An investigation into sex-differences in the regulation and function of Toll-like receptors in leukocyte trafficking *in vivo*

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By

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Abstract

Sexual dimorphisms exist in the incidence and severity of many diseases, with females demonstrating relative protection from inflammatory conditions. The extent and mechanisms by which excessive leukocyte recruitment underlies these differences are not well established, and better understanding is essential for the development of targeted therapies. Evidence suggests that variances in pathogen-sensing Toll-like receptors (TLRs) underlie sex-differences in leukocyte recruitment. This thesis aimed to investigate sex-differences in trafficking of leukocytes in the zymosan peritonitis murine model of acute inflammation and furthermore evaluate if these differences were accompanied by changes in TLR2 or TLR4 expression.

This work shows that female mice recruit fewer classical monocytes and neutrophils during zymosan induced peritonitis. It demonstrates female murine peritoneal macrophages are more numerous, whilst the peritoneal cytokine environments and zymosan-sensing receptors are similar between the sexes. Sex-differences were evident in the circulation as female mice showed reduced neutrophilia and monocytosis versus male counterparts, despite having similar mobilisation from bone marrow (BM) stores. The work further revealed that storage and trafficking of splenic leukocytes during acute inflammation is distinct between the sexes. Male mice have greater splenic stores of neutrophils, classical- and non-classical- monocytes, despite similar spleen sizes, signifying another source of potential pathogenic leukocytes. Furthermore, males but not females mobilise splenic classical monocytes in response to peritonitis. Conversely, neutrophils appear to traffic to the spleen in females, but not males, in this model. Whilst BM neutrophils from males displayed more TLR2 and TLR4 than females, no major differences under basal or inflamed conditions in TLR2 or TLR4 expression were evident on leukocyte subsets.

This work demonstrates that males and females have distinct leukocyte trafficking profiles in acute inflammation, and suggests that the spleen, not the BM, plays a role in determining sex-differences in the available pool of immune cells. Such dimorphisms demonstrate the importance of considering gender in assay development, drug design and clinical trials.

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Abbreviations

7-AAD	7-aminoactinomycin D
ACK	ammonium-calcium-potassium
ANOVA	analysis of variance
AP-1	activator protein 1
ApoE	apolipoprotein E
AR	androgen receptor
AT-1	angiotensin II receptor, type I
AU	arbitrary units
BCR	B cell receptor
BM	bone marrow
BPI	bactericidal/ permeability-increasing protein
BSA	bovine serum albumin
C5a(R)	complement component C5a (receptor)
CAR cells	CXCL12-abundant reticular cells
CCL	chemokine (C-C motif) ligand
CFSE	carboxyfluorescein succinimidyl ester
CHD	coronary heart disease
CMKLR1	chemokine receptor-like 1
COX2	cyclo-oxygenase 2
CR3	complement receptor 3
CVD	cardiovascular disease
CX_3CR1	chemokine (C-X3-C motif) receptor 1
CXCL	chemokine (C-X-C motif) ligand
CXCR	chemokine (C-X-C) receptor
DAMP	damage associated molecular pattern
DAPI	4’,6-diamidino-2-phenylindole dihydrochloride
DCs	dendritic cells
EAE	experimental autoimmune encephalomyelitis
ECs	endothelial cells
EDTA	ethylenediaminetetraacetic acid
eGFP	enhanced green fluorescent protein
ELISA	enzyme linked immunosorbent assay
ER	oestrogen receptor
fMLP	formyl-methionyl-leucyl-phenylalanine

FSC	forward scatter
G-CSF	granulocyte-colony stimulating factor
GM-CSF	granulocyte macrophage-colony stimulating factor
GPCR	G-protein coupled receptor
HAECs	human aortic endothelial cells
HASMCs	human arterial smooth muscle cells
HBP	heparin-binding protein
HCAECs	human coronary artery endothelial cells
HIF-1	hypoxia inducible factor-1
HMGB1	high-mobility group protein B1
Hsp	heat shock protein
HSPCs	hematopoietic stem and progenitor cells
HUVECs	human umbilical vein endothelial cells
(s)ICAM-1	soluble intracellular adhesion molecule 1
IFN	interferon
Ig	immunoglobulin
IKK	inhibitor κB kinase
(s) IL-1(R)	(soluble) interleukin-1 (receptor)
i.m.	intramuscular
i.p.	intraperitoneal
I/R	ischaemia/reperfusion
IRAK	IL-1 receptor associated kinase
IRF	interferon regulatory factor
JAM	junctional adhesion molecule
KO	knockout
LBP	lipopolysaccharide binding protein
Ldl(r)	low density lipoprotein (receptor)
LFA-1	lymphocyte function-associated antigen 1
LPS	lipopolysaccharide
LRR	leucine rich repeat
LTB_4	leukotriene B_4
MAPK	mitogen activated protein kinase
M-CSF	macrophage-colony stimulating factor
MDP	monocyte-macrophage-dendritic cell precursor
MFI	median fluorescence intensity
MHC	major histocompatibility complex

MI	myocardial infarction
MMP	matrix metalloproteinase
MS	multiple sclerosis
MSC	mesenchymal stem cells
MyD88	myeloid differentiation primary response gene 88
NADPH Ox	nicotinamide adenine dinucleotide phosphate oxidase
NETs	neutrophil extracellular traps
NFκB	nuclear factor kappa B
NGS	normal goat serum
NK	natural killer
NO	nitric oxide
(i)NOS	(inducible) nitric oxide synthase
oxLDL	oxidise low density lipoprotein
PAMP	pathogen associated molecular pattern
PBMCs	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PE	phycoerythrin
PECAM-1	platelet endothelial cell adhesion molecule 1
PRR	pattern recognition receptor
PSGL1	P-selectin glycoprotein ligand 1
RA	rheumatoid arthritis
RFI	relative fluorescence intensity
ROS	reactive oxygen species
SEM	standard error of the mean
siRNA	small interfering RNA
SLE	systemic lupus erythematosus
SSC	side scatter
TAK	transforming growth factor beta activated kinase
TCR	T cell receptor
TEM	transendothelial migration
TGFβ	transforming factor beta
TIR	Toll/interleukin-1 receptor
TIMP-1	tissue inhibitor of metalloproteinase-1
TipDCs	TNF/ iNOS-producing dendritic cells
TLR	Toll-like receptor
TNF	tumour necrosis factor

TRAF	TNF receptor associated factor
TRAM	TRIF-related adaptor molecule
TREM-1	triggering receptor expressed on myeloid cells 1
TRIF	TIR domain containing adaptor protein
VCAM	vascular cell adhesion molecule
VEGF	vascular endothelial growth factor
VLA-4	very late antigen-4
WT	wild type

Publications arising from this work

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http://www.pa2online.org/abstract/abstract.jsp?abid=31183&period=55

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Kay E, Nourshargh S, Whiteford JR, Scotland RS (2013). **Sex-differences in monocyte trafficking in a model of zymosan-induced peritonitis.** William Harvey Day (London, 16th October 2013). *Poster presentation.*

Kay E, Nourshargh S, Scotland RS (2012). **Temporal regulation of neutrophil Toll-like receptor expression in inflammation.** William Harvey Annual Review (London, 3rd July 2013). *Poster presentation.*

Kay E, Nourshargh S, Scotland RS (2012). **Temporal regulation of neutrophil Toll-like receptors 2 and 4 in acute inflammation.** British Pharmacological Society Winter Meeting (London, 18th-20th December 2012). *Poster presentation.*

Statement of originality

The approach and experiments presented here are novel. The author has personally undertaken all the work described here, unless stated otherwise.

Chapter 1: General Introduction

1.1. Inflammation

Inflammation is an essential biological response to infection or injury. Abnormalities in this process, however, can be harmful and ultimately result in disease. An inflammatory aetiology can underlie both immune disorders, such as rheumatoid arthritis (RA), and non-immune diseases like stroke, and together can account for a huge proportion of human diseases worldwide. The financial repercussions place a huge burden on both health systems and the workforce and therefore the need for new, more efficacious therapies to target aberrant inflammation must be addressed.

Manifested in the form of heat, pain, redness and swelling, inflammation represents the critical process the body adopts to restore homeostasis after infection or injury. Such changes are governed by inflammatory mediators released from activated leukocytes and endothelial cells (ECs) in response to the infection. Consequently, chemokine and cell adhesion molecules are upregulated, attracting circulating leukocytes towards the infected area and blood vessel wall, and aiding their migration into the inflamed tissue. These changes represent acute inflammation. It remains, however, a fine balance between eliminating the harmful pathogens or damaged tissue and causing harm to the host. If control over leukocyte influx or leukocyte effector functions is lost, the consequence can be chronic inflammation and damage to healthy tissues.

1.2. Leukocytes

Leukocytes are pivotal to the inflammatory response. They are required for immune surveillance, immune suppression, and detection and clearance of damaged tissues or invading pathogens. An array of leukocyte subsets exists, each with specific functions essential for the restoration and maintenance of tissue homeostasis.

1.2.1. Macrophages

First described by Ilya Mechnikov over 100 years ago, macrophages are resident cells found in all resting tissues at low levels (Tauber, 2003). Primarily the first sensors of pathogens or tissue damage, they are therefore considered inducers of the inflammatory response (Cailhier *et al.*,

2005). Detection by macrophages causes intracellular signalling which results in the activation of the cell, release of pro-inflammatory cytokines such as interleukin 1 (IL-1) and tumour necrosis factor (TNF), and the phagocytosis of pathogens or tissue debris.

Macrophages are thought to be derived from the differentiation of circulating monocytes that enter the tissue, and in doing so adopt either the 'M1' or 'M2' phenotype. M1 macrophages exhibit more 'killer' characteristics, being more pro-inflammatory, highly microbicidal, and promoting T_H1-type responses (Geissmann *et al.*, 2010). M2 macrophages on the other hand display 'repair' features and participate in wound healing, angiogenesis, resolution of inflammation, and more T_H2-type responses (Martinez *et al.*, 2009; Geissmann *et al.*, 2010; Randolph, 2011). Recent studies have suggested that whilst M1 macrophages derive from circulating monocyte precursors, M2 macrophages result from local proliferation of macrophages in vivo (Jenkins *et al.*, 2011). This topic remains controversial with other groups postulating monocyte-derived macrophages can shuttle between phenotypes depending on the cytokine environment, or arguing that terminally differentiated M1 or M2 macrophages derive from different subsets of circulating monocytes (Auffray *et al.*, 2007). Macrophages abundantly produce pro-inflammatory cytokines, growth factors and pro-angiogenic factors and their persistent presence at high levels and continual activation can be detrimental in chronic diseases such as asthma, atherosclerosis and cancer.

1.2.2. Neutrophils

Neutrophils arise from a common myeloid progenitor in the bone marrow (BM) and comprise between 40-75% of all leukocytes in mammals. There is a small but continual release into the circulation to retain the 10-25% present in murine blood under physiological conditions (Mestas *et al.*, 2004). In humans, 50-70% of circulating leukocytes are neutrophils. Mature neutrophils are also referred to as polymorphonuclear cells (PMNs) due to the multi-lobed nature of the nucleus and in mice are identifiable by the expression of Ly6G (recognised by the Gr1 antibody). In the circulation, these cells are between 7-10μm in diameter, and are highly granular. The lifespan of a circulating neutrophil is debated but has been reported to be between 1.5 and 12h (Kolaczkowska *et al.*, 2013). Numbers of circulating neutrophils are therefore regulated by rate of precursor proliferation, egress from the BM to the circulation, and clearance in the spleen, liver, and BM (Sadik *et al.*, 2011).

Neutrophils are innate immune cells recruited from the circulation to the tissue shortly after recognition of pathogens or tissue damage. They contain 3 main types of granules in the cellular cytoplasm that keep damaging or unstable molecules contained but ready for quick

release when required. Primary, secondary, and tertiary granules contain varying combinations of proteases and antimicrobial peptides to address the infection (Faurschou *et al.*, 2003). As professional phagocytes, neutrophils rapidly ingest bacteria or cell debris, removing them from the body and thereby limiting further damage (Phillipson *et al.*, 2011). The contents of the resulting phagosomes are degraded by reactive oxygen species (ROS) and enzymes using granule stores of NAPDH oxidase, and antibacterial proteins (e.g. cathepsins, defensins, lysozyme), respectively (Kolaczkowska *et al.*, 2013). Antibacterial proteins can also be released from the cell, thereby promoting killing of bacteria residing in the tissue. Finally, highly activated neutrophils can also expel neutrophil extracellular traps (NETs), consisting of core DNA elements, histones, proteins, and enzymes, which immobilise pathogens to prevent spreading and aid digestion (Brinkmann *et al.*, 2004). Despite the short half-life of circulating neutrophils, once activated and in the tissue during inflammation their lifespan extends, ensuring primed neutrophils are present at the infected site long enough to clear the pathogen and mount a sufficient inflammatory response.

1.2.3. Classical monocytes

Monocytes originate in the BM from hematopoietic stem cells via a common monocyte-macrophage-dendritic cell (DC) precursor (MDP) (Fogg *et al.*, 2006). Development of mature monocytes is dependent on macrophage colony stimulating factor (M-CSF) (Cecchini *et al.*, 1994). Although traditionally considered a BM-specific process, it has recently been demonstrated that BM-derived hematopoietic stem and progenitor cells (HSPCs) can circulate and relocate to the splenic red pulp in a murine model of atherosclerosis (Robbins *et al.*, 2012). Here, in a process termed 'extramedullary haematopoiesis', they proliferate and differentiate into classical monocytes in a manner dependent upon GM-CSF and IL-3. Peripheral proliferation of monocytes is not however believed to occur under resting conditions.

In mice approximately 4% of peripheral blood leukocytes are monocytes (Doeing *et al.*, 2003). In the blood these are mononuclear cells identifiable by a bean-shaped nucleus. Classical monocytes are defined by their expression of cell surface markers and as such are $CD14^{++}CD16^{-}$ in humans and $CD115^{+}Ly6C^{+}CCR2^{high}CX_3CR1^{dim}$ in mice (Figure 1.1A) (Ziegler-Heitbrock *et al.*, 2010). Of note, the Gr1 antibody that recognises both the Ly6G and Ly6C epitopes is often used to describe these monocytes as $Gr1^{+}$. In response to infection or tissue damage, classical monocytes exhibit innate effector function, killing pathogens by phagocytosis, producing ROS and myeloperoxidase (MPO), and propagating the inflammatory response by cytokine production (Saha *et al.*, 2011). The exact response however is dependent on the nature and

location of the inflammatory stimulus. Unlike neutrophils, monocytes do not store cytokines and so produce them *de novo*.

Classical monocytes are recruited en masse to the site of infection or injury. They are able to produce high amounts of TNF, IL-1, ROS, and NO during infection and have been referred to as 'inflammatory'. Recruited classical monocytes later give rise to inflammatory DCs, or TNF/ iNOS-producing DCs (TipDCs) which upregulate MHC class II antigens, uptake antigen and migrate to the lymphoid organs (Geissmann *et al.*, 2008; Shi *et al.*, 2011a). Furthermore, they have the potential to differentiate into 'classically-activated', or 'M1', macrophages, associated with pro-inflammatory and T_H1-type responses (Figure 1.2) (Geissmann *et al.*, 2010). In addition to roles in propagating inflammation and repopulating tissues, classical monocytes provide a small contribution to myeloid-derived suppressor cells which promote the tumour microenvironment by suppressing T cell function and thereby preventing immune cell attack (Movahedi *et al.*, 2008).

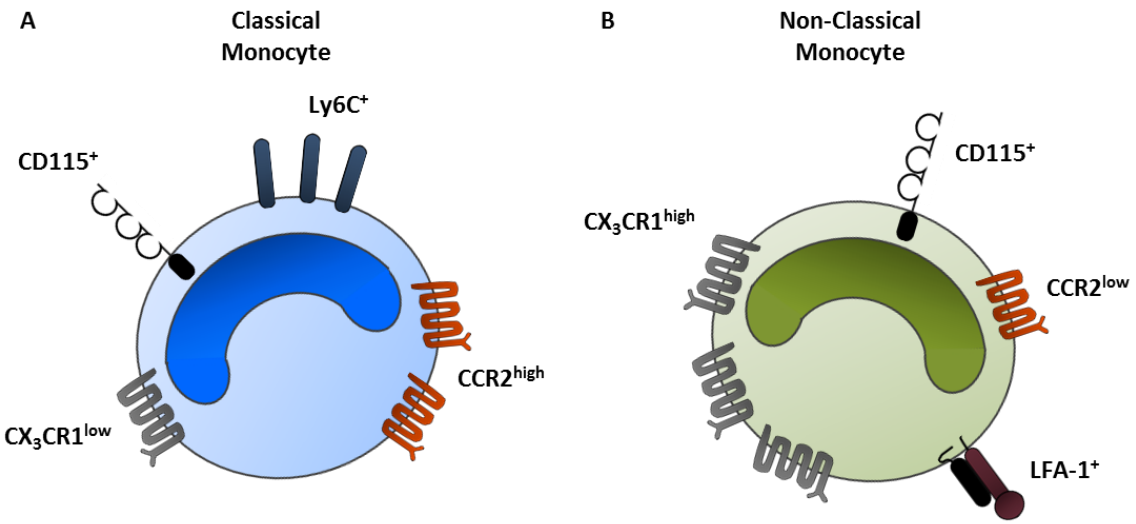


Figure 1.1. Phenotype of monocyte subsets. Presence of cell surface markers to distinguish (A) classical monocytes and (B) non-classical monocytes.

