The Role of the D6 Chemokine Receptor in Immunity and Inflammation

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Table of Contents

List of Tables and Figures

CHAPTER 4

CHAPTER 5

CHAPTER 6

List of Abbreviations

The following abbreviations are used in this thesis:

AAM	Alternatively Activated Macrophage(s)
Ag	Antigen
APC	Antigen Presenting Cell(s)
APC	Allophycocyanin
ApoE	Apolipoprotein E
BCR	B Cell Receptor
BM	Bone Marrow
Bio	Biotinylated
BrdU	Bromodeoxyuridine
CAM	Classically Activated Macrophage(s)
CCL	CC-chemokine ligand
CCR	CC-chemokine receptor
CCX-CKR	Chemocentryx Chemokine Receptor
CD	Cluster of Differentiation
CFA	Complete Freund's Adjuvant
CIPR	Chemokine Internalising Pseudoreceptor
CLP	Common Lymphoid Progenitor
CMP	Common Myeloid Progenitor
CMF-HBSS	Calcium/Magnesium Free Hank's Buffered Salt Solution
Con A	Concanavalin A

COX	Cyclooxygenase
CXCL	CXC-chemokine ligand
CXCR	CXC-chemokine receptor
CX_3CL	CX3C-chemokine ligand
CX_3CR	CX3C-chemokine receptor
DAG	Diacylglycerol
DARC	Duffy Antigen Receptor for Chemokines
DC	Dendritic Cell(s)
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DSS	Dextran Sulphate Sodium
DTH	Delayed Type Hypersensitivity
EAE	Experimental Autoimmune Encephalitis
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked ImmunoSorbent Assay
ER	Endoplasmic Reticulum
FACS	Fluorescence Activated Cell Sorting
FAK	Focal Adhesion Kinase
FCS	Foetal Calf Serum
FGF	Fibroblast Growth Factor
FITC	Fluorescein Isothiocyanate
FSC	Forward Scatter
Flt-3	FMS-like Tyrosine Kinase 3
GAG	Glycosaminoglycan

GALT	Gastrointestinal Associated Lymphoid Tissue
GC	Germinal Centre
GDP	Guanosine diphosphate
GEF	GDP Exchange Factor
GI	Gastrointestinal
GM-CSF	Granulocyte Macrophage Colony-Stimulating Factor
GRK	G-protein coupled Receptor Kinase
GTP	Guanosine triphosphate
HAO	Heat-aggregated Ovalbumin
HBSS	Hank's Buffered Salt Solution
HEV	High Endothelial Venule
HHV	Human Herpesvirus
HIV	Human Immunodeficiency Virus
IBD	Inflammatory Bowel Disease
IFN	Interferon
IPEX	Immunodysregulation Polyendocrinopathy Enteropathy X-linked
Ig	Immunoglobulin
IL	Interleukin
i.p.	Intraperitoneal
IP_3	Inositol triphosphate
IS	Immunological Synapse
i.v.	Intravenous
JAK	Janus Protein Tyrosine Kinase
KO	Knock-out

KGF	Keratinocyte Growth Factor
LP	Lamina Propria
LPS	Lipopolysaccharide
Mϕ	Macrophage(s)
MACS	Magnetically Activated Cell Sorter
MAPK	Mitogen-Activated Protein Kinase
M-CSF	Macrophage-Colony Stimulating Factor
MFI	Mean Fluorescence Intensity
MHC	Major Histocompatibility Complex
MLN	Mesenteric Lymph Node
MCMV	Murine Cytomegalovirus
MMP	Matrix Metalloproteinase(s)
mRNA	messenger RNA
NO	Nitric Oxide
NOD	Nuclear Oligomerization Domain
NK	Natural Killer
NSAID	Non-steroidal Anti-inflammatory Drug
OD	Optical Density
OVA	Ovalbumin
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PE	Phycoerythrin
PEC	Peritoneal Exudate Cells
PH	Pleckstrin Homology

PI	Propidium Iodide
PI3K	Phosphatidylinositol 3-kinase
PIP_2	Phosphatidyl inositol biphosphate
PLC	Phospholipase C
PLN	Peripheral Lymph Node
PP	Peyer's Patch(es)
PRR	Pattern Recognition Receptor(s)
RNA	Ribonucleic Acid
RPMI-1640	Roswell Park Memorial Institute-1640 Medium
RT-PCR	Reverse-transcriptase Polymerase Chain Reaction
SA	Streptavidin
SCID	Severe Combined Immunodeficiency
SSC	Side Scatter
STAT	Signal Transducers and Activators of Transcription
T_{CM}	Central Memory T cell
TCR	T Cell Receptor
T_{EM}	Effector Memory T cell
TPA	12-O-tetradecanoylphorbol 13-acetate
T_H1	T Helper 1
T_H2	T Helper 2
T_H17	T Helper 17
TLR	Toll Like Receptor(s)
T_{REG}	Regulatory T cell
TNF	Tumour Necrosis Factor

VEGF	Vascular Endothelial Growth Factor
WT	Wild type
XCL	XC-chemokine ligand
XCR	XC-chemokine receptor

SUMMARY

D6 is a novel chemokine receptor, homologous to other members of the CC-chemokine receptor family, which recognises a number of inflammatory CC-chemokines with high affinity. Unusually, D6 fails to induce any detectable intracellular signal in response to its ligands, but has been demonstrated to traffic constitutively to and from the cell surface, targeting any bound ligand for intracellular degradation. Due to this atypical behaviour, it has been suggested that D6 may serve as a decoy receptor for inflammatory CC-chemokines and, moreover, recent work on skin inflammation in D6 KO mice indicates that D6 may serve an important role in the resolution of inflammatory responses. However, the vast majority of evidence to support this claim has arisen from data derived *in vitro*: the true biological function of D6 remains unclear.

The aims of this thesis therefore, were to further our understanding of the biology of D6, chiefly through characterisation of immune responses in D6-deficient animals. Firstly, as described in Chapter 3, I analysed the cellular composition of lymphoid tissues of D6 KO mice. These studies revealed higher proportions of CD11c+ and F4/80+ cells in the D6 KO spleen compared with WT controls, suggesting that increased accumulation of myeloid lineage cells was occurring at this site. No other alterations in lymphoid populations were seen, but lymphocytes from D6 KO mice demonstrated higher levels of proliferation in response to mitogen, *in vitro*. In the same chapter I examined the induction of antigen-specific immune responses by immunising D6 KO animals with OVA/CFA. These results were variable, with D6 KO animals demonstrating both reduced and enhanced antigen-specific responses depending on the particular time-point analysed. This suggested that D6

may have a temporal affect on the development of antigen-specific immune responses, although this will need substantiated by further experimentation.

In Chapter 4, I examined the role of D6 in myeloid cell responses, by comparing monocyte recruitment to the inflamed peritoneum and dendritic cell development from bone-marrow (BM) cultures. I found that while the accumulation of inflammatory monocytes/macrophages appeared quantitatively similar in WT and D6 KO animals, D6 KO cells expressed greater levels of CD11c, suggesting preferential accumulation of DC-like cells in the inflamed peritoneum. Functional comparison of inflammatory PEC between WT and D6 KO mice showed reduced generation of pro-inflammatory cytokines by KO cells following *ex vivo* stimulation. DC generated from GM-CSF supplemented D6 KO BM cultures displayed higher levels of CD11c and of the CC-chemokine receptors, CCR2 and CCR5 compared with their WT counterparts. BM-derived DC from D6 KO mice showed no differences in endocytosis or in the upregulation of costimulatory molecules following LPS-induced activation. However, KO DC were less efficient in generating a mixed leukocyte reaction, and also demonstrated higher production of TNFα, VEGF and the CC-chemokines CCL3, CCL4 and CCL5 in response to LPS-stimulation. These data suggest that D6 may influence the development and function of myeloid lineage cells.

As D6 is expressed at high levels in the small and large intestine, I next investigated both tolerogenic and inflammatory intestinal responses in D6 KO animals. As detailed in Chapter 5 of this thesis, the induction of oral tolerance in response to a high dose feeding protocol was normal in D6 KO mice. However, D6 KO mice showed increased resistance to experimental colitis induced by the administration of dextran sulphate sodium (DSS) in

their drinking water. The reduced susceptibility to DSS colitis was found to correlate with increased *ex vivo* secretion of several pro-inflammatory type cytokines, including IFNγ and IL-17. There were also differences in basal epithelial proliferation rate between WT and D6-deficient animals, indicating, that altered inflammatory cytokine production and barrier function may be involved in protecting KO mice from DSS-induced disease.

Finally, as I had detected several differences in the expression of CCR2 and CCR5 by D6 KO leukocytes in the inflamed peritoneum and colon, I undertook a more thorough examination of the expression of these receptors by D6 KO leukocyte populations. As described in Chapter 6, various D6 KO populations displayed differential chemokine receptor profiles compared with their WT counterparts, the most significant of which was strikingly enhanced expression of CCR5 by D6 KO B cells: this increased CCR5 expression was seen by B cells isolated from all lymphoid tissues, as well as from the resting peritoneal cavity and colonic lamina propria. In addition, altered CCR5 expression was detected on B cells from the bone marrow of D6-deficient animals, suggesting that aberrant B cell differentiation may be occurring in the absence of D6. Purified B cells from D6 KO mice showed enhanced migration to CCL4 in chemotaxis assays, but responded comparably to WT B cells in response to *in vitro* mitogens. These results suggest a role for D6 in the normal development of leukocytes populations, with absence of this atypical receptor leading to the dysregulated expression of other chemokine receptors.

Taken together, my data suggest that the biological functions of D6 may be more complicated than previously appreciated. Indeed, I found no evidence for a decoy role of D6 in vivo, but D6-deficient animals were characterised by altered leukocyte development,

aberrant chemokine receptor expression and increased resistance to experimental colitis induction. Further study of these animals will hopefully identify the mechanisms leading to these phenotypical differences.

Chapter 1

General Introduction

Introduction

The innate and adaptive arms of the immune system have evolved in partnership over countless millennia, ensuring our protection from the hordes of potentially menacing agents that lurk in our air, food and water. Beyond the physical barriers of the skin and mucosal surfaces, a vast array of chemical mediators and a highly coordinated force of leukocytes defend us. In response to dangerous stimuli, these versatile cells mobilise to inflammatory sites, where they can attack and evict troublesome agents. However, as well as possessing potent pro-inflammatory mechanisms, the immune system must also be able to become tolerant to ensure that essential foodstuffs, commensal organisms and our own bodily tissues are not caught in the crossfire.

Be they of a tolerogenic or pro-inflammatory nature, the generation of effective immune responses requires the temporal and spatial coordination of leukocyte populations within tissues. The cells of the immune system are in constant motion, navigating between blood, lymphoid organs and non-lymphoid tissues. Multiple adhesion molecules, such as members of the selectin, integrin and immunoglobulin families, and chemotactic factors, including complement metabolites, lipid mediators and pathogen-derived products themselves, integrate to successfully guide immune populations (1-7). However, many of the most important mediators of motility belong to the chemokine superfamily.

CXC Family

Systematic Name	Human Ligand	Mouse Ligand	Chemokine Receptor(s)
CXCL1	GROα	GRO/MIP-2/KC	CXCR1, CXCR2
CXCL2	GROβ	GRO/MIP-2/KC	CXCR2
CXCL3	GROγ	GRO/MIP-2/KC	CXCR2
CXCL4	PF4	PF4	CXCR3B
CXCL5	ENA-78	GCP-2/LIX	CXCR2
CXCL6	GCP-2	GCP-2	CXCR1, CXCR2
hCXCL7	NAP-2	?	CXCR2
hCXCL8	IL-8	?	CXCR1, CXCR2
CXCL9	Mig	Mig	CXCR3
CXCL10	IP-10	IP-10	CXCR3
CXCL11	I-TAC	I-TAC	CXCR3, CXCR7
CXCL12	SDF-1	SDF-1	CXCR4, CXCR7
CXCL13	BCA-1	BLC	CXCR5
CXCL14	BRAK/bolekine	BRAK	?
mCXCL15		Lungkine/WECHE	?
CXCL16			CXCR6

CC Family

CCL1	I-309	TCA-3	CCR8
CCL2	MCP-1	JE	CCR2
CCL3	MIP-1α		CCR1, CCR5
CCL4	MIP-1β		CCR5
CCL5	RANTES		CCR1, CCR3, CCR5
mCCL6		C10	CCR1
CCL7	MCP-3		CCR1, CCR2, CCR3
CCL8	MCP-2		CCR3
mCCL9		MIP-1γ	CCR1
mCCL10		MIP-1γ	CCR1
CCL11	Eotaxin	Eotaxin	CCR3
mCCL12		MCP-5	CCR2
hCCL13	MCP-4		CCR2, CCR3
hCCL14	HCC-1		CCR1
hCCL15	HCC-2		CCR1, CCR3
hCCL16	HCC-4		CCR1, CCR2, CCR5
CCL17	TARC	TARC	CCR4
hCCL18	DC-CK1/PARC		?
CCL19	ELC/MIP-3β/exodus3	ELC/MIP-3β/exodus3	CCR7
CCL20	MIP3α/LARC/exodus1	MIP3α/LARC/exodus1	CCR6
CCL21	SLC/6Ckine/exodus2	SLC/6Ckine/exodus2	CCR7
CCL22	MDC	ABCD-1	CCR4
hCCL23	MPIF-1		CCR1
CCL24	Eotaxin-2/MPIF-2	Eotaxin-2/MPIF-2	CCR3
CCL25	TECK	TECK	CCR9
hCCL26	Eotaxin-3		CCR3
CCL27	C-TACK	CTACK/Eskine	CCR10
CCL28	MEC		CCR10

C Family

Systematic Name	Human Ligand	Mouse Ligand	Chemokine Receptor(s)
XCL1	Lyphotactin/SCM-1α	Lymphotactin	XCR1
hXCL2	SCM-1β		XCR1

CX3C Family

CX3CL1	Fractalkine	Fractalkine/Neurotactin	CX3CR1

Table 1.1

The Chemokine Superfamily

The systematic nomenclature for the chemokine family is shown, along with the previously used common names for human and mouse ligands. The far right column indicates the receptor(s) to which each chemokine normally binds. Chemokines that are present only in human or mouse are denoted with "h" or "m", respectively. This systematic nomenclature was adopted in the late nineties to clarify confusion in the literature, arising from the existence of multiple common names for singular chemokines. Four sub-families of chemokines have been identified, based on the configuration of a conserved N-terminal tetra-cysteine motif. CXC chemokines have a single amino acid between the first two cysteines of this conserved motif, while the initial cysteine residues are directly juxtaposed in members of the CC-chemokine sub-family. The sole member of the CX3C family, CXC3CL1/fractalkine, has three intervening amino acid residues between the first two cysteines, while members of the C family lack these initial cysteine residues altogether.

1.1 The Chemokine Family

Best known and named for their ability to induce chemotaxis, chemokines are small (6-11kDa), basic proteins that mediate their functions via G-protein coupled serpentine receptors (8, 9). Membership of the chemokine family is defined structurally rather than functionally, with all chemokines (with the exception of XCL1, XCL2) possessing a conserved tetra-cysteine motif. Although chemokines demonstrate relatively low homology at the primary sequence level (15-50%) (10), disulphide bonding between the cysteine residues results in a distinctive conserved three-dimensional structure.

To date, around 50 chemokines have been identified and classified into four major families (CXC, CC, XC and CX_3C) according to the precise positioning of the first two cysteine residues in the aforementioned motif (Table 1.1) (8, 9, 11). For the most part, chemokines are secreted as soluble proteins and are produced by many different cell types, including leukocyte, epithelial, endothelial and stromal cell populations. However, CXCL16 and CX_3CL1 can exist in transmembrane forms, with the chemokine domain associated with a mucin-like stalk (12, 13).

In addition to the systematic classification based on structure, chemokines can also be divided into two different functional categories, homeostatic and inducible, based upon their pattern of expression (14). Typically, homeostatic chemokines are expressed constitutively and are involved in processes such as lymphorganogenesis, immune surveillance and tissue-specific trafficking (15, 16). The inducible chemokines, on the other hand, tend to be upregulated during inflammation and are important for recruiting immune effectors to inflammatory sites (14).

However, the distinction between homeostatic and inducible chemokine classes is becoming increasingly unclear (17, 18). It is now apparent that many so-called inducible chemokines are constitutively expressed and participate in important homeostatic responses (19, 20). Conversely, homeostatic chemokines can be upregulated in inflamed tissues and perpetuate inflammatory responses (18, 21, 22).

1.2 Chemokine Receptors

Chemokines signal via 7-transmembrane spanning, G-protein coupled receptors, which have been classified in such a way as to parallel the systematic nomenclature of their ligands (See Table 1.1) (11). Thus CCR, CXCR, XCR and CX_3CR chemokine receptors recognise members of the CC, CXC, C and CX_3C chemokine subfamilies, respectively. As a general rule, members of each chemokine sub-family are recognised only by members of their corresponding receptor family. For example, CC-chemokines will bind only CC-chemokine receptors. However, there are some notable exceptions to this doctrine and these will be discussed further below (see section 1.17) (23-30).

Chemokine receptors are around 40 kDa in size and typically 340-370 amino acids long. The general chemokine receptor structure is highly conserved at the primary level (<80%) and chemokine receptors also share identity with other G-protein-coupling receptors, such as dopamine and beta-adrenergic receptors. The receptor molecule consists of an acidic extracellular amino terminus, 7 hydrophobic transmembrane domains, 3 intra- and extra-cellular loops and an intracellular tail at the carboxy terminus (Figure 1.1). Each of the extracellular loops contains a cysteine residue, and these enable formation of disulphide

bridges essential to maintain the 3-dimensional structure of the receptor. Additionally, the second intracellular loop contains a canonical DRYLAIV amino acid motif, which mediates coupling of the activated receptor to G-proteins and so is necessary for chemokine receptor signalling. With a few important exceptions, which will be discussed later in this chapter, ligand binding to chemokine receptors leads to the activation of several well-characterised signalling pathways. Although the complexities of chemokine receptor signalling cascades are beyond the scope of this thesis, the following section and Figure 1.1 give a brief overview of the best-known chemokine receptor signalling pathways (31-33).

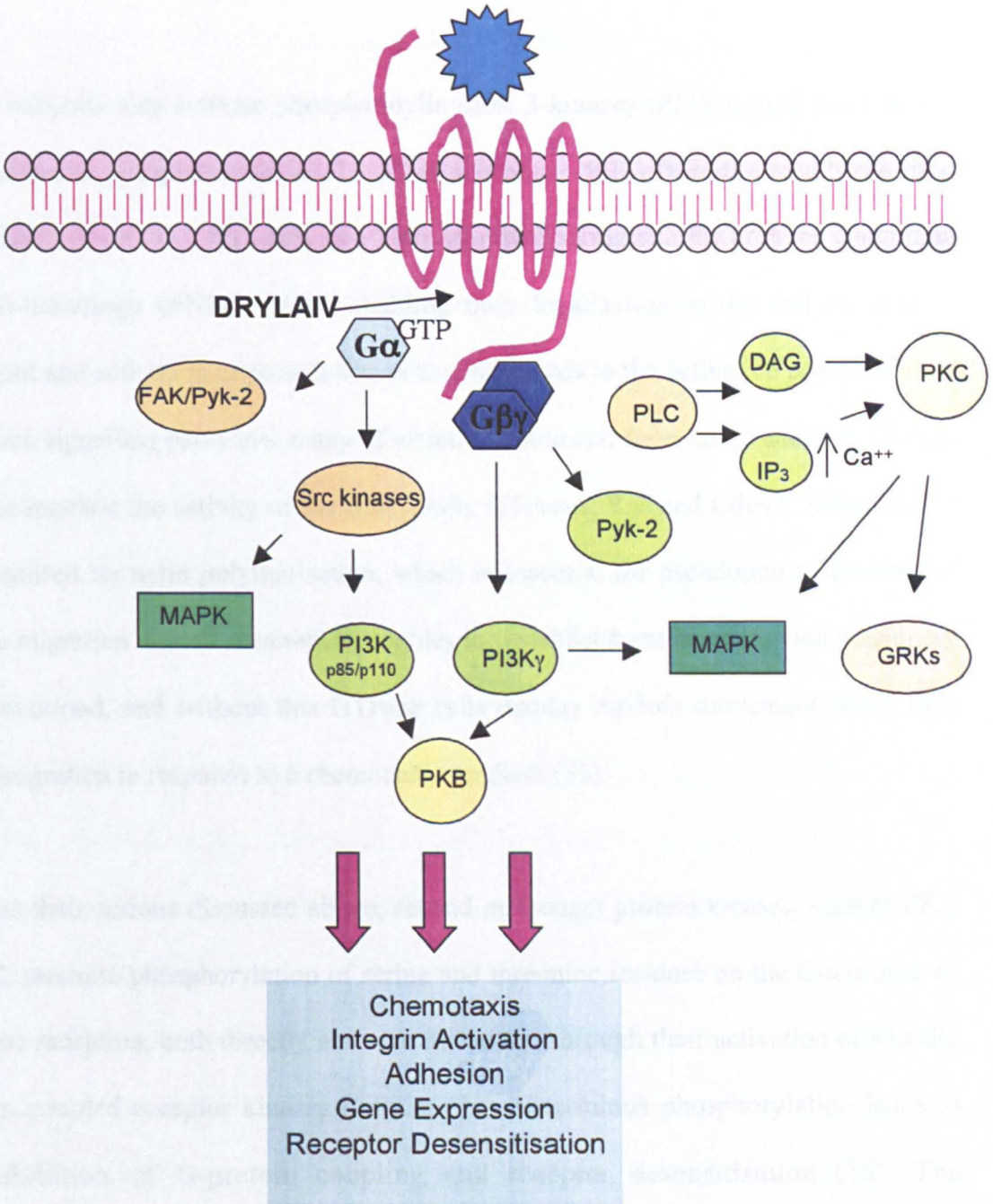
Chemokine Receptor Signal Transduction

Upon activation, chemokine receptors act as GDP exchange factors (GEF) for receptor-coupled G-proteins. This leads to dissociation of the heterotrimeric G-protein into G_{α} and $G_{\beta\gamma}$ subunits, both of which subsequently initiate a variety of signalling cascades (See Figure 1.1) (31-33). The response is terminated by G_{α} hydrolysis of bound GTP to GDP, leading to reassociation of the $G_{\alpha\beta\gamma}$ heterotrimer. $G_{\beta\gamma}$ subunits derived from G_i complexes are principally responsible for initiating the downstream signalling pathways that lead to chemotaxis. $G_{\beta\gamma}$ activates the phosphoinositide specific phospholipase C (PLC), which catalyses the cleavage of cell membrane phosphatidyl inositol biphosphate (PIP_2) into two parts, inositol triphosphate (IP_3) and diacylglycerol (DAG). IP_3 interacts with receptors on the endoplasmic reticulum (ER), causing the release of ER-stored Ca^{2+} into the cytosol, thus raising intracellular Ca^{2+} levels several-fold. Such increases in Ca^{2+} intracellular levels are commonly used to determine the ability of a particular chemokine to act as an agonist for a chemokine receptor. DAG and the released Ca^{2+} cooperate in the activation of the protein

Figure 1.1:

Schematic Representation of Chemokine Receptor Signalling

The figure overleaf gives an overview of some of the major signalling pathways initiated following chemokine receptor activation. Arrows indicate downstream targets.


DRYLAIV
Gα
GTP
Gβγ
FAK/Pyk-2
Src kinases
Pyk-2
PLC
DAG
PKC
IP3
Ca++
MAPK
PI3K
p85/p110
PI3Kγ
MAPK
GRKs
PKB
Chemotaxis
Integrin Activation
Adhesion
Gene Expression
Receptor Desensitisation

kinase C (PKC) family of serine/threonine kinases. PKC generated signals are important for responses such as integrin activation, through activation of the GTPase Rho, as well as for phosphorylation of the activated chemokine receptor promoting its desensitisation and internalisation (see below).

The Gβγ subunits also activate phosphatidylinositol 3-kinaseγ (PI3Kγ), and this enzyme rapidly generates phosphatidylinositol 3,4,5-triphosphate (PIP_3) from the membrane lipid phosphatidylinositol (34). PIP_3 acts as a high-affinity docking site for proteins containing pleckstrin-homology (PH) domains, enabling their localisation to the cell membrane. Recruitment and activation of protein kinase B (PKB) leads to the activation of a number of downstream signalling pathways, many of which promote cell survival. In addition, PI3K is thought to regulate the activity of the Rho family GTPases, Rac and Cdc42. Activation of Rac is required for actin polymerisation, which is essential for pseudopod extension and leukocyte migration. Cdc42 meanwhile, enables the establishment of orientation machinery at the pseudopod, and without this GTPase cells display random movement rather than directed migration in response to a chemotactic gradient (33).

As well as their actions discussed above, second messenger protein kinases, such as PKB and PKC, promote phosphorylation of serine and threonine residues on the C-terminus of chemokine receptors, both directly and also indirectly, through their activation of specific G-protein coupled receptor kinases (GRKs) (35). C-terminus phosphorylation leads to steric inhibition of G-protein coupling and receptor desensitisation (36). The phosphorylated receptors also provide binding sites for multifunctional intracellular adaptor and scaffold proteins, the β-arrestins. β-arrestins link the chemokine receptor complex to

the endocytic machinery, promoting receptor internalisation and can also act as a scaffold for downstream signal transduction (35, 36).

In addition to activation of membrane lipids, chemokine receptors trigger other intracellular signalling cascades. G_{α} subunits can target Src family kinases leading to the activation of proteins, such as focal adhesion kinase (FAK), that are important for regulating cytoskeletal adhesion to the extracellular matrix. Mitogen-activated protein kinase (MAPK) cascades are initiated in response to chemokine signalling, and this is suggested to be critical for T cell proliferation and the transcription of cytokine genes (37). There are also reports that chemokine receptors can act in a G-protein independent manner through janus-family tyrosine kinases (JAKs) and signal transducers and activators of transcription (STATs) (38-40). However, this issue remains controversial as others have found no involvement of the JAK/STAT pathway in chemokine receptor signalling (41).

1.3 Chemokine Receptor and Ligand Interactions

A key feature of the chemokine system is the highly promiscuous nature of the interactions between ligands and receptors. Many chemokines interact with two or more receptors, and most receptors can recognise multiple chemokines (11). This characteristic has led to suggestions of high redundancy in chemokine biology (42). However, it is more probable that as yet undetermined intricacies exist in the chemokine network, and that complexity ensures robust responses to a broad array of pathogens that may be attempting to manipulate the chemokine system (see section 1.19) (33). Chemokines show heterogeneous binding affinities and potencies for the same receptor and, consequently, triggering of a single receptor can lead to a different functional outcome depending on the ligand involved

(43-45). For instance, while both CCL19 and CCL21 act as agonists for CCR7, only CCL19 induces a reduction in surface receptor levels (46). Moreover, studies in genetically manipulated animals have revealed that deletion of a single ligand or receptor can lead to phenotypic changes in experimental models of infection or disease (19, 47-58). For example, during the induction of experimental autoimmune encephalitis (EAE), CCL2-deficient mice do not upregulate other CCR2 ligands in compensation and these animals are resistant to disease development (57). Such studies argue against the existence of a system where multiple ligands and receptors can be mere substitutes for one another.

Single chemokine and receptor partnerships do appear to exist in the chemokine system, usually involving homeostatic chemokines. Examples of these include the B-cell chemoattractant, CXCL13, recognised solely by CXCR5, and CCL25/CCR9, whose interactions are important in thymocyte development and intestinal homing (59, 60). However, it remains possible that additional receptors or ligands will be identified for such pairs. Indeed, the long thought monogamous relationship of CXCR4 and CXCL12 was recently challenged by the discovery of CXCR7 as a novel receptor for both CXCL12 and CXCL11(61).

Functional Outcomes of Chemokine Receptor Activation

1.4 Chemotaxis

The cardinal response of chemokine receptor activation is the induction of chemotaxis, the process by which cells show directed migration along a chemically imposed gradient. Almost all chemokines can induce chemotaxis *in vitro* and indeed, they were named for

their ability to induce this response: **chemo**tactic cyto**kines** (62). Directed leukocyte motion requires a number of cellular responses to occur, including cytoskeletal rearrangements and changes in integrin affinity. Migrating cells assume a polarised morphology, characterised by a pseudopod at the leading edge of the cell, and a trailing uropod at the rear. These changes are mediated by activation of G proteins coupled to chemokine receptors and initiation of downstream signalling cascades (See Figure 1.1) (31).

It has been argued however, that genuine chemotaxis may be an entirely *in vitro* phenomenon and it has been suggested that it may not be feasible to maintain true chemokine gradients in living tissues (63). Instead, it is proposed that *in vivo* leukocytes may be guided by chemokines immobilised on vascular or stromal cells (haptotaxis), or that chemokine receptor activation may increase the overall motility of cells in a gradient-independent manner (chemokinesis). In support of the latter, imaging of intact lymph nodes using two-photon microscopy has revealed apparently random migratory patterns for B and T lymphocytes despite the presence of substantial amounts of chemokines and known restriction of lymphocytes to specific anatomical niches (64-66). However, a recent report by Takada et al. provided evidence for the *in vivo* existence of chemokine gradients, demonstrating CCR7-dependent haptotaxis of activated B cells towards the B-zone/T-zone boundary in the lymph node (67). It is possible therefore that the ability of chemokines to guide cell trafficking involves a combination of chemotaxis, haptotaxis and chemokinesis.

Originally, CXC and CC chemokine receptors were proposed to mediate migration of neutrophils and monocytes, respectively. Although now realised to be a gross over-simplification, leukocyte subsets can exhibit differential chemokine receptor profiles, and

variations in chemokine receptor expression determine the migratory pathway undertaken by a particular leukocyte. This fine control of leukocyte trafficking is essential for the proper development of immune responses, as will be discussed later.

1.5 More Than Movers

While their synonymous function may be the activation of leukocyte locomotion, the scope of chemokine activity greatly exceeds this (8, 68). Chemokines upregulate integrin expression by blood-borne leukocytes, enabling their arrest at venular endothelium and subsequent tissue entry (Figure 1.2) (69). At the inflammatory site, triggering of chemokine receptors can induce leukocyte degranulation, phagocytosis and activation of the respiratory burst (70-78). Chemokines have also been shown to promote leukocyte proliferation, influence T-cell differentiation and to exert both positive and negative effects on apoptosis (61, 79-90). In addition, expression of CX_3CR1 by DC has been shown to mediate their protrusion of transepithelial dendrites into the intestinal lumen, which may facilitate the sampling and uptake of intestinal contents (91).

Chemokine receptors are also expressed by non-leukocyte populations, including epithelium, endothelium, smooth muscle cells, fibroblasts and neurons (57, 58). As a result, chemokines can directly influence many important physiological processes, such as angiogenesis, epithelial turnover, and wound healing (53, 92, 93) Additionally, the chemokine receptor CXCR4 has been demonstrated to play an essential role in the development of nervous, circulatory and immune networks (94-96).

Interestingly, the activity of chemokines is not just restricted to interactions with endogenous chemokine receptors. Chemokines share close structural similarity to cationic antimicrobial peptides, such as the defensins, and several chemokines have been found to kill a broad spectrum of micro-organisms *in vitro* (97-99). Thus chemokines not only promote immune responses through the recruitment and activation of leukocytes, but may also be directly employed to attack microbes. Interestingly, defensins can also induce chemotaxis via chemokine receptors and it has been shown that human β-defensins attract immature DC and memory T cells via CCR6 (100).

With such a comprehensive range of activities, it is hardly surprising that chemokines are involved at all stages of leukocyte life. The following sections aim to give an overview of the various ways in which chemokines coordinate leukocyte development and function to enable the generation of productive immune responses.

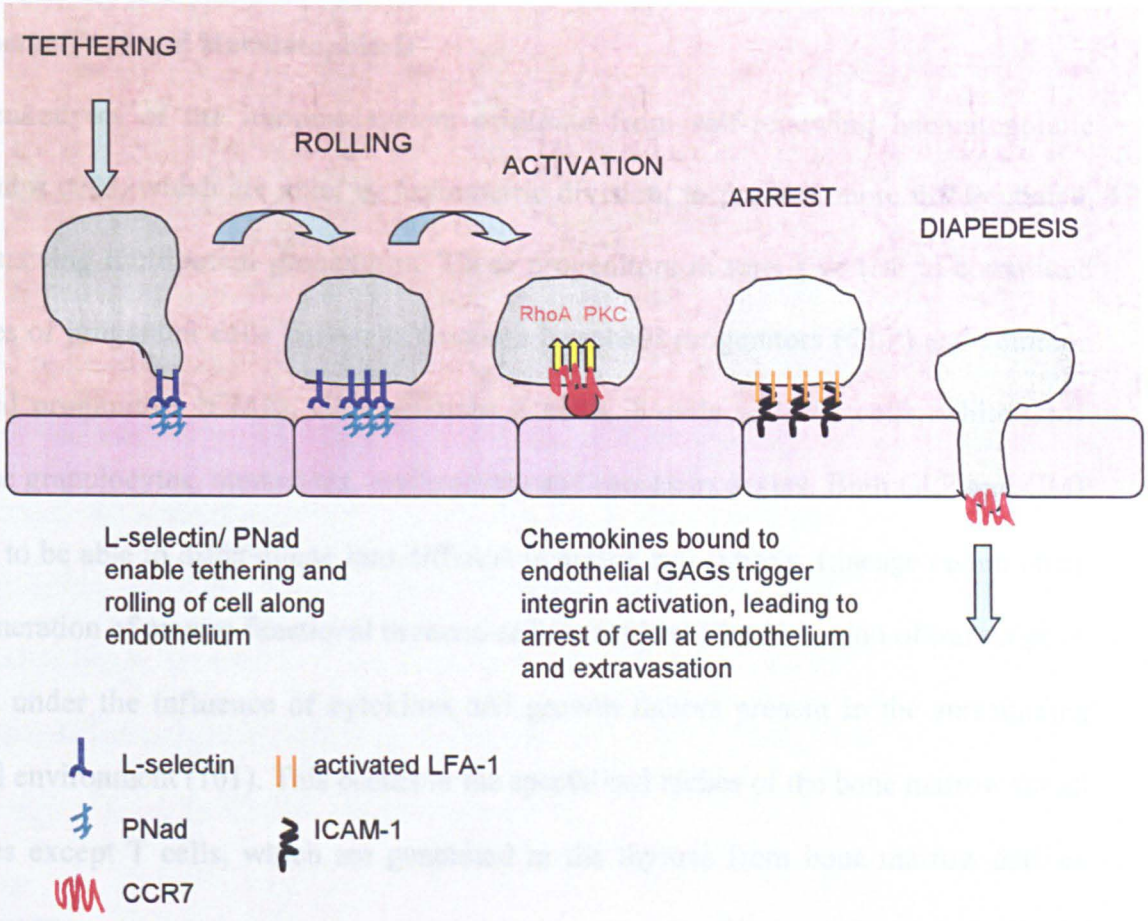


Figure 1.2:

Role of Chemokines in Lymphocyte Extravasation

Through their ability to induce rapid integrin activation, chemokines play an important role in leukocyte extravasation. For example, during the recruitment of naïve T cells to peripheral lymph nodes, weak affinity interactions between L-selectin and PNAd lead to the tethering and rolling of lymphocytes along the endothelium. Rolling cells are activated by endothelial-bound chemokines, leading to the conformational change of LFA-1 to its high-affinity state and the clustering of LFA-1 molecules. This increases both the affinity and avidity of LFA-1 and ICAM interactions, resulting in the arrest of T cells at the endothelium and subsequent extravasation to the lymph node. The induction of high-affinity LFA-1 is dependent on the downstream activation of the small GTPase, RhoA, while both RhoA and PKCζ are involved in controlling the lateral mobility of integrins.

1.6 Chemokines and Haematopoiesis

The leukocytes of the immune system originate from self-renewing haematopoietic progenitor cells, which are able, by asymmetric division, to produce more differentiated, non-renewing multipotent progenitors. These progenitors in turn give rise to committed lineages of progenitor cells known as common lymphoid progenitors (CLP) and common myeloid progenitors (CMP). CLP generate B cells, T cells and NK cells, while CMP produce granulocytes, monocytes, erythrocytes and megakaryocytes. Both CLP and CMP appear to be able to differentiate into different dendritic cell subsets. Lineage commitment and generation of mature functional immune cells is mediated by activation of transcription factors under the influence of cytokines and growth factors present in the surrounding stromal environment (101). This occurs in the specialised niches of the bone marrow for all lineages except T cells, which are generated in the thymus from bone marrow-derived immigrants.

For all these lineages, spatial positioning of precursors must be effectively controlled to ensure contact with supportive stroma and the other cell populations involved in the developmental process. Fittingly, a variety of chemokine ligands and receptors have been implicated in haematopoiesis, most notably CXCR4 and its ligand, CXCL12 (94, 96, 102-111). Mice deficient in either receptor or ligand exhibit gross abnormalities in B-lymphopoeisis and myelopoiesis, as well as in vascular, neuronal and cardiac development, and these animals die perinatally. It has been suggested that CXCR4-mediated signals induce retention of cells in the bone marrow, preventing the premature release of myeloid

and B-cell precursors during development (103, 109). In addition, in a comprehensive study of the chemotactic responses of B lineage cells, Bowman and coworkers revealed efficient migration of very early progenitor B cells to the CC-chemokines CCL5, CCL2 and CCL25 (112). The authors speculated that these chemokines may attract progenitor B cells to supportive bone marrow niches during development or, alternatively, may have direct effects on the growth or survival of these cells. Consistent with their ability to migrate to CCL25, pre-pro B cells were shown to express CCR9 and CCR9-deficient mice have reduced numbers of pre-pro B cells in the bone marrow (113). In spite of this, the peripheral B cell compartment appears normal in CCR9-deficient mice (113). To the best of my knowledge, B cell development has not been thoroughly assessed in CCR2- and CCR5-deficient animals. However, it has been reported that signalling via CCR5 on human progenitor B cells leads to heterologous desensitisation of CXCR4, allowing immature B cells to escape from the BM into the bloodstream (114).

Signalling via CXCR4 is additionally critical during generation of T cells in the thymus, with CXCR4-deficient progenitors showing complete arrest at the DN1 stage of development (115). The chemokine receptors CCR7 and CCR9 are also expressed at specific stages of T cell development and are thought to be important for the proper guidance and positioning of progenitor T cells throughout the thymus (116). While T cell development is not blocked in animals lacking these receptors, deficiency of CCR7 results in abnormal early progenitor development, characterised by accumulation of CD25+CD44+ thymocytes at the cortico-medullary junction and disruption of the thymic architecture (113, 117). In addition, a recent study has shown that CCR7 expression may be essential for establishment of central tolerance in the thymus (118). Kurobe et al. reported that the

failure of CCR7-deficient thymocytes to migrate from the thymic cortex to the medulla prevents their acquisition of tolerance to lachrymal and salivary glands. CCR9 KO BM precursors meanwhile, were shown by competitive transplantation experiments to repopulate the thymus of lethally irradiated Rag KO mice less efficiently than BM cells from WT animals (119).

Various other chemokines are expressed in primary lymphoid organs, including CCL5, CCL17 and CCL22, and the receptors for these ligands are found on progenitor lymphocyte populations. However, the exact role of these molecules in haematopoiesis remains to be defined. It should also be noted that the influence exerted by chemokines on cell development extends beyond precursor navigation: members of the chemokine family can suppress haematopoiesis or promote precursor survival (120) (102, 108, 121, 122).

1.7 Mobilisation and Circulation Of Haematopoietic Cells

In the absence of inflammation, constitutively produced chemokines can facilitate the steady-state release of haematopoietic cells into the bloodstream and their subsequent circulation throughout the body. However, the role of chemokines in this steady state traffic of myeloid populations remains poorly defined. The homeostatic-type chemokines CXCL14 and CX_3CL1 have been proposed to be involved in baseline trafficking of monocyte populations (123, 124). Geissmann and coworkers have described the existence of two distinct functional murine monocyte subsets one of which, the $CX_3CR1^{hi}CCR2^-Gr1^-$ subset, is recruited to noninflamed sites and is thought to represent the precursor of tissue resident macrophages and DC (124). The complementary $CX_3CR1^{lo}CCR2^+Gr1^+$ subset, on the other hand, is preferentially recruited to inflamed tissues.

Low-level production of inflammatory-type chemokines may also be involved in constitutive cell trafficking. For example, eotaxin enables the baseline recruitment of eosinophils to the small intestine and signals via CXCR2 mediate the homing of mast cell precursors to this same site (125, 126). Additionally, CCR2 promotes the release of a subset of monocytes from the bone marrow into the circulation in the absence of inflammation (127).

Constitutive recirculation of naïve lymphocyte populations between and within secondary lymphoid tissues is dependent on leukocyte expression of CCR7 and CXCR5. CCR7 is present on naïve T cells and enables their entry to lymph nodes in response to CCL19 and CCL21 presented on HEVs (128). Recruitment of dendritic cells from peripheral tissues to the draining lymph node also depends on CCR7, and it has been suggested that low-level CCR7-mediated traffic of DC may contribute to the development and maintenance of peripheral tolerance (129, 130). In addition, CCR7-dependent trafficking of DC from the intestinal lamina propria to mesenteric lymph nodes (MLN) has recently been shown to be essential for the induction of oral tolerance following antigen feeding (131).

Akin to their T cell counterparts, naïve B cells also recirculate between the blood and secondary lymphoid tissues in search of their specific antigens. Intravital microscopy imaging of chemokine-desensitised cells has revealed that CCR7 and CXCR4 are important for the arrest and extravasation of B cells at HEV (132). CXCR5 meanwhile, enables follicular homing of B cells within lymph nodes and spleen in response to CXCL13 (132, 133). In addition, CXCR5 and CXCL13 interactions are involved in B cell recruitment to small intestinal Peyer's patches (132).

This chemokine-controlled recirculation of lymphocyte populations is important as, although collectively the TCR and BCR repertoires are of enormous magnitude, only a relatively small proportion of naïve T and B cells will recognize any specific antigen. Thus by guiding naïve lymphocytes to sites at which tissue-derived antigen concentrate, chemokines help optimise the generation of productive adaptive immune responses.

1.8 Chemokines in Innate Immunity

In contrast to the relative paucity of information regarding steady-state leukocyte trafficking, an extensive body of work has focussed on the importance of chemokines for leukocyte infiltration to inflamed tissues (134). Typically, the ligands of CXCR2, CXCR3, CCR1, CCR2, CCR3 and CCR5 collaborate to bring granulocytes, monocytes, dendritic cells, NK cells and effector lymphocytes to the epicentre of inflammation (Figure 1.3) (11, 47, 48, 52, 135-149). Activation of chemokine receptors on endothelial cells can also augment leukocyte recruitment by increasing endothelial permeability (150, 151).

Animals deficient in the aforementioned chemokine receptors, or their ligands, have been shown to have reduced or delayed cellular infiltration following acute inflammatory challenge and several display increased susceptibility to infectious agents (48, 52, 137, 142, 144, 152-154). CCR2 KO mice, for instance, show impaired monocyte recruitment to inflamed tissues, are incapable of clearing *Listeria monocytogenes* infection and have reduced granuloma formation in response to yeast or *Mycobacterium* extracts (47, 48, 136-139, 155). Conversely, over-expression of inflammatory chemokine ligands can enhance immunity to pathogens (156, 157).

Acute inflammation typically occurs following triggering of pattern-recognition receptors (PRR), such as members of the toll-like receptor family (TLR) or the nucleotide-binding site leucine-rich repeat (NOD) proteins (158, 159). These receptors recognise highly conserved microbial products, such as LPS and flagellin, and thus represent an effective intruder alert system. Activation of PRR leads to the production of chemokines as well as inflammatory cytokines, such as IL-1, IL-6 and TNFα, which in turn stimulate further chemokine secretion (158).

Different PRR elicit distinct patterns of cytokine and chemokine expression and since leukocyte populations display distinct chemokine receptor profiles (Figure 1.3), such differential chemokine generation enables the immune system to tailor the cellular infiltrate to suit the precise nature of the challenge at hand (158).

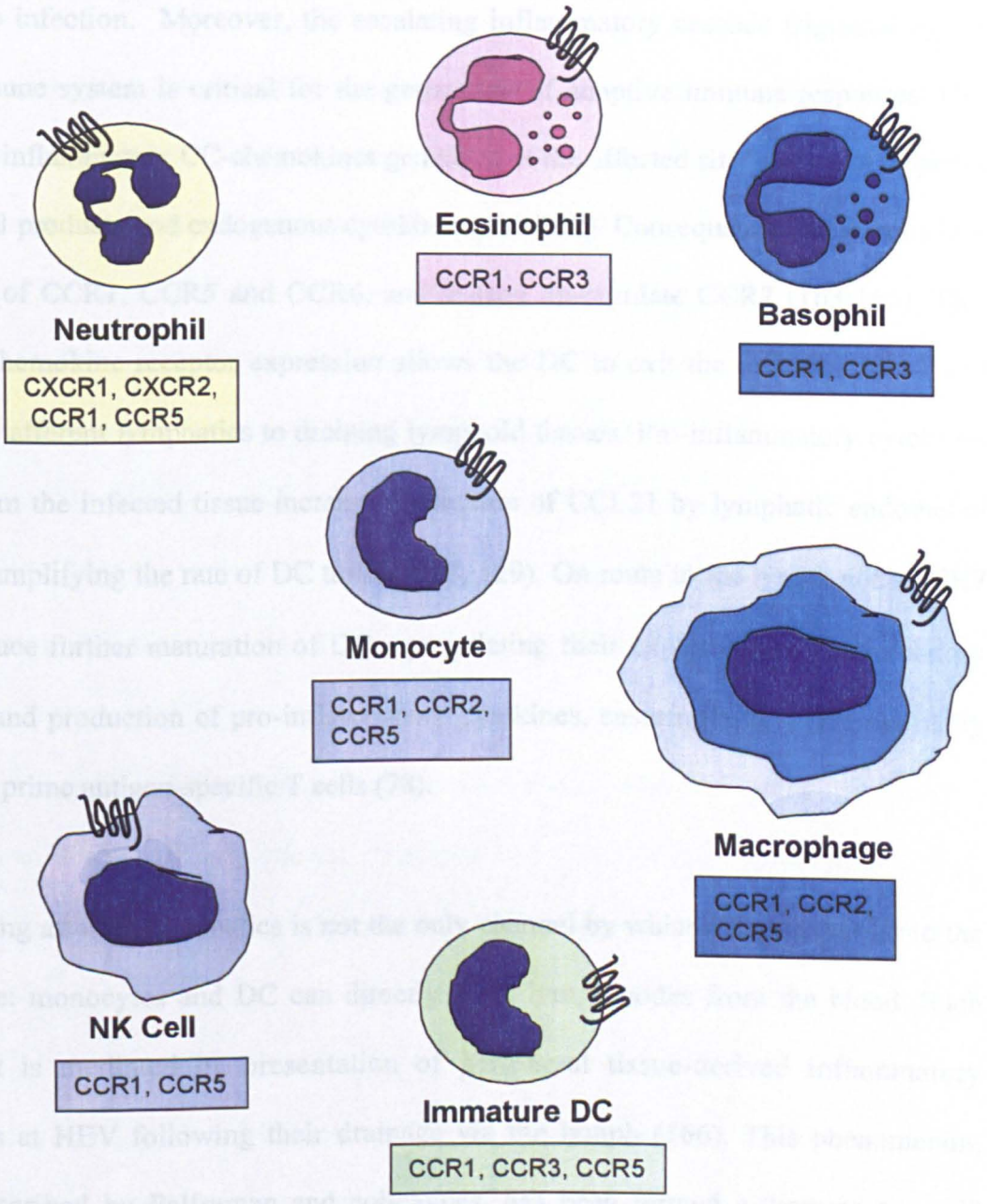


Figure 1.3

Chemokine Receptor Profiles of Innate Leukocyte Populations

1.9 Chemokines in the Generation of Adaptive Immunity

Chemokine-mediated recruitment of innate leukocyte populations is essential for a swift response to infection. Moreover, the escalating inflammatory cascade triggered by the innate immune system is critical for the generation of adaptive immune responses. DC, attracted to inflammatory CC-chemokines generated at the affected site, mature in response to microbial products and endogenous cytokines (160-162). Consequently, these cells lose expression of CCR1, CCR5 and CCR6, and instead up-regulate CCR7 (163-165). This switch in chemokine receptor expression allows the DC to exit the inflamed tissue and passage via afferent lymphatics to draining lymphoid tissues. Pro-inflammatory cytokines derived from the infected tissue increase expression of CCL21 by lymphatic endothelial cells, thus amplifying the rate of DC traffic (128, 129). On route to the lymph node CCR7 signals induce further maturation of DC, upregulating their expression of costimulatory molecules and production of pro-inflammatory cytokines, ensuring they arrive optimally prepared to prime antigen-specific T cells (78).

Passage along afferent lymphatics is not the only channel by which DC gain access to the lymph node: monocytes and DC can directly enter lymph nodes from the blood. Such recruitment is mediated by presentation of peripheral tissue-derived inflammatory chemokines at HEV following their drainage via the lymph (166). This phenomenon, initially described by Palframan and colleagues, has been termed a "remote control" mechanism for leukocyte recruitment to lymphoid tissues draining sites of inflammation. The rate of T cell entry to lymph nodes neighbouring inflamed tissue also increases in response to the higher levels of CCL21 and CCL19 present on HEV. Simultaneously, lymphoid organ "shutdown", induced by cytokines such as type 1 IFN or TNF, prevents

egress of lymphocytes from lymphoid tissue thus increasing the precursor frequency of antigen-specific T cells (167, 168).

The interactions that occur between antigen-loaded DC and antigen-specific T cells in the LN are vital for initiation of adaptive immune responses as well as for determining the nature of the ensuing response. Recent elegant studies utilising twin-photon microscopy have revealed apparently random migratory behaviour of T cells within the lymph node (66). However, chemokines within the lymph node will also influence the dynamics of T cells and DC interactions. For instance, encounters between T cells and DC are promoted by CCL21 and CCL19, expressed by stromal cells in the T cell dependent area of secondary lymphoid tissues as well as by a subset of DC at this location (169-171). Mature DC can directly attract activated T cells via generation of CCL22, which may increase the frequency of activated DC and antigen-specific T cells encounters (172). Additionally, it was shown recently that inflammation-induced upregulation of CCR5 guides naive CD8+ cells to clusters of CD4+ T cells and DCs in response to CCL3 and CCL4 produced at these sites, with these interactions being important for the development of CD8+ T cell memory (173).

Mature DC displaying peptide-MHC complexes engage antigen-specific T cells, forming a specialised site at which signalling molecules are compartmentalised, known as the immunological synapse (174). The multiple signals integrated at this site lead to activation and proliferation of antigen-specific T cells. Interestingly, the chemokine receptors CCR5 and CXCR4 are recruited into the immunological synapse where, by either promoting

stability of the IS or having direct activatory effects, they enhance T-cell proliferation and cytokine production (81, 175-177).

Following activation, naïve T cells acquire new chemokine receptor profiles that dictate their subsequent role in the immune response (Table 1.2). Some activated T cells can upregulate CXCR5 and migrate towards CXCL13-rich LN follicles (178). Here, they colocalise with antigen-specific B cells at the follicle boundary and providing help for antibody production (133, 178, 179). As B cells differentiate, they downregulate CXCR5 and instead express new chemokine receptors that enable them to localise to peripheral sites. Upregulation of CXCR4, for example, facilitates plasma cell homing to the bone marrow, while CCR9 and CCR10 enable IgA-secreting plasma cells to enter the lamina propria of the small and large intestine (180).

Subset	Chemokine Receptor Profile	Function
T_{NAIVE}	CCR7, CXCR4	Recirculate throughout secondary lymphoid tissue in search of specific antigen.
T_{FH}	CXCR5	Locate to follicles and provide help to B cells for antibody production.
T_H1	CCR5, CXCR3	Defence against intracellular pathogen, induction of cell-mediated immunity.
T_H2	CCR3, CCR4, CCR8	Defence against extra-cellular pathogens, induction of humoral immunity.
T_H17	Not yet defined.	Defence against acute infection, promote upregulation of pro-inflammatory cytokines and neutrophil recruitment.
T_{REG}	Many, including CCR2, CCR4, CCR5, CCR8, CCR5, CXCR4, CXCR5	Down-regulation of pro-inflammatory responses. Large repertoire of chemokine receptors may allow access to all sites at which effector cells can locate.
$T_{CUTANEOUS}$	CCR4, CCR10	Induced by DC from peripheral LN and home preferentially to the skin.
$T_{INTESTINE}$	CCR9	Induced by small intestinal DC and preferentially home to this site.
T_{CM}	CCR7	Traffic throughout secondary lymphoid tissue, involved in long-term protection.
T_{EM}	Negative for CCR7, express inflammatory chemokine receptors	Rapidly recruited to inflamed tissues and mount immediate effector response.

Table 1.2

Chemokine Receptor Profiles of Various Helper T Cell Subsets

1.10 Chemokine Receptor Expression By Effector T Cell Subsets

Newly generated effector T cells upregulate chemokine receptors that facilitate homing to peripheral sites of inflammation. The particular class of pathogen faced in the inflamed tissue will dictate the type of adaptive immune response generated. T_H1 cells produce IFNγ and promote macrophage activation and immunity against intracellular pathogens, whilst T_H2 cells characteristically secrete IL-4, IL-5 and IL-13 and are key to the development of humoral immune responses involving IgE, eosinophils and basophils. These subsets have been found to differ in their chemokine receptor profiles with T_H1 effectors predominantly expressing CCR5 and CXCR3, while T_H2 cells primarily display CCR3, CCR4 and CCR8 (181, 182). Distinct chemokines may themselves promote T_H 1 and T_H 2 development, with CCL3 and CCL2 reported to drive expression of IFNγ and IL-4, respectively, during T cell activation (86-90).

The distinctive chemokine receptor profiles of helper T cells may allow preferential recruitment of one subset over another according to the particular pattern of chemokines present at an inflamed site. Moreover, such particular patterns of chemokine receptor expression could enable co-localisation of effector T cells with those innate cells most suited to deal with the challenge at hand. However, a problem with a number of these studies is that analyses were performed on T_H1 and T_H2 cells derived from *in vitro* differentiation protocols. Indeed, analysis of peripheral CD4+ memory T cells by a single cell RT-PCR method did not reveal any correlation between cytokine and chemokine receptor expression (183). Thus whether chemokine receptor expression actually differs between T_H1 and T_H2 subsets remains a controversial issue.

1.11 Chemokine Receptor Profile of the T_H17 Subset

It has recently become evident that a separate effector T cell lineage, distinct from both T_H1 and T_H2 exists (184). This T-cell population secretes high levels of IL-17A, IL-17F, TNFα and IL-6, but not the signature cytokines typically associated with T_H1 or T_H2 responses. These cells, which have been termed T_H17, are characterised by the expression of distinct transcription factors, and the development of T_H1, T_H2 and T_H17 cells appears to be mutually exclusive (185-187). TGFβ, in conjunction with the inflammatory cytokines IL-1, IL-6 and TNFα, drives the development of T_H17 from naïve T cells, while IL-23 appears to be important for their expansion and maintenance, *in vivo* (188-190)

The T_H17 subset has been shown to be vital in protection against a number of infectious agents (191-193). This is due to their capacity to rapidly mobilise, recruit and activate neutrophils. However, it is now apparent that the IL-23/IL-17 pathway is also a major mediator of tissue inflammation in autoimmune disease models previously believed to be driven by an IL-12 induced T_H1 response (194, 195). As yet, no study has determined any particular chemokine receptor associations with these cells. However, this will be an important goal for future studies, as a better understanding of the trafficking properties of T_H17 cells may enable the development of therapeutics for the various diseases in which they have been implicated.

1.12 Chemokine Receptor Expression By Regulatory T cells

An important class of T-lymphocytes are regulatory T cells, commonly termed T_{REG}. Regulatory T cells secrete immunosuppressive cytokines such as IL-10 and TGF-beta and are important for preventing immunopathology through their control of both the innate and

the adaptive arms of the immune system (196, 197). Development of regulatory T cells is dependent on the expression of the forkhead family transcription factor FoxP3, and humans with null mutations in the gene encoding FoxP3 succumb to a severe and fatal autoimmune syndrome known as IPEX (Immunodysregulation, polyendocrinopathy, enteropathy, X-linked) syndrome (198).

A variety of chemokine receptors have been associated with T_{REG}; these include CCR1, CCR2, CCR4, CCR5, CCR8 and CXCR4 (199-201). During collagen-induced arthritis, it has been demonstrated that $CCR2^+$ T_{REG} play a protective role with blockade of CCR2 resulting in worse disease (202). Several studies have linked CCR5 and its ligands to T_{REG} biology, and CCR5 KO mice were recently shown to have enhanced clearance of Leishmania infection due to reduced T_{REG} recruitment (203-206). In addition, it has been shown that regulatory T cells can migrate to follicles via CXCR5 expression and suppress GC-B cell reactions (207).

However, perhaps logic dictates that T_{REG} should be able to locate to all sites to which pro-inflammatory cells traffic. In agreement with this supposition, Lim and colleagues recently reported the expression of a wide array of chemokine receptors by human regulatory T cells, with the exact receptor repertoire of the T_{REG} being determined by its state of activation and differentiation (208).

1.13 Chemokine Receptor Expression By Memory T cells

An important feature of adaptive immunity is the capacity to mount swifter and more effective responses following re-exposure to a previously encountered pathogen. This is

due to the maintenance of pools of antigen-specific T cells that mediate rapid secondary immune responses. It has been suggested that memory T cells can be divided into two different subsets, central memory (T_{CM}) and effector memory cells (T_{EM}), based upon their differential expression of CCR7 (209-211). T_{EM} are CCR7-negative, but display receptors for inflammatory chemokines and localise in non-lymphoid tissues where they can mount a rapid tissue response upon re-challenge. T_{CM} on the other hand, express CCR7 and recirculate throughout lymphoid tissue, where they proliferate and provide a source of fresh effector cells upon antigenic-stimulation. The presence of these two distinctly routed memory populations may enhance immunological memory by providing T cells that mount an immediate effector response (T_{EM}), as well as a subset that are involved in the secondary response and long-term protection (T_{CM}) (212).

1.14 Chemokines and the Control of Tissue Specific Migration

Subsets of memory and effector T-lymphocytes re-circulate through tissues associated with their initial site of antigen encounter. Evidence for such compartmentalisation of T cell responses came from early studies showing that cells collected from lymph draining the gut or the skin demonstrated a preference of at least two-fold to recirculate to the type of organ from whence they came (213). This phenomenon was observed in adult animals but not in neonates, suggesting that priming by a specific antigen in a particular environment leads to the acquisition of specific surface receptors that dictate the particular recirculatory route undertaken by a lymphocyte.

The molecular mechanisms behind tissue-specific trafficking are beginning to be unravelled, and have been shown to involve the acquisition of distinct adhesion molecules

and chemokine receptors by effector cells primed at different tissue sites (214, 215). Acquisition of such specific tissue homing phenotypes by effector T cells is due to "imprinting" by dendritic cells in the associated lymphoid tissues. T cells that are activated by DC in lymphoid tissue draining cutaneous sites acquire expression of CCR4 and CCR10 (216). Ligands of these receptors, CCL17 and CCL27, respectively, are expressed on dermal postcapillary venules and thus enable the specific homing of cutaneous effector T cells to the skin (216-218). On the other hand, effector T cells stimulated by DC in gut-associated lymphoid tissues upregulate CCR9 and are targeted to the lamina propria of the small intestine in response to CCL25, which is constitutively expressed at this site (219-223). The ability of GALT DC to upregulate lymphocyte expression of CCR9 and $\alpha 4\beta 7$, the mucosal homing integrin, was found to be due to their generation of retinoic acid, a vitamin A metabolite (223, 224). On the other hand, vitamin D3 metabolites appear to promote lymphocyte upregulation of the cutaneous-homing receptor, CCR10 (225).

Such compartmentalisation of immune responses is likely to increase the efficiency of immune responses, as effector T cells are targeted to the sites at which they are actually needed. Additionally, it may allow containment of inflammatory responses so as to reduce the risk of immunopathological damage at distant sites. Indeed, the extra-intestinal expression of gut-homing molecules has been associated with disease (226, 227)

1.15 Chemokine Receptor Expression By B Cells

Akin to T cells, B cells demonstrate differential patterns of chemokine receptor expression at distinct developmental stages. CXCR4 is expressed universally by B cells and, as already described in Section 1.7, signals via this receptor are thought to retain developing pre and

pro B cells in the supportive environs of the bone marrow. Expression of CCR5 and CCR9 has also been described on progenitor B cells, although the roles of these particular receptors in B cell development are less well understood (see Section 1.7) (112, 113, 228). Immature B cells exiting the bone marrow retain expression of CXCR4, and this receptor has been proposed to cooperate with CXCR5 and CCR7 to enable B cell homing to peripheral lymph nodes and Peyer's patches, as well as the peritoneal cavity (229-231). CCR6 meanwhile enables accumulation of B cells within Peyer's patches and isolated lymphoid follicles of the intestine (232).

Within the LN, CXCR5 expression enables the localisation of B cells to the follicles (233). During an immune response, follicular B cells activated by their specific antigen will proliferate, giving rise to low affinity IgM-producing plasma cells or, alternatively, forming a germinal centre. In the germinal centre, B cell-derived centroblasts proliferate extensively and undergo somatic hypermutation, affinity maturation and class switching. This process leads to the generation of high affinity centrocytes, which are positively selected to become class-switched memory and plasma cells. Proliferation of centroblasts occurs within the so-called "dark zone" of the germinal centre, and CXCR4 is required for the accumulation of centroblasts at this particular anatomical location (234). CXCR5 meanwhile, may be involved in guiding centrocytes from the dark to the light zone to interact with follicular helper T cells (234).

Subsets of memory B cells and plasma cells acquire distinct chemokine receptor profiles that, in combination with various adhesion molecules, contribute to the particular trafficking pathways these cells subsequently undertake. Class switched memory B cells

express CCR7, CXCR4, CXCR5 and CCR6, and may use these receptors to recirculate via secondary lymphoid organs (229). CXCR4 has also been shown to be involved in the accumulation of long-lived plasma cells in the bone marrow, a process thought to be important for the maintenance of serum antibody levels (235). IgG-secreting cells traffic mainly to the bone marrow or inflamed tissue, whereas IgA-producing cells are generated in mucosa- associated lymphoid tissues and targeted to mucosal sites (229). CXCR3 is upregulated by activated B cells and may enable accumulation of plasma cells at inflamed sites in response to the IFNγ-inducible chemokines, CXCL9, CXCL10 and CXCL11. However, ligands of CXCR3 are expressed constitutively in spleen and lymph nodes, raising the possibility that this receptor also contributes to homing to these sites.

IgA-secreting plasma cells, but not IgG- or IgM-secreting plasma cells express high levels of CCR9 and are recruited to the small intestine in response to CCL25 (236, 237). CCR9 KO mice have reduced number of IgA+ plasma cells in the small intestinal lamina propria and show a defective generation of IgA in response to orally administered antigen (237). It has recently been demonstrated that intestinal DC, as well as inducing T cell expression of CCR9 and α4β7, can similarly imprint B cells with these gut-homing receptors (224). The chemokine CCL28 and its receptor CCR10 are also involved in targeting IgA-plasmablasts to diverse mucosal surfaces, including the salivary glands, tonsils and both the small and large intestine (180).

This section has given an overview of the most well characterised roles of chemokine receptors and their ligands in B cell biology. However, in addition to the receptors described above, B cells have been shown to express various other chemokine receptors.

For example, CCR1 to CCR5 as well as CXCR6 have been reported to be expressed by human memory B cells or plasma cells, although the biological significance of this remains to be established (238, 239).

Regulation of The Chemokine System

The preceding passages have provided an overview of the role of chemokines in the development of immune responses. With such potent effects, it is imperative that appropriate methods exist to regulate chemokine responses. That increased chemokine levels are associated with a number of chronic inflammatory and autoimmune diseases stresses the importance of controlling these powerful mediators (22, 240, 241). A number of mechanisms have evolved to regulate the chemokine network and these are discussed in the following sections.

1.16 Chemokine Regulation by the Glycosaminoglycan Family

The glycosaminoglycan (GAG) family comprises long, sulphated polysaccharides, such as heparan sulphate and chondroitin sulfate, which are capable of binding soluble chemokines. GAGs are thought to be essential for the proper presentation of chemokines on activated endothelial cells and for creating haptotactic gradients to guide leukocytes (242, 243). In support of this, leukocytes have been shown to exhibit swifter integrin activation and arrest in response to immobilised and endothelium-bound chemokines as opposed to soluble chemokines (244).

While all chemokines bind GAGs, they do not do so uniformly. Chemokines differ in their affinity for various GAGs, which are themselves highly heterogeneous molecules, and they

can also interact with GAGs by diverse functional domains (245). Thus differential anatomical expression of GAGs can facilitate site-specific patterns of chemokine expression, enabling control of cellular recruitment and activation at these locations. In addition, chemokine binding to GAGs can mediate outside-in signalling and GAGs are also likely to facilitate the presentation of chemokines to modifying proteases (246).

1.17 Proteolytic Regulation of The Chemokine System

Enzymatic alteration of chemokines can alter their agonistic potential. A number of proteases have been identified that digest chemokines: these include the cell surface dipeptidyl-peptidase CD26, elastase, chymotrypsin, cathepsins and matrix metalloproteinases (MMPs). Chemokine editing by proteases can enhance or diminish their potency. Truncated CCL2 family members still bind, but cannot activate their respective receptors (247, 248). Conversely, chemokines with weak affinity for CCR1 can be converted into potent agonists following N-terminal truncation (249).

1.18 Chemokine Receptor Antagonism

Even in the absence of proteolytic modification some chemokines can act as receptor antagonists. CCL7 has been shown to act as a natural antagonist for CCR5 (23) , while CCL11 and CCL26 antagonise CCR2 (24-26). Additionally, CCR3 ligands antagonise CXCR3, and the ligands of CXCR3 antagonise CCR3 (25, 27-29). As CXCR3 and CCR3 are associated with T_H1 and T_H2 type responses respectively, their ability to act as functional antagonists may allow them to promote one arm of immunity while simultaneously suppressing the another.

1.19 Regulation of Chemokine Receptor Signalling

Cytokines can regulate chemokine-induced activities by altering receptor expression or coupling of receptors to downstream signalling pathways. Additionally, signalling via chemokine receptors may be modulated by homodimerisation or heterodimerisation. CCR2, CCR5, CXCR2 and CXCR4 have been shown to oligomerise. Also, CCR2 forms heterodimers with CCR5 and CXCR4 (250). However, controversy shrouds the functional consequences of this phenomenon, and both increased and decreased chemokine responses have been reported following CCR2/CCR5 heterodimerisation (251).

Chemokine receptor activation leads to exchange of GDP for GTP and the dissociation of Gα and Gβγ subunits, with each proceeding to trigger distinct signalling cascades. The response is terminated due to the intrinsic GTPase activity of the Gα subunits, which trigger hydrolysis of GTP, allowing the heterotrimeric G-protein units to reunite. A group of molecules called regulators of G-protein signalling (RGS proteins) can enhance or limit the rate of hydrolysis by the Gα subunits (252). Chemokine signalling can also be terminated by desensitisation and internalisation of the chemokine receptor, which typically occurs following ligand binding (33).

1.20 Microbial Regulation of The Chemokine System

Given the importance of the chemokine system in the development of protective immunity, it is not surprising that microbes have come to possess a remarkable array of proteins that interfere with chemokine networks (253). Although such molecules are not endogenous to the host, the pressures these pathogen-derived products have placed upon our immune

system is likely to have heavily influenced the evolution of the chemokine network, contributing to its complexity.

Herpes viruses generate chemokine analogues that can antagonise host chemokine receptors, allowing these pathogens to keep the cells of the immune system at bay. For example, Kaposi's sarcoma associated herpesvirus (HHV-8) encodes the vMIP-II chemokine, which binds with high affinity to a number of CC and CXC- chemokine receptors and prevents their activation by endogenous chemokines (254). Conversely, viruses also produce agonistic chemokine molecules and such tactics may facilitate viral dissemination by attracting cellular targets of infection, or by favouring the induction of non-protective host responses over anti-viral T_H1-type immunity. Murine cytomegalovirus (MCMV) for instance, attracts target monocyte populations by generating the CC-chemokine homologues MCK-1 and MCK-2, while the HHV-8-derived vMIP-I chemokine agonises CCR8, a chemokine receptor associated with Th2 and regulatory T cell populations (199, 255-258).

Pox and herpes viruses are able to induce the expression of virally encoded chemokine receptors in infected cells. Ligation of such receptors can result in increased cell proliferation, providing additional targets for viral replication (259). Viruses can also secrete chemokine-binding proteins and encode "decoy" receptors, enabling broad-spectrum chemokine neutralisation. For example, human cytomegalovirus induces infected cells to express a viral chemokine receptor, US28, which can internalise and facilitate destruction of a number of host CC-chemokines (260-262). The egg stage of the parasite

Schistosoma mansoni was also recently found to produce a chemokine-neutralising protein (263).

1.21 Regulation of the Chemokine Network by Decoy Receptors

It is well established that decoy receptors can play a role in preventing excessive accumulation of cytokines. Non-signalling decoy receptors have been identified for members of the IL-1/IL-18, TNF, IL-10 and IL-13 cytokine receptor families (264, 265). Recently, it has become apparent that decoy receptors may play an analogous role in maintaining homeostasis within the chemokine system.

Exposure of DC to IL-10 in combination with LPS can uncouple chemokine receptors from intracellular signalling pathways, effectively resulting in the generation of decoy receptors. These receptors may then "clean up" sites of inflammation by scavenging chemokines from the surrounding environment (266). Ariel and co-workers recently demonstrated upregulation of CCR5 by apoptotic leukocytes and showed that these receptors were not functional with regard to migration, but instead acted to sequester and clear CCL3 and CCL5 at inflammatory sites (267). In addition, it appears that a number of dedicated decoy receptors may regulate chemokine responses. To date, three chemokine binding receptors of this kind have been proposed: these are DARC (268, 269), D6 (270, 271) and CCX-CKR (272, 273).

DARC, D6 and CCX-CKR are capable of recognising multiple ligands with high affinity, but all appear unable to couple to major signalling pathways or to induce any functional response following ligand binding (274). In addition, unlike conventional chemokine

receptors, these atypical receptors appear to be predominantly expressed outwith the haematopoietic system, where they have been suggested to play a role in neutralisation or transport of chemokines (275). Ineligible for membership to the chemokine receptor super-family, due to their lack of signalling capabilities, it has been suggested that these molecules be classified into a separate family of "interceptors", abbreviated to CIPR 1-3 (**C**hemokine **I**nternalising **P**seudo**R**eceptors) (275).

1.22 The Duffy Antigen Receptor For Chemokines (DARC)

The Duffy blood group antigen was first identified in 1950 but attracted interest in the mid-1970s when it was found to act as an erythrocyte receptor for the malarial parasites *Plasmodium vivax* and *Plasmodium knowlesi*. Absence of Duffy expression by red blood cells provides resistance to these parasites, and the high prevalence of Duffy-negative individuals seen among black African populations suggests that this trait may have emerged due to evolutionary selection pressure. Later, it became apparent that Duffy could also bind a large variety of inflammatory CXC and CC chemokines and Duffy was re-christened DARC (**D**uffy **A**ntigen/**R**eceptor for **C**hemokines). In addition to erythrocytes, vascular endothelial cells were shown to express DARC and interestingly, Duffy-negative individuals were found to maintain venular DARC expression.

DARC completely lacks the DRYLAIV motif, which is found on the second intracellular loop of most chemokine receptors and required for coupling to G-proteins, and appears unable to transduce any detectable signal upon ligand binding (268, 276). Instead, it has been proposed to serve as a chemokine transporter or neutraliser at vascular endothelial barriers (277, 278). In favour of a transporter role, Lee et al. found that DARC could traffic

CXCL8 across endothelial cell monolayers *in vitro*, and DARC-deficient animals showed diminished neutrophil recruitment in response to CXCL8 or LPS administration (278, 279).

On the other hand, when Dawson et al. administered high doses of LPS, DARC KO mice were found to have increased neutrophil infiltration, suggesting an anti-inflammatory function for DARC (280). Also, transgenic mice that over-express venular DARC demonstrate reduced angiogenesis following administration of pro-angiogenic CXC chemokines (281). These conflicting data may be reconciled by the fact that when lower levels of chemokine are present, chemokine trancytosis by DARC may promote neutrophil influx while, when chemokine levels become excessive, DARC may act to sequester its ligands, thus down-regulating the inflammatory response.

Similar to this dual-purpose on vascular endothelial cells, erythrocyte DARC might not only act to sequester its ligands, but may also help to maintain sufficient levels of chemokines in the circulation. Injected chemokines disappear more rapidly from the plasma of DARC-deficient mice compared to WT animals, and Duffy-negative individuals have lower plasma levels of CCL2 compared with their Duffy-positive counterparts (282, 283). Therefore the true physiological importance of DARC may be as a chemokine "buffer" to maintain homeostatic levels of chemokines in the circulation.

1.23 CCX-CKR: A Regulator of Homeostatic Chemokines?

The most recently discovered of the three proposed interceptors, CCX-CKR (**C**hemo**C**entryx **c**hemokine **r**eceptor) binds three constitutive CC chemokines, CCL19, CCL21 and CCL25, as well as showing weak affinity for CXCL13 (272, 273). As with

DARC, CCX-CKR appears unable to connect to major signalling pathways following ligand recognition.

A recent report by Comerford et al. has suggested that, at least *in vitro*, CCX-CKR might function as a chemokine scavenger (284). Transfection of HEK293 cell lines with either CCX-CKR or the structurally related CCR7, a functional receptor for CCL19 and CCL21, revealed that both receptors could internalise CCL19, but uptake by CCX-CKR led to enhanced degradation of the chemokine. Moreover, while recognition of CCL19 led to desensitisation of CCR7, ligation of CCX-CKR led to even greater uptake of CCL19.

Little else is currently known regarding this receptor. However, ongoing studies in the recently generated CCX-CKR-deficient animals should help us to decipher its real biological relevance (284).

1.24 D6: Regulation of Inflammatory CC-Chemokines

D6, the final of these three atypical chemokine receptors, has the capacity to bind multiple inflammatory CC-chemokines (270, 271, 285, 286). The ligands of D6 are agonists of CCR1, CCR2, CCR3, CCR4 or CCR5; Figure 1.4 depicts the interactions between D6 ligands and CCR1-CCR5 and describes the leukocyte populations that typically express these chemokine receptors. The predominant site of D6 expression appears to be on lymphatic endothelial cells, with D6-positive lymphatics particularly abundant in barrier tissues, such as the skin, lung and intestine (270, 287). Additionally, D6 is highly expressed by placental trophoblasts and there is some evidence for leukocyte expression (270, 271).

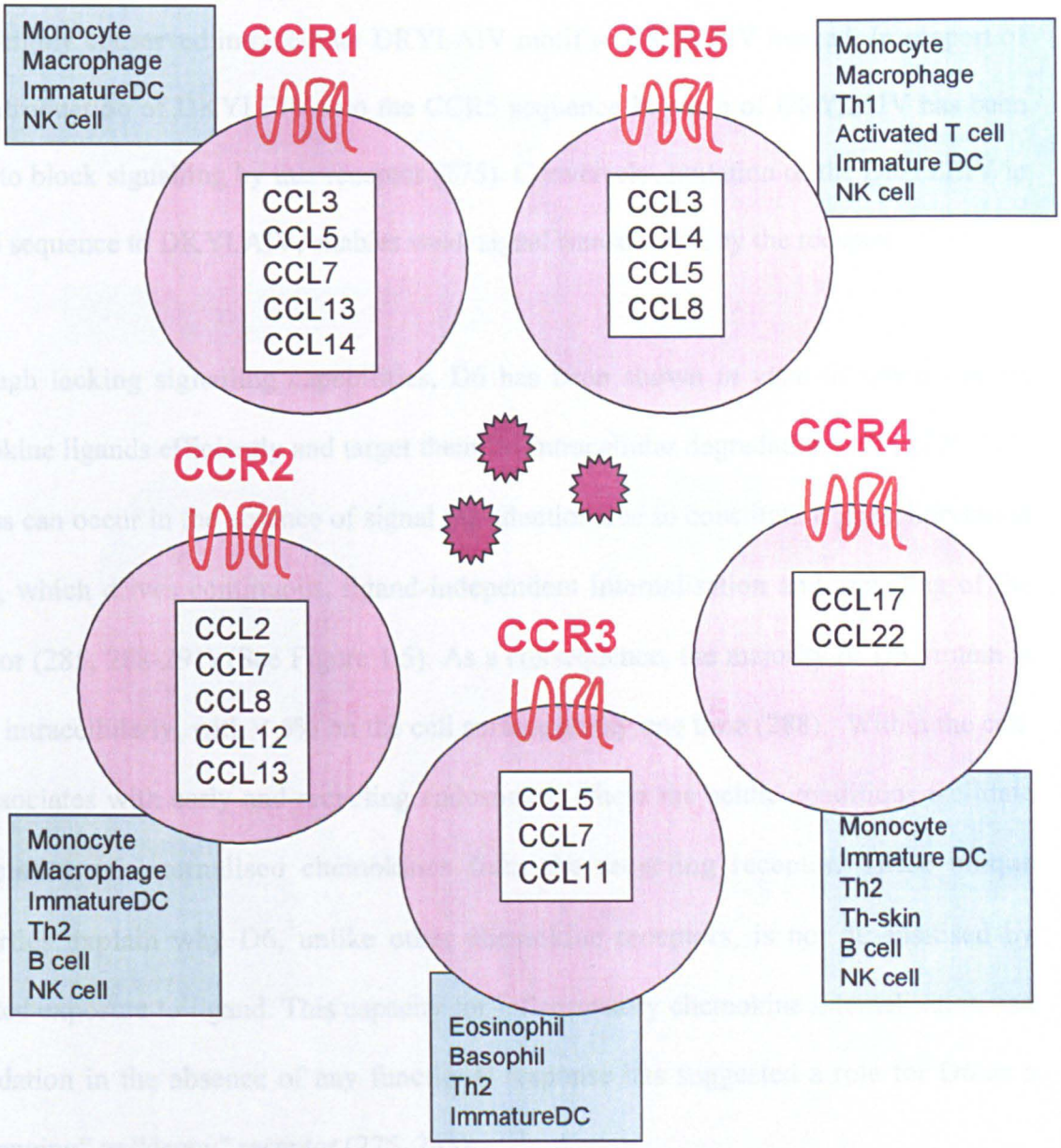


Figure 1.4

Chemokine Receptors Activated by D6 Ligands

The above diagram represents the chemokine receptors which are conventionally activated (i.e. induce productive signalling) in response to ligands of D6. The leukocytes on which these receptors are typically found are listed in the associated boxes.

