

Mononuclear Phagocytes in Intestinal Homeostasis and Inflammation

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Abstract

Changes to the composition and function of the gut mononuclear phagocyte (MNP) compartment are associated with the development of intestinal inflammation. Much work has focused on the role of MNPs in gut-associated lymphoid tissue in maintaining homeostasis, however little is known regarding the roles of MNPs during colitis. We have investigated MNPs in the large intestinal lamina propria during the steady state and inflammation. One of our primary aims was to determine the contribution of MNP subsets to intestinal pathology. For our studies of inflammation, we focused mainly on the *Helicobacter hepaticus* infection + anti-IL-10R model, which induces inflammation of the colon and caecum (typhlocolitis). We defined the composition of the MNP compartment alongside intestinal pathology scores throughout *Hh* + anti-IL-10R typhlocolitis. Peak pathology, 2-3 weeks after induction of colitis, coincided with peak frequencies of CX₃CR1^{int} Ly6C⁺ MNPs. Having observed the accumulation of CX₃CR1^{int} CD64⁺ monocyte/macrophage MNPs in the inflamed lamina propria, we conducted comparative whole genome microarray analysis of these cells isolated from the large intestine three weeks after *Hh* + anti-IL-10R treatment. CX₃CR1^{int} CD64⁺ MNPs selectively expressed a variety of pro- and anti-inflammatory genes, including a number of genes which individually can both promote and negatively regulate inflammation. IL-23 is essential for *Hh* + anti-IL-10R-induced intestinal pathology. We investigated the role of MNPs as a source of IL-23 which drives *Hh* + anti-IL-10R colitis. Unexpectedly, our results indicate that normally hyporesponsive CX₃CR1^{hi} macrophages may act as the initial source of IL-23, which induces development of colitis. Recruitment of Ly6C⁺ MHCII⁺ MNPs to the lamina propria was IL-23-dependent, and these cells also expressed IL-23, which may establish a positive feedback loop of immune cell recruitment, activation and IL-23 production. Finally, we also examined how MNPs might be recruited to the colonic lamina propria during inflammation. Our studies support the conclusion that CCR6 is not required for accumulation of monocyte-derived populations in the inflamed intestine. We cannot rule out a role for CCR2, however preliminary data from the *Hh* + anti-IL-10R colitis model suggest a potential role for CCR1 or its close relation CCRL2. Such pathways could represent new therapeutic targets in inflammatory bowel disease.

The harder the conflict, the more glorious the triumph.

What we obtain too cheap, we esteem too lightly:

it is dearness only that gives every thing its value.

– Thomas Paine, *The American Crisis*

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Abbreviations

7-AAD	7-aminoactinomycin D
ANOVA	analysis of variance
AOM	azoxymethane
APC	allophycocyanin (fluorochrome); antigen presenting cell
aRNA	antisense ribonucleic acid
AT-1	angiotensin II type 1 receptor
B6	C57BL/6
BATF3	basic leucine zipper transcription factor, activating transcription factor-like 3
BM	bone marrow
BMDC	bone marrow-derived dendritic cell
BMDM	bone marrow-derived macrophage
BrdU	bromo-2'-deoxyuridine
BSA	bovine serum albumin
C/EBP α	CCAAT/enhancer binding protein
CCL	C-C chemokine ligand
CCR	C-C chemokine receptor
CCRL	C-C chemokine receptor-like
CD	cluster of differentiation (e.g. CD11b); Crohn's disease
cDC	conventional dendritic cell, classical dendritic cell
cDNA	complementary deoxyribonucleic acid
CDP	common dendritic cell precursor
CFU	colony forming unit
CGAT	Computational Genomics Analysis Tools
CLEC	C-type lectin
cMoP	common monocyte progenitor
CMP	common myeloid progenitor
Cre	Cre recombinase
CXCL	C-X-C chemokine ligand
CXCR	C-X-C chemokine receptor
CX ₃ CL	C-X-X-X-C (CX ₃ C) chemokine ligand
CX ₃ CR	C-X-X-X-C (CX ₃ C) chemokine receptor
d	day
DAMP	damage-associated molecular pattern
DAPI	4',6-diamidino-2-phenylindole
DC	dendritic cell
DNA	deoxyribonucleic acid
DNBS	dinitrobenzene sulfonic acid
DSS	dextran sodium sulphate
DTR	diphtheria toxin receptor
EAE	experimental autoimmune encephalomyelitis
E-cad	E-cadherin
EDTA	ethylenediaminetetra-acetic acid
EGFP	enhanced green fluorescent protein

ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence-activated cell sorting
FAE	follicle-associated epithelium
FBS	foetal bovine serum
Fc	fragment crystallisable
FITC	fluorescein isothiocyanate
Flt3	Fms-like tyrosine kinase 3
Flt3L	Fms-like tyrosine kinase 3 ligand
FoxP3	forkhead box P3
FSC	forward scatter
G	gauge
G-CSF	granulocyte colony-stimulating factor
G-CSFR	granulocyte colony-stimulating factor receptor
GFP	green fluorescent protein
GITR	glucocorticoid-induced tumour necrosis factor receptor family-related protein
GITR-L	glucocorticoid-induced tumour necrosis factor receptor family-related protein ligand
GM-CSF	granulocyte-macrophage colony-stimulating factor
GM-CSFR	granulocyte-macrophage colony-stimulating factor receptor
GMP	granulocyte/macrophage precursor
GO	gene ontology
GPCR	G protein-coupled receptor
H&E	haematoxylin and eosin
HBSS	Hanks' balanced salt solution
h	hour
<i>Hh</i>	<i>Helicobacter hepaticus</i>
HSC	haematopoietic stem cell
HuR	Hu-antigen R (ELAV-like protein 1)
IBD	inflammatory bowel disease
iDTR	inducible diphtheria toxin receptor
IFN	interferon
Ig	immunoglobulin
IHC	immunohistochemistry
IL	interleukin
ILC	innate lymphoid cell
ILF	isolated lymphoid follicle
iNOS	inducible nitric oxide synthase
i.p.	intraperitoneal
IRF	interferon regulatory factor
iTreg	induced regulatory T cell
i.v.	intravenous
KC	keratinocyte chemoattractant
KLF	Krüppel-like factor
KO	knockout
L	ligand (e.g. CD40L = CD40 ligand)
L-CCR	lipopolysaccharide-inducible C-C chemokine receptor related
L/D	live/dead

LP	lamina propria
LPMC	lamina propria mononuclear cell
LPS	lipopolysaccharide
LysM	lysozyme M; lysin motif
Lyz2	lysozyme 2
M1	classical [macrophage/macrophage activation]
M2	alternative [macrophage/macrophage activation]
mAb	monoclonal antibody
MCP	monocyte chemotactic protein
M-CSF	macrophage colony-stimulating factor
M-CSFR	macrophage colony-stimulating factor receptor
MDP	macrophage-DC precursor
MerTK	tyrosine-protein kinase Mer
MHC	major histocompatibility complex
MIP	macrophage inflammatory protein
MLN	mesenteric lymph node
MMP	matrix metalloproteinase (a.k.a. matrix metalloproteinase)
MNP	mononuclear phagocyte
mRNA	messenger ribonucleic acid
NFκB	nuclear factor kappa B
NK	natural killer [cell]
NLRP	nucleotide-binding, oligomerisation domain-containing protein (NOD)-like receptor family pyrin domain-containing
NO	nitric oxide
NOD	nucleotide-binding, oligomerisation domain-containing [protein]
OD	optical density
PAMP	pathogen-associated molecular pattern
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
pDC	plasmacytoid dendritic cell
PE	phycoerythrin
PE-Cy7	phycoerythrin-cyanine7
PerCP-Cy5.5	peridinin chlorophyll-cyanine5.5
PG	prostaglandin
PMA	phorbol 12-myristate 13-acetate
PP	Peyer's patch
PRR	pattern recognition receptor
R	receptor
RAG	recombination-activating gene
RALDH	retinal dehydrogenase
RANTES	regulated on activation, normal T cell expressed
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute [medium]
RT-qPCR	quantitative reverse transcription polymerase chain reaction
s	second
SF	super family
SI	small intestine
Siglec	sialic acid-binding immunoglobulin-type lectin

SIRP	signal regulatory protein
SLC	solute carrier
SLPI	secretory leukocyte peptidase inhibitor
SS	steady state
SSC	side scatter
STAT	signal transducer and activator of transcription
TARM	T cell-interacting, activating receptor on myeloid cells
T-bet	T-box expressed in T cells
TFL	transformed follicular lymphoma
TGF	transforming growth factor
Th	helper T [cell]
TipDC	tumour necrosis factor- and inducible nitric oxide synthase-producing dendritic cell
TLR	Toll-like receptor
TNBS	2,4,6-trinitrobenzenesulfonic acid
TNF	tumour necrosis factor
TNF ^{ΔARE}	tumour necrosis factor AU-rich element deletion
Treg	regulatory T [cell]
TREM	triggering receptor expressed on myeloid cells
TRUC	<i>Tbx21</i> ^{-/-} <i>Rag2</i> ^{-/-} ulcerative colitis
TSB	tryptone soya broth
TSLP	thymic stromal lymphopoietin
TVP	trimethoprim, vancomycin, polymyxin B
UC	ulcerative colitis
VST	variance stabilising transformation
w	week
WT	wild-type
Zbtb	zinc finger and BTB domain-containing protein

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1 Introduction

1.1 The intestinal immune system

1.1.1 Intestinal architecture

The small intestine is comprised of three sections: the duodenum, jejunum and ileum. It is characterised by the presence of villi, finger-like projections which extend into the lumen and increase the absorptive surface area of the epithelium. The large intestine comprises the caecum and colon, which have a flat surface with no villi. Both the small and large intestines have invaginations, known as the crypts of Lieberkühn (crypts), from which the epithelium is constantly renewed from multipotent stem cells (van der Flier and Clevers, 2009). The intestine is lined by a single layer of columnar epithelium, which separates the underlying tissue from an enormous antigenic load derived from diet, 10^{14} commensal bacteria and occasionally pathogens (Hooper and Macpherson, 2010). Goblet cells in the epithelial layer produce mucus, which aids host protection against pathogenic infection. Underlying the epithelium is the lamina propria (LP), a layer of loosely packed connective tissue containing blood and lymphatic vessels, and nerve fibres which supply the mucosa. The mucosa consists of the epithelium, the lamina propria and the muscularis mucosa, a thin layer of muscle below the lamina propria. Beneath the muscularis mucosa lies the submucosa (connective tissue), a thicker external muscle layer, and the serosa, a fibrous cover separating the intestine from the peritoneal cavity (Mowat and Agace, 2014).

1.1.2 Gut-associated lymphoid tissue

The gut-associated lymphoid tissue (GALT) is the largest compartment of the immune system (Mowat and Viney, 1997). Immune responses are induced in organised secondary lymphoid tissue: the draining lymph nodes (LNs), such as the mesenteric lymph nodes (MLNs), and small intestinal Peyer's patches (PPs); and smaller lymphoid aggregates known as solitary isolated lymphoid tissues (SILTs), ranging in size from small cryptopatches to mature isolated lymphoid follicles (ILFs) in the small and large intestines (Kanamori et al., 1996) (note: cryptopatches have not yet been formally identified in humans (Pearson et al., 2012)). Cryptopatches mainly consist of innate lymphoid cells (ILCs) and dendritic cells (DCs), and can mature into ILFs containing B cell zones and germinal centres (Pearson et al., 2012). Contrary to popular belief, the MLNs do not drain the entire small and large intestines in mice. In addition to the MLNs, different sections of the intestines are drained by the pancreatic and caudal lymph nodes (Carter and Collins, 1974). The lamina propria and intestinal epithelium represent the effector sites of the intestinal immune system, and are comprised of scattered lymphocytes within the lamina propria, and between or closely associated with intestinal epithelial cells, respectively (Figure 1.1).

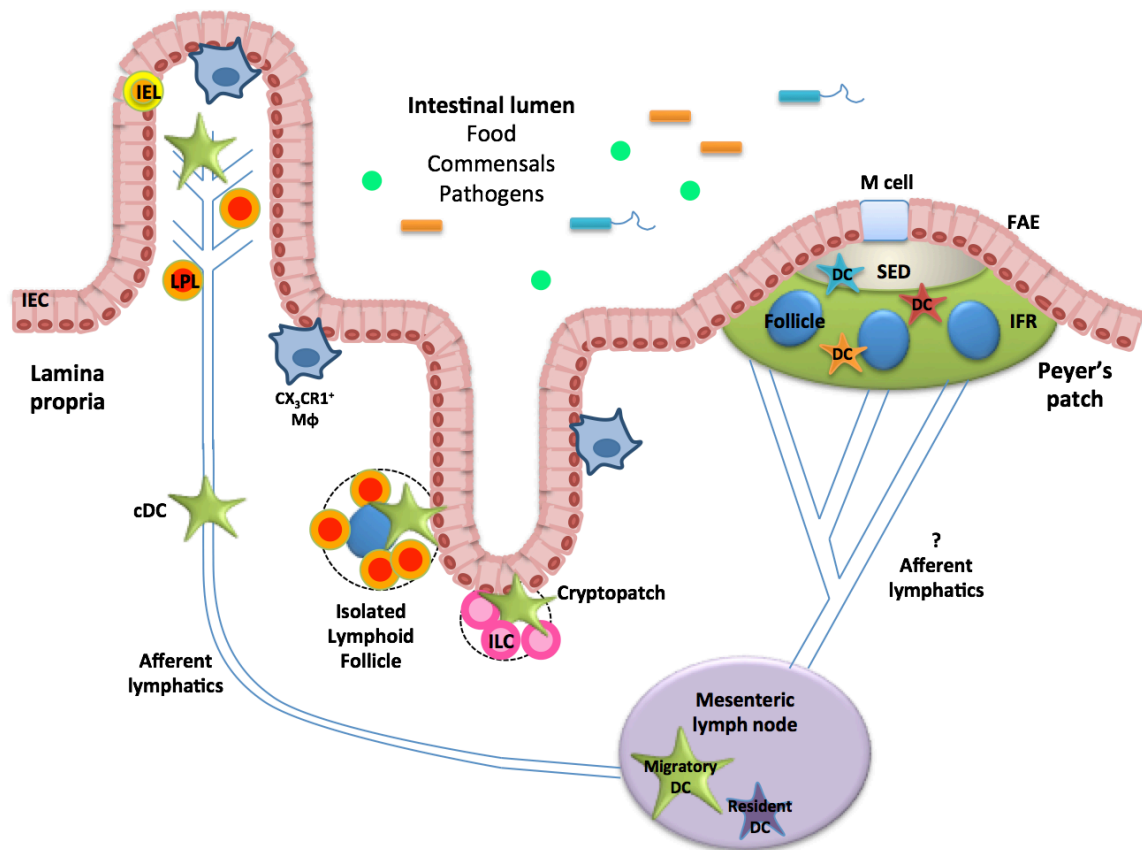


Figure 1.1: The intestinal immune system. Organised lymphoid tissues – the mesenteric lymph nodes, isolated lymphoid follicles, cryptopatches and, in the small intestine, Peyer's patches – are where induction of protective immune responses and tolerance occurs in the intestine. Effector sites consist of lymphoid cells scattered throughout the lamina propria and between the intestinal epithelial cells, a single layer of which separates the underlying tissue from the intestinal lumen. Some lamina propria dendritic cells migrate to the mesenteric lymph nodes in both steady state and inflammatory conditions. It is unclear at present whether dendritic cells migrate from the Peyer's patches to the mesenteric lymph nodes. (c)DC, (classical) dendritic cell; CX₃CR1, CX₃C chemokine receptor 1; FAE, follicle-associated epithelium; IEC, intestinal epithelial cell; IEL, intraepithelial lymphocyte; IFR, interfollicular region, also known as the T-dependent area; ILC, innate lymphoid cell; LPL, lamina propria lymphocyte, can also refer to lamina propria leukocyte; M cell, micro-fold cell; Mφ, macrophage; SED, sub-epithelial dome. Adapted from (Mowat, 2003).

1.1.3 Antigen access to the intestinal mucosa

Antigens from the intestinal lumen gain access to the underlying mucosa and immune cells through specialised routes, which vary by region. Micro-fold (M) cells, found in the follicle-associated epithelium (FAE) overlying PPs, ILFs and also in murine intestinal villi (Jang et al., 2004), are specialised epithelial cells which deliver particulate antigens and microorganisms to the underlying tissue (Nicoletti, 2000). DCs in PPs, including some in “pockets” in the M cell basolateral membrane, internalise this antigen and can then initiate immune responses, probably within PPs (Kelsall, 2008). CX₃C chemokine receptor 1 (CX₃CR1)⁺ MNPs in the lamina propria have been shown to acquire antigen directly from the lumen by means of transepithelial dendrite extension (Kim et al., 2011a; Niess et al., 2005; Rescigno et al., 2001). Although the cells in which this behaviour was observed were originally defined as DCs, they now seem more likely to be macrophages (discussed further in Section 1.2.5.2). CD103⁺ DCs have also been suggested to capture luminal bacteria after crawling into the epithelium (Farache et al., 2013). Additionally, CD103⁺ DCs in the small intestine but not colon have been shown to acquire antigen via goblet cell-associated passages (McDole et al., 2012). Antigen can also cross the non-follicular absorptive intestinal epithelium by paracellular or transcellular transport (including receptor-mediated transport, such as via the neonatal Fc receptor in the presence of immunoglobulin (Ig)G (Yoshida et al., 2004)), or when the epithelium is damaged, such as in inflammatory bowel disease (IBD) and certain infections (Kelsall, 2008). In addition, antigen may access the mucosa via exosomes released from epithelial cells (Karlsson et al., 2001). Luminal antigen which has entered the lamina propria can then be transported to the mesenteric and possibly other draining LNs, such as the caudal and pancreatic LNs (Carter and Collins, 1974), for initiation of immune responses.

The intestinal immune system must discriminate between pathogen-derived and innocuous antigens derived from commensal bacteria or food, in order to mount appropriate host-protective immunity but prevent unnecessary auto-inflammatory damage. How this delicate and essential balance is maintained is incompletely understood. Disruption of intestinal homeostasis can result in inflammatory bowel disease (Xavier and Podolsky, 2007), food allergy, or coeliac disease (Pabst and Mowat, 2012).

DCs and macrophages, both types of mononuclear phagocyte (MNP), are abundant in the intestine. They play crucial roles in maintaining tissue homeostasis, and in both innate and adaptive immune responses, working together to ensure tolerance to commensal microorganisms and food (Bain and Mowat, 2014; Coombes and Powrie, 2008). As such, MNPs are of interest as potential therapeutic targets to treat IBD; however, this requires detailed knowledge of their origins and functions, and the factors controlling these, during both homeostasis and disease.

1.2 Intestinal mononuclear phagocytes

The mononuclear phagocyte system is comprised of monocytes, macrophages and dendritic cells (van Furth et al., 1972). The phenotype and function of these types of cells often overlap. The difficulty of finding subset-specific markers and recent discoveries regarding the ontogeny of MNP subsets have led to the proposal that MNPs be defined differently, primarily according to their origin, and secondarily by their location, function and phenotype (Guilliams et al., 2014). The traditional view of the mononuclear

phagocyte system held that monocytes were the precursors of steady state tissue macrophages (van Furth et al., 1972). However, recent studies have suggested that in the steady state, most tissue macrophages are derived from embryonic precursors seeded before birth, which maintain themselves by self-renewal (Ginhoux and Jung, 2014). The newly developed scheme has therefore concluded there may be three “families” of MNPs: common DC precursor-derived *bona fide* classical dendritic cells (cDCs, which will be referred to here as “DCs”), embryonically-derived macrophages, and monocyte-derived cells, the last of which would include monocytes, some cells currently described as tissue macrophages and a population of “monocyte-derived DCs” (Guilliams et al., 2014). Here it is important to note that Ly6C^{hi} classical monocytes, and not embryonically-derived precursors, appear to be the primary source of most if not all mature macrophages in the intestine of adult mice (Bain et al., 2014). Although there is certainly a need to unify the MNP nomenclature to ensure understanding and interpretability of results from different laboratories and experimental systems, no consensus has yet been agreed. As such, I will continue to use the term “macrophage” to describe both embryonic precursor- and monocyte-derived cells with the phenotype and functions of macrophages, specifying their origin when the literature at the point of writing permits.

1.2.1 Traditional roles of mononuclear phagocytes

In addition to their role as the precursors of some tissue macrophages in the steady state, monocytes are typically described as circulating innate immune cells, which can be mobilised from the bone marrow in response to infection or inflammation (Serbina et al.,

2008). They migrate via the blood to tissues, where they typically give rise to macrophages or “dendritic cells” (such as tumour necrosis factor alpha (TNF α)- and inducible nitric oxide synthase (iNOS)-producing DCs, TipDCs) in response to environmental factors (Serbina et al., 2008).

As mentioned, intestinal macrophages derive from Ly6C^{hi} monocytes (Bain et al., 2014) whereas most other tissue macrophages derive from embryonically-derived precursors (Ginhoux and Jung, 2014). Macrophages are found in lymphoid tissue and throughout peripheral tissues. They exist in particularly abundant networks underlying mucosal surfaces, where they play a major role in the innate immune response. Macrophages express many pattern recognition receptors (PRRs) and are avidly phagocytic, poised to clear apoptotic or damaged host cells and invading microorganisms. Signals from their microenvironment appear to condition tissue macrophages, influencing their responses to microbes, which can in turn affect subsequent host immune responses. Macrophages are considered sessile, and do not migrate to the tissue-draining lymph nodes, which is a hallmark of DCs (Geissmann et al., 2010a). Following inflammation, injury or infection, macrophages can participate in the resolution of inflammation and tissue remodelling (Murray and Wynn, 2011).

DCs arise from common DC precursors and cDC-committed precursors (pre-cDCs) in a Flt3L-dependent manner (Liu et al., 2009). DCs are professional antigen presenting cells (APCs) found in both peripheral non-lymphoid tissues and lymphoid organs and can be divided into migratory and lymphoid organ-resident (“resident”) DCs (Alvarez et al., 2008). Migratory DCs differentiate from blood-derived pre-cDCs in peripheral tissues. There, they obtain antigen, whether directly or via accessory cells, in an “immature” state. Migratory DCs are sentinels of the immune system, migrating to the tissue-draining LNs

via afferent lymphatic vessels in a CCR7-dependent manner (Alvarez et al., 2008), both homeostatically and in response to inflammatory or infectious stimuli (Villadangos and Schnorrer, 2007). DCs can mature during this process and arrive in the lymphoid tissue capable of presenting peptides from processed antigen on major histocompatibility complex (MHC) class I (MHCI) and II (MHCII) molecules to CD8⁺ and CD4⁺ T cells, respectively. The presence or absence of pathogen- or damage-associated molecular patterns (PAMPs and DAMPs, respectively) or other signals such as cytokines during antigen encounter can alter DC phenotypes, including their own cytokine production or surface expression of co-stimulatory molecules. Via such mechanisms, DCs can shape subsequent immune responses (Coombes and Powrie, 2008). No migratory DCs are found in the spleen, and although it had previously been suggested that 50% of LN DCs were derived from upstream non-lymphoid tissues in the steady state (Villadangos and Schnorrer, 2007), it is now thought that only a minority of LN DCs are migratory (Jakubzick et al., 2008). During inflammation however, migratory DCs represent a substantial proportion of LN DCs (Jakubzick et al., 2008). Resident DCs develop in lymphoid tissues from precursors delivered in the blood (Geissmann et al., 2010b), and reside therein until death (Villadangos and Schnorrer, 2007).

1.2.2 Mononuclear phagocytes in the intestine

The intestine hosts the largest population of MNPs in the body. The largest subpopulation of MNPs is macrophages, which are the most abundant leukocytes in the resting intestine (Hume et al., 1984; Lee et al., 1985). Macrophages are found throughout the small and large intestines, mainly in the lamina propria relatively close to the

epithelium (Mowat and Agace, 2014). In both humans and mice, the absolute numbers and relative frequencies of macrophages are higher in the colon than the small intestine (Denning et al., 2011; Nagashima et al., 1996).

During the steady state, most murine lamina propria resident macrophages express high levels of CX₃CR1. They are also identified by their expression of the macrophage-selective marker CD64 (Langlet et al., 2012), F4/80, CD11b, MHCII and low or absent expression of Ly6C (Bain et al., 2013; Tamoutounour et al., 2012). CX₃CR1^{hi} resident intestinal macrophages display an anti-inflammatory phenotype, even when stimulated with Toll-like receptor (TLR) agonists (Monteleone et al., 2008; Smythies et al., 2005). Precisely how this phenotype is induced or maintained is unclear, however interleukin 10 (IL-10) may be involved (Fiorentino et al., 1991; Monteleone et al., 2008; Pils et al., 2010). The functions of CX₃CR1^{hi} macrophages, which do not migrate to the MLNs (Schulz et al., 2009), remain unclear.

DCs are found in the draining LNs, PPs, ILFs, cryptopatches and the small intestinal and colonic lamina propria (Coombes and Powrie, 2008; Kelsall, 2008; Rescigno and Di Sabatino, 2009). A number of subsets exist in the intestine, which tend to be subdivided by their expression of CD103 (integrin α E) and CD11b. CD103⁺ DCs are one of the best-characterised subsets of intestinal DCs. Both CD11b⁺ and CD11b⁻ CD103⁺ DCs exist, in differing proportions by location: CD103⁺ CD11b⁺ DCs are the major population of CD103⁺ DCs in the small intestinal lamina propria, while CD103⁺ CD11b⁻ DCs are more frequent in the colon (Denning et al., 2011). CD103⁺ DCs in the MLNs have been shown to possess an enhanced ability to induce Foxp3⁺ regulatory T cells (Tregs) that home to the intestine (Coombes et al., 2007; Jaensson et al., 2008; Sun et al., 2007). Thanks to this property, CD103⁺ DCs have become known as “tolerogenic” DCs. In contrast, CD103⁻ DCs in the

MLNs have been shown to induce type 1 helper T cell (Th1) responses (Annacker et al., 2005; Coombes and Powrie, 2008). DCs are thought to control the balance between tolerance and immunity in the intestine; environmental conditioning factors, such as TSLP, IL-10 and transforming growth factor β (TGF β), and the source of antigen seem to modulate their phenotype and shape the ensuing immune response (Coombes and Powrie, 2008), however the precise mechanisms controlling this balance are incompletely understood.

DCs have been shown to accumulate in the intestine in CD and experimental models of colitis (Hart et al., 2005; Uhlig et al., 2006b), however relatively little is known regarding which subsets may contribute to inflammation. Our laboratory has recently shown that despite their tolerogenicity in the steady state, CD103⁺ MLN DCs are pro-inflammatory during colitis (Laffont et al., 2010). Additional work found that a Gr1^{hi} (Ly6C/Ly6G^{hi}) monocyte-derived MNP population – which we called “E-cadherin⁺ DCs” due to their expression of E-cadherin, CD11c and MHCII – accumulated in the colonic lamina propria and MLNs during T cell transfer- and anti-CD40-induced colitis (Siddiqui et al., 2010). These cells were shown to express TLRs and pro-inflammatory cytokines, and exacerbated colitis when they were adoptively transferred to lymphocyte-deficient mice reconstituted with naïve CD4⁺ T cells (Siddiqui et al., 2010).

During the course of this thesis, in parallel with our unpublished work, Ly6C^{hi} monocyte-derived CX₃CR1^{int} MNPs were shown to accumulate during T cell transfer- and DSS-induced colitis (Bain et al., 2013; Rivollier et al., 2012; Tamoutounour et al., 2012; Varol et al., 2009; Weber et al., 2011; Zigmond et al., 2012). How E-cadherin⁺ DCs and CX₃CR1⁺ MNPs relate is unclear. However, the accumulation of monocyte-derived cells in the inflamed colonic lamina propria suggested that they might have a pro-inflammatory role.

The following sections discuss the identification, ontogeny and function of intestinal MNP subsets. Unless otherwise specified, the findings discussed relate to mice.

1.2.3 Defining mononuclear phagocyte subsets by surface marker expression: macrophages versus dendritic cells

How we define subpopulations of MNPs has evolved rapidly over recent years. This process can involve multiple properties, including surface marker expression, location, function, and ontogeny. Accurate distinction between sub-populations of MNPs is important if we are to establish their specific roles in intestinal immune responses.

The identification of macrophages and DCs by surface marker expression has become decidedly more complex, since they share many surface markers previously considered to be “specific” (Geissmann et al., 2010a). In many tissues, the expression of CD11c and MHCII is sufficient to identify genuine DC, but this is not the case in the intestine, where most macrophages also express these markers, therefore a larger panel is needed to distinguish between these cells, including CD11c, MHCII, F4/80, CX₃CR1, CD103, CD11b, CD64, MerTK, Ly6C and CD24 (Bain et al., 2013; Cerovic et al., 2013; Gautier et al., 2012; Geissmann et al., 2010a; Rivollier et al., 2012; Tamoutounour et al., 2012; Varol et al., 2009; Weber et al., 2011; Zigmond et al., 2012). The key monocyte/macrophage and DC markers that have now been identified are CD64 and MerTK (Gautier et al., 2012; Langlet et al., 2012), and CD24 (Schlitzer et al., 2013), respectively. Additionally, the transcription factor zinc finger and BTB domain-containing protein 46 (Zbtb46) is expressed only by cDCs (Meredith et al., 2012; Satpathy et al., 2012).

Standardisation of the markers and flow cytometry gating strategies used to identify cell populations is still lacking, however the discovery and adoption of these macrophage- and DC-selective markers is beginning to ease interpretation of results from different groups and experimental approaches.

1.2.4 Monocytes

1.2.4.1 Subsets

Murine monocytes are divided into two main subsets: classical monocytes, and non-classical monocytes, previously referred to as “inflammatory” and “resident” monocytes, respectively (Ziegler-Heitbrock et al., 2010). These subsets were first formally identified as distinct by Geissmann and colleagues (Geissmann et al., 2003), and can be distinguished by their differential expression of surface markers and functions (Ginhoux and Jung, 2014). Classical monocytes express high levels of Ly6C, intermediate levels of CX₃CR1, alongside CD62L (L-selectin) and C-C chemokine receptor 2 (CCR2; Ly6C^{hi} CX₃CR1^{int} CD62L⁺ CCR2⁺; they are referred to here as Ly6C^{hi} monocytes) (Geissmann et al., 2003). Non-classical monocytes express low levels of Ly6C and high levels of CX₃CR1, but no CD62L or CCR2 (Ly6C^{lo} CX₃CR1^{hi} CD62L⁻ CCR2⁻; referred to here as Ly6C^{lo} monocytes) (Geissmann et al., 2003). Both subsets express CD45, CD44, macrophage colony-stimulating factor receptor (M-CSFR, also called colony-stimulating factor 1 receptor (CSF-1R) or CD115), CD11b, variable levels of F4/80, and CD16/32 (FcγRIII/FcγRII); and are negative for T, B, natural killer (NK), DC and plasmacytoid dendritic cell (pDC) lineage markers including TCRβ, CD3, CD90 (Thy1); B220, CD19; NK1.1, DX5; CD11c and MHCII

(Geissmann et al., 2003). Neither population of blood monocytes proliferates (Geissmann et al., 2003; van Furth and Cohn, 1968).

Although important distinctions requiring further investigation remain between mouse and human monocyte subsets (Ingersoll et al., 2010), the Ly6C^{hi} and Ly6C^{lo} mouse monocyte subsets correspond with human CD14^{hi} and CD14^{lo} CD16⁺ monocyte subsets, respectively (Geissmann et al., 2003; Ginhoux and Jung, 2014; Ingersoll et al., 2010; Shi and Pamer, 2011). In contrast to those in mice, human monocytes express CD11c and MHCII (Geissmann et al., 2003). Human CD14^{hi} monocytes can be further subdivided into CD14^{hi} CD16⁺ and CD14^{hi} CD16⁻ subsets, which are described as intermediate and classical monocytes, respectively (Ziegler-Heitbrock et al., 2010). CD14^{lo} CD16⁺ human monocytes are referred to as non-classical (Ziegler-Heitbrock et al., 2010) and are similar to murine Ly6C^{lo} resident monocytes in terms of their patrolling function (Cros et al., 2010) (see below).

1.2.4.2 Ontogeny

Monocytes belong to the myeloid lineage and in adult mice arise in the bone marrow (BM) from haematopoietic stem cells (HSCs) via a series of multipotent progenitors, followed by the myeloid-restricted common myeloid progenitor (CMP), granulocyte/macrophage precursor (GMP), committed macrophage and DC precursor (MDP) (Varol et al., 2007) and a newly identified common monocyte progenitor (cMoP) (Hettinger et al., 2013) (Figure 1.2). MDPs also give rise to common DC precursors (CDPs), which can differentiate into classical DCs and plasmacytoid DCs (Onai et al., 2007). Unlike the MDP, the cMoP is unable to produce cDCs or pDCs, in accordance with its lack of Fms-

like tyrosine kinase 3 (Flt3) expression (Hettinger et al., 2013) which is required for cDC and pDC development (D'Amico and Wu, 2003; McKenna et al., 2000; Onai et al., 2006; Waskow et al., 2008). cMoPs go on to become Ly6C^{hi} monocytes (Hettinger et al., 2013), which in turn can give rise to Ly6C^{lo} monocytes (Sunderkotter et al., 2004; Varol et al., 2007; Yona et al., 2013) or other MNPs as described below. A recent study has prompted debate over the differentiation potential of the MDP, suggesting that rather than bipotent precursors of either cMoPs or CDPs, MDPs might also give rise to granulocytes (Sathe et al., 2014).

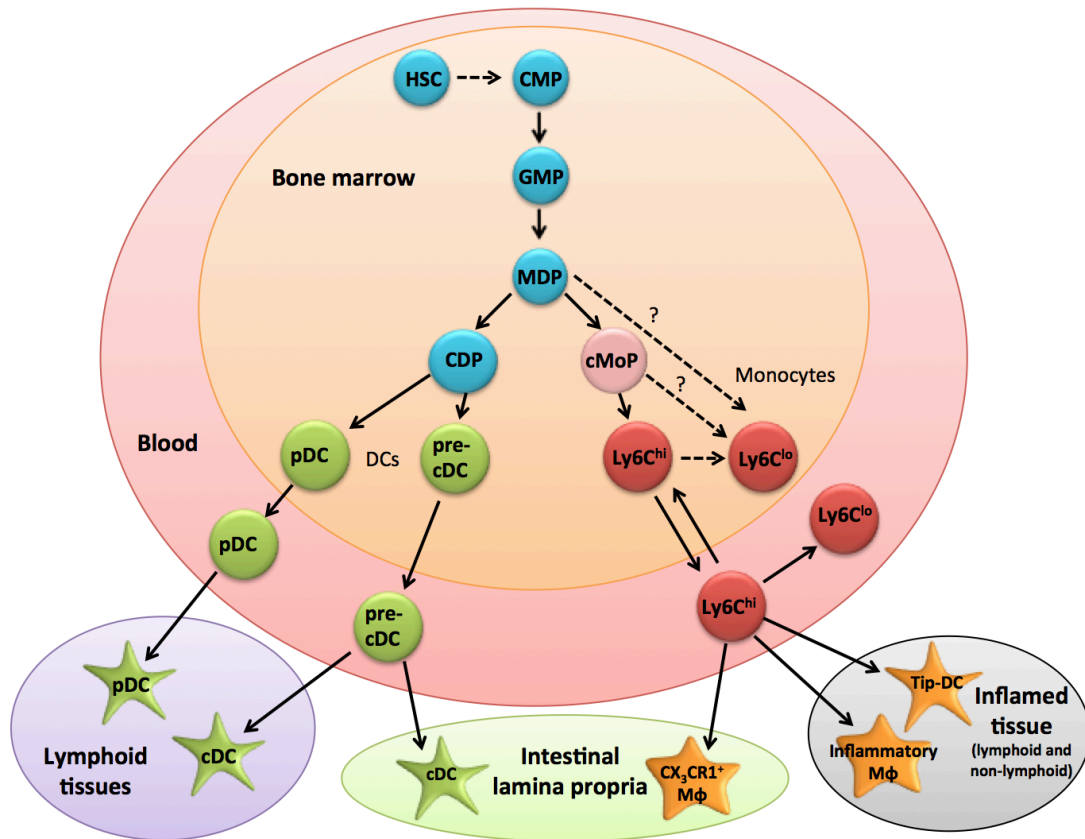


Figure 1.2: Mononuclear phagocyte development. In bone marrow, haematopoietic stem cells (HSC) give rise to common myeloid progenitors (CMP) and common lymphoid progenitors (not shown). CMPs produce granulocyte/macrophage precursors (GMP), followed by macrophage and dendritic cell precursors (MDP), which give rise to common dendritic cell precursors (CDP) and common monocyte progenitors (cMoP). CDPs produce plasmacytoid dendritic cells (pDC) and classical dendritic cell-committed precursors (pre-cDC). Some pDC remain in the bone marrow while others enter the blood and migrate to peripheral tissues. Pre-cDCs migrate via the blood to lymphoid tissues where they give rise to resident conventional dendritic cells (cDC). They also give rise to cDCs in the small intestinal and colonic lamina propria. Ly6C^{hi} monocytes give rise to Ly6C^{lo} monocytes in the bone marrow or after entering the blood. Ly6C^{hi} monocytes are able to shuttle back into the bone marrow in the absence of recruitment to peripheral tissues. Ly6C^{hi} monocytes can give rise to macrophages (Mφ) and monocyte-derived DCs upon entry into lymphoid and non-lymphoid tissues. Ly6C^{hi} monocytes differentiate into CX₃CR1^{hi} macrophages in the small intestinal and colonic lamina propria in the steady state, and give rise to CX₃CR1^{int} MNPs during inflammation. Ly6C^{hi} monocytes can also become TNFα- and inducible nitric oxide synthase (iNOS)-producing dendritic cells (Tip-DCs) or macrophages during inflammation. Adapted from (Geissmann et al., 2010b).

cMoPs were identified in BM and spleen (Hettinger et al., 2013), raising the possibility that Ly6C^{hi} monocytes might develop in the spleen in addition to the BM. The spleen does not appear to be a site of monocyte production in the steady state (Swirski et al., 2009). However, following macrophage depletion, or during thioglycollate-induced peritoneal inflammation, splenic cMoPs gave rise to liver and spleen tissue-resident macrophages (Hettinger et al., 2013). Additionally, during colitis, GMPs with the ability to become monocytes and neutrophils have been shown to accumulate in the spleen and colon (Griseri et al., 2012), further supporting the possibility that the spleen and other tissues may act as sites of monocyte generation during perturbation of homeostasis.

Ly6C^{lo} monocytes seem to arise from Ly6C^{hi} monocytes (Sunderkotter et al., 2004; Varol et al., 2007; Yona et al., 2013). A recent study suggests that this process occurs independently in the bone marrow and circulation, and that the latter pathway feeds the pool of Ly6C^{lo} monocytes found in the spleen (Yona et al., 2013). The differentiation of Ly6C^{lo} monocytes from Ly6C^{hi} monocytes in steady state mouse blood has been definitively shown (Yona et al., 2013), however it remains to be confirmed whether their generation in BM requires a Ly6C^{hi} monocyte intermediate, or if MDPs or cMoPs can give rise to Ly6C^{lo} BM monocytes directly.

Both monocyte subsets are entirely dependent upon the growth factor macrophage colony-stimulating factor (M-CSF, also known as colony-stimulating factor 1 or CSF-1), demonstrated by the severe monocytopenia which occurs in mice deficient in M-CSF- or M-CSFR (Cecchini et al., 1994; Dai et al., 2002; Wiktor-Jedrzejczak et al., 1982). The M-CSFR is expressed by monocytes, macrophages, DCs and their precursors (MacDonald et al., 2005; Sasmono et al., 2003), and its expression by myeloid progenitors requires the transcription factor PU.1 (DeKoter et al., 1998). M-CSF appears to be differentially

involved in multiple stages of monocyte/macrophage differentiation and to varying degrees between tissues (Wiktor-Jedrzejczak et al., 1992). M-CSFR also binds IL-34 (Lin et al., 2008), which might play an additional role in MNP development since M-CSF-deficient osteopetrotic (*Csf1^{op/op}*) mice (Marks and Lane, 1976) display less severe defects in monocytes, macrophages and their precursors than M-CSFR-deficient mice (Dai et al., 2002).

The half-life of circulating Ly6C^{hi} and Ly6C^{lo} monocytes in the steady state is approximately 17-20 hours and two days, respectively (Sunderkotter et al., 2004; van Furth and Cohn, 1968; van Furth et al., 1972; Yona et al., 2013). However, in the absence of renewal by Ly6C^{hi} monocytes, Ly6C^{lo} monocytes could survive for 11 days (Yona et al., 2013). Ly6C^{lo} monocyte survival was partially reduced by antibody-mediated M-CSFR blockade, and it was suggested that their extended survival in the absence of Ly6C^{hi} monocytes might be due to greater availability of M-CSF (Yona et al., 2013). This could indicate a mechanism by which the Ly6C^{lo} population maintains itself when Ly6C^{hi} monocytes must fulfil other functions. Ly6C^{lo} monocytes in the blood also depend upon CX₃CR1 and its sole ligand CX₃C ligand 1 (CX₃CL1, also known as fractalkine (Bazan et al., 1997)) for survival, and without this signal can undergo apoptosis (Geissmann et al., 2003; Kim et al., 2011a; Landsman et al., 2009). In contrast, Ly6C^{lo} monocytes in BM were unaffected in CX₃CR1-deficient mice (Auffray et al., 2007). Ly6C^{lo} monocytes are thought to remain in the blood vessel lumen and to monitor the integrity of the endothelium in a CX₃CR1-dependent manner (Auffray et al., 2007; Carlin et al., 2013).

PU.1 is an essential transcription factor during monocyte development. In addition to contributing to M-CSFR expression, PU.1 restricts the alternative differentiation potential of haematopoietic progenitors at multiple stages of myelopoiesis. PU.1 restricts these

alternate fates via carefully balanced antagonism of other transcription factors, such as GATA-1, GATA-2 and CCAAT/enhancer binding protein alpha (C/EBP α), thus restricting erythroid/megakaryocyte, mast cell and granulocyte development, respectively (Auffray et al., 2009; Dahl et al., 2003; Walsh et al., 2002). Interferon regulatory factor-8 (IRF-8) and Krüppel-like factor 4 (KLF4) can also induce differentiation of monocytes and macrophages (Feinberg et al., 2007; Tamura et al., 2000), as can MafB and c-Maf, which are highly expressed in these cells (Bakri et al., 2005; Eichmann et al., 1997; Gemelli et al., 2006; Hegde et al., 1999; Kelly et al., 2000; Sieweke et al., 1996). In addition, Ly6C^{lo} monocyte differentiation has been shown to require the transcription factor NR4A1 (Nur77) (Hanna et al., 2011). MDPs were unaffected by NR4A1-deficiency, suggesting that it plays a specific role in the differentiation and survival of Ly6C^{lo} monocytes (Hanna et al., 2011).

1.2.4.3 Egress of Ly6C^{hi} monocytes from bone marrow

Ly6C^{hi} monocyte emigration from the bone marrow into the circulation requires CCR2 (Serbina and Pamer, 2006). This was demonstrated using *Ccr2*^{-/-} mice which exhibited fewer circulating Ly6C^{hi} monocytes and accumulation of activated monocytes in BM following infection with *Listeria monocytogenes* (Serbina and Pamer, 2006). Crucially, this work discovered that CCR2-deficient monocytes transferred intravenously were unimpaired in their ability to migrate from the circulation to sites of infection, pinpointing the specific role of CCR2 in egress from the bone marrow.

Shortly after, CC chemokine ligand 2 (CCL2) and CCL7 (also known as monocyte chemoattractant protein (MCP)-1 (Yoshimura et al., 1989) and MCP-3 (Van Damme et al.,

1992), respectively) were shown to promote Ly6C^{hi} monocyte exit from the BM (Tsou et al., 2007). Intravenous administration of LPS and infection with *Listeria monocytogenes* have been shown to induce CCL2 expression in bone marrow vascular sinus-associated cells, including mesenchymal stem cells (MSCs) and their progeny such as CXCL12-abundant reticular (CAR) cells (Shi et al., 2011). CCL2 was also expressed to a lesser extent by vascular endothelial cells (Shi et al., 2011). Accordingly, MSC-restricted CCL2 deficiency impaired Ly6C^{hi} monocyte emigration to the blood and increased the susceptibility of mice to infection with *L. monocytogenes*, which requires Ly6C^{hi} monocyte-mediated defence (Serbina et al., 2012). This led to the proposal that MSCs and their progeny control circulating Ly6C^{hi} monocyte frequency during infection via CCL2 secretion in response to circulating TLR ligands (Shi et al., 2011). When MSCs were unable to produce CCL2, the frequency of circulating Ly6C^{hi} monocytes was also reduced in mice not administered LPS (Shi et al., 2011), indicating that constitutive expression of CCL2 might control the release of Ly6C^{hi} monocytes from the BM in the steady state. Low levels of CCL2 and CCL7 have been observed in normal mouse plasma (Wang et al., 2009) which could contribute to the homeostatic trafficking of Ly6C^{hi} monocytes from the BM into the circulation (Tsou et al., 2007).

Ly6C^{hi} monocytes transferred intravenously can be recovered from the BM of recipient mice (Varol et al., 2007). However, return of Ly6C^{hi} monocytes from the bloodstream to the BM was not observed when mice were also administered thioglycollate to induce peritoneal inflammation (Varol et al., 2007). This indicates that in the absence of recruitment to peripheral tissues or lymphoid organs stimulated by inflammation or infection, Ly6C^{hi} monocytes can traffic in both directions between the BM and bloodstream. How this process is controlled is unclear, although it is possible that the

CCL2 expressed by BM MSCs, CAR cells and vascular endothelium (Shi et al., 2011) could facilitate the return of Ly6C^{hi} monocytes to the BM in addition to their emigration.

Interestingly, the abundance of Ly6C^{hi} monocytes in the circulation and spleen follows a diurnal rhythm (Nguyen et al., 2013). When mice were maintained under standard animal facility conditions, exposed to light and dark in alternating 12 hour periods, numbers of Ly6C^{hi} monocytes in the blood and spleen peaked around 4 hours before the nocturnal mice awoke, approximately 8 hours into a 12 hour period of light exposure (Nguyen et al., 2013). The authors postulate that Ly6C^{hi} monocyte levels peaked at this time in “anticipatory inflammation”, readying the innate immune system for potentially greater exposure to pathogens during the daily active period of mice. This diurnal rhythm was overridden by administration of exogenous CCL2, representing a mechanism by which pathogen-directed responses could supersede the circadian system if necessary (Nguyen et al., 2013). These findings could affect the results of experiments conducted *in vivo* therefore it may be prudent to conduct experimental procedures and measurements at consistent and well-defined times.

1.2.4.4 Functions of Ly6C^{hi} monocytes

Ly6C^{hi} monocytes used to be referred to as “inflammatory” monocytes due to their propensity to migrate to inflamed tissues, however they are now known also to play important roles during the steady state and to avoid bias have been designated “classical” monocytes (Ziegler-Heitbrock et al., 2010). Ly6C^{hi} monocytes exhibit high plasticity, and can perform a variety of functions in a context-dependent manner, likely influenced by local environmental factors (Lauvau et al., 2014).

During inflammation, infection or injury, Ly6C^{hi} monocytes are rapidly mobilised from the BM, and in some cases the spleen (Swirski et al., 2009), into the blood through which they traffic to peripheral tissues (Shi and Pamer, 2011). Ly6C^{hi} monocytes have been shown to play protective roles in numerous infections including *Listeria monocytogenes* (Serbina et al., 2003); *Mycobacterium tuberculosis* (Peters et al., 2004; Peters et al., 2001; Samstein et al., 2013; Skold and Behar, 2008); *Leishmania major* (De Trez et al., 2009; Leon et al., 2007); *Plasmodium chabaudi*, used as a model of malaria (Sponaas et al., 2009); West Nile virus (Lim et al., 2011); *Cryptococcus neoformans* (Traynor et al., 2000); and *Aspergillus fumigatus* (Hohl et al., 2009; Rivera et al., 2011).

Ly6C^{hi} monocytes can also prove detrimental to the host during immune responses to pathogens including *Trypanosoma brucei* (Bosschaerts et al., 2010) and *M. tuberculosis* (Antonelli et al., 2010). Additionally, Ly6C^{hi} monocytes have been implicated in the pathogenesis of sterile inflammatory diseases, such as atherosclerosis (Swirski et al., 2007), and autoimmune diseases including multiple sclerosis and its murine model experimental autoimmune encephalomyelitis (King et al., 2009; Mildner et al., 2009).

Ly6C^{hi} monocytes exhibit a number of functions. The Ly6C^{hi} monocyte-derived cells required for protective immunity against *L. monocytogenes* expressed high levels of TNF α and inducible nitric oxide synthase (iNOS) and became known as TNF α - and iNOS-producing “DCs” (TipDCs) (Serbina et al., 2003). Their classification as DCs was based on expression of CD11c and MHCII, rather than on any functional characteristics of DCs such as migration to tissue-draining LNs or T cell priming, which we now know is insufficient to warrant such classification (Geissmann et al., 2010a). Numerous other studies have observed pro-inflammatory cytokine and reactive oxygen species production by Ly6C^{hi} monocytes and their derivatives (Bosschaerts et al., 2010; De Trez et al., 2009; Narni-

Mancinelli et al., 2007; Narni-Mancinelli et al., 2011). These cells have been shown to perform many additional functions, including phagocytosis and microbial killing (Narni-Mancinelli et al., 2011; Serbina et al., 2008; Sponaas et al., 2009); transport of antigen from sites of infection to draining LNs (Hohl et al., 2009; Samstein et al., 2013); and antigen presentation which, although they seem unlikely to prime naïve T cells *in vivo*, may drive restimulation of effector cells (Lauvau et al., 2014). Ly6C^{hi} monocytes can also give rise to macrophages with tissue repair functions, which promote the resolution of inflammation, such as during spinal cord injury (Shechter et al., 2009). Although their production of mediators and functions can overlap, a wide variety of combinations exists, illustrating the plasticity of Ly6C^{hi} monocytes and the difficulty in generalising about these cells (Lauvau et al., 2014; Shi and Pamer, 2011).

Although certain factors, such as IFN γ (Goldszmid et al., 2012; Kang et al., 2008; Soudja et al., 2014), CCL3 and TNF α (Narni-Mancinelli et al., 2007) amongst others, have been shown to induce Ly6C^{hi} monocyte activation *in vivo*, what controls their functional differentiation under many circumstances remains unclear. A variety of pattern recognition receptors, including TLR2, TLR4 and TLR9, have been implicated in the activation and differentiation of Ly6C^{hi} monocytes by studies in gene knockout mice (Lauvau et al., 2014). However, in the absence of confirmation by adoptive transfer of gene knockout Ly6C^{hi} monocytes to wild-type recipients, there is little direct evidence of cell-intrinsic requirements for specific PRRs *in vivo* (Lauvau et al., 2014). The mechanisms controlling Ly6C^{hi} monocyte recruitment to peripheral tissues are also incompletely understood, and vary in response to different stimuli (Lauvau et al., 2014; Shi and Pamer, 2011). For instance, CCR2 is probably important in recruiting Ly6C^{hi} monocytes to the colon during DSS-induced colitis (Bain et al., 2013; Zigmond et al., 2012); while CCR1 was

required for their recruitment to the ileum during *Toxoplasma gondii*-induced ileitis (Schulthess et al., 2012). Ly6C^{hi} monocyte recruitment to the inflamed intestine is discussed further below.

1.2.4.5 Functions of Ly6C^{lo} monocytes

Although initially thought to be the precursors of tissue resident macrophages, Ly6C^{lo} monocytes do not enter non-lymphoid organs after adoptive transfer (Bain et al., 2013; Varol et al., 2009) and are now thought to patrol the blood vessel lumen and to monitor the integrity of the endothelium in a CX₃CR1-dependent manner (Auffray et al., 2007; Carlin et al., 2013). Sensing of nucleic acids by Ly6C^{lo} monocytes via TLR7 has been shown to induce neutrophil recruitment, resulting in focal necrosis of endothelial cells which were then cleared by the Ly6C^{lo} monocytes (Carlin et al., 2013). Endothelial patrolling and TLR7/8-mediated sensing of nucleic acids has also been observed in the equivalent human CD14^{lo} monocyte population (Cros et al., 2010). Due to their functions in the bloodstream which are reminiscent of more mature MNPs, Ly6C^{lo} monocytes might rather be considered blood-resident macrophages (Ginhoux and Jung, 2014).

1.2.5 Intestinal monocyte-derived mononuclear phagocytes

1.2.5.1 Ly6C^{hi} monocytes are the precursors of intestinal macrophages

As in other tissues, Ly6C^{hi} monocytes were well known to be recruited to the intestine during inflammation or infection with pathogens. However, it is now clear that they are

also the precursors of intestinal tissue macrophages in the steady state (Bain et al., 2014; Bain et al., 2013; Tamoutounour et al., 2012).

Recent studies, using techniques including fate mapping, parabiosis and bone marrow chimeric mice, have shown that in a number of tissues, macrophage populations derive from embryonic precursors which maintain themselves during adult life by self-renewal (Ginhoux and Jung, 2014). These include microglia in the central nervous system (Ajami et al., 2007; Ginhoux et al., 2010; Kierdorf et al., 2013; Schulz et al., 2012; Yona et al., 2013), lung alveolar macrophages (Guilliams et al., 2013; Hashimoto et al., 2013; Yona et al., 2013), peritoneal macrophages (Hashimoto et al., 2013), Kupffer cells in the liver (Schulz et al., 2012; Yona et al., 2013) and epidermal Langerhans cells (Chorro et al., 2009; Hoeffel et al., 2012; Merad et al., 2002). However, the intestine has proved a rare exception to this model (Bain et al., 2014). Some cardiac macrophages (Molawi et al., 2014) and dermal macrophages in the skin also seem to derive from Ly6C^{hi} monocytes (Tamoutounour et al., 2013).

A number of findings indicate that intestinal macrophages arise from Ly6C^{hi} monocytes. The first studies to show that Ly6C^{hi} monocytes might be the precursors of small intestinal and colonic lamina propria macrophages did so by adoptive transfer of monocytes into [CD11c-DTR→WT] BM chimeric mice (in which CD11c⁺ cells express the diphtheria toxin receptor under control of the CD11c promoter) depleted of mononuclear phagocytes by diphtheria toxin treatment (Bogunovic et al., 2009; Varol et al., 2007; Varol et al., 2009). Although these studies showed the potential of Ly6C^{hi} monocytes to replenish more mature MNPs in the intestine after their severe depletion, it remained unclear whether the CX₃CR1⁺ cells which arose were DCs or macrophages, or whether Ly6C^{hi} monocytes could act as the precursors of intestinal MNPs during homeostatic conditions. This has

recently been clarified, by means of BrdU pulse-chase and adoptive transfer studies, which showed that Ly6C^{hi} monocytes were continually recruited to the unmanipulated colonic and small intestinal lamina propria where they differentiated into CX₃CR1^{hi} Ly6C⁻ F4/80^{hi} resident macrophages (Bain et al., 2013; Tamoutounour et al., 2012). CX₃CR1^{hi} macrophages were CD64^{hi} (Bain et al., 2013; Tamoutounour et al., 2012), a marker which allows discrimination between CD64⁺ macrophages and monocyte-derived DCs versus CD64⁻ cDCs (Langlet et al., 2012), and also expressed MHCII and variable levels of CD11c, explaining their previous misidentification as DCs (Bogunovic et al., 2009; Varol et al., 2009). The maturation of Ly6C^{hi} monocytes into CX₃CR1^{hi} resident macrophages was shown to take around five days (Bain et al., 2013) and occurred through a series of intermediary stages, which gradually lost or gained expression of various markers (Figure 1.3) (Bain et al., 2013; Tamoutounour et al., 2012). The surface-expressed molecules CX₃CR1, Ly6C, MHCII, CD64 and F4/80 can thus be used to distinguish between these stages (Table 1.1). Ly6C^{hi} monocytes and their CX₃CR1^{int} progeny are infrequent in the steady state intestinal lamina propria (Bain et al., 2013). In addition to phenotypic changes, differentiating MNPs progressively acquired other characteristics of steady state CX₃CR1^{hi} intestinal macrophages, such as phagocytic activity, vacuolar appearance, constitutive expression of IL-10 and TNF α , and hyporesponsiveness to TLR stimulation (Bain et al., 2013; Rivollier et al., 2012) (see below).

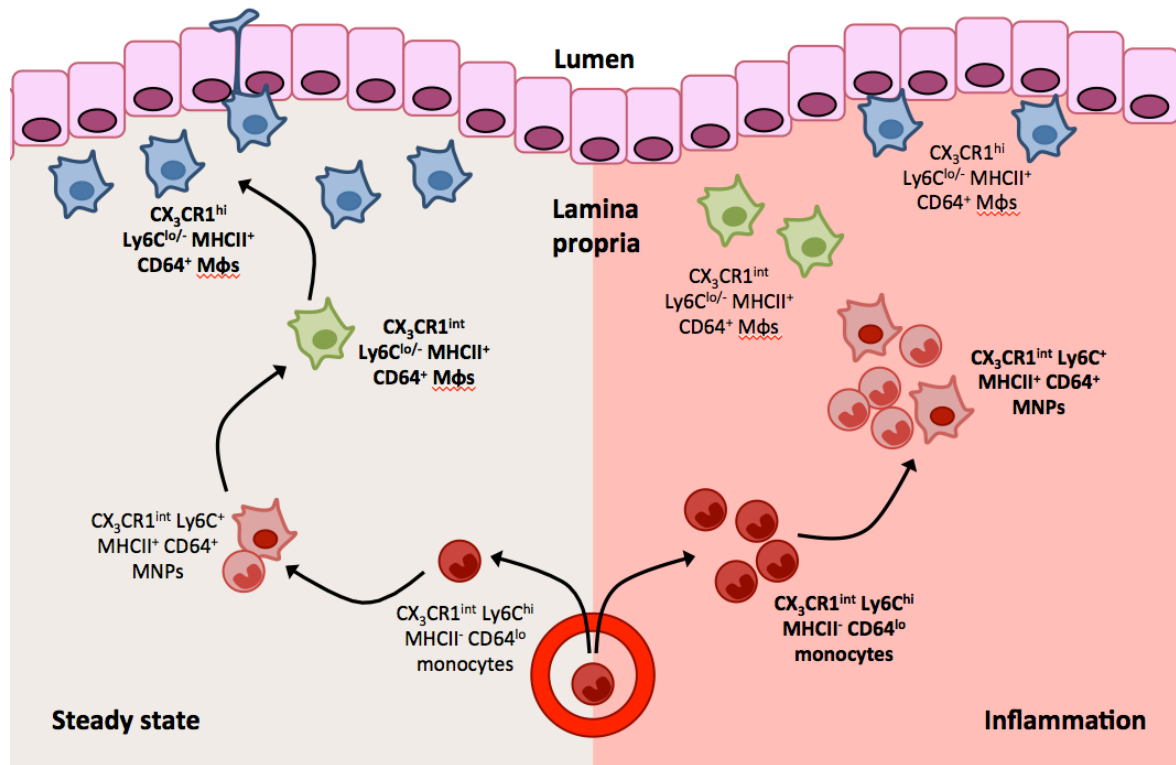


Figure 1.3: Differentiation of Ly6C^{hi} monocytes in the intestinal lamina propria.

During the steady state, Ly6C^{hi} monocytes are recruited from the blood into the intestinal lamina propria, where they differentiate into CX₃CR1^{hi} resident macrophages through a series of CX₃CR1^{int} intermediaries. During inflammation, Ly6C^{hi} monocyte recruitment to the lamina propria is enhanced. The normal differentiation of Ly6C^{hi} monocytes is disrupted by unknown factors, resulting in accumulation of CX₃CR1^{int} Ly6C⁺ pro-inflammatory MNPs during inflammation. Adapted from (Bain and Mowat, 2014).

Population: mouse (human)	Cell surface marker expression: mouse (human)
Ly6C ^{hi} monocytes (CD14 ^{hi} monocytes)	CX ₃ CR1 ^{int} Ly6C ^{hi} MHCII ⁻ CD64 ^{lo} (CD14 ^{hi} CD11c ^{hi} HLA-DR ^{lo} CD209 ^{lo} CD163 ^{lo})
Ly6C ⁺ MHCII ⁺ MNPs (?)	CX ₃ CR1 ^{int} Ly6C ⁺ MHCII ⁺ CD64 ⁺ (CD14 ^{hi} CD11c ^{hi/lo} HLA-DR ^{hi} CD209 ^{lo} CD163 ^{lo})
CX ₃ CR1 ^{int} Ly6C ^{lo/-} macrophages (?)	CX ₃ CR1 ^{int} Ly6C ^{lo/-} MHCII ⁺ CD64 ⁺ F4/80 ⁺ (CD14 ^{hi} CD11c ^{lo} HLA-DR ^{hi} CD209 ^{hi} CD163 ^{hi})
CX ₃ CR1 ^{hi} macrophages (CD14 ^{lo} macrophages)	CX ₃ CR1 ^{hi} Ly6C ^{lo/-} MHCII ⁺ CD64 ⁺ F4/80 ^{hi} (CD11c ⁻ HLA-DR ⁺ CD209 ⁺ CD163 ^{+/-})

Table 1.1: Cell surface marker expression of stages in intestinal macrophage differentiation. From (Bain et al., 2013; Tamoutounour et al., 2012).

The equivalent of Ly6C^{hi} monocytes, CD14^{hi} monocytes, might also be the source of human intestinal macrophages (Bain et al., 2013). Analysis of cells from healthy ileum showed that two main populations existed among CD14⁺ macrophages, distinguishable by their level of CD14 expression (Bain et al., 2013). The CD14^{lo} population was comprised of HLA-DR⁺ CD209⁺ CD163^{+/-} macrophages which were mainly CD11c⁻, presumed equivalents of murine CX₃CR1^{hi} macrophages (Table 1.1); and a smaller population expressing CD11c, which may represent less mature macrophages (Bain et al., 2013). The CD14^{hi} population contained three subsets defined by their expression of CD11c, HLA-DR, CD209 and CD163: CD11c^{hi} HLA-DR^{lo} CD209^{lo} CD163^{lo}, CD11c^{hi/lo} HLA-DR^{hi} CD209^{lo} CD163^{lo}, and CD11c^{lo} HLA-DR^{hi} CD209^{hi} CD163^{hi} (Bain et al., 2013). CD14^{hi} monocytes, which express CD11c, are the equivalent of Ly6C^{hi} monocytes in the mouse (Geissmann et al., 2003; Ginhoux and Jung, 2014; Ingersoll et al., 2010), therefore these CD14^{hi} populations might respectively represent the CX₃CR1^{int} Ly6C^{hi} MHCII⁻ monocytes, CX₃CR1^{int} Ly6C^{hi} MHCII⁺ MNPs and CX₃CR1^{int} Ly6C^{lo/-} MHCII⁺ macrophages observed during the differentiation of CX₃CR1^{hi} resident macrophages in the mouse intestine (Bain et al., 2013) (Table 1.1).

Following these studies, Bain et al. went on to show conclusively that intestinal macrophages in adult mice are maintained by input from circulating Ly6C^{hi} monocytes and not by self-renewal of embryonic precursors (Bain et al., 2014). Embryonic precursor-derived macrophages were present in the lamina propria of embryonic and young mice, however these cells were progressively lost and replaced by Ly6C^{hi} monocyte-derived macrophages, leaving less than 1,000 embryo-derived macrophages in the intestine of adult mice (Bain et al., 2014). Lamina propria macrophages present in the first few weeks of life proliferated *in situ*, but this diminished as Ly6C^{hi} monocytes began

to enter the colonic lamina propria and gave rise to macrophages 2-3 weeks after birth, coinciding with weaning (Bain et al., 2014). Comparison of *Ccr2*^{-/-} with wild-type mice confirmed that the Ly6C⁺ intermediaries and Ly6C⁻ macrophages appearing in the lamina propria were derived from CCR2-dependent Ly6C^{hi} monocytes, and showed that the contribution of Ly6C^{hi} monocytes was essential to maintain the intestinal macrophage pool in adult mice (Bain et al., 2014). Further evidence of the monocytic origin of adult intestinal macrophages came from parabiotic studies, which observed the same degree of chimerism among Ly6C^{hi} blood monocytes as among the monocyte-derived MNP populations in the intestine (Bain et al., 2014).

In contrast, Ly6C^{lo} monocytes have not been observed in the intestinal lamina propria following adoptive transfer (Bain et al., 2014; Bain et al., 2013; Varol et al., 2007; Varol et al., 2009). Furthermore, CX₃CR1-deficient mice – which have defects in Ly6C^{lo} blood monocytes due to the requirement of CX₃CR1-CX₃CL1 signals for their survival (Kim et al., 2011a; Landsman et al., 2009) – seem to have normal numbers of intestinal macrophages (Hadis et al., 2011), indicating that Ly6C^{lo} monocytes do not act as precursors of these cells.

The factors controlling the differentiation of Ly6C^{hi} monocytes into CX₃CR1^{hi} resident macrophages are unknown. Discovery of these factors is an important area of research, which could lead to advances in manipulating intestinal MNPs for the treatment of intestinal inflammation.

1.2.5.2 Functions of CX_3CR1^{hi} macrophages in the steady state

CX_3CR1^{hi} macrophages are found throughout the lamina propria in the small and large intestine, often in close proximity to the epithelium (Niess et al., 2005; Schulz et al., 2009). These cells, like most tissue macrophages, perform housekeeping functions such as clearance of apoptotic cells (Muller et al., 2012; Nagashima et al., 1996; Smythies et al., 2005), which is particularly important in the intestine due to continual renewal of the epithelium (van der Flier and Clevers, 2009). Intestinal macrophages also play an important role in maintaining the integrity of the epithelial barrier by stimulating the proliferation of epithelial progenitors via production of prostaglandin (PG) E_2 (Pull et al., 2005). Colitis induced in mice by administration of dextran sodium sulphate (DSS) in drinking water induces severe damage to the colonic epithelium (Okayasu et al., 1990). Susceptibility to and severity of DSS colitis are enhanced when $M-CSFR^{+}$ macrophages are depleted (Qualls et al., 2006), and epithelial regeneration in this model was found to require myeloid differentiation primary response gene 88 (MyD88) signalling in myeloid cells (Malvin et al., 2012). Intestinal MNPs have been shown to produce PGE_2 in response to commensals and various TLR ligands, including those for TLRs 2, 4, 5 and 9 (Grainger et al., 2013). All of these TLRs require MyD88 to signal, with the exception of TLR4 which can also signal via TRIF (Medzhitov, 2001). TLR-dependent production of PGE_2 might explain the requirement of MyD88 signalling in myeloid cells for intestinal epithelial repair during DSS colitis.

The better-known role of intestinal macrophages is as crucial innate immune effector cells. They are highly phagocytic and bactericidal (Smythies et al., 2005), allowing them to neutralise any breaches of the intestinal epithelial barrier to which they are so close. One of the characteristic features of intestinal resident macrophages is their

hyporesponsiveness to bacteria or bacterial components (Bain et al., 2013; Monteleone et al., 2008; Smythies et al., 2005). This feature prevents the development of deleterious inflammation that might otherwise be induced by the continual exposure of intestinal immune cells to the commensal microbiota, particularly in the large intestine and terminal ileum where the commensal burden is greatest (Mowat and Agace, 2014). Rather than lacking expression of TLRs, CX₃CR1^{hi} resident macrophages seem to downregulate expression of key TLR signalling molecules, such as MyD88 and TNF receptor-associated factor 6 (TRAF6) (Smythies et al., 2010; Zigmond et al., 2012), and to upregulate negative regulators of TLR and NFκB signalling, including A20 and IκBNS (Hirotsani et al., 2005; Smythies et al., 2010; Zigmond et al., 2012). Critically, CX₃CR1^{hi} macrophages maintain their anti-inflammatory phenotype even during intestinal inflammation (Bain et al., 2013; Tamoutounour et al., 2012; Weber et al., 2011; Zigmond et al., 2012) and might therefore provide an important counterbalance during intestinal inflammation.

A key mediator preventing intestinal macrophages from mounting pro-inflammatory responses is IL-10. Hyporesponsiveness to TLR stimulation was reversed in *ex vivo* small intestinal MNPs by treatment with anti-IL-10R prior to stimulation (Monteleone et al., 2008). The majority of CX₃CR1^{hi} macrophages in the colonic lamina propria constitutively produce high levels of IL-10 and low levels of TNFα, which they continue to co-express when stimulated by LPS *in vitro* (Bain et al., 2013). This suggests that these cells can act as an autocrine or paracrine source of inhibitory IL-10, which is further supported by previous findings regarding peritoneal macrophages and macrophage cell lines (Fiorentino et al., 1991; Pils et al., 2010). Production of IL-10 by intestinal macrophages appears to be induced by signals from the intestinal microbiota, since macrophages from

the colonic lamina propria of germ-free mice produce reduced levels of IL-10 (Rivollier et al., 2012; Ueda et al., 2010).

IL-10 has long been recognised as a potent anti-inflammatory cytokine in the intestine, defects in which lead to early onset IBD (Shah et al., 2012). The ability of IL-10 to maintain intestinal homeostasis seems to depend upon its actions on macrophages, as illustrated by the development of colitis in mice lacking this pathway. LysM-Cre-driven deficiency in STAT3, a signalling molecule downstream of the IL-10 receptor, results in aberrant macrophage activation and spontaneous T cell-dependent colitis in mice by 20-24 weeks of age (Takeda et al., 1999). Colitis in these mice was ameliorated by the transfer of wild-type CX₃CR1^{hi} macrophages (Kayama et al., 2012). CX₃CR1^{hi} macrophages were shown to suppress the activation and proliferation of CD4⁺ T cells in a cell contact-dependent manner via low macrophage expression of the costimulatory molecules CD80 and CD86, which required IL-10/STAT3 signalling (Kayama et al., 2012).

CX₃CR1⁺ cells in the LP have been shown to extend transepithelial dendrites into the lumen in a CX₃CR1-dependent manner, and although originally believed to be DCs, these now seem likely to be macrophages (Kim et al., 2011a; Niess et al., 2005; Rescigno et al., 2001). Investigation of this process in the small intestine showed that it occurred only in the terminal ileum (Niess et al., 2005), and whether CX₃CR1⁺ MNPs extend transepithelial dendrites in the caecum or colon is unclear. Although not entirely absent, internalisation of *Citrobacter rodentium* expressing the red fluorescent protein mRuby by colonic CX₃CR1⁺ MNPs was reduced in CX₃CR1-deficient mice (Manta et al., 2013), suggesting that a CX₃CR1-dependent mechanism such as transepithelial dendrite extension could also occur in the colon.

1.2.5.3 *CX₃CR1^{int} MNPs accumulate during intestinal inflammation*

During intestinal inflammation, the composition of the intestinal MNP compartment changes. Ly6C^{hi} MHCII⁺ monocytes and their CX₃CR1^{int} progeny – Ly6C⁺ MHCII⁺ MNPs and Ly6C^{lo/-} MHCII⁺ CD64⁺ F4/80⁺ macrophages – accumulate (Figure 1.3), resulting in a reversal of the relative proportions of CX₃CR1^{hi} and CX₃CR1^{int} MNPs (Bain et al., 2013; Platt et al., 2010; Rivollier et al., 2012; Tamoutounour et al., 2012; Weber et al., 2011; Zigmond et al., 2012). Accumulation of CX₃CR1^{int} MNPs has been observed in colitis induced by DSS (Bain et al., 2013; Platt et al., 2010; Zigmond et al., 2012) and by adoptive transfer of naïve CD4⁺ T cells into T cell-deficient hosts (Rivollier et al., 2012; Tamoutounour et al., 2012; Weber et al., 2011). Initially it was unclear whether these CX₃CR1^{int} MNPs were derived from newly arrived Ly6C^{hi} monocytes or if resident CX₃CR1^{hi} macrophages present in the steady state lamina propria altered their phenotype during inflammation. A number of studies have now shown that in the inflamed lamina propria, Ly6C^{hi} monocytes which enter the intestine fail to differentiate fully, leading to the accumulation of CX₃CR1^{int} MNPs; and that CX₃CR1^{hi} macrophages maintain their anti-inflammatory phenotype even during inflammation (Bain et al., 2013; Rivollier et al., 2012; Tamoutounour et al., 2012; Weber et al., 2011; Zigmond et al., 2012).

The factors controlling the differentiation of Ly6C^{hi} monocytes into CX₃CR1^{hi} macrophages in the steady state intestine are unknown, as is the cause of interruption to this process when the intestine becomes inflamed. It has been suggested that during inflammation, the normal conditioning of Ly6C^{hi} monocytes (which induces the acquisition of the characteristic phenotypic and functional features of anti-inflammatory CX₃CR1^{hi} macrophages) does not take place, resulting in the accumulation of Ly6C^{hi} monocytes and CX₃CR1^{int} MNPs which still respond to TLR stimulation (Bain and Mowat, 2014).

During resolution of DSS colitis, the frequency of CX₃CR1^{int} MNPs contracted to baseline levels (Zigmond et al., 2012). The mechanisms promoting this contraction and the resolution of colitis are unknown.

1.2.5.4 What should we call Ly6C⁺ MHCII⁺ monocyte-derived cells?

A current problem in the MNP field is that we lack sufficient terms to distinguish between similar yet apparently distinct subsets of cells. This applies particularly to a subset of Ly6C^{hi} MHCII⁺ cells now described in a number of tissues, the roles of which are beginning to be revealed (Bain et al., 2013; Grainger et al., 2013; Jakubzick et al., 2013; Tamoutounour et al., 2012). These cells have been referred to, potentially inappropriately, as “monocytes” (Grainger et al., 2013; Jakubzick et al., 2013).

In one such instance, these cells were observed in the inflamed ileum during *Toxoplasma gondii* infection, where they were shown to negatively regulate neutrophil activity and thus inflammation (Grainger et al., 2013); while Jakubzick *et al.* found that Ly6C^{hi} MHCII⁺ cells were present in steady state tissues including the skin and lung, and could transport antigen to lung-draining LNs (Jakubzick et al., 2013). In the latter instance the authors named these cells “monocytes” due to their gene expression profile, which was similar to that of blood monocytes and lacked a DC or macrophage signature (Jakubzick et al., 2013). However, as a recent review highlights, the cells had lost the ability to recirculate, which is a hallmark of monocytes (Ginhoux and Jung, 2014).

Bain and Mowat have suggested the alternative terms “maturing monocytes” and “effector monocytes” (Mildner et al., 2013) for the Ly6C⁺ MHCII⁺ cells found in the resting and inflamed intestinal lamina propria, respectively (Bain and Mowat, 2014). The term

maturing monocyte is logical considering that these cells represent a transient intermediate population during the differentiation of Ly6C^{hi} MHCII⁻ monocytes into CX₃CR1^{hi} resident macrophages in the steady state (Bain et al., 2013; Tamoutounour et al., 2012) (see below).

Similar, if not identical, Ly6C⁺ MHCII⁺ cells were studied in this thesis. The author remains unconvinced that these cells should be referred to as monocytes; therefore until the adoption of a more appropriate term, Ly6C⁺ MHCII⁺ cells will be referred to here as MNPs. Additionally, the term MNP may be applied in cases of ambiguity when reporting findings from the literature, for instance if definitive identification of macrophages or DCs cannot be made on the basis of reported characteristics.

1.2.5.5 Functions of CX₃CR1^{int} MNPs during inflammation

In contrast to CX₃CR1^{hi} resident macrophages, the CX₃CR1^{int} Ly6C⁺ MNPs (Ly6C^{hi} MHCII⁻ monocytes and Ly6C⁺ MHCII⁺ MNPs) which accumulate during colitis display pro-inflammatory features, including expression of pro-inflammatory cytokines, such as TNF α , IL-1 β , IL-6, IL-12, and IL-23; and iNOS (Bain et al., 2013; Platt et al., 2010; Rivollier et al., 2012; Weber et al., 2011; Zigmond et al., 2012). CX₃CR1^{int} Ly6C⁺ MNPs have been shown to express TLR2, TLR4 and nucleotide-binding, oligomerisation domain-containing protein (NOD)2, through which their expression of pro-inflammatory cytokines could be induced (Bain et al., 2013; Zigmond et al., 2012).

Depletion of Ly6C^{hi} blood monocytes and CX₃CR1^{int} MNPs in the lamina propria induced by treatment with an anti-CCR2 antibody (MC-21; (Mack et al., 2001)) was shown to ameliorate DSS colitis, highlighting CX₃CR1^{int} Ly6C⁺ MNPs as drivers of colonic

inflammation (Zigmond et al., 2012). *Ccr2*^{-/-} monocytopenic mice also exhibit less severe DSS colitis (Platt et al., 2010). TNF α production seems to exacerbate disease, since DSS-induced colitis was less severe in mice with TNF α -deficient Ly6C^{hi} monocytes (Varol et al., 2009). CX₃CR1^{int} Ly6C^{hi} CD11b⁺ F4/80⁺ MNPs (which probably includes the Ly6C⁺ MHCII⁺ MNP population described above) have also been reported to recruit eosinophils during DSS colitis via production of CCL11 (Waddell et al., 2011), an axis which also seems to exist in ulcerative colitis (UC) (Ahrens et al., 2008; Lampinen et al., 2013).

