Epigenetic changes in gut macrophages in health and disease

Aneta Katarzyna Kucik

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Centre for Immunobiology
Blizard Institute
Barts and The London School of Medicine and Dentistry
Queen Mary, University of London
United Kingdom

Statement of originality

I, Aneta Katarzyna Kucik, confirm that the research included within this thesis is my own work or that where it has been carried out in collaboration with, or supported by others, that this is duly acknowledged below and my contribution indicated.
Previously published material is also acknowledged below.
The research on identification of new markers for intestinal macrophage isolation and microarray study was performed in collaboration with Francesca Ammoscato.
The statistical analysis of microarray results was supported by Catriona Sharp (GSK, Stevenage).
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Abstract

A major feature of intestinal macrophages in the normal gut is inflammatory anergy, a state of tolerance essential for intestinal homeostasis, changes in which lead to inflammatory bowel disease (IBD).

Intestinal macrophages undergo a specific process of differentiation. Under homeostatic conditions, cytokines in the local environment drive functional differentiation of newly recruited monocytes into noninflammatory intestinal macrophage. This process is associated with downregulation of proinflammatory cytokines.

Growing evidence supports the idea that epigenetic changes contribute to macrophage reprogramming, and lead to tailored gene expression in response to gut environmental factors. However, current knowledge on how chromatin modification drives genes expression in human intestinal macrophages is still limited.

This project aimed to define the relationship between chromatin modification (histone methylation) and the repression of inflammatory genes in intestinal macrophages isolated from mucosa of control subjects and IBD patients. It was of particular interest to understand if the anergic state of macrophages in normal gut is associated with repressive marks. Also in IBD, if there are any differences in epigenetic modifications between resident and infiltrating macrophages. Finally, if by blocking histone methylation, it is possible to prevent/reduce TNFα production by macrophages from IBD mucosa.

TNF-α is an inflammatory cytokine that plays a critical role in innate and adaptive immune responses and its dysregulation has been implicated in the pathology of IBD. Considering its central role in IBD pathology, the *TNFA* gene was selected and different repressive and permissive histone modifications were investigated.

Silencing marks H3K27me3, H3K9me3 and H3K9me1, as well as activating marks H3K4me3, H3K4me1 and also RNAPII were selected and analysed using chromatin immunoprecipitation (ChIP) assays.

Based on data collected, it was speculated that a break of anergic phenotype in IBD macrophages might be associated with changes in level of silencing marks. Macrophages isolated from mucosa of CD patients showed decreased enrichment of H3K27me3 and H3K9me3, with H3K27me3 having the greatest reduction.

Additional analysis of peripheral blood monocytes suggested that in healthy gut, the differentiation of blood monocytes into resident intestinal macrophages is associated with deposition of H3K27me3 and H3K9me3 silencing marks at the *TNFA* TSS, and that this process fails in IBD environment.

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My Family have always inspired me, there is one thing that my parents thaught me (…)

"Your knowledge is something that nobody will take away from you"

My parents and my sister were truly a great support to me over these years. They always know what to say at the right time, when I need it… and for this reason (well… not only…) I am truly thankful.

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I dedicate this work to my father

Kazimierz Kucik

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List of abbreviations

APC	Allophycocyanin
APCs	antigen-presenting cells
AMP	antimicrobial peptide
CSF	Colony stimulating factor
EDTA	ethylenediaminetetraacetic acid
FACS	fluorescence activated cell sorting
FITC	fluorescein isothiocyanate
FSC	forward scatter
FoxP3	forkhead box P3
GALT	gastrointestinal associated lymphoid tissue
GM-CSF	granulocyte macrophage colony-stimulating factor
IBD	Inflammatory bowel disease
IFN	interferon
Ig	immunoglobulin
IL	interleukin
ILF	isolated lymphoid follicles
IMACs	Intestinal macrophages
IRAK	IL1 receptor-associated kinase
IRF	interferon regulatory factor
LP	lamina propria
LPMCs	lamina propria mononuclear cells
LPS	lipopolysaccharide
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
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
TRAF	TNF receptor activated factor
TREM	triggering receptor expressed by myeloid cells
TSS	Transcription start side
TH1	T helper 1
TH2	T helper 2
TH17	T helper 17
TNF	tumour necrosis factor
Treg	regulatory T cell

Chapter 1

General Introduction

1. Introduction

Through the means of innate and adaptive responses, the mammalian immune system has developed a complex of cellular and biochemical processes ensuring efficient detection and elimination of harmful agents from the body with the minimum damage to the host tissue.

Innate immunity provides a prompt non-specific response, whereas the adaptive immune system responds in a delayed antigen-specific manner resulting in immunological memory that allows rapid and specific response upon future re-exposure to the agents. The Innate and adaptive immunity work together in balance to provide an optimum protection against potential pathogens.

The intestine represents the largest surface for colonisation or entry of pathogens to challenge the immune system. It is also the site at which the body is exposed to food antigens and the antigens of the microbiota. Yet the vast majority of individuals do not react inappropriately to foods or the microbiota. However it is known that failure to induce tolerance to food proteins results in food allergies including celiac disease (Meresse et al., 2009). Likewise, although the intestine is colonised by large numbers of bacteria, which are responsible for inducing the mucosal immune system, in most individuals the immune response to these microbes is controlled and does not lead to pathology. It appears therefore that the mucosal immune system in health is tolerant to the antigens of foods and microbes. While it is clear that barrier function and active immune suppression are important in maintaining homeostasis, despite intense investigation, the pathways, which break down and result in disease are still unclear.

1.1. The Intestinal Immune system

The gastrointestinal tract provides the largest surface for microorganisms to challenge the immune system (Peterson and Artis, 2014). The gut also hosts a large diversity of commensal bacteria, many of which are mutualistic symbionts (Sonnenburg et al., 2004). Therefore, the intestinal immune system faces the challenge of responding to vast variety of pathogens while remaining relatively unresponsive to food antigens and commensal microbiota.

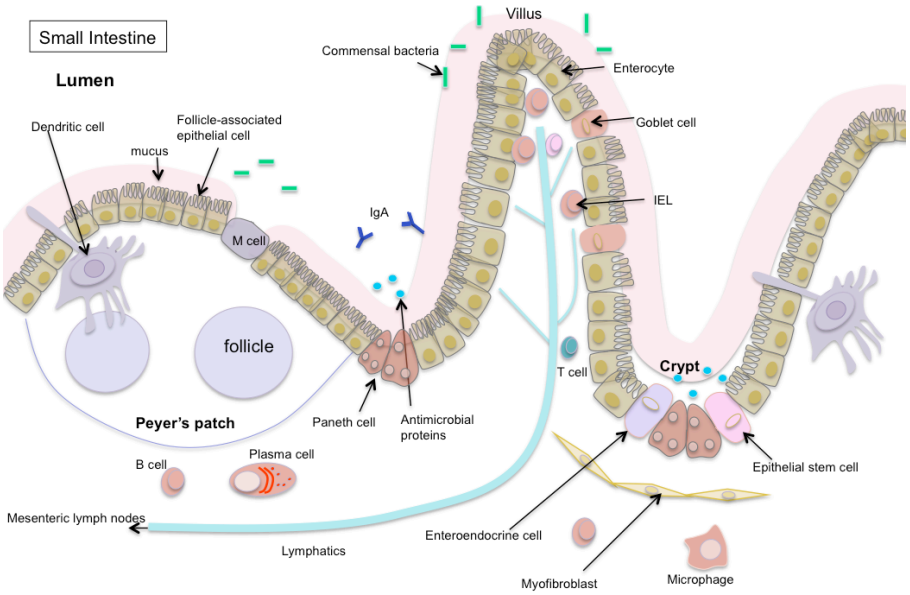
Moreover, continuous exposure to a variety of foreign antigens in the diet can also trigger inappropriate immune responses, such as coeliac disease or cows milk allergy. It is thought that unresponsiveness in the gut is due to the phenomenon of immune tolerance (Mowat, 2005).

The intestinal immune system can be divided into inductive and effector sites. The inductive sites include GALT as well as local and regional draining lymph nodes (LNs), whereas the lamina propria and epithelium are the main effector sites (Brandtzaeg et al., 2008).

1.1.1. The Intestine Epithelial Barrier

The intestinal epithelium provides a physical and biochemical barrier to commensal and pathogenic microorganisms (Peterson and Artis, 2014). It comprises a single layer of cells organised into crypts and villi in the small intestine (Figure 1.1) (Peterson and Artis, 2014). The mucosal surface is covered by a hydrated gel formed by mucins, secreted by specialised epithelial cells, such as gastric foveolar mucous cells and intestinal goblet cells, and provides an extracellular barrier preventing large particles, including most bacteria, from directly contacting the epithelial cell layer (Turner, 2009).

A).



B).

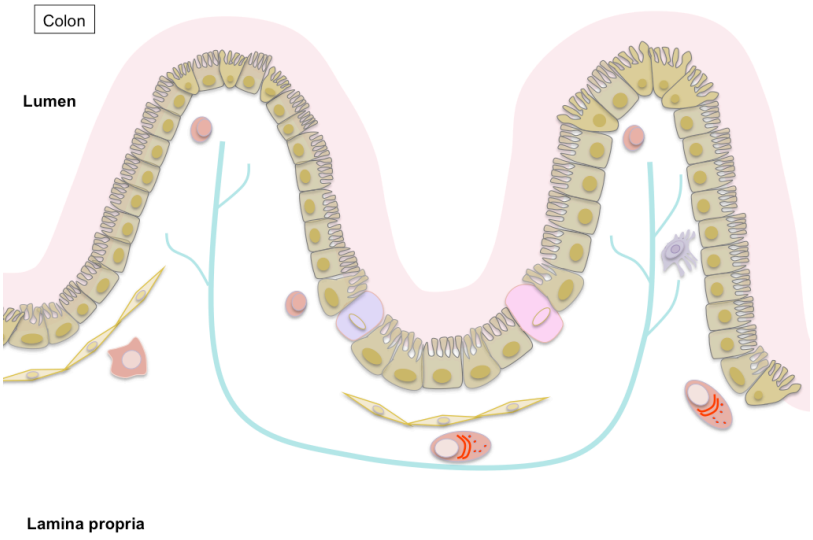


Figure 1.1: Anatomy of the intestinal immune system (Adapted from Abreu, 2010)

In the small intestine the mucus layer is thin, but in the colon the mucus layer is thicker and essentially sterile above the epithelial microvilli (Figure 1.1). The outer mucus layer is colonised by bacteria which use mucus as a food source.

An intact mucosal barrier depends on integrity of adjacent epithelial cells (the paracellular space). This function is mediated by the apical junctional complex, which is composed of tight junctions and subjacent adherens junctions. The adherens junctions are essential for the assembly of tight junctions (Figure 1.2) (Turner et al., 2009; Marchiando et al., 2010). Both tight and adherens junctions are supported by a dense perijunctional ring of actin and myosin that 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 (Marchiando et al., 2010). Loss of adherens junctions results in disruption of cell–cell and cell–matrix contacts, ineffective epithelial cell polarisation and differentiation, and premature apoptosis (Hermiston and Gordon, 1995; Turner, 2009). The adherens junctions are composed of cadherins (transmembrane proteins) that interact in a homotypic manner with cadherins on adjacent cells. The cytoplasmic tail of the epithelial cadherin, E-cadherin (cadherin-1) interacts directly with catenin δ1 (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 perijunctional ring of actin and myosin (Turner, 2009).

Tight junctions are multi-protein complexes composed of transmembrane proteins, such as claudins and occludin, (Turner, 2009). 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 (Turner, 2009).

The gut epithelial barrier does not completely prevent luminal antigens from entering the tissue. Antigens can cross the epithelial surface through gaps in tight junctions,

possibly at the villus tips or through follicle-associated epithelium (FAE) that overlies lymphoid tissues of intestinal wall (Neutra et al., 2001).

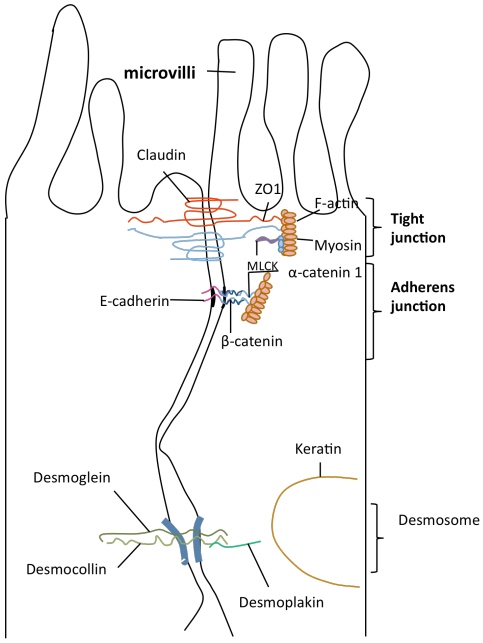


Figure 1.2: Anatomy of the mucosal barrier (Adapted from Turner, 2009)

The intestinal epithelial cell (IEC) layer is composed of four cell types, absorptive enterocytes, mucus-producing goblet cells, hormone-producing enteroendocrine cells and Paneth cells, which produce antimicrobial peptides (AMPs) such as defensins, serine leukocyte protease inhibitor (Figure 1.2) (Ma et al., 2004; Abreu, 2010). The renowal of adult intestinal epithelial layer depends on resident specialised stem cells localised within crypts (Barker et al., 2010). The luminal secretion of mucus and antimicrobial proteins forms a biochemical and a physical barrier against microbial encroachment (Johansson et al., 2008; Gallo and Hooper, 2012). Mucin 2 (MUC2) plays an essential role in the organisation of the intestinal mucous layer of the colon (Johansson et al., 2008). MUC2-deficient mice develop a colitis and also inflammation-induced colorectal cancer (Velcich et al., 2002; Van der Sluis et al., 2006). Goblet cells also produce other molecules, such as trefoil factor 3 (TFF3) and

resistin-like molecule-β (RELMβ). TFF3 acts as a signal to promote epithelial repair, increase IEC migration across damaged tissues and resistance to apoptosis (Dignass et al., 1994; Taupin et al., 2000; Aamann et al., 2014). In addition, RELMβ promotes MUC2 production (Peterson, 2014). The secretion of AMPs additionally reinforces the intestinal barrier. Paneth cells secrete antimicrobial components such as defensins, cathelicidins and lysozyme in the crypts of the small intestine (Bevins et al., 2011; Gallo et al., 2012).

Moreover, IECs are also able to directly transport secretary immunoglobulins across the epithelial barrier (Peterson, 2014). Upon their production by plasma cells in the lamina propria, dimeric IgA complexes are bound by polymeric immunoglobulin receptor (pIgR) on the basolateral membrane of IECs and the complex is actively transported into intestinal lumen (Johanssen et al., 2011).

In order to maintain homeostatic immune responses, IECs must appropriately respond to microbial stimuli and to integrate commensal bacteria-derived signals into antimicrobial and immunoregulatory responses. The IECs recognise microorganisms via pattern-recognition receptors (PRRs). The PRRs comprises of Toll-like receptors (TLRs), NOD-like receptors (NRs) and RIG-I-like receptors (RLRs) (Medzhitov et al., 1997; Hoshino et al., 1999; Akira et al., 2006). The IECs express a number of Toll-like receptors (Peterson, 2014). TLR3 expression is seen in a small intestine and colon, whereas TLR5 is expressed predominantly in the colon (Cario and Podolsky, 2000). Other types of receptors such as TLR1, TLR3, TLR5 and 9 have also been found in IECs of the human small intestine (Otte et al., 2004). In the human small intestine, the expression of TLR3, TLR4 and TLR5 has been shown on the basolateral surfaces of villus enterocytes (Cario and Podolsky, 2000 Otte et al., 2004). The Paneth cells were shown to express TLR4 and TLR9 (Otte et al., 2004).

In mice, the follicular-associated epithelial cells that neighbour microfold (M) cells express TLR2 and TLR9 on both, the apical and basolateral surfaces, and also TLR4 and TLR5 only on the apical surfaces (Chabot et al., 2007). Enteroendocrine cells also express several TLRs, including TLR1, TLR2 and TLR4, but it is not clear whether they are present on apical or basolateral surfaces (Bogunovic et al., 2007).

Under normal physiological conditions, TLR2 and TLR4 are expressed by IECs at low levels (Otte et al., 2004; Abreu et al., 2011). However, the expression of TLRs, particularly TLR4 is increased in IECs from patients with inflammatory bowel disease (Cario and Podolsky, 2000; Pedersen et al., 2005). Animal studies showed that inflammatory cytokines, especially IFN-γ and TNF promote the expression of TLR4 and its co-receptor MD-2, as opposed to IL-4 and IL-13 which decreases the responsiveness of IECs to TLR4 ligand (LPS) (Peterson, 2014).

The epithelial layer also contains large numbers of cells called intraepithelial lymphocytes (IELs) that are very heterogeneous with regard to their function and phenotype. The IELs are unique subsets of intestinal T-cells located in the epithelial layer, physically separated from lamina propria lymphocytes (van Wijk and Cheroutre, 2009). Most IEL are $CD8^+$ T cells, with large numbers of γδT cells (Spencer et al., 1991). Most IEL are enriched for $CD8\alpha\alpha^+$ cells in comparison to other $CD8^+$ T cells in the body, which are CD8αβ (Denning et al., 2007). IELs also have potent cytotoxic potential and help to clear infected or damaged IECs. TCRαβ+ IELs do not respond to conventional major histocompatibility complex (MHC)-peptide ligands, but to ligands expressed on IECs (e.g. the thymus leukemia antigen ligand for $CD8\alpha\alpha^+$ IELs) (Leishman et al., 2001). Additionally, IELs also function as regulatory cells by suppressing inflammation in animal models (Das et al., 2003).

TCR$\alpha\beta^+$ IELs have been shown to produce AMPs (Ismail et al, 2011). The presence of commensal bacteria influences the development and function of these cells, since in germ-free mice TCR$\alpha\beta^+$ IELs are almost absent (Kawaguchi-Miyashita et al., 1996; Hendricks and Fink, 2009).

1.1.2. The gut-associated lymphoid tissues (GALT)

GALT is an inductive site for intestinal B cells responses (Macpherson et al., 2008). The GALT consists of Peyer's patches (PPs), scattered along the anti-mesenteric site of the small intestine, the isolated lymphoid follicles (ILFs), the mesenteric lymph nodes (MLNs), and appendix (Brandtzaeg et al., 2008).

1.1.2.1. Development of PPs, MLNs and ILFs

The development of PPs and MLNs is initiated in fetal life and depends on interaction between mesenchymal organiser cells and hematopoietic inducer cells (Brandtzaeg and Johansen, 2005). This process involves lymphotoxins (LTs), members of the TNF superfamily (Eberl and Lochner, 2009).

An important differentiating event in PP formation is membrane expression of $LT\alpha_1\beta_2$ on IL-7Rα^+ $CD4^+$ lymphoid tissue inducer (LTi) cells (Yoshida et al., 2002). The expression of $LT\alpha_1\beta_2$ occurs after stimulation of the receptor activator of nuclear factor (NF)-KB (RANK)-TNF-associated factor family 6 (TRAF6) pathway and also through IL-7R signalling (Yoshida et al., 2002). The development of PP takes through three stages (Yoshida et al., 1999). Step one is the induction of organising centres for PP, through the production of IL-7 or thymic stromal-derived lymphopoietin (IL-7Rα ligand). The local source of IL-7 remains undetermined (Eberl and Lochner, 2009) and it is speculated that an activation of LTi cells through

IL-7RA can occur in the blood (Luther et al., 2003), on the way from the liver, where these cells are generated (Yoshida et al., 2001; Mebius et al., 2001). The activation of LTi through IL-7Rα subsequently induces PP-organising centres. The PP organisers (lymphoid tissue organiser (LTo) cells) are of mesenchymal origins (Honda et al., 2001) and also express CXCL13 (Cyster et al., 2000, Peduto et al., 2009). The CXCL13 is a ligand for CXCR5 receptor expressed by LTi, which additionally can promote the recruitment of these cells upon LTβR-mediated activation (Mebius et al., 2003). Additionally, CXCR5 regulates the activation of integrin $\alpha_4\beta_1$ on LTi cells, and binding of integrin $\alpha_4\beta_1$ to vascular cell adhesion molecule (VCAM)-1 allows the interaction of LTi cells with LTo cells and the subsequent activation of LTo cells (Eberl and Lochner, 2009). In step two, hematopoietic cells, including LTi cells, $F4/80^+$ cells (in mice), and $CD11c^+$ cells, accumulate in the organising centres, and high endothelial venules are formed. Final third step is characterised by B- and T-cell homing to the growing PP (Eberl and Lochner, 2009).

This three-step model of PP development was supported by numerous murine models. It has been shown that mice deficient in lymphotoxin LTα (De Togni et al., 1994) and LTβ (Koni et al., 1997), and mice treated with LTβR-Ig fusion protein (Rennert et al., 1996) all lack PPs. This suggests that membrane form of lymphotoxin, heterotrimer $LT\alpha_1\beta_2$ and ligand of LTβR are critical for PP development (Eberl and Lochner, 2009). Adachi and colleagues (1998) have shown that IL-7Rα-deficient mice failed to develop PPs. Additinally, it has been shown that in the absence of IL-7Rα signalling, LTi cells fail to upregulate $LT\alpha_1\beta_2$, thus cannot induce LTβR signalling and PP development (Luther et al., 2003).

Additionally, the nuclear hormone receptor RORγ is also required for LNs and PPs development (Sun et al., 2000). It has been shown that the RORγt is expressed by LTi cells in the fetus (Eberl et al., 2004a), and that in the absence of RORγt, LTi cells are not generated and PPs do not develop (Eberl et al., 2004a).

Although, the development of PPs and mLNs follows similar pattern, some distinct pathways between the two also exist. As described previously, the development of PPs and LNs depends on generation of LTi cells and the activation of LTβR-mediated signalling in LTo cells. Mice deficient in RORγt (Eberl et al., 2004a), LTβR (Rennert et al., 1998) and NF-KB-inducing kinases (Miyawaki et al., 1994), all fail to develop LNs and PPs. However, lack of TNF-α and TNF receptor-I affects only PP development (Neumann et al., 1996; Korner et al., 1997). Additionally, most LNs, but not PPs, do not develop in the absence of the TNF superfamily member TRANCE, its receptor TRANCE-R, or its associated signalling molecule TRAF6 (Kim et al., 2000). However, as shown by Yoshida et al. (2002), LN development can be rescued in TRAF6-deficient mice by the administration of IL-7. Therefore, it has been suggested that local activation of LTi involves local availability of IL-7 and TRANCE in PPs and LNs, respectively (Eberl and Lochner, 2009).

In addition, mice deficient in IL-7R (Cao et al., 1995) or its associated signalling molecule JAK3 (Park et al., 1995), or LTβ (Koni et al., 1997), CXCR5 (Forster et al., 1996) and CXCL13 (Ansel et al., 2000) lack PPs and most LNs, whereas MLNs still develop. Through an inhibition of LTβR signalling with LTβR-Ig fusion protein, it has been shown that MLNs are among the first to initiate development (Rennert et al., 1998).

The development of ILFs also requires $LT\alpha_1\beta_2$/LTβR interaction and TNFRI function (Lorenz et al, 2003; Yamamoto et al., 2004). However, in contrast to PPs, ILFs develop only post-natally in response to microbial stimulation (Hamada et al., 2002). The role of microbiota in initiation and development of ILFs has been supported by number of studies in knockout or germ free mouse models (Lorenz et al., 2003; Bouskra et al, 2008; Knoop et al., 2011; Baptista et al., 2013).

For example, it has been shown that germ-free (GF) mice have hypoplastic PPs and lack IFLs (MacDonald and Spencer, 1990; Bouskra et al., 2008). Additionally, in germ-free mice, ILFs with defined B follicle do not develop (Hamada et al., 2002; Lorenz et al., 2003; Pabst et al., 2006; Bouskra et al., 2008). Also, in mice deficient in RORt, LTi cells are absent from the intestine, and cryptopatches (CPs) and ILFs are not formed (Eberl and Littman, 2004). As proposed by Bouskra et al. (2008), gram-negative bacteria induce iILF development through the nucleotide-binding oligomerisation domain containing 1 (NOD1) receptor. The peptidoglycans released by bacteria are recognised by NOD1 innate receptor expressed in intestinal epithelial cells (Fritz et al., 2006), which consequently lead to the expression of CCL20 and β-defensin (both CCR6 ligands) on these cells. The CCL20 and β-defensin activate LTi in CPs, engage LTo cells and recruit $CCR6^+$ B cells to CPs (McDonald et al., 2007).

1.1.2.2. Peyer's patches

Peyer's patches are organised aggregates of lymphoid tissue scattered at intervals underneath the gut epithelium in the small intestine. The PPs are bound to the intestinal epithelium and connected to lymphatic system though efferent vessels. They collect antigens directly from the intestinal lumen though specialised epithelial cells called microfolds (M) cells (Eberl and Lochner, 2009) (function of M cells is

discussed later in this paragraph).The PPs consist of at least 5 aggregated lymphoid follicles, but can contain up to 200 such organised structures (Cornes, 1965; Brandtzaeg and Johansen, 2005). The number of PPs also increases from approximately 50 at the beginning of the last trimester to 100 at birth and 250 in the midteens, then diminishes to 100 at the age of 70 (Cornes, 1965; Brandtzaeg and Johansen, 2005).

The PPs can be seperated into three main domains, namely the follicular area, the interfollicular area and the follicle-associated epithelium (Neutra et al., 2001). The follicular and interfollicular area consists of PP lymphoid follicles with prominent germinal centre (GC) containing proliferating B lymphocytes, DCs and macrophages (Boursier et al., 2005; Jung et al., 2010). The germinal centre forms the core of each follicle. The formation of germinal centre occurs after the exposure to antigen at birth and most of adaptive secretory IgA response is generated in PPs, the progeny migrating to the lamina propria (MacDonald, 2003).

The follicle is surrounded by the corona, or subepithelial dome (SED) also containing populations of B and T cells, DCs and macrophages (Jung et al., 2010). In human PPs, T cells are present in high density in the area surrounding the high endothelial venules between follicles (Spencer et al., 1986), in areas surrounding the follicle but also in the mixed cell zone in the dome and in the lymphoepithelium (MacDonald et al., 2003). T cell zone also extends between the follicle centre and the muscularis mucosa, and occasionally T cells are present in the follicle centre (MacDonald, 2003). The majority of the T cells in PPs are $CD4^+$ (MacDonald, 2003). A single layer of epithelial cells known as follicle-associated epithelium

(FAE) seperates the PPs from the lumen. The FAE is also characterised by a large number of infiltrated B and T cells, macrophages and DCs (Jung et al., 2010).

The FAE contains specialised enterocytes called microfold (M) cells (Neutra et al., 2001). The M cells lack surface microvilli, and are not covered by a mucus layer. These cells function as antigen-transporting cells, providing the main way in which antigen can gain access to the PPs and be presented to professional APCs (e.g., DCs) in the epithelium or in the underlying SED (Brandtzaeg and Pabst, 2004). M cells do not express MHC class II molecules and do not process antigens, therefore they don't function as antigen-presenting cells (Mantis et al., 2002). However, M cells express IgA receptors allowing the capture and uptake of IgA trapped bacteria (Mantis et al., 2002).

1.1.2.3. Isolated lymphoid follicles (ILFs)

The ILFs are microscopic lymphoid aggregates localised along the small and large intestine, and they are developmentally independed of PPs (Lindner et al., 2012). Human intestinal mucosa harbours at least 30,000 ILFs that increases in density distally (Trepel, 1974), which correlated with increased bacterial load.

Just like PPs, the ILFs also have FAE and M cells, but comprise of only 1 or 2 B cell follicles, surrounded by DCs (Eberl and Lochner, 2009). The ILFs contain relatively few T cells and no distinct T-cell zones. Upon activation, more B cells are recruited to ILF to form germinal centres (Glaysher and Mabbott, 2007). Therefore, although ILFs are smaller that PPs, they also represent an important source of follicle B cells. The ILFs also serve as an inductive site for IgA synthesis, and a maturation to large

B cell follicle occurs in part in response to changes in bacterial composition and dietary products (Lorenz et al., 2003; Hooper, 2011; Kiss and Diefenbach, 2012).

1.1.2.4. Mesenteric lymph nodes

In contrast to PPs, the mesenteric lymph nodes (MLNs) are considered the largest lymph nodes in the body (Mowat, 2003), and together with PPs are essential for the initiation of protective immune responses in the intestine against microbial antigen. The MLNs contain an abundance of B cells. However, as demostared by Worbs and colleagues (2006), MLNs also play a critical role in the induction of oral tolerance. This finding was additionally supported by Pabst et al. (2007), who showed that a transport of antigen from the lamina propria into the MLNs by $CD103^+$ DCs is the key event for induction of oral tolerance. The migration of DCs into the draining LNs requires the expression of CCR7 (Forster et al., 2008), and as shown by Worbs et al. (2006), genetic CCR7 deficiency prevents the recognition of food antigen by T cells in the MLNs and impairs the induction of oral tolerance. Also, it was shown that oral tolerance can be abrogated in lymphotoxin α-deficient mice lacking all LNs and PPs, but can be consequently restored by selective rescue of MLN development (Spahn et al., 2002).

1.1.3. Migration of lymphocytes into the GALT

When released from the thymus, naïve lymphocytes express high levels of L-selectin (CD62L) and the chemokine receptor CCR7 (Butcher and Picker, 1996; Gunn et al., 1999). The migration of naïve lymphocytes into the GALT is highly regulated and occurs via specialised high endothelial venules (HEVs). Mucosal addressin cell adhesion molecule (MAdCAM-1) is a key player in intestinal trafficking of both T

and B cells (Wagner et al., 1996; Brandtzaeg et al., 1999). In human GALT, MAdCAM-1 is highly expressed by HEVs of the MLN, PP and the venules of the small and large intestine (Leung et al., 1996; Shyjan et al., 1996; Brandtzaeg et al., 1999a; 1999b). It is also displayed on follicular dendritic cells in PP at sites associated with microenvironmental homing decisions, and on follicular dendritic cells in peripheral lymph nodes after primary immunisation with antigen (Shabo et al., 1997).

In murine models, naïve lymphocytes can enter PP through glycosylation of the mucin-like domains of MAdCAM-1, which promotes binding of CD62L (Berg et al., 1993, Butcher et al., 1996).

Additionally, naïve lymphocytes also express low to intermediate levels of α4β7-integrin (the main receptor mediating interaction with MAdCAM-1). Interaction between CD62L and MAdCAM-1 provides initial tethering, however additional interaction between α4β7 and MAdCAM-1 is crucial for emigration of naïve lymphocytes into GALT (Berlin et al., 1993; Yong et al., 1995; Briskin et al., 1996).

Additional interaction between CCL21 that is expressed on the luminal surface of the HEVs and CCR7-expressed by naïve lymphocytes promotes firm adhesion and lymphocytes arrest, which leads to cells extravasation into PPs and MLNs (Luster et al., 2005; Brandtzaeg and Johansen, 2005). CCL21-CCR7 interaction activates the integrin $\alpha_L\beta_2$ on naïve T cells, leading to interaction between integrin $\alpha_L\beta_2$ and ICAM-1 (Sigmundsdottir and Butcher, 2008). It has been shown that CCL21 also stimulates α4β7-mediated lymphocyte adhesion to MAdCAM-1 (Pachynski et al., 1998).

1.1.4. Lymphocyte activation in the GALT

The DCs encountering antigen subsequently migrate into the T cell areas and B cell rich follicles of PPs or into MLN where they present antigen to naïve T and B lymphocytes (Newberry and Lorenz, 2005; Salzman et al., 2007). In PPs, DCs acquire antigen from M cells, whereas DCs that take up antigen directly from the lumen do not mediate antigen presentation in the lamina propria, but transfer antigen to DCs present in MLNs (Pabst et al., 2007; Pabst and Mowat, 2012) (Discussed more in section on DCs). The DCs can present antigen directly to naïve B cells driving their differentation into IgA^+ B cells or present antigen to naïve T cells which leads to expression of antigen specific TCRs on T cells. Additionally, activated T cells also interact with B cells to drive their maturation into IgA plasma blast (Discussed more in section on IgA). Upon activation, B cells start to proliferate and generate germinal centres within PP or MLN, where they undergo affinity maturation and isotype switch from IgM to IgA (Pabst et al., 2004). Most of fully differentiated B cells and effector T cells exit PP and MLN and migrate via the lymphatics and the thoracic duct into the blood and consequently into the lamina propria. In the lamina propria, IgA^+ B cells become fully developed IgA plasma cells (MacDonald et al., 2011).

1.1.5. Lymphocyte homing into the gut

After encountering the antigen in the GALT, naïve lymphocytes undergo antigen-driven priming/activation and polarise into effector cells, which consequently allows them to home to the target tissue in search for antigen. During this process, activated lymphocytes downregulate CD62L and CCR7 (Farstad et al., 1997) but enhance

expression of α4β7-integrin and chemokine receptor CCR9, the process that is driven by retinoic acid production by $CD103^+$ CDs (also discussed in section 1.1.2.3).

The interaction between integrin-α4β7 and MAdCAM-1, which is expressed on intestinal endothelial cells promote homing of lymphocytes into the lamina propria (Hamman et al., 1994). Additionally, the homing of lymphocytes specifically to the small intestine is enhanced by the expression of CCL25 by crypt and glandural epithelium, and its presence on small bowel venular endothelium (Kunkel et al., 2000). In the small intestine, the majority of lymphocytes express integrin-α4β7 and CCR9 (Papadakis et al., 2001). Interaction between CCR9 and CCL25 triggers conformational changes in α4β7-integrin and a firm adhesion to MAdCAM-1 (Berlin et al., 1993). In contrary, lymphocytes that home to the colon express low levels of CCR9 but upregulate CCR10. The interaction between CCR10 and CCL28 that is expressed on colonic mucosal vessels and epithelial cells triggers homing of lymphocytes to the colonic lamina propria (Pan et al., 2000).

1.1.6. Lamina propria

The lamina propria is an area below an epithelial barrier, which as opposed to FAE is secured by tight junctions and covered by a layer of mucins (discussed in section 1.1.1.). The lamina propria is considered as an effector site and consists of diffused connective tissue with a lymphoid constituent including cytokine producing T cells and IgA producing plasma cells, DCs, macrophages, eosinophils and mast cells. There are more lymphocytes in the mucosa than in any other part of the body (Farstad et al., 2000; Brandtzaeg et al., 2010; Spencer et al., 2012).

1.1.7. Mucosal T cells

Lymphocytes are found in three distinct anatomical and functional compartments in the gut, the organised gut-associated lymphoid tissue (GALT), the lamina propria, and the surface epithelium (Brandtzaeg, 2008). In the normal gastrointestinal (GI) tract, T cells constitute one-third of the cells in the intestinal lamina propria (MacDonald et al., 2011). The balance between effector T cells and regulatory T cells in the gut mucosa is important for the maintenance of a healthy gut (MacDonald et al., 2011). The phenotypic distribution of $CD4^+$ and $CD8^+$ T cells in lamina propria is similar to that of peripheral blood lymphocytes, with a dominance of $CD4^+$ T cells (Helgeland et al., 2000; MacDonald et al., 2011).

The $CD4^+$ T cells in the lamina propria are distributed more evenly throughout the villus-crypt unit. Phenotypically, most $CD4^+$ T lamina propria lymphocytes (LPLs) have an activated phenotype being L-selectin^{lo} HLA-DR, $\alpha4\beta7^+$, CD62^{lo}, CD25$^{hi/lo}$ and $CD45RO^+$ (MacDonald et al., 2011). The lamina propria $CD4^+$ T cells derive from PP T cells blasts which have extravasated from thr blood via $\alpha4\beta7$ integrin/MAdCAM-1 interaction (Butcher et al., 1996). In the lamina propria, $CD4^+$ TCR$\alpha\beta$ T cells predominate (Brandtzaeg et al., 1989; MacDonald and Pender, 1998), however, a minority (~40%) of T cells in the lamina propria are also $CD8^+$ (Lefrançois et al., 1999) and express the $\alpha E\beta7$ integrin, therefore are likely to be en route to the epithelium (Farstad et al., 1996). Some of these antigen-experienced lamina propria T cells may be true effector cells, and may help local B cells to produce IgA. The antigen-specific memory $CD4^+$ and $CD8^+$ T cells accumulate preferentially in non-lymphoid tissues, in particular in the intestinal mucosa (Sallusto et al., 1999; Reinhardt et al., 2001). The effector T cells that react to the microbial

flora or other GI antigens are kept in check by regulatory T cells (T_{Reg}) (Maloy et al., 2003; MacDonald et al., 2011). Th17 cells are also important in mucosal immune responses. Th17 cells secrete IL-17 and also IL-22, and play an important role in protecting against bacterial and fungal infections (Sonnenberg et al., 2009). In mice, IL-22 appears to have anti-inflammatory effects, since it targets epithelial cells and induces secretion of defensins and mucus (Sugimoto et al., 2008). The Th17 and Treg are both dependent on TGF-β for their differentation, but are also defined by the expression of the lineage-specific transcription factors RORγt and Foxp3, respectively (Hori et al., 2003; Mangan et al., 2006; Manel et al., 2008). The composition of the intestinal microbiota influences the presence T cell population in the gut. As an example, in the colon, but not in the small intestine, the function of $Foxp3^+$ T_{Reg} has been shown to be regulated by commensal bacteria (Clostridia clusters IV and XIVa) (Atarashi et al., 2011; Farache et al., 2013). In specific pathogen-free mice, Clostridia colonise preferentially the colon, occupying the mucus layer in close proximity to IECs. In humans, these bacteria are associated with IL-10 induction and protection from colitis (Sokol et al., 2008). In contrast, Th17 cells in the small intestine lamina propria, accumulate in the presence of specific commensal microbiota, namely segmented filamentous bacteria (SFB) (Atarashi et al., 2011). Most data on T_{Reg} cells and gut homeostasis has been collected from studies of cells that express FoxP3 (MacDonald et al., 2011). In mice, two types of Foxp3 expressing T cells have been identified. The first, so-called naturally occurring T_{Reg} cells were recognised as a subpopulation of $CD4^+$ T cells that develop in the thymus during the first days of postnatal life and express high levels of the IL-2 receptor α chain (Asano et al., 1996). The other group of regulatory T cells is represented by inducible T_{Reg} (iT_{Reg}) cells which also express FoxP3, but develop

from naïve $CD4^+$ T cells in the presence of TGF-β1 (Fantini et al., 2006) and retinoic acid (Coombes et al., 2007). The T_{Reg} are present throughout the GI tract, but especially are enriched in the colon, where they provide immune suppression and downregulate excessive inflammatory responses. In the colon, T_{Reg} can account for 40-50% of $CD4^+$ T cells (Atarashi et al., 2011).

A role of T lymphocytes in intestinal homeostasis was also discussed in section 1.3.5.

1.1.8. Mucosal B cells

The presence of a large quantitiy of IgA with a diverse antigen-binding repertoire is crucial for the maintenance of intestinal homeostasis (Brandtzaeg et al., 1999; Wei et al., 2011). The lamina propria contains large numbers of plasma cells located beneath the intestinal epithelium, which secrete dimeric IgA (mostly IgA2) and pentameric IgM (Johansen et al., 2000). The vast majority of lamina propria B cells are $CD19^+CD20^-$, which are the extravasated immunoblasts enroute to terminal differentation into plasma cells (Spencer et al., 2012).

The functional significance of sIgA is highlighted by a fact that 80-90% of plasma cells produce IgA (Brandtzaeg et al., 1999b). T cells activated by microbial and other antigens in GALT, supported by cytokines such as transforming growth factor (TGF)-β and IL-10, induce the development of antigen-specific B cells to predominantly IgA-committed plasma blasts (Helgeland et al., 2000; Brandtzaeg and Finn-Erik Johansen, 2005), which proliferate and differentiate further on their route through mesenteric lymph nodes and thoracic duct into the blood stream. The IgA-committed plasma blasts home preferentially to the intestinal mucosa, where they

complete their terminal differentiation to IgA-producing plasma cells, probably under continued influence of T cells and their cytokines in the lamina propria, and immunoregulatory factors from intraepithelial T cells (Macpherson et al., 2008; Spencer et al., 2009). The plasma cells destined for the colonic lamina propria express CCR10 and migrate towards CCL28, a chemokine secreted by colonic epithelial cells (Kunkel et al., 2000). However, plasma cells that are destined for the small intestine migrate via CCR9/CCL25 axis (Morteau et al., 2008).

Consequently, upon arrival to the lamina propria, IgA is carried through IECs into lumen as secretory IgA (SIgA), where it facilitates the clearance of bacteria in the lumen (Brandtzaeg, 2003). IgA production takes through T cell-dependent (Gardby et al., 2003) and T cell-independent pathways (Bergqvist et al., 2006; He et al., 2007; Cerutti and Rescigno, 2008). The T cell dependent class switch to IgA involves ligation of CD40 present on B cells and CD40L expressed by T cells and binding of TGF-β to its receptor on B cells (McIntyre at al., 1995; Zan et al., 1998; Spencer et al., 2012). The importance of T cell-independent pathway has been supported by the fact that patients with hyper-IgM syndrome, with a mutation in CD40 can still generate intestinal IgA responses despite the inability to recruit cognate T cell help via CD40/CD40L (Spencer et al., 2012).

Also DCs, through the production of APRIL may support class switch to IgA in the absence of T cell derived CD40 ligation (Cerutti et al., 2008). It has been also shown that murine small intestine lamina propria $CD103^{+}CD11b^{+}$ DCs can induce the differentation of naïve B cells into IgA^{+} lamina plasma cells in vitro after CpG (ODN) stimulation through T cell-independent pathway (Fujimoto et al., 2011). The

ability of $CD103^{+}CD11b^{+}$ DCs to induce IgA class switcg may be related to secretion of RA by these cells (Fujimoto et al., 2011).

Additionally, T cell-independent pathway of IgA production involves B-cell activating cytokines produced by IECs in response to commensal bacterial signals (Yanagibashi et al., 2013). However, not all commensal bacteria induce IgA production, and also the anatomical location influences the secretion of IgA (Yanagibashi et al., 2013). For example, introduction of *Bacteroides acidifaciens* in the colon, but not in the ileum of germ-free mice can induce IgA production (Yanagibashi et al., 2013). Based on mouse models, it has been suggested that T cell-dependent IgA responses are initiated in PPs, whereas T cell-independent IgA induction mostly occurs in ILFs (Tsuji et al., 2008; Suzuki and Fagarasan, 2009).

1.1.9. Mononuclear Phagocyte System

Macrophages (Mϕ) together with dendritic cells (DCs) and monocytes make up the mononuclear phagocyte system. Although deriving from a common bone marrow progenitor, Mϕ and DCs follow separate developmental pathways deriving from distinct precursors (Liu et al., 2009). However, the use of overlapping markers has created much confusion in distinguishing between Mϕ and DCs. It has also been suggested it may be better to classify mononuclear phagocytes based on function (Mosser and Edwards, 2009). For Mϕ, three functional subsets have been proposed, namely host defence, wound healing and immune regulatory, suggesting that by classifying Mϕ accordingly to function rather than phenotype, it is possible to capture traits shared by more than one population (Mosser and Edwards, 2009).

1.1.9.1. Macrophage Ontogeny and Development

1.1.9.1.1. Ontogeny of mouse intestinal macrophages

In adult mice, progenitors of Mϕ and DCs arise from haematopoietic stem cells (HSCs) through the series of intermediate stages in the bone marrow (Figure 1.5). Each stage is accompanied by a stepwise lineage commitment (Liu et al., 2009). The first stage gives rise to the common myeloid progenitor (CMP) and the common lymphoid progenitor (CLP) (Figure 1.5). The common myeloid progenitors have the potential to generate all myeloid cells, including Mϕ, DCs, granulocytes, neutrophils and eosinophils (Akashi et al., 2000). The CMPs differentiate into Mϕ/DC progenitors (MDPs) or granulocyte/Mϕ progenitors (GMPs). The GMPs maintain the potential to generate DCs, but preferentially differentiate into granulocytes and monocytes/Mϕs (Auffray et al., 2009). The MDPs have a potential to generate DCs and monocytes/Mϕs only (Fogg et al., 2006). These cells express CSF1R (CD115) and two subsets can be identified on the basis of Ly6C expression (Geissman et al., 2003).The MDPs generate either $CD11b^{+}$ $Ly6C^{hi}$ monocytes or DC committed progenitors (CDPs) or preDCs (Geissmann et al., 2010). The $Ly6^{hi}$ monocytes are short-lived and can mature to $Ly6C^{lo}$ monocytes, which circulate in the blood or give rise to tissue resident Mϕs (Bain et al., 2013).

The $Ly6C^{+}$ monocytes are continuously released into the circulation. They express the chemokine receptor CCR2 and emigrate out of the bone marrow in response to monocyte chemotactic protein-1 (MCP-1), the CCR2 ligand (CCL2) (Serbina and Pamer, 2006) produced by endothelial cells, fibroblasts, monocytes and Mϕ. Subsequently, circulating $Ly6C^{+}CCR2^{+}$ monocytes migrate into the mucosa in CCR2-dependent manner (Zigmond et al., 2012).

The additional recruitment of $Ly6C^+$ monocytes into lamina propria may be also induced by CCL1, which is produced by activated T cells, in the healthy mucosa (Zigmond and Jung, 2013). Circulating $Ly6C^+$ monocytes give rise to several $CX3CR1^+$ ($CD103^-$) mucosal cell populations in the presence of local macrophage colony stimulating factor (M-CSF) (Varol et al., 2007; Bogunovic et al., 2009). The absence of CD103, a DC-specific marker, and limited ability of these cells to migrate to the mesenteric lymph nodes (MLNs) (Shulz et al., 2009) and present antigen to naïve T cells, indicates that $CX3CR1^+$ ($CD103^-$) are not DCs, as previously assumed (Bain and Mowat, 2014). Constitutive expression of CX3CL1 (fractalkine), the CX3CR1 ligand, by gut epithelium plays a central role in the recruitment of $CX3CR1^+$ Mϕ to the subepithelial space and the formation of transendothelial processes by which the cells sample gut antigen (Niess et al., 2005; Vallon-Eberhard et al., 2006). The profound reduction of lamina propria Mϕ in CX3CR1 knock out mice emphasises the importance of the constitutive recruitment of $Ly6C^+CX3CR1^+$ macrophages to the mucosa (Medina-Contreras et al., 2011).

Despite the fact that terminally differentiated cells typically show diminished proliferative potential, there is evidence suggesting that tissue macrophages can divide in the peritoneum and tissues in response to IL-4 and in Th2 type inflammation, however it is not known if this happens in the gut (Chorro et al., 2009, Jenkins et al. 2011).

The $Ly6C^+$ monocytes give rise to both anti-inflammatory and inflammatory Mϕ. As shown by Rivollier et al. (2012), in the steady-state, mouse $Ly6C^{hi}$ monocytes are precursors of $CX3CR1^{hi}$ ($CD11c^+$) Mϕ, which are poor at antigen presentation, and constitutively release the anti-inflammatory cytokine IL-10 (Denning et al., 2007). $CX3CR1^{hi}$ cells display long-term persistence in intestinal tissue, do not migrate to

MLNs, either in the steady-state or during inflammation (Shulz et al., 2009). During inflammation Ly6C^{hi} monocytes differentiate into CX3CR1^{int} CD103^{-} cells that secrete proinflammatory cytokines, including IL-12 and IL-23, and over time these cells differentiate into CX3CR1^{int}Ly6C^{lo} with DCs features (Zigmond et al., 2012; Rivollier et al., 2012). The expression of CCR7 on CX3CR1^{int}Ly6C^{lo} cells, but not on CX3CR1^{hi} Mϕ enables these cells to migrate to MLNs and prime naïve T cells (Zigmond et al., 2012).

Recently the concept that tissue Mϕ derive from circulating monocytes that originate in the bone marrow has been re-evaluated (Wynn et al., 2013). Recent findings suggest that the origin of Mϕ in the steady state can vary between different tissues (Ginhoux et al., 2014), In some tissues such as the liver and the brain, Mϕ arise from embryonic precursors and are independent of blood monocytes (Shulz et al., 2012; Hashimoto et al., 2013, Yona et al., 2013). The fate-mapping studies in mice indicate that yolk sac precursors are the source of Mϕ in the liver and the brain (Ginhoux et al., 2010), where cells differentiate into long-lived Kuffer cells and microglia cells, respectively, with minimal replenishment from circulating monocytes. Other cells, such as Langerhans cells originate from both the yolk sac and the fetal liver (Guilliams et al., 2013). However, intestinal mucosa Mϕ are exception. Although seeded early in life by embryonic precursors colonic Mϕ become completely depended on constant replenishment by classical Ly6C^{hi} monocytes (Bain and Mowat, 2014). The *Csf1r*- and *Flt3*-driven fate mapping confirmed the presence of yolk sac-derived Mϕ in the intestine of neonatal mice, but they are completely absent from the mucosa of adult mice (Bain and Mowat et al., 2014).

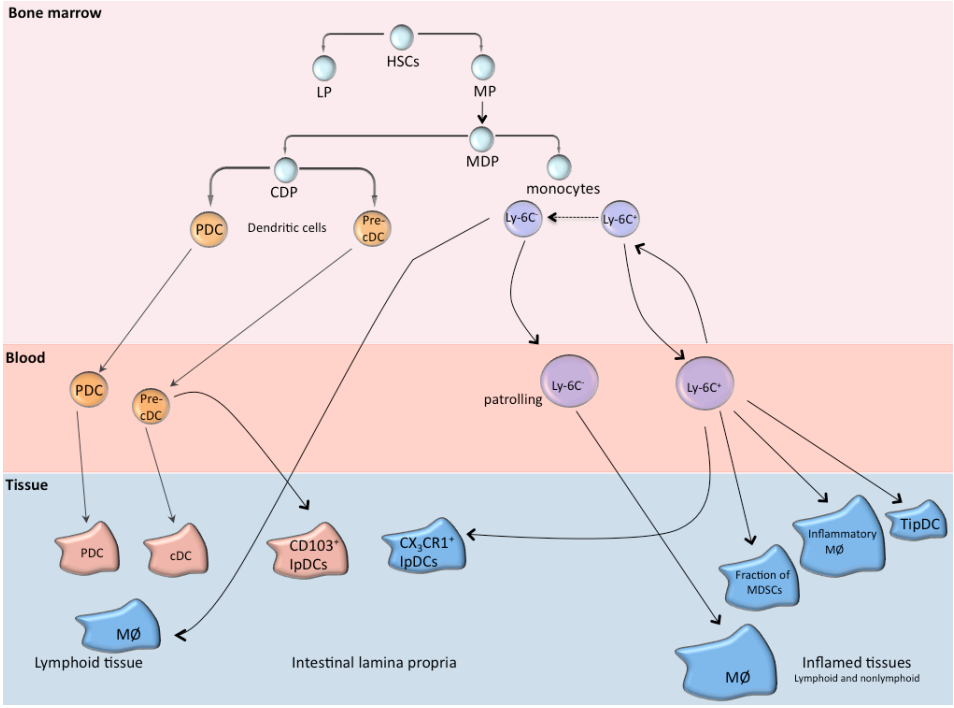


Figure 1.3: Ontogeny of macrophages and dendritic cells (Adapted from Geissmann *et al.*, 2010)

Hematopoietic stem cells (HSCs) give rise to lymphoid (LP) and myeloid (MP) committed precursors. MPs can differentiate into monocytes, macrophage and DC precursors (MDPs). MDPs are proliferating cells of phenotypic characteristics of myeloid precursors. MDPs give rise to many macrophages and DC subsets, but not granulocytes. From MDPs, two subsets of monocytes can be generated, Ly6C^{-} and Ly6C^{+}. After leaving the bone marrow, these cells can enter blood stream. CDPs can also give rise to pre-classical DCs (pre-cDCs) and plasmocytoid DCs (PDCs). Pre-cDCs after circulating in blood can enter lymphoid tissue and differentiate to CD8α^{-} and CD8α^{+} cDCs. Pre-cDCs can also travel to non-lymphoid tissue and there differentiate into CD103^{+} lamina propria DCs (lpDCs). Under homeostatic conditions, Ly-6C^{+} monocytes can become CX3CR1^{+} lpDCs in nonlymphoid tissues. During inflammation, Ly-6C^{+} monocytes give rise to monocyte-derived DCs, for example, tumor necrosis factor (TNF) and inducible nitric oxide synthase (iNOS)–producing dendritic cells (TipDCs), inflammatory macrophages, and may contribute to myeloid-derived suppressor cells (MDSCs) associated with tumors. HSCs can also leave their bone marrow niche (dashed arrow) and enter peripheral tissues, where they differentiate to myeloid cells during inflammation. It is unclear at this time whether LPs contribute substantially to PDCs and cDCs

1.1.9.1.2. Ontogeny of human intestinal macrophages

In humans, circulating $CD14^+$ blood monocytes (equivalent of murine $Ly6C^{hi}$ monocytes) are also the likely source of human gut Mϕ (Ziegler-Heitbrock et al., 2010). In the steady state, the chemokines transforming growth factor-β (TGF-β) and IL-8 are constitutively produced by gut epithelial cells and mast cells and then bind to and are released from the lamina propria extracellular matrix (stroma), allowing both bound and released forms of chemokines to recruit circulating blood $TGF\text{-}\beta R^+$ and $IL\text{-}8R^+$ monocytes (Smythies et al., 2005; 2006). These factors induce phenotypic and functional differentiation of newly recruited monocytes into non-inflammatory intestinal Mϕ (Smith, 2005).

The fact that intestinal Mϕ do not proliferate (Smythies et al., 2006) highlights the importance of the recruited blood monocytes to replace senescent and apoptotic lamina propria macrophages. After recruitment, monocytes take up residency in the lamina propria stroma, where they survive for weeks or months (Friedmann et al., 2007; Dale et al., 2008). The unique microenvironment of the lamina propria plays a fundamental role in the differentation of newly recruited $CD14^+$ blood monocytes into $CD14^-$ Mϕ (Smith et al., 2001; Smythies 2005; 2006). In humans, $CD14^+$ blood monocytes enter the intestinal lamina propria using CCR2, and lose CD14 as a consequence of differentiation into gut resident macrophages (Platt et al., 2010; Bain et al., 2013).

In mucosal inflammation, the potent chemotactic ligands MCP-1/CCL2 and macrophage inflammatory protein-1β/chemokine (C-C motif) ligand 4 (MIP-1β/CCL4) are up regulated in the lamina propria (Smith et al., 2011). These molecules are produced mainly by activated monocytes and macrophages, but also

by endothelial and epithelial cells (Yoshimura et al., 1989), and contribute to the accelerated recruitment of blood monocytes to the mucosa.

In humans, blood monocytes can also be separate in two groups: classical monocytes ($CD14^{hi}CD16^{-}$) and non-classical ($CD14^{+}CD16^{+}$) (Auffray et al., 2007). The $CD14^{hi}CD16^{-}$ monocytes share a phenotype similar to mouse inflammatory monocytes (Table 1.1) and $CD14^{+}CD16^{+}$ resembling mouse resident monocytes (Geissman et al., 2003).

Subset	Markers	Chemokine receptors	Function
Mouse			
$LY6C^{hi}$	$CD11b^{+}CD115^{+}LY6C^{hi}$	$CCR2^{hi}CXCR1^{low}$	Proinflammatory, antimicrobial role
$LY6C^{low}$	$CD11b^{+}CD115^{+}LY6C^{low}$	$CXCR1^{hi}CCR2^{low}$	Patrolling, early responses, tissue repair
Human			
Classical	$CD14^{++}CD16^{-}$	$CCR2^{hi}CXCR1^{low}$	Resemble $LY6C^{hi}$ monocytes
Intermediate	$CD14^{++}CD16^{+}$	$CXCR1^{hi}CCR2^{low}$	Proinflammatory roles
Non-classical	$CD14^{+}CD16^{++}$	$CXCR1^{hi}CCR2^{low}$	Patrolling, antiviral roles

Table 1.1: Mouse and human monocyte subsets (Adapted from Shi and Pamer, 2011)

In humans, the majority of blood monocytes are $CD14^{hi}CD16^{-}$, whereas in mice, both are represented equally (Shi and Pamer, 2011).

1.1.9.1.3. Phenotype of mouse intestinal macrophages

Mouse resident intestinal macrophages were initially defined by the expression of the macrophage-specific F4/80 antigen (Hume 1983; Pavli et al., 1990).

Mouse resident intestinal macrophages are most accurately defined as $CD45^{+}$ $MHCII^{+}F4/80^{+}CD11b^{+}CD11c^{+/-}CD103^{+}CX3CR1^{+}$ and mouse intestinal DCs as $CD45^{+}MHCII^{+}F4/80^{-}CD11b^{+/-}CD11c^{+}CD103^{+}CX3CR1^{-}$ (Denning et al., 2011). Mouse intestinal macrophages have been further separated into these that express high levels of CD11c and are present in the lamina propria and these that express low

or intermediate levels of CD11c and probably are serosal macrophages (Bogunovic et al., 2009).

Two additional surface antigens used to identify mouse intestinal macrophages are CD64 and Siglec F. The CD64 is a high-affinity IgG receptor (FcγRI), constitutively expressed by $CX3CR1^{+}$ intestinal macrophages in mice and has been used to further distinguish intestinal macrophages from DCs (Gautier et al., 2012). Due to overlap in expression of CD11b and F4/80 by mouse intestinal macrophages and eosinophils (Gautier et al., 2012), additional markers are used to distinguish these cells. These markers include MHCII and Siglec F, a sialic acid-binding lectin.

1.1.9.1.4. Phenotype of human intestinal macrophages

Human intestinal macrophages, similarly to mouse, express CD45 and MHCII (HLA-DR). However, few, if any, of other cells surface markers present on mouse mucosal macrophages can be extrapolated to human (Bain and Mowat, 2014). The surface antigen F4/80 defines intestinal macrophages in mice but is not expressed by human lamina propria macrophages (Bain and Mowat, 2014). Also, CX3CR1, which is expressed by mouse macrophages is absent on humans (Bain and Mowat, 2014).

In humans, the markers HLA-DR, CD13 and CD33 can be used to identify human intestinal macrophages (Smith et al., 1997; Smith et al., 2001; Smythies et al., 2006; 2006; Kamada et al., 2008, Mowat and Bain, 2011). Human gut macrophages do not express the dendritic cell markers CD21, CD34, CD83, CD123 (Smith et al., 2001). Additionally, CD68, CD1 can be used to identify intestinal macrophages in situ (Smith et al., 2001; Weber et al., 2009). Importantly, isolated human intestinal macrophages do not express CD11a, CD11b, CD11c, CD14, CD18, CD89, FcγRI-III

(CD64, CD32, CD16), whereas these receptors are strongly expressed on human blood monocytes (Smith et al., 2001; 2011; Smythies et al., 2005).

1.1.9.2. Function of intestinal macrophages

The intestine contains the largest reservoir of macrophages in the body (Lee et al., 1984; Smith et al., 2011) composed of terminally differentiated, non-migratory phagocytic cells (Mosser and Edwards, 2008). Intestinal macrophages play an active role in maintaining the integrity of the epithelial barrier through the production of prostaglandin E2 (PGE2), which promote the proliferation and survival of epithelial progenitors in intestinal crypts (Pull et al., 2005).

Strategically positioned in the subepithelial lamina propria, these cells are the first phagocytic cells of the innate immune system to interact with microorganisms and microbial products that have breached the epithelium (Smith et al., 2011). Morphological analysis of intestinal macrophages showed that intestinal macrophages have pseudopod projections from the cell membrane; curved in-like nucleus, phagocytic vacuoles and secondary lysosomes (Smith et al., 2001).

Under homeostatic conditions, mucosal macrophages are involved in recognition and the clearance of antigen, but they do so without triggering strong proinflammatory responses. Unlike macrophages from other tissues, resident mucosal macrophages in the normal colon are unable to mediate strong proinflammatory responses, and they do not respond to stimuli like TLR ligands by producing increased proinflammatory cytokines or chemokines such as TNF-α, IL-1, IL-6, IL-12, IL-23 and CXCL10 (IP-10) (Mowat et al., 2011).

Intestinal macrophages also lack a number of cell surface costimulatory molecules such as CD40, CD80 and CD86 (Rogler et al., 1998; Carlsen et al., 2006; Smith et al., 2011) and lack the Fc receptors for immunoglobulin G (CD16, CD32, CD64) (Smith et al., 2001; 2011) and the complement receptors CD3 (CD11b/CD18) and CR4 (CD11c/CD18) (Sheikh and Plevy, 2010).

Another specific characteristic of human intestinal macrophages is lack of responses through TLR stimulation, which is due to receptor downregulation (e.g., TLR2) or due to impaired receptor down stream signalling. This aspect of inflammatory anergy will be discussed in section 1.2. In contrast, TLR3 and TLR5-9 are retained by the macrophages, or are expressed at even higher levels than in blood monocytes (Smythies et al., 2010; Smith et al., 2011).

Unresponsiveness to LPS activation is not due to TLR downregulation, since TLR4 mRNA and protein can be detected in resident mucosal macrophages (Smith et al., 2001). However, in the normal gut, intestinal macrophages lack CD14, a high affinity receptor for complexes of LPS and LPS-binding protein (Smith et al., 1997) or they do express CD14 but at very low level (Rogler et al., 1998; Smythies et al., 2010). They also lack CD89, a transmembrane glycoprotein receptor for monomeric and polymeric IgA1 and IgA2 (FcαR) (Morton et al., 1996; Smith et al., 2001). The absence of CD89 on lamina propria macrophages, downregulates IgA-mediated phagocytosis which normally induce the release of proinflammatory mediators including reactive oxygen intermediates (Gorter et al., 1987; Smith et al., 2001).

The inflammatory responses to microbial products are also amplified by a pathway mediated by triggering receptor expressed on myeloid cell (TREM)-1 (Bouchon et al., 2001; Colonna et al., 2003). Normally, TREM-1 is an activating receptor highly expressed on neutrophils and monocytes that infiltrate human tissue (Bouchon et al.,

2001) and also on $CD11b^+$ myeloid cells (Cheng et al., 2011). However, in the normal intestine, as oppose to their monocyte precursors, human macrophages down-regulate TREM-1 (Schenk et al., 2005). Although the ligand is unknown, it has been shown that TREM-1 activates DAP12-signalling pathways, which amplify inflammatory responses (Lanier et al, 1998; Bouchon et al., 2000). Using murine models of sepsis, it was shown that the blockage of TREM-1 protects mice against LPS-activated shock and microbial sepsis caused by E.coli (Bouchon et al., 2001).

It is thought that the absence of these receptors and lack of proinflammatory mediator production contributes to intestinal immune homeostasis by preventing excessive responses to PAMPS of the microbial flora (Zhang and Mosser, 2008).

Normally, a downstream signalling of TLRs stimulation activates the master transcription factor NF-κB, which is activated through phosphorylation and degradation of IκBα, therefore translocating NF-κBp65 subunit from the cytosol to the nucleus, where it activates the proinflammatory responses. However, in the steady state in intestinal macrophages, NF-κB remains in the cytosol, due to reduced ability to phosphorylate IκBα (Smythies et al., 2010). In contrast to macrophages in other tissue, human intestinal macrophages also downregulate the expression of adapter protein MyD88 and TRIF, which together mediate TLR MyD88-dependent and –independent NF-κB signalling, do not phosphorylate NF-κBp65 or Smad-induced IκBα, and do not translocate NF-κB into nucleus (Smythies et al., 2010).

