Phenotypic and functional characterization of macrophages in the normal and inflamed human gut

by

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Statement of originality

I, Francesca Ammoscato, confirm that the research included within this thesis is my own work or that where it has been carried out in collaboration with, or supported by others, that this is duly acknowledged below and my contribution indicated.

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Details of collaboration

I declare that the work presented in this thesis is my own, with the exception of Chapter 6 where the experiments have been performed in collaboration with Aneta Kucik and the statistical analysis of the microarray was performed at GlaxoSmithKline (GSK, Stevenage) by Catriona Sharp under the supervision of Dr. Laurens Kruidenier.

Abstract

Intestinal macrophages in the lamina propria underlying the gut epithelium play an important role in gut homeostasis. They remove apoptotic cells and cellular debris and phagocytose and destroy invading pathogens and intestinal microbiota crossing the epithelium. Infiltrating macrophages also play an important role in the pathogenesis of inflammatory bowel disease (IBD) by producing pro-inflammatory molecules such as TNF-alpha and metallo-elastase.

In this work I developed a new method to isolate macrophages from human lamina propria mononuclear cells (LPMCs) using CD33 magnetic beads. I then carried out functional and phenotypic studies of these cells isolated from the lamina propria of control samples and from patients with either Crohn's disease or ulcerative colitis.

The use of additional marker CD14 enabled me to distinguish two populations of macrophages, namely $CD33^{+}CD14^{-}$ LPMCs, the main intestinal macrophage population in the normal colon and $CD33^{+}CD14^{+}$ LPMCs, predominant in the inflamed mucosa.

Intestinal macrophages were also characterized for their expression of CD68, CD206 and CD64. $CD33^{+}CD14^{-}$ LPMCs and $CD33^{+}CD14^{+}$ LPMCs expressed CD68, the gold standard macrophage marker.

CD206 is a macrophage mannose receptor and it is associated with a M2 macrophages phenotype. The numbers of $CD206^{+}$ cells was higher in $CD33^{+}CD14^{+}$ cells of control and CD samples and to a lesser extent in $CD33^{+}CD14^{+}$ cells from UC patients.

CD64 is the high affinity Fcgamma type I receptor (FcγRI) used to discriminates macrophages from dendritic cells. The numbers of $CD64^{+}$ cells were markedly increased in the $CD33^{+}CD14^{+}$ cells of IBD mucosa and control subjects compared to $CD33^{+}CD14^{-}$ cells.

The ability of different macrophage subsets to make TNF-α was also studied. The number of $CD33^{+}CD14^{+}$ TNF-α^{+} LPMCs was significantly increased in IBD patients compared to control subjects. Interestingly, using CD206, I found that the majority of $CD33^{+}CD14^{+}CD206^{+}$ cells in IBD were TNFα^{+}. Moreover $CD33^{+}CD64^{+}$ LPMCs produced significantly more TNF-α compared to $CD33^{+}CD64^{-}$ LPMCs which probably represent DCs.

Since TNF-α is first produced as a transmembrane molecule and then cleaved into soluble TNF-α by the ADAM17/10 proteases, CD33 LPMCs were cultured with ADAM17/10 inhibitor, GW280264X, which induced a significant increase in transmembrane TNF-α and reduced release of soluble TNF-α. Moreover reduced production of soluble TNF-α and soluble IL-6R where found when biopsies of UC and CD patients were cultured with GW280264X.

Finally, gene arrays on CD33 gut macrophages isolated from IBD patients and control subjects showed a dysregulated immune gene profile. IL-24 transcripts and protein were increased in IBD macrophages and when exogenous IL-24 was added to IBD mucosal biopsies cultured ex vivo there was an increase in promoted TNF-α release.

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I dedicate this thesis to my Angel,
Angela Cappiello

Table of contents

List of tables

List of figures

List of abbreviations

7-AAD	7-amino-actinomycin D
APC	Antigen presenting cells
CSF	Colony stimulating factor
DMSO	Dimethyl sulfoxide
DC	Dendritic cell
DSS	Dextran sulphate sodium
EDTA	ethylenediaminetetraacetic acid *
ELISA	Enzyme-linked immunosorbent assay
FACS	fluorescence activated cell sorting
FSC	forward scatter
GALT	gastrointestinal associated lymphoid tissue
GM-CSF	granulocyte macrophage colony-stimulating factor
HBSS	Hanks's balanced salt solution
IBD	Inflammatory bowel disease
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IRAK	IL1 receptor-associated kinase
IRF	interferon regulatory factor
LP	lamina propria
LPMCs	lamina propria mononuclear cells

LPS	lipopolysaccharide
M1	classically activated macrophage
M2	alternatively activated macrophage
MAPK	mitogen-activated protein kinase
MFI	Mean flow intensity
MHC	major histocompatibility complex
MLN	mesenteric lymph node
MP	mononuclear phagocyte
MPS	mononuclear phagocyte system
MyD88	myeloid differentiation factor 88
NADPH	nicotinamide adenine dinucleotide phosphate-oxidase
NK	natural killer
NLR	Nod-like receptor
NO	Nitric oxide
NOD	Nucleotide-binding oligomerization domain
PAMP	pathogen associated molecular pattern
PBMCs	peripheral blood monocytes cells
PBS	phosphate buffered solution
PCR	polymerase chain reaction
PMA	phorbol 12-myristate 13-acetate
PP	Peyer’s patches
PPAR	peroxisome proliferator-activated receptor
PRR	pattern recognition receptor
RPMI	Roswell Park Memorial Institute-1640 medium

RT-PCR	reverse transcriptase polymerase chain reaction
SD	standard deviation
SSC	side scatter
STAT	signal transducer and activator of transcription
TAM	tumour-associated macrophage
TGF	transforming growth factor
TH1	T helper 1
TH2	T helper 2
TH17	T helper 17
TLRs	Toll-like receptors
TNF	tumour necrosis factor
TRAF	TNF receptor activated factor
TREM	triggering receptor expressed by myeloid cells

CHAPTER 1

General Introduction

1.1 Immune system

1.1.1 The gut mucosal innate immune system

The mucosal immune system is an integrated network of tissues and cells which protects the host from infections and environmental insults at the surfaces of the body. The crosstalk between various cell types of the innate and adaptive immune response, as well as the combination of physical, biochemical and mechanical mechanisms all contribute to the maintenance of immune homeostasis. With regards to the human gastrointestinal (GI) tract, the mucosal immune system is responsible for maintaining tolerance to the wide array of innocuous antigens in food, for maintaining a symbiotic relationship with the microbiota, and for providing the protective inflammatory responses which protect against enteric pathogens. A breakdown in intestinal immune homeostasis may lead to the onset of both food hypersensitivity and gut inflammation.

The key components of the gut mucosal immune system are the various cell types in the epithelial layer and the immune cells populating the lamina propria, Peyer's patches (PP), isolated lymphoid folicles and the mesenteric lymph nodes (MLNs). It is now widely appreciated that central players in shaping the features of the mucosal immune system are the microbial species inhabiting the intestinal lumen and the mucus layer (Kamdar et al., 2013). Indeed, the human GI tract is colonized by a diverse microbial population, consisting of approximate 100 trillion micro-organisms, including bacteria, fungi and viruses (Hooper and Gordon, 2001). The microbial component including luminal or mucosal-associated commensal bacteria is composed of 6–10 *phyla* and approximately 5,000 distinct species (Carvalho et al., 2012). The density of the luminal

microbes increases along the gastrointestinal tract, reaching up to 10^{12} bacteria per gram of luminal content in the colon (MacDonald et al., 2011). The mucus layer, together with anti-microbial peptides such as α-defensins released by Paneth cells, collectively forms the glycocalyx which traps invading micro-organisms and enables their expulsion. This process is facilitated by peristaltic movement.

Below the glycocalyx, the first line of interaction with the environment is represented by the intestinal epithelium (Figure 1.1). This includes several cell types, including absorptive enterocytes, goblet cells which besides secreting mucus also produce trefoil peptides contributing to both barrier function and mucosal repair (Mashimo et al., 1996), enteroendocrine cells and Paneth cells. In addition to defensins and other anti-microbial peptides, Paneth cells secrete enzymes such as lysozyme and phospholipase A2, that can break down the cell walls of micro-organisms (Salzman et al., 2007).

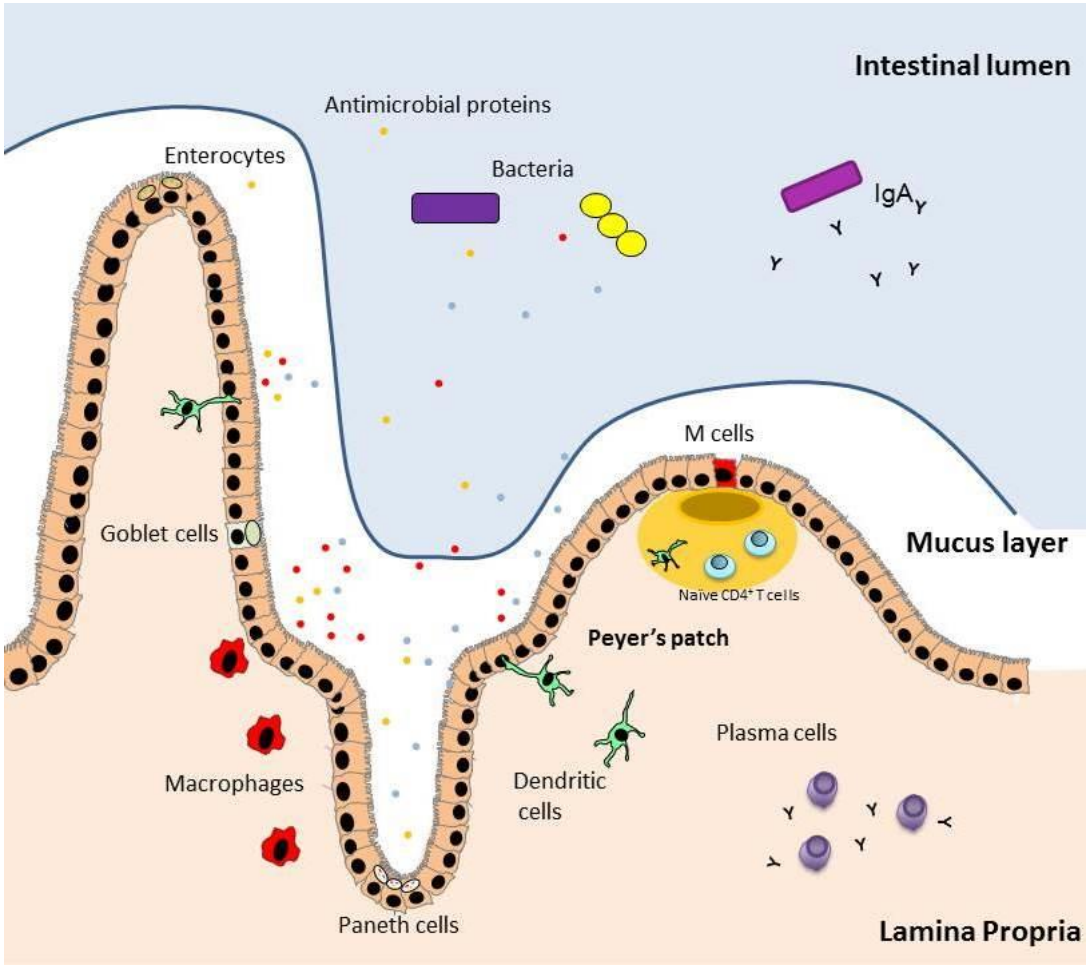

Figure 1.1: Schematic representation of the gut epithelial barrier: the intestinal lumen is inhabited by a diverse microbial population, consisting of approximate 100 trillion micro-organisms, including bacteria, fungi and viruses The mucus layer, together with anti-microbial peptides such as α-defensins released by Paneth cells, forms the glycocalyx which traps invading micro-organisms and enables their expulsion. The lamina propria lamina propria contain a large number of immunoglobulin A (IgA)+ plasma cells, T and B cells, macrophages, DCs. Lamina-propria DCs take up antigens from the lumen and present them directly to T cells and B cells, which can induce IgA class-switching and differentiation in situ. Secreted IgA is transported across the epithelium, where it serves as a first line of defence against pathogens and for the maintenance of gut-flora homeostasis.

The epithelium forms a physical barrier which is impermeable to most hydrophilic solutes in the absence of specific transporters. The function of tightly sealing the paracellular pathway between cells is mediated by the apical junctional complex, localized immediately below the microvilli of adjacent epithelial cells, by the adherens junctions and by the desmosomes (Turner, 2009).

Both tight and adherens junctions are supported by a dense peri-junctional ring of actin and myosin that can regulate barrier function. The adherens junctions, along with desmosomes, provide the strong adhesive bonds that maintain cellular proximity and are also a site of intercellular communication. Adherens junctions are composed of cadherins, a family of transmembrane proteins that form strong, homotypic interactions with molecules on adjacent cells. The cytoplasmic tail of the epithelial cadherin, E-cadherin (also known as cadherin-1), interacts directly with catenin δ1 (also known as p120 catenin) and β-catenin. In turn, β-catenin binds to α-catenin 1, which regulates local actin assembly and contributes to development of the peri-junctional ring of actin and myosin. Adherens junctions are required for the assembly of tight junctions, which in turn are multi-protein complexes composed of transmembrane, scaffolding and regulatory molecules that include kinases. Among the transmembrane proteins, claudins are the most important and are expressed in a tissue-specific manner. Occludin, another transmembrane protein of the tight junction, interacts directly with claudins and actin, but its role is less well understood. Amongst peripheral membrane proteins, zonula occludens (Zo)1 and Zo2 are crucial for the assembly and maintenance of tight junctions, at least in part as they contain multiple domains for the interaction with claudins, occludin and actin. Tight junctions limit solute flux along the paracellular pathway, which is typically more permeable than the transcellular pathway, therefore

they are the principal determinants of transepithelial transport and mucosal permeability (Turner, 2009).

Besides its mechanic action of defence, the epithelium has the important immunologic function of transporting immunoglobulin (Ig)A into the lumen using the polymeric Ig receptor, and can also produce anti-microbial peptides, cytokines and chemokines in response to bacterial and viral invasion. Epithelial cells express a specific class of proteins, namely the pattern recognition receptors (PRRs), specialized in the interaction with conserved microbial products structures commonly referred to as pathogen-associated molecular patterns (PAMPs). PRRs comprise a group of transmembrane proteins, the toll-like receptors (TLRs), and a class of intracellular proteins, the nucleotide-binding oligomerisation domain (NOD)-like receptors (NLRs), which play a key role in microbial recognition and subsequent induction of anti-microbial genes (Medzhitov and Janeway, 2002) and ultimately in the control of adaptive immune responses towards commensal and pathogenic bacteria. In particular, mammalian TLRs comprise a family of 11 individual type I transmembrane receptors which are characterised by three common structural features: a divergent ligand-binding extracellular domain with leucine-rich repeats (LRRs), a short transmembrane region, and a highly homologous cytoplasmic Toll/interleukin (IL)-1 receptor (TIR) domain, similar to that of the IL-1 receptor family and essential for initiation of downstream signalling cascades (Xu et al., 2000). TLRs are differentially (inducibly or constitutively) expressed by many distinct cell types throughout the whole GI tract, including mature and immature epithelial cells of the stomach, small intestine and colon, as well as intestinal lamina propria monocytes/macrophages (Cario et al., 2000), dendritic cells (DCs), myofibroblasts, endothelial cells and adipocytes.

Several PAMPs selectively activate specific PRRs. TLR2, for example, recognises bacterial lipopeptides and lipoteichoic acid, which are found abundantly in the cell wall of Gram-positive bacteria. TLR3 is activated by double-stranded RNA. TLR4 is the major receptor for lipopolysaccharide (LPS), however the interaction is not direct but occurs after the formation of a complex between LPS, serum LPS binding protein (LBP) and CD14 on the cell surface, which in turn delivers LPS to MD2 (Akira et al., 2006). TLR5 recognizes flagellin and flagellated bacteria, TLR7 interacts with ‘‘antisense’’ single-stranded viruses, TLR9 recognizes methylated CpG-DNA found in prokaryotic genomes and DNA viruses, and TLR11 is activated by pathogenic bacteria in the urinary tract (Cario, 2005). The NOD family comprises more than 20 different mammalian proteins which mostly contain three distinct functional domains: a carboxy-terminal LRR domain which mediates ligand recognition, a centrally located nucleotide binding domain (NBD), and a structurally variable amino-terminal effector-binding domain which consists of protein-protein interaction domains, such as caspase recruitment domains (CARDs) or pyrin domains (Inohara and Nuñez, 2003). Recent research has mostly focused on two cytosolic receptors of this family, NOD1 (also designated as CARD4) and NOD2 (also designated as CARD15), which both play a major role in intestinal regulation of pro-inflammatory signalling through nuclear factor TIR activation in response to distinct bacterial ligands. NOD2 shares significant homology with NOD1, but contains two, instead of one CARD domains at its amino-terminal end. NOD2 has been shown to be constitutively or inducibly expressed in monocytes, macrophages, DCs, T cells and B cells (Pauleau and Murray, 2003). It is also highly expressed in Paneth cells (Keshav, 2006).

TLR signalling is thought to occur through a series of homo- and heterotypic interactions between Toll/interleukin (IL)-1 receptor proteins (TIRs) to promote oligomerisation with the appropriate TIR-containing adaptor proteins: Myeloid differentiation protein 88 (MyD88), MyD88 adaptor-like (Mal), TIR domain-containing adaptor inducing interferon-β (IFN-β) (TRIF), and TRIF-related adaptor molecule (TRAM) (Oshiumi et al., 2003).

After ligand binding, the TLRs dimerize and undergo conformational changes required for the recruitment of TIR domain-containing adaptor molecules to the TIR domain of the TLR. MyD88 is critical for the signalling of most of the TLRs. Upon stimulation, MyD88 associates with the cytoplasmic portion of TLRs and then recruits IL-1R-associated kinase 4 (IRAK-4) and IRAK-1 through a homophilic interaction of the death domains (Figure 1.2) (Li et al., 2002). IRAK-4 then phosphorylates IRAK-1 which together with tumor necrosis factor (TNF)-α receptor associated factor-6 (TRAF6), dissociates from the receptor and then TRAF6 interacts with transforming growth factor (TGF)-β-activated kinase 1 (TAK1), TAB1, and TAB2. The complex of TRAF6, TAK1, TAB1, and TAB2 further forms a larger complex which induces the activation of TAK1. Activated TAK1 phosphorylates the IKK complex, consisting of IKKα, IKKβ, and NEMO/IKKγ, and MAP kinases, such as JNK, thereby inducing the activation of the transcription factors NF-kB and AP-1, respectively (Takeda and Akira, 2004).

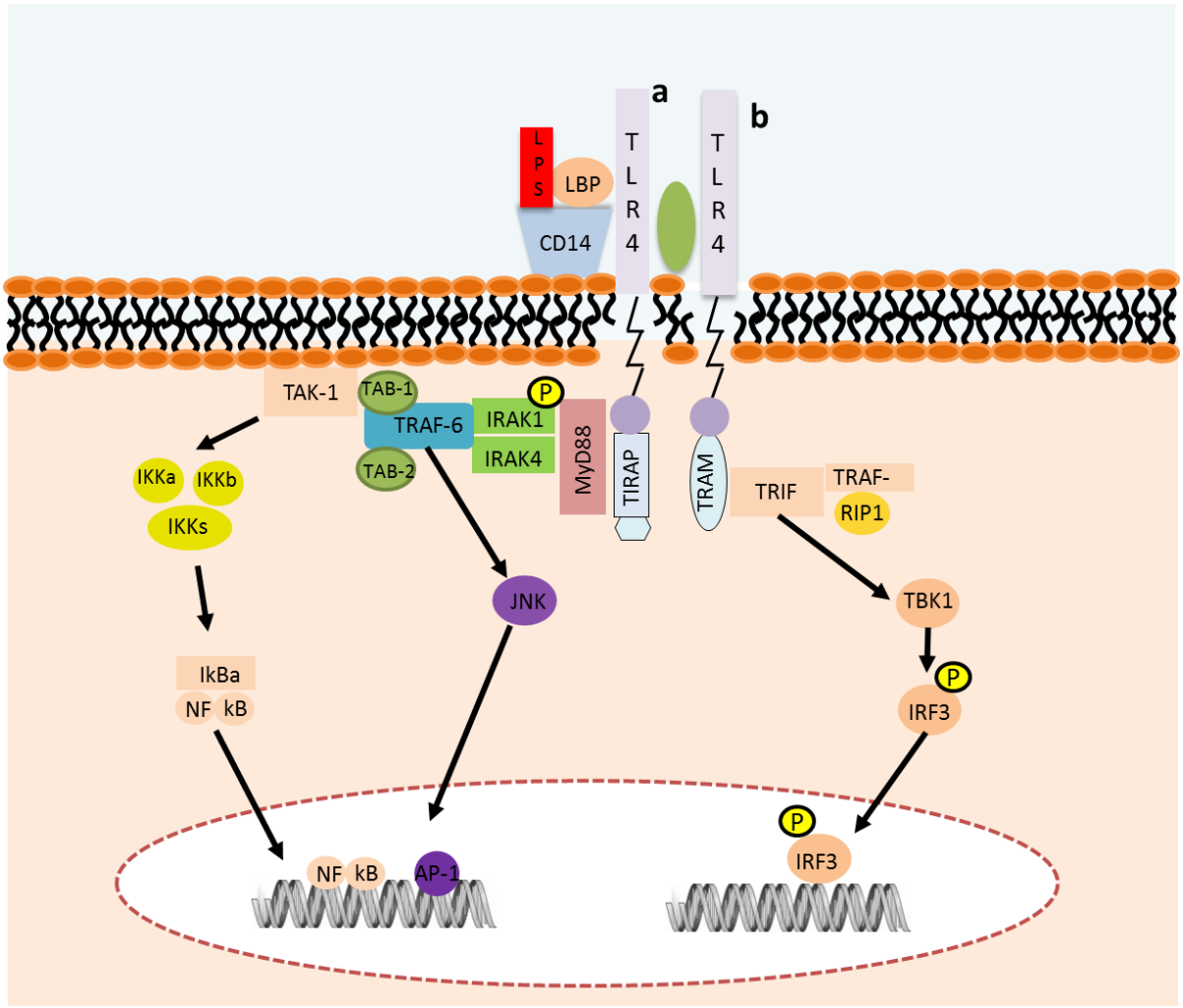

Figure 1.2:TLR4 signaling pathway. In the MyD88 dependent signalling pathway upon TLR4 stimulation with his ligands MyD88 is recruited and associate with the cytoplasmic portion of TLRs and then recruits IRAK-4 which then phosphorylate IRAK-1 and together with tumor necrosis factor (TRAF6), dissociates from TRAF6 interacts with TAK1, TAB1, and TAB2. The complex of TRAF6, TAK1, TAB1, and TAB2 further forms a larger complex which induces the activation of TAK1 which phosphorylates the IKK complex inducing the activation of the transcription factors NF-kB and AP-1, respectively resulting in the induction of proinflammatory cytokine genes. In the alternatively MyD88 independent TL4 signalling pathway, TRIF interacts with RIP1 and TRAF-6 which activate TBK1 that phosphorylate IRF3 that is responsible for the activation of NF-kB.

In the MyD88-independent TLR4 signalling pathway TRIF interacts with receptor-interacting protein 1 (RIP1), which is responsible for the activation of NF-kB (Meylan et al., 2004). On the other hand, TRIF activates TRAF family-member-associated NF-KB activator (TANK) binding kinase 1 (TBK1; also known as NAK or T2K) via TRAF3. TBK1 then phosphorylates IRF3 which translocate into the nucleus, and binds to the ISREs, resulting in the expression of a set of IFN-inducible genes (Doyle et al., 2002; Hoshino et al., 2002).

1.1.2 Macrophages and mononuclear phagocyte system

Macrophages were first described by Elie Metchnikoff in the 19th century as cells that, as part of mammalian immunity, guard the body and ingest and destroy foreign material. Macrophages exhibit variable morphology, express non-specific esterase, lysosomal hydrolases and ecto-enzymes, and contribute to non-specific uptake of particulate material (Hume, 2006). They also express a wide array of receptors for Ig Fc and complement components. Macrophages are highly efficient phagocytes, and play a central role in the maintenance of tissue homeostasis and wound healing through the clearance of apoptotic, senescent and damaged cells. The capability to scavenge dying material enables macrophages to play an important role also during organogenesis in embryonic development (Hopkinson-Woolley et al., 1994). Macrophages also have tissue-specific functions. For example in the liver, macrophages called Kupffer cells are present in the lumen of hepatic sinusoids and exhibit endocytic activity against blood-borne materials entering the liver (Naito et al., 2004). In the lungs, during the resolution phase of tissue damage, alveolar macrophages are initiators of parenchymal repair

mechanisms essential for return to homeostasis for normal gas exchange. Osteoclasts, the macrophages present in the bones, are essential for bone remodelling.

Macrophages are central members of the innate immune system and, together with neutrophils, eosinophils and natural killer cells, function as a first-line defence to recognize, eliminate or contain invading microbes and toxic macromolecules. Moreover, macrophages play an essential role in the maintenance of immune homeostasis by producing immunoregulatory cytokines such as IL-10 and TGF-β (Harrison and Maloy, 2011). Macrophage responses towards pathogens and microbes are triggered by PAMPs and are rapid. Moreover, when the innate immune system is unable to handle and eliminate an invading micro-organism, macrophages serve as effector cells of the adaptive immune system, after receiving the appropriate activation and information from antigen-specific T cells and B cells.

1.1.3 Origin of monocytes/macrophages

Tissue macrophages derive from bone marrow stem cells through a complex cascade of differentiation events that require the presence of IL-1, IL-3, and IL-6 (Ogawa, 1993). These cytokines give rise to a pluripotent myeloid cell type, referred to as granulocyte-erythrocyte-megakaryocyte-macrophage colony-forming unit (GEMM-CFU). The continued presence of IL-1 and IL-3 induces the GEMM-CFU to become progenitors of both macrophages and granulocytes known as granulocyte-macrophage colony-forming unit cells (GM-CFU) (Lopez et al., 1992). At this point, IL-3 and granulocyte-macrophage colony-stimulating factor (GM-CSF) induce the proliferation of myeloid precursors, whereas macrophage colony-stimulating factor (M-CSF) induces not only their proliferation but also their differentiation to the monocyte precursor (M-CFU). The

persistent presence of IL-3, M-CSF and GM-CSF leads to the differentiation of the monoblast, then to the promonocyte, which shows a limited phagocytic capability (Figure 1.3). Promonocyte differentiation into monocytes requires the presence of M-CSF. Monocytes are generally smaller than their immediate precursors, but they have a well-developed lysosomal system and enhanced phagocytic capability (Hamilton, 1993).

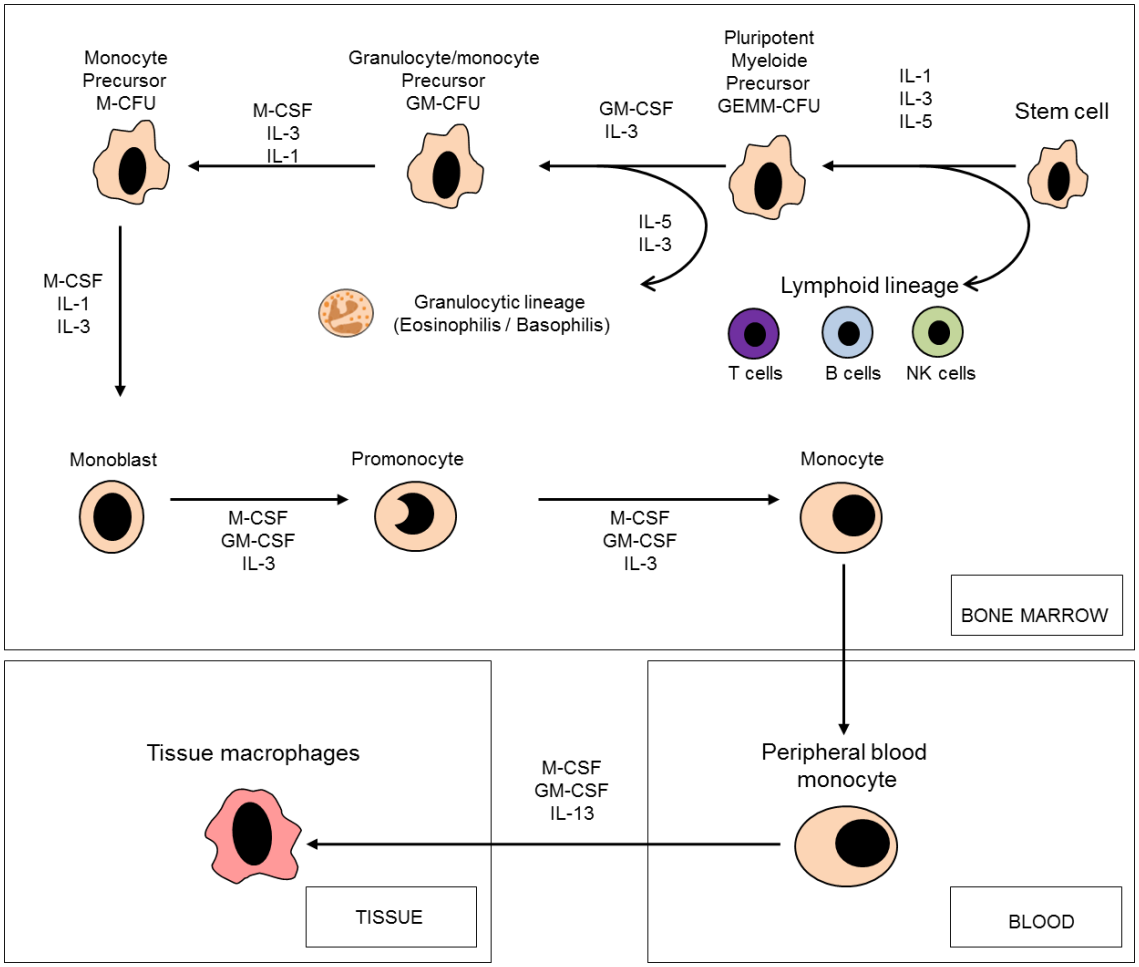

Figure 1.3: Differentiation of stem cells to monocyte/macrophages: Pluripotent stem cells differentiate into myeloid progenitors in the bone marrow where, under the influence of growth and colony stimulating factors, the cells differentiate into monoblasts. Monoblasts in turn develop into monocytes and migrate from the bone marrow into the blood where they circulate for several days and then are distributed to the tissues either as resident macrophages or as recruited macrophages at sites of infection and inflammation.

After leaving the bone marrow, monocytes enter the blood and are subsequently distributed to the various tissues either as resident macrophages or as recruited macrophages at the sites of infection and inflammation. The maturation of macrophages involves an increase in size and an increase in the lysosomal content and amount of hydrolytic enzymes.

1.2 Intestinal macrophages

1.2.1 Introduction

Intestinal macrophages represent the most abundant subpopulation of mononuclear phagocytes in the body and are usually localized in the lamina propria, underneath the superficial small blood vessels just below the sub-epithelial collagen layer, and in the *muscularis propria*, which faces the peritoneum. This strategic localization guarantees a first-line defence against the huge numbers of potentially harmful bacteria and antigenic stimuli that are present in the intestinal lumen (Sagaert et al., 2012).

1.2.2 Recruitment of blood monocytes to the gut

Little is known about the mechanisms regulating the recruitment of circulating monocytes and their differentiation into resident macrophages within the intestinal lamina propria. Among the many hypothesis, intestinal inflammatory macrophages develop from $CD14^{+}CD16_{+}$ monocytes and resident macrophages from $CD14^{+}CD16^{-}$ monocytes (Fiocchi, 2008). Compared with $CD14^{+}CD16^{+}$ monocytes (which are also $CD64^{+}$), $CD14^{+}CD16^{-}$ monocytes have a high phagocytic activity and produce large amounts of cytokines such as TNF-α and IL-6, and these features are not shared with the $CD14^{+}CD16^{+}CD64^{-}$ subset (Gordon and Taylor, 2005). It has been assumed that intestinal macrophages display functional plasticity under different conditions, an idea consistent with the paradigm that there are "resident" and "inflammatory" lineages of monocytes (Geissmann et al., 2010a).

Circulating blood monocytes then, under the influence of TGF-β and IL-8, are recruited into the lamina propria (Smythies et al., 2006), where during homeostasis they differentiate preferentially into inflammation-anergic intestinal macrophages (Smith et

al., 2011). This process is driven by TGF-β thereby down-regulating the subsequent production of pro-inflammatory cytokines (Smythies et al., 2010).

Other mechanisms apart from TGF-β and IL-8 have been reported to regulate monocyte migration to the lamina propria; after circulating in the bloodstream, monocytes extravasate into tissues using adhesion molecules such as L-selectin (CD62L), P-selectin glycoprotein ligand 1 (PSGL1), lymphocyte function-associated antigen 1 (LFA1) and platelet endothelial cell adhesion molecule (PECAM1) (Ley et al., 2007); (Shi et al., 2011), together with chemotactic factors such as CSF1 and the chemokines CX3CL1 and CXCL12. Once in tissues, monocytes differentiate into macrophages under control of CSF1 (Auffray et al., 2009); (Fong et al., 1998); (Pixley and Stanley, 2004). Amongst the chemokine receptors, it has been previously reported that CCR2 (CCL2 receptor) plays a key role in monocyte recruitment to the gut during inflammation. The best known role for CCR2 is in the accumulation of the $Ly6C^{hi}$ subset of monocytes at sites of inflammation. Moreover as noted above, the idea that CCR2 dependent accumulation of monocytes is only important in intestinal inflammation has been challenged by the recent work which indicates that $CCR2^{+}$ $Ly6C^{hi}$ monocytes appear to replenish steady state intestinal macrophages (Bain et al., 2013). This is supported a work showing that the accumulation of IL10 producing intestinal macrophages in normal intestine is dependent on CCR2 and its ligand CCL2 (Feterowski et al., 2004). Moreover it has also been suggested that CCR2 dependent monocytes actually play a protective role in the intestinal inflammation associated with *T. gondii* infection, by encouraging tissue repair and inhibiting pathogenic neutrophil activity (Grainger et al., 2013). Finally other receptors such as CCR1 and CCR5 have been implicated in monocyte recruitment (Lebre et al., 2011; Tokuyama et al., 2005).

Marker	Intestinal macrophages	Inflammatory macrophages	Monocyte
CD11b	+++	+++	+++
F4/80	+++	++	+/-
CD68	++	++	-
Class II MHC	+++	++/+	-
CD11c	++	++	-
CX3CR1	+++	++	-
CCR2	++	+++	++/-
CD40	+/-	+/-	-
CD80,CD86	+/-	+/-	
CD14	+	++	+++
TLR2	++	++	+++
TLR4	+/-	+	+++
Mannose R	?	?	-
Scavenger R	?	?	-
Phegocytosis	+++	+++	+
iNOS	-	++	+
ROI	-	++	+
TNF-α	±	+++	+
IL-10	++	±	-
Arginase	-	-/++	-
COX-2	++	?	-
IL-4R	-	-	-
IL-10R	+	-	-
CD33	++	+++	+

Table 1.1: Receptor expression on intestinal macrophages, inflammatory macrophages and monocytes. Resident mucosal macrophages in resting colon are compared with recruited macrophages which infiltrate during intestinal inflammation in IBD and blood monocytes.

Lamina propria macrophages are phenotypically distinct both from blood monocytes and from other tissue macrophages (Table 1.1). While tissue macrophages, such as Kupffer cells and alveolar macrophages, are able to proliferate locally (Tarling et al., 1987), it has been shown that gut lamina propria macrophages are unable to replicate, which suggests that intestinal macrophages are replenished solely by blood monocytes. Moreover, resident macrophages of the gut display an inflammation-anergic phenotype (Sagaert et al., 2012) with little or no expression of various PRRs, including CD14, CR3 component (CD11b/CD18), Ig receptors CD16 and CD32 (Smythies et al., 2005). Normal intestinal macrophages also lack receptors for IL-2 and IL-3, as well as the chemokine receptors CCR5 and CXCR4, the integrin leukocyte function-associated antigen (LFA)-1 (Platt and Mowat, 2008) and the triggering receptor expressed on myeloid cells (TREM)-1, an efficient amplifier of acute and chronic inflammatory reactions expressed on most monocytes and macrophages in secondary lymphoid organs. The lack of expression of IgA receptor (CD89) and the low expression of CD14 prevent resident macrophages from participating to the inflammatory cascade and thereby promote the maintenance of homeostasis present at the steady state in the intestinal mucosa (Smith et al., 2005).

Intestinal lamina propria macrophages, under physiological conditions, are not only refractory to the induction of pro-inflammatory cytokine production by PAMPs but also by other pro-inflammatory cytokines (e.g. TNF-α, IFN-γ), or upon phagocytosis of necrotic cells (Smith et al., 2005). This is in sharp contrast to most other tissue macrophages and blood monocytes.

However, intestinal macrophages retain their phagocytic activity, which is remarkably powerful. Compared to circulating monocytes, intestinal macrophages are highly

effective at killing phagocytosed microorganisms such as *S. typhimurium* and *E. coli* (Smythies et al., 2005). Phagocytosis of apoptotic cells by intestinal macrophages also induces the secretion of immunosuppressive cytokines such as IL-10 and TGF-β, whereas the secretion of pro-inflammatory cytokines such as TNF-α, IL-12, IL-6 and IL-1β is significantly inhibited (Qualls et al., 2006). Moreover, intestinal macrophages in homeostatic conditions continuously act as sentinel phagocytic cells, continuously sampling and surveying the commensal microbiota (Mowat and Bain, 2011).

1.2.3 Classical macrophage activation

Intestinal macrophages have the ability to change from one phenotype to another. The activation of macrophages in response to pathogenic stimuli has been termed "classical activation", resulting in macrophages identified as classically activated macrophages or M1 (Lin et al., 2006). Classical macrophage activation results from the recognition of PAMPs on the surface of Gram-positive and Gram-negative bacteria. Macrophages express a range of TLRs and other PRRs which are triggered during phagocytosis of pathogenic material. Classical activation of macrophages is dependent on the activation of signal transducer and activator of transcription (STAT)1 and interferon regulatory factor (IRF)5 (Krausgruber et al., 2011). This results in the production of pro-inflammatory cytokines, including TNF-α, IL-1β, IL-6, IL-12 and IL-23, and chemokines such as the Chemokine (C-X-C motif) ligand 9 (CXCL9) and C-X-C motif chemokine 10 (CXCL10) (Mosser and Edwards, 2008). The activation of TLR4 is one of the main pathways responsible for the production of pro-inflammatory cytokines, chemokines, adhesion molecules such as intracellular vascular adhesion molecule-1 and intracellular-1 adhesion molecule, and overexpression of co-stimulatory molecules such as CD80 and CD86 (Takeda and Akira, 2005). Pro-inflammatory mediators released by

classically activated macrophages facilitate the recruitment of other inflammatory leukocytes such as monocytes and neutrophils, as well as the polarisation of T lymphocytes to the pro-inflammatory T helper (Th)1 and/or Th17 phenotypes. As showed in Figure 1.4, classically activated macrophages also express inducible nitric oxide synthase (iNOS or NOS2), allowing them to generate nitric oxide (NO) from L-arginine. M1 macrophages also produce other cytotoxic mediators such as superoxide anions and other oxygen radicals through expression of the enzyme nicotinamide adenine dinucleotide phosphate-oxidase (NADPH).

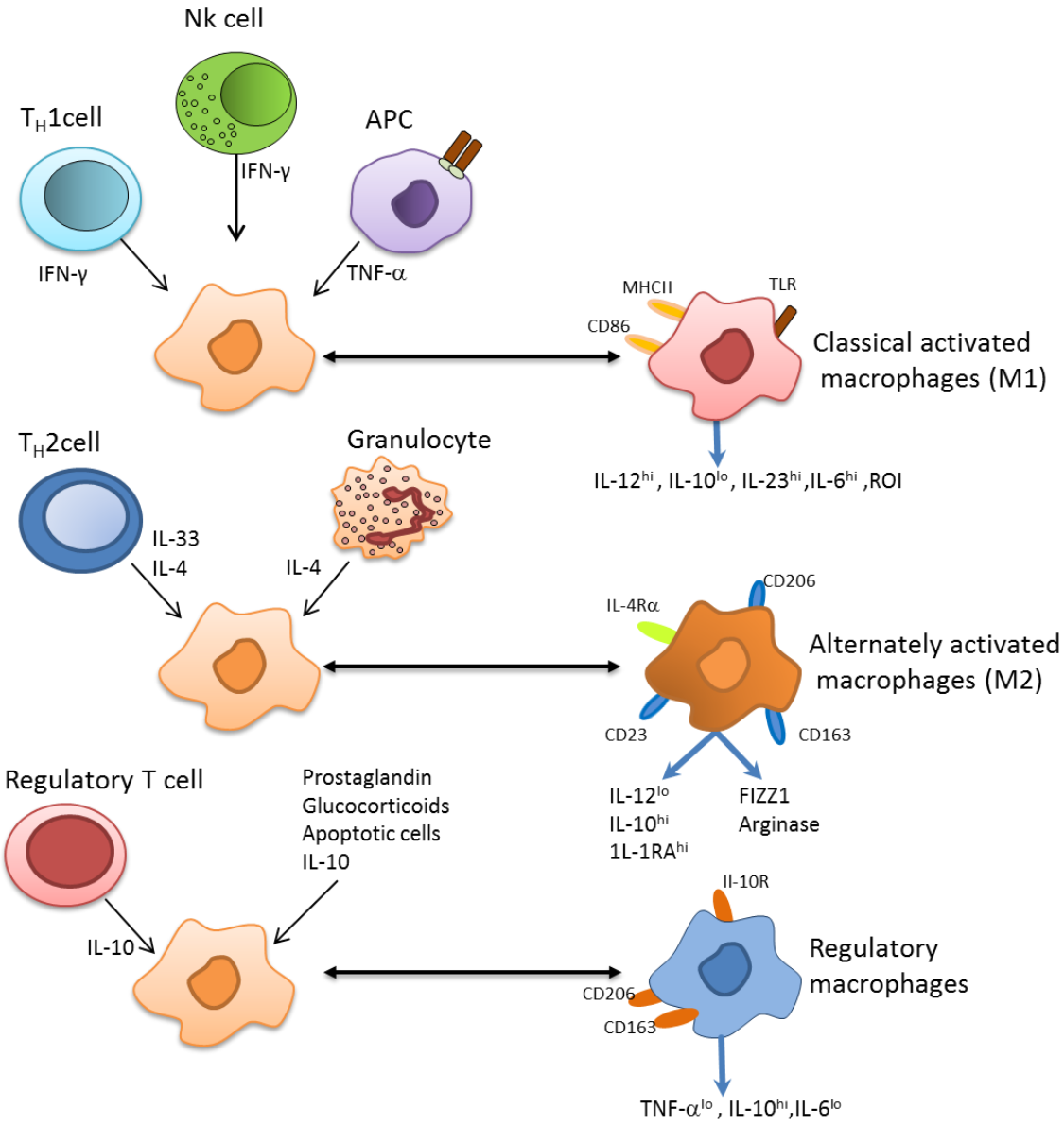

Figure 1.4: Macrophages activation: Cytokines produced by immune cells can give rise macrophages with distinct phenotypic and functional properties defined as classically activated, alternatively activated and regulatory macrophages.

Furthermore, their ability to up-regulate the expression of the major histocompatibility complex class II (MHC) and co-stimulatory molecules allows classically activated macrophages to act as antigen presenting cells (APC) for T cells. However, these potent pro-inflammatory properties also mean that classically activated macrophages must be tightly controlled, as aberrant or excessive activity can lead to tissue damage. Indeed, macrophages exhibiting M1 characteristics are commonly found in excess at the sites of chronic inflammation (Abraham and Medzhitov, 2011).

1.2.4 Alternative macrophage activation

Although in the past, the main feature of alternatively activated macrophages was considered to be the ability to inhibit classical macrophage activation (Ohmori and Hamilton, 1997), it now has become clear that alternatively wound-healing macrophages are involved in the tissue repair. Macrophages are polarised to M2 phenotype by Th2 cells. IL-4 and IL-13 are the prototypical direct inducers but other cytokines such as IL-33 and IL-25 amplify M2 induction indirectly, through Th2 cells (O'Shea and Paul, 2010). Th2 cells, macrophages, and B cells, as well as tumor cells, are potent down-regulators of macrophage gene expression, a process controlled by IRF4, STAT6 and the peroxisome proliferator-activated receptor gamma (PPARγ) (Satoh et al., 2010). M2 macrophages do not express pro-inflammatory cytokines and iNOS and are poor producers of reactive oxygen and nitrogen species. In contrast, they express the mannose receptor (CD206), FIZZ1, Ym1, and produce chemokines such as CCL22 (MDC), which attract $CCR4^+$ $CD4^+$ Th2 cells (Bonecchi et al., 1998). Ym1 belong to a family of chitinase-like molecules which are strongly induced by Th2 cell stimuli on macrophages as well as epithelium. Fizz1, is a secreted protein produced by macrophages, epithelium, and eosinophils, and it is highly induced by IL-13. Fizz1 in

lung allergy and asthma may inhibit inflammation (Nair et al., 2009). Fizz $^{-/-}$ M2 macrophages display an exaggerated ability to drive antigen-specific Th2 cell differentiation. Fizz1 protein binds to macrophages and effector $CD4^{+}$ Th2 cells, inhibiting Th2 cytokine production. For example, NO generated by IFN-γ-induced iNOS is shut down in macrophages (it is induced in epithelial targets), with a shift to Arginase-1 (Arg1) considered to have a possible role in switching to an alternative versus classical activation. Interactions with mast cells, basophils, eosinophils, and NKT cells, as well as IgE and selected subclasses of IgG, promote allergy and hypersensitivity. Together they influence vascular permeability, angiogenesis, cell recruitment, smooth muscle contraction, goblet cell secretion, mucus production, and collagen deposition by myofibroblasts (Locksley, 2010). They also express Arg1 (Loke et al., 2002), allowing them to convert L-arginine to L-ornithine, which is a precursor of polyamines and collagen, and facilitates tissue remodelling and encapsulation of helminths (Gordon and Martinez, 2010).

1.2.5 Regulatory macrophage activation

Similarly to the two populations above, a third population of macrophages, which exert marked effects during stress, can arise following innate or adaptive immune responses. Although stress responses are not typically considered part of innate immunity, when glucocorticoids are released by adrenal cells in response to stress, they can inhibit macrophage mediated host defence and inflammatory functions by inhibiting the transcription of pro-inflammatory cytokine genes and decreasing mRNA stability, giving rise to a population of regulatory macrophages (Sternberg, 2006). Regulatory macrophages can also arise during the later stages of adaptive immune responses, the primary role of which seems to be to dampen the immune response and limit

inflammation (Mosser, 2003). There are many different ways to generate regulatory macrophages *in vitro*, for example stimulation with TLRs agonists in the presence of IgG immune complexes promotes the development of a population of regulatory macrophages that produce high levels of IL-10 (Gerber and Mosser, 2001). Regulatory macrophages may also be activated by adenosine (Haskó et al., 2007), dopamine, histamine, sphingosine 1-phosphate, melanocortin, vasoactive intestinal peptide, adiponectin and Siglec-9 (Huang et al., 2008). Because IL-10 can inhibit the production and activity of various pro-inflammatory cytokines, these regulatory macrophages are potent inhibitors of inflammation, despite the fact that they retain the ability to produce many pro-inflammatory cytokines. Unlike wound-healing macrophages, regulatory macrophages do not contribute to the production of the extracellular matrix, and many of these regulatory cells express high levels of co-stimulatory molecules (CD80 and CD86) and therefore can present antigens to T cells (Edwards et al., 2006).

