INTESTINAL MACROPHAGES IN HEALTH AND INFLAMMATION

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Author's Declaration

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Abbreviations

7-AAD	7-amino-actinomycin D
AAM	alternatively activated macrophages
-APC	allophycocyanin
APC	antigen presenting cell
BLP	bacterial lipoprotein
BM	bone marrow
BMM	bone marrow-derived macrophages
BrdU	bromodeoxyuridine
CAM	classically activated macrophage
CD	clusters of differentiation
CMF	calcium magnesium free
CSF1	colony stimulating factor-1
CSF-1R	colony stimulating factor-1 receptor
DAPI	4',6-diamidino-2-phenylindole
DC	dendritic cell
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DSS	dextran sulphate sodium
EDTA	ethylendiaminetetraacitic acid
ELISA	enzyme-linked immunosorbent assay
EMA	ethidium monoazide
ER	endoplasmic reticulum

ES	excretory-secretory
FACS	fluorescence-activated cell sorter
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FSC	forward scatter
GALT	gut associated lymphoid tissues
GM-CSF	granulocyte-macrophage colony-stimulating factor
HBSS	Hank's buffered salt solution
HPRT	hypoxyanthine-guanine phosphoribosyltransferase
IBD	inflammatory bowel disease
IEC	intestinal epithelial cells
IFN	interferon
Ig	immunoglobulin
IKK	inhibitory κB kinase
IL	interleukin
i.p.	intraperitoneal
IP-10	IFNγ-induced protein-10
IRAK	IL-1 receptor-associated kinase
IRF	IFN-regulatory factor
i.v	intravenous
kD	kilo Daltons
KO	knock out
LDL	low density lipoprotein

LP	lamina propria
LPS	lipopolysaccharide
mϕ	macrophage
mAb	monoclonal antibody
MACS	magnetically activated cell sorter
MCP-1	macrophage chemoattractant protein-1
M-CSF	macrophage colony-stimulating factor
MDA-5	melanoma differentiation-associated protein-5
MDP	muramyl dipeptide
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
MIG	monokine induced by γIFN (CXCL9)
MIP-1α	macrophage inflammatory protein-1α
MIP-1β	macrophage inflammatory protein-1β
MLN	mesenteric lymph node
mRNA	messanger ribonucleic acid
MyD88	myeloid differentiation factor 88
NFκB	nuclear factor-κB
NK	natural killer
NLR	Nod-like receptor
NO	nitric oxide
NOD	nuclear oligomerisation domain
OD	optical density

PBS	phosphate buffered saline
PC	phosphorylcholine
PCR	polymerase chain reaction
PE	phycoerythrin
PEC	peritoneal exudate cells
PI	propidium iodide
PLN	peripheral lymph nodes
poly I:C	polyinosinic-polycytidylic acid
PP	Peyer's patch
PRR	pattern recognition receptor
RANTES	regulated upon activation, normal T-cell expressed, and secreted (CCL5)
RIG-1	retinoic acid-inducible gene-I
RLR	RIG-I-like receptor
RNA	ribonucleic acid
RPMI	roswell park memorial institute-1640 medium
RT	reverse transcriptase
RT-PCR	reverse transcriptase polymerase chain reaction
SA	streptavidin
s.c.	subcutaneous
SCID	severe combined immunodeficiency
SD	standard deviation
SPF	specific pathogen free
SSC	side scatter

STAT	signal transducers and activators of transcription
TAK1	transforming growth factor β-activated kinase
TCR	T cell receptor
TGF	transforming growth factor
Th	T helper cell
TLR	Toll-like receptor
TMB	3, 3′, 5, 5′-tetramethylbenzidine peroxidase
TNF	tumour necrosis factor
TNFR	tumour necrosis factor receptor
TRAF6	TNF receptor activated factor 6
Treg	regulatory T cell
TRIF	TIR domain-containing adaptor protein inducing IFNβ
WT	wild type

g	gram
mg	milligrams
μg	micrograms
ng	nanograms
M	molar
mM	millimolar
μM	micromolar
ml	millilitres

Summary

The healthy large intestinal mucosa contains a vast pool of macrophages (mϕ) that are close to the local bacterial flora and have several unique phenotypic and functional properties compared with other mϕ populations. Although human colonic mϕ retain some of the hallmark functions of mϕ, such as the ability to phagocytose particulate material and exert bactericidal activity, they are unable to produce pro-inflammatory mediators. Thus it has been suggested that intestinal mϕ are functionally adapted to the microbe-rich, immunostimulatory environment of the gut, where strong inflammatory responses to harmless commensal bacteria would lead to continuous inflammation and ultimately tissue pathology. Indeed, there is mounting evidence that mϕ play an essential role in maintaining homeostasis and epithelial renewal in the normal intestine. In contrast, mϕ from the intestine of patients with inflammatory bowel disease (IBD) differ markedly from those present physiologically, exhibiting heightened inflammatory and bactericidal activities, and contributing to the tissue damage. How these differing properties of colonic mϕ are controlled and how this potentially dangerous population is kept quiescent under physiological conditions are important questions. Most existing information comes from either simple, observational studies of human tissue, or from work on cell culture systems which aim to reproduce the unusual phenotype of resident intestinal mϕ *in vitro*. Importantly analogous experiments of resident and inflammatory mϕ have not been carried out in murine systems, where it would be possible to characterise the cells fully and explore their origin, function and role in inflammatory processes.

Therefore, the aims of this thesis were first to characterise mϕ in the resting murine colon both functionally and phenotypically, focussing particularly on their expression of toll-like receptors (TLR) and responsiveness to TLR ligation and on their population dynamics *in vivo*. By comparing colonic mϕ with other tissue mϕ populations, I hoped to gain an understanding of how resident gut mϕ might have adapted to their local environment. In the second part of my work, I examined how the properties of colonic mϕ altered in inflammation, employing a well-established experimental model of colitis, with the aim of determining how resident and inflammatory mϕ might relate to each other. Lastly, I explored the effects of the ES-62 parasite product, known to have potent anti-inflammatory effects on mϕ, on experimental colitis *in vivo*.

Experiments detailing my initial characterisation of the myeloid cells expressing the F4/80 mϕ marker in the colon of normal mice are described in Chapter 3. These revealed that the F4/80$^+$ population in the gut is extremely heterogeneous compared with other mϕ populations in the body. Virtually all *in vitro*-differentiated BM mϕ (BMM) and mϕ from the resting peritoneum (PEC mϕ) exhibited the conventional F4/80$^+$CD11b$^+$CD11c$^-$ phenotype of classical mϕ and upregulated costimulatory molecules in response to TLR ligation. In stark contrast, the colon contained three F4/80$^+$ subsets, one F4/80$^+$CD11b$^+$CD11c^{int}, one F4/80$^+$CD11b$^+$CD11c$^-$ and a smaller population of F4/80$^+$CD11b$^-$CD11c$^-$ cells. None of these subsets expressed co-stimulatory molecules, even after LPS stimulation, but unlike other mϕ, the majority of colonic mϕ expressed high levels of class II MHC without stimulation. BMM and PEC mϕ also produced several pro-inflammatory cytokines and chemokines following stimulation, whereas colonic mϕ showed no mediator production under these conditions. Nevertheless, colonic mϕ did retain avid endocytic and phagocytic activities, indicating that colonic mϕ may engulf bacteria without initiating inflammation.

In Chapter 4, I explored the unresponsiveness of resting colonic mϕ to microbial stimuli in more detail and found that the TLR refractoriness is associated with reduced expression of TLR2, 3, 4 and 9. Apart from a small proportion of mϕ that retained TLR2 expression, TLR expression was downregulated both at the protein level and to some extent also at the mRNA level; TLRs were not re-expressed following *ex vivo* culture of purified mϕ. This global downregulation of TLRs could not be reproduced in BMM by treatment with TLR ligands, and was also present in colonic mϕ taken from mice unable to signal via TLR2 or TLR4, suggesting it was not simply a form of endotoxin "tolerance". However, the mechanism seemed to involve IL-10, as colonic mϕ from IL-10-deficient animals displayed a heightened level of TLR expression and responsiveness, even prior to the onset of intestinal inflammation.

In Chapter 5, I examined the phenotype and function of mϕ during the experimental colitis induced by feeding dextran sodium sulphate (DSS). During inflammation, the absolute number of F4/80$^+$ mϕ increased 6-fold, the majority of which now expressed TLR, CD11b and low levels of CD11c. This new population of colitic mϕ also expressed class II MHC, low levels of co-stimulatory molecules and produced large amounts of TNFα. In Chapter 6, I went on to examine the population dynamics of colonic mϕ under resting conditions and during inflammation, showing that the overall turnover rate of the total mϕ population was increased during colitis, as assessed by uptake of BrdU *in vivo*. The increased turnover was mainly due to the TLR-expressing, TNFα$^+$ population of mϕ and more detailed analysis showed that the small number of these cells present in resting colon had identical turnover rates to those found in colitis. In contrast, the TLR negative mϕ had much lower turnover rates in resting and inflamed colon, suggesting that the TLR$^+$ and TLR$^-$ subsets may represent distinct mϕ populations with different population dynamics, and that during intestinal inflammation, the TLR$^+$ subset may display a preferential recruitment into the gut. Indeed, proliferation *in situ* was minimal, indicating that the recently divided, TLR-expressing mϕ proliferated outside the intestine before being recruited into the gut. My subsequent experiments suggested that this recruitment may involve the CCR2 chemokine receptor, which was expressed at high levels specifically by the TLR$^+$ subset of mϕ both in resting and inflamed colon.

Finally in Chapter 7, I treated colitic mice with ES-62, a phosphorylcholine (PC)-containing glycoprotein secreted by the filarial nematode, *Acanthocheilonema viteae*, which has been shown to modulate pro-inflammatory cytokine production by mϕ *in vitro*. ES-62 treatment had no significant effect on weight loss or pro-inflammatory cytokine production in the colon of mice with DSS colitis, although it slightly delayed the onset of the clinical signs of disease. Thus further studies of ES-62 as a modulator of mϕ-dependent intestinal inflammation may be warranted.

Taken together, my data suggest that under resting conditions, intestinal mϕ are heterogeneous and adapt to their microenvironment by being non-inflammatory via active downregulation of TLR expression and function, which may be partly dependent on IL-10. During inflammation, large numbers of TLR expressing, fully responsive mϕ appear, probably via CCR2-dependent recruitment of recently divided blood-derived monocytes. Interestingly, small numbers of these TLR-expressing, rapidly turning over mϕ are also present in normal colon and my data suggest that these pro-inflammatory mϕ may be quite distinct from the more sessile mϕ which are the dominant "resident" population in normal gut. A delicate balance between these two mϕ populations must ensure homeostasis and appropriate responses to inflammation.

Chapter 1

General Introduction

The immune system has evolved complex and stringent regulatory mechanisms to ensure that whilst it can defend the body against an endless array of invasive pathogens, it can also actively prevent immune effector responses against its own tissues and innocuous antigens. Furthermore, the immune system must ensure it can terminate responses once a pathogen has been eradicated to prevent ongoing inflammation and tissue damage. The innate and adaptive components of the immune system act in concert to ensure this vital balance is maintained.

There is no other tissue in the body that the importance of this balance is more apparent than in the intestine, which is home to a vast and complex microbiota, along with experiencing lifelong exposure to a large variety of food antigens. At the same time, the gut mucosa represents one of the main entry routes for potentially lethal pathogens. Therefore, the intestinal immune system has evolved many unique properties and a complex interplay of regulatory mechanisms to ensure homeostasis is maintained in this essential tissue.

1.1 The intestinal immune system

The gastrointestinal tract consists of the small intestine, caecum, the large intestine (colon) and rectum. The small intestine is divided into the duodenum, jejunum, and ileum, and is where the majority of digestion takes place, whereas the colon is primarily responsible for reabsorbing water. There is a marked gradient of the numbers of commensal bacteria going down the intestine, from the almost sterile jejunum, to the descending colon, which has a large resident population of microbiota, consisting of at least 10^{10}-10^{12}

organisms per gram of luminal contents (1). These organisms play essential roles in many physiological processes and the normal function of the intestinal immune system is dependent on colonisation by commensal microbes. These organisms, together with the antigenic load provided by the diet and the constant threat of potential pathogens, means the intestinal immune system encounters more antigen than any other part of the body. To deal with this onslaught, the intestine contains the largest compartment of the immune system. As many bacterial, parasitic and viral pathogens enter the body via the intestinal mucosa, it is vital the gut-associated lymphoid tissues (GALT) can provide strong and effective immune responses when necessary. However, inappropriate responses against innocuous food and commensal antigens can lead to inflammatory disorders such as coeliac disease and inflammatory bowel diseases (IBD).

The two main forms of IBD, Crohn's disease and ulcerative colitis, are chronic, relapsing inflammatory disorders that result in loss of intestinal architecture and tissue destruction. Ulcerative colitis is restricted to the colon and involves a superficial inflammation, whereas Crohn's disease can affect the entire gastrointestinal tract and is characterised by transmural inflammation and the formation of mϕ-rich granulomas. Both forms of IBD involve a substantial infiltrate of neutrophils, monocytes/mϕ, eosinophils and T cells into the intestine. However it is thought that Crohn's disease has a predominant Th1-type cytokine profile with elevated levels of IFNγ and TNFα, whereas ulcerative colitis is considered a more Th2 polarised disease, with increased levels of IL-13, IL-5 and TGFβ (2). There is considerable evidence that the host's commensal microbiota are crucial for the development of IBD, with virtually all experimental models of IBD being dependent on the presence of commensal bacteria (3, 4). In addition, broad-spectrum antibiotic therapy has proven beneficial in several trials in IBD patients (5, 6), and mutations in the gene encoding nucleotide-binding oligomerisation domain (Nod) 2, a cytosolic receptor for bacterial muramyl dipeptides (MDP) (7), are associated with 30% of familial cases of Crohn's disease (8, 9).

The lymphoid elements of the gut comprise organised lymphoid tissues such as the Peyer's patches (PP), which lie at regular intervals along the small bowel, and the mesenteric lymph nodes (MLN). In addition, isolated lymphoid follicles, whose architecture resembles that of PP, with the exception that they lack a discrete T zone, exist along the small intestine and colon (10). The role of all these tissues is in the induction phase of intestinal immune responses, as they represent the sites where antigens are taken up and presented to B and T lymphocytes. The effector sites of the intestine are the mucosal epithelium and underlying lamina propria (LP) in both the large and small intestine. Here there are many different immune cells including activated T cells, plasma cells, mast cells, dendritic cells (DCs) and mϕ even under normal conditions, giving rise to the idea that homeostasis in the gut is characterised by a state of physiological inflammation. That this does not result in overt tissue pathology reflects the fact that the effector cells present are actively held in check by potent regulatory mechanisms.

1.2 Mechanisms of immune protection and inflammation in the intestine

Although composed of only a single cell layer, the intestinal epithelium itself forms a barrier against penetration of microorganisms. Defects in barrier function are thought to be a major contributor to the development and perpetuation of inflammation in IBD (11, 12). Furthermore, the epithelial cells of the small intestine are coated in a glycocalyx of mucins and other glycoproteins that can interact with and trap bacteria in the mucus so that they are simply washed away (13). In addition, anti-microbial peptides such as defensins are secreted by Paneth cells located at the bottom of the intestinal crypts, providing another level of innate protection (14). Epithelial cells also act as microbial sensors by secreting chemoattractant factors such as IL-8, MCP-1, TNFα and IL-6 in response to bacterial entry (15-17). This results in the recruitment of neutrophils, eosinophils, monocytes/mϕ and T cells, and so enhances the induction of protective immunity. As mentioned above, the intestine is also characterised by the presence of many specific immune cells, including IgA-secreting plasma cells, $CD4^+$ and $CD8^+$ T cells, regulatory T cells and $\gamma\delta$T cells. The exact functions of mucosal $CD4^+$ and $CD8^+$ T cells remain unclear, but the majority have a 'memory' phenotype (18) and some show effector function such as pro-inflammatory cytokine production and cytotoxic T lymphocyte (CTL) activity (19, 20); others are hyporesponsive to TCR ligation (21), and may be regulatory T cells.

1.3 Mechanisms of immune tolerance in the intestine

A large number of immune tolerance mechanisms have been described in the intestine. These result in the usual response of the intestine to innocuous protein antigens, such as food antigens, being the induction of local and systemic immunological tolerance, termed oral tolerance (22). The mechanisms of oral tolerance include the deletion of specific T lymphocytes (23), induction of T cell anergy (24), and the differentiation of regulatory T cell populations (25). Many of these T cell properties may be determined by the presence of inhibitory DC in the gut. Our laboratory has shown that small bowel LP DC produce IL-10 and type 1 IFN, but not IL-12, and although they present antigen to specific $CD4^+$ T cells, this induces hyporesponsiveness to subsequent challenge in these cells (26). Similarly, regulatory DC populations have been described in the PP and MLN (27-30).

Effector immune responses also do not occur against commensal microbiota, but it is thought that there may be differences in the way immune responses against commensals are controlled, compared with protein antigens. Firstly, commensals can themselves actively downregulate host inflammatory responses. For example, *Bacteriodes thetaiotaomicron* inhibits NFκB actively in epithelial cells by enhancing its peroxisome proliferator activated receptor γ (PPARγ)-dependent nuclear export, thus antagonising NFκB (31). Furthermore, some non-pathogenic Salmonella strains can block the activation of NFκB via the inhibition of IκB-α ubiquitination (32). Another reason for the lack of inflammation in the normal intestine could be the nature of the pathogen-associated molecular patterns (PAMPs) found on commensal bacteria. The extent of acetylation of the lipid A component of LPS seems critical for activating the host innate immune response (33), and the lipid A of the *Bacteroides* genus, one of the predominant species in the lower intestine, is pentacylated and thus exerts weak endotoxicity (34, 35). Unlike the situation with food antigens, there may be no systemic immune recognition of, or tolerance to, commensal bacteria. Instead these antigens are taken up by mucosal DC and thereafter are limited to the gut and MLN (36). This may then lead to local secretory IgA production and the induction of regulatory T cells in the GALT and LP (36), indicating that the mucosal immune system lies in a mutual balance with commensals, restricting their penetration by inducing local IgA, and regulatory T cells that prevent inflammation. However, the systemic immune response remains ignorant of these antigens as is supported by the fact that normal mice have no serum antibodies to *Enterobacter cloacae*, but do have specific IgA in their intestinal washings (37). As a result, intravenous injection of mice with *E. cloacae* drives a normal immune response, in contrast to the tolerance found when soluble proteins are given orally (37).

1.4 Macrophages and intestinal immune responses

Given their number and proximity to the local microbial flora, it seems likely that mϕ in the colon may play an important part in the regulation of local immune responses to bacteria. Indeed, mice deficient in bactericidal mechanisms that generate reactive oxygen and nitric oxide (NO) radicals develop abscesses in the liver, spleen and intestine containing commensals (38), suggesting that active killing mechanisms are critical to maintain a steady level of commensals. One of the aims of this thesis was to investigate how resident mϕ may contribute to these processes.

Historically, mϕ were defined as scavenging and bactericidal tissue-resident cells with critical innate immune functions. They exhibit stellate morphology, express non-specific esterase, lysosomal hydrolases and ecto-enzymes, and contribute to non-specific uptake of particulate material (39). They also express an array of receptors for the Fc portions of immunoglobulin (Ig) and complement components. When activated, tissue mϕ phagocytose and kill microorganisms, and secrete pro-inflammatory cytokines. They can also play a role in adaptive immunity, as mϕ can degrade phagocytosed material and process antigens for presentation to T cells on MHC molecules, and may even be able to prime naïve T cells *in vivo* (40). In addition, the pro-inflammatory cytokines and chemokines they release upon activation contribute to the recruitment and activation of antigen-specific lymphocytes.

Resident mϕ are found throughout the body and can take on many different appearances and form depending on their tissue of origin. These include Kupffer cells in the liver, microglia in the central nervous system, osteoclasts in bone, alveolar mϕ in the lung, metallophilic mϕ in the splenic marginal zone, and the resident mϕ of the gastrointestinal tract (Table 1.1). Every epithelial and endothelial surface in the body has a significant mϕ population, generally found in the area underlying the basement membrane separating the surface layer from the rest of the tissue. In the intestine, mϕ are abundant in the lamina propria of the small and large intestines.

Location	**Name**
Liver	Kupffer cells
Bone	Osteoclasts
Central nervous system	Microglia
Splenic marginal zone	Metallophilic macrophages
Splenic marginal zone	Marginal zone macrophages
Lung	Alveolar macrophages
Germinal centre	Tingible body macrophages
Epidermis	Langerhans cells

Table 1.1 Macrophage populations throughout the body

Numerous markers for mϕ have been described, including F4/80, CD11b (also known as mϕ-1 (Mac-1); complement receptor 3 (CR3)), CD115, macrosialin (CD68), CD83 and CD11c, although the latter is generally considered to be a marker of murine DC. CD68 is used as the conventional marker of human mϕ, while F4/80 is the most widely used marker of tissue mϕ in mice. F4/80 is a member of the EGF-like 7 transmembrane spanning (EGF-TM7) family, which also includes human EGF module-containing mucin-like hormone receptor 1 (EMR1) and human CD97. No clear function has been described as yet for F4/80, but it has been suggested that it could be involved in the adhesion and/or retention of mϕ in tissues (41), as has been shown for epidermal Langerhans cells (LCs) which downregulate F4/80 following stimulation, before migrating to draining LNs (42). F4/80 is expressed on the cell surface of the majority of tissue mϕ populations, and at lower levels by blood monocytes (43). However, F4/80 is not expressed by mϕ in the marginal zone of the spleen, mϕ in the lung, or by osteoclasts (44, 45). Instead, marginal zone mϕ are identified by a monoclonal antibody, MOMA-1, which recognizes sialoadhesin (CD169, Siglec-1) and by their strong phagocytic capacity (46, 47). In addition, some other cell types such as eosinophils can express low levels of F4/80 (48).

1.5 Roles of macrophages in the immune system and beyond

The principal role of mϕ in the immune system is their unrivalled capacity to engulf and kill pathogenic microbes including bacteria, protozoa, helminths and fungi. These processes usually involve actin-dependent phagocytosis, whereby the mϕ extends part of its cell membrane around the bacteria as pseudopodia, internalising the resulting membrane-bound vesicle into a phagosome. The phagosome then fuses with a lysosome, forming a phagolysosome that contains the machinery responsible for killing microbes and degrading cellular material. During this process the mϕ becomes activated via pathogen recognition receptors by PAMPs such as TLR ligands present on the microbe (see below) and current evidence suggests that this may have to happen inside the phagosome itself (49). Mϕ activation is also enhanced by IFNγ released by NK cells and T cells.

An important function of mϕ under resting and inflammatory conditions is the rapid uptake of apoptotic tissue and inflammatory cells from tissues. The uptake of apoptotic cells is mediated by a range of receptors such as CD36 and this induces an anti-inflammatory response, as apoptosis drives the release of TGFβ (50). This process is important for tissue homeostasis (50), as uptake of apoptotic cells by mϕ prevents their contents leaking into the environment and hence further limits the release of toxic and pro-inflammatory mediators. This contrasts with the inflammatory reactions that occur after uptake of necrotic cells which do leak their contents into the local environment. Interestingly, the receptors involved in the recognition of apoptotic cells, such as CD36 and $\alpha v\beta 3$, may also be involved in the recognition of necrotic cells (51).

Mϕ are much more than just the 'big eaters' implied by their name, as it has long been known that they play important roles in tissue homeostasis, strongly influencing the function and differentiation of neighbouring cells. They play a non-redundant and pleiotropic role in wound healing and tissue homeostasis, and aid tissue repair following inflammation-induced damage (52, 53). They do this via the clearance of apoptotic and senescent cells, by inducing mesothelial cell proliferation (54), and by producing extracellular matrix regulators such as thrombospondin 1 and TGFβ (55-57). These are involved in the inhibition of neoangiogenesis and fibroblast accumulation, respectively, promoting collagen deposition (57). Mϕ also participate in the normal ontogeny of tissues and mϕ-deficient osteopetrotic op/op mice exhibit sensory neural dysfunction, infertility and defects in mammary gland development and in insulin-secreting cells of the pancreas (58-60).

Thus tissue resident mϕ play apparently contrasting roles in the body, maintaining tissue homeostasis via the clearance of apoptotic and senescent cells, and cellular debris, and by initiating inflammatory responses to pathogenic insult.

1.6 Features of activated macrophages

Mϕ activation is the induction of anti-microbial mechanisms in the cell and this occurs when mϕ are exposed to microbial moieties such as LPS, together with immune signals, particularly IFNγ. Mϕ activation is accompanied by the production of pro-inflammatory cytokines such as TNFα, IL-12, IL-6 and IL-1. Mϕ-derived TNFα and IL-1 act on the endothelium of small blood vessels at the site of infection, activating the endothelial cells to upregulate adhesion molecules (61) and thus promoting monocyte migration to sites of infection. This, together with the production of inflammatory chemokines such as MCP-1, IL-8 and MIP-3α by mϕ, results in further recruitment of mϕ, neutrophils and other inflammatory cells. Mϕ activation also results in the induction of several bactericidal mechanisms such as the NADPH oxidase enzyme in both the plasma membrane and the membrane of the phagosome. This converts oxygen into the superoxide anion and other free radicals, and these reactive oxygen intermediates (ROIs) are toxic to the ingested microbe via *inter alia*, DNA degradation and inactivation of metabolic enzymes. Patients with chronic granulomatous disease (CGD) have a defect in the gene encoding for NADPH oxidase and therefore their mϕ are defective in microbial killing, resulting in recurrent bacterial and fungal infections. Activated mϕ also release nitric oxide (NO), formed by the action of the enzyme inducible nitric oxide synthase (iNOS) on the substrate L-arginine. NO is toxic to bacteria, primarily by causing DNA damage and is critical in innate immunity, as evidenced by the susceptibility of iNOS KO mice to infection (62). Activated mϕ also upregulate the expression of class II MHC and co-stimulatory molecules, resulting in enhanced antigen-presenting capacity.

1.7 Microbial recognition by macrophages

Mϕ are remarkably well equipped to recognise microbes and microbial products, achieving this through the expression of germline-encoded pathogen recognition receptors (PRRs). These PRRs recognise conserved structural motifs found on prokaryotic and lower eukaryotic organisms, the so-called pathogen-associated molecular patterns (PAMPs). The best known PRRs are the 11 members of the TLR family, most of which are expressed by mϕ. The ligands for TLR1-9 are well defined, recognising PAMPs such as bacterial LPS, lipoprotein, lipoteichoic acid, flagellin, RNA and unmethylated CpG-containing DNA. The ligand for TLR10 has not been determined, whilst a profilin-like molecule from

Toxoplasma gondii has been shown to signal via TLR11 (63). Details of the recognition patterns and cellular location of the different members of the TLR family are shown in Table 1.2.

Some TLRs can also bind multiple ligands. For example, in addition to its well known ability to recognise bacterial LPS, TLR4 can also bind the fusion protein of respiratory syncytical virus (64, 65), as well as endogenous ligands such as HSP60 and HSP70 (66-68). TLR2 can also bind zymosan, a glucan present in yeast cell walls (69), and can form complexes with TLR1 and TLR6, conferring specificity for additional microbial components (70), as well as the ability to recognise subtle differences between triacyl and diacyl lipopeptides (71). This means that together, TLRs equip the innate immune system with the ability to detect pathogens of a bacterial, fungal and viral nature, as well as endogenous ligands associated with cell stress.

Receptor	Cellular location	Ligand(s)	Expression by mϕ	References
TLR1	Plasma membrane	Triacyl lipopeptides	Most populations	(72, 73)
TLR2	Plasma membrane	Lipoprotein, peptidoglycan, lipoteichoic acid	Most populations	(73, 74)
TLR3	Endosome	dsRNA	Most populations	(75)
TLR4	Plasma membrane	LPS	Most populations	(76, 77)
TLR5	Plasma membrane	Flagellin	Human monocytes, not mouse	(78-80)
TLR6	Plasma membrane	Lipoteichoic acid	Most populations	(70, 71)
TLR7,8	Endosome	ssRNA	Some populations	(81-83)
TLR9	Endosome	Unmethylated CpG containing DNA	Most populations	(84)

Table 1.2 TLRs, their location in the cell, their ligands and their expression by tissue macrophage populations

1.8 Cellular Distribution of TLR

Monocytes and mϕ have been shown to express mRNA for most TLRs except TLR3 (85), whereas TLR expression by DC varies with the subset (86). T cells and B cells also express several TLRs and the roles they play in lymphocyte responses are discussed briefly below. In addition, tissue cells such as epithelial cells can also express TLR, but expression by intestinal epithelial cells (IEC) is controversial. IEC may not express any on the apical surface, but may express TLR5 on their basolateral surface (87), thereby only inducing an a inflammatory response to those bacteria that have crossed the epithelial barrier. Finally, human dermal endothelial cells have been shown to express TLR4 (88). Thus there is a wide range of immune and non-immune cells that can recognise microbes via TLR, and the importance and the role of TLR expression by mϕ is discussed below.

1.9 TLR signalling

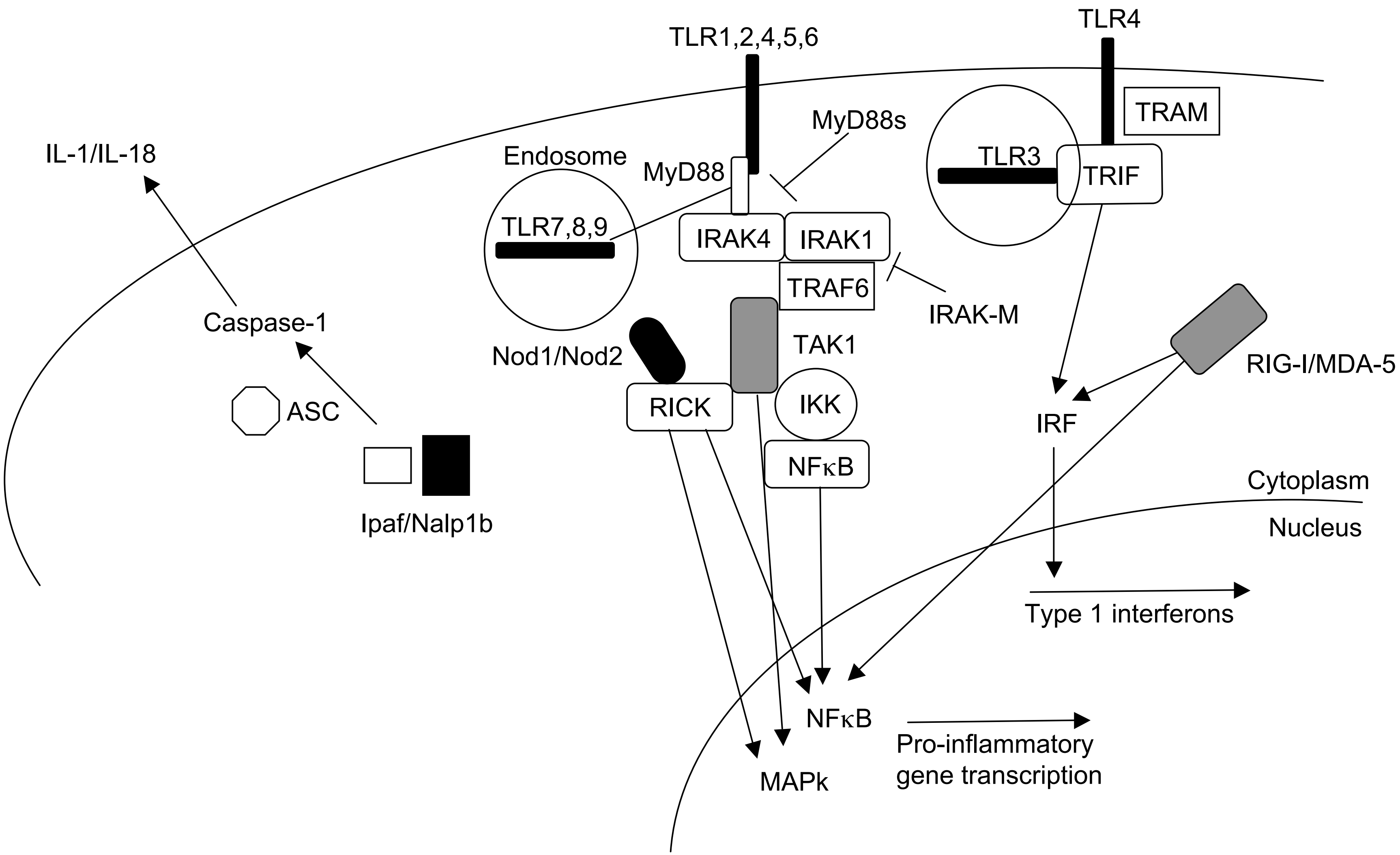
TLRs recognise their ligands via leucine-rich repeat elements (LRRs) in their extracellular domain and activate cellular responses by signalling through their intracellular Toll-interleukin-1 receptor (TIR) domain, which recruits TIR-containing adaptor proteins (Figure 1.1). For most TLRs, the relevant adaptor protein is MyD88, which then recruits IL-1 receptor-associated kinase 4 (IRAK4), allowing the association of IRAK1 (89). After phosphorylation by IRAK4, IRAK1 then binds TNF receptor activated factor 6 (TRAF6), an event blocked by the negative regulator, IRAK-M (90). Phosphorylated IRAK1 and TRAF6 then disassociate from MyD88 and activate transforming growth factor β-activated kinase (TAK1), which phosphorylates MAP kinases and the inhibitory κB kinase (IKK) complex. This activates IKK which then phosphorylates IκB, promoting its degradation and liberating NFκB from inhibition and allowing nuclear translocation of active NFκB (p50 and p65), with resulting transcription of genes for pro-inflammatory cytokines and co-stimulatory molecules (91). MyD88-dependent TLR signalling can also activate the activating protein-1 (AP-1) family of transcription factors (92), which have been shown to be involved in processes such as cell proliferation and survival (93).

In contrast to other TLRs, TLR3 utilises the TRIF adaptor protein (TIR domain-containing adaptor protein inducing IFNβ) in a MyD88-independent pathway, culminating in the activation of IRF3 and the IFNβ gene (94). TLR4 can also activate this MyD88-independent, TRIF-dependent pathway, but unlike TLR3, TLR4 uses TRIF-related adaptor molecule (TRAM) which interacts with TRIF (95, 96), leading to both the activation of IRF3 and a later phase of NFκB activation (97, 98) (Fig 1.1).

Figure 1.1 Recognition of bacterial moieties and non-self nucleic acids

PAMPs are recognised by numerous families of receptors expressed by mϕ in different cellular locations. **TLRs**: TLR1, 2, 4, 5 and 6 are expressed on the cell surface, whereas TLR3, 7, 8 and 9 are expressed in intracellular compartments. With the exception of TLR3, triggering of TLRs results in binding of the adaptor protein MyD88, which then recruits and activates IRAK4 and IRAK1, an event blocked by the negative regulator MyD88s. IRAK1 then disassociates from MyD88 and binds TRAF6 (blocked by IRAK-M), which activates TAK1. TAK1 phosphorylates and activates both MAP kinases and the IKK complex, liberating NFκB from the inhibitor IκB, allowing its nuclear translocation and transcription of pro-inflammatory genes. In contrast, triggering of TLR3 recruits TRIF, which results in the activation of IRF3 and activation of the IFNβ gene. TLR4 also signals via a MyD88-independent, TRAM- and TRIF-dependent pathway, activating IRF3 and the late phase activation of NFκB. **Nod-like receptors**: NLRs are expressed in the cytosol and activation of Nod1/Nod2 by iE-DAP/MDP recruits the adaptor RICK and activates the NFκB and MAPk pathways. Ipaf and Nalp1b form an inflammasome and use ASC to recruit caspase-1, which then cleaves pro-IL-1 and pro-IL-18 into their active forms. **RIG-I-like receptors**: RIG-I and MDA-5 activation by viral RNA results in the activation of the IRF and NFκB pathways.

Figure 1.1 Recognition of bacterial moieties and non-self nucleic acids



1.10 Regulation of TLR expression and function

The regulation of TLR expression is not well understood, nor are the transcription factors involved in the control of TLR mRNA expression. However, the transcription factor PU. 1 and the IFN consensus sequence-binding protein are involved in the basal regulation of TLR4 in human mϕ (99). In addition, the expression of TLR2, 4 and 9 mRNA by murine mϕ is increased following stimulation with LPS in a ERK MAP kinase and NFκB-dependent manner (100).

In addition, other microbial components and cytokines have been shown to modulate TLR expression. For example, LPS has been shown to upregulate TLR2 but downregulate TLR4 in mϕ (101, 102). Indeed, one of the commonest forms of TLR hyporesponsiveness is endotoxin tolerance, in which initial exposure to LPS inhibits subsequent responses via TLR4. This is accompanied by downregulation of TLR4 (102), decreased association of TLR4 with the MyD88 adaptor protein and decreased association of MyD88 with IRAK (103, 104). This phenomenon has not been described as extensively with other TLR ligands, but BLP tolerance has been associated with reduced TLR2 expression in the THP-1 mϕ cell line (105). In addition, prolonged stimulation of monocyte-derived mϕ with the Nod2 ligand, MDP, results in similar tolerance to subsequent stimulation via both Nod2 and TLR (106), although the expression of TLR was not examined in this study. The mϕ growth factor, colony-stimulating factor (CSF)-1, can downregulate TLR9 expression in mϕ and suppress CpG-induced pro-inflammatory cytokine production (107).

The endoplasmic reticulum (ER)-associated heat shock protein, gp96, has been demonstrated to be an essential chaperone for allowing the shuttling of TLR1, 2, 4, 5, 7 and 9 to the cell surface or correct intracellular compartment of mφ (108, 109). Although little is known of the mechanisms, recycling and degradation are also likely to play a role in controlling TLR expression and an E3 ubiquitin ligase, Triad3A, has been shown to enhance the ubiquitination and proteasomal degradation of TLR4 and TLR9 (110). TLR can also be regulated at the transcriptional level, as shown by the ability of LPS and inflammatory cytokines such as IL-1β, IFNγ and TNFα to induce the expression of the TLR2 gene in mφ (101).

TLR function can also be regulated at the level of intracellular signalling. IRAK-M lacks kinase activity, but is induced in response to TLR ligation and prevents the dissociation of IRAK1 and IRAK4 from MyD88, thus inhibiting the formation of active IRAK-TRAF6 complexes (90). IRAK-M-deficient mice show an impaired induction of LPS tolerance. The alternatively spliced variant of MyD88, MyD88s, is induced in monocytes in response to LPS and blocks the association of IRAK4 with MyD88, and subsequent IL-1/LPS-induced NFκB activation (111) (Fig 1.1). In addition, the ubiquitin-modifying enzyme, A20, regulates TLR responses by terminating TLR-induced IKK activation and NFκB transcriptional activity by deubiquitinating TRAF6 (112). IL-10 and TGFβ can negatively regulate TLR responses. Indeed, peripheral blood mononuclear cells (PBMCs) cultured with TGFβ and IL-10 show a reduced TNFα response to TLR4 ligation, comparable to that of LPS desensitisation (113). Furthermore, neutralisation of these cytokines during primary culture with LPS prevents the reduced TNFα response to subsequent challenge. How TGFβ and IL-10 mediate these effects is not completely understood, but it is likely to reflect altered downstream signalling. IL-10 can inhibit TLR-mediated NFκB activation by inhibiting IKK and NFκB DNA-binding activity (114), and by inducing nuclear expression of the inhibitory IκB family members, IκBNS and Bcl-3 (115, 116). By associating with the p50 subunit of NFκB, these negative regulators inhibit the activation of the IL-6 and TNFα promoters, respectively. TGFβ can block NFκB activation in response to TLR2, 4, and 5 ligands by facilitating ubiquitination and proteosomal degradation of MyD88 (117).

Therefore, TLR expression can be regulated at the transcriptional level, at the level of protein folding, delivery to the cell surface or correct endosomal compartment, or by changes in recycling. In addition, there may also be alterations in the downstream signalling pathways used by TLRs, even when the proteins are still expressed at normal levels. It will be critical to delineate the mechanisms involved in the regulation of TLR expression by mϕ, due to the functional impact this will have for mϕ and for homeostasis in tissues like the intestine.

1.11 The Role of TLR in immune responses

TLR activation has wide ranging effects on both innate and adaptive immunity. The downstream effects of TLR ligation include pro-inflammatory cytokine and chemokine production, microbial killing and enhanced expression of class II MHC and co-stimulatory molecules. The importance of TLR in immunity is demonstrated by MyD88 KO animals, which exhibit increased susceptibility to *Mycobacterium avium* and *Listeria monocytogenes* infection (118, 119), and abrogated Th1 differentiation and increased susceptibility during Toxoplasma infection (120, 121). Activation of DCs via TLRs drives their production of cytokines, such as IL-12, and the upregulation of co-stimulatory molecules, events which are critical for the activation and differentiation of naïve T cells (122). TLR2 KO and MyD88 KO mice both show increased susceptibility to infection with *Staphylococcus aureus* (123). TLR2 activation leads to NO-dependent and –independent killing of *M. tuberculosis* in mϕ from mice and humans, respectively (124). In addition, TLR2 drives the induction of β–defensin-2 in a human lung epithelial cell line (125), whilst LPS drives the production of murine β-defensin-2 and -6 (126, 127). TLR2 has also been shown to confer lipoprotein-induced apoptosis of mϕ (74), suggesting a potential role of TLRs in infection-induced cell death. Moreover, activation of mϕ with LPS, but not TLR2 agonists, mediates IFNβ production and in turn, STAT-1-dependent gene expression and anti-viral immunity (128).

In addition to their effects on innate cells such as mϕ, TLR expression on $CD4^+$ T cells can act as a co-stimulatory signal for their activation (129). Similarly, activated/memory B cells can express most TLR (130, 131) and TLR9 expression induced by BCR triggering can assist differentiation into Ig-secreting plasma cells (131).

TLRs also interact with other activation stimuli such as IFNs. One of the best described pathways that integrates with TLRs is the IFNγ receptor (132). Associated with the ability of IFNγ to upregulate TLR4 expression by mϕ (133), IFNγ has long been known to 'prime' mϕ for heightened LPS responses and this has been shown more recently for CpG DNA (134, 135). TLRs also synergise with other families of PRRs in the recognition of microbial products. For example, Dectin-1, the β–glucan receptor, and TLR2 are both involved in the recognition of yeasts via zymosan (136, 137). Similarly, bacterial flagellin is recognised by both TLR5 and the Nod-like receptor, Ipaf, inducing divergent signalling pathways via NFκB and caspase-1, respectively (78, 138). Meanwhile, another C-type lectin, the mannose receptor, inhibits LPS-induced IL-12 production (139).

1.12 Other pathogen recognition receptors

Another family of PRRs receiving increasing interest are the Nod-like receptors (NLRs), characterised by a conserved Nod domain and LRRs. Whereas TLRs recognise microbes on the cell surface and in endosomes, NLRs recognise microbial moieties in the cytosol. 23 NLR genes have been identified in the human genome, but the best known are Nod1 and Nod2, which recognise bacterial peptidoglycan-related molecules containing meso-diaminopimelic acid (iE-DAP) and MDP, respectively (140, 141). Whereas Nod1 is expressed in many cell types, Nod2 is expressed primarily by immune cells, including mϕ, and by Paneth cells in the small intestine (142). Ligation of Nod1 and Nod2 by their respective ligands recruits the adaptor protein, RICK (143), which binds and promotes polyubiquitylation of IKKγ and activation of TAK1 and NFκB (144). Like TLRs, Nod1 and Nod2 stimulation also results in the activation of the MAP kinases p38, ERK and JNK (145). As mentioned above, mutations in Nod2 are associated with Crohn's disease, and functional studies have shown that the Crohn's disease-associated variants exhibit reduced or loss of activity when stimulated with MDP (7). This suggests that Nod2 mutations may result in impaired clearance of commensal bacteria, allowing inappropriate inflammatory responses in the gut. However, when the human disease-associated variant was introduced into the mouse Nod2 locus, the murine mϕ produced increased IL-1β in response to MDP (146), and so the exact mechanism by which Nod2 mutations increase the susceptibility to Crohn's disease is not clear.

A further set of NLRs include Ipaf and Nalp1b that are activated by bacterial flagellin in the cytosol (138) and by the *Bacillus anthracis* lethal toxin, respectively (147). These receptors are involved in the formation of the 'inflammasome', a molecular platform assembled by NLRs upon ligand binding. The inflammasome-associated, caspase recruitment domain (CARD)-containing adaptor, apoptosis-associated speck-like protein containing a CARD (ASC), is required for recruitment of caspase-1, which then mediates the proteolytic maturation of IL-1β and IL-18 (148). Another set of cytosolic PRRs include the retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs), including RIG-I and melanoma differentiation-associated protein-5 (MDA-5), which are involved in anti-viral responses (149). These receptors recognise different types of dsRNA, but both result in activation of IRF3 and NFκB, and the production of IFNs (150).

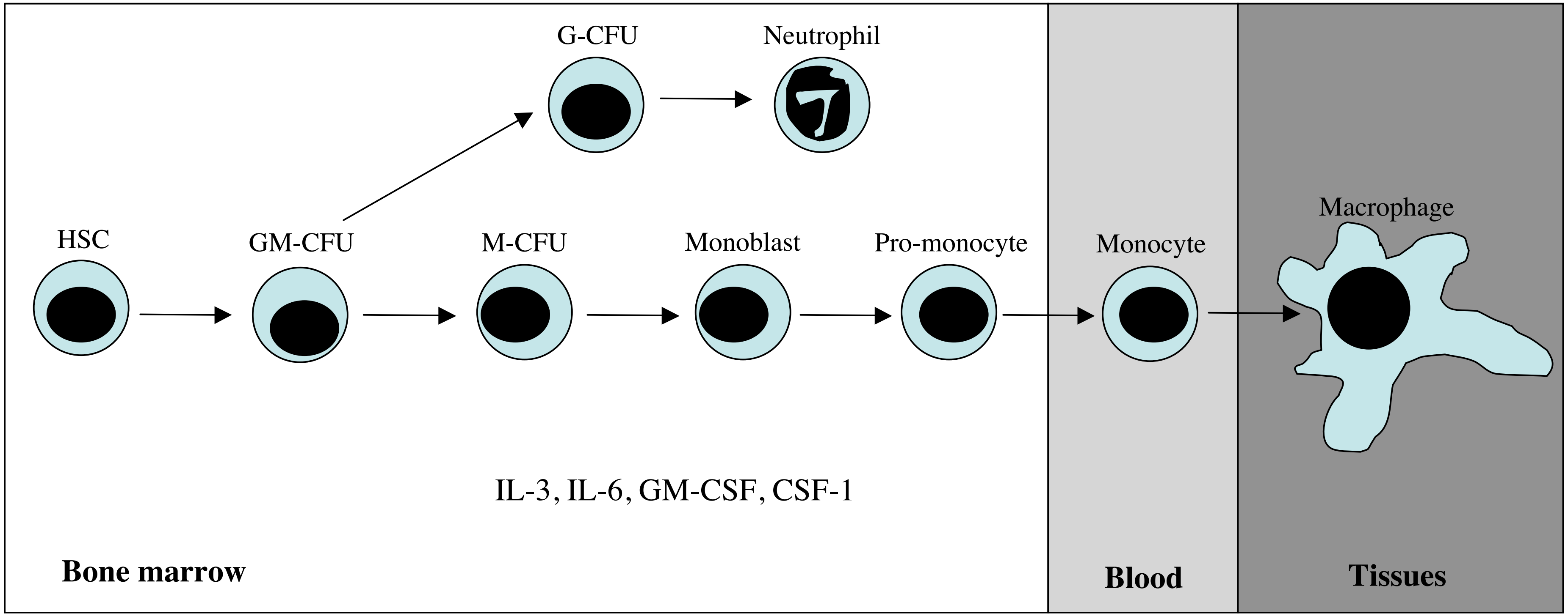
1.13 Ontogeny of myeloid cells

Tissue mϕ are derived from myeloid precursors in the BM. In the adult BM, haematopoietic stem cells (HSC) first give rise to granulocyte/mϕ colony-forming unit precursors (GM-CFU), which have the ability to differentiate into either monocytes or granulocytes. During monocyte development, GM-CFU differentiate into mϕ colony-forming unit precursors (M-CFU) which then differentiate into monoblasts, promonocytes and finally monocytes (Figure 1.2). The same progenitor cell population at the M-CFU stage of development is thought to give rise to both mϕ and DC (151, 152). These then enter the bloodstream and circulate for 1-3 days, from where they are recruited into tissues as mϕ. Under resting conditions, tissue mϕ probably survive without dividing for up to several weeks before undergoing apoptosis, to be replenished by newly recruited monocytes. Monoblasts have a cell cycle time of <12 hours, while that of monocytes is ~16 hours (153), and mature monocytes then enter the blood within 24 hours of their formation (154). Thus the processes of development and replenishment of mϕ are rapid, meaning these cells can make an immediate response to infection or tissue trauma.

Figure 1.2 Macrophage development

Haematopoeitic stem cells (HSC) in the BM give rise to granulocyte/mϕ colony-forming units (GM-CFU), which can become granulocyte colony-forming units (G-CFU) or mϕ colony-forming units (M-CFU). G-CFU can then differentiate into mature granulocytes such as neutrophils and eosinophils, whereas M-CFU differentiate into monoblasts, pro-monocytes, monocytes and then finally tissue mϕ. Growth factors such as IL-3, IL-6, GM-CSF and particularly M-CSF are critical in this developmental pathway.

Figure 1.2 Development of macrophages



A combination of growth factors is required for the differentiation of myeloid cells, including mϕ colony-stimulating factor (M-CSF; also known CSF-1), granulocyte/mϕ colony-stimulating factor (GM-CSF), IL-3, IL-6, stem cell factor (SCF; c-kit ligand) and leukaemia inhibitory factor (LIF) (155-157). Although the exact role of the individual growth factors at the distinct stages of development are unclear, CSF-1 and its receptor (CSF-1R; CD115), encoded by the *c-fms* proto-oncogene, play a particularly crucial role in mϕ proliferation, differentiation and survival (158). Indeed op/op mice, which have a point mutation in the gene encoding CSF-1 (159), are severely mϕ-deficient apart from retaining some subsets of splenic mϕ, BM monocytes and mϕ in LNs and the thymus (160-162). They exhibit reduced body weight and almost a complete lack of osteoclasts, resulting in bone-remodelling defects and skeletal deformities.

The molecular mechanisms that govern the mobilisation of mature monocytes from the BM, are also not well defined. After exiting the blood, monocytes differentiate into mature tissue mϕ under the influence of ill defined, local tissue factors including signals received when crossing the endothelium of blood vessels. Thereafter, most tissue mϕ do not divide *in situ* and are thought to be replenished continuously by blood monocytes under resting conditions. However some mϕ undergo self-renewal in tissues, including Kupffer cells, alveolar mϕ and microglial cells, all of which are replenished by both local proliferation and from blood monocytes (163-167).

1.14 Origin of tissue macrophages and the heterogeneity of blood monocytes

As discussed above, the consensus is that tissue mϕ derive from monocytes in the blood, which extravasate continuously at a low rate to replenish the resident mϕ populations. However, recent studies indicate that distinct subsets of monocytes may give rise to different myeloid cell populations in tissues. In mice, DCs appear to derive from a short-lived $CX_3CR1^{lo}CCR2^+Gr1(Ly6C)^+$ 'inflammatory' subset of blood monocytes that has a propensity to home to inflamed tissues, such as the thioglycollate-inflamed peritoneum, where they can also give rise to inflammatory mϕ (168). In contrast, the $CX_3CR1^{hi}CCR2^-Gr1^-$ 'non-inflammatory' subset of monocytes is recruited to non-inflamed tissues and gives rise to long-lived resident myeloid cells in the liver, lung, brain and spleen, with a proportion acquiring a DC ($CD11c^+$class II MHC^+) phenotype in the spleen in the absence of inflammation. Analogous populations may occur in humans, where blood monocytes can be separated into at least two subsets based on their expression of CD14 and CD16 (169). The $CD14^+CD16^-$ subset are also $CCR2^+$ and are considered to be the equivalent of the 'inflammatory' monocyte, whereas the $CD14^+CD16^+$ subset, which constitute about 10% of all blood monocytes, is considered to be the 'resident' or 'non-inflammatory' population, expressing CCR5 and higher levels of class II MHC (170). However the $CD14^+CD16^+$ monocytes also give rise to DCs in an *in vitro* model of endothelial reverse transmigration (171), mimicking entry into lymphatic vessels.

The exact relationship between these subsets and at what stage they diverge during development is unclear. Although phenotypically distinct monocyte subsets exist in the BM and blood (172), some studies suggest that these subsets actually represent the same cells at different stages of maturation. When mononuclear phagocytes were depleted by injection of toxic liposomes, Ly6C^{hi} monocytes reappeared in the circulation first but then downregulated Ly6C expression while still in the bloodstream, suggesting that Ly6C^{hi} monocytes may be the precursors for Ly6C^{lo} monocytes (173). Furthermore, grafted Gr-1^{hi} monocytes can home back to the BM in the absence of inflammation, differentiate into Gr-1^{lo} monocytes, and return to the bloodstream (174). It will be important to define these processes in detail, as it could prove possible to target one subset selectively and so modulate the function of inflammatory mϕ, without affecting the potential homeostatic role of the other subset. Indeed the Ly6C^{lo} monocyte subset has been shown to be recruited into the infarcted heart, promoting tissue healing by expressing high levels of vascular endothelial growth factor (VEGF) (175). However it has to be noted that such studies have rarely examined the role of monocyte subsets in health and disease in the same tissue and in particular, nothing is known of how intestinal mϕ fit into the scheme outlined.

1.15 Functional heterogeneity of macrophages

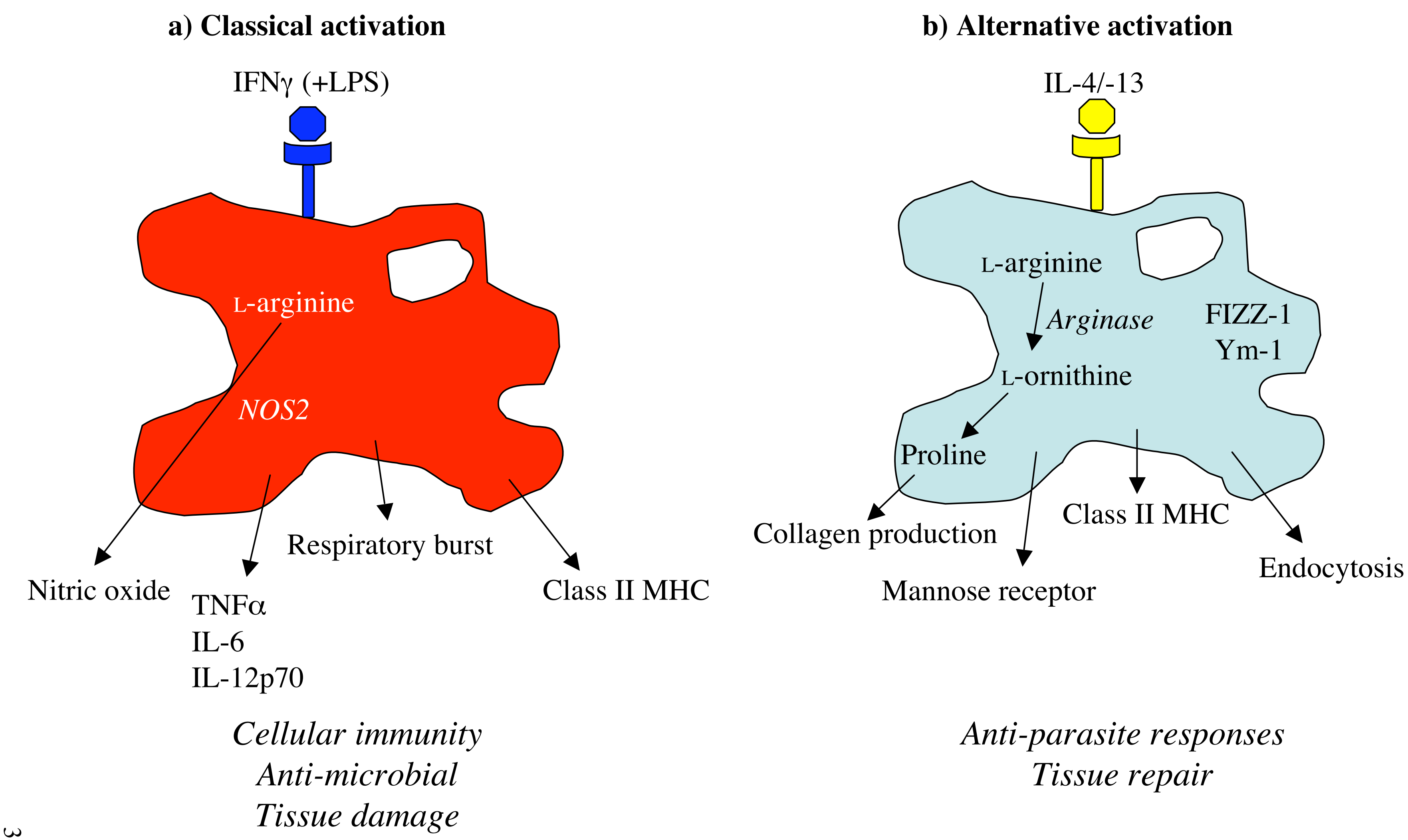
There is considerable heterogeneity of function within the mature mϕ population during inflammatory responses. ‘Classically’ activated mϕ are triggered by stimuli such as TLR ligands and IFNγ, leading to the upregulation of class II MHC and the production of pro-inflammatory cytokines and NO. These are important for combating intracellular infections such as *Mycobacterium tuberculosis*.

In contrast, ‘alternatively’ activated mϕ (AAM) develop in response to Th2 cytokines such as IL-4 and/or IL-13, and this subset of mϕ is thought to be involved in allergic and anti-parasite responses, as well as in tissue repair (Figure 1.3). AAM are also class II MHC^+, but are characterised by the lack of pro-inflammatory cytokine production. In contrast, they express the mannose receptor, FIZZ1, Ym-1 and produce chemokines such as CCL22 (MDC), which attract $CCR4^+$ $CD4^+$ Th2 cells (176, 177). In addition, whereas classically-activated mϕ (CAM) use L-arginine to generate iNOS-dependent NO, AAM express arginase which converts L-arginine to L-ornithine and then proline, a precursor of collagen, resulting in fibroblast proliferation and collagen production (178). Arginase plays a critical role in schistosome egg-induced granuloma formation (179) and together with the fact that mature AAM express mRNA for angiogenic factors such as TGFα and insulin-like growth factor (IGF-1) (180), it appears that AAM may play a role in fibrosis, re-vascularisation and tissue remodelling during Th2 responses. Moreover, as they do not produce pro-inflammatory mediators such as IL-12, TNFα, NO or ROIs, mϕ of this kind could contribute to protective immunity and tissue repair without driving substantial immunopathology.

Figure 1.3 Macrophage differentiation and heterogeneity

Macrophages exposed to Th1 or Th2 cytokines develop into ‘classically’ (CAM) or ‘alternatively’ activated mϕ (AAM), respectively. A) Following stimulation by LPS and IFNγ, mϕ upregulate class II MHC, produce pro-inflammatory cytokines and show respiratory burst activity. In addition, they convert L-arginine to NO using inducible nitric oxide synthase (iNOS). B) In contrast, following stimulation with IL-4 or IL-13, AMM upregulate class II MHC, but do not make pro-inflammatory cytokines. In addition, AAM express the mannose receptor, Ym-1, FIZZ-1 and using arginase, convert L-arginine to L-ornithine and proline, leading to collagen production.

Figure 1.3 Macrophage differentiation and heterogeneity



A group of BM-derived cells termed myeloid-derived suppressor cells (MDSCs) has been shown to be involved in the suppression of immune responses during cancer. These express F4/80, CD11b and Gr-1 and represent 20-30% of BM cells in normal mice (181). Injection of tumour cells, or the development of spontaneous tumours results in a dramatic expansion of MDSC (182), which exert immunosuppressive effects in both an antigen-specific and non-specific manner, via factors such as arginase and reactive oxygen and nitrogen species. In this respect, arginase decreases L-arginine levels, which blocks translation of the CD3ζ signalling protein in T cells (183), while NO inhibits the IL-2 signalling cascade (184). Thus, MDSCs exhibit characteristics of both classically and alternatively activated mϕ, but have the unique property of suppressing T cell function.

1.16 Intestinal macrophages

The healthy intestinal mucosa is home to one of the largest populations of mϕ in the body (185), yet relatively little is known about their function. In the colon and small intestinal mucosa, mϕ are located in the LP just below the epithelium and in the underlying muscularis mucosa (Fig 3.1). In the small intestine, which contains slightly lower numbers of mucosal mϕ compared with the colon (185), they are also present in the sub-epithelial dome region of PP. Murine intestinal mϕ express a number of characteristic markers found on other mϕ populations, including F4/80, CD11b, and some class II MHC (115, 186). Human colonic mϕ express CD68, and low levels of CD11c, but unlike blood monocytes, express only low levels of class II MHC and CD11b (187). In contrast, human small intestinal mϕ have been reported to express high levels of class II MHC, but unlike blood monocytes, fail to express CD11b, CD11c, and the integrin LFA-1 (CD11a/CD18) (188).

By virtue of their location, mucosal mϕ are in an ideal position to interact directly with bacteria in the lumen and to detect any microbes or microbial products that may cross the epithelial monolayer. This could occur via several routes. One possibility is that mϕ could behave like mucosal DCs, which have been reported to send processes out between IEC and into the lumen in response to signals from epithelial cells that have recognised bacteria (189, 190). Indeed, it is now thought that the myeloid cells that do this are actually mϕ (Agace, WW and Pabst, O; personal communication). Other possibilities include antigen acquisition through breaches of the intestinal barrier, or indirectly via uptake of dying epithelial cells that have acquired antigen (18). It is also possible that mϕ may acquire bacteria or their products indirectly, following uptake and transfer from intact IEC. Pro-inflammatory mediators released by IEC in response to bacterial products such as LPS, which can be detected intracellularly (191, 192), could also act as ‘danger signals’ for adjacent mϕ (15, 193).

1.17 Unique functional specialisation of intestinal macrophages

As mϕ are highly phagocytic cells, it seems likely that their close positioning to intestinal bacteria may allow them to contribute to intestinal homeostasis simply by clearing organisms from the vicinity. Indeed, mϕ from the small intestine have been shown to be very efficient at phagocytosis and killing bacteria even without prior activation (36, 188). As a consequence, it would seem reasonable to predict that intestinal mϕ would be highly activated *in situ*. However, this is not the case. Although they are highly phagocytic with prominent phagocytic vacuoles, secondary lysosomes and pseudopodia (194), as well as expressing some class II MHC, intestinal mϕ express only low levels of co-stimulatory molecules such as CD40, CD80, and CD86 (115). In addition, unlike their counterparts in other tissues, human small intestinal mϕ do not produce pro-inflammatory cytokines or bactericidal reactive oxygen and nitrogen intermediates in response to TLR ligation (see below).

Human small intestinal mϕ fail to make IL-1, IL-6 or TNFα, and produce only low levels of IL-8, in response to numerous microbial products, including LPS, *Helicobacter pylori* urease and heat-killed *Staphylococcus aureus* (188). Similarly murine colonic mϕ, identified on the basis of CD11b expression, fail to produce pro-inflammatory cytokines or upregulate co-stimulatory molecules in response to microbial stimuli (115, 195). Instead, in response to stimulation by whole bacteria, intestinal mϕ may produce the anti-inflammatory cytokine, IL-10 (195). IFNγ or Nod2 stimulation and phagocytosis of FITC-labelled beads by intestinal mϕ also fails to induce pro-inflammatory cytokine release (106, 188), nor do these cells show a respiratory burst after stimulation with phorbol myristate acetate (PMA) or opsonised zymosan (196). Furthermore, colonic mϕ fail to produce pro-inflammatory cytokines after stimulation with whole *Escherichia coli* (195), suggesting that these cells are unresponsive to an plethora of PRR ligands. Staining of sections from healthy human biopsies has also failed to reveal any iNOS expression by intestinal mϕ (197, 198).

Thus there is a profound, global state of refractoriness in intestinal mϕ, in which downregulation of pro-inflammatory functions allows uptake and killing of microbes without initiating an inflammatory cascade. This would appear to be an ideal behavioural adaptation in such a microbe-rich environment where inflammation must be avoided. It remains to be determined how intestinal mϕ can kill bacteria without being able to recruit the mechanisms normally associated with this function, although it may involve other anti-microbial mechanisms such as lysosomal acidification, acid hydrolases, lysozyme, and nutrient competitors such as lactoferrin. However none of these have been studied directly in intestinal mϕ.

1.18 Pro-inflammatory receptor expression on intestinal macrophages

As discussed above, a number of different mechanisms have been described which can regulate TLR function and expression, and which need to be considered as reasons for the hyporesponsiveness of intestinal mϕ to TLR ligation. However, this has not been explored yet and although one group has reported that human large intestinal mϕ may express reduced levels of TLR mRNA compared with monocytes (199), this has not been confirmed in other species. In addition, not all TLRs were examined and only TLR2 and 4 were measured at the protein level, but these were absent in the resting state and increased during IBD (199). Most human small and large intestinal mϕ also lack surface expression of CD14, the glycosylphosphatidyl inositol (GPI)-linked glycoprotein which forms part of the high affinity complex essential for LPS recognition (187, 194, 200, 201), although CD14 may be expressed at low levels intracellularly in murine colonic mϕ (202).

Transcriptional control is one obvious way in which TLR expression could be regulated and it has been reported that human colonic mϕ fail to express mRNA for TLR1-5 (199). However others have found that human small intestinal mϕ express mRNA for TLR2 and 4 (194), meaning which TLRs are expressed by intestinal mϕ and at what level this expression is regulated, remains to be elucidated.

If correct, this pattern of decreased TLR expression by intestinal mϕ contrasts with another myeloid cell population in the intestine, DCs in the LP of the small bowel. These cells are also hyporesponsive to TLR stimulation, but in this case, retain full expression of TLRs (203). It is also unusual given that intestinal mϕ are considered to derive from circulating monocytes which express a range of functional TLRs (199). However intestinal mϕ also fail to express a variety of other receptors present on other mϕ populations which could be involved in activation by local bacteria. Human small intestinal mϕ lack expression of the stimulatory FcγR1 and FcγRIII receptors for IgG, as well as the CR3 and CR4 complement receptors (188). In addition, they lack the human Fcα receptor (FcαR) (194). IgA is the most abundant antibody isotype in the GALT and IgA-mediated phagocytosis can induce a respiratory burst in polymorphonuclear cells (204). Although not shown directly, if IgA-mediated phagocytosis does induce a similar effect in mϕ, lack of this receptor could thus contribute to the absence of pro-inflammatory responses in intestinal mϕ. Finally, in contrast to blood monocytes, the majority of mϕ from the human small and large intestine do not express the triggering receptor expressed on myeloid cells-1 (TREM-1) (205). Although the natural ligand for TREM-1 remains elusive, its ligation on monocytes with agonistic anti-TREM-1 antibodies leads to upregulation of co-stimulatory molecules and pro-inflammatory cytokine production (206). Furthermore, during active IBD and experimental colitis, TREM-1 is upregulated in the intestine and administration of an antagonistic peptide blocking TREM-1 activity ameliorates experimental murine colitis (207), suggesting it may play an important role during intestinal inflammation.

1.19 Origin of the intestinal macrophage pool

The functional hyporesponsiveness of intestinal mϕ correlates with the downregulation of a number of different receptors which could be involved in mediating inflammatory responses. However, some studies suggest that not all mϕ in the normal intestine are identical in terms of responsiveness to pro-inflammatory stimuli, with small numbers of resident mϕ expressing CD14 (201). These could represent recently arrived monocytes recruited as part of homeostatic renewal. Experiments in humans with IBD show that peripheral blood monocytes can be recruited to the inflamed intestine (208, 209), and it is generally assumed that this may also occur constitutively under resting conditions. As discussed above, some tissue mϕ populations have also been shown to proliferate *in situ*, as well as being replenished by blood monocytes, although this has never been studied in the intestine. Alternatively, there may be separate subsets of intestinal mϕ with distinct functions in the resting intestine. These issues remain to be addressed and it is also not known how mϕ populations vary between health and disease. In addition, the nature of the precursors and the molecular mechanisms that govern their recruitment into the mucosa in the resting state and in response to intestinal inflammation are not well understood. These are vital questions, as effective blocking of mononuclear cell infiltration into the gut may prove to be an effective route for therapeutic intervention.

1.20 Mechanisms underlying unresponsiveness of intestinal macrophages

That the distinctive properties of intestinal mϕ may be conditioned after arrival in the local microenvironment would be consistent with the heterogeneity and plasticity of cells within the myeloid lineage, all of which can adopt highly specialised functional profiles suited to their role in different anatomical locations. Recent studies also suggest that mediators produced by epithelial and other mucosal cells can condition intestinal DCs to become tolerogenic and to imprint T cells with gut homing properties. These mediators include TGFβ, IL-10, thymic stromal lymphopoietin (TSLP), vasoactive intestinal peptide (VIP), prostaglandin (PG) E2 and retinoic acid (210-218). Similar conditioning of blood monocytes could occur following their arrival in the intestinal microenvironment. Indeed co-culturing monocytes with IEC *in vitro* induces an intestinal mϕ-like phenotype, with reduced CD14 expression and abrogated IL-1β responses to stimulation with LPS (219). Furthermore, prolonged culture of blood monocytes with intestinal stromal-cell conditioned medium results in reduced expression of CD14 and decreased TNFα production, but has no effect on phagocytic ability (188). These latter effects are dependent on TGFβ, produced by chymase$^+$ c-kit$^+$ mast cells and IEC. Importantly, it appears that continuous exposure to such factors may not be required, as intestinal mϕ conditioned in this way fail to re-express receptors such as CD14 when cultured *ex vivo* even for long periods (188).

IL-10 is another cytokine which could play an important role in desensitising intestinal mϕ. Recent work in our laboratory shows that IL-10 is responsible for the mucosal partial refractoriness of DCs to TLR stimulation (203), and colonic mϕ from IL-10 null mice have enhanced IL-12p70 production in response to stimulation with whole bacteria and LPS (115, 195). There are several possible sources of IL-10 in the gut, including mϕ themselves, which may produce IL-10 constitutively and after stimulation with whole bacteria (115, 195). Alternatively, IL-10 may be provided by another cell source, such as the T_R-1-like regulatory T cell populations which are particularly abundant in the intestine (220).

The possibility that IL-10 can affect the expression of a range of TLRs or responses against a range of purified TLR agonists remains unexplored. However, IL-10 and TGFβ have a synergistic ability to downregulate TREM-1 and CD89 on monocytes *in vitro* (205), and as discussed earlier, can reproduce TLR unresponsiveness *in vitro* (113). The ability of IL-10 to inhibit NFκB activity (114) could also potentially explain the failure to respond to PMA and the lack of TREM expression which are features of intestinal mϕ (221). In support of this role for IL-10, the inhibitory IκB family members IκBNS and Bcl-3 are expressed constitutively by mϕ from the normal intestine, but are absent in colonic mϕ from IL-10 KO mice which develop colitis (115). Whether NFκB expression and/or its nuclear translocation is downregulated in normal resident intestinal mϕ remains unclear.

Not all the features of intestinal mϕ can be explained by TGFβ and/or IL-10, particularly the lack of TLR expression, as neither of these mediators have been found to do this. Thus, additional conditioning factors would need to be considered. VIP has been shown to downregulate iNOS, and pro-inflammatory cytokine and chemokine production by mϕ in response to LPS (222-225), by inhibiting the reduction of cytoplasmic IκBα, thus preventing nuclear translocation of NFκB (226). One further group of molecules that warrant investigation as potential mediators of intestinal mϕ unresponsiveness are inhibitory receptors such as CD200R. Although the role of CD200R has not been examined in the gut, it has recently been shown to be expressed at high levels by alveolar and small intestinal mϕ and it is known to be important for regulating mϕ activation in tissues such as the eye and the lung (227-229). How CD200R mediates inhibition of myeloid cell activation is not completely understood, and unlike many immune inhibitory receptors, CD200R lacks an ITIM motif in its cytoplasmic tail. However it has been reported that CD200R-mediated inhibition of mast cell degranulation occurs via inhibition of Ras/MAPK pathways (230). However, CD200R agonists have been shown to inhibit IFNγ-, but not LPS-mediated TNFα production in peritoneal mϕ (231), perhaps suggesting CD200R may not be involved in the regulation of intestinal mϕ function.

Therefore, it seems that the regulation of mϕ function in the gut may reflect a modulation of pro-inflammatory receptor function that biases the behaviour of these cells toward a non-inflammatory phenotype. In this thesis, I assessed the level of expression of TLRs and the level at which expression is regulated, as well as exploring what factors may be involved in this regulation. This information is critical to assess the mechanisms involved in gut mϕ homeostasis in the resting state, and may also provide information as to how inflammatory responses are usually controlled to prevent inflammation.

1.21 Role of intestinal macrophages in health and disease

Homeostatic Effects

As I have discussed, intestinal mϕ may be critical for maintaining homeostasis in the face of continuous exposure to commensal bacteria, and this may be due to decreased expression and/or function of activating receptors. In support of this, a breakdown in this adapted response of mϕ is sufficient to provoke intestinal inflammation. It is well known that mice lacking IL-10-producing regulatory T cells develop colitis spontaneously (232) and the target for this IL-10 appears to be mϕ, as intestinal inflammation also occurs in mice with myeloid cell-specific deletion of STAT-3 (233), the transcription factor which mediates IL-10R signalling. In the absence of IL-10 signalling, mϕ are induced to produce pro-inflammatory mediators such as IL-12 or IL-23 which promote the generation of pathogenic, Th1 or Th17 T cells (195). However, it is important to note that the STAT-3 signalling pathway is shared by other cytokines such as IL-6, which has been shown to be important for epithelial homeostasis (234). Thus IL-10 hyperresponsiveness may not be the only role for STAT-3 in homeostatic control of gut mϕ function.

Intestinal mϕ are not simply passive targets of IL-10-mediated regulation, as there is accumulating evidence that they may also play an active role in controlling intestinal immune responses. Small intestinal F4/80$^+$CD11b$^+$CD11c^{dull} mϕ-like cells have recently been shown to induce the differentiation of FoxP3$^+$ T regulatory cells *in vitro* (235).

Moreover, mice lacking F4/80 do not develop oral tolerance after feeding protein antigens, and this is associated with defective induction of $CD8^+$ T regulatory cells (236). Several findings also indicate that intestinal mϕ can prevent intestinal inflammatory responses in an active manner. Depletion of intestinal mononuclear phagocytes by clodronate liposomes or using transgenic mice expressing a drug-inducible suicide gene under the control of the c-fms (CD115) promoter, exacerbates DSS-induced colitis, possibly secondary to increased chemokine-induced neutrophil infiltration (237). Colonic LP mϕ from schistosome-infected mice also transfer protection against DSS-induced colitis (186).

Somewhat paradoxically, the anti-inflammatory effects of mϕ appear to require commensal bacteria-dependent TLR signalling, as deletion of TLRs or MyD88 in BM-derived cells exacerbates experimental colitis (238, 239). In addition, colitis induced by infection with *Citrobacter rodentium*, is exacerbated in TLR2 null mice; this is associated with impaired barrier function, suggesting that TLR2 on mϕ may be involved in maintaining mucosal integrity (240). The principal protective role of mϕ in these circumstances may be to modulate the epithelial cell response to injury, with mϕ in the peri-cryptal stem cell niche contacting epithelial progenitors, and increasing the local concentration of cytoprotective factors such as cyclooxygenase (COX)-2-dependent PGE2 (241). These TLR-mediated protective effects of mϕ may be additional and/or overlapping with a similar protective role for TLR signalling in epithelial cells (242, 243). Collectively, these results suggest that TLR signalling in intestinal mϕ is important for actively maintaining mucosal function and/or repair. Although this is most apparent during inflammation, it could be that mϕ play similar roles in the resting state given the high numbers of bacteria present. This is paradoxical in view of the usual lack of TLR on these cells and therefore it may be that such functions occur before newly arrived mϕ lose TLR expression. Alternatively, the small $CD14^+$ subset found in the resting state, which are likely to express TLRs, may play this role. However, this needs to be examined by more detailed phenotypic and functional analyses of mϕ subsets in the resting intestine.

Protective Immunity

In addition to their role in homeostasis, intestinal mϕ are also central to protective immunity and immunopathology when pathogens or inflammation are present. As I have noted, intestinal mϕ have constitutive phagocytic activities even in the healthy state (188). Intestinal mϕ phagocytose and kill pathogenic *Salmonella typhimurium* and *Escherichia coli in vitro* (188), supporting the view that they may contribute to clearance of pathogens. Indeed animals infected with *Shigella* and treated with antagonistic anti-CD14 antibodies display higher levels of bacterial invasion and more severe tissue damage (244). In addition to their ability to kill bacteria directly, the fact that intestinal mϕ undergo apoptosis when infected by some intestinal bacteria such as *Salmonella*, facilitates uptake and presentation of bacterial antigen by the local DCs (245). This process is known to be crucial for protective immunity in such infections. In parallel, like IEC, intestinal mϕ secrete CCL20 (246), the ligand for CCR6. This chemokine receptor is needed to recruit DCs into *Salmonella*-infected PP, a process which is essential for specific immunity to develop (247). Human intestinal mϕ have also been shown to produce the neutrophil chemoattractant, IL-8, in response to LPS, albeit at low levels (188).