During T cell transfer colitis, Ly6C^{hi} monocytes were also recruited to the MLNs where they differentiated into pro-inflammatory iNOS⁺ Ly6C⁺ MHCII⁺ CD64⁺ macrophages (Tamoutounour et al., 2012). These cells were found in the T cell zone, drove the differentiation of IFN γ ⁺ Th1 cells *in vitro* and *in vivo*, and were sufficient to drive T cell transfer colitis in mice lacking CCR7 (Tamoutounour et al., 2012), which is required for DC migration from the lamina propria to the MLNs (Jang et al., 2006). However, these cells were shown to migrate into the MLNs directly from the bloodstream, rather than from the LP (Tamoutounour et al., 2012). Ly6C^{hi} monocytes have also been shown to give rise to a population of E-cadherin⁺ DCs (Siddiqui et al., 2010). These cells were also CD11b⁺ and displayed heterogeneous expression of Gr-1 (Ly6C/Ly6G). E-cadherin⁺ DCs accumulated in the inflamed colon and MLNs during T cell transfer and anti-CD40 colitis, but not during the innate model of *Helicobacter hepaticus* (*Hh*)-induced inflammation, indicating dependence on an undefined CD40L-mediated signal (Siddiqui et al., 2010). Adoptive transfer of E-cadherin⁺ DCs exacerbated T cell transfer colitis, suggesting that these cells play a pro-inflammatory role in intestinal inflammation (Siddiqui et al., 2010).

Ly6C^{hi} monocytes and their progeny are also involved in protective immunity in the intestine. Studies in CCR2- and CCL2-deficient mice have shown that Ly6C^{hi} monocytes

are essential for control of parasite growth during infection with the protozoan parasite *Toxoplasma gondii* (Dunay et al., 2008; Robben et al., 2005). Both strains of mice succumb to lethal toxoplasmosis, which can be averted by the transfer of CCR2-sufficient Ly6C^{hi} monocytes (Dunay et al., 2008). Oral inoculation with *T. gondii* induces acute ileitis during which Ly6C^{hi} monocytes and Ly6C⁺ MHCII⁺ MNPs accumulate and exhibit expression of IL-12, TNF α and iNOS (Dunay et al., 2008), similar to their phenotype during colitis. Alongside *T. gondii* outgrowth, lower levels of IL-12p70 were observed in the serum of CCR2-deficient infected mice while levels of TNF α and IFN γ remained intact, suggesting that IL-12 production by Ly6C^{hi} monocytes or their progeny helps control *T. gondii* expansion (Serbina et al., 2008). Interestingly, recent results suggest that Ly6C⁺ MHCII⁺ MNPs can also inhibit neutrophil activity during *T. gondii*-induced ileitis via commensal-dependent production of PGE₂ (Grainger et al., 2013), highlighting the functional plasticity of Ly6C^{hi} monocytes. This negative regulation of neutrophils appeared to be important in limiting intestinal damage, which was exacerbated in its absence (Grainger et al., 2013).

It is unclear whether the macrophages involved in the immune response to intestinal helminth infection are derived from Ly6C^{hi} monocytes, as this has not been directly investigated (Bain and Mowat, 2014). However, CCL2-deficient mice were susceptible to infection with the gastrointestinal nematode *Trichuris muris* (deSchoolmeester et al., 2003). CCL2-deficient mice were completely unable to expel worms, which was associated with a reduced Th2 response and impaired recruitment of macrophages, defined as CD11b⁺ F4/80⁺ cells, to the intestine and MLNs (deSchoolmeester et al., 2003). This suggests that Ly6C^{hi} monocytes could contribute to immune responses during intestinal helminth infection.

CD14^{hi} MNPs, the human equivalents of Ly6C^{hi} monocytes and their CX₃CR1^{int} progeny (see Table 1.1), accumulate in the inflamed intestine of Crohn's disease (CD) and ulcerative colitis patients (Bain et al., 2013; Kamada et al., 2008; Thiesen et al., 2014). As in mice, these cells are responsive to microbial stimulation (Kamada et al., 2008; Ogino et al., 2013; Thiesen et al., 2014), express pro-inflammatory cytokines such as TNF α , IL-1 β , IL-6 and IL-23 (Ogino et al., 2013; Rugtveit et al., 1997) and exhibit respiratory burst activity (Rugtveit et al., 1995). During ulcerative colitis, intestinal macrophages induce recruitment of eosinophils via production of CCL11 (Ahrens et al., 2008; Lampinen et al., 2013), as observed in mice. CD14⁺ CD163^{lo} MNPs from the mucosa of CD patients also had increased ability to induce Th17 cells relative to their counterparts from normal intestine (Ogino et al., 2013). Collectively, these results suggest that the functions of monocyte-derived MNPs during intestinal inflammation are relatively conserved between mouse and human.

1.2.5.6 Recruitment of Ly6C^{hi} monocytes to the intestine

1.2.5.6.1 CCR2

A number of recent studies report a link between CCR2 and the recruitment of Ly6C^{hi} monocytes and macrophages to the resting and inflamed intestine during DSS-, hapten- and *Citrobacter rodentium*-induced colitis (Bain et al., 2013; Khan et al., 2006; Kim et al., 2011b; Platt et al., 2010; Popivanova et al., 2009; Takada et al., 2010; Zigmond et al., 2012). As CCR2 controls monocyte egress from the bone marrow (Serbina and Pamer, 2006), defects in CCR2-deficient monocyte accumulation in the lamina propria (Bain et al., 2014; Bain et al., 2013; Platt et al., 2010; Zigmond et al., 2012) could be due to their

inability to exit the bone marrow, rather than an inability to enter the intestine from the bloodstream. The strongest evidence that CCR2 might mediate Ly6C^{hi} monocyte entry into the intestinal lamina propria during the steady state comes from a study using wild-type:*Ccl2*^{-/-} parabiotic mice in which CCR2-sufficient Ly6C⁺ MHCII⁺ MNPs were not found in the colonic lamina propria of *Ccl2*^{-/-} mice (Takada et al., 2010). In BM chimaeras reconstituted with a mixture of wild-type and *Ccr2*^{-/-} BM, although 4-5% of Ly6C^{hi} monocytes in the blood came from *Ccr2*^{-/-} BM, only less than 1% of each Ly6C^{hi} monocyte-derived MNP population in the lamina propria derived from these cells (Bain et al., 2014). This indicates that, despite the fact that CCR2-deficient MNPs were not completely absent from the lamina propria, *Ccr2*^{-/-} Ly6C^{hi} monocytes were impaired in their ability to enter the lamina propria from the bloodstream (Bain et al., 2014). Intravenous co-transfer of *Ccr2*^{-/-} and wild-type Ly6C^{hi} BM monocytes into mice with DSS-induced colitis also illustrated that CCR2-sufficient monocytes have a competitive advantage in homing to the inflamed lamina propria, since virtually all monocytes found in the colon were of wild-type origin (Zigmond et al., 2012).

Since Ly6C^{hi} monocyte-derived MNPs are not entirely absent from the lamina propria of *Ccr2*^{-/-} (Bain et al., 2013) and *Ccl2*^{-/-} mice (Takada et al., 2010), it is possible that other mechanisms also control Ly6C^{hi} monocyte recruitment. CCR1 was recently shown to be essential for the recruitment of Ly6C^{hi} monocytes to the inflamed ileum during *T. gondii*-induced ileitis (Schulthess et al., 2012). During inflammation, IL-15-dependent NKp46⁺ innate lymphoid cells produced the CCR1 ligand CCL3 in response to stromal cell-derived IL-18, leading to CCR1-mediated recruitment of Ly6C^{hi} monocytes (Schulthess et al., 2012). Whether other inflammation-inducing stimuli recruit monocytes via further mechanisms is unknown.

1.2.5.6.2 Influence of the microbiota on recruitment of intestinal monocytes

The recruitment of Ly6C^{hi} monocytes to the intestine of neonatal mice parallels the acquisition of the microbiota, peaking at weaning. In addition, antibiotic treatment of *Ccr2*^{-/-}:wild-type parabionts resulted in reduced chimerism of lamina propria macrophages, suggesting that commensal bacteria may stimulate monocyte recruitment to the lamina propria (Bain et al., 2014). Accordingly, studies of germ-free versus conventionally housed mice showed that there were lower numbers of Ly6C⁺ MHCII⁻ monocytes, Ly6C⁺ MHCII⁺ MNPs and Ly6C⁻ macrophages in the lamina propria of adult and 3 week-old germ-free mice, and that fewer of their Ly6C⁻ macrophages had upregulated MHCII (Bain et al., 2014). Antibiotic treatment of wild-type mice for two weeks also reduced the number of Ly6C⁺ MNPs, however mature Ly6C⁻ macrophages were unaffected, which may reflect the ~4-6 week half-life of the latter (Bain et al., 2014). Although previous studies have produced conflicting evidence on the numbers of intestinal macrophages in germ-free mice (Niess and Adler, 2010; Rivollier et al., 2012; Ueda et al., 2010), collectively these more recent results strongly suggest that the commensal microbiota stimulate the recruitment of Ly6C^{hi} monocytes. Circulating TLR ligands have been described to induce expression of CCL2 by MSCs in BM, which led to the release of Ly6C^{hi} monocytes into the blood (Shi et al., 2011). The bacterial cell wall component peptidoglycan, which can be recognised by the pattern recognition receptors NOD1, NOD2, TLR2 and the peptidoglycan recognition proteins (Cloud-Hansen et al., 2006), has been described to translocate from the gut to the bone marrow via the bloodstream, where it induced activation of neutrophils via NOD1 (Clarke et al., 2010). This peptidoglycan was derived from the intestinal microbiota, confirming that microbiota-derived substances can access the BM. It is therefore possible that

microbiota-derived TLR ligands might be stimulating the homeostatic recruitment of Ly6C^{hi} monocytes from the BM to the intestinal lamina propria.

1.2.6 Intestinal dendritic cells

Intestinal DCs are found dispersed throughout the lamina propria, or in the GALT, including small intestinal Peyer's patches; isolated lymphoid follicles and cryptopatches in both the small intestine and colon; and intestine-draining LNs such as the MLNs.

Dendritic cells play crucial roles in maintaining the balance between intestinal homeostasis and immunity (Coombes and Powrie, 2008). The cardinal functional features of DCs are their ability to migrate from peripheral tissues to draining lymphoid tissue via lymphatic vessels, and a superior ability to prime naïve T cells (Geissmann et al., 2010a). Intestinal DCs initiate adaptive immune responses by priming naïve T cells in the GALT, and are additionally specialised to direct the migration of responding cells to the intestine, where the effector or regulatory T cells are required (Coombes and Powrie, 2008).

DCs are regularly defined by high expression of CD11c and MHCII, which allows clear identification in lymphoid tissues but not in peripheral tissues such as the intestinal lamina propria, where macrophages also express these markers (Geissmann et al., 2010a). At the outset of this project, CD103 and CX₃CR1 were believed to be mutually exclusively expressed by DCs and macrophages, respectively (Bogunovic et al., 2009; Varol et al., 2009). This is now known not to be the case, since although macrophages do not express CD103 (Bain et al., 2013), a population of CD103⁻ CX₃CR1^{int} CD11b⁺ CD64⁻ F4/80⁻ CD11c⁺ MHCII⁺ DCs has been identified (Bain et al., 2013; Cerovic et al., 2013; Scott et al., 2014; Zigmond et al., 2012). The discoveries of CD64 as a macrophage-selective surface marker

(Langlet et al., 2012), and CD24 and CD26 as DC-selective molecules (Miller et al., 2012; Schlitzer et al., 2013), have made distinction between DCs and macrophages in peripheral tissues much more reliable, and renewed investigations into the features and functions of the many MNP subsets are ongoing.

There are four subsets of DCs in the intestinal immune system: CD103⁺ CD11b⁺ DCs, CD103⁺ CD11b⁻ CD8α⁺ DCs (henceforth referred to as CD103⁺ CD11b⁻ DCs), and CD103⁻ CD11b⁺ CX₃CR1^{int} DCs, all of which can be found in the lamina propria and intestinal lymph, suggesting that they migrate to the MLNs; and CD103⁻ CD11b⁻ DCs, the smallest subset, which is present in intestinal lymphoid tissue (Cerovic et al., 2014). All of these populations are *bona fide* DCs: they are derived from pre-cDCs in a Flt3-dependent manner, without a monocytic intermediate; they lack expression of CD64 and F4/80; and they migrate in intestinal lymph in a CCR7-dependent manner (Cerovic et al., 2014). Additional features of the individual subsets are discussed below.

1.2.6.1 CD103⁺ DCs

1.2.6.1.1 CD103⁺ CD11b⁺ DCs

CD103⁺ CD11b⁺ DCs are the major population of DCs in the small intestinal lamina propria, however they are only a minor component of the colonic lamina propria DC pool (Denning et al., 2011). CD103⁺ CD11b⁺ DCs have been shown to constitutively migrate to the MLNs (Cerovic et al., 2013). CD103⁺ CD11b⁺ DCs are dependent on the transcription factors IRF4 (Persson et al., 2013; Schlitzer et al., 2013) and Notch2 (Lewis et al., 2011), and are also selectively affected by GM-CSF deficiency (Greter et al., 2012).

In the absence of CD103⁺ CD11b⁺ DCs, frequencies of Th17 cells are reduced in the intestinal lamina propria and MLNs (Lewis et al., 2011; Persson et al., 2013; Welty et al., 2013), and indeed CD103⁺ CD11b⁺ DCs have been shown to drive the differentiation of Th17 cells via IL-6 production (Persson et al., 2013). Following TLR-mediated activation, CD103⁺ CD11b⁺ DCs have also been shown to induce differentiation of Th1 cells, IFN γ ⁺ CD8⁺ T cells, and IgA-producing B cells (Cerovic et al., 2013; Fujimoto et al., 2011; Uematsu et al., 2008).

1.2.6.1.2 CD103⁺ CD11b⁻ DCs

CD103⁺ CD11b⁻ DCs are functionally and developmentally related to splenic CD8 α ⁺ DCs, and are dependent on the transcription factors Batf3 (basic leucine zipper transcription factor, activating transcription factor-like 3), IRF8 (Edelson et al., 2010) and Id2 (inhibitor of DNA-2; Ginhoux et al., 2009)). In contrast to the small intestine, the majority of CD103⁺ DCs in the colon are CD11b⁻ (Denning et al., 2011). Only a small population of CD103⁺ CD11b⁻ DCs exists in the small intestinal lamina propria (Fujimoto et al., 2011; Jakubzick et al., 2008). CD103⁺ CD11b⁻ DCs are found in PPs and ILFs, and it was previously thought that their presence in lamina propria cell preparations could be due to contamination with these lymphoid tissues (Jakubzick et al., 2008). However, CD103⁺ CD11b⁻ DCs are present in normal proportions in the lamina propria and lymph of mice lacking PPs and ILFs due to deficiency in the transcription factor retinoic acid receptor-related orphan receptor (ROR) γ t (Cerovic et al., 2013), indicating that they are lamina propria-derived. CD103⁺ CD11b⁻ DCs are found in intestinal lymph in the steady state and are almost absent from the MLNs of CCR7-deficient mice (Jang et al., 2006), supporting that they are a lamina propria-derived migratory DC population. Following TLR

stimulation, intestinal CD103⁺ CD11b⁻ DCs have been shown to efficiently cross-present soluble antigen and prime CD8⁺ T cells (Cerovic et al., 2013; Fujimoto et al., 2011).

1.2.6.1.3 CD103⁺ “tolerogenic” DCs

Intestinal CD103⁺ DCs are uniquely able to drive the differentiation of naïve CD4⁺ T cells into Foxp3⁺ Tregs, by a retinoic acid (RA)- and TGFβ-dependent mechanism (Coombes et al., 2007; Sun et al., 2007). CD103⁺ DCs are also preferentially able to induce the gut-homing molecules, CCR9 and integrin α4β7, on responding T cells (Johansson-Lindbom et al., 2005), which occurs in a RA-dependent manner (Iwata et al., 2004). CCR9 and α4β7 direct the migration of leukocytes to the small intestine, where CCL25 and MAdCAM, their respective ligands, are expressed (Griffith et al., 2014). CD103⁺ DCs are able to metabolise vitamin A into retinoic acid via their expression of aldehyde dehydrogenase (ALDH) enzymes (Coombes et al., 2007) and expression of the integrin αvβ8 allows them to activate latent TGFβ (Travis et al., 2007; Worthington et al., 2011).

Their ability to induce gut-homing Foxp3⁺ Tregs has implicated CD103⁺ DCs in the development of oral tolerance, a phenomenon which leads to local and systemic immunological unresponsiveness to orally administered innocuous antigen (Pabst and Mowat, 2012; Weiner et al., 2011). Oral tolerance does require the migration of intestinal DCs to the MLNs, and as such is abolished in the absence of MLNs, lymphatic drainage, or CCR7 (Worbs et al., 2006). However, which subset of CD103⁺ DCs is required for the generation of oral tolerance is unknown. Absence of all CD103⁺ intestinal DCs resulted in fewer Foxp3⁺ Treg cells in the lamina propria (Welty et al., 2013), however these were unaffected by depletion of either CD103⁺ CD11b⁺ or CD103⁺ CD11b⁻ intestinal

DCs (Edelson et al., 2010; Persson et al., 2013; Welty et al., 2013), suggesting that both subsets may be able to induce gut-homing Tregs. CD103⁺ CD11b⁻ DCs in intestinal lymph have been shown to express ALDH (Cerovic et al., 2013), which could enable them to induce Foxp3⁺ Tregs in the MLN. The specific contribution of CD103⁺ CD11b⁺ DCs is unknown.

Their involvement in Foxp3⁺ Treg induction and the generation of oral tolerance led to CD103⁺ DCs being referred to as “tolerogenic” DCs. However, during colitis, CD103⁺ DCs in the MLNs were impaired in their ability to induce Foxp3⁺ Tregs, instead favouring an IFN γ ⁺ Th1 response (Laffont et al., 2010). This was associated with lower expression of *Tgfb2* (encoding TGF β -2) and *Aldh1a2* (encoding retinal dehydrogenase 2, RALDH2), lower ALDH activity, and increased expression of *Tbx21* (encoding T-bet, T-box expressed in T cells) by CD103⁺ MLN DCs from colitic mice relative to their steady state counterparts (Laffont et al., 2010). This study showed that CD103⁺ DCs are not limited to performing tolerogenic roles, however surprisingly little is known about the factors influencing the “decision” of DCs to drive regulatory versus effector T cells.

1.2.6.2 CD103⁻ CD11b⁺ CX₃CR1^{int} DCs

A small population of CD103⁻ CD11b⁺ CX₃CR1^{int} DCs has been identified in the small intestinal and colonic lamina propria, and the MLNs (Bain et al., 2013; Cerovic et al., 2013), and was found to migrate in intestinal lymph (Cerovic et al., 2013). These DCs do not express the macrophage markers CD64 or F4/80 (Bain et al., 2013) and have recently been shown to derive from pre-cDCs in a Flt3L-dependent manner (Scott et al., 2014). This population may explain the presence of CX₃CR1^{int} cells observed in the colonic lamina

propria following transfer of pre-cDCs into mice depleted of CD11c⁺ cells (Varol et al., 2009). CD103⁻ CD11b⁺ CX₃CR1^{int} DCs in the lamina propria were depleted by treatment with an anti-CCR2 monoclonal antibody (mAb) and were therefore thought to derive from Ly6C^{hi} monocytes (Zigmond et al., 2012), however a subset of these *bona fide* DCs expresses CCR2 (Scott et al., 2014), which could explain the observed depletion. CD103⁻ CD11b⁺ CX₃CR1^{int} DCs were found to induce IFN γ and IL-17 expression by T cells *in vitro*, even in the absence of stimulation (Cerovic et al., 2013), while the CCR2⁺ subset was shown to promote Th17 polarisation of naïve CD4⁺ T cells (Scott et al., 2014).

1.2.6.3 CD103⁻ CD11b⁻ DCs

CD103⁻ CD11b⁻ DCs are the smallest population of intestinal DCs. This population expanded in response to Flt3L, suggesting that they might also be pre-cDC-derived. CD103⁻ CD11b⁻ DCs may reside in PPs or ILFs, since they are absent from the small intestine and lymph of ROR γ t-deficient mice, and are also rare in the colonic lamina propria (Cerovic et al., 2013).

In summary, much of the research on intestinal MNPs prior to this project was focused on the role of DCs in inducing adaptive immune responses in the GALT, while definitive roles for MNPs in the lamina propria were under investigated. Previous work also focused on DCs from the MLNs and small intestinal lamina propria. Consequently, relatively little has been shown regarding MNPs in the colon, or their roles during colitis.

1.3 Inflammatory bowel disease

Inflammatory bowel disease comprises Crohn's disease and ulcerative colitis, chronic relapsing-remitting diseases of the gastrointestinal tract affecting approximately 0.1% of Western populations (Bouma and Strober, 2003; Shanahan, 2002). CD can affect any part of the gastrointestinal tract, from mouth to anus, but frequently occurs in the ileum and colon. Inflammation in CD is discontinuous, with unaffected areas between lesions, and can be transmural. Associated complications can include strictures, abscesses, and fistulae. UC exhibits continuous inflammation confined to the colon and rectum, invariably arising in the latter from which inflammation spreads proximally. UC exhibits more superficial inflammation compared to CD, being typically confined to the mucosa and submucosa (Khor et al., 2011).

The aetiology of IBD remains unclear, however interactions between several factors are thought to be involved. These fall into three main categories: genetic susceptibility, the host immune system and environmental factors, such as the intestinal microbiota (Maloy and Powrie, 2011). Available evidence suggests that intestinal pathology might arise as a consequence of aberrant immune responses towards the microbiota in genetically susceptible hosts (Khor et al., 2011). In support of this hypothesis, genome-wide association studies (GWAS) have identified a number of genetic risk loci associated with IBD, many of which relate to epithelial barrier function, bacterial handling, and innate and adaptive immune regulation (Anderson et al., 2011; Franke et al., 2010; Khor et al., 2011). These include *CDH1*, *GNA12*; *NOD2*, *CARD9*; *IL23R* and *IL10*, respectively.

1.3.1 The microbiota and inflammatory bowel disease

IBD is associated with changes to the composition of the microbiota, characterised by reductions in *Firmicutes* and *Bacteroidetes*, and an increase in the proportion of *Enterobacteriaceae* (Sartor, 2008). Whether dysbiosis induces disease or occurs as a result of immune responses against the microbiota is unclear. Mice with innate immune defects, such as genetic deficiency in T-bet or NOD-like receptor family pyrin domain-containing 6 (NLRP6), have been shown to develop colitis associated with a change in the microbiota (Elinav et al., 2011; Garrett et al., 2010; Garrett et al., 2007). This colitis was communicable, meaning that intestinal pathology could be induced in healthy recipients by the transfer of the dysregulated microbiota.

In mice, Tregs are most abundant in the colonic lamina propria (Atarashi et al., 2011), where the commensal burden is relatively high (Mowat and Agace, 2014). The emergence of Tregs in the colonic lamina propria is impaired in germ-free mice, and was shown to be promoted particularly by clusters IV and XIVa of the genus *Clostridium* (Atarashi et al., 2011). *Clostridium* species also enhanced production of IL-10 by Tregs. Interestingly, Clostridial clusters IV and XIVa are reduced in IBD patients, suggesting that these components of the microbiota might also promote intestinal homeostasis in humans (Frank et al., 2007; Sokol et al., 2008).

Some evidence indicates that antigen-specific immune responses against the microbiota might drive intestinal pathology. Studies using lamina propria mononuclear cells (LPMCs) isolated from inflamed and adjacent uninfamed tissue from patients with active IBD, uninfamed tissue from IBD patients in remission, and uninfamed controls, showed that immune tolerance towards the microbiota exists in uninfamed intestine, but is broken

during active IBD (Duchmann et al., 1995). LPMCs from inflamed IBD tissue strongly proliferated in response to stimulation with autologous bacterial sonicate, while those from uninfamed tissue did not; and proliferation could be prevented by the addition of an anti-MHCII mAb, indicating that it was driven by antigen presentation (Duchmann et al., 1995). Antigen-specific immune responses against *Helicobacter hepaticus* have also been shown to drive colitis induced in immune- or IL-10-deficient mice by infection with this bacterium (see Section 1.4.1, below), further supporting the hypothesis that intestinal inflammation might arise due to aberrant immune responses against the microbiota.

1.3.2 IL-23 and inflammatory bowel disease

Studies using samples from IBD patients and experimental models have shown that the cytokine IL-23 is linked to intestinal inflammation. IL-23 is a heterodimeric cytokine composed of the specific subunit IL-23p19, and IL-12/23p40, which is shared with IL-12 (Oppmann et al., 2000). The ability to target IL-23 specifically using an anti-IL-23p19 mAb (Chen et al., 2006) and gene-targeted mice lacking IL-23p19 (*IL23a*^{-/-}) (Cua et al., 2003), which are thus deficient in IL-23 but not IL-12, allowed reassessment of diseases previously thought to be driven by IL-12. This led to the discovery that IL-23 was responsible for driving inflammation in experimental models of a number of diseases, including multiple sclerosis (Cua et al., 2003), rheumatoid arthritis (Murphy et al., 2003), and IBD (Hue et al., 2006; Kullberg et al., 2006; Uhlig et al., 2006b; Yen et al., 2006).

IL-23 alone was shown to be essential for colitis, but not systemic disease, in the anti-CD40 model ((Uhlig et al., 2006b); see Section 1.4.3, below). IL-23 has also been shown to

drive typhlocolitis in IL-10-deficient mice, and *Hh*-induced disease mediated by innate and adaptive mechanisms ((Hue et al., 2006; Kullberg et al., 2006; Yen et al., 2006); see Section 1.4.1). Intestinal pathology in IBD, particularly Crohn's disease, is also strongly associated with IL-23. Elevated expression of IL-23 is found in the intestinal mucosa of CD patients and to a lesser extent in those with UC (Liu et al., 2011b; Schmidt et al., 2005). In addition multiple variants of the gene *IL23R*, which encodes the IL-23-specific subunit of the IL-23 receptor, are associated with both ileal CD and UC (Duerr et al., 2006). One such variant, Arg381Gln, confers strong protection against CD (Duerr et al., 2006). The link between IL-23 and its receptor and IBD, and experimental models thereof, plus the discovery that it drives local intestinal immunopathology but not systemic inflammation, highlights IL-23 as a potentially specific target for the treatment of IBD.

The mechanisms by which IL-23 causes pathology have been well studied (Maloy and Powrie, 2011). IL-23 contributes to a pro-inflammatory cascade, inducing expression of other cytokines such as IFN γ , IL-17, TNF α , IL-1 β , and IL-23 and IL-12 themselves, in addition to distinct classes of inflammatory mediators, such as matrix metalloproteinases (MMPs) (Cua et al., 2003; Vignali and Kuchroo, 2012). In terms of adaptive immunity, IL-23 is particularly associated with Th17 cell responses. Although IL-23 is not required for the initial differentiation of Th17 cells, it is associated with maintenance of the Th17 lineage (Mangan et al., 2006). During their differentiation, TGF β upregulates IL-23R expression rendering Th17 cells responsive to IL-23, which then acts to maintain their IL-17-secreting phenotype (Stritesky et al., 2008). IL-23 can act on both Th17 cells and innate lymphoid cells to induce production of cytokines including IL-17A and IL-22 (Buonocore et al., 2010; Maloy and Powrie, 2011).

The source of IL-23 which drives intestinal inflammation has not been definitively shown. Constitutive IL-12/23p40 subunit promoter activity, alongside expression of IL-23 protein, has been observed in the terminal ileum of mice (Becker et al., 2003). These phenomena were localised to CD11c⁺ CD11b⁻ CD8α⁻ cells in the lamina propria and were absent in germ-free mice, indicating that expression was driven by commensal bacterial stimulation. Some studies have suggested that mononuclear phagocytes may produce IL-23 during intestinal inflammation. During anti-CD40 colitis, CD11c^{hi} (“DCs”) and CD11b⁺ CD11c⁻ (“monocytic”) cells sorted from the colon were shown to express IL-23p19 mRNA (Uhlir et al., 2006b). IL-23p19 mRNA expression was also observed in CD14⁺ CX₃CR1⁺ macrophages found in the lamina propria of CD patients (Kamada et al., 2008). Despite these reports, a functional source of IL-23 required for the development of colitis has not been demonstrated *in vivo*.

1.4 Animal models of intestinal inflammation

Many experimental models of inflammatory bowel disease have been developed (Hoffmann et al., 2002). These fall into five broad categories: antigen-specific, inducible (mainly chemically), genetic, adoptive transfer, and spontaneous (Blumberg et al., 1999; Hoffmann et al., 2002). Often, “spontaneous” development of colitis in genetically targeted mouse strains requires the presence of a hidden genetic or environmental factor, such as a particular commensal species (Blumberg et al., 1999). Although no single model recapitulates all of the features of either Crohn’s disease or ulcerative colitis, experimental models allow dissection of the cellular and molecular mechanisms which

contribute to the development of intestinal inflammation. The models used in this thesis are described below.

1.4.1 *Helicobacter hepaticus* + anti-IL-10R colitis

Helicobacter hepaticus is a flagellated, curved-to-spiral, Gram-negative, microaerophilic bacterium, which colonises the caecal and colonic crypts of mice (Fox et al., 1994). *Hh*, along with other members of its genus, can be found as a commensal bacterial species in conventionally housed mice (Fox et al., 1994; Shames et al., 1995; Ward et al., 1996). *Hh* is referred to as a “pathobiont”: while *Hh* infection does not induce disease in wild-type mice, it has been shown to cause severe inflammation of the caecum and colon (typhlocolitis) in immunodeficient (Ward et al., 1996) and IL-10-deficient mice (Kullberg et al., 1998). Similarly, intestinal pathology can be induced in wild-type mice by *Hh* infection with concomitant IL-10R-blocking mAb treatment (*Hh* + anti-IL-10R; (Kullberg et al., 2006)). These studies illustrate that IL-10 is a crucial regulatory cytokine, which normally prevents intestinal inflammation following *Hh* infection. Indeed, although *Hh* infection in wild-type mice induces IL-23p19 expression (Kullberg et al., 2006), the dominant cytokine induced is IL-10 (Kullberg et al., 1998). Importantly, IL-10R blockade alone does not induce colitis in our *Helicobacter* species-negative, specific pathogen-free colony, although this may not be the case in other animal facilities which do carry *Helicobacter* strains. This shows that the endogenous microbiota is not sufficient to induce colitis in the absence of IL-10, and that *Hh* infection is necessary to trigger disease.

Pathology induced by *Hh* infection in lymphocyte-sufficient or -reconstituted mice is driven by CD4⁺ T cell responses against *Hh* (Kullberg et al., 2003) and innate immune

mechanisms (Maloy et al., 2003). Infection of wild-type mice induces a *Hh*-specific regulatory T cell response, which prevents *Hh*-induced colitis via IL-10 production (Kullberg et al., 2002). Regulatory T cells have also been shown to prevent T cell-dependent and -independent *Hh*-induced intestinal pathology via IL-10- and TGF β -dependent mechanisms, however the antigen specificity of these Tregs was unclear since *Rag*^{-/-} mice were reconstituted with naïve CD4⁺ T cells +/- Tregs prior to *Hh* infection (Maloy et al., 2003). These studies indicate that microbiota-specific Tregs may be a mechanism preventing the development of IBD.

In the *Hh* + anti-IL-10R model, the Treg-derived IL-10 which would normally be capable of preventing inflammation is rendered ineffective, resulting in intestinal pathology. IL-23, and not IL-12, is essential for *Hh* + anti-IL-10R-induced typhlocolitis since IL-12p40-deficient but not IL-12p35-deficient mice do not develop pathology (Kullberg et al., 2006). IL-23 was found to drive both IFN γ and IL-17 responses, which synergised to drive intestinal inflammation.

Hh + anti-IL-10R-induced colitis is therefore relevant to IBD in two important ways. *Hh* is a normal commensal bacterial species, which does not cause pathology due to immune regulation by Treg-derived IL-10. Aberrant immune responses against the commensal microbiota, rather than pathogenic species, are thought to play a key role in the development of IBD (Xavier and Podolsky, 2007). Secondly, genetic defects in IL-10 or its receptor can lead to early onset IBD (Shah et al., 2012), and single nucleotide polymorphisms in IL-10 have also been associated with adult UC and CD (Amre et al., 2009; Franke et al., 2008). Thus the *Hh* + anti-IL-10R model may accurately reflect aspects of the breakdown in tolerance towards the commensal microbiota which can occur during IBD.

1.4.2 T cell transfer-induced colitis

Adoptive transfer of CD4⁺ CD45RB^{hi} naïve T cells into lymphocyte-deficient *Rag*^{-/-} or severe combined immunodeficiency (SCID) recipients induces colitis, splenomegaly and systemic wasting disease (Leach et al., 1996; Morrissey et al., 1993; Powrie et al., 1993). Inflammation occurs along the length of the colon, with histological characteristics including epithelial hyperplasia, goblet cell depletion and significant accumulation of mononuclear cells in the lamina propria. Intestinal inflammation is associated with high levels of IFN γ , TNF α and IL-17A. Antibody-mediated neutralisation of IFN γ or TNF α prevents induction of colitis, indicating that these cytokines play an important functional role (Hue et al., 2006; Izcue et al., 2008). T-bet-deficient CD4⁺ T cells did not induce colitis upon adoptive transfer (Neurath et al., 2002), which led to the conclusion that Th1 cells drive T cell transfer-induced colitis. However, Th17 cells also play a pathogenic role in this model, since ROR γ t-deficient CD4⁺ T cells also did not induce colitis although disease could be restored by administration of IL-17A (Leppkes et al., 2009). This study also showed that IL-17A and IL-17F mediate redundant biological effects in this model.

As in *Hh*-induced colitis, IL-23 is essential for intestinal pathology but not systemic disease induced by T cell transfer (Hue et al., 2006). T cell transfer colitis is also bacterially driven, since recipients maintained in germ-free conditions do not develop disease (Stepankova et al., 2007).

Co-transfer of CD4⁺ CD45RB^{lo} T cells prevents the colitis and wasting disease induced by CD4⁺ CD45RB^{hi} T cells (Morrissey et al., 1993; Powrie et al., 1993). Similarly, transfer of unfractionated CD4⁺ T cells does not induce colitis, due to the presence of this suppressive population. Subsequent work showed that the suppressive capacity was

enriched among a population of CD4⁺ CD45RB^{lo} CD25⁺ T cells (Read et al., 2000). These cells require expression of OX40 (Griseri et al., 2010), CTLA-4 (Read et al., 2006; Read et al., 2000) and CCR6 (Kitamura et al., 2010); and responsiveness of CD4⁺ CD45RB^{hi} T cells to TGFβ (Fahlen et al., 2005), in order to suppress colitis. Additionally, transfer of CD4⁺ CD45RB^{lo} CD25⁺ T cells can cure established colitis (Mottet et al., 2003; Uhlig et al., 2006a), demonstrating their immunoregulatory potency.

The T cell transfer model allows dissection of the contribution of innate versus adaptive immune cells to the development of colitis, since genetically altered mice can be used as lymphopenic recipients or T cell donors. This model also has drawbacks: all lymphocytes except the reconstituted populations are absent in *Rag*^{-/-} hosts, which may affect the mechanisms and requirements for T cell transfer colitis; and lymphopenia induces the transferred T cells to rapidly proliferate and acquire effector functions in an unphysiological manner (Min et al., 2005).

1.4.3 Anti-CD40 colitis

Colitis, splenomegaly and wasting disease can be induced in lymphocyte-deficient mice by treatment with an agonistic anti-CD40 mAb (Rolink et al., 1996; Uhlig et al., 2006b). CD40 is a co-stimulatory molecule expressed by activated antigen presenting cells, which normally interacts with CD40L (also known as CD154) found on activated T cells (Diehl et al., 2000). In the inflamed mucosa of both IBD patients and mice with experimental colitis, CD40⁺ APCs have been observed in association with CD4⁺ CD40L⁺ T cells (Liu et al., 1999; Polese et al., 2002), and crucially, blockade of the interaction between CD40 and CD40L prevents development of colitis and can ameliorate established pathology in mouse

models (Cong et al., 2000; De Jong et al., 2000; Kelsall et al., 1996; Liu et al., 2000). This interaction establishes a positive feedback loop of both T cell and APC activation, with both cell types contributing to disease (Diehl et al., 2000; Strober et al., 2002; van Kooten and Banchereau, 2000). The anti-CD40 model is induced in *Rag*^{-/-} or SCID lymphocyte-deficient mice: anti-CD40 mAb mimics ligation by the CD40L which would normally be found on activated T cells. The absence of lymphocytes during anti-CD40 colitis means that pathology is mediated by innate mechanisms, therefore this model can be used to investigate the contribution of innate immune cells to colitis.

Anti-CD40 colitis is induced by a single intraperitoneal injection of anti-CD40 mAb (Uhlir et al., 2006b). Recipients typically show peak weight loss 3-4 days after administration, which then begins to recover. Seven days after anti-CD40 treatment, peak intestinal pathology occurs. Histological signs of anti-CD40 colitis include epithelial hyperplasia, leukocytic infiltrate in the lamina propria, depletion of goblet cells and destruction of epithelial cells. Lamina propria infiltration by mononuclear cells remained apparent at day 10, however all histological signs of colitis were reported to have resolved by three weeks after anti-CD40 treatment. Interestingly, anti-CD40 treatment induced colitis in both SPF and germ-free mice, indicating that bacterial stimulation is not required for intestinal pathology in this model.

Elevated levels of TNF α , IL-6, IFN γ , CCL2, and IL-12p70 are observed in serum and in the intestine following anti-CD40 treatment. Blockade of specific cytokines by co-injection of neutralising antibodies with anti-CD40, and studies in cytokine-deficient mice, have revealed some of the specific roles of distinct cytokines in driving anti-CD40-induced disease. For instance, treatment with anti-IFN γ or anti-TNF α ameliorated wasting, but not colitis or pro-inflammatory cytokine levels in serum, indicating that these cytokines

drive systemic but not intestinal disease (Uhlir et al., 2006b). Treatment of mice with anti-IL-12/23p40 mAb significantly ameliorated all measures of anti-CD40-induced disease, as was the case in p40-deficient mice. Following the discovery that IL-12 and IL-23 share the p40 subunit (Oppmann et al., 2000), mice deficient in the IL-23-specific subunit IL-23p19 were developed to examine the specific functions of IL-12 and IL-23 (Cua et al., 2003). Anti-CD40 treatment of mice deficient in the IL-12-specific subunit IL-12p35 did not suffer wasting disease and exhibited reduced splenomegaly, but did develop colitis associated with elevated pro-inflammatory cytokines in the intestine (Uhlir et al., 2006b). On the contrary, mice deficient in the IL-23-specific subunit IL-23p19 did not develop colitis but still exhibited splenomegaly and wasting disease associated with elevated serum pro-inflammatory cytokines, highlighting the specific role of IL-23 in driving intestinal pathology (Uhlir et al., 2006b). These results also demonstrate the independence of colitis, splenic enlargement and weight loss.

CD11c⁺ CD40⁺ cells, presumed to be DCs, were found in the colonic lamina propria of untreated *Rag*^{-/-} and SCID mice. Accumulation of these cells occurred after anti-CD40 treatment, when they also upregulated expression of MHCII and the co-stimulatory molecules CD80 and CD86. Quantitative reverse transcription polymerase chain reaction (RT-qPCR) performed on CD11c^{hi} ("DCs") and CD11b⁺ CD11c⁻ ("monocytic cells") sorted from the colon and spleen of anti-CD40-treated mice revealed expression of IL-23p19 mRNA by both of these populations (Uhlir et al., 2006b).

Although the anti-CD40 model can be a useful tool for investigating the contribution of innate immune cells to colitis, as with the T cell transfer model, the absence of adaptive immune cells presents a substantial caveat. While anti-CD40 mimics one type of interaction with activated T cells, T cells produce additional mediators such as cytokines,

which can influence the behaviour of APCs. For instance, as previously mentioned, IL-10 and TGF β produced by Tregs have been shown to inhibit not only T cell activity but also that of innate immune cells during *Hh*-induced colitis (Maloy et al., 2003).

1.5 Aims

The composition of the gut MNP compartment changes during colitis. Much research has focused on the roles of MNPs in maintaining intestinal homeostasis, however little is known about specific roles MNPs may play during inflammation, particularly in the colonic lamina propria. It is widely accepted in the field that MNPs are the source of IL-23 during intestinal inflammation, however neither the specific population(s) which produce this mediator, nor a functional cellular population, have been defined *in vivo*. It is also generally accepted that CCR2 controls Ly6C^{hi} monocyte recruitment to the intestine, however other mechanisms might also play an important role. Therefore the aims of this thesis were to:

- 1) Investigate whether MNP populations which accumulate during colitis promote intestinal inflammation;
- 2) Define the source of pro-inflammatory IL-23 during colitis;
- 3) Investigate the mechanisms controlling recruitment of MNPs to the colon during colitis.

2 Materials and Methods

2.1 Mice

C57BL/6 (B6), B6 *Rag1*^{-/-}, B6 CCR6^{GFP/GFP} *Rag1*^{-/-} (generated in our facility by crossing B6 CCR6^{GFP/GFP} mice (Kucharzik et al., 2002) with B6 *Rag1*^{-/-} mice), B6 CX₃CR1^{GFP/+} mice (generated in our facility by crossing B6 CX₃CR1^{GFP/GFP} mice (Jung et al., 2000) with wild-type B6 mice), B6 CX₃CR1^{GFP/+} CD45.1⁺ CD45.2⁺ mice (generated in our facility by crossing B6 CX₃CR1^{GFP/GFP} (CD45.2⁺) mice with B6 CD45.1⁺ congenic mice), B6 CD11c-Cre.*Il23a*^{fl/fl} mice (generated in our facility by crossing B6 CD11c-Cre mice (Stranges et al., 2007) with B6 *Il23a*^{fl/fl} mice (Thakker et al., 2007)), B6 *Batf3*^{-/-} (Hildner et al., 2008), Balb/c *Rag2*^{-/-} and Balb/c *Tbx21*^{-/-} *Rag2*^{-/-} mice (generated in our facility by crossing Balb/c *Tbx21*^{-/-} mice (Szabo et al., 2002) with Balb/c *Rag2*^{-/-} mice) were bred and maintained under specific pathogen-free conditions in accredited animal facilities at the University of Oxford.

CX₃CR1^{GFP/GFP} mice were originally obtained from Professor Steffen Jung, Weizmann Institute of Science, Israel; B6 CD11c-Cre mice were originally obtained from Dr Paul Kaye, University of York, UK; B6 *Il23a*^{fl/fl} mice were originally obtained from Dr Paresh Thakker, Wyeth Research Corporation, USA; B6 CCR6^{GFP/GFP} mice (stock number 013061), B6 *Batf3*^{-/-} (stock number 013755) and Balb/c *Tbx21*^{-/-} (stock number 004432) mice were originally purchased from the Jackson Laboratory.

Experiments were conducted in accordance with the UK Scientific Procedures Act (1986) under a Project License (PPL) authorised by the UK Home Office. Mice were routinely screened for the absence of pathogens and *Helicobacter* species and were over 6 weeks of age when used.

2.2 Antibodies

2.2.1 Flow cytometry

2.2.1.1 Surface molecules

The following directly conjugated fluorescent anti-mouse monoclonal antibodies were used for cell surface staining at a concentration of 1-2 µg/ml (1:200-1:100 dilution, respectively) unless otherwise stated [Antigen: *Clone*, Fluorochrome(s) (Supplier)]:

B220: *RA3-6B2*, Brilliant Violet 421 (BioLegend), eFluor 605 Nanocrystal and PerCP-Cy5.5 (both eBioscience).

CD3: *145-2C11*, PerCP-Cy5.5 (eBioscience).

CD4: *RM4-5*, APC, PE-Cy7, PerCP-Cy5.5 (all eBioscience) and QDot 605 (Invitrogen).

CD11b: *M1/70*, V500 (BD Horizon), APC (BD Pharmingen), eFluor 450 and PerCP-Cy5.5 (both eBioscience).

CD11c: *N418*, APC, eFluor 450, PerCP-Cy5.5, PE (all eBioscience), PE-Cy7 (eBioscience, BioLegend) and Brilliant Violet 605 (BioLegend).

CD25: *PC61.5*, PE (eBioscience).

CD45: *30-F11*, eFluor 650 Nanocrystal (eBioscience), PE (BD Pharmingen) and PerCP-Cy5.5 (BioLegend).

CD45.1: *A20*, PE (eBioscience) and Brilliant Violet 650 (BioLegend).

CD45.2: *104*, FITC (BD Pharmingen), PE and PerCP-Cy5.5 (both eBioscience).

CD45RB: *C363.16A*, FITC (eBioscience).

CD49b, integrin alpha 2: *DX5*, PerCP-Cy5.5 (BioLegend).

CD64: *X54-5/7.1*, Alexa Fluor 647 (BD Pharmingen) and PE (BioLegend).

CD103: *M290*, PE (BD Pharmingen); *2E7*, Brilliant Violet 421 and Pacific Blue (BioLegend).

c-Kit (CD117): *2B8*, PE (eBioscience) and PerCP-Cy5.5 (BioLegend).

E-cadherin: *36*, FITC (BD Transduction Labs) and Alexa Fluor 647 (BD Pharmingen);
DECMA-1, Alexa Fluor 647 (eBioscience).

F4/80: *BM8*, APC and PerCP-Cy5.5 (both eBioscience).

Gr-1: *RB6-8C5*, APC, PerCP-Cy5.5 (eBioscience) and PE (BD Pharmingen).

Ly6C: *HK1.4*, PE-Cy7 (BioLegend).

Ly6G: *1A8*, Brilliant Violet 570 (BioLegend).

MHCII: *M5/114.15.2*, Alexa Fluor 700 and eFluor 450 (both eBioscience).

NKp46: *29A1.4*, eFluor 450 (eBioscience) and PerCP-Cy5.5 (BioLegend).

Siglec F: *E50-2440*, PE (BD Pharmingen) and Brilliant Violet 421 (BD Horizon).

TCR β : *H57-597*, PerCP-Cy5.5 (eBioscience).

The following biotinylated anti-mouse monoclonal antibodies were used for enrichment of cell subsets and/or cell surface staining in combination with a secondary reagent at a concentration of 1-2 μ g/ml (1:200-1:100 dilution, respectively) unless otherwise stated

[Antigen: *Clone* (Supplier)]:

CD4 enrich cocktail - B220: *RA36B2*; CD8: *YTS169*; CD11b: *M1/70*; MHCII: *TIB120* (~ 10 µg/mL, all in-house).

CD11b: *M1/70* (eBioscience).

CD11c: *N418* (eBioscience).

CD115: *AFS98* (eBioscience).

Secondary reagent: Streptavidin-Brilliant Violet 605 (BioLegend).

Fc block (anti-CD16/CD32, 93, eBioscience) was included during surface staining steps to block Fc receptors to minimise non-specific antibody binding.

Fixable Viability Dye eFluor 780 (eBioscience) or Live/Dead Aqua (Invitrogen) was included at 1:1000 dilution during surface staining; alternatively unfixed cells were incubated with 7-AAD (Sigma-Aldrich) for 10 minutes at room temperature, or DAPI (Sigma-Aldrich) was added immediately prior to acquisition, to allow exclusion of dead cells during cell sorting or analysis.

2.2.1.2 Intracellular molecules

The following directly conjugated fluorescent anti-mouse antibodies were used for intracellular staining at a concentration of 1-2 µg/ml (1:200-1:100 dilution, respectively)

unless otherwise stated [Antigen: *Clone*, Fluorochrome(s) (Supplier)]:

IFN γ : *XMG1.2*, eFluor 450, PE-Cy7 (both eBioscience), APC (BD Biosciences and eBioscience) and PE (BD Biosciences).

IL-17A: *eBio17B7*, Alexa Fluor 647 (eBioscience); *TC11-18H10*, PE (BD Biosciences).

2.2.2 *In vivo* antibody treatment

The following unconjugated anti-mouse monoclonal antibodies were administered *in vivo* at doses described in the appropriate sections: CD40 (agonistic, *FGK45*, in-house); IL-10R (blocking, *1B1.2*, in-house); CCR2 (depleting/blocking, *MC-21*, kindly provided by Prof Matthias Mack (Universitätsklinikum Regensburg, Germany)).

2.3 *Helicobacter hepaticus* culture and assays

2.3.1 Growth of *Helicobacter hepaticus*

Helicobacter hepaticus NCI-Frederick isolate 1A (Hh1A; strain 51449; American Type Culture Collection) was grown at 37°C on blood agar plates containing trimethoprim (5 µg/mL), vancomycin (10 µg/mL) and polymyxin B (25 IU/mL) (TVP; all from Oxoid) under microaerophilic conditions (10% CO₂, 10% H₂, balance N₂), which were maintained in vented CampyPak jars (Oxoid) as previously described (Maloy et al., 2003). After two days, bacteria were transferred to and maintained in liquid cultures of tryptone soya broth (TSB, Oxoid; 30 g TSB dissolved in 1000 mL H₂O and autoclaved) supplemented with 2 mL TVP and 10% foetal bovine serum (FBS; Gibco, Life Technologies) in vented

Erlenmeyer flasks (Corning) under microaerophilic conditions at 37°C, shaking at 180 rpm. Cultures were inoculated at OD₆₀₀ 0.05 and maintained at OD₆₀₀ 0.1 by daily dilution as necessary to maintain optimal growth conditions. Viability of bacteria was assessed with a fluorescent Live/Dead assay kit (Molecular Probes, Life Technologies) according to the manufacturer's protocol, using an Axioskop 2 fluorescence microscope (Zeiss).

2.3.2 Assessment of bacterial colonisation

Hh colonisation levels were quantified in caecal contents collected upon sacrifice. DNA was isolated from caecal contents using Stool DNA Isolation kit (Qiagen) according to the manufacturer's instructions. RT-qPCR with *Hh*-specific primers against the *cdtB* gene was performed as previously described (Maloy et al., 2003) using a CFX96 detection system (Bio-Rad Laboratories). Standard curves were constructed using *Hh* DNA purified from bacterial cultures using the DNeasy kit (Qiagen). 20 ng total caecal DNA was added to reaction mixtures in a volume of 4 µL in a final volume of 25 µL and *Hh* concentration expressed as [Hh DNA]/20 ng total caecal DNA.

2.4 Mouse models of intestinal inflammation

2.4.1 *Helicobacter hepaticus* + anti-IL-10R

Helicobacter hepaticus NCI-Frederick isolate 1A (strain 51449) was grown as described in section 2.3.1. Bacteria were harvested by ultracentrifugation at 7000 rpm for 15 minutes. The pellet was washed and resuspended at approximately 5 OD₆₀₀/mL, corresponding to

approximately 5×10^8 bacteria/mL, in sterile phosphate-buffered saline (PBS; PAA). Mice were administered $5 \times 10^7 - 1 \times 10^8$ CFU *Hh* by oral gavage in a volume of 100-300 μ L PBS using a 22G curved feeding needle (Popper and Sons) on d0 and d1. Fresh bacteria were harvested for each infection. Starting on d0 of *Hh* infection, mice were administered 1 mg of a blocking anti-IL-10R monoclonal antibody (clone 1B1.2) by intraperitoneal (i.p.) injection in a volume of 100-200 μ L once per week for up to four consecutive weeks. Mice were monitored weekly for signs of colitis development and sacrificed up to 4 weeks post-infection, typically after 2-3 weeks.

Upon sacrifice, pieces of colon, ~ 0.5 -1.0 cm in length, were taken from the proximal, mid and distal colon. Samples were rinsed in PBS/0.1% BSA and fixed in a 3.6% (w/v) formaldehyde saline solution (stock formaldehyde at 36% w/v, GPR Rectapur). The caecum was opened, caecal contents were collected in 1 mL PBS/0.1% BSA and stored at -20°C . Excess caecal contents were washed away in PBS/0.1% BSA and a section of the caecum adhered to filter paper and fixed as per colon sections. Sections were processed and analysed as described in section 2.4.4.

Additional tissue was collected for cell isolation as described in section 2.5.

2.4.2 T cell transfer

Naïve $\text{CD4}^+ \text{CD45RB}^{\text{high}} \text{CD25}^-$ T cells were purified and sorted from wild-type B6 or Balb/c spleens by fluorescence-activated cell sorting (FACS) as described in sections 2.5.4, 2.5.5 and 2.6.5.1. Naïve T cells were washed in sterile PBS then 4×10^5 cells transferred to sex-matched lymphocyte-deficient B6 *Rag1*^{-/-}, B6 *CCR6*^{GFP/GFP} *Rag1*^{-/-}, Balb/c *Rag2*^{-/-} or Balb/c *Tbx21*^{-/-} *Rag2*^{-/-} recipients by i.p. injection in a volume of 100-200 μ L sterile PBS. Weight

loss and body condition were monitored at least once a week for the duration of experiments and mice were sacrificed upon development of clinical signs of disease, namely weight loss (typically 10-20% of body weight at initiation of experiment) and/or diarrhoea. Sacrifice typically occurred 4-8 weeks post-T cell transfer. Colon sections were prepared and fixed as described in section 2.4.1. Additional tissue was collected for cell isolation as described in section 2.5.

2.4.3 Anti-CD40

B6 *Rag1*^{-/-} and B6 CCR6^{GFP/GFP} *Rag1*^{-/-} mice were administered once dose of 100 µg agonistic anti-CD40 mAb (FGK45) by i.p. injection in a volume of 100-200 µL. Mice were weighed and monitored for signs of colitis development daily and sacrificed at day 7 post-injection. Colon sections were prepared and fixed as described in section 2.4.1. Additional tissue was collected for cell isolation as described in section 2.5.

2.4.4 Histological assessment of intestinal inflammation

At sacrifice pieces of the proximal, mid and distal colon, ~ 0.5-1.0 cm, and caecum were prepared as described in section 2.4.1. 4.0 µm sections were prepared using a microtome and stained with haematoxylin and eosin (H&E) by Richard Stillion (Sir William Dunn School of Pathology, University of Oxford). Sections of colon and caecum were scored in a blinded fashion by at least two researchers according to the criteria described below (Izcue et al., 2008). Images of colon were captured using a CoolScope microscope (Nikon).

A. EPITHELIUM HYPERPLASIA and/or GOBLET CELL DEPLETION

0	None	None
1	Mild (1.5x)	Mild (25%)
2	Moderate (2-3x)	Marked (25-50%)
3	Severe (>3x)	Substantial (>50%)

B. INFLAMMATION IN LAMINA PROPRIA

- 0 None - few leukocytes
- 1 Mild - some increase in leukocytes at tips of crypts OR many lymphoid follicles
- 2 Moderate - marked infiltrate (notable broadening of crypt)
- 3 Severe - dense infiltrate throughout

C. AREA AFFECTED (% of section)

- 0 None
- 1 Up to 25%
- 2 25-50%
- 3 >50%

D. MARKERS OF SEVERE INFLAMMATION

- 0 None
- 1 Submucosal inflammation OR Few crypt abscesses (<5)
- 2 Submucosal inflammation & Few crypt abscesses (<5)
Many crypt abscesses (>5) OR Extensive submucosal inflammation OR Crypt branching (*Hh*)
- 3 Many crypt abscesses (>5) & Extensive submucosal inflammation / Crypt branching (*Hh*)
Ulceration OR Extensive fibrosis

Scores for each individual region of the colon were assigned as above and the average score plotted as mean colitis score. Caecum scores were calculated separately according to the above system.

2.5 Cell isolation

2.5.1 Isolation of colonic and caecal lamina propria leukocytes

The protocol for intestinal lamina propria cell isolation was continually improved over the course of this thesis. Alterations were made to the base media of buffers (Roswell Park Memorial Institute- (RPMI-) 1640 medium; Hanks' balanced salt solution (HBSS)), type of collagenase (type II, type VIII), inclusion (dispase) and concentration of enzymes, volume of digest medium, length of incubations, and density gradient fractions (30/40/75 vs. 40/80). The final protocol is described here, which was used for the majority of experiments described in this thesis.

For LP cell isolation, after collection of tissue for histological assessment as described in section 2.4.1, the caecum and colon were opened longitudinally, washed by agitation in a petri dish containing PBS/0.1% BSA, cut into pieces and stored on ice in 50 mL tubes (Falcon, BD Biosciences) containing RPMI-1640 supplemented with 4% FBS (both Sigma-Aldrich)/100 U/mL penicillin/100 µg/mL streptomycin (PAA). To remove epithelial cells, pieces were washed twice in 25 mL pre-warmed HBSS (Gibco, Invitrogen) supplemented with 4% FBS/100 U/mL penicillin/100 µg/mL streptomycin/5 mM ethylenediaminetetraacetic acid (EDTA; 1 L 0.5M stock made in-house by dissolving 186.12g of Na₂EDTA.2H₂O (ethylenediaminetetra-acetic acid disodium salt dihydrate; VWR BDH Prolabo) in 800ml H₂O, adjusted to pH 8.0 with NaOH (VWR BDH Prolabo), volume adjusted to 1 L with H₂O, autoclaved, stored at room temperature) for 30 minutes at 37°C and 200 rpm in a shaking incubator. Epithelial cell washes were either aspirated, leaving tissue pieces in the tube, or entire samples were decanted into petri dishes and tissue pieces were returned to their tubes with tweezers. Following the second wash step, to remove residual EDTA, tissue pieces were washed by addition of RPMI/4% FBS/100 U/mL penicillin/100 µg/mL

streptomycin, rested for a few minutes, then transferred to fresh tubes. The remaining tissue was digested in 15-25 mL of pre-warmed RPMI/4% FBS/100 U/mL penicillin/100 µg/mL streptomycin containing 1 mg/mL collagenase type VIII (Sigma-Aldrich) and 40 µg/mL DNase I (Roche) for 45-60 minutes at 37°C, 200 rpm. 20 mL digest medium is sufficient for 1-2 pooled colons and caeca. To quench enzyme activity, 200 µL of 0.5 M EDTA was added directly to samples. Remaining intact tissue was disaggregated using a 10 mL syringe (BD Biosciences) without a needle. Samples were filtered into fresh tubes through 70-100 µm cell strainers (Falcon, BD Biosciences) which were washed with PBS/0.1% BSA. Cells were centrifuged for 5 minutes at 4°C, 1500 rpm. Cells were then resuspended in 4 mL 80% Percoll (GE Healthcare) over which 4 mL 40% Percoll was layered in 15 mL tubes (Falcon, BD Biosciences), to make a 40/80% gradient. Gradients were centrifuged for 15 min at 10°C, 3000 rpm *without brake*, and the interface, containing mostly leukocytes, was recovered, washed thoroughly in PBS/0.1% BSA and resuspended in appropriate buffer for further enrichment, staining for flow cytometry, or isolation of mRNA for RT-qPCR. Cells which were not to be used immediately were resuspended in RPMI/4% FBS/100 U/mL penicillin/100 µg/mL streptomycin/5 mM EDTA and stored on ice or at 4°C until required. Cells were counted using a Casy Cell Counter and Analyser Model TT (Innovatis, Roche Diagnostics).

2.5.2 Isolation of blood cells

Blood was collected by cardiac puncture post-mortem with a 1 mL 29G fixed needle insulin syringe (Terumo UK Ltd.) or by tail bleeding, in which case a scalpel (no. 24 blade; Swann-Morton Ltd.) was used to make an incision in the tail vein and blood was collected into 1.5 mL tubes (Eppendorf) containing 100 µL EDTA to prevent clotting. Blood

collected by cardiac puncture was decanted into 1.5 mL or 15 mL tubes containing 100 μ L EDTA. Tubes were topped up with ice-cold PBS/0.1% BSA and cells were pelleted by centrifugation for 5 min at 1500 rpm. Red blood cells were lysed by resuspension in ACK (ammonium chloride potassium) lysis buffer (150 mM NH_4Cl , 10 mM KHCO_3 , 0.1 mM Na_2EDTA , pH 7.2-7.4) followed by incubation for 3 min at room temperature. Samples were flooded with 15 ml PBS/0.1% BSA to quench ACK activity. Cells were then filtered through 70-100 μ m cell strainers and washed in PBS/0.1% BSA. Cells were resuspended in ice-cold PBS/0.1% BSA and stored on ice or at 4°C until required.

2.5.3 Isolation of bone marrow cells

Femur, tibia and fibula were collected from one or both hind legs of individual mice. Whole legs were stored briefly in 70% ethanol (absolute ethanol from Sigma-Aldrich) to reduce potential bacterial contamination, and then washed in PBS/0.1% BSA. After removal of muscle tissue, the ends of each bone were cut using a scalpel and bone marrow flushed out with cold PBS using a 5 mL syringe (BD Biosciences) with 26G needle (Terumo UK Ltd.) under sterile conditions. Cells were filtered into a 50ml tube and centrifuged at 1500 rpm for 5 min. Red blood cells were lysed as described in section 2.5.2. Cells were washed by addition of excess PBS/0.1% BSA and centrifugation at 1500 rpm for 5 min. Cells were resuspended in ice-cold PBS/0.1% BSA and stored on ice or at 4°C until required.

2.5.4 Isolation of spleen cells

Sterile single cell suspensions were prepared from spleen by mashing through 70-100 μm cell strainers using the end of a 5 mL syringe plunger. Cells were washed by addition of ice-cold PBS/0.1% BSA followed by centrifugation for 5 minutes at 1500 rpm. Red blood cells were lysed as described in section 2.5.2. Cells were resuspended in ice-cold PBS/0.1% BSA and stored on ice or at 4°C until required.

2.5.5 Enrichment of CD4⁺ T cells

Sterile cell suspensions were prepared from spleen as described in section 2.5.4. Cells were incubated on ice for 20 minutes with a CD4-enrich antibody cocktail made in-house (a panel of rat antibodies directed against mouse CD8, MHCII, CD11b, B220; described in section 2.2.1.1). Cells were washed in PBS/0.1% BSA and resuspended at a concentration of 1×10^8 cells/ml. Magnetic beads coated with anti-rat IgG antibodies (Dyna, Invitrogen) were resuspended at a concentration of 1×10^8 beads/ml. Cells and beads were combined and incubated at 4°C on a rotator for 20 minutes. Cell/bead suspensions were then placed on a magnet for 30-60 seconds to allow separation of magnetic bead-labeled cells and unlabeled cells. Cell suspensions enriched for CD4⁺ T cells, depleted of bead-labeled cells, were removed by pipette to a fresh tube. Cell suspensions were then washed in ice-cold PBS/0.1% BSA and resuspended at a concentration of 1×10^8 cells/ml.

2.5.6 Enrichment of mononuclear phagocyte populations

2.5.6.1 *Microarray (Chapter 3)*

Single cell suspensions were prepared from lamina propria as described in section 2.5.1. After recovery from density gradient centrifugation and washing, cells were resuspended in 100 μ L MACS buffer (PBS/0.5% BSA/2mM EDTA) in a 50 mL tube. For positive selection of cells expressing CD11c and/or CD11b, 50 μ L anti-mouse CD11c MicroBeads (MACS, Miltenyi Biotec), 50 μ L anti-mouse CD11b MicroBeads (MACS, Miltenyi Biotec) and 50 μ L Fc block were added to each cell sample. Samples were incubated upright for 15 minutes at 4°C. Following incubation, samples were washed with 20 mL MACS buffer and centrifuged at 1500 rpm for 10 minutes. Supernatant was aspirated and samples resuspended in 500 μ L MACS buffer. Samples were filtered and applied to pre-prepared LS columns (MACS, Miltenyi Biotec) used with a QuadroMACS Separator attached to a MultiStand (both MACS, Miltenyi Biotec) as per manufacturer's instructions. Anti-CD11c and anti-CD11b MicroBead-labelled cells were retained on column in the separator by magnetism. After three washes with 3 mL MACS buffer, the column was removed from the separator and placed over a new collection tube. 5 mL buffer was applied to the column and immediately flushed out using a plunger to elute the magnetically labelled fraction. Cells were washed and resuspended in FACS buffer for surface staining and sorting. The sorting strategy is described in detail in Chapter 3.

2.5.6.2 *Ly6C^{hi} monocyte transfer (Chapter 4)*

Bone marrow cells from CX₃CR1^{GFP/+} CD45.1⁺ CD45.2⁺ mice were prepared under sterile conditions as described in section 2.5.3. Cells were pooled in a 50 mL tube and incubated

in FACS buffer containing Fc block and biotinylated antibodies specific for CD115 and CD11c (see section 2.2.1.1) for 15 minutes at 4°C. Following incubation, cells were washed with excess FACS buffer and centrifuged at 1500 rpm for 10 minutes. Supernatant was aspirated and labelled cells were resuspended in 100 µL MACS buffer per 10^7 cells. Anti-biotin MicroBeads (MACS, Miltenyi Biotec; 1:50, 2 µL per 10^7 cells) were added and samples were incubated for 15 minutes at 4°C. Following incubation, cells were treated as above (section 2.5.6.1) prior to staining and sorting.

2.6 Flow cytometry

For acquisition using a flow cytometer, up to 1×10^6 cells were stained in 50 µL FACS buffer (PBS/0.1% BSA/5mM EDTA) containing combinations of the antibodies detailed in section 2.2.1 in round-bottomed 96 well plates (Corning). Cells were washed by resuspension in 200 µL FACS buffer followed by centrifugation at 1700 rpm for 3 minutes to pellet cells.

2.6.1 Restimulation of cells for intracellular cytokine staining

For intracellular cytokine staining, freshly isolated cells were incubated in round-bottomed 96 well plates in 200 µL complete medium (RPMI-1640/10% FBS/2mM L-glutamine/100 U/mL penicillin/100 µg/mL streptomycin/0.05 mM β-mercaptoethanol) containing 0.1 µM PMA, 1 µM ionomycin and 10 µg/ml Brefeldin A (all Sigma-Aldrich), for 3.5 h at 37°C. Cells were washed three times before surface and then intracellular staining.

2.6.2 Surface staining

For surface staining, cells were stained in the dark for 30 minutes at 4°C in FACS buffer containing a viability dye, Fc block to minimise non-specific antibody binding, and combinations of the antibodies detailed in section 2.2.1. In the majority of experiments, especially those utilising GFP-reporter expression, cells were washed twice, resuspended in FACS buffer, and analysed immediately without fixation. Alternatively, cells were washed twice in FACS buffer, fixed in 2% paraformaldehyde or Cytofix/Cytoperm Fixation and Permeabilisation Solution (BD Biosciences) for 15-30 minutes or overnight at 4°C, washed twice more and resuspended in FACS buffer for analysis. Cells to be stained for intracellular proteins were washed twice and fixed as described below.

2.6.3 Intracellular staining

After viability and surface staining, cells were fixed for 10 minutes by resuspension in 50 µL Cytofix/Cytoperm Fixation and Permeabilisation Solution (BD Biosciences). Cells were either washed and resuspended in FACS buffer for storage overnight at 4°C, or permeabilised and stained immediately. Cells were washed and permeabilised in 50 µL permeabilisation buffer (eBioscience) for 30 minutes. Cells were washed again and stained in the dark for 30 minutes at 4°C in 50 µL permeabilisation buffer containing combinations of the antibodies detailed in section 2.2.1. Following incubation, cells were washed once in permeabilisation buffer, then again in FACS buffer, and resuspended in FACS buffer for acquisition.

2.6.4 Acquisition and analysis

Data were acquired on a SORP LSRII using FACSDiva software (BD Biosciences). Single stained cells or CompBeads (BD Biosciences) were used for single colour samples for set-up and compensation. Prior to sample acquisition or at the outset of data analysis, compensation was checked relative to fluorescence minus one (FMO) control samples, in each of which cells were stained with the full panel of antibodies minus one fluorochrome. Analysis and, when required, compensation, were performed using FlowJo software (Tree Star, Inc.). Figure 3.4 was created using FACSDiva software (BD Biosciences).

2.6.5 Fluorescence-Activated Cell Sorting (FACS)

For fluorescence-activated cell sorting (FACS), cells were stained for surface markers in FACS buffer containing Fc block and combinations of antibodies (see section 2.2.1), at 1×10^8 cells/mL in 15 or 50 mL tubes, for 30 minutes at 4°C in the dark. Cells were washed in an excess of FACS buffer and centrifuged at 1500 rpm for 5-10 minutes to pellet cells. Cells were sorted on a FACSARIAIII (BD Biosciences) by Drs Helen Ferry, Kate Alford, Claire Pearson or Julie Schulthess, or with a MoFlo cell sorter (Dako Cytomation) by Nigel Rust. For sorting with FACSARIAIII, cells were resuspended in 1.5 mL tubes and were collected in 1.5 mL tubes prepared with ~150 µL FBS; for sorting with MoFlo, cells were resuspended in 5 mL polypropylene round-bottomed tubes and were collected in 5 mL polypropylene round-bottomed tubes prepared with ~500 µL FBS.

Sorted populations described in this thesis typically showed >95% purity during reanalysis.

2.6.5.1 Sorting naïve CD4⁺ T cells

Spleen cells were isolated and enriched for CD4⁺ T cells under sterile conditions as described in sections 2.5.4 and 2.5.5. CD4⁺ T cell-enriched cell suspensions were stained for surface expression of CD4, CD25 and CD45RB as described in section 2.6.5. Stained cells were resuspended in FACS buffer at a concentration of 5×10^7 cells/ml. Immediately prior to sorting DAPI (final concentration 1 μ M) was added to cell suspensions to assess viability. Cells were sorted using a FACSARIAIII or MoFlo cell sorter. Naïve CD4⁺ T cells were purified as DAPI⁻ CD4⁺ CD25⁻ CD45RB^{hi} cells.

2.6.5.2 Sorting mononuclear phagocyte populations

2.6.5.2.1 Microarray (Chapter 3)

Single cell suspensions were prepared from lamina propria as described in section 2.5.1, followed by enrichment for CD11c- and CD11b-expressing cells as described in section 2.5.6.1. Cells were incubated with a viability marker and Fc block for 15 minutes at 4°C, washed, then stained for surface markers (see Chapter 3) as described in section 2.6.5. After washing, cells were resuspended in 400 μ L FACS buffer containing 40 μ g/mL DNase I, and transferred to 1.5 mL tubes for immediate sorting using a FACSARIAIII.

2.6.5.2.2 Ly6C^{hi} monocyte transfer (Chapter 4)

Bone marrow cells from CX₃CR1^{GFP/+} CD45.1⁺ CD45.2⁺ mice were prepared under sterile conditions as described in section 2.5.3 and cells expressing CD115 and/or CD11c were positively selected as described in section 2.5.6.2. Cells were stained with a viability dye (Fixable Viability Dye eFluor 780, eBioscience) and fluorescently labelled antibodies

specific for a panel of markers (CD11c; MHCII; lineage markers = CD3, TCR β , B220, NKp46, c-Kit; Siglec F; Ly6G; CD11b; CD45.1; CD45.2; Ly6C) as described in section 2.6.5. After washing, cells were resuspended in 400 μ L FACS buffer containing 40 μ g/mL DNase I, and transferred to 1.5 mL tubes for immediate sorting using a FACSAriaIII. Total CD11c⁺ cells and Ly6C^{hi} monocytes (CD11c⁻ MHCII⁻ lineage⁻ Siglec F⁻ Ly6G⁻ CD11b⁺ CD45.1⁺ CD45.2⁺ Ly6C^{hi} CX₃CR1^{int}) were sorted.

2.7 Analysis of cytokine secretion

2.7.1 Preparation of samples

At sacrifice, cells were isolated from the colon $\pm$ caecum lamina propria as described in section 2.5.1 and 5×10^5 cells were cultured for 48h in round-bottomed 96 well plates in complete medium (see section 2.6.1). After culture, plates were centrifuged at 1500 rpm for 5 minutes to pellet cells, and supernatants were collected and stored at -20°C or -80°C.

2.7.2 Measurement of cytokines in supernatant

Samples were prepared as described in section 2.7.1. IL-23 protein levels were quantified using Mouse IL-23 ELISA Ready-SET-Go! (second generation assay; eBioscience) according to manufacturer's instructions. All other cytokine (CCL2, CCL3, CCL7, IFN γ , IL-1 β , IL-17A, IL-22, TNF α) protein levels were determined by FlowCytomix (Bender Medsystems, eBioscience) flow cytometric multiplex assay as per manufacturer's instructions.

2.8 Analysis of gene expression

2.8.1 Preparation of whole intestinal tissue for gene expression analysis

At termination of experiment, mice were sacrificed and ~ 0.5 cm pieces of the proximal, mid and distal colon were pooled in 2 mL cryotubes (Sarstedt), snap frozen in liquid nitrogen and stored at -80°C. Tissue was homogenised in 700 µL Buffer RLT (Qiagen) supplemented with 1% β-mercaptoethanol (Sigma-Aldrich) in Lysing Matrix D Tubes (MP Biomedicals) at 4.0 m/s for 40 s, twice, in a FastPrep 24 homogeniser (MP Biomedicals). Samples were centrifuged at 13,000 rpm for 5 minutes, and homogenate was collected and centrifuged again to remove debris. For RNA isolation, homogenate was loaded onto a QIamp column (Qiagen) and total RNA was purified as described below.

2.8.2 Preparation of RNA and cDNA

RNA was isolated from whole colonic or caecal tissue or from isolated cells using the RNeasy Mini kit (Qiagen) according to manufacturer's instructions, including an on-column DNase I (RNase-Free DNase Set, Qiagen) digestion step. RNA concentration was determined using a Nanodrop spectrophotometer (Thermo Scientific).

cDNA was synthesised in 20 µL reactions, using a maximum of 1 µg total RNA (quantity normalised across samples), by incubating 12 µL RNA (balance sterile distilled H₂O) with 0.5 µL oligo dT primers (Invitrogen) and 1 µL 10mM dNTP (Bioline) at 65°C for 5 minutes and subsequently on ice for 1 minute. This was followed by addition of 0.5 µL SuperScript III reverse transcriptase, 4 µL 5X First Strand buffer, 1 µL 0.1M DTT and 1 µL RNase OUT (all Invitrogen), incubation at 50°C for 60 minutes, and inactivation of the reaction by heating at 70°C for 15 minutes. In cases of very low starting RNA concentration, reactions

were performed in a volume of 40 µL to allow use of all starting RNA in a volume of approximately 24 µL (all volumes described above for 20 µL reaction were doubled).

cDNA synthesis was performed using a G-Storm GS2 thermal cycler (Gene Technologies Ltd.). RNA and cDNA were stored between -80°C and -20°C.

2.8.3 Quantitative reverse transcription PCR (RT-qPCR)

Quantitative reverse transcription PCR (RT-qPCR) was performed for candidate genes using TaqMan Gene Expression Assays for mouse (Applied Biosystems):

Ccl2 (Mm00441242_m1); *Ccl3* (Mm00441259_g1); *Ccl4* (Mm00443111_m1);

Ccr1 (Mm00438260_s1); *Ccr2* (Mm00438270_m1); *Ccr5* (Mm01963251_s1);

Ccr6 (Mm99999114_s1); *Ccr7* (Mm01301785_m1); *Ccl2* (Mm00516914_g1);

Fcgr1 (Mm00438874_m1); *Hprt* (Mm01545399_m1); *Il17a* (Mm00439618_m1);

Il22 (Mm01226722_m1); *Il23a* (Mm01160011_g1, Mm00518984_m1). cDNA samples

were assayed in duplicate, in a final volume of 20 µL (0.4 µL TaqMan Assay + 10 µL qPCR

Mastermix (Eurogentec) + 6.6 µL H₂O + 3 µL cDNA), in Microseal 96-well skirted PCR

plates (Bio-Rad Laboratories) using a CFX96 real-time PCR detection system (Bio-Rad

Laboratories). RNA that was not reverse transcribed was used as a negative control to

assess DNA contamination. Mean relative gene expression was determined and

differences calculated using the $2^{-\Delta C(t)}$ method (Pfaffl, 2001). Gene expression values

were normalised to *Hprt*.

2.8.4 Microarray sample preparation and analysis

RNA was prepared as described in section 2.8.2. RNA quality was assessed using the Agilent RNA 6000 Pico kit with the Agilent 2100 Bioanalyser (both from Agilent, now Keysight Technologies Inc.). RNA samples were amplified and labelled with biotin using the TargetAmp 2-Round Biotin-aRNA Amplification Kit (Epicentre, Cambio Ltd.) and assessed for quality as before. Dr Dilair Baban (microarray core facility, Wellcome Trust Centre for Human Genetics, University of Oxford) hybridised the samples to Illumina MouseWG-6 v2.0 Expression BeadChips (Illumina), which were washed and scanned using a HiScan scanner (Illumina) to generate expression profiles for each condition (CX₃CR1^{int} CD64⁺ during steady state, n = 3; CX₃CR1^{int} CD64⁻ during steady state, n = 3; CX₃CR1^{int} CD64⁺ during inflammation, n = 3; and CX₃CR1^{int} CD64⁻ during inflammation, n = 3).