Despite high structural homology to other chemokine receptors, D6, akin to DARC, fails to initiate intracellular signalling following ligand recognition. This may be due to alteration of the highly conserved intracellular DRYLAIV motif to DKYLEIV instead. In support of this, introduction of DKYLEIV into the CCR5 sequence in place of DRYLAIV has been found to block signalling by this receptor (275). Conversely, mutation of the DKYLEIV in the D6 sequence to DKYLAIV, enables weak signal transduction by the receptor (275).

Although lacking signalling capabilities, D6 has been shown *in vitro* to internalise its chemokine ligands efficiently and target them for intracellular degradation (285, 288). This process can occur in the absence of signal transduction due to constitutive phosphorylation of D6, which drives continuous, ligand-independent internalisation and recycling of the receptor (285, 288-291) (See Figure 1.5). As a consequence, the majority of D6 protein is found intracellularly, with < 5% on the cell surface at any one time (288). Within the cell, D6 associates with early and recycling endosomes, where the acidic conditions facilitate dissociation of internalised chemokines from the recycling receptor. These unique properties explain why D6, unlike other chemokine receptors, is not desensitised by repeated exposure to ligand. This capacity for inflammatory chemokine internalisation and degradation in the absence of any functional response has suggested a role for D6 as a "scavenging" or "decoy" receptor (275, 285).

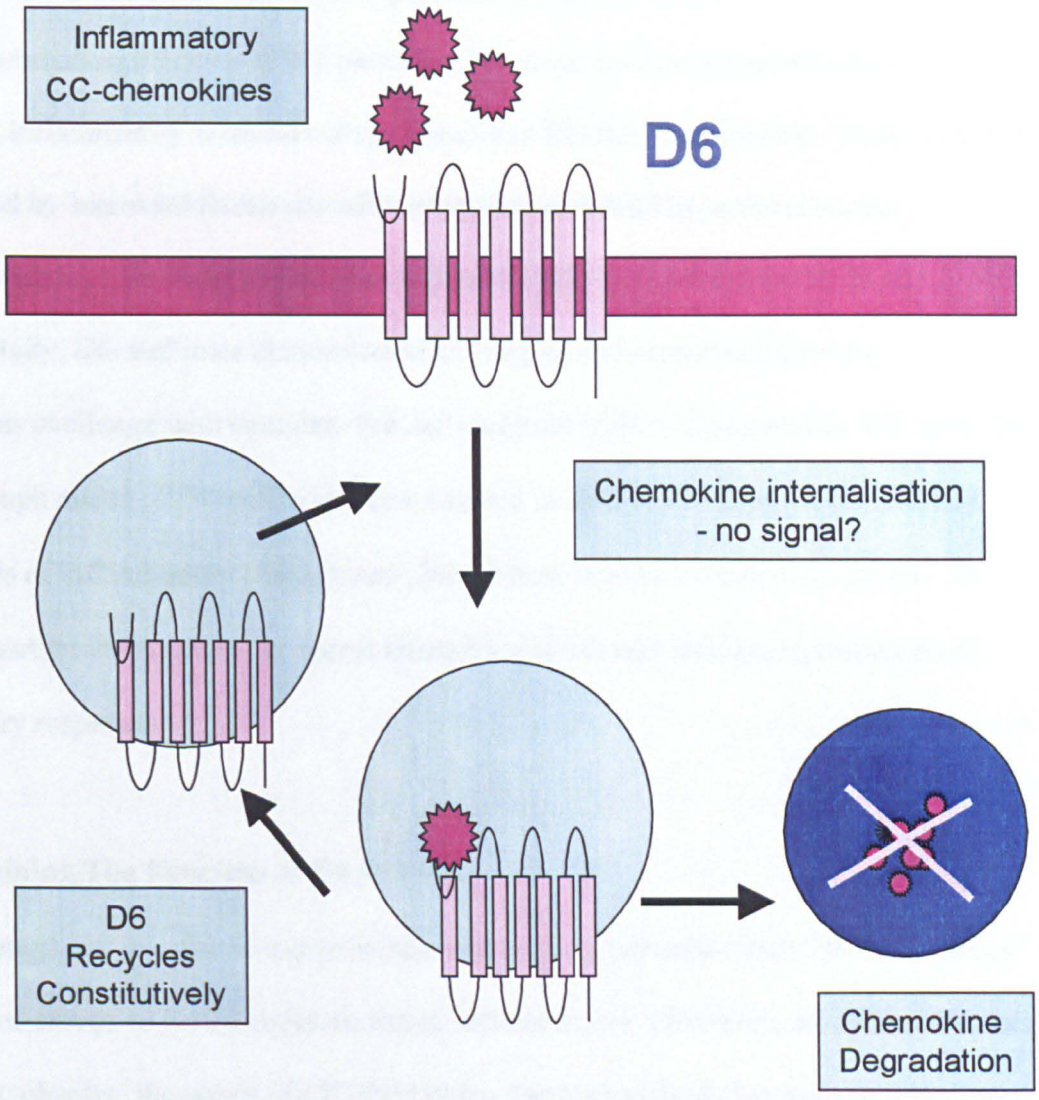


Figure 1.5

Schematic of the D6 Chemokine Receptor

D6 is an atypical chemokine receptor that binds inflammatory CC-chemokines but fails to initiate classical intracellular signalling pathways. Instead, D6 traffics constitutively to and from the cell surface, targeting it ligands for degradation. It has been proposed that through this capacity to effectively scavenge CC-chemokines, D6 may play an important role in the resolution of inflammation.

The recent generation of D6-deficient mice lends further support to the "decoy" hypothesis. Compared to wild-type controls, D6-knockout animals show enhanced pathology in a model of skin inflammation induced by repeated application of phorbol ester (292). While dermal inflammation subsides in WT animals a few days following phorbol ester application, inflammatory responses are prolonged in D6-deficient animals. These mice are characterised by increased leukocyte infiltration and epidermal hyperproliferation, associated with high levels of chemokines within the inflamed dermal tissue. In an additional study, D6-null mice demonstrated an exaggerated response following subcutaneous challenge with complete Freund's adjuvant (291). Compared to WT mice, the draining lymph nodes of D6-null mice demonstrated increased cellularity and contained higher levels of inflammatory chemokines (291). These studies indicate that, *in vivo*, D6 may be important for neutralising excess chemokine levels and facilitating resolution of inflammatory responses.

1.25 Examining The Function of D6 In Vivo

On the strength of the above experiments, it has been proposed that D6 is a "decoy" receptor that serves to down-regulate tissue inflammation. However, as detailed in this introductory chapter, the scope of CC-chemokine function extends beyond the recruitment of innate effector cells. Members of the CC-chemokine family can affect the development of adaptive immune responses, for example, by inducing DC maturation, promoting interactions at the immunological synapse and determining the developmental pathway undertaken by naïve T cells; therefore D6 may influence such responses. In addition, D6 could affect immune responses independent of active infection and inflammation. Regulation of CC-chemokines in primary lymphoid tissue may be essential for proper

lymphocyte development, for instance, by controlling T and B cell precursors localisation with stromal cells in the thymus and bone marrow. Levels of D6 ligands in the blood could also impinge upon steady-state leukocyte mobilisation, as has been shown for the release of $Ly6C^{hi}$ monocytes into the bloodstream (127). Indeed, as expression of D6 is constitutive at many sites, it is likely that its function may extend beyond the elimination of its ligands during inflammation. Thus, theoretically, the function of D6 may impact upon multiple aspects of immunity. However, these particular facets of D6 biology have not been investigated, and it will be important to thoroughly examine the role of D6 in homeostatic immune interactions, as well as this atypical receptor's role in the generation of adaptive immune responses.

1.26 Exploring the Function of D6 in the Intestine

As already mentioned above, expression of D6 is particularly abundant in the intestinal mucosa, but its function at these sites remains unexplored (287). The mucosal surfaces are continuously exposed to a vast array of antigens from the external milieu and at such sites the immune system faces a particularly delicate challenge in managing pro- and anti-inflammatory type responses (293). Mucosal infections are the major killer before the age of five, emphasising the need for strong effector immune responses to deal with potentially deadly pathogens (294). On the other hand, it is vital that the immune system is tolerant towards foodstuffs and commensals, which are essential for life (295). Breakdown in these tolerance mechanisms in the gut can lead to the development of intestinal pathology. Coeliac disease is caused by an aberrant immune response against dietary gluten, whilst Crohn's disease and ulcerative colitis are believed be driven by T cell dependent attacks directed against the intestinal flora (296).

The CC-chemokine ligands of D6 have been implicated in both inflammatory and tolerogenic intestinal immune responses, suggesting that this atypical receptor could play a role in immune homeostasis in the intestine. For example, Karpus and coworkers have described a role for both CCR5 and CCR2 in oral tolerance, the process by which the immune system becomes systemically tolerant towards fed antigens (19, 20, 297). By regulating levels of the CC-chemokine ligands of these receptors, D6 could contribute to this essential physiological phenomenon.

On the other hand, it is possible that chronic immunopathology could ensue from excessive chemokine accumulation in the gut. In support of this, increased levels of CCL2, CCL3, CCL4, CCL5, CCL7 and CCL8 are detected in the colonic mucosa of patients with ulcerative colitis and Crohn's disease (298-300), with a strong correlation seen between CCL7 expression and the extent of epithelial destruction in biopsies from patients with IBD (300). Additionally, mice with deletion of the CCR5 or CCR2 receptors are protected from experimental colitis induced by dextran sulphate sodium (DSS) administration (301). Studies such as these have led to increasing interest in targeting chemokines as a possible therapy for IBD (302). However, our limited understanding of chemokine networks within the intestine hampers this goal.

Accordingly, a better appreciation of the role of D6 will be important for extending our knowledge of chemokine responses in the intestine. With its potential capacity to scavenge CC-chemokines, D6 may serve to limit excessive inflammation developing in the intestine, in an analogous manner to that described for cutaneous inflammation. Moreover, as the

intestine is faced with the greatest antigenic load in the entire body, the role of D6 may be particularly important at this site.

One chief aim of this thesis therefore, was to characterise the role of D6 in intestinal tolerance and inflammation, by examining the induction of oral tolerance and colitis in D6-deficient animals. It was planned to adopt a model of experimental colitis induced by the oral administration of dextran-sulphate sodium (DSS) (303). Mice subjected to DSS develop inflammation of the colon, characterised by diarrhoea, rectal bleeding, weight loss and significant shortening of colon length (303, 304). Histologically, epithelial erosion and ulceration, a marked infiltration of inflammatory cells, and destruction of crypt architecture are seen (303, 304). DSS has been shown to be directly toxic to intestinal epithelial cells and its administration leads to disruption of the colonic epithelium (305). Following breakdown of the epithelial barrier, colonic microflora may gain access to the underlying lamina propria and drive the colitic response. It was anticipated that, due to increased levels of inflammatory CC-chemokines, D6 KO mice might display increased susceptibility to this model of colitis.

Importantly though, despite the current belief that D6 is a chemokine scavenger, its true biological function remains to be established. It is possible that D6 may exert pro-inflammatory effects or may even signal through as yet unidentified pathways. Dissecting the intricacies of D6 action and of the chemokine network as a whole is an important goal; such knowledge may enable manipulation of the chemokine system to either enhance efficacy of vaccines or curtail immunopathological responses.

THESIS AIMS

Regulation of the chemokine family is essential for proper immune homeostasis and the CC-chemokine system has a role at each stage of leukocyte life, not merely during inflammation. Thus, with its ability to recognise multiple inflammatory CC-chemokines, D6 could potentially influence numerous stages of immune development and function. Initial experimental data suggested an anti-inflammatory "scavenging" role for D6; however the vast majority of these studies had been conducted *in vitro*.

The aims of this thesis therefore, were to conduct a thorough characterisation of the immune system of D6 KO mice. As described in this introductory chapter, chemokines facilitate the development and baseline trafficking of haematopoietic cells. Thus, I first explored a possible homeostatic role for D6 by analysing lymphoid tissues of resting D6-null animals. Subsequently, utilising appropriate experimental models, I sought to examine the influence of D6 on both tolerogenic and inflammatory-type immune responses. Due to the abundant expression of D6 in the intestine, I planned to place significant emphasis on exploring the role of D6 at this site. It was anticipated that my studies would either fortify the decoy hypothesis or shed light on as yet unappreciated functions of D6, thus aiding a more complete understanding of this atypical chemokine receptor.

Chapter 3 of this thesis chronicles my preliminary studies in which I assessed the cellular composition of various lymphoid tissues from resting D6 KO animals and examined the ability of the D6 KO animals to respond to specific immunisation with antigen and adjuvant. These early data suggested differences in the myeloid cell compartment of the D6

KO mice, and Chapter 4 describes how I explored myeloid cell responses in D6 KO mice, by examining monocyte and macrophage recruitment to the inflamed peritoneum and by characterising D6 KO dendritic cells derived from bone marrow cultures. In chapter 5, I detail the impact of D6 in intestinal immunity, by describing the induction of oral tolerance and experimental colitis in D6-deficient mice. Finally, Chapter 6 follows up on unexpected observations from the preceding chapter, in which I found that intestinal leukocytes, most notably B cells, from D6 KO animals show aberrant chemokine receptor expression, and I describe how I uncovered this to be a universal feature of leukocytes in D6-deficient mice.

Chapter 2

Materials and Methods

2.1 Animals

D6 knock out (KO) and wild-type (WT) mice were originally obtained from the Beatson Institute for Cancer Research, Glasgow and subsequently bred and maintained on a 129 x C57Bl/6 background under specific pathogen free conditions in the Central Research Facility, University of Glasgow. For some experiments, D6 KO animals and WT controls on a DBA/2 background, also obtained from the Beatson Research for Cancer Institute were used. BALB/c mice were obtained from Harlan Olac, Bicester, U.K.

All mice used in experiments were male and first used between 6-12 weeks of age. All procedures were performed in accordance with United Kingdom Home Office regulations.

2.2 Preparation of Genomic DNA from Tailtips for Genotyping

Tailtips were removed and incubated in 1.5ml screwcap tubes at 55°C overnight in 100µl lysis buffer (100mM Tris.HCl pH 8.5, 5mM EDTA, 0.2% SDS, 200mM NaCl, 0.1mg/ml Proteinase K). Tubes were heated to 96°C on a heating block for 5 min before 500µl of distilled H_2O was added. Tubes were then spun in a microcentrifuge at 13,000 rpm for 5 min and samples stored at 4°C.

2.3 Isolation of RNA

Mouse MLN, PP, small intestinal and large intestinal tissue were dissected out, rinsed in PBS, snap frozen in liquid nitrogen and stored at –70°C. Tissue was ground up whilst frozen using a mortar and pestle under liquid nitrogen. Ground tissue was collected in a sterile RNAse free microcentrifuge tube and homogenised in 10ml of TRIZOL (Invitrogen) by pipetting vigorously up and down. RNA was then isolated from cell extracts according

to the manufacturer's instructions. Briefly, samples were incubated at room temperature for 5 min, spun at 3000g for 5 min at 4°C, and the supernatant collected in fresh tubes. Next, chloroform (0.2ml per ml of TRIZOL) was added to each sample and the samples were shaken vigorously for 15 sec and incubated at room temperature for 2 min. Samples were then spun in a microcentrifuge at 3000g at 4°C for 15 min before the aqueous phase was removed and placed in a fresh tube. Propan-2-ol (0.5ml per 1ml of TRIZOL) was added to each sample and the samples were incubated at RT for 10 min to enable RNA precipitation. Samples were then centrifuged at 3000g for 10 min at 4°C and the pellets of RNA were washed in 75% EtOH, air-dried and resuspended in distilled water. The optical absorbance at 260nm and 280nm were measured using a Beckman DU 650 spectrophotometer and the readings used to determine the RNA concentration. Purity was estimated from the OD_{260}/OD_{280}.

2.4 Removal of DNA from RNA samples

RNA samples were DNAase treated using the DNA-*free* kit (Ambion) according to the manufacturer's instructions. In brief, 10μg of each RNA sample was diluted in 1 x DNAaseI buffer containing 1μl of DNAaseI with the total volume made up to 50μl with diethylpyrocarbonate (DEPC) treated water. Samples were mixed and incubated at 37°C for 30min. 5μl of DNAase Inactivation Reagent was then added and samples mixed thoroughly and incubated at RT for 2min. Samples were centrifuged at 10,000g for 1.5 min and the supernatant, containing the DNAase treated RNA, placed in a fresh tube.

2.5 Synthesis of cDNA from RNA

cDNA was reverse transcribed from DNAase-treated RNA using the Superscript First-Strand Synthesis System for RT-PCR (Invitrogen) according to the manufacturer's instructions. Briefly, 5μg of each RNA sample was mixed with 1μg/ml of random hexamers, 25μg/ml Oligo$(dT)_{12-18}$, 1mM dNTP mix, and the volume made up to 10μl with DEPC treated water. Samples were incubated at 65°C for 5 min and then placed on ice for > 1 minute. 9μl of a mixture containing the following components was added to each sample: 1 x RT-buffer, 5mM $MgCl_2$, 10nmDTT and 40 units of RNase OUT Recombinant RNase Inhibitor. Samples were incubated at 42°C for 2 min and then 1 μl (50 units) of Superscript II Reverse Transcriptase added to each sample, or 1μl of DETC-treated water added to –RT controls. Samples were incubated at room temperature for 10 min, and then heated to 42°C for 50 min before termination of the reaction by heating samples at 70°C for 15 mins. Samples were collected by centrifugation and 1μl of RNAaseH added to each of the samples, which were then incubated at 37°C for 20 min and stored at -20°C prior to PCR amplification.

2.6 Polymerase Chain Reaction (PCR)

PCR was performed using Reddymix PCR Master Mix Tubes according to the manufacturer's instructions. Briefly, 2μl of an oligonucleotide primer mixture comprising 1μg of forward and 1 μg of reverse primers (all purchased from Sigma, see **Table 2.1** for sequences) was added to each of the tubes along with 3μl of cDNA sample. Tubes were then incubated in a thermocycler under the following conditions: 94°C for 2 mins, 35 cycles of 94°C for 20 seconds + 57°C for 1 min + 72°C for 1 min, then 72°C for 10 min

and 4°C until end. PCR products were analysed by agarose gel electrophoresis and visualised with ethidium bromide under UV illumination.

2.7 Isolation of Leukocytes from Lymphoid Tissue

Single cell suspensions from spleen, peripheral lymph node (PLN), mesenteric lymph node (MLN), Peyer's Patch (PP) and thymus were prepared in sterile RPMI 1640 medium (Gibco BRL) by homogenisation of organs through Nitex mesh (Cadisch, London, UK). Cells were washed by centrifugation in RPMI 1640 at 400g for 5 minutes, resuspended in fresh RPMI 1640 and viable cell counts were performed in a Neubauer haemocytometer using a phase contrast microscope (Nikon Labophot, UK). Cells were stored on ice until required.

2.8 Proliferative Responses of Lymphocytes

The proliferative capacity of lymphocytes was assessed by culturing 2 x 10^5 cells in 200μl of complete RPMI (RPMI containing 2mM L-glutamine (Gibco), 100 U/ml penicillin (Gibco), 100 U/ml streptomycin (Gibco), 1.25 μg/ml fungizone (Gibco) and 10% foetal calf serum) in the presence or absence of 1-10 μg/ml concanavalin-A (ConA), 1 μg/ml *E. Coli* LPS (Sigma) or anti-CD3 (2μg/ml). Cells were cultured for 48-120h in triplicate in flat-bottomed 96-well tissue culture plates (Costar) at 37°C in 5% CO_2. The cells were pulsed with 1μCi/well thymidine (^{3}H-TdR) (West of Scotland Radionucleotide Dispensary) for the last 18-24 hours of culture and cell bound DNA was harvested onto glass-fibre filter mats (Wallac). ^{3}H-TdR uptake was measured by a Betaplate counter (Wallac).

2.9 Staining of Leukocytes for Analysis by Flow Cytometry

Aliquots of cells (< 1 x 10^6) were added to 5ml conical, round-bottomed tubes (Becton Dickinson, UK) and washed by adding ice cold FACS buffer (PBS, 2% FCS) and centrifuging at 400g for 5 min at 4°C. Cells were incubated with anti-CD16/CD32 (BD Pharmingen) for 15 min at 4°C to prevent binding of antibody to cells via Fc regions. Subsequently, cells were stained for 45 min with fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, allophycoerythrin (APC)-, or biotin-conjugated antibodies specific for the surface markers of interest (see Table 2.1 for antibody clones used). Biotinylated antibodies were recognised by streptavidin (SA)-APC (BD Pharmingen). All antibodies were purchased from BD Pharmingen, except anti-F4/80-PE and anti-F4/80-bio, which were obtained from Caltag.

2.10 Chemokine Receptor Staining

For flow cytometric analysis of chemokine receptor expression, monoclonal antibodies against CCR2 (MC-21) and CCR5 (MC-68), were generously provided by Prof M. Mack (Department of Internal Medicine, Ludwig-Maximilians University, Munich, Germany). Monoclonal antibodies specific for CCR9 (7E7), CXCR4 (2B11) and CXCR5 (2F18) were a kind gift of Dr Oliver Pabst (Institute of Immunology, Hannover Medical School, Hannover, Germany). Cells were incubated in PBS/ 2% FCS/ 10% mouse serum for 1h at 4°C, washed in 2% FCS/PBS three times and then stained with 5μg/ml of anti-chemokine receptor antibody or isotype control antibody (purified rat IgG2b) for 1 h at 4°C. Cells were washed three times in 2% FCS/ PBS and stained with biotinylated polyclonal anti-rat IgG (BD Pharmingen) for 30 min at 4°C. Following a further set of washes, cells were stained with SA-APC for 15 min at 4°C and then washed again. Expression of cell surface markers

was assessed as described in the previous section, except that the anti-CD16/CD32 blocking step was omitted.

2.11 Intracellular Cytokine Staining

To assess expression of IFNγ and IL-17, leukocytes were stimulated for 4-5 hours with 50 ng/ml PMA, 500 ng/ml ionomycin and 10μg/ml Brefeldin A or with 10μg/ml Brefeldin A alone, in complete RPMI. Cells were washed in ice-cold FACS buffer as described above and stained with F4/80-bio for 30 min at 4°C and, following a further wash, were stained with SA-FITC for 15 min at 4°C. The cells were then washed twice in FACS buffer and resuspended in 200μl Cytofix/Cytoperm (BD Pharmingen) for 30 min at 4°C before washing twice with Perm Wash (BD Pharmingen). Permeabilised cells were next stained in 50μl Perm wash containing FITC- or PE-labelled cytokine specific antibody (BD Pharmingen), or isotype control antibody, for 30 min at 4°C. Following cytokine staining, the cells were washed with Perm wash and then with FACS buffer before being resuspended and analysed for intracellular cytokine staining by flow cytometry.

2.12 Analysis of Stained Cells By Flow Cytometry

Cellular fluorescence data was acquired using a dual-laser (488-nm argon laser, 635-nm red diode laser) Becton Dickinson FACSCalibur flow cytometer and CELLQuest software (BD Biosciences). Dead cells were excluded by addition of 9μl of a 50μg/ml propidium iodide solution (Sigma-Aldrich) to each tube immediately before analysis. Following acquisition, data files were analysed using FlowJo software (Tree Star Inc, OR, USA).

2.13 Measurement of Cytokine and Chemokines by ELISA

To detect IL-1, IL-6 and TNFα, Immulon-4 plates (Corning-Costar) were coated with anti-IL-1 (4μg/ml, R&D systems), anti-IL-6 (1μg/ml, BD Biosciences) or anti-TNFα (2μg/ml, BD Biosciences) detection antibodies overnight at 4°C. Plates were washed at least three times with PBS/0.05% Tween (Sigma) before being blocked with PBS/ 10% FCS for 1h at room temperature. The plates were washed and incubated with serially diluted recombinant cytokine standards (BD Pharmingen) and samples for 2 h before being again washed and incubated with biotinylated-detection antibody (300ng/ml IL-1 (R&D Systems), 1μg/ml IL-6 (BD Pharmingen), 2μg/ml TNFα (BD Pharmingen)) for 1 h. Plates were then washed and incubated with Extravidin-Peroxidase (1/1000 dilution, Sigma) for 1 h. Following a final series of washes, plates were developed using tetramethylbenzidine (TMB) substrate (KPL) and read at 630 nm on a MRX II microplate reader (Dynex).

MCP-1 and IL-23 supernatant levels were assessed by ELISA using commercial kits (from R&D Systems and E-Biosciences, respectively) according to the manufacturer's instructions.

2.14 Measurement of Cytokine and Chemokine Levels by Luminex

The following cytokines and chemokines were analysed simultaneously using Multiplex Bead Assay (BioSource, UK) according to the manufacturer's instructions: 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. The principle of the method is a sandwich immunoassay where specific antibodies for the cytokines/chemokines

of interest have been coated onto the surface of fluorescently encoded microspheres. Each microsphere is labelled with a distinguishable fluorophore that enables its assignment to a particular region by the scanner. Cytokine capture microspheres were incubated in a 96 well Luminex plate with test samples or cytokine standards and, following washing of the plate, biotinylated detection antibody added. At the end of the incubation period, the plate was washed and samples incubated with Streptavidin-PE. After a final wash, 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 present.

2.15 Antigens

Ovalbumin (OVA, Grade V) was obtained from Sigma (Poole, Dorset, UK) as a lyophilised powder and stored at 4°C until required. Heat-aggregated OVA (HAO) was prepared by heating a 2% solution of OVA in sterile saline at 70°C for 60 minutes in a waterbath. The precipitated OVA was then centrifuged for 10 minutes at 450g, 4°C and the supernatant removed. The pelleted HAO was resuspended in sterile saline at a concentration of 20mg/ml and stored in aliquots at -20°C until required. For use as an *in vivo* challenge, HAO was resuspended in sterile saline at a concentration of 2mg/ml and sonicated for 20 minutes until a colloidal suspension was obtained.

2.16 Assessment of Oral Tolerance

Oral tolerance was induced by feeding mice 25mg of OVA dissolved in 0.2 ml of saline using a rigid steel gavage tube. Control mice received oral saline alone. One week later, mice were challenged by subcutaneous injection in the hind footpad with 100μg OVA

dissolved in saline and emulsified at a 1:1 ratio in Complete Freund's Adjuvant (Sigma-Aldrich) in a total volume of 50μl.

2.17 Induction and Measurement of antigen-specific proliferation *in vitro*

Single cell suspensions of draining popliteal lymph node cells were prepared in sterile RPMI 1640 medium by homogenisation of organs through nitex mesh as described above and cultured in complete RPMI alone or in complete RPMI containing 1mg/ml OVA or 5μg/ml con A (Sigma) at 37°C in 5% CO_2 for 120h. Culture supernatants were collected at 48h, centrifuged at 13000g and stored at -20°C until use for cytokine analysis by ELISA (see above). Proliferation was assessed by addition of 1μCi/well thymidine for the last 18-24 hours of culture as described above.

2.18 Measurement of Antigen-specific DTH Responses

Systemic OVA-specific DTH responses were assessed 21 days after OVA/CFA footpad challenge by using callipers (Kroplin Ltd, Kingston-upon-Thames, Surrey, UK) to measure the increment in footpad thickness 24 hours after subcutaneous challenge of the previously unimmunised hind footpad with 100μg heat-aggregated OVA. Antigen-specific DTH responses were calculated by subtracting the footpad increments found after challenge of naïve mice.

2.19 Measurement of Antigen-specific Antibodies

Serum levels of OVA-specific total IgG were measured by ELISA 21 days after immunisation with OVA/CFA. Briefly, plates were coated overnight at 4°C with10μg/ml OVA in 50mM carbonate buffer. The plates were washed 3 times with PBS/0.05% Tween

and blocked with 10%FCS/PBS to prevent non-specific binding. Following 3 washes with PBS/Tween, aliquots of test sera in doubling dilutions in 10%FSC/PBS were added to the plates and incubated overnight at 4°C. Following a further series of washes, peroxidase conjugated anti-IgG antibody (Sigma) diluted 1:1000 in 10%FCS/PBS was added to the plates and incubated for 1h at 37°C. The plates were washed 5 times before addition of TMB substrate to each well. Plates were read at 630nm on a MRXII microplate reader (Dynex).

OVA-specific IgG1 and IgG2a antibody levels in serum were measured as described above, except that following incubation of test samples, plates were washed in PBS/Tween and either biotinylated-anti-IgG1 (1/1000) or biotinylated-anti-IgG2 (1/2000) added to the plate for 1h. Subsequently, plates were washed 3 times and incubated with Extravidin-Peroxidase (1/1000 dilution, Sigma) for 1 h. Plates were developed and read as described above for total IgG.

2.20 Measurement of Total Serum Antibodies

Total serum levels of IgG and IgM were measured by ELISA. Briefly, plates were coated overnight at 4°C with 100μl of 1 μg/ml capture anti-IgG (Sigma) or 100μl of 2 μg/ml capture anti-IgM (Sigma) in 50mM carbonate buffer. The plates were washed 3 times with PBS/0.05% Tween and blocked with 200μl of 10%FCS/PBS to prevent non-specific binding. Following 3 washes with PBS/Tween, 100 μl of test sera, serially diluted in 10%FSC/PBS, were added to the plates and incubated overnight at 4°C. Following a further series of washes, biotin-conjugated anti-IgG antibody diluted 1:200,000 in 10%FCS/PBS (Sigma) or 100μl of 2μg/ml anti-IgM (Sigma) diluted in 10%FCS/PBS was added to the

plates and incubated for 1h at 37°C. Plates were washed 3 times and incubated with Extravidin-Peroxidase (diluted 1:1000 in 10% FCS/PBS) for 1 h. The plates were washed 5 times before addition of TMB substrate to each well. Plates were read at 630nm on a MRXII microplate reader (Dynex).

2.21 Induction of Peritonitis by Injection of Thioglycollate

Mice were injected intraperitoneally with 2ml of 4% Thioglycollate broth (Sigma) and, at the time-points indicated, mice were euthanised by CO_2 inhalation. To obtain peritoneal exudate cells (PEC), sacrificed mice were injected with 6 ml of PBS/1mM EDTA, the abdomen was gently massaged and the fluid withdrawn using a 21-gauge needle. Cells were washed by centrifugation in RPMI 1640 at 400g for 5 minutes, resuspended in fresh RPMI 1640 and counted before being stored on ice until required. The lavage fluids were stored at -20°C until use.

To analyse PEC in cytospins, aliquots of 1-5 x 10^5 cells were spun onto glass microscope slides at 600 RPM for 4 min using a cyto-centrifuge (Shandon, Runcorn, UK) and fixed in acetone for 5-10 min. The slides were allowed to air dry and washed in PBS before immersion in 1:10 Geimsa stain (Sigma) for 30 minutes. Next, the slides were rinsed in distilled water, allowed to air dry and then mounted in DPX mountant (BDH, UK) under glass. Slides were sealed using clear nail varnish (Rimmel, Boots The Chemist, UK) and the stained cells assessed histologically at 400x magnification using a Nikon Labophot microscope. To perform analysis of cell numbers, 10 fields per cytospin were assessed and the average calculated.

2.22 Measurement of Nitric Oxide Production

Isolated PEC were resuspended in complete RPMI-1640 and $1x10^5$ cells were stimulated with IFNγ (25U/ml, Biosource) and/or LPS (1μg/ml) for 48h. The concentration of nitric oxide (NO) present in culture supernatants was determined by detection of nitrite, a stable metabolite of NO, using the Griess reaction. Supernatants from cultured cells (50μl) or standard dilutions of sodium nitrite were added to 96-well Immulon 4 plates in triplicate and then 50μl of Griess reagent A (1% sulphanilamide/ 5% H_3PO_4) and 50 μl of Griess reagent B (0.1% napthyethylenediamine/ 5% H_3PO_4) added. Plates were read at 570nm using a microplate reader.

2.23 Induction of Intestinal Inflammation by Administration of Indomethacin

Stock solution was prepared by dissolving 100mg of indomethacin (Sigma) in 100μl DMSO + 900μl of absolute ethanol. The solution was then diluted in pre-warmed PBS and mice received an intraperitoneal dose of 2.5mg/kg daily for 14 days. Control mice received PBS injections. At the end of the experiment, animals were sacrificed and the small and large intestines of each mouse removed for histological analysis.

2.24 Analysis of Intestinal Architecture

The method of Clarke was used to assess intestinal architecture. $1cm^2$ pieces of duodenum, jejunum, ileum and colon were opened longitudinally and fixed for 6-24h in Clarke's Fixative (75% ethanol, 25% glacial acetic acid) before being stored in 75% alcohol. After hydrolysis in 1 N HCl at 60°C, the tissues were stained with Schiff reagent (Sigma). Using a dissecting microscope, the muscularis mucosae was removed and sections one villus/crypt unit thick were cut using a cataract knife (Weiss). The sections were placed on a

microscope slide in 30% acetic acid and villus and crypt lengths measured. The tissues were then gently squashed under the coverslip and the number of mitotic bodies per crypt determined.

2.25 Assessment of Epithelial Cell Turnover by Uptake of Bromodeoxyuridine

Mice were injected intraperitoneally with 1 mg bromodeoxyuridine (BD Pharmingen) diluted in PBS and sacrificed 24 hours later. Excised colons were frozen with liquid nitrogen in cryomoulds containing OCT embedding medium (Bayer, Newbury, Berkshire, England) and stored at -70°C. Tissue sections 6μm thick were cut on a cryostat (ThermoShandon, Chesire, UK) and stored at -20°C. Brd-U incorporation was assessed immunohistochemically using a commercial kit (BD biosciences) in accordance with the manufacturer's instructions.

2.26 Induction and Assessment of Dextran Sodium Sulphate Colitis

Mice received dextran sodium sulphate (molecular weight 36-50 kDa, ICN Biomedicals) dissolved in sterile drinking water, *ad libitum,* for up to 14 days. Control mice received water without DSS. Animals were monitored daily for clinical signs of colitis, namely weight loss, diarrhoea, rectal bleeding and general morbidity. Weight change was calculated as percent change in weight compared with body weight at start of experiment. Any animal that lost > 20% of its original body weight was immediately sacrificed by cervical dislocation. At the end of the experiment, colons were excised and their length recorded.

Histological scoring of colitis was performed on samples of colon, which were fixed in 10% formalin, embedded in paraffin and stained with haematoxylin and eosin. Scoring was performed in a blinded fashion utilising a combined score of inflammatory cell infiltration (0-5) and tissue pathology (0-5) (see table 2.3).

2.27 Colon Organ Culture

Colons were opened longitudinally and washed in sterile PBS (Gibco BRL, Paisley) supplemented with 1% penicillin/streptomycin (Gibco BRL, Paisley). Three segments from the distal colon of approximately 1cm in length were placed in 24 flat-bottom well culture plates (Costar) containing fresh RPMI 1640 (Gibco BRL, Paisley) supplemented with 1% penicillin/streptomycin and incubated at 37°C for 24 h. Culture supernatants were then harvested, centrifuged at 13,000g and stored at -20°C before measurement of cytokines and chemokines by ELISA and Luminex.

2.28 Isolation of Colonic Lamina Propria Cells

Whole colons were washed in CMF-HBSS (Gibco), opened longitudinally and cut into segments of ~ 1 cm. Epithelial cells were removed by incubating the colon segments in 2mM EDTA/ CMF-HBSS for 15 minutes at 37°C with shaking. The tissue was then washed in CMF-HBSS, the supernatant discarded and fresh EDTA/ CMF-HBSS added. A total of three EDTA/HBSS incubations were performed, after which the tissue was washed thoroughly in sterile PBS and digested in 20% FCS/ CMF-HBSS containing 100 U/ml collagenase-A (Sigma) and 30μg/ml DNase (Roche) for 3 x 45 minute incubations at 37°C. Liberated cells were harvested by shaking, washed in fresh CMF-HBSS, counted and stained for analysis by flow cytometry.

2.29 Generation of Bone Marrow Dendritic Cells

To obtain dendritic cells (DC), mouse femurs were washed out with RPMI 1640 using a syringe and a 21g needle. Aliquots of 3 x 10^6 bone marrow cells were seeded into 90cm low-adherence Petri dishes (Bibby Sterilin, UK) and grown in complete RPMI at 37°C in 5% CO_2. On days 0, 3 and 6 of culture, the medium was supplemented with 10% sterile, filtered supernatant from X-63, a GM-CSF-transfected fibroblast cell line. Non-adherent DC were harvested by gentle washing of the Petri dishes after 5-12 days of culture.

2.30 Activation of DC *in vitro*

BM-DC (1 x 10^6) were cultured overnight in ultra low adherence 24-well flat-bottom plates (Corning, NY) either in complete medium alone, or with 1-2μg/ml *Escherichia Coli* LPS (Sigma). Non-adherent cells were analysed by flow cytometry and supernatants harvested for analysis of cytokine production.

2.31 Assessment of Antigen Presenting Cell (APC) Activity of DC

The ability of WT and D6 KO DC to stimulate allogeneic T-cell responses was assessed by culturing 1-2 x 10^5 BM derived DC, pre-treated with 50 μg/ml mitomycin C (Sigma) at 37°C for 40 min, with 1-2 x 10^5 BALB/c lymphocytes in V-bottom well plates (Corning) for 48-72 hours. The cells were pulsed with 1μCi/well thymidine for the last 18-24 hours of culture.

2.32 Assessment of Endocytic Activity of DC

BM-derived DC were cultured with complete RPMI 1640 containing 1 mg/ml FITC-dextran (Sigma) in ultra low adherence 24-well flat-bottom plates at 4°C or 37°C. Cells

were then washed twice in ice-cold FACS buffer (2% FCS/PBS) and stained for 30 min with anti-CD11c-PE (BD Pharmingen). Cells were analysed by flow cytometry and the uptake of FITC-dextran (Δ MFI) was calculated by subtracting the FITC-channel Mean Fluorescence Intensity (MFI) of cells cultured with FITC-dextran at 4°C from the FITC-channel MFI of cells cultured at 37°C.

2.33 Negative Selection of Naïve B Cells

Naïve B cells were purified from the spleen by negative selection. Single cell suspensions were prepared and red blood cells lysed by addition of 5ml Red Blood Cell Lysis Solution (Sigma) for 5 minutes at room temperature. The remaining cells were washed in MACS medium (2% FCS/ PBS), resuspended and counted. Following centrifugation, cells were resuspended at a concentration of 10^7 cell/100μl MACS medium before the addition of 10μl/1x10^7 cells anti-CD43 (Ly-48) coated microbeads (Miltenyi Biotech, UK) for 15 min at 4°C. After washing, a maximum of 2 x10^8 cells in 1ml MACS medium were applied through Nitex to a CS column (Miltenyi Biotec, UK), which had previously been equilibrated with 8 column volumes of cold MACS medium. To elute naïve B-cells, a further 5 column volumes of cold MACS medium were passed through the CS column and the eluent collected. All solutions and cells were kept at 4°C throughout the procedure. B cell purity was assessed by FACS analysis of B220 expression and was in the range of 90-94%.

2.34 Assessment of B cell Activation *in vitro*

A total of 2 x 10^6 MACS-purified B cells were cultured in 24-well plates (Costar) in complete RPMI alone or complete RPMI containing anti-IgM (10μg/ml) ± anti-CD40

(10μg/ml), dextran sulphate sodium (DSS) (50μg/ml) or LPS (1μg/ml) at 37°C in 5% CO_2. Cells were harvested 48h later and stained for expression of costimulatory molecules and chemokine receptors as described above. Proliferative responses were assessed by pulsing with ^{3}H-TdR as described previously.

2.35 RNA-isolation from Purified B cells

Following MACS purification, $7x10^6$ B cells were added to eppendorfs and lysed by vortexing in 350μl of RNA lysis buffer (provide by Dr Clive McKimmie, University of Glasgow). Samples were then added to QIAshredder Mini Columns (Qiagen) and centrifuged at top speed on a microcentrifuge for 2 min at room temperature. The spin through was kept and stored at -80°C.

2.36 **Statistical Analysis**

The results are represented as the mean ± 1 SD unless otherwise stated. Normally distributed data were analyzed using Student's t test, whereas other datasets were compared using a Mann-Whitney U test. Values of $p < 0.05$ were considered to be statistically significant. Statistically significant differences between data sets are indicated throughout this thesis using the following key:

* Statistically significant with a p value < 0.05

** Statistically significant with a p value < 0.01

*** Highly significant with a value of p < 0.001

Table 2.1: Primers Used For PCR

cDNA	Forward Primer (5'-3')	Reverse Primer (5'-3')
CCL2	TGATCCCAATGAGTAGGCTGG	AGAAGTGCTTGAGGTGGTTGTG
CCL3	CTGCCCTTGCTGTTCTTCTCTG	TCAGGCATTCAGTTCCAGGTC
CCL4	GCTGTTTCTCTTACACCTCCCG	AGCAAGGACGCTTCTCAGTGAG
CCL5	CTCACCATCATCCTCACTGCAG	TGCAAGATTGGAGCACTTGC
CCL7	TTCTCCACCATGAGGATCTCTG	TATAGCCTCCTCGACCCACTTC
CCL8	TGCTCATAGCTGTCCCTGTCAG	TCAAGGCTGCAGAATTTGAGAC
CCL11	CCCCAACACACTACTGAAGAGC	TTCCCTCAGAGCACGTCTTAGG
CCL12	TCCACACTTCTATGCCTCCTGC	TGGCTGCTTGTGATTCTCCTG
CCL19n	CTGCCTCAGATTATCTGCCATG	TGGAGGTGCACAGAGCTGATAG
CCL19p	TGCCTCAGATCGTCTGCCAC	TGGAGGTGCACAGAGCTGATAG
CCL21	TCCCTACAGTATTGTCCGAGGC	TCAGTTCTCTTGCAGCCCTTG
CCL22	CACCCTCTGCCATCACGTTTAG	GTGAGTAAAGGTGGCGTCGTTG
CCL25	CTTTTTGCCTGCCTGGTTG	GCTGTCAAGATTCTCATCGCC
D6	AGCTTTAGCTGCTGAACCTGG	AAGAAGATCATCGCCAAGAGTG
β-actin	TCCATCATGAAGTGTGACGT	TACTCCTGCTTGCTGATCCA

Table 2.2: Antibodies used to detect Cell Surface Markers by Flow Cytometry

ANTIBODY	CLONE	SUPPLIER	ISOTYPE	CELLS +ve	NOTES	LIGANDS
CD3e	145-2C11	BD Biosciences	HamIgG1	T cells NK-T cells	TCR-signalling complex, Ig SF	
CD4	RM4-5	BD Biosciences	RatIgG2b	T cell subset DC subset	T CR co-receptor, Ig SF	MHC II
CD5	53-7.3	BD Biosciences	RatIgG2a	T cells NK-T cells B1 B cells	cysteine-rich scavenger-R	CD72
CD8a	53-6.7	BD Biosciences	Rat IgG2b	T cell subset DC subset IEL	T CR co-receptor Ig SF	MHC I
CD11b	M1/70	BD Biosciences	Rat IgG2b	Granulocytes Macrophages DC NK cells B-1 cells	α_M integrin adhesion molecule	C3bi CD54
CD11c	HL3	BD Biosciences	Ham IgG1	DC IEL Macrophages	Integrin α_x	iC3b
CD19	1D3	BD Biosciences	RatIg2a	B cells	BCR co-receptor Ig SF	
CD25	7D4	BD Biosciences	Rat IgMκ	Activated T cells, activated B cells, macrophages, Treg	Low affinity IL-2Rα chain	IL-2
CD40	3/23	BD Biosciences	Rat IgG2b	APCs	Costimulatory molecule TNF-R SF	CD40L
CD80	16-10A1	BD Biosciences	HamIgG2	DC Monocytes Macrophages B cells	Costimulatory molecule Ig SF	CD28 CD152
CD86	GL1	BD Biosciences	Rat IgG2a	DC Monocytes Macrophages B cells T cells	Costimulatory molecule Ig SF	CD28 CD152
IA/IE	2G9	BD Biosciences	Rat IgG2a	APCs	Antibody reacts with following class II MHC: I-A^b, I-A^d, I-A^q, I-E^d, I-E^k	TCR CD4

ANTIBODY	CLONE	SUPPLIER	ISOTYPE	CELLS +ve	NOTES	LIGANDS
Ly6G	1A8	BD Biosciences	Rat IgG2a	Granulocytes, predominantly neutrophils	GPI-anchored protein	
Ly6C	AL-21	BD Biosciences	Rat IgM	Monocytes Macrophages CD8 T cells NK cells Plasma cells	GPI-linked cell surface protein Promotes endothelial adhesion via LFA-1 clustering	
Gr1	RB6-8C5	BD Biosciences	Rat IgG2b	Neutrophils Eosinophils monocytes	Recognises a common epitope on Ly6C and Ly6G	
γδ TCR	V65	BD Biosciences	Mouse IgG1	γδ T cells IEL DETC	TCR	
NK1.1	PK136	BD Biosciences	Mouse IgG2a	NK cells NK T cells	Recognises killer cell lectin-like Rs: NKR-P1C (activatory) on C57Bl6, FVB and NZB and NKR-P1B (inhibitory) on Swiss NIH and SJL	
B220 (CD45)	RA3-6B2	BD Biosciences	Rat IgG2a	B cells T cell subset NK cells	Protein Tyrosine Phosphatase Regulation of TCR/BCR signalling	
F4/80	BM8	Caltag (Now Invitrogen)	Rat IgG2a	Monocytes/ Macrophages	EGF-TM7 family member	

Chapter 3

Characterisation of the Immune System in D6 KO Mice

Introduction

Chemokines are of fundamental importance for the trafficking and positioning of leukocyte populations (9). The atypical chemokine receptor D6 has been shown to act as a scavenger for a number of inflammatory-type CC-chemokines, *in vitro,* (see Figure 1.4) and, interestingly, immunohistochemical studies have identified lymphatic endothelial cells as a prominent site of D6 expression (271, 286-288). These data pointed to a potential role for D6 in controlling leukocyte recruitment to lymphoid tissues, possibly through regulating bioavailable levels of CC-chemokines at these sites. To explore this idea, I first compared the composition of various lymphoid tissues from resting WT and D6 KO animals (292) by flow cytometry, and assessed lymphocyte functions. Then, I immunised mice with specific antigen in adjuvant and induced inflammation in skin.

3.1 Analysis of Lymphoid Tissue Composition in D6 KO Mice

Cells isolated from primary lymphoid tissue (thymus) and secondary lymphoid tissue (peripheral lymph nodes, mesenteric lymph nodes and spleen) were stained with antibodies specific for CD4, CD8, B220, F4/80 and CD11c to identify CD4 T cells, CD8 T cells, B cells, macrophages and dendritic cells, respectively (Figures 3.1 - 3.2). As shown in Figure 3.1, there were no differences between WT and D6 KO mice in the proportions of $CD4^+$, $CD8^+$ or $B220^+$ cells found in any of the tissues analysed. Additionally, the percentages of $F4/80^+$ and $CD11c^+$ cells retrieved from PLN, MLN and thymuses of WT and KO mice were similar (Figures 3.2 A-B).

However, analysis of spleen revealed small, but significant increases in the proportions of $F4/80^+$ and $CD11c^+$ cells in D6 KO animals compared with WT controls (Figures 3.2 C-D).

Thus the absence of the D6 chemokine receptor could affect population of the spleen by myeloid lineage cells.

3.2 Mitogen-Induced Proliferative Responses of D6 KO Cells

I next compared the functional responses of WT and D6 KO cells when stimulated with mitogen *in vitro*. Cells isolated from peripheral LN or spleen were cultured in complete medium supplemented with 5μg/ml concanavalin A (Con A) or 1μg/ml LPS and proliferative responses measured by ^{3}H-Tdr incorporation 48, 72, 96 and 120 hours later.

As shown in Figure 3.3, cells harvested from peripheral LN of D6 KO mice demonstrated higher proliferation responses *in vitro* to Con A compared with WT controls at all time-points examined. A similar effect was also seen at 48 and 72 hours of culture when spleen cells were stimulated with Con A (Figures 3.4 A-B). By 96h there were no statistically significant differences in the Con A responses between the groups, although the proliferative response of the KO spleen cells still exceeded that of WT cells at this time (Figure 3.4 C).

The proliferative responses of KO LN cells in response to LPS also exceeded those of WT at each time point assessed, but the differences were not statistically significant and the responses, in general, were relatively low. Spleen cells responded better to LPS stimulation and there was a significant enhancement of the KO response compared with that of WT cells at 48 and 72 hours of culture.

To confirm the increased responsiveness of D6 KO cells to Con A, I stimulated PLN, MLN and spleen cells from WT and KO mice with different concentrations of Con A for 48-96h. As shown in Figures 3.5, 3.6 and 3.7, markedly higher proliferation rates were observed in the cultures of lymphoid tissues at each time-point and at all Con A concentrations investigated.

3.3 Antigen Specific Immunisation of D6 KO Mice

These initial experiments suggested that there were few differences in the compartmentalisation of lymphoid tissue in D6 KO mice under steady-state conditions. However, due to the great complexity of the chemokine system and the redundant nature of interactions between its family members, the effects of deficiency in an individual receptor are often only apparent when KO animals are subjected to specific immunological stimuli (11, 306). Therefore I next explored a role for D6 in the generation of antigen-specific immune responses and inflammation.

D6 KO and WT mice were immunised in the footpad with OVA emulsified in complete Freund's adjuvant (CFA) and their ability to respond to this challenge was compared 1-6 weeks later by measuring the antigen-specific proliferation and cytokine production of cells isolated from the draining popliteal lymph nodes. Serum levels of OVA-specific IgG antibody, IgG1/IgG2a isotypes, and specific delayed-type hypersensitivity responses following challenge with heat-aggregated OVA were also assessed.

Initial experiments showed that both WT and D6 KO mice on the 129/B6 background developed effective antigen-specific immunity, *in vivo* and *in vitro*, in response to

OVA/CFA, which was maximal at 2-3 weeks (Figures 3.8 – 3.11). At weeks 1 and 6 post immunisation, primed WT cells appeared to proliferate better than cells from KO mice upon culture with OVA *in vitro*, but these differences were not statistically significant (Figs 3.8 A and D). Conversely, cells harvested from KO mice at 2 and 3 weeks after priming, displayed increased proliferative responses and produced significantly greater levels of IFNγ upon re-exposure to antigen compared with their WT counterparts (Figs 3.8 B and C and 3.9).

D6 KO animals generally had higher DTH responses than WT controls when assessed 2 or 3 weeks after immunisation (Figures 3.10 A and B). However, these changes did not reach statistical significance and there were no differences between the DTH responses 6 weeks after immunisation (Fig 3.10 C). Measurement of specific antibody responses at weeks 2 and 3 post immunisation showed that WT and D6 KO mice had similar levels of OVA-specific IgG, and there were also no statistically significant differences found between IgG1 and IgG2a antibody subclasses (Figure 3.11).

In view of the variability of these findings depending on the time of assessment, I decided to repeat the experiment and measured responses at one and two weeks following immunisation. On this occasion, there were no significant differences between the strains in terms of OVA-specific proliferation or IFNγ production, *in vitro* (Figure 3.12) at both time points examined. Specific DTH responses were also comparable between WT and KO mice (Figures 3.13 A and 3.14 A). At week one, similar levels of OVA-specific IgG, IgG1 and IgG2a were measured in serum from immunised WT and KO mice (Figures 3.13 B - D). However, at two weeks after immunisation, D6 KO mice had significantly lower levels

of serum OVA-specific IgG compared with WT animals, and this appeared to be due to a specific reduction in the IgG1 subclass (Figures 3.14 B – D).

As my data were variable, I decided to examine the contribution of D6 to the generation of antigen specific immune responses on a different genetic background, and therefore conducted similar experiments using mice on a DBA/2 background. In these mice, lymph node cells isolated from D6 KO mice showed significantly reduced antigen-specific responses *in vitro* compared with WT cells at one week after immunisation with OVA/CFA (Fig 3.15 A and B). However, at two weeks post-challenge this phenotype had reversed, with the KO cells responding better upon re-exposure to OVA (Fig 3.15 C and D). DTH responses were comparable between WT and KO DBA/2 mice when measured at 1 or 2 weeks (Figures 3.16 A and 3.17 A), although OVA-specific antibody levels were lower in KO mice one week after immunisation, with a striking defect in OVA-specific IgG2a in KO mice at this time point (Figures 3.16 B and D). By two weeks post challenge, however, there were no differences between WT and KO animals in terms of OVA-specific antibody production (Fig 3.17 C and D).

Taken together, these data suggested that, whilst not necessary for the generation of effective antigen-specific immune responses, the D6 chemokine receptor might influence the kinetics of such immune responses, although the exact nature of this control needs to be defined. Unfortunately, due to time limitations and a shortage of animals, I was unable to follow up these experiments.

3.4 Analysis of Lymphoid Tissue Draining Inflamed Skin

To assess further how D6 might regulate immune responses, I examined the draining lymph nodes of B6/129 WT and D6 KO mice subjected to application of the phorbol ester, 12-O-tetradecanoylphorbol 13-acetate (TPA). TPA treatment evokes an inflammatory response characterised by epidermal hyperplasia, hyperkeratosis, the development of rete ridges and increased cellular recruitment to the dermis of the skin. Recent work in the laboratory had shown that D6 knockout mice display increased epidermal thickening, excessive dermal infiltration and failure to resolve the inflammatory response generated by multiple TPA application (292). I therefore compared draining lymph nodes from TPA-painted WT and KO mice for absolute cell numbers, composition of leukocyte subsets, and for the proliferative capacity of isolated LN cells *in vitro*.

In the first experiment, I examined lymph nodes from WT and D6 KO mice which had been given 3 doses of TPA at 48-hour intervals. TPA treatment led to a marked increase in the cellularity of the draining inguinal nodes of both WT and KO mice when compared with controls treated with acetone alone (Figure 3.18). This peaked at four days after the last application, but at all times from day 1-6, more cells were obtained from the nodes of TPA-treated KO mice than from their WT counterparts, with the differences reaching statistical significance at both 2 and 6 days (Fig 3.18). There were no differences in the numbers of cells obtained from WT and KO control nodes (Fig 3.18).

To determine which particular leukocyte population(s) accounted for the increased cellularity of the nodes, the cells were analysed by flow cytometry for expression of CD4, CD8, CD25, B220, CD11c, MHCII and F4/80. Overall, there were no consistent

differences between the individual subsets in the lymph nodes of TPA-treated KO and WT mice (Figure 3.19) suggesting that the increased total cellularity noted in the KO nodes was not due to selective expansion of any one individual cell type.

To explore further the increased cellularity in D6 KO nodes, I measured the proliferative responses of cells harvested from TPA-painted WT and KO mice when stimulated with Con A, anti-CD3 or LPS (Fig. 3.20). Cells from KO mice proliferated better in response to anti-CD3 stimulation compared with WT cells 4 days post TPA application (Fig. 3.20 A), although the differences were not statistically significant. There were no differences in proliferation between WT and KO cells following culture in medium alone or with LPS, but KO cells showed significantly higher proliferative responses to Con A than their WT counterparts at both 4 and 6 days following TPA-treatment (Figures 3.20 A and B). These effects were most marked in TPA-treated mice, although they were also seen in acetone treated control mice. The data thus correlated with my earlier experiments examining mitogen responses, in which I found D6 KO lymphocytes to be more responsive to Con-A induced proliferation (Section 3.2, Figures 3.3 – 3.7).

In conclusion, TPA-treated D6-deficient mice showed a tendency to have intense lymphadenopathy compared to WT counterparts. This did not appear to be due to the selective expansion of any particular cell type, but was associated with increased proliferation rates *in vitro* following stimulation with mitogen.

Summary

In this chapter, I conducted an initial characterisation of the immune system of D6 KO animals, comparing the leukocyte composition of lymphoid tissues from WT and D6-deficient mice and the ability of cells isolated from these tissues to respond to mitogen. Higher proportions of $F4/80^+$ and $CD11c^+$ cells were retrieved from the spleens of D6 KO animals compared with WT, suggesting a possible role for D6 in controlling myeloid cell population of this site. While the absence of D6 did not appear to alter the lymphocyte composition of spleen or lymph nodes, compared with WT counterparts, D6 KO lymphocytes showed increased proliferative responses following culture with Con A or LPS. Immunisation of WT and KO mice with OVA/CFA revealed a reduction or enhancement of *in vitro* responses and serum antibody production, depending on the particular time-point analysed, suggesting that these animals might differ kinetically in their ability to generate antigen-specific immune responses. Finally, TPA-induced skin inflammation was found to be associated with increased cellularity of draining lymph nodes in D6 KO mice, as well as elevated proliferative responses of KO lymph node cells in response to mitogen. Taken together, these data provided preliminary evidence that D6 may be involved in the steady state population of lymphoid tissues and in regulating the amplitude of inflammatory immune responses.

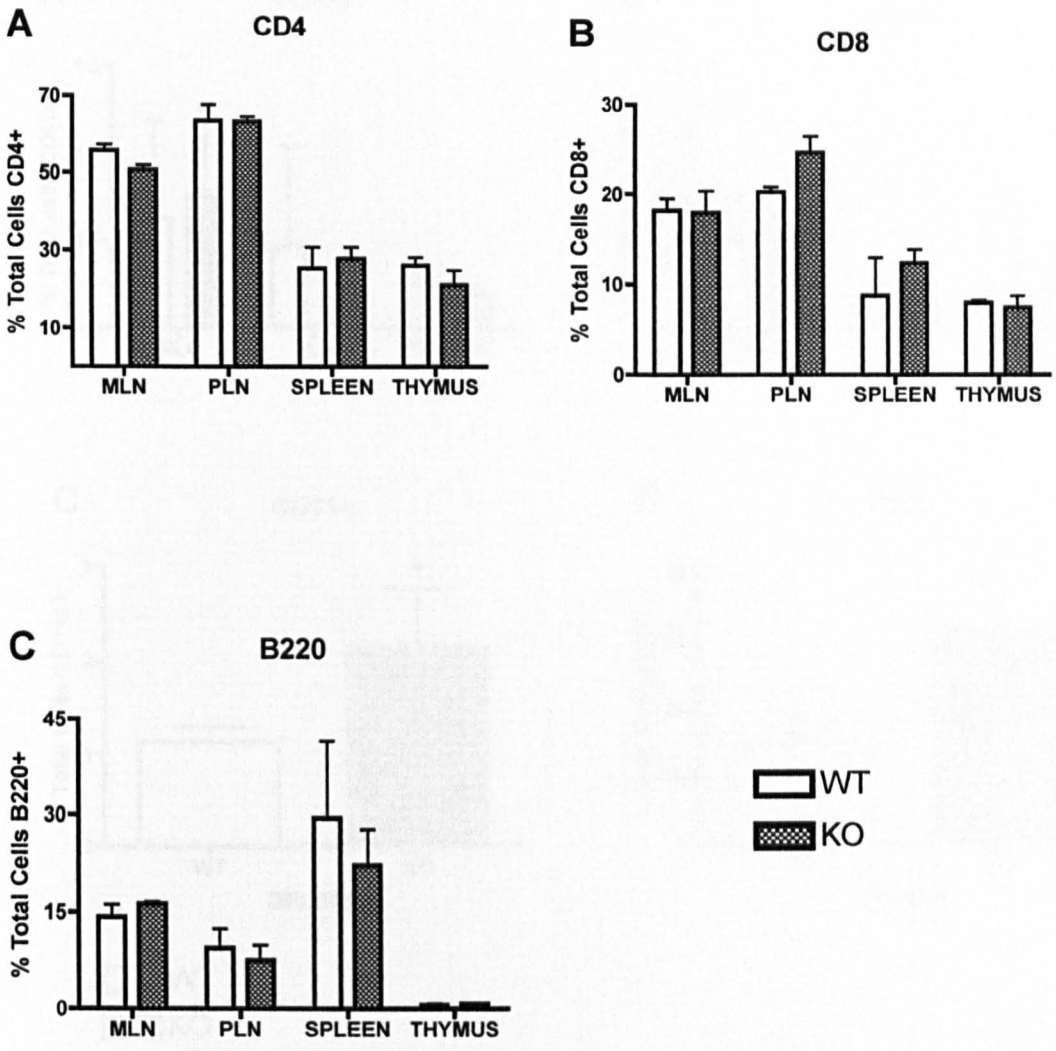


Figure 3.1

Cellular Composition of D6 KO Lymphoid Tissues

Single cell suspensions obtained from MLN, PLN, spleen and thymus of WT and D6 KO animals were analysed by flow cytometry for the relative proportions of (A) CD4+, (B) CD8+ and (C) B220+ cells. Dead cells were excluded by PI-staining. Results represent 3 animals per group and are presented as means + 1 SD . Similar results were obtained from two additional experiments.

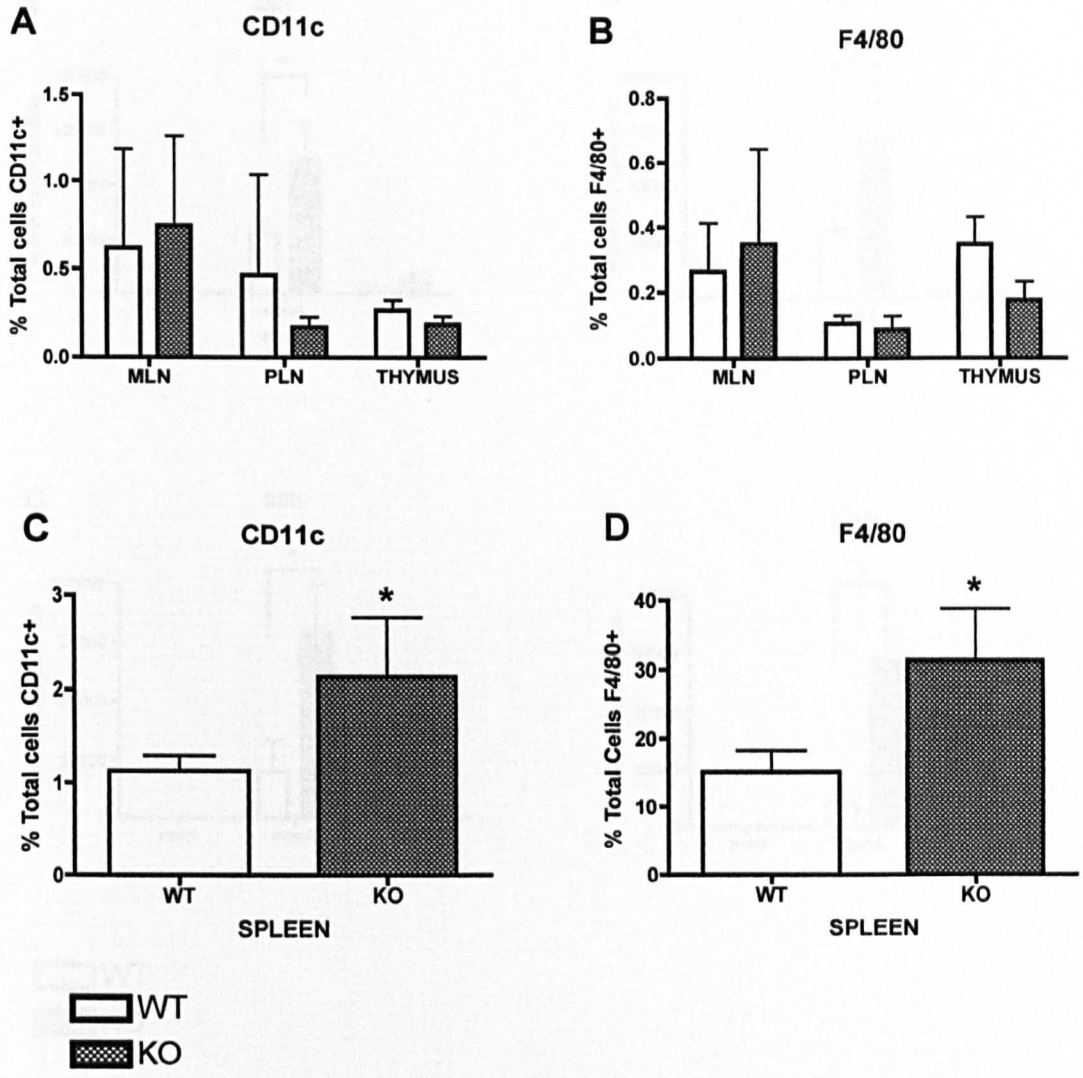


Figure 3.2

Cellular Composition of D6 KO Lymphoid Tissues

Single cell suspensions obtained from MLN, PLN, and thymus (A, B) and spleen (C, D) of WT and D6 KO animals were analysed by flow cytometry for the relative proportions of CD11c+ and F4/80+ cells. Dead cells were excluded by PI-staining. Results represent 3 animals per group and are presented as means + 1 SD. Similar results were obtained from two additional experiments.

* $p < 0.05$

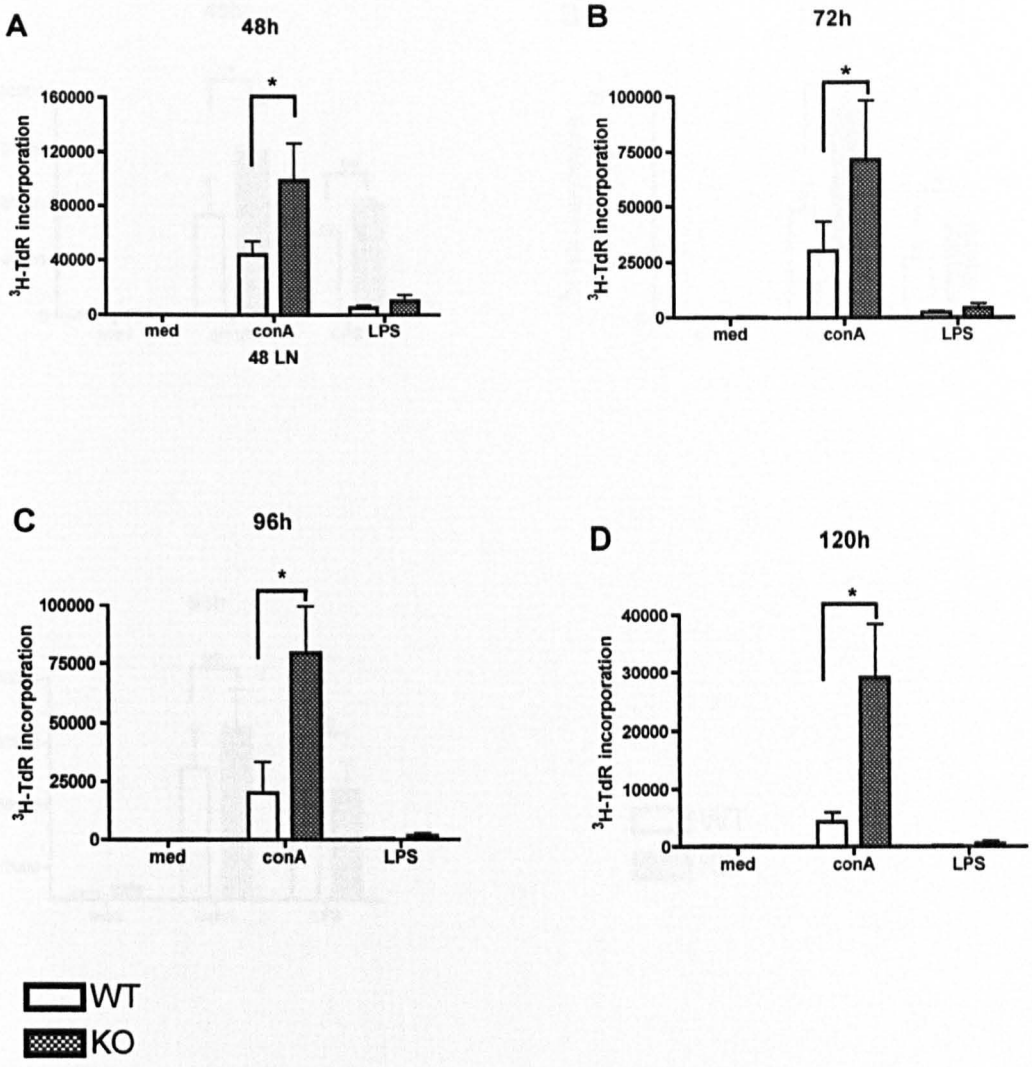


Figure 3.3

Proliferative Responses of D6 KO Lymph Node Cells

Single cell suspensions from peripheral lymph nodes of WT and D6 KO animals were cultured in medium ± 5 μg/ml Con A or 1 μg/ml LPS for 48-120 hr and levels of proliferation determined by ^{3}H-TdR incorporation. Similar results were obtained from two experiments. Results represent the mean of 3 animals per group + 1 SD.

* $p < 0.05$

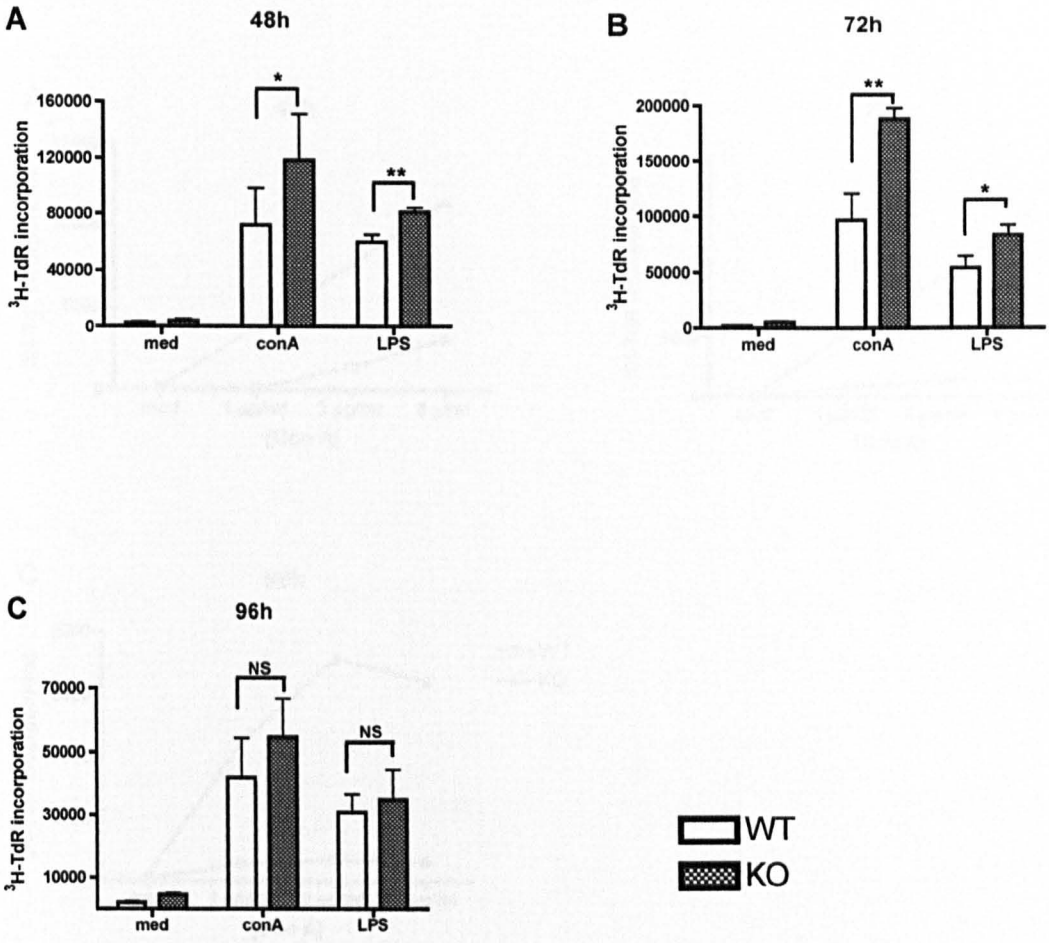


Figure 3.4

Proliferative Responses of D6 KO Splenocytes

Single cell suspensions from spleens of WT and D6 KO animals were cultured in medium ± 5 μg/ml Con A or 1 μg/ml LPS for 48-96 hr and levels of proliferation determined by ^{3}H-TdR incorporation. Similar results were obtained from two experiments. The data represent the mean of 3 animals per group + 1 SD.

* $p < 0.05$, ** $p < 0.001$, NS = not significant

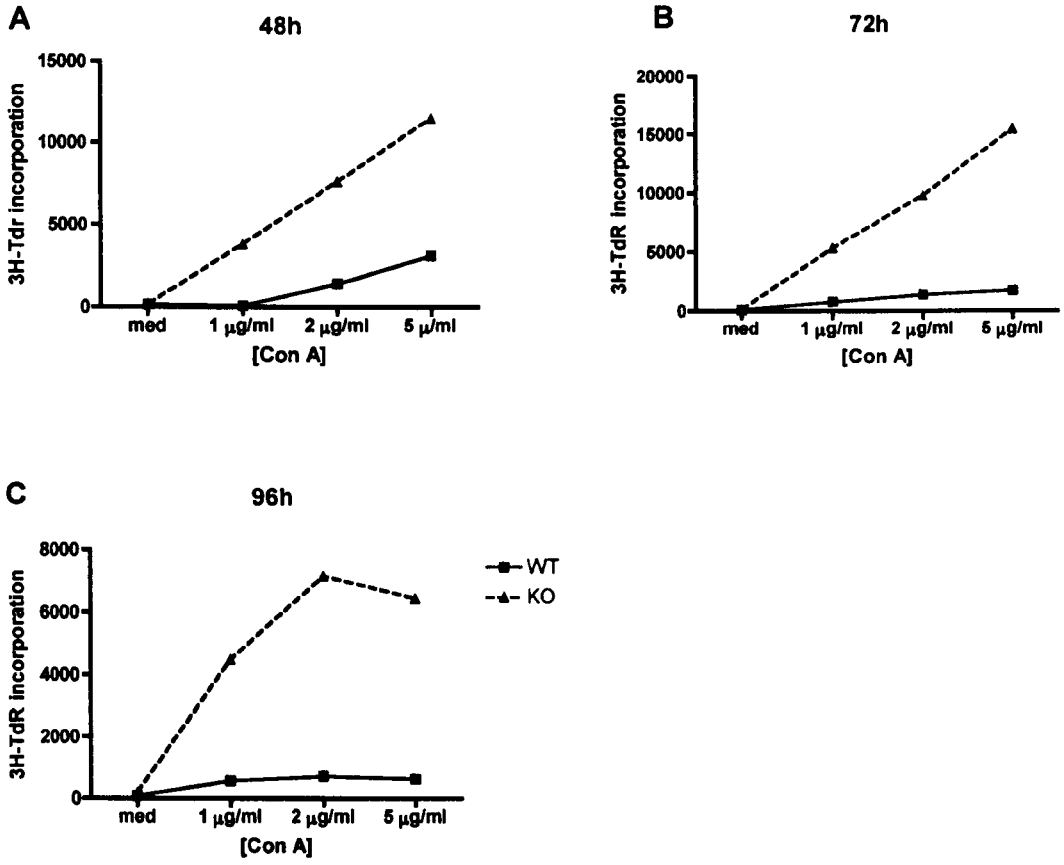


Figure 3.5

Dose Response of Peripheral LN Cells Stimulated With Con A

Single cell suspensions obtained from peripheral lymph nodes of a single WT mouse and a single D6 KO animal were cultured in medium ± 1, 2 or 5 µg/ml of Con A for 48-96 hr and levels of proliferation determined by ^{3}H-TdR incorporation.

