings clearly show that non-T sources of Th-17 cytokines are present in the intestinal immune response even in the absence of any pathological inflammation. Transcript levels of IL22 are comparable in intestinal T and non-T cells, while *IL17A*, *IL17F* and *IL26* are significantly lower in $CD3^-$ compared to $CD3^+$ cells suggesting that only a minority of cells express these genes in the $CD3^-$ compartment.

We then investigated the role of the IL23/IL17 pathway in patients with IBD. Intestinal over-expression of this axis was confirmed in our cohort of patients. We found increased levels of *IL17A*, *IL17F* and *IL22* in patients with IBD, both CD and UC, in accordance with the literature [308-310, 312]. *IL26* appeared to be only significantly increased in the intestinal mucosa of patients with UC, differently from previous data by Dambacher *et al.*, which showed increased levels of IL26 in CD, but not UC [272]. IL26 has been shown to induce IL10, IL8, TNF-α and ICAM1 expression by IEC and to inhibit cell proliferation, but its role in IBD remains to be elucidated [271]. Noteworthy, not all patients with IBD present high transcript levels of Th-17 cytokines and large variability of expression is observed between patients. This may suggest that different pathways could play a relevant role in inducing and propagating inflammation in subgroups of patients. In addition, stratification of patients in accordance to activity and behaviour of disease, concomitant treatment or genetic background was not performed due to the limited number of patients in our study, but all these factors may affect the expression of the IL23/IL17 axis.

Recent studies have suggested that Th-17 cells play a role in inflammation in IBD. In our study we found no difference in Th-17 cytokine expression in intestinal T-cells in patients with IBD compared to controls. This apparent discrepancy with the literature may result from different experimental settings. We examined Th-17 gene expression in intestinal sorted $CD3^+$ cells *ex vivo*. On the other hand, Cosmi *et al* have described the presence of intestinal $IL17^+$ and $IL17^+IFN\text{-}\gamma^+$ Th-17 cells in CD after *in vitro* expansion of T-cells in the presence of anti-CD3 and anti-CD28 antibodies and restimulation with PMA and ionomycin [163]. Kleinschek *et al* observed increased frequencies of PB IL17-producing cells amongst $CD4^+CD161^+$ cells and higher numbers of $CD4^+CD161^+$ cells in the intestine of patients with CD [164]. In another

study, IL17A expression was found to be increased in LP $CD4^+$ cells in UC, but not in CD [311]. Our data represent Th-17 gene expression in the whole CD3 compartment *ex vivo* and cannot be directly compared with data on different subsets of T-cells or on *in vitro* manipulated cells.

Our findings that *IL17A* and *IL17F* are over-expressed in the intestinal $CD3^-$ compartment in IBD suggest that IL23 dependent non-T sources of IL17 might participate in chronic intestinal inflammation in IBD. These data appear of particular interest in light of our recent work in the anti-CD40- and *H. hepaticus*-induced innate models of colitis, where Buonocore *et al.* found that intestinal inflammation is dependent on the presence of an IL23 responsive innate lymphoid source of IL17A, IL22 and IFN-γ. Similar populations of $CD56^+$ and $CD56^-$ ILC have been identified in the human adult MALT and have been shown to produce Th-17 cytokines and IL23 responsiveness [259, 288, 289]. To further investigate the human non-T source of IL17 in the inflamed gut, we sorted different populations of ILC on the basis of lack of Lin markers and on the expression of the adhesion molecule CD56. Interestingly, our data show different cytokine profiles in intestinal $Lin^-CD45^+CD56^+$ and $CD56^-$ cells. Expression of IL10 related cytokines, such as *IL22* and *IL26*, is induced by IL23 in $Lin^-CD45^+CD56^+$, but not $CD56^-$ cells. Conversely, *IL17A* and *IL17F* expression is restricted to the $CD56^-$ compartment. No difference between $Lin^-CD45^+CD56^+$ and $CD56^-$ cells was observed in other Th-17 gene expression and in the expression of Th-1, Th-2 and LTi-related cytokine genes. It must be taken into consideration that, due to cell number limitations, $Lin^-CD45^+CD56^-$ and $CD56^+$ cells were sorted regardless of the expression of CD127, which is a marker of human LTi-like cell populations. Higher levels of LTα, LTβ and TNF-α were instead observed in one experiment in sorted colonic $Lin^-CD45^+CD127^+$ cells compared to the $CD127^-$ counterpart, in

accordance with an LTi-like phenotype.

Strikingly, we observed that majority of Lin^- cells producing the inflammatory cytokines IL17A and IFN-γ in the inflamed intestine in CD showed a $CD127^+CD56^-$ phenotype and accumulation of $CD56^-$ ILC was found in both the inflamed small bowel and colon from CD patients.

Recent murine studies have shown a clear contribution of ILC populations to the mucosal immune response against infections. In particular $ROR\gamma\text{-}t^+$ ILC are the main source of IL22, which controls C. rodentium infection in mice [259, 260, 283, 412]. Moreover, type 2 cytokine producing ILC participate to the immune response towards helminths [292]. A contribution of ILC to autoimmunity and chronic immune disorders is also emerging. We have identified IL23-responsive ILC in innate murine models of intestinal inflammation, where they play a fundamental pathogenic role through the production of IL17A and IFN-γ [214]. The $CD56^-$ ILC that accumulate in the intestinal lesions of CD patients strongly resemble their murine counterpart. They also respond to IL23 stimulation and secrete IL17A and IFN-γ. It is notable that an imbalance between $CD56^+$ ILC populations has also been observed in CD, with increased IL23-responsive IFN-γ-producing $NKp44^-NKp46^+CD3^-CD56^+$ cells and reduced IL22-producing $NKp44^+NKp46^-CD3^-CD56^+$ cells [413]. These data indicate that ILC with a more pathogenic phenotype accumulate in the inflamed intestine of CD patients.

$CD56^-$ ILC also display features shared by LTi-cells and could therefore be involved in the recruitment and topographical organisation of inflammatory cells in the inflamed gut. It has been shown that ILC isolated from human adult tissue maintain their LTi activity *in vitro*, but if they exert this function *in vivo* needs to be demonstrated [40, 288]. In fact, the relationship between adult IL17-producing $CD56^-$

ILC, $CD56^+$ ILC and foetal LTi is controversial. Human foetal LTi are known to produce IL17A, while ILC isolated from adult tissues were found to express mainly IL22 *ex vivo*, even if clones of ILC expressing both cytokines have been observed [288]. It is currently unknown if IL17-producing and IL22-producing ILC are developmentally distinct cell subsets or whether they represent different stages of maturation [414]. In accordance with the latter hypothesis, ILC from tonsils can be induced to express IL17 by stimulation with IL1β and IL7, while IL2 can induce IFN-γ, and IL2 and TLR2 stimulation promotes IL13 secretion [291, 415]. These observations suggest the presence of some degree of plasticity between ILC populations, as previously shown for Th-cell subsets [414]. In this light, the accumulation of IL17-producing ILC in CD may be secondary to the exposure to environmental factors and subsequent epigenetic changes.

The factors driving ILC accumulation in CD lesions are currently unknown. Altered composition of the bacterial flora together with disrupted epithelial barrier, bacterial breaching and tissue damage may be responsible for the recruitment and activation of ILC to the inflamed intestine. Studies in mice have shown that NOD1-mediated recognition of bacterial peptidoglycan by IEC induce expression of CCL20 and β-defensins, which activate CCR6 and promote ileal ILF formation probably through their activity on LTi-like cells [416]. Moreover adult LTi are responsible for lymph node regeneration after murine infection with lymphocytic choriomeningitis virus, representing an important mechanism of restoration of lymphoid structure integrity after tissue damage [417]. In addition, microbial components, but also stress responses can trigger the inflammasome resulting in secretion of active IL1β, which is increased in CD [418] and has been shown to expand ILC populations and sustain their function [415, 419, 420]. Finally, it needs to be elucidated whether the CD

associated genetic mutations affecting innate pathways, such as NLR activity, autophagy, ER stress responses and the IL23/IL17 axis may influence the accumulation of ILC populations to the inflamed intestine.

All together the data presented in this chapter show that innate sources of IL17 are present in the human intestine and that they may participate in chronic intestinal inflammation in patients with IBD. In particular, we have identified a specific subset of IL17-producing $CD56^-$ ILC that accumulates in the inflamed intestine in patients with CD. It remains to be established if the observed accumulation of ILC in CD represents a primary or secondary mechanism of intestinal inflammation and to what extent ILC contribute to IBD pathogenesis through production of cytokines or recruitment of other inflammatory cells.

Chapter 5- Characterization of DC subsets in the intestinal and systemic immune response and their contribution to IBD

5.1 Introduction

DC are professional APC that derive from BM precursors and are characterized by their ability to capture, process and present antigen on MHC molecules. They migrate to specialized areas of lymphoid organs and induce proliferation and differentiation of naïve and memory T-cells, which bear a specific receptor for the peptide-MHC complex. DC form a very heterogeneous family of cells, which have been mostly characterized in the murine peripheral and systemic immune system, but are still largely undefined in humans [421]. They are classically divided into pDC, which secrete type I IFN and contribute to anti-viral responses, and cDC that can be further subdivided on the basis of their migratory or tissue-resident phenotype and on their maturation state. In fact, immature DC undergo maturation after PRR-mediated recognition of microbial components or cytokine exposure, which induce expression of MHC and co-stimulatory molecules on their cell surface, allowing optimal presentation and activation of T-cells. On the other hand, during homeostasis, DC presenting innocuous or self antigens contribute to maintain a tolerogenic immune response through induction of T-cell anergy and $Foxp3^+$ Treg differentiation [70].

cDC isolated from the murine spleen are defined by the expression of high levels of the integrin CD11c and can be classified in $CD11b^+$ and $CD8\alpha^+$ cells. $CD11b^+$ DC are dependent on the transcription factors RelB, IRF4 and RBP-J, secrete IL10 and mainly induce Th-2 responses. On the other hand, $CD8\alpha^+$ DC depend on BATF3, but

also IRF8 and ID2 transcription factors and are characterized by high levels of TLR3, CD103, CD36, Necl2 and DNGR-1 expression. Functionally, $CD8\alpha^+$ DC are able to cross-present exogenous antigen on MHC class I molecules, ingest material from dead cells and produce IL12. They have also been shown to induce the differentiation of antigen-specific T cells into Treg [422]. The study of intestinal DC populations has been hampered by the difficult isolation of these cells from the gut, which requires long enzymatic digestion. However, recent progress in isolation and imaging techniques has led to great advance in this field. Intestinal DC are particularly skewed towards a more regulatory phenotype, allowing immune tolerance towards the huge number of harmless antigens that are present in the gut lumen, derived from both ingested food or the commensal flora. Their regulatory properties have been linked to the ability to secrete immunosuppressive cytokines, such as IL10 and TGF-β, and are influenced by both dietary factors and epithelial cell products, such as RA, TSLP and TGF-β [75, 76]. In the GALT, DC can be found in organised lymphoid structures, such as MLN, PP and ILF, but they are also scattered through the LP. Murine intestinal DC have been classically defined by the expression of the CD11c integrin and further classified on the base of other markers such as CD11b and CD8α. In PP, $CD11b^+CD8\alpha^-$ DC are characterized by high production of IL10, while $CD11b^-CD8\alpha^+$ and $CD11b^-CD8\alpha^-$ produce IL12 and induce Th-1 differentiation [75, 421]. However, it has been suggested that CD11c may not represent an adequate marker for defining intestinal tissue DC, since it is also expressed by some tissue macrophages, and the definition of intestinal mononuclear phagocytes has recently been re-evaluated. A new classification has been proposed, which includes four main populations of intestinal mononuclear phagocytes, but their identification as macrophages or DC remain controversial. These populations include $CD11c^+$ DC, which express the α_E integrin

$CD103^+$ and can be further subdivided in $CD11b^+$ and $Cd11b^-$ cells. $CD103^+CD11b^+$ DC represent the main population in the LP while $CD103^+CD11b^-$ DC prevail in the GALT. The other two populations are represented by $CD11c^{mid}CD11b^+CD103^-$ cells expressing the fraktalkine receptor CX3CR1 and $CD11c^-F4/80^+$ classical macrophages. The classification of $CX3CR1^+$ cells as macrophages or DC is controversial, since these cells are characterized by properties of both cell types [423]. Recent studies have shown that $CD103^+$ DC and $CX3CR1^+$ cells differ in their developmental origin. $CD103^+$ DC differentiate from the common-DC progenitor via DC-committed precursors in a Flt3-dependent manner. $CD103^+CD11b^+$ DC differentiation is further controlled by GM-CSF, while $CD103^+CD11b^-$ DC also depend on BATF3, ID2 and IRF8 showing similarities with splenic $CD8\alpha^+$ DC. On the other hand, $CX3CR1^+$ cells derive from $LY6C^{hi}$ monocytes mostly under GM-CSF stimulation [81, 82] (Figure 5.1) . These subsets not only have distinct developmental origins, but also exert different functional properties. $CD103^+CD11b^+$ DC, but not $CX3CR1^+$ cells, express CCR7 and constantly migrate to the MLN both at steady state and in the presence of inflammation [424]. We and others have shown that $CD103^+$ MLN DC, but not their $CD103^-$ counterpart, can induce peripheral differentiation of $Foxp3^+$ Treg through a TGF-β- and RA-dependent mechanism [79, 80]. Furthermore, $CD103^+$ MLN DC are able to induce the expression of gut-homing receptors, such as CCR9 and α4β7, on T-cells, conferring them the ability to recirculate to the intestinal mucosa [77, 78]. On the other hand, $CX3CR1^+$ cells can induce the differentiation of Th-17 cells in an ATP-dependent fashion, in accordance with a more inflammatory phenotype [425]. Mice reconstituted solely with LP $CX3CR1^+$ mononuclear phagocytes were found to develop a more severe colitis after DSS oral administration, compared to mice reconstituted with both $CD103^+$ DC and

CX3CR1$^+$ cells [82]. This observation indicates that the presence of both subsets is crucial in order to keep the right balance between inflammatory and regulatory responses. CX3CR1$^+$ cells have also been shown to represent the major mononuclear phagocyte population performing intraepithelial dendrite sampling of luminal antigens [426]. However, since they cannot migrate to the MLN, the functional role of antigen uptake by CX3CR1$^+$ cells remains to be elucidated. Many speculative hypotheses have been formulated, which include the possibility that they might transfer the antigen to CD103$^+$ DC. Alternatively, transepithelial sampling by CX3CR1$^+$ cells may represent an early innate protective mechanism against bacterial invasion [427].