Dr Nicholas Iltott performed data analysis as follows: Two samples (1 x CX₃CR1^{int} CD64⁺ during steady state and 1 x CX₃CR1^{int} CD64⁻ during steady state) were removed from further analysis due to general low signal intensity across the arrays. Probes were used for downstream processing and analysis if they were expressed significantly above background (detection p value < 0.05) in at least two samples. This resulted in the analysis of 15,529 probes (out of a total of 45,288). Array signal intensities were background adjusted, transformed using the variance stabilising transformation (VST) and quantile normalised using Lumi from Bioconductor (Du et al., 2008). Differential expression analysis was performed for each condition contrast using the empirical Bayes method implemented in LIMMA from Bioconductor (Smyth, 2004). Significant differences for each contrast were identified at a Benjamini-Hochberg adjusted p value < 0.05. To ensure cleaner clustering, a two-fold ($\log_2(\text{fold change [FC]}) = 1$) threshold was applied.

The union of significantly differentially regulated probes (adjusted p value < 0.05; $\log_2(\text{FC})$ > 1) were visualised as a heatmap and probes were clustered into distinct sets using k-means clustering ($k = 7$) as implemented in R (`kmeans()` function in R3.1.0).

We performed functional enrichment analysis on the genes in each cluster against gene ontology (GO) categories using the hypergeometric test implemented in the `runGO.py` script from Computational Genomics Analysis Tools (CGAT; <https://github.com/CGATOxford/cgat>).

2.9 Statistics

Mann-Whitney U test, Student's t test, Kruskal-Wallis test with Dunn's post-test correction for multiple comparisons, or one-way ANOVA with Bonferroni's post-test correction for multiple comparisons, were performed as indicated, using GraphPad Prism (GraphPad Software).

Statistical analysis of microarray data was performed in R by Dr Nicholas Illott, as described in section 2.8.4.

Differences were considered statistically significant when $p < 0.05$.

3 Characterisation of mononuclear phagocytes in the large intestine during homeostasis and inflammation

3.1 Introduction

The intestine experiences an enormous antigenic load derived from food, commensal bacteria and occasionally pathogens, which are separated from the underlying tissue by a single epithelial cell layer (Hooper and Macpherson, 2010). The intestinal immune system must discriminate between pathogen-derived and innocuous antigens in order to mount appropriate host-protective immunity but prevent unnecessary auto-inflammatory damage. Disruption of this delicate balance can result in inflammatory bowel disease (Xavier and Podolsky, 2007).

Dendritic cells and macrophages found throughout the intestine and associated lymphoid tissue are essential to the maintenance of intestinal homeostasis (Bain and Mowat, 2014; Bekiaris et al., 2014). They are ideally situated for this purpose, and via their abilities to sense and internalise both exogenous and endogenous stimuli, produce cytokines and present antigen, these cells influence innate and adaptive immune responses to the diverse sources of antigen encountered in the gastrointestinal tract. Macrophages also play crucial roles in clearing dead host cells and microbial debris, in the resolution of inflammation and infection, and in wound healing (Davies et al., 2013; Murray and Wynn, 2011).

Given the important role of mononuclear phagocytes in controlling the balance between intestinal homeostasis, protective immunity and damaging inflammation, it has been of great interest to investigate which MNP populations are present under each condition

and to determine their respective functions. The roles of DCs in induction of gut homing T cells and oral tolerance have been well-studied (Bekiaris et al., 2014; Coombes and Powrie, 2008) but the contributions of MNP populations to intestinal inflammation are still not fully understood (Bain and Mowat, 2014; Bekiaris et al., 2014).

We have previously reported that CD103⁺ MLN DCs exhibit pro-inflammatory functions during colitis, in contrast to their tolerogenic role in the steady state (Laffont et al., 2010). The switch to a pro-inflammatory phenotype was associated with greater expression of T-bet (encoded by *Tbx21*) (Laffont et al., 2010). However, T-bet has also been shown to suppress development of spontaneous colitis and its progression to colorectal cancer (Garrett et al., 2007; Garrett et al., 2009). The role of T-bet in colitis thus remains uncertain.

This chapter shows the characterisation of large intestinal MNP populations present in the steady state and throughout *Helicobacter hepaticus*-induced colitis. We present whole genome microarray analysis of CX₃CR1^{int} CD64⁺ and CX₃CR1^{int} CD64⁻ MNPs from resting and inflamed large intestinal lamina propria. Finally, we investigated the role of T-bet expressed by the innate immune system during T cell transfer-induced colitis using *Tbx21*^{-/-} *Rag2*^{-/-} mice.

3.2 Results

3.2.1 Intestinal mononuclear phagocyte populations during homeostasis and

Helicobacter hepaticus-induced inflammation

An experimental model of chronic colitis used throughout this thesis will be the *Helicobacter hepaticus* + anti-IL-10R model (Kullberg et al., 2006). This involves infection of mice with *Helicobacter hepaticus* by oral gavage and weekly treatment with an IL-10R-blocking monoclonal antibody (anti-IL-10R) to disrupt IL-10 signalling (Figure 3.1a). A timecourse analysis of tissue pathology during this model was performed by Drs Chris Schiering and Matthew Shale, and is reproduced here with their permission. Both *Hh* infection and IL-10 signalling blockade are required to induce disease in wild-type lymphocyte-replete mice (Figure 3.1b) (Kullberg et al., 2006). *Hh* + anti-IL-10R treatment induces mild-to-severe colitis and typhlitis – inflammation of the caecum – within ten days. However, at this timepoint considerable biological variation exists in the severity of disease and we have found that not all mice are reliably colitic by day ten (our unpublished observations). By day 14 mice display more uniform histological signs of colitis, which peaks between two and three weeks post-*Hh* + anti-IL-10R (Figure 3.1b). The first publication of this model (Kullberg et al., 2006), and in its subsequent use by different research groups, focused on assessing disease four weeks after *Hh* + anti-IL-10R treatment. However, we observed that histopathology scores at day 28 were lower than at day 14 (Figure 3.1b), suggesting partial resolution of inflammation by four weeks after disease induction. At day 28 we also observed greater biological variability, as at day ten (Figure 3.1b).

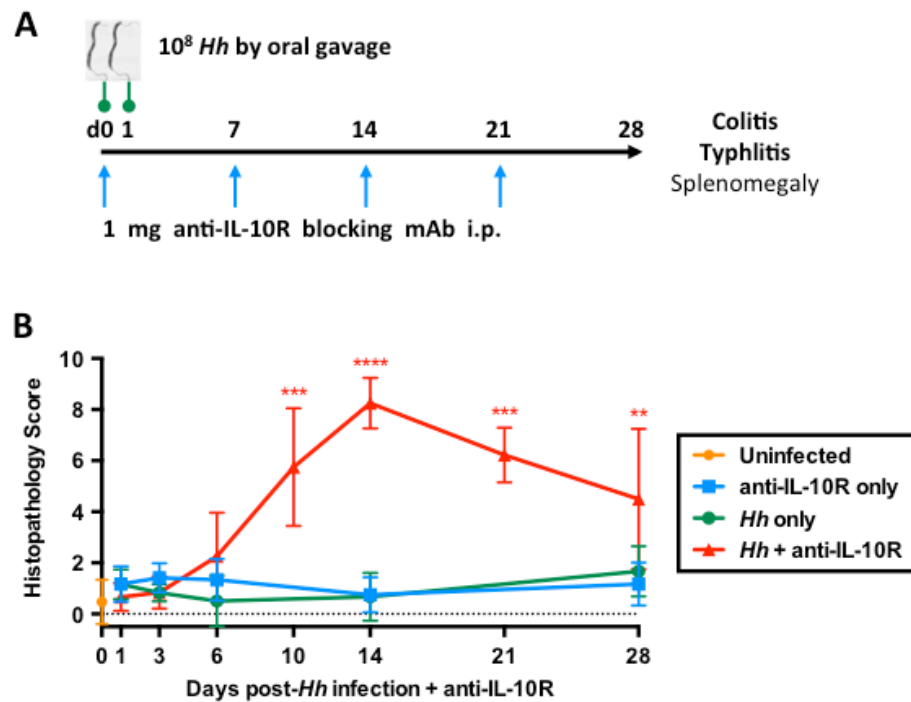


Figure 3.1 Intestinal pathology during *Helicobacter hepaticus* + anti-IL-10R-induced colitis.

(A) Schematic of *Hh* + anti-IL-10R colitis model. Mice were infected with *Helicobacter hepaticus* (*Hh*) and treated weekly with anti-IL-10R blocking mAb prior to analysis at the indicated times. (B) Pieces of colon and caecum were collected at sacrifice. Fixed sections were stained with haematoxylin and eosin, and scored for pathology by assessment of histological features. Histopathology scores are the whole colon mean score, determined as mean of proximal + mid + distal colon section scores. Data represent group mean $\pm$ SD. Data from uninfected mice are plotted at day 0 to indicate steady state. Uninfected, orange circle, $n=5$ mice; anti-IL-10R only, blue squares, $n=4$ mice per timepoint; *Hh* only, green circles, $n=4$ mice per timepoint; *Hh* + anti-IL-10R, red triangles, $n=4$ mice per timepoint except d21, $n=3$ mice. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$; as determined by one way ANOVA with Bonferroni's post-test correction for multiple comparisons; each group was compared per timepoint with the uninfected control. Experiments performed and histology scored by Drs Chris Schiering and Matthew Shale. Analysis performed by Stephanie Mathisen. Data are representative of at least three experiments per timepoint.

Distinction between mononuclear phagocyte subsets based on their expression of surface markers has caused persistent problems in the field and the approaches we used evolved during the project. Based on the emerging literature, we eventually came to use gating strategies in which a combination of CD11b, MHCII, Ly6C, Ly6G, Siglec F, CD11c, CD103, CD64 and/or F4/80 was used to distinguish between granulocytes, monocytes, macrophages and dendritic cells. CX₃CR1 expression was employed as an additional refinement in mice expressing one copy of a GFP reporter gene under control of the myeloid cell marker CX₃CR1 (CX₃CR1^{GFP/+} mice) (Bain et al., 2013; Jung et al., 2000; Rivollier et al., 2012; Tamoutounour et al., 2012; Zigmond et al., 2012). Figure 3.2 shows how this strategy was applied in CX₃CR1^{GFP/+} mice. An example of inflamed colonic and caecal lamina propria three weeks following induction of *Hh* + anti-IL-10R colitis is shown to illustrate the MNP populations which accumulate during inflammation, as these cells were of most interest to us during this project. A table summarising the surface markers defining each population follows the description below (Table 3.1).

Following exclusion of debris by size, and selection of live (viability dye⁻) CD45⁺ cells, neutrophils were excluded as CD11b^{hi} Ly6G⁺ cells (CD45⁺ CD11b^{hi} Ly6G⁺, population 1, Figure 3.2, Table 3.1). The remaining CD45⁺ Ly6G⁻ cells were distinguished by differential expression of CX₃CR1 and CD11b to yield CX₃CR1⁻, CX₃CR1^{int} CD11b⁺ and CX₃CR1^{hi} CD11b⁺ cells. Eosinophils are CD45⁺ CD11b⁺ SSC^{hi} F4/80⁺ Ly6C^{int} CX₃CR1⁻ cells, and were distinguished from macrophages by their lack of CX₃CR1 expression (Bain et al., 2013). CX₃CR1^{int} CD11b⁺ cells were then assessed for expression of Ly6C and MHCII, where a “waterfall” of differentiating monocytes/macrophages can be seen (Bain et al., 2013; Tamoutounour et al., 2012). Ly6C^{hi} monocytes are found in the Ly6C^{hi} MHCII⁻ quadrant (CD45⁺ Ly6G⁻ CX₃CR1^{int} CD11b⁺ Ly6C^{hi} MHCII⁻, population 2, Figure 3.2, Table 3.1). Cells

expressing both Ly6C and MHCII (CD45⁺ Ly6G⁻ CX₃CR1^{int} CD11b⁺ Ly6C^{hi} MHCII⁺, population 3, Figure 3.2, Table 3.1) represent a transitional population during the differentiation of Ly6C^{hi} monocytes into CX₃CR1^{hi} macrophages (Bain et al., 2013; Tamoutounour et al., 2012), which have been shown to accumulate during intestinal inflammation (Bain et al., 2013; Tamoutounour et al., 2012). The next stage in this differentiation pathway involves downregulation of Ly6C (Tamoutounour et al., 2012), producing a population of CX₃CR1^{int} CD11b⁺ CD64⁺ F4/80⁺ macrophages that are found among the Ly6C^{lo/-} MHCII⁺ cells at the bottom of the waterfall. These CX₃CR1^{int} Ly6C^{lo/-} macrophages are also a transitional population during CX₃CR1^{hi} macrophage development (Bain et al., 2013; Tamoutounour et al., 2012). However, these macrophages need to be distinguished from the DCs that are also present within the CX₃CR1^{int} CD11b⁺ Ly6C^{lo/-} MHCII⁺ gate on the basis of CD64 or F4/80 expression. This reveals two populations, with the macrophages expressing both of these markers and variable levels of CD11c (CX₃CR1^{int} CD11b⁺ Ly6C^{lo/-} MHCII⁺ CD64⁺ F4/80⁺, population 5, Figure 3.2, Table 3.1) (Bain et al., 2013; Tamoutounour et al., 2012). The remaining Ly6C^{lo/-} MHCII⁺ MNPs are CD11c⁺, but are CD64⁻ F4/80⁻ (CX₃CR1^{int} CD11b⁺ Ly6C^{lo/-} MHCII⁺ CD64⁻ F4/80⁻, population 4, Figure 3.2, Table 3.1) and have recently been shown to comprise *bona fide* CD11b⁺ CD103⁻ DCs (Scott et al., 2014).

The CX₃CR1^{hi} CD11b⁺ population consists almost exclusively of CD64⁺ F4/80⁺ macrophages (CD45⁺ Ly6G⁻ CX₃CR1^{hi} CD11b⁺ CD64⁺ F4/80⁺, population 6, Figure 3.2, Table 3.1).

We also examined CX₃CR1⁻ cells for the presence of the two CD103⁺ intestinal DC subsets that can be distinguished by CD11b expression. CD11c⁺ MHCII⁺ cells among this gate were selected, and CD64⁻ and/or F4/80⁻ cells were assessed for expression of CD103 and CD11b to yield CD103⁺ CD11b⁻ DCs (CD45⁺ Ly6G⁻ CX₃CR1⁻ CD11c⁺ MHCII⁺ CD64⁻ F4/80⁻ CD103⁺ CD11b⁻, population 7, Figure 3.2, Table 3.1) and CD103⁺ CD11b⁺ DCs (CD45⁺ Ly6G⁻

CX₃CR1⁻ CD11c⁺ MHCII⁺ CD64⁻ F4/80⁻ CD103⁺ CD11b⁺, population 8, Figure 3.2, Table 3.1).

Of these two populations, CD103⁺ CD11b⁻ DCs are predominant in the colon in contrast to the small intestine (Figures 3.2 and 3.3) (Annacker et al., 2005).

These populations and the cell surface markers used to identify them are summarised in Table 3.1.

Population (number in Figure 3.2)	Cell surface marker expression
Neutrophils (1)	CD45 ⁺ Ly6G ⁺ CD11b ^{hi}
Ly6C ^{hi} monocytes (2)	CD45 ⁺ Ly6G ⁻ CX ₃ CR1 ^{int} CD11b ⁺ Ly6C ^{hi} MHCII ⁻
Ly6C ⁺ MHCII ⁺ MNP (3)	CD45 ⁺ Ly6G ⁻ CX ₃ CR1 ^{int} CD11b ⁺ Ly6C ^{hi} MHCII ⁺
CX ₃ CR1 ^{int} Ly6C ^{lo/-} CD64 ⁻ MNP (4)	CD45 ⁺ Ly6G ⁻ CX ₃ CR1 ^{int} CD11b ⁺ Ly6C ^{lo/-} MHCII ⁺ CD64 ⁻ F4/80 ⁻ (CD11c ⁺)
CX ₃ CR1 ^{int} Ly6C ^{lo/-} CD64 ⁺ macrophages (5)	CD45 ⁺ Ly6G ⁻ CX ₃ CR1 ^{int} CD11b ⁺ Ly6C ^{lo/-} MHCII ⁺ CD64 ⁺ F4/80 ⁺ (CD11c ^{variable})
CX ₃ CR1 ^{hi} CD64 ⁺ macrophages (6)	CD45 ⁺ Ly6G ⁻ CX ₃ CR1 ^{hi} CD11b ⁺ (Ly6C ^{lo/-} MHCII ⁺) CD64 ⁺ F4/80 ⁺ (CD11c ⁺)
CD103 ⁺ CD11b ⁻ DCs (7)	CD45 ⁺ Ly6G ⁻ CX ₃ CR1 ⁻ CD11c ⁺ MHCII ⁺ CD64 ⁻ F4/80 ⁻ CD103 ⁺ CD11b ⁻
CD103 ⁺ CD11b ⁺ DCs (8)	CD45 ⁺ Ly6G ⁻ CX ₃ CR1 ⁻ CD11c ⁺ MHCII ⁺ CD64 ⁻ F4/80 ⁻ CD103 ⁺ CD11b ⁺

Table 3.1: Cell surface marker expression used to identify populations in CX₃CR1^{GFP/+} mice. Markers in brackets were not included in the gating strategy shown in Figure 3.2 but are known from our unpublished work and that of others (Bain et al., 2013; Tamoutounour et al., 2012).

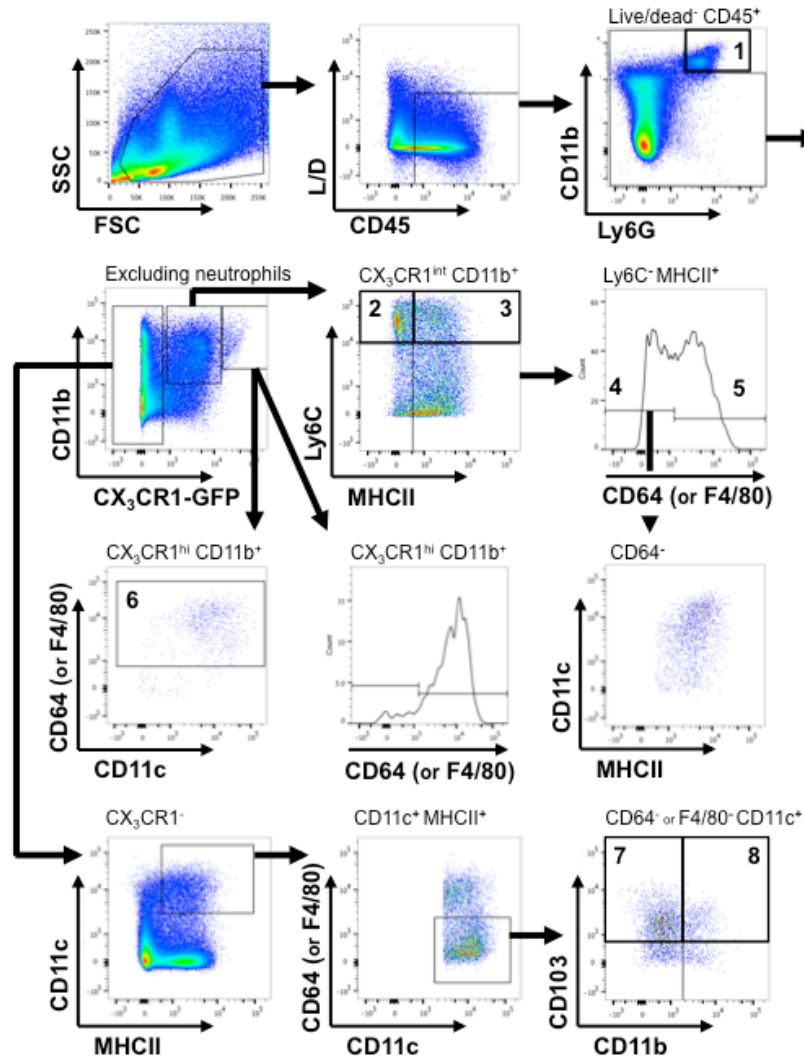


Figure 3.2 FACS gating strategy for myeloid populations in the caecal and colonic lamina propria of CX₃CR1^{GFP/+} mice.

Full gating strategy used to identify neutrophils (1), Ly6C^{hi} monocytes (2), CX₃CR1^{int} Ly6C⁺ MHCII⁺ pro-inflammatory intermediate stage MNPs (3), CX₃CR1^{int} Ly6C^{lo} MHCII⁺ CD64⁻ (or F4/80⁻) MNPs (4), CX₃CR1^{int} Ly6C^{lo} MHCII⁺ CD64⁺ (or F4/80⁺) macrophages (5), CX₃CR1^{hi} Ly6C^{lo} MHCII⁺ CD64⁺ (or F4/80⁺) macrophages (6), CD103⁺ CD11b⁻ DCs (7) and CD103⁺ CD11b⁺ DCs (8) by flow cytometric analysis of stained single cell preparations of combined caecal and colonic lamina propria from individual mice. Example from a representative CX₃CR1^{GFP/+} mouse infected with *Hh* and treated with anti-IL-10R for three weeks. Data are representative of at least three experiments.

Using the gating strategy in Figure 3.2, we examined the MNP compartment in the combined colonic and caecal lamina propria of CX₃CR1^{GFP/+} mice at different times during the *Hh* + anti-IL-10R model of intestinal inflammation (Figure 3.3). We observed infiltration of CX₃CR1^{int} CD11b⁺ Ly6C^{hi} inflammatory monocytes and Ly6C^{hi} MHCII⁺ intermediates within five days of *Hh* infection and treatment with anti-IL-10R (Figure 3.3a). Neutrophils followed the same pattern of accumulation. Consistent with the peak of disease, the frequencies of these three populations peaked 2-3 weeks after colitis induction, after which they declined. At three weeks, the frequency of CX₃CR1^{int} CD11b⁺ Ly6C^{lo/-} macrophages had increased, correlating with the decline in the Ly6C^{hi} monocytes and Ly6C^{hi} MHCII⁺ MNPs, which are the precursors of this population (Bain et al., 2013; Tamoutounour et al., 2012) (Figure 3.3a). The frequencies of CX₃CR1^{hi} macrophages (Figure 3.3a), CD103⁺ CD11b⁻ DCs and CD103⁺ CD11b⁺ DCs (Figure 3.3b) remained relatively stable throughout the course of the model, but had decreased slightly two and three weeks after induction of colitis, likely reflecting the infiltration of inflammatory populations. By contrast, the frequency of CX₃CR1^{int} CD11b⁺ Ly6C^{lo/-} CD64⁻ F4/80⁻ MNPs had increased by day five, remaining elevated two weeks post-*Hh* + anti-IL-10R treatment, and had returned to steady state levels by week three (Figure 3.3b), supporting the hypothesis that resolution of inflammation has begun by 3-4 weeks after *Hh* + anti-IL-10R treatment.

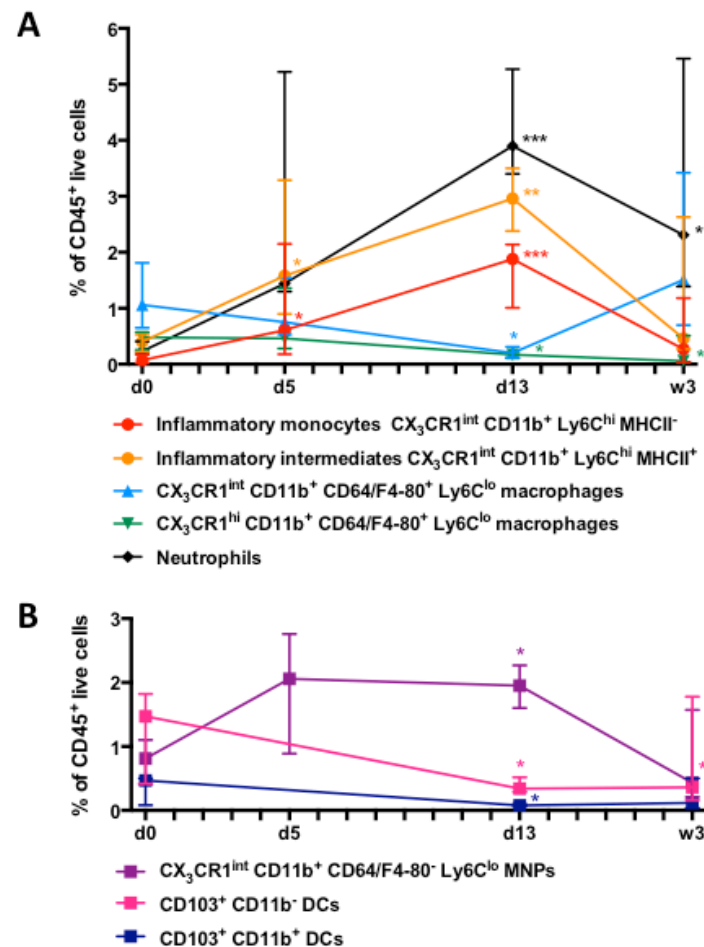


Figure 3.3 Mononuclear phagocyte populations in the colonic and caecal lamina propria of CX₃CR1^{GFP/+} mice at various timepoints during *Helicobacter hepaticus*-induced colitis.

CX₃CR1^{GFP/+} mice were infected with *Helicobacter hepaticus* (*Hh*) and treated weekly with anti-IL-10R blocking mAb prior to analysis at the indicated times. Single cell preparations of combined colonic and caecal lamina propria from individual mice were stained and analysed by flow cytometry. Frequency among CD45⁺ live lamina propria cells of (A) neutrophils (black), CX₃CR1^{int} Ly6C^{hi} MHCII⁻ monocytes (red), CX₃CR1^{int} Ly6C^{hi} MHCII⁺ MNPs (orange), CX₃CR1^{int} CD64/F4/80⁺ Ly6C^{lo} macrophages (light blue), and CX₃CR1^{hi} CD64/F4/80⁺ Ly6C^{lo} macrophages (green); and (B) CX₃CR1^{int} CD11b⁺ CD64/F4/80⁺ Ly6C^{lo} MNPs (purple), CD103⁺ CD11b⁻ DCs (pink), and CD103⁺ CD11b⁺ DCs (dark blue). Day 0 data are from uninfamed mice infected with *Hh* but not treated with anti-IL-10R, sacrificed at three weeks post-infection. Data represent group median + range. Data for day 0 are from one experiment (CX₃CR1^{GFP/+} *Hh*, n=5 mice); data for day 5 are from one experiment (CX₃CR1^{GFP/+} *Hh* + anti-IL-10R, n=5 mice); data for day 13 are from one experiment (CX₃CR1^{GFP/+} *Hh* + anti-IL-10R, n=7 mice); data for week 3 are pooled from two independent experiments (CX₃CR1^{GFP/+} *Hh* + anti-IL-10R, n=9 mice). * p<0.05, ** p<0.01, *** p<0.001; as determined by Kruskal-Wallis test with Dunn's post-test correction for multiple comparisons; population frequency at each timepoint was compared to the frequency of the same population at d0.

3.2.2 Microarray analysis of CX₃CR1^{int} CD64⁺ and CX₃CR1^{int} CD64⁻ MNPs

Having observed their accumulation during intestinal inflammation, we wanted to assess the potential functions of colitis-associated CX₃CR1^{int} CD64⁺ MNPs, and how they might be recruited to the large intestine. We sorted MNP subsets from the resting and inflamed combined colonic and caecal lamina propria, three weeks after *Hh* + anti-IL-10R treatment, to conduct microarray analysis. This study was performed before the gating strategy shown in Figure 3.2 had been developed and the relationships between the various populations of monocytes, macrophages and DCs had not yet been published (Bain et al., 2013; Tamoutounour et al., 2012).

3.2.2.1 Isolation of MNP populations

Figure 3.4 shows the different gating strategy we used to identify and isolate MNP populations from the steady state (Figure 3.4a-b) and inflamed (Figure 3.4c) lamina propria by fluorescence-activated cell sorting (FACS) for microarray analysis. Since our cells of interest expressed CD11c and/or CD11b, and cell numbers were limiting, we enriched the lamina propria preparations for cells expressing CD11b or CD11c by magnetic cell separation prior to sorting. As we focused on DCs and macrophages, we started by selecting MHCII⁺ cells (Figure 3.4), which excluded Ly6C^{hi} monocytes from our analysis. At the time, CD103 and CX₃CR1 were thought to be mutually exclusively expressed by DCs and macrophages, respectively (Schulz et al., 2009), so we gated CD103⁺ CX₃CR1⁻, CX₃CR1^{int} CD103⁻ and CX₃CR1^{hi} CD103⁻ cells. The CX₃CR1^{int} cells included both CD64⁺ and CD64⁻ subsets, which we compared in our microarray study (CD64⁺: orange back-gating in Figure 3.4a-b, light blue back-gating in Figure 3.4c; CD64⁻: green back-gating in Figure 3.4a-b, orange back-gating in Figure 3.4c), while the CX₃CR1^{hi} cells were

uniformly CD64⁺ (red orange back-gating in Figure 3.4a-b, dark blue back-gating in Figure 3.4c). The CD103⁺ CX₃CR1⁻ cells included both CD11b⁺ and CD11b⁻ DCs (blue back-gating in Figure 3.4a-b, fuchsia back-gating in Figure 3.4c). All sorted populations showed >95% purity upon reanalysis. Unfortunately we were unable to include the CX₃CR1^{hi} macrophage and CD103⁺ DC populations in our microarray study.

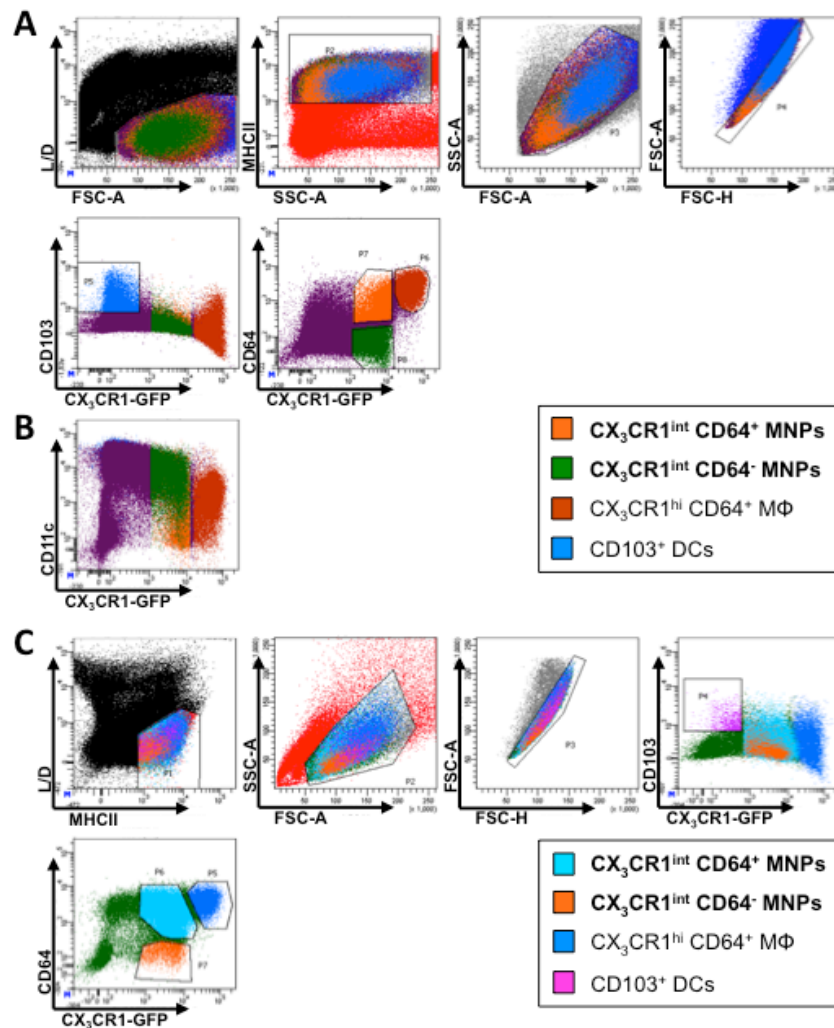
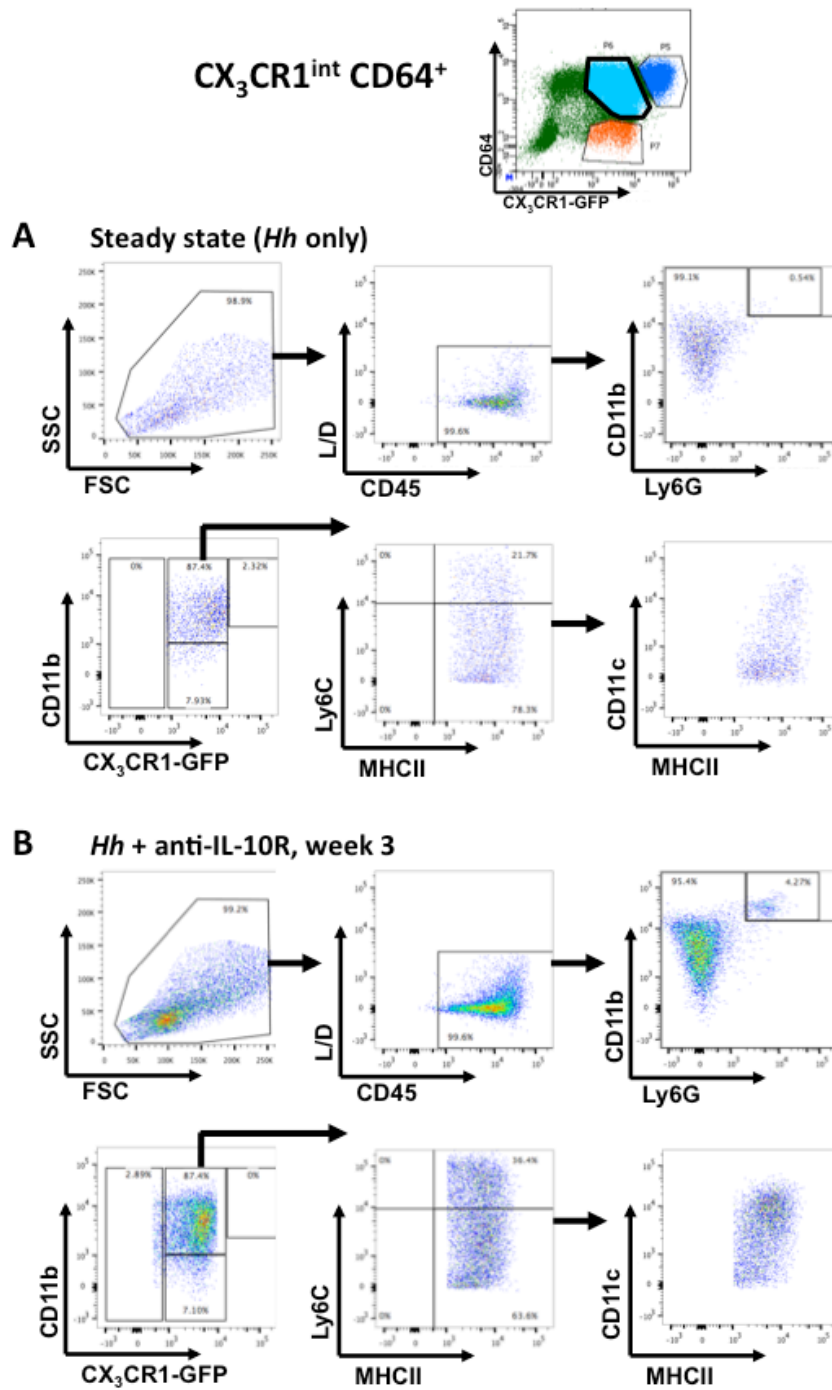


Figure 3.4 Gating strategy for isolation of CX₃CR1^{int} CD64⁺ and CX₃CR1^{int} CD64⁻ MNPs from the colonic and caecal lamina propria of CX₃CR1^{GFP/+} mice for analysis by microarray.

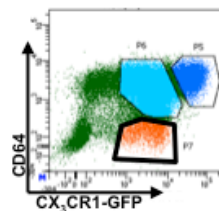
CX₃CR1^{GFP/+} mice were infected with *Hh* and treated with anti-IL-10R for three weeks or were left uninfected and untreated. Three weeks after induction of colitis, single cell preparations of combined colonic and caecal lamina propria pooled from groups of 4-13 mice were CD11c- and CD11b-enriched by magnetic cell separation, stained and sorted by FACS. Populations typically showed >95% purity upon reanalysis. (A, C) Full gating strategy used to sort CX₃CR1^{int} CD64⁺ MNPs (orange in A-B, light blue in C), CX₃CR1^{int} CD64⁻ MNPs (green in A-B, orange in C), CX₃CR1^{hi} CD64⁺ macrophages (red orange in A-B, dark blue in C) and CD103⁺ DCs (blue in A-B, fuchsia in C) illustrated with representative samples from (A) an uninfected mouse and (C) a mouse at day 21 of *Hh* + anti-IL-10R colitis. (B) Shows CD11c expression by all populations from (A). Data are representative of at least three independent experiments per condition.

We were primarily interested in the differences between the major subsets of MNPs – DCs and macrophages – found in the lamina propria during inflammation; therefore we chose to compare gene expression in $CX_3CR1^{int} CD64^+$ MNPs (macrophages) and $CX_3CR1^{int} CD64^-$ MNPs (DCs). To correlate the populations we sorted for microarray analysis with the subsequent advances made in MNP subset identification, we later analysed the contents of each population studied by microarray using the more precise gating strategy shown in Figure 3.2. During both steady state and inflammation, the majority of sorted $CX_3CR1^{int} CD64^+$ cells were $CD11b^+$ and included $Ly6C^+ MHCII^+$ MNPs and $Ly6C^{lo/-}$ macrophages displaying a range of CD11c expression (Figure 3.5a-b). The small proportion of cells which fell in the $CX_3CR1^{int} CD11b^-$ gate showed the same pattern of $Ly6C$, $MHCII$ and $CD11c$ expression (data not shown). These cells expressing lower levels of $CD11b$ could represent $CD11b^{int}$ monocyte-derived “TNF- and iNOS-producing DCs” (Serbina et al., 2003) or embryonic precursor-derived macrophages, which express high levels of $F4/80$ but only low levels of $CD11b$ (Schulz et al., 2012). In steady state, approximately 80% of $CX_3CR1^{int} CD64^+$ cells were $Ly6C^{lo/-} MHCII^+$, but during inflammation, approximately one-third of the cells were $Ly6C^{hi} MHCII^+$, and the remaining two-thirds were $Ly6C^{lo/-} MHCII^+$; the latter population showed greater $CD11c$ expression than in the steady state (Figure 3.5a-b).

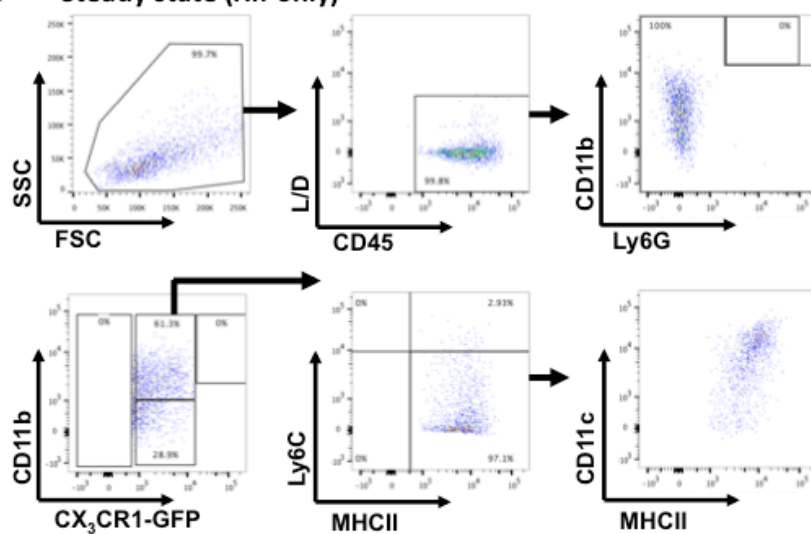
Whether sorted from resting or inflamed lamina propria, the $CX_3CR1^{int} CD64^-$ population used for the microarrays appeared to contain both $CD11b^+$ and $CD11b^-$ cells (Figure 3.5c-d). Both the $CD11b^+$ and $CD11b^-$ fractions comprised predominantly $Ly6C^{lo/-} CD11c^+ MHCII^+$ cells.



$CX_3CR1^{int} CD64^{-}$



C Steady state (*Hh* only)



D *Hh* + anti-IL-10R, week 3

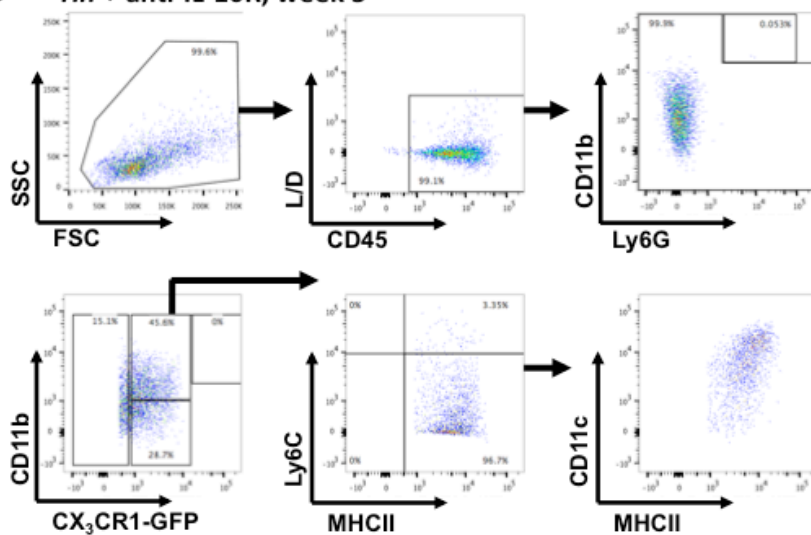


Figure 3.5 Further analysis of the contents of CX₃CR1^{int} CD64⁺ and CX₃CR1^{int} CD64⁻ gates used to sort MNPs for analysis by microarray.

CX₃CR1^{GFP/+} mice were infected with *Hh* (steady state) with or without treatment with anti-IL-10R (*Hh* + anti-IL-10R) for three weeks. Single cell preparations of combined colonic and caecal lamina propria from individual mice were stained and analysed by flow cytometry. Cells were pre-gated on each MNP population as shown in Figure 3.4, and analysed for the presence of more specifically defined MNP populations using the gating strategy shown in Figure 3.2. (A-D) Representative FACS plots showing surface marker expression by (A-B) CX₃CR1^{int} CD64⁺ MNPs and (C-D) CX₃CR1^{int} CD64⁻ MNPs from uninfamed *Hh*-infected mice (A, C) and colitic mice at day 21 of *Hh* + anti-IL-10R colitis (B, D). Numbers in gates represent the frequency of cells in that gate among the parent population. Data are representative of at least three independent experiments per condition.

3.2.2.2 Microarray

We performed a microarray study of CX₃CR1^{int} CD64⁺ and CX₃CR1^{int} CD64⁻ cells isolated from both resting and inflamed combined colonic and caecal lamina propria three weeks after induction of *Hh* + anti-IL-10R colitis. RNA was isolated, and samples were amplified and labelled with biotin using the TargetAmp™ 2-Round Biotin-aRNA Amplification Kit (Epicentre, Cambio Ltd). Expression profiles for each condition (CX₃CR1^{int} CD64⁺ during steady state (n = 3), CX₃CR1^{int} CD64⁻ during steady state (n = 3), CX₃CR1^{int} CD64⁺ during inflammation (n = 3) and CX₃CR1^{int} CD64⁻ during inflammation (n = 3)) were obtained using Illumina MouseWG-6-V2 Expression BeadChips by Dr Dilair Baban (Wellcome Trust Centre for Human Genetics, University of Oxford). Data analysis was performed by Dr Nicholas Illott. Two samples (1 x CX₃CR1^{int} CD64⁺ during steady state and 1 x CX₃CR1^{int} CD64⁻ during steady state) were removed from further analysis due to general low signal intensity across the arrays. Probes were used for downstream processing and analysis if they were expressed significantly above background (detection p value < 0.05) in at least two samples. This resulted in the analysis of 15,529 probes (out of a total of 45,288). Array signal intensities were background adjusted, transformed using the variance stabilising transformation (VST) and quantile normalised using Lumi from Bioconductor (Du et al., 2008). Differential expression analysis was performed for each condition contrast using the empirical Bayes method implemented in LIMMA from Bioconductor (Smyth, 2004). Significant differences for each contrast were identified at a Benjamini-Hochberg adjusted p value < 0.05. To ensure cleaner clustering, a two-fold (log₂(fold change [FC]) = 1) threshold was applied. The union of significantly differentially regulated probes (adjusted p value < 0.05; log₂(FC) > 1) were visualised as a heatmap and probes

were clustered into distinct sets using k-means clustering ($k = 7$) as implemented in R (`kmeans()` function in R3.1.0).

Complete lists of genes showing significant differential expression (adjusted p value < 0.05) in each comparison performed can be found in the Appendix (Table 1: CX₃CR1^{int} CD64⁺ versus CX₃CR1^{int} CD64⁻ MNPs from inflamed lamina propria; Table 2: CX₃CR1^{int} CD64⁺ versus CX₃CR1^{int} CD64⁻ MNPs from resting lamina propria; Table 3: CX₃CR1^{int} CD64⁺ MNPs from inflamed versus resting lamina propria; and Table 4: CX₃CR1^{int} CD64⁻ MNPs from inflamed versus resting lamina propria). Figure 3.6 shows a heatmap of all probes which were at least two-fold ($\log_2(\text{FC}) > 1$) differentially regulated with an adjusted p value < 0.05, in at least one of the comparisons performed. Each probe was assigned to a cluster by k-means clustering ($k = 7$). Clusters are indicated in the heatmap by the coloured sidebar and numbering (Figure 3.6). Tables 3.2-3.8 (presented at the end of the chapter) list the genes in each cluster, which exhibit the following general patterns of gene regulation:

1, grey) genes upregulated in CD64⁺ cells during inflammation resulting in expression similar to steady state- and inflammation-derived CD64⁻ cells (Table 3.2);

2, green) genes downregulated in CD64⁺ cells during inflammation resulting in expression similar to steady state- and inflammation-derived CD64⁻ cells (Table 3.3);

3, purple) genes downregulated in CD64⁻ cells during inflammation resulting in expression similar to steady state- and inflammation-derived CD64⁺ cells (Table 3.4);

4, yellow) genes downregulated during inflammation regardless of CD64 status (Table 3.5);

5, orange) genes downregulated in CD64⁺ cells during inflammation (Table 3.6);

6, brown) genes induced in CD64⁺ cells during inflammation (Table 3.7);

7, red) genes induced during inflammation regardless of CD64 status (Table 3.8).

Graphs in Figure 3.7 show expression of example genes from each cluster to illustrate these distinct patterns of gene regulation.

For the genes in each cluster, we performed functional enrichment analysis against gene ontology (GO) categories using the hypergeometric test implemented in the runGO.py script from Computational Genomics Analysis Tools (CGAT; <https://github.com/CGATOxford/cgat>). The only clusters showing significant enrichment for any biological processes, molecular functions or cell locations were clusters 2 and 6.

In cluster 2 (genes downregulated in CD64⁺ cells during inflammation resulting in expression similar to steady state- and inflammation-derived CD64⁻ cells), 17 genes from the “respiratory electron transport chain” and 10 genes from the “mitochondrial electron transport, NADH to ubiquinone” GO biological processes were enriched (data not shown). The same 10 genes were also enriched with the annotation “NADH dehydrogenase (ubiquinone) activity” during analysis for enrichment of molecular functions, and with the cell location GO term “mitochondrial respiratory chain complex I” (data not shown). 16 genes with the molecular function annotation “structural constituent of ribosome” were also enriched in cluster 2 (data not shown).

The biological processes enriched in cluster 6 (genes induced in CD64⁺ cells during inflammation) were “immune response” (18 genes), “inflammatory response” (23 genes), “retinoid metabolic process” (6 genes) and “phototransduction, visible light” (6 genes)

(Table 3.9, shown at the end of the chapter). A number of genes, such as *Il23a*, *Ccl5*, *Cxcl1* and *Cxcl2*, were annotated with both “immune response” and “inflammatory response”, while there was complete overlap in the 6 genes labelled with “retinoid metabolic process” and “phototransduction, visible light”. No molecular functions were enriched in cluster 6.

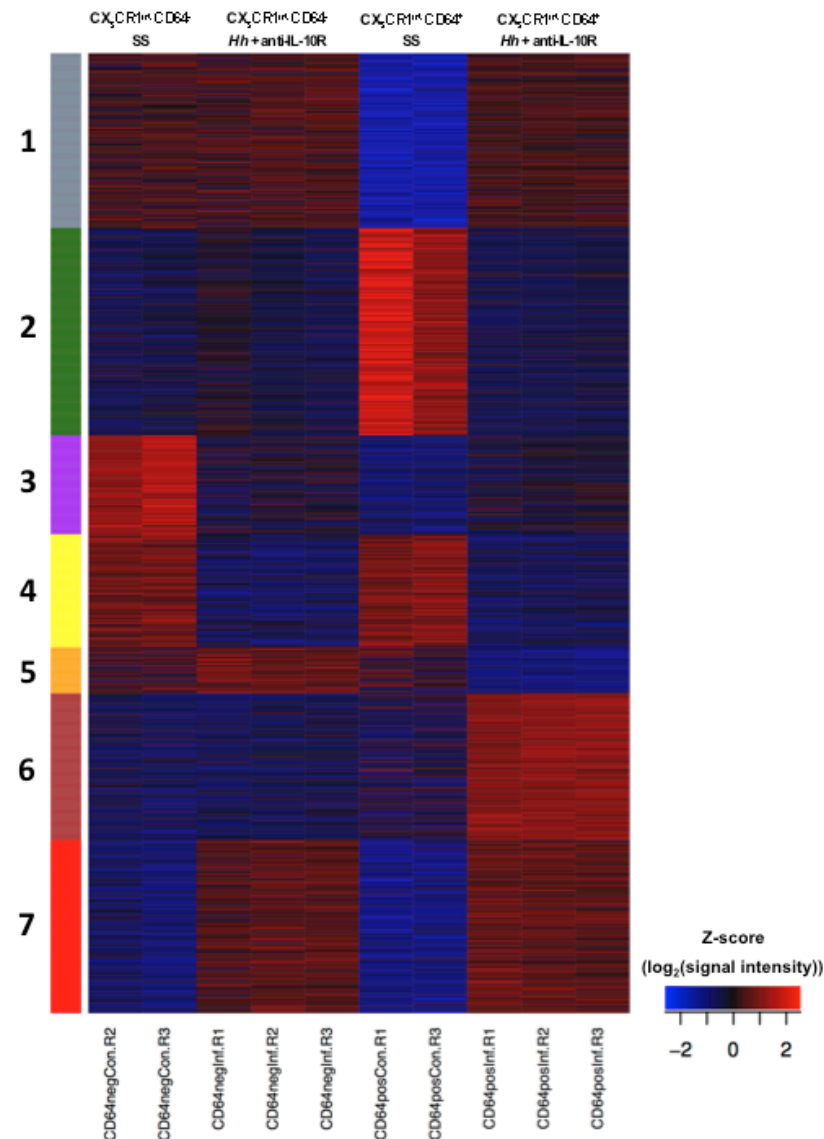


Figure 3.6 Heatmap of genes which were differentially expressed in at least one comparison between CX₃CR1^{int} CD64⁺ and CX₃CR1^{int} CD64⁺ MNPs from resting and inflamed lamina propria.

CX₃CR1^{GFP/+} mice were infected with *Helicobacter hepaticus* (Hh) and treated weekly with anti-IL-10R blocking mAb (Hh + anti-IL-10R) prior to analysis at three weeks. Single cell preparations of combined colonic and caecal lamina propria were stained and the indicated cell subsets isolated by FACS as described in Figure 3.4. Illumina microarray and data analysis in R were performed. Heatmap shows row-normalised (z-score) log₂(signal intensity) for each of the 1994 probes which showed significant differential expression (adjusted p value < 0.05; log₂(fold change) > 1, two-fold) in at least one comparison (CX₃CR1^{int} CD64⁺ inflammation vs. CX₃CR1^{int} CD64⁺ inflammation; CX₃CR1^{int} CD64⁺ control vs. CX₃CR1^{int} CD64⁺ control; CX₃CR1^{int} CD64⁺ inflammation vs. CX₃CR1^{int} CD64⁺ control; CX₃CR1^{int} CD64⁺ inflammation vs. CX₃CR1^{int} CD64⁺ control). Seven clusters could be seen, so *k*-means clustering analysis (*k*=7) was performed to cluster probes in distinct sets. Clusters are distinguished by colour on the left of the heatmap and are numbered 1-7. Heatmap produced by Dr Nicholas Iliott.

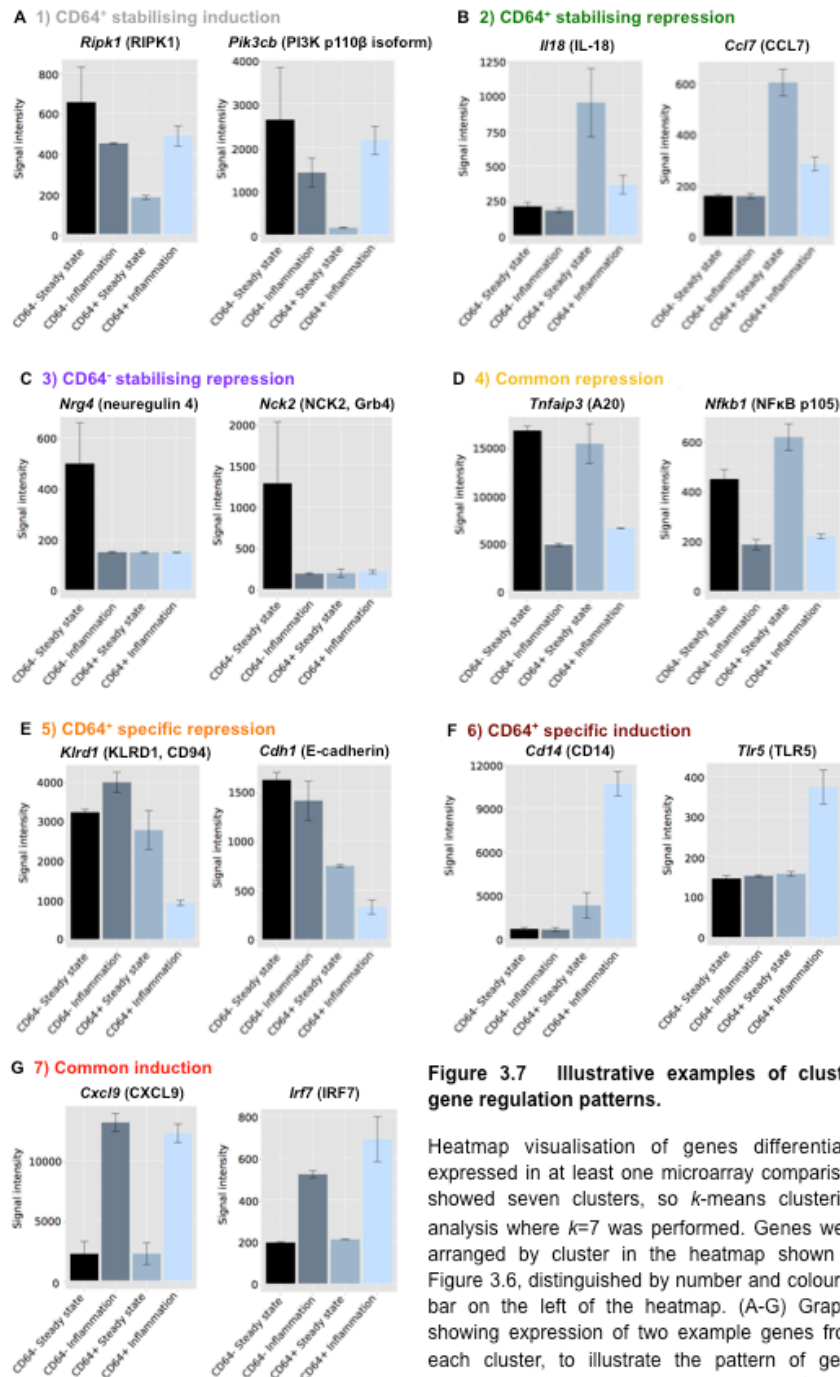


Figure 3.7 Illustrative examples of cluster gene regulation patterns.

Heatmap visualisation of genes differentially expressed in at least one microarray comparison showed seven clusters, so k -means clustering analysis where $k=7$ was performed. Genes were arranged by cluster in the heatmap shown in Figure 3.6, distinguished by number and coloured bar on the left of the heatmap. (A-G) Graphs showing expression of two example genes from each cluster, to illustrate the pattern of gene regulation observed in each cluster. Graphs produced by Dr Nicholas Iltott.

3.2.2.3 *CX₃CR1^{int} CD64⁺ versus CX₃CR1^{int} CD64⁻ MNP from inflamed lamina propria*

Due to space limitations, only the comparison of gene expression by CX₃CR1^{int} CD64⁺ versus CX₃CR1^{int} CD64⁻ MNPs from inflamed intestine three weeks after induction of *Hh* + anti-IL-10R colitis is discussed in detail. This comparison found that 174 genes were expressed at greater levels by CX₃CR1^{int} CD64⁺ MNPs while 47 genes were expressed at greater levels by CX₃CR1^{int} CD64⁻ MNPs (adjusted p value < 0.05, log₂(FC) > 1.5; full list of significant differentially expressed genes shown in Appendix, Table 1). Table 3.10 (shown at the end of the chapter) lists these genes, and highlights which were also upregulated by CX₃CR1^{int} CD64⁺ MNPs during inflammation versus the steady state (gene name in bold; for full list see Appendix, Table 3). The latter comparison showed that CX₃CR1^{int} CD64⁺ MNPs upregulated 619 genes during inflammation, while 230 genes were more highly expressed in the steady state (Appendix, Table 3).

Figure 3.8 shows a volcano plot of significance versus fold change in gene expression by CX₃CR1^{int} CD64⁺ versus CX₃CR1^{int} CD64⁻ MNPs during intestinal inflammation. Most of the differentially expressed genes show positive fold change values and were thus more highly expressed by CX₃CR1^{int} CD64⁺ MNPs, indicating that the differences between CX₃CR1^{int} CD64⁺ and CX₃CR1^{int} CD64⁻ MNPs during colitis seem to be due to greater gene expression in CX₃CR1^{int} CD64⁺ MNPs.

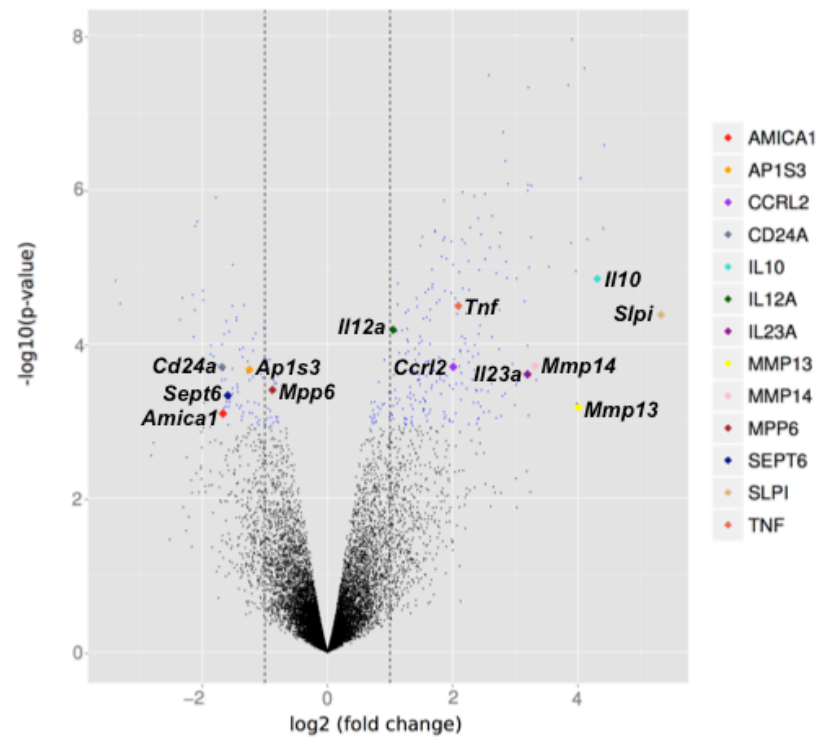


Figure 3.8 Volcano plot showing gene expression by CX₃CR1^{int} CD64⁺ vs. CX₃CR1^{int} CD64⁻ MNPs from inflamed lamina propria.

CX₃CR1^{GFP/+} mice were infected with *Helicobacter hepaticus* (*Hh*) and treated weekly with anti-IL-10R blocking mAb (*Hh* + anti-IL-10R) prior to analysis at three weeks. Single cell preparations of combined colonic and caecal lamina propria were stained and CX₃CR1^{int} CD64⁺ and CX₃CR1^{int} CD64⁻ MNPs were isolated by FACS as described in Figure 3.4. Illumina microarray and data analysis in R were performed. Volcano plot shows the significance ($-\log_{10}(\text{p value})$) and magnitude ($\log_2(\text{fold change})$) of differences in gene expression between CX₃CR1^{int} CD64⁺ and CX₃CR1^{int} CD64⁻ MNPs isolated from inflamed lamina propria. Blue dots represent probes that were significant (adjusted p value < 0.05) with an absolute fold change > 1.5. Large coloured dots highlight genes of interest as outlined in the key. Dotted lines represent absolute fold change -1.5 and +1.5. Positive $\log_2$ (fold change) indicates higher expression by CX₃CR1^{int} CD64⁺ MNPs, negative $\log_2$ (fold change) indicates higher expression by CX₃CR1^{int} CD64⁻ MNPs. Graph produced by Dr Nicholas Iltott.

3.2.2.3.1 Genes enriched in CX₃CR1^{int} CD64⁺ MNPs

3.2.2.3.1.1 Macrophage-selective genes

As expected, among the genes more highly expressed by CX₃CR1^{int} CD64⁺ versus CX₃CR1^{int} CD64⁻ MNPs during colitis were a number that have recently been described to be specific to or upregulated in macrophages relative to their expression in DCs (Gautier et al., 2012; Xue et al., 2014). These include *Cd14*, *Fcgr3* (CD16), *Sqrdl*, *Dok3* and *Pecr*. Additional genes that Gautier *et al.* (Gautier et al., 2012) noted were upregulated in three out of the four macrophage populations they investigated were also enriched in our CX₃CR1^{int} CD64⁺ population, such as *Slpi*, *Klra2*, *Lpl*, *Fcrl3* (FcγR4), *Pilrb*, *Lrp1*, *Itgb5*, *Mafb*, *Slc11a1*, *Slc15a3*, *C1qb* and *Apoe*. CX₃CR1^{int} CD64⁺ cells also showed higher expression of *Csf1r* and the transcription factor *Mafb*, which is required for monocyte differentiation (Kelly et al., 2000). Genes associated with both classical macrophage activation (e.g. *Il12a*, *Il23a*, and *Smox* and *Sod2*, components of the reactive oxygen species generation pathway) and alternative macrophage activation (e.g. *Chi3l3* (encoding Ym1), *Chi3l4* (encoding Ym2) and *Il1rn*) (Martinez and Gordon, 2014) were also upregulated in inflammation-associated CX₃CR1^{int} CD64⁺ relative to CX₃CR1^{int} CD64⁻ MNPs. These results confirm the presence of macrophages among CX₃CR1^{int} CD64⁺ MNPs. With the exception of *Fcrl3*, probes specific for all of the aforementioned genes were found in cluster 6, which represents genes induced in CD64⁺ cells during inflammation (Table 3.7). *Fcrl3* and one additional *Smox*-specific probe (probe ID 620008) were found in cluster 7 (genes induced during inflammation regardless of CD64 status (Table 3.8)).

3.2.2.3.1.2 Secretory leukocyte peptidase inhibitor (SLPI)

Slpi, which encodes secretory leukocyte peptidase inhibitor (SLPI), came top of the list of differentially regulated genes. SLPI is a serine protease inhibitor which can be induced in macrophages by LPS and may be partially responsible for hyporesponsiveness of macrophages to LPS (Jin et al., 1998; Jin et al., 1997). *Slpi* was expressed 40.15-fold ($\log_2(\text{FC}) = 5.33$) more by CX₃CR1^{int} CD64⁺ versus CX₃CR1^{int} CD64⁻ MNPs at day 21 of *Hh* + anti-IL-10R colitis (Table 3.10 and Appendix, Table 1). CX₃CR1^{int} CD64⁺ MNPs from inflamed lamina propria also expressed *Slpi* 39.76-fold ($\log_2(\text{FC}) = 5.31$) more than their steady state counterparts (Appendix, Table 3). These results suggest that *Slpi* expression is induced among CX₃CR1^{int} CD64⁺ MNPs during inflammation, which is reflected by its presence in cluster 6 (genes induced in CD64⁺ cells during inflammation; Table 3.7).

3.2.2.3.1.3 Pattern recognition receptors and innate immunity

CX₃CR1^{int} CD64⁺ MNPs expressed a number of molecular pattern recognition receptors at higher levels than CX₃CR1^{int} CD64⁻ MNPs. These included TLR5 (*Tlr5*), and a number of lectin and scavenger receptor genes such as *Clec4d* (*Clecsf8*), *Clec4e* (*Clecsf9*), *Clec5a* (*Clecsf5*), *Klra2* and *Scarf1*. CX₃CR1^{int} CD64⁺ MNPs also expressed *Trem3*, a macrophage-activating receptor (Chung et al., 2002) the functions and role of which remain unknown; numerous Fc receptors (*Fcgr3*, *Fcgr2b*, *Fcrl3*, *Fcer1g*); *Sirpb1a* (9930027N05RIK), which has been shown to promote phagocytosis in macrophages (Hayashi et al., 2004), and components of the complement cascade (*C1qb*, *C1qc* (*C1qg*), *C4*, *Cfb* (*H2-Bf*)). All of the genes mentioned here, including two *Fcgr2b*-specific probes (IDs 1110326 and 5700220), were found in cluster 6 (genes induced in CD64⁺ cells during inflammation; Table 3.7). An additional *Fcgr2b*-specific probe (ID 1780500) was found in cluster 2, indicating

downregulation in CD64⁺ cells during inflammation resulting in expression similar to steady state- and inflammation-derived CD64⁻ cells (Table 3.3; Figure 3.7b).

3.2.2.3.1.4 Cytokines and chemokines

CX₃CR1^{int} CD64⁺ MNPs expressed higher levels of the pro-inflammatory cytokine genes *Il23a*, *Il12a* (CX₃CR1^{int} CD64⁺ vs. CX₃CR1^{int} CD64⁻ log₂(FC) = 1.05, 2.07-fold; Appendix, Table 1) and *Tnf*. They also expressed *Tnfsf13*, which encodes a proliferation-inducing ligand (APRIL), a cytokine that controls T cell-independent IgA class switching (Castigli et al., 2005; Hardenberg et al., 2008; Litinskiy et al., 2002). Of particular relevance is the finding that Ly6C⁺ CD11b⁺ CD11c^{lo} MHCII⁺ TNFα⁺ iNOS⁺ cells, “TipDCs”, in the small intestinal lamina propria have been shown to induce such class switching via iNOS-induced expression of APRIL (Tezuka et al., 2007). In addition, CX₃CR1^{int} CD64⁺ MNPs expressed *Fzd1*, a marker of inflammatory macrophages, through which Wnt3a induced Wnt/β-catenin signalling can negatively regulate release of TNFα (Neumann et al., 2010). CX₃CR1^{int} CD64⁺ cells also expressed *Il10* and *Il1rn*, which encodes IL-1R antagonist, further suggesting that these cells may negatively regulate inflammatory cytokine responses in addition to producing these cytokines. CX₃CR1^{int} CD64⁺ cells expressed greater levels of the chemokines *Ccl5*, *Ccl9*, *Cxcl1* and *Cxcl2*. All of the genes mentioned here were found in cluster 6 (genes induced in CD64⁺ cells during inflammation; Table 3.7) except *Tnfsf13*, which was in cluster 7 (genes induced during inflammation regardless of CD64 status; Table 3.8).

3.2.2.3.1.5 Reactive oxygen species generation, lipid metabolism, tissue remodelling

We observed that genes encoding components of the pathways governing reactive oxygen species generation (*Smox*, *Sod2*), lipid metabolism (*Apoe*, *Lrp1*), and tissue remodelling and angiogenesis (*Mmp13*, *Mmp14*, *Spon1*) were also differentially expressed by CX₃CR1^{int} CD64⁺ compared to CX₃CR1^{int} CD64⁻ MNPs during inflammation. This pattern of expression is again consistent with these cells belonging to the monocyte-macrophage lineage (Murray and Wynn, 2011). Probes specific for all of these genes were found in cluster 6 (genes induced in CD64⁺ cells during inflammation; Table 3.7), although an additional *Mmp13*-specific probe (ID 2060040) was found in cluster 2 (probes downregulated in CD64⁺ cells during inflammation resulting in expression similar to steady state- and inflammation-derived CD64⁻ cells (Table 3.3; Figure 3.7b)), and an additional *Smox*-specific probe was present in cluster 7 as previously mentioned.

3.2.2.3.1.6 Chemokine receptors

The only chemokine receptor genes enriched among CX₃CR1^{int} CD64⁺ MNPs during inflammation were *Ccr12* (CX₃CR1^{int} CD64⁺ vs. CX₃CR1^{int} CD64⁻ log₂(FC) = 2.10, 4.29-fold), which shares homology with *Ccr1* (Fan et al., 1998), and *Ccr5* (CX₃CR1^{int} CD64⁺ vs. CX₃CR1^{int} CD64⁻ log₂(FC) = 0.76, 1.69-fold; and log₂(FC) = 0.73, 1.66-fold for different probes). *Ccr12* was found in cluster 6 (genes induced in CD64⁺ cells during inflammation; Table 3.7), however no *Ccr5*-specific probes met both of our clustering criteria (adjusted p value < 0.05 and log₂(FC) > 1) therefore *Ccr5* was not represented in any cluster.

3.2.2.3.1.7 Solute carrier family members

A number of solute carrier family members (*Slc11a1*, *Slc31a2*, *Slc2a1*, *Slc15a3*, *Slc25a28*, *Slc7a11*) were more greatly expressed by CX₃CR1^{int} CD64⁺ MNPs, including *Slc11a1* and *Slc15a3*, which were recently described to be expressed by multiple macrophage populations (Gautier et al., 2012). All of these genes were found in cluster 6, confirming their upregulation in CD64⁺ MNPs during inflammation (Table 3.7). The human SLC family contains at least 384 proteins (Fredriksson et al., 2008), making it the second largest family of membrane proteins after the GPCRs, which are encoded by approximately 800 human genes (Fredriksson et al., 2003). SLCs control the entry of numerous substances into cells, including sugars, amino acids and nucleotides, along with inorganic ions and drugs (Hoglund et al., 2011). The genes upregulated in CX₃CR1^{int} CD64⁺ MNPs fell into three categories: transporters of amino acids and oligopeptides (*Slc7a11*, *Slc15a3*), inorganic ions (*Slc11a1*, *Slc25a28*, *Slc31a2*), and solutes (*Slc2a1*). These proteins may provide ways to specifically target CX₃CR1^{int} CD64⁺ MNPs, for instance with substances engineered for uptake according to the SLCs they selectively express.

3.2.2.3.2 Genes enriched in CX₃CR1^{int} CD64⁻ MNPs

Genes which were expressed more by CX₃CR1^{int} CD64⁻ MNPs (represented by negative fold change values in Table 3.10; log₂(FC) < -1.5) and were also not upregulated by CX₃CR1^{int} CD64⁺ MNPs in response to inflammation, included *Cd24a*, *Amica1*, *Sept6*, *Itgae* (encoding CD103) and *Cdh1* (encoding E-cadherin) (Table 3.10). CD24 is a DC-selective surface marker (Schlitzer et al., 2013) and *Amica1* and *Sept6* have been identified as cDC-selective core genes (Miller et al., 2012). Some of the other cDC-selective genes identified by the latter study were also more highly expressed by CX₃CR1^{int} CD64⁻ MNPs

during colitis but did not make the $\log_2(\text{FC}) < -1.5$ cut-off, such as *Mpp6* ($\text{CX}_3\text{CR1}^{\text{int}} \text{CD64}^+$ vs. $\text{CX}_3\text{CR1}^{\text{int}} \text{CD64}^-$ $\log_2(\text{FC}) = -0.88$) and *Ap1s3* ($\text{CX}_3\text{CR1}^{\text{int}} \text{CD64}^+$ vs. $\text{CX}_3\text{CR1}^{\text{int}} \text{CD64}^-$ $\log_2(\text{FC}) = -1.24$) (Appendix, Table 1). Additional genes related to specific subpopulations of DCs were also more highly expressed by $\text{CX}_3\text{CR1}^{\text{int}} \text{CD64}^-$ MNPs during colitis, including the transcription factor *Irf4* ($\text{CX}_3\text{CR1}^{\text{int}} \text{CD64}^+$ vs. $\text{CX}_3\text{CR1}^{\text{int}} \text{CD64}^-$ $\log_2(\text{FC}) = -1.25$) associated with $\text{CD103}^+ \text{CD11b}^+$ DCs (Persson et al., 2013; Schlitzer et al., 2013), $\text{CD11b}^{\text{hi}} \text{CD4}^+ \text{CD8}\alpha^-$ DCs and GM-CSF-mediated *in vitro* DC differentiation (Suzuki et al., 2004; Tamura et al., 2005), and the chemokine receptor *Xcr1* ($\text{CX}_3\text{CR1}^{\text{int}} \text{CD64}^+$ vs. $\text{CX}_3\text{CR1}^{\text{int}} \text{CD64}^-$ $\log_2(\text{FC}) = -0.99$) associated with $\text{CD8}\alpha^+ \text{CD103}^+$ DCs (Bachem et al., 2010; Dorner et al., 2009; Miller et al., 2012) (Appendix, Table 1). Despite the heterogeneity of the sorted $\text{CX}_3\text{CR1}^{\text{int}} \text{CD64}^-$ population, these results indicate the presence of dendritic cells. With the exception of *Irf4*, *Mpp6* and *Xcr1*, all of the genes mentioned here were found in cluster 5 (Table 3.6). Cluster 5 represents genes which were enriched in $\text{CX}_3\text{CR1}^{\text{int}} \text{CD64}^-$ relative to $\text{CX}_3\text{CR1}^{\text{int}} \text{CD64}^+$ MNPs during inflammation due to their downregulation by $\text{CX}_3\text{CR1}^{\text{int}} \text{CD64}^+$ cells (Figure 3.7e). *Irf4* was found in cluster 4 (genes downregulated during inflammation regardless of CD64 status (Table 3.5) and *Mpp6* in cluster 1 (genes upregulated in CD64^+ cells during inflammation resulting in expression similar to steady state- and inflammation-derived CD64^- cells (Table 3.2; Figure 3.7a)). No *Xcr1*-specific probes met both of our clustering criteria (adjusted p value < 0.05 and $\log_2(\text{FC}) > 1$) therefore *Xcr1* was not represented in any cluster.

3.2.2.4 Validation of genes by RT-qPCR

We selected some of the genes enriched in $\text{CX}_3\text{CR1}^{\text{int}} \text{CD64}^+$ MNPs during inflammation for validation by RT-qPCR. We included *Fcgr1*, encoding CD64, which according to our

microarray study was not differentially expressed at the mRNA level by CX₃CR1^{int} CD64⁺ MNPs versus CX₃CR1^{int} CD64⁻ MNPs (Appendix, Table 1) despite obvious differences in protein expression (Figure 3.4). Figure 3.9 shows RT-qPCR confirmation of greater expression of *Fcgr1* (CD64), *Il23a* (IL-23p19) and *Ccr12* (CCRL2) by CX₃CR1^{int} CD64⁺ relative to CX₃CR1^{int} CD64⁻ MNPs from the inflamed lamina propria three weeks after induction of *Hh* + anti-IL-10R-induced colitis.