1.2.6 Endotoxin tolerance

Prolonged or excessive macrophage activation can be deleterious to the host due to the potential for macrophages to cause pathology. Therefore, multiple mechanisms exist to ensure that macrophage activity is appropriately regulated. One of the most effective protective mechanisms that suppress inflammatory cytokine production by macrophages is endotoxin tolerance. Monocytes and macrophages are the principal cells involved in endotoxin tolerance *in vivo*, and tolerization of these cells has been studied extensively (Biswas and Mantovani, 2010). Endotoxin tolerance selectively prevents inflammation associated with excessive cytokine production while allowing beneficial TLR-induced responses, such as the production of anti-microbial peptides. Endotoxin tolerance is accompanied by the down-regulation of TLR4 (Nomura et al., 2000), decreased

association of TLR4 with MyD88 adaptor protein, and decreased association of MyD88 with IRAK (Medvedev et al., 2002). Besides promoting MyD88 activation, LPS stimulation is known to cause the induction of splice variants of the MyD88 protein that lack the necessary domain to interact with IRAK4, thereby inhibiting positive TLR signalling (Burns et al., 2003). Moreover, TLR activation induces the activation of IRAK-M, a molecule that inhibits the dissociation of IRAK1-IRAK4 from the TLR signalling complex, by inhibiting their phosphorylation and thereby preventing further signalling. IRAK-M expression is restricted by macrophages and monocytes and its levels are increased when cells are stimulated with LPS (Kobayashi et al., 2002). Toll-interacting protein (Tollip) represents another adaptor protein which maintains immune cells in a quiescent state in the absence of infection. Tollip was found to associate with the cytoplasmic TIR domain of IL-1 receptors (IL-1Rs) after IL-1 stimulation. Within resting cells Tollip forms a complex with IRAK and inhibits IL-1-induced signalling by blocking IRAK phosphorylation (Burns et al., 2000). Because of the significant homology in the intracellular portion of TLRs, IL-1R, and IL-18R, Tollip might also inhibit TLR-mediated signalling by interacting with TLRs through the TIR domain (Zhang and Mosser, 2008). Suppressor of cytokine signalling (SOCS)-1 was discovered independently on the basis of its ability to interact with the kinase domain of Janus kinase (JAK)2 and was named JAK-binding protein (JAB) (Endo et al., 1997). It was also discovered because of similarity of the SOCS-1 SH2 domain to a sequence motif in the STAT3 SH2 domain, and was referred to as STAT-induced STAT inhibitor (SSI)-1 (Narazaki et al., 1998) . Transcription of the SOCS-1 gene is induced in response to a number of cytokines, such as IL-6, IL-4 and IFN-γ, and the protein has been shown to inhibit the tyrosine phosphorylation of each member of the JAK family of protein tyrosine kinases (Nicholson et al., 1999).

1.3 Induction of adaptive immune responses in the gut

The gut-associated lymphoid tissue (GALT) can be classified into effector sites, which consist of lymphocytes scattered along the epithelium and lamina propria of the mucosa, and organized structures that are responsible for the induction of the immune response (Mowat, 2003). Primary sites for the induction of immunological responses are small intestinal Peyer's patches, which consist of a collection of large B cell follicles and T cell areas, isolated lymphoid follicles (ILFs) in the small and large intestine, and MLNs (Kraehenbuhl and Neutra, 2000).

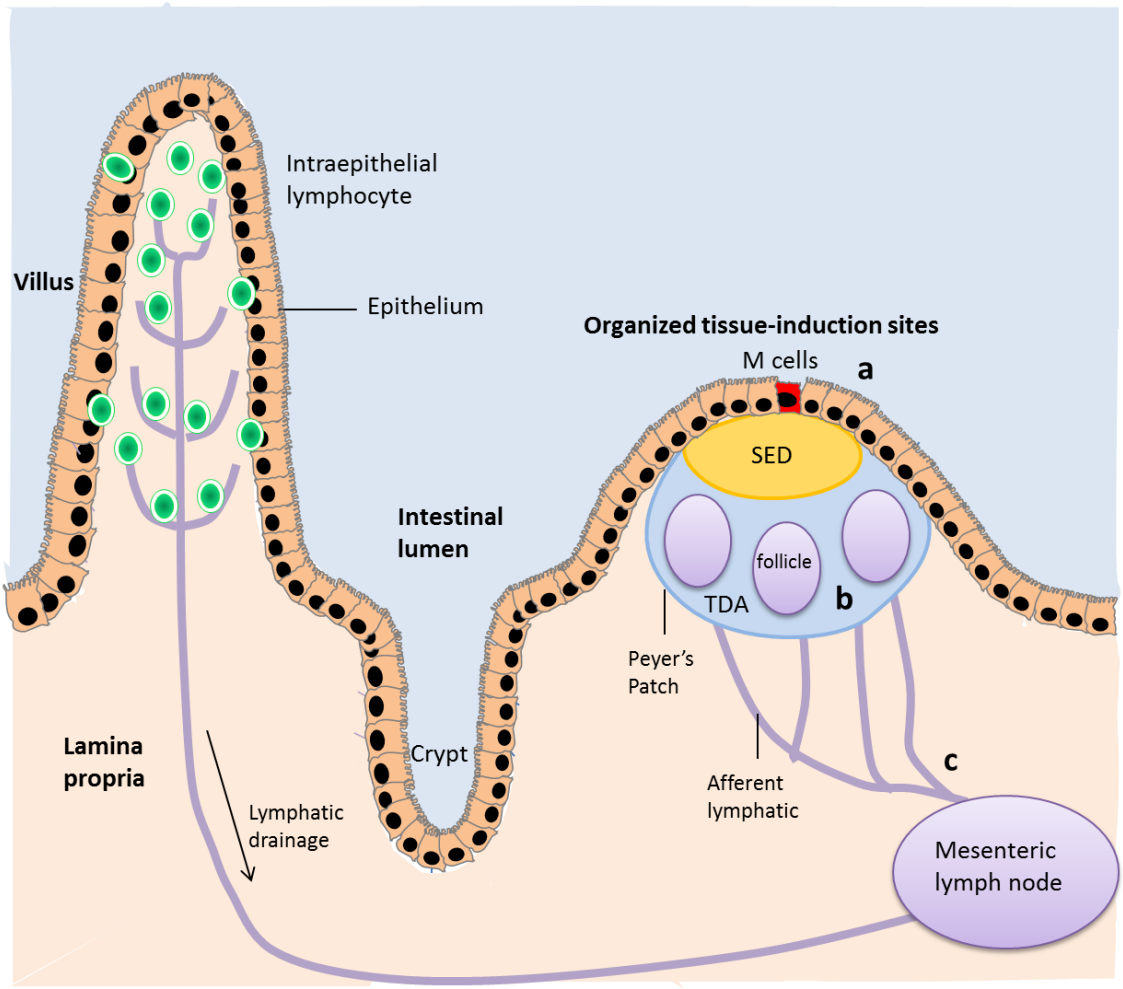

Figure 1.5: Schematic representation of the lymphoid elements of the intestinal immune system. Antigen might enter through the microfold (M) cells in the follicle-associated epithelium (FAE) (**a**), and after transfer to local DCs, might then be presented directly to T cells in the Peyer's patch (**b**). Alternatively, antigen or antigen-loaded DCs from the Peyer's patch might gain access to draining lymph (**c**), with subsequent T-cell recognition in the MLNs.

The organized gut lymphoid structures are separated from the intestinal lumen by a single layer of columnar epithelial cells, also known as follicle-associated epithelium (FAE) (figure 1.5), and by a more diffuse area below the epithelium known as the subepithelial dome (SED). The most notable feature of the FAE is the presence of microfold (M) cells, which are specialized antigen-transporting enterocytes lacking surface microvilli (Debard et al., 2001) (Golovkina et al., 1999). M cells provide the main way in which complex antigens can gain access to the intestinal immune system. M cells do not express MHC class II molecules and do not process antigens (Mantis et al., 2002), however they are believed to be essential in passing on intact antigens to professional APCs, either in the epithelium or in the underlying SED. APCs subsequently move to the T cell areas and/or B cell follicles in the Peyer's patches, where they can interact with naïve lymphocytes (Salzman et al., 2007). Following the interaction with the antigens the lymphocytes primed in the Peyer's patches migrate through the draining lymphatic vessels to the MLNs, where they reside for an undefined period of further differentiation, before further migrating into the bloodstream from where they are recruited and redistributed to the effector sites of the intestinal mucosa.

The efficient homing and retention of lymphocytes to the gut is dependent on the β7-containing integrins, α4β7 and αEβ7 integrin in presence of retinoic acid, made by DCs, as well as the chemokine CCL25 and its receptor CCR9 (Salmi and Jalkanen, 2005). α4β7 integrin binds Mucosal Addressin Cell Adhesion Molecule-1 (MAdCAM-1), which is constitutively expressed on high endothelial venules (HEVs) of PPs and MLNs as well as on postcapillary venules of gut LP. Both naïve and effector lymphocytes use α4β7 integrin to extravasate from blood to gut mucosal tissues of GALT, MLN and lamina propria via interactions with MAdCAM-1.

Next, lymphocytes entering the mucosa are redistributed to distinct compartments. B-cell blasts mature into IgA-producing plasma cells and remain in the lamina propria. $CD4^+$ T cells also remain in the lamina propria, but are distributed more evenly throughout the *villus*-crypt unit. $CD8^+$ T cells migrate preferentially to the epithelium, although ~40% of T cells in the lamina propria are also $CD8^+$, where they can have potent cytotoxic activity (Lefrançois et al., 1999). Some of these antigen-experienced lamina propria T cells may be true effector cells, and may help local B cells to produce IgA. Alternatively, they may be 'effector memory' cells (Sallusto et al., 1999), as indicated by the observations that antigen-specific memory $CD4^+$ and $CD8^+$ T cells accumulate preferentially in non-lymphoid tissues, in particular in the intestinal mucosa (Reinhardt et al., 2001). $CD4^+$ T cells in the lamina propria are particularly important in local immune regulation. They produce large amounts of pro-inflammatory and regulatory cytokines, particularly IFN-γ, but also IL-4 and IL-10. Due to the presence of such a vast range of immune cells under normal conditions, the intestine has been often described as being in a state of physiological inflammation, emphasising the delicate balance needed to maintain homeostasis.

1.4 Mechanisms of immune tolerance in gut

The mucosa of the small and large intestine is constantly exposed to a large variety of TLR ligands, to a great antigenic load from luminal bacteria, and to potential mitogens. Many pathways and cell types are implicated in the regulation of host-environment interaction and in the generation and maintenance of immune tolerance in the GI tract, including inhibitory macrophages and DCs, regulatory T cells (Tregs), T cell apoptosis, and immunoregulatory cytokines (Macpherson et al., 2008) (Rescigno et al., 2001). The exact mechanisms that support the maintenance of oral tolerance remain to be established with certainty, but anergy of reactive T cells may be involved. $CD4^+$ Tregs expressing the transcription factor forkhead box P3 (Foxp3) are present at higher frequencies in the gut lamina propria, particularly in the colon, than in other organs (Hall et al., 2008). It has been postulated that $Foxp3^+$ Tregs are induced by luminal antigens and prevent antigen-specific responses through cell-cell interactions and regulatory cytokine production. Furthermore, colonization of mice with human commensal *Bacteroides fragilis* facilitates Treg differentiation and IL-10 production (Round and Mazmanian, 2010). Atarashi and colleagues showed that mice inoculated with chloroform-treated feces displayed an increased number of Tregs , comparable to that in germ-free mice gavaged with untreated feces. *Clostridia* species were shown as having the most important role in the induction of colonic Tregs by activating TGF-β and by inducing the production of other Treg-inducing factors from intestinal epithelial cells, which presumably cooperate with DCs to induce a general accumulation of Tregs in the colon and at the same time affect the proportions of individual Treg subsets through the preferential induction of $IL\text{-}10^+$ inducible Tregs (Atarashi et al., 2011). More recently, the same group identified Treg-cell-inducing bacterial strains derived from the human microbiota. Stool samples from a healthy Japanese volunteer were

untreated or treated with chloroform and orally inoculated into IQI/Jic germ-free mice, in which they induced a significant increase in the percentage of $Foxp3^+$ Treg cells among $CD4^+$ T cells in the colon. These findings suggested that the human intestinal microbiota contains Treg-cell-inducing bacteria (Atarashi et al., 2013).

Within the gut mucosa, not only the Tregs but several other cell types, including macrophages and myofibroblasts, can produce and respond to TGF-β. However a major source of TGF-β is mainly produced from epithelial cells in a latent complex formed by three proteins: TGF-β, the processed TGF-β propeptide, and a member of the latent TGF-β binding protein (LTBP) family and is activated by an epithelium-restricted integrin αvβ6 (Munger and Sheppard, 2011). In the gut TGF-β and IL-8 act as chemokines on circulating blood monocytes by inducing the recruitment of blood monocytes in the lamina propria where, like described above, during homeostasis they differentiate preferentially into inflammation-anergic intestinal macrophages (Smith et al., 2011). This process is driven by TGF-β, which prevents the translocation of NF-KB into the nucleus and, therefore, down-regulates the subsequent production of pro-inflammatory cytokines (Smythies et al., 2010).

TGF-β blocks NF-KB activation in macrophages in response to TLR2, TLR4 and TLR5 stimulation also by facilitating the proteasomal degradation of MyD88 (Naiki et al., 2005). As a result, a breach in epithelial integrity normally triggers host defence activity (Maheshwari et al., 2011) but not an inflammatory response by macrophages in the gut (Smythies et al., 2006).

Epithelial cells play an important role in the activation and communication with the cells underlying; the interaction of macrophages and DCs with the epithelial cells is thought to be integral for the conditioning of the immune cells to their local

environment. The first evidence for this came from co-colture studies with epithelial cells and human monocyte derived DCs, whereby epithelial cells treated with different bacterial strain produced different pro-inflammatory mediators which modulated DCs function (Rimoldi et al., 2005).

In addition to TGF-β and prostaglandin E2, also thymic stromal lymphopoietin (TSLP) production by epithelial cells contribute to the ability of these cells to condition DC to express pro-inflammatory cytokines subsequently affecting their ability to induce naïve T cell polarization. TSLP although constitutively expressed can be up-regulated in response to infection, inflammation and tissue injury (Allakhverdi et al., 2007; Bogiatzi et al., 2007; Kato et al., 2007). Consistent with the role of TSLP in the skin and lung, epithelial cells-derived TSLP has been show to condition DCs to drive Th2-like anti-inflammatory T cell differentiation, while limiting their production of IL-12 (Rimoldi et al., 2005).

1.5 Inflammatory bowel disease

1.5.1 Introduction

The inflammatory bowel diseases are a group of chronic and relapsing inflammatory conditions of the gastrointestinal tract, believed to be caused by a complex interplay between genetic, immunologic, microbial and environmental factors. In particular, IBD is thought to be driven by an inappropriate immune response directed against the enteric microbiota in a genetically susceptible host. The two major forms of IBD are Crohn's disease (CD) and ulcerative colitis (UC). CD can affect the whole gastrointestinal tract but is mainly localised to the colon and terminal ileum, whereas UC exclusively affects the colon (Blumberg, 2009). Both forms of IBD are characterised by a substantial infiltrate of neutrophils, monocytes, macrophages, eosinophils and T cells into the intestinal wall. The inflammatory infiltrate in CD is mostly mononuclear with granulomata, hallmarks of classical cell-mediated immunity. CD is characterised by a predominant Th1 mucosal cytokine profile with elevated levels of IFN-γ, IL-2, IL-12 and TNF-α (Macdonald, 2010). It has been suggested that UC is driven by an atypical Th2 response, with IL-5 and IL-13 being produced by NK T cells (Kaser et al., 2010). However studies from our lab failed to find increased IL-13 in UC (Biancheri et al., 2013a). The failure of anti-IL-13 in 2 recent trials of patients with UC reinforces the notion that the atypical Th2 response is incorrect (Tilg and Kaser, 2015).

In the last few years, other factors have been recognised to contribute to the pathogenesis of IBD, and the paradigms formulated some years ago to explain the mechanisms underlying these conditions have undergone a rapid and, in some cases, radical change in the light of more recent knowledge. The Th1/Th2 paradigm has in fact been replaced by the Th1/Th17 paradigm since high levels of IL-17A are present in both UC and CD inflamed mucosa. In order to determine whether a particular cytokine is

important in IBD it is critical to take into account the data on the effect of its *in vivo* neutralisation in humans. Anti-TNF-α therapies are a clear example of the relevance of this approach. In fact, infliximab induces clinical improvement and clinical remission in 80% and 50% of CD patients, respectively, thus proving the importance of TNF-α in CD. However, blocking IFN-γ, IL-17A, CD80/B7-1 and IL-12/23 have all failed in clinical trials in unselected patients with CD (Biancheri et al., 2013b).

1.5.2 TLRs in IBD

The role and influence of TLRs in the pathogenesis of IBD has become increasingly evident and widely acknowledged (Atreya et al., 2011). Among the TLRs, TLR2, TLR4, TLR5 and TLR9 have been shown to have important role in intestinal inflammation. Mice deficient in TLR2, TLR4 or TLR9 have increased susceptibility to both 2,4,6-trinitrobenzene sulfonic acid (TNBS)- and dextran sulfate sodium (DSS)-induced murine colitis (Kopprasch et al., 2002). TLR2 appears to play an essential pro-inflammatory role in the pathogenesis of IBD. Indeed, Hausmann *et al*. observed a marked increase in TLR2 expression on mucosal and submucosal $CD68^+$ intestinal macrophages from inflamed areas of IBD patients in comparison to those of control subjects, which instead exhibited little to no expression of TLR2 (Hausmann et al., 2002). Interestingly, TLR2 was highly expressed compared to control subjects in colonic biopsies taken from both children with newly diagnosed IBD and from patients with long-standing disease (Szebeni et al., 2008). A novel study by Canto *et al.* demonstrated that colonic lamina propria monocytes from active IBD patients exhibit high levels of TLR2 on their cell surface, and that TLR2 stimulation on their surface induced markedly high levels of TNF-α production in comparison to monocytes from inactive IBD patients and control subjects (Cantó et al., 2006). TLR4 has also been

implicated in the pathogenesis of IBD. Both TLR4 and MyD88 knock-out mice display significantly impaired intestinal barrier function and show defective mucosal healing in DSS-induced colitis. Moreover, mucosal TLR4 expression is markedly up-regulated in both CD and UC patients compared to normal subjects (Levin and Shibolet, 2008). Despite some conflicting evidence, TLR5 is generally believed to contribute to the pathogenesis of IBD (Lodes et al., 2004). One study has reported that intra-rectal administration of flagellin directly into DSS-treated mice greatly enhanced colitis symptoms relative to control mice (Levin and Shibolet, 2008). Accordingly, TLR5 knock-out mice develop spontaneous colitis and display markedly higher mucosal pro-inflammatory cytokine production (Vijay-Kumar et al., 2007). Furthermore, sera from a substantial proportion of CD patients contains a marked elevation of anti-flagellin antibodies relative to healthy control subjects (Heinsbroek and Gordon, 2009). Also TLR9 seems to be involved in intestinal inflammation. In particular, some studies revealed that the apparent success of treating colitic mice with bacterial probiotics was in part, facilitated via CpG activated TLR 9 signalling (Pedersen et al., 2005). However in CD, probiotics have been completely ineffective in either inducing or maintaining remission. The effects of CpG oligo-deoxynucleotides able to stimulate TLR9 were investigated on colonic mucosal biopsies from active UC patients (Hornung et al., 2002). CpG significantly reduced colonic expression of IL-1β and TNF-α by UC colonic biopsies cultured *ex vivo*, with a mechanism dependent on TLR9 (Lodes et al., 2004).

1.5.3 Intestinal macrophages in IBD

The anergic phenotype of intestinal macrophages represent the principal mechanisms that prevent the inappropriate reactions to commensal microbes that cause IBD (Bain

and Mowat, 2011). However, resident mucosal macrophages may fail in maintaining the anergic phenotype and can produce pro-inflammatory mediators in response to stimuli such as the TLRs (Geissmann et al., 2010b). More specifically, the release of these pro-inflammatory cytokines induces epithelial cell apoptosis, lesions in the mucosal barrier, compromises the integrity and permeability of the barrier, and promotes necrosis, fibrosis, and the formation of granuloma (Goto and Kiyono, 2012)

Both in experimental colitis and in human IBD, activated macrophages play an important role in the initiation of the inflammatory process (Strauss-Ayali et al., 2007). In patients with active IBD macrophage numbers are increased and many of these macrophages display a different phenotypic and functional profile than under homeostatic conditions. Macrophages in the inflamed intestine express functional T cell co-stimulatory molecules such as CD40, CD80 and CD86, and they also express TLR2, TLR4, CD89 and TREM-1 at the site of intestinal inflammation. Similarly, a high proportion of the macrophages from patients with IBD express CD14 (Kamada et al., 2008; Smith et al., 2001). CD14-expressing macrophages infiltrating the mucosa in IBD promote the recruitment of other effector cells producing inflammatory mediators such as TNF-α, IL-1β, IL-6 and NO (MacDonald et al., 2011). They also produce large amounts of IL-12, IL-23 and IFN-γ (Kamada et al., 2008). Moreover, the production of TNF-α and other mediators by these macrophage population plays a crucial role in the tissue damage (Mowat, 2003).

$CD14^+$ intestinal macrophages from CD patients respond vigorously to *in vitro* microbial stimulation by producing even more TNF-α, IL-12 and IL-23 compared to $CD14^-$ intestinal macrophages and peripheral blood mononuclear cells (PBMCs) (Kamada et al., 2008).

1.5.4 The role of TNF-α in IBD

Human TNF-α is a member of a large family of proteins and receptors which are critically involved in immune regulation (MacDonald et al., 2011). TNF-α exists in two forms: membrane-bound (m)TNF-α and a soluble form (sTNF-α) (Kollias et al., 1999). Upon stimulation by for example LPS, newly synthesized TNF rapidly accumulates in the Golgi complex as a type II transmembrane protein (Shurety et al., 2000) and the molecule is then transported to recycling endosomes before delivery to the plasma membrane (Murray et al., 2005). Upon further stimulation membrane TNF can be cleaved by a metalloprotease domain 17 (ADAM17) between residues Ala76 and Val77 (Gearing et al., 1994). Soluble TNF is released from the cell surface as a homotrimer in the form of three mature 17 kDa TNF molecules (Black et al., 1997).

Soluble TNF-α can signal through type 1 and 2 TNF receptors (TNFR1 and TNFR2), which are expressed as pre-assembled trimers on the cell surface (Chan et al., 2000). While TNFR1 is present in all nucleated cells, TNFR2 is found solely on immune cells, endothelial cells and neurons (Hodgkinson et al., 2008).

These receptors can act as ligands or signalling molecules. TNFR1 is capable of directly inducing apoptosis through its death domain (DD) (Nakao et al., 2005). TNFR2 does not contain a DD and can be fully stimulated only by the membrane integrated form of the ligand (Grell et al., 1995). Upon stimulation by soluble TNF, TNFR2 is thought to be a ligand passing receptor that after capturing soluble TNF, rapidly transfers the molecule to TNFR1 (Tartaglia et al., 1993).

Upon contact with their ligand TNFR1 undergo to a conformational change, thus leading to the dissociation of the inhibitory protein silencer of death domains (SODD) from the intracellular death domain. The dissociation enables the adaptor protein tumor necrosis factor receptor 1-associated death domain protein (TRADD) to bind to the

death domain, serving as a platform for subsequent protein binding (figure 1.6). TRADD then recruits TRAF2 and RIP. TRAF2 in turn recruits the multicomponent protein kinase IKK, the IkB kinase, causing IKK complex to phosphorylate IkB, which enables the translocation of NFkB to the nucleus, activating NF-kβ (Gewirtz et al., 2001). Upon NF-κb activation, TNF-α and other pro-inflammatory cytokines are produced. This cytokine production in turn activates other inflammatory pathways, such as arachidonic acid metabolites, superoxides, nitric oxide and growth factors (Viscido et al., 2005). Other effects of TNF-α include recruitment of neutrophils and monocytes via upregulation of adhesion molecules, alteration of cell barrier permeability and regulation of apoptosis in target cells through TNF-receptor I binding (Haraoui, 2005).

Numerous reports throughout the literature demonstrate an important role of cytokines with anti-inflammatory properties (e.g., IL-10, IL-4 or TGF-β) as negative regulators of TNF-α production. For instance, a study by Ebert et al. investigated the release of TNF-α by resting T cells and monocytes in the presence of antibodies neutralising IL-10, IL-4 or TGF-β and demonstrated elevated release of TNF-α in such conditions (Ebert, 2005). Interestingly, the authors of this study reported that when IL-4 or TGF-β were neutralised with appropriate antibodies, elevated levels of IL-10 were produced by monocytes, providing an additional protection against the release of exceptionally high amounts of TNF-α by these cells. The importance of IL-10 as an endogenous regulator of TNF-α is further supported by the findings that, in humans, subjects deficient in serum IL-10 levels are more prone to develop chronic inflammatory diseases, e.g. UC (MacDonald et al., 2012).

In particular, recent work has found that loss-of-function mutations in the genes encoding IL-10 and IL-10R are associated with a very early-onset form of IBD that is characterized by severe intractable enterocolitis in children (Glocker et al., 2009).

It has been consistently shown that TNF-α is increased in the mucosa of IBD patients (MacDonald et al., 1990). Increased TNF-α expression occurs both in CD and UC, however the distribution of producing cells differs: higher levels are seen in deep lamina propria of CD than in UC (Breese and MacDonald, 1995). Although many other cytokines are involved in the progression of disease, TNF-α represent the central cytokine that induces the expression of other important mediators of inflammation (Tracey and Cerami, 1993). The strong correlation between increased TNF-α production and IBD has been made clear via numerous studies on murine colitis models. Mice over-expressing TNF-α (TNFΔARE mice) spontaneously develop an enteritis (Kontoyiannis et al., 1999).

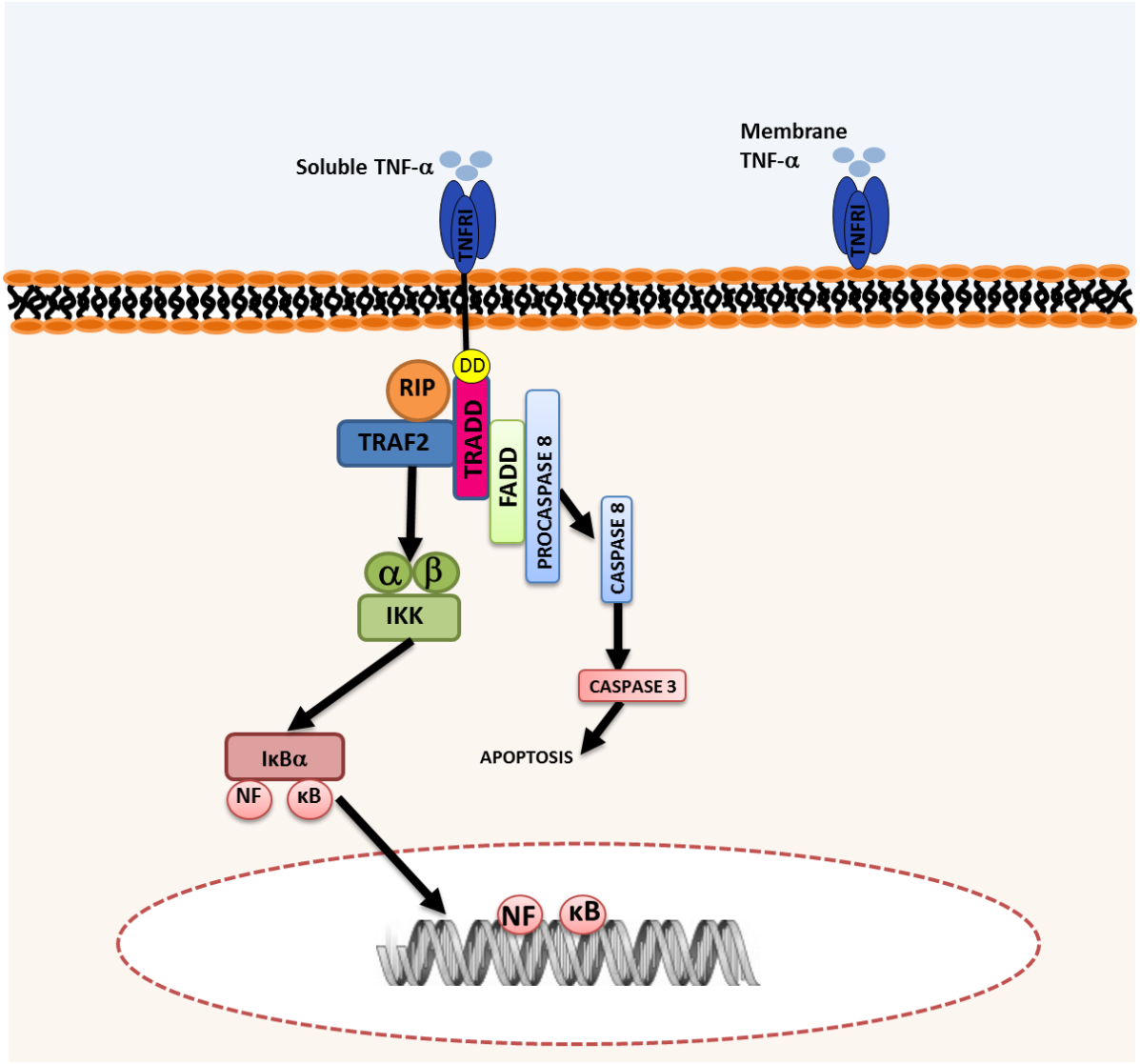

Figure 1.6: Proposed interaction mechanism of TNFR1 and soluble TNF-α. Soluble and membrane TNF-α activates the classical TNF-α signaling cascade by interacting with TNFRI displayed on the surface of immune cells resulting in chemokine and pro-inflammatory cytokine expression. Upon contact with their ligand, TNF receptors form trimers, which causes TRADD to recruit TRAF2 and RIP which recruit the IkB kinase, causing the phosphorylation of IKK complex enabling the translocation of NFkB to the nucleus, activating NF-kβ.

1.5.5 Transmembrane TNF-α and soluble TNF-α

Soluble TNF-α is released after the proteolytic cleavage via the metalloproteinase ADAM 17 (also known as TACE) between residues Ala76 and Val 77 (Gearing et al., 1994). This process is termed ectodomain shedding and represents a critical regulatory mechanism, not only for the release of cytokines (e.g. TNF-α), but also cytokine receptors (e.g. TNFR1 and IL-6R), chemokines (e.g. CXCL16 and CX3CL1), adhesion molecules (e.g. L-selectin) and surface proteoglycans. Membrane TNF-α is expressed on the cell surface of resting natural killer cells as well as of activated T cells and macrophages.

Since membrane TNF-α can function in cell-to-cell contact, it is considered to play a critical role in ontogenesis and in local inflammation. Similar to soluble TNF, membrane TNF-α exists as a homotrimer of uncleaved monomers and binds to both subtypes of TNF receptors on target cells, however biological activities are mediated mainly through TNFR2 (Grell et al., 1995). By cell death or activation of the target cells, expression of membrane TNF-α contributes to physiological and pathological responses. Cytotoxic activities of membrane TNF has been reported for various tumor cells (Horiuchi et al., 2010) and HIV-infected lymphocytes (Lazdins et al., 1997). The importance of membrane TNF-α in inhibition of intracellular pathogens has been also reported (Allenbach et al., 2008). In addition, membrane TNF- α has been shown to initiate T cell and macrophage migration, granuloma formation and to be effective against acute *Mycobacterium tuberculosis* infection(Saunders et al., 2005). Direct cell-to-cell contacts through membrane TNF-α trigger activation processes in endothelial cells, B cells, T cells, monocytes and NK cells (Horiuchi et al., 2010).

In contrast to the well characterized functions of membrane TNF-α as a ligand, the biological functions as a receptor still remain to be clarified. TNF-α reverse signaling by anti-TNF antibodies has been reported to trigger activation in human T cells and NK cells. In addition to its co-stimulatory role and $Ca2^{+}$ signaling inducing capacity in T cells (Higuchi et al., 1997), membrane TNF-α stimulation can induce the production of high amounts of IL-2 and adhesion molecules such as E-selectin (Harashima et al., 2001).

Recent clinical and experimental studies have shown that membrane-bound TNF-α, rather than soluble TNF-α, has a major role in driving intestinal inflammation. Consistent with this, neutralization of membrane-bound TNF-α has been shown to induce T cell apoptosis and was effective in suppressing experimental colitis in mice, whereas activation of TNFR2 (induced by membrane bound but not soluble TNF) on T cells was found to aggravate colitis activity (Perrier et al., 2013).

Moreover, a recent study has used fluorescently labelled TNF-specific antibodies and in vivo imaging to determine the frequency of immune cells that express membrane-bound TNF-α in the mucosa of patients with Crohn's disease (Atreya et al., 2014). It was found that the presence of high numbers of immune cells expressing membrane-bound TNF predicted a positive clinical response to subsequent therapy with TNF specific antibodies. Patients in which few immune cells expressed membrane-bound TNF-α showed little or no response to this type of therapy.

The first data with ADAM-17 conditional knockout mouse were published in 2007 by Blobel et al , who inactivated ADAM-17 in myeloid cells and prevented endotoxin-

induced lethality of the animals by inhibiting TNF-α shedding and preventing increased serum TNF-α (Horiuchi et al., 2007).

1.5.6 TREM-1 expression by intestinal macrophages in IBD

Triggering receptor expressed on myeloid cells (TREM)-1 belongs to a family of molecules related to natural killer cell-receptors and is expressed on neutrophils, mature monocytes and macrophages and has been previously categorized as a mediator of acute inflammation (Derive et al., 2010). While circulating serum monocytes express TREM-1, most intestinal macrophages do not express TREM-1 in healthy gut (Golovkina et al., 1999). However, intestinal macrophages express TREM-1 at a significantly higher level in the inflamed intestinal mucosa of IBD patients (Reinhardt et al., 2001). This aberrant TREM-1 expression mediates enhanced secretion of pro-inflammatory chemokines and cytokines (Schenk et al., 2007). Moreover blocking TREM-1 upon induction of experimental colitis attenuates intestinal inflammation. The activation of TREM-1 in the presence of TLR2 or TLR4 ligands amplifies the production of pro-inflammatory cytokines TNF-α, IL-1β, granulocyte-macrophage colony stimulating factor, together with the inhibition of IL-10 release (Bleharski et al., 2003), thus TREM-1 and TLRs appear to cooperate in producing an inflammatory response. The role of TREM-1 as an amplifier of the inflammatory response has been confirmed in a mouse model of septic shock in which blocking signaling through TREM-1 partially protected animals from death (Gibot et al., 2004).

TREM-1 is one of several TREM and TREM-like molecules, all of which are members of the Ig super family (Ig-sf), it contains only a short cytoplasmic tail, and thus signals upon association with the promiscuous adaptor molecule DAP12, which possesses two immunoreceptor tyrosine-associated activation motifs (ITAMs). Upon ligand binding of

TREM-1 the ITAMs of the associated DAP12 become phosphorylated. This allows binding of protein tyrosine kinases (such as Syk) via the Src homology 2 (SH2) domains. The kinase domain of such tyrosine kinases mediates downstream effects of TREM-1 by phosphorylating many proteins. This phosphorylation process may lead to Ca^{2+} ion mobilization or activation of extracellular signal-regulated kinases (ERK) leading to the transcription of target genes (Colonna and Facchetti, 2003; Wang et al., 2012). Therefore TREM-1 both directly and indirectly drives a pro-inflammatory response by the release of pro-inflammatory cytokines and by increasing the expression of receptors of other inflammation pathways.

The levels of the soluble form of TREM-1 (sTREM-1) correlate with a release of cultured human monocytes after stimulation with LPS. sTREM-1 was also detectable in the serum of endotoxemic mice as early as 1 h after LPS challenge. This is consistent with the role of TREM-1 in the very early phases of the innate immune response to infection (Brandl et al., 2007; Putsep et al., 2000). The mechanism by which sTREM-1 is released is not clearly elucidated but seems to be related to an increased transcription of the TREM-1 gene. Nevertheless, although incubation with a protease inhibitor cocktail does not alter the sTREM-1 release, cleavage of the surface TREM-1 from the membrane cannot be totally excluded. Interestingly, stimulation of human monocytes with such proinflammatory cytokines as TNF-α, IL-1β and IFN-γ induced very small sTREM-1 release unless LPS is added as a co-stimulus (Bleharski et al., 2003).

1.6 Aims of the PhD project:

1. To set up a robust and reliable method for the isolation of intestinal macrophages from healthy and inflamed gut resection specimens.

2. To perform a detailed phenotypic characterisation of intestinal lamina propria macrophages in comparison to circulating monocytes in control subjects and IBD patients.

3. To identify markers able to distinguish the populations of resident and recruited, resting and activated macrophages in healthy control and in IBD mucosa.

4. To study the production of TNF-α by intestinal macrophages in control subjects and IBD patients.

5. To study the behaviour of intestinal macrophages compared to circulating monocytes with regards to their key functions such as cytokine release.

6. To perform a gene expression array of intestinal macrophages in healthy control subjects and IBD patients.

CHAPTER 2

Materials and methods

2.1 Patients and tissues

Colonic biopsies or surgical specimens were taken from macroscopically and microscopically inflamed or uninflamed mucosa of patients affected by CD and UC. Diagnosis of CD and UC was ascertained according to the usual clinical criteria, and the site and extent of the disease were confirmed by endoscopy and histology. In CD patients, disease activity was assessed by Crohn's Disease Activity Index (Best et al., 1976). Patients with scores below 150 were classified as being in remission, whereas those with scores over 450 had severe disease (Best et al., 1976). In UC patients, disease activity was assessed according to the Clinical Activity Index of Rachmilewitz (Rachmilewitz, 1989). Clinical remission was defined as a score below 4. Surgical specimens were also taken from uninflamed areas of strictured and non-strictured ileum of patients with fibrostenosing CD. Moreover, mucosal samples were collected perendoscopically from the ileum or the colon of subjects who had functional diarrhoea at the end of their diagnostic work-up. Intestinal samples were also collected from macroscopically and microscopically unaffected ileal or colonic areas of patients undergoing intestinal resection for colon cancer. Some samples were used to isolate lamina propria mononuclear cells (LPMCs), others for organ culture experiments, and others were snap frozen in liquid nitrogen and stored at -70°C for subsequent analyses.

2.2 Buffers, media and solutions

Buffers and stock solutions were prepared according to standard procedures using water (H_2O) or phosphate buffered solution (PBS). Ready-to-use Roswell Park Memorial Institute-1640 medium (RPMI-1640) was bought from Invitrogen. Antibiotics (Aldrich, Poole, UK) were prepared as stock solutions, filter-sterilized (0.2 μm) and added to media.

2.3 *Ex vivo* organ culture

Perendoscopic mucosal biopsies from inflamed or uninflamed areas of CD and UC patients were placed (one biopsy per well) in 24-well plates (VWR International, Lutterworth, UK) in 300 µl serum-free HL-1 medium (Cambrex BioScience, Wokingham, UK) supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin, and cultured at 37°C, 5% CO_2 in order to evaluate spontaneous pro-inflammatory cytokine production. After 24h *ex vivo* culture, supernatants of mucosal biopsies were collected and stored at -70°C until cytokines production was quantified using DuoSet Enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Abingdon, UK).

2.4 Measurement of cytokine production by ELISA

Ninety-six-well microplates (VWR International) were coated with anti-IL-6 (360 µg/mL; R&D Systems), anti-IL-8 (720 µg/mL; R&D Systems) anti-IL-24 (360; µg/mL R&D Systems) anti-TNF-α (480 µg/mL; R&D Systems) IL-1β (480 µg/mL; R&D Systems) and sIL-6R (360 µg/mL; R&D Systems) overnight at room temperature. The plates were washed 3 times with PBS 0.05% Tween (Sigma-Aldrich, Poole, UK) before being blocked with PBS 0.1% bovine serum albumin (BSA) (IL-6, IL-24, TNF-α, IL-1β and sIL-6R) or 0.01% BSA (IL-8) for 1h at 37°C. The plates were then washed and incubated with detection antibodies for 2 h and washed again, incubated with serially diluted recombinant cytokine standards and diluted samples (1:10 for IL-6, 1:50 for IL-8, undiluited for IL-24, 1:2 for TNF-α 1:5 for IL-1β and 1:5 for sIL-6R) for 2h at 37°C, before being washed again and incubated with biotinylated anti-IL-6 (9µg/ml), anti-IL-8 (3.6 µg/ml), anti-IL-24 (72 µg/ml) anti-TNF-α (4 µg/ml), anti- IL-1β (4 µg/ml) and anti- sIL-6R (2 µg/ml).

Plates were then washed and incubated with Streptavidin-HRP (1:200) for 20 min. After washing, substrate solution was added for 20 minutes at room temperature and finally, without washing 50 µl of stop solution was added to each well. The optical density of each well was determined immediately after using a microplate reader at 450 nm.

2.5 LPMCs isolation and culture

LPMCs were isolated as previously described (Di Sabatino et al., 2007). Briefly, in order to remove both epithelial cells and intraepithelial lymphocytes, the mucosa was incubated for 10 min at 37°C in calcium-and magnesium-free Hanks's balanced salt solution (HBSS) (Life Life Technologies, Paisley, Scotland) with 1 mM EDTA (Sigma-Aldrich, Poole, UK) for three times. After stirring for 1h at 37°C, the supernatant was removed and the remaining tissue was disrupted mechanically into smaller pieces of approximately 3-4 mm and incubated, for1 h, with continuous agitation at 37 °C in RPMI medium (Sigma-Aldrich, Poole, UK) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, Poole, UK), 100 U/ml penicillin and 100 µg/ml streptomycin, and treated with 1A collagenase (1 mg/ml; Sigma-Aldrich, Poole, UK) and DNase I (10 U/ml; Roche, Mannheim, Germany). After washing, LPMCs were isolated with Ficoll density centrifugation using a Ficoll-Paque Plus gradient (Amersham Pharmacia Biotech, Uppsala, Sweden) following the manufacturer's protocol, and kept on ice until used.

Freshly isolated LPMCs were cultured (1×10^6 cells/well) in complete medium, at 37◦C, 5% CO2 for 4 h in 24-well plates (VWR International) with medium alone or with Lipopolysaccharide (LPS) (100 ng/m; Sigma-Aldrich, Poole, UK) or with GW280264X (10µM/ml) or dimethyl sulfoxide (DMSO) (1µg/ml; SignaAlrich, Pool,UK) or phorbol 12-myristate 13-acetate (PMA) (50 ng/ml; Sigma-Aldrich, Poole,

UK) + Ionomycin. After culture, LPMCs supernatants were collected and stored at −70◦C.

2.6 PMA + ionomycin stimulation

Two million cells from normal colon were incubated with and without PMA and ionomycin for 4 h. After 2h, 2μm of monensin (BioLegend, Cambridge, England) were added in order to prevent cytokine secretion. Cells were washed in PBS and then incubated with live/dead® fixable blue dead cell stain kits (Molecular Probes; Life Technologies, Paisley, Scotland) as per the manufacturer's guidelines. From this point supernatant was collected and stored at -80°C and cells were stained.

2.7 Isolation of PBMCs and cell culture

Peripheral blood mononuclear cells (PBMCs) were obtained from healthy volunteers, CD and UC patients after informed consent was obtained. Briefly, the blood was diluted 1:1 in PBS (without Ca^{2+}, Mg^{2+}) and 25 ml loaded on Ficoll in 1:1 ration. After centrifugation at 21°C for 20 minutes at 1500 rpm the buffy coat was collected into a 50 ml tube and washed twice in PBS (without Ca^{2+}, Mg^{2+}). Finally, the pellet was resuspended in 1 ml PBS (without Ca^{2+}, Mg^{2+}) and cells were counted using haemocytometer. Cells were then cultured in RPMI 10% human serum/penicillin/streptomycin/L-glutamine.

$CD14^{+}$ human monocytes were purified from PBMCs by positive selection using anti-CD14 magnetic beads following manufacture instruction (Miltenyi Biotec Bergisch Gladbach, Germany).

2.8 Flow cytometry and antibodies

2.8.1 Surface staining

After isolation of LMPCs, $1x10^6$ cells were washed in 1 ml of FACS buffer, resuspended in 100 µl of FACS buffer (PBS + 2mM EDTA + 2% BSA), and then incubated at 4 °C with 20 µl of Human Fc Receptor Binding Inhibitor (eBioscience; Hatfield, England) to reduce non-specific binding via Fc receptors, then incubated with the relevant primary antibodies (as detailed in Table 2.1) for 20-30 minutes at 37°C protected from the light. Cells were then washed three times in ice cold FACS buffer and analysed using LSRII flow cytometry (BD Bioscience Oxford, England). Dead cells were excluded from analysis by adding 0.25 µg/test of 7-aminoactinomycin D (7-AAD; BD Biosciences, Oxford, England) to each sample immediately before analysis. Appropriate isotype controls were included in all experiments. As detailed in Table 2.1, isotype controls were purchased from the manufacturer of the antibodies. All data generated were analysed using FlowJo software (Tree Star Inc, OR, USA).

ANTIBODY	Clone	Isotype	Company	Format
CD3 CD3	HIT3a WM53	Mouse IgG2a, κ Mouse IgG1, κ	Biolegend Biolegend	PE APC
CD45 CD45	HI30 HI30	Mouse IgG1, κ Mouse IgG1, κ	BD Biosciences Biolegend	APC Pacific Blue™
HLA-DR HLA-DR HLA-DR	TU36 G46-6 (RUO) L243	Mouse IgG2b, κ Mouse IgG2a, κ Mouse IgG2a, κ	BD Biosciences BD Biosciences Biolegend	PE PE-Cy5 PerCP-Cy5.5
CD33 CD33	WM53 WM53	Mouse IgG1, κ Mouse IgG1, κ	Biolegend Biolegend	APC PE
CD68 CD68	Y1/82A Y1/82A	Mouse IgG2b, κ Mouse IgG2b, κ	Biolegend Biolegend	FITC PerCP-Cy5.5
CD14 CD14 CD14 CD14	HCD14 HCD14 M5E2 M5E2 (RUO)	Mouse IgG1, κ Mouse IgG1, κ Mouse IgG2a, κ Mouse IgG2a, κ	Biolegend Biolegend Biolegend BD Biosciences	FITC PerCP-Cy5.5 Pacific Blue™ BV650
CD206	15-2	Mouse IgG1, κ	Biolegend	APC-Cy7
CD64	10.1	Mouse IgG1, κ	Biolegend	PE
TNF-α	MAb11	Mouse IgG1, κ	Biolegend	PE-Cy7
Membrane TNF-α	Cl 6401	Mouse IgG1	R&D	Fluorescein
Lineage cocktail	UCHT1, HCD14, 3G8, HIB19, 2H7, HCD56	Mouse IgG1, κ; Mouse IgG2b,κ	Biolegend	Lineage cocktail

Table 2.1: List of Monoclonal Antibodies for Flow Cytometry: Primary antibodies were conjugated to either APC, FITC, PE, PerCP-Cy5.5, PE-Cy5, PE-Cy7, APC, APC-Cy7, Alexa fluor 488, BD Horizon V650, PE-Cy5, Pacific blue, Fluorescein.