Intestinal macrophages are highly phagocytic, and express the scavenger receptor CD36, which facilitates uptake of apoptotic cells (Smythies et al., 2005). Phagocytosis occurs independently of immune cell signalling, thus it results in little or no inflammatory response (Smythies et al., 2011).

Human gut macrophages are also highly bacteriocidal, suggesting that these cells are programmed to kill bacteria without a proinflammatory response (Smith et al., 2011).

Table 1.2: represents the major phenotypic differences between intestinal macrophages intestine and their precursors, blood monocytes.

Cell population	HLA-DR CD13 CD36	TGFβRI/II	CD14 CD25	CD89 CD16	CD80 CD86 CD40	C5aR FMLPR	TREM-1	CCR5; CXCR4
Monocytes	+	+	+	+	+	+	+	+
Intestinal Mo	+	+	-	-	-	-	-	-
Cell population	**Phagocytosis**	**Killing**	**Bacterial activity**		**Co-stimulation**	**Proinflammatory cytokines**		
Monocytes	+	+	+		+	+		
Intestinal Mo	+	+	+		-	-		

Table 1.2: Phenotypic and functional differences between intestinal macrophages and blood monocytes (Adapted from Schenk et al., 2007)

High expression of MHCII on intestinal Mϕ, suggests that these cells may also serve as antigen-presenting cells, however, as intestinal Mϕ do not migrate to the MLNs (Schulz et al., 2009), and since the intestinal mucosa is free from naïve $CD4^+$ T cells (MacDonald et al., 1998), it is unlikely that they are involved in the initial priming of naïve T cells (Bain and Mowat, 2014). However, intestinal Mϕ could be involved in establishment of lymphocytes that have returned to the mucosa after being first primed in the GALT (Bain and Mowat, 2014). Hadis and colleagues (2011) showed that intestinal Mϕ facilitate the secondary expansion and maintenance of antigen-specific $FoxP3^+$ Treg through IL-10 production. As shown in mice models of colitis (Murai et al., 2009), deletion of the IL-10R drives the loss of FoxP3 expression. The finding that intestinal Mϕ may contribute to the generation of homeostatic Treg is

also supported by a fact that mice lacking TRAF6 expression in $CD11c^+$ mononuclear phagocytes develop a spontaneous Th2 cell-mediated enteritis (Bain and Mowat, 2014). Intestinal Mϕ may also maintain other T cell populations in the mucosa (Bain and Mowat, 2014), through the production of IL1β in response to TLR stimulation, which may be relatively preserved in intestinal Mϕ and therefore supports the development of Th17 cells in the steady state (Shaw et al., 2012). Recent studies also proposed that mucosal Mϕ indirectly contribute to T-cell priming through connexin-43-mediated transfer of soluble antigen obtained from the lumen to neighbouring $CD103^+$ DCs (Mazzini et al., 2014). It still remains unknown how Mϕ in the lamina propria acquire luminal antigen. It may happen through extension of transepithelial dendrites. Originally, this phenomenon was assigned to DCs on the basis of MHCII and CD11c expression (Niess et al., 2005; Chieppa et al., 2006). However, it is now known that transepithelial dendrites originate from $CX3CR1^+$ Mϕ. The transepithelial dendrites formation requires CX3CR1 expression, since it has been shown that induction of oral tolerance to protein antigen is impaired in CX3CR1 deficient mice (Hadis et al., 2011; Mazzini et al., 2014).

	Human		Mouse	
	Intestinal Macrophages	Monocytes	Intestinal Macrophages	Monocytes
Markers				
CD13	+++	+++	**ND**	**ND**
HLA-DR	+++	+++	+++	-
CD11c	-	++	++	-
CD11b	-	+++	+++	++
CD14	-	+++	+	++
CD16	-	+	-/+	-/+
CD32	-	++	-/+	-/+
CD64	+	++	++	-/+
CXCR1	-	-/+	+++	+
TREM-1	-	-	-	-/+
F4/80	-	-	+++	++
Ly6C	-	-	-	-/+
TLR1	-/+	-/+	+	+
TLR2	-	-/+	+	+
TLR4	-/+	+	+	+
TLR3, 5-9	+++	++	+	+

Table 1.3: Phenotype of normal human and mouse intestinal macrophages and blood monocytes

"-" – Absent; "+/-" – variable but low; "+" – positive but low; "++"– moderate; "+++"– high. Data obtained from Smythies et al., 2005; 2006; 2010; Smith et al., 2001; De Calisto et al., 2012; Mowat and Bain, 2011; Bain and Mowat, 2011; Varol et al., 2009; Weber et al., 2009; Geissmann et al., 2003; Ancuta et al., 2003; Sunderkotter et al., 2004, Denning et al., 2007.

1.1.9.3. Function of Dendritic cells (DCs)

Just like Mϕ, DCs greatly contribute to homeostatic immune responses in the intestine. Depending on signals received from the local environment, DCs function is to ensure that pathological immune responses to harmless antigens do not develop. However, at the same time, DCs are involved in the initation of active immune responses in the steady state (Coombes and Powrie, 2008). Macrophages and DCs share many functional features (Bain and Mowat, 2014). However, there are certain functional aspects unique to DCs.

The DCs are important players in initiating and polarising intestinal adaptive immune responses (Farache et al., 2013). These cells are found deep inside the lamina propria, in association with villous epithelium, close to the lumen, or in the GALT (Kelsall, 2008; Rescigno and Di Sabatino, 2009).

In mice, intestinal DCs are lineage negative and express the integrins CD11c (α_X) and CD103 (α_E), and have further been subdivided accordingly to expression of CDC11b (α_M) (Jang et al., 2006; Schulz et al., 2009; Varol et al., 2009). In the colonic mucosa and the GALT, $CD103^+CD11b^-$ DCs make up the majority of DCs (Denning et al., 2011; Cerovic et al., 2014). In the small intestine, a population of DCs expressing $CD103^+$ and $CD11b^+$ are present in the cores of the villi, deep in the lamina propria (Shultz et al., 2009; Farache et al., 2013). Other studies have also reported the presence of these cells in the intestinal epithelium (Edele et al., 2008). The epithelial location could explain why these cells express CD103, an αE integrin which functions as a receptor for epithelial E-cadherin, and is upregulated by TGF-β (Edele et al., 2008). Farache and colleagues (2013) have shown in mice that at steady state, a small population of $CD103^+$ DCs migrate between the lamina propria and the intraepithelial compartment of the small intestine. Additionally, they showed that after entering the epithelium, $CD103^+CD11b^+$ DCs migrate above the basement membrane and extend thick finger-like projections (dendritis), which can capture luminal *Salmonella*. However, not all mucosal DCs express CD103 and bone fide $CD103^-$ DCs subset has recently been identified (Cerovic et al., 2013; Scott et al., 2015). The $CD103^-$ DCs constitutively migrate to MLN, and are highly effcient at presenting antigen to naïve T cells and induce CCR9 expression on dividing T cells (Cerovic et al., 2013; Scott et al., 2015). Additionally, $CD103^-$ DCs were shown to express CCR2, constitutively express IL-12/Il-23p40 and are involved in priming

mucosal Th17 responses (Scott et al., 2015). After taking up antigens, DCs migrate to draining MLNs through CCR7/CCL21 interaction (Jang et al., 2006), to present antigen from the intestinal lumen on their surface via major histocompatibility complex molecules (MHC) to naïve T lymphocytes, hence driving their proliferation and polarisation into antigen specific effector or regulatory T cell (Bogunovic et al., 2009; Schulz et al., 2009).

The homeostatic state that limits proinflammatory potential of DCs is maintained by signals from the epithelium and stroma such as thymic stromal lymphopoietin (TSLP) and prostaglandin E2 (PGE2) (Hammerschmidt et al., 2008; Stock et al., 2011). A particular property of DCs is their ability to induce $Foxp3^+$ T_{Reg} cells, through production of TGF-β and retinoic acid (RA) (Coombes et al., 2007). Spadoni and colleagues (2012) have shown that intestinal $CD103^+$ DCs can also produce TSLP, which limits Th17 responses and promotes T_{Reg} cells differentiation. Moreover, intestinal $CD103^+$ DCs shape the homing potential of recently activated T and B cells by induction of molecules CCR9 and α4β7 integrin in a process also dependent on retinoic acid (RA) signalling (Stagg et al., 2002; Johansson-Lindbom et al., 2005; Hall et al., 2011). For example, T cells primed with antigen derived from MLNs or PP DCs, but not with DCs from the spleen or peritheral lymph nodes, express CCR9, the receptor for CCL25, a chemokine constitutively produced by epithelial cells of the small intestine (Johanssen-Lindbom et al., 2005). Additionally, these T cells also express high level of α4β7, the integrin that mediates localisation to the GI tract (DeNucci et al., 2010) and interacts with MAdCAM-1 (discussed more in section 1.1.5).

Many aspects of homeostatic potential of intestinal DCs, mentioned above is driven by the ability of these cells to produce retinoic acid (RA) via enhanced expression of retinal dehydrogenase aldh1a2 (Denning et al., 2007; Coombes et al., 2007; Sun et al., 2007; Hammerschmidt et al., 2011). The beneficial role of retinoic acid is supported by studies, where vitamin A deficiency shifts the balance between Th1 and Th2 responses in favour of Th1 (Stephenesen, 2001). Additionally, RA enhances the TGF-β-driven generation of $Foxp3^+$ T_{reg} (Denning et al., 2007; Sun et al., 2007; Mucida et al., 2007), as was demonstarted by retinoic acid receptor (RAR) antagonist studies (Sun et al., 2007; Mucida et al., 2007). It has also been shown that RA leads to a reduction in IFN-γ production by T cells (Annacker et al., 2005), hence may be implemented in establishing the balance between IFN-γ and TGF-β.

Although, $CD103^+$ DCs are tolerogenic under intestinal homeostatic conditions, they may switch to become potent activators of T cells in inflammatory setting. These cells also express higher levels of co-stimulatory molecules (e.g CD70, CD80, and CD86), produce higher amounts of tumor necrosis factor (TNF)–α, indicating their role in activation of effector T cells (Coombes et al., 2008; Rescigno and Sabatino, 2009). The DCs are involved in T-helper (Th)1 and Th17 T cell induction via IL-12/IL-23 production. $CD103^+$ DCs with proinflammatory potential have been shown to accumulate in MLNs in experimental colitis (Laffont et al., 2010) and rather than inducing $Foxp3^+$ T_{Reg} cells differentiation, they promote Th1 responses.

1.2. Endotoxin tolerance

Activation of the TLR pathway in monocytes/macrophages drives strong inflammatory responses, hence the process needs to be tightly regulated, as uncontrolled inflammation will cause tissue damage. One mechanism for host

protection against uncontrolled immune responses is the phenomenon of endotoxin tolerance (Biswas et al, 2007). The establishment of endotoxin tolerance has been observed in vitro as well in vivo in animal models and in humans (Medvedev et al., 2000; Dobrovolskaia et al., 2003; del Fresco et al., 2009).

Endotoxin tolerance is a state whereby pre-exposure to a low concentration of LPS induces a state of hyporesponsiveness to subsequent LPS stimulation (Biswas et al., 2009). The establishment of the tolerance is a result of a complex interplay at the level of TLR signal transduction and also involves changes in transcriptional targets of two distinct pathways involving Myd88 and Trif adapter proteins (Akida and Takeda, 2004).

1.2.1. TLR4 signalling pathway in recognition of microbial components

Toll- like receptors (TLRs) play a central role in the recognition of and response to microbial pathogens in the hosts (Akira and Takeda, 2004). Ten functional TLRs have been identified in humans and twelve in mice, with TLR1-9 being conserved in both species (Figure 1.8) (Shinya et al., 2012).

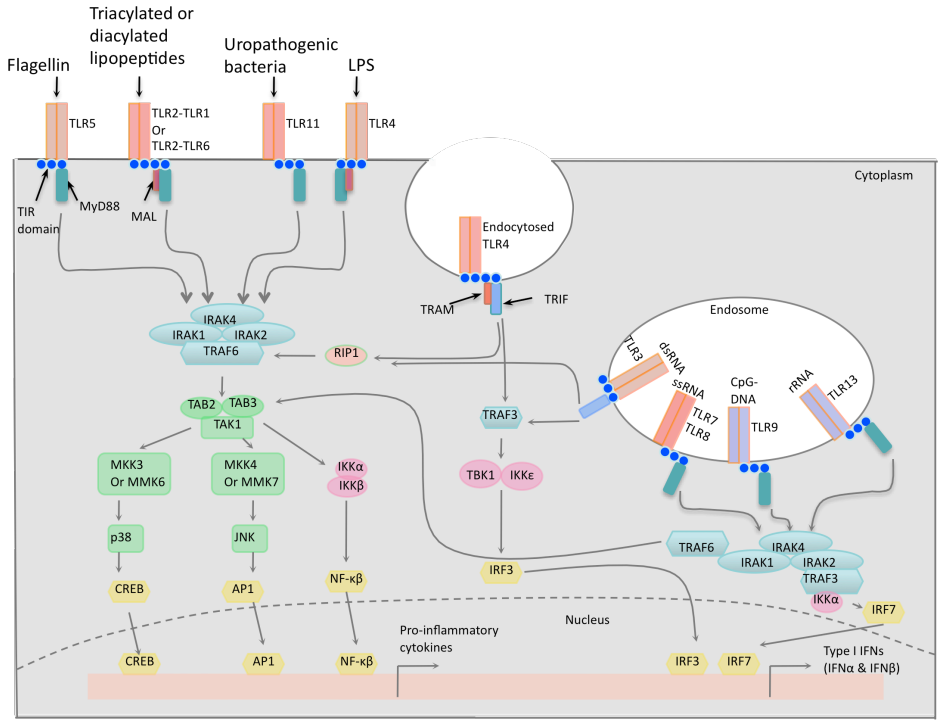


Figure 1.4: Schematic diagram of TLR4 signalling and ligand specificities of TLRs (Adapted from Silverman and Fitzgerald, 2004)**.**

Toll- like receptor 4 (TLR4) is the major PRR involved in the detection of Gram-negative bacteria by recognising LPS (Biswas et al., 2009). LPS recognition by TLR4 requires the adaptor proteins MD-2 and CD14. LPS first binds to serum LPS-binding protein, and the complex is next recognised by CD14/TLR4. Activation of the downstream signalling pathway of TLR4 results in production of cytokines/chemokines and type I interferon through two independent signalling pathways: MyD88-dependent and MyD88-independent, respectively (Figure 1.5) (Akira et al., 2006). Upon a ligand binding, the cytoplasmic domain of TLR4 associates with four toll-interleukin 1 receptor (IL-1R) domain (TIR domain)-containing adaptor proteins: MyD88, Mal (TIRAP), TIR-domain-containing adapter-inducing interferon-β (Trif) (TICAM-1) and TRIF-related adaptor molecule (TRAM) (Vogel et al., 2003). MyD88 and MAL are needed for the initial rapid activation of

transcription factor activation after LPS binding, whereas Trif and TRAM activate a second alternative pathway with much slower kinetics (Silverman and Fitzgerald, 2004). Additionally, Trif-TRAM is required for activation of IRF3 (a transcription factor essential for type I interferon induction). Both pathways lead to TRAF6 activation, with MyD88-MAL acting via IRAK kinases, and Trif-TRAM activation receptor-interacting protein 1 (RIP1) (Meylan et al., 2005). Subsequent activation of TRAF6 mediates the recruitment of TAK1, which additionally interacts with TAB2 and TAB3. It is known that TAK1-TAB complex associates with ubiquinated TRAF6 to activate TAK1 kinase, which next activates IKK complex and JNK kinase (MKKs) (Sun and Chen, 2004).

Consequently, a downstream signalling of TLRs stimulation activates the master transcription factor NF-κB, which is activated through phosphorylation and degradation of IκBα, therefore translocating NF-κB from the cytosol to the nucleus, where it activates the proinflammatory responses.

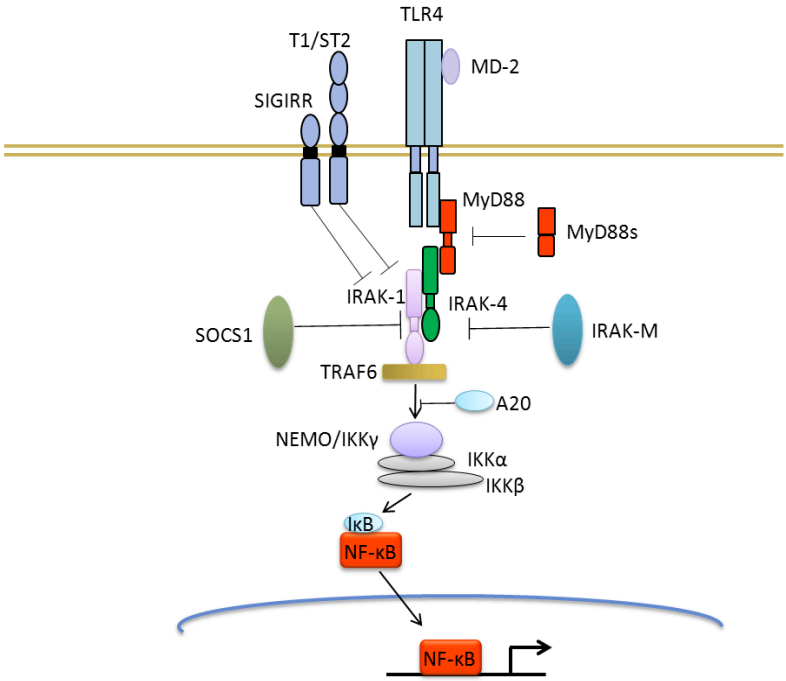


Figure 1.5: Negative regulation of TLR4 signalling pathways

The negative regulators of TLR4 signalling include IRAK-M that inhibits dissociation of IRAK-1/IRAK-4 complex from the receptor. MyD88s blocks association of IRAK-4 with MyD88. SOCS1 is associated with IRAK-1 and inhibits its activity. TRIAD3A induces ubiquitination-mediated degradation of TLR4. TIR domain-containing receptors, SIGIRR and T1/ST2 were shown to have a negative modulation on TLR signalling.

During the establishment of endotoxin tolerance, there is reduced surface expression of TLR4 and MD2 (Li et al., 2000; Siedlar et al., 2004). Additionally, TLR signalling pathways are negatively regulated by inhibitory molecules, for example IRAK-M (a member of IRAK family of serine/theonine kinases) (Figure 1.5) (Kabayashi et al., 2002). Based on studies, in mice it is known that IRAK-M prevents dissociation of IRAK-1 and IRAK-4 (positive regulators of TLR signalling) from MyD88 and formation of IRAK-TRAF6 complexes (Figure 1.5) (Kabayashi et al., 2002; Burns et al., 2003). The negative regulation of TLR signalling pathway also involves

overexpression of MyD88s, a spliced variant of MyD88 (Burns et al., 2003). MyD88s is induced after LPS stimulation, impairing LPS-induced NF-κβ activation by inhibiting IRAK-4- mediated IRAK-1 phosphorylation (Burns et al., 2003).

Other molecules, such as SOCS1, SIGIRR and T1/ST2 have also been shown to be negative regulators of TLR activation. SOCS1-deficient mice also showed defective induction of LPS tolerance (Nakawaga et al., 2002; Liew et al., 2005). Membrane-bound proteins harbouring the TIR domain, such as SIGRR (single immunoglobulin IL-1 receptor-related molecule) and T1/ST2, have also been shown to be involved in negative regulation, since SIGRR- and T1/ST2-deficient mice have been reported to have elevated LPS-induced inflammatory responses (Wald et al., 2003; Brint et al., 2004). Toll-interacting protein (Tollip) represents another adaptor protein, which maintains immune cells in an inactive 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). Due to 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).

1.3. Pathogenesis of Inflammatory Bowel disease (IBD)

1.3.1. General information about IBD

Inflammatory bowel disease (IBD) is a polygenic disease involving complex interactions between genes and the environment. IBD is chronic, relapsing and remitting inflammation of gastrointestinal tract (Sheikh and Plevy, 2010; Khor et al., 2011; Lees et al., 2011), also associated with increased risk of intestinal cancer. IBD

is most commonly diagnosed between the third and fourth decades of life, can occur at any age, but often begins in younger people aged 10-40.

Approximately 20% of all patients with IBD develop symptoms during childhood (Rogers, Clark, Kirsner, 1971; Kelsen and Baldassano, 2008), with about 5% being diagnosed before 10 years of age (Mir-Madjlessi et al., 1986). In addition to the common GI symptoms, children with IBD often experience growth failure, malnutrition, puberty delay and bone demineralisation (Lahad and Weiss, 2015). There are two main types of IBD, namely Crohn's disease (CD) and ulcerative colitis (UC). Both diseases are characterised by damage to the epithelium and mucosa due to an imbalance between pro- and anti-inflammatory processes (Reinecker et al., 1993; Reimund et al., 1996).

Crohn's disease is characterised by a mononuclear cell infiltrate, granulomas, thickened submucosa, patchy, transmural inflammation (Khor et al., 2011) that can occur anywhere in the digestive tract, however it usually affects terminal ileum and the beginning of the colon (Podolsky et al., 2002; Abraham et al., 2009).

The symptoms of CD include recurrent abdominal pain and diarrhea. The majority of patients with CD will develop complications including strictures, penetration of the bowel wall with obstruction, fistulas and abscess (Louis et al., 2001; Cosnes et al., 2002).

In contrast, UC is characterised by superficial inflammation of mucosa and submucosa, affecting only colon, usually around rectum. In UC, the infiltrate is dominated by neutrophils with crypt abscesses, loss of goblet cells and epithelial damage (Abraham et al., 2009).

Systemic symptoms are also common in IBD and include weight loss, vomiting and nausea, fever and sweats.

The prevalence of IBD is around 1 in 1000 in Europe, with higher prevalence and incidence rate observed in westernised countries (Loftus, 2004). In the last 50 years, the prevalence of IBD, particularly Crohn's disease, has increased dramatically, especially in children and adolescents, indicating that environmental factors play an important role (Xavier et al., 2007).

A substantial amount of research has identified the genetic and environmental factors, which may predispose and contribute to the onset of IBD. Appendectomy before the age of 20, and smoking strongly protect against UC (Calkins, 1989), whereas smoking is a risk factor for CD (Odes et al., 2001). The reasons for these powerful effects are unknown (MacDonald et al., 2011).

The etiology of IBD is unknown, however it is believed that IBD is caused by an abnormal immune response against the intestine microflora, in genetically susceptible individuals. Both, dysregulated innate and adaptive immune pathways contribute to the uncontrolled inflammatory responses in IBD (Geremia et al., 2014).

1.3.2. Genetics and IBD

Genetics is a strong factor in IBD. Within the relatives of patients with CD or UC, the odds ratio to develop IBD is in the range of 15-42 over the normal population for CD, and 7-17 for UC (Spehlmann et al., 2008). It has been shown that monozygotic twins exhibit phenotypic concordance in 50-70% of CD patients, and in 10-20% of UC (Halme et al., 2006). Additionally, the relative risk factor of developing CD is 800-fold greater than in the general population (Halme et al., 2006). The epidemiologic studies of twin pairs allow the evaluation of heritable factors to IBD, discriminating between the genetic and environmental factors to phenotype variance (Thompson et al., 1996; Orholm et al., 2000; Halfvarson et al., 2003; Spehlmann et

al., 2008). Based on these data, a stronger genetic influence was proposed in CD than in UC (Spehlmann et al., 2008), additionally emphasising the role of environmental trigger factors in UC than in CD (Khor et al., 2011).

Large number of IBD risk alleles is associated with either mucosal barrier function or innate and adaptive responses to microbial organisms, highlighting the fact that mucosal immune responses to commensal gut bacteria underlie the IBD pathogenesis (Khor et al., 2011; Jostins et al., 2012).

The majority (110 IBD loci) of susceptibility genes are associated with both, Crohn's disease and ulcerative colitis, indicating that pathogenesis of IBD derives from shared genetic mechanisms. Of the remaining loci, 30 are classified as Crohn's-disease-specific and 23 as ulcerative-colitis-specific (Jostins et al., 2012).

GWAS studies enabled the identification of pathways previously known through immunologic studies (e.g., IL-23 and T helper (Th) 17 cells) (Duerr et al., 2006; Kaser et al., 2010), but have also discovered completely new pathways such as autophagy (Hampe et al., 2007). The magnitude of risk associated with each polymorphism is very small, but provides a potential insight into the particular pathways that may be involved in the pathogenesis of IBD.

Early studies identified mutations in nucleotide oligomerisation domain-2 (NOD2), also designated as CARD15, as the first strong genetic association between an individual gene and Crohn's disease, but not UC (Ogura et al., 2001; Hampe et al., 2001). Three known NOD2 mutations are associated with susceptibility to ileal location and structuring disease (Economou et al., 2004) with an odds ratio of 2.4 and 17 in heterozygotes and homozygotes, respectively, representing the strongest link to IBD to date (Economou et al., 2004). Additionally, these three risk alleles have demonstrated a remarkable amount of heterogeneity across ethnicities and

geographic localisation. As an example, NOD2 risk factors are more commonly associated with individuals of European ancestors, while in African Americans are less common (Shivananda et al., 1996; Kugathasan et al., 2005). Although, it still remains to be determined, most probably NOD2 risk alleles confer loss of function mutation (Abraham and Cho, 2006).

NOD2 is expressed in myeloid cells, IECs, Paneth cells and also T cells and act as a receptor for muramyl dipeptide (MDP), a component of peptidoglycan leading to NF-KB activation and consequent production of proinflammatory cytokines (Gutierrez et al., 2002; Hisamatsu et al., 2003; Shaw et al., 2009). The *Nod2* mutation in immune cells, such as dendritic cells, macrophages results in impaired ability to process bacterial antigen and to present bacteria-derived peptides (Conney et al., 2010). Additionally, murine model studies have shown that $Nod2^{-/-}$ mice exhibit decreased α-defensin expression in Paneth cells and increased overall bacterial load in the intestinal lumen (Petnicki-Ocwieja et al., 2009).

Another gene that has been strongly associated with CD is *ATG16L1,* which encodes the protein component of the autophagy complex (Levine and Deretic, 2007).

A process of autophagy is a lysosomal degradation pathway of cell components for subsequent turnover, and by which, internalised bacteria are degraded (Levine and Kroemer, 2008). Variants in *ATG16L1* are implicated in CD via dysregulation of Paneth cell function, as with impaired autophagy the degradation of accumulated long-lived proteins is also altered (Cadwell et al., 2008). This may lead to increased bacterial translocation due to reduced production of anti-microbial peptides. Moreover, it has been shown that activation of NOD2 recruits ATG16L1 protein to the cell membrane to facilitate the internalisation of bacteria (Travassos et al., 2010). Stimulation of $Atg16l^{-/-}$ macrophages with LPS results in high production of IL-1β

and IL-18 via TRIF dependent activation of caspase-1, showing that ATG16L1 regulate LPS-induced proinflammatory cytokine production (Saitoh et al., 2008).

Interestingly, NOD2 is also involved in the autophagic responses against invading bacteria, as it induces the recruitment of ATG16L1 protein to the entry site of bacteria at the plasma membrane (Travassos et al., 2009). Thus, these two genetic risk factors seem to function in a common pathway regulating inflammatory responses in epithelium and myeloid cells.

A 20-kb deletion polymorphism upstream of *IRGM* has also been identified as CD risk variant involved in autophagy (Parkes et al., 2007; McCarroll et al., 2008). The IRGM is a member of IFN-γ-induced p47 immunity-related GTPase family (Feng et al., 2009). The murine model studies on its mouse homolog, LRG-47 revealed that IRGM controls pathogen invasion via a process of autophagy, since *Irgm1*$^{-/-}$ mice showed higher sensibility to *Toxoplasma gondii, Listeria monocytogenes and Mycobacterium tuberclosis* infection due to decreased bacterial killing in *Irgm1*$^{-/-}$ macrophages (MacMicking et al., 2003).

In contrast, UC-specific genes link to intestinal epithelial cell (IEC) function, where two genes in particular are strongly predisposing factors in UC, namely *ECM1* (Fisher et al., 2008) and variants in the region encoding *HNF4A* (Barrett et al., 2008). The ECM1 is thought to be involved in the maintenance of the epithelial barrier of the gut, through the interaction with matrix metalloproteinase 9 (known as MMP9) (Chan et al., 2007), which is known to remodel extracellular matrix and tight junctions in response to injury, and immunologically by modulating Th2 cells migration (Li et al., 2011). The *HNF4A* encodes the transcription factor called hepatocytes nuclear factor 4, which regulates the expression of tight junction proteins in the intestinal epithelium (Battle et al., 2006).

GWASs have identified several IBD susceptibility loci that contain genes encoding proteins involved in cytokine and chemokine receptor signalling, and T helper (T_H) responses Anderson et al., 2009), for example signal transducer and activator of transcription 1 (STAT1), STAT3, STAT4, CC-chemokine receptor 6 (CCR6), CC-chemokine ligand 2 (CCL2), CCL13, IL-12 receptor (IL-12R), IL-23R and Janus kinase 2 (JAK2) (Neurath et al., 2014). Further studies have identified IBD risk loci that contain genes that encode cytokines (for example, IL-2, IL-21, interferon-γ (IFN-γ), IL-10 and IL-27)), hence highlighting a potentially major role for these cytokines in disease pathogenesis (Jostins et al., 2012). In particular, it has been suggested that loss of function mutations in the genes encoding *IL10* and *IL10R* are associated with a very early onset form of IBD (Kotlarz et al., 2012). All together, these observations confirmed that cytokines have a fundamental role in controlling mucosal inflammation in IBD. Studies in mouse models of IBD have shown that the modulation of cytokine function can be used for therapy and have identified new cytokines as potential therapeutic targets.

IBD risk loci vary remarkably between different populations (Kaser et al., 2010). For example, *NOD2* and other autophagy genes are the major risk loci in the Caucasian population, but do not represent susceptibility factors in the Asian population (Ng et al., 2012). Hence, despite common genetic basis between CD and UC, substantial genetic heterogeneity exists within and between populations (Zhernakova et al., 2009).

Overall, variants of genes associated with innate immunity (e.g. *NOD2, NCX5, IRGM, ATG16L1*) are seen in Crohn's disease, suggesting that defects in the ability to handle gut bacterial products are integral to the development of this disease (Khor et al., 2011).

However, despite a huge amount of investment it is clear that the genetic variants only account for approximately 14 % of CD and 8 % of UC cases, respectively (*Jostins et al.,* 2012).

1.3.3. Intestinal Macrophages in Inflammation

The phenotype and consequently the behaviour of intestinal macrophages change dramatically during intestinal inflammation. As opposed to the homeostatic state, in IBD, increased influx of $CD14^+$ monocytes is observed (Bain et al., 2013). During the state of inflammation, $CD14^+$ blood monocytes rather than differentiating into resident anti-inflammatory macrophages, are a source of macrophages with pro-inflammatory potential (Kamada et al., 2008). The inflammatory state of the intestinal mucosa defines, if $CD14^+$ blood monocytes will become anti- or pro-inflammatory macrophages (Rivollier et al., 2012). This is supported by a fact that increased proportion of intestinal macrophages expressing CD14 is detected in IBD mucosa (Kamada et al., 2008; Thiesen et al., 2014).

As opposed to normal gut macrophages, IBD macrophages express T cell co-stimulatory molecules, such as CD40, CD80, CD86, but also Toll-like receptors TLR2, TLR4, and CD89 or TREM-1 (Schenk et al., 2007; Smith et al., 2011). Therefore, $CD14^+$ macrophages infiltrating the mucosa during IBD, upon activation with commensal bacteria, secrete large amount of proinflammatory mediators such as IL-1β, IL-6, Il-8, TNFα, IL-12/IL-23p40, nitric oxide, reactive oxygen intermediates, metalloproteases but also monocytes chemotactic protein-1 (MCP-1), which additionally facilitates the recruitment of $CD14^+$ monocytes to the inflamed mucosa (Mahida et al., 1989; Rugtveit et al., 1995; 1997; Rogler et al., 1998; Kamada et al., 2008; Ogino et al., 2013). Monocytes additionally recruit other innate

effector cells, such as $CCR3^+$ eosinophils, through the production of CCL11 (eotaxin-1) (Wadell et al., 2011; Lampinen et al., 2013). Furthermore, since intestinal macrophages have enhanced expression of CD40 (Carlsen et al., 2006), they are fully capable of interacting with effector T cells, therefore initiating adaptive immune responses. As shown by Kamada et al. (2008), in Crohn's disease, apart from TNF-α, $CD14^+$ intestinal macrophages are the main source of IL-23, which additionally induces IFN-γ immune responses by lamina propria $CD4^+$ T cells. Therefore the importance of intestinal macrophages in IBD pathogenesis is mirrored by the importance of IL-23, as a key cytokine linking innate and adaptive immunity (Discussed in Section 1.3.5.).

The important role of macrophages in IBD is also supported by studies using neutralising anti-CCR2 antibody or CCR2-deficient mice models (Platt et al., 2010; Zimond et al., 2012). Is has been shown that blocking CCR2 can prevent differentation of macrophages into proinflammatory phenotype, and result in less severe colitis in mice (Zimond et al., 2012).

Also genome wide association studies in IBD identified risk alleles associated with intestinal macrophage dysfunction. For example, NOD2, which is expressed in intestinal macrophages to facilitate the recognition of bacterial wall compartment muramyl dipeptide. Approximately, 30% of CD patients have no-functional mutation in NOD2 gene (Cho and Weaver, 2007). Additionally, a number of identified autophagy genes mutations in CD, like ATG16L and IRGM (Stappenbeck et al., 2011) also point out on important position of intestinal macrophages in IBD pathogenesis. The importance of intestinal macrophages in CD pathogenesis is also supported by the presence of granulomas, typical characteristics of CD patients. Macrophage activation is therefore a central feature of IBD, and the activated state of

these cells plays a central role in Th1 or Th1/Th17-skewed intestinal inflammation in CD. Therefore, targeting pathways that are regulated by a function of intestinal macrophages represents an attractive therapeutic target.

1.3.4. Cytokines in IBD

Activation of T cell responses plays a fundamental role in immunopathology of IBD, as indicated by a fact that CD has been considered as Th1-driven immune responses, whereas UC as non-conventional Th2 (Fuss et al., 1996). Additionally, a role of other subsets of T cell, namely Th17 has emerged as an important player (Geremia et al., 2012). Based on the levels of T cell-derived cytokines, CD and UC are characterised by different proinflammatory immune profile.

Immunologically, CD is characterised by a predominant Th1/Th17 mucosal cytokine profile with elevated levels of IFN-γ, IL-17, TNF-α, IL-12/23 and IL-2 (Gordon et al., 2005; MacDonald, 2011).

In CD, an abnormal Th1 immune responses are triggered by increased mucosal levels of IL-18 and IL-12 (Monteleone et al., 1997; Podolsky, 2002). Macrophage and DC-derived IL-12 is crutial in induction of Th1 immune resposnes, as shown by numerous reports (Monteleone et al., 1997; Gordon et al., , 2005). As a consequence of this activation, Th1 cells produce increased levels of IFN-γ (Fuss et al., 1996). A number of studies reported increased IFN-γ production in CD mucosa, as oppose to UC and control T cells (Camoglio et al., 1998; Heller et al., 2005). The recognision of IFN-γ, as an important player in CD, led to the development of antibodies blocking IFN-γ or IL-12/p40. However, the results of three clinical studies testing the effect of anti-IFN-γ antibody (fontalizumab) showed no beneficial effect in active CD (Hommes et al., 2006; Reinisch et al., 2006; 2010), even though neutralising

IFN-γ was effective in mouse models of colitis (Powrie et al., 1994). Also neutralisation of IL-12 with monoclonal anti-IL-12p40 antibodies turned out to be weakly superior to placebo (Mannon *et al*., 2004; Sandborn et al., 2008).

The role of Th17 cells in IBD immunopathogenesis has also been recognised, as increased IL-17A levels have been detected in both CD and UC lamina propria (Monteleone et al., 2005; Rovedatti et al., 2009; MacDonald et al., 2011). Th17 cells are important source of IL-21 (Monteleone et al., 2005; Sarra et al., 2010). The IL-21 protein levels are increased in the inflamed intestine in patients with CD compared to UC or controls (Fantini et al., 2008). The IL-21 may also synergise with IL-6 and TGF-β to induce a differentation of Th17 cells in mouse models (Zhou et al., 2007). The role of IL-21 in IBD pathogenesis was supported by murine models of colitis, showing that IL-21-deficient mice are resistant to Th1/Th17-cell-driven colitis and that IL-21 blocking with an IL-21 receptor fusion protein inhibits experimental colitis in mice (Fina et al., 2008). However, despite the potential of Th1 and Th17 cytokines to trigger and amplify immune responses in the gut, anti-IL17A treatment with blocking antibody secukinumab was deleterious in patients with CD (Huebert et al., 2012).

One theory for the deveopment of IBD is a failure of regulatory T cells to dampen immune responses to the microbiota. T_{Regs} play a major protective role by secreting antiinflammatory cytokines, such as IL-10 and transforming growth factor-β (TGF-β) (Powrie et al., 1996; Asseman et al., 1999; Huber et al., 2011). These findings were supported by studies in mice model with T cell-specific inactivation of genes encoding IL-10 and TGF-β. In these mice, T_{Regs} were functionally inactive, failed to suppress proinflammatory cytokines produced by APCs and effector T cells, and

spontaneously developed chromic intestinal inflammation (Davidson et al., 1996; Marie et al., 2006). Moreover, T_{Regs} are also targets of IL-10, and additional studies have revealed that mucosal T_{Reg} cells express the key transcription factor forkhead box P3 (FOXP3) (Maynard et al., 2007). Interestingly, T_{regs} are increased in IBD mucosa, and their function is normal, as was demonstrated by a potential of these cells to dampen the proliferation of effector T cells (Maul et al., 2005; Eastaff-Leung et al., 2010). However, effector T cells from patients with IBD have been shown to overexpress SMAD7, which inhibits TGFβ signalling, hence resulting in resistant to TGFβ-mediated suppression (Monteleone et al., 2001). The functional relevance of this finding was shown by studies of mice in which transgenic overexpression of *Smad7* resulted in the resistance of T cells to T_{Reg} cell-mediated suppression. Based on this concept, *SMAD7* antisense oligonucleotides have been tested as a new therapy in patients with Crohn's disease and have shown promising effects (Monteleone et al., 2015).

Many proinflammatory cytokines are overexpressed in IBD mucosa. However the most important of these is TNF-alpha. TNF-α is a master proinflammatory cytokine present in elevated quantities in the inflamed mucosa of CD and UC patient (MacDonald et al., 1990; Breese et al., 1994). Macrophages and T cells are the main source of TNF-α (Reinecker et al., 1993). Subepithelial macrophages are the main source of TNF-α in UC, however in CD, TNF producting cells are evently scattered within lamina propria, extending into the submucosa (Murch et al., 1993).

The key role of cytokines, as a therapeutic target for IBD, is supported by great efficacy of the neutralising monoclonal antibodies targeting TNF-α (infliximab and adalimumab), at inducing clinical remission in patients with active CD (Colombel et

al., 2010). However, etanercept turned out to be ineffective in the treatment of CD (Sandborn et al., 2001). Both, etanercept and infliximab neutralise TNF-α, however only the latter is able to bind surface TNF-α on activated T cells (Scallon et al., 1995; Van Den Brande et al., 2003). A recent study from Biancheri et al. (2015) however showed that etanercept loses its ability to neutralise TNF when exposed to the proteases in inflamed gut, so the failure of the drug to work in the clinic could be due to its degradation in inflamed mucosa. Neutralisation of TNF-α by infliximab induces apoptosis of blood monocytes (Lugering et al., 2001) and lamina propria T cells (ten Hove et al., 2002). Additionally, it was also shown that infliximab caused monocytes to increase their release of soluble TNFR2, which serves to neutralise TNF-α, hence amplify action of infliximab (Ebert, 2009). Infliximab also enhances production of IL-10, thereby promoting an anti-inflamamtory microenvironment (Ebert, 2009). The IL-23 is a key cytokine linking innate and adaptive immunity, and plays a central role in driving early responses to microbes (Geremia et al., 2011). It has been shown that, unconventional, innate-like T cell populations, which are particularly present at mucosa, such as γδT cells, invariant natural killer T (iNKT) cells and mucosa-associated invariant T cells, respond to IL-23 stimulation by production of Th17-associated cytokines (Cua and Tato, 2010). Additionally, IL-23 also induces Th17 cytokine production by innate lymphoid cells (ILCs) (Takatori et al., 2009; Cella et al., 2009).

The functional role of IL-23 has also been supported by GWAS study, as *Il23R* polymorphisms are associated with both, CD and UC (Duerr et al., 2006). Additionally, IL-23 belongs to IL-12 cytokine family (Goriely et al., 2008). The IL-12p35/p40 produced by CD8α dendritic cells or macrophages is a pivotal cytokine that controls Th1 cell differentation that also involves activation of STAT4 (Magram

et al., 1996, Moser and Murphy, 2000), and consequently upregulation of INF-γ, as already mentioned in this section.

Several studies have also reported that IL-27 may drive proinflammatory responses leading to chronic intestinal inflammation. It has been reported that *IL10*-deficient animals that normally develop spontaneous colitis, with *IL27R* deficiency in T cells showed reduced colitis activity (Villarino et al., 2008). In another study, it has been reported that *IL27R* deficient T cells failed to induce disease in T cell transfer model of colitis due to impaired T_H1-type cytokine production and the expansion of T_{reg} cell populations, and p28-deficient mice did not develop colitis upon transfer of T cells due to the reduced production of IL-1 and IL-6 by APCs (Cox et al., 2011; Visperas et al., 2014). However, other investigators have found that IL-27 has an anti-inflammatory effect (Troy et al., 2009; Wirtz et al., 2011; Hanson et al., 2014). For instance, IL-27 was not required for the development of spontaneous colitis in mice with a myeloid-specific deletion of STAT3 (Wirtz et al., 2011), which suggests that the functions of this cytokine are dependent on the model that is used.

The IL-6 is also overexpressed and although clinical efficacy of an anti-IL-6R antibody was shown over 10 years ago, there have been no further studies (Heinrich et al., 1990). The IL-6 production by lamina propria macrophages and $CD4^+$ T cells is increased in experimental colitis and in patients with IBD (Atreya et al., 2000; Kai et al., 2005). In particular, $CD14^+CD33^+CD68^+CD163^{lo}$ myeloid cells that express some macrophage-associated and DC-associated markers were found to produce high amounts of IL-6 (Kamada et al., 2008). The IL-6 binds to the soluble IL-6R (sIL-6R), and the IL-6–sIL-6R complex then activates intestinal target cells by binding to the gp130 surface molecule (also known as IL-6R subunit-β). Therefore,

IL-6 can exert proinflammatory functions by activating multiple target cells, including APCs and T cells. IL-6–sIL-6R complex prevents programmed cell death (apoptosis) of mucosal T cells and activates proinflammatory cytokine production by these cells (Atreya et al., 2000). However, IL-6 may also have important homeostatic functions by stimulating the proliferation and expansion of intestinal epithelial cells (IECs). Interestingly, blockade of IL-6 signalling with monoclonal antibodies was effective in suppressing chronic intestinal inflammation in mouse models (Yamamoto et al., 2000; Kamada et al., 2008).

As mentioned, UC is more Th-2 like driven disease, with a predominant, signature synthesis of IL-4, IL-5, IL-13 by non-conventional NKT cells (Fuss et al., 1996). The UC-related inflammation is also driven by the excessive production of Th17 cytokines and macrophage-derived cytokines (IL-6, IL-1, TNF) (Fuss et al., 1996; Heller et al., 2005), and transcription factor GATA-binding protein 3 (GATA3) (Neurath et al., 2002). However, in UC, T cells do not display a full classical T_H2 profile, as low level of IL-4 is produced (Fuss et al., 1996).

Initially IL-13 was identified as a key cytokine driving the pathogenesis of UC. Based on results from oxazolone-induced models of colitis, it was observed that IL-13, produced by CD1-reactive natural T (NKT) cells, is a key cytokine involved in UC pathogenesis (Heller et al., 2002; Fuss et al., 2008). Moreover, through experimental settings, it was shown that elimination of NKT cells or neutralisation of IL-13 dampens the development of colitis (Fuss et al., 2008). The fact that the treatment with IFN-β is associated with a reduction of IL-13 production (Mannon et al., 2011) additionally supported the fact that IL-13 may be an important therapeutic target in UC.

Additional functional studies have shown that IL-13 promotes fibrosis and disturbs a function of tight junctions, hence driving mucosal ulceration (Heller et al., 2005). The therapeutic potential of anti-IL-13 antibodies was tested, first in models of colitis and eventually in clinical trials. However, recently reported two clinical trials with anrukinzumab and tralokinumab showed no efficacy for anti-IL-13 blockage as a therapeutic strategy in UC (Reinisch et al., 2015; Danese et al., 2015).

The role of IL-13, as a key cytokine in UC was also challenged by Biancheri and colleagues (2014), who were unable to show increased secretion of IL-5 or IL-13 by UC mucosal biopsies ex vivo or anti-CD3/CD28 activation of LPMCs. Therefore, further investigation is required to understand the role of IL-13 in UC immunopathology.

Another cytokine that might play an important role in UC is IL-9 (Gerlach et al., 2014), however its role in IBD pathogenesis is unknown. Recently, increased expression of *IL9* was reported in patients with active UC (Gerlach et al., 2014). The same authors also demonstated that IL-9-producing cells in these patients are $CD4^+$ T cells. These results were also additionally supported by murine models of colitis, where colitis was reduced in both cases by blocking IL-9 with neutralising antibody or using *IL9*-deficient mouse (Gerlach et al., 2014).

Innate lymphoid cells (ILCs) are seen to be important in intestinal mucosa immunity (Artis and Spits, 2015). These cells might perform protective role, but also contribute to chronic intestinal inflammation (Satoh-Takayama et al., 2008; Sonnenberg et al., 2011). The ILCs are also considered as an important source of IFN-γ and IL-23-inducible proinflammatory cytokines, such as IL-17A and IL-17F, which mediate experimental innate immune-mediated colitis (Buonocore et al., 2010). In human

IBD, an expansion of IL-17-producing ILCs was noted in the inflamed mucosa of patients with Crohn's disease but not in patients with ulcerative colitis (Geremia et al., 2011). Other studies have also reported that upon IL-12 and IL-15 stimulation, the expansion of a human IFN-γ producing intraepithelial ILC1 was observed in patients with Crohn's disease (Bernink et al., 2013). Additional functional studies in a model of innate colitis that is induced by CD40-specific antibodies have revealed that ILC1s that express T-bet contribute to pathology, and thus might be a new therapeutic target (Fuchs et al., 2013). Given that immunopathology of IBD includes dysregulation of a rather complex cytokine profile, it might be that simultaneous inhibition of multiple cytokines provides more efficient clinical outcome (Monteleone et al., 2014). This is supported by a recent clinical outcome of tofacitinib in treatment of active UC (Sandborn et al., 2012), which was shown to inhibit the activity of Janus kinases (JAK) 1, 2, 3, tyrosine kinases that mediate signal-transduction activity involving the common chain of the surface receptors for multiple cytokines, including IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 (Furumoto and Gadina, 2013). In vitro, tofacitinib inhibits IL-2-dependent differentation of type 2 and type 17 helper T cells, and also LPS-induced innate immune responses (Ghoreschi et al., 2011). The inhibitory effect on JAK1 also fine-tunes the signalling by proinflammatory cytokines, including IL-6 and IFN-γ (Meyer et al., 2010). Effectively, by blocking these signalling pathways, a suppression of both T and B cells is achieved with a concurrent maintenance of regulatory T cells (Changelian et al., 2008; Sewgobind et al., 2010; Ghoreschi et al., 2011).

In IBD, the increased recruitment of inflammatory cells (T cells, monocytes/macrophages, neutrophils) into the gut tissue is enhanced by production of chemoattractants within the inflammatory environment. One such chemoattractant

is the chemokine ligand 25 (CCL25), of which increased production is detected in the inflamed epithelium of CD patients and is known to promote homing of CC chemokine receptor 9 (CCR9)-expressing T cells to the small intestine (Nishimura et al., 2009). The biological implication of CCR9 blockage was demonstrated in SAMP1/YitFc mice, a spontaneous model of CD (Rivera-Nieves et al., 2006). Additionally, some benefit of CCR9 inhibition was also observed in patients with active CD (Eksteen et al., 2010).

Additionally, the trafficking of lymphocytes from the circulation into the gut tissue is also mediated by adhesive interactions between the lymphocytes and endothelial cells (Van Assche and Rutgeerts, 2005).

Among all adhesive molecules, integrins, especially α4β7 have a key role in T-cell trafficking into the inflamed gut. The integrin α4β7 binds to mucosal addressin-cell adhesion molecule 1 (MAdCAM-1) (Sandborn and Yednock, 2003). MAdCAM-1 is typically linked with gut-associated lymphoid tissue (Briskin et al., 1997), and increased expression of MAdCAM-1 is detected in inflamed IBD mucosa (Souza et al., 1999). Additionally, it has been shown that blocking of either the integrin β7 or MAdCAM-1 inhibits the homing of intestinal seeking lymphocytes (Hamann et al., 1994). Monoclonal antibodies directed against α4 (natalizumab) and β7 (vedolizumab) integrins were also tested in IBD patients with beneficial outcome (Gordon et al., 2001; Sandborn et al., 2005).

1.4. Epigenetics

Epigenetic regulation of gene expression has emerged as a potentially important determinant in the development of many diseases, including IBD (Scarpa et al., 2012). Epigenetics refers to modifications in gene expression that are controlled by heritable, but potentially reversible changes in DNA methylation and chromatin

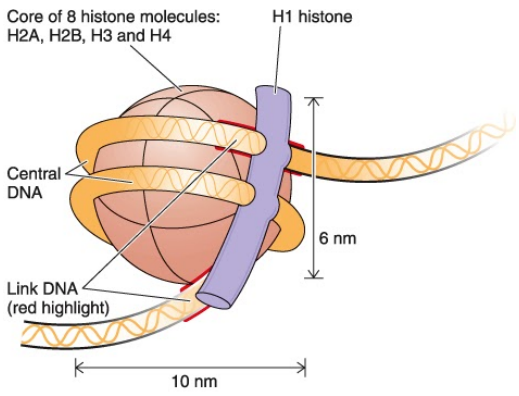
structure (histone modifications, chromatin remodelling) without altering DNA sequence (Herman et al., 2003; Natoli, 2010). Phenotypic differences are likely to be initiated by dynamic epigenetic modifications of the genome, thus the epigenetic status can have a crucial effect on the tissue-specific phenotype of a cell (Huang et al., 2012). The stability of epigenetic regulation provides a new opportunity for the explanation of a number of features in complex diseases that traditional DNA sequence-based genetics could not (Tan et al., 2011).

Epigenetic modifications can be driven by DNA methylation, histone modification and chromatin remodelling (Jin et al., 2011). In this respect, only histone modifying changes will be discussed in detail with the emphasis on histone methylation.

1.4.1. Chromatin structure

1.4.1.1. Nucleosome

Chromatin comprises of polymers of nucleosomes (Figure 1.6). The nucleosome consists of 146 basepair (bp) DNA wrapped around building blocks composed of a histone octamer made of two heterodimers of the core histones H2A, H2B and a tetramer of the core histones H3, H4 (Luger et al., 1997; D'Arcy et al., 2013). Chromatin provides a structural skeleton allowing compression of DNA within the nucleus (Cloos et al., 208). Its structure is highly dynamic, which consequently allows the regulation of biological processes essential for correct cellular function such as transcription, DNA replication and repair. Depending on the degree of condensation, chromatin can be present in inactive (heterochromatin) or active (euchromatin) form (Naughton et al., 2010).



(http://dehistology.blogspot.co.uk/2011/06/chromatin.html).

Figure 1.6: Schematic representation of nucleosome

The basic structural unit of chromatin is the nucleosome, which consists of a core of four types of histones: two copies each of histones H2A, H2B, H3, and H4, around which are wrapped 146 DNA base pairs. An additional 48-base pair segment forms a link between adjacent nucleosomes, and another type of histone (H1 or H5) is bound to this DNA.

1.4.2. Histone modifications and their functional role in establishing chromatin environment

Histones can be modified at many sites, and hundreds of enzymes are involved in establishing the epigenetic state (Sterner and Berger, 2000; Zhang and Reinberg, 2006; Kouzarides, 2007; Cloos et al., 2008).

The posttranslational modification of histones affects biological processes either by making changes to chromatin structure (loosing or tightening the DNA-histone interaction) or by contributing to the recruitment of additional regulatory elements (Cloos et al., 2008). As oppose to DNA modification, which is associated only with methylation, histones can be affected by methylation, acetylation, phosphorylation, biotylation, ubiquitination, sumoylation and ADP-ribosylation (Kouzarides, 2007).

Histone modifications affect higher order chromatin structure by influencing the contact between different histones in nearby nucleosomes or histones and DNA interactions, thus establishing a global chromatin environment and DNA-based biological processes (Kouzarides, 2007). These modifications enable partition of the

genome into individual areas where DNA is either accessible or blocked for transcription (Lubelsky et al., 2014).

Histone acetylation and methylation represent the most common modifications, and result in different nucleasome–DNA interactions (Cloos et al., 2008). These changes occur at the final 15-38 amino acids of the histone tails (Choi and Friso, 2010). Lysine residues in the histone tail can be either methylated (tri-, di-, mono) or acetylated, whereas arginine can undergo only methylation. Histone acetylation deposits a negative charge on a modified histone residue, hence decreasing interaction between the histone and DNA, influencing inter-nucleosomal contacts and destabilising higher order chromatin structure, and is associated with active transcription (Kouzarides, 2007; Cloos et al., 2008). In contrast, methylation does not influence the net charge, and hence has no effect on DNA-histone interaction. Histone methylation acts as a recognition template for effector proteins, which can modify chromatin and lead to repression or activation of transcription depending on the effector protein being recruited (Cloos et al., 2008; Estarás et al., 2013).

Different enzymes, termed writers, readers and erasers also can modulate the level of histone modification (Tarakovsky, 2010). Acetylation can be influenced by histone acetylases (HAT) and deacetylases (HDAC), whereas methylation is maintained by methyltransferases (HMTs) and demethylases (HDMs) ((Däbritz et al., 2014).

Individual as well as the combination of DNA and histone modifications can establish the dynamics of epigenetic changes affecting the chromatin status.

For example, DNA methylation is strongly linked with the methylation levels at histone H3 lysine 4 (H3K4) and histone H3 lysine 9 (H3K9), which have reciprocal effects on gene expression. The H3K4 methylation (active methyl mark) usually increases the gene expression and H3K9 (repressive methyl mark) is associated with

repression (El Gazzar et al., 2008). Enzymatic and structural studies suggest that Jumonji demethylases, which demethylase H3K4 or H3K9, H3K27 and histone methyltransferases are also important players in reciprocal methylation of these marks (Cheng and Blumenthal, 2010).

1.4.2.1. Histone acetylation

Acetylation is one of the most studied forms of histone modification (Däbritz et al., 2014). Changes of N-terminal lysine residues at position 9, 14, 18 and 23 of histone H3, as well as 5, 8, 12 and 16 of histone 4 mediate decondensation of the nucleosome structure, hence altering histone and DNA interaction, facilitating access of transcription factors. An increased histone acetylation at H4K5 or H4K8 is found in euchromatin regions where transcription is potentially active, whereas acetylation of H4K12 is increased in heterochromatin regions, indicating a potentially transcriptionally inactive state. Additionally, modifications associated with acetylation status are not only restricted to histone proteins, since for example HDAC3 has been shown to enhance STAT3 phosphorylation (Togi et al., 2009).

Several studies have shown that HDACs have a role in autoimmune disease (Bhavsar et al., 2008) and in inflammatory regulation (Blanchard and Chipoy, 2005). Supporting this concept, a number of inhibitors have emerged as a therapy for autoimmune disease (Mishra et al., 2001; Pankaj et al., 2008) and regulation of immune tolerance (Tao and Hancock, 2007). It is known that the role of HDACs in immune cells involves complex regulatory mechanisms that are dependent on a stage of cellular differentation or the enzyme's tissue expression (Glozak et al., 2005). For instance, HDAC6 is a key regulator of the cytoskeleton, cell migration and cell-cell interactions (Valenzuela-Fernandez et al., 2008). However, HDAC6 is also known to

be involved in a formation of antigen presenting cells (APCs) and T cell synapse (Serrador et al., 2004).

A number of studies show that histone acetylation is highly associated with inflammation. The involvements of HDACs in cytokine regulation is not restricted to any specific class of cytokines, and HDACs play an essential role in transcription of pro- and anti-inflammatory cytokines (Villagra et al., 2009). The HDACs regulate proinflammatory genes (e.g. *Il1*, *Il5*, *Il8*, *Il12*) and anti-inflammatory genes such as *Il10* (Villagra et al., 2010) as well as NF-κB binding and NF-κB-mediated transcriptional activation. It has been shown that a number of HDACs have anti-inflammatory effects, for example Sirt1 was shown to have an anti-inflammatory effect in mouse colitis, potentially by inhibiting iNOS, COX-2, NF-κB (Cui et al., 2010). HDAC11 has been shown to function as a transcriptional repressor of *Il10* gene expression in APCs (Villagra et al., 2009). It has also been documented that transcriptional activation of *Il8* involves promoter acetylation by p300, a process facilitated by a transient decrease in HDAC1 and HDAC5 within promoter region (Schmeck et al., 2008). However, at a certain point HDAC1 and HDAC5 are recruited back to *Il8* promoter in order to terminate transcription (Schmeck et al., 2008). Another cytokine whose expression is regulated via HDAC1 is IL-12, where promoter modification also involves histone acetylation by p300, but in contrast to *IL8*, at the *IL12* promoter HDAC1 represses transcription (Lu et al., 2005).

Histone deacetylases have also been reported to modulate IL-2, a key cytokine involved in differentation and homeostasis of T cells, which expression is inhibited by HDAC1 (Wang et al., 2009). Additionally, FOXP3 is an additional repressor of *IL2* expression that directly associates with acetyltransferase protein TIP60 (Tat-interacting protein), HDAC7 and HDAC9 (Li et al., 2007).

Without doubt, histone modifications provide a means for regulation of gene expression associated with either inflammatory responses or cell differentation, which leads to an establishment of immune homeostasis.

1.4.2.2. Histone methylation and a role of JMJD3-H3K27me3 demethylase in gene expression

In contrast to other histone modifications such as acetylation and phosphorylation, methylation (particularly trimethylation) was initially considered to be an irreversible change, until the discovery of enzymes, which regulate the methylation of histones (Shi et al., 2004; Allis et al., 2007; Cloos et al., 2008). Now, it is known that histone methylation on specific lysine residues is controlled by the opposite forces of lysine methyltransferases (KTMs; e.g. polycomb group proteins) and demethylases (histone lysine demethylases (KDMs), e.g., lysine-specific histone demethylase 1 (LSD1) or the family of Jumonji (Jmj) C domain-containing enzymes) (Pereira et al., 2011). The methylated amino residues of histone tails constitute regulatory marks that define transcriptionally active and inactive chromatin.

Multiple lysine residues (e.g. H3K4, H3K27, H3K36, H3K79) are methylated and demethylated by histone methyltransferases and demethylases, respectively (Chen et al., 2012). As an example; tri-, di-methylation of Lys 9 on histone H3 (H3K9me3; me2) or trimethylation of Lys 27 (H3K27me3) mark a silent state, whereas tri-, demethylated Lys 4 or Lys 36 on the same histone (H3) is connected with active transcription (Cloos et al., 2008; Ishii et al., 2009). The co-occupancy of these marks additionally states the silent/active transcription profile. The methylation of Lys 27 of histone H3 (H3K27me3) is considered a key epigenetic regulator of cell homeostasis and development (Margueron et al., 2011). The methylation patterns of

H3K4 and H3K27 have been shown to affect the function of effector $CD4^+$ helper T cell subsets (Mucasa et al., 2010). This evidence strongly supports a key role for histone demethylases and methyltransferases in gene transcription and chromatin-depended processes. In particular, the importance of Jumonji C proteins, and their role in chromatin regulation, cellular differentation, and involvement in human diseases (Cloos et al., 2008; Estarás et al., 2013).

The discovery of group of enzymes; the amide oxidase LSD1 (KDM1) and enzymes with Jumonji C (JmjC)- catalytic domain, acting as a histone demethylase defined a new thinking on how methyl state can be reverted (Yamane et al., 2006; Tsukada et al., 2006). The majority of JmjC enzymes are capable of demethylating mono-, demethylated lysine, particularly favouring trimethyl state, and they show specific substrate affinity (Bannister et al., 2002; Tsucada et al., 2006). Additionally, some histone demethylases can also act as dual enzymes, acting on different lysine residues (e.g. KDM7A for H3K9 and H3K27) (Tsucada et al., 2010).

Methylation on lysine residues of histone 3 lysine 27 (H3K27me) driven by EZH1/2 plays important role in regulating gene activity, since it acts as a silencing mark through the polycomb-repressive complex 2 (PRC2) (Schuettengruber and Cavalli, 2009; Simon et al., 2009). The core PRC2 complex itself consists of several components: histone-lysine N-methyltransferase (EZH1/2), SUZ12 polycomb repressive complex 2 subunit (SUZ12), embryonic ectoderm development (EED) and RbAp46/48 (RBBP7/4) and the recently identified AEBP2, PCLs and Jumonji/ARID Domain-Containing Protein 2 (JARID2). Additionally other elements can transiently bind to the complex (e.g. DNA methyltransferase (DNMTs), histone deacetylases 1 (HDAC1) and sirtuin 1 (SIRT1) (Margueron and Reinberg, 2011). The PRC2-EZH2 drives H3K27me2/3 state via its EZH2-mediated methyltransferases activity,

whereas PRC2-EZH1 restores the silencing mark (Margueron and Reinberg, 2011). The EZH1 and EZH2 target the same group of genes, participating in common silencing pathway. The EZH1 is present in dividing as well as differentiated cells, whereas EZH2 is found only in actively dividing cells (Margueron and Reinberg, 2011).

Lysine residues can be mono-, di- or trimethylated, with each methylation state being functionally different and complementary, hence providing new means for kinetics.

The methylated H3K27 is very abundant, for example, nearly 50% of all H3 histone are being dimethylated, 15% trimethylated and 15% monomethylated in embryonic stem (ES) cells (Peters et al., 2003).

The H3K27me2 is responsible for indirect gene repression, since it is important intermediary PRC2 product and consequently a substrate for a subsequent H3K27me3 formation (Tie et al., 2009). Additionally, H3K27me2 prevents H3K27me3 from being acetylated, since the acetylated state of H3K27 functions as antagonist to PcG mediated silencing (Margueron and Reinberg, 2011).

The deposition of H3K27me1 in actively transcribed genes is driven by enzymatic activity other than PRC2, such as UTX or JMJD3 that demethylase H3K27me2 to monomethyl state (Aggar et al., 2007; Swigut et al., 2007; De Santa et al., 2007).

The H3K27me3 occupancy potentially prevents transcriptional factors from binding to chromatin; hence enrichment of this mark correlates with a silent state of the gene (Mikkelsen et al., 2007). The trimethyl K27 modification is essential in transmitting epigenetic information during development (Jepsen et al., 2007).