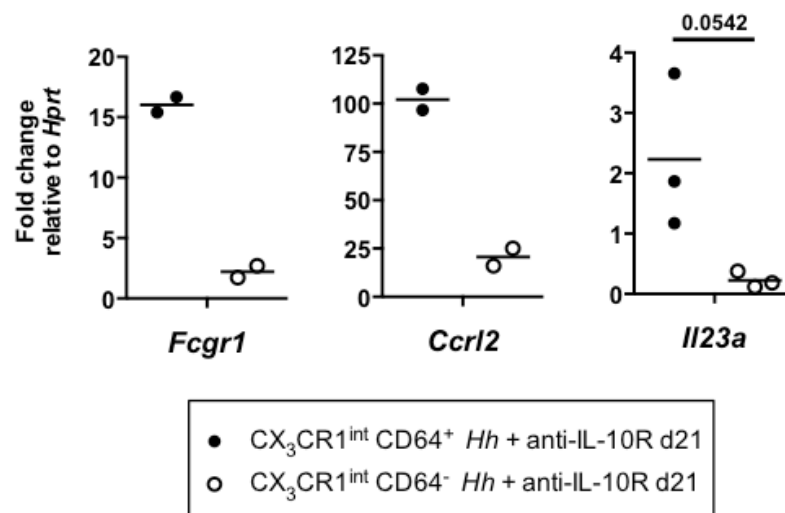


Figure 3.9 RT-qPCR assessment of CD64 expression and validation of selected microarray data.

$CX_3CR1^{GFP/+}$ mice were infected with *Helicobacter hepaticus* (*Hh*) and treated weekly with anti-IL-10R blocking mAb (*Hh* + anti-IL-10R) prior to analysis at three weeks. Single cell preparations of combined colonic and caecal lamina propria were stained and the indicated cell subsets isolated by FACS as described in Figure 3.4. RNA was extracted and RT-qPCR used to measure expression of the genes encoding CD64 (*Fcgr1*), CCRL2 (*Ccr12*) and IL-23p19 (*Il23a*). Each data point represents a biological replicate of cells sorted from colonic and caecal lamina propria pooled from 4-7 mice, horizontal line indicates group mean. Data represent two biological replicates obtained from one experiment (*Fcgr1*, *Ccr12*) or three biological replicates pooled from two independent experiments (*Il23a*) ($CX_3CR1^{int} CD64^+$, *Hh* + anti-IL-10R day 21, filled circles, $n=2-3$; $CX_3CR1^{int} CD64^-$, *Hh* + anti-IL-10R day 21, open circles, $n=2-3$). $p = 0.0542$ calculated by unpaired two-tailed Student's *t* test.

3.2.3 Role of T-bet in innate immune cells during intestinal inflammation

Previous work by our group found that CD103⁺ DCs in the MLNs were pro-inflammatory during colitis (Laffont et al., 2010). In the steady state these cells are tolerogenic, and the change to a pro-inflammatory phenotype was associated with increased expression of the transcription factor T-bet (encoded by *Tbx21*) (Laffont et al., 2010). Our microarray analysis indicated that lamina propria-derived CX₃CR1^{int} CD64⁻ MNPs upregulate expression of *Tbx21* during inflammation relative to the steady state, although this was not statistically significant after p value adjustment (Appendix, Table 4). We did not observe differential expression of *Tbx21* by CX₃CR1^{int} CD64⁺ versus CX₃CR1^{int} CD64⁻ MNPs when both were isolated from either inflamed or resting colonic lamina propria (Appendix).

To examine whether T-bet expressed by DCs might influence intestinal inflammation, we utilised lymphocyte-deficient *Tbx21*^{-/-} *Rag2*^{-/-} mice. Although this strain lacks T-bet not only in DCs but in all innate immune cells, it allowed us to assess initially whether T-bet expressed by the innate immune system, of which DCs are an important component, may play a role in colitis.

First, we determined whether *Tbx21*^{-/-} *Rag2*^{-/-} mice developed spontaneous colitis in our animal facility, following contradictory reports on this from different laboratories (Garrett et al., 2007; Powell et al., 2012). We monitored *Tbx21*^{-/-} *Rag2*^{-/-} mice until 12 weeks of age and did not observe spontaneous colitis development (data not shown). Our animal facility was negative for *Klebsiella pneumoniae*, *Proteus mirabilis* and *Helicobacter* bacterial species, which have all recently been associated with the development of colitis in these mice (Garrett et al., 2010; Powell et al., 2012).

T-bet expression by DCs has been suggested to promote induction of IFN γ expression in naïve T cells (Lipscomb et al., 2009; Lugo-Villarino et al., 2003), so we examined whether deficiency of T-bet in the innate immune system affected colitis during the T cell transfer model. We induced colitis in *Tbx21*^{-/-} *Rag2*^{-/-} mice by reconstitution with 4 x 10⁵ wild-type naïve CD4⁺ CD45RB^{hi} CD25⁻ T cells to ensure that any effects were not due to the absence of T-bet expressed by T cells. In contrast to Balb/c *Rag2*^{-/-} controls, *Tbx21*^{-/-} *Rag2*^{-/-} mice reconstituted with wild-type naïve CD4⁺ T cells continued to gain weight throughout the model (Figure 3.10a-b). Although *Tbx21*^{-/-} *Rag2*^{-/-} mice were initially lighter than Balb/c *Rag2*^{-/-} controls (Figure 3.10b), their weight did not differ from that of the control group at any point following adoptive transfer of T cells. T-bet-deficient and T-bet-sufficient T cell recipients developed identical splenomegaly (Figure 3.10c) and intestinal pathology (Figure 3.10d). Due to the absence of any effect on the severity of intestinal disease, we did not proceed further with investigation of the role of T-bet in mononuclear phagocytes.

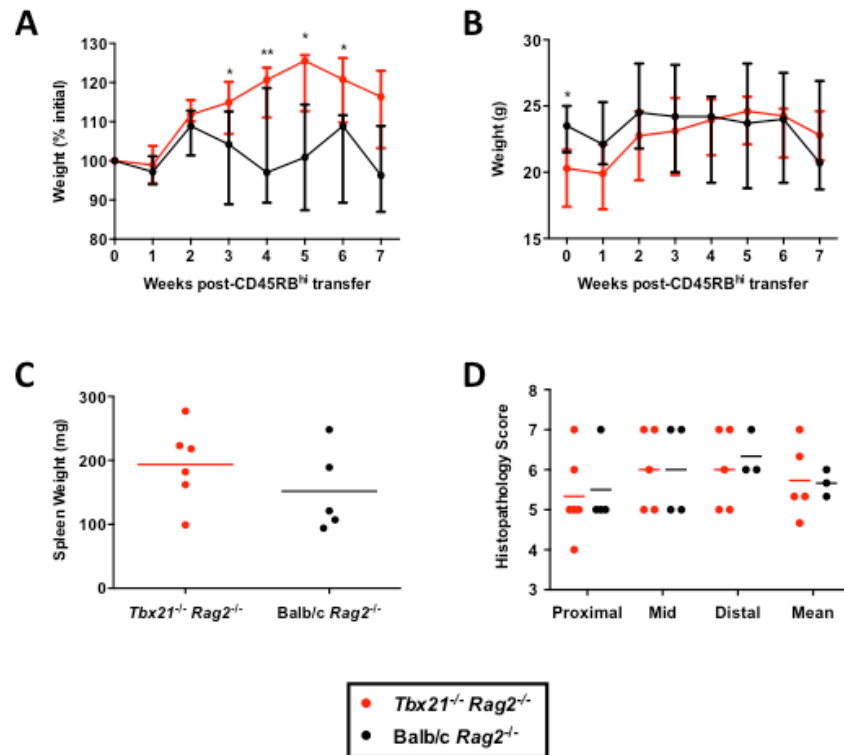


Figure 3.10 T-bet deficiency lessens T cell transfer colitis-associated weight loss but does not affect splenomegaly or intestinal pathology.

T-bet-deficient *Tbx21*^{-/-} *Rag2*^{-/-} mice or Balb/c *Rag2*^{-/-} controls received 4×10^5 naïve CD4⁺ CD45RB^{hi} CD25⁻ T cells (>95% purity) by i.p. injection, prior to analysis 7 weeks later. (A) Weight loss over the duration of the experiment, shown as a percentage of initial weight prior to T cell transfer. (B) Absolute weights throughout the experiment. (A-B) Data represent median + range. * $p < 0.05$, ** $p < 0.01$; *Tbx21*^{-/-} *Rag2*^{-/-} + naïve CD4⁺ CD45RB^{hi} T cells versus Balb/c *Rag2*^{-/-} + naïve CD4⁺ CD45RB^{hi} T cells, per timepoint, determined by Mann Whitney U test. (C) Spleen weight at termination 7 weeks post-T cell transfer. (D) Histopathology scores for proximal, mid and distal colon, and whole colon mean, determined as mean of proximal + mid + distal colon scores, where all were available. (C-D) Each data point represents an individual mouse, horizontal line indicates group mean. Data are from one experiment (*Tbx21*^{-/-} *Rag2*^{-/-} + naïve CD4⁺ CD45RB^{hi} T cells, red filled circles, $n=5-6$; Balb/c *Rag2*^{-/-} + naïve CD4⁺ CD45RB^{hi} T cells, black filled circles, $n=3-5$. Variety in “n” due to inadequacy of some tissue sections for scoring in (D)).

3.3 Discussion

Mononuclear phagocytes are abundant in the intestine, where they play vital roles in the maintenance of homeostasis by balancing tolerance to harmless antigens with appropriate protective immunity. During the course of this project, significant advances were made in our understanding of the diverse MNP subsets found in the intestine and of their origins and functions, although much remains unknown.

Biological variability can be seen among the histopathology scores and frequencies of cells infiltrating the lamina propria during *Hh* + anti-IL-10R colitis presented here. This could be explained by the fact that *Hh* + anti-IL-10R colitis is triggered by bacterial infection and that individual mice may vary in how they respond to *Hh*, especially early after colonisation with *Hh*. We found that frequencies of MNP subsets in the lamina propria were more uniform two weeks after induction of colitis, perhaps indicating that by that point an inflammatory “equilibrium” had been reached. Interestingly, MNP frequencies were more variable three weeks post-*Hh* + anti-IL-10R treatment. This could reflect a shift in MNP phenotype around this time, when the immune response switches to resolve inflammation, something that our microarray results (discussed below) seem to suggest. We used the three-week timepoint for analysis of various experiments prior to discovering that robust intestinal pathology is also apparent two weeks after *Hh* + anti-IL-10R treatment.

We observed lower frequencies of CX₃CR1⁺ MNP subsets in the lamina propria during inflammation than those published in other studies using the DSS model of colitis (Zigmond et al., 2012). We also observed very low frequencies of CX₃CR1^{hi} macrophages in the uninfamed lamina propria. These frequencies were representative of our other experiments. Differences between our results and those of other groups may therefore

reflect the influence of the microbiota, which varies between animal facilities, or potentially a sub-optimal isolation protocol. Despite continued optimisation of the lamina propria cell isolation protocol, macrophage retrieval remains difficult as these cells can be highly associated with the connective tissue and are thus hard to disassociate efficiently.

3.3.1 MNP subsets in the lamina propria during steady state and inflammation

We investigated the mononuclear phagocyte compartment of the large intestine during colitis induced by *Helicobacter hepaticus* in the absence of functional IL-10 signalling (Kullberg et al., 2006). Our results from *Hh* + anti-IL-10R colitis are in agreement with published work which used the T cell transfer model (Tamoutounour et al., 2012; Weber et al., 2011). *Hh* + anti-IL-10R and T cell transfer colitis are both models of chronic intestinal inflammation, in contrast to the acute DSS-induced colitis model commonly used in important recent investigations of intestinal MNPs (Bain et al., 2013; Zigmond et al., 2012).

CX₃CR1^{hi} macrophages represent the fate of Ly6C^{hi} monocytes in the colonic lamina propria during the steady state (Bain et al., 2014; Bain et al., 2013; Rivollier et al., 2012; Tamoutounour et al., 2012; Varol et al., 2009; Zigmond et al., 2012). Ly6C^{hi} monocytes themselves express intermediate levels of CX₃CR1 and develop into CX₃CR1^{hi} macrophages via a series of CX₃CR1^{int} populations (Bain et al., 2013; Tamoutounour et al., 2012) (Figure 1.3). However, in the presence of inflammation, differentiation of Ly6C^{hi} monocytes into resident macrophages is interrupted and CX₃CR1^{int} MNPs accumulate (Bain et al., 2013; Rivollier et al., 2012; Tamoutounour et al., 2012; Weber et al., 2011;

Zigmond et al., 2012). We found that frequencies of $CX_3CR1^{int} Ly6C^{hi} MHCII^{-}$ monocytes and $CX_3CR1^{int} Ly6C^{hi} MHCII^{+}$ MNPs in the lamina propria increased until at least two weeks and peaked between two and three weeks after *Hh* + anti-IL-10R treatment. These two populations rose in frequency in parallel. It is possible that our earliest timepoint – day 5 – was too late to observe the sequential appearance of these populations (Bain et al., 2013). We have observed in other studies that $CX_3CR1^{int} Ly6C^{hi}$ MNPs were recruited to the lamina propria within 16 hours of *Hh* + anti-IL-10R treatment (Dr Isabelle Arnold, unpublished observations). By three weeks, frequencies of $CX_3CR1^{int} Ly6C^{hi} MHCII^{-}$ monocytes and $CX_3CR1^{int} Ly6C^{hi} MHCII^{+}$ MNPs had returned almost to baseline levels. However, it was not until three weeks after colitis induction that we observed an increase in the frequency of $CX_3CR1^{int} Ly6C^{lo/-} MHCII^{+} CD64/F4/80^{+}$ macrophages, the relative frequency of which had been in decline until that point, likely reflecting the influx of cells into the lamina propria during inflammation. Whether these alterations in frequency are relative or reflect absolute changes in cell numbers is unclear and could be confirmed in future. These results suggest that *Hh* + anti-IL-10R-induced inflammation may halt the differentiation of $Ly6C^{hi}$ monocytes into CX_3CR1^{hi} macrophages at the $CX_3CR1^{int} Ly6C^{hi} MHCII^{+}$ stage, as indicated by other models including acute DSS (Bain et al., 2013; Platt et al., 2010; Zigmond et al., 2012) and chronic T cell transfer (Tamoutounour et al., 2012) colitis.

Although we did not study the developmental relationship between these MNP populations, from the timing of their arrival in the lamina propria, it appears that $CX_3CR1^{int} Ly6C^{hi} MHCII^{+}$ MNPs or newly recruited monocytes may resume differentiation into $CX_3CR1^{int} Ly6C^{lo/-}$ macrophages 2-3 weeks after induction of colitis. Whether the same cells that were present during colitis differentiate further into $Ly6C^{lo/-}$ macrophages,

or whether newly recruited cells are able to differentiate beyond CX₃CR1^{int} Ly6C⁺ MNPs as in the steady state is currently unclear.

Our timecourse analysis of both intestinal histopathology and the lamina propria MNP compartment during *Hh* + anti-IL-10R colitis indicates that peak pathology coincided with the highest frequencies of CX₃CR1^{int} Ly6C⁺ MNP populations. This suggests that these populations – Ly6C^{hi} MHCII⁻ monocytes and Ly6C^{hi} MHCII⁺ MNPs – may be pro-inflammatory and contribute to pathology during *Hh* + anti-IL-10R colitis, in agreement with studies of other models (Bain et al., 2013; Platt et al., 2010; Tamoutounour et al., 2012; Zigmond et al., 2012). We also observed that the return in frequency of CX₃CR1^{int} CD64⁺ Ly6C^{lo/-} macrophages after peak pathology correlated with reduced intestinal pathology scores. This suggests that CX₃CR1^{int} CD64⁺ Ly6C^{lo/-} macrophages might play a role in the resolution of inflammation.

We also observed elevated frequencies of CX₃CR1^{int} CD11b⁺ CD64⁻ MNPs at days 5 and 13, which had declined by week 3. It is interesting that their frequency follows the same pattern as the CX₃CR1^{int} Ly6C⁺ populations. A recent study has identified a population of *bona fide* DCs which would fall in our CX₃CR1^{int} CD11b⁺ CD64⁻ gate, some of which express CCR2 and promote Th17 polarisation (Scott et al., 2014). If these cells are indeed present during *Hh* + anti-IL-10R colitis, they might thus contribute to pathology. To confirm the presence of DCs, future staining panels should include additional DC-selective markers such as Flt3, CD26 (Miller et al., 2012) or CD24 (Schlitzer et al., 2013). Ly6C^{hi} monocyte recruitment to the colon is reported to be mediated by CCR2 in the steady state and during DSS-, hapten- and *Citrobacter rodentium*-induced colitis (Bain et al., 2013; Khan et al., 2006; Kim et al., 2011b; Platt et al., 2010; Popivanova et al., 2009; Takada et al., 2010; Zigmond et al., 2012). Ly6C^{hi} monocytes and CX₃CR1^{int} CD11b⁺ CD64⁻ MNPs may mirror

each other in recruitment during *Hh* + anti-IL-10R colitis due to shared CCR2-mediated recruitment.

It would also be important to try to identify all populations of DCs simultaneously in such experiments. This could be achieved by selecting all CD11c^{hi} MHCII⁺ cells, followed by exclusion of CD64⁺ F4/80⁺ macrophages, before assessment of CD103 and CD11b expression to identify CD103⁺ CD11b⁻, CD103⁺ CD11b⁺ and CD103⁻ CD11b⁺ DCs (Scott et al., 2014). These subsets could then be further assessed for expression of other markers such as CX₃CR1 and Ly6C.

3.3.2 Microarray analysis

Using a more precise staining panel, we found that the CX₃CR1^{int} CD64⁺ population we used for microarray analysis contained both Ly6C^{hi} MHCII⁺ MNPs and Ly6C^{lo/-} MHCII⁺ macrophages expressing a range of CD11c. During inflammation, approximately one-third of the cells were Ly6C^{hi} MHCII⁺, and the remaining two-thirds Ly6C^{lo/-} MHCII⁺. During the steady state, approximately 80% of CX₃CR1^{int} CD64⁺ cells were Ly6C^{lo/-} MHCII⁺ and expressed lower levels of CD11c than their counterparts during colitis. The CX₃CR1^{int} CD64⁻ microarray population was almost entirely comprised of Ly6C^{lo/-} MHCII⁺ CD11c⁺ MNPs during both steady state and inflammation, although this population showed heterogeneous expression of CD11b. In addition to *bona fide* CD103⁻ CD11b⁺ DCs (Scott et al., 2014), it is possible that some CD103⁺ CD11b⁻ DCs could have been present in this population particularly during inflammation, which is supported by our observation of higher *Itgae* expression by CX₃CR1^{int} CD64⁻ versus CX₃CR1^{int} CD64⁺ MNPs from inflamed lamina propria. The heterogeneity of the CX₃CR1^{int} CD64⁻ population means that caution

needs to be exercised in interpreting exactly which cell type may be responsible for differential gene expression by these cells.

3.3.2.1 $CX_3CR1^{int} CD64^+$ MNP

Our microarray analysis revealed genes that were differentially expressed by $CX_3CR1^{int} CD64^+$ and $CX_3CR1^{int} CD64^-$ MNPs during the presence and absence of inflammation. We found enrichment of numerous genes recently described to be expressed by macrophages (Gautier et al., 2012; Xue et al., 2014) among $CX_3CR1^{int} CD64^+$ cells.

However, we did not observe enrichment of all of the so-called “core” macrophage genes reported by Gautier *et al.* (Gautier et al., 2012), most notably *Mertk* which is increasingly used as a macrophage-selective marker. The reason for this discrepancy is unclear. If MerTK is not expressed or only expressed at lower levels by monocytes and is upregulated during macrophage differentiation, as is the case for a number of macrophage markers including CD64 (Bain et al., 2013; Tamoutounour et al., 2012), then *Mertk* transcripts may have been diluted by the presence of the less mature macrophages which were abundant in our microarray population obtained from inflamed intestine. This hypothesis is supported by work carried out by Mowat *et al.*, who compared the four monocyte-to-macrophage stages found in steady state colon by microarray and observed their progressive upregulation of *Mertk* (Professor Allan Mowat, personal communication).

Some genes typically associated with alternative activation, or M2 polarisation, of macrophages were upregulated in $CX_3CR1^{int} CD64^+$ MNPs, such as *Chi3l3* and *Chi3l4*, which encode Ym1 and Ym2, respectively. However, these cells also expressed genes

associated with classically activated M1 macrophages, including *Il23a*, *Il12a*, and two components of the reactive oxygen species generation pathway, *Smox* and *Sod2*. We do not know whether individual cells expressed both “types” of gene, or if they were expressed by distinct cells within a heterogeneous population. Given the abundance and variety of stimuli in the intestine, our results are not necessarily surprising, as it is now clear that macrophage stimulation by different combinations of factors produces a diverse spectrum of activation which is not as polarised as the M1 versus M2 classification implies (Xue et al., 2014). Our results may also reflect the plasticity of the Ly6C⁺ MHCII⁺ cells among CX₃CR1^{int} CD64⁺ MNPs, whose ultimate differentiation and function varies depending on the presence of inflammation (Bain et al., 2013; Tamoutounour et al., 2012).

Some of the genes expressed more highly by CX₃CR1^{int} CD64⁺ MNPs during colitis are discussed in greater detail below, selected on the basis that they could be particularly relevant to the functions of CX₃CR1^{int} CD64⁺ MNPs during *Hh* + anti-IL-10R colitis.

3.3.2.1.1 *SLPI*

Sipi, encoding secretory leukocyte peptidase inhibitor (SLPI), was differentially expressed by CX₃CR1^{int} CD64⁺ relative to CX₃CR1^{int} CD64⁻ MNPs at day 21 of *Hh* + anti-IL-10R colitis. It was also upregulated by CX₃CR1^{int} CD64⁺ cells during inflammation. SLPI is a serine protease inhibitor which can be induced in macrophages by LPS and may be partially responsible for hyporesponsiveness of macrophages to LPS (Jin et al., 1998; Jin et al., 1997), potentially by interfering with binding of LPS to CD14 or by inhibiting NFκB activation (reviewed in (Williams et al., 2006)). SLPI is constitutively expressed by

mucosal epithelia and can be induced further by pro-inflammatory stimuli, such as TLR ligands (Williams et al., 2006; Xu et al., 2007).

By interfering with NF κ B activation, SLPI has been shown to inhibit IgG and IgA class switching by B cells from human tonsils which were exposed to viral RNA, IL-10 and B cell-activating factor (BAFF) (Xu et al., 2007). CX₃CR1^{int} CD64⁺ MNPs expressed *Tnfsf13*, encoding APRIL, which also induces T cell-independent IgA class switching (Castigli et al., 2005; Hardenberg et al., 2008; Litinskiy et al., 2002). Ly6C⁺ CD11b⁺ CD11c^{lo} MHCII⁺ TNF α ⁺ iNOS⁺ cells, “TipDCs”, have been shown to induce such class switching via iNOS-induced expression of APRIL (Tezuka et al., 2007). Some cells in our CX₃CR1^{int} CD64⁺ MNP population expressed very similar surface markers to these TipDCs, and the expression of *Tnfsf13* by CX₃CR1^{int} CD64⁺ MNPs during *Hh* + anti-IL-10R colitis suggests they might be capable of inducing T cell-independent IgA class switching in the lamina propria. Whether SLPI also inhibits APRIL-induced class switching is unknown, however co-expression of SLPI and APRIL could represent a negative regulatory system controlling this process.

As an anti-protease, SLPI inactivates a number of proteases including human neutrophil elastase, cathepsin G, trypsin, chymotrypsin and chymase (Williams et al., 2006). This is in keeping with the recent description of a role for Ly6C^{hi} MHCII⁺ cells in the regulation of neutrophils during *Toxoplasma gondii* infection (Grainger et al., 2013), and suggests that CX₃CR1^{int} CD64⁺ MNPs could also regulate the neutrophils which accumulate during *Hh* + anti-IL-10R colitis. SLPI displays additional anti-microbial activity against bacteria, including *E. coli*, and fungi, and can prevent transmission of HIV by blocking its entry into monocytes (Williams et al., 2006).

SLPI also has anti-inflammatory properties, including prevention of inflammatory cell recruitment to sites of inflammation, inhibition of mast cell and neutrophil degranulation, and promotion of tissue repair (Williams et al., 2006). Of particular interest is the suggestion that SLPI might control the switch between inflammation and resolution (Williams et al., 2006). SLPI can be upregulated in macrophages following recognition of apoptotic cells (Odaka et al., 2003), and promotes macrophage production of TGF β and IL-10 – which was also differentially expressed by CX₃CR1^{int} CD64⁺ versus CX₃CR1^{int} CD64⁻ MNPs from inflamed lamina propria – in response to LPS (Sano et al., 2000). Via its ability to prevent inflammatory cell recruitment and to induce anti-inflammatory mediator production by macrophages, SLPI could promote the reductions in both inflammatory MNP subsets and intestinal pathology that we observed three weeks after induction of colitis, when our microarray populations were sorted.

SLPI is also relevant to IBD. Immunohistochemistry and RT-qPCR revealed that SLPI expression is elevated in inflamed tissue from UC but not CD patients (Schmid et al., 2007). SLPI expression did not differ significantly between inflamed CD, uninfamed CD, uninfamed UC, and uninfamed controls (Schmid et al., 2007). It is thought that the balance of proteases and protease inhibitors plays an important role in IBD histopathology, and that the defective induction of protease inhibitors in CD might contribute to the more severe transmural inflammation observed in CD relative to the mucosa-restricted inflammation seen in UC (Wehkamp et al., 2007). SLPI is an extremely interesting candidate. Following validation of its expression by CX₃CR1^{int} CD64⁺ MNPs, its potential roles in macrophage function and in promoting the shift from inflammation to resolution of colitis could be investigated.

3.3.2.1.2 *Pro-inflammatory cytokines*

Three weeks after induction of *Hh* + anti-IL-10R colitis, CX₃CR1^{int} CD64⁺ MNPs differentially expressed the pro-inflammatory cytokines *Tnf*, *Il23a* (IL-23p19) and *Il12a* (IL-12p35), indicating that these cells may play a role in prolonging intestinal and systemic disease. IL-23 and IL-12 both contribute to a pro-inflammatory cascade, inducing expression of other cytokines such as IFN γ , IL-17, TNF α , IL-1 β , and IL-23 and IL-12 themselves, in addition to distinct classes of inflammatory mediators, such as matrix metalloproteinases (MMPs) (Cua et al., 2003; Vignali and Kuchroo, 2012). IL-23 has been shown to drive the intestinal pathology observed during innate and adaptive *Hh*-induced colitis models (Hue et al., 2006; Kullberg et al., 2006), the anti-CD40 model (Uhlir et al., 2006b), and in IL-10-deficient mice (Yen et al., 2006). IL-12 is not required for colitis, but is responsible for the systemic symptoms often seen alongside intestinal pathology, such as splenomegaly and wasting disease (Uhlir et al., 2006b).

Unfortunately *Il12b*, encoding IL-12p40, was not represented in our results because it did not contain a probe that reached a signal intensity significantly above background in at least 2 samples, and was therefore filtered out prior to analysis. Expression of *Il12b* could be determined by RT-qPCR. However, it has been suggested that regulation of IL-12p35 and IL-23p19 controls production of functional IL-12 versus IL-23 (Croxford et al., 2012) therefore knowledge of the expression of *Il23a* and *Il12a* might be more informative regarding the balance of these two cytokines. Expression of the individual protein subunits, or of IL-12p70 and IL-23, could be confirmed by ELISA or multiplex assay on supernatant from *ex vivo* cultures of sorted CX₃CR1^{int} CD64⁺ MNPs. IL-12p40 protein expression could also be measured by intracellular flow cytometry staining.

3.3.2.1.3 *Proteases*

We observed expression of *Mmp13* (MMP13; collagenase 3) and *Mmp14* (MMP14; membrane type MMP 1, MT-MMP1) by CX₃CR1^{int} CD64⁺ MNPs during *Hh* + anti-IL-10R colitis. These enzymes play both pro-and anti-inflammatory roles in the intestine and several MMPs are associated with IBD (Ravi et al., 2007). MMP13 degrades multiple forms of collagen and their cleavage products during extracellular matrix remodelling in chronic wounds (Knauper et al., 1996). Expression of MMP13 has been observed in fibroblasts and macrophage-like cells in and beneath the beds of chronic gastrointestinal (Vaalamo et al., 1998) and cutaneous (Vaalamo et al., 1997) ulcers. MMP14, which activates MMP13 (Kostadinova et al., 2014) and seems to promote macrophage migration and basement membrane invasion (Sakamoto and Seiki, 2009), was also elevated in ulcerated but not mildly inflamed colonic tissue from IBD patients (von Lampe et al., 2000). Since expression of MMP13 – and MMP14 – was not observed at sites of superficial intestinal damage, nor in all samples from UC and CD patients, MMP13 was suggested to be upregulated relatively late during inflammation and intestinal remodelling (Vaalamo et al., 1998). This is in accordance with the timepoint at which our microarray samples were sorted, three weeks after induction of *Hh* + anti-IL-10R colitis, when we observed that intestinal pathology had begun to decrease in severity, suggesting a shift towards resolution of inflammation.

Additionally, MMP13 can induce shedding of transmembrane TNF α , and accordingly MMP13-deficient mice are slightly protected from DSS colitis and resistant to LPS-induced shock (Vandenbroucke et al., 2013). However, MMP13 and MMP14 can also activate latent TGF β (D'Angelo et al., 2001; Mu et al., 2002), a potent anti-inflammatory cytokine.

These functions indicate mechanisms through which expression of MMP13 and MMP14 could also limit inflammation.

3.3.2.1.4 Chemokines

CX₃CR1^{int} CD64⁺ MNP^s expressed greater levels of the chemokines *Ccl5* (CCL5, RANTES), *Ccl9* (CCL9), *Cxcl1* (CXCL1, KC) and *Cxcl2* (CXCL2, MIP2). CCL5 induces macrophage and NK cell migration and facilitates interactions between T cells and DCs via CCR1, CCR3 and CCR5 (Griffith et al., 2014). CXCL1 and CXCL2 bind CXCR2 through which they mediate neutrophil recruitment (Burdon et al., 2005). The function of CCL9 remains largely unknown (Griffith et al., 2014). CCL9 was found to be secreted by the follicle-associated epithelium overlying Peyer's patches, and recruited CD11b⁺ DCs to the dome region (Zhao et al., 2003). CCL9 is reported to bind weakly to CCR1 in its native form, when it may act as an antagonist, but induced greater calcium signalling and chemotactic activity via CCR1 following cleavage by proteases (Berahovich et al., 2005). In contrast, CCL7 (MCP-3) – a ligand for CCR1, CCR2 and CCR3 involved in monocyte mobilisation (Jia et al., 2008; Rot and von Andrian, 2004; Tsou et al., 2007) – can be inactivated by cleavage by MMP13 and MMP14 which produces a truncated chemokine that acts as an antagonist of its receptors (McQuibban et al., 2002). Once again, CX₃CR1^{int} CD64⁺ MNP^s were found to express genes which can both promote and negatively regulate inflammation, consistent with the plasticity of Ly6C⁺ MHCII⁺ MNP^s.

3.3.2.1.5 CCRL2

We were interested in how pro-inflammatory MNPs might be recruited to the large intestine during colitis. CX₃CR1^{int} CD64⁺ MNPs expressed greater levels of *Ccr12* than CX₃CR1^{int} CD64⁻ MNPs during *Hh* + anti-IL-10R colitis. CCRL2 is an atypical chemokine receptor that shares high homology with CCR1, CCR2, CCR3 and CCR5 (Fan et al., 1998). Its expression has been reported in macrophages (Brouwer et al., 2004; Migeotte et al., 2002; Oostendorp et al., 2004; Shimada et al., 1998; Zuurman et al., 2003), DCs (Otero et al., 2010), mast cells (Zabel et al., 2008) and microglia and astrocytes in the central nervous system (Brouwer et al., 2004). CCRL2 has been shown to bind and concentrate chemerin, a chemotactic ligand for ChemR23 (Wittamer et al., 2003), although chemerin does not elicit a functional response via CCRL2 (Zabel et al., 2008). This is also the case when CCRL2 binds CCL19, which it internalises in the manner of a scavenger or decoy chemokine receptor, leading to the proposal that CCRL2 be classified as such (Leick et al., 2010). Expression of CCL19 is augmented in both ileal and colonic CD (Kawashima et al., 2005; McNamee et al., 2013) and UC (McNamee et al., 2013), over its absence from uninfamed intestine (Kawashima et al., 2005). CCL19 was also elevated in a murine model of Crohn's-like ileitis, the TNF^{ΔARE} model, where it was expressed primarily in inducible lymphoid tissue (McNamee et al., 2013). CCL19 itself is capable of inducing lymphoid tissue development (Luther et al., 2002) and has been suggested to mediate retention of CD4⁺ T cells (McNamee et al., 2013) and mature CCR7⁺ DCs (Middel et al., 2006) in the inflamed intestine, which may contribute to pathology during IBD. CCL19 has also been shown to enhance the release of IL-8 and TNFα, and MMP activity, in human THP-1 cell line-derived macrophages via CCR7 (Damas et al., 2007). By internalising and thus limiting the availability of CCL19, CCRL2 might negatively regulate the development

of inducible lymphoid tissue, MNP-T cell interactions, and the pro-inflammatory activity of CCR7⁺ macrophages in the lamina propria (see Chapter 5).

CCRL2 is reported to provide chemotactic responsiveness to CCL2, CCL5, CCL7, and CCL8 (Biber et al., 2003; Zuurman et al., 2003), the latter three of which are also CCR1 ligands (Rot and von Andrian, 2004). *Ccr12*^{-/-} bone marrow-derived dendritic cells (BMDCs) also show reduced chemotaxis towards CCL3, another CCR1 ligand (Otero et al., 2010). CCRL2-mediated binding and functional responses to CCL2 have also been described in microglia (Zuurman et al., 2003), which display features of conventional macrophages but arise from self-renewing embryonic precursors rather than circulating haematopoietic stem cell-derived monocytes (Ginhoux et al., 2010; Schulz et al., 2012).

The exact functions of CCRL2 are not fully understood. Via an unknown mechanism, CCRL2 plays a non-redundant role in the migration of antigen-bearing lung DCs to draining lymph nodes, but does not affect their initial entry into the lung; nor does it affect skin DC trafficking (Otero et al., 2010). CCRL2 expression was also observed on activated macrophages, in accordance with the initial description of this receptor which was expressed by RAW264.7 macrophages following LPS treatment, inspiring its original name, LPS-inducible C-C chemokine receptor-related gene (L-CCR) (Shimada et al., 1998). Human monocytes and macrophages also express CCRL2 and upregulate its expression upon activation (Migeotte et al., 2002). Another study of ovalbumin (OVA)-induced airway inflammation also revealed CCRL2 expression by macrophages, which was upregulated following OVA challenge (Oostendorp et al., 2004). CCRL2 has recently been implicated in ischemia-induced brain injury in mice (Douglas et al., 2013), which might be related to its expression by microglia (Zuurman et al., 2003). CCRL2 seems to exhibit functional features of traditional chemokine receptors, and warrants further investigation

as a candidate for controlling inflammatory MNP recruitment to the intestine during colitis.

3.3.2.1.6 CX_3CR1^{int} $CD64^+$ MNPs microarray analysis conclusion

Collectively, our microarray results show that CX_3CR1^{int} $CD64^+$ MNPs found in the lamina propria three weeks after induction of *Hh* + anti-IL-10R colitis express genes which could endow them with both pro- and anti-inflammatory functions. Whether the respective genes which may promote inflammation versus its resolution were expressed by the same cells or by distinct subpopulations, which do exist among CX_3CR1^{int} $CD64^+$ MNPs, is not known. Having sorted our microarray populations from the lamina propria three weeks after *Hh* + anti-IL-10R treatment clouds this issue further, as by this point the severity of intestinal pathology had begun to decrease following peak colitis 2-3 weeks after induction of disease, and this correlated with altered composition of the CX_3CR1^{int} $CD64^+$ population. These results are all consistent with the plasticity of $Ly6C^+$ $MHCII^+$ MNPs, whose differentiation and subsequent functions are influenced by the presence or absence of inflammation (Bain et al., 2013; Tamoutounour et al., 2012). Further validation of interesting genes and pathways is required and should be combined with the developments that have been made in MNP subset identification to ensure that results refer to specific subpopulations as much as possible.

3.3.2.2 CX_3CR1^{int} $CD64^-$ MNPs

The cells in our CX_3CR1^{int} $CD64^-$ microarray population may be related to a recently described population of *bona fide* DCs which can be found in the small and large intestinal

lamina propria (Scott et al., 2014). The described cells were CX₃CR1^{int} CD11b⁺ CD103⁻ CD11c⁺ MHCII⁺ and derived from pre-cDCs in a Flt3L-dependent manner. Interestingly we observed greater expression of *Itgae*, encoding CD103, by CX₃CR1^{int} CD64⁻ versus CX₃CR1^{int} CD64⁺ MNPs, despite no apparent protein expression by the CD64⁻ cells (Figure 3.4).

Although our CX₃CR1^{int} CD64⁻ microarray population expressed the DC-selective genes *Cd24a*, *Amica1*, *Sept6*, *Ap1s3* and *Mpp6* (Miller et al., 2012; Schlitzer et al., 2013), we did not observe enrichment of a number of key DC-selective genes, such as *Flt3*, *Zbtb46* or *Ccr7*. *Zbtb46* expression has previously been reported in colonic MNPs using similar gating strategies (Bain et al., 2013; Scott et al., 2014; Zigmond et al., 2012). It is possible that our microarray analysis did not effectively detect differential expression of *Zbtb46* and perhaps RT-qPCR might be more sensitive in this case. In future, inclusion in our staining panel of more markers expressed by dendritic cells, such as CD26 (Miller et al., 2012) or CD24 (Schlitzer et al., 2013), could make results easier to interpret by selecting a population of DCs rather than “non-macrophages”.

3.3.3 T-bet

We also examined the role of T-bet expressed by innate immune cells during colitis. Shortly after the two-month period over which we monitored *Tbx21*^{-/-} *Rag2*^{-/-} mice for signs of spontaneous colitis, it was reported that the previously observed colitis (Garrett et al., 2007) correlated with infection with *Klebsiella pneumoniae* and *Proteus mirabilis* (Garrett et al., 2010). These species were able to transmit colitis to T-bet-sufficient *Rag2*^{-/-} and wild-type mice with an endogenous microbiota (i.e. not germ-free) either by

infection of mice with cultured bacteria or by maternal transmission from *Tbx21*^{-/-} *Rag2*^{-/-} ulcerative colitis (TRUC) mothers during cross-fostering experiments (Garrett et al., 2010). Another more recent study identified isogenic mice, derived from those used in the original work, which did not develop colitis unless infected with the bacterial species *Helicobacter typhlonius* (Powell et al., 2012). Our animal facility has been consistently negative for these species, which likely explains the absence of spontaneous colitis in our colony. It seems likely that infection with *Helicobacter hepaticus* would have induced colitis in *Tbx21*^{-/-} *Rag2*^{-/-} mice. It could be of interest to assess the effect of T-bet-deficiency during *Hh* + anti-IL-10R colitis in *Tbx21*^{-/-} *Rag2*^{+/+} mice, and to test whether *Helicobacter hepaticus* infection alone is capable of inducing disease in these mice.

T-bet also seems to regulate ILC-mediated intestinal inflammation in TRUC mice by influencing ILC numbers, via repressing IL-7R α expression, and by controlling the balance of IFN γ and IL-17A produced by ILCs (Powell et al., 2012). The importance of T-bet in ILC regulation could have been a confounding factor in our examination of the role of T-bet in MNPs using *Tbx21*^{-/-} *Rag2*^{-/-} mice, since these mice also lack T-bet in ILCs (Powell et al., 2012).

We found that reconstitution with naïve CD4⁺ T cells induced equivalent intestinal pathology and splenomegaly in *Tbx21*^{-/-} *Rag2*^{-/-} mice and Balb/c *Rag2*^{-/-} controls. However, consistent with the IFN γ -dependence of wasting disease associated with colitis (Ito and Fathman, 1997; Uhlig et al., 2006b), *Tbx21*^{-/-} *Rag2*^{-/-} mice displayed higher weight than their T-bet-sufficient counterparts throughout the study. Although we did not confirm reduced IFN γ in the T-bet-deficient mice, the absence of weight loss could have been due to impaired production of IFN γ by CD4⁺ T cells or innate immune cells, such as DCs (Lugo-

Villarino et al., 2003) or intestinal ILCs (Powell et al., 2012), as occurs when these cells lack functional T-bet.

Despite indications that T-bet expressed by innate immune cells might both promote (Laffont et al., 2010; Lugo-Villarino et al., 2003) and prevent (Garrett et al., 2010; Garrett et al., 2007; Garrett et al., 2009; Powell et al., 2012) intestinal inflammation, collectively our results do not indicate any effect of innate immune cell T-bet deficiency on the susceptibility of mice to T cell transfer-induced colitis in the absence of particular bacterial species.

3.3.4 Summary

In summary, we have shown that during *Hh* + anti-IL-10R colitis, inflammatory CX₃CR1^{int} Ly6C⁺ MNP populations accumulate in the large intestinal lamina propria in a pattern similar to those observed in other models of colitis. Our results indicate that by three weeks after induction of disease a shift towards resolution may be occurring, as evidenced by the genes expressed by CX₃CR1^{int} CD64⁺ MNPs and by the reductions in the presence of inflammatory cells and tissue pathology. However, biological variability in disease severity at three weeks indicates that some inflammatory processes may be ongoing, and we have confirmed that CX₃CR1^{int} CD64⁺ MNPs differentially express IL-23 at this timepoint. Expression of both pro- and anti-inflammatory genes by CX₃CR1^{int} CD64⁺ MNPs during *Hh* + anti-IL-10R colitis is consistent with the plasticity of the Ly6C⁺ MHCII⁺ cells within this population. Our study of T-bet expressed by innate immune cells did not indicate a major role in intestinal pathology and was not investigated further.

Cluster 1 (grey)

Probe ID	gene	Probe ID	gene	Probe ID	gene
5910372	0610012D17RIK	7150349	2310079N02RIK	7040133	AVPI1
4120017	1110001J03RIK	4040184	2410003K15RIK	4150669	AW742319
3830020	1110001K21RIK	3450309	2410004B18RIK	290632	AXIN
7200491	1110007A13RIK	3830707	2610101N10RIK	5570537	B930004C15RIK
2680612	1110007L15RIK	7570291	2700023B17RIK	3710598	BC003236
5270747	1110007L15RIK	2490762	2700029M09RIK	6100719	BC003236
3420075	1110018G07RIK	4540278	2700061N24RIK	4860382	BC003965
5130609	1110019J04RIK	3060139	2700082D03RIK	240487	BC017643
5290475	1110020P15RIK	4780681	2700084L06RIK	6520154	BC027231
3710678	1110032A13RIK	2570176	2810025O06RIK	2760349	BCAP29
1430647	1110032N12RIK	4200047	2810037C03RIK	1500274	BCAP31
2370138	1110032N12RIK	610670	4930453N24RIK	3610424	BCAP31
510608	1110032O16RIK	3130129	4932414K18RIK	7510253	BCAS2
1110563	1110034O24RIK	3710470	5133401N09RIK	5080338	BCL2L2
7150753	1190005P17RIK	2680528	5730538E15RIK	630674	BFAR
2850082	1200003I07RIK	1050424	5930416I19RIK	4200753	BFAR
4230470	1200011O22RIK	3710079	6030457N17RIK	3390053	BNIP3L
5310722	1200015F23RIK	1470475	6330412F12RIK	4830110	C130067A03RIK
2000274	1300006C06RIK	1710332	6430706D22RIK	5900561	C230050J15RIK
620113	1300006C19RIK	6620554	6720456B07RIK	5910113	CANT1
3390152	1500001M20RIK	630546	8430408H12RIK	1190068	CASP8
3390259	1500003D12RIK	4900678	9030619K07RIK	3710014	CCNG1
2760615	1500012F01RIK	1410730	9430023L20RIK	360301	CCT6A
460632	1600002K03RIK	2940600	A630082K20RIK	4560703	CCT6A
3450544	1700001A24RIK	7160202	ACADL	4060767	CD47
60056	1700022N24RIK	6380095	ADK	2970452	CDC5L
5820242	1700022N24RIK	4730646	AGGF1	1050170	CDCA3
780121	1700027M01RIK	7570315	AGPAT4	4920468	CDCA4
1010356	1700027M01RIK	3420020	AGPAT6	650437	CEPT1
5690228	1700037H04RIK	6110240	AHSA1	110138	CHIC2
3440093	1810006K21RIK	2470609	AI450540	4540154	CHIC2
4760041	1810008O21RIK	1430091	ANAPC7	3610379	CLASP1
5260438	1810011O16RIK	2340121	AP1B1	2710653	COMMD6
5870504	1810030A06RIK	3120253	APG3L	2140605	COMMD7
7040286	1810046J19RIK	4280592	APPBP1	70731	COPS5
130382	1810059G22RIK	6620767	ARID1A	2970563	COPS5
7380546	2010005O13RIK	6060564	ARRDC4	1500463	CORO1A
7000112	2010007E07RIK	3850450	ATG5	4290661	CORO1A
1740497	2310006J04RIK	4810064	ATG5	4150196	COX10
6650040	2310010I15RIK	4480603	ATP1B3	540037	CREB3
3710136	2310033F14RIK	2570672	ATP2A3	290685	CRELD2
6620735	2310036D04RIK	4540554	ATP5O	4860035	CRIP1
2340598	2310039H08RIK	2350170	AV340375	7560411	CRSP2

Probe ID	gene	Probe ID	gene	Probe ID	gene
6480168	CSNK1G2	4830138	GAS7	6510152	LOC654842
7160477	CSNK2A2	4880674	GLG1	240403	LRRC59
7320390	CSNK2A2	3440243	GNG12	3830022	LSM3
1190037	CSPG6	3420300	GORASP2	6860543	MAPRE2
4060474	CSTF1	4880411	GORASP2	670719	MFAP3
1030368	CTSC	5050162	GORASP2	1850706	MFN1
7550059	CYFIP2	4670156	GOSR2	2450066	MFN2
270730	D030051N19RIK	2750543	GPR18	7320731	MFNG
6040037	D19ERTD721E	6520725	GPR43	6650097	MISC12
6290356	D19WSU12E	620682	GPS1	510438	MORF4L1
620360	D530030D03RIK	7380273	GRPEL1	2810673	MPP6
3800437	D7WSU128E	5290358	GSTP1	5870193	MRE11A
990671	D930015E06RIK	6280070	GTF2F2	4640553	MRPL12
6100014	DAP3	3940605	GTF3A	6350332	MRPL17
6200176	DAP3	3850142	GTPBP2	1440010	MRPL27
4290170	DDX52	3130240	H2-Q5	6060014	MRPL36
2350139	DENR	2600541	HAGH	3120689	MRPL43
1260692	DHX30	6370678	HEATR3	2100427	MRPL46
1450066	DNAJC15	4060703	HPRT	5090332	MRPS15
580706	DNAJC5	3780463	HRB	5960491	MRPS15
990056	DYRK1A	2340382	HTATIP2	4070543	MRPS24
6330575	E030003N15RIK	2710152	HTATIP2	3780022	MRPS30
1190020	EFHD2	4610373	ICSBP1	7610338	MSH6
6960730	EIF2S3Y	2570441	IDH3B	2650538	MTCH1
6110519	ELK3	3940575	IFT20	460497	NCL
5860491	ESD	4610167	IFT20	630088	NCL
6760255	EVI2A	7560671	IFT52	1260450	NDUFS3
6520192	EXOSC7	4220546	ILF2	6840670	NDUFS4
2630605	F730031O20RIK	3800452	KPNB1	70286	NDUFV1
4640754	F730045P10RIK	7040348	LAIR1	1850564	NFE2L1
610274	FADS1	4200327	LASS2	2070520	NME1
2850609	FAIM	2470528	LEMD2	360050	NMT1
4150440	FBXL12	4050039	LEPROTL1	6180437	NSF
3800630	FBXL20	770259	LLGL1	3390468	NT5C
5910300	FBXO4	2120110	LLGL1	6510706	NUCB2
2690102	FNTB	2030376	LMTK2	2900291	NUDC
1850403	FRAP1	3850008	LOC223594	1690291	NUDT5
3420632	FXC1	4560646	LOC235857	5560180	OAZ2
5910189	GABARAPL2	4280482	LOC245828	6270022	OSBPL2
460767	GAPVD1	6860307	LOC380692	3420500	OXCT
4220088	GAPVD1	5870692	LOC381297	1240538	P2RY14
4250681	GART	4280519	LOC384710	6580379	P2RY14
3830577	GAS7	1300204	LOC385662	6100162	PACS1

Probe ID	gene	Probe ID	gene	Probe ID	gene
6180341	PARL	6760017	RP9	7650653	TXNDC4
3310538	PCBD2	6250291	RPIA	1410113	UAP1L1
4210538	PEA15	4640239	RPL22	1780168	UBXD1
5670326	PHC1	6520598	SAS	2600050	UCK1
4200471	PHF12	70386	SATB1	3130750	UNG
2230228	PIGF	7330181	SCL000911.1_939	110747	VAMP3
3440286	PIK3CB	5670100	SDHC	5700168	VAMP3
1940255	PLCG2	6350603	SDHD	1230543	VAMP4
4890113	PNP	4830703	SEC15L1	6840731	VDAC1
10372	PNPT1	620048	SEC24C	6370553	WBSCR5
580356	POLDIP3	2680487	SEPT15	1260341	WDR45L
1660593	POLR2H	5820056	SERPINB6B	2190437	WDR68
6350446	POLR2K	2900592	SFXN1	7040681	XPO6
6290075	POLR2L	4180577	SFXN1	3190609	ZC3HDC5
7210280	POM121	1090037	SLC25A20	1440685	ZDHHC4
2000669	PPA1	6660079	SLC25A38	5220301	ZFP511
3830592	PPIH	5870092	SLC25A5	4180647	ZFP535
1510088	PPP2R4	6550500	SLC41A3	6560040	ZMYM2
4250372	PPP2R5C	5890564	SNRK		
990064	PPT1	630215	SNX1		
1690753	PRDX2	4560528	SNX2		
1990152	PRMT3	4830129	SREBP2		
6770379	PRNP	2370632	SRI		
4390672	PSMC2	7650451	SRI		
4640128	PSMC2	450195	SRP14		
3450041	PSMD10	5080192	STAG2		
6510136	PSMD10	6480131	TAPBPL		
4230215	PSMD13	4560047	TBCA		
1440750	PSMD4	2760601	TEX2		
5290474	PSMD7	4040343	TEX292		
6330309	PTTG1IP	4560379	THTPA		
4260019	PYCR2	2350204	TIMM17A		
1030142	PYGL	4780239	TMEM160		
3610193	RAB11B	5260097	TMEM63A		
3190735	RAB2	160519	TMEM65		
1780619	RAB32	990692	TMOD3		
3850239	RAB35	6290554	TOR2A		
1230747	RALB	5220482	TRAF6		
4010243	RAMP1	730092	TRAK2		
2120653	RBM14	7150292	TRAP1		
1300402	RIPK1	4570037	TRAPPC1		
1230138	RNF26	5870241	TXN1		
6100593	RNF34	7550711	TXN2		

Table 3.2 Cluster 1: Genes upregulated in CD64⁺ cells during inflammation resulting in expression similar to steady state- and inflammation-derived CD64⁻ cells.

Alphabetical list of genes in Cluster 1 (grey bar, Figure 3.6) from k-means clustering analysis of genes significantly differentially expressed (adjusted p value < 0.05; log₂(fold change) > 1) in at least one comparison of CX₃CR1^{int} CD64⁺ and CX₃CR1^{int} CD64⁻ MNP from resting and inflamed lamina propria (results of each comparison are presented in the Appendix).

Cluster 2 (green)

Probe ID	gene	Probe ID	gene	Probe ID	gene
4230019	1110007C24RIK	5820767	4930477M19	4390440	ANG1
3390678	1110020P09RIK	1170014	4930544D05RIK	5310746	APG7L
4920543	1110030J09RIK	2630523	4930565B19RIK	1110088	APOBEC1
3370338	1110058L19RIK	270603	4932439E07RIK	4810497	AQR
4670386	1190002A17RIK	110242	4933404K08RIK	5270601	ARID4A
7550026	1300019J08RIK	60463	4933409N07RIK	3420022	ARL6IP2
3780601	1700013L23RIK	1820672	4933417K04RIK	4570709	ATF7IP
110601	1700020C11RIK	360709	4933428I03RIK	3450356	ATP8B2
5910056	1700020O03RIK	2370519	5330427M13RIK	2000047	ATXN7L2
2450747	1700025B16RIK	6590070	5730402C02RIK	2000156	AU014645
7550692	1700028D13RIK	2140441	5830411E10RIK	3930195	AYTL2
4570475	1700028I04RIK	1740736	5830415F09RIK	6980735	B230209C24RIK
6450008	1700040I03RIK	5090162	5830434K11RIK	2970040	B930011P16RIK
4730288	1700085B03RIK	1660209	5830468K18RIK	3170152	BC016188
3610672	1700087I21RIK	3290554	5930402A21	5220170	BC019977
3310291	1700109H08RIK	4060762	6230412O07RIK	2810575	BC034054
1940392	1810009A16RIK	5700681	6330400D04	6560273	BC046418
1820451	1810030N24RIK	2710400	6330505F04RIK	520709	BC056485
4390278	1810037O03RIK	5550307	6430596G11RIK	4570739	BRF1
5960475	1810045K06RIK	1450053	9030418K01RIK	3710477	C030016K15RIK
4850544	1810062O14RIK	630408	9530013D10RIK	6290386	C130023A14RIK
7210672	2010001M09RIK	1820162	A330080J22RIK	6040019	C230060F13RIK
3840475	2310014L17RIK	50138	A430034D21RIK	4070017	C330016O10RIK
3360241	2310016C08RIK	3060736	A430083B19RIK	2510131	C330018L13RIK
20431	2310066N05RIK	2480711	A430108B07RIK	6290753	C530047L02RIK
290735	2410001H17RIK	1050138	A630084D02RIK	3940592	C630028F04RIK
2320414	2510015F01RIK	3130553	A830008O07	5910672	C730026J16
4480193	2610020N02RIK	3400544	A830054O07RIK	4880736	C8B
1440059	2610022G08RIK	3310736	A930006D11	2630600	CAMK2A
5080553	2610511M17RIK	6450600	A930008A22RIK	1820133	CAMK2G
5860168	2810003O05RIK	5700750	ABCA14	5290017	CASP4
3400767	2810011L19RIK	6370270	ABHD4	6620437	CCL17
3370176	2810409K11RIK	4040504	ABP1	4670674	CCL7
3780672	2810449M09RIK	5090414	ACAA2	2190164	CCNB1
540382	2810457I06RIK	2470025	ACOT2	7550156	CCNB1
60601	2900024C23RIK	6840392	ACOT7	1570056	CD209C
6420088	3110001A13RIK	2650398	ACTL6	3190634	CD209C
2070239	3110047P20RIK	3370746	ACTR3B	6250497	CD209D
6220044	4432409M07RIK	2690544	ADAM22	1340524	CD70
3170743	4631434O19RIK	830736	ADRA2A	6450066	CDC2L2
1500519	4632434I11RIK	1710674	AK3	1570367	CEACAM2
4670072	4732441L20RIK	2750079	AKAP1	6420594	CLDN15
2600324	4921511C16	5820133	AMPD2	5700037	CLDN9

Probe ID	gene	Probe ID	gene	Probe ID	gene
6510519	CLEC1A	1820327	E230022H04RIK	2030372	IGK-V1
4810445	CLOCK	2000343	E330022B15RIK	130162	IGLC2_J00595_IG _LAMBDA_CONST ANT_2_14
7040601	CNGA1	1770639	E330036I19RIK	6130767	IKBKE
3840315	CNOT2	4850373	E330036I19RIK	5290215	IKZF5
4560347	CNOT2	3990376	EEF1E1	2690768	IL15
2900725	CNOT6L	2850338	EFHA1	3420301	IL18
5390072	CNP1	6420273	EG384814	6330601	IL18BP
4490053	COG4	3170088	ELAVL4	1850402	IL2RG
7050056	CRTAP	4290075	ELF3	6840703	ILDR1
5820528	CSF2RB2	360402	EVI1	1990440	INPPL1
4390468	CST11	6960451	F13A1	5670072	INPPL1
4490324	CTRL	1740369	F830004M19RIK	510068	INVS
3520044	D030022P06RIK	1780500	FCGR2B	6520730	KCNMB4
2230324	D10ERTD641E	5390288	FIZ1	4150224	KCNN4
160437	D12ERTD647E	5720040	FKBPL	3180164	KIF18A
6960026	D2BWG0891E	6580682	FOXO1	2470070	KLF16
610301	D330004F16RIK	3370154	FRG1	7320138	KLRA17
2360347	D330004O07RIK	4560195	G630058F05RIK	7380390	KLRB1B
1470180	D430042L13RIK	2600288	GARNL1	3850274	KLRB1D
4920564	D4ERTD22E	290358	GCNT1	1850037	KNSL5
510709	D630002J18RIK	3610707	GJA6	6960672	KPNA6
6620528	D7ERTD462E	4050209	GLS	6330438	KRTAP5-1
1440500	D930020L01	2190687	GLT8D1	3170010	LMAN1
5890746	D930024N12RIK	2000672	GM166	6940086	LMAN2L
5340114	D930046M13RIK	3190121	GMPPA	1580445	LMYC1
6290279	D9ERTD280E	4880369	GNAO	2140324	LOC208166
5690102	DAPK1	1260364	GPR85	3120369	LOC211208
3310487	DAPP1	7550601	GRIK2	1110441	LOC211895
2650136	DCAMKL2	2690762	HARS	5810128	LOC212815
5390128	DCI	670164	HBS1L	6650601	LOC218476
1780411	DES	1030296	HBS1L	6290592	LOC226691
4070136	DGKZ	2810541	HCPH	110653	LOC227092
4670678	DHX40	4060240	HCPH	6860500	LOC228790
6060278	DMTF1	3390546	HNRPH1	150519	LOC234187
2900747	DNAHC2	1690113	HNRPK	650348	LOC235997
2970328	DNAJA3	6200673	HNRPK	4880672	LOC236060
4480022	DOCK4	1510672	HOMER1	2260392	LOC245666
4540564	DOK2	270731	HOXA13	6520114	LOC276809
2490682	DPP7	5690519	IAN1	4040082	LOC328526
2970279	DSPP	6200692	IDB1	3060369	LOC331336
2370494	DTX3	4070047	IFNA1	1050338	LOC380920
2810653	E130001F20RIK	4780072	IFNA1	6660669	LOC381196
10746	E230008N13RIK	4180411	IFT140	6110576	LOC381255

Probe ID	gene	Probe ID	gene	Probe ID	gene
20736	LOC382406	1240148	NTE	2030424	RBM12
5910079	LOC382765	6020601	NUP88	4290019	RBM12
4590762	LOC383293	240187	OASL1	1030725	RBM1A1
1440349	LOC383873	1820767	OC90	1110709	RETNLA
5820189	LOC384100	4900204	OLFR1384	1690008	RHOBTB2
4230519	LOC384384	1500349	OLFR206	6980551	RHOBTB2
6380575	LOC384636	1010431	OLFR270	730113	RHOX12
6860669	LOC384943	1500600	OLFR272	6370278	RHPN1
3460338	LOC385642	2710692	OLFR378	6400403	RIPK2
7570040	LOC385843	3850634	OLFR503	1170022	RMND1
3460601	LOC386205	2900672	OLFR538	3290689	RNASEN
1470519	LOC433224	7050601	OLFR618	5360647	RNASEN
4730612	LOC434858	6280369	OLFR724	4490600	RNF135
360767	LOC632687	6060632	OLFR748	6200736	RNF148
5890553	LSS	1500136	OLFR786	4260730	RNF31
1820088	LYRM5	1440754	OLFR910	2750356	RNPS1
4640528	MACF1	70279	OLFR982	4570292	RNU6
6270500	MAP4K6-PENDING	830601	OVOL2	7320608	RPL30
730059	MAPK13	360132	OXT	1510647	RPPH1
1440671	MAPK13	1740332	P2RX5	3890064	RPS6KA2
4220326	MCM10	6330451	P2RX5	5550035	RRAD
5820369	MEF2C	6560075	PAK1	2450553	RSHL2
1010047	MGL2	5310059	PAK2	150164	SAMD11
2350451	MGLL	4150148	PANX1	6590369	SCL0001379.1_70
630519	MID1	2260414	PCDHB8	1820402	SCL0002855.1_1056
3850519	MJ-6000-172_3824	2260520	PENK1	5810184	SDCCAG33
2060040	MMP13	2000044	PIRA2	990689	SEC31L1
6380427	MMP1A	360014	PLEC1	5290274	SEMA5A
4540148	MON1B	3930086	POLR2E	5550195	SEPM
4730220	MRC1	7000026	POLR2E	4590431	SF1
1820156	MRGPRA2	3450519	POU5F1	5490008	SIAT4A
3170167	MRGPRG	3140673	PPARBP	4780747	SKD3
940541	MSR2	1440762	PPEF2	2070309	SLC1A7
5220279	MT1	1820132	PREI4	1340240	SLC20A1
5490068	MTHFS	4390021	PRPF39	4290367	SLC29A3
6370674	MYST1	5820070	PTBP1	2750746	SLC30A4
3990114	NAPA	1430487	QRICH1	1820538	SLC4A3
6280672	NEU2	2190519	R3HDM	3120546	SLCO2B1
4560739	NIT1	6840347	RABL4	4040672	SLITRK1
7560300	NIT1	5390482	RAD23B	60523	SMURF2
3310672	NR1D1	3290736	RAD51L3	6590672	SOX12
1260039	NR1D2	5700215	RAI12	430392	SP8
4150634	NRAS	2450735	RAI14	4220673	SPATA13

Probe ID	gene
5560066	STAT4
7650575	STAT5A
4280138	STIM1
7380669	STIM2
6450438	STK38L
4590672	STXBP5L
6380750	SYT9
6330725	TCF19
3420372	TGOLN1
3520630	TGOLN2
3520711	THOC6
610040	THRAP4
6220450	TIMM44
4280296	TNFRSF13B
7200601	TNFRSF19L
1450731	TOR1B
2690431	TRCG1
5260201	TRP53INP1
4890022	TTC3
770653	UBE2D3
6330736	ULK1
60278	UNC119
6510279	USP12
1850672	V1RA2
4290746	VDP
2690369	WDR13
2690224	WDR34
580537	WFDC6A
3990743	WHSC1L1
3450373	XIST
4280551	YTHDF2
2360291	ZBTB33
2630546	ZBTB33
6250519	ZDHC14
6510278	ZFP212
6840504	ZFP264
7560112	ZFP265
5670154	ZFP296
1440224	ZFP622
3130255	ZFP661
3830128	ZFP709
3120228	ZRANB1
5080259	ZRANB2

Table 3.3 Cluster 2: Genes downregulated in CD64⁺ cells during inflammation resulting in expression similar to steady state- and inflammation-derived CD64⁻ cells.

Alphabetical list of genes in Cluster 2 (green bar, Figure 3.6) from k-means clustering analysis of genes significantly differentially expressed (adjusted p value < 0.05; log₂(fold change) > 1) in at least one comparison of CX₃CR1^{int} CD64⁺ and CX₃CR1^{int} CD64⁻ MNPs from resting and inflamed lamina propria (results of each comparison are presented in the Appendix).

Cluster 3 (purple)

Probe ID	gene	Probe ID	gene	Probe ID	gene
7150386	1110014J01RIK	3400440	A430106J12RIK	2370707	CPEB2
6130575	1110054N06RIK	6280521	A530060O05RIK	4640367	CUL1
3710050	1200008O12RIK	1050440	A630056H20RIK	1070603	D030026J11RIK
7400475	1300007B12RIK	3400152	A930006D20RIK	3170494	D17H6S56E-5
4040689	1700041B20RIK	4920746	AA408296	2140114	D5ERTD591E
2480022	1810014F10RIK	5900653	ADAM17	3450689	DARS
4060377	2010205O06RIK	3390450	ANKRD10	6960148	DCUN1D4
1430170	2310001A20RIK	3180475	AP3M2	5310288	DDX26
5340609	2310051F07RIK	6550382	APEX1	2350487	DDX50
5270309	2410118P20RIK	3360403	ARAF	6840435	DFNA5H
2350470	2610021K21RIK	5570280	ARF3	6060215	DHODH
4120685	2610028H07RIK	7550333	ARHGAP6	630053	DMWD
5260528	2610529H08RIK	870753	ARL6	2000128	DNAJA3
4010670	2700033B16RIK	4280072	ARL6IP1	2710703	E130306D19RIK
6760521	2810012G08RIK	4570168	ARMCX3	2510079	EIF2B1
730300	2810403A07RIK	1190328	ASE1	830176	FBXO25
4920647	2810474O19RIK	4010021	AW549877	1340491	FKBP1A
4780711	2900036G02RIK	4900725	BC005537	10609	G630041M05RIK
6350139	3010027N08RIK	1400685	BC008163	270431	GAB3
2490274	3010031K01RIK	3130142	BC010304	4830487	GAB3
1240707	3110040M04RIK	7200386	BC016198	7610669	GMFB
2470164	3632431M01RIK	870048	BC023823	4540482	GOLGA3
7610139	3930401E15RIK	50576	BC035537	2100129	GOSR2
1660520	4632411B12RIK	6560347	BCAS1	4280020	GOSR2
3390608	4732471D19RIK	5810181	BICD2	1510201	GPIAP1
7510427	4832412D13RIK	4850669	BLM	5890605	GTF2H1
2340670	4921506I22RIK	5960189	BLM	6130692	HNRPM
4480494	4930487N19RIK	3370382	BRD9	6840754	HNRPM
2450707	4930562C03RIK	2320369	C430003P19RIK	4220270	IARS
1440142	4931406P16RIK	6060315	C920005C14RIK	4230093	IARS
110594	4933434E20RIK	7330196	CARD10	1660538	IER2
6480711	5330401P04RIK	670561	CCT4	5360685	IGFBP7
7050594	5530400K22RIK	6020630	CD209E	160176	ING1L
4060014	5730527J01RIK	1190630	CDC27	650168	IVNS1ABP
4230377	5930412E23RIK	7160066	CDCA4	1450482	IVNS1ABP
4640685	6230401I02RIK	7570025	CHCHD7	4390026	LOC218814
5340446	6330439P19RIK	6290731	CHD9	4040500	LOC245251
460242	6430540M20RIK	6900048	CHD9	780328	LOC382790
5960450	6430604K15RIK	4570209	CKN1	5900059	MAK3
3180274	8030462N17RIK	4060609	CLEC9A	4390255	MAP3K7IP3
2570494	8430410K20RIK	2370093	CLK2	270379	MCM6
6100470	9430008C03RIK	2600019	CLSPN	4390075	MFAP1B
1940097	A130062D16RIK	7570278	CLSPN	5310240	MGAT4A

Probe ID	gene	Probe ID	gene
1780474	MSN	1820296	SMC6L1
6330170	MYNN	6860082	SNAPC3
1110273	NAP1L1	360097	SNORA65
3120091	NCK2	6450056	SQLE
6840433	NPM1	1240070	SRP72
5860500	NRG4	4640468	ST7L
2490369	NSMAF	160753	STAG3
2970554	NSMAF	6560477	STK2
1440440	NUP160	6290242	SUV420H1
1300477	NUP85	7000209	TANK
6370097	NXT1	1770471	THOC3
5290403	PABPC1	3800228	TMEM23
2680170	PCBP2	6250523	TMEM32
4220670	PCBP2	2710520	TNFRSF13C
6350240	PCNA	4250403	TOP2A
2970592	PELI1	6510431	TRAK2
7160458	PIGA	5220056	TRIM59
3840561	PIGF	7570196	TRIM59
4060441	POLE	3170102	TRIT1
6840170	PRDM2	6940181	TRPS1
7320441	PUM1	1030133	TUBB2B
7560131	PUM1	6480368	UBE2N
1660682	PYCRL	3610731	VRK1
3890224	QARS	1240270	WASF2
5340487	QARS	7200296	ZDHHC13
5870184	RAB6	3140735	ZDHHC3
4880040	RABGGTB	5360414	ZDHHC3
1340326	RALA	4490025	ZFP236
7000373	RBBP6	4730086	ZFP236
5900017	RBM18	2120689	ZFP597
10739	RG9MTD1	1070053	ZFP68
610278	RPO2TC1	3400600	ZFP715
60639	SBK	4670138	ZFX
3060360	SCL000121.1_106		
3120138	SCL0003876.1_26		
6100286	SCL0003903.1_2		
6100408	SCP2		
4480411	SET7		
5700541	SFRS7		
4150670	SIAT10		
110064	SLA		
6290747	SLC25A14		
1230379	SMARCAD1		

Table 3.4 Cluster 3: Genes downregulated in CD64⁻ cells during inflammation resulting in expression similar to steady state- and inflammation-derived CD64⁺ cells.

Alphabetical list of genes in Cluster 3 (purple bar, Figure 3.6) from k-means clustering analysis of genes significantly differentially expressed (adjusted p value < 0.05; log₂(fold change) > 1) in at least one comparison of CX₃CR1^{int} CD64⁺ and CX₃CR1^{int} CD64⁻ MNP from resting and inflamed lamina propria (results of each comparison are presented in the Appendix).

Cluster 4 (yellow)

Probe ID	gene	Probe ID	gene	Probe ID	gene
3420347	1110020A09RIK	6660528	BRD8	7400044	FOSL2
1980470	1300013B24RIK	2100719	BXDC2	6270739	GCNT2
3400136	1810026B05RIK	3610192	CALU	4120356	GP49A
150196	2010305C02RIK	5700424	CCNL1	5820739	GP49A
4290152	2310016C08RIK	4780739	CD209A	3140500	H2AFZ
7610594	2500003M10RIK	2230538	CD44	6510575	H2AFZ
1260609	2610039C10RIK	6560301	CD53	7200242	HAK
2680202	2610039C10RIK	540647	CD69	60603	HAVCR2
3370349	3230401D17RIK	2370364	CD81	870066	HAVCR2
1510435	3632413B07RIK	990280	CD97	670133	HMGN3
3180678	4122402O22RIK	2810632	CD97	4250121	HMGN3
2750575	5730405I09RIK	3310196	CDCA5	380196	HNRPD
4260279	5730453I16RIK	630446	CDK2	4540370	HPS3
1430315	5830435K17RIK	1510753	CENTD1	990685	HSD17B4
1470156	8030448M07RIK	360725	CHORDC1	3290270	HSP105
6330048	8430410A17RIK	2190220	CLK1	2350017	HSPA1A
4780561	9330180L10RIK	4220239	COQ10B	6270685	HSPA1A
6060681	9430065L19RIK	5560288	CTLA4	4040670	HSPA1B
4540603	9630055N22RIK	70753	CTNNA1	1110241	HSPCA
5360202	A730098D12RIK	6100523	CUGBP2	5870142	HSPCA
3940242	AA175286	510440	CUL3	4120671	HSPD1
1400170	AA407930	540634	D12ERTD771E	1450095	ICAM1
2470315	ACOT7	4570400	D12WSU95E	1660253	IFRD1
2750168	ADNP	5220142	D3UCLA1	240242	IGH-VJ558
4210487	ADNP	1050609	DAB2	6380762	IGH-VJ558
1820360	AL022779	1940347	DAB2	4250162	IGJ
5890768	ARHGAP25	4250537	DAPP1	630730	IGK-C
1690243	ARIH1	1010528	DDEF1	3400100	IGK-C
1090438	ATF3	4670768	DHX15	4730180	IGL-V1
4900743	ATP10D	4060047	DHX36	5080091	IGL-V1
2260592	AW228700	4540020	DNAJB1	6400184	IGSF9
630091	AY078069	5550086	DUSP16	460301	ILF3
160717	B230114J08RIK	7160397	DUSP16	1780132	IMPACT
1010669	B230114J08RIK	6270181	DUSP4	1010397	IRF4
4490189	B3GNT5	6620079	EGR1	3710717	IVNS1ABP
6660653	BAG3	1300491	EIF3S10	2760431	JAK2
6940477	BC039282	4150184	ELOVL5	5310050	JAK2
6060189	BC051080	7160167	EMP1	5130241	JMJD1C
6330201	BC051080	1770424	EPB4.1L2	4670100	KIF21B
1780735	BCL2L11	1300347	FBXL10	3840523	KIF23
5820168	BCL2L11	2060646	FBXL10	5490358	KLHL6
1780253	BCLAF1	510368	FOS	2320079	LMNA
1230341	BHLHB2	6420520	FOSB	4880360	LMNA

Probe ID	gene	Probe ID	gene	Probe ID	gene
6350682	LMNA	5260600	PPP1R10	4900136	TP120A-PENDING
2120372	LOC212399	5550092	PPRC1	730543	TPP2
2370189	LOC218482	4060543	PRKWNK1	5490204	TRA2A
1050047	LOC219145	4860180	PSMD1	3780403	TRERF1
1570750	LOC225927	2490537	PTGS2	6290053	TRERF1
1110131	LOC242051	990687	QPCT	6840021	TUBA6
1230681	LOC268569	520121	RAB6IP1	6860717	TUG1
7100224	LOC277016	3180114	RAB6IP1	6980639	UBAP2
4890114	LOC328703	2470392	REL	1690121	USP52
5900370	LOC381140	4830072	RGS1	3370692	USP52
1820242	LOC385392	6840594	RGS1	4540682	USP52
4220386	LOC386192	670440	RGS2	4120703	VCL
7050433	LPXN	1050541	RHEB	5560497	VIL2
3830021	LRRC5	3780192	RHOF	1230722	WSB1
2000112	LTA4H	5960348	RIOK3	2190544	YWHAH
7160709	LTA4H	4040072	SCL000636.1_46	2690114	ZC3H7A
4780445	LUC7L	2680753	SCL000819.1_3	380240	ZFP131
4890603	LYPLA1	780678	SDCBP2	7330435	ZFP131
5670240	MAP3K6	4670577	SERTAD2	1440270	ZFP263
7200025	MAP3K6	4200497	SFPQ	2070079	ZFP473
1820224	MARCKS	5130612	SFRS2		
1580187	MAT2A	2630445	SFRS6		
670068	MDM2	3170129	SH3BGRL		
3310243	MDM2	460327	SLC2A3		
3870273	MOBK1B	2350594	SLC35B1		
990008	MORC3	20066	SLC38A2		
2190239	MORF4L2	3420154	SMARCA2		
6960022	MTDNA_CYTB	6900288	SMCR8		
3120520	MTDNA_ND5	1470242	SMEK1		
6110242	MUM1	7380768	SMEK1		
1570138	NCAPH2	7160433	SPAG9		
4180669	NFKB1	4560619	SQSTM1		
6650458	NFKBIZ	4200392	SRGAP2		
6980722	NOL5	1430368	ST6GAL1		
3460468	NPTN	3180367	STARD7		
1510523	PCF11	6270091	STK17B		
3360220	PELI1	870427	TACC3		
5900497	PER2	2350270	TEC		
6960201	PLEC1	4850682	TGIF		
110220	PLEKHE1	5360608	TJP2		
6980021	PLP2	610341	TLE1		
6130328	PLSCR1	430523	TNFAIP3		
1430445	PPP1R10	380348	TNFSF9		

Table 3.5 Cluster 4: Genes downregulated during inflammation regardless of CD64 status.

Alphabetical list of genes in Cluster 4 (yellow bar, Figure 3.6) from k-means clustering analysis of genes significantly differentially expressed (adjusted p value < 0.05; $\log_2(\text{fold change}) > 1$) in at least one comparison of CX₃CR1^{int} CD64⁺ and CX₃CR1^{int} CD64⁻ MNP from resting and inflamed lamina propria (results of each comparison are presented in the Appendix).

Cluster 5 (orange)

Probe ID	gene	Probe ID	gene	Probe ID	gene
130735	1110007C02RIK	130754	HIST1H2BK	2810451	SNX22
2140450	1110012O05RIK	2190047	HSPCB	3120392	STMN1
6450634	1110017L21RIK	520196	ITGAE	7150121	TALDO1
1300092	1600029D21RIK	1300059	ITGAE	160703	TIMELESS
1980446	2410015N17RIK	7100687	ITGAE	3850070	TIMELESS
7200719	2810046M22RIK	3370288	KEO4	2260066	TNNI2
1780338	4833427B12RIK	6330114	KIF15	3140707	TPX2
1260519	5033403D15RIK	4210246	KIF22	3830735	TYMS-PS
2900307	5830405N20RIK	4890279	KLRD1	4560397	UHRF1
520500	AARSD1	780037	KLRI1		
4590136	ACTN1	1240639	KLRI1		
3180068	AKR7A5	5360152	LOC268695		
3850692	AMICA1	6520088	LOC269355		
6840338	AP1S3	4060411	LOC331539		
2470259	ARMC8	380482	LSP1		
6270059	ATAD3A	1770612	MCM2		
6370739	BC065078	6220270	MCM5		
870603	BEX6	1110541	MDH2		
6980672	BTLA	6510465	MFGE8		
5890110	CBFA2T3H	3890102	MRPS6		
3390110	CD24A	1090524	MYBBP1A		
4150739	CDC42EP3	380369	NAPSA		
5820184	CDH1	5690500	NAPSA		
2470356	COX6A2	4220519	NDUFA8		
2810072	CYB5	360491	NGFRAP1		
7560639	CYB5	3170731	NME2		
3060546	DPP4	3830025	NME2		
940170	DTYMK	1230703	NUP210		
5700722	ECE1	6400070	P2RY10		
3830241	EPHB2	2900300	PBK		
4670451	EXOSC8	2900333	PDIR		
2140113	FBL	1740379	PLP2		
1240019	FGF22	4180575	PPM1M		
2060433	FLNB	780475	PRC1		
5700093	FLNB	1090035	PRKAR2A		
4670270	FPGS	3170110	PYGB		
4860041	GCAP27	4830528	RANGAP1		
3870113	GOT2	5130487	RGS12		
3840040	GRASP	430703	RNASE6		
6760167	H2-OB	6380370	RPO1-4		
6520446	H2AFV	2340243	RRM1		
1090327	H2AFX	5260762	SEPT6		
50129	HERPUD1	5550554	SIRT3		

Table 3.6 Cluster 5: Genes downregulated in CD64⁺ cells during inflammation.