In addition to these anticipated roles in protective immunity during classical Th1-type responses, there is also a striking increase in the number of colonic mϕ in mice infected with the helminth parasite, *Trichuris muris*, which peaks at the time of Th2-mediated worm expulsion (248). During infection with helminths and related pathogens, intestinal mϕ exhibit the functional and phenotypic features of the AAM discussed earlier. Although much remains to be discovered about the functional role of AAM in the gut and of the factors driving their differentiation, these studies highlight the plasticity of intestinal mϕ, and their ability to adapt appropriately to local conditions. Alternatively, these observations may suggest the presence of different subsets of mϕ in the gut in different situations. However, studies using models of intestinal infection in mϕ-deficient animals are needed to provide definitive answers to the role these cells play during infection.

Pathological roles of macrophages in intestinal inflammation

During human and experimental IBD, the intestinal mucosa is massively disrupted, with compromised epithelial barrier function and secondary invasion by commensal bacteria. This is accompanied by an intense influx of leukocytes, including monocyte-derived mϕ (249), presumably attracted by chemokines released by epithelial cells and other inflammatory cells in the mucosa (15, 193). There is increased production of mϕ-derived NADPH oxidase and of pro-inflammatory cytokines and chemokines such as TNFα, IL-6, IL-18 and IL-8 (250-253). Moreover, treatment with TNFα depleting antibodies is beneficial in IBD patients (254, 255) and in reducing tumour development in a mouse model of chronic colitis associated with colon carcinogenesis (256). In addition, non-T cell-derived TNFα is essential for development of disease in the $CD4^{+}CD45RB^{hi}$ T cell transfer model of colitis in mice (257). The intestinal inflammation found during *Citrobacter rodentium* infection in mice is also dependent on the characteristic mϕ products, TNFα and IL-12 (258). Depletion of mϕ ameliorates colitis in IL-10KO mice (259) and the fact that DSS colitis can be induced in the absence of lymphocytes in NK cell-deficient SCID mice (260, 261), suggests that mϕ may play a direct pathogenic role in this model. Depletion of mϕ also reduces mucosal damage in a model of intestinal ischaemia reperfusion injury (262). Thus it is clear that mϕ activation is a major feature of intestinal inflammation and plays a central pathogenic role under these conditions.

Mϕ may contribute to tissue pathology in a number of ways, including the release of pro-inflammatory chemoattractants, such as IL-8, which induce further recruitment of mononuclear and granulocytic cells. These effects will be enhanced by TNFα-induced upregulation of adhesion molecules on local blood vessels (61). Moreover, IL-12 production by activated mϕ drives IFNγ production from T cells, which then increases epithelial permeability (263, 264) and further mϕ activation (265). TNFα and IL-1 from activated mϕ drive epithelial cell apoptosis and barrier dysfunction, vascular damage, and necrosis (266-268). These mϕ-derived mediators also drive production of matrix metalloproteinases (MMP) by fibroblasts, leading to the degradation of collagen and other components of the extracellular matrix (269). Expression of tissue-degrading cathepsins by intestinal mϕ is also seen in IBD (270), and increased release of aggressive metabolites such as NO, and oxygen radicals which attack and destroy DNA, all contribute further to the mϕ-dependent tissue damage (271).

Colonic mϕ in clinical colitis show increased expression of CD14, TLR2 and 4 (199, 201, 272), as well as of co-stimulatory molecules, scavenger receptors such as CD163, pro-inflammatory cytokines, and greater respiratory burst activity (195, 273-278). Most available evidence suggests that these inflammatory cells represent a distinct population of mϕ which have recently arrived in the mucosa, rather than a change in the resident population. As noted above, human blood monocytes are recruited very efficiently into the inflamed gut (208). Moreover, blockade of chemokine receptors CCR2, CCR5 and CXCR3 ameliorates DSS colitis by inhibiting the recruitment of inflammatory cells into the mucosa (279). Thus it may be that mϕ-dependent intestinal inflammation requires replacement of the environmentally conditioned resident population by newly recruited, fully responsive monocyte-derived cells. These recently recruited monocytes may not be in the local milieu for a long enough period of time for adaptive differentiation to occur, or it may be that there are heightened levels of immunostimulatory signals, which overcome the usual inhibitory processes.

Thus, intestinal mϕ behave very differently during inflammation and in physiological conditions, but it is not known whether the inflammatory mϕ in pathology are resident cells that have altered their behaviour, or if they are newly recruited cells. If the latter is true, it may be possible to ameliorate intestinal inflammation by blocking inflammatory monocyte infiltration into the gut. However if resident mϕ can alter their properties *in situ*, a greater understanding of the control of gut mϕ function must be gained in order to manipulate mϕ behaviour *in vivo*.

1.22 Animal models of inflammatory bowel disease

Several animals models have been developed to study the pathogenesis of human IBD. These include the administration of exogenous agents such as DSS, trinitrobenzene sulphonic acid (TNBS) or oxazalone (280-282). Models of intestinal inflammation caused by defects in epithelial barrier function include the DSS model, and the multi-drug resistant (*mdr*)*1a*-deficient mouse model (283). In addition, disruption of genes such as IL-10, IL-2/Rα, TGFβ and TCRα/β in mice results in the spontaneous development of intestinal inflammation (232, 284-287), while transfer of CD4^{+}CD45RB^{hi} T cells drives colitis in SCID or RAG-deficient recipients (288). Some of these models are caused by primary abnormalities in immune function or regulation, while others are caused by defects in barrier function and both specific and innate effector mechanisms are involved.

One model which is used widely is the DSS-induced model. DSS can be administered in the drinking water and is thought to be directly toxic to the colonic epithelium (289), inhibiting epithelial cell proliferation (290) and impairing barrier function and repair. This allows the translocation of commensal bacteria into the underlying submucosa, driving a rapid inflammatory response and ultimately loss of intestinal architecture, rectal bleeding, diarrhoea and weight loss usually within 5 days. The inflammatory cell infiltrate in DSS colitis, primarily mϕ, neutrophils and eosinophils (291), focuses in the distal colon, culminating in upregulation of pro-inflammatory cytokines (292), oedema, mucosal ulceration, epithelial disruption and crypt necrosis.

That bacterial invasion may be critical for the development of DSS colitis is supported by the fact that germ-free mice have reduced inflammation compared with their conventionally reared counterparts (293). The TLR5 ligand, flagellin, can also exacerbate DSS colitis (294). However, two other reports have indicated that DSS colitis can develop under germ-free conditions (295, 296), indicating that the role of bacteria and/or their products remains contentious. In addition, as discussed above, TLR-mediated recognition of commensal bacteria by mϕ may be critical for maintaining intestinal homeostasis and this has been shown to be required for restoring epithelial homeostasis and recovery from DSS-induced injury (238).

DSS colitis has several advantages as a model. It can be induced in normal mice by a readily available exogenous agent. It develops rapidly, follows a relatively well-defined pattern and produces a consistent and characteristic form of colitis (281). In addition, cessation of DSS administration allows recovery, thus providing a model for investigation of tissue repair. In addition to this acute form of inflammation, cyclical administration of DSS allows the induction of a more chronic form of colonic inflammation. Therefore this is the model I decided to use to study intestinal mϕ during inflammation.

1.23 Modulation of macrophage inflammatory activity and immune responses by helminths and their products

One of the most potent examples of how intestinal pathogens can modulate the host immune response is that of helminths. These produce chronic infection in most mammalian species by virtue of their ability to inhibit host inflammation and tissue pathology. For example, the presence of the gastrointestinal nematode, *Nippostrongylus brasiliensis*, is prolonged in mice previously infected with *Nematospiroides dubius* (297), and *Nippostrongylus* infection can prolong the survival of kidney allografts in rats (298). Remarkably, although the trematode, *Schistosoma mansoni*, activates Langerhans cells, it also retains them in the epidermas via parasite-derived prostaglandin D2 (299), thus inhibiting subsequent T cell activation. The ability of helminths and their products to modulate immune responses has been shown to be associated with the development of regulatory T cells and the production of IL-10 and/or TGFβ (300, 301).

These immunomodulatory effects reflect a number of properties, but one of the most important is the secretion of regulatory products. One such molecule is ES-62, a phosphorylcholine (PC)-containing glycoprotein secreted by the rodent filarial nematode, *Acanthocheilonema viteae* (302). Homologues of ES-62 have also been discovered in human filarial nematodes (303, 304). As ES-62 is a parasite-derived glycoprotein, it would be predicted that the host's immune system would recognise it as foreign. However, PC-containing molecules of this kind persist for long periods in the bloodstream of patients with filariasis (305).

Recent studies have attempted to exploit the availability of purified ES-62 for use as a therapeutic agent. As a result, ES-62 has been shown to suppress the development of collagen-induced arthritis (CIA), a mouse model of rheumatoid arthritis, with associated inhibition of collagen-specific TNFα, IL-6, IFNγ and IgG2a antibody production (306). In parallel, ES-62 can suppress pro-inflammatory cytokine production by synovial cells from human RA patients *in vitro*. Importantly, ES-62 has also been shown to suppress a Th2-like response in a murine model of airway inflammation, where it reduced airway eosinophilia and IL-4 production (307). The exact mechanism(s) of action of ES-62 is unclear, but it has effects on a range of cell types including B cells, T cells, mast cells, DCs and mϕ. PC is considered the active moiety, as it mimics many of the effects of ES-62 on a range of cell types (308-310) and it skews the antibody response to a T helper (Th2)-type, generating anti-ES-62 IgG1 responses, but not IgG2a responses, via an IL-10-dependant mechanism (311).

The effects of ES-62 on T cells have been demonstrated using Jurkat T cells, where it suppressed anti-CD3 induced proliferation by modulating the activation of tyrosine kinases such as ZAP-70, and so disrupting signalling downstream of the T cell receptor (305). ES-62 has also been demonstrated to modulate a specific immune response to a heterologous antigen, ovalbumin (OVA), *in vivo*, where ES-62-treated OVA-specific T cells produced lower levels of IL-2 and proliferated less upon antigen rechallenge *ex vivo* (312). Pre-exposure to ES-62 also suppressed antigen-specific production of IFNγ, IL-13 and IL-4, and inhibited clonal expansion of transferred OVA-specific T cells, as well as reducing follicular migration of T cells. In conventional B2 B cells, ES-62 inhibits B cell receptor

(BCR)-driven proliferation by targeting key signalling events such as downregulating protein kinase C (309, 313), and renders them hyporesponsive to stimulation (314). In contrast, ES-62 activates peritoneal B1 B cells *in vivo* and induces them to produce the anti-inflammatory cytokine, IL-10 (314). Together these findings indicate that ES-62 may suppress conventional lymphocyte activation and proliferation, whilst enhancing the production of anti-inflammatory mediators.

ES-62 induces a low and transient production of IL-12, IL-6 and TNFα by mϕ and DCs, but these cells are then rendered refractory to stimulation by LPS and IFNγ (315). Furthermore, BM-DC and BMM derived *ex vivo* from BM precursors exposed to ES-62 *in vivo* have an anti-inflammatory phenotype upon maturation, even in the presence of LPS, indicating such effects are long-lived (310). ES-62 inhibits IL-12, IL-6 and TNFα, but not NO production by mϕ in response to stimulation (315), and decreases co-stimulatory molecule expression by DCs, imparting on them a phenotype that drives the development of Th2 cells (316). The low level cytokine induction and suppression of subsequent activation by DCs and mϕ requires TLR4 and MyD88, perhaps reflecting the ability of ES-62 to complex with TLR4 (317-319). ES-62 also prevents Fcε receptor I (FcεRI)-induced release of allergic mediators from human mast cells and can prevent mast cell-dependent hypersensitivity in the skin and airways (319).

By modulating pro-inflammatory cytokine production and co-stimulatory molecule expression by APCs in response to bacterial products, as well as lymphocyte responsiveness and production of anti-inflammatory mediators, ES-62 can induce an anti-inflammatory/Th2-like response. However, the fact that ES-62 can suppress both a Th1- and Th2-model of *in vivo* inflammation indicates that ES-62 does not simply reverse the polarisation of the immune response, but rather acts to regulate inflammation in general and suggests that it may induce a Th3/regulatory-type response, although this possibility has not yet been formally determined. Because of this general ability to suppress a range of specific and non-specific effector cells including mϕ, one part of my project aimed to examine how ES-62 might modulate mϕ-dependent pathology seen in the murine model of DSS colitis.

1.24 Thesis Aims

Regulation of mϕ function is clearly essential for intestinal immunity and maintenance of homeostasis. Studies on humans thus far suggest that monocytes arrive in the resting gut mucosa and differentiate into non-inflammatory cells to prevent aberrant responses against innocuous commensal bacteria. However, although the existence of different monocyte/mϕ subsets has been described in other tissues, this has not been studied extensively during intestinal inflammation. Furthermore, although mϕ from the human intestine have been shown to lack expression of activating receptors such as TLRs, there are contradictory reports. In addition, all previous work has failed to look at a large number of TLR family members, and has not compared expression both at the protein and mRNA levels. There has also been a paucity of studies examining mϕ in the resting versus the inflamed intestine in experimental models of colitis, where it is possible to conduct a more detailed characterisation of these cells at different timepoints of disease *in vivo*.

The main aims of this thesis therefore, were to characterise murine colonic mϕ both phenotypically and functionally under resting conditions. Using inbred mice under SPF conditions allows steady-state *in vivo* experimentation, and there are mutant mice lacking immunomodulatory genes available along with a multitude of immunological cell markers. As described in this introductory chapter, small intestinal mϕ in humans are non-inflammatory, but retain some of the hallmark functions of mϕ, such as phagocytosis. If a similar altered phenotype was observed in murine colonic mϕ, I planned to explore the reasons behind this hyporesponsiveness, by examining the expression of PRRs, particularly TLRs, and assessing what factors might modulate their expression.

Furthermore, by employing an experimental model of colitis by DSS administration, I sought to investigate how mϕ from the inflamed colon differed phenotypically and functionally from those found under physiological conditions. I anticipated that my studies would shed further light on the role mϕ, and possibly distinct mϕ subsets, play during the course of intestinal inflammation. Finally, I wished to use a parasite-derived immunomodulator, which has known effects on mϕ-dependent inflammation, to try to inhibit DSS colitis.

Chapter 3 of this thesis provides information about the phenotypic heterogeneity of the resident mϕ population in the normal colon, and describes how these differ phenotypically and functionally from other mϕ populations in the body. These initial studies suggested that colonic mϕ had many distinctive properties, and Chapter 4 describes how I then explored the reasons for these unusual phenotypic and functional properties.

Chapter 5 describes my studies of how intestinal mϕ may differ during inflammation, and provides an insight into the role these cells play in the disease process. In Chapter 6, the origin of the mϕ in the resting and inflamed gut is explored by investigating the differences in cell turnover kinetics of distinct mϕ subsets, as well as differences in the expression of chemokine receptors which could be involved in recruitment of these cells into the intestine. Finally, Chapter 7 investigates the effects of ES-62, the immunomodulatory parasite product that dampens pro-inflammatory mϕ functions, on the severity of intestinal inflammation.

Chapter 2

Materials and Methods

2.1 Mice

C57Bl/6 (B6 mice) mice were obtained from Harlan Olac (Bicester, Oxfordshire) and maintained on conventional diets under SPF conditions in the Central Research Facility at the University of Glasgow, Scotland, until use. Ly5.1 mice were bred in the veterinary research facility at the University of Glasgow. TLR2 (C57Bl/6 background) and IL-10 (BALB/c background) null mice were kindly provided by Professor F. Y. Liew (University of Glasgow), and Professor Fiona Powrie (University of Oxford), respectively. All mice were first used at 6 to 8 weeks of age, unless stated otherwise.

2.2 Generation of bone marrow-derived dendritic cells and macrophages

Bone marrow (BM) was flushed out of the femurs and tibias of adult C57Bl/6 mice in RPMI 1640 (Gibco BRL, Paisley, Scotland) using a syringe and a 21G needle. The BM cells were passed through Nitex mesh (Cadisch and Sons, London, UK) into a sterile 15ml tube and counted using a haemocytometer and phase contrast microscope. To generate BM-derived dendritic cells (BMDC), 1ml of cells were transferred into 90cm Petri dishes (Sterilin, UK) at $3x10^6$ cells/ml with 8ml complete medium (RPMI 1640, 2mM L-glutamine, 100μg/ml penicillin, 100μg/ml streptomycin, 1.25μg/ml Fungizone, and 10% foetal calf serum (FCS)- all Gibco), and 10% GM-CSF (supernatant from the X-63 cell line) and incubated at 37^oC in 5% CO_2. After 3 and 6 days, the medium was supplemented with 5ml complete medium and 0.5ml GM-CSF. The BM cells were used after 7-9 days and were typically >75% $CD11c^+$ as assessed by flow cytometry. For generation of BM-derived macrophages (BMM), BM cells were obtained as above and cultured in complete medium RPMI 1640 containing 1mM sodium pyruvate and 20% FCS- all Gibco), and 20% M-CSF (supernatant from the L929 cell line) at 37^{o}C in 5% CO_2. After 3 days, the medium was supplemented with 5ml complete medium and 20% M-CSF and the cells were used on day 6 of culture. Non-adherent cells were removed by washing with RPMI 1640 and the adherent cells were then collected by adding ice cold 1mM EDTA/PBS for 5 minutes and then displacing them with cell scrapers (Costar). The purity of BM cells was assessed by flow cytometry and was typically >90% F4/80 positive.

2.3 Activation of macrophages and DC *in vitro*

In most cases, single cell suspensions were resuspended at a final concentration of 5x10^{5}/ml cells and cultured overnight in 1ml aliquots in ultra low attachment, 24-well tissue culture plates (Costar). For macrophages purified from the colonic lamina propria, cells were plated at 1x10^{5} cells/200μl. Cells were incubated either in medium alone, or with varying concentrations of lipopolysaccharide (LPS) from *Salmonella typhimurium* (Sigma), 10μg/ml flagellin (Autogen Bioclear UK Ltd), 25μg/ml polyinosinic-polycytidylic acid (poly I:C) (Sigma), 1μg/ml bacterial lipoprotein (BLP; Pam_3CSK_4) (Alexis Biochemicals, Axxora LTD, Nottingham, UK), 3.2μg/ml CpG oligonucleotide (ODN 1826) (Autogen Bioclear UK Ltd), 10μg/ml MDP (Invivogen), 100nM retinoic acid (Sigma), 40ng/ml IL-4 (Biosource International, CA, USA) or 10^{-8}M vasoactive intestinal peptide (Calbiochem, San Diego, CA) at 37^{o}C in 5% CO_2. In some experiments, macrophages were cultured with TLR ligands together with 100U/ml recombinant mouse interferon γ (IFNγ) (BioSource).

2.4 Isolation of resting peritoneal macrophages (PEC mϕ)

To obtain resting peritoneal macrophages, euthanased mice were injected intraperitoneally with 10ml 1mM ice cold EDTA/PBS and peritoneal exudate cells (PEC) were retrieved by harvesting the solution from the peritoneal cavity.

2.5 Adoptive transfer of macrophages

BMM from Ly5.1 mice were derived as described above, counted and washed twice in RPMI. $1x10^6$ cells in a volume of 0.2ml RPMI were injected intravenously into congenic C57Bl/6 Ly5.2 recipients. Organs were then harvested from recipient mice at various timepoints and transferred cells were identified by expression of Ly5.1 by flow cytometry.

2.6 Isolation of lymph node and spleen cells

Lymph nodes and spleens from C57Bl/6 mice were removed, mashed through 100μm Nitex mesh in 5ml RPMI 1640, washed twice, counted by phase contrast microscopy and resuspended at $1x10^6$ cells/ml. For spleen cell preparations, red blood cells were removed by resuspending the cell pellet with 1ml Red Blood Cell Lysing Buffer Hybri-Max (Sigma), gently mixing for 1 minute, washing, and resuspending in complete medium.

2.7 Isolation of colonic lamina propria cells

The large intestines of mice were removed and placed on paper towels soaked in PBS (Gibco), and the fat was removed. The intestines were opened longitudinally, washed in Hank's balanced salt solution (HBSS) 2% FCS, and cut into 0.5cm sections. The tissue was then shaken vigorously in 10 ml HBSS 2% FCS, and the supernatant was discarded. To remove the epithelial layer, 10 ml fresh CMF HBSS (Gibco) containing 2mM EDTA (Sigma) was then added, the tube placed in a shaking water bath for 15mins at 37^{o}C, before being shaken vigorously and the supernatant discarded. 10ml fresh CMF HBSS was then added, the tube shaken again and the supernatant discarded. After a second incubation in 2mM EDTA CMF HBSS, the washes were repeated and the remaining tissue was digested with pre-warmed 1.25mg/ml collagenase D (Roche), 0.85mg/ml collagenase V (Sigma), 1mg dispase (Gibco), and 30U/ml DNase (Roche Diagnostics GmbH, Mannheim, Germany) in complete medium for 30-40 minutes in a shaking water bath at 37^{0}C until complete digestion of the tissue. At the start of the incubation, and at 5-10 minute intervals thereafter, the tube was shaken vigorously and finally the supernatant (containing lamina propria cells) was removed and passed through Nitex. The cells were spun down, resuspended in complete medium, passed through Nitex, counted, and kept on ice until use.

In Chapter 7 a different method for the isolation of colonic LP cells was used and details are as follows: The tissue was placed in 50ml tubes containing 20ml PBS and incubated in a shaker for 15mins at 37^{o}C, shaken gently, and the supernatant discarded. 20 ml fresh CMF HBSS containing 2mM EDTA was then added, the tube placed in the shaker for 15mins at 37^{o}C, and the supernatant discarded. 10-15ml CMF HBSS (pre-warmed to 37^{o}C) was added and the tube was shaken vigorously and the supernatant discarded. This step was repeated once before a further 20-30ml EDTA-containing CMF HBSS was added and the tube returned to the shaker for 15mins at 37^{o}C. The last three steps were repeated four times. The excess EDTA was removed by washing the tissue with 30-40ml sterile PBS and the remaining tissue was digested with 100U/ml collagenase Type VIII from *Clostridium histolyticum* (Sigma), 30U/ml Dnase (Roche Diagnostics GmbH, Mannheim, Germany) and 20% FCS (Gibco) in CMF HBSS for three 45min incubations at 37^{o}C. Following each incubation, the tube was shaken gently and the supernatant (containing the cells) removed and passed through Nitex. The cells were spun down, resuspended in CMF HBSS, passed through Nitex, counted, and kept on ice until use.

2.8 Purification of macrophages from the colon and peritoneum

Macrophages were purified from the colon lamina propria (LP) and peritoneal cell preparations by positive selection, by MACS according to the manufacturer's instructions. Single cell suspensions of colonic LP and peritoneal cells were prepared, resuspended at 1x10^6 cells/100µl PBS 2% FCS and incubated for 15mins at 4^{0}C with purified anti-mouse CD16/CD32 (Fc block), and then with an allophycocyanin (APC)-conjugated antibody to F4/80 (Caltag Laboratories) (1 in 100 dilution) for 30 minutes at 4^{0}C. The cells were then washed in ice cold sterile MACS medium (PBS, 2 mM EDTA, 0.5% BSA (Sigma) in PBS) and incubated with 20µl anti-APC microbeads (Miltenyi Biotec, UK) per 10^7 total cells for 15 minutes at 4^{0}C in the dark. Cells were then washed and resuspended in 500µl MACS buffer and passed through a Large Cell Separation column, and then an MS column previously equilibrated by adding 3 aliquots of 500μl of cold MACS medium in the magnetic field of a MACS separator. Unbound cells were then eluted by washing with three aliquots of 500μl MACS buffer, the column removed from the magnetic field and the positively selected cells eluted. The purity of the cells was assessed by flow cytometry and was typically >90% F4/80 positive. In some experiments, macrophages were positively selected on the FACS Aria (BD Biosciences). Cells were incubated with Fc block and APC-conjugated anti-F4/80 antibody for 30mins as before, passed through Nitex and $10x10^6$cells/ml run through the FACS aria. Isotype controls were used to set up gates for positive APC staining and purity was typically >90% F4/80 positive.

2.9 Flow Cytometry

200μl aliquots containing $1x10^5$ cells in 12 x 75mm polystyrene tubes (Falcon BD, Oxford, UK) were washed in FACS buffer (PBS/2mM EDTA/2% FCS) and incubated for 15mins at 4^0C in the dark with purified anti-mouse CD16/CD32 (Fc block) to prevent non-specific binding via Fc receptors. The cells were washed and incubated with the appropriate fluorochrome-conjugated or biotinylated primary antibodies for 30mins at 4^oC in the dark. All antibodies and appropriate isotype controls were used at a 1:200 dilution and their details are shown in Table 2.1. The cells were washed and resuspended in FACS buffer and, where appropriate, biotinylated antibodies were detected by fluorochrome-conjugated streptavidin for a further 15 minutes. Cells were then washed in FACS buffer and analysed on a FACScalibur Flow Cytometer (Becton Dickinson) using Flowjo software. In most experiments, 10ng/ml propidium iodide (PI; Sigma-Aldrich) or 0.25μg/sample 7-amino-actinomycin D (7-AAD; BD Pharmingen) was added to the cells a few minutes before acquisition to enable exclusion of dead cells from the analyses. FSC (forward scatter) and SSC (side scatter) were adjusted to allow gating on relevant cell types. Unstained samples were used as controls for auto-fluorescence. For detection of chemokine receptor expression, cells were incubated with 10% mouse serum (Biosera) in FACS buffer for 1 hour at 4^0C, and washed three times with FACS buffer. Cells were then incubated with 5μg/ml monoclonal antibody against CCR2 (MC-21) (a kind gift from Professor M. Mack, Dept of Internal Medicine, Ludwig-Maximilians University, Munich, Germany) or isotype control (purified rat IgG2b) for 1 hour at 4^oC, followed by three washes. Staining with biotinylated polyclonal anti-rat Ig (BD Pharmingen) for 30 minutes at 4^oC was then conducted, followed by a further set of three washes. Cells were then incubated with SA-FITC for 15-20 minutes at 4^oC, and then washed. Cells were then incubated with Fc block and stained for other markers as before.

2.10 Assessment of endocytosis by macrophages

To measure endocytosis, single cell suspensions were resuspended at a final concentration of $5x10^5$/ml cells and cultured in 1ml aliquots in ultra low attachment, 24-well tissue culture plates (Costar) with 1mg/ml FITC-dextran (MW 4400; Sigma) at either 4^0C or 37^0C in 5% CO_2. The cells were harvested and the uptake of FITC-dextran by F4/80$^+$ mϕ was assessed by flow cytometry. The results are expressed as the ΔMFI (mean fluorescent intensity calculated by subtracting the MFI obtained after incubation at 4^0C from that obtained at 37^0C).

2.11 Assessment of phagocytosis by macrophages

Cells were plated out at $2.5x10^5$/ml (BMM and purified PEC mϕ) and $1x10^6$/ml (unpurified colonic mϕ) in complete medium and allowed to adhere to a 24-well plate for 2 hours at 37^0C in 5% CO_2. The adherent cells were washed once with complete medium and then incubated with $2.5x10^7$ 3μm FITC-conjugated zymosan bioparticles (Molecular Probes/Invitrogen) per well for 1.5hr at 37^0C in 5% CO_2, or at 4^oC in the presence of 5mM NaN_3. Phagocytosis was arrested by washing twice in ice cold PBS/5mM NaN_3 and the phagocytic index was measured by calculating the percentage of F4/80$^+$ cells that were FITC positive under both conditions. Phagocytosis was also visualised using fluorescence microscopy (see below) and here, FACS-sorted F4/80$^+$ colonic mϕ were used.

2.12 Fluorescence microscopy

To image phagocytosis of fluorescent bioparticles, single cell fluorescence staining was conducted using FACS-sorted F4/80$^+$ colonic macrophages. 1ml aliquots of $5x10^5$cells/ml complete medium were added to a circular glass cover slip (VWR) placed in a flat bottomed 24-well plate. After culture, the cells were fixed with 4% formaldehyde in PBS for 15 mins, washed with PBS for 5 mins and then incubated with permeabilisation buffer (2% FCS, 2mM EDTA, 0.1% saponin in PBS), to allow phalloidin to enter the cell for 5 mins. The samples were then blocked in PBS/3% BSA/0.1% saponin for 10 mins, before 0.12μg/ml Phalloidin-Alexa Fluor® 594 (Molecular Probes, Eugene, OR) in PBS/3% BSA/0.1% saponin was added for 30 mins. The cells were then washed three times in TNT buffer (100mM TRIZMA base, 150mM NaCl and 0.05% Tween-20 in dH_2O) for 3 mins, and incubated with 300nM DAPI (Invitrogen) for 5 mins. After 3 more washes in TNT buffer, the cover slips were removed and allowed to air dry for 5 mins before being mounted in Vectashield (Vector Labs), sealed with clear nail varnish and stored in the dark at 4^oC. Samples were kept in a darkened, humidified chamber at room temperature throughout staining, and all staining steps were performed in PBS/3% BSA/0.1% saponin. Fluorescent images were captured using a 3CCD colour vision camera (regulated by a Hamamatsu and Orbit controller) and analysed using Openlab version 3.0.9 digital imaging programme (Improvision, Warwick, UK) connected to an Olympus BX50 microscope.

2.13 Immunofluorescence microscopy of tissue sections

To visualise $F4/80^+$ macrophages in the intestine, colons were removed, opened longitudinally and rolled around a needle, before being snap-frozen in liquid nitrogen in O.C.T. (Tissue-Tek, Sakura Finetek Europe) and stored at -80^oC. 6μm sections were cut on a cryostat (ThermoShandon, Cheshire, UK) and stored in a sealed box at -20^oC until used for immunofluorescence staining. All staining was performed in a darkened, humidified chamber at room temperature. The sections were fixed in acetone for 10 mins, re-hydrated in PBS for 15min and endogenous peroxidase activity was quenched with three washes of PBS/0.1% sodium azide/3% hydrogen peroxide for 10 min. Avidin (Avidin/Biotin

Blocking Kit, Vector Laboratories, Burlingame, CA) was then added in PBS/3% BSA for 12min to block endogenous biotin, followed by another wash in PBS for 5min. Biotin was then added in PBS/3% BSA for 12min to block excess avidin, followed by another wash step. The sections were then incubated with 1:200 biotinylated anti-F4/80 (Caltag Laboratories) for 30 min, washed in TNT buffer and then incubated with streptavidin-HRP (diluted 1:100 in PBS/3% BSA) for 25 min. After washing, the sections were treated with biotinylated-tyramide (diluted 1:50 in amplification buffer; Perkin Elmer Life Sciences, Boston, MA) for 10 min, washed and then incubated with Streptavidin-Alexa Fluor 647 (2 mg/ml in PBS/3% BSA; Molecular Probes) for 30 min. Details of the tyramide-based amplification are provided in Fig 2.1. The sections were then washed twice in TNT buffer, permeabilised in PBS/3% BSA/0.1% Triton X for 30min to optimise staining, washed, and then incubated with 300nM DAPI for 5min. After three washes in TNT buffer, the sections were air dried for 10 mins before being mounted in Vectashield and a coverslip sealed onto the slide with clear nail varnish and stored in the dark at 4^{0}C. Fluorescent images were captured as before.

2.14 Induction of DSS colitis

To induce acute colitis, C57Bl/6 mice received between 1.5 and 2.5% dextran sodium sulphate (DSS) salt (reagent grade; MW 36,000-50,000 kDa; MP Biomedicals, Ohio), *ad libitum* in sterile drinking water for up to 10 days. For the induction of chronic DSS colitis, animals were given three cycles of DSS for 5-7 days, with rest periods of 7 days on sterile water alone in between. Water intake was measured daily for each group and the volume of water consumed per day per mouse was estimated by dividing the total volume of water consumed per cage by the number of animals in the cage. Mice were also monitored daily for weight change, diarrhoea and rectal bleeding, and the clinical score for each mouse calculated based on the presence of weight loss, rectal bleeding and diarrhoea was as follows:

Points	Weight Loss (%)	Rectal bleeding	Diarrhoea
0	no weight loss	no blood	well-formed pellet
1	1-5		
2	5-10	blood stain around anus	pasty/semi-formed pellets that did not adhere to anus
3	10-20		
4	>20	gross bleeding	diarrhoea that adhered to anus

Total clinical disease score was determined by adding all the individual scores. Immediately following sacrifice, each colon length was measured to assess the extent of colon shortening. Furthermore, H&E histology of colons was conducted to assess the extent of tissue pathology.

2.15 Colon organ culture

1cm segments of the proximal and distal colon were opened longitudinally, washed in PBS supplemented with penicillin and streptomycin and cultured in 24-well flat bottom culture plates in 1ml RPMI 1640 medium supplemented with penicillin and streptomycin for 24 hours at 37^0C in 5% CO_2. The cultures were then centrifuged at 13,000 rpm and the supernatants were harvested and stored at $–20^0$C until assayed.

2.16 Detection of intracellular cytokines and TLRs by flow cytometry

Aliquots containing 1×10^5 cells were washed in FACS buffer and intracellular expression of cytokines or TLR determined after permeabilisation. Cells were washed and incubated in 200µl for 15mins at 4^oC with purified anti-mouse CD16/CD32 (Fc block), washed, and then stained for cell surface markers for 30mins at 4^oC. After washing, the cells were fixed in 1% formaldehyde (BDH Laboratory Supplies, Poole, England) in PBS for 10 minutes at 4^oC. Cells were then washed with FACS buffer and permeabilised with Perm stain (PBS/0.1% NaN_3 (Sigma)/0.1% BSA/1% FCS/0.1% saponin (Sigma)) and stained with Fc block as before, to block antibody binding to intracellular Fc receptors. The cells were washed in Perm wash (PBS/0.1% NaN_3/0.1% BSA/0.2% FCS/0.1% saponin) and fluorochrome-conjugated anti-cytokine or anti-TLR antibodies, or appropriate isotype controls, were added at 1:200 dilutions in Perm stain at 4^oC in the dark for 30mins. The cells were washed and resuspended in FACS buffer for flow cytometric analysis as before. To allow dead cells to be excluded, cells were treated with 0.11µg/ml ethidium monoazide (EMA; Molecular Probes) prior to permeabilisation. Cells were incubated with EMA in the dark at RT for 10 minutes, and then exposed to bright light for a further 10 minutes. To detect intracellular cytokines, cells were first activated *in vitro* by TLR ligands in the presence of 10µg/ml Brefeldin A (Sigma) for 4.5 hours before being washed and stained.

2.17 Assessment of cell turnover *in vivo*

Mice were injected i.p. with 1mg BrdU (BD Pharmingen) in PBS, culled 24hrs later, and the uptake of BrdU by cells isolated from the colon was measured using the BrdU Flow Kit (BD Pharmingen) as per the manufacturer's instructions. Briefly, dead cells were stained with EMA and cell surface antigen expression as described above. The cells were then fixed and permeablised twice, and treated with 30μg DNase/tube to expose incorporated BrdU, and stained with FITC-conjugated anti-BrdU monoclonal antibody. Mice receiving long-term BrdU administration were injected with 1mg BrdU i.p. and then 0.8mg/ml BrdU was administered in the drinking water and this was kept in the dark and changed daily. Cells in cell division were detected by permeabilisation and staining with PE-conjugated anti-Ki-67 antigen (BD Pharmingen), using the same method as used for the detection of intracellular cytokines.

2.18 Measurement of cytokine production by ELISA

Supernatants from cell cultures were harvested and stored at –20^{o}C until cytokine production was quantified using sandwich ELISA. Immulon-4 plates (Corning) were coated with anti-IL-1 (4μg/ml, R&D systems), anti-IL-6 (1μg/ml, BD Biosciences), anti-IL-12p70 (2μg/ml, BD Biosciences) or anti-TNFα (2μg/ml, BD Biosciences) detection antibodies overnight at 4^{0}C. The plates were washed 3 times with PBS/0.05% Tween (Sigma), before being blocked with PBS/10% FCS for 1hr at 37^{0}C. The plates were then washed and incubated with serially diluted recombinant cytokine standards (BD Biosciences), and with undiluted samples for 2hrs at 37^{0}C, before being washed again and incubated with biotinylated anti-IL-1 (300ng/ml, R&D systems), anti-IL-6 (1μg/ml, BD Biosciences), anti-

IL-12p40/70 (1μg/ml, BD Biosciences), anti-TNFα (2μg/ml, BD Biosciences) for 1hr at 37^0C. Plates were washed and incubated with extravidin-peroxidase (1/1000, Sigma) for 1hr. Finally, following washing, the plates were developed using tetramethylbenzidine (TMB) substrate (KPL) and read at 630nm on a MRX II microplate plate reader (Dynex).

2.19 Measurement of chemokine and cytokine production by Luminex

Supernatants from cell cultures were harvested and stored at -20^0C until assayed. CCL2, CCL3, CCL4, CCL5, CCL19, CXCL10, FGF, GM-CSF, IFNγ, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12 (p40 and p70), IL-13, IL-17, KC, MIG, and TNFα production was quantified simultaneously using the Multiplex Bead Assay (Biosource, UK) according to the manufacturer's instructions. This assay uses specific antibodies for the cytokines/chemokines of interest that have been coated on the surface of fluorescently encoded microspheres. Each microsphere is labelled with a distinct fluorophore, which can be recognised individually by the scanner. The microspheres were incubated in a 96-well Luminex plate with test samples or standards and, after washing, biotinylated detection antibodies are added for 1hr. After incubation the plate was washed and the samples incubated with SA-PE for 30mins. After washing, the fluorescence bound to the microspheres was analysed using a Luminex XMAP system with the intensity of the fluorescence being directly proportional to the concentration of cytokine/chemokine present.

2.20 RNA extraction

Single cell suspensions were washed twice with PBS, spun down and the supernatant was completely removed by aspiration. RNA was then isolated using the RNeasy Micro or Mini Kit (Qiagen) (depending on cell number) according to the manufacturer's instructions. Contaminating genomic DNA was removed on-column during RNA isolation with the RNase-free DNase Set (Qiagen) according to the manufacturer's instructions and the amounts of RNA were measured using the BioPhotometer (Eppendorf, Germany). Samples were stored at -80^0C until use.

2.21 cDNA synthesis from RNA

cDNA was reverse transcribed from DNAse-treated RNA using Superscript II Reverse Transcriptase (RT) (Invitrogen) according to the manufacturer's instructions. Briefly, 5µg of RNA, 1µl Oligo$(dT)_{12\text{-}18}$ (500µg/ml; Invitrogen), 1µl dNTP mix (25mM each; Invitrogen), and nuclease-free water (Ambion) were added to a nuclease-free microcentrifuge tube (ABgene, Surrey, UK) in a total volume of 12µl. The mixture was heated at 65^0C for 5 minutes, and then quick-chilled on ice. 4µl 5X First-Strand Buffer (Invitrogen), 2µl 0.1M DTT (Invitrogen), and 1µl RNaseOUT (40 units/ml; Invitrogen) were added and incubated at 42^0C for 2 minutes. 1µl (200 units) Superscript II RT was then added and the RNA was reverse transcribed at 42^0C for 50 minutes. Superscript II RT was then inactivated by heating at 70^0C for 15 minutes. Negative control samples were incubated in the absence of Superscript II, and cDNA was stored at -20^0C until use.

2.22 End-product polymerase chain reaction (PCR)

A 1 in 5 dilution of cDNA was added to a 1.1x Pre-Aliquoted ReddyMix PCR Master Mix, 50µl Reaction (ABgene) and used according to the manufacturer's instructions. Briefly, 2µl cDNA (1 in 5 dilution), 2µl of forward and reverse primer mix (primers at a final concentration of 1µM), and 1µl nuclease-free water was added to the Master Mix. For end-product PCR, amplications were performed using the FTGENE5D thermocycler (Techne (Cambridge) Ltd; Duxford, Cambridge, UK) using the primers, annealing temperatures and cycle numbers shown in Table 2.2.

The PCR products were then run on a 2% agarose gel (2% ultra pure agarose electrophoresis grade (Gibco) containing 800ng/ml ethidium bromide (Sigma)) in 0.5x TBE buffer in a Horizon 58 (Life Technologies) gel tank. Gels were run at 90V powered by a Pharmacia Electrophoresis Constant Power Supply ECPS 3000/150, and analysed using the Gel Logic 200 imaging system.

2.23 Quantitative/real time PCR

Relative levels of mRNA were quantified by RT-PCR using the Taqman system, using the primers and fluorogenic probes described in Table 2.3. RNA was extracted and cDNA synthesised as before, and cDNA was used at a 1 in 5 dilution. The fluorogenic probes contained a reporter dye (FAM) covalently attached at the 5' end and a quencher dye (TAMRA) covalently attached at the 3' end. PCR reactions were performed in a 96-

well plate in the ABI-prism 7900 Sequence Detector (ABI). PCR reactions contained 2μl of the diluted cDNA sample, 10μl Taqman Real-Time PCR Universal Master Mix (2X) (ABI), 900nM of each primer, 200nM of the detection probe, and nuclease-free water (Ambion) to a total volume of 20μl. Each PCR reaction was performed in triplicate using the following cycle conditions: 2 min at 50^0C and 10 min at 94^0C, followed by a total of 40 cycles of 15 sec at 94^0C and 1 min at 60^0C. Threshold cycle (CT) values were calculated and data analysed using RQ Manager software (ABI) and samples were normalised by reference to the hypoxyanthine-guanine phosphoribosyltransferase (HPRT) as a reporter gene.

2.24 Statistical Analysis

Results are shown as means ± 1 standard deviation unless stated otherwise, and groups were compared using a Student's two tailed unpaired t-test. When comparing multiple groups, a one-way ANOVA was performed (see specific figure legends for repeated measures examples), followed by a Bonferroni multiple comparison test. Values of $p<0.05$ were considered to be statistically significant. The daily rate of macrophage turnover in Chapter 6 was calculated using linear regression analysis, where best-fit values were used to calculate the difference between slopes.

Antigen	Clone	Isotype	Company
CD4	GK1.5	Rat IgG2b	BD Pharmingen
CD8	53-6.7	Rat IgG2a	BD Pharmingen
CD11b	M1/70	Rat IgG2b	BD Pharmingen
CD11c	HL3	Hamster IgG1	BD Pharmingen
CD40	3/23	Rat IgG2a	BD Pharmingen
B220	RA3-6B2	Rat IgG2a	BD Pharmingen
CD80	16-10A1	Hamster IgG2a	BD Pharmingen
CD86	GL1	Rat IgG2a	BD Pharmingen
CD103	M290	Rat IgG2a	BD Pharmingen
CD115	AFS98	Rat IgG2a	Ebioscience
Class II MHC (I-A^b)	25-9-17	Murine IgG2a	BD Pharmingen
Ly6G	1A8	Rat IgG2a	BD Pharmingen
F4/80	BM8	Rat IgG2b	Caltag Laboratories
Gr-1	RB6-8C5	Rat IgG2b	BD Pharmingen
TLR2	6C2	Rat IgG2b	Ebioscience
TLR3	TLR3.7	Murine IgG1	Ebioscience
TLR4	MTS510	Rat IgG2a	Ebioscience
TLR9	M9.D6	Rat IgG2a	Ebioscience
TNFα	MP6-XT22	Rat IgG1	BD Pharmingen
Ly5.1	A20	Murine IgG2a	BD Pharmingen
Ki-67	B56	Murine IgG1	BD Pharmingen

Table 2.1 Monoclonal antibodies and isotype controls used for flow cytometry

Gene	Forward Primer 5'-3'	Reverse Primer 5'-3'	Ta (^{0}C)	Cycles	Amplicon (bp)
GAPDH	AACTCCCACTCTTCCACCTT	GCCCCTCCTGTTATTATGG	60	30	270
TLR1	TACAGTTCCTGGGGTTGAGC	TAGTGCTGACGGACACATCC	60	30	216
TLR2	CGTTGTTCCCTGTGTTGCT	AAAGTGGTTGTCGCCTGCT	60	35	119
TLR3	TTGCGTTGCGAAGTGAAG	TAAAAAGAGCGAGGGGACAG	60	35	406
TLR4	TTCACCTCTGCCTTCACTACA	GGGACTTCTCAACCTTCTCAA	60	35	225
TLR5	CAGGATGTTGGCTGGTTTCT	CGGATAAAGCGTGGAGAGTT	60	35	169
TLR6	ATGGCACAGCGGACTTACTT	ATGAGAGCCCAGGTTGACAG	60	30	170
TLR7	GCTGTGTGGTTTGTCTGGTG	CCCCTTTATCTTTGCTTTCC	60	35	270
TLR8	GACTTCATCCACATCCCAAA	TCCCAATCCCTCTCCTCTAA	60	35	158
TLR9	GAAAGCATCAACCACACCAA	ACAAGTCCACAAAGCGAAGG	60	35	304
iNOS	GCCACCAACAATGGCAACA	CGTACCGGATGAGCTGTGAA	60	28	103
Arginase	CAGAAGAATGGAAGAGTCAG	CAGATATGCAGGGAGTCACC	55	30	250
Ym-1	CATGAGCAAGACTTGCGTGAC	GGTCCAAACTTCCATCCTCCA	59	35	101

Table 2.2 Sequences, annealing temperatures and cycle numbers for primers, and amplicon sizes of products used in end-product PCR reactions.

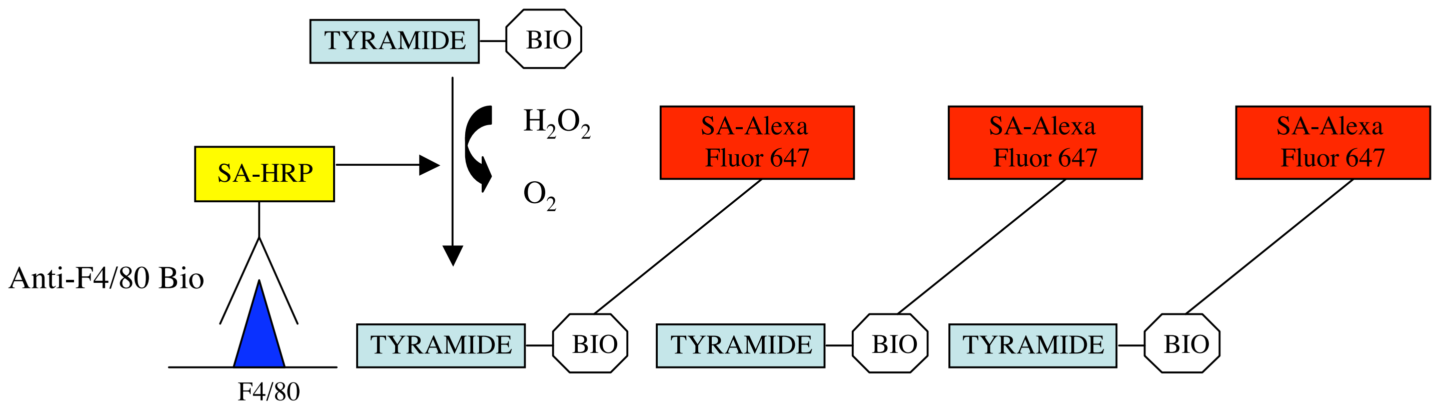
Gene	Forward Primer 5'-3'	Reverse Primer 5'-3'	Probe
HPRT	CTTCCTCCTCAGACCGCTTTT	AACCTGGTTCATCATCGCTAATC	AGCCGACCGGTCCCGTCATG
TLR2	AAGATGCGCTTCCTGAATTTG	TCCAGCGTCTGAGGAATGC	CGTTTTTACCACCCGCATCCCTGTACTG
TLR4	GCCAAGTTTCTCTGGACTAACAAGTTTAGA	GCCAAGTTTCTCTGGACTAACAAGTTTAGA	GCCAAGTTTCTCTGGACTAACAAGTTTAGA
TLR9	AGGCTGTCAATGGCTCTCA	TGAACGATTTCCAGTGGTA	TGCCGCTGACTAATCTGCA

Table 2.3 Sequences of primers and probes used in Q-PCR reactions

Figure 2.1 Tyramide signal amplification

Tyramide signal amplification is an enzyme-based system that enhances fluorescent labelling of target proteins. Biotinylated anti-mouse F4/80 or isotype control antibody is used to detect the target protein. Steptavidin-horse radish peroxidase (SA-HRP) then binds via biotin and catalyses the oxidation of tyramide and deposition of multiple biotin labels in the immediate vicinity of the target protein. Biotin labels were then detected by SA-Alexa Fluor 647.

Figure 2.1 Tyramide signal amplification



Chapter 3

Phenotypic and functional characterisation of macrophages in the resting colonic lamina propria

Introduction

Tissue mϕ play a number of essential roles in the body. In addition to characteristic inflammatory functions, such as phagocytosis and the production of pro-inflammatory cytokines and chemokines, and presentation of antigen to T cells, mϕ are also involved in tissue homeostasis, tissue repair, and in clearing senescent cells. Mϕ are abundant in the healthy human intestine, but unlike most other mϕ populations in the body, human small intestinal mϕ fail to produce pro-inflammatory cytokines in response to a variety of stimuli, despite exhibiting some other properties normally associated with mϕ, such as phagocytic and bactericidal functions (188). As a result, it has been suggested that mϕ may play a central role in regulating intestinal immune responses, possibly by maintaining the level of the resident microbiota without initiating inflammation. In addition, local mϕ are thought to play a critical role in protection from intestinal infection and contribute to inflammation during IBD. It is not known how these functions of intestinal mϕ are controlled, nor has the biology of intestinal mϕ been compared directly in inflamed or resting colon under controlled experimental conditions. In addition, their relationship to other myeloid cells is not well characterised, partly because of difficulties in isolating them, nor is it known if mϕ in the colon constitute a homogeneous population, or if there are individual subsets with distinct functions. No detailed experiments of this kind have been carried out using murine colonic mϕ, and so the aim of my project was to examine the properties of resident and inflammatory murine colonic mϕ.

To characterise this population, I explored their location, phenotype, function and lineage in normal mice, before investigating their properties in mice with colitis. In this chapter, I examined the location of mϕ in the resting intestine *in situ*, before establishing and optimising methods for isolating them by tissue digestion. Having established an appropriate method, I used it to conduct a detailed phenotypic analysis of resident myeloid cells in the normal mouse colon, before comparing their functions with those of mϕ in other tissues, by examining the production of pro-inflammatory cytokines and chemokines, upregulation of co-stimulatory molecules, endocytosis and phagocytosis.

3.1 Macrophages are abundant in the healthy mouse colon

As others have shown in mice and humans, the distal colon of healthy C57Bl/6 mice contained large numbers of mϕ as defined by the expression of F4/80 (Fig 3.1). These mϕ were found mainly in the LP underlying the epithelium. In order to conduct a more detailed characterisation, I established methods for isolating and phenotyping these cells. I first compared several previously described isolation methods to assess which method provided the best and most consistent yields with optimal cell viability. The percentage of F4/80$^+$ cells obtained and the levels of F4/80 expression varied markedly depending on the isolation method, and many techniques produced wide variations from experiment to experiment. As a result of these experiments, I finally devised a protocol using two types of collagenase, dispase and DNase which allowed effective tissue digestion with high cell viability, and good yields of F4/80-expressing cells which were consistent from experiment to experiment. This protocol is described in the Materials and Methods on p62.

Using this procedure, a heterogeneous population of cells was obtained with varying FSC and SSC properties (Fig 3.2a) and to begin my phenotypic analyses, I selected a gate which was likely to include as wide a range of mononuclear cells as possible. Dead cells were excluded on the basis of 7-AAD uptake (Fig 3.2b) and approximately 40% of the resulting, live-gated cells expressed CD45, indicating they were of haematopoietic origin (Fig 3.2c). 10.9±1.9% of the total cells expressed the F4/80 marker of mature mouse mϕ (Fig 3.2d). The CD45$^-$ population was not characterised, but is likely to contain stromal, epithelial and other mesenchymal cells.

To gain an insight into the phenotypic heterogeneity of the resident mϕ population, I first assessed the expression of myeloid cell markers, which display different patterns of expression in mϕ and DC subsets in mice. Classical tissue mϕ are F4/80$^+$CD11b$^+$CD11c$^-$, while mature classical DCs express high levels of CD11c and although some co-express CD11b, they are usually negative for the expression of F4/80 (43).

Throughout my experiments I used BMM as a representative population of immature mϕ, whilst resident PEC mϕ were used as representative mature tissue mϕ. As expected and as reported by others, 86.2±6.8% and 96.9±1.6% of BMM and PEC mϕ respectively had the F4/80$^+$CD11b$^+$CD11c$^-$ phenotype of conventional mϕ (Fig 3.3a). Just over 10% of F4/80$^+$ BMM expressed the DC marker, CD11c, but this was absent from PEC mϕ (Fig 3.3a).

As expected, the majority (75.5±6.3%) of F4/80$^+$ cells in the colon expressed CD11b; this was at slightly higher levels than BMM, but much less than that of PEC mϕ (Fig 3.3b). 35.4±10.5% of colonic F4/80$^+$ cells also expressed the DC marker, CD11c (Fig 3.3+3.4b). These 'triple positive' cells expressed slightly lower levels of CD11c compared with the small (2-3%) subset of CD11c$^+$ F4/80$^-$ cells present in the same preparations, which are likely to be conventional DCs (Fig 3.4b). There was also a smaller population (22.4±8.5%) of F4/80$^+$ CD11b$^-$ cells, most of which were also negative for CD11c (Figure 3.4a), as well as some F4/80$^-$CD11b$^+$ cells. 48.6±4.2% of these latter cells were also CD11c$^+$, and are also likely to be a subset of DCs. Together these data show that there are a number of different myeloid cell populations present in the healthy intestine, with overlapping characteristics of mϕ and DCs.

Despite their phenotypic heterogeneity, there was little evidence of morphological heterogeneity among colonic F4/80$^+$ subsets, as revealed by FSC/SSC analysis, apart from the presence of cells with slightly higher granularity among the F4/80$^+$CD11b$^+$CD11c$^-$ subset (Fig 3.5b). These may include eosinophils, which have been shown to express these markers at low levels (48). I carried out a more detailed morphological comparison of BMM, PEC mϕ and FACS-sorted F4/80$^+$ colonic mϕ. After being allowed to adhere to plastic for 2 hrs, the cells were stained with DAPI and with phalloidin to visualise actin filaments. All these F4/80$^+$ populations were large cells with several cytoplasmic processes, typical of the mϕ lineage and no granular cells were evident (Fig 3.6). In general, PEC mϕ and colonic mϕ exhibited a rounder morphology with more numerous processes compared with BMM, which seemed to exhibit a more irregular morphology.

Due to the fact the F4/80$^+$CD11c$^+$ cells expressed lower levels of CD11c compared with F4/80$^-$CD11c$^+$ classical DCs, I considered the total F4/80$^+$ population of myeloid cells as colonic mϕ for the remainder of this thesis. However, the possible relationships between these different subsets is discussed in detail in Chapter 8.

I next attempted to characterise colonic mϕ in more detail by examining the expression of other myeloid cell markers. Consistent with previous findings (320), all BMM expressed high levels of intracellular CD68 (macrosialin), a scavenger receptor for oxidised low density lipoprotein (LDL), and also had moderate surface expression of this marker (Fig 3.7a). In contrast, although PEC mϕ and colonic mϕ both expressed CD68 intracellularly, this was at a lower level than that of BMM and a small population of colonic F4/80$^+$ cells lacked any expression of intracellular CD68. A proportion of this population were larger than the cells present within the F4/80$^+$CD68$^+$ population (Fig 3.7b). There was little or no surface expression of CD68 by PEC mϕ or colonic mϕ. As anticipated, the immature mϕ found in BM expressed uniformly high levels of the M-CSF receptor, CSF1R (CD115) and this was also present on all PEC mϕ, although at substantially lower levels (Fig 3.7c). In contrast, colonic F4/80$^+$ cells expressed little or no CD115.

Almost all BMM and PEC mϕ were negative for the granulocytic marker Gr-1, but around 30% of F4/80$^+$ cells in the colon expressed this marker (Fig 3.8). There was also a substantial population of F4/80$^-$ Gr-1$^+$ cells in the colon, indicating the presence of a granulocytic cell population in the colon under resting conditions (Fig 3.8b).

Finally, I examined the expression of CD103, the $\alpha_E\beta7$ integrin expressed on a high proportion of small intestinal DCs and on CD11c^{hi} class II MHC^{hi} DCs in the colon (321). A small proportion (2%) of total colonic LP cells expressed CD103, of which ~27% expressed CD11c, indicating that these may be DCs, an idea supported by the fact they were F4/80$^-$. The remainder of the CD103$^+$ cells lacked CD11c expression and had the FSC/SSC properties of lymphocytes, consistent with the known expression of CD103 by mucosal T cells (322). None of the F4/80$^+$ cells in the colon expressed CD103, although a small subset of BMM and around 40% of PEC mϕ expressed low levels of this marker (Fig 3.9a+b).

3.2 Expression of class II MHC and co-stimulatory molecules by colonic mϕ

Previous findings have shown that human colonic mϕ express low levels of co-stimulatory molecules and class II MHC (187). To examine if this also applied to mϕ in the mouse colon, F4/80$^+$ cells were analysed for the expression of CD40, CD80, CD86 and class II MHC. Freshly isolated BMM expressed only low levels of CD80 and CD86 and no CD40, while PEC mϕ expressed higher levels of these markers, especially CD40 and CD86 (Fig 3.10). Both BMM and PEC mϕ lacked expression of class II MHC. In stark contrast, the vast majority of colonic mϕ expressed high levels of class II MHC, but had little or no CD40, CD80 or CD86.

When the different subsets of colonic F4/80$^+$ cells were analysed for the expression of class II MHC, almost all of the F4/80$^+$CD11b$^-$ population were found to lack class II expression, whereas >80% of the F4/80$^+$CD11b$^+$ subset expressed high levels of class II MHC (Fig 3.11a). Expression of class II MHC by the F4/80$^-$CD11b$^+$ cells was heterogeneous, with most of these being negative and the remainder expressing intermediate levels. All the F4/80$^+$CD11b$^+$CD11c^{int} cells expressed high levels of class II MHC, whereas most of the cells with the phenotype of classical DCs (F4/80$^-$CD11c$^+$) expressed intermediate levels (Fig 3.11b). The F4/80$^+$CD11c$^-$ cells show bimodal levels of class II MHC, with ~60% being class II MHC^{hi} and the others being class II MHC$^-$.

These findings allow three major phenotypic subsets of F4/80$^+$ cells to be identified in the colon of normal mice (Fig 3.12). These are an F4/80$^+$CD11b$^+$CD11c^{int} subset, an F4/80$^+$CD11b$^+$CD11c$^-$ subset and a smaller F4/80$^+$CD11b$^-$CD11c$^-$ subset. These subsets are not present in PEC mϕ or BMM and are therefore unique to the intestine. In addition, there is an F4/80$^-$ CD11c^{hi} population, some of which expressed CD11b or CD103, which are likely to be classical mucosal DCs.

3.3 Upregulation of co-stimulatory molecules and class II MHC following activation of mϕ

I next set out to analyse the functional capabilities of colonic mϕ. To do this, I first purified F4/80$^+$ cells from PEC and colon by MACS or FACS and examined the expression of CD40, CD80, CD86 and class II MHC after stimulation with LPS ± IFNγ for 8hrs. 40-50% of freshly isolated PEC were F4/80$^+$, and following MACS or FACS-based positive selection (see figure legends), this was increased to almost 95% (Fig 3.13b). F4/80$^+$ mϕ constituted approximately 10% of the total colonic LP cell population and following MACS or FACS-based purification, this was increased to >90% F4/80$^+$ (Fig 3.13c). Cells harvested from *in-vitro* BM cultures were >90% F4/80$^+$ and therefore further purification was not necessary (Fig 3.13a).

As described above, freshly harvested BMM expressed low levels of CD40, CD80, CD86 and class II MHC, and these markers were increased slightly following stimulation with LPS, especially CD40 and CD86 (Fig 3.14). These changes were even greater after stimulation with LPS + IFNγ. As described above, freshly isolated PEC mϕ expressed intermediate levels of all the co-stimulatory molecules, but failed to express any class II MHC. None of these molecules were upregulated markedly on PEC mϕ by stimulation with LPS (Fig 3.14a-d). However, CD86 expression by PEC mϕ was upregulated after culture in medium alone. The addition of IFNγ induced a greater upregulation of co-stimulatory molecules by PEC mϕ in the presence of LPS, but there was still little effect on class II MHC expression. As shown earlier, freshly isolated colonic mϕ expressed high levels of class II MHC, but failed to express any co-stimulatory molecules. Stimulation with LPS or with LPS + IFNγ had no effect on CD40, CD80 and CD86 expression by colonic mϕ, while class II MHC expression was slightly lower than on unstimulated colonic mϕ (Fig 3.14).

3.4 Production of pro-inflammatory cytokines and chemokines following stimulation of colonic macrophages

To examine if murine colonic mϕ produced pro-inflammatory cytokines in a similar manner to other mϕ populations in the body, BMM and MACS-purified PEC or colonic mϕ were examined for the production of TNFα, IL-6 and IL-1β after culture in medium, LPS, IFNγ, or LPS + IFNγ. Following stimulation with LPS or IFNγ alone, BMM produced moderate amounts of TNFα as assessed by ELISA and there was a synergistic effect of adding both stimuli together (Fig 3.15a). Although TNFα production by PEC mϕ was lower compared with BMM under all conditions, a similar pattern was observed, with some production in response to either stimulus alone and higher levels being observed following culture with LPS + IFNγ together (Fig 3.15a). In stark contrast, colonic mϕ produced little or no TNFα under any conditions (Fig 3.15a).

A similar pattern was seen for the production of IL-6, with BMM producing high levels of cytokine following culture with LPS + IFNγ together, and some IL-6 after stimulation with LPS alone (Fig 3.15b). Unlike TNFα production, treatment of BMM with IFNγ alone failed to induce production of IL-6. Treatment of PEC mϕ with either LPS or IFNγ alone induced moderate production of IL-6, which was increased further after culture with LPS + IFNγ. As with TNFα production, colonic mϕ produced negligible levels of IL-6 under any conditions (Fig 3.15b). Consistent with previous findings (323, 324), the production of IL-12p70 by BMM was only observed following stimulation with both LPS + IFNγ (Fig 3.15c). However, PEC mϕ produced small but detectable amounts of IL-12p70 in response to either IFNγ alone, or with LPS + IFNγ. Again there was no IL-12p70 production by colonic mϕ under any conditions (Fig 3.15c).

I next set out to confirm and extend the findings of functional unresponsiveness of colonic mϕ by examining this at the cellular level using intracellular cytokine analysis and by employing additional innate stimuli. These included the TLR2 ligand, BLP, and MDP, the ligand for intracellular NOD2. As before, LPS stimulation of BMM induced intracellular TNFα production, with 95.6% of cells being positive for this cytokine (Fig 3.16). BLP and MDP also induced significant TNFα production when used alone (67.7% and 51.4% cells positive, respectively) and this was increased further when these agents were used together (82.6%). In contrast to BMM and consistent with the results shown in

Fig 3.15, colonic mϕ were almost completely unresponsive to LPS, with only 3.2% expressing TNFα, compared with 3.7% when cultured in medium alone. A somewhat larger proportion of colonic mϕ expressed TNFα following culture with the TLR2 ligand, BLP (14.2%), but very few cells responded to MDP (6.5%) and these numbers were markedly less than BMM under the same conditions (Fig 3.16).

Next I compared the ability of BMM, PEC mϕ and colonic mϕ to produce pro-inflammatory chemokines, namely IP-10 (CXCL10), KC (CXCL1), MCP-1 (CCL2), MIG (CXCL9), MIP-1α (CCL3), RANTES (CCL5) and MIP-1β (CCL4) following stimulation with LPS, IFNγ or LPS + IFNγ using Luminex. Stimulation of BMM and PEC mϕ with LPS, IFNγ, or LPS + IFNγ induced similar levels of IP-10, although these were always lower for PEC mϕ compared with BMM (Fig 3.17a). Overall, BMM produced little or no KC except after culture with LPS alone, whereas PEC mϕ produced higher amounts of KC under all conditions, especially when LPS was used alone (Fig 3.17b). High levels of MCP-1 production by BMM and even higher levels by PEC mϕ were observed following stimulation with LPS alone, or with LPS + IFNγ (Fig 3.17c). In contrast, treatment with IFNγ and LPS + IFNγ induced higher levels of MIG production by BMM than by PEC mϕ (Fig 3.17d). High levels of MIP-1α production by BMM and PEC mϕ were induced by treatment with LPS alone (Fig 3.18a). Both BMM and PEC mϕ produced RANTES after treatment with LPS or with LPS + IFNγ, with higher levels seen with BMM (Fig 3.18b). High levels of MIP-1β production were induced by treatment of BMM and PEC mϕ with LPS alone (Fig 3.18c). In stark contrast to these active responses by BMM and PEC mϕ, colonic mϕ produced virtually none of the inflammatory chemokines under any condition, the only exception being that some KC was found after stimulation with LPS (Fig 3.17b).

Together these results show that colonic mϕ are virtually incapable of producing pro-inflammatory mediators in response to any stimulus.

3.5 Endocytic and phagocytic activities of colonic macrophages

As human small intestinal mϕ have been shown to phagocytose FITC-labelled beads despite their impaired functional responses in other respects, I assessed the abilities of murine colonic mϕ to phagocytose particles and to endocytose soluble material. I first optimised an endocytosis assay using FITC-dextran and BMM. BMM were cultured with FITC-labelled dextran for 10, 30 or 60 minutes either at 37^0C in 5% CO_2, or at 4^0C as a negative control. As shown in Fig 3.19a, temperature-dependent dextran uptake increased with time, with active endocytosis peaking at 60mins and there was little background uptake at 4^0C at this time. Therefore I used this timepoint in subsequent experiments. Under these conditions, BMM endocytosed higher levels of dextran than PEC mϕ, which showed little activity above background (MFI = 136.2±9.1 vs 8.3±0.8; Fig 3.19b). Colonic mϕ exhibited significantly higher endocytic activity than PEC mϕ, but lower than that of BMM (MFI = 35.8±2.4).

I next assessed the ability of colonic mϕ to phagocytose FITC-labelled zymosan-coated bioparticles *in vitro*. Zymosan is a yeast cell wall component that can interact with

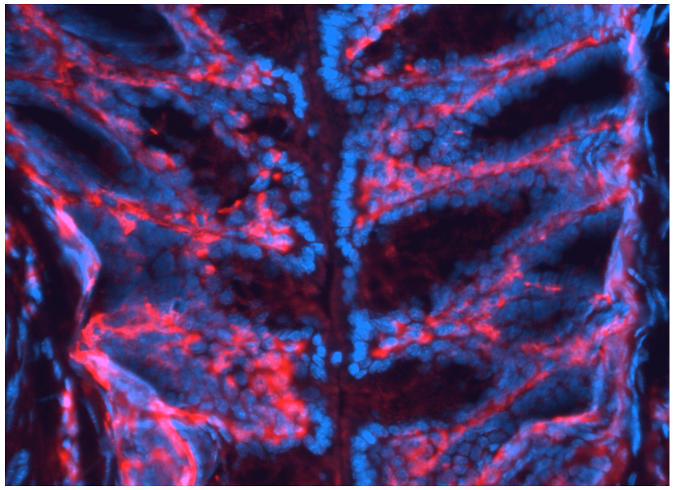
TLR2 and TLR6, thus providing an innate immune stimulus to trigger phagocytic activity (70). As negative controls for passive adhesion of particles, I used non-phagocytic L929 cells and mϕ cultured at 4^{o}C in the presence of sodium azide. As shown in Fig 3.20, 42.9±0.7% and 52.8±2.5% of BMM and PEC mϕ phagocytosed particles in a temperature-dependent manner, respectively. Colonic mϕ exhibited similar levels of phagocytosis to BMM, but these were slightly but significantly lower than that of PEC mϕ (37.3±3.9%). I confirmed these findings at the cellular level by fluorescence microscopy and as shown in Fig 3.21, BMM, PEC mϕ and colonic mϕ, but not L929 cells, all internalised large numbers of zymosan-coated beads per cell, indicating that all three populations exhibit avid phagocytic capacity.

Summary

In this chapter, I demonstrated that there is a large resident population of F4/80$^+$ mϕ in the colon that is located predominantly in the LP just under the epithelium. Comprehensive phenotypic analysis of this myeloid cell population in the healthy colon demonstrated that it is extremely heterogeneous, with three main subsets being identifiable on the basis of the expression of F4/80, CD11b and CD11c. There were also cells with the phenotype of classical DCs and such heterogeneity was not apparent in other tissues such as BM or the resting peritoneum. In addition, unlike other mϕ, the majority of resident colonic mϕ expressed high levels of class II MHC, particularly the F4/80$^+$CD11b$^+$ subset, some of which also expressed intermediate levels of CD11c. However colonic mϕ failed to express the CD40, CD80 or CD86 co-stimulatory molecules, even after stimulation with LPS and/or IFNγ, which induced increased expression of these molecules on other mϕ.

Furthermore, following culture with a variety of innate immune stimuli, colonic mϕ failed to produce the pro-inflammatory mediators, TNFα, IL-6, IL-12p70, IP-10, MCP-1, MIG, MIP-1α, RANTES and MIP-1β in response to stimuli which induced their secretion by the other mϕ. However, colonic mϕ produced low levels of the chemokine, KC, in response to LPS, and a small proportion retained responsiveness to the TLR2 ligand, BLP. In contrast to their inability to produce pro-inflammatory mediators, colonic mϕ showed strong endocytic and phagocytic activities, both hallmark functions of mϕ. Together these results support the idea that resident colonic mϕ are in a partially refractory state, with some functions being retained, but other, pro-inflammatory functions being impaired. This suggests that colonic mϕ may be able to take up and possibly clear commensal bacteria, but importantly, this does not result in upregulation of co-stimulatory molecules or the production of pro-inflammatory cytokines and chemokines. Therefore I next sought to investigate how these pro-inflammatory functions were regulated in colonic mϕ and these studies are described in the next chapter.

A



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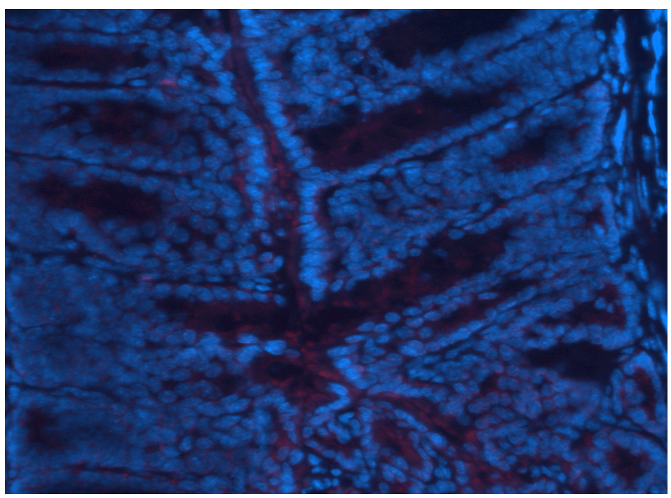


Figure 3.1 Immunofluorescence staining of macrophages in the healthy intestine

Immunofluorescence images show staining for F4/80$^+$ mϕ (red) (A) and the nuclear stain DAPI (blue), and staining with isotype control antibody (B), on sections of distal colon from C57Bl/6 mice.

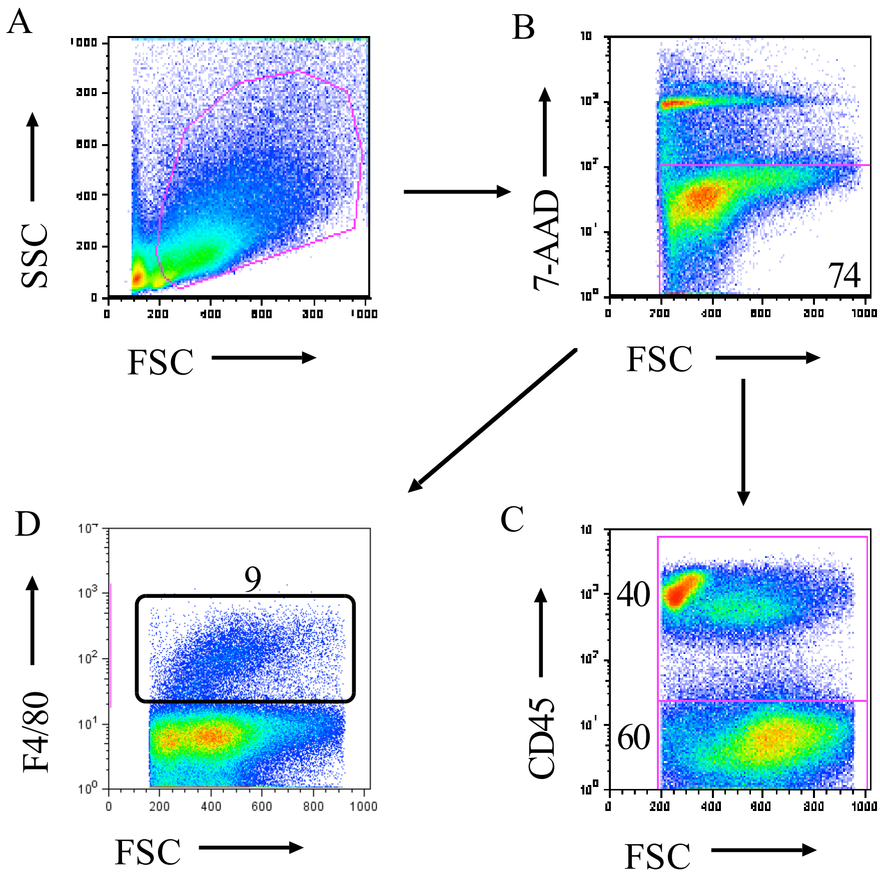
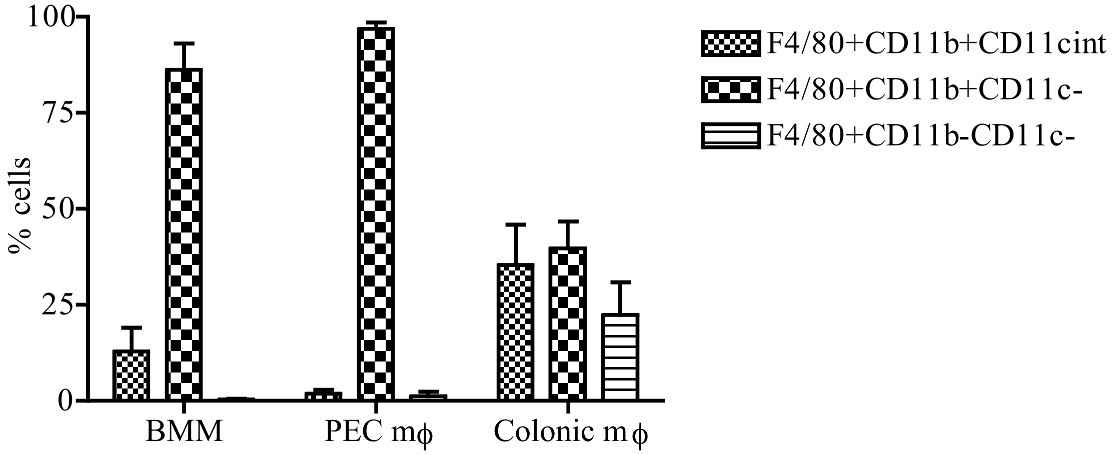


Figure 3.2 Colonic LP cell preparations from mice

A) Colons pooled from 3 C57Bl/6 mice were digested and the cell preparations were examined for granularity (SSC) and size (FSC). B) To assess dead cell content, gated cells were stained with 7-AAD and the resulting, live-gated cells were assessed for the expression of CD45 (C) and F4/80 (D). Numbers in plots represent percentages of the total population and the results are representative of at least five experiments.

A



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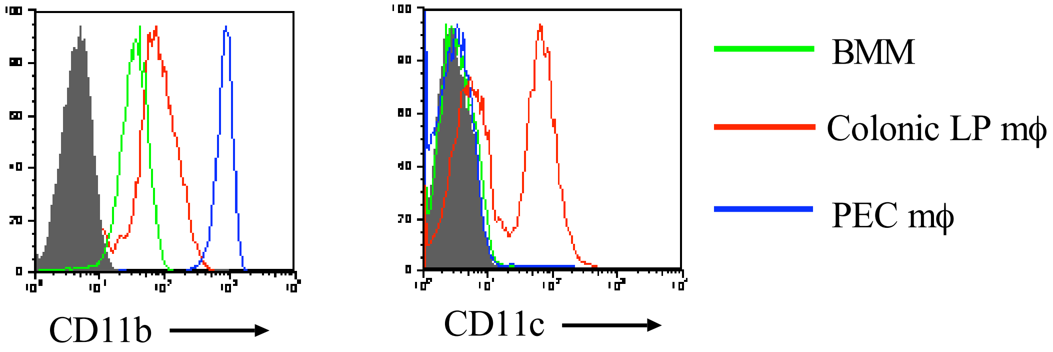


Figure 3.3 Comparative expression of myeloid markers by mϕ populations

$F4/80^+$ BMM, PEC mϕ and colonic mϕ from C57Bl/6 mice were assessed for the expression of F4/80, CD11b and CD11c by flow cytometry (A). Results are shown as the mean percentage of $F4/80^+$ cells in each subset ± SD for 3 individual mice. B) Histograms show marker expression on live-gated $F4/80^+$ cells, and the filled histograms represent staining with isotype controls. Data are representative of at least three individual experiments.