2.8.2 Intracellular cytokine staining

One million LPMCs were washed with 3 times with FACS buffer and incubated (with or without extracellular markers) for 30 min with 1 µl of Fixable Blue Dead Cell stain kit (for UV excitation, Molecular Probes; Life Technologies Paisley, Scotland) as the manufacturer's guidelines, for 20-30 minutes protected from light.

After 20 min the cells were washed and fixed with 100 µl of Fixation Buffer, (containing 4% formaldehyde from the Intracellular Fixation & Permeabilization Buffer Set, eBioscience; Hatfield, England) in the dark for 15 minutes in ice. Without washing, 1 ml of 1X Permeabilization Buffer (containing 0.1% saponin and 0.09% sodium azide) (Intracellular Fixation & Permeabilization Buffer Set, e Bioscience; Hatfield, England), was added to each tube. Prior to a further incubation with Human Fc Receptor Binding Inhibitor, cells were then resuspended in Permeabilization buffer 1X together with the appropriate fluorochrome-conjugated cytokine-specific antibodies for 20 minutes protected from light. Cells were then washed with 1X Permeabilization Buffer, resuspended in FACS buffer and analysed by flow cytometry.

Data from about 250,000 cells was collected, FSC and SSC were analysed on linear scales. All data generated were analysed using FlowJo software (Tree Star Inc, OR, USA).

2.8.3 Flow cytometry gating strategy

Events were gated to eliminate artefacts caused by poor flow and 7-aminoactinomycin D (7-AAD; BD Pharmingen) was used to exclude dead cells or LIVE/DEAD Fixable Blue Dead Cell (depending on whether the analysis was for extracellular or intracellular staining).

Seven-AAD is a membrane impermeant dye, generally excluded from viable cells which bind to double stranded DNA by intercalating between base pairs in G-C-rich regions. The LIVE/DEAD® Fixable Blue Stain is an amine reactive dye that binds covalently to intracellular and extracellular amines, preserving the staining pattern following formaldehyde fixation.

The doublets corresponding to cell aggregates were also excluded by the FSC-A/FSC-H and SSC-A/SSC-H profile and single cells were further analysed.

2.9 RNA extraction

RNA was extracted using trizol-chloroform isolation method. The cells were lysed by adding 200 µl of trizol per 10^6 cells after removal of culture media. If tissue material was used for RNA extraction, 400 µl of trizol was used per biopsy size tissue material. The lysates were stored at -70°C for subsequent RNA extraction or used immediately The RNA extraction was performed using the Qiagen RNeasy kit (Qiagen, Manchester UK). First, the trizol lysates were mixed with $1/5^{th}$ volume of chloroform and incubated for 5 minutes on a shaker (650 rpm) at room temperature and centrifuges at 16,000xg for 3 minutes. The clear aqueous phase was transferred to a clean eppendorf and an equal volume of 70% ethanol was added to each sample at the time. The samples were applied to a Qiagen RNeasy mini-column and centrifuged for 30 seconds at 9,000xg and washed two times with RW1 buffer (provided in the kit). Additionally, DNase digestion was included for more complete DNA removal using Qiagen RNase-free DNase Set. The DNase-1 stock was initially prepared following the manufacturer's instruction and 10 µl of DNase-1 stock solution was dissolved in 70 µl of RDD buffer (provided in the kit) and 80 µl of final volume was applied onto each column and incubated for maximum 15 minutes at room temperature. Next, the columns were washed twice with

RPE buffer (provided in the kit) and eventually columns were transferred to fresh eppendorfs and RNA was eluted using RNase-free water, also provided in the kit. RNA was quantified using a Nanodrop spectrophotometer (Thermo Scientific) according to the manufacturer's instruction. Samples were stored at -70°C.

2.10 Reverse Transcription and RT2 Profiler Array PCR

Prior to RT^2 Profiler Array PCR, reverse transcription was performed with the RT2 First Strand Kit (Qiagen, Manchester Limited, UK) with 340 ng of RNA per sample accordingly to manufacturer's instructions in a maximum volume of 8 µl. A preliminary genomic elimination step was performed by adding 2 µl of GE buffer/sample (included in the kit). Samples were incubated at 42°C for 5 minutes. Next, to set up the reverse transcription 4 µl of 5XBC3 buffer, 1 µl of P2 buffer, 2 µl RE3 buffer and 3 µl of RNase-free water were added to each RNA sample (all buffers were included in a kit). Prepared samples were incubated at 42°C for 15 minutes and then 95°C for 5 minutes on the thermal cycle. After incubation, samples were cooled on ice for 1 minute and 91 µl of RNase-free water was added to each sample and all was mixed very gently. Next, 102 µl of cDNA material was added to PCR premix (448 µl of RNase-free water mixed with 550 µl of RT2 Syber Green ROX qPCR Mastermix (Qiagen, cat no: 330401). Finally, the master mix (10 µl/well) was loaded onto Human Common Cytokine RT2 Profiler PCR Array plate (Qiagen PAHS-021E-4). The plate was centrifuged for 5 minutes at 524xg and run on ABI7900HT (Applied Biosystems) with cycling conditions: 1 hot start cycle at 95°C for 10 minutes; 40 PCR cycles of 95°C for 15 seconds, and 60°C for 1 minute, then thermal denaturation cycle of 95°C for 15 seconds

(ramp rate 100%), 60°C for 15 minutes (ramp rate 100%), and 95°C for 15 seconds (ramp rate 2%).

The raw Ct values were normalized to GAPDH and converted to copy numbers. Undetected genes were eliminated from further analysis. Fold changes and P-values were obtained by fitting a linear model to the normalized copy number for each gene. Table 2.2 represent the set of all 84 genes included in RT-PCR Assay.

BMP1	BMP2	BMP3	BMP4	BMP5	BMP6	BMP7	BMP8B	CSF1	CSF2	FAM3B	FASLG
FIGF	GDF10	GDF11	GDF2	GDF3	GDF5	MSTN	GDF9	IFNA1	IFNA2	IFNA4	IFNA5
IFNA8	IFNB1	IFNG	IFNK	IL10	IL11	IL12A	IL12B	IL13	TXLNA	IL15	IL16
IL17A	IL17B	IL17C	IL25	IL18	IL19	IL1A	IL1B	IL1F10	IL1F5	IL1F6	IL1F7
IL1F8	IL1F9	IL2	IL20	IL21	IL22	IL24	IL3	IL4	IL5	IL6	IL7
IL8	IL9	INHA	INHBA	LEFTY2	LTA	LTB	NODAL	PDGFA	TGFA	TGFB1	TGFB2
TGFB3	TNF	TNFRSF11B	TNFSF10	TNFSF11	TNFSF12	TNFSF13	TNFSF13B	TNFSF14	TNFSF4	CD70	TNFSF8
B2M	HPRT1	RPL13A	GAPDH	ACTB	HGDC	RTC	RTC	RTC	PPC	PPC	PPC

Table 2.2: List of 84 genes panel for RT-PCR Array Fields in gray and green represent housekeepers and controls, respectively. The reaction was set for 340 ng per sample/genes panel.

2.11 Statistical analysis

Statistical analyses were performed using GraphPad Prism version 4 (GraphPad Software, San Diego, CA) using the unpaired t test, some samples were also analysed using paired T-test when appropriate. Statistical analyses was assumed if the p value was <0.05.

CHAPTER 3

Results: Phenotypic characterization of intestinal macrophages in the inflamed and normal colon

3.1 Introduction

It is known that inflammatory macrophages are recruited to the intestine in CD and UC (Heinsbroek and Gordon, 2009). A variety of different receptors play a role in generating and facilitating inflammatory macrophages in pathogenesis of IBD. However, the roles of macrophages and their receptors in IBD are still poorly understood (Mowat and Bain, 2011).

This chapter describes the strategies I developed to isolate different populations of macrophages from the gut of IBD patients and those with colon cancer.

3.2 Gating strategy

Phenotypic analysis of intestinal macrophages was conducted in order to have a more detailed characterization of these populations. Macrophages were isolated from LPMCs prepared from resected bowel of patients with CD, UC or colon cancer by collagenase digestion and enrichment using density gradient centrifugation. Cells were then analysed by flow cytometry in order to determine their surface antigen, intracellular antigen and cytokine expression.

Using this procedure, a heterogeneous population of cells was identified by adjustment of FSC and SSC properties (Figure 3.1, G5). These two parameters highlighted the difference in size and granularity allowing the exclusion of lymphocytes from the rest of the cells. The same gating procedures were applied on control, CD and UC samples.

Approximately more than 50% of the live-gated cells expressed CD45, indicating they were populations of haematopoietic origin (Figure 3.2). The other cells were probably fibroblasts and a few contaminating enterocytes. A control isotype matched antibody labelled with the same fluorochrome as the specific anti-CD45 mAb was used to identify $CD45^+$ population within the LPMCs.

3.3 CD33 expression by control and IBD LPMCs

To increase the understanding into the phenotypic heterogeneity of the different macrophage populations, the expression of various myeloid cell markers was assessed. The marker CD33 was used to identify the entire myeloid compartment of $CD45^+$ 7-ADD^- cells in the LPMCs of control, CD and UC patients.

CD33 is a 67 kDa glycoprotein member of the Ig superfamily, expressed in early myeloid progenitors. It is a member of the sialoadhesin family of sialic acid-dependent cell adhesion molecules (Munday et al., 1999) but the function and binding properties of CD33 are still unknown.

As shown in Figure 3.3 there is no significant difference in the percentages of $CD33^+$ cells between the control, CD and UC groups. Seventeen ± 2.4% of LPMCs in normal colon expressed CD33, and in the LPMCs of CD and UC patients, 15.6 ± 2.7% and 17.4 ± 2.5% were respectively $CD33^+$.

To exclude lymphocytes which may be $CD33^+$ a gating strategy was adopted in the initial experiments for CD3 and CD33 expression. Using LPMCs from normal colon, total myeloid cells were identified amongst live leukocytes ($CD45^+$ live/$dead^-$) as $CD33^+$ and $CD3^+$ lymphocytes within the same gated cells ($CD45^+$ Live $dead^-$) (Figure 3.4). The absence of double-positive expression on LPMCs shows that the majority of CD33 myeloid cells are $CD3^-$. Moreover a much smaller population (Figure 3.4 B) of $CD3^+$ cells are $CD33^+$ which can be clearly distinguished from the larger myeloid $CD33^+$ cells (Figure 3.4 C).

3.4 CD68 and CD33 expression by control and IBD LPMCs

The intracellular marker CD68 (the human homologue of mouse macrosialian) is the gold standard macrophage marker in man, CD68 is a heavily glycosylated protein

localised in the lysosomal membranes of macrophages (Smythies et al., 2005). Although the biologic functions of CD68 have not been fully defined, it is known that CD68 serves as a scavenger receptor for oxidized low density lipoproteins and may also be involved in cell-cell interactions (Ramprasad et al., 1996). Consistent with previous findings, some cells in live^{+} gated LPMCs (Figure 3.5 A) express intracellular CD68, in contrast to the CD3^{+} population, which is negative for CD68.

To determine whether CD68-expressing macrophages were increased in UC and CD, LPMCs from 13 patients with CD, 10 patients with UC and 10 control patients were analysed. Control LPMCs expressed a markedly lower percentage of CD68^{+} cells than the LPMCs from IBD patients (Figure 3.5 C).

The next studies were therefore to determine in more detail other markers on CD45, CD33 and CD68 cells. Double-staining for intracellular CD68 and CD33 was performed in LPMCs from IBD patients and controls (Figure 3.6). The percentage of double positive CD68^{+}CD33^{+} macrophages in control LPMC was significantly lower (5.7 ± 0.9%) than in the CD LPMCs (11.4 ± 2.1%). Moreover the frequency of CD33^{+}CD68^{+} LPMCs in UC (11.77% ± 2.2%) was significantly higher than normal mucosa.

3.5 Identifying dendritic cells in the gut

One of the main difficulties in achieving a better understanding of intestinal macrophages during steady state and inflammatory conditions is discriminating them from dendritic cells which also express CD33.

To do this, were first identified macrophages as lineage^{+} HLA-DR^{+} and dendritic cells as lineage^{-} HLA-DR^{+} cells. Dendritic cells and macrophages show two distinct populations with differential expression of CD33 (Figure 3.7). While 78.2 ± 5.5% of

cells in the lineage$^+$ HLA-DR$^+$ population expressed CD33, the percent of dendritic cells expressing CD33 was 36.0 ± 4.3%.

3.6 CD33 as an alternative to CD68 for functional application

Functional studies need the development of relatively rapid isolation of pure populations of cells. Therefore the next experiments were designed to compare MACS as opposed to FACS methodology.

CD33 and CD68 were used to stain LPMCs and the cells were sorted by FACS Aria. It took 3-4 hours to run all the cells and ended up isolating an average of 15% of CD33$^+$CD68$^+$ cells, with a purity of 85 ±0.8%. Thus, FACS provided a relatively high purity of CD33$^+$ CD68$^+$ cells but the sort time was too long and expensive. A major issue however is also that CD68 is an intracellular marker and so cells have to be permeabilised, which renders them unusable for functional studies.

Also, since this project is part of a more extensive study conducted with fellow PhD student Aneta Kucik, who performs Chromatin Immunoprecipitation (ChIP) on isolated intestinal macrophages, cell isolation techniques were optimised in order to obtain a higher cell yield.

MACS technology was tested next, using CD33 as a marker for macrophages. For intestinal macrophages MACS separation overall was better than FACS, although it reached only 5.6% ± 0.5 of the LPMCs, but a greater degree of purity was achieved as compared to sorting (close to 98±0.6%) after ~ 1.5 h (Figure 3.8).

Hence, MACS sorting achieves the highest purity of cells in a shorter time but has poorer yields. For the purpose of functional studies it was also important for us to determine the cell yields from control, CD and UC tissues by MACS sorting. Table 3.1 shows the number of cells obtained after isolation of LPMCs using CD33$^+$ magnetic

beads. It was possible to obtain an average of 5.4 (±1) x10^6 $CD33^+$ cells, starting from 100(±17) x10^6 LPMCs cells in control tissues, 4.5(±0.4) x10^6 $CD33^+$ cells from 67.8 (±11) x10^6 LPMCs in CD, and 6.8 (±2.2) x10^6 $CD33^+$ cells starting from 114.1(±28.4) x10^6 LPMCs) in UC.

3.7 CD14 and CD33 expression by control and IBD LPMCs

CD14 is the classical surface marker used to identify macrophages and it was therefore of interest to determine if the LPS unresponsive macrophages in normal LPMC express CD14 in control tissues and in IBD tissues (Figure 3.9).

In normal LPMC, $CD33^+$ cells were mostly $CD14^-$. However in inflamed and uninflamed IBD tissues there was a large increase in $CD14^+$ $CD33^+$ cells.

As shown in the figure 3.9 B there was a significant increase of $CD33^+CD14^+$ LPMCs in the inflamed (9.9% ± 2.7, p=0.01) colon of UC patient compared to controls. Also, the mean percentage of $CD33^+CD14^+$ LPMCs in the inflamed colon of CD patients (5.2% ± 0.9) was significantly (p= 0.04) higher compared to controls.

3.8 Discussion

The results presented in the first results chapter describe the approach of distinguish intestinal macrophages by studying the best gating strategy in order to exclude most of the cells of the lamina propria which were not macrophages. In more detail it was showed that the CD33 myeloid marker is expressed both in control and IBD tissues, that high upregulation of $CD68^+$ LPMCs is seen in the LPMCs of IBD patients, and that the frequency of $CD33^+CD14^+$ LPMCs was greater in the inflammatory intestine of active CD and UC patients relative to non-IBD control patients. Of interest the proportion of $CD33^+CD14^+$ LPMCs was higher in UC patients.

Previous studies have described the significantly increase of inflammatory macrophages in the patients with IBD (Kamada et al., 2008). It was also shown that $CD14^+$ and TLR^+ myeloid cells are increased in the lamina propria of patients with IBD (Rogler et al., 1998) (Smythies et al., 2005) and that proinflammatory macrophages, such as TREM-1^+ macrophages, are increased in intestinal mucosa and contributed to the intestinal inflammation (Schenk et al., 2007).

The characterization of intestinal macrophages has been extremely hard for many reasons. In this chapter I set out to optimise a protocol for the purification and characterisation of myeloid cells in the human colon using multi-parameter flow cytometry.

At the beginning of the project I was specifically interested in testing CD33 as a marker, based on previous findings by Kamada and colleagues (Kamada et al., 2008). By using anti-CD33 I was able to define heterogeneous myeloid subsets in control and inflammatory conditions. Hence myeloid cells from LPMCs of control, CD and UC patients, inflamed or non-inflamed, were identified amongst live leukocytes

($CD45^+Live^+$) as $CD33^+$ and were seen in LPMCs from control, CD and UC tissues (Figure 3.3). In contrast to CD33, CD68 expression by LPMCs was higher in inflamed IBD, especially in CD (Figure 3.5) compared to normal mucosa. This is consistent with CD showing a more mononuclear cell infiltrate. Moreover neither $CD33^+$ nor $CD68^+$ populations expressed CD3, ensuring that the population of myeloid cells were likely to represent just myeloid cells with no lymphocyte contamination (Figure 3.3 and 3.5).

The presence of $CD68^+$ cells in control mucosa has been recognised for many years. In the colon, these cells reside just below the epithelium, optimally sited to phagocytose invading pathogens and commensals that leak through the barrier. Of special interest was the number of macrophages which significantly increase in IBD. The increased numbers of $CD68^+$ cells in inflamed mucosa was shown by immunofluorescence many years ago and their co-expression of CD14 suggests recruitment of new monocytes/macrophages from peripheral blood (Rugtveit et al., 1994).

CD33 is not a specific marker for macrophages but it is also expressed on granulocytes and dendritic cells (Lock et al., 2004). However the methodology used to process immune cells from lamina propria require the step of using a density gradient centrifugation which prevents the inclusion of the granulocyte compartment. Hence the only major adversity in using CD33 as unique marker for macrophages is that dendritic cells, express CD33. However our results using lineage markers and HLA-DR showed that most of the CD33+ cells were macrophages.

It has been reported that human intestinal macrophages do not express typical macrophage innate-immune receptors, such as CD14 or TLRs, and that they exhibit anti-inflammatory anergic phenotypes (Smith et al., 2001). Consistent with the anergic nature of intestinal macrophages, my data showed that some $CD33^+$ macrophages in

control mucosa express CD14 but the proportion of cells expressing CD14 is lower in comparison to cells from IBD patients (Figure 3.9). The number of $CD33^{+}CD14^{+}$ LPMCs subsets was increased not only in inflamed mucosa but also in uninflamed mucosa of CD and UC. The significant accumulation of $CD14^{+}$ LPMCs that expands preferentially in IBD inflamed tissue may suggest two possibilities; the first is that these two different intestinal subsets are derived from different subsets of monocyte, such as inflammatory or resident monocytes as previously reported (Geissmann et al., 2003; Gordon and Taylor, 2005). The second suggests that monocytes enter the colonic mucosa and differentiate in macrophages in situ with acquisition of certain markers and loss of others such as the typical macrophage innate-immune receptors, such as CD14 or TLRs (Smythies et al., 2005).

	Number of Cells before Sorting ±SD (*10^6)	Number of Cells after Sorting(*10^6)	% of Cells Yields after Sorting
Control	100 ±17.0	5.4 (±1.0)	6.0 (±0.8)
CD	67.8 ±11.0	4.5 (±0.4)	6.1 (±0.7)
UC	114.1(±28.3)	6.8 (±2.2)	4.8 (±0.9)

Table 3.1: Numbers and percentage of $CD33^+$ LPMCs obtained after MACS sorting.

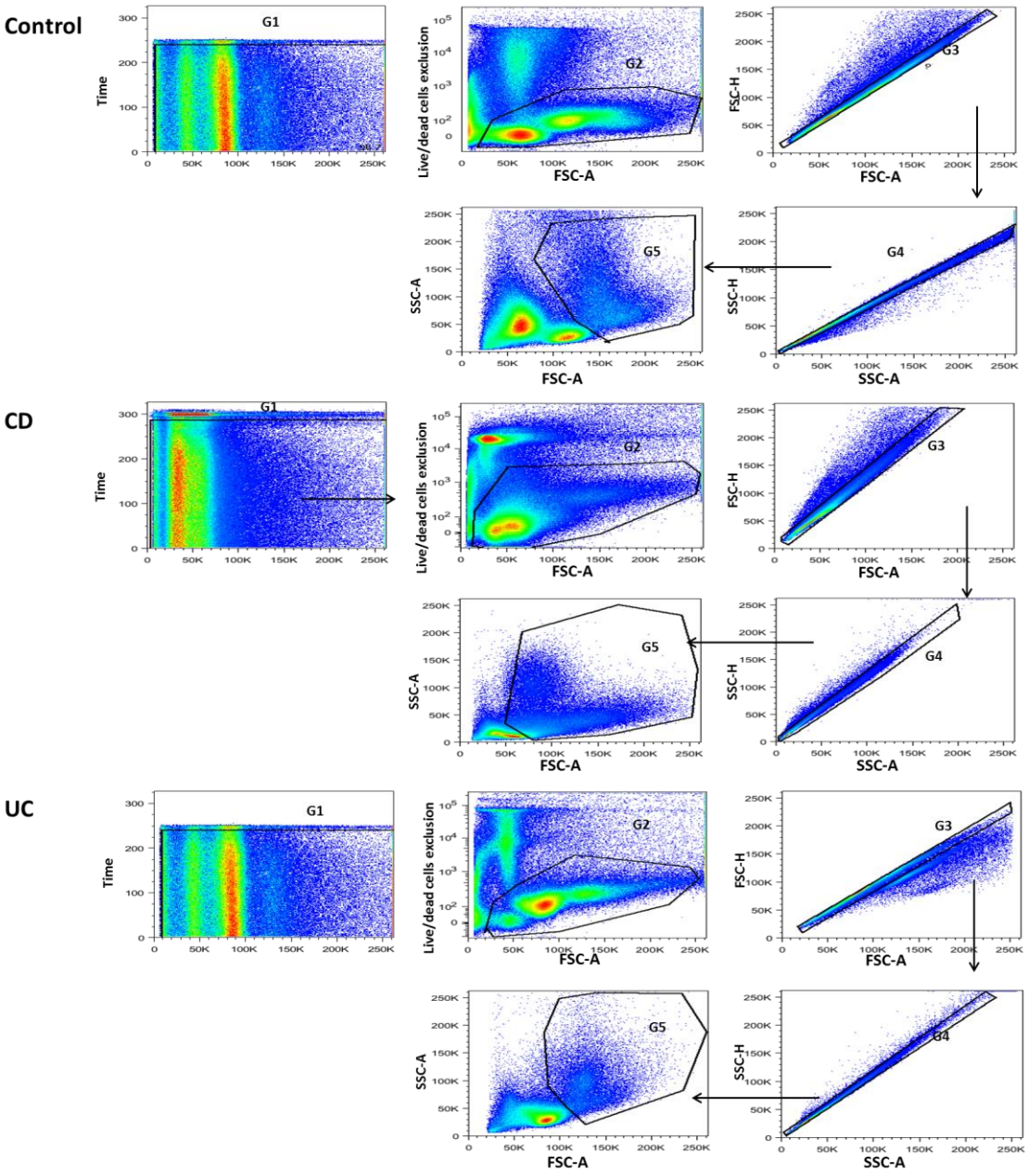

Figure 3.1: Gating strategy. LPMCs were isolated from control, Crohn's disease (CD) and ulcerative colitis (UC) tissues. Dead cells were excluded using 7-aminoactinomycin (7-AAD) or LIVE/DEAD Fixable Blue Dead Cell (**G2**). FSCA/FSC-H and SSC-A/SSC-H were used to discriminate doublets (**G3-G4).** Representative dot plots showing the identification of single cells based on SSC-A vs FSC-A was used to discriminate lymphocytes from the rest of the cells (**G5**).

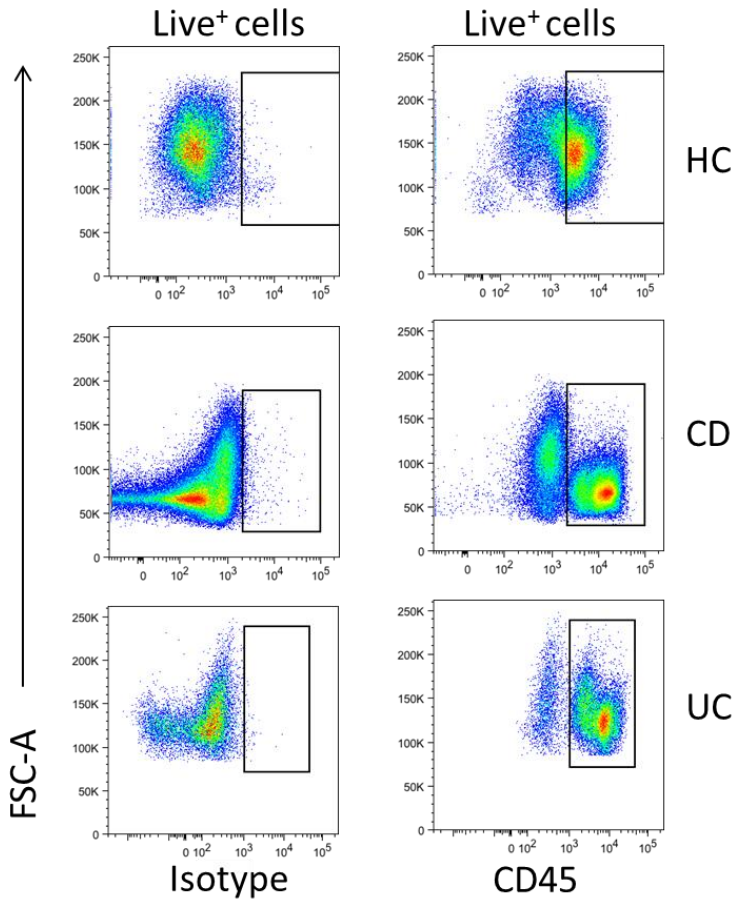

Figure 3.2: Refined gating strategy for analysis of LPMCs. Representative dot plot showing the expression of CD45 by $FSC^{+}SSC^{+}$ live LPMCs from control , CD and UC tissues . Gating strategy employed to define $CD45^{+}$ cells by comparing specific anti mAb staining with a matched fluorochrome conjugated isotype.

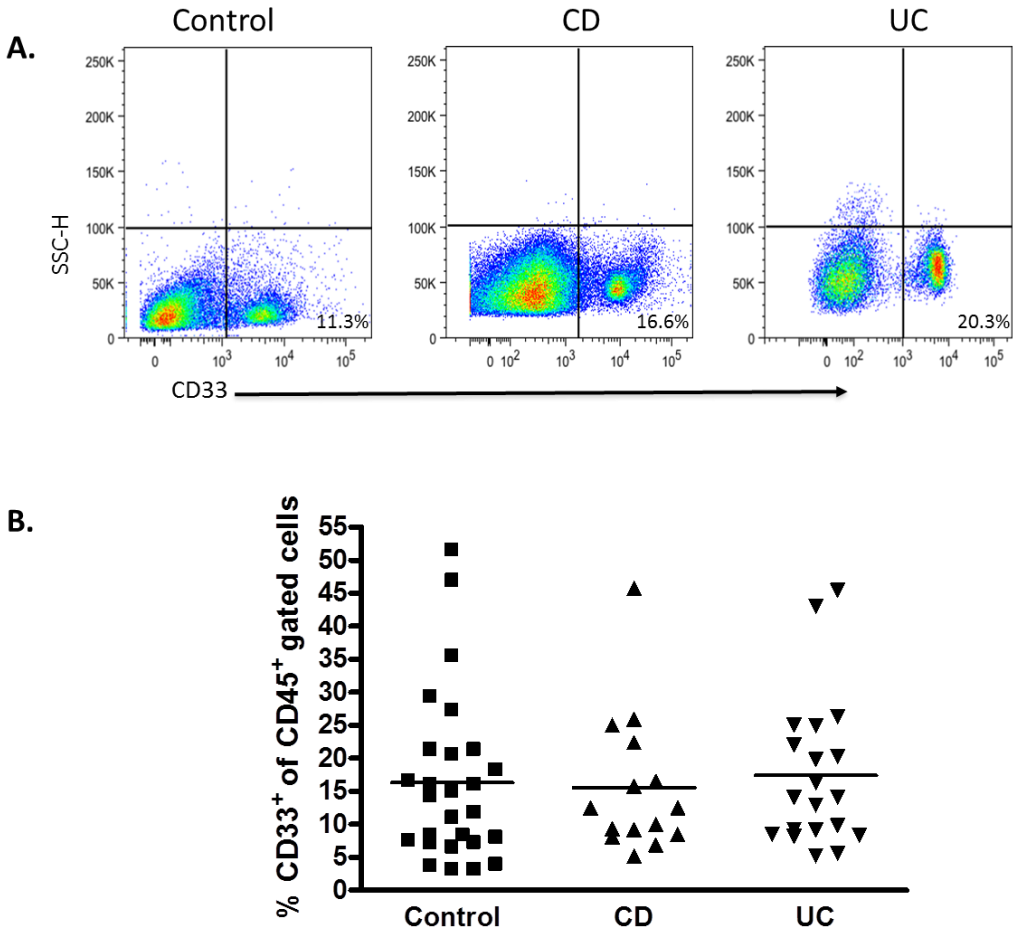

Figure 3.3: Myeloid cell populations defined by CD33 expression. A. Representative dot plots of CD33 staining on $CD45^+$ live LPMCs cells. **B.** The mean proportion of $CD33^+$ cells within each population. The percentage of $CD33^+$ cells shows no significative difference between control, CD and UC tissues. Results are representative of three individual experiments with N=27 for control experiment, N=15 for CD and N=20 for UC.

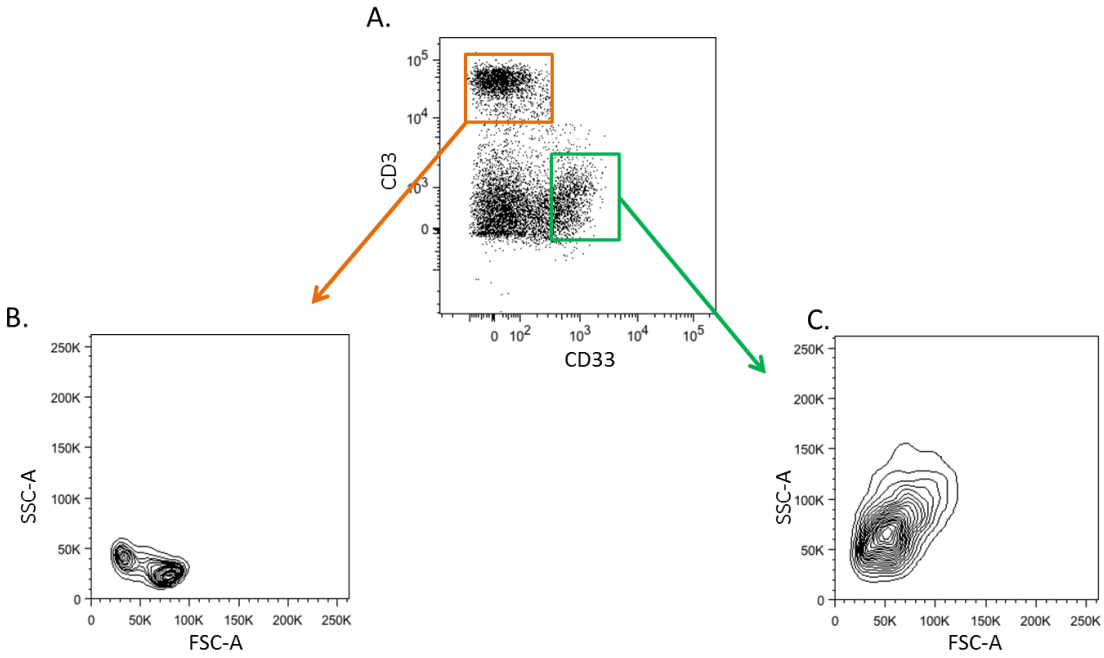

Figure 3.4: FSC/SSC properties of LPMCs. LPMCs pooled from 3 control patients were analysed for the expression of CD33 and CD3 by flow cytometry. **A.** Representative dot plot of CD33 and CD3 expression by live-gated $CD45^+$ cells and FSC and SSC properties of the individual subsets are shown. **B.** Results are representative of at least three experiments.

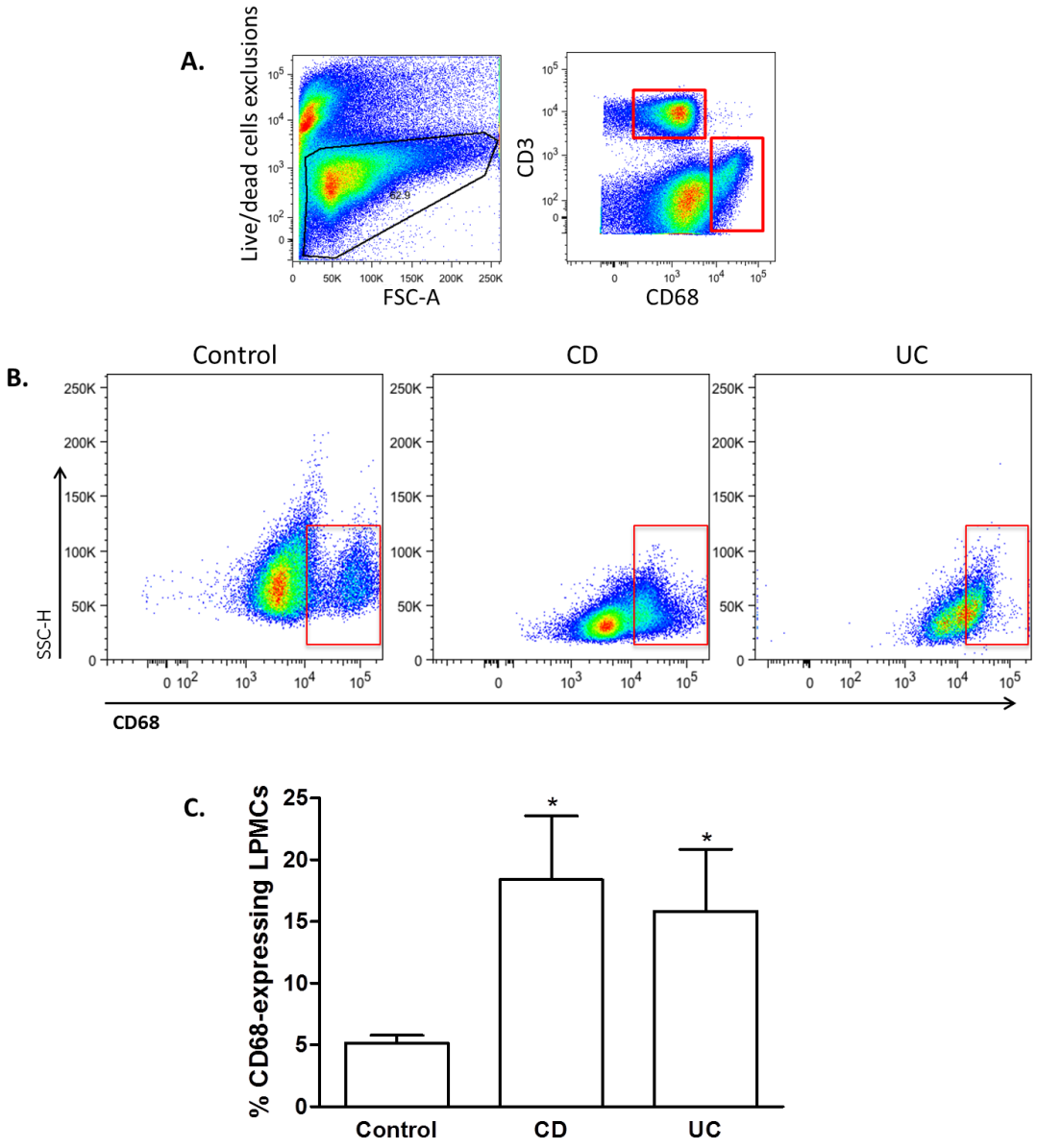

Figure 3.5: CD68^{+} cells in normal and IBD mucosa. Live-gated LPMCs **A.** from control patients were assessed for the expression of surface CD3 and cytoplasmic CD68 expression. **B.** Representative plots showing CD68 expression by control, CD and UC tissues. **C.** The data shown are representative of n=14 control subjects, n=14 inflamed mucosa of CD and n=10 inflamed mucosa of UC patients.*p (CD versus Control) = 0.0143; *p (UC versus Control) =0.0203.

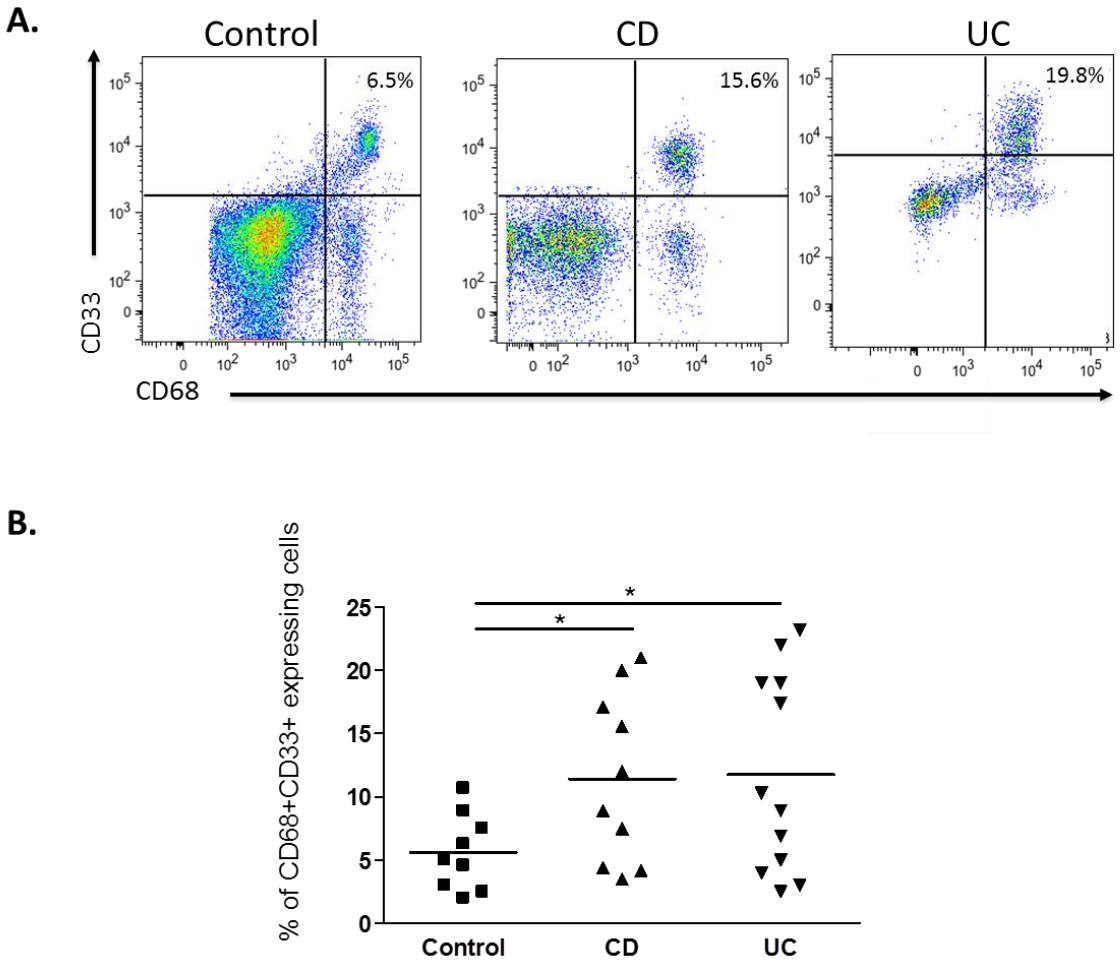

Figure 3.6: Percentage of CD33^{+}CD68^{+} LPMCs in control and IBD mucosa. LPMCs from control subjects and from patients with active IBD were analysed by FACS for CD68 and CD33 expression. **A.** Representative dot plots showing CD68 and CD33 expression by live-gated (CD45^{+}) LPMCs obtained from control, CD and UC tissues. **B.** CD33^{+}CD68^{+} expression in controls (n=9) was lower than in in IBD samples CD (n=10) and UC (n=12) ($p<0.05$).

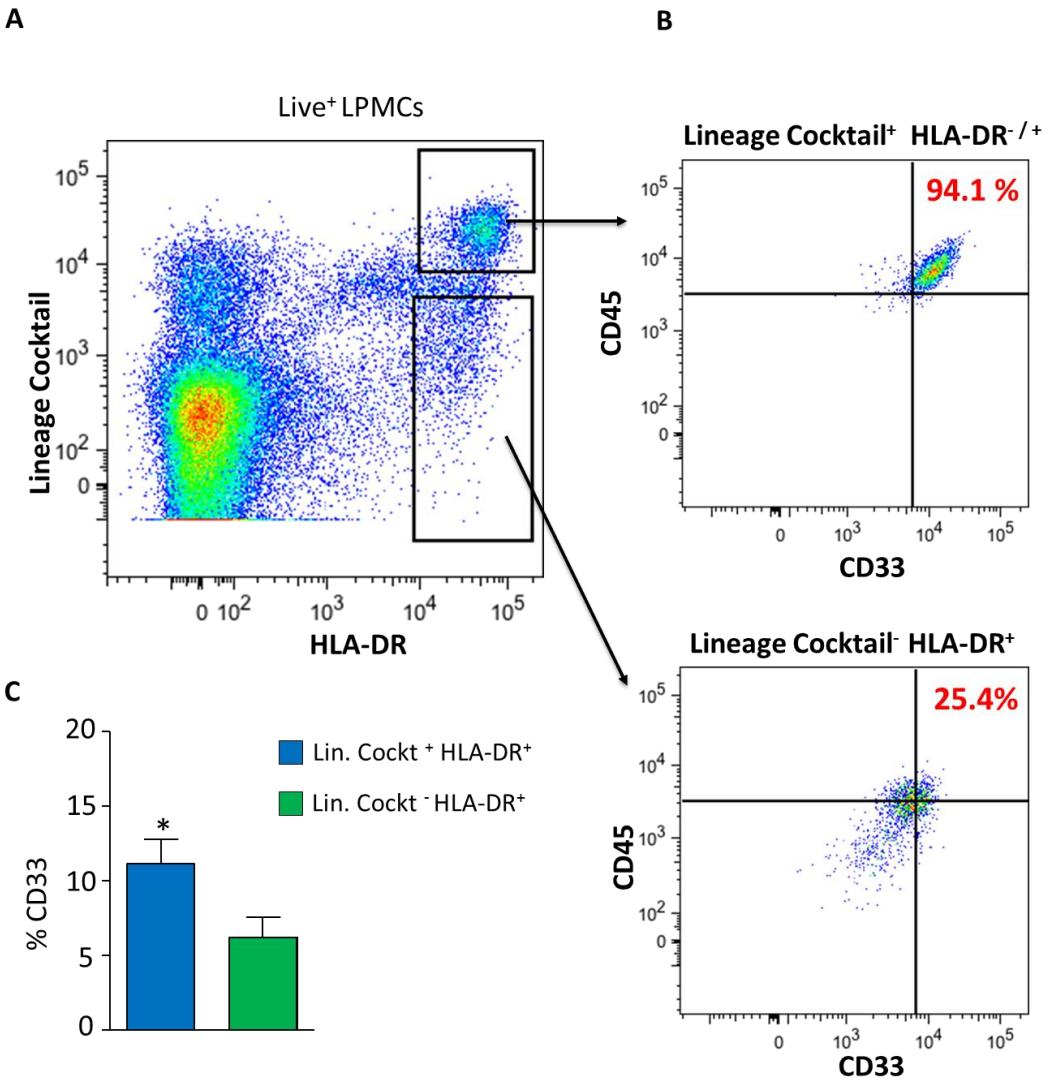

Figure 3.7: DCs and macrophages in LPMCs: LPMCs from control samples were analysed by FACS for Lineage markers, HLA-DR, CD33 and CD45. **A.** Representative lineage cocktail and HLA-DR expression by LPMCs. **B.** Representative CD45 and CD33 expression by DC and macrophages. **C.** Percentage of Lin^{+} $HLA\text{-}DR^{+}$ and Lin^{-} $HLA\text{-}DR^{+}$cells expressing CD33. Data are representative of n=6 independent experiments. Bars represent mean percentage of expression and error bars denote SEM.

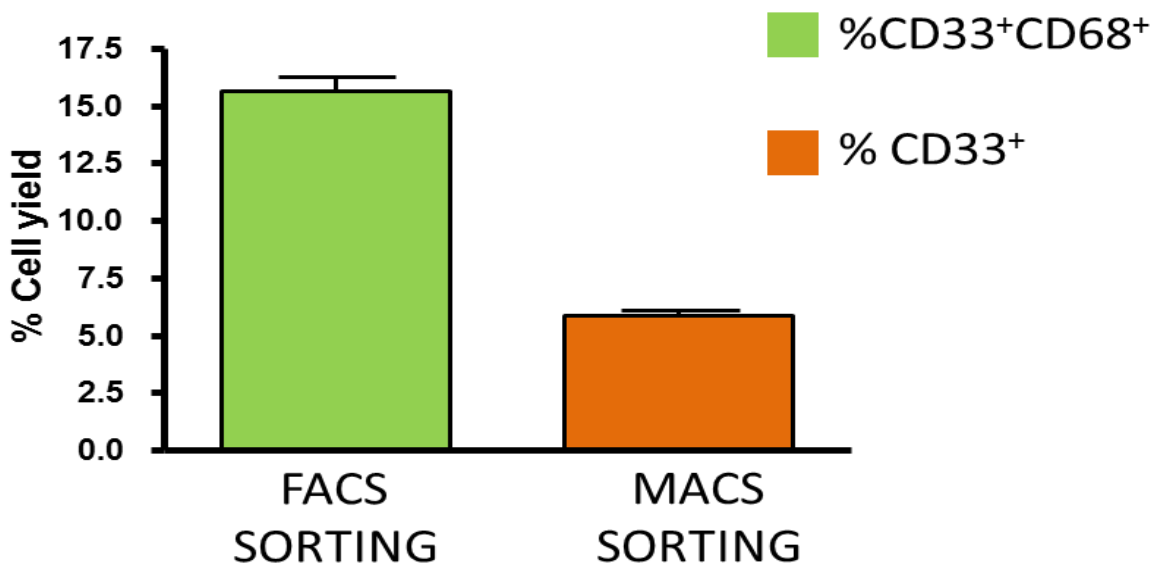

Figure 3.8: FACS sorting of overall LPMCs compared to MACS sorting. Following isolation of LPMCs from control , CD and UC mucosa, $CD33^+$ cells were positively selected by MACS sorting or $CD33^+CD68^+$ by FACS-based purification. The percentage showed for each method is the result of control, CD and UC pooled together (n=5).