Alphabetical list of genes in Cluster 5 (orange bar, Figure 3.6) from k-means clustering analysis of genes significantly differentially expressed (adjusted p value < 0.05; log₂(fold change) > 1) in at least one comparison of CX₃CR1^{int} CD64⁺ and CX₃CR1^{int} CD64⁻ MNP from resting and inflamed lamina propria (results of each comparison are presented in the Appendix).

Cluster 6 (brown)

Probe ID	gene	Probe ID	gene	Probe ID	gene
5810274	0610007H07RIK	5340500	ABHD1	3870242	CHI3L3
430068	0610041G09RIK	5260253	ACP2	2600180	CHI3L4
5090368	1110012E06RIK	1770468	ADSSL1	6280356	CHMP7
5270100	1200002N14RIK	3190689	ADSSL1	6840634	CIB2
3310215	1200016E24RIK	4040270	AGRN	6200433	CLCN7
4570368	1300002F13RIK	5390364	APBB3	5390463	CLECSF5
5080528	1600010D10RIK	7210687	APOE	6580162	CLECSF5
2470402	1600013P15RIK	670600	ARG2	10170	CLECSF8
2680475	1600029O10RIK	270309	ASB8	1780437	CLECSF9
3610491	1700051E09RIK	2030725	ATP6VOC	4590458	CLECSF9
2750184	1810054O13RIK	4610291	ATP6V1E1	2000719	CLIC4
2940044	1810059A23RIK	780274	AUH	2140338	CLIP1
7570612	2310022A10RIK	7100577	AXL	3370609	CPT2
1690497	2310043N10RIK	6110736	B2M	7050255	CRYBA4
4490008	2410039M03RIK	2320390	B430201A12RIK	3400369	CSF1R
840291	2600001B17RIK	2750594	BC003277	5550437	CSF2RA
150646	2600005C20RIK	2760259	BC049354	5900259	CSF3R
6420196	2600005C20RIK	2510376	BC060631	6840040	CSF3R
6100132	2610024H22RIK	2060674	BIRC1E	5550360	CSNK1D
1940368	2810432D09RIK	5360278	BIRC1E	3120465	CSTB
730228	4922504H04RIK	1440717	BLVRB	110121	CTSB
3140021	4930438M06RIK	4010446	BLVRB	990246	CTSC
3170619	4930555L03RIK	1690170	BST1	1710754	CTSC
2810091	4932439K10RIK	580332	C1QB	3610082	CXCL1
430360	4933405A16RIK	3710170	C1QG	4150750	CXCL2
4850021	4933407C03RIK	6060050	C1QR1	5390639	CXCL2
4060037	4933411K20RIK	5960717	C230009C22RIK	2120392	CYP4F18
5900682	5730528L13RIK	5860347	C3	6020487	CYP4F18
4920491	6720458D17RIK	7510243	C4	3140471	D8ERTD587E
2480768	9030216K14RIK	3190468	CAMK2D	2120202	D930007N19RIK
2850458	9130019I15RIK	20064	CAPG	610291	DOK3
4290324	9130404D14RIK	3400082	CAPG	6250148	DPEP2
50242	9430080K19RIK	4210762	CASP4	7150333	DPEP2
6550523	9630037P07RIK	1690768	CCL5	1710242	ECM1
1090647	9830148G24RIK	7050538	CCL9	5080184	EHD4
5050086	9930022N03RIK	5130156	CCRL2	2190445	ELL
3420044	9930027N05RIK	6020674	CD14	5490026	EMILIN2
5560204	9930031P18RIK	3780338	CDC42EP2	5910164	EMILIN2
3400521	9930108O06RIK	4010133	CDC42EP4	1030411	EMR1
6840181	A130086G11RIK	4230348	CEBPB	2710110	EMR1
5050240	A430073A17RIK	5860139	CHCHD8	1710603	EMR4
5690192	A630077B13RIK	150114	CHI3L1	5690463	ENTPD4
7560475	ABCC5	2570240	CHI3L3	3360189	ERN1

Probe ID	gene	Probe ID	gene	Probe ID	gene
2000474	ESRRA	1770152	ITGB5	610463	NPN3
7510154	ETS2	2970286	ITM2B	4900630	NR1H3
1230639	F10	6380086	ITPKC	2490039	NRG1
50128	F830005D05RIK	1980521	JMJD2B	4780253	OASL1
5890221	FAS	5860717	JUNDM2	5870040	P4HA1
3990739	FBXO42	4640338	KLF7	2360475	PAPD1
2230039	FCER1G	1190102	KLRA2	3870561	PDCD1LG1
4220603	FCGR1	5080685	LAIR1	2940615	PDLIM4
1110326	FCGR2B	1570131	LAMP1	2260561	PECR
5700220	FCGR2B	5270358	LAMP2	6650711	PFKL
3830678	FCGR3	240259	LDH2	70630	PGD
6960189	FURIN	4210300	LENG9	5690341	PGK1
4290593	FVT1	5360300	LPL	4570204	PILRB
5570309	FZD1	2570615	LRP1	3780291	PKM2
7200678	GAB1	5860154	LST1	4120255	PLA2G7
5690246	GBP1	5550671	LY6C	5720497	PLAUR
60553	GBP4	2630577	LY6F	610414	PLTP
3130546	GLB1L	5810672	LY6I	1570139	PRDM4
1340446	GP38	2360719	LYZ	3850433	PRDX5
5700161	GPR109A	830240	LYZS	4920100	PRDX5
7000259	GPR84	7380022	LYZS	2680050	PRM1
2140753	GRINA	3190026	MAD	3440753	PRM1
6860369	GSR	6060403	MAFB	6620445	PTGS1
3850129	GSTM1	3400050	MAN2A1	3370537	RAI16
5260541	H2-BF	5360477	MAPKAPK3	5860296	RASGRP1
2970324	HDC	7040161	MAPKAPK3	4050121	RBPM5
2350300	HIVP3	4810546	MCF2L	5670224	RDH11
840044	HP	60048	MCL1	430181	RHOU
2470242	HSD3B7	4220437	MEFV	5810398	RNASE4
620646	HVCN1	7610114	MGST1	6900091	RSN
110471	IDB3	4890500	MIR16	1190465	RUSC2
6520075	IER3	5270709	MIR16	6100471	RUSC2
3400747	IGFBP4	5690131	MMP13	6400719	SAA3
5670382	IGSF6	3520066	MMP14	110692	SCARF1
1260632	IL10	3870072	MMP2	6980102	SCL0002883.1_98
2070468	IL12A	6480201	MOCOS	5810767	SDC1
3390131	IL1RN	60600	MOCS1	6550603	SEPN1
7650747	IL1RN	3180025	MS4A6D	5260670	SESTD1
5820670	IL23A	2320553	MSCP	6020725	SETD8
5080458	ILVBL	2760475	MSCP	3850497	SLC11A1
3060458	INHBA	1050553	MXI1	6940735	SLC11A1
1030497	IRAK3	6420450	NFKBIE	3290427	SLC15A3
7560307	IRG1	6040224	NPC2	6130445	SLC15A3

Probe ID	gene	Probe ID	gene
290504	SLC22A4	6510112	YBX3
6590093	SLC25A28	6060349	ZC3H12A
6350044	SLC2A1	7510240	ZSWIM4
6560270	SLC31A2		
520470	SLC7A11		
3800458	SLC7A11		
60368	SLP		
130348	SLP		
2810487	SLPI		
1660367	SMOX		
5050008	SMOX		
60544	SNX8		
1570594	SOCS3		
4860224	SOD2		
1470091	SORT1		
2810468	SORT1		
2100762	SPON1		
2600424	SQRDL		
2060050	TCE4		
4760324	TCIRG1		
450133	TGFB1		
4060327	TGFB1		
7000187	TGM2		
6940719	TLR1		
2100468	TLR5		
1570487	TLR7		
4540626	TMEM38B		
4050626	TNF		
4830097	TNF		
1070630	TNFAIP2		
3170204	TNIP1		
1230673	TPD52		
1820414	TPD52		
3990477	TPI1		
6110170	TPI1		
270025	TRAF3		
5420441	TRAF3		
4290035	TREM3		
630259	TXNL4B		
1580338	TXNRD1		
4120243	UPP1		
2850575	VCAM1		
4830475	VCAM1		

Table 3.7 Cluster 6: Genes induced in CD64⁺ cells during inflammation.

Alphabetical list of genes in Cluster 6 (brown bar, Figure 3.6) from k-means clustering analysis of genes significantly differentially expressed (adjusted p value < 0.05; log₂(fold change) > 1) in at least one comparison of CX₃CR1^{int} CD64⁺ and CX₃CR1^{int} CD64⁻ MNPs from resting and inflamed lamina propria (results of each comparison are presented in the Appendix).

Cluster 7 (red)

Probe ID	gene	Probe ID	gene	Probe ID	gene
3390088	0610010E21RIK	3060152	4930563C06RIK	1500608	ATP6V1D
7160209	0610010E21RIK	7160246	4933430F08RIK	5670075	BC013529
6550632	0910001L09RIK	5690735	6330578E17RIK	430358	BC022145
2900681	1110002M09RIK	1770477	6720456B07RIK	4780047	BC025206
1570554	1110003O08RIK	1190647	9030624J02RIK	2480025	BC031181
5390021	1110012M11RIK	2810424	9130017C17RIK	6450059	BC037034
870382	1110020P15RIK	2060164	9430029K10RIK	4050360	BC060631
870408	1110039B18RIK	4900685	9430029K10RIK	2370326	BNIP2
3370398	1190002C06RIK	7560170	A930029N17RIK	870437	BRI3
540138	1500012F01RIK	7160047	AA467197	4590164	BSCL2
450161	1500032D16RIK	6220438	ABCD1	3610634	C130020C07RIK
6180092	1500032D16RIK	6450687	ABI3	2630463	C2TA
4590377	1500034E06RIK	6180463	ABR	290048	C730025P13RIK
4540070	1600019D15RIK	3180022	ACAA1	7100014	CAMK1
5900360	1700012G19RIK	5570497	AES	5690021	CAPN1
1110021	1700029G01RIK	1980014	AI316787	4150040	CCNH
650328	1700064K09RIK	2230386	AI481100	5700528	CD72
6590382	1700112E06RIK	4490239	AI481105	6220091	CENPB
670014	1810017G16RIK	4010722	AI837181	4880554	CNIH4
4560148	1810027O10RIK	290068	AIF1	7400343	COG2
7210019	1810035L17RIK	1400672	AIF1	6510296	COG8
7160086	1810037J08RIK	940435	AIP	3840092	CORO1B
2230184	1810048P08RIK	5720136	ALDH3B1	1070762	COX4I1
5260356	1810055D05RIK	6400669	ALDH3B1	2570373	COX5B
130270	1810058I14RIK	5870754	ALDH4A1	4900379	COX6B
7100634	1810063B05RIK	7650438	AP2M1	5490050	COX6C
2750612	2010005J08RIK	6100020	AP4S1	7100632	COX8A
4560386	2300009A05RIK	1400735	APBA3	6620377	CTDSP1
6020747	2310008H09RIK	4220189	APOB48R	2190215	CXCL10
1400398	2310014H01RIK	7210349	APOBEC3	10598	CXCL9
4060056	2310016E02RIK	5570093	ARBP	7330333	CYFIP2
1300411	2310056P07RIK	1090180	ARHGDIB	5720397	D11LGP2E
2370408	2310061J03RIK	1110382	ARL10B	6330592	D11LGP2E
4830543	2310061N23RIK	20037	ARL6IP4	1110280	D12ERTD647E
1780348	2410001E19RIK	770682	ARL6IP4	2810040	D14ERTD668E
5720630	2410005K17RIK	6180288	ARL6IP4	2810088	D15MG127
3830288	2410015M20RIK	5900253	ARPC5L	7050603	D2ERTD217E
4670689	2410025L10RIK	1110451	ARRB2	4780192	DAZAP1
3800091	2510003E04RIK	7560338	ASS1	1990731	DDT
4880639	2810021O14RIK	7100292	ATP13A1	2810139	DEADC1
5960086	2900008M13RIK	6280612	ATP5E	4180392	DMAP1
6370707	3110003A17RIK	650161	ATP5G1	2070543	DNAJC19
150053	4930432N22RIK	610681	ATP5J2	7210719	DNASE1L1

Probe ID	gene	Probe ID	gene	Probe ID	gene
6520193	DNASE1L3	780221	H2-T9	2600097	LYRM5
2710504	DOT1L	6560564	HELB	2680095	MAPKAP1
1230538	DRAP1	5690561	HIST1H3I	2100678	MGC18837
580196	ECD	5220689	HIST1H4A	5810541	MGC18837
7200403	EG622339	7050546	HIST2H2AA1	650347	MGC68323
2370187	EIF2B5	2340315	HIST2H2BE	4040093	MIF
240615	EIF4EBP1	4810575	HK3	1030731	MIPEP
6450050	FADD	6980274	HMGA1	990148	MLF2
1260128	FBXO6B	4290100	HPCAL1	7380725	MRPL14
830632	FCRL3	2470193	HSPC171	6590767	MRPL23
1710162	FER1L3	1710619	IFI205	10630	MRPL52
3870100	FH1	1090139	IFI47	1070358	MRPL54
4890750	FIS1	360041	IFIT2	1090121	MRPS18A
4220050	FLII	4010019	IFITM1	6330041	MRPS18C
2480719	FLOT1	5820068	IFITM1	2690376	MRPS21
7650048	FPR-RS2	5960577	IFITM1	990630	MRPS33
2630008	FXVD5	840349	IFITM2	3610286	MS4A6B
460221	G1P2	3840292	IFITM3	4390184	NCOA5
7560326	G431001I09RIK	6290037	IGTP	4040435	NDG2
240653	G6PDX	4480735	IL15	3890739	NDUFA1
3290020	GAPD	6940402	IL15	770221	NDUFA10
5870154	GAPD	4890041	ILVBL	4830187	NDUFA3
6940475	GAPD	6590653	IRF7	6330477	NDUFA3
1400041	GBP2	870523	LOC208092	6560152	NDUFA5
2100068	GBP2	4610309	LOC215678	2100386	NDUFB10
2140433	GI_6679936_REF_NM_008084.1__328	2940544	LOC224137	1260370	NDUFB2
7400132	GIT2	4670133	LOC269365	2900301	NDUFB3
1070091	GMFG	5390088	LOC327957	4900538	NDUFB5
7050450	GMFG	5340133	LOC333830	1300544	NDUFS4
6840521	GNA13	60497	LOC381292	4490154	NDUFS4
2320041	GNAI2	6660044	LOC382061	5910475	NDUFS6
4220753	GNAI2	110400	LOC382127	5890164	NEDD8
5720615	GNGT2	3400452	LOC383706	2230403	NENF
7330379	GNGT2	3290477	LOC383850	270026	NFIX
7210039	GPC1	2630192	LOC383883	2070014	NFKB2
6370100	GPI1	7040438	LOC384593	6550367	NMI
4760154	GPR35	6420605	LSM5	4810246	NOSIP
2000246	GRN	1090221	LSM6	6940187	NRP
1980102	GSDMDC1	1690187	LY6A	6450220	NUBP1
870446	H2-T10	4780184	LY6E	4830010	OASL2
2230731	H2-T17	6550376	LY6E	2570468	ODF2
4480685	H2-T22	430167	LY86	5690717	OGFR
2350181	H2-T23	3170577	LYPLA3	4670670	PDCD5

Probe ID	gene	Probe ID	gene	Probe ID	gene
650598	PFDN5	6760762	SDC3	770086	UQCR
3140246	PGLYRP1	4540129	SEC61G	7560687	USMG5
650564	PGM2	780554	SEMA7A	2490014	VPS28
4200575	PGM2	4010343	SEPX1	3870332	VRK2
4490592	PIP5K1C	1050092	SERPINA3G	4260739	VTI1B
5860243	PLAC8	3310187	SIAT7D	4780437	VTI1B
240717	PPARD	2340044	SLAMF8	2350397	WARS
6350309	PRKRIP1	5270500	SLC10A3	2100360	WTAP
7050753	PRKRIP1	620008	SMOX	5340577	XDH
4120711	PRUNE	3440132	SMPDL3B	540762	XPA
990682	PSEN2	4010091	SNAPAP	7320246	ZCRB1
5870398	PSMB10	1030463	SNRPD2	2190543	ZDHHC12
6350343	PSMB10	6510070	SNRPF	1010195	ZFAND2B
3060537	PSMB7	2230278	SRD5A2L	2750037	ZFP277
3360376	PSMB7	5260451	SRD5A2L	1820739	ZMYND15
2450064	PSMB9	5910397	SRD5A2L		
2100497	PSME2B	2100327	STARD5		
1430110	PTPN1	240725	STAT1		
650064	RAB5C	3710332	STX8		
2480152	RBAK	3990441	STX8		
540603	RBMS1	1070152	STXBP2		
2370370	RBX1	580152	SUGT1		
2630703	RHOG	3360132	SYF2		
5910427	RIT1	2480373	TAF10		
6650088	RNF135	2630044	TES3		
730528	RNUXA	270376	THAP7		
4900703	RPL19	2320296	TIMM10		
510674	RPL24	2490491	TMEM1		
2140296	RPL36	4210228	TMEM49		
6020343	RPLP1	6480458	TMEM49		
1030612	RPS13	3710278	TMSB10		
5570129	RPS15	7040592	TMUB1		
4850519	RPS23	2340543	TNFSF13		
6420242	RPS27	1710202	TNFSF5IP1		
5270543	RPS29	4570575	TRAK1		
5890064	RRAS	6350189	TREX1		
3850477	S100A11	6580241	TRIOBP		
5720523	S100A11	7210196	TRIOBP		
3190577	SAMHD1	3890202	TRPC4AP		
5310743	SAMHD1	1410554	TYKI		
6860278	SAMHD1	2100689	UNC93B1		
1260673	SCOTIN	1050095	UPP1		
6960674	SCOTIN	3520594	UPP1		

Table 3.8 Cluster 7: Genes induced during inflammation regardless of CD64 status.

Alphabetical list of genes in Cluster 7 (red bar, Figure 3.6) from k-means clustering analysis of genes significantly differentially expressed (adjusted p value < 0.05; $\log_2$ (fold change) > 1) in at least one comparison of CX₃CR1^{int} CD64⁺ and CX₃CR1^{int} CD64⁻ MNP from resting and inflamed lamina propria (results of each comparison are presented in the Appendix).

Gene	Biological Process
B2M	immune response
C3	immune response
CCL5	immune response
CEBPB	immune response
CTSC	immune response
CXCL1	immune response
CXCL2	immune response
FAS	immune response
FCGR2B	immune response
IGSF6	immune response
IL10	immune response
IL12A	immune response
IL1RN	immune response
IL23A	immune response
LST1	immune response
SLC11A1	immune response
TLR1	immune response
TNF	immune response
AXL	inflammatory response
C3	inflammatory response
CCL5	inflammatory response
CCRL2	inflammatory response
CD14	inflammatory response
CEBPB	inflammatory response
CHI3L1	inflammatory response
CSF1R	inflammatory response
CXCL1	inflammatory response
CXCL2	inflammatory response
ECM1	inflammatory response
IGFBP4	inflammatory response
IL10	inflammatory response
IL23A	inflammatory response
LYZ	inflammatory response
MEFV	inflammatory response
PRDX5	inflammatory response
SLC11A1	inflammatory response
TLR1	inflammatory response
TLR5	inflammatory response
TLR7	inflammatory response
TNF	inflammatory response
TNIP1	inflammatory response

Gene	Biological Process
AGRN	retinoid metabolic process
APOE	retinoid metabolic process
LPL	retinoid metabolic process
LRP1	retinoid metabolic process
RDH11	retinoid metabolic process
SDC1	retinoid metabolic process
AGRN	phototransduction, visible light
APOE	phototransduction, visible light
LPL	phototransduction, visible light
LRP1	phototransduction, visible light
RDH11	phototransduction, visible light
SDC1	phototransduction, visible light

Table 3.9 Cluster 6 gene set enrichment analysis.

List of genes in each gene ontology (GO) biological process which was significantly enriched in Cluster 6. Analysis performed by Dr Nicholas Ilott.

log₂ Fold Change	Adj. p value	Gene Symbol	Aliases	Probe ID
5.33	0.0071	SLPI	secretory leukocyte peptidase inhibitor	2810487
4.42	0.0006	SLC11A1	solute carrier family 11 (proton-coupled divalent metal ion transporter)	3850497
4.4	0.0021	F10	coagulation factor X	1230639
4.31	0.0039	IL10	interleukin 10	1260632
4.15	0.0021	CD14		6020674
4.11	0.0001	CHI3L1	chitinase 3-like 1; chitinase-like 1 (Chil1)	150114
4.04	0.0011	SAA3	serum amyloid A 3	6400719
4.01	0.0365	MMP13	matrix metalloproteinase 13	5690131
3.99	0.0346	EMR1	EGF-like module containing, mucin-like, hormone receptor-like sequence 1	1030411
3.98	0.0343	IRAK3	Interleukin 1 receptor-associated kinase 3	1030497
3.97	0.0034	KLRA2	killer cell lectin-like receptor, subfamily A, member 2	1190102
3.91	0.0001	C1QB	complement component 1, q subcomponent, beta polypeptide	580332
3.89	0.0021	INHBA	inhibin, beta A	3060458
3.85	0.0001	LPL	lipoprotein lipase	5360300
3.54	0.0032	PLA2G7	phospholipase A2, group VII (platelet-activating factor acetylhydrolase, plasma)	4120255
3.34	0.0222	GAB1	growth factor receptor bound protein 2-associated protein 1	7200678
3.32	0.0181	MMP14	matrix metalloproteinase 14 (membrane-inserted); MT1-MMP	3520066
3.26	0.0011	CLECSF8	C-type lectin superfamily member 8; C-type lectin domain family 4, member d (Clec4d)	10170
3.26	0.0021	9930027N05RIK	signal-regulatory protein beta 1A (Sirpb1)	3420044
3.22	0.0032	FCGR3	Fc receptor, IgG, low affinity III; CD16	3830678
3.21	0.0426	EMR1	EGF-like module containing, mucin-like, hormone receptor-like sequence 1	2710110
3.21	0.0001	SLP	sex-limited protein; complement component 4A (C4a)	60368
3.21	0.0011	APOE	apolipoprotein E	7210687
3.2	0.0012	CSF1R	colony stimulating factor 1 receptor; macrophage colony-stimulating factor receptor (M-CSFR); CD115	3400369
3.2	0.013	ITGB5	integrin beta 5	1770152
3.2	0.0203	IL23A	interleukin 23, alpha subunit p19	5820670
3.18	0.0203	RASGRP1	RAS guanyl releasing protein 1	5860296
3.15	0.0058	CLECSF9	C-type lectin superfamily member 9; C-type lectin domain family 4, member e (Clec4e); Mincle	1780437
3.06	0.0205	H2-BF	complement factor B (Cfb)	5260541
3.03	0.0227	ARG2	arginase 2	670600

3.02	0.0039	SLC11A1	solute carrier family 11 (proton-coupled divalent metal ion transporter)	6940735
3.01	0.024	BLVRB	biliverdin reductase B (flavin reductase (NADPH))	4010446
2.99	0.0351	SLC7A11	solute carrier family 7 (anionic amino acid transporter light chain, xc- system), member 11	520470
2.96	0.0175	CHI3L3	chitinase 3-like 3; chitinase-like 3 (Chil3); Ym1	2570240
2.88	0.0011	HP	haptoglobin	840044
2.87	0.0039	FCGR2B	Fc receptor, IgG, low affinity IIb; CD32	1110326
2.85	0.0029	CAPG	capping protein (actin filament), gelsolin-like	20064
2.84	0.0008	FPR-RS2	formyl peptide receptor 2 (Fpr2)	7650048
2.84	0.0283	C1QR1	complement component C1q receptor; Ly68; CD93	6060050
2.81	0.0005	SPON1	spondin 1, (f-spondin) extracellular matrix protein	2100762
2.79	0.0299	FCRL3	Fc receptor-like 3; Fc receptor-like 3 (Fcgr4)	830632
2.77	0.0428	YBX3	Y box protein 3	6510112
2.76	0.0021	GP38	glycoprotein 38; podoplanin (Pdpn)	1340446
2.75	0.0022	CXCL1	chemokine (C-X-C motif) ligand 1; KC; Gro1	3610082
2.75	0.0064	VCAM1	vascular cell adhesion molecule 1	2850575
2.74	0.0141	BC049354	C-type lectin domain family 4, member a1 (Clec4a1)	2760259
2.7	0.0035	RHOU	ras homolog gene family, member U	430181
2.7	0.0439	CCL5	chemokine (C-C motif) ligand 5; RANTES	1690768
2.63	0.0018	MS4A6D	membrane-spanning 4-domains, subfamily A, member 6D	3180025
2.63	0.0111	ELL	elongation factor RNA polymerase II	2190445
2.62	0.0236	LYZ	lysozyme	2360719
2.59	0.0022	FCGR2B	Fc receptor, IgG, low affinity IIb; CD32	5700220
2.58	0.0394	SPATA13	spermatogenesis associated 13	4220673
2.58	0.0001	TGFBI	transforming growth factor, beta induced	450133
2.56	0.0273	SQRDL	sulphide quinone reductase-like (yeast)	2600424
2.54	0.0058	NRG1	neuregulin 1	2490039
2.53	0.0071	IL1RN	interleukin 1 receptor antagonist (IL-1RA)	3390131
2.52	0.0029	SMOX	spermine oxidase	1660367
2.52	0.0012	PRDX5	peroxiredoxin 5	4920100
2.5	0.0404	KLF7	Krüppel-like factor 7 (ubiquitous)	4640338
2.46	0.0264	MXI1	MAX interacting protein 1	1050553
2.45	0.0021	LYZS	lysozyme 2 (Lyz2)	7380022
2.45	0.0361	CHI3L3	chitinase 3-like 3; chitinase-like 3 (Chil3); Ym1	3870242
2.44	0.0089	IGSF6	immunoglobulin superfamily, member 6	5670382
2.42	0.0034	SMOX	spermine oxidase	5050008

2.42	0.0439	TNFSF13	tumour necrosis factor (ligand) superfamily, member 13; a proliferation-inducing ligand (APRIL)	2340543
2.41	0.0105	PDLIM4	PDZ and LIM domain 4	2940615
2.41	0.0151	LY6C	lymphocyte antigen 6 complex, locus C1 (Ly6c1)	5550671
2.41	0.0018	TGFBI	transforming growth factor, beta induced	4060327
2.41	0.0338	TRAF3	TNF receptor-associated factor 3	270025
2.4	0.0446	TREM3	triggering receptor expressed on myeloid cells 3	4290035
2.39	0.0058	C1QG	complement component 1, q subcomponent, C chain (C1qc)	3710170
2.39	0.0271	CDC42EP2	CDC42 effector protein (Rho GTPase binding) 2	3780338
2.38	0.0479	CHI3L4	chitinase 3-like 4; chitinase-like 4 (Chil4); Ym2	2600180
2.37	0.0264	CIB2	calcium and integrin binding family member 2	6840634
2.35	0.0129	1300002F13RIK	ERBB receptor feedback inhibitor 1 (Errfi1)	4570368
2.35	0.0194	CSF3R	colony stimulating factor 3 receptor (granulocyte) (G-CSFR); CD114	6840040
2.35	0.0012	ETS2	E26 avian leukemia oncogene 2, 3' domain	7510154
2.33	0.0338	TRAF3	TNF receptor-associated factor 3	5420441
2.32	0.016	1600010D10RIK	family with sequence similarity 102, member B (Fam102b)	5080528
2.31	0.0222	CYP4F18	cytochrome P450, family 4, subfamily f, polypeptide 18	6020487
2.28	0.0021	2410039M03RIK		4490008
2.26	0.0048	CLECSF9	C-type lectin superfamily member 9; C-type lectin domain family 4, member e (Clec4e); Mincle	4590458
2.26	0.0484	CDC42EP4	CDC42 effector protein (Rho GTPase binding) 4	4010133
2.26	0.0173	CCL9	chemokine (C-C motif) ligand 9	7050538
2.25	0.0029	LY6F	lymphocyte antigen 6 complex, locus F	2630577
2.25	0.0145	ECM1	extracellular matrix protein 1	1710242
2.22	0.026	HDC	histidine decarboxylase	2970324
2.21	0.0069	PRDM4	PR domain containing 4	1570139
2.2	0.0126	IL1RN	interleukin 1 receptor antagonist (IL-1RA)	7650747
2.2	0.0114	5730528L13RIK	Myb/SANT-like DNA-binding domain containing 3 (Msantd3)	5900682
2.2	0.0021	4933405A16RIK	sphingomyelin synthase 2 (Sgms2)	430360
2.16	0.0048	CYP4F18	cytochrome P450, family 4, subfamily f, polypeptide 18	2120392
2.16	0.0012	9030216K14RIK		2480768
2.15	0.0021	NPN3	sulfiredoxin 1 homolog (S. cerevisiae) (Srxn1)	610463

2.15	0.0236	4932439K10RIK	sperm antigen with calponin homology and coiled-coil domains 1-like (Specc1l)	2810091
2.14	0.0111	GPR84	G protein-coupled receptor 84	7000259
2.14	0.0199	CEBPB	CCAAT/enhancer binding protein (C/EBP), beta	4230348
2.13	0.0326	ENTPD4	ectonucleoside triphosphate diphosphohydrolase 4	5690463
2.12	0.0145	GP49A	glycoprotein 49 A	5820739
2.12	0.0143	TNF	tumor necrosis factor; TNF α	4050626
2.11	0.0184	HIVEP3	human immunodeficiency virus type I enhancer binding protein 3	2350300
2.11	0.0034	ADSSL1	adenylosuccinate synthetase like 1	3190689
2.09	0.006	TNF	tumor necrosis factor; TNF α	4830097
2.07	0.007	CXCL2	chemokine (C-X-C motif) ligand 2; macrophage inflammatory protein 2 alpha (MIP-2a); Gro2	4150750
2.06	0.0236	ZMYM2	zinc finger, MYM-type 2	6560040
2.05	0.0409	2310022A10RIK	RIKEN cDNA 2310022A10 gene	7570612
2.04	0.0055	MDM2	transformed mouse 3T3 cell double minute 2; E3 ubiquitin-protein ligase Mdm2	670068
2.01	0.0168	GP49A	glycoprotein 49 A	4120356
2.01	0.0186	CCRL2	chemokine (C-C motif) receptor-like 2; lipopolysaccharide-inducible C-C chemokine receptor (L-CCR)	5130156
2.01	0.0031	MIR16	membrane-interacting protein of RGS16; glycerophosphodiester phosphodiesterase 1 (Gde1)	5270709
2.01	0.0262	LYZS	lysozyme 2 (Lyz2)	830240
2	0.0468	MOCS1	molybdenum cofactor synthesis 1	60600
1.98	0.0021	SCARF1	scavenger receptor class F, member 1	110692
1.98	0.005	MAFB	V-maf musculoaponeurotic fibrosarcoma oncogene homolog B	6060403
1.96	0.0461	CSF3R	colony stimulating factor 3 receptor (granulocyte) (G-CSFR); CD114	5900259
1.95	0.0242	SLC2A1	solute carrier family 2 (facilitated glucose transporter), member 1; glucose transporter type 1 (Glut1)	6350044
1.94	0.0447	1600029O10RIK	poly (ADP-ribose) polymerase family, member 14 (Parp14)	2680475
1.91	0.0207	LY6I	lymphocyte antigen 6 complex, locus I	5810672
1.9	0.0029	HAK	heart alpha-protein kinase; alpha-kinase 2 (Alpk2)	7200242
1.89	0.0428	ADSSL1	adenylosuccinate synthetase like 1	1770468
1.88	0.0173	9830148G24RIK	pyrin domain containing 3 (Pydc3)	1090647
1.88	0.0048	IRG1	immunoresponsive gene 1	7560307
1.87	0.0021	9930022N03RIK	T cell-interacting, activating receptor on myeloid cells 1 (Tarm1)	5050086

1.87	0.0439	GBP1	guanylate nucleotide binding protein 1; guanylate binding protein 2b (Gbp2b)	5690246
1.86	0.0401	ZSWIM4	zinc finger SWIM-type containing 4	7510240
1.86	0.0012	C4	complement component 4 (within H-2S); complement component 4B (C4b)	7510243
1.86	0.0225	SLC31A2	solute carrier family 31, member 2	6560270
1.85	0.0021	CAPG	capping protein (actin filament), gelsolin-like	3400082
1.84	0.05	GDF15	growth differentiation factor 15	650524
1.83	0.0028	SCL0002883.1_98		6980102
1.81	0.0285	SLC15A3	solute carrier family 15, member 3	6130445
1.81	0.0175	RSN	Reed-Steinberg cell-expressed intermediate filament-associated protein (Restin); CAP- GLY domain containing linker protein 1 (Clip1)	6900091
1.8	0.0222	SLC15A3	solute carrier family 15, member 3	3290427
1.8	0.0207	MEFV	Mediterranean fever; pyrin	4220437
1.79	0.0057	PRDX5	peroxiredoxin 5	3850433
1.79	0.0161	SOD2	superoxide dismutase 2, mitochondrial	4860224
1.75	0.0199	2600005C20RIK	ribosomal RNA processing 1 homolog B (S. cerevisiae) (Rrp1b)	150646
1.74	0.0058	FZD1	frizzled homolog 1 (Drosophila)	5570309
1.74	0.0227	PFKL	phosphofructokinase, liver, B-type	6650711
1.72	0.0021	CLECSF5	C-type lectin superfamily member 5; C-type lectin domain family 5, member a (Clec5a)	5390463
1.72	0.0021	IER3	immediate early response 3	6520075
1.71	0.0194	2600005C20RIK	ribosomal RNA processing 1 homolog B (S. cerevisiae) (Rrp1b)	6420196
1.69	0.0197	DOK3	docking protein 3	610291
1.68	0.0044	FCER1G	Fc receptor, IgE, high affinity I, gamma polypeptide	2230039
1.68	0.0048	CSTB	cystatin B	3120465
1.67	0.0033	GRINA	glutamate receptor, ionotropic, N-methyl D- aspartate-associated protein 1 (glutamate binding)	2140753
1.67	0.0242	ERN1	endoplasmic reticulum (ER) to nucleus signalling 1	3360189
1.66	0.0259	DPEP2	dipeptidase 2	6250148
1.64	0.038	DPEP2	dipeptidase 2	7150333
1.64	0.0064	RUSC2	RUN and SH3 domain containing 2	1190465
1.64	0.0078	TPD52	tumor protein D52	1230673
1.64	0.0128	MDM2	transformed mouse 3T3 cell double minute 2; E3 ubiquitin-protein ligase Mdm2	3310243
1.63	0.0199	1200016E24RIK		3310215
1.62	0.0407	PLEC1	plectin (Plec)	6960201
1.61	0.0048	9930031P18RIK	RIKEN cDNA 9930031P18 gene	5560204

1.61	0.0173	BLVRB	biliverdin reductase B (flavin reductase (NADPH))	1440717
1.61	0.0172	SEPN1	selenoprotein N, 1	6550603
1.59	0.046	A430073A17RIK		5050240
1.59	0.0074	RUSC2	RUN and SH3 domain containing 2	6100471
1.58	0.0207	MAN2A1	mannosidase 2, alpha 1	3400050
1.57	0.0428	CXCL2	chemokine (C-X-C motif) ligand 2; macrophage inflammatory protein 2 alpha (MIP-2a); Gro2	5390639
1.57	0.0277	MAPKAPK3	mitogen-activated protein kinase-activated protein kinase 3	5360477
1.57	0.0173	EMILIN2	elastin microfibril interfacer 2	5910164
1.57	0.005	MOCOS	molybdenum cofactor sulfurase	6480201
1.56	0.0338	CRYBA4	crystallin, beta A4	7050255
1.56	0.033	CD81		2370364
1.54	0.0376	2310016C08RIK	hypoxia inducible lipid droplet associated (Hilpda)	4290152
1.53	0.0391	TPD52	tumor protein D52	1820414
1.53	0.0076	GLB1L	galactosidase, beta 1-like	3130546
1.53	0.0376	6720458D17RIK	integrin alpha 9 (Itga9)	4920491
1.53	0.0316	F830005D05RIK	RIKEN cDNA F830005D05 gene	50128
1.52	0.0021	CLECSF5	C-type lectin superfamily member 5; C-type lectin domain family 5, member a (Clec5a)	6580162
1.51	0.012	PLAUR	plasminogen activator, urokinase receptor; CD87	5720497
-1.51	0.0031	1600029D21RIK	placenta expressed transcript 1 (Plet1)	1300092
-1.51	0.0283	H2AFX	H2A histone family, member X	1090327
-1.51	0.0259	NGFRAP1	nerve growth factor receptor (TNFRSF16) associated protein 1	360491
-1.52	0.0193	OXCT	3-oxoacid CoA transferase 1 (Oxct1)	3420500
-1.52	0.006	BC065078	cytochrome b561 family, member A3 (Cyb561a3)	6370739
-1.56	0.0338	H2-OB	histocompatibility 2, O region beta locus; histocompatibility 2, class II antigen A, beta 2 (H2-Ab2)	6760167
-1.56	0.0316	BEX6	brain expressed gene 6	870603
-1.57	0.01	CBFA2T3H	core-binding factor, runt domain, alpha subunit 2, translocated to, 3 (human)	5890110
-1.58	0.0362	RNASE6	ribonuclease, RNase A family, 6	430703
-1.58	0.0489	SIRT3	sirtuin 3	5550554
-1.59	0.0285	ECE1	endothelin converting enzyme 1	5700722
-1.59	0.0296	SEPT6	septin 6	5260762
-1.61	0.0338	LMYC1	lung carcinoma myc related oncogene 1; v-myc myelocytomatosis viral oncogene homolog, lung carcinoma derived (avian) (Myc1)	1580445

-1.62	0.0079	RGS12	regulator of G-protein signalling 12	5130487
-1.62	0.0361	CYB5	cytochrome b5; cytochrome b5 type A (microsomal) (Cyb5a)	7560639
-1.63	0.0222	CYB5	cytochrome b5; cytochrome b5 type A (microsomal) (Cyb5a)	2810072
-1.65	0.0105	CDCA3	cell division cycle associated 3	1050170
-1.65	0.037	PRC1	protein regulator of cytokinesis 1	780475
-1.66	0.0194	PPM1M	protein phosphatase 1M	4180575
-1.67	0.0409	AMICA1	adhesion molecule, interacts with CXADR antigen 1	3850692
-1.68	0.0128	COX6A2	cytochrome c oxidase subunit VIa polypeptide 2	2470356
-1.68	0.0186	CD24A	CD24; heat stable antigen (HSA); lymphocyte antigen 52 (Ly52)	3390110
-1.68	0.0222	PLP2	proteolipid protein 2	1740379
-1.76	0.0177	ITGAE	integrin alpha E; CD103	1300059
-1.78	0.0012	FPGS	folylpolyglutamyl synthetase	4670270
-1.82	0.0159	LMTK2	lemur tyrosine kinase 2	2030376
-1.83	0.0175	PIIH	peptidyl prolyl isomerase H	3830592
-1.87	0.013	SNX22	sorting nexin 22	2810451
-1.87	0.0151	1110012005RIK	CAAX box 1A (Cxx1a)	2140450
-1.96	0.0168	AKR7A5	aldo-keto reductase family 7, member A5 (aflatoxin aldehyde reductase)	3180068
-1.97	0.0338	CDC42EP3	CDC42 effector protein (Rho GTPase binding) 3	4150739
-1.99	0.0057	MCM5	minichromosome maintenance deficient 5, cell division cycle 46 (<i>S. cerevisiae</i>); Cdc46	6220270
-1.99	0.0048	2410015N17RIK	dCTP pyrophosphatase 1 (Dctpp1)	1980446
-2.08	0.0019	PBK	PDZ binding kinase	2900300
-2.11	0.0021	KLRD1	killer cell lectin-like receptor, subfamily D, member 1; CD94	4890279
-2.12	0.0064	1110017L21RIK		6450634
-2.13	0.0474	2810046M22RIK	WD repeat domain 86 (Wdr86)	7200719
-2.14	0.0111	CDH1	cadherin 1; E-cadherin	5820184
-2.15	0.0052	NUP210	nucleoporin 210	1230703
-2.21	0.0176	STMN1	stathmin 1	3120392
-2.24	0.0194	TNNI2	troponin I type 2 (skeletal, fast)	2260066
-2.32	0.0055	PRNP	prion protein	6770379
-2.36	0.0078	UHRF1	ubiquitin-like with PHD and ring finger domains 1	4560397
-2.53	0.0197	TIMELESS	timeless circadian clock 1	160703
-2.56	0.0173	KIF22	kinesin family member 22	4210246
-3.31	0.0059	ITGAE	integrin alpha E; CD103	7100687
-3.39	0.004	ITGAE	integrin alpha E; CD103	520196

Table 3.10 Microarray analysis of CX₃CR1^{int} CD64⁺ and CX₃CR1^{int} CD64⁻ MNPs from inflamed large intestine.

List of genes which were significantly differentially expressed (adjusted p value < 0.05; log₂(fold change) > 1.5 or < -1.5) by CX₃CR1^{int} CD64⁺ vs. CX₃CR1^{int} CD64⁻ MNPs both isolated from inflamed colonic and caecal lamina propria 21 days after induction of *Hh* + anti-IL-10R colitis. Positive log₂(fold change) indicates higher expression by CX₃CR1^{int} CD64⁺ MNPs, negative log₂(fold change) indicates higher expression by CX₃CR1^{int} CD64⁻ MNPs. Where some genes were identified as significantly differentially expressed by more than one probe, all probes are listed. Genes and probes highlighted in bold text were also upregulated in CX₃CR1^{int} CD64⁺ MNPs from d21 *Hh* + anti-IL-10R vs. CX₃CR1^{int} CD64⁺ MNPs from uninfected controls (full results from this comparison can be found in Appendix, Table 3).

4 The source of IL-23 during *Helicobacter hepaticus*-driven intestinal inflammation

4.1 Introduction

Helicobacter hepaticus is a flagellated, curved-to-spiral Gram-negative bacterium which along with other members of its genus can be found as a commensal bacterial species in conventionally housed mice (Fox et al., 1994; Shames et al., 1995). *Hh* is referred to as a “pathobiont”: in wild-type mice infection does not cause disease, however in genetically susceptible strains, such as IL-10 knockout (Kullberg et al., 1998) and immunodeficient mice (Ward et al., 1996), infection with *Hh* causes inflammation of the caecum and colon (typhlocolitis). *Hh*-induced typhlocolitis can also occur in wild-type mice when the anti-inflammatory IL-10 pathway is inhibited using a monoclonal antibody (mAb) to block the IL-10R (anti-IL-10R; clone 1B1.2) (Kullberg et al., 2006).

Studies using samples from inflammatory bowel disease patients and experimental models have shown that the cytokine IL-23 is linked to intestinal inflammation. IL-23 is a heterodimeric molecule composed of the specific subunit IL-23p19, and IL-12/23p40, which is shared with IL-12 (Oppmann et al., 2000). Subsequently, IL-23 – and not IL-12 – was found to be responsible for driving inflammation in experimental models of a number of diseases, including multiple sclerosis (Cua et al., 2003), rheumatoid arthritis (Murphy et al., 2003), and inflammatory bowel disease (Hue et al., 2006; Kullberg et al., 2006; Uhlig et al., 2006b; Yen et al., 2006). IL-23 alone was shown to be essential for colitis in the anti-CD40 model (Uhlig et al., 2006b). This study found that IL-12 drove the systemic

symptoms often seen alongside intestinal pathology, such as splenomegaly and wasting disease, thus highlighting the independence of colitis, splenic enlargement and weight loss. IL-23 has also been shown to drive typhlocolitis in IL-10-deficient mice, and *Hh*-induced disease mediated by innate and adaptive mechanisms (Hue et al., 2006; Kullberg et al., 2006; Yen et al., 2006). Intestinal pathology in IBD, particularly Crohn's disease, is also strongly associated with IL-23. Elevated expression of IL-23 is found in the intestinal mucosa of CD patients and to a lesser extent in those with UC (Liu et al., 2011b; Schmidt et al., 2005). In addition multiple variants of the gene *IL23R*, which encodes a subunit of the IL-23 receptor, are associated with both ileal CD and UC (Duerr et al., 2006). One such variant, Arg381Gln, confers strong protection against CD (Duerr et al., 2006). The link between IL-23 and its receptor and IBD, and experimental models thereof, plus the discovery that it drives local intestinal immunopathology but not systemic inflammation, highlights IL-23 as a potentially specific target for the treatment of IBD.

The mechanisms by which IL-23 causes pathology have been well studied (Maloy and Powrie, 2011). However, its source during colitis has not been definitively shown. Some studies have suggested mononuclear phagocytes may produce IL-23 during colitis (Becker et al., 2003; Kamada et al., 2010; Kamada et al., 2008; Liu et al., 2011b; Rivollier et al., 2012; Uhlig et al., 2006b; Zigmond et al., 2012). Some of this work cannot be directly aligned with recent developments in characterisation of intestinal MNPs, however they commonly describe the accumulation of a population of MNPs which expresses IL-23p19 mRNA during experimental colitis in mice (Rivollier et al., 2012; Uhlig et al., 2006b; Zigmond et al., 2012) and in Crohn's disease (Kamada et al., 2010; Kamada et al., 2008), in which MNP-expressed IL-23 protein has also been observed by ELISA (Kamada et al., 2008) and immunohistochemistry (Liu et al., 2011b). Three of these studies identified IL-

IL-23 expression by a CX₃CR1^{int} CD11b⁺ population during Crohn's disease (Kamada et al., 2008), and T cell transfer- (Rivollier et al., 2012) and DSS-induced experimental colitis (Zigmond et al., 2012). We have also observed accumulation of CX₃CR1^{int} CD11b⁺ MNPs in the lamina propria during T cell transfer- (data not shown) and *Helicobacter hepaticus*-driven colitis, described in Chapter 3.

MNPs are abundant and widely dispersed throughout the intestinal lamina propria and in the gut-associated lymphoid tissue. With only a single columnar epithelial cell layer separating the lamina propria from the intestinal lumen, lamina propria MNPs capable of antigen presentation are in close proximity to the many sources of antigen found in the gastrointestinal tract. During T cell priming, thought to be restricted to organised lymphoid tissue, antigen presenting cells provide instructive cytokines and co-stimulatory molecules which shape the adaptive immune response (Coombes and Powrie, 2008; Varol et al., 2010). DCs and macrophages have also been shown to support and control T cell responses in non-lymphoid tissues, including the intestine, following initial T cell priming in lymphoid organs (Hadis et al., 2011; McLachlan et al., 2009; Rossini et al., 2013). Thanks to their ideal position and ability to influence immune responses at multiple stages, MNPs would be a logical source of IL-23 in the intestine.

However, despite numerous claims that DCs and macrophages are capable of IL-23 production following a variety of stimuli and in different disease settings, no population has been shown to act as a functional or necessary source of IL-23 during colitis *in vivo*. Therefore we sought to determine the source of IL-23 in the *Hh* + anti-IL-10R model of intestinal inflammation.

4.2 Results

4.2.1 CD11c⁺ cell-derived IL-23 is necessary for *Hh* + anti-IL-10R colitis

Mononuclear phagocytes can influence innate and adaptive immune responses and are ideally situated throughout the intestine, which led to the hypothesis that a population of MNPs may be the source of IL-23 during *Hh* + anti-IL-10R colitis. The two major populations of MNPs, DCs and macrophages, both express CD11c to varying degrees (Geissmann et al., 2010a) (Figures 3.2, 3.4 and 3.9). We used transgenic mouse lines to conditionally knock out IL-23 only in cells expressing CD11c. Mice which express Cre recombinase and GFP under the control of the *Cd11c* promoter (CD11c-Cre) (Stranges et al., 2007) were crossed to those with “floxed” alleles of *Il23a*, which encodes the p19 subunit specific to IL-23 (Thakker et al., 2007). Two loxP sequences, which are recognised by the Cre recombinase enzyme, were placed around four exons of the *Il23a* locus (Thakker et al., 2007), which results in excision of the intervening genomic DNA when cells possess these floxed alleles and co-express CD11c-Cre. The resulting CD11c-Cre⁺ IL-23^{fl/fl} mice (which will be referred to as CD11c^{IL-23-}) lack IL-23 production by cells expressing CD11c, while production by all other cell types remains intact.

To determine whether CD11c⁺ cells are a necessary source of IL-23 in colitis, we infected CD11c^{IL-23-} mice and CD11c-Cre⁻ (CD11c^{IL-23+}) littermate controls with *Hh* and treated them with anti-IL-10R for three weeks, at which point typhlitis and colitis can be observed (Figure 3.1). Indeed, we saw inflammation in the caecum and colon of CD11c^{IL-23+} mice infected with *Hh* and treated with anti-IL-10R, but not in *Hh*-infected and anti-IL-10R-treated CD11c^{IL-23-} mice, or in control CD11c^{IL-23+} mice infected with *Hh* but not treated with anti-IL-10R (Figure 4.1). We also did not see intestinal inflammation in uninfected

CD11c^{IL-23+} mice treated with anti-IL-10R (data not shown; see Figure 3.1). Since *Hh* can be found as part of the commensal microbiota of normal mice, and *Hh* infection induces IL-10 expression (Kullberg et al., 1998) possibly in addition to other changes, we used *Hh* infected CD11c^{IL-23+} mice not treated with anti-IL-10R as the most appropriate control group. We confirmed that the lack of inflammation in CD11c^{IL-23-} mice was not due to differences in *Hh* colonisation between the groups (Figure 4.2).

We expected that IL-23 levels would be reduced in CD11c^{IL-23-} mice, given our hypothesis that a subset of CD11c⁺ cells is the source of IL-23 which drives colitis. We measured the level of IL-23 expressed by intestinal lamina propria cells by performing RT-qPCR on *ex vivo* cells frozen directly after isolation, and the level of IL-23 secreted by lamina propria cells by ELISA on supernatants from *ex vivo* cells cultured without stimulation for 48 hours (Figure 4.3). This confirmed our hypothesis, showing significantly lower levels of IL-23 expression by lamina propria cells from treated CD11c^{IL-23-} mice than IL-23-sufficient controls at the mRNA (Figure 4.3a) and protein (Figure 4.3b) level, indicating that CD11c⁺ lamina propria cells are the main source of IL-23.

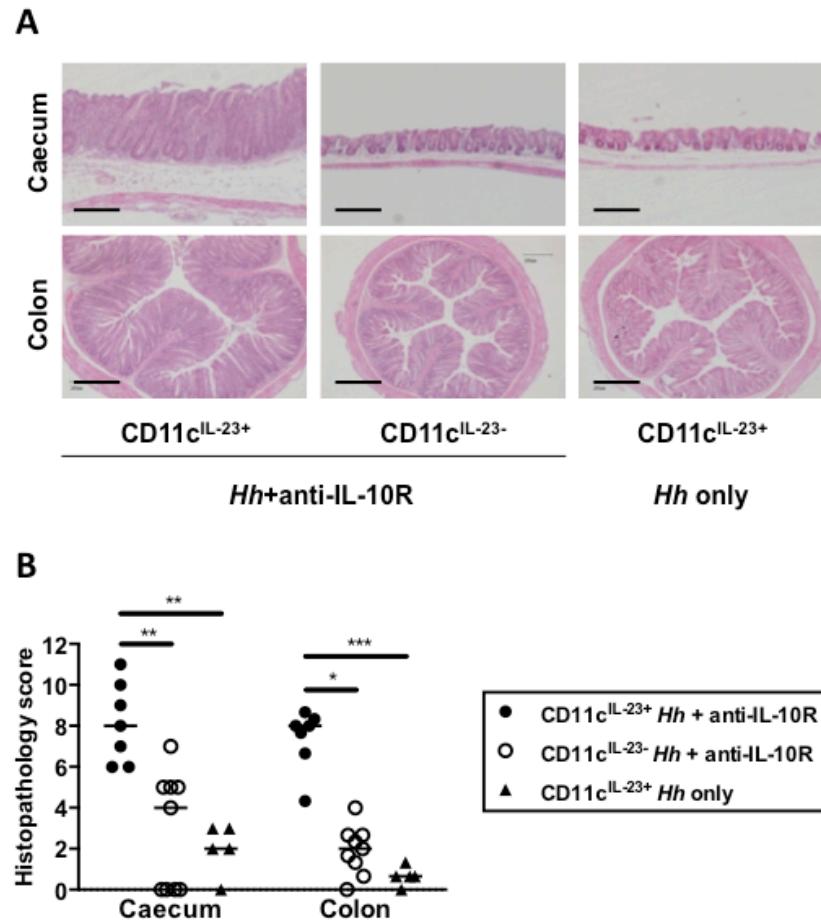


Figure 4.1 CD11c⁺ cell-derived IL-23 drives *Helicobacter hepaticus*-induced colitis.

CD11c-Cre⁺ *Il23a*^{fl/fl} (CD11c^{IL-23-}) mice or pooled CD11c-Cre⁻ *Il23a*^{fl/+} and CD11c-Cre⁻ *Il23a*^{fl/fl} (CD11c^{IL-23+}) littermate controls were infected with *Helicobacter hepaticus* (*Hh*) and treated weekly with anti-IL-10R blocking mAb prior to analysis after three weeks. (A) Representative photomicrographs of haematoxylin and eosin stained sections of caecum and colon. Bar, 200µm. (B) Caecum and colon histopathology scores. Data are pooled from two independent experiments (CD11c^{IL-23+} *Hh*+anti-IL-10R, filled circles, n=7; CD11c^{IL-23-} *Hh*+anti-IL-10R, open circles, n=9; CD11c^{IL-23+} *Hh* only, filled triangles, n=5) and are representative of four experiments (two further experiments performed by Dr Isabelle Arnold). Each data point represents an individual mouse, horizontal line indicates group median. * p<0.05, ** p<0.01, *** p<0.001; as determined by Kruskal-Wallis test with Dunn's post-test correction for multiple comparisons.

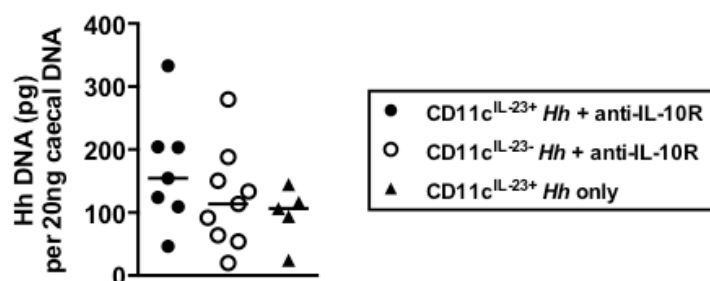


Figure 4.2 *Helicobacter hepaticus* colonisation does not differ between infected CD11c^{IL-23+} and CD11c^{IL-23-} mice.

CD11c-Cre⁺ *Il23a*^{fl/fl} (CD11c^{IL-23+}) mice or pooled CD11c-Cre⁻ *Il23a*^{fl/fl} and CD11c-Cre⁻ *Il23a*^{fl/fl} (CD11c^{IL-23-}) littermate controls were infected with *Helicobacter hepaticus* (*Hh*) and treated weekly with anti-IL-10R blocking mAb prior to analysis after three weeks. *Helicobacter hepaticus* colonisation level was quantified by RT-qPCR performed on caecal DNA using *Hh*-specific primers for the bacterial gene *cdtB*. Data are pooled from two independent experiments (CD11c^{IL-23+} *Hh*+anti-IL-10R, filled circles, n=7; CD11c^{IL-23-} *Hh*+anti-IL-10R, open circles, n=9; CD11c^{IL-23+} *Hh* only, filled triangles, n=5) and are representative of four experiments (two further experiments performed by Dr Isabelle Arnold). Each data point represents an individual mouse, horizontal line indicates group median.

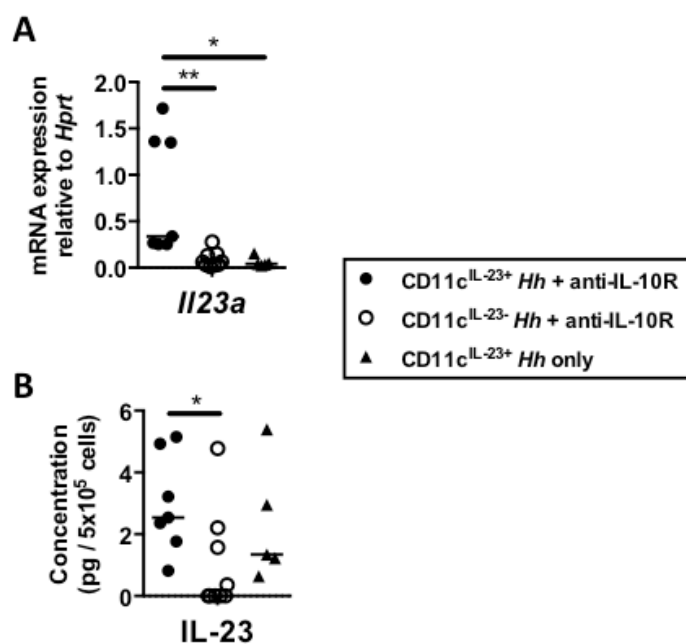


Figure 4.3 CD11c⁺ cells are the major source of IL-23 during *Helicobacter hepaticus* + anti-IL-10R colitis.

CD11c-Cre⁺ *IL23a*^{fl/fl} (CD11c^{IL-23+}) mice or pooled CD11c-Cre⁺ *IL23a*^{fl/+} and CD11c-Cre⁻ *IL23a*^{fl/fl} (CD11c^{IL-23-}) littermate controls were infected with *Helicobacter hepaticus* (*Hh*) and treated weekly with anti-IL-10R blocking mAb prior to analysis after three weeks. Expression of IL-23 was assessed by (A) RT-qPCR on RNA extracted from 500,000 freshly isolated lamina propria cells and (B) ELISA on supernatants from 500,000 *ex vivo* lamina propria cells cultured for 48 hours without stimulation. All data are pooled from two independent experiments (CD11c^{IL-23+} *Hh*+anti-IL-10R, filled circles, n=7; CD11c^{IL-23-} *Hh*+anti-IL-10R, open circles, n=9; CD11c^{IL-23+} *Hh* only, filled triangles, n=5) and are representative of four experiments (two further experiments performed by Dr Isabelle Arnold). Each data point represents an individual mouse, horizontal line indicates group median. * p<0.05; ** p<0.01; ns, not significant; as determined by Kruskal-Wallis test with Dunn's post-test correction for multiple comparisons.

Most models of colitis, including *Hh* + anti-IL-10R, exhibit some symptoms of disease outside the intestine, such as increased spleen weight, wasting disease and/or liver pathology. In mice infected with *Hh* and treated with anti-IL-10R, CD11c^{IL-23+} but not CD11c^{IL-23-} animals exhibited increased spleen weight (Figure 4.4), suggesting that in this model splenomegaly requires IL-23. This is supported by our finding that full IL-23p19 knockout mice also do not exhibit splenomegaly following *Hh* + anti-IL-10R treatment (Dr Philip Ahern, unpublished results).

Inflammatory cytokine levels in intestinal tissue correlate well with tissue pathology. We tested for protein expression of a range of cytokines using a cytometric bead assay. In agreement with our histological assessment, supernatants of *ex vivo* lamina propria cells cultured without stimulation for 48 hours contained significantly lower concentrations of TNF α , IL-1 β , IL-17A and IFN γ produced by cells from treated CD11c^{IL-23-} mice than from IL-23-sufficient controls (Figure 4.5a). Interestingly, we found that *Il17a* mRNA transcripts were elevated in both CD11c^{IL-23+} and CD11c^{IL-23-} *Hh*-infected, anti-IL-10R-treated mice (Figure 4.5b), suggesting that the lower levels of IL-17A protein might reflect IL-23-dependent post-transcriptional regulation of *Il17a* expression. Although a relationship with IL-23 has not been established, post-transcriptional regulation of IL-17A production by the RNA binding proteins HuR and TFL has been described (Chen et al., 2013; Minagawa et al., 2014). All other measured mRNA levels mirrored those of the corresponding protein (data not shown). We found no differences in IL-22 mRNA or protein expression (Figure 4.5c), indicating that IL-22 expression seems not to be driven by IL-23 during *Hh* + anti-IL-10R colitis, in contrast to colitis driven by *Citrobacter rodentium*.

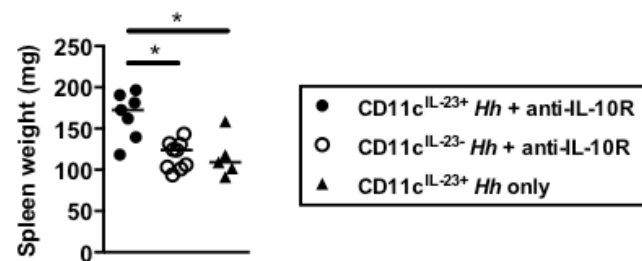


Figure 4.4 Splenomegaly requires CD11c⁺ cell-derived IL-23 during *Helicobacter hepaticus* + anti-IL-10R colitis.

CD11c-Cre⁺ *Il23a*^{fl/fl} (CD11c^{IL-23+}) mice or pooled CD11c-Cre⁻ *Il23a*^{fl/+} and CD11c-Cre⁻ *Il23a*^{fl/fl} (CD11c^{IL-23+}) littermate controls were infected with *Helicobacter hepaticus* (*Hh*) and treated weekly with anti-IL-10R blocking mAb prior to analysis after three weeks. Spleen weight as measured after sacrifice. Data are pooled from two independent experiments (CD11c^{IL-23+} *Hh*+anti-IL-10R, filled circles, n=7; CD11c^{IL-23-} *Hh*+anti-IL-10R, open circles, n=9; CD11c^{IL-23+} *Hh* only, filled triangles, n=5) and are representative of four experiments (two further experiments performed by Dr Isabelle Arnold). Each data point represents an individual mouse, horizontal line indicates group median. * p<0.05, as determined by Kruskal-Wallis test with Dunn's post-test correction for multiple comparisons.

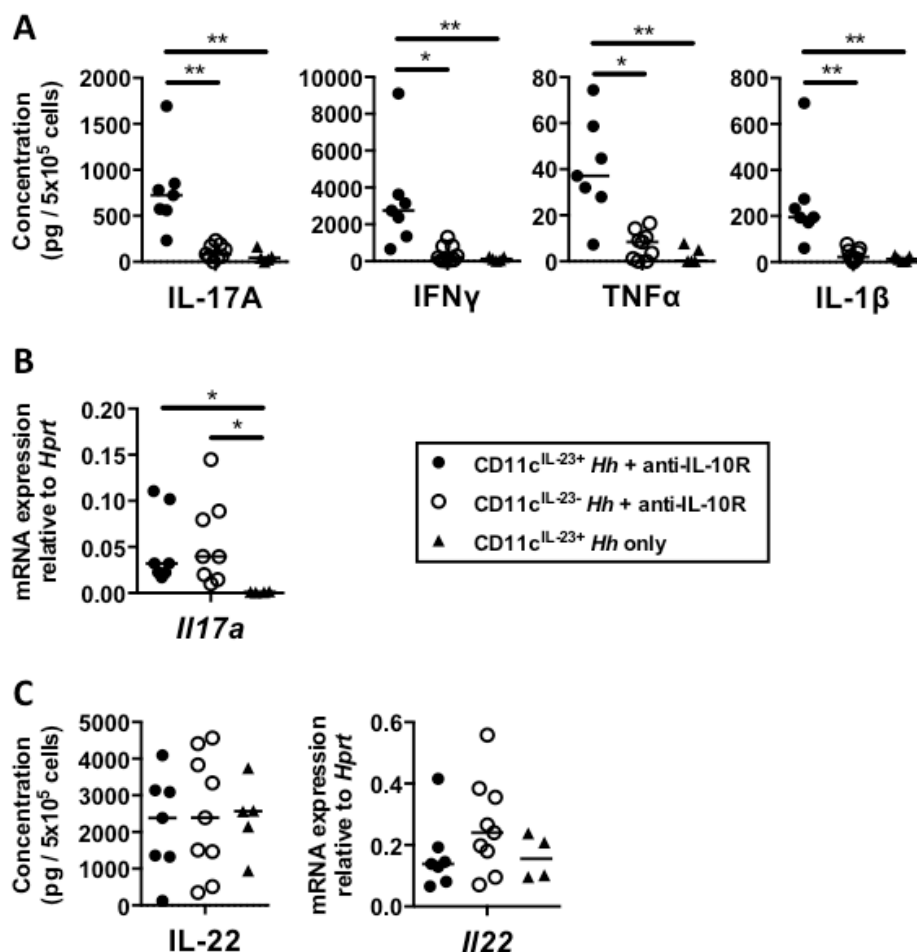


Figure 4.5 CD11c⁺ cell-derived IL-23 is necessary for elevated expression of inflammatory cytokines during *Helicobacter hepaticus* + anti-IL-10R colitis.

CD11c-Cre⁺ *Il23a*^{fl/fl} (CD11c^{IL-23-}) mice or pooled CD11c-Cre⁺ *Il23a*^{fl/+} and CD11c-Cre⁻ *Il23a*^{fl/fl} (CD11c^{IL-23+}) littermate controls were infected with *Helicobacter hepaticus* (*Hh*) and treated weekly with anti-IL-10R blocking mAb prior to analysis after three weeks. Expression of cytokines was assessed by (A) multiplex flow cytometric assay on supernatants from 500,000 *ex vivo* lamina propria cells cultured for 48 hours without stimulation and (B, C) RT-qPCR on 500,000 freshly isolated lamina propria cells. All data are pooled from two independent experiments (CD11c^{IL-23+} *Hh*+anti-IL-10R, filled circles, n=7; CD11c^{IL-23-} *Hh*+anti-IL-10R, open circles, n=9; CD11c^{IL-23+} *Hh* only, filled triangles, n=5) and are representative of four experiments (two further experiments performed by Dr Isabelle Arnold). Each data point represents an individual mouse, horizontal line indicates group median. * p<0.05; ** p<0.01; as determined by Kruskal-Wallis test with Dunn's post-test correction for multiple comparisons.

In a lymphocyte-replete inflammatory setting such as in *Hh* + anti-IL-10R colitis, common sources of IL-17A and IFN γ are Th17 and Th1 cells, respectively. By flow cytometry we observed that, in line with fewer total cells in the lamina propria (Figure 4.6a), *Hh*-infected, anti-IL-10R-treated CD11c^{IL-23-} mice possessed fewer intestinal lamina propria CD4⁺ T cells than did controls (Figure 4.6b). To characterise the CD4⁺ T cell response, we performed intracellular staining for the cytokines IL-17A and IFN γ . Although the pattern of CD4⁺ T cell cytokine expression observed was consistent between the two experiments performed, differences in intracellular cytokine staining intensity lead to generally higher frequencies of cytokine-positive T cells in one of the experiments, hence we decided not to pool the results. Due to the resulting low sample number in Figure 4.6c-d, in addition to comparing all three experimental groups using the Kruskal-Wallis test with Dunn's post-test correction for multiple comparisons, we performed Mann Whitney U tests comparing only CD11c^{IL-23+} and CD11c^{IL-23-} mice treated with *Hh* + anti-IL-10R.

Intracellular staining for IL-17A and IFN γ revealed lower frequencies of IFN γ ⁺ single-positive and IL-17A⁺ IFN γ ⁺ double-positive CD4⁺ T cells in *Hh*-infected, anti-IL-10R-treated CD11c^{IL-23-} mice, compared with colitic CD11c^{IL-23+} controls (Figure 4.6c-d). Interestingly, the frequency of IL-17A⁺ single-positive CD4⁺ T cells in *Hh* + anti-IL-10R-treated CD11c^{IL-23-} mice exhibited only a non-significant trend towards reduction, suggesting that IL-17A⁺ CD4⁺ T cells were less affected by reduced levels of IL-23 than were IL-17A⁺ IFN γ ⁺ double-positive and IFN γ ⁺ single-positive populations.

We also examined the myeloid populations present in the lamina propria, both as correlates of disease and as potential sources of IL-23. As the genetic construct used to create the mice expressing Cre recombinase under the control of the *Cd11c* promoter also included GFP, we could not cross these mice to the CX₃CR1-GFP line used previously

to identify myeloid cells in the intestine. In the absence of CX₃CR1-GFP to identify MNP populations, we devised a new flow cytometry staining panel incorporating recent developments from our own unpublished observations and the literature. This gating strategy is illustrated using a representative sample from an *Hh*-infected, anti-IL-10R-treated CD11c^{IL-23+} mouse in Figure 4.7. Following exclusion of debris and doublets by size, and selection of live (viability dye⁻) CD45⁺ cells, neutrophils were excluded as CD11b^{hi} Ly6G⁺ cells (CD45⁺ CD11b^{hi} Ly6G⁺, population 1, Figure 4.7). The remaining cells were divided on the basis of CD11b expression. The CD11b⁻ fraction was gated on CD11c⁺ MHCII⁺ cells, which were analysed for expression of CD103 to identify CD103⁺ CD11b⁻ DCs (CD45⁺ Ly6G⁻ CD11b⁻ CD11c⁺ MHCII⁺ CD103⁺, population 2, Figure 4.7). The CD11b⁺ fraction was further gated on the basis of size and granularity (forward scatter (FSC) vs. side scatter (SSC)) to exclude SSC^{hi} eosinophils, then examined for Ly6C and MHCII expression, where a “waterfall” of differentiating monocytes/macrophages can be seen (Bain et al., 2013; Tamoutounour et al., 2012). Ly6C^{hi} MHCII⁻ monocytes (CD45⁺ Ly6G⁻ CD11b⁺ SSC^{low} Ly6C^{hi} MHCII⁻, population 3, Figure 4.7) and Ly6C^{hi} MHCII⁺ MNPs (CD45⁺ Ly6G⁻ CD11b⁺ SSC^{low} Ly6C^{hi} MHCII⁺, population 4, Figure 4.7) were identified at this stage. Ly6C^{lo/-} MHCII⁺ cells were then divided on the basis of expression of CD64 or F4/80 and CD11c, as shown previously (Bain et al., 2013), into CD64⁺ F4/80⁺ macrophages, which also express variable levels of CD11c (CD45⁺ Ly6G⁻ CD11b⁺ SSC^{low} Ly6C^{lo/-} MHCII⁺ CD64⁺ F4/80⁺, population 5, Figure 4.7), and CD64⁻ F4/80⁻ CD11c⁺ MNPs. The main difference between these gating strategies is that in the absence of CX₃CR1-GFP, this macrophage gate contains both CX₃CR1^{int} and CX₃CR1^{hi} Ly6C^{lo/-} macrophages (Bain et al., 2013; Tamoutounour et al., 2012). The CD64⁻ F4/80⁻ CD11c⁺ population contains CD103⁺ CD11b⁺ DCs (CD45⁺ Ly6G⁻ CD11b⁺ SSC^{low} Ly6C^{lo/-} MHCII⁺ CD64⁻ F4/80⁻ CD11c⁺ CD103⁺,

population 6, Figure 4.7) and CD103⁻ CD11b⁺ DCs (CD45⁺ Ly6G⁻ CD11b⁺ SSC^{low} Ly6C^{lo/-} MHCII⁺ CD64⁻ F4/80⁻ CD11c⁺ CD103⁻, population 7, Figure 4.7).

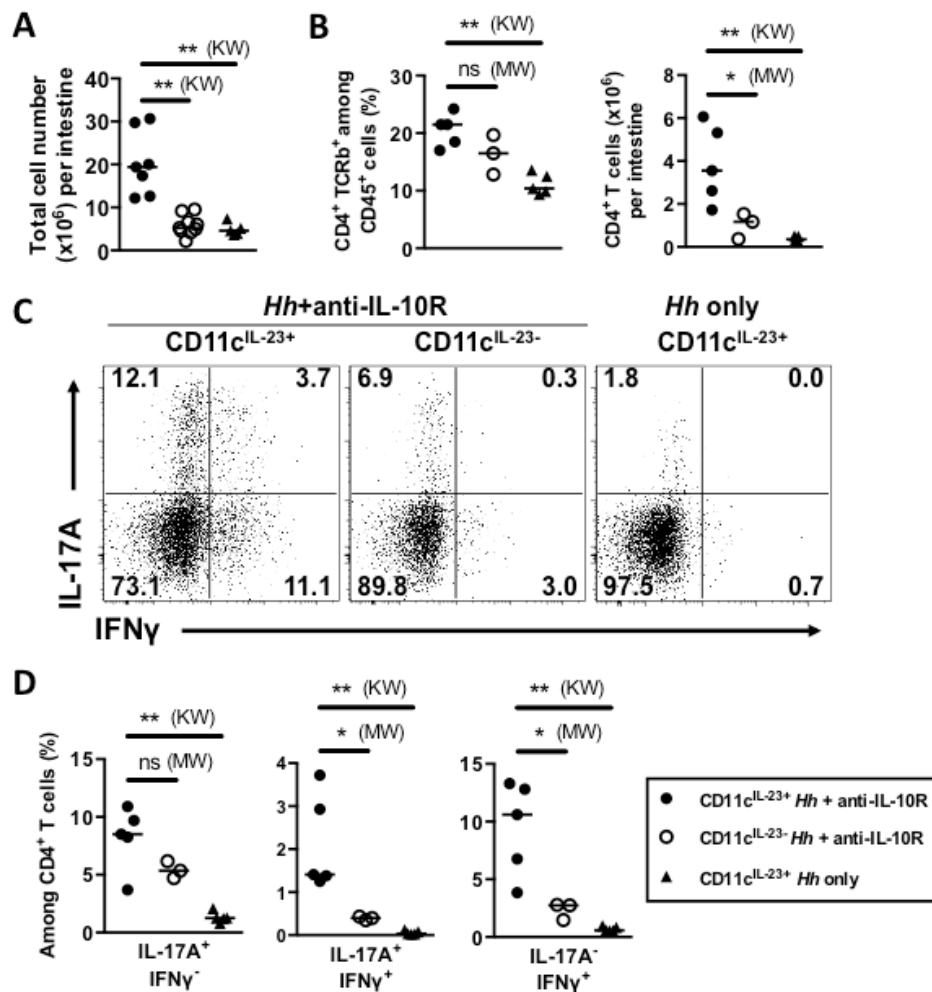


Figure 4.6 CD11c⁺ cell-derived IL-23 is required for the emergence of pathogenic CD4⁺ T cells during *Helicobacter hepaticus* + anti-IL-10R colitis.

CD11c-Cre⁺ *Il23a*^{fl/fl} (CD11c^{IL-23-}) mice or pooled CD11c-Cre⁻ *Il23a*^{fl/fl} and CD11c-Cre⁻ *Il23a*^{fl/fl} (CD11c^{IL-23+}) littermate controls were infected with *Helicobacter hepaticus* (*Hh*) and treated weekly with anti-IL-10R blocking mAb prior to analysis after three weeks. Single cell suspensions of combined caecal and colonic lamina propria from individual mice were prepared. (A) Total number of viable lamina propria cells per mouse. (B-D) Cells were stained and analysed by flow cytometry. (B) Frequency among CD45⁺ cells and absolute number of CD4⁺ T cells. (C and D) Intracellular cytokine staining was performed after 3.5h PMA/ionomycin/Brefeldin A stimulation of 500,000 lamina propria cells. Representative FACS plots (C; pre-gated on single live CD45⁺ CD4⁺ TCR β ⁺ cells) and frequencies (D) of IL-17A⁺ IFN γ ⁻, IL-17A⁺ IFN γ ⁺ and IL-17A⁻ IFN γ ⁺ CD4⁺ T cells. Numbers in quadrants represent frequencies among parent population. Data are pooled from two independent experiments (A; CD11c^{IL-23+} *Hh*+anti-IL-10R, filled circles, n=7; CD11c^{IL-23-} *Hh*+anti-IL-10R, open circles, n=9; CD11c^{IL-23+} *Hh* only, filled triangles, n=5) or are from one experiment representative of two independent experiments (B-D; CD11c^{IL-23+} *Hh*+anti-IL-10R, filled circles, n=5; CD11c^{IL-23-} *Hh*+anti-IL-10R, open circles, n=3; CD11c^{IL-23+} *Hh* only, filled triangles, n=5). Two further experiments performed by Dr Isabelle Arnold showed similar results. Each data point represents an individual mouse, horizontal line indicates group median. * p<0.05, ** p<0.01, as determined by Kruskal-Wallis test with Dunn's post-test correction for multiple comparisons or Mann Whitney U test as indicated.