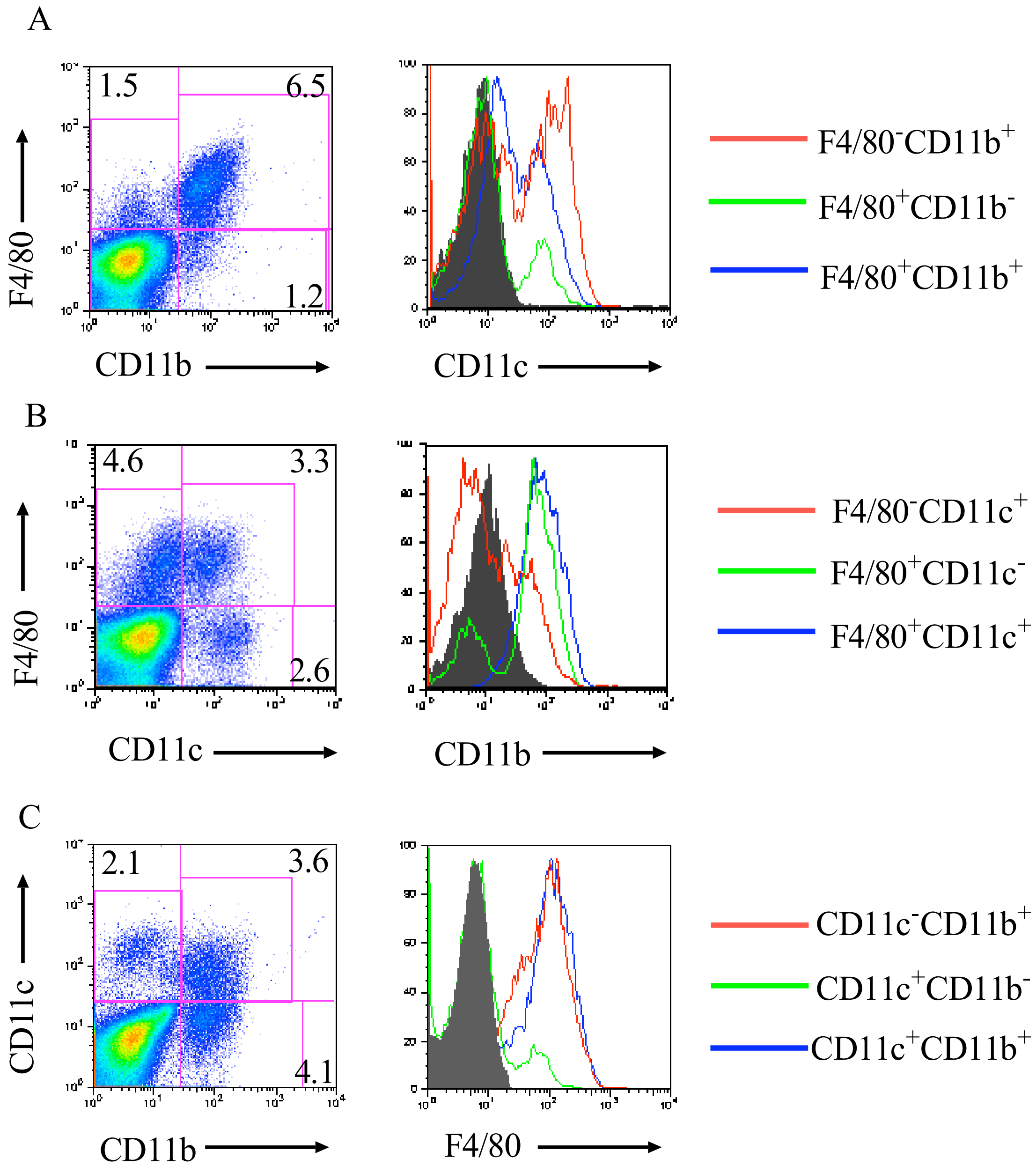


Figure 3.4 Populations of myeloid cells in the colonic LP of mice

Colons pooled from 3 C57Bl/6 mice were digested and live-gated LP cells were analysed for the expression of F4/80, CD11b and CD11c by flow cytometry. The histograms are gated on live cells and show the levels of CD11c (A), CD11b (B) and F4/80 (C) expression by the different subsets. The filled histograms represent staining with isotype controls. Numbers in quadrants represent percentages of the total population and the plots are representative of at least four individual experiments.

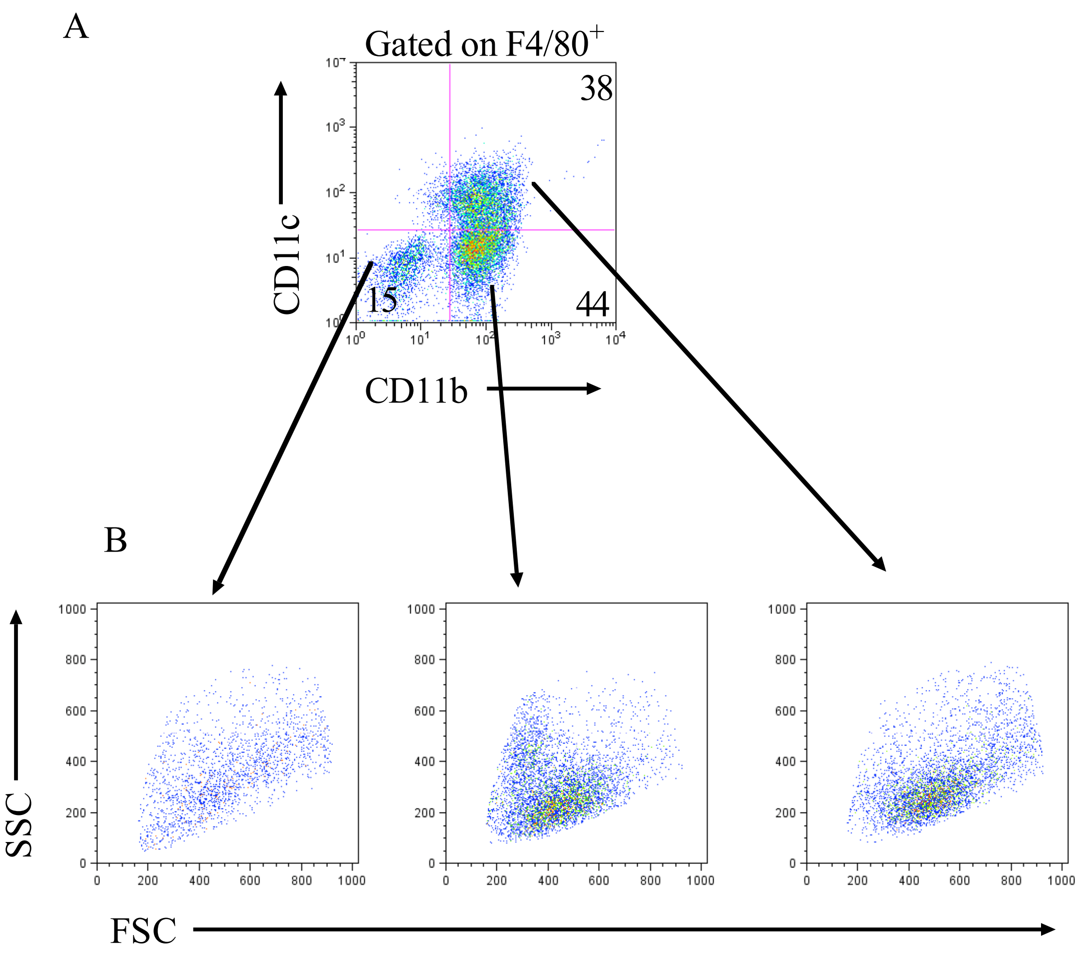
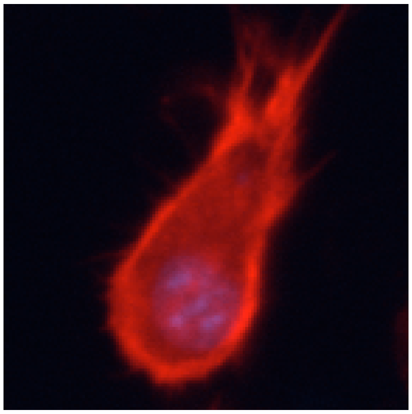


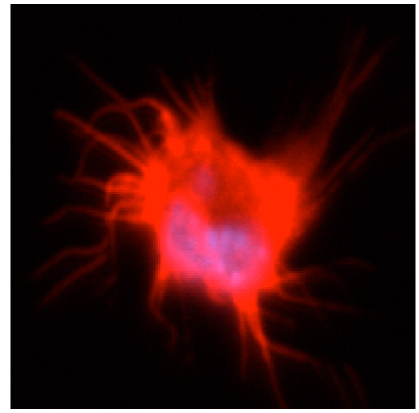
Figure 3.5 FSC/SSC properties of colonic F4/80$^+$ subsets

F4/80$^+$ colonic LP cells pooled from 3 C57Bl/6 mice were analysed for the expression of CD11b and CD11c by flow cytometry. A representative dot plot of CD11b and CD11c expression by live-gated F4/80$^+$ cells (A) and FSC and SSC properties of the individual subsets are shown (B). Results are representative of at least three experiments.

A **BMM**



B **PEC mϕ**



C **Colonic mϕ**

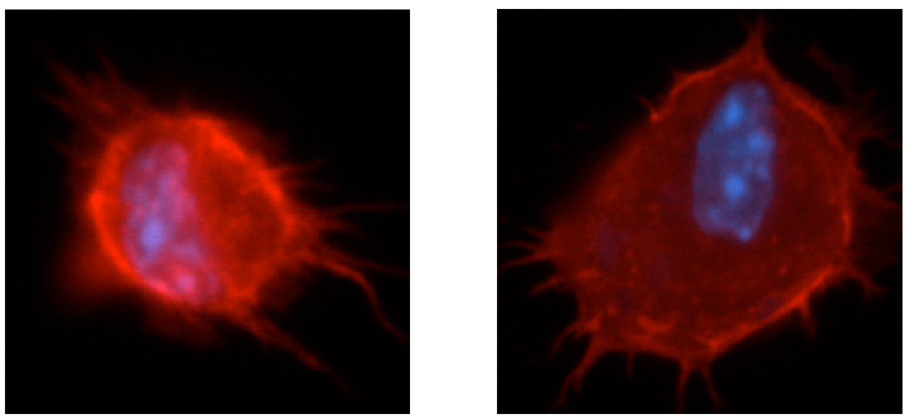


Figure 3.6 Immunofluorescence images of purified macrophages

Representative images of adherent BMM (A), and MACS-purified F4/80^{+} PEC mϕ (B) and colonic F4/80^{+} mϕ (C). Cells were allowed to adhere for 2.5hrs and stained for phalloidin (red) and DAPI, before being visualised by fluorescence microscopy.

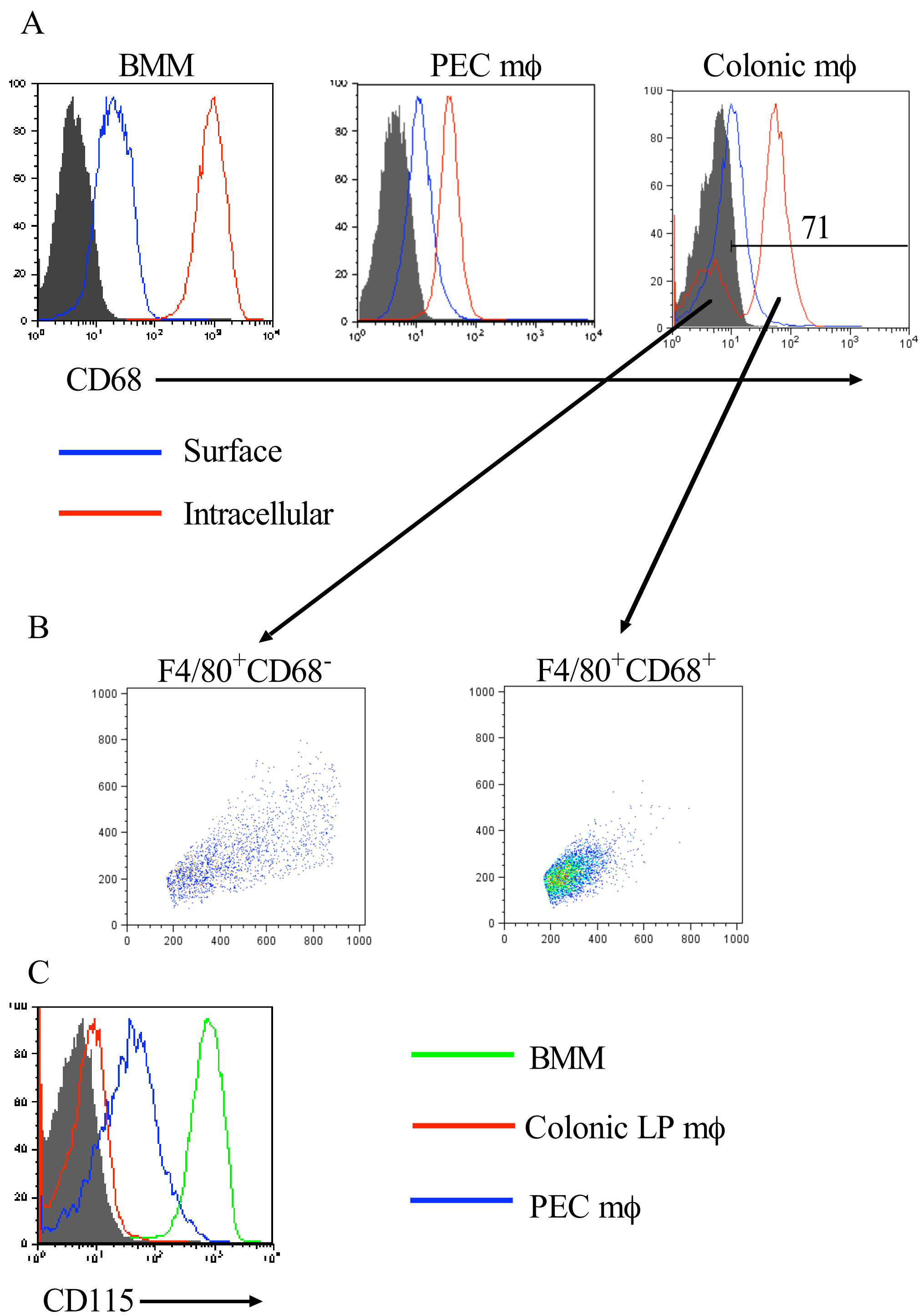


Figure 3.7 Expression of myeloid markers by different macrophage populations

Live-gated F4/80$^+$ BMM, PEC mϕ and colonic mϕ from C57Bl/6 mice were assessed for the expression of surface (blue) and cytoplasmic (red) CD68 (A). The FSC/SSC properties of colonic F4/80$^+$CD68$^-$ and F4/80$^+$CD68$^+$ cells are shown in B. C) Surface expression of CD115 by live-gated F4/80$^+$ BMM, PEC mϕ and colonic mϕ. Histograms are gated on live F4/80$^+$ cells and filled histograms represent staining with isotype controls.

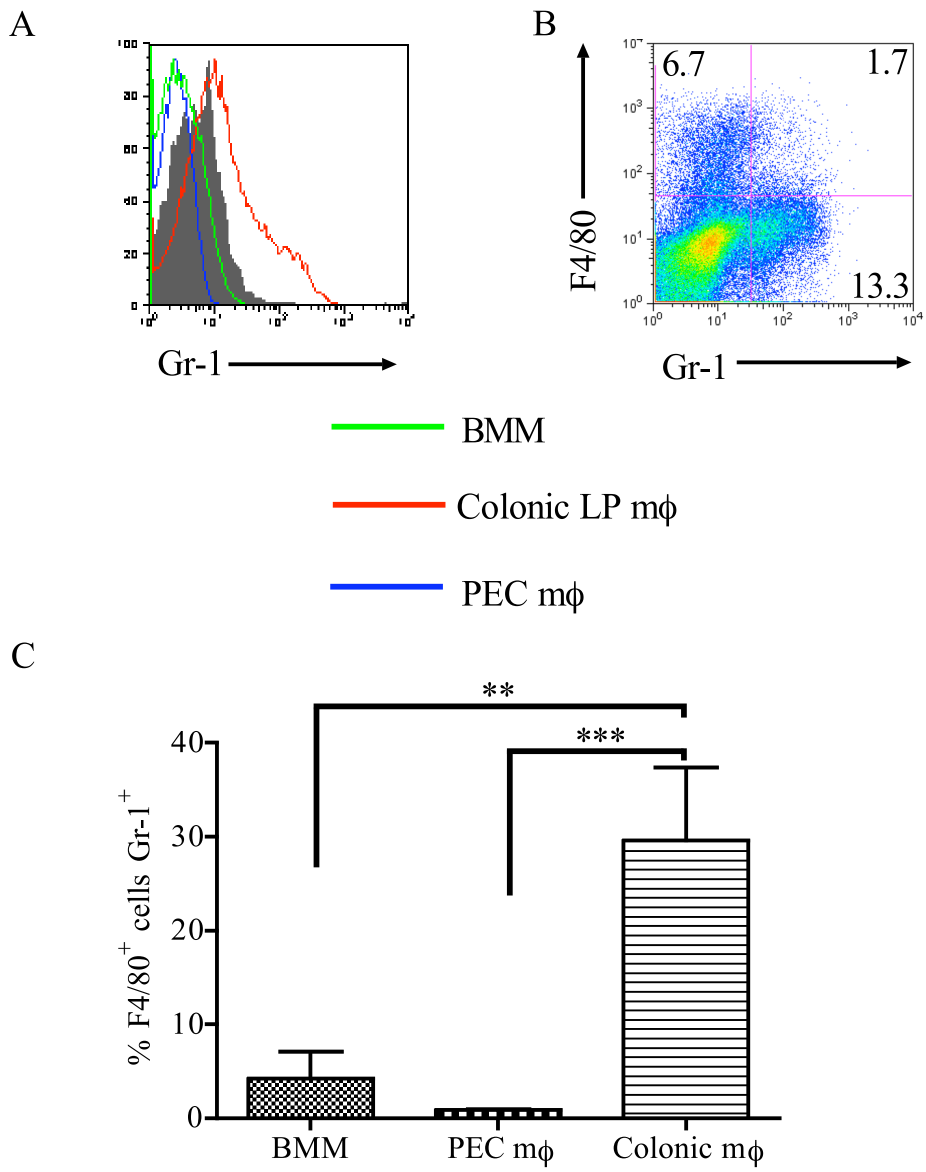


Figure 3.8 Expression of Gr-1 by different macrophage populations

Live-gated F4/80$^+$ BMM, PEC and colonic LP cells from C57Bl/6 mice were analysed for the expression of F4/80 and Gr-1 by flow cytometry. A) The histogram shows the level of Gr-1 expression on live-gated F4/80$^+$ cells and the shaded histogram represents staining with the isotype control. B) Representative dot plot of colonic LP cells stained for F4/80 and Gr-1 expression. Numbers in quadrants represent percentages of the total population. C) Results shown are the mean percentage ± SD of F4/80$^+$ cells positive for Gr-1 expression for 3 mice/group. A one-way ANOVA revealed a significant difference, $F(2,6) = 32.38$, $p<0.001$. Planned comparisons which were significant showed that a higher proportion of colonic mϕ expressed Gr-1 compared with BMM (**$p<0.01$) and PEC mϕ (***$p<0.001$), Bonferroni's multiple comparison test.

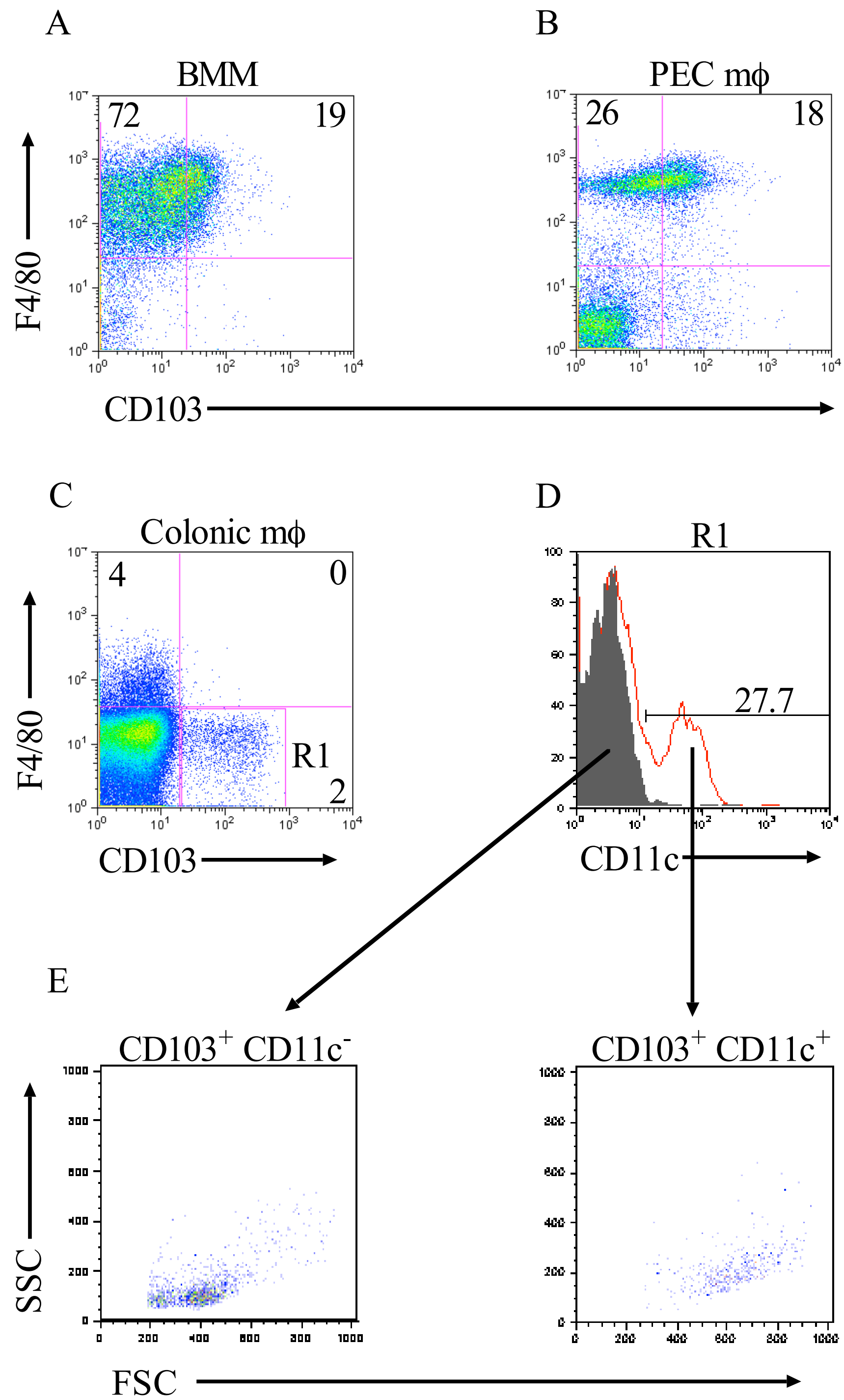


Figure 3.9 Expression of CD103 by different macrophage populations

Live-gated BMM (A), PEC mϕ (B) and colonic mϕ (C) were assessed for the expression of CD103 by flow cytometry. Numbers in quadrants represent percentages of the total population. D) Expression of CD11c by colonic CD103$^+$ cells and FSC/SSC properties of CD103$^+$CD11c$^-$ and CD103$^+$CD11c$^+$ cells (E). The shaded histogram represents staining with the isotype control antibody.

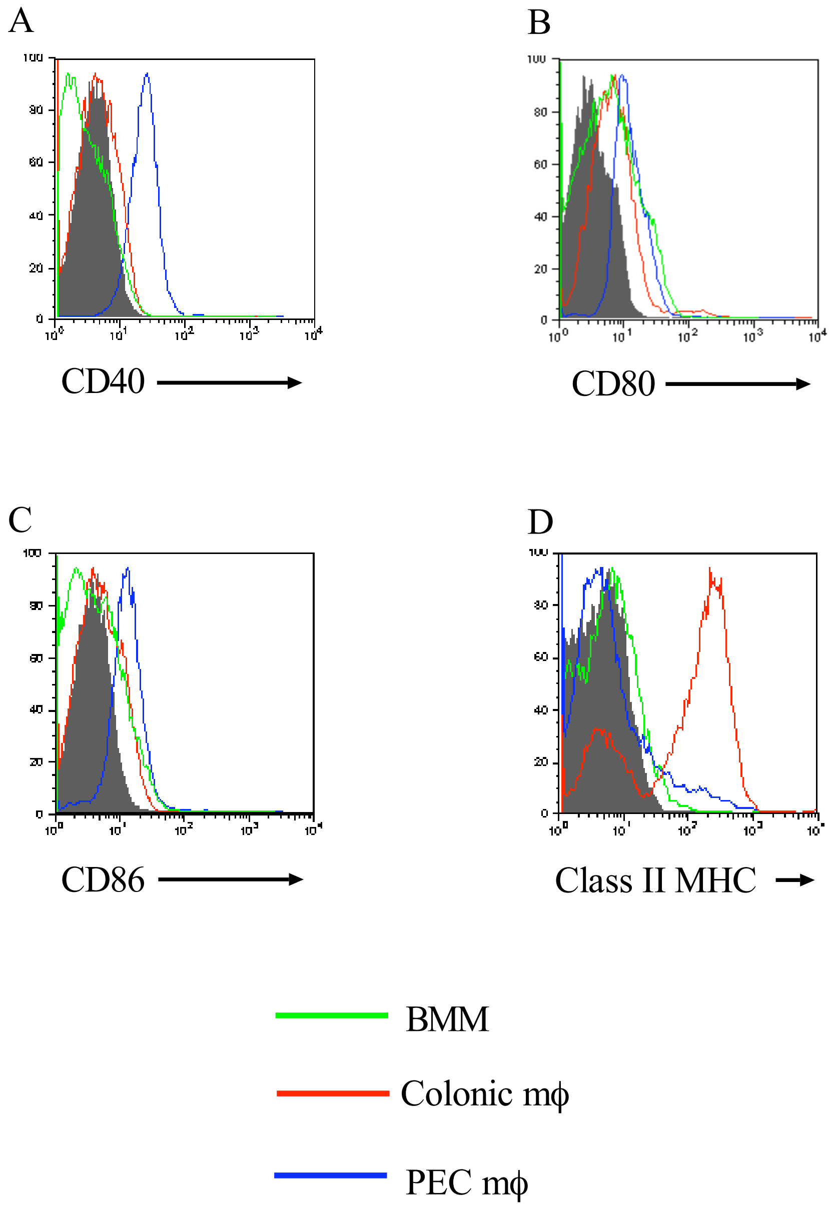


Figure 3.10 Expression of class II MHC and co-stimulatory molecules by resting mϕ populations

BMM, PEC mϕ and colonic mϕ pooled from 3 C57Bl/6 mice were assessed for the expression of CD40 (A), CD80 (B), CD86 (C) and class II MHC (D) by flow cytometry. The histograms show the expression of each marker by live-gated F4/80$^+$ cells, with the filled histograms representing staining with isotype control antibodies.

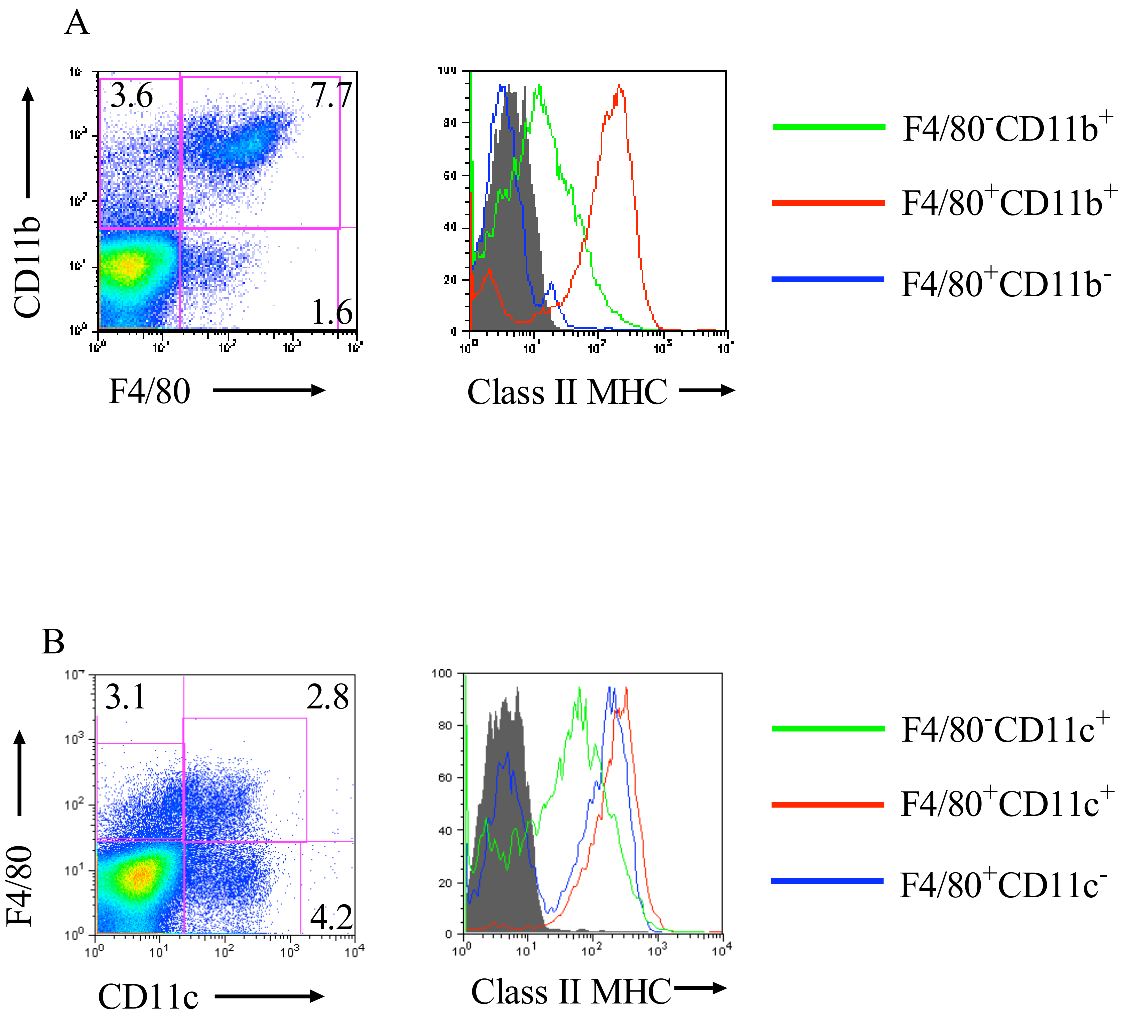


Figure 3.11 Expression of class II MHC by colonic mϕ subsets

Colonic LP cells pooled from 3 C57Bl/6 mice were analysed for the expression of F4/80, class II MHC, and CD11b (A) or CD11c (B). Numbers in quadrants represent percentages of the total population. Histograms show the levels of class II MHC expression by live-gated subsets, with the shaded histograms representing staining with the isotype control antibody. Data are representative of at least two individual experiments.

Subset	**F4/80$^+$CD11b$^+$CD11c^{int}**	**F4/80$^+$CD11b$^+$CD11c$^-$**	**F4/80$^+$CD11b$^-$CD11c$^-$**
% of total F4/80$^+$ population	35.4±10.5	39.7±6.9	22.4±8.5
Class II MHC expression	+++	++	**-**
What these cells may represent	Mϕ/DC intermediate	Conventional mϕ	?

Figure 3.12 F4/80$^+$ subsets in the resting colonic LP

A summary of the F4/80$^+$ subsets present in the resting colon and their relative contribution to the total F4/80$^+$ population.

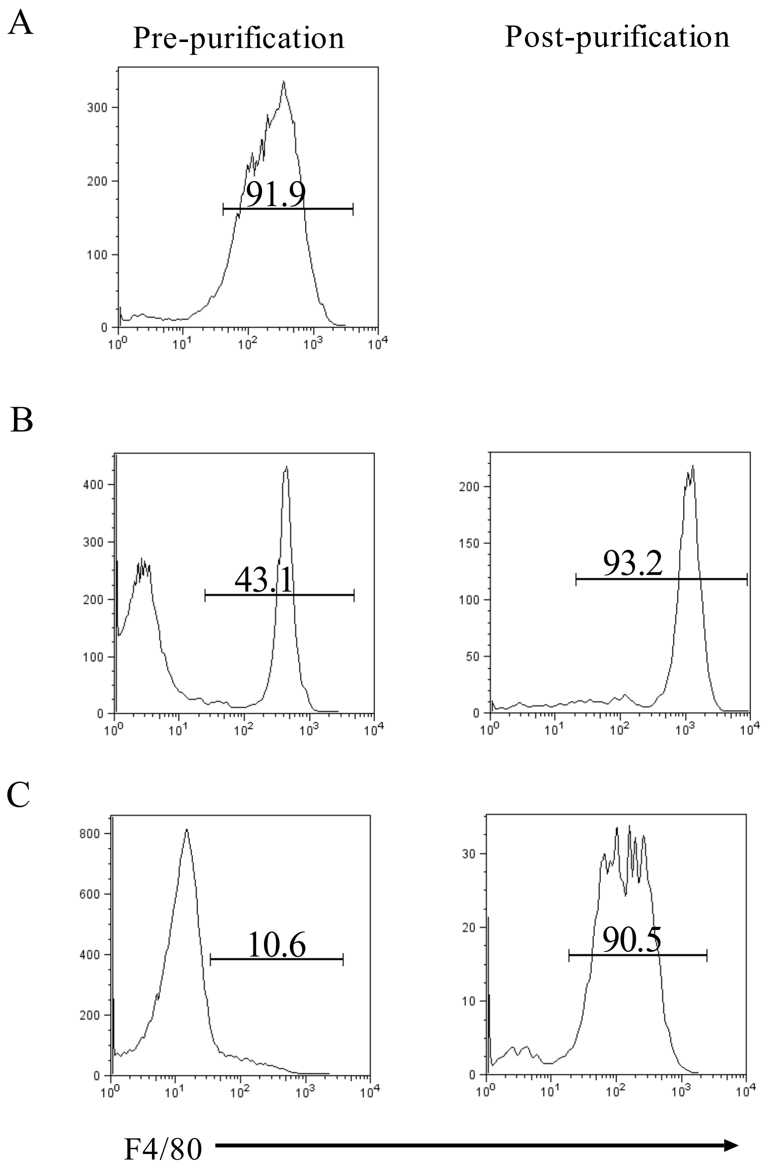


Figure 3.13 Purification of macrophage populations by positive selection

Following isolation, PEC and colonic LP cells were positively selected for F4/80$^+$ cells by MACS or FACS-based purification (see figure legends). The purity of fresh BMM (A), or PEC mϕ (B) and colonic mϕ (C) was assessed before and after purification. Histograms show the percentage of F4/80$^+$ cells among total live-gated cells.

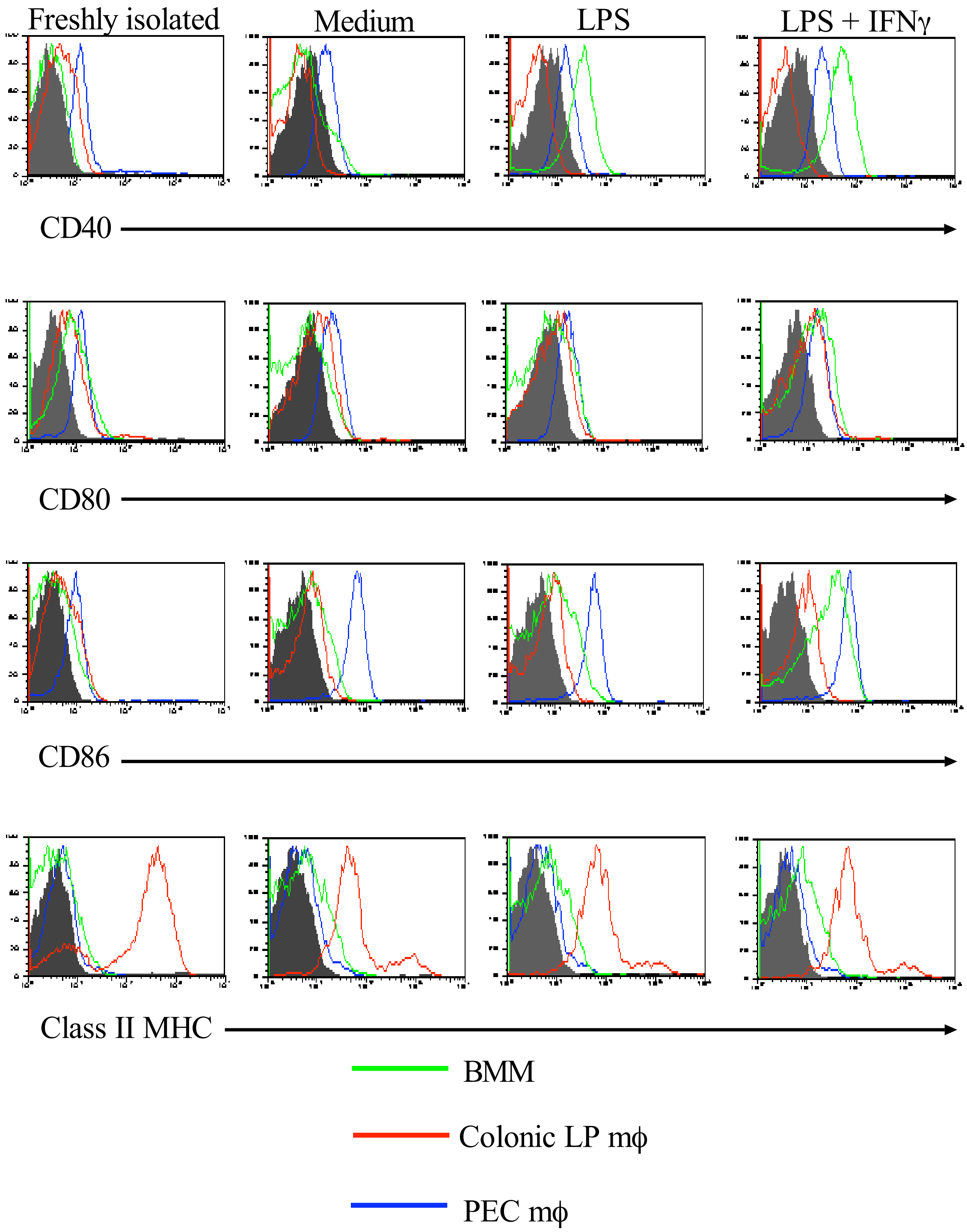


Figure 3.14 Expression of co-stimulatory molecules and class II MHC by different macrophage populations after stimulation

BMM, MACS-purified F4/80$^+$ PEC mϕ and FACS-purified F4/80$^+$ colonic mϕ were cultured in medium, 1µg/ml LPS or LPS + IFNγ for 8hrs and assessed for the expression of CD40, CD80, CD86 and class II MHC by flow cytometry. Histograms show marker expression by live-gated F4/80$^+$ cells and shaded histograms represent staining with isotype control antibodies.

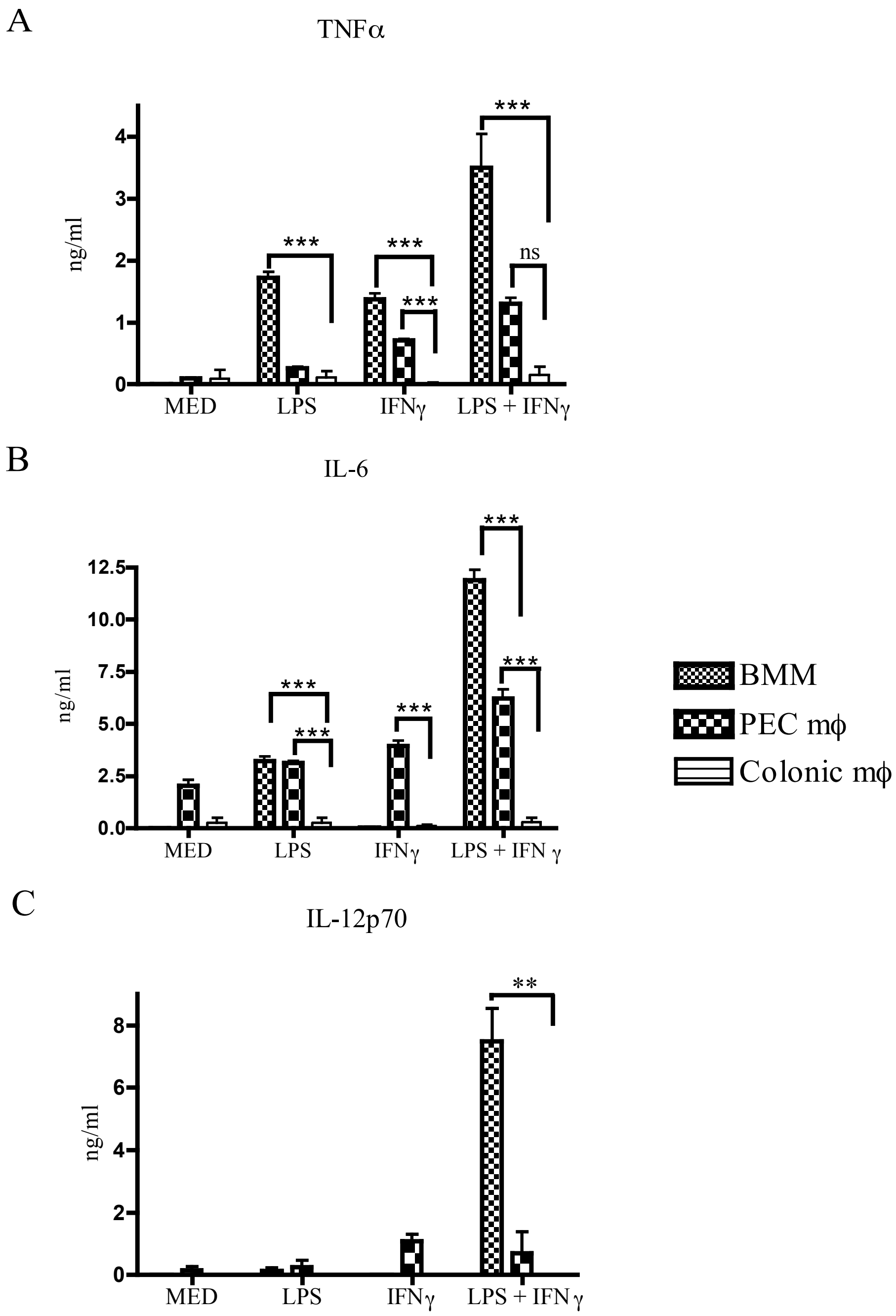


Figure 3.15 Production of pro-inflammatory cytokines by macrophages

BMM and MACS-purified F4/80$^+$ mϕ from PEC and colon were cultured for 20hrs in medium, 1μg/ml LPS, 100U/ml IFNγ or LPS and IFNγ, and TNFα (A), IL-6 (B) and IL-12p70 (C) production was assessed by ELISA. Results are shown as the mean cytokine concentration ± SD for 3 replicates/group. One-way ANOVAs to compare cells within each condition revealed significant differences, Bonferroni's multiple comparison test. ns=not significant; **$p<0.01$; ***$p<0.001$.

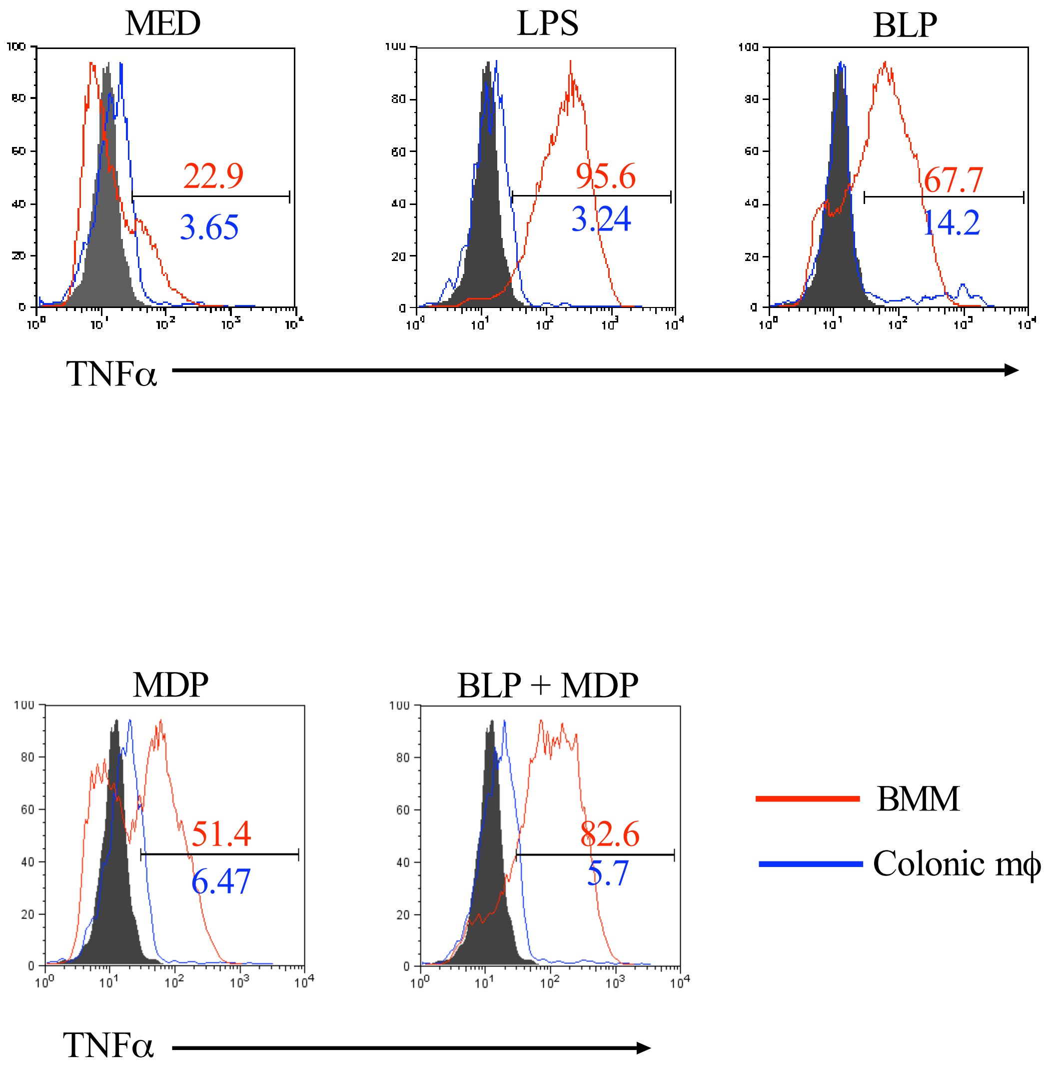


Figure 3.16 Production of TNFα by BMM and colonic mφ in response to LPS, BLP and MDP

BMM and colonic LP cells were cultured in medium, 1μg/ml LPS, 1μg/ml BLP, 10μg/ml MDP or BLP + MDP together for 4.5hrs in the presence of Brefeldin-A and intracellular TNFα expression was assessed by flow cytometry. Histograms are gated on live F4/80$^+$ cells and the shaded histograms represent staining with isotype control antibody. The numbers represent the percentages of F4/80$^+$ cells that were positive for TNFα.

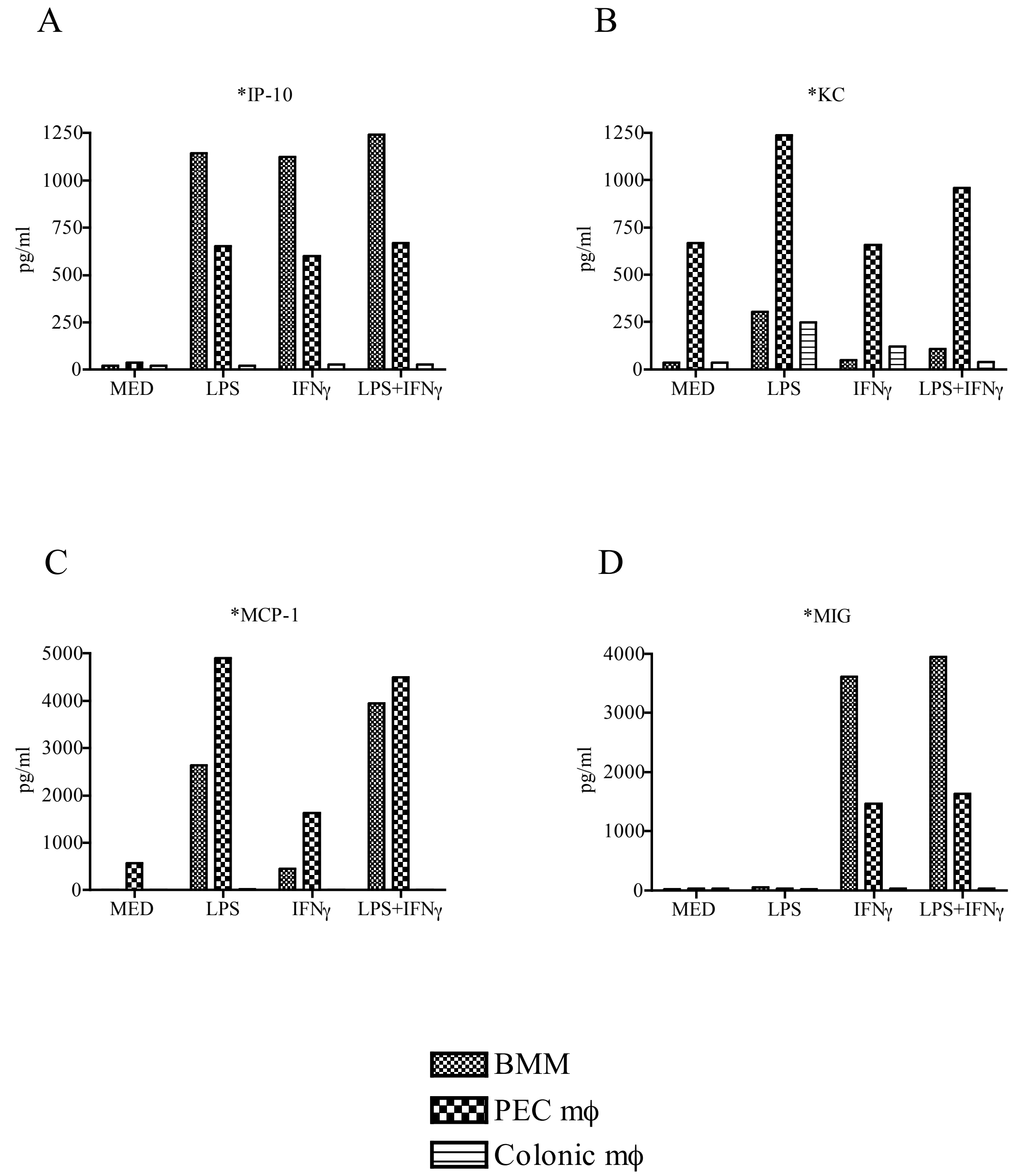


Figure 3.17 Production of pro-inflammatory chemokines by mϕ populations

BMM and MACS-purified F4/80$^+$ mϕ from PEC or colon were cultured for 20hrs in medium, 1μg/ml LPS, 100U/ml IFNγ or LPS + IFNγ. The production of IP-10 (A), KC (B), MCP-1 (C) and MIG (D) was assessed by Luminex and the results are pg/ml for single cultures.

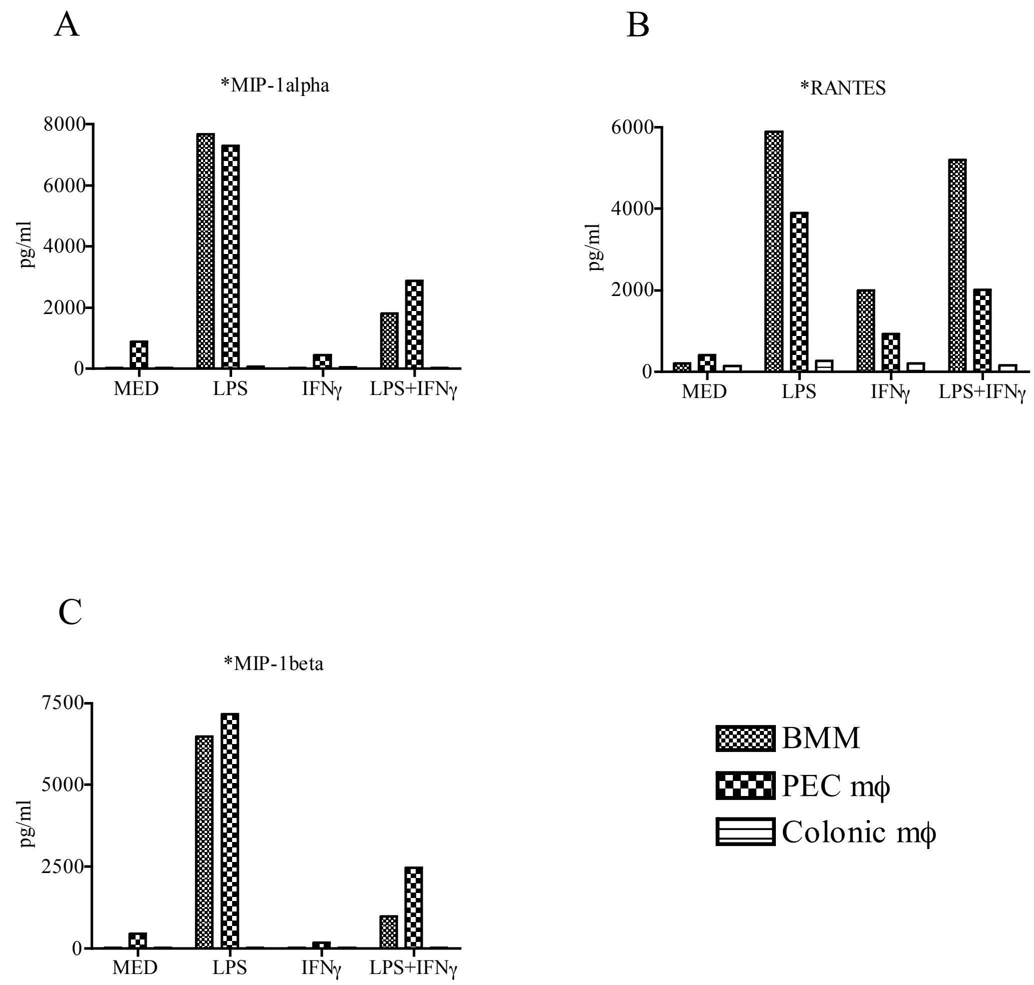
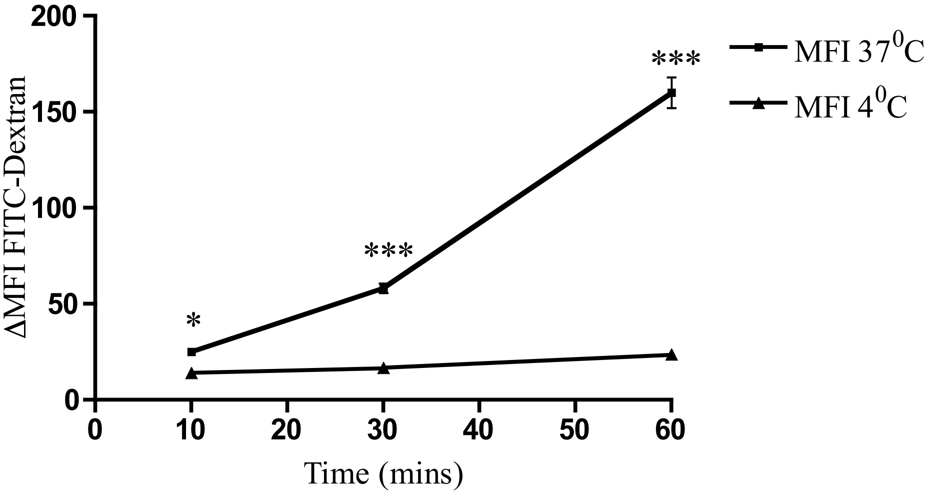


Figure 3.18 Production of pro-inflammatory chemokines by mϕ populations

BMM and MACS-purified F4/80^{+} mϕ from PEC or colon were cultured for 20hrs in medium, 1μg/ml LPS, 100U/ml IFNγ or LPS + IFNγ. The production of MIP-1α (A), RANTES (B) and MIP-1β (C) was assessed by Luminex and the results are pg/ml for single cultures.

A



B

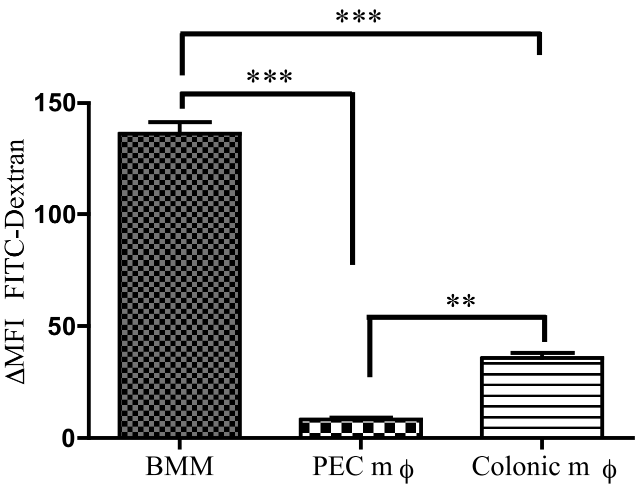


Figure 3.19 Endocytic activity of macrophage populations

A) BMM were assessed for their capacity to endocytose 1mg/ml FITC dextran (Mol. Wt 40kD) after culture at 4^0C or 37^0C. Results are shown as the MFI ± SD for FITC at the different points at 4^0C or 37^0C. A two-way ANOVA (repeated measures) revealed a significant difference, $F(2,2)=378.1$, $p<0.0001$. B) BMM, PEC and colonic LP cells were incubated with 1mg/ml FITC-dextran for 60 minutes and the uptake by live-gated F4/80$^+$ cells was assessed by flow cytometry. The results are expressed as the differences in mean fluorescence intensity (ΔMFI) calculated by subtracting the uptake at 4^0C from that at 37^0C for 3 replicates/group. A one-way ANOVA revealed a significant difference, $F(2,6)=458.1$, $p<0.0001$. Planned comparisons which were significant showed that a higher proportion of BMM were FITC$^+$ compared with PEC mϕ and colonic mϕ (***p<0.01), and colonic mϕ compared with PEC mϕ (**p<0.01), Bonferroni's multiple comparison test.

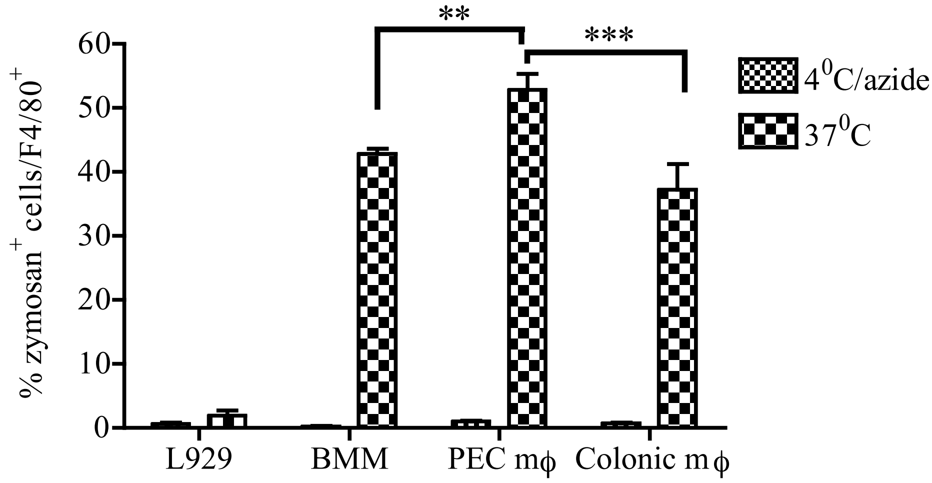


Figure 3.20 Phagocytosis activity of macrophage populations

L929 cells, BMM, PEC and colon LP cells were cultured for 1.5hr with FITC-labelled, zymosan-coated beads at 37^0C or 4^0C in azide as a control and enumerated by flow cytometry. Results shown are the mean percentage ± SD of live-gated F4/80$^+$ cells positive for FITC for 3 replicates/group. Results are representative of two individual experiments. A one-way ANOVA revealed a significant difference, $F(3,8) = 249.1$, $p<0.0001$. Planned comparisons which were significant showed that a higher proportion of PEC mϕ were FITC$^+$ compared with BMM (**$p<0.01$) and colonic mϕ (***$p<0.001$), Bonferroni's multiple comparison test.

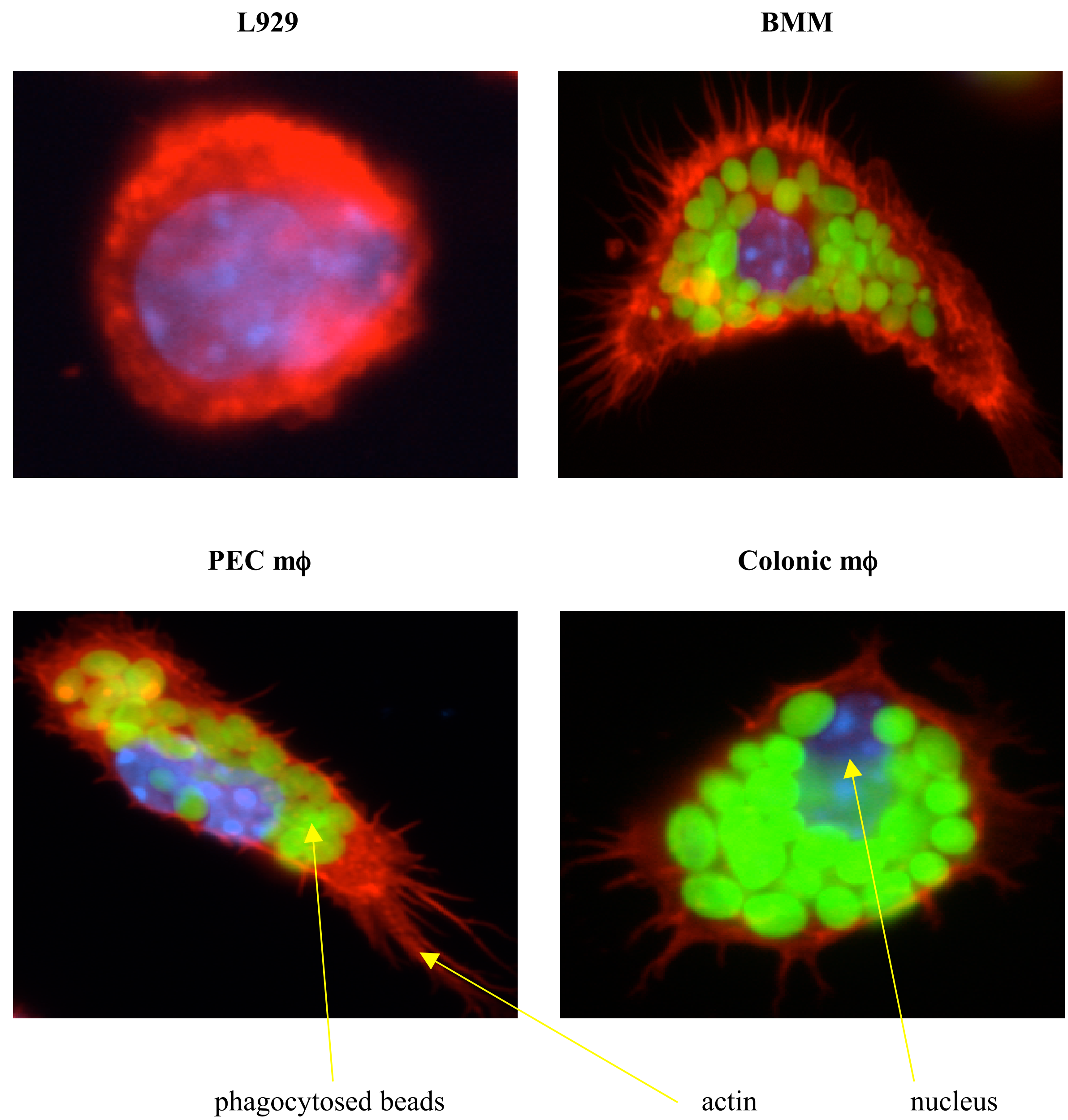


Figure 3.21 Phagocytic activity of macrophage populations

L929 cells, BMM, adherance-separated PEC mϕ and FACS-purified F4/80^{+} colonic mϕ were assessed for their capacity to phagocytose FITC-labelled, zymosan-coated beads (green). Cells were incubated with beads for 1.5hr, washed, and stained for phalloidin (red) and DAPI (blue) using fluorescence microscopy. Results shown are representative images of the four cell populations.

Chapter 4

Mechanisms of TLR hyporesponsiveness in colonic macrophages

Introduction

The experiments in Chapter 3 showed that colonic mϕ exhibit a profound refractoriness to stimulation and one way this could occur is via modulation of PRR expression. In humans, mϕ from resting intestine have been shown to lack expression of TLR1-5 mRNA, but there are also contradictory reports suggesting that these cells can express TLR mRNA. In addition, similar analysis has not been carried out in the murine system and it is unclear at what level expression of TLR may be modulated. Thus to try and understand the cause of the hyporesponsiveness of resident colonic mϕ, in this Chapter I assessed the expression of TLR protein compared with mϕ from other tissues in the body, and tried to determine at what level this was regulated in colonic mϕ. In addition, I have explored whether intestinal mϕ arrive in the mucosa as fully responsive cells which are subsequently conditioned to become refractory by the local microenvironment, and have investigated some of the possible factors involved.

4.1 Expression of surface and intracellular TLR proteins by colonic macrophages

To examine the cause of the hyporesponsiveness of intestinal mϕ, I first carried out a detailed study of the expression of a number of TLR proteins using BMM and PEC mϕ as examples of mϕ from other sites. 93.0±1.2% of BMM and 98.6±1.4% of PEC mϕ expressed surface TLR2, but in stark contrast, only 29.1±5.1% of colonic mϕ expressed TLR2 (Fig 4.1a). Similarly, 94.1±4.8% of BMM and 95.8±1.4% of PEC mϕ expressed surface TLR4, but only 3.4±0.9% of colonic mϕ were positive for TLR4 (Fig 4.1c). As expected from their predominantly intracellular expression, all three mϕ populations failed to express TLR3 or TLR9 on the cell surface (Fig 4.1b+d), with the exception of a small number of colonic mϕ that expressed surface TLR3 (Fig 4.1b).

I next assessed the expression of intracellular TLR3 and TLR9 after cell permeabilisation and found that 94.4±2.4% of BMM and 97.3±1.2% of PEC mϕ expressed TLR3, while 94.2±5.9% of BMM and 97.7±1.8% of PEC mϕ expressed TLR9 intracellularly (Fig 4.2a+b). In contrast, virtually no colonic mϕ were positive for either TLR3 or 9 (0.3±0.4% and 0.8±0.7%, respectively; Fig 4.2a+b). I also examined whether TLR2 and TLR4 could be detected intracellularly in colonic mϕ, as this could indicate there was active internalisation of the receptors. However, the proportions of colonic mϕ expressing TLR2 or TLR4 after permeabilisation were identical to those found when surface TLR was examined, suggesting there had not been significant internalisation of these proteins (Fig 4.2c+d). As expected, virtually all BMM and PEC mϕ were positive for

TLR2 and TLR4 after permeabilisation, but it was not possible to determine whether this was surface or intracellular (data not shown).

One trivial explanation for the apparent failure of colonic mφ to express TLR could be the enzymatic digestion protocol used to obtain colonic mφ. To investigate this, I treated PEC mφ in exactly the same way as colonic mφ before examining them for the expression of F4/80, CD11b, CD11c, TLR and class II MHC by flow cytometry. Enzyme treatment did not affect the expression of any of the myeloid markers by PEC mφ (Fig 4.3a), nor did it affect the expression of surface TLR2, TLR4 or class II MHC (Fig 4.3b+c). Thus, the unusual phenotype of colonic mφ appears to be an inherent property of these cells and is not due to the extraction protocol.

4.2 Expression of TLR2 by phenotypic subsets of colonic macrophages

As I had found there were different phenotypic subsets of F4/80$^+$ cells, I thought it important to investigate whether the expression of TLR differed between the subsets I had identified on the basis of F4/80, CD11b and CD11c expression. Due to the fact that a proportion of colonic mφ retained expression of TLR2, I analysed its expression by different subsets. As shown in Fig 4.4a, ~57% of the cells with the F4/80$^+$CD11b$^+$ phenotype typical of classical mφ expressed TLR2 at intermediate levels. A higher proportion of the F4/80$^+$CD11c^{int} cells expressed TLR2 (~73%), whereas the F4/80$^+$CD11c$^-$ cells showed biphasic expression of TLR2 (Fig 4.4b). Only 23% of the F4/80$^-$CD11c$^+$ cells with the phenotype of classical DC, and ~20% of CD11b$^+$ F4/80$^-$ cells expressed TLR2 (Fig

4.4a+b. In contrast, almost none (5%) of the F4/80$^+$CD11b$^-$ cells expressed TLR2 (Fig 4.4a).

To investigate how TLR expression might correlate with maturation status of the cells, I examined the presence of class II MHC on TLR2$^+$ and TLR2$^-$ subsets, reasoning that immature mϕ would express class II MHC at low levels. Interestingly, almost all (>93%) TLR2-expressing mϕ in the resting colon expressed high levels of class II MHC, whereas only ~38% of TLR2$^-$ mϕ expressed class II MHC (Fig 4.4c).

4.3 Expression of mRNA for TLRs by colonic macrophages

Given the low TLR protein expression by colonic mϕ, I sought to investigate the level at which TLR protein expression is regulated. Therefore, I first performed end-product PCR analysis to assess if colonic mϕ expressed mRNA for TLR1-9. BMM and PEC mϕ expressed mRNA for all these TLRs, although TLR5 expression appeared low, especially in PEC mϕ (Fig 4.5). Colonic mϕ showed a similar pattern of TLR expression, with the possible exception of TLR7, which was virtually absent (Fig 4.5). All three mϕ populations appeared not to express mRNA for TLR8, but I could not draw conclusions from this analysis, as I was unable to obtain a positive control for this TLR, despite using a variety of different cell populations and three different TLR8 primer pairs. Overall, these results demonstrated that colonic mϕ expressed mRNA for most TLRs, and I went on to explore this further by quantitative PCR analysis using Taqman.

I used this approach to assess the levels of TLR2, 4 and 9 as representative surface and intracellular TLRs. BMM expressed high levels of mRNA for all these TLRs, and PEC mϕ also expressed mRNA for these receptors, but at lower levels (Fig 4.6). In contrast, colonic mϕ expressed virtually no TLR2 or TLR4 mRNA, but expressed high levels of TLR9 mRNA, similar to those found in BMM. Thus TLR expression appears to be regulated at the transcriptional level for TLR2 and TLR4, but at a post-transcriptional level for TLR9.

4.4 Irreversible downregulation of TLR expression by colonic macrophages

As discussed earlier, intestinal mϕ are thought to arise from the monocyte pool which normally expresses a wide range of TLRs, suggesting that downregulation of these receptors might occur following their arrival in the gut microenvironment. To assess whether constant exposure to immunomodulatory factors in the local microenvironment is necessary for maintenance of this altered phenotype, F4/80$^+$ colonic mϕ were FACS-sorted, cultured overnight in medium and examined for TLR expression. As shown in Fig 4.7a, there was no upregulation of TLR2 expression on colonic mϕ after overnight culture, with only a small proportion (~11%) being TLR2 positive in these experiments. Similarly, colonic mϕ failed to re-express TLR4 following 24hr culture in medium (Fig 4.7b). Thus TLR expression cannot be re-induced on colonic mϕ simply by short-term withdrawal from the local environment of the gut.

4.5 Effects of TLR ligands on TLR expression and responsiveness by macrophages

Given the large numbers of commensal bacteria located in the colon which express numerous TLR ligands, I hypothesised that constant ligation of TLRs by these materials might result in downregulation of the cognate or non-cognate receptors. This process has been described in the phenomenon known as endotoxin tolerance, where LPS-treated cells are refractory to subsequent stimulation with LPS (102). To try and replicate these conditions *in vitro*, BMM were cultured overnight with various TLR ligands and then assessed for the expression of TLR2, 3, 4 and 9 by flow cytometry. As shown in Figs 4.8 and 4.9, treatment with BLP, poly I:C, LPS or CpG had no effect on the expression of surface TLR2 or intracellular TLR3 and 9 compared with medium-treated BMM. Similarly, treatment with BLP or CpG did not affect surface TLR4 expression by BMM (Fig 4.8b). However treatment with LPS completely downregulated surface expression of TLR4 on virtually all BMM (Fig 4.8b), consistent with previous data on PEC mϕ treated with LPS. Interestingly, treatment with poly I:C also resulted in a partial downregulation of TLR4 from the surface of BMM, but around 60% of cells remained $TLR4^+$. Thus LPS was the only stimulus that markedly affected expression of any of the TLRs, and this was specific to its corresponding receptor. To examine this profound effect of LPS in more detail, I carried out a dose response study. BMM were cultured with 20ng/ml, 200ng/ml or 2μg/ml of LPS overnight before assessing the level of TLR4 expression. All of the concentrations of LPS completely downregulated surface TLR4 expression (Fig 4.10a). To examine whether the downregulation from the cell surface was due to receptor internalisation, I assessed intracellular TLR4 expression after treatment with 2μg/ml LPS. In contrast to the complete absence of TLR4 when surface expression was examined, when the intracellular staining protocol was used, intermediate levels of TLR4 could be detected intracellularly following treatment with LPS, suggesting that TLR4 may indeed be internalised after ligand treatment (Fig 4.10b).

Next I explored at what level TLR expression might be regulated by TLR ligation, by carrying out quantitative PCR to determine mRNA levels in BMM treated overnight with medium, BLP or LPS. I used these stimuli as BLP failed to affect TLR protein expression, whereas LPS downregulated TLR4 protein expression. Culture of BMM in medium alone increased the expression of mRNA for TLR2, 4 and 9 compared with freshly harvested BMM (Fig 4.11). Treatment of BMM with BLP or LPS abrogated this increase in TLR4 mRNA expression, but had no effect on the induction of TLR2 mRNA (Fig 4.11a+b). BLP also reduced TLR9 mRNA levels to below the baseline found in freshly harvested BMM, but LPS had no effect on TLR9 mRNA expression after overnight culture (Fig 4.11c). Thus BLP specifically targeted the transcription of TLR9 mRNA, rather than at the protein level where TLR9 may be more stable, suggesting different TLR ligands might regulate TLR expression at distinct levels. In conclusion, LPS has a selective effect on the expression of its own TLR protein and mRNA, while BLP appears to have somewhat more wide ranging effects, especially at the mRNA level. However none of the TLR ligands themselves can reproduce the global downregulation of TLRs seen in resident colonic mϕ.

Finally, I thought it important to explore the functional consequences of prior exposure to TLR ligands on the responsiveness of BMM to subsequent stimulation with the same or different TLR ligands. Thus BMM were cultured for 20hrs in medium alone, or with BLP, poly I:C or LPS, washed and re-stimulated with the same or different TLR ligands. All three stimuli induced TNFα and IL-6 production by freshly isolated BMM after the initial overnight culture, with the weakest stimulus being BLP (Fig 4.12). Interestingly, overnight culture in medium alone led to significantly increased subsequent cytokine responses to all stimuli, which corresponds with the increase in TLR mRNA levels I found in BMM cultured overnight in medium. In addition, consistent with the dramatic reduction in TLR4 protein expression found under the same conditions, BMM pre-treated with LPS and re-stimulated with the same ligand failed to produce any TNFα and IL-6 at all, despite the higher levels seen after culture in medium (Fig 4.12). Although BLP had failed to affect the expression of TLR2 protein, BMM cultured with BLP produced significantly lower levels of TNFα and IL-6 upon subsequent BLP stimulation compared with control BMM cultured in medium overnight (Fig 4.12). Similarly, poly I:C treatment abrogated subsequent TLR3-mediated stimulation, even though it did not affect TLR3 protein expression (Fig 4.12). Thus all three ligands profoundly inhibit subsequent responsiveness via their own TLR.

To investigate whether these effects extended to responsiveness to other ligands, I also examined cytokine production by pre-treated BMM after re-stimulation with different TLR ligands. As shown in Fig 4.13, BMM cultured initially with poly I:C or LPS failed to produce TNFα or IL-6 after re-stimulation with BLP. BMM treated with BLP also showed a significantly reduced TNFα response upon re-stimulation with poly I:C, but IL-6 responses were normal. However, poly I:C-induced TNFα and IL-6 production by BMM was completely abrogated by pre-treatment with LPS. Pre-treatment with BLP reduced the subsequent TNFα response to LPS, but did not affect the production of IL-6 significantly. In contrast, pre-treatment of BMM with poly I:C completely abrogated the subsequent LPS-induced production of both TNFα and IL-6. These data demonstrate that treatment of BMM with poly I:C or LPS prevents subsequent TNFα and IL-6 responses to the other ligands. However, BLP has a similar, if somewhat lesser effect, as culture with it only decreased the production of TNFα, not IL-6, in response to subsequent stimulation with poly I:C or LPS.

4.6 Effects of *in vivo* TLR2 and TLR4 signalling on TLR expression by colonic macrophages

The data thus far indicated that ligation of individual TLR *in vivo* may not provide the explanation for the global downregulation of TLRs observed in colonic mϕ. To examine this *in vivo*, the expression of TLR3, 4 and 9 by colonic mϕ from TLR2- and TLR4-unresponsive mice was examined. As shown in Fig 4.14a-c, colonic mϕ from both WT and TLR2KO animals expressed no surface TLR4 or 9 and had similarly low levels of surface TLR3. TLR2 KO mϕ also expressed the same low levels of intracellular TLR3 and TLR9 as found in WT mϕ and appeared to express even lower levels of intracellular TLR4 than WT controls.

I next examined the expression of TLRs by colonic mϕ from C3H/HeJ mice, which have a point mutation in the cytoplasmic tail of TLR4, and thus cannot respond to TLR4 ligation (76). This is confirmed by the fact that BMM from C3H/HeJ mice produced TNFα after stimulation with BLP and poly I:C, but not with LPS (Fig 4.15). Although no C3H/HeN mice were available as WT controls, the expression of surface TLR2 and 4, and intracellular TLR3 and 9 by C3H/HeJ colonic mϕ were at the same low levels as I normally found on control C57Bl/6 mϕ (Fig 4.16). Together these results indicate that TLR2 or TLR4 signalling *in vivo* is not required for the lack of TLR expression by colonic mϕ.