Among the histone modifying enzymes, histone demethylase; JmjD3 and its homologue UTX (belonging to the KDM group) play important role in cellular differentiation and development, since many key developmental promoters are often

marked by H3K27me3 in combination with H3K4me3 (Boyer et al., 2006; Pan et al., 2007; Chen et al., 2012). It has been shown that H3K27me3 may coexist with the activating mark H3K4me3 to form a bivalent state (at high occupancy of H3K4me3 and H3K27me3) of the gene, resulting in poised transcription (Huang et al., 2012). The bivalent state may be established by JMJD3 and KDM7A (a novel histone demethylase specific for H3K9/H3K27) (Bernstain et al., 2006). Additionally, JmjD3 and KDM7A are upregulated in differentiating cells and can bind directly to a target gene acting in both a demethylases-dependent and-independent fashion (Huang et al., 2010; Chen et al., 2012). The interaction between JMJD3 and KDM7A provides a dynamic and robust mechanism for rapid conversion of H3K27me3 to H3K27me0 (Chen et al., 2012).

Agger et al. (2009) showed that upon cell differentiation, JmJD3 is recruited at the promoter, transcription start sites (TSS) for the activation of target gene. Hence the removal of H3K27me3 mark is proposed to be an important step in the resolution of the bivalent domains (Kim et al., 2011).

Although the importance of H3K27me3 and JMJD3 has been broadly stressed, the molecular mechanism by which the enzymes function remains incomplete. The JMJD3 regulates a subset of its target genes by demethylating H3K27me3. It has been noted that although JMJD3 and KDM7A have different substrate preferences H3K27me3/H3K27me2 and H3K27me2/H3K27me1, respectively, both enzymes show the same binding affinity to H3K4me3 (Chen et al., 2012). The JMJD3 is involved in the inflammatory responses (De Santa et al., 2007; 2009). However, it still remains unknown if transcriptional regulation is driven via demethylation of H3K27me3 at the gene promoter alone.

The JMJD3 has also been shown to regulate proinflammatory gene expression in activated macrophages. De Santa and colleagues (2009) have shown that upon LPS stimulation, JMJD3 is recruited to the gene promoter through NF-κB activation. Additionally, Ishii and colleagues (2009) have showed that acquisition of M2 macrophage phenotype is epigenetically regulated by reciprocal changes in H3K4 and H3K27 methylation, and that the latter methyl mark is removed by JMJD3. They also reported that increased *Jmjd3* expression was IL-4 dependent and STAT6 mediated. Moreover, JMJD3 is involved in retinoic acid-induced cell differentiation, since JMJD3, together with UTX (JMJD3 homologue) have been shown to be a direct retinoic-acid-receptor target in neural differentiation (Jespen et al., 2007).

Of interest, JMJD3 was shown to bind not only to promoters but also to the coding regions of the gene (De Santa et al., 2009; Hawkins et al., 2010). Estarás and colleagues (2013) have shown that JMJD3 associates with the 90% of methylated genes, suggesting that H3K27 demethylase is recruited to methylated regions upon signal activation, hence resolving the H3K27me3 repressed state and consequently contributing to RNA elongation. Since JMJD3 has been shown to interact with RNAPII-S2p, as opposed to unphosphorylated RNAPII, it may form an element of elongating complex, allowing the progression of RNAPII through gene bodies (Estarás et al., 2013). It is possible that JMJD3 drives elongation by changing the chromatin state at the region being transcribed by interacting with e.g., Brg1, which is a transcription activator belonging to SWI/SNF family (Estarás et al., 2013).

1.4.3. Epigenetics in IBD

Epigenetics has emerged as an important aspect of IBD etiology (Jenke and Zilbauer, 2012; Kellermayer, 2013; Low et al., 2013).

The preliminary knowledge on IBD epigenetics was restricted to information on epigenome-wide DNA methylation analysis, with a focus on predisposition to cancer in IBD (Ventham et al., 2013). For example, it has been shown that methylation of a CpG island in the androgen receptor (AR) gene was increased with age in nonneoplastic colorectal epithelium, and was also highly methylated in colorectal epithelium from UC (Issa et al., 2001). The DNA methylation of IBD susceptibility genes (e.g., gene-specific methylation of mutator L homolog 1 (MLH1) and hyperplastic polyposis 1 (HPP1) was also linked to colon cancer and IBD (Chan et al., 2006). Additionally, study by Nimmo et al. (2012) showed different pattern of DNA methylation in genes involved in immune system activation (e.g., MAPK, RPIK3, IL21R) between IBD patients and controls. Several key epigenetic regulatory enzymes, such as DNA methyltransferases (DNMT) 3a and 3b were also linked to CD susceptibility genes (Franke et al., 2010; Jostins et al., 2012). These findings are additionally supported by a recent study. Adams and colleagues (2014) provided evidence of DNA hypermethylation at different sites across genome, including HLA region and MIR21 among children with recently diagnosed Crohn's disease, therefore providing a new insight into gene-environmental interactions at the early onset of disease.

Epigenetic aspect of IBD pathogenesis links to interaction between commensal microbiota and chromatin architecture of intestinal immune responses. For instance, the expression of TLR2 and TLR4 on IEC, in a large but not in a small intestine, is

regulated by DNA methylation and histone deacetylation, which in turn depends on the presence of commensal gut microbiota.

Many recent reviews additionally cover the aspect of epigenetic regulation of intestinal immune responses (Scarpa et al., 2012; Däbritz et al., 2014). Alterations in chromatin environment are potentially important by fine-tuning of the inflammatory responses, through regulation of inflammation-induced transcription, tolerance and T-cell lineage commitment as well (Scarpa et al., 2012). Recently, Schmolka and colleagues (2013) have described the epigenetic landscape of Th1 and Th17-related loci in mouse γδ T cells. By performing genome-wide analysis of methylation pattern of histone H3, they showed that dual ability of γδ T cells to produce IL-17 and IFN-γ is regulated by H3K4me2 and H3K4me3 methylation at the loci encoding both cytokines and their regulatory transcription factors.

1.4.4. Epigenetic modifications in macrophages

The process of monocytes differentation into macrophages involves morphological, functional changes taking place without actual cell proliferation. Moreover, the process of differentiation involves regulation at the level of transcription factor and the relevant epigenetic marks (deposition or removal of e.g. histone methylation, acetylation, DNA methylation) (Shi, 2007; Ivashkiv, 2013).

Cytokine signals and other microenvironmental factors, through epigenetic modifications can influence phenotype and consequently a function of immune cells. The interplay between signalling pathways and therefore its effect on chromatin state regulates the transcription of specific genes, hence defining cellular identity (Esterás et al., 2013). In vitro and in vivo studies in mice and humans have demonstrated that

IFN-γ can resolve macrophages anergy and restore the inflammatory cytokine production (Bundschuh, 1997; Randow et al., 1997; Medzhitov et al., 2009).

The molecular understanding of INF-γ-driven reversal of macrophage tolerance, although still remain to be elucidated is believed to be driven by promoting recruitment of transcription factors; e.g., NF-κB, CCAAT/enhancer-binding protein β (C/EBPβ) and RNA polymerase II (Pol II) to gene promoter, without altering upstream TLR pathway signalling (Chen, Ivashkiv, 2010). Additionally, increased expression of secondary responses genes, such as IL-6 upon pretreatment with IFN-γ, was associated with a synthesis of new proteins and recruitment of nucleosome remodelling complexes, such as Brahma-related gene 1 (Brg1)-containing switch/sucrose nonfermenting (SWI/SNF) complex (Chen, Ivashkiv, 2010). All these changes are known to increase chromatin accessibility to transcription factors (Ramirez-Carrozzi et al., 2009). Interestingly, no changes in H3 and H4 acetylation and H3K4 trimethylation at the IL-6 promoter were observed upon LPS stimulation and tolerisation.

Chen et al. (2010) also showed that studied cytokines had different kinetics for IFN-γ response, showing that TNF-α was more resistant to inhibition by IFN-γ at higher LPS doses. More recent data also suggest gene-specific TLR-induced regulations in tolerant macrophages, as some set of genes (e.g. antimicrobial effectors) is highly expressed after a second stimulation with inflammatory stimuli (Medzhitov et al., 2009; Foster et al., 2010). An anergic state has been shown to be facilitated by TLR-induced chromatin remodelling, consequently allowing the "shutting down" of the expression of proinflammatory genes (Foster et al., 2007). Presumably, gene silencing in anergic intestinal macrophages is associated with acquisition of nonpermissive histone modifications and reduction of activating marks present at

gene promoter, consequently blocking TLR-induced nucleosome remodelling and resulting in decreased accessibility of gene loci to transcription factors (El Gazzar et al., 2007; Foster et al., 2010). Foster and colleagues have proposed a system where they explained how genes of opposing functions could be regulated upon the same stimuli (e.g., LPS). They have proposed that upon LPS stimulation of naïve macrophages transcription factors are recruited to both, proinflammatory and antimicrobial class of genes. These genes also show increased histone acetylation, H3K4 trimethylation and chromatin remodelling. In tolerant macrophages, H3K4 trimethylation is elevated at both classes of genes, and histone acetylation is only increased in a group of antimicrobial genes. Following stimulation with LPS, proinflammatory genes promoter remains deacetylated and inaccessible, whereas antimicrobial genes become even more acetylated with even greater kinetics. Thus TLR4 stimulation leads to induction of negative and positive factors that lead to silencing of one group of genes and activation of others (Foster et al., 2007).

Additionally to T cells, upon activation, monocytes and macrophages are the main source of TNF-α (MacDonald et al., 1993; 2011).

The *TNF* promoter and its regulatory DNA elements (not discussed in this work), such as enhancer, silencers have been shown to control transcriptional activation in response to the stimuli (Gross and Garrard, 1988; Falvo et al., 2010). The *TNFA* is an immediate early response gene, and is transcribed within minutes after stimulation (Goldfeld et al., 1993; Falvo et al., 2013).

In numerous studies it was shown that the activation of *TNF* transcription is associated with multiple HATs such as CBP/p300 co-activators (Tsai et al., 2000; Brthel et al., 2003). The activating transcription factor-2 (ATF-2) was the first sequence-specific DNA-binding transcription factor (TF) to be identified as a HAT

(Kawasaki et al., 2000). The ATF-2 was shown to bind a conserved variant cyclic AMP response element (CRE) in the *TNF* proximal promoter, which activates the *TNF* gene expression in many cell types in response to multiple stimuli (Barthel et al., 2003).

Additionally, HDAC1 and HDAC3, and also HDAC-recruiting co-repressors NCoR and CoREST associate with the *TNF* promoter in unstimulated immune cells (as shown in BMDMs), however this association is reduced upon LPS stimulation (Hargreaves et al., 2009). Histone acetylation therefore was linked to activated transcription in many genes, including the *TNF* promoter. For example, in Jurkat T cells, it was shown that PHA/PMA stimulation leads to induced histone H3 acetylation but not H4 (Ranjbar et al., 2006). Furthermore, in murine primary $CD4^+$ T cells, anti-CD3/CD28 stimulation increases acetylation of histone H3 and H4 at the *TNF* promoter (Tsytsykova et al., 2007).

Additional studies supported the observation that increased H3 and H4 acetylation at the *TNF* promoter also correlates with LPS-induced *TNF* transcription in primary human monocytes and TH-1 cells (Sullivan et al., 2007; Garret et al., 2008), and is linked to maturation of monocytes into macrophages (Lee et al., 2003). Moreover, IFN- priming of primary human monocytes leads to persistent histone H4 acetylation and recruitment of ATF-2 and RNAPII at the *TNF* promoter, which consequently leads to even more enhanced H3/H4 acetylation in response to LPS stimulation (Garrett et al., 2008).

DNA methylation at the *TNF* gene also regulates its transcription and correlates with cellular differentation. A number of studies indicate that DNA methylation within TNF regions, including the promoter, is associated with transcriptional repression (Falvo et al., 2013). It has been reported that *TNF* promoter and first exon are

demethylated in primary monocytes and macrophages, where the gene is expressed (Sullivan et al., 2007). DNA methylation profile at the *TNF* gene undergoes modification during myeloid commitment, leading to elevated methylation at CpG sites flanking the *TNF* promoter as a differentation process is initiated (Falvo et al., 2013).

Histone methylations are also involved in regulation of the *TNF* transcription. The histone marks, such as H3K4me3, -me2, -me1 were shown to be highly enriched at the *TNF* promoter following LPS or TNF stimulation and PMA/ionomycin activation of TH-1 cells or Jurkat cells, respectively (Taylor et al., 2008). By contrast, in LPS-tolerant TH-1 cells, LPS stimulation fails to induce H3K4 methylation and H3K9 demethylation at the *TNF* promoter, as it happens in LPS-responsive cells (El Gazzar et al., 2008).

As an immediate early response gene, the *TNF* gene is set at poised state, ready for immediate activation if needed. This is supported by the observation that in unstimulated murine bone marrow derived macrophages (BMDMs), high levels of H3K4me3 and H3ac together with RNAPII, CBP/p300, are present at the *TNF* promoter (Hargreaves at al., 2009; Falvo et al., 2013). The poised state of the gene is also established by a bivalent, high enrichment of H3K4me3 and H3K27me3 (Lesch et al., 2013). As shown by Kruidenier and colleagues (2012), in primary human macrophages, LPS stimulation activates *TNFA* transcription, which is associated with reduced binding of H3K27me3 to the gene promoter (discussed more in section 1.4.2.2).

In summary, much of the work on epigenetic regulation of immune responses comes from work on mice models or human cell lines. Although the data provide a valuable

platform of information, it may not be possible to extrapolate these findings to human studies of relevant disease.

1.5. PhD hypothesis and Aims

Under homeostatic conditions intestinal macrophages undergo a unique process of differentation. This process is associated with the establishment of anergic phenotype also known as hyporesponsivness. The state of anergy is driven by down-regulating TLR4 signalling pathway through recruitment of inhibitory molecules, for example IRAK-M or MyD88s (discussed in Section 1.2). However, growing evidence supports the idea that also epigenetic changes contribute to macrophage reprogramming and the establishment of anergic phenotype. Current knowledge on how chromatin modification drives genes expression in human intestinal macrophages is still very limited. Intestinal macropahges are an important players in driving homeostatic immune response. However, these cells represent a small component of immune cell population in normal and inflamed gut and no previous studies have looked into how their phenotype might be established through epigenetic modifications.

The major goal of this thesis project was to look at the relationship between histone methylation and repression of proinflammatory gene expression. We have hypothesised that the suppressed gene expression in human intestinal macrophages under homeostatic condition is associated with abundant occupancy of repressive histone marks. We have also hypothesised that the lost of anergy, as seen in intestinal macrophages of patients with inflammatory bowel disease (IBD) is associated with loss of silencing marks and possibly associated with increased occupancy of activating marks. The intestinal macrophages are highly plastic cells and therefore their phenotypic characterisation is not straightforward. Hence, the first aim of this

project was to optimise a method for isolation of sufficient number of intestinal macrophages. Only then it was possible to focus on epigenetic studies.

The next aim of this project was to investigate, if the anergic state of macrophages in normal gut associated with repressive marks. The third aim was to test, if in IBD, are there differences in epigenetic modifications between resident and infiltrating macrophages. The fourth aim was to test, if macrophages from normal and inflamed mucosa have different transcroption profile. Finally it was of interest to invstigate, if it is possible to prevent/reduce TNF-α production in IBD macrophages by blocking histone methylation at the gene promoter.

Chapter 2

Materials and Methods

2. Materials and Methods

2.1. Materials

Antibodies against specific modifications of histone 3 (H3), transcription factors and controls used for ChIP assays and flow cytometry analyses are listed in Table 2.1 and 2.2.

Table 2.1: Antibodies used for chromatin immunoprecipitation (ChIP) assays

Description	Antibody Cat. No:	Company
Anti-RNA polymerase II, CTD, cl8WG16	05-952	Millipore Ltd. Watford, UK
Anti-RNA polymerase II CTD repeat YSPTSPS (phosphoS2)	ab5095	Abcam Ltd. Cambridge, UK
Anti-RNA polymerase II CTD repeat YSPTSPS (phosphoS5)	ab5131	Abcam Ltd. Cambridge, UK
Rabbit IgG – ChIP Grade	ab37415	Abcam Ltd. Cambridge, UK
Rabbit polyclonal to Histone3 – ChIP Grade	ab1791	Abcam Ltd. Cambridge, UK
Anti-H3K27me3	07-449	Millipore Ltd. Watford, UK
JMJD3	ab85392	Abcam Ltd. Cambridge, UK
Anti-H3K4me3	07-473	Millipore Ltd. Watford, UK
Anti-H3K4me2	07-030	Millipore Ltd. Watford, UK
Anti-H3K4me1	ab8895	Abcam Ltd. Cambridge, UK
Anti-H3K9me3	07-442	Millipore Ltd. Watford, UK
Anti-H3K9me1	07-450	Millipore Ltd. Watford, UK

Table 2.2: Antibodies and Isotype controls used for flow cytometry analysis

Description	Clone	Isotype	Cat. No.	flourochrome	Company
CD45	HI30	Mouse IgG1, κ	555485	APC	Biosciences Cambridge, UK
CD45	HI30	Mouse IgG1, κ	304021	Pacific Blue™	Biolegend Cambridge, UK
CD33	#6C5/2	Mouse IgG1	FAB1137 A-025	APC	R&D Systems Europe Ltd. Abingdon, UK
CD14	M5E2	Mouse IgG2a, κ	301816	Pacific Blue™	Biolegend Cambridge, UK
CD68	Y1/82A	Mouse IgG2b, κ	333813	PerCP-Cy5.5	Biolegend Cambridge, UK
CD3	HIT3a	Mouse IgG2a, κ	300307	PE	Biolegend Cambridge, UK
TNF-α	MAb11	Mouse IgG1, κ	502930	PE-Cy7	eBioscience, Cambridge, UK
Anti-Histone H3 (tri methyl K27), unconjugated	mAbcam 6147	Mouse IgG1, κ	ab6147	N/A	Abcam Ltd. Cambridge, UK
Secondary Ab to H3K27me3	N/A	Goat anti-mouse IgG	ab96879	DyLight488	Abcam Ltd. Cambridge, UK

2.1.1. Reagents

Reagents used in this study are listed in Tables 2.3 unless stated elsewhere.

Table 2.3: An index of Reagents

Product	Cat. No.	Company
Hank's Balanced Salt Solution (HBSS) (w/o Ca^{2+}, Mg^{2})	14175	Life Technologies Ltd. Paisley, UK
Collagenase Ia	C2674	Sigma-Aldrich Co Ltd. Poole, UK
DNase I	D4263	Sigma-Aldrich Co Ltd. Poole, UK
RPMI1640 medium [+] L-Glutamine	31870-025	Life Technologies Ltd. Paisley, UK
HL-1 medium, completely defined Serum Free w/o L-Glutamine	BE 344017	LONZA, Cambridge BioSciecne, UK
Penicillin	15140-122	Life Technologies Ltd. Paisley, UK
Gentamicin solution (10mg/ml)	G1272-100ML	Sigma-Aldrich Co Ltd. Poole, UK
hrGM-CSF	215-GM-010	R&D Systems Europe Ltd., Abington, UK
LPS from *E. coli* (O111:B4)	L4391-1MG	Sigma-Aldrich Co Ltd. Poole, UK
GSKJ4 compound	4594	R&D Systems Europe Ltd., Abington, UK
Ficoll-Paque Premium	17-5442-02	GE Healthcare Life Sciences
Phosphate buffered saline (PBS) (w/o Ca^{2+}, Mg^{2+})	14190-094	Life Technologies Ltd. Paisley, UK
CD33 MicroBeads	130-045-501	Miltenyi Biotec Bisley, UK
CD14 MicroBeads	130-050-201	Miltenyi Biotec Bisley, UK
0.5M EDTA	E7889-100ML	Sigma-Aldrich Co Ltd., Poole, UK
Tris-EDTA buffer x 100	T9285	Sigma-Aldrich Co Ltd., Poole, UK
Formaldehyde Solution (37%)	F1635	Sigma-Aldrich Co Ltd., Poole, UK
Glycine	G8898	Sigma-Aldrich Co Ltd., Poole, UK
Protein A-coated paramagnetic beads (Dynabeads ProteinA) (30 mg/ml)	100-01D	Life Technologies Ltd., Paisley, UK
ChIP DNA Clean and Concentrator	D5201	ZymoResearch, Cambridge Bioscience, UK

Trizol Reagent	15596026	Life Technologies Ltd., Paisley, UK
Chloroform	2432	Sigma-Aldrich Co Ltd., Poole, UK
RNase-Free DNase Set (rxn 50)	79254	Qiagen Ltd., Crawley, UK
Superscript III First-Strand synthesis Supermix	11752-050	Life Technologies Ltd., Paisley, UK
hTNF-alpha DuoSet ELISA kit	DY210	R&D Systems Europe Ltd., Abington, UK

2.1.2. Patients and Samples

All human tissues were obtained during routine surgery or endoscopy at Barts Health NHS Trust with appropriate ethical approval from the local research ethics committee and consent given by the patient in all cases. The surgical specimens were collected from patients undergoing intestinal resection for colorectal cancer and were used as controls or from macroscopically and microscopically inflamed or sporadically uninflamed area of intestine of patients with either Crohn's disease (CD) or ulcerative colitis (UC). The surgical specimens from cancer patients were collected from a distal part of the bowel away from cancer.

Additionally, colonic biopsies were also collected from patients undergoing colonoscopy due to either abnormal bowel habits or from patients diagnosed with IBD. The biopsies or small mucosal fragment cuts from resected tissue were used for ex vivo organ culture. The tissue resection samples were also used to isolate lamina propria mononuclear cells (LPMCs) and used for ChIP assays and array studies. The blood samples were also collected and used for isolation of peripheral blood mononuclear cells (PBMCs) for ChIP analysis. Detailed clinical features of IBD patients are presented in Figure 2.4.

Table 2.4: Clinical features of patients with IBD (Crohn's disease; n= 38 and Ulcerative colitis; n= 37). The table shows the patients records diagnosed with IBD and the location of the tissue material taken during endoscopy or surgical resections.

IFX- Infliximab; AZA-Azathioprine; ADA-Adolimumab; 6-MP-Mercaptopurine; 5-ASA-Mesalamine.

Crohn's disease			Ulcerative colitis		
		Number			Number
AGE			**AGE**		
	Average	36		Average	45
	Median (range)	29 (15-83)		Median (range)	42 (16-76)
	15≤25	16		15≤25	4
	26≤35	8		26≤35	8
	36≤50	5		36≤50	9
	51≤65	7		51≤65	8
	66≤83	2		66≤83	6
Gender			**Gender**		
	Female	17		Female	16
	Male	21		Male	21
Presentation			**Presentation**		
	Inflamed	22		Inflamed	20
	Mild	12		Mild	13
	Uninflamed	4		Uninflamed	4
Intestinal location			**Intestinal location**		
	Colon	23		Colon	31
	Ileum	10		Ileum	0
	Rectum	1		Rectum	6
Treatment			**Treatment**		
Currently no medication		4	**Currently no medication**		4
Other medication			**Other medication**		
(Combination/or alone)	IFX	34	(Combination/or alone)	IFX	33
	AZA			AZA	
	ADA			ADA	
	6-MP			6-MP	
	5-ASA			5-ASA	

2.2. Methods

2.2.1. Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were obtained from heparinized blood by density gradient centrifugation using Ficoll-Paque Premium. Briefly, the blood was diluted 1:1 in phosphate buffered saline (PBS) (w/o Ca^{2+}, Mg^{2+}) and 25 ml of the blood was layered over Ficoll in 50 ml Falcon tubes in 1:1 ratio. Next, tubes were centrifuged for 30 minutes at ~ 450x g at 20°C. The buffy coat formed at the

interface was collected into a 50 ml tube and washed twice in PBS (w/o Ca^{2+}, Mg^{2+}, 5 minutes at ~ 450 xg). Finally, the pellet was resuspended in 1 ml PBS (w/o Ca^{2+}, Mg^{2+}) and cells were counted using haemocytometer.

2.2.2. Isolation of monocytes from PBMCs and differentation into human primary macrophages

$CD14^{+}$ human monocytes were purified from PBMCs by positive selection using anti-CD14 magnetic beads following the manufacturer's instructions (Miltenyi Biotec Ltd., Surrey, UK). Next, monocytes were cultured in macrophage media (RPMI1640, 10% heat-inactivated fetal bovine serum (FBS), 2mM L-glutamate, 100units/ml penicillin/100µg/ml streptomycin) supplemented with 5ng/mL human macrophage granulocyte colony-stimulating factor (hrGM-CSF) (R&D Systems Europe Ltd, Abingdon, UK) for M1 macrophage differentiation. Monocytes at $1x10^{6}$/ml/well were seeded into 24-well plates and incubated at 37°C in a humidified atmosphere with 5% CO_2 for 5 days. Finally, cells were harvested by scraping and used for chromatin immunoprecipitation assay and mRNA analysis.

Additionally, monocytes were also isolated from blood (10 ml) of IBD patients following the same protocol. Freshly isolated cells were immediately prepared for chromatin immunoprecipitation assay as described in section 2.2.3.1.

2.2.3. Isolation of lamina propria mononuclear cells (LPMCs) from resected human gut

The mucosa layer was removed from surgical specimens and cut into 3-4 mm size fragments. Next, the tissue was washed 3 times (for 10 minutes each time) with Hank's balanced salt solution (HBSS) (Life Technologies Ltd., Paisley, UK) supplemented with 1 mM EDTA and penicillin/streptomycin/gentamycin at 37°C,

5% CO_2 to remove the epithelial layer. The tissue was then mechanically fragmented using scalpels and incubated in medium (RPMI1640, 10% FBS, 100units/ml penicillin/100μg/ml streptomycin) containing 1mg/ml collagenase Ia (Sigma-Aldrich, Co Ltd., Poole, UK), 10U/ml DNase I (Sigma-Aldrich, Co Ltd., Poole, UK)) with agitation for 1 h at 37°C. After incubation, the tissue fragments and isolated cells were washed once with medium, resuspended in 25 ml of medium and finally layered over 20 ml of Ficoll. The LPMCs were isolated by density gradient centrifugation, as described in section 2.2.1. Finally, gut macrophages were isolated from LPMCs by positive selection using anti-CD33 magnetic beads following the manufacture's instructions (Miltenyi Biotec Ltd., Bisley, UK). Additionally, tissue resident monocytes were isolated using anti-CD14 magnetic beads (Miltenyi Biotec Ltd., Bisley, UK).

2.2.4. Flow Cytometry

2.2.4.1. Cell Surface Staining

Freshly isolated cells ($1x10^6$) were transferred into 5 ml polystyrene FACS tube, washed twice in a cold FACS buffer (PBS w/o Ca^{2+}, Mg^{2+}, 2% FBS, 2mM EDTA) and finally resuspended in 100 μl of cold FACS buffer. To prevent non-specific binding of antibodies, Fc receptor was blocked using Hu FcR binding inhibitor (Cat no: 14-9161-73, eBioscience, Hatfield, UK) for 15 mins at 4°C prior to extracellular staining. Next, cells were incubated with required primary antibody or isotype controls (Table 2.2) at the dilution specified by manufacturer at 4°C for 30-40 mins in the dark. After incubation, cells were washed twice in cold FACS buffer. Just before analysis, 3 μl of 7-aminoactinomycin D (7-AAD, BD Bioscience) was added

into each sample to identify dead cells. Cells were analysed on a BD LSRII and data was analysed using FlowJo software (Tree Star Inc, Oregon, USA).

2.2.4.2. Intracellular Staining

The protocol was modified for a simultaneous analysis of surface molecules and intracellular antigens. The staining protocol was amended using a fixable dead cell exclusion dye (Zombie Aqua ^{TM} Dye, Biolegend, Cambridge, UK). Following the extracellular staining (as described in section 2.2.4.1), cells were washed in cold FACS buffer and then fixed and permeabilised using intracellular (IC) fixation and permeabilisation buffers, respectively (eBioscience Ltd., Hatfield, UK) following the manufacture's instruction. Subsequently, cells were incubated with the relevant antibodies in permeabilisation buffer for 30 mins at 4°C in the dark. After incubation, cells were washed in permeabilisation buffer and finally resuspended in permeabilisation buffer and analysed by flow cytometry.

2.2.4.2.1. H3K27me3 Staining

Cells were first fixed and then permeabilised for 30 mins at 4°C in the dark using a Foxp3 fixation/permeabilisation Set (cat no. 900-552, eBioscience Ltd., Hatfield, UK,). After indicated times, cells were washed in permeabilisation buffer (eBioscience, cat no. 00-8333) and incubated with primary, unconjugated H3K25me3 antibody for 1 h at 4°C in a dark. Subsequently, cells were washed in permeabilisation buffer and stained for 1 h at 4°C in a dark with secondary antibody, together with other intracellular antibodies, or isotype controls. Finally, cells washed in permeabilisation buffer, resuspended in FACS buffer and analysed by flow cytometry as described previously.

2.2.5. Chromatin Immunoprecipitation Assay (ChIP assay)

Chromatin immunoprecipitation assay is described in three sections: 2.2.5.1-3.

Antibodies used for ChIP Assays were listed in section 2.1. (Table 2.1).

2.2.5.1. Chromatin preparation

All reagents used for chromatin preparation are listed in Table 2.5.

Table 2.5: Buffers and reagents used for chromatin preparation and immunoprecipitation

Buffer	Composition
Nuclei Preparation Buffer	50 mM Tris-HCl, pH 8, 10 mM EDTA, 1% SDS
Shearing Buffer	0.1% SDS, 3% NaCl, 1% Triton X-100, 2% Tris-EDTA
Dilution Buffer	0.102% Tris, 0.51% NaCl, 0.102% Tris HCl, 0.018% EDTA
RIPA Buffer	10 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.1% SDS, 0.1% Na-deoxycholate, 140 mM NaCl
Elusion Buffer	20 mM Tris-HCl, pH 7.5, 5 mM EDTA, 50 mM NaCl, 1% SDS, 50 µg/ml proteinase K

Briefly, 0.1–0.25 x 10^6 cells were used per immunoprecipitation (IP) under cross-linking conditions. Cells were cross-linked with 1% formaldehyde for 10 minutes at room temperature on a shaker. Following quenching with 125 mM glycine, cells were harvested by scraping and washed once with cold PBS (w/o Ca^{2+}, Mg^{2+}). Next, the cells were resuspended in nuclei preparation buffer and incubated on ice for 10 minutes. After incubation, samples were vortex vigorously for 10 seconds and centrifuged for 10 minutes at ~ 180x g. Finally, the nuclear pellet was resuspended in shearing buffer supplemented with proteinase inhibitor cocktail (PIC, Roche Diagnostics Ltd., cat no: 11836170001) and incubated on ice for 10 minutes, vortexing occasionally. Next, samples were sonicated with an Ultrasonics Sonicator for 20 cycles (10 seconds/cycle) to obtain the chromatin length of approximately 1000 base pair (bp). After sonication, samples were centrifuged for 10 minutes at

13300x g and supernatant was collected, diluted 1:1 with dilution buffer and used for subsequent ChIP assays or stored at – 70 °C.

2.2.5.2. Preparation of antibody–Protein A magnetic beads complex and chromatin immunoprecipitation

The required amount of Protein A-coated paramagnetic beads (0.3 mg/0.1x10^6 cells) were washed twice in cold RIPA buffer and finally resuspended to desired concentration. The beads were then incubated with the specific antibody (2.5 µg) for at least 4 hours at 4°C on a rotator. After incubation, the RIPA buffer was aspirated, 100 µl of chromatin was added to the beads-antibody complex and all samples were further incubated overnight.

2.2.5.3. DNA elution, cross-link reversal and proteinase K digestion

The elution of the DNA from IP complex (chromatin/antibody/ Protein A beads), reversal of the DNA-protein cross-link, and digestion of the proteins were combined into a single 2 h step.

Briefly, after overnight incubation of chromatin with the antibody/Protein A bead complex, the chromatin was aspirated and the beads were washed 4 times with cold RIPA buffer and once with cold TE buffer. Next, 50 µg/ml proteinase K (Life Technology, cat no: AM2546) was added to elution buffer and beads were incubated for 2 h at 68°C on a shaker at 1300 rpm using Thermomixer. After incubation, the eluate was collected into eppendorfs and precipitated DNA fragments were isolated using ChIP DNA Clean and Concentrator (ZymoResearch, Cambridge bioscience, UK).

2.2.5.4. Genomic DNA quantification

The precipitated DNA material was analysed by Taqman quantitative real time (qRT)-PCR using a set of primers and a probe designed for *TNFA* transcription start site (*TNFA* TSS) with forward: GGGACATATAAAGGCAGTTGTTGG and reverse: TCCCTCTTAGCTGGTCCTCTGC primers in a combination with a probe: AGCCAGCAGACGCTCCCTCAGCAAG. The quantification performed in duplicate on an ABI PRISM 7900HT Sequence Detection System for absolute quantification of the amount of a specific histone modification or transcription factor associated with the transcription start side of *TNFA* gene. The plate was run using the following thermal conditions: stage I: 50°C for 2 minutes; stage II: 95°C for 10 minutes, stage III: 95°C for 15 seconds, 60°C for 1 minute for 40 PCR cycles.

The results obtained from Taqman qPCR were expressed as quantity mean (Qty mean) values. The Qty mean values were used to calculate percentage Input according to the formula:

Percent Input = Qty mean of IP sample/Qty mean of total chromatin sample * 100

IP – immunoprecipitation

The percent input value was calculated for each histone mark analysed including total chromatin sample and also IgG sample. Finally each histone mark sample was additionally normalised to IgG.

2.2.6. RNA extraction

RNA was extracted using trizol-chloroform. Cells were lysed by adding 200 µl of trizol per 10^6 cells. If tissue material was used for RNA extraction, 400 µl of trizol was used per biopsy. The lysates were stored at -70°C for subsequent RNA extraction or used immediately The RNA extraction was performed using the Qiagen RNeasy kit (cat no: 74104, Qiagen Ltd., Crawley, UK,). First, the trizol lysates were

mixed with 1/5th volume of chloroform and incubated for 5 minutes on a shaker at room temperature and centrifuged 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. 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) according to the manufacturer's instruction. Samples were stored at -70°C.

2.2.7. Reverse Transcription PCR (RT-PCR)

Reverse transcription was performed with SuperScript® III First-Strand Synthesis SuperMix following manufacturer's instructions (cat no: 11752250, Invitrogen Ltd., Paisley, UK). Each reaction was set up for no more than 1000 ng of RNA/sample. The RT-PCR cycles were set as follows: Stage 1: 10 minutes at 25°C, 30 minutes at 50°C, 5 minutes at 85°C, forever at 1°C. After adding *E.coli* RNaseH, all samples were additionally run through stage 2: 20 minutes at 37°C, forever at 1°C. All cDNA samples were stored at -20°C for future work.

2.2.8. Quantitative real-time PCR (qRT-PCR)

The RNA was extracted using the method described in section 2.2.6 and reverse transcribed with SuperScript® III First-Strand Synthesis SuperMix (Invitrogen Cat#11752250) as described in section 2.2.7. Quantitative real time RT-PCR was performed in duplicate on an ABI PRISM 7900HT Sequence Detection System, using primers and probes as listed in Table 2.6. The plate was run at the following thermal conditions: stage I: 50°C for 2 minutes; stage II: 95°C for 10 minutes, stage III: 95°C for 15 seconds, 60°C for 1 minute for 40 PCR cycles. Results were normalized to the abundance of B-Actin, GAPDH, UBB and RPL13A.

Table 2.6: Primers and probes used for analysis of RNA expression level

Gene of interest ID	**Forward primer**	**Reverse primer**	**Probe**
TNFα 3px	TCCTCTCTGCCATCAAGAGCC	GTCGGTCACCCTTCTCCAGC	TGGAAGACCCCTCCCAGATAGATGGGC
Housekeeping genes ID	**Forward primer**	**Reverse primer**	**Probe**
ACTB	GCTGTCCACCTTCCAGCAGA	CGCCTAGAAGCATTTGCGGT	AGCAAGCAGGAGTATGACGAGTCCGGC
GAPDH	CAAGGTCATCCATGACAACTTTG	GGGCCATCCACAGTCTTCTG	ACCACAGTCCATGCCATCACTGCCAT
UBB	CGGCAAGACCATCACTCTGG	AAAGAGTGCGGCCATCTTCC	TGGAGCCCAGTGACACCATCGAAAATG
RPL13A	GGGACTGCAGGTGGTGAC	GGCCTCAGATGGTAGTGCAT	AGTTTCCCGACCATGAGATG

2.2.9. Reverse Transcription and RT2 Profiler Array PCR

Prior RT^2 Profiler Array PCR, reverse transcription was performed with the RT2 First Strand Kit (Qiagen, cat no: 330401) 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 sample and gently mixed. 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 (cat no: 330401, Qiagen Ltd., Crawley, UK). Finally, the master mix (10 µl/well) was loaded onto Human Common Cytokine RT2 Profiler PCR Array plate (PAHS-021E-4, Qiagen Ltd., Crawley, UK). The plate was centrifuges 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 normalised 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 normalised copy number for each gene. Table 2.7 represents the set of all 84 genes included in RT-PCR Assay.

Table 2.7: Index of 84 genes panel for Human Common Cytokine RT2 Profiler PCR Array plate (Qiagen, PAHS-021E-4)

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

Fields in gray and green represent housekeepers and controls, respectively. The reaction was set for 340 ng per sample/gene panel

2.2.10. JMJD3 inhibition

2.2.10.1. Cell culture with GSK-J4 compound

Human blood-derived primary macrophages (M1) (methods 2.2.1.) and intestinal macrophages (method 2.2.2.) were cultured with H3K27me3 histone demethylase inhibitor at a concentration of 30 μM with or without E. coli LPS (100 ng/ml) for 1 h. The experimental set up is shown in Figure 2.1.

2.2.10.2. Organ culture with GSK-J4 compound

If mucosal resection tissue was used, small biopsy sized fragments were obtained using fine scissors. Next, biopsies or biopsy size fragments were placed in 300 μl serum-free HL-1 medium (supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin) with or without the compound (30 μM) in 24-well plate format. All samples were cultured for 12h or 24 h at 37°C, 5% CO_2. Supernatants were collected and stored at -70°C for future analysis. Additionally, the tissues were stored in Trizol at - 70°C.

2.2.11. Cytokine Assay

Human recombinant TNF-alpha, IL-6, IL8, IL-24 DuoSet ELISA kit (R&D Systems) were used accordingly to the manufacture‘s instruction.

2.2.12. Statistical analysis

Except for common human cytokine profiler RT2 PCR Array, Graph Pad Prism 4.03 version was used. Data analysed using Student *t*-test. Gene microarray analysis was performed by collaborators at GSK using one-way ANOVA analysis.

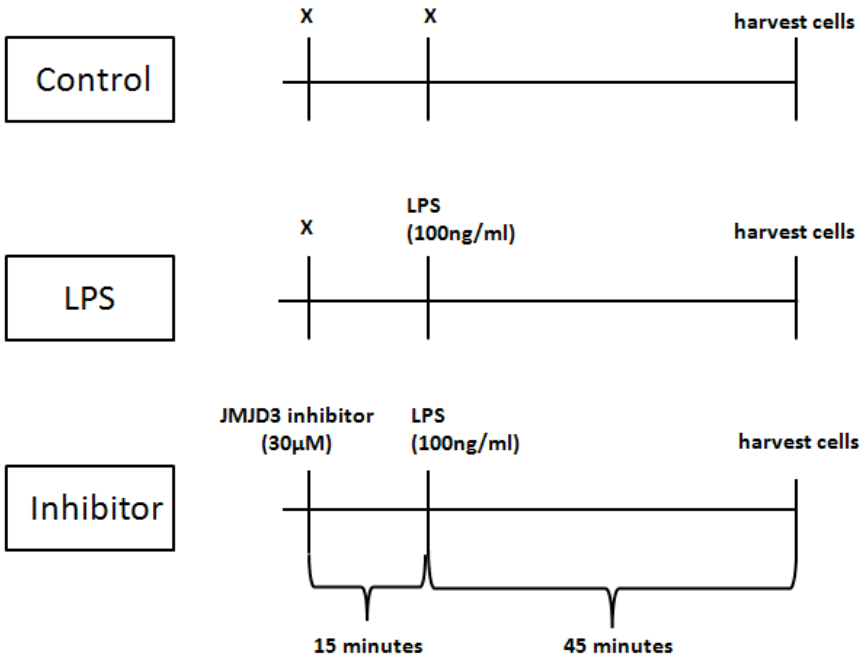


Figure 2.1: The study of JMJD3 inhibitor experiment set up.

Chapter 3

Identification of phenotypic markers of intestinal macrophages for chromatin immunoprecipitation (ChIP) assay

3.1. Introduction

Intestinal macrophages (IMACs) are essential players in the innate immune system of the gut. However, the fact that they are largely anergic to activation with pro-inflammatory cytokines and PAMPS is a unique feature. The molecular basis for intestinal macrophage anergy is not known.

In recent years, it has become clear that chromatin accessibility plays an important role in gene expression. Epigenetic regulatory mechanisms involve complex crosstalk between different regulatory elements, and those processes can be gene- or inducer-specific. Hence, there is great interest in understanding how chromatin remodelling regulates gene activation/repression. Much of the work on epigenetic regulation of immune responses comes from the work on mouse models or human cell lines. However epigenetic regulation of gene expression has emerged as a potentially important determinant in the development of many diseases, including inflammatory bowel disease (IBD) (Scarpa et al., 2012). Chromatin Immunoprecipitation (ChIP) is the most widely used procedure to examine histone modification and DNA/protein interaction.

Standard ChIP assay requires a large number of cells ($10^6/10^7$) per immunoprecipitation (IP) and is time consuming. Intestinal macrophages are only a small component of mononuclear cells in normal and inflamed gut and no previous studies have looked at their epigenetic modifications. Therefore the first studies involved optimising the method of intestinal macrophages isolation. This work was done in collaboration with Francesca Ammoscato.

3.2. CD33 marker as an alternative to CD68 for functional studies

Intestinal macrophages comprise only around 5% of total immune cells in lamina propria. The differentiation of monocytes into intestinal macrophages progresses through stages associated with acquisition or loss of certain markers (e.g., CD14) (Smith *et al*., 2011). The gold standard macrophage marker CD68 has been used to identify macrophages in various tissue types (Smythies et al., 2005). CD68 is a heavily glycosylated protein localised in the lysosomal membranes of macrophages (Smythies *et al*., 2005), and it functions as a scavenger receptor for oxidised low density lipoproteins (Ramprasad et al., 1996; Song et al., 2011). Even though separation of cells using CD68 is potentially the best way to isolate intestinal macrophages it is an intracellular marker and cells have to be permeabilised to allow the antibody to enter the cells. This makes CD68 a rather poor marker for any functional studies. Hence, the first part of this project aimed to identify other marker(s), which would allow recovery of sufficient cells for functional studies.

We chose to investigate the potential of CD33 marker, a member of the Ig superfamily. In many publications CD33 has been broadly referred as a marker for macrophages co-expressed with CD68 (Rogler et al., 1998; Kamada et al., 2008). CD33 belongs to the sialoadhesin family of sialic acid-dependent cell adhesion molecules (Munday et al., 1999; Lajaunias et al., 2005). To date, sialic acid (α2,3-linkage) is the only known cell surface ligand (Crocker and Varki, 2001). CD33 expression is largely restricted to the myeloid lineage (Crocker and Varki, 2001). Thus, it is highly expressed on myeloid committed cells in the bone marrow and circulating monocytes (Hernandez-Caselles et al., 2006). Some other data also suggest that CD33 can be expressed on activated $CD4^+$ T cells, NKT and B cells (Hermandez-Caselles *et al.*, 2006). However, lymphoid specific CD33 was shown to

display lower molecular weight than myeloid CD33 (Hernandez-Caselles et al., 2006).

By performing flow cytometry analysis we were able to show that about 15% of $CD45^+$ LPMCs were $CD33^+$ and this level remained constant across control subjects and patients with IBD (Figure 3.1), although with considerable inter-individual variation.

Since CD68 is the gold standard marker for macrophages, we next performed double staining of CD33 and CD68. The analysis revealed that the majority of LPMCs also expressed macrophage marker $CD68^+$ (Figure 3.2A). Moreover, CD68 expression was significantly ($p<0.05$) increased in patients with IBD, again with donor variability between analysed groups (Figure 3.2B).

It was previously shown that human intestinal macrophages do not express innate response receptors (Smith et al., 2001). Although, many previous studies have indicted that CD14 is downregulated in intestinal macrophages (Smith et al., 2001; Smythies *et al*., 2005), a small number of $CD14^+$ intestinal macrophages were also reported in normal human intestine (Kamada et al., 2008). Therefore, CD14 marker was also analysed on LPMCs.

In the normal human intestine, the majority of $CD33^+$ LPMCs were $CD14^-$ and there was no significant difference between normal and inflamed and uninflamed IBD mucosa (Figure 3.3B). However, a small population (~2%) of $CD14^+CD33^+$ cells was present in normal human intestine (Figure 3.3C), and these cells were significantly increased in inflamed IBD mucosa. The highest increase of $CD14^+$ intestinal macrophages was observed in inflamed UC mucosa samples ($p< 0.01$). There were no significant differences in the number of $CD14^+CD33^+$ cells between healthy and uninflamed IBD mucosa (Figure 3.3C).

Finally, we also studied the CD68 co-expression together with CD33 and CD14 markers. As shown in Figure 3.4, in $CD33^{+}CD14^{-}$ LPMCs, the majority of cells were also $CD68^{+}$ (80% for HC, 97% and 86% for CD and UC) (Figure 3.4B). A similar proportion was also observed in $CD33^{+}CD14^{+}$ LPMCs (70% for HC and 90% for IBD) (Figure 3.3A). In both cases, there was no significant difference between controls and IBD patients.

Finally, knowing that lymphocytes also co-express CD33 (Hernandez-Casselles *et al*., 2006), although of lower molecular weight, we also checked the co-expression of CD33 and CD3 (Figure 3.5). We could not detect CD33 expression on $CD3^{+}$ LPMCs, however only mucosa of healthy control subjects was analysed.

3.3. Optimising cell yields and speed of isolation by FACS and MACS

The first approach was to sort the intestinal macrophages with anti-CD33 antibodies using fluorescence activated cell sorting (FACS). By FACS it was possible to obtain ~15% of $CD33^{+}$ LPMCs (Figure 3.1). Unfortunately, the method turned out to be time-consuming and expensive. We then moved to the magnetic cell sorting (MACS) method, which enabled us to isolate around 5-6% of $CD33^{+}$ cells from the total numbers of LPMCs (Table 3.1). In terms of actual numbers, by using CD33 positive magnetic beads sorting method, it was possible to obtain around 2-3 million cells for subsequent experiments. The isolated cells population had also a high purity and importantly the procedure was much faster (~ 1.5 h). Table 3.1 shows the overall numbers of cells isolated for functional studies.

3.4. Optimisation of Chromatin Immunoprecipitation (ChIP) method

When these experiments were commenced, a standard ChIP assay required ~1x10^6 million of cells per immunoprecipitation (IP). For the purpose of this project, fewer cells per IP would greatly enhance the numbers of experiments that could be done with the limited number of $CD33^+$ cells we could isolate from the gut. Optimisation of ChIP protocol was therefore performed using GM-CSF differentiated human primary macrophages. The ChIP method that was used involved the formaldehyde cross-linking in cell culture medium, thus providing a rapid fixation of the chromatin structure, and also eliminating the additional loss of cells. Crosslinking with formaldehyde requires subsequent chromatin shearing by sonication. As tested on 1.2% agarose electrophoresis (Figure 3.6A) the sonication regime 20 x 0.10 minute impulse on (amplitude 10) using SonicPrep 150 sonicator provided the chromatin fragment of between 200-400 bp.

For ChIP assay, antibodies are the most important factor for obtaining a good level of precipitation. In all cases we have decided to work with ChIP grade, polyclonal antibodies rather than monoclonal antibodies, since the polyclonal recognises larger numbers of epitopes on the target. To optimise ChIP protocol, two negative controls were used; rabbit IgG antibody and also 'no antibody-beads only' control sample. First, two concentrations of antibody against chosen target of interest (e.g., RNAPII and H3K27me3) were tested. As shown in Figure 3.6B, at the lowest concentration of antibody (2.5 µg) only a strong signal for RNAPII was detected (~ 22% input). The H3K27me3 pull down was at the same level as for IgG and "beads only" negative controls (~ 2% input). However two-fold increase of H3K27me3 signal (~ 10% input) was obtained at the higher antibody concentration (5 µg) (Figure 3.6C).

Additional increase in the number of washes even further improved the signal by reducing the background noise (Figure 3.6C-D).

3.5. Discussion

Given their crucial role in driving the innate and adaptive immune responses, for a long time now many studies have focused on accurate identification and classification of intestinal mucosa macrophages. It is truly difficult task considering that macrophages are highly plastic cells and that an environmental milieu greatly influences their phenotype and function. This is especially true in human, unlike the mouse where lineage tracers can be used. Additionally, macrophages and DC phenotypic profile also overlaps complicating their identification even more (Kamada et al., 2009; Bain et al., 2014).

At the time when I started my PhD, to my knowledge, it was a first attempt made to investigate how gut macrophages regulate inflammatory responses through epigenetic modification. In this chapter I set out a task to optimise two methods essential to the project. Firstly, it was necessary to develop a method for isolation of intestinal macrophages from collected tissue material. Next to optimise a technique to study the chromatin architecture of these cells.

Primarily, it was shown that $CD33^+$ cells constituted about 15% of total LPMCs, and that this level remained constant in healthy and IBD mucosa as assessed by FACS.

Moreover, it was possible to obtain a sufficient number of $CD33^+$ intestinal macrophages for consequent functional analysis. Using MACS sorting method, on average about 2-4 million of $CD33^+$ macrophages were isolated, which constitute 5-6% of total LPMCs (Table 3.1).

The potential of CD33 was evaluated in combination with CD68; a well defined marker of tissue macrophages. By exploiting this, we have shown that CD33 marker highly co-expressed with CD68 (Figure 3.4).

We also investigated co-expression of CD33 with CD14 marker (Figure 3.3). Under normal homeostatic conditions, the majority of $CD33^+$ LPMCs did not express CD14. However, a small subset of $CD14^+$ intestinal macrophages was also detected and this population increased significantly in inflamed IBD mucosa. Those findings were consistent with previous reports (Smith et al., 2001; Kamada et al., 2008, 2009; Lampinen et al., 2013).

Although we have not performed additional studies of $CD33^+CD14^+$ cells, analysis of these cells by Grimm et al., (1995) and Kamada et al., (2008) suggested that $CD14^+$ intestinal macrophages in the inflamed intestinal mucosa were newly recruited proinflammatory blood monocytes (Grimm et al., 1995) and that this subset of macrophages was a source of pro-inflammatory cytokines (e.g., IL-23, TNF-α) (Kamada et al., 2008, 2009). The accelerated recruitment of monocytes to the intestinal mucosa was observed in response to bacterial and viral infections (Orenstein et al., 1997; Hale-Donze et al., 2002; Smith et al., 2011). Therefore, it is likely that $CD14^+$ intestinal macrophages represent monocyte-derived macrophages that have left the circulation at the site of infection and this pro-inflammatory cytokine/chemokine profile was dysregulated by the environmental factors (Greenwell-Wild et al., 2002; Vazquez et al., 2006; Smith et al., 2011). Indeed, additional FACS analysis revealed that these two subsets of intestinal macrophages; $CD33^+CD14^-$ and $CD33^+CD14^+$ cells also co-expressed macrophage marker CD68 (Figure 3.4) at a constant and comparable level, which was consistent with previous reports (Kamada et al., 2008). Hence, these results suggest that $CD14^+$ cells denote a

new subset of intestinal macrophages present in inflamed mucosa, rather than freshly recruited blood monocytes. Interestingly, Kamada et al. (2008) also showed that the distinct differences between healthy and IBD $CD14^+$ intestinal macrophages also exist.

In conclusion, it was determined that CD33 is a good alternative to CD68 and could be used for intestinal macrophage isolation for this project. However, since other cells of myeloid lineage (e.g., monocytes, DCs) (Kamada et al., 2008), and also activated T cells, NKT and B cells express this molecule, we would expect to have some level of contamination coming from these cells in our isolated population. However, the level of this contamination could reach no more than 10-15% of total $CD33^+$ LPMCs (Figure 3.4). Therefore, it was concluded that at this point, using CD33 was the only way of macrophage isolation.

These experiments allowed us to establish a technique for isolation of intestinal macrophages, for consequent functional studies involving a use of chromatin immunoprecipitation method. Subsequent optimisation of ChIP method allowed reducing the number of cells to $0.25x10^6$ per IP, which was sufficient for the project.

In the next chapter, I set out to implement these methods to investigate chromatin architecture of intestinal macrophages and its involvement in inflammatory responses.

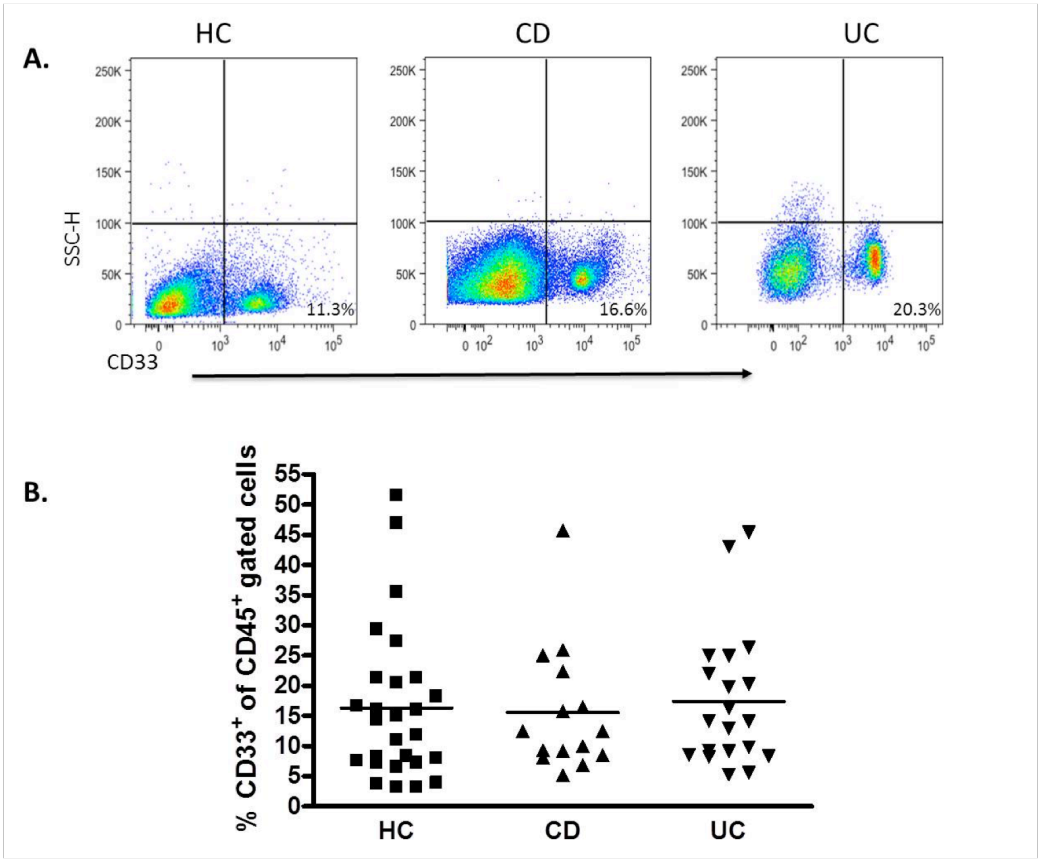


Figure 3.1: The analysis of CD33 expression on LPMCs

LPMCs were isolated from colonic tissue resection from IBD patients and controls using collagenase-Ficoll method as described in Chapter 2. **A).** Representative dot plots of CD33 expression by CD45-gated LPMCs. **B).** The mean proportion of $CD33^+$ cells within each population. About 15% of $CD45^+$ LPMCs were $CD33^+$, and this level remained constant across control and patients with IBD, although with considerable inter-individual variation. No significant difference was observed between analysed individuals (HC n=27, CD n=15 and UC n=20).

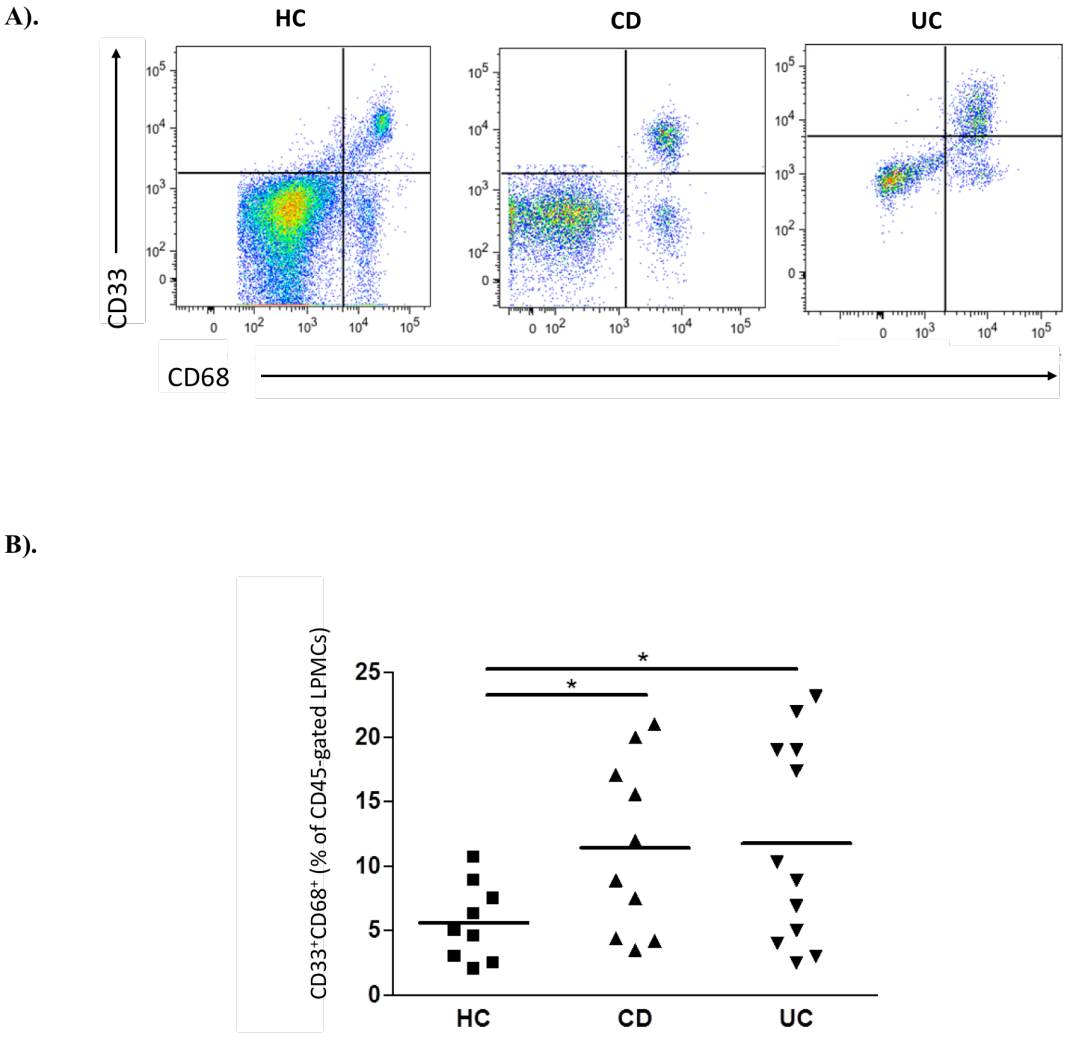


Figure 3.2: The expression of CD33 and CD68 on CD45-gated LPMCs isolated from HC and IBD mucosa

LPMCs isolated from normal mucosa specimens and of IBD patients were analysed by FACS for CD68 and CD33 expression. **(A).** Representative dot plots showing CD68 and CD33 expression by CD45-gated LPMCs. **(B).** $CD33^{+}CD68^{+}$ expression among groups: HC (n=9) CD (n=10) and UC (n=12). Two-fold increase in $CD33^{+}CD68^{+}$ proportion was observed in IBD mucosa (*) $p<0.05$.

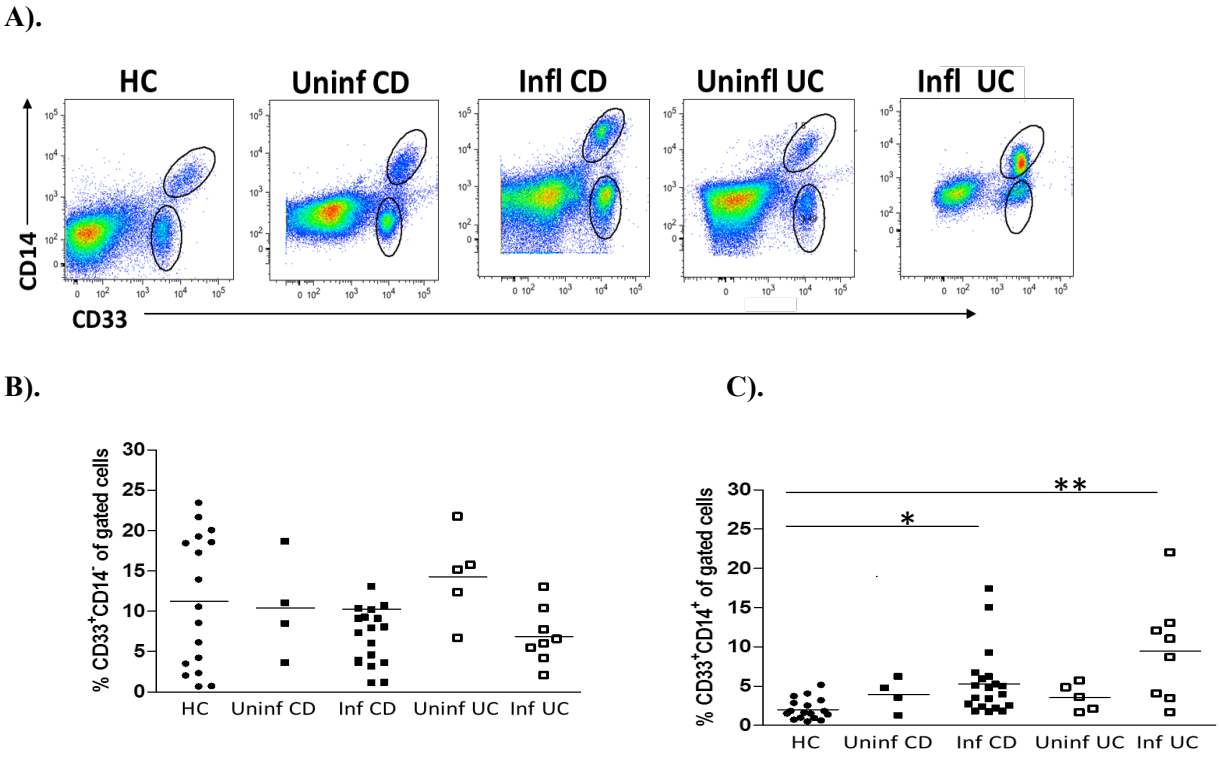


Figure 3.3: The expression of CD33 and CD14 markers on LPMCs

A). Representative dot plots showing the expression of CD33 and CD14 markers by CD45-gated LPMCs using flow cytometry analysis. **(B)** The graph showing the expression of $CD33^{+}CD14^{-}$ by LPMCs between control subjects and inflamed and uninflamed IBD samples. The percentage of $CD33^{+}CD14^{-}$ LPMCs was not significantly different between HC, CD and UC individuals. **(C)**. The graph showing the expression of $CD33^{+}CD14^{+}$ by LPMCs between control subjects and inflamed and uninflamed Crohn's and ucelerative colitis samples. The proportion of recruited $CD33^{+}CD14^{+}$ LPMCs increased significantly in inflamed IBD. There was no significant difference between controls and uninflamed IBD mucosa. Data is a representative of HC (n= 18), uninf. CD (n=4), inf. CD (n=17), uninf. UC (n=5), inf. UC (n=8), (*)$p< 0.05$, (**)$p<0.05$.