1.2.4. Non-classical monocytes

Also called 'resident' due to their longer half-life, non-classical monocytes are defined as $CD14^+CD16^{++}$ in humans (Ziegler-Heitbrock, 2007) and $CD115^+Ly6C(Gr1)^-CX_3CR1^{high}LFA\text{-}1^{high}$ in mice (Figure 1.1B) (Ingersoll *et al.*, 2010). The development of these cells is however unclear with some suggestions they differentiate from the classical subset, upregulating CX_3CR1 and downregulating Ly6C, whilst others suggest they develop within the BM independently of classical monocytes (Figure 1.2) (Geissmann *et al.*, 2010). Their function was originally

considered one of resident macrophage and DC repopulation due to their presence in both inflamed and resting tissues (Geissmann *et al.*, 2003). More recently however, the function of murine non-classical monocytes has been elucidated in immune surveillance. These monocytes exhibit 'patrolling' behaviour, undertaking long-range crawling along the luminal side of the endothelium under resting conditions (Auffray *et al.*, 2007). Not only does this behaviour allow

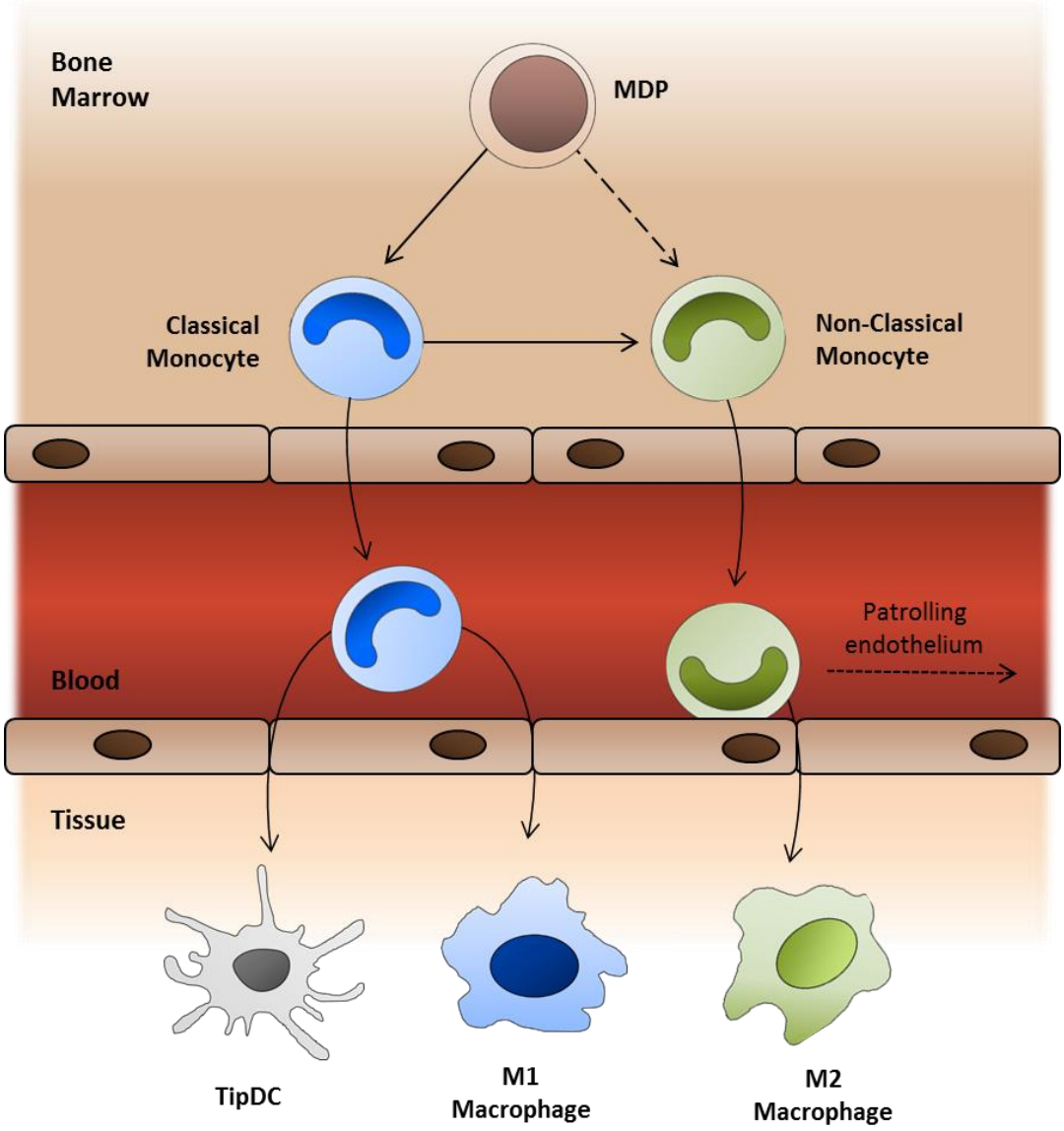


Figure 1.2. Development and fate of monocyte subsets. Hematopoietic stem cells give rise to monocyte-macrophage-DC precursors (MDPs) in the bone marrow. MDPs give rise to classical monocytes which are then able to differentiate into non-classical monocytes in the bone marrow. It is unclear if MDPs can directly give rise to non-classical monocytes. Mature monocytes can then enter the blood and under naïve conditions non-classical monocytes in the circulation patrol the endothelium. In inflamed conditions both classical and non-classical monocytes enter the tissue and differentiate, with classical monocytes giving rise to 'M1' inflammatory macrophages and TNF and iNOS-producing DCs (TipDCs), and non-classical monocytes giving rise to alternatively activated 'M2' macrophages.

detection of pathogens or tissue damage, or subsequently released cytokines, it is also suggestive of oxidized lipid and dead cell scavenging, although this has yet to be demonstrated in vivo (Auffray *et al.*, 2009a). Non-classical monocytes differentiate into 'alternatively activated' or 'M2' macrophages, associated with T_H2-type responses such as tissue remodelling, wound repair, and immunomodulation. In the myocardium after infarction, non-classical monocytes are involved in collagen deposition, express growth factors indicative of angiogenesis and are thought to promote myofibroblast accumulation, all suggestive of their function in wound healing (Nahrendorf *et al.*, 2007).

1.2.5. Lymphocytes

Derived from the common lymphoid progenitor in the BM, T cells, B cells, and natural killer (NK) cells, are the three major types of lymphocyte. T cells, defined by the presence of the T cell receptor (TCR), have their name due to their maturation in the thymus (Alberts *et al.*, 2002). A highly heterogeneous leukocyte with diverse subtypes, T cells play a key role in cell-mediated immunity with T helper (T_H) cells involved in B cell maturation, differentiating into a number of subtypes including T_H1, T_H2, and T_H17 cells. Cytotoxic T cells are also involved in cytokine and granule production that result in the killing of infected cells whilst regulatory T cells (T_{reg}) have important immune suppressive functions. B cells are defined by the presence of a B cell receptor (BCR) and are primarily involved in the production of antibodies, otherwise called the humoral response (Alberts *et al.*, 2002). Both cell types are components of the adaptive immune system and participate later in the inflammatory response. NK cells, named due to their response to stressed cells in the absence of opsonisation, are located in the BM, blood, lymphoid, and non-lymphoid tissues. After activation by interferons and macrophage-derived cytokines, NK cells participate in immune responses to viral infections and tumours with both cytotoxic and cytokine-producing responses, thereby limiting spread and tissue damage (Vivier *et al.*, 2008). The characterisations of these lymphocytes are, however, vastly oversimplified as the exact function and phenotype of lymphocytes is outside the scope of this thesis.

1.3. Inflammatory cell recruitment

One of the key pro-inflammatory strategies the body adopts in response to infection or injury is the recruitment of circulating immune cells into tissues. Recognition of pathogens or damaged tissue by macrophages enables recruitment of neutrophils and monocytes from their sites of storage to the circulation and the tissue. This process involves a plethora of

inflammatory molecules operating in a highly regulated and sequential manor which together act to eliminate the infection, remove damaged tissue, and restore homeostasis.

1.3.1. Chemokines

The process by which inflammatory cells are recruited to the tissue involves cellular activation and subsequent secretion of cytokines that provide a gradient for immune cells towards the site of infection. This gradient is achieved by specific chemotactic cytokines, or 'chemokines'. Chemokines are distributed into families depending on the number and location of cysteine residues at the amino terminus: C, CC, CXC, and CX_3C, whereby a succeeding 'L' indicates ligand, and 'R' indicated receptor (Luster, 1998). As a generalisation, CC-chemokines attract mononuclear cells, whilst CXC-chemokines attract granulocytes (Charo *et al.*, 2006). Once released, chemokines bind to glycosaminoglycans on the luminal side of ECs, anchoring them in a gradient and preventing removal in the bloodstream or potential proteolytic cleavage (Mantovani *et al.*, 2006). Chemokines are bound by chemokine receptors; G-protein coupled receptors (GPCRs) that are differentially expressed amongst leukocyte subsets. Binding allows activation of complex intracellular signalling events that trigger cellular activation, and thereby orchestration of the inflammatory response to recruit specific cell types to the site of infection at the correct time.

1.3.2. Neutrophil recruitment

Neutrophils are considered the cells that extravasate most rapidly out of the circulation into the tissue. They respond from remote locations to a plethora of chemotactic agents, acting on only a handful of cell surface receptors (Table 1.1). Interestingly, neutrophils have been shown to traffic to the site of inflammation not by the shortest route through the tissue, but via the vasculature, ensuring a quick and close delivery (McDonald *et al.*, 2010). Sentinel cell-derived inflammatory mediators such as IL-1β, TNF, LTB_4, and C5a, activate nearby vascular ECs, inducing expression of adhesion molecules and vascular permeability, thereby promoting adhesion and transmigration of neutrophils, respectively (Soehnlein *et al.*, 2010). Activated ECs can propagate the inflammatory signal, also producing pro-inflammatory mediators. Movement along the endothelium exposes neutrophils to increasing concentrations of chemokines acting on CXCR2, which in turn induces cell activation and migration. CXCR2 ligands, including CXCL1 (Bozic *et al.*, 1995), CXCL2 (Wuyts *et al.*, 1998) and CXCL5 (Soehnlein *et al.*, 2010), are highly important for neutrophil chemoattraction and activation. Neutralisation of CXCL1 and CXCL2 resulted in a 4-fold reduction in infiltrating neutrophils in a

peritonitis model (Wengner *et al.*, 2008). The activities of CXCL1 and CXCL2 are increased by exposure to MMP-9 and MMP-8, respectively, released from activated neutrophil granules to elevate the inflammatory response. The actions of both chemokines are reportedly terminated by MMP12 cleavage, an important step in preventing excess cytokine storm and neutrophil influx (Soehnlein *et al.*, 2010). CXCL8 is the human analogue to the murine CXCR2 ligands, acting via CXCR2 to induce the same potent neutrophil recruitment (Baggiolini *et al.*, 1989). As with ECs, activation of neutrophils triggers adhesion molecule upregulation promoting stronger cell-to-cell contact.

Chemokine	Alternative Name	Receptor	Species	Source	Reference
CXCL1	KC	CXCR2	human, mouse	macrophages	(Bozic *et al.*, 1995)
CXCL2	MIP-2α	CXCR2	human, mouse	macrophages	(Wuyts *et al.*, 1998)
CXCL5	ENA-78	CXCR2	human, mouse	macrophages, eosinophils, epithelial cells	(Soehnlein *et al.*, 2010)
CXCL7	NAP-2	CXCR2	human, mouse	platelets	(Brandt *et al.*, 1991)
CXCL8	IL-8	CXCR1	human	macrophages neutrophils	(Baggiolini *et al.*, 1989)
CXCL12	SDF-1	CXCR4	human, mouse	stromal cells (BM)	(Martin *et al.*, 2003a)

Table 1.1. Chemokines involved in murine neutrophil migration.

Interestingly chemoattractants released from, or located at, intermediate sites such as the endothelium (i.e. CXCL2, LTB_4), are reportedly overridden by more localised 'end-target' chemoattractants such as fMLP and C5a located close to the pathogen to which the neutrophils preferentially respond to (Kolaczkowska *et al.*, 2013). It has also been proposed that in order for neutrophils to be fully activated, they must first be primed by cytokines such as TNF or IL-1β, or by contact with activated ECs, and then once in the tissue their activation heightens by exposure to bacteria or damaged tissues (Summers *et al.*, 2010). Whether this is a method of rapidly recruiting close-by neutrophils, or a method for better homing precision is unknown. In addition to recruitment of already circulating neutrophils, chemokines also promote mobilisation of the BM store, another method to ensure a sufficient supply of circulating neutrophils (Martin *et al.*, 2003a). Taken together, these mechanisms ensure arrival

of neutrophils *en masse* to rapidly eliminate the bacteria or damaged tissue by phagocytosis, ROS, and protease production. It is worth noting however, many of the neutrophil functions can potentially also be detrimental to the host and thus have a potential causative role in disease (Sadik *et al.*, 2011).

1.3.3. Monocyte recruitment

Like neutrophils, monocytes respond to specific sets of chemokines that are sensed by receptors on their cell surface and as a result are recruited towards the site of inflammation. CCL2 is a key monocyte chemokine produced primarily by macrophages and ECs, which exerts its chemotactic effect via the CCR2 receptor highly expressed on the classical monocyte subset. CCL7 also acts on CCR2 to induce monocyte recruitment. The importance of CCR2 activation by CCL2 and CCL7 has been demonstrated in mice as knockout of either chemokine almost halves the number of monocytes recruited in response to bacteria (Jia *et al.*, 2008). There are however, many other chemokines involved in the chemotaxis of monocytes from various stores during inflammation (Table 1.2).

Infiltration of classical monocytes typically begins around the height of neutrophil accumulation at 3h and peaks at approximately 12h after bacterial infection (Auffray *et al.*, 2009a). Once recruited, classical monocytes participate in inflammatory 'M1-type' responses including phagocytosis, inflammatory cytokine release, and production of bacterial killing tools such as ROS and iNOS (Auffray *et al.*, 2009a). Non-classical monocyte infiltration peaks later, approximately 18h post infection, and 'M2-type' responses including tissue remodelling and wound healing are employed (Auffray *et al.*, 2009a).

Classical monocyte recruitment is considered to be neutrophil-dependent. The extent of dependency on neutrophils is not however clear. Both during and after transmigration, activated neutrophils release a series of granules containing proteins able to stimulate subsequent classical monocyte influx (Soehnlein *et al.*, 2010). Granule proteins such as azurocidin are released from secretory vesicles of transmigrating neutrophils and deposited on the endothelium. This interaction causes further activation of ECs, inducing expression of adhesion molecules vascular cell adhesion molecule 1 (VCAM-1) and E-selectin that aid monocyte activation and migration (Soehnlein *et al.*, 2009b). Activated transmigrated neutrophils also shed their surface IL-6 receptor, forming a soluble sIL-6R that binds to IL-6 present at the site of inflammation. The IL-6/sIL-6R complex is then able to interact with endothelial gp130 co-receptor resulting in enhanced CCL2 and adhesion molecule expression and further promotion of monocyte recruitment (Hurst *et al.*, 2001). Such signalling also turns

off neutrophil recruitment due to the loss of the IL-6R and is therefore referred to as a 'switch' in leukocyte recruitment.

Chemokine	Alternative Name	Receptor	Species	Source	Target
CCL1	I-309	CCR8	human, mouse	T cells	classical monocytes (Qu *et al.*, 2004)
CCL2	MCP-1	CCR2	human, mouse	macrophages, ECs, MSCs, CAR cells	classical monocytes (Boring *et al.*, 1997)
CCL3	MIP-1α	CCR1 CCR5	human, mouse	macrophages, ECs, monocytes	classical monocytes (Soehnlein *et al.*, 2010)
CCL4	MIP-1β	CCR1	human, mouse	neutrophils	classical monocytes (Soehnlein *et al.*, 2009a)
CCL5	RANTES	CCR1 CCR3 CCR5	human, mouse	macrophages	classical monocytes (Shi *et al.*, 2011a), neutrophils (Soehnlein *et al.*, 2010)
CCL6	C10	CCR1	mouse	macrophages neutrophils	classical monocytes (Berahovich *et al.*, 2005)
CCL7	MCP-3	CCR1 CCR2 CCR3	human, mouse	macrophages	classical monocytes (Tsou *et al.*, 2007)
CCL12	MCP-5	CCR2	mouse	macrophages	classical monocytes (Sarafi *et al.*, 1997)
CCL20	MIP-3A	CCR6	human, mouse	macrophages neutrophils lymphocytes	classical monocytes (Le Borgne *et al.*, 2006)
CX_3CL1	fractalkine	CX_3CR1	human, mouse	ECs macrophages	mainly non-classical, also classical monocytes (Ingersoll *et al.*, 2011)

Table 1.2. Chemokines involved in the migration of monocytes

Granule proteins act to induce and enhance chemokine release and potency. Proteinase 3, alongside its ability to produce antimicrobial peptides by cleavage, induces EC CCL2 production (Sugawara, 2005). Azurocidin on the other hand induces the production of monocyte-expressing CCR1 ligand, CCL3 from monocytes and macrophages (Soehnlein *et al.*, 2010). Interestingly, the other CCR1 ligands (CCL6, CCL9, CCL15, and CCL23) are also released by

macrophages but have only weak chemotactic capabilities. The action of neutrophil-derived serine proteases can however increase the potency of these CCR1 ligands by up to 1000-fold *in vitro* (Berahovich *et al.*, 2005). Therefore, the release of specific granule contents by neutrophils has the potential to modulate the extent and strength of monocyte infiltration by proteolytic cleavage of certain chemokines (Soehnlein *et al.*, 2009b). Furthermore, certain granule contents such as bactericidal/permeability-increasing protein (BPI) or heparin-binding protein (HBP) are capable of promoting vessel permeability, additionally aiding the extravasation of monocytes (Borregaard *et al.*, 2007).

The classical multistep paradigm of macrophage activation, neutrophil migration, and classical monocyte recruitment followed later by non-classical monocyte recruitment has however recently been challenged. Non-classical monocytes reportedly continually patrol the endothelium in a CX_3CR1-dependent manner, rapidly extravasating within 1h in response to a range of inflammatory stimuli (Auffray *et al.*, 2007). CX_3CR1 expression differs between the monocyte subsets with non-classical monocytes CX_3CR1^{high} (Geissmann *et al.*, 2003). Its ligand, CX_3CL1 is found in both soluble and membrane-anchored forms, contributing to potent non-classical monocyte chemoattractive and adhesive abilities, respectively. Non-classical monocyte recruitment therefore precedes that of neutrophils in certain situations, suggesting both monocyte and macrophage activation is able to induce neutrophil recruitment.

1.3.4. Leukocyte adhesion cascade

Additional factors besides chemokine gradients are required to recruit immune cells to the tissue. Leukocytes must cross the blood vessel wall, a process requiring induction of machinery to physically aid the extravasation process. In addition to leukocyte-targeted chemokines, activated tissue macrophages also release inflammatory mediators that activate and alter the phenotype of the endothelium. Mediators such as TNF and IL-1β induce such changes to aid leukocyte extravasation (Nourshargh *et al.*, 2010). The exact mechanism by which inflammatory cells migrate from the circulation, through the endothelium, into the tissue is a well characterised phenomenon comprising 4 main stages: rolling, adhesion, crawling, and transmigration (Figure 1.3) (Ley *et al.*, 2007). Collectively, these processes are termed the 'leukocyte adhesion cascade'. Circulating leukocytes are captured and begin rolling along the wall of the blood vessel by interaction of leukocyte L-selectin and activated endothelial E- and P-selectin, with the P-selectin glycoprotein ligand 1 (PSGL1) (Ley *et al.*, 2007). As rolling slows, integrins take over, resulting in subsequent arrest and firm adhesion. This can occur via leukocyte lymphocyte function-associated antigen 1 (LFA-1, $\alpha L\beta 2$ integrin) – endothelial

intracellular adhesion molecule 1 (ICAM-1) interaction in the case of neutrophils, or very late antigen 4 (VLA-4, α4β1 integrin) – VCAM-1 interaction in the case of monocytes and lymphocytes (Ley *et al.*, 2007). Firmly adhered leukocytes begin intraluminal crawling, a process of membrane protrusion and filopodia formation that interact with activated adhesion-molecule expressing ECs, thus seeking permissive sites for transendothelial migration (TEM). Neutrophils use Mac-1 (αMβ2 integrin) expressing filopodia to crawl, interacting with endothelial ICAM-1 whilst lymphocytes use those expressing LFA-1 (Nourshargh *et al.*, 2010). Luminal endothelial chemokines activate leukocytes, inducing conformational changes that allow adhesion, whilst chemotactic gradients along the endothelium provide direction for crawling and TEM. TEM, the final step in the adhesion cascade, involves additional adhesion molecules including platelet/endothelial cell adhesion molecule 1 (PECAM1), and junctional adhesion molecules (JAMs) (Nourshargh *et al.*, 2010). TEM can occur either by the paracellular route (between ECs), or the transcellular route (through individual cells), allowing the subsequently extravasated leukocyte to contribute to the inflammatory response within the tissue (Kolaczkowska *et al.*, 2013).