4.7 Effects of candidate immunomodulatory factors on TLR expression by macrophages

As TLR signalling itself does not seem to account for the lack of TLR expression by colonic mϕ, I went on to explore some of the factors which could be present in the intestinal microenvironment and have the potential to downregulate pro-inflammatory functions by mϕ. The first mediator I investigated was vasoactive intestinal peptide (VIP), which has been shown to modulate DC function and downregulate TLR4 expression by murine mϕ (217, 218). To confirm these previous findings on TLR4 expression and to assess whether VIP affected expression of other TLRs by mϕ, BMM were cultured overnight with a dose of VIP others in the laboratory had used to treat DCs. However this did not affect the surface expression of TLR2 or TLR4 (Fig 4.17a). I also included IL-4 in this experiment, as IL-4 has been shown to decrease the expression of TLR in human IEC (325). However IL-4 had no effects on TLR expression by BMM (Fig 4.17b).

As described previously, retinoic acid has been shown to be critical for the ability of intestinal $CD103^+$ DCs to drive $FoxP3^+$ regulatory T cells and to impart T cells with gut-homing properties (30, 211). Retinoic acid also downregulates TLR2 expression in human monocytes (326). For these reasons, I cultured BMM with retinoic acid at the same dose as found to be optimal for driving T cells to express FoxP3, for 7 or 24hrs to assess any short and longer term effects, and examined the expression of TLR2, 3, 4 and 9. As shown in Fig 4.18, retinoic acid had no effect on the expression of surface TLR2 or TLR4, or intracellular TLR3 or TLR9 after 7hrs (left panel) or 24hrs (right panel). Together these preliminary results suggest that VIP, IL-4 and retinoic acid may not contribute to the phenotype of colonic mϕ, but time did not permit me to perform more detailed experiments using different concentrations, combinations and time courses to confirm these conclusions.

4.8 Effects of IL-10 on TLR expression and function by colonic macrophages *in vivo*

I decided to concentrate the remainder of these studies on IL-10. IL-10 is a potent immunomodulatory cytokine that downregulates pro-inflammatory mϕ functions. IL-10KO mice develop colitis spontaneously (232), as do mice with a myeloid-specific defect in the IL-10R signalling molecule, STAT-3 (233). As intestinal mϕ from IL-10KO mice have been shown to produce increased IL-12p70 following stimulation with whole bacteria (195), I assessed whether intestinal mϕ from these mice were hyperresponsive to purified TLR ligands and if this heightened responsiveness was associated with an increased expression of TLRs. I examined the expression of TLRs by colonic mϕ from 2 and 4 month-old IL-10KO mice, first assessing whether either of these groups had colitis. Neither group of IL-10KO mice had blood in their faeces as assessed by the Haemoccult test. Furthermore, 2 month-old IL-10KO mice also had no histological signs of colitis, but one of the two 4 month-old KO mice examined had early signs of inflammatory cell infiltrates in the colon (Fig 4.19). For these reasons, I used 2 month-old mice as a pre-colitic group and 4 month old mice as animals with early signs of inflammation.

The total number of mϕ in the colon was not significantly different between KO and WT animals of either age (Fig 4.20a-c) and there were also no differences in the proportions of F4/80$^+$ subsets between KO and WT animals (Fig 4.21a). It should be noted that in this experiment, the F4/80$^+$CD11b$^+$CD11c^{int} subset constituted a lower proportion of the F4/80$^+$ fraction compared with the C57Bl/6 mice described in Chapter 3, producing concomitant increases in the other two subsets. There was also no difference in the proportion of Gr-1-expressing F4/80$^+$ cells between KO and WT mice (Fig 4.21b).

As shown in Fig 4.22a, significantly higher percentages of colonic mϕ from 2 and 4 month-old IL-10KO mice expressed surface TLR2 (70.7±3.7% and 61.1±1.8% compared with 26.2±11.2% and 30.1±5.8% in WT controls, respectively). Similarly, a significantly higher proportion of colonic mϕ from 2 and 4 month-old IL-10KO mice expressed surface TLR4 compared with WT controls (51.2±8.2% and 42.5±11.2% compared with 15.2±3.3% and 12.1±1.9%, respectively; Fig 4.22b). There were no differences in the expression of intracellular TLR3 or TLR9 between IL-10KO and WT mice with virtually no expression in any group (Fig 4.22c+d).

As shown in Fig 4.23a, the increased expression of TLR2 and TLR4 correlated with increased responsiveness of colonic mϕ from IL-10KO mice to stimulation with LPS. In contrast to the experiments described in Chapter 3 using C57Bl/6 mice, a small proportion (~15%) of colonic mϕ from Balb/c WT mice in this experiment produced TNFα spontaneously, but this was not increased further by TLR stimulation (Fig 4.23b+c). In contrast, significantly higher proportions of colonic mϕ from pre-colitic IL-10KO mice produced TNFα spontaneously (28.0±1.0%) and this was increased even further by stimulation with BLP or LPS (44.4±4.3% and 59.5±5.7%, respectively). An identical pattern of results was seen when colonic mϕ from 4 month-old IL-10KO mice were analysed, with 29.20±2.26% producing TNFα spontaneously, increasing to 39.4±5.4% and 36.3±3.6% after BLP and LPS stimulation, respectively. To try and assess whether enhanced pro-inflammatory cytokine production was an intrinsic property of mϕ from IL-10KO mice, I examined the capacity of BMM from these mice to produce cytokines. Although there was no difference in TNFα or IL-6 production, *in vitro*-differentiated BMM derived from IL-10KO mice displayed a significantly heightened IL-12p70 response to stimulation compared with BMM from WT mice (Fig 4.24).

To examine the activation status of colonic mϕ from IL-10KO mice further, the expression of co-stimulatory molecules and class II MHC was assessed. The levels of these molecules were low, but colonic mϕ from IL-10KO mice expressed lower levels of CD40 and this was significantly different from WT in 4 month-old mice (Fig 4.25a). Colonic mϕ from IL-10KO mice also expressed significantly lower levels of CD80 (Fig 4.25b), but had a small but significant increase in CD86 expression at 4 months (Fig 4.25c). Class II MHC expression was significantly higher in IL-10KO colonic mϕ compared with WT controls at both ages (Fig 4.25d).

Summary

The results of this chapter demonstrate that the hyporesponsiveness of colonic mϕ to TLR ligation is associated with a lack of TLR protein expression. However, colonic mϕ do express mRNA for TLRs, albeit at reduced levels for some TLRs when compared with other mϕ populations and removal of mϕ from the intestinal microenvironment failed to allow expression of TLRs by gut mϕ. However a small proportion of colonic mϕ retained expression of TLR2 in the resting intestine and it is possible that these represent recently recruited monocytes, which have not yet acquired the refractory phenotype which characterises the resident population. Alternatively, these mϕ may represent a distinct subset of monocyte-derived cells, which are present in lower numbers under resting conditions.

Further experiments suggested that continuous TLR ligation is probably not the full explanation for the global defect in TLR expression by colonic mϕ, as exogenous TLR ligands such as BLP, poly I:C, LPS and CpG did not reproduce the phenotype in BMM and colonic mϕ from TLR2KO or C3H/HeJ mice still showed no expression of TLRs. The immunomodulatory mediators IL-4, VIP or retinoic acid did not seem to account for the TLR phenotype of colonic mϕ. However, IL-10 may be involved in the regulation of TLR expression by mϕ in the intestine, as colonic mϕ from IL-10KO mice, which had no signs of inflammation, expressed higher levels of TLR2 and TLR4 compared with WT controls. Importantly, colonic mϕ from IL-10KO animals also displayed a heightened TNFα response to TLR ligation. Whether the role for IL-10 is in the regulation of mϕ function or in the recruitment of monocyte subsets into the intestine, it represents a crucial checkpoint in preventing excessive inflammatory responses to commensal bacteria in the gut.

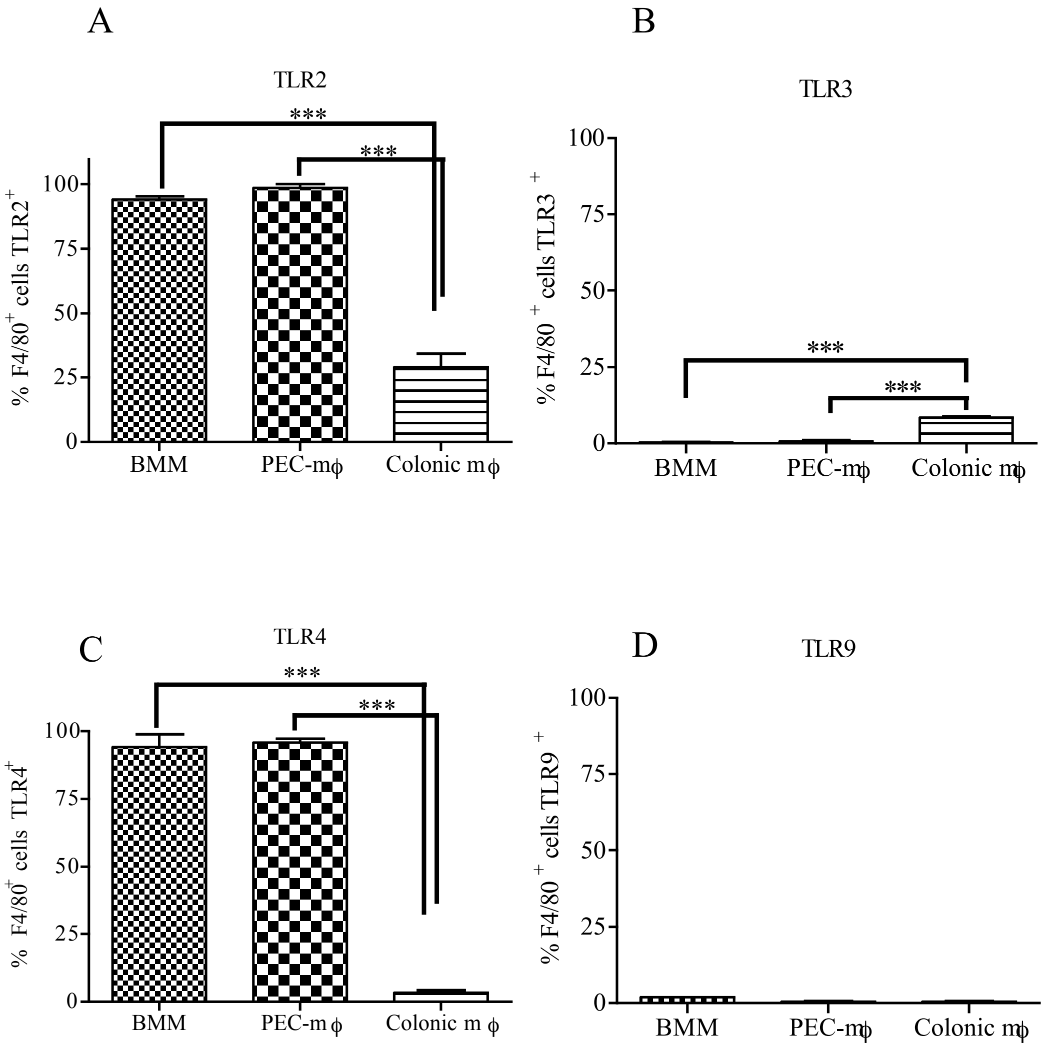


Figure 4.1 Surface TLR protein expression by macrophage populations

BMM, PEC mϕ and colonic mϕ were examined for the expression of surface TLR2 (A), TLR3 (B), TLR4 (C) and TLR9 (D) by flow cytometry. The results shown are the mean percentages ± SD of live-gated F4/80$^+$ cells for 3 mice/group and the results are representative of at least 3 individual experiments. A one-way ANOVA revealed a significant difference; (A) $F(2,6) = 454.9$, $p<0.0001$; (B) $F(2,6)=723.4$, $p<0.0001$; (C) $F(2,6)=952.3$, $p<0.0001$. Planned comparisons were significant (***$p<0.001$), Bonferroni's multiple comparison test.

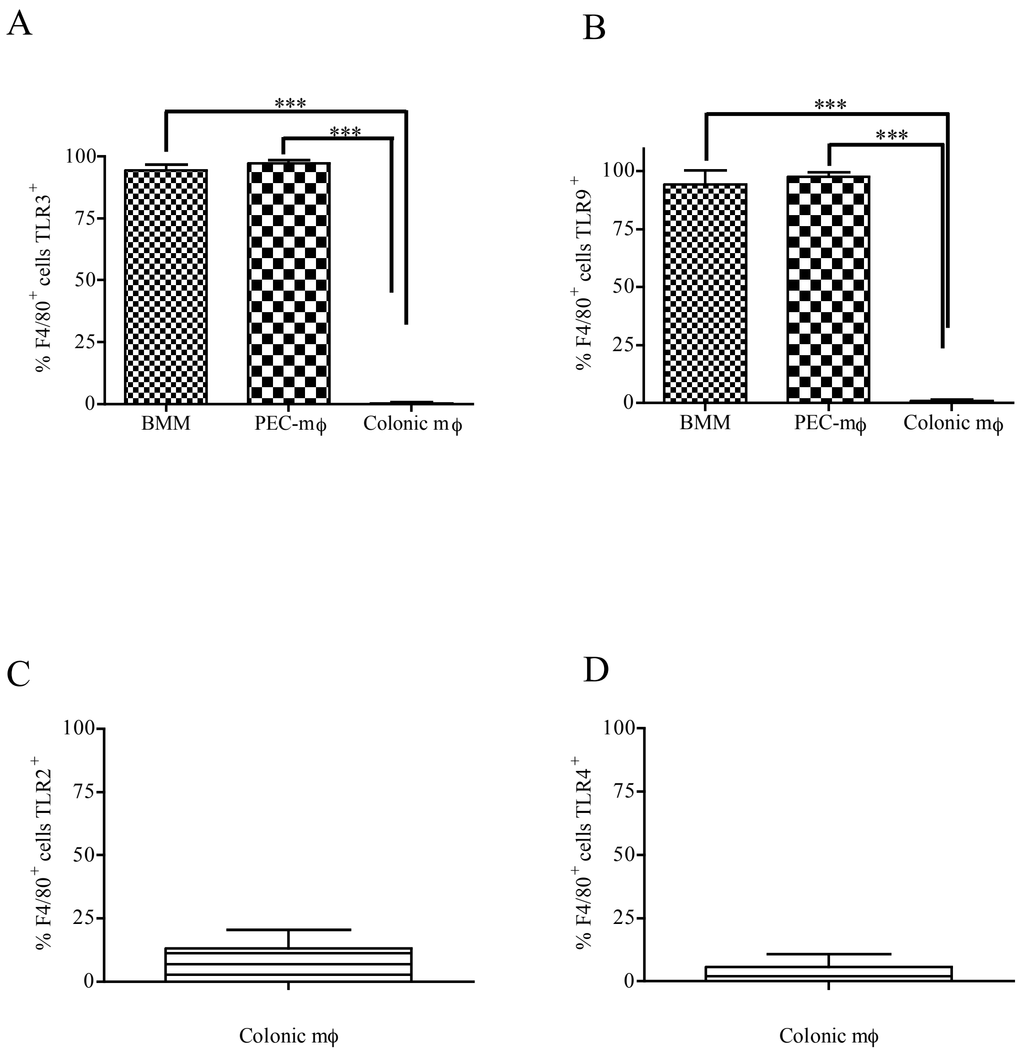


Figure 4.2 Intracellular TLR protein expression by macrophage populations

BMM, PEC mϕ and colonic mϕ were examined for intracellular expression of TLR3 (A) and TLR9 (B), and colonic mϕ were examined for intracellular expression of TLR2 (C) and TLR4 (D) by flow cytometry. The results shown are the means ± SD of live-gated F4/80^{+} cells for 3 mice/group and are representative of at least three individual experiments. A one-way ANOVA revealed a significant difference; (A) $F(2,6) = 3603$, $p<0.0001$; (B) $F(2,6)=696.6$, $p<0.0001$; Planned comparisons were significant (***$p<0.001$), Bonferroni's multiple comparison test.

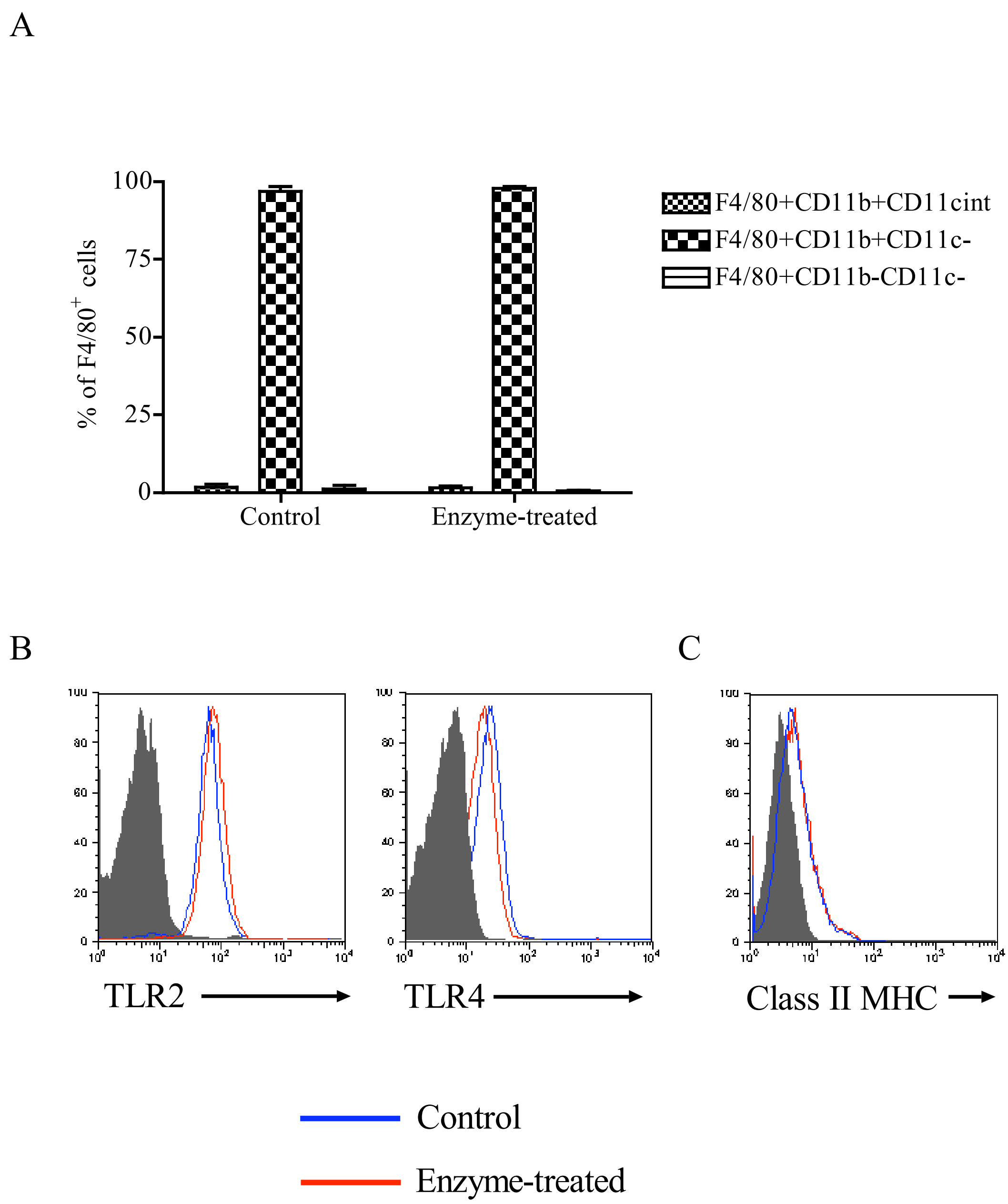


Figure 4.3 Effects of enzyme treatment on the expression of myeloid markers, TLRs and class II MHC by PEC mϕ

PEC mϕ were isolated by wash out and some were treated with the same enzyme mixture and isolation protocol as colonic LP cells, before being examined for the expression of F4/80, CD11b, CD11c (A), surface TLR2 and TLR4 (B) and class II MHC (C) by flow cytometry. The results shown are the mean percentages ± SD for 3 mice/group. The histograms show marker expression levels on live-gated F4/80$^+$ cells and the shaded histograms represent staining with isotype control antibodies.

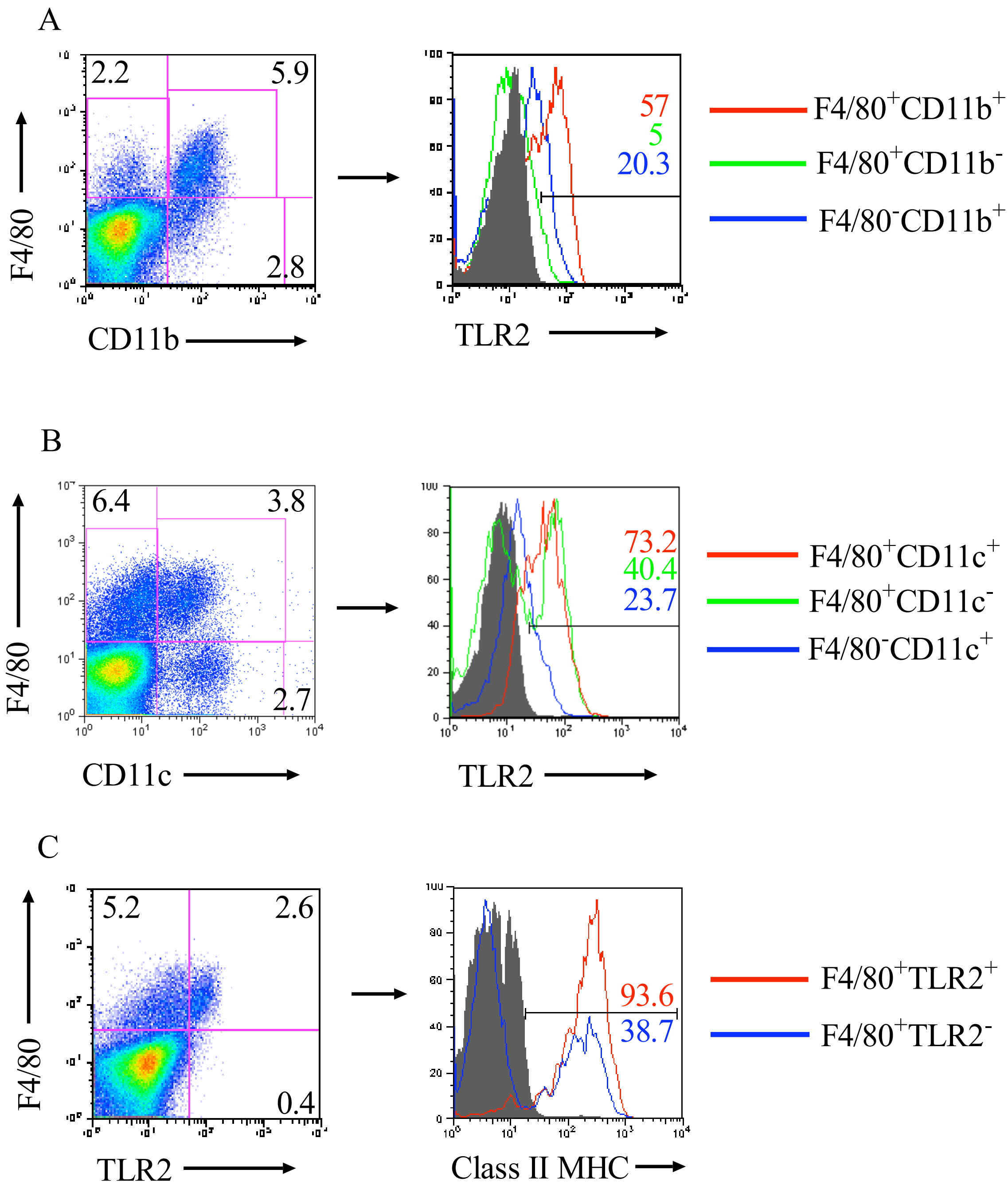


Figure 4.4 Expression of TLR2 by colonic macrophage subsets

Colon LP cells from C57Bl/6 mice were analysed for the expression of F4/80, TLR2, CD11b (A), CD11c (B) and class II MHC (C) by flow cytometry. Numbers in quadrants represent the percentages of the total population positive for each marker. Shaded histograms represent staining with isotype controls and the data are representative of two individual experiments.

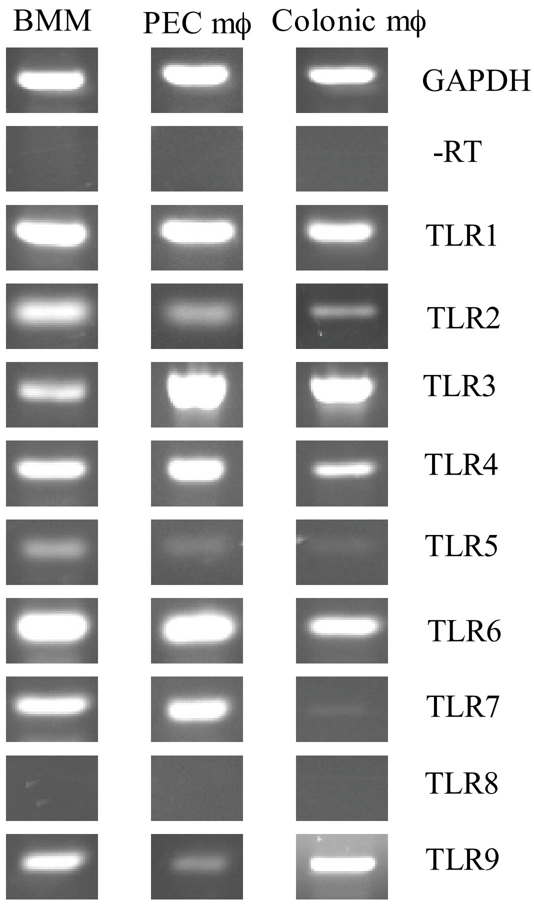


Figure 4.5 Expression of TLR mRNA by macrophages

mRNA was extracted from BMM or from MACS-purified F4/80$^+$ PEC mϕ and colonic mϕ before being examined for the expression of TLR1-9 by end-product PCR, using the primer sequences detailed in Table 2.2.

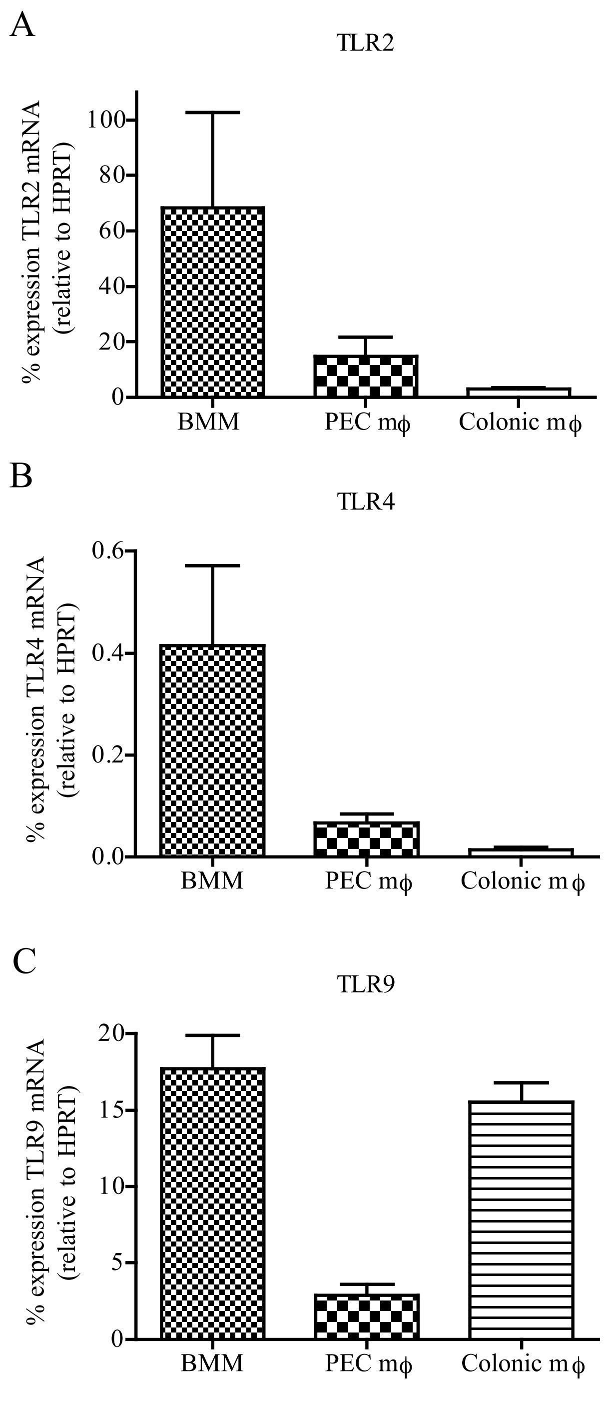


Figure 4.6 Quantitative analysis of TLR mRNA expression by macrophages

mRNA from freshly isolated BMM, and from FACS-sorted F4/80$^+$ PEC or colonic mϕ was isolated and mRNA levels for TLR2 (A), TLR4 (B) and TLR9 (C) were analysed by Taqman real-time PCR. Levels of mRNA are expressed as the percentage expression relative to HPRT and the data are representative of two individual experiments. Primer and probe sequences are detailed in Table 2.3.

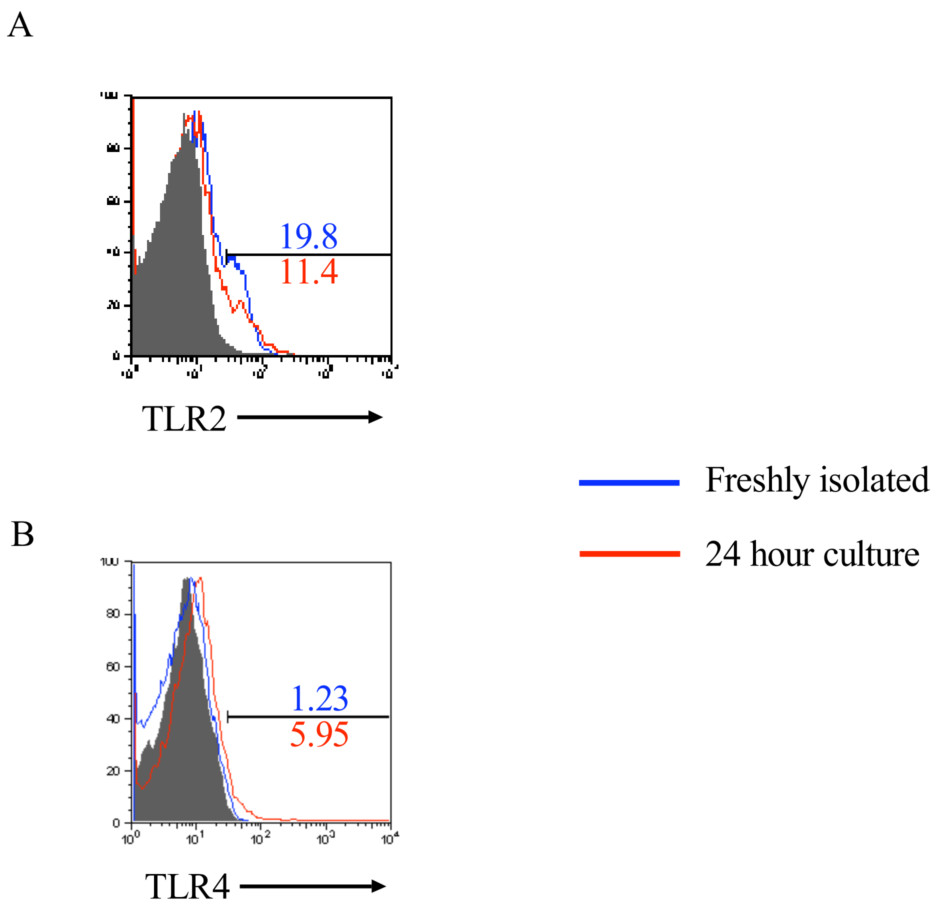
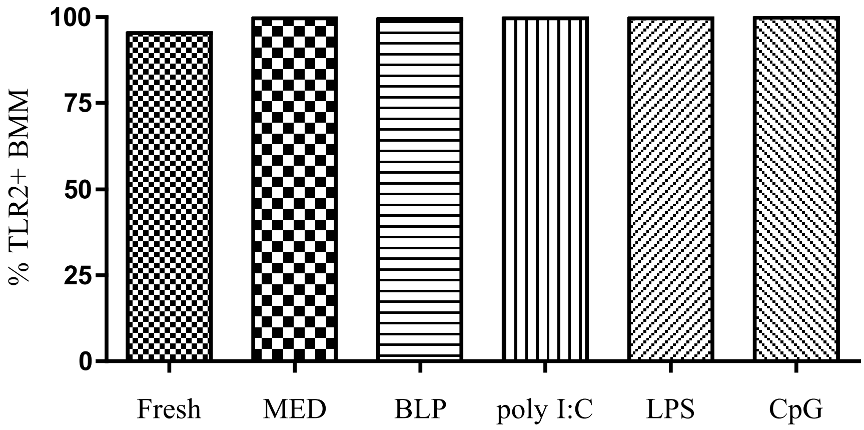


Figure 4.7 Expression of TLR2 and TLR4 by colonic mϕ following *ex vivo* culture

F4/80$^+$ cells from colonic LP were FACS-sorted and examined for the expression of surface TLR2 (A) and TLR4 (B) by flow cytometry before or after culture in medium for 24 hrs. The histograms show marker expression levels on live-gated F4/80$^+$ cells and the shaded histograms represent staining with isotype controls.

A



B

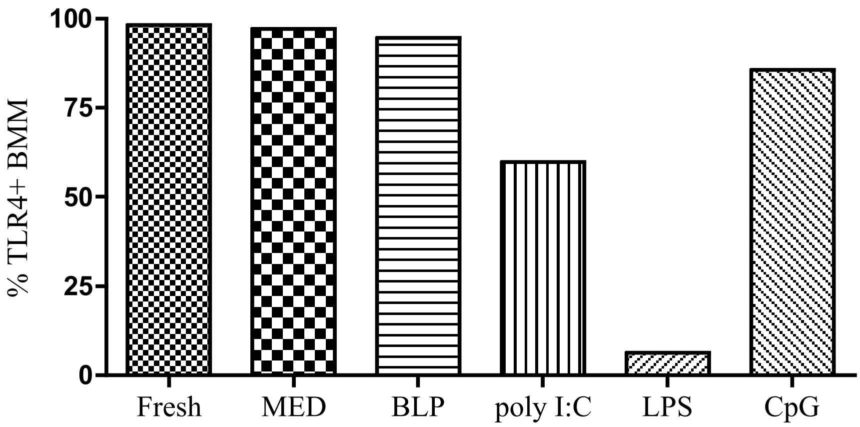


Figure 4.8 Effects of TLR ligands on surface TLR expression by BMM

Freshly isolated BMM or BMM that had been cultured overnight in medium, 1μg/ml BLP, 25μg/ml poly I:C or 1μg/ml LPS were analysed for surface expression of TLR2 (A) and TLR4 (B) by flow cytometry. The results shown are the percentage of live-gated F4/80^{+} cells positive for each TLR.

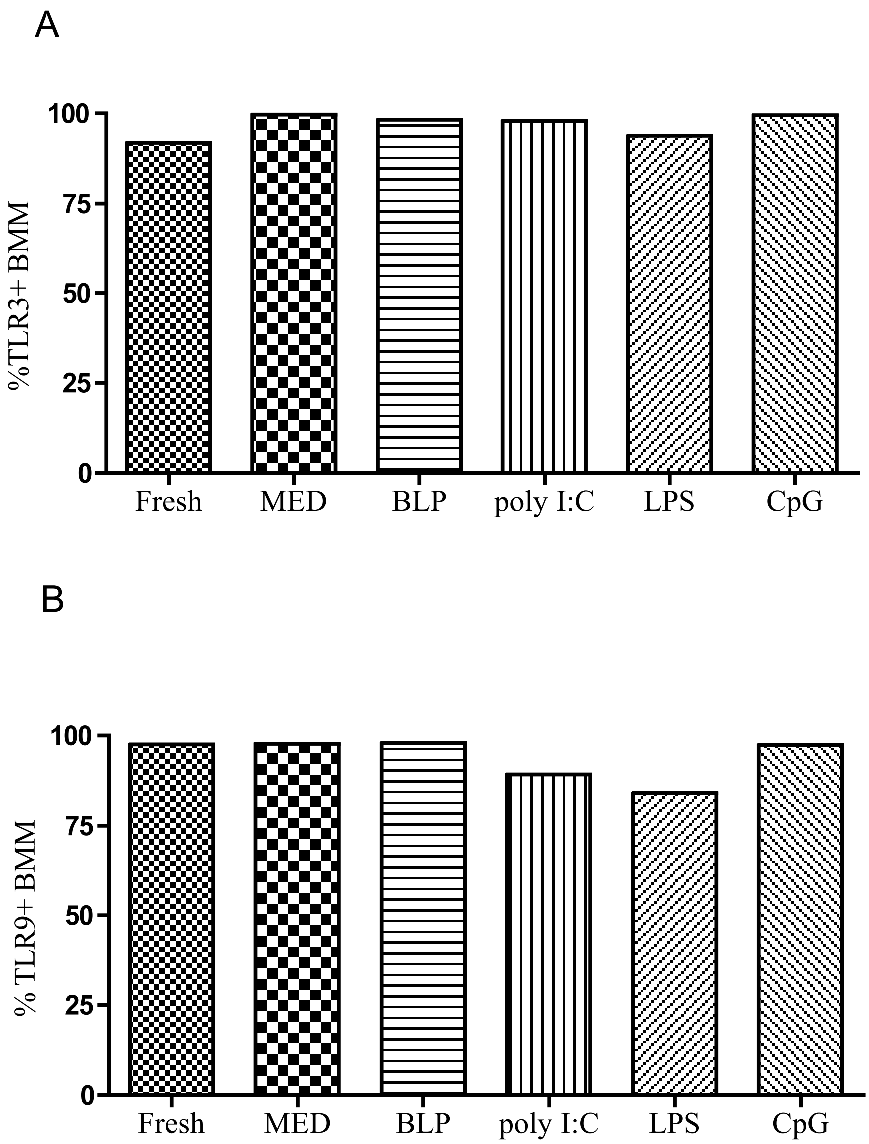
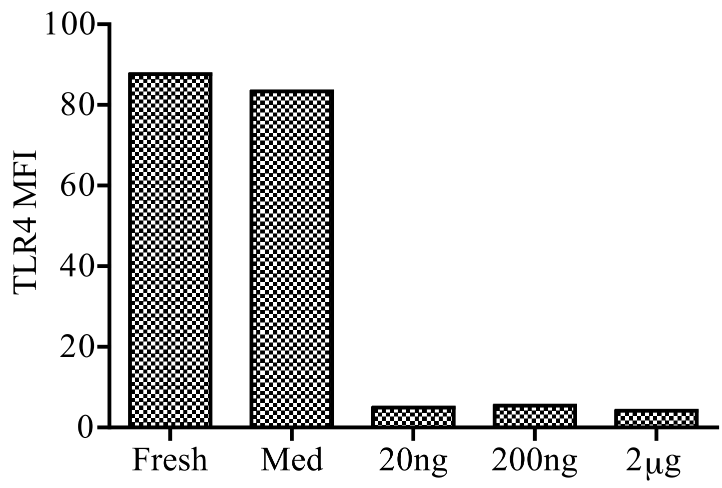


Figure 4.9 Effects of TLR ligands on intracellular TLR expression by BMM

Freshly isolated BMM or BMM that had been cultured overnight in medium, 1μg/ml BLP, 25μg/ml poly I:C or 1μg/ml LPS were analysed for intracellular expression of TLR3 (A) and TLR9 (B) by flow cytometry. The results shown are the percentage of live-gated F4/80^{+} cells positive for each TLR.

A



B

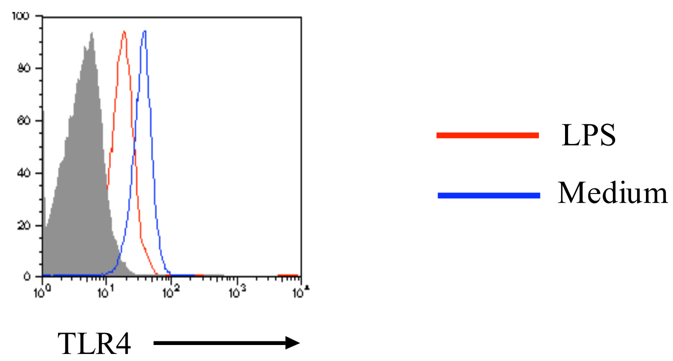


Figure 4.10 Effects of LPS concentration on TLR4 expression by BMM

Freshly harvested BMM or BMM cultured overnight in medium or 2μg/ml, 200ng/ml or 20ng/ml LPS were assessed for surface TLR4 expression. A) The results shown are the MFI for TLR4 expression by live-gated F4/80$^+$ cells. B) Intracellular TLR4 expression by live-gated F4/80$^+$ cells following culture with or without 2μg/ml LPS. The shaded histogram represents staining with isotype control antibody.

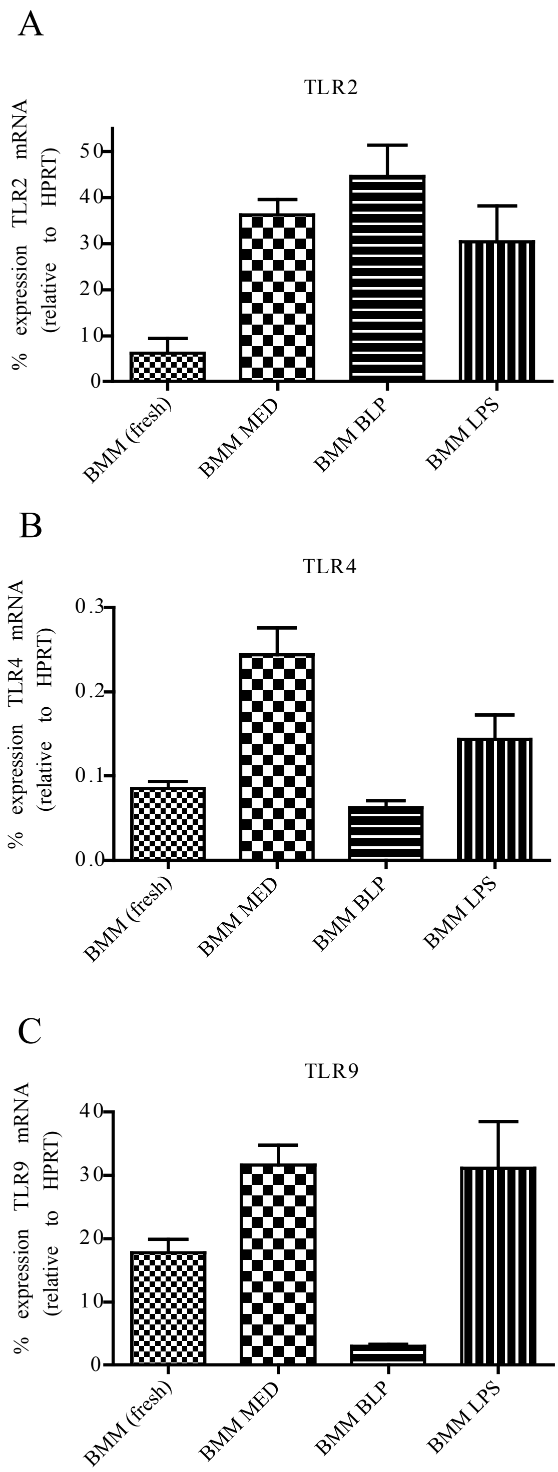


Figure 4.11 Effects of TLR ligands on TLR mRNA expression by BMM

Freshly harvested BMM or BMM cultured in medium, 1μg/ml BLP or 1μg/ml LPS for 18hrs were analysed for the levels of expression of mRNA for TLR2 (A), TLR4 (B) and TLR9 (C) by Taqman real-time PCR. The levels of mRNA are shown as the percentage expression relative to HPRT ± SEM of triplicate wells.

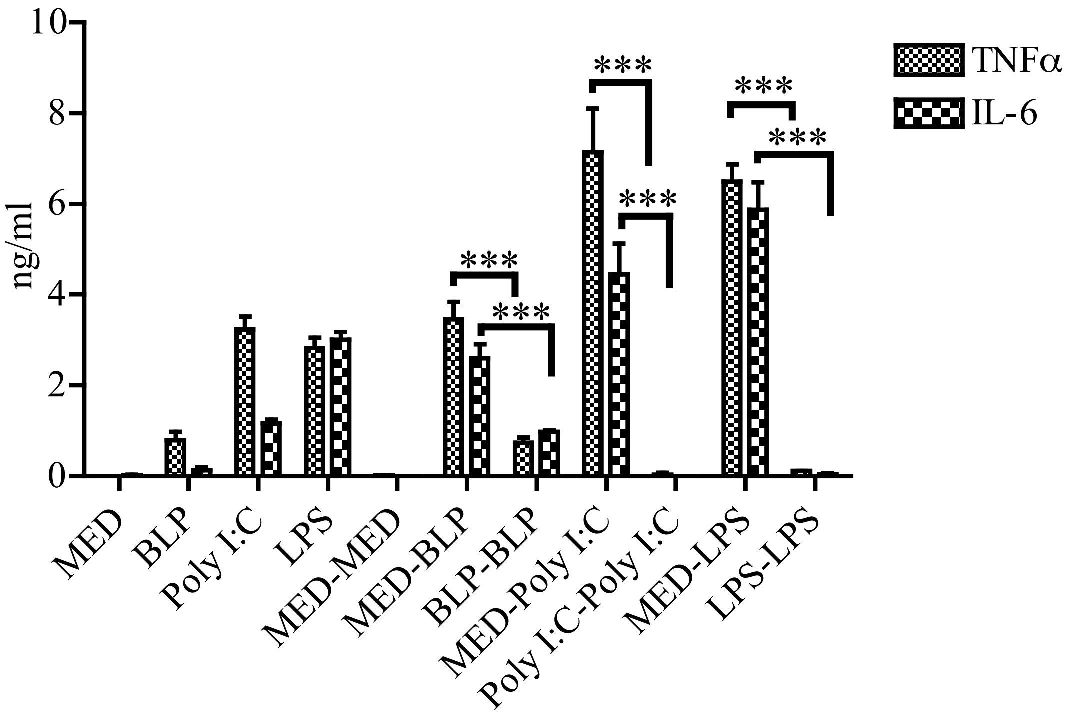


Figure 4.12 Induction of functional tolerance to TLR ligation in BMM

BMM were cultured in medium, 1μg/ml LPS, 1μg/ml BLP or 25μg/ml poly I:C overnight and the supernatants were analysed for the production of TNFα and IL-6 by ELISA. BMM were then re-stimulated with the homologous TLR ligand for a further 24hrs. The results shown are the mean cytokine concentration ± SD for triplicate wells. Two-way ANOVAs revealed significant differences, Bonferroni-corrected. ns=not significant; ***p<0.001.

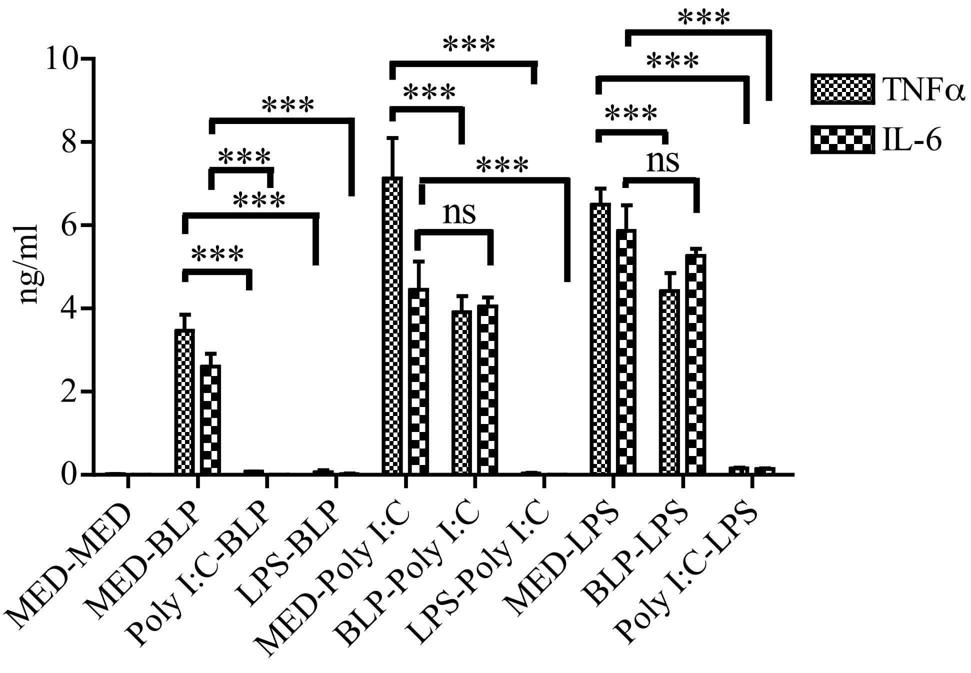


Figure 4.13 Induction of functional tolerance to TLR ligation in BMM

BMM were cultured in medium, 1μg/ml LPS, 1μg/ml BLP or 25μg/ml poly I:C overnight and the supernatants were analysed for the production of TNFα and IL-6 by ELISA. BMM were then re-stimulated with the heterologous TLR ligands for a further 24hrs. The results shown are the mean cytokine concentration ± SD for triplicate wells. Two-way ANOVAs revealed significant differences, Bonferroni-corrected. ns=not significant; ***p<0.001.

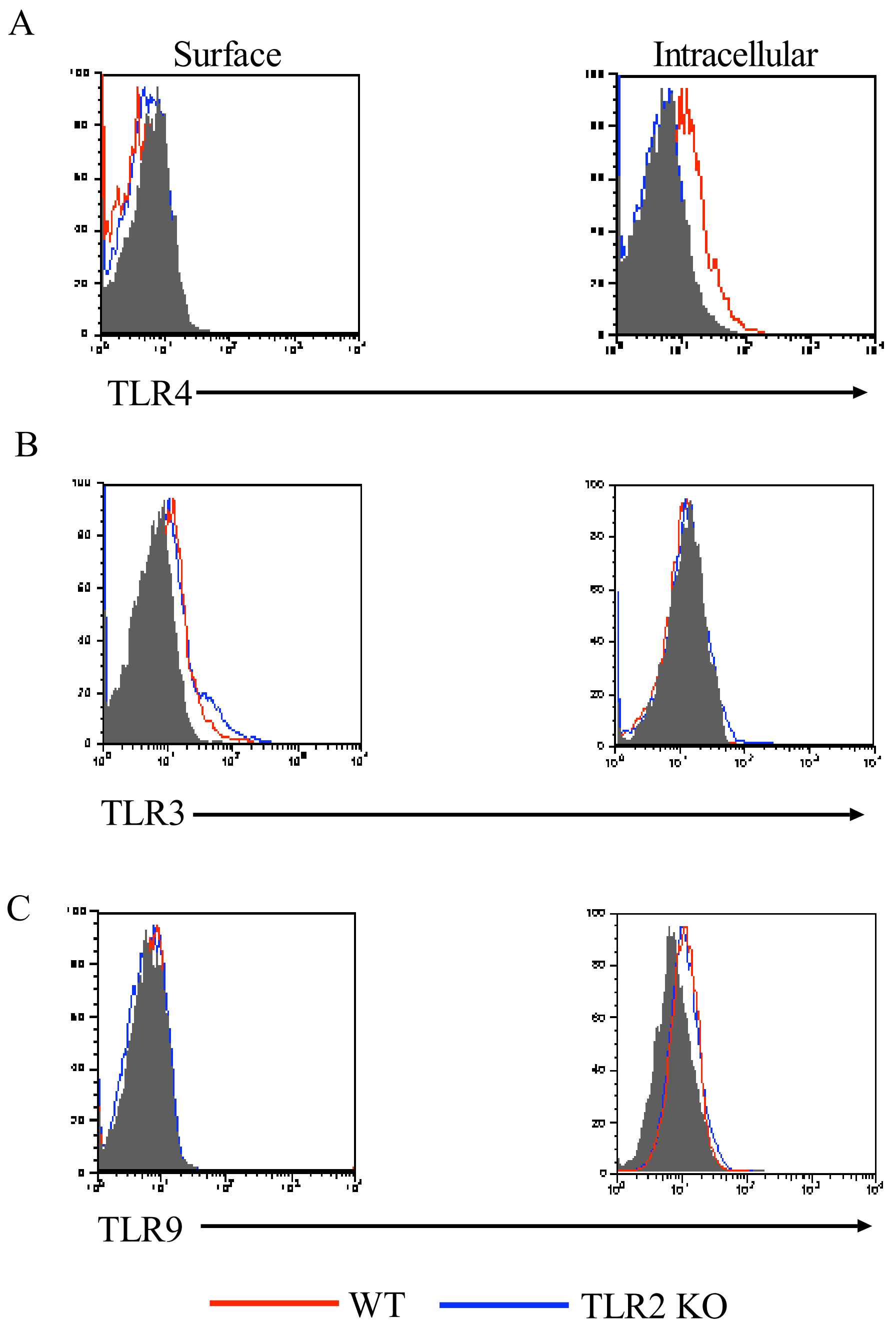


Figure 4.14 TLR expression by colonic macrophages from TLR2 KO mice

Colonic LP cells from TLR2 KO and control mice were assessed for the expression of surface (left panels) and intracellular (right panels) TLR4 (A), TLR3 (B) and TLR9 (C). Histograms show the level of marker expression on live-gated F4/80$^+$ cells and the shaded histograms represent staining with isotype control antibodies.

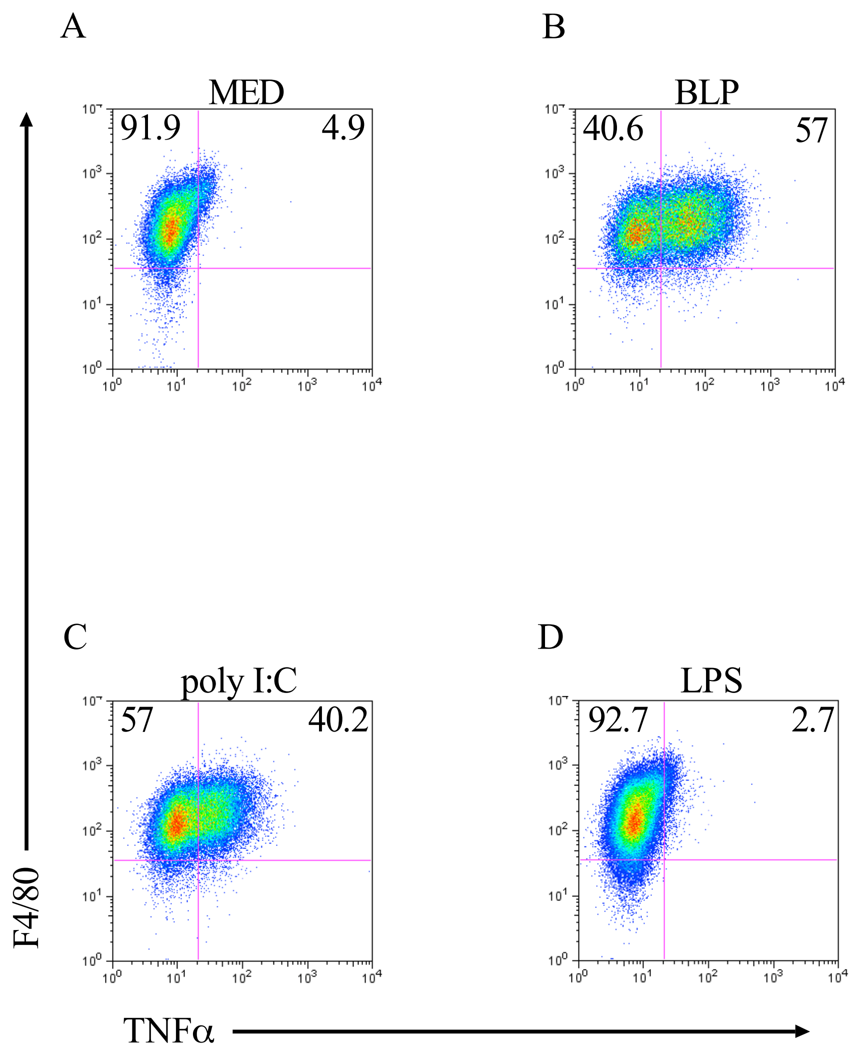


Figure 4.15 Responsiveness of C3H/HeJ bone marrow macrophages to TLR ligands

BMM from C3H/HeJ mice were cultured in medium (A), 1μg/ml BLP (B), 25μg/ml poly I:C (C) or 1μg/ml LPS (D) in the presence of brefeldin-A for 4.5hrs. The cells were then assessed for the expression of intracellular TNFα by flow cytometry. Numbers in quadrants represent the percentages of the total live-gated population.

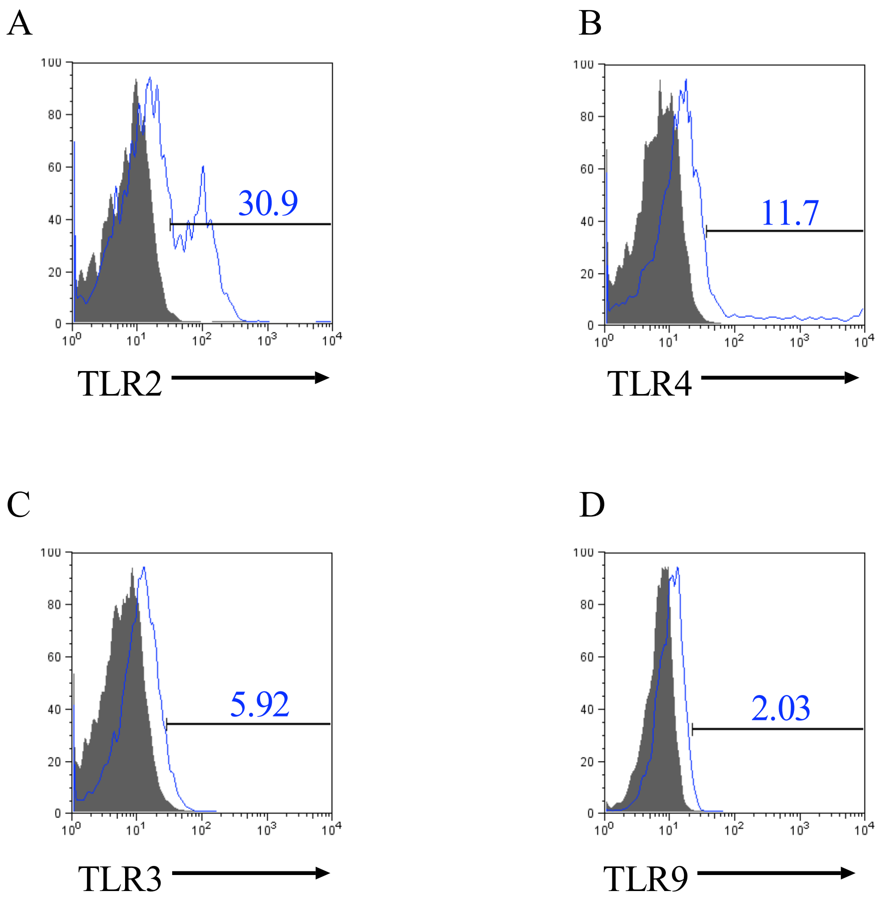


Figure 4.16 TLR expression by colonic macrophages from C3H/HeJ mice

Colonic LP cells from C3H/HeJ mice were assessed for the expression of surface TLR2 (A) and TLR4 (B), or intracellular TLR3 (C) and TLR9 (D) by flow cytometry. The histograms show the levels of marker expression on live-gated F4/80$^+$ cells and the shaded histograms represent staining with isotype controls. Numbers represent the percentages of live-gated F4/80$^+$ cells positive for each TLR.

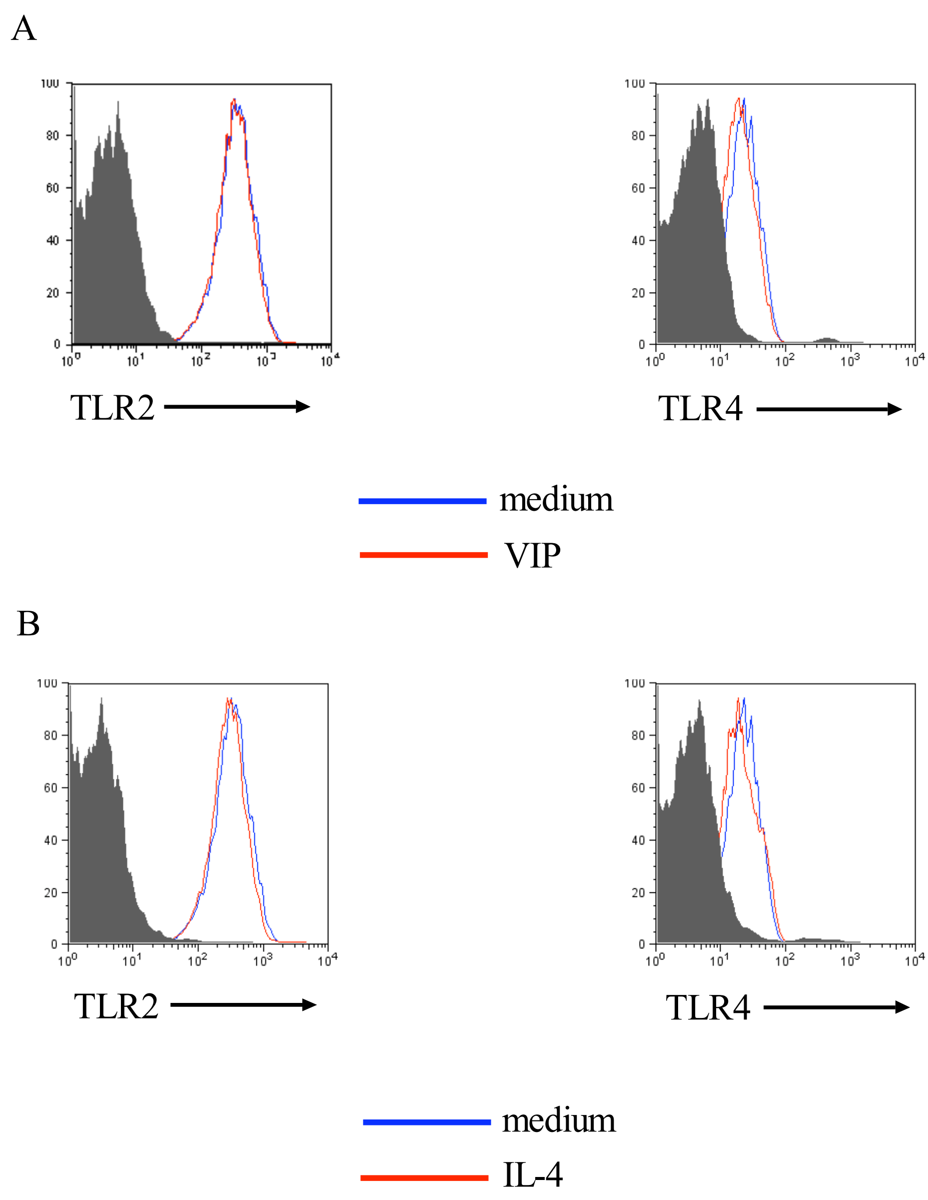


Figure 4.17 Effects of VIP and IL-4 on TLR expression by macrophages

BMM were cultured overnight in medium, 10^{-8}M VIP (A) or 40ng/ml IL-4 (B) and examined for the expression of TLR2 (left panels) and TLR4 (right panels) by flow cytometry. The histograms show the levels of marker expression on live-gated F4/80^{+} cells and the shaded histograms represent staining with isotype control antibodies.

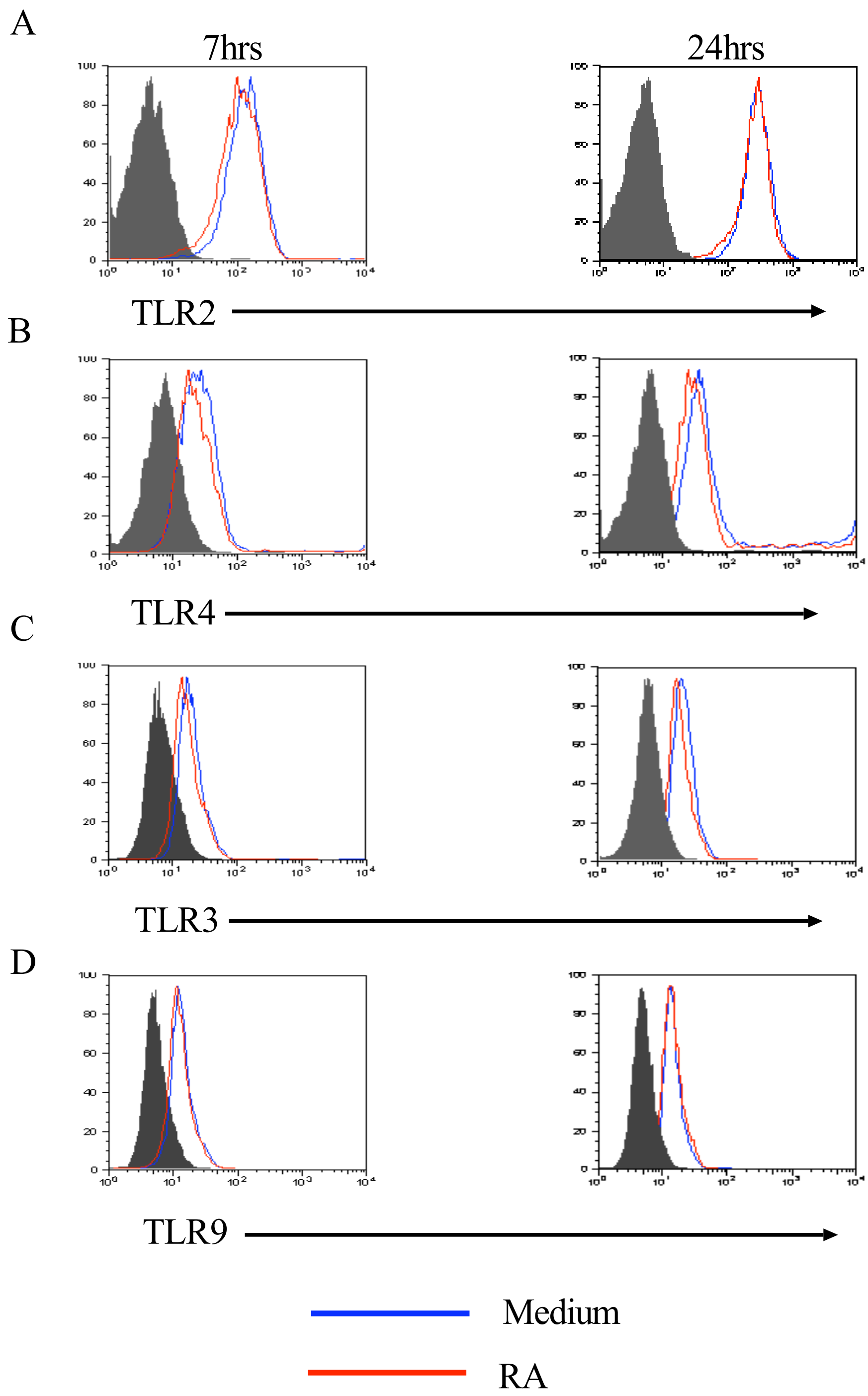


Figure 4.18 Effects of retinoic acid on TLR expression by macrophages

BMM were cultured in medium alone or with 100nM RA for 7hrs (left panels) or 24hrs (right panels) and assessed for the expression of surface TLR2 (A) and TLR4 (B), or intracellular TLR3 (C) and TLR9 (D) by flow cytometry. The histograms show the levels of marker expression on live-gated F4/80$^+$ cells and the shaded histograms represent staining with isotype control antibodies.

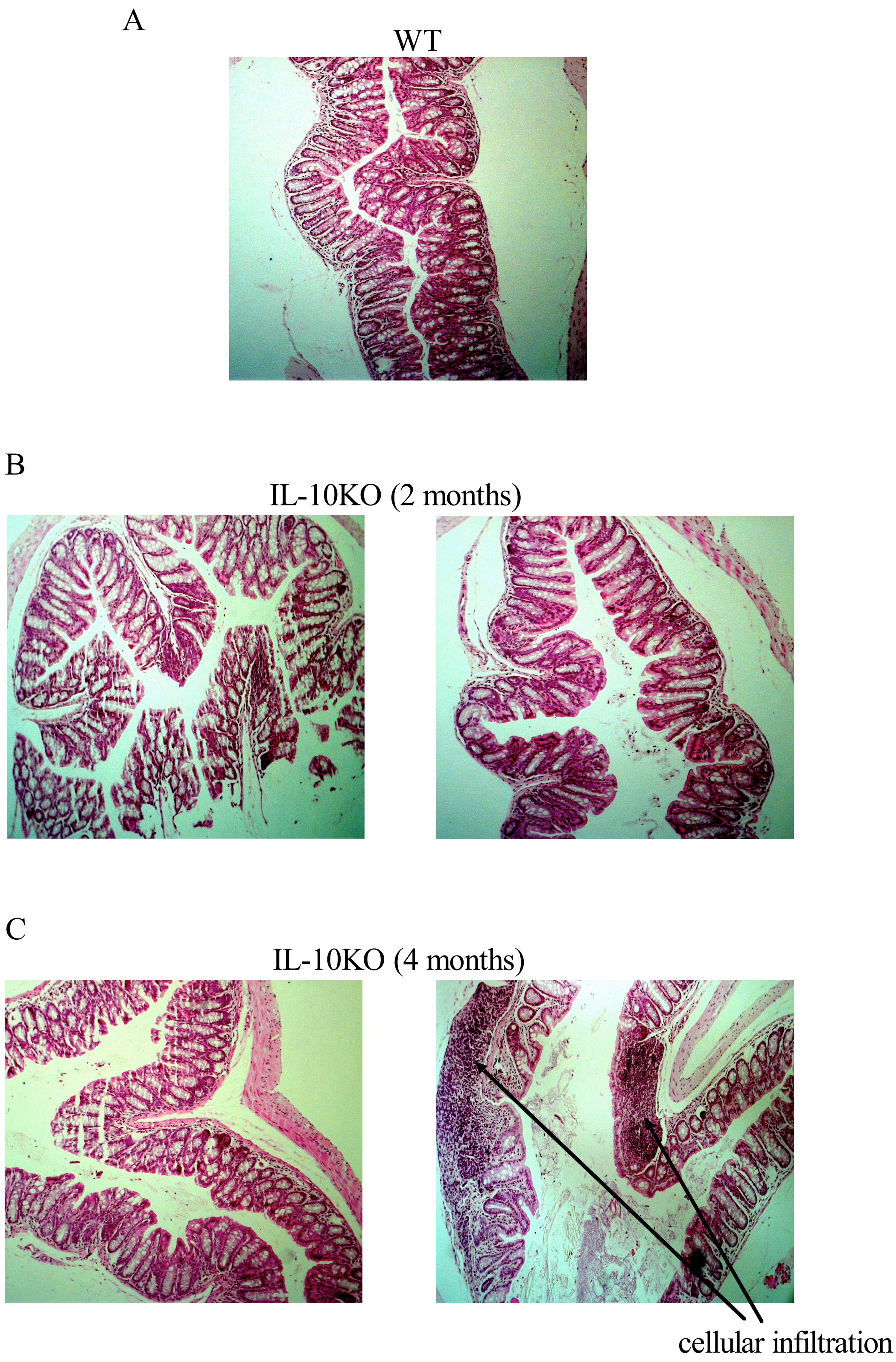
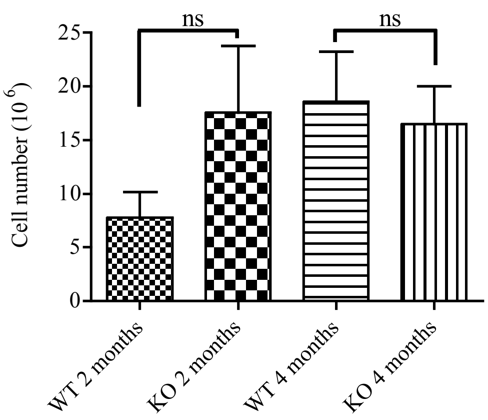


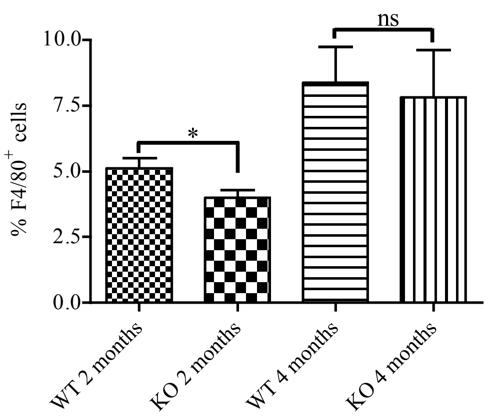
Figure 4.19 Histology of the distal colon from IL-10KO mice

The distal colons from 4 month-old WT (A), or 2 month-old (B) and 4 month-old (C) IL-10KO mice were excised and fixed in 10% formalin, embedded in paraffin, and stained with haematoxylin and eosin (final magnification x100).

A



B



C

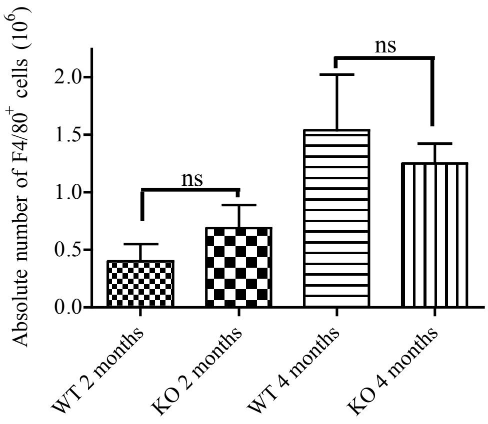


Figure 4.20 Total cellularity, proportion and absolute numbers of macrophages in the colon of IL-10KO mice

Colonic LP cells from IL-10KO and WT control mice were isolated and enumerated. Results shown are the mean total cell numbers (A), percentage F4/80 positive (B) and absolute numbers of F4/80$^+$ cells (C) ± SD for 3 mice/group. ns=not significant; *p<0.05.

A

B

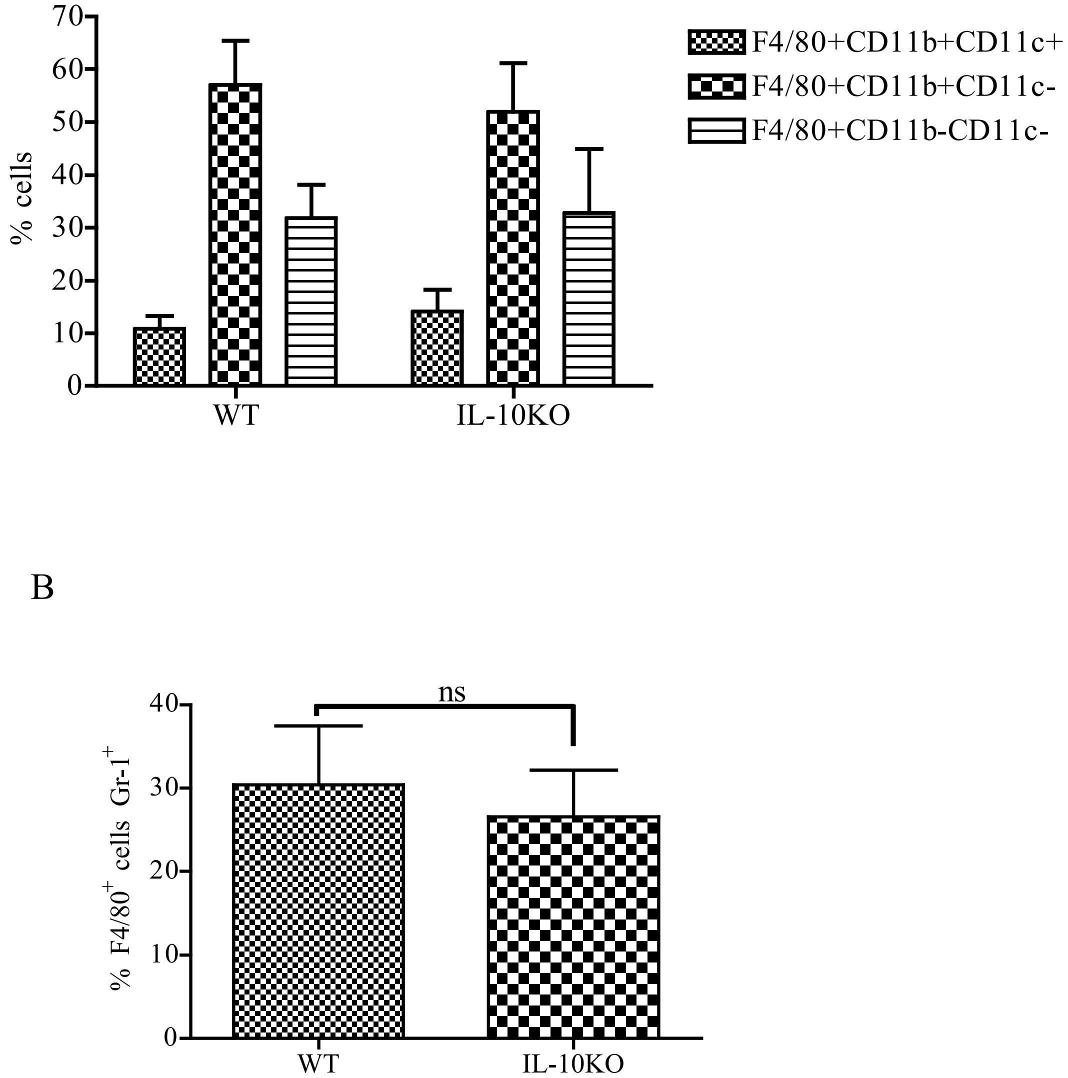


Figure 4.21 Macrophage subsets in the colon of IL-10KO mice

Colonic LP cells from 4 month-old IL-10KO and WT mice were analysed for the expression of F4/80, CD11b and CD11c (A) and Gr-1 (B) by flow cytometry. Results show the mean percentage ± SD of F4/80$^+$ cells in each subset (A) or the proportion of F4/80$^+$ cells that co-express Gr-1 (B) for 3 mice/group. ns=not significant.