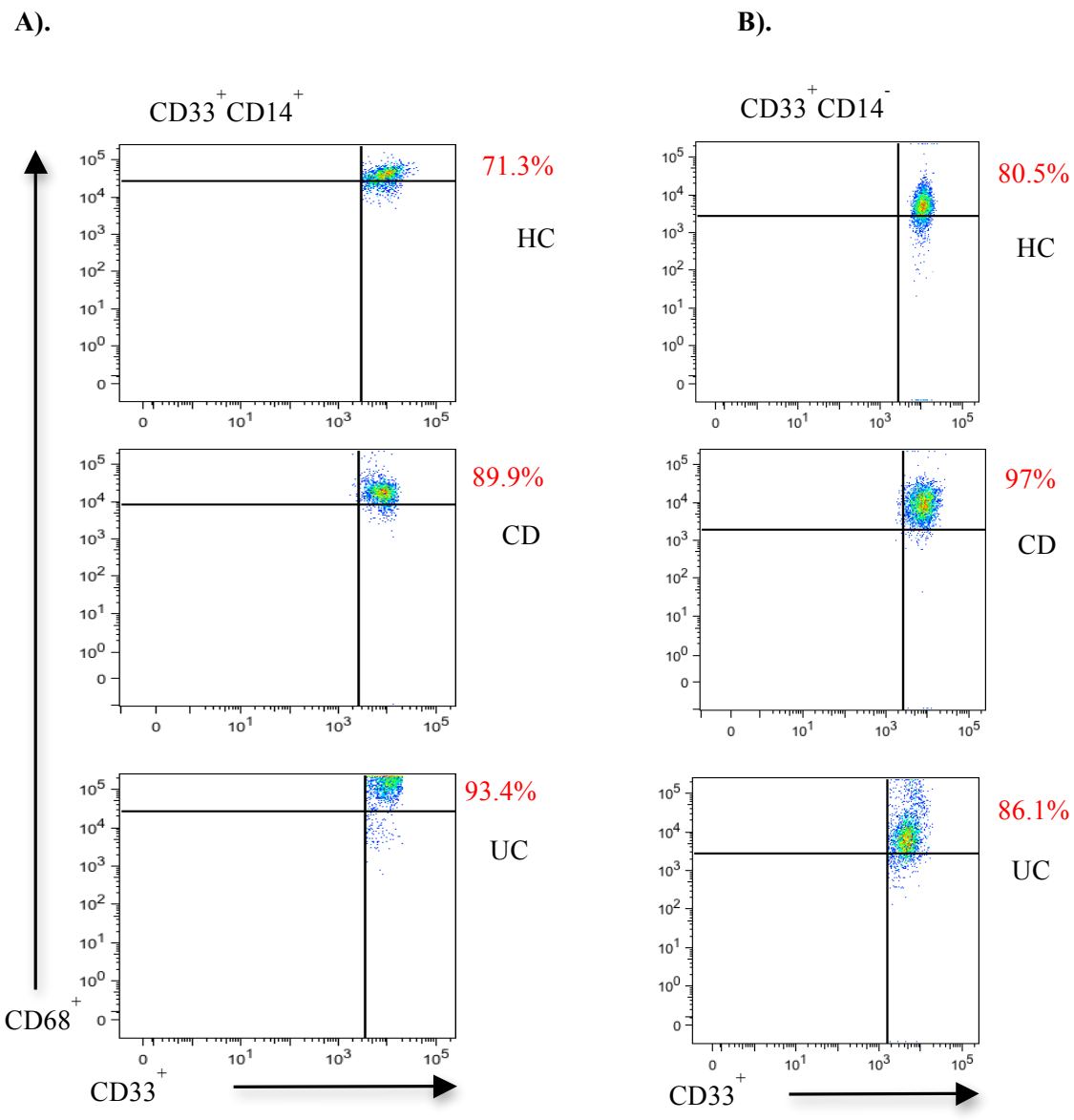


Figure 3.4: The expression of CD68 marker by recruited (A) and resident (B) intestinal macrophages

Representative dot plots showing the expression of CD68 marker by **(A)** $CD33^{+}CD14^{+}$ and **(B)** $CD33^{+}CD14^{-}$ intestinal macrophages isolated from IBD patients and controls as assessed by flow cytometry analysis. Both subsets of intestinal macrophages expressed comparable level of CD68 marker across different tissue type (IBD vs. control). In both cases, there was no significant difference between controls and IBD patients.

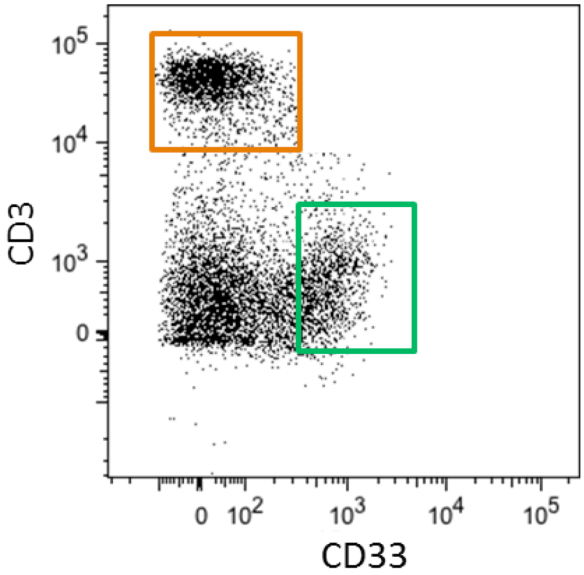


Figure 3.5: The CD33 and CD3 co-expression in LPMCs isolated from normal mucosa

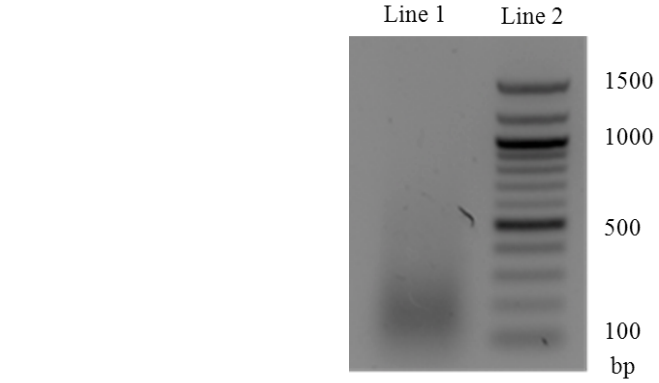
LPMCs pooled from 3 HC patients were analysed for the expression of CD33 and CD3 by FACS. Representative dot plot of CD33 and CD3 expression by live-gated cells is shown.

	LPMCs [Average cell number]	$CD33^+$ LPMCs [Average cell number]	%
	$x10^6$	$x10^6$	
HC	100±17	**5.4±1.0**	6.0±0.8
UC	68±11	**4.5±0.4**	6.1±0.7
CD	114±28	**6.8±2.2**	4.8±0.9

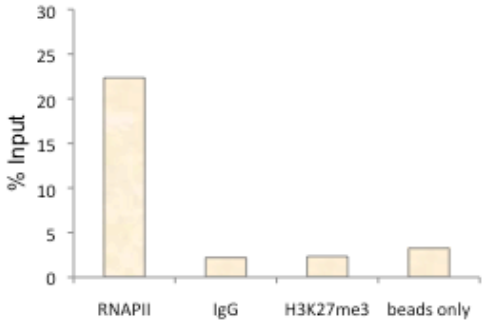
Table 3.1: The total number of $CD33^+$ LPMCs isolated from collected intestinal mucosa using MACS method

Using MACS sorting method, it was possible to isolate about 4-5 million of $CD33^+$ cells, which constitute 5-6% of total LPMCs.

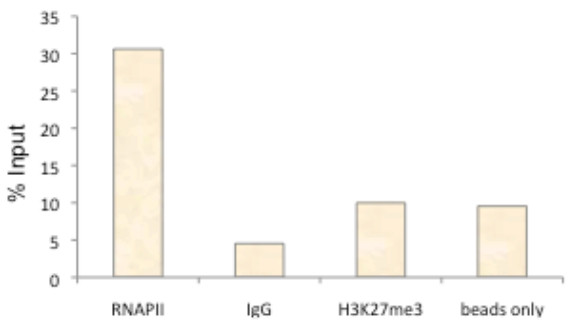
A).



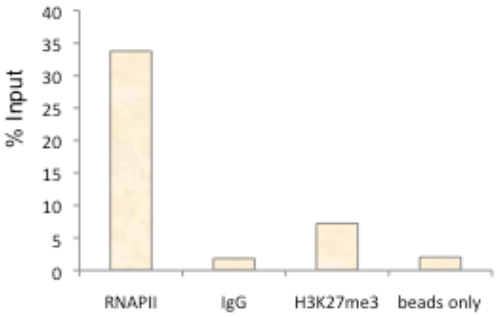
B).



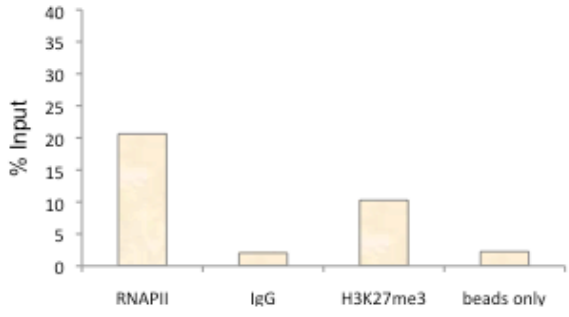
C).



D).



E).



3.6: Optimisation of chromatin immunoprecipitation protocol

A). Agarose (1.2%) gel electrophoresis of DNA isolated from cell lysate. Samples prepared with sonication regime: 20x0.10 minute impulse on/(amplitude 10). The sonication was optimised using GM-CSF differentiated human primary macrophages ($2x10^6$). The chromatin length between 200-400bp was recovered. **Line1:** 10 µl of cell lysate was loaded to assess the chromatin length. **Line 2:** 100 bp ladder. **B-E). ChIP protocol was optimised for antibody concentration using GM-CSF differentiated human primary macrophages ($0.25x10^6$ per IP).** Antibody concentrations used; **B).** 2.5µg, (n=1) **C).** 5.0µg, (n=1) **D/E).** 5.0µg with increased number of washes to eliminate a background noise (n=2).

Chapter 4

Characterisation of chromatin architecture at the *TNFA* promoter in human intestinal macrophages

4.1. Introduction

Intestinal macrophages undergo a specific process of differentiation, which is believed to be essential for their function in the innate immune system of the gut. Under homeostatic conditions cytokines in the local environment, such as TGF-β made by stromal cells and epithelial cells, induce phenotypic and consequently functional differentiation of newly recruited monocytes into non-inflammatory intestinal macrophages (Smith et al., 2005). Macrophages isolated from mucosa of healthy gut show tissue-specific features associated with downregulation of proinflammatory cytokines (Smythies et al., 2005). For example, most lack CD14 needed for responses to LPS through TLR4.

A state of anergy or transient hyporesponsiveness can be achieved through repeated or prolonged stimulation of monocytes or primary macrophages with inflammatory stimuli (e.g., LPS). Consequently, this leads to downregulation of acute proinflammatory genes, such as *TNFA*. The drive of the tolerant state is thought to prevent excessive tissue damage. In the context of the gut, where there is stimulation by bacterial PAMPs from the lumen, this anergy prevents cytokine production, but the cells remain highly phagocytic and with the capacity to kill invading microorganisms. Notably, IFN-γ priming has been shown to abrogate endotoxin tolerance, thus restoring responses to LPS stimulation (Chen et al., 2010).

The proximal region of *TNFA* promoter (spanning -200 bp upstream of the TSS) mediates initiation of TNF transcription in response to a wide range of stimuli (Tsytsykova and Goldfeld, 2002). To understand the epigenetic architecture of intestinal macrophages in health and disease, TNF-α was selected due to its crucial role in driving pathology in the gut.

4.2. Sustained silencing of acute proinflammatory genes (e.g. *TNFA*) is regulated by chromatin alteration

To investigate the epigenetic state of the *TNFA* gene in intestinal macrophages, $CD33^+$ LPMCs were isolated from mucosa of 13 IBD patients and 10 control subjects and RNA was analysed for *TNFA* transcripts. As shown in Figure 4.1A, only intestinal macrophages isolated from CD patients showed increased *TNFA* mRNA levels. A significant (p=0.05) 3-fold increase in the *TNFA* expression was observed in CD macrophages when compared with controls or even UC samples (Figure 4.1A). Additionally, intestinal macrophages were also compared with blood monocytes and GM-CSF derived primary human macrophages. Monocytes and GM-CSF derived macrophages were activated with LPS (100ng/ml) for 1h. As shown in Figure 4.1B-C and Figure 4.2A, LPS stimulation significantly increased *TNF* transcript levels in each cell type. However, intestinal macrophages isolated from healthy mucosa did not respond to activation with LPS (Figure 4.2B).

Next, to investigate whether histone modifications contribute to the *TNFA* silencing state, macrophages from intestinal mucosa of controls were isolated and chromatin immunoprecipitation (ChIP) assay was performed. Since a small proportion of intestinal macrophages also express CD14, some of the cells were stimulated with LPS (100ng/ml/2h). Genomic DNA was analysed by Taqman qPCR for epigenetic changes of interest around the transcription start site (TSS) region of the *TNFA* gene. Since H3K27me3 is a silencing mark associated with the gene promoter, it was of interest to study this particular histone mark. Additionally, RNAPII, and its phosphorylated forms RNAPII-pS5 and RNAPII-pS2 as well as total H3 (tH3) were analysed. The ChIP assay revealed high prevalence (50% input) of H3K27me3 at the *TNFA* TSS (Figure 4.3A). Additional LPS activation did not alter H3K27me3

enrichment at this region (Figure 4.3A). The same was true for tH3 (Figure 4.3B). Also, RNAPII level did not change upon LPS stimulation and remained at relatively low levels (2.5−3.0% input, Figure 4.4A). The LPS treatment also did not influence the phosphorylation level at serine-5 and -2 of RNAPII, indicating that neither initiation of transcription nor elongation occurred (Figure 4.4B-C). The RNAPII-pS5 showed the highest enrichment at the *TNFA* TSS (40% input) when compared with – pS2 (~ 20% input) or RNAPII (3.0%). Together these data show a silent state of the *TNFA* promoter of intestinal macrophages isolated from control individuals and that LPS stimulation does not prime the gene for active transcription. Unfortunately, it proved impossible to isolate sufficient number of $CD14^{+}CD33^{+}$ macrophages from normal gut to carry out additional ChIP analysis to determine if this minor subset was LPS responsive.

4.3. In macrophages isolated from IBD patients there are changes in a chromatin architecture of the *TNFA* promoter

Next, to investigate if increased the *TNFA* mRNA expression level correlated with epigenetic changes, ChIP assay was performed on macrophages isolated from intestinal mucosa of 11 IBD patients and 7 control subjects. A number of repressive and permissive histone modifications were investigated which are known to be associated with a gene promoter. Silencing marks such as H3K27me3, H3K9me3 and H3K9me1, as well as activating marks such as H3K4me3, H3K4me1 and also RNAPII were selected. Results of analysis are presented in Figure 4.5A-G. The increased levels of the *TNFA* transcripts in Crohn's disease might be associated with a removal of silencing marks from the *TNFA* TSS region. Among all silencing modifications assessed in normal intestinal macrophages, H3K27me3 showed the

highest occupancy at the *TNFA* TSS (~ 40% input) (Figure 4.5A) as oppose to methylation at H3K9 (~ 20% input for H3K9me3 and H3K9me1) (Figure 4.5B-C). Moreover, in macrophages isolated from mucosa of patients with CD but not UC, H3K27me3 mark showed a significant 2.5-fold reduction (from 40 to 15% input) compared to macrophages from normal gut.

In CD, an active transcription could also be a result of reduced binding of other silencing marks such as H3K9me3 and –me1, however these changes were not significant (Figure 4.5B-C). Other histone methylations were also analysed, such as H3K4me3 and –me1 (Figure 4.5D-E). In IMACs isolated from control mucosa, the highest binding level was observed for H3K4me1 (~70% input). The occupancy of H3K4me3 was about ~40% input (Figure 4.5D-E). The reduced binding of H3K4me1 (50% input) was observed in both CD and UC macrophages (Figure 4.5E), however the difference was not signifcant when compated with controls. H3K4me3 also was reduced, but only in macropahges isolated from CD mucosa (Figure 4.5D). The chromatin state was also assessed by looking at total H3 (tH3) (Figure 4.5F). The 2-fold reduction (from 30% to 15% input) in tH3 was observed in CD macrophages but not from UC or control samples.

Interestingly, no change in binding was observed for RNAPII, which remained at very low and constant level in all cells (~1% input) (Figure 4.5G). Unfortunately due to insufficient DNA we did not have a chance to investigate the state of RNAPII phosphorylated forms to fully understand the level of active transcription.

We also compared the chromatin architecture of intestinal macrophages with GM-CSF derived primary human macrophages. The GM-CSF macrophages were activated with LPS (100ng/ml/1h). The comparison between these groups revealed that macrophages isolated from mucosa of control subjects resemble the naïve GM-

CSF macrophage profile (Figure 4.6). High enrichment of H3K27me3 was detected in naïve GM-CSF macrophages (~25% input), and in intestinal macrophages isolated from control samples (~ 40% input) and UC mucosa (~ 20% input) (Figure 4.6A). The LPS activation (100ng/ml/1h) drove a reduction of H3K27me3 at the *TNFA* TSS to the same level as seen in macrophages isolated from CD mucosa (Figure 4.6A). Interestingly, the same trend was observed for H3K4me3 mark (Figure 4.6B). However, LPS stimulation did not affect H3K4me1 binding in GM-CSF macrophages (50% input) (Figure 4.6C).

We also analysed the recruitment of RNAPII to the *TNFA* TSS upon LPS activation. The LPS stimulation activated RNAPII recruitment to the *TNFA* TSS, since a 2-fold increase in binding was detected (from 20% to 40% input) (Figure 4.6E). Additional analysis of RNAPII at *TNFA* TSS revealed that intestinal macrophages, whether isolated from control or IBD mucosa, are rather unique, since RNAPII level was at surprising low level (1% input) (Figure 4.6E).

The removal of the methyl mark from H3K27me3 is facilitated by JmjD3 demethylase upon inflammatory stimuli. Jumonji D3 (JmjD3) and its paralog, UTX are two related histone demethylases specific to H3K27me3 which regulate inflammatory responses in the context of LPS macrophage activation (Agger et al., 2007; De Santa et al., 2007, Kruidenier et al., 2012). JmjD3 is preferentially recruited to the transcription start sites of activated genes (De Santa et al., 2009), hence it was of interest to investigate, if increased mRNA levels of *JMJD3/UTX* are seen in intestinal macrophages from IBD patients. *JMJD3* and *UTX* expression was observed in normal and IBD macrophages (Figure 4.7). Increased *JMJD3* and decreased *UTX* transcript level were detected in intestinal macrophages isolated from Crohn's disease patients, although the difference was not significant (Figure 4.7).

Moreover, *UTX* expression was significantly higher than *JMJD3* in all tissue types (Figure 4.7).

4.4 . Differentiation of blood monocytes into resident gut macrophages in the healthy intestine is associated with the acquisition of repressive histone marks

The precursors of intestinal macrophages are blood monocytes, which are attracted to the lamina propria upon exposure to the endogenous chemoattractants in the non-inflamed mucosa or by inflammatory chemokines during inflammation (Bain and Mowat, 2014).

Since we observed that increased *TNFA* transcript level in CD intestinal macrophages was associated with a removal of repressive marks, it was of interest to investigate, if the establishment of repressive chromatin architecture is set during blood monocytes differentiation into resident intestine macrophages. To do so, we compared histone modification profile between blood monocytes and intestinal macrophages.

As shown in Figure 4.8A, in healthy gut, the differentiation of blood monocytes into resident intestinal macrophages is associated with deposition of H3K27me3 and H3K9me3 silencing marks at the *TNFA* TSS.

In monocytes isolated from blood of control individuals and patients with CD, H3K27me3 prevalence was at the same level (~10% input). Blood monocytes isolated from UC patients showed higher (2-fold) enrichment of this mark (~30% input) (Figure 4.8A). However, the difference between groups did not reach a significant difference.

The increased binding (from 2% to 20% input, p = 0.06) of H3K9me3 was also more profound in macrophages isolated from normal mucosa than in macrophages isolated from IBD mucosa when compared with blood monocytes (Figure 4.8C). Additionally, H3K9me3 could be hardly detected in blood monocytes isolated from control and IBD samples (Figure 4.8C). Also, there were no differences between blood monoctyes and intestinal macrophages in H3K9me1 binding as the occupancy of this mark was within range of 10-20% input (Figure 4.8D).

We speculated that a recruitment of silencing marks seems to be tissue specific, since there was no difference in H3K27me3 and H3K9me3 binding between controls and IBD blood monocytes, and also IBD mucosa macrophages (Figure 4.8). Only blood monocytes undergoing differentiation into intestinal resident macrophages (control macrophages), under homeostatic conditions acquired H3K27me3 and H3K9me3 repressive marks. Interestingly, no differences were observed in H3K4me3 and H3K9me1 binding between blood monocytes and IBD intestinal macrophages (Figure 4.9B-D). The enrichment of those marks remained constant across all analysed cell types. However, changes in H3K4me1 were detected between healthy control and UC monocytes and intestinal macrophages (Figure 4.9). We have also observed that in healthy individuals, the differentiation was associated with significant ($p \leq 0.05$) increase in H3K4me1 binding (from 40% to 80% input) (Figure 4.9A). In CD, H3K4me1 remained constant at 40% input in blood monocytes and also intestinal macrophages. Whereas, in UC, macrophages lost H3K4me1, since a significant ($p \leq 0.001$) 3-fold reduction of this mark was detected.

Additionally, we have also analysed the enrichment of RNAPII and its phosphorylated forms, as well as JMD3 direct binding to the *TNFA* TSS. The data suggests that increased JMJD3 binding to the *TNFA* promoter can occur in IBD

environment (Figure 4.9C). However, this is only preliminary conclusion. The same can be speculated for RNAPII, since increased enrichment of RNAPII, -pS5 and -pS2 was detected in blood monocytes of IBD patients (Figure 4.10).

4.5. Discussion

Chromatin modification plays an important role in macrophage polarisation and function, although a current knowledge in this field is still limited (Ivashkiv et al., 2013). Growing evidence supports the idea that epigenetic changes contribute to macrophage reprogramming and lead to tailored gene expression in response to gut environmental factors. There is still ongoing search to identify epigenetic mechanism regulating macrophage tolerisation (Ivashkiv et al., 2013). It has been recognised that a better understanding of IBD epigenetics, especially mechanisms that mediate repression of inflammatory cytokine gene expression in macrophages in human disease setting, represents an important aspect of search for new therapeutic targets.

The *TNFA* gene was selected since it is the master regulator of innate and adaptive immune responses. It was demonstrated that the *TNFA* is an immediate early response gene and is transcribed within minutes after stimulation (Goldfeld et al., 1993; Falvo et al., 2013). Depending on cell type and triggering stimuli, a range of transcription factors and co-activators are recruited to the proximal TNF promoter, which drive transcription of the gene (Falvo et al., 2008). This cell type- and stimuli-specific activation of the TNF gene transcription is a key element of epigenetic regulation of the gene (Biglione et al., 2011).

The main phenotypic characterisation of intestinal macrophages is their unique anergic potential. Under homeostatic conditions, intestinal macrophages undergo a specific process of differentation associated with down regulation of

proinflammatory cytokines (Smythies et al., 2005). These cells also become hyporesponsive to continuous LPS stimulation, a state known as endotoxin tolerance. It is known that this stage of transient non-responsiveness is driven by stimuli- and gene-specific regulatory mechanisms. These mechanisms involve alterations in TLR-signal induction pathways, but also change in chromatin environment, as was shown here.

The aim of this part of the project was to study the chromatin architecture of intestinal macrophages isolated from mucosa of control subjects and IBD patients. We wanted to understand how the disease environment influences chromatin state and transcription profile of these cells.

To do so, we first looked into the *TNFA* mRNA level in the gut macrophages. Among all samples recruited for this study, only macrophages isolated from colonic mucosa of patients with Crohn's disease showed increased *TNFA* transcription. Interestingly, macrophages isolated from ulcerative colitis patients and control subjects had the same *TNFA* transcript level. In 8 out of 10 control subjects, the *TNFA* mRNA level was lower than in representative Crohn's mucosa, but not in ulcerative colitis. Among all UC patients recruited in this study, only 2 out of 8 had 2-fold increase in the *TNFA* level. However, the same was observed in control donors.

The general opinion is that in IBD, TNF-α production is increased at mRNA and protein level and that excessive TNF production correlates with the severity of the disease (Olsen et al., 2007; Yarur et al., 2015). However no increase in *TNF* mRNA production was detected in ulcerative colitis samples used in this study.

In UC, the role of TNF has been less characterised, although a *TNFA* gene polymorphism was linked with susceptibility to UC. For example, the frequency of

carriers for polymorphism (-308A and -238G) is significantly increased in Japanese UC patients (Wilson et al., 1993), but only weakly associated among Chinese UC patients (Cao et al., 2006) compared with controls (Levin and Shibolet, 2008). In contrast, in Dutch UC patients, the frequency of the same polymorphism site was reduced (Bouma et al., 1996; Levin and Shibolet, 2008). Additionally, increased prevalence of TNF-α polymorphism site (-857C) was linked with ulcerative colitis among UK Caucasian patients (van Heel *et al.,* 2002), while homozygosity for a TNF-α haplotype (TNF-α, -1031T, -8363C, -857C, 380G, -308G, -238G) was linked to low TNF-α production (Ahmad et al., 2003; Levin and Shibolet, 2008). A number of studies have also shown significant increase in TNF-α levels in colonic mucosa of UC patients (Breese et al., 1994, MacDonald et al., 1990), but also that the increased TNF-α production is more apparent in inflamed but not in noninflamed sites (van Heel et al., 2002). The above examples highlight the fact of great heterogeneity of TNF-α production in UC patients. In fact, additional single sample analysis of samples that were used in this study revealed that increased *TNFA* mRNA level was also associated with the disease activity. The lack of evidence in increased averaged TNF-α transcript can be due to the fact that most of UC samples for tis sudy were collected from nonactive sites.

Next, it was speculated that a break of anergic phenotype in IBD macrophages might be associated with changes in a level of silencing marks. By assessing the *TNFA* TSS we noted that macrophages isolated from mucosa of CD patients showed decreased enrichment of H3K27me3, H3K9me3 and –me1, with H3K27me3 having the greatest significant reduction. Also, some level of reduction was observed for H3K4me1 in both, CD and UC macrophages and H3K4me3 but only in CD macrophages. However, these changes did not reach significant values. Nevertheless,

this finding suggests that loss of tolerance might be associated with reduced occupancy of H3K27me3 silencing mark.

The phenomenon of endotoxin tolerance has been extensively studied using various in vitro models (Foster et al., 2007; De Santa et al., 2009; Chen and Ivashkiv, 2010). Along the process of tolerisation, cells become transiently hyporesponsive upon repeated or prolonged exposure to LPS. Based on these data, it is known that chromatin remodelling plays an important part (El Gazzar et al., 2007). In respect to immune cell activation that involves increased TNF production, histone marks such as H3K4me3, -me2, -me1 were shown to be highly enriched at the *TNF* promoter following LPS or TNF stimulation and PMA/ionomycin activation of TH-1 cells or Jurkat cells, respectively (Taylor et al., 2008). However, Sullivan et al. (2007) showed that LPS activation of TH-1 cells results in loss of H3K4me2 and in increased H3K4me3 (Sullivan et al., 2007). In unstimulated murine bone marrow derived macrophages (BMDMs), high levels of H3K4me3 and H3ac (but not H4ac) together with RNAPII, CBP/p300, are present at the *TNFA* promoter, consistent with a primary response gene poised for transcription (Hargreaves at al., 2009; Falvo et al., 2013). By contrast, in LPS-tolerant TH-1 cells, LPS stimulation fails to induce H3K4 methylation and H3K9 demethylation at the *TNF* promoter, as it happens in LPS-responsive cells (El Gazzar et al., 2008).

In reference to histone methylation, none of the mentioned examples included the H3K27me3 silencing mark in their study, therefore we decided to investigate the role of this mark in the process of tolerisation using human primary macrophages (GM-CSF). Our preliminary experiment suggests that LPS stimulation of naïve macrophages results in enrichment of activating mark H3K4me3 and that this failed to happen in LPS-tolerant cells (Foster et al., 2007; El Gazzar et al., 2008) (Figure

Appendix 0.1C). LPS activation of naïve macrophages also triggered the recruitment of RNAPII to the *TNFA* TSS and a decrease in silencing mark; H3K27me3. Of note, LPS stimulation of tolerant macrophages did not influence the RNAPII level. Moreover, an enrichment of RNAPII remained constant and comparable to the level of LPS stimulated naïve cells (Figure Appendix 0.1A). Interestingly, it was possible to detect even higher enrichment of H3K27me3 in tolerant cells stimulated with LPS, suggesting that indeed H3K27me3 contributes to the gene tolerisation (Figure Appendix 0.1AB).

The reduced H3K27me3 signal after LPS stimulation may reflect enzymatic demethylation, histone exchange or nucleosome loss (De Santa et al., 2009). Indeed, H3K27me3 reduced occupancy mirrored the decrease in the total H3 (tH3) level. This suggests that nucleasome loss rather than enzymatic demethylation may be the mechanism underlying the observed reduction of H3K27me3. As reported by others, nucleosome depletion at inducible genes is a common phenomenon in LPS-stimulated macrophages, possibly because of the nucleosome displacement linked to Poll II elongation (De Santa et al., 2009). It should be remembered that what we see here is just a piece of the puzzle and that epigenetic modifications also involve dynamic changes involving protein complexes.

It may be speculated that in resident gut macrophages, the enrichment of silencing marks at the *TNFA* TSS is driven by environmental factors within the gut. The main source of gut macrophages is peripheral blood monocytes, which are attracted to lamina propria during inflammation (Bain et al., 2014). However, inflammatory environment, the one seen in IBD, drives these cells to gain pro-inflammatory phenotype.

In fact, it has been shown that IFN-γ abrogates endotoxin tolerance by chromatin remodelling in *in vitro* model (Chen et al., 2010). The increased IFN-γ level is a characteristic feature of Crohn's disease lamina propria, hence the above scenario can most likely happen.

The results presented in section 4.4 also suggest that the homeostatic environment of lamina propria drives the enrichment of silencing marks to the *TNFA* TSS along the process of cell differentation. Peripheral blood monocytes from controls and IBD patients displayed similar levels of H3K27me3 mark. However, only control macrophages eventually acquired H3K27me3 at the *TNFA* TSS together with H3K9me3. Additionally, no difference in H3K4me3 was observed between blood monocytes and intestinal macrophages from control and IBD patients, although higher binding of this mark was detected in monocytes and intestinal marophages isolated from UC patients that in other groups.

It is possible that during the process of cell differentiation, the bivalent state of the *TNFA* promoter is being established through interplay between H3K27me3 and H3K4me3 in healthy gut macrophages and this is disturbed in IBD environment. This additionally supports the role of these silencing marks, H3K27me3 especially, as important in establishment of anergic phenotype. Therefore, an acquisition of repressive mark by anergic resident intestinal macrophages is most likely a tissue-type specific process. However, the results presented here are still preliminary as statements made can not be supported by a significant outcome.

Possibly future work will establish the exact role and dynamics between histone methylation marks, especially H3K27me3 in driving the endotoxin tolerance in IBD intestinal macrophages.

Additionally, JmjD3 was also studied. As the only known substrate of JmjD3 is H3K27me3 (De Santa et al., 2007; 2009), therefore it was of interest to explore JmjD3 in IBD. The JmjD3 is an enzyme belonging to histone demethylase group involved in a control of gene expression in LPS-activated macrophages (De Santa et al., 2009). The exact functions of JmjD3 and its paralog UTX are unclear. Elevated transcripts of *JMJD3* were detected in CD macrophages, however without a significant difference. Interestingly, *UTX* expression was at much higher significant level than *JMJD3*, which came as surprise, considering that in previous studies, *UTX* level was detected at lower and constant levels that *JMJD3* (De Santa et al., 2007). It has been shown that in *Drosophila melanogaster,* UTX colocalises with the elongation form of RNAPII, thus suggesting an active role in ongoing transcription (Smith et al., 2008).

Noting that *JMJD3* transcript level was increased in CD macrophages, we also looked into its direct binding to the *TNF* gene. Based on genomic studies of JMJD3 distribution, it was shown that the enzyme preferentially occupies TSS±0.5kbp (De Santa et al., 2009). Analysis of JmjD3 occupancy at the *TNF* TSS using ChIP data generated from blood monocytes and intestinal macrophages from control and IBD samples showed increased prevalence of JmjD3 in IBD monocytes and UC macrophages. Although, these data are preliminary, it may be possible that increased JmjD3 binding associates with pro-inflammatory profile of cells.

The JmjD3 involvement in tuning the transcription output of LPS-stimulated macrophages was investigated by De Santa et al. (2009). Considering the high substrate specificity and the fact that JmjD3 is recruited to the gene promoter upon LPS stimulation, it is believed that JmjD3 functions to reduce H3K27me3 occupancy and enhance transcriptional activity (De Santa et al., 2009).

However, De Santa and collegues (2009) also showed that JmjD3 recruitment to a target gene does not rely on pre-existing H3K27me3 and at most recruitment sites, JmjD3 will not encounter H3K27me3. They showed that some of the genes showing the highest level of JmjD3 recruitment were these undergoing massive H3K4me3 increase after stimulation.

In summary, in this chapter, we set out to characterise the chromatin architecture at a selected proinflammatory cytokine gene in the intestinal macrophages. We have decided to study a number of repressive and permissive histone modifications associated with the gene promoter (TSS) and which have been described to be important in regulating gene expression.

We first looked at the chromatin state of resident 'anergic' intestinal macrophages isolated from a mucosa of control subjects. It allowed us to understand that chromatin architecture in a normal intestinal macrophages is such that active *TNFA* transcription can not be initiated and possibly this is due to a high occupancy of silencing marks, especially H3K27me3. Additionally, we have also speculated that under homeostatic conditions, the *TNFA* gene is set at a bivalent state, since we have observed a concurrent high binding of H3K27me3 and H3K4me3 at the gene transcription start site. Next, we went on to investigate what is a chromatin state of the *TNFA* TSS in IBD intestinal macrophages. We have observed that CD macrophages displayed significantly lower binding of the silencing mark H3K27me3 to the *TNFA* TSS than controls and even UC macrophages. Moreover, activated state of the *TNFA* promoter in CD macrophages was additionally associated with decreased binding of other repressive marks H3K9me3 and –me1, although with no significant difference.

In conclusion, these results suggest that the establishment of tolerogenic phenotype of the intestinal macrophages as seen through down-regulation of proinflammatory cytokine gene expression is driven by epigenetic modifications associated with an establishment of different repressive histone 3 (H3) environment. Potentially, a silencing mark H3K27me3 may play an important role in driving this phenotype, along with other repressive marks, such as H3K9me3. We have also documented that acquisition of repressive mark by anergic resident intestinal macrophages is most likely a tissue specific process and that this process is disturbed in IBD.

Unfortunately, the results presented here in many aspects could not reach a statistical significance due to sample's variability and low number of patients recruited. Hence, future work is required to further confirm these preliminarly data.

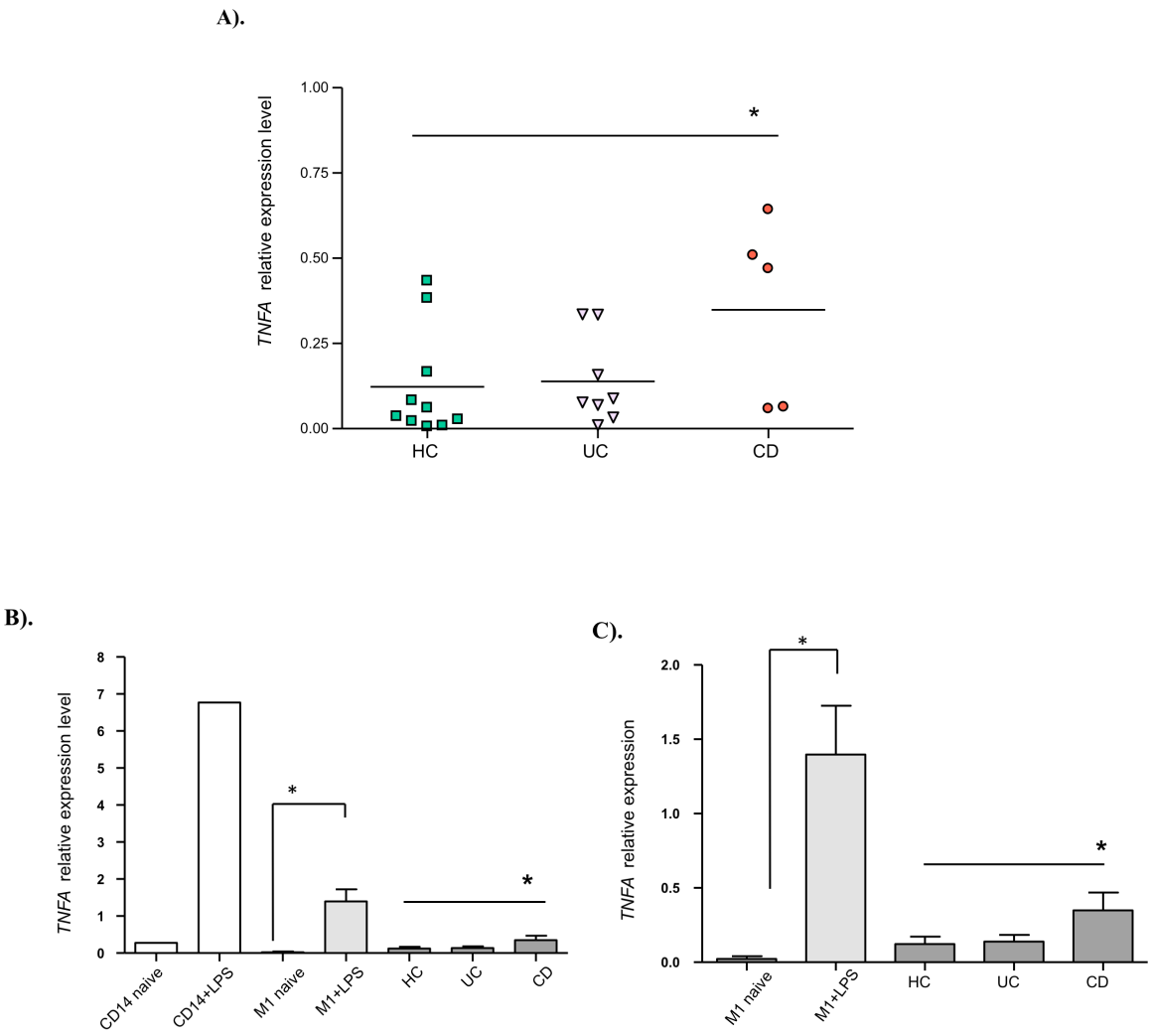
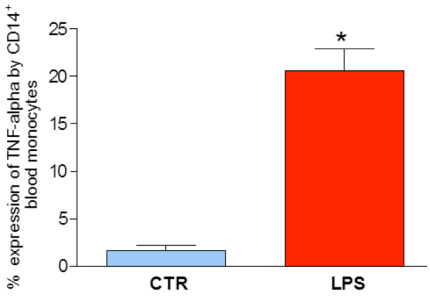


Figure 4.1: Characterisation of anergic phenotype of resident intestinal macrophages based on potential to express *TNFA*

A). *TNFA* expression level in intestinal macrophages ($CD33^{+}$LPMCs) isolated from control subjects and IBD patients HC n = 10; UC n = 8; CD n = 5 **B).** *TNFA* expression level as in Figure 4.1A in comparison with GM-CSF derived primary human macrophages (n=3) and peripheral blood monocytes from control subjects (n=1). Cells stimulated with LPS (100ng/ml/1h). **C).** The *TNFA* expression level as in Figure 4.1B, excluding control peripheral blood monocytes. Increased *TNFA* transcript level (3-fold) was detected in intestinal macrophages isolated from Crohn's patients, but not ulcerative colitis or control subjects. *TNFA* relative expression level was normalised to *GAPDH*, *ACTB*, *UBB* and *RPL13A* as housekeepers. Data are shown as mean ± SEM from independent experiments (*) $p \leq 0.05$.

A).



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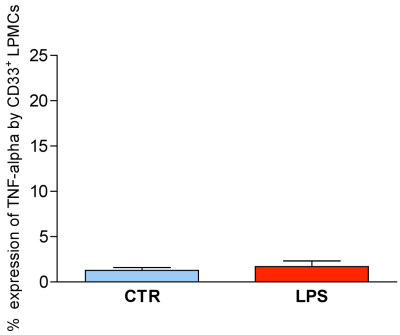


Figure 4.2: Characterisation of anergic phenotype of resident intestinal macrophages based on potential to express *TNFA*

TNF-α production by (A) human blood monocytes (n=4) and (B) $CD33^+$ LPMCs isolated from mucosa of control subjects (n=2). Cells were analysed by flow cytometry. Cells were additionally stimulated with LPS (100ng/ml/1h) (*) p≤ 0.05.

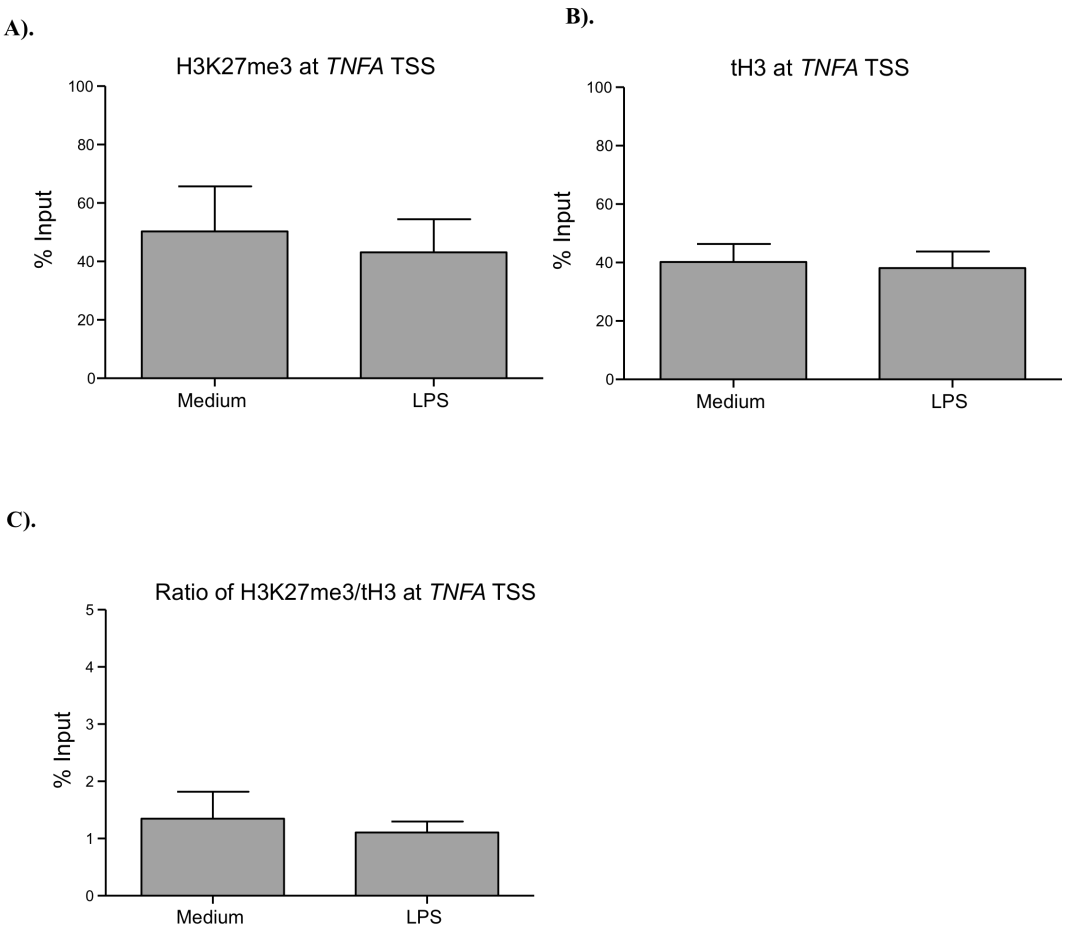
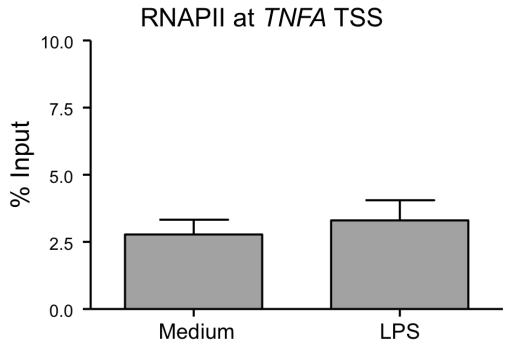


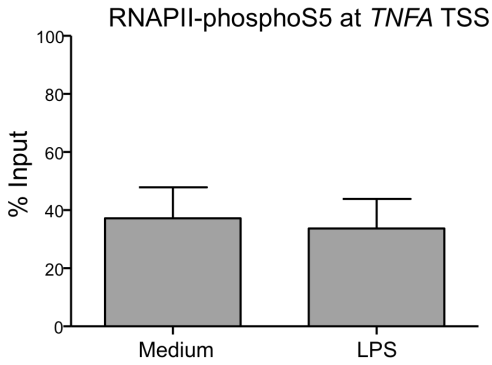
Figure 4.3: H3K27me3 and tH3 occupancy at the *TNFA* TSS

ChIP assay performed on intestinal macrophages isolated from control subjects. Since a small fraction of resident macrophages also express CD14, a half of cells were stimulated with LPS (100ng/ml) for 2h in 24 well-plate format. Data are presented as mean ± SEM of 5 independent experiments. Data are presented as % input normalised to IgG signal. Additional LPS stimulation did not influence the binding of H3K27me3 or did not change the chromatin state.

A).



B).



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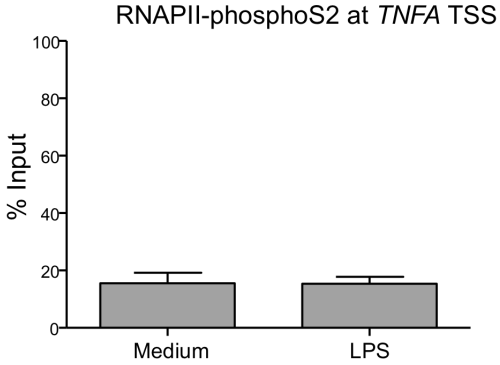


Figure 4.4: RNAPII and phosphorylated –S5 and –S2 level at the *TNFA* transcription start site (TSS)

ChIP assay performed on intestinal macrophages isolated from control subjects. Experimental setting were as in Figure 4.3. Data are presented as mean ± SEM of 5 independent experiments. Data are presented as % input normalised to IgG signal. Additional LPS stimulation did not influence the binding of RNAPII forms.

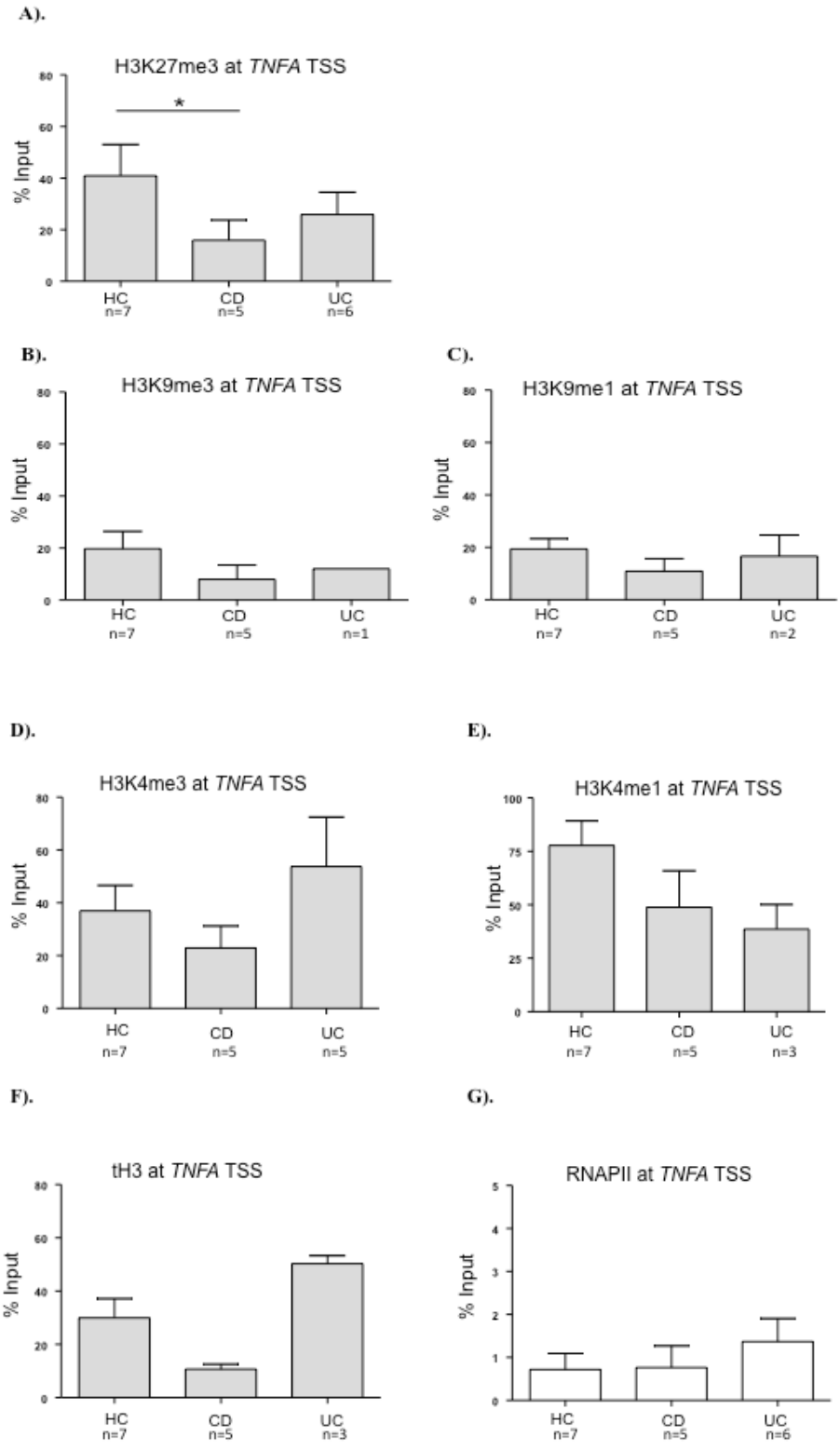


Figure 4.5: The assessment of repressive and permissive histone modifications, tH3 and RNAPII levels at *TNFA* TSS between IMACs isolated from IBD patients and control subjects

ChIP assay performed on intestinal macrophages isolated from control subjects (HC), Crohn's (CD) and Ulcerative colitis (UC) patients. LPMCs were sorted using $CD33^{+}$MACS method. Cells were fixed with 1% formaldehyde and sonicated to shear a chromatin. Data are presented as mean ± SEM of independent experiments. Data are presented as % input normalised to IgG signal (*) $p \leq 0.05$.

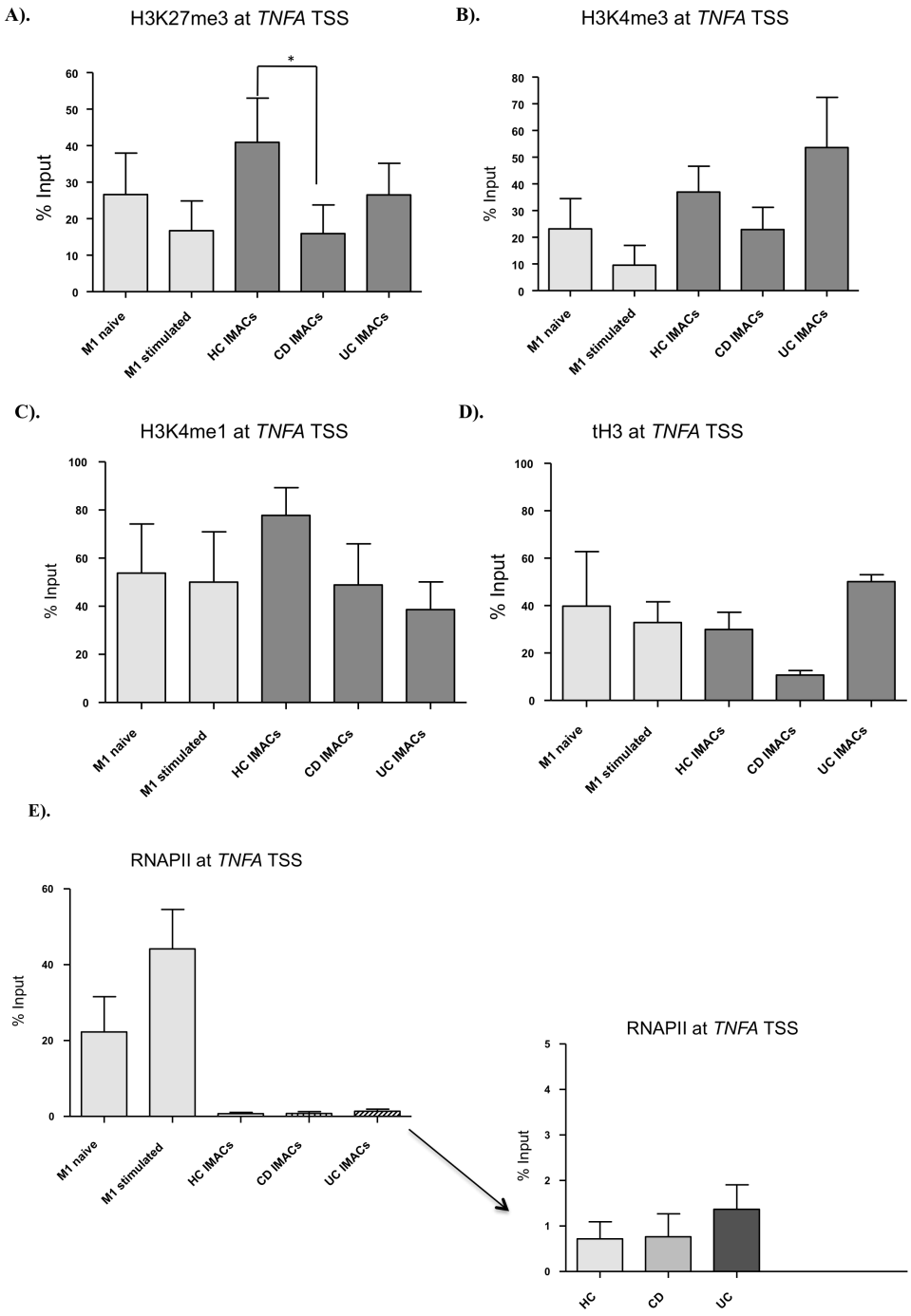


Figure 4.6: The assessment of repressive and permissive modifications histone modification, tH3 and RNAPII levels at the *TNFA* TSS between IMACs isolated from IBD patients, control subjects and GM-CSF macrophages

ChIP Assay performed on IMACs isolated from control subjects (HC), Crohn's disease (CD) and Ulcerative colitis (UC) patients as stated in Figure 4.5 and GM-CSF derived human macrophages (n=3). Data are presented as mean ± SEM of independent experiments. Data are presented as % input normalised to IgG signal (*) p≤ 0.05.

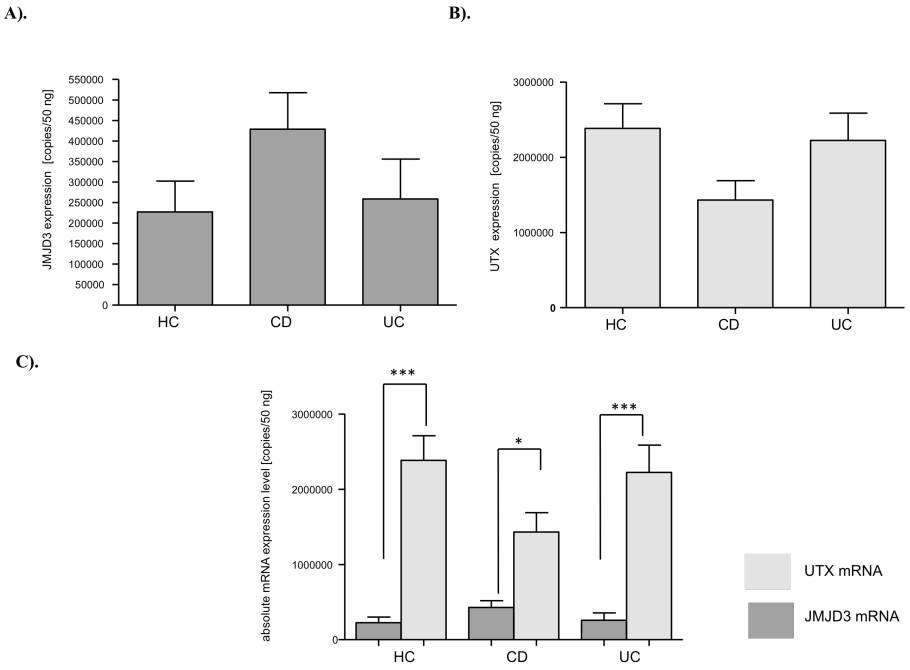


Figure 4.7: The *JMJD3* and *UTX* mRNA expression level in intestinal macrophages ($CD33^+$ LPMCs) isolated from control subjects (HC) and IBD patients

The *JMD3* and UTX absolute mRNA expression level was normalised to *ACTB* and *UBB* as housekeepers. Data are presented as copies number/50 ng. Data are shown as mean ± SEM from HC n=13; UC n=6; CD n=8 (*) $p \leq 0.05$, (***) $p \leq 0.001$.

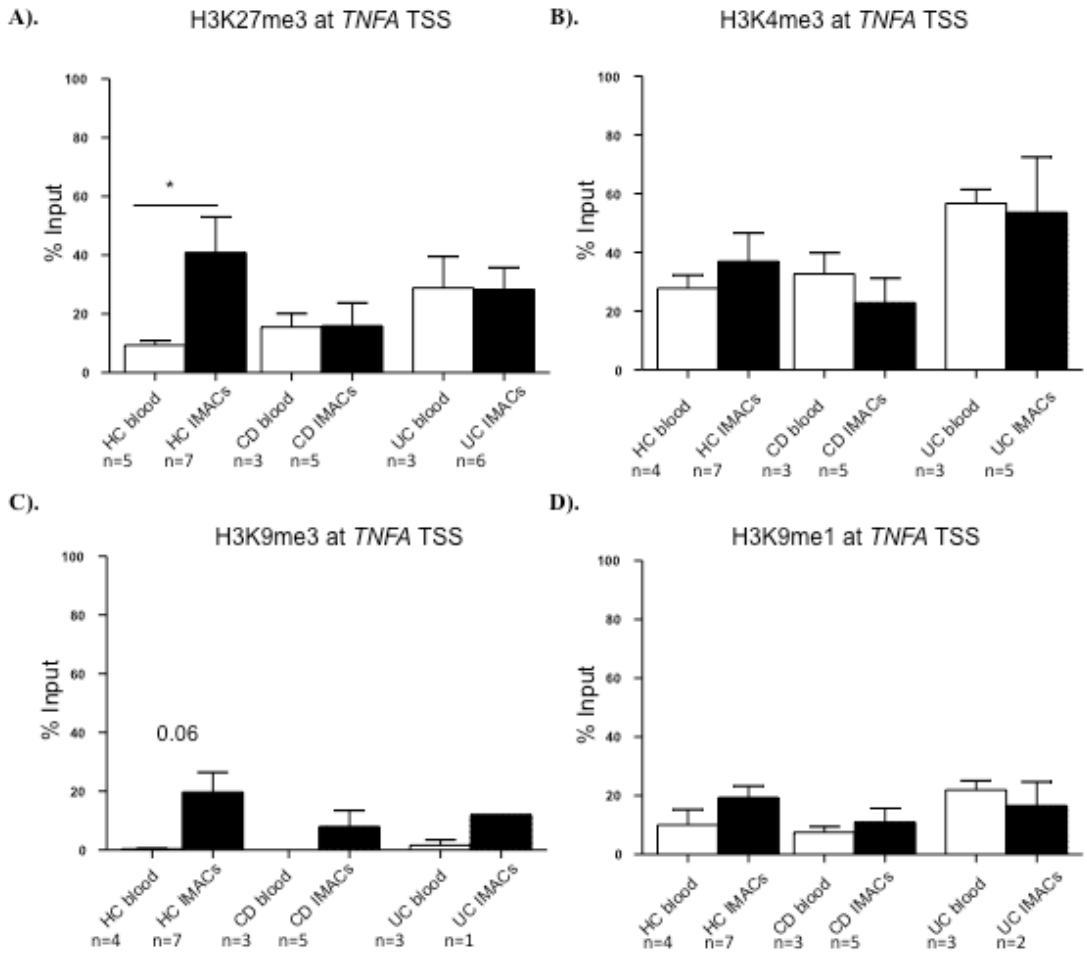


Figure 4.8: The assessment of repressive modifications; H3K27me3, H3K9me3 and H3K9me1 and permissive histone modification; H3K4me3 at the *TNFA* TSS between IMACs isolated from IBD patients, control subjects (as in Figure 4.5) and blood monocytes from IBD patients and control subjects (*) p≤ 0.05.