Studies in mice have suggested that DC may play a central role in the development of IBD. In fact, increased frequencies of DC with an activated phenotype have been found in both MLN and LP in different murine models of colitis [204, 428]. In addition to DC accumulation, we recently described an alteration of DC subset composition in the presence of colitis. In the T-cell transfer model of colitis, we found reduced frequency of CD103$^+$ DC in MLN, colon and especially in the small intestine. MLN CD103$^+$ DC from colitic mice also show an altered phenotype with reduced expression of the immune regulatory genes *tgfβ2* and *aldh1a2* and induction of *tbet* and *IL12p35*. This correlates with their reduced ability to induce differentiation of Foxp3$^+$ Treg and with increased induction of IFN-γ-producing CD4$^+$ T-cells compared to CD103$^+$ DC at steady state. These data indicate that during colitis CD103$^+$ DC may loose their regulatory properties and contribute to intestinal inflammation. Even in the presence of colitis, CD103$^+$ DC originate independently from CD103$^-$ DC, suggesting that exposure to the inflammatory cytokine milieu in colitis might be responsible for this phenotypic change. Otherwise, in the presence of colitis CD103$^+$ DC might be directly recruited from the circulation to the MLN,

therefore escaping gut conditioning, but both these hypotheses remain to be confirmed [429]. In another study we have described a population of monocyte-derived CD103$^-$ E-cadherin$^+$ DC, that represents only a minority of intestinal DC at steady state, but accumulate in the colon and MLN of colitic mice in both the T-cell dependent and anti-CD40-induced innate model of colitis [83]. E-cadherin is a transmembrane glycoprotein, which is mainly expressed by IEC, and represents the only known ligand for CD103 [430]. E-cadherin$^+$ DC are characterized by expression of pro-inflammatory genes, such as various TLRs and the chemokine receptors CCR2 and CCR6. While CCR2 is required for monocyte recruitment to the inflamed tissue, CCR6 was previously shown to mediate DC activation of pathogen specific T cells in PP [431, 432]. CD103$^-$E-cadherin$^+$ DC also induce higher transcript levels of IL12p40 and IL23p19 after *in vitro* stimulation than their CD103$^-$E-cadherin$^-$ counterpart and are able to exacerbate T-cell mediated colitis after adoptive transfer *in vivo*. In these settings, increased severity of colitis is associated with higher frequencies of Th-17 cells.

Another subset of pro-inflammatory mononuclear phagocytes is represented by CD103$^-$ signal regulatory protein (SIRP)α$^+$ DC which accumulate in the LP and MLN of mice with TNBS-induced colitis and drive Th-17 responses. Mice deficient for CD47, the ligand of SIRPα, are protected from colitis and the reconstitution of these mice with WT but not CD47 KO CD103$^-$SIRPα$^+$ DC result in Th-17 driven wasting disease [433]. Furthermore, SIRPα mutant mice, which lack the cytoplasmic region of the protein, are resistant to IL10 deficiency-induced colitis [434].

Human studies have proven to be more challenging due to the difficult accessibility of intestinal DC, their low frequency and consequent limitations in isolating cells and performing functional studies. Furthermore, the lack of an agreement in the definition

of human intestinal DC and the resulting use of different markers in different studies have led to controversial results. In one study, an increased frequency of intestinal immature Langerin$^+$ DC was found in the intestine of patients with IBD by immunofluorescence. This accumulation of immature DC in the inflamed intestine was related to the over-expression of CCL20 in the FAE, which would lead to the recruitment of immature CCR6$^+$ DC to the inflamed gut [408]. Higher numbers of DC-specific ICAM3 grabbing non-integrin (DC-SIGN)$^+$ DC and CD83$^+$ DC were also observed by immunohistochemistry in CD, in the LP and lymphoid aggregates, respectively [435]. Conversely, no difference in the frequency and maturation stage of LIN$^-$HLADR$^+$CD11c$^+$ cDC has been found by FACS analysis in LPMC isolated from the colon of patients and controls after enzymatic digestion [85]. Other studies have shown that intestinal DC have a more activated phenotype in IBD, with higher expression of inflammatory and co-stimulatory molecules, such as TLR2 and TLR4, CD40, CD80 and CD86 [404, 435, 436]. Furthermore, LP and MLN DC from IBD patients produce higher amounts of pro-inflammatory cytokines, such as IL6, IL12, IL18 and IL23 in response to different stimuli [404, 435, 437].

The role of different DC subsets in the pathogenesis of IBD has not been fully elucidated. CD103$^+$ DC are present in human MLN in both uninflamed individuals and CD patients and have been shown to induce the expression of CCR9 on CD8$^+$ T-cells in a RA-dependent fashion, similarly to what is described in mice [78]. Moreover, data from Rescigno's group have shown that CD103$^+$ DC isolated from human MLN have increased ability to drive differentiation of Treg compared to their CD103$^-$ counterpart, suggesting that CD103$^+$ DC have the capacity to promote Treg in humans, as well as in mice. Furthermore conditioning of DC in the presence of human IEC supernatants induces CD103 expression and the ability to induce Treg.

Interestingly, incubation with IEC isolated from patients with CD resulted in reduced Treg differentiation, suggesting that a secondary impairment in tolerogenic DC function might contribute to intestinal inflammation in these patients [86].

In this chapter we report our phenotypical analysis of different DC subsets in the peripheral and systemic immune responses in patients with IBD and controls.

We first evaluated the ability of intestinal and systemic human DC to modulate the activity of TGF-β, a key cytokine in maintaining intestinal homeostasis and regulating tolerogenic and inflammatory immune responses. We then wanted to investigate if an altered phenotype of intestinal DC populations is associated with the presence of chronic intestinal inflammation. In particular we aimed to assess whether impairment in $CD103^+$ DC frequency and function is involved in IBD. We also investigated if inflammatory DC populations are present in the human systemic and mucosal immune system and if they may contribute to intestinal inflammation in IBD.

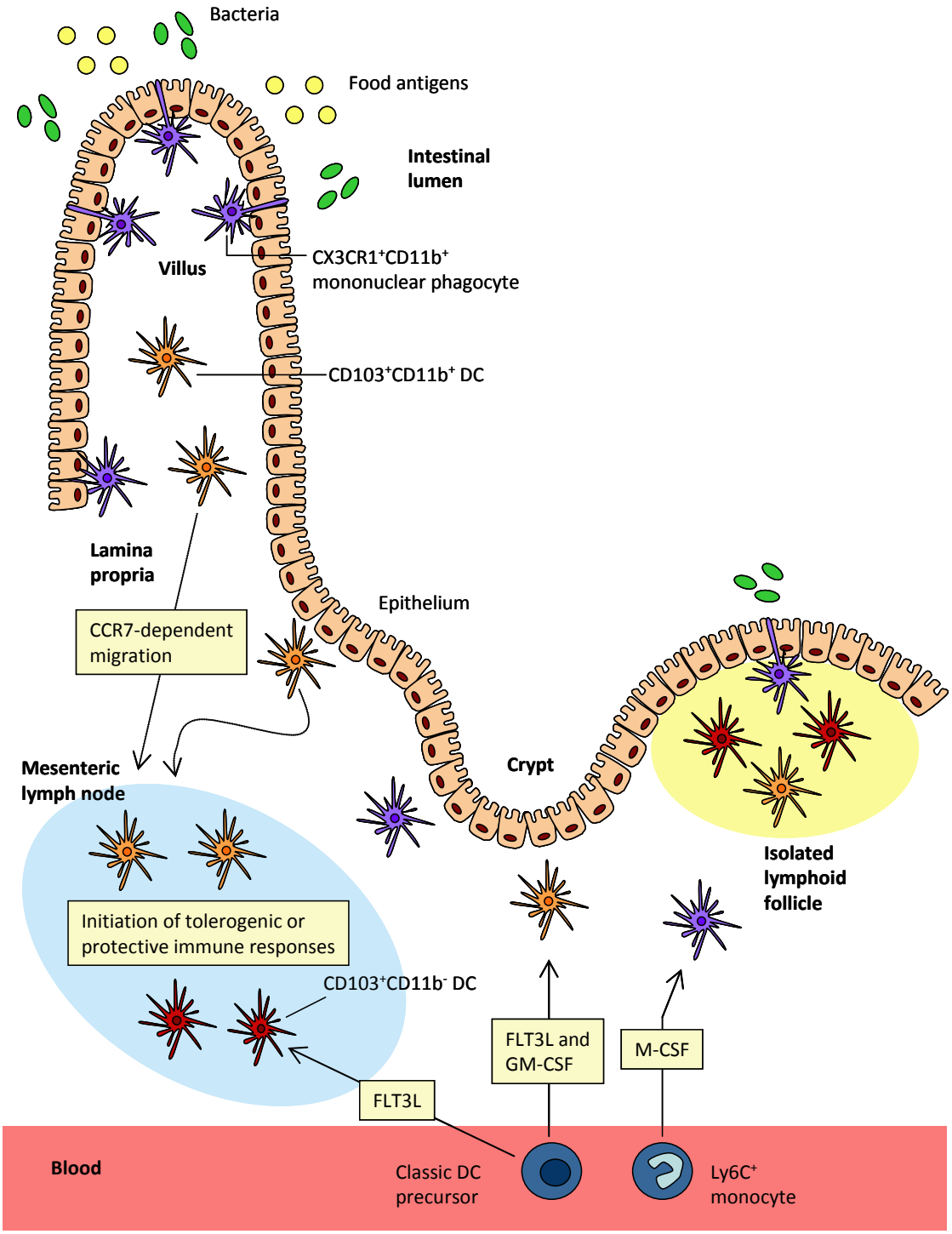

Figure 5.1 Murine intestinal DC subsets and their precursors.

Blood classic DC precursors home to the gut and give rise to GALT $CD103^+CD11b^-$ and LP $CD103^+CD11b^+$ DC via FLT3L and FLT3L plus GM-CSF stimulation, respectively. $CD103^+CD11b^+$ DC migrate to the MLN in a CCR7-dependent manner and induce antigen-specific T-cell responses. $CX3CR1^+$ mononuclear phagocytes derive from $Ly6C^+$ monocytes under M-CSF stimulation. $CX3CR1^+$ cells do not migrate to the MLN, but sample luminal antigens with their trans-epithelial dendrites.

Adapted from Varol. Nature Reviews Immunology 10: 2010.

5.2 Results

5.2.1 TGF-β is highly expressed in the intestine

TGF-β is a pleiotropic, multipotent cytokine that is involved in cell-growth, apoptosis, matrix synthesis and inflammation. In the immune response TGF-β is known to participate in tolerance and immune suppression, but in conjunction with other factors can also promote inflammatory pathways, such as differentiation of Th-17 cells. This cytokine is abundantly expressed in the intestine, where it is secreted as a large latent complex (LLC) with the latency-associated protein (LAP) and the LTBP and it is covalently bound to the extra-cellular matrix (Figure 5.2). Therefore, it appears that TGF-β activity is mainly regulated through activation of latent TGF-β [438].

TGF-β has previously been shown to be increased in the intestinal tissue in patients with IBD and we were able to confirm these findings in our cohort of patients (Figure 5.3, A) [439]. However, as discussed above, the evaluation of total TGF-β in the intestine has intrinsic limitations, since it cannot distinguish between active and latent TGF-β. Interestingly, we also observed increased protein expression of plasminogen activator inhibitor (PAI-1) in mucosal homogenates from IBD patients (Figure 5.3, B). PAI-1 is a member of the serine proteinase inhibitor superfamily and the main inhibitor of PLAT, which is involved in the activation of latent TGF-β [440]. These findings suggested that, even if higher concentrations of total TGF-β are present in the inflamed intestine in IBD, activation of latent TGF-β might be compromised in these patients. Therefore, we calculated the concentration of active TGF-β and latent TGF-β in colon homogenates from patients with IBD and controls, as explained in Chapter 2. Strikingly only the latent form of TGF-β appeared to be over-expressed in the inflamed intestine in IBD, while no difference was observed in intestinal concentration of active TGF-β (Figure 5.3, C-D).

All together these observations highlight the importance of evaluating the complex pathways involved in secretion and activation of TGF-β, together with the analysis of TGF-β signalling and the presence of other cofactors in the microenvironment, before making any conclusion on the role of this cytokine in intestinal homeostasis and inflammation.

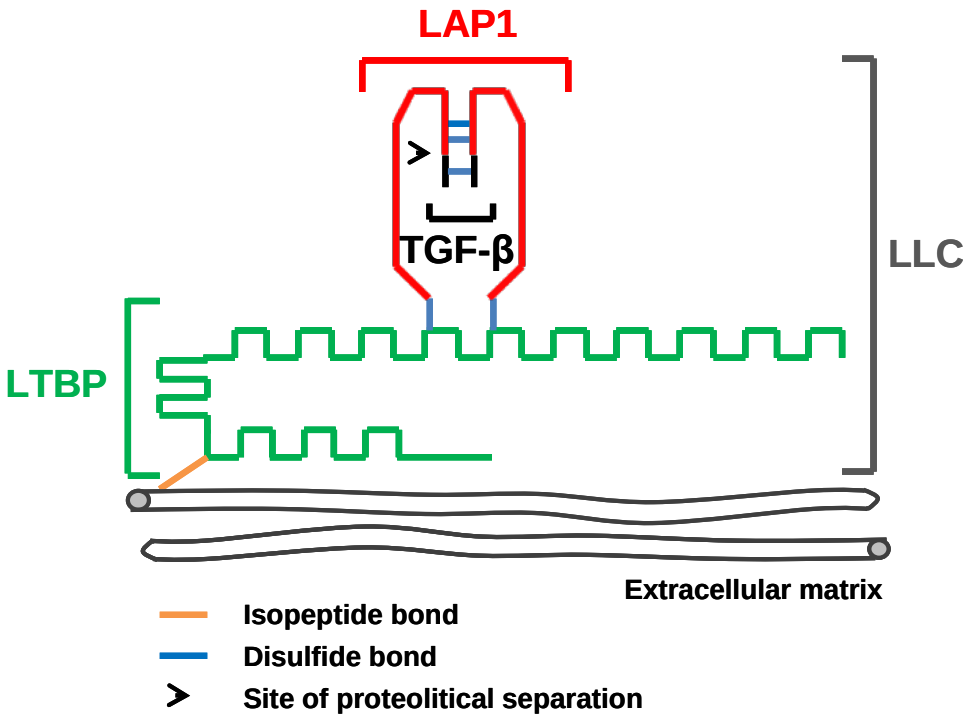

Figure 5.2 Structural organisation of TGF-β large latent complex

The LLC comprises TGF-β, LAP and LTBP and it is covalently bound to the extracellular matrix.