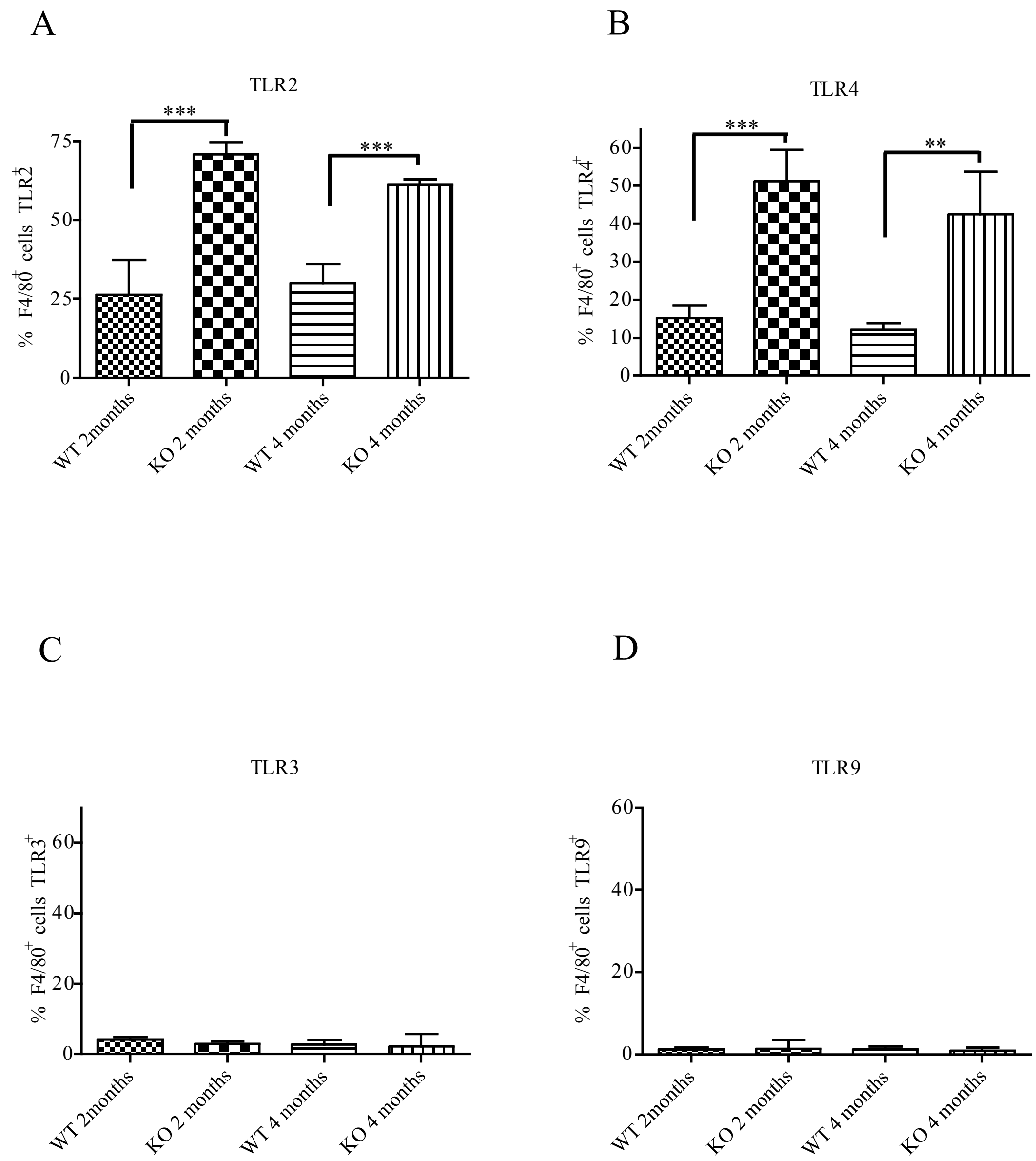


Figure 4.22 Expression of TLRs by colonic macrophages from IL-10KO mice

Colonic mϕ from IL-10KO mice and control mice were assessed for the expression of surface TLR2 (A) and TLR4 (B), or intracellular TLR3 (C) and TLR9 (D) by flow cytometry. The results shown are the mean percentages ± SD of F4/80$^+$ cells positive for each TLR for 3-4 mice/group. **p<0.01; ***p<0.001.

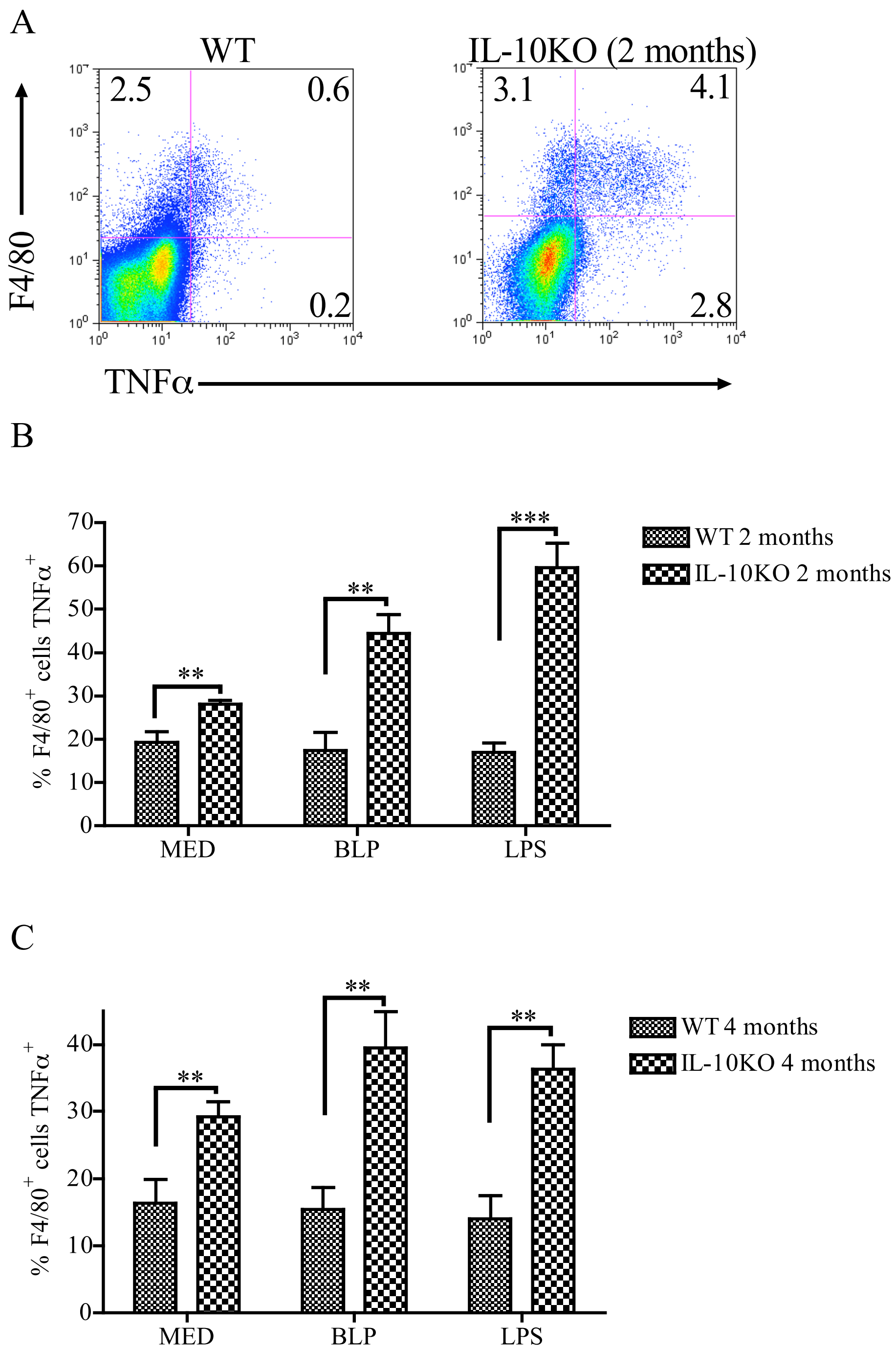


Figure 4.23 TNFα production by colonic macrophages from IL-10KO mice

Colonic LP cells from IL-10KO and WT mice were cultured for 4.5hrs in medium, 1μg/ml BLP or 1μg/ml LPS in the presence of brefeldin-A. A) Representative dot plots of TNFα expression by cells cultured with LPS. B) and C) show the mean percentage ± SD of F4/80^{+} cells positive for TNFα in 2 month- (B) and 4 month-old (C) mice for 3 mice/group. **$p<0.01$; ***$p<0.001$.

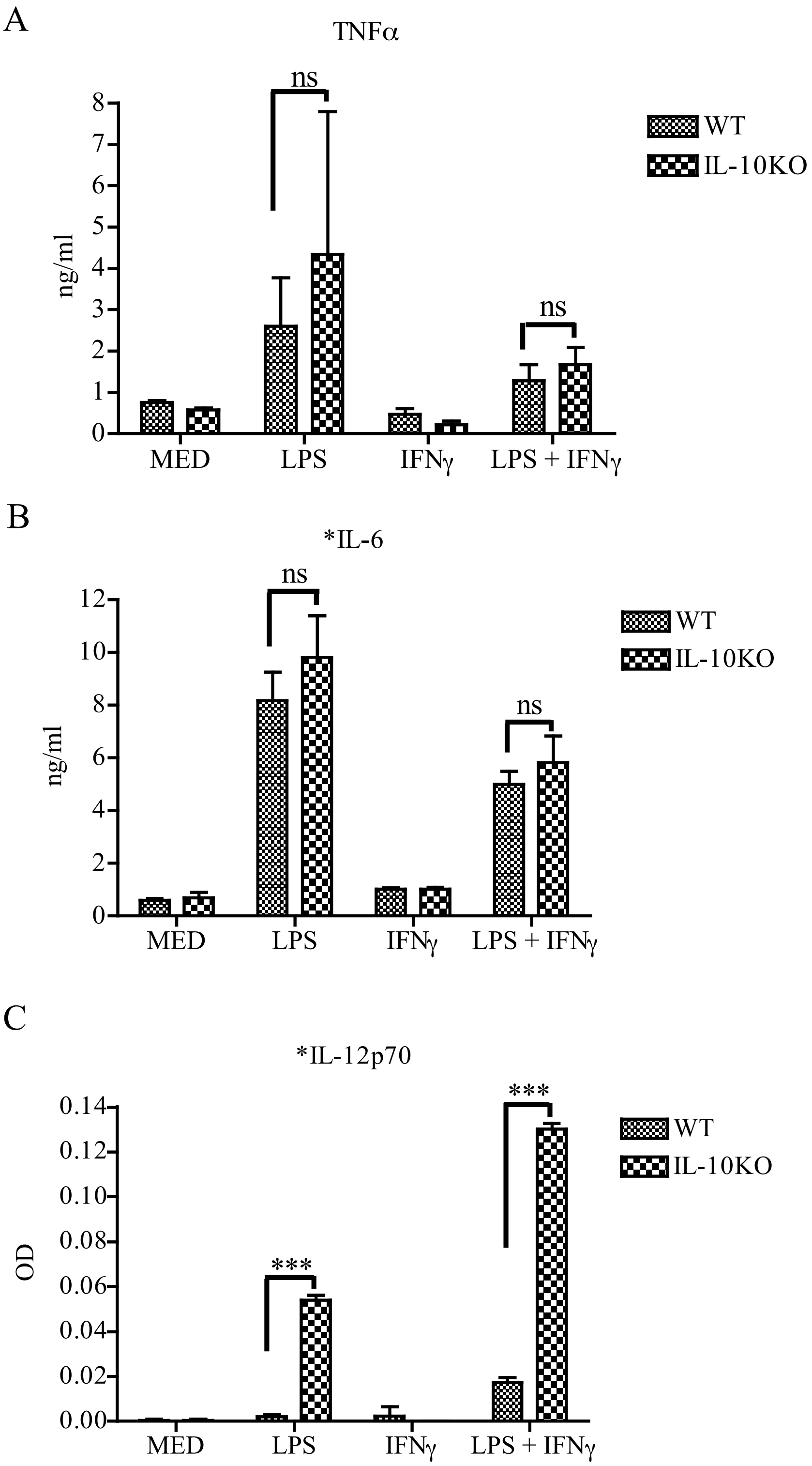


Figure 4.24 Production of pro-inflammatory cytokines by BMM from IL-10KO mice

BMM from WT and IL-10KO mice were cultured in medium, 1μg/ml LPS, 100U/ml IFNγ or LPS + IFNγ for 18hrs and the supernatants assessed for the production of TNFα (A), IL-6 (B) and IL-12p70 (C) by ELISA. The results shown are the mean cytokine concentration or OD ± SD for triplicate wells. ns=not significant; ***$p<0.001$.

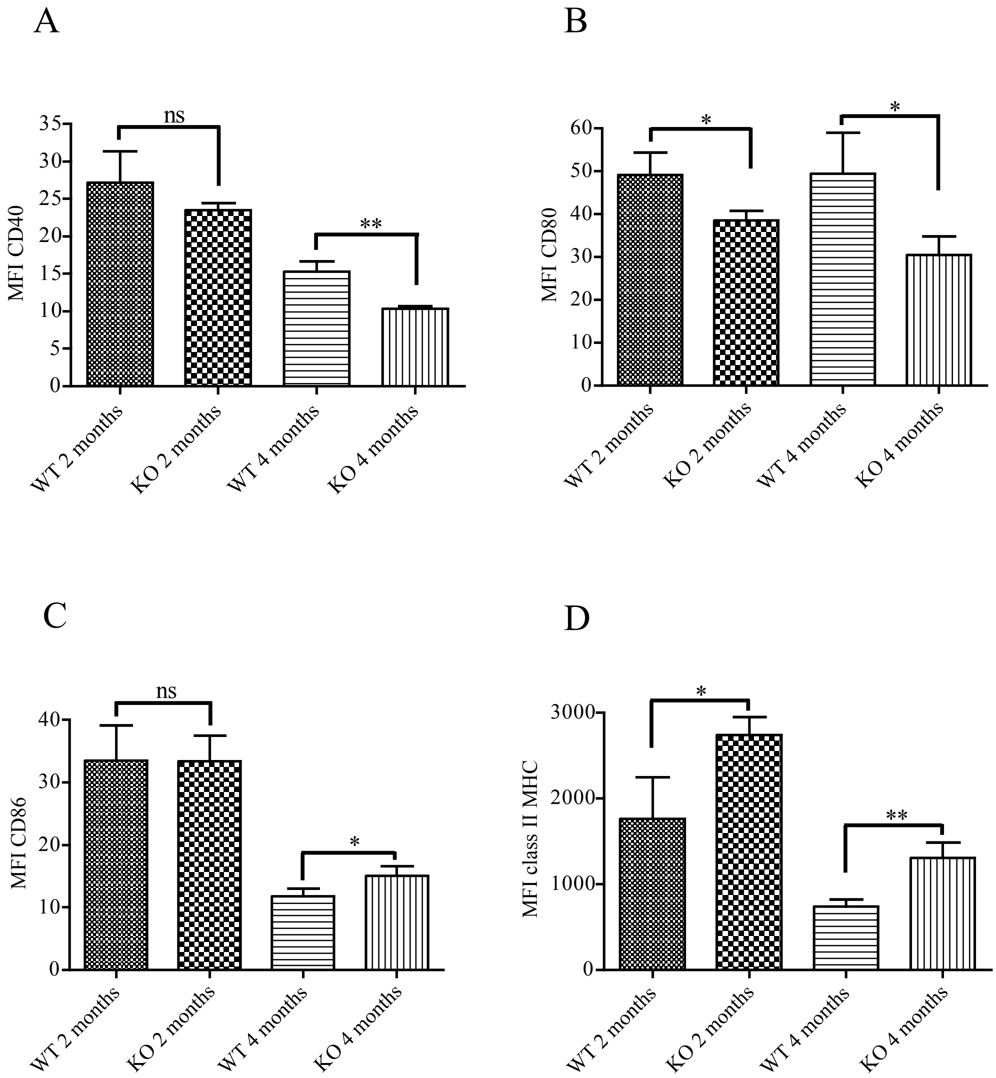


Figure 4.25 Expression of co-stimulatory molecules and class II MHC by colonic macrophages from IL-10KO mice

Colonic LP cells from IL-10KO and WT mice were analysed for the expression of F4/80 and CD40 (A), CD80 (B), CD86 (C) and class II MHC (D) by flow cytometry. Results shown are the mean MFI of marker expression on live-gated F4/80$^+$ cells ± SD for 3 mice/group. ns=not significant; *p<0.05; **p<0.01.

Chapter 5

Phenotypic and functional characterisation of macrophages in the inflamed gut

Introduction

It has been demonstrated that during intestinal inflammation, there is an increase in the number of mφ present in the colon. In addition, these mφ have been shown to exhibit distinct functional properties compared with the resident population present in the healthy gut. In humans with active IBD, intestinal mφ exhibit heightened inflammatory and bactericidal functions, producing pro-inflammatory cytokines and chemokines, and expressing higher levels of NADPH oxidase, compared with mφ under resting conditions. Furthermore, mφ from IBD biopsies express higher levels of TLR2 and TLR4, and higher levels of costimulatory molecules. For these reasons, intestinal mφ are considered to be one of the main contributors to ongoing tissue pathology, possibly via production of inflammatory mediators such as TNFα. To obtain a more detailed insight into how mφ might contribute to intestinal inflammation, I performed a detailed phenotypic and functional analysis of mφ from the colon of mice with DSS-induced colitis. Thus, I quantified these cells, examined their location *in situ*, and investigated their expression of TLRs, as well as the production of pro-inflammatory mediators.

5.1 Induction of intestinal inflammation and tissue pathology

Following administration of 2% DSS in drinking water, C57Bl/6 mice began to lose body weight after 5 days, which continued to fall rapidly in the days thereafter (Fig 5.1a). Weight loss was accompanied by severe, fibrotic shortening of the colon, which is one of the standard parameters of disease severity in this model. As shown in Fig 5.1b, following administration of 2% DSS for 7 days, the length of the colon decreased by almost 50% in the majority of animals, from 9.85±0.75cm in water fed control mice to 6.2±0.45cm. The clinical severity of disease was assessed by monitoring diarrhoea and rectal bleeding, which were evident in some animals as early as 4-5 days after beginning DSS administration (Fig 5.2b+c). As shown in Fig 5.2d, the total clinical disease score continued to increase steadily up to day 7, although there was marked variability between individual animals. Histological analysis showed that following 7 days of DSS administration, there was severe intestinal pathology with oedema, crypt lengthening, epithelial damage, areas of ulceration and complete crypt loss, as well as an influx of inflammatory cells into the mucosa and submucosa (Fig 5.3). To assess the contribution mϕ make to intestinal pathology, I next performed immunofluorescence staining for F4/80-expressing cells in the inflamed intestine. As shown in Fig 5.4, there were numerous F4/80$^+$ cells in the normal distal colon, mainly scattered throughout the LP and around the crypts. By day 7 of colitis, there was a large increase in the numbers of F4/80$^+$ cells in the inflamed colon, and these were now found throughout the mucosa and submucosa (Fig 5.4).

I then isolated colonic LP cells in order to conduct detailed phenotypic and functional analyses of the infiltrating mϕ. The total number of cells obtained following tissue digestion increased significantly from $5.7 \times 10^6 \pm 0.9$ per colon under resting conditions, to $10.5 \times 10^6 \pm 3.4$ and $15 \times 10^6 \pm 2.3$ cells following administration of DSS for 4 or 7 days, respectively (Fig 5.5a). As shown in Fig 5.5b, morphometric analysis by flow cytometry showed increased populations of mononuclear and granulocytic cells during colitis compared with resting conditions. This was confirmed by a signifcant increase in the proportion of F4/80-expressing cells, with 16.9±1.6% and 21.6±3.8% cells being F4/80$^+$ after 4 and 7 days of DSS administration respectively, compared with 11.7±2.3% in the resting colon (Fig 5.6a). This corresponded to increases in the absolute number of mϕ from $0.61 \times 10^6 \pm 0.3$ per colon under resting conditions, to $1.38 \times 10^6 \pm 0.2$ and $3.44 \times 10^6 \pm 0.4$ following 4 and 7 days of DSS administration, respectively (Fig 5.6b).

To extend the phenotypic characterisation of the inflammatory infiltrates, I examined the expression of Ly6C, a non-specific marker present on some monocyte/mϕ populations, including the 'inflammatory' subset of blood monocytes which express Gr-1, a shared epitope on Ly6C and Ly6G. Unlike the Ly6C^{bright} population which did not change, there was a rapid and substantial increase in the percentage of Ly6C^{int} cells in the DSS inflamed colon, from 10.5±4.4% of total colonic LP cells under resting conditions to 26.7±5.6% (Fig 5.7a+c). This occurred as early as 3 days after DSS administration was commenced and most of the cells were mononuclear in terms of size and granularity, suggesting they were of myeloid origin (Fig 5.7b). However, the majority of these cells failed to express F4/80 or CD11b both in normal mice and in colitis (Fig 5.7a+d), suggesting that these may be an unusual population of mononuclear cells. This was supported by the fact that they did not express the Ly6G marker of neutrophils, which were completely absent in the healthy gut and on day3 of colitis (0.1±0.1% and 0.2±0.1%, respectively), and constituted only 1.5±0.3% of total live-gated cells by d7 of colitis (Fig 5.8a+b). As shown above in the morphometric analyses, there was an increase in granulocytic cells by day 3 of colitis, suggesting that these may represent another population of granulocytes, such as eosinophils.

5.2 TLR-expressing macrophages become dominant in the inflamed colon

To assess further how mϕ present in the inflamed colon differed from those present under resting conditions, I next conducted a detailed phenotypic analysis of TLR expression by mϕ during colitis. In stark contrast to the healthy colon, large numbers of $TLR2^+$ $F4/80^+$ cells were observed during colitis (Fig 5.9a). This $TLR2^+$ population accounted for 29.3±5.0% of $F4/80^+$ cells in the healthy colon, but it constituted the majority of F4/80-expressing cells in the inflamed colon, increasing to 69.1±10.6% and 75.9±15.1% on days 4 and 7 of DSS administration (Fig 5.9b). There was also an increase in the proportion of $F4/80^+$ cells expressing surface TLR4 during colitis, but no intracellular TLR3 and 9 expression could be detected in control or colitic intestine (Fig 5.10a-c). Unfortunately, due to the unavailability of antibodies, I was unable to assess the expression of these TLRs on the total $TLR2^+$ population. The high levels of surface TLR2 on these cells during colitis allowed two clearly distinct populations to be identified and analysed on the basis of TLR2 expression. Interestingly, although the majority (>85%) of the total TLR-expressing cells in colitis were CD11b$^+$ and these CD11b$^+$TLR$^+$ cells had the size and granularity consistent with monocytic cells, around 50% of the TLR-expressing cells in colitic mucosa had low or absent expression of F4/80 (Fig 5.11a+b). In contrast, the majority of TLR2$^+$ cells in resting mucosa were F4/80$^+$ (Fig 5.11a). As blood monocytes express lower levels of F4/80 than tissue mϕ (43), this suggests that these F4/80^{lo}TLR2$^+$CD11b$^+$ cells in the inflamed colon may be recently derived from blood monocytes. However, to ensure that the cells I was characterising were definitely mϕ, I based my studies strictly on the F4/80$^+$ population. A greater proportion of the TLR2$^+$F4/80$^+$ cells expressed CD11c both under resting conditions and in colitis, compared with the TLR negative subset (Fig 5.11d). Therefore this F4/80$^+$TLR$^+$CD11b$^+$CD11c^{lo} population is somewhat similar to the minor population of triple positive F4/80$^+$ cells I defined in the normal colon in Chapter 3, but they are now the dominant population in the inflamed intestine.

To assess the activation status of the F4/80$^+$ cells present during colitis, total F4/80$^+$ cells were assessed for the expression of CD40, CD80, CD86 and class II MHC. As shown in Fig 5.12a-c, F4/80$^+$ cells in the resting and inflamed colon expressed low levels of costimulatory molecules. However a lower proportion of F4/80$^+$ cells expressed class II MHC in the inflamed colon, compared with this population in the resting colon (Fig 5.12d). As I had found that a significant proportion of mϕ in the inflamed colon expressed TLR2, I next assessed the presence of activation markers directly on the TLR$^-$ and TLR$^+$ mϕ subsets. Whereas the TLR2$^-$ F4/80$^+$ cells from DSS inflamed colon expressed little or no class II MHC, no CD40 or CD86 and only low levels of CD80, the majority of

$TLR2^+F4/80^+$ cells were strongly class II MHC^+ and expressed detectable amounts of CD40, CD80 and CD86 (Fig 5.13). Interestingly a similar dichotomy based on TLR expression was seen in resting colon, with the majority (>90%) $TLR2^+F4/80^+$ cells being class II MHC^+ compared with <40% of $TLR2^-$ cells and they also expressed higher levels of the co-stimulatory molecules. Two marked differences were that a small subset (~20%) of $TLR2^+$ class II MHC^- mϕ appeared for the first time in colitis and there were also very few $TLR2^-$ class II MHC^+ mϕ in colitis, whereas almost 40% of these cells are present in resting colon.

Due to the fact that the inflammatory response in this disease model is focused at the distal end of the colon, I conducted an anatomical analysis of TLR expression by mϕ in the resting and inflamed colon. As shown in Fig 5.14, a significantly higher percentage of mϕ expressed TLR2 in the proximal colon compared with the distal colon under resting conditions (63.96±7.9% compared with 34.0±16.56%). On day 5 of colitis, the percentage of TLR-expressing mϕ in the distal colon increased significantly to 78.06±6.47%, while the proportion of TLR^+ mϕ in the proximal colon did not change during intestinal inflammation. These results suggest that TLR expression by mϕ is anatomically determined under resting conditions, but its increase during colitis may be driven locally by the inflammatory process.

5.3 Production of pro-inflammatory TNFα by colonic macrophages during colitis

To assess whether this expanded population of TLR-expressing mϕ may contribute to the inflammation of the colon seen after administration of DSS, I examined spontaneous production of TNFα by intracellular staining. Compared with the healthy colon, which contained almost no TNFα-producing cells, the inflamed colon contained a substantial population of TNFα-expressing cells (~12%), the majority of which were F4/80$^+$ (Fig 5.15a). As before, colitis in this experiment was associated with an increased percentage of F4/80$^+$ cells that expressed TLR2 and it was notable that the majority of TNFα-expressing cells were also TLR2$^+$ (Fig 5.15b+c). As I found with TLR expression, a substantial proportion of the TNFα$^+$ cells expressed little or no F4/80, but all expressed CD11b at high levels and resembled mononuclear cells in terms of size and granularity (Fig 5.15d), again suggesting these may be derived from recently recruited blood monocytes. Notably, there were very few TNFα$^+$ cells in the inflamed colon which were not CD11b$^+$, indicating that these infiltrating myeloid cells account for most of the production of this cytokine during colitis (Fig 5.15).

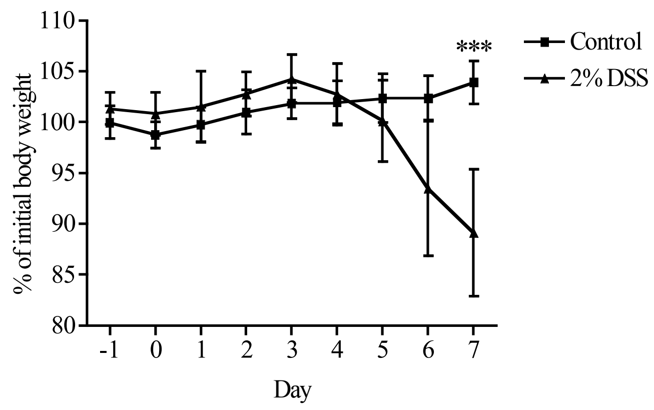
The results from replicate mice in these experiments are summarised in Fig 5.16. This shows that in resting colon, there was minimal spontaneous production of TNFα, with no significant difference between the TLR$^-$ and the TLR$^+$ mϕ, although some TNFα could be detected in the TLR$^+$ cells (4.9±4.2% vs 0.2±0.2%). In stark contrast, a substantial percentage (up to 60% on d5 and d7) of the TLR2$^+$ F4/80$^+$ cells produced TNFα spontaneously during colitis. However very few TLR2$^-$ cells produced TNFα even in colitis (4.1±1.8% and 6.1±6% on d5 and d7, respectively). Due to the fact that a large proportion of F4/80$^+$ cells in the inflamed intestine expressed TLR2 and TLR4, I next assessed whether I could further increase TNFα expression via stimulation with the corresponding ligands, BLP and LPS. Consistent with the presence of small numbers of TLR2-expressing mϕ in the resting colon, up to 30% of these cells expressed TNFα after culture with BLP (Fig 5.17). However this increased markedly to 60-70% of $TLR2^+$ cells on days 5 and 7 of colitis. Stimulation of resting cells with LPS induced little production of TNFα by $TLR2^+$ cells, but again this was greatly increased in colitis, where similar proportions of TLR^+ mϕ stimulated with LPS expressed TNFα as found after stimulation with BLP. $TLR2^-$ mϕ from resting colon produced little or no TNFα in response to BLP or LPS and this was not significantly increased during colitis (Fig 5.17).

Summary

In this chapter, I have demonstrated that following administration of DSS, mice begin to lose weight rapidly, along with shortening of the colon, diarrhoea and rectal bleeding. The colon loses its architecture and becomes infiltrated by large numbers of inflammatory cells, a large proportion of which are F4/80-expressing monocytic cells and are now present throughout all layers of the colon. In addition there is a delayed increase in both the proportion and absolute number of $F4/80^+$ cells. Furthermore, there is also a rapid influx of $Ly6C^{int}$ cells and a more delayed influx of $Ly6G^+$ neutrophils. The majority of the former $Ly6C^{int}$ cells were mononuclear in terms of FSC/SSC properties and a similar population of $Ly6C^{int}$ monocytes has been found in the inflamed peritoneum (173). In this model, the monocytes are also $CD11b^{hi}$, but in contrast, the majority of $Ly6C^{int}$ cells I found in the inflamed colon failed to express F4/80 or CD11b, suggesting these are an unusual population of mononuclear cells. Furthermore, a large increase in the proportion of granulocytic cells was apparent before the appearance of the Ly6G$^+$ neutrophil population, suggesting the infiltration of another granulocytic population such as eosinophils.

A substantial proportion of the increased numbers of mϕ which appeared in the inflamed gut now expressed TLR2 and TLR4 and this change was most obvious at the distal end of the colon, where the inflammatory response is focused. The TLR$^+$ mϕ expressed CD11b, class II MHC and low levels of CD11c, CD40, CD80 and CD86 and accounted for virtually all the TNFα producing cells in the intestine of mice with DSS-induced colitis. Interestingly, the smaller proportion of TLR2-expressing mϕ in the normal colon were also capable of expressing TNFα in response to the corresponding ligand, BLP, but unlike mϕ from the inflamed colon, these cells did not express TNFα spontaneously and did not respond to TLR4 stimulation. The TLR2-expressing, TNFα-producing mϕ present in the inflamed colon are likely to represent a population of blood monocytes that has been recruited to the inflamed intestine under the influence of pro-inflammatory chemokines. However, to try and answer this question and gain a further insight into the origin of these inflammatory mϕ, I went on to carry out BrdU pulse-chase experiments which would provide me with information regarding the turnover kinetics of the TLR$^-$ and TLR$^+$ mϕ subsets. These experiments are reported in the next Chapter.

A



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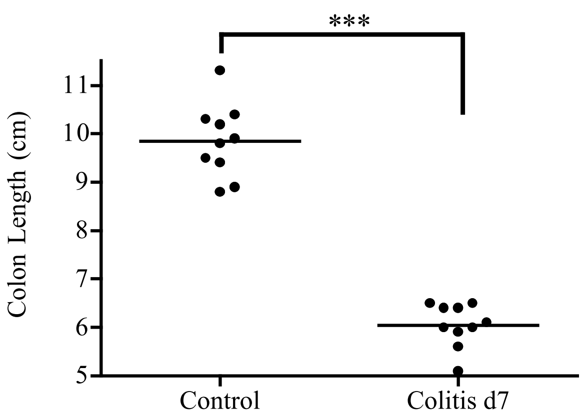


Figure 5.1 Weight loss and colon lengths in DSS colitis

C57Bl/6 mice were given water or 2% DSS for 7 days, starting on day 0, and monitored daily for weight loss, calculated as % of initial body weight (A). A one-way ANOVA revealed a significant difference, $F(8,8)=34.86$, $p<0.0001$, Bonferroni-corrected. B) After 7 days, colons were excised and the lengths were measured. The results shown are the means ± SD for 10 mice/group and similar results were obtained in repeat experiments. ***$p<0.001$.

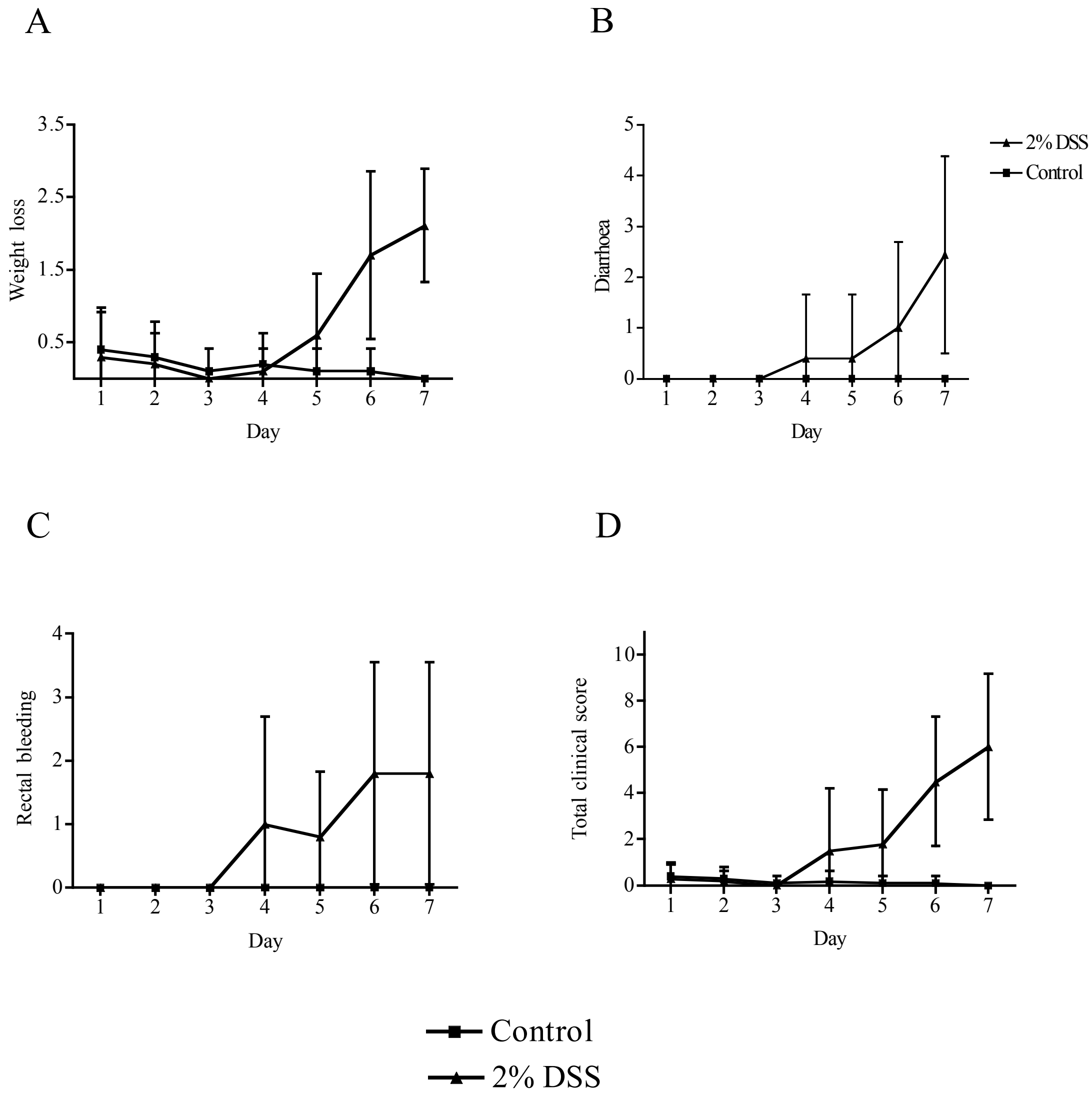


Figure 5.2 Clinical aspects of DSS-induced colitis

C57Bl/6 mice were given water or 2% DSS and were monitored daily for weight change (A), diarrhoea (B), rectal bleeding (C), and total clinical score (D), calculated as described in the Materials and Methods. The results shown are the means ± SD for 10 mice/group and are representative of at least five experiments.

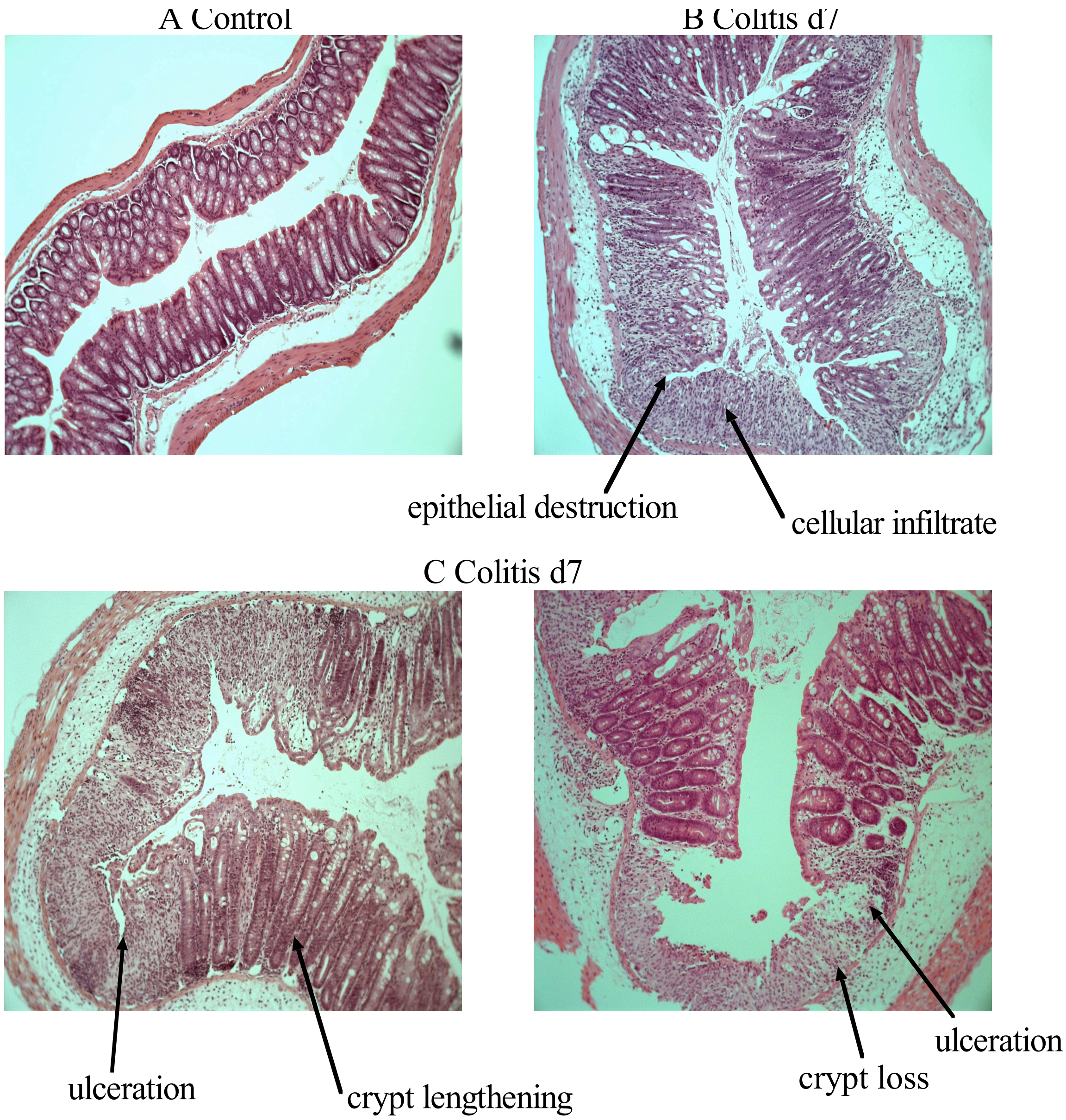


Figure 5.3 Histology of the distal colon from control and colitic mice

Colons from control C57Bl/6 mice (A) or mice fed DSS for 7 days (B + C) were excised and fixed in 10% formalin, embedded in paraffin, and stained with haematoxylin and eosin. Results are representative histological images (magnification x100).

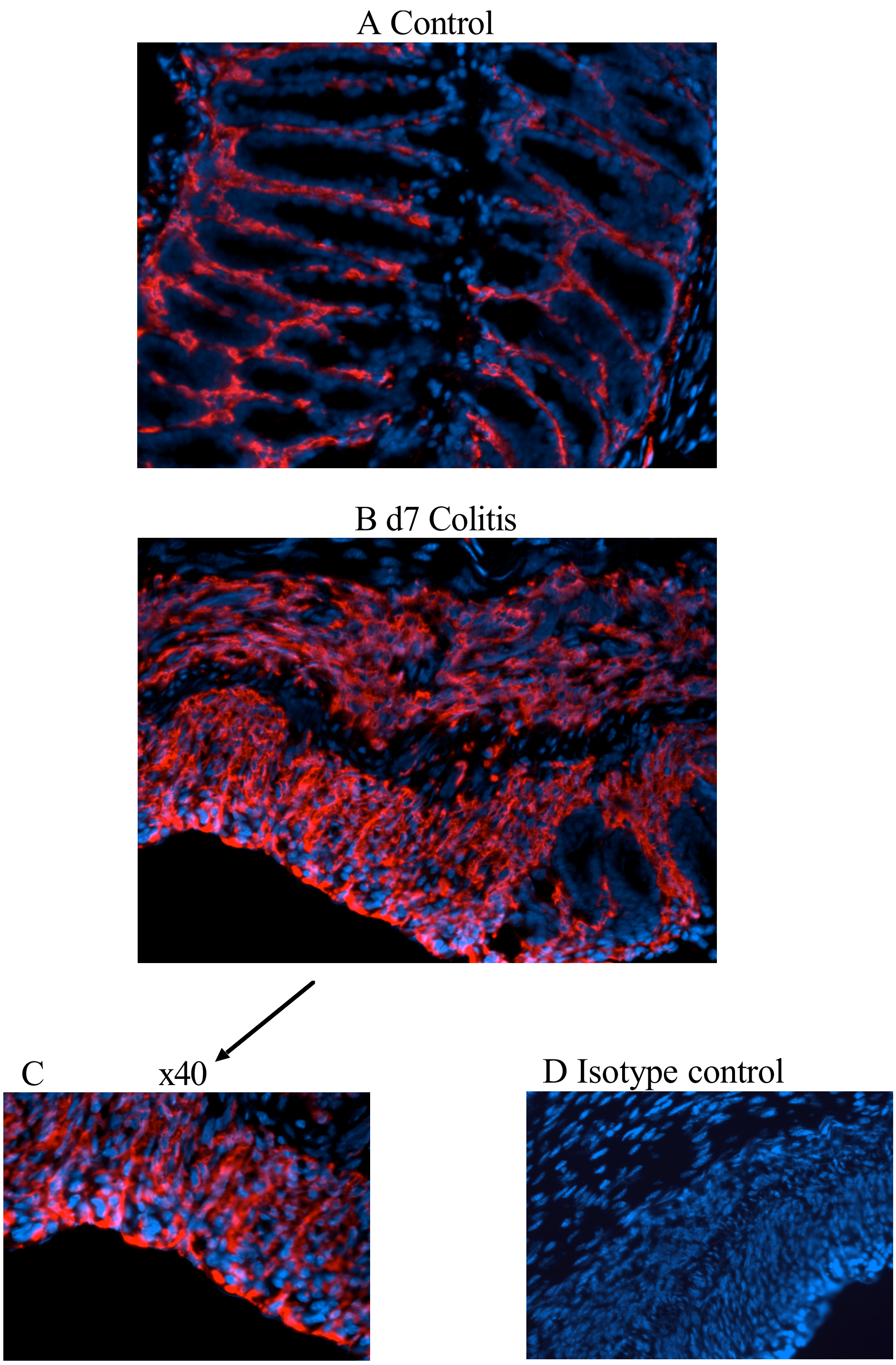
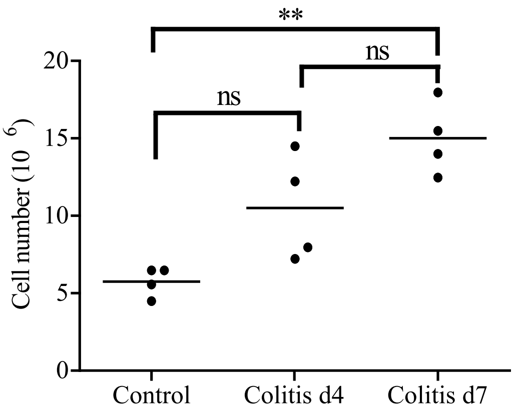


Figure 5.4 Localisation of macrophages in the resting and inflamed colon

Immunofluorescence images show staining with anti-F4/80 (red) and the nuclear stain DAPI (blue) on sections of distal colon from control C57Bl/6 mice (A) or mice fed DSS for 7 days (B). High power images (x40) of inflamed colon were taken after staining with anti-F4/80 (C) or isotype control antibody (D). Images are representative of colons from three individual mice.

A



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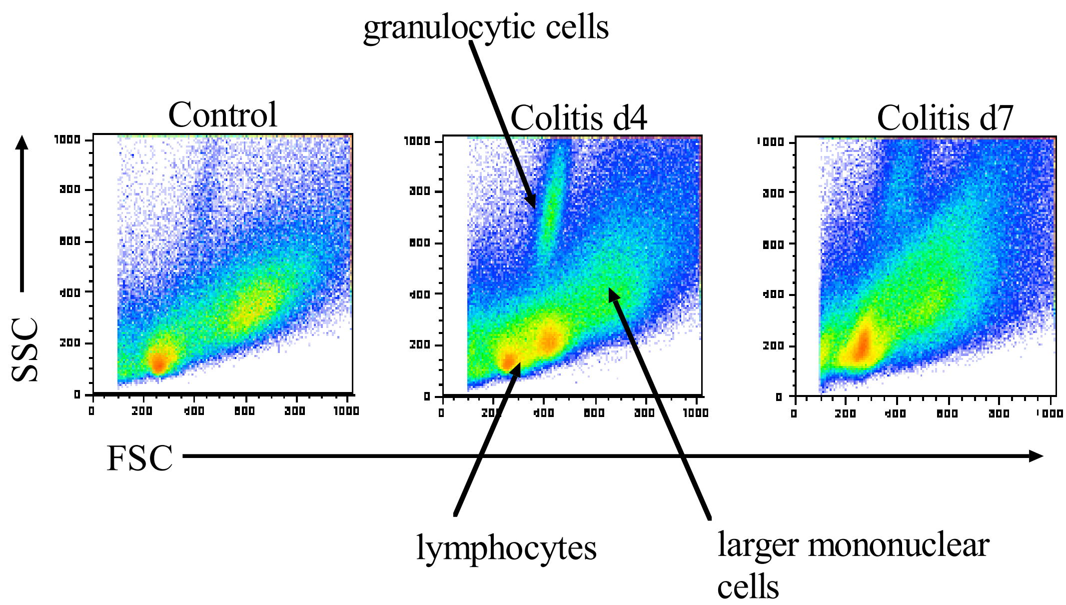
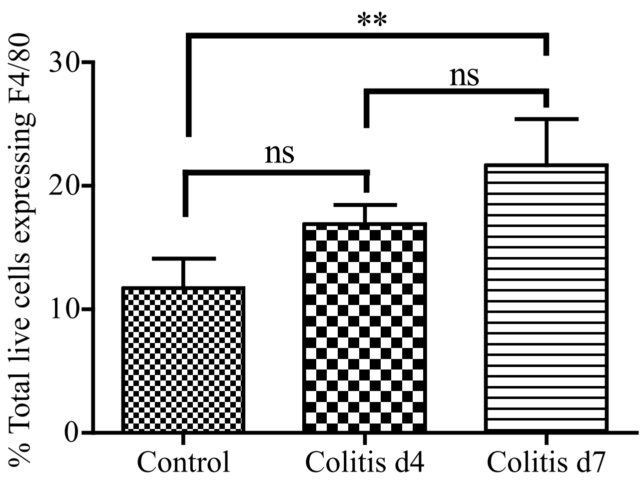


Figure 5.5 Total cellularity of the LP of control and inflamed colon

Live-gated colonic LP cells were isolated and enumerated from C57Bl/6 mice given water or DSS for 4 and 7 days (A). The means ± SD of total numbers of cells for 4 mice/group are shown and are representative of at least four individual experiments. B) Representative FSC vs SSC analysis of LP cells taken on day 4 and 7 of colitis. A one-way ANOVA revealed a significant difference, $F(2,9)=13.91$, $p=0.0018$. Planned comparisons which were significant showed a higher number of cells on colitis d7 compared with control colon (**$p<0.01$), Bonferroni's multiple comparison test. ns=not significant.

A



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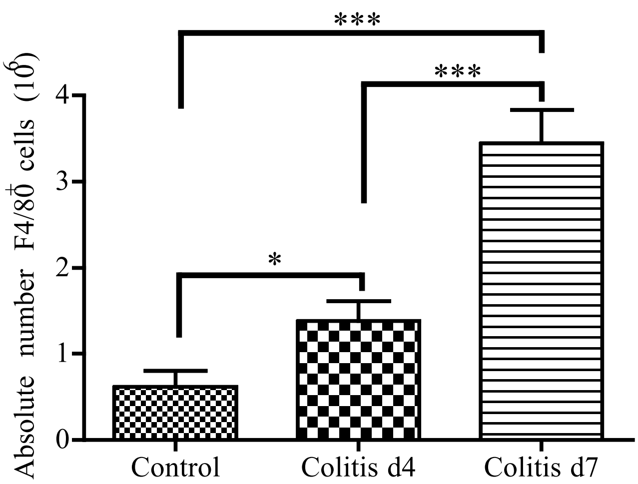


Figure 5.6 Proportions and total number of macrophages in the inflamed colon

Colonic LP cells from C57Bl/6 mice fed water or DSS for 4 or 7 days were examined for the expression of F4/80 by flow cytometry. Percentages (A) and absolute numbers of F4/80$^+$ cells (B) are shown as the means ± SD for 4 mice/group. A one-way ANOVA revealed a significant difference; (A) $F(2,9) = 13.33$, $p<0.0020$; (B) $F(2,9)=108.7$, $p<0.0001$; Planned comparisons were significant (ns=not significant; *$p<0.05$; **$p<0.01$; ***$p<0.001$), Bonferroni's multiple comparison test.

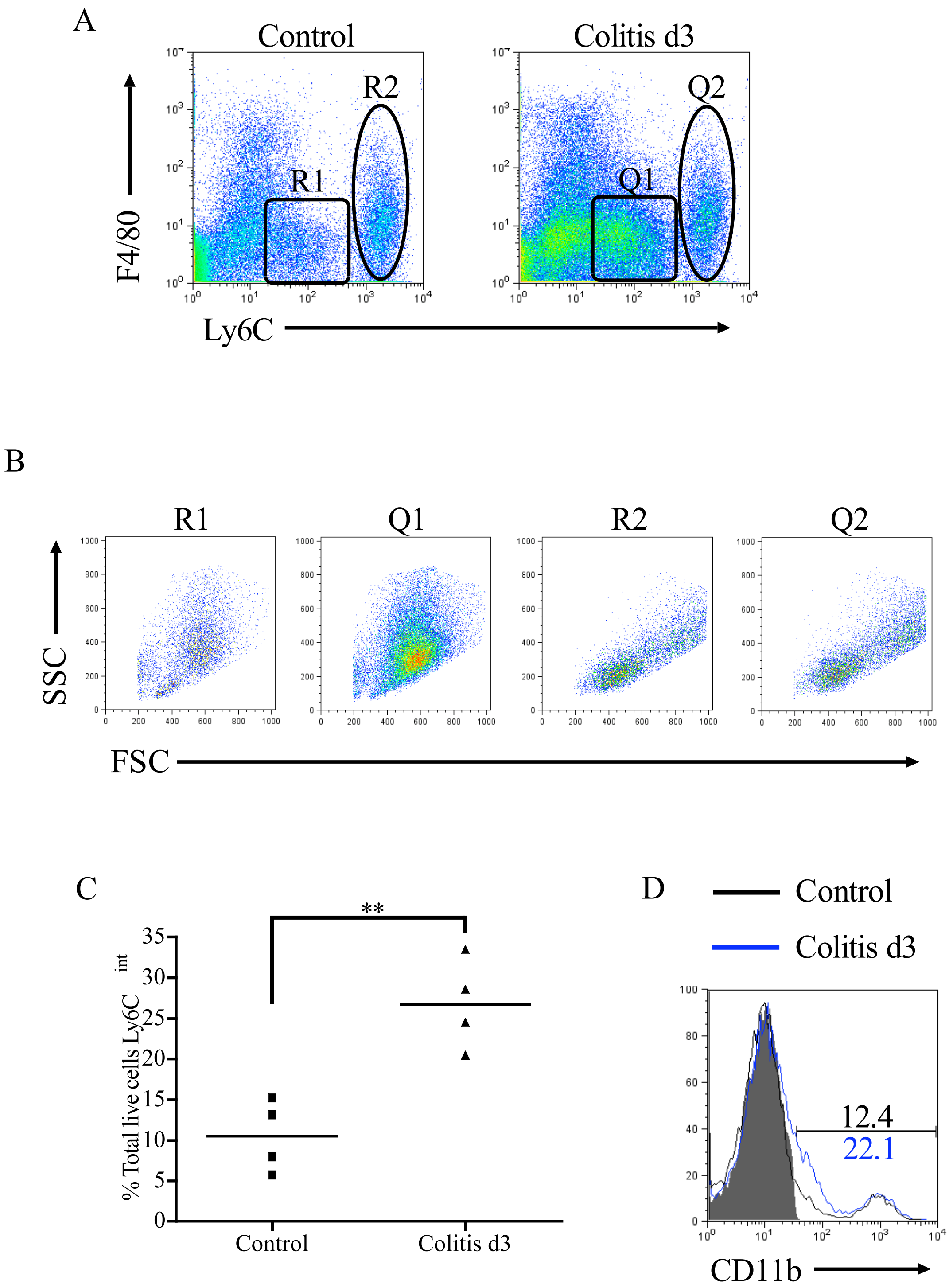


Figure 5.7 Expression of Ly6C by cells in the normal and inflamed colon

Colonic LP cells from C57Bl/6 mice fed water or DSS for 3 days were assessed for the expression of F4/80 and Ly6C by flow cytometry. Representative dot plots obtained from the different groups (A) and FSC and SSC properties (B) of the total Ly6C^{int} and Ly6C^{bright} cell population are shown. C) The mean percentage ± SD of total Ly6C^{int} cells for 4 mice/group and CD11b expression by total Ly6C^{int} cells (D). Data are representative of two individual experiments. **p<0.01.

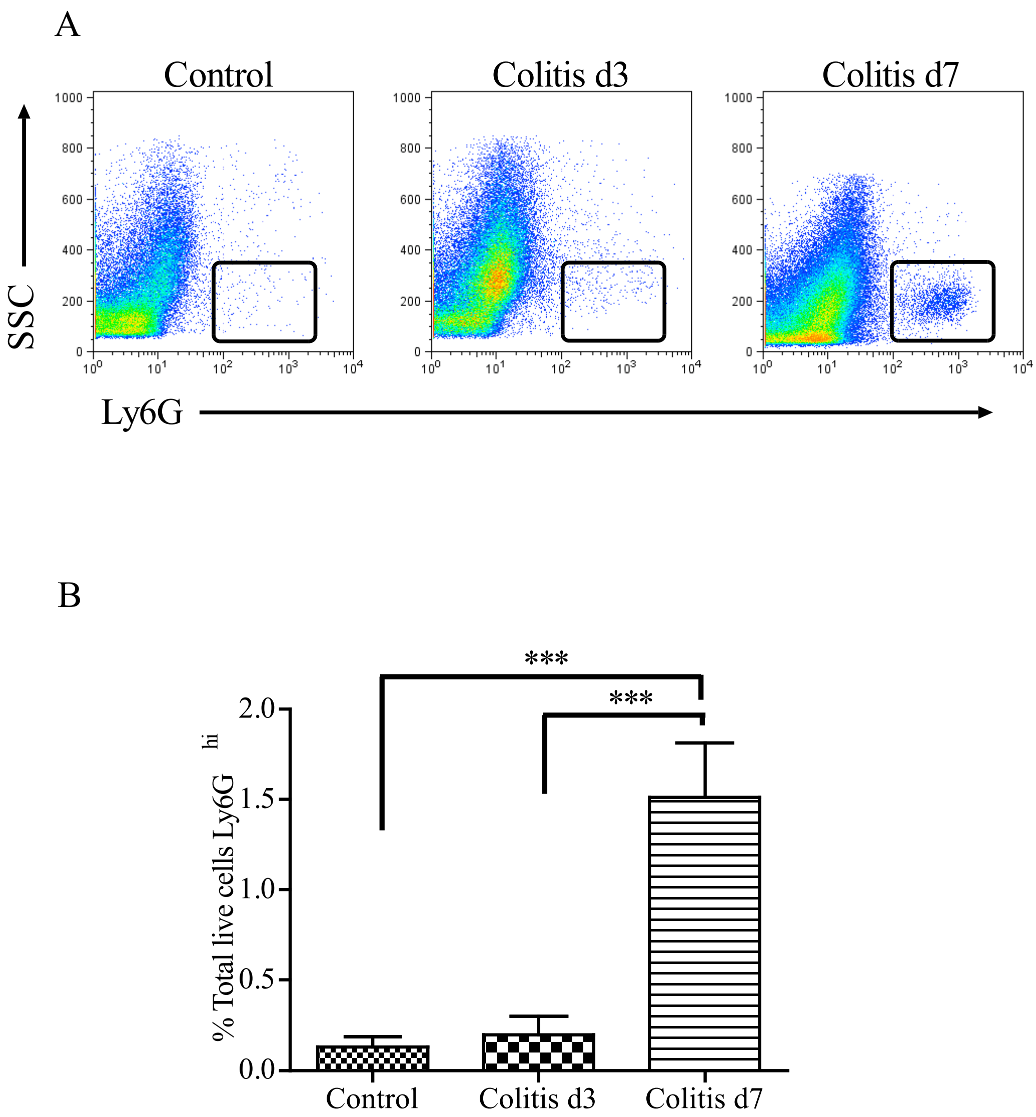
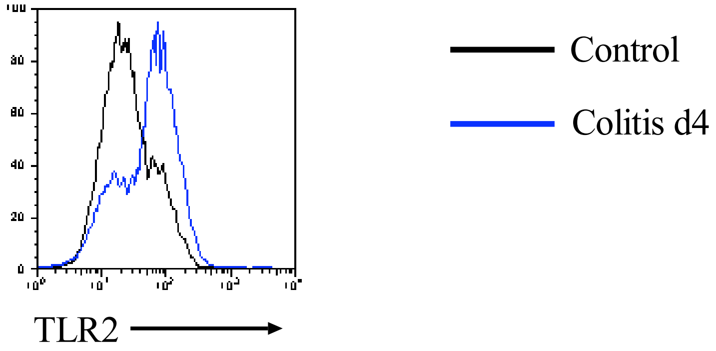


Figure 5.8 Infiltration of colon by Ly6G^{hi} cells during colitis

Colonic LP cells from C57Bl/6 mice fed water or DSS for 3 or 7 days were assessed for the expression of Ly6G by flow cytometry. Representative dot plots (A) and the mean percentages ± SD (B) of total Ly6G^{hi} cells for 4 mice/group. Data are representative of two individual experiments. A one-way ANOVA revealed a significant difference, $F(2,9) = 69.91$, $p<0.0001$. Planned comparisons which were significant showed that there was a higher proportion of Ly6G^{hi} cells on colitis d7 compared with colitis d3 or control colon (***$p<0.001$), Bonferroni's multiple comparison test.

A



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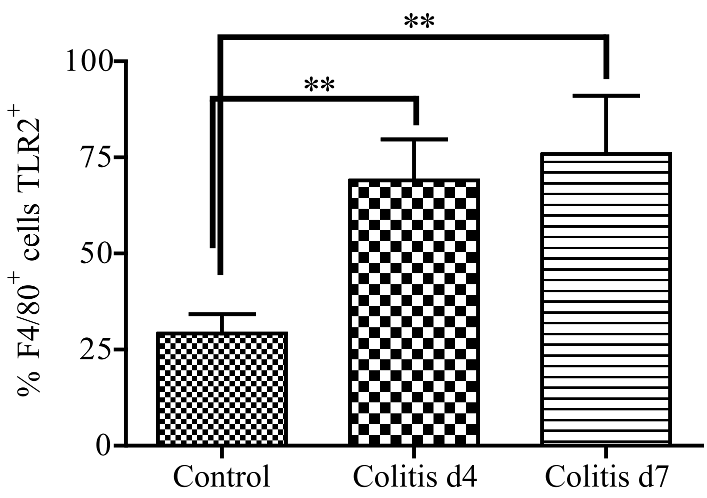


Figure 5.9 Infiltration of colon by TLR2^{+} macrophages during colitis

Colonic F4/80^{+} cells from C57Bl/6 mice fed water or DSS for 4 or 7 days were assessed for the expression of TLR2 by flow cytometry. A) Levels of TLR2 expression on live-gated F4/80^{+} cells from control and colitic mice. B) The mean percentages ± SD of F4/80^{+} cells that express TLR2 for 4 mice/group. The data are representative of at least three individual experiments. A one-way ANOVA revealed a significant difference, $F(2,8) = 15.62$, $p<0.0017$. Planned comparisons which were significant showed that there was a higher proportion of TLR^{+} F4/80^{+} cells on colitis d4 and d7 compared with control colon (**$p<0.01$), Bonferroni's multiple comparison test.

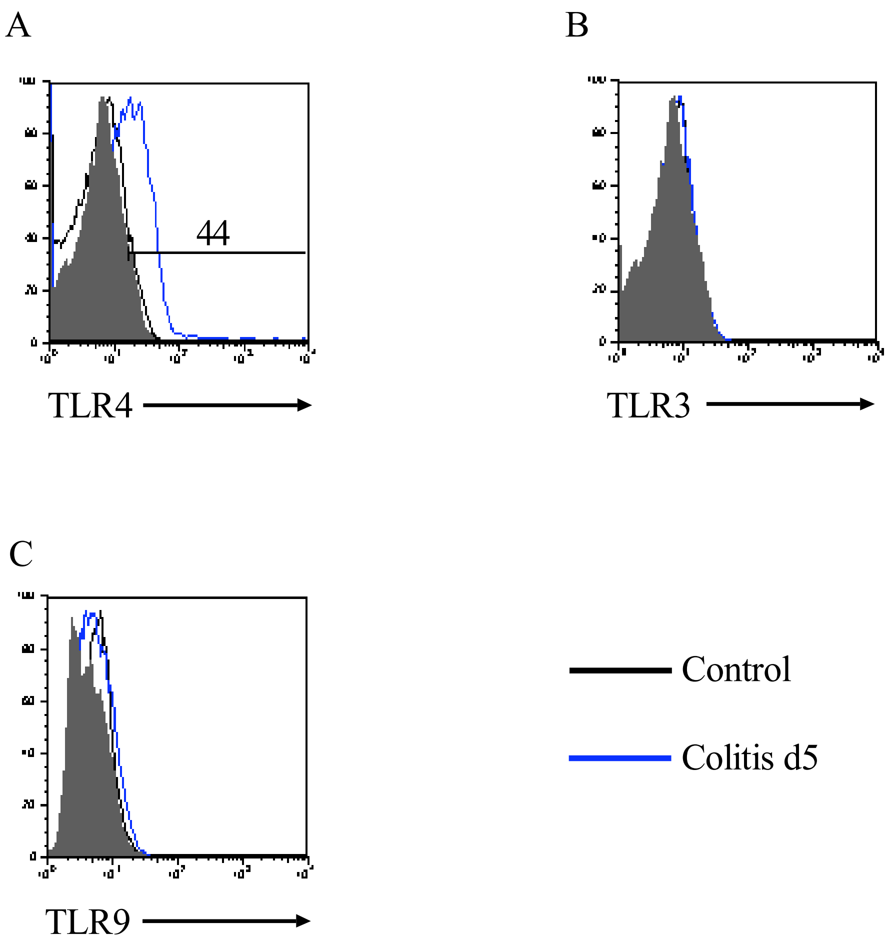


Figure 5.10 Expression of TLRs by intestinal mϕ during colitis

Colonic LP cells from mice fed water or DSS for 5 days were assessed for the expression of surface TLR4 (A), and intracellular TLR3 (B) and TLR9 (C). The data show the proportion of TLR positive cells among live-gated F4/80$^+$ cells and are representative of three individual experiments. Shaded histograms represent staining with isotype controls.

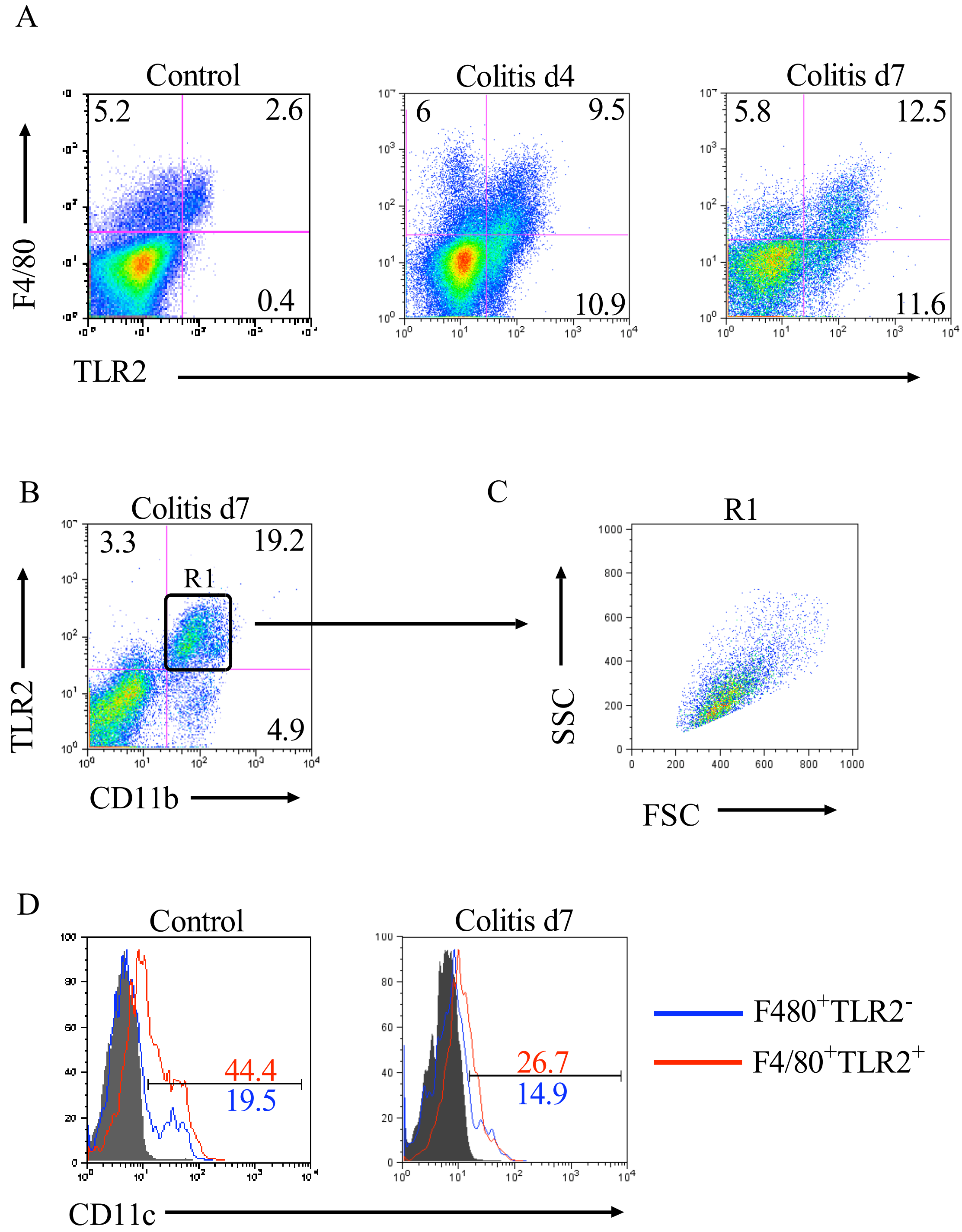


Figure 5.11 Phenotype of TLR2$^+$ cells in the colon

Mice were fed water or DSS for 4 or 7 days and the co-expression of F4/80 (A) and CD11b (B) by live-gated TLR2$^+$ cells was examined. Numbers in quadrants represent the percentages of the total population. C) Forward and side scatter of total CD11b$^+$TLR2$^+$ cells. D) Expression of CD11c by live-gated F4/80$^+$TLR2$^+$ and F4/80$^+$TLR2$^-$ cells. The shaded histograms represent isotype control staining. Results are representative of at least three individual experiments.

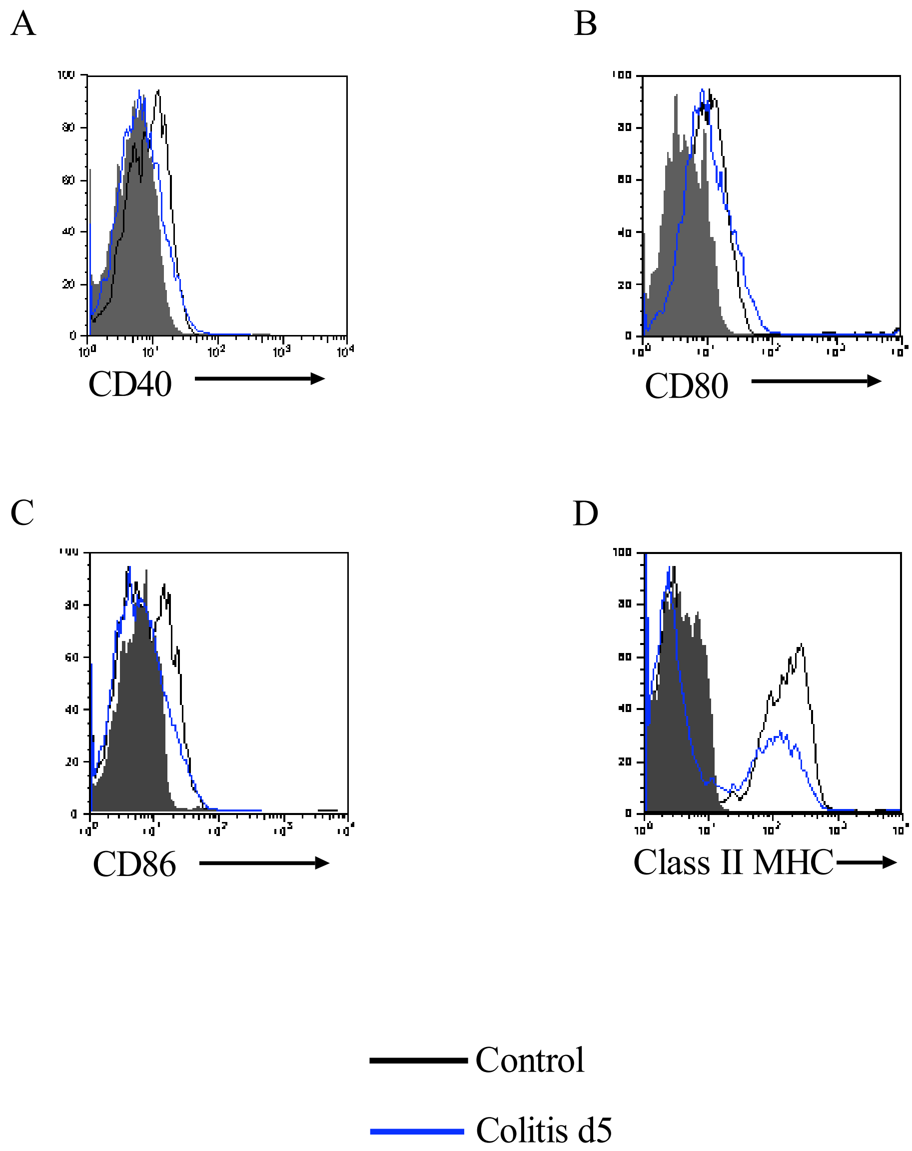


Figure 5.12 Expression of class II MHC and co-stimulatory molecules by total F4/80^{+} cells

Colonic LP cells from C57Bl/6 mice fed water or DSS for 5 days were assessed for the expression of F4/80 and CD40 (A), CD80 (B), CD86 (C) and class II MHC (D) by flow cytometry. Histograms show the proportions of live-gated F4/80^{+} cells positive for each marker, and shaded histograms represent staining with isotype controls. Data are representative of two experiments.

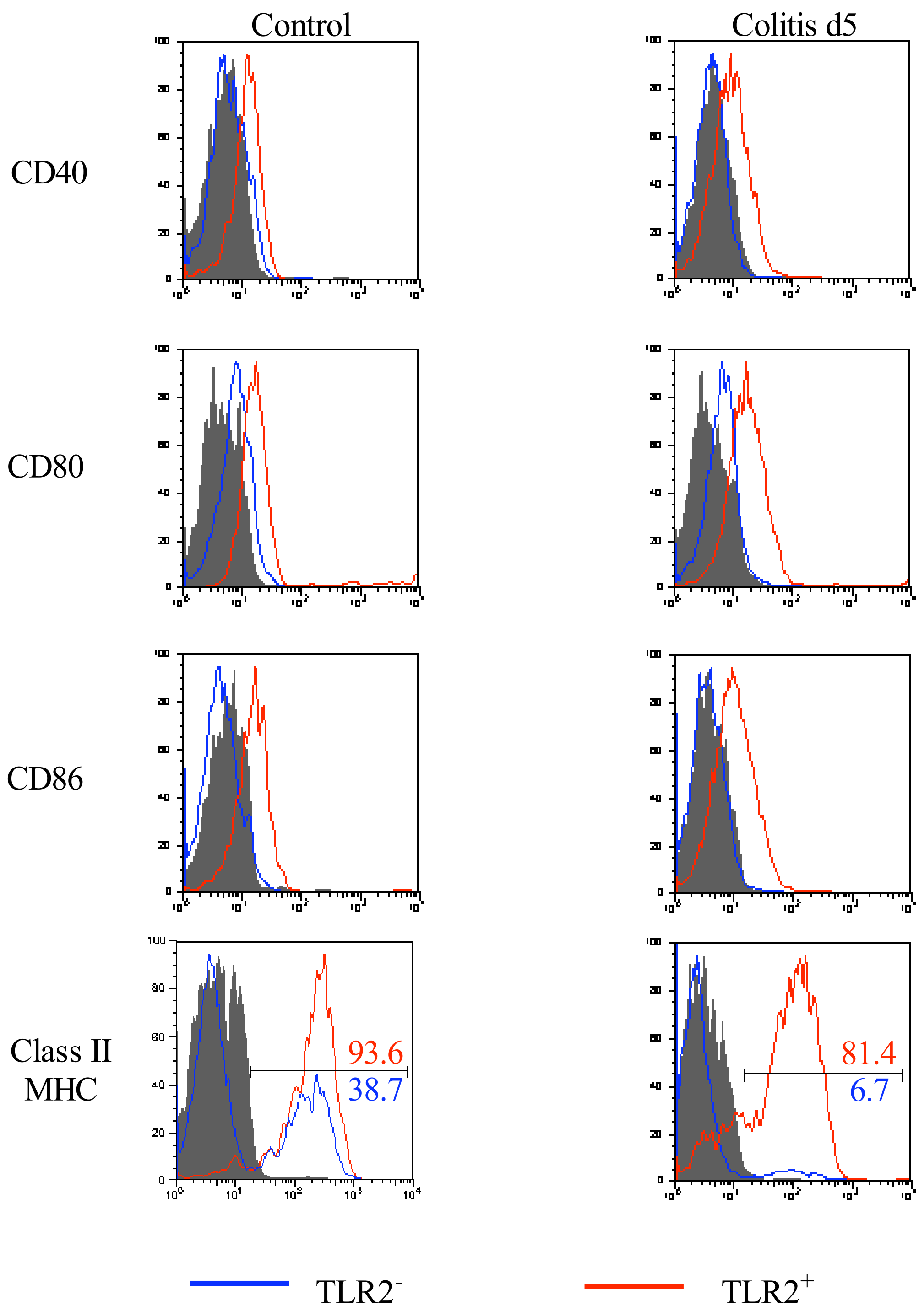


Figure 5.13 Expression of co-stimulatory molecules and class II MHC by TLR2$^-$ and TLR2$^+$ mϕ populations

Colonic LP cells from mice fed water or DSS for 5 days were analysed for the expression of CD40, CD80, CD86 and class II MHC by flow cytometry. Histograms show the proportions of live-gated F4/80$^+$ cells positive for each marker, and shaded histograms represent staining with isotype controls. Data are representative of two experiments.