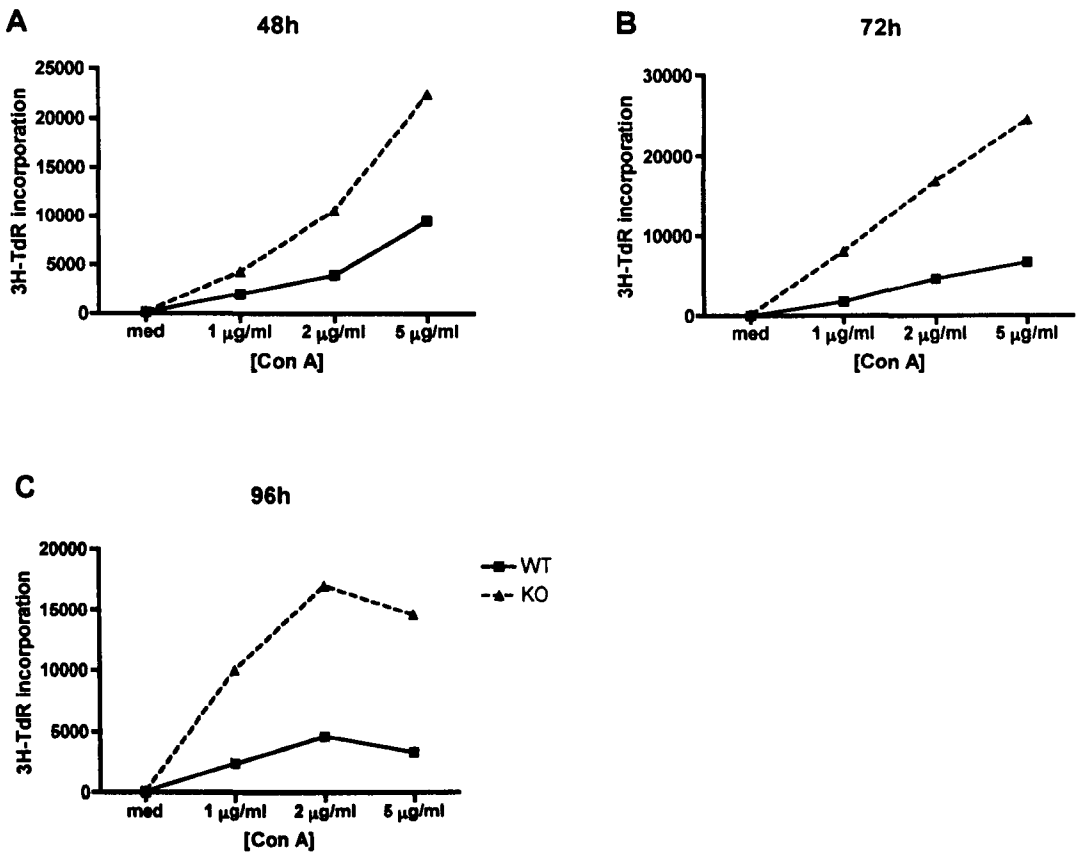


Figure 3.6

Dose Response of MLN Cells Stimulated With Con A

Single cell suspensions obtained from mesenteric lymph nodes of a single WT mouse and a single D6 KO animal were cultured in medium ± 1, 2 or 5 µg/ml of Con A for 48-96 hr of culture and the levels of proliferation determine by ^{3}H-TdR incorporation.

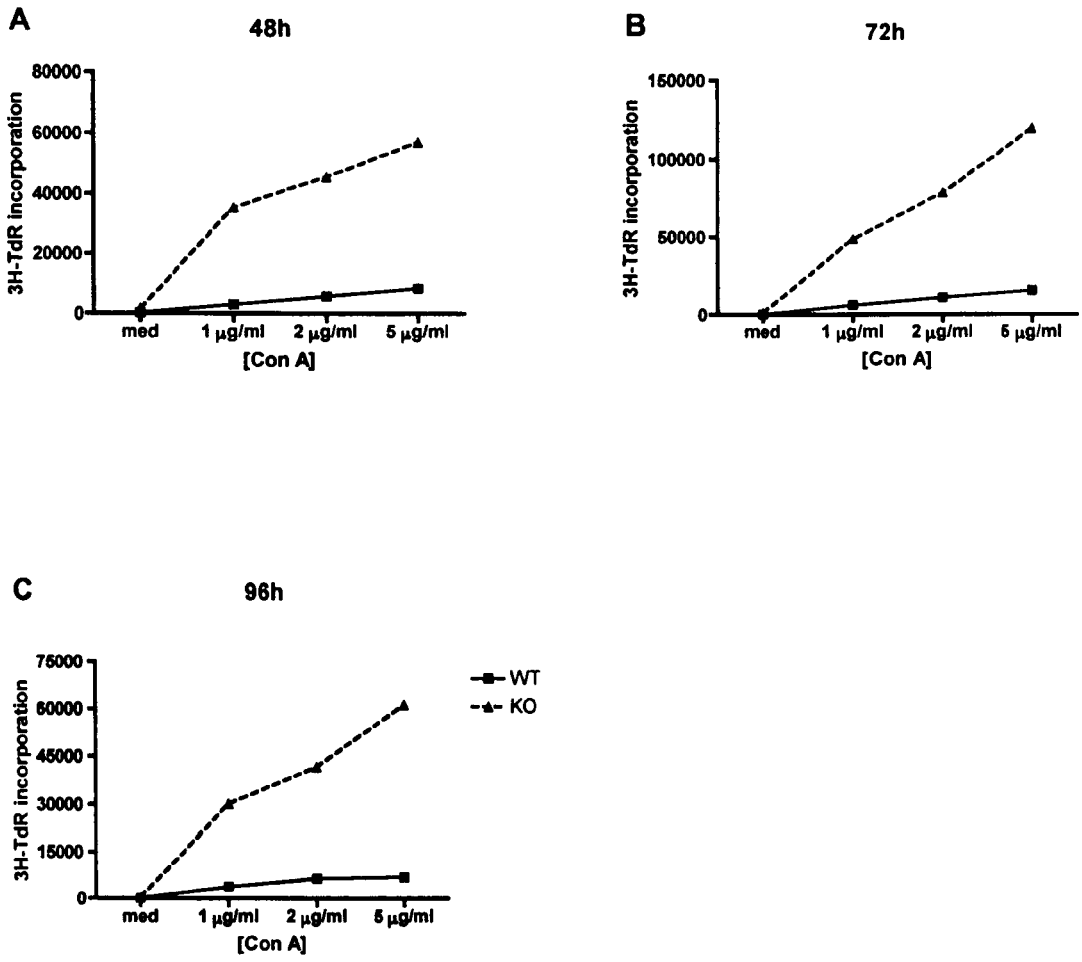


Figure 3.7

Dose Response of Spleen Cells Stimulated With Con A

Single cell suspensions obtained from the spleens of a single WT mouse and a single D6 KO animal were cultured in medium ± 1, 2 or 5 µg/ml of Con A for 48-96 hr and levels of proliferation determined by ^{3}H-TdR incorporation.

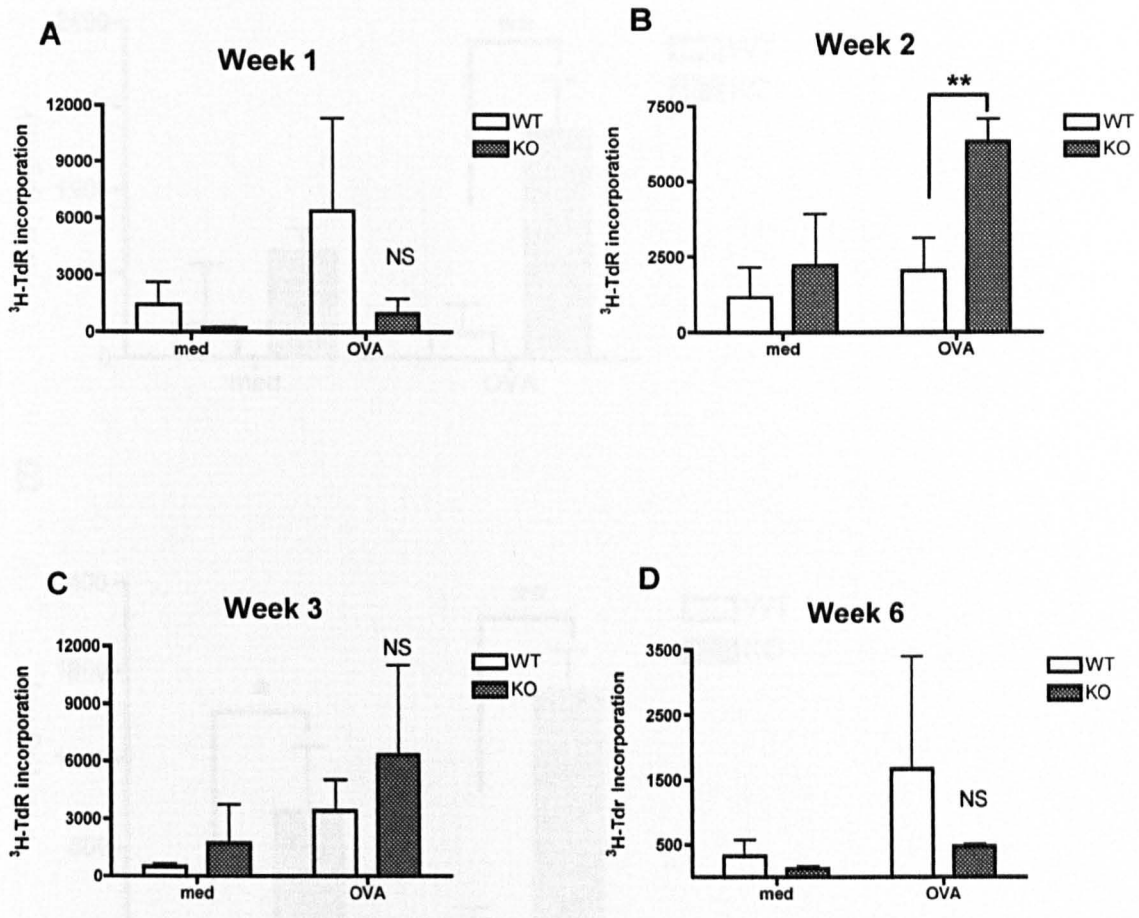


Figure 3.8

Priming of Antigen-Specific Proliferative Responses In D6 KO Mice

WT and D6 KO mice were immunised in the footpad with OVA/CFA and 1 (A), 2 (B), 3 (C) and 6 (D) weeks later, draining popliteal lymph node cells were cultured in medium ± OVA (1 mg/ml) for 120 hours before assessment of ^{3}H-TdR incorporation. The results are from an individual experiment and are the means + 1 SD of 3-4 animals per group.

** $p < 0.001$, NS = not significant

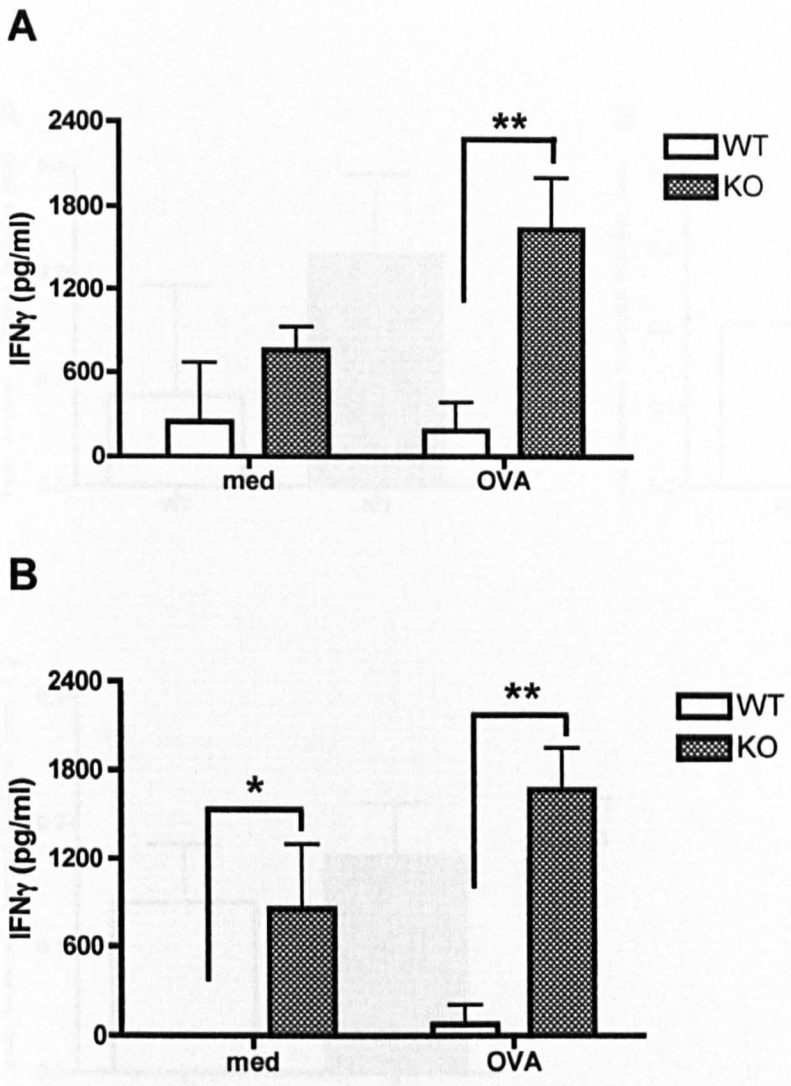


Figure 3.9

Priming of Antigen-Specific IFNγ production in D6 KO mice

WT and D6 KO mice were immunised in the footpad with OVA/CFA, and 2 (A) and 3 (B) weeks later, draining popliteal lymph node cells were cultured in medium ± OVA (1 mg/ml) for 48 hr and levels of IFNγ in culture supernatants were determined by specific ELISA. Results are from an individual experiment and are the mean + 1 SD of 3-4 animals per group.

* $p < 0.05$, ** $p < 0.001$

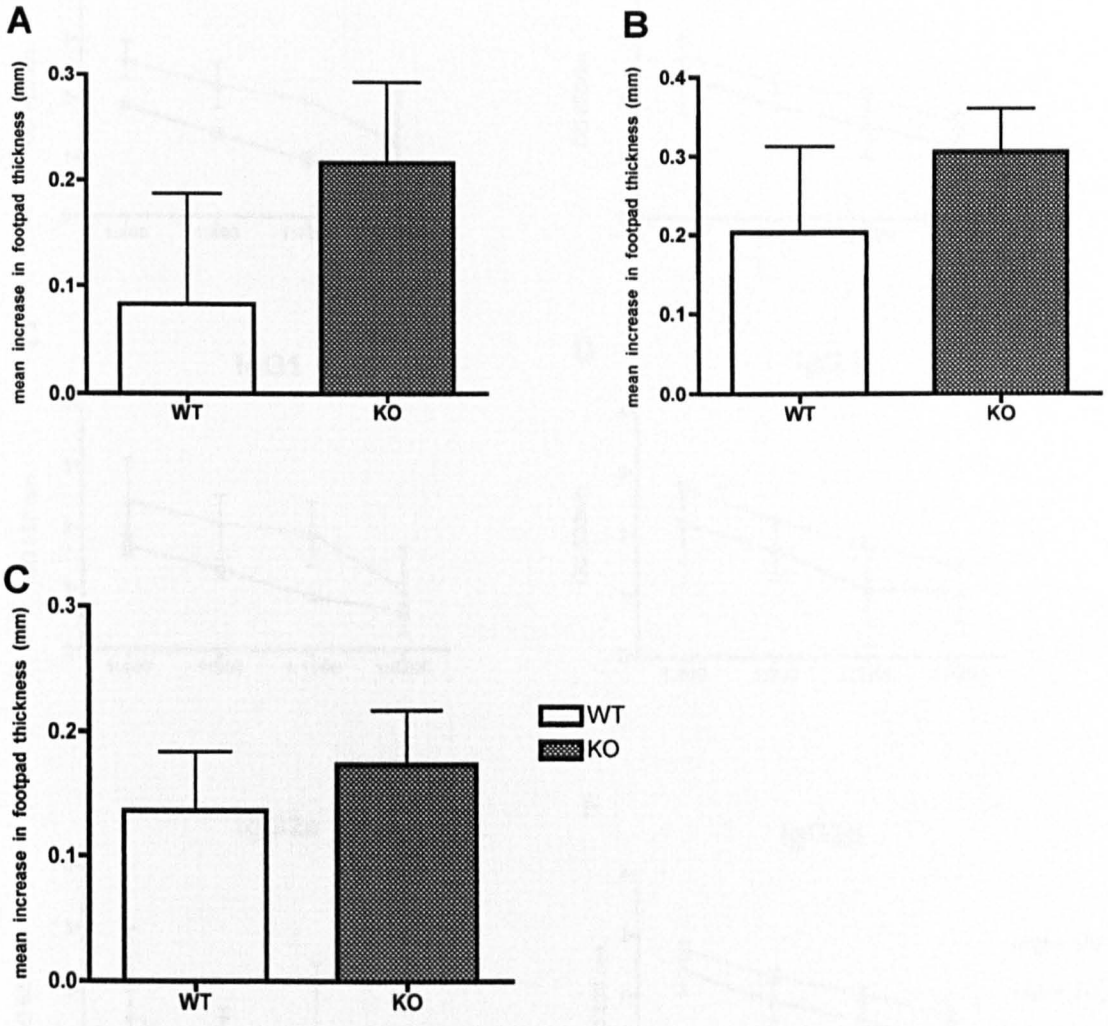


Figure 3.10

Priming of antigen-specific DTH Responses in D6 KO Mice

WT and D6 KO mice were immunised in the footpad with OVA/CFA and re-challenged 2 (A), 3 (B), and 6 (C) weeks later with heat-aggregated OVA. The mean increase in footpad thickness was measured 24h later. Results are from an individual experiment and are the mean + 1 SD of 3-4 animals per group.

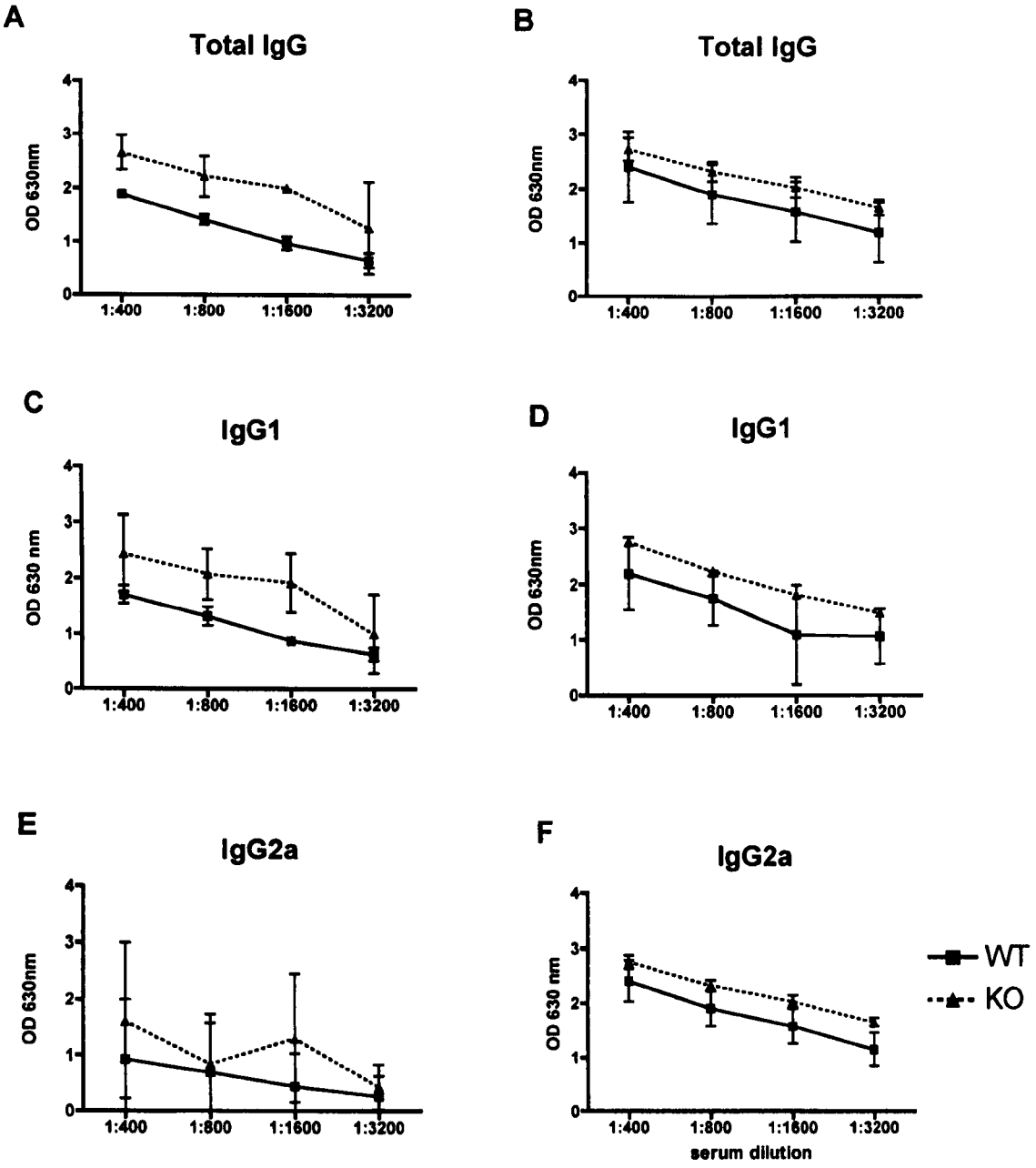


Figure 3.11

Priming of antigen-specific antibody responses in D6 KO Mice

WT and D6 KO mice were immunised in the footpad with OVA/CFA, and 2 (A, C, E) and 3 (B, D, F) weeks later, serum levels of OVA-specific IgG (A, B), IgG1 (C, D), and IgG2a (E, F) antibodies were assessed by ELISA. The data represent the mean + 1 SD of 3-4 animals per group.

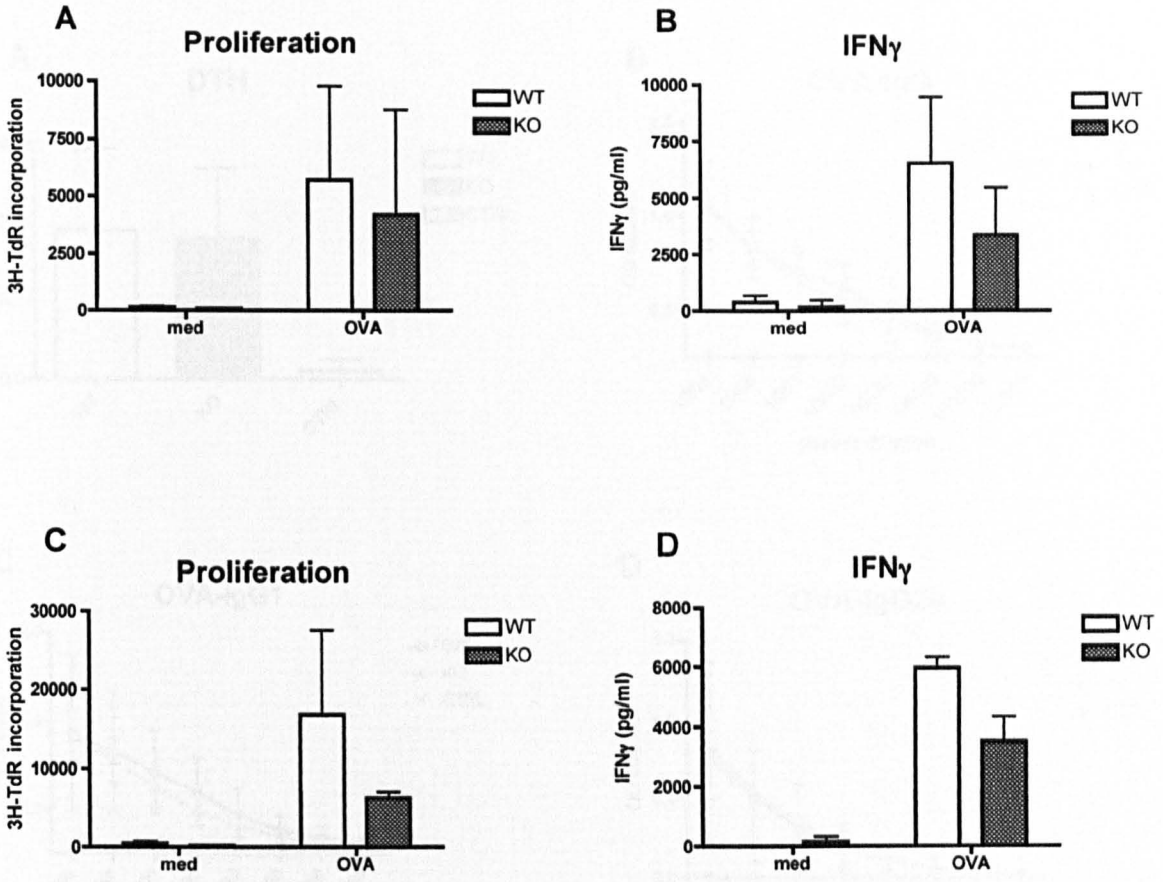


Figure 3.12

Priming of Antigen-Specific Proliferation and IFNγ Production in D6 KO Mice

WT and D6 KO mice were immunised in the footpad with OVA/CFA and 1 (A, B) and 2 (C, D) weeks later, draining popliteal lymph node cells were cultured in medium ± OVA (1 mg/ml) for 120 hours before assessment of ^{3}H-TdR incorporation (A, C). Culture supernatants were harvested at 48h and the levels of IFNγ determined by ELISA (B, D). Results represent the mean + 1 SD of 3-4 animals per group.

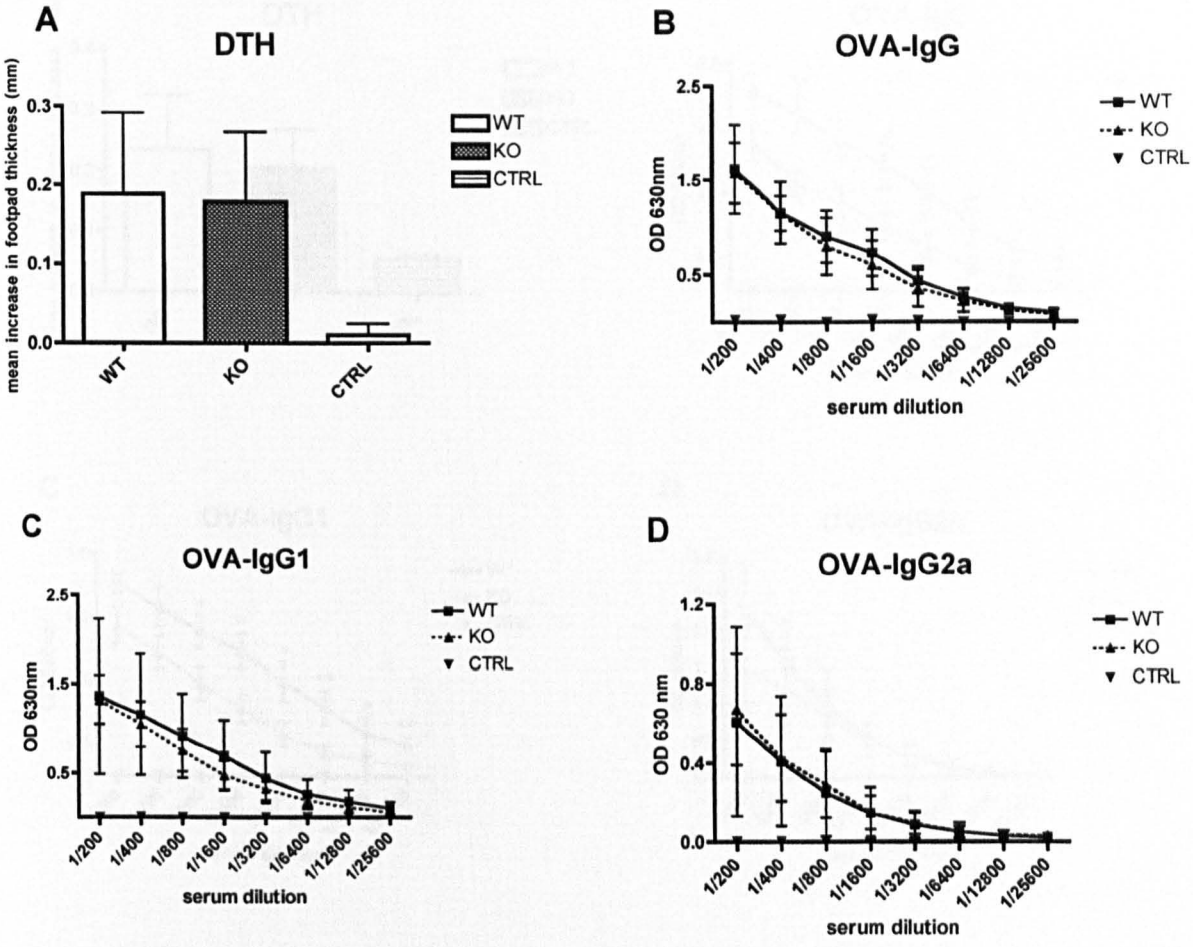


Figure 3.13

Priming of antigen-specific DTH and Antibody Responses in D6 KO Mice

WT and D6 KO mice were immunised in the footpad with OVA/CFA and challenged one week later with heat-aggregated OVA (HAO). The mean increase in footpad thickness was measured 24 hr later and serum levels of OVA-specific IgG, IgG1 and IgG2a antibodies were determined by ELISA. The control group represents unimmunised animals that were injected with HAO to calculate non-specific footpad swelling. Results shown are the means + 1 SD of 3-4 animals per group.

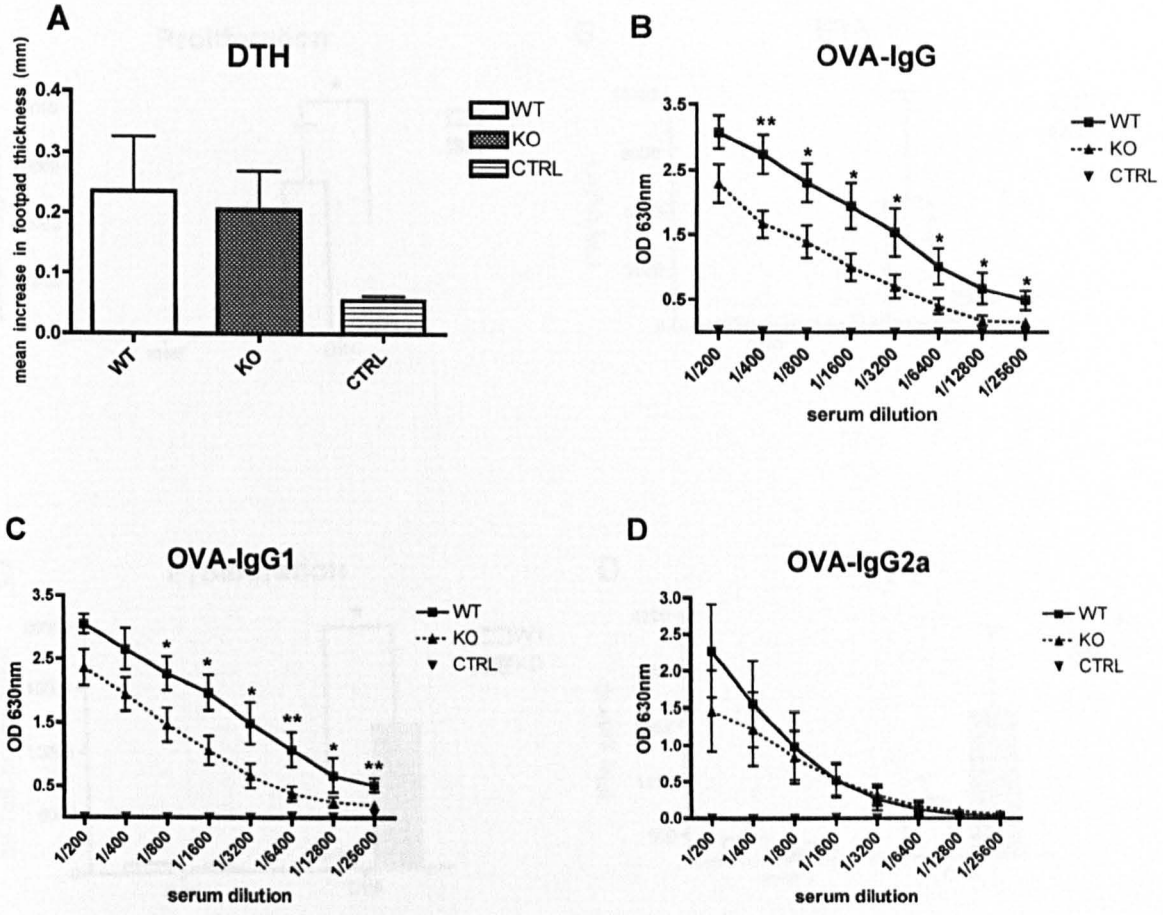


Figure 3.14

Priming of DTH and Antibody Responses in D6 KO Mice

WT and D6 KO mice were immunised in the footpad with OVA/CFA and challenged two weeks later with heat-aggregated OVA. The mean increase in footpad thickness was measured 24 hr later and serum levels of OVA-specific IgG, IgG1 and IgG2a antibodies were determined by ELISA. The control group represents unimmunised animals that were injected with HAO to calculate non-specific footpad swelling. Results shown are the means + 1 SD of 3-4 animals per group.

* $p < 0.05$, ** $p < 0.01$

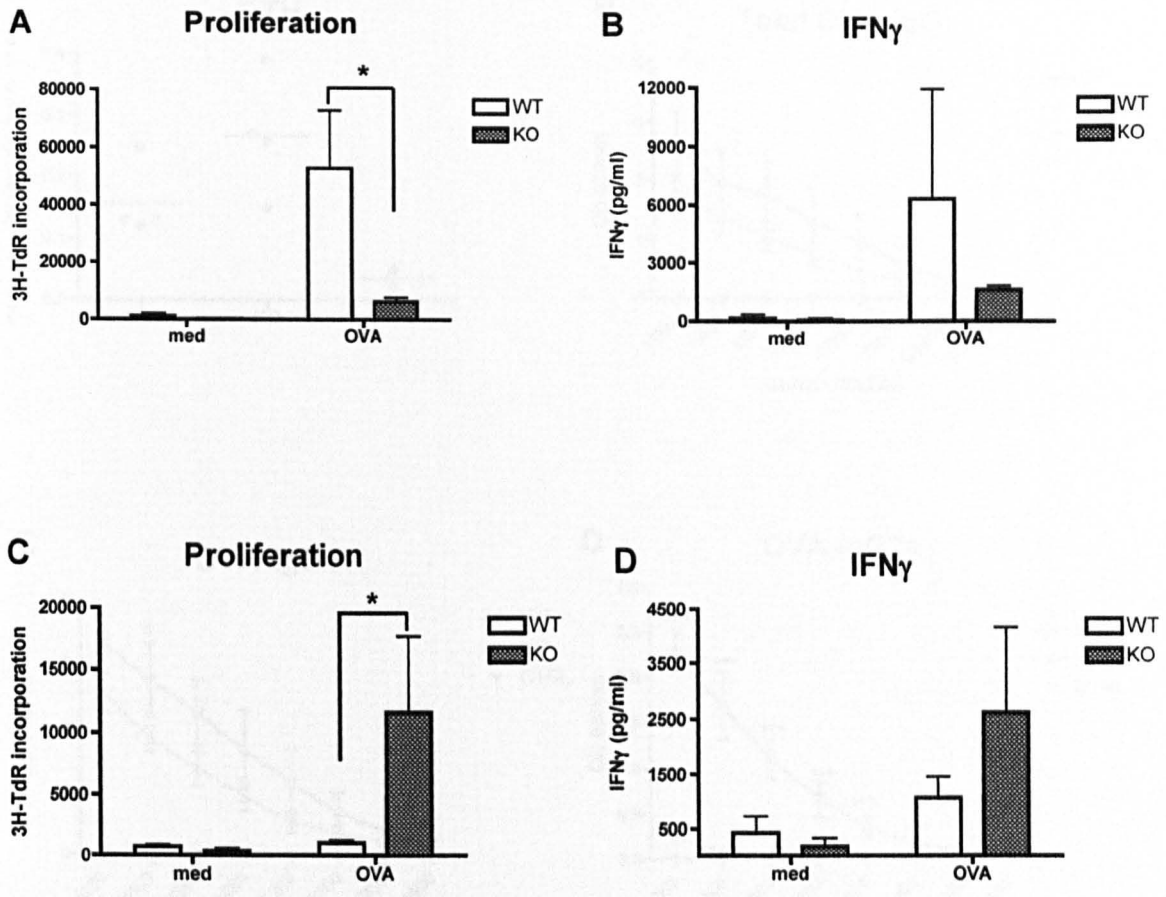


Figure 3.15

Priming of Proliferation and IFNγ Responses in DBA/2 D6 KO Mice

WT and D6 KO animals on a DBA/2 genetic background were immunised in the footpad with OVA/CFA and 1 (A, B) and 2 (C, D) weeks later, draining popliteal lymph nodes cells were cultured in medium ± OVA (1 mg/ml) for 120 hours and the levels of proliferation determined by ^{3}H-TdR incorporation. Culture supernatants were harvested at 48h and IFNγ levels were measured by ELISA. Results are the means + 1 SD of 3 mice per group.

* $p < 0.05$

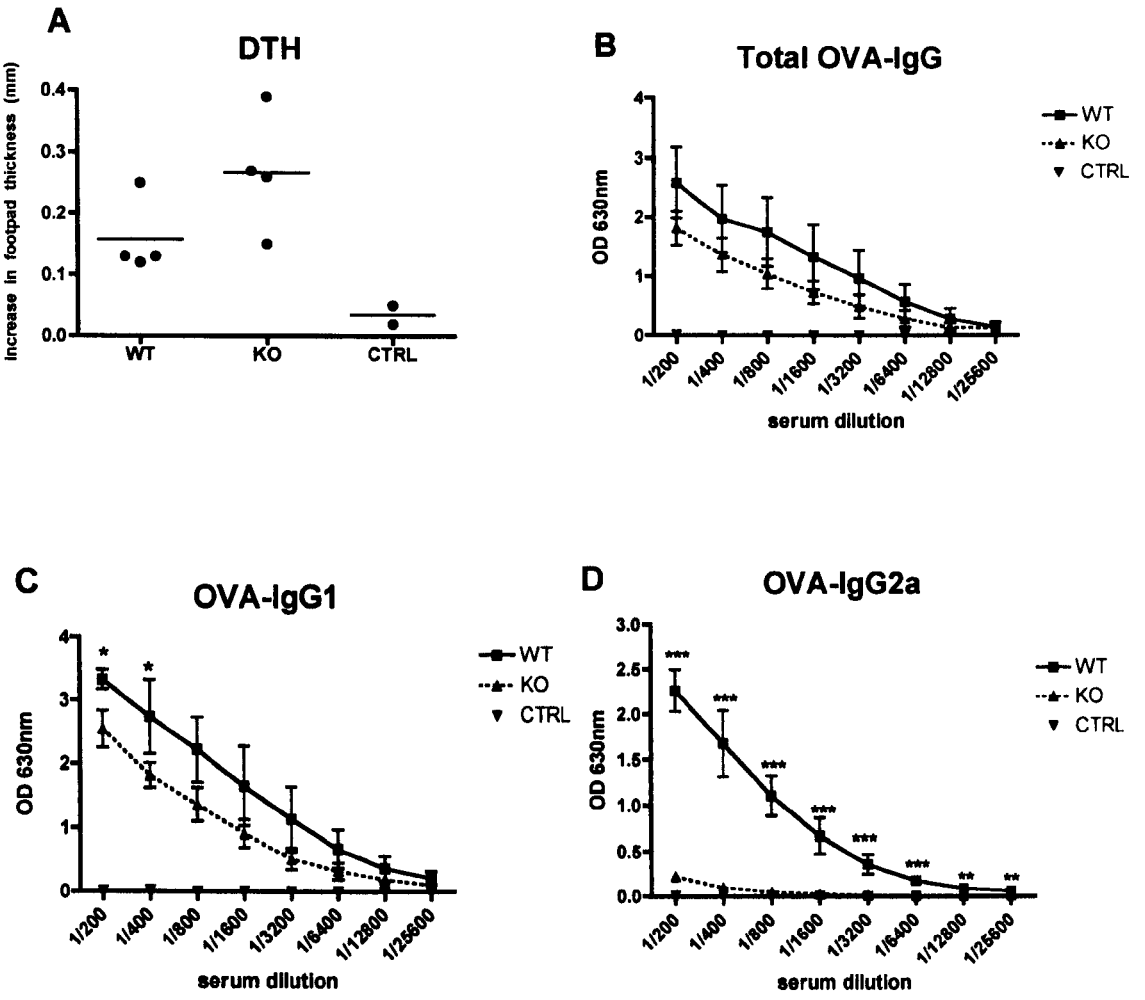


Figure 3.16

Priming DTH and Antibody Responses in DBA/2 D6 KO Mice

WT and D6 KO mice on a DBA/2 genetic background were immunised in the footpad with OVA/CFA and challenged one week later with heat-aggregated OVA. The mean increase in footpad thickness was measured 24hr later (A) and serum levels of OVA-specific IgG (B), IgG1(C) and IgG2a (D) antibodies were assessed by ELISA. The control group represents unimmunised animals that were injected with HAO to calculate non-specific footpad swelling. Results are the mean + 1 SD of 4 mice per group.

* $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$

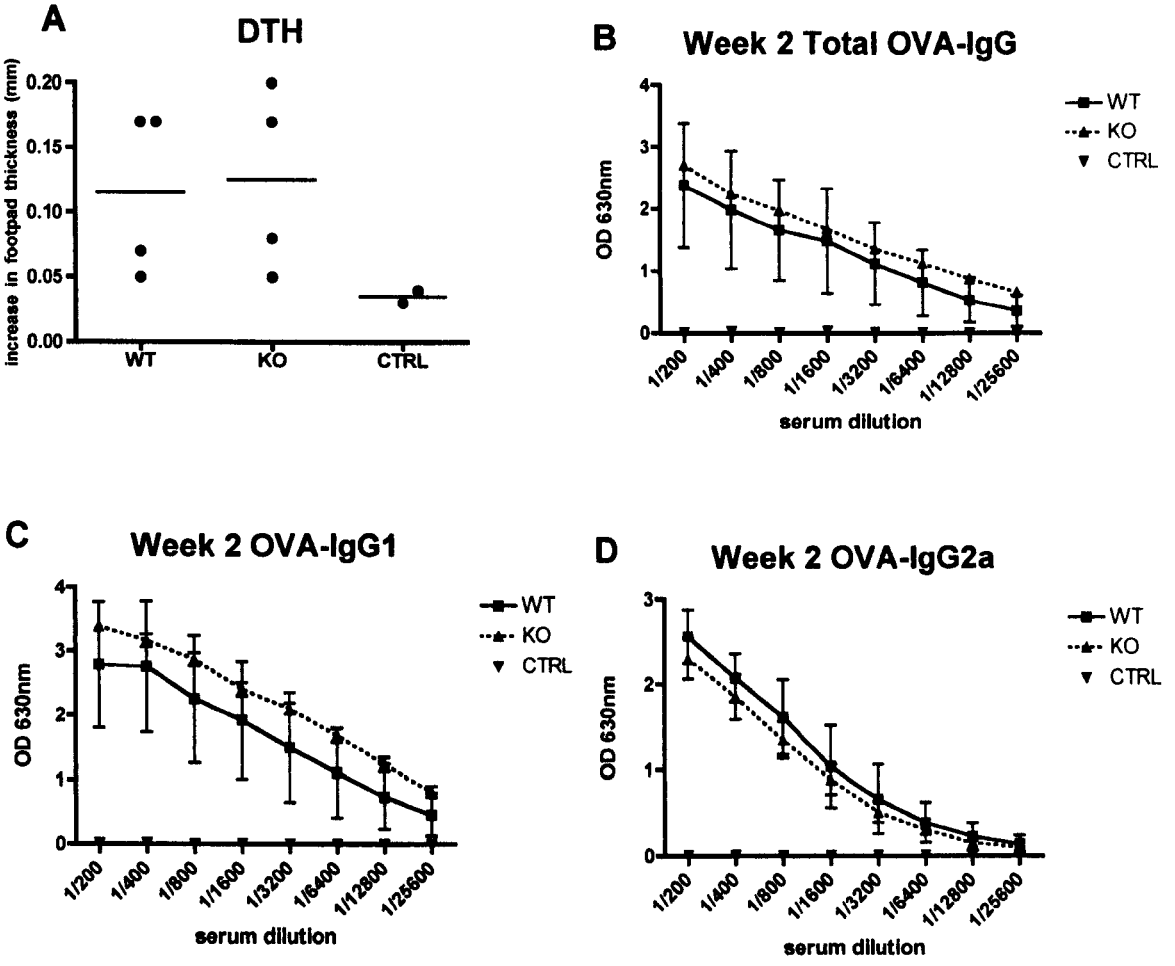


Figure 3.17

Priming DTH and Antibody Responses in DBA/2 D6 KO Mice

WT and D6 KO mice on a DBA/2 genetic background were immunised in the footpad with OVA/CFA and challenged two weeks later with heat-aggregated OVA. The mean increase in footpad thickness was measured 24hr later (A) and serum levels of OVA-specific IgG (B), IgG1(C) and IgG2a (D) antibodies were assessed by ELISA. The control group represents unimmunised animals that were injected with HAO to calculate non-specific footpad swelling. Results are the mean + 1 SD of 4 mice per group.

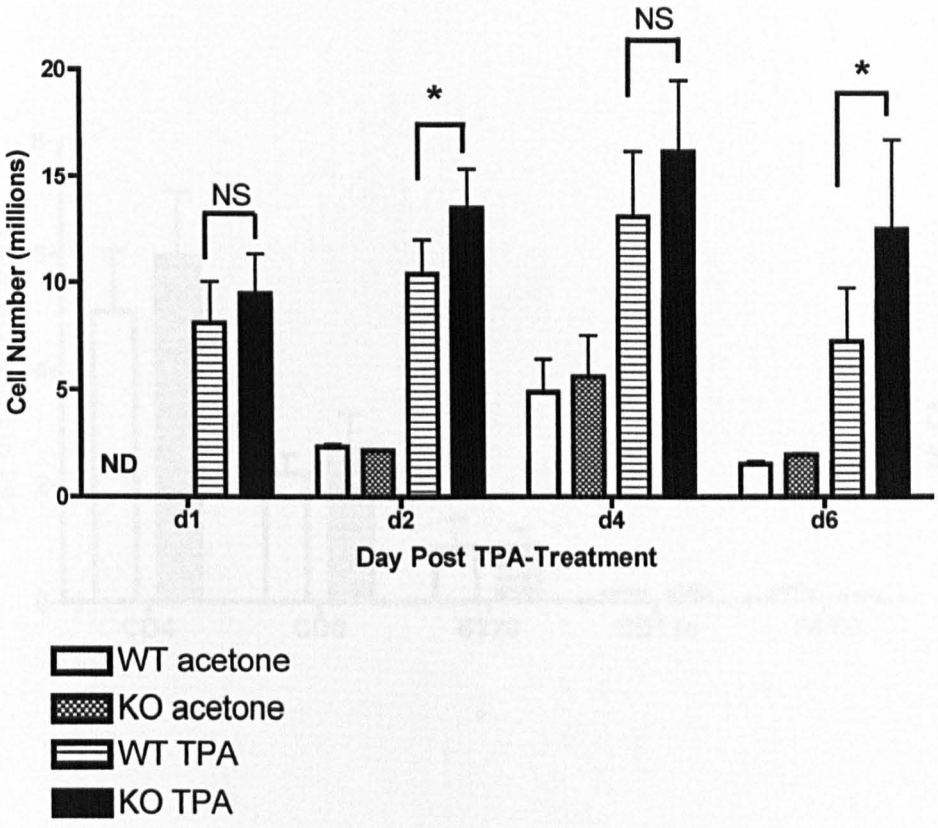


Figure 3.18

Lymph Node Cellularity in TPA-Treated D6 KO Mice

Single cell suspensions were obtained from the inguinal lymph nodes of WT and D6 KO mice that had received 3 topical applications of TPA (50μM solution in acetone) and from controls that were treated with acetone alone. The results represent the mean viable cell counts for 5 (TPA) or 2-3 animals per group (control) + 1 SD.

* $p < 0.05$
NS = not significant
ND = not determined

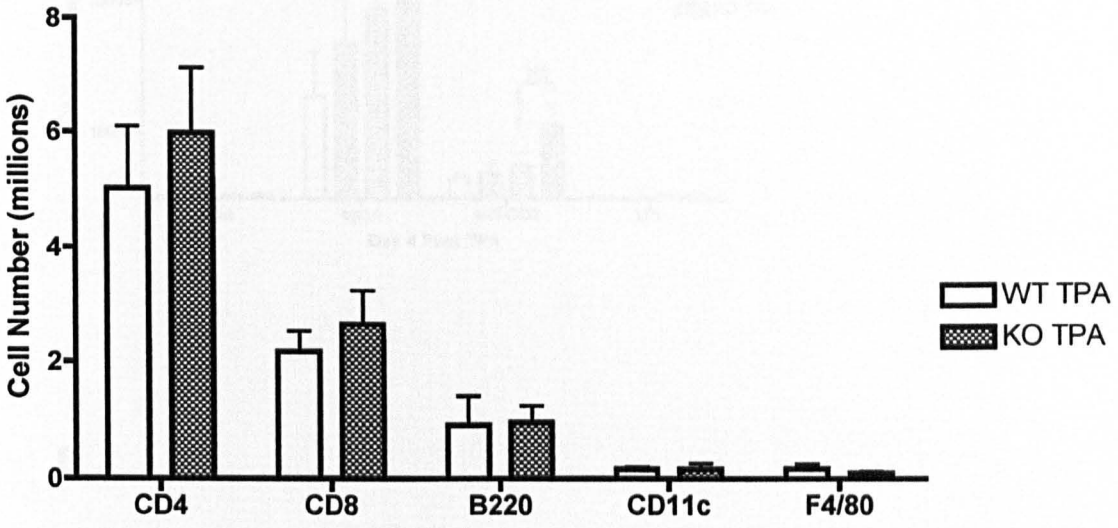


Figure 3.19

Cellular Composition of Lymph Nodes of TPA-Treated D6 KO Mice

WT and D6 KO mice received 3 topical applications of TPA and 48h later single cell suspensions were made from the draining inguinal lymph nodes. The proportions of CD4+, CD8+, B220+, CD11c+ and F4/80+ cells were determined by flow cytometry and the results represent the means + 1 SD for 5 mice per group.

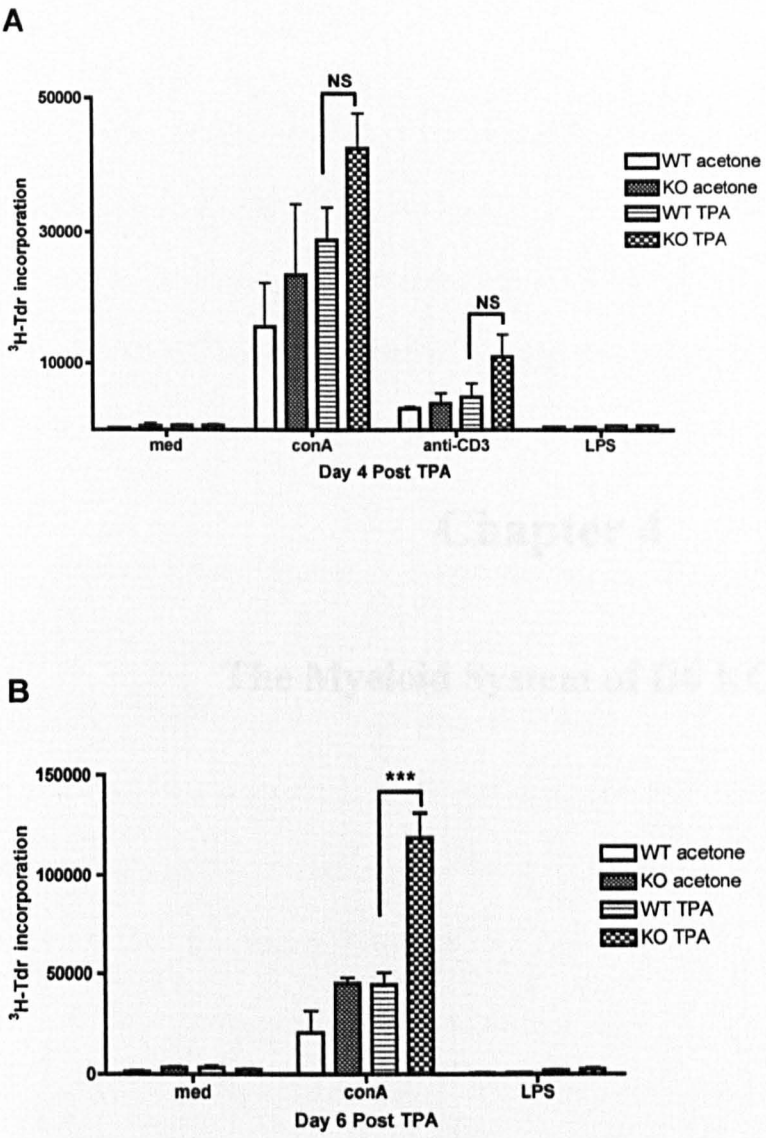


Figure 3.20

Mitogen-Induced Proliferative Responses of Lymph Node Cells from TPA-Treated Mice

WT and D6 KO mice received 3 topical applications of TPA and at 4 (A) and 6 (B) days later, draining inguinal lymph node cells were stimulated, in vitro, in medium ± 5μg/ml Con A or 1μg/ml LPS for 72 hr. The results respresent the mean incorporation of ^{3}H-TdR for 4-5 animals per group (TPA) or 2-3 animals per group (acetone controls) + 1 SD.

*** $p < 0.001$, NS = not significant

Chapter 4

The Myeloid System of D6 KO Mice

Introduction

My initial characterisation studies of the D6 KO mice revealed minor changes in the proportions of $F4/80^+$ and $CD11c^+$ cells in the spleen under steady state conditions and had also pointed to deviations in the numbers of $F4/80^+$ cells in lymphoid organs draining the inflamed skin (See Chapter 3, Figure 3.3). These data suggested that D6 could play a role in the development, recruitment and/or functions of myeloid lineage cells.

The peritoneal cavity houses a large macrophage population that is essential for maintaining the sterility of this compartment (307). D6 ligands have been shown to play critical roles in both the recruitment and activation of cells to this site during inflammation (47, 136, 137, 155, 308). Thus to further explore a role for D6 in myeloid responses, I decided to analyse the cellular composition of the peritoneum of D6 KO mice, both under normal conditions and during inflammation.

4.1 Myeloid and Lymphoid Populations in Resting Peritoneum of D6 KOs

Peritoneal cells were isolated from untreated WT and KO mice by lavage and assessed for the relative proportions of macrophages, dendritic cells, T cells, B cells and neutrophils using flow cytometry. As shown in Figure 4.1 A, comparable total cell yields were retrieved from WT and KO peritoneum and there were no differences between WT and D6 KO PEC in the proportions of $CD4^+$, $CD8^+$, $CD11c^+$ and $B220^+$ cells (Fig. 4.1 B). However, higher proportions of $F4/80^+$ cells were detected in the PEC of KO mice compared with WT controls (Fig. 4.1 B). Further analysis revealed that this was due to a specific increase in an $F4/80^{hi}$ subpopulation in D6 KO mice, while there were similar

numbers of cells expressing lower levels of F4/80 (Fig. 4.2). This alteration was similar to the increase in $F4/80^+$ cells I observed in the spleen of D6 KO mice (See Chapter 3).

4.2 Inflammatory Infiltrates in Peritoneal Exudates

Next, to explore the effect of D6 on myeloid cell responses in more detail, I employed a model of sterile peritonitis induced by thioglycollate injection. Following thioglycollate administration, monocytes are recruited in a CCR2-dependent fashion to the peritoneum, where they differentiate into macrophages. Roles for several other inflammatory CC-chemokines have also been demonstrated in murine models of peritonitis (47, 136, 137, 155, 308). Thus it seemed possible that altered levels of CC-chemokines, such as CCL2, resulting from absence of D6 scavenging function, could lead to abnormal recruitment of inflammatory cells to the KO peritoneum.

Peritoneal administration of thioglycollate induced a large inflammatory infiltrate which peaked at 24hr and the total numbers of cells recruited were not significantly different between WT and KO mice at any of the time points analysed (Fig 4.3). As others have shown (309), thioglycollate injection induced a significant influx of neutrophils and $F4/80^+$ monocytes/macrophages to the peritoneal cavities of WT and KO mice at 24h post challenge (Fig. 4.4 A). However, by later times (48h-96h) isolated PEC comprised primarily $F4/80^+$ cells (Fig. 4.4 B-D). In contrast to the $F4/80^+$ cells in resting PEC, which were mostly $F4/80^{hi}$ (Fig. 4.1), the vast majority of $F4/80^+$ cells which appeared at later times in the inflamed peritoneum demonstrated low to intermediate expression of this surface antigen (Fig. 4.5). Although there was a slight increase in $F4/80^+$ cells in the D6 KO PEC harvested at 24-48 hours, this was not seen later and, overall, there were no

statistically significant differences in proportions of total $F4/80^+$ cells or neutrophils in WT and KO PEC at any of the time-points investigated, (Fig. 4.4).

Compared with WT animals, D6 KO mice had an increased proportion of $CD11c^+MHCII^+$ cells in the inflamed peritoneum at all time points after thioglycollate administration (Fig. 4.4 and 4.6). Further analysis indicated that the majority of these $CD11c^+MHCII^+$ cells also expressed F4/80 (Fig. 4.7). Thus the absence of D6 appeared to alter myeloid cell sub-populations during peritoneal inflammation, with D6 KO mice exhibiting an increase in $F4/80^+CD11c^+$ cells and an accompanying decrease in the numbers of $F4/80^+CD11c^-$ cells, which was most obvious at 72 and 96h post-injection (Fig. 4.7). However, by 96h after thioglycollate challenge, there was also a small but significant increase in the $CD11c^+F4/80^-$ population in the KO PEC compared with WT PEC (Fig 4.7 D).

Closer examination of PEC indicated that at least five different cell populations could be defined in terms of their intensity of CD11c and F4/80 expression (Figure 4.8). These were therefore divided PEC into $F4/80^{int}$ $CD11c^-$, $F4/80^{int}$ $CD11c^{int}$, $F4/80^{int}$ $CD11c^{hi}$, $F4/80^-$ $CD11c^+$ and $F4/80^-$ $CD11c^-$ subsets, all of which expressed CD11b. I therefore compared the relative proportions of each of these populations in WT and KO peritoneum following thioglycollate injection.

As shown in figure (4.8 A), the proportion of $F4/80^-CD11c^-$ cells present in both WT and KO PEC was greatest at 24h and this decreased sharply at each time point thereafter. Higher proportions of $F4/80^-CD11c^-$ cells were found in WT PEC compared with KO PEC at 24 and 48h after thioglycollate challenge, but by later time-points the proportions of

F4/80$^-$CD11c$^-$ cells present in PEC were equivalent in the two strains. The F4/80^{int} CD11c^{int} population became gradually more prevalent in inflamed peritoneum, increasing steadily from 24h to 96h, but similar proportions of this cell type were detected in both WT and KO exudates (Figure 4.8 B). Thus the increase in F4/80$^+$CD11c$^+$ cells I had detected in the inflamed peritoneum from D6 KO mice appeared to be due to expansion of the F4/80^{int} CD11c^{hi} subset (Figure 4.8 C). Although similar proportions of these F4/80^{int} CD11c^{hi} cells were detected in WT PEC at all times analysed, this population continued to increase in KO PEC and became significantly higher in KO exudates from 48h onwards, being most marked at 96h. Conversely, as shown in Figure 4.8 D, there were lower proportions of F4/80^{int} CD11c$^-$ cells in KO PEC at 72h and 96h post thioglycollate. There were no differences between WT and KO PEC in the frequency of this subset at 24h and 48h time-points. The proportions of F4/80$^-$ CD11c$^+$ cells in PEC (Figure 4.8 E) were generally low (<7%), but by 96h following thioglycollate challenge, a statistically significant increase in this population was noted in KO compared with WT PEC (6.44±0.21 KO vs. 4.09±.71 WT), suggesting that this subset might also contribute to the increased numbers of CD11c$^+$ cells found in KO exudates (Fig 4.8 E).

Further analysis of the different F4/80/CD11c fractions revealed that the majority of the F4/80^{int} CD11c^{int} and F4/80^{int} CD11c^{hi} subpopulations were large, granular cells (Fig 4.9). This was particularly the case for the F4/80^{int} CD11c^{hi} cells, but in both cases their forward scatter and side scatter properties were characteristic of macrophage or DC-like cells. The F4/80^{int} CD11c$^-$ cells were generally smaller and less granular cells, similar to lymphocytes or monocytes, but there was also a population of these cells which had forward and side scatter profiles indicative of eosinophils (310). The F4/80$^-$ CD11c$^+$ and F4/80$^-$ CD11c$^-$

populations both consisted of small, fairly agranular cells, again, likely to be lymphocytes or monocytes (Figure 4.9).

In light of the differences in myeloid cell subsets defined by CD11c and F4/80 in inflamed D6 KO and WT peritoneum, I went back and examined resting PEC for the same markers. Interestingly, the $F4/80^{hi}$ cells that were the predominant F4/80 subset in resting PEC did not co-express CD11c (Figure 4.10). Additionally, very few $F4/80^{lo/int}CD11c^{+}$ cells were detected in resting PEC, and there were no significant differences in these populations between WT and KO mice (figure 4.10). Therefore the $F4/80^{int}$ $CD11c^{int}$ and $F4/80^{int}$ $CD11c^{hi}$ populations appeared to be a specific result of peritoneal inflammation.

Overall, these data suggested that the absence of the D6 chemokine receptor could support increased recruitment and/or differentiation of a large, granular $F4/80^{+}CD11c^{+}$ population in the inflamed peritoneum.

4.3 Functional Comparison of WT and D6 KO PEC

Next, I examined whether the phenotypic differences I had observed in PEC had any functional consequences. Classical macrophage activation is associated with high microbicidal activity and production of pro-inflammatory cytokines: I therefore compared the capacity of WT and KO inflammatory PEC to generate the free radical nitric oxide and various cytokines upon *in vitro* stimulation with LPS and IFNγ (311). WT and KO PEC isolated at 6, 48 and 96 h produced NO following stimulation with LPS and IFNγ, with the greatest generation of NO seen by 96h PEC (Figure 4.11). However, there were no

differences in NO-generation by WT and KO PEC isolated at either 6, 48h or 96 hours following thioglycollate administration (Fig 4.11).

To compare the cytokine and chemokine production of WT and KO infiltrates, cells were isolated from the inflamed peritoneum at 96h following thioglycollate injection, as this was the time-point at which WT and KO PEC appeared to differ most. Cells were stimulated overnight with IFNγ and LPS and the culture supernatants were assessed by Luminex technology, enabling simultaneous analysis of multiple cytokines and chemokines. Supernatants from LPS/IFNγ-stimulated PEC contained substantial amounts of IL-12 p40/p70 and IL-10 as well as low levels of IL-4 and IL-2, but there were no quantitative differences in the amounts produced in WT and KO cultures (Figure 4.12 A - D). As shown in Figures 4.12 E – H, high levels of IL-1, IL-6 and TNFα were detected in stimulated culture supernatants and greater amounts of these cytokines were found in WT culture supernatants compared with KO cultures, with the increases in IL-1α and TNFα levels being statistically significant. Substantial amounts of the inflammatory CC-chemokines, CCL2, CCL3, CCL4 and CCL5, all of which are ligands of D6, were found in culture supernatants, with lower levels of CCL4 being seen in KO cultures (Figure 4.13 D - G). The levels of CCL3 and CCL5 also appeared to be lower in KO cultures, although these differences did not reach statistical significance (Figures 4.13 E and G). There were also detectable levels of CXCL1 and IFNγ-inducible CXCL9 and CXCL10, with significantly less CXCL10 in D6 KO cultures compared to WT. Overall, these data suggested that D6 KO PEC were either less efficient than WT counterparts in the generation of pro-inflammatory cytokines and chemokines following activation, or showed enhanced uptake or degradation of these cytokines and chemokines.

4.4 Assessment Of Chemokine Levels in Inflamed Peritoneal Fluid

As D6 is proposed to be a decoy receptor for CCL2 and is vital in mediating macrophage recruitment to the inflamed peritoneum (47, 136, 137, 155, 308), I decided to analyse levels of this particular D6 ligand in peritoneal fluid at different times after thioglycollate injection. As shown in figure 4.14, levels of CCL2 increased in peritoneal lavage fluid of WT mice from 24 to 96 hours after thioglycollate injection (61.3 ± 3.2 pg/ml at 24h and 112.5 ± 17.2 pg/ml at 96h). However the levels of CCL2 in D6 KO lavage did not increase over time, so that by 96 hours, significantly increased levels of CCL2 were detected in WT peritoneal fluid.

4.5 Chemokine Receptor Expression by WT and D6 KO PEC during Inflammation

CCR2 and CCR5 are activatory receptors for inflammatory CC-chemokine ligands of D6 (CCL2, CCL3, CCL5, CCL7) and have been shown to be involved in monocyte trafficking to inflamed sites (47, 155, 312). As such, it was possible that their expression could be affected by D6 function and so to further compare WT and KO peritoneal exudates, I examined the expression of the CCR2 and CCR5 chemokine receptors on resident and inflammatory PEC of D6 KO and WT mice, analysing particularly the different subsets defined by CD11c and F4/80 that I had identified in earlier experiments.

In resident PEC, a higher proportion of KO F4/80$^+$ cells expressed CCR2 and CCR5 compared with WT F4/80$^+$ cells, although only CCR2 expression was significantly different (Figure 4.15 A). In contrast, resident KO F4/80$^+$ PEC expressed higher levels of CCR5 per cell than WT F4/80$^+$ PEC, as shown by MFI (Figure 4.15 B).

Next, in order to compare chemokine receptor expression in the inflammatory response, I analysed the chemokine receptor profile of the various F4/80/CD11c subsets at 24 and 96 hours after thioglycollate challenge to represent early and late time points. As shown in Figures 4.16 A and B, at 24h after thioglycollate injection, almost all (~98%) $F4/80^{+}CD11c^{+}$ PEC from both WT and KO mice expressed CCR2 and CCR5, but the levels of expression of these receptors were higher on D6 KO $F4/80^{+}CD11c^{+}$ PEC (Fig. 4.16 C and D), although this was only statistically significant for CCR5. The vast majority (~90%) of $F4/80^{-}CD11c^{+}$ cells at this time were also positive for both of these chemokine receptors, but levels of expression were similar between WT and KO cells (Fig 4.16 A - D). Similar percentages of WT and KO $F4/80^{+}CD11c^{-}$ cells expressed CCR2 (~75%) and CCR5 (~50%) (Fig. 4.16 A and B); however the $F4/80^{+}CD11c^{-}$ cells that were CCR2 or CCR5 positive in D6 KO PEC expressed significantly higher levels of these receptors compared with their WT counterparts (Fig. 4.16 C and D). Only around 10% of $F4/80^{-}CD11c^{-}$ cells from both WT and KO expressed CCR2 or CCR5, and there were no differences in levels of receptor expression between WT and D6 KO cells.

At 96h post-thioglycollate, most (>90%) of the $F4/80^{+}CD11c^{+}$ subset expressed CCR2 and CCR5 (Figures 4.17 A and B). Similar to 24 PEC, D6 KO $F4/80^{+}CD11c^{+}$ cells showed higher levels of CCR5 expression compared with WT $F4/80^{+}CD11c^{+}$ PEC (Fig. 4.17 D). However, at this time point levels of CCR2 expression were lower on the KO $F4/80^{+}CD11c^{+}$ cells (Fig. 4.17 C). Expression of CCR2 and CCR5 was comparable between WT and D6 KO $F4/80^{+}CD11c^{+}$ and $F4/80^{-}CD11c^{+}$ subsets harvested at 96h (Fig. 4.17 A – B). Although high proportions of 24h $F4/80^{+}CD11c^{-}$ cells were found to express CCR2 and CCR5, at 96h only around a quarter of this subset at most expressed either of

these receptors. In addition, although CCR5 expression levels were similar between WT and D6 KO $F4/80^{+}CD11c^{+}$ PEC at this time, compared with their WT counterparts, the expression of CCR2 was lower on 96h $F4/80^{+}CD11c^{-}$ KO PEC (Fig. 4.17 A and C).

Taken together, these data demonstrated that myeloid populations retrieved from both resting and inflamed peritoneum of D6 KO mice differed from their WT counterparts in terms of CC-chemokine receptor expression.

4.6 Investigation of DC Development In Bone Marrow Cultures from D6 KO and WT Mice

The increased prevalence of $CD11c^{+}$ cells in the inflamed D6 KO peritoneum suggested that absence of D6 either favoured the recruitment of DC to sites of inflammation, or that there was enhanced differentiation of myeloid precursors towards the DC lineage. To examine the latter idea in more detail, I compared the generation and function of dendritic cells derived from WT and KO bone marrow.

Bone marrow cells isolated from femurs of WT and KO mice were cultured in GM-CSF for seven days in order to generate BM-derived DC. At day 7, non-adherent cells were harvested and cultured overnight with or without LPS, before assessment of differentiation and activation markers. D6 KO BM incubated overnight with medium contained significantly greater proportions of $CD11c^{+}$ cells compared with WT controls (Fig 4.18 A). Moreover, the $CD11c^{+}$ cells in KO cultures expressed higher levels of CD11c than WT $CD11c^{+}$ cells (Fig 4.18 B). LPS had no effects on the numbers or levels of CD11c expression in either strain, but, as expected, led to increased levels of the activation markers

CD40, CD80, CD86, as well as some increase in class II MHC expression (Fig 4.19). WT and KO cells did not differ significantly in these responses to LPS (Fig 4.19).

As these results suggested that there might be unusual differentiation of DC in GM-CSF stimulated BM-cultures in D6 KO mice, I examined this in more detail by investigating $CD11c^+$ cell development in BM cultures taken after days 4, 6, 8, 10 and 12 of culture with GM-CSF. As shown in Figure 4.20 A, the proportion of cells in BM-cultures expressing CD11c gradually increased throughout the duration of culture, and by day 12, almost all cells in both WT and KO cultures were $CD11c^+$. Greater proportions of $CD11c^+$ cells were found in KO cultures at most times, although this was only statistically significant at days 4 and 8 of culture. Additionally, $CD11c^+$ cells from D6 KO BM-cultures expressed higher levels of CD11c than their WT counterparts at all times, as determined by increased MFI and was statistically significant on days 8 and 10 of culture (Fig 4.20 B).

In my earlier experiments examining the cellular composition of exudates from inflamed peritoneum, I had been unable to determine the identity of the $F4/80^+CD11c^+$ subset that prevailed in the KO peritoneum. Therefore I next examined if BM-generated DC expressed the F4/80 antigen and if this differed between D6 KO and WT DC. F4/80 expression could be detected on around 20% of BM cells by day 4 of culture, and the proportions of $F4/80^+$ cells increased with the duration of culture, with around 60-70% of cells being $F4/80^+$ by day 12 (Fig. 4.20 C). However, no significant differences in F4/80 expression were seen between WT and KO cells at any of the times analysed.

4.7 Chemokine Receptor Profile of BM-derived DC

My studies of the peritoneal exudates had revealed differences in chemokine receptor expression between WT and D6 KO cells. Therefore I decided to examine chemokine receptor expression by WT and KO BM-DC.

Consistent with existing reports (163), the vast majority of $CD11c^+$ cells in both WT and KO BM cultures expressed CCR2 and CCR5 on day 7 (Fig 4.21). However, in keeping with my previous studies, I found increased expression of both CCR2 and CCR5 by D6 KO $CD11c^+$ cells, although this was statistically significant only for CCR5 (Fig 4.21). In contrast, CXCR4 expression was equivalent between WT and KO BM-DCs (Figure 4.21).

I next assessed whether CCR2 and CCR5 showed similar downregulation in response to LPS. Following overnight culture with LPS, DC derived from both WT and KO mice showed significant down-regulation of CCR5, although there was little or no effect on CCR2 (Fig 4.22). There were no differences between WT and KO DC in terms of chemokine receptor down-regulation in response to LPS (Fig 4.22).

4.8 Functional Activity of BM-derived DC

To examine the functional properties of BM-derived DC from D6 KO and WT mice, I first compared their endocytic activity. BM DC were harvested after 4, 6, 8, 10 and 12 days of culture with GM-CSF and the uptake of FITC-dextran assessed by flow cytometry. In all cases, uptake of FITC-dextran increased with the time of culture with greatest uptake seen at 60 min, but no significant differences were observed between WT and KO endocytic responses regardless of the day at which these cells were harvested (Figure 4.23).

Next, I assessed the APC activity of WT and KO DC by measuring their ability to stimulate allogeneic T cells in a mixed-leukocyte reaction (MLR). WT and D6 KO BM-derived DC, harvested at day 7 or 9, were treated with mitomycin C and co-cultured with BALB/c lymphocytes. Under these conditions, both DC sources stimulated allogeneic T cells, with maximal responses being obtained using DC:T ratios of 1:2. As shown in Figure 4.24, D6 KO DC generated lower MLR reactions than their WT counterparts and this difference was most apparent in cultures of DC harvested at day 9.

Finally, I compared cytokine production by WT and KO DC. DC isolated on d7 of culture, were cultured overnight in medium or with LPS and the culture supernatants analysed by Luminex. Culture with LPS led to substantial production of IL-1, IL-6 and TNFα by WT and KO DC (Figures 4.25 A-D). While the levels of IL-1α, IL-1β and IL-6 did not differ between WT and KO LPS-treated DC cultures, significantly higher levels of TNFα were detected in the D6 KO cultures. IL-4 and IL-10 were detected when DC were cultured with medium alone and at higher levels in LPS cultures, but no differences were observed between WT and KO cultures (Figures 4.25 E and F). LPS-treatment induced significant IL-12 p40/p70 secretion (Figure 4.25 G), but again, the levels of IL-12 p40/p70 did not differ between WT and KO cultures. The angiogenic cytokine, VEGF, was present at high levels in cultures of both WT and KO DC in medium alone, and these were reduced in the presence of LPS (Figure 4.25 H). Increased levels of VEGF were found in KO DC cultures compared with WT, both in the presence and absence of LPS. LPS-stimulated DC produced marked amounts of the CXC-chemokines CXCL1, CXCL9 and CXCL10, but there were no differences between WT and KO DC (Figures 4.26 A-C). The D6 ligands CCL2, CCL3, CCL4 and CCL5 were barely detectable when DC were cultured in medium alone, but were

found at high levels in LPS-treated DC cultures (Figure 4.26 D-G). The production of CCL2 was similar for both WT and KO DC, but KO DC produced higher levels of CCL3, CCL4 and CCL5 in response to LPS culture compared with WT DC (Figure 4.26 E-F).

Overall, my data indicated that D6 KO BM-derived DC differ from WT DC in terms of CD11c, CCR2 and CCR5 expression. In spite of this altered surface phenotype, I failed to detect any functional differences in D6 KO DC in terms of costimulatory molecule upregulation or endocytosis of antigen. However, D6 KO DC generated poorer MLR responses compared to WT counterparts. In addition, higher levels of TNFα, VEGF and a variety of inflammatory CC-chemokines were found in culture supernatants from LPS-stimulated D6 KO BM-DCs compared to their WT counterparts. Taken together, these data suggested that absence of D6 might result in both phenotypically and functionally altered BM-DC.

Summary

In this chapter I explored the myeloid cell compartment of D6 KO animals. I detected increased numbers of resident $F4/80^+$ macrophages in the peritoneal cavity of resting D6 KO mice and these cells showed higher expression of CCR2 and CCR5 compared with WT controls. Following injection of thioglycollate, D6 KO mice showed normal levels of total cell recruitment to the peritoneum, but there was preferential accumulation of an $F4/80^+CD11c^+$ cell population. Inflammatory peritoneal exudates from D6 KO animals could not be distinguished from WT cells in terms of NO generation, but generated less pro-inflammatory cytokines following *ex vivo* stimulation. Generation of DC revealed that

D6 KO BM-derived DC were characterised by more rapid and higher expression of CD11c, as well as of the CC-chemokine receptors, CCR2 and CCR5. These differences did not alter the capacity of D6 KO BM-DC to upregulate costimulatory molecules in response to LPS-activation, and KO DC were equally efficient at endocytosis compared with WT DC. However, D6 KO DC were poorer in their capacity to generate mixed leukocyte reactions compared with WT DC and KO DC also demonstrated higher production of TNFα, VEGF and a number of inflammatory CC-chemokines in response to LPS. Overall, these data suggest that the D6 chemokine receptor may influence the development and function of myeloid lineage cells.