Adapted from Annes. Journal of Cell Science. 116: 2003.

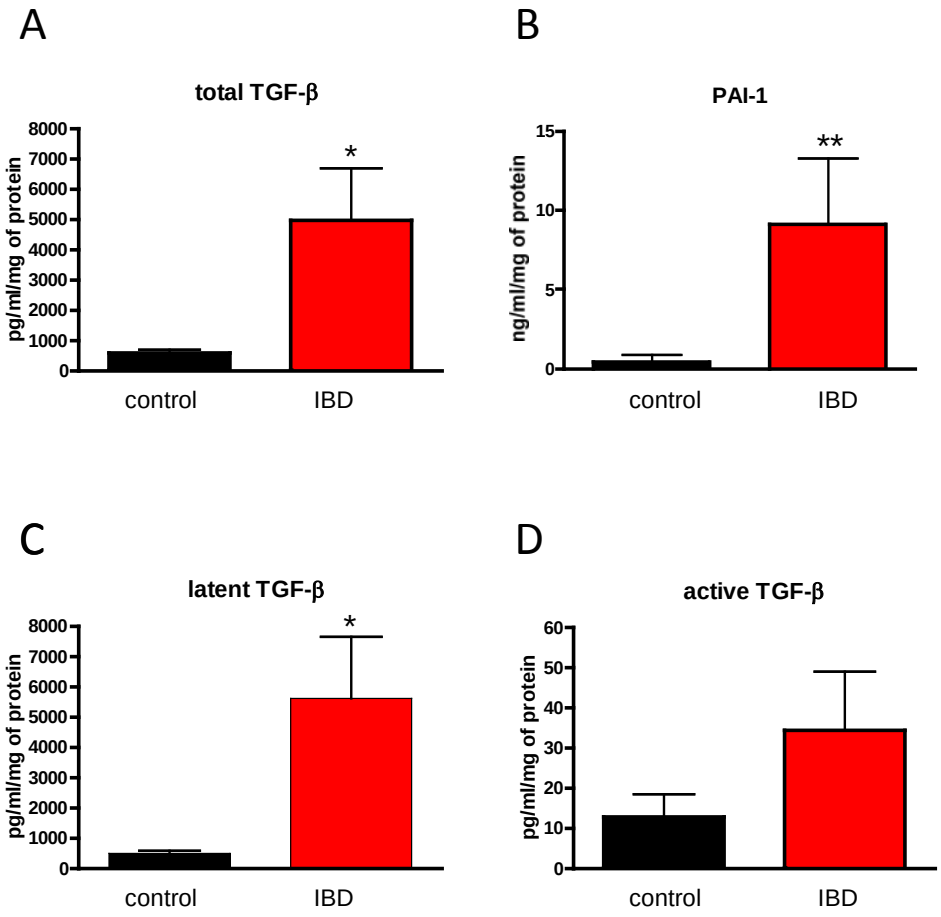

Figure 5.3 Active TGF-β is not increased in IBD

(A) Total TGF-β protein levels measured after activation with HCl, in intestinal mucosa homogenates from control individuals (n=9) and IBD patients (n=11). (B) PAI-1 protein levels in intestinal mucosa homogenates from control individuals (n=9) and IBD patients (n=14). (C) Latent TGF-β protein levels in intestinal mucosa homogenates from control individuals (n=8) and IBD patients (n=9). Latent TGF-β concentration was calculated subtracting total TGF-β concentration and active TGF-β concentration measured without acidification with HCl. (D) Active TGF-β protein levels in intestinal mucosa homogenates from control individuals (n=9) and IBD patients (n=13). Bars represent mean +/- SEM. *$p<0.05$, **$p<0.01$

5.2.2 Intestinal DC are specialized in regulation of TGF-β activity

In initial experiments we aimed to characterize intestinal and systemic human DC in particular in relation to their ability to modulate TGF-β-activity.

We analysed the expression of TGF-β related genes, such as *LTBP1*, *PLAT* and *SMAD7* in sorted $LIN^{-}HLADR^{+}CD11c^{high}$ cDC from the blood and colon of control subjects. LTBP1 belongs to the family of the LTBPs, which form part of the LLC and mediates LLC binding to the extracellular matrix, while *PLAT* encodes for a serine protease, which is involved in the activation of latent TGF-β. SMAD7 is induced by TGF-β through its activity on the TGFBR1, but inhibits TGF-β signalling, via inhibition of SMAD2/3 phosphorilation and induction of TGFBR1 degradation thus representing a negative feedback loop. Interestingly, studies from Monteleone's group have shown that SMAD7 is over-expressed in the intestine of IBD patients, suggesting that reduced TGF-β activity might play a role in IBD pathogenesis [441, 442].

No difference was observed in the expression of *LTBP1* between LP and PB DC. Interestingly, we found increased expression of *PLAT* in sorted LP DC compared to PB DC, which indicates that DC may contribute to the activation of latent TGF-β in the intestine. Furthermore intestinal DC also express higher transcript levels of *SMAD7*, in accordance with a higher exposure, but also a reduced responsiveness of intestinal DC to local TGF-β (Figure 5.4).

All together these observations show that gut-conditioned DC develop tissue-specific functions associated with modulation of TGF-β- activity.

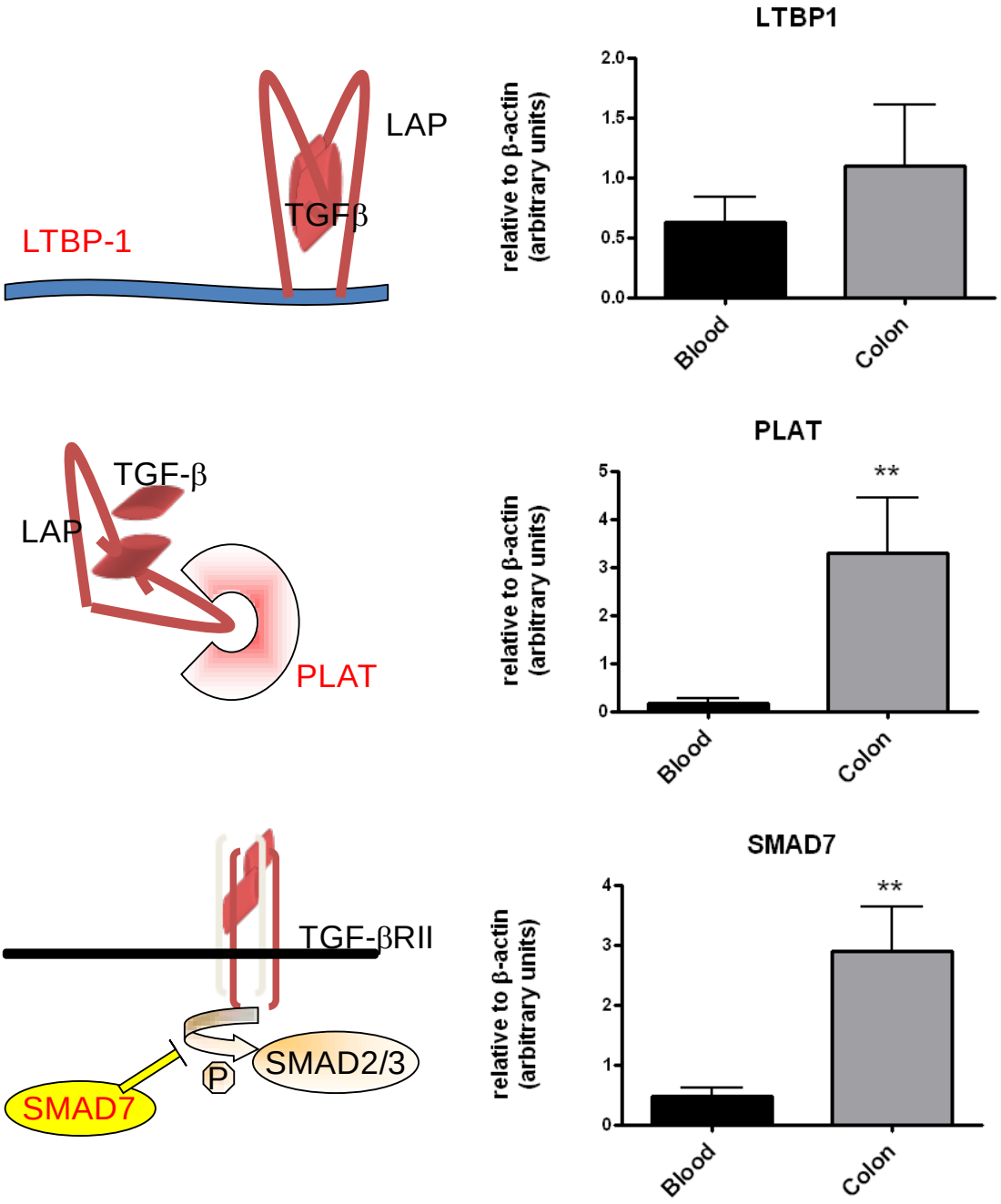

Figure 5.4 TGF-β-related genes are over-expressed in intestinal DC

Relative mRNA expression of *LTBP1*, *PLAT* and *SMAD7* in sorted LIN$^-$ HLADR$^+$CD11c^{high} cDC from blood (n=12) and colon (n=10) of control individuals. Bars represent mean +/- SEM. **p<0.01

5.2.3 Compartmentalization of CD103$^+$ DC

We and others have shown that a specific subset of murine mucosal CD103$^+$ DC induce the differentiation of Foxp3$^+$ Treg through a TGF-β- and RA-dependent mechanism. RA is a metabolite of dietary vitamin A and derives from oxidation of retinol via retinal. Different forms of RA bind to the nuclear receptors RA receptor (RAR) and the retinoid X receptor (RXR) which form heterodimers and regulate gene transcription. Accordingly with their activity and function, we have shown that murine CD103$^+$ DC preferentially express *tgfb2*, which encodes for one of the isoforms of TGF-β, and genes involved in TGF-β secretion and activation such as *ltbp3* and *plat*. Furthermore, they preferentially express *aldh1a2*, the enzyme responsible for the conversion of retinal to RA (Figure 5.5).

In accordance with the higher expression of TGF-β-related genes, we found a trend to a higher expression of *CD103* in sorted human cDC isolated from the colon compared to the blood of uninflamed controls, suggesting that the human intestine is enriched for CD103 expressing DC (Figure 5.6, A). We then analysed the frequency of CD103$^+$ cells amongst PB and LP cDC in non-inflammatory controls by FACS, using the gating strategy shown in Figure 5.6, B. Strikingly, we found preferential expression of CD103$^+$ DC in the colon, where they represented an average 18% of cDC (range=2.2-60.6). On the contrary CD103$^+$ DC were not found in most individuals in the blood or represented only a very low frequency of cDC (average=1, range=0-6.6) (Figure 5.6, C). CD103$^+$ DC were also identified *in situ* by immunofluorescence analysis, in close proximity to the intestinal crypts (Figure 5.6, D).

These findings suggest that human CD103$^+$ DC are compartmentalised to the intestine, compared to systemic sites.

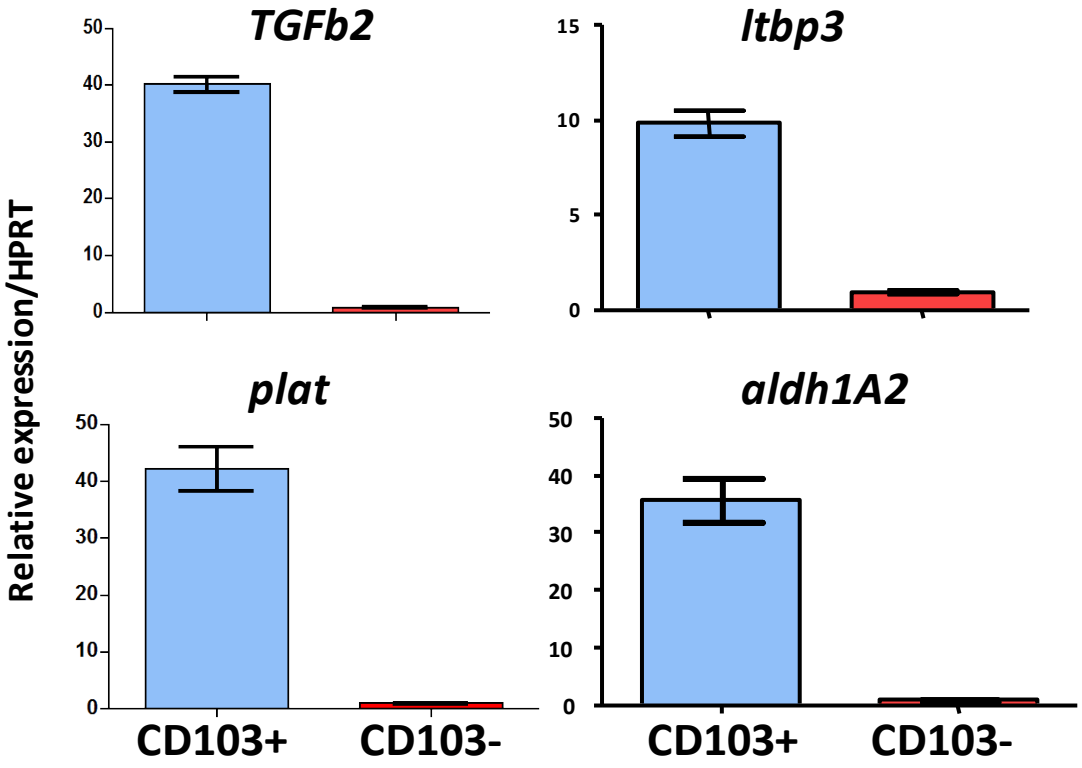

Figure 5.5 CD103^{+} DC from murine MLN over-express TGF-β and RA-related genes.