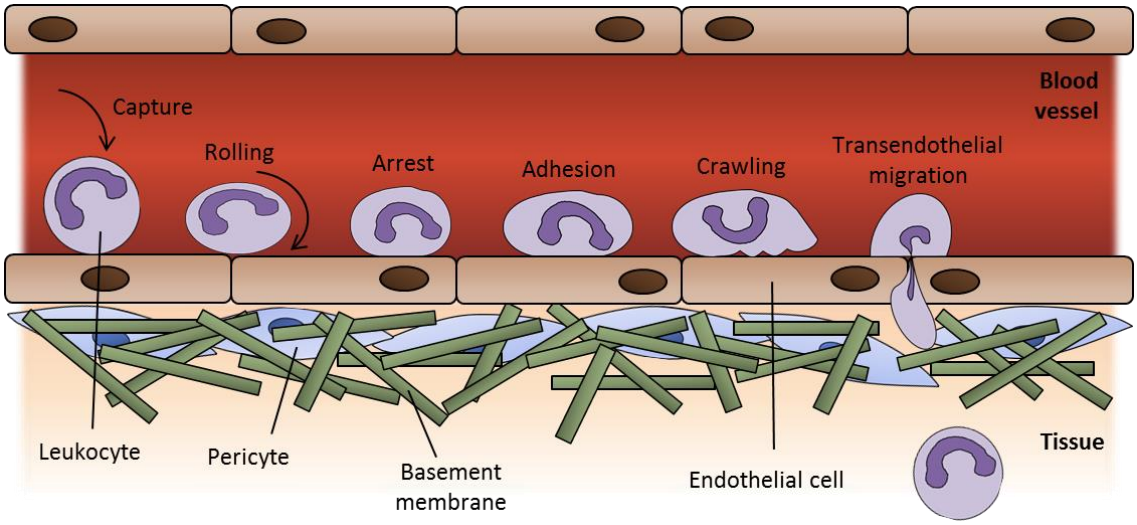


Figure 1.3. The leukocyte adhesion cascade. In response to inflammatory stimuli, circulating leukocytes migrate into the tissue in response to released cytokines and chemokines. Leukocytes are first captured onto the endothelium, and begin rolling, a process mediated by selectins. Chemokines drive activation of rolling and leukocyte activation, which allows arrest and adhesion to the endothelium with subsequent crawling. The leukocyte finally transmigrates by either the transcellular or paracellular route. (Figure adapted from Nourshargh et al., 2010).

1.3.5. Leukocyte recruitment in disease

Leukocyte recruitment to the site of infection or injury is essential for clearance of the pathogen or damaged tissue and the subsequent restoration of tissue homeostasis. Immune cells involved in the response clearly have powerful functions that if not tightly regulated can be highly damaging to the host. Not only must the functions of these cells be controlled, the number recruited must be tightly regulated to ensure adequate clearance of the stimuli, thus maintaining control of the inflammatory response. Whilst the pathogenesis of many diseases is highly complex and not well characterised, there is increasing evidence that dysregulation of immune cell infiltration and response is in fact the underlying cause of many diseases. Understanding the exact context of dangers versus importance of leukocyte infiltration may provide potential new targets for treating inflammatory disorders.

1.3.5.1. Reperfusion Injury

Ischaemia reperfusion (I/R) injuries are an example of leukocyte recruitment being causative in tissue damage. Ischemic insult such as during myocardial infarction (MI), stroke, or organ transplantation is followed by the essential reperfusion phase, whereby the tissue is once again exposed to oxygen. Without oxygenation, tissue death would result. This restoration of blood flow however is accompanied by release of cytokines and chemokines, activation of the endothelium, upregulation of adhesion molecules, and accumulation of activated neutrophils (Eltzschig *et al.*, 2011; Timmers *et al.*, 2012). The presence of activated neutrophils consequently causes ROS generation and a subsequent imbalance of superoxide ($O_2^{-\bullet}$) and nitric oxide (NO). This imbalance promotes accumulation of highly toxic oxygen products such as peroxynitrite ($OONO^-$), hydrogen peroxide (H_2O_2), and hypochlorus acid (HOCl) that have the ability to oxidise DNA, proteins and lipids, inevitably destroying the cell (Thannickal *et al.*, 2000). It is suggested that disruption of the cell membrane by oxidation may also result in more ROS and cytokine release, propagating the potential for tissue damage. Release of reperfusion-induced inflammatory mediators and activated leukocytes in the blood results in remote organ damage via the circulation, and is a serious problem after ischaemic insult potentially causing multi organ dysfunction (Carden *et al.*, 2000). The key role for leukocyte infiltration accelerating disease progression has been demonstrated using neutrophil-depletion protocols that effectively suppressed neutrophil recruitment, ROS generation, phagocytosis, and degranulation in rat myocardial I/R (Kohtani *et al.*, 2002). Further evidence for association of neutrophils with the injuries obtained during reperfusion is seen in kidney (Klausner *et al.*, 1989), cerebral (Matsuo *et al.*, 1995), liver (Jaeschke *et al.*, 1990), and skeletal

muscle I/R injuries (Carden *et al.*, 1990) where depletion of neutrophils has further proved suppressive.

1.3.5.2. Atherosclerosis

Unlike the acute nature of I/R injury, atherosclerosis is a chronic condition involving gradual lesion formation and thickening of the arterial wall as a consequence of immune cell and lipid infiltration. Comparable to I/R however, leukocyte recruitment underlies the pathology. Primarily monocyte-driven, these cells accumulate and differentiate into macrophages once in the vessel wall, appearing as fatty streaks. An atheroma develops when macrophages take up lipid droplets to become foam cells and the plaque matures. A smooth muscle cell and collagen matrix 'fibrous cap' covers a plethora of infiltrated immune cells (DCs, mast cells, T cells, monocytes), and a core of cholesterol and cell debris (Hansson *et al.*, 2006). Lesions can grow so large as to limit the blood flow however the real danger arises when the plaque ruptures and a sudden thrombotic event is triggered, resulting in a blood clot, occlusion of the artery and often the formation of an unstable new atheroma. In the heart this causes MI, and in the brain, stroke. The importance of monocyte recruitment in atherosclerosis is demonstrated by the positive correlation of lesion size and numbers of circulating monocytes (Combadiere *et al.*, 2008). Furthermore, combined deletion of key monocyte chemokines and receptors, CCL2, CX_3CR1, and CCR5 limited numbers of circulating monocytes and markedly reduced lesion progression in atherosclerosis-prone mice.

1.3.5.3. Sepsis

Acute infection leads to the activation of sentinel cells, release of cytokines and chemokines and infiltration of immune cells in order to restore homeostasis of the tissue. In some cases the host response becomes dysregulated and cell, tissue, and organ damage can result. The clinical hallmark of sepsis is multi-organ failure, an indication the immune response has lost control, entered the bloodstream, and is injuring organs it reaches. The pathogenesis of sepsis is not clear, but in most cases is initiated from gram-negative bacterial infection of the lungs, abdomen, urinary tract or blood (Cohen, 2002). Activation of leukocytes and the endothelium by bacteria results in activation of complement, imbalance of coagulation pathways, and production of cytokines and subsequent oxygen and lipid intermediates. These cause a combination of vascular instability and occlusion that produce the classical signs of shock (fever, vasodilation, capillary leakage), and culminate in sepsis and multi organ failure (Angus *et al.*, 2013). It is therefore clear that dysregulation of neutrophil and monocyte responses can

result in excessive respiratory burst, cytokine release, and promotion of further immune cell recruitment, however exactly how these responses become both aberrant and systemic to culminate in sepsis is considered a complex web of events that are not fully clear.

I/R injuries, atherosclerosis, and sepsis are three diverse examples of inflammatory conditions where leukocyte infiltration and function no longer become helpful, but instead turn against the host. They are the result of lost regulation and therefore subsequent amplification of response. These examples only scratch the surface but demonstrate the importance of understanding mechanisms of leukocyte recruitment in the clearance of infection and damaged tissue, and the restoration of the surrounding environment homeostasis.

1.4. Bone marrow leukocyte mobilisation

The BM is both a large storage pool for mature neutrophils, and the site of neutrophil production: in mice the neutrophil BM reserve is thought to be in the region of $1.2x10^8$ cells (Furze *et al.*, 2008). Cells are continually released into the circulation and upon inflammatory insult the BM reserve rapidly mobilises, amplifying neutrophil release. The molecular mechanism of sequestration and release is thought to be via cytokine and chemokine actions. Mature neutrophils are retained in the BM via the CXCL12/CXCR4 chemokine axis (Ma *et al.*, 1999; Furze *et al.*, 2008). Low levels of CXCR4 on BM neutrophils are unlikely to support chemotaxis, however blockade of CXCR4 by small molecule antagonist AMD3100, or repopulation of irradiated WT mouse BM with CXCR4-/- cells, resulted in relocation of neutrophils from the BM to the circulation (Ma *et al.*, 1999; Martin *et al.*, 2003a). These observations, combined with high levels of CXCL12 in the resting BM, suggest CXCL12 functions via CXCR4 as a retention factor for the BM reserve neutrophils.

Whilst CXCR4 blockade leads to the release of neutrophils from the BM, the mechanism by which this occurs is not passive and is reported to be via CXCR2. Chemokines CXCL1 and CXCL2 are produced both remotely and locally and act on neutrophil CXCR2 to promote mobilisation (Eash *et al.*, 2010). Thus whilst CXCL12/CXCR4 acts as a retention axis for BM neutrophils, the CXCL1-CXCL2/CXCR2 axis acts antagonistically to regulate neutrophil release (Figure 1.4). The CXCR4 axis is however dominant, thus granulocyte colony stimulating factor (G-CSF) also acts similarly to CXC chemokines and down regulates BM neutrophil CXCR4 and CXCL12 so that the CXCR2 axis can predominate (Semerad *et al.*, 2002; Kim *et al.*, 2006; Eash *et al.*, 2010). Combined, the actions of CXCL1, CXCL2, and G-CSF disrupt the CXCL12/CXCR4 retention axis and facilitate neutrophil mobilisation. Whilst high CXCR4 is indicative of BM sequestration, and low expression is suggestive of mobilisation, it is worth noting that under homeostatic

conditions neutrophil CXCR2 is down regulated and CXCR4 is again upregulated in senescence representing an indicator for old circulating neutrophils to return back to the BM to die (Martin *et al.*, 2003a; Rankin, 2010).

The CXCL12/CXCR4 BM retention axis has also been described in the context of murine classical monocytes. As with neutrophils, BM classical monocytes reportedly express CXCR4 (Wang *et al.*, 2009). With the use of small molecule inhibitors, monocyte CXCR4 is shown to play a role in sequestration in the BM during CCR2 blockade, however the physiological relevance of such a mechanism is not clear. Mobilisation of classical monocytes on the other hand involves the CCL2-CCL7/CCR2 axis (Serbina *et al.*, 2006; Wang *et al.*, 2009). CCR2 null

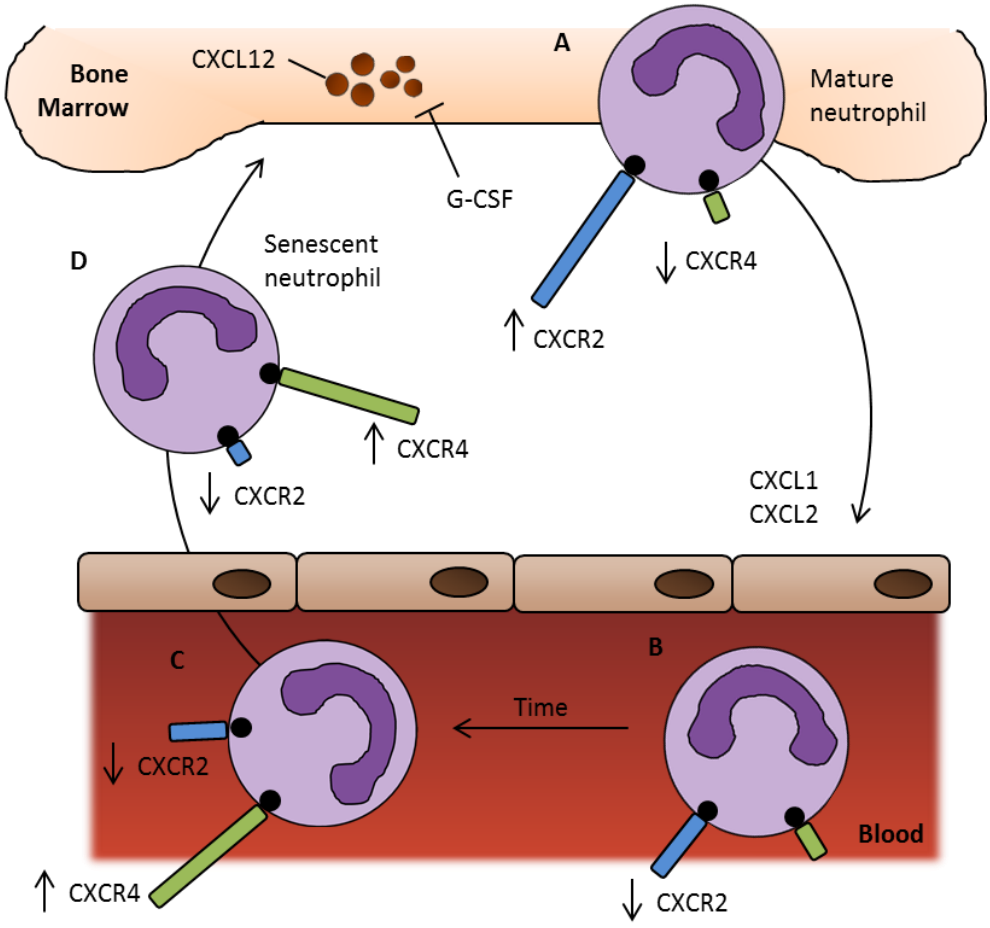


Figure 1.4. Changes in neutrophil chemokine receptors in inflammation. (A) Neutrophils are produced and stored in the bone marrow (BM) via the CXCL12-CXCR4 interaction. Mature BM neutrophils upregulate CXCR2 and are mobilised in response to CXCL1 and CXCL2 produced during inflammatory conditions. G-CSF also produced in inflammation aids this process by inhibiting the CXCL12/CXCR4 axis. (B) Neutrophils with high CXCR2 enter the circulation from the BM during inflammation. (C) Aging neutrophils downregulate CXCR2 and upregulate CXCR4. (D) High CXCR4 causes homing of senescent neutrophils back to the BM via a CXCL12 chemotactic gradient.

mice display accumulation of monocytes in the BM, with fewer cells in the circulation and peripheral organs (Serbina *et al.*, 2006). In response to bacteria, monocytes already present in the blood were able to traffic to the site of infection suggesting the importance of CCR2 in BM egress but not recruitment.

Classical monocyte mobilisation under both homeostatic and inflammatory conditions is thought to be dependent on the constitutively produced CCR2 ligands CCL2 and CCL7, as mice deficient in either chemokine displayed elevated BM monocytes and reduced circulating monocytes, whilst pharmacological blockade of CCR2 led to elevated blood CCL2 and CCL7 levels (Tsou *et al.*, 2007; Wang *et al.*, 2009). The mechanism of CCR2-dependent classical monocyte egress is reportedly governed by BM mesenchymal stem cells (MSCs) and their CXCL12-abundant reticular (CAR) cell progeny. These cells rapidly express CCL2 in response to circulating TLR ligands, inducing monocyte mobilisation into the bloodstream (Shi *et al.*, 2011b). CCR2 is clearly important for mobilisation, however CCL2 deficiency does not affect monocyte egress from the BM in a model of atherosclerosis suggesting that whilst CCR2 is of utmost importance, CCL2 action can be compensated for by other chemokines (Combadiere *et al.*, 2008). The importance of CCR2 in classical monocyte egress for both homeostasis and inflammation is becoming clear; the mechanisms for the non-classical subset however are not so well documented. Whilst some groups refer to non-classical monocytes as $CCR2^{-}$, others have demonstrated that although this is characteristic for human monocytes, mice indeed express low levels of CCR2 and are therefore considered $CCR2^{low}$ (Ingersoll *et al.*, 2010). As a result, it would be fair to speculate that mobilisation of non-classical monocytes may also occur via the CCL2/CCR2 axis, however unlike the classical subset, non-classical monocytes are not reduced in the circulation of CCR2-/- mice (Qu *et al.*, 2004), and the differing role of each subset suggests an alternative mechanism may be more likely.

Monocyte subsets are also distinguished by expression of CX_3CR1, with classical and non-classical monocytes CX_3CR1^{low} and CX_3CR1^{high}, respectively. In the absence of CX_3CR1, classical monocytes were reduced in the BM and accumulated in the circulation of mice exposed to chemotherapy agent cyclophosphamide (Jacquelin *et al.*, 2013). This indicates CX_3CR1 acts in an opposing manner to CCR2 to retain classical monocytes in the BM. More specifically, CX_3CR1 reduces monocyte movement within the BM parenchyma, and adherence to the endothelium. In contrast, CX_3CR1 deficiency did not affect mobilisation of BM monocytes after thioglycollate treatment, suggesting the CCL2/CCR2 axis is dominant over that of CX_3CL1/CX_3CR1 (Jacquelin *et al.*, 2013). The role of CX_3CR1 in the fate of BM CX_3CR1^{high} non-classical monocytes has not however been explored. Indeed, the very existence of BM non-

classical monocytes is a disputed topic. Some believe only Ly6C^{high} classical monocytes exist in the BM and once in the periphery differentiate in Ly6C^{low} non-classical monocytes (Sunderkotter *et al.*, 2004). Whether MDPs directly give rise to either subset, or whether non-classical Ly6C^{low} cells are precursors of classical Ly6C^{high} cells is unclear. However generally accepted is that both subsets exist in the BM and exit to the circulation, with classical monocytes considered to shuttle back and forth from the BM to the circulation (Yona *et al.*, 2010).

1.5. Spleen leukocyte reservoir

The spleen consists of white and red pulp, and functions as a filter of the blood, removing old erythrocytes, recycling iron, and metabolising haemoglobin of senescent cells (Mebius *et al.*, 2005). As a lymphoid organ, it also synthesises antibodies in the white pulp and disposes of antibody-coated pathogens or cells via the blood and lymphatic circulations. In mice, the spleen has a further role as a leukocyte reservoir holding half of the body's monocytes within the red pulp, a store that far outnumbers circulating counterparts (Swirski *et al.*, 2009). This splenic reservoir was shown to hold mature but undifferentiated monocytes indistinguishable and at the same subset ratio as those in the blood. The relative contribution of splenic leukocytes to disease is poorly characterised, however in response to myocardial ischemic injury in mice, monocytes reportedly exit the spleen, not the BM, and accumulate in the injured myocardium (Swirski *et al.*, 2009). The spleen did not however contribute neutrophils to the extent of monocytes, leaving the BM as the primary site of mature neutrophil storage. This indicates a role for the spleen as another store of readily available inflammatory cells, and the same phenomenon has also been documented in human acute MI patients (van der Laan *et al.*, 2014). In non-inflamed conditions, both monocyte subsets have been shown to accumulate in the spleen after adoptive transfer (Geissmann *et al.*, 2003). Whilst the non-classical subset also accumulated in the lung, circulation and liver, classical monocytes homed solely to the spleen, suggesting that while the non-classical cells are likely to be involved in tissue homeostasis, classical monocytes are awaiting deployment in response to infection.

More recently the spleen has been shown to supplement the haematopoietic function of the BM by producing classical monocytes that are released into the circulation and intrude lesions in atherosclerosis-prone mice (Robbins *et al.*, 2012). Interestingly, despite splenectomy not being lethal, it is reported to enhance both ischaemic heart disease and risk of infection, and human patients are required to be on lifelong antibiotics (Robinette *et al.*, 1977). Considering the physical properties of the spleen, i.e. location, high vascularisation, and the plethora of

adhesive ligands suggestive of cell retention, these reports add increasing evidence towards the spleen as an ideal store of inflammatory cells (Mebius *et al.*, 2005). Whilst it is known that B cells are retained in the follicles by CXCL13/CXCR5 interaction, and T cells to the T-cell zone by the CCL19-CCL21/CCR7 axis, the nature by which monocytes and neutrophils are arranged in the spleen is not well documented (Mebius *et al.*, 2005). CX_3CR1 is reportedly required for classical monocyte recruitment to the murine spleen after infection and irradiation: Evidence is provided by increased circulating classical monocytes and reduced spleen recruitment in CX_3CR1-deficient mice after splenic *L.monocytogenes* infection compared to WT counterparts (Auffray *et al.*, 2009b). Although its ligand CX_3CL1 has indeed been identified in the spleen, a possible involvement of the CX_3CL1/CX_3CR1 axis in the naïve state has been refuted (Auffray *et al.*, 2009b; Jacquelin *et al.*, 2013).