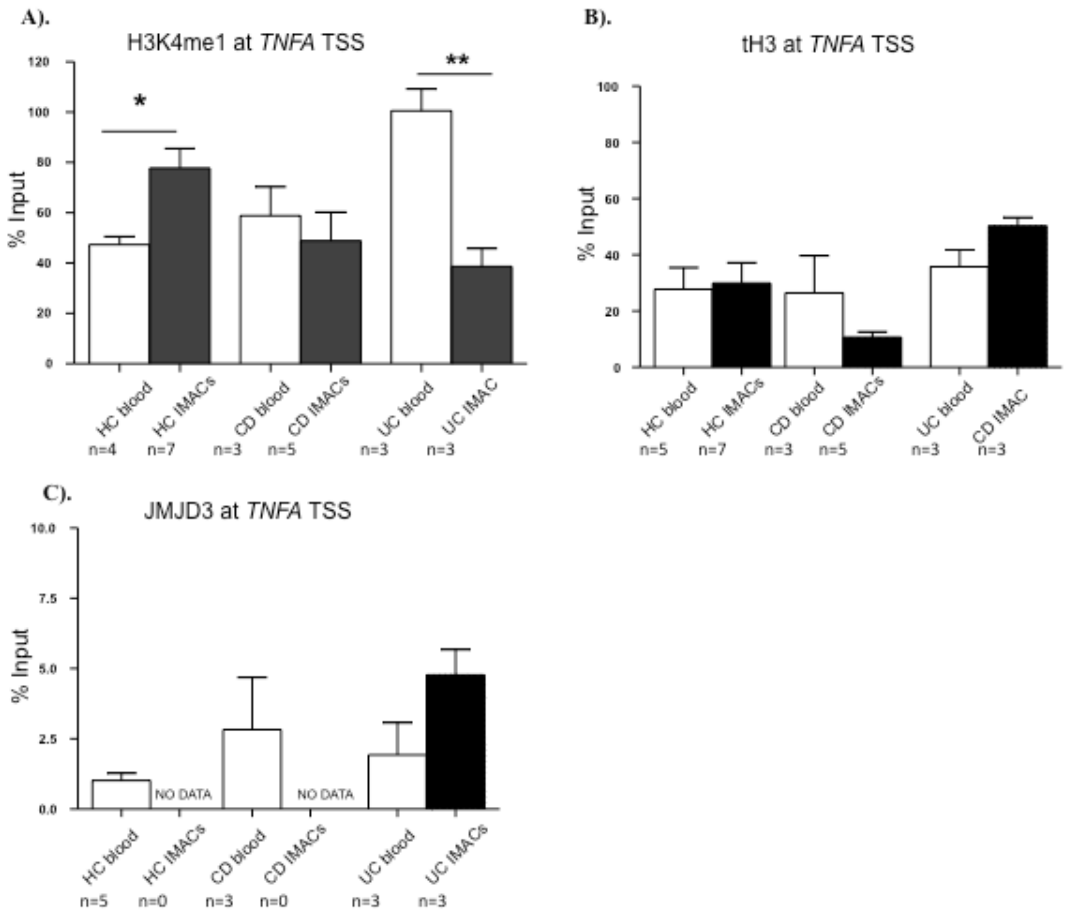


Figure 4.9: The assessment of histone methylation marks H3K4me1 and histone 3 (tH3) and JMJD3 direct binding at the *TNFA* TSS.

ChIP assay was performed on blood monocytes from IBD patients, control subjects and intestinal macrophages (IMACS) from IBD patients (CD and UC) and controls (*) p≤ 0.05, (**) p≤ 0.01.

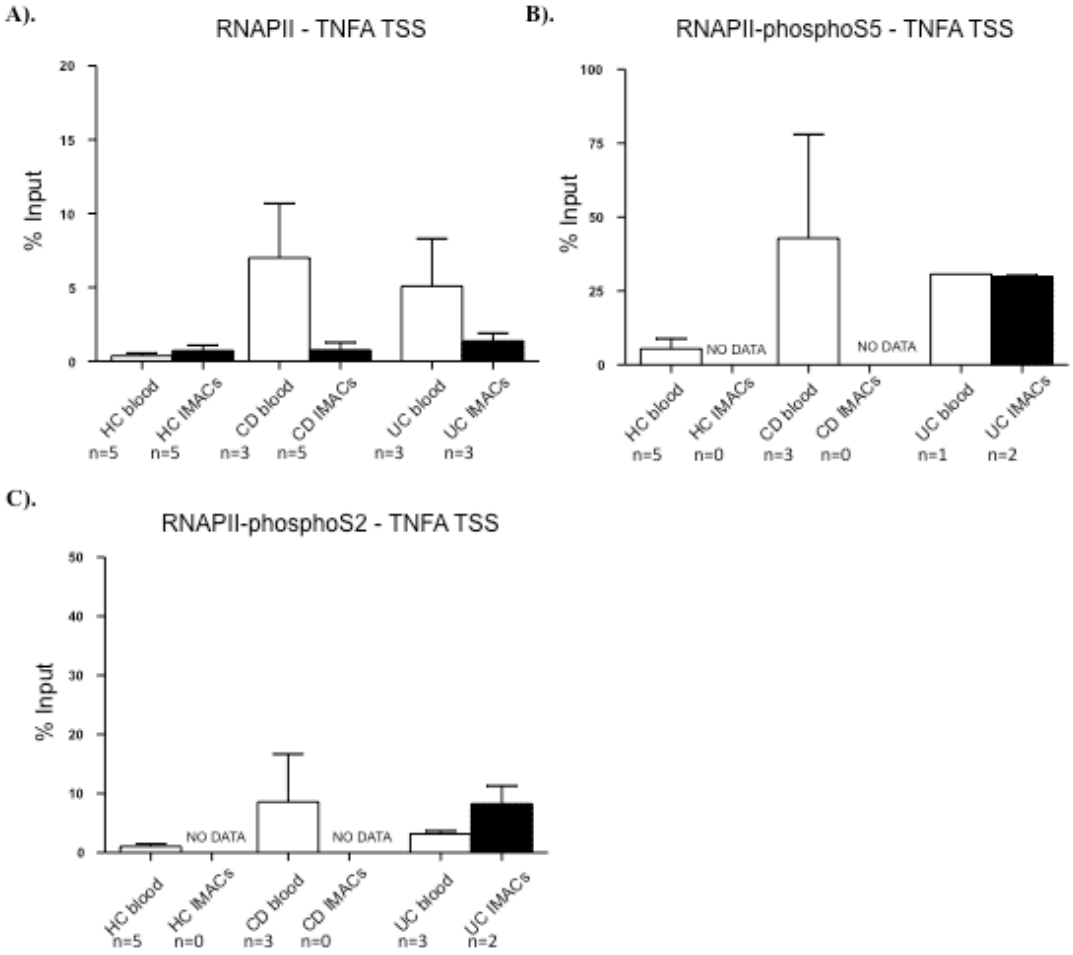


Figure 4.10: The assessment of RNAPII and its phosphorylated forms RNAPII-phosphoS5 and RNAPII-phosphoS2 at the *TNFA* TSS

As in Figure 4.9, ChIP assay was performed on blood monocytes from IBD patients and control patients as well as from intestinal macrophages (IMACS) from IBD patients (CD and UC) and control subjects to assess RNAPII and its phosphorylated forms.

Chapter 5

Investigating the inhibitory effect of GSKJ4 (a JMJD3 inhibitor) on human primary GM-CSF macrophages and intestinal macrophages in IBD

5.1. Introduction

The methylation of H3K27me3 is thought to be a key epigenetic regulator of cell homeostasis and development (Margueron et al., 2011). Among all histone demethylases, the enzymes involved in an establishment or resolution of the H3K27me3 state play an important role in cellular differentation. As described in Chapter 1, JMJD3 (H3K27me3 demethylase) has been shown to function in regulation of LPS-inducible gene expression as well as in establishment of the M2 macrophage phenotype (Satoh et al., 2010). De Santa and colleagues (2009) have shown that JMJD3 is induced by the transcription factor NF-κB in response to microbial stimuli upon which H3K27me3, a histone mark associated with transcriptional repression is removed. They also showed that 70% of lipopolysaccharide (LPS)-inducible genes are indeed JMJD3 targets (De Santa et al., 2009). Moreover, Chen and colleagues (2012) showed that JMJD3 activates bivalent gene transcription. JMJD3 is also essential for RNAPII elongation, as proposed by Estaras and colleagues (2013), since colocalisation of JMJD3 with RNAPII-S2p stimulates gene transcription.

In Chapter 4, it was shown that the process of differentation from blood monocytes into anergic resident intestinal macrophages is associated with acquisition of the H3K27me3 repressive mark at the *TNFA* TSS (Chapter 4, Figure 4.8A). It was also shown that in patients with Crohn's disease, intestinal macrophages failed to establish H3K27me3 at the *TNFA* promoter. Therefore, H3K27me3 might play an essential role in driving the anergic phenotype. Since, the removal of the silencing mark H3K27me3 is associated with increased *TNFA* transcripts it was interesting to determine, if targeting the enzyme responsible for elimination of this silencing mark

could restore the silent state of the *TNFA*. The recent development of a selective JMJD3 inhibitor; GSK-J4 enabled to investigate this hypothesis.

5.2. Inhibiting JMJD3 results in the restoration of silencing mark H3K27me3 at the *TNFA* transcription start site (TSS) in human primary macrophages

The efficacy of GSK-J4 at inhibiting the TNFA production was first tested at the transcript level using GM-CSF human primary macrophages. First, human blood monocytes were induced to differentiate into M1 macrophages using GM-CSF (5ng/ml). On day 6, macrophages were stimulated with LPS (100 ng/ml) for 1 h. To test the effect of GSK-J4 some cells were also treated with the compound (30μM) 15 min before LPS stimulation.

Administration of GSK-J4 (JMJD3 inhibitor) decreased the expression of the *TNFA* transcripts after LPS activation, but the reduction was not significant (Figure 5.1). Next, ChIP analysis was performed to understand the epigenetic modulation associated with changes in the *TNFA* expression. As shown in Figure 5.2A, pretreatment with GSK-J4 preserved H3K27me3 binding to the *TNFA* TSS upon LPS stimulation.

Two other histone marks, H3K4me3 and H3K4me1 were also analysed, as well as binding of JMJD3 and RNAPII to the *TNF* TSS. Upon LPS activation, reduced binding of H3K4me3 to the *TNF* TSS was observed, which was maintained by pre-incubation with GSK-J4 (Figure 5.2D). The stimulation with LPS led to 2-fold reduction (from 20% to 10%) of H3K4me3 binding, however again this was not significant. In contrast, H3K4me1 occupancy at the *TNFA* TSS remained unchanged upon LPS stimulation and GSK-J4 pre-treatment (~50% input) (Figure 5.3A).

Therefore it was concluded that in addition to H3K27me3, GSK-J4 also might have an effect on H3K4me3 but not on H3K4me1 (Figure 5.2D and 5.3A, respectively).

To understand the dynamics of epigenetic changes, the level of total histone 3 (tH3) was also measured (Figure 5.2B). The activation of M1 macrophages with LPS or the pre-treatment with JMJD3 inhibitor (GSK-J4) did not influenced the tH3 level (~40% input) at the *TNF* TSS region. Moreover, the GSK-J4 compound did not seem to affect the recruitment of JMJD3 to the *TNFA* TSS, (10% input) (Figure 5.2C). Additionally, the RNAPII recruitment to the *TNF* TSS was also studied. The 2-fold increase in RNAPII recruitment to the *TNF* TSS was observed after LPS stimulation from 20% to 40% input (Figure 5.3B). Conversely, pre-treatment with GSK-J4 decreased recruitment of RNAPII to the *TNF* TSS, however this change was not as profound (Figure 5.3B). Although it was possible to detect a trend in modulatory effect of LPS stimulation or pre-treatment with GSK-J4 on RNAPII recruitment, these changes were not significant. Nevertheless, the trend seen in this study is in an agreement with Kruidenier *et al*. 2012, where GSK-J4 was also tested in human primary macrophages.

Lastly, the effect of GSK-J4 on human primary macrophages was examined by flow cytometry. It was possible to confirm ChIP analysis for H3K27me3 by showing that GSK-J4 also targets global H3K27me3 demethylation (Figure 5.4). Using flow cytometry analysis, a 3-fold decrease (from 6.5% of total cells population to 2%) in proportion of macrophages stained for H3K27me3 was seen upon LPS activation (Figure 5.4). The pre-treatment with GSK-J4 restored the H3K27me3 level to even higher than an initial level (from 2% to 11%), however the difference was not significant (Figure 5.6).

The TNF-α expression was also analysed (Figure 5.5). The activation of human primary macrophages with LPS caused a 3-fold increase in proportion of cells expressing TNF-α (from ~24% to 76%) (Figure 5.5). Additionally, a significant 30% reduction (from ~76% to 53%) in TNF-α levels was also seen in macrophages pretreated with the compound upon subsequent LPS activation, however not to the level seen before LPS stimulation (Figure 5.5).

5.3. Assessing the effect of the JMJD3 inhibitor in intestinal macrophages isolated from Crohn's disease mucosa and in IBD mucosa in ex vivo organ culture

Having established the epigenetic effects of GSK-J4 in human primary macrophage setting, GSK-J4 was investigated on intestinal macrophages isolated from Crohn's disease mucosa. First, intestinal macrophages were cultured with or without GSK-J4 (30μM) for 1h and analysed using ChIP assay. Again, H3K27me3, H3K4me3, tH3, JMJD3 were analysed. No effect of GSK-J4 was seen on any of assessed epigenetic marks (Figure 5.6-7).

The occupancy of H3K27me3 was established at the highest level of 40% input, comparing to H3K4me3, which was approximately 20% input (Figure 5.6). Unfortunately, due to limited amounts of DNA only two other histone 3 (H3) modifications were analysed. The H3 (tH3) level was found to be at 40% input as well. Interestingly, the direct binding of JMJD3 to the *TNF* TSS in intestinal macrophages isolated from Crohn's mucosa was 5 times lower that seen in GM-CSF differentiated macrophages (2% and 10%, respectively) (Figure 5.6D and Figure 5.2D). Finally, RNAPII and also, RNAPII-pS5 and RNAPII-pS2 were assessed (Figure 5.7). Interestingly, the level of RNAPII was surprisingly low at 2% input level (Figure 5.7A). Between the two RNAPII phosphorylated forms, pS5 showed

the highest binding to the *TNF* TSS (~15% input), whereas, pS2 was only 4% input (Figure 5.7B-C).

Next, the effect of GSK-J4 was also assessed on TNF protein production in IBD biopsies. Biopsies were incubated for 24 hours in serum-free media and supernatants analysed for TNF production (Figure 5.8A-B). In both, CD and UC biopsies the level of TNF-α was measured at around 100 pg/ml. Although, some level of cytokines reduction was seen in individuals patients, the overall group analysis showed no significant reduction (Figure 5.8).

Additionally, the effect of GSK-J4 was also tested on IL-6 and IL-8 production (Figure 5.9). The highest IL-6 and IL-8 production was observed in UC samples when compared with CD (7500 pg/ml and 50 000 pg/ml, respectively). In UC, IL-6 level was 3 times higher and IL-8 production 2 times higher than in CD (Figure 5.9). However, incubation of IBD biopsies samples with GSK-J4 compound did not affect cytokine production (Figure 5.9). Some level of reduction in IL-6 protein level was observed in UC samples, however the different was not significant (Figure 5.8A). In CD biopsies no reduction was observed.

5.4. Discussion

Since the removal of silencing marks could be associated with increased *TNF* transcripts in intestinal macrophages isolated from patients with Crohn's disease it was interesting to investigate, if by targeting these marks it was possible to restore the silent state of the gene.

In this chapter JMJD3 inhibitor, GSK-J4 was examined. GSK-J4 is known to be a selective Jumonji H3K27me3 demethylase inhibitor. Initially, the effect of the compound was tested on human primary macrophages (GM-CSF). By doing so, it

was speculated that GSK-J4 could modulate the proinflammatory macrophage responses, as also shown by Kruidenier et al. (2012) Indeed, pretreatment with the JMJD3 inhibitor prevented increased *TNF* transcript levels seen upon subsequent LPS stimulation. This was associated with an epigenetic modulatory effect, as seen by GSK-J4-preserved H3K27me3 enrichment upon LPS activation.

Moreover, in this study two additional histone marks; H3K4me3, H3K4me1 and also JMJD3 were investigated to fully understand the effect of the inhibitor. It was speculated that in addition to H3K27me3, GSK-J4 compound also showed an effect on H3K4me3 level but not on H3K4me1. A recent paper by Heinemann et al. (2014) confirmed this finding by showing that the compound is a potent inhibitor of Jumonji proteins with activity towards H3K27me3/me2 (KDM6) and also H3K4me3/me2 (KDM5) in vitro.

Additionally, since no differences were seen in the level of JMJD3 at the *TNF* TSS after LPS activation or GSK-J4 treatment, it was speculated that GSK-J4 did not affect the recruitment of JMJD3 to the *TNFA* TSS. It was concluded that the compound influenced demethylase properties of JMJD3 rather than its direct binding to the gene. Kruidenier et al. (2012) showed that the compound binds endogenous JMJD3, therefore H3K27me3 reduction is a result of enzymatic demethylation, also because no decrease of total H3 (tH3) was detected.

Having established that the compound could exert its effect in human primary macrophages, the next step was to study intestinal macrophages isolated from Crohn's disease tissue resections. Analysis of GSK-J4 on intestinal macrophages isolated from mucosa of Crohn's patients showed no effect of JMJD3 inhibitor, as analysed by ChIP assay and on transcript level. The compound was also tested in ex-

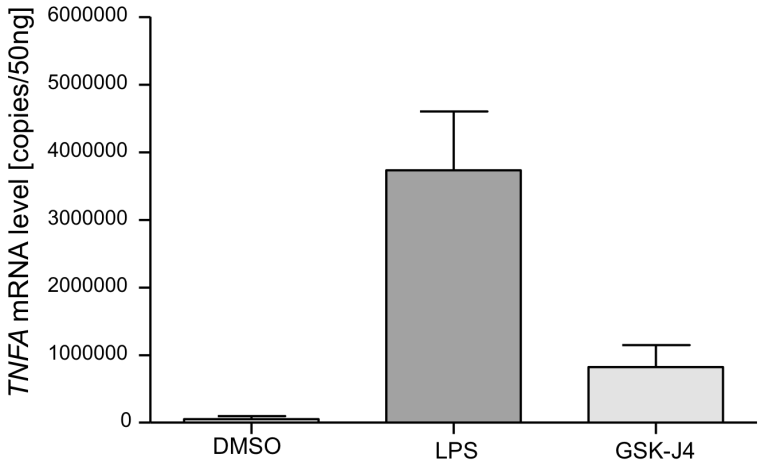
vivo organ culture and supernatants were collected for proinflammatory cytokines production (TNF, IL-6, IL-8). However no effect was detected.

At this stage it is hard to speculate on possible reasons of why GSK-J4 did not exert its effect on CD intestinal macrophages or did not influence the cytokine production on a protein level. The study by Kruidenier et al. (2012) and the results of this study, although with insignificant outcome showed that indeed GSK-J4 has a modulatory potential at least in GM-CSF differentiated macrophages.

GSK-J4 is delivered to the target cell in a form of pro-drug, which upon uptake by macrophages is converted into an active form through macrophage esterase activity (Kruidenier et al., 2012). Since, the activity of GSK-J4 was initially only tested on GM-CSF differentiated macrophages, it was unknown how intestinal macrophages would respond to the drug treatment. Due to lack of DNA it was impossible to extend the experimental setting of this study to fully understand the effect of the treatment. Also due to lack of samples it was problematic to investigate the effect of GSK-J4 on intestinal macrophages isolated from ulcerative colitis patients.

Also, no effect of GSK-J4 was seen in ex-vivo organ culture. This was a first attempt to investigate the effect of the GSK-J4 on IBD mucosa samples in ex-vivo organ culture. Since the GSK-J4 activity depends on macrophage esterase action, it might be that the concentration of the enzyme was not sufficient to convert the GSK-J4 pro-drug into an active form. Among all samples collected in this study, some level of cytokine reduction was only observed for IL-6 in UC samples.

In summary, despite the initial data on GSK-J4 on GM-CSF differentiated macrophages, it was impossible to show the treatment effect on IBD intestinal macrophages.



	LPS stimulation	GSK-J4	
	Fold Change	**Fold Change**	Fold Reduction
Donor 1	**38**	**10**	4
Donor 2	**391**	**52**	8
Donor 3	**384**	**78**	5

Figure 5.1: Pretreatment of macrophages with GSK-J4 prevents increased TNFA transcripts upon subsequent LPS activation

Macrophages were differentiated from human blood monocytes in a presence of GM-CSF (5ng/ml). On day 6, cells were stimulated with LPS (100ng/ml) for 1h with or without GSK-J4 (JMJD3 inhibitor) (30μM). GSK-J4 compound was added 15 min before LPS stimulation. Data represent *TNFA* absolute expression from 3 independent experiments. The expression level was normalised to GAPDH and ACTB housekeepers. Although considerable increase in the *TNFA* transcript level was observed upon LPS stimulation and reduction was detected after GSK-J4 administration (average 6-fold reduction), the data analysis using Mann-Whitney two-tailed test showed no significant difference between medians.

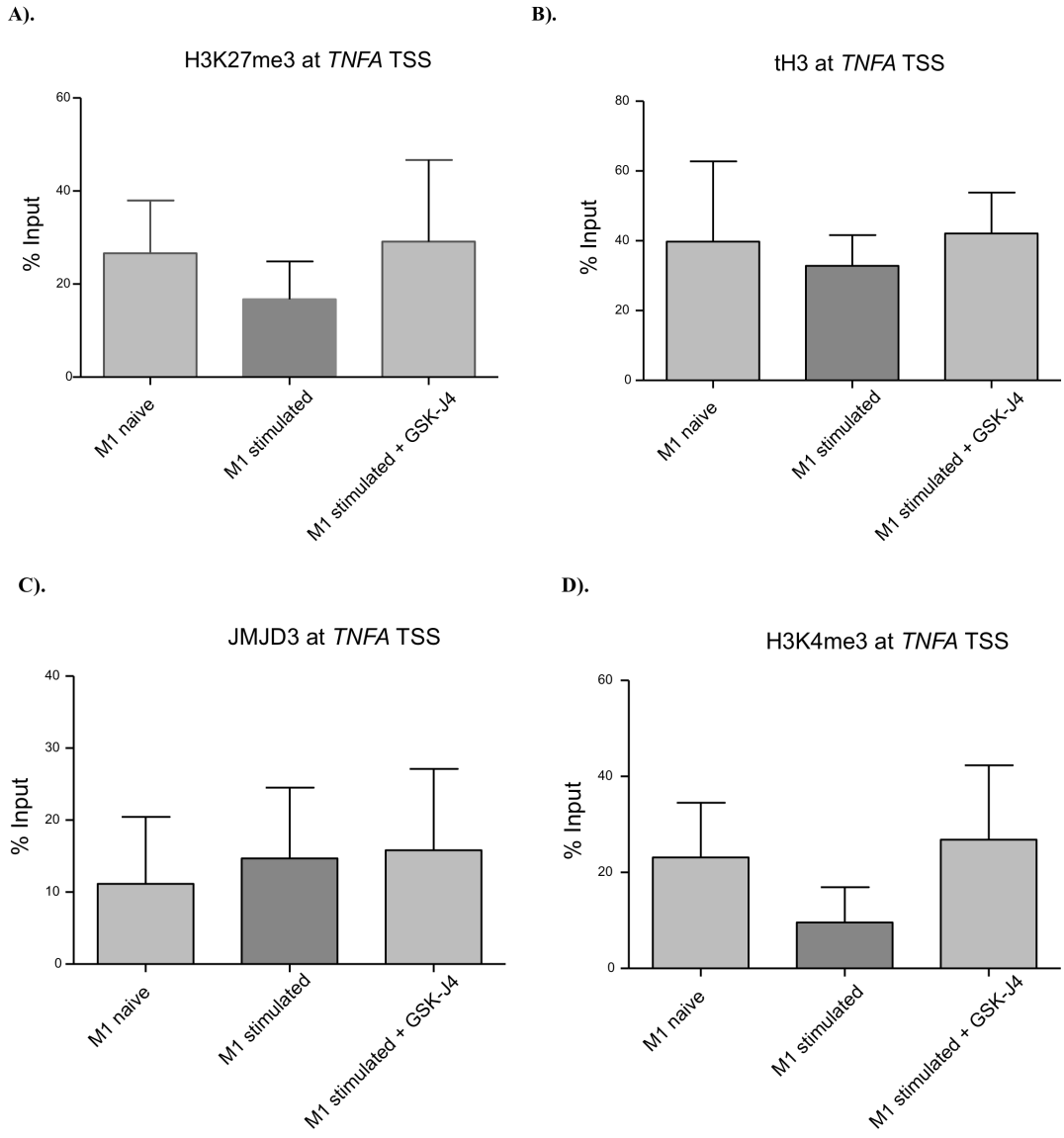


Figure 5.2: The assessment of direct binding of H3K27me3, H3K4me3 and JMJD3 to *TNFA* TSS after a treatment with GSK-J4 compound in human primary macrophages

ChIP assays were performed on human primary macrophages (GM-CSF). Macrophages were differentiated as in Figure 5.1. For H3K27me3 and tH3 data are presented as mean ± SEM of 3 experiments. For H3K4me3 and JMJD3 data are presented as mean ± SEM of 2 experiments. All data are presented as % input normalised to IgG. Data analysis showed no significant difference between groups. However, LPS stimulation and pre-treatment with GSK-J4 showed a modulatory effect on H3K27me3 and H3K4me3.

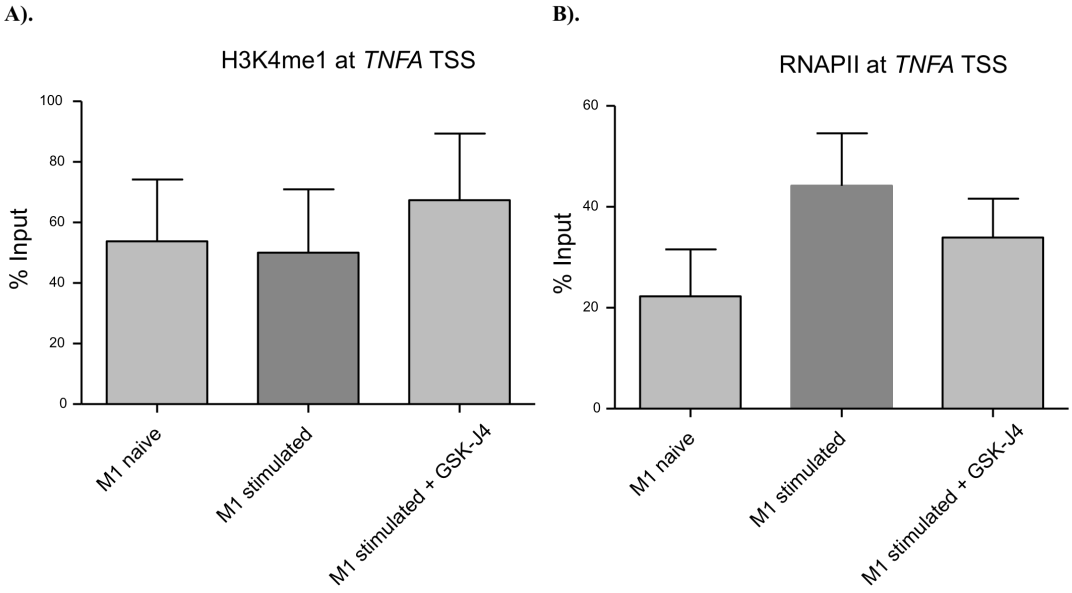
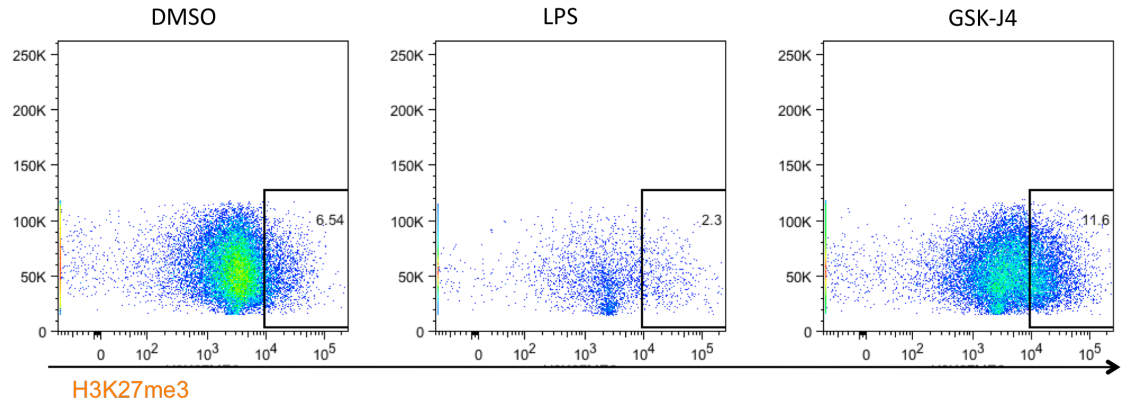


Figure 5.3: The assessment of direct binding of H3K4me1 and RNAPII to *TNFA* TSS after a treatment with GSK-J4 compound in human primary macrophages

ChIP assay performed on human primary macrophages (GM-CSF). Macrophages were differentiated as in Figure 5.1-2. For RNAPII data are presented as mean ± SEM of 3 experiments. For H3K4me1 data are presented as mean ± SEM of 2 experiments. All data are presented as % input normalised to IgG. Data analysis showed no significant difference between groups. LPS stimulation and pre-treatment with GSK-J4 showed a modulatory effect on RNAPII recruitment but not on H3K4me1.

A).



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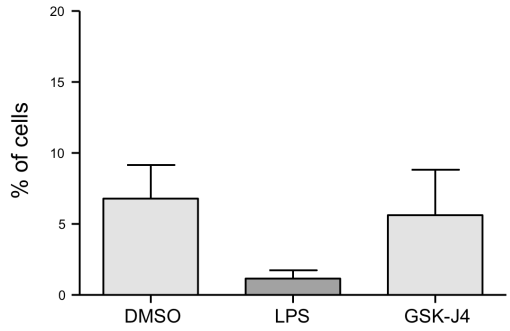
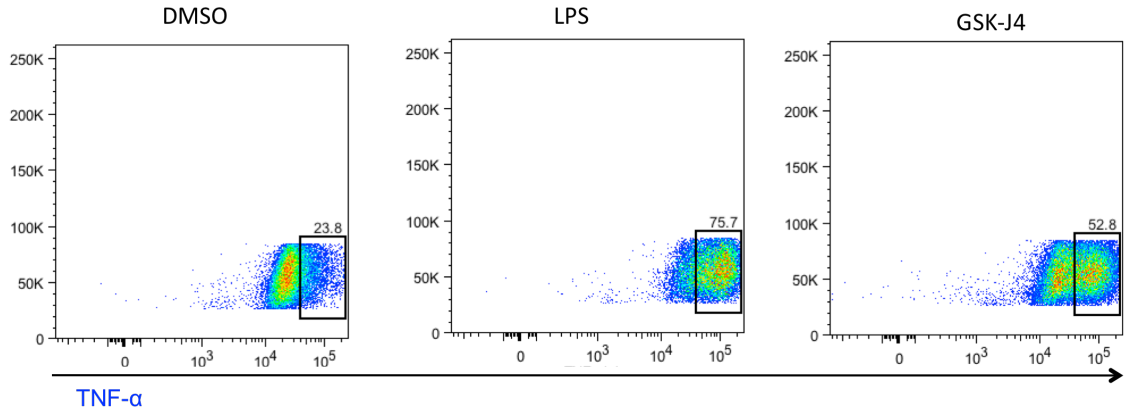


Figure 5.4: The assessment of global H3K27me3 level after a treatment with GSK-J4 compound in human primary macrophages (GM-CSF)

Flow cytometry analysis was used to assess H3K27me3 level. Macrophages were differentiated from human blood monocytes in a presence of GM-CSF (5ng/ml) as stated previously. A). Representative dot plot of H3K27me3 staining on human primary macrophages. B). The mean proportion of macrophages stained for H3K27me3. Data are presented as mean ± SEM of 3 donors. Data analysis showed no significant difference between groups.

A).



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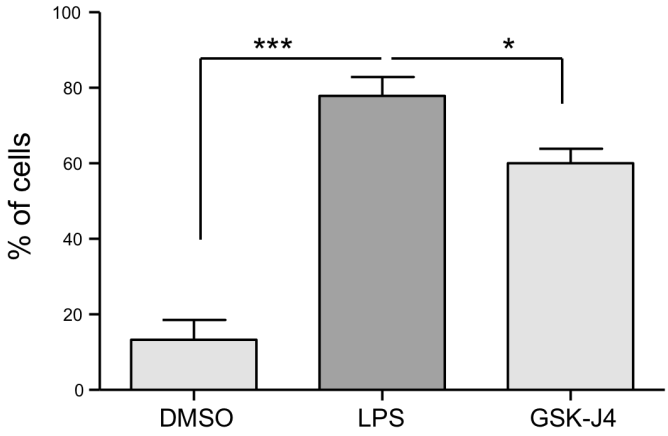


Figure 5.5: The assessment of TNF-α level after a treatment with GSK-J4 compound in human blood-derived (GM-CSF) macrophages

Flow cytometry analysis was used to assess TNF-α level. M1 macrophages were differentiated from human blood monocytes in a presence of GM-CSF (5ng/ml) as described previously. Data are presented as mean ± s.e.m. of 3 donors. A). Representative graph of TNF-α on GM-CSF macrophages. B). A graph showing a percentage of cells expressing TNF-α, (*) p≤ 0.05, (***) p≤ 0.001.

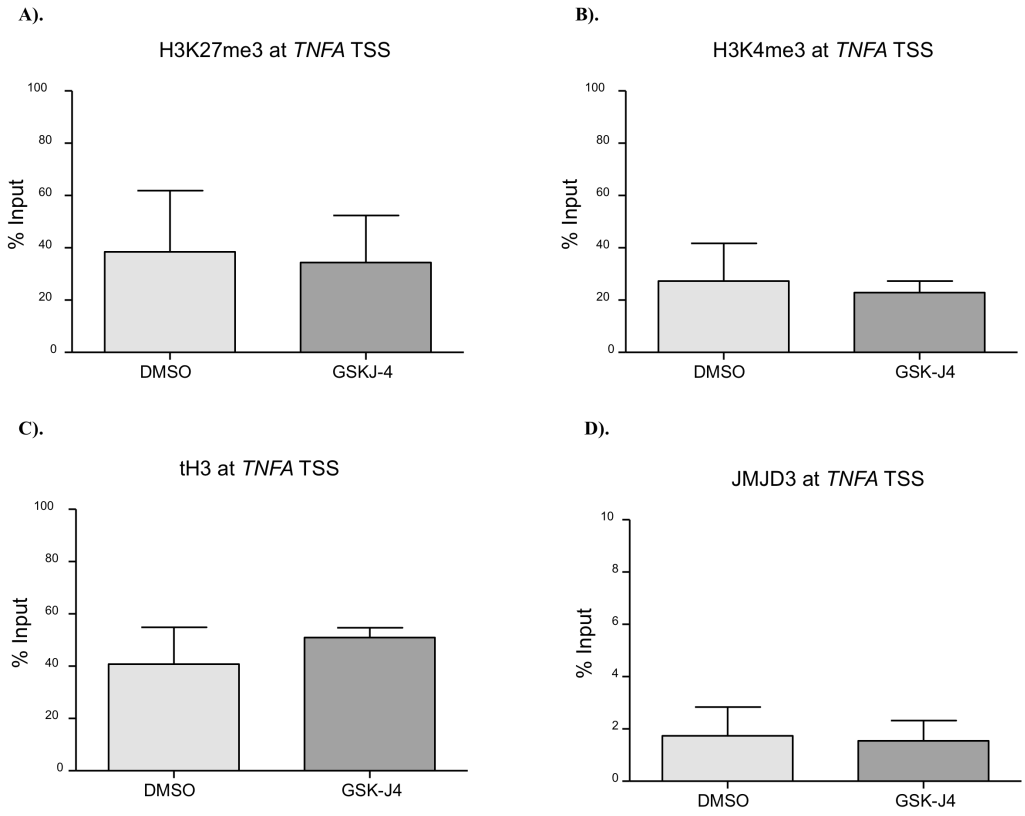


Figure 5.6: The assessment of H3K27me3, H3K4me3 and JMJD3 direct binding to *TNFA* TSS after a treatment with GSK-J4 compound in intestinal macrophages isolated from Crohn's patients

ChIP assay performed on intestinal macrophages isolated from Crohn's disease patients (n=3) treated with JMJD3 inhibitor. $CD33^{+}$MACS sorted LPMCs were incubated with JMJD3 inhibitor (30 μM) for 1h in 24 well plate format. Data are representative of 3 independent experiments (mean ± s.e.m.). Data are expressed as % input normalised to IgG. P value calculated using paired two-tailed Student's *t*-test.

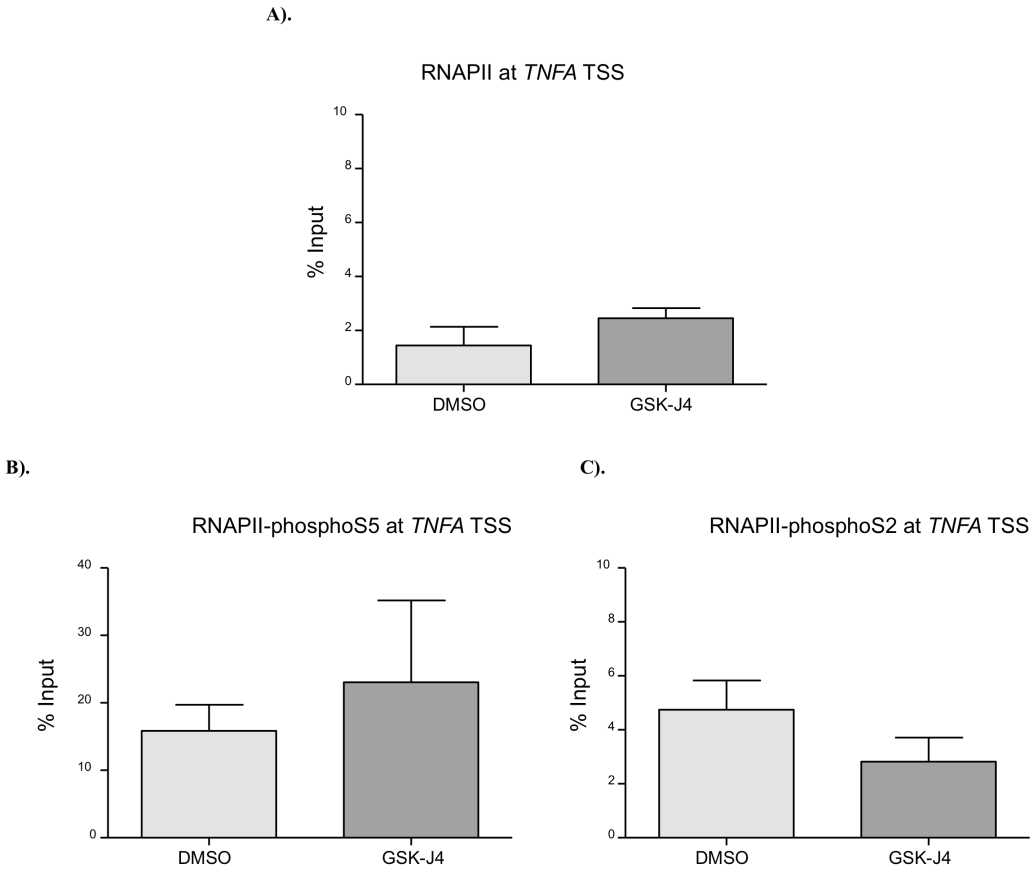


Figure 5.7: The assessment of RNAPII, RNAPII-phosphoS5 and RNAPII-phosphoS2 recruitment to *TNFA* TSS after a treatment with GSK-J4 compound in intestinal macrophages isolated from Crohn's patients

ChIP assay performed on intestinal macrophages isolated from Crohn's disease patients (n=3) treated with JMJD3 inhibitor. $CD33^{+}$MACS sorted LPMCs were incubated with JMJD3 inhibitor (30 μM) for 1h in 24 well plate format. Data are representative of 3 independent experiments (mean ± s.e.m.). Data are presented as % input normalised to IgG. P value calculated using paired two-tailed Student's *t*-test.

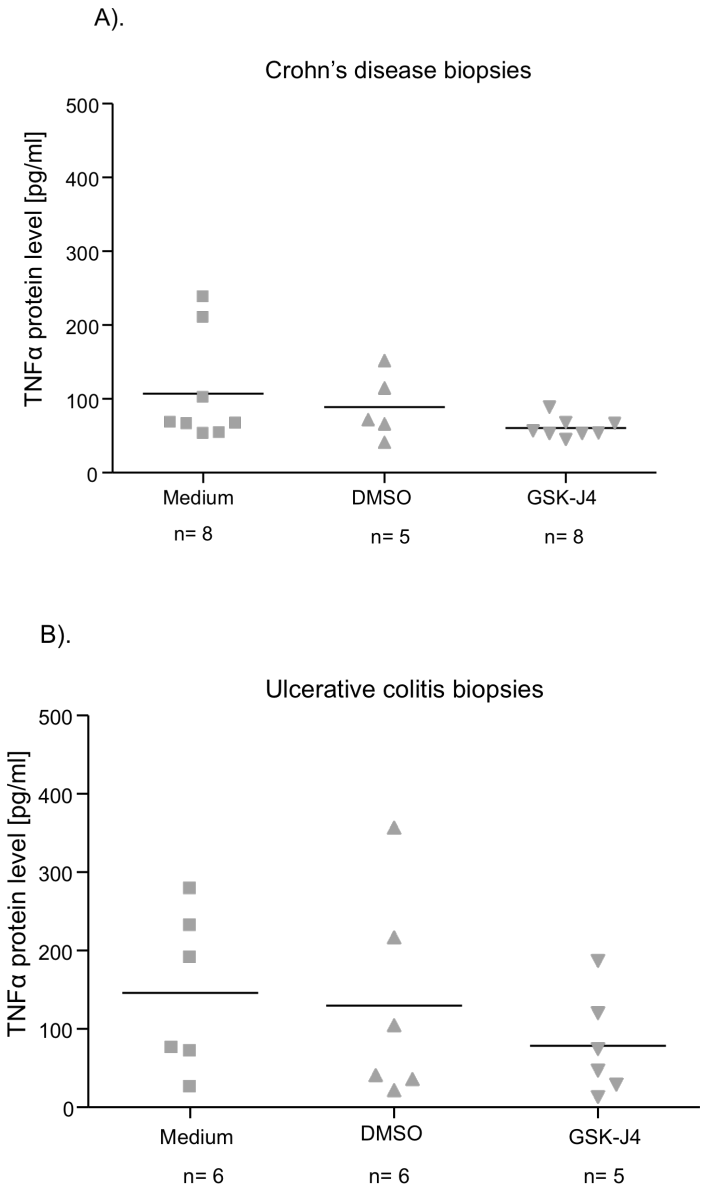


Figure 5.8: TNFA protein levels measured in Crohn's biopsies and ucelerative colitis after incubation with GSK-J4 compound for 24h

TNF-α protein level was measured using ELISA assay. Data are representative of independent experiments (mean ± s.e.m.). Data analysed using paired two-tailed Student's *t*-test. No significant difference was observed between treated and untreated groups.

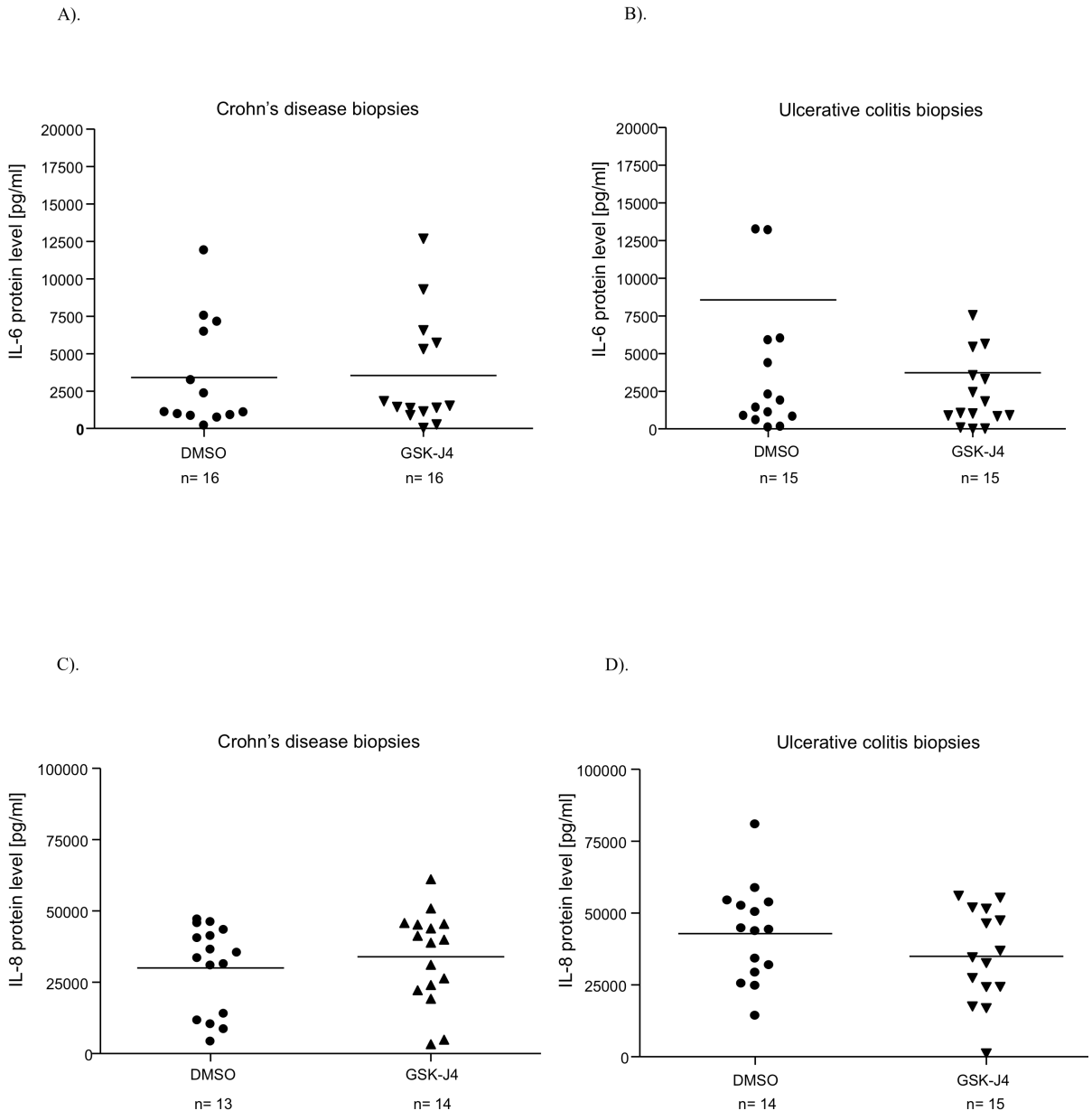


Figure 5.9: IL-6 and IL-8 protein levels measured in Crohn's disease and ucelerative colitis biopsies after incubation with GSK-J4 compound for 24h

Data are representative of independent experiments (mean ± s.e.m.). Data analysed using paired two-tailed Student's *t*-test. No significant difference was observed between treated and untreated groups. However, some degree of IL-6 reduction was observed for UC biopsy samples only.

Chapter 6

Characterisation of the gene expression pattern in intestinal macrophages isolated from the mucosa of patients with inflammatory bowel disease

6.1. Introduction

In the healthy gut, under homeostatic conditions, macrophages acquire their tissue-specific anergic phenotype as a consequence of downregulation of proinflammatory response cytokines, such as TNF-α, IL-6, IL-8, IL-1β (Sheikh and Plevy, 2010), and upregulation of antimicrobial properties. However, in an inflammatory surrounding, these cells remain activated, and therefore contribute to IBD pathogenesis, resulting in elevated level of proinflammatory mediators. In Chapter 1, the aspect of immune anergy was discussed in detail.

Physiologically, the state of anergy, also known as endotoxin tolerance, is considered to be a negative feedback response that protects against uncontrolled inflammatory responses, as observed in septic patients or during severe tissue injuries. *In vitr*o studies have shown that it is possible to induce endotoxin tolerance in human monocytes and macrophages with prolonged or repeated LPS stimulation.

In previous chapters work has focused primarily on the TNF, however it is likely that there are many different changes in gene expression between normal gut macrophages and those in patients with IBD. Some of these molecules could be potential targets for therapy. Therefore we undertook a microarray analysis of gene expression in macrophages from normal and inflamed human gut.

6.2. A specific subset of pro-inflammatory genes fails to undergo tolerisation in intestinal macrophages from IBD patients

The genes expression pattern in intestinal macrophages ($CD33^+$ LPMCs) was assessed using human common cytokine profiler array (84 genes). The study used gut macrophages ($CD33^+$ LPMCs) isolated from the colonic mucosa of 11 IBD patients (CD, n=4 and UC, n=7) and from the colon of 10 control subjects.

Initial data analysis aimed to group all genes into three categories; genes up- or down-regulated or those showing no change.

In total, 23 genes were downregulated and 36 were upregulated in UC and CD samples when compared with control subjects (Figure 6.1).

Among all genes analysed, *Il21*, *Il24*, *TNFSF8* were significantly ($p \leq 0.05$) upregulated in IBD patients (Table 6.1; Figure 6.2-3). The *TNFSF8* transcript levels increased by 9- and 13-fold, respectively in UC and CD macrophages. The *Il24* transcripts showed on average 5-fold increase in expression level and *Il21* transcripts showed a 52-fold change in expression compared to the control group. Additionally, *Il8*, *Il10*, *Il13*, *INHBA* were increased in IBD, however only in ulcerative colitis macrophages were the fold changes in expression levels significant (Table 6.1). *Il13* and *INHBA* showed the highest increase in expression (17-fold and 22-fold change in UC) comparing to controls (Table 6.1; Figure 6.3). The expression level of *Il8* and *Il10* increased at similar level (4- and 5-fold change in UC) (Figure 6.3). Also *LTB* was upregulated in IBD macrophages (9- and 17-fold changes in UC and CD, respectively), but only in CD was the fold change significant (Figure 6.1; Table 6.1).

Respectively, a significant ($p \leq 0.05$) 3- and 4-fold decrease in *BMP2* transcript levels was detected in both ulcerative colitis and Crohn's disease macrophages (Figure 6.2-3). Furthermore, *GDF5* and *Il1F5* were significantly upregulated (7- and 4-fold change) and *BMP5* and *Il1F7* were significantly downregulated (6- and 11-fold change) only in ulcerative colitis (Table 6.1; Figure 6.3). In contrast, *Il15* was only upregulated in CD (3-fold change) (Table 6.1, Figure 6.2-3).

Finally, all genes that were upregulated in IBD were also additionally analysed for their potential to undergo tolerisation and are highlighted in red font (Figure 6.5).

The gene expression pattern was also assessed in reference to *in vitro* tolerised GM-CSF differentiated human primary macrophages, which following repeated LPS stimulation show downregulation of number of proinflammatory cytokine genes. Colleagues at EpiNova, at GSK in Stevenage performed the study on tolerogenic human primary macrophages and the data was kindly provided for this analysis (Appendix 0.3).

For that purpose, genes which were downregulated in IBD, were excluded from analysis. It was observed that only some of upregulated genes such as *TNFA, LTA, TNFSF8, IL10, IL1β, IL1F5, IL24, INHBA* which are LPS-tolerised in human primary macrophages (GM-CSF-differentiated) failed to become tolerised in intestinal macrophages from IBD patients (Figure 6.5).

6.3. A role for IL-24 in proinflammatory responses in IBD

Performing gene array analysis on intestinal macrophages enabled to identify *IL24* (belonging to IL-10 cytokine family) as a candidate gene potentially involved in the pathophysiology of IBD. A significant 4-fold increase in *IL24* mRNA levels was observed in macrophages isolated from IBD mucosa when compared with controls (Table 6.1). Therefore, additional analysis of IL24 was performed on protein level. A significant increase in IL-24 protein was detected in both inflamed ($p \leq 0.001$) and uninflamed ($p \leq 0.05$) CD biopsies, and also in inflamed ($p \leq 0.05$) UC biopsies (Figure 6.6).

To investigate a proinflammatory potential of IL-24, IBD biopsies were stimulated with recombinant human (rh)IL-24 (100ng/ml). After 24h stimulation, supernatants were collected and analysed for TNF-α, IL-6, IL-8 levels. Stimulation with (rh)IL-24

triggered increased ($p \leq 0.05$) production of TNF-α only in CD ex-vivo organ culture (Figure 6.7). No change in other cytokines (IL-6, IL-8) was detected.

6.4. Discussion

Mucosal immune cells respond to microbial products or antigens by producing cytokines that promote inflammatory reaction in the gastrointestinal tract. These inflammatory responses need to be tightly regulated to prevent any uncontrolled reaction and subsequent tissue destruction. The causative molecular aspect of IBD pathogenesis still remains poorly understood. Increased knowledge on IBD suggests that number of effector molecules are involved in maintaining the homeostatic intestinal immunity. However, dysregulated production of these molecules leads to abnormal immune responses towards intestinal flora (Jump et al., 2004). Hence, cytokine production underlies IBD pathogenesis and targeting these molecules has successfully led to a reduced disease activity (MacDonald et al., 2011).

Macrophages play a fundamental role in regulating controlled gut immunity. However, activated macrophages are thought to be the major contributor to a production of proinflammatory cytokines in the gut, which consequently drives the differentation of many subsets of T cells and activates the adaptive immune responses. Under homeostatic conditions, macrophages acquire a rather unique phenotype, as they become hyporesponsive to activation. This state is known as endotoxin tolerance. The concept of endotoxin tolerance in reference to unique phenotype of intestinal macrophages was already discussed.

To investigate novel aspects of IBD pathogenesis, gene microarray analysis was performed to determine changes in gene expression in macrophages isolated from mucosa of Crohn's and ulcerative colitis patients. To provide more comprehensive

data on the aspect of endotoxin tolerance, gene expression profile of human intestinal macrophages was analysed with reference to LPS-tolerised human primary macrophages. It allowed studying the level and the pattern of expression of a large number of genes simultaneously. The cDNA microarray representing 84 genes was used to compare gene pattern of intestinal macrophages (IMACs) from 11 IBD patients (CD= 4; UC= 7) and 10 control individuals. In total, 59 genes were identified to have different expression pattern between disease and control groups. Among all, the majority (n= 36) of assessed genes were up regulated in IBD samples. Additionally, some genes showed opposing expression pattern between CD and UC and were categorised as UC- or CD-specific genes (for UC, genes such as *GDF5*, *Il1F5*, *BMP5*, *Il1F7* and for CD, *Il15*) (Figure 6.4).

The gene expression profile was also classified accordingly to gene function or based on different family group (Table 6.1). A large number of these genes have been shown to function in proliferation and differentation pathways.

Among genes belonging to TGF-β family, all analysed BMPs were downregulated in IBD macrophages except BMP7, with BMP5 showing UC-specific expression pattern, and BMP2 characteristic for IBD (Table 6.1).

The role of BMPs in the gut is just beginning to be elucidated. The BMPs are important morthogens crucial in developmental processes that regulate the maintenance of adult tissue homeostasis (Wang et al., 2014). It is known that many processes in early development are dependent on BMP signalling, including cell growth, apoptosis and differentation (Kishigami and Mishina, 2005; Lowery and de Caestecker, 2010). The BMP signalling involves JAK/STAT and Notch pathways (Guo *et al*., 2009).

The BMPs are important in normal cell development in the intestine (Batts et al., 2006), as a possible malfunction of how BMPs exert its function may result in the development of cancerous tissue (Hardwick et al., 2008; Wang et al., 2014). For example, BMP2 has a crucial role during the embryonic development of digestive organs (Yuvaraj et al., 2012). Also the colon is characterised by high expression of *BMP2* (Hardwick et al., 2004; Kosinski et al., 2007) and genetic loss of *BMP2* signalling is associated with the development of sporadic cancer (Kodach et al., 2008). It has been shown that BMP2 is an important and powerful tumor suppressor in the colon (Batts et al., 2006). Batts and colleagues (2006) demonstrated that *BMP2* transcripts are present in epithelial cells at the villus tips and in the surface epithelium of the large intestine. They also showed that inhibiting BMP signalling leads to abnormal villus morphogenesis, stromal and epithelial hyperplasia. Additionally, mesenchynal loss of BMP signalling has been implemented in colorectal carcinogenesis (Hardwick et al., 2008).

However, *BMP2* expression is not only restricted to epithelial cells. A recent paper by Muller (2014) highlights an interesting concept of interplay between BMP2 signalling and a distinct population of macrophages distributed in the intestinal muscularis externa. They showed that, in the steady state, these macrophages regulate peristaltic activity of the colon by secreting BMP2, which activates BMP2 receptor by enteric neurons. Additionally, they also showed that microbial commensals regulate BMP2 expression by macrophages (Muller et al., 2014). The muscularis macrophages (MMs) population resembles the $CD103^{-}CD11b^{+}CX3CR1^{+}$ macrophages, which are present in the intestinal lamina propria (Bugonovic et al., 2009). However, the function of MMs is less characterised compared to mucosal counterparts (Muller et al., 2014).

Changes in the cytokine profile, between CD and UC were also detected for *BMP5*, *GDF5* and *INHBA*. The expression level of all three genes was significantly different in UC, but not in CD macrophages (Figure 6.2-3). The role of BMP5 is less known. The GDF5 and INHBA are also members of TGF-β superfamily and are closely related to BMPs (Miyamoto et al., 2007). The GDF5 was shown to be a part of LPS receptor cluster, functioning as a signal transducer and that GDF5 blocking may limit the response to LPS affecting immune responses and macrophage functions (Daans at al., 2009; Triantafilou et al., 2001; 2002). The *INHBA* was linked with the autoimmune disease, such as rheumatoid arthritis (Dong et al., 2014). Also, *INHBA* over-expressed transcript was reported to be associated with different cancers including colorectal cancer (Okano et al., 2013) and gastric cancer (Wang et al., 2012).

The *INHBA* gene encodes for Activin A that is produced upon macrophage activation and is involved in the early phases of inflammatory responses (Dong et al., 2014). Activin A also promotes the acquisition of GM-CSF-dependent macrophage polarisation markers (Escribese et al., 2012) and was shown to regulate macrophage polarisation.

Additionally, the array analysis showed an increase in expression of both *LTA* and *LTB* (lymphotoxin, type II membrane protein of the TNF family), together with *TNF* genes, but only *LTB* was significantly upregulated in CD. The *TNF* locus encodes factors that are the key components of the immediate innate immune responses, and *LTA* and *LTB* constitute *TNF* locus. The transcriptional orientation of *LTB* is opposite to that of *TNF* and *LTA* (Appendix 0.2) (Cross et al., 2005; Deakin et al., 2006). Of interest, among all three genes, *TNF* and *LTA* were shown to become tolerised in human primary macrophages (data provided by Epinova, GSK). This

finding suggests potentially another mechanism of *TNF* transcription involved in IBD.

Interestingly, among all genes belonging to TNF superfamily, *TNFSF8* was also highly upregulated ($p \leq 0.05$) in both CD (13 fold increase) and UC (9-fold increase) intestinal macrophages (Table 6.1, Figure 6.2-3). The *TNFSF8* gene encodes a ligand (CD30L) for TNFRSF8 (CD30) receptor (Sun et al., 2013). The CD30-CD30L pathway tightly controls immune responses by providing positive signals that promote activation and proliferation of B and T cells (especially an activation of interleukin 17A (IL-17A) producing γδ T cells)) (Sun et al., 2013). Whereas, CD30 and CD30L are both present on activated B and T cells, CD30L is also expressed on mature dendritic cells and macrophages (Foks et al., 2012). Also recently, *TNFSF8* was identified as a novel risk factor for Crohn's disease (Hong et al., 2015).

The IL-2 family of cytokines plays an important role in T cell function. There are 6 cytokines belonging to this family and include IL-2, IL-4, IL-7, IL-9 IL-15 and IL-21. Each IL-2 family cytokine has unique functions, and due to differences in the expression patterns of each cytokine and their receptors, different family members regulate the T cell physiology at different maturation stages (Marrack et al., 2000; Kelly et al., 2002). Cytokines belonging to IL-2 family play a major role in regulating development, survival, proliferation and differentation (Malek et al., 2010). For example, IL-7 is required to maintain an optimum number of immature T cells (Grabstein et al., 1993; Ku et al., 2000), but also just like IL-4 is involved in regulating the number of naïve T cells (Kelly et al., 2002).

In this study, cytokines of IL-2 family also differed in their expression pattern. Among all IL-2 family cytokines, IL-7 was not expressed in both UC and CD intestinal macrophages, as is expressed by epithelial cells in thymus and bone

marrow (Schluns and Lefrancois, 2003; Fry and Mackall, 2005). Also *Il4* was not up-regulated in UC and down regulated in CD (15-fold change), however not significantly. Additionally, *I15* showed CD-specific expression pattern, as 3-fold increase ($p \leq 0.05$) in expression was only observed in these cells only. In UC, the expression level of *Il15* was the same as in control group. Several studies have demonstrated that IL-7 and IL-15 are essential in maintaining memory T cells (Kim et al., 2007) and it was shown that animals genetically lacking IL-15 or its receptor do not have memory phenotype $CD8^+$ T cells (Kennedy et al., 2000; Kim et al., 2007). IL-15 is secreted mainly by APCs (DCs and macrophages) and monocytes and it is involved in promoting cytotoxicity and maintenance of memory $CD8^+$ T cells and also proliferation and differentation of NK and NK T cells (Waldmann et al., 2006; Kim et al., 2007).

The position of IL-21 in the pathogenesis of IBD has long been recognised (MacDonald et al., 2011). IL-21 is increased in biopsies from patients with IBD. Recent Genome-Wide Association studies have shown an association between the locus containing IL-2/IL-21 and IBD (Jostins et al., 2012; Neurath, 2014).

In the study presented here, IL-21 had the highest increase in expression (50-fold change) ($p \leq 0.05$) in both, UC and CD (Table 6.1). IL-21 is a pleiotropic cytokine that has both proinflammatory (Fantini et al., 2008) and anti-inflammatory activities (Spolski et al., 2009). IL-21 is a cytokine with close structural similarities to IL-15, IL-2 and IL-4 (Parrish-Novak et al., 2000; Ruckert et al., 2007). IL-21 binds to a heterodimeric receptor composed of a high-affinity α-chain and the common γ-chain which is shared by all IL-2 cytokine family (Brandt et al., 2007). The expression of complete IL-21 receptor was reported in dendritic cells (DCs) and in synovial macrophages (Jungel et al., 2004). IL-21Rα was also found in bone marrow (BM)

cells and it has been shown that IL-21 modulates the differentation of murine myeloid DCs. IL-21 might also be involved in the interaction of antigen presenting cells (APCs), like macrophages with T cells at inflammatory site (Ruckert et al., 2007). Additionally, IL-21 is involved in the cytokine-driven proliferation of $CD4^+$ helper T cells synergically with IL-7 and IL15 (Onoda et al., 2007), and that the cytokine induces apoptosis of antigen-specific $CD8^+$ T cell (Barker et al., 2007).

Among genes encoding cytokines belonging to IL-10 cytokine family, *Il10* was increased in UC (5-fold change) and CD (4-fold change) groups, but only in UC with a significant difference ($p \leq 0.05$) (Table 6.1). Additionally, a significant 4- and 5-fold increase in expression level of *Il24* was observed in intestinal macrophages isolated from mucosa of UC and CD patients, respectively.

Gene array analysis on intestinal macrophages enabled to identify *Il24* as a candidate gene involved in the pathophysiology of IBD, which was additionally supported by functional studies on protein level. The role of IL-24 in driving immune responses has already been reported in number of studies (Poindexter et al., 2005; Andoh et al., 2009; Sahoo et al., 2011). IL-24 expression has been identified in dermal keratinocytes, LPS-stimulated monocytes and macrophages or Th2-polarised T cells (Wolk et al., 2002; Poindexter et al., 2005; Kunz et al., 2006).