Relative mRNA expression of *tgfb2*, *ltbp3*, *plat* and *aldh1A2* in sorted CD103^{+} and CD103^{-} DCs from the MLN of BALB/c mice. Bars represent mean +/- SD

Adapted from Coombes. Journal of Experimental Medicine 204: 2007.

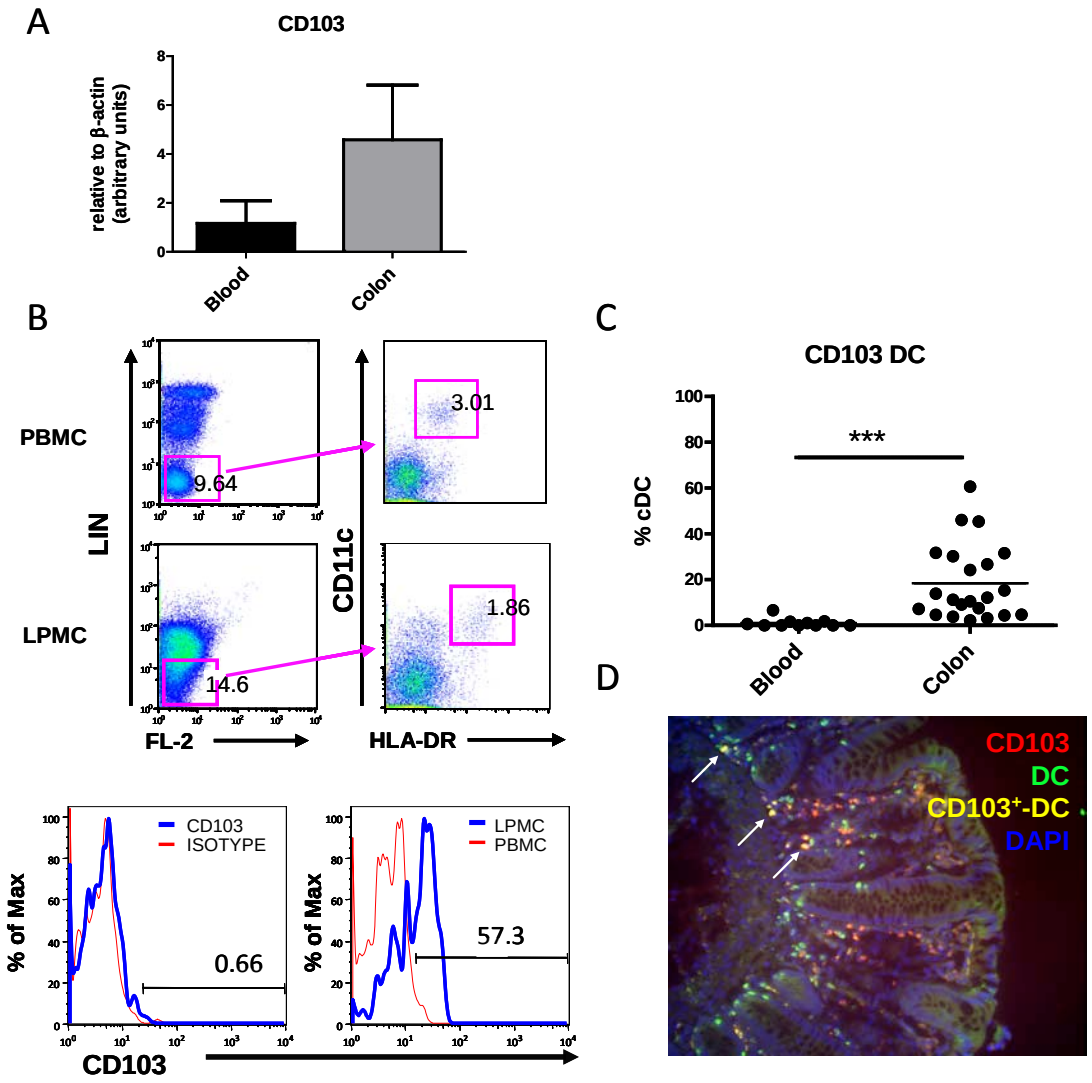

Figure 5.6 CD103$^+$ DC are compartmentalized to the human intestine

(A) Relative mRNA expression of *CD103* in sorted LIN$^-$HLADR$^+$CD11c^{high} cDC from blood (n=6) and colon (n=6) of control individuals. (B) Representative FACS staining of CD103 on LIN$^-$HLADR$^+$CD11c^{high} cDC from LPMC and PBMC. Gates were set using the appropriate isotype control, as shown. (C) Frequency of CD103$^+$ cells in the LIN$^-$HLADR$^+$CD11c^{high} cDC from blood (n=11) and colon (n=22) of controls. (D) Immunofluorescent staining of CD103$^+$ DC in PFA-fixed, frozen tissue from an uninflamed colon. Three-colour immunofluorescence was used to determine CD103 expression (Cy5-tyramide, red fluorescence), DC LAMP expression (FITC, green fluorescence), nuclear counterstaining (DAPI, blue fluorescence). Examples of CD103$^+$ DC are shown by arrows.***p<0.001

5.2.4 Human gut CD103$^+$ DC have a regulatory phenotype in homeostatic conditions

We then wanted to characterize human CD103$^+$ DC and evaluate whether they have the same tolerogenic profile observed in murine mucosal CD103$^+$ DC.

CD103$^+$ and CD103$^-$ LIN$^-$HLADR$^+$CD11c^{high} DC were sorted from the intestine of control individuals using the gating strategy shown in Figure 5.6, B. Expression of genes previously associated with murine regulatory and inflammatory DC subsets were then analysed in the two compartments. Our preliminary results show higher expression of the *ALDH1A2* gene in human intestinal CD103$^+$ DC compared to their CD103$^-$ counterpart, suggesting they are specialized in converting retinal to RA. We also observed a trend to higher expression levels of TGF-β related genes, such as *LTBP1*, *SMAD7* and *PLAT* in the human intestinal CD103$^+$ DC. Another significant mechanism of activation of latent TGF-β is mediated by integrins, such as $\alpha_v\beta_6$, whose expression is restricted to some epithelial cells, and $\alpha_v\beta_8$, which is instead expressed on immune and non immune cells [443-446]. Conditional loss of β_8 in DC in *(CD11c-cre)Itgb8$^{fl/fl}$* mice leads to the development of systemic autoimmune disease and severe intestinal inflammation. Colitis in these mice is associated with reduced frequencies of intestinal Treg. Furthermore β_8-deficient DC present impaired ability to induce Treg differentiation *in vitro*, which can be restored by addition of exogenous active TGF-β [446]. These data suggest that expression of $\alpha_v\beta_8$ on DC is key for TGF-β activation and peripheral induction of Treg cells. Recent studies have shown that murine MLN CD103$^+$ DC preferentially express $\alpha_v\beta_8$ and that their enhanced ability to induce Treg differentiation depends entirely on $\alpha_v\beta_8$-mediated activation of latent TGF-β [447]. In accordance with these findings, we observed a trend to an increase in the expression of *ITGB8*, encoding for the human β_8 integrin, in intestinal CD103$^+$

DC relative to their $CD103^-$ counterpart (Figure 5.7, A). The presence of β_8 integrin on human intestinal $CD103^+$ DC was confirmed by FACS staining in preliminary experiments. However, β_8 expression was not restricted to $CD103^+$ DC (Figure 5.7, B).

By contrast, genes previously associated with inflammatory subsets of murine DC, such as *TBX21* (encoding for T-bet) *IL1B* and *CCR6* showed a higher trend of expression in the $CD103^-$ DC (Figure 5.7, A).

These data suggest that human intestinal $CD103^+$ DC are enriched for TGF-β related gene expression compared to their $CD103^-$ counterpart in the absence of intestinal inflammation, in agreement with previous findings in the murine mucosal immune system.

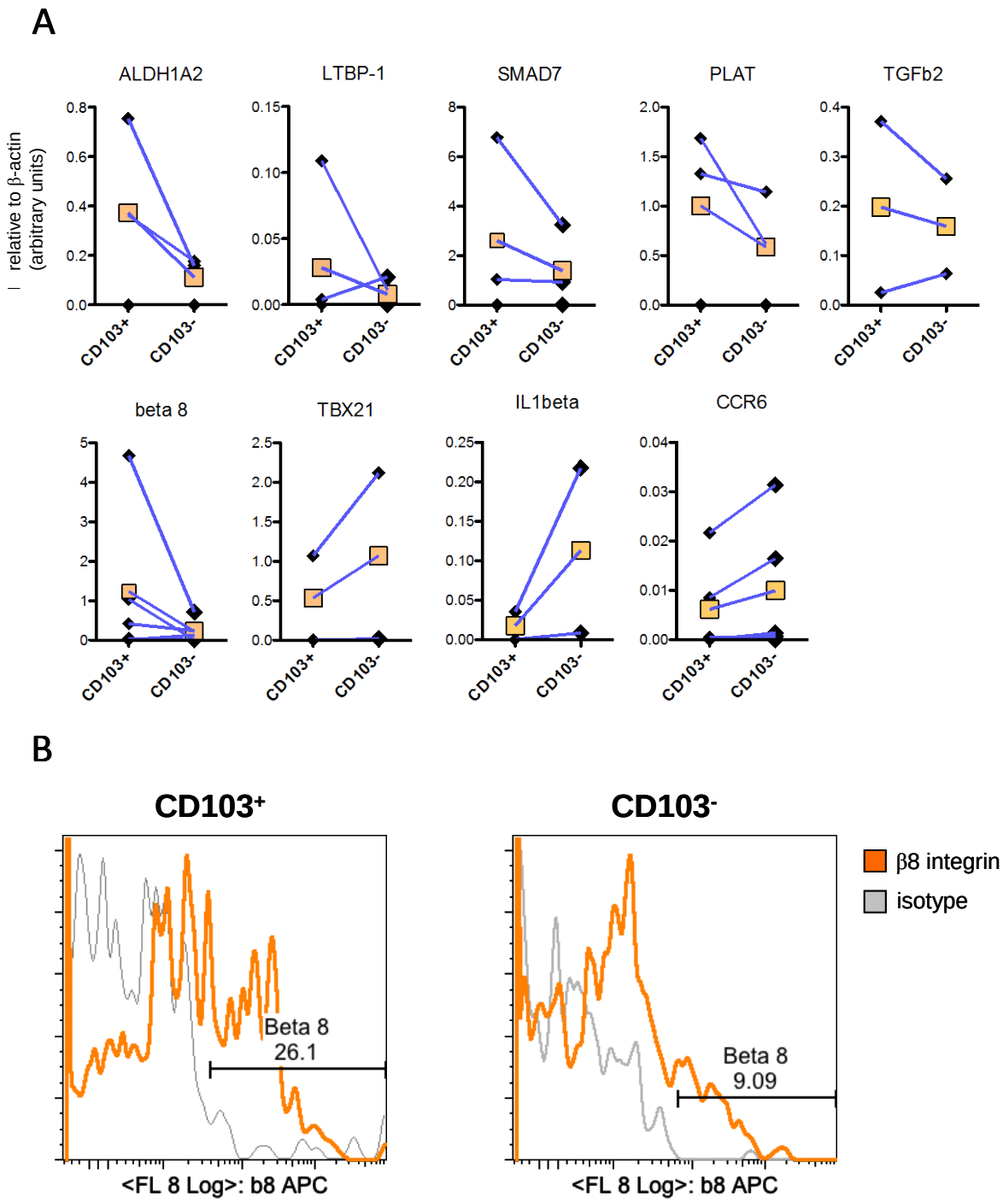

Figure 5.7 CD103^{+} DC show a regulatory gene expression profile

(A) mRNA expression of *ALDH1A2* (n=3), *LTBP1* (n=4), *SMAD7* (n=3), *PLAT* (n=3), *TGFB2* (n=2), *ITGB8* (n=5), *TBX21*(n=2), *IL1B* (n=2) and *CCR6* (n=3) in sorted CD103^{+} and CD103^{-} DC from the colon of control individuals. Mean relative gene expression was expressed as $2^{-\Delta CT}$($\Delta CT=CT_{gene}-CT_{\beta\text{-actin}}$) for *TGFB2*, *IL1B* and *CCR6;* $2^{-\Delta CT}$($\Delta CT=CT_{gene}-CT_{\beta\text{-actin}}$) x1000 for *ALDH1A2*, *LTBP1*, *SMAD7* and *PLAT*, and x100 for *ITGB8* and *TBX21*. The yellow squares represent the average values in each group. (B) FACS staining for β8 on CD103^{+} and CD103^{-} DC. Cells were gated on HLADR^{+}CD11c^{high} CD103^{+} and HLADR^{+}CD11c^{high}CD103^{-} cells.

5.2.5 The frequency of gut CD103$^+$ DC is reduced in IBD

In studies conducted in our laboratory in the T-cell transfer model of colitis, we have shown that DC subset composition is altered in the presence of intestinal inflammation, with reduced frequencies of CD103$^+$ DC. Furthermore, in these settings, CD103$^+$ DC also show an altered phenotype with decreased expression of immune regulatory genes associated to over-expression of inflammatory genes. Accordingly, CD103$^+$ DC from colitic mice show reduced ability to induce differentiation of Foxp3$^+$ Treg, while they are better inducer of IFN-γ-producing CD4$^+$ T-cells compared to CD103$^+$ DC from steady state [429].

We wanted to investigate if a defect in frequency and function of regulatory CD103$^+$ DC can also be found in patients with IBD. Strikingly, decreased frequencies of CD103$^+$ DC were found amongst cDC in LPMC isolated from the inflamed colon of patients with IBD, both UC and CD, compared to non-inflammatory controls, suggesting that a disturbed composition of the intestinal DC pool is indeed associated with intestinal inflammation in IBD (Figure 5.8, A).