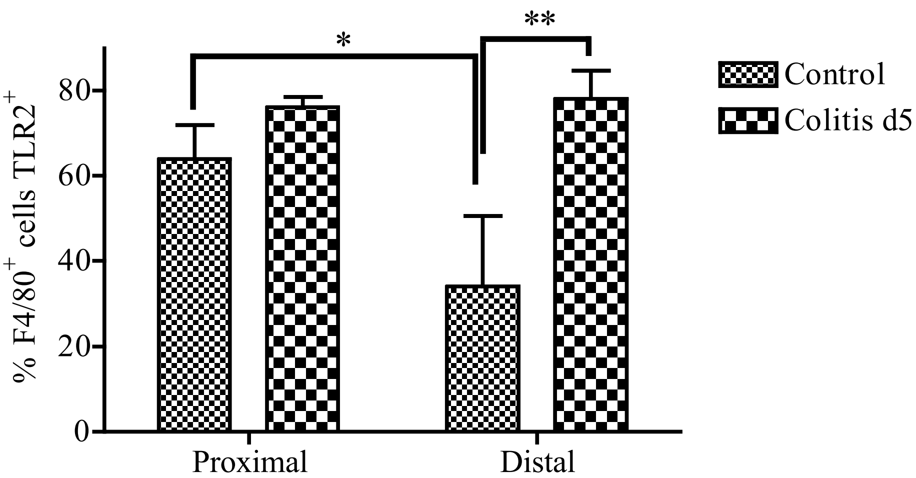


Figure 5.14 Anatomical expression of TLR by mϕ in the resting and inflamed colon

Proportions of TLR2$^+$ F4/80$^+$ cells in the proximal and distal colon of control and mice fed DSS for 5 days. The data shown are the mean percentages ± SD of live-gated F4/80$^+$ cells positive for TLR2 for 3 mice/group. Two-way ANOVAs revealed significant differences, $F(1,1)=6.101$, $p=0.0387$ for control proximal vs distal, $F(1,1)=24.61$, $p=0.0011$ for control vs colitis, Bonferroni-corrected. *$p<0.05$; **$p<0.01$.

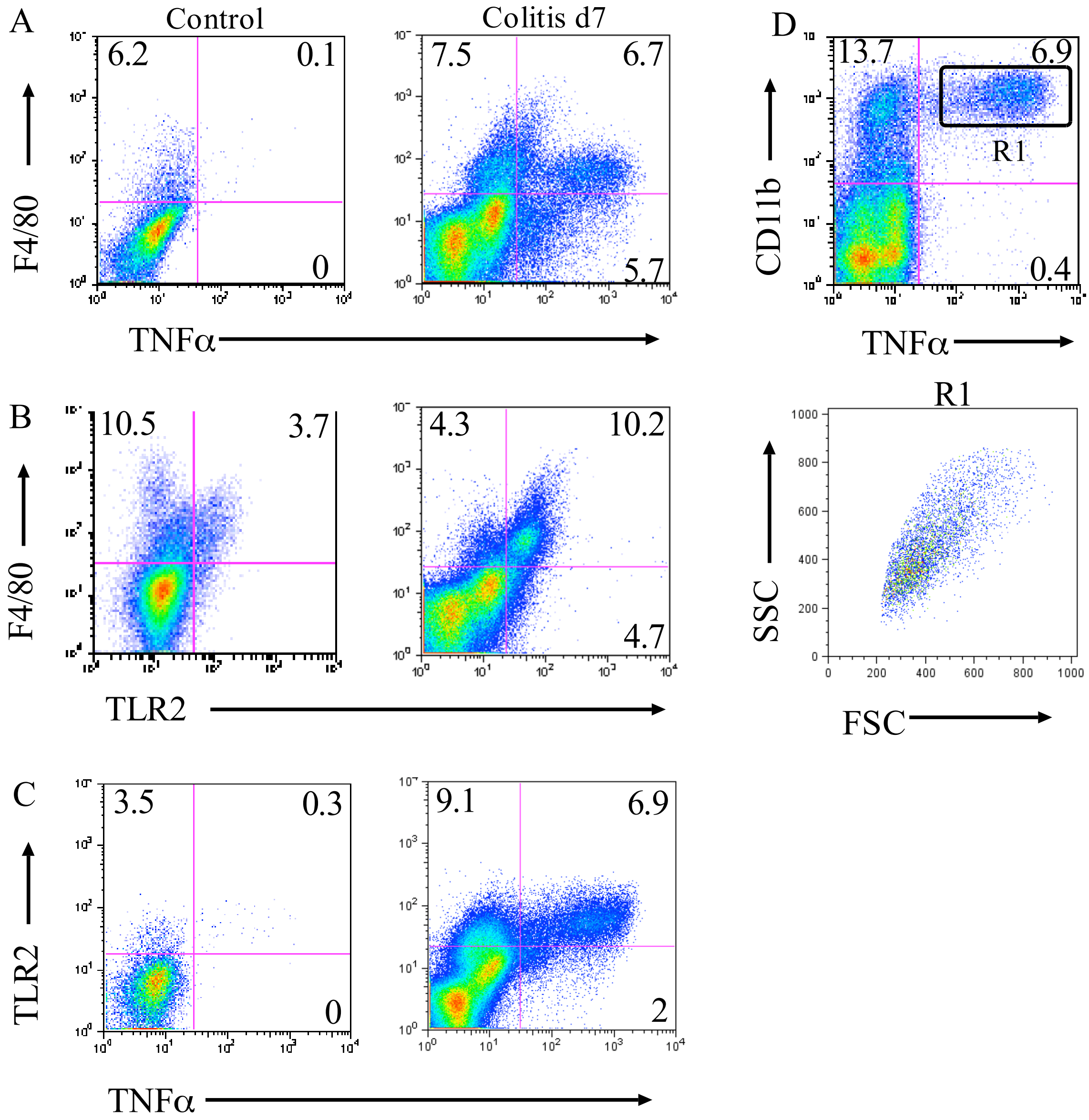


Figure 5.15 Spontaneous TNFα expression by mϕ from the control and inflamed colon

Control C57Bl/6 mice (left panel) or mice given DSS for 7 days (middle panel) were examined for spontaneous expression of cytoplasmic TNFα by flow cytometry. LP cells were cultured for 4.5 hours with Brefeldin-A before analysing the expression of F4/80 vs TNFα (A), F4/80 vs TLR2 (B) and TLR2 vs TNFα (C). D) Cells were also stained for CD11b and TNFα following DSS administration for 7 days, and double positive cells (R1) were assessed for granularity and size. Numbers in quadrants represent percentages of the total population and plots are representative of at least three individual experiments.

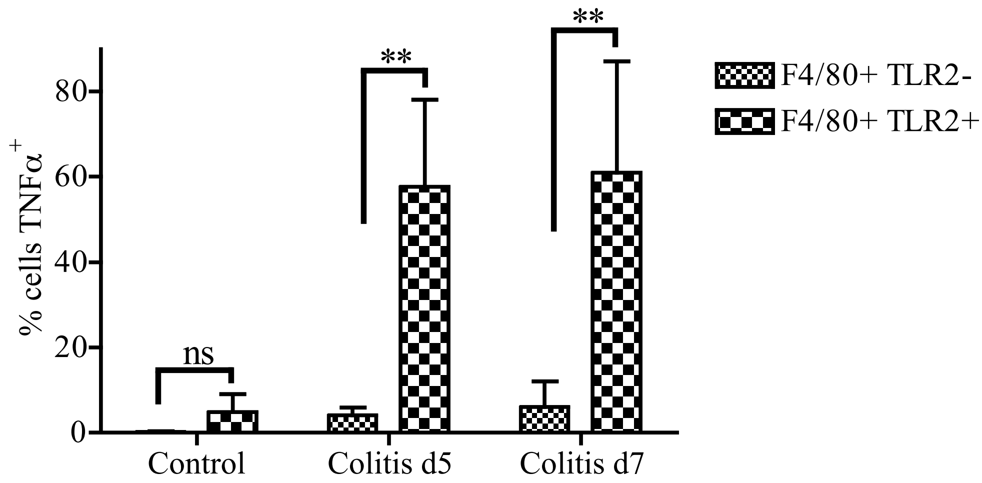


Figure 5.16 Spontaneous TNFα expression by macrophage populations during colitis

Colonic LP cells from control C57Bl/6 mice or mice given DSS for 5 or 7 days were examined for spontaneous expression of cytoplasmic TNFα by flow cytometry. Total LP cells were isolated and cultured for 4.5 hours in the presence of Brefeldin-A before being stained for F4/80, TLR2 and TNFα. The results shown are the mean percentage ± SD for 4 mice/group and are representative of three experiments. ns=not significant; **$p<0.01$.

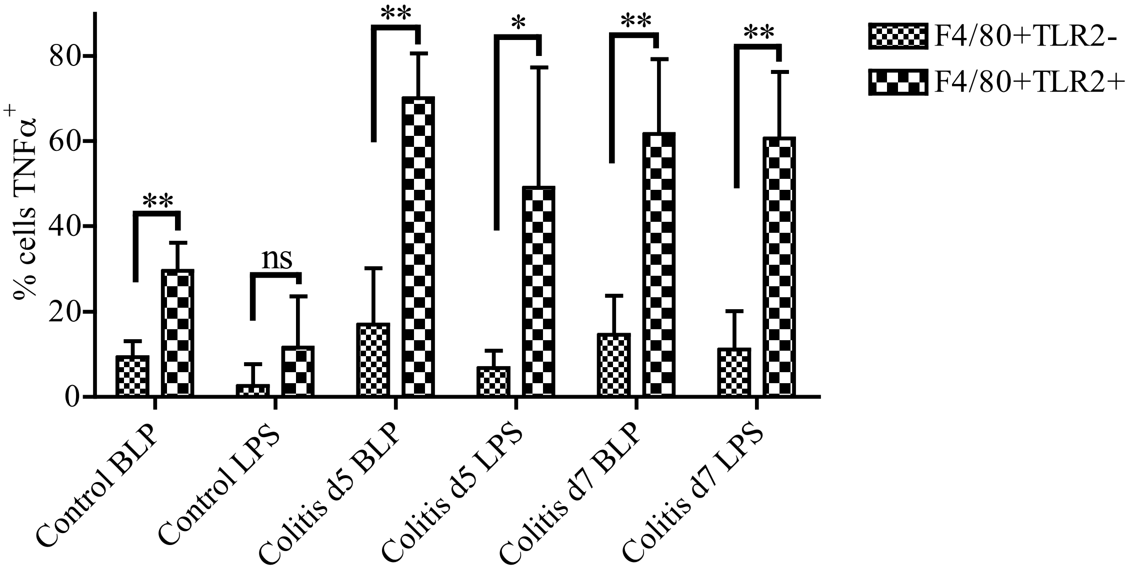


Figure 5.17 TLR-induced TNFα expression by macrophage populations during colitis

Colonic LP cells from control C57Bl/6 mice or mice given DSS for 5 or 7 days were examined for TLR-induced expression of cytoplasmic TNFα by flow cytometry. Total LP cells were cultured in either 1μg/ml BLP or 1μg/ml LPS for 4.5hrs in the presence of Brefeldin-A, before being stained for F4/80, TLR2, and TNFα. The results shown are the mean percentages ± SD for 4 mice/group and are representative of two experiments. ns=not significant; *p<0.05; **p<0.01.

Chapter 6

Origin of F4/80-expressing cells in the inflamed intestine

Introduction

The large increase in mϕ numbers in colitic mucosa could reflect rapid recruitment of new inflammatory F4/80$^+$ monocytic cells into the colon, or could be due to local proliferation and differentiation of the resident mϕ population. During human IBD, it has been suggested that blood monocytes are recruited into the inflamed gut mucosa (208). However, this is difficult to prove under clinical conditions and in particular, it is not known exactly how the inflammatory cells which appear in colitis relate to the normal resident population. In this chapter I have investigated the turnover and population dynamics of the different subsets of F4/80$^+$ cells in the healthy and inflamed gut, concentrating on the TLR$^+$ and TLR$^-$ subsets I defined in previous chapters. In addition, I investigated the expression of the CCR2 inflammatory chemokine receptor which could be involved in recruitment of these cells and attempted to follow the recruitment of adoptively transferred mϕ into the colon of healthy and colitic recipients.

6.1 Turnover of macrophages in the resting and inflamed colon

To explore the relationship between the different subsets of mϕ, I first examined the turnover kinetics in normal and inflamed colon by measuring the uptake of BrdU *in vivo*. Firstly, I assessed the turnover of monocyte precursors in the BM by injecting BrdU i.p. and measuring its uptake by flow cytometry, as I postulated that this population would be proliferating actively and thus allow me to assess the usefulness of the technique, and also give me an idea about the dynamics of monocyte precursors. Within 2hrs after injection, over 50% of F4/80-expressing cells in the BM incorporated BrdU and this increased somewhat 24hrs after injection (Fig 6.1). Thus even under physiological conditions, there is rapid turnover of myeloid cells and their precursors in the BM.

To examine intestinal myeloid cell turnover in the resting state and during inflammation, control and DSS-fed mice were injected i.p. with BrdU, and culled 24 hours later. 4.1±0.6% of the total F4/80$^+$ mϕ population in the resting colonic LP had proliferated in the 24-hour period (Fig 6.2a). As discussed in Chapter 4, a low percentage of F4/80$^+$ cells in the resting colon express TLR2 (Fig 6.2b) and interestingly, more of the TLR2$^+$ subset had proliferated than the TLR2$^-$ subset, which represents the majority of the mϕ in the resting state (6.0±0.8% BrdU$^+$ compared with 1.3±0.5% of the TLR2$^-$ subset; Fig 6.3e). Indeed, >70% of the F4/80$^+$BrdU$^+$ cells expressed TLR2. The proportions of recently divided F4/80$^+$ mϕ increased dramatically during colitis to 16.8±5.1% and 18.9±4.7% of the total F4/80$^+$ population after a 24-hour pulse with BrdU on days 3→4 and 5→6, respectively (Fig 6.3). As in the control colon, the proportions of TLR2$^+$F4/80$^+$ cells incorporating BrdU were significantly higher than those among the TLR$^-$ subset, with 21.0±5.7% and 21.9±2.4% of TLR2$^+$ cells being BrdU$^+$ on days 3→4 and 5→6 of colitis, respectively (Fig 6.3e). Indeed >96% of the F4/80$^+$BrdU$^+$ cells present on day 5→6 of colitis expressed TLR2. In contrast, BrdU uptake by the TLR2$^-$ fraction from the same animals increased very little in colitis, to 4.7±1.4% and 5.9±5.0% on days 3→4 and 5→6 of colitis, respectively (Fig 6.3e).

A significant amount of BrdU incorporation occurred in the F4/80$^-$ fraction of colonic cells in control mice and approximately 70% of these cells were negative for CD45, indicating they were of non-haematopoietic origin, possibly epithelial cells (Fig 6.2d). During colitis, less than 30% of F4/80$^-$ BrdU$^+$ cells were CD45$^-$, suggesting that there is a decrease in proliferation of non-haematopoietic cells and/or a proportional increase in the proliferation of CD45$^+$ F4/80$^-$ cells (Fig 6.3d).

When analysing BrdU incorporation by mϕ in colitic mice, I noticed that a substantial number of BrdU$^+$ cells expressed lower levels of F4/80 compared with the BrdU$^+$ cells in the control colon, which either expressed high levels of F4/80, or were completely negative (Fig 6.4a+b). Thus the proportion of CD45$^+$F4/80^{lo} cells almost doubled from ~20% under resting conditions to ~40% during inflammation (Fig 6.4a). It seems likely that these BrdU$^+$F4/80^{lo} cells represent the F4/80^{lo}TLR2$^+$TNFα^+ cells I found in colitis in Chapter 5. Despite their low levels of F4/80 expression, the majority (90%) of these F4/80^{lo} BrdU$^+$ cells expressed high levels of CD11b and had the FSC/SSC characteristics of mononuclear cells (Fig 6.4c+d). These are probably cells recently derived from blood monocytes/BM precursors, which are known to express lower levels of F4/80 than tissue mϕ (43).

To investigate whether there was *in situ* proliferation of F4/80$^+$ cells in the colon, I assessed the expression of the nuclear antigen, Ki-67, in colonic mϕ from the resting and inflamed intestine. Ki-67 protein is present during all active phases of cell cycle, so is an ideal marker to examine when trying to identify cells within a population that are actively dividing. As shown in Fig 6.5, Ki-67 expression was virtually undetectable among F4/80$^+$ cells from the resting colon (0.5±0.1%). Although this increased significantly on d5 of colitis to 1.5±0.3% of the F4/80$^+$ population, this was much less than the number of BrdU$^+$F4/80$^+$ cells at similar timepoints (~20%), indicating that local proliferation is unlikely to account for either the BrdU uptake, or for the dramatic rise in mϕ numbers seen during inflammation.

To gain a better idea of the turnover of mϕ subsets in the normal and inflamed colon, I used a long-term BrdU administration protocol. Thus control and DSS-fed mice received BrdU throughout the experiment starting with an i.p. injection on day 0, followed by administration in the drinking water for 6 days. Mice were culled 1 day, 4 days and 6 days after the initiation of BrdU treatment. As shown in Fig 6.6a, the percentage of total F4/80$^+$ BrdU$^+$ mϕ in normal colon rose steadily from 4.0±0.2% after 1 day of feeding, to 18.4±2.9% and 24.1±2.7% after 4 and 6 days, respectively. In the group receiving DSS, the uptake of BrdU by F4/80$^+$ cells was similar to that in controls (4.6±0.8%) after 1 day, but by d4 and d6 of colitis, 26.7±3.3% and 39.8±3.8% of F4/80$^+$ mϕ had incorporated BrdU.

Linear regression analysis of these data showed that the turnover rate of mϕ rose significantly from 4.1±0.4% mϕ per day under resting conditions, to 7.1±0.5% mϕ per day during colitis (Fig 6.6b).

Dramatic differences were seen when BrdU incorporation by the F4/80$^+$ population was compared on the basis of TLR2 expression. As with the total population of F4/80$^+$ cells, the uptake of BrdU by the TLR2$^+$ F4/80$^+$ subset from control mice rose from 7.8±0.8% on day 1, to 37.3±2.6% and 44.0±5.9% on days 4 and 6, respectively (Fig 6.7a). However, there was no significant increase in the rate of proliferation by these TLR2$^+$F4/80$^+$ cells during colitis, which showed identical values to those in control mice at all times rising from 7.5±0.9% after 1 day, to 41.2±3.0% and 48.5±4.6% after 4 and 6 days, respectively. Notably, although the levels of BrdU incorporation by the TLR2$^-$ subset of F4/80$^+$ cells also rose in control colon over time, from 1.9±0.3% after 1 day, to 8.8±3.3% and 16.6±3.2% after 4 and 6 days, respectively (Fig 6.7a), these levels were significantly lower than the equivalent values among the TLR2$^+$ cells at all times ($p=0.0003$). The TLR2$^-$ population also showed increased BrdU incorporation over time during colitis, rising from 2.5±0.7% after 1 day, to 5.0±1.0% and 15.5±3.3% after 4 and 6 days, respectively. Like the TLR2$^+$ population, the overall turnover of the TLR2$^-$ cells did not significantly change during colitis compared with control mice, but at all times these levels were significantly less than the TLR2$^+$ subset in colitic mice ($p=<0.0001$). Linear regression analysis confirmed these differences between TLR2$^+$ and TLR2$^-$ mϕ, with overall turnover rates of 7.4±0.9% and 8.4±0.9% TLR2$^+$ mϕ per day in resting and inflamed colon versus 2.9±0.4%

and 2.5±0.5% TLR2$^-$ mϕ per day. These analyses also confirmed that the overall turnover of the individual subsets was not different in the resting and inflamed intestine (Fig 6.7b).

To ensure that any differences in the levels of BrdU incorporation were not due to differences in uptake of the BrdU-containing water between the groups, I measured daily water consumption in water and DSS-fed groups. As shown in Fig 6.8, water intake in the healthy group averaged around 4-6ml per mouse per day. This decreased to <3ml by d3-4 of colitis and then increased again in subsequent days to similar levels consumed by the water control group, suggesting increased BrdU intake did not account for the increased level of BrdU incorporation observed in the DSS-fed group.

6.2 Expression of CCR2 and Gr-1 inflammatory markers by colonic mϕ in healthy and inflamed intestine

As the lack of Ki-67 expression indicated that the F4/80$^+$ cells that had incorporated BrdU had done so outside the intestine, I assessed whether these cells might express the CCR2 chemokine receptor which is known to be involved in recruitment of inflammatory mϕ to the intestine (327). Strikingly, 73.4±5.5% of the TLR2-expressing population of mϕ in the resting intestine expressed high levels of CCR2, compared with only 22.6±4.5% of the dominant TLR2$^-$ population (Fig 6.9a). Similar differences concerning the CCR2 expression by each subset were seen during colitis, with 74.4±8.4% and 76.8±5.3% of the TLR2$^+$ mϕ expressing CCR2 on days 3 and 7, respectively, compared with 27.5±3.0% and 16.6±4.3% of the TLR2$^-$ population. The TLR2-expressing mϕ also expressed higher levels of the receptor than the TLR2$^-$ subset under both resting and inflammatory conditions (Fig 6.9b). Thus TLR2$^+$ mϕ in the colon have constitutively higher levels of CCR2 expression than their TLR2$^-$ counterparts and this does not change during colitis.

As CCR2 and the Gr-1 granulocytic marker have been shown to be co-expressed on the subset of inflammatory monocytes described in other studies, I examined if this applied to the mϕ subpopulations in the inflamed intestine by assessing the expression of Gr-1. As shown in Chapter 3, 29.6±7.8% of total F4/80$^+$ mϕ in the resting intestine expressed Gr-1 and this rose during colitis, particularly at later timepoints (Fig 6.10). Significantly higher proportions of the TLR2$^+$ subset expressed Gr-1 at all timepoints compared with the TLR2$^-$ subset, and although the proportion of Gr-1-expressing F4/80$^+$TLR2$^+$ cells decreased slightly on day 3 of colitis, this rose again by day 7. These results are further support for the TLR2$^+$ subset being derived from inflammatory CCR2$^+$Gr-1$^+$ monocytes in both the resting and inflamed colon.

6.3 Adoptive transfer of macrophages

I next set out to follow the recruitment of myeloid cell precursors into the intestine by more direct means, with the ultimate aims of determining their phenotype and monitoring their adaptation upon arrival in the normal or inflamed gut. To try and do this, I transferred *in vitro*-differentiated BMM from Ly5.1$^+$ donors into normal Ly5.2$^+$ recipients and analysed the appearance of donor cells in the BM, PLN, MLN, peritoneum, spleen and colonic LP 24 and 48 hours later. However donor BMM could not be detected in any tissue apart from the peritoneum (Fig 6.11), where they were found in very low numbers at both 24 and 48 hours after transfer (Fig 6.12). I tried to repeat the experiment to obtain sufficient cells in the intestine for analysis, but this was unsuccessful.

Summary

In this chapter, I have demonstrated that the appearance of increased numbers of phenotypically and functionally distinct mφ during intestinal inflammation involves substantial proliferation of myeloid cells, as measured by the incorporation of BrdU by total F4/80$^+$ cells. However, there was minimal proliferation of mφ *in situ*, suggesting that colonic mφ had proliferated outside the intestine before being recruited into the tissue. Although there was a significantly increased rate of mφ turnover throughout the course of experimental colitis, this proliferation was restricted primarily to the TLR2-expressing population of mφ. Interestingly, this population showed identical levels of turnover in the resting and inflamed intestine, while the TLR2$^-$ population had much lower turnover kinetics under both conditions. A substantial population of the BrdU$^+$ TLR2$^+$ cells expressed low-intermediate levels of F4/80 and high levels of CD11b, suggesting these may be recently derived from blood monocytes. In addition, these cells expressed high levels of CCR2 and some Gr-1, markers of the inflammatory monocyte subset. Together with my earlier finding that the TLR2$^+$ mφ are the principal source of TNFα in colitis, these results suggest that the functionally competent TLR2$^+$ mφ that appear during inflammation represent recruitment of recently divided inflammatory monocytes. As a small population of TLR2$^+$ CCR2$^+$ BrdU$^+$ mφ is also present in the resting colon, there may be constant turnover of these cells even in the absence of inflammation. Importantly, these cells are quite distinct from their $TLR2^-$ counterparts which are not turning over rapidly and are $CCR2^-$.

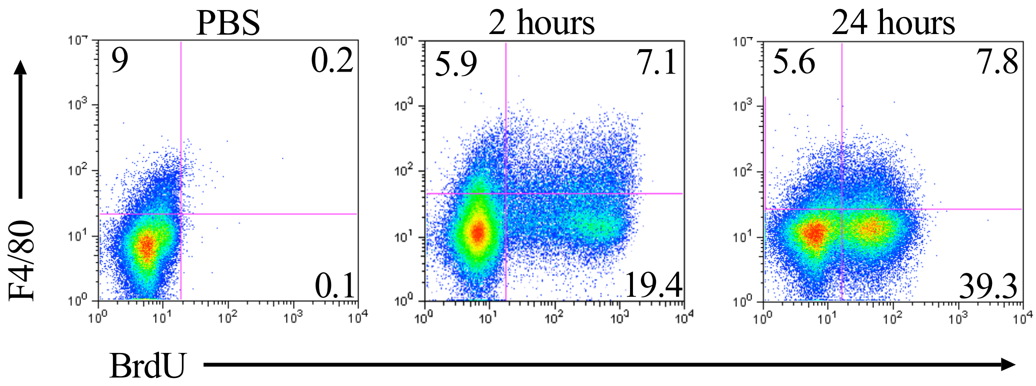


Figure 6.1 Turnover of monocyte precursors in the BM

Control C57Bl/6 mice were injected with PBS (left panel) or 1mg BrdU i.p., culled 2hrs (middle panel) or 24hrs later (right panel), and F4/80$^+$ BM cells were analysed for BrdU incorporation by flow cytometry. Numbers in quadrants represent percentages of the total, live-gated population.

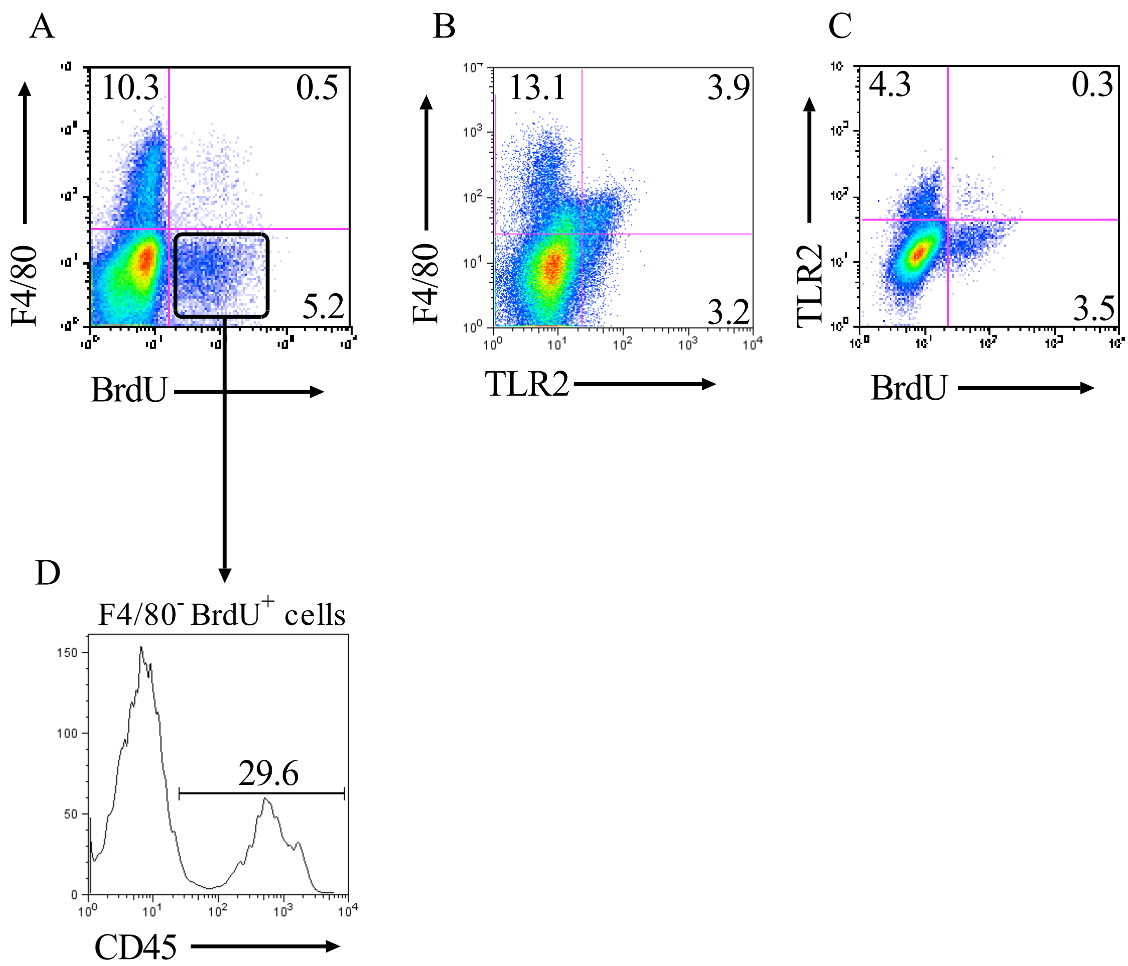


Figure 6.2 Macrophage turnover in the resting colon

Control C57Bl/6 mice were injected with 1mg BrdU i.p, and culled 24 hours later, when colonic LP cells were harvested and stained for the expression of F4/80, TLR2 and BrdU incorporation (A, B, C). Numbers in quadrants represent percentages of the total, live-gated population. D) The expression of CD45 by live F4/80$^-$ BrdU$^+$ cells. The results are representative of at least three individual experiments.

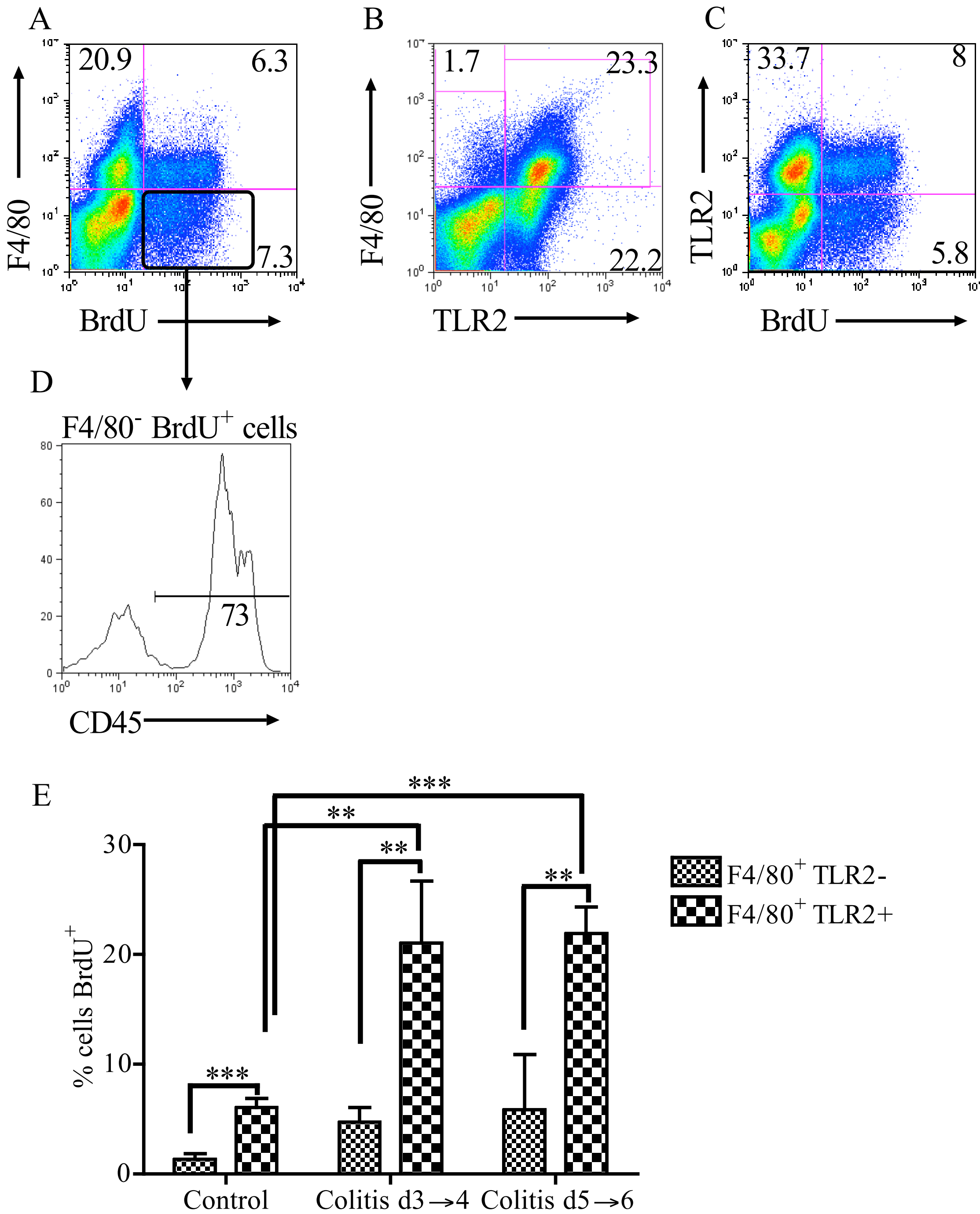


Figure 6.3 Macrophage turnover in the inflamed colon

C57Bl/6 mice were fed water or DSS and injected with 1mg BrdU i.p. on day 3 or 5, and culled a day later. A, B, and C show representative plots of colonic LP cells from mice fed DSS for 6 days and stained for the expression of F4/80, TLR2 and BrdU incorporation. D) The expression of CD45 by live F4/80^{-} BrdU^{+} cells. E) Mean percentages ± SD of F4/80^{+} TLR2^{+} and F4/80^{+} TLR2^{-} cells which have incorporated BrdU from 4 mice/group at different stages of colitis. ** $p<0.01$; *** $p<0.001$.

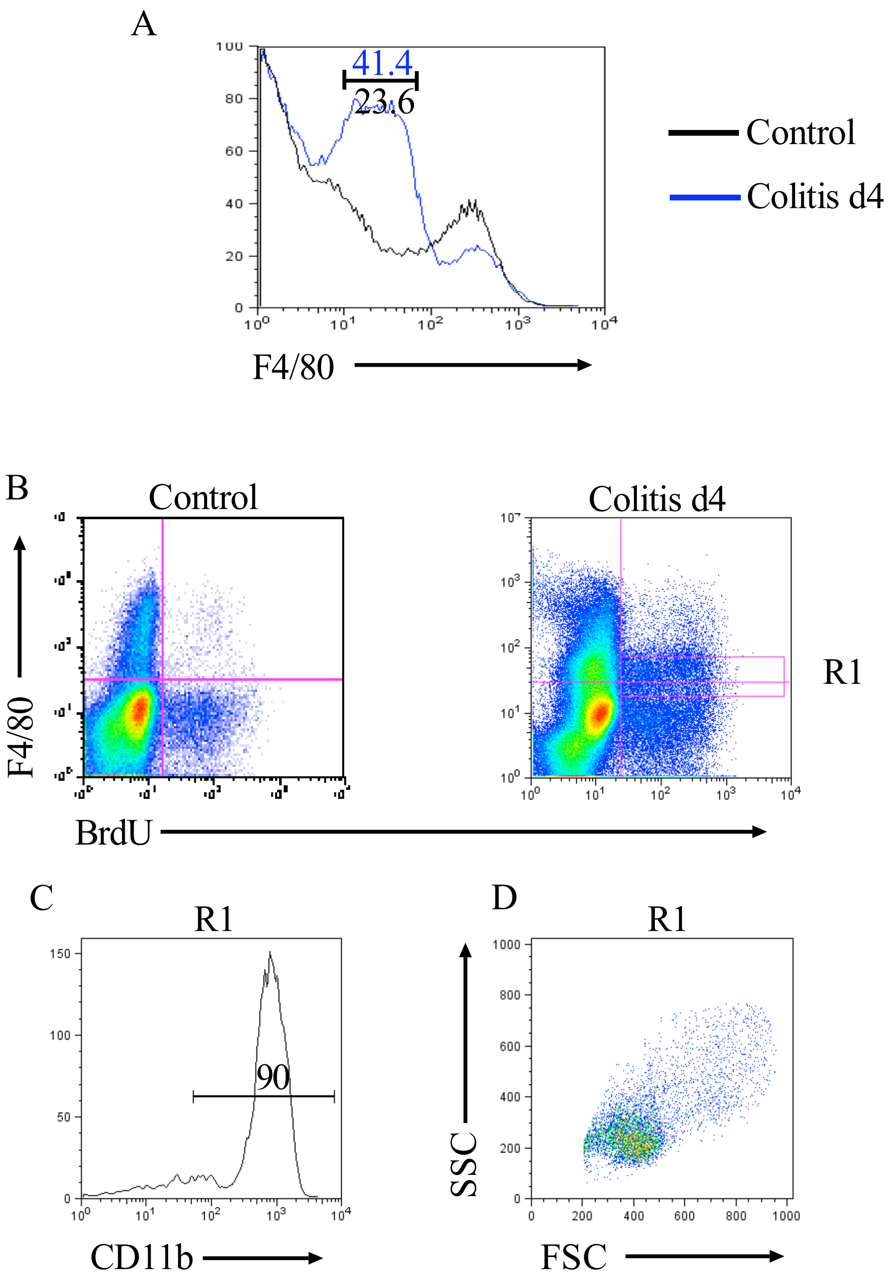


Figure 6.4 Turnover and phenotype of F4/80^{int} mϕ in colon

C57Bl/6 mice were fed water or DSS for 4 days and colonic LP cells were examined for the expression of CD45 and F4/80. A) Levels of F4/80 expression by live CD45^{+} cells. B) Mice fed water or DSS for 4 days were injected with 1mg BrdU i.p. on d3, culled 24 hours later and colonic LP cells harvested. Live-gated F4/80^{int} BrdU^{+} cells (R1) were examined for the expression of CD11b (C) and for FSC and SSC properties (D).

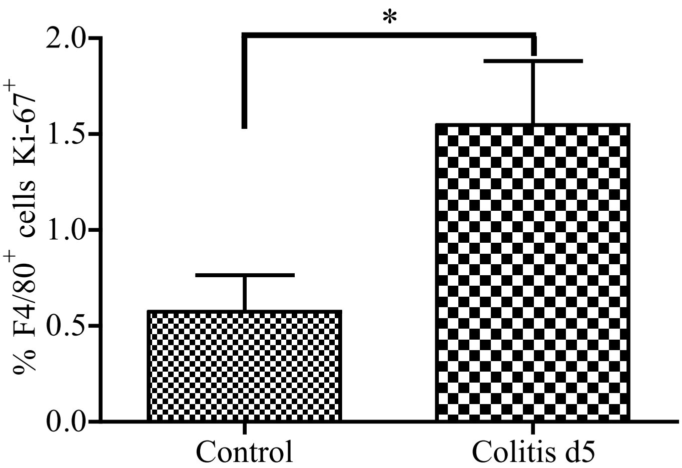
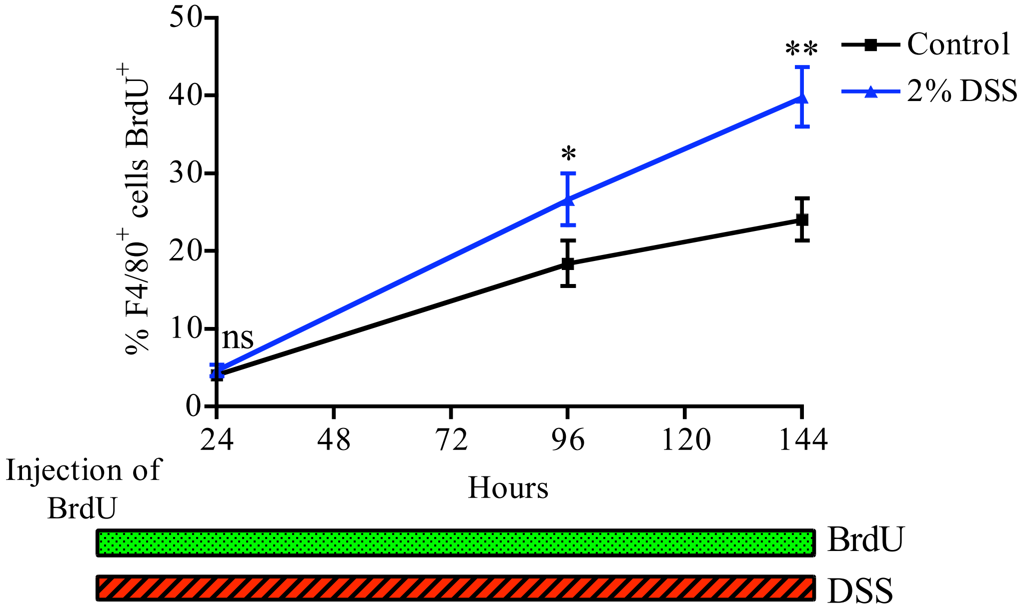


Figure 6.5 ***In situ*** **proliferation of mϕ in the resting and inflamed intestine**

Colonic LP cells from C57Bl/6 mice fed water or DSS for 5 days were stained for F4/80 and permeabilised for the detection of the nuclear antigen, Ki-67, by flow cytometry. Results are shown as the mean percentages ± SD of live F4/80$^+$ cells that are Ki-67$^+$ from 3 mice/group and are representative of two individual experiments. *p=0.011.

A



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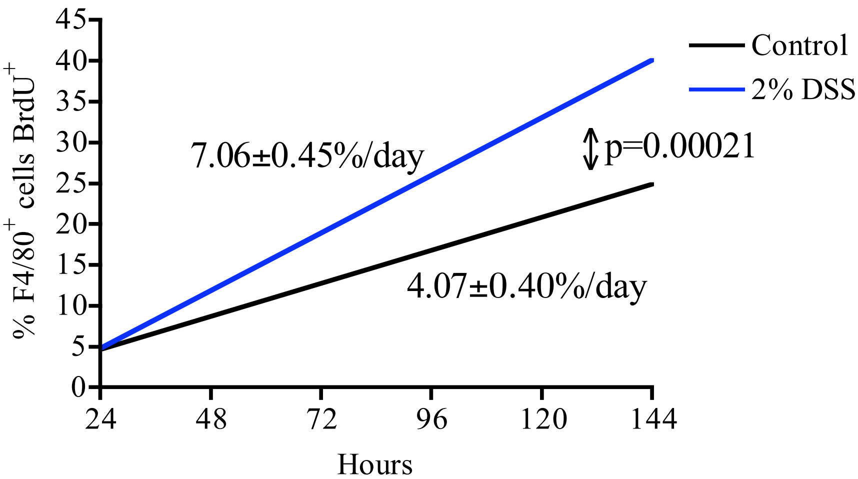


Figure 6.6 Turnover rates of colonic mϕ in the resting and inflamed colon

C57Bl/6 mice were given a single injection of 1mg BrdU i.p. on day 0 and drinking water containing 0.8mg/ml BrdU was administered from day 0 onwards. Some mice then had DSS added to the water, while controls received BrdU in water alone. Colon LP cells were harvested 24 hrs, 4 days or 6 days later and analysed for the expression of F4/80 and BrdU incorporation by flow cytometry. A) The mean percentages ± SD of total, live-gated F4/80^{+} cells positive for BrdU from 3 mice/group. B) Rate of turnover of F4/80^{+} cells calculated by linear regression analysis of BrdU incorporation. ns=not significant; *p<0.05; **p<0.01.

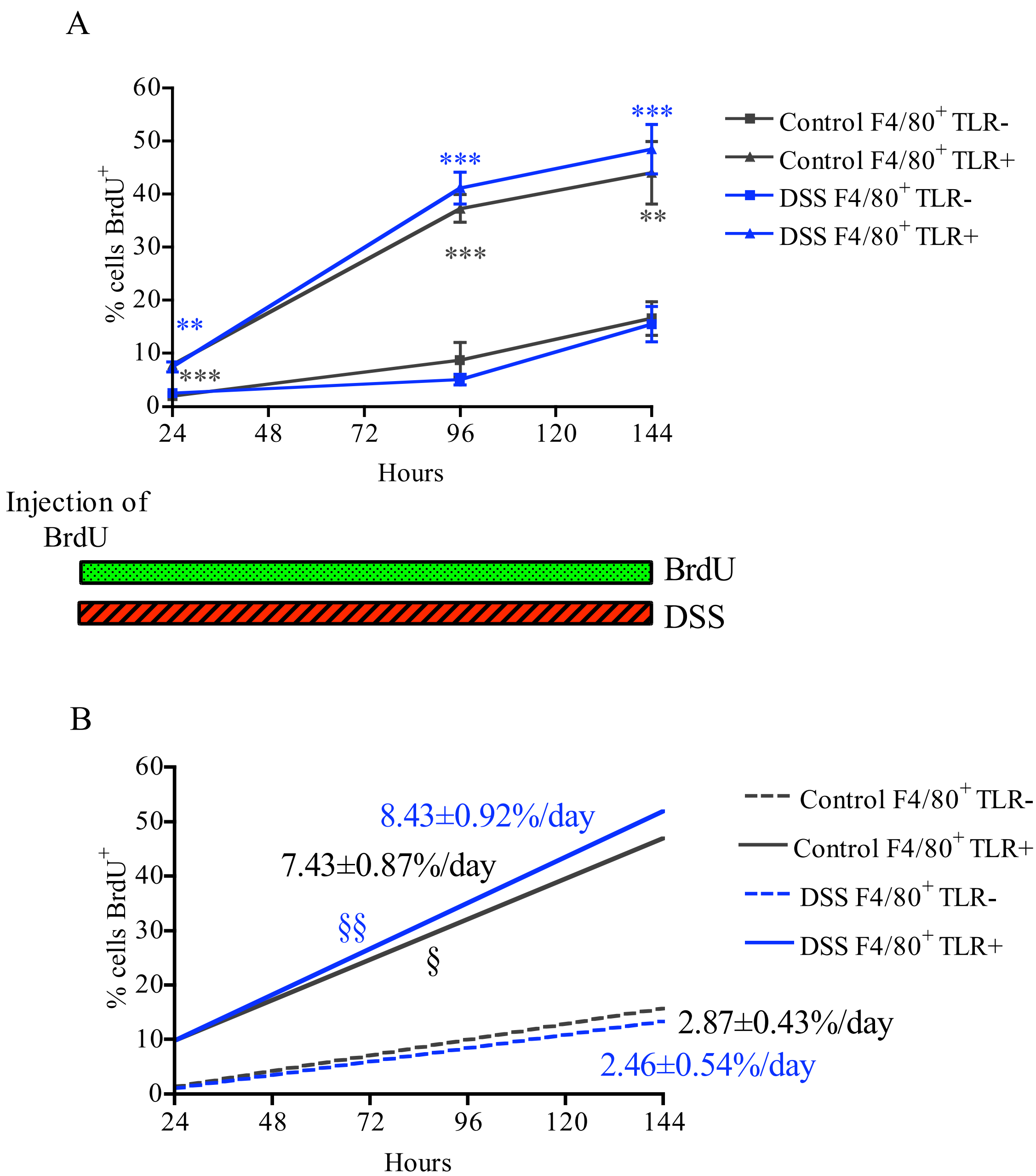


Figure 6.7 Turnover rates of colonic mϕ subsets in the resting and inflamed colon

C57Bl/6 mice were given a single injection of 1mg BrdU i.p. on day 0 and drinking water containing 0.8mg/ml BrdU was administered from day 0 onwards. Some mice then had DSS added to the water, while controls received BrdU in water alone. Colon LP cells were harvested 24 hrs, 4 days or 6 days later and analysed for the expression of F4/80, TLR2 and BrdU incorporation by flow cytometry. A) The mean percentages ± SD of total, live-gated F4/80$^+$TLR2$^+$ and F4/80$^+$TLR2$^-$ cells positive for BrdU from 3 mice/group. B) Rate of turnover of the two subsets calculated by linear regression analysis of BrdU incorporation. **p<0.01; ***p<0.001; §§p<0.0001; §p=0.0003.

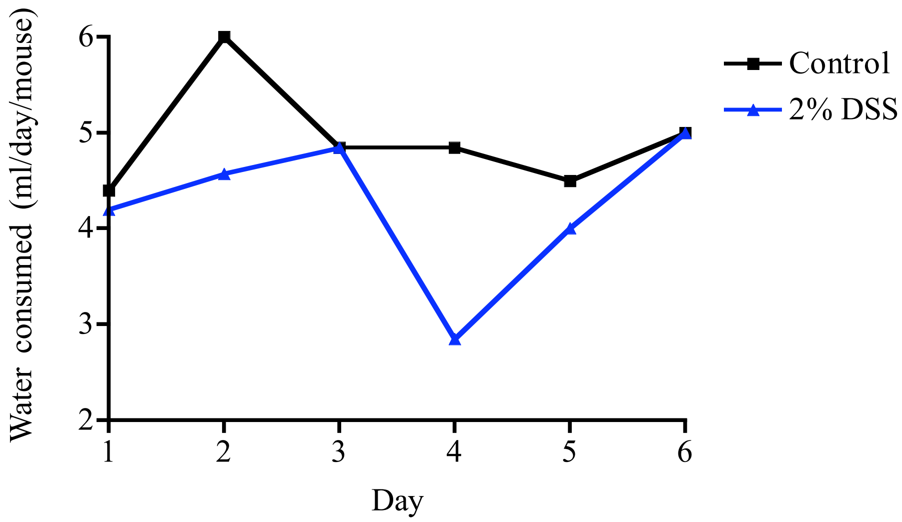
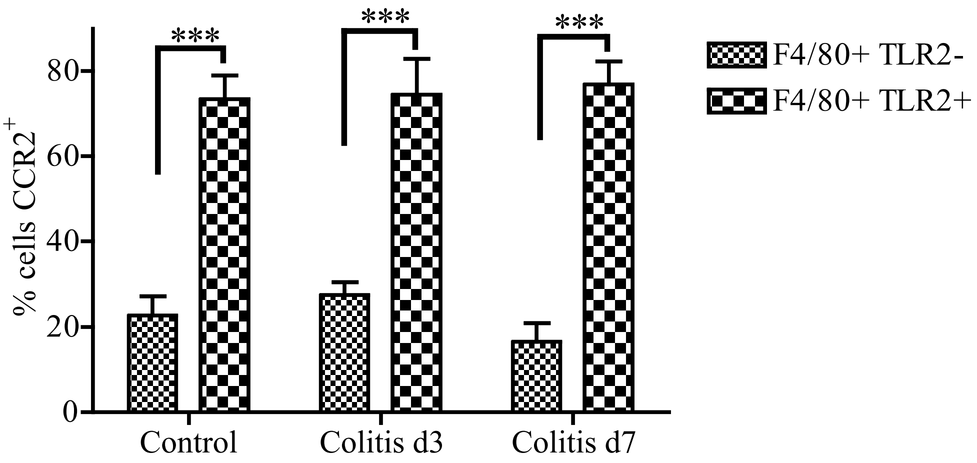


Figure 6.8 Consumption of BrdU-containing drinking water by control and colitic mice

C57Bl/6 mice were given 0.8mg/ml BrdU in their drinking water and some mice also received 2% DSS. Results are shown as the estimated volume of water consumed per day per mouse in each group calculated as described in the Materials and Methods.

A



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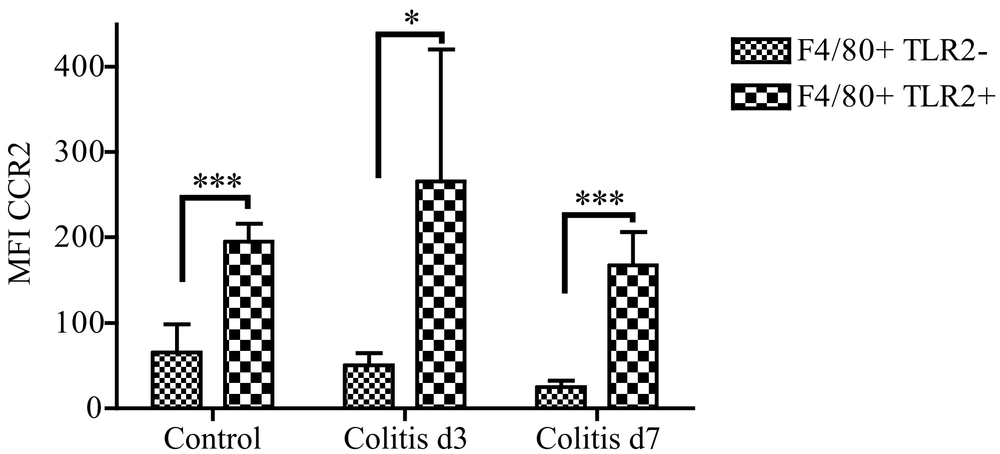


Figure 6.9 Expression of CCR2 by macrophages in the resting and inflamed intestine

C57Bl/6 mice were fed water or DSS for 3 or 7 days, and total live-gated colonic LP cells were analysed for the expression of F4/80, TLR2 and CCR2 by flow cytometry. Results are shown as the mean percentages (A) and mean MFI (B) ± SD of $F4/80^+$ $TLR2^-$ and $F4/80^+$ $TLR2^+$ cells expressing CCR2 from 4 mice/group. The results are representative of two individual experiments. *p=0.031; ***p<0.001.

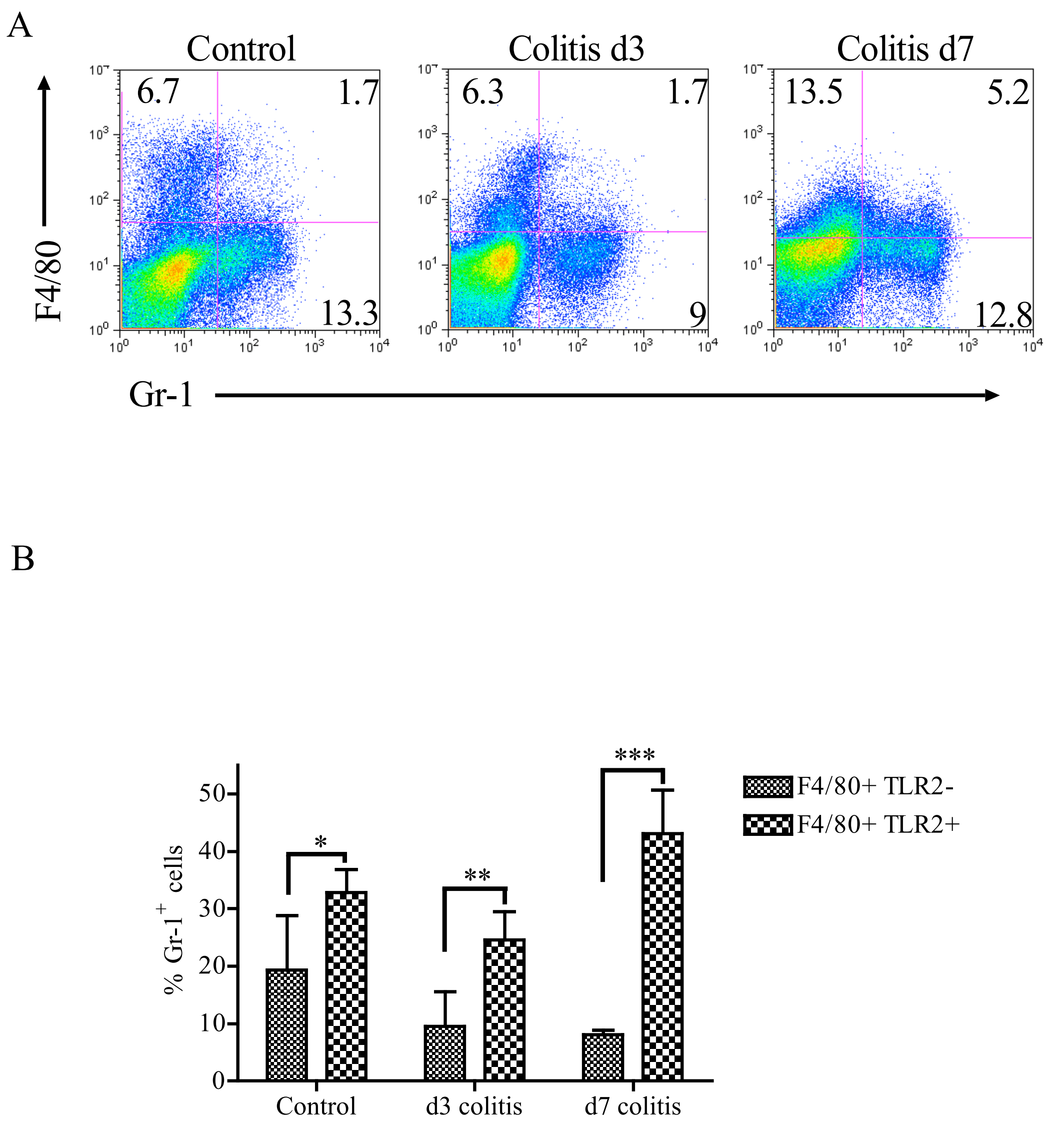


Figure 6.10 Expression of Gr-1 by macrophages in the resting and inflamed intestine

C57Bl/6 mice were fed water or DSS for 3 or 7 days and total live-gated colon LP cells were analysed for the expression of F4/80, TLR2 and Gr-1 by flow cytometry (A). B) The mean percentage ± SD of F4/80^{+}TLR2^{-} and F4/80^{+}TLR2^{+} cells positive for Gr-1 from 4 mice/group. The results are representative of two individual experiments. ns=not significant; *p<0.05; **p<0.01; ***p<0.001.

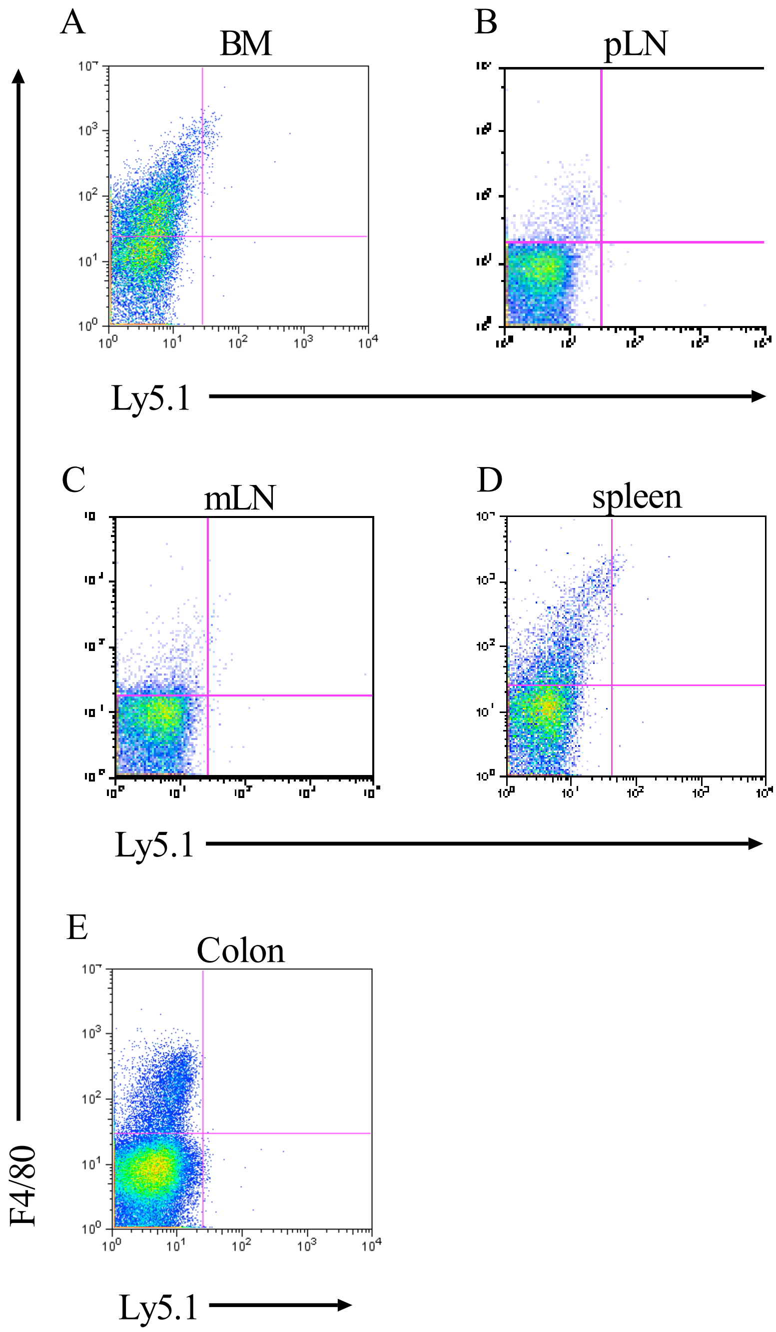


Figure 6.11 Analysis of recipient tissues after adoptive transfer of BMM

1x10^6 Ly5.1 BMM were transferred i.v. into congenic Ly5.2 recipients and BM (A), pLN (B), mLN (C), spleen (D) and colon (E) cells were harvested from recipient mice 24 hrs later. The presence of Ly5.1$^+$ F4/80$^+$ cells was assessed by flow cytometry.

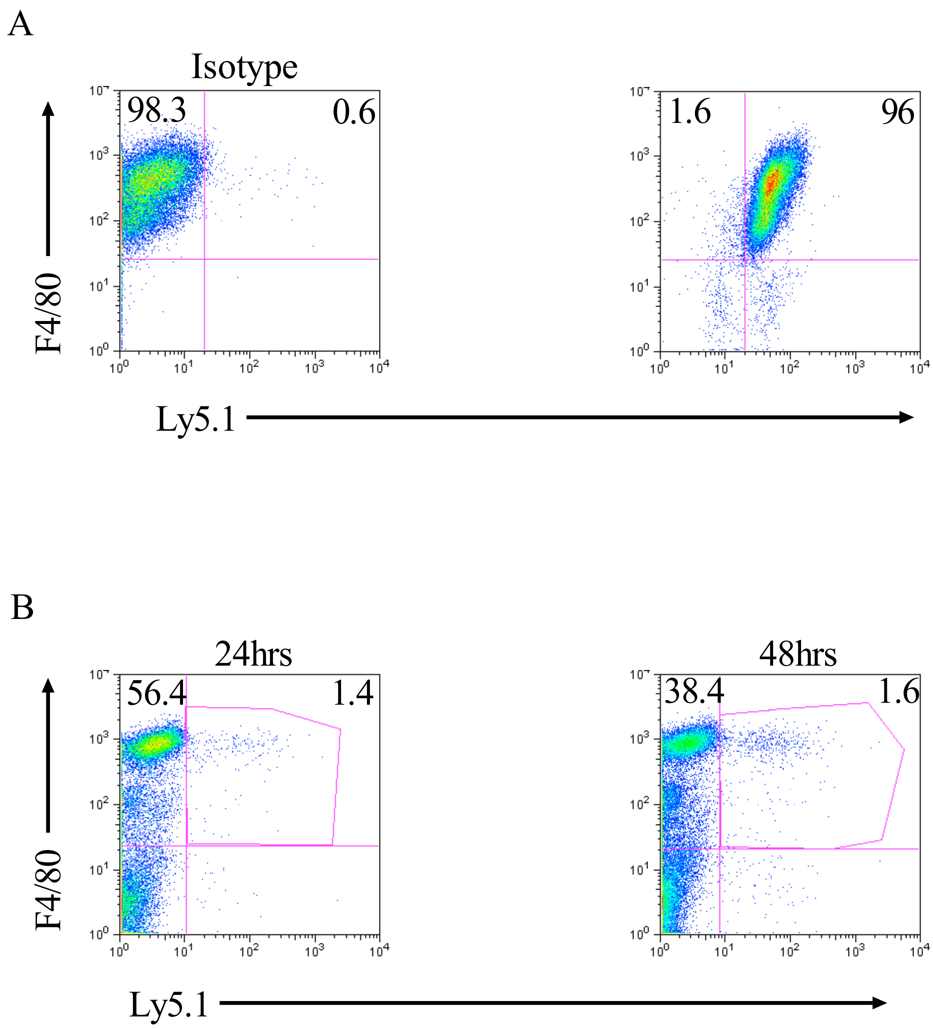


Figure 6.12 Repopulation of PEC by adoptively transferred BMM

1x10^6 Ly5.1 BMM were transferred i.v. into congenic Ly5.2 recipients and PEC were harvested from recipient mice 24 hrs (left panel) and 48 hrs (right panel) later. The presence of Ly5.1$^+$ F4/80$^+$ cells was assessed by flow cytometry.

Chapter 7

Effects of ES-62 on macrophage function and intestinal inflammation

Introduction

The findings in my previous chapters, together with other published studies, have demonstrated the critical role mϕ play during the DSS-induced model of intestinal inflammation. DSS colitis does not require T or B cells and as I have shown, mϕ are the principal producers of TNFα in this disease. As blockade of TNFα has already proven beneficial in patients with Crohn's disease (328), there is currently considerable interest in targeting mϕ and their products as therapies for IBD. One molecule which is currently in pre-clinical testing as a treatment for arthritis and other inflammatory disorders is ES-62 (306), a secreted phosphorylcholine (PC)-containing glycoprotein of the rodent filarial nematode, *Acanthocheilonema viteae.* ES-62 modulates pro-inflammatory cytokine production by mϕ in response to LPS and IFNγ (315) and although it initially induces some production of TNFα and IL-12 by mϕ, it then renders these cells hyporesponsive to subsequent stimulation with LPS and IFNγ. The low level cytokine induction by ES-62 is abrogated in TLR4-deficient and MyD88-deficient mice (318), suggesting the effects are TLR4/MyD88-dependent. However, the same study showed that LPS-unresponsive, TLR4-mutant C3H/HeJ mice respond normally to ES-62, suggesting TLR4 must be present, but does not need to be fully functional for ES-62 to exert its effects. Indeed it was recently shown that binding of TLR4 by ES-62 prevents FcεRI-induced release of allergic mediators from human mast cells and can prevent mast cell-dependent hypersensitivity in the skin and airways (319). ES-62 also suppresses the response of DCs and mϕ to other TLR ligands, such as BLP and CpG (318), indicating that ES-62 may inhibit a signalling component common to TLRs. Together these findings indicate that ES-62 can inhibit mϕ responses to bacterial products and other stimuli, and that this property may allow it to suppress inflammatory disease *in vivo*. Therefore I examined whether the modulatory effects of ES-62 on mϕ would influence DSS colitis *in vivo*.

7.1 Effects of ES-62 on BMM function

I first wanted to confirm previous findings that ES-62 could modulate the pro-inflammatory functions of mϕ and DCs. In these experiments, treatment of BMM with LPS, but not BLP, increased the expression of CD40 and CD80 compared with medium alone, but both LPS and BLP had little effect on the expression of CD86 or class II MHC (Fig 7.1). In contrast, treatment with IFNγ alone, or with LPS + IFNγ or BLP + IFNγ markedly increased the expression of all co-stimulatory molecules, with IFNγ treatment alone inducing the highest levels of class II MHC expression. ES-62 alone had no effects on the expression any of these molecules by BMM compared with medium alone. When supernatants from these cultures were analysed for the production of pro-inflammatory cytokines, treatment with either LPS or BLP alone induced production of intermediate levels of TNFα and IL-6 (Fig 7.2a+b). However, treatment of BMM with BLP or LPS plus IFNγ induced high levels of TNFα and IL-6 production, and only these combined stimuli induced any production of IL-12p70 (Fig 7.2c). No pro-inflammatory cytokines could be detected after treatment of BMM with ES-62 alone.

These results indicated that ES-62 itself had no ability to induce a classical pattern of mϕ activation, but as it has been suggested that helminths can induce polarisation of mϕ toward the AAM subtype, I examined if ES-62 could affect the expression of markers associated with this subtype. In this experiment, I used IL-4 as a positive control for alternative activation of mϕ, as well as LPS + IFNγ to produce classical activation. PCR was used to assess the expression of mRNA for arginase and Ym-1 as markers of alternative activation, and for iNOS as the marker for classical activation. BMM cultured with medium showed induction of iNOS, and if anything LPS treatment seemed to decrease this (Fig 7.3a). However, as expected, the strongest induction of iNOS was seen after treatment with LPS + IFNγ. iNOS expression was also seen after treatment with ES-62 or IL-4, but it is difficult to interpret this finding, as this was also seen with cells treated in medium alone. Next, I examined the expression of the AAM marker, arginase, and found that BMM expressed low levels of arginase mRNA when freshly isolated and this appeared to increase following overnight culture in medium alone (Fig 7.3b). Interestingly, arginase expression seemed to be downregulated when LPS or IFNγ were present in the culture, whereas culture with ES-62 or IL-4 induced levels similar to or even higher than those in medium alone. The second AAM marker, Ym-1, was expressed by freshly isolated BMM, and this expression was not affected by treatment with medium alone, ES-62 or IL-4. However, Ym-1 expression seemed to be downregulated when LPS or IFNγ were present. Therefore, ES-62 seems to favour the development of an AMM rather than a CAM phenotype, and it would be important to confirm these results by Q-PCR or at the protein level.

7.2 Effects of ES-62 on responsiveness of DCs to re-stimulation

As ES-62 has also been reported to downregulate pro-inflammatory functions of DCs, I used these cells to assess whether ES-62 would affect subsequent responsiveness to stimulation. Thus BMDCs were cultured with ES-62 overnight, washed and re-stimulated with the TLR ligands, BLP, poly:IC, LPS, flagellin and CpG and assessed for the expression of co-stimulatory molecules and class II MHC, as well as for the production of pro-inflammatory cytokines. As shown in Fig 7.4, ligation of all TLRs increased expression of most co-stimulatory molecules and class II MHC, although the levels were somewhat variable depending on the marker examined. Most importantly, none of these effects were altered by pre-treatment with ES-62 in this experiment, although no formal statistical analysis could be performed. When production of pro-inflammatory cytokines from the same cultures was assessed, ligation of TLR2, 3, 4, 5 and 9 by their respective ligands induced the production of TNFα and IL-6 above the levels seen with medium alone (Fig 7.5a+b). Although only duplicate wells were used and so no statistical analysis could be carried out, pre-treatment of BMDC with ES-62 slightly reduced the subsequent TNFα and IL-6 responses to BLP, but it had no effect on the responses to the other TLR ligands.

7.3 Effects of ES-62 on acute intestinal inflammation

To assess whether ES-62 is able to modulate acute intestinal inflammation, I administered ES-62 i.p. to mice with DSS-induced colitis, beginning two days before DSS was given (day-2) and every two days thereafter. The dose of ES-62 was based on previous findings demonstrating the beneficial effect of ES-62 during CIA. As in the experiments described in previous chapters, mice began to lose weight after 4-5 days of DSS administration and this was not significantly affected by treatment with ES-62 (Fig 7.6a). Similarly, the colon shortening which occurred in colitis was not reduced by ES-62 (Fig 7.6b). However, when the individual components of clinical disease were evaluated individually, ES-62 was seen to significantly reduce rectal bleeding on days 6 and 7 of colitis, as well as the total clinical score on day 5 (Fig 7.7). However it had no significant effect on diarrhoea or on any of the other parameters at other times.

To assess the effects of ES-62 further, I measured pro-inflammatory cytokines in supernatants of explants taken from the proximal and distal colon. Consistent with the histological pattern of DSS colitis, explants of distal colon from PBS-treated colitic animals produced significantly increased levels of TNFα and IL-6 compared with explants of inflamed proximal or control colon, which both produced very little of either cytokine (Fig 7.8). ES-62 treatment had no significant effect on the enhanced production of cytokines seen in the distal colon of colitic PBS-treated controls, although the levels were highly variable and not significantly above control mice (Fig 7.8). I next assessed whether treatment of colitic mice with ES-62 affected cellular infiltration into the colon, by examining the expression of Ly6G, B220, CD4, CD8 and F4/80 to assess the presence of neutrophils, B cells, $CD4^+$ T cells, $CD8^+$ T cells and mϕ, respectively, among isolated LP cells. As described in Chapter 5, the proportion of $Ly6G^+$ cells increased during colitis compared with untreated control mice, and this was not affected by treatment with ES-62 (Fig 7.9). Consistent with the fact that SCID mice are susceptible to DSS-induced colitis, the proportions of B cells, $CD4^+$ and $CD8^+$ T cells did not increase significantly during colitis and none of these were affected by ES-62 treatment. ES-62 also did not affect mϕ infiltration in colitis, but in contrast to the data shown in Chapter 5, the proportion of $F4/80^+$ mϕ was not increased during colitis in this experiment. It should be noted that these experiments were carried out much earlier and with a different enzymatic isolation protocol, and thus are not directly comparable to results in previous chapters.

To ensure that any differences between PBS- and ES-62-treated mice were not due to the amount of water consumed and therefore DSS, water intake was monitored daily and although DSS-fed animals consumed less water as the experiment progressed, there were no differences between the PBS and ES-62 groups (Fig 7.10).

Due to the fact that ES-62 reduced the clinical aspects of colitis, but exerted little effect on the other parameters measured, I postulated that this acute model of DSS colitis might be too short-lived and severe for ES-62 to have had any effect. Therefore, I decided to lower the dose of DSS from 2.5% to 2% to try and reduce the severity of disease. The ES-62 dose used was the same as in the previous experiment, but ES-62 was injected s.c. on this occasion to try and slow absorption into the bloodstream and maintain a more constant level of ES-62 between injections. This was also the route used for ES-62 administration in the CIA experiments conducted by McInnes *et al* (306). Under these conditions, mice began to lose weight after 5-6 days of DSS administration and again ES-62 had no effect (Fig 7.11a). However ES-62 did significantly reduce the colon shortening (Fig 7.11b) found in this experiment and also reduced the diarrhoea and rectal bleeding on day 8 (Fig 7.12). Overall these parameters were generally lower in ES-62-treated mice, with the result that the total clinical score was significantly reduced on day 8 (Fig 7.12d). As before there was increased pro-inflammatory cytokine production by explants of distal colon from colitic mice compared with control mice, but ES-62 failed to reduce the levels of these cytokines (Fig 7.13). Ly6G^{hi} neutrophils again infiltrated the inflamed intestine, but ES-62 had no effect on this (Fig 7.14a). As before, the proportions of B cells and CD4^{+}

T cells were unchanged during this model of colitis and were unaffected by treatment with ES-62 (Fig 7.14). Although the numbers were extremely low, there was a small but significant increase in the proportion of $CD8^+$ T cells in the ES-62-treated group. Unusually, the proportion of $F4/80^+$ cells was actually lower during colitis in this experiment, and this was not altered by ES-62 treatment (Fig 7.14).

As ES-62 seemed to have a variable and rather subtle ability to modify colitis in the previous two experiments, I assessed whether a higher dose of ES-62 (10μg every two days) could modulate the pathology more substantially. In addition, I used female mice, which are considered to be less susceptible to DSS-induced colitis. However the disease was not notably less severe in terms of time of onset or clinical aspects of disease, and even at high doses, ES-62 had no significant effect on weight loss or colon shortening in this experiment (Fig 7.15). Although there was significant reduction in rectal bleeding and the total clinical scores on day 4 in the ES-62 treated animals, there were no effects at other times or on other parameters of disease (Fig 7.16). Non-colitic control animals were not used in this particular experiment due to lack of availability of ES-62.

7.4 Effects of ES-62 on chronic intestinal inflammation

As ES-62 had only minor and inconsistent effects on acute colitis, I next assessed its effects in a chronic form of the disease which I postulated might allow any modulatory effects of ES-62 to be observed more clearly. Thus, female mice received three 5-7 day cycles of DSS administration with increasing DSS concentration in each cycle and a rest period of 7 days water administration in between each cycle. As shown in Fig 7.17, mice began to lose weight after 5-6 days of the first cycle, but began to gain weight again 2-3 days after DSS was stopped on day 7. Thereafter they did not lose significant amounts of weight when DSS administration was recommenced in the following cycles. In addition, when measured on d33, colon shortening was less severe in the majority of colitic animals (Fig 7.18) when compared with the acute colitis model (Fig 7.6+7.11+7.15). Clinical signs of disease were also most apparent during the first cycle of DSS feeding and most animals failed to show any signs of disease thereafter (Fig 7.19-7.22). ES-62 had little effect during the first cycle of DSS treatment or at later timepoints when the disease was minimal. ES-62 also had no effect on colon shortening and it should be noted that three ES-62-treated mice had to be culled on day 8 of the experiment due to severe disease, whereas all the DSS treated control mice survived. Again the proportion of $Ly6G^+$ neutrophils in colonic LP isolates was increased on d33 of disease compared with control animals (Fig 7.23a), but to a lesser degree than in the acute model of colitis (Fig 7.9+7.14) and ES-62 treatment did not affect this. The proportions of B cells, $CD4^+$ T cells, $CD8^+$ T cells and mϕ present in the colon were not altered in chronic colitis and ES-62 did not affect these features (Fig 7.23). Thus, ES-62 appeared to have no beneficial effect on chronic DSS colitis.