IL-24 is a member of IL-10 family of cytokines, together with IL-10, IL-19, IL-20, IL-22, IL-26, IL-28, IL-29 (Jiang et al., 1996; Langer et al., 2004). IL-24 has been shown to bind two different heterodimeric receptor complexes, IL-20R1/IL-20R2 and IL-22R1/IL20R2, which activates STAT3 (Wang et al., 2005). As oppose to non-immune tissues, immune cells do not express IL-24 receptors, suggesting that IL-24 cannot stimulate the acquired immune responses (Nagalakshmi et al., 2004; Wang et al., 2005; Kunz et al., 2006). The biological function of IL-24 is still poorly

understood, but IL-24 can function as an intracellular cell death-inducing factor or as a classical cytokine through cell surface receptor (Andoh et al., 2013). Through receptor binding, IL-24 has been reported to induce expression of proinflammatory cytokines from monocytes, such as TNF-α and IL-6 (Caudell et al., 2002). Just recently, Andoh and collegues (2013) have reported increased IL-24 expression level in the inflamed mucosa of patients with inflammatory bowel disease. Our preliminary data on IL-24 have confirmed these findings. Andoh and collegues (2013) have also identified that colonic subepithelial myofibroblasts (SEMFs) were a major source of lL24 in the mucosa. Interestingly they have also demonstrated that IL-24 was expressed in infiltrating immune cells (Andoh et al., 2013).

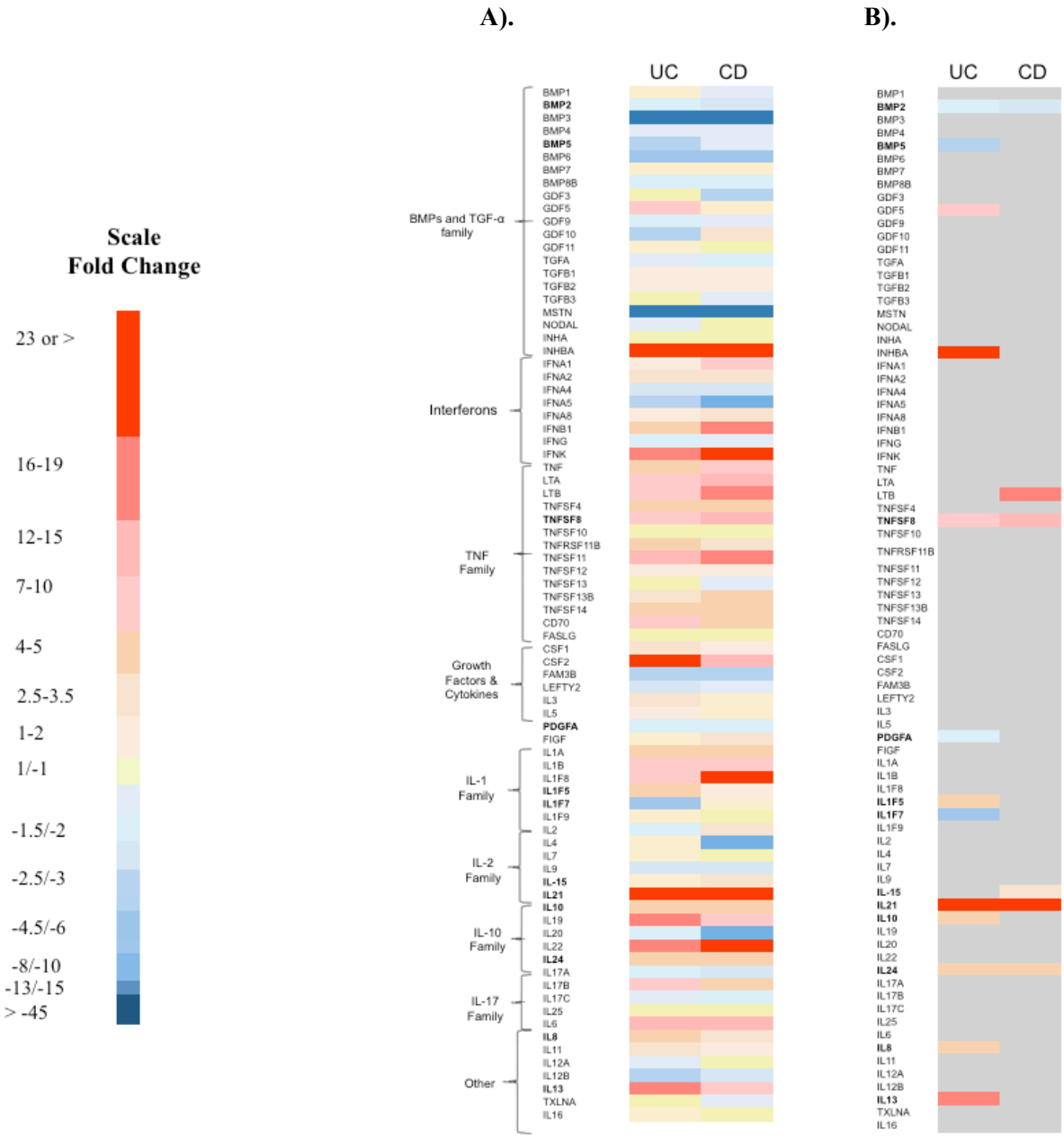


Figure 6.1: Heat map representation of cytokine gene expression pattern by intestinal macrophages isolated from mucosa of IBD patients

The study included human gut macrophages ($CD33^{+}$ LPMCs) isolated from the colonic mucosa of 11 IBD patients (CD, n=4 and UC, n=7) and from colon of 10 control subjects. **A).** Gene expression was assessed using human common cytokine profiler array (84 genes). **B).** Heat map representing only genes with significant difference. Data are represented as fold change in reference to control samples (red: high expression; blue: low expression). Data analysed using two-way ANOVA of variance.

	Ulcerative colitis		Crohn's disease	
	Fold Change	P-value	Fold Change	P-value
TGF-β Family:				
BMP2	-3	***0.0291***	-4	***0.0221***
BMP3	-268	*0.2496*	-274	*0.3328*
BMP4	-2	*0.243*	-2	*0.2588*
BMP5	-6	***0.0061***	-2	*0.3694*
BMP6	-10	*0.176*	-8	*0.3059*
BMP7	1	*0.7213*	1	*0.8585*
BMP8B	-3	*0.1357*	-3	*0.2667*
GDF3	-1	*0.824*	-5	*0.5224*
GDF5	7	***0.0518***	1	*0.7841*
GDF9	-3	*0.1091*	-2	*0.3807*
GDF10	-5	*0.2627*	3	*0.4829*
GDF11	1	*0.803*	-1	*0.8811*
TGFA	-2	0.3243	-3	0.1418
TGFB1	2	0.0871	2	0.2473
TGFB2	2	0.3585	2	0.2864
TGFB3	-1	0.7887	-2	0.3097
MSTN	-45	*0.0835*	-138	*0.0644*
NODAL	-2	0.0905	-1	0.4407
INHA	-1	0.7178	-1	0.7729
INHBA	22	**0.0553**	24	0.099
Metalloproteases:				
BMP1	1	*0.5547*	-2	*0.1276*
Growth factors/Cytokines:				
CSF1	3	*0.1042*	2	*0.3197*
CSF2	28	*0.1022*	13	*0.2871*
FAM3B	-6	*0.5619*	-6	*0.6155*
LEFTY2	-4	*0.1149*	-2	*0.4837*
IL3	3	*0.1286*	1	*0.7778*
IL5	2	*0.3301*	1	*0.665*
PGDF/VEGF family:				
PDGFA	-3	**0.0422**	-3	0.0849
FIGF	1	0.6658	3.5	0.1574
Interferons:				
IFNA1	2	*0.5552*	7	0.0945
IFNA2	3	*0.2489*	3	0.4174
IFNA4	-4	*0.0752*	-4	0.1531
IFNA5	-5	*0.4877*	-14	0.373
IFNA8	2	*0.4136*	3	0.1253
IFNB1	4	*0.2683*	18	*0.0643*
IFNG	-3	*0.2202*	-2	*0.547*
IFK	16	*0.4926*	36	*0.4625*

continued

	Ulcerative colitis		Crohn's disease	
	Fold Change	P-value	Fold Change	P-value
TNF Superfamily:				
TNF	4	*0.1847*	8	*0.0935*
LTA	10	*0.0862*	12	*0.116*
LTB	9	*0.0661*	17	***0.0534***
TNFSF4	4	*0.1125*	4	*0.1721*
TNFSF8	9	***0.0461***	13	***0.0497***
TNFSF10	-1	*0.3731*	-1	*0.803*
TNFRSF11B	4	*0.1956*	3	*0.4646*
TNFSF11	12	*0.2494*	18	*0.2538*
TNFSF12	2	*0.148*	2	*0.2751*
TNFSF13	-1	*0.7888*	-2	*0.119*
TNFSF13B	3	*0.1007*	4	*0.0797*
TNFSF14	4	*0.0605*	4	*0.153*
CD70	7	*0.0912*	4	*0.3124*
FASLG	-1	*0.9311*	-1	*0.9368*
IL-1 cytokine family:				
IL1A	4	*0.1289*	5	*0.1746*
IL1B	9	*0.0611*	9	*0.1228*
IL1F8	8	*0.3989*	194	*0.0785*
IL1F5	4	***0.037***	2	*0.247*
IL1F7	-11	***0.0352***	1	*0.9714*
IL1F9	1	*0.9712*	-1	*0.8978*
IL-2 cytokine family:				
IL2	-3	*0.2272*	3	*0.3391*
IL4	1	*0.9349*	-15	*0.4073*
IL7	1	*0.2846*	-1	*0.8762*
IL9	-4	*0.4718*	-4	*0.5997*
IL15	1	0.5405	3	***0.0486***
IL21	52	***0.0216***	52	***0.0498***
IL-10 cytokine family:				
IL10	5	***0.0307***	4	*0.1107*
IL19	17	*0.3481*	8	*0.5722*
IL20	-3	*0.5413*	-14	*0.2517*
IL22	16	*0.1087*	50	*0.0634*
IL24	4	***0.0194***	5	***0.0227***
IL-17 cytokine family:				
IL17A	-3	*0.6126*	-4	*0.5794*
IL17B	7	*0.1969*	4	*0.4065*
IL17C	-2	*0.369*	-3	*0.3479*
IL25	-1	*0.6445*	-1	*0.4799*

continued

	Ulcerative colitis		Crohn's disease	
	Fold Change	P-value	Fold Change	P-value
Other interleukins:				
IL6	14	*0.1249*	12	*0.2239*
IL8	4	***0.0415***	3	*0.1622*
IL11	3	*0.2226*	2	*0.4866*
IL12A	-2	*0.1933*	-1	*0.7163*
IL12B	-5	*0.5537*	-4	*0.6909*
IL13	17	***0.0393***	6	*0.2392*
TXLNA (IL14)	-1	*0.38*	-2	*0.0792*
IL16	1	*0.594*	1	*0.6663*

Table 6.1: Data represents gene microarray analysis as in Figure 6.1. Annotated genes were classified accordingly to cytokine family

The study included human gut macrophages ($CD33^{+}$ LPMCs) isolated from the colonic mucosa of 11 IBD patients (CD, n=4 and UC, n=7) and from colon of 10 control subjects. Data are represented as Log2-fold change in gene expression in reference to control samples. Genes with significant difference are highlighted in red. Data analysed using one-way ANOVA of variance.

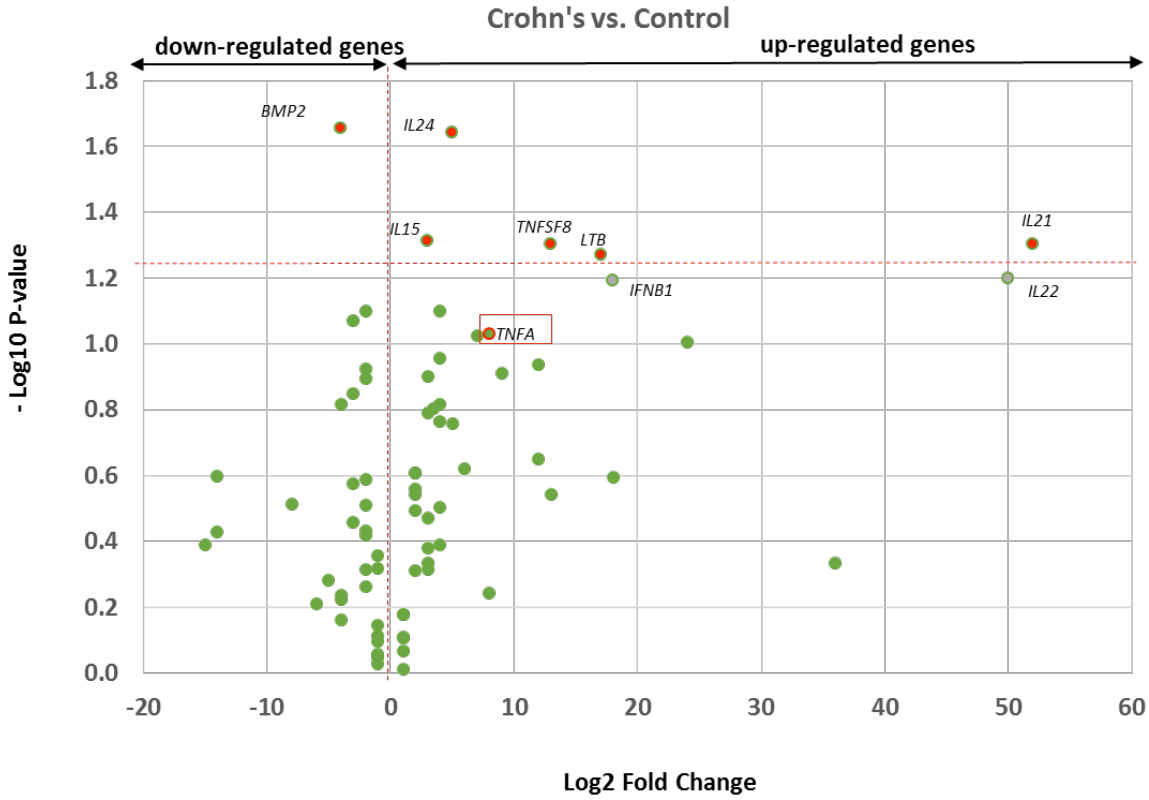


Figure 6.2: Volcano plot showing a summary of qRT-PCR array (Table 6.1) performed on human intestinal macrophages (CD33$^+$ LPMCs) isolated from patients with Crohn's disease (CD) and control subjects

The gut macrophages were isolated from the colonic mucosa of 4 CD and from colon of 10 control subjects. Expression level was normalised to RPL13A housekeeper gene. Data are presented as log2 fold change vs. –log10 p-value. Red dots above dashed horizontal line highlight genes with significant values ($p \leq 0.05 = -\log10 \geq 1.2$). Assessed genes were grouped into two categories; genes up- or down-regulated (marked by a vertical dashed red line).

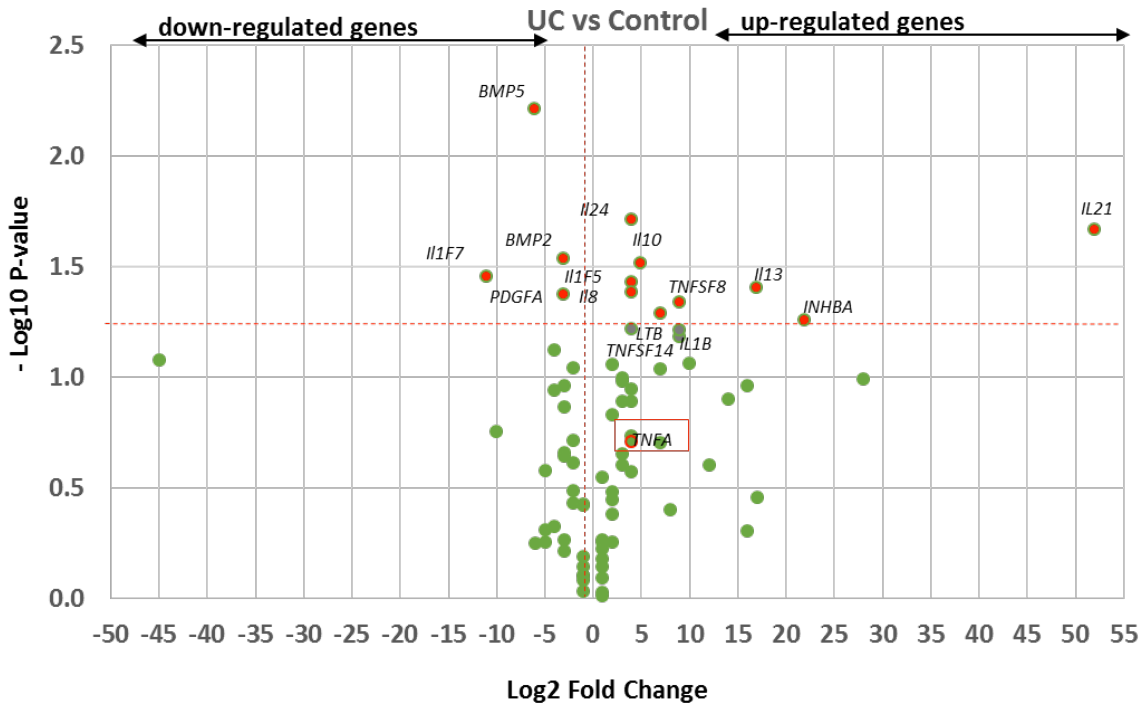
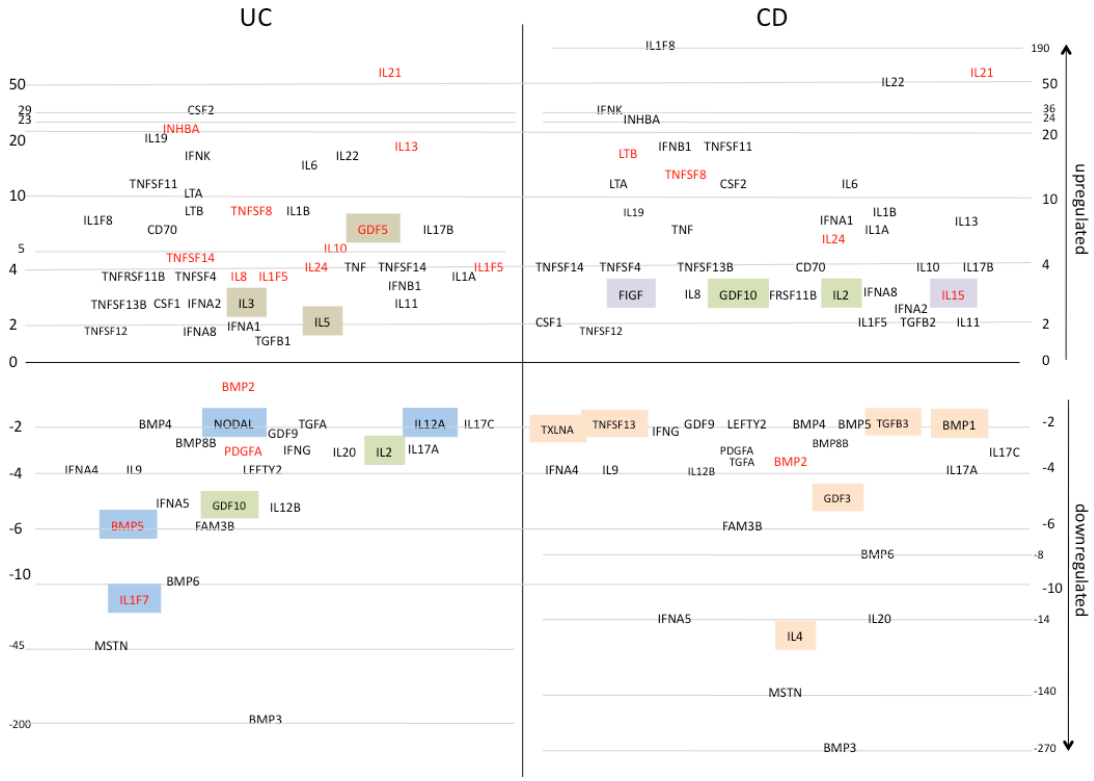


Figure 6.3: Volcano plot showing a summary of qRT-PCR array (Table 6.1) performed on human intestinal macrophages ($CD33^+$ LPMCs) isolated from patients with ulcerative colitis (UC) and control subjects

The gut macrophages were isolated from the colonic mucosa of 7 UC and from colon of 10 control subjects. As in Figure 6.2 expression level was normalised to RPL13A housekeeper gene. Data are presented as log2 fold change vs. –log10 p-value. Red dots above dashed horizontal line highlight genes with significant values ($p \leq 0.05 = -\log10 \geq 1.2$). Assessed genes were grouped into two categories; genes up- or down-regulated (marked by a vertical dashed red line).



Upregulated CD-specific genes	*IL15, FIGF*
Upregulated UC-specific genes	*IL3, IL5, GDF5*
Downregulated CD-specific genes	*BMP1, GDF3, TGFB3, TNFSF13, TXLNA, IL4*
Downregulated UC-specific genes	*IL1F7, BMP5, NODAL, IL12A*
Common genes significantly upregulated in CD and UC	*IL21, IL24, TNFSF8*
Common genes significantly downregulated in CD and UC	*BMP2*
Genes with reversed expression pattern	*GDF10, IL2*

Figure 6.4: A summary of qRT-PCR array (Table 6.1) performed on intestinal macrophages (CD33^{+} LPMCs) isolated from IBD patients and control subjects

Assessed genes were grouped into 5 subcategories; 1: ( ) upregulated CD-specific genes, 2: ( ) upregulated UC-specific genes, 3: ( ) downregulated CD-specific genes, 4: ( ) downregulated UC-specific genes, 5: ( ) genes with reversed expression in CD and UC. Expression level was normalised to RPL13A housekeeper gene. Data are presented as fold change in reference to control group. Red font highlights genes with significant values ($p \leq 0.05$).

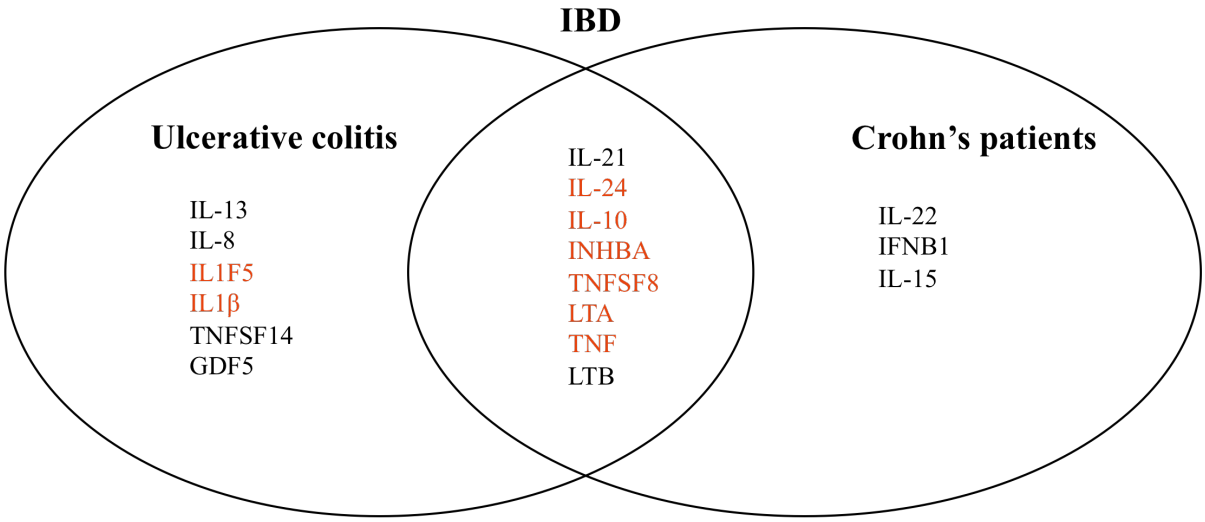


Figure 6.5: Musocal expression of genes, which are tolerised in human primary macrophages fail to be down regulated in IBD vs. control intestinal macrophages ($CD33^+$ LPMCs)

Genes highlighted in red are tolerised in *in vitro* model and failed to undergo tolerisation in IBD samples.

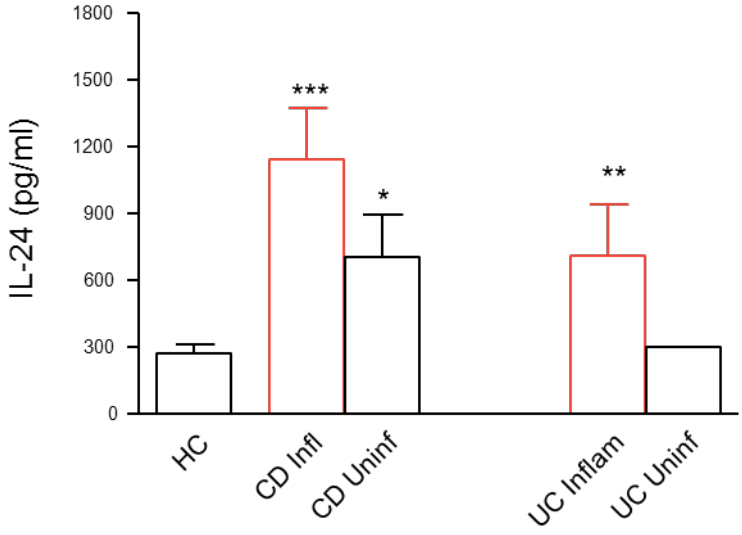


Figure 6.6: IL-24 protein level measured in IBD and control subjects, in ex-vivo organ culture

IBD (CD inflamed, n= 4; CD uninflamed, n= 7; UC inflamed, n= 4; UC uninflamed, n= 3) and control (n= 11) biopsies were incubated for 24h in 24-well plate format. Next, supernatants were collected and used for ELISA analysis to detect IL-24 protein level. A significant increase in IL-24 protein level was detected in both inflamed ($p \leq 0.001$) and uninflamed ($p \leq 0.05$) CD biopsies, and also in inflamed ($p \leq 0.05$) UC biopsies.

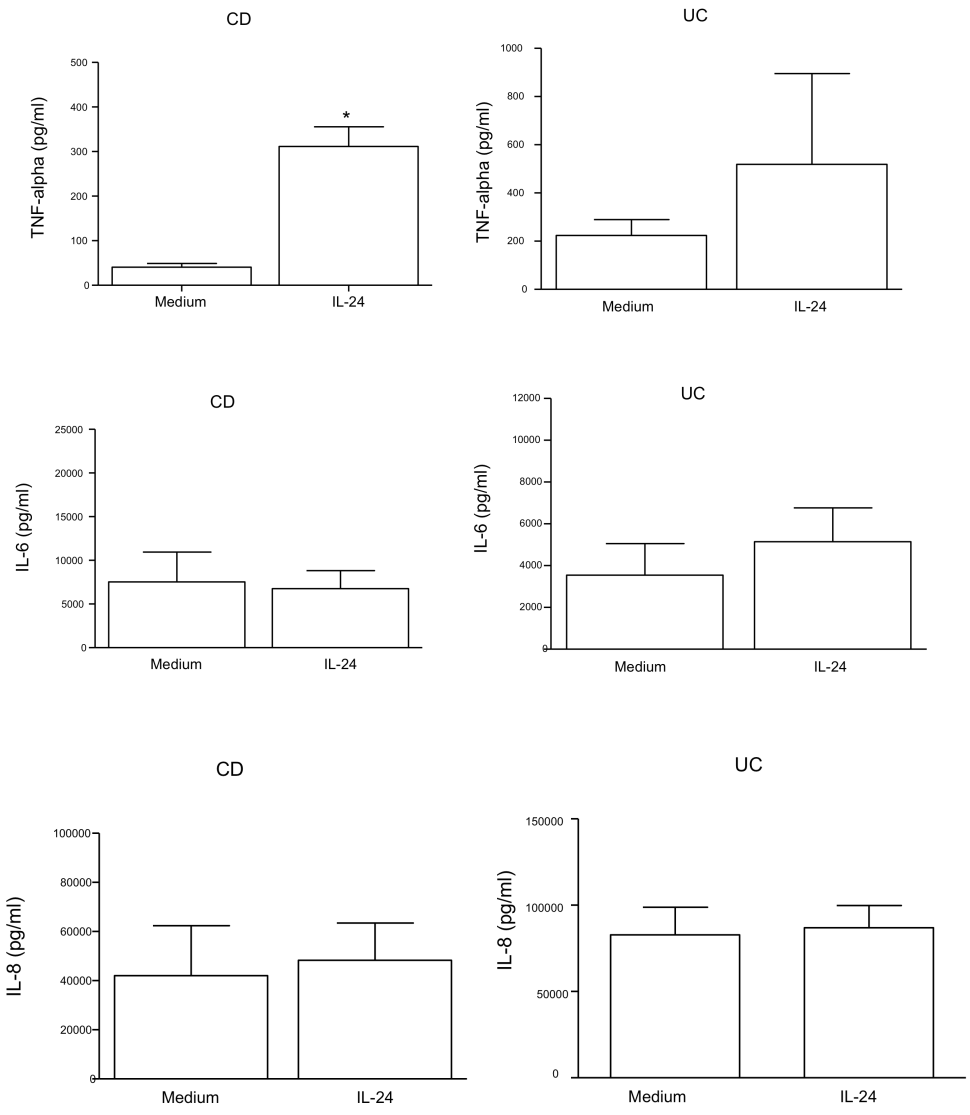


Figure 6.7: The effect of (rh)IL-24 stimulation on pro-inflammatory cytokine production

TNFα, IL-6 and IL-8 protein level was measured in IBD *ex-vivo* organ culture (CD, n= 5; UC, n= 6). Biopsies were incubated with (rh)IL-24 (100 ng/ml) for 24h in 24-well plate format and supernatant was collected for ELISA analysis. Significant increase of TNF protein level was detected in CD biopsies only. No change of IL-6, IL-8 was detected.

Chapter 7

General Discussion and Future work

In recent years, it has become clear that epigenetic regulation of gene expression is an important determinant in the development of many diseases, including inflammatory bowel disease (IBD) (Scarpa et al., 2012).

In general there is a great interest in understanding how chromatin remodelling regulates gene activation/repression. Much of the work on epigenetic regulation of immune responses comes from the work on mouse models or human cell lines.

The work presented in this thesis aimed to investigate the relationship between chromatin modification (histone methylation) and the repression of inflammatory genes in human intestinal macrophages. It was of particular interest to understand, if the anergic state of macrophages in normal gut is associated with repressive marks. Also, if in IBD there are any differences in epigenetic modifications between resident and infiltrating macrophages. Finally, if by blocking histone methylation it is possible to prevent/reduce TNFα production by macrophages from IBD mucosa.

Although, the intestine represents the largest reservoir of macrophages in the body, these cells are only a small component of mononuclear cells in normal and inflamed gut and no previous studies have looked at their epigenetic modifications.

Hence, it became apparent that the availability of sufficient cells for ChIP assay analysis might be a limiting factor for this study. Therefore the first part of the project involved optimising the method for the isolation of intestinal macrophages to obtain sufficient numbers of cells for functional analysis.

The gold standard macrophage marker, CD68 has been used to identify macrophages in various tissue types including human intestine (Smythies et al., 2005). However, as discussed in Chapter 3, due to its intracellular localisation, it was impossible to use CD68 for macrophage isolation. Therefore there was a need to identify other marker(s), which would allow recovery of sufficient number of cells.

By performing flow cytometry analysis it was determined that about 15% of $CD45^+$ LPMCs were $CD33^+$ and that this level remained constant across control subjects and patients with IBD. CD33 has been widely used as a marker for macrophages and is largely co-expressed with CD68 (Rogler et al., 1998; Kamada et al., 2008).

In this work it was confirmed that the majority of $CD33^+$ cells isolated from normal and inflamed mucosa co-expressed CD68.

The co-expression of CD33 with another macrophage marker, CD14 was also analysed. In uninflamed gut the majority of $CD33^+$ LPMCs did not express CD14. A small subset of $CD33^+$ cells however co-expressed $CD14^+$ (~ 2%) in healthy gut and this population increased significantly in IBD mucosa. Additional FACS analysis revealed that two subsets of intestinal macrophages, namely $CD33^+CD14^-$ and $CD33^+CD14^+$ cells also co-expressed CD68. It is highly likely that the $CD33^+CD14^+$ cells in healthy gut are recently recruited from blood, although there is the possibility that they represent a stable resident population. It is also likely that the increase in $CD14^+$ cells seen in IBD mucosa is due to recruitment from blood.

As CD33 is also expressed by other cells of myeloid lineage such monocytes, DCs and activated T cells, the use of CD33 as a marker may not yield a pure population of macrophages. However in the accompanying thesis of Francesca Ammoscato it was shown that $CD33^+$ cells were $CD3^-$ and $CD64^-$. By MACS sorting it was possible to isolate about 2-4 million $CD33^+$ cells for subsequent functional analysis using ChIP. Additional optimisation of ChIP allowed a reduction in the number of cells used per immunoprecipitation from $1x10^6$ to $0.25x10^6$.

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To define the relationship between chromatin modification (histone methylation) and the repression of inflammatory genes in intestinal macrophages, the *TNFA* gene was selected.

Different repressive and permissive histone modifications were investigated. Silencing marks H3K27me3, H3K9me3 and H3K9me1, as well as activating marks H3K4me3, H3K4me1 and also tH3 and RNAPII were selected and analysed by ChIP assay. The first major observation was that the silencing marks H3K9me3 and H3K27me3 were abundant at the *TNFA* TSS in macrophages isolated from normal gut. This agrees well with previous studies that normal intestinal macrophages do not produce pro-inflammatory cytokines (Smythies et al., 2005). However, macrophages isolated from mucosa of IBD patients showed decreased levels of H3K27me3, H3K9me3, with H3K27me3 having the greatest significant reduction. Interestingly, there was no difference in RNAPII enrichment at the *TNFA* TSS between normal and IBD intestinal macrophages. However, additional work would be needed to investigate, if the same applies for RNAPII phosphorylated forms, as due to cells limitation RNAPIIpS5 and RNAPIIpS2 occupancy was not studied in this project.

The enrichment of silencing marks at the *TNF* TSS seen in resident macrophages isolated from normal colon could be driven by environmental factors within the gut. The main source of gut macrophages is peripheral blood monocytes, which are attracted to lamina propria during inflammation (Bain et al., 2014). The homeostatic environment of lamina propria drives the enrichment of silencing marks to the *TNF* TSS along the process of cell differentation. Results obtained in this study suggest that peripheral blood monocytes from controls and IBD patients displayed similar levels of H3K27me3 and H3K9me3 marks. However, only macrophages in the normal gut eventually acquired both H3K27me3 and H3K9me3 at the *TNFA* TSS.

Interestingly, no change in H3K4me3 was shown between blood monocytes and intestinal macrophages. It is possible that during the process of cell differentiation the bivalent state of the *TNFA* promoter is being established through interplay between H3K27me3 and H3K4me3 in healthy gut macrophages and this is disturbed in IBD environment. Of note, the change in H3K4me1 enrichment was observed between blood monocytes and intestinal macrophages isolated from UC mucosa.

Nevertheless, the results presented in this thesis represent the preliminary outcome of how potentially chromatin state can regulate the gene expression and the establishment of endotoxin tolerance in human intestinal macrophages. The future work should be carried out to further investigate the epigenetic regulation in these cells. The more comprehensive data generated on donor-matched monocytes and intestinal macrophages would validate these preliminary results.

The phenomenon of endotoxin tolerance has been extensively studied using various *in vitro* models (Foster et al., 2007; De Santa et al., 2009; Chen and Ivashkiv, 2010). The state of tolerance is associated with hyporesponsivness upon repeated or prolonged exposure to LPS and chromatin remodelling plays an important part in this phenomenon (El Gazzar et al., 2007). Using the model of LPS-induced endotoxin tolerance in human primary macrophages (GM-CSF) I showed that LPS stimulation of naïve macrophages results in enrichment of activating mark H3K4me3 and that this does not happen in LPS-tolerant cells. The LPS activation of naïve macrophages also triggered the recruitment of RNAPII to the *TNFA* TSS and decreased silencing mark H3K27me3. However activation of tolerant macrophages with LPS did not influence the recruitment of RNAPII. Moreover, an enrichment of RNAPII remained constant and comparable to the level of LPS stimulated naïve cells. Interestingly, it was possible to detect even higher enrichment of H3K27me3 in tolerant cells

stimulated with LPS, suggesting that indeed H3K27me3 may contribute to endotoxin tolerance. However, these results are very preliminary and more experiments are needed to validate this outcome.

The removal of methyl group from H3K27 is driven by the H3K27me3 demethylase JmjD3 (De Santa et al., 2007; 2009). Analysis of JmjD3 occupancy at the *TNFA* TSS generated from blood monocytes and intestinal macrophages from control and IBD samples showed unexpectedly the increased abundance of JmjD3 in IBD blood monocytes and UC intestinal macrophages. Unfortunately due to material limitation it was impossible to investigate JMJD3 binding in CD macrophages. Although, these data are preliminary, it may be possible that increased JmjD3 binding associates with proinflammatory profile of cells. This was additionally supported by detection of elevated transcripts for *Jmjd3* in CD macrophages.

In summary, results obtained here suggest that chromatin architecture in normal intestinal macrophages is such that active *TNFA* transcription cannot be initiated, possibly this is due to a high occupancy of silencing marks, especially H3K27me3. Additionally, under homeostatic conditions, the *TNFA* gene is set at a bivalent state, since concurrent high binding of H3K27me3 and H3K4me3 at the gene transcription start site was observed and that this process is disturbed in IBD.

Due to tissue limitation and consequently lack of sufficient number of isolated macrophages, it was impossible to expend this study to the $CD33^{+}CD14^{-}$ and $CD33^{+}CD14^{+}$ subsets of intestinal macrophages. Analysis of these cells would provide more in depth understanding of how the process of macrophage differentiation occurs in homeostatic and inflammatory milieu in terms of chromatin remodelling.

The methylation of H3K27me3 is thought to be a key epigenetic regulator of cell homeostasis and development (Margueron et al., 2011). Also JMJD3 has been shown to function in regulation of LPS-inducible gene expression (Satoh et al., 2010). Since, the removal of the silencing mark H3K27me3 was associated with increased *TNFA* transcripts it was interesting to determine, if targeting the enzyme responsible for elimination of this silencing mark could restore the silent state of the *TNFA*.

The efficacy of JMJD3 inhibitor (GSK-J4) was first tested using GM-CSF differentiated human primary macrophages. Administration of GSK-J4 resulted in decreased expression of the *TNFA* transcripts after LPS activation and this was associated with preserved H3K27me3 binding to the *TNFA* TSS upon LPS stimulation. These findings were in agreement with previously published data by Kruidenier and colleagues (2012) where GSK-J4 was also tested in human primary macrophages. Interestingly, results presented in this study additionally suggest that GSK-J4 may exert its effect on H3K4me3 but not on H3K4me1.

Subsequent analysis of GSK-J4 on intestinal macrophages isolated from mucosa of Crohn's patients, showed no effect on JMJD3 inhibition. It is difficult to speculate on possible reasons why the inhibitor did not exert its effect, despite positive results on GM-CSF macrophages. Since the GSK-J4 activity depends on macrophage esterase action, it might be that the concentration of the enzyme was not sufficient to convert the GSK-J4 pro-drug form into an active form.

Figure 7.1 represents a proposed model of chromatin remodelling at the *TNFA* promoter in human intestinal macrophages.

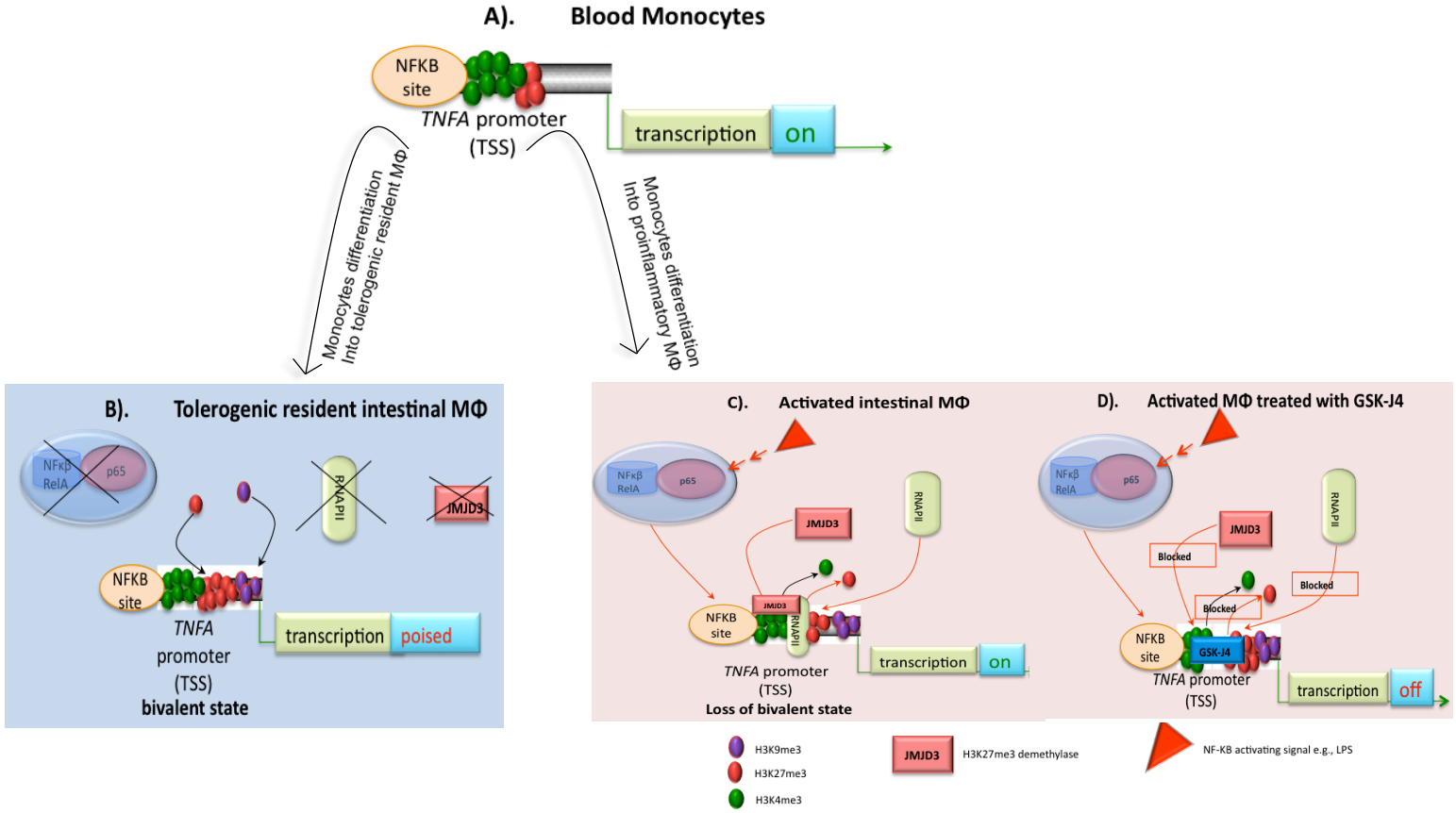


Figure 7.1: Proposed model of how epigenetic changes may occur during the process of differentation from blood monocytes into intestinal macrophages under (B) homeostatic conditions and in (C) inflamed intestine. Under homeostatic conditional, along the process of differentation into resident MΦ, blood monocytes acquire the repressive histone marks (H3K27me3 and H3K9me3). Together with already prebound H3K4me3 (activating mark), H3K27me3 sets the *TNFA* promoter at a bivalent, poised state. However, in IBD the process is disturbed. In inflamed intestine, MΦ fail to recruit H3K27me3 and H3K9me3. **(D).** Based on data collected using GM-CSF derived human macrophages, it was speculated that GSK-J4 (JMJD3 inhibitor) prevents the loss of H3K27me3 and reduces the *TNF* transcription

To investigate novel aspects of IBD pathogenesis, gene microarray analysis was performed to determine changes in gene expression in intestinal macrophages isolated from mucosa of Crohn's and ulcerative colitis patients compared to cells isolated from normal gut. To provide more comprehensive data on the aspect of endotoxin tolerance, gene expression profile of human intestinal macrophages was also analysed with reference to LPS-tolerised human primary macrophages.

The majority of assessed genes were up regulated in IBD samples. The gene expression profile was also classified accordingly to gene function or based on different family group. A large number of upregulated genes was associated with proinflammatory responses and have been shown to function in cell proliferation and differentation pathways, such as *Il1β*, *Il1F5*, *TNFSF8*, *TNFA*, *LTA*, *GDF5*, *Il8*, *Il13*, *Il15, INHBA* and *Il24*.

Some genes showed opposing expression pattern between ulcerative colitis and Crohn's disease, such as *GDF5*, *Il1F5*, *BMP5*, *Il1F7* and *Il15*, respectively.

The gene array analysis identified *IL24* as being particularly increased. IL-24 protein level was also increased in both inflamed and uninflamed CD biopsies and also in inflamed UC biopsies. Additionally, it was also shown that IL-24 stimulation increases TNF-α production in Crohn's disease *ex-vivo* organ culture.

The role of IL-24 in driving immune responses has been reported in number of studies (Poindexter et al., 2005; Andoh et al., 2009; Sahoo et al., 2011) and its expression has been identified in LPS-stimulated monocytes and macrophages or Th2-polarised T cells (Wolk et al., 2002; Poindexter et al., 2005; Kunz et al., 2006).

The function of IL-24 is still poorly understood, but IL-24 can function as an intracellular cell death-inducing factor, or as a classical cytokine through cell surface receptor (Andoh et al., 2013).

Thus preliminary analysis of gene expression pattern presented in this thesis identified IL-24 as a new proinflammatory cytokine potentially involved in IBD. Further investigation of IL-24 could be a very exiting project for the future.

In summary, epigenetics has emerged as a promising area of research, especially in reference to understanding a very complex and multifactorial diseases like IBD. Much of work on epigenetics and its involvement in regulating immune responses comes from the work on mouse models or human cell lines. Although providing valuable information, these studies not always can be extrapolated into human disease setting. Therefore, the main focus of this project was to investigate the epigenetic regulation of pro-inflammatory immune responses in human intestinal macrophages. The results presented here, although preliminary, provide a valuable insight into how the anergic phenotype is being established in human intestinal macropahges. These results could form a platform for any future study in this area of research.

References

Aamann L, Vestergaard EM, Grønbæk H. Trefoil factors in inflammatory bowel disease. World J Gastroenterol 2014; 20:3223-30.

Abraham C, Cho J. Interleukin-23/Th17 pathways and inflammatory bowel disease. Inflamm Bowel Dis 2009; 15:1090-100.

Abraham C, Cho JH. Functional consequences of NOD2 (CARD15) mutations. Inflamm Bowel Dis 2006; 12:641-50.

Abraham C, Cho JH. Inflammatory bowel disease. N Engl J Med 2009; 361:2066-78.

Abreu MT. Toll-like receptor signalling in the intestinal epithelium: how bacterial recognition shapes intestinal function. Nat Rev Immunol 2010; 10:131-44.

Adachi S, Yoshida H, Honda K, Maki K, Saijo K, Ikuta K, et al. Essential role of IL-7 receptor alpha in the formation of Peyer's patch anlage. Int Immunol 1998; 10:1-6.

Adams AT, Kennedy NA, Hansen R, Ventham NT, O'Leary KR, Drummond HE, et al. Two-stage genome-wide methylation profiling in childhood-onset Crohn's Disease implicates epigenetic alterations at the VMP1/MIR21 and HLA loci. Inflamm Bowel Dis 2014; 20:1784-93.

Agger K, Cloos PA, Christensen J, Pasini D, Rose S, Rappsilber J, et al. UTX and JMJD3 are histone H3K27 demethylases involved in HOX gene regulation and development. Nature 2007; 449:731-4.

Agger K, Cloos PA, Rudkjaer L, Williams K, Andersen G, Christensen J, et al. The H3K27me3 demethylase JMJD3 contributes to the activation of the INK4A-ARF locus in response to oncogene- and stress-induced senescence. Genes Dev 2009; 23:1171-6.

Ahmad T, Armuzzi A, Neville M, Bunce M, Ling KL, Welsh KI, et al. The contribution of human leucocyte antigen complex genes to disease phenotype in ulcerative colitis. Tissue Antigens 2003; 62:527-35.

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

Allis CD, Berger SL, Cote J, Dent S, Jenuwien T, Kouzarides T, et al. New nomenclature for chromatin-modifying enzymes. Cell 2007; 131:633-6.

Ancuta P, Rao R, Moses A, Mehle A, Shaw SK, Luscinskas FW, et al. Fractalkine preferentially mediates arrest and migration of CD16+ monocytes. J Exp Med 2003; 197:1701-7.

Anderson CA, Massey DC, Barrett JC, Prescott NJ, Tremelling M, Fisher SA, et al. Investigation of Crohn's disease risk loci in ulcerative colitis further defines their molecular relationship. Gastroenterology 2009; 136:523-9.e3.

Andersson RE, Olaison G, Tysk C, Ekbom A. Appendectomy and protection against ulcerative colitis. N Engl J Med 2001; 344:808-14.

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

Andrian E, Mostefaoui Y, Rouabhia M, Grenier D. Regulation of matrix metalloproteinases and tissue inhibitors of matrix metalloproteinases by Porphyromonas gingivalis in an engineered human oral mucosa model. J Cell Physiol 2007; 211:56-62.

Annacker O, Coombes JL, Malmstrom V, Uhlig HH, Bourne T, Johansson-Lindbom B, et al. Essential role for CD103 in the T cell-mediated regulation of experimental colitis. J Exp Med 2005; 202:1051-61.

Ansel KM, Ngo VN, Hyman PL, Luther SA, Förster R, Sedgwick JD, et al. A chemokine-driven positive feedback loop organizes lymphoid follicles. Nature 2000; 406:309-14.

Artis D, Spits H. The biology of innate lymphoid cells. Nature 2015; 517:293-301.

Asseman C, Mauze S, Leach MW, Coffman RL, Powrie F. An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation. J Exp Med 1999; 190:995-1004.

Atarashi K, Tanoue T, Shima T, Imaoka A, Kuwahara T, Momose Y, et al. Induction of colonic regulatory T cells by indigenous Clostridium species. Science 2011; 331:337-41.

Atreya R, Mudter J, Finotto S, Müllberg J, Jostock T, Wirtz S, et al. Blockade of interleukin 6 trans signaling suppresses T-cell resistance against apoptosis in chronic intestinal inflammation: evidence in crohn disease and experimental colitis in vivo. Nat Med 2000; 6:583-8.

Auffray C, Fogg D, Garfa M, Elain G, Join-Lambert O, Kayal S, et al. Monitoring of blood vessels and tissues by a population of monocytes with patrolling behavior. Science 2007; 317:666-70.

Auffray C, Sieweke MH, Geissmann F. Blood monocytes: development, heterogeneity, and relationship with dendritic cells. Annu Rev Immunol 2009; 27:669-92.

Bäckdahl L, Bushell A, Beck S. Inflammatory signalling as mediator of epigenetic modulation in tissue-specific chronic inflammation. Int J Biochem Cell Biol 2009; 41:176-84.

Bain CC, Bravo-Blas A, Scott CL, Gomez Perdiguero E, Geissmann F, Henri S, et al. Constant replenishment from circulating monocytes maintains the macrophage pool in the intestine of adult mice. Nat Immunol 2014; 15:929-37.

Bain CC, Mowat AM. Intestinal macrophages - specialised adaptation to a unique environment. Eur J Immunol 2011; 41:2494-8.

Bain CC, Scott CL, Uronen-Hansson H, Gudjonsson S, Jansson O, Grip O, et al. Resident and pro-inflammatory macrophages in the colon represent alternative context-dependent fates of the same Ly6Chi monocyte precursors. Mucosal Immunol 2013; 6:498-510.

Bannister AJ, Schneider R, Kouzarides T. Histone methylation: dynamic or static? Cell 2002; 109:801-6.

Baptista AP, Olivier BJ, Goverse G, Greuter M, Knippenberg M, Kusser K, et al. Colonic patch and colonic SILT development are independent and differentially regulated events. Mucosal Immunol 2013; 6:511-21.

Barker BR, Parvani JG, Meyer D, Hey AS, Skak K, Letvin NL. IL-21 induces apoptosis of antigen-specific CD8+ T lymphocytes. J Immunol 2007; 179:3596-603.

Barker N, Bartfeld S, Clevers H. Tissue-Resident Stem Cell Populations of Rapidly of Self-Renewing Organs. Cell Stem Cell 2010; 7:656-70.

Barrett JC, Hansoul S, Nicolae DL, Cho JH, Duerr RH, Rioux JD, et al. Genome-wide association defines more than 30 distinct susceptibility loci for Crohn's disease. Nat Genet 2008; 40:955-62.

Battle MA, Konopka G, Parviz F, Gaggl AL, Yang C, Sladek FM, et al. Hepatocyte nuclear factor 4alpha orchestrates expression of cell adhesion proteins during the epithelial transformation of the developing liver. Proc Natl Acad Sci U S A 2006; 103:8419-24.

Batts LE, Polk DB, Dubois RN, Kulessa H. Bmp signaling is required for intestinal growth and morphogenesis. Dev Dyn 2006; 235:1563-70.

Baumgart DC, Sandborn WJ. Crohn's disease. Lancet 2012; 380:1590-605.

Becker C, Wirtz S, Blessing M, Pirhonen J, Strand D, Bechthold O, et al. Constitutive p40 promoter activation and IL-23 production in the terminal ileum mediated by dendritic cells. J Clin Invest 2003; 112:693-706.
Berg EL, McEvoy LM, Berlin C, Bargatze RF, Butcher EC. L-selectin-mediated lymphocyte rolling on MAdCAM-1. Nature 1993; 366:695-8.

Bergqvist P, Gärdby E, Stensson A, Bemark M, Lycke NY. Gut IgA class switch recombination in the absence of CD40 does not occur in the lamina propria and is independent of germinal centers. J Immunol 2006; 177:7772-83.

Berlin C, Berg EL, Briskin MJ, Andrew DP, Kilshaw PJ, Holzmann B, et al. Alpha 4 beta 7 integrin mediates lymphocyte binding to the mucosal vascular addressin MAdCAM-1. Cell 1993; 74:185-95.

Bernink JH, Peters CP, Munneke M, te Velde AA, Meijer SL, Weijer K, et al. Human type 1 innate lymphoid cells accumulate in inflamed mucosal tissues. Nat Immunol 2013; 14:221-9.

Bernstein BE, Mikkelsen TS, Xie X, Kamal M, Huebert DJ, Cuff J, et al. A bivalent chromatin structure marks key developmental genes in embryonic stem cells. Cell 2006; 125:315-26.

Bettelli E, Oukka M, Kuchroo VK. T(H)-17 cells in the circle of immunity and autoimmunity. Nat Immunol 2007; 8:345-50.

Bevins CL, Salzman NH. Paneth cells, antimicrobial peptides and maintenance of intestinal homeostasis. Nat Rev Microbiol 2011; 9:356-68.

Bhavsar P, Ahmad T, Adcock IM. The role of histone deacetylases in asthma and allergic diseases. J Allergy Clin Immunol 2008; 121:580-4.

Biancheri P, Brezski RJ, Di Sabatino A, Greenplate AR, Soring KL, Corazza GR, et al. Proteolytic Cleavage and Loss of Function of Biologic Agents that Neutralize Tumor Necrosis Factor in the Mucosa of Patients with Inflammatory Bowel Disease. Gastroenterology 2015.

Biancheri P, Di Sabatino A, Ammoscato F, Facciotti F, Caprioli F, Curciarello R, et al. Absence of a role for interleukin-13 in inflammatory bowel disease. Eur J Immunol 2014; 44:370-85.

Biglione S, Tsytsykova AV, Goldfeld AE. Monocyte-specific accessibility of a matrix attachment region in the tumor necrosis factor locus. J Biol Chem 2011; 286:44126-33.

Biswas SK, Lopez-Collazo E. Endotoxin tolerance: new mechanisms, molecules and clinical significance. Trends Immunol 2009; 30:475-87.

Björkbacka H, Fitzgerald KA, Huet F, Li X, Gregory JA, Lee MA, et al. The induction of macrophage gene expression by LPS predominantly utilizes Myd88-independent signaling cascades. Physiol Genomics 2004; 19:319-30.

Blanchard F, Chipoy C. Histone deacetylase inhibitors: new drugs for the treatment of inflammatory diseases? Drug Discov Today 2005; 10:197-204.

Bogunovic M, Davé SH, Tilstra JS, Chang DT, Harpaz N, Xiong H, et al. Enteroendocrine cells express functional Toll-like receptors. Am J Physiol Gastrointest Liver Physiol 2007; 292:G1770-83.

Bogunovic M, Ginhoux F, Helft J, Shang L, Hashimoto D, Greter M, et al. Origin of the lamina propria dendritic cell network. Immunity 2009; 31:513-25.

Bouma G, Xia B, Crusius JB, Bioque G, Koutroubakis I, Von Blomberg BM, et al. Distribution of four polymorphisms in the tumour necrosis factor (TNF) genes in patients with inflammatory bowel disease (IBD). Clin Exp Immunol 1996; 103:391-6.

Boursier L, Gordon JN, Thiagamoorthy S, Edgeworth JD, Spencer J. Human intestinal IgA response is generated in the organized gut-associated lymphoid tissue but not in the lamina propria.

Gastroenterology 2005; 128:1879-89.

Bouskra D, Brézillon C, Bérard M, Werts C, Varona R, Boneca IG, et al. Lymphoid tissue genesis induced by commensals through NOD1 regulates intestinal homeostasis. Nature 2008; 456:507-10.

Boyer LA, Plath K, Zeitlinger J, Brambrink T, Medeiros LA, Lee TI, et al. Polycomb complexes repress developmental regulators in murine embryonic stem cells. Nature 2006; 441:349-53.

Brandt K, Singh PB, Bulfone-Paus S, Rückert R. Interleukin-21: a new modulator of immunity, infection, and cancer. Cytokine Growth Factor Rev 2007; 18:223-32.

Brandtzaeg P, Bosnes V, Halstensen TS, Scott H, Sollid LM, Valnes KN. T lymphocytes in human gut epithelium preferentially express the alpha/beta antigen receptor and are often CD45/UCHL1-positive. Scand J Immunol 1989; 30:123-8.

Brandtzaeg P, Farstad IN, Haraldsen G. Regional specialization in the mucosal immune system: primed cells do not always home along the same track. Immunol Today 1999; 20:267-77.

Brandtzaeg P, Farstad IN, Johansen FE, Morton HC, Norderhaug IN, Yamanaka T. The B-cell system of human mucosae and exocrine glands. Immunol Rev 1999; 171:45-87.

Brandtzaeg P, Kiyono H, Pabst R, Russell MW. Terminology: nomenclature of mucosa-associated lymphoid tissue. Mucosal Immunol 2008; 1:31-7.

Brandtzaeg P, Pabst R. Let's go mucosal: communication on slippery ground. Trends Immunol 2004; 25:570-7.

Breese EJ, Michie CA, Nicholls SW, Murch SH, Williams CB, Domizio P, et al. Tumor necrosis factor alpha-producing cells in the intestinal mucosa of children with inflammatory bowel disease. Gastroenterology 1994; 106:1455-66.

Brint EK, Xu D, Liu H, Dunne A, McKenzie AN, O'Neill LA, et al. ST2 is an inhibitor of interleukin 1 receptor and Toll-like receptor 4 signaling and maintains endotoxin tolerance. Nat Immunol 2004; 5:373-9.

Briskin M, Winsor-Hines D, Shyjan A, Cochran N, Bloom S, Wilson J, et al. Human mucosal addressin cell adhesion molecule-1 is preferentially expressed in intestinal tract and associated lymphoid tissue. Am J Pathol 1997; 151:97-110.

Brown EM, Sadarangani M, Finlay BB. The role of the immune system in governing host-microbe interactions in the intestine. Nat Immunol 2013; 14:660-7.

Budarf ML, Labbé C, David G, Rioux JD. GWA studies: rewriting the story of IBD. Trends Genet 2009; 25:137-46.

Bulger M, Groudine M. Enhancers: the abundance and function of regulatory sequences beyond promoters. Dev Biol 2010; 339:250-7.

Bundschuh DS, Barsig J, Hartung T, Randow F, Döcke WD, Volk HD, et al. Granulocyte-macrophage colony-stimulating factor and IFN-gamma restore the systemic TNF-alpha response to endotoxin in lipopolysaccharide-desensitized mice. J Immunol 1997; 158:2862-71.

Buonocore S, Ahern PP, Uhlig HH, Ivanov II, Littman DR, Maloy KJ, et al. Innate lymphoid cells drive interleukin-23-dependent innate intestinal pathology. Nature 2010; 464:1371-5.

Burns K, Janssens S, Brissoni B, Olivos N, Beyaert R, Tschopp J. 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 2003; 197:263-8.

Butcher EC, Picker LJ. Lymphocyte homing and homeostasis. Science 1996; 272:60-6.

Cabal-Hierro L, Lazo PS. Signal transduction by tumor necrosis factor receptors. Cell Signal 2012; 24:1297-305.
Cadwell K, Liu JY, Brown SL, Miyoshi H, Loh J, Lennerz JK, et al. A key role for autophagy and the autophagy gene Atg16l1 in mouse and human intestinal Paneth cells. Nature 2008; 456:259-63.

Calkins BM. A meta-analysis of the role of smoking in inflammatory bowel disease. Dig Dis Sci 1989; 34:1841-54.

Camoglio L, Te Velde AA, Tigges AJ, Das PK, Van Deventer SJ. Altered expression of interferon-gamma and interleukin-4 in inflammatory bowel disease. Inflamm Bowel Dis 1998; 4:285-90.

Cao H, Crocker PR. Evolution of CD33-related siglecs: regulating host immune functions and escaping pathogen exploitation? Immunology 2011; 132:18-26.

Cao Q, Zhu Q, Wu ML, Hu WL, Gao M, Si JM. Genetic susceptibility to ulcerative colitis in the Chinese Han ethnic population: association with TNF polymorphisms. Chin Med J (Engl) 2006; 119:1198-203.

Cao X, Shores EW, Hu-Li J, Anver MR, Kelsall BL, Russell SM, et al. Defective lymphoid development in mice lacking expression of the common cytokine receptor gamma chain. Immunity 1995; 2:223-38.

Capaldo CT, Nusrat A. Cytokine regulation of tight junctions. Biochim Biophys Acta 2009; 1788:864-71.

Cario E, Podolsky DK. Differential alteration in intestinal epithelial cell expression of toll-like receptor 3 (TLR3) and TLR4 in inflammatory bowel disease. Infect Immun 2000; 68:7010-7.

Carlsen HS, Yamanaka T, Scott H, Rugtveit J, Brandtzaeg P. The proportion of CD40+ mucosal macrophages is increased in inflammatory bowel disease whereas CD40 ligand (CD154)+ T cells are relatively decreased, suggesting differential modulation of these costimulatory molecules in human gut lamina propria. Inflamm Bowel Dis 2006; 12:1013-24.

Casini-Raggi V, Kam L, Chong YJ, Fiocchi C, Pizarro TT, Cominelli F. Mucosal imbalance of IL-1 and IL-1 receptor antagonist in inflammatory bowel disease. A novel mechanism of chronic intestinal inflammation. J Immunol 1995; 154:2434-40.

Caudell EG, Mumm JB, Poindexter N, Ekmekcioglu S, Mhashilkar AM, Yang XH, et al. The protein product of the tumor suppressor gene, melanoma differentiation-associated gene 7, exhibits immunostimulatory activity and is designated IL-24. J Immunol 2002; 168:6041-6.

Cella M, Fuchs A, Vermi W, Facchetti F, Otero K, Lennerz JK, et al. A human natural killer cell subset provides an innate source of IL-22 for mucosal immunity. Nature 2009; 457:722-5.

Cerovic V, Bain CC, Mowat AM, Milling SW. Intestinal macrophages and dendritic cells: what's the difference? Trends Immunol 2014; 35:270-7.