Although CCR2 has been implicated as important for monocyte mobilisation from the BM, its role in the spleen is not yet defined. After cardiac ischaemia monocytes were mobilised from the spleen to the circulation in WT and CCR2-/- mice despite a defect in accumulation of cells in the CCR2-/- myocardium (Swirski *et al.*, 2009). This indicated a redundant role for the CCL2/CCR2 axis in splenic monocyte mobilisation. In contrast, angiotensin II (ang II), acting via the angiotensin type I receptor (AT-1) on monocytes is reported to control the egress of these cells from the spleen. Evidence for this is provided by the failure of AT-1 receptor null mice (*Atrgr1a-/-*) to both mobilise and recruit splenic monocytes in the ischaemia model, whilst naïve WT mice infused with ang II are able to promote egress from the spleen (Swirski *et al.*, 2009). The splenic reserve pool of monocytes are therefore mobilised during MI via an ang II-dependent, CCR2-independent mechanism (Kintscher *et al.*, 2001; Swirski *et al.*, 2009; Shi *et al.*, 2011a). The exact mechanism of ang II-mediated classical monocyte release is currently unknown however effects on the cytoskeletal arrangement and on vasoconstriction have been proposed. Whether ang II is pivotal to classical monocyte recruitment in other diseases or non-sterile inflammatory models however is as yet unknown. Monocytes also express CCR1, CCR5, CCR6, CCR7, CCR8, and CXCR2 however any contribution of the chemokines acting via these receptors has yet to be described in the context of the spleen.

Unlike the lung, liver, and BM, under physiological conditions the spleen was not thought to contain any neutrophils (Peters *et al.*, 1985). Conversely, it is reported as a site of neutrophil clearance in addition the BM and liver, suggesting basal splenic neutrophils are indeed there to die (Furze *et al.*, 2008). Under basal and inflammatory conditions the action of so-called splenic reticular endothelial macrophages clears these neutrophils (Mebius *et al.*, 2005). The mechanisms of senescent neutrophil homing to the spleen are poorly characterised, however

it could be speculated that a CXCL12/CXCR4 axis may be exploited as it is required for homing to the BM. More recently, neutrophils have been identified for the first time in the marginal zone of the resting spleen, increasing the controversy over their presence or absence (Puga *et al.*, 2012). The presence of so-called B cell-helper neutrophils in this area specifically enhanced production of B cell immunoglobulin production and class switching in inflammatory conditions, providing one of the first detailed demonstrations of the involvement of neutrophils in adaptive immunity.

1.6. Pattern Recognition

Sections 1.6, 1.7 and 1.8 have also been published as a review (Kay et al., (2013) BioFactors vol. 40, (3) 284-294).

The complex series of events that culminate in the inflammatory response must first be initiated. Objects foreign or damaging to the body contain unique highly conserved sequences detectable by innate immune receptors, which alert the immune system to mount a rapid inflammatory response. Such sequences on invading pathogens are termed pathogen-associated molecular patterns (PAMPs) and allow discrimination between self and non-self, harmful and non-harmful (Medzhitov *et al.*, 2000). Tissue damage also requires the inflammatory response, and stressed, dying or injured cells expose self-patterns, termed damage-associated molecular patterns (DAMPs). The pattern recognition receptor (PRR) family function to detect PAMPs and DAMPs and instruct the immune system to respond accordingly.

The concept of pattern recognition does not however account for the highly varied responses to the diverse number of stimuli, whilst remaining 'self'-tolerant, and thus has been deemed overgeneralised by some. Alternative theories have therefore been proposed opposing the idea the immune system works to discriminate between 'self' and 'non-self', protecting the body from the 'non-self'. The *Danger Theory* suggested the immune response was not orchestrated as classically thought, and instead is driven by the need to detect the difference between what is dangerous, such as tissue and cell damage, independent of how it arose, and what is not, consequently protecting the body against the danger. Controversially, as part of the danger theory, Matzinger proposed PRRs did not evolve to recognise pathogenic PAMPs, instead the pathogens evolved to activate PRRs (Medzhitov, 2009). Despite initial popularity, danger theory was not widely accepted as microbial cells can be detected whether they cause damage or not.

1.6.1. Toll-like receptors

1.6.1.1. Structure

One family of PRRs termed Toll-like receptors (TLRs) function to recognise PAMPs and DAMPs. First identified as the human homologue to drosophila *Toll*, 10 human and 12 murine functional TLRs have since been discovered (Kawai *et al.*, 2010). TLRs demonstrate stark similarities to the mammalian IL-1 pathway, both in terms of receptor structure and signalling. Both receptors consist of a single membrane-spanning glycoprotein, and a highly conserved region of around 200 amino acids in their C-terminal, termed the Toll/IL-1R (TIR) domain (Figure 1.5) (Akira *et al.*, 2004). Markedly distinct from the three immunoglobulin-like domains the IL-1R family exhibits in its extracellular region, TLRs contain leucine-rich repeat (LRR) motifs, 19-25 in tandem. The LRRs have been described as forming a 'horseshoe' structure with speculation this surface forms the basis for ligand binding.

The first mammalian family member to be discovered was TLR4, identified as the receptor for lipopolysaccharide (LPS), a component of Gram-negative bacterial outer membranes, however

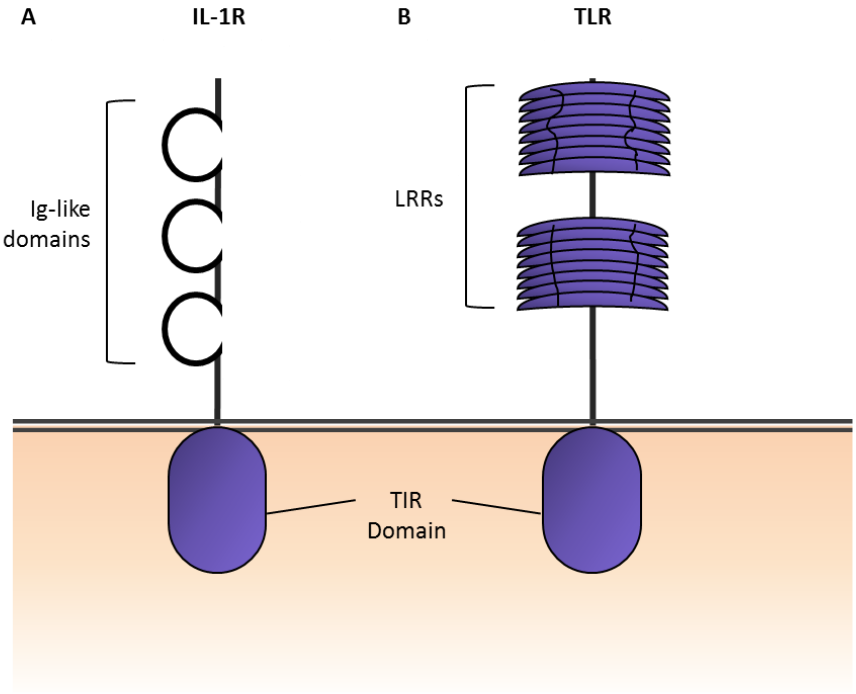


Figure 1.5. Structural homology of Toll-like receptors and the interleukin-1 receptor. (A) The IL-1 receptor (IL-1R) and (B) TLR families exhibit a conserved cytoplasmic domain termed the Toll/IL-1R (TIR) domain. The TIR domain contains highly homologous regions. Both receptors have a single membrane-spanning domain, however differ in their extracellular portion with TLRs containing leucine-rich region repeats (LRRs), compared to IL-1Rs that have three repeating immunoglobulin (Ig)-like domains. (Figure adapted from Akira *et al.,* 2004).

subsequently each TLR has been described to recognise specific sets of PAMPs. The TLR family is divided into two groups based on subcellular localisation (Figure 1.6): TLR1, TLR2, TLR4, TLR5 and TLR6 are cell surface receptors, principally focused on detecting bacterial PAMPs. Conversely, TLR3, TLR7, TLR8, TLR9 and murine TLR11 are located intracellularly in endosomal compartments, primarily sensing viral PAMPs and DNA, and uropathogenic bacteria in the case of TLR11 (Broz *et al.*, 2013). TLR2 is considered the most promiscuous, recognising a range of patterns from peptidoglycan and lipoteichoic acid from Gram-positive bacteria, to lipoproteins and lipopeptides from various pathogens (Takeda *et al.*, 2003). Certain TLRs also identify DAMPs. In addition to its role as the LPS receptor, TLR4 recognises a number of DAMPs including heat shock proteins (HSP60, HSP70), high-mobility group protein B1 (HMGB1), and components of the extracellular matrix such as hyaluronic acid, heparan sulphate, fibronectin, and tenascin-C (Takeda *et al.*, 2003; Midwood *et al.*, 2009; Bauer *et al.*, 2013). TLR7, TLR8 and TLR9 detect RNA immune complexes, and chromatin and DNA complexes, respectively. TLR2 and TLR4 together are the most widely characterised of the TLR family.

Greater research into pattern recognition, particularly TLR4, found the PAMP-DAMP/receptor interaction to be oversimplified. Cells with TLR4 only were unresponsive to LPS, leading to the

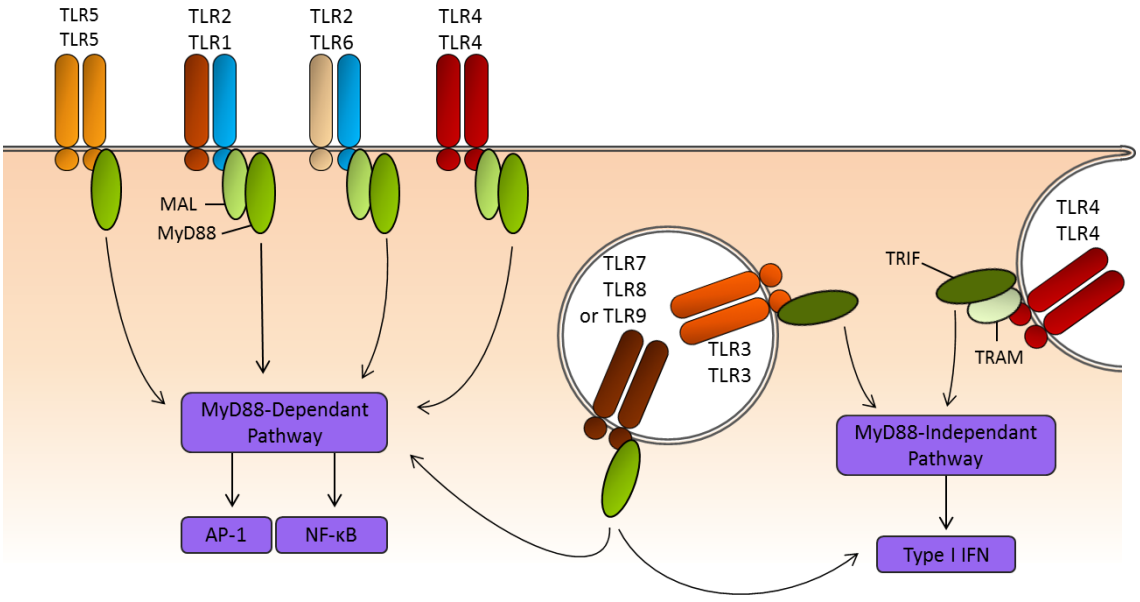


Figure 1.6. Subcellular localisation of Toll-like receptors. TLRs are located either at the cell surface, as is the case for TLR2, TLR4, TLR5, and TLR6, or intracellularly, for TLR3, TLR7, TLR8, TLR9. Intracellular TLRs reside in endosomes, where they detect both foreign and host-derived nucleic acids. Uniquely, TLR4 is found both at the cell surface and intracellularly. To function, TLRs dimerise which allows recruitment of adaptor proteins crucial for the TLR signalling pathways. (From Kay *et al.,* 2013).

discovery of MD-2, a glycosylated soluble protein required to bind extracellularly to TLR4 and link receptor and ligand (Akira *et al.*, 2006). LPS-binding protein (LBP) is also an important cofactor in ligand delivery to surface TLRs. By binding LPS, lipoteichoic acid, peptidoglycan and lipopeptides, amongst others, LBP transfers TLR ligands to CD14, another cofactor of several TLRs with the ability to bind multiple TLR ligands and enhance their activation of the receptor (Stewart *et al.*, 2010). It is emerging that TLR signalling requires a plethora of accessory molecules which may prove important in the development of novel therapeutics that target initiation of innate immune signalling in inflammatory disorders.

1.6.1.2. Signalling

Despite existing at the plasma membrane as monomers, TLR dimerisation is required for signalling, and both homo- and hetero- dimerisations occur. TLR2 acts only as a heterodimer with either TLR1 or TLR6, whilst the remaining TLRs are traditionally considered to function as homodimers (Kawai *et al.*, 2010). TLR4 has also been reported to form heterodimers with TLR6 and as so act as a receptor for both oxidised low-density lipoproteins (oxLDL), and amyloid-β (Shimazu *et al.*, 1999). More recently, TLR11 has been shown to dimerise with TLR12 for recognition of profilin (Broz *et al.*, 2013). TLR ligation and dimerisation activates signalling cascades and subsequent production of pro-inflammatory cytokines, interferons, ROS, and proteases. Signalling is dependent on recruitment of adaptor proteins MyD88, MAL, TRIF or TRAM (Figure 1.7), differential recruitment of which is thought to confer some discrimination of the ligand (Lee *et al.*, 2012). The MyD88-dependent pathway is required for all TLRs except TLR3, and signalling constitutes a serine kinase IL-1R-associated kinase (IRAK), TNFR-associated factor 6 (TRAF6), TGFβ-activated kinase 1 (TAK-1) sequence followed by activation of nuclear factor (NF)κB and activator protein 1 (AP-1) transcription factors via the IKK and MAPK pathways, respectively (Akira *et al.*, 2006). Translocation of NFκB to the nucleus results in expression of proinflammatory cytokines such as TNF, IL-1β and IL-6. The MyD88-independent signalling pathway is activated with TLR3 ligation, and is also an alternative pathway for TLR7, TLR8, TLR9, in addition to endocytosed TLR4, with recruitment of TRIF and subsequent type II interferon (IFN) production via translocation of nuclear factors IRF3 and 7 to the nucleus.

1.6.1.3. Expression: Immune cells, non-immune cells, vasculature, disease

Immune Cells TLRs are widely accepted to be present on immune cells and vast numbers of studies report the presence of TLR message in leukocytes. Despite all TLRs being present on specific leukocyte subsets, most widely investigated are TLR2 and TLR4, therefore from

hereon, only these will be discussed. High TLR mRNA is present in human monocytes, particularly TLR2, followed by TLR4, whilst T cells, B cells, and NK cells have low mRNA levels (Muzio *et al.*, 2000; Hornung *et al.*, 2002; Zarember *et al.*, 2002). Human neutrophils and mast cells also express TLR2 and TLR4 mRNA (Muzio *et al.*, 2000; Supajatura *et al.*, 2001). Human

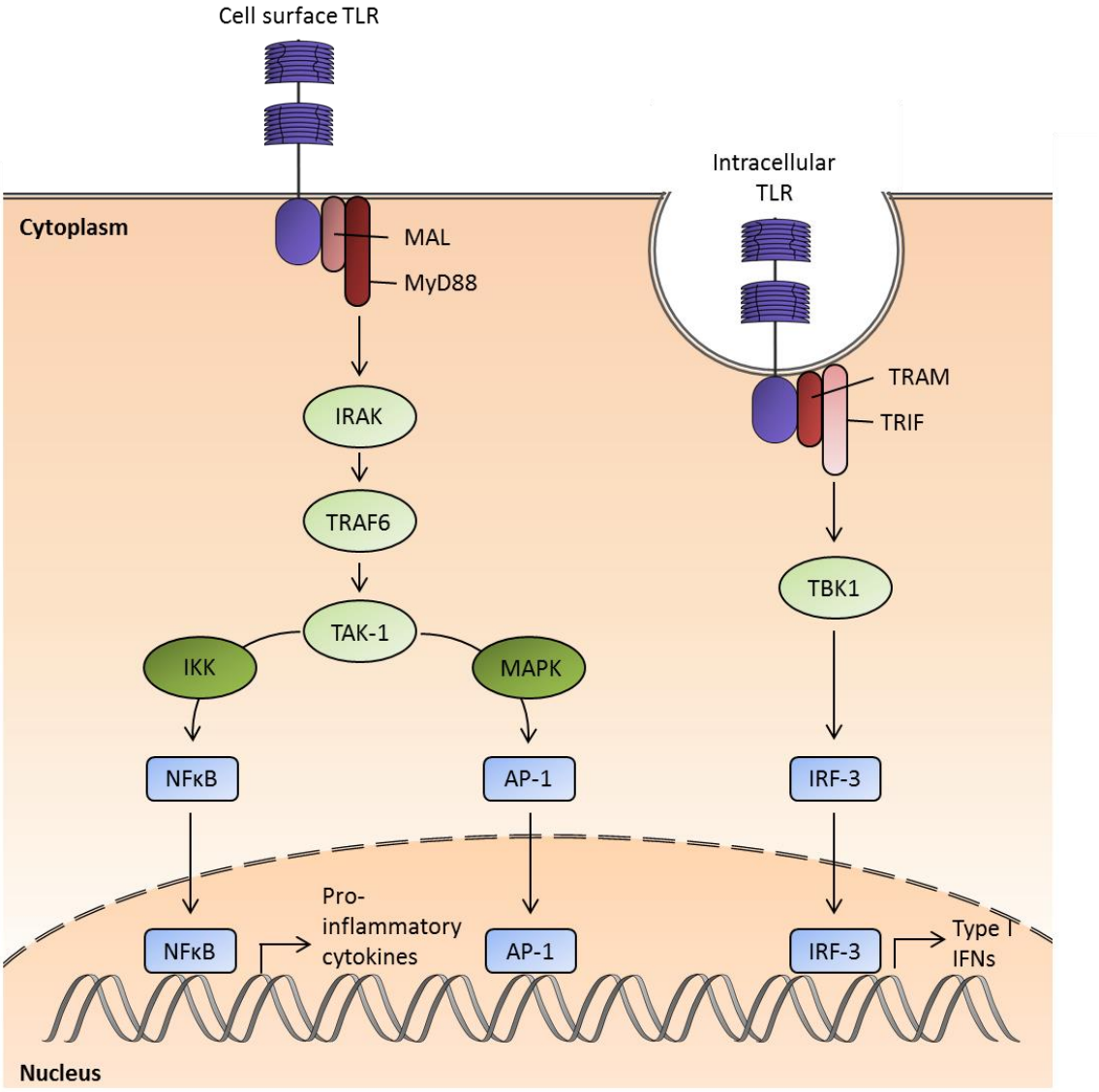


Figure 1.7. TLR signalling pathways. Ligation of TLRs leads to recruitment of adaptor proteins MAL and MyD88, or TRAM and TRIF. The MyD88-dependent pathway involves an IRAK- TRAF6-TAK-1 cascade with subsequent activation and translocation to the nucleus of NFκB via IKK, and AP-1 via MAPK pathways. Translocation results in inflammatory gene expression. MyD88-independent/ TRIF-dependent signalling results in IRF-3 translocation to the nucleus via TBK1 activation and type I interferon production. IRAK, IL-1R-associated kinase; TRAF, TNFR-associated factor; TAK1, TGFβ-activated kinase; IKK, inhibitor kB kinase; MAPK, mitogen active protein kinase; NFκB, nuclear factor kB; AP-1, activator protein 1; TRAM, TRIF-related adaptor molecule; TRIF, TIR-domain-containing adaptor protein-inducing IFN-β; IRF, interferon regulatory factor. (Figure adapted from Akira *et al*., 2006).

blood DCs express various TLRs depending on the subset, however only myeloid DCs express TLR2 and TLR4 (Kadowaki *et al.*, 2001). TLR protein has also been demonstrated on leukocytes by flow cytometry. Monocytes and macrophages are reported to express high levels of TLR2 and TLR4 protein on their cell surface, compared to low levels on neutrophils, and no expression on eosinophils (Sabroe *et al.*, 2002; Henning *et al.*, 2008). A handful of studies confirm leukocyte TLR expression by immunofluorescence microscopy. Both flow cytometry and immunohistochemistry were used to show RAW264.7 macrophages express TLR4 (Jiang *et al.*, 2005), whilst human alveolar macrophages were shown to express TLR4 by confocal microscopy (Thorley *et al.*, 2011), and monocyte-like THP-1 cells express TLR2 also by immunofluorescence (Faure *et al.*, 2000). Neutrophil TLRs have yet to be demonstrated by immunofluorescence, and unlike PCR and flow cytometry data, the extent of immune cell imaging of TLR2 and TLR4 is very limited. Moreover, despite the vast amount of literature into immune cell TLR expression, a comprehensive study into the expression of protein on leukocytes subsets has yet to be carried out.