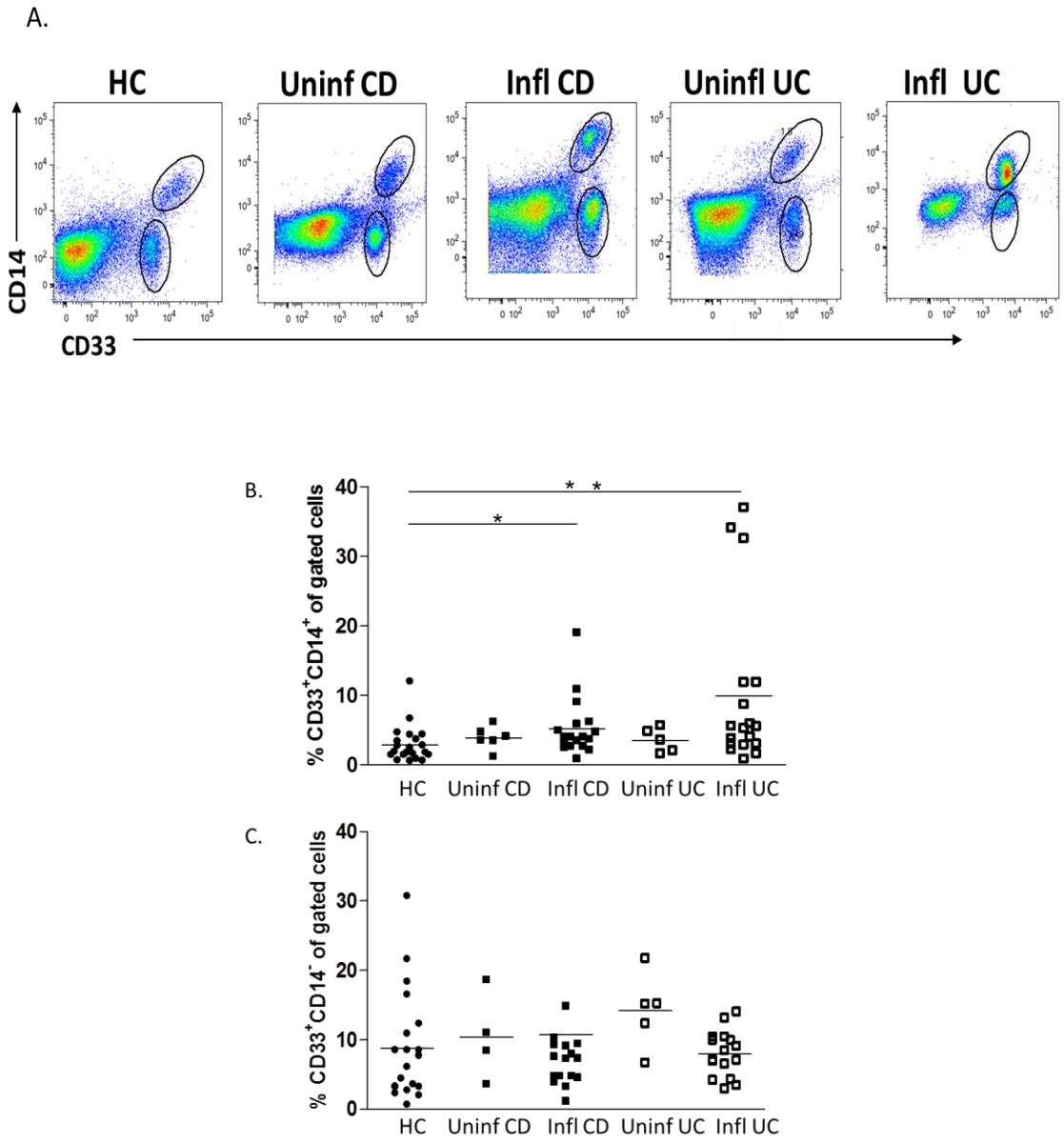

Figure 3.9: $CD33^{+}CD14^{+}$ cells and $CD33^{+}CD14^{-}$ cells analysed for expression of $CD45^{+}$. LPMCs were analysed by FACS for CD14 and CD33 cell-surface expression; two subsets were identified, $CD33^{+}CD14^{+}$ and $CD33^{+}CD14^{-}$. **A.** The plots are representative data of the average values for each experimental group. **B.** Percentage of $CD14^{+}$ intestinal macrophages among $CD33^{+}$ cells from control tissues, uninflamed tissue from UC patients, inflamed tissue from patients with UC, uninflamed mucosa from CD tissues, and inflamed tissue from patients with CD. *P < 0.05, **P < 0.01. CD14-expressing CD33 LPMCs were increased in the inflamed intestinal mucosa of patients with IBD. Recruited $CD33^{+}CD14^{+}$ LPMCs show a significative increase in inflamed IBD (C) The percentage of $CD33^{+}CD14^{-}$ show no significative difference between control , CD and UC.

CHAPTER 4

Results: Phenotypic characterization of resident and recruited intestinal macrophages in the inflamed and normal colon

4.1 Introduction

In the previous chapter, it was shown the CD33 cells in the gut comprise two main populations that differ in the expression of CD14. Those with low levels of CD14 dominated the normal mucosa, whereas $CD14^{+}$ cells were expanded in the tissues in IBD.

In vitro, macrophages can be categorized into two subgroups namely M1 pro-inflammatory macrophages and alternatively activated M2 macrophages involved in repair processes. Within the M2 population there is also a group of macrophages with immunoregulatory and anti-inflammatory properties that are very similar to those of resident mucosal macrophages (Biswas and Mantovani, 2010; Geissmann et al., 2010b). These "M2-like' macrophages typically express the scavenger receptor CD163 and the mannose receptor CD206, and produce IL-10 as well as tissue remodelling factors such as vascular endothelial growth factor (VEGF) and metalloproteinases (Etzerodt et al., 2010; Fabriek et al., 2007). Initial studies identified CD163, the scavenger receptor for hemoglobin-haptoglobin complexes, as a distinctive M2 marker for macrophages (Kristiansen et al., 2001). More recently it has been demonstrated that CD206, the macrophage mannose receptor, is up-regulated following IL-4 stimulation (Stein et al., 1992). M2 macrophages expressing CD206 produce high levels of IL10, but low levels of TNF-α and IL12 (Fong et al., 2008).

Moreover recent studies have refined the characterization of intestinal mononuclear phagocytes, based on the mutually exclusive expression of CD64 (FcγRI), a marker that discriminates macrophages and monocyte-derived dendritic cells from conventional dendritic cells (Langlet et al., 2012). It was therefore of interest to analyse the expression of these markers on intestinal macrophages in health and disease.

4.2 Expression of CD68 by recruited and resident macrophages

CD68 is the gold-standard marker for macrophages in man. Therefore CD68 expression was analysed on $CD33^+CD14^+$ and $CD33^+CD14^-$ cells. As shown in Figure 4.1, both $CD33^+$ $CD14^+$ and $CD33^+$ $CD14^-$ express CD68. In the normal gut 82.4 ± 4.4% of $CD33^+$ $CD14^+$ cells were $CD68^+$, in CD 63.5 ± 5.1% were $CD68^+$ and in UC 78.3± 9.0% were $CD68^+$. In the normal colon and UC, most samples were over 80% $CD68^+$, but the number was consistently lower in CD. Seventy six ± 7.2% of $CD33^+CD14^-$ cells were $CD68^+$ in normal colon, 60.0 ± 5.4% were $CD68^+$ in CD and 81.2± 9.0% were $CD68^+$ in UC.

4.3 CD206 expression on intestinal macrophages

To explore the hypothesis that there might be a population of M2 macrophages, I therefore assessed the expression of CD206 in the $CD33^+CD14^+$ and $CD33^+CD14^-$ subsets of intestinal macrophages.

As shown in Figure 4.2, 48.0±10.0% of the $CD33^+CD14^+$ cells in control LPMCs and 52.0 ± 15.1% in CD samples expressed $CD206^+$, whereas in UC 35.0 ± 17.3% of the $CD33^+CD14^+$ cells expressed CD206.

In comparison to $CD33^+CD14^+$ population, the $CD33^+CD14^-$ cells had low expression of CD206 with 13.4 ± 5.0% of CD206-expressing $CD33^+CD14^-$ cells in control tissues , 12.0 ± 6.3% in CD samples and 6.0 ± 4.0 % in UC samples.

4.4 CD206 expression on blood monocytes

As was shown in the previous section, in IBD tissue, CD206-expressing $CD33^+CD14^+$ were increased compared to control subject, therefore the expression of CD206 was examined on peripheral blood monocytes from IBD patients and controls (Figure 4.3). Blood monocytes from CD and UC patients showed an increased expression of CD206. In particular the percentage of $CD206^+$expressing $CD14^+CD33^+$ monocytes in CD was 6 times higher than in controls (control: 10.0 ± 4.0%; CD: 59.0 ± 17.8%) whereas in UC, CD206-expressing monocytes were almost 4 times higher than control (37.0±7.0%). Moreover the number of $CD33^+CD14^+CD206^+$ monocytes was significantly different between CD ($p=0.0283$) and UC ($p=0.0127$) and controls.

4.5 Expression of CD64 by intestinal macrophages

The majority of $CD33^+CD14^+$ cells expressed high levels of CD64, as shown in Figure 4.4. Sixty four ± 8.9% of the cells expressed CD64 in control tissues, 99.6 ± 1.0% expressed CD64 in CD tissues and 35.0±17.3% expressed CD64 in UC samples. There was a significant difference in the CD64-expressing $CD33^+CD14^+$ between control and CD ($p=0.0085$) and between CD and UC ($p=0.0064$).

On the other hand, the analysis of $CD33^+CD14^-$ cells showed a significant increase of expression of CD64 in LPMCs from control patients (45.9 ± 10.3%;) compared to CD (14.0 ± 2.5%; $p=0.0298$) and UC (6.0± 2.2%; $p=0.0107$).

Since CD64 has been used in both mice and human to distinguish macrophages from DCs, CD33 negative cells were also investigated for the expression of CD64.

The graph in figure 4.5 shows the percentage of CD64-expressing $CD33^-$ cells in control, CD and UC samples. CD64 is virtually absent on the $CD33^-$ LPMCs in IBD

(CD, 0.5 ± 0.2%; UC, 0.7 ± 0.1%) and is slightly expressed by $CD33^-$ cells from normal colon (3.8 ± 1.2%). So we can conclude that the majority of $CD33^-$ cells in LPMC are not DC's .

4.6 CD64 expression on blood monocytes

In control subjects, nearly 100% of $CD33^+CD14^+$ peripheral blood monocytes displayed surface expression of CD64 (Figure 4.6) and there was no difference in the percentage of CD64-expressing circulating $CD14^+$ monocytes between IBD patients and healthy volunteers.

4.7 Discussion

The aim of this chapter was to study phenotypic diversity in gut macrophage markers in IBD patients compared to normal subjects and also to study diversity between different subsets of macrophages. Therefore CD68, CD206 and CD64 were studied as these are well recognised to delineate functional subsets.

Results showed that intracellular CD68 was highly expressed by $CD33^{+}CD14^{+}$ and $CD33^{+}CD14^{-}$ cells both in control samples and IBD samples. CD206 was equally highly expressed on the surface of $CD33^{+}CD14^{+/-}$ cells in control subjects and CD compared to UC LPMCs but was down-modulated on blood monocytes of healthy volunteers compared to IBD patients. A higher percentage of cells co-expressing CD64 were found in the $CD33^{+}CD14^{+}$ cells from control tissues and CD tissues and in lower part in UC patients compared to the $CD33^{+}CD14^{-}$ cells and there were no differences in the CD64-expressing blood monocytes between the groups.

$CD33^{+}CD14^{+}$ cells and $CD33^{+}CD14^{-}$ cells were mostly $CD68^{+}$ in the normal mucosa and in the inflamed mucosa of UC patients, however in Crohn's disease mucosa, the number of $CD68^{+}$ cells was significantly lower. In view of the fact that in the previous chapter it was shown that CD68 dominated both in CD and UC mucosa (Figure 3.5), the lower proportion of CD68-expressing CD33+ cells, found in CD is not easily explained. However examination of the data in Figure 4.1 shows that in all groups there was considerable variation between individual patients which may have skewed the mean values. We are confident that the $CD33^{+}$ cells were not T cells or DC's.

Macrophages are an essential component of the innate immune system and key players in the regulation of inflammation. They are also involved in other physiological processes such as tissue remodelling. Although they have been studied for many years,

the division of these cells into distinct M1 and M2 subpopulations has been investigated only recently. The dynamic M1 *versus* M2 ratio plays an important role in the development and resolution of inflammation. M1 cells dominate during the early inflammatory phase, whereas M2 cells dominate in the late/repair phase. In chronic disease, this normal progression is arrested during the M1 dominant phase, leading to a chronic inflammatory response and tissue damage. However studies in the human gut are few.

The classical M2 macrophage CD206 marker was highly expressed in the population of $CD33^{+}CD14^{+}$ cells, IBD and control mucosa. Gordon proposed to redefine the concept of alternative macrophage activation limiting this term to activation by IL-4 and IL-13, suggesting that other anti-inflammatory molecules should be regarded as factors which deactivate macrophages (Geissmann et al., 2010b).

Hence, it seems evident that despite the effort to classify in vitro sub-populations of macrophages, with different functions, *in vivo* due to the complexity of intestinal immune environment the situation is much more complex.

$CD33^{+}CD14^{+}CD206^{+}$ cells could be considered classically activated macrophages which lost the pro-inflammatory capacity taking on features of the alternatively activated anti-inflammatory macrophages or they could represent a new population of cells which do not share any feature between the pro- and the anti-inflammatory feature of macrophages.

Conversely $CD33^{+}CD14^{-}$ cells, which do not produce pro-inflammatory cytokines but are highly phagocytic in normal gut (Smith et al., 2001), showed a lower proportion expressing CD206, hence most likely these cells may be anti-inflammatory macrophages in the gut (Sica and Mantovani, 2012).

Finally blood monocytes in healthy controls do not express CD206 but blood monocytes from CD and UC patients contained a significant proportion of CD206 cells. Therefore CD206 expression levels on circulating blood monocytes were significantly increased in patients with active IBD compared to blood monocytes from control subject. This may reflect the general immune activation seen in IBD patients.

For the first time the present study reports an increased proportion of $CD206^{+}CD33^{+}CD14^{+}$ cells in the mucosa of control subjects, CD patients and UC patients and as will be shown in the next chapter, we demonstrate that these CD206+ cells make TNF-alpha.

One of the aims of this project was selecting a combination of useful markers in order to distinguish macrophages population within LPMCs, therefore the markers CD33, CD14 and CD68 seemed ideal candidates. However CD33 and CD14 markers, although if convenient for many reasons, are not completely specific for macrophages, so we were aware that $CD33^{+}$ DCs could be contaminating the gut macrophage populations. This could also explain why the $CD33^{+}CD14^{+}$ and $CD33^{+}CD14^{-}$ cells in all patients were not entirely $CD68^{+}$.

The use of CD64 helped to clarify the sub-population within the LPMCs in control and IBD subjects. The importance of CD64 as a target molecule to recognise macrophages selectivity was showed by Thepen and colleagues (2000), when elimination of CD64-positive macrophages using the IT H22-RicinA was shown to resolve chronic skin inflammation in transgenic mice within 24 h (Thepen et al., 2000). Numerous other studies showed that CD64 is constitutively expressed only on macrophages, monocytes and their progenitors (van de Winkel and Anderson, 1991). In humanized mice and humans, CD64 was highly expressed by $HLA\text{-}DR^{+}$ cells that co-expressed CD14; a

finding strongly suggesting that high CD64 expression marks cells of monocytic origin in humans (Tamoutounour et al., 2012).

Consistent with these observations, the results presented here showed how a large number of $CD33^{+}CD14^{+}$ cells in CD and, at less extend, in normal colon express CD64. However the fewer percentage of CD64 expressing-$CD33^{+}CD14^{+}$ cells found in UC is perplexing since it is well documented that this marker is preferentially expressed in inflammatory $CD14^{+}$ macrophages in human IBD (Bain et al., 2013).

Moreover $CD14^{lo}$ cells uniformly expressed CD64 in control tissues in contrast to $CD14^{lo}$ cells from IBD subjects, which contained fewer cells which expressed CD64. This suggest that the $CD33^{+}CD14^{-}CD64^{+}$ cells in the normal gut are resident anergic macrophages but that in the inflamed gut they are replaced by CD64- cells.

In addition blood monocytes ($CD14^{+}$) expressed CD64 both in normal controls and IBD patients, hence it can be speculated that while in normal colon and CD, blood monocytes in the gut do not lose CD64, in UC once monocytes enter the intestinal compartment they may decrease the expression of CD64. While the reason for this discrepancy is unclear, it might be related to differences between CD and UC that are still, nowadays, poorly understood. Further evidence confirming the specificity of this marker for CD64 is showed by the small number of $CD64^{+}$ cells found in the CD33 negative gates in normal controls and IBD patients.

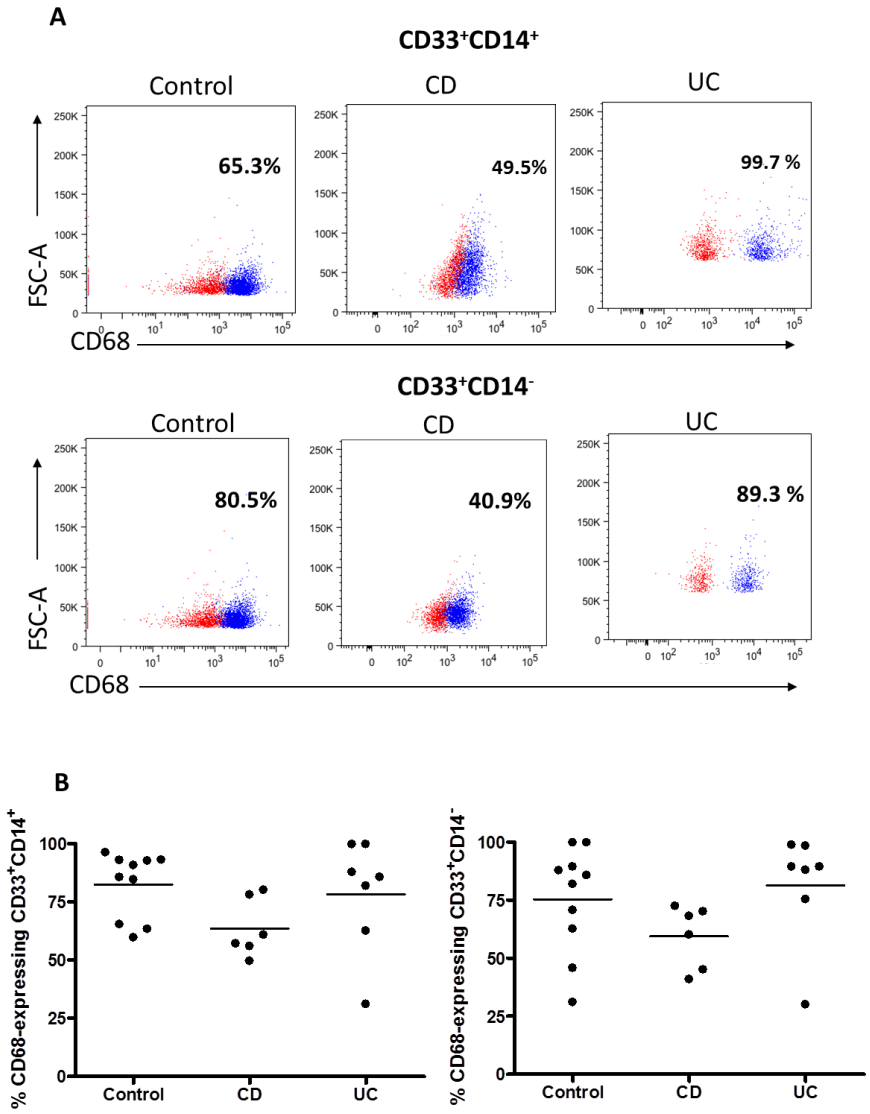

Figure 4.1: Expression of CD68 by $CD33^{+}CD14^{+}$ and $CD33^{+}CD14^{-}$ cells. $CD33^{+}CD14^{+}$ and $CD33^{+}CD14^{-}$ intestinal macrophages were analysed for the expression of the intracellular marker CD68. **A** Representative plots identifying CD68 (blue) or control isotype staining (red) among $CD33^{+}CD14^{+}$ and $CD33^{+}CD14^{-}$ in Control ,CD and UC. **B** Proportion of CD68 cells shown as a percentage of $CD33^{+}CD14^{+}$ and $CD33^{+}CD14^{-}$ in Control (n=10), CD (n=6) and UC (n=7). Horizontal bars are median values.

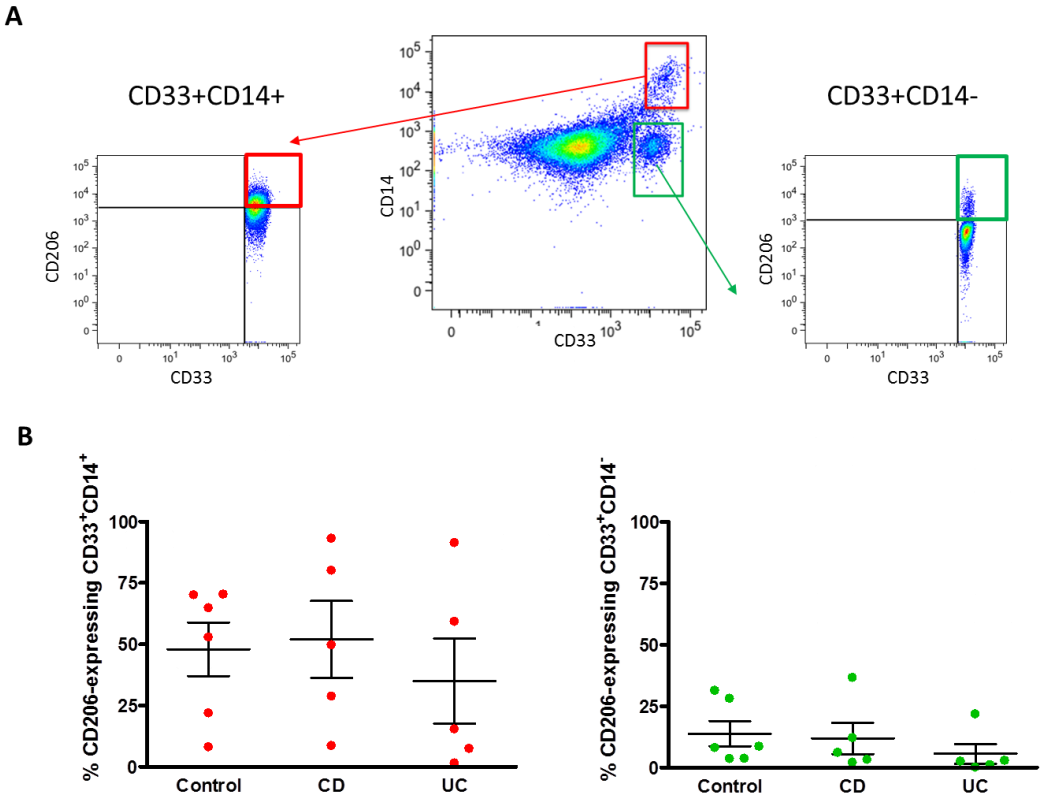

Figure 4.2: Expression of CD206 by defined subsets of intestinal macrophages. LPMCs were isolated from control mucosa and mucosa from CD and UC patients and examined for the expression of CD206 (mannose receptor) by flow cytometry. **A.** Representative dot plots showing the $CD14^{+}$ cells (red panel) and resident $CD14^{-}$ cells (green panel) populations. **B.** Results are mean ± SEM and are representative of n=6 (Control), n=5 (CD) and n=5 (UC). Horizontal bars are median values.

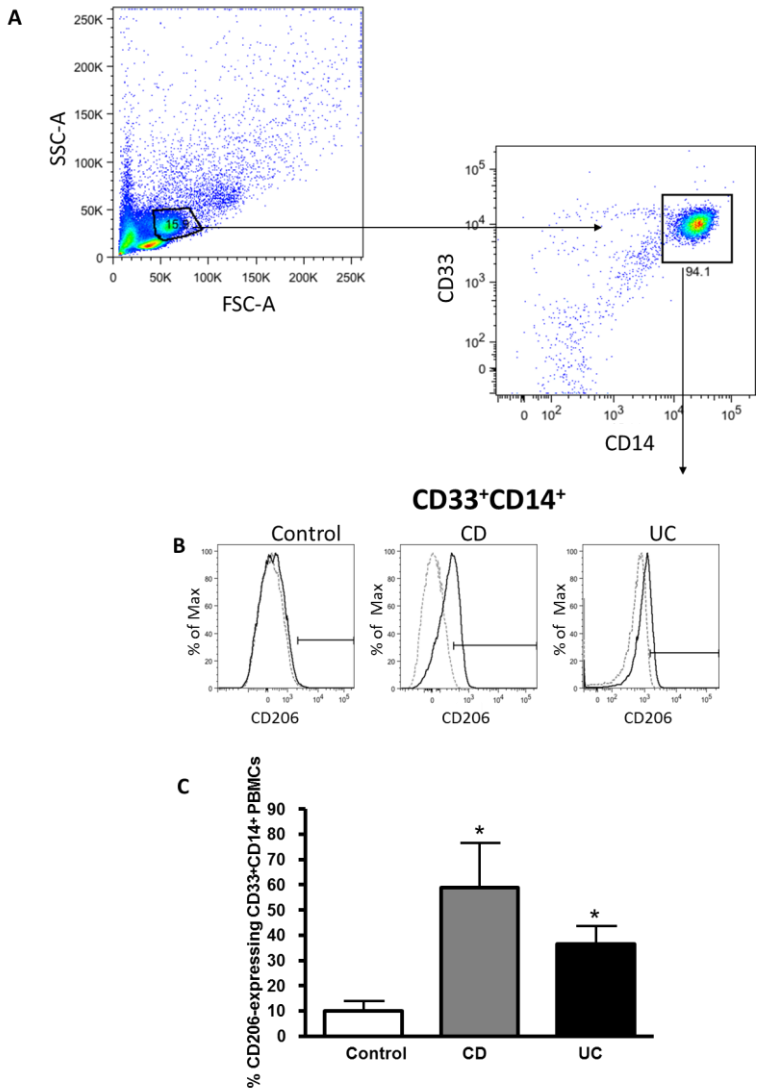

Figure 4.3: CD206-expressing cells are increased in the blood of IBD patients. PBMCs were isolated from control, CD and UC peripheral blood. **A.** Representative dot plots of CD33 and CD14. **B.** Shaded histograms represent staining with the appropriate isotype control. **C.** Proportion of CD206 as a percentage of total $CD33^{+}CD14^{+}$. Results are mean ± SEM and are representative of of n=5 (Control and CD) and n=3 (UC). *p<0.05. CD *versus* Control: *p= 0.0127 and UC *versus* Control: *p=0.2144.

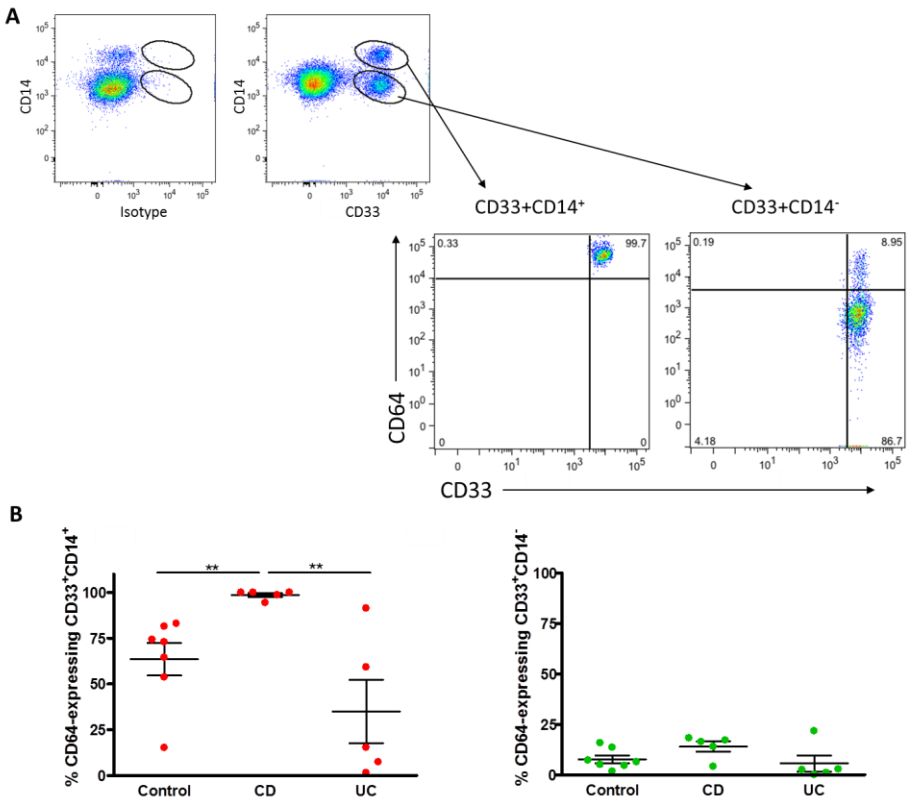

Figure 4.4: CD64-expressing CD33^{+}CD14^{+} and CD33^{+}CD14^{-} populations. A. Representative dot plots of CD33^{+}CD14^{+} and CD33^{+}CD14^{-} by live-gated CD45^{+} **A.** Representative plots showing CD64 expression by live-gated CD33^{+} CD14^{+} and CD33^{+}CD14^{-} LPMCs. **B.** Mean percentage of CD64 as a proportion of total live CD33^{+} CD14$^{+/-}$ LPMCs. Results are mean ± SEM and are representative of n=7 (Control), n=5 (CD) and n=5 (UC), *p(Control *versus* CD)= 0.0085; ***p(CD *versus* UC)=0.0064. Horizontal bars are median values.

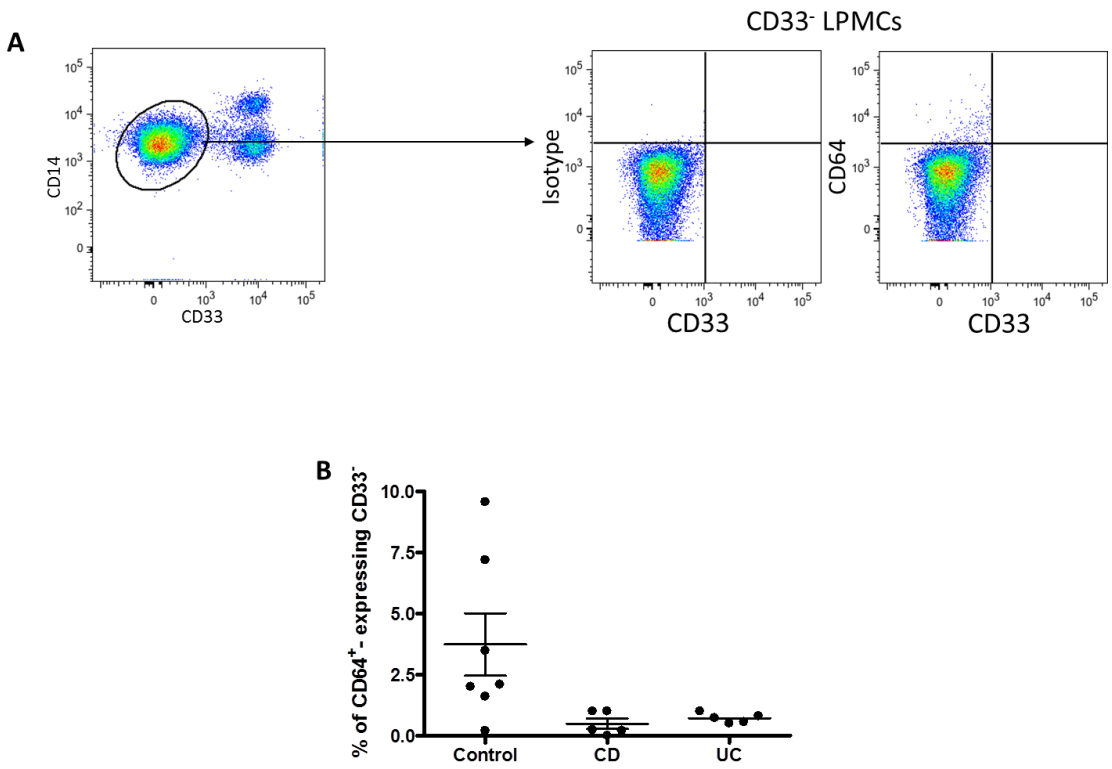

Figure 4.5: CD64-expressing CD33$^-$ in control and IBD. A. Representative dot plots of CD33$^-$ by live-gated CD45$^+$ **B.** Mean percentage of CD64 as a proportion of total live CD33$^-$ LPMCs. Results are mean ± SEM and are representative of n=7 (Control), n=5 (CD) and n=5 (UC). Horizontal bars are median values.

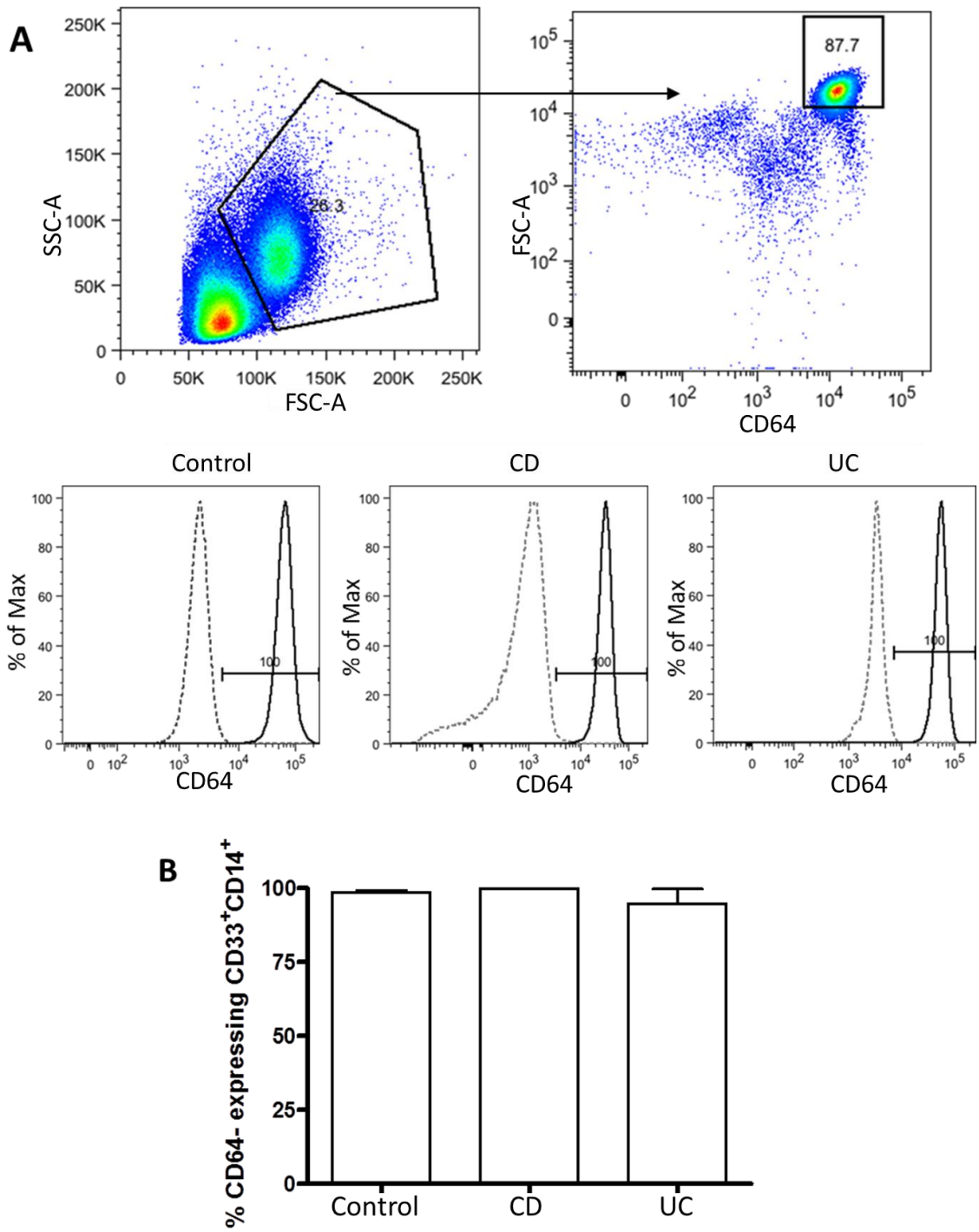

Figure 4.6: CD64-expressing blood monocytes. PBMCs were isolated from Control, CD and UC peripheral blood. **A.** Representative dot plots of CD33 and CD14. Shaded histograms represent staining with the appropriate isotype control. **B.** Proportion of CD64 as a percentage of total $CD33^{+}CD14^{+}$. Results are mean ± SEM and are representative of n=3 (Control and CD) and n=2 (UC).

CHAPTER 5

Results: Functional analysis of intestinal $CD33^+$ LPMCs

5.1 Introduction

It is known that in normal human colon, macrophages are unresponsive to stimuli that evoke pro-inflammatory cytokine production in monocytes (Smythies et al., 2005). Various reasons have been proposed for this anergy, including lack of CD14, rendering the cells unresponsive to LPS, or TGF-β made by stromal cells dampening pro-inflammatory responses. In IBD, there is a large influx of $CD14^+$ monocytes into the tissues and interestingly, by immunohistochemistry, Brandtzaeg's group showed that the highest expression of intracellular TNF-α was in $CD14^+$ cells (Bjerre et al., 2000; Bjerre et al., 2003). However many feature of intestinal macrophages remain unknown. It is not clear if in inflamed gut $CD14^+$ infiltrating monocytes lose CD14 in the tissues and become unresponsive. Likewise with the appreciation that there are also wound healing macrophages which express CD206, their role in the gut remains unclear. The relative contribution of DCs versus macrophages to TNF-α production is not known. Finally whether inhibition of ADAM17 increases mTNF-α with a reduction in secreted TNF-α is not known. In this chapter all of these issues have been addressed.

5.2 TNF-α production by peripheral blood monocytes from healthy individuals in response to LPS stimulation

As a positive control, $CD14^+$ blood monocytes were stimulated with LPS (100 ng/ml) for 4 hours and the expression of the intracellular TNF-α in $CD14^+$ cells was determined by flow cytometry.

As shown in Figure 5.1, the number of cells expressing intracellular TNF-α in $CD14^+$ PBMCs cells increased to 20.6 ± 2.3% compared to unstimulated cells (1.7 ± 0.5%).

5.3 TNF-α production by $CD33^+$ LPMCs in response to LPS stimulation

The percentage of intracellular TNF-α expressing $CD33^+$ LPMCs from the normal and inflamed colon after activation with LPS was next examined.

LPMCs from normal colon and inflamed colon were stimulated with LPS for 4 hours and assessed for $CD33^+$ and TNF-α expression by flow cytometry. The results showed no significant increase in TNF-α expression in cells from control tissues after LPS (1.3 ± 0.3% without LPS and 1.7 ± 0.6% with LPS, Figure 5.2). On the other hand 4.2 ± 1.0% of unstimulated $CD33^+$ LPMCs from inflamed IBD mucosa showed intracellular staining, which significantly increased to 19.0 ± 4.6% when the cells were stimulated with LPS for 4 hours (Figure 5.3).

5.4 TNF-α production by $CD33^+$ and $CD3^+$ LPMCs in response to PMA + ionomycin stimulation

PMA and ionomycin was also used to stimulate LPMCs from control and IBD mucosa and TNF-α expression was measured in $CD33^+$ macrophages and on $CD3^+$ lymphocytes.

As expected, unstimulated $CD33^+$ LPMCs from control tissues (Figure 5.4 A) showed less than 1.0 ±0.0 % of cells expressing TNF-α. Activation of $CD33^+$ LPMCs by PMA and ionomycin increased the mean expression of TNF-α (15.0 ± 3.2%).

On the other hand, when inflamed IBD mucosa was studied, $CD33^+$ LPMCs which at basal level already expressed an higher percentage of TNF-α (CD: 8.5 ± 2.1%; UC: 11.2± 3.4%), compared to $CD33^+$ from normal colon (0.8±0.1%), increased the intracellular percentage of TNF-α after PMA+ionomycin stimuli to 17.0 ±2.2% in CD

and 16.0 ± 4.2% in UC (Figure 5.4B), notably also LPMCs from control patients increased the expression of TNF-α to 13.2±4.8%.

$CD3^+$ lymphocytes showed a significant increase in intracellular TNF-α expression when LPMCs were stimulated with PMA and ionomycin (25.0 ± 0.8%) compared to unstimulated LPMCs (1.3 ±0.4%) (Figure 5.4 C).

5.5 TNF-α production by $CD33^+CD14^+$ and $CD33^+CD14^-$ LPMCs

Having identified two different populations of macrophages which differed in the expression of CD14, I hypothesised that there may be heterogeneity in TNF-α expression between the different subpopulations.

As shown in Figure 5.5, LPMCs from IBD patients expressed intracellular TNF-α without the addition of exogenous stimuli. Notably, the $CD33^+CD14^+$ but not the $CD33^+CD14^-$ cells represented the major source of spontaneous TNF-α production. As shown in Figure 5.5 A, the frequency of $CD33^+CD14^+$ $TNF\text{-}\alpha^+$ cells in LPMCs from both CD and UC inflamed mucosa was significantly higher (47.3 ± 7.2 % and 29.6 ± 6.5%) compared to control $CD33^+CD14^+$ $TNF\text{-}\alpha^+$ LPMCs (3.1 ± 1.6%).

The $CD33^+CD14^+$ populations in uninflamed CD mucosa showed a slight increase in the number of TNF-α containing cells (8.4 ± 3.4%) when compared to control (0.8 ± 0.5%). Similarly 23.6 ±16.4 % of $CD33^+CD14^+$ from uninflamed mucosa in UC expressed TNF-α.

$CD33^+CD14^-$ LPMCs also produced TNF-α, but at lower levels (Figure 5.5B). In particular, $CD33^+CD14^-TNF\text{-}\alpha^+$ LPMCs were significantly increased in inflamed CD

mucosa (9.8 ± 2.5%) as well as in inflamed (13.36 ± 4.9%) and uninflamed UC mucosa (5.6 ± 1.93%) when compared to controls (0.75± 0.51%). Moreover there was no significant difference between control and uninflamed CD (2.8 ± 1.5%). Also in order to have an overall indication of the IBD patient's medical diagnosis, table 5.1 shows the demographics and medical records of the patient populations in the inflammatory bowel disease group.

5.6 Correlation between TNF-α expression and CD14 expression in IBD

To determine if intracellular TNF-α expression was related to surface CD14 levels**,** correlation analysis was assessed between the mean fluorescence intensity (MFI) of TNF-α and the MFI of $CD14^+$ in IBD in a group of 18 IBD patients (11 CD and 7 UC). TNF-α expression was positively correlated (r=0.63, P= 0.0054) with the expression of $CD14^+$ in the LPMCs (Figure 5.6).

5.7 TNF-$\alpha^+$$CD206^+$-expressing macrophages subsets in IBD

CD206 is the best known phenotypic marker for M2 macrophages but there is little data on intracellular TNF-α within these cells in IBD tissues.

Surprisingly in CD patients all of the CD206-expressing $CD33^+CD14^+$ cells (which represent 48.0 ±15.0 % of overall $CD33^+CD14^+$) were positive for intracellular TNF-α (Figure 5.7). In UC patients, the mean percentage of TNF-α-expressing $CD33^+CD14^+$ $CD206^+$ LPMCs was 22.0 ±11.2%. Moreover TNF-α-expression on $CD33^+CD14^-$ $CD206^+$ LPMCs in CD was only 4.0 ± 1.5% and in UC was 3.9 ± 2.4%.

5.8 TNF-α^+CD64$^+$-expressing macrophages subsets in IBD

Using CD64 expression, macrophages can be distinguished from DCs in the intestine of both mice and humans and as CD33 is present on both these cell types in the gut, it was felt that it would be helpful to look at TNF-α expression in CD33$^+$ cells which were CD64$^+$, being presumably macrophages, or CD64$^-$, which represent DCs (Figure 5.8 B). In CD, 26.8 ±13.6% of CD33$^+$ CD64$^-$ cells were TNF-α-producing moreover a significant larger proportion of TNF-α (63.3 ± 19.0%) in CD was produced by CD64$^+$ LPMCs (Figure 5.8 C).

In UC there was a more profound significant difference between the numbers of TNF-α containing cells in CD64$^+$ and CD64$^-$ cells (Figure 5.8 D). TNF-α containing cells were in fact 4 times higher in the CD64$^+$ cells (84.2 ± 7.0%) as opposite as CD64$^-$ cells (21.0 ±12.4%).

5.9 Membrane TNF-α expression on resting CD3$^+$ PBMCs

The role of ectodomain shedding of TNF-α was assessed by the use of an ADAM17-specific inhibitor, GW280264X, in CD3$^+$ PBMCs from healthy volunteers based on the measurement of surface membrane TNF-α expression. Hence, to test whether GW280264X was a substrate for ADAM 17 protease, PBMCs were stimulated with PMA+ionomycin in presence or absence of ADAM 17 inhibitor GW280264X or left untreated followed by FACS analysis for mTNF-α expression.

Treatment of PBMCs with PMA+ionomycin resulted in a significant increase of membrane TNF-α on CD3 lymphocytes. Moreover membrane TNF-α^+CD3$^+$ cells were significantly increased when the cells were stimulated with PMA+ionomycin+

GW280264X (36.8 ± 6.8%) compared to those unstimulated (0.6 ± 0.2%) or stimulated with PMA+ionomycin alone (7.4 ± 2.5%) (Figure 5.9).

I next determined the level of released soluble TNF-α in the supernatants after 4 hours of culture medium alone, PMA+ionomycin and PMA+ionomycin+GW280264X. PMA+ionomycin increased the release of soluble TNF-α into the supernatant (465.4 ± 85.6 pg/ml) compared to unstimulated cells which do not release TNF-α (0%). On the contrary PBMCs stimulation with PMA+Ionomycin+GW280264X showed significantly (p=0.0135) less TNF-α production (191 ± 36 pg/ml).

In addition, the level of soluble IL-8 and IL-6 in the same supernatant was investigated. Figure 5.10 show that addition of GW280264X did not inhibit release of these cytokines. However it has to be considered that monocytes population, as well as lymphocytes, may release TNF-α after PMA+ionomycin incubation.