To evaluate if intestinal CD103$^+$ DC maintain their tolerogenic phenotype or whether they present an altered phenotype, we analysed the expression of regulatory and inflammatory genes in sorted CD103$^+$ and CD103$^-$ DC isolated from the intestine of patients with IBD. Interestingly, our preliminary data show a lower gene expression of regulatory molecules, such as *ALDH1A2*, *LTBP* and *SMAD7* in CD103$^+$ DC compared to CD103$^-$ DC in IBD, with a trend opposite to what observed in controls. *ITGB8* expression was higher in the CD103$^+$ DC in IBD, similarly to what observed in controls. The β_8 integrin was also analysed by FACS on intestinal CD103$^+$ DC, using the gating strategy showed in Figure 5.7, B, and no significant difference was observed between patients and controls (Figure 5.8, C). The expression of

inflammatory genes, such as *TBX21* and *CCR6*, was increased in $CD103^+$ DC from IBD patients compared to their expression in $CD103^-$ DC, while *IL1B* was higher in $CD103^-$ DC (Figure 5.8, B).

All together these data suggest that both a reduced frequency of regulatory $CD103^+$ DC and a disturbed gene expression profile of $CD103^+$ DC with a more inflammatory signature are present in patients with IBD. It remains to be elucidated whether this finding represents a primary event contributing to chronic intestinal inflammation or if the observed alteration in DC phenotype is the result of cell conditioning in the presence of a pro-inflammatory intestinal milieu.

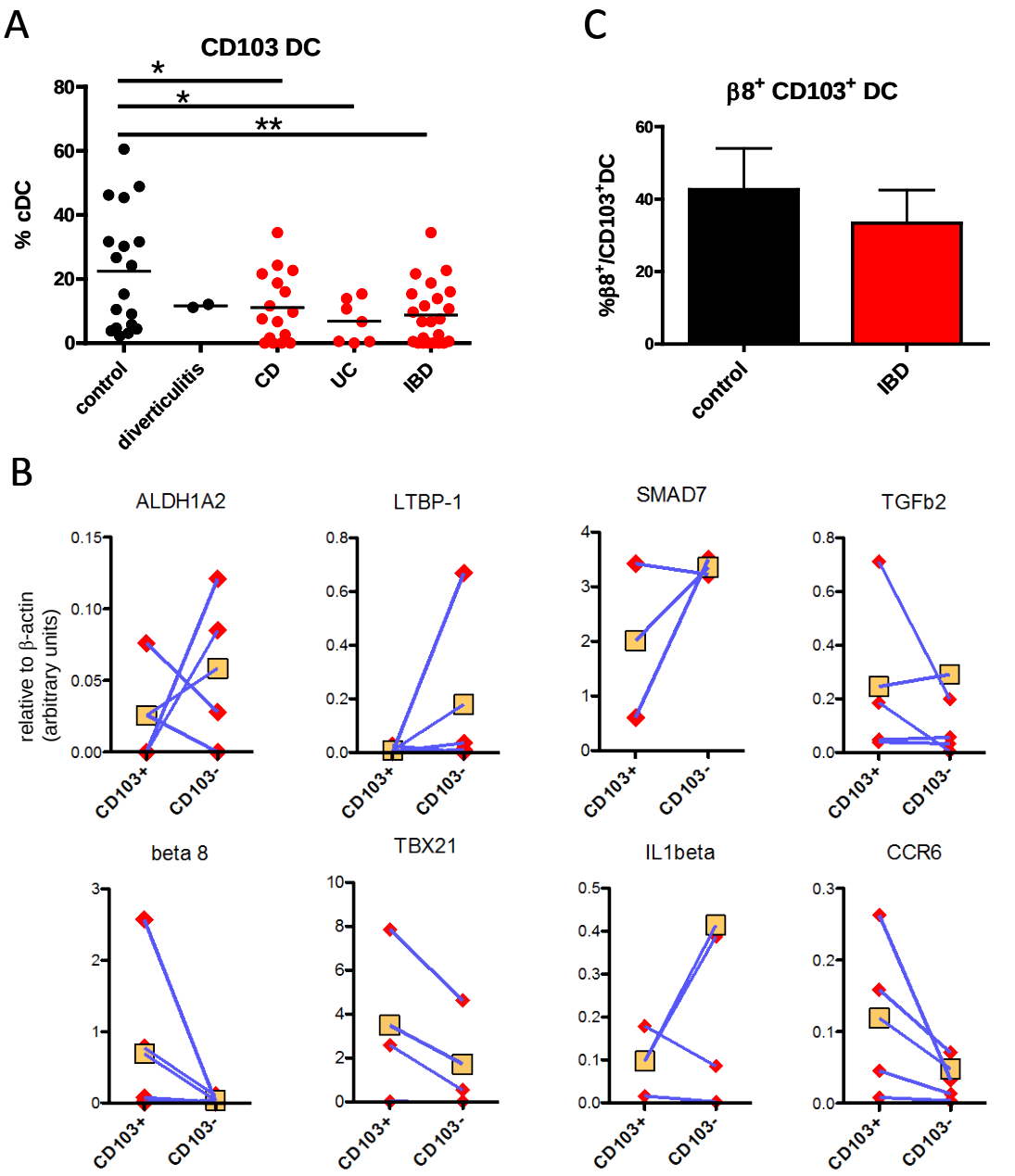

Figure 5.8 Intestinal CD103$^+$ DC are decreased in IBD and show a more inflammatory phenotype

(A) Frequency of CD103$^+$ cells amongst intestinal LIN$^-$HLADR$^+$CD11c^{high} cDC from controls (n=18) and patients with CD (n=16), UC (n=7), diverticulitis (n=2) and IBD (UC+CD, n=23). (B) mRNA expression of *ALDH1A2* (n=4), *LTBP1* (n=4), *SMAD7* (n=2), *TGFB* (n=4), *ITGB8* (n=5), *TBX21* (n=3), *IL1B* (n=3) and *CCR6* (n=6) in intestinal CD103$^+$ and CD103$^-$ DC from IBD patients. Mean relative gene expression was expressed as $2^{-\Delta CT}$($\Delta CT=CT_{gene}-CT_{\beta\text{-actin}}$) for *TGFB2*, *IL1B* and *CCR6*; $2^{-\Delta CT}$ ($\Delta CT=CT_{gene}-CT_{\beta\text{-actin}}$) x1000 for *ALDH1A2*, *LTBP1*, *SMAD7*, and x100 for *ITGB* and *TBX21*. (C) Frequency of β8$^+$ cells amongst intestinal CD103$^+$ DC from controls (n=6) and IBD patients (n=3). Cells were gated as shown in Figure 5.7, B. Bars represent mean +/- SEM.

5.2.6 Circulating CCR6$^+$ DC are increased in IBD

We next wanted to evaluate if DC subsets with an inflammatory phenotype can be found in the human intestinal and systemic immune response and if they contribute to intestinal inflammation in patients with IBD. As previously mentioned, we have recently described a population of monocyte-derived CD103$^-$ E-cadherin$^+$ DC, that accumulate in the colon and MLN of colitic mice in both the T-cell dependent and anti-CD40-induced innate model of colitis [83]. We have shown that murine E-cadherin$^+$ DC express the chemokine receptors CCR2 and CCR6 and an inflammatory subset of CCR6$^+$ DC was previously described in infectious models [431, 432]. We analysed the expression of CCR6 on intestinal and systemic LIN$^-$HLA-DR$^+$CD11c^{high} cDC and observed that CCR6$^+$ DC were significantly increased in the blood of patients with IBD compared to control individuals. No significant difference was observed in the frequency of intestinal CCR6$^+$ DC in initial experiments, but more samples will need to be evaluated to address if they contribute to intestinal inflammation (Figure 5.9, A-C).

Further studies are necessary to investigate the origin and function of circulating CCR6$^+$ DC to evaluate if they play a role in the pathogenesis of IBD.

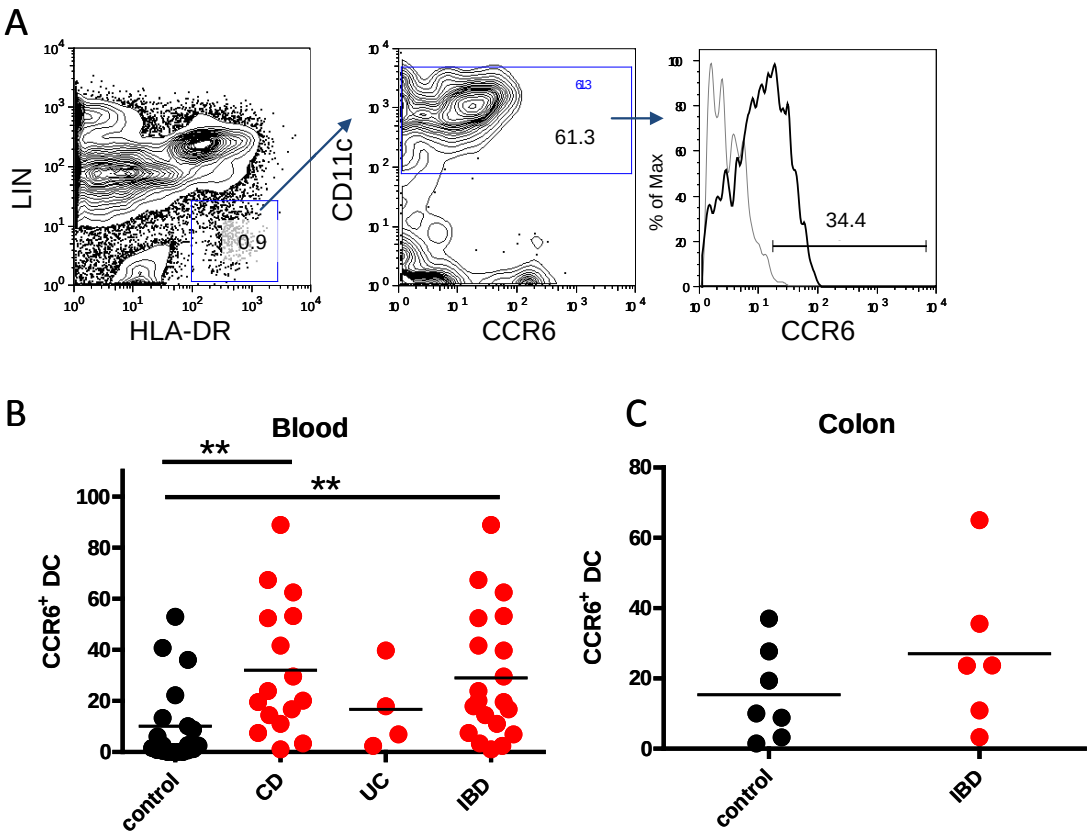

Figure 5.9 Circulating CCR6^{+} DC are increased in IBD

(A) Representative staining for CCR6^{+} DC. Cells were gated on the LIN^{-} HLADR^{+}CD11c^{high} cDC. Gates were set using the appropriate isotype control. (B) Frequency of CCR6^{+} cells amongst LIN^{-}HLADR^{+}CD11c^{high} cDC in the blood from controls (n=20), CD (n=16), UC (n=4) and IBD (UC+CD, n=20) patients. (C) Frequency of CCR6^{+} cells amongst LIN^{-}HLADR^{+}CD11c^{high} cDC in the colon from controls (n=7) and IBD (n=6) patients. **p<0.01

5.3 Conclusion

In this chapter we have presented our phenotypic analysis of DC subsets in the intestinal and systemic human immune responses.

We identified $CD103^+$ DC in the human non-inflamed intestine, where they represent around 20% of cDC. On the contrary, $CD103^+$ DC are virtually non-existent in the human PB, suggesting that $CD103^+$ DC may differentiate in the presence of gut micro-environmental factors, such as the presence of dietary factors or the intestinal microflora. In order to further characterize the function of different human DC subsets, we were able to sort $CD103^+$ and $CD103^-$ DC from LPMC. Isolation of DC populations has proven to be very challenging due to the very low frequencies of DC in the gut and only small numbers of cells could be obtained by FACS sorting. Nevertheless, this allowed us to perform initial studies on the expression of genes involved in regulatory and inflammatory pathways in the two cell compartments. Interestingly, we found a trend to a higher expression of genes involved in TGF-β activation, secretion and signalling and in RA conversion in $CD103^+$ DC in the absence of intestinal inflammation, in accordance with their more tolerogenic phenotype. On the contrary the expression of inflammatory genes, such as *CCR6*, *IL1B* and *TBX21* was found to be higher in the $CD103^-$ counterpart. These observations resemble what previously described in mice.

A complex network of DC subtypes involved in immunity and tolerance has emerged from murine studies. However, the translation of these findings into human studies and clinical applications has been hampered by the failure in identifying human DC subsets with phenotype and function similar to their murine counterpart [448]. The description of human intestinal $CD103^+$ DC, which resemble murine $CD103^+$ DC is very promising and may help us to understand the human DC network. Similarly, a

step forward in this translation has been represented by the recent identification of the human equivalent of murine CD8α$^+$ DC. CD8α$^+$ DC are characterized by the ability of cross-presenting exogenous antigens on MHC I, ingest debris from dead cells, produce IL12 and induce Th-1 responses thus representing an interesting target for vaccination and anti tumour strategies [449]. Disappointingly, DC expressing CD8 were not found in men. However, recent studies have identified a population of human DC expressing the blood DC antigen BDCA3 and the C-type lectin DNGR-1, which share many functional and phenotypical properties with murine CD8α$^+$ DC. These cells were found by different groups in the human spleen, blood and tonsils and equivalent DC were generated from cord blood stem cells, opening new exciting possibilities for the development of clinical applications [450-453].

We then evaluated if a modification in frequency or phenotype of CD103$^+$ DC was present in patients with IBD as previously suggested by our studies in murine models of colitis [429]. Strikingly, we observed a decreased frequency of CD103$^+$ DC in the inflamed intestine of patients with IBD compared to controls. Furthermore, the expression of regulatory genes appeared to be reduced in preliminary experiments in CD103$^+$ DC isolated from IBD patients, which on the contrary presented a more inflammatory gene expression profile. These data are intriguing and suggest that impaired DC-mediated regulatory mechanisms may play a role in the pathogenesis of IBD. It remains to be elucidated if CD103$^+$ DC isolated from the intestine of patients with IBD present reduced ability to induce Foxp3$^+$ Treg and whether they induce differentiation of inflammatory T-cell subsets such as Th-17 or Th-1 cells.