Non-Immune Cells In comparison to leukocytes, little is known about expression of TLRs on non-immune cells. TLR mRNA in healthy human tissues was comprehensively investigated in one study, demonstrating out of 15 different tissues all express at least one isoform, with the spleen expressing all 10 (Zarember *et al.*, 2002). Other studies focus on a specific tissue or cell type, for example functional TLR4 is reportedly expressed intracellularly in the Golgi of the m-IC_{c12} mouse epithelial cell line (Hornef *et al.*, 2002). Similarly, human pulmonary epithelial cells exhibit intracellular, LPS-responsive TLR4, although LPS concentrations required for activation (i.e. inflammatory cytokine secretion) (0.1-1µg/ml) do not compare to phagocytic leukocytes (1-10ng/ml) (Guillot *et al.*, 2004). TLR4 is also expressed on epithelial cells of the lung. Both mRNA and protein has been shown to be present in human alveolar (A549) and tracheobronchial (BEAS-2B and 16-HBE) epithelial cell lines. Alongside the aforementioned cell types, gastric epithelial cells, intestinal enteroendocrine cells, cystic fibrosis bronchial epithelial cells, tubular kidney cells, and lymphatic ECs have been shown to express TLR4 (Schmausser *et al.*, 2004; Palazzo *et al.*, 2007; Sawa *et al.*, 2008; John *et al.*, 2010; Chen *et al.*, 2011). TLR2 is also reportedly expressed on non-immune cells including isolated small airway epithelial cells, astrocytes, hepatocytes and cultured keratinocytes (Mempel *et al.*, 2003; Ritter *et al.*, 2005; Visvanathan *et al.*, 2007; Stridh *et al.*, 2011). These lists suggest examples of the diverse localisation and the cell types that TLR2 and TLR4 protein have been identified in, however this is in no way comprehensive, and in many cases, highly disputed.

Vasculature There remains much debate as to the expression profile of TLRs in the vasculature. As yet, there is little evidence for TLR2 or TLR4 protein on ECs or associated vascular structures like pericytes or basement membrane. PCR and Western blot revealed no TLR4 was present in unstimulated human umbilical vein ECs (HUVECs) and human aortic ECs (HAECs), however both mRNA and protein were induced when treated with LPS (Wang *et al.*, 2011). Another study further shows TLR4 protein by immunoblotting in HAECs and human dermal microvascular ECs (Lu *et al.*, 2012). A more comprehensive study reported TLR1-9 mRNA expression in primary human vascular cells, showing TLR4 was expressed in HUVECs, HAECs, human coronary artery ECs (HCAECs) and human arterial smooth muscle cells (HASMCs), whilst TLR2 was only expressed in HCAECs (Erridge *et al.*, 2008). Amongst the only studies to demonstrate TLR4 on ECs directly by fluorescence microscopy showed LPS-responsive TLR4, but not TLR2, to be located intracellularly in HCAECs (Dunzendorfer *et al.*, 2004). Likewise, it has been shown by en face confocal microscopy of atherosclerosis-prone Ldlr-/- mouse aortas that TLR2 expression co-localised with ECs, specifically those in regions of disturbed blood flow (Mullick *et al.*, 2008). This aligns with previous reports of reduced TLR2 expression in ECs subjected to chronic laminar flow (Dunzendorfer *et al.*, 2004).

Functional data provides the majority of evidence for the expression of TLRs in the vasculature. Leukocyte transmigration was significantly reduced in TLR2-/- mice subjected to cremaster ischaemia-reperfusion injury. In addition, post-ischaemic vascular leakage was significantly attenuated in both the TLR2-/- and TLR4 mutant mice in the same model indicating a role for TLR2 and TLR4 in post-ischemic EC integrity, and for TLR2 in recruitment (Khandoga *et al.*, 2009). Despite this, adhesion, motility and polarisation of TLR2-/- and TLR4 mutant migrating leukocytes displayed no difference to wild-type mice. TLR4 has however been shown to be involved in maintaining endothelial function as TLR4-/- mice reportedly have reduced endothelium-dependent dilator responses in mesenteric resistance arteries, despite a normal appearance (Harrington *et al.*, 2007). A major factor contributing to sepsis-related death is neutrophil accumulation in the lung leading to pulmonary failure. Using chimeric mice in a systemic LPS model of sepsis, it was shown that endothelium TLR4-/- mice show largely attenuated neutrophil sequestration in the lung compared that of leukocyte TLR4-/- mice, suggesting the importance of endothelial TLR4 (Andonegui *et al.*, 2003). Evidence of TLR4 expression in the endothelium further stems from in vitro studies of response to PAMPs and DAMPs, with cultured human lung microvascular ECs producing IL-8 on treatment with LPS (Gatheral *et al.*, 2012), and cultured human pulmonary artery EC migration inhibited by HMGB1, however not when treated with siRNA to TLR4 (Bauer *et al.*, 2013)

Therefore, despite mounting functional evidence for TLR2 and TLR4 expression in the vasculature, few groups have produced robust studies displaying the expression of TLR protein associated with vascular structures such as ECs or pericytes, and no studies show a detailed analysis of TLR expression and localisation in different kinds of blood vessels, information which would be highly beneficial when studying vascular diseases.

Disease Many of the pathophysiological processes involved in cardiovascular, autoimmune, and other inflammatory conditions involve TLR activation, and there is increasing evidence for modulation of TLR expression in such diseases. Many TLR isoforms have been implicated in a wide variety of diseases however an extensive list of these is beyond the scope of this thesis (for a more detailed review see (Cook *et al.*, 2004)), therefore focus will be kept to TLR2 and TLR4. Hypoxia is a characteristic of multiple cardiovascular diseases (CVD) such as MI and stroke, and is a known modulator of TLR expression. RAW264.7 macrophages upregulate TLR4 message and protein in response to <1% O_2, and this occurs via hypoxia-inducible factor 1 (HIF-1) (Kim *et al.*, 2010). In patients with acute MI, there are multiple reports of modulation of TLR expression, with one study showing leukocyte TLR4 mRNA and protein expression is increased versus healthy controls, and another exploring further to specifically show elevated monocyte TLR4 (Ishikawa *et al.*, 2008; Kashiwagi *et al.*, 2012). PBMCs from patients with both unstable and stable angina had higher TLR4 than those from healthy controls, whilst TLR2 was highest in those with unstable angina (Ashida *et al.*, 2005). In patients undergoing cardiopulmonary bypass, blood monocyte TLR2 and TLR4 was significantly elevated at 20h post-surgery in comparison to pre-surgery (Hadley *et al.*, 2007). Furthermore, both monocytes and neutrophils from patients with sepsis exhibited significantly more TLR2 and TLR4 than those from healthy controls (Härter *et al.*, 2004). Contrary to the majority of studies detailing upregulation of TLRs in disease, patients with acute anterior uveitis, a common but dangerous intraocular inflammatory condition, had reduced monocyte and neutrophil TLR2 protein (Chang *et al.*, 2007).

Much less is known however regarding regulation of TLR expression on non-immune cells. TLR2 and TLR4 are present in the tubules, loop of Henle and collecting ducts and expression is upregulated in all areas in a mouse model of renal I/R injury (Wolfs *et al.*, 2002). Similarly, in a study of human kidney transplants, TLR4 expression was significantly higher on organs from deceased donors versus living donors. Elevated TLR4 correlated with increased TNF, CCL2, haem oxygenase 1, and lower rate of immediate graft function (Kruger *et al.*, 2009). TLR2 expression is also elevated on aortic ECs in areas of disturbed blood flow in Ldlr-/- atherosclerosis-prone mice fed a high fat diet versus chow (Mullick *et al.*, 2008), however

whether human aorta exhibit such a phenotype is still unknown, likely due to difficulties in obtaining tissue samples. TLR2 and TLR4 have also been shown to traffic in response to LPS or peptidoglycan from the apical membrane to the cytoplasmic compartments of intestinal epithelium, indicating that TLRs can be regulated both in terms of expression and subcellular localisation (Cario *et al.*, 2002).

TLRs are also considered to play a role orchestrating the tumour microenvironment via regulation of both pro- and anti-tumorigenic agents. The inflammatory and hypoxic nature of the tumour microenvironment produces DAMPs that act both on immune cell and tumour cell TLRs to induce recruitment of immunosuppressive cells such as T_{Regs}, T_H17, and myeloid derived suppressor cells (for a detailed review see (Ridnour *et al.*, 2013)). Multiple studies have both directly and indirectly linked alteration in TLR expression to tumour outcome. Directly, TLR4 was overexpressed on tumours from 67% of oesophageal, and 69% of pancreatic cancer patients (Ridnour *et al.*, 2013), whilst TLR4 was also upregulated on tumour-infiltrating monocytes from patients. Similarly, knockdown of TLR2 reduced metastases in a mouse pulmonary tumour model (Yang *et al.*, 2009). Downstream of receptor upregulation, consequent enhanced TLR signalling is reported to increase NOS2 and COX2 production with subsequent IL-10, TGFβ and VEGF that further promote tumour progression via suppression of immune cell action and consequently chemoresistance.

1.7. Leukocyte TLRs and the deleterious effects of inflammation

Activation of TLR signalling, proinflammatory mediators and adhesion molecule upregulation, and subsequent infiltration of leukocytes into the tissue is essential for clearance of pathogens, however if this process becomes aberrant, damage to the host can result. Although TLRs are considered to be present on both immune and non-immune cells, several lines of evidence suggest leukocyte TLRs are the main contributor in the pathogenesis of vascular diseases. Atherosclerosis is one such disease, characterised by accumulation of lipids leading to thickening of the arterial wall, a process largely macrophage-mediated. Knockout of TLR4 and to a lesser degree TLR2 was protective in early intimal foam cell accumulation and chemokine (CCL2) production in the aorta of ApoE-/- atherosclerosis-prone mice (Higashimori *et al.*, 2011). In another model of atherosclerosis, Ldlr-/- mice on a high fat diet demonstrated reduced aortic lesions when also deficient in TLR2 (Mullick *et al.*, 2005). Whether deletion of leukocyte or tissue TLR provided protection against atherosclerosis is unknown, however many would postulate leukocytes as the main culprits in pathogenic inflammation associated with such diseases. Indeed, a distinct TLR expression profile is observed in diseased human arteries,

fundamentally due to population of the tissue with TLR-expressing immune cells (i.e. DCs), providing additional fire for such inflammatory vascular conditions (Pryshchep *et al.*, 2008).

Ischaemia-reperfusion injury is a common problem with solid organ transplantation and is the major event in trauma, stroke, septic shock and MI (Arslan *et al.*, 2010a). Ischemia deprives cells of oxygen, therefore exposes them to periods of hypoxia, causing accumulation of metabolic products. However, when blood flow is restored, the tissue is reperfused and a series of inflammatory events occur including complement deposition, adhesion molecule upregulation, and inflammatory cell accumulation. This can cause substantial damage to the vasculature and surrounding tissue, predominantly via proinflammatory cytokine, ROS, and chemokine release from infiltrated leukocytes. TLR2 and TLR4 are being increasingly linked to the detrimental inflammatory effects of these conditions, and their involvement has been reported in I/R injury in the kidney, liver, brain, heart, and intestine (Zhai *et al.*, 2004; Arslan *et al.*, 2010b; Hyakkoku *et al.*, 2010; Rusai *et al.*, 2010; Victoni *et al.*, 2010). Beneficial effects of TLR-blocking antibodies prior to ischaemia provide further evidence to cement this theory.

Whether TLRs are specifically involved in facilitating leukocyte transmigration any more so than simply sensing DAMPs and PAMPs and activating immune cells in not known. The process of leukocyte recruitment is significantly attenuated in TLR2 knockout mice subjected to I/R injury, but no effect on adhesion was observed (Khandoga *et al.*, 2009). Decreased permeability was observed in both TLR2 and TLR4 knockouts, indicating a potential role in regulation of junctional molecules. This area of TLR function is little explored however a handful of studies investigate the link, for example showing TLR3 agonist poly(I:C), but not other TLR ligands, reduces JAM-A expression in nasal epithelial cells (Ohkuni *et al.*, 2011), whilst TLR2 activation induces calcium-dependent proteases that cleave E-cadherin and occludin in airway epithelial cells (Chun *et al.*, 2009).

1.8. TLRs as therapeutic targets

Based on the fundamental role TLRs play in the innate immune response, plus evidence for modulation of their expression in disease, there have been numerous therapeutics designed to target these receptors. Much research is being carried out on TLRs as therapeutic targets, yet to date only one compound is in the clinic: Imiquimod is a TLR7 agonist used to treat papillomavirus infection (Hennessy *et al.*, 2010). A large number of other compounds are however in clinical trials each targeting various members of the TLR family (for detailed list see review (Connolly *et al.*, 2012)), however the majority target the intracellular TLRs and are indicated primarily for viral infections and as adjuvants and therefore will not be discussed in

this thesis. Few successful compounds have emerged targeting the surface TLRs. One such example is TLR4 antagonist Eritoran, a synthetic lipodisaccharide that has proved beneficial in animal models of inflammation. Mice treated with Eritoran were protected at least partially from lethal influenza challenge (Shirey *et al.*, 2013), endotoxemia (Ehrentraut *et al.*, 2011b) and cardiac hypertrophy after organ injury (Ehrentraut *et al.*, 2011a). Eritoran blockade of TLR4 improved the course of rat kidney I/R injury (Liu *et al.*, 2010). Based on success in rodents, Eritoran also proved beneficial in Phase II trials, showing a trend in two trials of lower mortality in severely septic patients, and in a trial of patients with sepsis, shock or respiratory failure (Tidswell *et al.*, 2010; Opal *et al.*, 2013). Neither trial however showed significance in mortality rate or in any suppression of the cytokine storm and the compound has therefore been unable to progress through to phase III trials. Other TLR4-targetting therapeutics includes AV411, a TLR4 antagonist in Phase II trials for chronic pain and withdrawal, and NI0101, a TLR4 antagonistic biologic in the preclinical phase indicated for acute and chronic inflammation (Connolly *et al.*, 2012).

Data from animal studies using anti-human/mouse TLR2 antibodies demonstrated the protection achieved from TLR2 blockade. OPN-301 treated mice have a functional graft and preserved renal function and structure in a model of renal transplantation I/R injury (Farrar *et al.*, 2012). In addition, antagonising TLR2 with OPN-301 only 5 minutes before reperfusion decreases infarct size and preserves cardiac structure and function in a murine model of cardiac I/R injury (Arslan *et al.*, 2010b). The cardiac protection obtained was attributed to reduced leukocyte infiltration, and therefore reduced associated cytokine production and proapoptotic signalling. This strong evidence linking TLR2 as detrimental in I/R injuries led to the development of a fully humanised monoclonal anti-TLR2 antibody, OPN-305, that blocks both TLR2/1 and TLR2/6 signalling. In a porcine myocardial I/R model, OPN-305 given during the period of ischemia reduced infarct size by up to 50% at the highest dose, and significantly preserved cardiac function (Arslan *et al.*, 2012). Promising results from both rodent and porcine studies led to movement into Phase I trials that showed infusion of OPN-305 was well tolerated in healthy subjects with no adverse effects (Reilly *et al.*, 2013). Phase II clinical trials are therefore due to commence imminently in renal transplant patients at high risk of delayed graft function.

1.9. Sex-differences

1.9.1. Disease

The prevalence of numerous autoimmune diseases (Sjogren's syndrome, systemic lupus erythematosus (SLE), autoimmune thyroid diseases and scleroderma) is remarkably sex-biased with female patients accounting for over 80% of disease presentation (Whitacre, 2001). More common autoimmune diseases such as multiple sclerosis (MS) and RA exhibit a 3:1 female: male ratio. Despite sex-bias in disease incidence, the severity of human autoimmune conditions is considered similar between males and females (Lockshin, 2006). Conversely, innate immune conditions have higher incidence in aged-matched men. After trauma, male patients show higher frequency of pneumonia and multi organ failure (Kher *et al.*, 2005). Males also demonstrate around 25% higher incidence of sepsis than females, however whilst men are more likely to gain bacterial infections, the mortality outcomes among sepsis patients are highly debated. In multiple studies including the largest cohort to date, mortality rates were not different between male and female sepsis patients (Angus *et al.*, 2001; Martin *et al.*, 2003b; Berkowitz *et al.*, 2007). Yet in other reports of severe septic shock, male patients had far greater rates of mortality after hospitalisation versus matched females (Schroder *et al.*, 1998; Adrie *et al.*, 2007). It therefore continues to remain unclear whether sex underlies mortality outcome of sepsis patients. Sex is also shown to affect innate disease severity: of patients with severe sepsis, a lower risk of hospital mortality is observed in postmenopausal women than age-matched men (Adrie *et al.*, 2007). Furthermore, male MI patients show poor life expectancy pre-hospitalisation but equal survival rates after 1 month suggesting women show early, but not late, protection (Kher *et al.*, 2005).

Premenopausal women are considered at less risk of CVD than aged-matched men. Women develop CVD 7-10 years later than males (Maas *et al.*, 2010) and demonstrate better cardiac contractility and wall thickness compared to disease-matched men (Leinwand, 2003). 39% more males than females die from heart disease between the ages of 45-64 whilst after 65 women outnumber men (Leinwand, 2003). Females continue to have a lower death rate due to CVD, coronary heart disease (CHD), stroke and heart failure when adjusted for age than males, and at the age of 40 have a 1 in 2 remaining lifetime risk versus 2 in 3 for males (Mosca *et al.*, 2011). In 2007, the total age-adjusted CVD death rate was nearly 50% greater in males than females. Prevalence of atherosclerotic lesions and associated risk factors were moreover consistently lower in females independent of age, despite a rise post-menopause (Kroger *et al.*, 1999). Sex-bias in CVD have frequently been attributed to variances in risk factors including

lower blood pressure, acute MI, hypercholesterolaemia and atherosclerosis, however it is also thought disease presentation may vary between males and females (Reckelhoff, 2001; Maas *et al.*, 2010).

The protection that women appear to demonstrate in the context of innate immune conditions is not constrained to sepsis and CVD, these purely provide examples and others are demonstrated in Table 1.3. In humans this protection is commonly contested, nevertheless the majority of studies are suggestive towards female protection in acute injury. Further investigation using animal studies is clearer, with female animals consistently demonstrating better outcomes than aged-matched males in inflammatory models (Kher *et al.*, 2005). Male mice for example exhibit poorer survival rates after LPS induced sepsis as well as that induced by cecal ligation, puncture and trauma-haemorrhage (Marriott *et al.*, 2006a). Clear differences in both the incidence and severance of human and animal immune disease therefore suggest an influence of inherent differences most likely stemming from both sex hormones and X chromosome genes. The effects of other factors such as the environmental, socio-economic status and race are also however likely to have a role in shaping the responses of males and females in inflammation.