5.10 Membrane TNF-α expression on $CD3^+$ and $CD33^+$ IBD LPMCs

The relative expression of membrane TNF-α and sTNF-α in LPMCs was next examined. Increased expression of membrane TNF-α was seen in CD3 T $cells^+$ in inflamed IBD LPMCs when activated with PMA+ION+DMSO (Figure 5.11).

In CD and in UC the percentage of membrane TNF-α expressing $CD3^+$ T cells stimulated with PMA+Ionomycin+GW280264X (Figure 5.11) was higher (CD: 31.3 ± 3.3% and UC: 20.2±3.0%) compared to the cells stimulated with PMA+Ionomycin+DMSO (CD: 7.6 ± 4.9%; p=0.0576 and UC: 5.1± 1.0% ; p=0.041) (Figure 5.11 B-C).

$CD33^+$ LPMCs expressed high level of membrane TNF-α when the cells where incubated with the ADAM17 inhibitor. After 4 hours, about 13.0 ± 4.4% of the $CD33^+$

cells in CD and 23.5 ± 4.5% in UC expressed membrane TNF-α^+ compared to PMA+Ionomycin+DMSO (CD: 3.7 ± 1.3% and UC: 4.0 ± 0.5%) (Figure 5.12 B-C).

5.11 Pro-inflammatory cytokine secretion in the supernatants of LPMCs

Next, I verified if the increased expression of membrane TNF-α on lymphocytes and macrophages in LPMC led to a reduced release of the soluble form of the TNF-α.

Stimulation with PMA+ionomycin+DMSO significantly enhanced TNF-α production by IBD LPMCs (858 ±118 pg/ml) (Figure 5.13). Moreover the addition of GW280264X induced a significant decrease of TNF-α in the supernatant of LPMCs (32 ± 6 pg/ml) compared to PMA+ionomycin+DMSO alone. Conversely addition of GW280264X did not induce any significant change in IL-6 and IL-8 production (888.3 ± 36.5 pg/ml; mean 8213 ± 5845 pg/ml respectively) compared to the culture with PMA+ionomycin+DMSO (890 ± 14 pg/ml; 10160 ± 7834 pg/ml respectively).

5.12 Pro-inflammatory cytokines in colon explant cultures from IBD patients

Since GW280264X was observed to inhibit sTNF production by PBMCs and LPMCs supernatants, the hypothesis that ADAM10/17 would reduce sTNF and sIL-6R production in CD and UC mucosal biopsy cultured ex vivo was tested.

The spontaneous release of soluble TNF-α, sIL-6R, IL-8, IL-6 and IL-1β in the organ culture supernatants was measured. GW280264X reduced the concentration of sTNF-α in CD (67.5 ± 25.5 pg/ml) and UC (117.9 ± 33.9 pg/ml) explant culture supernatant compared with DMSO (119.5 ± 57.2 pg/ml and 214.8 ± 43.8 pg/ml) (Table 5.2, Figure 5.14). Spontaneous sIL-6R production was also measured in culture supernatants of CD and UC treated with GW280264X or DMSO.

GW280264X treated biopsies had a significant reduction in spontaneous sIL-6R biopsies production both CD (133.3 ± 36.6 pg/ml) and UC samples (277.9 ± 92.7 pg/ml) compared with DMSO (CD: 609.2 ± 151.1 pg/ml; UC: 839.2 ± 267.2 pg/ml).

On the contrary, no significant differences were found between CD and UC in the production of IL-8, IL-1β and IL-6, both in biopsies cultured in vehicle [(IL-8: CD (35620 ± 7868 pg/ml) and UC (50100 ± 14030 pg/ml); IL-1β: CD (39.20 ± 17.58 pg/ml) and UC (277.5 ± 137.8 pg/ml)]; IL-6: CD (1900 ± 892 pg/ml) and UC (2957 ± 1042 pg/ml) and biopsies cultured with GW 280264X vehicle [(IL-8: CD (41210 ± 15020 pg/ml) and UC (49880 ± 11780 pg/ml); IL-1β: CD (47.42 ± 2.260 pg/ml) and UC (277.5 ± 137.8 pg/ml)]; IL-6: CD (2926 ± 1238 pg/ml) and UC (2774 ± 739.0 pg/ml) (Table 5.2).

5.13 Discussion

Results presented in this chapter suggested that in contrast to monocytes, $CD33^+$ LPMCS from control colon do not make intracellular TNF-α when activated with LPS. In contrast, LPMCs obtained from affected IBD mucosa spontaneously produced TNF-α and were further stimulated by LPS to produce greater amounts of this cytokine. On the other hand $CD33^+$ LPMCs from control colon and IBD colon responded to PMA+Ionomycin stimulation, increasing intracellular TNF-α production, in the same way as lymphocytes.

In IBD tissues there was a lot more TNF-α, both spontaneous and inducible, and the highest producers were the $CD14^{hi}$ cells. In addition $CD206^+$ cells in IBD expressed intracellular TNF-α. The $CD64^+$ marker, used to distinguish DCs and macrophages, was highly expressed on the $CD33^+CD14^+$ LPMCs of IBD patients and these cells frequently expressed intracellular TNF-α.

Results from the second part of the chapter showed that addition of GW280264X, an ADAM17 inhibitor, induced increase of membrane-TNF-α, both on activated $CD3^+$ lymphocytes and $CD33^+$ LPMCs, and in parallel sTNF-α was reduced in the supernatant of cells stimulated with PMA+Ionomycin+GW280264X. IBD biopsies in culture produced high levels of TNF-α and sIL-6R which decreased by the addition of GW280264X while IL-6, IL-8 and IL-1β production were unaffected.

It has previously been shown that blood monocytes $CD14^+$ are the principle source of macrophages which in healthy gut mucosa are thought to slowly replace senescent resident macrophages and to acquire at the same time a profound inflammatory anergy (Smythies et al., 2005). Conversely, in IBD patients $CD14^+$ cells are abundant in the

gut (Grimm et al., 1995), therefore the data presented in this thesis support this notion, since $CD14^+$ cells, as was shown in the previous chapter, almost absent in normal but abundant in IBD mucosa.

In this study, intracellular TNF-α production was compared between $CD14^+$ blood monocytes and $CD33^+$ LPMCs from normal and inflamed mucosa after stimulation with LPS or PMA+Ionomycin.

Blood monocytes, from healthy volunteers as expected, expressed intracellular TNF-α after LPS stimulation. However $CD33^+$ LPMCs from normal mucosa, which contained a number of $CD14^{low}$ cells, under the same condition did not increase the percentage of intracellular TNF-α. This suggests that although expressing CD14, these cells may be on the pathway to becoming anergic. It is still unclear the mechanism which bring intestinal macrophages in a state of 'inflammatory anergy' when they are situated in such close proximity to a vast quantities of immunostimulatory material. One of the mechanisms proposed, with no clear answer being offered, is that human resident intestinal macrophages lack receptors involved in macrophages activation, TREM-1 (Schenk et al., 2005) , complement receptors (CR) 3 and 4, as well as the Fc receptors for IgA and IgG (Smith et al., 2001). Moreover, they express anti-inflammatory molecules, such as IL-10, and contribute to the differentiation of Treg cells, while suppressing DC-derived Th1 and Th17 immunity (Denning et al., 2007).

However when LPMCs from IBD patients, which contain large numbers of $CD14^{hi}$ cells were stimulated with LPS, the number of cells expressing intracellular TNF-α increased. Thus $CD14^+$ blood monocytes and $CD33^+$ LPMCs from inflamed gut are potential responders to the increased inflammatory stimuli as opposite as the $CD33^+$ cells from normal colon.

Notably when $CD33^+$ LPMCs from control colon were stimulated with PMA+Ionomycin, the number of cells expressing intracellular TNF-α dramatically increased. This was expected for T cells but not for $CD33^+$ cells which were unresponsive to LPS. These results suggested that the pathway involving the production of TNF-α by PMA+Ionomycin-stimulated $CD33^+$ cells is probably not related to the pathway activated by LPS.

It is known that TNF-α expression is regulated by transcriptional and posttranscriptional mechanisms (Han et al., 1990). The adenine and uridine–rich element (ARE) regulates mRNA stability and translation in a way that prevents the pathologic overexpression of TNF-α (Caput et al., 1986). The importance of this element has been demonstrated in transgenic "knockin" mice that lack this cis element (Kontoyiannis et al., 1999) and develop chronic inflammatory polyarthritis and inflammatory bowel disease as a consequence of TNF-α overproduction.

In a study reported by Saito et al it was shown that TIA-1 regulates the production of TNF in LPS–activated macrophages, but not in PMA/ionomycin, or anti-CD3/anti-CD28–activated T cells (Saito et al., 2001). Therefore the activation cascades triggered by LPS and TCR ligation or PMA/Ionomycin might differentially affect the ability of TIA-1 to function differently on the $CD33^+$ cells.

Analysis of the spontaneous production of TNF-α by intracellular cytokine staining revealed that the numbers of $CD33^+$ TNF-α^+ cells were increased in the mucosa of inflamed IBD. However there were marked differences in the percentage of TNF-α containing cells in the different CD33-defined populations. The number of $CD33^+CD14^+$ macrophages expressing TNF-α was radically increased not only in inflamed mucosa but also in uninflamed mucosa of IBD patients. This is perhaps not

surprising since it is well established that during the course of IBD there is dramatic immune change with intense infiltration of immune cells.

The present study demonstrated that the $CD33^{+}CD14^{+}CD206^{+}$ cells, both in CD and UC, showed high expression of TNF-α. This contrasts with previous studies where CD206 macrophages produce high levels of IL10 and low levels of TNFα and IL12 (Gordon and Martinez, 2010). The mechanisms of macrophage phenotypic changes are unclear, especially in inflamed human gut. The difficulty is also enhanced by the fact that the macrophages activation depend on which stimulation is provided first and also whether the activation will be pro- or anti-inflammation is subject to a broad range of factors. Stout and Suttles (Stout and Suttles, 2004) raised the hypothesis that macrophages could reverse their phenotype and functions depending on their microenvironment, hence the results of this study suggest that macrophage activation is plastic and that in IBD mucosa $CD33^{+}CD14^{+}TNF\text{-}\alpha^{+}$ cells represented recruited pro-inflammatory cells with high activated inflammatory state which expressed also TNF-α. On the other hand CD33+CD14- cells, may represent a small anti-inflammatory percentage of CD206-expressing cells with a little contribute of TNF-α expression.

Another important finding was that $CD33^{+}CD64^{+}$ LPMC cells, in CD and UC, were the larger producers of TNF-α compared to $CD33^{+}CD64^{-}$ cells. This was particularly interesting in the light of the finding that CD64-expressing cells were exclusively macrophages (Tamoutounour et al., 2012). Indeed the Wojtal group hypothesised that Infliximab, one of the anti-TNF-α therapeutics approved for use in IBD, by binding to CD64 limits the ability of this drug to block soluble TNF-α (Wojtal et al., 2012). This

study was in agreement with the finding that Adalimumab and Infliximab bind to Fc receptor-expressing THP-1 cells (Arora et al., 2009).

Is likely that IFX-TNF complexes, which form during neutralization of TNF-α, are responsible for the limited anti-inflammatory effect via direct activation of Fc receptor downstream signalling. Moreover elevated soluble forms of CD64 have been also detected in gut lavage fluid of IBD patients (Hommes et al., 1996), so there is a possibility that anti-TNF antibodies which are thought to act in part by interacting with Fc receptors and trigger ADCC may be inhibited because the therapeutic antibody is complexed to soluble CD64.

Additionally a conspicuous population of $CD33^{+}CD64^{-}$ cells in IBD mucosa, which probably were DCs, expressed TNF-α. DCs, in contrast to macrophages, are specialised antigen-presenting cells that can prime naïve T cells and induce their differentiation in inflammatory (eg, Th1, Th17) or Treg phenotypes and similar to macrophages. They also accumulate in the mucosa of IBD patients (Hart et al., 2005) and experimental models of colitis (Uhlig et al., 2006). Inflammatory DCs also play a critical role in the $Tbx21^{-/-}$ $Rag2^{-/-}$ ulcerative colitis (TRUC) model (Garrett et al., 2007). These mice spontaneously develop colitis closely reminiscent of human UC.

Specifically, TNFα secreted from $CD103^{-}$ DCs in TRUC mice potentiated IL-23-induced IL-17 expression in ILCs, (Powell et al., 2012) whereby neutralisation of TNFα, IL-23p19 or IL-17A ameliorated disease (Garrett et al., 2007).

In human, M-DC8+ DCs, detected in the subepithelial dome of ileal PP M-DC8+ DCs, secrete large amounts of TNF-α but not IL-10 upon stimulation LPS suggests that these cell types might contribute to the pathogenesis of IBD (de Baey et al., 2003).

TNF-α is considered to be centrally involved in the pathogenesis of IBD and to play a pivotal role in the inflammatory cascade (Papadakis and Roubelakis-Angelakis, 2005).

As was described in the introduction, to generate soluble TNF-α, the membrane-associated TNF-α is cleaved through the proteolytic activity of ADAM17 (Horiuchi et al., 2007). Because many biological effects exerted by TNF-α agents, including apoptosis and cell-cycle arrest, are mediated by outside-to-inside signalling through mTNF (Mitoma et al., 2005) the expression of mTNF, with or without ADAM17 inhibitor, on PBMCs and LPMCs was examined.

Although it has been suggested that other proteinases are capable of TNF-α cleavage, it has been shown that ADAM17 has the highest affinity for TNF-α ectodomain shedding among the known substrates (Armstrong et al., 2006).

Several lines of investigation mainly based on TIMP-3, a family member of the matrix metalloproteinase (MMP) family, have showed that TIMP-3 is an endogenous inhibitor of ADAM 17 (Schlöndorff et al., 2000). In vitro experiment on timp-3 $^{-/-}$ mice have shown that TIMP-3 is able to inhibit inflammatory responses (Smookler et al., 2006), and that TIMP-3 deficiency is associated with increased hepatic inflammation due to abnormal TACE activity (Murthy et al., 2012). However TIMP-3 is essential for normal innate immune function and the loss of TIMP-3 impacts innate immunity by dysregulating cleavage of TNF-α and its receptors. .

Nevertheless a series of TIMP-3-based therapies were considered a novel approaches to control inflammation (Newton et al., 2001), in the context of the emergence of new agents able to block TNF- α (Billiet et al., 2014).

In the present study PMA and Ionomycin treatment increased membrane TNF-α in cultured PBMCs ($CD3^+$) and LPMCs ($CD3^+$ and $CD33^+$) respectively. Moreover TNF-

α increased on the surface of the cells when the pharmacological inhibitor GW280264X was added, as a consequence of the inhibition of ADAM 17 cleavage.

In addition, the lower systemic inflammatory state generated by GW280264X on the LPMCs was reflected by significant reduced level of sTNF-α in the supernatants of the same cells. Therefore as pre-treatment with GW280264X, but not DMSO alone, inhibited the release of sTNF-α, was clear that these cells expressed functional ADAM17 activity which is responsible for processing mature TNF-a protein.

There is growing evidence implicating ADAM 17 as an important target in IBD. It has previously been shown dysregulation of ADAM17 on the level of gene transcription in endothelial barrier dysfunction (Dreymueller et al., 2012) , atherosclerosis (Canault et al., 2006) and in in various models of inflammation (Chalaris et al., 2010a; Horiuchi et al., 2007; Li et al., 2006; Long et al., 2010).

Organ culture experiments performed with CD and UC biopsies specimens showed that decreased amounts of soluble TNF-α were detected in IBD biopsies cultured with GW280264X. This is in accord with previous finding where increased levels of mucosal mRNA ADAM17 were observed in patients with ulcerative colitis (Brynskov et al., 2002) suggesting that higher amount of ADAM17 protease could induce a more prominently inhibition by GW280264X.

The reason why GW280264X increased membrane TNF-α in UC biopsies but failed in CD biopsies remains unclear but it is reasonable to assume that in most cases of complex, immune-mediated disease-including IBD-several pathways are activated and contribute to disease and the relative role of these pathways may vary from one patient to another.

ADAM17 has also been implicated in shedding of a variety of other cell membrane substrates including IL-6R (Chalaris et al., 2010b) which is expressed by monocytes, macrophages, neutrophils, B-cells, subpopulations of T-cells and non-lymphoid cells such as hepatocytes (Chalaris et al., 2010a; Oberg et al., 2006; Scheller and Rose-John, 2006). In the present study as expected the levels of sIL-6R were significantly decreased in the supernatants of IBD biopsies cultured with GW280264X.

These studies suggested that the regulation of ADAM 17 activity and subsequent alteration of the soluble TNF-α and sIL-6R could have a great impact on IBD considering the impact of TNF-α on the persistence of the diseases.

Several monoclonal TNF-α antibodies and a soluble TNF-α receptor are used for clinical practice in immune mediated inflammatory diseases, where they bind to soluble and membrane TNF-α and can neutralize the pathological effects of TNF-α. Unfortunately, almost half of IBD patients do not respond to anti-TNF-α antibodies and the response diminishes with time (Caprioli et al., 2012). Interestingly, clinical therapy with adalimumab revealed that patients with high amounts of mTNF cells in the mucosa were significantly more likely to respond to anti-TNF-α therapy than patients with low amounts of mTNFα cells (Atreya et al., 2014). This observation opens new paths for individualized therapy in IBD suggesting that failure to anti-TNF therapy may occur in patients with low amounts of mTNFα lamina propria cells due to TNF-independent gut inflammation. With this new finding, it might be of interest to target in the future the mTNF/TNFR2 interaction rather than total TNF (mTNF plus sTNF) as it has been done until now.

Disease	Gender	Age	Presentation	Disease location
CD	M	19	Uninflamed	Ileum
CD	M	32	Uninflamed	colon
CD	F	30	Uninflamed	Rectum
CD	M	19	Uninflamed	Ileum
CD	F	58	Inflamed	Ileum
CD	M	60	Inflamed	Rectum
CD	F	29	Inflamed	Ileum
CD	F	57	Inflamed	Ileum
CD	M	28	Inflamed	Ileum
CD	F	58	Inflamed	Ileum
CD	F	23	Inflamed	Ileum
CD	M	19	Inflamed	Ileum
CD	F	23	Inflamed	Ileum
CD	F	54	Inflamed	Ileum
CD	M	52	Inflamed	Ileum
UC	M	32	Uninflamed	Rectum
UC	M	30	Uninflamed	Colon
UC	M	67	Uninflamed	Colon
UC	M	18	Uninflamed	Colon
UC	F	47	Inflamed	Colon
UC	M	51	Inflamed	Colon
UC	M	49	Inflamed	Colon
UC	F	23	Inflamed	Colon
UC	M	30	Inflamed	Ileum
UC	F	63	Inflamed	Colon
UC	M	32	Inflamed	Colon
UC	M	22	Inflamed	Rectum
UC	M	18	Inflamed	Colon

Table 5.1: Demographic and clinical characteristics of IBD patients: The table shows the medical records of patients diagnosed with sever or mild IBD and the location of the tissue material taken during endoscopy or surgical resections

	CD		UC	
	DMSO	**GW280264X**	**DMSO**	**GW280264X**
TNF-α	67.50 ± 25.5	119.5 ± 57.1	214.8 ± 43.8	117.9 ± 33.9**
sIL-6R	609.2 ± 155.8	133.3 ± 36.6*	839.2 ± 267.2	277.9 ± 92.7*
IL-8	35620 ± 7868	41210 ± 15020	50100 ± 14030	49880 ± 11780
IL-1β	39.20 ± 17.5	47.42 ± 2.260	277.5 ± 137.8	163.1 ± 77.9
IL-6	1900 ± 892.3	2926 ± 1238	2957 ± 1042	2774 ± 739.0

Table 5.2: Pro-inflammatory cytokines levels in the supernatants of IBD biopsies: TNF-α, IL-8, IL-1β and IL-6 levels (pg/ml) in the supernatants of DMSO and GW280264X stimulated biopsies taken from the inflamed mucosa of CD and UC and cultured for 24 h. Results are mean ± SEM (**p=0.0016 versus biopsies cultured with DMSO only).

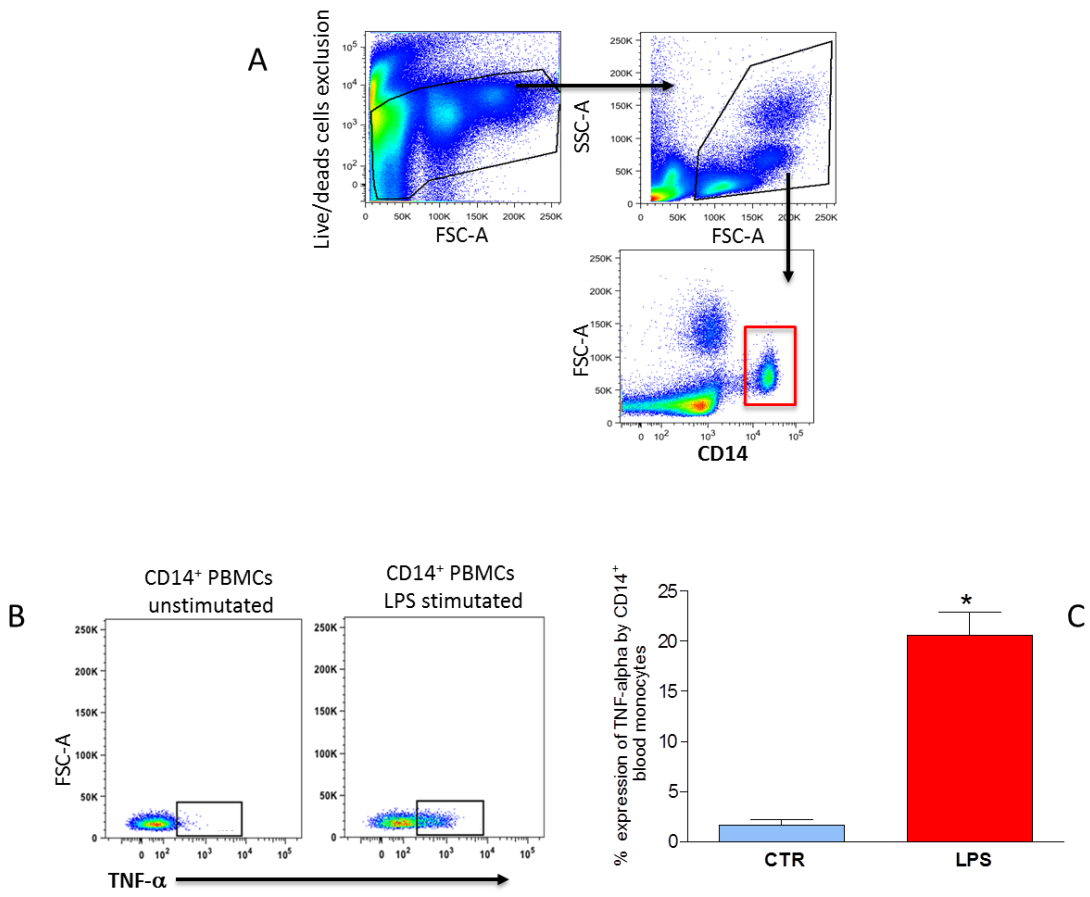

Figure 5.1: TNF-α expression by CD14$^+$ PBMCs with or without LPS stimulation: CD14$^+$ blood monocytes were cultured in medium and LPS (100 ng/ml) in the presence of brefeldine for 4 hours. **A.** Gating strategy used to identify CD14$^+$ PBMCs.

B. Representative flow cytometry dot plot of TNF-α among four samples analyzed. **C.** Bar chart displaying significatively ($p<0.05$) higher expression of intracellular TNF-α on the surface of CD14$^+$ blood monocytes after LPS stimulation for 4 hours. Data are representative of n=4 experiments.*p=0.0195 *versus* CTR (medium alone). Bars represent mean percentage of expression and error bars denote SEM.

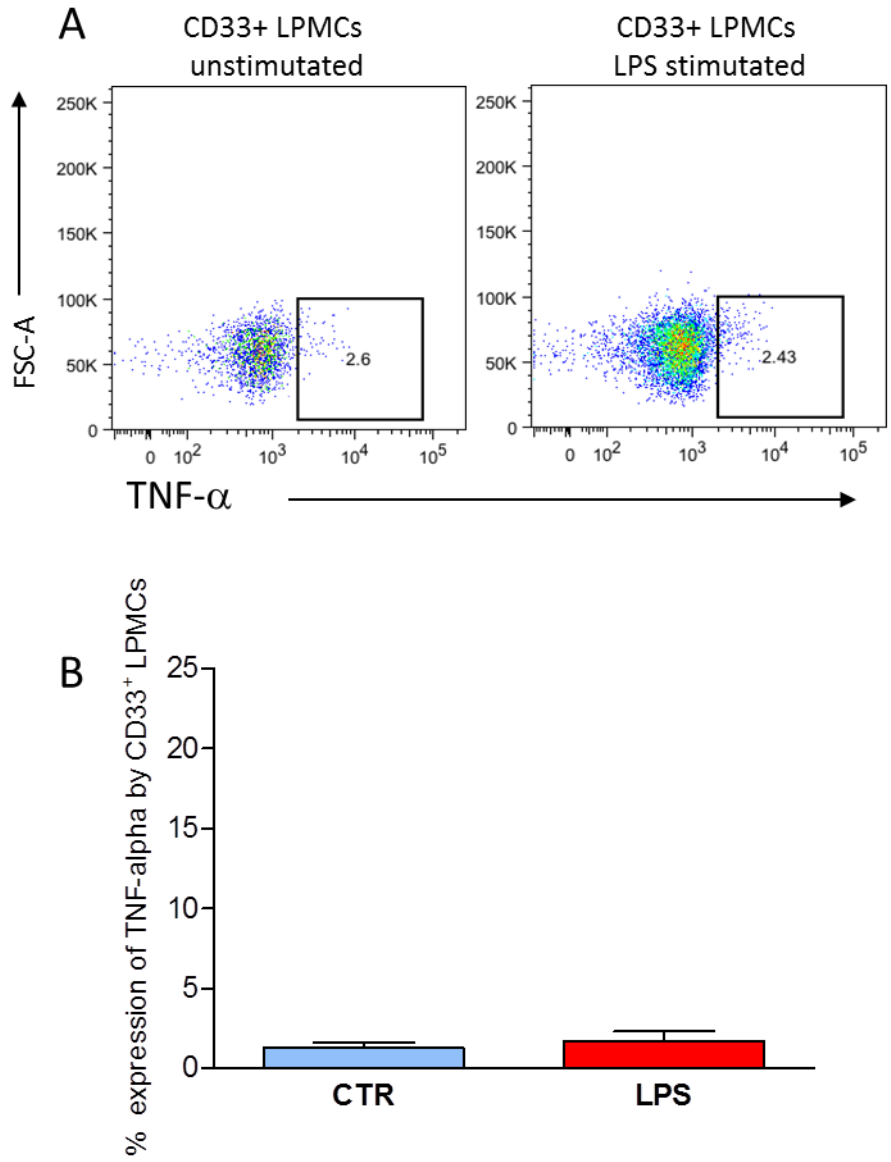

Figure 5.2: TNF-α expression by control CD33$^+$ LPMCs with or without LPS stimulation: LPMCs from control where cultured in medium and LPS (100ng/ml) in presence of brefeldin for 4 hours. **A.** Representative plots showing TNF-α expression by live-gated CD33$^+$ LPMCs unstimulated and stimulated with LPS. **B.** Percentage of CD33$^+$ cells expressing TNF-α in only medium and LPS stimulation. Data are representative of two independent experiments with n=4 per experiment. Bars represent mean percentage of expression and error bars denote SEM.

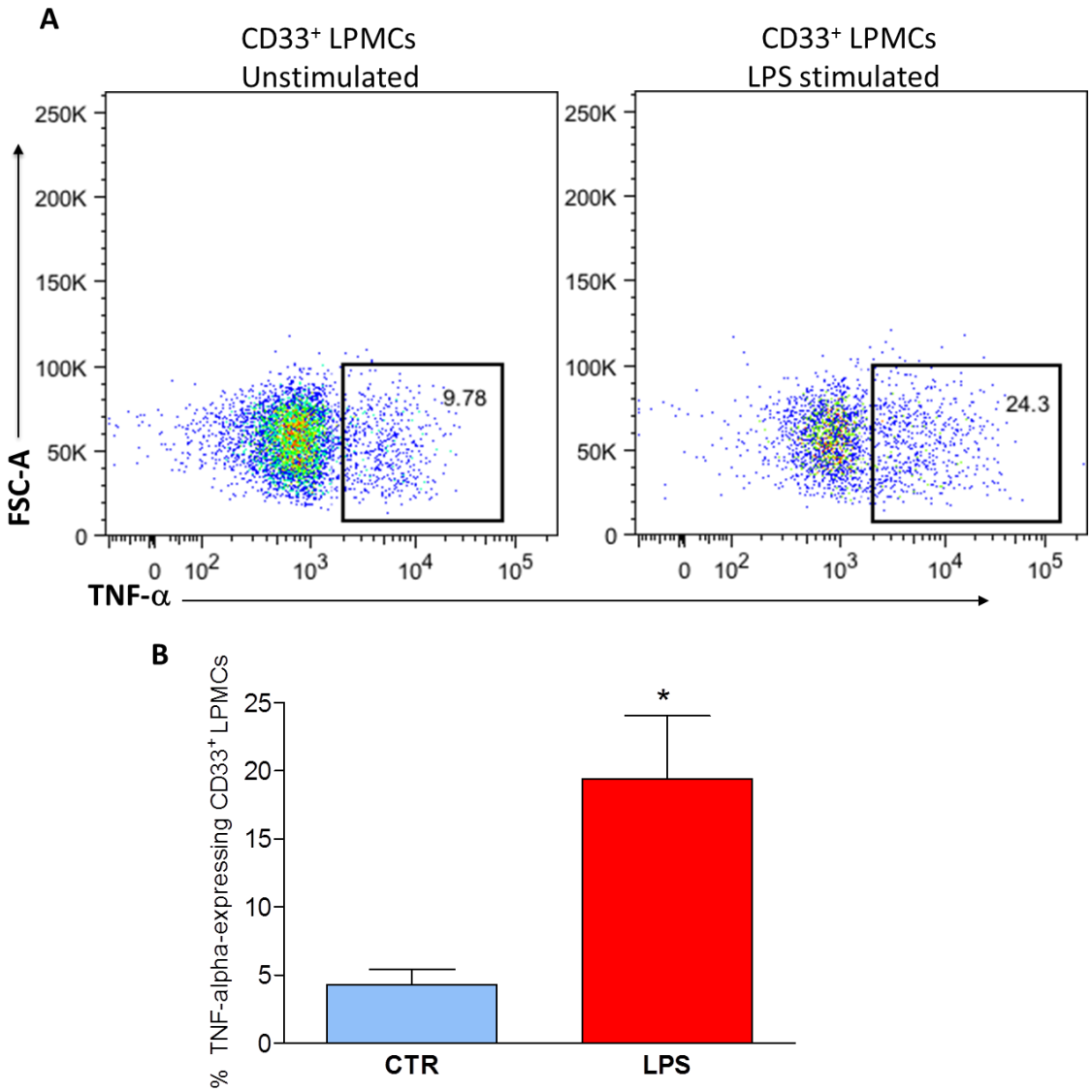

Figure 5.3: TNF-α expression by IBD CD33$^+$ LPMCs with or without LPS stimulation: LPMCs from IBD where cultured in medium and LPS (100ng/ml) in presence of brefeldin for 4 hours. **A.** Representative plots showing TNF-α expression by live-gated CD33$^+$ LPMCs unstimulated and stimulated with LPS. **B.** Percentage of CD33$^+$ cells expressing TNF-α in only medium and LPS stimulation. Data are representative of two independent experiments with n=4 per experiment. Bars represent mean percentage of expression and error bars denote SEM.

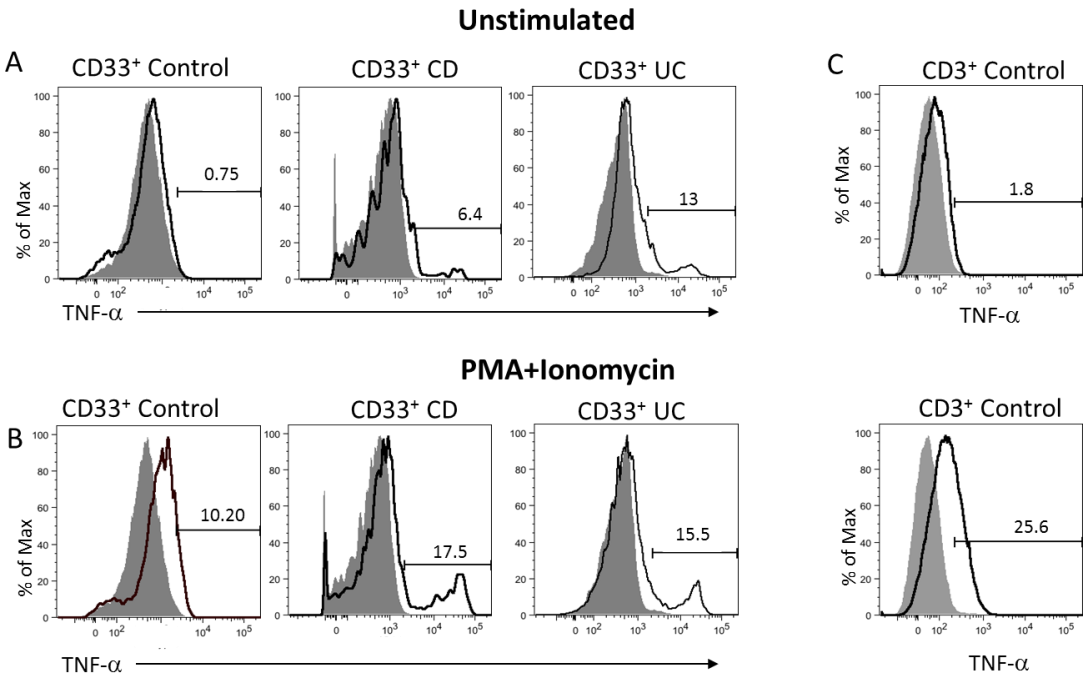

Figure 5.4: CD3$^+$ and CD33$^+$ LPMCs stimulated by PMA+ionomycin. Representative histograms expressing percentage of TNF-α as a proportion of total live LPMCs in different populations: **A**. CD33$^+$ LPMCs from normal mucosa and IBD mucosa cultured with medium **B**. CD33$^+$ LPMCs from normal mucosa and IBD mucosa cultured with PMA+Ionomycin. TNF-α expressed in each subsets in presence (continue black line) or absence (solid line) of PMA+ionomycin. **C.** CD3$^+$ LPMCs from normal mucosa cultured with medium or with PMA+ION

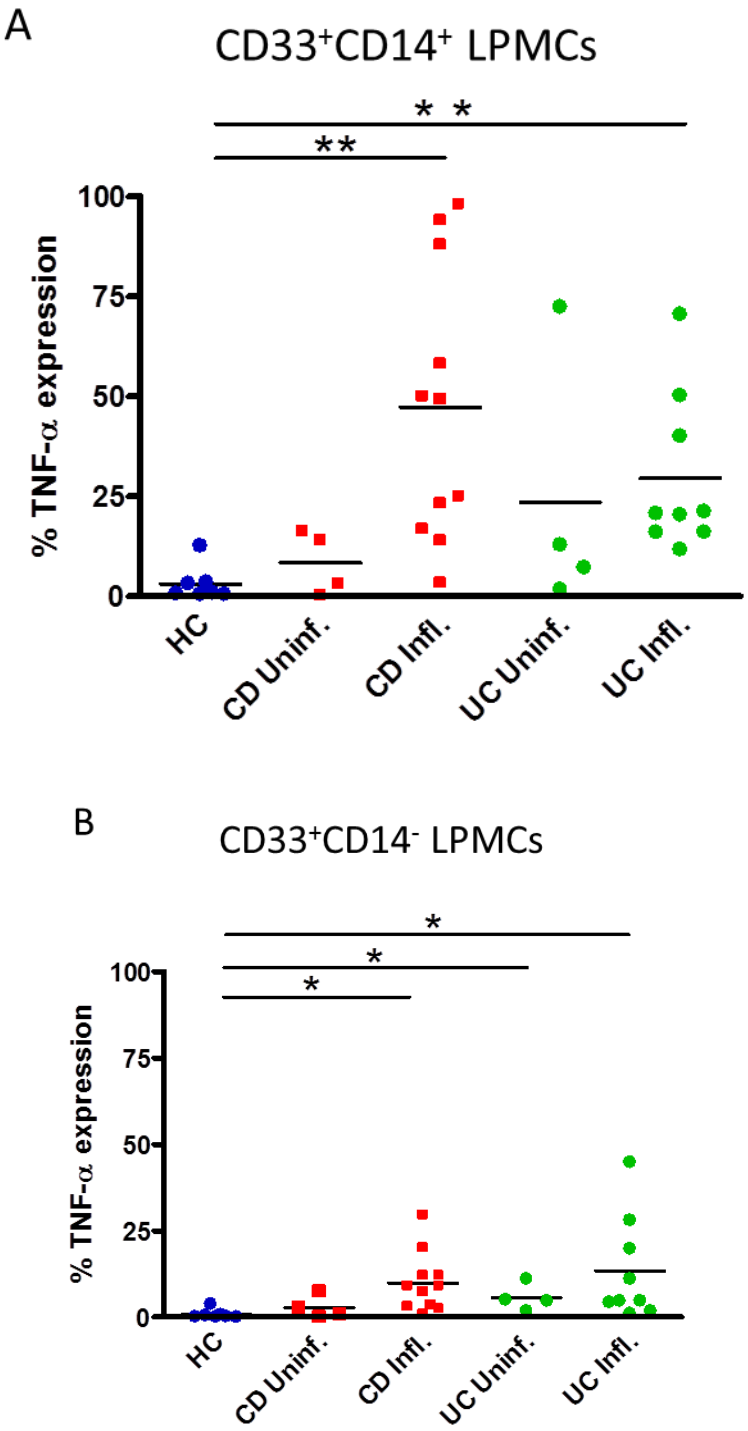

Figure 5.5: **TNF-α expression by CD33$^+$CD14$^+$ and CD33$^+$CD14$^-$. A.** Percentage of intracellular TNF-α among CD33$^+$CD14$^+$ population in control (n=7) uninflamed CD (n=4), Inflamed CD (n=11) Uninflamed UC (n=4) and Inflamed UC (n=4). **p (control *versus* Infl CD) =0.0039; **p (control *versus* Infl UC) =0.0032. **B.** Percentage of intracellular TNF-α among CD33$^+$CD14$^-$ in the same groups of graph A. *p (control *versus* Infl CD) = 0.0135; *p (control *versus* Uninf UC) = 0.0122; *p (control *versus* Infl UC) = 0.0437

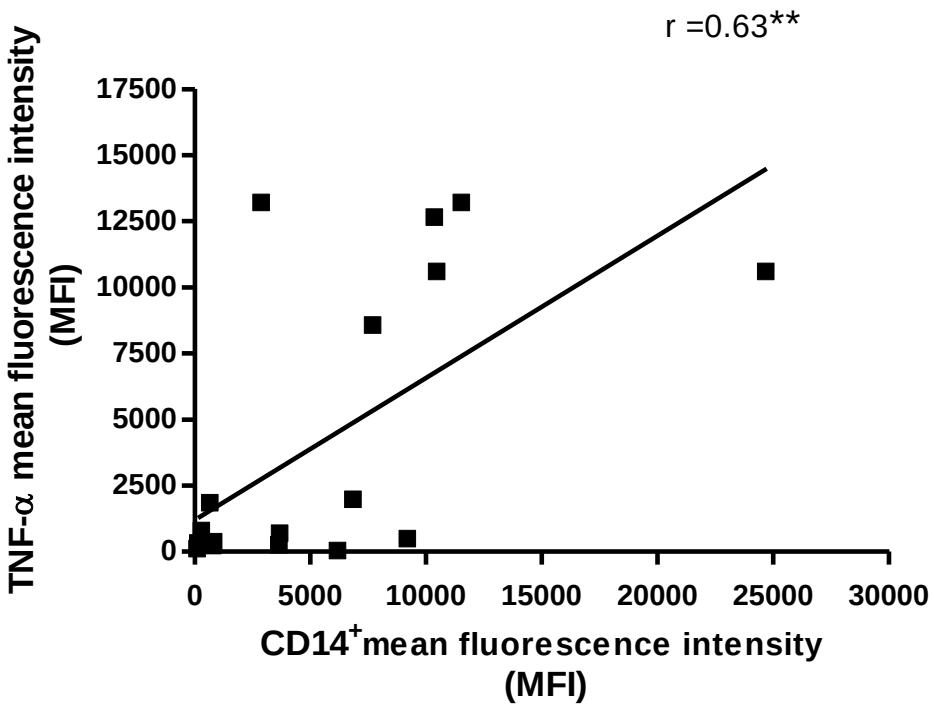

Figure 5.6: **Correlation between TNF-α and CD14$^+$:** Pearson's correlation analysis between the MFI of TNF-α and the MFI of CD14$^+$ .18 IBD mucosa samples were considered (n=11 inflamed CD and n=7 inflamed UC). **p=0.0054

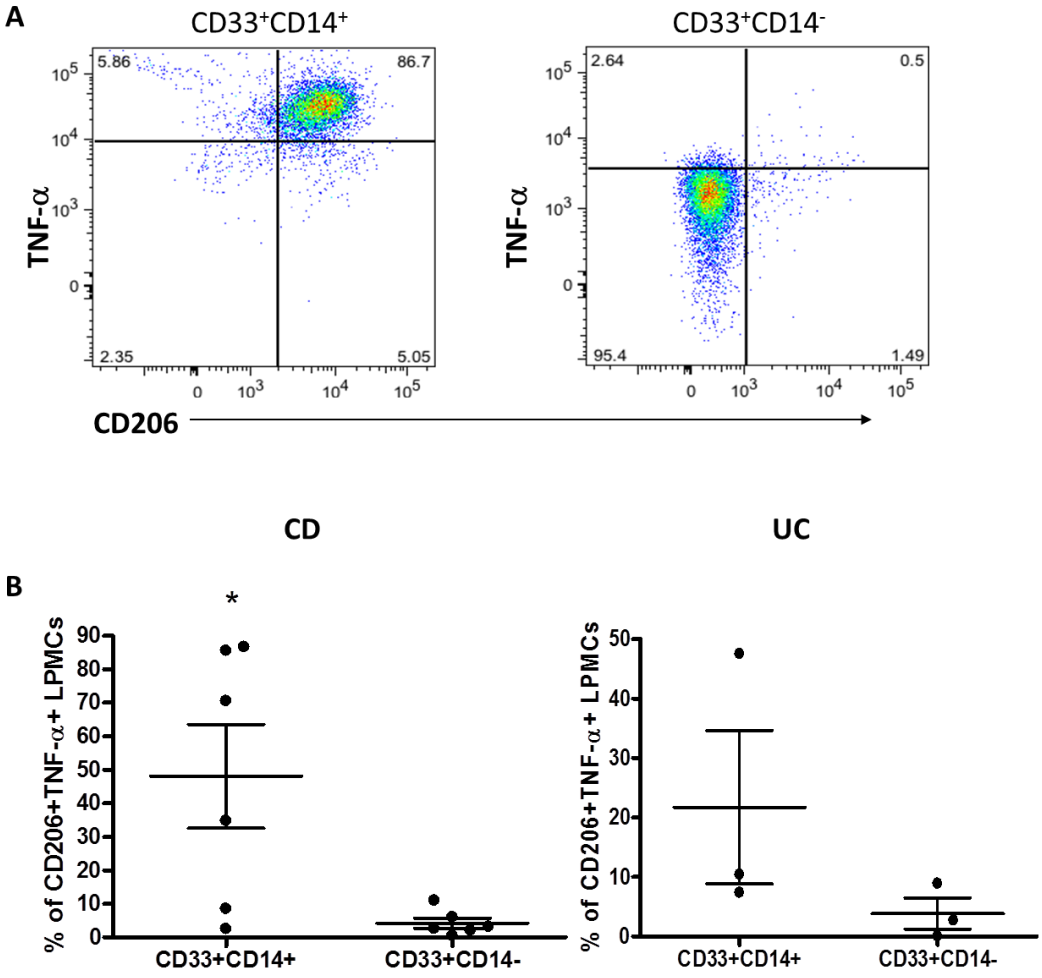

Figure 5.7: Recruited population of macrophages co-express the M2 marker CD206 and TNF-α: A. Representative plots showing $CD206^+$ expressing TNF-α by live-gated $CD33^+CD14^+$. **B.** Percentage of $CD33^+CD14^+CD206^+$ and $CD33^+CD14^-CD206^+$ expressing TNF-α. Data are representative of n=6 per experiment. Bars represent mean percentage of expression and error bars denote SEM.

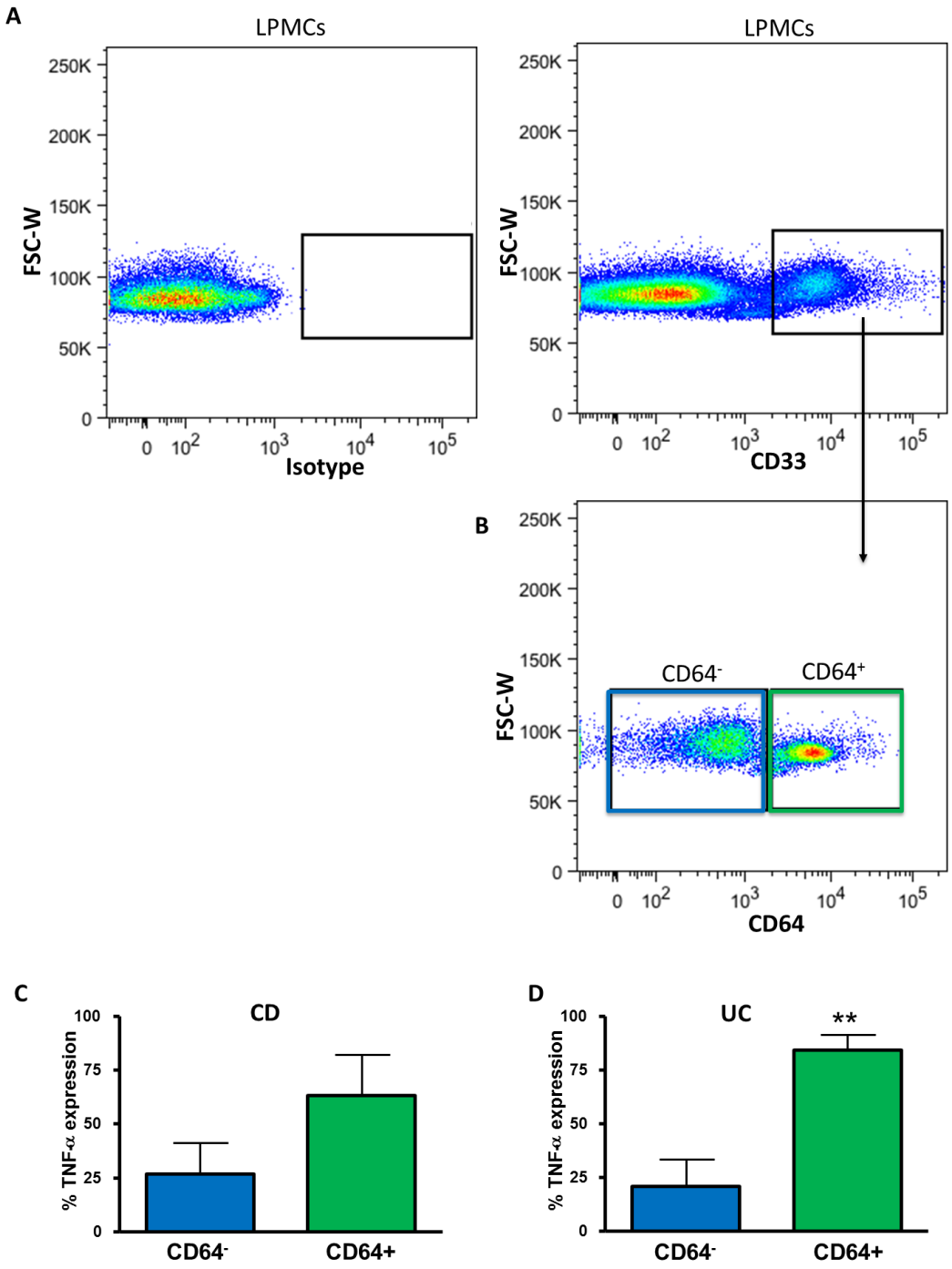

Figure 5.8: TNF-α-expressing CD64$^+$ and CD64$^-$ LPMCs in IBD: A. Representative gating strategy showing CD33$^+$LPMCs gate. **B.** Representative plot showing CD64$^-$ and CD64$^+$ **C.** Percentage of TNF-α$^+$-expressing CD64$^-$ and TNF-α$^+$-expressing CD64$^+$ in CD and UC. Data are representative of n=4 per experiment. Bars represent mean percentage of expression and error bars denote SEM. *p CD64$^+$ cells versus CD64$^-$ = 0.0045.