We also presented here some initial work on the characterization of inflammatory DC subsets in the human intestinal and systemic immune response. Interestingly, we observed increased frequencies of CCR6$^+$ DC in the PB of patients with IBD

compared to controls. No difference was observed in the colon, but more samples will need to be evaluated in order to confirm these preliminary results. CCR6 is expressed by murine E-cadherin$^+$ DC and CCR6$^+$ DC with an inflammatory phenotype have been described in murine infectious models [431]. Human immature BM-derived CD34$^+$ DC, immature monocyte-derived DC and Langerhans cells are also known to express CCR6 [454-456]. It has been suggested that CCR6$^+$ immature DC are recruited to inflamed mucosal sites through a CCR6-CCL20 interaction. Antigen uptake and exposure to inflammatory stimuli induce DC maturation, associated with CCR6 down-regulation and CCR7 induction. CCR7$^+$ DC can enter the lymphatic vessels and migrate to the local lymph nodes where they induce antigen-specific T-cell activation [457, 458]. The recruitment of CCR6$^+$ immature DC and DC precursors from the blood may participate to the induction and propagation of chronic intestinal inflammation in IBD and interfering with this chemokine-driven migration may represent a possible strategy for patient treatment.

Chapter 6- Discussion

6.1 Summary

The work presented in this thesis has required a thorough process of optimization of methods in order to establish an effective system for the study of human intestinal and systemic immune responses. This preliminary work has been instrumental for the achievement of our specific project aims and will represent a resource for the development of further human studies in our laboratory, with the aim of translating results from animal research into better understanding and enhanced treatment of human diseases.

In the present work, our interest has focused on the evaluation of the newly described IL23/IL17 pathway in the human intestinal and systemic immune response and on its contribution to chronic intestinal inflammation in patients with IBD. In particular, following our recent identification of pathogenic IL17 producing ILC in innate murine models of colitis, we wanted to investigate the role of IL23-responsive innate sources of IL17 in the pathogenesis of IBD [214]. Interestingly, we were able to identify IL23-responsive ILC populations in the human intestine and we observed accumulation of a specific subset of IL17 producing ILC in the inflamed intestine of patients with CD. These cells may participate to the initiation and propagation of inflammation through production of cytokines and recruitment of other inflammatory cells, therefore representing a novel promising target for treatment in a subset of patients with IBD.

As part of this study, we also aimed to characterize human intestinal and systemic DC populations. In particular, we wanted to evaluate if in the context of the intestinal microenvironment DC develop specific regulatory features, as observed in the

described $CD103^+$ DC subset in mice [79, 80]. Our results show that human intestinal DC populations indeed exhibit specific regulatory properties compared to their systemic counterpart, associated with their ability to regulate TGF-β and RA tissue-availability. Furthermore, we were able to identify a subset of $CD103^+$ DC in the human gut, which express TGF-β-related genes. Interestingly, our data also suggest that both reduced frequency of intestinal $CD103^+$ DC and alteration of their phenotype are present in the inflamed intestine of patients with IBD. A modification in DC subset composition and phenotype may result in a distort balance between immune effector and regulatory responses, promoting the development of intestinal inflammation.

6.2 Establishing a human translational project

Studies conducted in animal models of intestinal inflammation have greatly advanced our knowledge on the immunological pathogenesis of IBD. Work from our laboratory in T-cell dependent and independent murine models of colitis has allowed evaluating the role of adaptive and innate intestinal immune responses in intestinal inflammation. Nevertheless, in order to translate results from animal research into human disease, it appears fundamentally important to perform studies in human tissue, which ultimately represent an essential step in order to introduce changes in patient care and prognosis and alter disease progression. However, human studies have been hampered by the many limitations associated with low availability of tissue and poor experimental flexibility, together with frequently inadequate communication and collaboration between scientists and clinicians. These factors have resulted in an insurmountable gap between the growing amount of data originating from animal studies and their translation into enhanced treatment of human diseases.

Aim of this thesis was to translate findings from animal work into human pathophisiology. This has required the organisation of a multidisciplinary team work and the optimization of laboratory techniques (presented in Chapter 3).

A thorough process of optimization of human protocols was necessary to obtain efficient and reliable experimental tools for our studies. Isolation of haematopoietic cells from human intestinal tissue, MLN and blood was successfully achieved. Initially, LPMC were isolated from surgical specimens, which allowed us to obtain cell yields in accordance with what published in the literature. More recently, we have optimized a protocol for the isolation of LPMC from biopsy samples collected from endoscopy. We believe that biopsies represent a huge resource for human studies, for their easier accessibility compared to surgical specimens. In fact, more than 12000

procedures are performed each year in the endoscopy unit at the John Radcliffe Hospital, with various endoscopic sessions being held on a daily basis, representing a huge reservoir for intestinal tissue collection. Furthermore, recruitment of patients from endoscopy allows the evaluation of IBD patients with a much wider spectrum of disease activity than patients undergoing surgery. Endoscopic evaluation is performed on IBD patients at different stages of their disease. A first endoscopy is usually carried out at diagnosis on treatment naïve patients and then other examinations can be required during the course of the disease for assessment in the presence of clinical relapse, but also for surveillance in complete clinical remission. Furthermore, biopsies can also be obtained from subjects with a big range of other inflammatory and non-inflammatory intestinal disorders and even from healthy controls, such as patients undergoing endoscopy for CRC screening. This way, we are able to include different inflammatory and non-inflammatory control populations in our studies. We now have ethical approval to collect up to 10 biopsies from each individual which have proven to be adequate to get satisfactory cell yields to perform our experiments, such as cell phenotyping by FACS, gene expression analysis or cell cultures. As exposed in the result chapters, the characterization of human intestinal and systemic immune responses was achieved through the combination of various experimental techniques that required protocol optimization. We have now reliable FACS staining protocols, using up to 7 different colours in single experiments, to analyse immune cell populations, intracellular cytokine (IL17A and IFN-γ) and chemokine receptor (CCR6) expression. With these tools, we were able to phenotype the profile of αβ and γδ T-cell, B-cell, NK, ILC and DC populations from human blood, intestine and MLN. Furthermore, cell populations, such as T-cells, ILC and DC subsets, were successfully isolated from blood, intestine and MLN by FACS and MACS sorting and

analysed *ex vivo* or after *in vitro* culture in the presence of different stimuli. We have optimized the analysis of gene and protein expression of a vast range of molecules in the whole mucosal tissue and in sorted cell populations. Finally, ILC and DC populations could be visualized *in situ* by immunofluorescence analysis on frozen and paraffin-embedded tissue (work performed by Carolina Arancibia, Holm Uhligh and Ana Lena Schaupp). On this regard, we envisage that immunohistochemistry and immunofluorescence techniques for *in situ* analysis will represent a particularly useful tool for future studies to be conducted in the TGU, where we have access to a large archive of several thousands of paraffin-embedded samples from well characterized IBD patients and controls.

All together these methodologies allow a comprehensive evaluation of human systemic and tissue-specific immune responses in health and disease (Figure 6.1). Future studies would also greatly benefit of a comprehensive patient database, which is currently being created in the TGU. This will enable us to link experimental immunological data with patient clinical information in order to perform cross-sectional and longitudinal studies.

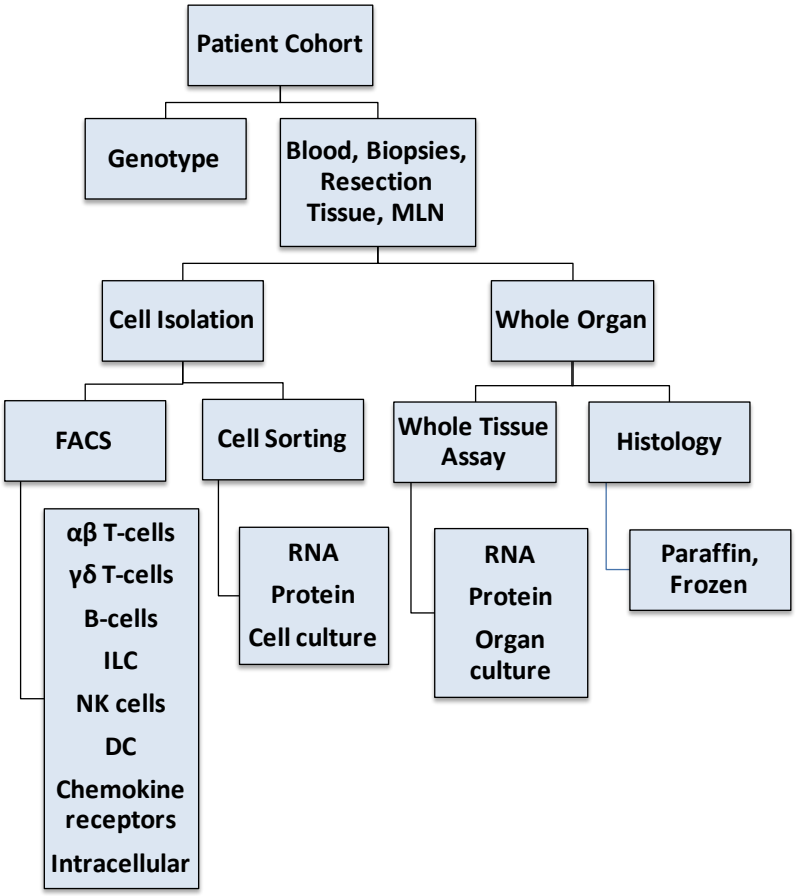

Figure 6.1 Oxford TGU capabilities for human studies in IBD

The Oxford IBD cohort includes over 2000 IBD patients. As part of the Wellcome Trust consortium, genotyping information is available for many IBD susceptibility SNPs in a proportion of patients. We currently have ethical approval for collection of blood, biopsies (up to 10 per patient), surgical intestinal specimens and MLN from IBD patients, uninflamed controls and inflammatory controls. Cells can be isolated from blood, intestine and MLN and FACS staining protocols for the characterization of different immune cell types have been optimized. Intracellular staining for cytokines such as IL17A and IFN-γ and the Foxp3 transcription factor are successfully performed. Specific cell subsets can be sorted by MACS and FACS and characterized *ex vivo* and after *in vitro* culture. Gene and protein expression of a vast range of molecules can be assessed in both isolated cells and whole tissue. We are also able to analyse the inflammatory infiltrate *in situ* on frozen and paraffin-embedded tissue by well established immunohistochemistry and immunofluorescence techniques.

6.3 The role of the IL23/IL17 pathway in IBD

Results deriving from both murine studies and recent GWAS strongly support a role for the IL23/IL17 axis in the pathogenesis of IBD [181, 205, 296, 300-304]. A main contribution of this thesis is represented by the evaluation of the IL23/IL17 pathway in the human intestinal and systemic immune response in the absence and presence of chronic intestinal inflammation (presented in Chapter 4).

Results from our laboratory and others have revealed that IL23 is the key cytokine driving intestinal inflammation in both adaptive and innate models of colitis [181, 205, 296, 300]. Furthermore, results from the anti-CD40 model have shown that intestinal inflammation is dependent on IL23, while the associated systemic disease depends on the related cytokine IL12 [204]. We aimed to investigate if tissue-specific compartmentalization of the IL23/IL17 pathway was also present in the human immune response. In accordance with this hypothesis, we observed induction of Th-17 genes in human intestinal cells, but not PBMC, after IL23 stimulation *in vitro*. As a readout of IL23 activity we have shown the induction of *IL22*, which appears to be tightly regulated by IL23. We also observed that Th-17 signature genes are over-expressed in LP compared to PB cells *ex vivo*, even in the absence of any inflammatory changes in the gut. We intended to analyse IL23R expression on human intestinal and blood cells by FACS, but in our hands staining with the mouse anti-human IL23R monoclonal antibody (clone 218213, R&D Systems, Abingdon, UK) did not give specific results. Future work should focus on optimizing the IL23R staining that would represent an extremely informative tool for the evaluation of this axis. Collectively, our findings confirm the presence of compartmentalization of the IL23/IL17 pathway to the human intestine and suggest that therapeutic strategies aiming to selectively inhibit this axis would result in lower occurrence of systemic

side-effects compared to conventional immunosuppressive treatments. The compartmentalization of the IL23 axis to the gut may be the result of tissue imprinting. Several factors, such as PGE2, TSLP, RA and TGF-β, have been implicated in the intestinal conditioning of the immune cells. Most studies have focused on the role of these factors in promoting intestinal immune tolerance in order to maintain homeostasis despite the exposure to a huge load of foreign antigens [76, 79, 80, 459, 460]. However, gut-conditioning molecules have also been implicated in the promotion of IL23 and Th-17 mediated responses. PGE2 has been shown to induce DC production of IL23 [212]. Furthermore, TGF-β, which is highly enriched in the mucosal tissue and is the main factor driving Treg differentiation in the periphery, can also induce the development of Th-17 cells in the presence of inflammatory cytokines, such as IL6, IL21 and IL1 [138-140, 159]. Finally, the intestinal microbiota itself can contribute to the induction of Th-17 responses in the gut. Bacteria are necessary for the development of intestinal Th-17 responses and specific components of the bacterial flora have been found to favour Th-17 differentiation [461].

In accordance with previous reports, we found increased mucosal expression of Th-17 genes in IBD patients compared to controls in our cohort [309, 405, 406]. In particular higher expression of *IL17A* and *IL17F* was found in IBD, while *IL26* appeared to be selectively increased in UC. IL17A and IL17F may contribute to intestinal inflammation and tissue damage through the induction of inflammatory cytokines, chemokines, G-CSF, GM-CSF and MMP [226-230]. However, the pathogenic role of these cytokines in IBD is controversial. In fact, IL17A can also promote intestinal barrier integrity through the induction of antimicrobial peptides [226]. Colitis is associated with IL17A over expression in murine models [181, 182, 204] and IL17A

has been shown to play a central pathogenic role in the *H. Hepaticus* innate model of colitis [214]. On the contrary, IL17A-production from T-cells appears to be dispensable for induction of intestinal inflammation in T-cell dependent colitis [300, 462]. IL17A was even found to play a protective role in the DSS-induced model of colitis, whilst IL17F was shown to be pathogenic [229]. Results from a double-blind, placebo-controlled, proof-of-concept study on the use of secukinumab (a monoclonal anti-IL17A antibody, AIN457) for treatment of moderate-severe CD clearly showed no efficacy of treatment *versus* placebo. In fact, administration of secukinumab appeared to exacerbate disease in a subset of patients with increased inflammatory markers [463]. A redundant role for IL17A and IL17F in intestinal inflammation has been suggested by results in animal models [464]. The outcome of this first clinical study may therefore reflect the selective inhibition of IL17A.