More prevalent in males	More prevalent in females
Coronary heart disease	Sjogren syndrome
Stroke	SLE
Sepsis	Thyroid disease
Trauma	Dementia
Heart failure	Myasthenia gravis
Atherosclerosis	Rheumatoid arthritis
Bacterial infection	Multiple Sclerosis

Table 1.3. Differences in disease prevalence in males and females.

1.9.2. Sex-differences in the inflammatory response

Sexual dimorphism in the severity and incidence of diseases are likely to stem, at least in part, from inherent differences in the immune system and the inflammatory response. In humans, females have more $CD4^+$ T cells (Amadori *et al.*, 1995), and are shown to have more circulating IgM and IgG than aged-matched men although the latter is debated (Bouman *et al.*, 2005). Similarly, in mice and rats more tissue-resident leukocytes, namely macrophages, T- and B-

lymphocytes, are present in the peritoneal and pleural cavities of females (Scotland *et al.*, 2011). In addition to being more numerous, resident macrophages of the mouse female peritoneum exhibited higher surface TLR2 and TLR4, and had greater NADPH oxidase and phagocytosis activity when stimulated compared to male macrophages (Scotland *et al.*, 2011). Male neutrophils have also been shown to express more TLR4 under basal conditions than female equivalents, a difference exaggerated during IFNγ stimulation (Aomatsu *et al.*, 2013). Differences in PPRs have been linked both to oestrogen increasing- and testosterone reducing-expression (D'Agostino *et al.*, 1999; Scotland *et al.*, 2011).

In addition to inherent differences in the cellular make-up and phenotype of the immune system, sex-differences have also been described with respect to cytokines. In a study of X-linked genes in healthy adults, males were found to have more monocytes and greater TNF production than females (Lefevre *et al.*, 2012). Indeed, of genes present on the human X chromosome, over 60 have the potential to directly influence immunity including immune receptors, immune-response related proteins, and transcriptional and translational effectors (Fish, 2008). Female sepsis patients produced higher immunosuppressive IL-10 and lower pro-inflammatory TNF levels than males (Schroder *et al.*, 1998), whilst hearts from female rats had reduced expression of TNF, IL-1β, and IL-6 after acute I/R compared to males (Wang *et al.*, 2005). The translations of such observations were lower mortality and improved post-ischaemic myocardial functions, respectively. Furthermore, despite no difference in severity of the condition, male trauma patients had higher plasma IL-1β than females following haemorrhage (Ertan *et al.*, 2007). In vitro studies mimic such in vivo observations as post LPS challenge adult male peripheral immune cells produce higher levels of TNF whilst similarly treated male rat neutrophils produce more CXCL1 than matched females (Marriott *et al.*, 2006a; Aomatsu *et al.*, 2013). Likewise, isolated macrophages from male mice are shown to produce more IL-1β and IL-6, and lower amounts of immunosuppressive prostaglandin E_2 following LPS challenge than those from females, further suggestive of reduced pro-inflammatory responses coupled with stronger immunosuppressive responses in females (Kahlke *et al.*, 2000; Marriott *et al.*, 2006b; Marriott *et al.*, 2006a). Reports of lower cytokines are however mixed as low dose LPS induced a greater proinflammatory response in the form of TNF and IFNγ in female versus male volunteers (van Eijk *et al.*, 2007). Females were also shown to produce more neutrophil attracting IL-8 per cell, possibly explaining the higher neutrophil count in girls with acute inflammatory disorders.

Sexual dimorphisms in the immune system do not end at initiation of the inflammatory response and production of cytokines, but continue to chemokine production and importantly,

leukocyte recruitment. In a model of hepatic I/R, female mice had rapid accumulation of serum CXCL1, CXCL2, and IL-6, with male responses generally peaking later, and being slightly greater in TNF production versus females (Crockett *et al.*, 2006). In addition, male rats exhibited significantly more CC chemokines (CCL3, CCL5, CCL24 and CCL28), and more neutrophil accumulation than females during the development of abdominal aortic aneurysms (Hannawa *et al.*, 2006). The presented examples of greater chemokine production in males is not always the case however, as macrophages produced equal levels of CCL2 upon stimulation with LPS, despite male derived cells producing more CXCL10 than those derived from females (Marriott *et al.*, 2006b). It may be fair to speculate that sex-difference in cytokine and chemokine responses connect the earlier addressed topics of the role of aberrant leukocyte recruitment and sex-differences in disease.

1.9.3. Sex hormones

Sex-hormones including progestins, androgens and oestrogens are capable of mediating many aspects of the immune response (Pennell *et al.*, 2012). Oestrogens are primarily produced in the ovaries and exert their effects via activation of intracellular nuclear oestrogen receptors (ERs) ERα and ERβ (Garcia-Gomez *et al.*, 2013). High expression of ERs in the female reproductive tract is indicative of their role in reproduction and development, however ERs are also expressed in immune cells, including neutrophils, DCs, macrophages, and lymphocytes, suggestive of a further role of sex hormones in regulation of the inflammatory response (Fish, 2008). Although no comprehensive studies of sex-differences in leukocyte hormone receptor expression exist, ER expression in human granulocytes is reported to be the same in men and women (Wolf *et al.*, 2012). In addition to higher $CD4^+$ T cells numbers in females, effects of 17β-oestradiol on leukocyte populations are exemplified with the increase in T_{Reg} cell number during the follicular phase of the menstrual cycle, the phase whereby oestrogen levels are highest (Arruvito *et al.*, 2007). Alteration of T_{Regs} consequentially effects the regulation of the peripheral T cell pool, immune response, and immune suppression. In addition to its effects on T_{Regs}, 17β-oestradiol increases neutrophil anti-inflammatory nitric oxide (NO) production whilst reducing chemotactic activity, and reduces IL-1β, IL-6, IL-8 and TNF production by monocytes (Kramer *et al.*, 2004; Pioli *et al.*, 2007; Fish, 2008). Effects on other leukocyte populations include reduced NK cell cytotoxicity, decreased T cell CCL1 and CCL5 production, and increased differentiation and cytokine production from DCs (Fish, 2008). Adhesion molecules are also affected as 17β-oestradiol inhibits IL-1-mediated EC E-selectin and VCAM-1 induction (Caulin-Glaser *et al.*, 1996), and LPS-mediated ICAM-1 and VCAM-1 expression (Simoncini *et al.*, 2000;

Thor *et al.*, 2010). Such changes thereby influence activation of leukocytes and ECs themselves, in addition to numbers of circulating and recruited cells. For example, numbers of both monocytes and neutrophils in the circulation during different phases of the oestrous cycle are altered, suggestive of alterations in mobilisation and recruitment mechanisms (Pennell *et al.*, 2012).

ERs have been described to influence the phenotype and activity of leukocytes, and variations in ER expression have been observed in SLE and RA patients (Pennell *et al.*, 2012). The greater resistance to CVD in pre-menopausal women compared to men is commonly attributed to 17β-oestradiol, the principle oestrogen during the female reproductive years. Challenging this hypothesis however, is the finding in one study that oestrogen replacement in postmenopausal women increased heart disease (Leinwand, 2003). Similarly, females are considered at lower risk of sepsis than males, yet experimental sepsis severity was found to be increased with 17β-oestradiol treatment (Rettew *et al.*, 2009). Despite the association of oestrogen in the protection that females are considered to have in CVD and sepsis, 17β-oestradiol reportedly enhances production of proinflammatory cytokines (IL-1, IL-6 and TNF) whilst inhibiting immune suppressive cytokines (IL-4, IL-10 and TGFβ) (Garcia-Gomez *et al.*, 2013). Furthermore, in an experimental autoimmune encephalomyelitis (EAE) mouse model of MS, treatment with either 17β-oestradiol or ERα ligands provided both anti-inflammatory and delayed EAE onset effects (Tiwari-Woodruff *et al.*, 2007). Therefore although females are generally accepted to be more resistant to infection and CVD, and more prone to autoimmune diseases, it is unclear how exactly sex hormones play a role in this.

Testosterone is part of the androgen family and is present at around 7 times greater levels in males than females. It is produced predominantly in the testes and acts via the androgen receptor (AR), another nuclear receptor with transcription factor properties similar to ERs (Garcia-Gomez *et al.*, 2013). The role of testosterone, like oestrogens, is considered to extend beyond reproduction and development into immune regulation. Testosterone reportedly reduces TNF whilst increasing IL-10 synthesis in murine macrophages, shifting towards an anti-inflammatory response (D'Agostino *et al.*, 1999), whilst its metabolites have similarly been shown to decrease cytokine and immunoglobulin production by lymphocytes (Bouman *et al.*, 2005). Testosterone treatment also demonstrated potential protective effects in men with MS, in line with males being more protected in autoimmune conditions (Sicotte *et al.*, 2007). ARs are expressed on lymphocytes but not macrophages, unlike ERs, suggesting a more dominant role in the adaptive rather than innate immune response (Marriott *et al.*, 2006a). The numerous reports of the influence of 17β-oestradiol and testosterone on leukocyte responses

therefore demonstrate the important role sex hormones play, either directly or indirectly, on the inflammatory response. Klein fully reviews both agreeing and conflicting reports of the effects of sex hormones (Klein, 2004), nevertheless it is possible that libraries of contradictory results are the consequence of the diverse function and sites of action of oestrogens, which in addition alter with age and disease.

Pattern recognition is also influenced by sex hormones as demonstrated by reduced murine macrophage TLR4 expression after endogenous oestrogen removal by ovariectomy (Rettew *et al.*, 2008; Rettew *et al.*, 2009). Removal of oestrogens also decreased levels of circulating PRR accessory molecules namely LBP levels, which was rescued alongside TLR4 after 17β-oestradiol administration (Rettew *et al.*, 2009). Similarly, in response to LPS, male mice had increased LBP levels compared to females (Marriott *et al.*, 2006b). In the same study, male-derived macrophages expressed greater levels of TLR4 both pre- and post-LPS stimulation compared to those derived from females. Conversely, testosterone is shown to reduce macrophage TLR4 (Rettew *et al.*, 2008). Expression of ERs on immune cells, influence on pattern recognition receptor expression, and regulation of EC adhesion molecules are all suggestive of a role for oestrogens in leukocyte recruitment in inflammation. Reports of sex hormones regulating the immune response are not however limited to oestrogens. The influences of progesterone, testosterone and prolactin have also been reported, however mainly with reference to T cell responses and adaptive immunity.

1.9.4. Anti-inflammatory treatment

Sexual dimorphism is not constrained to the inflammatory response and disease. More recently, questions have been raised with regard to anti-inflammatory drug dynamics. Due to inherent difference between males and females including metabolism, body composition, cardiac output and tidal volume, both pharmacokinetic and pharmacodynamic differences exist in response to drug treatment (Soldin *et al.*, 2011). Some indications however suggest there are sex differences over and above this, and specifically in responses anti-inflammatory treatments due to the described differences in the immune systems and inflammatory responses. For example, statins reduced the risk of mortality and stroke in males, however lack of efficacy in females meant no such protection was observed (Dale *et al.*, 2007). Similarly, low dose aspirin reduced the risk of first MI in males but not in females, whilst contrastingly lowering the risk of stroke in females but not in males (Ridker *et al.*, 2005). This evidence again highlights the distinct differences in the male and female response, something that is consistently overlooked both in basic research with male animals most commonly used, and in

drug development whereby females are vastly underrepresented in clinical trials. It is imperative that sex-differences are fully understood and properly considered in future approaches to targeting the immune system in the treatment of inflammatory diseases both in terms of drug development and study design.

1.10. Aims

The incidence and severity of inflammatory conditions that are characterised by excessive leukocyte recruitment are more prominent in males. However at present little is known about the mechanisms behind this difference. It was hypothesised that male mice recruit more leukocytes to the site of inflammation due to sex-differences in the expression and regulation of leukocyte TLRs in acute inflammation. The overall aim of this study was therefore to comprehensively investigate leukocyte subset trafficking in male and female mice and evaluate a possible role for differential TLR expression and modulation in this dimorphism. Therefore, the specific aims were to:

Characterise sex-differences in the numbers of murine leukocyte subsets and their TLR2 and TLR4 expression.

Female mice are reported to have greater numbers of peritoneal and pleural resident macrophages. Furthermore, peritoneal macrophages were also higher in TLR2 and TLR4 expression in female versus male mice. To date no study has characterised such sex-differences further. Therefore, leukocyte subsets from various leukocyte-containing pools were compared in naïve male and female mice for dimorphisms in their number and TLR2 and TLR4 expression.

Investigate sex-differences in leukocyte subset trafficking in the murine zymosan peritonitis model of acute inflammation.

To elucidate the extent of differential inflammatory responses in males and females *in vivo*, trafficking responses of leukocyte subsets were investigated using the well-established murine zymosan peritonitis model of acute inflammation. Numbers of leukocyte subsets in the circulation, peritoneum, BM and spleen were investigated throughout the time course of the inflammation.

Study the influence of inflammation on leukocyte TLR2 and TLR4 expression and examine the potential of temporal receptor modulation to influence differential leukocyte recruitment in male and female mice.

The expression of TLR2 and TLR4 are differentially expressed on male and female peritoneal macrophages and modulated in certain inflammatory diseases. In addition, therapies targeting both receptors are currently in clinical trials for treatment of inflammatory conditions. Whether sex-differences in the expression of TLR2 or TLR4 in inflammation influence dimorphic leukocyte recruitment responses is unclear, therefore a temporal expression profile of both receptors on the different leukocyte subsets in the zymosan peritonitis model of acute inflammation will be carried out.

Using this experimental approach this project will also elucidate new information relating to sex-differences in the way in which leukocytes are stored and mobilised in males and females.

Chapter 2: Materials & Methods

2.1. Animals

All animal procedures were subject to Queen Mary ethical approval (Animal Welfare Ethical Review Body) and carried out in agreement with the United Kingdom Home Office Animals Scientific Procedures Act 1986 under project licence 70/7884 and personal licence 70/24053. Both wild type (WT) and genetically modified strains used were on a C57BL/6 background. Mice were age-matched (8-12 weeks) and 22-30g (males) and 18-25g (females) in weight. Those from the same litter were equally distributed between experimental groups and male and females were treated at the same time. Mice were housed in individually ventilated cages with a controlled 12h light/dark cycle (lights on 0700-1900h), and constant temperature (21±2°C). Animals were killed humanely by cervical dislocation in accordance with Home Office regulations

2.1.1. Strains

C57BL/6 Male and female WT C57BL/6 mice were purchased from Charles River Laboratories (Margate, UK)

$CX_3CR1^{+/gfp}$ Mice heterozygote for targeted replacement of the CX_3CR1 fractalkine gene with cDNA encoding eGFP ($CX_3CR1^{+/gfp}$) (Jung *et al.*, 2000), resulting in the expression of eGFP in all monocytes, and certain natural killer and T cells, were used for certain peritonitis experiments. Mice were bred *in house* by crossing $CX_3CR1^{gfp/gfp}$ homozygotes with WT C57BL/6s resulting in heterozygote $CX_3CR1^{+/gfp}$ offspring. No differences in leukocyte transmigration have been shown in these heterozygotes as compared with WT (Jung *et al.*, 2000), and this was confirmed in this thesis also (Appendix 1).

2.1.2. Anaesthetics

Ketamine	Ketaset® solution, 100mg/ml, Cat #SH4401C, Fort Dodge Animal Health Ltd (Southampton, UK)
Xylazine	Rompun® 2% w/v, 20mg/ml, Cat #79769161, Bayer plc (Berkshire, UK)

In experiments where blood was taken, mice were anaesthetised by intramuscular (i.m.) injection of 1ml/kg anaesthetic (40mg/kg ketamine and 3mg/kg xylazine in saline) in order to preserve the integrity of the carotid vessels after Schedule 1 killing by cervical dislocation.

2.2. Reagents

2.2.1. Antibodies

Primary antibodies:

B220	Rat monoclonal anti-mouse CD45R (B220) PE/Cy7, clone RA3-6B2, 0.2mg/ml, used at 0.25µg/ml, BioLegend (San Diego, USA)
C5aR	Rat monoclonal anti-mouse C5aR (CD88) PE, clone 20/70, 0.2mg/ml, used at 1.0µg/ml, BioLegend (San Diego, USA)
CD3	Rat monoclonal anti-mouse CD3 PerCP/Cy5.5, clone 17A2, 0.2mg/ml, used at 0.67µg/ml, BioLegend (San Diego, USA)
CCR1	Rat monoclonal anti-mouse CCR1 PE, clone 643854, 25µg/ml, used at 1.0µg/ml, R&D Systems (Minneapolis, USA)
CCR2	Rat monoclonal anti-mouse/rat CCR2 PE, clone 475301, 25µg/ml, used at 1.0µg/ml, R&D Systems (Minneapolis, USA)
CCR3	Rat monoclonal anti-mouse CCR3 (CD193) PE, clone J073E5, 0.2mg/ml, used at 0.5µg/ml, BioLegend (San Diego, USA)
CCR5	Hamster monoclonal anti-mouse CD195 PE, clone HM-CCR5, 0.2mg/ml, used at 1.0µg/ml, BioLegend (San Diego, USA)
CD115	Rat monoclonal anti-mouse CD115 (CSF-1R) PE, clone AFS98, 0.2mg/ml, used at 0.67µg/ml, eBioscience (Hatfield, UK)
CXCR2	Rat monoclonal anti-mouse CXCR2 (CD182) Alexa Fluor® 647, clone TG11, 0.5mg/ml, used at 1.0µg/ml, BioLegend (San Diego, USA)
CXCR4	Rat monoclonal anti-mouse CXCR4 (Fusin/CD184) PerCP-eFluor®710, clone 2B11, 0.2mg/ml, used at 1.0µg/ml, eBioscience (Hatfield, UK)
Dectin1	Rat monoclonal anti-mouse Dectin-1 PerCP-eFluor®710, clone bg1fpj, 0.2mg/ml, 2.0µg/ml, eBioscience (Hatfield, UK)

Fc Block™	Purified monoclonal rat anti-mouse CD16/CD32 (FcγIII/II receptor), clone 2.4G2, 0.5mg/ml, used at 5µg/ml, BD Pharmingen (Oxford, UK)
F4/80	Rat monoclonal anti-mouse F4/80 PE/Cy7, Alexa Fluor® 700 or PE, clone BM8, 0.2mg/ml, used at 0.67µg/ml, eBioscience (Hatfield, UK)
Gr1	Rat monoclonal anti-mouse Ly-6G/Ly-6C (Gr1) Alexa Fluor® 647, clone RB6-8C5, 0.2mg/ml, used at 0.1µg/ml, BioLegend (San Diego, USA)
TLR2	Mouse monoclonal anti-human/mouse TLR2 PE, clone T2.5, 0.2mg/ml. Selected for study, used at 1µg/ml, BioLegend (San Diego, USA)
TLR2	Rat monoclonal anti-mouse TLR2 PE, clone 6C2, 0.2mg/ml, eBioscience (Hatfield, UK)
TLR4	Rat monoclonal anti-mouse TLR4/MD2 PE, clone MTS510, 0.2mg/ml. Selected for study, used at 1.0µg/ml, eBioscience (Hatfield, UK)
TLR4	Rat monoclonal anti-mouse TLR4 PE, clone UT41, 0.2mg/ml, eBioscience (Hatfield, UK)
TLR6	Rat monoclonal anti-mouse TLR6 PE, clone 4186-1, 25µg/ml, used at 0.5µg/ml, R&D Systems (Minneapolis, USA)