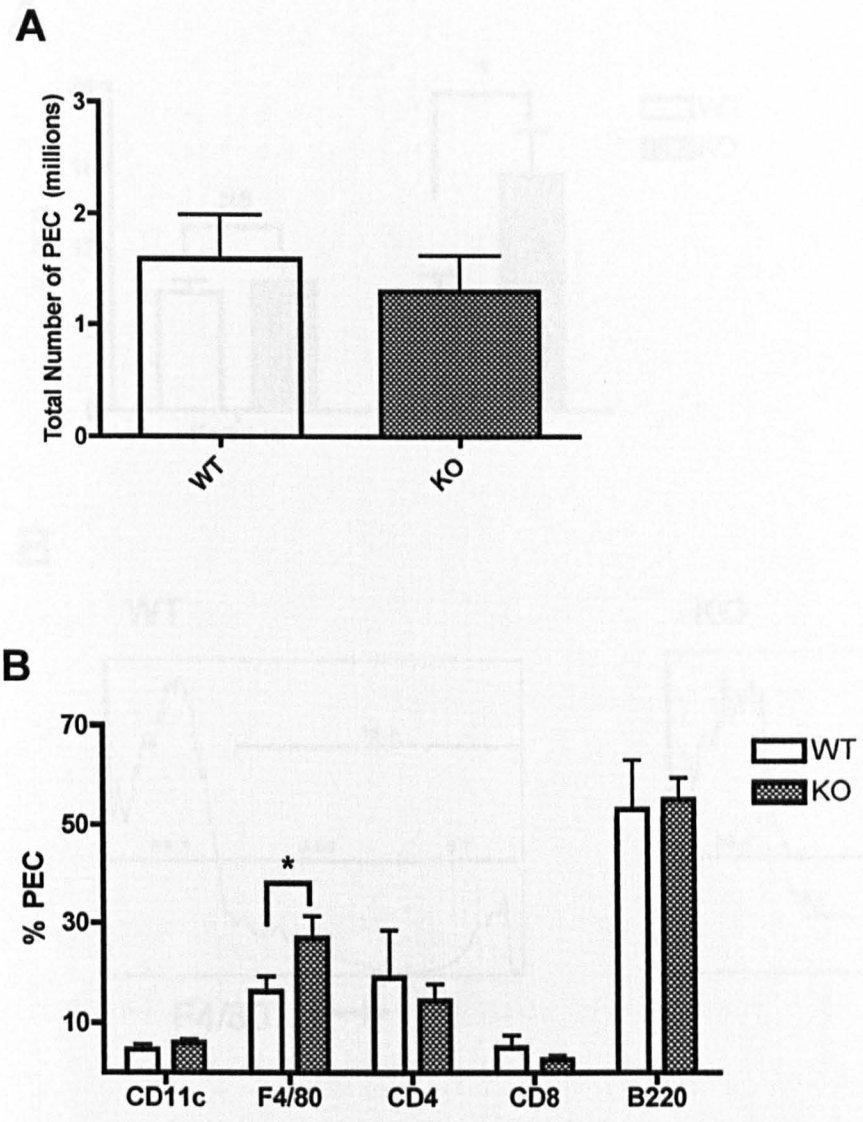


Figure 4.1

Cellular Composition of Resting D6 KO Peritoneum

Cells were obtained from the peritoneal cavity of WT and D6 KO mice by lavage and the relative proportions of CD11c+, F4/80+, CD4+, CD8+ and B220+ cells were determined by flow cytometry. Figure A represents the total number of cells retrieved from the peritoneum and figure B shows the relative proportions of the different cell populations in peritoneal exudates. The data are means + 1 SD for 3 animals per group. Similar results were obtained from three individual experiments.

* $p < 0.05$

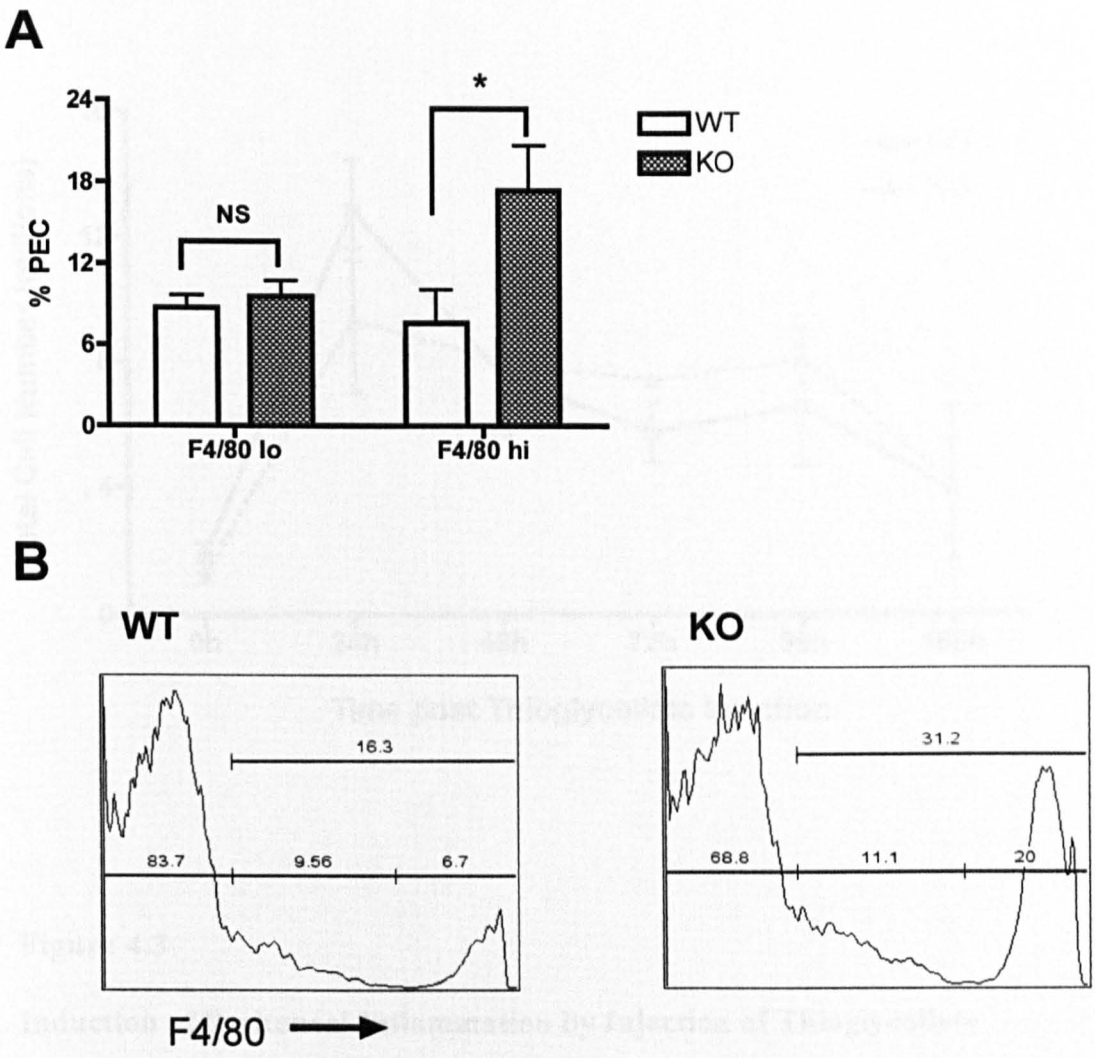


Figure 4.2

Cellular Composition of Resting D6 KO Peritoneum

Figure A shows the relative proportions of F4/80-lo and F4/80-high cell subsets in peritoneal exudate cells of WT and D6 KO mice, as determined by flow cytometry. Representative flow analyses from a WT (left) and D6 KO (right) mouse are shown in figure B. The data in (A) are the mean + 1 SD for 3 animals per group. Similar results were obtained from three individual experiments.

* $p < 0.05$

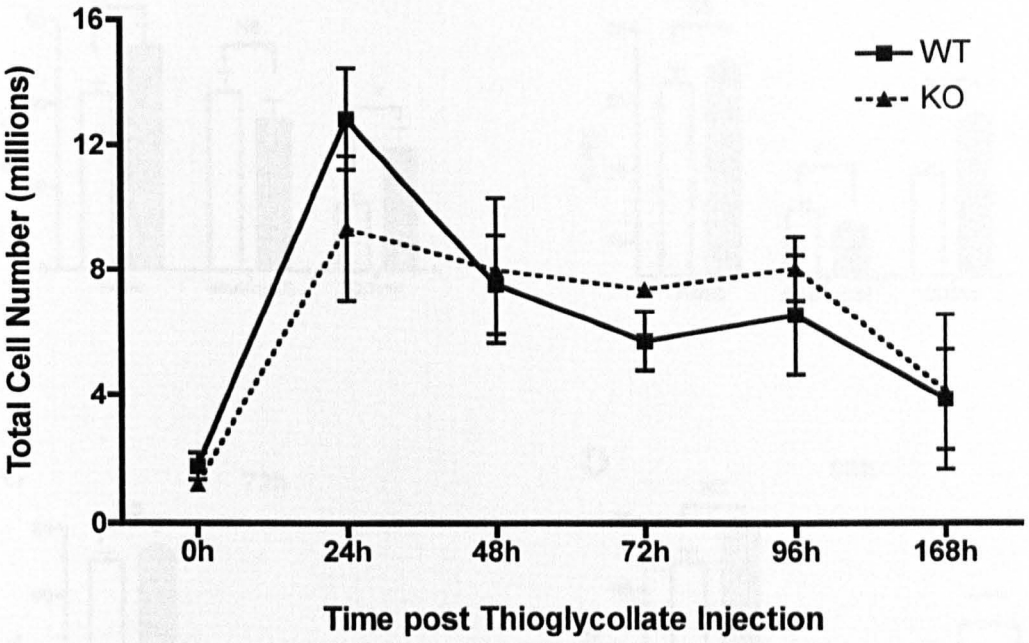


Figure 4.3

Induction of Peritoneal Inflammation by Injection of Thioglycollate

The above figure represents the total number of cells retrieved from the peritoneal cavities of WT and D6 KO mice at various times following intraperitoneal injection of 4% thioglycollate. The data represent the mean ± 1 SD for 3-4 animals per group.

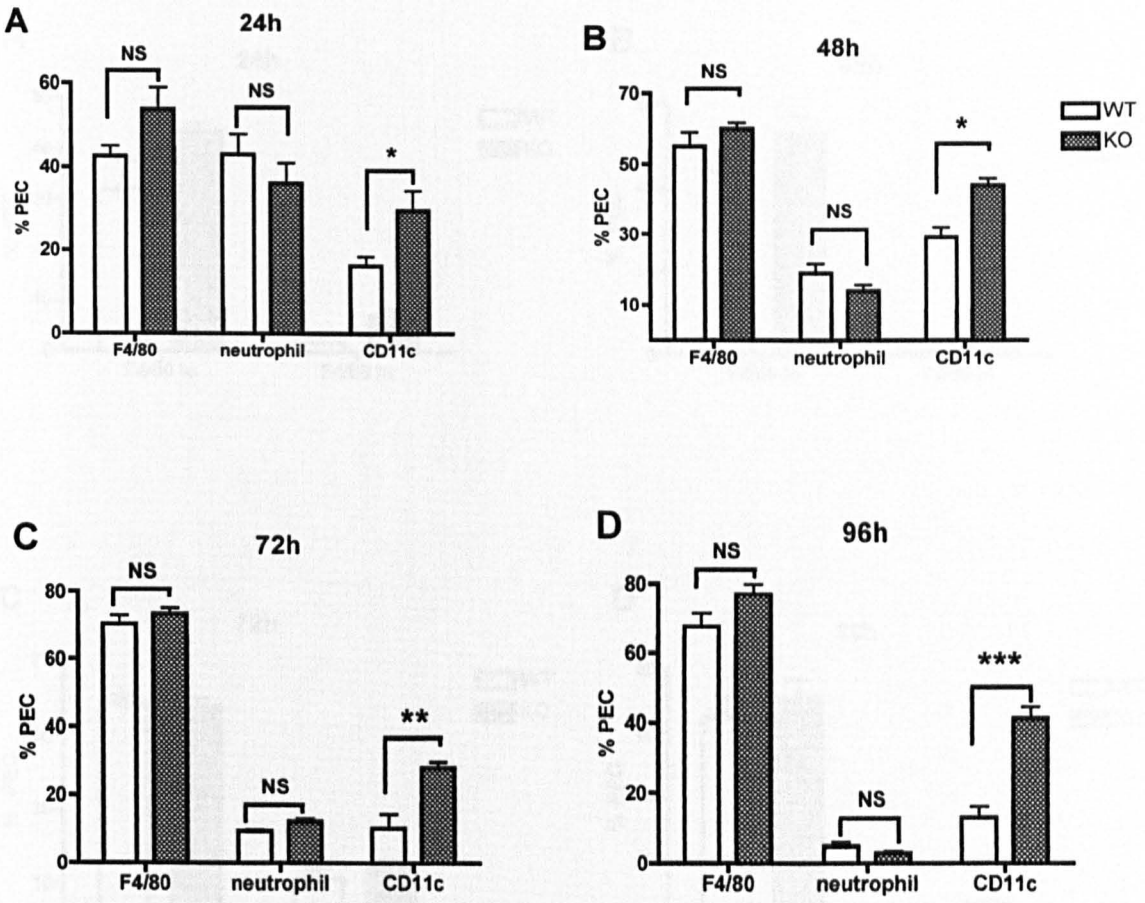


Figure 4.4

Induction of Peritoneal Inflammation by Thioglycollate Injection

Cells were obtained from the peritoneal cavity of WT and D6 KO mice by lavage at different times following injection of thioglycollate and the relative proportions of F4/80+ cells, CD11c+ cells and Gr1+ neutrophils determined by flow cytometry. Figures A, B, C and D show the composition of exudates obtained at 24, 48, 72 and 96 hours, respectively. The data represent the mean + 1 SD for 3-4 animals per group and similar results were obtained from two or three individual experiments at each time point.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, NS = not significant

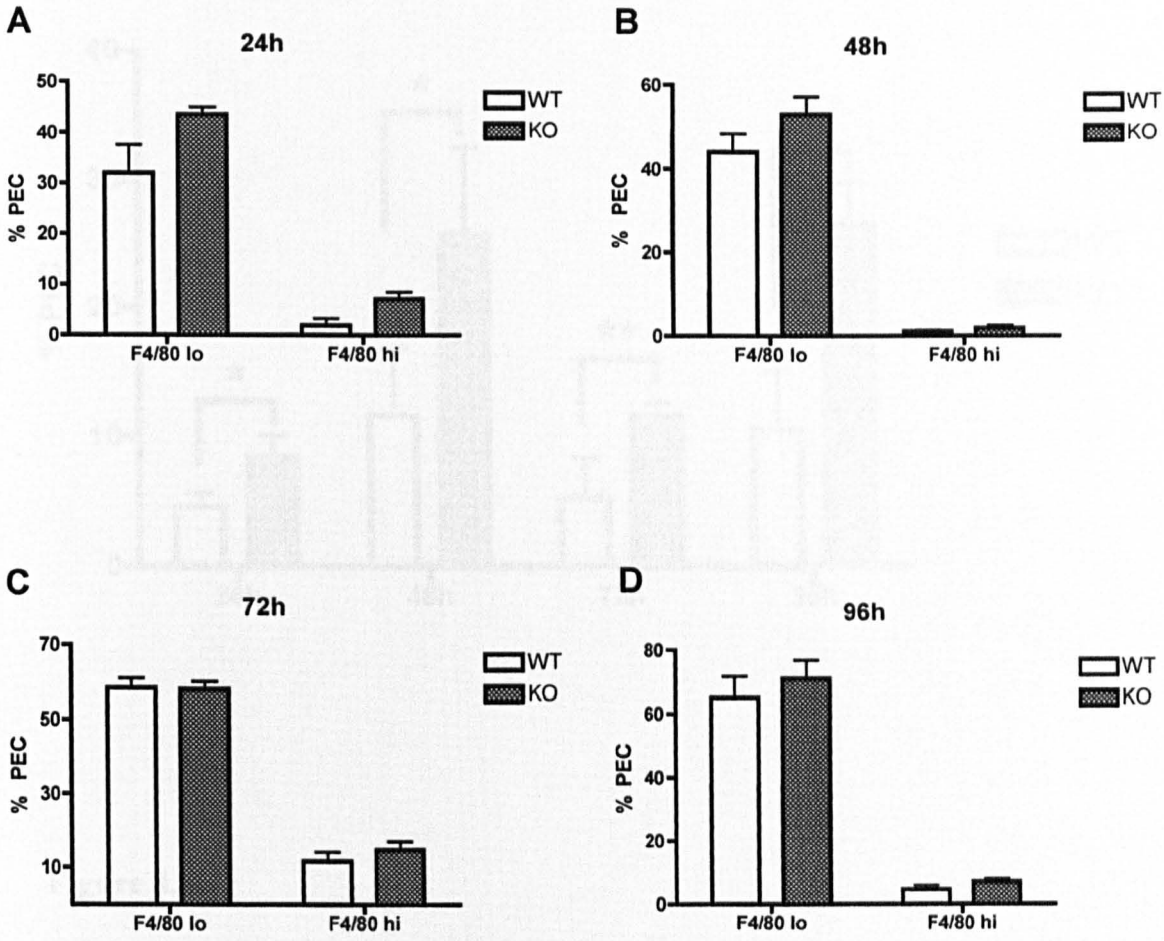


Figure 4.5

Effects of Thioglycollate on F4/80 Expression by Peritoneal Exudate Cells

Cells were obtained from the peritoneal cavity of WT and D6 KO mice by lavage at different times after injection of thioglycollate and the expression of F4/80 determined by flow cytometry. Figures A, B, C and D show the percentages of $F4/80^{hi}$ and $F4/80^{lo}$ obtained at 24, 48, 72 and 96 hours, respectively. The data represent the mean + 1 SD for 3-4 animals per group and similar results were obtained from two or three individual experiments at each time point.

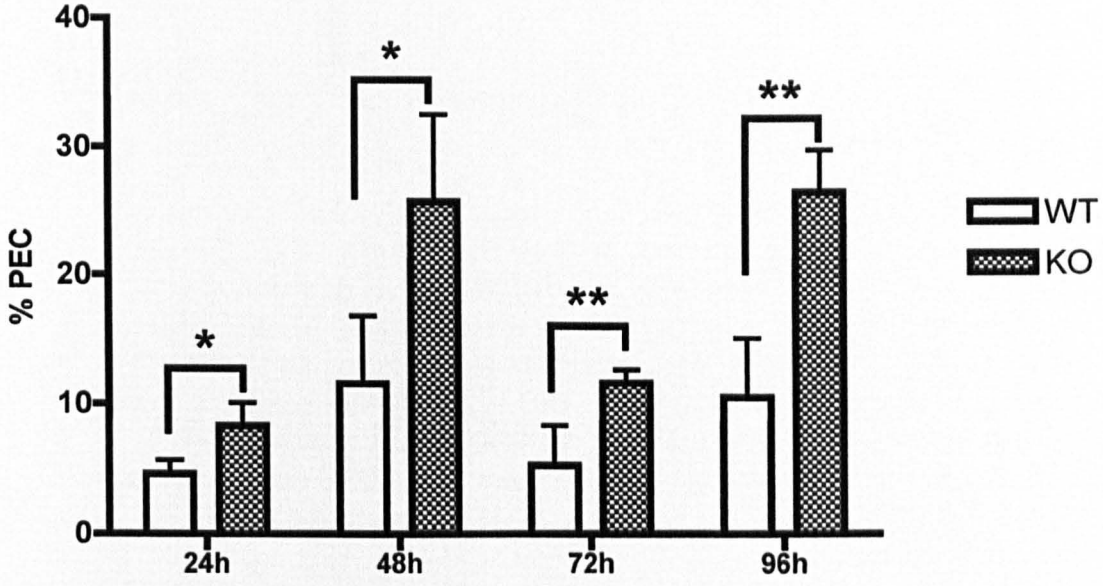


Figure 4.6

Relative Proportions of CD11c+MHCII+ Cells in Peritoneum Following Injection of Thioglycollate

Cells were obtained from the peritoneal cavity of WT and D6 KO mice by lavage at different times after injection of thioglycollate and the relative proportions of CD11c+MHCII+ cells determined by flow cytometry. Figures A, B, C and D show the percentages of CD11c+MHCII+ cells obtained at 24, 48, 72 and 96 hours, respectively. The data represent the mean + 1 SD for 3-4 animals per group and similar results were obtained from two or three individual experiments at each time point.

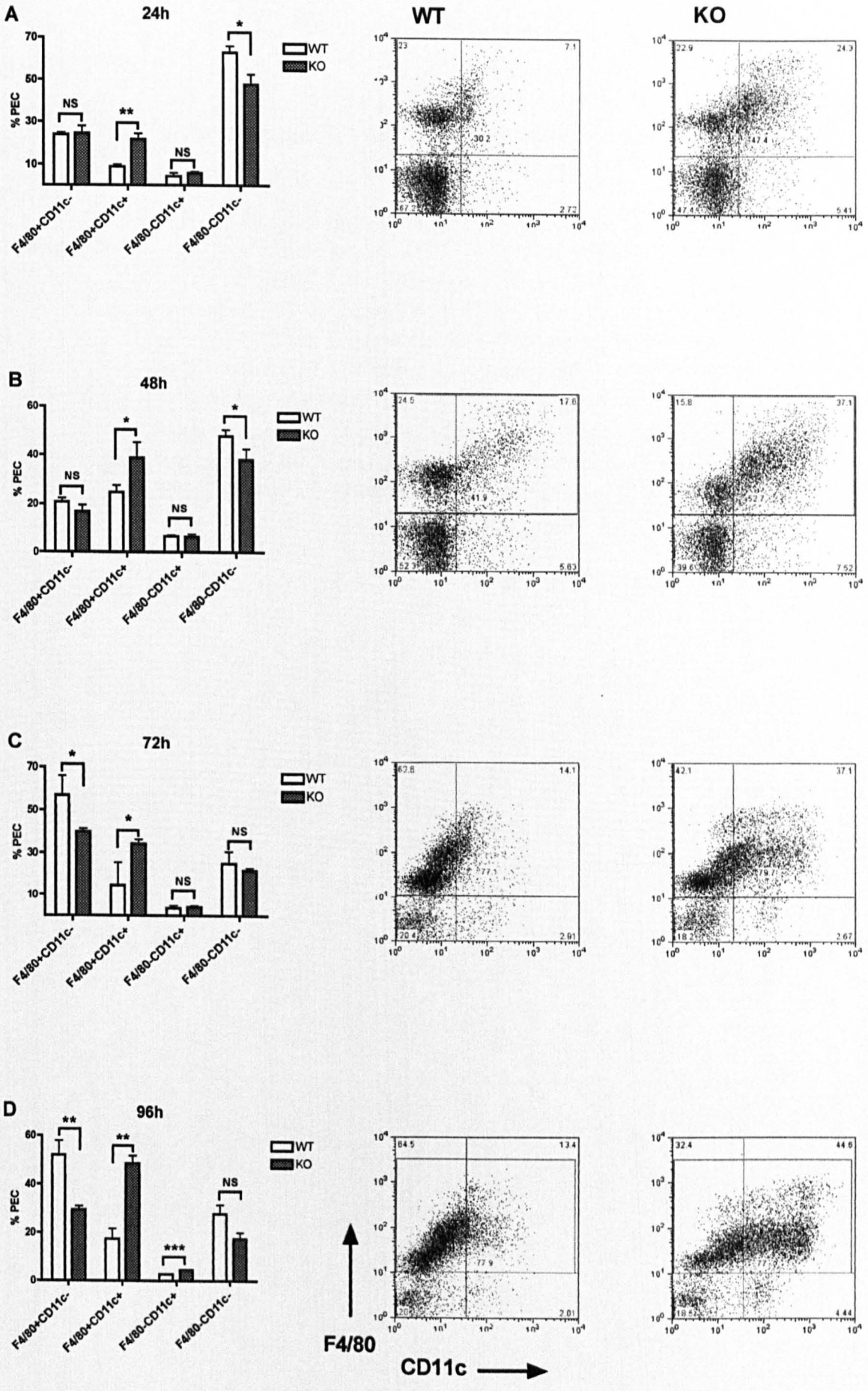
* $p < 0.05$, ** $p < 0.01$

Figure 4.7

Subsets of CD11c+ and F4/80+ Cells in Exudates From Thioglycollate Challenged Peritoneum

Cells were obtained from the peritoneal cavity of thioglycollate-injected WT and D6 KO mice by lavage and the relative proportions of F4/80+CD11c-, F4/80+CD11c+, F4/80-CD11c+ and F4/80-CD11c- cells determined by flow cytometry. Figures A, B, C and D show the proportions of each subset retrieved at 24, 48, 72 and 96 hours, respectively, following thioglycollate injection. The data represent the mean + 1 SD of 3-4 animals per group. Also shown are representative FACS plots from a thioglycollate-challenged WT mouse (centre) and D6 KO mouse (right) at each time point. Similar results were obtained from two or three individual experiments at each time point.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, NS = not significant


A
24h
WT
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% PEC
NS
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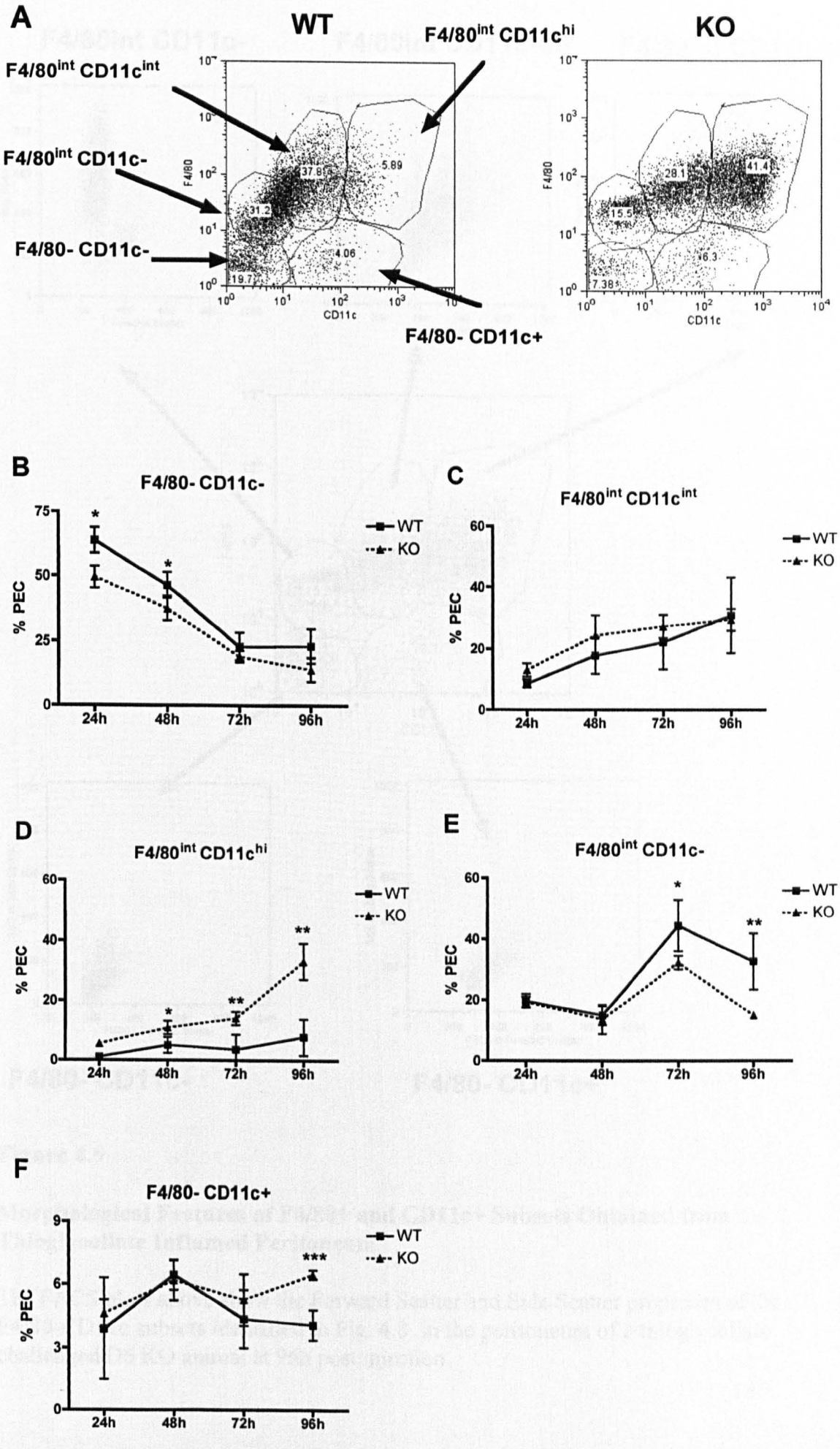
NS
*
F4/80+CD11c-
F4/80+CD11c+
F4/80-CD11c+
F4/80-CD11c-
WT
KO
B
48h
C
72h
D
96h
***
F4/80
CD11c

Figure 4.8

Phenotypic Characterisation of CD11c+ and F4/80+ Cells in Thioglycollate Induced Peritoneal Exudates

Cells were obtained from the peritoneal cavity of thioglycollate-injected WT and D6 KO mice by lavage and the relative proportions of CD11c-F4/80-, F4/80^{int} CD11c^{int}, F4/80^{int} CD11c^{hi}, F4/80^{int} CD11c- and F4/80-CD11c+ subsets determined by flow cytometry. (A) Representation of the different subsets in one WT animal (left) and one D6 KO animal (right) 96h post thioglycollate injection. Figures B, C, D, E and F show the proportions of CD11c-F4/80-, F4/80^{int} CD11c^{int}, F4/80^{int} CD11c^{hi}, F4/80^{int} CD11c- and F4/80-CD11c+, respectively, at 24, 48, 72 and 96 hours following thioglycollate injection. The data represent 3-4 animals per group and are presented as the means + SD. Similar results were obtained from at least two individual experiments at each time point.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$


A
WT
KO
F4/80int CD11chi
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F4/80int CD11c-
F4/80- CD11c-
F4/80- CD11c+
F4/80
CD11c
37.8
5.89
31.2
4.06
19.7
28.1
41.4
15.5
6.3
7.38
B
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C
F4/80int CD11cint
D
F4/80int CD11chi
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48h
72h
96h

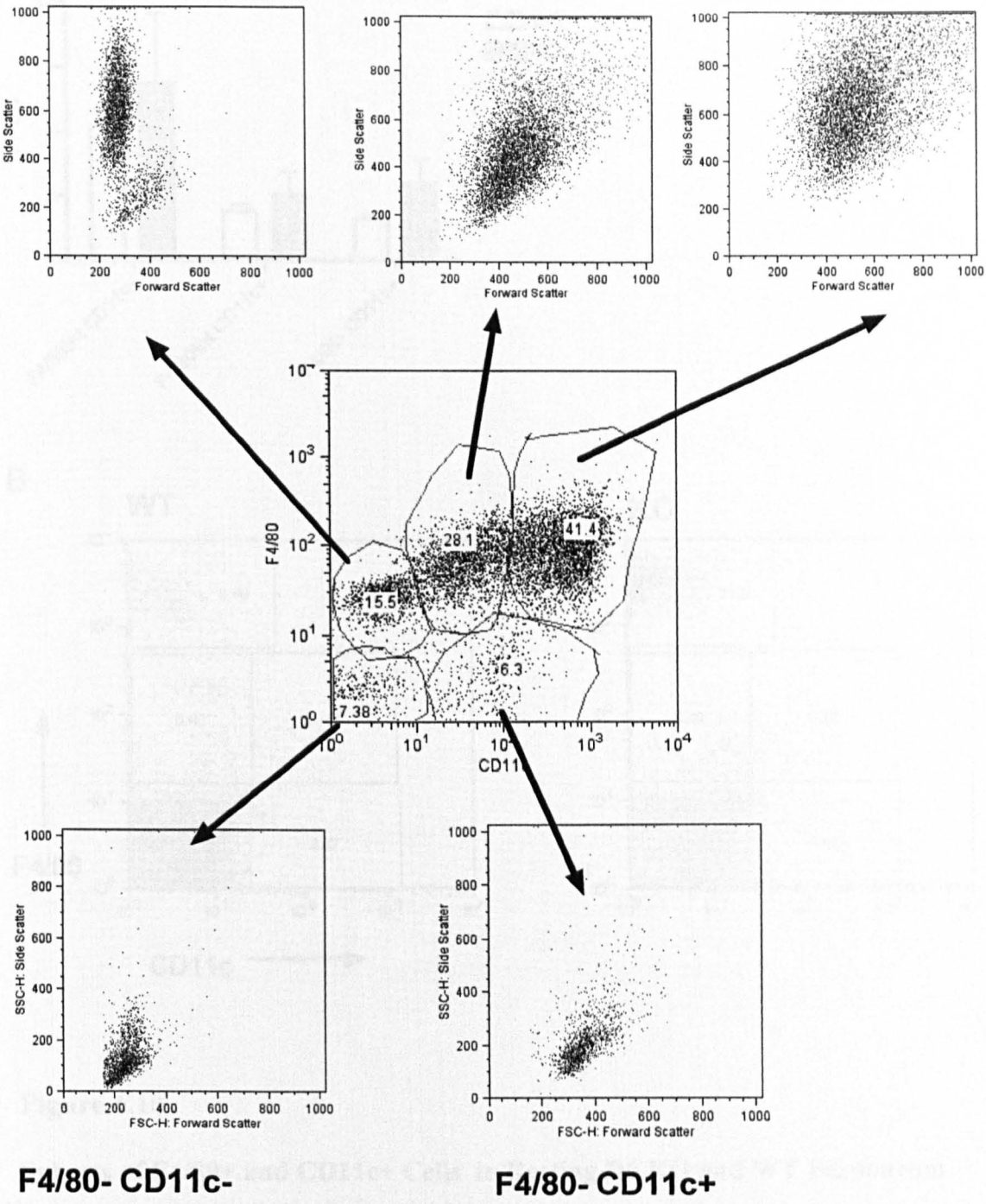


Figure 4.9

Morphological Features of F4/80+ and CD11c+ Subsets Obtained from Thioglycollate Inflamed Peritoneum

The FACS plots above show the Forward Scatter and Side Scatter properties of the F4/80-CD11c subsets identified in Fig. 4.8 in the peritoneum of a thioglycollate challenged D6 KO animal at 96h post injection.

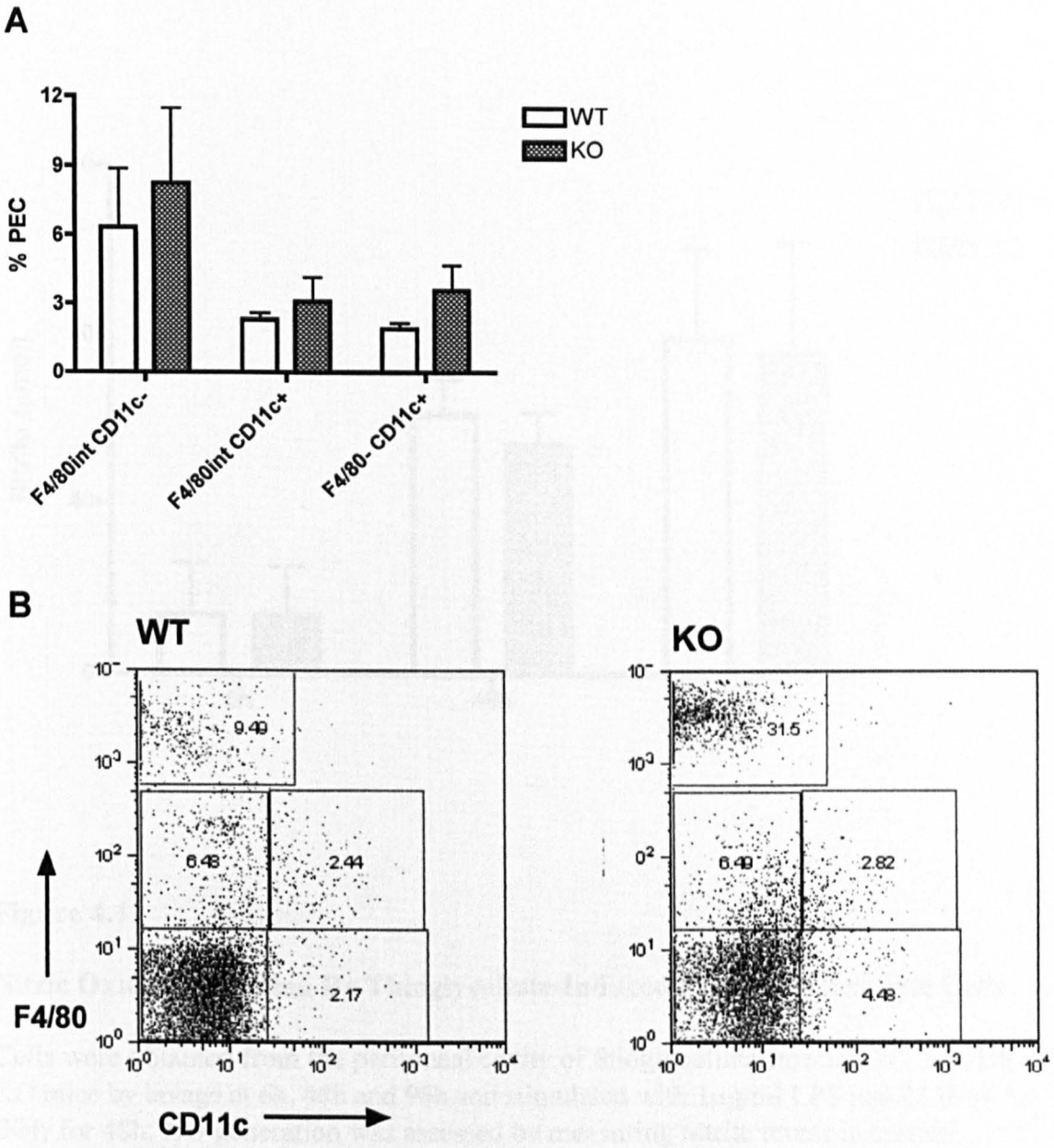


Figure 4.10

Subsets of F4/80+ and CD11c+ Cells in Resting D6 KO and WT Peritoneum

(A) The proportions of resting F4/80int-CD11c-, F4/80int-CD11c+ and F4/80-CD11c+ subsets were determined in the resting peritoneum of unmanipulated WT and D6 KO animals. The data are the mean + 1 SD of 3 mice per group. (B) Representative FACS plots from an unchallenged WT (left) and KO mouse (right).

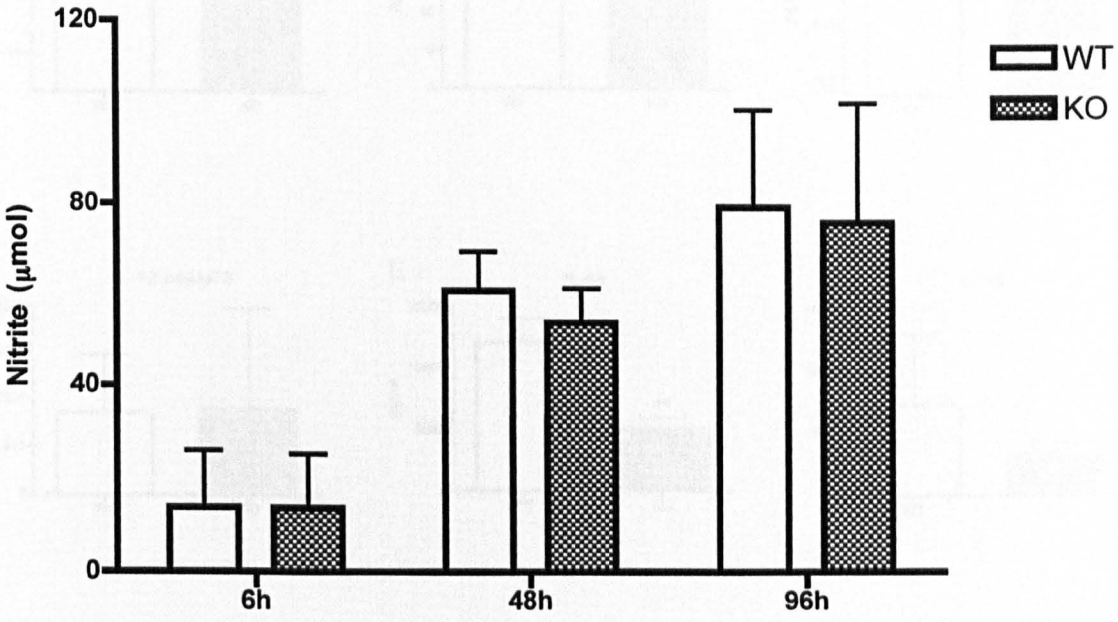


Figure 4.11

Nitric Oxide Production By Thioglycollate-Induced Peritoneal Exudate Cells

Cells were obtained from the peritoneal cavity of thioglycollate-injected WT and D6 KO mice by lavage at 6h, 48h and 96h and stimulated with 1µg/ml LPS and 25 U/ml IFNγ for 48h. NO generation was assessed by measuring nitrite levels in culture supernatants using the Griess reaction. The data represent the mean + 1 SD for 3-4 animals per group.

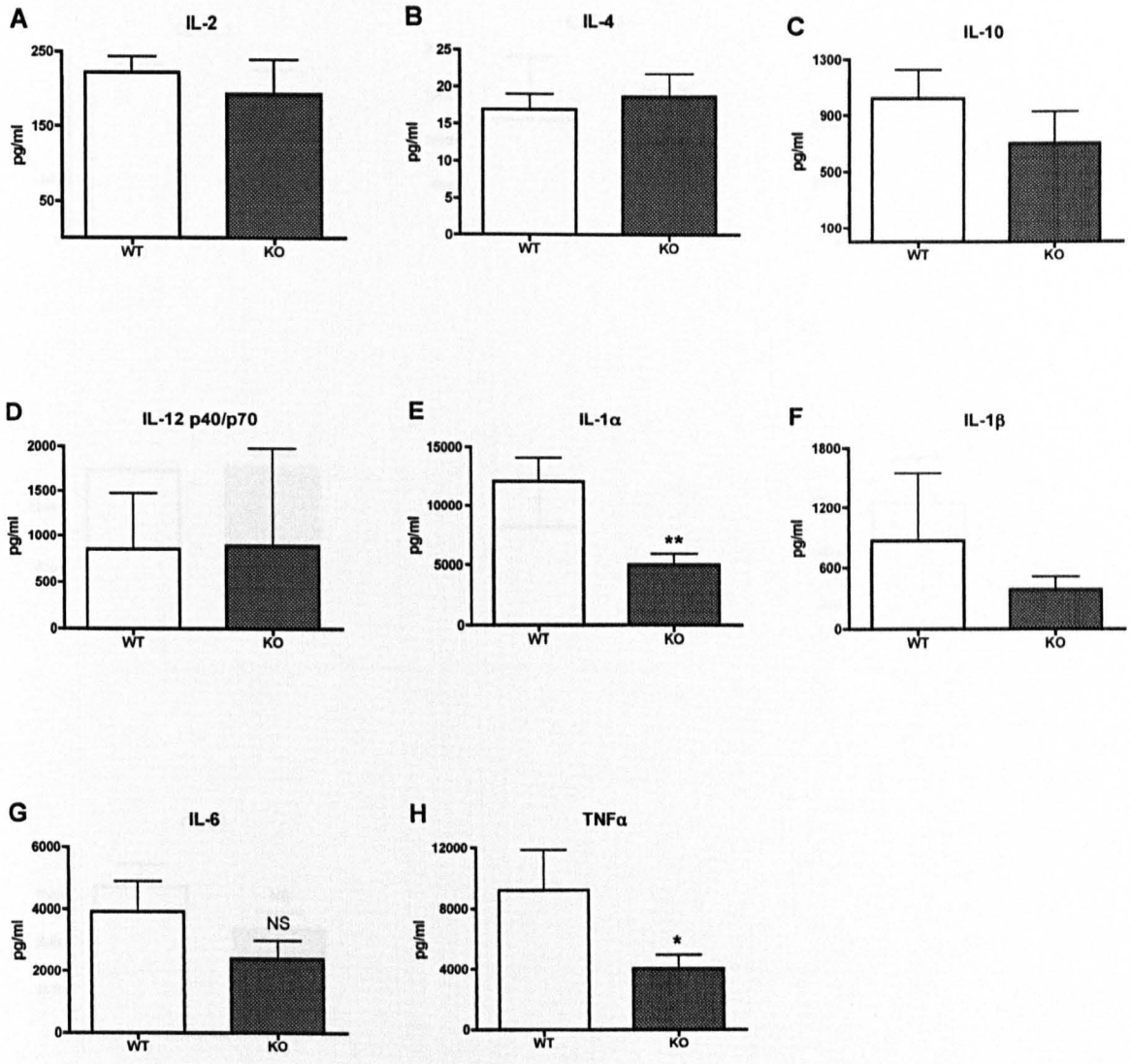


Figure 4.12

Cytokine Production By Thioglycollate-Induced Peritoneal Exudate Cells

Cells were obtained from the peritoneal cavity of thioglycollate-injected WT and D6 KO mice by lavage at 96h and stimulated with 1μg/ml LPS and 25U/ml IFNγ in complete medium for 24h. The levels of (A) IL-2, (B) IL-4, (C) IL-10,(D) IL-12 p40/ p70, (E) IL-1α, (F) IL-1β, (G) IL-6 and (H) TNFα were determined by Luminex analysis. The data represent the mean + 1 SD of 3 animals per group.

* $p < 0.05$, ** $p < 0.01$

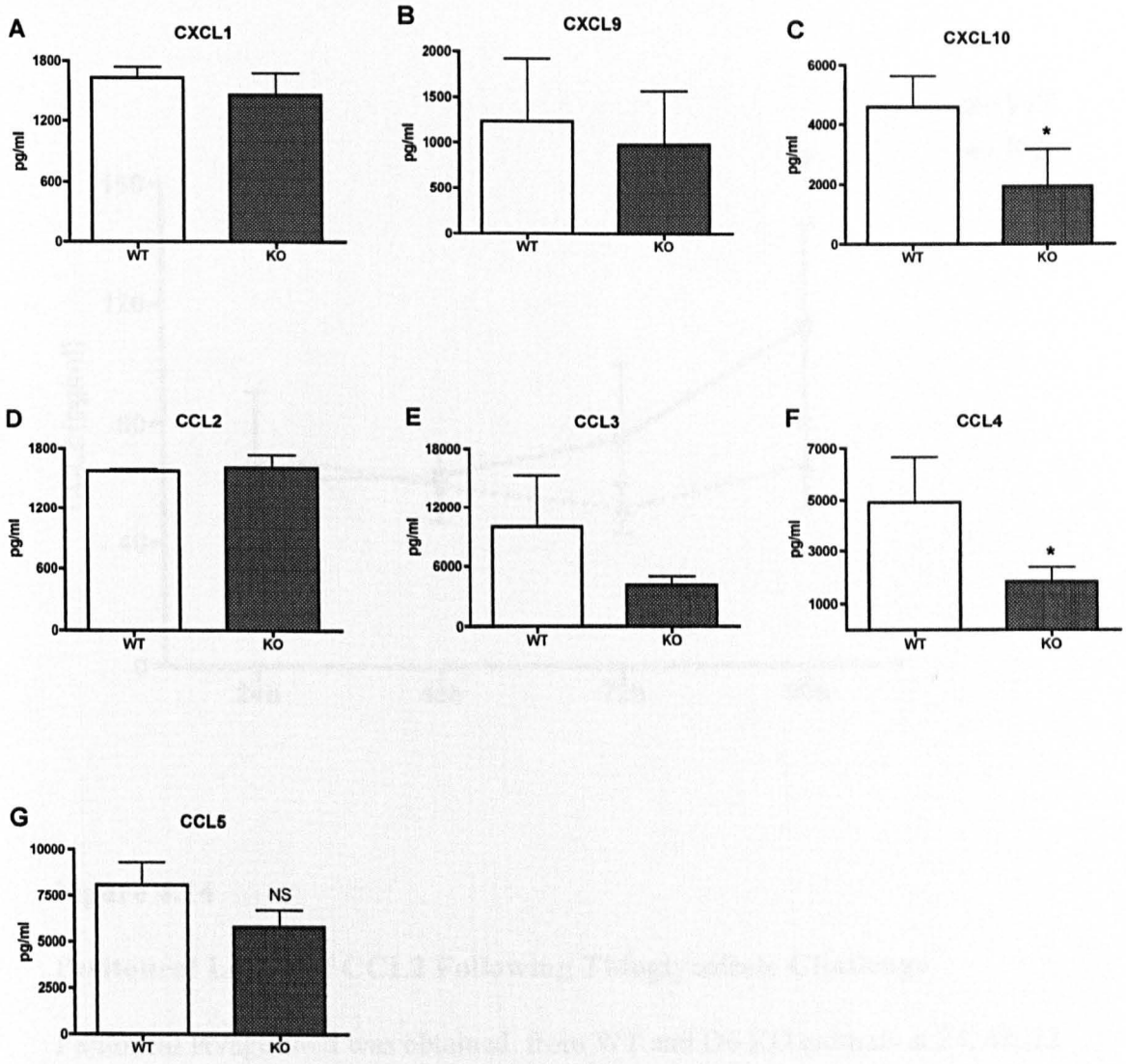


Figure 4.13

Chemokine Production By Thioglycollate-Induced Peritoneal Exudate Cells

Cells were obtained from the peritoneal cavity of thioglycollate-injected WT and D6 KO mice by lavage at 96h and stimulated with 1μg/ml LPS and 25U/ml IFNγ for 24h. The levels of (A) CXCL1, (B) CXCL9, (C) CXCL10,(D) CCL2, (E) CCL3, (F) CCL4 and (G) CCL5 were determined by Luminex analysis. The data represent the mean + 1 SD of 3 animals per group.

* $p < 0.05$

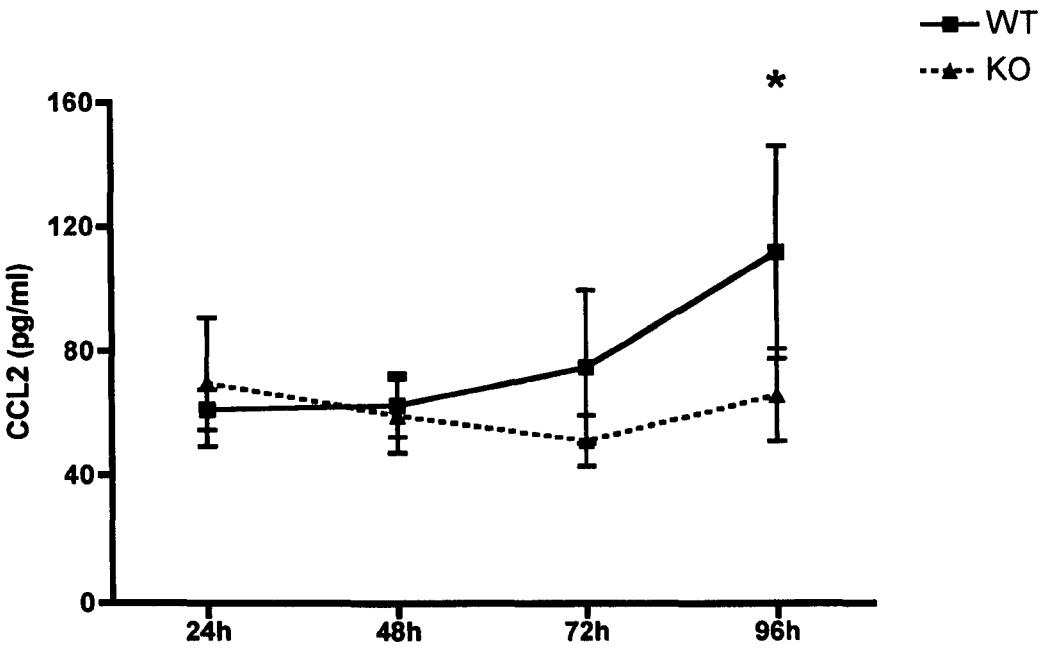


Figure 4.14

Peritoneal Levels of CCL2 Following Thioglycollate Challenge

Peritoneal lavage fluid was obtained from WT and D6 KO animals at 24, 48, 72 and 96h after injection of thioglycollate and CCL2 levels assayed by luminex. The data represent 3 animals per group and are presented as the mean ± SD.

* $p < 0.05$ (96h WT vs KO)

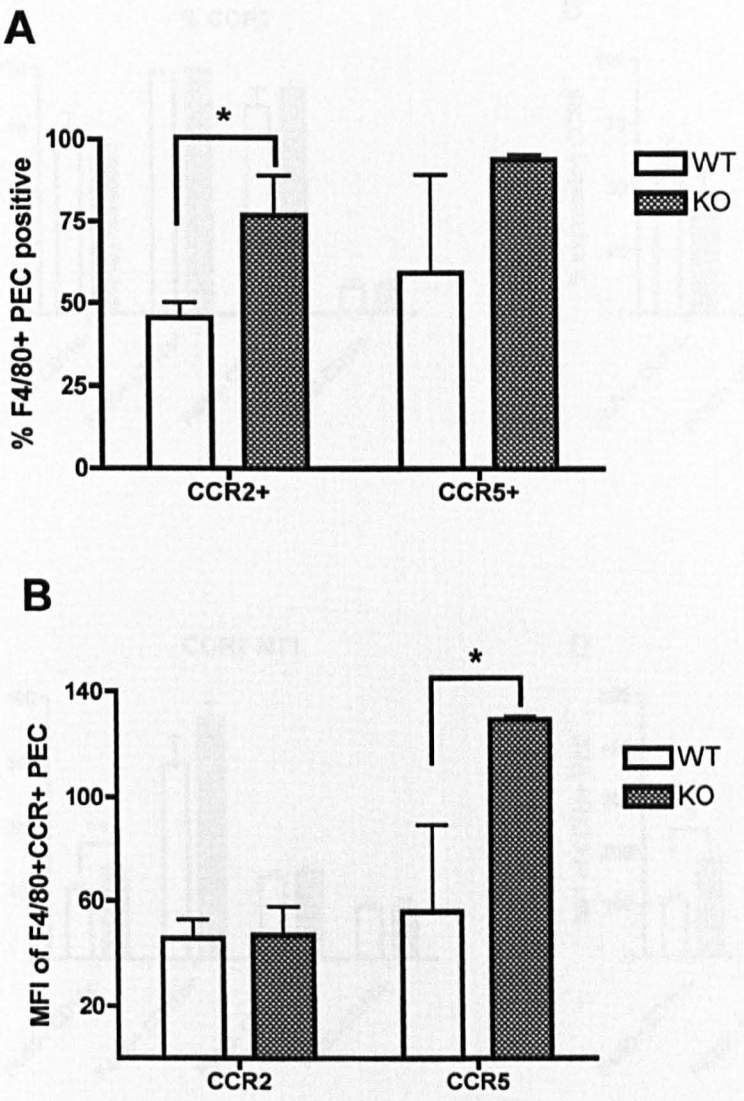


Figure 4.15

Expression of CCR2 and CCR5 by Resident F4/80+ Cells in Peritoneum

Cells were obtained from the peritoneal cavity of unmanipulated WT and D6 KO mice by lavage and the expression of CCR2 and CCR5 on F4/80+ cells was assessed. (A) The proportions of F4/80+ cells postive for chemokine receptor expression and (B) the MFI of CCR2 and CCR5 on F4/80+CCR2+ cells and F4/80+CCR5+ cells are shown. The data represent the mean + 1 SD for 3 animals per group.

* $p < 0.05$

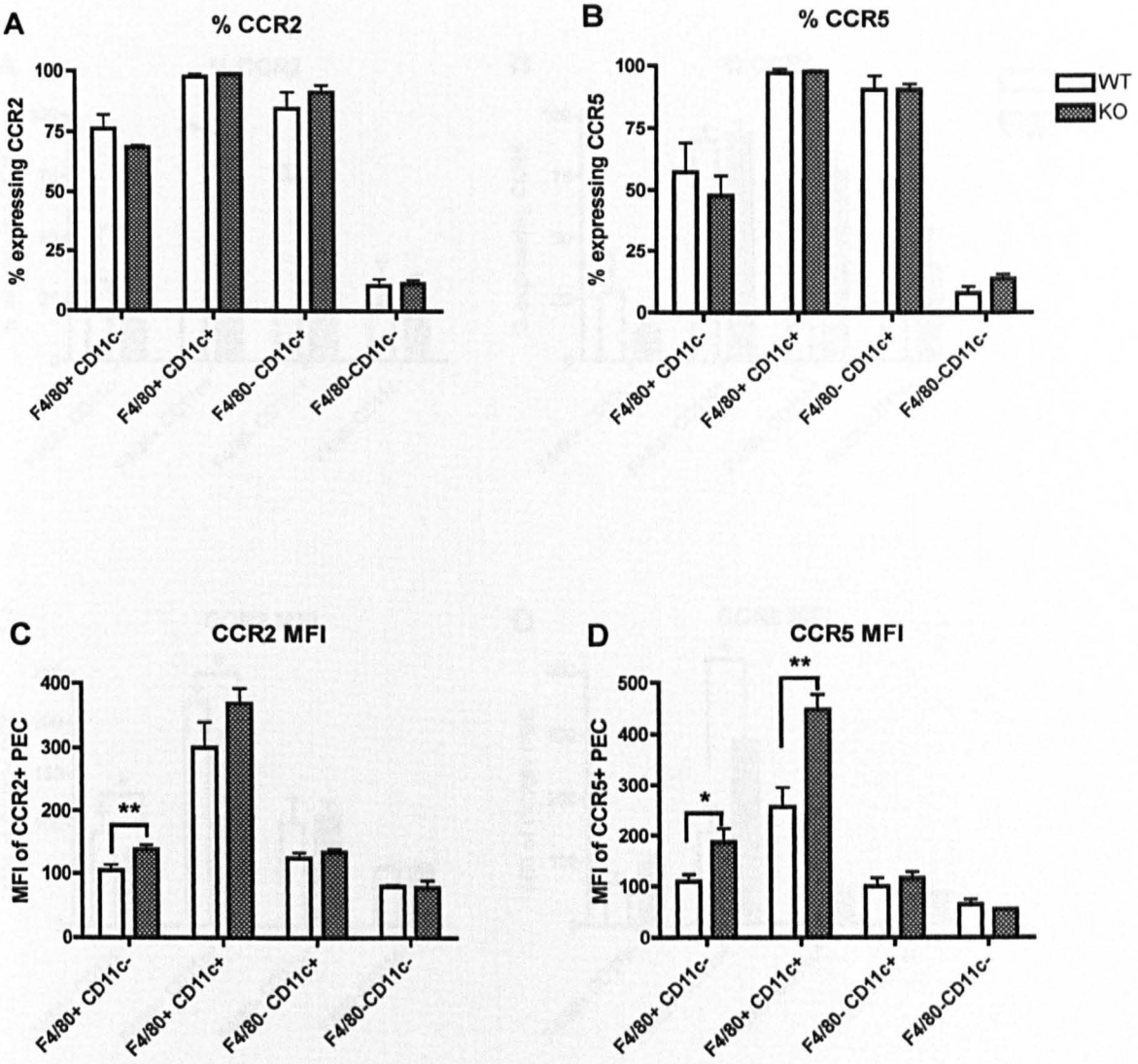


Figure 4.16

Chemokine Receptor Expression By Thioglycollate-Induced Peritoneal Exudate Cells

F4/80+ and CD11c+ cells obtained from the peritoneal cavity of WT and D6 KO mice at 24h post thioglycollate-injection were assessed by flow cytometry for the expression of (A, C) CCR2 or (B, D) CCR5. Figures A and B show the proportion of each subset positive for CCR2 or CCR5 expression, and figures C and D represent the MFI of the CCR2 or CCR5 positive cells. The data are the mean + 1 SD for 3 mice per group.

* $p < 0.05$, ** $p < 0.01$

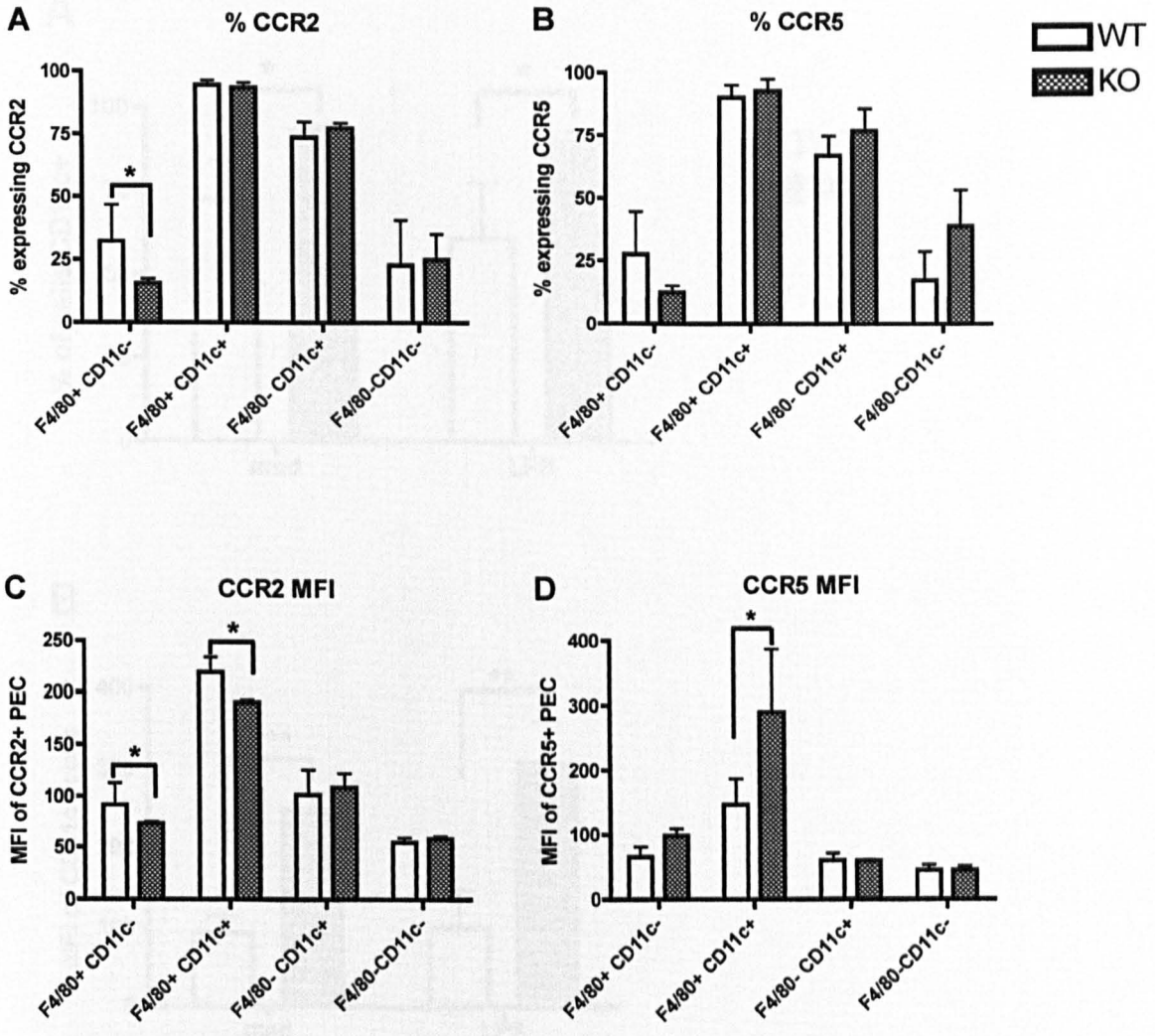


Figure 4.17

Chemokine Receptor Expression By Thioglycollate-Induced Peritoneal Exudate Cells

F4/80+ and CD11c+ cells obtained from the peritoneal cavity of WT and D6 KO mice at 96h post thioglycollate-injection were assessed by flow cytometry for the expression of (A, C) CCR2 or (B, D) CCR5. Figures A and B show the total percent of each subset positive for CCR2 or CCR5 expression, and figures C and D represent the MFI of the CCR2 or CCR5 positive cells. The data are the mean + 1 SD for 3 mice per group.

* $p < 0.05$

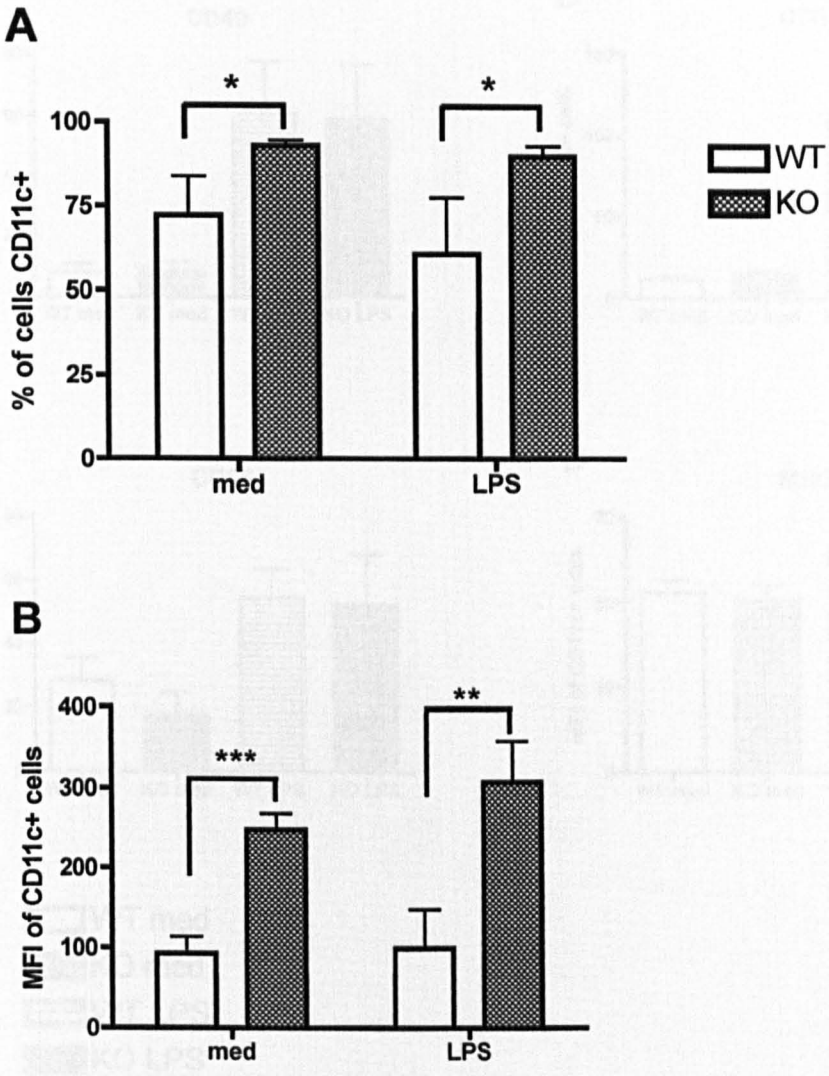


Figure 4.18

Development of BM Derived DC in D6 KO and WT Mice

BM cells from WT and D6 KO mice were cultured in GM-CSF for 7 days and harvested and cultured overnight in medium alone or in medium containing 1 μg/ml of LPS and the expression of CD11c was determined by flow cytometry. The proportion (A) and MFI (B) of CD11c+ cells is presented as the mean + 1 SD for 3 animals per group.

* $p < 0.05$, ** $p < 0.01$

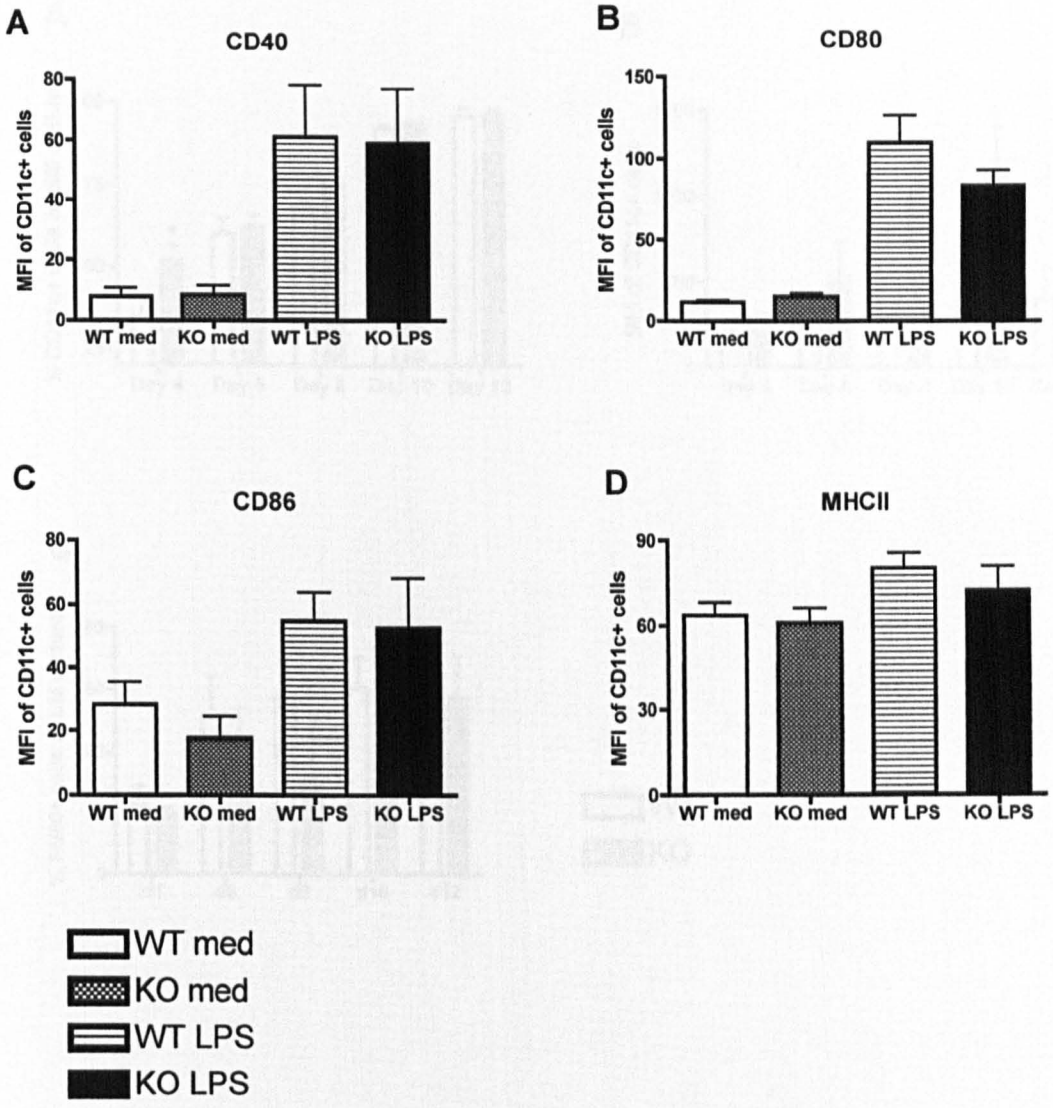


Figure 4.19

Activation of BM Derived D6 KO and WT DC by LPS

BM cells from WT and D6 KO mice were cultured in GM-CSF for 7 days and harvested and cultured overnight in medium alone or in medium containing 1 μg/ml LPS. The expression of (A) CD40, (B) CD80, (C) CD86 and (D) class II MHC on CD11c+ cells was determined by flow cytometry. The data represent the mean MFI + 1 SD of 3 animals per group.

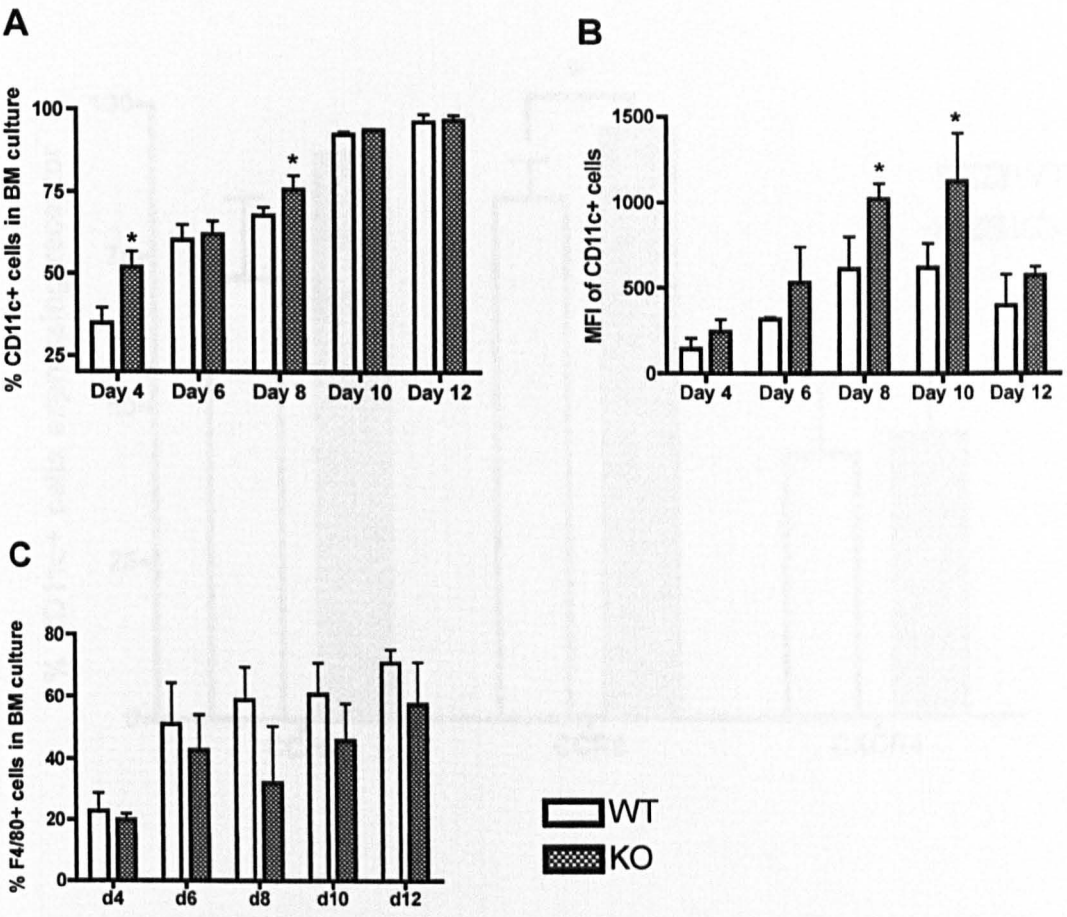


Figure 4.20

Time Course of Development of BM Derived DC in D6 KO and WT Mice

BM cells from WT and D6 KO mice cultured in GM-CSF were harvested at days 4, 6, 8, 10 and 12 of culture and the expression of CD11c and F4/80 was determined by flow cytometry. The results shown are the mean proportion (A) and MFI (B) of CD11c expression and the proportion of F4/80 expression (C) + 1 SD for 3 animals per group.

* $p < 0.05$

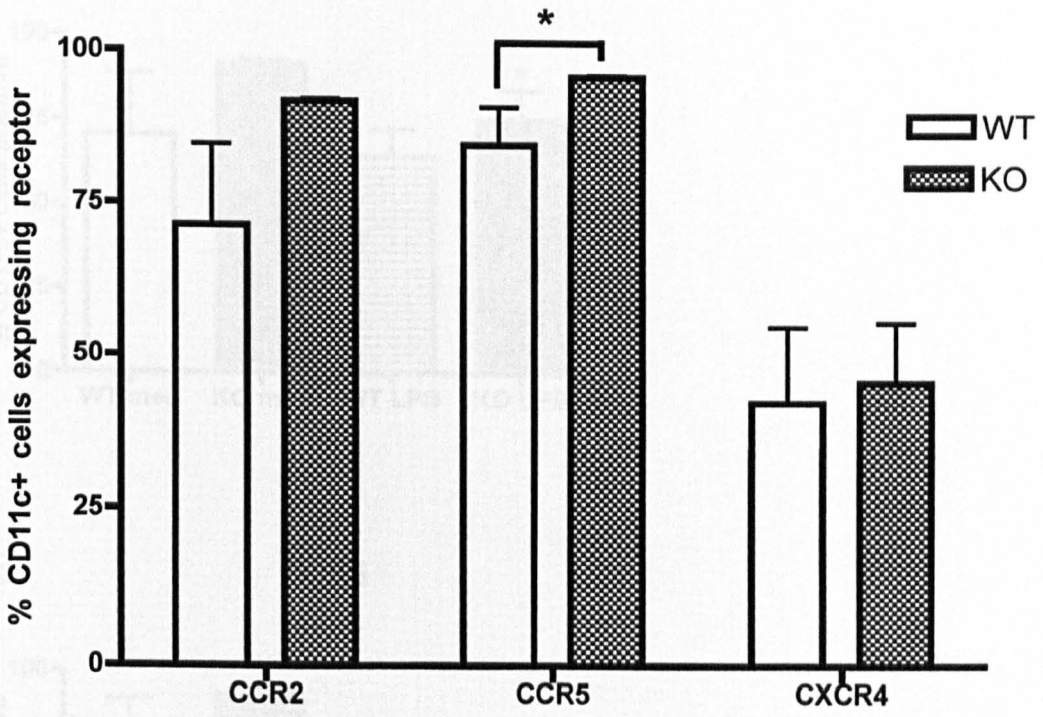


Figure 4.21

Chemokine Receptor Expression By BM Derived DC From D6 KO and WT Mice

BM cells from WT and D6 KO mice were cultured in GM-CSF for 7 days and the expression of CCR2, CCR5 and CXCR4 determined by flow cytometry. The results shown are the proportions of positive CD11c+ cells and are the means + 1 SD for 3 animals per group.