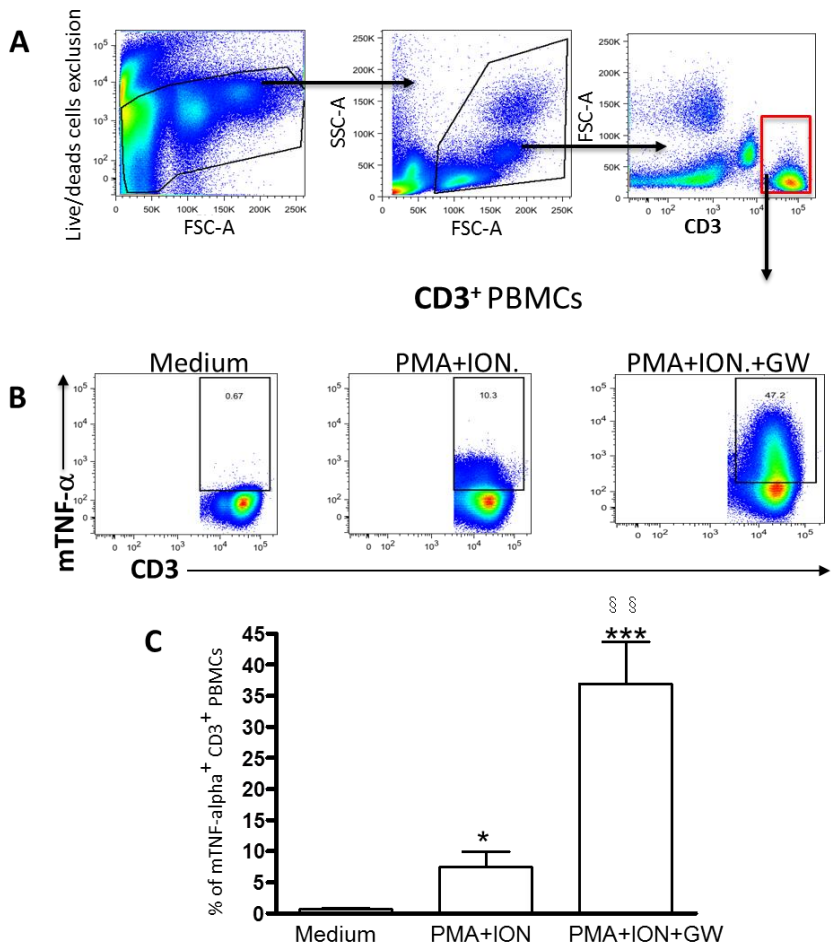

Figure 5.9: Expression of membrane TNF-α on unstimulated CD3$^+$, PMA+Ionomycin and PMA+Ionomycin+ GW280264X (GW) activated CD3$^+$ cells: PBMCs from healthy individuals were stimulated with 50 ng/ml of PMA and 500 ng/ml of ionomycin and 10µg/ml of GW for 4 hours. **A.** Gating strategy used to gate positive CD3$^+$. **B.** Representative dot plots showing expression of membrane TNF-α by FITC-conjugated mouse anti-TNF-α monoclonal antibody using FACS analysis. **C.** Proportion of membrane TNF-α expression on unstimulated CD3$^+$ (medium), PMA+Ionomycin+GW and PMA+ION+GW CD3 stimulated. Data are representative of n=6. *p= 0.0298; §§p= 0.0017 *versus* P+I; ***p=0.0008 *versus* DMSO.

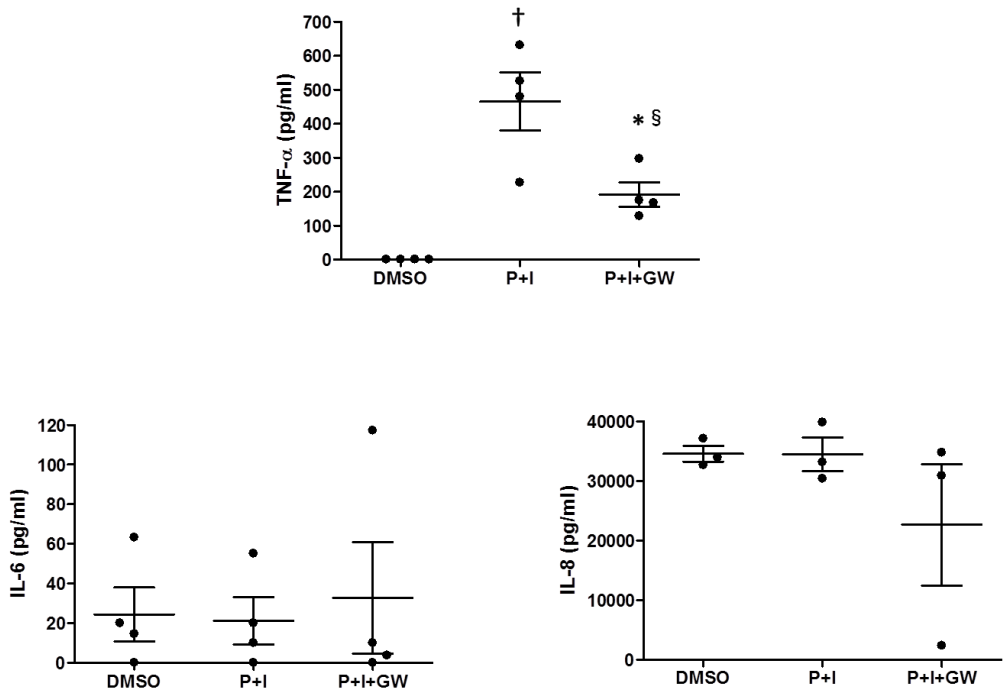

Figure 5.10: Expression of soluble TNF-α in the supernatant of stimulated PBMCs: TNF-α, IL-6 and IL-8 cytokines concentration (measured by ELISA) in the culture supernatants following PBMCs culture incubated, for 4h, in the presence of medium with DMSO (0.1%) or PMA+ionomycin (P+I) or PMA+ionomycin+GW (P+I+GW) at final concentration of DMSO 0.1%. Results are mean ± SEM and are representative of n=4 (TNF-α and IL-6) and n=3 (IL-8).*p= 0.0135 *versus* DMSO; †p= 0.0122 versus DMSO; §p=0.0188 *versus* P+I.

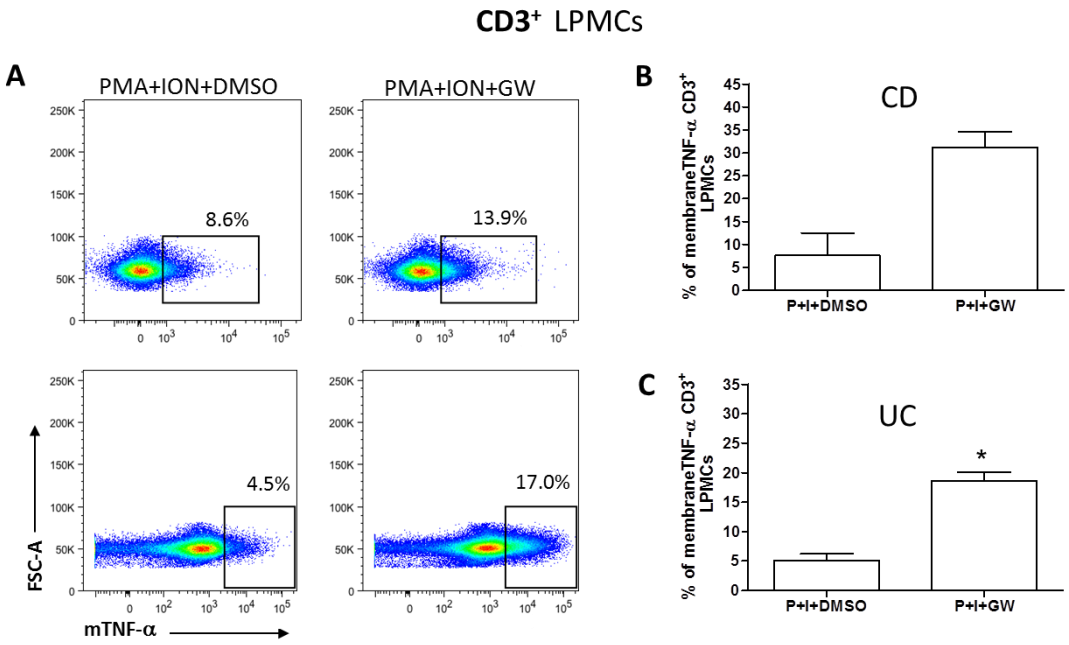

Figure 5.11: Expression of membrane TNF-α on activated CD3$^+$ T cells in CD and UC LPMCs. A. Representative dot plots showing expression of membrane TNF-α by inflamed CD (plots above) and UC (plots below) T cells LPMCs. Cells were first stimulated in the presence of PMA, ionomycin and DMSO (P+I+DMSO) and PMA ionomycin and GW (P+I+GW) for 4 hours. **A**. Proportion of membrane TNF-α expression on P+I+DMSO and P+I+GW CD stimulated cells. **B.** Proportion of membrane TNF-α expression on P+I+DMSO and P+I+GW UC stimulated cells. * p=0.0175. Data are representative of n=2.

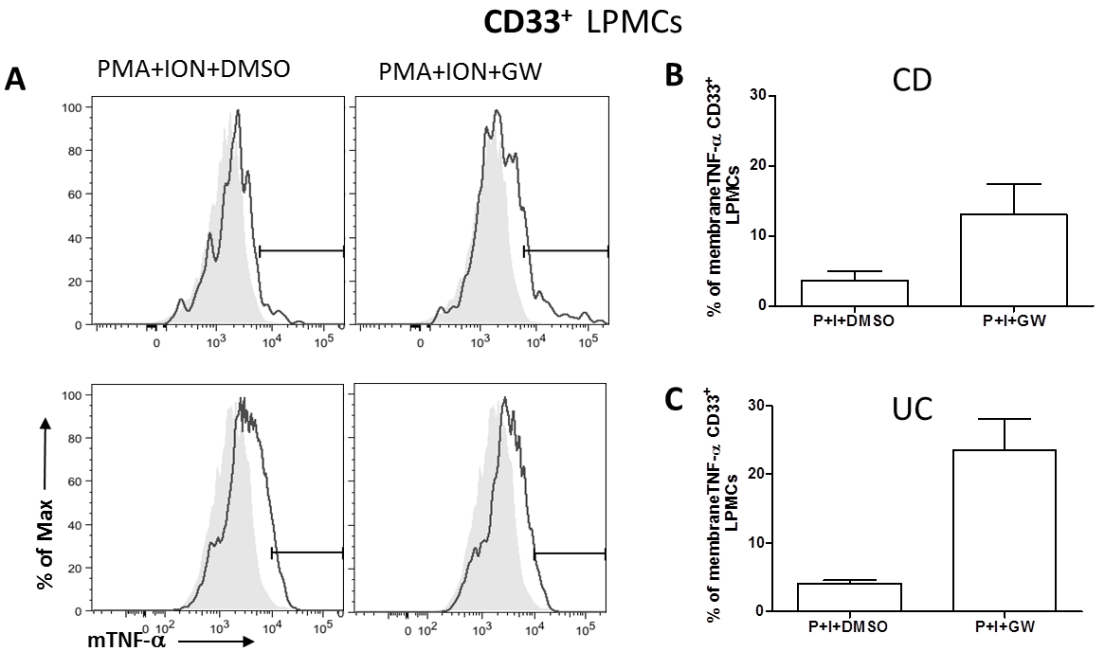

Figure 5.12: Expression of membrane TNF-α on activated IBD CD33$^+$ LPMCs. A. Flow cytometry histogram showing membrane TNF-α staining (continue black line) in comparison with isotype control antibody (solid line) in CD (upper plots) and UC (lower plots). Cells were gated on CD33 LPMCs. Gating strategy for CD33$^+$ has been described previously. **B.** Proportion of membrane TNF-α expression by P+I+DMSO and P+I+GW stimulated cells in CD. **C.** Proportion of membrane TNF-α expression by P+I+DMSO and P+I+GW stimulated cells in UC. Data are representative of n=2.

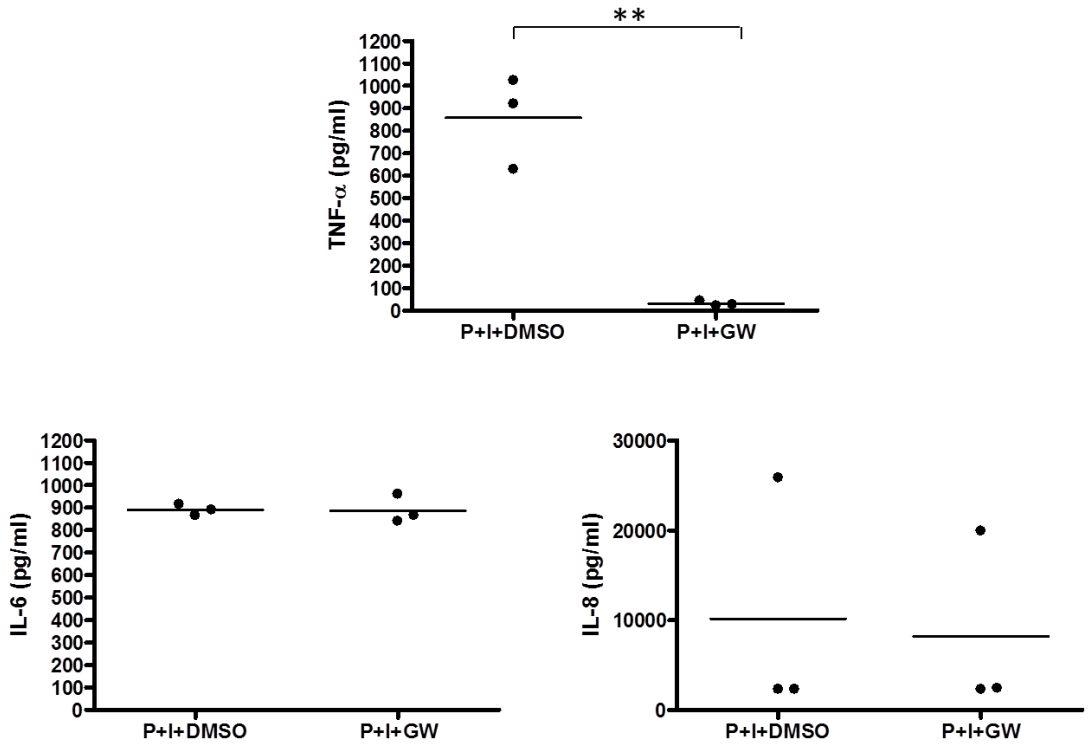

Figure 5.13: Effect of ADAM10 and ADAM17 inhibition on cytokine secretion in PMA+ionomycin stimulated LPMCs. LPMCs from mucosa of IBD patients were isolated and cultured in the presence of PMA+ionomycin+DMSO (P+I+DMSO) or PMA+ionomycin+GW (P+I+GW) at final concentration of DMSO 0.1% for 4 hours. TNF-α, IL-6 and IL-8 cytokines concentration were measured in culture supernatants by ELISA. Results are mean ± SEM and are representative of n=3 **p= 0.0022 *versus* PMA+ionomycin+DMSO.

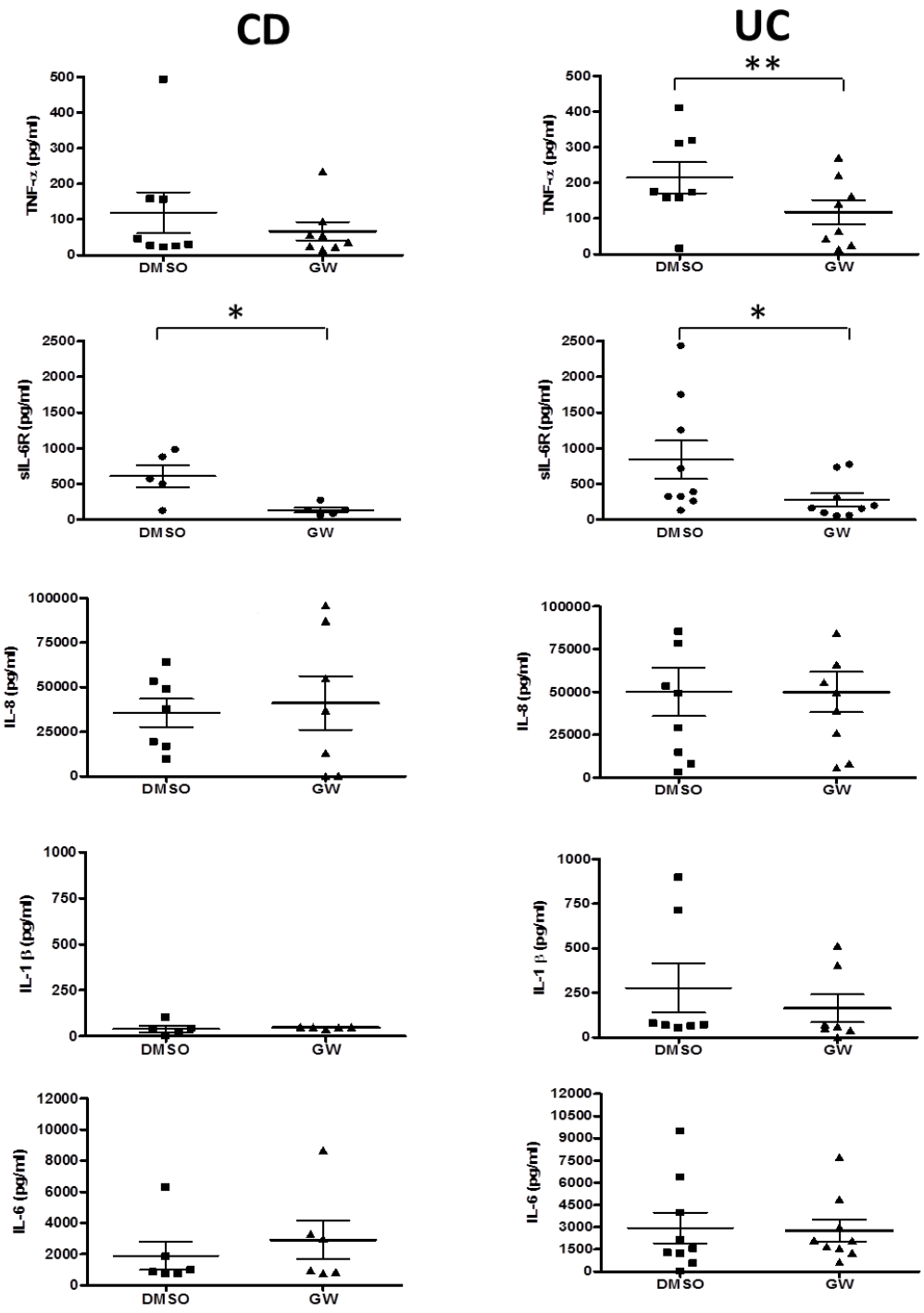

Figure 5.14: *Ex vivo* production of TNF-α, sIL-6R, IL-8, IL-1β and IL-6. Levels of TNF-α sIL-6R,IL-8, IL-1β and IL-6 expressed in pg/ml, in the supernatants of intestinal biopsies taken from the inflamed mucosa of CD (TNF-α n=8; sIL-6R n=5; IL-8 n=7; IL-1β n=5; IL-6 n=6) and UC (TNF-α n=8; sIL-6R n=9; IL-8 n=8; IL-1β n=7; IL-6 n=9) and cultured for 24h with DMSO only or with 10 μM/ml of GW.

CHAPTER 6

Results: Gene expression profile of $CD33^+$ LPMCs in IBD

6.1 Introduction

$CD14^+$ $CD33^+$ macrophages are increased in the gut mucosa of IBD patients. Therefore to elucidate possible functional differences, a gene expression array was performed on $CD33^+$ LPMCs from normal colon and the colon of patients with IBD. LPMCs were isolated from the inflamed colonic mucosa of 11 IBD patients (7 UC and 4 CD) and from normal colon of 10 patients and $CD33^+$ cells were purified by MACS.

Transcripts were grouped in 6 categories: Interferons, Bone Morphogenic Proteins (BMPs) and TGF-β Family, PDGF/VEGF Family, TNF Superfamily, and other Growth Factors/Cytokines and Interleukins.

Using +2 and -2 fold change cutoff to define the genes up-regulated or down-regulated, 71 genes were up-regulated (33 in UC and 38 in CD) and 45 down-regulated (23 in UC and 22 in CD) in IBD samples compared to controls. A complete list of all the genes including fold change and p value is listed in the Table 6.1. On the basis of the array results, we focused on the genes which were significantly ($p < 0.05$) down- or up-regulated. Twelve genes were differentially expressed in IBD patients compared to control subjects (Figure 6.1). These include BMPs and TGF-β Family (*BMP2* both in UC and CD; *BMP5* only in UC), PDGF/VEGF Family (*PDGFA* only in UC), *TNF* Superfamily (*TNFSF8* both in UC and CD), the IL-10 cytokine family (*IL10* only in UC and *IL24* both in UC and CD), the IL1 cytokine family (*IL1F5* and *IL1F7* both in UC) and also *IL13* and *IL8* in UC.

6.2 Up-regulated genes in CD33^{+} LPMCs from patients with ulcerative colitis

IL21 was significantly up-regulated in UC samples (p=0.0216) compared to controls, as well as *IL24* (UC p=0.0194) and the anti-inflammatory cytokine *IL-10* (p=0.0307). Regarding the TNF superfamily, the only gene in the group to be significantly upregulated in UC was *TNFSF8* (p=0.046). Finally *IL13*, *IL8* and *IL1F5* genes were significantly up-regulated (*IL13* p=0.0393; *IL8* p=0.0415 and *IL1F5* p=0.037) (Table 6.2).

6.3 Up-regulated genes in CD33^{+} LPMCs from patients with Crohn's disease

Four genes were up-regulated in CD, particularly *IL21* with a 52 fold increase (p=0.0498). *IL15* was also up-regulated in CD (p=0.0486), while *IL-24* (p=0.0227) and *TNFSF8* (p=0.049) were also up-regulated (Table 6.2).

6.4 Down-regulated genes in CD33^{+} LPMCs from patients with ulcerative colitis

Two genes belonging to BMPs family were down-regulated in UC, *BMP2* (p=0.0291) and BMP5 (p= 0.0061). *PDGFA* and *IL1F7*, were also significantly down-regulated in UC compared to healthy mucosa (p=0.0422 and p=0.0352 respectively) (Table 6.3).

6.5 Down-regulated genes in CD33^{+} CD LPMCs

Only one gene, belonging to the BMPs family; BMP2, was significantly down-regulated in CD (p=0.0221) compared to control (Table 6.3).

6.6 IL-24 mRNA expression in the $CD33^+$ LPMCs

The array analysis unexpectedly highlighted that IL24 transcripts were highly over-expressed in both CD and UC. RT-PCR for IL-24 on the same samples also showed significantly higher ($p<0.05$) expression of mRNA in UC and CD compared to normal colon (Figure 6.2).

IL24 gene is located in chromosome 1, within a 195-kb cytokine cluster containing the IL-10, IL-19, IL-20, and IL-24 genes (Huang et al., 2001) and it shares a 20–30% amino acid homology with IL-10, IL-20, and IL-22 and interacts with two different heterodimeric receptors, IL-20R1/IL-20R2 and IL-22R1/IL-20R2. Binding to both receptors leads to the activation of STAT-3, similar to other members of the IL-10 family of cytokines (Dumoutier and Renauld, 2002) (Dumoutier et al., 2001). Transgenic mice that overexpress *IL20, IL22* or *IL24* develop skin lesions similar to those seen in human psoriasis (Blumberg et al., 2001; Wolk et al., 2009). Moreover, in vivo, IL-24 is predominantly expressed in skin during inflammatory conditions, such as psoriasis (Kunz et al., 2006). IL-24 gene expression is also greatly increased at the edge of excisional skin wounds (Soo et al., 1999).

IL-24 protein was next measured by ELISA in the supernatants of mucosal biopsies from normal colon, inflamed and uninflamed IBD cultured overnight. Higher concentrations of IL-24 were found in the supernatants of inflamed biopsies from IBD patients (928.5 pg/ml ± 171.3) compared to uninflamed IBD (584.2 pg/ml ± 145.1) and normal samples (273.2 pg/ml ± 37.79) (Figure 6.3).

6.7 Effect of recombinant human IL-24 on TNF-α production by IBD biopsies

Biopsies from control and inflamed IBD were cultured for 24 h with recombinant human IL-24 and the concentrations of IL-6, IL-8 and TNF-α were measured in the supernatants. IL-24 significantly increased TNF-α in the supernatants of biopsies from IBD patients (436.9 pg/ml ± 115.7) compared to biopsies incubated with medium only (119.9 pg/ml± 40.99). IL-8 and IL-6 were unchanged when biopsies were cultured with IL-24 (Figure 6.4).

6.8 TNF-α mRNA expression in $CD33^+$ LPMCs

By PCR array, *TNF* was increased 3.91 fold in UC and 8.2 in CD fold but this result was not statistically significant, so TNF transcripts were quantified in the same $CD33^+$ cells. There was no statistically significant difference in the abundance of this transcript between UC and control ($p=0.8226$). On the contrary *TNF* was significantly up-regulated in CD ($p<0.05$) compare to the control (Figure 6.5).

6.9 Discussion

The main focus of this part of the project was to analyze changes in gene expression in macrophages from the inflammatory bowel disease tissues. CD33 was used as a marker to purify the cells because the numbers of cells obtained using CD33 and CD14, yielded too few cells to perform the experiments.

Among the genes up-regulated in IBD, *IL24* expression was significantly elevated both at mRNA and protein level. *Ex vivo* experiments showed that IL-24 protein was higher increased in the supernatant of inflamed mucosa of IBD and also recombinant human IL-24 induced a significant increase of TNF-α by IBD biopsies cultured ex vivo.

To interpret these findings more clearly, the genes up- and down-regulated will be discussed by organizing them into four groups (the groups which did not show dysregulated genes are excluded) namely: Bone Morphogenic Proteins (BMPs) and TGF-β Family, PDGF/VEGF Family, TNF Superfamily and Interleukins.

While BMP2 expression was down-regulated in both UC and CD, BMP5 decreased only in UC. BMPs and their signaling pathway are expressed in normal intestine and colon. BMP2, phosphorylated Smad1 and Smad4 are present in mature colonocytes at the epithelial surface of normal human and mouse colon (Hardwick et al., 2004). Moreover an increase in BMP2 expression is seen in TNBS-induced colonic inflammation resulting in increased epithelial cell proliferation (Maric et al., 2012).

The array showed that most of the genes belonging to the TNF superfamily were up-regulated in UC and CD, but only *TNFSF8*, was significantly up-regulated.
In vivo administration of a TNFSF8 agonist (anti-CD30 mAb) ameliorates oxazolone (OXA)-induced colitis, but aggravates TNBS-induced colitis in CD30LKO mice (Sun et

al., 2008). The analysis of some of the genes of TNF superfamily which were up-regulated in CD or UC showed increased expression of TNFSF4 (OX40L). This molecule is expressed by dendritic cells and macrophages and serves as a co-stimulatory molecule by binding to OX40 on T cells. Although much of the activity of OX40-OX40L interactions can be attributed to OX40 signalling, OX40L clearly can produce functional effects as the production of cytokines such as IL-12, IL-6, IL-1, TNF, and IFN-α. How OX40L transmits signals to promote proinflammatory cytokines is not known, but some studies have found alterations in PKCβ2, c-Jun, and c-Fos after OX40L is cross-linked (Burgess et al., 2004; Matsumura et al., 1999). Moreover, blocking the interaction OX40-OX40L ameliorates TNBS colitis in mice and the spontaneous colitis seen in IL-2 null mice (Higgins et al., 1999).

The increased production of TNF-α protein level by flow cytometry in $CD33^{+}CD14^{+}$ was not confirmed by the results of the array which showed no significant increase for UC and CD compared to control. This apparent contradiction can be ascribed to an important feature of TNF transcript which are that they are very short lived (< 45 min) independent of the activation state of the macrophages, (Mijatovic et al., 2000).

Genes from the IL-1 cytokine family as IL-1F5 (IL-36Ra) and IL-1F7 (IL-37), were respectively up-regulated and down-regulated in UC and although little is known regarding the potential contributions of IL-1 family members in chronic intestinal inflammation and gut health, IL-36Ra has been shown to possess an anti-inflammatory effect in the brain mediated through a unique TIR8/SIGIRR-dependent pathway (Costelloe et al., 2008).

Interestingly while in UC, IL-37 was significantly down-regulated, in CD it was up-regulated suggesting that IL-37 dysregulation may play a different role in the two

diseases. IL-37b (which is the most abundant form of IL-37 and the most studied), is expressed in the lamina propria of normal colon (Chen and Fang, 2004) and *in vitro* studies on macrophages and epithelial cells overexpressing IL-37b, as well as in vivo experiments in transgenic mice overexpressing human IL-37b, show reduced DC activation and decreased production of pro-inflammatory and Th1/Th17 cytokines, including IL-1β, IL-6, IFN-γ, and IL-17 following LPS stimulation (Nold et al., 2010).

IL15 gene expression was significantly increased in CD array. IL-15 belongs to the IL-2 cytokine family. The role of IL-15 has been previously investigates in IBD and it has been suggested that macrophages may be the main producers of IL-15 in IBD patients (Liu et al., 2000). Moreover IL-15 together with IL-12 is responsible for the expansion of the human intraepithelial ILC1 which produce IFNγ in CD patients (Bernink et al., 2013).

IL-21 also belongs to the IL-2 family cytokine and in the array *IL21* gene expression was significantly highly up-regulated both in UC and CD. IL-21 and IL-17, T_H17-type cytokines were found to mediate pro-inflammatory functions including the up-regulation of TNF, IL‑1β, IL‑6 and IL‑8, the recruitment of neutrophils and the secretion of matrix metalloproteinase by intestinal fibroblasts (Monteleone et al., 2006; Monteleone et al., 2005; Siakavellas and Bamias, 2012).

The up-regulation of *IL13* in UC was not surprising considered how several studies in recent years have highlighted a crucial role of IL-13 in the development of UC. Targeting IL-13 has received particular attention as blockade of IL-13 expression and function was highly effective in the treatment of experimental colitis. Moreover IL‑13

has been shown to promote fibrosis and to cause altered tight junction function and apoptosis of IECs thereby driving mucosal ulceration (Heller et al., 2005).

However the importance of IL-13 as driven cytokine in the disease of UC has recently been questioned by Biancheri et al in our lab. It was shown that IL-13 is not produced in excess by mucosal explants or LPMCs from inflamed areas of UC patients in comparison to inflamed areas of CD patients and healthy mucosa (Biancheri et al., 2013a).

The arrays showed also that *IL10* was highly expressed in UC but not in CD. The major sources of IL-10, an anti-inflammatory cytokine, are macrophages and dendritic cells (Paul et al., 2012). Although some other authors showed increased levels of IL-10 mRNA in patients with UC but not CD (Peña and Crusius, 1998) it remains unclear why IL-10 is not significantly up-regulated in CD. In UC, IL-10 mRNA expression was found to be highly increased in mucosal T cells (Melgar et al., 2003)..

The increased expression of *IL24* in the active lesions of patients with UC and CD suggested that IL24 can have a role in mucosal inflammation in IBD; furthermore the results from microarray were confirmed by IL-24 PCR, confirming others previous results (Ghoreschi et al., 2003). There were also higher levels of IL-24 protein in supernatants of IBD biopsies.

Only a few reports describe the *in vivo* expression of IL-24 under normal and pathological conditions (Soo et al., 1999). One of these studies showed that IL-24 expression is greatly increased at the edge of cutaneous wounds in an animal model. Another study by Kunz et al. (Kunz et al., 2006) showed that IL-24 was expressed by keratinocytes from patients with psoriasis.

The precise cytokine regulation and molecular mechanisms of IL-24 induction still remain unclear but it is known that IL-24 can function either as an intracellular cell death-inducing factor in cancer cells, or as a classical pro-inflammatory cytokine signaling through its cell surface receptors, IL-20R1/IL-20R2 and IL-22R1/IL-20R2. These receptors complexes have been studied in the colonic epithelial cells, neutrophils, $CD4^+$ cells, $CD8^+$ cells and neutrophils (Ma et al., 2011).

Interestingly, in IBD biopsies cultured with recombinant human IL-24, among various cytokines, TNF-α was greatly increased, confirming a possible mechanism of interaction between IL-24 and the receptors IL-20R1/IL-20R2 and IL-22R1/IL-20R2.

The use of biopsy organ culture system may more closely approximate ongoing, in vivo cytokine production by allowing undisrupted dialogue between intestinal immune cells. Moreover, organ cultures include cytokines production from several different intestinal sources hence is only possible to speculate the mechanism involving the productions of TNF-α by IL-24. Binding of IL-24 with two heterodimeric receptor complexes, IL-20R1/IL-20R2 and, preferentially, IL-10R2/IL-22R1 results in the activation of STAT1 and STAT3 signalling pathways (Dumoutier et al., 2001).

Another possible mechanism requires the binding of IL-24 to the receptor dimers expressed on Th1 cells which activate and induce TNF-α production.

In conclusion this study provides for the first time a global analysis of human macrophages gene expression profiles where genes from inflamed intestinal mucosa were compared to uninflamed non-IBD area, providing an important reference point for further studies focusing on modulating macrophage activity in chronic inflammatory conditions.

	UC vs control		CD vs control		UC vs CD	
	Fold change	**P value**	**Fold change**	**P value**	**Fold change**	**P value**
Interferons:						
IFNB1	4.0597	*0.2683*	18.2213	*0.0643*	4.4883	*0.3485*
IFNG	-3.3452	*0.2202*	-2.0148	*0.547*	1.6603	*0.68*
IFNK	16.2625	*0.4926*	36.1688	*0.4625*	2.2241	*0.8763*
IFNA1	1.7583	*0.5552*	7.3097	0.0945	4.1572	*0.2482*
IFNA8	1.6053	*0.4136*	2.9779	0.1253	1.8551	*0.4015*
IFNA5	-5.4572	*0.4877*	-13.8291	0.373	-2.5341	*0.7637*
IFNA4	-3.9717	*0.0752*	-3.6978	0.1531	1.0741	*0.9395*
IFNA2	3.1455	*0.2489*	2.607	0.4174	-1.2066	*0.8797*
Bone Morphogenic Proteins (BMPs) and TGF-β Family:						
BMP1	1.1837	*0.5547*	-1.711	*0.1276*	-2.0253	*0.0631*
BMP2	-3.0781↓	***0.0291***	-4.1605 ↓	***0.0221***	-1.3517	*0.6234*
BMP3	267.9016	*0.2496*	-274.5393	*0.3328*	-1.0248	*0.9968*
BMP4	-2.0221	*0.243*	-2.2628	*0.2588*	-1.1191	*0.8812*
BMP5	-6.0531↓	***0.0061***	-1.8972	*0.3694*	3.1906	*0.1328*
BMP6	-9.7945	*0.176*	-7.763	*0.3059*	1.2617	*0.9114*
BMP7	1.298	*0.7213*	1.1692	*0.8585*	-1.1102	*0.9103*
BMP8B	-3.1975	*0.1357*	-2.7843	*0.2667*	1.1484	*0.8854*
GDF10	-4.6131	*0.2627*	3.1185	*0.4829*	14.3862	*0.1303*
GDF11	1.0875	*0.803*	-1.0622	*0.8811*	-1.1551	*0.7361*
GDF3	-1.6331	*0.824*	-5.4854	*0.5224*	-3.3588	*0.6664*
GDF5	6.832	*0.0518*	1.3608	*0.7841*	-5.0206	*0.186*
MSTN	-45.4962	*0.0835*	-137.9421	*0.0644*	-3.0319	*0.6805*
GDF9	-3.2657	*0.1091*	-2.1327	*0.3807*	1.5313	*0.639*
INHA	-1.422	0.7178	-1.4011	0.7729	1.0149	0.9905
NODAL	-2.4468	0.0905	-1.6056	0.4407	1.5239	0.5163
TGFA	-2.0179	0.3243	-3.5883	0.1418	-1.7783	0.5218
TGFB1	2.0782	0.0871	1.7865	0.2473	-1.1633	0.7719
TGFB2	1.828	0.3585	2.3263	0.2864	1.2726	0.7706
TGFB3	-1.1237	0.7887	-1.7126	0.3097	-1.5241	0.4497
INHBA	22.625	0.0553	24.0037	0.099	1.0609	0.976
PDGF/VEGF Family						
PDGFA	-2.9957↓	**0.0422**	-2.999	0.0849	-1.0011	0.9986
FIGF	1.3612	0.6658	3.4683	0.1574	2.548	0.3089

TNF Superfamily:

LTA	10.0344	*0.0862*	12.411	*0.116*	1.2368	*0.8968*
LTB	9.1674	*0.0661*	16.6262	*0.0534*	1.8136	*0.6843*
TNF	3.9069	*0.1847*	8.1705	*0.0935*	2.0913	*0.5644*
TNFRSF11B	4.4563	*0.1956*	2.7107	*0.4646*	-1.644	*0.7293*
TNFSF10	-1.4276	*0.3731*	-1.1258	*0.803*	1.2681	*0.6376*
TNFSF11	11.6094	*0.2494*	18.4421	*0.2538*	1.5886	*0.8617*
TNFSF12	2.0318	*0.148*	1.8847	*0.2751*	-1.078	*0.9012*
TNFSF13	-1.0957	*0.7888*	-1.935	*0.119*	-1.7661	*0.1997*
TNFSF13B	2.6571	*0.1007*	3.5222	*0.0797*	1.3256	*0.6994*
TNFSF4	4.1705	*0.1125*	4.3101	*0.1721*	1.0335	*0.9762*
TNFSF8	9.1882 ↑	***0.0461***	13.4177 ↑	***0.0497***	1.4603	*0.7713*
CD70	7.056	*0.0912*	3.92	*0.3124*	-1.8	*0.6779*
TNFSF14	4.5082	*0.0605*	3.8455	*0.153*	-1.1723	*0.8698*
FASLG	-1.0533	*0.9311*	-1.0588	*0.9368*	-1.0052	*0.9945*

Other Growth Factors/Cytokines:

LEFTY2	-4.3709	*0.1149*	-2.1477	*0.4837*	2.0352	*0.5382*
CSF1	2.8581	*0.1042*	2.1254	*0.3197*	-1.3448	*0.7088*
CSF2	28.5522	*0.1022*	12.9832	*0.2871*	-2.1992	*0.7539*
FAM3B	-5.9222	*0.5619*	-6.3419	*0.6155*	-1.0709	*0.9859*

Interleukins:

IL-10 Family

IL10	4.8668 ↑	***0.0307***	3.8929	*0.1107*	-1.2502	*0.7977*
IL19	18.662	*0.3481*	8.1478	*0.5722*	-2.2904	*0.8326*
IL22	16.166	*0.1087*	50.2084	*0.0634*	3.1058	*0.5956*
IL24	4.1236 ↑	***0.0194***	5.2046 ↑	***0.0227***	1.2621	*0.7439*
IL20	-3.17	*0.5413*	-13.9291	*0.2517*	-4.394	*0.5378*

IL-1 cytokine family

IL1A	4.4201	*0.1289*	4.8766	*0.1746*	1.1033	*0.935*
IL1B	9.255	*0.0611*	8.7156	*0.1228*	-1.0619	*0.9667*
IL1F8	7.6429	*0.3989*	194.634	*0.0785*	25.4659	*0.2937*
IL1F5	4.1726 ↕	***0.037***	2.4866	*0.247*	-1.678	*0.5291*
IL1F7	-10.9475 ↕	***0.0352***	1.047	*0.9714*	11.4619	*0.0846*
IL1F9	1.0501	*0.9712*	-1.232	*0.8978*	-1.2938	*0.8811*
IL1F10	1	*1*	1	*1*	1	*1*
IL1F6	-1.549	*0.3761*	-1.549	*0.4595*	1	*1*

IL-2 cytokine family

IL-15	1.3053	0.5405	2.9583 ↑	***0.0486***	2.2664	*0.1493*
IL2	-2.8877	*0.2272*	2.718	*0.3391*	7.8488	*0.0722*
IL21	51.6207 ↑	***0.0216***	52.3679 ↑	***0.0498***	1.0145	*0.9943*
IL7	1.4648	*0.2846*	-1.0678	*0.8762*	-1.5641	*0.323*

IL-17 cytokine family						
IL17A	-2.875	*0.6126*	-4.0099	*0.5794*	-1.3948	*0.8998*
IL17B	6.9292	*0.1969*	4.3675	*0.4065*	-1.5865	*0.8045*
IL17C	-2.1501	*0.369*	-2.6148	*0.3479*	-1.2161	*0.8551*
IL25	-1.1596	*0.6445*	-1.3143	*0.4799*	-1.1334	*0.7587*
Other Interleukins:						
IL13	17.5468	***0.0393***	6.576	*0.2392*	-2.6683	*0.5568*
IL6	14.4195	*0.1249*	12.2707	*0.2239*	-1.1751	*0.9399*
IL4	1.2459	*0.9349*	-14.9238	*0.4073*	-18.5931	*0.3979*
IL9	-4.4086	*0.4718*	-3.6494	*0.5997*	1.208	*0.9421*
IL5	1.9338	*0.3301*	1.4166	*0.665*	-1.3652	*0.7146*
IL16	1.4207	*0.594*	1.4057	*0.6663*	-1.0107	*0.9899*
IL12B	-5.3743	*0.5537*	-3.8641	*0.6909*	1.3908	*0.9269*
IL12A	-2.0517	*0.1933*	-1.2658	*0.7163*	1.6209	*0.4843*
IL8	4.1188	***0.0415***	3.0906	*0.1622*	-1.3327	*0.7303*
IL11	3.3663	*0.2226*	2.269	*0.4866*	-1.4836	*0.7506*
IL7	1.4648	*0.2846*	-1.0678	*0.8762*	-1.5641	*0.323*
TXLNA	-1.4416	*0.38*	-2.4787	*0.0792*	-1.7195	*0.3082*
IL3	3.1982	*0.1286*	1.2853	*0.7778*	-2.4882	*0.3391*

Table 6.1: Gene profiling of the human common cytokines pathway. The expression of the 84 genes related to the human common cytokines pathway was analyzed employing a qRT-PCR based RT2 Profiler TM PCR Array in 11 IBD patients (7 UC and 4 CD) and 10 healthy control subjects. The gene expression was normalized to the average expression of 5 housekeeping genes (B2 M, HPRT1, RPL13A, GAPDH, ACTB) and directly calculated using the Web-Based PCR Array Data Analysis. The table indicates the name and the fold change of all 84 genes, a fold change of ≤ 2 indicates a down-regulation, while fold change of ≥ 2 indicates an up-regulation. The level of statistical significance is set at <0.005. In red and in bold are indicated the genes whose expression was statistically significant with respect to the control group. Array data were analyzed using average values for control *versus* UC and CD sample and/or UC *versus* CD.

	UC vs control		CD vs control	
	Fold change	**P value**	**Fold change**	**P value**
TNFSF8	9.1882 ↑	***0.0461***	13.4177 ↑	***0.0497***
IL10	4.8668 ↑	***0.0307***	3.8929	*0.1107*
IL24	4.1236 ↑	***0.0194***	5.2046 ↑	***0.0227***
IL1F5	4.1726 ↑	***0.037***	2.4866	*0.247*
IL-15	1.3053	*0.5405*	2.9583 ↑	***0.0486***
IL21	51.6207 ↑	***0.0216***	52.3679 ↑	***0.0498***
IL13	17.5468 ↑	***0.0393***	6.576	*0.2392*
IL8	4.1188 ↑	***0.0415***	3.0906	*0.1622*

Table 6.2: Genes up-regulated in UC and CD. Genes showing significantly higher expression (indicated by a fold change of ≥ 2) in UC and CD compared to control. In red and in bold are indicated the genes whose expression was statistically significant ($p \leq 0.05$) with respect to the control group.

	UC vs control		CD vs control	
	Fold change	P value	Fold change	P value
BMP2	-3.0781 ↓	***0.0291***	-4.1605 ↓	***0.0221***
BMP5	-6.0531 ↓	***0.0061***	-1.8972	*0.3694*
PDGFA	-2.9957 ↓	**0.0422**	-2.999	*0.0849*
IL1F7	-10.9475 ↓	***0.0352***	1.047	*0.9714*

Table 6.3: Genes down-regulated in UC and CD. Genes showing significantly lower expression (indicated by a fold change of ≤ 2) in UC and CD compared to control. In red and in bold are indicated the genes whose expression was statistically significant ($p \leq 0.05$) with respect to the control group.