IL26 expression was increased in UC patients in our cohort, while a previous study had shown higher levels of *IL26* in CD [272]. A role for IL26 in UC pathogenesis is supported by the identification of a UC-specific susceptibility SNP in the *IL26* gene [465]. Similarly to IL17A and IL17F, IL26 can induce expression of pro-inflammatory cytokines, chemokines and adhesion molecules in IEC. Moreover, the inhibitory activity of IL26 on epithelial cell proliferation may impair the restitution of the intestinal epithelial barrier integrity. The described activities of IL26 has been suggested by *in vitro* experiments with human cell lines, however *in vivo* studies are lacking due to the absence of a murine homologue of IL26 [271, 272].

We found higher expression of *IL17A* and *IL17F* in sorted intestinal $CD3^-$ cells from patients with IBD, indicating a role for innate sources of these cytokines in the pathogenesis of the disease. This observation is in agreement with our data from the innate models of colitis, where higher levels of IL17A are present in the inflamed

colon in mice that completely lack T-cells [204, 214]. The pro-inflammatory activity of IL23 has been mainly linked to its effects on Th-17 cells. However, here we have shown that IL23 induces *IL17A* in intestinal $CD3^-$ cells isolated from patients with IBD. We were able to further characterize the IL23 dependent non-T source of IL17A and IL17F present in IBD, which is enriched in the $Lin^-CD45^+CD56^-$ intestinal cell compartment. On the contrary expression of other Th-17 cytokine genes, such as *IL22* and *IL26* was found in the $Lin^-CD45^+CD56^+$ counterpart. Higher frequencies of $CD56^-$ ILC were observed in the inflamed intestine of patients with CD compared to controls. This feature appears to be specific of CD patients, since no increase in $CD56^-$ ILC was found in patients with UC. All together, these data indicate that specific accumulation of a functionally distinct subset of $CD56^-$ ILC, that produces IL17 in response to IL23, is present in patients with CD.

There has been an explosion of interest in ILC as modulators of the immune response in various mouse models of tissue immunity. Sofia Buonocore from our group has recently described a population of Sca^+ $Thy1^+$ ILC that secrete IL17A, IFN-γ and IL22 in response to IL23 and are responsible for intestinal inflammation in the *H. hepaticus* and anti CD40-induced models of colitis [214]. Similarly, ILC populations that respond to IL23 and secrete IL22 have been identified in human intestinal adult tissue [259]. Here we describe the involvement of an ILC population in a human disease raising possibilities for the treatment of patients with inflammatory disorders. However, the functional relevance of such a small subset of cells in the pathogenesis of CD needs to be elucidated.

Further studies will be needed in order to characterize human intestinal ILC populations and evaluate their functional activity. For this purpose, *in vitro* expansion of intestinal $CD56^-$ and $CD56^+$ ILC might be desirable, due to the low frequencies of

these cells in the gut. Phenotypic analysis should include the expression of RORγ-t and IL23R on different ILC populations. RORγ-t expression is a common trait of IL-17 producing cells, from Th-17 cells to IL17-producing γδ-T cells, MAIT-cells and ILC and it induces the expression of the IL23R on Th-17 cells [56, 67, 141, 145, 259, 281-284]. We were able to detect gene transcripts for *RORC* and *IL23R* in both intestinal $Lin^{-}CD45^{+}CD56^{-}$ and $CD56^{+}$ sorted cells. In accordance with our mRNA data, both $CD56^{-}$ and $CD56^{+}$ LTi-like cells isolated from human tonsils were found to express RORγ-t by intracellular FACS staining [466]. However, as previously discussed, specific staining for RORγ-t was not achieved in this study and similarly we had unspecific results for the IL23R staining. Alternative approaches could also be explored, such as *in situ* analysis by immunofluorescence. $CD3^{-}CD127^{+}$RORγ-t^{+} cells have been recently identified by immunofluorescence in human and murine lymph nodes [466]. Tissue-staining would allow localization of ILC in different compartments of the intestinal mucosa, such as the LP and the specialized lymphoid structures as well as insight into their interaction with other immune cell types. We have speculated that through a CCR6-mediated mechanism, ILC might preferentially localize in ILF or PP. In fact CCL20, the main ligand for CCR6, is preferentially expressed on the FAE [408]. Interestingly, CCR6 expression is also shared by Th-17 cells and IL17-producing unconventional lymphocytes, such as γδ-T-cells, MAIT-cells and ILC populations, which are preferentially recruited at mucosal sites [51, 56, 67, 166, 259, 281] (Figure 6.1). The observation of a common RORγ-t/CCR6 axis shared by innate and adaptive sources of IL17 is particularly fascinating and suggests that these cells may be developmentally linked. In this light, the described innate lymphoid sources of IL17 may represent ancestral precursors of the adaptive Th17 response. The phenotypic characterization of intestinal ILC should also include

CD161, whose expression also appears to be linked to IL17 production and RORγ-t expression in human Th-17 and IL17-secreting unconventional T-cells [67, 163, 281, 467].

ILC might themselves play a role in promoting the formation of ILF, which are increased in the inflamed intestine in IBD, through the induction of adhesion molecules on mesenchymal cells. This possibility is suggested by the phenotypical similarities between $CD56^-$ ILC and human foetal LTi, which are the orchestrators of secondary lymphoid structure organisation during development [40, 288, 289]. However, further work is required to investigate if adult intestinal LTi-like ILC populations retain this functional activity. Co-culture of intestinal derived ILC with mesenchymal cells and analysis of adhesion molecule induction could be performed to address this question. The evaluation of ILC populations in children with IBD, who could be recruited in the TGU, would also provide invaluable insight into the role of ILC in lymphoid tissue development and organisation. Accumulation of ILC might be most marked in the intestine of children with IBD, where they may contribute to lymphoid tissue hyperplasia and to the development of aberrant intestinal immune responses. This scenario would suggest that therapeutic targeting of this pathway might be particularly indicated in treatment naïve patients and in early phases of the disease.

LTi cells have been shown to sustain memory T-cell survival in adult mice providing OX40 and CD30 signals [468]. Accordingly, a recent study has shown that LTi-like cells isolated from human tonsils and gut express high levels of OX40L and sustain memory T-cell survival *in vitro* [466]. The OX40-mediated signal on activated T-cells may represent another mechanism contributing to the perpetration of chronic

intestinal inflammation in IBD and further studies should include the analysis of OX40L expression in $CD56^-$ and $CD56^+$ ILC (Figure 6.1).

Our finding of a specific accumulation of ILC in the inflamed intestine in CD and not in UC is very intriguing. As already discussed in the introduction, inflammation differs largely in the two forms of IBD, in its localization along the gastrointestinal tube, its depth throughout the bowel wall and its evolution into fibrosis or fistulisation. The identification of a CD-specific feature appears therefore of particular interest since it could be related to a CD-specific disease manifestation. However, more samples will need to be analysed in order to confirm these preliminary data and to allow a comprehensive stratification of patients according to other variables, such as clinical activity, disease location, disease behaviour and concomitant treatments. The flexibility of ILC function in the intestinal immune response should also be investigated. The tissue innate lymphoid response may extend beyond Th-17-like responses as ILC have been shown to be a source of Th-2-type cytokines in response to different stimuli in both mice and men [291-293]. Recently, a population of Lin^- $CD127^+CRTH2$(chemoattractant receptor–homologous molecule expressed on T_H2 lymphocytes)$^+$ ILC, which secrete IL13 in response to IL25 and IL33, have been identified in the foetal gut. Similar cells are also present in the adult gut and lung and accumulate in the nasal polyps of patients with chronic rhinosinusitis [469]. While both Th-17 and Th-1 inflammatory responses have been described in patients with CD, Th-17 and non-typical Th-2 responses are associated with UC. It should be investigated whether ILC from UC patients respond to other stimuli (such as IL33, IL25, IL2 and TLR2 ligands) with increased production of IL13 or IL5. In particular, it would be interesting to analyse if $CRTH2^+$ ILC are increased in the intestine in UC.

Finally, it remains to be addressed how the genetic variants that have been described in the IL23/IL17 pathway can affect the IL23 response in both T-cells and non-T cells, such as ILC. The genetic variant R381Q in the IL23R has been associated with protection against IBD, while other polymorphisms in the IL23R have been shown to confer increased susceptibility [301]. Despite being identified and replicated in many different studies, the functional consequences of these polymorphisms remain to date unknown. In order to address this question, analysis of cytokine induction or signalling pathway activation by phospho-flow or phosphoproteomics could be performed after IL23 stimulation in both T-cell and ILC populations isolated from individuals carrying different genetic variants. These approaches would allow us to understand the impact of the IL23R susceptibility SNPs on the pathogenesis of IBD and other IBD- and IL23R polymorphism-associated diseases, such as psoriasis and ankilosing spondylitis [346, 347].

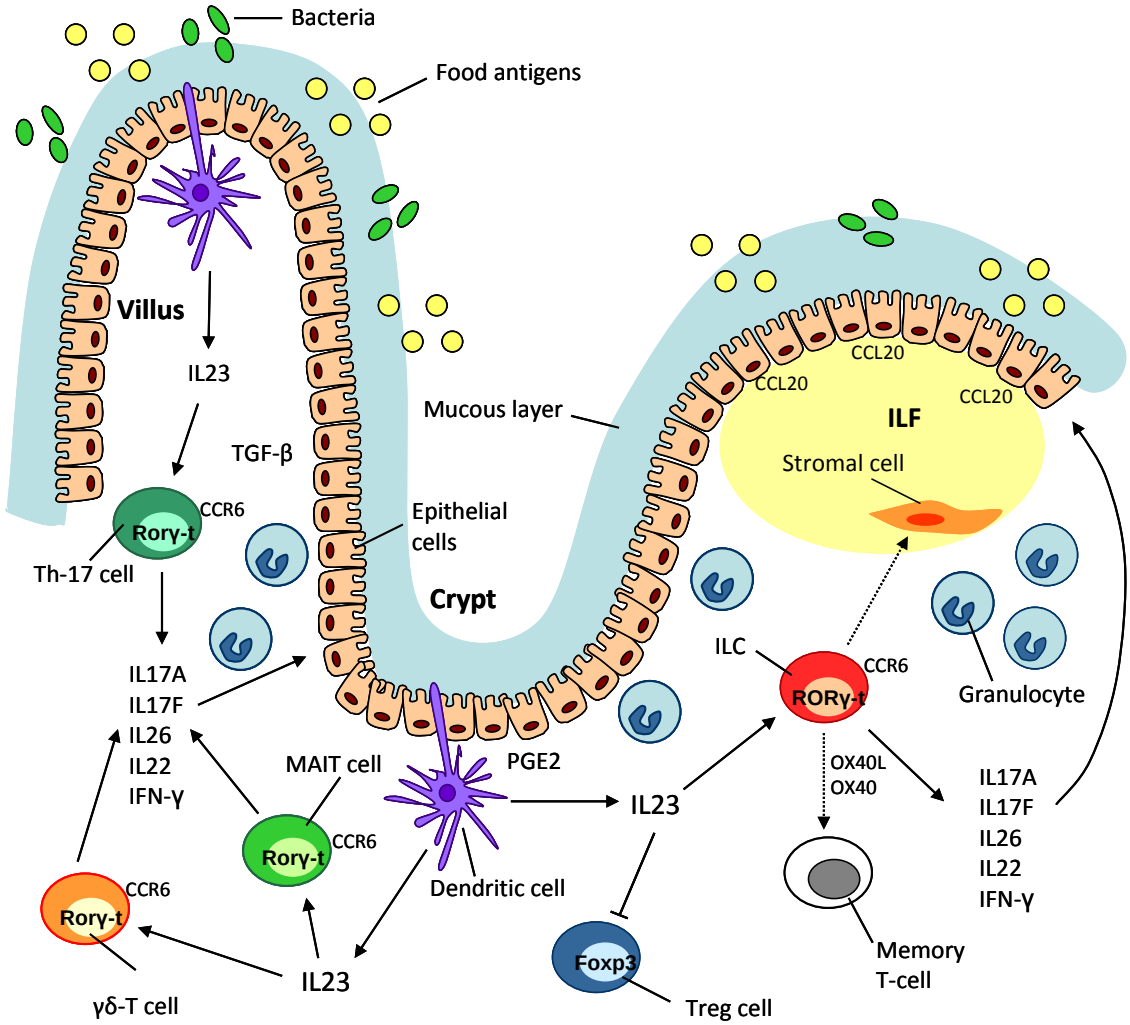

Figure 6.2 RORγ-t$^+$CCR6$^+$ cells accumulate in the gut and respond to IL23. RORγ-t$^+$CCR6$^+$ cells accumulate in the intestine where they are attracted by expression of CCL20, which is induced in presence of inflammation. After sampling of microbial antigens gut-conditioned DC are activated and secrete IL23, which acts on RORγ-t$^+$CCR6$^+$ cells. In response to IL23, Th-17 cells produce IL17A, IL17F and IL26, which induce expression of chemokines and adhesion molecules on IEC, leading to neutrophil recruitment. RORγ-t$^+$CCR6$^+$ unconventional T-cells, such as γδ-T-cells and MAIT-cells also respond to IL23 and secrete Th-17 signature cytokines. IL23 activity is necessary for IFN-γ secretion by Th-17 cells, which may contribute to their pathogenicity. On the other hand, IL23 restrains regulatory mechanisms, preventing the accumulation of Treg cells. RORγ-t$^+$CCR6$^+$ ILC secrete Th-17 cytokines and IFN-γ in response to IL23 and may participate to inflammation and tissue damage. IL22 is also produced by both Th-17 cells and ILC and may mediate protective mechanisms and IEC regeneration. Other potential pro-inflammatory activities of ILC include induction of adhesion molecules on stromal cells, resulting in ILF formation, and promotion of memory T-cell responses by OX40 stimulation.