Isotype controls:

Hamster IgG	Armenian hamster immunoglobulin IgG PE isotype control antibody, clone HTK888, 0.2mg/ml, BioLegend (San Diego, USA)
Mouse IgG1	Mouse immunoglobulin IgG1, κ PE isotype control antibody, clone MOPC-21, 0.2mg/ml, BioLegend (San Diego, USA)
Rat IgG2a	Rat immunoglobulin IgG2a, κ PE or PerCP-eFluor®710 isotype control antibody, 0.2mg/ml, clone eBR2a, eBioscience (Hatfield, UK)
Rat IgG2b	Rat immunoglobulin IgG2b, κ PE isotype control antibody, clone eB149/10H5, 0.2mg/ml, eBioscience (Hatfield, UK)

2.2.2. Fluorescent cell markers

7-AAD	7-aminoactinomycin D, 5mg/ml, Cat#A9400, Sigma (St Louis, USA)
DAPI	4',6-diamidino-2-phenylindole dihydrochloride, 1mg, Cat #D9542, Sigma (St Louis, USA)

2.2.3. Inflammatory stimuli, cytokines, chemoattractants

Zymosan	Zymosan A from *Saccharomyces cerevisiae*, Cat #Z4250, lot #BCBF4506V used at 1mg, Sigma (St Louis, USA)

2.2.4. Kits

Proteome Profiler™ Mouse Chemokine Array	Cat #ARY020, R&D Systems (Minneapolis, USA)
Proteome Profiler™ Mouse Cytokine Array	Panel A, Cat #ARY006, R&D Systems (Minneapolis, USA)
BCA™ Protein Assay Kit	Cat #23227, Thermo Scientific (Rockford, USA)

2.2.5. Buffers

ACK lysis buffer	150mM NH_4Cl, 10mM $KHCO_3$, 0.1mM EDTA, pH 7.3 in H_2O
FACS buffer	PBS supplemented with 1% heat-inactivated goat's serum
Lavage buffer	PBS with 0.25% BSA and 2mM EDTA
Spleen lysis buffer	PBS with 1% triton X-100 and 1% protease and phosphate inhibitor cocktail

2.2.6. Other reagents

Bovine serum albumin (BSA)	Cohn fraction V, 95% standard powder pH7.0, Cat #BPE9701, Fisher Scientific (Leicestershire, UK)
BD™ CompBeads	Anti-Rat Ig, κ / Negative control compensation particles set, Cat #552844, BD Biosciences (San Diego, USA)
EDTA	Ethylenediaminetetraaceticacid disodium salt dehydrate, Cat #E5134, Sigma (St Louis, USA)
Halt™ protease inhibitor cocktail	Halt protease and phosphatase inhibitor cocktail (100X), Cat #78440 (Thermo Scientific, Rockford, USA)
Normal goat serum (NGS)	Goat serum, Cat #G6767, Sigma (St Louis, USA)
Phosphate buffered saline (PBS)	Dulbecco A tablets, 1 tablet dissolved in 100ml distilled water pH7.2, Cat #BR0014G, Oxoid (Hampshire, UK)

Saline	Sodium chloride 0.9%w/v pH5.5, Cat #FKE1323, Baxter healthcare (Norfolk, UK)
Triton™ X-100	Laboratory grade, Cat #X100, Sigma (St Louis, USA)
Trypan blue	0.4% trypan blue solution, Cat #T8154, Sigma (St Louis, USA)

2.3. Characterisation of male and female leukocyte subset numbers and TLR expression

2.3.1. Leukocyte isolation

Leukocytes were isolated from various compartments of male and female mice post-schedule 1 killing by cervical dislocation. Circulating cells were obtained by cardiac puncture using a 27 gauge needle and 1ml syringe filled with 50µl 0.5M EDTA. 0.5-1ml of blood was obtained per mouse. Peritoneal cells were collected by lavage of the peritoneum with 6ml PBS supplemented with 0.25% BSA and 2mM EDTA (lavage buffer) injected with a 27 gauge needle, followed by 10 seconds shaking of the mouse by its tail and subsequent withdrawal of lavage fluid with a 19 gauge needle and 6ml syringe. At least 5.8ml was recovered per mouse. Splenic leukocytes were obtained by organ dissection followed by mechanical dissociation in PBS with 1% goat's serum (FACS-buffer) with the end of a 5ml syringe, through a 40µm cell strainer to obtain a single cell suspension. Bone marrow cells were isolated from the right femur by flushing with 4ml cold FACS-buffer. Cells were passed through a 40µm cell strainer for a single-cell suspension. Blood and spleen samples were centrifuged at 300g for 5mins at 4°C, serum supernatant discarded, and erythrocytes were removed by incubation (7min, room temperature) with ACK lysis buffer (150mM NH_4Cl, 10mM $KHCO_3$, 0.1mM EDTA, pH 7.3). All samples were then washed twice by centrifugation (300g, 5mins, 4°C) with resuspension in FACS-buffer.

2.3.2. Determination of cell numbers

Total leukocytes were stained with 0.4% trypan blue (Sigma) included for live/dead cell discrimination (1:1 ratio) and counted using a Neubauer haemocytometer (Optik Labor, Germany). 10µl of mixed cell suspension was loaded onto the haemocytometer and cells counted under a light microscope (Olympus BH2-RFCA, 20X lens). Dead cells appear blue due

to uptake of dye and were excluded. Leukocyte number was calculated by inclusion of trypan blue dilution, pre-dilutions made of counting, and haemocytometer adjustment factor:

Leukocyte number/ml = No. cells counted x 2 (x pre-dilution) x 10^4

Blood leukocyte number was expressed at cells/ml of blood (as determined by measuring volume of blood obtained), and peritoneal cavity, spleen, and BM number was expressed as total cells. Leukocytes were adjusted to a concentration of $1x10^6$ cells/ml in FACS-buffer.

2.3.3. Immunofluorescence labelling of leukocytes

$1x10^5$ cells were incubated with 0.5µg FcγIII/II receptor blocking antibody (Fc-block™) for 20mins at 4°C to prevent unspecific Fc-receptor binding, followed by addition of fluorescently-labelled primary antibody cocktail containing Gr1, B220, CD3, CD115 and F4/80 combinations to label leukocyte subsets. Antibodies to TLR2, TLR4, TLR6, dectin-1, CXCR2, CXCR4, CCR1, CCR2, CCR3, CCR5, C5aR or isotype control antibodies were also included in certain studies. Samples were incubated for 30mins in the dark at 4°C with the antibody cocktail.

2.3.4. Flow cytometry

Antibody-labelled cells were washed twice with FACS-buffer by centrifugation at 300g for 5mins at 4°C and resuspend in 200µl cold FACS-buffer to achieve a single cell suspension. Samples were run on LSRFortessa flow cytometer (BD) using FACSDiva software. Samples were compensated for multi-colour fluorescence overlap using either single-stained cells or CompBeads. At least 20,000 events were acquired per sample. Periodically a live/dead cell marker (1µg/ml DAPI or 1.25µg/ml 7-ADD) was added just before data acquisition to check the protocol had not resulted in decreased viability.

2.3.5. Identification of leukocyte subsets

Data was analysed using FlowJo version 7.6.4 (Treestar) and leukocytes were identified from the sample data by forward scatter (FSC) and side scatter (SSC) profile (Figure 2.1A). Neutrophils were identified as $Ly6G(Gr1)^{high}$ cells (Figure 2.1B), and monocytes by the expression of CX_3CR1 and subdivided as $CX_3CR1^{low}Gr1^{med}$ cells (classical monocytes) and $CX_3CR1^{high}Gr1^{-}$ cells (non-classical monocytes) (Figure 2.1B). CX_3CR1^{+} cells were confirmed as monocytes by CD115 expression (Figure 2.2).

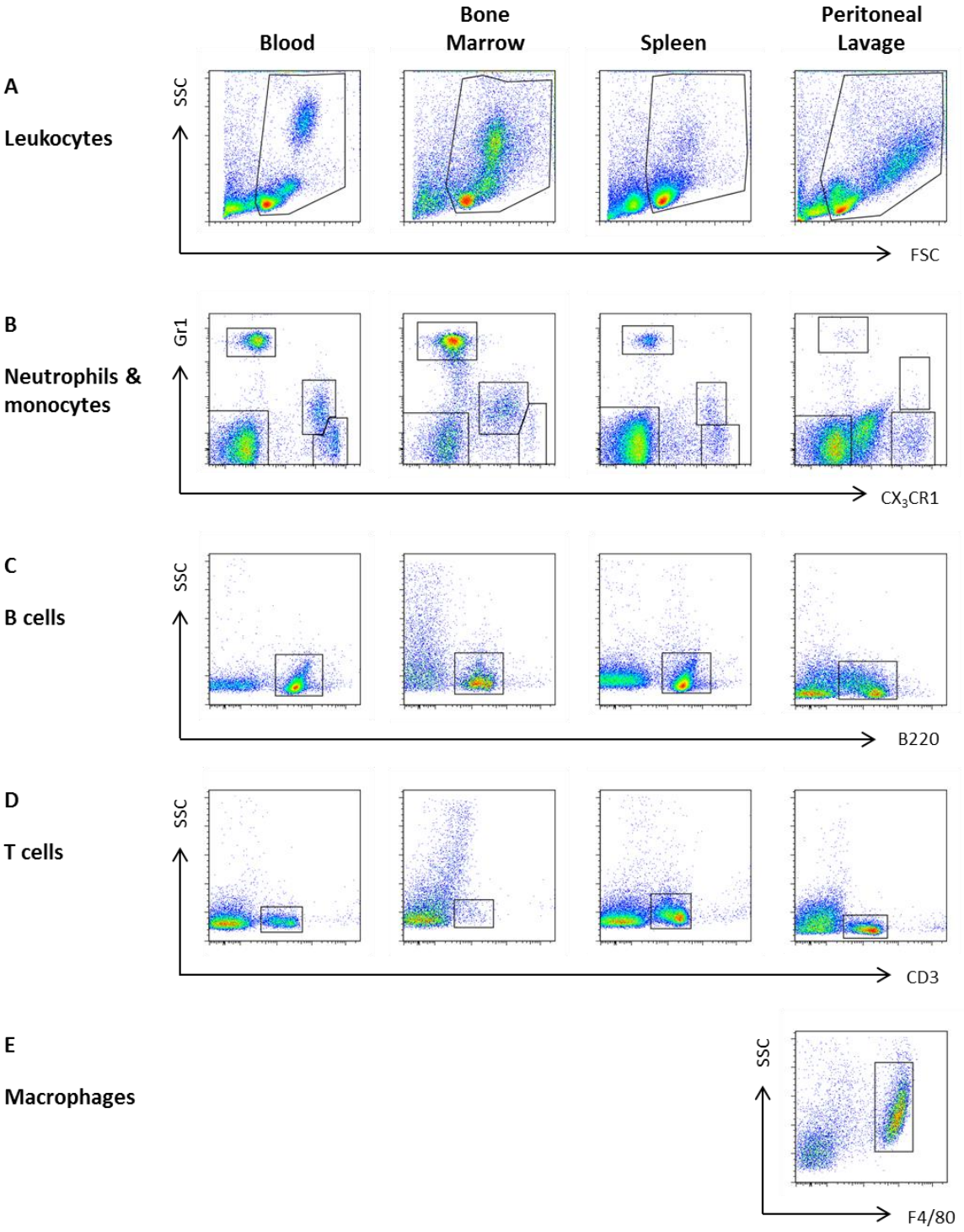


Figure 2.1. Leukocyte subset gating strategies. Leukocytes were isolated from murine blood, bone marrow, spleen and peritoneal cavity and subsets were analysed by flow cytometry. (A) Total leukocytes were first gated based on FSC/SSC profile. (B) After selection on the leukocyte gate, neutrophils were classified as $Gr1^{high}$, classical monocytes as $CX_3CR1^{low}Gr1^{+}$, and non-classical monocytes as $CX_3CR1^{high}Gr1^{-}$. $CX_3CR1^{-}Gr1^{-}$ population was selected with subsequent gating on (C) $B220^{+}$ B cell and (D) $CD3^{+}$ T cell populations. (E) Peritoneal lavage samples were also stained for macrophages ($F4/80^{+}$ cells).

$CX_3CR1^-Gr1^-$ populations (Figure 2.1B) were gated and subsequent $B220^+$ populations identified as B cells (Figure 2.1C), and $CD3^+$ populations as T cells (Figure 2.1D). Macrophages were identified as $F4/80^{high}$ (Figure 2.1E). Populations were checked for the negative expression of the other markers (Table 2.1). All subsets were also back-gated and FSC/SSC profiles compared with that of single stained cells.

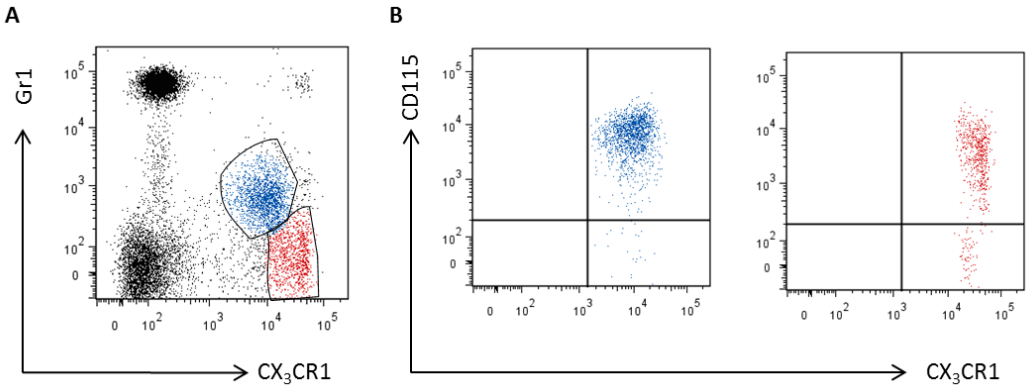


Figure 2.2. Confirmation of CX_3CR1-GFP^+ cells as $CD115^+$ monocytes. (A) Classical- and non-classical monocytes were defined as $CX_3CR1^{low}Gr1^+$ and $CX_3CR1^{high}Gr1^-$ respectively. (B) Both CX_3CR1^+ populations were confirmed as monocytes by expression of CD115.

Leukocyte Subset	Positive Markers	Negative Markers
Neutrophils	$Gr1^{high}$	$CX_3CR1^-B220^-CD3^-$
Classical monocytes	$CX_3CR1^{low}Gr1^{med}CD115^+$	$B220^-CD3^-$
Non-classical monocytes	$CX_3CR1^{high}Gr1^-CD115^+$	$B220^-CD3^-$
B cells	$B220^+$	$Gr1^-CX_3CR1^-CD3^-$
T cells	$CD3^+$	$Gr1^-CX_3CR1^-B220^-$
Macrophages	$F4/80^+$	$Gr1^-CX_3CR1^-B220^-CD3^-$

Table 2.1. Identification of leukocyte subsets by the expression cell-specific markers.

2.3.6. Calculation of leukocyte subset numbers

Percentage of leukocyte subsets was calculated using acquired flow cytometry data as:

Subset % = No. events positive for subset marker/ No. events in leukocyte gate

Numbers of leukocyte subsets were expressed $x10^4$ for peritoneal, spleen and BM cells, and $x10^4$/ml for blood cells and calculated using percentage of events and total cells counted on the haemocytometer:

Subset no.($x10^4$) = (No. total cells/ subset %) $x10^4$

2.3.7. Determination of cell surface receptor expression on leukocyte subsets

Cell surface TLR2 (Figure 2.3), TLR4, TLR6 and dectin-1 on specific leukocyte subsets were determined by flow cytometry. Expression was calculated as a ratio of test antibody median fluorescence intensity (MFI) versus that of an isotype control antibody (mouse IgG1 (TLR2), rat IgG2a (all others)) to achieve the relative fluorescence intensity (RFI). Cell surface CXCR2, CXCR4, CCR1, CCR2, CCR3, CCR5 and C5aR were also determined by flow cytometry and expressed as MFI. Where MFI was used, samples were run on the same cytometer, on the same day, with the same cytometer settings for the most accurate comparisons.

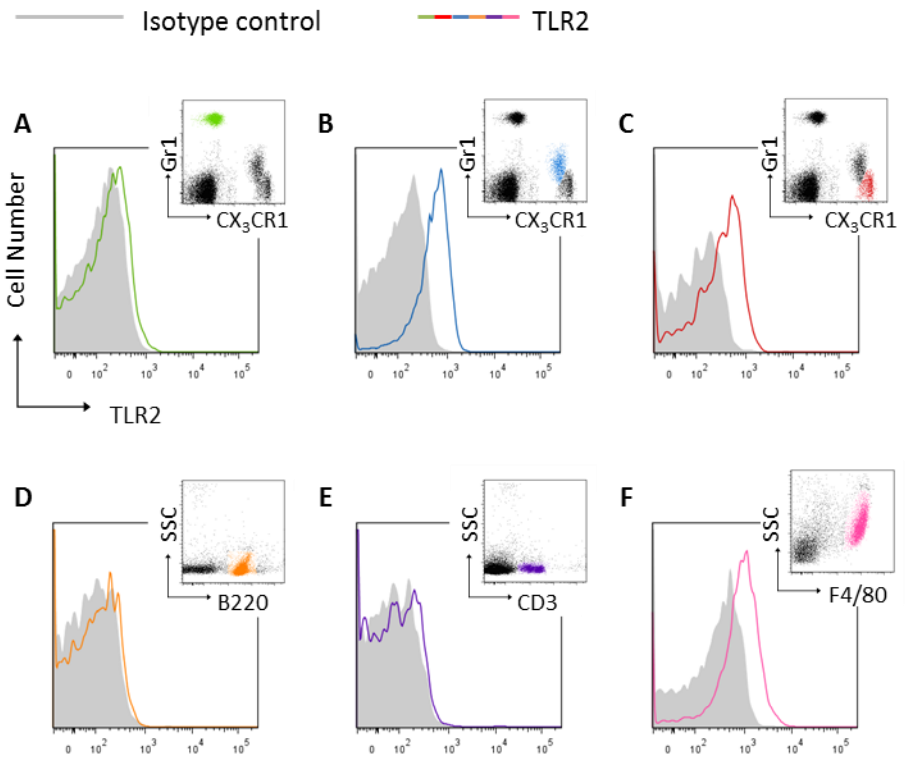


Figure 2.3. Representative flow cytometry plots showing expression of TLR2 on leukocyte subsets. Subsets were identified based on cell markers and the expression of TLR2 was assessed on gated populations of (A) neutrophils, (B) classical monocytes, (C) non-classical monocytes, (D) B cells, (E) T cells and (F) macrophages (shown as insets). TLR2 expression was calculated as a ratio of the medium fluorescence intensity (MFI) of anti-TLR2 antibody (coloured histograms) versus that of an isotype control antibody (grey filled histograms). Results were expressed as relative fluorescence intensity (RFI). This method was also applied to TLR4, TLR6, dectin-1 and CXCR4 expression.

2.3.8. Preparation of zymosan

Zymosan A (lot #BCBF4506V, Sigma) was prepared by transferring 250mg of zymosan powder into 50ml of sterile PBS. The mixture was heated and stirred for 30mins until boiling and allowed to cool for 1-2hrs, keeping covered. After cooling, the mixture was divided into 5x 10ml aliquots on ice and centrifuged at 300g for 10min at 4°C. Supernatants were removed and zymosan was washed with 5ml sterile PBS on ice. Centrifuge and washing was repeated 3 times. The mixture was then divided into 2 tubes of 12.5ml that were again centrifuged (300g, 10mins, 4°C) and washed 3 times with 5ml sterile PBS. The remaining tube contained 250mg of zymosan in 5ml PBS. Aliquots of 10mg of zymosan were obtained by aliquoting 200µl of mixture into eppendorf tubes. Aliquots were centrifuged at 1000g for 5mins, supernatants removed, and dry pellets were stored at -20°C until use.