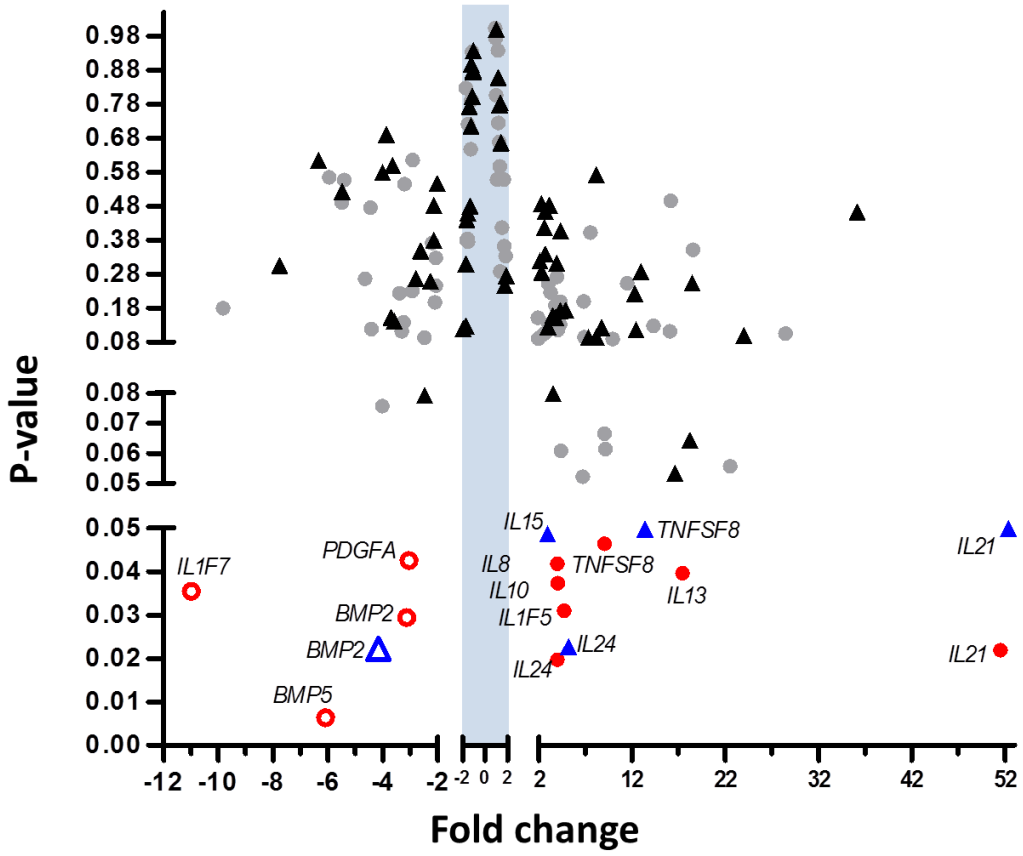

Figure 6.1: RNA isolated from CD33+LPMCs from normal colon and IBD were characterized on the Human Common Cytokines RT2 Profiler PCR Array. Fold changes in gene expression between $CD33^{+}$ LPMCs are plotted against p-value to produce a volcano plot. In the lower position are the genes which showed to be significantly higher (UC●; CD▲) and significantly lower (UC○ ; CD△) compared to control. In the higher position are shown all the other genes. Genes plotted farther from the central blue line have larger changes in gene expression. The plot doesn't include the gene up-regulated more than 52 and down-regulated less than -12 fold change. See table 6.3 for all the list of genes.

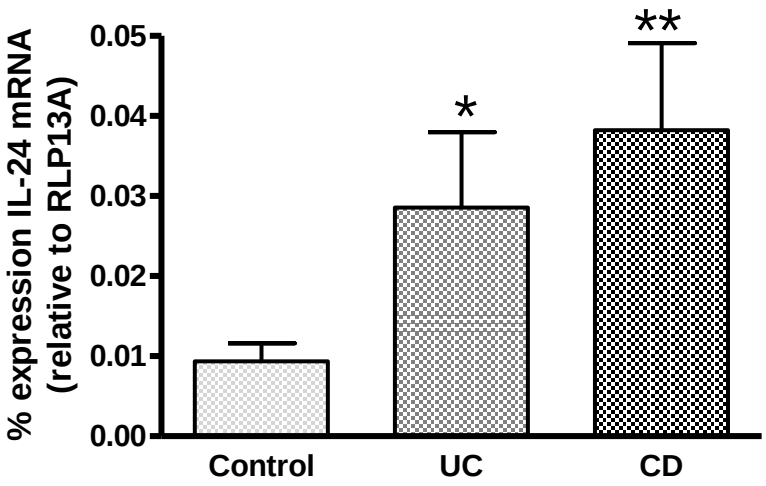

Figure 6.2: Quantitative analysis of IL-24 mRNA expression by CD33^{+} cells. Levels of mRNA for IL-24 were analysed by qRT-PCR in control (n=10) UC (n=8) and CD (n=5). Results shown are mean expression relative to RLP13A (using the $2^{-\Delta C(t)}$ method) ± SEM.

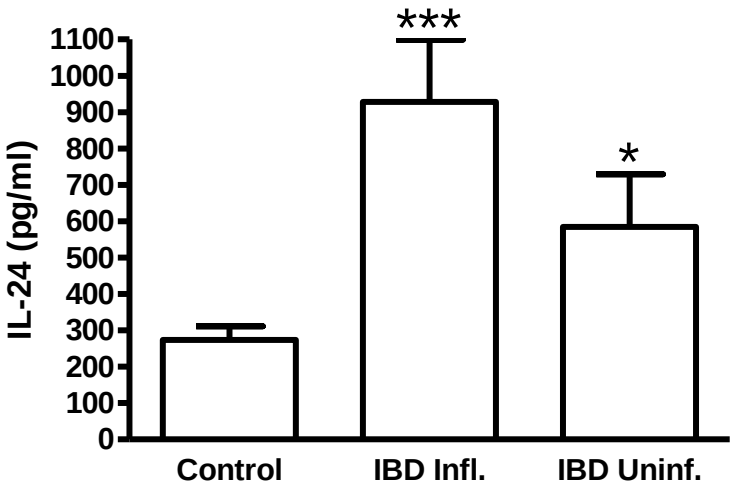

Figure 6.3: IL-24 production assessed by ELISA. Level of IL-24 expressed in pg/ml in the supernatants of intestinal biopsies taken from normal colon (n=11), inflamed IBD (CD (n=4) + UC (n=4)), Uninflamed IBD (CD (n=7) + UC (n=3)) and cultured for 24h with medium only. Results are mean ± SEM (unpaired T-test). All the p value are referred to control (***p=0.0005, *p= 0.0432).

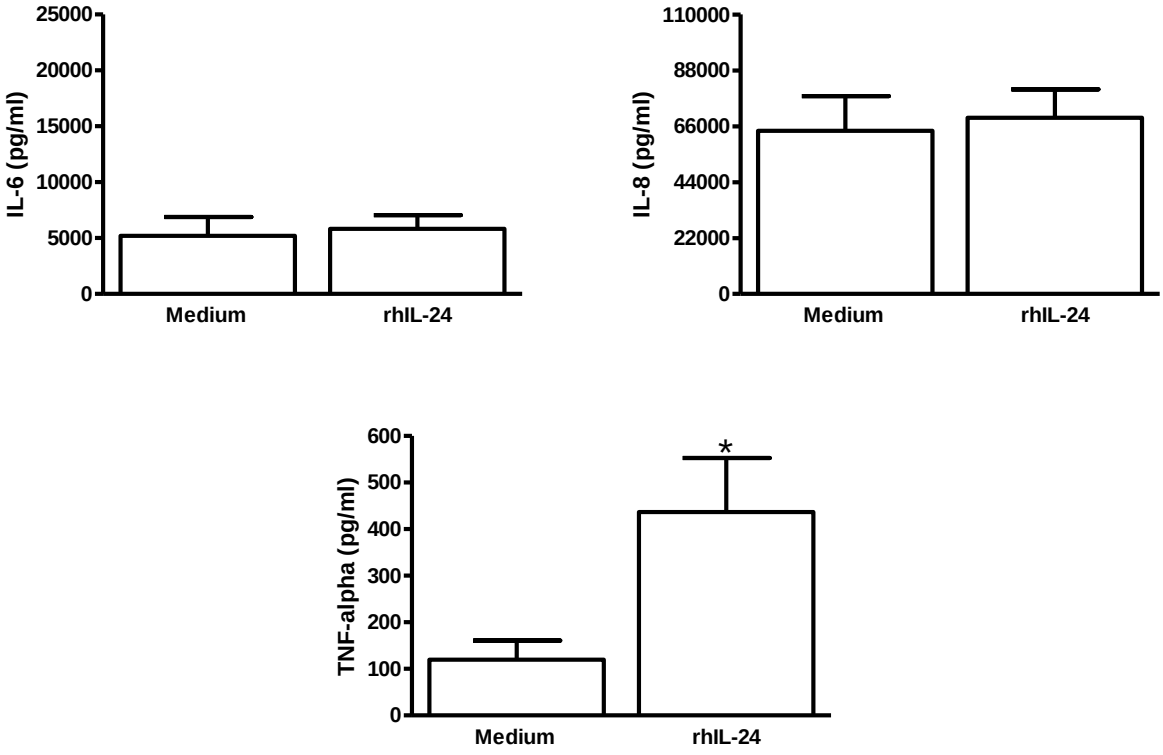

Figure 6.4: Recombinant human IL-24 increase TNF-α release in *ex vivo* culture: Levels of TNF-α, IL-6 and IL-8 expressed in pg/ml, in the supernatants of intestinal biopsies taken from the inflamed mucosa of 5 CD patients and 6 UC patients (IBD), and cultured for 24h in presence of recombinant human IL-24 or with medium only. Bars represent mean ± SEM, values relative to medium only *p=0.0273.

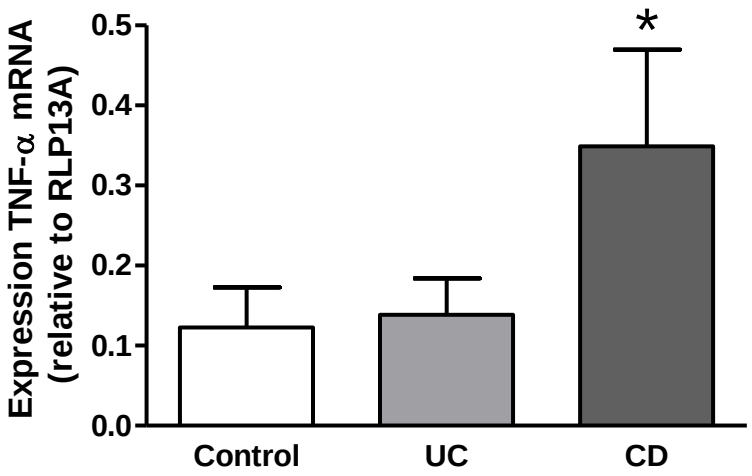

Figure 6.5: Quantitative analysis of TNF-α mRNA expression by CD33^{+} cells. Levels of mRNA for TNF-α were analysed by qRT-PCR in control (n=10) UC (n=7) and CD (n=4). Results shown are mean expression relative to RLP13A (using the $2^{-\Delta C(t)}$ method) ± SEM.

CHAPTER 7

General discussion and future work

The data presented in this thesis provide new information on the changes in colonic mucosal macrophages in patients with IBD. I performed phenotypic marker studies, functional experiments on the ability of macrophages to produce TNF-α, and an unbiased gene expression array which altogether allowed to make a number of novel observations.

Firstly, I was able to show that CD33, the pan-myeloid marker, can be used to identify macrophages both in normal and inflamed IBD mucosa. The additional staining with CD14 identified two populations of cells, namely $CD33^{+}CD14^{+}$ cells, which were rare in normal mucosa but abundant in IBD mucosa, and $CD33^{+}CD14^{-}$ cells, which were, the majority in control mucosa. Based on this and previous work, it is likely that the $CD33^{+}CD14^{+}$ cells are a cell population recently extravasated from blood into the inflamed mucosa (Brandtzaeg et al., 1997). Other surface markers were also studied, included CD68, CD206 and CD64. CD68 (macrosialin) is a highly glycosylated transmembrane protein (Holness and Simmons, 1993), located in the endosomal compartment of the cytoplasm. Its function is not known and it is a marker restricted to the monocyte/macrophage lineage (Pulford et al., 1990). I showed that CD68 was expressed in the majority of $CD33^{+}$ cells both in control tissues and IBD tissues. We were also particularly interested in the expression of M2 marker CD206, which other studies have found to be associated with M2 macrophages. M2 macrophages have high phagocytic capacity, produce extracellular matrix components and IL-10 (Bohlson et al., 2014; Fuentes-Duculan et al., 2010). M2 macrophages clear apoptotic cells, can mitigate inflammatory responses, and promote wound healing (Ferrante and Leibovich, 2012; Sica and Mantovani, 2012). CD206 is a mannose receptor involved in phagocytosis of mannose-coated particles, the endocytosis of mannosylated

glycoproteins, and in receptor-mediated facilitated antigen presentation (Sallusto et al., 1995; Tan et al., 1997). There were large numbers of $CD206^+$ cells in the $CD33^+CD14^+$ cells of control and CD mucosa. On the contrary, the number of $CD206^+$ cells was fewer among the $CD33^+CD14^+$ cells of UC mucosa. There were very few $CD206^+$ cells among $CD33^+CD14^-$ cells, both in control subjects and in IBD patients.

According to current views, monocyte-derived macrophages are characterized by the expression of CD64 (Tamoutounour et al., 2012), the high affinity Fcγ type I receptor. Consistently with these observations, the results presented here showed that in IBD mucosa the number of $CD64^+$ cells was greater in the $CD33^+CD14^+$ cells compared to a lower number of $CD64^+$ cells found in $CD33^+CD14^-$ cells. In normal controls the number of $CD64^+$ cells was higher both in the $CD33^+CD14^+$ cells than in the $CD33^+CD14^-$ cells. Also, CD64 was present on all blood $CD14^+$ monocytes from control subjects and IBD patients, which suggest that the down-regulation of CD64 marker observed in the $CD33^+CD14^-$ cells IBD mucosa may take place when blood monocyte enter the intestinal mucosa.

The next part of the thesis focused on the ability of different macrophage subsets to make TNF-α. The overall conclusion that emerges from this work is that in normal mucosa $CD33^+$ cells, which were mostly $CD14^-$, do not make TNF-α when activated with LPS. The low intracellular expression of TNF-α in these $CD33^+CD14^-$ cells may be the result of endotoxin tolerance. In contrast, $CD33^+$ cells from IBD mucosa, which contain large numbers of $CD14^+$ cells, displayed an increased expression of intracellular TNF-α after activation with LPS. Also, we observed a significant statistical positive correlation between the MFIs for TNF-α and $CD14^+$, in cells from IBD mucosa,

suggesting that loss of CD14 may be a way by which infiltrating macrophages regulate excessive TNF production.

Of particular interest is the observation that $CD33^{+}CD14^{+}CD206^{+}$ cells in inflamed mucosa from both CD and UC patients showed high levels of intracellular TNF-α. On the contrary, $CD33^{+}CD14^{-}CD206^{+}$ cells showed only little expression of intracellular TNF-α. Furthermore, the increasing $CD206^{+}$ cells in $CD14^{+}$ blood monocytes may indicate that in IBD there is selective recruitment of blood $CD14^{+}CD206^{+}$ monocytes leading to an overall increase in the proportion of intestinal $CD14^{+}CD206^{+}$ macrophages which make TNF-α in IBD. This is clearly different from previous studies where $CD206^{+}$ macrophages were shown to produce high levels of IL-10 and low levels of TNF-α and IL-12 (Gordon and Martinez, 2010). Hence, despite the effort to define the function of $CD206^{+}$ cells, the data presented here showed that CD206 does not represent a reliable marker to identify M2 macrophages in tissues.

Another important finding is that in CD and UC patients, $CD33^{+}CD64^{+}$ cells were more frequently positive for TNF-α compared to $CD33^{+}CD64^{-}$ cells. Additionally, in IBD mucosa a conspicuous population of $CD33^{+}CD64^{-}$ cells, which were probably DCs, expressed TNF-α. The role of DCs in human IBD has not been fully elucidated. Studies have found an increased number and maturation of DCs within inflamed IBD tissue (Kaser et al., 2004) and others have suggested enhanced recruitment of immature DC into inflamed tissue (te Velde et al., 2003). DC from inflamed Crohn's disease lamina propria express higher levels of CD40 than DC from non-inflamed Crohn's disease or from healthy control tissue (Stagg et al., 2003). Moreover elevated levels of CD40 returned to normal following treatment with anti-TNF-α. These findings on isolated

cells are consistent with immunohistological studies showing increased numbers of DC expressing CD40, CD86, (Vuckovic et al., 2001), CD83 (Ikeda et al., 2001) and CD80 (te Velde et al., 2003) in mucosal tissue from Crohn's disease and ulcerative colitis patients.

In addition, M-DC8^{+} monocytes, which were possible precursor of DCs, secrete large amounts of TNF-α but not IL-10 upon stimulation with LPS suggested that DCs may contribute to the pathogenesis of IBD (de Baey et al., 2003).

In mice, inflammatory CD11c^{high}HLA-II^{+} CD11b^{+}CD103^{-} DCs predominantly produced TNF-α which synergises with IL-23 to drive IL- 17A production by ILCs (Powell et al., 2012). Additionally, colitis in TRUC mice is characterized by dysregulated transcription of TNFα by colonic DCs (Garrett et al., 2007).

Proteolytic cleavage of membrane-anchored TNF-α (mTNF-α) into soluble TNF-α by ADAM17/TACE (TNF-α converting enzyme), the most important sheddase for mTNF-α, plays an important role in regulating TNF-α activity (Monteleone et al., 2012). Work in this thesis showed that culturing LPMCs with an ADAM10/17 inhibitor increased membrane TNF-α in activated CD3^{+} lymphocytes and CD33^{+} LPMCs, and in parallel reduced the release of soluble TNF-α into the supernatants of the same cells. T cells from ADAM17 knock-out mice produce 90% less soluble TNF-α than T cells from wild type mice (Locksley et al., 2001), and consistent with this in our study, treatment with an ADAM10/17 inhibitor strongly suppressed the level of TNF-α and sIL-6R (another substrate of ADAM17) released into the supernatant of inflamed biopsies from CD and UC patients cultured *ex vivo*. Hence, ADAM17 inhibition has the ability to inhibit the cleavage process of mTNF-α and suppress the production of soluble TNF-α, therefore it might be a promising strategy to prevent TNF-α-related diseases. However, further

studies are needed to investigate all the consequences of ADAM17 inhibition, as a loss-of-function mutation in ADAM17 was identified in two of three children born to consanguineous parents and was associated to inflammatory skin and bowel disease (Blaydon et al., 2011).

In the last part of the thesis we investigated the gene expression profile of macrophages isolated from IBD samples. Among all the genes up- (*TNFSF8, IL21, IL10, IL1F5, IL13* and *IL-8*) or down-regulated (*BMP2, BMP5, PDGFA* and *IL1F7*) in IBD, *IL24* was one of the most highly up-regulated gene in IBD macrophages. In addition, IL-24 protein was also increased in supernatants of IBD biopsies cultured *ex vivo*. The precise cytokine regulation and molecular mechanisms of IL-24 induction is still unknown. IL-24 mRNA expression is significantly increased in the inflamed mucosa of patients with IBD (Andoh et al., 2009). The up-regulation of IL-24 in patients with CD and patients with UC could reflect a similar pathophysiological mechanism which accounts for the induction of this cytokine in the two forms of IBD. Other studies showed that IL-24 expression is greatly increased at the edge of cutaneous wounds in an animal model or in keratinocytes from patients with psoriasis (Bernink et al., 2013; Kunz et al., 2006). Furthermore, data in this thesis showed that TNF-α was increased in the supernatants of biopsies cultured with recombinant human IL-24. In contrast, treatment of IBD biopsies with recombinant human IL-24 did not change the production of other cytokines, such as IL-6 and IL-8. However, whether IL-24 can selectively induce the secretion of TNF-α awaits further studies. Although IL-24 is a member of the IL-10 superfamily (Chada et al., 2004), it differs from IL-10 in that the latter inhibits NF-κβ activation (Wang et al., 1995), whereas IL-24 enhances TNF-induced NF-κβ activation. Since NF-κβ is hyper activated in IBD LPMCs (Atreya et al., 2008; Coskun et al., 2011), it is likely that

induction of IL-24 in the gut of patients with CD and patients with UC is driven by these signaling pathways. Overall, these results show that IL-24 may have an important role in mucosal inflammation in IBD.

Future work

Possible future studies should focus on determining if neutralizing IL-24 in mucosal biopsies cultured ex vivo inhibits pro-inflammatory cytokine production and/or investigating some of the other up-regulated genes identified in the array. For example, IL-21 produced by T cells has been shown to be important in mouse and human gut inflammation (Monteleone et al., 2005), and yet it was markedly increased in macrophages.

Moreover the gene expression array provides new evidence regarding the IL-1F5 (Interleukin 36 Receptor Antagonist; IL-36Ra) gene which was significantly up-regulated in UC and CD $CD33^{+}$ cells. Among the few published papers regarding IL-36Ra function, it has been shown that IL-36Ra has an anti-inflammatory effect localized to the brain and mediated through a unique TIR8/SIGIRR-dependent pathway (Costelloe et al., 2008). Hence, on the basis of the limited availability of published data and our preliminary findings, IL-36Ra may potentially play an important role in IBD.

References

Abraham, C., and R. Medzhitov, 2011, Interactions between the host innate immune system and microbes in inflammatory bowel disease: Gastroenterology, v. 140, p. 1729-37.

Akira, S., S. Uematsu, and O. Takeuchi, 2006, Pathogen recognition and innate immunity: Cell, v. 124, p. 783-801.

Allakhverdi, Z., M. R. Comeau, H. K. Jessup, B. R. Yoon, A. Brewer, S. Chartier, N. Paquette, S. F. Ziegler, M. Sarfati, and G. Delespesse, 2007, Thymic stromal lymphopoietin is released by human epithelial cells in response to microbes, trauma, or inflammation and potently activates mast cells: J Exp Med, v. 204, p. 253-8.

Allenbach, C., P. Launois, C. Mueller, and F. Tacchini-Cottier, 2008, An essential role for transmembrane TNF in the resolution of the inflammatory lesion induced by Leishmania major infection: Eur J Immunol, v. 38, p. 720-31.

Andoh, A., M. Shioya, A. Nishida, S. Bamba, T. Tsujikawa, S. Kim-Mitsuyama, and Y. Fujiyama, 2009, Expression of IL-24, an activator of the JAK1/STAT3/SOCS3 cascade, is enhanced in inflammatory bowel disease: J Immunol, v. 183, p. 687-95.

Armstrong, L., S. I. Godinho, K. M. Uppington, H. A. Whittington, and A. B. Millar, 2006, Contribution of TNF-alpha converting enzyme and proteinase-3 to TNF-alpha processing in human alveolar macrophages: Am J Respir Cell Mol Biol, v. 34, p. 219-25.

Arora, T., R. Padaki, L. Liu, A. E. Hamburger, A. R. Ellison, S. R. Stevens, J. S. Louie, and T. Kohno, 2009, Differences in binding and effector functions between classes of TNF antagonists: Cytokine, v. 45, p. 124-31.

Atarashi, K., T. Tanoue, K. Oshima, W. Suda, Y. Nagano, H. Nishikawa, S. Fukuda, T. Saito, S. Narushima, K. Hase, S. Kim, J. V. Fritz, P. Wilmes, S. Ueha, K. Matsushima, H. Ohno, B. Olle, S. Sakaguchi, T. Taniguchi, H. Morita, M. Hattori, and K. Honda, 2013, Treg induction by a rationally selected mixture of Clostridia strains from the human microbiota: Nature, v. 500, p. 232-6.

Atarashi, K., T. Tanoue, T. Shima, A. Imaoka, T. Kuwahara, Y. Momose, G. Cheng, S. Yamasaki, T. Saito, Y. Ohba, T. Taniguchi, K. Takeda, S. Hori, I. I. Ivanov, Y. Umesaki, K. Itoh, and K. Honda, 2011, Induction of colonic regulatory T cells by indigenous Clostridium species: Science, v. 331, p. 337-41.

Atreya, I., R. Atreya, and M. F. Neurath, 2008, NF-kappaB in inflammatory bowel disease: J Intern Med, v. 263, p. 591-6.

Atreya, R., H. Neumann, C. Neufert, M. J. Waldner, U. Billmeier, Y. Zopf, M. Willma, C. App, T. Münster, H. Kessler, S. Maas, B. Gebhardt, R. Heimke-Brinck, E. Reuter, F. Dörje, T. T. Rau, W. Uter, T. D. Wang, R. Kiesslich, M. Vieth, E. Hannappel, and M. F. Neurath, 2014, In vivo imaging using fluorescent antibodies to tumor necrosis factor predicts therapeutic response in Crohn's disease: Nat Med, v. 20, p. 313-8.

Atreya, R., M. Zimmer, B. Bartsch, M. J. Waldner, I. Atreya, H. Neumann, K. Hildner, A. Hoffman, R. Kiesslich, A. D. Rink, T. T. Rau, S. Rose-John, H. Kessler, J. Schmidt, and M. F. Neurath, 2011, Antibodies against tumor necrosis factor (TNF) induce T-cell apoptosis in patients with inflammatory bowel diseases via TNF receptor 2 and intestinal $CD14^{+}$ macrophages: Gastroenterology, v. 141, p. 2026-38.

Auffray, C., D. K. Fogg, E. Narni-Mancinelli, B. Senechal, C. Trouillet, N. Saederup, J. Leemput, K. Bigot, L. Campisi, M. Abitbol, T. Molina, I. Charo, D. A. Hume, A. Cumano, G. Lauvau, and F. Geissmann, 2009, CX3CR1+ CD115+ CD135+ common macrophage/DC precursors and the role of CX3CR1 in their response to inflammation: J Exp Med, v. 206, p. 595-606.

Bain, C. C., and A. M. Mowat, 2011, Intestinal macrophages - specialised adaptation to a unique environment: Eur J Immunol, v. 41, p. 2494-8.

Bain, C. C., C. L. Scott, H. Uronen-Hansson, S. Gudjonsson, O. Jansson, O. Grip, M. Guilliams, B. Malissen, W. W. Agace, and A. M. Mowat, 2013, Resident and pro-inflammatory macrophages in the colon represent alternative context-dependent fates of the same Ly6Chi monocyte precursors: Mucosal Immunol, v. 6, p. 498-510.

Bernink, J. H., C. P. Peters, M. Munneke, A. A. te Velde, S. L. Meijer, K. Weijer, H. S. Hreggvidsdottir, S. E. Heinsbroek, N. Legrand, C. J. Buskens, W. A. Bemelman, J. M. Mjösberg, and H. Spits, 2013, Human type 1 innate lymphoid cells accumulate in inflamed mucosal tissues: Nat Immunol, v. 14, p. 221-9.

Best, W. R., J. M. Becktel, J. W. Singleton, and F. Kern, 1976, Development of a Crohn's disease activity index. National Cooperative Crohn's Disease Study: Gastroenterology, v. 70, p. 439-44.

Biancheri, P., A. Di Sabatino, F. Ammoscato, F. Facciotti, F. Caprioli, R. Curciarello, S. S. Hoque, A. Ghanbari, I. Joe-Njoku, P. Giuffrida, L. Rovedatti, J. Geginat, G. R. Corazza, and T. T. Macdonald, 2013a, Absence of a role for interleukin-13 in inflammatory bowel disease: Eur J Immunol.

Biancheri, P., N. Powell, G. Monteleone, G. Lord, and T. T. MacDonald, 2013b, The challenges of stratifying patients for trials in inflammatory bowel disease: Trends Immunol, v. 34, p. 564-71.

Billiet, T., P. Rutgeerts, M. Ferrante, G. Van Assche, and S. Vermeire, 2014, Targeting TNF-α for the treatment of inflammatory bowel disease: Expert Opin Biol Ther, v. 14, p. 75-101.

Biswas, S. K., and A. Mantovani, 2010, Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm: Nat Immunol, v. 11, p. 889-96.

Bjerre, A., B. Brusletto, E. Rosenqvist, E. Namork, P. Kierulf, R. Øvstebø, G. B. Joø, and P. Brandtzaeg, 2000, Cellular activating properties and morphology of membrane-bound and purified meningococcal lipopolysaccharide: J Endotoxin Res, v. 6, p. 437-45.

Bjerre, A., B. Brusletto, R. Øvstebø, G. B. Joø, P. Kierulf, and P. Brandtzaeg, 2003, Identification of meningococcal LPS as a major monocyte activator in IL-10 depleted shock plasmas and CSF by blocking the CD14-TLR4 receptor complex: J Endotoxin Res, v. 9, p. 155-63.

Black, R. A., C. T. Rauch, C. J. Kozlosky, J. J. Peschon, J. L. Slack, M. F. Wolfson, B. J. Castner, K. L. Stocking, P. Reddy, S. Srinivasan, N. Nelson, N. Boiani, K. A. Schooley, M. Gerhart, R. Davis, J. N. Fitzner, R. S. Johnson, R. J. Paxton, C. J. March, and D. P. Cerretti, 1997, A metalloproteinase disintegrin that releases tumour-necrosis factor-alpha from cells: Nature, v. 385, p. 729-33.

Blaydon, D. C., P. Biancheri, W. L. Di, V. Plagnol, R. M. Cabral, M. A. Brooke, D. A. van Heel, F. Ruschendorf, M. Toynbee, A. Walne, E. A. O'Toole, J. E. Martin, K. Lindley, T. Vulliamy, D. J. Abrams, T. T. MacDonald, J. I. Harper, and D. P. Kelsell, 2011, Inflammatory skin and bowel disease linked to ADAM17 deletion: N Engl J Med, v. 365, p. 1502-8.

Bleharski, J. R., V. Kiessler, C. Buonsanti, P. A. Sieling, S. Stenger, M. Colonna, and R. L. Modlin, 2003, A role for triggering receptor expressed on myeloid cells-1 in host defense during the early-induced and adaptive phases of the immune response: J Immunol, v. 170, p. 3812-8.

Blumberg, H., D. Conklin, W. F. Xu, A. Grossmann, T. Brender, S. Carollo, M. Eagan, D. Foster, B. A. Haldeman, A. Hammond, H. Haugen, L. Jelinek, J. D. Kelly, K. Madden, M. F. Maurer, J. Parrish-Novak, D. Prunkard, S. Sexson, C. Sprecher, K. Waggie, J. West, T. E. Whitmore, L. Yao, M. K. Kuechle, B. A. Dale, and Y. A. Chandrasekher, 2001, Interleukin 20: discovery, receptor identification, and role in epidermal function: Cell, v. 104, p. 9-19.

Blumberg, R. S., 2009, Inflammation in the intestinal tract: pathogenesis and treatment: Dig Dis, v. 27, p. 455-64.

Bogiatzi, S. I., I. Fernandez, J. C. Bichet, M. A. Marloie-Provost, E. Volpe, X. Sastre, and V. Soumelis, 2007, Cutting Edge: Proinflammatory and Th2 cytokines synergize to induce thymic stromal lymphopoietin production by human skin keratinocytes: J Immunol, v. 178, p. 3373-7.

Bohlson, S. S., S. D. O'Conner, H. J. Hulsebus, M. M. Ho, and D. A. Fraser, 2014, Complement, c1q, and c1q-related molecules regulate macrophage polarization: Front Immunol, v. 5, p. 402.

Bonecchi, R., S. Sozzani, J. T. Stine, W. Luini, G. D'Amico, P. Allavena, D. Chantry, and A. Mantovani, 1998, Divergent effects of interleukin-4 and interferon-gamma on macrophage-derived chemokine production: an amplification circuit of polarized T helper 2 responses: Blood, v. 92, p. 2668-71.

Brandl, K., G. Plitas, B. Schnabl, R. P. DeMatteo, and E. G. Pamer, 2007, MyD88-mediated signals induce the bactericidal lectin RegIII gamma and protect mice against intestinal Listeria monocytogenes infection: J Exp Med, v. 204, p. 1891-900.

Brandtzaeg, P., G. Haraldsen, and J. Rugtveit, 1997, Immunopathology of human inflammatory bowel disease: Springer Semin Immunopathol, v. 18, p. 555-89.

Breese, E., and T. T. MacDonald, 1995, TNF alpha secreting cells in normal and diseased human intestine: Adv Exp Med Biol, v. 371B, p. 821-4.

Brynskov, J., P. Foegh, G. Pedersen, C. Ellervik, T. Kirkegaard, A. Bingham, and T. Saermark, 2002, Tumour necrosis factor alpha converting enzyme (TACE) activity in the colonic mucosa of patients with inflammatory bowel disease: Gut, v. 51, p. 37-43.

Burgess, J. K., S. Carlin, R. A. Pack, G. M. Arndt, W. W. Au, P. R. Johnson, J. L. Black, and N. H. Hunt, 2004, Detection and characterization of OX40 ligand expression in human airway smooth muscle cells: a possible role in asthma?: J Allergy Clin Immunol, v. 113, p. 683-9.

Burns, K., J. Clatworthy, L. Martin, F. Martinon, C. Plumpton, B. Maschera, A. Lewis, K. Ray, J. Tschopp, and F. Volpe, 2000, Tollip, a new component of the IL-1RI pathway, links IRAK to the IL-1 receptor: Nat Cell Biol, v. 2, p. 346-51.

Burns, K., S. Janssens, B. Brissoni, N. Olivos, R. Beyaert, and J. Tschopp, 2003, Inhibition of interleukin 1 receptor/Toll-like receptor signaling through the alternatively spliced, short form of MyD88 is due to its failure to recruit IRAK-4: J Exp Med, v. 197, p. 263-8.

Canault, M., F. Peiretti, F. Kopp, B. Bonardo, M. F. Bonzi, J. C. Coudeyre, M. C. Alessi, I. Juhan-Vague, and G. Nalbone, 2006, The TNF alpha converting enzyme (TACE/ADAM17) is expressed in the atherosclerotic lesions of apolipoprotein E-deficient mice: possible contribution to elevated plasma levels of soluble TNF alpha receptors: Atherosclerosis, v. 187, p. 82-91.

Cantó, E., E. Ricart, D. Monfort, D. González-Juan, J. Balanzó, J. L. Rodríguez-Sánchez, and S. Vidal, 2006, TNF alpha production to TLR2 ligands in active IBD patients: Clin Immunol, v. 119, p. 156-65.

Caprioli, F., R. Caruso, M. Sarra, F. Pallone, and G. Monteleone, 2012, Disruption of inflammatory signals by cytokine-targeted therapies for inflammatory bowel diseases: Br J Pharmacol, v. 165, p. 820-8.

Caput, D., B. Beutler, K. Hartog, R. Thayer, S. Brown-Shimer, and A. Cerami, 1986, Identification of a common nucleotide sequence in the 3'-untranslated region of mRNA molecules specifying inflammatory mediators: Proc Natl Acad Sci U S A, v. 83, p. 1670-4.

Cario, E., 2005, Bacterial interactions with cells of the intestinal mucosa: Toll-like receptors and NOD2: Gut, v. 54, p. 1182-93.

Cario, E., I. M. Rosenberg, S. L. Brandwein, P. L. Beck, H. C. Reinecker, and D. K. Podolsky, 2000, Lipopolysaccharide activates distinct signaling pathways in intestinal epithelial cell lines expressing Toll-like receptors: J Immunol, v. 164, p. 966-72.

Carvalho, F. A., J. D. Aitken, M. Vijay-Kumar, and A. T. Gewirtz, 2012, Toll-like receptor-gut microbiota interactions: perturb at your own risk!: Annu Rev Physiol, v. 74, p. 177-98.

Chada, S., R. B. Sutton, S. Ekmekcioglu, J. Ellerhorst, J. B. Mumm, W. W. Leitner, H. Y. Yang, A. A. Sahin, K. K. Hunt, K. L. Fuson, N. Poìndexter, J. A. Roth, R. Ramesh, E. A. Grimm, and A. M. Mhashilkar, 2004, MDA-7/IL-24 is a unique cytokine--tumor suppressor in the IL-10 family: Int Immunopharmacol, v. 4, p. 649-67.

Chalaris, A., N. Adam, C. Sina, P. Rosenstiel, J. Lehmann-Koch, P. Schirmacher, D. Hartmann, J. Cichy, O. Gavrilova, S. Schreiber, T. Jostock, V. Matthews, R. Häsler, C. Becker, M. F. Neurath, K. Reiss, P. Saftig, J. Scheller, and S. Rose-John, 2010a, Critical role of the disintegrin metalloprotease ADAM17 for intestinal inflammation and regeneration in mice: J Exp Med, v. 207, p. 1617-24.

Chalaris, A., J. Gewiese, K. Paliga, L. Fleig, A. Schneede, K. Krieger, S. Rose-John, and J. Scheller, 2010b, ADAM17-mediated shedding of the IL6R induces cleavage of the membrane stub by gamma-secretase: Biochim Biophys Acta, v. 1803, p. 234-45.

Chan, F. K., H. J. Chun, L. Zheng, R. M. Siegel, K. L. Bui, and M. J. Lenardo, 2000, A domain in TNF receptors that mediates ligand-independent receptor assembly and signaling: Science, v. 288, p. 2351-4.

Chen, P. H., and S. Y. Fang, 2004, The expression of human antimicrobial peptide LL-37 in the human nasal mucosa: Am J Rhinol, v. 18, p. 381-5.

Colonna, M., and F. Facchetti, 2003, TREM-1 (triggering receptor expressed on myeloid cells): a new player in acute inflammatory responses: J Infect Dis, v. 187 Suppl 2, p. S397-401.

Coskun, M., J. Olsen, J. B. Seidelin, and O. H. Nielsen, 2011, MAP kinases in inflammatory bowel disease: Clin Chim Acta, v. 412, p. 513-20.

Costelloe, C., M. Watson, A. Murphy, K. McQuillan, C. Loscher, M. E. Armstrong, C. Garlanda, A. Mantovani, L. A. O'Neill, K. H. Mills, and M. A. Lynch, 2008, IL-1F5 mediates anti-inflammatory activity in the brain through induction of IL-4 following interaction with SIGIRR/TIR8: J Neurochem, v. 105, p. 1960-9.

de Baey, A., I. Mende, G. Baretton, A. Greiner, W. H. Hartl, P. A. Baeuerle, and H. M. Diepolder, 2003, A subset of human dendritic cells in the T cell area of mucosa-associated lymphoid tissue with a high potential to produce TNF-alpha: J Immunol, v. 170, p. 5089-94.

Debard, N., F. Sierro, J. Browning, and J. P. Kraehenbuhl, 2001, Effect of mature lymphocytes and lymphotoxin on the development of the follicle-associated epithelium and M cells in mouse Peyer's patches: Gastroenterology, v. 120, p. 1173-82.

Denning, T. L., Y. C. Wang, S. R. Patel, I. R. Williams, and B. Pulendran, 2007, Lamina propria macrophages and dendritic cells differentially induce regulatory and interleukin 17-producing T cell responses: Nat Immunol, v. 8, p. 1086-94.

Derive, M., F. Massin, and S. Gibot, 2010, Triggering receptor expressed on myeloid cells-1 as a new therapeutic target during inflammatory diseases: Self Nonself, v. 1, p. 225-230.

Di Sabatino, A., K. M. Pickard, J. N. Gordon, V. Salvati, G. Mazzarella, R. M. Beattie, A. Vossenkaemper, L. Rovedatti, N. A. Leakey, N. M. Croft, R. Troncone, G. R. Corazza, A. J. Stagg, G. Monteleone, and T. T. MacDonald, 2007, Evidence for the role of interferon-alfa production by dendritic cells in the Th1 response in celiac disease: Gastroenterology, v. 133, p. 1175-87.

Doyle, S., S. Vaidya, R. O'Connell, H. Dadgostar, P. Dempsey, T. Wu, G. Rao, R. Sun, M. Haberland, R. Modlin, and G. Cheng, 2002, IRF3 mediates a TLR3/TLR4-specific antiviral gene program: Immunity, v. 17, p. 251-63.

Dreymueller, D., C. Martin, T. Kogel, J. Pruessmeyer, F. M. Hess, K. Horiuchi, S. Uhlig, and A. Ludwig, 2012, Lung endothelial ADAM17 regulates the acute inflammatory response to lipopolysaccharide: EMBO Mol Med, v. 4, p. 412-23.

Dumoutier, L., C. Leemans, D. Lejeune, S. V. Kotenko, and J. C. Renauld, 2001, Cutting edge: STAT activation by IL-19, IL-20 and mda-7 through IL-20 receptor complexes of two types: J Immunol, v. 167, p. 3545-9.

Dumoutier, L., and J. C. Renauld, 2002, Viral and cellular interleukin-10 (IL-10)-related cytokines: from structures to functions: Eur Cytokine Netw, v. 13, p. 5-15.

Ebert, E. C., 2005, Endogenous inhibitory cytokines repress TNFalpha secretion: Cell Immunol, v. 237, p. 106-14.

Edwards, J. P., X. Zhang, K. A. Frauwirth, and D. M. Mosser, 2006, Biochemical and functional characterization of three activated macrophage populations: J Leukoc Biol, v. 80, p. 1298-307.

Endo, T. A., M. Masuhara, M. Yokouchi, R. Suzuki, H. Sakamoto, K. Mitsui, A. Matsumoto, S. Tanimura, M. Ohtsubo, H. Misawa, T. Miyazaki, N. Leonor, T. Taniguchi, T. Fujita, Y. Kanakura, S. Komiya, and A. Yoshimura, 1997, A new protein containing an SH2 domain that inhibits JAK kinases: Nature, v. 387, p. 921-4.

Etzerodt, A., M. B. Maniecki, K. Møller, H. J. Møller, and S. K. Moestrup, 2010, Tumor necrosis factor α-converting enzyme (TACE/ADAM17) mediates ectodomain shedding of the scavenger receptor CD163: J Leukoc Biol, v. 88, p. 1201-5.

Fabriek, B. O., H. J. Møller, R. P. Vloet, L. M. van Winsen, R. Hanemaaijer, C. E. Teunissen, B. M. Uitdehaag, T. K. van den Berg, and C. D. Dijkstra, 2007, Proteolytic shedding of the macrophage scavenger receptor CD163 in multiple sclerosis: J Neuroimmunol, v. 187, p. 179-86.

Ferrante, C. J., and S. J. Leibovich, 2012, Regulation of Macrophage Polarization and Wound Healing: Adv Wound Care (New Rochelle), v. 1, p. 10-16.

Feterowski, C., M. Mack, H. Weighardt, B. Bartsch, S. Kaiser-Moore, and B. Holzmann, 2004, CC chemokine receptor 2 regulates leukocyte recruitment and IL-10 production during acute polymicrobial sepsis: Eur J Immunol, v. 34, p. 3664-73.

Fiocchi, C., 2008, What is "physiological" intestinal inflammation and how does it differ from "pathological" inflammation?: Inflamm Bowel Dis, v. 14 Suppl 2, p. S77-8.

Fong, A. M., L. A. Robinson, D. A. Steeber, T. F. Tedder, O. Yoshie, T. Imai, and D. D. Patel, 1998, Fractalkine and CX3CR1 mediate a novel mechanism of leukocyte capture, firm adhesion, and activation under physiologic flow: J Exp Med, v. 188, p. 1413-9.

Fong, C. H., M. Bebien, A. Didierlaurent, R. Nebauer, T. Hussell, D. Broide, M. Karin, and T. Lawrence, 2008, An antiinflammatory role for IKKbeta through the inhibition of "classical" macrophage activation: J Exp Med, v. 205, p. 1269-76.

Fuentes-Duculan, J., M. Suárez-Fariñas, L. C. Zaba, K. E. Nograles, K. C. Pierson, H. Mitsui, C. A. Pensabene, J. Kzhyshkowska, J. G. Krueger, and M. A. Lowes, 2010, A subpopulation of CD163-positive macrophages is classically activated in psoriasis: J Invest Dermatol, v. 130, p. 2412-22.

Garrett, W. S., G. M. Lord, S. Punit, G. Lugo-Villarino, S. K. Mazmanian, S. Ito, J. N. Glickman, and L. H. Glimcher, 2007, Communicable ulcerative colitis induced by T-bet deficiency in the innate immune system: Cell, v. 131, p. 33-45.

Gearing, A. J., P. Beckett, M. Christodoulou, M. Churchill, J. Clements, A. H. Davidson, A. H. Drummond, W. A. Galloway, R. Gilbert, and J. L. Gordon, 1994, Processing of tumour necrosis factor-alpha precursor by metalloproteinases: Nature, v. 370, p. 555-7.

Geissmann, F., S. Gordon, D. A. Hume, A. M. Mowat, and G. J. Randolph, 2010a, Unravelling mononuclear phagocyte heterogeneity: Nat Rev Immunol, v. 10, p. 453-60.

Geissmann, F., S. Jung, and D. R. Littman, 2003, Blood monocytes consist of two principal subsets with distinct migratory properties: Immunity, v. 19, p. 71-82.

Geissmann, F., M. G. Manz, S. Jung, M. H. Sieweke, M. Merad, and K. Ley, 2010b, Development of monocytes, macrophages, and dendritic cells: Science, v. 327, p. 656-61.

Gerber, J. S., and D. M. Mosser, 2001, Reversing lipopolysaccharide toxicity by ligating the macrophage Fc gamma receptors: J Immunol, v. 166, p. 6861-8.

Gewirtz, A. T., T. A. Navas, S. Lyons, P. J. Godowski, and J. L. Madara, 2001, Cutting edge: bacterial flagellin activates basolaterally expressed TLR5 to induce epithelial proinflammatory gene expression: J Immunol, v. 167, p. 1882-5.

Ghoreschi, K., P. Thomas, S. Breit, M. Dugas, R. Mailhammer, W. van Eden, R. van der Zee, T. Biedermann, J. Prinz, M. Mack, U. Mrowietz, E. Christophers, D. Schlöndorff, G. Plewig, C. A. Sander, and M. Röcken, 2003, Interleukin-4 therapy of psoriasis induces Th2 responses and improves human autoimmune disease: Nat Med, v. 9, p. 40-6.

Gibot, S., M. N. Kolopp-Sarda, M. C. Béné, P. E. Bollaert, A. Lozniewski, F. Mory, B. Levy, and G. C. Faure, 2004, A soluble form of the triggering receptor expressed on myeloid cells-1 modulates the inflammatory response in murine sepsis: J Exp Med, v. 200, p. 1419-26.

Glocker, E. O., D. Kotlarz, K. Boztug, E. M. Gertz, A. A. Schäffer, F. Noyan, M. Perro, J. Diestelhorst, A. Allroth, D. Murugan, N. Hätscher, D. Pfeifer, K. W. Sykora, M. Sauer, H. Kreipe, M. Lacher, R. Nustede, C. Woellner, U. Baumann, U. Salzer, S. Koletzko, N. Shah, A. W. Segal, A. Sauerbrey, S. Buderus, S. B. Snapper, B. Grimbacher, and C. Klein, 2009, Inflammatory bowel disease and mutations affecting the interleukin-10 receptor: N Engl J Med, v. 361, p. 2033-45.

Golovkina, T. V., M. Shlomchik, L. Hannum, and A. Chervonsky, 1999, Organogenic role of B lymphocytes in mucosal immunity: Science, v. 286, p. 1965-8.

Gordon, S., and F. O. Martinez, 2010, Alternative activation of macrophages: mechanism and functions: Immunity, v. 32, p. 593-604.

Gordon, S., and P. R. Taylor, 2005, Monocyte and macrophage heterogeneity: Nat Rev Immunol, v. 5, p. 953-64.

Goto, Y., and H. Kiyono, 2012, Epithelial barrier: an interface for the cross-communication between gut flora and immune system: Immunol Rev, v. 245, p. 147-63.

Grainger, J. R., E. A. Wohlfert, I. J. Fuss, N. Bouladoux, M. H. Askenase, F. Legrand, L. Y. Koo, J. M. Brenchley, I. D. Fraser, and Y. Belkaid, 2013, Inflammatory monocytes regulate pathologic responses to commensals during acute gastrointestinal infection: Nat Med, v. 19, p. 713-21.