Summary

In this chapter, I found that ES-62 did not activate BMM either in terms of upregulation of co-stimulatory molecules and class II MHC, or production of pro-inflammatory cytokines. Furthermore, in contrast to LPS + IFNγ, ES-62 favoured the expression of molecules expressed by the AAM subtype, such as Ym-1. However, I was unable to confirm previous findings that pre-treatment with ES-62 could inhibit the subsequent activation of BMDC in response to TLR ligation, although there may have been slightly reduced pro-inflammatory cytokine production in response to the TLR2 ligand, BLP. ES-62 had a subtle and rather variable effect on the development of acute DSS colitis, chiefly by reducing some of the clinical signs of disease at earlier timepoints. However ES-62 did not affect weight loss or colonic pro-inflammatory cytokine production, or the cellular infiltrate during intestinal inflammation. Similarly, it had no effect clinical disease, colon shortening or cellular infiltration during chronic intestinal inflammation. Therefore ES-62 seems to provide some protection from the acute form of DSS colitis but not during chronic intestinal inflammation.

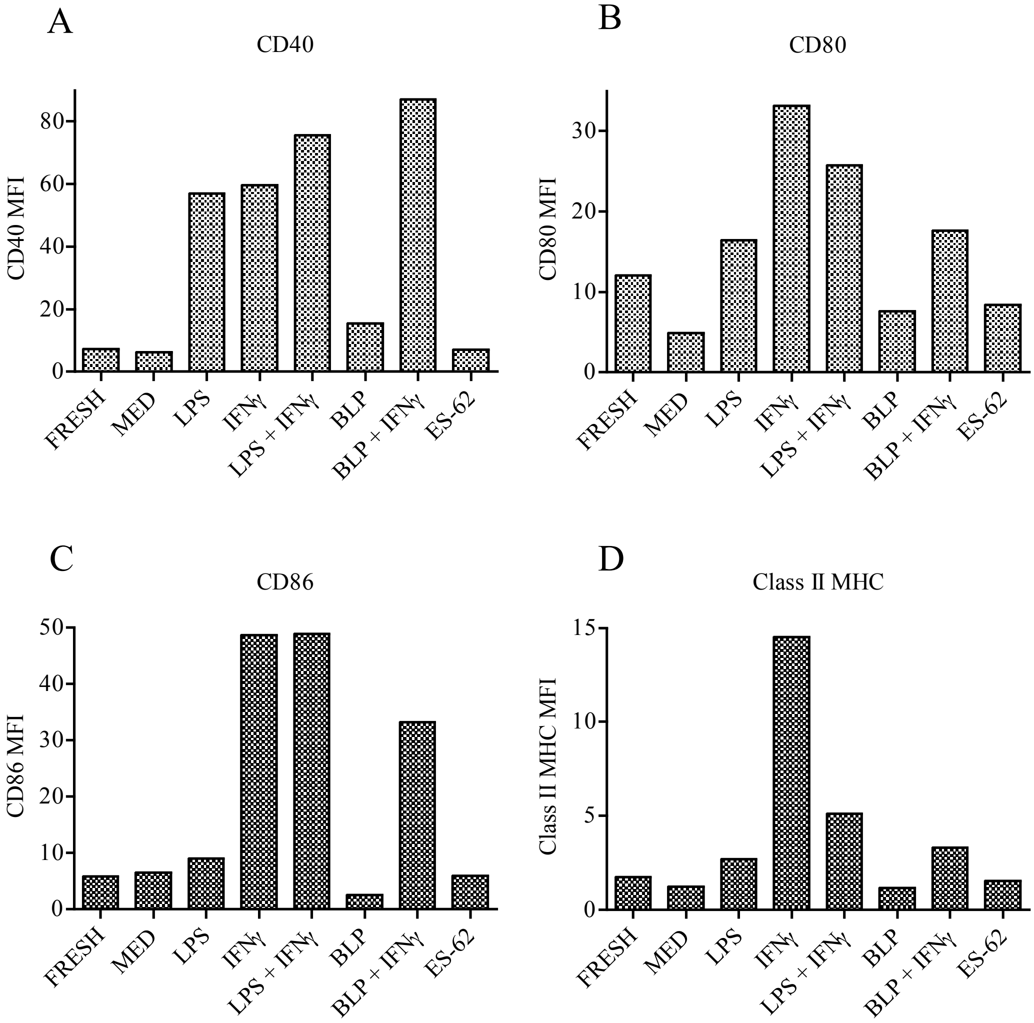


Figure 7.1 Effects of ES-62 on class II MHC and co-stimulatory molecule expression by BMM

BMM pooled from triplicate cultures were analysed immediately after harvesting, or after culture in medium, 1μg/ml LPS, 100U/ml IFNγ, LPS + IFNγ, 1μg/ml BLP, BLP + IFNγ or 2μg/ml ES-62 for 18hrs and assessed for the expression of CD40 (A), CD80 (B), CD86 (C) and class II MHC (D) by flow cytometry. Results are shown as the MFI for each marker.

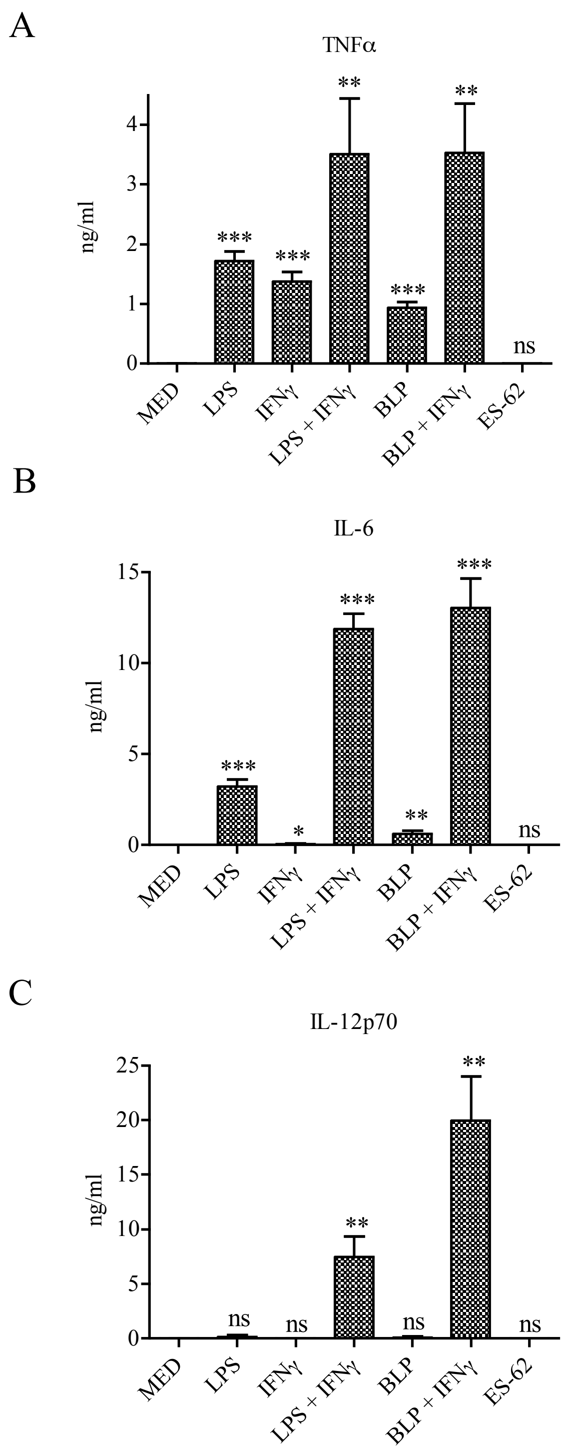


Figure 7.2 Effects of ES-62 on pro-inflammatory cytokine production by BMM

BMM were cultured in medium, 1μg/ml LPS, 100U/ml IFNγ, LPS + IFNγ, 1μg/ml BLP, BLP + IFNγ or 2μg/ml ES-62 for 18hrs and assessed for the production of TNFα (A), IL-6 (B) and IL-12p70 (C) by ELISA. The results shown are the mean concentration of cytokine ± SD for 3 replicates/group. ns=not significant; *p<0.05; **p<0.01; ***p<0.001 versus medium control.

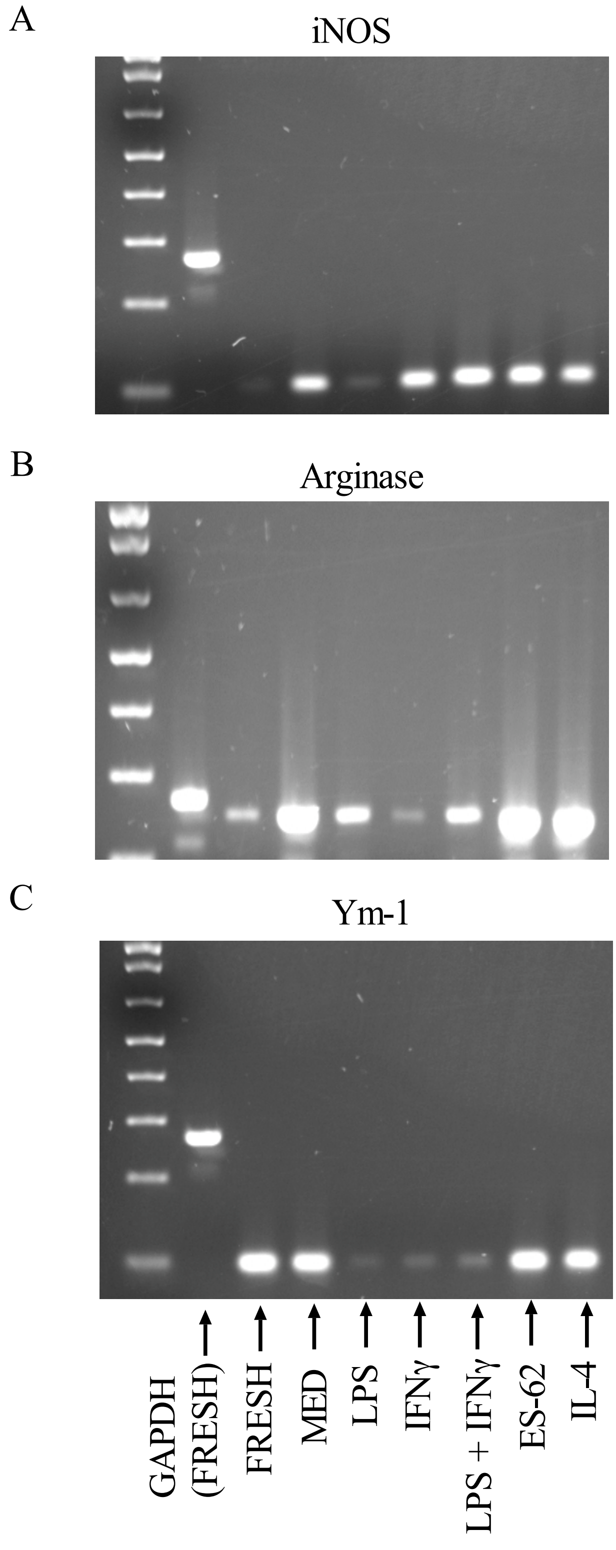


Figure 7.3 Effects of ES-62 on expression of iNOS, Arginase and Ym-1 mRNA

Freshly harvested BMM or BMM pooled from triplicate wells cultured in medium, 1μg/ml LPS, 100U/ml IFNγ, LPS + IFNγ, 2μg/ml ES-62 or 40ng/ml IL-4 for 18hrs were assessed for the expression of iNOS (A), arginase (B) and Ym-1 (C) mRNA by PCR.

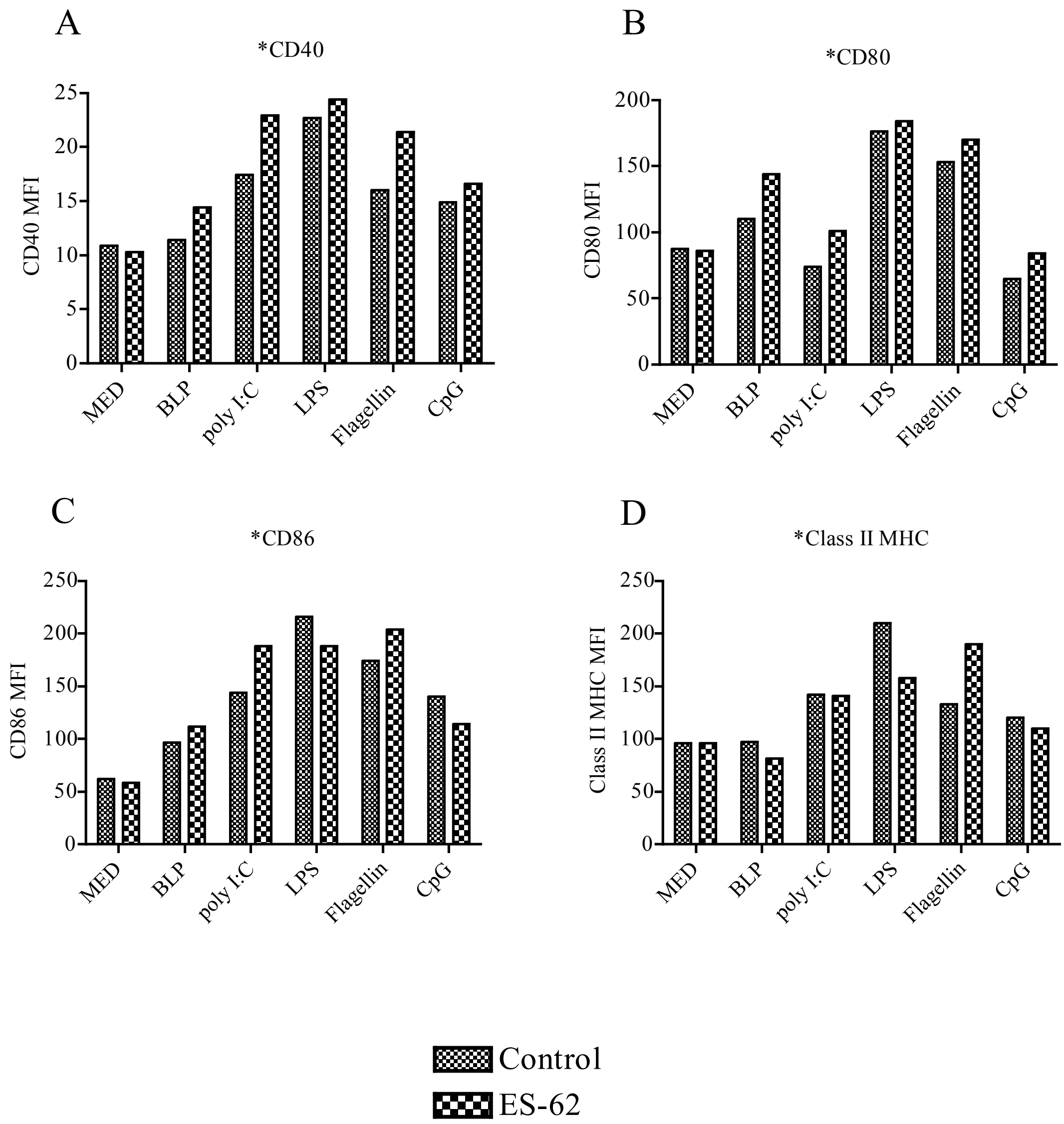
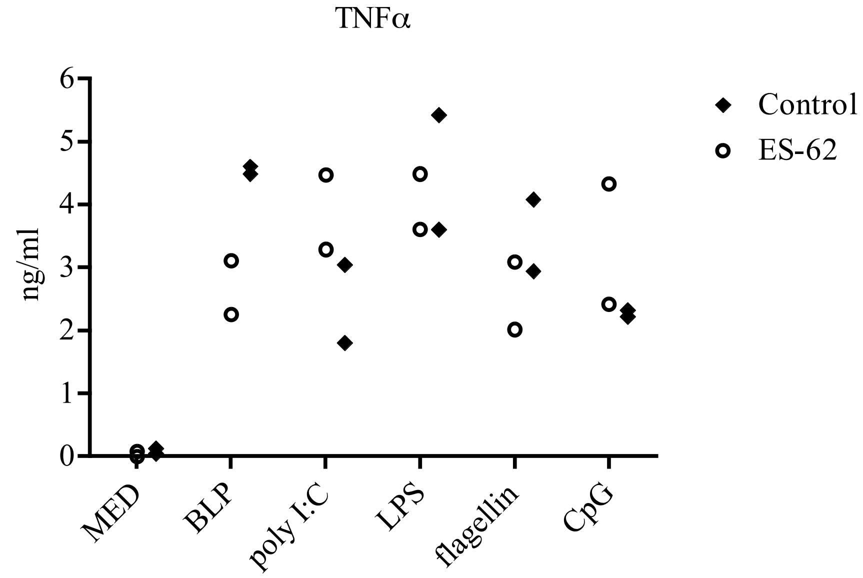


Figure 7.4 Effects of ES-62 on class II MHC and co-stimulatory molecule expression by BMDC

BMDC were cultured in medium or 2μg/ml ES-62 for 24hrs, washed and re-stimulated in medium or 1μg/ml BLP, 25μg/ml poly I:C, 2μg/ml LPS, 20 μg/ml flagellin or 3.2μg/ml CpG for a further 24hrs, before the expression of CD40 (A), CD80 (B), CD86 (C) and class II MHC (D) was assessed by flow cytometry. Results are shown as the MFI of expression using BMDC pooled from duplicate wells.

A



B

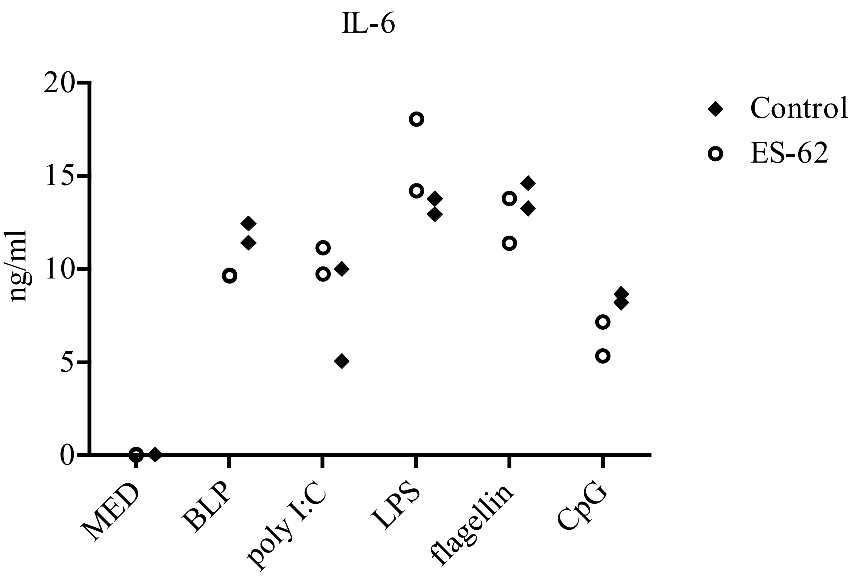


Figure 7.5 Effects of ES-62 on pro-inflammatory cytokine production by BMDC

BMDC were cultured in medium or 2μg/ml ES-62 for 24hrs, washed and re-stimulated in medium or 1μg/ml BLP, 25μg/ml poly I:C, 2μg/ml LPS, 20 μg/ml flagellin or 3.2μg/ml CpG for a further 24hrs. The production of TNFα (A) and IL-6 (B) was then analysed by ELISA. Results are shown as the concentration of cytokine for two replicates/group.

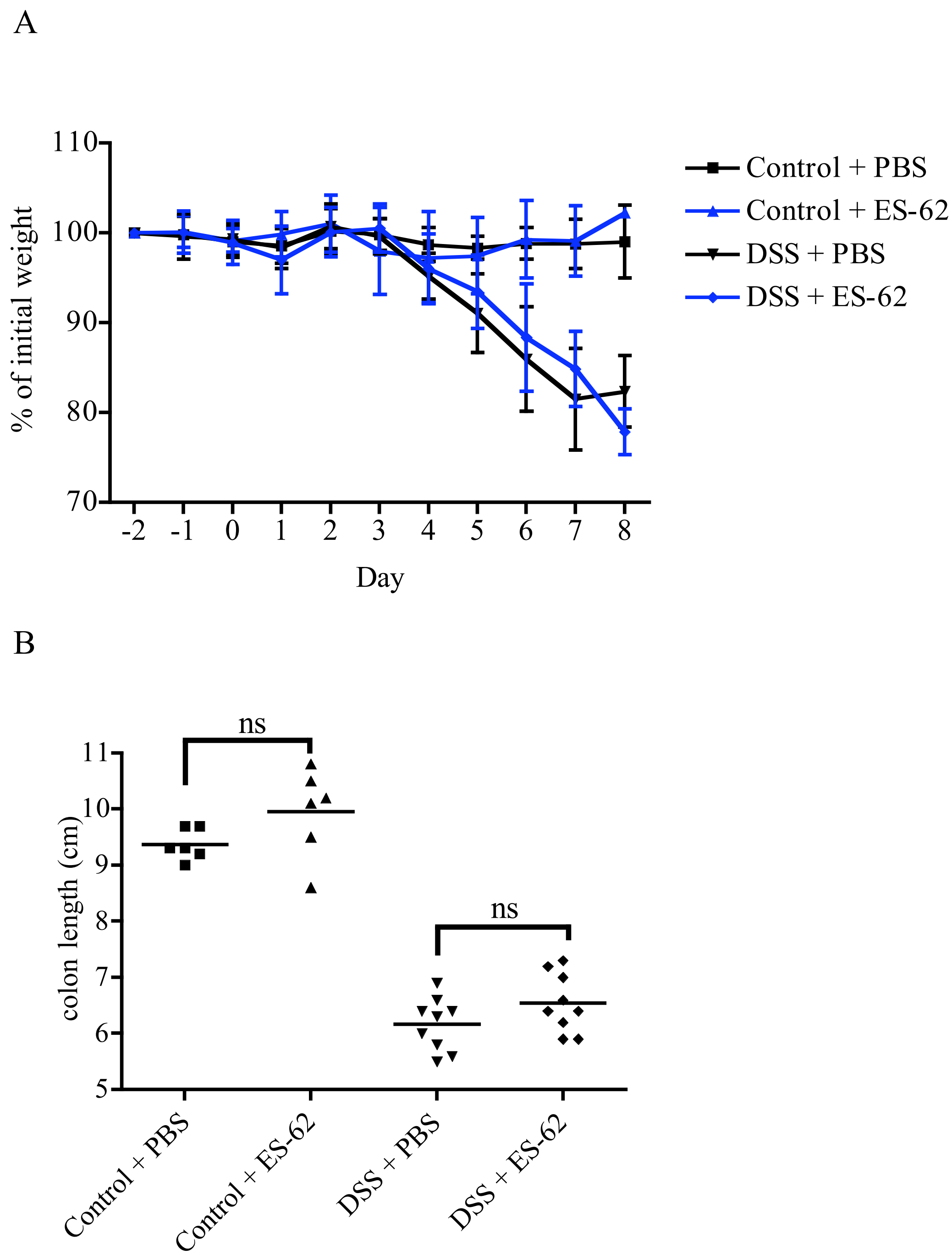


Figure 7.6 Effects of ES-62 on weight loss and colon shortening during acute colitis

Male C57Bl/6 mice received water or 2.5% DSS for 8 days and were injected with PBS or 2µg ES-62 i.p. every second day starting two days before DSS was given. Mice were weighed daily and the results in (A) are the mean percentage ± SD change from initial weight. Colon lengths were also recorded (B). ns=not significant.

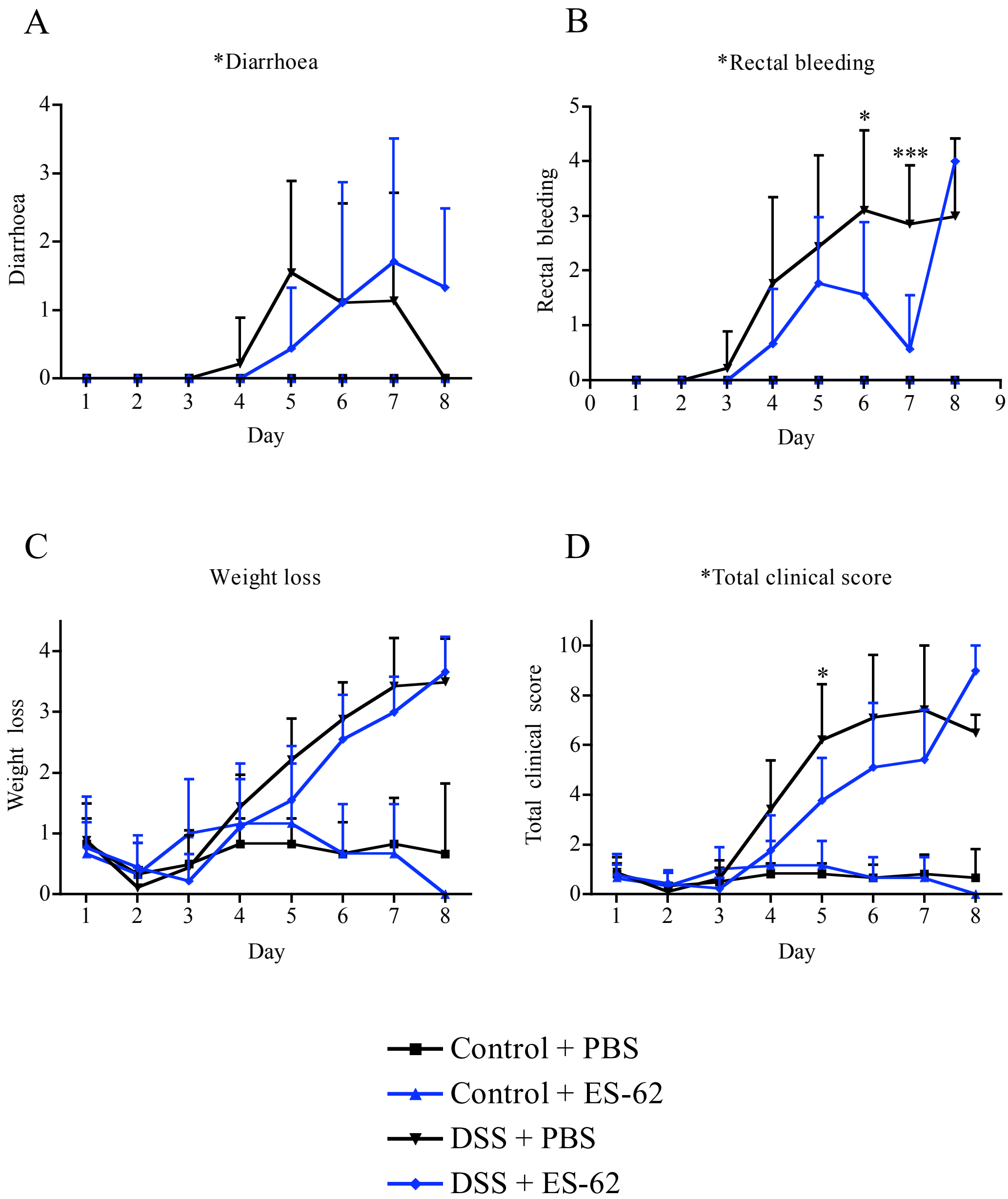
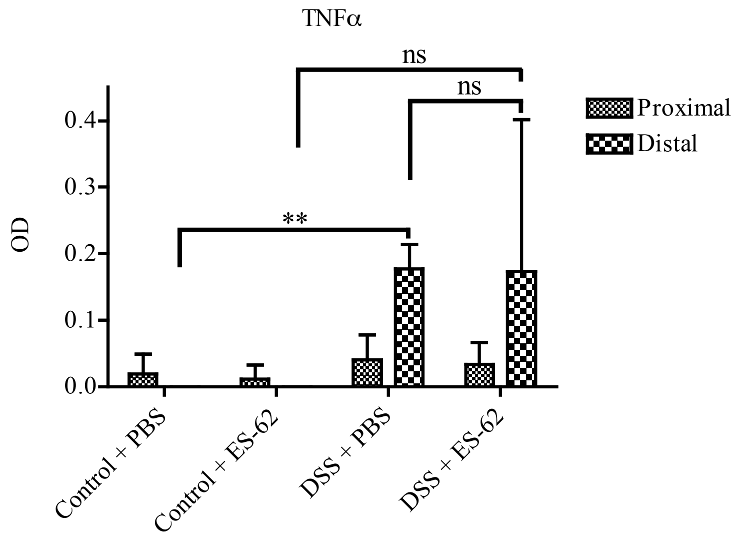


Figure 7.7 Effects of ES-62 on clinical aspects of colitis

Male C57Bl/6 mice were given water or 2.5% DSS and injected with PBS or 2μg/ml ES-62 i.p. every second day (starting on day-2) and were monitored daily for diarrhoea (A), rectal bleeding (B), weight loss (C) and total clinical score (D), calculated as described in the Materials and Methods. n=6 for water control groups and n=9 for colitis groups. Two-way ANOVAs revealed significant differences, Bonferroni-corrected. *p<0.05; ***p<0.001.

A



B

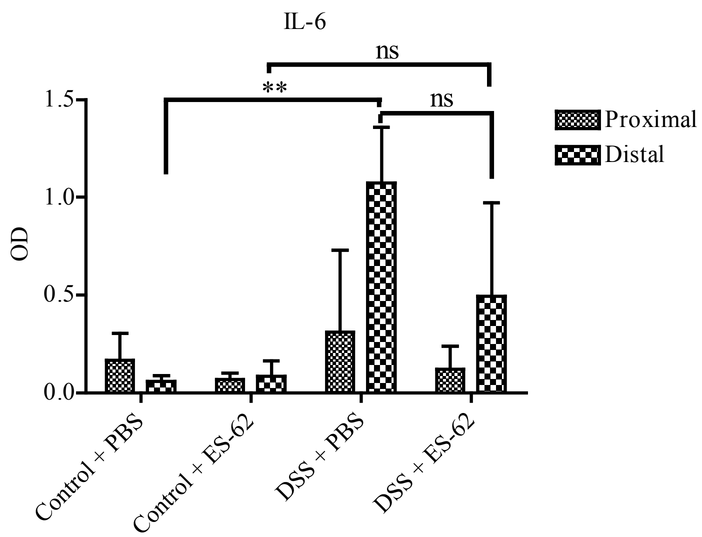


Figure 7.8 Effects of ES-62 on colonic pro-inflammatory cytokine production during colitis

1cm segments of proximal or distal colon from male C57Bl/6 mice fed water or 2.5% DSS and injected with PBS or 2μg/ml ES-62 i.p. every second day were cultured for 24hrs and the production of TNFα (A) and IL-6 (B) was measured by ELISA. Results are shown as the mean OD ± SD for 3 mice/group. Two-way ANOVAs revealed significant differences in distal control vs distal colitis for TNFα and IL-6, but not PBS vs ES-62, Bonferroni-corrected. ns=not significant; **p<0.01.

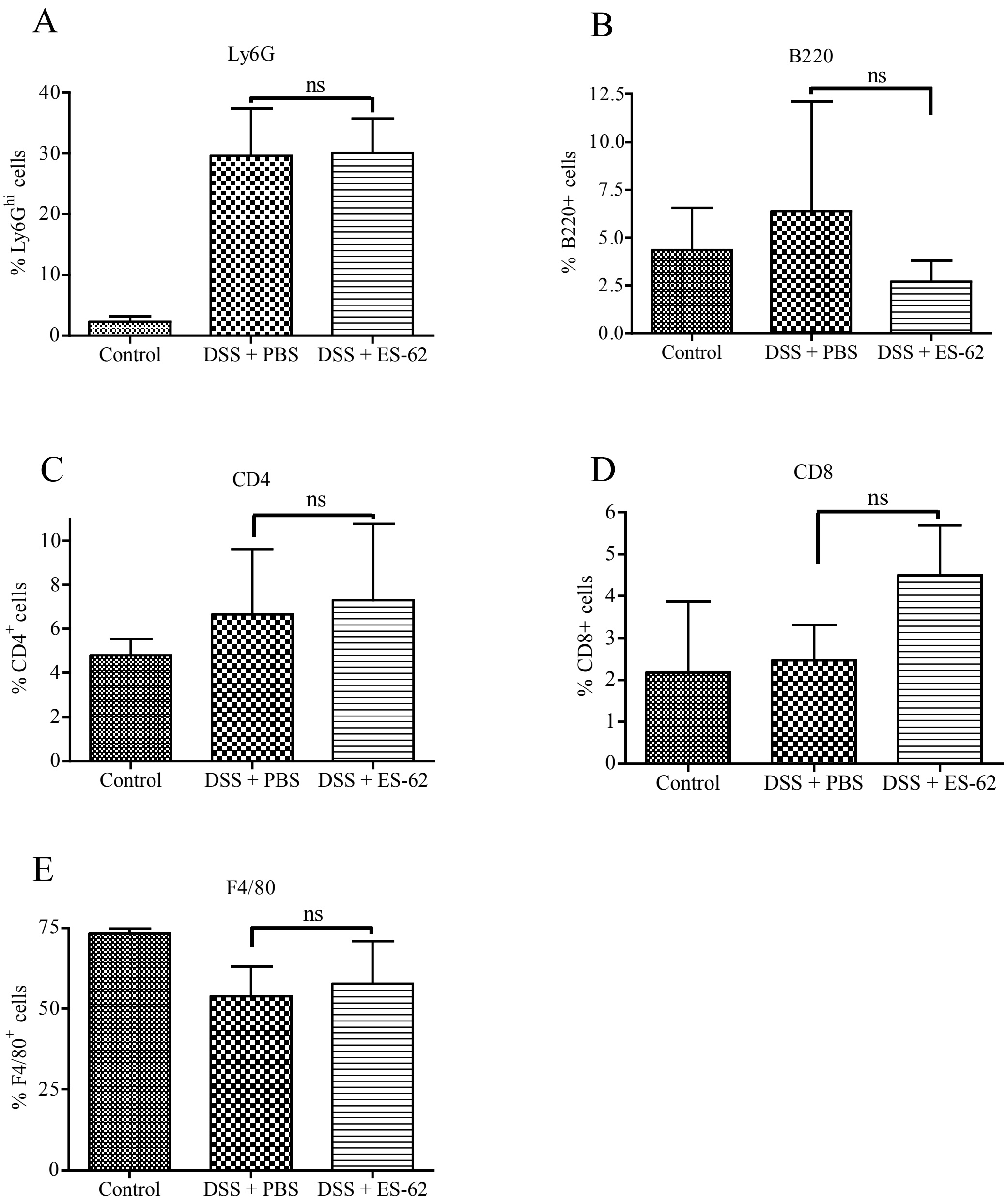


Figure 7.9 Effects of ES-62 on the cellular composition of the inflamed colon

Colons from male C57Bl/6 mice given water or 2.5% DSS for 7 days and injected with PBS or 2μg/ml ES-62 i.p. every second day (starting on day-2) were digested and the cells examined for the expression of Ly6G (A), B220 (B), CD4 (C), CD8 (D) and F4/80 (E) by flow cytometry. Results are shown as the mean percentage ± SD for 3 mice/group. ns=not significant.

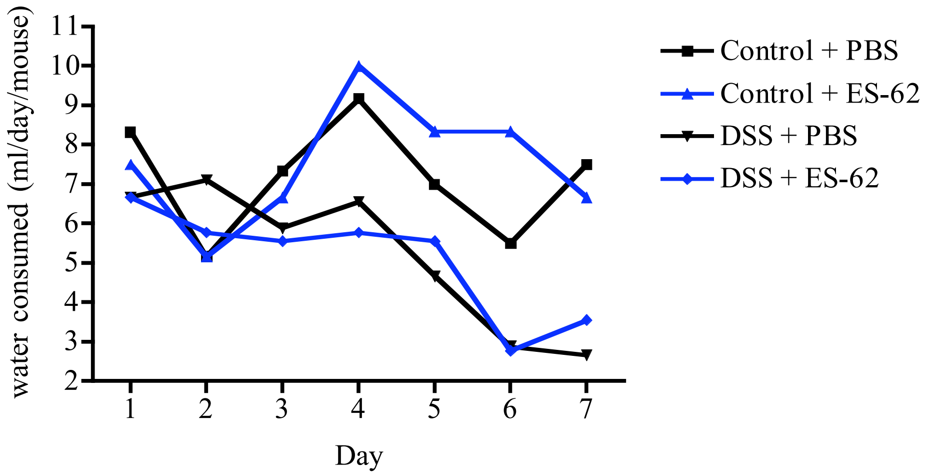
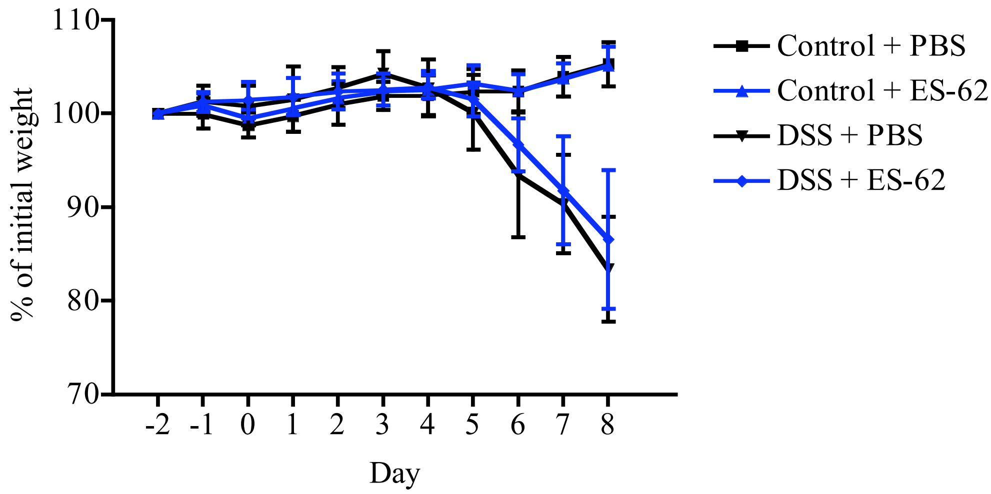


Figure 7.10 Consumption of DSS-containing water during colitis

C57Bl/6 mice were given water or 2.5% DSS and injected with PBS or 2μg/ml ES-62 i.p. every second day (starting at day-2) and the volume of water consumed per day per mouse in each group was calculated, as described in the Material and Methods.

A



B

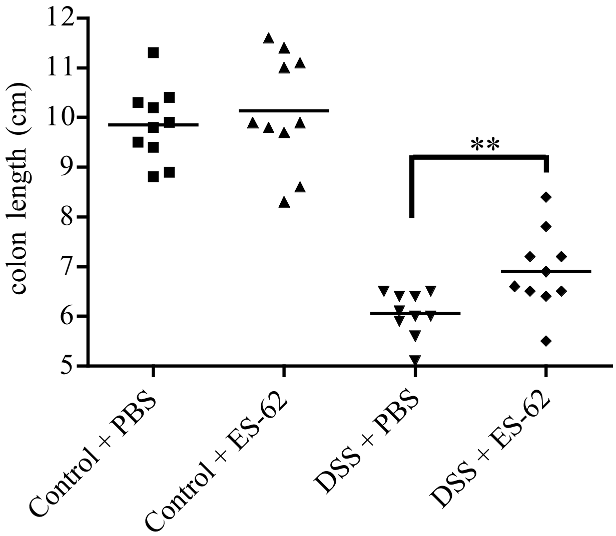


Figure 7.11 Effects of ES-62 on severity of acute DSS colitis

Male C57Bl/6 mice received water or 2% DSS for 8 days and were injected with PBS or 2μg ES-62 s.c. every second day starting two days before DSS was given. Mice were weighed daily and the results in (A) are the mean percentage ± SD change from initial weight for 10 mice/group. Colon lengths were also recorded (B). **$p<0.01$.

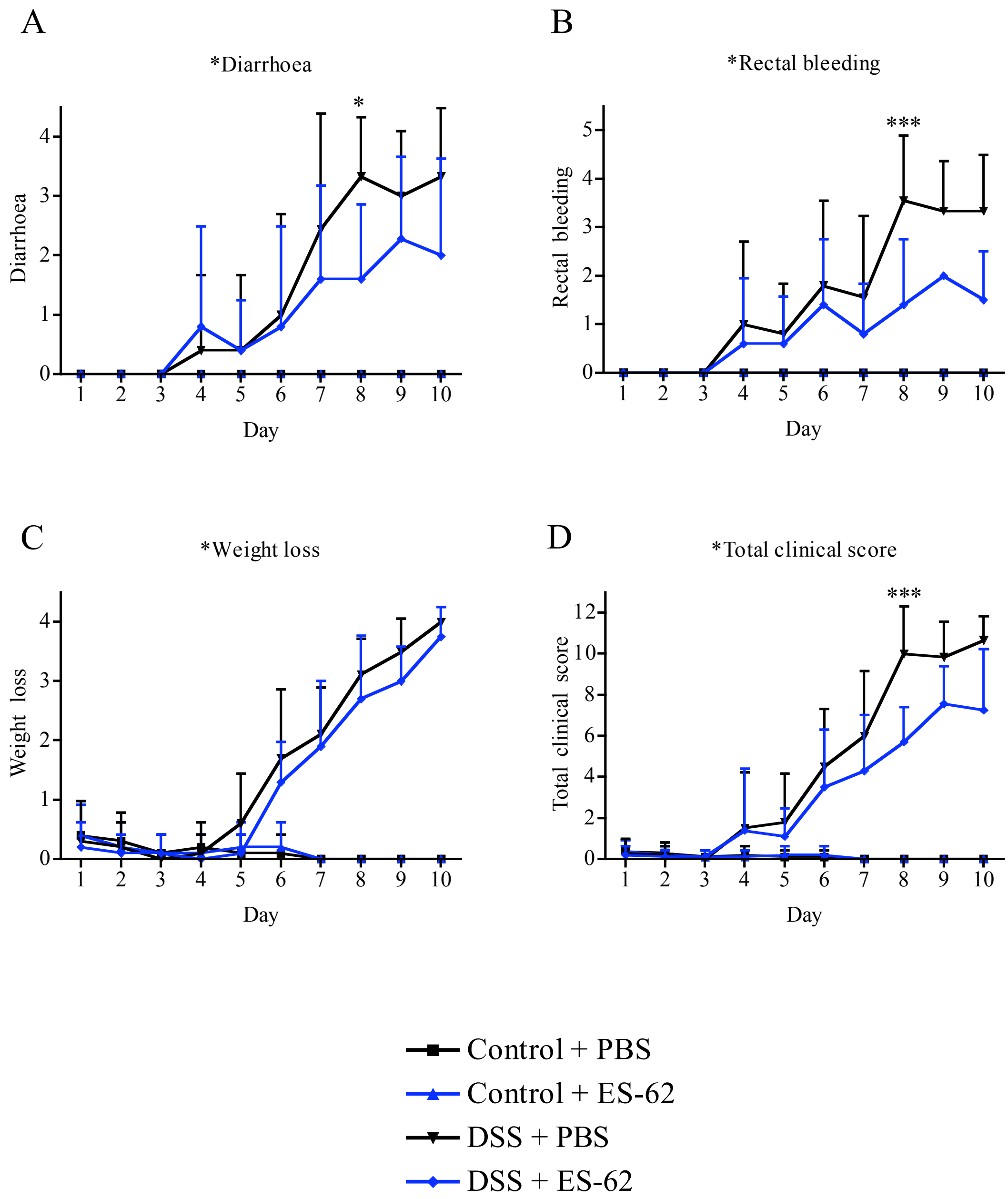


Figure 7.12 Effects of ES-62 on clinical aspects of colitis

Male C57Bl/6 mice were given water or 2% DSS and injected with PBS or 2μg/ml ES-62 s.c. every second day (starting on day-2) and were monitored daily for diarrhoea (A), rectal bleeding (B), weight loss (C) and total clinical score (D), calculated as described in the Materials and Methods, for 10 mice/group. Two-way ANOVAs revealed significant differences, Bonferroni-corrected. *p<0.05; ***p<0.001.

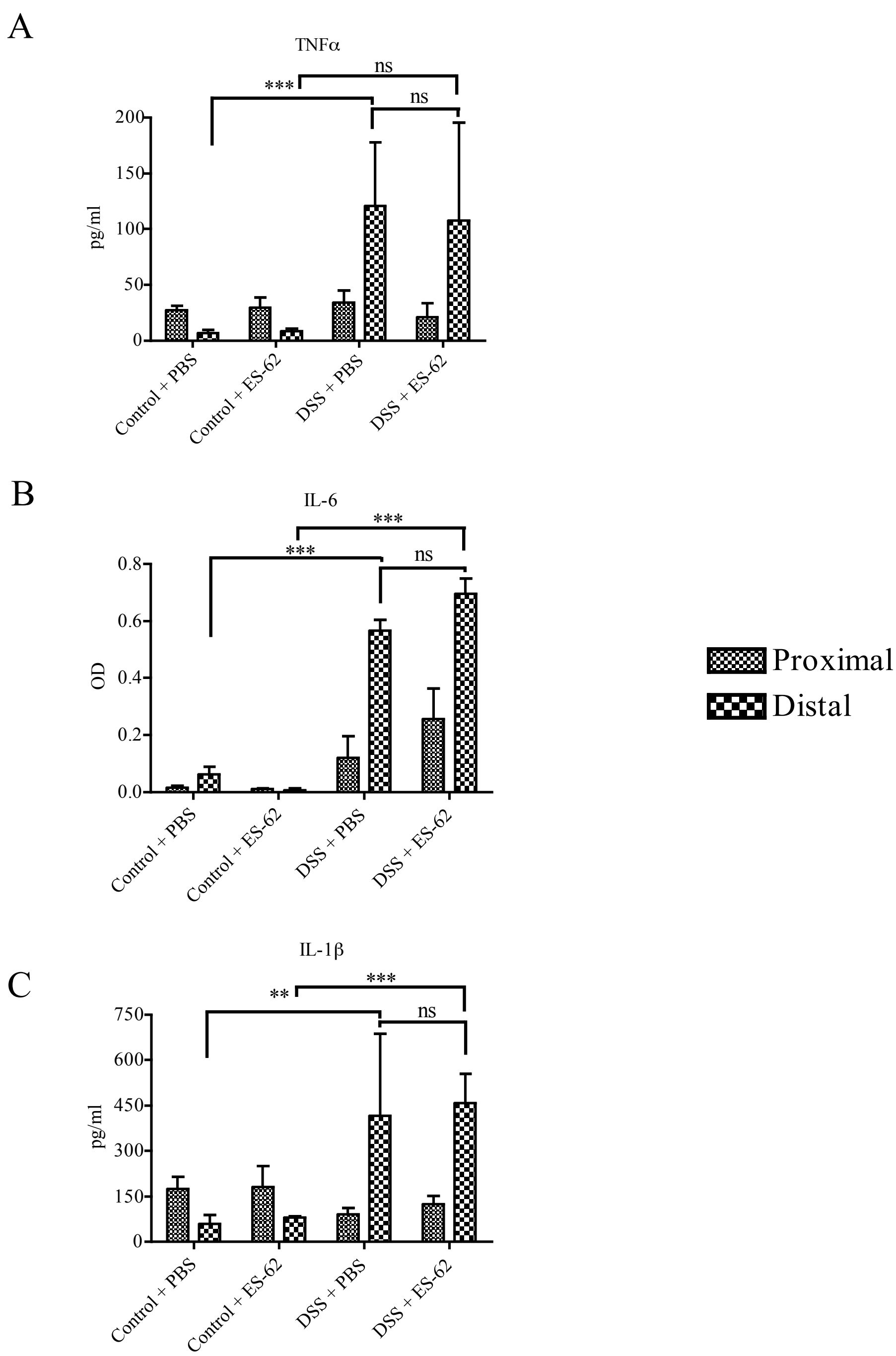


Figure 7.13 Effects of ES-62 on colonic pro-inflammatory cytokine production during colitis

1cm segments of proximal or distal colon from mice fed water or 2% DSS and injected with PBS or 2μg/ml ES-62 s.c. every other day were cultured for 24hrs and the production of TNFα (A), IL-6 (B) and IL-1β (C) was measured by ELISA. Results are shown as the mean OD or cytokine concentration ± SD for 3 mice/group. Two-way ANOVAs revealed significant differences, Bonferroni-corrected. ns=not significant; *p<0.05, **p<0.01; ***p<0.001.

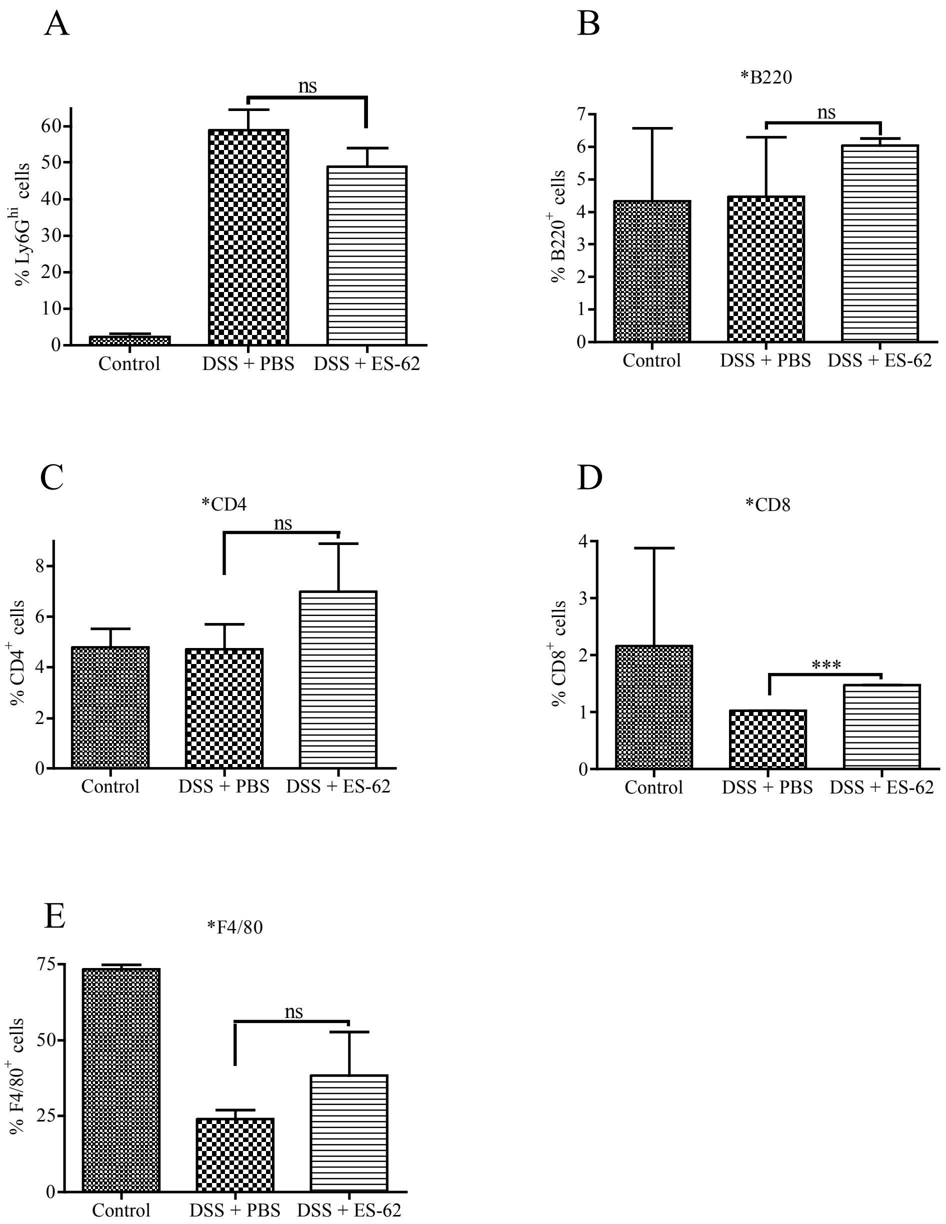


Figure 7.14 Effects of ES-62 on the cellular composition of the inflamed colon

Colons from male C57Bl/6 mice given water or 2% DSS for 7 days and injected with PBS or 2μg/ml ES-62 s.c. every second day (starting on day-2) were digested and the cells examined for the expression of Ly6G (A), B220 (B), CD4 (C), CD8 (D) and F4/80 (E) by flow cytometry. Results are shown as the mean percentage ± SD for 3 mice/group. ns=not significant; ***$p<0.001$.

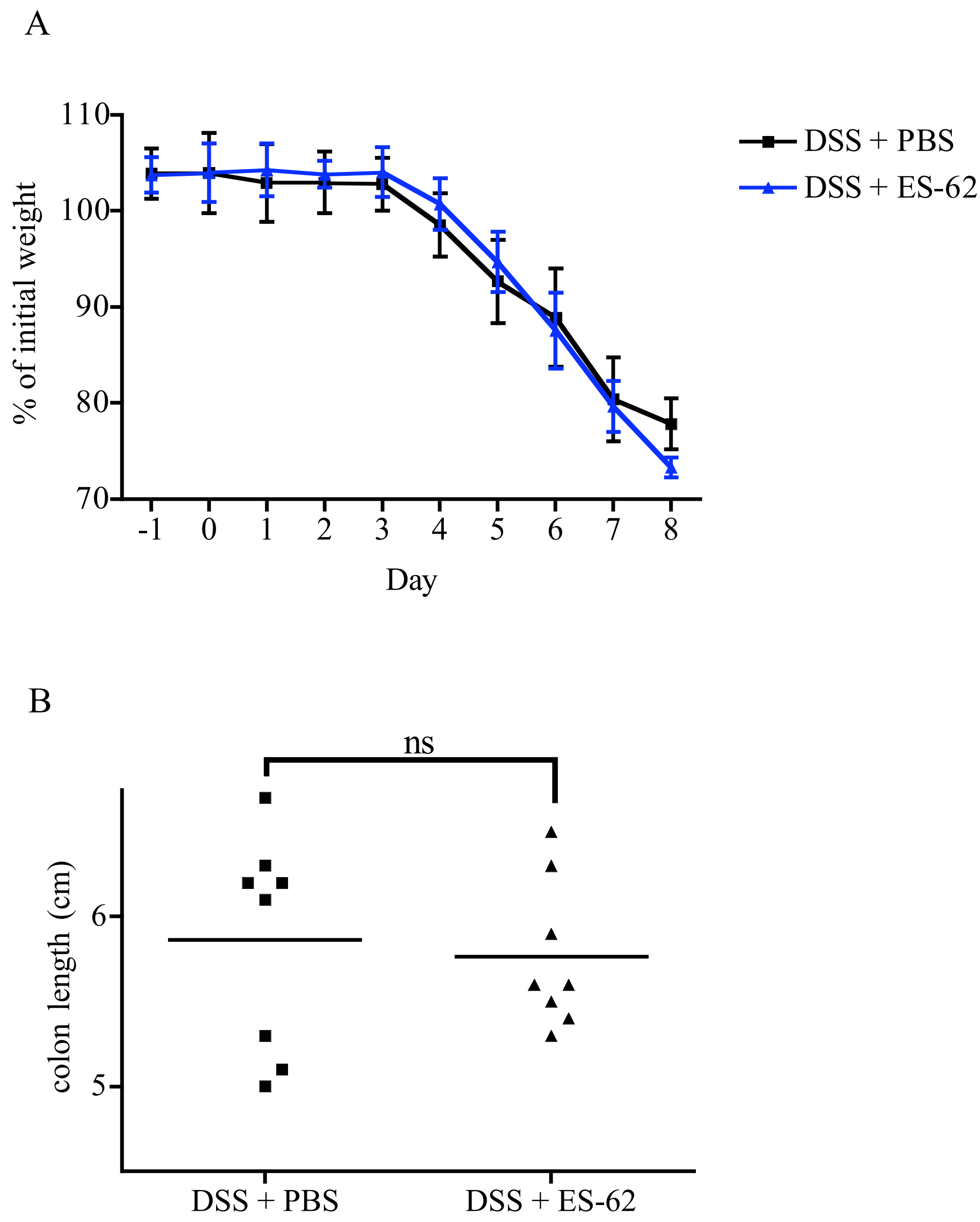


Figure 7.15 Effects of high dose ES-62 on weight loss and colon shortening during acute colitis

Female C57Bl/6 mice received water or 2% DSS for 8 days and were injected with PBS or 10μg ES-62 s.c. every second day starting two days before DSS was given. Mice were weighed daily and the results in (A) are shown as the mean percentage ± SD change from initial weight for 8 mice/group. Colon lengths were also recorded (B). ns=not significant.

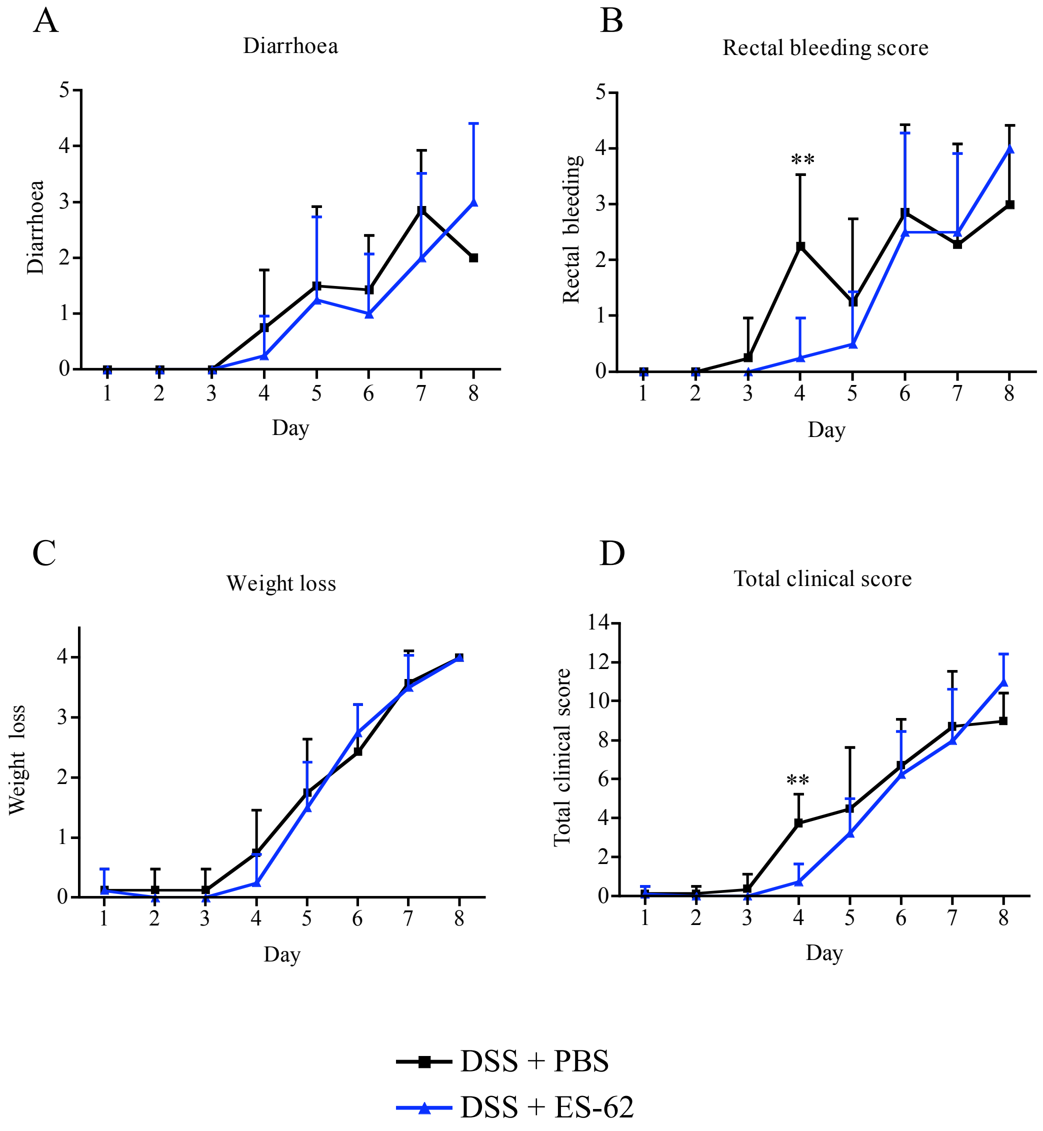
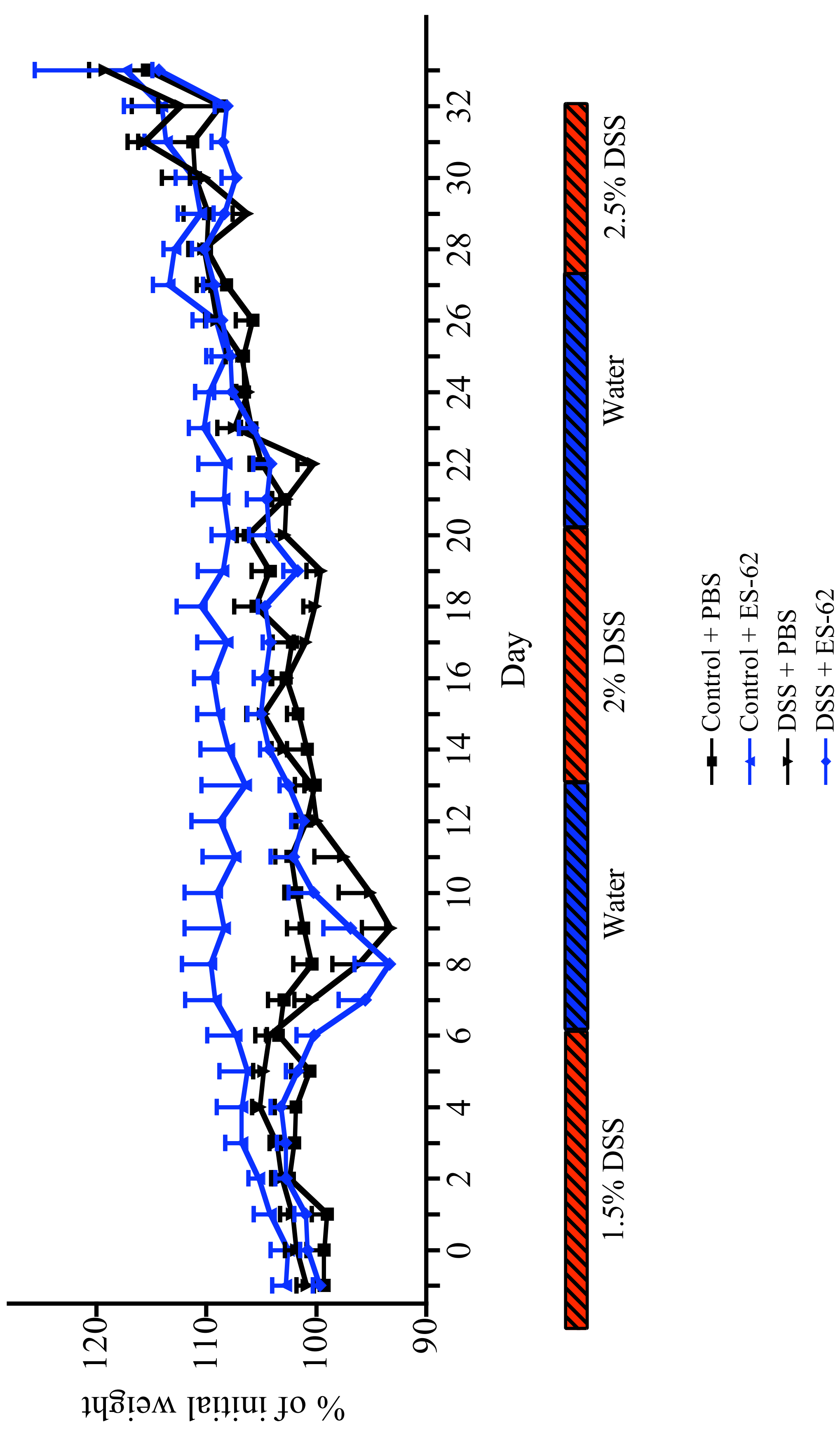


Figure 7.16 Effects of high dose ES-62 on clinical aspects of colitis

Female C57Bl/6 mice were given 2% DSS and injected with PBS or 10μg ES-62 s.c. every second day (starting on day-2). Mice were monitored daily for diarrhoea (A), rectal bleeding (B), weight loss (C) and total clinical score (D), calculated as described in the Materials and Methods, for 8 mice/group. Two-way ANOVAs revealed significant differences, Bonferroni-corrected. **$p<0.01$.

Figure 7.17 Effects of ES-62 on weight loss during chronic DSS colitis

Female C57Bl/6 mice were given three cycles of DSS for 5-7days, with a 7-day rest period in between each cycle when they received water. The first cycle used DSS at 1.5%, the second at 2% and the third at 2.5%. 2μg/ml ES-62 s.c was administered every second day throughout the experiment commencing two days before DSS administration began. Mice were weighed daily and results are shown as the mean percentage ± SEM of change from initial body weight. n=4 for water control groups and n=10 for the colitis groups. Two-way ANOVA (Bonferroni-corrected) revealed no significant difference between the PBS vs ES-62 colitis groups.



% of initial weight
90
100
110
120
Day
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
1.5% DSS
Water
2% DSS
Water
2.5% DSS
Control + PBS
Control + ES-62
DSS + PBS
DSS + ES-62

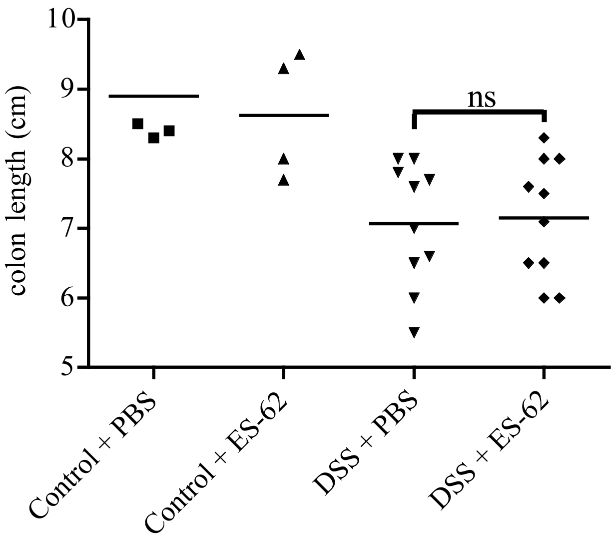
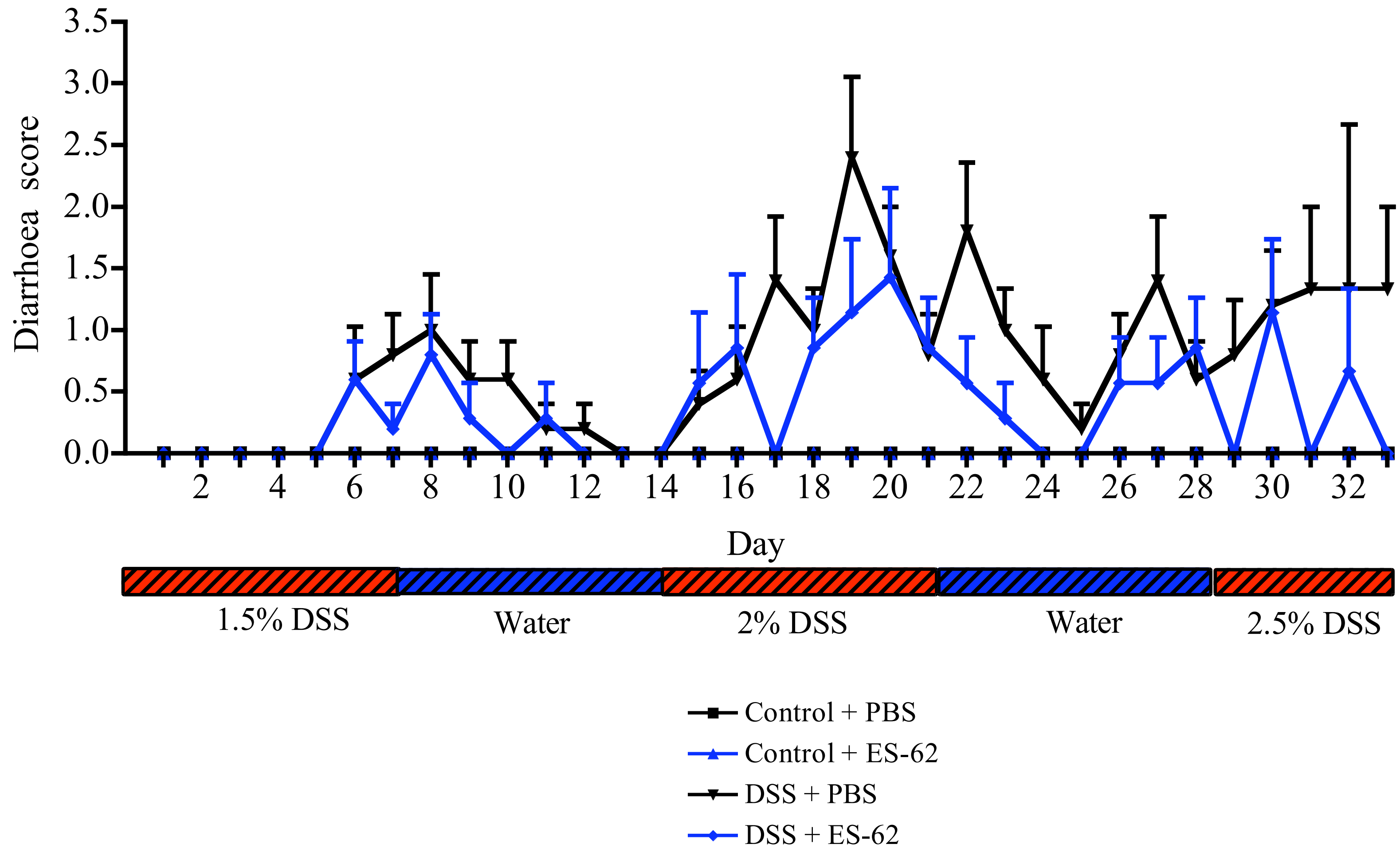


Figure 7.18 Effect of ES-62 on colon length during chronic colitis

Female C57Bl/6 mice were given three cycles of DSS for 5-7days, with a 7-day rest period in between when they received water. The first cycle used DSS at 1.5%, the second at 2% and the third at 2.5%. 2μg/ml ES-62 s.c. was administered every second day throughout the experiment commencing two days before DSS administration began. Colon lengths were recorded on d33 (except from 3 mice in the DSS + ES-62 group which were measured on day 8) and n=4 for water control groups and n=10 for the colitis groups. ns=not significant.

Figure 7.19 Effects of ES-62 on diarrhoea during chronic DSS colitis

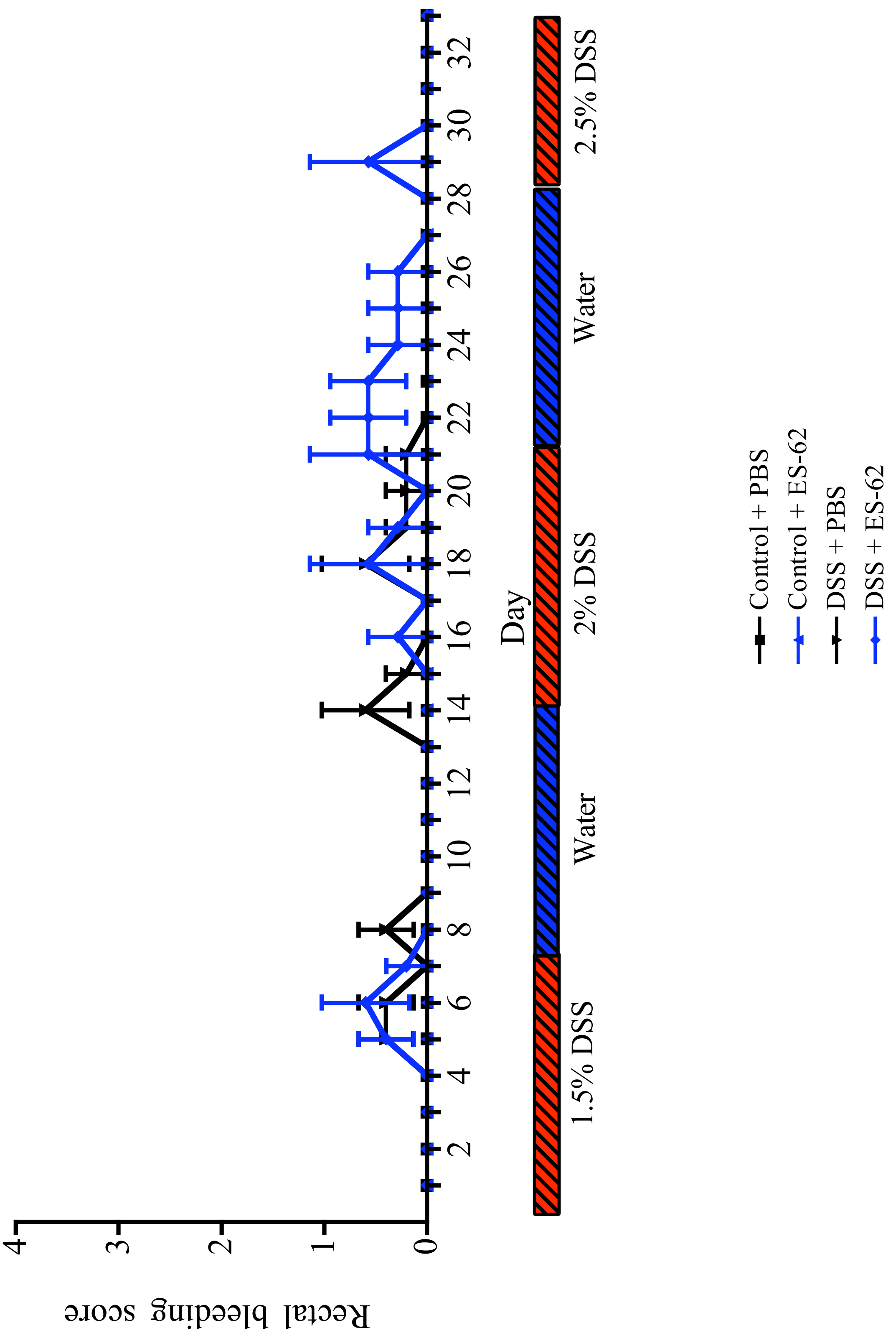
Female C57Bl/6 mice were given three cycles of DSS for 5-7days, with a 7-day rest period in between each cycle when they received water. The first cycle used DSS at 1.5%, the second at 2% and the third at 2.5%. 2μg/ml ES-62 s.c. was administered every second day throughout the experiment commencing two days before DSS administration began. Mice were monitored daily for the presence of diarrhoea and results are shown as the mean percentage ± SEM of diarrhoea score. n=4 for water control groups and n=10 for the colitis groups. Two-way ANOVA (Bonferroni-corrected) revealed no significant differences between the PBS and ES-62 colitis groups.



Diarrhoea score
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
Day
1.5% DSS
Water
2% DSS
Water
2.5% DSS
Control + PBS
Control + ES-62
DSS + PBS
DSS + ES-62

Figure 7.20 Effects of ES-62 on rectal bleeding during chronic DSS colitis

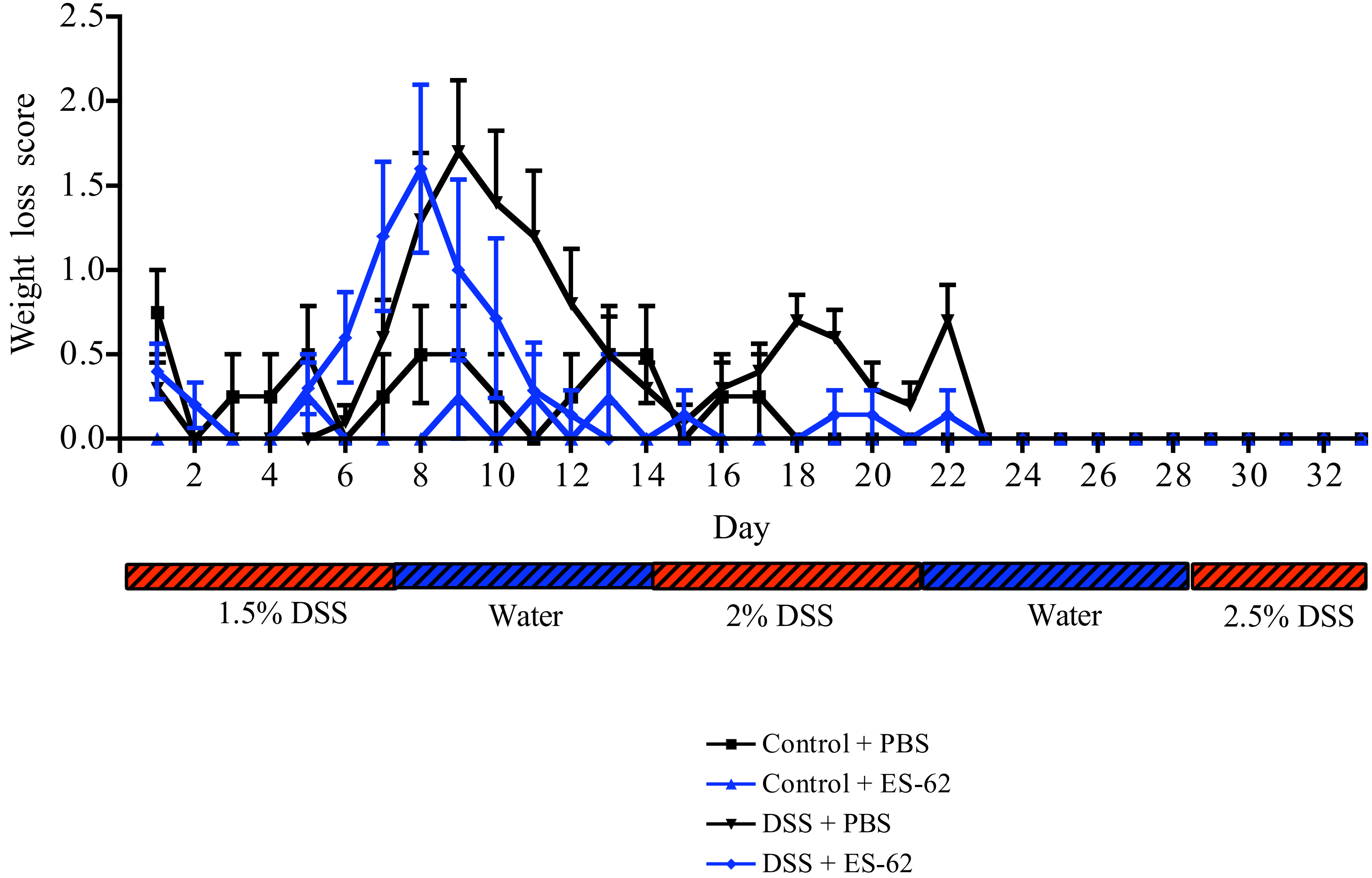
Female C57Bl/6 mice were given three cycles of DSS for 5-7days, with a 7-day rest period in between each cycle when they received water. The first cycle used DSS at 1.5%, the second at 2% and the third at 2.5%. 2μg/ml ES-62 s.c was administered every second day throughout the experiment commencing two days before DSS administration began. Mice were monitored daily for the presence of rectal bleeding and results are shown as the mean percentage ± SEM of bleeding score. n=4 for water control groups and n=10 for the colitis groups. Two-way ANOVA (Bonferroni-corrected) revealed no significant differences between the PBS and ES-62 colitis groups.