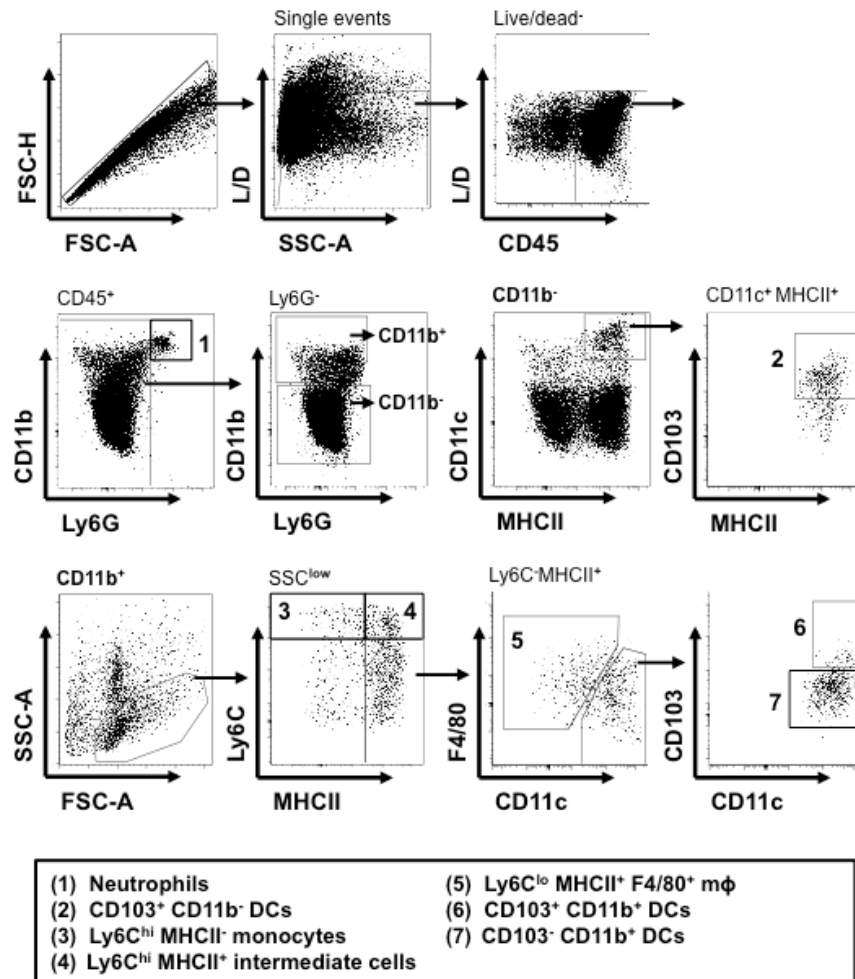


Figure 4.7 FACS gating strategy for myeloid populations in the caecal and colonic lamina propria.

Full gating strategy used to identify neutrophils (1), CD103⁺ CD11b⁻ DCs (2), Ly6C^{hi} MHCII⁻ monocytes (3), Ly6C^{hi} MHCII⁺ intermediate cells (4), Ly6C^{lo} MHCII⁺ F4/80⁺ macrophages (5), CD103⁺ CD11b⁺ DCs (6) and CD103⁻ CD11b⁺ DCs (7) by flow cytometric analysis of stained single cell preparations of combined caecal and colonic lamina propria from individual mice. Example from a representative CD11c^{IL-23+} mouse infected with *Hh* and treated with anti-IL-10R for three weeks.

It has recently been shown that monocyte-derived MNPs in the steady state lamina propria develop from Ly6C^{hi} monocytes, through a Ly6C⁺ MHCII⁺ intermediate, into macrophages and possibly DCs (Bain et al., 2013; Rivollier et al., 2012; Zigmond et al., 2012). During inflammation, this developmental process is halted at the Ly6C⁺ MHCII⁺ intermediate stage, resulting in accumulation of these cells in the inflamed intestine. The combination of comparing three groups using the non-parametric Kruskal-Wallis test, which was deemed most appropriate since not all data appeared to be normally distributed, and relatively low sample sizes, may have affected the statistical significance of some of our results. Therefore, as above, we also used the Mann Whitney U test to compare only CD11c^{IL-23+} and CD11c^{IL-23-} mice treated with *Hh* + anti-IL-10R. We saw increased frequencies of Ly6C⁺ MHCII⁺ MNPs and the Ly6C^{hi} MHCII⁻ monocytes from which they derive in the colonic and caecal lamina propria during intestinal inflammation in IL-23-sufficient mice, which was abrogated in CD11c^{IL-23-} mice (Figure 4.8a-b). Whether IL-23 drives monocyte recruitment directly, or via inflammatory pathways downstream of IL-23, would require further investigation. Among other myeloid cells associated with intestinal inflammation, we also found that the frequency of neutrophils was partially reduced in CD11c^{IL-23-} mice following treatment with *Hh* + anti-IL-10R (Figure 4.8c).

In contrast to cell types associated with inflammation, frequencies of Ly6C^{lo} F4/80⁺ macrophages and CD103⁺ CD11b⁺ DCs, both of which are found in the steady state lamina propria, were unaffected in *Hh* + anti-IL-10R-treated CD11c^{IL-23-} mice (Figure 4.8d-e). There did appear to be a reduction in the frequency of CD103⁺ CD11b⁻ DCs in CD11c^{IL-23-} mice following *Hh* + anti-IL-10R treatment relative to CD11c^{IL-23+} *Hh*-infected control mice, however no significant difference was observed between CD11c^{IL-23+} and CD11c^{IL-23-} mice treated with *Hh* + anti-IL-10R (Figure 4.8e). Other studies indicate that there is no defect

in CD103⁺ CD11b⁻ DC frequency in uninfected CD11c^{IL-23⁻} versus CD11c^{IL-23⁺} mice (Dr Isabelle Arnold, unpublished results). A three-way comparison did not show any effect of CD11c-Cre-driven IL-23 deficiency on CD103⁻ CD11b⁺ DC frequency following *Hh* + anti-IL-10R treatment, which elevated their frequency in IL-23-sufficient mice (Figure 4.8f). However, a comparison of only CD11c^{IL-23⁺} and CD11c^{IL-23⁻} mice treated with *Hh* + anti-IL-10R suggested a partial reduction in the frequency of these cells in CD11c^{IL-23⁻} mice (Figure 4.8f).

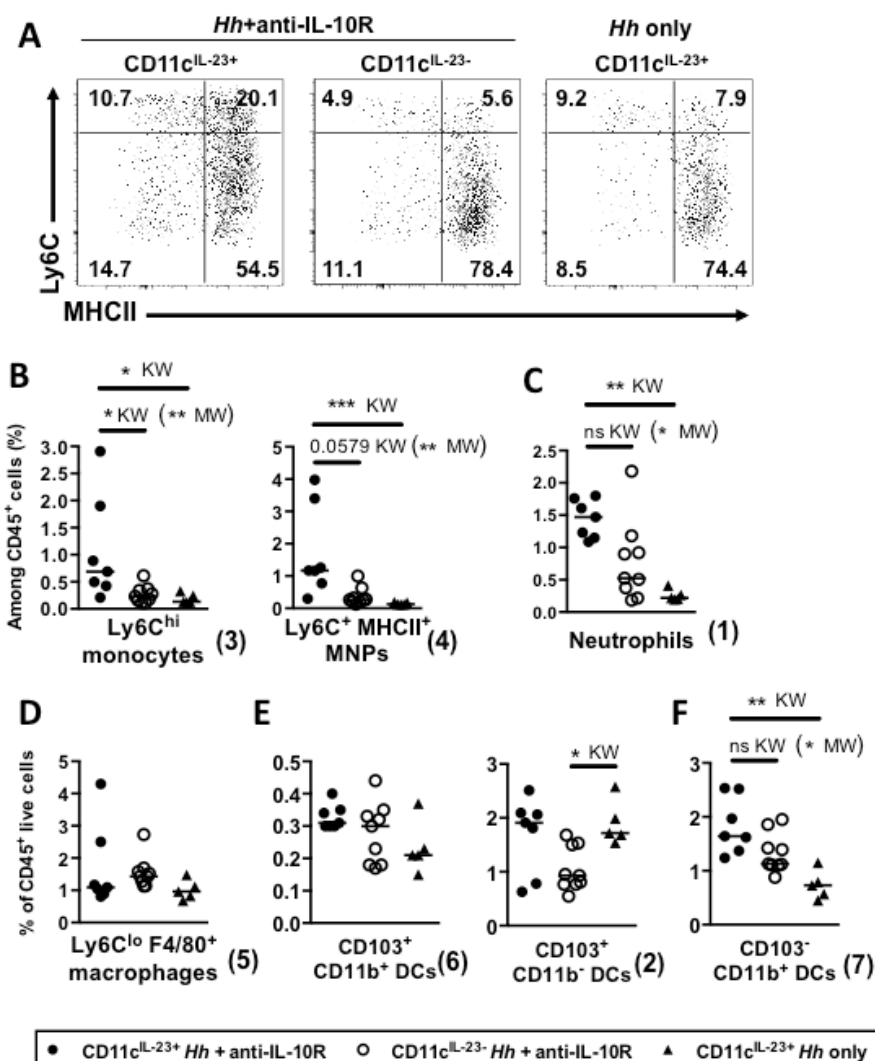


Figure 4.8 CD11c⁺ cell-derived IL-23 controls intestinal inflammatory myeloid cell influx.

CD11c-Cre⁺ *Il23a*^{fl/fl} (CD11c^{IL-23-}) mice or pooled CD11c-Cre⁺ *Il23a*^{fl/+} and CD11c-Cre⁻ *Il23a*^{fl/fl} (CD11c^{IL-23+}) littermate controls were infected with *Helicobacter hepaticus* (*Hh*) and treated weekly with anti-IL-10R blocking mAb prior to analysis after three weeks. Single cell suspensions of combined caecal and colonic lamina propria from individual mice were prepared, stained and analysed by flow cytometry. (A) Representative FACS plots (pre-gated on single live CD45⁺ Ly6G⁻ CD11b⁺ SSC^{low} cells as per Figure 4.7) and (B) frequencies of Ly6C^{hi} MHCII⁺ monocytes and Ly6C^{hi} MHCII⁺ MNPs among CD45⁺ live cells. (C-F) Frequency of neutrophils (C), Ly6C^{lo} MHCII⁺ F4/80⁺ macrophages (D), CD103⁺ CD11b⁺ and CD103⁺ CD11b⁻ DCs (E) and CD103⁻ CD11b⁺ DCs (F) among CD45⁺ live cells, gated as per Figure 4.7. Numbers in quadrants in (A) represent frequency among parent population, numbers in brackets in (B-F) denote population number from gating strategy. Data are pooled from two independent experiments (CD11c^{IL-23+} *Hh* + anti-IL-10R, filled circles, n=7; CD11c^{IL-23-} *Hh* + anti-IL-10R, open circles, n=9; CD11c^{IL-23+} *Hh* only, filled triangles, n=5). Each data point represents an individual mouse, horizontal line indicates group median. * p<0.05, ** p<0.01, *** p<0.001 as determined by Kruskal-Wallis test with Dunn's post-test correction for multiple comparisons or Mann Whitney U test as indicated.

4.2.2 Expression of IL-23 by MNP subsets during *Hh* + anti-IL-10R colitis

We sought to determine which specific population or populations of cells expressing CD11c might be the source of IL-23 in this model. To be a relevant source of IL-23 affected by our experimental system, cells must express both CD11c-Cre and IL-23. First, we examined CD11c-Cre-GFP expression to determine which cellular populations should be affected by the conditional gene knockout. At 3 weeks post-infection, CD11c-Cre-GFP expression was highest in CD103⁺ DCs, but was also present in a proportion of Ly6C^{lo} F4/80⁺ macrophages and Ly6C^{hi} MHCII⁺ MNPs, correlating with the relative CD11c expression of these populations (Figure 4.9; (Bain et al., 2013)).

We then sought to determine which of these populations expressed IL-23 during inflammation. First we analysed IL-23 expression in gross colonic tissue across a timecourse of the *Hh* + anti-IL-10R model using RT-qPCR to establish the optimal time for harvesting the myeloid cells. As *Il23a* mRNA levels peaked between days 3 and 5 of colitis (Figure 4.10), we sorted cell subsets from mice 5 days after *Hh* infection and treatment with anti-IL-10R mAb.

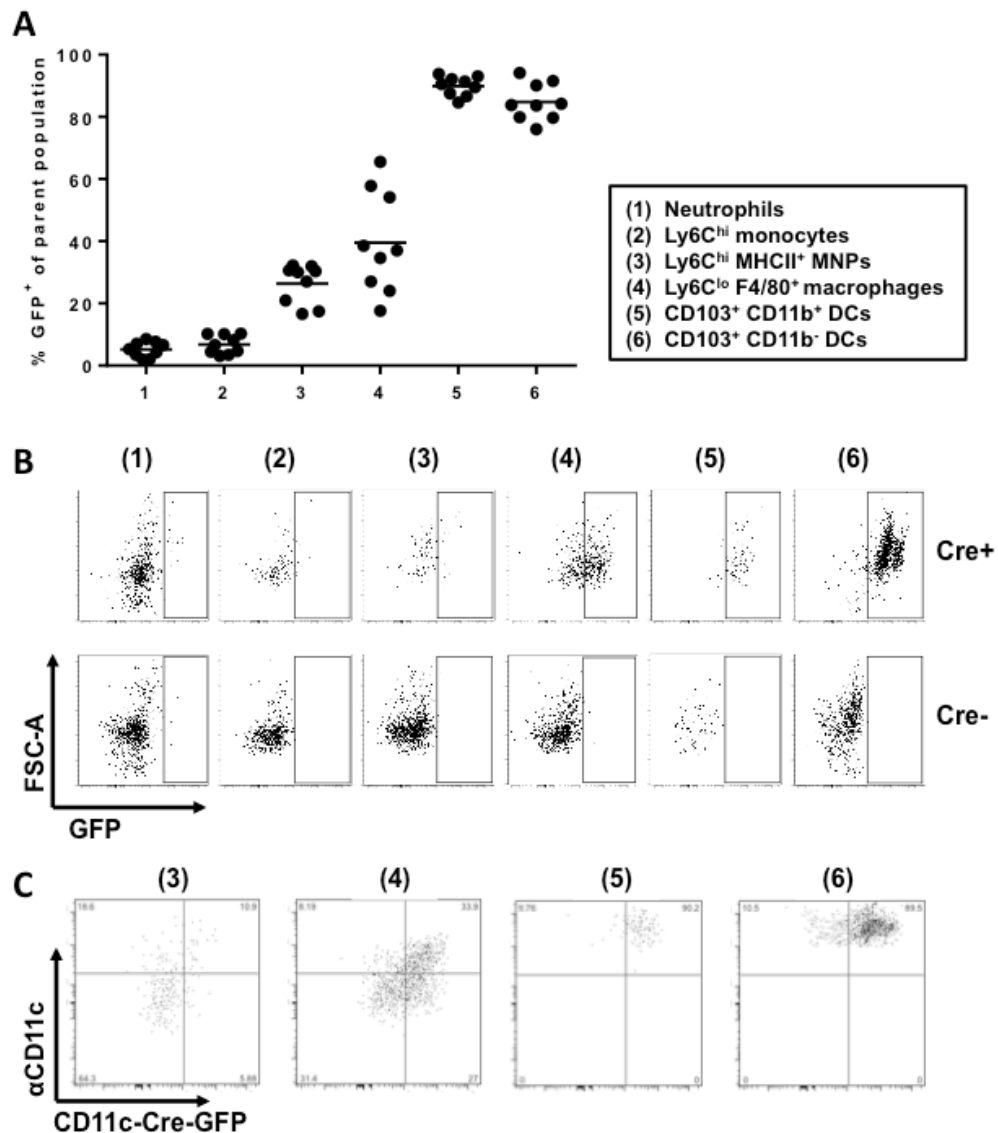


Figure 4.9 CD11c-Cre-associated GFP expression by intestinal myeloid populations.

CD11c-Cre⁺ *Il23a^{fl/fl}* (CD11c^{IL-23^{-/-}) mice were infected with *Helicobacter hepaticus* (*Hh*) and treated weekly with anti-IL-10R blocking mAb prior to analysis three weeks post-infection. Single cell suspensions of combined caecal and colonic lamina propria from individual mice were prepared, stained and analysed by flow cytometry. (A) Frequency of CD11c-Cre-GFP⁺ cells in each population (gated as per Figure 4.7). Data are pooled from two independent experiments (CD11c^{IL-23^{-/-} *Hh*+anti-IL-10R, n=9). Each data point represents an individual mouse, horizontal line indicates group mean. A further experiment performed by Dr Isabelle Arnold showed similar results. (B and C) Representative FACS plots showing CD11c-Cre-GFP versus (B) FSC and (C) CD11c expression by each population. (B) Top row and (C) show plots from a CD11c-Cre⁺ (CD11c^{IL-23^{-/-}) mouse treated with *Hh*+anti-IL-10R; (B) bottom row shows plots from a CD11c-Cre⁻ (CD11c^{IL-23^{+/+}) mouse treated with *Hh*+anti-IL-10R which were used to set CD11c-Cre-GFP⁺ gates for each population.}}}}

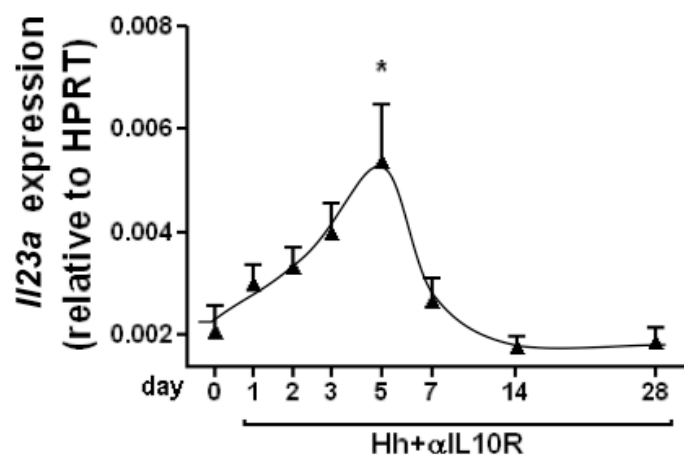


Figure 4.10 IL-23 gene expression during *Hh* + anti-IL-10R colitis

RT-qPCR analysis of *Il23a* gene expression in colonic tissue collected from 5 individual mice at each indicated time point. Data are plotted as mean + SD. * $p < 0.05$, as determined by Mann-Whitney U test relative to day 0. Graph produced by Dr Isabelle Arnold.

We were able to sort four populations per sample (Table 4.1; see Figure 4.7 for gating strategy, however we used Siglec F rather than SSC to exclude eosinophils, and CD64 in place of F4/80 to identify macrophages, as described in Chapter 3). We sorted both populations of CD103⁺ DCs – CD11b⁺ and CD11b⁻ – despite the paucity of the former population in the colon, which contains ten-fold more CD11b⁻ than CD11b⁺ CD103⁺ DCs (Figure 4.8e), since CD103⁺ CD11b⁺ DCs have been reported to produce IL-23 (Kinnebrew et al., 2012; Satpathy et al., 2013; Schlitzer et al., 2013). We also sorted the Ly6C⁺ MHCII⁺ MNPs, which accumulate within 16 hours of *Hh* infection and anti-IL-10R treatment (Dr Isabelle Arnold, unpublished results). The final population sorted was macrophages, expressing CD64 and only low levels of, if any, Ly6C. This population would have included both CX₃CR1^{hi} and CX₃CR1^{int} Ly6C^{lo/-} macrophages. As we were limited to four populations we were unable to include CD103⁻ CD11b⁺ DCs, some of which express intermediate levels of CX₃CR1 and may be pro-inflammatory (Cerovic et al., 2013; Scott et al., 2014). Table 4.1 lists the sorted populations and the cell surface marker expression used to define and isolate them. Eosinophils, another CD11b⁺ myeloid cell population which accumulates during colitis (Bain et al., 2013), can be partially identified by high SSC during flow cytometry analysis (Thurau et al., 1996), hence our selection of SSC^{low} cells during gating for MNP populations (Figure 4.7). For this experiment we included an additional antibody specific for Siglec F, a murine eosinophil marker (Zhang et al., 2004), to allow these cells to be definitively excluded from our sorted populations.

Population	Cell surface marker expression
CD103 ⁺ CD11b ⁺ DCs	CD45 ⁺ Ly6G ⁻ CD11b ⁺ Siglec F ⁻ Ly6C ^{neg-lo} MHCII ⁺ CD64 ⁻ CD11c ⁺ CD103 ⁺
CD103 ⁺ CD11b ⁻ DCs	CD45 ⁺ Ly6G ⁻ CD11b ⁻ Siglec F ⁻ Ly6C ^{neg-lo} MHCII ⁺ CD64 ⁻ CD11c ⁺ CD103 ⁺
Ly6C ⁺ MHCII ⁺ MNPs	CD45 ⁺ Ly6G ⁻ CD11b ⁺ Siglec F ⁻ Ly6C ⁺ MHCII ⁺
CD64 ⁺ macrophages	CD45 ⁺ Ly6G ⁻ CD11b ⁺ Siglec F ⁻ Ly6C ^{neg-lo} MHCII ⁺ CD64 ⁺

Table 4.1: Cell surface marker expression used to identify populations for sorting from CD11c^{IL-23+} and CD11c^{IL-23-} mice.

When we performed RT-qPCR on these populations sorted from CD11c^{IL-23+} mice 5 days post-*Hh* + anti-IL-10R we found expression of *Il23a* only in the monocyte-derived MNP populations – the Ly6C⁺ MHCII⁺ MNPs and Ly6C^{neg-lo} CD64⁺ macrophages – and this was reduced in cells from CD11c^{IL-23-} mice (Figure 4.11a), indicating CD11c-Cre activity in these populations. These results are representative of a further experiment performed by Dr Isabelle Arnold, which found statistically significant reductions in *Il23a* expression by Ly6C⁺ MHCII⁺ MNPs and Ly6C^{neg-lo} macrophages sorted from CD11c^{IL-23-} mice relative to CD11c^{IL-23+} mice.

Macrophages and dendritic cells are best distinguished by differential expression of CD64 (Gautier et al., 2012; Tamoutounour et al., 2012) and can be further subdivided by levels of CX₃CR1 expression (Bain et al., 2013; Rivollier et al., 2012; Weber et al., 2011; Zigmond et al., 2012). Therefore, having confirmed expression of CD11c-Cre in CD11c-Cre-GFP mice (Figure 4.9), we turned to CX₃CR1^{GFP/+} mice, which are essentially wild-type, to extend our results. We sorted CX₃CR1^{int} CD64⁺ MNPs, which include Ly6C^{hi} MHCII⁺ MNPs and Ly6C^{lo/-} MHCII⁺ macrophages; CX₃CR1^{int} CD64⁻ MNPs, which include DCs; and CX₃CR1^{hi} CD64⁺ macrophages from CX₃CR1^{GFP/+} mice 5 days after *Hh* infection and anti-IL-10R treatment (see Figure 3.4 for gating strategy and Figure 3.5 for analysis of contents of

CX₃CR1^{int} CD64⁺ and CX₃CR1^{int} CD64⁻ MNP populations). We found that among these populations, CX₃CR1^{hi} macrophages preferentially expressed *Il23a*, while CX₃CR1^{int} CD64⁺ MNPs expressed low levels (Figure 4.11b). We then determined *Il23a* mRNA levels in cells sorted from CX₃CR1^{GFP/+} mice at day 21 post-*Hh* + anti-IL-10R treatment to determine whether the source of IL-23 varies throughout the course of disease. At this timepoint, CX₃CR1^{hi} macrophages continued to express *Il23a*, though now at levels similar to CX₃CR1^{int} CD64⁺ MNPs, which accumulate during the course of disease (Figures 3.3 and 3.5) and maintained *Il23a* expression (Figure 4.11c), suggesting a role for the latter inflammation-associated subset in perpetuating pathology.

Notably, we found no expression of *Il23a* by either CD103⁺ DC population (Figure 4.11a). In the large intestinal lamina propria, the majority of CD103⁺ DCs are CD11b⁻. CD8α⁺ and CD103⁺ CD11b⁻ DCs require the transcription factor BATF3 for their development and are therefore absent from *Batf3*^{-/-} mice (Hildner et al., 2008) (Figure 4.12a). We found that these mice developed *Hh* + anti-IL-10R colitis in exactly the same way as wild-type controls, confirming that CD103⁺ CD11b⁻ DCs are not a source of the IL-23 needed for *Hh* + anti-IL-10R colitis (Figure 4.12b-c; experiments and analysis performed by Dr Isabelle Arnold).

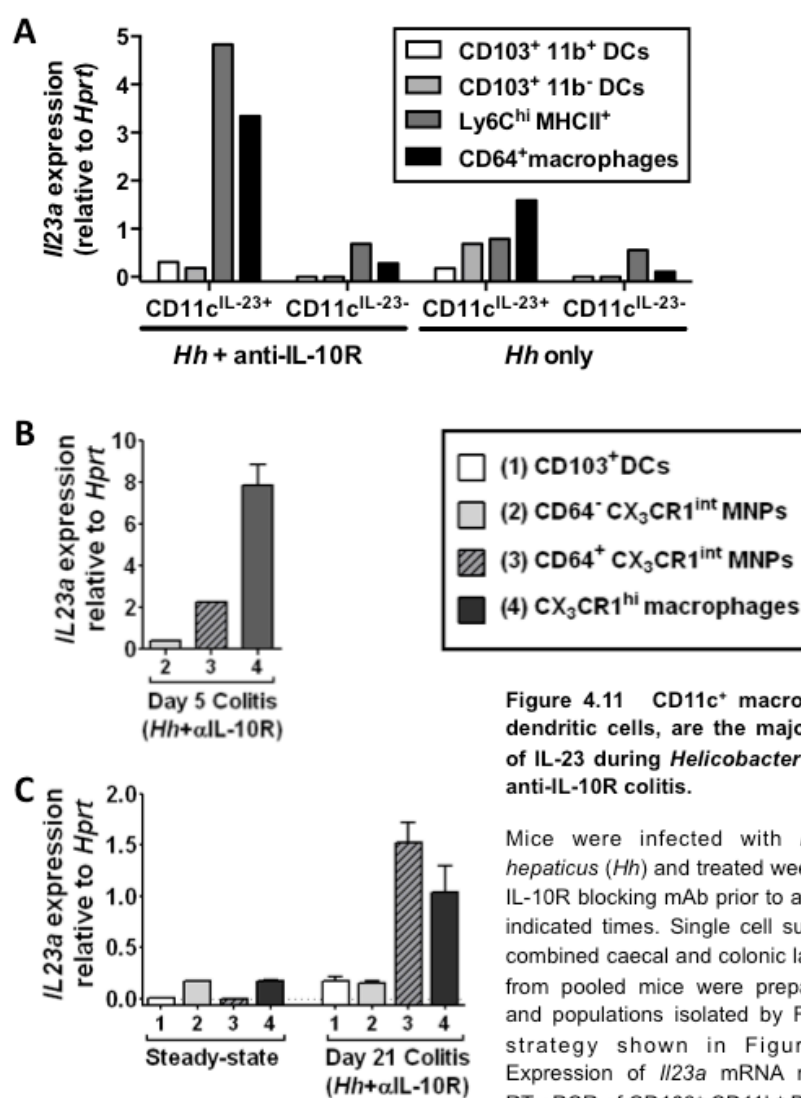


Figure 4.11 CD11c⁺ macrophages, not dendritic cells, are the major producers of IL-23 during *Helicobacter hepaticus* + anti-IL-10R colitis.

Mice were infected with *Helicobacter hepaticus* (*Hh*) and treated weekly with anti-IL-10R blocking mAb prior to analysis at the indicated times. Single cell suspensions of combined caecal and colonic lamina propria from pooled mice were prepared, stained and populations isolated by FACS. Gating strategy shown in Figure 4.7. (A) Expression of *IL23a* mRNA measured by RT-qPCR of CD103⁺ CD11b⁺ DCs, CD103⁺

CD11b⁻ DCs, Ly6C^{hi} MHCII⁺ cells and CD64⁺ macrophages sorted from CD11c-Cre⁺ *IL23a*^{fl/fl} (CD11c^{IL-23+}) mice or pooled CD11c-Cre⁺ *IL23a*^{fl/+} and CD11c-Cre⁺ *IL23a*^{fl/fl} (CD11c^{IL-23+}) littermate controls five days after *Hh* + anti-IL-10R treatment. One biological replicate of each population was sorted from lamina propria pooled from 5-6 mice per group. Data represent the mean of two technical replicates. A further experiment performed by Dr Isabelle Arnold showed similar results. (B-C) Expression of *IL23a* mRNA measured by RT-qPCR of populations sorted from CX₃CR1^{GFP/+} mice. Single cell suspensions were MACS enriched for CD11b⁺ and CD11c⁺ subsets prior to isolation by FACS. Populations were pre-gated on single live CD45⁺ MHCII⁺ cells and sorted using the following markers: (1) CD11c⁺ MHCII⁺ CX₃CR1⁻ CD103⁺ DCs, (2) CX₃CR1^{int} CD64⁺, (3) CX₃CR1^{int} CD64⁺ (4) CX₃CR1^{hi} CD64⁺ as described in Figure 3.4. Cells were sorted from pools of 4-13 mice per group. Number of biological replicates per timepoint: (B) *Hh* + anti-IL-10R day 5, n=2; (C) steady state, n=1; *Hh* + anti-IL-10R day 21, n=3, pooled from two independent experiments. Data are plotted as mean + SEM.

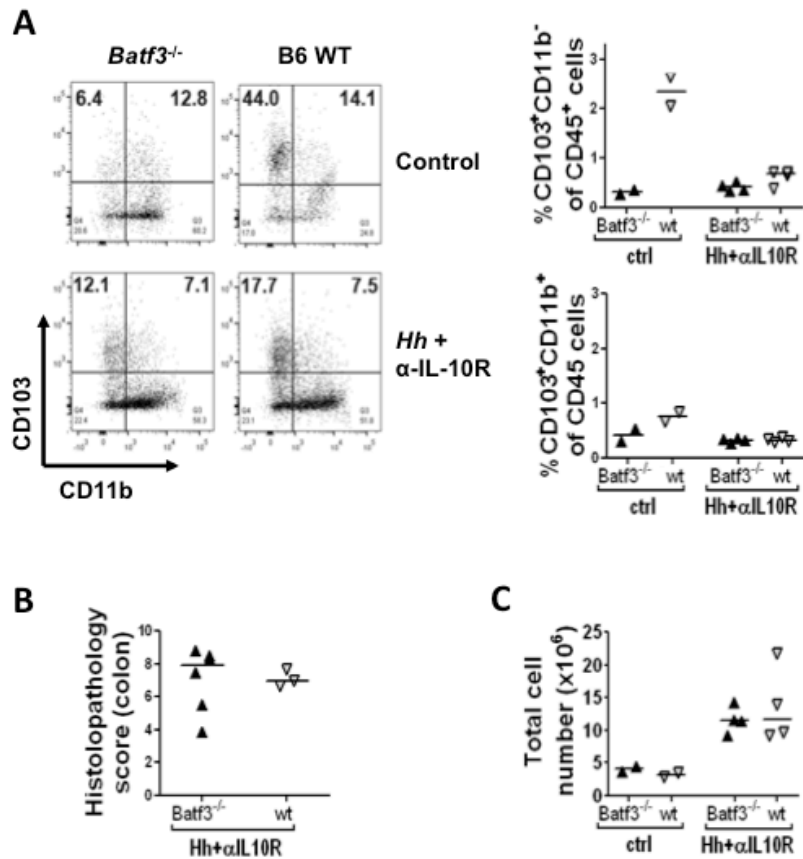


Figure 4.12 CD103⁺ CD11b⁻ dendritic cells are not required for pathology during *Helicobacter hepaticus* + anti-IL-10R colitis.

Batf3^{-/-} mice were infected with *Helicobacter hepaticus* (*Hh*) and treated weekly with anti-IL-10R blocking mAb prior to analysis three weeks post-infection. Single cell suspensions of combined caecal and colonic lamina propria from individual mice were prepared, stained and analysed by flow cytometry. (A) Representative FACS plots and frequencies of indicated subsets. All populations were pre-gated on single live CD45⁺ MHCII⁺ CD11c⁺ cells. Numbers in quadrants indicate frequency among parent population. (B) Colon histopathology scores. (C) Total number of viable lamina propria cells per mouse. Data are representative of two independent experiments. Horizontal line represents group median. Experiments were performed and analysed by Dr Isabelle Arnold.

Once we had identified the source of IL-23, we made a preliminary examination of the contributions of *Hh* and anti-IL-10R in inducing its expression *in vivo*. Early work on *Hh* + anti-IL-10R colitis reported that at the whole colon level, *Il23a* transcripts were induced equally by *Hh* infection with or without concomitant anti-IL-10R treatment (Kullberg et al., 2006). On one occasion, we sorted MNP populations from the lamina propria of IL-23-sufficient mice which were uninfected, infected with *Hh*, or infected with *Hh* and treated with anti-IL-10R, five days post-infection, which is prior to the development of robust intestinal pathology (Figure 3.1). These preliminary results suggest that IL-23 expression might be induced in Ly6C^{neg-lo} CD64⁺ macrophages by *Hh* infection alone, although expression of IL-23 was enhanced in the additional absence of IL-10 signalling (Figure 4.13). Consistent with the hypothesis that they become an important IL-23-producing cell population as disease develops, Ly6C⁺ MHCII⁺ MNPs appeared only to express IL-23 during *Hh* infection with concomitant anti-IL-10R treatment (Figure 4.13). These results indicate that IL-10 limits expression of IL-23 in monocyte-derived intestinal MNPs.

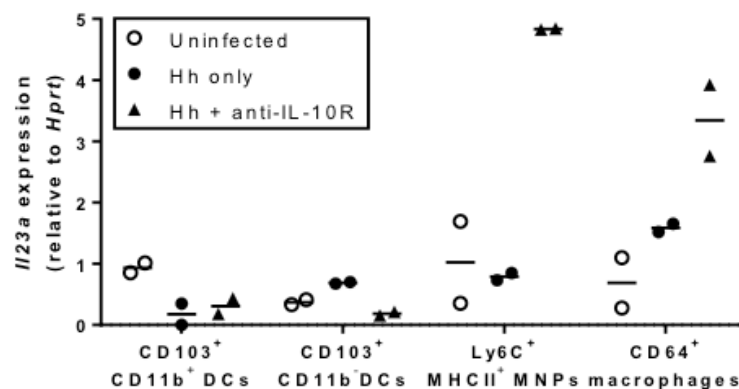


Figure 4.13 Induction of IL-23 expression by *Helicobacter hepaticus* and anti-IL-10R treatment.

Expression of *Il23a* measured by RT-qPCR performed on sorted CD103⁺ CD11b⁺ DCs, CD103⁺ CD11b⁻ DCs, Ly6C^{hi} MHCII⁺ MNPs and CD64⁺ macrophages. CD11c-Cre⁻ *Il23a*^{fl/+} and *Il23a*^{fl/fl} (CD11c^{IL-23+}) mice were infected with *Helicobacter hepaticus* (*Hh*) with or without anti-IL-10R blocking mAb treatment prior to sacrifice 5 days post-infection. Single cell suspensions of combined caecal and colonic lamina propria from pooled mice (n = 5-6 per group) were prepared, stained, and the indicated populations were isolated by FACS. Data are from one experiment; each data point indicates a technical replicate of *Il23a* expression by the indicated population sorted from a pool of 5-6 mice; horizontal bar indicates mean.

As the CX₃CR1^{hi} CD64⁺ macrophages and CX₃CR1^{int} CD64⁺ MNPs which produce IL-23 are both derived from Ly6C^{hi} monocytes (Bain et al., 2013; Rivollier et al., 2012; Zigmond et al., 2012), we attempted to restore disease in CD11c^{IL-23-} mice by adoptive transfer of 2 x 10⁶ IL-23-sufficient Ly6C^{hi} monocytes (CD11c⁻ MHCII⁻ lineage⁻ (lineage markers = CD3, TCRβ, B220, NKp46, c-Kit) Siglec F⁻ Ly6G⁻ CD11b⁺ CD45.1⁺ CD45.2⁺ Ly6C^{hi} CX₃CR1^{int}) sorted from CX₃CR1^{GFP/+} CD45.1⁺ CD45.2⁺ mouse bone marrow. We transferred 1.4 x 10⁶ CD11c⁺ bone marrow cells sorted from the same mice as a positive control. These cells were chosen as they fulfilled two criteria: that they should be bone marrow-derived, as were the Ly6C^{hi} monocytes; and express CD11c, which controlled Cre recombinase-mediated IL-23 deficiency in CD11c^{IL-23-} mice. A population of CD11c⁺ cells also expressing Ly6C and CD31 is found in the bone marrow and peripheral blood of mice, and is capable of giving rise to macrophages and multiple populations of DCs (Bruno et al., 2001). As they would include this multipotent population, CD11c⁺ bone marrow cells seemed to be a suitable positive control for our experiment, which would allow the restoration of many types of MNPs. This preliminary study showed trends toward greater caecal pathology and increased frequency of IL-17A⁺ CD4⁺ T cells in *Hh* + anti-IL-10R-treated CD11c^{IL-23-} mice which received Ly6C^{hi} monocytes compared with those which received no cells (Figure 4.14).

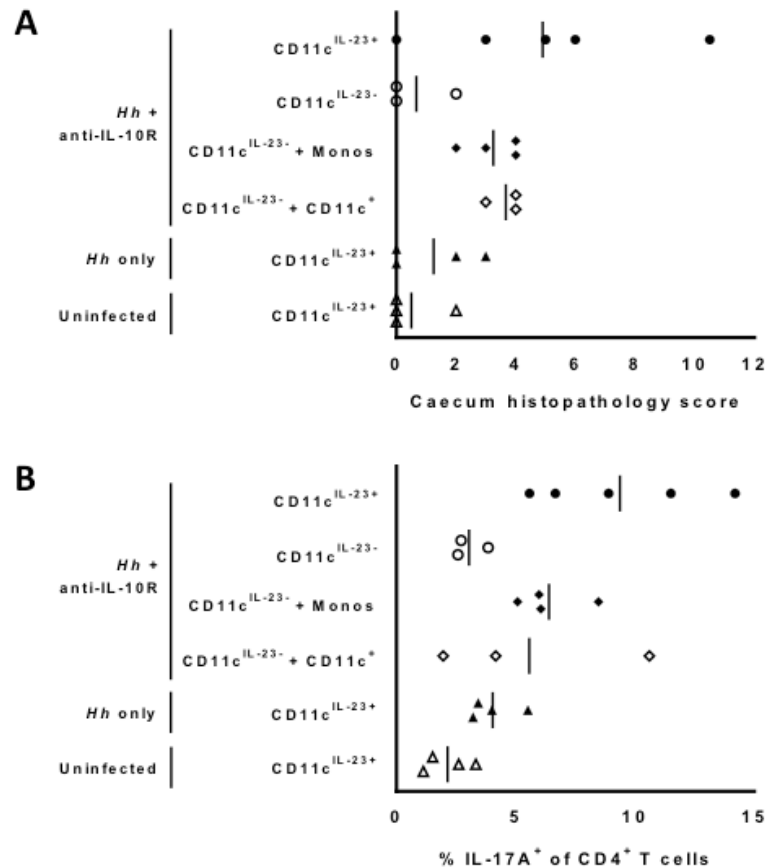


Figure 4.14 Partial restoration of *Helicobacter hepaticus* + anti-IL-10R-associated caecal pathology and IL-17A⁺ CD4⁺ T cell frequency in CD11c^{IL-23-} mice by Ly6C^{hi} monocyte transfer.

CD11c-Cre⁺ *Il23a*^{fl/fl} (CD11c^{IL-23-}) mice or pooled CD11c-Cre⁻ *Il23a*^{fl/+} and CD11c-Cre⁻ *Il23a*^{fl/fl} (CD11c^{IL-23+}) littermate controls were infected with *Helicobacter hepaticus* (*Hh*) and treated weekly with anti-IL-10R blocking mAb prior to analysis after two weeks. CD11c^{IL-23-} mice received 2×10^6 Ly6C^{hi} monocytes or 1.4×10^6 CD11c⁺ cells isolated from pooled CD11c^{IL-23+} bone marrow as indicated. Sorted populations showed >95% purity upon reanalysis. Sections of intestine were collected and single cell preparations of combined caecal and colonic lamina propria were prepared and analysed as described. (A) Caecal histopathology scores. (B) Frequency of IL-17A⁺ IFN γ ⁻ CD4⁺ T cells as determined by intracellular cytokine staining performed after 3.5h PMA/ionomycin/Brefeldin A stimulation of 500,000 lamina propria cells. Data are from one experiment (CD11c^{IL-23+} *Hh*+anti-IL-10R, filled circles, n=5; CD11c^{IL-23-} *Hh*+anti-IL-10R, open circles, n=3; CD11c^{IL-23-} *Hh*+anti-IL-10R + 2×10^6 Ly6C^{hi} monocytes, filled diamonds, n=4; CD11c^{IL-23-} *Hh*+anti-IL-10R + 1.4×10^6 CD11c⁺ cells, open diamonds, n=4; CD11c^{IL-23+} *Hh* only, filled triangles, n=4; CD11c^{IL-23+} uninfected, filled triangles, n=4). Each data point represents an individual mouse, vertical line indicates group mean.

4.3 Discussion

These experiments aimed to determine the source of IL-23 that is required for the colitis caused by *Helicobacter hepaticus* in lymphocyte-replete mice with disrupted IL-10 signalling. Using conditional gene knockout techniques to generate mice in which only CD11c⁺ cells lack the ability to produce IL-23, we have shown that CD11c⁺ cells are a crucial source of IL-23 which drives *Hh* + anti-IL-10R colitis. Mice lacking IL-23 production in CD11c⁺ cells showed significant reductions in all parameters of disease, including colonic and caecal tissue pathology, splenomegaly, pro-inflammatory cytokine levels, recruitment of Ly6C^{hi} monocytes and neutrophils, and pathogenic CD4⁺ T cell subsets. Importantly, these results phenocopy those from experiments performed with full IL-23p19 knockout mice (Dr Philip Ahern, unpublished results). Since CD11c^{IL-23-} mice also showed an absence of intestinal *Il23a* mRNA transcripts and significantly reduced IL-23 secretion by cultured *ex vivo* lamina propria cells, it seems that CD11c⁺ cells are the main, possibly only, source of the IL-23 required to drive *Hh* + anti-IL-10R colitis.

Although IL-23 is clearly a major driver of inflammation in the intestine, it can be hard to measure due to its low concentration. When we examined IL-23 production by colonic and caecal lamina propria cells, we used a relatively small number of cells, which could have contributed to the variability in our ELISA results (Figure 4.3b). In future we could culture intestinal explants which may yield a higher concentration of secreted cytokines.

Interestingly, we observed that *Hh*-infected CD11c^{IL-23-} mice treated with anti-IL-10R showed no increase in spleen weight, which usually accompanies *Hh* + anti-IL-10R-induced colitis. During the recent delineation of the functions of IL-23 and IL-12, further to the realisation that they share the IL-12/23p40 subunit (Oppmann et al., 2000), it was shown that in the anti-CD40 (Uhlir et al., 2006b) and T cell transfer (Hue et al., 2006)

models of colitis, IL-23 is responsible for intestinal disease but dispensable for systemic symptoms including splenomegaly. However, in the innate *Hh* model where *Hh* infection alone drives pathology in immunodeficient 129/SvEv *Rag2*^{-/-} mice, both intestinal disease and splenomegaly were abrogated when IL-23 was blocked with an antibody against IL-23p19 (Hue et al., 2006). IL-23p19 knockout mice also do not develop colitis, typhlitis or splenomegaly following *Hh* + anti-IL-10R treatment (Dr Philip Ahern, unpublished results). Combined with our finding that in the absence of IL-23 from CD11c⁺ cells splenomegaly does not develop following *Hh* + anti-IL-10R treatment of lymphocyte-replete mice, these results suggest that dependency of splenomegaly on IL-23 might be common to models of intestinal inflammation driven by *Hh* regardless of the presence of lymphocytes, but not to non-bacterial models such as anti-CD40 colitis.

Most readouts positively associated with colitis, including protein levels of the pro-inflammatory cytokines IL-17A, IFN γ , TNF α , and IL-1 β ; accumulation of inflammatory MNPs; and splenomegaly, were almost completely abrogated in *Hh* + anti-IL-10R-treated CD11c^{IL-23-} mice. However, we observed only partial reductions in some aspects of the IL-17 axis, the frequency of IL-17A⁺ single-positive CD4⁺ T cells and neutrophil recruitment.

Although IL-23 is not required for the initial differentiation of Th17 cells, our finding is interesting because IL-23 is associated with maintenance of the Th17 lineage (Mangan et al., 2006). During their differentiation, TGF β upregulates IL-23R expression rendering Th17 cells responsive to IL-23, which then acts to maintain their IL-17-secreting phenotype (Stritesky et al., 2008). IL-23 also controls the generation of IL-17A⁺ IFN γ ⁺ double-positive CD4⁺ T cells from IL-17A⁺ single-positive Th17 cells (Ahern et al., 2010), which occurs during *Hh*-induced colitis (Morrison et al., 2013). In agreement, the results

presented here show almost complete abrogation of this pathogenic double-positive subset of CD4⁺ T cells in CD11c^{IL-23⁻} mice.

IL-17A is important in the innate immune response by promoting the early recruitment of neutrophils to sites of inflammation (Amulic et al., 2012), which we have observed in the lamina propria within 8 hours of *Hh* + anti-IL-10R treatment (Dr Isabelle Arnold, unpublished results). Populations of innate lymphoid cells serve as an early source of IL-17A in the intestine (Pearson et al., 2012). As for CD4⁺ T cells, stimulation with IL-23 has been shown to induce expression of IL-17A by ILCs (Pearson et al., 2012). However, in CD11c^{IL-23⁻} mice the frequency of neutrophils found in the lamina propria following *Hh* + anti-IL-10R treatment was only partially abrogated, potentially suggesting an IL-23-independent mechanism of promoting neutrophil recruitment. Expression of IL-17A by innate immune cells can be induced in the absence of IL-23, for instance by IL-6, IL-1 β or PRR stimulation (Rubino et al., 2012), however, we observed complete abrogation of IL-17A secretion by lamina propria cells from *Hh* + anti-IL-10R-treated CD11c^{IL-23⁻} mice, suggesting that the observed neutrophil recruitment was also IL-17A-independent.

Interestingly, although IL-17A protein levels were abrogated in *Hh* + anti-IL-10R-treated CD11c^{IL-23⁻} mice, *Il17a* mRNA levels were equal to those in *Hh* + anti-IL-10R-treated CD11c^{IL-23⁺} mice. Our results suggest that in *Hh* + anti-IL-10R colitis, induction of IL-17A expression at the mRNA level may be partially IL-23-independent, yet IL-23-dependent mechanisms of post-transcriptional regulation could control expression of functional IL-17A protein. At least two RNA binding proteins, HuR and TFL, are described to post-transcriptionally regulate IL-17A expression, by stabilising and promoting degradation of *Il17a* transcripts, respectively (Chen et al., 2013; Minagawa et al., 2014). Although a relationship with IL-23 has not been established, mice deficient in either of these proteins

experienced altered EAE (Chen et al., 2013; Minagawa et al., 2014), in which IL-23 plays a pro-inflammatory role (Chen et al., 2006; Cua et al., 2003; Thakker et al., 2007).

Unexpectedly, we found that IL-23 was required for the recruitment of Ly6C^{hi} monocytes to the large intestinal lamina propria, and thus for the accumulation of inflammatory macrophages. A recent paper has similarly described that IL-23 is necessary for the optimal recruitment of TNF α - and NO-producing Ly6C^{hi} monocytes to the spleen during experimental *Listeria monocytogenes* infection (Indramohan et al., 2012). Exactly how IL-23 controls monocyte recruitment to the lamina propria during *Hh* + anti-IL-10R colitis is unknown, but is discussed in further detail in Chapter 5.

Our results indicate that monocyte-derived MNPs, and not classical or monocyte-derived dendritic cells, are the source of IL-23 during *Hh* + anti-IL-10R colitis. We found that macrophages, in addition to DCs, expressed CD11c-Cre as indicated by GFP expression. When we sorted CD11c-Cre⁺ populations from CD11c^{IL-23⁺} and CD11c^{IL-23⁻} mice at the peak of *Il23a* expression 5 days post-*Hh* infection and anti-IL-10R treatment, we found that only the two monocyte-derived populations – Ly6C⁺ MHCII⁺ MNPs and Ly6C^{neg-lo} CD64⁺ macrophages, which include both CX₃CR1^{int} and CX₃CR1^{hi} macrophages (Bain et al., 2013) – expressed IL-23. This was reduced in cells from CD11c^{IL-23⁻} mice, indicating that these populations express reduced levels of IL-23 in these mice, and are therefore functionally relevant sources of IL-23 which drive *Hh* + anti-IL-10R colitis. To further characterise the populations, we turned to CX₃CR1^{GFP/+} mice and found that early in colitis (day 5) IL-23 was expressed by fully mature CX₃CR1^{hi} CD64⁺ macrophages and to a lesser extent by CX₃CR1^{int} CD64⁺ MNPs, which include Ly6C⁺ MHCII⁺ MNPs and Ly6C^{lo/-} MHCII⁺ macrophages. Later in the course of disease (week 3), near the peak of tissue pathology, we found that IL-23 was expressed by both of these populations and was elevated in

CX₃CR1^{int} CD64⁺ MNPs. CX₃CR1^{int} Ly6C⁺ CD64⁺ MNPs have been described to express pro-inflammatory genes in a number of colitis models including DSS (Bain et al., 2013; Zigmond et al., 2012) and T cell transfer (Rivollier et al., 2012; Weber et al., 2011). CD11c^{hi} and CD11c^{low} CD11b^{hi} cells in the colonic lamina propria have previously been shown to be potential sources of IL-23 during anti-CD40 colitis (Uhlig et al., 2006b). Macrophages have also previously been described as a source of IL-23 in the mucosa of IBD patients (Kamada et al., 2010; Kamada et al., 2008; Liu et al., 2011b), a murine colitis-associated colorectal cancer model (Grivennikov et al., 2012) and *in vitro* (Kamada et al., 2005; Sheikh et al., 2010), however none of these studies thoroughly addressed the relative contribution of distinct MNP subsets.

Our results showing expression of IL-23 by CX₃CR1^{hi} macrophages are highly unusual, as these cells are normally hypo-responsive to TLR stimulation and display an anti-inflammatory phenotype which is maintained even during colitis (Bain et al., 2013; Weber et al., 2011; Zigmond et al., 2012). A crucial difference between those studies and ours is blockade of the IL-10R, which may release CX₃CR1^{hi} macrophages from regulation by IL-10, discussed below, allowing them to initiate colitis.

Notably, we did not find expression of IL-23 in either CD11b⁺ or CD11b⁻ CD103⁺ DCs sorted from the colonic and caecal lamina propria. In the large intestine, most CD103⁺ DCs are CD11b⁻. *Batf3*^{-/-} mice, which lack CD8α⁺ and CD103⁺ CD11b⁻ DCs (Hildner et al., 2008), developed pathology indistinguishable from *Hh* + anti-IL-10R colitis in wild-type mice, indicating that CD103⁺ CD11b⁻ DCs are not necessary for intestinal disease in this model. Recent studies have identified CD103⁺ CD11b⁺ DCs as a source of IL-23 in the small intestine within 60 minutes of systemic administration of flagellin by intravenous injection (Kinnebrew et al., 2012), and as producers of IL-23 during *Citrobacter rodentium*

infection (Satpathy et al., 2013). IL-23 in these instances led to induction of IL-22, and RegIII expression by intestinal epithelial cells, an axis which is important for host defence in *Citrobacter rodentium* infection (Manta et al., 2013; Satpathy et al., 2013; Zheng et al., 2008). IL-22 was unaffected by the absence of IL-23 in our system during *Hh* + anti-IL-10R colitis. In contrast with *Citrobacter rodentium*-induced intestinal inflammation, IL-23 plays a pathogenic role in *Helicobacter hepaticus*-driven colitis. It is possible that, in addition to whether it plays a pathogenic or protective role, the source of IL-23 also differs between models. This should be borne in mind when interpreting results from different models of colitis, and also from *in vitro* experiments.

Additionally, in contrast to the small intestine, CD103⁺ CD11b⁺ DCs are infrequent in the colon and caecum. This alone does not rule out this population as a potential cytokine source, since small amounts of IL-23 seem to trigger a cascade which results in intestinal pathology. It is possible that small intestinal, rather than colonic, CD103⁺ CD11b⁺ DCs could provide the IL-23 required to induce *Hh* + anti-IL-10R colitis. A recent paper described expression of *Il23a* by unstimulated IRF4-dependent CD103⁺ CD11b⁺ DCs, but not CD11b⁺ macrophages, from the small intestine (Schlitzer et al., 2013). Constitutive IL-12/23p40 subunit promoter activity, alongside expression of IL-23 protein, has also been observed in the terminal ileum of mice (Becker et al., 2003). These phenomena were localised to CD11c⁺ CD11b⁻ CD8α⁻ cells in the lamina propria and were absent in germ-free mice, indicating that expression was driven by commensal bacterial stimulation. Since *Hh* is administered by oral gavage and thus must transit through the small intestine *en route* to the caecum and colon, it is possible that small intestinal CD103⁺ CD11b⁺ DCs could encounter *Hh* and be stimulated to produce IL-23. This possibility could be assessed by performing RT-qPCR for *Il23a* on CD103⁺ CD11b⁺ DCs purified from the terminal ileum and

other regions of the small intestine, which is commonly ignored in this model due to the absence of inflammation. In future it would be prudent to test the requirement for CD103⁺ CD11b⁺ DCs during *Hh* + anti-IL-10R colitis using CD11c-Cre x IRF4-floxed mice (Persson et al., 2013; Schlitzer et al., 2013) or CD11c-Cre x Notch2-floxed mice (Satpathy et al., 2013), as both of these strains lack CD103⁺ CD11b⁺ DCs due to the dependency of this population on the transcription factor IRF4 and the signalling receptor Notch2. Given the broad expression of CD11c-Cre in our system, our results do not strictly rule out the possibility that other CD11c⁺ subsets might contribute as sources of IL-23 at diverse stages of colitis.

Only when *Hh*-infected mice were treated with anti-IL-10R did we observe IL-23 expression by Ly6C⁺ MHCII⁺ MNPs. However, this population is present only at very low frequencies during homeostasis, and in the inflamed intestine the majority of Ly6C⁺ MHCII⁺ MNPs are descendants of recently arrived Ly6C^{hi} monocytes (Bain et al., 2013; Zigmond et al., 2012). It is therefore possible that we compared disparate populations – a small resident population, and newly recruited cells – rather than the same population in the presence and absence of inflammation. Preliminary results from our *in vivo* studies indicate that IL-23 may be expressed constitutively, or at low levels following *Hh* infection, by lamina propria Ly6C^{neg-lo} CD64⁺ macrophages. In such absence of inflammation, this population consists mainly of CX₃CR1^{hi} macrophages (Bain et al., 2013). Expression of IL-23 was greatly increased in Ly6C^{neg-lo} CD64⁺ macrophages by concomitant anti-IL-10R treatment, suggesting that IL-10 may limit the production of IL-23 by macrophages in the lamina propria *in vivo*.

As IL-23 is a potent inflammatory cytokine, regulatory pathways are required to prevent unnecessary damage to self. *Hh* infection in wild-type mice upregulates IL-23p19

expression (Kullberg et al., 2006), however the dominant cytokine induced is IL-10 (Kullberg et al., 1998). This IL-10 prevents the development of colitis, demonstrated by the fact that spontaneous colitis develops when *Hh*-infected mice are genetically deficient in IL-10 (Kullberg et al., 1998). In the model of colitis we have used – in IL-10-sufficient mice – the IL-10 pathway must be blocked by anti-IL-10R monoclonal antibody treatment in order to induce disease (Kullberg et al., 2006). Importantly, IL-10R blockade alone does not induce colitis in our *Helicobacter* species-negative, specific pathogen-free colony, although this may not be the case in other animal facilities which do carry *Helicobacter* strains.

Precisely how IL-10 prevents colitis has been unclear due to the expression of IL-10 and its receptor by many cell types, including both haematopoietic and non-haematopoietic populations (Moore et al., 2001). However, it seems to be the action of IL-10 on macrophages that is crucial for the maintenance of intestinal homeostasis. LysM-Cre-driven deficiency in STAT3, a signalling molecule downstream of IL-10, results in aberrant macrophage activation and spontaneous colitis in mice by 20-24 weeks of age (Takeda et al., 1999). This has recently been corroborated by Zigmond *et al.* who found that *Cx3cr1*^{Cre} *Il10ra*^{fl/fl} mice, which lack IL-10R expression in intestinal macrophages and a proportion of DCs, also develop spontaneous colitis at 6 months of age (Zigmond et al., 2014). It should be noted that this publication states the presence of *Helicobacter* species within their colony (Zigmond et al., 2014). A similar study which used LysM-Cre mice to induce conditional IL-10Rα deficiency did not observe spontaneous colitis, most likely due to the absence of *Helicobacter* species in their facility (Li et al., 2014). These results highlight the importance of IL-10 as a macrophage-conditioning factor in the intestine, one which appears to be even more critical in the presence of *Helicobacter* infection.

CX₃CR1^{hi} intestinal lamina propria macrophages are hyporesponsive to bacterial stimuli (Bain et al., 2013; Monteleone et al., 2008), an important characteristic given their proximity to an enormous and varied microbial load. IL-10 has been shown to contribute to their hyporesponsiveness. In addition to previously discussed results, *Cx3cr1*^{Cre} *Il10*^{fl/fl} and *Cx3cr1*^{Cre} *Il10ra*^{fl/fl} bone marrow-derived macrophages deficient in IL-10 and IL-10R α , respectively, have been shown to produce greater levels of IL-6 and TNF α following LPS stimulation *in vitro*, which could be returned to wild-type levels by IL-10 addition in the case of IL-10-deficient but not IL-10R α -deficient cells (Zigmond et al., 2014). Comparative transcriptome analyses of CX₃CR1^{hi} colonic macrophages isolated from *Cx3cr1*^{Cre} *Il10ra*^{fl/fl} mice prior to spontaneous colitis development and CX₃CR1^{GFP/+} control mice from a *Helicobacter*-positive colony, revealed elevated *Il23a* mRNA expression in IL-10R-deficient macrophages (Zigmond et al., 2014). Additionally, Rivollier and colleagues found that colonic MNP subsets isolated from IL-10-deficient mice expressed greater levels of IL-23 than those isolated from wild-type controls (Rivollier et al., 2012). These results support the hypothesis that in macrophages, expression of IL-23 and other pro-inflammatory cytokines is limited by IL-10. Despite indications that macrophages could act as auto- or paracrine sources of IL-10 (Bain et al., 2013; Fiorentino et al., 1991; Monteleone et al., 2008; Pils et al., 2010), macrophage-derived IL-10 was not absolutely required to prevent the development of spontaneous colitis since this did not occur in *Cx3cr1*^{Cre} *Il10*^{fl/fl} mice (Zigmond et al., 2014).

Importantly, colonic lamina propria macrophages from *Helicobacter*-negative LysM-Cre *Il10ra*^{fl/fl} mice – which do not develop spontaneous colitis – expressed equal levels of *Il23a* compared with macrophages from Cre-negative *Il10ra*^{fl/fl} control mice (Li et al., 2014). These results indicate that *Helicobacter* or another infection may be required to

enhance expression of IL-23 by macrophages in the absence of IL-10-mediated regulation.

IL-10 appears to be a key factor maintaining the anti-inflammatory nature of lamina propria CX₃CR1^{hi} macrophages in the face of bacterial challenge, which prevents them from inducing colitis via expression of IL-23.

In addition to suppressing IL-23 expression, IL-10 might function by suppressing APC maturation: CX₃CR1^{hi} CD11b⁺ CD11c⁺ cells were found to prevent colitis development by suppressing CD4⁺ T cell activation and proliferation, via low expression of the co-stimulatory molecules CD80 and CD86, which was caused by IL-10/STAT3 signalling in the APCs (Kayama et al., 2012). Shouval *et al.* also observed elevated expression of CD86 and MHCII in *Il10rb*^{-/-} bone marrow-derived macrophages, which induced greater proliferation of naïve CD4⁺ T cells during co-culture (Shouval et al., 2014). Interestingly, wild-type Tregs were less able to suppress naïve CD4⁺ T cell proliferation in co-culture with *Il10rb*^{-/-} bone marrow-derived macrophages than wild-type macrophages (Shouval et al., 2014), indicating that Treg-derived IL-10 may suppress CD4⁺ T cell responses partially via action on macrophages. Additionally, treatment of APCs with anti-IL-10R during pulsing with commensal bacteria prior to co-culture with CD4⁺ T cells has been shown to induce greater production of IL-12/23p40 and IFN γ by the T cells (Liu et al., 2011a), indicating that IL-10 diminishes pro-inflammatory adaptive responses against the microbiota via its action on macrophages. This could be of great importance during the regulation of CD4⁺ T cell responses in the intestinal lamina propria by CX₃CR1^{hi} macrophages (Hadis et al., 2011; Rossini et al., 2013).

As intestinal macrophages express MHCII and can acquire luminal protein antigen (Chang et al., 2013; Farache et al., 2013; Mazzini et al., 2014; Schulz et al., 2009) and bacteria (Manta et al., 2013; Muller et al., 2012; Rossini et al., 2013), they may be able to present

antigen to T cells. However, CX₃CR1^{hi} macrophages do not migrate to the MLNs and are relatively weak stimulators of T cell proliferation (Schulz et al., 2009), therefore they are unlikely to prime naïve T cells, which are rare in the mucosa. The CX₃CR1⁺ MNPs that have been reported to migrate to the MLNs (Cerovic et al., 2013; Diehl et al., 2013; Schulz et al., 2009) likely represent a population of *bona fide* DCs expressing intermediate levels of CX₃CR1 (Cerovic et al., 2013; Scott et al., 2014). Nevertheless intestinal macrophages could provide secondary antigen stimulation to T cells which have migrated to the lamina propria following priming in secondary lymphoid tissues. Indeed, CX₃CR1^{hi} macrophages have been shown to induce expansion and maintenance of Foxp3⁺ Tregs in the lamina propria via CX₃CR1-dependent IL-10 production, which was necessary for the generation of oral tolerance (Hadis et al., 2011). Similarly, IL-10 production by CD11b⁺ cells was required to maintain Foxp3 expression by Tregs, which was necessary for prevention of T cell transfer colitis (Murai et al., 2009).

Intestinal macrophages also appear to support IFN γ ⁺ and IFN γ ⁺ IL-17⁺ CD4⁺ T cell responses in the lamina propria during infection with *Citrobacter rodentium* (Schreiber et al., 2013), an enteric pathogen that resembles enteropathogenic and enterohaemorrhagic *E. coli* (Mundy et al., 2005). Although neither population was required for T cell priming, monocytes and macrophages in the lamina propria again acted as a source of IL-12, inducing IFN γ expression in CD4⁺ T cells (Schreiber et al., 2013). CX₃CR1⁺ MNPs have also been shown to co-localise with CD4⁺ T cells in the lamina propria during an ovalbumin (OVA)-driven model of T cell transfer colitis (Rossini et al., 2013). These results indicate that intestinal macrophages influence CD4⁺ T cell responses in the lamina propria during both homeostasis and inflammation. IL-23 produced by CX₃CR1^{hi} macrophages or Ly6C⁺ MHCII⁺ MNPs might thus contribute to the generation of IL-17A⁺

IFN γ ⁺ double-positive CD4⁺ T cells in the lamina propria (Ahern et al., 2010; Morrison et al., 2013), which were almost completely absent in *Hh* + anti-IL-10R-treated CD11c^{IL-23-} mice.

Antigen acquired by intestinal macrophages could be presented directly to primed T cells in the LP as described above; alternatively it could be transferred to antigen presenting CD103⁺ DCs via gap junctions in a Connexin 43-dependent manner (Mazzini et al., 2014). Deletion of Connexin 43 in CD11c⁺ cells resulted in defects in Foxp3⁺ Treg differentiation and the generation of oral tolerance (Mazzini et al., 2014), suggesting that gap junction-mediated transfer of antigen from CX₃CR1⁺ MNPs to CD103⁺ migratory DCs could be important for the immunoregulatory functions of intestinal macrophages.

It would be informative to confirm expression of IL-10R by intestinal CX₃CR1^{hi} macrophages and CX₃CR1^{int} Ly6C⁺ MHCII⁺ MNPs specifically. Shouval *et al.* observed reduced frequencies of Ly6C^{lo} MHCII⁺ MNPs and a corresponding increase in Ly6C⁺ MHCII⁺ MNPs in *Il10rb*^{-/-} mice in the absence of colitis, suggesting that IL-10 might control the differentiation of Ly6C⁺ MHCII⁺ MNPs into Ly6C^{lo} macrophages (Shouval et al., 2014). Expression of *Il10ra* was found to be enriched in CD11c⁺ CD11b⁺ CD64⁺ macrophages among colonic lamina propria cells (Zigmond et al., 2014) and colonic CX₃CR1⁺ macrophages have also been described to express *Il10rb* (Rivollier et al., 2012).

Unfortunately these studies did not distinguish between subpopulations of macrophages. Functions such as gene and protein expression of IL-23, antigen presentation, T cell priming, and regulation of neutrophils, in the presence and absence of *Hh* and anti-IL-10R could be investigated to confirm results reported in other models (Grainger et al., 2013; Kayama et al., 2012; Liu et al., 2011a; Monteleone et al., 2008; Rivollier et al., 2012; Shouval et al., 2014; Zigmond et al., 2014; Zigmond et al., 2012) in the *Hh* + anti-IL-10R

model of colitis. *In vitro* experiments using *ex vivo* cells would be conducted in the absence of environmental factors which may influence MNPs *in situ*, but could nonetheless be informative regarding the roles of *Hh* and the anti-IL-10R mAb in induction of colitis, and conversely that of IL-10 in its prevention. Alternatively, the use of intestinal organoids (Sato et al., 2011; Sato et al., 2009; Yui et al., 2012) might allow a more physiological yet still tractable system.

Early work on the role of IL-23 in the *Hh* + anti-IL-10R model of colitis found that IL-23p19 transcription in whole colonic tissue was induced equally by *Hh* alone and *Hh* + anti-IL-10R treatment (Kullberg et al., 2006), indicating that it is infection with *Hh* which triggers IL-23 expression. The pathogenic effects of this IL-23 expression, however, are counteracted by the additional induction of IL-10 (Kullberg et al., 1998). Gram-negative non-pathogenic bacteria, which include *Hh*, have been reported to prime human monocyte-derived DCs for enhanced IL-23 expression (Smits et al., 2004). Compared to *Hh* infection alone, Kullberg and colleagues (Kullberg et al., 2006) found that *Hh* + anti-IL-10R treatment resulted in a substantial increase in transcription of IL-12/23p40, and decreased transcription of the IL-12-specific subunit IL-12p35. This supports the idea that there are two components in the regulation of functional IL-23 expression: firstly, expression of the specific IL-23p19 subunit, which appears to be induced by *Hh*; and secondly, availability of the shared IL-12/23p40 subunit to combine with IL-23p19 to create IL-23, rather than with IL-12p35 to produce IL-12. Expression of IL-23p19 and IL-12p35 might be reciprocally regulated, akin to IFN γ and IL-4 in CD4⁺ T cells (Kastelein et al., 2007). It would be interesting to test whether these gene expression dynamics, observed in whole colonic tissue, hold when applied to sorted populations of cells from the lamina propria of uninfected, *Hh*-infected, and *Hh* + anti-IL-10R-treated mice.

To confirm whether CX₃CR1^{hi} macrophages act as the source of IL-23 which initiates *Hh* + anti-IL-10R colitis, and whether CX₃CR1^{int} CD64⁺ macrophages prolong inflammation by providing a sustained source of IL-23, would require an *in vivo* system allowing depletion of each population at the appropriate timepoint during disease. Specific ablation of these subsets is a persistent problem in the field. Ly6C^{hi} monocytes and CX₃CR1^{int} CD64⁺ Ly6C⁺ MNPs express CCR2 and are reported to depend upon this receptor for recruitment to the colon during DSS-mediated colitis (Bain et al., 2013; Platt et al., 2010; Zigmond et al., 2012). In this setting, CX₃CR1^{hi} macrophages were found not to express CCR2, thus it is possible that CCR2-DTR mice, which express the diphtheria toxin receptor (DTR) under the control of the CCR2 promoter (Hohl et al., 2009), could be used to selectively deplete recruited CCR2-expressing Ly6C^{hi} monocytes and CX₃CR1^{int} CD64⁺ MNPs during colitis. One paper reports depletion of intestinal CX₃CR1^{int} Ly6C⁺ MNPs using an antibody directed against CCR2, MC-21 (Zigmond et al., 2012), a strategy discussed in more detail in Chapter 5.

Other approaches are likely to be less specific and to target both CX₃CR1^{hi} and CX₃CR1^{int} subsets of MNPs. Transgenic mice expressing Cre recombinase under the control of a selected promoter, crossed with mice possessing an inducible simian diphtheria toxin receptor (iDTR mice) under the control of a ubiquitous promoter can be used to conditionally ablate specific cells via diphtheria toxin treatment (Buch et al., 2005). The iDTR transgene contains a loxP-flanked transcriptional stop codon, preventing iDTR transcription unless Cre is co-expressed to excise the floxed stop codon. When Cre and thus the iDTR are expressed, cells are rendered susceptible to apoptosis induced by diphtheria toxin, allowing their depletion. Numerous relevant Cre-expressing mouse lines exist which could be used in this system. Unfortunately, CX₃CR1-Cre mice (Yona et al.,

2013) were not the advent researchers in the field were hoping for, showing Cre recombinase activity in almost all macrophages, but also in a third of CD11c⁺ CD64⁻ CD24⁺ CD11b⁻ DCs, and in two thirds of CD103⁺ CD11b⁺ and CD103⁻ CD11b⁺ DCs in the colon (Zigmond et al., 2014). Transgenic mice possessing LysM-Cre (Clausen et al., 1999) or CSF-1R-Cre (Deng et al., 2010) are other well-established tools used to target macrophages, however both can have additional effects on granulocytes and in the case of CSF-1R-Cre, some lymphocytes (Clausen et al., 1999; Deng et al., 2010). CD64-Cre mice are in development, and although this transgene would affect all macrophage subsets, not to mention some less mature macrophages and monocytes as they upregulate CD64 expression (Bain et al., 2013; Tamoutounour et al., 2012), it is hoped that its effects would be confined to macrophages (Gautier et al., 2012; Tamoutounour et al., 2012).

Other strategies for macrophage depletion include antibodies directed against CSF-1R (M-CSFR, CD115), which have recently shown promise *in vivo* (clone AFS98 (Hashimoto et al., 2011); clone M279 (Hume and MacDonald, 2011)). Liposome-encapsulated clodronate administered intra-rectally have also been utilised to deplete macrophages locally in the descending colon and rectum (Qualls et al., 2006), however these can affect all phagocytic cells, including dendritic cells. This method of local depletion might be of particular use in experimental models involving inflammation of the distal colon, such as oxazolone-induced colitis, used to model ulcerative colitis which invariably begins in the distal colon.

The strategies described here illustrate the obvious problems involved in targeting specific MNP subsets. An approach which would eliminate all macrophage subsets, preferably CD64-Cre x iDTR mice when available, could be used prior to *Hh* infection and anti-IL-10R treatment, when CX₃CR1^{int} CD64⁺ MNPs are infrequent in the lamina propria,

to deplete the resident CX₃CR1^{hi} macrophage population and test their posited role as a source of IL-23 which initiates colitis. Alternatively, these strategies could be used at various timepoints after establishment of disease to show the requirement for these macrophage populations for continuing inflammation.

Given that our data suggest that during *Hh* + anti-IL-10R colitis IL-23 is produced by two populations which both derive from Ly6C^{hi} monocytes, we attempted to restore disease by supplying IL-23-sufficient monocytes to CD11c^{IL-23-} mice. Although this was a small pilot experiment, some of our preliminary results were encouraging. This experiment should be repeated, with some changes to the protocol. An alternative to CD11c⁺ bone marrow cells, such as macrophage-DC precursors (MDPs), as the positive control is required. CD11c⁺ cells isolated from the bone marrow were chosen for their source and expression of CD11c. However, the latter should have taken into account which cells will eventually express CD11c in the intestinal lamina propria rather than in the bone marrow, as not all of the former necessarily express CD11c in the bone marrow. In this regard, MDPs could be used. It would also be appropriate to include a group of CD11c^{IL-23-} mice transferred with Ly6C^{hi} monocytes sorted from CD11c^{IL-23-} mice as a negative control, however this was unfeasible in this study due to limited availability of CD11c^{IL-23-} mice. Adjustments to the transfer of monocytes might also be required. The Ly6C^{hi} monocytes we transferred were CD11c⁻ MHCII⁻ lineage⁻ (lineage markers = CD3, TCRβ, B220, NKp46, c-Kit) Siglec F⁻ Ly6G⁻ CD11b⁺ CD45.1⁺ CD45.2⁺ Ly6C^{hi} CX₃CR1^{int} cells, and therefore did not include Ly6C^{hi} c-Kit⁺ CD11b⁻ cMoPs, which have been described to proliferate (Hettinger et al., 2013). Initially, we transferred 2 x 10⁶ monocytes at the onset of *Hh* infection and anti-IL-10R treatment. However, this single provision of a non-proliferative cell type may not have been adequate. Administration of monocytes every two days, for instance, may

be required to provide a consistent source of IL-23-sufficient cells. Our approach also did not address the potential role of cells resident in the lamina propria prior to *Hh* infection. To test whether CX₃CR1^{hi} resident macrophages are a sufficient source of IL-23 which begins the cascade leading to intestinal inflammation, these cells would need to be replaced with IL-23-proficient macrophages before the onset of *Hh* + anti-IL-10R treatment in CD11c^{IL-23-} mice. This may be possible to achieve by depleting the resident macrophages as described above, followed by transfer of wild-type Ly6C^{hi} monocytes to seed the lamina propria with IL-23-sufficient macrophages in their stead, prior to inducing colitis. Successful differentiation of lamina propria macrophages from adoptively transferred Ly6C^{hi} monocytes has been reported (Bain et al., 2013; Varol et al., 2009). To ensure that no other CD11c⁺ cells were capable of IL-23 production, CD11c^{IL-23-} mice could be used as Ly6C^{hi} monocyte recipients. If successful, further transfers of Ly6C^{hi} monocytes could be administered following disease induction to test the additional requirement of these cells for continuing inflammation.

A sophisticated genetically engineered system could provide an alternative strategy. Varol and colleagues (Varol et al., 2009) showed that CX₃CR1^{int} and CX₃CR1^{hi} macrophages could be depleted from the intestinal lamina propria by diphtheria toxin treatment of [CD11c-DTR → WT] bone marrow chimeric animals. If CD11c-DTR mice could be crossed with the CD11c^{IL-23-} line, to generate mice expressing both the DTR and Cre recombinase under the control of the CD11c promoter, alongside floxed alleles of *Il23a*, then not only would CD11c⁺ MNPs be unable to produce IL-23, they could also be depleted to empty the lamina propria resident macrophage niche. In the steady state, Ly6C^{hi} monocytes give rise to CX₃CR1^{hi} resident macrophages, but inflammatory conditions halt this process resulting in accumulation of CX₃CR1^{int} Ly6C⁺ MNPs (Bain et al., 2013; Rivollier et al., 2012;

Zigmond et al., 2012). Provided that the mass depletion of resident macrophages did not cause sufficient inflammation to interfere with the development of transferred monocytes into replacement resident macrophages, perhaps this strategy could be used to generate mice with a larger proportion of IL-23-proficient macrophages in the steady-state lamina propria. Unfortunately, this strategy is unlikely to be straightforward.