* $p < 0.05$

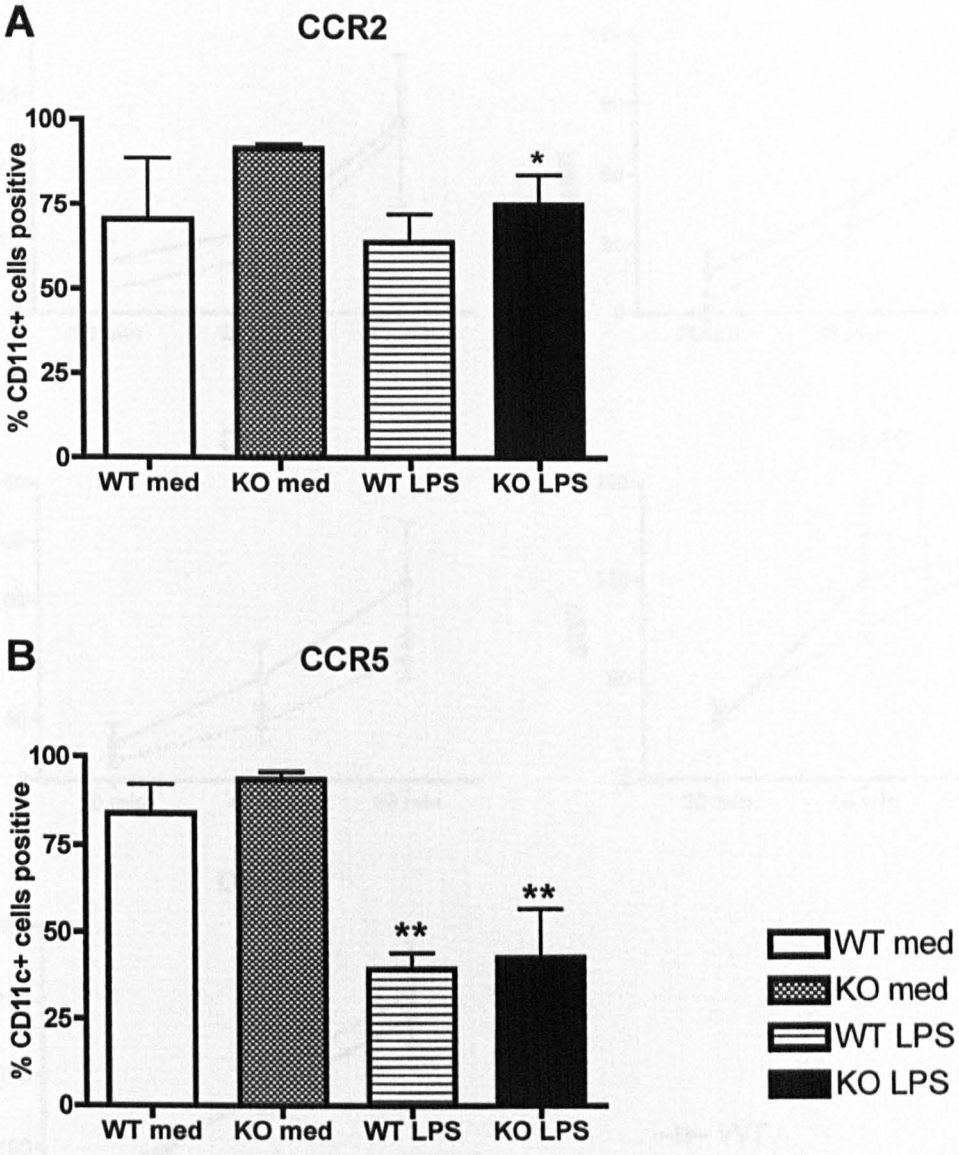


Figure 4.22

Chemokine Receptor Expression on BM Derived DC from D6 KO and WT Mice

BM-derived DC from WT and D6 KO mice harvested after 7 days of culture in GM-CSF were cultured overnight in medium or medium containing 1μg/ml LPS and the expression of (A) CCR2 and (B) CCR5 determined by flow cytometry. The results shown are the mean proportions of chemokine receptor positive cells in the CD11c+ population + 1 SD for 3 animals per group.

* $p < 0.05$ (med vs LPS),
** $p < 0.005$ (med vs LPS)

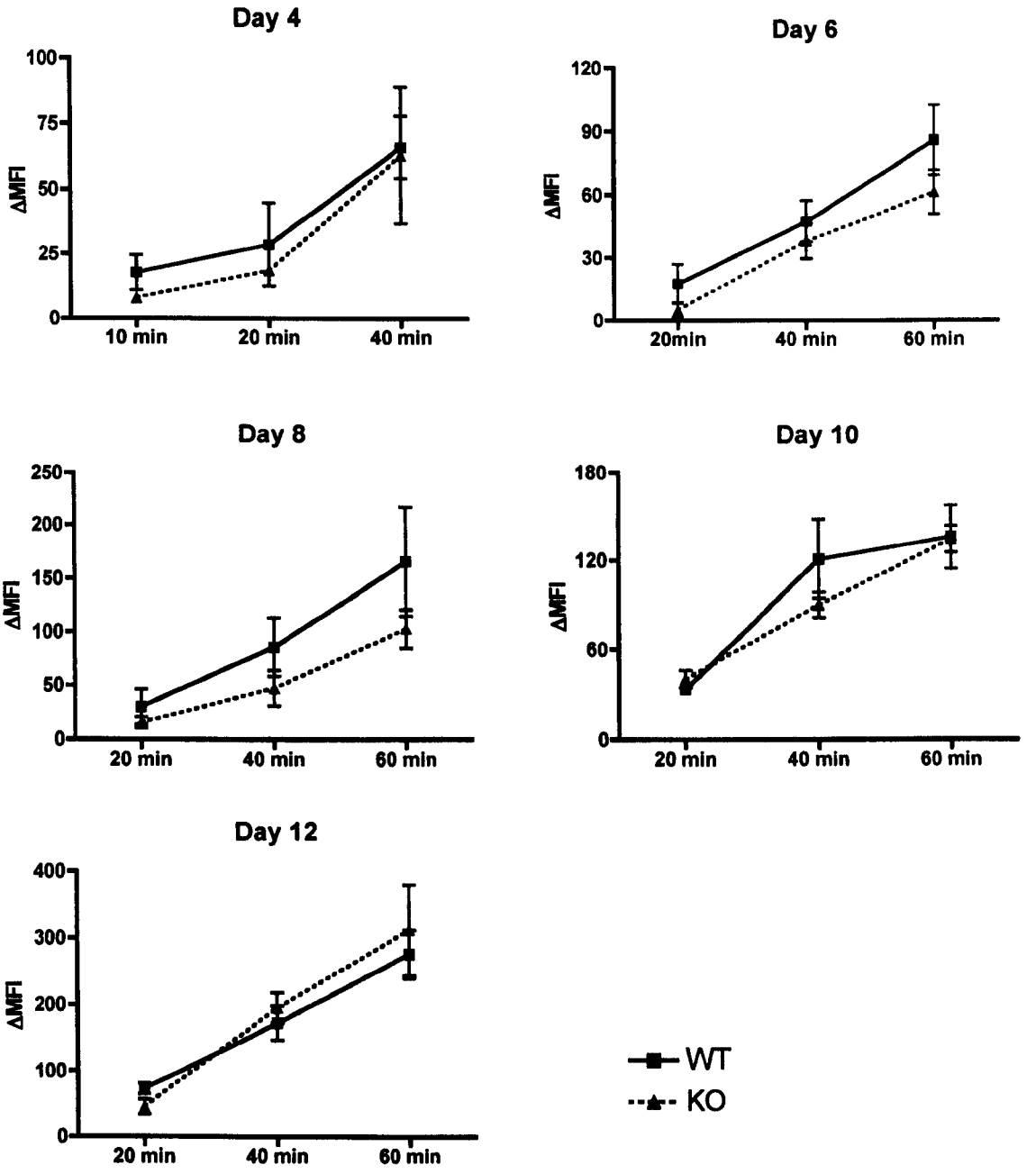


Figure 4.23

Endocytic Activity of BM Derived Dendritic Cells

BM cells from WT and D6 KO mice were cultured in GM-CSF for 4, 6, 8, 10 and 12 days, and then harvested and cultured with 1 mg/ml FITC-dextran at 37°C for 20 - 60 minutes. The specific uptake of FITC-dextran (ΔMFI) was calculated by subtracting the MFI of controls cultured with FITC-dextran at 4°C from the MFI readings obtained from the 37°C FITC-dextran cultures. The data are the means + 1 SD for 3 animals per group.

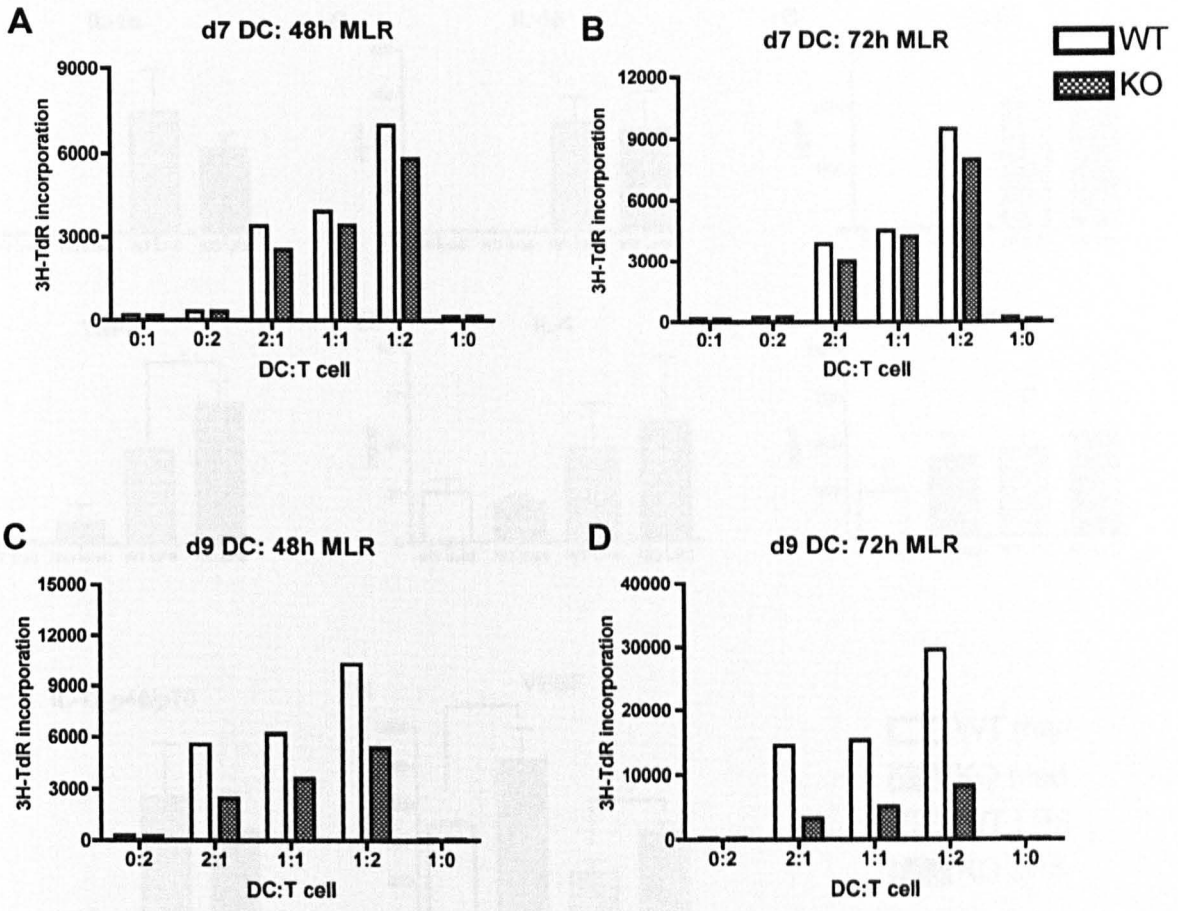


Figure 4.24

Antigen Presenting Cell Activity of BM Derived DC in MLR

BM-derived DC from B6/129 WT and D6 KO mice were harvested after 7 (A, B) and 9 (C, D) days of culture in GM-CSF, pre-treated with mitomycin C and then cultured for 48h (A, C) or 72h (B, D) with BALB/c responder lymphocytes (T) at the ratios indicated. ^{3}H-TdR was added for the last 20 hours of culture and the results shown are the mean for 2 animals per group.

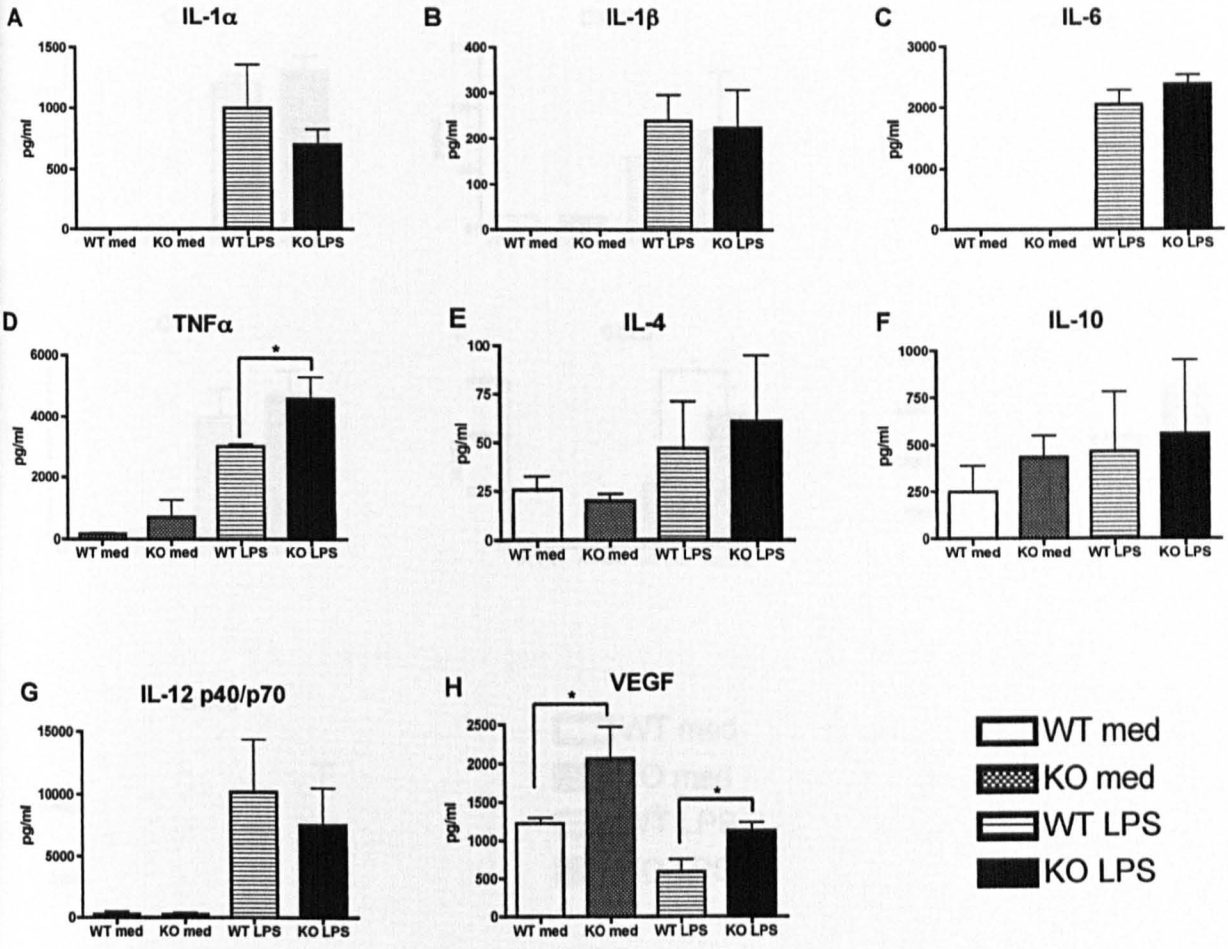


Figure 4.25

Cytokine Production by D6 KO and WT BM Derived DC

BM-derived DC from WT and D6 KO mice harvested after 7 days of culture in GM-CSF were cultured overnight in medium or medium containing 1μg/ml LPS. Levels of (A) IL-1α, (B) IL-1β, (C) IL-6, (D) TNFα, (E) IL-4, (F) IL-10, (G) IL-12 p40/p70 and (H) VEGF in culture supernatants were determined by Luminex analysis. The data are the means + 1 SD for 3 animals per group.

* $p < 0.05$

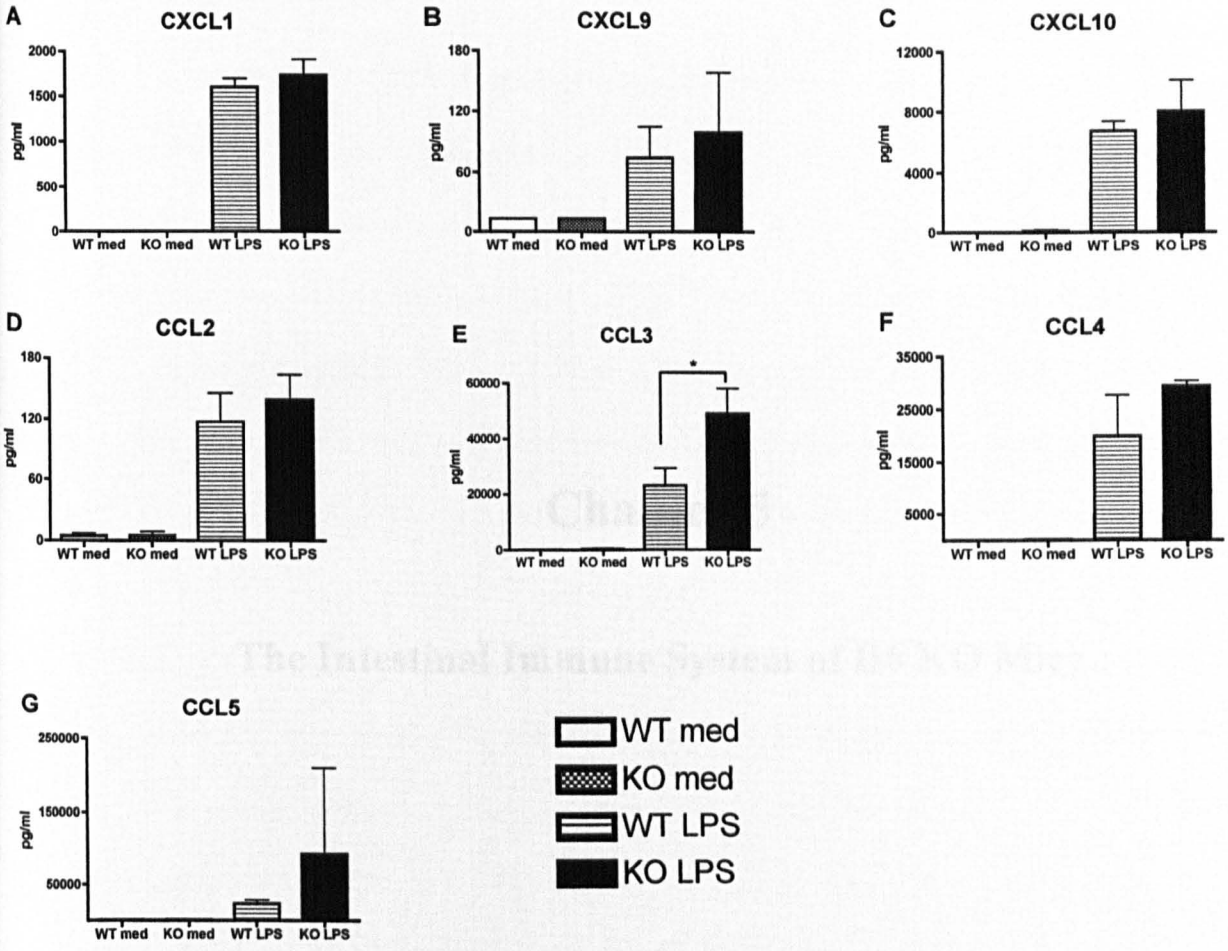


Figure 4.26

Chemokine Production by D6 KO and WT BM Derived DC

BM-derived DC from WT and D6 KO mice harvested after 7 days of culture in GM-CSF were cultured overnight in medium or medium containing 1μg/ml LPS. Levels of (A) CXCL1, (B) CXCL9, (C) CXCL10, (D) CCL2, (E) CCL3, (F) CCL4 and (G) CCL5 in culture supernatants were determined by Luminex analysis. The data are the means + 1 SD for 3 animals per group.

* $p < 0.05$

Chapter 5

The Intestinal Immune System of D6 KO Mice

Introduction

It is possible that excessive chemokine accumulation in the intestine may drive chronic immunopathology. Increased levels of inflammatory-type CC-chemokines have been detected in the colonic mucosa of ulcerative colitis and Crohn's patients (313-315). Moreover, mice with deletion of CCR2 or CCR5 are protected from experimental colitis (301). Conversely, these receptors have also been reported to be essential for development of tolerance following antigen feeding (19, 20, 297). Therefore proper regulation of the CC-chemokine family seems likely to be essential for homeostasis in intestinal tissue.

Although D6 is expressed abundantly by human intestinal lymphatics (287), the functional consequence of this remained unexplored. I thus set out to determine a role for D6 in the control of intestinal immunity, by comparing both tolerogenic and inflammatory responses in the intestines of WT and D6 KO animals. To achieve these aims, I utilised a high dose model of oral tolerance and attempted to induce intestinal inflammation by treating mice with indomethacin or dextran sulphate sodium (DSS).

5.1 Expression of D6 and D6 Ligands in the Murine Intestine

Initially, it was important to establish the sites of D6 expression in murine intestine. RNA was isolated from stomach, small intestine, mesenteric lymph nodes and Peyer's patches of WT mice and assessed for presence of D6 mRNA using RT-PCR. As shown in Figure 5.1, D6 mRNA was detected in stomach, jejunum, ileum, colon, MLN and PP, but not in duodenum. These data, whilst not quantitative, confirmed the presence of D6 in the mouse intestine.

5.2 Assessment of Oral Tolerance in D6 KO Mice

CCR2 and CCR5 have been reported to be essential for high dose oral tolerance (19, 20, 316), and since these receptors recognise several D6 ligands, I decided to examine whether the D6 chemokine receptor could influence this phenomenon. D6-deficient mice and control animals were fed either a single high dose of OVA (25mg) or saline, and seven days later, animals were challenged in the footpad with OVA /CFA. Tolerance induction was assessed by measurement of *in vitro* proliferation responses and cytokine production after restimulation of draining lymph node cells with OVA, and by examining the ability of animals to mount delayed-type hypersensitivity responses to OVA *in vivo*. Proliferation and in vivo responses were determined at two and three weeks following footpad challenge, respectively.

As shown in Figure 5.2, both WT and KO animals fed OVA prior to OVA/CFA challenge showed reduced OVA-specific proliferation and IFNγ production compared with saline controls. Responses of OVA-fed KO animals were comparable to those of the OVA-fed WT mice and there were also no differences between the control responses of both WT and KO mice (Figure 5.2).

In both WT and KO groups, DTH responses were reduced in animals fed OVA when compared with saline-fed controls (Figure 5.3 A). OVA-fed mice also had reduced levels of circulating OVA-specific IgG antibody, of both IgG1 and IgG2a classes. (Figures 5.3 B-D). Again, no differences were noted between the DTH and antibody responses in either saline or OVA fed KO and WT mice. Overall, these data indicated that the induction of oral

tolerance was normal in both D6 KO and WT animals, suggesting that oral tolerance induction is not dependent on the presence of the D6 chemokine receptor.

Investigation of Intestinal Inflammation in D6 KO Mice

Increased chemokine expression correlates with disease activity in IBD patients and a number of D6 ligands have been detected in the inflamed mucosa of mouse and man (302, 317, 318). By modulating CC-chemokine availability, D6 may play a role in regulating intestinal inflammatory responses. To explore this idea, I attempted to induce intestinal inflammation in D6 KO mice by administration of indomethacin or dextran sulphate sodium (DSS).

5.3 Indomethacin-Induced Enteritis

Indomethacin is a non-steroidal anti-inflammatory drug (NSAID) that can damage the GI tract of humans and rodents, leading to mucosal erosions and ulceration secondary to inhibition of the normally protective effect of prostaglandins on the epithelial barrier (319-324). WT and D6 KO mice received daily i.p. injections of indomethacin for two weeks, following which, sections of duodenum, jejunum, ileum and colon were assessed by microdissection for villus length, crypt length and mitotic activity. Control samples from PBS-challenged mice were also included.

As shown in Figure 5.4, there were no significant differences between WT and KO control mice in any of the parameters of intestinal morphology in any part of the intestine. These results suggested that no overt abnormalities in intestinal architecture arise as a result of D6-deficiency. Unfortunately, administration of indomethacin mice did not induce any

signs of damage in any part of the intestine, with villus lengths, crypt lengths and mitotic activity being similar to those in the same parts of the intestine in appropriate controls groups (Fig 5.4). There were also no differences between WT and KO mice given indomethacin. Thus WT and KO mice on the 129/B6 background were not susceptible to this particular model of intestinal inflammation.

5.4 Induction of DSS Colitis in D6 KO Mice

As neither WT nor KO mice showed susceptibility to indomethacin-induced injury, I next adopted a model of chemically induced colitis to explore the role of D6 in intestinal inflammation in these animals. Administration of the sulphated polysaccharide dextran sulphate sodium (DSS) to mice in drinking water results in the development of severe colonic inflammation with some of the characteristics of human ulcerative colitis (303). Increased levels of several CC-chemokines have been detected in colonic tissue of patients with inflammatory bowel disease (IBD) and animals deficient for CCR2 or CCR5 are reportedly resistant to DSS-mediated colitis ((325),(313),(318),(301)). I therefore predicted that D6 KO animals might show increased susceptibility to colitis following addition of DSS to their drinking water.

As anticipated, wild type mice given 2% DSS in the drinking water developed colitis, with most mice displaying clinical symptoms of diarrhoea and rectal bleeding (Figures 5.5 and 5.7 A). Moreover, DSS-treated WT mice demonstrated marked weight loss and most mice were moribund by day 7 (Figure 5.6 A). When these mice were sacrificed on day 8, they had significantly shorter colons than controls. (Fig 5.7 B)

Unexpectedly, the D6 KO recipients of DSS appeared to be less susceptible to the development of colitis compared with their WT counterparts (Figures 5.6 and 5.7). Diarrhoea and rectal bleeding became evident later and the clinical scores were significantly lower in D6 KO mice at all times (Figure 5.7 A). D6 KO animals also had significantly less weight loss than WT mice and more KO mice survived the duration of the experiment (Figures 5.6 A and B). Additionally, there was significantly less colonic shortening in D6 KO animals (Fig 5.7 B). Histological examination confirmed these results, as although both groups demonstrated colonic pathology and inflammation, pathology was significantly reduced in D6 KO mice, as determined by scoring of mucosal damage and inflammation (Figures 5.7 C and 5.8). Throughout the course of the experiment, water consumption was equivalent in both WT and KO groups, indicating that the reduced disease observed in the D6 KO mice was not a result of decreased DSS intake (Fig 5.6 C).

Repeated experiments, using either 2% or 2.5% gave similar results, although in some cases the differences observed between WT and D6 KO mice were less marked (Figures 5.6, 5.13 and 5.19). Taken together however, my data demonstrated that the absence of D6 appeared to ameliorate disease in this model of colitis.

5.5 Characterisation of Lamina Propria Infiltrates In Colitic Mice

The increased resistance of CCR2 and CCR5 KO mice to colitis development is associated with an altered mucosal infiltrate (301). I therefore set out to determine if differential cell recruitment, possibly resulting from an altered chemokine environment, might account for the reduced severity of colitis in D6 KO animals. Lamina propria cells were obtained from control and inflamed colons of both WT and KO mice by collagenase digestion, and the

relative proportions of CD4 T cells, CD8 T cells, B cells, neutrophils, macrophages and dendritic cells determined by flow cytometric analysis.

As shown in Figure 5.9 A, the total number of leukocytes retrieved from the colons of colitic mice increased markedly between days 2 and 7 after induction of disease. However, there were no significant differences between the total number of cells isolated from WT and KO mice at either time point following DSS administration (Fig 5.9 A). Phenotypic analysis of WT and KO LP cells from colitic mice revealed that, in both cases, F4/80+ macrophages were the predominant cell type, with dramatic increases between days 2 and 7 of disease (Fig 5.9 B). There were also significantly increased numbers of Ly6G+ neutrophils, T-cells and B-cells in the inflamed colon compared with controls (Fig 5.9 B). There were no statistically significant differences between the numbers of any of these populations in the lamina propria of WT and D6 KO mice at either day 2 or day 7.

5.6 Chemokine Receptor Expression by Lamina Propria Infiltrate Cells

I next assessed whether the inflammatory infiltrates of WT and KO mice might differ in terms of chemokine receptor expression, by analysing the expression of two functional receptors for D6 ligands, CCR2 and CCR5. As shown in Figure 5.10, the infiltrates of d7 colitic mice contained higher proportions of CCR2+ and CCR5+ cells compared with day 2 infiltrates. There were increases in the proportions of F4/80-negative CCR5+ cells in KO infiltrates compared with WT infiltrates, at both day 2 (4.5% ± 3.3 WT vs. 8.5% ± 4.2 KO, mean ± SD) and day 7 (10.2% ± 6.6 WT vs. 39.3 ± 24.9 KO), although these were not statistically significant.

To identify the particular cell subset responsible for the increased CCR5 positivity in KO cells, I next compared expression of CCR2 and CCR5 by CD4+, CD8+, B220+, F4/80+, CD11c and Ly6G+ cells from day 7 colitic mice. As shown in Figure 5.11 A, around half of all CD4+ cells from WT and KO colons expressed CCR2 or CCR5. Most CD8+ cells from both WT and KO colitic colons expressed CCR2 and CCR5 (Figure 5.11 B), and there were slightly higher proportions of CCR2+ and CCR5+ cells among the CD8+ cells from KO mice compared with WT (74.6 ± 6.7 vs. 64.4 ± 8.5) and (92.4 ± 3.6 vs. 82.4 ± 5.1), respectively, although the differences were not statistically significant.

Conversely, analysis of F4/80+, CD11c+, and Ly6G+ populations revealed higher expression of CCR2 and CCR5 by WT cells compared with KO cells (Figures 5.11 C-E). As seen in Figure 5.11 C, many F4/80+ cells from WT and D6 KO colons stained positive for CCR2, but significantly fewer KO F4/80+ cells were CCR2+ compared with WT F4/80+ cells (80.5% ± 7.9 vs. 62.6% ± 2.3). Less than half as many D6 KO F4/80+ cells expressed CCR5 compared with WT F4/80+ cells (56.9% ± 29.6 vs. 22.2% ± 5.8), although this difference was not statistically significant (Figure 5.11 A). Similarly, a consistently reduced proportion of CD11c+ lamina propria cells from colitic KO mice expressed CCR2 and CCR5 compared with their WT counterparts, although only the differences in CCR2 expression were statistically significant. Most Ly6G+ neutrophils from colitic mice were found to express CCR2, but again, fewer KO Ly6G+ cells were positive for this receptor, as well as for CCR5 compared with the WT Ly6G+ fraction, although these differences were not significant.

As shown in Figures 5.12A and B, almost all B220+ cells, from both WT and KO colon, expressed CCR2 (95.2% ± 0.5 cf. 95.8% ± 1.5, respectively). However, analysis of CCR5 expression by B220+ cells revealed a dramatic and significant increase in the expression of this receptor by KO B cells compared with WT B cells (Figure 5.12 A and C). Whilst less than half of B220+ cells from the colons of colitic WT mice expressed CCR5, almost all B220+ cells from KO animals were CCR5 positive. Analysis of LP cells from resting colons revealed that this phenotype was not specific to colitis, as B220+ cells isolated from untreated KO animals also showed increased expression of CCR5 compared with WT controls. These findings are explored in more depth in Chapter 6.

In summary, although WT and KO mice appeared to have similar overall proportions of T cells, B cells, macrophages and neutrophils in the DSS-subjected colon, the populations appeared to differ in terms of chemokine receptor expression, suggesting existence of more subtle differences between WT and KO colitic infiltrates.

5.7 Analysis of Cytokine Response to DSS

My studies so far had revealed altered chemokine receptor profiles of WT and D6 KO lamina propria cells retrieved from colitic mice. I next set out to determine whether the milder disease in D6 KO mice was associated with a different pattern of inflammatory cytokine production. Using Luminex technology, I measured the levels of multiple cytokines and chemokines in supernatants of colon explants taken from control and colitic mice. This was done at days 3 and 6 following DSS administration, when the experiment had to be terminated due to severe disease in the WT mice (Figure 5.13).

Consistent with previous studies (326), I found that the colons of colitic mice released significant quantities of the pro-inflammatory cytokines IL-1α, IL-1β, IL-6 and TNFα. These cytokines were not released, or released only in very low quantities by control colons, but were detectable after 3 days of DSS treatment and were present at high levels in d6 DSS colon cultures (Figure 5.14). Supernatants from the colons of DSS-treated mice also contained increased levels of IL-2, IL-12 (p40/p70), IL-13, IL-17 and IFNγ (Figure 5.15) as well as of the chemokines CXCL1, CXCL9, CCL2, CCL3, CCL4 and CCL5 (Figure 5.16) and the growth factors GM-CSF, FGF and VEG-F (Figure 5.17).

There were significantly elevated levels of TNFα, IFNγ and IL-17 in day 6 DSS KO cultures compared with WT cultures (Figures 5.14 D, 5.15 D and 5.15 E, respectively) and the higher levels of IFNγ and IL-17 correlated with reduced weight loss (Figure 5.18 A and B). A similar trend was seen for TNFα and weight loss although this was not statistically significant (Figure 5.18 C). These results suggested that the increased resistance to DSS-induced colitis in D6 KO mice was associated with higher production of the pro-inflammatory cytokines IFNγ, TNFα and IL-17. No statistically significant differences were seen in the levels of the other mediators between WT and KO supernatants in either baseline, day 3 DSS or day 6 DSS cultures (Figures 5.14, 5.15, 5.17).

As IL-23 has been reported to drive the development of IL-17 producing effector T cells (327), I examined the levels of IL-23 in WT and KO colon cultures by ELISA. As shown in Figure 5.15 F, substantial quantities of IL-23 were detected in supernatants of control colons from untreated WT and KO mice as well as in d3 DSS colons. However, the levels

of IL-23 then fell markedly on d6 of colitis and there were no differences between WT and KO colon cultures in terms of IL-23 production.

Following these initial findings, I carried out a repeat experiment to attempt to confirm the different pattern of cytokine production. On this occasion, I included an additional group that were given DSS for 3 days and then returned to water for 8 days, as I wished to compare the recovery response of D6 WT and KO animals. Under these conditions, D6 KO mice were again generally less susceptible than WT mice to colitis (Figure 5.19 A), although this effect was not as great as had previously been observed (Figures 5.6 and 5.13).

The 3 days DSS/ 8 days water group suggested some differences between WT and KO mice in their ability to recover from DSS-mediated intestinal damage (Figure 5.19 B). Out of 6 WT mice subjected to this protocol, one mouse died on day 6 and the other WT mice demonstrated maximum weight loss at days 6 and 7. However, by days 7 and 8, these mice began to recover weight and by the end of the experiment had virtually all returned to their original body weight.

In the D6 KO group, 3 out of 6 mice demonstrated no significant weight loss throughout the duration of the experiment. One mouse showed signs of weight loss by day 5 but recovered weight thereafter to reach its original body weight by the end of the experiment. The remaining two KO mice showed signs of weight loss by day 5 and unlike their WT counterparts, these animals continued to lose weight until the end of the experiment on day 11. Whilst the group sizes were relatively small, these data suggested that although D6 KO

mice may be less susceptible to induction of DSS-colitis, once ill, these animals might show poorer recovery from DSS-mediated damage compared with their WT counterparts.

Supernatants were collected from d3 and d7 colon cultures (normal DSS protocol) and from d11 (DSS recovery protocol) colon cultures and, as before, analysed by Luminex for cytokine and chemokine levels. In agreement with my initial findings, DSS treatment led to high levels of IL-1α, IL-1β, IL-6 and TNFα in d7 DSS colon cultures, as well as IL-2, IL-12 (p40/p70), IL-13, IL-17, IFNγ, CXCL1, CXCL9, CCL2, CCL3, CCL4, CCL5 and the growth factors GM-CSF, FGF and VEG-F (Figures 5.20 – 5.23). The levels of cytokines and chemokines were comparable in the two experiments.

However, in contrast to my earlier analysis, there were no significant differences in the levels of any of the mediators secreted from the colon explants by WT and KO mice on day 7. Specifically, although I had previously detected significantly higher IFNγ, IL-17 and TNFα levels in D6 KO colon cultures taken on d6 compared with WT, the levels of all these cytokines were comparable in WT and KO cultures in this second experiment (Figures 5.21 E and F and 5.20 D).

It was possible that the failure to detect differences in these colon cultures was due to the greater variability between disease in WT and KO mice in this particular DSS experiment. In the initial cytokine analysis experiment all WT mice had developed severe disease and extensive weight loss in response to DSS, while KO animals showed only minimal weight loss and few clinical symptoms. On the other hand, in my repeat experiment some of the WT mice remained relatively healthy following DSS treatment, while some of the D6 KO

mice developed more severe colitis (Fig. 5.19). Therefore I decided to pool the luminex results from all colitic mice and compare cytokine production with disease activity, using body weight as an indicator of the latter.

These analyses revealed statistically significant inverse correlations between the levels of IL-17, IFNγ and IL-12 (p40/p70) in colon cultures and weight loss, with the highest levels of these cytokines being present in the mice that showed the least weight loss following DSS administration (Figures 5.24 A - C). A similar trend was seen with TNF production, although this did not achieve statistical significance (Figure 5.25 D). On the other hand, the levels of GM-CSF showed a significant positive correlation with weight loss, as did IL-6 production, although again this was not statistically significant (Figure 5.24 D). There was no association between weight loss and IL-1 levels in colon cultures (Figure 5.25 A and B), or with weight loss and the levels of IL-2, IL-13, IL-23, FGF and VEGF (data not shown). When chemokine levels were analysed in this way, higher CXCL1 correlated significantly with weight loss (Figure 5.26 A) and similar but non-significant trends were found between the CC-chemokines CCL2, CCL3, CCL4 and CCL5and weight loss (Figures 5.26 C – F). There was no correlation between CXCL9 levels and weight loss (Figure 5.26 B).

In summary, my data from the cytokine analysis revealed that higher secretion of the pro-inflammatory cytokines IL-17, IFNγ and IL-12 (p40/p70) was associated with less weight loss in response to acute DSS-colitis and provided some preliminary evidence that the increased resistance demonstrated by D6 KO animals might be associated with an increased ability to generate these cytokines.

5.8 Intracellular Cytokine Analysis of LP Infiltrate

To extend these findings regarding IFNγ and IL-17 levels in colon cultures, I next attempted to analyse intracellular production of these cytokines by colonic LP cells. Cells isolated from the lamina propria of day 7 colitic WT and KO mice were cultured with ionomycin, PMA and Brefeldin A, and following permeabilisation and staining with fluorescently-conjugated antibodies, they were assessed by flow cytometry for presence of intracellular IFNγ or IL-17. As shown in Figure 5.27, only a small percentage of colonic LP cells stained positively for intracellular IL-17 and no difference was found between WT and KO samples. A higher proportion of LP cells were found to stain positive for IFNγ expression, but again there were no differences between WT and KO samples.

5.9 Assessment of Colonic Epithelial Cell Turnover

My cytokine analyses had demonstrated a trend for increased production of certain inflammatory type cytokines, most notably IL-17 and IFNγ, in the colons of mice which were relatively resistant to DSS colitis. However, it was still unclear if or why this altered cytokine environment reduced susceptibility to DSS. Although the exact mechanism by which DSS invokes experimental colitis has not been defined, it is thought to be directly toxic to intestinal epithelial cells, with the inflammatory response developing secondary to a loss of barrier function (304, 305). Therefore, one possible explanation for my findings could be that the epithelium of D6 KO mice was inherently less susceptible to DSS-induced damage, or repaired more efficiently. I therefore compared epithelial cell turnover in WT and D6 KO mice by analysing the incorporation of bromodeoxyuridine (BrdU) over a period of 24 hours. This was assessed in untreated mice, mice that had received DSS for 3 days, mice that had received DSS for 5 days followed by water for 2 days, and mice that

had received DSS for 3 days and water for 8 days. The latter group was included to examine the recovery phase from colitis.

As previously reported (328-330), DSS administration for 3 days resulted in a marked reduction of dividing cells in the colonic crypts of WT mice (Figures 5.28 and 5.29). Recovery was evident, although variable, in WT animals by day 7 and the rates of BrdU incorporation had returned to normal by day 11 despite evidence of continuing severe architectural damage. D6 KO mice showed significantly lower epithelial cell turnover than WT mice under resting conditions and only a small further reduction on day 3, which was not statistically significant. Rates of epithelial turnover remained low in D6 KO animals at day 7, but higher epithelial proliferation, comparable to that observed in WT controls, was evident in KO colons by day 11.

Overall, these data suggest that the absence of D6 can lead to aberrant epithelial turnover in the large intestine. Since DSS can be directly toxic to dividing epithelium, a reduced baseline rate of epithelial proliferation might lessen the negative impact of DSS administration (331). Therefore lower turnover of the epithelium in the resting D6 KO colon might account for the reduced susceptibility of these animals to DSS-induced damage.

Summary

In this chapter, I explored the function of the D6-chemokine receptor in both tolerogenic and inflammatory intestinal responses. D6 and at least 7 of its ligands were expressed in the normal murine intestine, but the absence of D6 did not affect the induction of oral

tolerance. Somewhat unexpectedly, D6 KO mice demonstrated increased resistance to acute colitis induction following oral administration of DSS. Whilst no differences were detected between WT and D6 KO mice in the recruitment of various leukocyte populations to the inflamed colon, cellular infiltrates of WT and KO animals were found to differ in terms of CC-chemokine receptor expression. Reduced susceptibility to acute-DSS colitis was found to correlate with increased *ex vivo* secretion of several inflammatory cytokines, most notably IL-17 and IFNγ, by cultured explanted colons. Additionally, there were significant differences between the proliferation of epithelial cells in resting colonic tissue of D6 KO mice compared to WT mice. If confirmed, these data suggest that alterations in inflammatory-cytokine production and innate barrier function may account for the increased resistance of D6 KO animals to DSS-induced colitis.

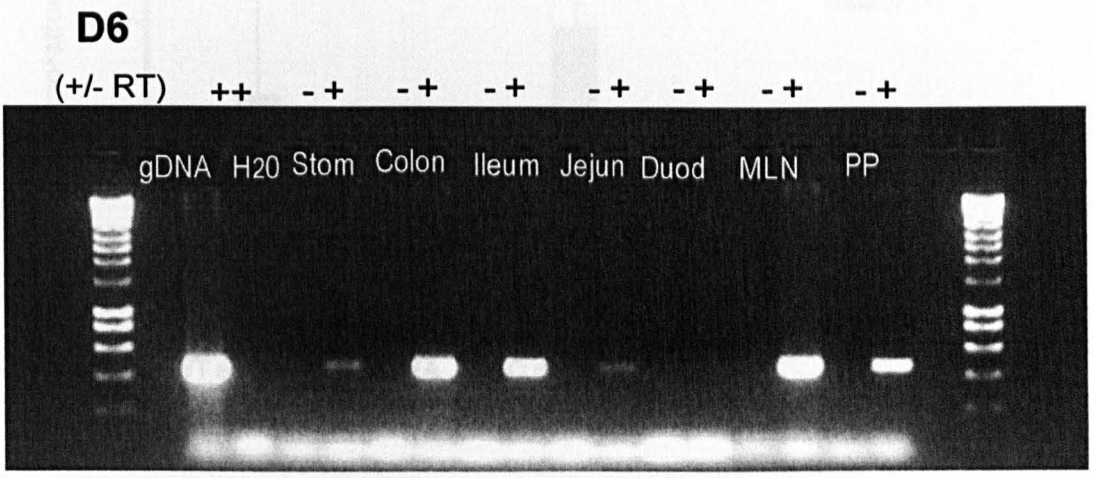


Figure 5.1

D6 mRNA Expression In Intestinal Tract

RNA was isolated from stomach, colon, ileum, jejunum, duodenum, MLN and PP of 129/B6 WT mice and the presence of D6 mRNA determined by RT-PCR in the presence (+) or absence (-) of reverse transcriptase(RT). Mouse genomic DNA (gDNA) was also analysed by RT-PCR as a positive control.

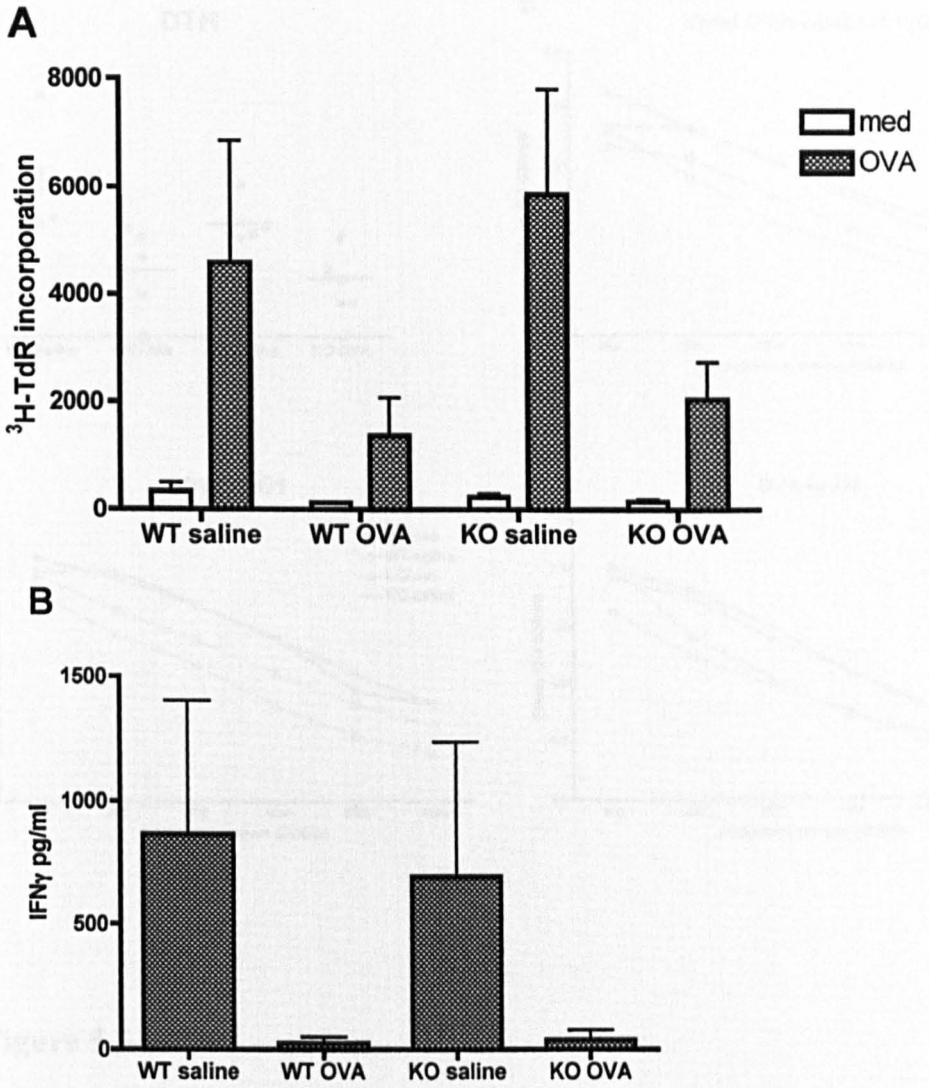


Figure 5.2

Induction of Oral Tolerance in D6 KO and WT Mice

WT and D6 KO mice were fed 25mg OVA or saline alone and seven days later were immunised in the footpad with OVA/CFA. Two weeks later, the popliteal lymph nodes draining the site of immunisation were harvested and 2 x 10^5 LN cells stimulated with 1 mg/ml OVA in complete medium for 120 hours. Antigen-specific proliferation responses were determined after 120 hr by ^{3}H-Tdr incorporation (A) and the levels of IFNγ in culture supernatants were measured by ELISA after 48h (B). The data represent the means + 1 SD for 3-4 mice per group.

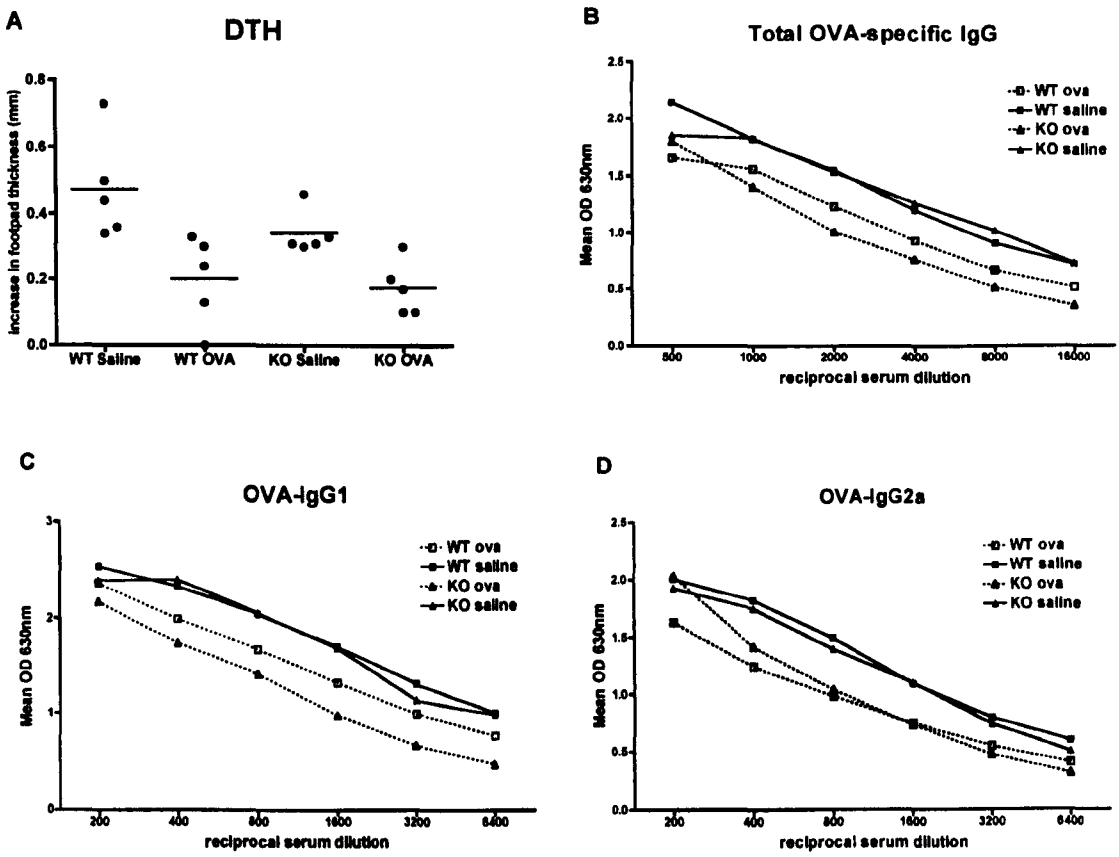


Figure 5.3

Assessment of Oral Tolerance Induction in D6 KOs

WT and D6 KO mice were fed 25mg OVA or saline alone and seven days later were immunised in the footpad with OVA/CFA. Two weeks later, mice were challenged in the opposite footpad with heat-aggregated OVA and their ability to mount DTH responses assessed by the increase in footpad thickness 24 hr later (A). The levels of OVA-specific IgG (B), IgG1 (C) and IgG2a (D) antibodies were assessed in serum by ELISA. The data are the means + 1 SD for 5 animals per group.

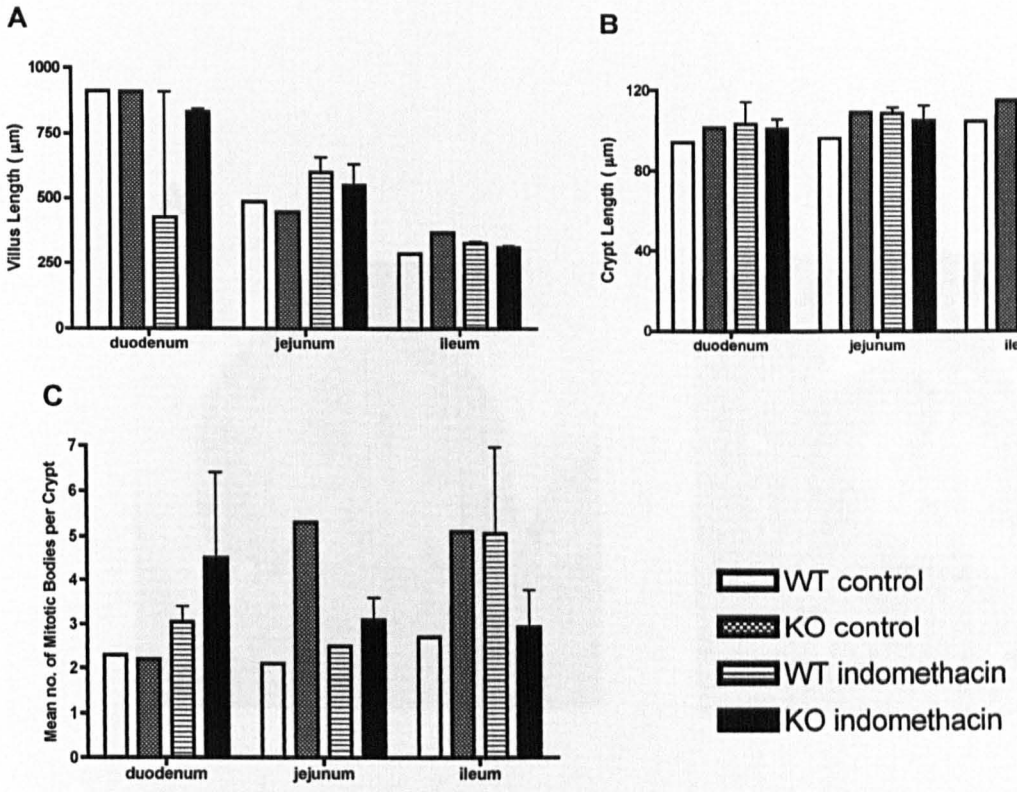


Figure 5.4

Intestinal Architecture in Indomethacin Treated Mice

WT and D6 KO mice received daily i.p. injections of 2.5 mg/kg indomethacin for 14 days and sections of duodenum, jejunum, ileum and colon were assessed by microdissection for (A) villus length, (B) crypt length and (C) mitotic activity. The data shown are the means + 1 SD for 3 animals per group (indomethacin treated). Control samples from 1 PBS fed WT and KO are also shown.

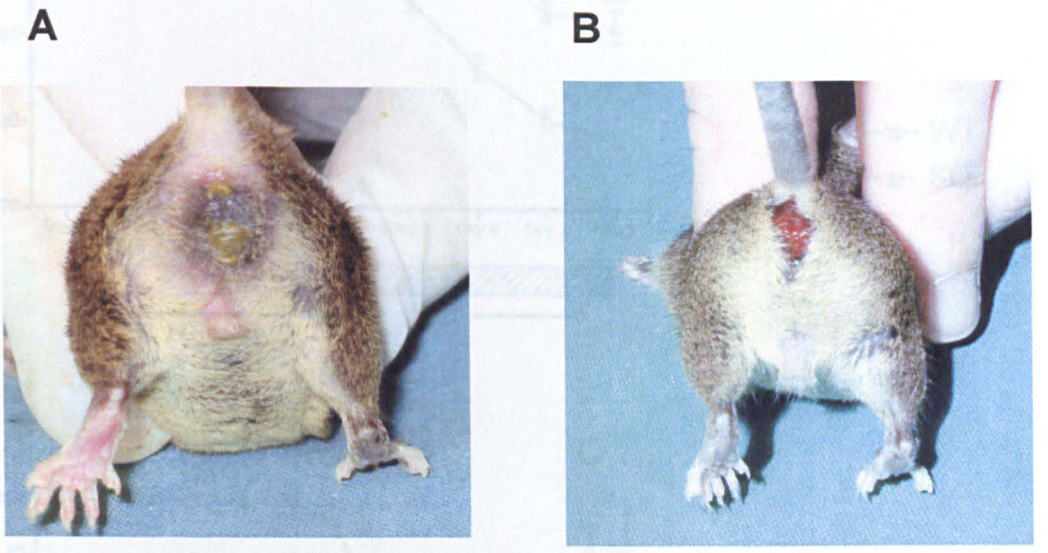


Figure 5.5

Clinical Disease In DSS Colitis

WT mice that received 2% DSS in their drinking water typically showed clinical symptoms of colitis, namely diarrhoea (A) and rectal bleeding (B), by day 4. The development of clinical symptoms of colitis was delayed in DSS-treated D6 KO mice and, overall, diarrhoea and rectal bleeding were less pronounced in D6 KO mice compared to their WT counterparts.

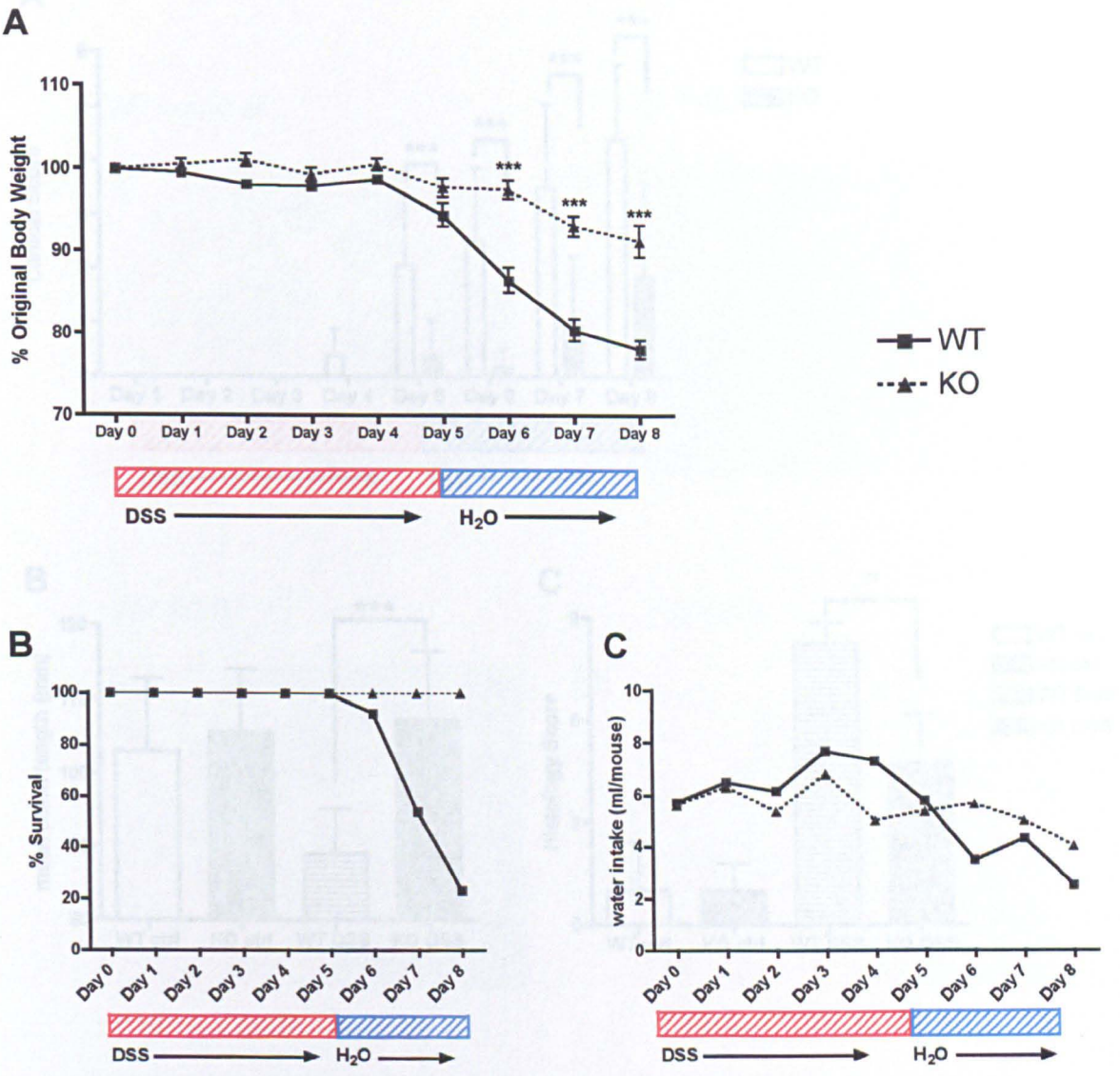


Figure 5.6

Induction of DSS Colitis in D6 KO and WT Mice

WT and D6 KO mice received 2% DSS in their drinking water for 5 days and normal water thereafter. Body weight (A), survival (B) and water intake (C) were assessed daily. The data in (A) are the means ± 1 SEM for 13 animals per group. The data in (B) and (C) represent the means for 13 mice per group.

*** $p < 0.001$

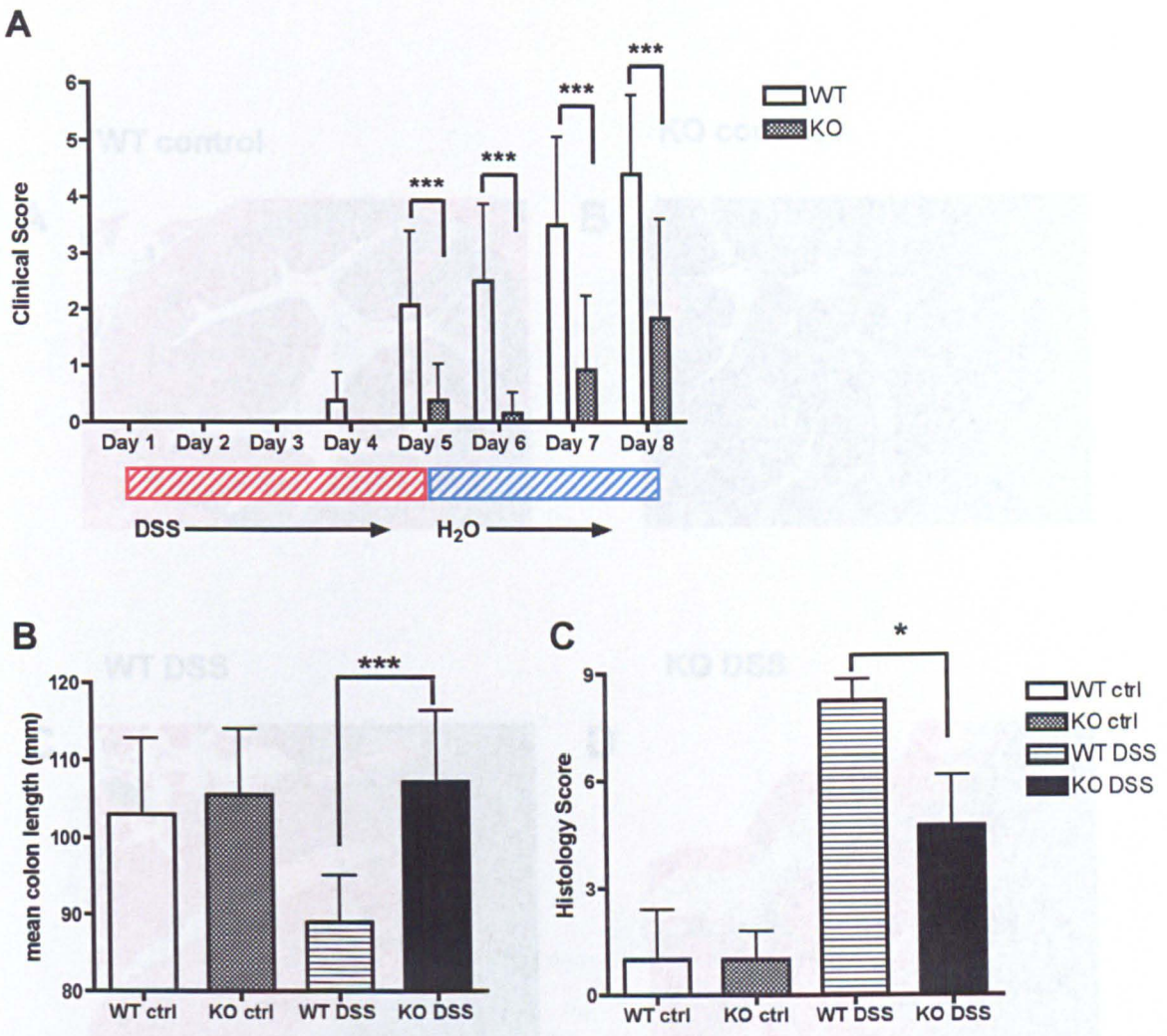


Figure 5.7

Induction of DSS Colitis in D6 KO and WT Mice

WT and D6 KO mice received 2% DSS in their drinking water for 5 days and normal water thereafter. Clinical disease (A) was assessed daily by monitoring diarrhoea (score of 0-2) rectal bleeding (score of 0-2) and general appearance (score of 0-2). After 8 days, colons were excised, their lengths were recorded (B) and the tissues were fixed in 10% formalin, embedded in paraffin and stained with haemtoxylin and eosin for histological scoring (C). Scoring was performed in a blinded fashion using a combined score of inflammatory cell infiltration (0-5) and tissue pathology (0-5) The data are the mean + 1 SD for 13 animals per group (A,B) and for 4-8 animals per group (C).

* $p < 0.05$, *** $p < 0.001$

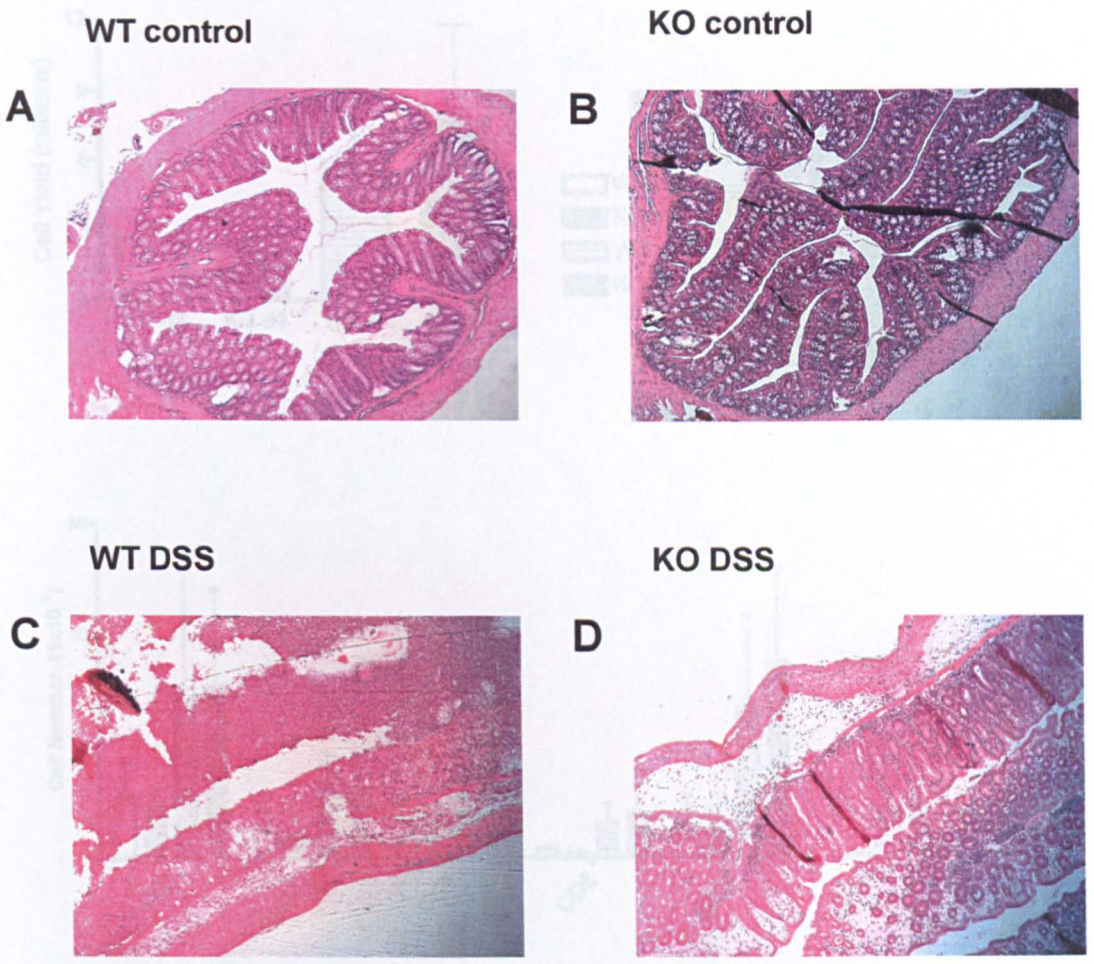
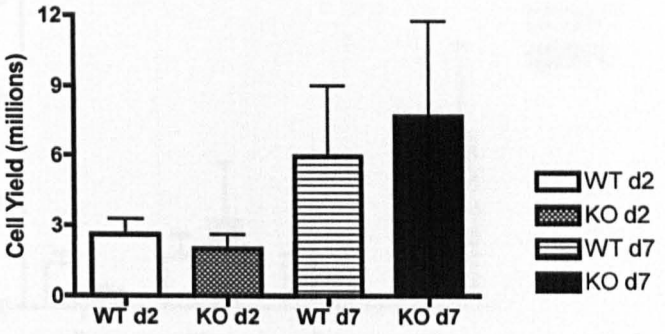


Figure 5.8

Histology of Colons from DSS-treated WT and D6 KO Mice

WT and D6 KO mice received 2% DSS in their drinking water for 5 days and normal water thereafter (see Fig. 5.7). Control mice received water alone. At day 8, excised colons were fixed in 10% formalin, embedded in paraffin and stained with haematoxylin and eosin for histological analysis. Shown above are representative histology from WT and D6 KO control mice (A and B) and from DSS-treated WT and D6 KO mice (C and D). The epithelium of the DSS-treated WT mouse (C) is characterised by complete ulceration and crypt loss. By contrast, the pathology observed in the D6 KO colonic epithelium (D) is less severe, although crypt hyperplasia, goblet cell loss and inflammatory cell infiltration are evident and indicate DSS-induced damage. Magnification x 200.

A



B

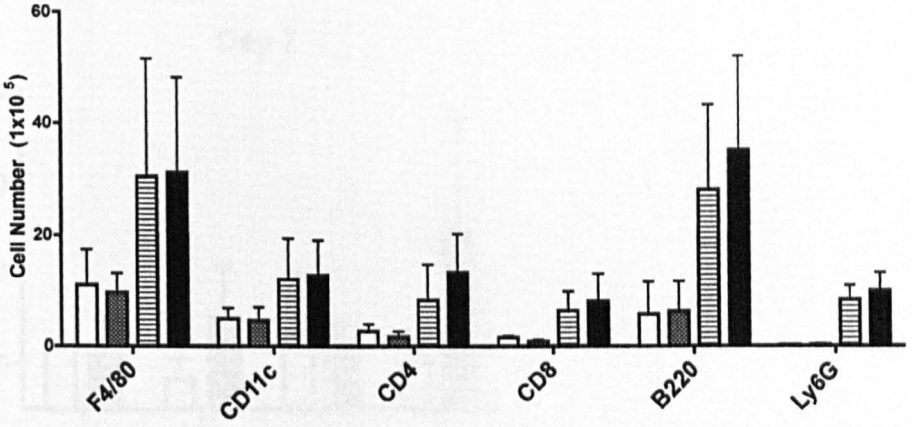


Figure 5.9

Lamina Propria Infiltrates During DSS Colitis in D6 KO and WT Mice

WT and D6 KO mice received 2% DSS in their drinking water for 5 days and normal water thereafter. At days 2 and 7, lamina propria cells were isolated, total cell counts determined (A) and the proportions of F4/80+, CD11c+, CD4+, CD8+, B220+ and Ly6G+ cells assessed by flow cytometry (B). The results are the means + 1 SD for 3 mice per group.

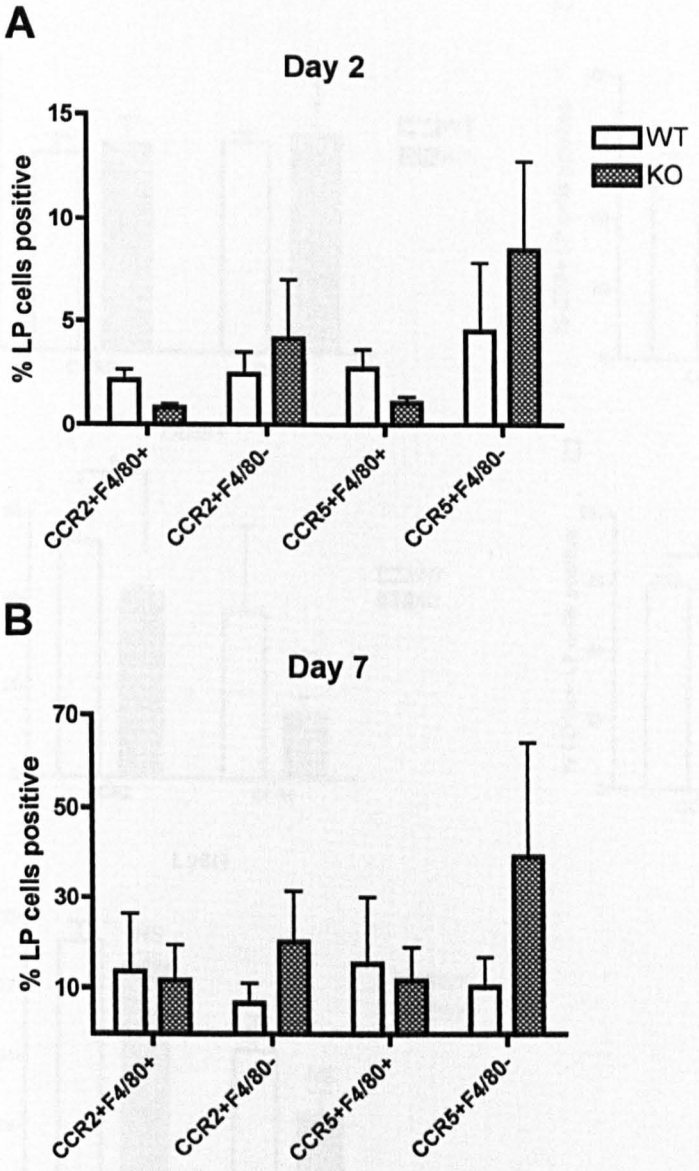


Figure 5.10

Chemokine Receptor Expression By Lamina Propria Cells in DSS Colitis

WT and D6 KO mice received 2% DSS in the drinking water for 5 days and normal water thereafter. At days 2 and 7 (A and B, respectively) lamina propria cells were isolated and the expression of F4/80, CCR2 and CCR5 assessed by flow cytometry. The data are the means + 1 SD for 3 mice per group.