Rectal bleeding score
0
1
2
3
4
Day
1.5% DSS
Water
2% DSS
Water
2.5% DSS
Control + PBS
Control + ES-62
DSS + PBS
DSS + ES-62

Figure 7.21 Effects of ES-62 on weight loss during chronic DSS colitis

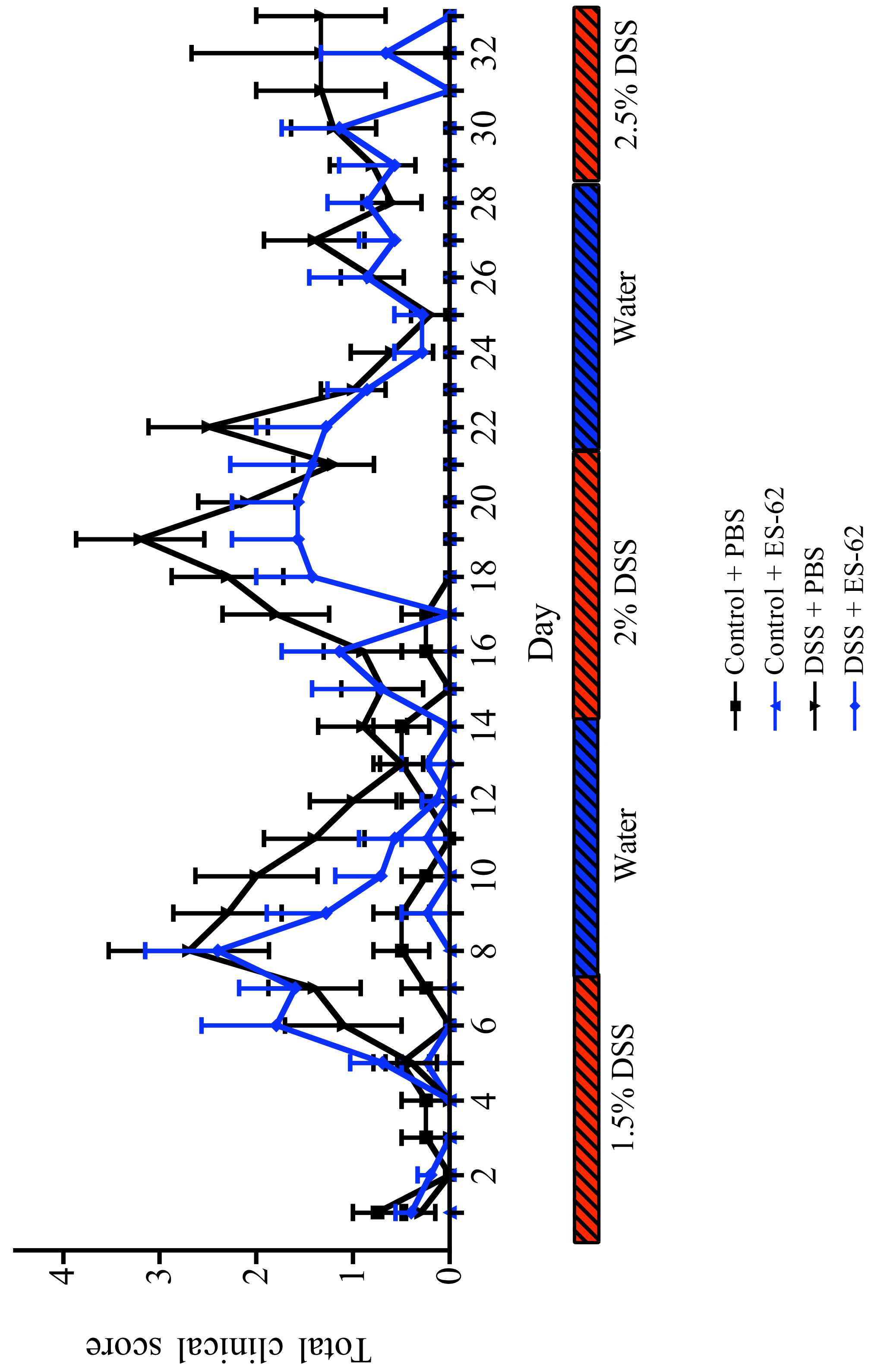
Female C57Bl/6 mice were given three cycles of DSS for 5-7days, with a 7-day rest period in between each cycle when they received water. The first cycle used DSS at 1.5%, the second at 2% and the third at 2.5%. 2μg/ml ES-62 s.c. was administered every second day throughout the experiment commencing two days before DSS administration began. Mice were monitored daily for weight loss and results are shown as the mean percentage ± SEM of weight loss score. n=4 for water control groups and n=10 for the colitis groups. Two-way ANOVA (Bonferroni-corrected) revealed no significant differences between the PBS and ES-62 colitis groups.



Weight loss score
2.5
2.0
1.5
1.0
0.5
0.0
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
Day
1.5% DSS
Water
2% DSS
Water
2.5% DSS
Control + PBS
Control + ES-62
DSS + PBS
DSS + ES-62

Figure 7.22 Effects of ES-62 on total clinical score during chronic DSS colitis

Female C57Bl/6 mice were given three cycles of DSS for 5-7days, with a 7-day rest period in between each cycle when they received water. The first cycle used DSS at 1.5%, the second at 2% and the third at 2.5%. 2μg/ml ES-62 s.c was administered every second day throughout the experiment commencing two days before DSS administration began. Mice were monitored daily for the total clinical score and results are shown as the mean percentage ± SEM of total clinical score. n=4 for water control groups and n=10 for the colitis groups. Two-way ANOVA (Bonferroni-corrected) revealed no significant differences between the PBS and ES-62 colitis groups.



Total clinical score
0
1
2
3
4
Day
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
1.5% DSS
Water
2% DSS
Water
2.5% DSS
Control + PBS
Control + ES-62
DSS + PBS
DSS + ES-62

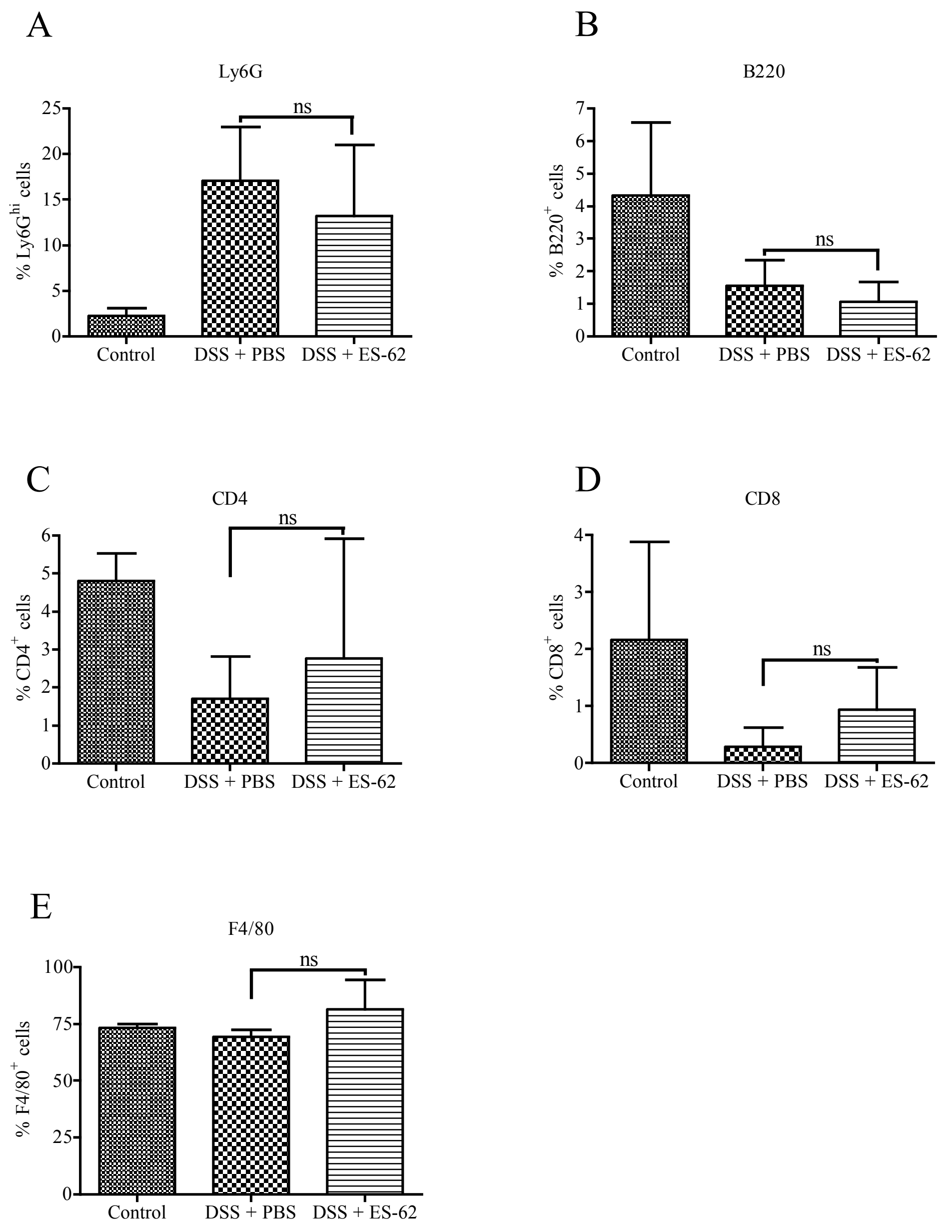


Figure 7.23 Effects of ES-62 on the cellular composition of the chronically inflamed colon

Female C57Bl/6 mice were given three cycles of DSS for 5-7days, with a 7-day rest period in between each cycle when they received water. The first cycle used DSS at 1.5%, the second at 2% and the third at 2.5%. 2μg/ml ES-62 s.c. was administered every second day throughout the experiment commencing two days before DSS administration began. Colons were digested on day 33 and the cells examined for the expression of Ly6G (A), B220 (B), CD4 (C), CD8 (D) and F4/80 (E) by flow cytometry. Results are shown as the mean percentage ± SD for 3 mice/group. ns=not significant.

Chapter 8

Discussion

The intestinal immune system faces the onerous task of protecting the body against pathogens whilst regulating responses directed at self-tissues, food antigens and the vast number of commensal microbes that reside in this tissue. Thus, cellular immune responses in the intestine have to be tightly regulated to ensure protection against infection without immunopathology. It has long been known that mϕ contribute to tissue destruction in a number of inflammatory diseases via the production of pro-inflammatory and tissue-destroying mediators. This is particularly true in the gut, where mϕ-derived pro-inflammatory cytokines are central mediators of disease and can be targeted therapeutically. However, mϕ are also known to have apparently paradoxical roles in protective immunity and tissue repair in the inflamed gut, stressing the need for further investigation into the roles intestinal mϕ play in health and inflammation.

When I started my project, there was a paucity of information on mϕ biology in the resting intestine, and the studies which had been published had focused primarily on small bowel mϕ from human biopsies *ex vivo*. This work had suggested that small bowel mϕ had dramatically downregulated pro-inflammatory functions, such as cytokine production, but retained the ability to engulf and kill bacteria (188). Furthermore, despite their potent phagocytic and bactericidal activity, human intestinal mϕ fail to show respiratory burst activity (196), or NO production via iNOS (197, 198). The basis of this was relatively unexplored, as was the possibility that different subsets of mϕ might account for these distinct homeostatic and/or pathological roles. Phenotypic studies of human colonic mϕ had demonstrated a similar phenotype, with low levels of the LPS co-receptor, CD14, class II MHC and co-stimulatory molecules, and they are also phagocytic, but no further functional studies were carried out (187). Thus the aims of my thesis were to conduct a more thorough characterisation of colonic mϕ in the resting and inflamed intestine using established systems in inbred mice. In this way, I hoped to understand more clearly the role mϕ, and possibly distinct subsets of mϕ, play in intestinal inflammation and homeostasis. In particular, I sought to investigate whether there were mϕ subsets with different functions in health and disease, to try to assess the origin of these cells and how they relate to the resident mϕ found in the resting state. The studies described in this thesis have generated a number of novel observations with regard to the function, regulation, origin and turnover of mϕ subsets in the resting and inflamed intestine.

8.1 Phenotype of resident colonic macrophages in normal mice

My initial experiments, described in Chapter 3, examined the phenotypic and functional characteristics of resident mϕ in the colon of normal mice. Mϕ have been shown to be abundant in the human intestine, suggesting an important role of these cells in intestinal homeostasis, and I found a similarly large population of F4/80^{+} mϕ in the resting colonic LP of mice. Interestingly, during my studies in the IL-10KO model, I observed that the proportion and absolute numbers of mϕ were slightly higher in older WT and KO mice, but that the level of class II MHC expression was lower compared with their younger counterparts. This could suggest that the colonic mϕ pool may expand with age and/or exposure to microbes, but that this is associated with lower basal levels of class II MHC.

I next set out to explore the phenotype of these cells in more detail as this had not been done in mice to any extent. Although mϕ in the human small and large intestine have been examined phenotypically, several differences were observed between these sites. Human colonic mϕ express the human mϕ marker, macrosialin (CD68) and low levels of CD11c, but unlike blood monocytes, express only low levels of class II MHC and CD11b (187). In contrast, human small intestinal mϕ express the zinc metalloproteinase, CD13, and high levels of class II MHC, but unlike blood monocytes, fail to express CD11b, CD11c, and the integrin LFA-1 (CD11a/CD18) (188). Previous studies in mice have shown that mϕ in the normal and helminth-infected colon express F4/80 and CD11b, but lack CD11c expression (186).

In view of the discrepancies and the lack of information on murine intestinal mϕ, I first performed a detailed phenotypic characterisation of the myeloid cell population in the colon of resting mice, using F4/80 to identify mϕ (44, 185). Like mϕ in other tissues, isolated colonic F4/80^{+} cells were large cells with cytoplasmic processes and the majority (70%) expressed intracellular CD68. Identical F4/80^{+}CD68^{+} cells were found in BMM and PEC mϕ, and all had FSC/SSC properties of mϕ. However, the smaller subset of F4/80^{+}CD68^{-} cells in the colon appeared heterogeneous in terms of FSC/SSC properties, and included some large cells not seen in the F4/80^{+}CD68^{+} gate. The identity of these cells is unclear and I was unable to find a previous description of cells with a similar phenotype. It would be interesting to examine whether these cells were included among the F4/80^{+} population that was negative for CD11b expression (see below). One possibility is that they are eosinophils, as these have been shown to express F4/80 (48) and this could be assessed by histochemical analysis of purified F4/80$^+$CD68$^-$ cells. However, my preliminary assessment of the cytospins suggested that there were no granular cells of this kind in the total colonic F4/80$^+$ population.

I also assessed the expression of CD115, the M-CSFR, which is critical for the development of mϕ (158) and is commonly used as a marker for monocytes in the mouse. In contrast to PEC mϕ and to an even greater extent BMM, colonic mϕ expressed very low levels of CD115. This could reflect differentiation status, or could be due to ligand-induced receptor internalisation (329), secondary to the high levels of M-CSF present in the colon (195). To discriminate between these ideas, it would be interesting to examine if culture of colonic F4/80$^+$ cells in the absence of M-CSF allowed expression of CD115, as this would presumably reverse the effects of M-CSF. In addition, it would be important to assess the expression of CD115 mRNA in normal colonic mϕ. Alternatively, the *c-fms* (CSF-1R)-GFP reporter mice could be used to determine if this receptor has been expressed at any time during colonic mϕ development, as this may provide an insight into the requirement for CD115 signalling in the survival and/or differentiation of colonic mϕ.

Unlike BMM and PEC mϕ, a significant proportion of colonic F4/80$^+$ cells (~30%) expressed the granulocytic marker, Gr-1. As Gr-1 expression has been associated with the 'inflammatory' subset of blood monocytes (168), it could be that the Gr-1-expressing and Gr-1$^-$ F4/80$^+$ cells in the colon derive from distinct precursors. Adoptive transfer studies using different monocyte subsets would be needed to resolve this issue fully, although functional studies of the sorted subsets could also be useful. It is also possible that at least some of these Gr-1^+ cells in the colon are granulocytes such as eosinophils which are known to express Gr-1 (330).

Compared with mϕ from the resting peritoneum and BMM, which almost all exhibited the $F4/80^+CD11b^+CD11c^-$ phenotype of conventional mouse mϕ, colonic $F4/80^+$ cells showed a strikingly high degree of heterogeneity and could be separated into three distinct subsets on the basis of these markers. These included an $F4/80^+CD11b^+CD11c^{int}$ subset, an $F4/80^+CD11b^+CD11c^-$ subset and a smaller F4/80 single positive subset. There were no consistent differences in SSC/FSC properties or in terms of morphology when the subsets were examined in preparations of purified $F4/80^+$ cells from the colon. All the $F4/80^+$ cells were large cells with cytoplasmic processes and no granular cells were apparent. It would be useful to conduct more detailed histological staining and/or electron microscopy on the different $F4/80^+$ subsets to explore any morphological differences such as numbers of lysosomes or granules, as this may indicate functional differences. Importantly, the phenotypic characteristics of colonic mϕ appear not to be due to the isolation procedure, as PEC mϕ treated with the same enzymes and protocol showed no detectable differences in the expression of the myeloid markers.

I next assessed the activation status of colonic mϕ and found that in contrast to BMM and PEC mϕ, the majority of freshly isolated colonic mϕ expressed high levels of class II MHC. These levels were higher than had been described in previous work (115), but as the earlier study had examined CD11b-expressing cells in the mouse colon, the findings may not be directly comparable. However in agreement with the previous study, I found low expression of the co-stimulatory molecules CD40, CD80 and CD86 on resting colonic mϕ. Freshly isolated BMM also expressed low levels of co-stimulatory molecules, whereas PEC mϕ expressed higher levels of these markers, especially CD40 and CD86. Although co-stimulatory molecule expression by the individual subsets was not examined, the subsets differed in class II MHC expression, with the F4/80^{+}CD11b^{-}CD11c^{-} subset being almost completely negative, while the other subsets expressed high levels. On the basis of their conventional mϕ phenotype, I would conclude that the F4/80^{+}CD11b^{+}CD11c^{-} cells in the colon are bone fide tissue mϕ, but the nature of the other two subsets is less clear. The identity of the F4/80^{+}CD11b^{-} cells I found in the colon is also unknown and I have been unable to find any published evidence of cells with a similar phenotype. As discussed above, they have a similar mononuclear morphology to the F4/80^{+}CD11b^{+} cells, which would argue against them being eosinophils that can express low levels of F4/80 (48). Nevertheless, it would be useful to examine this more directly by assessing the expression of CCR3 or Siglec F, both of which are expressed preferentially by eosinophils (331, 332).

The F4/80^{+}CD11b^{+}CD11c^{int} subset is particularly interesting, given the fact that they co-express mϕ and DC markers. Similar cells have been seen in the small intestine (Palendran, B and Agace, WW; personal communication), kidney (333), lung (229) and the inflamed peritoneum (work in our laboratory by Bordon, Y). These are probably not classical DCs, which are likely to be the F4/80^{-}CD11c^{hi} cells I found. These expressed higher levels of CD11c, as well as class II MHC and were the only cells that expressed CD103, the marker of small intestinal DC (30, 334). Nevertheless, epidermal LCs, which may represent an immature population of myeloid DCs, express F4/80 and therefore the $F4/80^{+}CD11b^{+}CD11c^{int}$ cells in the colon could also represent an early stage of myeloid cell differentiation with the potential to develop into either mϕ or DCs. This is consistent with the small number of $F4/80^{+}CD11c^{lo}$ present in BMM, although I could find no formal evidence that myeloid cell precursors co-express F4/80 and CD11c. Rather, it seems more likely to be a mature cell of some kind, given the high levels of F4/80 and class II MHC expression (335). Nevertheless, it should be noted that plasmacytoid DC-like cells in melanoma-bearing mice have also been reported to be $F4/80^{+}CD11c^{+}$ (336), and therefore the nature of these cells in the colon warrants further study.

Studies over the last 10-15 years have supported the idea that DC and mϕ are distinct lineages of myeloid cell with separate progenitors and functions. However, there is now increasing confusion over their exact relationship, with more and more overlap being found between their markers and precursors. Thus, although it was thought initially that DC development occurred independently of M-CSF (160), which is essential for mϕ development, more recent studies suggest that all monocytes, DCs and mϕ derive from a CX_3CR1^{+} $CSF\text{-}1R^{+}$ $CD117^{+}$ Lin^{-} common precursor (152). In addition, DC appear to have expressed the *c-fms* (CSF-1R) promoter (337) and it has been shown that myeloid DC numbers are dependent on CSF-1 *in vivo* (338).

Therefore DCs and mϕ may share a common circulating precursor that may differentiate separately once in tissues, under the control of the local environment. This is supported by the ability to generate conventional DC from human and murine PBMC monocytes, and from findings from *in vitro* transendothelial migration and *in vivo* studies (171, 339). Thus the unusual subset of F4/80$^+$CD11c^{int} cells in the colon could represent an unusually activated form of either DC or mϕ.

The best way to distinguish DCs and mϕ is probably functionally, by comparing antigen-presenting capacity, CCR7-dependent LN migration or bactericidal capacity. DCs are usually considered to be the only APC capable of priming naïve CD4$^+$ T cells in LN, perhaps reflecting the presence of a less proteolytic environment within DC compared with mϕ, allowing sustained antigen presentation (340). In addition, tissue DCs upregulate CCR7 after taking up antigen, and migrate to the draining LN under the influence of ELC (CCL19) and SLC (CCL21) (341, 342). Although these are usually thought to be unique properties of DCs, it has been shown that CD11c$^-$ mϕ can upregulate class II MHC and may be able to migrate to LN and present antigen to naïve CD8$^+$ T cells *in vivo* (40), suggesting they can initiate antigen presentation in some situations. However, it has not been shown that the migration of mϕ to LN is CCR7-dependent, nor if such mϕ can prime naïve CD4$^+$ T cells *in vivo*. Therefore their APC activity may not be similar to, or as efficient as DCs, but this needs to be addressed directly. Thus it would be interesting to examine the expression of CCR7 on colonic mϕ, test their ability to migrate *in vitro* in response to CCR7 ligands and assess their ability to present antigen to naïve CD4$^+$ T cells.

On the other hand, mϕ are generally considered to be more bactericidal than DC, being capable of producing high levels of toxic mediators such as ROI, NO and lysosomal proteases. Although some DCs may produce TNFα and iNOS during microbial infection

(343), this is an unusual feature and therefore the high bactericidal capacity of intestinal mϕ (188) supports the idea that the colonic F4/80$^+$ cells may be mϕ. It would be important to determine if the F4/80$^+$CD11c^{int} subset show such properties.

8.2 Functional characterisation of macrophages in the resting colon

Although I did not have time to assess the functions of the individual F4/80$^+$ subsets, I did examine the whole population of F4/80$^+$ cells. Here, in contrast to BMM and PEC mϕ, which upregulated CD40, CD80 and CD86 to some extent following stimulation with LPS or LPS + IFNγ, colonic F4/80$^+$ mϕ failed to upregulate these co-stimulatory molecules. However, unlike BMM and PEC mϕ which failed to express class II MHC when freshly isolated, or after stimulation with LPS + IFNγ, colonic mϕ expressed class II MHC when freshly isolated and after overnight culture.

As expected, BMM and PEC mϕ stimulated with LPS + IFNγ produced the pro-inflammatory cytokines TNFα, IL-6 and IL-12p70. In stark contrast and in agreement with previous studies (188), there was negligible production of any of these cytokines by the total colonic F4/80$^+$ population after overnight stimulation with LPS + IFNγ. However, there was some response to BLP by colonic mϕ. The hyporesponsive phenotype of colonic mϕ extended to Nod2 stimulation via MDP, whereas BMM produced TNFα under the same conditions. This is consistent with previous findings that human intestinal mϕ fail to make pro-inflammatory cytokines in response to Nod2 ligation (106). My findings that MDP induced TNFα production by BMM, especially when used together with BLP support some findings, but not others (344, 345). In contrast, TNFα production by colonic mϕ in response to BLP was lower when MDP + BLP were used together. Although this could mean that the interplay between the Nod/TLR pathways differs in colonic mϕ compared with other mϕ populations, these experiments were only performed once and would thus have to be repeated.

In contrast to BMM and PEC mϕ, colonic mϕ failed to produce the pro-inflammatory chemokines IP-10, KC, MCP-1, MIG, MIP-1α, RANTES and MIP-1β following stimulation with LPS + IFNγ. However, low levels of the neutrophil chemoattractant, KC, were produced. This suggests that mϕ resident in the normal colon are profoundly impaired in their ability to influence the chemokine-mediated infiltration of inflammatory cells. I was only able to do this experiment once and therefore it needs repeated, but these results are consistent with the idea that colonic mϕ have a generalised inability to react in a pro-inflammatory manner (Table 8.1). Nevertheless, it is important to note that the viability of the F4/80$^+$ population decreased to 80-85% after overnight culture, and thus the presence of dead or dying cells could influence my interpretation as the normal function of the viable mϕ could be affected.

Marker/Function	$F4/80^+$ BMM	$F4/80^+$ PEC mϕ	$F4/80^+$ colonic mϕ
CD11b	+	+++	75% (+)
CD11c	-	-	35% (+)
TLR2	++	++	30% (+)
TLR3	++	++	-
TLR4	++	++	-
TLR9	++	++	-
Class II MHC	-	-	75% (+++)
CD40	-	+	-
CD80	+/-	+	+/-
CD86	+/-	+	+/-
TNFα production	+++	++	-
Chemokine production	++	++	-
Phagocytosis	++	+++	++
Endocytosis	+++	+	++

Table 8.1 Comparison of different macrophage populations

For markers:

- - No expression; +/- very low; + low; ++ moderate; +++ high
- % The proportion of the total $F4/80^+$ population expressing marker

For cytokines/chemokines:

- - No production; ++ moderate production; +++ high production

For endocytosis/phagocytosis:

- + Low uptake; ++ moderate uptake; +++ high uptake

Despite their lack of pro-inflammatory functions, I found that colonic mϕ retained avid endocytic and phagocytic activities comparable to the other mϕ populations. Similar phagocytic activity has been demonstrated for human small bowel mϕ, which were also shown to kill internalised bacteria (188). It is unclear what mediators are used by human small bowel mϕ to kill bacteria given that they lack iNOS expression and respiratory burst activity (196-198). Thus it would be important to determine the bactericidal capacity of colonic mϕ and, if they lack these anti-microbial mechanisms, to investigate what mechanisms may be involved in this process, such as acidification, acid hydrolases, lysozyme, and nutrient competitors such as lactoferrin.

In view of their non-inflammatory properties, I considered the possibility that colonic mϕ may be alternatively activated mϕ (AAM). Like colonic mϕ, IL-4 and/or IL-13 induced AAM do not produce pro-inflammatory cytokines or NO, but they express class II MHC, are phagocytic and are involved in Th2-type anti-parasite responses (178). In addition, co-infection of mice with *Heligmosomoides polygyrus* exacerbates *C. rodentium* colitis and this is associated with the induction of AAM (346). It would be important to examine the expression of other markers associated with AMM, such as the mannose receptor and Ym-1, or the production of the AMM-associated chemokines, CCL17 and CCL22, in colonic mϕ. Due to time constraints, I was unable to carry out these experiments.

Despite lacking the expression of CD40, CD80 or CD86, colonic mϕ retained class II MHC expression, suggesting they could act as tolerogenic APCs, producing T cell anergy, or possibly the differentiation of regulatory T cells (347, 348). This would be consistent with the recent finding that small intestinal F4/80$^+$CD11b$^+$CD11c^{dull} mϕ-like cells can induce the differentiation of FoxP3$^+$ T regulatory cells *in vitro* (235). In addition, mice lacking F4/80 do not develop oral tolerance after feeding protein antigens, and this is associated with defective induction of CD8$^+$ T regulatory cells (236). To test the tolerogenic capacity of colonic mϕ, I could purify F4/80$^+$ cells from the resting colon, load them *in vitro* with ovalbumin (OVA) and examine their ability to stimulate T cells by culturing them with OVA-specific OT-II transgenic T cells and assessing the functional profile of the responding T cells, such as proliferation, cytokine production and FoxP3 expression.

For all of these functional assays, it would be important to assess the properties of the individual subsets I had identified, by first FACS-sorting the cells into individual subsets to define whether they differ in these functions.

8.3 Regulation of colonic macrophage responsiveness

In Chapter 4, I went on to examine the mechanisms of hyporesponsiveness in colonic mϕ by examining their expression of the TLRs, which mediate the recognition of microbial stimuli. Colonic mϕ showed a complete lack of surface TLR4 and intracellular TLR3 and 9 protein expression, in contrast to BMM and PEC mϕ which all expressed these TLRs. However, a small proportion of F4/80$^+$ cells in the resting colon retained the expression of surface TLR2, consistent with their partial responsiveness to the TLR2 ligand, BLP. The only previous study examining the expression of TLR proteins on mϕ from the normal human intestinal mucosa found no expression of TLR4 or TLR2, and did not examine surface or intracellular TLR3 or 9 (199). Although contested, some workers have reported that TLR4 is also absent on mucosal DC (203, 349). In that work, it was also suggested that small bowel DCs express TLR5 and respond to its ligand, flagellin. However I was unable to examine the expression of TLR5 on F4/80$^+$ colonic mϕ due to a lack of an effective antibody, and due to time constraints, was unable to examine the colonic mϕ response to flagellin stimulation.

Unusually, I found that a small proportion of F4/80$^+$ cells in the colon (~8%) expressed TLR3 on their surface. Surface TLR3 expression has been shown on human endothelial cells (350), and experiments culturing colonic mϕ with TLR3 ligand would be required to assess if this expression is functionally significant. Thus, the overall TLR hyporesponsiveness of intestinal mϕ is associated with a lack of most of the relevant receptor proteins confirming previous studies showing the lack of CD14 expression (194).

As with their other characteristics, the lack of TLR proteins in colonic mϕ was not due to the isolation procedure, as PEC mϕ treated with the same enzymes and protocol showed no detectable differences in the expression of TLRs. It would be useful to confirm my results from isolated mϕ by using immunohistochemistry to assess TLR expression by colonic mϕ *in situ*, and by measuring TLR proteins by Western blotting. However it should be noted that the absence of CD14 and CD89 on isolated human small bowel mϕ was confirmed with immunohistological analysis, and was also not a function of the isolation procedure (194), supporting the idea that these cells genuinely lack TLR expression *in vivo*.

Due to the fact that a proportion of colonic mϕ retained expression of TLR2, I assessed whether this was associated with one or other of the phenotypic subsets I had previously identified. As a result, I found that the unusual F4/80$^+$CD11b$^+$CD11c^{int}class II MHC$^+$ subset contained the highest proportion of TLR2-expressing cells (73%). In contrast, the F4/80$^+$CD11b$^-$ cells, which failed to express class II MHC, also failed to express any TLR2. As I will discuss later, it appears that this pattern of TLR2 expression may define a functionally distinct lineage of mϕ in the resting and inflamed colon.

Several mechanisms of TLR hyporesponsiveness have been described, including inhibition of TLR transcription, synthesis and signalling (114-116). I therefore tried to assess the level at which TLR expression was modulated in colonic mϕ. Although there was no previous evidence to suggest it might occur, I hypothesised that enhanced internalisation of TLRs could be a way of regulating TLR ligation and signalling. I therefore examined whether TLR2 and 4 could be detected intracellularly in colonic mϕ. This was not the case, suggesting that these TLRs are not simply internalised by the cells. This contrasts with findings that murine colonic mϕ may retain low levels of intracellular CD14 expression (202) and therefore it could be that the TLR proteins themselves are still being synthesised in colonic mϕ, but are then rapidly recycled/degraded. It would be interesting to conduct formal experiments on the biosynthesis of TLR proteins in colonic mϕ and examine the effects of blocking degradative processes such as proteasome activity or lysosomal digestion.

Although I was unable to do such experiments, I did examine TLR transcriptional activity in colonic mϕ by PCR analysis. Non-quantitative PCR first showed that BMM, PEC mϕ and colonic mϕ all expressed mRNA for TLR1, 2, 3, 4, 6 and 9. However in contrast to BMM and PEC mϕ, colonic mϕ appeared to express only low levels of TLR7 mRNA. This is in contrast to previous studies showing that human colonic mϕ from the normal mucosa fail to express TLR1-5 mRNA (199), although human small intestinal mϕ have been shown to express TLR2 and 4 mRNA (194). This has not been studied previously in mice, but these results suggested TLR mRNA synthesis might be regulated differentially in mϕ depending on the region of the gastrointestinal tract and/or the species. Because of these apparent contradictions, I performed Q-PCR analysis of TLR mRNA expression. These experiments showed that colonic mϕ expressed reduced amounts of mRNA for TLR2 and 4 compared with the other mϕ populations, indicating that control of their expression may occur at both the transcriptional and post-transcriptional levels. Little is known about the transcriptional control of TLR expression, although LPS and inflammatory cytokines such as IL-1β, IFNγ and TNFα can induce the expression of the TLR2 gene in mϕ via NFκB activation (101). The transcription factor PU. 1 and the IFN consensus sequence-binding protein are also involved in the basal regulation of TLR4 in human mϕ (99), and therefore it would be interesting to examine their expression in colonic mϕ. In contrast, TLR9 mRNA levels were normal in colonic mϕ, showing that it is regulated post-transcriptionally, either during translation, protein folding or chaperoning to the correct cellular locale. As discussed above, functional TLR9 could be synthesised in colonic mϕ but then be degraded rapidly. To examine the level of expression of other TLRs and examine how this is controlled in colonic mϕ, it would also be important to perform Q-PCR for other TLRs such as TLR7, which appeared reduced in the end-product PCR experiments, and also for the TLRs for which protein expression could not be analysed due to a lack of available antibodies.

If TLR proteins could be detected in colonic mϕ by Western blotting, it is possible that these cells have a defect in the ability to fold TLR proteins correctly and/or deliver them to the appropriate cellular compartment. Indeed murine colonic mϕ have been reported to lack the TLR chaperone, gp96, which controls TLR2, 4, 5, 7 and 9 expression (351). However gp96 is expressed in normal human intestinal mϕ, suggesting this is either not the explanation, or again that distinct mechanisms may be involved in the two species. Therefore it would be interesting to determine whether gp96 expression correlates with TLR expression in murine colonic mϕ. A further candidate regulator I could examine might be Triad3A, which targets TLR4 and 9, but not TLR2, for degradation (110). Triad3A has never been examined in intestinal mϕ, but as it may explain the specific pattern of TLR expression I observed, it would be important to determine its expression.

To investigate whether the lack of TLR expression was a permanent feature, or was dependent on constant exposure to the intestinal environment, I cultured purified F4/80$^+$ cells overnight and found no differences in the expression of TLR2 or 4. This is consistent with the finding that CD14 and CD89 were not expressed by human intestinal mϕ cultured for long periods *ex vivo* (194). This suggests that if the intestinal microenvironment is conditioning these cells, then the altered phenotype may be irreversible. It would be important to culture purified colonic mφ for longer periods to see if a longer withdrawal from the intestinal microenvironment could induce the expression of TLRs, but I found such experiments difficult due to the poor viability of colonic mφ after prolonged culture.

8.4 Effects of TLR signalling on TLR expression by macrophages

Given that colonic mφ are thought to be derived mostly from circulating monocytes, which express a range of functional TLRs (199), it could be that the unusual phenotype of colonic mφ is due to a conditioning effect by the intestinal microenvironment. Thus, I hypothesised that this lack of TLR expression by colonic mφ could reflect the presence of TLR ligands in the colon, as constant ligation of certain TLR on mφ has been shown to result in the downregulation of the corresponding receptor. This accounts for the well known phenomenon of LPS tolerance, in which exposure to LPS downregulates TLR4 (102) and it has also been described for some other TLRs, such as TLR2 (105).

To examine if TLR ligation might explain the global lack of TLR on colonic mφ, I first examined whether culture of TLR^+ BMM with various TLR ligands would alter the expression of the cognate and non-cognate TLRs. These experiments confirmed that LPS dramatically reduced the surface expression of TLR4, but some intracellular TLR4 remained detectable and LPS had no effects on other TLR proteins. In addition, the other TLR ligands, BLP, poly I:C and CpG did not affect the expression of their corresponding

TLRs, or of the other TLRs. This is in contrast to previous findings that BLP treatment reduces TLR2 expression in the THP-1 monocyte cell line (105), and that low dose CpG reduces intracellular TLR9 in RAW mϕ cell line (352). Similar studies have not been performed previously for TLR3. Given that I used similar culture periods, the reasons for these discrepancies could include the use of cell lines and different doses of TLR agonists in the previous studies. The inability of LPS to affect the expression of the other TLR proteins, together with the fact that I could not detect TLR4 in colonic mϕ following permeablisation, suggests that LPS itself is not the only factor involved in the regulation of TLR in colonic mϕ. In addition, as none of the other TLR agonists affected TLR expression, they themselves also seem unlikely to be responsible for the phenotype of colonic mϕ.

To follow up the effects of TLR ligation on TLR expression in more detail, I next assessed the effect of prior TLR stimulation on the expression of TLR mRNA in BMM, as colonic mϕ retained mRNA for TLR which were not expressed at the protein level. When I cultured BMM overnight in medium alone, I found increased expression of mRNA for TLR2, 4 and 9 compared with freshly harvested BMM. To my knowledge this phenomenon has not been looked at previously, but it could reflect differentiation of the BMM during the additional culture period and is clearly an essential control for any experiments of this kind. Treatment of BMM with BLP or LPS abrogated the increase in TLR4 mRNA expression, but had no effect on the induction of TLR2 mRNA. This is in contrast to a previous finding showing that LPS increases TLR2, but not TLR4 mRNA in murine splenic mϕ, although this study did not compare TLR levels in freshly isolated mϕ with those cultured in medium alone (101). Others have reported that treatment of murine peritoneal mϕ with LPS results in a decrease in TLR4 mRNA within a few hours, but this returned to normal by 24 hours (102). However LPS had no effect on TLR9 mRNA expression. BLP also reduced TLR9 mRNA levels to below the baseline found in freshly harvested BMM, despite having no effect on TLR9 protein expression. I could not find any previous evidence for the effects of BLP on TLR9 mRNA expression in mϕ, but these results suggest that TLR2 ligation selectively inhibits the transcription of TLR9 mRNA, rather than at the protein level where TLR9 may be more stable, suggesting different TLR ligands might regulate TLR expression at distinct levels. Specifically, LPS has a selective effect on the expression of its own TLR protein and mRNA, while BLP appears to have somewhat more wide ranging effects, especially at the mRNA level. These results also indicate that TLR mRNA expression does not necessarily correlate with the regulation of protein levels.

I found that treatment of BMM with poly I:C or LPS prevented subsequent TNFα and IL-6 responses to all the other ligands I used. The hyporesponsiveness of LPS-treated BMM to subsequent LPS was expected as a result of endotoxin tolerance. In addition, the phenomenon of functional cross-tolerance between TLR2 and 4 has been documented previously (353). BLP had a similar, if somewhat lesser effect, as it decreased the production of TNFα, but not IL-6, in response to subsequent stimulation with poly I:C or LPS. Cross-tolerance between the other TLR ligands has not been documented previously, but it should be noted that TLR4 ligation reduces the association of MyD88 with IRAK (103, 104), which could explain the effects of LPS (and other TLR ligands) on signalling via other TLRs which share the same signalling cascade. Altogether, these *in vitro* findings show that TLR function can be modulated without affecting expression of the receptor and that individual TLR ligands can have different effects at the level of protein/mRNA expression and function. Importantly, they also show that individual TLR ligands cannot reproduce the overall phenotype of colonic mϕ.

Together these findings indicate that the global downregulation of TLRs seen in resident colonic mϕ is not simply ligation of individual TLRs, suggesting this involves additional mechanisms/factors. However, the actual level of TLR protein would be important to measure, for example by Western blot analysis, to assess whether TLR ligands affected the quantity of protein rather than the percentage of cells expressing TLR. In addition, my *in vitro* culture system does not replicate the long-term exposure to multiple TLR agonists and other PRR ligands that intestinal mϕ encounter *in vivo*, and therefore experiments combining TLR agonists and with longer culture periods could reveal a role for TLR ligation in the regulation of TLR expression.

To address some of the artefacts that might be involved in the *in vitro* system, I used an *in vivo* approach in which I assessed TLR expression by colonic mϕ from TLR2KO and C3H/HeJ mice, which have a non-functional point mutation in TLR4 (76). Colonic mϕ from both strains showed the usual absence of TLR2, 3, 4 and 9 and although these studies should be repeated using additional KO mice and appropriate C3H/HeN congenic controls, they support my *in vitro* experiments that TLR2 and TLR4 signalling are not involved in the regulation of TLR expression by colonic mϕ *in vivo*. Again these *in vivo* studies do not preclude the possibility that the combined effects of multiple TLR ligands and possibly related PRRs such as Nod receptors, might reproduce the overall phenomenon. Therefore, it would be important to assess the expression of TLRs by colonic mϕ from MyD88KO or MyD88/TRIF double KO mice, or in germ-free animals where the majority of PRR signalling would be abrogated. Surprisingly, such experiments have not been published, although interestingly it has been shown that intestinal epithelial cells actually exhibit reduced expression of TLR9 in germ-free animals compared with mice colonised with microflora (354).

Interestingly, prolonged stimulation of monocyte-derived mϕ with MDP also results in tolerance to subsequent Nod2 and TLR stimulation. Although the levels of Nod2/TLR expression were not examined in this study, the phenomenon was associated with failure to activate IRAK-1 and/or over-expression of inhibitory IRAK-M (106). However it has been demonstrated that Nod1 and Nod2 play an important role in bacterial recognition after mϕ have been stimulated via TLR (355). That the colonic mϕ that I have described were unresponsive to both LPS and MDP could suggest therefore that they have been exposed chronically to MDP in the intestine. Supporting this argument, it has been shown that Nod2-deficient mϕ exhibit heightened TLR2 responses (344), and it would be interesting to examine TLR expression by Nod2-deficient colonic mϕ.

As TLR signalling alone did not seem to account for the lack of TLR expression by colonic mϕ, I went on to explore the effects of some of the potentially immunomodulatory factors that are present in the intestinal microenvironment. VIP has been shown to downregulate iNOS, as well as pro-inflammatory cytokine and chemokine production by mϕ in response to LPS (222-225), and it has been shown to downregulate TLR4 in murine mϕ (217). Retinoic acid is required for the ability of intestinal DCs and mϕ to drive regulatory T cell differentiation (30, 235), and it has also been shown to downregulate TLR2 in human monocytes (326). As noted above, IL-4 and IL-13 drive the differentiation of AAM, and can also decrease TLR3 and TLR4 mRNA expression in intestinal epithelial cells (325). Therefore I decided to examine the effects of these immunomodulatory factors on TLR expression by mϕ. However, the concentrations of VIP, IL-4 and retinoic acid I used all failed to have any effect on TLR expression by BMM. Nevertheless, given the findings from previous studies, it would be interesting to repeat these experiments using different doses and culture regimens, including combining the various mediators.

In view of these negative findings, I went on to examine the role of IL-10, by characterising colonic mϕ in IL-10-deficient mice. These mice develop spontaneous colitis in which colonic mϕ produce increased levels of pro-inflammatory cytokines in response to stimulation with whole bacteria or LPS (115, 195). Furthermore, STAT-3-deficiency in myeloid cells results in the development of colitis (233), suggesting that IL-10-mediated control of mϕ function is critical for gut homeostasis. I found that colonic mϕ from IL-10-deficient animals expressed increased levels of TLR2 and TLR4 even when examined at an age before colitis had appeared, consistent with the colitis in IL-10KO mice being TLR-dependent (239). Interestingly, increased expression of intracellular TLR3 or 9 was not detected in colonic mϕ from IL-10KO mice, suggesting that these TLRs may be regulated by distinct mechanisms. The increased expression of TLR2 and 4 by colonic mϕ from pre-colitic IL-10-deficient mice was associated with increased production of TNFα in response to the corresponding ligands, BLP and LPS. There were no substantial differences in co-stimulatory molecule expression between WT and KO mice, but the expression of class II MHC was significantly higher on IL-10KO colonic mϕ than on WT mϕ. These results indicate that in the absence of IL-10, mϕ in the colon are more susceptible to stimulation by microbial products even before overt disease develops. Although there was little spontaneous cytokine production by colonic mϕ, the fact that BMM from IL-10KO mice showed a dramatic increase in IL-12p70 production compared with WT BMM, suggests that mϕ from these mice may be intrinsically hyper-responsive. These findings from pre-colitic mice support the idea that the unusual colonic mϕ found in IL-10KO mice are not simply an additional population that has been recruited in response to inflammation.

There were no differences between the individual subsets of F4/80$^+$ cells, or Gr-1$^+$F4/80$^+$ mϕ in the WT and IL-10KO mice used in these studies, although the proportion of F4/80$^+$CD11b$^+$CD11c^{int} cells was lower in both of these BALB/c background strains compared with the C57Bl/6 mice used in my earlier studies. This suggests that although the proportion of the subsets may differ between mouse strains, IL-10 does not play a direct role in establishing the heterogeneity of resident colonic mϕ populations.

I based my interpretation that there was no colitis in the young IL-10KO mice, on the absence of clinical signs of colitis, normal histology, lack of blood in faeces as measured by the Haemoccult test, and normal numbers/proportions of mϕ. However, it is difficult to rule out completely the possibility that a low level of inflammation was present in the younger IL-10-deficient animals that was not detectable by these means. Indeed, two reports have demonstrated that IL-10-deficient mice show increased epithelial permeability in the small intestine before 4 weeks of age and prior to histological inflammation (356, 357). Therefore it remains possible that there was already minor inflammation that could have affected mϕ function, and this would also need more detailed examination of inflammatory cell infiltration at these early timepoints.

Thus, IL-10 appears to condition TLR2 and 4 expression and function by intestinal mϕ, without markedly affecting the nature of the resident colonic population. However the hyper-responsive phenotype of IL-10KO BMM may argue that all mϕ are intrinsically altered in these mice. The source of the IL-10 is unknown, but could include mϕ themselves (195), DCs (26) and regulatory T cells (220). Blocking IL-10R in cultures of purified mϕ would help assess whether this is an autocrine effect, and would also help investigate any active conditioning effects of IL-10 on TLR expression or function. It would also be important to explore how IL-10 might be acting and to what extent it can account for the phenotype of colonic mϕ. As IL-10 is involved in the differentiation of regulatory T cells (358), colonic mϕ could contribute to T regulatory cell-dependent tolerance. Furthermore, IL-10 can inhibit TLR-mediated NFκB activation by inhibiting IKK and NFκB DNA-binding activity (114), and by inducing nuclear expression of the inhibitory IκB family members, IκBNS and Bcl-3 (115, 116). Interestingly, one report found that IL-10 could induce LPS tolerance in human monocytes without modulating TLR4 expression (359). Thus, it remains unclear if IL-10 is the factor ultimately responsible for the colonic mϕ phenotype, but is certainly involved either directly or indirectly in their unresponsiveness.

8.5 Phenotype and function of macrophages in the inflamed colon

During intestinal inflammation, there is a large infiltration of mϕ that differ from the resident population in many respects, exhibiting heightened bactericidal and pro-inflammatory properties (251-253). To gain a greater insight into the nature and role of mϕ in colonic inflammation, I used the DSS model of colitis as this develops rapidly, follows a relatively well-defined pattern and produces a consistent and characteristic form of colitis (281). In my hands, DSS fed mice developed consistent weight loss by day 5, accompanied by colon shortening, diarrhoea and rectal bleeding. Histological analysis revealed crypt loss, epithelial denudation, ulceration, loss of intestinal architecture and cellular infiltration of the LP and submucosa. These histological changes were accompanied by a large influx of F4/80^{+} cells, which was first apparent by day 4, and was approximately 6-fold higher than WT by day 7 of colitis. There was also an earlier infiltration of Ly6C^{int} cells into the colon which was seen by day 3 of colitis. The majority of these cells failed to express F4/80 or CD11b, but resembled mononuclear cells in terms of FSC/SSC properties. A similar Ly6C^{int} population of monocytic cells has been described in the inflamed peritoneum, but these expressed high levels of CD11b (173) and I was unable to find any previous description of mononuclear Ly6C^{+} cells that failed to co-express CD11b. Further phenotypic analysis of these cells would be needed to identify this population definitively. During these experiments, I also found a substantial population of Ly6C^{hi} cells in the resting colon, the proportion of which did not change during inflammation. These cells were quite heterogeneous in terms of FSC/SSC properties, with most appearing to be mononuclear in nature. However some of these Ly6C^{hi} cells were small enough to fall within the lymphocyte gate, and indeed Ly6C is expressed by a subset of memory $CD8^+$ T cells and small intestinal LP IgA^+ plasma cells (360, 361). However, the identity of the larger $Ly6C^{hi}$ cells is unclear and would require further characterisation.

There was also infiltration of $Ly6G^{hi}$ neutrophils into the inflamed colon, but this was delayed with respect to the monocytic cells, not being seen until day 7. This was unexpected given that neutrophils are usually one of the first cells to appear in inflammatory sites. Nevertheless, a population of $Ly6G^-$ cells with the FSC/SSC appearance of granulocytes did appear by day 4 of colitis and these could be eosinophils. Eosinophils and mϕ are sometimes considered mutually exclusive in models of colitis, with eosinophils being found in Th2-type models, such as that induced by oxazalone, whereas mϕ are associated with the Th1-like TNBS model of colitis (282). However, during DSS colitis and human ulcerative colitis, eosinophils and mϕ are present together and in fact, intestinal mϕ have been shown to express the eosinophil chemoattractant, eotaxin-1 (291, 362). Again, it would be interesting to assess the expression of eosinophil-specific markers such as Siglec F and CCR3 to confirm the nature of these granulocytic cells that appear early in DSS colitis.

The increase in the number of mϕ during colitis accounted for a substantial proportion of the increase in total cellularity, and was associated with a switch in the phenotype of the dominant mϕ population, with 70% now being $F4/80^+CD11b^+CD11c^{int}TLR2^+$. The majority of this population also expressed class II MHC, but still expressed only low levels of co-stimulatory molecules. There was also an increase in the proportion of colonic mϕ that expressed TLR4 during colitis and I assume that these were the mϕ that also expressed TLR2$^+$, although there were insufficient colours available to determine this directly by flow cytometry. The increased proportion of TLR-expressing mϕ was particularly apparent at the distal end of the colon, reflecting the pattern of disease severity in this model (281), presumably due to the higher level of microbiota in this location. TLR expression by colonic mϕ has not been studied previously in mouse models of colitis, but the TLR2$^+$ mϕ I found may be similar to the 'inflammatory' CD14$^+$ mϕ subset recently described in biopsies from Crohn's disease patients, that produce TNFα, IL-6 and IL-23 (272). Consistent with this and in stark contrast to the resting state, a large proportion of the TLR$^+$ mϕ population I found in the inflamed colon produced TNFα spontaneously, and this was increased slightly by stimulation with BLP or LPS. In contrast, the TLR2$^-$ mϕ remained unable to produce TNFα under any conditions. Although some of the TNFα-producing cells in the inflamed colon expressed little or no F4/80, they all expressed high levels of CD11b, and were mononuclear in terms of FSC/SSC properties. These were not present in the resting colon and it is possible that they may represent recently recruited monocytes, which have been shown to express reduced levels of F4/80 (43). Their ability to produce TNFα 'spontaneously' without addition of exogenous stimulus suggests that these mϕ had been exposed to pro-inflammatory stimuli *in situ*.

My findings support the idea that monocytes/mϕ are the major source of TNFα in this model of colitis and suggest that they play a central role in TNFα-dependent pathologies in the intestine. However, the role of TNFα in DSS colitis is not clear, as anti-TNFα has been shown to aggravate acute DSS colitis but ameliorate chronic colitis, (363).

Furthermore, acute DSS colitis is exacerbated in TNFα null mice (364). It would be interesting to assess whether the $TLR2^+$ mϕ expressed other pro-inflammatory mediators such as IL-6, IL-23 and iNOS, which can play a detrimental role in experimental models of colitis (365-367), but I was unable to do this due to time constraints.

8.6 Turnover of macrophages in the resting and inflamed colon

To explore the relationship between the TLR^+ and TLR^- subsets of mϕ, I investigated the turnover kinetics of the two subsets in the resting and inflamed state. BrdU pulse-chase experiments would provide some insight into the origin of the TLR^+, TNFα-producing mϕ population present during colitis. By comparing BrdU uptake during pulse-chase experiments with *in situ* cell division, I could assess whether the appearance of the TLR^+, TNFα-producing mϕ reflected differentiation of the resident population, or active proliferation of F4/80$^+$ cells and whether these cells had divided outside the intestine and been recruited. This was important to investigate as the manipulation of mϕ behaviour or alternatively, effective blocking of mononuclear cell infiltration into the gut, could prove to be effective therapeutic approaches.

I first assessed the turnover of monocyte precursors in the BM by measuring BrdU uptake, as I postulated that this population would be proliferating actively and thus allow me to assess the usefulness of the technique, and also give me an insight into the dynamics of monocyte precursors. Remarkably, within 2 hours after BrdU administration, >50% of

$F4/80^+$ cells in the BM had divided, indicating that a substantial turnover of monocyte/mϕ precursors occurs in the BM in the steady state.

In the colon, I found that ~5% of the total $F4/80^+$ population in control animals had incorporated BrdU during the 24 hour period, and there was also considerable uptake among the non-haematopoietic cell compartment, probably epithelial cells. However, during colitis, less than 30% of these $F4/80^-$ $BrdU^+$ cells were $CD45^-$, suggesting that there was a decrease in proliferation of non-haematopoietic cells and/or a proportional increase in the proliferation of $CD45^+$ $F4/80^-$ cells. More significantly, during colitis, ~17% and ~19% of the total $F4/80^+$ population incorporated BrdU on days 3→4 and 5→6, respectively. Together with the substantial increase in total mϕ numbers, these results show there was a considerable increase in the accumulation of recently divided mϕ during colitis.

Interestingly, when colonic mϕ were divided on the basis of TLR2 expression, the recently divided mϕ belonged to the $TLR2^+$ population, with negligible proliferation being seen among the TLR^- subset in both the resting and inflamed colon. The proportion of $BrdU^+$ cells among the $TLR2^+$ population also increased during intestinal inflammation (~20% versus ~6% in resting colon), but the low levels of BrdU uptake by the TLR^- subset did not alter substantially during inflammation. Together these findings suggested that TLR^+ mϕ might behave differently even in the resting colon.

To explore this further and to gain a better idea of the turnover of mϕ subsets in the normal and inflamed colon, I assessed the turnover rates of colonic mϕ over a prolonged period during the development of colitis and in control mice. This confirmed the increased accumulation of recently divided mϕ during colitis and again, the TLR^+ subset showed higher levels of turnover both in the resting and inflamed colon. Although I did not prove it directly, the $BrdU^+$ cells are probably the spontaneous TNFα-producing cells present during colitis which also have the capacity to produce TNFα when stimulated via TLR2 in the resting state. In addition, some of the $BrdU^+$ cells I found in the colitic mucosa were $F4/80^{lo}$. Like the $F4/80^{lo}$ TNFα-producing cells I had identified earlier, these $F4/80^{lo}BrdU^+$ cells were only present in the inflamed colon, expressed high levels of CD11b and exhibited mononuclear FSC/SSC properties. This together with their considerable level of BrdU uptake, supports the idea that these cells are recently derived from the monocyte pool which is known to express a lower level of F4/80 (43). The TLR^+ subset of colonic $F4/80^+$ cells also differed from their TLR^- counterparts in their expression of Gr-1 in both the resting and inflamed state, with a higher proportion of the TLR^+ subset positive for this marker. In addition, the $TLR2^+$ subset of colonic mϕ, but not the $TLR2^-$ subset, expressed CCR2 in the resting and inflamed colon. Together these differences in phenotype and population kinetics suggest that the TLR^+ and TLR^- mϕ may represent independent lineages of mϕ, rather than the effects of a local conditioning event in which one subset differentiates into the other.

The increased number of TLR^+BrdU^+ mϕ during colitis compared to the resting state could reflect increased local proliferation, increased recruitment to the colon, possibly under the influence of increased chemokines, and/or increased survival. The increased number of TLR^+ mϕ in colitis appeared not to be due to increased local proliferation, as although Ki-67 expression was slightly increased in colitic mϕ, this was too small to account for the differences in BrdU uptake.

Increased CCR2-dependent recruitment could be a critical mechanism underlying the expanded population of TLR^+ mϕ during colitis. The CCR2 chemokine receptor is known to be involved in recruitment of inflammatory mϕ to the intestine (327), and the fact that the subset of colonic mϕ that expressed TLR2 also expressed high levels of CCR2 suggests ligands for this receptor may be involved in the recruitment of these cells into the colon in the resting and inflamed state. Their increased numbers in colitis would be consistent with the increased expression of one of the CCR2 ligands, MCP-1 in IBD mucosa (368). The production of CCR2 ligands in colitic mucosa would help test the hypothesis that increased recruitment is important for the increased accumulation of TLR^+ mϕ during DSS colitis. In addition, the accumulation of adoptively transferred $CCR2^+$ versus $CCR2^-$ monocytes in the colon could be determined. Alternatively, measuring $F4/80^+TLR2^+$ cell infiltration into the colon of control or DSS-fed CCR2-deficient mice, or WT mice treated with anti-CCR2 blocking antibody would allow the importance of CCR2 in monocyte recruitment during colitis to be addressed.

Interestingly, the release of $Ly6C^{hi}$ monocytes from the BM into the circulation in response to *L. monocytogenes* infection, but not from the circulation into the infected spleen, is dependent on CCR2 (172). In addition, analysis of uninfected CCR2KO mice demonstrated a paucity of $Ly6C^{hi}$ monocytes in the blood, suggesting CCR2 contributes to monocyte emigration from the BM even under physiological conditions (172, 369), however this may be different for recruitment into tissues. Therefore, by comparing the accumulation of transferred, fluorescently labelled $CCR2^{+/+}CD45.1^{+}$ and $CCR2^{-/-}CD45.2^{+}$ monocytes into the colon of normal or colitic WT recipients, with monocyte recruitment in CCR2 KO mice, I could distinguish between a role for CCR2 in BM egress versus recruitment from blood into the tissue in this model. It may also be interesting to investigate whether the absolute numbers of monocytes and the proportions of monocyte subsets are altered in the blood of colitic mice compared with control animals. Furthermore, repeating the BM monocyte experiment during colitis to investigate how monocyte precursor turnover in the BM changes during colitis would be interesting.

However, as discussed below, I was unable to establish an effective method to investigate monocyte recruitment to the colon, and the exact contribution of CCR2-dependent mϕ recruitment to the pathogenesis of colitis is unclear. Although antibody-mediated triple blockade of CCR2, CCR5 and CXCR3 reduces DSS-induced colitis (279), this study did not examine the effect of blocking CCR2 function alone and indeed, CCR2-deficient mice are still susceptible to DSS colitis (370). However they exhibit reduced mucosal ulceration, suggesting that different aspects of pathology may involve different cells and mechanisms. It is important to emphasise that as the majority of TLR2-expressing mϕ are also $CCR2^{+}$ in the resting colon, this process must also be occurring in the absence of inflammation. These TLR^{+} mϕ in the resting colon may therefore represent recently recruited, BM-derived monocytes and resemble the small proportion of $CD14^{+}$ mϕ seen in the normal human intestine (201, 272). Again it would be interesting to investigate directly the role of CCR2 in the recruitment of these cells to the normal colon.

The increased number of TLR2$^+$ mϕ in the inflamed colon could also reflect increased survival of this subset under these conditions, possibly via heightened immunostimulatory signals present in the inflamed intestine. Their survival could be investigated by long term BrdU incorporation studies in which the rate of loss of labelled mϕ subsets could be compared, along with examining the survival of the subsets *ex vivo*. However, the long term BrdU studies could still be complicated by emigration of cells to other tissues, and it would also be difficult to exclude conversion of phenotype over long periods. It would therefore also be interesting to assess the presence of apoptotic mϕ directly using methods such as the TUNEL assay.

8.7 Colonic macrophage subsets in the resting and inflamed state

I found several differences between the TLR$^+$ and TLR$^-$ mϕ that suggest that these cells are two distinct subsets (Table 8.2). The TLR$^-$ subset is F4/80$^+$CD11b$^{+/-}$CD11c$^-$ and lacks CCR2 and Gr-1 expression. These cells turn over slowly and are unresponsive to stimulation, even during inflammation. In contrast, the TLR$^+$ subset is F4/80$^+$CD11b$^+$CD11c^{int}, expresses some Gr-1 and high levels of CCR2. These turn over rapidly and produce pro-inflammatory mediators such as TNFα.

Marker/Function	TLR$^-$ ‘resident’ subset	TLR$^+$ ‘inflammatory’ subset
Description	Dominant in resting (70%)	Dominant in colitis (70%)
TLR	-	TLR2++, TLR4+
Class II MHC	++	+++
Co-stimulatory molecules	-	+
CCR2	-	+++
Gr-1	-	(30%) +
CD11b	+/-	+
CD11c	-	+
In vivo cell turnover	+	+++
Pro-inflammatory cytokine production	-	+++

Table 8.2 Comparison of TLR$^-$ and TLR$^+$ macrophage subsets in the colon

<u>For markers:</u>

- - No expression; +/- proportion negative and positive for CD11b; + low; ++ moderate; +++ high
- % The proportion of the population expressing marker

<u>For cell turnover:</u>

- + Low turnover; +++ high turnover

<u>For cytokine production:</u>

- - No production; +++ high production

As discussed above, I propose these represent independent lineages of monocyte-derived mϕ and when I cultured total purified F4/80$^+$ cells from the resting colon *ex vivo*, TLR2 expression did not alter, indicating conversion did not occur *in vitro*. However it would be necessary to repeat these experiments using sorted TLR$^+$ and TLR$^-$ subsets to address this directly. Furthermore, there was no evidence from the long term BrdU studies that the BrdU uptake slopes of the two subsets converge in either the resting or inflamed state, as might be expected if one subset were the precursor of the other. Furthermore, the fact that the TLR$^-$ mϕ subset remains unresponsive during colitis suggests that these cells cannot simply convert to the TLR$^+$, responsive phenotype in response to inflammatory signals.

My hypothesis is consistent with current views on monocyte/mϕ precursor heterogeneity. Specifically, it could be that the TLR$^+$ subset are derived from the short-lived CCR2$^+$CX$_3$CR1$^-$Gr-1$^+$ inflammatory monocyte subset, whereas the TLR$^-$ subset may be derived from the long lived CCR2$^-$CX$_3$CR1$^+$Gr-1^{lo} monocyte subset.

I did attempt to study this by examining precursor cell migration into the intestine using BMM, which were used as it was impossible to obtain sufficient numbers of blood monocytes. Adoptively transferred BMM were undetectable in all tissues examined apart from the peritoneum, where they were only present in small numbers. Therefore, this model of adoptive transfer proved unsuitable for tracking the migration and/or behavioural adaptation of mϕ in the colon, possibly because BMM are different from blood monocytes and may not have the ability to migrate into tissues. Thus to track the migration and behaviour of mϕ in the colon, it would be necessary to repeat these experiments using monocytes from RAG KO donors, where there is a higher proportion of blood monocytes making isolation of sufficient monocyte numbers a more feasible approach. Alternatively, an approach pioneered by Randolph and colleagues involves intravenous injection of particulate tracers that are taken up preferentially by the Gr-1^{lo} monocyte subset, allowing differential fluorescent labelling and tracking of monocyte subsets (371).

A crucial question is what the mϕ subsets do in the healthy and inflamed intestine. Interestingly, following myocardial infarction, Ly6C^{hi} and Ly6C^{lo} monocytes are recruited sequentially to the heart via CCR2 and CX_3CR1, respectively (175). Ly6C^{hi} monocytes dominate the early stages of the reaction in the myocardium and exhibit inflammatory and proteolytic functions, driving tissue destruction. Conversely, non-inflammatory Ly6C^{lo} monocytes do not appear until later, express vascular-endothelial growth factor (VEGF), and promote myofibroblast accumulation, angiogenesis, collagen deposition and tissue healing. Thus in the inflamed colon, it could be that the two subsets are responsible for different phases of inflammation. The TLR^{+} mϕ could drive inflammation by producing pro-inflammatory mediators such as TNFα, whilst the TLR^{-} mϕ may promote tissue healing by producing cytoprotective factors. However this raises the interesting question of why both subsets are also present in the normal colon, what they are doing there and how their activities are balanced. The TLR^{-} mϕ subset may be able to clear bacteria without initiating any inflammation, and so may be present solely to regulate the levels of commensal flora in the colon. It could be that these mϕ gain access to commensals via temporary breaches in the epithelial barrier, or could send out cellular processes between epithelial cells and into the lumen, as has been described for DCs. Another role for the TLR^- population of mϕ could be the protective functions mϕ play in regulating epithelial renewal and integrity under physiological and inflammatory conditions (235, 237, 241). However this would seem difficult to reconcile with the fact that TLR signalling in BM-derived cells appears to be necessary for epithelial homeostasis and repair after DSS-induced injury (238, 239). In addition, colitis induced by infection with *Citrobacter rodentium* is exacerbated in TLR2 null mice due to impaired barrier function (240, 372). Here it is thought that mϕ send out processes to contact epithelial progenitors, and that MyD88 signalling in mϕ drives the repositioning of prostaglandin-endoperoxide synthase-2 (Ptgs2)-expressing stromal cells from the upper/middle crypts to the crypt base adjacent to the progenitor cells (241, 373). If $TLR2^-$ mϕ are truly protective, these findings raise the question of whether TLR expression is downregulated after arrival in the mucosa, and if the TLR^+ and TLR^- mϕ populations are actually distinct lineages. In addition, due to the lack of CCR2 expression, the question remains if, and how, the TLR^- mϕ subset is actively recruited to the intestine. It could be that these cells have expressed TLR and CCR2 at the time of entry into the gut, before downregulating TLR and CCR2, but my findings in the cell turnover experiments suggest that this may not be the case.

In contrast, $TLR2^+$ mϕ may be recruited continuously from recently divided $CCR2^+$ precursors and patrol the mucosa constantly for pathogens. Although potentially responsive to stimulation, these cells may not persist for long periods in the gut in the absence of invasive pathogens or other exogenous stimuli and so are unable to generate significant inflammation. However, it is quite possible that this may contribute to the low level of 'physiological inflammation' that appears to characterise the normal intestine. The possibility of reduced survival of these cells is supported by the fact that TLR2 signalling has been shown to deliver pro-apoptotic signals in TLR2-transfected human embryonic kidney (HEK) 293 cells (374). In addition, the $TLR2^+$ mϕ may be situated in a part of the mucosa distant from the epithelium, sequestered from non-invasive commensal bacteria, and so may only respond to pathogenic organisms that breach the epithelial barrier. Thus, it would be interesting to compare the relative localisation of the TLR^- and TLR^+ mϕ subsets with respect to the epithelium in the resting and inflamed colon by immunohistochemistry. In addition, to address the impact of CCR2-mediated signals on the location of mϕ in the intestine, it would interesting to look at what happens to the physiology of the gut in CCR2KO mice.

Thus, further studies focusing on the functional characterisation of TLR^+ and TLR^- mϕ during intestinal inflammation, epithelial repair and homeostasis are required. Indeed, it would be interesting to compare the gene expression profiles of the mϕ subsets in the healthy colon and at different stages of DSS colitis to explore the ways in which these cells contribute to homeostasis and pathology. As discussed, the differences between the TLR^+ and TLR^- mϕ may reflect a distinct nature and/origin of these two subsets. To test if the mϕ subsets have distinct precursors and if one subset is preferentially recruited to the inflamed colon, I could use the same system as that used by Littman and colleagues (168). These authors co-injected the two monocyte subsets, separated on the basis of CX_3CR1-GFP expression, into congenically different mice, before tracking the migration of these subsets into inflamed and non-inflamed tissues. By comparing control and DSS-fed recipients, I could track and compare the ability of the two subsets of monocytes to accumulate in the colon under both conditions. It would also be important to assess the phenotype, and the expression of TLR2, of monocyte subsets before transfer and following their arrival in the resting or inflamed colon.

8.8 Effects of ES-62 on macrophage function

The nematode glycoprotein, ES-62, has been shown to modulate pro-inflammatory functions of mϕ, reducing the production of cytokines such as TNFα and IL-12, but not NO (315). Therefore I attempted to use ES-62 in the DSS model of colitis where mϕ play a central role. Culture with ES-62 did not result in classical activation of mϕ, in that there was no upregulation of class II MHC, co-stimulatory molecule expression, iNOS or production of pro-inflammatory cytokines. In my hands, there was also no cytokine production induced by ES-62 alone, in contrast to earlier reports (315). I also planned to confirm the previous findings that ES-62 treatment could inhibit subsequent activation of mϕ by LPS and IFNγ (315), but due to time constraints I was unable to do this.

As discussed earlier, it has been suggested that helminths can induce the polarisation of mϕ toward the AAM subtype (375). Unlike LPS and IFNγ, ES-62 did not reduce the expression of the AMM markers, arginase and Ym-1, which were both present in freshly harvested BMM and arginase appeared to increase during culture in medium alone. Although this could suggest that ES-62 favours the differentiation of mϕ into an AMM-like phenotype, the fact that control BMM also expressed these markers makes it difficult to know whether ES-62 is actively driving this phenotype, or is simply just not preventing the loss of these properties. Repeat experiments and further quantitative PCR analyses, together with measurement of the markers at the protein level would provide a greater insight into the ability of ES-62 to drive an AAM phenotype.

When I carried out these experiments using ES-62, my project had not yet focused specifically on mϕ, so I also conducted some of the studies examining the effects of ES-62 on DCs. I first attempted to replicate work that had shown ES-62 to reduce TLR responsiveness in DCs (318). However, I could not confirm the findings that ES-62 inhibits TLR-mediated upregulation of class II MHC or co-stimulatory molecules, or the production of pro-inflammatory cytokines. Although TNFα and IL-6 responses to BLP were somewhat reduced by ES-62, this was not statistically significant and repeat experiments would be necessary to address properly the ability of ES-62 to modulate TLR-induced functions in DCs. Therefore, it is clear that ES-62 does not drive the full classical activation of mϕ, and instead may favour the differentiation of AMM and although I was unable to show this, it has previously been shown to induce hyporesponsiveness in DCs and mϕ.

8.9 Effects of ES-62 on intestinal inflammation

For these reasons, I went on to examine the effects of ES-62 on DSS colitis to determine if it would have a similar ability to inhibit this form of inflammation, as it does in other models such as joint and lung inflammation (306, 319). ES-62 treatment had no effect on weight loss, colon shortening, pro-inflammatory cytokine production or cellular infiltrates in the colon during acute DSS colitis. However, ES-62 did seem to have a subtle effect on some of the clinical aspects of disease, particularly rectal bleeding. Because of this partial effect, I set up another experiment, in which I used a lower dose of DSS to try and produce a less severe disease. The ES-62 dose used was the same as in the previous experiment, but the ES-62 was injected s.c. to slow absorption into the bloodstream and maintain a more constant level of ES-62 in the circulation. Colon shortening was significantly reduced in the ES-62-treated group, as were the clinical aspects of disease. However, again there were no effects of ES-62 on weight loss and pro-inflammatory cytokine production, and although there was a significant increase in the proportion of $CD8^+$ T cells in the ES-62-treated group, this was very small and was not seen in the other experiments. To try to modulate the pathology more substantially, I repeated the experiment using a higher dose of ES-62 and using female mice, thought to have reduced susceptibility to DSS-induced colitis (376). However, this disease was not less severe in terms of time of onset and severity, and the only difference was that ES-62 delayed the onset of clinical disease by 24hrs.

As ES-62 had only minor and inconsistent effects on acute colitis, I next assessed its effects in a chronic form of the disease which I postulated might allow any modulatory effects of ES-62 to be seen more clearly. However, after the first cycle of DSS and body weight had recovered, further cycles of DSS treatment induced little overt disease in ES-62-treated or control animals. There was also reduced infiltration by $Ly6G^+$ neutrophils and colon shortening compared with what was seen in acute DSS colitis. Here, ES-62 had no effect on clinical scores, rectal bleeding, colon shortening or the cellular infiltrates.

Taken together, my data suggest that ES-62 may have some protective effects in acute colonic inflammation, but these were subtle and I was unable to develop a suitable model of chronic disease in which the effects of ES-62 could be investigated. It is possible that the inability of ES-62 to substantially modulate DSS-induced pathology could reflect an insufficient concentration reaching the colon. Although I used the same or higher doses of ES-62 used in other tissue-specific models of inflammation, oral administration of ES-62 might be needed to obtain sufficiently high local levels in the intestine. Alternatively, ES-62 has been shown to complex with TLR4 and PKCα, to be internalised into vesicular compartments (319). Thus it may be that colonic mϕ, even in the inflamed colon, do not express TLR4 at sufficient levels for ES-62 to be internalised in sufficient concentration to exert its effects. I was unable to explore these possibilities further due to the need for large quantities of ES-62 and mice, and the fact that I had begun to obtain interesting data on mϕ characteristics in the resting and inflamed colon, and thus my project changed focus to this area.

8.10 Concluding Remarks

Taken together, my results show that colonic mϕ retain some characteristics of activated mϕ, such as class II MHC expression, endocytosis and phagocytosis, but they have lost the ability to produce pro-inflammatory mediators. This would imply that intestinal mϕ could potentially clear bacteria without initiating an inflammatory cascade. In addition, unlike other mϕ populations, colonic F4/80$^+$ cells were extremely heterogeneous and could be split into three subsets on the basis of CD11b and CD11c expression. Their lack of co-stimulatory molecule expression, but high phagocytic capacity and expression of class II MHC, suggests that mϕ may take up commensals and induce tolerance. If mϕ in the resting colon were found to be tolerogenic, it could provide a mechanism for the maintenance of peripheral tolerance in the intestine, whereby uptake and presentation of innocuous antigens to T cells in a non-inflammatory manner could maintain tolerance to these antigens. In addition, intestinal mϕ are likely to play an important role in epithelial renewal in homeostasis and inflammation.

The apparent stimulus-independent downmodulation of pro-inflammatory activities in the dominant population of resting intestinal mϕ indicates a global reprogramming of cellular function under physiological conditions. That mucosal mϕ are quiescent and refractory to inflammatory signals seems somewhat paradoxical given their continuous exposure to microbial stimuli. However, by being specifically and exquisitely adapted to a unique and extreme microenvironment, this population is critical for the maintenance of tissue homeostasis. These are actively functioning cells which not only reflect their microenvironment, but can also influence the milieu and other cell populations around them.

However, under resting conditions, there is also a small population of $TLR^{+}CCR2^{+}$ mϕ that more closely resemble conventional monocytes/mϕ, and which are potentially responsive to inflammatory stimuli. These turn over rapidly, being derived from recently divided precursors and may not have yet undergone ‘conditioning’ by the intestinal microenvironment, or alternatively they may represent a distinct subset of mϕ. These potentially responsive mϕ may not be present in sufficient numbers to elicit inflammatory responses in the normal intestine, but during intestinal inflammation or infection, this balance is altered by increased recruitment and/or survival of $TLR^{+}CCR2^{+}TNF\alpha^{+}$ mϕ to the mucosa. Together with the altered microenvironment of heightened immunostimulatory signals, this may shift the overall balance in favour of mϕ activation and overt inflammation. The inertia of resident mϕ is physiologically crucial, as failure of intestinal mϕ to become tolerant to TLR ligands and other pro-inflammatory stimuli can produce inflammation in the intestine. Whatever the explanation for the hyporesponsiveness of the dominant resident population, it appears that health and inflammation in the intestine reflect a delicate balance between these two mutually opposed populations of mϕ, whose numbers and behaviour are intimately related to the state of the local environment. Understanding the mechanism(s) of immunoregulation in intestinal mϕ has clear implications for the understanding of oral tolerance, oral vaccine development, and treatment of diseases such as IBD.

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Publications

1. **Platt AM**, Mowat AM “Mucosal macrophages and the regulation of immune responses in the intestine” *Immunology Letters* 2008 August 15;119(1-2):22-31.

2. Monteleone I, **Platt AM**, Jaensson E, Agace WW and Mowat AM “IL-10-dependent partial refractoriness to toll-like receptor stimulation modulates gut mucosal dendritic cell function” *European Journal of Immunology* 2008 May 6;38(6):1533-154.