We favour the hypothesis that CX₃CR1^{hi} macrophages, which are abundant in the steady state lamina propria, are the source of IL-23 which initiates inflammation in the *Hh* + anti-IL-10R model of colitis. CX₃CR1^{hi} lamina propria resident macrophages are ideally situated to act as environmental sensors, which can direct appropriate subsequent immune responses, transfer antigen to migratory DCs, potentially migrate to draining lymph nodes themselves, and support ongoing adaptive immunity in the periphery. Infection with *Hh* seems to induce IL-23 expression by these resident macrophages, in addition to the IL-10 they produce constitutively. This IL-10 may be suppressing expression of IL-23 and perhaps maturation of APCs, thus preventing the development of colitis. When IL-10 signalling is blocked however, IL-23 production increases, and drives recruitment of neutrophils and Ly6C^{hi} monocytes, of which the latter differentiate into CX₃CR1^{int} Ly6C⁺ CD64⁺ macrophages. Later in the course of disease, this population has dramatically accumulated in the lamina propria and also expresses IL-23, which may maintain the ongoing intestinal inflammation, by supporting and expanding Th17 responses and driving conversion of these cells into pathogenic IL-17⁺ IFNγ⁺ Th cells and IFNγ⁺ “ex-Th17” cells, which drive *Hh* + anti-IL-10R colitis.

5 Recruitment of mononuclear phagocytes to the large intestine during inflammation

5.1 Introduction

We have shown in Chapter 4 that monocyte-derived MNPs are the major source of the pathogenic IL-23 which drives *Helicobacter hepaticus*-induced colitis. Inflammation seems to be initiated via IL-23 produced by CX₃CR1^{hi} resident macrophages, which are present in the steady state lamina propria, however CX₃CR1^{int} CD64⁺ MNPs predominate in the inflamed intestine when they also express IL-23. Both of these populations arise from Ly6C^{hi} monocytes as part of a developmental continuum: under homeostatic conditions complete differentiation into CX₃CR1^{hi} macrophages occurs, however during inflammation this process is halted at an intermediate stage MNP expressing Ly6C, MHCII, CD64 and intermediate levels of CX₃CR1 (CX₃CR1^{int} Ly6C⁺ CD64⁺ MNPs; Figure 1.3) (Bain et al., 2013; Tamoutounour et al., 2012). Substantial accumulation of Ly6C^{hi} monocytes and their CX₃CR1^{int} Ly6C⁺ CD64⁺ progeny occurs in the inflamed colonic lamina propria (Figure 3.3; (Bain et al., 2013; Zigmond et al., 2012)). Further to previous publications, we found that the accumulation of these populations in the colon during *Helicobacter hepaticus*-induced colitis is dependent on IL-23, however the downstream mechanism controlling their recruitment remains unknown. Due to their expression of IL-23 (Chapter 4), in the absence of which colitis fails to develop, we believe that Ly6C^{hi} monocyte-derived CX₃CR1^{int} CD64⁺ MNPs play a role in maintaining intestinal inflammation following their arrival in the lamina propria, and might represent a therapeutic target in inflammatory bowel disease.

One approach to targeting cellular populations that accumulate during inflammation is to prevent their recruitment, for instance by blocking the appropriate chemokine receptor or its ligands. Previous work from our group has described expression of CCR6 by a pro-inflammatory, monocyte-derived MNP population found in the lamina propria and MLN during T cell transfer- and anti-CD40-induced colitis (Siddiqui et al., 2010). Most research on CCR6 expressed by intestinal MNPs regards the small intestinal Peyer's patches (PPs) since expression of its only chemokine ligand, CCL20 (Greaves et al., 1997), is concentrated in the follicle-associated epithelium (FAE) overlying PPs (Cook et al., 2000; Iwasaki and Kelsall, 2000). CCR6 might play a role in other local intestinal lymphoid tissue such as isolated lymphoid follicles (ILFs) (Eberl and Lochner, 2009; McDonald et al., 2007), which unlike Peyer's patches are also found in the large intestine (Kanamori et al., 1996).

CCR6 was initially thought to be necessary for localisation of CCR6⁺ CD11b⁺ DCs to the PP subepithelial dome (Cook et al., 2000; Iwasaki and Kelsall, 2000). However, side-by-side analysis of multiple CCR6-deficient mice showed that CCR6⁺ CD11b⁺ DCs could be found in the subepithelial dome in the absence of functional CCR6 (Lugering et al., 2005; Zhao et al., 2003). It has been suggested that CCR6 may play a greater role in CD11b⁺ PP DC migration during acute immune responses than in the steady state (Williams, 2006).

Following invasion of the Peyer's patches by *Salmonella* Typhimurium, CCR6⁺ DCs are recruited to the subepithelial dome where they activate pathogen-specific CD4⁺ T cells in a CCR6-dependent manner (Salazar-Gonzalez et al., 2006). Although a role for MNP-expressed CCR6 is evident during invasive intestinal infection, its role during colitis remains unclear.

Genome-wide association studies have found that *CCR6* is located within a Crohn's disease susceptibility locus (Barrett et al., 2008). Indeed, CD patients show higher levels

of expression of CCR6 and CCL20 in the colon (Kwon et al., 2002), which is also observed in experimental models of IBD (Scheerens et al., 2001). However, the role of CCR6 in intestinal inflammation has not been easy to interpret, as findings from experimental models of acute colitis are conflicting. Following DSS treatment, CCR6-deficient mice developed less severe colitis than wild-type controls (Varona et al., 2003). However, CCR6-deficiency rendered B6 mice susceptible to TNBS-induced colitis, in contrast to disease-resistant wild-type controls (Varona et al., 2003). Conversely, wild-type Balb/c mice are naturally susceptible to TNBS-induced colitis, which was ameliorated by treatment with an anti-CCL20 neutralising antibody (Katchar et al., 2007), highlighting the importance of genetic background when working with animal models of disease. In contrast to experimental models of autoimmune diseases such as multiple sclerosis, psoriasis and arthritis, in which CCR6 plays a pathogenic role by controlling Th17 cell recruitment (Hedrick et al., 2009; Hirota et al., 2007; Liston et al., 2009; Reboldi et al., 2009), chronic T cell transfer colitis induced by CCR6-deficient naïve CD4⁺ T cells was exacerbated, and associated with an enhanced IFN γ ⁺ T cell response, compared to disease induced by wild-type T cells in Balb/c *Rag2*^{-/-} mice (Kitamura et al., 2010). Although induction of Tregs from CCR6-deficient naïve T cells was unaffected, Treg migration to the colon was impaired in its absence, hence co-transferred Treg cells in the protection model required CCR6 to mediate suppression of T cell transfer colitis (Kitamura et al., 2010). CCR6-deficient mice are also described to lack a CD4⁺ CD62L⁻ CD45RB^{lo} regulatory T cell population normally found in the Peyer's patches (Lugering et al., 2005). Whether CCR6 also influences intestinal MNP populations, which are crucial for direction and maintenance of adaptive immune responses, during colitis is unknown.

In addition to CCR6, the pro-inflammatory, monocyte-derived MNP population previously described by our lab also expresses CCR2 (Siddiqui et al., 2010). A number of recent studies report a link between CCR2 and Ly6C^{hi} monocyte and macrophage recruitment to the resting and inflamed intestine during DSS-, hapten- and *Citrobacter rodentium*-induced colitis (Bain et al., 2013; Khan et al., 2006; Kim et al., 2011b; Platt et al., 2010; Popivanova et al., 2009; Takada et al., 2010; Zigmond et al., 2012). CCR2 controls monocyte egress from the bone marrow (Serbina and Pamer, 2006). Therefore, observed defects in CCR2-deficient monocyte accumulation in the lamina propria (Bain et al., 2013; Platt et al., 2010; Takada et al., 2010; Zigmond et al., 2012) could be due to their inability to exit the bone marrow (Serbina and Pamer, 2006), rather than an inability to enter the intestine from the bloodstream which has not always been confirmed. However, at least one study found that when CCR2-deficient and wild-type Ly6C^{hi} bone marrow monocytes were co-transferred intravenously to mice with DSS-induced colitis, virtually all of the monocytes recruited to the inflamed lamina propria were of wild-type origin (Zigmond et al., 2012), illustrating the competitive advantage bestowed by CCR2 expression. The extent to which CCR2 is involved in Ly6C^{hi} monocyte recruitment to the intestine during other experimental models of colitis is unclear.

We set out to determine whether CCR6, CCR2 or other chemokine receptors might play a role in the accumulation of Ly6C^{hi} monocytes and MNPs in the large intestine during different experimental models of inflammatory bowel disease, including the anti-CD40-induced form of innate immune-driven acute colitis, T cell transfer- and *Helicobacter hepaticus*-induced chronic colitis.

5.2 Results

5.2.1 Role of CCR6 in recruitment of MNPs to the inflamed colon

To examine whether CCR6 might play a role in the accumulation of pro-inflammatory monocyte-derived MNPs in colitis, we used CCR6-EGFP knock-in mice, in which CCR6 expression is traceable but non-functional (Kucharzik et al., 2002). To exclude effects caused by a lack of CCR6 expression on Treg and Th17 cells, we used CCR6^{GFP/GFP} *Rag1*^{-/-} mice to examine the function of CCR6 within the MNP compartment. We investigated the role of CCR6 in the anti-CD40 and T cell transfer models of colitis. These models were chosen as they involve a T cell-MNP interaction: either real, as in T cell transfer, or simulated, as in the anti-CD40 model, which uses a mAb specific for CD40 (anti-CD40; clone FGK45 (Rolink et al., 1996)) to emulate ligation of this APC-expressed co-stimulatory molecule by CD40L, which is usually expressed by activated T cells (Uhlig et al., 2006b). When we treated CCR6^{GFP/GFP} *Rag1*^{-/-} mice with anti-CD40, we found no difference in the resulting weight loss (Figure 5.1a), splenomegaly (Figure 5.1b) or colonic pathology (Figure 5.2) relative to B6 *Rag1*^{-/-} controls, indicating that disease in this model is not affected by CCR6 deficiency in non-lymphocytes.

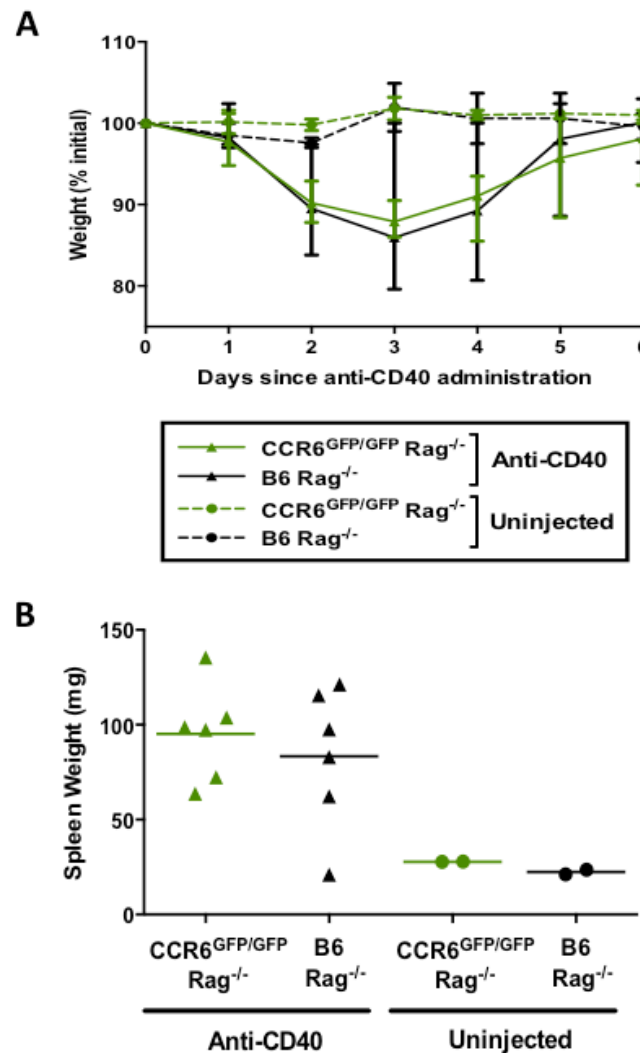


Figure 5.1 CCR6 deficiency does not affect anti-CD40-induced disease.

CCR6^{GFP/GFP} Rag1^{-/-} mice or B6 Rag1^{-/-} controls were given one i.p. injection of 100 μ g anti-CD40 mAb prior to analysis 7 days later. (A) Weight loss over the duration of the experiment. Data represent median (+ range) weight of mice shown as a percentage of initial weight prior to anti-CD40 treatment. (B) Spleen weight at sacrifice 7 days post-anti-CD40 administration. Each data point represents an individual mouse, horizontal line indicates group mean. Data are from one experiment (CCR6^{GFP/GFP} Rag1^{-/-} + anti-CD40, green filled triangles, n=6; B6 Rag1^{-/-} + anti-CD40, black filled triangles, n=6; CCR6^{GFP/GFP} Rag1^{-/-} uninjected, green filled circles, n=2; B6 Rag1^{-/-} uninjected, black filled circles, n=2).

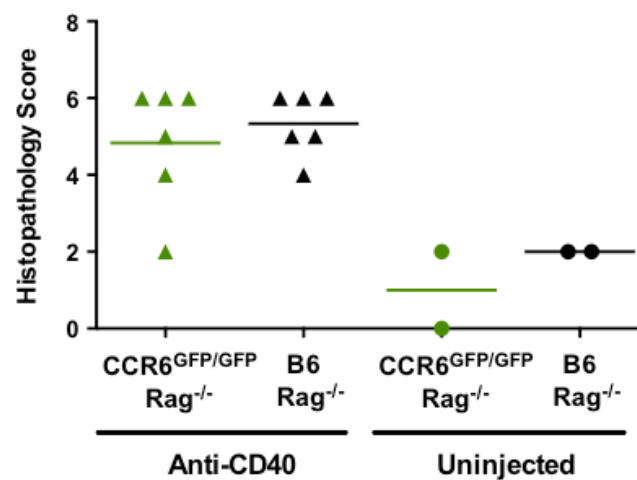


Figure 5.2 CCR6 deficiency does not affect colonic pathology induced by anti-CD40 monoclonal antibody treatment.

CCR6^{GFP/GFP} Rag1^{-/-} mice or B6 Rag1^{-/-} controls were given one i.p. injection of 100 µg anti-CD40 mAb prior to analysis 7 days later. Histopathology scores for proximal colon. Each data point represents an individual mouse, horizontal line indicates group mean. Data are from one experiment (CCR6^{GFP/GFP} Rag1^{-/-} + anti-CD40, green filled triangles, n=6; B6 Rag1^{-/-} + anti-CD40, black filled triangles, n=6; CCR6^{GFP/GFP} Rag1^{-/-} uninjected, green filled circles, n=2; B6 Rag1^{-/-} uninjected, black filled circles, n=2).

Due to the insertion of GFP into the CCR6 gene, we were unable to cross these mice to CX₃CR1-GFP mice and therefore could not include this marker in our flow cytometry analysis. As this work was also undertaken prior to the major developments in distinguishing MNP subsets outlined in earlier chapters, we used a previously described panel of markers to identify MNP populations (Siddiqui et al., 2010). We observed no differences between CCR6-deficient mice and controls in the frequency of CD11c⁺ MHCII⁺ colonic lamina propria cells in resting or anti-CD40-treated mice, nor any differences in E-cadherin⁺ or CD103⁺ subsets of CD11c⁺ MHCII⁺ cells (Figure 5.3). Respectively, these populations represent the Ly6C^{hi} monocyte-derived MNPs that we previously described (Siddiqui et al., 2010), and all CD103⁺ DCs, including both CD11b⁻ and CD11b⁺ subsets. Our results indicate that CCR6 does not play a major role in the accumulation of Ly6C^{hi} monocyte-derived MNPs in the colon during anti-CD40-induced colitis.

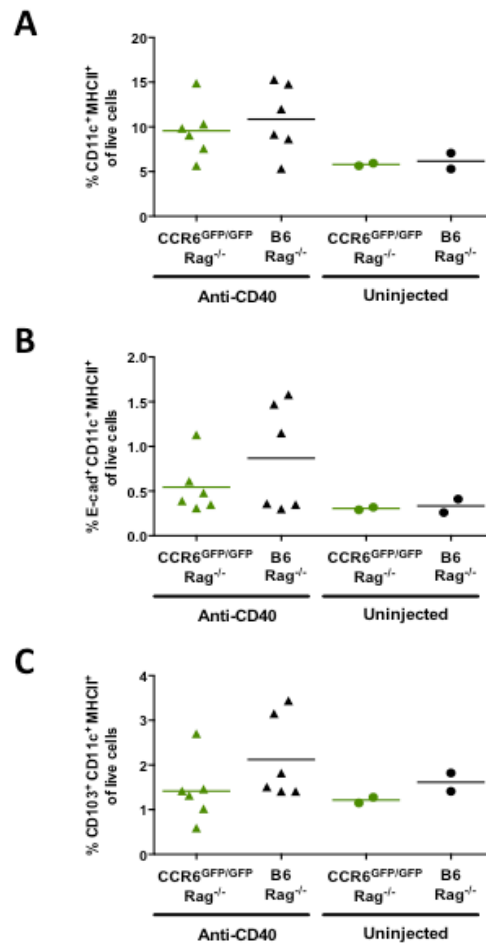


Figure 5.3 CCR6 deficiency does not affect anti-CD40-induced accumulation of CD11c⁺ MHCII⁺ cells in the colon lamina propria.

CCR6^{GFP/GFP} Rag1^{-/-} mice or B6 Rag1^{-/-} controls were given one i.p. injection of 100 µg anti-CD40 mAb prior to analysis 7 days later. Single cell preparations from colon lamina propria of individual mice were stained and analysed by flow cytometry. (A) Percentage of live cells expressing CD11c and MHCII. (B-C) Percentage of live CD11c⁺ MHCII⁺ cells expressing (B) E-cadherin (E-cad) or (C) CD103. During analysis, samples were pre-gated to exclude debris by FSC/SSC. Each data point represents an individual mouse, horizontal line indicates group mean. Data are from one experiment (CCR6^{GFP/GFP} Rag1^{-/-} + anti-CD40, green filled triangles, n=6; B6 Rag1^{-/-} + anti-CD40, black filled triangles, n=6; CCR6^{GFP/GFP} Rag1^{-/-} uninjected, green filled circles, n=2; B6 Rag1^{-/-} uninjected, black filled circles, n=2).

We investigated the role of CCR6 in the T cell transfer model. To ensure that any effects of CCR6 deficiency were not due to its absence on CD4⁺ T cells, we transferred 2×10^5 naïve CD4⁺ CD45RB^{hi} T cells sorted from wild-type CCR6-sufficient B6 mice. 5 weeks post-transfer, CCR6^{GFP/GFP} *Rag1*^{-/-} recipients were protected from colitis-associated weight loss (Figure 5.4a), but not splenomegaly (Figure 5.4b), relative to B6 (CCR6^{+/+}) *Rag1*^{-/-} controls. Interestingly, CCR6-deficient recipients also exhibited significantly less severe colitis in the proximal colon (Figure 5.5a), with trends towards the same in other regions (Figure 5.5b-c), and their whole colon mean score was significantly reduced (Figure 5.5d) compared to B6 *Rag1*^{-/-} T cell recipients. These results indicate that CCR6 deficiency among cells other than lymphocytes may partially protect mice from T cell transfer-induced colitis.

We hypothesised that these differences could be related to the presence or function of antigen presenting cells, which play an essential role in T cell responses during priming and effector stages. However, when we examined the effect of CCR6 deficiency on the frequencies of CD11c⁺ MHCII⁺, E-cadherin⁺ CD11c⁺ MHCII⁺, and CD103⁺ CD11c⁺ MHCII⁺ MNP populations in the inflamed colonic lamina propria, we found no major differences between CCR6^{GFP/GFP} *Rag1*^{-/-} and control B6 *Rag1*^{-/-} T cell recipients (Figure 5.6). Our results therefore indicate that CCR6 does not play a major role in the recruitment of MNPs to the colon during T cell transfer or anti-CD40-induced colitis.

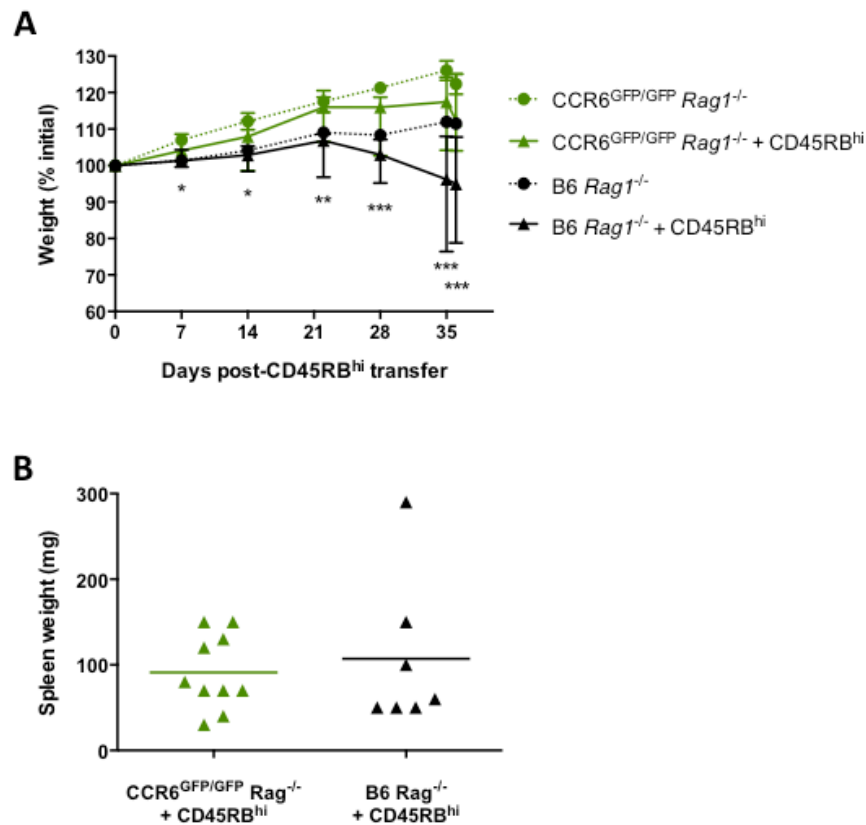


Figure 5.4 CCR6 deficiency prevents naïve CD4⁺ T cell transfer colitis-associated weight loss but not splenomegaly.

CCR6^{GFP/GFP} *Rag1*^{-/-} mice or B6 *Rag1*^{-/-} controls received 2×10^5 naïve CD4⁺ CD45RB^{hi} T cells by i.p. injection, prior to analysis 5 weeks later. (A) Weight loss over the duration of the experiment. Data represent median (+ range) weight of mice shown as a percentage of initial weight prior to T cell transfer. (B) Spleen weight at termination 5 weeks post-T cell transfer. Each data point represents an individual mouse, horizontal line indicates group mean. Data are from one experiment (CCR6^{GFP/GFP} *Rag1*^{-/-} + naïve CD4⁺ CD45RB^{hi} T cells, green filled triangles, solid line, n=10; B6 *Rag1*^{-/-} + naïve CD4⁺ CD45RB^{hi} T cells, black filled triangles, solid line, n=7-8; CCR6^{GFP/GFP} *Rag1*^{-/-} untransferred, green filled circles, dotted line, n=2 (A only); B6 *Rag1*^{-/-} untransferred, black filled circles, dotted line, n=2 (A only)). * p<0.05, ** p<0.01, *** p<0.001; CCR6^{GFP/GFP} *Rag1*^{-/-} + naïve CD4⁺ CD45RB^{hi} T cells versus B6 *Rag1*^{-/-} + naïve CD4⁺ CD45RB^{hi} T cells, per timepoint, determined by Mann Whitney U test.

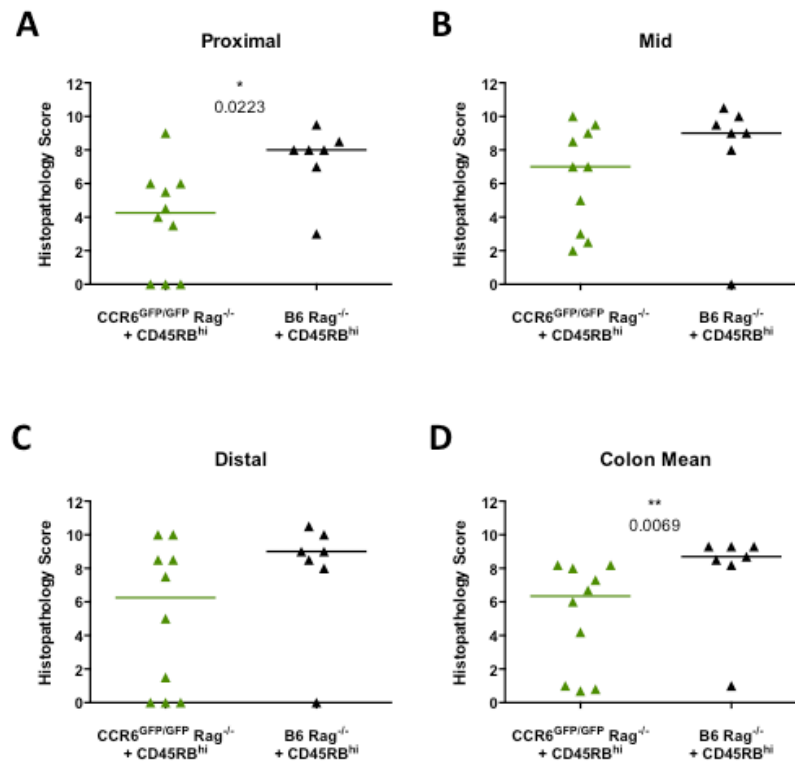


Figure 5.5 CCR6 deficiency lessens the severity of colonic pathology induced by naïve CD4⁺ T cell transfer.

CCR6^{GFP/GFP} Rag1^{-/-} mice or B6 Rag1^{-/-} controls received 2×10^5 naïve CD4⁺ CD45RB^{hi} T cells by i.p. injection, prior to analysis 5 weeks later. (A-D) Histopathology scores for (A) proximal, (B) mid, and (C) distal colon. (D) Mean histopathology score for whole colon (calculated as mean of proximal, mid and distal colon scores per mouse). Each data point represents an individual mouse, horizontal line indicates group median. Data are from one experiment (CCR6^{GFP/GFP} Rag1^{-/-} + naïve CD4⁺ CD45RB^{hi} T cells, green filled triangles, n=10; B6 Rag1^{-/-} + naïve CD4⁺ CD45RB^{hi} T cells, black filled triangles, n=7). * p<0.05, ** p<0.01, determined by Mann Whitney U test.

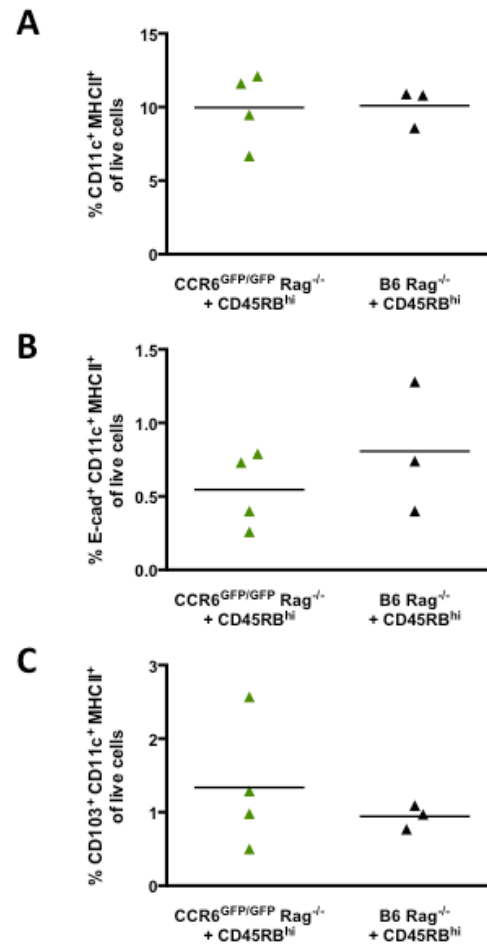


Figure 5.6 CCR6 deficiency does not affect accumulation of CD11c⁺ MHCII⁺ cells in the colon lamina propria during T cell transfer colitis.

CCR6^{GFP/GFP} Rag1^{-/-} mice or B6 Rag1^{-/-} controls received 2×10^5 naïve CD4⁺ CD45RB^{hi} T cells by i.p. injection, prior to analysis 5 weeks later. Single cell preparations from colon lamina propria of 2-4 pooled mice were stained and analysed by flow cytometry. (A) Percentage of live cells expressing CD11c and MHCII. (B-C) Percentage of live CD11c⁺ MHCII⁺ cells expressing (B) E-cadherin (E-cad) or (C) CD103. During analysis, samples were pre-gated to exclude debris by FSC/SSC. Each data point represents 2-4 pooled mice, horizontal line indicates group mean. Data are from one experiment (CCR6^{GFP/GFP} Rag1^{-/-} + naïve CD4⁺ CD45RB^{hi} T cells, green filled triangles, n=4; B6 Rag1^{-/-} + naïve CD4⁺ CD45RB^{hi} T cells, black filled triangles, n=3).

We next used the *Hh* + anti-IL-10R colitis model to explore the expression of CCR6 and other chemokine receptors by the subpopulations of MNPs that can be defined on the basis of CX₃CR1 expression. To do this, we sorted MNP populations from the colonic and caecal lamina propria of *Hh* + anti-IL-10R-treated CX₃CR1^{GFP/+} mice with colitis, using untreated CX₃CR1^{GFP/+} mice as controls, and measured chemokine receptor expression by RT-qPCR (for gating strategy see Figure 3.4). In steady state colon, CCR6 was expressed by the CX₃CR1^{int} CD64⁻ subset, which contains *bona fide* DCs (Scott et al., 2014), and by CX₃CR1^{hi} CD64⁺ resident macrophages, but at much lower levels by CD103⁺ DCs (Figure 5.7). We were unable to sort sufficient CX₃CR1^{int} CD64⁺ MNPs from untreated mice to carry out this analysis. None of the subsets expressed CCR6 in inflamed intestine. Our results indicate that monocytes and their progeny may express CCR6 under homeostatic conditions but not during *Hh* + anti-IL-10R colitis, consistent with the idea that this chemokine receptor is not involved in the accumulation of pro-inflammatory MNPs in any of the investigated models of colitis.

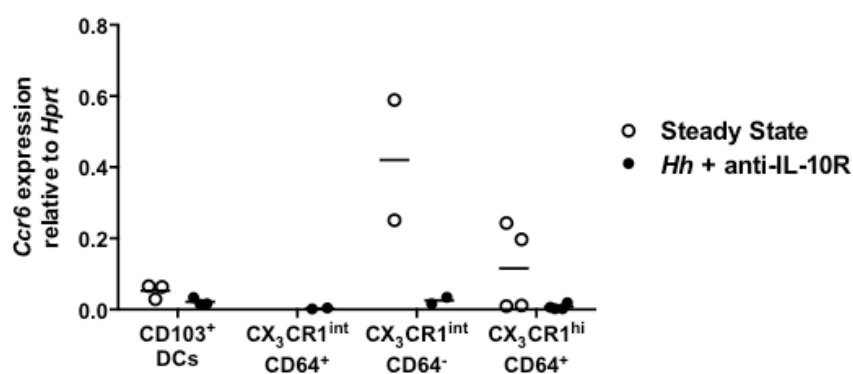


Figure 5.7 CCR6 expression by large intestinal mononuclear phagocyte subsets during homeostasis and *Helicobacter hepaticus* + anti-IL-10R colitis.

CX₃CR1^{GFP/+} mice were left untreated (steady state) or infected with *Helicobacter hepaticus* (*Hh*) and treated weekly with anti-IL-10R blocking mAb (*Hh* + anti-IL-10R) prior to analysis at three weeks. Single cell preparations of combined colonic and caecal lamina propria were stained and the indicated cell subsets isolated by FACS. For gating see Figure 3.4. RNA was extracted and RT-qPCR used to measure expression of *Ccr6*. Each data point represents a biological replicate of cells sorted from 4-7 pooled mice, horizontal line indicates group mean. Data are pooled from five independent experiments (steady state, open circles; *Hh* + anti-IL-10R, filled circles. CD103⁺ DCs, steady state, n=3-4; CD103⁺ DCs, *Hh* + anti-IL-10R, n=2-3; CX₃CR1^{int} CD64⁺, steady state, unavailable; CX₃CR1^{int} CD64⁺, *Hh* + anti-IL-10R, n=2; CX₃CR1^{int} CD64⁻, steady state, n=2; CX₃CR1^{int} CD64⁻, *Hh* + anti-IL-10R, n=2; CX₃CR1^{hi} CD64⁺, steady state, n=4; CX₃CR1^{hi} CD64⁺, *Hh* + anti-IL-10R, n=4-5).

5.2.2 Role of CCR2 in recruitment of MNPs to the colon during *Helicobacter hepaticus*-induced inflammation

Recent studies indicate that CCR2 can control Ly6C^{hi} monocyte migration to the intestine during homeostasis and DSS-, hapten- and *Citrobacter rodentium*-induced colitis (Bain et al., 2013; Khan et al., 2006; Kim et al., 2011b; Platt et al., 2010; Popivanova et al., 2009; Takada et al., 2010; Zigmond et al., 2012). We used RT-qPCR to measure expression of CCR2 by MNP subsets sorted from untreated and colitic *Hh* + anti-IL-10R-treated CX₃CR1^{GFP/+} mice as described above. We observed some expression of CCR2 by all subsets (Figure 5.8). CCR2 was most highly expressed by CX₃CR1^{int} CD64⁺ cells – which include Ly6C⁺ MHCII⁺ MNPs and Ly6C^{lo/-} MHCII⁺ macrophages (Figure 3.5; (Bain et al., 2013)) – from the inflamed colonic and caecal lamina propria during *Hh* + anti-IL-10R-induced colitis (Figure 5.8).

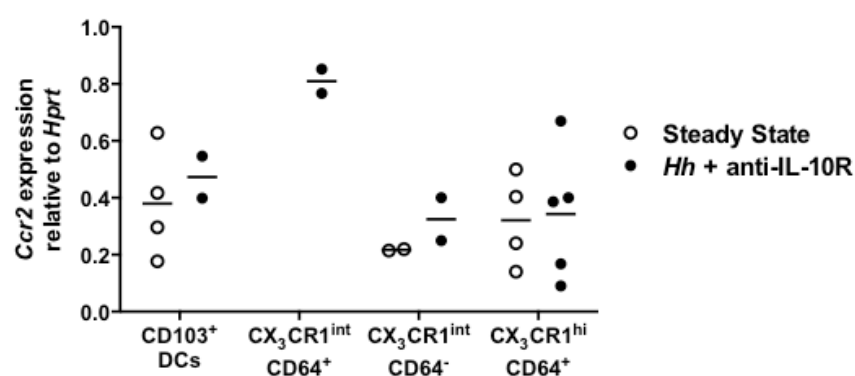


Figure 5.8 CCR2 expression by large intestinal mononuclear phagocyte subsets during homeostasis and *Helicobacter hepaticus* +anti-IL-10R colitis.

CX₃CR1^{GFP/+} mice were left untreated (steady state) or infected with *Helicobacter hepaticus* (*Hh*) and treated weekly with anti-IL-10R blocking mAb (*Hh* + anti-IL-10R) prior to analysis at three weeks. Single cell preparations of combined colonic and caecal lamina propria were stained and the indicated cell subsets isolated by FACS. For gating see Figure 3.4. RNA was extracted and RT-qPCR used to measure expression of *Ccr2*. Each data point represents a biological replicate of cells sorted from 4-7 pooled mice, horizontal line indicates group mean. Data are pooled from five independent experiments (steady state, open circles; *Hh* + anti-IL-10R, filled circles. CD103⁺ DCs, steady state, n=3-4; CD103⁺ DCs, *Hh* + anti-IL-10R, n=2-3; CX₃CR1^{int} CD64⁺, steady state, unavailable; CX₃CR1^{int} CD64⁺, *Hh* + anti-IL-10R, n=2; CX₃CR1^{int} CD64⁻, steady state, n=2; CX₃CR1^{int} CD64⁻, *Hh* + anti-IL-10R, n=2; CX₃CR1^{hi} CD64⁺, steady state, n=4; CX₃CR1^{hi} CD64⁺, *Hh* + anti-IL-10R, n=4-5).

Having confirmed its expression, we investigated whether CCR2 is involved in the recruitment of Ly6C^{hi} monocytes to the large intestine during *Helicobacter hepaticus*-induced colitis. To do this we used MC-21, a monoclonal antibody which blocks CCR2 specifically *in vivo* (Mack et al., 2001).

MC-21 can be administered up to a maximum of five times, usually daily, after which it is no longer effective most likely due to a humoral immune response against the antibody (Bruhl et al., 2007). Peak intestinal pathology caused by *Hh* infection and anti-IL-10R treatment occurs within two to three weeks (Figure 3.1). In order to observe any effect of CCR2 blockade on pathology, it was necessary to allow sufficient time for colitis to fully develop, therefore we conducted a pilot experiment to determine whether the period of MC-21 treatment could be extended to eight days by injecting the antibody every second day. To do so, we measured the frequency of Ly6C^{hi} monocytes in the blood of CX₃CR1^{GFP/+} mice following treatment with one 20 µg dose of MC-21. We found that MC-21 treatment induced a reduction in the frequency of circulating Ly6C^{hi} monocytes two days after treatment, which had returned to baseline by day 4 (Figure 5.9a). We observed no effect of MC-21 treatment on the CX₃CR1^{hi} Ly6C^{lo} resident monocyte subset (Figure 5.9b), which does not express CCR2 (Geissmann et al., 2003). Since we observed a reduction in circulating Ly6C^{hi} monocytes two days after antibody administration, we proceeded to administer MC-21 by intraperitoneal injection every second day.

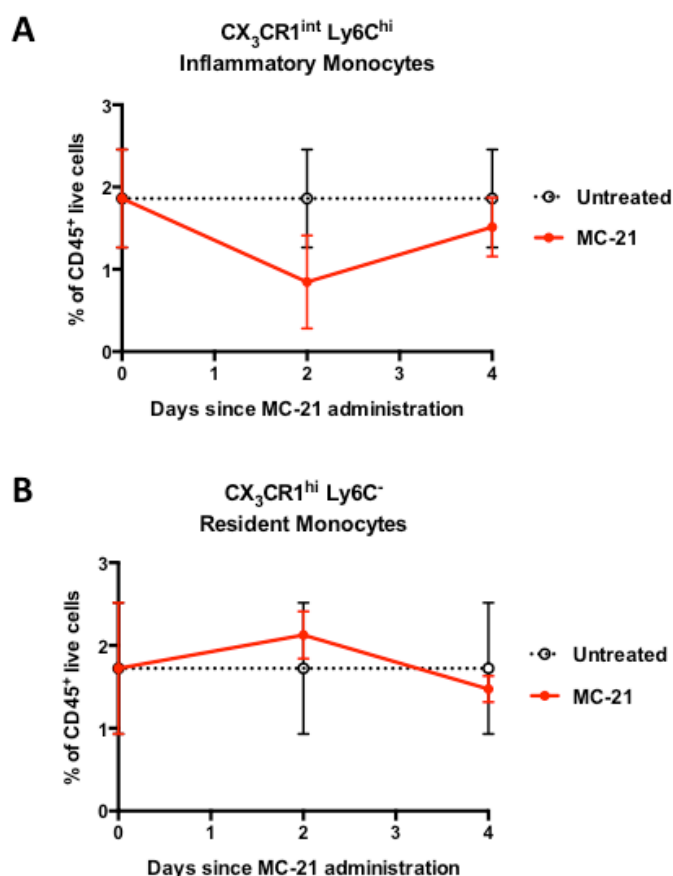


Figure 5.9 Effect of one 20 µg dose of MC-21 on blood monocyte frequencies.

$CX_3CR1^{GFP/+}$ mice were treated once with 20 µg CCR2-specific mAb (MC-21) by i.p. injection. Blood was collected from the tail vein 2 days after MC-21 treatment and by cardiac puncture following sacrifice on day 4. Blood was collected from untreated mice to generate a baseline measurement of blood monocyte frequencies. Blood samples were treated with ACK lysis buffer to remove red blood cells and were subsequently stained and analysed by flow cytometry. Frequency of (A) $CX_3CR1^{int} Ly6C^{hi}$ inflammatory monocytes and (B) $CX_3CR1^{hi} Ly6C^{-}$ resident monocytes among CD45⁺ live cells. Populations were pre-gated on live/dead⁻ CD45⁺ CD11b⁺ Ly6G⁻ Siglec F⁻ cells. Results represent the group mean $\pm$ SD. Data are from one experiment ($CX_3CR1^{GFP/+}$ untreated, black open circles and dotted line $n=3$ mice; $CX_3CR1^{GFP/+}$ + MC-21, red filled circles and solid line, $n=3$ mice).

To test the role of CCR2 in the recruitment of Ly6C^{hi} monocytes to the colon during the initial phase of *Hh* + anti-IL-10R-induced colitis, we administered MC-21 to CX₃CR1^{GFP/+} mice from day 0 until day 8 after *Hh* infection and anti-IL-10R treatment, the latter continuing weekly until analysis at three weeks post-infection. In an initial experiment, we observed less severe pathology in MC-21-treated versus isotype control-treated mice. However, when pooled with the results of a repeat experiment, there were no overall differences in colonic or caecal disease severity between MC-21-treated mice and controls (Figure 5.10). We also observed no differences in the frequencies of CX₃CR1^{int} Ly6C^{hi} monocytes, CX₃CR1^{int} Ly6C⁺ MHCII⁺ MNPs, CX₃CR1^{int} Ly6C^{lo} macrophages, CX₃CR1^{hi} resident macrophages, neutrophils, CX₃CR1^{int} Ly6C^{lo} CD64⁻ MNPs, CD103⁺ CD11b⁻ DCs, or CD103⁺ CD11b⁺ DCs in the colonic and caecal lamina propria following MC-21 treatment (see Figure 5.11 for gating strategy; Figures 5.12 and 5.13).

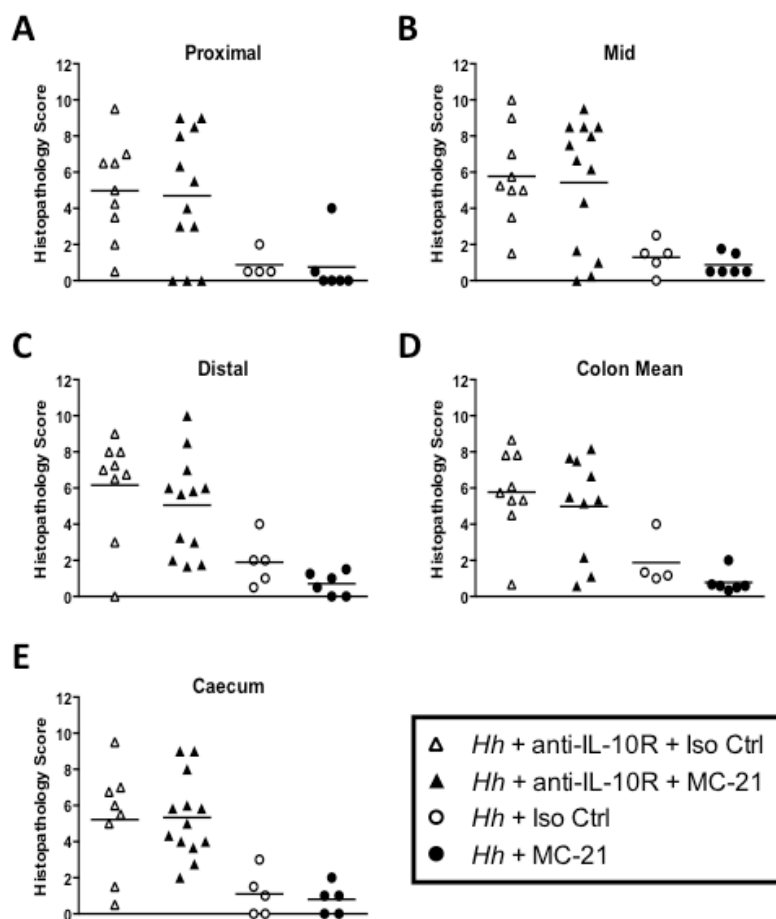


Figure 5.10 MC-21 treatment does not affect large intestinal pathology at week 3 of *Helicobacter hepaticus* + anti-IL-10R colitis.

CX₃CR1^{GFP/+} mice were infected with *Helicobacter hepaticus* (*Hh*) with or without weekly anti-IL-10R blocking mAb treatment ("*Hh* + anti-IL-10R" and "*Hh*", respectively). Mice were also treated 5 times with a CCR2-specific mAb (MC-21), or isotype control (Iso Ctrl), once every second day from d0 to d8, prior to analysis at three weeks. (A-E) Histopathology scores for (A) proximal, (B) mid, and (C) distal colon; (D) whole colon, determined as mean of proximal + mid + distal colon scores, where all were available; and (E) caecum. Each data point represents an individual mouse, horizontal line indicates group mean. Data are pooled from two independent experiments (CX₃CR1^{GFP/+} *Hh* + anti-IL-10R + isotype control, open triangles, n=8-9; CX₃CR1^{GFP/+} *Hh* + anti-IL-10R + MC-21, filled triangles, n=10-13; CX₃CR1^{GFP/+} *Hh* + isotype control, open circles, n=4-5; CX₃CR1^{GFP/+} *Hh* + MC-21, filled circles, n=5-6. Variety in "n" due to inadequacy of some tissue sections for scoring).

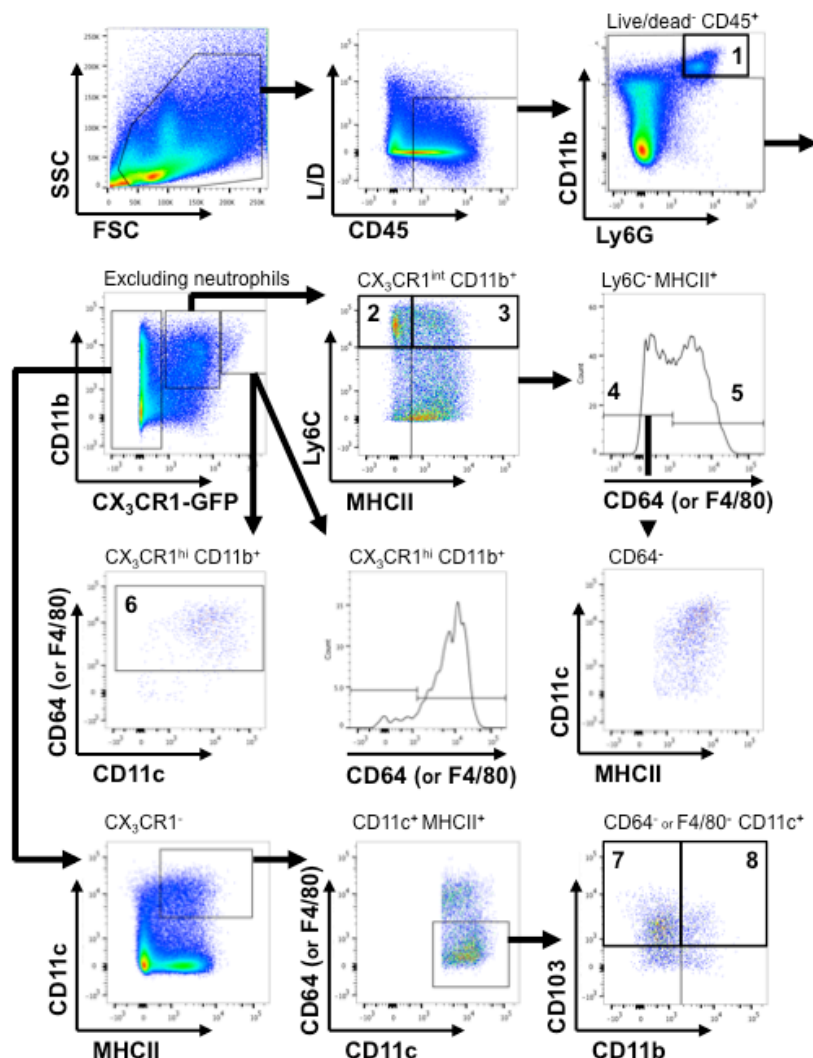


Figure 5.11 FACS gating strategy for myeloid populations in the caecal and colonic lamina propria of $CX_3CR1^{GFP/+}$ mice.

Full gating strategy used to identify neutrophils (1), CX_3CR1^{int} Ly6C⁺ MHCII⁻ monocytes (2), CX_3CR1^{int} Ly6C⁺ MHCII⁺ MNPs (3), CX_3CR1^{int} Ly6C^{lo} MHCII⁺ CD64⁻ (or F4/80⁻) MNPs (4), CX_3CR1^{int} Ly6C^{lo} MHCII⁺ CD64⁺ (or F4/80⁺) macrophages (5), CX_3CR1^{hi} Ly6C^{lo} MHCII⁺ CD64⁺ (or F4/80⁺) macrophages (6), CD103⁺ CD11b⁻ DCs (7) and CD103⁺ CD11b⁺ DCs (8) by flow cytometric analysis of stained single cell preparations of combined caecal and colonic lamina propria from individual mice. Example from a representative $CX_3CR1^{GFP/+}$ mouse infected with *Hh* and treated with anti-IL-10R for three weeks.

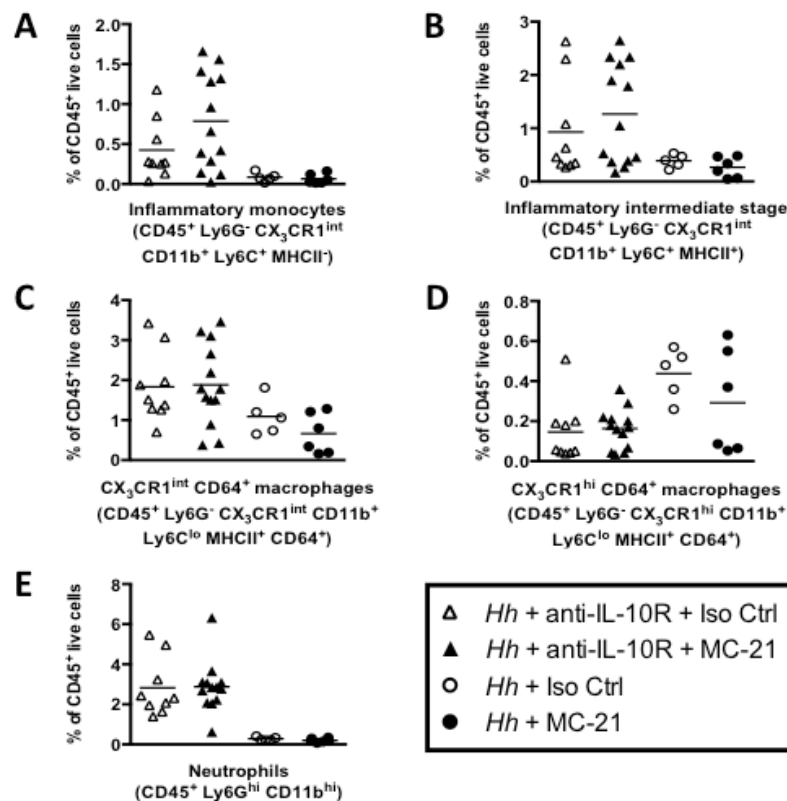


Figure 5.12 MC-21 treatment does not affect large intestinal mononuclear phagocyte subset frequencies at week 3 of *Helicobacter hepaticus* + anti-IL-10R colitis.

CX₃CR1^{GFP/+} mice were infected with *Helicobacter hepaticus* (*Hh*) with or without weekly anti-IL-10R blocking mAb treatment ("*Hh* + anti-IL-10R" and "*Hh*", respectively). Mice were also treated five times with a CCR2-specific mAb (MC-21), or isotype control, once every second day from d0 to d8, prior to analysis at three weeks. (A-F) Single cell preparations of combined colonic and caecal lamina propria from individual mice were stained and analysed by flow cytometry. Frequency of (A) inflammatory monocytes, (B) pro-inflammatory CX₃CR1^{int} intermediate stage MNPs, (C) CX₃CR1^{int} CD64⁺ Ly6C^{lo} macrophages, (D) CX₃CR1^{hi} CD64⁺ Ly6C^{lo} macrophages, and (E) Neutrophils. Each data point represents an individual mouse, horizontal line indicates group mean. Data are pooled from two independent experiments (CX₃CR1^{GFP/+} *Hh* + anti-IL-10R + isotype control, open triangles, n=9; CX₃CR1^{GFP/+} *Hh* + anti-IL-10R + MC-21, filled triangles, n=13; CX₃CR1^{GFP/+} *Hh* + isotype control, open circles, n=5; CX₃CR1^{GFP/+} *Hh* + MC-21, filled circles, n=6).

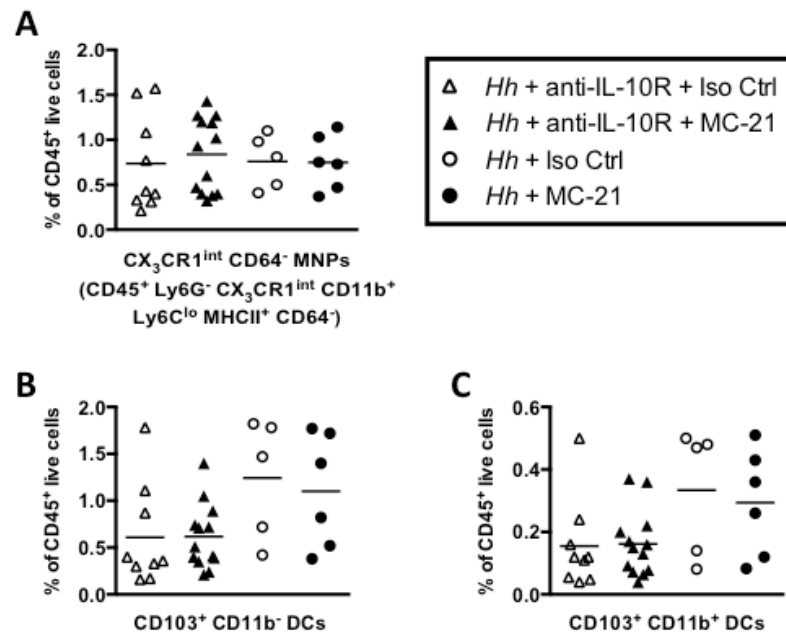


Figure 5.13 MC-21 treatment does not affect large intestinal mononuclear phagocyte subset frequencies at week 3 of *Helicobacter hepaticus* + anti-IL-10R colitis.

CX₃CR1^{GFP/+} mice were infected with *Helicobacter hepaticus* (*Hh*) with or without weekly anti-IL-10R blocking mAb treatment ("*Hh* + anti-IL-10R" and "*Hh*", respectively). Mice were also treated five times with a CCR2-specific mAb (MC-21), or isotype control, once every second day from d0 to d8, prior to analysis at three weeks. (A-C) Single cell preparations of combined colonic and caecal lamina propria from individual mice were stained and analysed by flow cytometry. Frequency of (A) CX₃CR1^{int} CD64⁻ MNPs, (B) CD103⁺ CD11b⁻ DCs, and (C) CD103⁺ CD11b⁺ DCs. Each data point represents an individual mouse, horizontal line indicates group mean. Data are pooled from two independent experiments (CX₃CR1^{GFP/+} *Hh* + anti-IL-10R + isotype control, open triangles, n=9; CX₃CR1^{GFP/+} *Hh* + anti-IL-10R + MC-21, filled triangles, n=13; CX₃CR1^{GFP/+} *Hh* + isotype control, open circles, n=5; CX₃CR1^{GFP/+} *Hh* + MC-21, filled circles, n=6).

It is possible that we observed no differences in MNP population frequencies because these were measured almost two weeks after the final dose of MC-21, during which time Ly6C^{hi} monocyte recruitment could have resumed. We investigated an earlier timepoint – day 13 post-*Hh* infection, 5 days after cessation of MC-21 administration – but one still sufficient to allow development of colitis (Figure 3.1). At day 13, although we observed a trend towards fewer cells, both total and CD45⁺, in the lamina propria of MC-21-treated mice (Figure 5.14), again we did not observe significant differences in the frequencies of MNP subsets or neutrophils between MC-21- and isotype control-treated mice (Figures 5.15 and 5.16).

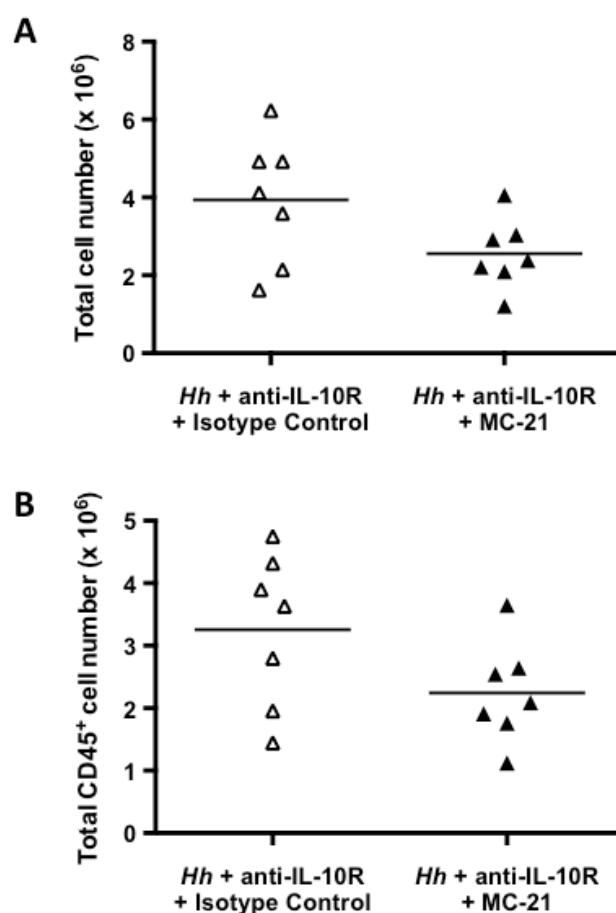


Figure 5.14 Total number of viable cells in large intestinal single cell preparations at day 13 of *Helicobacter hepaticus* + anti-IL-10R colitis.

CX₃CR1^{GFP/+} mice were infected with *Helicobacter hepaticus* (*Hh*) and treated weekly with anti-IL-10R blocking mAb. Mice were also treated five times with a CCR2-specific mAb (MC-21), or isotype control, once every second day from d0 to d8, prior to sacrifice and analysis on d13. Single cell preparations of combined colonic and caecal lamina propria from individual mice were stained and analysed by flow cytometry. (A) Total number of live cells per intestine. (B) Total number of CD45⁺ cells per intestine. Each data point represents an individual mouse, horizontal line indicates group mean. Data are from one experiment (CX₃CR1^{GFP/+} *Hh* + anti-IL-10R + isotype control, open triangles, n=7; CX₃CR1^{GFP/+} *Hh* + anti-IL-10R + MC-21, filled triangles, n=7).

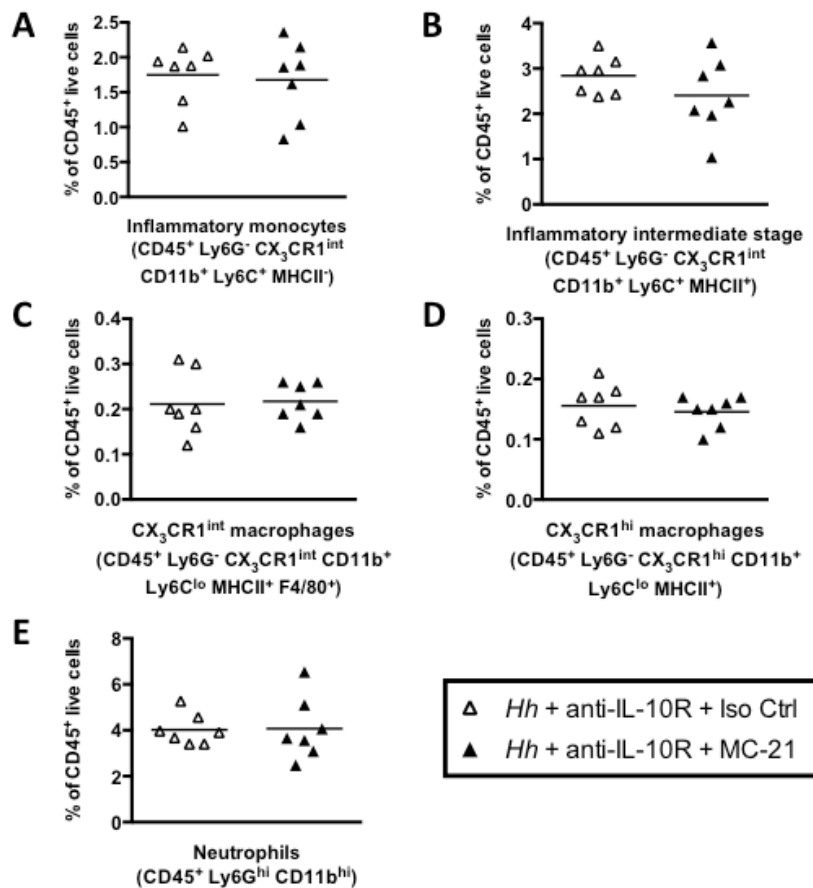


Figure 5.15 MC-21 treatment does not affect large intestinal mononuclear phagocyte subset frequencies at day 13 of *Helicobacter hepaticus* + anti-IL-10R colitis.

CX₃CR1^{GFP/+} mice were infected with *Helicobacter hepaticus* (*Hh*) and treated weekly with anti-IL-10R blocking mAb. Mice were also treated five times with a CCR2-specific mAb (MC-21), or isotype control, once every second day from d0 to d8, prior to sacrifice and analysis on d13. (A-F) Single cell preparations of combined colonic and caecal lamina propria from individual mice were stained and analysed by flow cytometry. Frequency of (A) inflammatory monocytes, (B) pro-inflammatory CX₃CR1^{int} intermediate stage MNPs, (C) CX₃CR1^{int} Ly6C^{lo} macrophages, (D) CX₃CR1^{hi} Ly6C^{lo} macrophages, and (E) Neutrophils. Each data point represents an individual mouse, horizontal line indicates group mean. Data are from one experiment (CX₃CR1^{GFP/+} *Hh* + anti-IL-10R + isotype control, open triangles, n=7; CX₃CR1^{GFP/+} *Hh* + anti-IL-10R + MC-21, filled triangles, n=7).

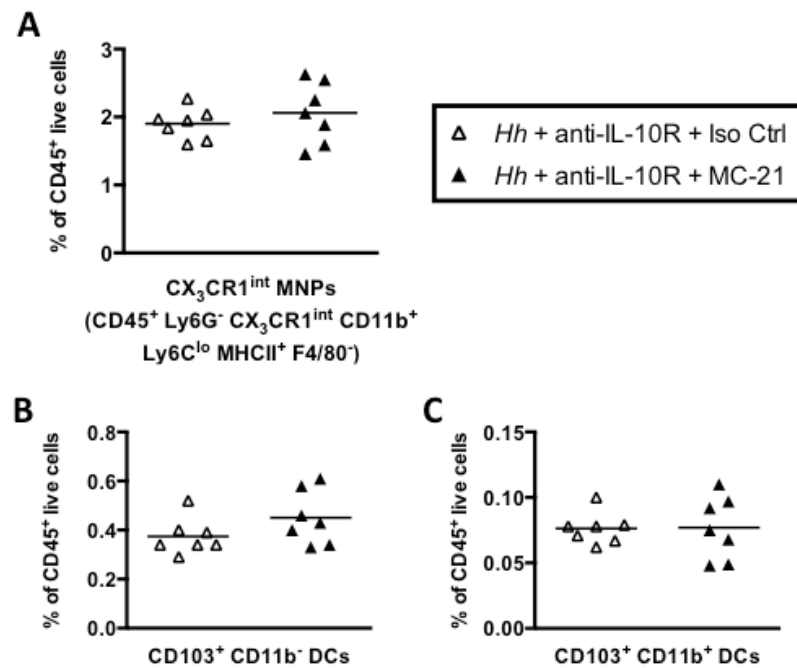


Figure 5.16 MC-21 treatment does not affect large intestinal mononuclear phagocyte subset frequencies at day 13 of *Helicobacter hepaticus* + anti-IL-10R colitis.

CX₃CR1^{GFP/+} mice were infected with *Helicobacter hepaticus* (*Hh*) and treated weekly with anti-IL-10R blocking mAb. Mice were also treated five times with a CCR2-specific mAb (MC-21), or isotype control, once every second day from d0 to d8, prior to sacrifice and analysis on d13. (A-C) Single cell preparations of combined colonic and caecal lamina propria from individual mice were stained and analysed by flow cytometry. Frequency of (A) CX₃CR1^{int} F4/80⁻ MNPs, (B) CD103⁺ CD11b⁻ DCs, and (C) CD103⁺ CD11b⁺ DCs. Each data point represents an individual mouse, horizontal line indicates group mean. Data are from one experiment (CX₃CR1^{GFP/+} *Hh* + anti-IL-10R + isotype control, open triangles, n=7; CX₃CR1^{GFP/+} *Hh* + anti-IL-10R + MC-21, filled triangles, n=7).

Having seen no differences in colitis severity or frequencies of myeloid populations in the lamina propria when analysis was carried out at two timepoints following cessation of MC-21 treatment, we analysed the lamina propria MNP compartment *during* MC-21 treatment when we should have been able to detect any effect on early Ly6C^{hi} monocyte recruitment to the colon. Five days following *Hh* infection and anti-IL-10R treatment, after three doses of MC-21 on days 0, 2 and 4, we observed trends towards reduced frequencies of CX₃CR1^{int} Ly6C^{hi} monocytes, CX₃CR1^{int} Ly6C⁺ MHCII⁺ MNPs, CX₃CR1^{int} Ly6C^{lo} F4/80⁻ MNPs and to a lesser extent CX₃CR1^{int} Ly6C^{lo} macrophages in MC-21-treated versus isotype control-treated mice, although the differences were not statistically significant (Figure 5.17a-c, f). Importantly, the frequency of CX₃CR1^{hi} resident macrophages was unaffected (Figure 5.17d), as was the frequency of neutrophils (Figure 5.17e). We also observed a downward trend in the frequency of Ly6C^{hi} monocytes in the blood at this timepoint, though this too was not statistically significant likely due to the variability in the MC-21-treated group (Figure 5.18).

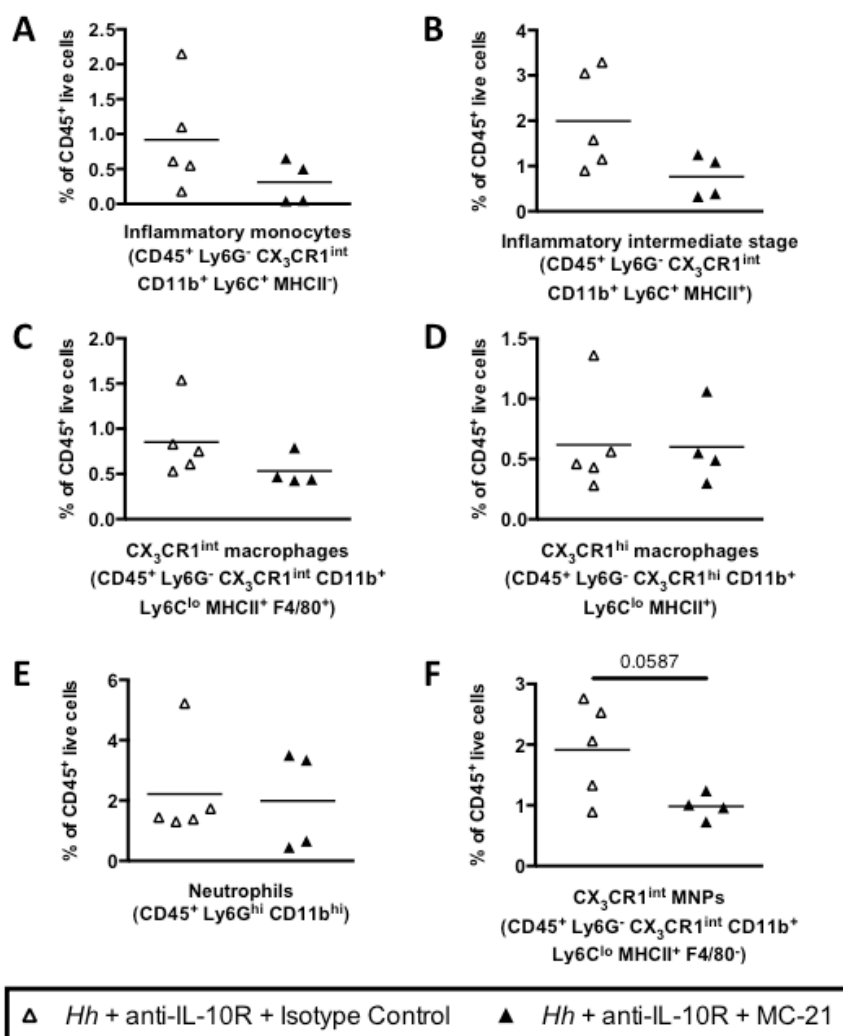


Figure 5.17 Large intestinal mononuclear phagocyte subset frequencies at day 5 of *Helicobacter hepaticus* + anti-IL-10R colitis during MC-21 treatment.

CX₃CR1^{GFP/+} mice were infected with *Helicobacter hepaticus* (*Hh*) and treated once with anti-IL-10R blocking mAb. Mice were also treated three times with a CCR2-specific mAb (MC-21), or isotype control, once every second day from d0 to d3, prior to sacrifice and analysis on d5. (A-F) Single cell preparations of combined colonic and caecal lamina propria from individual mice were stained and analysed by flow cytometry. Frequency of (A) inflammatory monocytes, (B) pro-inflammatory CX₃CR1^{int} intermediate stage MNPs, (C) CX₃CR1^{int} Ly6C^{lo} macrophages, (D) CX₃CR1^{hi} Ly6C^{lo} macrophages, (E) Neutrophils, and (F) CX₃CR1^{int} F4/80⁻ MNPs. Each data point represents an individual mouse, horizontal line indicates group mean. Data are from one experiment (CX₃CR1^{GFP/+} *Hh* + anti-IL-10R + isotype control, open triangles, n=5; CX₃CR1^{GFP/+} *Hh* + anti-IL-10R + MC-21, filled triangles, n=4). p=0.0587 determined by unpaired Student's t test.

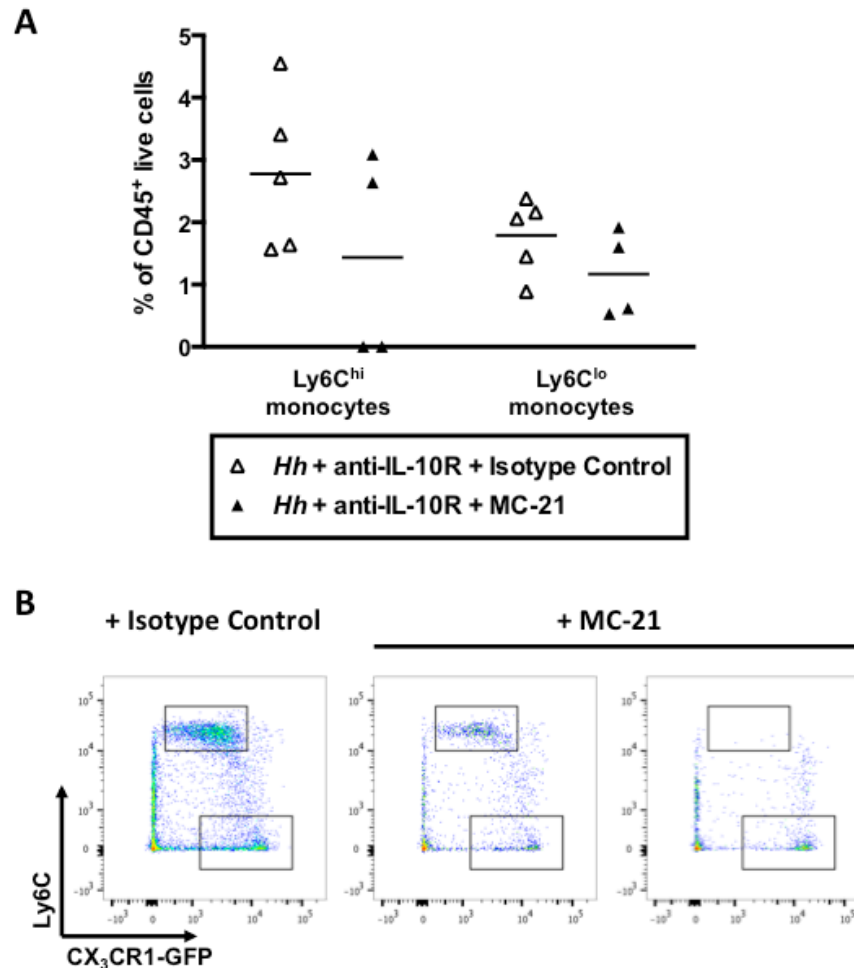


Figure 5.18 Blood monocyte subset frequencies at day 5 of *Helicobacter hepaticus* + anti-IL-10R colitis during MC-21 treatment .

CX₃CR1^{GFP/+} mice were infected with *Helicobacter hepaticus* (*Hh*) and treated once with anti-IL-10R blocking mAb. Mice were also treated three times with a CCR2-specific mAb (MC-21), or isotype control, once every second day from d0 to d4, prior to sacrifice and analysis on d5. Blood samples, collected by cardiac puncture following sacrifice, were ACK lysed, stained and analysed by flow cytometry. (A and B) Pre-gated on live CD45⁺ Ly6G⁻ CD11b⁺ Siglec F⁻ cells. (A) Frequency of CX₃CR1^{int} Ly6C^{hi} monocytes and CX₃CR1^{hi} Ly6C^{lo} monocytes. Each data point represents an individual mouse, horizontal line indicates group mean. Data are from one experiment (CX₃CR1^{GFP/+} *Hh* + anti-IL-10R + isotype control, open triangles, n=5; CX₃CR1^{GFP/+} *Hh* + anti-IL-10R + MC-21, filled triangles, n=4). (B) Representative FACS plots showing blood monocytes from one isotype control-treated and two MC-21-treated mice.

5.2.3 Recruitment of MNPs to the colon downstream of IL-23 during *Helicobacter hepaticus*-induced inflammation

Following inconclusive results regarding CCR2, we moved on to investigate the possible involvement of other chemokine receptors in the accumulation of inflammatory MNPs during *Hh* + anti-IL-10R colitis. We showed in Chapter 4 that CD11c⁺ cell-derived IL-23 is required for the development of colonic and caecal pathology and for the accumulation of Ly6C^{hi} monocytes and Ly6C⁺ MHCII⁺ MNPs during this model (Figures 4.1 and 4.8a-b). In this series of experiments we compared CD11c^{IL-23⁻} *Hh*-infected anti-IL-10R-treated mice which did not exhibit colitis due to the absence of IL-23 production by CD11c⁺ cells, with colitic *Hh* + anti-IL-10R-treated IL-23-sufficient (CD11c^{IL-23⁺}) controls, and with *Hh* only or uninfected CD11c^{IL-23⁺} uninflamed control mice, as described in Chapter 4. To investigate a possible link between the reduced accumulation of inflammatory MNPs in the absence of IL-23 and a chemokine-chemokine receptor axis, we measured the expression of various chemokines and their receptors by RT-qPCR on total colonic and caecal lamina propria cells isolated from these groups. We found no difference in *Ccr2* expression between CD11c^{IL-23⁺} and CD11c^{IL-23⁻} mice three weeks after *Hh* infection and anti-IL-10R treatment, which respectively showed a trend towards and significantly increased *Ccr2* expression relative to *Hh* only and uninfected CD11c^{IL-23⁺} controls (Figure 5.19a). Interestingly, while preliminary results indicate that expression of CCL2 mRNA was unaffected by CD11c-Cre-driven IL-23 deficiency, levels of CCL2 protein secreted by lamina propria cells were reduced during inflammation (*Hh* + anti-IL-10R-treated CD11c^{IL-23⁺} mice) (Figure 5.19b-c). CCL2 protein secretion by cells from CD11c^{IL-23⁻} mice treated with *Hh* + anti-IL-10R appeared to be at an intermediate level, which was not statistically significantly different from CD11c^{IL-23⁺} inflamed or uninflamed controls (Figure

5.19c). The same was true for secretion of another CCR2 ligand, CCL7 (Figure 5.19d).

These results suggest that CCL2 and CCL7 could be negatively regulated during inflammation.