Cerovic V, Houston SA, Scott CL, Aumeunier A, Yrlid U, Mowat AM, et al. Intestinal CD103(-) dendritic cells migrate in lymph and prime effector T cells. Mucosal Immunol 2013; 6:104-13.

Cerutti A. The regulation of IgA class switching. Nat Rev Immunol 2008; 8:421-34.

Chabot S, Wagner JS, Farrant S, Neutra MR. TLRs regulate the gatekeeping functions of the intestinal follicle-associated epithelium. J Immunol 2006; 176:4275-83.

Chan I, Liu L, Hamada T, Sethuraman G, McGrath JA. The molecular basis of lipoid proteinosis: mutations in extracellular matrix protein 1. Exp Dermatol 2007; 16:881-90.

Changelian PS, Moshinsky D, Kuhn CF, Flanagan ME, Munchhof MJ, Harris TM, et al. The

specificity of JAK3 kinase inhibitors. Blood 2008; 111:2155-7.

Chaudhry A, Rudra D, Treuting P, Samstein RM, Liang Y, Kas A, et al. CD4+ regulatory T cells control TH17 responses in a Stat3-dependent manner. Science 2009; 326:986-91.

Chen CJ, Kono H, Golenbock D, Reed G, Akira S, Rock KL. Identification of a key pathway required for the sterile inflammatory response triggered by dying cells. Nat Med 2007; 13:851-6.

Chen G, Shaw MH, Kim YG, Nuñez G. NOD-like receptors: role in innate immunity and inflammatory disease. Annu Rev Pathol 2009; 4:365-98.

Chen J, Ivashkiv LB. IFN-γ abrogates endotoxin tolerance by facilitating Toll-like receptor-induced chromatin remodeling. Proc Natl Acad Sci U S A 2010; 107:19438-43.

Chen S, Ma J, Wu F, Xiong LJ, Ma H, Xu W, et al. The histone H3 Lys 27 demethylase JMJD3 regulates gene expression by impacting transcriptional elongation. Genes Dev 2012; 26:1364-75.

Chen X, El Gazzar M, Yoza BK, McCall CE. The NF-kappaB factor RelB and histone H3 lysine methyltransferase G9a directly interact to generate epigenetic silencing in endotoxin tolerance. J Biol Chem 2009; 284:27857-65.

Cheng X, Blumenthal RM. Coordinated chromatin control: structural and functional linkage of DNA and histone methylation. Biochemistry 2010; 49:2999-3008.

Cho JH. The genetics and immunopathogenesis of inflammatory bowel disease. Nat Rev Immunol 2008; 8:458-66.

Cho JH, Weaver CT. The genetics of inflammatory bowel disease. Gastroenterology 2007; 133:1327-39.

Choi SW, Friso S. Epigenetics: A New Bridge between Nutrition and Health. Adv Nutr 2010; 1:8-16.

Chorro L, Sarde A, Li M, Woollard KJ, Chambon P, Malissen B, et al. Langerhans cell (LC) proliferation mediates neonatal development, homeostasis, and inflammation-associated expansion of the epidermal LC network. J Exp Med 2009; 206:3089-100.

Clayburgh DR, Shen L, Turner JR. A porous defense: the leaky epithelial barrier in intestinal disease. Lab Invest 2004; 84:282-91.

Clemente JC, Ursell LK, Parfrey LW, Knight R. The impact of the gut microbiota on human health: an integrative view. Cell 2012; 148:1258-70.

Cloos PA, Christensen J, Agger K, Helin K. Erasing the methyl mark: histone demethylases at the center of cellular differentiation and disease. Genes Dev 2008; 22:1115-40.

Coccia M, Harrison OJ, Schiering C, Asquith MJ, Becher B, Powrie F, et al. IL-1β mediates chronic intestinal inflammation by promoting the accumulation of IL-17A secreting innate lymphoid cells and CD4(+) Th17 cells. J Exp Med 2012; 209:1595-609.

Cohavy O, Zhou J, Granger SW, Ware CF, Targan SR. LIGHT expression by mucosal T cells may regulate IFN-gamma expression in the intestine. J Immunol 2004; 173:251-8.

Colombel JF, Sandborn WJ, Reinisch W, Mantzaris GJ, Kornbluth A, Rachmilewitz D, et al. Infliximab, azathioprine, or combination therapy for Crohn's disease. N Engl J Med 2010; 362:1383-95.

Commins S, Steinke JW, Borish L. The extended IL-10 superfamily: IL-10, IL-19, IL-20, IL-22, IL-24, IL-26, IL-28, and IL-29. J Allergy Clin Immunol 2008; 121:1108-11.

Coombes JL, Powrie F. Dendritic cells in intestinal immune regulation. Nat Rev Immunol 2008;

8:435-46.

Coombes JL, Siddiqui KR, Arancibia-Cárcamo CV, Hall J, Sun CM, Belkaid Y, et al. A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. J Exp Med 2007; 204:1757-64.

Cooney R, Baker J, Brain O, Danis B, Pichulik T, Allan P, et al. NOD2 stimulation induces autophagy in dendritic cells influencing bacterial handling and antigen presentation. Nat Med 2010; 16:90-7.

Cornes JS. Peyer's patches in the human gut. Proc R Soc Med 1965; 58:716.

Corredor J, Yan F, Shen CC, Tong W, John SK, Wilson G, et al. Tumor necrosis factor regulates intestinal epithelial cell migration by receptor-dependent mechanisms. Am J Physiol Cell Physiol 2003; 284:C953-61.

Cosnes J, Cattan S, Blain A, Beaugerie L, Carbonnel F, Parc R, et al. Long-term evolution of disease behavior of Crohn's disease. Inflamm Bowel Dis 2002; 8:244-50.

Costello EK, Stagaman K, Dethlefsen L, Bohannan BJ, Relman DA. The application of ecological theory toward an understanding of the human microbiome. Science 2012; 336:1255-62.

Cox JH, Kljavin NM, Ramamoorthi N, Diehl L, Batten M, Ghilardi N. IL-27 promotes T cell-dependent colitis through multiple mechanisms. J Exp Med 2011; 208:115-23.

Crocker PR, Varki A. Siglecs, sialic acids and innate immunity. Trends Immunol 2001; 22:337-42.

Cross JG, Harrison GA, Coggill P, Sims S, Beck S, Deakin JE, et al. Analysis of the genomic region containing the tammar wallaby (Macropus eugenii) orthologues of MHC class III genes. Cytogenet Genome Res 2005; 111:110-7.

Cua DJ, Tato CM. Innate IL-17-producing cells: the sentinels of the immune system. Nat Rev Immunol 2010; 10:479-89.

Cui K, Zang C, Roh TY, Schones DE, Childs RW, Peng W, et al. Chromatin signatures in multipotent human hematopoietic stem cells indicate the fate of bivalent genes during differentiation. Cell Stem Cell 2009; 4:80-93.

Cui X, Jin Y, Hofseth AB, Pena E, Habiger J, Chumanevich A, et al. Resveratrol suppresses colitis and colon cancer associated with colitis. Cancer Prev Res (Phila) 2010; 3:549-59.

Cyster JG, Ansel KM, Reif K, Ekland EH, Hyman PL, Tang HL, et al. Follicular stromal cells and lymphocyte homing to follicles. Immunol Rev 2000; 176:181-93.

D'Arcy S, Martin KW, Panchenko T, Chen X, Bergeron S, Stargell LA, et al. Chaperone Nap1 shields histone surfaces used in a nucleosome and can put H2A-H2B in an unconventional tetrameric form. Mol Cell 2013; 51:662-77.

Daans M, Lories RJ, Luyten FP. Absence of GDF5 does not interfere with LPS Toll-like receptor signaling. Clin Exp Rheumatol 2009; 27:495-8.

Däbritz J, Menheniott TR. Linking immunity, epigenetics, and cancer in inflammatory bowel disease. Inflamm Bowel Dis 2014; 20:1638-54.

Dambacher J, Beigel F, Zitzmann K, De Toni EN, Göke B, Diepolder HM, et al. The role of the novel Th17 cytokine IL-26 in intestinal inflammation. Gut 2009; 58:1207-17.

Danese S, Fiocchi C. Ulcerative colitis. N Engl J Med 2011; 365:1713-25.
Danese S, Rudziński J, Brandt W, Dupas JL, Peyrin-Biroulet L, Bouhnik Y, et al. Tralokinumab for moderate-to-severe UC: a randomised, double-blind, placebo-controlled, phase IIa study. Gut 2015; 64:243-9.

Das G, Augustine MM, Das J, Bottomly K, Ray P, Ray A. An important regulatory role for CD4+CD8 alpha alpha T cells in the intestinal epithelial layer in the prevention of inflammatory bowel disease. Proc Natl Acad Sci U S A 2003; 100:5324-9.

Das ND, Jung KH, Choi MR, Yoon HS, Kim SH, Chai YG. Gene networking and inflammatory pathway analysis in a JMJD3 knockdown human monocytic cell line. Cell Biochem Funct 2012; 30:224-32.

Davidson NJ, Leach MW, Fort MM, Thompson-Snipes L, Kühn R, Müller W, et al. T helper cell 1-type CD4+ T cells, but not B cells, mediate colitis in interleukin 10-deficient mice. J Exp Med 1996; 184:241-51.

Dayer Schneider J, Seibold I, Saxer-Sekulic N, Paredes BE, Saurer L, Mueller C. Lack of TNFR2 expression by CD4(+) T cells exacerbates experimental colitis. Eur J Immunol 2009; 39:1743-53.

De Calisto J, Villablanca EJ, Mora JR. FcγRI (CD64): an identity card for intestinal macrophages. Eur J Immunol 2012; 42:3136-40.

De Santa F, Narang V, Yap ZH, Tusi BK, Burgold T, Austenaa L, et al. Jmjd3 contributes to the control of gene expression in LPS-activated macrophages. EMBO J 2009; 28:3341-52.

De Santa F, Totaro MG, Prosperini E, Notarbartolo S, Testa G, Natoli G. The histone H3 lysine-27 demethylase Jmjd3 links inflammation to inhibition of polycomb-mediated gene silencing. Cell 2007; 130:1083-94.

De Togni P, Goellner J, Ruddle NH, Streeter PR, Andrea F, Mariathasan S, et al. Pillars article: Abnormal development of peripheral lymphoid organs in mice deficient in lymphotoxin. Science. 1994. 264: 703-707. J Immunol 2014; 192:2010-4.

Deakin JE, Papenfuss AT, Belov K, Cross JG, Coggill P, Palmer S, et al. Evolution and comparative analysis of the MHC Class III inflammatory region. BMC Genomics 2006; 7:281.

Denning TL, Wang YC, Patel SR, Williams IR, Pulendran B. Lamina propria macrophages and dendritic cells differentially induce regulatory and interleukin 17-producing T cell responses. Nat Immunol 2007; 8:1086-94.

DeNucci CC, Pagán AJ, Mitchell JS, Shimizu Y. Control of alpha4beta7 integrin expression and CD4 T cell homing by the beta1 integrin subunit. J Immunol 2010; 184:2458-67.

Dideberg V, Théâtre E, Farnir F, Vermeire S, Rutgeerts P, De Vos M, et al. The TNF/ADAM 17 system: implication of an ADAM 17 haplotype in the clinical response to infliximab in Crohn's disease. Pharmacogenet Genomics 2006; 16:727-34.

Dignass A, Lynch-Devaney K, Kindon H, Thim L, Podolsky DK. Trefoil peptides promote epithelial migration through a transforming growth factor beta-independent pathway. J Clin Invest 1994; 94:376-83.

Dobrovolskaia MA, Vogel SN. Toll receptors, CD14, and macrophage activation and deactivation by LPS. Microbes Infect 2002; 4:903-14.

Dong F, He X. Activin A: a potential therapeutic target for characterizing and stopping joint pain early in rheumatoid arthritis patients. Inflammation 2014; 37:170-6.

Duerr RH, Taylor KD, Brant SR, Rioux JD, Silverberg MS, Daly MJ, et al. A genome-wide association study identifies IL23R as an inflammatory bowel disease gene. Science 2006; 314:1461-3.

Eastaff-Leung N, Mabarrack N, Barbour A, Cummins A, Barry S. Foxp3+ regulatory T cells, Th17 effector cells, and cytokine environment in inflammatory bowel disease. J Clin Immunol 2010; 30:80-9.

Eberl G, Littman DR. Thymic origin of intestinal alphabeta T cells revealed by fate mapping of RORgammat+ cells. Science 2004; 305:248-51.

152. Eberl G, Lochner M. The development of intestinal lymphoid tissues at the interface of self and microbiota. Mucosal Immunol 2009; 2:478-85.

Eberl G, Marmon S, Sunshine MJ, Rennert PD, Choi Y, Littman DR. An essential function for the nuclear receptor RORgamma(t) in the generation of fetal lymphoid tissue inducer cells. Nat Immunol 2004; 5:64-73.

Ebert EC. Infliximab and the TNF-alpha system. Am J Physiol Gastrointest Liver Physiol 2009; 296:G612-20.

Economou M, Trikalinos TA, Loizou KT, Tsianos EV, Ioannidis JP. Differential effects of NOD2 variants on Crohn's disease risk and phenotype in diverse populations: a metaanalysis. Am J Gastroenterol 2004; 99:2393-404.

Edelblum KL, Goettel JA, Koyama T, McElroy SJ, Yan F, Polk DB. TNFR1 promotes tumor necrosis factor-mediated mouse colon epithelial cell survival through RAF activation of NF-kappaB. J Biol Chem 2008; 283:29485-94.

Edelblum KL, Washington MK, Koyama T, Robine S, Baccarini M, Polk DB. Raf protects against colitis by promoting mouse colon epithelial cell survival through NF-kappaB. Gastroenterology 2008; 135:539-51.

Edele F, Molenaar R, Gütle D, Dudda JC, Jakob T, Homey B, et al. Cutting edge: instructive role of peripheral tissue cells in the imprinting of T cell homing receptor patterns. J Immunol 2008; 181:3745-9.

Edwards JP, Zhang X, Frauwirth KA, Mosser DM. Biochemical and functional characterization of three activated macrophage populations. J Leukoc Biol 2006; 80:1298-307.

Eken A, Singh AK, Treuting PM, Oukka M. IL-23R+ innate lymphoid cells induce colitis via interleukin-22-dependent mechanism. Mucosal Immunol 2014; 7:143-54.

Eksteen B, Adams DH. GSK-1605786, a selective small-molecule antagonist of the CCR9 chemokine receptor for the treatment of Crohn's disease. IDrugs 2010; 13:472-81.

El Gazzar M, McCall CE. MicroRNAs distinguish translational from transcriptional silencing during endotoxin tolerance. J Biol Chem 2010; 285:20940-51.

El Gazzar M, Yoza BK, Chen X, Hu J, Hawkins GA, McCall CE. G9a and HP1 couple histone and DNA methylation to TNFalpha transcription silencing during endotoxin tolerance. J Biol Chem 2008; 283:32198-208.

El Gazzar M, Yoza BK, Hu JY, Cousart SL, McCall CE. Epigenetic silencing of tumor necrosis factor alpha during endotoxin tolerance. J Biol Chem 2007; 282:26857-64.

Escribese MM, Sierra-Filardi E, Nieto C, Samaniego R, Sánchez-Torres C, Matsuyama T, et al. The prolyl hydroxylase PHD3 identifies proinflammatory macrophages and its expression is regulated by activin A. J Immunol 2012; 189:1946-54.

Estarás C, Fueyo R, Akizu N, Beltrán S, Martínez-Balbás MA. RNA polymerase II progression through H3K27me3-enriched gene bodies requires JMJD3 histone demethylase. Mol Biol Cell 2013; 24:351-60.

167. Fais S, Capobianchi MR, Pallone F, Di Marco P, Boirivant M, Dianzani F, et al. Spontaneous release of interferon gamma by intestinal lamina propria lymphocytes in Crohn's disease. Kinetics of in vitro response to interferon gamma inducers. Gut 1991; 32:403-7.

Falvo JV, Jasenosky LD, Kruidenier L, Goldfeld AE. Epigenetic control of cytokine gene expression: regulation of the TNF/LT locus and T helper cell differentiation. Adv Immunol 2013; 118:37-128.

Falvo JV, Tsytsykova AV, Goldfeld AE. Transcriptional control of the TNF gene. Curr Dir Autoimmun 2010; 11:27-60.

Fantini MC, Becker C, Tubbe I, Nikolaev A, Lehr HA, Galle P, et al. Transforming growth factor beta induced FoxP3+ regulatory T cells suppress Th1 mediated experimental colitis. Gut 2006; 55:671-80.

Fantini MC, Monteleone G, MacDonald TT. IL-21 comes of age as a regulator of effector T cells in the gut. Mucosal Immunol 2008; 1:110-5.

Farache J, Koren I, Milo I, Gurevich I, Kim KW, Zigmond E, et al. Luminal bacteria recruit CD103+ dendritic cells into the intestinal epithelium to sample bacterial antigens for presentation. Immunity 2013; 38:581-95.

Farstad IN, Carlsen H, Morton HC, Brandtzaeg P. Immunoglobulin A cell distribution in the human small intestine: phenotypic and functional characteristics. Immunology 2000; 101:354-63.

Farstad IN, Halstensen TS, Lien B, Kilshaw PJ, Lazarovits AI, Brandtzaeg P, et al. Distribution of beta 7 integrins in human intestinal mucosa and organized gut-associated lymphoid tissue. Immunology 1996; 89:227-37.

Farstad IN, Norstein J, Brandtzaeg P. Phenotypes of B and T cells in human intestinal and mesenteric lymph. Gastroenterology 1997; 112:163-73.

Feng CG, Zheng L, Lenardo MJ, Sher A. Interferon-inducible immunity-related GTPase Irgm1 regulates IFN gamma-dependent host defense, lymphocyte survival and autophagy. Autophagy 2009; 5:232-4.

Fina D, Sarra M, Fantini MC, Rizzo A, Caruso R, Caprioli F, et al. Regulation of gut inflammation and th17 cell response by interleukin-21. Gastroenterology 2008; 134:1038-48.

Finotto S, Neurath MF, Glickman JN, Qin S, Lehr HA, Green FH, et al. Development of spontaneous airway changes consistent with human asthma in mice lacking T-bet. Science 2002; 295:336-8.

Fisher SA, Tremelling M, Anderson CA, Gwilliam R, Bumpstead S, Prescott NJ, et al. Genetic determinants of ulcerative colitis include the ECM1 locus and five loci implicated in Crohn's disease. Nat Genet 2008; 40:710-2.

Flanagan P, Campbell BJ, Rhodes JM. Bacteria in the pathogenesis of inflammatory bowel disease. Biochem Soc Trans 2011; 39:1067-72.

Foks AC, Bot I, Frodermann V, de Jager SC, Ter Borg M, van Santbrink PJ, et al. Interference of the CD30-CD30L pathway reduces atherosclerosis development. Arterioscler Thromb Vasc Biol 2012; 32:2862-8.

Förster R, Davalos-Misslitz AC, Rot A. CCR7 and its ligands: balancing immunity and tolerance. Nat Rev Immunol 2008; 8:362-71.

Förster R, Mattis AE, Kremmer E, Wolf E, Brem G, Lipp M. A putative chemokine receptor, BLR1, directs B cell migration to defined lymphoid organs and specific anatomic compartments of the spleen. Cell 1996; 87:1037-47.

Foster SL, Hargreaves DC, Medzhitov R. Gene-specific control of inflammation by TLR-induced chromatin modifications. Nature 2007; 447:972-8.

Fritz JH, Ferrero RL, Philpott DJ, Girardin SE. Nod-like proteins in immunity, inflammation and disease. Nat Immunol 2006; 7:1250-7.

Fry TJ, Mackall CL. Interleukin-7: from bench to clinic. Blood 2002; 99:3892-904.

Fuchs A, Vermi W, Lee JS, Lonardi S, Gilfillan S, Newberry RD, et al. Intraepithelial type 1 innate lymphoid cells are a unique subset of IL-12- and IL-15-responsive IFN-γ-producing cells. Immunity 2013; 38:769-81.

Fujimoto K, Karuppuchamy T, Takemura N, Shimohigoshi M, Machida T, Haseda Y, et al. A new subset of CD103+CD8alpha+ dendritic cells in the small intestine expresses TLR3, TLR7, and TLR9 and induces Th1 response and CTL activity. J Immunol 2011; 186:6287-95.

Furumoto Y, Gadina M. The arrival of JAK inhibitors: advancing the treatment of immune and hematologic disorders. BioDrugs 2013; 27:431-8.

Fuss IJ, Neurath M, Boirivant M, Klein JS, de la Motte C, Strong SA, et al. Disparate CD4+ lamina propria (LP) lymphokine secretion profiles in inflammatory bowel disease. Crohn's disease LP cells manifest increased secretion of IFN-gamma, whereas ulcerative colitis LP cells manifest increased secretion of IL-5. J Immunol 1996; 157:1261-70.

Fuss IJ, Strober W. The role of IL-13 and NK T cells in experimental and human ulcerative colitis. Mucosal Immunol 2008; 1 Suppl 1:S31-3.

Galli SJ, Borregaard N, Wynn TA. Phenotypic and functional plasticity of cells of innate immunity: macrophages, mast cells and neutrophils. Nat Immunol 2011; 12:1035-44.

Gallo RL, Hooper LV. Epithelial antimicrobial defence of the skin and intestine. Nat Rev Immunol 2012; 12:503-16.

Geissmann F, Jung S, Littman DR. Blood monocytes consist of two principal subsets with distinct migratory properties. Immunity 2003; 19:71-82.

Geissmann F, Manz MG, Jung S, Sieweke MH, Merad M, Ley K. Development of monocytes, macrophages, and dendritic cells. Science 2010; 327:656-61.

Geremia A, Arancibia-Cárcamo CV, Fleming MP, Rust N, Singh B, Mortensen NJ, et al. IL-23-responsive innate lymphoid cells are increased in inflammatory bowel disease. J Exp Med 2011; 208:1127-33.

Geremia A, Jewell DP. The IL-23/IL-17 pathway in inflammatory bowel disease. Expert Rev Gastroenterol Hepatol 2012; 6:223-37.

Gerlach K, Hwang Y, Nikolaev A, Atreya R, Dornhoff H, Steiner S, et al. TH9 cells that express the transcription factor PU.1 drive T cell-mediated colitis via IL-9 receptor signaling in intestinal epithelial cells. Nat Immunol 2014; 15:676-86.

Ghoreschi K, Jesson MI, Li X, Lee JL, Ghosh S, Alsup JW, et al. Modulation of innate and adaptive immune responses by tofacitinib (CP-690,550). J Immunol 2011; 186:4234-43.

Glaysher BR, Mabbott NA. Isolated lymphoid follicle maturation induces the development of follicular dendritic cells. Immunology 2007; 120:336-44.

Glozak MA, Sengupta N, Zhang X, Seto E. Acetylation and deacetylation of non-histone proteins. Gene 2005; 363:15-23.

Goldfeld AE, McCaffrey PG, Strominger JL, Rao A. Identification of a novel cyclosporin-sensitive element in the human tumor necrosis factor alpha gene promoter. J Exp Med 1993; 178:1365-79.

Gordon FH, Lai CW, Hamilton MI, Allison MC, Srivastava ED, Fouweather MG, et al. A randomized placebo-controlled trial of a humanized monoclonal antibody to alpha4 integrin in active Crohn's disease. Gastroenterology 2001; 121:268-74.

Gordon JN, Di Sabatino A, Macdonald TT. The pathophysiologic rationale for biological therapies in inflammatory bowel disease. Curr Opin Gastroenterol 2005; 21:431-7.

Gordon S. Macrophage heterogeneity and tissue lipids. J Clin Invest 2007; 117:89-93.

Gordon S. The macrophage: past, present and future. Eur J Immunol 2007; 37 Suppl 1:S9-17.

Gordon S, Martinez FO. Alternative activation of macrophages: mechanism and functions. Immunity 2010; 32:593-604.

209. Gordon S, Plűddemann A. Tissue macrophage heterogeneity: issues and prospects. Semin Immunopathol 2013; 35:533-40.

Gordon S, Taylor PR. Monocyte and macrophage heterogeneity. Nat Rev Immunol 2005; 5:953-64.

Goriely S, Goldman M. Interleukin-12 family members and the balance between rejection and tolerance. Curr Opin Organ Transplant 2008; 13:4-9.

Grabstein KH, Waldschmidt TJ, Finkelman FD, Hess BW, Alpert AR, Boiani NE, et al. Inhibition of murine B and T lymphopoiesis in vivo by an anti-interleukin 7 monoclonal antibody. J Exp Med 1993; 178:257-64.

Greenwell-Wild T, Vázquez N, Sim D, Schito M, Chatterjee D, Orenstein JM, et al. Mycobacterium avium infection and modulation of human macrophage gene expression. J Immunol 2002; 169:6286-97.

Gregersen PK, Olsson LM. Recent advances in the genetics of autoimmune disease. Annu Rev Immunol 2009; 27:363-91.

Grimm MC, Pavli P, Van de Pol E, Doe WF. Evidence for a CD14+ population of monocytes in inflammatory bowel disease mucosa--implications for pathogenesis. Clin Exp Immunol 1995; 100:291-7.

Gross DS, Garrard WT. Nuclease hypersensitive sites in chromatin. Annu Rev Biochem 1988; 57:159-97.

Gunn MD, Kyuwa S, Tam C, Kakiuchi T, Matsuzawa A, Williams LT, et al. Mice lacking expression of secondary lymphoid organ chemokine have defects in lymphocyte homing and dendritic cell localization. J Exp Med 1999; 189:451-60.

Guo X, Wang XF. Signaling cross-talk between TGF-beta/BMP and other pathways. Cell Res 2009; 19:71-88.

Gupta S. Molecular signaling in death receptor and mitochondrial pathways of apoptosis (Review). Int J Oncol 2003; 22:15-20.

Gutierrez O, Pipaon C, Inohara N, Fontalba A, Ogura Y, Prosper F, et al. Induction of Nod2 in myelomonocytic and intestinal epithelial cells via nuclear factor-kappa B activation. J Biol Chem 2002; 277:41701-5.

Hale-Donze H, Greenwell-Wild T, Mizel D, Doherty TM, Chatterjee D, Orenstein JM, et al. Mycobacterium avium complex promotes recruitment of monocyte hosts for HIV-1 and bacteria. J Immunol 2002; 169:3854-62.

Halfvarson J, Bodin L, Tysk C, Lindberg E, Järnerot G. Inflammatory bowel disease in a Swedish twin cohort: a long-term follow-up of concordance and clinical characteristics. Gastroenterology 2003; 124:1767-73.

Hall JA, Cannons JL, Grainger JR, Dos Santos LM, Hand TW, Naik S, et al. Essential role for retinoic acid in the promotion of CD4(+) T cell effector responses via retinoic acid receptor alpha. Immunity

2011; 34:435-47.

Halme L, Paavola-Sakki P, Turunen U, Lappalainen M, Farkkila M, Kontula K. Family and twin studies in inflammatory bowel disease. World J Gastroenterol 2006; 12:3668-72.

Hamann A, Andrew DP, Jablonski-Westrich D, Holzmann B, Butcher EC. Role of alpha 4-integrins in lymphocyte homing to mucosal tissues in vivo. J Immunol 1994; 152:3282-93.

Hamilton KE, Simmons JG, Ding S, Van Landeghem L, Lund PK. Cytokine induction of tumor necrosis factor receptor 2 is mediated by STAT3 in colon cancer cells. Mol Cancer Res 2011; 9:1718-31.

Hammerschmidt SI, Ahrendt M, Bode U, Wahl B, Kremmer E, Förster R, et al. Stromal mesenteric lymph node cells are essential for the generation of gut-homing T cells in vivo. J Exp Med 2008; 205:2483-90.

Hammerschmidt SI, Friedrichsen M, Boelter J, Lyszkiewicz M, Kremmer E, Pabst O, et al. Retinoic acid induces homing of protective T and B cells to the gut after subcutaneous immunization in mice. J Clin Invest 2011; 121:3051-61.

Hampe J, Franke A, Rosenstiel P, Till A, Teuber M, Huse K, et al. A genome-wide association scan of nonsynonymous SNPs identifies a susceptibility variant for Crohn disease in ATG16L1. Nat Genet 2007; 39:207-11.

Hanson ML, Hixon JA, Li W, Felber BK, Anver MR, Stewart CA, et al. Oral delivery of IL-27 recombinant bacteria attenuates immune colitis in mice. Gastroenterology 2014; 146:210-21.e13.

Hardwick JC, Kodach LL, Offerhaus GJ, van den Brink GR. Bone morphogenetic protein signalling in colorectal cancer. Nat Rev Cancer 2008; 8:806-12.

Hardwick JC, Van Den Brink GR, Bleuming SA, Ballester I, Van Den Brande JM, Keller JJ, et al. Bone morphogenetic protein 2 is expressed by, and acts upon, mature epithelial cells in the colon. Gastroenterology 2004; 126:111-21.

Hargreaves DC, Horng T, Medzhitov R. Control of inducible gene expression by signal-dependent transcriptional elongation. Cell 2009; 138:129-45.

Hawkins RD, Hon GC, Lee LK, Ngo Q, Lister R, Pelizzola M, et al. Distinct epigenomic landscapes of pluripotent and lineage-committed human cells. Cell Stem Cell 2010; 6:479-91.

He B, Xu W, Santini PA, Polydorides AD, Chiu A, Estrella J, et al. Intestinal bacteria trigger T cell-independent immunoglobulin A(2) class switching by inducing epithelial-cell secretion of the cytokine APRIL. Immunity 2007; 26:812-26.

Heinemann B, Nielsen JM, Hudlebusch HR, Lees MJ, Larsen DV, Boesen T, et al. Inhibition of demethylases by GSK-J1/J4. Nature 2014; 514:E1-2.

Heinrich PC, Castell JV, Andus T. Interleukin-6 and the acute phase response. Biochem J 1990; 265:621-36.

Heinsbroek SE, Gordon S. The role of macrophages in inflammatory bowel diseases. Expert Rev Mol Med 2009; 11:e14.

Heintzman ND, Stuart RK, Hon G, Fu Y, Ching CW, Hawkins RD, et al. Distinct and predictive chromatin signatures of transcriptional promoters and enhancers in the human genome. Nat Genet 2007; 39:311-8.

Heinz S, Benner C, Spann N, Bertolino E, Lin YC, Laslo P, et al. Simple combinations of lineage-determining transcription factors prime cis-regulatory elements required for macrophage and B cell identities. Mol Cell 2010; 38:576-89.

Heller F, Florian P, Bojarski C, Richter J, Christ M, Hillenbrand B, et al. Interleukin-13 is the key effector Th2 cytokine in ulcerative colitis that affects epithelial tight junctions, apoptosis, and cell restitution. Gastroenterology 2005; 129:550-64.

Heller F, Fuss IJ, Nieuwenhuis EE, Blumberg RS, Strober W. Oxazolone colitis, a Th2 colitis model resembling ulcerative colitis, is mediated by IL-13-producing NK-T cells. Immunity 2002; 17:629-38.

Hendricks DW, Fink PJ. Uneven colonization of the lymphoid periphery by T cells that undergo early TCR{alpha} rearrangements. J Immunol 2009; 182:4267-74.

Herman JG, Baylin SB. Gene silencing in cancer in association with promoter hypermethylation. N Engl J Med 2003; 349:2042-54.

Hermiston ML, Gordon JI. In vivo analysis of cadherin function in the mouse intestinal epithelium: essential roles in adhesion, maintenance of differentiation, and regulation of programmed cell death. J Cell Biol 1995; 129:489-506.

Hernández-Caselles T, Martínez-Esparza M, Pérez-Oliva AB, Quintanilla-Cecconi AM, García-Alonso A, Alvarez-López DM, et al. A study of CD33 (SIGLEC-3) antigen expression and function on activated human T and NK cells: two isoforms of CD33 are generated by alternative splicing. J Leukoc Biol 2006; 79:46-58.

Herrlinger KR, Witthoeft T, Raedler A, Bokemeyer B, Krummenerl T, Schulzke JD, et al. Randomized, double blind controlled trial of subcutaneous recombinant human interleukin-11 versus prednisolone in active Crohn's disease. Am J Gastroenterol 2006; 101:793-7.

Hisamatsu T, Suzuki M, Reinecker HC, Nadeau WJ, McCormick BA, Podolsky DK. CARD15/NOD2 functions as an antibacterial factor in human intestinal epithelial cells. Gastroenterology 2003; 124:993-1000.

Hoffmann A, Baltimore D. Circuitry of nuclear factor kappaB signaling. Immunol Rev 2006; 210:171-86.

Hommes DW, Mikhajlova TL, Stoinov S, Stimac D, Vucelic B, Lonovics J, et al. Fontolizumab, a humanised anti-interferon gamma antibody, demonstrates safety and clinical activity in patients with moderate to severe Crohn's disease. Gut 2006; 55:1131-7.

Honda K, Nakano H, Yoshida H, Nishikawa S, Rennert P, Ikuta K, et al. Molecular basis for hematopoietic/mesenchymal interaction during initiation of Peyer's patch organogenesis. J Exp Med 2001; 193:621-30.

Hong SN, Park C, Park SJ, Lee CK, Ye BD, Kim YS, et al. Deep resequencing of 131 Crohn's disease associated genes in pooled DNA confirmed three reported variants and identified eight novel variants. Gut 2015.

Hooper LV. You AhR what you eat: linking diet and immunity. Cell 2011; 147:489-91.

Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. Science 2003; 299:1057-61.

Hoshino K, Takeuchi O, Kawai T, Sanjo H, Ogawa T, Takeda Y, et al. Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. J Immunol 1999; 162:3749-52.

Hu X, Ivashkiv LB. Cross-regulation of signaling pathways by interferon-gamma: implications for immune responses and autoimmune diseases. Immunity 2009; 31:539-50.

Huang C, Xiang Y, Wang Y, Li X, Xu L, Zhu Z, et al. Dual-specificity histone demethylase KIAA1718 (KDM7A) regulates neural differentiation through FGF4. Cell Res 2010; 20:154-65.

Huang Y, Min S, Lui Y, Sun J, Su X, Liu Y, et al. Global mapping of H3K4me3 and H3K27me3 reveals chromatin state-based regulation of human monocyte-derived dendritic cells in different environments. Genes Immun 2012; 13:311-20.

Huber S, Gagliani N, Esplugues E, O'Connor W, Huber FJ, Chaudhry A, et al. Th17 cells express interleukin-10 receptor and are controlled by Foxp3⁻ and Foxp3+ regulatory CD4+ T cells in an interleukin-10-dependent manner. Immunity 2011; 34:554-65.

Hueber W, Sands BE, Lewitzky S, Vandemeulebroecke M, Reinisch W, Higgins PD, et al. Secukinumab, a human anti-IL-17A monoclonal antibody, for moderate to severe Crohn's disease: unexpected results of a randomised, double-blind placebo-controlled trial. Gut 2012; 61:1693-700.

Ishii M, Wen H, Corsa CA, Liu T, Coelho AL, Allen RM, et al. Epigenetic regulation of the alternatively activated macrophage phenotype. Blood 2009; 114:3244-54.

Ismail AS, Severson KM, Vaishnava S, Behrendt CL, Yu X, Benjamin JL, et al. Gammadelta intraepithelial lymphocytes are essential mediators of host-microbial homeostasis at the intestinal mucosal surface. Proc Natl Acad Sci U S A 2011; 108:8743-8.

Ivanov AI, Young C, Den Beste K, Capaldo CT, Humbert PO, Brennwald P, et al. Tumor suppressor scribble regulates assembly of tight junctions in the intestinal epithelium. Am J Pathol 2010; 176:134-45.

Ivanov II, Atarashi K, Manel N, Brodie EL, Shima T, Karaoz U, et al. Induction of intestinal Th17 cells by segmented filamentous bacteria. Cell 2009; 139:485-98.

Ivashkiv LB. Epigenetic regulation of macrophage polarization and function. Trends Immunol 2013; 34:216-23.

Jaensson E, Uronen-Hansson H, Pabst O, Eksteen B, Tian J, Coombes JL, et al. Small intestinal CD103+ dendritic cells display unique functional properties that are conserved between mice and humans. J Exp Med 2008; 205:2139-49.

Jang MH, Sougawa N, Tanaka T, Hirata T, Hiroi T, Tohya K, et al. CCR7 is critically important for migration of dendritic cells in intestinal lamina propria to mesenteric lymph nodes. J Immunol 2006; 176:803-10.

268. Jenke AC, Zilbauer M. Epigenetics in inflammatory bowel disease. Curr Opin Gastroenterol 2012; 28:577-84.

Jenkins SJ, Ruckerl D, Cook PC, Jones LH, Finkelman FD, van Rooijen N, et al. Local macrophage proliferation, rather than recruitment from the blood, is a signature of TH2 inflammation. Science 2011; 332:1284-8.

Jepsen K, Solum D, Zhou T, McEvilly RJ, Kim HJ, Glass CK, et al. SMRT-mediated repression of an H3K27 demethylase in progression from neural stem cell to neuron. Nature 2007; 450:415-9.

Jiang H, Lin JJ, Su ZZ, Goldstein NI, Fisher PB. Subtraction hybridization identifies a novel melanoma differentiation associated gene, mda-7, modulated during human melanoma differentiation, growth and progression. Oncogene 1995; 11:2477-86.

Jiang H, Su ZZ, Lin JJ, Goldstein NI, Young CS, Fisher PB. The melanoma differentiation associated gene mda-7 suppresses cancer cell growth. Proc Natl Acad Sci U S A 1996; 93:9160-5.

Jin B, Li Y, Robertson KD. DNA methylation: superior or subordinate in the epigenetic hierarchy? Genes Cancer 2011; 2:607-17.

274. Johansson ME, Larsson JM, Hansson GC. The two mucus layers of colon are organized by the MUC2 mucin, whereas the outer layer is a legislator of host-microbial interactions. Proc Natl Acad

Sci U S A 2011; 108 Suppl 1:4659-65.

Johansson ME, Phillipson M, Petersson J, Velcich A, Holm L, Hansson GC. The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria. Proc Natl Acad Sci U S A 2008; 105:15064-9.

Johansson ME, Sjövall H, Hansson GC. The gastrointestinal mucus system in health and disease. Nat Rev Gastroenterol Hepatol 2013; 10:352-61.

Johansson-Lindbom B, Svensson M, Pabst O, Palmqvist C, Marquez G, Förster R, et al. Functional specialization of gut CD103+ dendritic cells in the regulation of tissue-selective T cell homing. J Exp Med 2005; 202:1063-73.

Jostins L, Ripke S, Weersma RK, Duerr RH, McGovern DP, Hui KY, et al. Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease. Nature 2012; 491:119-24.

Jump RL, Levine AD. Mechanisms of natural tolerance in the intestine: implications for inflammatory bowel disease. Inflamm Bowel Dis 2004; 10:462-78.

Jung C, Hugot JP, Barreau F. Peyer's Patches: The Immune Sensors of the Intestine. Int J Inflam 2010; 2010:823710.

Jüngel A, Distler JH, Kurowska-Stolarska M, Seemayer CA, Seibl R, Forster A, et al. Expression of interleukin-21 receptor, but not interleukin-21, in synovial fibroblasts and synovial macrophages of patients with rheumatoid arthritis. Arthritis Rheum 2004; 50:1468-76.

Kai Y, Takahashi I, Ishikawa H, Hiroi T, Mizushima T, Matsuda C, et al. Colitis in mice lacking the common cytokine receptor gamma chain is mediated by IL-6-producing CD4+ T cells. Gastroenterology 2005; 128:922-34.

Kamada N, Hisamatsu T, Honda H, Kobayashi T, Chinen H, Kitazume MT, et al. Human CD14+ macrophages in intestinal lamina propria exhibit potent antigen-presenting ability. J Immunol 2009; 183:1724-31.

Kamada N, Hisamatsu T, Honda H, Kobayashi T, Chinen H, Takayama T, et al. TL1A produced by lamina propria macrophages induces Th1 and Th17 immune responses in cooperation with IL-23 in patients with Crohn's disease. Inflamm Bowel Dis 2010; 16:568-75.

Kamada N, Hisamatsu T, Okamoto S, Chinen H, Kobayashi T, Sato T, et al. Unique CD14 intestinal macrophages contribute to the pathogenesis of Crohn disease via IL-23/IFN-gamma axis. J Clin Invest 2008; 118:2269-80.

Kamdar K, Nguyen V, DePaolo RW. Toll-like receptor signaling and regulation of intestinal immunity. Virulence 2013; 4:207-12.

Kanai T, Watanabe M, Okazawa A, Sato T, Yamazaki M, Okamoto S, et al. Macrophage-derived IL-18-mediated intestinal inflammation in the murine model of Crohn's disease. Gastroenterology 2001; 121:875-88.

288. Kaser A, Zeissig S, Blumberg RS. Genes and environment: how will our concepts on the pathophysiology of IBD develop in the future? Dig Dis 2010; 28:395-405.

Katakura K, Lee J, Rachmilewitz D, Li G, Eckmann L, Raz E. Toll-like receptor 9-induced type I IFN protects mice from experimental colitis. J Clin Invest 2005; 115:695-702.

Kawaguchi-Miyashita M, Shimizu K, Nanno M, Shimada S, Watanabe T, Koga Y, et al. Development and cytolytic function of intestinal intraepithelial T lymphocytes in antigen-minimized mice. Immunology 1996; 89:268-73.

Kellermayer R. Epigenetics and the developmental origins of inflammatory bowel diseases. Can J

Gastroenterol 2012; 26:909-15.

Kelly E, Won A, Refaeli Y, Van Parijs L. IL-2 and related cytokines can promote T cell survival by activating AKT. J Immunol 2002; 168:597-603.

Kelsall B. Recent progress in understanding the phenotype and function of intestinal dendritic cells and macrophages. Mucosal Immunol 2008; 1:460-9.

Kennedy MK, Glaccum M, Brown SN, Butz EA, Viney JL, Embers M, et al. Reversible defects in natural killer and memory CD8 T cell lineages in interleukin 15-deficient mice. J Exp Med 2000; 191:771-80.

Kharbanda S, Nakamura T, Stone R, Hass R, Bernstein S, Datta R, et al. Expression of the early growth response 1 and 2 zinc finger genes during induction of monocytic differentiation. J Clin Invest 1991; 88:571-7.

Khor B, Gardet A, Xavier RJ. Genetics and pathogenesis of inflammatory bowel disease. Nature 2011; 474:307-17.

Kim D, Mebius RE, MacMicking JD, Jung S, Cupedo T, Castellanos Y, et al. Regulation of peripheral lymph node genesis by the tumor necrosis factor family member TRANCE. J Exp Med 2000; 192:1467-78.

Kim HR, Hwang KA, Kang I. Dual roles of IL-15 in maintaining IL-7RalphalowCCR7- memory CD8+ T cells in humans via recovering the phosphatidylinositol 3-kinase/AKT pathway. J Immunol 2007; 179:6734-40.

Kim SW, Yoon SJ, Chuong E, Oyolu C, Wills AE, Gupta R, et al. Chromatin and transcriptional signatures for Nodal signaling during endoderm formation in hESCs. Dev Biol 2011; 357:492-504.

Kinjyo I, Hanada T, Inagaki-Ohara K, Mori H, Aki D, Ohishi M, et al. SOCS1/JAB is a negative regulator of LPS-induced macrophage activation. Immunity 2002; 17:583-91.

Kishigami S, Mishina Y. BMP signaling and early embryonic patterning. Cytokine Growth Factor Rev 2005; 16:265-78.

Kiss EA, Diefenbach A. Role of the Aryl Hydrocarbon Receptor in Controlling Maintenance and Functional Programs of RORγt(+) Innate Lymphoid Cells and Intraepithelial Lymphocytes. Front Immunol 2012; 3:124.

Kleinschek MA, Boniface K, Sadekova S, Grein J, Murphy EE, Turner SP, et al. Circulating and gut-resident human Th17 cells express CD161 and promote intestinal inflammation. J Exp Med 2009; 206:525-34.

Klose RJ, Zhang Y. Regulation of histone methylation by demethylimination and demethylation. Nat Rev Mol Cell Biol 2007; 8:307-18.

Knoop KA, Butler BR, Kumar N, Newberry RD, Williams IR. Distinct developmental requirements for isolated lymphoid follicle formation in the small and large intestine: RANKL is essential only in the small intestine. Am J Pathol 2011; 179:1861-71.

Kobayashi K, Hernandez LD, Galán JE, Janeway CA, Medzhitov R, Flavell RA. IRAK-M is a negative regulator of Toll-like receptor signaling. Cell 2002; 110:191-202.

Kobayashi T, Okamoto S, Hisamatsu T, Kamada N, Chinen H, Saito R, et al. IL23 differentially regulates the Th1/Th17 balance in ulcerative colitis and Crohn's disease. Gut 2008; 57:1682-9.

Kodach LL, Wiercinska E, de Miranda NF, Bleuming SA, Musler AR, Peppelenbosch MP, et al. The bone morphogenetic protein pathway is inactivated in the majority of sporadic colorectal cancers. Gastroenterology 2008; 134:1332-41.

Komatsu M, Kobayashi D, Saito K, Furuya D, Yagihashi A, Araake H, et al. Tumor necrosis factor-alpha in serum of patients with inflammatory bowel disease as measured by a highly sensitive immuno-PCR. Clin Chem 2001; 47:1297-301.

Koni PA, Sacca R, Lawton P, Browning JL, Ruddle NH, Flavell RA. Distinct roles in lymphoid organogenesis for lymphotoxins alpha and beta revealed in lymphotoxin beta-deficient mice. Immunity 1997; 6:491-500.

Konkel JE, Maruyama T, Carpenter AC, Xiong Y, Zamarron BF, Hall BE, et al. Control of the development of CD8αα+ intestinal intraepithelial lymphocytes by TGF-β. Nat Immunol 2011; 12:312-9.

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

Körner H, Cook M, Riminton DS, Lemckert FA, Hoek RM, Ledermann B, et al. Distinct roles for lymphotoxin-alpha and tumor necrosis factor in organogenesis and spatial organization of lymphoid tissue. Eur J Immunol 1997; 27:2600-9.

Kosinski C, Li VS, Chan AS, Zhang J, Ho C, Tsui WY, et al. Gene expression patterns of human colon tops and basal crypts and BMP antagonists as intestinal stem cell niche factors. Proc Natl Acad Sci U S A 2007; 104:15418-23.

Kotlarz D, Beier R, Murugan D, Diestelhorst J, Jensen O, Boztug K, et al. Loss of interleukin-10 signaling and infantile inflammatory bowel disease: implications for diagnosis and therapy. Gastroenterology 2012; 143:347-55.

Kouzarides T. Chromatin modifications and their function. Cell 2007; 128:693-705.

Kruidenier L, Chung CW, Cheng Z, Liddle J, Che K, Joberty G, et al. A selective jumonji H3K27 demethylase inhibitor modulates the proinflammatory macrophage response. Nature 2012; 488:404-8.

Ku CC, Murakami M, Sakamoto A, Kappler J, Marrack P. Control of homeostasis of CD8+ memory T cells by opposing cytokines. Science 2000; 288:675-8.

Kunkel EJ, Campbell JJ, Haraldsen G, Pan J, Boisvert J, Roberts AI, et al. Lymphocyte CC chemokine receptor 9 and epithelial thymus-expressed chemokine (TECK) expression distinguish the small intestinal immune compartment: Epithelial expression of tissue-specific chemokines as an organizing principle in regional immunity. J Exp Med 2000; 192:761-8.

Kunz S, Wolk K, Witte E, Witte K, Doecke WD, Volk HD, et al. Interleukin (IL)-19, IL-20 and IL-24 are produced by and act on keratinocytes and are distinct from classical ILs. Exp Dermatol 2006; 15:991-1004.

Kurowska-Stolarska M, Stolarski B, Kewin P, Murphy G, Corrigan CJ, Ying S, et al. IL-33 amplifies the polarization of alternatively activated macrophages that contribute to airway inflammation. J Immunol 2009; 183:6469-77.

Laffont S, Siddiqui KR, Powrie F. Intestinal inflammation abrogates the tolerogenic properties of MLN CD103+ dendritic cells. Eur J Immunol 2010; 40:1877-83.

Lajaunias F, Dayer JM, Chizzolini C. Constitutive repressor activity of CD33 on human monocytes requires sialic acid recognition and phosphoinositide 3-kinase-mediated intracellular signaling. Eur J Immunol 2005; 35:243-51.

Lampinen M, Waddell A, Ahrens R, Carlson M, Hogan SP. CD14+CD33+ myeloid cell-CCL11-eosinophil signature in ulcerative colitis. J Leukoc Biol 2013; 94:1061-70.

Lan F, Bayliss PE, Rinn JL, Whetstine JR, Wang JK, Chen S, et al. A histone H3 lysine 27 demethylase regulates animal posterior development. Nature 2007; 449:689-94.

Langer JA, Cutrone EC, Kotenko S. The Class II cytokine receptor (CRF2) family: overview and patterns of receptor-ligand interactions. Cytokine Growth Factor Rev 2004; 15:33-48.

Latham JA, Dent SY. Cross-regulation of histone modifications. Nat Struct Mol Biol 2007; 14:1017-24.

Lazarevic V, Glimcher LH, Lord GM. T-bet: a bridge between innate and adaptive immunity. Nat Rev Immunol 2013; 13:777-89.

Lees CW, Barrett JC, Parkes M, Satsangi J. New IBD genetics: common pathways with other diseases. Gut 2011; 60:1739-53.

Leishman AJ, Naidenko OV, Attinger A, Koning F, Lena CJ, Xiong Y, et al. T cell responses modulated through interaction between CD8alphaalpha and the nonclassical MHC class I molecule, TL. Science 2001; 294:1936-9.

Leon F, Smythies LE, Smith PD, Kelsall BL. Involvement of dendritic cells in the pathogenesis of inflammatory bowel disease. Adv Exp Med Biol 2006; 579:117-32.

Leppkes M, Becker C, Ivanov II, Hirth S, Wirtz S, Neufert C, et al. RORgamma-expressing Th17 cells induce murine chronic intestinal inflammation via redundant effects of IL-17A and IL-17F. Gastroenterology 2009; 136:257-67.

Lesch BJ, Dokshin GA, Young RA, McCarrey JR, Page DC. A set of genes critical to development is epigenetically poised in mouse germ cells from fetal stages through completion of meiosis. Proc Natl Acad Sci U S A 2013; 110:16061-6.

Leung JM, Davenport M, Wolff MJ, Wiens KE, Abidi WM, Poles MA, et al. IL-22-producing CD4+ cells are depleted in actively inflamed colitis tissue. Mucosal Immunol 2014; 7:124-33.

Levin A, Shibolet O. Infliximab in ulcerative colitis. Biologics 2008; 2:379-88.

Levine B, Kroemer G. Autophagy in the pathogenesis of disease. Cell 2008; 132:27-42.

Levine M. Transcriptional enhancers in animal development and evolution. Curr Biol 2010; 20:R754-63

Li B, Samanta A, Song X, Iacono KT, Bembas K, Tao R, et al. FOXP3 interactions with histone acetyltransferase and class II histone deacetylases are required for repression. Proc Natl Acad Sci U S A 2007; 104:4571-6.

Li MO, Sanjabi S, Flavell RA. Transforming growth factor-beta controls development, homeostasis, and tolerance of T cells by regulatory T cell-dependent and -independent mechanisms. Immunity 2006; 25:455-71.

Li Z, Zhang Y, Liu Z, Wu X, Zheng Y, Tao Z, et al. ECM1 controls T(H)2 cell egress from lymph nodes through re-expression of S1P(1). Nat Immunol 2011; 12:178-85.

Liew FY, Xu D, Brint EK, O'Neill LA. Negative regulation of toll-like receptor-mediated immune responses. Nat Rev Immunol 2005; 5:446-58.

Liu Z, Feng BS, Yang SB, Chen X, Su J, Yang PC. Interleukin (IL)-23 suppresses IL-10 in inflammatory bowel disease. J Biol Chem 2012; 287:3591-7.

Liu Z, Yadav PK, Xu X, Su J, Chen C, Tang M, et al. The increased expression of IL-23 in inflammatory bowel disease promotes intraepithelial and lamina propria lymphocyte inflammatory responses and cytotoxicity. J Leukoc Biol 2011; 89:597-606.

Lorenz RG, Chaplin DD, McDonald KG, McDonough JS, Newberry RD. Isolated lymphoid follicle formation is inducible and dependent upon lymphotoxin-sufficient B lymphocytes, lymphotoxin beta receptor, and TNF receptor I function. J Immunol 2003; 170:5475-82.

Louis E, Collard A, Oger AF, Degroote E, Aboul Nasr El Yafi FA, Belaiche J. Behaviour of Crohn's disease according to the Vienna classification: changing pattern over the course of the disease. Gut 2001; 49:777-82.

Low D, Mizoguchi A, Mizoguchi E. DNA methylation in inflammatory bowel disease and beyond. World J Gastroenterol 2013; 19:5238-49.

Lowery JW, de Caestecker MP. BMP signaling in vascular development and disease. Cytokine Growth Factor Rev 2010; 21:287-98.

Lu J, Sun H, Wang X, Liu C, Xu X, Li F, et al. Interleukin-12 p40 promoter activity is regulated by the reversible acetylation mediated by HDAC1 and p300. Cytokine 2005; 31:46-51.

Lubelsky Y, Prinz JA, DeNapoli L, Yulong L, Belsky JA, MacAlpine DM. DNA replication and transcription programs respond to the same chromatin cues. Genome Research 2014; 24(7):1102-1114.

Luger K, Mäder AW, Richmond RK, Sargent DF, Richmond TJ. Crystal structure of the nucleosome core particle at 2.8 A resolution. Nature 1997; 389:251-60.

Lügering A, Schmidt M, Lügering N, Pauels HG, Domschke W, Kucharzik T. Infliximab induces apoptosis in monocytes from patients with chronic active Crohn's disease by using a caspase-dependent pathway. Gastroenterology 2001; 121:1145-57.

Luo D, Luo Y, He Y, Zhang H, Zhang R, Li X, et al. Differential functions of tumor necrosis factor receptor 1 and 2 signaling in ischemia-mediated arteriogenesis and angiogenesis. Am J Pathol 2006; 169:1886-98.

Luster AD, Alon R, von Andrian UH. Immune cell migration in inflammation: present and future therapeutic targets. Nat Immunol 2005; 6:1182-90.

Luther SA, Ansel KM, Cyster JG. Overlapping roles of CXCL13, interleukin 7 receptor alpha, and CCR7 ligands in lymph node development. J Exp Med 2003; 197:1191-8.

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

MacDonald TT, Monteleone I, Fantini MC, Monteleone G. Regulation of homeostasis and inflammation in the intestine. Gastroenterology 2011; 140:1768-75.

MacDonald TT, Pender SL. Lamina propria T cells. Chem Immunol 1998; 71:103-17.

MacDonald TT, Spencer J. Ontogeny of the mucosal immune response. Springer Semin Immunopathol 1990; 12:129-37.

MacMicking JD, Taylor GA, McKinney JD. Immune control of tuberculosis by IFN-gamma-inducible LRG-47. Science 2003; 302:654-9.

Macpherson AJ. IgA adaptation to the presence of commensal bacteria in the intestine. Curr Top Microbiol Immunol 2006; 308:117-36.

Macpherson AJ, Geuking MB, McCoy KD. Immune responses that adapt the intestinal mucosa to commensal intestinal bacteria. Immunology 2005; 115:153-62.

Mages J, Dietrich H, Lang R. A genome-wide analysis of LPS tolerance in macrophages. Immunobiology 2007; 212:723-37.

Magram J, Connaughton SE, Warrier RR, Carvajal DM, Wu CY, Ferrante J, et al. IL-12-deficient mice are defective in IFN gamma production and type 1 cytokine responses. Immunity 1996; 4:471-81.

Malek TR, Castro I. Interleukin-2 receptor signaling: at the interface between tolerance and immunity. Immunity 2010; 33:153-65.

Maloy KJ, Salaun L, Cahill R, Dougan G, Saunders NJ, Powrie F. CD4+CD25+ T(R) cells suppress innate immune pathology through cytokine-dependent mechanisms. J Exp Med 2003; 197:111-9.

Manel N, Unutmaz D, Littman DR. The differentiation of human T(H)-17 cells requires transforming growth factor-beta and induction of the nuclear receptor RORgammat. Nat Immunol 2008; 9:641-9.

Mangan PR, Harrington LE, O'Quinn DB, Helms WS, Bullard DC, Elson CO, et al. Transforming growth factor-beta induces development of the T(H)17 lineage. Nature 2006; 441:231-4.

Mannon PJ, Fuss IJ, Mayer L, Elson CO, Sandborn WJ, Present D, et al. Anti-interleukin-12 antibody for active Crohn's disease. N Engl J Med 2004; 351:2069-79.

Mannon PJ, Hornung RL, Yang Z, Yi C, Groden C, Friend J, et al. Suppression of inflammation in ulcerative colitis by interferon-β-1a is accompanied by inhibition of IL-13 production. Gut 2011; 60:449-55.

Mantis NJ, Cheung MC, Chintalacharuvu KR, Rey J, Corthésy B, Neutra MR. Selective adherence of IgA to murine Peyer's patch M cells: evidence for a novel IgA receptor. J Immunol 2002; 169:1844-51.

Marchiando AM, Shen L, Graham WV, Weber CR, Schwarz BT, Austin JR, et al. Caveolin-1-dependent occludin endocytosis is required for TNF-induced tight junction regulation in vivo. J Cell Biol 2010; 189:111-26.

Margueron R, Reinberg D. The Polycomb complex PRC2 and its mark in life. Nature 2011; 469:343-9.

Marie JC, Liggitt D, Rudensky AY. Cellular mechanisms of fatal early-onset autoimmunity in mice with the T cell-specific targeting of transforming growth factor-beta receptor. Immunity 2006; 25:441-54.

Marrack P, Bender J, Hildeman D, Jordan M, Mitchell T, Murakami M, et al. Homeostasis of alpha beta TCR+ T cells. Nat Immunol 2000; 1:107-11.

Maul J, Loddenkemper C, Mundt P, Berg E, Giese T, Stallmach A, et al. Peripheral and intestinal regulatory CD4+ CD25(high) T cells in inflammatory bowel disease. Gastroenterology 2005; 128:1868-78.

Maynard CL, Harrington LE, Janowski KM, Oliver JR, Zindl CL, Rudensky AY, et al. Regulatory T cells expressing interleukin 10 develop from Foxp3+ and Foxp3- precursor cells in the absence of interleukin 10. Nat Immunol 2007; 8:931-41.

Maynard CL, Harrington LE, Janowski KM, Oliver JR, Zindl CL, Rudensky AY, et al. Regulatory T cells expressing interleukin 10 develop from Foxp3+ and Foxp3- precursor cells in the absence of interleukin 10. Nat Immunol 2007; 8:931-41.

McCarroll SA, Huett A, Kuballa P, Chilewski SD, Landry A, Goyette P, et al. Deletion polymorphism upstream of IRGM associated with altered IRGM expression and Crohn's disease. Nat Genet 2008; 40:1107-12.

McDonald KG, McDonough JS, Wang C, Kucharzik T, Williams IR, Newberry RD. CC chemokine receptor 6 expression by B lymphocytes is essential for the development of isolated lymphoid follicles. Am J Pathol 2007; 170:1229-40.

Mebius RE, Miyamoto T, Christensen J, Domen J, Cupedo T, Weissman IL, et al. The fetal liver counterpart of adult common lymphoid progenitors gives rise to all lymphoid lineages, CD45+CD4+CD3- cells, as well as macrophages. J Immunol 2001; 166:6593-601.

Medzhitov R, Horng T. Transcriptional control of the inflammatory response. Nat Rev Immunol 2009; 9:692-703.

3Medzhitov R, Preston-Hurlburt P, Janeway CA. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. Nature 1997; 388:394-7.

Medzhitov R, Shevach EM, Trinchieri G, Mellor AL, Munn DH, Gordon S, et al. Highlights of 10 years of immunology in Nature Reviews Immunology. Nat Rev Immunol 2011; 11:693-702.

Meresse B, Ripoche J, Heyman M, Cerf-Bensussan N. Celiac disease: from oral tolerance to intestinal inflammation, autoimmunity and lymphomagenesis. Mucosal Immunol 2009; 2:8-23.

Meyer DM, Jesson MI, Li X, Elrick MM, Funckes-Shippy CL, Warner JD, et al. Anti-inflammatory activity and neutrophil reductions mediated by the JAK1/JAK3 inhibitor, CP-690,550, in rat adjuvant-induced arthritis. J Inflamm (Lond) 2010; 7:41.

Micheau O, Tschopp J. Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes. Cell 2003; 114:181-90.

Mielke LA, Jones SA, Raverdeau M, Higgs R, Stefanska A, Groom JR, et al. Retinoic acid expression associates with enhanced IL-22 production by γδ T cells and innate lymphoid cells and attenuation of intestinal inflammation. J Exp Med 2013; 210:1117-24.

Mikkelsen TS, Ku M, Jaffe DB, Issac B, Lieberman E, Giannoukos G, et al. Genome-wide maps of chromatin state in pluripotent and lineage-committed cells. Nature 2007; 448:553-60.

389. Miller SA, Mohn SE, Weinmann AS. Jmjd3 and UTX play a demethylase-independent role in chromatin remodeling to regulate T-box family member-dependent gene expression. Mol Cell 2010; 40:594-605.

Milling SW, Yrlid U, Jenkins C, Richards CM, Williams NA, MacPherson G. Regulation of intestinal immunity: effects of the oral adjuvant Escherichia coli heat-labile enterotoxin on migrating dendritic cells. Eur J Immunol 2007; 37:87-99.

Mishra N, Brown DR, Olorenshaw IM, Kammer GM. Trichostatin A reverses skewed expression of CD154, interleukin-10, and interferon-gamma gene and protein expression in lupus T cells. Proc Natl Acad Sci U S A 2001; 98:2628-33.

Miyamoto Y, Mabuchi A, Shi D, Kubo T, Takatori Y, Saito S, et al. A functional polymorphism in the 5' UTR of GDF5 is associated with susceptibility to osteoarthritis. Nat Genet 2007; 39:529-33.

Mizoguchi E, Mizoguchi A, Takedatsu H, Cario E, de Jong YP, Ooi CJ, et al. Role of tumor necrosis factor receptor 2 (TNFR2) in colonic epithelial hyperplasia and chronic intestinal inflammation in mice. Gastroenterology 2002; 122:134-44.

Moelants EA, Mortier A, Van Damme J, Proost P. Regulation of TNF-α with a focus on rheumatoid arthritis. Immunol Cell Biol 2013; 91:393-401.

Mogensen TH. Pathogen recognition and inflammatory signaling in innate immune defenses. Clin Microbiol Rev 2009; 22:240-73, Table of Contents.

Monteleone G, Biancone L, Marasco R, Morrone G, Marasco O, Luzza F, et al. Interleukin 12 is expressed and actively released by Crohn's disease intestinal lamina propria mononuclear cells. Gastroenterology 1997; 112:1169-78.

Monteleone G, Caruso R, Fina D, Peluso I, Gioia V, Stolfi C, et al. Control of matrix metalloproteinase production in human intestinal fibroblasts by interleukin 21. Gut 2006; 55:1774-80.
Monteleone G, Caruso R, Pallone F. Targets for new immunomodulation strategies in inflammatory bowel disease. Autoimmun Rev 2014; 13:11-4.

Monteleone G, Fantini MC, Onali S, Zorzi F, Sancesario G, Bernardini S, et al. Phase I clinical trial of Smad7 knockdown using antisense oligonucleotide in patients with active Crohn's disease. Mol Ther 2012; 20:870-6.