6.4 DC regulatory and inflammatory subsets in IBD

Murine studies from our laboratory and others have highlighted the importance of environmental conditioning on DC phenotype and function [470]. In homeostasis DC expressing the integrin CD103 represent the majority of cDC in the murine intestine and MLN and they are characterized by the unique ability to induce expression of gut homing receptors, such as CCR9 and α4β7, on T-cells and to promote peripheral differentiation of $Foxp3^+$ Treg [77-80]. In murine models of colitis, we have shown that altered frequency and phenotype of intestinal regulatory and inflammatory DC subsets can contribute to intestinal inflammation, through their influence on the balance between Treg and T effector cell populations [83, 429]. Based on these emerging data from mice, we aimed to evaluate if subsets of DC with regulatory or inflammatory properties are present in the human systemic and intestinal immune responses and if a dysregulation in the DC pool composition or in DC function might contribute to the development of intestinal inflammation in patients with IBD.

In Chapter 5, we have presented our data on the characterization of regulatory and inflammatory DC subsets in the human intestinal and systemic immune response. Although still work in progress, this has provided us with some very promising preliminary results. The study of intestinal DC has proven to be very challenging due to the low frequency of these cells and their unstable phenotype, which hamper the isolation of DC subsets in order to evaluate their functional activity. This is reflected by most human studies that have been carried out on monocyte-derived DC, which may not reproduce tissue-specific cell functions. Here we have obtained successful isolation of intestinal and circulating cDC, defined as $LIN^-HLA\text{-}DR^+CD11c^{high}$ cells, which has allowed us to evaluate regulatory marker expression in the two compartments. Similarly to what observed in mice, human intestinal DC exhibited

distinct properties compared to PB DC, suggesting a role for environmental factors in conditioning the differentiation of gut-specific DC subsets. In particular, our analysis showed increased expression of TGF-β-related genes in intestinal DC compared to PB DC. TGF-β is a pleiotropic cytokine with known immune suppressive activity, but in the presence of other cofactors it can also induce inflammatory pathways, such as differentiation of Th-17 cells [138-140, 158-160].

Several factors have been shown to act in concert to condition intestinal DC populations. These include IEC-derived molecules, such as TSLP, TGF-β and RA [76, 86, 471]. However, intestinal macrophages, fibroblasts, Treg cells and $CD103^+$ DC themselves can also contribute to TGF-β secretion [472]. RA is a metabolite of Vitamin A (retinol), which is introduced with the diet and metabolized to retinal by alcohol dehydrogenases and to RA by the enzyme ALDH, which is preferentially expressed by $CD103^+$ DC [79]. Recent studies have shown that high concentrations of retinol are present in the bile and may play a role in driving small intestinal $CD103^+$ DC differentiation [473]. Other molecules that have been implicated in the imprinting of intestinal DC include IL4, GM-CSF and the peroxisome proliferator-activated receptor (PPAR)-γ lipid ligands [474, 475]. Finally microbial components, such as the polysaccharide A from the *Bacteroides fragilis* and the yeast wall component zymosan can also induce DC-mediated regulatory responses [476, 477] (Figure 6.3).

In accordance with other reports from the literature, we found increased protein levels of TGF-β in the inflamed intestine of patients with IBD compared to controls. However, this correlated with higher expression of latent TGF-β, with no difference in the active cytokine. Furthermore, PAI-1, an inhibitor of TGF-β activation by PLAT, was also increased in the inflamed intestine, indicating that a defect of TGF-β activation might be present in IBD. These observations together with previous reports

of reduced TGF-β signalling due to SMAD7 over-expression suggest that a defect in TGF-β activity might contribute to the development of chronic intestinal inflammation in IBD [441]. Murine studies have shown that TGF-β is required for suppression of T-cell induced colitis by Treg cells. Treg-mediated protection is reversed by administration of anti TGF-β antibodies and naïve $CD4^+$ T-cells that express a dominant negative mutant of TGF-βRII and cannot respond to TGF-β, escape suppression by Tregs [478, 479]. Whether Tregs represent the source of TGF-β remains controversial. TGF-$\beta^{-/-}$ Tregs were shown to still suppress T-cell transfer-induced colitis, when the recipient is TGF-β competent, suggesting that other sources of TGF-β may contribute to Treg mediated suppression [479]. However, in another study Treg cells isolated from mice with T-cell specific deletion of TGF-β were shown to be incapable of suppressing colitis [480]. Li and Flavell have proposed a "three cell" model of TGF-β-mediated regulation, which involves Treg cells, DC and naïve T cells. After antigen-presentation by DC, Treg cells produce latent TGF-β, which is then activated by the αvβ8 integrin expressed on DC and regulates activation and differentiation of naïve T-cells [481]. Conversely, Treg cells may also participate to TGF-β activation or induce TGF-β production or activation by other cell-sources. In this respect, it would be interesting to study the interactions between DC and Treg cells and their role in the activation of TGF-β in health and IBD. These questions could be addressed by *in vitro* co-culture of Treg and DC populations isolated from different immune compartments and from IBD patients and controls.

Consistent with their more tolerogenic phenotype, DC isolated from the human colon also presented higher transcript levels of *CD103* compared to their PB counterpart. Accordingly, $CD103^+$ DC could be identified by FACS amongst intestinal cells, where they accounted for around 20% of cDC in uninflamed individuals, and they

could also be visualized in the LP by immunofluorescence. On the contrary, CD103 expressing DC were not found in the human blood, representing a distinctive feature of the mucosal immune response. We were able to FACS-sort intestinal DC subsets on the base of CD103 expression and analyse the expression of regulatory and inflammatory markers in the two populations. In initial experiments, $CD103^+$ DC expressed higher transcript levels of TGF-β- and RA-related genes than their $CD103^-$ counterpart and this suggested they might exert regulatory properties in men as well as in mice. Interestingly, we observed a reduction in frequency of $CD103^+$ DC in the intestine of patients with IBD. This observation may reflect either the recruitment of $CD103^-$ DC or the downregulation of CD103 by intestinal DC in IBD. Furthermore, our preliminary data show that $CD103^+$ DC isolated from IBD patients may present a reduced expression of genes involved in TGF-β regulation and RA activation and increased expression of pro-inflammatory genes. It remains to be elucidated if intestinal $CD103^+$DC change their phenotype in presence of inflammation or whether inflammatory DC are recruited to the intestine where they upregulate CD103. These data support our hypothesis that an altered frequency and function of intestinal $CD103^+$ DC might be involved in the pathogenesis of chronic intestinal inflammation in patients with IBD. (Figure 6.3). Further work will be necessary to assess the functional activity of human intestinal $CD103^+$ DC and their ability to imprint T-cell function. It has been shown that $CD103^+$ DC isolated from human MLN can induce expression of gut-homing receptors on T-cells and differentiation of Treg [78, 86]. CD103 expression and the ability to induce Treg were promoted in monocyte-derived DC by conditioning with human IEC supernatants. Interestingly, incubation with IEC isolated from patients with CD resulted in reduced Treg differentiation [86]. However it remains to be elucidated whether $CD103^+$ DC isolated from the inflamed intestine

of patients with IBD present an impaired ability to induce differentiation of Treg. This question could be addressed *in vitro* by co-culturing sorted intestinal $CD103^+$ DC isolated from IBD patients or controls, with naïve PB T-cells from normal subjects (to exclude any alteration due to the responding T-cell population), in the presence of anti-CD3 activation. This approach would also allow evaluating if DC from IBD patients acquire the capacity to induce differentiation of T effector cell subsets, such as Th-17 cells. We predict that a dysregulation in $CD103^+$ DC function could determine an altered balance between regulatory and effector responses.

We have also presented the initial characterization of intestinal inflammatory DC subsets in the human immune response. Work from our laboratory has described a murine population of E-cadherin$^+$ DC, which are characterized by expression of pro-inflammatory genes and exacerbate intestinal inflammation in models of colitis [83]. Interestingly, we observed higher frequencies of circulating $CCR6^+$ DC in patients with IBD compared to controls. CCR6 had been previously shown to be expressed by inflammatory DC subsets in mice [83, 432]. However, our analysis showed no difference in CCR6 expression on intestinal DC between patients and controls. CCR6 is a chemokine receptor expressed by many immune cell types, including majority of B-cells, $CD4^+$ and $CD8^+$ T-cell subsets, LTi cells and DC populations [166, 454, 482-486]. In fact, it has been reported that pDC, Langerhans cells, immature BM-derived and monocyte-derived DC express CCR6 [456, 458, 487-489]. In the latter, CCR6 expression requires the presence of TGF-β together with IL4 and GM-CSF, indicating a role for TGF-β in maintaining an immature DC phenotype. CCL20, the main ligand for CCR6, is constitutively expressed in the GALT and can be induced on IEC in the presence of inflammation and infection. A CCR6-CCL20 mediated mechanism is thought to participate to the migration of immature DC to mucosal sites. After antigen

uptake $CCR6^+$ DC undergo maturation, which is associated with down-regulation of CCR6 and up-regulation of CCR7. $CCR7^+$ DC can then migrate to the local draining lymph nodes and present antigen to T-cells [457]. We could speculate that the higher frequency of $CCR6^+$ circulating DC in patients with IBD may reflect an increased release of monocyte-derived immature DC from the BM. $CCR6^+$ DC would then be recruited to the inflamed intestine, where upon maturation they would down-regulate CCR6 (Figure 6.3). This would be reflected in our analysis by the absence of any increase in $CCR6^+$ DC in the intestinal tissue in IBD. However, further studies will be necessary to elucidate the role and functional activity of circulating $CCR6^+$ DC in patients with IBD. In particular, their relationship to inflammatory monocyte populations will need to be investigated.

For their central role in both first-lines innate immunity and initiation of adaptive immune responses DC represent strong suspects in orchestrating the aberrant intestinal immune response responsible for IBD. Therefore, studies aiming to characterize features and function of distinct human DC subsets have the potential of improving our understanding of this chronic inflammatory disorder and may lead to the identification of novel therapeutic targets for the treatment of patients.

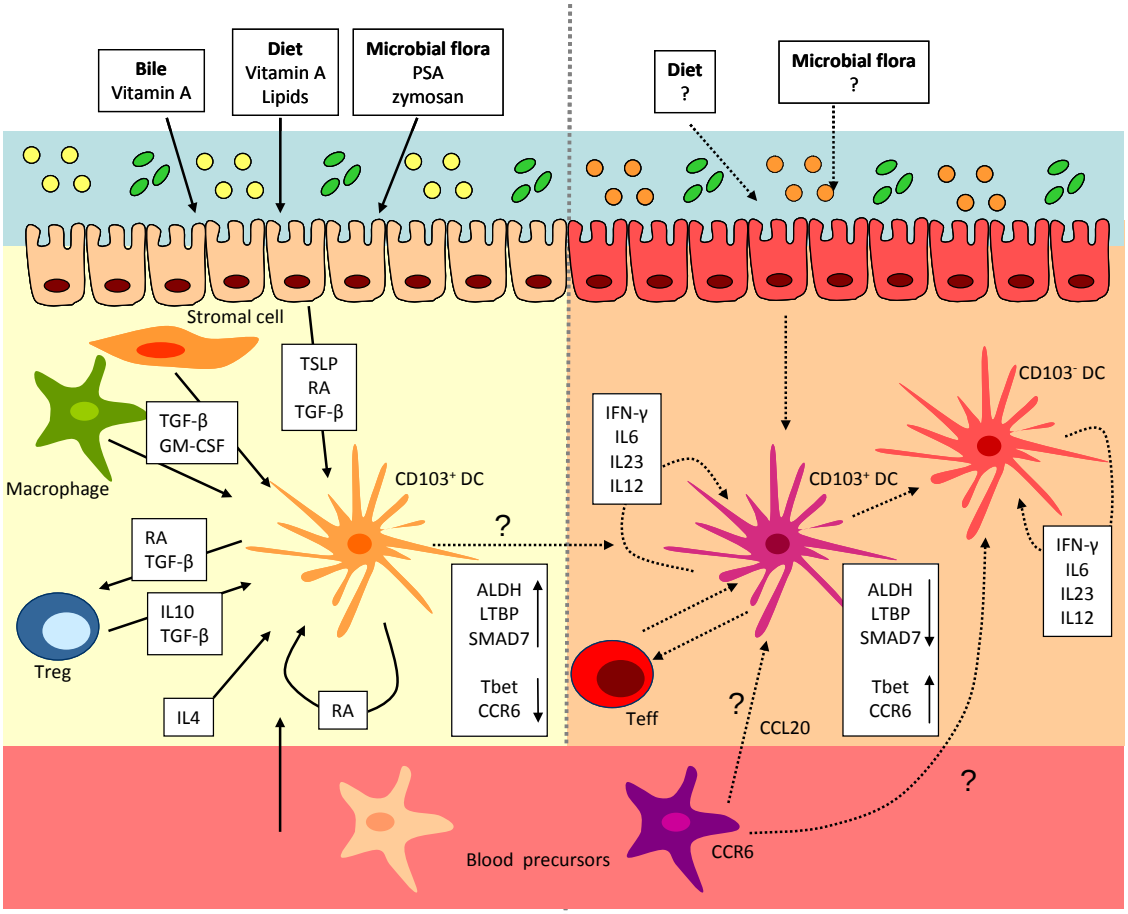

Figure 6.3 Altered frequency and function of intestinal DC may contribute to intestinal inflammation in IBD

DC precursors continuously migrate from the blood to the intestine. In homeostasis (left) several conditioning factors induce the differentiation of regulatory CD103$^+$ DC, which promote tolerance *versus* the plethora of harmless antigens deriving from both resident commensal flora and food components. The tolerogenic phenotype of CD103$^+$ DC is characterized by over-expression of genes involved in regulation of TGF-β and RA and low expression of pro-inflammatory genes. In IBD (right) lower frequency of CD103$^+$ DC are found in the inflamed intestine, which also show pro-inflammatory features, such as induction of inflammatory genes and reduced transcript levels of TGF-β- and RA-related molecules. The reduced frequency of CD103$^+$ DC in IBD may result from loss of CD103 expression on DC or recruitment of CD103$^-$ DC from blood precursors. The inflammatory milieu may be responsible for the phenotypic change of CD103$^+$ DC in IBD. Alternatively, a distinct population of inflammatory DC may be recruited to the intestine, where they upregulate the expression of CD103.

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