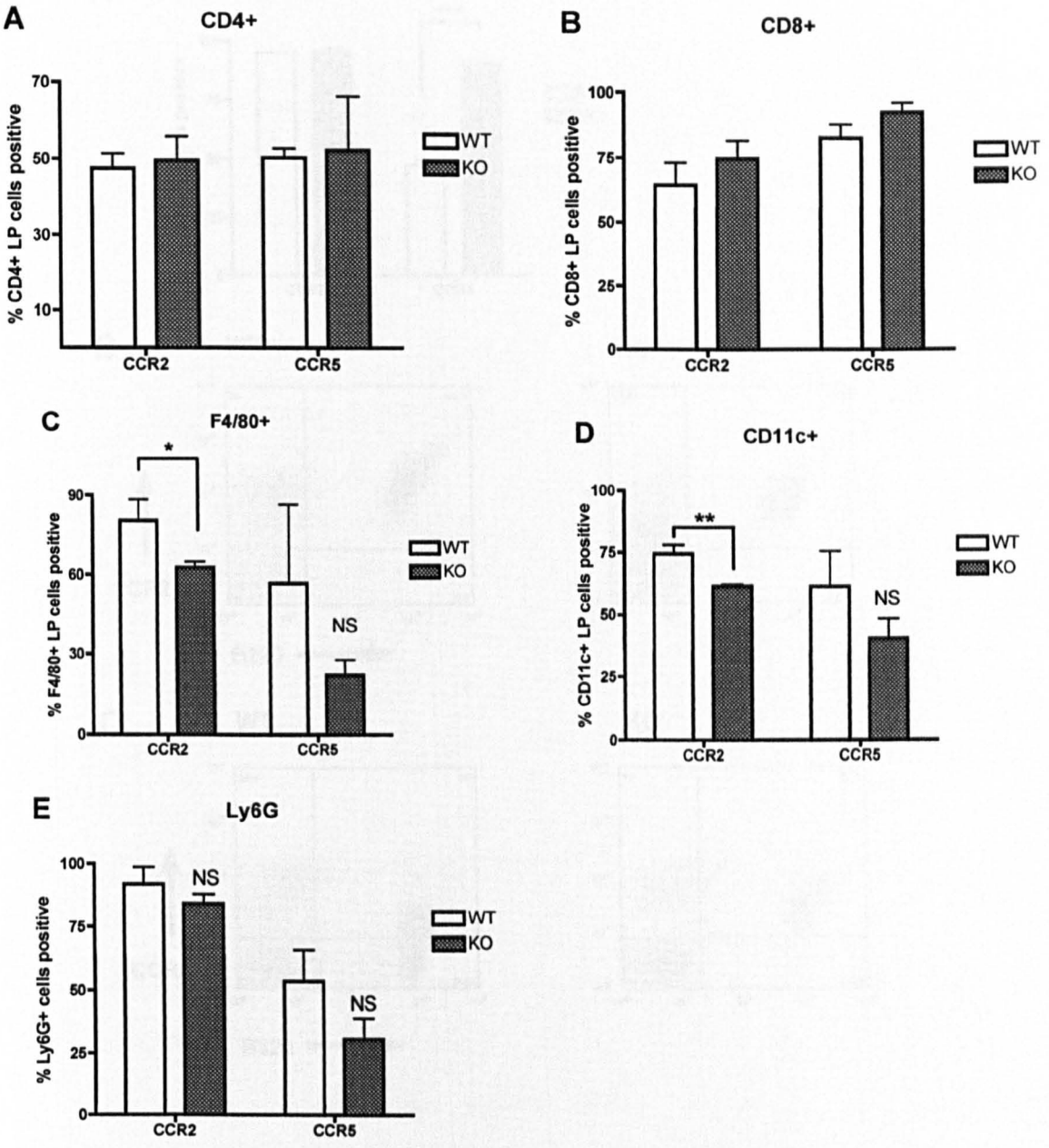


Figure 5.11

Chemokine Receptor Expression By Colonic Lamina Propria Infiltrates of DSS Colitic Mice

WT and D6 KO mice received 2% DSS in their drinking water for 5 days and normal water thereafter. Colonic lamina propria cells were isolated on day 7 and the expression of CCR2 and CCR5 by (A) CD4+, (B) CD8+, (C) F4/80+, (D) CD11c+ and (E) Ly6G+ cell populations was assessed by flow cytometry. Data are presented as means + 1 SD for 3 mice per group.

* $p < 0.005$, ** $p < 0.001$

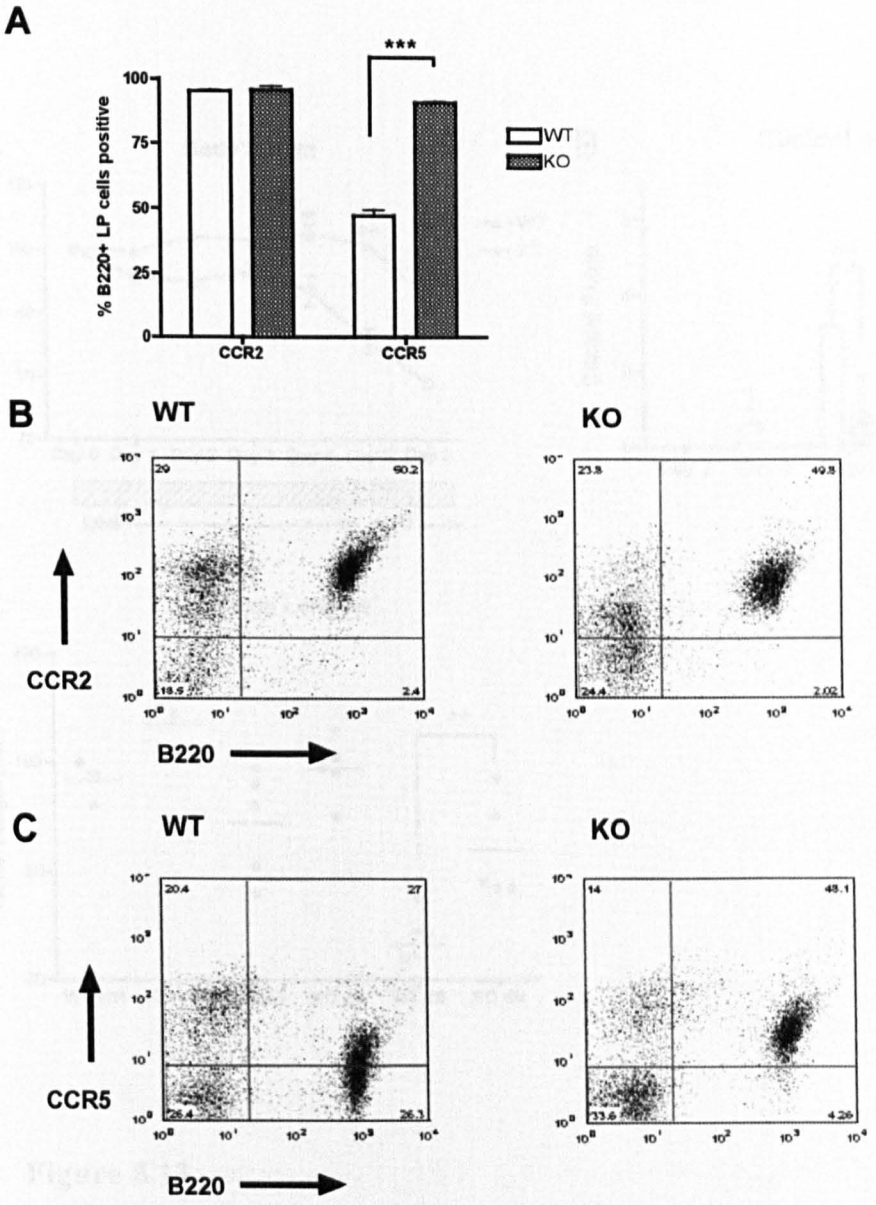


Figure 5.12

Chemokine Receptor Expression By Colonic Lamina Propria B cells during DSS Colitis

WT and D6 KO mice received 2% DSS in their drinking water for 5 days and normal water thereafter. Colonic lamina propria cells were isolated on day 7 and the expression of CCR2 and CCR5 on B220+ B cells was assessed by flow cytometry. (A) The mean proportions + 1 SD of chemokine receptor expressing B cells for 3 mice per group. (B, C) Representative FACS plots of CCR2 (B) and CCR5 (C) expression by WT (left) and KO (right) B220+ lamina propria B cells.

*** $p < 0.0001$

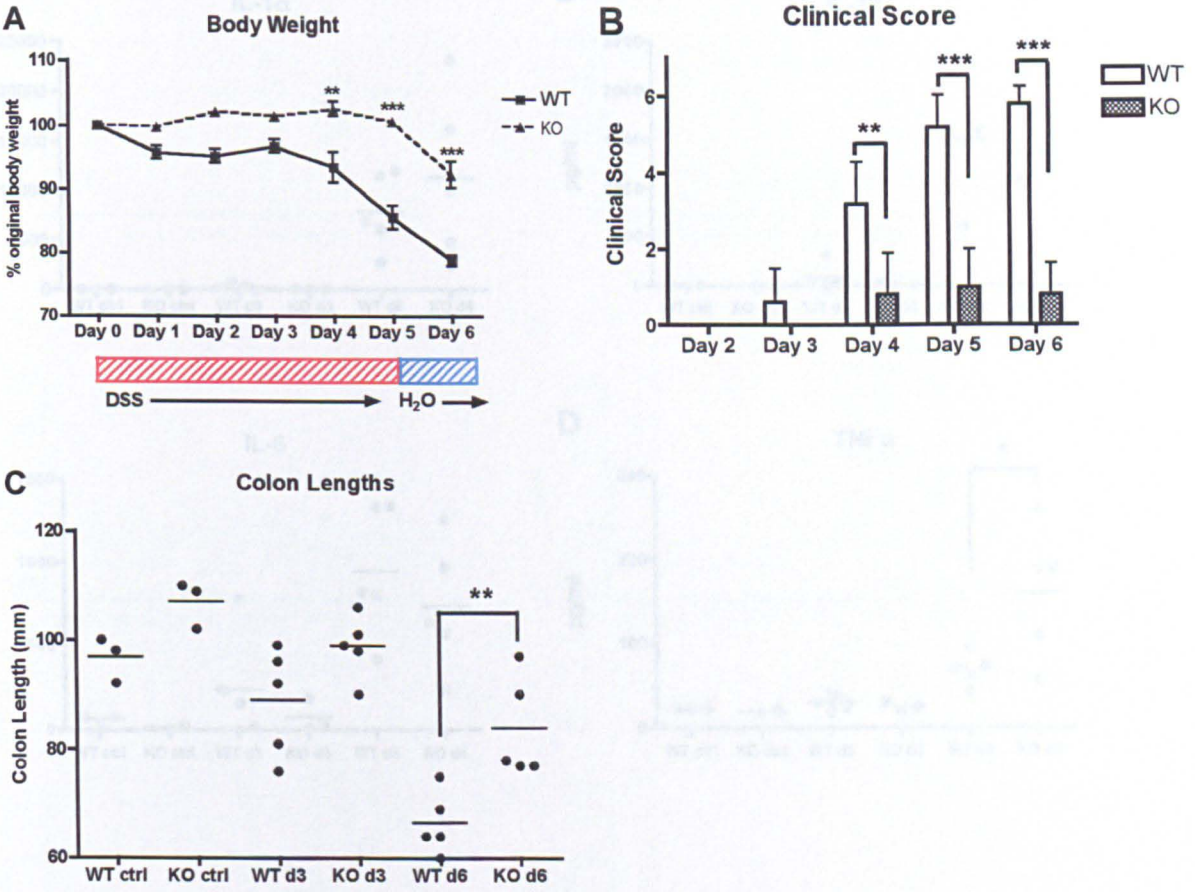


Figure 5.13

Induction of DSS Colitis in D6 KO Mice

WT and D6 KO mice (n = 10) received 2% DSS in their drinking water for 5 days and normal water thereafter. Body weight (A) and clinical disease (B) were monitored daily and are presented as the means ± 1 SEM (A) and as the means + 1 SD (B) for 5-10 mice. On days 3 and 6, five mice from each group were culled and whole colon lengths measured (C).

** $p < 0.01$, *** $p < 0.001$

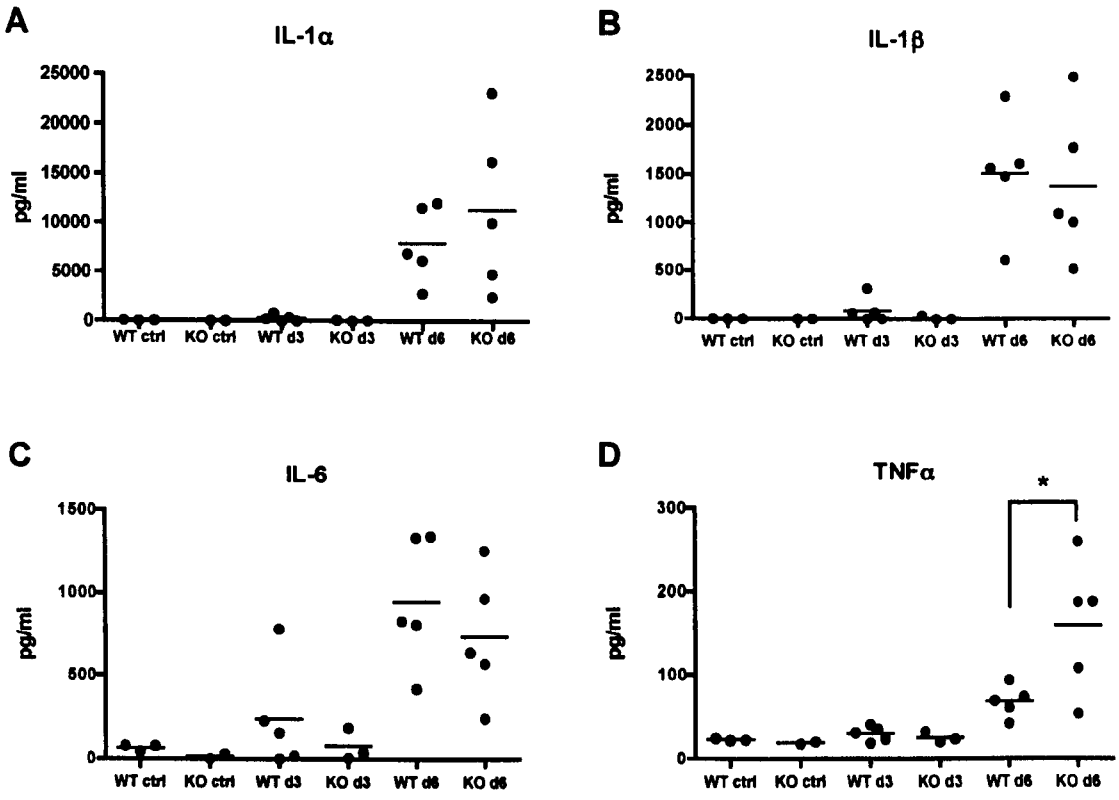


Figure 5.14

Production of Cytokines by Colon Explants from D6 KO and WT Mice with DSS Colitis

WT and D6 KO mice received 2% DSS in the drinking water for 5 days and normal water thereafter. Control mice received normal drinking water for the duration of the experiment. At days 3 and 7, individual explants of colon were cultured for 24 hours and the levels of (A) IL-1α, (B) IL-1β, (C) IL-6 and (D) TNFα in culture supernatants were determined by Luminex analysis.

* $p < 0.05$

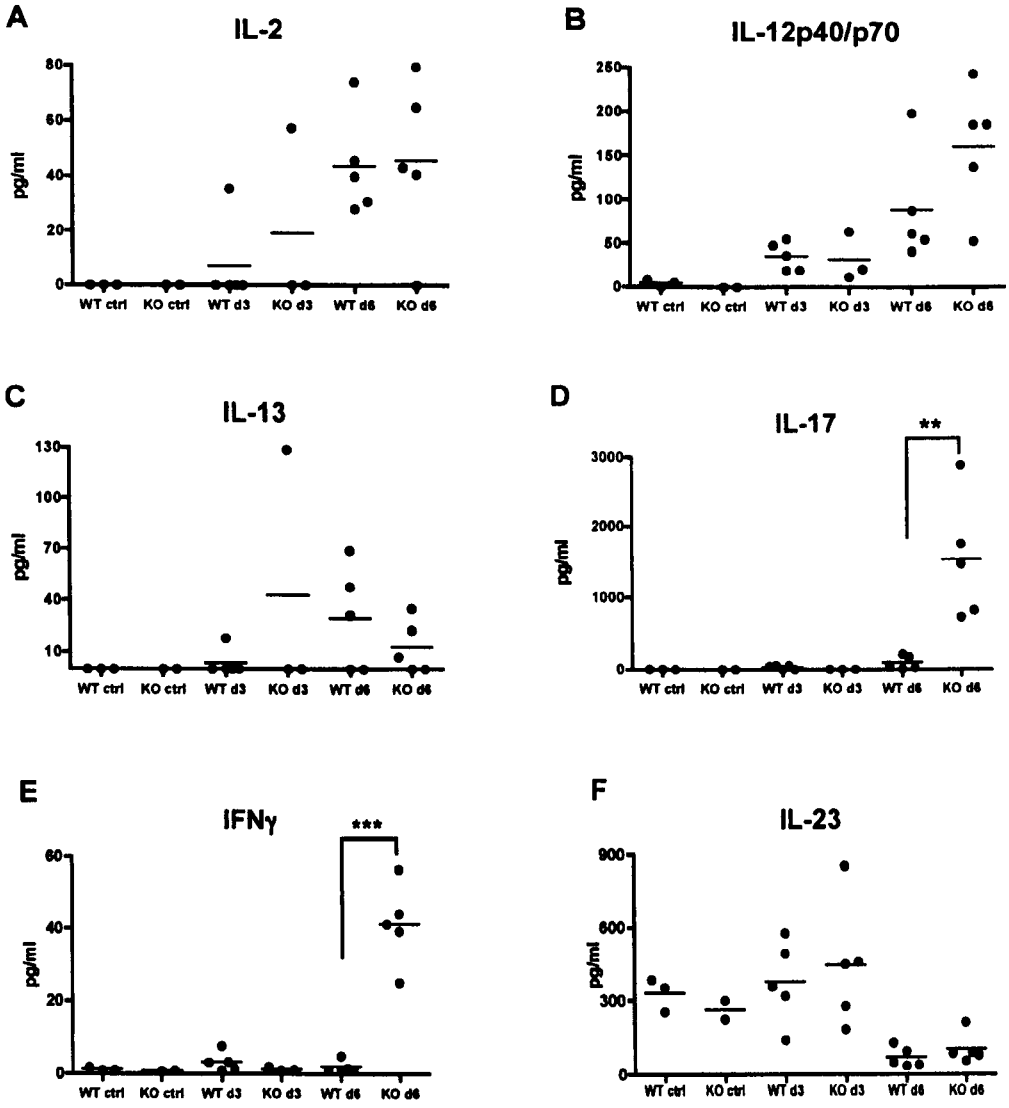


Figure 5.15

Production of Cytokines by Colon Explants from D6 KO and WT Mice with DSS Colitis

WT and D6 KO mice received 2% DSS in the drinking water for 5 days and normal water thereafter. Control mice received normal drinking water for the duration of the experiment. At days 3 and 7, explants of colon were cultured for 24 hours and the levels of (A) IL-2, (B) IL-12, (C) IL-13, (D) IL-17 and (E) IFNγ in culture supernatants were determined by Luminex analysis. Levels of IL-23 (F) were assessed by ELISA.

** $p < 0.01$, ** $p < 0.001$

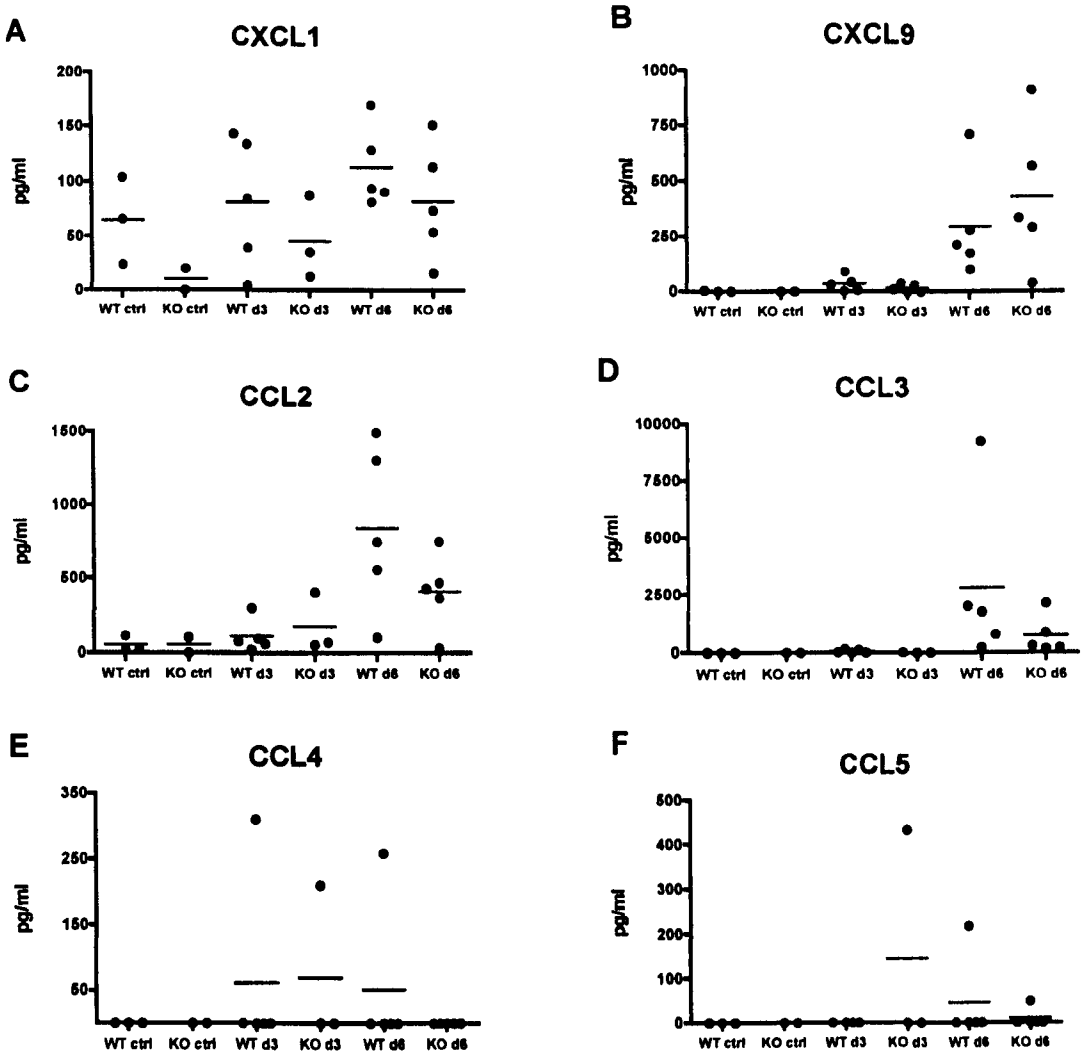


Figure 5.16

Production of Chemokines by Colon Explants from D6 KO and WT Mice with DSS Colitis

WT and D6 KO mice received 2% DSS in the drinking water for 5 days and normal water thereafter. Control mice received normal drinking water for the duration of the experiment. At days 3 and 7, explants of colon were cultured for 24 hours and the levels of (A) CXCL1, (B) CXCL9, (C) CCL2, (D) CCL3, (E) CCL4 and (F) CCL5 in culture supernatants were determined by Luminex analysis.

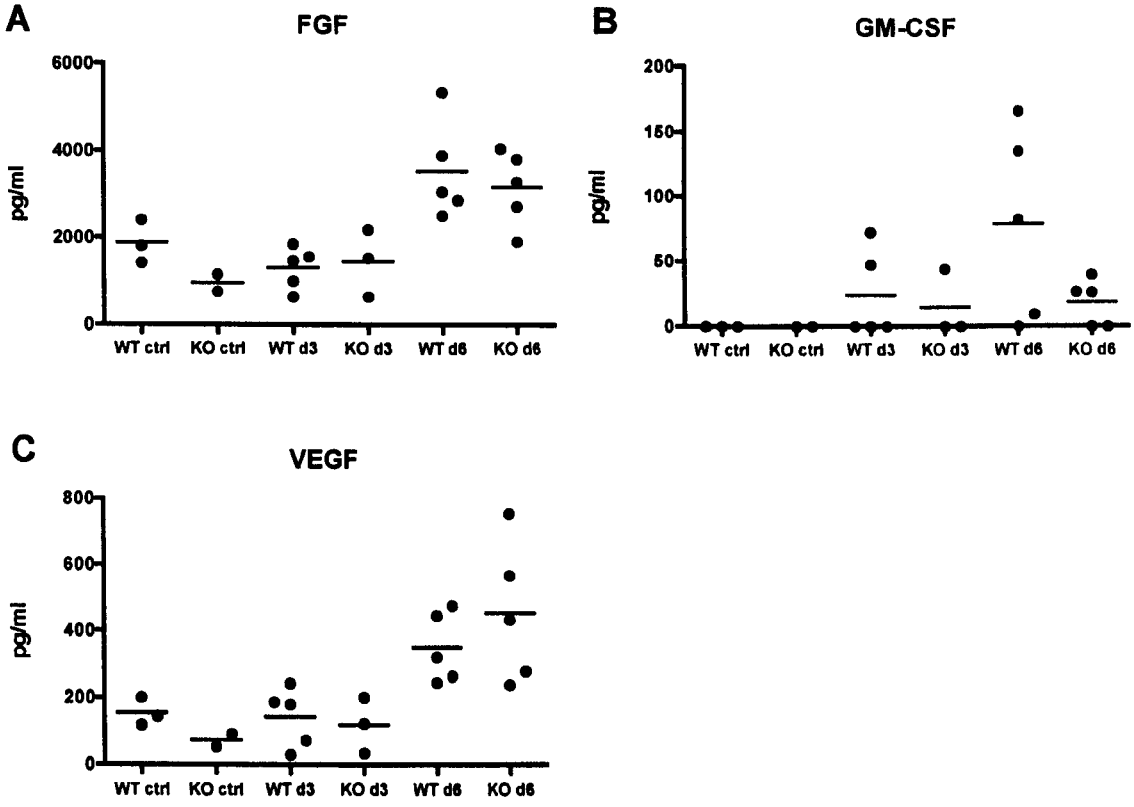


Figure 5.17

Production of Cytokines by Colon Explants from D6 KO and WT Mice with DSS Colitis

WT and D6 KO mice received 2% DSS in the drinking water for 5 days and normal water thereafter. Control mice received normal drinking water for the duration of the experiment. At days 3 and 7, explants of colon were cultured for 24 hours and the levels of (A) FGF, (B) GM-CSF and (C) VEGF in culture supernatants were determined by Luminex analysis.

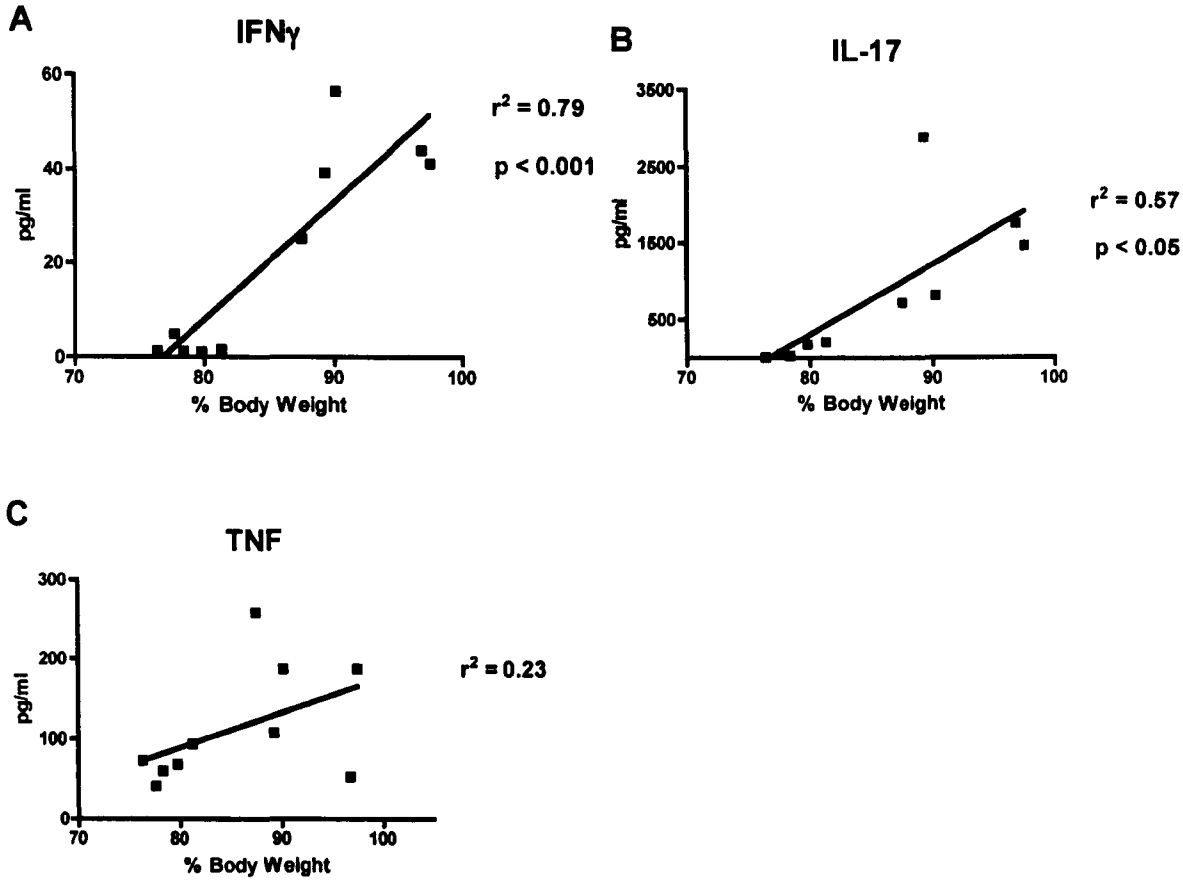


Figure 5.18

Correlation Between Colonic Cytokine Production and Weight Loss in Mice with DSS Colitis

Linear regression analysis of weight loss and IFNγ (A), IL-17 (B) and TNFα (C) production by day 7 colon explants from individual D6 KO and WT mice with DSS colitis (see Figure 5.14).

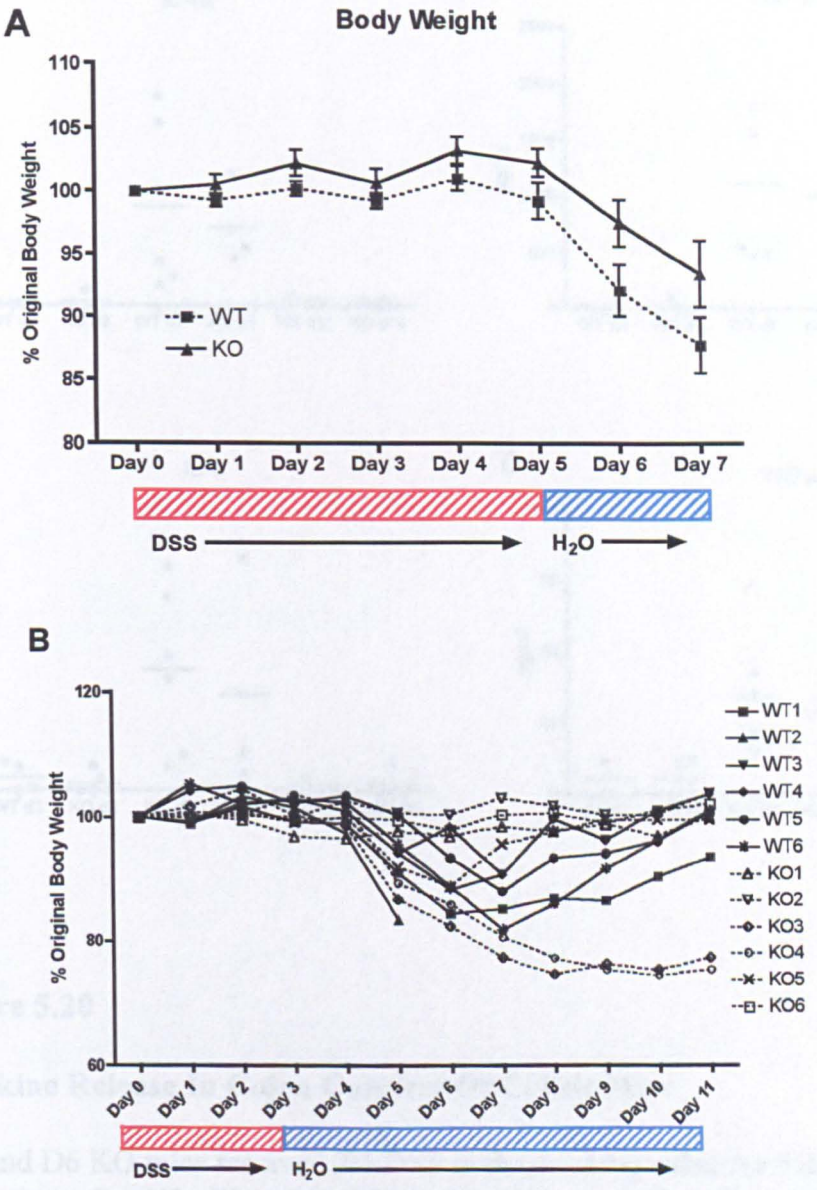


Figure 5.19

Induction of Acute DSS Colitis and Recovery From DSS-Induced Injury in WT and D6 KO Mice

WT and D6 KO mice received (A) 2% DSS in the drinking water for 5 days and normal water thereafter or (B) 2% DSS in the drinking water for 3 days only followed by normal water for 7 days to monitor recovery from DSS-induced injury. The data shown in A are the mean body weights + 1 SD for 9-10 animals per group.

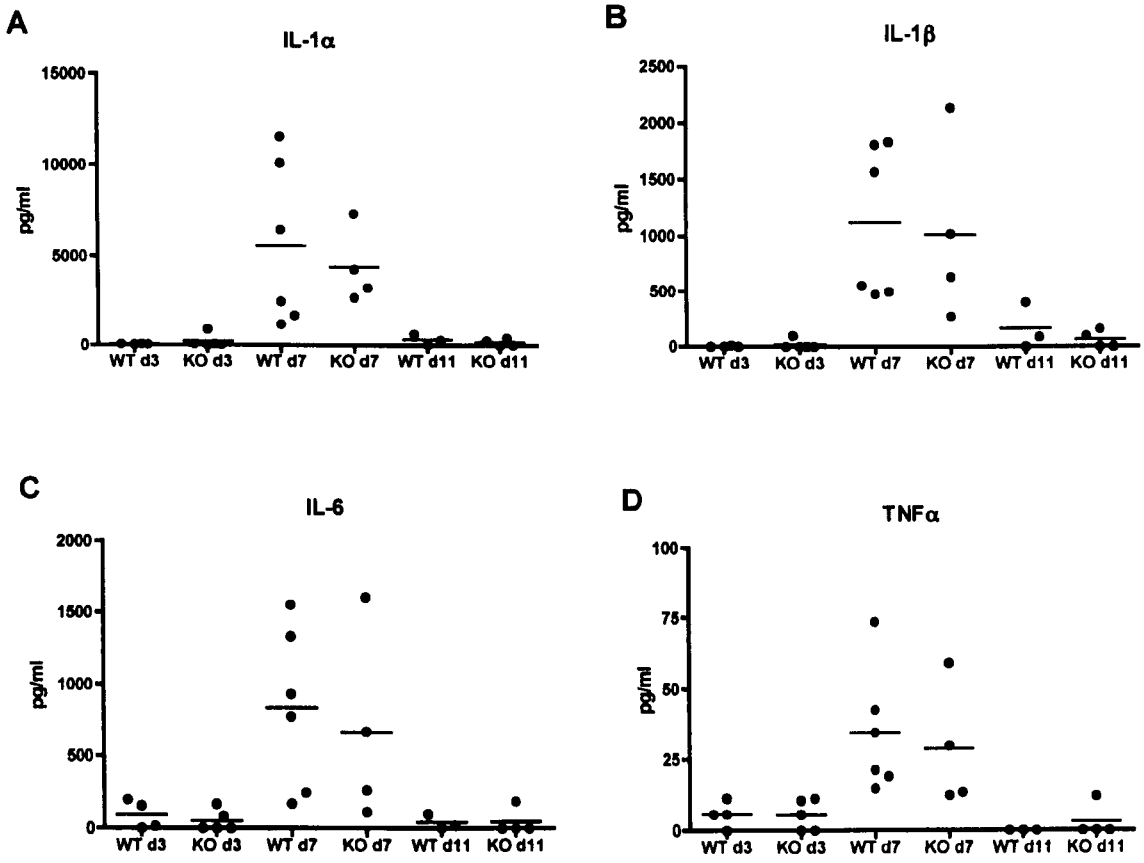


Figure 5.20

Cytokine Release In Colon Cultures Of Colitic Mice

WT and D6 KO mice received 2% DSS in the drinking water for 5 days and normal water thereafter (d3, d7) or 2% DSS in drinking water for 3 days only followed by normal water for 7 days to monitor recovery from DSS-induced injury (d11). Control mice received normal drinking water for the duration of the experiment. At days 3, 7 and 11 the levels of (A) IL-1α, (B) IL-1β, (C) IL-6 and (D) TNFα in culture supernatants from colon explants were determined by Luminex analysis.

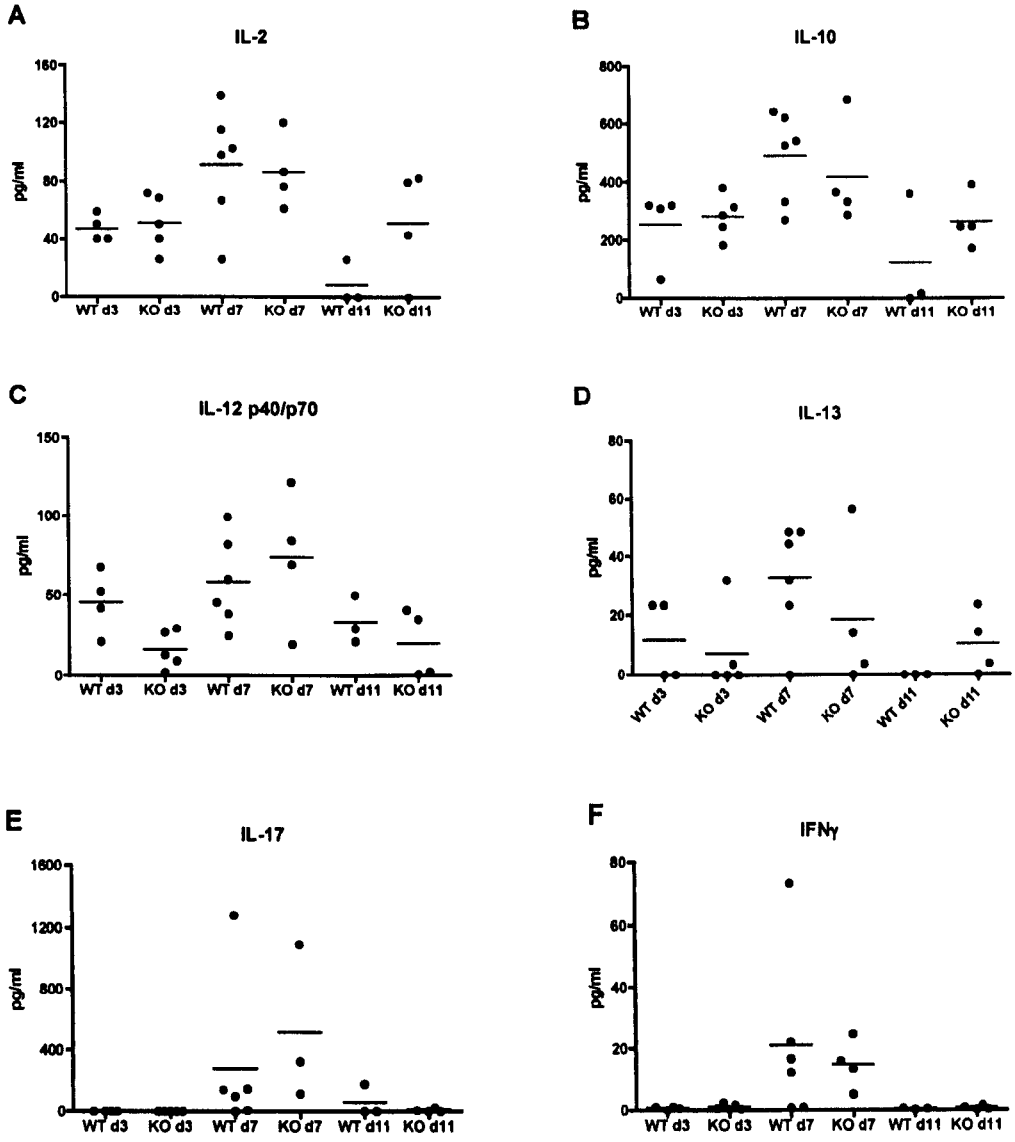


Figure 5.21

Cytokine Release In Colon Cultures Of Colitic Mice

WT and D6 KO mice received 2% DSS in their drinking water for 5 days and normal water thereafter (d3, d7) or 2% DSS in their drinking water for 3 days only followed by normal water for 7 days to monitor recovery from DSS-induced injury (d11). Control mice received normal drinking water for the duration of the experiment. At days 3, 7 and 11 the levels of (A) IL-2, (B) IL-10, (C) IL-12 p40/p70, (D) IL-13, (E) IL-17 and (F) IFNγ in the culture supernatants were determined by Luminex analysis.

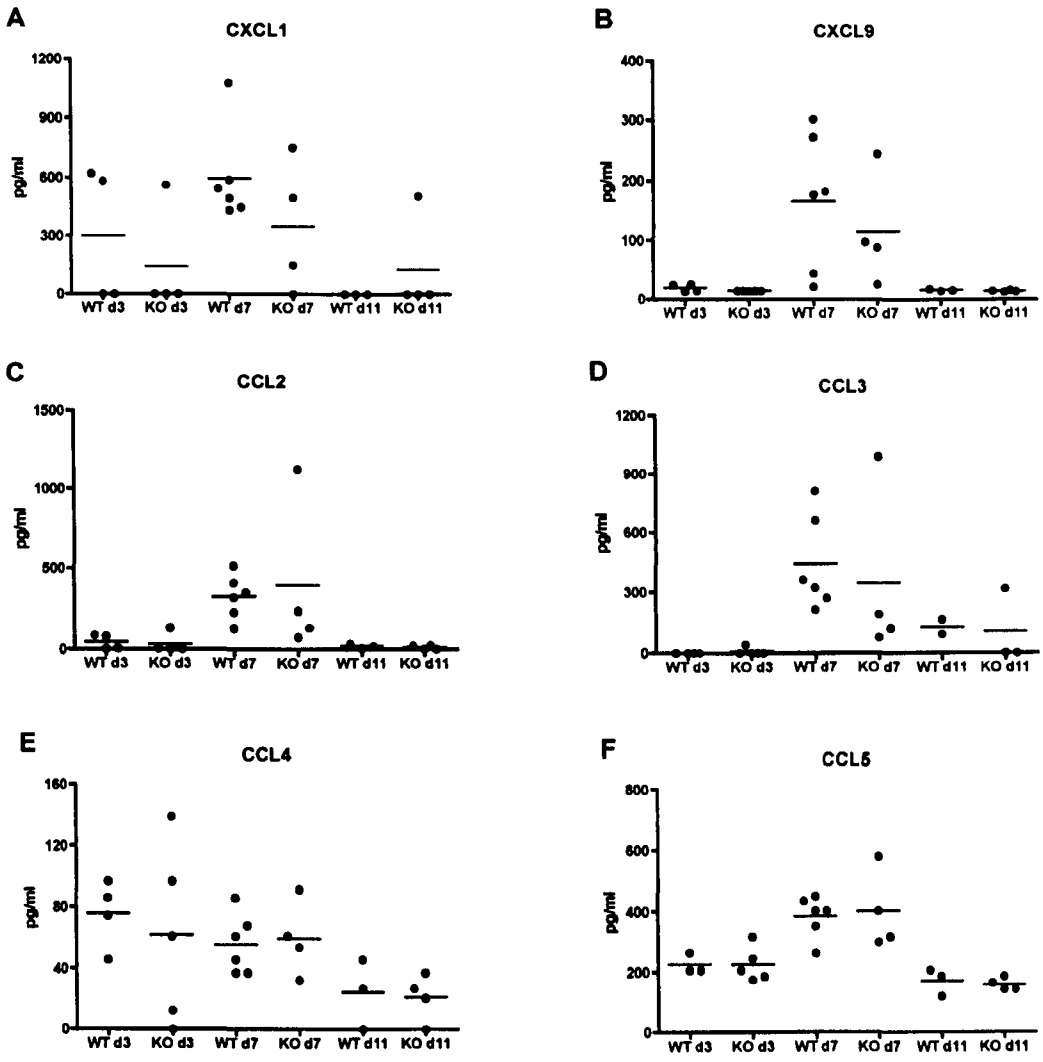


Figure 5.22

Chemokine Release In Colon Cultures Of Colitic Mice

WT and D6 KO mice received 2% DSS in drinking water for 5 days and normal water thereafter (d3, d7) or 2% DSS in drinking water for 3 days only followed by normal water for 7 days to monitor recovery from DSS-induced injury (d11). Control mice received normal drinking water for the duration of the experiment. At days 3, 7 and 11 the levels of (A) CXCL1, (B) CXCL9, (C) CCL2, (D) CCL3, (E) CCL4 and (F) CCL5 in culture supernatants were determined by Luminex analysis.

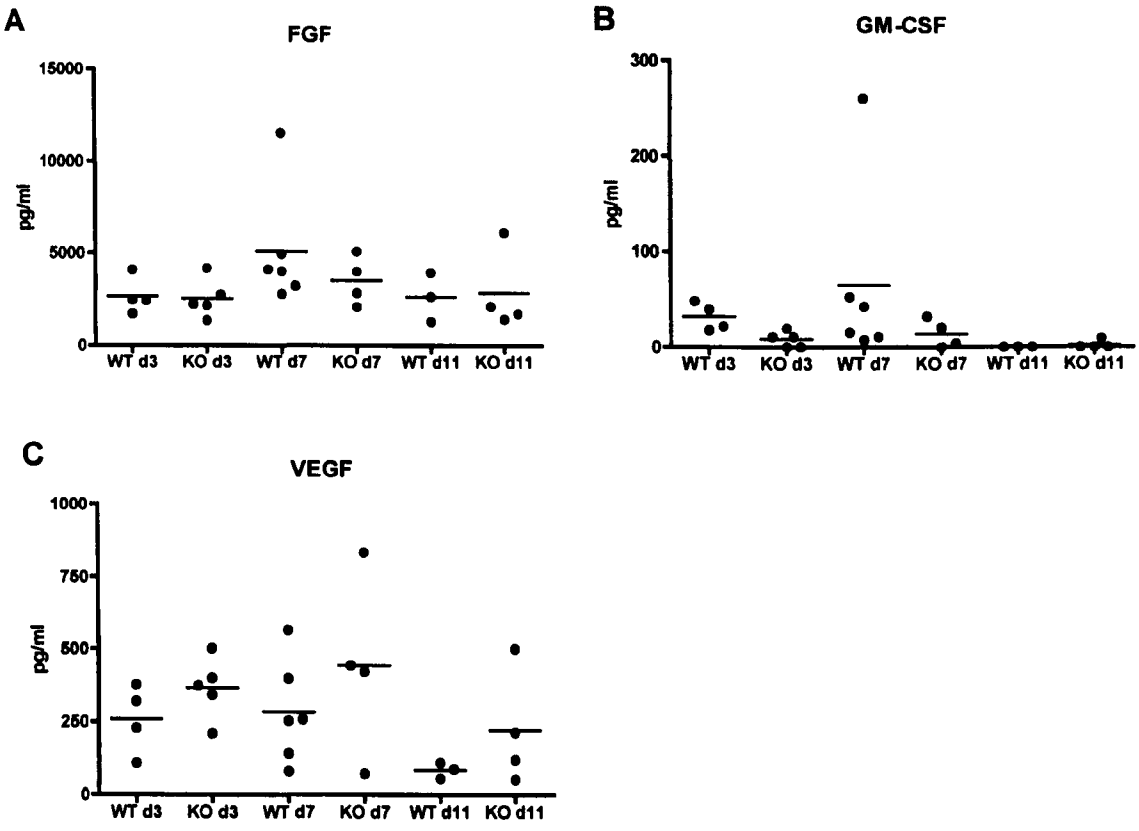


Figure 5.23

Production of Cytokines by Colon Explants from D6 KO and WT Mice with DSS Colitis

WT and D6 KO mice received 2% DSS in drinking water for 5 days and normal water thereafter (d3, d7) or 2% DSS in drinking water for 3 days only followed by normal water for 7 days to monitor recovery from DSS-induced injury (d11). Control mice received normal drinking water for the duration of the experiment. At days 3, 7 and 11 the levels of (A) FGF, (B) GM-CSF, and (C) VEGF in culture supernatants from colon explants were determined by Luminex analysis.

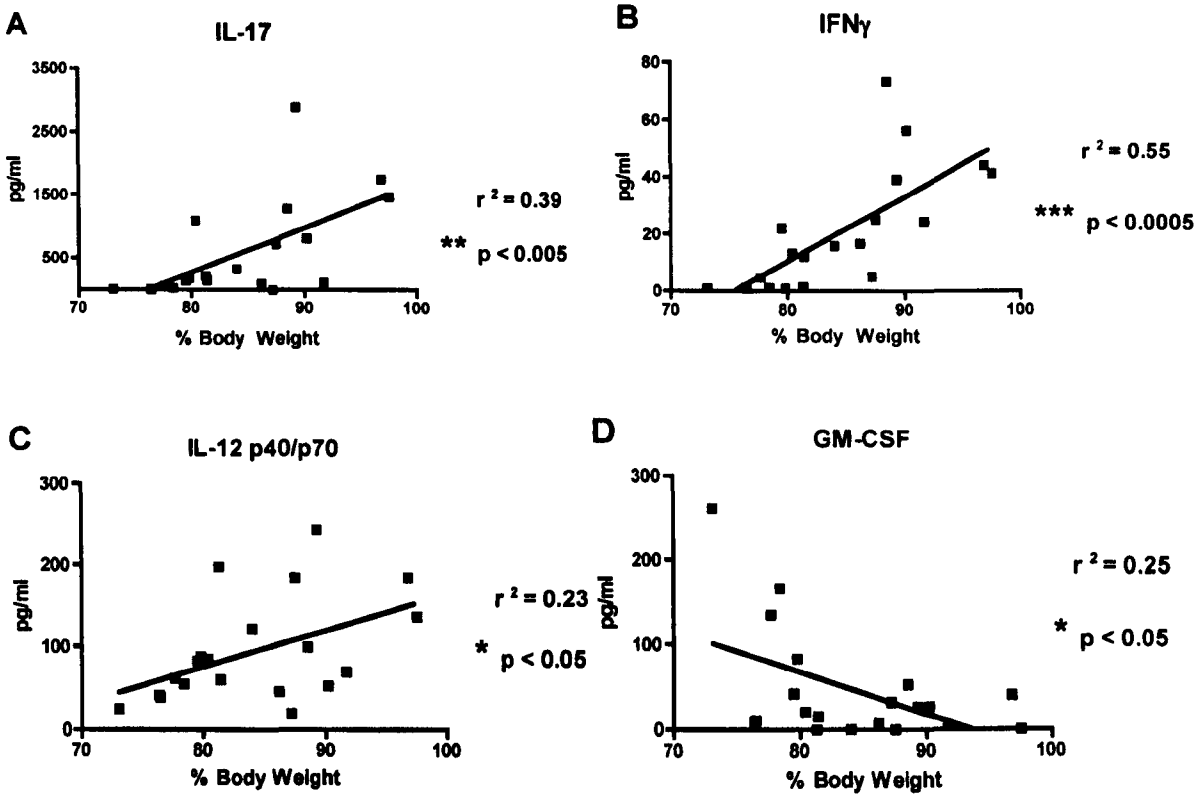


Figure 5.24

Correlation Between Colonic Cytokine production and Weight Loss in Mice with DSS Colitis

Linear regression analysis of weight loss and IL-17 (A), IFNg (B), IL-12 p40/p70 (C), and GM-CSF (D) production by day 6 or 7 colon explants from all individual D6 KO and WT mice with DSS colitis examined in two separate experiments.

* $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$

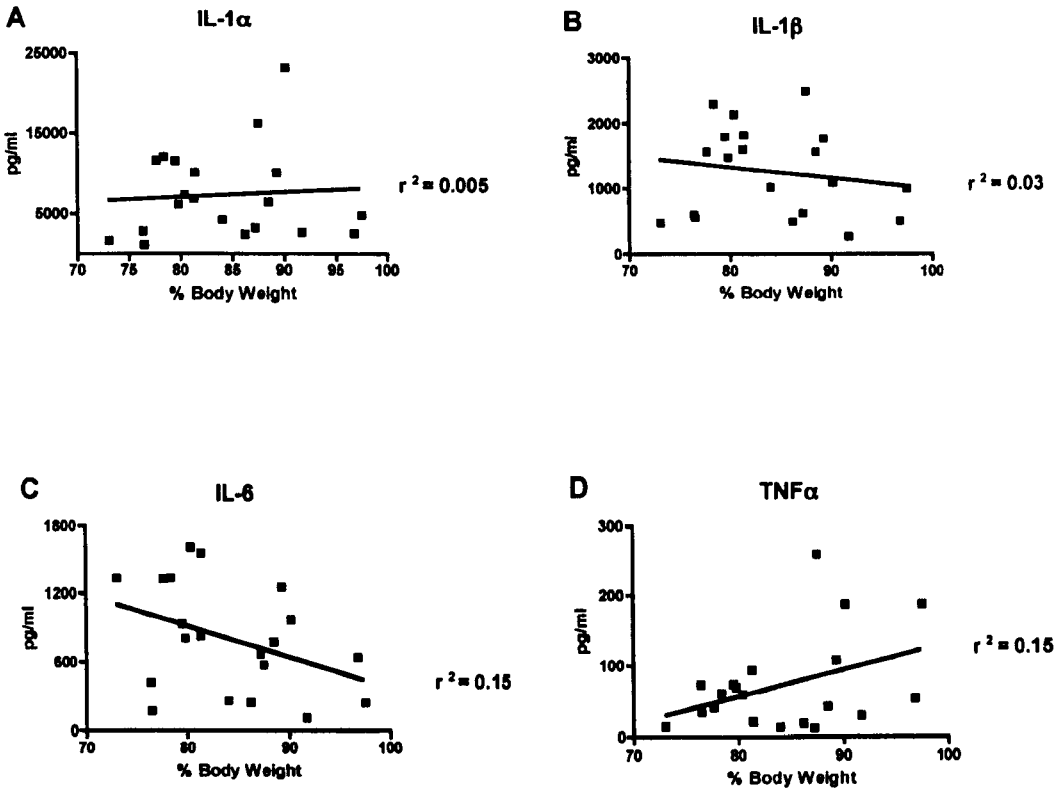


Figure 5.25

Correlation Between Colonic Cytokine Production and Weight Loss in Mice with DSS Colitis

Linear regression analysis of weight loss and IL-1α (A), IL-1β (B), IL-6 (C), and TNFα (D) production by day 6 or 7 colon explants from all individual D6 KO and WT mice with DSS colitis examined in two separate experiments.

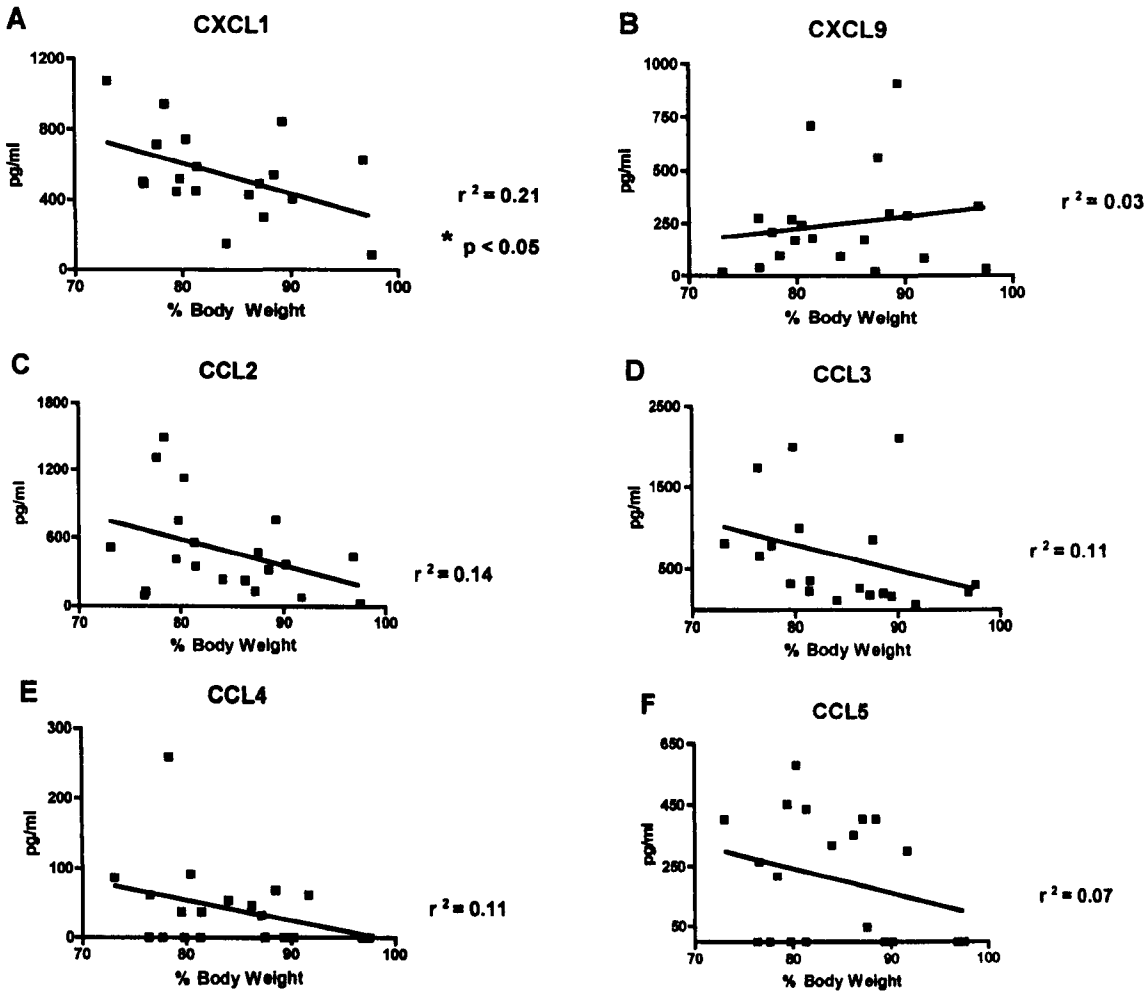


Figure 5.26

Correlation Between Colonic Chemokine Production and Weight Loss in Mice with DSS Colitis

Linear regression analysis of weight loss and CXCL1 (A), CXCL9 (B), CCL2 (C), CCL3 (D), CCL4 (E) and CCL5 (F) production by day 6 or 7 colon explants from all individual D6 KO and WT mice with DSS colitis examined in two separate experiments.

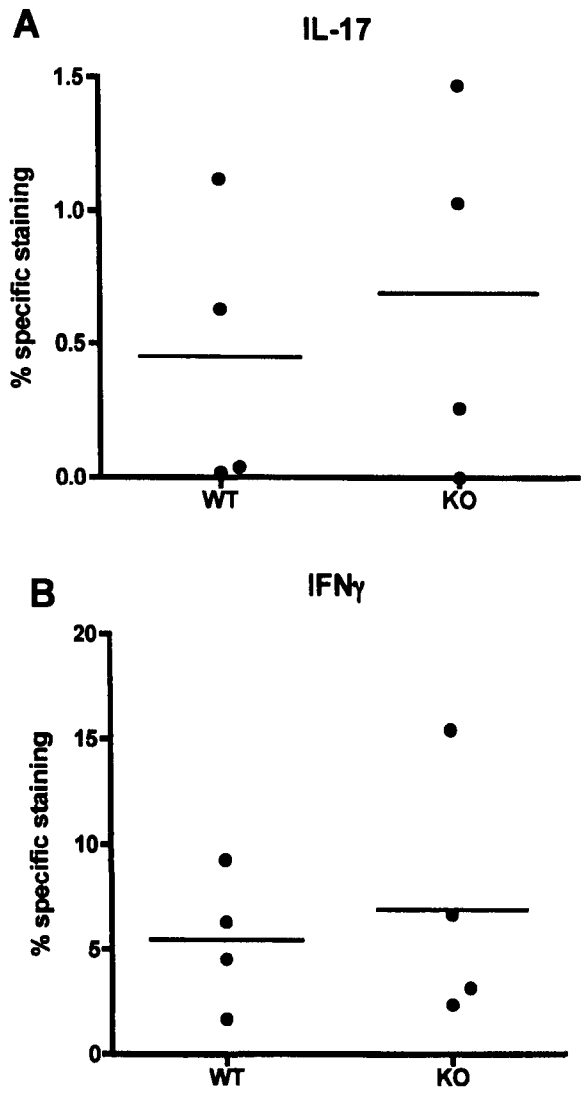


Figure 5.27

Intracellular Cytokine Production by Colonic Lamina Propria Cells

WT and D6 KO mice received 2% DSS in the drinking water for 5 days and normal water therafter. On day 7, colonic lamina propria cells were isolated and cultured with ionomycin, PMA and Brefeldin A for 4-5 hr. The cells were then permeabilised and the presence of intracellular IFNγ (A) and IL-17 (B) within cells lying in a lymphocyte gate were assessed by flow cytometry. Specific staining was calculated by subtracting isotype control values.

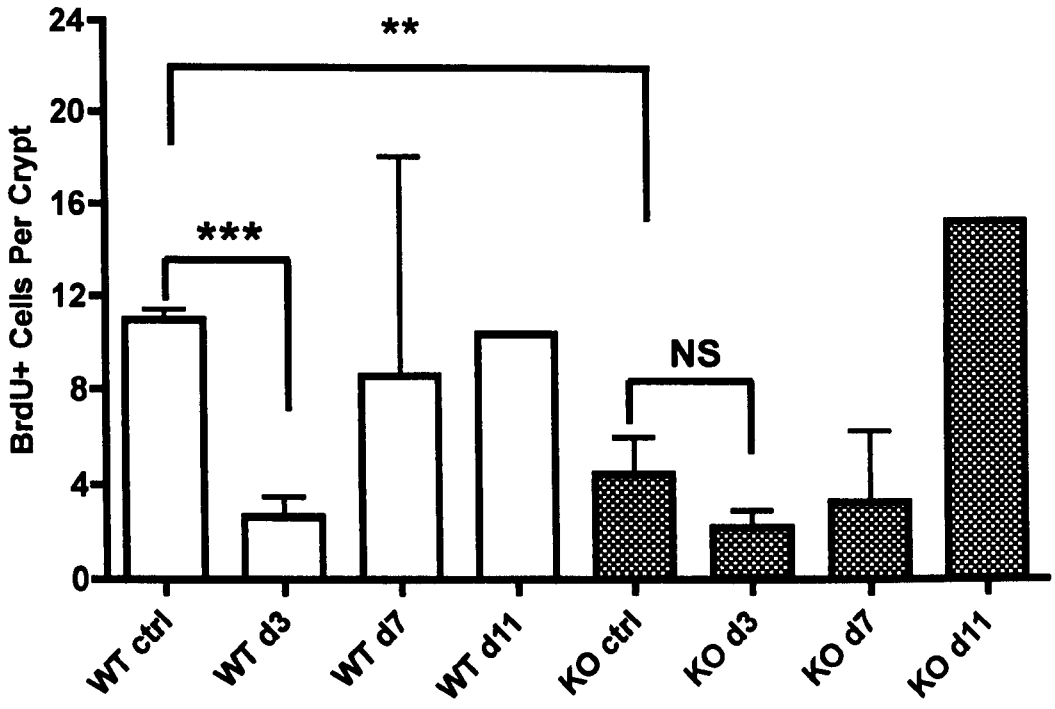


Figure 5.28

Proliferative Activity Of Colonic Epithelial Cells in D6 KO and WT Mice with Colitis

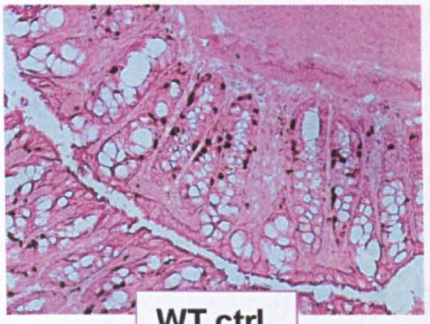
WT and D6 KO mice received 2% DSS in the drinking water for 5 days and normal water thereafter, or 2% DSS in the drinking water for 3 days only followed by normal water for 7 days to monitor recovery from DSS-induced injury. Control mice received normal drinking water for the duration of the experiment. On days 3, 7 and 11, mice that had been administered BrdU solution (50mg/kg) by intraperitoneal injection 24h previously, were sacrificed, sections of colons were snap-frozen and the number of BrdU+ cells assessed by immunohistochemistry. The results shown are the mean numbers of BrdU+ cells per crypt + 1 SD for 3 animals per group (ctrl, d3 and d7) and the means of 2 animals per group (d11).

** $p < 0.005$, *** $p < 0.0001$

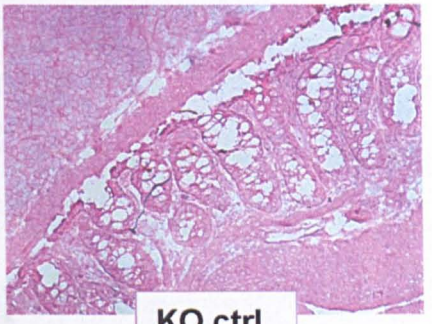
Figure 5.29

Proliferative Activity Of Colonic Epithelial Cells in D6 KO and WT Mice with Colitis

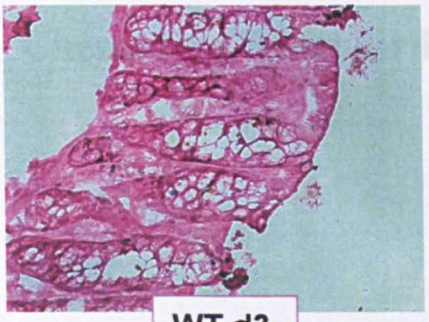
WT and D6 KO mice received 2% DSS in the drinking water for 5 days and normal water thereafter, or 2% DSS in the drinking water for 3 days only followed by normal water for 7 days to monitor recovery from DSS-induced injury. Control mice received normal drinking water for the duration of the experiment. On days 3, 7 and 11, mice that had been administered BrdU solution (50mg/kg) by intraperitoneal injection 24h previously, were sacrificed, sections of colons were snap-frozen and the number of BrdU+ cells assessed by immunohistochemistry. Representative images from WT and D6 KO mice taken at the various time-points indicated are shown. Magnification x 400.



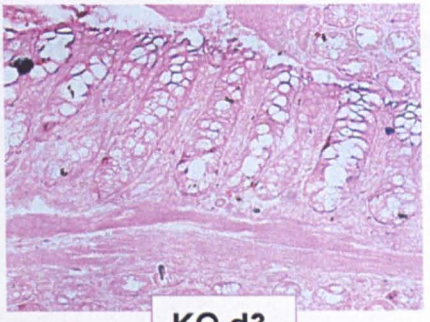
WT ctrl



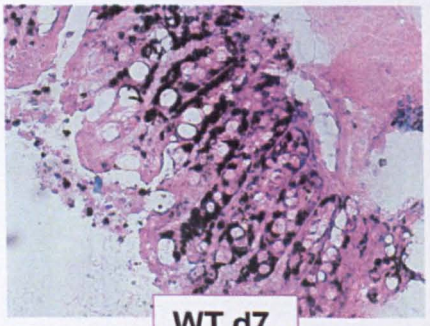
KO ctrl



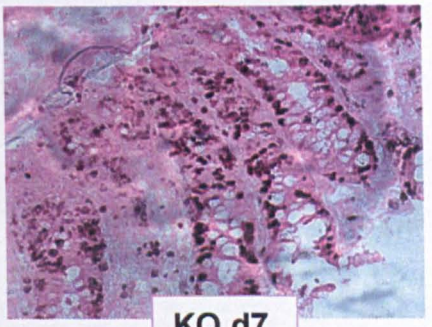
WT d3



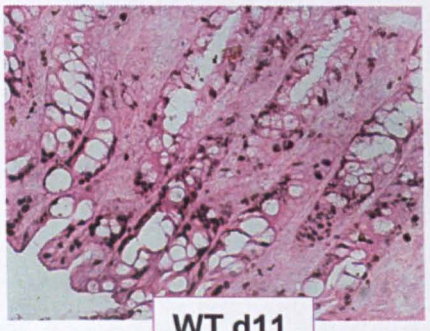
KO d3



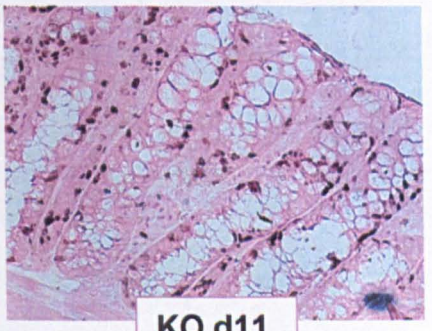
WT d7



KO d7



WT d11



KO d11

Chapter 6

Chemokine Receptor Profile of D6 KO Leukocytes

Introduction

Several of the experiments described in previous chapters suggested that there were differences in chemokine receptor expression between WT and D6 KO leukocytes. Thioglycollate-induced peritoneal macrophages, bone marrow DC and colonic lamina propria cells from colitic D6 KO animals all displayed altered chemokine receptor profiles relative to WT counterparts. In light of these findings, I decided to conduct a more thorough examination of chemokine receptor expression by D6 KO leukocytes from unmanipulated D6 KO mice.

6.1 Chemokine Receptor Profile Of Colonic Leukocytes

Analysis of lamina propria cells from mice with DSS colitis had revealed higher proportions of $CCR5^+$ leukocytes in the inflamed colonic lamina propria of D6 KO mice compared with WT mice with colitis (Chapter 5). This appeared to be mainly due to greater numbers of $CCR5^+$ $B220^+$ cells in the KO lamina propria and to explore this in more detail, I first assessed chemokine receptor expression by cells isolated from the colon of untreated WT and KO mice.

Lamina propria cells from the colons of three WT and three KO mice were pooled and assessed for expression of CCR2 and CCR5, two of the CC-chemokine receptors that are activated by a number of D6 ligands. As a control, I also examined expression of CXCR4, a CXC-receptor family member not activated by ligands of D6. As shown in Figure 6.1, similar proportions of WT and KO lamina propria cells expressed CCR2 and CXCR4, with around 70% of isolated cells positive for these receptors in both cases. However, while only 20% of WT lamina propria cells expressed CCR5, this chemokine receptor was found on

56% of KO LP cells (Figure 6.1). This was similar to what I had found in colitic mice and in further agreement with these previous findings, the increase in $CCR5^+$ cells in D6 KO colonic tissue was due to a large increase in the proportion of $CCR5^+$ B cells. While only 19.2% of WT $B220^+$ cells expressed CCR5, 77.6% of KO B cells were CCR5-positive (Figure 6.2 A). On the other hand, CCR2 and CXCR4 expression did not differ between WT and KO B cells.

WT and KO $CD8^+$ cells demonstrated a similar chemokine receptor profile, with CCR2, CCR5 and CXCR4 being found on approximately 40%, 80% and 70% of each population of $CD8^+$ cells, respectively (Figure 6.2 C). CXCR4 expression was also equivalent on the WT and KO $CD4^+$ populations, but only around half as many D6 KO $CD4^+$ cells expressed CCR2 and CCR5 compared with WT $CD4^+$ cells (19% vs. 48% and 22% vs. 42%, respectively, Figure 6.2 B). In contrast to my findings with colitic mice, more $F4/80^+$ lamina propria cells from untreated D6 KO mice expressed CCR2 and CCR5 compared with their WT counterparts (90% vs. 77% and 40% vs. 16%, respectively, Figure 6.2 D). There were also slightly increased numbers of $F4/80^+CXCR4^+$ cells in KO lamina propria isolates compared with WT (63% $CXCR4^+$ WT vs. 72% KO). These data suggested that the differential chemokine receptor profiles of WT and KO lamina propria isolates was not disease related, as differences in CCR2 and CCR5 expression by $B220^+$, $CD4^+$, $CD8^+$ and $F4/80^+$ populations were seen in both normal and colitic colon. In both cases, the most striking difference was an increased proportion of $CCR5^+$ B cells in D6 KO colon.

6.2 Chemokine Receptor Profile of D6 KO Leukocytes

Previously, I had found no differences between the overall numbers of leukocytes in WT and KO lymphoid organs (Chapter 3). However, having detected altered chemokine receptor profiles of normal D6 KO colonic leukocytes, I went on to determine if there were similar alterations in organised lymphoid tissues of D6 KO mice.

Cells isolated from peripheral lymph nodes, mesenteric lymph nodes and spleen of WT and D6 KO mice were examined for the expression of CCR2, CCR5, CCR9, CXCR4 and CXCR5. There were similar proportions of total CCR2, CCR9, CXCR4 and CXCR5 positive cells in WT and KO PLN (Figure 6.3) and in spleen (Figure 6.4). However, significantly increased percentages of KO PLN cells and KO splenocytes expressed CCR5 compared with their WT counterparts. Greater proportions of $CCR5^+$ cells were also detected in the MLN of D6 KO mice (Figure 6.5). Interestingly, while there were no differences in CCR2, CXCR4 and CXCR5 expression by WT and KO MLN cells, there was a small but significant increase in the numbers of $CCR9^+$ cells in the D6 KO MLN (Figure 6.5).

In contrast to my findings in the colon, higher proportions of $CD3^+$ T cells were found to be $CCR5^+$ from all lymphoid tissues (Figure 6.6) of D6 KO animals (Figures 6.7 A - C). However, in accordance with my earlier results, the most dramatic differences between WT and KO lymphoid tissues were seen with $B220^+$ cells, which showed much higher expression of CCR5 in PLN, spleen and MLN. Additionally, in each of the three KO tissues > 85% of KO B cells were $CCR5^+$ compared with < 25% of WT.

In all three tissues, almost all $B220^+$ B cells were found to be $CCR2^+$, while fairly low numbers of $CD3^+$ T cells expressed this chemokine receptor and there were no differences between WT and KO expression (Figures 6.8 A - C). However, there was an increase in the proportion of $CD3^-B220^-$ cells, most likely representing non-lymphoid populations, expressing CCR2 in KO PLN compared with WT PLN (Figure 6.8 A).

There were no differences in CCR9 expression by any of these T and B cell subsets in WT and KO PLN (Figure 6.9 A), but analysis of MLN cells revealed increased proportions of $CCR9^+$ cells in the CD3+ and B220+ populations of KO mice (Figure 6.9 B). Additionally, $CD3^+$ T cells and $CD3^-B220^-$ cells in the KO spleen showed increased expression of CCR9 compared with WT cells (Figure 6.9 C). There were no differences in the expression of CXCR4 and CXCR5 by the various B220/CD3 subsets from PLN, MLN and spleen of WT and D6 KO mice (Figures 6.10 A - C and 6.11 A – C).

Finally, I examined chemokine receptor expression by T- and B- lymphocytes from the peritoneal cavity, as a representative extra-lymphoid site. As shown in Figures 6.12 A and B, the expression of CCR2, CCR5, CCR9 and CXCR4 was similar on WT and D6 KO peritoneal $CD4^+$ and $CD8^+$ T cells. Additionally, similar proportions of WT and KO peritoneal $B220^+$ B cells expressed CCR2, CCR9 and CXCR4 (Figure 6.12 C). However, in agreement with my findings from the colon and secondary lymphoid tissues, more D6 KO peritoneal B cells expressed CCR5 compared with WT control B cells (Figure 6.12 C).

Taken together, these data demonstrate that increased expression of CCR5 is not specific to colonic lamina propria B cells of D6 KO animals, but also characterises B cells from PLN,

MLN, spleen and peritoneum of D6 KO mice. Moreover, differential chemokine receptor expression was also apparent in some other D6 KO leukocytes.

6.3 Increased CCR5 Transcription in D6 KO Cells

To confirm whether the increased levels of CCR5 surface expression reflected increased levels of CCR5 transcripts, I next examined CCR5 mRNA levels. In collaboration with Dr. Clive McKimmie, levels of transcripts for CCR5 were compared between purified B cells from WT and KO spleen by real-time PCR. As shown in Figure 6.13, around a three-fold increase in expression of CCR5 mRNA was detected in purified D6 KO B cells compared with WT controls. Thus the increased level of CCR5 protein expression by D6 KO B cells correlated with increased CCR5 transcription. Increased expression of CCR5 mRNA was also found in purified T cell, neutrophil and macrophage populations from D6 KO animals, although these differences were less dramatic than with the B cells. Altogether, these experiments revealed that absence of D6 affected transcription of CCR5 and not merely surface protein expression.

6.4 Chemokine Responsiveness of D6 KO B Cells

To determine the functional consequences of the increased CCR5 expression by D6 KO B cells, I compared the chemotaxis responses of WT and D6 KO B cells to CCL4 in a Transwell assay. Splenocytes from WT and KO mice were added to the top chamber of Transwell plates and the migration of B220+ cells towards CCL4 in the lower chamber was assessed by flow cytometry. As shown in Figure 6.14, D6 KO B cells showed greater specific migration to CCL4 compared with WT B cells, with optimal migration of D6 KO B cells occurring in response to 10 ng/ml of CCL4.

6.5 Chemokine Receptor Profile of Developing B Cells

As there were increased numbers of $CCR5^+$ B cells in all D6 KO tissues, I decided to examine at what stage of B cell differentiation this phenotype arose. Early B cell development occurs in bone marrow, where sequential interactions between lymphoid progenitors and the surrounding stroma culminate in the export of functional B-cells to the periphery (333-335). Various chemokines and adhesion molecules have been implicated in this process (102, 333-335) and I therefore compared chemokine receptor expression by WT and D6 KO bone marrow B cells.