Monteleone G, Kumberova A, Croft NM, McKenzie C, Steer HW, MacDonald TT. Blocking Smad7 restores TGF-beta1 signaling in chronic inflammatory bowel disease. J Clin Invest 2001; 108:601-9.

Monteleone G, Monteleone I, Fina D, Vavassori P, Del Vecchio Blanco G, Caruso R, et al. Interleukin-21 enhances T-helper cell type I signaling and interferon-gamma production in Crohn's disease. Gastroenterology 2005; 128:687-94.

Monteleone G, Pallone F. Mongersen, an Oral SMAD7 Antisense Oligonucleotide, and Crohn's Disease. N Engl J Med 2015; 372:2461.

Monteleone I, Rizzo A, Sarra M, Sica G, Sileri P, Biancone L, et al. Aryl hydrocarbon receptor-induced signals up-regulate IL-22 production and inhibit inflammation in the gastrointestinal tract. Gastroenterology 2011; 141:237-48, 48.e1.

Morteau O, Gerard C, Lu B, Ghiran S, Rits M, Fujiwara Y, et al. An indispensable role for the chemokine receptor CCR10 in IgA antibody-secreting cell accumulation. J Immunol 2008; 181:6309-15.

Moser M, Murphy KM. Dendritic cell regulation of TH1-TH2 development. Nat Immunol 2000; 1:199-205.

Mosser DM, Edwards JP. Exploring the full spectrum of macrophage activation. Nat Rev Immunol 2008; 8:958-69.

Mowat AM. Anatomical basis of tolerance and immunity to intestinal antigens. Nat Rev Immunol 2003; 3:331-41.

Mucida D, Cheroutre H. TGFbeta and retinoic acid intersect in immune-regulation. Cell Adh Migr 2007; 1:142-4.

Mudter J, Amoussina L, Schenk M, Yu J, Brüstle A, Weigmann B, et al. The transcription factor IFN regulatory factor-4 controls experimental colitis in mice via T cell-derived IL-6. J Clin Invest 2008; 118:2415-26.

Muller PA, Koscsó B, Rajani GM, Stevanovic K, Berres ML, Hashimoto D, et al. Crosstalk between muscularis macrophages and enteric neurons regulates gastrointestinal motility. Cell 2014; 158:300-13.

Munday J, Floyd H, Crocker PR. Sialic acid binding receptors (siglecs) expressed by macrophages. J Leukoc Biol 1999; 66:705-11.

Murch SH, Braegger CP, Walker-Smith JA, MacDonald TT. Location of tumour necrosis factor alpha by immunohistochemistry in chronic inflammatory bowel disease. Gut 1993; 34:1705-9.

Műzes G, Molnár B, Tulassay Z, Sipos F. Changes of the cytokine profile in inflammatory bowel diseases. World J Gastroenterol 2012; 18:5848-61.

Nagalakshmi ML, Murphy E, McClanahan T, de Waal Malefyt R. Expression patterns of IL-10 ligand and receptor gene families provide leads for biological characterization. Int Immunopharmacol 2004; 4:577-92.

Natoli G. Maintaining cell identity through global control of genomic organization. Immunity 2010; 33:12-24.
Naughton C, Sproul D, Hamilton C, Gilbert N. Analysis of active and inactive X chromosome architecture reveals the independent organization of 30 nm and large-scale chromatin structures. Mol Cell 2010; 40:397-409.

Neumann B, Luz A, Pfeffer K, Holzmann B. Defective Peyer's patch organogenesis in mice lacking the 55-kD receptor for tumor necrosis factor. J Exp Med 1996; 184:259-64.

Neurath MF. Cytokines in inflammatory bowel disease. Nat Rev Immunol 2014; 14:329-42.

Neurath MF, Fuss I, Kelsall BL, Stüber E, Strober W. Antibodies to interleukin 12 abrogate established experimental colitis in mice. J Exp Med 1995; 182:1281-90.

Neurath MF, Weigmann B, Finotto S, Glickman J, Nieuwenhuis E, Iijima H, et al. The transcription factor T-bet regulates mucosal T cell activation in experimental colitis and Crohn's disease. J Exp Med 2002; 195:1129-43.

Neutra MR, Mantis NJ, Kraehenbuhl JP. Collaboration of epithelial cells with organized mucosal lymphoid tissues. Nat Immunol 2001; 2:1004-9.

Ng SC, Benjamin JL, McCarthy NE, Hedin CR, Koutsoumpas A, Plamondon S, et al. Relationship between human intestinal dendritic cells, gut microbiota, and disease activity in Crohn's disease. Inflamm Bowel Dis 2011; 17:2027-37.

Ng SC, Tsoi KK, Kamm MA, Xia B, Wu J, Chan FK, et al. Genetics of inflammatory bowel disease in Asia: systematic review and meta-analysis. Inflamm Bowel Dis 2012; 18:1164-76.

Niess JH, Brand S, Gu X, Landsman L, Jung S, McCormick BA, et al. CX3CR1-mediated dendritic cell access to the intestinal lumen and bacterial clearance. Science 2005; 307:254-8.

Nishimura M, Kuboi Y, Muramoto K, Kawano T, Imai T. Chemokines as novel therapeutic targets for inflammatory bowel disease. Ann N Y Acad Sci 2009; 1173:350-6.

Noguchi E, Homma Y, Kang X, Netea MG, Ma X. A Crohn's disease-associated NOD2 mutation suppresses transcription of human IL10 by inhibiting activity of the nuclear ribonucleoprotein hnRNP-A1. Nat Immunol 2009; 10:471-9.

O'Carroll C, Fagan A, Shanahan F, Carmody RJ. Identification of a unique hybrid macrophage-polarization state following recovery from lipopolysaccharide tolerance. J Immunol 2014; 192:427-36.

O'Connor W, Kamanaka M, Booth CJ, Town T, Nakae S, Iwakura Y, et al. A protective function for interleukin 17A in T cell-mediated intestinal inflammation. Nat Immunol 2009; 10:603-9.

O'Shea JJ, Murray PJ. Cytokine signaling modules in inflammatory responses. Immunity 2008; 28:477-87.

Odes HS, Fich A, Reif S, Halak A, Lavy A, Keter D, et al. Effects of current cigarette smoking on clinical course of Crohn's disease and ulcerative colitis. Dig Dis Sci 2001; 46:1717-21.

Ogino T, Nishimura J, Barman S, Kayama H, Uematsu S, Okuzaki D, et al. Increased Th17-inducing activity of CD14+ CD163 low myeloid cells in intestinal lamina propria of patients with Crohn's disease. Gastroenterology 2013; 145:1380-91.e1.

Ogura Y, Bonen DK, Inohara N, Nicolae DL, Chen FF, Ramos R, et al. A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. Nature 2001; 411:603-6.

Ogura Y, Lala S, Xin W, Smith E, Dowds TA, Chen FF, et al. Expression of NOD2 in Paneth cells: a possible link to Crohn's ileitis. Gut 2003; 52:1591-7.

Okano M, Yamamoto H, Ohkuma H, Kano Y, Kim H, Nishikawa S, et al. Significance of INHBA expression in human colorectal cancer. Oncol Rep 2013; 30:2903-8.

Olsen T, Goll R, Cui G, Husebekk A, Vonen B, Birketvedt GS, et al. Tissue levels of tumor necrosis factor-alpha correlates with grade of inflammation in untreated ulcerative colitis. Scand J Gastroenterol 2007; 42:1312-20.

Olszak T, An D, Zeissig S, Vera MP, Richter J, Franke A, et al. Microbial exposure during early life has persistent effects on natural killer T cell function. Science 2012; 336:489-93.

Ong CT, Corces VG. Enhancer function: new insights into the regulation of tissue-specific gene expression. Nat Rev Genet 2011; 12:283-93.

Onizawa M, Nagaishi T, Kanai T, Nagano K, Oshima S, Nemoto Y, et al. Signaling pathway via TNF-alpha/NF-kappaB in intestinal epithelial cells may be directly involved in colitis-associated carcinogenesis. Am J Physiol Gastrointest Liver Physiol 2009; 296:G850-9.

Onoda T, Rahman M, Nara H, Araki A, Makabe K, Tsumoto K, et al. Human CD4+ central and effector memory T cells produce IL-21: effect on cytokine-driven proliferation of CD4+ T cell subsets. Int Immunol 2007; 19:1191-9.

Orenstein JM, Fox C, Wahl SM. Macrophages as a source of HIV during opportunistic infections. Science 1997; 276:1857-61.

Orholm M, Binder V, Sørensen TI, Rasmussen LP, Kyvik KO. Concordance of inflammatory bowel disease among Danish twins. Results of a nationwide study. Scand J Gastroenterol 2000; 35:1075-81.

Otte JM, Cario E, Podolsky DK. Mechanisms of cross hyporesponsiveness to Toll-like receptor bacterial ligands in intestinal epithelial cells. Gastroenterology 2004; 126:1054-70.

Pabst O, Bernhardt G, Förster R. The impact of cell-bound antigen transport on mucosal tolerance induction. J Leukoc Biol 2007; 82:795-800.

Pabst O, Herbrand H, Friedrichsen M, Velaga S, Dorsch M, Berhardt G, et al. Adaptation of solitary intestinal lymphoid tissue in response to microbiota and chemokine receptor CCR7 signaling. J Immunol 2006; 177:6824-32.

Pabst O, Mowat AM. Oral tolerance to food protein. Mucosal Immunol 2012; 5:232-9.

Pachynski RK, Wu SW, Gunn MD, Erle DJ. Secondary lymphoid-tissue chemokine (SLC) stimulates integrin alpha 4 beta 7-mediated adhesion of lymphocytes to mucosal addressin cell adhesion molecule-1 (MAdCAM-1) under flow. J Immunol 1998; 161:952-6.

Pan G, Tian S, Nie J, Yang C, Ruotti V, Wei H, et al. Whole-genome analysis of histone H3 lysine 4 and lysine 27 methylation in human embryonic stem cells. Cell Stem Cell 2007; 1:299-312.

Pan J, Kunkel EJ, Gosslar U, Lazarus N, Langdon P, Broadwell K, et al. A novel chemokine ligand for CCR10 and CCR3 expressed by epithelial cells in mucosal tissues. J Immunol 2000; 165:2943-9.

Papadakis KA, Prehn J, Moreno ST, Cheng L, Kouroumalis EA, Deem R, et al. CCR9-positive lymphocytes and thymus-expressed chemokine distinguish small bowel from colonic Crohn's disease. Gastroenterology 2001; 121:246-54.

Parameswaran N, Patial S. Tumor necrosis factor-α signaling in macrophages. Crit Rev Eukaryot

Gene Expr 2010; 20:87-103.

Park SY, Saijo K, Takahashi T, Osawa M, Arase H, Hirayama N, et al. Developmental defects of lymphoid cells in Jak3 kinase-deficient mice. Immunity 1995; 3:771-82.

Parkes M, Barrett JC, Prescott NJ, Tremelling M, Anderson CA, Fisher SA, et al. Sequence variants in the autophagy gene IRGM and multiple other replicating loci contribute to Crohn's disease susceptibility. Nat Genet 2007; 39:830-2.

Parkes M, Cortes A, van Heel DA, Brown MA. Genetic insights into common pathways and complex relationships among immune-mediated diseases. Nat Rev Genet 2013; 14:661-73.

Parrello T, Monteleone G, Cucchiara S, Monteleone I, Sebkova L, Doldo P, et al. Up-regulation of the IL-12 receptor beta 2 chain in Crohn's disease. J Immunol 2000; 165:7234-9.

Parrish-Novak J, Dillon SR, Nelson A, Hammond A, Sprecher C, Gross JA, et al. Interleukin 21 and its receptor are involved in NK cell expansion and regulation of lymphocyte function. Nature 2000; 408:57-63.

Peduto L, Dulauroy S, Lochner M, Späth GF, Morales MA, Cumano A, et al. Inflammation recapitulates the ontogeny of lymphoid stromal cells. J Immunol 2009; 182:5789-99.

Pereira F, Barbáchano A, Silva J, Bonilla F, Campbell MJ, Muñoz A, et al. KDM6B/JMJD3 histone demethylase is induced by vitamin D and modulates its effects in colon cancer cells. Hum Mol Genet 2011; 20:4655-65.

Pérez-Oliva AB, Martínez-Esparza M, Vicente-Fernández JJ, Corral-San Miguel R, García-Peñarrubia P, Hernández-Caselles T. Epitope mapping, expression and post-translational modifications of two isoforms of CD33 (CD33M and CD33m) on lymphoid and myeloid human cells. Glycobiology 2011; 21:757-70.

Peters AH, Kubicek S, Mechtler K, O'Sullivan RJ, Derijck AA, Perez-Burgos L, et al. Partitioning and plasticity of repressive histone methylation states in mammalian chromatin. Mol Cell 2003; 12:1577-89.

Peterson LW, Artis D. Intestinal epithelial cells: regulators of barrier function and immune homeostasis. Nat Rev Immunol 2014; 14:141-53.

Petnicki-Ocwieja T, Hrncir T, Liu YJ, Biswas A, Hudcovic T, Tlaskalova-Hogenova H, et al. Nod2 is required for the regulation of commensal microbiota in the intestine. Proc Natl Acad Sci U S A 2009; 106:15813-8.

Pickert G, Neufert C, Leppkes M, Zheng Y, Wittkopf N, Warntjen M, et al. STAT3 links IL-22 signaling in intestinal epithelial cells to mucosal wound healing. J Exp Med 2009; 206:1465-72.

Pizarro TT, Michie MH, Bentz M, Woraratanadharm J, Smith MF, Foley E, et al. IL-18, a novel immunoregulatory cytokine, is up-regulated in Crohn's disease: expression and localization in intestinal mucosal cells. J Immunol 1999; 162:6829-35.

Platt AM, Bain CC, Bordon Y, Sester DP, Mowat AM. An independent subset of TLR expressing CCR2-dependent macrophages promotes colonic inflammation. J Immunol 2010; 184:6843-54.

Podolsky DK. Inflammatory bowel disease. N Engl J Med 2002; 347:417-29.

467. Poindexter NJ, Walch ET, Chada S, Grimm EA. Cytokine induction of interleukin-24 in human peripheral blood mononuclear cells. J Leukoc Biol 2005; 78:745-52.

Powrie F, Carlino J, Leach MW, Mauze S, Coffman RL. A critical role for transforming growth factor-beta but not interleukin 4 in the suppression of T helper type 1-mediated colitis by CD45RB(low) CD4+ T cells. J Exp Med 1996; 183:2669-74.

Powrie F, Leach MW, Mauze S, Menon S, Caddle LB, Coffman RL. Inhibition of Th1 responses prevents inflammatory bowel disease in scid mice reconstituted with CD45RBhi CD4+ T cells. Immunity 1994; 1:553-62.

Ramirez-Carrozzi VR, Braas D, Bhatt DM, Cheng CS, Hong C, Doty KR, et al. A unifying model for the selective regulation of inducible transcription by CpG islands and nucleosome remodeling. Cell 2009; 138:114-28.

Ramprasad MP, Terpstra V, Kondratenko N, Quehenberger O, Steinberg D. 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 1996; 93:14833-8.

Randolph GJ, Inaba K, Robbiani DF, Steinman RM, Muller WA. Differentiation of phagocytic monocytes into lymph node dendritic cells in vivo. Immunity 1999; 11:753-61.

Randolph GJ, Sanchez-Schmitz G, Liebman RM, Schäkel K. The CD16(+) (FcgammaRIII(+)) subset of human monocytes preferentially becomes migratory dendritic cells in a model tissue setting. J Exp Med 2002; 196:517-27.

Randow F, Döcke WD, Bundschuh DS, Hartung T, Wendel A, Volk HD. In vitro prevention and reversal of lipopolysaccharide desensitization by IFN-gamma, IL-12, and granulocyte-macrophage colony-stimulating factor. J Immunol 1997; 158:2911-8.

Rangnekar VM, Aplin AC, Sukhatme VP. The serum and TPA responsive promoter and intron-exon structure of EGR2, a human early growth response gene encoding a zinc finger protein. Nucleic Acids Res 1990; 18:2749-57.

Rani R, Smulian AG, Greaves DR, Hogan SP, Herbert DR. TGF-β limits IL-33 production and promotes the resolution of colitis through regulation of macrophage function. Eur J Immunol 2011; 41:2000-9.

Rath T, Roderfeld M, Blöcher S, Rhode A, Basler T, Akineden Ö, et al. Presence of intestinal Mycobacterium avium subspecies paratuberculosis (MAP) DNA is not associated with altered MMP expression in ulcerative colitis. BMC Gastroenterol 2011; 11:34.

Reinecker HC, Steffen M, Witthoeft T, Pflueger I, Schreiber S, MacDermott RP, et al. Enhanced secretion of tumour necrosis factor-alpha, IL-6, and IL-1 beta by isolated lamina propria mononuclear cells from patients with ulcerative colitis and Crohn's disease. Clin Exp Immunol 1993; 94:174-81.

Reinisch W, de Villiers W, Bene L, Simon L, Rácz I, Katz S, et al. Fontolizumab in moderate to severe Crohn's disease: a phase 2, randomized, double-blind, placebo-controlled, multiple-dose study. Inflamm Bowel Dis 2010; 16:233-42.

Reinisch W, Hommes DW, Van Assche G, Colombel JF, Gendre JP, Oldenburg B, et al. A dose escalating, placebo controlled, double blind, single dose and multidose, safety and tolerability study of fontolizumab, a humanised anti-interferon gamma antibody, in patients with moderate to severe Crohn's disease. Gut 2006; 55:1138-44.

Reinisch W, Panés J, Khurana S, Toth G, Hua F, Comer GM, et al. Anrukinzumab, an anti-interleukin 13 monoclonal antibody, in active UC: efficacy and safety from a phase IIa randomised multicentre study. Gut 2015; 64:894-900.

Rennert PD, Browning JL, Mebius R, Mackay F, Hochman PS. Surface lymphotoxin alpha/beta complex is required for the development of peripheral lymphoid organs. J Exp Med 1996; 184:1999-2006.

Rescigno M. The intestinal epithelial barrier in the control of homeostasis and immunity. Trends Immunol 2011; 32:256-64.

Rescigno M, Di Sabatino A. Dendritic cells in intestinal homeostasis and disease. J Clin Invest 2009; 119:2441-50.

Rescigno M, Urbano M, Valzasina B, Francolini M, Rotta G, Bonasio R, et al. Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. Nat Immunol 2001; 2:361-7.

Rimoldi M, Chieppa M, Salucci V, Avogadri F, Sonzogni A, Sampietro GM, et al. Intestinal immune homeostasis is regulated by the crosstalk between epithelial cells and dendritic cells. Nat Immunol 2005; 6:507-14.

Rivera-Nieves J, Ho J, Bamias G, Ivashkina N, Ley K, Oppermann M, et al. Antibody blockade of CCL25/CCR9 ameliorates early but not late chronic murine ileitis. Gastroenterology 2006; 131:1518-29.

Rodríguez M, Cabal-Hierro L, Carcedo MT, Iglesias JM, Artime N, Darnay BG, et al. NF-kappaB signal triggering and termination by tumor necrosis factor receptor 2. J Biol Chem 2011; 286:22814-24.

Rogler G, Hausmann M, Vogl D, Aschenbrenner E, Andus T, Falk W, et al. Isolation and phenotypic characterization of colonic macrophages. Clin Exp Immunol 1998; 112:205-15.

Romagnani S. Lymphokine production by human T cells in disease states. Annu Rev Immunol 1994; 12:227-57.

Rothe M, Wong SC, Henzel WJ, Goeddel DV. A novel family of putative signal transducers associated with the cytoplasmic domain of the 75 kDa tumor necrosis factor receptor. Cell 1994; 78:681-92.

Rovedatti L, Kudo T, Biancheri P, Sarra M, Knowles CH, Rampton DS, et al. Differential regulation of interleukin 17 and interferon gamma production in inflammatory bowel disease. Gut 2009; 58:1629-36.

Rückert R, Bulfone-Paus S, Brandt K. Interleukin-21 stimulates antigen uptake, protease activity, survival and induction of CD4+ T cell proliferation by murine macrophages. Clin Exp Immunol 2008; 151:487-95.

Rugtveit J, Nilsen EM, Bakka A, Carlsen H, Brandtzaeg P, Scott H. Cytokine profiles differ in newly recruited and resident subsets of mucosal macrophages from inflammatory bowel disease. Gastroenterology 1997; 112:1493-505.

Sahoo A, Lee CG, Jash A, Son JS, Kim G, Kwon HK, et al. Stat6 and c-Jun mediate Th2 cell-specific IL-24 gene expression. J Immunol 2011; 186:4098-109.

Saitoh T, Fujita N, Jang MH, Uematsu S, Yang BG, Satoh T, et al. Loss of the autophagy protein Atg16L1 enhances endotoxin-induced IL-1beta production. Nature 2008; 456:264-8.

Sakuraba A, Sato T, Kamada N, Kitazume M, Sugita A, Hibi T. Th1/Th17 immune response is induced by mesenteric lymph node dendritic cells in Crohn's disease. Gastroenterology 2009; 137:1736-45.

Sandborn WJ, Colombel JF, Enns R, Feagan BG, Hanauer SB, Lawrance IC, et al. Natalizumab induction and maintenance therapy for Crohn's disease. N Engl J Med 2005; 353:1912-25.

Sandborn WJ, Feagan BG, Fedorak RN, Scherl E, Fleisher MR, Katz S, et al. A randomized trial of Ustekinumab, a human interleukin-12/23 monoclonal antibody, in patients with moderate-to-severe Crohn's disease. Gastroenterology 2008; 135:1130-41.

Sandborn WJ, Gasink C, Gao LL, Blank MA, Johanns J, Guzzo C, et al. Ustekinumab induction and maintenance therapy in refractory Crohn's disease. N Engl J Med 2012; 367:1519-28.

Sandborn WJ, Ghosh S, Panes J, Vranic I, Su C, Rousell S, et al. Tofacitinib, an oral Janus kinase inhibitor, in active ulcerative colitis. N Engl J Med 2012; 367:616-24.

Sandborn WJ, Hanauer SB, Katz S, Safdi M, Wolf DG, Baerg RD, et al. Etanercept for active Crohn's disease: a randomized, double-blind, placebo-controlled trial. Gastroenterology 2001; 121:1088-94.

Sandborn WJ, Yednock TA. Novel approaches to treating inflammatory bowel disease: targeting alpha-4 integrin. Am J Gastroenterol 2003; 98:2372-82.

Sarra M, Monteleone G. Interleukin-21: a new mediator of inflammation in systemic lupus erythematosus. J Biomed Biotechnol 2010; 2010:294582.

Sarra M, Monteleone I, Stolfi C, Fantini MC, Sileri P, Sica G, et al. Interferon-gamma-expressing cells are a major source of interleukin-21 in inflammatory bowel diseases. Inflamm Bowel Dis 2010; 16:1332-9.

Sarra M, Pallone F, Macdonald TT, Monteleone G. IL-23/IL-17 axis in IBD. Inflamm Bowel Dis 2010; 16:1808-13.

Sasaki M, Jordan P, Welbourne T, Minagar A, Joh T, Itoh M, et al. Troglitazone, a PPAR-gamma activator prevents endothelial cell adhesion molecule expression and lymphocyte adhesion mediated by TNF-alpha. BMC Physiol 2005; 5:3.

Satoh T, Takeuchi O, Vandenbon A, Yasuda K, Tanaka Y, Kumagai Y, et al. The Jmjd3-Irf4 axis regulates M2 macrophage polarization and host responses against helminth infection. Nat Immunol 2010; 11:936-44.

Satoh-Takayama N, Vosshenrich CA, Lesjean-Pottier S, Sawa S, Lochner M, Rattis F, et al. Microbial flora drives interleukin 22 production in intestinal NKp46+ cells that provide innate mucosal immune defense. Immunity 2008; 29:958-70.

Scallon BJ, Moore MA, Trinh H, Knight DM, Ghrayeb J. Chimeric anti-TNF-alpha monoclonal antibody cA2 binds recombinant transmembrane TNF-alpha and activates immune effector functions. Cytokine 1995; 7:251-9.

Scarpa M, Stylianou E. Epigenetics: Concepts and relevance to IBD pathogenesis. Inflamm Bowel Dis 2012; 18:1982-96.

Schaible TD, Harris RA, Dowd SE, Smith CW, Kellermayer R. Maternal methyl-donor supplementation induces prolonged murine offspring colitis susceptibility in association with mucosal epigenetic and microbiomic changes. Hum Mol Genet 2011; 20:1687-96.

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

Schieferdecker HL, Ullrich R, Hirseland H, Zeitz M. T cell differentiation antigens on lymphocytes in the human intestinal lamina propria. J Immunol 1992; 149:2816-22.

Schluns KS, Lefrançois L. Cytokine control of memory T-cell development and survival. Nat Rev Immunol 2003; 3:269-79.

Schmolka N, Serre K, Grosso AR, Rei M, Pennington DJ, Gomes AQ, et al. Epigenetic and transcriptional signatures of stable versus plastic differentiation of proinflammatory γδ T cell subsets. Nat Immunol 2013; 14:1093-100.

Schmeck B, Lorenz J, N'Guessan PD, Opitz B, van Laak V, Zahlten J et al., Histone acetylation and flallin are essential for *Legionella pneumophila*-induced cytokine expression. J immunol 2008;181:940-947.

Schuettengruber B, Cavalli G. Recruitment of polycomb group complexes and their role in the dynamic regulation of cell fate choice. Development 2009; 136:3531-42.

Schulz O, Jaensson E, Persson EK, Liu X, Worbs T, Agace WW, et al. Intestinal CD103+, but not CX3CR1+, antigen sampling cells migrate in lymph and serve classical dendritic cell functions. J Exp Med 2009; 206:3101-14.

Scott CL, Aumeunier AM, Mowat AM. Intestinal CD103+ dendritic cells: master regulators of tolerance? Trends Immunol 2011; 32:412-9.

Scott CL, Bain CC, Wright PB, Sichien D, Kotarsky K, Persson EK, et al. CCR2(+)CD103(-) intestinal dendritic cells develop from DC-committed precursors and induce interleukin-17 production by T cells. Mucosal Immunol 2015; 8:327-39.

Seder RA, Paul WE. Acquisition of lymphokine-producing phenotype by CD4+ T cells. Annu Rev Immunol 1994; 12:635-73.

Sen R, Grosschedl R. Memories of lost enhancers. Genes Dev 2010; 24:973-9.

Serbina NV, Jia T, Hohl TM, Pamer EG. Monocyte-mediated defense against microbial pathogens. Annu Rev Immunol 2008; 26:421-52.

Serrador JM, Cabrero JR, Sancho D, Mittelbrunn M, Urzainqui A, Sánchez-Madrid F. HDAC6 deacetylase activity links the tubulin cytoskeleton with immune synapse organization. Immunity 2004; 20:417-28.

Sewgobind VD, Quaedackers ME, van der Laan LJ, Kraaijeveld R, Korevaar SS, Chan G, et al. The Jak inhibitor CP-690,550 preserves the function of CD4CD25FoxP3 regulatory T cells and inhibits effector T cells. Am J Transplant 2010; 10:1785-95.

Shaw MH, Reimer T, Sánchez-Valdepeñas C, Warner N, Kim YG, Fresno M, et al. T cell-intrinsic role of Nod2 in promoting type 1 immunity to Toxoplasma gondii. Nat Immunol 2009; 10:1267-74.

Sheikh SZ, Plevy SE. The role of the macrophage in sentinel responses in intestinal immunity. Curr Opin Gastroenterol 2010; 26:578-82.

Shi C, Pamer EG. Monocyte recruitment during infection and inflammation. Nat Rev Immunol 2011; 11:762-74.

Shi Y. Histone lysine demethylases: emerging roles in development, physiology and disease. Nat Rev Genet 2007; 8:829-33.

Shi Y, Lan F, Matson C, Mulligan P, Whetstine JR, Cole PA, et al. Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. Cell 2004; 119:941-53.

Shih DQ, Targan SR. Immunopathogenesis of inflammatory bowel disease. World J Gastroenterol 2008; 14:390-400.

Shivananda S, Lennard-Jones J, Logan R, Fear N, Price A, Carpenter L, et al. Incidence of inflammatory bowel disease across Europe: is there a difference between north and south? Results of the European Collaborative Study on Inflammatory Bowel Disease (EC-IBD). Gut 1996; 39:690-7.

Siakavellas SI, Bamias G. Role of the IL-23/IL-17 axis in Crohn's disease. Discov Med 2012; 14:253-62.

Siegmund B, Fantuzzi G, Rieder F, Gamboni-Robertson F, Lehr HA, Hartmann G, et al. Neutralization of interleukin-18 reduces severity in murine colitis and intestinal IFN-gamma and TNF-alpha production. Am J Physiol Regul Integr Comp Physiol 2001; 281:R1264-73.

Sieweke MH, Graf T. A transcription factor party during blood cell differentiation. Curr Opin Genet Dev 1998; 8:545-51.

Sigmundsdottir H, Butcher EC. Environmental cues, dendritic cells and the programming of tissue-selective lymphocyte trafficking. Nat Immunol 2008; 9:981-7.

Simon JA, Kingston RE. Mechanisms of polycomb gene silencing: knowns and unknowns. Nat Rev Mol Cell Biol 2009; 10:697-708.

Smith ER, Lee MG, Winter B, Droz NM, Eissenberg JC, Shiekhattar R, et al. Drosophila UTX is a histone H3 Lys27 demethylase that colocalizes with the elongating form of RNA polymerase II. Mol Cell Biol 2008; 28:1041-6.

Smith PD, Ochsenbauer-Jambor C, Smythies LE. Intestinal macrophages: unique effector cells of the innate immune system. Immunol Rev 2005; 206:149-59.

Smith PD, Smythies LE, Mosteller-Barnum M, Sibley DA, Russell MW, Merger M, et al. Intestinal macrophages lack CD14 and CD89 and consequently are down-regulated for LPS- and IgA-mediated activities. J Immunol 2001; 167:2651-6.

Smith PD, Smythies LE, Shen R, Greenwell-Wild T, Gliozzi M, Wahl SM. Intestinal macrophages and response to microbial encroachment. Mucosal Immunol 2011; 4:31-42.

Smith PM, Howitt MR, Panikov N, Michaud M, Gallini CA, Bohlooly-Y M, et al. The microbial metabolites, short-chain fatty acids, regulate colonic Treg cell homeostasis. Science 2013; 341:569-73.

Smythies LE, Maheshwari A, Clements R, Eckhoff D, Novak L, Vu HL, et al. Mucosal IL-8 and TGF-beta recruit blood monocytes: evidence for cross-talk between the lamina propria stroma and myeloid cells. J Leukoc Biol 2006; 80:492-9.

Smythies LE, Sellers M, Clements RH, Mosteller-Barnum M, Meng G, Benjamin WH, et al. Human intestinal macrophages display profound inflammatory anergy despite avid phagocytic and bacteriocidal activity. J Clin Invest 2005; 115:66-75.

Smythies LE, Shen R, Bimczok D, Novak L, Clements RH, Eckhoff DE, et al. Inflammation anergy in human intestinal macrophages is due to Smad-induced IkappaBalpha expression and NF-kappaB inactivation. J Biol Chem 2010; 285:19593-604.

Smythies LE, Wahl LM, Smith PD. Isolation and purification of human intestinal macrophages. Curr Protoc Immunol 2006; Chapter 7:Unit 7.6B.

Soler Palacios B, Estrada-Capetillo L, Izquierdo E, Criado G, Nieto C, Municio C, et al. Macrophages from the synovium of active rheumatoid arthritis exhibit an activin A-dependent pro-inflammatory profile. J Pathol 2015; 235:515-26.

Song L, Lee C, Schindler C. Deletion of the murine scavenger receptor CD68. J Lipid Res 2011; 52:1542-50.

Sonnenberg GF, Fouser LA, Artis D. Border patrol: regulation of immunity, inflammation and tissue homeostasis at barrier surfaces by IL-22. Nat Immunol 2011; 12:383-90.

Sonnenburg JL, Angenent LT, Gordon JI. Getting a grip on things: how do communities of bacterial symbionts become established in our intestine? Nat Immunol 2004; 5:569-73.

Souza HS, Elia CC, Spencer J, MacDonald TT. Expression of lymphocyte-endothelial receptor-ligand pairs, alpha4beta7/MAdCAM-1 and OX40/OX40 ligand in the colon and jejunum of patients with inflammatory bowel disease. Gut 1999; 45:856-63.

Spadoni I, Iliev ID, Rossi G, Rescigno M. Dendritic cells produce TSLP that limits the differentiation

of Th17 cells, fosters Treg development, and protects against colitis. Mucosal Immunol 2012; 5:184-93.
Spahn TW, Weiner HL, Rennert PD, Lügering N, Fontana A, Domschke W, et al. Mesenteric lymph nodes are critical for the induction of high-dose oral tolerance in the absence of Peyer's patches. Eur J Immunol 2002; 32:1109-13.

Spehlmann ME, Begun AZ, Burghardt J, Lepage P, Raedler A, Schreiber S. Epidemiology of inflammatory bowel disease in a German twin cohort: results of a nationwide study. Inflamm Bowel Dis 2008; 14:968-76.

Spencer J, Isaacson PG, MacDonald TT, Thomas AJ, Walker-Smith JA. Gamma/delta T cells and the diagnosis of coeliac disease. Clin Exp Immunol 1991; 85:109-13.

Spolski R, Kim HP, Zhu W, Levy DE, Leonard WJ. IL-21 mediates suppressive effects via its induction of IL-10. J Immunol 2009; 182:2859-67.

Stagg AJ, Kamm MA, Knight SC. Intestinal dendritic cells increase T cell expression of alpha4beta7 integrin. Eur J Immunol 2002; 32:1445-54.

Stappenbeck TS, Rioux JD, Mizoguchi A, Saitoh T, Huett A, Darfeuille-Michaud A, et al. Crohn disease: a current perspective on genetics, autophagy and immunity. Autophagy 2011; 7:355-74.

Stefater JA, Ren S, Lang RA, Duffield JS. Metchnikoff's policemen: macrophages in development, homeostasis and regeneration. Trends Mol Med 2011; 17:743-52.

Stephensen CB. Vitamin A, infection, and immune function. Annu Rev Nutr 2001; 21:167-92.

Sterner DE, Berger SL. Acetylation of histones and transcription-related factors. Microbiol Mol Biol Rev 2000; 64:435-59.

Stock A, Booth S, Cerundolo V. Prostaglandin E2 suppresses the differentiation of retinoic acid-producing dendritic cells in mice and humans. J Exp Med 2011; 208:761-73.

Stock A, Napolitani G, Cerundolo V. Intestinal DC in migrational imprinting of immune cells. Immunol Cell Biol 2013; 91:240-9.

Strahl BD, Allis CD. The language of covalent histone modifications. Nature 2000; 403:41-5.

Strober W, Fuss IJ, Blumberg RS. The immunology of mucosal models of inflammation. Annu Rev Immunol 2002; 20:495-549.

Stylianou E. Epigenetics: the fine-tuner in inflammatory bowel disease? Curr Opin Gastroenterol 2013; 29:370-7.

Sugimoto K, Ogawa A, Mizoguchi E, Shimomura Y, Andoh A, Bhan AK, et al. IL-22 ameliorates intestinal inflammation in a mouse model of ulcerative colitis. J Clin Invest 2008; 118:534-44.

5Sullivan KE, Reddy AB, Dietzmann K, Suriano AR, Kocieda VP, Stewart M, et al. Epigenetic regulation of tumor necrosis factor alpha. Mol Cell Biol 2007; 27:5147-60.

Sun L, Chen ZJ. The novel functions of ubiquitination in signaling. Curr Opin Cell Biol 2004; 16:119-26.

Sun X, Shibata K, Yamada H, Guo Y, Muta H, Podack ER, et al. CD30L/CD30 is critical for maintenance of IL-17A-producing γδ T cells bearing Vγ6 in mucosa-associated tissues in mice. Mucosal Immunol 2013; 6:1191-201.

Sun Z, Unutmaz D, Zou YR, Sunshine MJ, Pierani A, Brenner-Morton S, et al. Requirement for RORgamma in thymocyte survival and lymphoid organ development. Science 2000; 288:2369-73.

Sunderkötter C, Nikolic T, Dillon MJ, Van Rooijen N, Stehling M, Drevets DA, et al. Subpopulations of mouse blood monocytes differ in maturation stage and inflammatory response. J Immunol 2004; 172:4410-7.

Suzuki K, Fagarasan S. Diverse regulatory pathways for IgA synthesis in the gut. Mucosal Immunol 2009; 2:468-71.

Swidsinski A, Loening-Baucke V, Theissig F, Engelhardt H, Bengmark S, Koch S, et al. Comparative study of the intestinal mucus barrier in normal and inflamed colon. Gut 2007; 56:343-50.

Swigut T, Wysocka J. H3K27 demethylases, at long last. Cell 2007; 131:29-32.
Swirski FK, Nahrendorf M, Etzrodt M, Wildgruber M, Cortez-Retamozo V, Panizzi P, et al. Identification of splenic reservoir monocytes and their deployment to inflammatory sites. Science 2009; 325:612-6.

Szabo SJ, Sullivan BM, Stemmann C, Satoskar AR, Sleckman BP, Glimcher LH. Distinct effects of T-bet in TH1 lineage commitment and IFN-gamma production in CD4 and CD8 T cells. Science 2002; 295:338-42.

Takahashi K, Sugi Y, Nakano K, Tsuda M, Kurihara K, Hosono A, et al. Epigenetic control of the host gene by commensal bacteria in large intestinal epithelial cells. J Biol Chem 2011; 286:35755-62.

Takatori H, Kanno Y, Watford WT, Tato CM, Weiss G, Ivanov II, et al. Lymphoid tissue inducer-like cells are an innate source of IL-17 and IL-22. J Exp Med 2009; 206:35-41.

Takeda K, Akira S. Toll receptors and pathogen resistance. Cell Microbiol 2003; 5:143-53.

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

Takeuchi O, Hoshino K, Kawai T, Sanjo H, Takada H, Ogawa T, et al. Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. Immunity 1999; 11:443-51.

Tamoutounour S, Henri S, Lelouard H, de Bovis B, de Haar C, van der Woude CJ, et al. 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 2012; 42:3150-66.

Tan M, Luo H, Lee S, Jin F, Yang JS, Montellier E, et al. Identification of 67 histone marks and histone lysine crotonylation as a new type of histone modification. Cell 2011; 146:1016-28.

Tao R, de Zoeten EF, Ozkaynak E, Chen C, Wang L, Porrett PM, et al. Deacetylase inhibition promotes the generation and function of regulatory T cells. Nat Med 2007; 13:1299-307.

Tarakhovsky A. Tools and landscapes of epigenetics. Nat Immunol 2010; 11:565-8.

Tartaglia LA, Pennica D, Goeddel DV. Ligand passing: the 75-kDa tumor necrosis factor (TNF) receptor recruits TNF for signaling by the 55-kDa TNF receptor. J Biol Chem 1993; 268:18542-8.

Taupin DR, Kinoshita K, Podolsky DK. Intestinal trefoil factor confers colonic epithelial resistance to apoptosis. Proc Natl Acad Sci U S A 2000; 97:799-804.

Taylor JM, Wicks K, Vandiedonck C, Knight JC. Chromatin profiling across the human tumour necrosis factor gene locus reveals a complex, cell type-specific landscape with novel regulatory elements. Nucleic Acids Res 2008; 36:4845-62.

Teles RM, Teles RB, Amadeu TP, Moura DF, Mendonça-Lima L, Ferreira H, et al. High matrix metalloproteinase production correlates with immune activation and leukocyte migration in leprosy reactional lesions. Infect Immun 2010; 78:1012-21.

ten Hove T, van Montfrans C, Peppelenbosch MP, van Deventer SJ. Infliximab treatment induces

apoptosis of lamina propria T lymphocytes in Crohn's disease. Gut 2002; 50:206-11.

Theiss AL, Simmons JG, Jobin C, Lund PK. Tumor necrosis factor (TNF) alpha increases collagen accumulation and proliferation in intestinal myofibroblasts via TNF receptor 2. J Biol Chem 2005; 280:36099-109.

Thiesen S, Janciauskiene S, Uronen-Hansson H, Agace W, Högerkorp CM, Spee P, et al. CD14(hi)HLA-DR(dim) macrophages, with a resemblance to classical blood monocytes, dominate inflamed mucosa in Crohn's disease. J Leukoc Biol 2014; 95:531-41.

598. Thompson NP, Driscoll R, Pounder RE, Wakefield AJ. Genetics versus environment in inflammatory bowel disease: results of a British twin study. BMJ 1996; 312:95-6.

Tie F, Banerjee R, Stratton CA, Prasad-Sinha J, Stepanik V, Zlobin A, et al. CBP-mediated acetylation of histone H3 lysine 27 antagonizes Drosophila Polycomb silencing. Development 2009; 136:3131-41.

Tilg H, Ulmer H, Kaser A, Weiss G. Role of IL-10 for induction of anemia during inflammation. J Immunol 2002; 169:2204-9.

Togi S, Kamitani S, Kawakami S, Ikeda O, Muromoto R, Nanbo A, et al. HDAC3 influences phosphorylation of STAT3 at serine 727 by interacting with PP2A. Biochem Biophys Res Commun 2009; 379:616-20.

Travassos LH, Carneiro LA, Ramjeet M, Hussey S, Kim YG, Magalhães JG, et al. Nod1 and Nod2 direct autophagy by recruiting ATG16L1 to the plasma membrane at the site of bacterial entry. Nat Immunol 2010; 11:55-62.

Trepel F. Number and distribution of lymphocytes in man. A critical analysis. Klin Wochenschr 1974; 52:511-5.

Triantafilou K, Triantafilou M, Dedrick RL. A CD14-independent LPS receptor cluster. Nat Immunol 2001; 2:338-45.

Triantafilou M, Miyake K, Golenbock DT, Triantafilou K. Mediators of innate immune recognition of bacteria concentrate in lipid rafts and facilitate lipopolysaccharide-induced cell activation. J Cell Sci 2002; 115:2603-11.

Troy AE, Zaph C, Du Y, Taylor BC, Guild KJ, Hunter CA, et al. IL-27 regulates homeostasis of the intestinal CD4+ effector T cell pool and limits intestinal inflammation in a murine model of colitis. J Immunol 2009; 183:2037-44.

Tserel L, Kolde R, Rebane A, Kisand K, Org T, Peterson H, et al. Genome-wide promoter analysis of histone modifications in human monocyte-derived antigen presenting cells. BMC Genomics 2010; 11:642.

Tsukada Y, Fang J, Erdjument-Bromage H, Warren ME, Borchers CH, Tempst P, et al. Histone demethylation by a family of JmjC domain-containing proteins. Nature 2006; 439:811-6.

Tsukada Y, Ishitani T, Nakayama KI. KDM7 is a dual demethylase for histone H3 Lys 9 and Lys 27 and functions in brain development. Genes Dev 2010; 24:432-7.

Tsytsykova AV, Goldfeld AE. Inducer-specific enhanceosome formation controls tumor necrosis factor alpha gene expression in T lymphocytes. Mol Cell Biol 2002; 22:2620-31.

Turner JR. Intestinal mucosal barrier function in health and disease. Nat Rev Immunol 2009; 9:799-809.

Tysk C, Lindberg E, Järnerot G, Flodérus-Myrhed B. Ulcerative colitis and Crohn's disease in an unselected population of monozygotic and dizygotic twins. A study of heritability and the influence of

smoking. Gut 1988; 29:990-6.

Van Assche G, Rutgeerts P. Physiological basis for novel drug therapies used to treat the inflammatory bowel diseases. I. Immunology and therapeutic potential of antiadhesion molecule therapy in inflammatory bowel disease. Am J Physiol Gastrointest Liver Physiol 2005; 288:G169-74.

Van den Brande JM, Braat H, van den Brink GR, Versteeg HH, Bauer CA, Hoedemaeker I, et al. Infliximab but not etanercept induces apoptosis in lamina propria T-lymphocytes from patients with Crohn's disease. Gastroenterology 2003; 124:1774-85.

Van der Sluis M, De Koning BA, De Bruijn AC, Velcich A, Meijerink JP, Van Goudoever JB, et al. Muc2-deficient mice spontaneously develop colitis, indicating that MUC2 is critical for colonic protection. Gastroenterology 2006; 131:117-29.

van Dullemen HM, van Deventer SJ, Hommes DW, Bijl HA, Jansen J, Tytgat GN, et al. Treatment of Crohn's disease with anti-tumor necrosis factor chimeric monoclonal antibody (cA2). Gastroenterology 1995; 109:129-35.

van Heel DA, Udalova IA, De Silva AP, McGovern DP, Kinouchi Y, Hull J, et al. Inflammatory bowel disease is associated with a TNF polymorphism that affects an interaction between the OCT1 and NF(-kappa)B transcription factors. Hum Mol Genet 2002; 11:1281-9.

van Wijk F, Cheroutre H. Intestinal T cells: facing the mucosal immune dilemma with synergy and diversity. Semin Immunol 2009; 21:130-8.

Vandenabeele P, Declercq W, Beyaert R, Fiers W. Two tumour necrosis factor receptors: structure and function. Trends Cell Biol 1995; 5:392-9.

Varol C, Landsman L, Fogg DK, Greenshtein L, Gildor B, Margalit R, et al. Monocytes give rise to mucosal, but not splenic, conventional dendritic cells. J Exp Med 2007; 204:171-80.

Varol C, Vallon-Eberhard A, Elinav E, Aychek T, Shapira Y, Luche H, et al. Intestinal lamina propria dendritic cell subsets have different origin and functions. Immunity 2009; 31:502-12.

Vázquez N, Greenwell-Wild T, Rekka S, Orenstein JM, Wahl SM. Mycobacterium avium-induced SOCS contributes to resistance to IFN-gamma-mediated mycobactericidal activity in human macrophages. J Leukoc Biol 2006; 80:1136-44.

Velcich A, Yang W, Heyer J, Fragale A, Nicholas C, Viani S, et al. Colorectal cancer in mice genetically deficient in the mucin Muc2. Science 2002; 295:1726-9.

Ventham NT, Kennedy NA, Nimmo ER, Satsangi J. Beyond gene discovery in inflammatory bowel disease: the emerging role of epigenetics. Gastroenterology 2013; 145:293-308.

Villagra A, Cheng F, Wang HW, Suarez I, Glozak M, Maurin M, et al. The histone deacetylase HDAC11 regulates the expression of interleukin 10 and immune tolerance. Nat Immunol 2009; 10:92-100.

Villagra A, Sotomayor EM, Seto E. Histone deacetylases and the immunological network: implications in cancer and inflammation. Oncogene 2010; 29:157-73.

Villarino AV, Artis D, Bezbradica JS, Miller O, Saris CJ, Joyce S, et al. IL-27R deficiency delays the onset of colitis and protects from helminth-induced pathology in a model of chronic IBD. Int Immunol 2008; 20:739-52.

Visperas A, Do JS, Bulek K, Li X, Min B. IL-27, targeting antigen-presenting cells, promotes Th17 differentiation and colitis in mice. Mucosal Immunol 2014; 7:625-33.

Vogel SN, Fitzgerald KA, Fenton MJ. TLRs: differential adapter utilization by toll-like receptors mediates TLR-specific patterns of gene expression. Mol Interv 2003; 3:466-77.

Waddell A, Ahrens R, Steinbrecher K, Donovan B, Rothenberg ME, Munitz A, et al. Colonic eosinophilic inflammation in experimental colitis is mediated by Ly6C(high) CCR2(+) inflammatory monocyte/macrophage-derived CCL11. J Immunol 2011; 186:5993-6003.

Wajant H, Scheurich P. TNFR1-induced activation of the classical NF-κB pathway. FEBS J 2011; 278:862-76.

Wald D, Qin J, Zhao Z, Qian Y, Naramura M, Tian L, et al. SIGIRR, a negative regulator of Toll-like receptor-interleukin 1 receptor signaling. Nat Immunol 2003; 4:920-7.

Waldmann TA. The biology of interleukin-2 and interleukin-15: implications for cancer therapy and vaccine design. Nat Rev Immunol 2006; 6:595-601.

Wang F, Schwarz BT, Graham WV, Wang Y, Su L, Clayburgh DR, et al. IFN-gamma-induced TNFR2 expression is required for TNF-dependent intestinal epithelial barrier dysfunction. Gastroenterology 2006; 131:1153-63.

Wang J, Lee S, Teh CE, Bunting K, Ma L, Shannon MF. The transcription repressor, ZEB1, cooperates with CtBP2 and HDAC1 to suppress IL-2 gene activation in T cells. Int Immunol 2009; 21:227-35.

Wang M, Liang P. Interleukin-24 and its receptors. Immunology 2005; 114:166-70.

Wang Q, Wen YG, Li DP, Xia J, Zhou CZ, Yan DW, et al. Upregulated INHBA expression is associated with poor survival in gastric cancer. Med Oncol 2012; 29:77-83.

Wang RN, Green J, Wang Z, Deng Y, Qiao M, Peabody M, et al. Bone Morphogenetic Protein (BMP) signaling in development and human diseases. Genes Dis 2014; 1:87-105.

Warner LE, Mancias P, Butler IJ, McDonald CM, Keppen L, Koob KG, et al. Mutations in the early growth response 2 (EGR2) gene are associated with hereditary myelinopathies. Nat Genet 1998; 18:382-4.

640. Weber B, Saurer L, Mueller C. Intestinal macrophages: differentiation and involvement in intestinal immunopathologies. Semin Immunopathol 2009; 31:171-84.

Wei G, Wei L, Zhu J, Zang C, Hu-Li J, Yao Z, et al. Global mapping of H3K4me3 and H3K27me3 reveals specificity and plasticity in lineage fate determination of differentiating CD4+ T cells. Immunity 2009; 30:155-67.

Wei M, Shinkura R, Doi Y, Maruya M, Fagarasan S, Honjo T. Mice carrying a knock-in mutation of Aicda resulting in a defect in somatic hypermutation have impaired gut homeostasis and compromised mucosal defense. Nat Immunol 2011; 12:264-70.

Wilson AG, de Vries N, Pociot F, di Giovine FS, van der Putte LB, Duff GW. An allelic polymorphism within the human tumor necrosis factor alpha promoter region is strongly associated with HLA A1, B8, and DR3 alleles. J Exp Med 1993; 177:557-60.

Wirtz S, Billmeier U, Mchedlidze T, Blumberg RS, Neurath MF. Interleukin-35 mediates mucosal immune responses that protect against T-cell-dependent colitis. Gastroenterology 2011; 141:1875-86.

Wirtz S, Finotto S, Kanzler S, Lohse AW, Blessing M, Lehr HA, et al. Cutting edge: chronic intestinal inflammation in STAT-4 transgenic mice: characterization of disease and adoptive transfer by TNF- plus IFN-gamma-producing CD4+ T cells that respond to bacterial antigens. J Immunol 1999; 162:1884-8.

Wolk K, Kunz S, Asadullah K, Sabat R. Cutting edge: immune cells as sources and targets of the IL-10 family members? J Immunol 2002; 168:5397-402.

Worbs T, Bode U, Yan S, Hoffmann MW, Hintzen G, Bernhardt G, et al. Oral tolerance originates in the intestinal immune system and relies on antigen carriage by dendritic cells. J Exp Med 2006; 203:519-27.

Xavier RJ, Podolsky DK. Unravelling the pathogenesis of inflammatory bowel disease. Nature 2007; 448:427-34.

Yamamoto M, Kweon MN, Rennert PD, Hiroi T, Fujihashi K, McGhee JR, et al. Role of gut-associated lymphoreticular tissues in antigen-specific intestinal IgA immunity. J Immunol 2004; 173:762-9.

Yamamoto M, Yoshizaki K, Kishimoto T, Ito H. IL-6 is required for the development of Th1 cell-mediated murine colitis. J Immunol 2000; 164:4878-82.

Yamane K, Toumazou C, Tsukada Y, Erdjument-Bromage H, Tempst P, Wong J, et al. JHDM2A, a JmjC-containing H3K9 demethylase, facilitates transcription activation by androgen receptor. Cell 2006; 125:483-95.

Yanagibashi T, Hosono A, Oyama A, Tsuda M, Suzuki A, Hachimura S, et al. IgA production in the large intestine is modulated by a different mechanism than in the small intestine: Bacteroides acidifaciens promotes IgA production in the large intestine by inducing germinal center formation and increasing the number of IgA+ B cells. Immunobiology 2013; 218:645-51.

Yarur AJ, Jain A, Sussman DA, Barkin JS, Quintero MA, Princen F, et al. The association of tissue anti-TNF drug levels with serological and endoscopic disease activity in inflammatory bowel disease: the ATLAS study. Gut 2015.

Yen D, Cheung J, Scheerens H, Poulet F, McClanahan T, McKenzie B, et al. IL-23 is essential for T cell-mediated colitis and promotes inflammation via IL-17 and IL-6. J Clin Invest 2006; 116:1310-6.

Yoshida H, Kawamoto H, Santee SM, Hashi H, Honda K, Nishikawa S, et al. Expression of alpha(4)beta(7) integrin defines a distinct pathway of lymphoid progenitors committed to T cells, fetal intestinal lymphotoxin producer, NK, and dendritic cells. J Immunol 2001; 167:2511-21.

Yoshida H, Naito A, Inoue J, Satoh M, Santee-Cooper SM, Ware CF, et al. Different cytokines induce surface lymphotoxin-alphabeta on IL-7 receptor-alpha cells that differentially engender lymph nodes and Peyer's patches. Immunity 2002; 17:823-33.

Yoshimura T, Robinson EA, Tanaka S, Appella E, Leonard EJ. Purification and amino acid analysis of two human monocyte chemoattractants produced by phytohemagglutinin-stimulated human blood mononuclear leukocytes. J Immunol 1989; 142:1956-62.

Yuvaraj S, Al-Lahham SH, Somasundaram R, Figaroa PA, Peppelenbosch MP, Bos NA. E. coli-produced BMP-2 as a chemopreventive strategy for colon cancer: a proof-of-concept study. Gastroenterol Res Pract 2012; 2012:895462.

Zenewicz LA, Yancopoulos GD, Valenzuela DM, Murphy AJ, Stevens S, Flavell RA. Innate and adaptive interleukin-22 protects mice from inflammatory bowel disease. Immunity 2008; 29:947-57.

Zhang X, Mosser DM. Macrophage activation by endogenous danger signals. J Pathol 2008; 214:161-78.

Zhang Y, Kinkel S, Maksimovic J, Bandala-Sanchez E, Tanzer MC, Naselli G, et al. The polycomb repressive complex 2 governs life and death of peripheral T cells. Blood 2014.

Zhang Y, Reinberg D. Transcription regulation by histone methylation: interplay between different covalent modifications of the core histone tails. Genes Dev 2001; 15:2343-60.

Zhernakova A, van Diemen CC, Wijmenga C. Detecting shared pathogenesis from the shared genetics of immune-related diseases. Nat Rev Genet 2009; 10:43-55.

Zhou L, Ivanov II, Spolski R, Min R, Shenderov K, Egawa T, et al. IL-6 programs T(H)-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways. Nat Immunol 2007; 8:967-74.

Ziegler SF, Artis D. Sensing the outside world: TSLP regulates barrier immunity. Nat Immunol 2010; 11:289-93.

Zigmond E, Varol C, Farache J, Elmaliah E, Satpathy AT, Friedlander G, et al. Ly6C hi monocytes in the inflamed colon give rise to proinflammatory effector cells and migratory antigen-presenting cells. Immunity 2012; 37:1076-90.
Zindl CL, Lai JF, Lee YK, Maynard CL, Harbour SN, Ouyang W, et al. IL-22-producing neutrophils contribute to antimicrobial defense and restitution of colonic epithelial integrity during colitis. Proc Natl Acad Sci U S A 2013; 110:12768-73.

Appendix

Supplemental Figures and Table

Supplemental Figure for Chapter 4

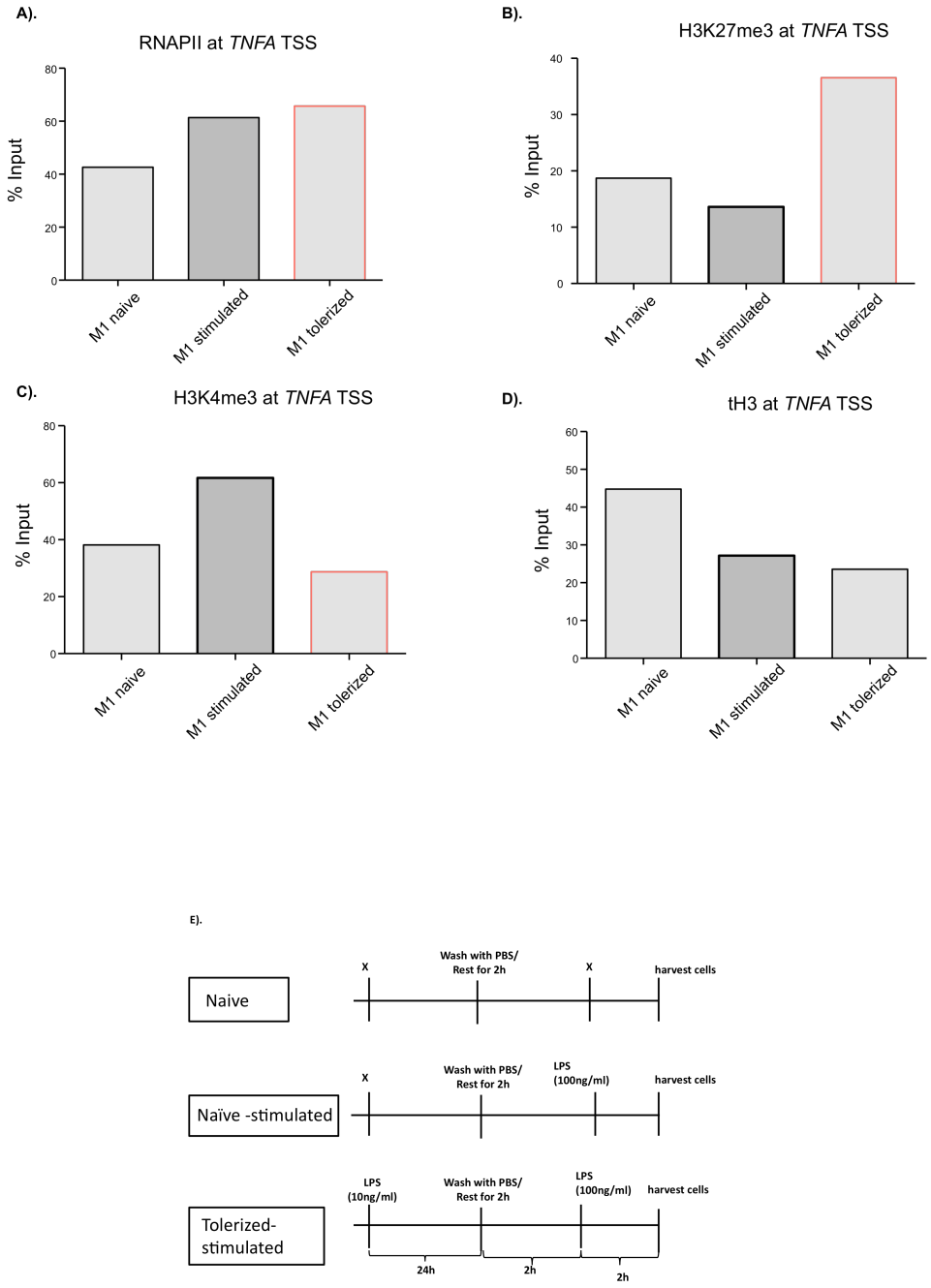
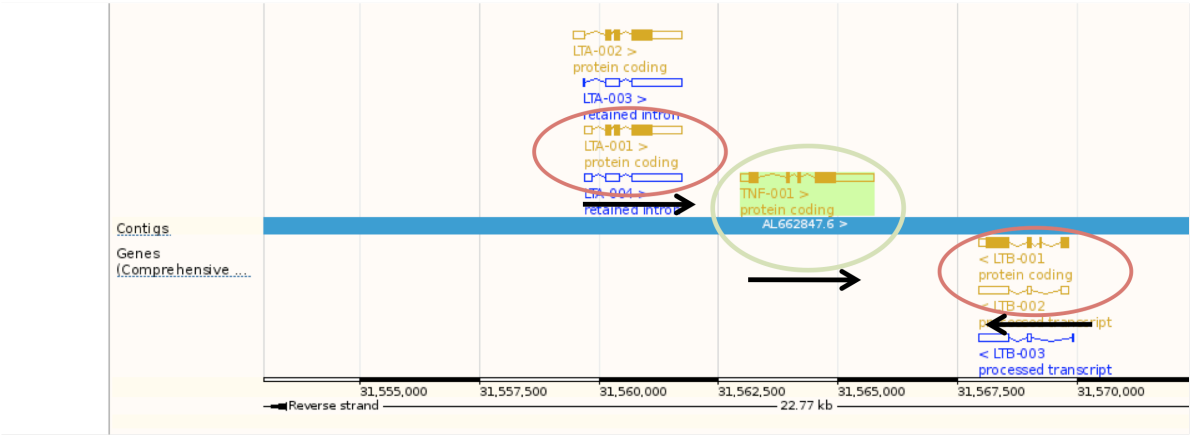


Figure A.01: ChIP assay analysis on tolerised human primary macrophages (GM-CSF - M1)

Supplemental figure for Chapter 6



http://www.ensembl.org/Homo_sapiens/Gene/Summary?
db=core;g=ENSG00000204490;r=CHR_HSCHR6_MHC_COX_CTG1:31562973-31565742;t=ENST00000376122

Figure A.02: TNF/LT locus

Supplemental table for Chapter 6

	GM-CSF macrophages	M-CSF macrophages
	Tolerised/Non-tolerised	
Gene:		
BMP1	YES	YES
BMP2	YES	YES
BMP6	YES	NO
BMPR2	YES	YES
CD70	NO	YES
CSF1	YES	NO
CSF2	YES	YES
IFNB1	YES	YES
IL10	YES	YES
IL1A	YES	YES
IL1B	YES	NO
IL1F5	YES	YES
IL24	YES	YES
IL8	NO	NO
IL8RB	NO	NO
IL6	YES	YES
IL6R	YES	YES
IL7	YES	YES
IL7R	YES	NO
INHBA	YES	YES
LTB	NO	NO
LTBR	NO	NO
LTA	YES	YES
TNF	YES	YES
TNFSF8	YES	YES

Table A.0.3. Gene expression pattern of *in vitro* tolerised GM-CSF/M-CSF differentiated human primary macrophages

continued

	GM-CSF macrophages	M-CSF macrophages
	Tolerised/Non-tolerised	
Gene:		
BMP2K	NO	NO
BMP8A	NO	NO
BMP8B	NO	NO
BMR1A	NO	NO
CSF1R	NO	NO
CSF2RA	NO	NO
CSF2RB	NO	NO
IL10RA	NO	NO
IL10RB	NO	NO
IL16	NO	NO
IL21R	NO	NO
IL3RA	NO	NO
SIRT1	NO	NO
SIRT2	NO	NO
TGFB1	NO	NO
TGFB2	NO	NO
TGFBR1	NO	NO
TNFSF4	NO	NO
TNFSF12	NO	NO
TNFSF13	NO	NO
TNFSF13B	NO	NO
TNFSF14	NO	NO
TXLNA	NO	NO

Table A.0.3. Gene expression pattern of *in vitro* tolerised GM-CSF/M-CSF differentiated human primary macrophages