Grell, M., E. Douni, H. Wajant, M. Löhden, M. Clauss, B. Maxeiner, S. Georgopoulos, W. Lesslauer, G. Kollias, K. Pfizenmaier, and P. Scheurich, 1995, The transmembrane form of tumor necrosis factor is the prime activating ligand of the 80 kDa tumor necrosis factor receptor: Cell, v. 83, p. 793-802.

Grimm, M. C., P. Pavli, E. Van de Pol, and W. F. Doe, 1995, Evidence for a CD14+ population of monocytes in inflammatory bowel disease mucosa--implications for pathogenesis: Clin Exp Immunol, v. 100, p. 291-7.

Hall, J. A., N. Bouladoux, C. M. Sun, E. A. Wohlfert, R. B. Blank, Q. Zhu, M. E. Grigg, J. A. Berzofsky, and Y. Belkaid, 2008, Commensal DNA limits regulatory T cell conversion and is a natural adjuvant of intestinal immune responses: Immunity, v. 29, p. 637-49.

Hamilton, J. A., 1993, Colony stimulating factors, cytokines and monocyte-macrophages--some controversies: Immunol Today, v. 14, p. 18-24.

Han, J., T. Brown, and B. Beutler, 1990, Endotoxin-responsive sequences control cachectin/tumor necrosis factor biosynthesis at the translational level: J Exp Med, v. 171, p. 465-75.

Haraoui, B., 2005, The anti-tumor necrosis factor agents are a major advance in the treatment of rheumatoid arthritis: J Rheumatol Suppl, v. 72, p. 46-7.

Harashima, S., T. Horiuchi, N. Hatta, C. Morita, M. Higuchi, T. Sawabe, H. Tsukamoto, T. Tahira, K. Hayashi, S. Fujita, and Y. Niho, 2001, Outside-to-inside signal through the membrane TNF-alpha induces E-selectin (CD62E) expression on activated human CD4+ T cells: J Immunol, v. 166, p. 130-6.

Hardwick, J. C., G. R. Van Den Brink, S. A. Bleuming, I. Ballester, J. M. Van Den Brande, J. J. Keller, G. J. Offerhaus, S. J. Van Deventer, and M. P. Peppelenbosch, 2004, Bone morphogenetic protein 2 is expressed by, and acts upon, mature epithelial cells in the colon: Gastroenterology, v. 126, p. 111-21.

Harrison, O. J., and K. J. Maloy, 2011, Innate immune activation in intestinal homeostasis: J Innate Immun, v. 3, p. 585-93.

Hart, A. L., H. O. Al-Hassi, R. J. Rigby, S. J. Bell, A. V. Emmanuel, S. C. Knight, M. A. Kamm, and A. J. Stagg, 2005, Characteristics of intestinal dendritic cells in inflammatory bowel diseases: Gastroenterology, v. 129, p. 50-65.

Haskó, G., P. Pacher, E. A. Deitch, and E. S. Vizi, 2007, Shaping of monocyte and macrophage function by adenosine receptors: Pharmacol Ther, v. 113, p. 264-75.

Hausmann, M., S. Kiessling, S. Mestermann, G. Webb, T. Spöttl, T. Andus, J. Schölmerich, H. Herfarth, K. Ray, W. Falk, and G. Rogler, 2002, Toll-like receptors 2 and 4 are up-regulated during intestinal inflammation: Gastroenterology, v. 122, p. 1987-2000.

Heinsbroek, S. E., and S. Gordon, 2009, The role of macrophages in inflammatory bowel diseases: Expert Rev Mol Med, v. 11, p. e14.

Heller, F., P. Florian, C. Bojarski, J. Richter, M. Christ, B. Hillenbrand, J. Mankertz, A. H. Gitter, N. Bürgel, M. Fromm, M. Zeitz, I. Fuss, W. Strober, and J. D. Schulzke, 2005, Interleukin-13 is the key effector Th2 cytokine in ulcerative colitis that affects epithelial tight junctions, apoptosis, and cell restitution: Gastroenterology, v. 129, p. 550-64.

Higgins, L. M., S. A. McDonald, N. Whittle, N. Crockett, J. G. Shields, and T. T. MacDonald, 1999, Regulation of T cell activation in vitro and in vivo by targeting the OX40-OX40 ligand interaction: amelioration of ongoing inflammatory bowel disease with an OX40-IgG fusion protein, but not with an OX40 ligand-IgG fusion protein: J Immunol, v. 162, p. 486-93.

Higuchi, M., K. Nagasawa, T. Horiuchi, M. Oike, Y. Ito, M. Yasukawa, and Y. Niho, 1997, Membrane tumor necrosis factor-alpha (TNF-alpha) expressed on HTLV-I-infected T cells mediates a costimulatory signal for B cell activation--characterization of membrane TNF-alpha: Clin Immunol Immunopathol, v. 82, p. 133-40.

Hodgkinson, C. P., R. C. Laxton, K. Patel, and S. Ye, 2008, Advanced glycation end-product of low density lipoprotein activates the toll-like 4 receptor pathway implications for diabetic atherosclerosis: Arterioscler Thromb Vasc Biol, v. 28, p. 2275-81.

Holness, C. L., and D. L. Simmons, 1993, Molecular cloning of CD68, a human macrophage marker related to lysosomal glycoproteins: Blood, v. 81, p. 1607-13.

Hommes, D. W., J. Meenan, M. de Haas, F. J. ten Kate, A. E. von dem Borne, G. N. Tytgat, and S. J. van Deventer, 1996, Soluble Fc gamma receptor III (CD 16) and eicosanoid concentrations in gut lavage fluid from patients with

inflammatory bowel disease: reflection of mucosal inflammation: Gut, v. 38, p. 564-7.

Hooper, L. V., and J. I. Gordon, 2001, Commensal host-bacterial relationships in the gut: Science, v. 292, p. 1115-8.

Hopkinson-Woolley, J., D. Hughes, S. Gordon, and P. Martin, 1994, Macrophage recruitment during limb development and wound healing in the embryonic and foetal mouse: J Cell Sci, v. 107 (Pt 5), p. 1159-67.

Horiuchi, K., T. Kimura, T. Miyamoto, H. Takaishi, Y. Okada, Y. Toyama, and C. P. Blobel, 2007, Cutting edge: TNF-alpha-converting enzyme (TACE/ADAM17) inactivation in mouse myeloid cells prevents lethality from endotoxin shock: J Immunol, v. 179, p. 2686-9.

Horiuchi, T., H. Mitoma, S. Harashima, H. Tsukamoto, and T. Shimoda, 2010, Transmembrane TNF-alpha: structure, function and interaction with anti-TNF agents: Rheumatology (Oxford), v. 49, p. 1215-28.

Hornung, V., S. Rothenfusser, S. Britsch, A. Krug, B. Jahrsdörfer, T. Giese, S. Endres, and G. Hartmann, 2002, Quantitative expression of toll-like receptor 1-10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides: J Immunol, v. 168, p. 4531-7.

Hoshino, K., T. Kaisho, T. Iwabe, O. Takeuchi, and S. Akira, 2002, Differential involvement of IFN-beta in Toll-like receptor-stimulated dendritic cell activation: Int Immunol, v. 14, p. 1225-31.

Huang, E. Y., M. T. Madireddi, R. V. Gopalkrishnan, M. Leszczyniecka, Z. Su, I. V. Lebedeva, D. Kang, H. Jiang, J. J. Lin, D. Alexandre, Y. Chen, N. Vozhilla, M. X. Mei, K. A. Christiansen, F. Sivo, N. I. Goldstein, A. B. Mhashilkar, S. Chada, E. Huberman, S. Pestka, and P. B. Fisher, 2001, Genomic structure, chromosomal localization and expression profile of a novel melanoma differentiation associated (mda-7) gene with cancer specific growth suppressing and apoptosis inducing properties: Oncogene, v. 20, p. 7051-63.

Huang, H., P. H. Park, M. R. McMullen, and L. E. Nagy, 2008, Mechanisms for the anti-inflammatory effects of adiponectin in macrophages: J Gastroenterol Hepatol, v. 23 Suppl 1, p. S50-3.

Hume, D. A., 2006, The mononuclear phagocyte system: Curr Opin Immunol, v. 18, p. 49-53.

Ikeda, Y., F. Akbar, H. Matsui, and M. Onji, 2001, Characterization of antigen-presenting dendritic cells in the peripheral blood and colonic mucosa of patients with ulcerative colitis: Eur J Gastroenterol Hepatol, v. 13, p. 841-50.

Inohara, N., and G. Nuñez, 2003, NODs: intracellular proteins involved in inflammation and apoptosis: Nat Rev Immunol, v. 3, p. 371-82.

Kamada, N., T. Hisamatsu, S. Okamoto, H. Chinen, T. Kobayashi, T. Sato, A. Sakuraba, M. T. Kitazume, A. Sugita, K. Koganei, K. S. Akagawa, and T. Hibi, 2008, Unique CD14 intestinal macrophages contribute to the pathogenesis of Crohn disease via IL-23/IFN-gamma axis: J Clin Invest, v. 118, p. 2269-80.

Kamdar, K., V. Nguyen, and R. W. Depaolo, 2013, Toll-like receptor signaling and regulation of intestinal immunity: Virulence, v. 4, p. 207-12.

Kaser, A., O. Ludwiczek, S. Holzmann, A. R. Moschen, G. Weiss, B. Enrich, I. Graziadei, S. Dunzendorfer, C. J. Wiedermann, E. Mürzl, E. Grasl, Z. Jasarevic, N. Romani, F. A. Offner, and H. Tilg, 2004, Increased expression of CCL20 in human inflammatory bowel disease: J Clin Immunol, v. 24, p. 74-85.

Kaser, A., S. Zeissig, and R. S. Blumberg, 2010, Inflammatory bowel disease: Annu Rev Immunol, v. 28, p. 573-621.

Kato, A., S. Favoreto, P. C. Avila, and R. P. Schleimer, 2007, TLR3- and Th2 cytokine-dependent production of thymic stromal lymphopoietin in human airway epithelial cells: J Immunol, v. 179, p. 1080-7.

Keshav, S., 2006, Paneth cells: leukocyte-like mediators of innate immunity in the intestine: J Leukoc Biol, v. 80, p. 500-8.

Kobayashi, K., L. D. Hernandez, J. E. Galán, C. A. Janeway, R. Medzhitov, and R. A. Flavell, 2002, IRAK-M is a negative regulator of Toll-like receptor signaling: Cell, v. 110, p. 191-202.

Kollias, G., E. Douni, G. Kassiotis, and D. Kontoyiannis, 1999, The function of tumour necrosis factor and receptors in models of multi-organ inflammation, rheumatoid arthritis, multiple sclerosis and inflammatory bowel disease: Ann Rheum Dis, v. 58 Suppl 1, p. I32-9.

Kontoyiannis, D., M. Pasparakis, T. T. Pizarro, F. Cominelli, and G. Kollias, 1999, Impaired on/off regulation of TNF biosynthesis in mice lacking TNF AU-rich elements: implications for joint and gut-associated immunopathologies: Immunity, v. 10, p. 387-98.

Kopprasch, S., J. Pietzsch, E. Kuhlisch, K. Fuecker, T. Temelkova-Kurktschiev, M. Hanefeld, H. Kühne, U. Julius, and J. Graessler, 2002, In vivo evidence for increased oxidation of circulating LDL in impaired glucose tolerance: Diabetes, v. 51, p. 3102-6.

Kraehenbuhl, J. P., and M. R. Neutra, 2000, Epithelial M cells: differentiation and function: Annu Rev Cell Dev Biol, v. 16, p. 301-32.

Krausgruber, T., K. Blazek, T. Smallie, S. Alzabin, H. Lockstone, N. Sahgal, T. Hussell, M. Feldmann, and I. A. Udalova, 2011, IRF5 promotes inflammatory macrophage polarization and TH1-TH17 responses: Nat Immunol, v. 12, p. 231-8.

Kristiansen, M., J. H. Graversen, C. Jacobsen, O. Sonne, H. J. Hoffman, S. K. Law, and S. K. Moestrup, 2001, Identification of the haemoglobin scavenger receptor: Nature, v. 409, p. 198-201.

Kunz, S., K. Wolk, E. Witte, K. Witte, W. D. Doecke, H. D. Volk, W. Sterry, K. Asadullah, and R. Sabat, 2006, Interleukin (IL)-19, IL-20 and IL-24 are produced by and act on keratinocytes and are distinct from classical ILs: Exp Dermatol, v. 15, p. 991-1004.

Langlet, C., S. Tamoutounour, S. Henri, H. Luche, L. Ardouin, C. Grégoire, B. Malissen, and M. Guilliams, 2012, CD64 expression distinguishes monocyte-derived and conventional dendritic cells and reveals their distinct role during intramuscular immunization: J Immunol, v. 188, p. 1751-60.

Lazdins, J. K., M. Grell, M. R. Walker, K. Woods-Cook, P. Scheurich, and K. Pfizenmaier, 1997, Membrane tumor necrosis factor (TNF) induced cooperative signaling of TNFR60 and TNFR80 favors induction of cell death rather than virus production in HIV-infected T cells: J Exp Med, v. 185, p. 81-90.

Lebre, M. C., C. E. Vergunst, I. Y. Choi, S. Aarrass, A. S. Oliveira, T. Wyant, R. Horuk, K. A. Reedquist, and P. P. Tak, 2011, Why CCR2 and CCR5 blockade failed and why CCR1 blockade might still be effective in the treatment of rheumatoid arthritis: PLoS One, v. 6, p. e21772.

Lefrançois, L., S. Olson, and D. Masopust, 1999, A critical role for CD40-CD40 ligand interactions in amplification of the mucosal CD8 T cell response: J Exp Med, v. 190, p. 1275-84.

Levin, A., and O. Shibolet, 2008, Toll-like receptors in inflammatory bowel disease-stepping into uncharted territory: World J Gastroenterol, v. 14, p. 5149-53.

Ley, K., C. Laudanna, M. I. Cybulsky, and S. Nourshargh, 2007, Getting to the site of inflammation: the leukocyte adhesion cascade updated: Nat Rev Immunol, v. 7, p. 678-89.

Li, S., A. Strelow, E. J. Fontana, and H. Wesche, 2002, IRAK-4: a novel member of the IRAK family with the properties of an IRAK-kinase: Proc Natl Acad Sci U S A, v. 99, p. 5567-72.

Li, Y., J. Brazzell, A. Herrera, and B. Walcheck, 2006, ADAM17 deficiency by mature neutrophils has differential effects on L-selectin shedding: Blood, v. 108, p. 2275-9.

Lin, E. Y., J. F. Li, L. Gnatovskiy, Y. Deng, L. Zhu, D. A. Grzesik, H. Qian, X. N. Xue, and J. W. Pollard, 2006, Macrophages regulate the angiogenic switch in a mouse model of breast cancer: Cancer Res, v. 66, p. 11238-46.

Liu, Z., K. Geboes, S. Colpaert, G. R. D'Haens, P. Rutgeerts, and J. L. Ceuppens, 2000, IL-15 is highly expressed in inflammatory bowel disease and regulates local T cell-dependent cytokine production: J Immunol, v. 164, p. 3608-15.

Lock, K., J. Zhang, J. Lu, S. H. Lee, and P. R. Crocker, 2004, Expression of CD33-related siglecs on human mononuclear phagocytes, monocyte-derived dendritic cells and plasmacytoid dendritic cells: Immunobiology, v. 209, p. 199-207.

Locksley, R. M., 2010, Asthma and allergic inflammation: Cell, v. 140, p. 777-83.

Locksley, R. M., N. Killeen, and M. J. Lenardo, 2001, The TNF and TNF receptor superfamilies: integrating mammalian biology: Cell, v. 104, p. 487-501.

Lodes, M. J., Y. Cong, C. O. Elson, R. Mohamath, C. J. Landers, S. R. Targan, M. Fort, and R. M. Hershberg, 2004, Bacterial flagellin is a dominant antigen in Crohn disease: J Clin Invest, v. 113, p. 1296-306.

Loke, P., M. G. Nair, J. Parkinson, D. Guiliano, M. Blaxter, and J. E. Allen, 2002, IL-4 dependent alternatively-activated macrophages have a distinctive in vivo gene expression phenotype: BMC Immunol, v. 3, p. 7.

Long, C., Y. Wang, A. H. Herrera, K. Horiuchi, and B. Walcheck, 2010, In vivo role of leukocyte ADAM17 in the inflammatory and host responses during E. coli-mediated peritonitis: J Leukoc Biol, v. 87, p. 1097-101.

Lopez, A. F., M. J. Elliott, J. Woodcock, and M. A. Vadas, 1992, GM-CSF, IL-3 and IL-5: cross-competition on human haemopoietic cells: Immunol Today, v. 13, p. 495-500.

Ma, Y., H. D. Chen, Y. Wang, Q. Wang, Y. Li, Y. Zhao, and X. L. Zhang, 2011, Interleukin 24 as a novel potential cytokine immunotherapy for the treatment of Mycobacterium tuberculosis infection: Microbes Infect, v. 13, p. 1099-110.

Macdonald, T. T., 2010, Inside the microbial and immune labyrinth: totally gutted: Nat Med, v. 16, p. 1194-5.

MacDonald, T. T., P. Hutchings, M. Y. Choy, S. Murch, and A. Cooke, 1990, Tumour necrosis factor-alpha and interferon-gamma production measured at the single cell level in normal and inflamed human intestine: Clin Exp Immunol, v. 81, p. 301-5.

MacDonald, T. T., I. Monteleone, M. C. Fantini, and G. Monteleone, 2011, Regulation of homeostasis and inflammation in the intestine: Gastroenterology, v. 140, p. 1768-75.

MacDonald, T. T., A. Vossenkaemper, M. Fantini, and G. Monteleone, 2012, Reprogramming the immune system in IBD: Dig Dis, v. 30, p. 392-5.

Macpherson, A. J., K. D. McCoy, F. E. Johansen, and P. Brandtzaeg, 2008, The immune geography of IgA induction and function: Mucosal Immunol, v. 1, p. 11-22.

Maheshwari, A., D. R. Kelly, T. Nicola, N. Ambalavanan, S. K. Jain, J. Murphy-Ullrich, M. Athar, M. Shimamura, V. Bhandari, C. Aprahamian, R. A. Dimmitt, R. Serra, and R. K. Ohls, 2011, TGF-β2 suppresses macrophage cytokine production and mucosal inflammatory responses in the developing intestine: Gastroenterology, v. 140, p. 242-53.

Mantis, N. J., M. C. Cheung, K. R. Chintalacharuvu, J. Rey, B. Corthésy, and M. R. Neutra, 2002, Selective adherence of IgA to murine Peyer's patch M cells: evidence for a novel IgA receptor: J Immunol, v. 169, p. 1844-51.

Maric, I., N. Kucic, T. Turk Wensveen, I. Smoljan, B. Grahovac, S. Zoricic Cvek, T. Celic, D. Bobinac, and S. Vukicevic, 2012, BMP signaling in rats with TNBS-induced colitis following BMP7 therapy: Am J Physiol Gastrointest Liver Physiol, v. 302, p. G1151-62.

Mashimo, H., D. C. Wu, D. K. Podolsky, and M. C. Fishman, 1996, Impaired defense of intestinal mucosa in mice lacking intestinal trefoil factor: Science, v. 274, p. 262-5.

Matsumura, Y., T. Hori, S. Kawamata, A. Imura, and T. Uchiyama, 1999, Intracellular signaling of gp34, the OX40 ligand: induction of c-jun and c-fos mRNA expression through gp34 upon binding of its receptor, OX40: J Immunol, v. 163, p. 3007-11.

Medvedev, A. E., A. Lentschat, L. M. Wahl, D. T. Golenbock, and S. N. Vogel, 2002, Dysregulation of LPS-induced Toll-like receptor 4-MyD88 complex formation and IL-1 receptor-associated kinase 1 activation in endotoxin-tolerant cells: J Immunol, v. 169, p. 5209-16.

Medzhitov, R., and C. A. Janeway, 2002, Decoding the patterns of self and nonself by the innate immune system: Science, v. 296, p. 298-300.

Melgar, S., M. M. Yeung, A. Bas, G. Forsberg, O. Suhr, A. Oberg, S. Hammarstrom, A. Danielsson, and M. L. Hammarstrom, 2003, Over-expression of interleukin 10 in mucosal T cells of patients with active ulcerative colitis: Clin Exp Immunol, v. 134, p. 127-37.

Meylan, E., K. Burns, K. Hofmann, V. Blancheteau, F. Martinon, M. Kelliher, and J. Tschopp, 2004, RIP1 is an essential mediator of Toll-like receptor 3-induced NF-kappa B activation: Nat Immunol, v. 5, p. 503-7.

Mijatovic, T., L. Houzet, P. Defrance, L. Droogmans, G. Huez, and V. Kruys, 2000, Tumor necrosis factor-alpha mRNA remains unstable and hypoadenylated upon stimulation of macrophages by lipopolysaccharides: Eur J Biochem, v. 267, p. 6004-12.

Mitoma, H., T. Horiuchi, N. Hatta, H. Tsukamoto, S. Harashima, Y. Kikuchi, J. Otsuka, S. Okamura, S. Fujita, and M. Harada, 2005, Infliximab induces potent anti-inflammatory responses by outside-to-inside signals through transmembrane TNF-alpha: Gastroenterology, v. 128, p. 376-92.

Monteleone, G., R. Caruso, D. Fina, I. Peluso, V. Gioia, C. Stolfi, M. C. Fantini, F. Caprioli, R. Tersigni, L. Alessandroni, T. T. MacDonald, and F. Pallone, 2006, Control of matrix metalloproteinase production in human intestinal fibroblasts by interleukin 21: Gut, v. 55, p. 1774-80.

Monteleone, G., I. Monteleone, D. Fina, P. Vavassori, G. Del Vecchio Blanco, R. Caruso, R. Tersigni, L. Alessandroni, L. Biancone, G. C. Naccari, T. T. MacDonald, and F. Pallone, 2005, Interleukin-21 enhances T-helper cell type I signaling and interferon-gamma production in Crohn's disease: Gastroenterology, v. 128, p. 687-94.

Monteleone, I., M. Federici, M. Sarra, E. Franzè, V. Casagrande, F. Zorzi, M. Cavalera, A. Rizzo, R. Lauro, F. Pallone, T. T. MacDonald, and G. Monteleone, 2012, Tissue inhibitor of metalloproteinase-3 regulates inflammation in human and mouse intestine: Gastroenterology, v. 143, p. 1277-87.e1-4.

Mosser, D. M., 2003, The many faces of macrophage activation: J Leukoc Biol, v. 73, p. 209-12.

Mosser, D. M., and J. P. Edwards, 2008, Exploring the full spectrum of macrophage activation: Nat Rev Immunol, v. 8, p. 958-69.

Mowat, A. M., 2003, Anatomical basis of tolerance and immunity to intestinal antigens: Nat Rev Immunol, v. 3, p. 331-41.

Mowat, A. M., and C. C. Bain, 2011, Mucosal macrophages in intestinal homeostasis and inflammation: J Innate Immun, v. 3, p. 550-64.

Munday, J., H. Floyd, and P. R. Crocker, 1999, Sialic acid binding receptors (siglecs) expressed by macrophages: J Leukoc Biol, v. 66, p. 705-11.

Munger, J. S., and D. Sheppard, 2011, Cross talk among TGF-β signaling pathways, integrins, and the extracellular matrix: Cold Spring Harb Perspect Biol, v. 3, p. a005017.

Murray, R. Z., J. G. Kay, D. G. Sangermani, and J. L. Stow, 2005, A role for the phagosome in cytokine secretion: Science, v. 310, p. 1492-5.

Murthy, A., Y. W. Shao, V. Defamie, C. Wedeles, D. Smookler, and R. Khokha, 2012, Stromal TIMP3 regulates liver lymphocyte populations and provides protection against Th1 T cell-driven autoimmune hepatitis: J Immunol, v. 188, p. 2876-83.

Naiki, Y., K. S. Michelsen, W. Zhang, S. Chen, T. M. Doherty, and M. Arditi, 2005, Transforming growth factor-beta differentially inhibits MyD88-dependent, but

not TRAM- and TRIF-dependent, lipopolysaccharide-induced TLR4 signaling: J Biol Chem, v. 280, p. 5491-5.

Nair, M. G., Y. Du, J. G. Perrigoue, C. Zaph, J. J. Taylor, M. Goldschmidt, G. P. Swain, G. D. Yancopoulos, D. M. Valenzuela, A. Murphy, M. Karow, S. Stevens, E. J. Pearce, and D. Artis, 2009, Alternatively activated macrophage-derived RELM-{alpha} is a negative regulator of type 2 inflammation in the lung: J Exp Med, v. 206, p. 937-52.

Naito, M., G. Hasegawa, Y. Ebe, and T. Yamamoto, 2004, Differentiation and function of Kupffer cells: Med Electron Microsc, v. 37, p. 16-28.

Nakao, Y., K. Funami, S. Kikkawa, M. Taniguchi, M. Nishiguchi, Y. Fukumori, T. Seya, and M. Matsumoto, 2005, Surface-expressed TLR6 participates in the recognition of diacylated lipopeptide and peptidoglycan in human cells: J Immunol, v. 174, p. 1566-73.

Narazaki, M., M. Fujimoto, T. Matsumoto, Y. Morita, H. Saito, T. Kajita, K. Yoshizaki, T. Naka, and T. Kishimoto, 1998, Three distinct domains of SSI-1/SOCS-1/JAB protein are required for its suppression of interleukin 6 signaling: Proc Natl Acad Sci U S A, v. 95, p. 13130-4.

Newton, R. C., K. A. Solomon, M. B. Covington, C. P. Decicco, P. J. Haley, S. M. Friedman, and K. Vaddi, 2001, Biology of TACE inhibition: Ann Rheum Dis, v. 60 Suppl 3, p. iii25-32.

Nicholson, S. E., T. A. Willson, A. Farley, R. Starr, J. G. Zhang, M. Baca, W. S. Alexander, D. Metcalf, D. J. Hilton, and N. A. Nicola, 1999, Mutational analyses of the SOCS proteins suggest a dual domain requirement but distinct mechanisms for inhibition of LIF and IL-6 signal transduction: EMBO J, v. 18, p. 375-85.

Nold, M. F., C. A. Nold-Petry, J. A. Zepp, B. E. Palmer, P. Bufler, and C. A. Dinarello, 2010, IL-37 is a fundamental inhibitor of innate immunity: Nat Immunol, v. 11, p. 1014-22.

Nomura, F., S. Akashi, Y. Sakao, S. Sato, T. Kawai, M. Matsumoto, K. Nakanishi, M. Kimoto, K. Miyake, K. Takeda, and S. Akira, 2000, Cutting edge: endotoxin tolerance in mouse peritoneal macrophages correlates with down-regulation of surface toll-like receptor 4 expression: J Immunol, v. 164, p. 3476-9.

O'Shea, J. J., and W. E. Paul, 2010, Mechanisms underlying lineage commitment and plasticity of helper CD4+ T cells: Science, v. 327, p. 1098-102.

Oberg, H. H., D. Wesch, S. Grüssel, S. Rose-John, and D. Kabelitz, 2006, Differential expression of CD126 and CD130 mediates different STAT-3 phosphorylation in CD4+CD25- and CD25high regulatory T cells: Int Immunol, v. 18, p. 555-63.

Ogawa, M., 1993, Differentiation and proliferation of hematopoietic stem cells: Blood, v. 81, p. 2844-53.

Ohmori, Y., and T. A. Hamilton, 1997, IL-4-induced STAT6 suppresses IFN-gamma-stimulated STAT1-dependent transcription in mouse macrophages: J Immunol, v. 159, p. 5474-82.

Oshiumi, H., M. Matsumoto, K. Funami, T. Akazawa, and T. Seya, 2003, TICAM-1, an adaptor molecule that participates in Toll-like receptor 3-mediated interferon-beta induction: Nat Immunol, v. 4, p. 161-7.

Papadakis, A. K., and K. A. Roubelakis-Angelakis, 2005, Polyamines inhibit NADPH oxidase-mediated superoxide generation and putrescine prevents programmed cell death induced by polyamine oxidase-generated hydrogen peroxide: Planta, v. 220, p. 826-37.

Paul, G., V. Khare, and C. Gasche, 2012, Inflamed gut mucosa: downstream of interleukin-10: Eur J Clin Invest, v. 42, p. 95-109.

Pauleau, A. L., and P. J. Murray, 2003, Role of nod2 in the response of macrophages to toll-like receptor agonists: Mol Cell Biol, v. 23, p. 7531-9.

Pedersen, G., L. Andresen, M. W. Matthiessen, J. Rask-Madsen, and J. Brynskov, 2005, Expression of Toll-like receptor 9 and response to bacterial CpG oligodeoxynucleotides in human intestinal epithelium: Clin Exp Immunol, v. 141, p. 298-306.

Perrier, C., G. de Hertogh, J. Cremer, S. Vermeire, P. Rutgeerts, G. Van Assche, D. E. Szymkowski, and J. L. Ceuppens, 2013, Neutralization of membrane TNF, but not soluble TNF, is crucial for the treatment of experimental colitis: Inflamm Bowel Dis, v. 19, p. 246-53.

Peña, A., and J. B. Crusius, 1998, Food allergy, coeliac disease and chronic inflammatory bowel disease in man: Vet Q, v. 20, p. 49-52.

Pixley, F. J., and E. R. Stanley, 2004, CSF-1 regulation of the wandering macrophage: complexity in action: Trends Cell Biol, v. 14, p. 628-38.

Platt, A. M., and A. M. Mowat, 2008, Mucosal macrophages and the regulation of immune responses in the intestine: Immunol Lett, v. 119, p. 22-31.

Powell, N., A. W. Walker, E. Stolarczyk, J. B. Canavan, M. R. Gökmen, E. Marks, I. Jackson, A. Hashim, M. A. Curtis, R. G. Jenner, J. K. Howard, J. Parkhill, T. T. MacDonald, and G. M. Lord, 2012, The transcription factor T-bet regulates intestinal inflammation mediated by interleukin-7 receptor+ innate lymphoid cells: Immunity, v. 37, p. 674-84.

Pulford, K. A., A. Sipos, J. L. Cordell, W. P. Stross, and D. Y. Mason, 1990, Distribution of the CD68 macrophage/myeloid associated antigen: Int Immunol, v. 2, p. 973-80.

Putsep, K., L. G. Axelsson, A. Boman, T. Midtvedt, S. Normark, H. G. Boman, and M. Andersson, 2000, Germ-free and colonized mice generate the same products from enteric prodefensins: J Biol Chem, v. 275, p. 40478-82.

Qualls, J. E., A. M. Kaplan, N. van Rooijen, and D. A. Cohen, 2006, Suppression of experimental colitis by intestinal mononuclear phagocytes: J Leukoc Biol, v. 80, p. 802-15.

Rachmilewitz, D., 1989, Coated mesalazine (5-aminosalicylic acid) versus sulphasalazine in the treatment of active ulcerative colitis: a randomised trial: BMJ, v. 298, p. 82-6.

Ramprasad, M. P., V. Terpstra, N. Kondratenko, O. Quehenberger, and D. Steinberg, 1996, Cell surface expression of mouse macrosialin and human CD68 and their role as macrophage receptors for oxidized low density lipoprotein: Proc Natl Acad Sci U S A, v. 93, p. 14833-8.

Reinhardt, R. L., A. Khoruts, R. Merica, T. Zell, and M. K. Jenkins, 2001, Visualizing the generation of memory CD4 T cells in the whole body: Nature, v. 410, p. 101-5.

Rescigno, M., M. Urbano, B. Valzasina, M. Francolini, G. Rotta, R. Bonasio, F. Granucci, J. P. Kraehenbuhl, and P. Ricciardi-Castagnoli, 2001, Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria: Nat Immunol, v. 2, p. 361-7.

Rimoldi, M., M. Chieppa, V. Salucci, F. Avogadri, A. Sonzogni, G. M. Sampietro, A. Nespoli, G. Viale, P. Allavena, and M. Rescigno, 2005, Intestinal immune homeostasis is regulated by the crosstalk between epithelial cells and dendritic cells: Nat Immunol, v. 6, p. 507-14.

Rogler, G., M. Hausmann, D. Vogl, E. Aschenbrenner, T. Andus, W. Falk, R. Andreesen, J. Schölmerich, and V. Gross, 1998, Isolation and phenotypic characterization of colonic macrophages: Clin Exp Immunol, v. 112, p. 205-15.

Round, J. L., and S. K. Mazmanian, 2010, Inducible Foxp3+ regulatory T-cell development by a commensal bacterium of the intestinal microbiota: Proc Natl Acad Sci U S A, v. 107, p. 12204-9.

Rugtveit, J., P. Brandtzaeg, T. S. Halstensen, O. Fausa, and H. Scott, 1994, Increased macrophage subset in inflammatory bowel disease: apparent recruitment from peripheral blood monocytes: Gut, v. 35, p. 669-74.

Sagaert, X., T. Tousseyn, G. De Hertogh, and K. Geboes, 2012, Macrophage-related diseases of the gut: a pathologist's perspective: Virchows Arch, v. 460, p. 555-67.

Saito, K., S. Chen, M. Piecyk, and P. Anderson, 2001, TIA-1 regulates the production of tumor necrosis factor alpha in macrophages, but not in lymphocytes: Arthritis Rheum, v. 44, p. 2879-87.

Sallusto, F., M. Cella, C. Danieli, and A. Lanzavecchia, 1995, Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: downregulation by cytokines and bacterial products: J Exp Med, v. 182, p. 389-400.

Sallusto, F., D. Lenig, R. Förster, M. Lipp, and A. Lanzavecchia, 1999, Two subsets of memory T lymphocytes with distinct homing potentials and effector functions: Nature, v. 401, p. 708-12.

Salmi, M., and S. Jalkanen, 2005, Lymphocyte homing to the gut: attraction, adhesion, and commitment: Immunol Rev, v. 206, p. 100-13.

Salzman, N. H., M. A. Underwood, and C. L. Bevins, 2007, Paneth cells, defensins, and the commensal microbiota: a hypothesis on intimate interplay at the intestinal mucosa: Semin Immunol, v. 19, p. 70-83.

Satoh, T., O. Takeuchi, A. Vandenbon, K. Yasuda, Y. Tanaka, Y. Kumagai, T. Miyake, K. Matsushita, T. Okazaki, T. Saitoh, K. Honma, T. Matsuyama, K. Yui, T. Tsujimura, D. M. Standley, K. Nakanishi, K. Nakai, and S. Akira, 2010, The Jmjd3-Irf4 axis regulates M2 macrophage polarization and host responses against helminth infection: Nat Immunol, v. 11, p. 936-44.

Saunders, B. M., S. Tran, S. Ruuls, J. D. Sedgwick, H. Briscoe, and W. J. Britton, 2005, Transmembrane TNF is sufficient to initiate cell migration and granuloma formation and provide acute, but not long-term, control of Mycobacterium tuberculosis infection: J Immunol, v. 174, p. 4852-9.

Scheller, J., and S. Rose-John, 2006, Interleukin-6 and its receptor: from bench to bedside: Med Microbiol Immunol, v. 195, p. 173-83.

Schenk, M., A. Bouchon, S. Birrer, M. Colonna, and C. Mueller, 2005, Macrophages expressing triggering receptor expressed on myeloid cells-1 are underrepresented in the human intestine: J Immunol, v. 174, p. 517-24.

Schenk, M., A. Bouchon, F. Seibold, and C. Mueller, 2007, TREM-1--expressing intestinal macrophages crucially amplify chronic inflammation in experimental colitis and inflammatory bowel diseases: J Clin Invest, v. 117, p. 3097-106.

Schlöndorff, J., J. D. Becherer, and C. P. Blobel, 2000, Intracellular maturation and localization of the tumour necrosis factor alpha convertase (TACE): Biochem J, v. 347 Pt 1, p. 131-8.

Shi, C., T. M. Hohl, I. Leiner, M. J. Equinda, X. Fan, and E. G. Pamer, 2011, Ly6G+ neutrophils are dispensable for defense against systemic Listeria monocytogenes infection: J Immunol, v. 187, p. 5293-8.

Shurety, W., A. Merino-Trigo, D. Brown, D. A. Hume, and J. L. Stow, 2000, Localization and post-Golgi trafficking of tumor necrosis factor-alpha in macrophages: J Interferon Cytokine Res, v. 20, p. 427-38.

Siakavellas, S. I., and G. Bamias, 2012, Role of the IL-23/IL-17 axis in Crohn's disease: Discov Med, v. 14, p. 253-62.

Sica, A., and A. Mantovani, 2012, Macrophage plasticity and polarization: in vivo veritas: J Clin Invest, v. 122, p. 787-95.

Smith, P. D., C. Ochsenbauer-Jambor, and L. E. Smythies, 2005, Intestinal macrophages: unique effector cells of the innate immune system: Immunol Rev, v. 206, p. 149-59.

Smith, P. D., L. E. Smythies, M. Mosteller-Barnum, D. A. Sibley, M. W. Russell, M. Merger, M. T. Sellers, J. M. Orenstein, T. Shimada, M. F. Graham, and H. Kubagawa, 2001, Intestinal macrophages lack CD14 and CD89 and consequently are down-regulated for LPS- and IgA-mediated activities: J Immunol, v. 167, p. 2651-6.

Smith, P. D., L. E. Smythies, R. Shen, T. Greenwell-Wild, M. Gliozzi, and S. M. Wahl, 2011, Intestinal macrophages and response to microbial encroachment: Mucosal Immunol, v. 4, p. 31-42.

Smookler, D. S., F. F. Mohammed, Z. Kassiri, G. S. Duncan, T. W. Mak, and R. Khokha, 2006, Tissue inhibitor of metalloproteinase 3 regulates TNF-dependent systemic inflammation: J Immunol, v. 176, p. 721-5.

Smythies, L. E., A. Maheshwari, R. Clements, D. Eckhoff, L. Novak, H. L. Vu, L. M. Mosteller-Barnum, M. Sellers, and P. D. Smith, 2006, Mucosal IL-8 and TGF-beta recruit blood monocytes: evidence for cross-talk between the lamina propria stroma and myeloid cells: J Leukoc Biol, v. 80, p. 492-9.

Smythies, L. E., M. Sellers, R. H. Clements, M. Mosteller-Barnum, G. Meng, W. H. Benjamin, J. M. Orenstein, and P. D. Smith, 2005, Human intestinal macrophages display profound inflammatory anergy despite avid phagocytic and bacteriocidal activity: J Clin Invest, v. 115, p. 66-75.

Smythies, L. E., R. Shen, D. Bimczok, L. Novak, R. H. Clements, D. E. Eckhoff, P. Bouchard, M. D. George, W. K. Hu, S. Dandekar, and P. D. Smith, 2010, Inflammation anergy in human intestinal macrophages is due to Smad-induced IkappaBalpha expression and NF-kappaB inactivation: J Biol Chem, v. 285, p. 19593-604.

Soo, C., W. W. Shaw, E. Freymiller, M. T. Longaker, C. N. Bertolami, R. Chiu, A. Tieu, and K. Ting, 1999, Cutaneous rat wounds express c49a, a novel gene with homology to the human melanoma differentiation associated gene, mda-7: J Cell Biochem, v. 74, p. 1-10.

Stagg, A. J., A. L. Hart, S. C. Knight, and M. A. Kamm, 2003, The dendritic cell: its role in intestinal inflammation and relationship with gut bacteria: Gut, v. 52, p. 1522-9.

Stein, M., S. Keshav, N. Harris, and S. Gordon, 1992, Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation: J Exp Med, v. 176, p. 287-92.

Sternberg, E. M., 2006, Neural regulation of innate immunity: a coordinated nonspecific host response to pathogens: Nat Rev Immunol, v. 6, p. 318-28.

Stout, R. D., and J. Suttles, 2004, Functional plasticity of macrophages: reversible adaptation to changing microenvironments: J Leukoc Biol, v. 76, p. 509-13.

Strauss-Ayali, D., S. M. Conrad, and D. M. Mosser, 2007, Monocyte subpopulations and their differentiation patterns during infection: J Leukoc Biol, v. 82, p. 244-52.

Sun, X., S. Somada, K. Shibata, H. Muta, H. Yamada, H. Yoshihara, K. Honda, K. Nakamura, R. Takayanagi, K. Tani, E. R. Podack, and Y. Yoshikai, 2008, A critical role of CD30 ligand/CD30 in controlling inflammatory bowel diseases in mice: Gastroenterology, v. 134, p. 447-58.

Szebeni, B., G. Veres, A. Dezsõfi, K. Rusai, A. Vannay, M. Mraz, E. Majorova, and A. Arató, 2008, Increased expression of Toll-like receptor (TLR) 2 and TLR4 in the colonic mucosa of children with inflammatory bowel disease: Clin Exp Immunol, v. 151, p. 34-41.

Takeda, K., and S. Akira, 2004, TLR signaling pathways: Semin Immunol, v. 16, p. 3-9.

Takeda, K., and S. Akira, 2005, Toll-like receptors in innate immunity: Int Immunol, v. 17, p. 1-14.

Tamoutounour, S., S. Henri, H. Lelouard, B. de Bovis, C. de Haar, C. J. van der Woude, A. M. Woltman, Y. Reyal, D. Bonnet, D. Sichien, C. C. Bain, A. M. Mowat, C. Reis e Sousa, L. F. Poulin, B. Malissen, and M. Guilliams, 2012, CD64 distinguishes macrophages from dendritic cells in the gut and reveals the Th1-inducing role of mesenteric lymph node macrophages during colitis: Eur J Immunol, v. 42, p. 3150-66.

Tan, M. C., A. M. Mommaas, J. W. Drijfhout, R. Jordens, J. J. Onderwater, D. Verwoerd, A. A. Mulder, A. N. van der Heiden, D. Scheidegger, L. C. Oomen, T. H. Ottenhoff, A. Tulp, J. J. Neefjes, and F. Koning, 1997, Mannose receptor-mediated uptake of antigens strongly enhances HLA class II-restricted antigen presentation by cultured dendritic cells: Eur J Immunol, v. 27, p. 2426-35.

Tarling, J. D., H. S. Lin, and S. Hsu, 1987, Self-renewal of pulmonary alveolar macrophages: evidence from radiation chimera studies: J Leukoc Biol, v. 42, p. 443-6.

Tartaglia, L. A., D. Pennica, and D. V. Goeddel, 1993, Ligand passing: the 75-kDa tumor necrosis factor (TNF) receptor recruits TNF for signaling by the 55-kDa TNF receptor: J Biol Chem, v. 268, p. 18542-8.

te Velde, A. A., Y. van Kooyk, H. Braat, D. W. Hommes, T. A. Dellemijn, J. F. Slors, S. J. van Deventer, and F. A. Vyth-Dreese, 2003, Increased expression of DC-SIGN+IL-12+IL-18+ and CD83+IL-12-IL-18- dendritic cell populations in the colonic mucosa of patients with Crohn's disease: Eur J Immunol, v. 33, p. 143-51.

Thepen, T., A. J. van Vuuren, R. C. Kiekens, C. A. Damen, W. C. Vooijs, and J. G. van De Winkel, 2000, Resolution of cutaneous inflammation after local elimination of macrophages: Nat Biotechnol, v. 18, p. 48-51.

Tilg, H., and A. Kaser, 2015, Failure of interleukin 13 blockade in ulcerative colitis: Gut, v. 64, p. 857-8.

Tokuyama, H., S. Ueha, M. Kurachi, K. Matsushima, F. Moriyasu, R. S. Blumberg, and K. Kakimi, 2005, The simultaneous blockade of chemokine receptors CCR2, CCR5 and CXCR3 by a non-peptide chemokine receptor antagonist protects mice from dextran sodium sulfate-mediated colitis: Int Immunol, v. 17, p. 1023-34.

Tracey, K. J., and A. Cerami, 1993, Tumor necrosis factor, other cytokines and disease: Annu Rev Cell Biol, v. 9, p. 317-43.

Turner, J. R., 2009, Intestinal mucosal barrier function in health and disease: Nat Rev Immunol, v. 9, p. 799-809.

Uhlig, H. H., B. S. McKenzie, S. Hue, C. Thompson, B. Joyce-Shaikh, R. Stepankova, N. Robinson, S. Buonocore, H. Tlaskalova-Hogenova, D. J. Cua, and F. Powrie, 2006, Differential activity of IL-12 and IL-23 in mucosal and systemic innate immune pathology: Immunity, v. 25, p. 309-18.

van de Winkel, J. G., and C. L. Anderson, 1991, Biology of human immunoglobulin G Fc receptors: J Leukoc Biol, v. 49, p. 511-24.

Vijay-Kumar, M., C. J. Sanders, R. T. Taylor, A. Kumar, J. D. Aitken, S. V. Sitaraman, A. S. Neish, S. Uematsu, S. Akira, I. R. Williams, and A. T. Gewirtz, 2007, Deletion of TLR5 results in spontaneous colitis in mice: J Clin Invest, v. 117, p. 3909-21.

Viscido, A., A. Aratari, F. Maccioni, A. Signore, and R. Caprilli, 2005, Inflammatory bowel diseases: clinical update of practical guidelines: Nucl Med Commun, v. 26, p. 649-55.

Vuckovic, S., T. H. Florin, D. Khalil, M. F. Zhang, K. Patel, I. Hamilton, and D. N. Hart, 2001, CD40 and CD86 upregulation with divergent CMRF44 expression on blood dendritic cells in inflammatory bowel diseases: Am J Gastroenterol, v. 96, p. 2946-56.

Wang, F., S. Liu, S. Wu, Q. Zhu, G. Ou, C. Liu, Y. Wang, Y. Liao, and Z. Sun, 2012, Blocking TREM-1 signaling prolongs survival of mice with Pseudomonas aeruginosa induced sepsis: Cell Immunol, v. 272, p. 251-8.

Wang, P., P. Wu, M. I. Siegel, R. W. Egan, and M. M. Billah, 1995, Interleukin (IL)-10 inhibits nuclear factor kappa B (NF kappa B) activation in human monocytes. IL-10 and IL-4 suppress cytokine synthesis by different mechanisms: J Biol Chem, v. 270, p. 9558-63.

Wojtal, K. A., G. Rogler, M. Scharl, L. Biedermann, P. Frei, M. Fried, A. Weber, J. J. Eloranta, G. A. Kullak-Ublick, and S. R. Vavricka, 2012, Fc gamma receptor CD64 modulates the inhibitory activity of infliximab: PLoS One, v. 7, p. e43361.

Wolk, K., H. S. Haugen, W. Xu, E. Witte, K. Waggie, M. Anderson, E. Vom Baur, K. Witte, K. Warszawska, S. Philipp, C. Johnson-Leger, H. D. Volk, W. Sterry, and R. Sabat, 2009, IL-22 and IL-20 are key mediators of the epidermal alterations in psoriasis while IL-17 and IFN-gamma are not: J Mol Med (Berl), v. 87, p. 523-36.

Xu, Y., X. Tao, B. Shen, T. Horng, R. Medzhitov, J. L. Manley, and L. Tong, 2000, Structural basis for signal transduction by the Toll/interleukin-1 receptor domains: Nature, v. 408, p. 111-5.

Zhang, X., and D. M. Mosser, 2008, Macrophage activation by endogenous danger signals: J Pathol, v. 214, p. 161-78.