As shown in Figure 6.15, around 20-30% of BM cells in WT and D6 KO mice expressed the B220 antigen. Mature $CD3^+$ T cells were rare. Based on the level of B220 expression, two distinct populations could be discerned: a larger population which was $B220^{int}$ (~15-20% of BM cells) and a minor $B220^{hi}$ subset (<5%). B220 is one of the earliest identifiable markers to appear on B-lineage cells, and is present throughout B-cell development, with the highest expression evident on mature B cells (333, 336-338). Thus the $B220^{int}$ and $B220^{hi}$ populations most likely comprised developing progenitor subsets and mature B cells, respectively. Interestingly, the $B220^{int}$ subset appeared somewhat reduced in D6 KO mice (15.1% total BM cells ± 1.8) compared with WT (22.8 ± 6.8), although this difference was not statistically significant.

Consistent with its essential role in B cell development (102), virtually all $B220^+$ cells in both strains expressed CXCR4 (Figure 6.16 A). In keeping with my previous findings in secondary lymphoid organs, highly statistical differences in CCR5 expression were noted

between WT and KO $B220^+$ cells. While less than a fifth of WT $B220^+$ BM cells were CCR5-positive, around half of all $B220^+$ KO cells expressed this chemokine receptor. The vast majority of $B220^+$ cells were also $CCR2^+$, but this was more so for D6 KO $B220^+$ cells compared with WT (85.9% vs. 71.5%, respectively). Expression of CCR9 by WT $B220^+$ BM cells was low (~9%) and this was increased in D6 KO BM $B220^+$ cells, although the difference was not statistically significant. Few $B220^+$ cells were found to express CXCR5 (< 2%).

Next, I compared chemokine receptor expression by the $B220^{int}$ and $B220^{hi}$ subsets of WT and D6 KO mice. Both these subsets expressed CXCR4 at high levels in both strains (Figure 6.16 E), while the few $CXCR5^+$ cells identified were found to reside in the $B220^{hi}$ subset (6.16F). CCR5 was expressed at higher levels on the $B220^{hi}$ subset compared with $B220^{int}$ cells, but this was increased on both subsets from D6 KO BM compared with the WT subsets (Figure 6.16 D). Interestingly, virtually all $B220^{hi}$ cells expressed CCR2 (Figure 6.16B), revealing that the increased CCR2-positivity of KO $B220^+$ cells was accounted for by the $B220^{int}$ subset (66.5% ± WT vs. 80.6% ± 7.8 KO). Although comparable percentages of WT $B220^{lo}$ and $B220^{hi}$ cells expressed CCR9, more CCR9-postive cells were found in both equivalent KO subsets, with the most dramatic increase witnessed in the $B220^{hi}$ subset (Figure 6.16 C).

In addition to comparing the overall proportions of cells positive for the various chemokine receptors, I also determined the MFI of the positive cells in order to evaluate the levels of receptor expression. While all $B220^+$ cells expressed CXCR4, greater levels of expression were found on $B220^{hi}$ BM subsets of both WT and D6 KO animals (Figure 6.17). CCR2

expression levels were also higher in $B220^{hi}$ subsets and D6 KO $B220^{+}$ cells from both subsets had higher surface levels of CCR2 than WT counterparts (Figure 6.18). As shown in Figure 6.19, $CCR5^{+}B220^{hi}$ KO cells demonstrated slightly higher levels of CCR5 expression compared with WT counterparts. Strikingly, although I had found less $CCR5^{+}$ cells in the WT $B220^{int}$ subsets compared with KOs, WT $B220^{int}CCR5^{+}$ cells demonstrated significantly higher levels of CCR5 expression compared with their KO counterparts (Figure 6.19). Similar trends were seen with respect to CCR9 expression (Figure 6.20). Thus, although there were more $CCR5^{+}$ and $CCR9^{+}$ cells in D6 KO $B220^{int}$ populations, individually, the $CCR5^{+}B220^{int}$ and $CCR9^{+}B220^{int}$ cells of WT mice demonstrated higher receptor expression than their KO counterparts (Figure 6.19 and 6.20). Taken together, these data indicated that in the absence of D6, aberrant chemokine receptor expression occurs during B-lymphopoiesis.

6.6 Analysis of B Cell Function in D6 KO Mice

My studies had revealed dramatically enhanced CCR5 expression by D6 KO B cells both at mRNA and protein levels, and this appeared to be from an early stage of development. Therefore I decided to examine if these abnormalities had any functional consequences. To address this point, I first compared the levels of total immunoglobulins in the sera of WT and D6 KO mice. As shown in Figure 6.21 A, serum IgM levels were comparable in WT and KO mice but there appeared to be somewhat lower total IgG levels in the D6 KO animals, although this was only significant at one serum dilution (Figure 6.21 B).

Next, I compared the functional and phenotypic responses of WT and KO B cells following stimulation with mitogenic factors *in vitro*. Purified splenic B cells were cultured for 48h in

medium alone, or medium supplemented with αCD40 ± αIgM, DSS or LPS. The expression of costimulatory molecules and chemokine receptors, cytokine production, and proliferation responses were then examined.

B cells from both WT and KO mice showed significant proliferation above background in response to anti-CD40/anti-IgM or DSS, and even greater proliferation was seen in cultures stimulated with LPS (Figure 6.22). WT and KO B cells showed similar levels of proliferation in all culture conditions examined.

B cells stimulated with DSS or LPS showed some upregulation of CD80, whereas anti-CD40 had no effect (Figure 6.23 A). On the other hand, cultured B cells upregulated CD86 in response to all mitogens, most notably LPS (Figure 6.23 B). Again, no differences were detected between WT and KO cells in any of the culture conditions.

Confirming my results with B cells from lymphoid tissues, higher proportions of D6 KO B cells expressed CCR5 when cultured in medium for 2 days and similar differences were seen when B cells were stimulated with DSS or LPS (Figure 6.24 C). KO B cells also expressed higher levels of CCR5 in all treatment conditions (Figure 6.24 D). LPS-treatment increased CCR5 expression levels in both WT and D6 KO cultures (Figure 6.24 D). Conversely, culture with DSS appeared to have no effect on CCR5 expression by WT cells and, interestingly, DSS treatment reduced CCR5 expression levels of purified KO B cells (Figures 6.24 C and D).

As shown in Figure 6.24 A, the vast majority of $B220^+$ cells were CCR2 positive (>90%) after culture, irrespective of the stimulus. D6 KO B cells cultured in medium alone had higher levels of CCR2 expression compared with WT $B220^+$ cells (Figure 6.24 B), but the intensity of CCR2 expression was comparable on WT and KO B cells stimulated with DSS or LPS cultures. Due to the use of a polyclonal anti-rat IgG in the staining procedure, I was unable to examine CCR2 and CCR5 expression by WT and KO B cells cultured with anti-CD40 and anti-IgM.

In conjunction with the above analyses, I also determined chemokine levels in WT and KO B cell culture supernatants. As shown in Figures 6.25 A - C, B cells cultured in medium alone produced little or none of the D6 ligands CCL3, CCL4 and CCL5, but higher levels of these chemokines were detected after stimulation with DSS and, notably, with LPS. No differences were seen between the levels of chemokines produced by WT or D6 KO B cells in medium alone or in response to DSS, and although LPS stimulated KO B cells produced generally lower levels of these CC-chemokines than WT B cells, these differences did not reach statistical significance. There was no production of the non-D6 ligand CXCL10 by WT or KO B cells cultures in medium or with DSS, but stimulation with LPS induced some production of CXCL10, which was equivalent in the two strains (Figure 6.25 D).

Summary

In this chapter, I examined chemokine receptor expression by D6 KO leukocytes, and I found that various D6 KO populations displayed differential chemokine receptor profiles compared with their WT counterparts. Most strikingly, D6 KO B cells from all tissues examined, demonstrated increased CCR5 expression. Despite this altered chemokine

receptor expression, serum immunoglobulin levels and *in vitro* mitogen-induced responses were comparable between WT and D6 KO B cells. However, presumably as a result of increased CCR5 expression, KO B cells demonstrated increased migration to CCL4 in chemotaxis assays. Altogether, these data suggest a role for D6 in the proper development of leukocytes populations, with absence of this atypical receptor resulting in deregulated expression of other chemokine receptors.

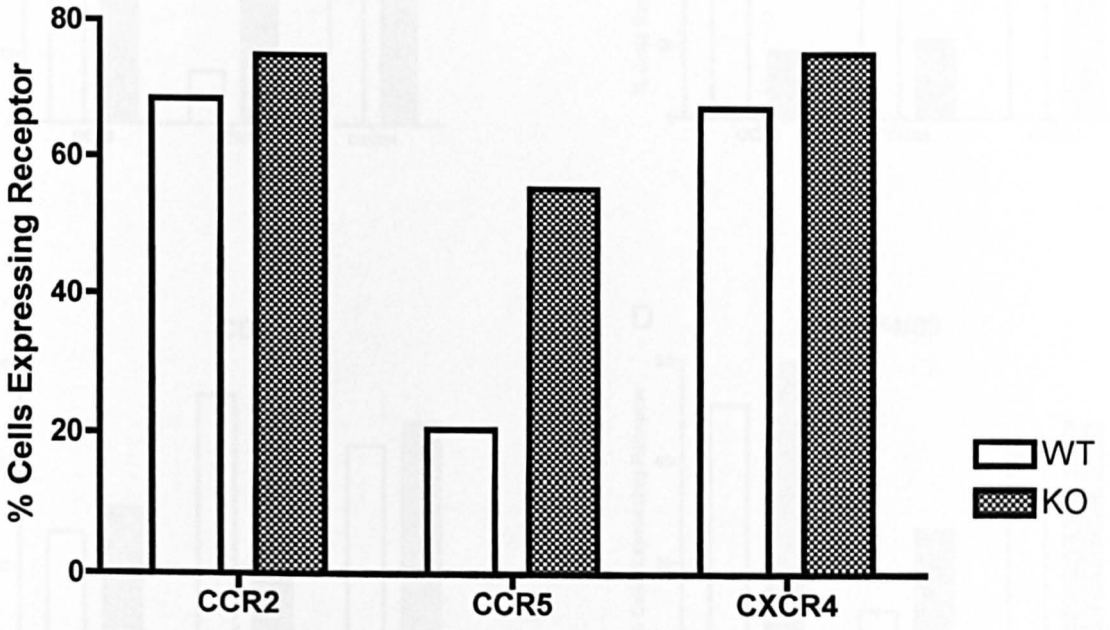


Figure 6.1

Chemokine Receptor Profile of Colonic Lamina Propria Cells From D6 KO Mice

Lamina propria cells pooled from the colons of 3 WT and 3 D6 KO mice were analysed by flow cytometry for expression of CCR2, CCR5 and CXCR4.

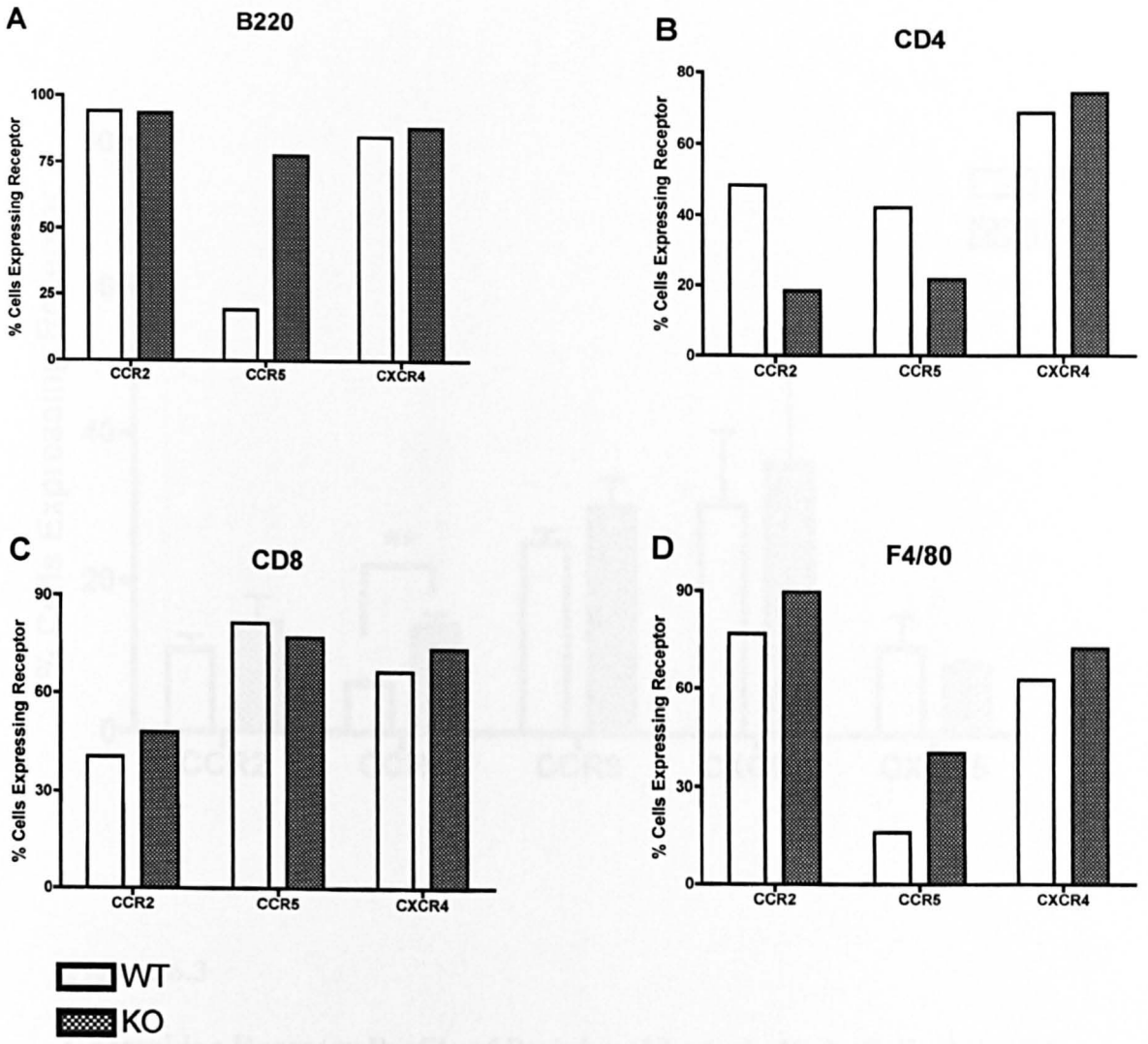


Figure 6.2

Chemokine Receptor Profile of Colonic Lamina Propria Cells From D6 KO Mice

Lamina propria cell populations pooled from the colons of 3 WT and 3 D6 KO mice were analysed by flow cytometry for expression of CCR2, CCR5 and CXCR4. Figures A, B, C and D show the chemokine receptor profiles of B220+, CD4+, CD8+ and F4/80+ lamina propria cells, respectively.

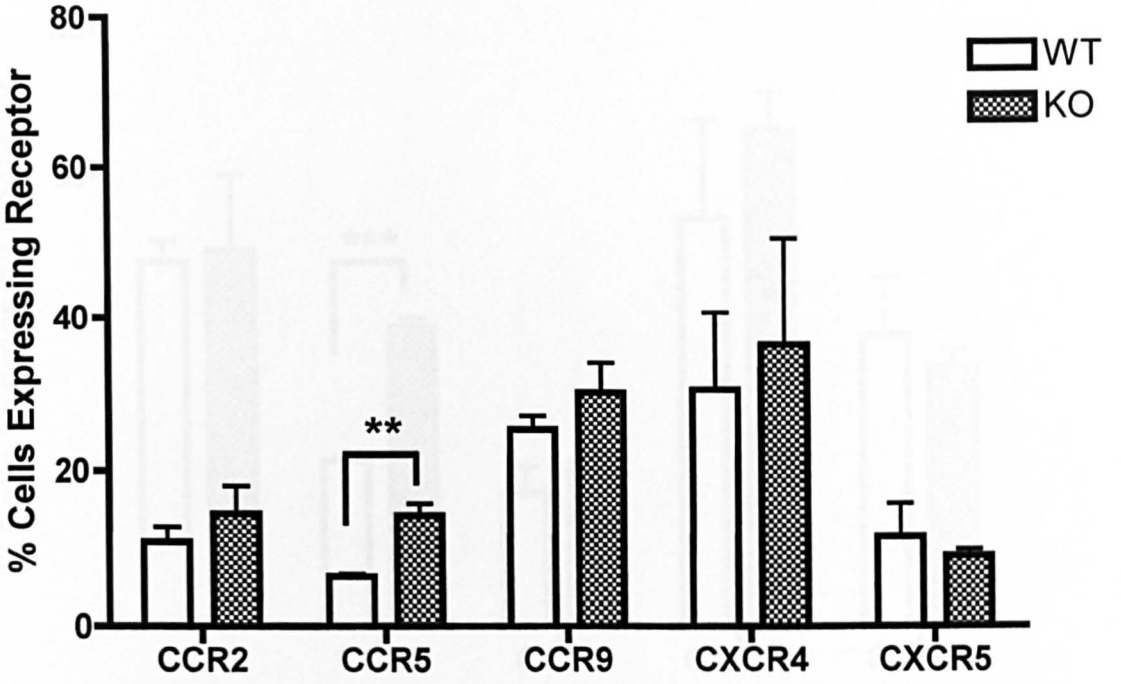


Figure 6.3

Chemokine Receptor Profile of Peripheral Lymph Node Cells From D6 KO Mice

Cells isolated from peripheral lymph nodes of WT and D6 KO mice were analysed by flow cytometry for the expression of CCR2, CCR5, CCR9, CXCR4 and CXCR5. Data are presented as mean + SD of 3 animals per group.

** $p < 0.01$

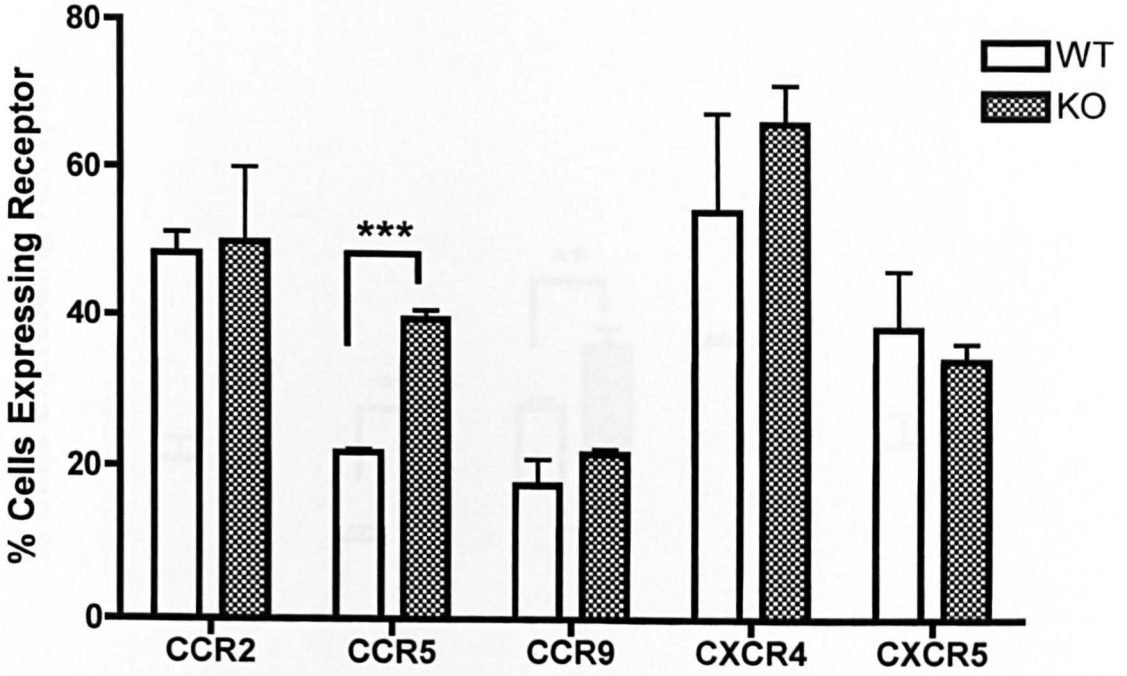


Figure 6.4

Chemokine Receptor Profile of Splenocytes From D6 KO Mice

Cells isolated from peripheral lymph nodes of WT and D6 KO mice were analysed by flow cytometry for the expression of CCR2, CCR5, CCR9, CXCR4 and CXCR5. Data are presented as mean + SD of 3 animals per group.

*** $p < 0.001$

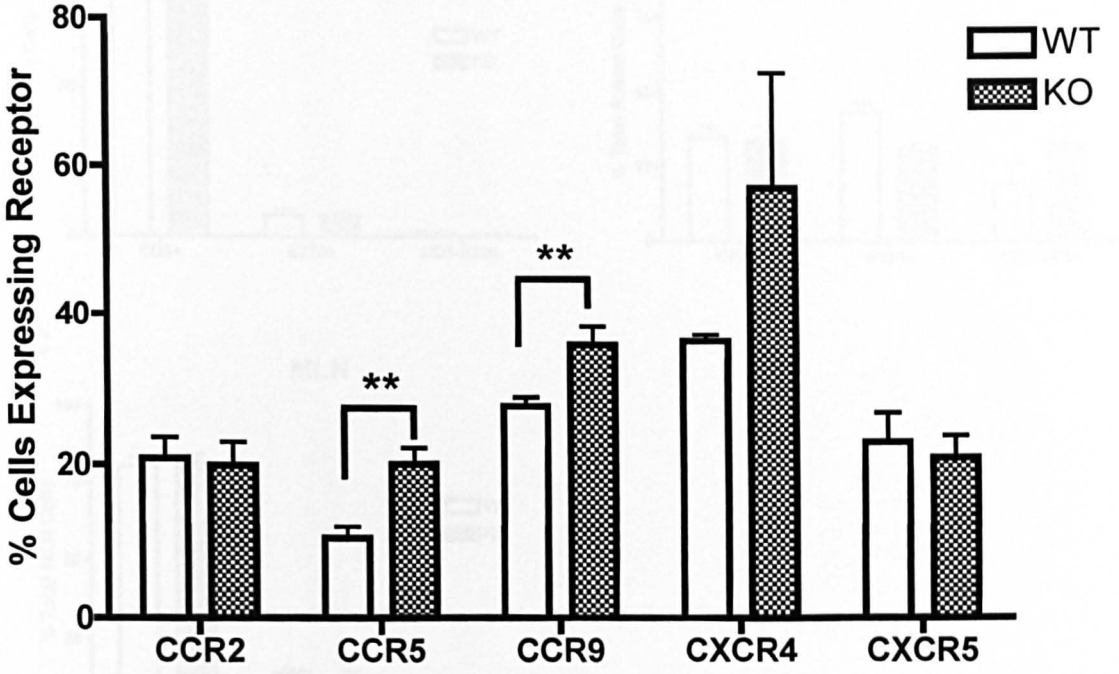


Figure 6.5

Chemokine Receptor Profile of Mesenteric Lymph Node Cells Of D6 KO Mice

Cells isolated from mesenteric lymph nodes of WT and D6 KO mice were analysed by flow cytometry for the expression of CCR2, CCR5, CCR9, CXCR4 and CXCR5. Data are presented as mean + SD of 3 animals per group.

** $p < 0.01$

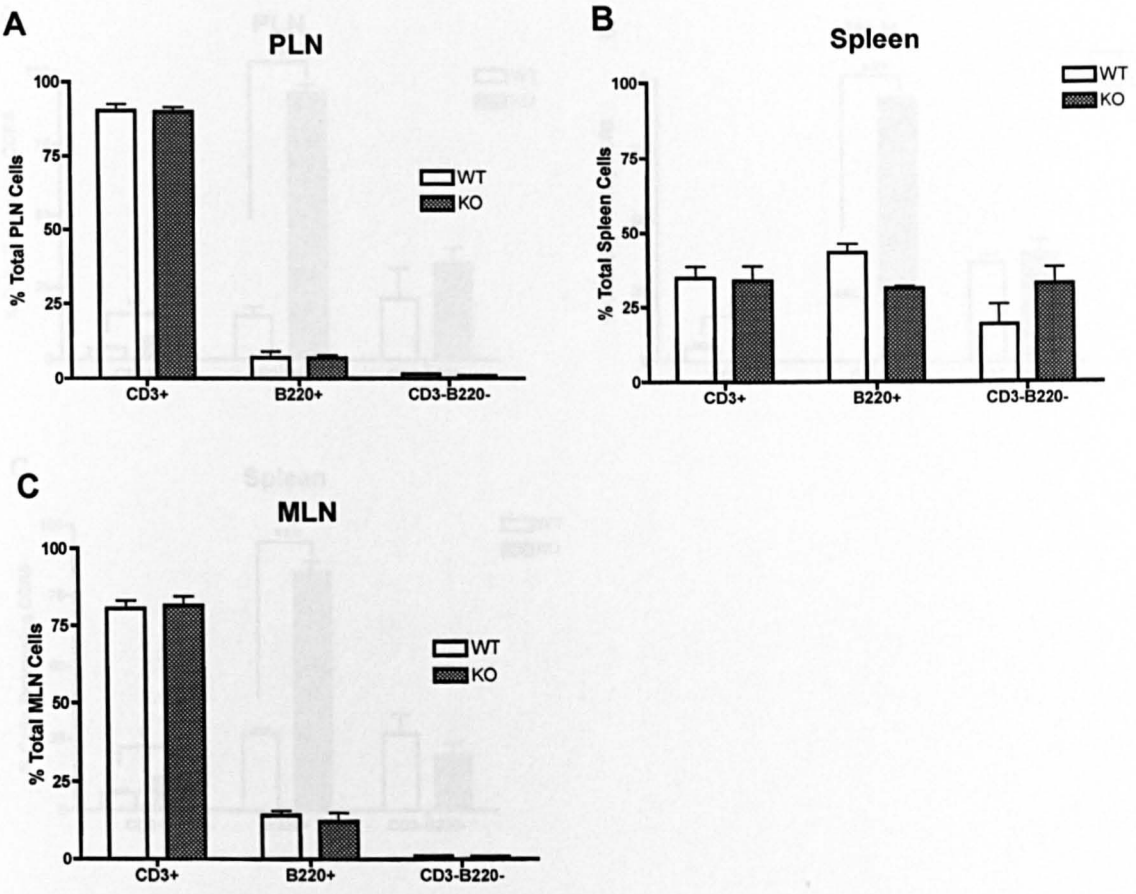


Figure 6.6

Cellular Composition of Lymphoid Tissues in D6 KO Mice

Cells isolated from (A) peripheral lymph nodes, (B) spleen and (C) mesenteric lymph nodes of WT and D6 KO mice were assessed by flow cytometry for the proportions of CD3+ and B220+ cells. Data are presented as mean + SD of 3 animals per group.

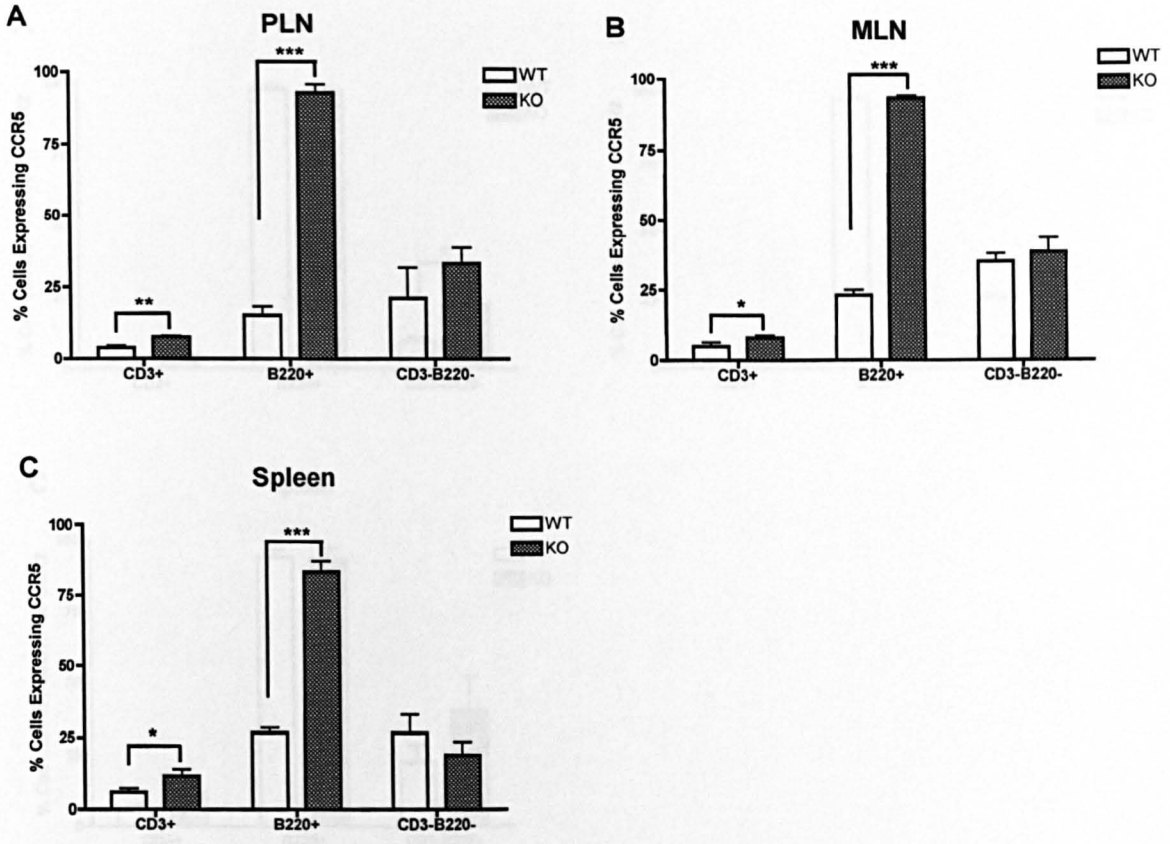


Figure 6.7

CCR5 Expression By CD3+ and B220+ Cell Subsets From D6 KO Mice

Cells were isolated from (A) PLN, (B) MLN and (C) spleens of WT and D6 KO mice and the expression of CCR5 by CD3/B220 subsets assessed by flow cytometry. Data are presented as the mean + SD of 3 animals per group.

*** $p < 0.001$

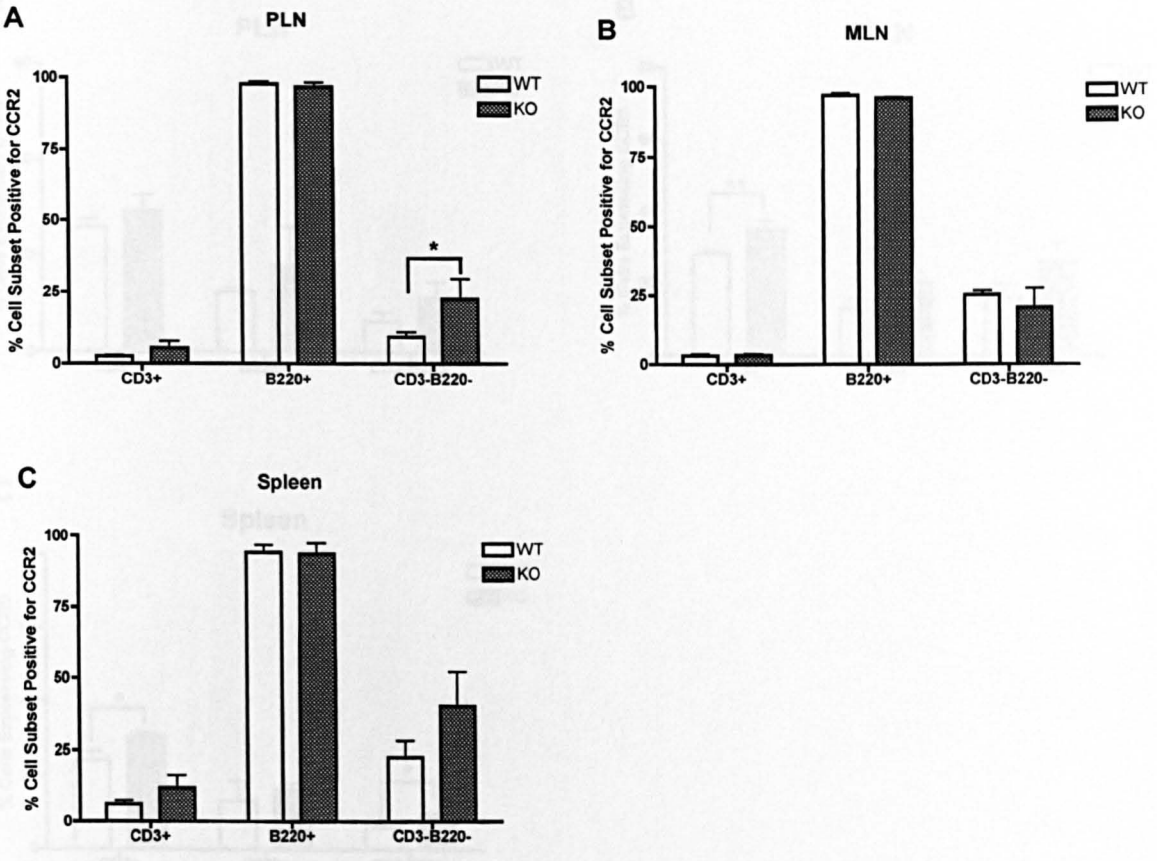


Figure 6.8

CCR2 Expression By CD3+ and B220+ Cell Subsets From D6 KO Mice

Cells were isolated from (A) PLN, (B) MLN and (C) spleens of WT and D6 KO mice and the expression of CCR2 by CD3/B220 subsets was assessed by flow cytometry. Data are presented as mean + SD for 3 animals per group.

* $p < 0.05$

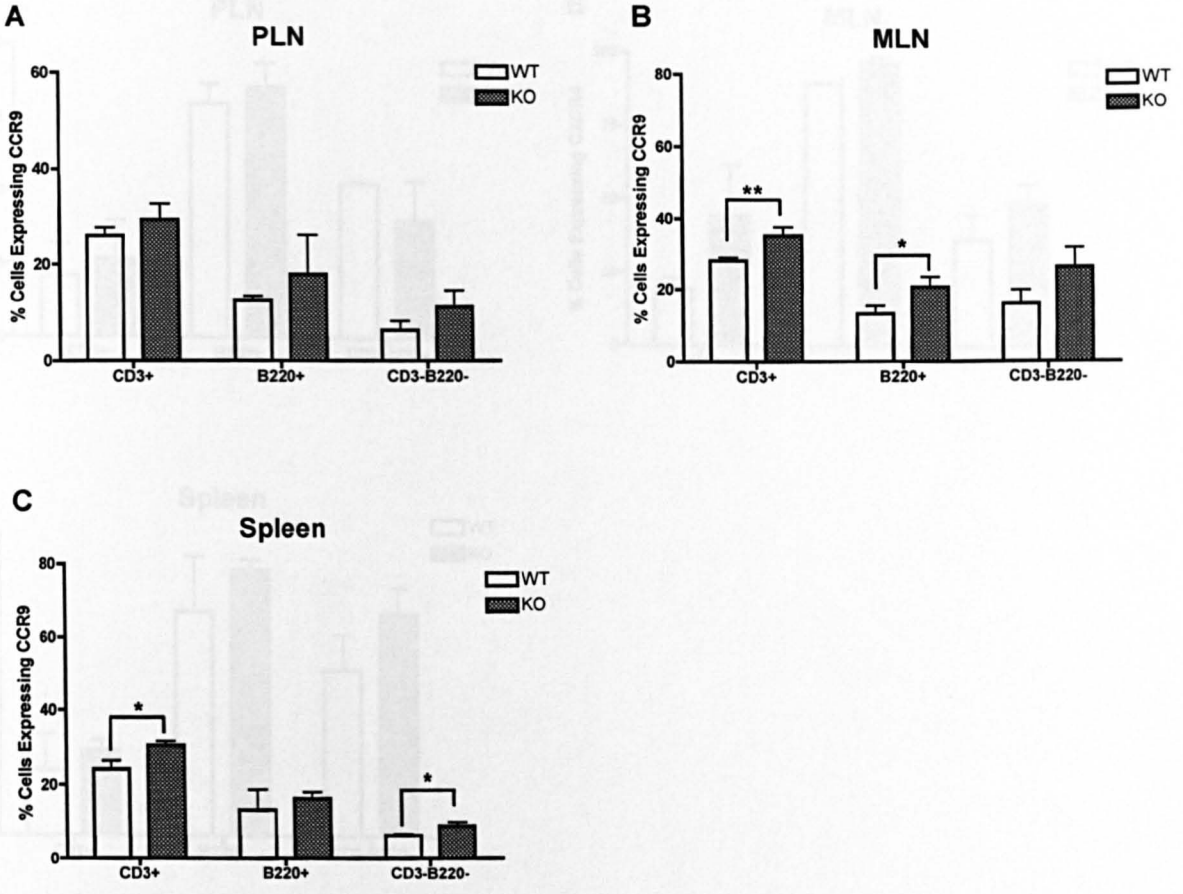


Figure 6.9

CCR9 Expression By CD3+ and B220+ Cell Subsets From D6 KO Mice

Cells were isolated from (A) PLN, (B) MLN and (C) spleens of WT and D6 KO mice and the expression of CCR9 by CD3/B220 subsets was assessed by flow cytometry. Data are presented as mean + SD for 3 animals per group.

* $p < 0.05$, ** $p < 0.01$

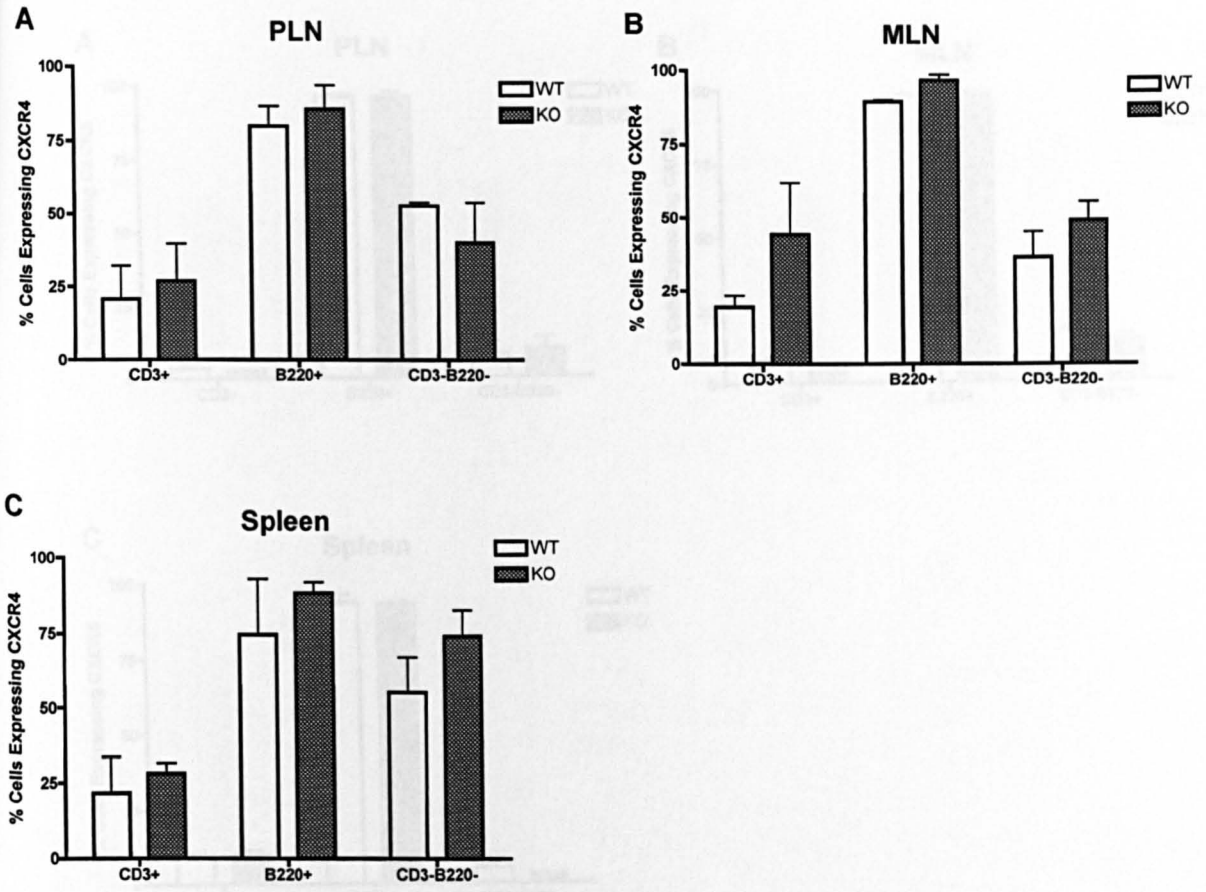


Figure 6.10

CXCR4 Expression By CD3+ and B220+ Cell Subsets From D6 KO Mice

Cells were isolated from (A) PLN, (B) MLN and (C) spleens of WT and D6 KO mice and the expression of CXCR4 by CD3/B220 subsets was assessed by flow cytometry. Data are presented as mean + SD for 3 animals per group.

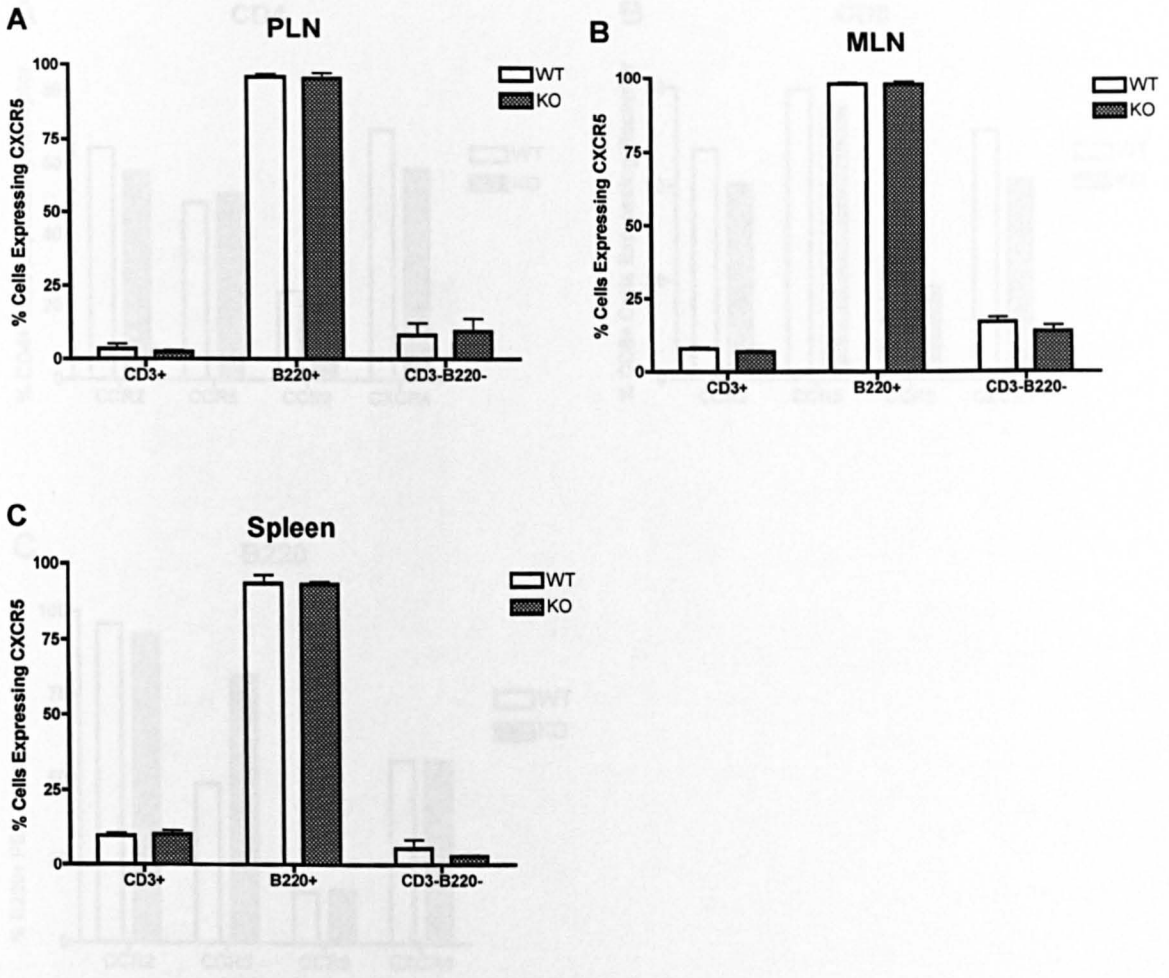


Figure 6.11

CXCR5 Expression By CD3+ and B220+ Cell Subsets From D6 KO Mice

Cells were isolated from (A) PLN, (B) MLN and (C) spleens of WT and D6 KO mice and the expression of CXCR5 by CD3/B220 subsets was assessed by flow cytometry. Data are presented as mean + SD for 3 animals per group.

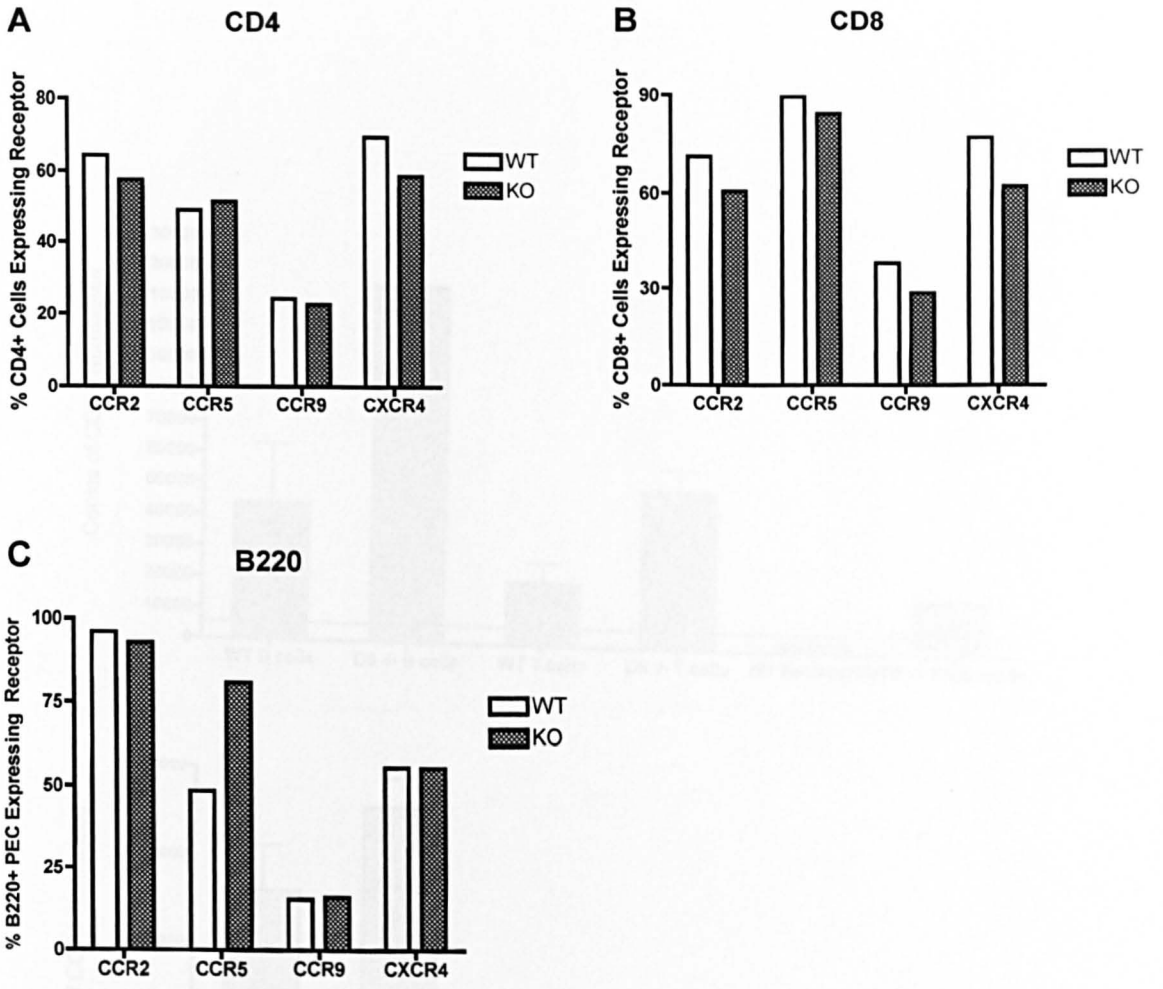


Figure 6.12

Chemokine Receptor Profile of Peritoneal Exudate Cells From Resting D6 KO Mice

Cells were isolated from the peritoneum of 3 WT and 3 D6 KO mice, pooled and the expression of CCR2, CCR5, CCR9 and CXCR4 by (A) CD4+, (B) CD8+ and (C) B220+ cells determined by flow cytometry.

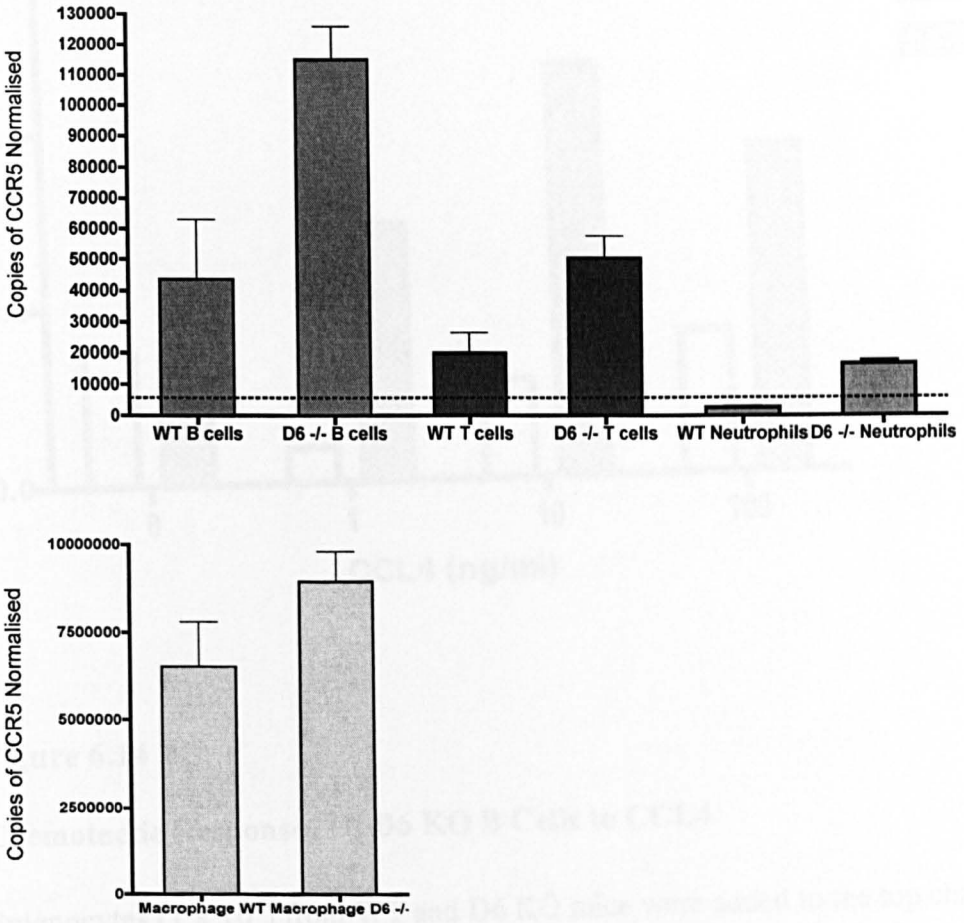


Figure 6.13

Quantification of CCR5 mRNA From D6 KO Cell Populations

RNA was extracted from purified T and B cells, bone marrow neutrophils, and M-CSF BM-derived macrophages. Levels of CCR5 mRNA were determined by real time PCR and normalised to the housekeeping gene, GAPDH. The data represent the means of three mice ± SEM. Dotted line represents the limit of sensitivity. The data appears courtesy of Dr Clive McKimmie.

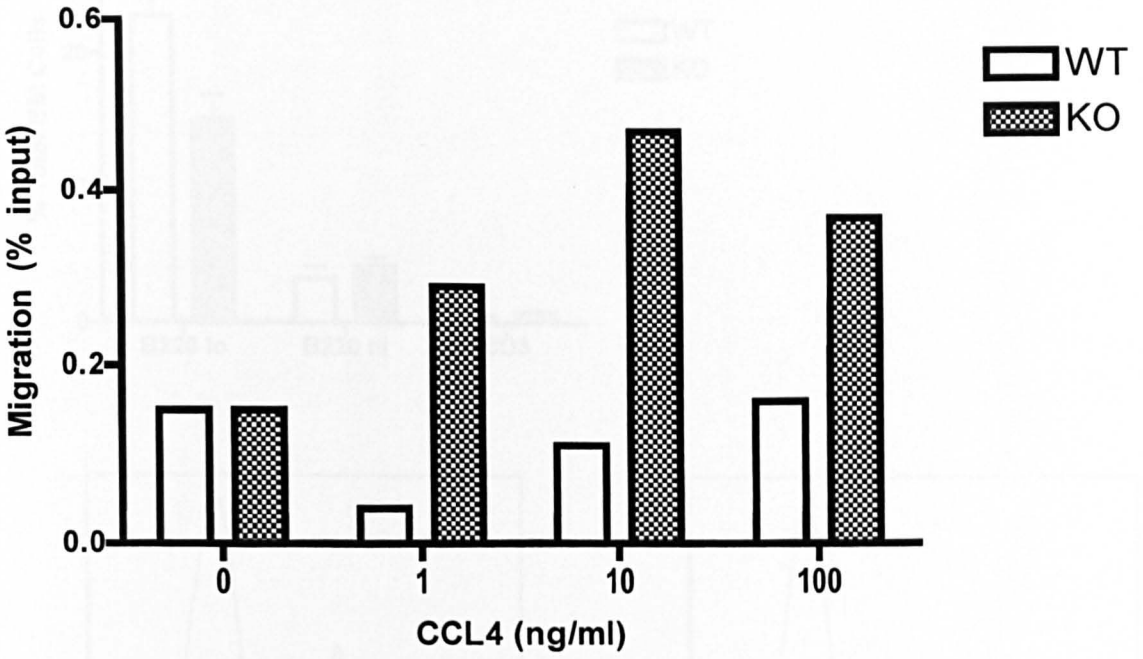


Figure 6.14

Chemotactic Responses Of D6 KO B Cells to CCL4

Splenocytes (1 x 10^5) from WT and D6 KO mice were added to the top chamber of Transwell plates containing 0, 1, 10 or 100 ng/ml of CCL4 in the lower chamber. Plates were cultured at 37°C for 3 hrs and then cells were collected from the lower wells. The index of migration was calculated by dividing the number of CD19+ cells found in the lower chamber by the original number of CD19+ cells added to the top chamber. The data are representative of 2 individual experiments.

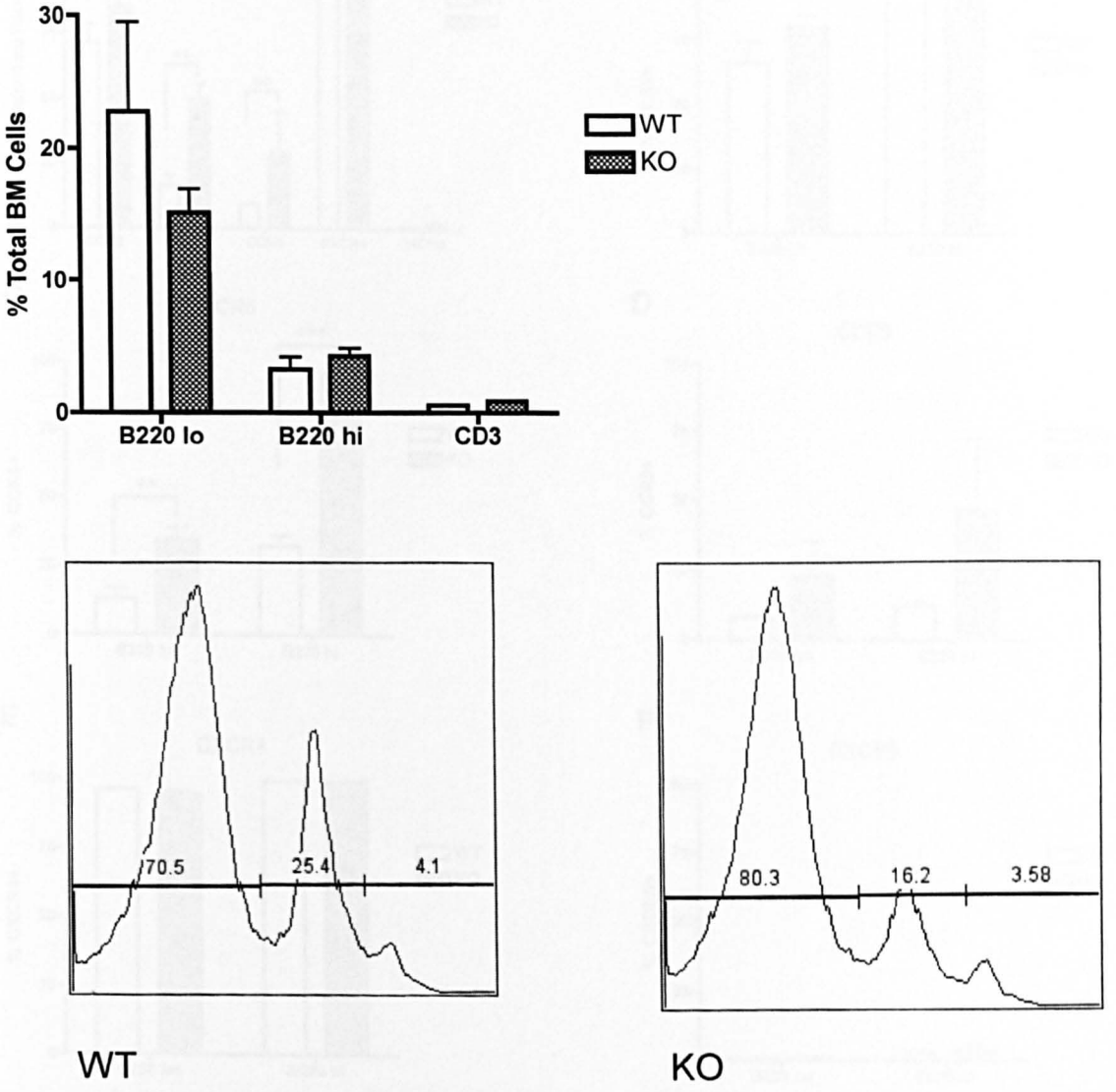


Figure 6.15

B220 and CD3 Expression by Bone Marrow Cells in D6 KO Mice

Bone marrow cells were retrieved from the femurs of WT and D6 KO mice and the proportions of $B220^{lo}$, $B220^{hi}$ and CD3+ cells determined by flow cytometry. Data are presented as mean + SD and represent 3 animals per group. Below are shown representative B220-FACS profiles of WT(left) and D6 KO (right) mice.

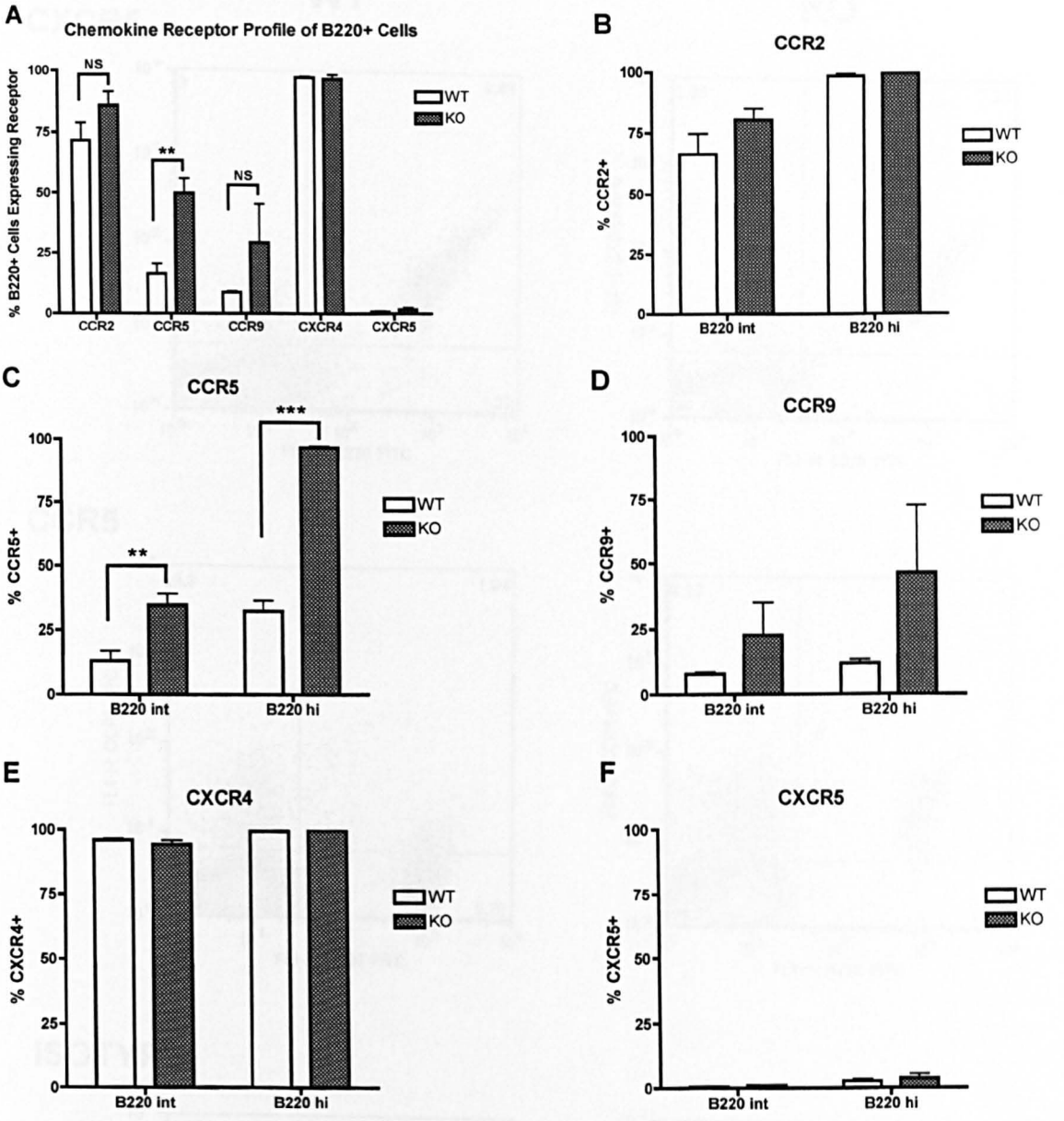


Figure 6.16

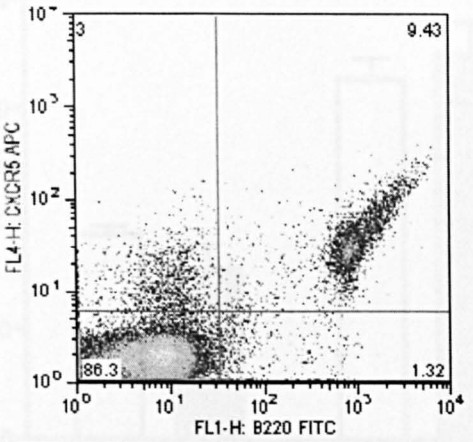
Chemokine Receptor Profile of B220+ BM Cells in D6 KO Mice

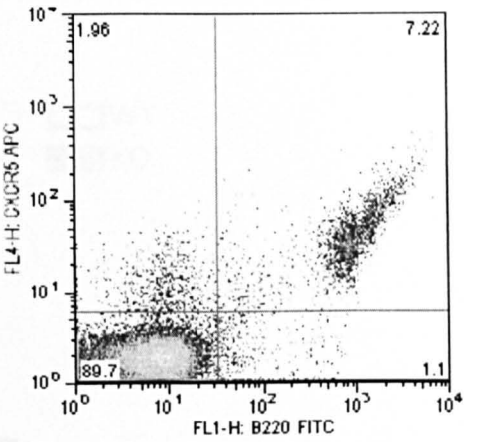
Bone marrow cells were retrieved from the femurs of WT and D6 KO mice and the expression of CCR2, CCR5, CCR9, CXCR4 and CXCR5 by B220+ cells determined by flow cytometry (A). The expression of (B) CCR2, (C) CCR5, (D) CCR9, (E) CXCR4 and (F) CXCR5 by $B220^{int}$ (left) and $B220^{hi}$ (right) was also assessed. Data are the mean + SD of 3 animals per group.

** $p < 0.01$, *** $p < 0.001$

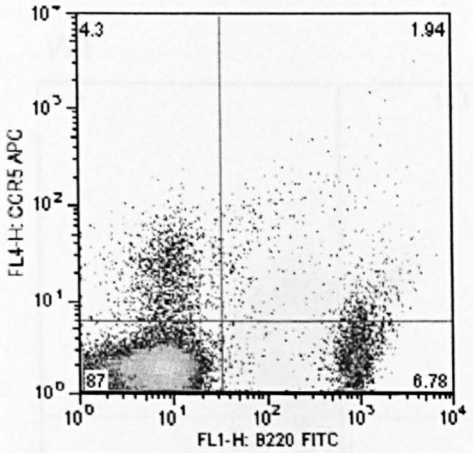
WT KO

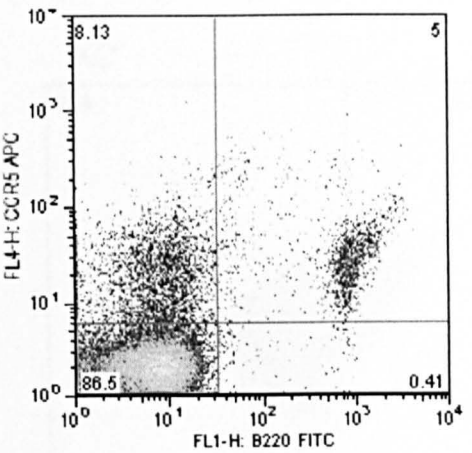
CXCR5



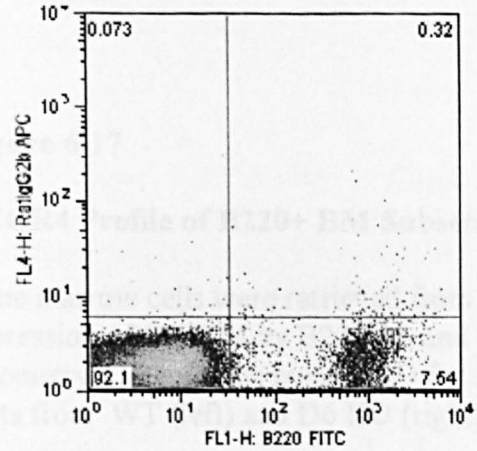


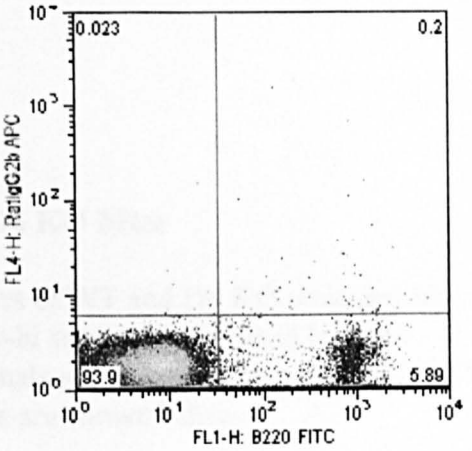
CCR5





ISOTYPE





Chemokine Receptor Profile of D6 KO B Cells

Shown above are representative flow cytometry plots of chemokine receptor-stained B cells from PLN of WT and D6 KO mice.

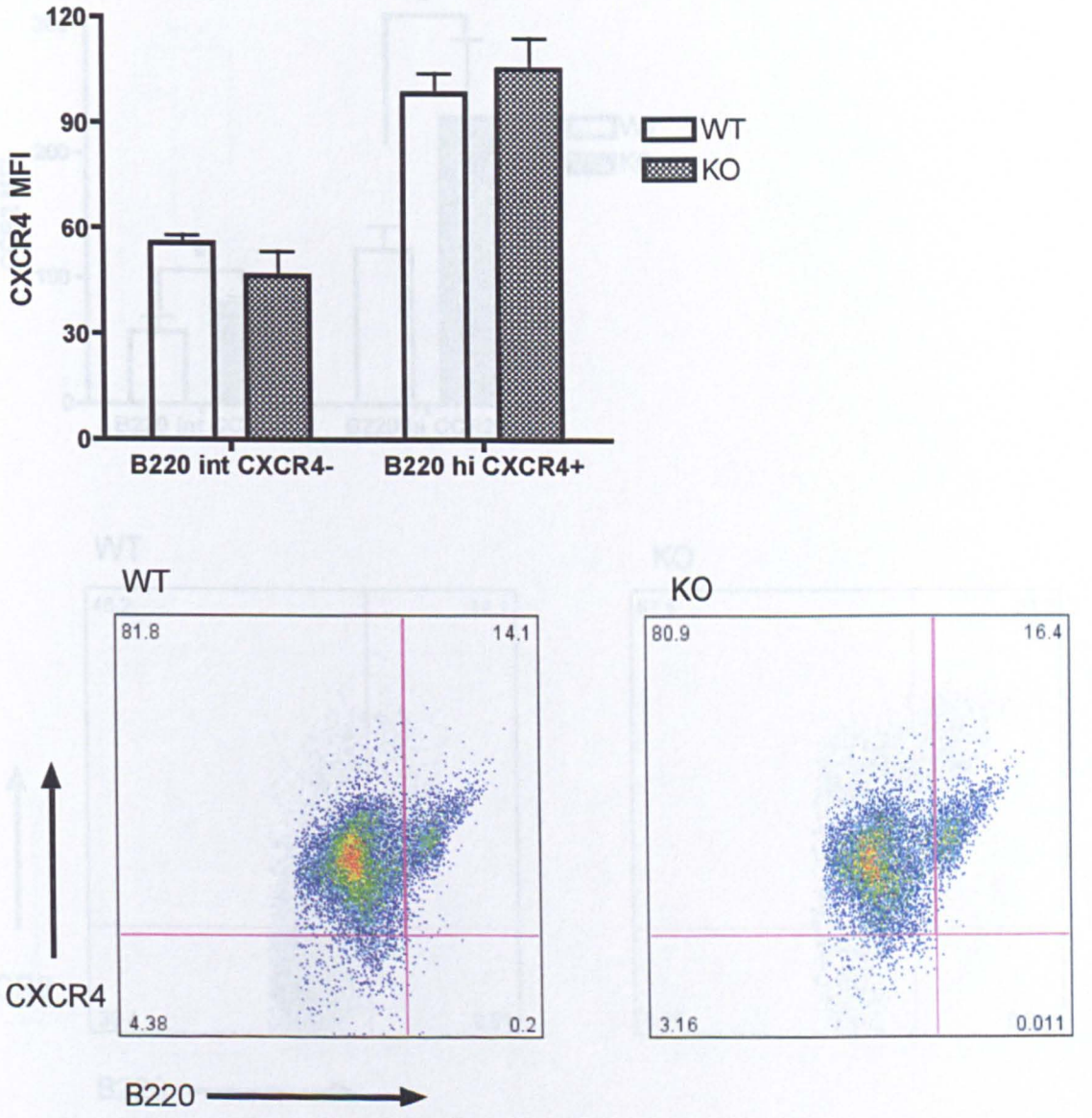


Figure 6.17

CXCR4 Profile of B220+ BM Subsets in D6 KO Mice

Bone marrow cells were retrieved from femurs of WT and D6 KO mice and the expression of CXCR4 by B220-int and B220-hi subsets determined by flow cytometry. Data are the mean + SD for 3 animals per group. Representative FACS plots from WT (left) and D6 KO (right) mice are shown below.

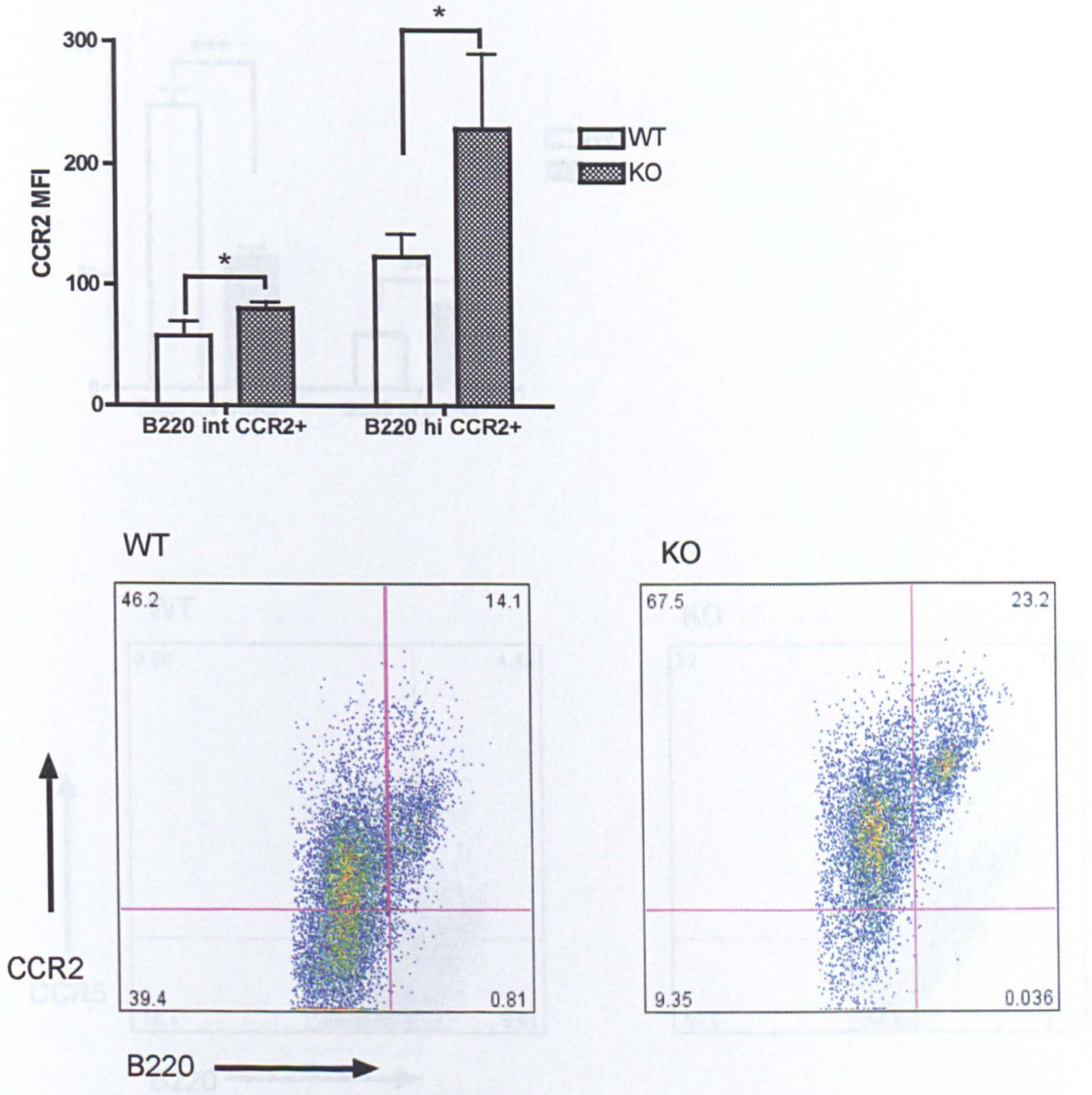


Figure 6.18

CCR2 Profile of B220+ BM Subsets in D6 KO Mice

Bone marrow cells were retrieved from femurs of WT and D6 KO mice and the expression of CCR2 by $B220^{int}$ and $B220^{hi}$ subsets determined by flow cytometry. Data are the mean + SD for 3 animals per group. Representative FACS plots from WT (left) and D6 KO (right) mice are shown below.