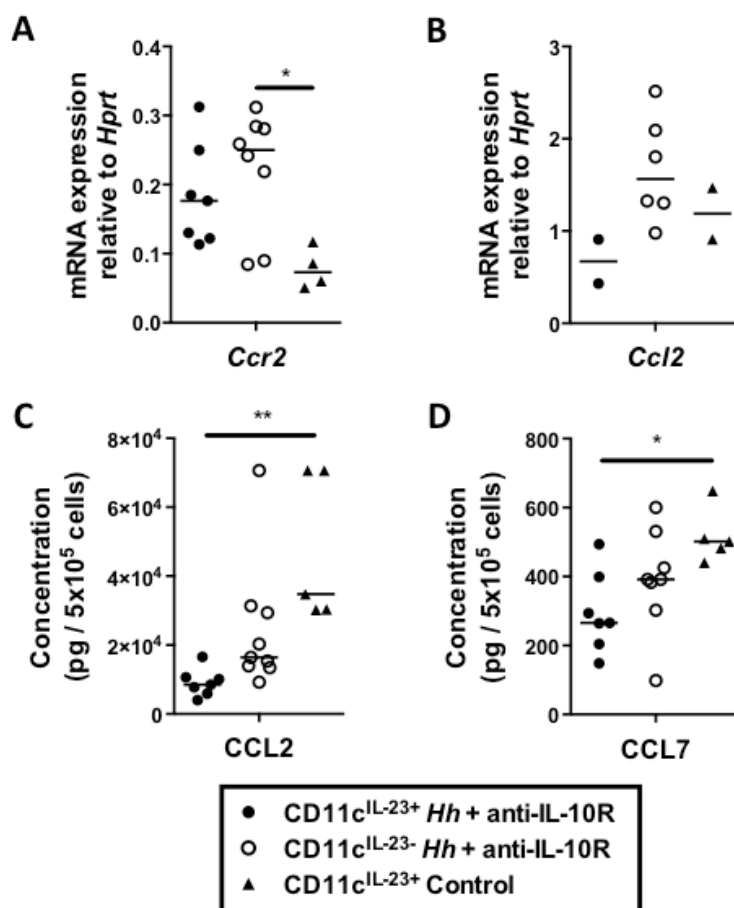


Figure 5.19 CCR2 chemokine receptor and ligand expression during *Helicobacter hepaticus* + anti-IL-10R colitis.

CD11c-Cre⁺ *Il23a*^{fl/fl} (CD11c^{IL-23+}) mice or pooled CD11c-Cre⁻ *Il23a*^{fl/fl} and CD11c-Cre⁻ *Il23a*^{fl/fl} (CD11c^{IL-23+}) littermate controls were infected with *Helicobacter hepaticus* (*Hh*) and treated weekly with anti-IL-10R blocking mAb prior to analysis after three weeks. Expression of (A) *Ccr2* and (B) *Ccl2*, and production of (C) CCL2 and (D) CCL7 were assessed by (A, B) RT-qPCR on 500,000 freshly isolated lamina propria cells, or (C, D) multiplex flow cytometric assay on supernatants from 500,000 *ex vivo* lamina propria cells cultured for 48 hours without stimulation. Data are from one or two pooled independent experiments (CD11c^{IL-23+} *Hh* + anti-IL-10R, filled circles, n=2-7; CD11c^{IL-23-} *Hh* + anti-IL-10R, open circles, n=6-9; CD11c^{IL-23+} *Hh* only or uninfected control, filled triangles, n=2-5). Each data point represents an individual mouse, horizontal line indicates group median. * p<0.05, ** p<0.01; determined by Kruskal-Wallis test with Dunn's post-test correction for multiple comparisons.

CCR1 and one of its ligands, CCL3, have been shown to control monocyte recruitment to the small intestine during an experimental model of ileitis caused by oral infection with the parasite *Toxoplasma gondii* (Schulthess et al., 2012). We observed high expression of *Ccr1* by Ly6C^{hi} monocyte-derived CX₃CR1^{int} CD64⁺ MNPs in the lamina propria during *Hh* + anti-IL-10R colitis (Figure 5.20). CX₃CR1^{int} CD64⁻ MNPs and CX₃CR1^{hi} CD64⁺ macrophages also expressed *Ccr1* under both homeostatic and inflammatory conditions, though slightly higher expression was exhibited by the steady state resident macrophages. During inflammation, but not in its absence, total CD103⁺ DCs also expressed *Ccr1*. During this model, significantly lower expression of *Ccr1* and secretion of CCL3 were observed among lamina propria cells from uninflamed *Hh* + anti-IL-10R-treated CD11c^{IL-23-} mice than among those from colitic CD11c^{IL-23+} counterparts (Figure 5.21a, c), while a trend towards the same was observed in *Ccl3* mRNA expression (Figure 5.21b). These results suggest that the CCR1-CCL3 axis may be augmented downstream of IL-23.

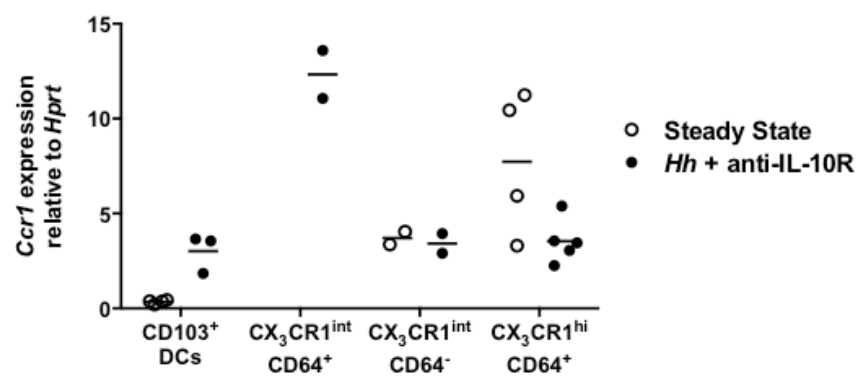


Figure 5.20 CCR1 expression by large intestinal mononuclear phagocyte subsets during homeostasis and *Helicobacter hepaticus* + anti-IL-10R colitis.

CX₃CR1^{GFP/+} mice were left untreated (steady state) or infected with *Helicobacter hepaticus* (*Hh*) and treated weekly with anti-IL-10R blocking mAb (*Hh* + anti-IL-10R) prior to analysis at three weeks. Single cell preparations of combined colonic and caecal lamina propria were stained and the indicated cell subsets isolated by FACS. For gating see Figure 3.4. RNA was extracted and RT-qPCR used to measure expression of *Ccr1*. Each data point represents a biological replicate of cells sorted from 4-7 pooled mice, horizontal line indicates group mean. Data are pooled from five independent experiments (steady state, open circles; *Hh* + anti-IL-10R, filled circles. CD103⁺ DCs, steady state, n=3-4; CD103⁺ DCs, *Hh* + anti-IL-10R, n=2-3; CX₃CR1^{int} CD64⁺, steady state, unavailable; CX₃CR1^{int} CD64⁺, *Hh* + anti-IL-10R, n=2; CX₃CR1^{int} CD64⁻, steady state, n=2; CX₃CR1^{int} CD64⁻, *Hh* + anti-IL-10R, n=2; CX₃CR1^{hi} CD64⁺, steady state, n=4; CX₃CR1^{hi} CD64⁺, *Hh* + anti-IL-10R, n=4-5).

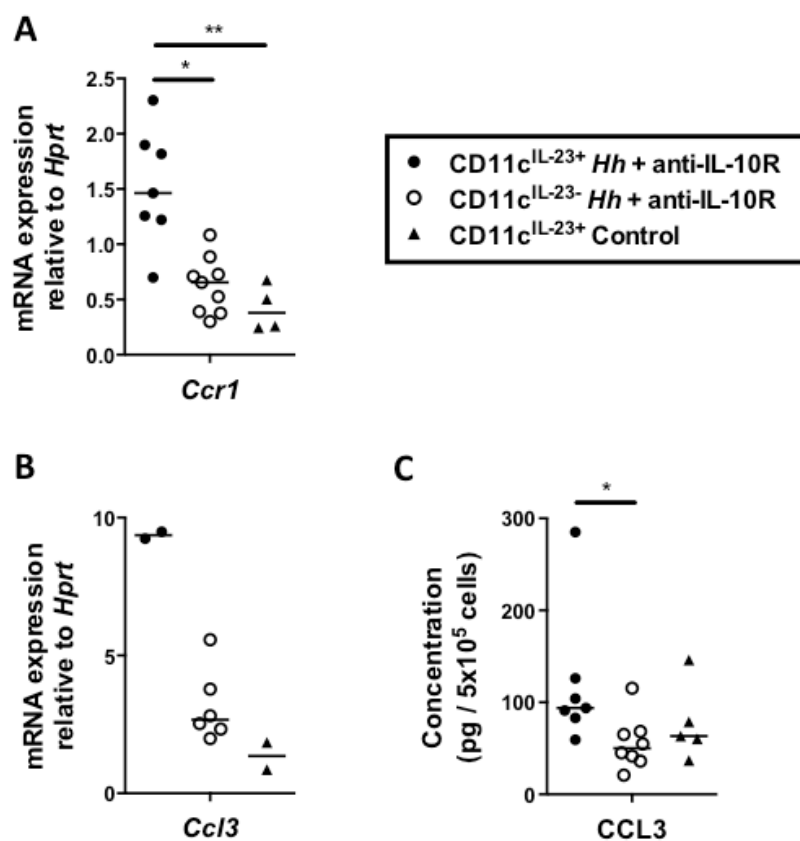


Figure 5.21 CCR1 chemokine receptor and ligand expression during *Helicobacter hepaticus* + anti-IL-10R colitis.

$CD11c-Cre^+ Il23a^{fl/fl}$ ($CD11c^{IL-23-}$) mice or pooled $CD11c-Cre^- Il23a^{fl/fl}$ and $CD11c-Cre^- Il23a^{fl/fl}$ ($CD11c^{IL-23+}$) littermate controls were infected with *Helicobacter hepaticus* (*Hh*) and treated weekly with anti-IL-10R blocking mAb prior to analysis after three weeks. Expression of (A) *Ccr1* and (B) *Ccl3*, and production of (C) CCL3 were assessed by (A, B) RT-qPCR on 500,000 freshly isolated lamina propria cells, or (C) multiplex flow cytometric assay on supernatants from 500,000 *ex vivo* lamina propria cells cultured for 48 hours without stimulation. Data are from one or two pooled independent experiments ($CD11c^{IL-23+} Hh + anti-IL-10R$, filled circles, $n=2-7$; $CD11c^{IL-23-} Hh + anti-IL-10R$, open circles, $n=6-9$; $CD11c^{IL-23+} Hh$ only or uninfected control, filled triangles, $n=2-5$). Each data point represents an individual mouse, horizontal line indicates group median. * $p<0.05$; ** $p<0.01$; determined by Kruskal-Wallis test with Dunn's post-test correction for multiple comparisons.

CCL3 also binds to CCR5 (Rot and von Andrian, 2004), which we observed to be preferentially expressed by CX₃CR1^{int} CD64⁺ MNPs in the lamina propria of CX₃CR1^{GFP/+} mice with *Hh* + anti-IL-10R colitis (Figure 5.22). *Ccr5* was essentially not expressed by total CD103⁺ DCs, and was expressed only at low levels by CX₃CR1^{int} CD64⁻ MNPs and CX₃CR1^{hi} CD64⁺ macrophages. While these are preliminary results, *Ccr5* expression appeared to be mildly increased among CX₃CR1^{int} CD64⁻ MNPs during inflammation. Preliminary data indicate no difference between CD11c^{IL-23+} and CD11c^{IL-23-} *Hh* + anti-IL-10R-treated mice in their expression of *Ccr5* among total lamina propria cells, despite the respective presence and absence of inflammation and inflammatory MNPs (Figure 5.23a). Preliminary results suggest that expression of *Ccl4*, encoding CCL4 which binds solely to CCR5 (Rot and von Andrian, 2004), by lamina propria cells from uninflamed CD11c^{IL-23-} *Hh* + anti-IL-10R-treated mice may remain at steady state levels, in contrast to its potential elevation in colitic IL-23-sufficient controls (Figure 5.23b), suggesting that expression of CCL4 could be induced downstream of IL-23.

Although it relates to migration away from rather than to the intestine, we also measured expression of CCR7, as a functionally important chemokine receptor. CCR7 expression is considered a hallmark of migratory DCs as it controls cellular migration to tissue-draining lymph nodes, including the migration of DCs from the small intestine to the MLNs (Jang et al., 2006; Johansson-Lindbom et al., 2005). In agreement with a recent study (Zigmond et al., 2012) and the description of *bona fide* DCs among this population (Scott et al., 2014), we observed expression of *Ccr7* by CX₃CR1^{int} CD64⁻ MNPs (Figure 5.24). Surprisingly, we also found expression of *Ccr7* by CX₃CR1^{int} CD64⁺ MNPs from the inflamed lamina propria, and only very low expression of *Ccr7* by CD103⁺ DCs, both CD11b⁺ and CD11b⁻, relative to

CX₃CR1^{int} CD64⁺ and CX₃CR1^{int} CD64⁻ MNPs. As expected, *Ccr7* expression was also very low among CX₃CR1^{hi} CD64⁺ resident macrophages.

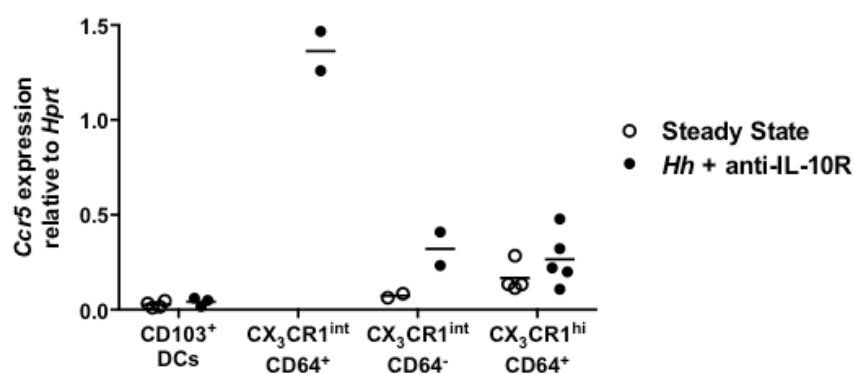


Figure 5.22 CCR5 expression by large intestinal mononuclear phagocyte subsets during homeostasis and *Helicobacter hepaticus* + anti-IL-10R colitis.

CX₃CR1^{GFP/+} mice were left untreated (steady state) or infected with *Helicobacter hepaticus* (*Hh*) and treated weekly with anti-IL-10R blocking mAb (*Hh* + anti-IL-10R) prior to analysis at three weeks. Single cell preparations of combined colonic and caecal lamina propria were stained and the indicated cell subsets isolated by FACS. For gating see Figure 3.4. RNA was extracted and RT-qPCR used to measure expression of *Ccr5*. Each data point represents a biological replicate of cells sorted from 4-7 pooled mice, horizontal line indicates group mean. Data are pooled from five independent experiments (steady state, open circles; *Hh* + anti-IL-10R, filled circles. CD103⁺ DCs, steady state, n=3-4; CD103⁺ DCs, *Hh* + anti-IL-10R, n=2-3; CX₃CR1^{int} CD64⁺, steady state, unavailable; CX₃CR1^{int} CD64⁺, *Hh* + anti-IL-10R, n=2; CX₃CR1^{int} CD64⁻, steady state, n=2; CX₃CR1^{int} CD64⁻, *Hh* + anti-IL-10R, n=2; CX₃CR1^{hi} CD64⁺, steady state, n=4; CX₃CR1^{hi} CD64⁺, *Hh* + anti-IL-10R, n=4-5).

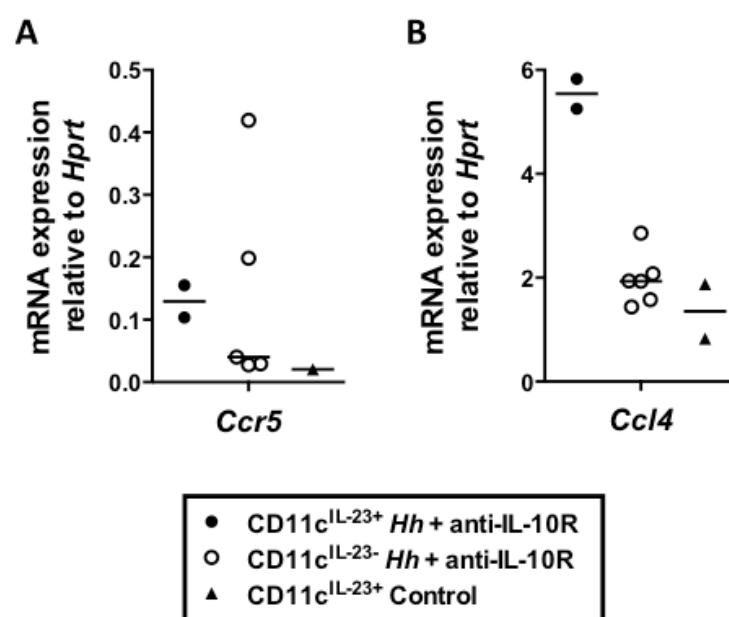


Figure 5.23 CCR5 chemokine receptor and ligand expression during *Helicobacter hepaticus* + anti-IL-10R colitis.

CD11c-Cre⁺ *Il23a*^{fl/fl} (CD11c^{IL-23+}) mice or pooled CD11c-Cre⁻ *Il23a*^{fl/fl} and CD11c-Cre⁻ *Il23a*^{fl/fl} (CD11c^{IL-23-}) littermate controls were infected with *Helicobacter hepaticus* (*Hh*) and treated weekly with anti-IL-10R blocking mAb prior to analysis after three weeks. Expression of (A) *Ccr5* and (B) *Ccl4* was assessed by RT-qPCR on 500,000 freshly isolated lamina propria cells. Data are from one experiment (CD11c^{IL-23+} *Hh* + anti-IL-10R, filled circles, n=2; CD11c^{IL-23-} *Hh* + anti-IL-10R, open circles, n=5-6; CD11c^{IL-23+} *Hh* only or uninfected control, filled triangles, n=1-2). Each data point represents an individual mouse, horizontal line indicates group median.

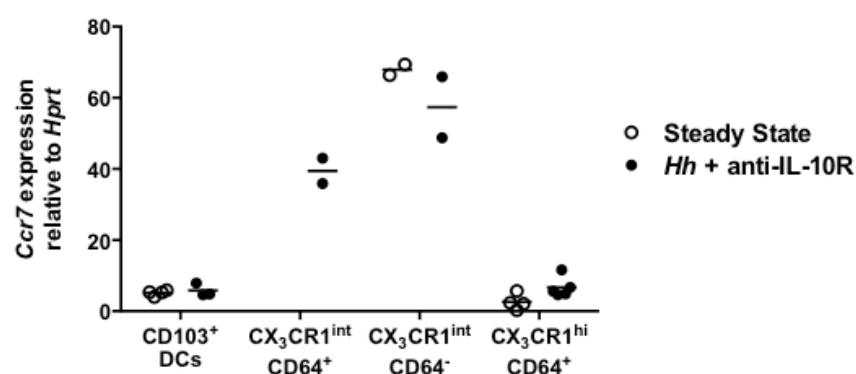


Figure 5.24 CCR7 expression by large intestinal mononuclear phagocyte subsets during homeostasis and *Helicobacter hepaticus* + anti-IL-10R colitis.

CX₃CR1^{GFP/+} mice were left untreated (steady state) or infected with *Helicobacter hepaticus* (*Hh*) and treated weekly with anti-IL-10R blocking mAb (*Hh* + anti-IL-10R) prior to analysis at three weeks. Single cell preparations of combined colonic and caecal lamina propria were stained and the indicated cell subsets isolated by FACS. For gating see Figure 3.4. RNA was extracted and RT-qPCR used to measure expression of *Ccr7*. Each data point represents a biological replicate of cells sorted from 4-7 pooled mice, horizontal line indicates group mean. Data are pooled from five independent experiments (steady state, open circles; *Hh* + anti-IL-10R, filled circles. CD103⁺ DCs, steady state, n=3-4; CD103⁺ DCs, *Hh* + anti-IL-10R, n=2-3; CX₃CR1^{int} CD64⁺, steady state, unavailable; CX₃CR1^{int} CD64⁺, *Hh* + anti-IL-10R, n=2; CX₃CR1^{int} CD64⁻, steady state, n=2; CX₃CR1^{int} CD64⁻, *Hh* + anti-IL-10R, n=2; CX₃CR1^{hi} CD64⁺, steady state, n=4; CX₃CR1^{hi} CD64⁺, *Hh* + anti-IL-10R, n=4-5).

5.3 Discussion

The intestinal mononuclear phagocyte compartment changes dramatically following the onset of inflammation due to an influx of Ly6C^{hi} monocytes and macrophages into the lamina propria. However, the mechanisms controlling the recruitment of MNPs under these conditions and during homeostasis are not fully understood.

Using CCR6-EGFP knock-in mice, we observed that colitis induced by adoptive transfer of wild-type naïve CD4⁺ T cells was significantly less severe in the proximal colon of CCR6^{GFP/GFP} *Rag1*^{-/-} mice compared to CCR6-sufficient mice. Trends toward reduced pathology were also observed in the mid and distal regions of CCR6-deficient mice, and their mean colon score was significantly reduced relative to wild-type controls. Less severe colitis in the absence of functional CCR6 could be linked to the suggestion that clinical and experimental IBD are initiated in the Peyer's patches and isolated lymphoid follicles, which both require CCR6 for full development (Lugering et al., 2005; McDonald et al., 2007; Varona et al., 2001). Assessment of cryptopatches and isolated lymphoid follicles in the colon by immunofluorescence or immunohistochemistry could provide further insight.

Despite less severe pathology, we found unaltered accumulation of CD11c⁺ MHCII⁺ MNPs, which include macrophages and DCs, in the colon of CCR6-deficient mice during anti-CD40- and T cell transfer-induced colitis. Neither E-cadherin⁺ nor CD103⁺ subsets of CD11c⁺ MHCII⁺ MNPs were affected by the loss of CCR6. Staining for E-cadherin and CD103 was used to identify monocyte-derived pro-inflammatory MNPs (Siddiqui et al., 2010) and CD103⁺ DCs, respectively, however this could be refined in light of recent advances in the identification of MNP subsets which had not been made when this work was carried out. These populations could now be defined more precisely using additional

markers such as CD64, CD11b, and Ly6C to distinguish Ly6C^{hi} monocytes and macrophages, and populations of DCs as described in more detail in Chapters 1 and 3. Among MNP populations defined in separate studies by CX₃CR1-GFP, which we could not combine with CCR6-EGFP, we did not observe *Ccr6* expression in CX₃CR1^{int} CD64⁺ MNPs which accumulate in the lamina propria during *Hh* + anti-IL-10R colitis. It is possible that CCR6 expression had been downregulated following their activation, as occurs during DC maturation (Dieu et al., 1998; Iwasaki and Kelsall, 2000). However, our results seem more likely to suggest that CCR6 is not involved in the recruitment of pro-inflammatory MNPs during multiple models of acute and chronic colitis.

In contrast, we observed *Ccr6* expression by CX₃CR1^{hi} resident macrophages and CX₃CR1^{int} CD64⁻ MNPs sorted from the uninfamed lamina propria. Combined with the expression of *Ccr7* by CX₃CR1^{int} CD64⁻ MNPs from both resting and inflamed large intestine, these results support findings that migratory DCs exist among this population (Bain et al., 2013; Cerovic et al., 2013; Scott et al., 2014; Zigmond et al., 2012). Collectively our results suggest a potential role for CCR6 in homeostatic recruitment of Ly6C^{hi} monocytes and maintenance of their progeny within the lamina propria, but do not indicate that CCR6 is required for inflammatory MNP accumulation during colitis.

Although the overall recruitment of CD11c⁺ MHCII⁺ MNPs to the colon was unaffected by CCR6 deficiency, it is possible that CCR6 could play a role in positioning MNP populations and other immune cells within the intestine. CCR6 controls the migration of Tregs to the colon and their ability to suppress T cell transfer-induced colitis (Kitamura et al., 2010), while CCR6-deficient mice have been described to lack a CD4⁺ CD62L⁻ CD45RB^{lo} regulatory T cell population normally found in the Peyer's patches (Lugering et al., 2005). Why this population was missing is unclear, but the proliferative capacity of CCR6-deficient splenic

CD4⁺ memory T cells was shown to be impaired (Louis et al., 2003). Effective induction of oral tolerance requires interactions between CX₃CR1⁺ lamina propria-resident macrophages and Tregs that have arrived in the lamina propria after priming in the MLNs, with such interactions leading to Treg expansion (Hadis et al., 2011). A second antigen presentation event in the periphery is also required for fully developed CD4⁺ T cell cytokine secretion in the skin (McLachlan et al., 2009), indicating that secondary presentation of antigen at effector sites is necessary for both expansion and full functional activation of CD4⁺ T cell populations. We found that CX₃CR1^{hi} resident macrophages in the uninflamed, but not inflamed, lamina propria expressed CCR6, which could facilitate their interaction with CCR6⁺ CD4⁺ T cells in the colon. Unfortunately poor intracellular cytokine staining prevented the measurement of CD4⁺ T cell responses in our T cell transfer colitis experiments. The role of CCR6 in the positioning and function of MNPs and CD4⁺ T cells could be explored in CCR6-EGFP knock-in mice, which would allow for *in situ* or *in vivo* visualisation of CCR6⁺ cells by immunofluorescence or intravital microscopy.

A number of studies report a predominant role for CCR2 and its canonical ligand CCL2 in the recruitment of Ly6C^{hi} monocytes to the resting and inflamed large intestine during DSS-, hapten-, and *Citrobacter rodentium*-induced colitis (Bain et al., 2013; Khan et al., 2006; Kim et al., 2011b; Platt et al., 2010; Popivanova et al., 2009; Takada et al., 2010; Zigmond et al., 2012). However, our results suggest that other chemokine-chemokine receptor axes, such as CCL3-CCR1, may be involved during *Helicobacter hepaticus* + anti-IL-10R colitis.

The results we obtained using MC-21 to investigate the role of CCR2 in Ly6C^{hi} monocyte recruitment to the large intestine were inconclusive. Observations such as reduced Ly6C^{hi}

monocyte frequency in the blood following one dose of MC-21, and in the colonic and caecal lamina propria and blood during ongoing MC-21 treatment early in *Hh* + anti-IL-10R colitis, indicate partial efficacy of MC-21 treatment. Had we not observed less severe intestinal pathology three weeks post-*Hh* infection and anti-IL-10R treatment in our first experiment, we would have tested higher doses of MC-21, but this was not feasible as we were limited by availability of MC-21 and other work using this regime had been reported to be successful (Bruhl et al., 2007). As a positive control for the use of MC-21 with effects in the intestine, we attempted to induce acute colitis with DSS which MC-21 treatment has been shown to ameliorate via ablation of Ly6C^{hi} monocytes (Zigmond et al., 2012). Unfortunately we were unable to replicate this experiment due to the unexpected failure of both MC-21- and isotype control-treated mice to develop colitis following DSS administration, and we were unable to try again using a greater dose of DSS due to a lack of further MC-21.

We observed trends towards reduced frequencies of Ly6C^{hi} monocytes and their progeny in the large intestine and blood of MC-21-treated mice during ongoing MC-21 treatment in the first week of *Hh* + anti-IL-10R colitis, but did not observe differences at later times after MC-21 administration had ceased. We believe that this may have been due to monocyte recruitment occurring during the period between the end of MC-21 treatment and our analysis, which ranged from 18 hours to 12 days per experiment. Ly6C^{hi} monocytes turn over relatively quickly, developing into intestinal macrophages and DCs through a series of CX₃CR1^{int} intermediary stages within a few days of entering the colonic lamina propria (Bain et al., 2013; Zigmond et al., 2012). During colitis, the proportion of Ly6C^{hi} monocytes in the lamina propria increases rapidly and remains elevated (Figure 3.3; (Bain et al., 2013)), indicating their continued recruitment. In other studies, we have

observed increases in the frequency and number of Ly6C^{hi} monocytes in the large intestine within 16 hours of *Hh* infection and anti-IL-10R treatment (Dr Isabelle Arnold, unpublished observations), demonstrating how quickly these cells are recruited during inflammation. This supports the idea that breakthrough accumulation of Ly6C^{hi} monocytes in the lamina propria could have taken place after final administration of MC-21 and before mice were sacrificed. However, we observed reduced frequencies of Ly6C^{hi} monocytes in blood collected two days after a single injection of MC-21, suggesting that any such breakthrough recruitment could only be partial when early timepoints were analysed. When mice were left for 12 days following final MC-21 administration however, there may have been sufficient time for “full” recruitment of Ly6C^{hi} monocytes to the lamina propria. In intestinal studies, MC-21 has only previously been used during acute DSS-induced colitis (Zigmond et al., 2012). The success observed in this model may reflect the short duration of DSS colitis, which could preclude potential problems due to the time/dose limitations of MC-21 administration. Unfortunately these limitations may make the use of MC-21 unfeasible in longer models such as *Hh* + anti-IL-10R colitis.

We also observed a trend towards reduction of CX₃CR1^{int} Ly6C^{lo/-} MHCII⁺ F4/80⁻ MNPs in the lamina propria during ongoing MC-21 treatment. This population is now known to contain *bona fide* CD103⁻ CD11b⁺ DCs, a subset of which express CCR2 (Scott et al., 2014). It is possible that these CCR2⁺ cells could have been depleted by MC-21 treatment, which could explain the observed reduction in frequency of CX₃CR1^{int} Ly6C^{lo/-} MHCII⁺ F4/80⁻ MNPs.

MC-21 was initially reported to block the ability of CCR2 to bind its canonical ligand CCL2, thus preventing responses to the chemokine (Mack et al., 2001). In this study, a 500 µg dose of MC-21 was used to prevent the development of peritonitis by blocking the CCR2-

dependent recruitment of monocytes and macrophages to the peritoneum. However, one of the recent studies supporting a role for CCR2 in inflammatory monocyte recruitment to the colon during DSS-induced colitis reported antibody-mediated depletion of CX₃CR1^{int} Ly6C⁺ MNPs by MC-21 (Zigmond et al., 2012). Although the dose of MC-21 administered was not disclosed in this publication, other references have also described depletion of inflammatory monocytes by MC-21 at doses ranging from 10 µg to 50 µg (Biburger et al., 2011; Bruhl et al., 2007; Mildner et al., 2007; Reich et al., 2013; Ribes et al., 2013; Wang et al., 2012). Depletion of cells by MC-21 has been shown to be mediated by antibody-dependent cell-mediated cytotoxicity, since it can be blocked by anti-CD16/32 (low affinity IgG Fc receptor (FcγR) III/II) antibody treatment (Mildner et al., 2009). By administering 20 µg MC-21, it is therefore possible that our strategy partially depleted CCR2⁺ inflammatory monocytes rather than blocked CCR2.

We also examined Ly6C^{hi} monocyte recruitment during *Hh* + anti-IL-10R colitis by comparing wild-type mice with CD11c^{IL-23-} mice, which we have shown do not develop intestinal pathology or accumulate pro-inflammatory MNPs in the lamina propria following *Hh* + anti-IL-10R treatment (Chapter 4). We found that total *Ccr2* expression by lamina propria cells was elevated following *Hh* + anti-IL-10R treatment regardless of the ability of CD11c⁺ cells to produce IL-23, despite significantly fewer Ly6C^{hi} monocytes and CX₃CR1^{int} CD64⁺ MNPs among the lamina propria cells of *Hh* + anti-IL-10R-treated CD11c^{IL-23-} mice. It might not be reasonable to expect to pick up subtle differences driven by changes in some cell populations by RT-qPCR, however in contrast, we did observe reduced expression of *Ccr1* in the lamina propria of *Hh* + anti-IL-10R-treated CD11c^{IL-23-} mice which indicates that it is possible.

Since one would expect to find elevated levels of the chemokines responsible for cell recruitment, further evidence that CCR2 may not be involved in Ly6C^{hi} monocyte accumulation during *Hh* + anti-IL-10R colitis came from our finding that levels of its ligands CCL2 and CCL7 were reduced in colitic (CD11c^{IL-23+} *Hh* + anti-IL-10R) relative to uninflamed (CD11c^{IL-23-} *Hh* + anti-IL-10R and CD11c^{IL-23+} control) lamina propria cell cultures. However, chemokines bound to cognate receptors are typically internalised and can be degraded by responding cells (Marchese, 2014). It is therefore possible that levels of CCL2 and CCL7 are lower in the inflamed lamina propria due to the presence of CCR2⁺ Ly6C^{hi} monocytes and macrophages.

In contrast to *Ccr2*, we observed reduced expression of *Ccr1* and production of its ligand CCL3 by lamina propria cells from *Hh* + anti-IL-10R-treated CD11c^{IL-23-} mice, in correlation with the absence of Ly6C^{hi} monocytes and CX₃CR1^{int} CD64⁺ MNPs, relative to wild-type counterparts. Ly6C^{hi} monocyte recruitment to the small intestine during experimental ileitis induced by the parasite *Toxoplasma gondii* is controlled by CCR1 (Schulthess et al., 2012). This receptor was highly expressed by CX₃CR1^{int} CD64⁺ MNPs from the inflamed lamina propria during *Hh* + anti-IL-10R colitis, supporting the potential of CCR1 to control the recruitment and accumulation of Ly6C^{hi} monocytes and their progeny in this model.

To confirm a role for CCR1 in monocyte recruitment to the colon, CCR1 knockout (*Ccr1*^{-/-}) mice (Gao et al., 1997) could be used. Mixed bone marrow chimaeras with 50:50 wild-type:*Ccr1*^{-/-} bone marrow could be used to exclude any potential effects of CCR1 deficiency among other cells which may express CCR1 such as neutrophils, DCs or Th1 cells (Griffith et al., 2014), while the contribution of CCR1 to monocyte recruitment could still be assessed by the proportion of CCR1-deficient and -sufficient cells found in the colon. We hypothesise that we would see a reduced ability of CCR1-deficient Ly6C^{hi}

monocytes to migrate to the lamina propria following *Hh* + anti-IL-10R treatment.

Alternatively, the role of CCR1 could be tested in CX₃CR1^{GFP/+} mice by blocking its ligands *in vivo* using monoclonal antibodies, as performed by Schulthess and colleagues during *Toxoplasma gondii* infection (Schulthess et al., 2012).

Finally, we also measured expression of CCR7 by intestinal MNPs. Interestingly, we observed expression of *Ccr7* by CX₃CR1^{int} CD64⁺ MNPs isolated from the inflamed lamina propria. CCR7 binds CCL19 and CCL21, through which it controls migration of tissue-derived DCs to draining lymph nodes, localisation to T cell areas of lymphoid tissue and lymphocyte recirculation (Griffith et al., 2014). In addition to these canonical functions, CCL19 stimulation via CCR7 has been shown to enhance the release of IL-8 and TNF α , and MMP activity, in human THP-1 cell line-derived macrophages (Damas et al., 2007). Whether CCL19 might have the same effect on CX₃CR1^{int} CD64⁺ MNPs, which expressed genes encoding TNF α and MMPs in our microarray (Chapter 3), is unknown. CCL19 levels are elevated in the inflamed intestine (Kawashima et al., 2005; McNamee et al., 2013) therefore investigation of its effects on CCR7⁺ primary intestinal MNPs could be of interest.

Surprisingly we found only very low expression of *Ccr7* among total (CD11b⁺ and CD11b⁻) CD103⁺ DCs from both the resting and inflamed large intestinal lamina propria. However, the published work showing CCR7-dependent migration of intestinal CD103⁺ DCs to the MLNs, where they promote gut-homing iTreg differentiation via retinoic acid (Coombes et al., 2007; Jaensson et al., 2008; Johansson-Lindbom et al., 2005; Sun et al., 2007), has focused on CD103⁺ DCs from the small intestine. In addition to this potential difference in expression of CCR7, CD103⁺ DCs from the large intestinal lamina propria differ from their small intestinal counterparts in other respects: in the colon, most CD103⁺ DCs are CD11b⁻

(Annacker et al., 2005; Varol et al., 2009) whereas those in the small intestine are predominantly CD11b⁺ (Bogunovic et al., 2009), and only small intestinal CD103⁺ DCs exhibit retinal dehydrogenase type 2 (RALDH2) activity as measured by Aldefluor assay (Dr Andrew Johnson, unpublished observations). Furthermore, the migration of CD103⁺ DCs from the colonic lamina propria to draining lymph nodes has not been extensively studied. Since the colon is drained by the caudal and pancreatic lymph nodes in addition to the MLNs (Carter and Collins, 1974), studies of cells collected by thoracic duct cannulation of mesenteric lymphadenectomised mice may have been inadequate to determine which cells migrate from the colonic lamina propria. There is also the problem that both the small intestine and regions of the colon drain to the MLNs, which could make it difficult to distinguish the specific source of migratory cells. It therefore remains possible that the lack of *Ccr7* expression by colonic and caecal CD103⁺ DCs means that CD103⁺ DCs do not migrate from the large intestine. This could be investigated by comparative analysis of MNP populations in the caudal and pancreatic lymph nodes of wild-type and CCR7-deficient mice (Forster et al., 1999) or, ideally, by selectively labelling colonic or caecal cells prior to examination of draining lymph nodes for the presence of labelled cells. This experiment could be possible using a system such as the Kaede transgenic mouse (Shand et al., 2014; Tomura et al., 2008) which possesses a fluorescent protein that can be stably converted from green to red by exposure to violet light (Ando et al., 2002). Colonoscopy or surgical exposure of the colon or caecum could allow violet light-induced labelling of large intestinal cells for analysis of migration patterns.

Collectively, our results highlight a potential role for CCR1 in the IL-23-dependent accumulation of Ly6C^{hi} monocytes and macrophages in the colonic and caecal lamina propria during *Hh* + anti-IL-10R typhlocolitis. In contrast to acute models of colitis,

chronic *Hh* + anti-IL-10R colitis may not involve a major role for CCR2 in MNP recruitment.

Our results indicate that IL-23 – directly or indirectly – induces expression of chemokines, such as CCL3, which could control the recruitment of Ly6C^{hi} monocytes via CCR1, in turn influencing the abundance of Ly6C^{hi} monocytes and pro-inflammatory CX₃CR1^{int} CD64⁺ MNPs in the lamina propria during inflammation.

6 General Discussion

6.1 Mononuclear phagocytes in the lamina propria during *Hh* + anti-IL-10R colitis

We found that Ly6C⁺ MNP populations – Ly6C^{hi} monocytes and Ly6C⁺ MHCII⁺ MNPs – accumulated in the large intestinal lamina propria during inflammation induced by *Helicobacter hepaticus* infection and anti-IL-10R treatment (Figure 3.3). These populations were rapidly recruited to the large intestine and their frequencies remained elevated until 2-3 weeks after induction of colitis. Our results confirm that Ly6C⁺ MNPs accumulate in colitis, as shown in the DSS (Bain et al., 2013; Platt et al., 2010; Zigmond et al., 2012) and T cell transfer (Rivollier et al., 2012; Tamoutounour et al., 2012; Weber et al., 2011) models during the course of this project.

Severity of *Hh* + anti-IL-10R colitis and Ly6C⁺ MNP frequencies peaked around two weeks after induction of disease, after which both pathology scores and Ly6C⁺ MNP frequencies began to decrease (Figures 3.1 and 3.3). The correlation between intestinal pathology and the presence of Ly6C⁺ MNPs suggests that these cells may play a pro-inflammatory role during *Hh* + anti-IL-10R intestinal inflammation, as indicated in other models of colitis (Platt et al., 2010; Rivollier et al., 2012; Tamoutounour et al., 2012; Weber et al., 2011; Zigmond et al., 2012). Indeed, these cells expressed *Il23a* at day five post-*Hh* + anti-IL-10R treatment, which was abrogated in CD11c-Cre x IL-23p19-floxed mice (Figure 4.11) that do not develop *Hh* + anti-IL-10R colitis (Figure 4.1).

CX₃CR1^{int} Ly6C⁺ MHCII⁺ MNPs could also be involved in inducing a protective immune response. *Hh* infection induces colitis in mice without functional IL-10, which is driven by a *Hh*-specific IFN γ ⁺ and IFN γ ⁺ IL-17A⁺ CD4⁺ T cell response (Kullberg et al., 2006; Kullberg

et al., 1998; Morrison et al., 2013). $CD4^+$ T cell responses might contribute to reducing bacterial burden, as observed by two weeks after *Hh* infection in IL-10-deficient mice (Morrison et al., 2013). Reduced bacterial stimulation might eventually lead to resolution of inflammation as pathogenic $CD4^+$ T cells cease to be induced (Morrison et al., 2013). Recent studies have shown that CX_3CR1^+ MNPs co-localise with $CD4^+$ T cells in the intestinal lamina propria (Hadis et al., 2011; Rossini et al., 2013). These interactions were necessary for the expansion of Tregs required to generate oral tolerance (Hadis et al., 2011) and may induce expansion of antigen-specific colitogenic $CD4^+$ T cells during colitis (Rossini et al., 2013). Our studies show that intestinal inflammation peaks at the same time as $CX_3CR1^{int} Ly6C^+ MHCII^+$ MNP frequencies in the lamina propria, which coincides with peak $IFN\gamma^+$ and $IFN\gamma^+ IL-17A^+ CD4^+$ T cell responses observed in *Hh*-infected IL-10-deficient mice (Morrison et al., 2013). $CX_3CR1^{int} Ly6C^+ MHCII^+$ MNPs in the lamina propria could enhance the $CD4^+$ T cell response, which might control levels of *Hh*. In turn, this could promote a shift towards resolution of inflammation.

Such a shift, away from inflammation and towards its resolution, might allow differentiation of $Ly6C^{hi}$ monocytes into $Ly6C^{lo/-} CX_3CR1^{int}$ and CX_3CR1^{hi} macrophages, as occurs in the steady state (Bain et al., 2013; Tamoutounour et al., 2012), to resume. Our studies revealed that the lower colitis scores observed three weeks after *Hh* + anti-IL-10R treatment coincided with increased frequency of $CX_3CR1^{int} Ly6C^{lo/-}$ macrophages (Figures 3.1 and 3.3). Their reappearance in the lamina propria could reflect the release of a brake that prevented their differentiation during inflammation (Bain et al., 2013; Rivollier et al., 2012; Tamoutounour et al., 2012), or might additionally indicate a role for these cells in promoting the resolution of colitis. Although we are unable to determine whether genes were expressed by $Ly6C^+$ or $Ly6C^{lo/-}$ cells among $CX_3CR1^{int} CD64^+$ MNPs, the results of our

microarray indicate that a response associated with the resolution of inflammation is indeed underway by three weeks after induction of colitis (Chapter 3).

6.2 Sources of pathogenic IL-23 during *Hh* + anti-IL-10R colitis

We have shown that IL-23 produced by CD11c⁺ cells is essential for the development of *Hh* + anti-IL-10R-induced colitis and typhlitis (Figure 4.1). Our results phenocopy full IL-23p19 knockout mice (Dr Philip Ahern, unpublished observations), suggesting that CD11c⁺ cells are the main source of IL-23, which drives intestinal pathology in this model.

Interestingly, splenomegaly was also absent in CD11c^{IL-23⁻} mice infected with *Hh* and treated with anti-IL-10R (Figure 4.4). The IL-23-dependence of splenic enlargement may be specific to models of colitis driven by *Hh*, as splenomegaly is otherwise reported to be IL-23-independent and rather driven by IL-12 (Hue et al., 2006; Uhlig et al., 2006b). We found that IL-23 was required for the accumulation of Ly6C^{hi} monocytes and Ly6C⁺ MHCII⁺ MNPs normally observed in the lamina propria following *Hh* + anti-IL-10R treatment (Figure 4.8), and for the emergence of pathogenic IL-17- and IFN γ -producing CD4⁺ T cells (Figure 4.6).

Our studies in CD11c-Cre x IL-23p19-floxed mice showed that Ly6C⁺ MHCII⁺ MNPs and Ly6C^{lo/-} macrophages – which importantly include both CX₃CR1^{int} and CX₃CR1^{hi} macrophages (Bain et al., 2013) – expressed IL-23p19 transcripts five days after *Hh* + anti-IL-10R treatment (Figure 4.11), when expression in whole colon tissue is at its peak (Figure 4.10). Crucially, IL-23p19 expression was abrogated in both Ly6C⁺ MHCII⁺ MNPs and Ly6C^{lo/-} macrophages sorted from CD11c^{IL-23⁻} mice (Figure 4.11a). When we sorted populations from CX₃CR1^{GFP/+} mice five days after *Hh* + anti-IL-10R treatment, we found

that CX₃CR1^{hi} macrophages showed the greatest expression of IL-23p19, while CX₃CR1^{int} CD64⁺ MNPs expressed low levels (Figure 4.11b). Collectively, these results indicate that CX₃CR1^{hi} resident macrophages and newly recruited pro-inflammatory Ly6C⁺ MHCII⁺ MNPs represent the early sources of IL-23 during *Hh* + anti-IL-10R colitis. Three weeks after induction of colitis, CX₃CR1^{int} CD64⁺ MNPs continued to express low levels of IL-23p19, by which point expression by CX₃CR1^{hi} macrophages had reduced to a similar level (Figure 4.11c). This low level of expression was consistent with whole large intestinal tissue levels of IL-23p19 transcripts three weeks after *Hh* + anti-IL-10R treatment (Figure 4.10). Neither five days nor three weeks after induction of colitis did we observe IL-23p19 expression by CD103⁺ CD11b⁻ DCs, CD103⁺ CD11b⁺ DCs or CX₃CR1^{int} CD64⁻ MNPs (Figure 4.11). Our microarray analysis confirmed that *Il23a* expression was enriched in CX₃CR1^{int} CD64⁺ monocyte/macrophage MNPs versus CX₃CR1^{int} CD64⁻ DCs three weeks after *Hh* + anti-IL-10R treatment (Table 3.10; Appendix, Table 1).

We performed a preliminary experiment transferring Ly6C^{hi} monocytes to CD11c^{IL-23-} mice to determine whether these cells were sufficient as a source of IL-23 to drive colitis. Following *Hh* + anti-IL-10R treatment, caecal pathology scores in CD11c^{IL-23-} mice which received Ly6C^{hi} monocytes displayed a trend towards scores observed in IL-23-sufficient mice, which was encouraging. However, this experiment needs to be refined and repeated before any conclusions can be drawn about sources of IL-23 sufficient to drive colitis, as discussed in detail in Chapter 4.

Hh infection in wild-type mice induces IL-23p19 expression (Kullberg et al., 2006), and indeed we observed expression of IL-23p19 by CX₃CR1^{hi} macrophages sorted from *Hh*-infected mice with intact IL-10 signalling (Figure 4.11). Compared to *Hh* infection alone, Kullberg and colleagues found that *Hh* + anti-IL-10R treatment resulted in a substantial

increase in transcription of IL-12/23p40, and decreased transcription of the IL-12-specific subunit IL-12p35 (Kullberg et al., 2006). This supports the idea that there are two components in the regulation of functional IL-23 expression: firstly, expression of the specific IL-23p19 subunit, which appears to be induced by *Hh*; and secondly, availability of the shared IL-12/23p40 subunit to combine with IL-23p19 to create IL-23, rather than with IL-12p35 to produce IL-12. Expression of IL-23p19 and IL-12p35 might be reciprocally regulated, akin to IFN γ and IL-4 in CD4⁺ T cells (Kastelein et al., 2007). It would be interesting to test whether these gene expression dynamics, observed in whole colonic tissue, hold when applied to sorted populations of cells from the lamina propria of uninfected, *Hh*-infected, and *Hh* + anti-IL-10R-treated mice.

We have not investigated the mechanism by which IL-23 expression is induced, however numerous papers support the conclusion that commensal bacterial stimulation drives colitis via actions on intestinal MNPs. MyD88 signalling in colonic MNPs was required for development of colitis in IL-10-deficient mice (Hoshi et al., 2012). This colitis was driven by the commensal microbiota, which contained numerous *Helicobacter* species including *Hh*. The study concluded that it was likely a CD11c⁺ CD11b⁺ CX₃CR1⁺ macrophage population which was responsible for inducing inflammation and subsequently recruiting inflammatory macrophages after microbial sensing. These cells expressed TLRs 1, 2, 4, 5, 6, 9 and MyD88; and IL-1 β , IL-6, IL-23p19 and IL-10 in wild-type mice. An earlier study found that the inflammatory monocyte-derived CX₃CR1^{int} Ly6C^{hi} MNPs which accumulate during DSS colitis are TLR2⁺ (Platt et al., 2010). This was confirmed by another study, which additionally found that these cells express the intracellular pattern recognition receptors NOD1 and NOD2 (Zigmond et al., 2012). The latter work noted expression of IL-23p19 mRNA during DSS colitis, and showed that IL-23 expression was greatly reduced in

cells isolated from [MyD88/TICAM1 (TRIF) double KO → WT] bone marrow chimeras, which are unable to respond to all TLR stimulation, relative to single TLR2 KO or NOD2 KO bone marrow chimeras. TLR5 was shown to be required for production of IL-23 by CD103⁺ CD11b⁺ DCs *in vivo* following intravenous injection of flagellin, a TLR5 ligand (Kinnebrew et al., 2012). Although we do not see expression of *Il23a* by CD103⁺ DCs, *Hh* is a flagellated bacterium and might therefore be recognised via TLR5. *Tlr5* expression was enriched in CX₃CR1^{int} CD64⁺ versus CX₃CR1^{int} CD64⁻ MNPs from the inflamed lamina propria in our microarray study (Table 3.10; Appendix, Table 1). The endoplasmic reticulum stress response has also been shown to regulate IL-23 expression, in combination with TLR stimulation (Goodall et al., 2010). Collectively, these results indicate that TLR signalling could induce *Il23a* expression in macrophages with disrupted IL-10 signalling. This may occur downstream of multiple TLRs, potentially in concert with other molecular response pathways, and requires further investigation.

Hh colonises the caecum and colon, where it resides in the mucus layer and less often can be found in the intestinal crypts (Chan et al., 2005; Zhang et al., 2006). CX₃CR1⁺ MNPs localise to the intestinal epithelium (Rossini et al., 2013; Schulz et al., 2009) and have been shown to extend transepithelial dendrites into the lumen in a CX₃CR1-dependent manner (Kim et al., 2011a; Niess et al., 2005; Rescigno et al., 2001). This phenomenon was demonstrated in the small intestine, where it was found only to occur in the terminal ileum (Niess et al., 2005). Whether CX₃CR1⁺ MNPs extend transepithelial dendrites in the caecum or colon, and the relevance of this process to human disease, are unclear.

However, colonic CX₃CR1⁺ MNPs have been shown to internalise *C. rodentium* expressing the red fluorescent protein mRuby (Manta et al., 2013) and *E. coli* expressing fluorescently labelled OVA (Rossini et al., 2013) *in situ*. *C. rodentium* uptake was reduced

in CX₃CR1-deficient mice (Manta et al., 2013) suggesting a partially CX₃CR1-dependent mechanism. Transepithelial dendrite extension could therefore represent a mechanism by which CX₃CR1⁺ MNPs sense *Hh*.

6.3 Recruitment of MNPs to the inflamed large intestinal lamina propria

We examined how MNPs might be recruited to the lamina propria during inflammation, focusing on chemokine receptors because these are well known to play an important role in tissue-specific cell recruitment (Griffith et al., 2014; Rot and von Andrian, 2004). Our studies support the conclusion that CCR6 is not required for accumulation of monocyte-derived populations in the inflamed intestine, however future studies could address a potential role for CCR6 in localisation of immune cells within resting tissues (discussed in Chapter 5). Multiple studies in other models of colitis and inflammation in various tissues support a role for CCR2 in the recruitment of inflammatory monocytes (Bain et al., 2013; Khan et al., 2006; Kim et al., 2011b; Platt et al., 2010; Popivanova et al., 2009; Serbina et al., 2008; Takada et al., 2010; Zigmond et al., 2012). CCR2 is required for Ly6C^{hi} monocyte egress from the bone marrow (Serbina and Pamer, 2006), but may not be the only mechanism controlling their recruitment into the intestine. During DSS colitis, although adoptively transferred CCR2-sufficient Ly6C^{hi} monocytes possessed a competitive advantage in homing to the colon over those without functional CCR2, Ly6C^{hi} monocyte-derived MNPs were not completely absent from the colonic lamina propria of CCR2-deficient mice (Platt et al., 2010; Zigmond et al., 2012). *Ccl2*^{-/-} mice with DSS- or dinitrobenzene sulfonic acid (DNBS)-induced colitis also displayed some accumulation of macrophages in the colonic lamina propria, though this was impaired relative to wild-type mice (Khan et al., 2006; Takada et al., 2010). In contrast, adoptively transferred *Ccr2*^{-/-}

Ly6C^{hi} monocytes were unimpaired in their ability to migrate into the colon of *Citrobacter rodentium*-infected mice (Kim et al., 2011b). This suggests that the predominant mechanism controlling recruitment of Ly6C^{hi} monocytes may differ in response to diverse inflammatory stimuli. In the steady state, CCR2- and CCL2-deficient mice also retain some Ly6C^{hi} monocyte-derived MNPs in the lamina propria (Bain et al., 2013; Takada et al., 2010) indicating that multiple mechanisms may control their recruitment in the presence and absence of inflammation.

Although our results do not rule out a role for CCR2 in inflammatory monocyte recruitment during *Hh* + anti-IL-10R colitis, our findings suggest that other chemokine receptor pathways could also be involved in this model, particularly CCR1 and CCRL2. We have shown that CX₃CR1^{int} CD64⁺ MNPs from inflamed lamina propria express CCR1 (Figure 5.20) and CCRL2 (Table 3.10; Appendix, Table 1; Figure 3.9). In the absence of IL-23 production by CD11c⁺ MNPs, Ly6C⁺ pro-inflammatory MNPs were absent from the lamina propria and expression of CCR1 and CCL3 at the whole tissue level was significantly reduced three weeks after *Hh* + anti-IL-10R treatment (Figure 5.21). Our results indicate that CD11c⁺ MNP-derived IL-23 induces – whether directly or indirectly – the expression of chemokines such as CCL3 which could control the recruitment of inflammatory monocytes via CCR1 and/or CCRL2, in turn influencing the abundance of pro-inflammatory CX₃CR1^{int} Ly6C⁺ MNPs in the large intestine during *Hh* + anti-IL-10R colitis.

CCR1 was recently shown to control the recruitment of inflammatory monocytes to the intestine during *Toxoplasma gondii*-induced ileitis (Schulthess et al., 2012). Perhaps inflammation driven by microbial stimuli, such as bacteria including *Helicobacter hepaticus* and *Citrobacter rodentium* (Kim et al., 2011b), and the protozoan parasite

Toxoplasma gondii (Schulthess et al., 2012), may induce recruitment of Ly6C^{hi} monocytes primarily via mechanisms other than CCR2-CCL2, in contrast to other models of intestinal damage such as DSS and DNBS (Khan et al., 2006; Platt et al., 2010; Takada et al., 2010; Zigmond et al., 2012).

Our microarray analysis revealed that CX₃CR1^{int} CD64⁺ MNPs expressed greater levels of the chemokine genes *Ccl5*, *Ccl9*, *Cxcl1* and *Cxcl2* than their CD64⁻ counterparts three weeks after *Hh* + anti-IL-10R treatment (Table 3.10; Appendix, Table 1). This suggests that these cells themselves could be involved in the recruitment of monocytes, and other cells such as neutrophils and T cells, to the lamina propria during colitis, as has been previously suggested regarding lamina propria resident macrophages (Zigmond et al., 2012). Resident Ly6C⁻ macrophages have recently been described to induce recruitment of Ly6C^{hi} monocytes to the uroepithelium via CCL2, and of neutrophils via CXCL1 and MIF (Schiwon et al., 2014). Crosstalk between resident Ly6C⁻ and recruited Ly6C⁺ macrophages was also described to control neutrophil activation: Ly6C⁺ macrophages produced TNF α , which induced CXCL2 expression by Ly6C⁻ macrophages, which in turn activated MMP9 expressed by neutrophils, allowing their invasion into the uroepithelium to combat bacterial infection (Schiwon et al., 2014). The uroepithelium might be a similar environment to the intestine in that it is also exposed to the host exterior, and thus must maintain a barrier against potential infection. Our microarray analysis also found that CX₃CR1^{int} CD64⁺ MNPs expressed *Cxcl2* (Table 3.10; Appendix, Table 1), suggesting that these cells could play a similar role in the intestinal lamina propria during *Helicobacter hepaticus*-induced inflammation. There are a number of other potential sources of chemokines, such as NK cells, which were recently described as a source of CCL3 responsible for inflammatory monocyte recruitment during ileitis (Schulthess et al., 2012),

or stromal cells, which have been shown to recruit monocytes to the colon via CCL2 production (Kim et al., 2011b).

It is possible that the relative roles of different chemokine-chemokine receptor pathways might vary depending on the context of cellular recruitment, for instance whether to resting or inflamed tissue. In the case of the latter, the particular stimuli inducing inflammation – which differ between experimental models of colitis – may differentially induce particular pathways. The resting intestine is also a special case: it is possible that constant stimulation by the intestinal microbiota induces tonic inflammation, which may result in a physiologically different state of homeostasis relative to sterile tissues.

Microbiota-derived PRR ligands have been shown to access the BM (Clarke et al., 2010), which could induce release of Ly6C^{hi} monocytes into the blood (Shi et al., 2011).

The spleen has also recently been described as a source of Ly6C^{hi} monocytes recruited to the intestine during T cell transfer- and anti-CD40-induced colitis, the peritoneum following thioglycollate administration (Liao et al., 2014), and the myocardium following myocardial infarction (Swirski et al., 2009). Unlike bone marrow egress, CCR2 was not required for monocytes to exit the spleen (Swirski et al., 2009). Instead, monocyte release from the spleen seems to be controlled by an angiotensin II/AT-1 signalling pathway (Leuschner et al., 2010; Swirski et al., 2009). Angiotensin II induces homodimerisation of AT-1, the angiotensin II type 1 receptor (Murphy et al., 1991; Sasaki et al., 1991), which stimulates cytoskeletal reorganisation and cellular motility (Kintscher et al., 2001). Liao *et al.* have shown that glucocorticoid-induced tumour necrosis factor receptor family-related protein (GITR) ligand (GITR-L) positively regulates Ly6C⁺ MHCII⁺ MNPs in the lamina propria and MLN during colitis, since GITR-L-deficient mice exhibited 50% reductions in these populations due to Ly6C^{hi} monocyte retention in the spleen (Liao

et al., 2014). Treatment with an anti-GITR-L antibody enhanced AT-1 dimerisation in thioglycollate-elicited macrophages stimulated with anti-CD40, and induced monocyte egress from the spleen when co-administered during anti-CD40 colitis (Liao et al., 2014). Whether stimulation of GITR-L induces monocyte egress directly, or indirectly via effects on other cells in the spleen is unclear. The role of the splenic reservoir as a source of Ly6C^{hi} monocytes for the intestine during the steady state is also unknown. However, this CCR2-independent source of Ly6C^{hi} monocytes might explain the residual monocyte/macrophage populations found in the lamina propria of CCR2- and CCL2-deficient mice.

Another mechanism which may be involved in the accumulation of Ly6C^{hi} monocytes and their progeny in the intestinal lamina propria is extramedullary haematopoiesis. Our lab has recently shown that granulocyte-monocyte progenitors (GMPs) accumulated in the inflamed colonic lamina propria during T cell transfer colitis (Griseri et al., 2012). Transfer of splenic GMPs isolated from colitic mice also exacerbated T cell transfer colitis, with the transferred GMPs giving rise to Gr1^{hi} CD11b⁺ neutrophils and a smaller population of Gr1^{int} CD11b⁺ monocytes in the lamina propria (Griseri et al., 2012). Whether local differentiation from GMPs plays a significant role in the accumulation of Ly6C^{hi} monocytes and later stage macrophages during colitis is yet to be determined.

6.4 Microarray analysis of CX₃CR1^{int} CD64⁺ and CX₃CR1^{int} CD64⁻ MNPs during *Hh* + anti-IL-10R colitis

We conducted microarray analysis of CX₃CR1^{int} CD64⁺ MNPs and CX₃CR1^{int} CD64⁻ MNPs sorted from the resting and inflamed large intestinal lamina propria during *Hh* + anti-IL-

10R colitis. We compared these two populations to determine the differences between the two major subsets of MNPs, monocyte/macrophages (CD64⁺) and “DCs” (CD64⁻), during colitis. CX₃CR1^{int} CD64⁺ MNPs expressed genes confirming their identity as macrophages, such as those encoding lysozyme, and Fc and other endocytic receptors. They also expressed numerous chemokines and pro-inflammatory cytokines, suggesting that these cells could perpetuate inflammation by recruiting and activating leukocytes in the lamina propria. Additionally, CX₃CR1^{int} CD64⁺ MNPs expressed MMP genes, suggesting that they might induce or exacerbate tissue damage. However, we also observed enrichment of *Slpi*, encoding SLPI, an anti-protease which could limit enzymatic tissue degradation. SLPI possesses additional anti-microbial activity and might also promote resolution of inflammation by limiting leukocyte recruitment, inhibiting mast cell and neutrophil degranulation (Williams et al., 2006), and by inducing greater expression of IL-10 and TGFβ by macrophages in response to LPS (Sano et al., 2000). Other genes expressed by CX₃CR1^{int} CD64⁺ MNPs which could reduce inflammation included *Il10* and *Il1rn* (IL-1R antagonist). CX₃CR1^{int} CD64⁺ MNPs also expressed *Tnfsf13*, encoding APRIL, through which they might be able to induce T cell-independent class switching (Castigli et al., 2005; Hardenberg et al., 2008; Litinskiy et al., 2002; Tezuka et al., 2007).

Our microarray analysis revealed enrichment of both pro- and anti-inflammatory genes in CX₃CR1^{int} CD64⁺ MNPs relative to CX₃CR1^{int} CD64⁻ MNPs found in the lamina propria three weeks after induction of *Hh* + anti-IL-10R colitis (Table 3.10; Appendix, Table 1). Pro-inflammatory genes included *Il23a*, *Il12a*, *Smox*, *Sod2*, *Trem3*, *Ccl5*, *Ccl9*, *Cxcl1* and *Cxcl2*; anti-inflammatory genes included *Il10*, *Il1rn*, *Slpi* and *Fzd1*; and genes individually described both to promote and inhibit inflammation included *Mmp13* and *Mmp14*. The CX₃CR1^{int} CD64⁺ population contained both Ly6C⁺ MHCII⁺ MNPs and Ly6C^{lo/-} macrophages

(Figure 3.5): whether the respective genes which may promote inflammation versus its resolution were expressed by the same individual cells, by subpopulations of each cellular subset identified by surface marker expression, or separately by the distinct cellular subsets is unknown. It is possible that one population or even an individual cell might have both pro-inflammatory and regulatory functions, as shown recently for Ly6C⁺ MHCII⁺ MNPs during *Toxoplasma gondii*-induced ileitis (Grainger et al., 2013). These cells accumulated in the inflamed small intestinal lamina propria and expressed pro-inflammatory mediators, such as TNF α , iNOS and IL-6, alongside the immunoregulatory molecules Arginase-1, IL-10 and PGE₂ (Grainger et al., 2013). Ly6C⁺ MHCII⁺ MNPs showed co-expression of IL-10 with TNF α , IL-1 α , IL-1 β , IL-6 and iNOS by intracellular staining, indicating that individual cells expressed both pro- and anti-inflammatory mediators (Grainger et al., 2013). Expression of IL-10 and PGE₂ were induced in Ly6C⁺ MHCII⁺ MNPs by exposure to commensal bacteria, and their production of PGE₂ was shown to limit the activation of neutrophils and therefore intestinal pathology *in vivo* (Grainger et al., 2013). Large intestinal MNPs have also been shown to co-express TNF α and IL-10 (Bain et al., 2013), further supporting the possibility that both the pro- and anti-inflammatory genes upregulated in our CX₃CR1^{int} CD64⁺ microarray population could be expressed by the same individual cells.

Reduced severity of intestinal pathology three weeks after *Hh* + anti-IL-10R treatment suggests that a shift from inflammation towards resolution had occurred by that point (Figure 3.1). Analysing cells sorted at this stage, which may be a turning point between inflammation and its resolution, might also have contributed to the enrichment of both pro- and anti-inflammatory genes in our CX₃CR1^{int} CD64⁺ MNP population.

Ideally, we would also have analysed CX₃CR1^{hi} CD64⁺ macrophages by microarray analysis, however this was unfeasible. In any potential therapeutic strategies targeting MNPs, it may be crucial to leave the functions of these constitutively anti-inflammatory resident cells intact. Now that their developmental pathway has been established (Bain et al., 2013; Tamoutounour et al., 2012), a comparative analysis of each stage during monocyte/macrophage differentiation could help to determine the precise functions of each population, whether specific populations could be targeted, and could also shed light on the factors controlling this differentiation pathway, which are currently unclear.

6.4.1 Microarray candidates for future investigation

Our microarray results highlight various potential avenues for future research. A number of these were discussed in detail in Chapter 3. Below are two candidates that may provide ways in which pro-inflammatory MNPs could be targeted during intestinal inflammation.

6.4.1.1 Solute carrier family members

Multiple members of the solute carrier family were significantly enriched in CX₃CR1^{int} CD64⁺ MNPs compared with CX₃CR1^{int} CD64⁻ cells from the inflamed lamina propria during *Hh* + anti-IL-10R colitis (Table 3.10; Appendix, Table 1), including *Slc11a1*, which has been identified by GWAS as a risk locus for UC (Anderson et al., 2011). SLC proteins are involved in the transport of diverse substances across cellular membranes. The genes upregulated in CX₃CR1^{int} CD64⁺ MNPs fell into three categories: transporters of amino acids and oligopeptides (*Slc7a11*, *Slc15a3*), inorganic ions (*Slc11a1*, *Slc25a28*, *Slc31a2*),

and solutes (*Slc2a1*). SLC family members might provide a way to target therapeutic drugs to pro-inflammatory macrophages by engineering agents to promote their uptake via selectively expressed SLCs. Targeting specific cells using drugs conjugated to monoclonal antibodies which recognise cell surface-expressed proteins has in some cases proven very successful in the treatment of cancer (Panowski et al., 2014). However, toxicity can occur when the monoclonal antibody in question is cross-reactive or recognises an antigen which it transpires is expressed by normal cells in addition to tumours (Panowski et al., 2014). Combining SLC-mediated uptake of molecules with delivery to specific cells via monoclonal antibodies could potentially allow specific targeting of substances to MNP subsets, which either method alone may be unable to achieve.

6.4.1.2 CCRL2

Another way to target pro-inflammatory cells, and reduce their contribution to pathology, is to prevent their recruitment to sites of inflammation in the first place. This can be achieved by inhibition of relevant chemokine receptors, chemokine ligands, integrins or cellular adhesion molecules (Danese and Panes, 2014; Thomas and Baumgart, 2012). Inhibiting cell recruitment has potential drawbacks, since some inflammatory cells and processes are necessary to clear damaged host cells and infection if present. However, chronic inflammation might be perpetuated by continued recruitment of pro-inflammatory cells (Kumar, 2004) and can cause unnecessary damage to the host. This may pose an additional problem in the intestine, where Ly6C^{hi} monocytes give rise to both pro-inflammatory effector cells during inflammation, and to constitutively anti-inflammatory resident macrophages during the steady state (Bain et al., 2014; Bain and

Mowat, 2014; Bogunovic et al., 2009; Rivollier et al., 2012; Tamoutounour et al., 2012; Varol et al., 2009).

We observed that *Ccr1* expression was enriched in CX₃CR1^{int} CD64⁺ MNPs from resting and inflamed lamina propria, and appeared to be upregulated by these cells during colitis (Table 3.10; Appendix, Tables 1-3; Figure 3.9). CCRL2 shares homology with CCR1 (Fan et al., 1998), which we also observed was expressed by CX₃CR1^{int} CD64⁺ MNPs three weeks after *Hh* + anti-IL-10R treatment (Figure 5.20). Our studies show that IL-23 was necessary for the recruitment of Ly6C⁺ pro-inflammatory MNPs to the lamina propria after *Hh* + anti-IL-10R treatment, and in the absence of IL-23, transcript levels of *Ccr1* and *Ccl3* and secretion of CCL3 protein were reduced among combined colonic and caecal lamina propria cells (Figure 5.21). CCRL2 seems to be a functional receptor for CCL3, since *Ccr1*^{-/-} BMDCs show reduced chemotaxis towards this chemokine (Otero et al., 2010). Our findings from multiple studies support further investigation of the role of the CCRL2/CCR1-CCL3 axis in recruitment of Ly6C^{hi} monocytes and Ly6C⁺ MHCII⁺ MNPs to the inflamed large intestine.

6.5 Summary of findings

Our results from *Hh* + anti-IL-10R colitis confirm that Ly6C⁺ MNPs accumulate in colitis, as recently shown in the DSS (Bain et al., 2013; Platt et al., 2010; Zigmond et al., 2012) and T cell transfer (Rivollier et al., 2012; Tamoutounour et al., 2012; Weber et al., 2011) models. Furthermore our data support that Ly6C⁺ MHCII⁺ MNPs play a pro-inflammatory role, at least through their production of IL-23. Although we cannot specify whether genes enriched in CX₃CR1^{int} CD64⁺ MNPs were expressed by Ly6C⁺ MHCII⁺ MNPs versus Ly6C^{lo/-} macrophages, our microarray results suggest numerous other mediators which could drive inflammation during *Hh* + anti-IL-10R colitis. We have shown that IL-23 produced by Ly6C⁺ MHCII⁺ MNPs and CX₃CR1^{hi} macrophages is required for the development of intestinal pathology and the accumulation of pro-inflammatory immune cells following *Hh* + anti-IL-10R treatment. Preliminary data suggest a role for CCR1 or CCRL2 in the recruitment of inflammatory monocytes during colitis and are deserving of further investigation. These findings are summarised visually in Figure 6.1. In addition to pro-inflammatory mediators, our microarray results suggest that CX₃CR1^{int} CD64⁺ MNPs expressed a number of genes that might suppress inflammation or promote its resolution. This was consistent with our studies of intestinal pathology and the composition of the lamina propria MNP compartment throughout the *Hh* + anti-IL-10R model of colitis, which suggest at the point of sorting cells for our microarray, three weeks after induction of colitis, a shift towards resolution may have occurred. Further studies are necessary to determine the factors controlling differentiation of MNPs in the intestine, as well as the exact contributions of specific MNP subsets to driving and resolving intestinal inflammation. Understanding these processes could allow us to therapeutically

manipulate the balance of pro- and anti-inflammatory MNP populations to improve the treatment of IBD.

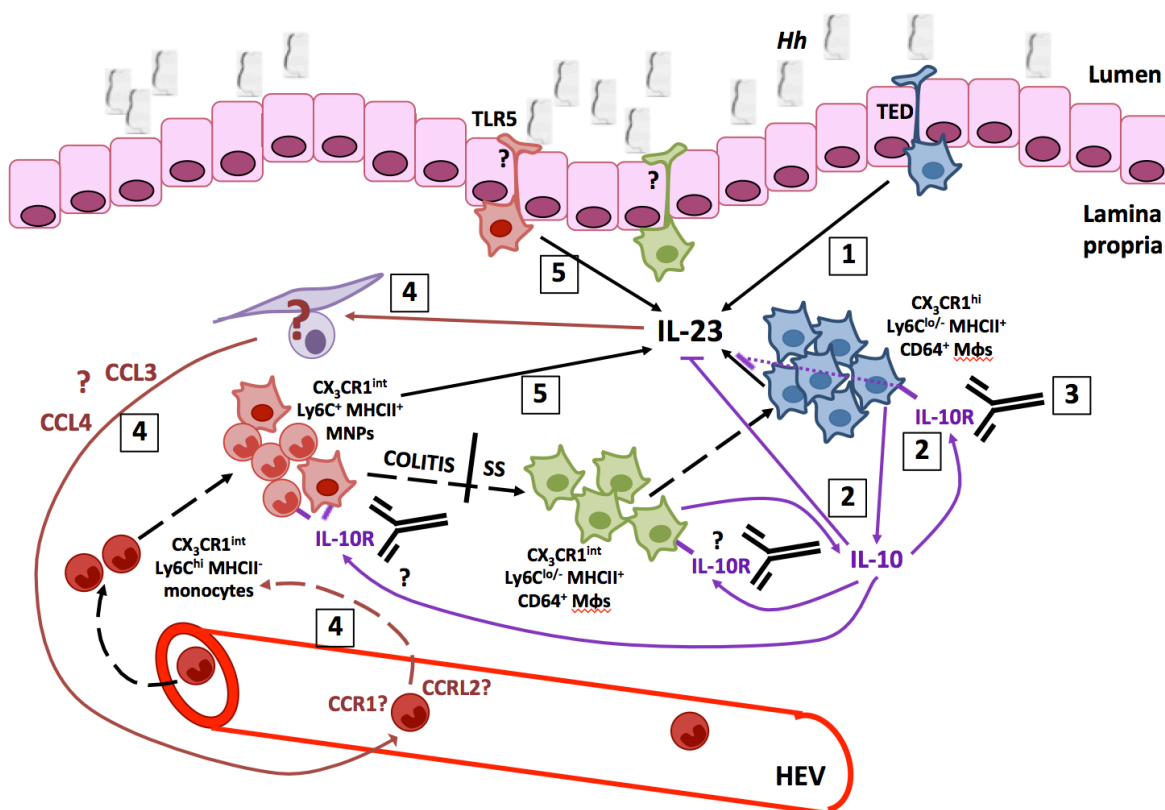


Figure 6.1 Summary of results: onset of inflammation in *Hh* + anti-IL-10R model.

Hh infection alone induces intestinal expression of IL-23 (1), which we observed among CX₃CR1^{hi} lamina propria macrophages, however the dominant cytokine produced in the lamina propria is IL-10 (Kullberg et al., 1998) which prevents the development of colitis (2). Blockade of IL-10R during *Hh* infection enhances IL-23 expression by CX₃CR1^{hi} macrophages (3) and results in intestinal inflammation. CX₃CR1^{hi} macrophages are abundant in the uninfamed lamina propria, and our results suggest that these cells may be the initial source of IL-23 which triggers the pro-inflammatory cascade leading to colitis. Subsequently, CX₃CR1^{int} Ly6C⁺ MHCII⁺ MNPs newly recruited to the lamina propria – perhaps via CCR1 or CCRL2, following induction of chemokines such as CCL3 downstream of IL-23 (4) – amplify this inflammatory pathway by producing more IL-23 (5), potentially in addition to other pro-inflammatory cytokines, chemokines, reactive oxygen species and proteases. Not illustrated are other events such as neutrophil recruitment, and the initiation of a Th17/Th1 response, as discussed in Chapter 4.

6.6 Directions and considerations for future study of intestinal MNPs

6.6.1 MNP subset identification

It is crucial that we unify how researchers identify and describe MNP populations. This includes our definitions by surface marker expression, and ideally also what we call them, to minimise confusion and to allow better interpretation of results from different research groups and experimental systems. While the author does not necessarily agree with all aspects of the suggested system, a unified nomenclature has recently been proposed (Guilliams et al., 2014). Whether we will find truly specific surface markers or transcription factors is unclear. Monocyte/macrophage MNPs in the intestine exist in a continuum of differentiation, and seem to display changes in levels of expression of common molecules rather than distinct markers (Tamoutounour et al., 2012).

Determining the factors controlling the differentiation of Ly6C^{hi} monocytes into macrophages, and how to promote certain stages, might yield more useful results (Bain and Mowat, 2014). What controls this process during the steady state, inflammation and the resolution of inflammation is incompletely understood.

6.6.2 Experimental models of intestinal inflammation

When the specific effects of a gene or microbial agent are not being investigated, certain models of colitis tend to be used more often than others, such as DSS-induced colitis, perhaps due to the relative ease of use. It is important when interpreting the literature to remember which model has been used; although different stimuli inducing inflammation may produce the same end result, they may do so via distinct pathways. For instance, distinct chemokine-chemokine receptor pathways might be involved in

Ly6C^{hi} monocyte recruitment to the resting versus inflamed lamina propria, and in their recruitment during different models of inflammation (Bain et al., 2014; Bain et al., 2013; Khan et al., 2006; Kim et al., 2011b; Platt et al., 2010; Popivanova et al., 2009; Takada et al., 2010; Zigmond et al., 2012). Additionally, different stimuli inducing apparently similar intestinal pathology might do so by activating the same cell type in distinct ways. It is clear from recent studies that macrophages isolated from different tissues, or generated *in vitro* and stimulated in different ways, express diverse sets of genes (Gautier et al., 2012; Xue et al., 2014). These factors must be kept in mind when designing experiments; interpreting results; and comparing results using different models, tissues (region of GI tract), *in vitro* cellular sources (bone marrow, PBMCs, cell lines), or stimulation. We are now beginning to understand the influence of the microbiota on the development of IBD (Huttenhower et al., 2014), which highlights the importance of such considerations.

Different models of intestinal inflammation might represent different phases of IBD. For example, a model of acute colitis might be more useful to study mechanisms of inflammation involved in disease onset or relapse, while chronic models could allow investigation of how inflammation is maintained or exacerbated. Models in which resolution occurs could be used to identify factors promoting remission. The type and phase – the start of a flare, chronic inflammation, approaching or in remission – of a patient's IBD plays an important part in the decision of treatment strategy (Mowat et al., 2011). When testing novel therapeutic agents in pre-clinical studies using experimental models, it could be worthwhile assessing each agent in diverse models which best recapitulate distinct phases of disease.

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