* $p < 0.05$

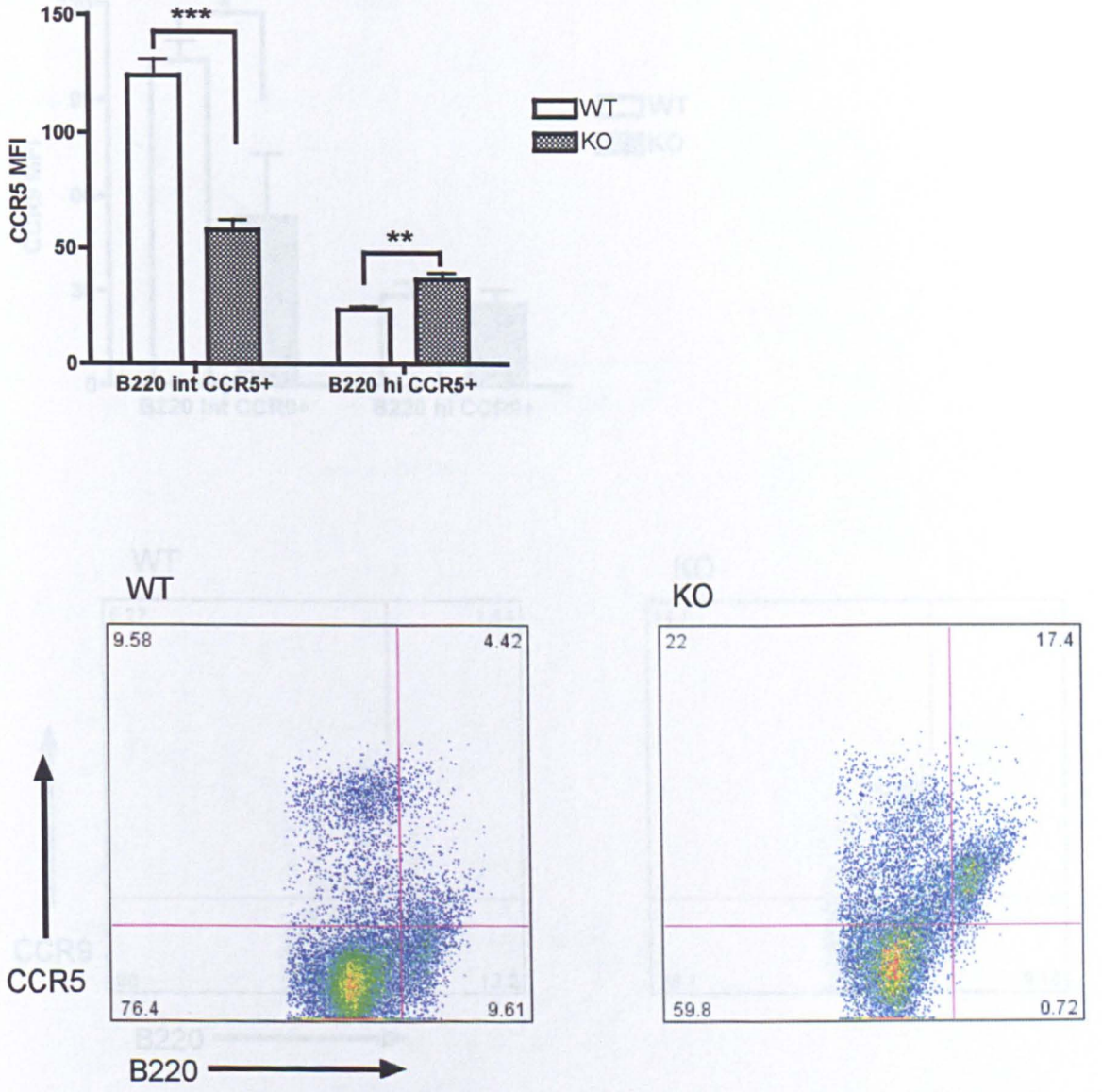


Figure 6.19

CCR5 Profile of B220+ BM Subsets in D6 KO Mice

Bone marrow cells were retrieved from femurs of WT and D6 KO mice and the expression of CCR5 by $B220^{int}$ and $B220^{hi}$ subsets was determined by flow cytometry. Data are the mean + SD for 3 animals per group. Representative FACS plots from WT (left) and D6 KO (right) mice are shown below.

** $p < 0.01$, *** $p < 0.001$

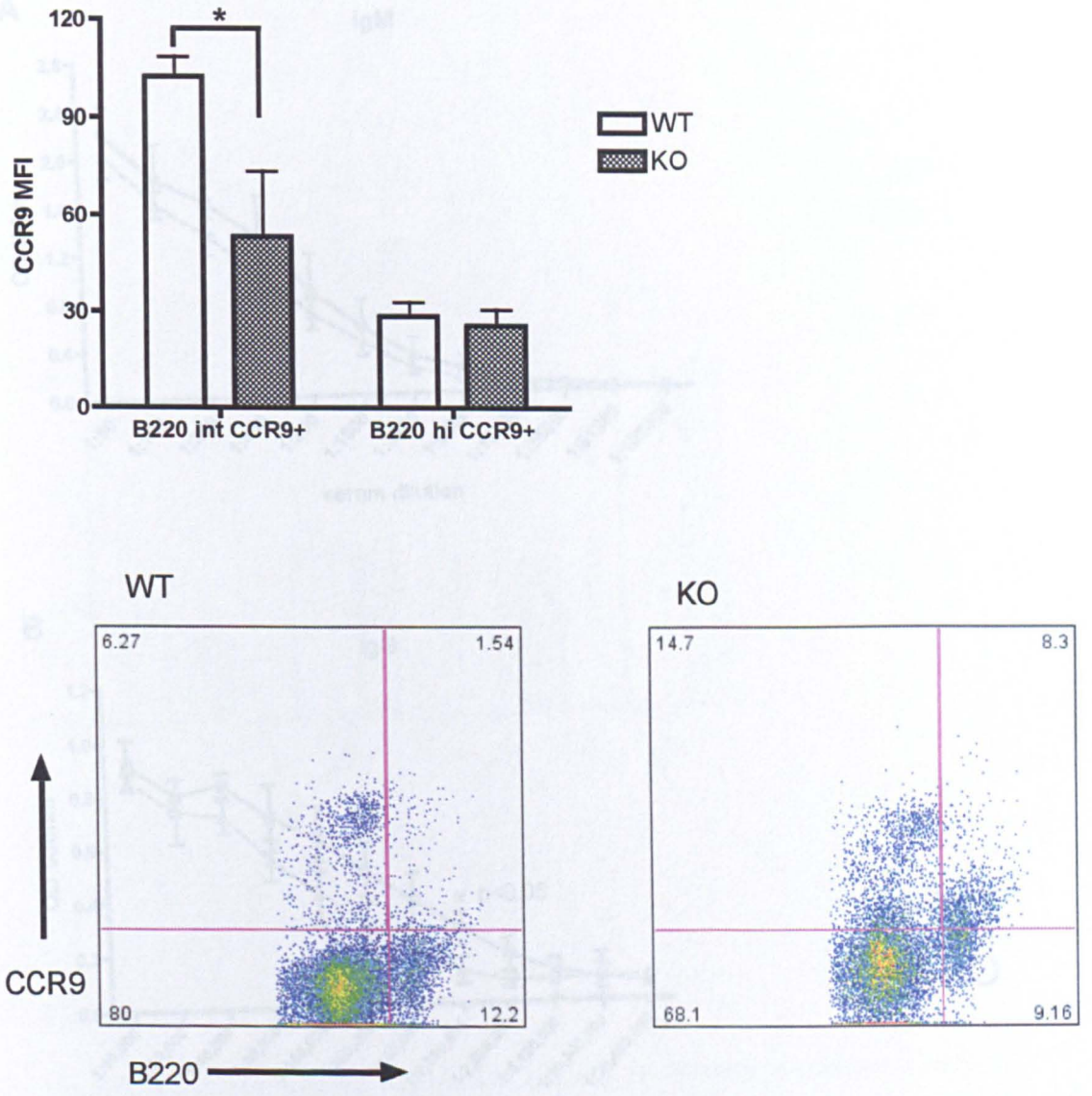


Figure 6.20

CCR9 Profile of B220+ BM Subsets in D6 KO Mice

Bone marrow cells were retrieved from femurs of WT and D6 KO mice and the expression of CCR5 by $B220^{int}$ and $B220^{hi}$ subsets determined by flow cytometry. Data are the mean + SD for 3 animals per group. Representative FACS plots from WT (left) and D6 KO (right) mice are shown below.

* $p < 0.05$

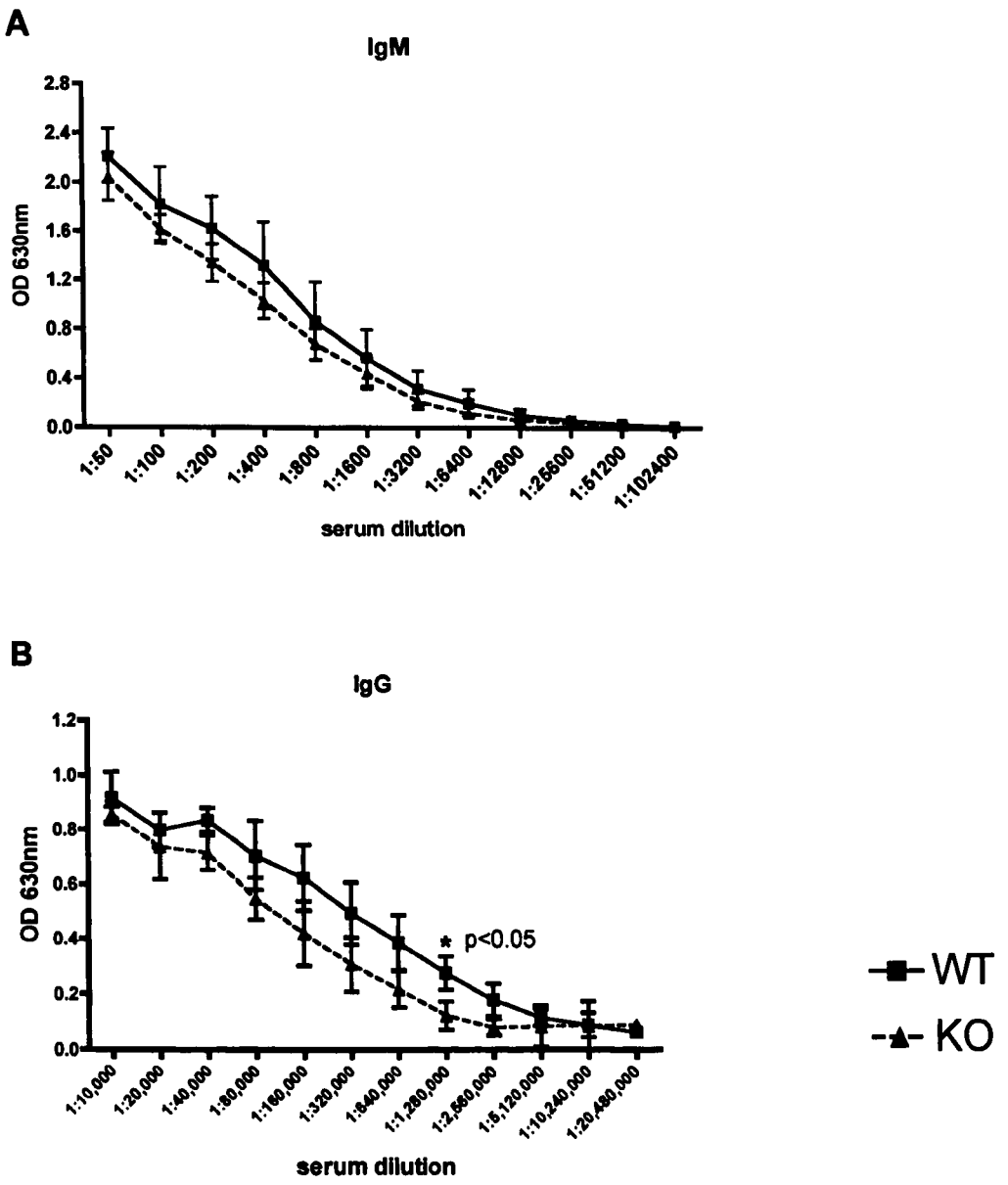


Figure 6.21

Serum IgM and IgG Levels in D6 KO Mice

Total levels of (A) IgM and (B) IgG were measured in sera of WT and D6 KO mice by ELISA. Data are the mean + SD of 3 animals per group.

* $p < 0.05$

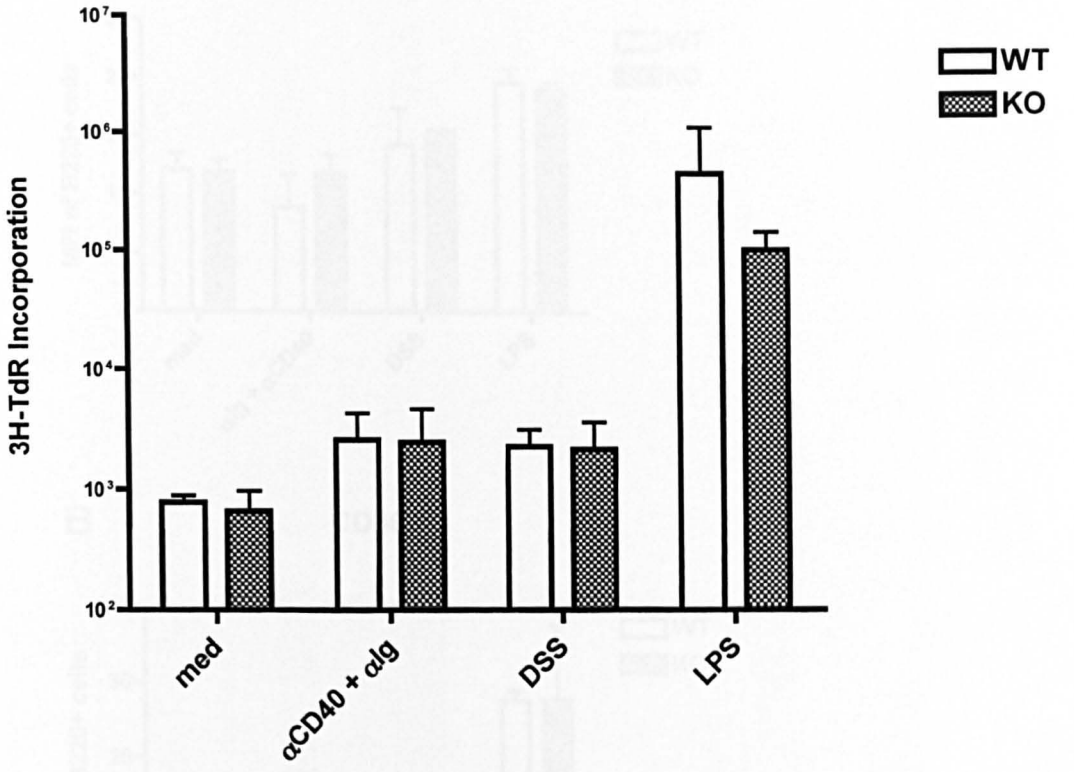


Figure 6.22

Proliferative Responses of D6 KO B Cells to Mitogenic Stimulation

B cells purified from spleens of WT and D6 KO mice were cultured in complete medium alone, or complete medium containing αCD40 + αIgM (10μg/ml), DSS (50μg/ml) or LPS (1μg/ml) for 48 hours. ^{3}H-Tdr was added for the last 20 hours of culture and levels of proliferation determined by ^{3}H-Tdr incorporation. Data are the means + 1 SD for 3 mice per group.

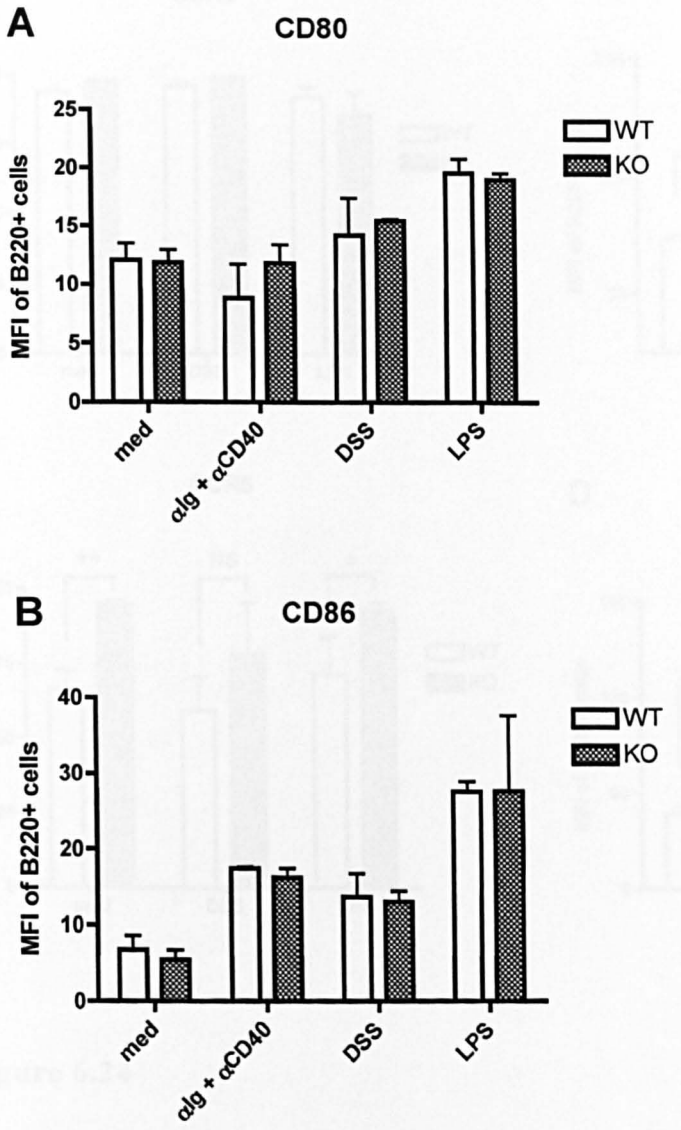


Figure 6.23

Costimulatory Molecule Expression By D6 KO B Cells in Response to Mitogenic Stimuli

B cells purified from spleens of WT and D6 KO mice were cultured in complete medium alone, or complete medium containing αCD40 + αIg (10μg/ml), DSS (50μg/ml) or LPS (1μg/ml). Cells were harvested after 48h and the expression of (A) CD80 and (B) CD86 determined by flow cytometry. Data are the means + 1 SD for 3 mice per group.

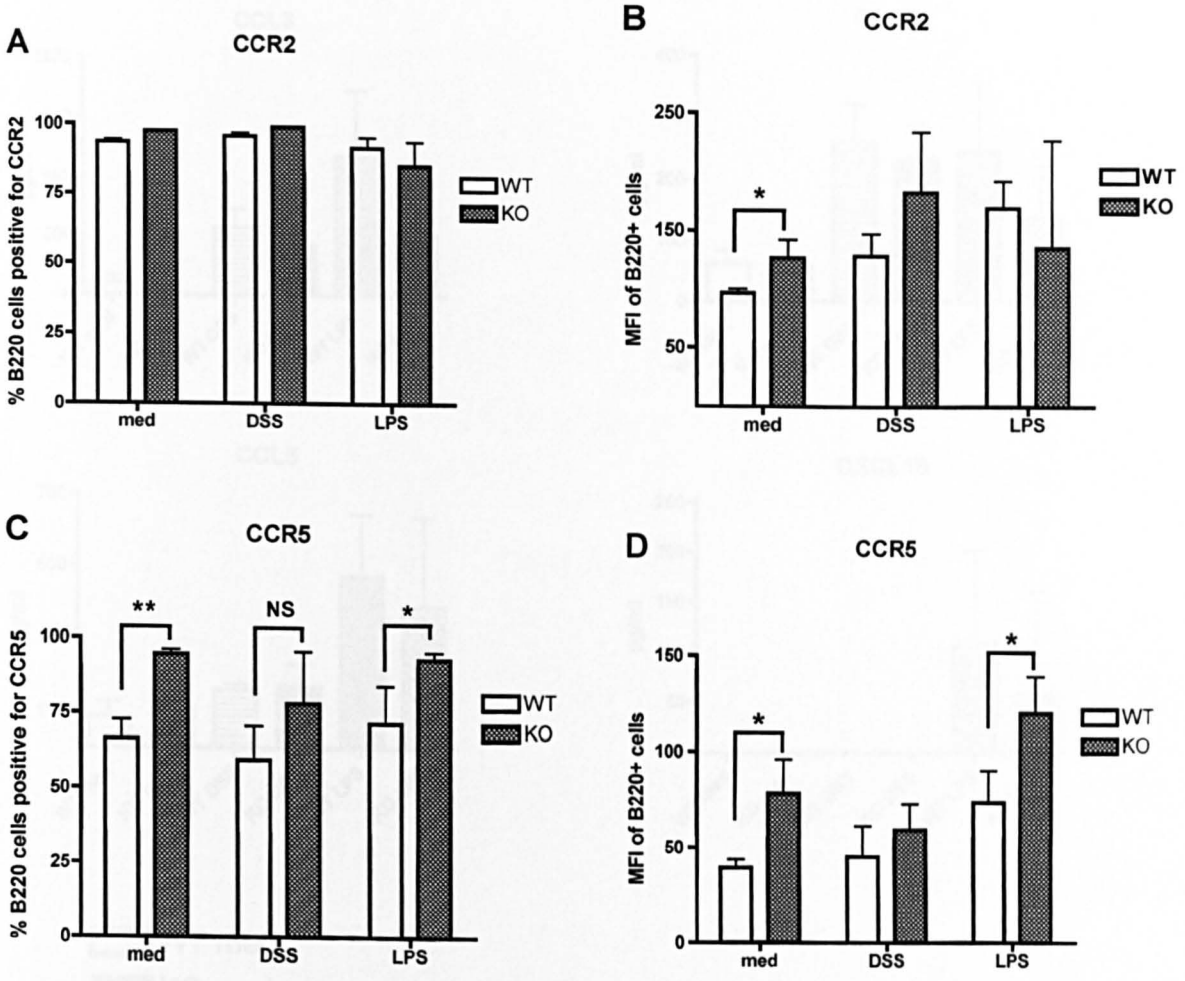


Figure 6.24

Chemokine Receptor Expression By D6 KO B Cells in Response to Mitogenic Stimulation

B cells purified from spleens of WT and D6 KO mice were cultured in complete medium alone, or complete medium containing DSS (50μg/ml) or LPS (1μg/ml). Cells were harvested after 48h and expression of CCR2 (A + B) and CCR5 (C + D) determined by flow cytometry. Percentage of B220+ cells positive (A + C) and MFI of B220+ cells (B + D) are both shown. Data are the means + 1 SD for 3 animals per group.

* $p < 0.05$, ** $p < 0.01$

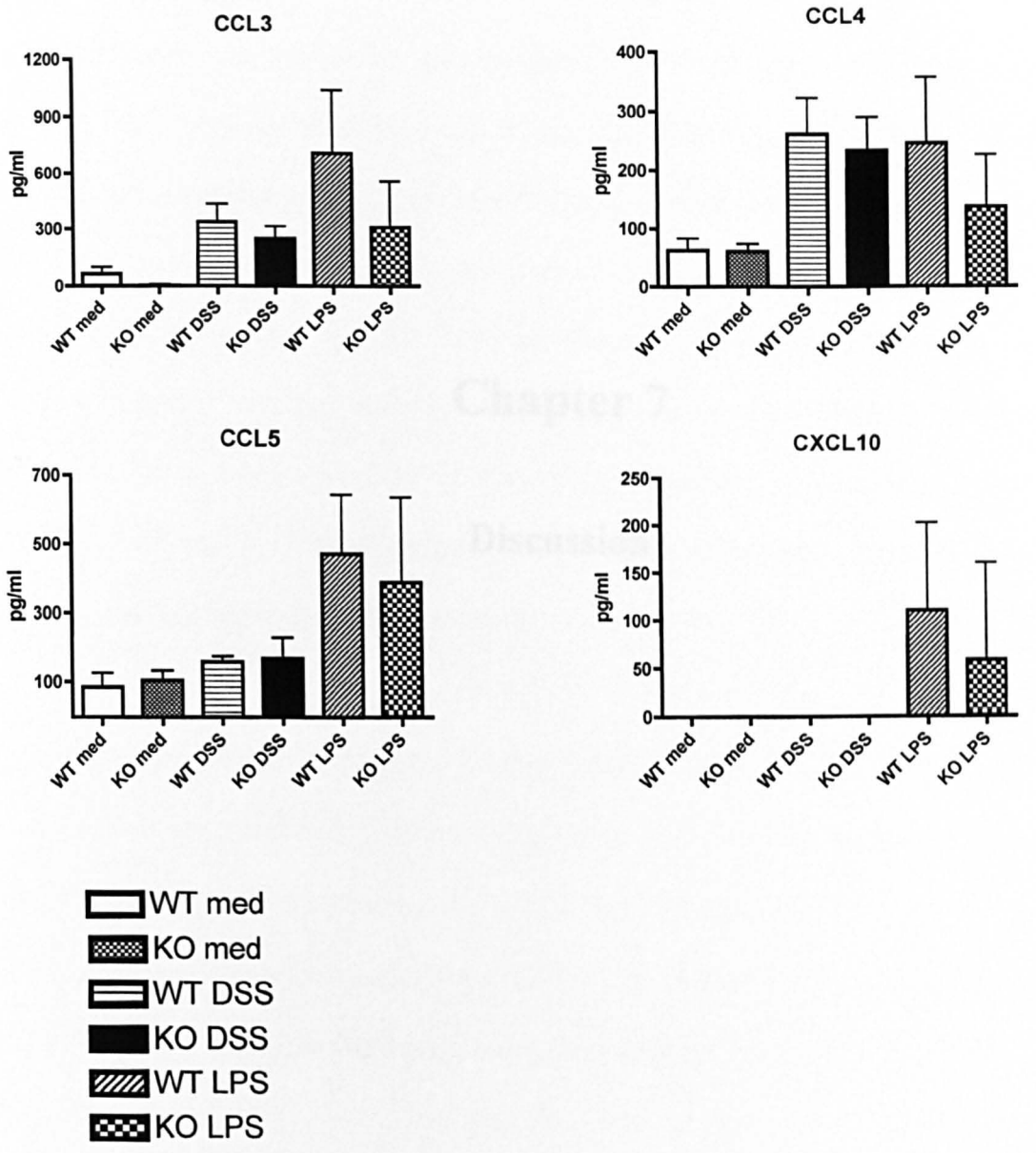


Figure 6.25

Chemokine Production By D6 KO B Cells in Response to Mitogenic Stimulation

B cells purified from spleens of WT and D6 KO mice were cultured in complete medium alone, or complete medium containing (50μg/ml) DSS or LPS (1μg/ml). Culture supernatants were harvested at 48h and assessed for levels of CCL2, CCL3, CCL5 and CXCL10 by Luminex. Data are presented as mean + SD and represent 3 animals per group.

Chapter 7

Discussion

The immune system faces the daunting task of protecting the organism against pathogens while simultaneously preserving host integrity. Therefore multiple regulatory mechanisms exist to fine-tune inflammatory responses, enabling protection against foreign microbes to develop in the absence of overt immunopathology. More than two decades ago, the study of the IL-1 type II receptor (IL-1RII) suggested a novel biological function for this molecule as a "decoy receptor" (339). The decoy paradigm describes a receptor that is capable of binding its ligand with high affinity, but lacks the capacity to transduce a productive signal following ligand recognition (265). Decoy receptors have been described for members of the IL-1, TNF and IL-10 cytokine families, and it is has been proposed that these molecules enable tight regulation of cytokine networks (265).

Recently, it has become apparent that decoy receptors may also play a role in chemokine biology. First described by Nibbs and colleagues in 1997, D6 is homologous to members of the CC-chemokine receptor family and shows high affinity for an unusually large number of inflammatory-type CC-chemokines (270, 271). However, unlike conventional CC-chemokines receptors, D6 expressed in cell lines does not induce any detectable signal or chemotaxis in response to ligand binding (270). Also atypically, D6 constitutively recycles to and from the cell surface independently of ligand, but is extremely efficient at internalising any ligand detected at the cell surface and targeting it for intracellular degradation (285, 286, 288). Due to this ability to scavenge ligands in the absence of any detectable functional response, it has been proposed that D6 may serve as a decoy receptor for inflammatory CC-chemokines (264, 274, 275).

When I started this thesis, all of the work previously published to support the "D6 decoy" hypothesis had been performed *in vitro*. However, others in the Division had recently generated D6-deficient mice and, strikingly, found that these animals developed excessive and prolonged cutaneous inflammatory responses when subjected to topical application of phorbol ester (292). These studies therefore represented the first evidence that D6 might normally have an anti-inflammatory function *in vivo*. Despite this, our understanding of the *in vivo* function of D6 remained limited at the time I started. Thus the aims of my thesis were to conduct a more thorough characterisation of the immune system of D6-deficient animals, in an attempt to understand the true biological significance of this molecule. I sought to investigate the role of D6 in lymphoid and extra-cutaneous tissues of both normal and immunologically challenged animals. In particular, due to the high levels of D6 expression that had been reported in intestinal tissue, I aimed to explore the function of D6 at this particular site. The studies described in this thesis have generated a number of novel observations with regard to the D6-deficient animals.

The Role of D6 in Steady State Leukocyte Trafficking

My initial experiments, described in Chapter 3, examined the cellular composition of various lymphoid tissues from D6-deficient animals under resting conditions. Although the ligands of D6 are traditionally associated with inflammation, CC-chemokines also influence haematopoiesis and govern leukocyte recruitment to non-inflamed sites (115, 121, 125, 135, 340). Moreover, immunohistochemical studies have shown D6 to be expressed at high levels by lymphatic endothelial cells. Therefore I wished to examine if D6-deficient mice would demonstrate altered leukocyte trafficking to lymphoid tissues in the steady state.

Compared to their WT counterparts, D6 KO mice did not show any aberrations in any lymphocyte populations of the thymus, spleen or lymph nodes. However, I detected a marginal increase in the proportions of $F4/80^+$ and $CD11c^+$ cells in the D6 KO spleen. These early experiments suggested that the absence of D6 does not affect the gross population of lymphoid tissues by T cells and B cells. On the other hand, my data do not exclude the possibility that different functional leukocyte populations, for example altered effector T cell subsets, are present in the D6 KO nodes. This could be addressed in future experiments by comparing cytokine production by WT and D6 KO T cells. Indeed, I observed that lymph node cells and splenocytes from D6 KO mice showed higher levels of proliferation in response to Con A stimulation, *in vitro*, compared with WT controls and this could reflect differences in leukocyte sub-populations in D6 KO lymphoid tissue. Alternatively, as CCL2 and CCL3 have been shown to enhance Con A induced activation, another explanation for my findings is that in the absence of D6 scavenging, increased levels of CC-chemokines amplified the mitogenic effects of Con A (89). Comparing levels of CC-chemokines in WT and KO cultures would be necessary to address this point. It would also be interesting in future to investigate whether Con A induced responses of WT mice can be heightened by addition of exogenous CC-chemokines, or if the Con A responses of D6 KO animals can be reduced by addition of antibodies targeted against various CC-chemokine ligands of D6.

A further explanation for these findings could be that greater numbers of antigen-presenting cells, which are needed to sustain Con-A induced responses of T cells, were present in the D6 KO cultures (341). As already stated, I found that the spleen of D6 KO mice contained

slightly increased proportions of F4/80$^+$ and CD11c$^+$ cells, taken to be macrophages and dendritic cells, respectively. Although I did not detect similar increases in CD11c$^+$ and F4/80$^+$ cells in other lymphoid tissues of D6 KO mice, the extremely low numbers of CD11c$^+$ or F4/80$^+$ cells obtained from these sites may have complicated these analyses. Other experimental approaches, for example, quantification of CD11c$^+$ cells *in situ* by laser-scanning cytometry, may enable better comparison of DC and macrophage populations in WT and D6 KO lymph nodes. On the other hand, as distinct mechanisms control the myeloid cell recruitment and maintenance at different tissues (342), it is possible that a lack of chemokine regulation by D6 might only affect myeloid populations in the spleen. An increase in splenic CD11c$^+$ and F4/80$^+$ populations in D6 KO mice could have arisen from higher levels of monocyte-attracting CC-chemokines in the absence of D6 scavenging. Also, as the maintenance of DC and macrophage populations in spleen has been proposed to be controlled by *in situ* proliferation and to be independent of monocyte input (343, 344), CC-chemokine-driven proliferation or survival may have caused the phenotype observed in the KO mice (122, 345). This could be investigated by comparing the rates of cell division and apoptosis in spleens from WT and KO animals, using BrdU labelling or TUNEL analysis, respectively. In addition, comparison of CC-chemokine levels in spleen homogenates of WT and D6 KO animals would be necessary to determine if lack of D6 scavenging leads to higher chemokine levels at this site.

Investigating the Myeloid Compartment of D6 KO Mice

These early data suggested minor deviations in the myeloid compartment of the D6 KO mice. However, as it was difficult to obtain sufficient cells of this kind in other organs, I decided to investigate the cellular composition of the peritoneum under resting and

inflamed conditions (Chapter 4). The peritoneal cavity houses a large resident macrophage population that can be easily retrieved by lavage and thus represents an excellent source of these cells. Analysis of peritoneal exudate cells (PEC) from resting WT and D6 KO mice revealed comparable numbers of $CD4^+$ T cells, $CD8^+$ T cells, $B220^+$ cells and $CD11c^+$ cells. However, I found that increased proportions of $F4/80^+$ cells were present in the non-inflamed peritoneum of D6 KO mice compared with WT controls. Therefore altered myeloid population was not restricted to the spleen of the D6 KO animals. The $F4/80^+$ cells could be divided into two subsets, namely $F4/80^{hi}$ and $F4/80^{lo}$, based on their expression levels of this surface antigen. The augmented $F4/80^+$ compartment in D6 KO mice was found to result from a specific increase in $F4/80^{hi}$ cells, while both WT and KO mice had similar proportions of $F4/80^{lo}$ cells. Further analysis also revealed that $F4/80^+$ cells isolated from the normal peritoneum of D6 KO mice showed higher expression of CCR2 and CCR5 compared with their WT counterparts.

This expansion of the $F4/80^{hi}$ subset in D6 KO mice suggested an increase in resident tissue macrophages, which are characterised by high levels of F4/80 expression (346-348). Ligands of D6 have been demonstrated to play a role in monocyte emigration from bone marrow and trafficking of eosinophils to tissues in the absence of inflammation (125, 127). It is possible therefore, that D6 ligands in non-inflamed sites mediate the constitutive recruitment of resident macrophages and that a lack of D6 function enables greater baseline macrophage recruitment. On the other hand, D6 has been found, at least in humans, to be expressed predominantly by lymphatic endothelium and does not appear to be expressed by vascular endothelial cells (287). This, as well as the fact that equal numbers of cells were found in the inflamed peritoneum of both WT and D6 KO mice following thioglycollate

challenge, suggests that D6 may not regulate the recruitment of cells to the peritoneum. An alternative explanation is that the increased prevalence of $F4/80^+$ cells in the KO peritoneal cavity may have been due to greater *in situ* proliferation, or prolonged persistence of resident macrophages in the D6 KO peritoneum. To address these points, BrdU labelling experiments could be used to compare the turnover rates of peritoneal macrophages in WT and D6 KO mice. CCR5 has been shown to deliver anti-apoptotic signals to both human and murine macrophages (82), and as already mentioned, I found that resident macrophages from the peritoneal cavity of D6 KO mice demonstrated enhanced CCR2 and CCR5 expression. Therefore D6 KO resident macrophages may be recruited normally to the resting peritoneum, but might have a longer tissue lifespan there, perhaps as a result of increased CCR5 expression. In this regard, it would be interesting to examine if antagonising CCR5, for instance through administration of Met-RANTES, could reduce levels of peritoneal macrophages (349, 350). As in other aspects of understanding the biology of D6, a concern is the lack of information regarding the sites of D6 expression. Work by others in our group has revealed that various murine leukocyte populations, including macrophages and DC, express D6, at least at the mRNA level (351). Therefore it is possible that the absence of D6 has an autonomous effect on tissue macrophages. It will be important to determine if the altered CCR2 and CCR5 expression arises within the peritoneum or if circulating monocytes in D6 KO mice have aberrant chemokine receptor expression.

Examination of Cell Populations in the Inflamed Peritoneum of D6 KOs

To further investigate the role of D6 in myeloid cell responses, I employed a model of sterile peritonitis induced by thioglycollate injection. Administration of thioglycollate

results in the recruitment of inflammatory monocytes to the peritoneum in a CCR2-dependent fashion (47, 48, 136, 137, 308). It was therefore envisaged that absence of D6 activity might lead to higher levels of CCR2 ligands and result in increased influx of monocytes to the D6 KO peritoneal cavity. Despite these predictions, WT and KO mice recruited similar numbers of $F4/80^+$ cells to the inflamed peritoneum at all times from 24-96h after thioglycollate challenge. As discussed above, this finding may reflect the absence of D6 activity at vascular endothelium. Although I found no differences in the total number of isolated PEC, $F4/80^+$ PEC from D6 KO mice co-expressed higher levels of CD11c than their WT counterparts. This phenotype became more distinct at later time-points following thioglycollate injection. I considered the possibility that some of these $F4/80^+$ PEC may have been eosinophils, which have been described previously as $F4/80^{lo}$ expressing cells with distinct high side-scatter properties (310, 352, 353). However cells with such characteristics appeared to be present in equal numbers in WT and KO mice. Moreover, the $F4/80^+CD11c^+$ cells were also found to co-express class II MHC and these were more likely a monocyte-derived population.

Similar to my findings with resident $F4/80^+$ PEC, I detected differences in the levels of expression of CCR2 and CCR5 by WT and KO inflammatory F4/80/CD11c subsets. Virtually all of the $F4/80^+CD11c^+$ cells in both WT and KO PEC expressed CCR5, but the levels of CCR5 expression were greater in D6 KO PEC. On the other hand, $F4/80^+CD11c^+$ PEC harvested from KO mice at 24h expressed CCR2 at comparable levels to WT PEC, and showed reduced levels of CCR2 expression by 96h. Chemokine receptor expression also differed between F4/80-single positive cells in inflammatory exudates of WT and D6

KO mice, with enhanced CCR2 and CCR5 expression seen by D6 KO PEC at 24h post-thioglycollate injection, although by 96h this difference was no longer apparent.

These phenotypic differences between WT and KO inflammatory PEC suggested that aberrant trafficking or differentiation of inflammatory monocytes may have been occurring in the absence of D6 function. Since the $F4/80^{+}CD11c^{+}$ subset also expressed high levels of class II MHC, it is possible that greater numbers of DC were appearing in the inflamed peritoneal cavity of the D6 KO mice. Circulating blood monocytes are a highly heterogeneous population, which can give rise to both macrophages and DCs in peripheral tissues. Recently, Geissmann et al. described the existence of two distinct murine monocyte subsets, which can be distinguished based on differential chemokine receptor profiles (124, 354). The major monocyte subset to infiltrate the inflamed peritoneum is the "inflammatory" $CCR2^{+}CX_3CR1^{lo}Gr1^{hi}$ monocyte, but "constitutive" $CCR2^{-}CX_3CR1^{hi}Gr1^{lo}$ monocytes are also recruited in response to thioglycollate administration (124). Although both monocyte subsets have been reported to be capable of differentiating into antigen-presenting DC (124), it has been suggested that the $CCR2^{-}CX_3CR1^{hi}Gr1^{lo}$ monocyte population may be more predisposed to DC-development, as adjudged by an increased propensity to upregulate CD11c (312). Interestingly, in ApoE KO mice, this subset is characterised by higher expression of CCR5, a phenotype reminiscent to that demonstrated by D6 KO PEC (312). Therefore one explanation for my observations could be that D6 KO mice were preferentially recruiting monocytes of the $Gr1^{lo}$ subset, thus giving rise to more DC in the peritoneum. However, against this hypothesis, the increased tendency for the $CCR2^{-}CX_3CR1^{hi}Gr1^{lo}$ monocytes to differentiate into DC-like cells has been observed only in ApoE-deficient KO mice. Tacke and coworkers reported CCR5 mRNA expression to be

similar between both monocyte subsets in WT mice, while Sunderkotter and colleagues suggested that in WT animals CCR5 is in fact expressed at greater levels by $Gr1^{hi}$ "inflammatory" monocytes compared to the $Gr1^{lo}$ subset (312, 354). Lang et al. additionally reported that the appearance of $F4/80^{+}CD11c^{+}$ cells in the ApoE KO model of atherosclerosis is dependent on CCR2, suggesting that inflammatory monocytes are the precursors of the $F4/80^{+}CD11c^{+}$ cells. Indeed, I found PEC isolated from both WT and KO thioglycollate-subjected animals to demonstrate high CCR2-positivity, arguing that the vast majority of recruited cells in response to thioglycollate administration were "inflammatory" monocytes.

An alternative explanation for my findings could therefore be that inflammatory monocytes recruited to the D6 KO peritoneum following thioglycollate injection are more prone to give rise to DC. CC-chemokines have been shown to upregulate CD11c expression by monocytes (355) and so it is possible that they may promote monocyte differentiation into DC. In support of this, although macrophages generated from BM by M-CSF and IL-6 show poor APC function, addition of CCR5 ligands during maturation increases their ability to stimulate T cell proliferation (356). It may therefore be the case that the increased levels of CCR5 expressed by freshly recruited D6 KO monocytes results in greater DC-differentiation. This would be consistent with the findings by Tacke and colleagues that the subset of monocytes expressing highest levels of CCR5 demonstrated greater expression of CD11c (312). Notably, thioglycollate-induced $F4/80^{+}CD11c^{+}$ cells from both WT and KO mice demonstrated higher levels of CCR5 expression than all other F4/80/CD11c subsets.

On the other hand, certain macrophages, including lung and splenic marginal zone populations, have also been reported to express CD11c and I was unable to rule out the possibility that the $F4/80^{+}CD11c^{+}$ cells represented a macrophage subset that was expanded in the D6 KO peritoneum (357, 358). As there are few, if any, reliable markers which can differentiate DC from macrophages absolutely (359), I next attempted to use functional assays to try and distinguish the subsets of PEC in WT and KO mice. I compared the capacity of WT and KO inflammatory PEC to induce the free radical nitric oxide and examined inflammatory cytokine production by both populations. Neither population differed in terms of nitric-oxide generation, but PEC taken from D6 KO mice after thioglycollate injection were characterised by lower production of the pro-inflammatory cytokines IL-1α and TNFα. The maturation of monocytes into inflammatory macrophages is associated with a switch from IL-1β production to IL-1α (360). In addition, TNFα is predominantly produced by activated macrophages. Therefore the lower levels of IL-1α and TNFα in D6 KO PEC cultures could support my conclusions that the increased $F4/80^{+}CD11c^{+}$ cells in D6 KO mice were not mature macrophages.

However, it remains possible that these cells represent a distinctive macrophage, such as the "alternatively activated" subset. Macrophages demonstrate great heterogeneity, but it has been proposed that these cells can be roughly categorised as "classically" or "alternatively" activated populations. Classically activated macrophages "CAM" are associated with the production of pro-inflammatory/ T_H1-type cytokines and chemokines, such as IL-1, TNFα, IL-12, IL-23, CXCL10 and CCL3 (361, 362). Alternatively activated macrophages (AAM) populations produce high levels of anti-inflammatory/T_H2-type cytokines, such as IL-10, CCL17 and CCL22, and negatively regulate T_H1 and CAM

generation (361-363). In response to stimulation with LPS and IFNγ, D6 KO PEC produced lower levels of CCL3, CXCL10, IL-1, TNF and IL-6 compared with WT PEC. Interestingly, the population of AAM recruited to the peritoneal cavity during helminth infection have also been shown to express higher levels of CCR5 similar to my findings in D6 KO mice (364). Therefore it is possible that AAM, rather than DC, were the cellular type which was expanded in the inflamed peritoneum of the D6 KO mice. As CCR4 and its ligands have been implicated in the generation of AAM, it is possible that deregulated chemokine networks in the D6 KO animals may have led to alternative macrophage activation (363, 365). Comparison of the levels of CCR4 ligands in peritoneal lavages of WT and KO mice would be necessary to determine this.

With the exception of CCL2, I was not able to detect CC-chemokines in peritoneal lavage fluid from WT and D6 KO animals. This may have reflected the absence of other ligands of D6 or may indicate that the volumes I used to retrieve the lavage fluids were too great, leading to dilution of chemokines below the levels of detection. Measurement of CCL2 levels in peritoneal lavage fluid provided no evidence that D6 scavenging activity occurred in normal peritoneum and, in fact, at late-time points (72-96h post thioglycollate), lower levels of CCL2 were found in D6 KO lavages. This may indicate that D6 is not expressed in the peritoneal cavity, or is expressed at insufficient levels to function as a decoy for inflammatory chemokines generated at this site. As a recent study examining the role of D6 in pulmonary inflammation by Whitehead and coworkers reported the scavenging ability of D6 to be concentration dependent, another explanation for these findings is that the levels of CCL2 did not fall within those concentration ranges susceptible to D6 regulation. Absence of D6 scavenging in the peritoneum does not account for my findings that levels

of CCL2 were actually higher in D6 KO lavages at later time-points. However, monocyte recruitment to inflamed lung has been shown to lead to a reduction in CCL2 levels as a result of CCR2-mediated uptake (140). Therefore the heightened expression of CCR2 observed in the early D6 KO PEC may have led to greater consumption of CCL2 in KO lavages.

Characterisation of BM-DC from D6 KO Mice

As I was unable to be sure if the expanded $CD11c^+$ subsets in the KO peritoneum were DC or macrophages, I decided to compare the generation of DC from precursor cells in WT and KO bone marrow cultures. Compared with identically treated WT BM cultures, GM-CSF-expanded BM cultures from D6 KO mice generated greater numbers of $CD11c^+$ cells. In addition, CD11c positive cells in KO cultures expressed CD11c at higher levels than $CD11c^+$ cells in WT cultures, as assessed by measuring MFI. This was consistent with my findings from the myeloid cells in the peritoneal cavity and suggested that monocytes may be more predisposed to DC development in D6 KO mice. Interestingly, many BM $CD11c^+$ cells from both WT and KO cultures also expressed the F4/80 antigen, supporting my idea that the CD11c+$F4/80^+$ cells I found in the inflamed peritoneum could be DC rather than a macrophage subset. It has been suggested that the DC generated from monocyte/GM-CSF cultures reflect inflammatory type DC, as opposed to the steady-state, circulating DC that can be expanded by Flt-3 ligand (366). Therefore dual F4/80/ CD11c expression may be a characteristic of DC populations recruited during inflammation. Similar to my findings with thioglycollate-induced PEC, D6 KO BM DC also showed increased expression of CCR5 when compared with WT BM-DC. The levels of CCR2 also seemed marginally

higher on D6 KO DC, but this did not reach statistical significance. On the other hand, both WT and KO DC expressed CXCR4 at comparable levels.

CC-chemokines are not only involved in the trafficking of DC, but have also been shown to affect cytokine production, costimulatory molecule expression and antigen presentation by DC (77, 367-369). Therefore I next compared the functional responses of WT and D6 KO BM-DC. Although higher yields of DC could be obtained from D6 KO cultures, the KO DC did not differ from WT counterparts in terms of costimulatory molecule expression, with comparable expression of CD40, CD80 and CD86 in WT and KO DC both at baseline and after LPS-stimulation. In addition, both WT and KO DC demonstrated comparable endocytosis of FITC-dextran and similar downregulation of CCR5 in response to LPS treatment. However, D6 KO DC were less efficient at inducing MLR responses than WT DC and, compared to their WT counterparts, LPS-treated D6 KO DC also produced greater levels of TNFα, CCL3, CCL4, CCL5, and VEGF. D6 KO DC produced greater levels of VEGF even in the absence of LPS-treatment, although no differences were found between levels of any other cytokine or chemokine under these conditions. The increased levels of CC-chemokines detected in the D6 KO cultures may have reflected lack of scavenging of these ligands by DC-expressed D6 (351). This in turn may have led to greater generation of TNFα by D6 KO DC, as it has previously been shown that CCL5 can induce TNFα production by murine BM-DC (367). Increased levels of CC-chemokines may also have accounted for the increased levels of VEGF detected in the D6 KO cultures, as G-protein signalling and ligands of D6 have been shown to lead to VEGF production (370-372). Furthermore, as VEGF has been demonstrated to decrease the stimulatory activity of BM-derived DC, this may explain the reduced MLR generated by D6 KO DC (373-376).

Overall, these studies support the idea that D6 can affect the differentiation and function of myeloid cells.

Adaptive Immune Responses in D6-deficient Mice

Although others in our group had found a role for D6 in the down-regulation of cutaneous inflammation, the role of D6 in the generation of antigen-specific immune responses remained unexplored (292). Therefore, as described in Chapter 3, WT and D6 KO animals were immunised in the footpad with OVA/CFA to address this point. Chemokines enhance the development of adaptive immune responses by promoting the recruitment, activation and interaction of leukocyte populations (33, 158). Ligands of D6 have also been shown to have direct stimulatory effects on T proliferation and cytokine production, and more recently, Castellino and coworkers reported a role for CCL3 and CCL4 in promoting the generation of CD8+ T cell memory by enhancing T cell and DC clustering (173, 175-177) It was therefore possible that heightened levels of CC-chemokines in D6 KO mice might lead to amplified adaptive immune responses in these animals.

In my initial experiments, performed on WT and D6 KO mice that had been maintained on a mixed 129/B6 background, *in vitro* antigen specific responses of D6 KO mice appeared reduced at weeks 1 and 6 following immunisation but increased at weeks 2 and 3, although only the differences seen at week 2 were statistically significant. On the other hand, DTH responses were comparable in WT and D6 KO mice at each time point. In a repeat set of experiments on this strain, I did not find any significant differences in the generation of antigen-specific immune responses between WT and D6 KO mice. However, in a similar set of experiments conducted on WT and D6 KO mice on a DBA/2 background, D6 KO

mice showed reduced antigen-specific proliferative responses one week after priming with OVA/CFA, but then demonstrated enhanced proliferative responses by two weeks following immunisation. Levels of OVA-specific antibodies were also lower in the sera of immunised D6 KO DBA/2 mice at week one, with the levels of OVA-IgG2a antibodies being particularly diminished. By week 2 however, similar levels of specific antibody were detected in the sera of WT and D6 KO DBA/2 mice. No significant differences in DTH responses were noted at either time-point.

While these data were somewhat variable, the first set of experiments with the 129/B6 mice and those conducted on the DBA/2 background suggested there might be a reduced antigen-specific immune response in D6 KO mice at early times after priming with OVA/CFA, followed by increased responses later on. This suggests that D6 might be important in controlling the kinetics of the developing primary immune response after immunisation with antigen in adjuvant. However, by themselves, these data are inconclusive and repeat analysis of antigen-specific responses, preferably using a wider range of time-points following immunisation would be necessary to confirm the differences. It would also be useful to employ an adoptive transfer system, using TCR transgenic T cells, in order to compare antigen-specific T cell expansion directly in WT and D6 KO animals. Unfortunately, D6 KO mice were not available on the correct genetic background to allow such experiments during my project. Of note, a recently published study reported reduced antigen-specific priming of D6 KO T cells in D6 KO mice subjected to experimental autoimmune encephalitis (EAE) (377). This defective T cell behaviour was sufficient to confer protection from disease in these animals. These findings, along with my own observations, suggest that D6 may be involved in the generation of adaptive immune

responses and point to a more complex function for D6 than simply as a negative regulator of inflammation, as was originally suggested.

As DC are key players in the initiation of adaptive immunity, aberrant DC responses in the absence of D6 might explain these altered Ag-specific responses observed in D6 KO mice (377). Reduced T cell priming could result from a lower stimulatory capacity of D6 KO DC, and in support of this, I found D6 KO BM DC to be less effective than WT DC in stimulating a MLR. Further experiments, such as examination of OVA-specific transgenic T cell priming by OVA-pulsed WT and D6 KO DC would enable a better assessment of APC capacity. On the other hand, D6 KO DC showed similar upregulation of costimulatory molecules and high production of inflammatory cytokines following LPS stimulation, which would be inconsistent with defective function. In addition, I found that the responses of D6 KO mice were equivalent or greater to those of WT animals at later times following immunisation. An alternative explanation therefore could be that altered DC trafficking in the absence of D6 leads to kinetic differences in adaptive immune responses. The magnitude of the CD4+ T cell response generated during immunisation has been reported to be proportional to the number of antigen-loaded DC that traffic to the node, and decreased DC recruitment to LN could have resulted in reduced T cell priming (129). One explanation for this could be that the higher expression of CCR5 by D6 KO DC retains these cells in inflamed tissues for a longer period of time, resulting in delayed T cell priming. Supporting this argument, several independent studies of D6 KO mice, have reported increased accumulation of $CD11c^+$ cells at the site of tissue inflammation (377, 378). Although I did not examine this directly, this would also be consistent with my findings of higher numbers of $CD11c^+$ cells in the inflamed peritoneum of D6 KO mice,

which could reflect enhanced retention of DC. This could result in a delay of DC trafficking to lymphoid tissues draining the site of inflammation and so might account for the initial decrease, but subsequent increase in antigen-specific responses that appeared to occur in the D6 KO mice. In future, it would be interesting to address these points by using FITC skin painting experiments to compare the rates of WT and D6 KO DC trafficking to draining lymph nodes.

Exploring the Role of D6 in Intestinal Immune Responses

D6 is expressed at high levels in the small and large intestine of both humans and mice, yet the function of D6 at these sites is not known. The intestinal immune system must be capable of responding to invasive pathogens but remain tolerant to foodstuffs and commensal organisms. As chemokines are involved in both tolerogenic and inflammatory intestinal immune responses, regulation of intestinal chemokine networks by D6 may be important for the maintenance of homeostasis at this site. Oral tolerance is the process by which the immune system becomes systemically tolerant to fed antigens, and is thought to be a physiologically important phenomenon for preventing aberrant responses to dietary proteins (379). The induction of high dose oral tolerance is reported to be dependent on signalling via CCR2 and CCR5 (19, 20, 297) and as D6 is expressed at high levels in human and murine intestine, I wished to explore if regulation of these ligands by D6 might play a role in maintaining intestinal homeostasis (19, 20, 270, 271, 287, 297). However, I found the induction of tolerance in response to a single high feed of OVA to be normal in D6 KO animals, with comparable suppression of all systemic antigen-specific responses in both WT and KO mice. These data suggest that D6 is not essential for the induction of oral tolerance, at least in response to a high dose of antigen. Different mechanisms are thought

to be responsible for the development of systemic tolerance following different feeding regimes. Specifically, it has been suggested that low-dose oral tolerance may involve induction of regulatory T-cell populations, while high dose feeding reputedly leads to deletion or anergy of antigen-specific T cells. My findings indicate that high dose oral tolerance is not impaired in the absence of D6. However, I did not explore low dose oral tolerance in the D6 KO mice, and these may be interesting experiments to conduct in future.

The Role of D6 in Intestinal Immunity

Another possible function for D6 at the intestinal mucosa might be to dampen inflammation at this site and prevent the development of immunopathological responses. Increased levels of CC-chemokines are found in the inflamed tissues of inflammatory bowel disease (IBD) patients and CC-chemokine receptor deletion or blockade has been reported to have beneficial effects in murine models of colitis (301, 302, 313, 317, 318). It was therefore predicted that, due to increased levels of CC-chemokines, D6 KO animals might display greater susceptibility to the induction of intestinal inflammation. As detailed in Chapter 5, my initial attempts to explore intestinal inflammation in D6 KO mice involved administration of indomethacin. This drug has previously been shown to damage the GI tract by preventing the synthesis of prostaglandins, which are important for maintaining homeostasis of the intestinal epithelium (319-324). However, I found no evidence of intestinal pathology in either WT or D6 KO mice following indomethacin treatment and I thus abandoned this model.

Instead, I adopted a model of colitis caused by oral administration of dextran sulphate sodium (DSS). DSS is thought to induce colitis by impairing barrier function, possibly due to the direct toxicity of DSS to intestinal epithelial cells, and the subsequent invasion of local bacteria, as well as direct activation of macrophages by DSS, may drive the ensuing inflammatory response (304, 328, 380, 381). Consistent with previous studies in this model, I found that administration of DSS to mice induced a colitis characterised by bloody diarrhoea, weight loss and colonic shortening (303). Histological analysis revealed extensive cellular infiltration and epithelial damage, including ulceration and crypt abscesses with crypt necrosis and fibrosis. Surprisingly, compared with their WT counterparts, D6 KO mice showed fewer clinical symptoms, reduced weight loss and had increased survival over the course of the colitis studies. This pattern was found consistently, although there was some variability in the susceptibility of individual animals to DSS and the time-course of development of clinical symptoms also varied between different colitis experiments. This variability may have been due to differences in intestinal bacterial colonisation in the mice used in different conditions, as the microflora play a major role in influencing the course of disease in all experimental colitides (382). However, despite this variability of colitis development, overall, D6 KO mice consistently showed reduced susceptibility to DSS. These findings were unexpected, given that previous work had proposed an anti-inflammatory role for D6, and could suggest that D6 may function in a different manner in intestinal tissue.

Due to the importance of chemokines in cell recruitment, I decided to examine whether the different course of disease observed in the D6 KO mice might have been due to altered leukocyte infiltration to the inflamed colonic tissue. As expected, DSS administration led to

a marked increase in lamina propria leukocytes in the colon. However, similar proportions of $CD4^+$ and $CD8^+$ T cells, B cells, monocytes/macrophages and neutrophils were retrieved from the inflamed colons of WT and D6 KO mice. Interestingly though, I detected higher proportions of CCR2 and CCR5 positive cells in the colonic lamina propria (CLP) of D6 KO mice compared to WT controls. Initially, I thought this might reflect higher CCR2 and CCR5 expression by infiltrating macrophages but, surprisingly, it was due mainly to increased expression of CCR5 by D6 KO B cells. In fact, $F4/80^+$ and $CD11c^+$ myeloid cells from D6 KO colons expressed lower levels of CCR2 and CCR5, perhaps suggesting that although similar overall proportions of $F4/80^+$ and $CD11c^+$ cells were detected in CLP from WT and KO mice, different myeloid subsets might be present in the two strains. CCR5 expression was also found to be higher on $CD8^+$ T cells in D6 KO isolates, although this difference did not reach statistical significance. Subsequent experiments showed the same phenotype of higher CCR5 expression in LP B cells from non-colitic D6 KO mice, and as discussed in more detail below, I found increased CCR5 expression to be a striking and universal feature of D6 KO B cells.

As CCR5 ligands have been shown to affect B cell expression of costimulatory molecules and antibody generation (383), it is possible that the altered B-cell compartment of the D6 KO animals might have contributed to the ameliorated disease in D6 KO mice. In favour of this hypothesis, B cells have been shown to have a protective impact in a number of murine colitis models. Mizoguchi and coworkers showed that IL-10 producing B cells reduced spontaneous intestinal inflammation in TCRα KO mice (384, 385) while B cells were reported by Wei and coworkers to have a protective role in the Gαi-deficient and $CD4^+CD45RB^{hi}$ → SCID transfer models of colitis, due to an effect on the expansion of

regulatory T cell populations (386). Homologous IgG has also been shown to have a suppressive effect on DSS-induced inflammation (387). Generation of intestinal antibody may protect mice from disease by preventing microbes permeating the epithelial barrier, by enhancing the clearance of apoptotic epithelial cells, or by triggering anti-inflammatory Fc receptors, such as FcγRIIB (388, 389). Therefore enhanced B cell function of any of these kinds may be responsible for the reduced colitis in D6 KO mice. However, I detected no differences in the levels of IL-10 produced by colon cultures from WT and D6 KO animals and my subsequent experiments in Chapter 6 did not uncover any differences in the function of WT and D6 KO B cells after activation. On the other hand, these latter functional studies used splenic B cells, which are likely to differ markedly from the B cell populations present in the colonic lamina propria, and I did not assess local B cell function. Therefore a role for mucosal B cells in protecting D6 KO animals from colitis development cannot be completely excluded. To address this issue, it would be important to compare mucosal antibody levels between WT and D6 KO mice and to examine the numbers of antibody-secreting plasma cells in the colonic lamina propria. Additionally, it would be interesting to compare functional responses of MLN and lamina propria B cells from WT and D6 KO mice. Of note, it has been shown that the behaviour of mucosal B cells is influenced by the dose of DSS used to induce colitis. Stevceva and coworkers reported that high doses of DSS (5%) reduced the number of colonic B cells while lower doses (<2.5%) resulted in increased numbers of lamina propria B cells (390). Therefore one way to test the importance of B cells in the relative protection from colitis observed in the D6 KO animals, might be to compare colitis development in WT and KO mice in response to high and low doses of DSS. If B cells protect D6 KO animals, it would be predicted that greater resistance of D6 KO animals would be seen following administration of lower doses of

DSS, while at B-cell depleting high doses, both WT and KO animals would show similar susceptibility to DSS.

Inflammatory Cytokine Production in Mucosa of Colitic D6 KO Mice

Inflammatory bowel diseases are thought to arise partly from dysregulated cytokine networks, with pro-inflammatory cytokines overcoming regulatory mediators to drive immunopathological responses (296, 391). Accordingly, a number of inflammatory cytokines and chemokines have been associated with the colitic response that develops in response to DSS administration (301, 326, 391-400). Although I did not detect significant differences in the cell types found in the inflamed colon of WT and D6 KO mice, it was possible that the lack of D6 function might have led to an altered cytokine or chemokine response that allowed protection against DSS-induced disease. To examine this idea I collected supernatants from colon cultures of WT and KO mice at various times after administration of DSS and analysed a broad array of cytokines and chemokines by Luminex technology. I found no significant differences in the levels of IL-1α, IL-1β, IL-2, IL-6, IL-12 (p40/p70), IL-13, GM-CSF, FGF and VEGF in WT and KO culture supernatants. The levels of all chemokines analysed (CXCL1, CXCL9, CCL2, CCL3, CCL4 and CCL5) were also comparable in WT and KO cultures. However, I detected significantly increased levels of IL-17, TNFα and IFNγ in the colon cultures of colitic D6 KO mice compared with WT cultures. Moreover, linear regression analysis revealed that higher levels of IL-17 and IFNγ in colon cultures correlated significantly with increased resistance to DSS-induced weight loss.

Initially it seemed somewhat counter-intuitive that increased production of inflammatory cytokines might be associated with reduced colitis susceptibility, especially given the considerable evidence that most models of IBD are associated with exaggerated production of pro-inflammatory cytokines (296, 382, 401-403). However, a new concept emerging from the study of both human and experimental colitis is that IBD may reflect a dysregulated immune response, as opposed simply to an exaggerated one (404, 405). This reflects the delicate balancing act the intestinal immune response must perform, ensuring that proper pro-inflammatory mechanism to protect against infection are complemented with anti-inflammatory measures to prevent immunopathology. In particular, failure to mount an effective inflammatory response may allow persistence of bacteria that then trigger the development of adaptive immune responses. Defective expression of defensins is associated with Crohn's disease (406, 407) and it has been proposed that a diminished ability to generate defensins explains the association of NOD2 mutations with CD (408-410). In addition, several studies have reported a protective role for TLR-2 and TLR-4 in DSS colitis (331, 411, 412). TLR signalling can trigger the production of pro-inflammatory cytokines, which may contribute to these protective effects by recruiting inflammatory neutrophils and macrophages that engulf and destroy bacteria, so preventing penetration of the epithelial barrier (413, 414). Although I did not find any differences in neutrophil or macrophage recruitment to the inflamed D6 KO colon, the increased levels of inflammatory cytokines may have promoted the anti-bacterial functions of any cells which had arrived, thus mediating protection in D6 KO mice.

An alternative possibility is that the increased inflammatory response in D6 KO animals may have protected the epithelial barrier directly against DSS-mediated damage. The

protective effect of TLR ligands in colitis has been attributed to the induction of factors that promote the protection and repair of mucosal epithelium. These include IL-6, TNF, KGF, VEGF, TGFβ and COX-2 (331, 411, 412, 415-419). Interestingly, one of the most striking differences I detected in the Luminex analyses of colon cultures, was heightened IL-17 levels in D6 KO colons. IL-17 has attracted great interest recently, as it has become apparent that this cytokine, rather than IFNγ, may be the main culprit in several models of autoimmune disease (420). However, IL-17 is also important for driving acute anti-microbial responses, by inducing chemokine release and recruiting neutrophils to inflamed sites, and this could be important for promoting a rapid response upon breach of mucosal barriers (327, 421). In addition, it has been shown that IL-17 can directly protect the integrity of intestinal epithelium, either secondary to upregulation of claudin expression, which promotes tight-junction formation, or through the induction of COX-2 expression, which generates epithelial-protective prostaglandins (422-424). Importantly, anti-IL-17 treatment has been demonstrated to exacerbate DSS colitis, supporting the argument that the increased levels of this cytokine in the KO colons may have mediated protection from disease (425). Unfortunately, I was unable to identify the cells responsible for producing IL-17 and this would be an important point to address in future experiments. Mutually exclusive subsets of CD4+ T cells are believed to be responsible for producing IL-17 and IFNγ, but I found both these cytokines were increased in KO colon cultures. However, a recent study reported the presence of high frequencies of IFNγ-secreting cells in the inflamed colons of colitic rodents, despite high levels of IL-17 (426), suggesting that IFNγ and IL-17 responses can coexist in this condition.

As signalling via CC-chemokine receptors can enhance the generation of pro-inflammatory cytokines, greater bioavailability of CC-chemokines in the absence of D6 scavenging, could have explained the higher production of pro-inflammatory cytokines in D6-deficient animals (49, 87, 367, 427-429). However, I did not detect increased chemokine levels in colon cultures from D6 KO mice. This may indicate that D6 does not serve as a chemokine scavenger at this site, or that chemokines did not fall within a range susceptible to D6 activity (378). On the other hand, my inability to detect increased CC-chemokines in the KO cultures could have been due to the increased CCR-expression by cells infiltrating the D6 KO colon leading to greater ligand consumption and so reducing the levels of ligand available for measurement. In addition, increased signalling via these chemokine receptors may have downregulated further chemokine generation (140, 430). IL-17 has also been shown to have a negative impact on CC-chemokine production, and so the elevated levels of this cytokine in inflamed D6 KO colons may have prevented further chemokine generation (423, 431, 432). Therefore to address these issues and gain a better understanding of the dynamic interplay of chemokine and cytokine networks, it would be useful to measure their levels at a greater range of time points during the course of DSS-induced disease.

An important caveat to these conclusions is that although my initial findings suggested a greater inflammatory response in D6 KO animals, a separate Luminex experiment found similar levels of all cytokines in WT and KO colon cultures. Interestingly, in this latter experiment, although D6 KO mice still showed decreased susceptibility to DSS colitis, the absence of a stronger inflammatory response in these KO mice was associated with lesser protection from colitis. This could support the idea that increased levels of pro-

inflammatory cytokines such as IL-17 and other factors may be involved in protection of the D6-deficient animals. However, further work, for example antibody-mediated neutralisation of IL-17 *in vivo*, will be necessary to establish whether any of these cytokines are involved in protecting D6 KO mice from DSS-induced disease.

A Role For D6 in Controlling Intestinal Turnover

The colonic epithelial barrier is a single layer of non-dividing, terminally differentiated cells, which is continuously and rapidly renewed by proliferating colonic epithelial precursors located at the base of colonic crypts (433). The colonic epithelial precursors migrate upwards toward the luminal surface, differentiating into enterocytes as they progress. It is essential that this process in properly regulated, as impairment of epithelial integrity has been suggested to be a causative factor in the development of inflammatory bowel diseases (405). In support of this, a number of murine models of intestinal inflammation result from defects in epithelial barrier function (382).

I therefore wished to assess if altered barrier function was associated with the increased resistance to DSS colitis observed in D6 KO animals. Measurement of steady-state epithelial turnover by BrdU incorporation revealed apparently decreased rates of proliferating cells in the normal colon of D6 KO mice compared with their WT counterparts. Consistent with previous reports, few BrdU+ cells were detected in the colonic crypts of WT and D6 KO mice immediately after DSS administration, presumably reflecting toxicity, while removal of DSS from the drinking water led to markedly increased rates of epithelial cell proliferation (328-330). It is known that higher baseline rates of epithelial cell proliferation increase susceptibility to intestinal damage (434). In

addition, a number of studies have shown that animals with intrinsically increased rates of baseline epithelial turnover have increased susceptibility to DSS colitis (331, 435, 436). Thus the altered epithelial turnover observed in the D6 KO mice could help explain the reduced colitis development in these animals.

Time constraints meant that I was unable to address the mechanism behind the altered epithelial turnover observed in the KO mice. However, colonic epithelial cells constitutively express an array of chemokine receptors, including those for ligands of D6 and there is evidence that D6 itself is expressed by intestinal epithelium (287, 437). As ligands of D6 are expressed constitutively in the murine colon under normal housing conditions, it is possible that D6 is involved in epithelial homeostasis (125, 438). Chemokine signalling could influence epithelial turnover in a number of ways, for instance, by driving the expression of growth and survival factors involved in barrier maintenance (93). In addition, D6 ligands could help drive movement of epithelial precursors upwards from colonic crypts. Upregulation of integrins, another known function of chemokines, can inhibit enterocyte migration, offering a further possible mechanism for chemokine-control of the epithelial escalator (439). Chemokines also activate molecules, such as the small GTPases Rac, Rho, and Cdc42, which are involved in actin cytoskeletal reorganization and can regulate tight junction formation (440), and CC-chemokines may promote differentiation of colonic epithelial cells (441). Interestingly, it has been reported that a CCL3 analogue reduces the number of mitotic cells in intestinal crypts and can protect mice from radiation-induced intestinal damage (442, 443).

In summary, my studies of intestinal inflammation revealed that the absence of D6 offers some protection against the disease induced by oral DSS, and suggest that this may be due to differences in epithelial homeostasis and/or an altered local inflammatory response. As development of DSS colitis is highly strain dependent it will be important to characterise the development of colitis in D6 KO mice on other genetic backgrounds (444, 445). In addition, it will be important to substantiate my findings by exploring other models of intestinal inflammation in D6 KO animals. For example, induction of TNBS-colitis or transfer of $CD25^{+}$-depleted T cell populations into RAG KO mice would enable exploration of the importance of D6 in T-cell mediated colitis (382). Moreover, as pro-inflammatory cytokines such as IL-17 have been reported to exacerbate disease in such models, they could allow direct determination of the link between IL-17 and protection from colitis (446, 447).

Altered Chemokine Receptor Expression by D6 KO Leukocytes

The altered CCR5 expression by intestinal LP cells of D6 KO mice was an unexpected finding that caused me to undertake a more extensive analysis of chemokine receptor expression by leukocytes in these mice (Chapter 6). These studies revealed increased CCR5 expression by T cell and B cell populations isolated from the PLN, MLN and spleen of D6 KO animals under resting conditions. The most dramatic effects were evident with B cells: in all lymphoid tissues analysed, WT B cells showed fairly low levels of CCR5 expression, whereas B cells from D6 KO animals were almost universally CCR5-positive. Expression of CCR2, CXCR4 and CXCR5 did not appear to be altered between WT and D6 KO lymphocytes. However, while CCR9 expression was comparable between WT and KO spleen and PLN cells, slightly increased levels of CCR9 were detected on D6 KO

leukocytes isolated from the MLN. Further analysis revealed that the aberrant expression of CCR5 and CCR9 in D6 KO B cells was already apparent in bone marrow B cell populations, indicating that absence of D6 was affecting chemokine receptor expression during B lymphopoiesis.

The expression of a chemokine receptor does not always predict responsiveness to ligands and it has been demonstrated that chemokine receptors can be uncoupled from their signalling components (266, 448, 449). However, I found that D6 KO B cells demonstrated greater chemotactic responses to CCL4 *in vitro*, suggesting that CCR5 was functional on these cells. The specificity of CCL4 for CCR5 ruled out the possibility that other functional CC-chemokine receptors accounted for the increased chemotactic response of the D6 KO B cells (11). On the other hand, one caveat of these experiments could be that expression of D6 by WT B cells may have competed with CCR5 for binding to CCL4, diminishing their chemotaxis responses to this chemokine.

To examine if WT and D6 KO B cells differed functionally, I compared their responses to a number of mitogenic stimuli. However, I did not detect any differences between WT and D6 KO B in terms of their ability to proliferate or upregulate costimulatory molecules following culture with LPS, DSS or anti-CD40+anti-IgM. Analysis of chemokine levels in these cultures also revealed no differences, except lower levels of CCL3 and CCL5 in D6 KO LPS supernatants compared with WT LPS supernatants. It is possible that this reflected higher uptake of these chemokines by KO B cells as a consequence of their increased CCR5 expression. Levels of total serum IgM antibodies were comparable between WT and KO mice, and although D6 KO mice appeared to have slightly reduced levels of total serum

IgG, this difference was not statistically significant. Taken together, my data suggested that WT and D6 KO B cells were functionally similar, at least in terms of the parameters I measured. On the other hand, I only measured B cell activation *in vitro*, and it could be argued that aberrant chemokine receptor expression would be more likely to affect the development of B cell responses in vivo, for example, by altering the trafficking and positioning of these cells during development of immune responses. Indeed, some of the differences in specific antibody generation that I observed in D6 WT and KO following footpad immunisation may have been attributable to dysregulated B cell localisation. It would be interesting to examine if the positioning of D6 KO B cells, for example within the follicles of the lymph node, is affected both during resting state and following immunisation.

The increased expression of CCR5 by D6 KO B cells pointed to a potentially novel function of D6 in controlling levels of chemokine receptor expression in an autocrine manner. Although I did not explore this idea in a mechanistic way, it is possible that D6 and CCR5 compete for anchorage sites at the cell surface, and the absence of D6 may lead to more space for CCR5. However, the fact that the levels of CCR5 mRNA were also higher in D6 KO leukocytes, argued against this idea, and it is therefore possible that D6 and CCR5 are in transcriptional competition. In favour of this hypothesis, others in our laboratory have found that WT B cells express higher levels of D6 mRNA than other leukocyte populations (Dr Clive McKimmie, unpublished data). Therefore the absence of D6 may lead to inherently greater levels of transcription of CCR5 by this population. Recent findings show that LPS decreases D6 mRNA expression by B cells (205), whereas I

found that LPS had the opposite effect on CCR5 protein expression, supporting the idea that CCR5 and D6 expression may be mutually inhibitory.

Another possible explanation is that lack of D6 decoy function results in dysregulated bioavailability of CC-chemokines, leading to changes in cellular chemokine receptor expression. Therefore, D6 may play a role in maintaining levels of CC-chemokines, and in its absence, decreased levels of CCR5 ligands would lead to upregulation of CCR5. It would be interesting to explore this idea by examining the effects of increasing or decreasing levels of its ligands on CCR5 expression. On the other hand, this would not seem to explain why the B cell compartment was most affected by the altered CCR5 expression. It will be important in future to define whether defects in CCR5 expression are due to cell autonomous defects in D6 expression, or due to the lack of regulation of chemokines in the external milieu by other D6-positive cells.

It is important to note that I did not determine any definitive link between dysregulated CCR5 expression and the other abnormalities I observed in the D6 KO mice. However, aberrant CCR5 expression has been associated with disease states in both mouse and man (450-454). Therefore it will be interesting to explore if altered CCR5 expression by D6 KO is responsible for some of the phenotypical changes that occur in these animals (291, 292, 377, 378). In addition, targeting of this receptor represents a major pharmacological goal, both for treatment of immune pathologies and prevention of HIV infection. Deciphering the mechanisms regulating CCR5 expression will be important for effective drug development, and further study of how D6 affects B cell expression of CCR5 may add to our understanding of this subject.

CONCLUDING REMARKS:

In summary, the studies in this thesis have revealed a number of novel phenotypical changes in D6-deficient animals. Importantly, my studies suggest previously unappreciated roles for D6 and its CC-chemokines in influencing monocyte differentiation and colonic epithelial turnover. Whether D6 shapes both of these biological processes through regulation of CCR5 expression remains to be determined. My results and the recently reported work of others also suggests that D6 may have a role over and above that of a simple chemokine scavenger (377, 378). Due to the extreme promiscuity of D6, and the hugely diverse roles of its many ligands, it is perhaps not surprising that both pro- and anti-inflammatory functions are emerging for this receptor. The specific consequences of D6-deficiency are almost certain to differ from tissue to tissue, depending on the particular chemokine networks present.

The data from the thioglycollate peritonitis experiments suggested that the D6 KO mice were recruiting different myeloid cell populations to the inflamed peritoneum, or that the differentiation of recruited monocytes was skewed, possibly towards a DC-like phenotype. Despite evidence for the existence of a common macrophage/ dendritic cell precursor, the mechanisms that control the particular developmental pathway undertaken by tissue-recruited monocytes remain unknown. Does a key role therefore exist for CC-chemokines in monocyte differentiation as well as in their recruitment?

My studies represent the first investigation of D6 function in the context of intestinal biology, and suggest significant functions for D6 in both resting and inflamed colon. The role for D6 in promoting susceptibility to DSS colitis was an unexpected finding, giving its

proposed anti-inflammatory nature in other circumstances. It was also intriguing that this might be related to a protective effect of inflammatory mediators, such as IL-17, in maintaining epithelial barrier integrity. It will now be important to determine the link between CC-chemokines and IL-17 generation. In addition, the altered epithelial proliferation noted in the uninflamed D6 KO colon points to as yet unappreciated roles for D6 and its ligands in epithelial homeostasis.

Aberrations in monocyte differentiation, increased IL-17 generation or altered epithelial proliferation occurring in D6-deficient mice could account for many of the other phenotypes that are beginning to emerge from the study of these animals. However, many key questions remain unanswered. The exact locations and the regulation of D6 expression under different conditions remain unknown and the generation of a reliable antibody, as well as study of recently generated lacZ-D6-reporter mice will hopefully help address these issues.

The challenge now is to define the exact mechanisms of D6 function *in vivo*. Such investigations will hopefully set a precedent for future study of CCX-CKR and any other atypical receptors that may emerge. A better appreciation of the true physiological role of these molecules will assist our understanding of the chemokine system and, hopefully, provide a framework for effectively targeting chemokine networks for therapeutic purposes.

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