2.3.9. Induction of peritonitis

10mg zymosan aliquots (as prepared in 2.3.8) were resuspended in 5ml sterile PBS to achieve a 2mg/ml concentration. Resuspended zymosan was sonicated for 30s and vortexed as previously described (Navarro-Xavier *et al.*, 2010). Peritonitis was induced by injecting 1mg zymosan intraperitoneally (i.p.) in 0.5ml sterile PBS.

2.4. Characterisation of male and female peritoneal cytokine and chemokine environment in naïve and inflamed conditions

2.4.1. Preparation of peritoneal and spleen samples for cytokine array

Lavage of the peritoneal cavity of naïve, 1h- or 3h-zymosan treated mice was carried out as described in section 2.3.1, after schedule 1 killing. Samples were centrifuged at 300g for 5mins at 4°C and the lavage fluid supernatant harvested and stored at -80°C until analysis. Cell pellets were resuspended in FACS-buffer, pre-incubated with anti-mouse Fc-block™ (20mins, 4°C), followed by incubation with fluorescently labelled anti-mouse Gr1 antibody in FACS-buffer for 30mins at 4°C in the dark (as described in section 2.3.3). Samples were then prepared as in 2.3.4 and run on the LSRFortessa flow cytometer to determine $Gr1^{high}$ neutrophil numbers as an assessment of the inflammation. Naïve lavage fluid supernatant was used for cytokine array if <1% neutrophils were present, and zymosan-treated samples used if >50% present.

Spleen lysates were also analysed by array. To prepare the lysates, whole spleens were mixed with 1ml PBS supplemented with 1% triton X-100 and 1% protease and phosphatase inhibitor

cocktail (spleen lysis buffer) in 2ml homogenisation tubes with 2.8mm ceramic beads (Stretton Scientific, Derbyshire, UK). Spleens were subject to homogenisation using a Precellys®24 homogeniser (Stretton Scientific) at 5000rpm for 3x30seconds. Samples were then centrifuged at 3500g at room temperature for 5mins, and the supernatants transferred to fresh tubes. Spleen lysate supernatants were then centrifuged at 17000g for 10mins and the supernatants harvested and stored at -80°C until analysis.

2.4.2. Proteome Profiler™ array

Peritoneal lavage fluids or spleen lysates were thawed and protein content determined using Pierce™ BCA protein assay kit (Thermo Scientific) as described by the manufacturers. Cytokines and chemokines in lavage fluid were compared between naïve and peritonitis-induced male and female mice using Proteome Profiler™ Array kits for mouse cytokine array panel A, and mouse chemokine array (R&D Systems). Arrays were performed as per the manufacturer's instructions probing pre-coated membranes with equal amounts of male and female protein. Briefly, nitrocellulose membranes had duplicate spots of specific panels of capture antibodies. Equal amounts of peritoneal lavage supernatants or spleen lysates were mixed with a cocktail of biotinylated detection antibodies and the sample/antibody mixture incubated overnight with the capture antibody-containing membrane. Cytokine and detection antibody complexes that were present bound to respective capture antibodies on the membrane. Membranes were then washed to remove unbound material and Streptavidin-HRP was added to bind to biotinylated detection antibodies. Chemokine levels were measured by the subsequent addition of chemiluminescent detection reagents with exposure of membranes for equal times. The light produced at each spot was proportional to the amount of cytokine bound.

2.4.3. Array quantification

Array blots were scanned simultaneously using a HP colour LaserJet CM2320Fx MFP printer scanner (Hewlett Packard) and the resultant scans were converted to jpeg format using Adobe Photoshop. The jpeg images used for quantification were all adjusted to a pixel density of 300dpi and the dot intensity quantified by densitometry using ImageJ (NIH, Bethesda, USA). Cytokine and chemokine levels were presented as pixel density (arbitrary units (AU)). Test dots were also compared to control references dots on the membranes.

2.5. Statistics

Data was expressed as arithmetic mean ± standard error of the mean (SEM). Analysis was carried out using GraphPad Prism 6.03 (GraphPad, San Diego, CA). Statistical significance was evaluated by unpaired two-tailed Student's *t*-test, or 1- or 2-way ANOVA followed by Bonferroni's post-test for multiple comparisons. Data was classed as significant when *p* values were below 0.05.

Chapter 3: Sex-differences in leukocyte subsets and TLR expression in naïve mice

3.1. Introduction

Detection of pathogens or damaged tissue by TLR-mediated PAMP or DAMP recognition represents the first step in mounting the inflammatory response. The presence of TLRs on immune cells is widely accepted and vast numbers of studies report the presence of TLR message in leukocytes. Most well described are TLR4, as the LPS receptor, and TLR2 due to its highly promiscuous nature and hence plethora of ligands. High TLR2 and TLR4 mRNA has been demonstrated in human monocytes, whilst conversely message for these TLRs is also described in low levels in T cells, B cells, and NK cells (Muzio *et al.*, 2000; Hornung *et al.*, 2002; Zarember *et al.*, 2002). Human neutrophils and mast cells also express TLR2 and TLR4 mRNA (Muzio *et al.*, 2000; Supajatura *et al.*, 2001). Human blood DCs express various TLRs depending on the subset, however only myeloid DCs express TLR2 and TLR4 (Kadowaki *et al.*, 2001). Surprisingly, only a handful of groups have detailed TLR protein on leukocytes. Monocytes are reported to express relatively high levels of TLR2 and TLR4 protein on their cell surface, compared to low levels on neutrophils, and no expression on eosinophils (Sabroe *et al.*, 2002), whilst macrophages also express TLR2 and TLR4 protein (Henning *et al.*, 2008). Despite the vast amount of literature describing immune cell TLR expression, a comprehensive study into the expression of protein on leukocytes subsets from different storage compartments has yet to be carried out. Sex-differences in leukocyte TLR phenotype have previously been reported with female murine peritoneal macrophages exhibiting greater TLR2 and TLR4 message and protein (Scotland *et al.*, 2011). The magnitude of such differences with regards to other leukocyte subsets and in the context of other storage compartments has been little explored.

The extent of readily deployable leukocyte pools is another highly important factor in the ability to mount an inflammatory response and clear an infection. The number of leukocytes in the tissue, circulation, and storage pools (i.e. BM or spleen), represents an instrumental factor in the capacity of the immune system to remove the pathogen or damaged tissue and restore homeostasis. Conversely, mass mobilisation of leukocytes and their subsequent accumulation in the tissue, if not finely regulated, can represent a series of events that are no longer protective, but instead become detrimental to the host. Sex differences in the extent of leukocyte pools are surprisingly poorly reported. One study however, shows female mice have more resident macrophages, T lymphocytes and B lymphocytes in the peritoneum than male

littermates (Scotland *et al.*, 2011). Dimorphisms in resting leukocyte numbers may pose implications for disease, certain types of which are known to clinically exhibit sex-bias both in terms of occurrence and severity (Marriott *et al.*, 2006a). Sex-differences in TLR phenotypes are likely to add to such implications.

3.2. Aims

Despite reports of sexual dimorphism in peritoneal leukocyte numbers, it is first important to characterise this further in different leukocyte compartments in order to fully understand trafficking in males and females in inflammation. Furthermore, despite previous characterisation of both leukocyte TLR2 and TLR4 protein, further investigations are required to understand the expression of these receptors between compartments, and on other specific leukocyte subsets. Differences in leukocyte TLRs between compartments may indeed elude to a potential role of TLRs themselves in leukocyte mobilisation and trafficking beyond PAMP recognition. Therefore, the specific aims were to:

- Investigate sex differences in the leukocyte subset composition of various leukocyte pools extending upon previous reports in the peritoneal cavity to include the circulation, BM, and spleen.
- Assess expression of TLR2 and TLR4 cell surface protein expression on leukocyte subsets from each source to achieve a comprehensive basal leukocyte subset expression profile in male and female mice.

3.3. Results

3.3.1. Male and female basal total leukocyte numbers

To further explore the extent of physiological sex-differences in peritoneal leukocytes seen previously, total basal cells in other major leukocyte-containing compartments were first investigated. In naïve mice, no significant difference in total circulating, BM or spleen leukocytes were observed (p>0.05) (Figure 3.1). In agreement with previously published data, female mice had significantly (p<0.001) more peritoneal leukocytes than males (4.4±0.20 x10^6 vs 3.3±0.20 x10^6, Figure 3.1) (Scotland *et al.*, 2011).

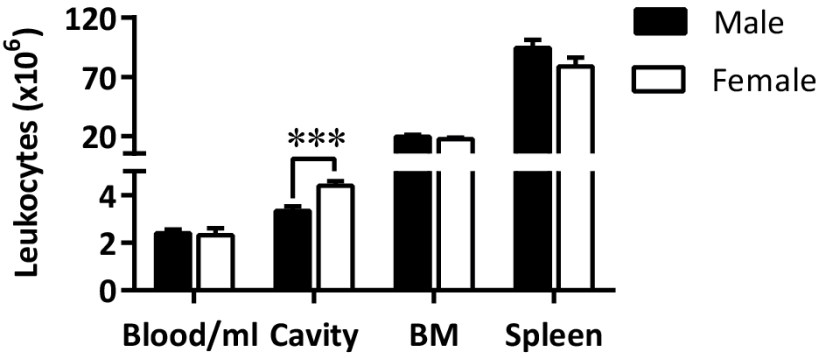


Figure 3.1. Total leukocyte numbers in naïve male and female mice. Total numbers of leukocytes isolated from male (■) and female (□) mouse blood (n=14/13), peritoneal cavity (n=24/26), bone marrow (BM) (n=9/8) and spleen (n=12/15) were determined using trypan blue exclusion and a haemocytometer. Bars represent the mean ± SEM. Significant differences between the sexes were determined by Student's *t*-test, *** (p<0.001).

3.3.2. Male and female mouse spleen and BM characteristics

Male and female comparisons in this study were made using aged-matched mice, and the male mice were therefore larger than female counterparts (p<0.05) (Figure 3.2A). To eliminate the possibility of animal size effecting significant cell count differences, circulating cells were displayed as a concentration (per ml of blood), and femurs and spleens were weighed (Figure 3.2A). No difference in femur (p>0.05) and spleen (p>0.05) weights were observed in those obtained from male and female mice (Figure 3.2). This suggests the available store of BM and splenic leukocytes is similar for both sexes. Of note, female mice have bigger spleens in relation to their body size than aged-matched males (p<0.05) (Figure 3.2).

A

	Male	Female	*p* value
Weight (g)	25.0 ± 2.58 (n=99)	20.2 ± 2.16 (n=94)	<0.001
Femur (mg)	81.6 ± 17.00 (n=9)	76.4 ± 27.18 (n=6)	NS
Femur: body weight (mg/g)	3.2 ± 0.74 (n=9)	3.80 ± 1.42 (n=6)	NS
Leukocytes: femur (x10^5/mg)	2.2 ± 0.78 (n=6)	2.76 ± 0.60 (n=5)	NS
Spleen (mg)	90.2 ± 19.24 (n=15)	87.38 ± 25.12 (n=17)	NS
Spleen: body weight (mg/g)	3.7 ± 0.62 (n=17)	4.4 ± 1.01 (n=17)	<0.05
Leukocytes: spleen (x10^5/mg)	10.4 ± 3.11 (n=12)	9.0 ± 3.37 (n=14)	NS

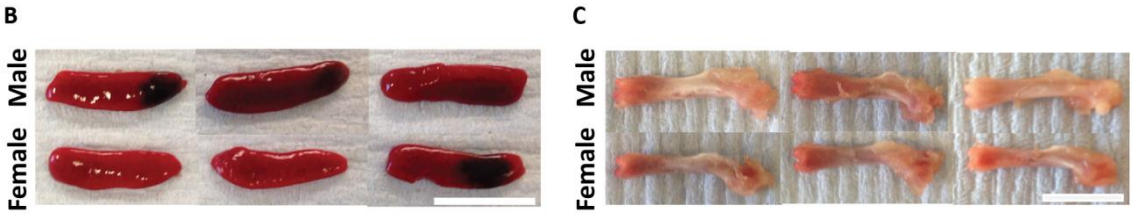


Figure 3.2. Murine body and organ characteristics. (A) Table of measurements from naïve male and female mice. Data are displayed as mean ± SEM of n number of experiments, as displayed in parentheses. Female data were compared to male by Student's *t*-test and the *p* value displayed. NS, not significant. (B) Representative spleens from male and female naïve mice. Scale bar, 1cm. (C) Representative femurs from male and female naïve mice. Scale bar, 1cm.

3.3.3. Sex-differences in peritoneal cavity and spleen, but not blood or BM, leukocyte subsets

Neutrophils were present in the circulation, BM and spleen of naïve male and female mice. As expected in uninflamed conditions, in the peritoneum there was an absence of neutrophils (Figure 3.3A). Classical monocytes, non-classical monocytes, B cells, and T cells were found in all compartments, although only a negligible number of classical monocytes were found in the naïve peritoneum (Figure 3.3B-E). In naïve mice, males exhibited over 2-fold more neutrophils (p<0.01), classical- (p<0.01) and non-classical monocytes (p<0.001) in their spleens versus females (neutrophils: 44.7±7.94 x10^5 vs 18.7±2.00 x10^5; classical monocytes: 16.1±2.49 x10^5 vs 7.7±0.82 x10^5; non-classical monocytes: 14.5±1.75 x10^5 vs 6.6±0.75 x10^5, Figure 3.3A-C). Despite constituting over 70% of splenic leukocytes, no significant differences in B cell (p>0.05) and T cell (p>0.05) numbers were observed (Figure 3.3D-E).

Confirming previous reports, the naïve murine female peritoneal cavity contained more macrophages (p<0.001) and B cells (p<0.01) than male counterparts, although no difference in T cells was observed (Figure 3.3D-F). Male and female mice had similar numbers of peritoneal non-classical monocytes (p>0.05), and both had an absence of neutrophils and very few classical monocytes (Figure 3.3A-C). No sex-differences were seen in BM (p>0.05) or circulating (p>0.05) subset numbers, though unexpectedly male mice displayed slightly more blood neutrophils than females (p<0.05).

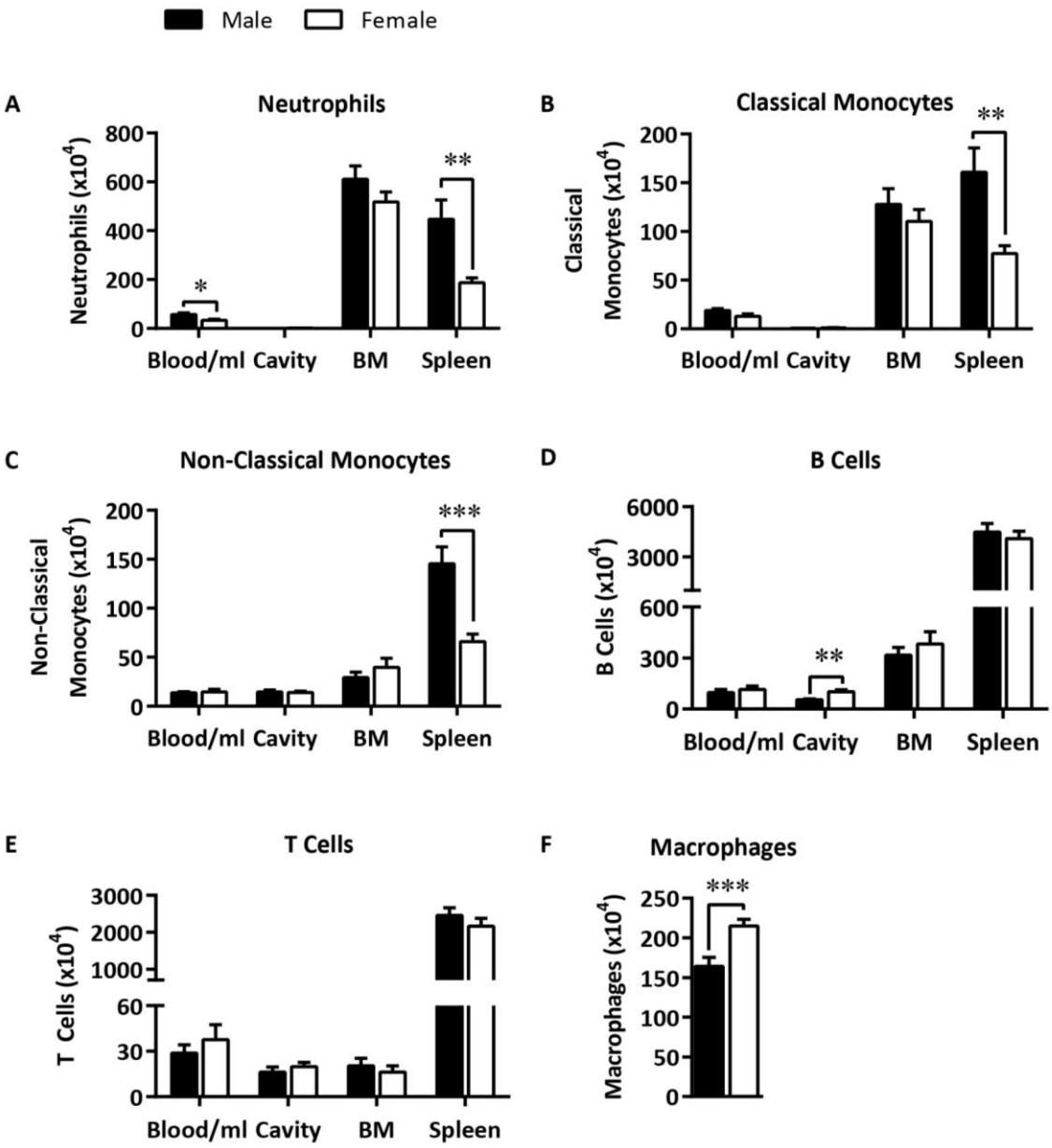


Figure 3.3. Basal leukocyte populations from different compartments of naïve male and female mice. Leukocytes isolated from blood, peritoneal cavity, bone marrow (BM) and spleen of male (■) and female (□) mice. (A) Neutrophils, (B) classical monocytes, (C) non-classical monocytes, (D) $B220^+$ B cells, (E) $CD3^+$ T cells, and (F) peritoneal macrophages were identified by flow cytometry. Data are presented as mean ± SEM of n animals where n≥7 (blood), n≥7 (cavity), n≥5 (BM), and n≥11 (spleen) animals. Differences between males versus females were determined using Student's *t*-test and indicated by * (p<0.05), ** (p<0.01) and *** (p<0.001).

3.3.4. No difference in TLR2 and TLR4 expression on leukocyte subsets from different compartments

To obtain a comprehensive TLR2 and TLR4 expression profile for male and female murine leukocytes, subsets from different compartments of naïve mice were assessed for presence of receptors on the cell surface by flow cytometry. Monocytes expressed highest amounts of TLR2, with both classical and non-classical subsets comparable in expression. Neutrophils, peritoneal macrophages and B cells expressed moderate amounts of TLR2, whilst T cells expressed a very small amount of the receptor (Figure 3.4). TLR4 on the other hand was most abundant on peritoneal macrophages, followed by the monocyte subsets and neutrophils with comparable expression and finally B cells, with T cells again demonstrating very low expression of TLR4 (Figure 3.5). Leukocyte subsets showed no differences in expression of TLR2 ($p>0.05$) (Figure 3.4) or TLR4 ($p>0.05$) (Figure 3.5) if obtained from blood, tissue, BM, or spleen.

Contrary to previous work, no significant difference in expression of TLR2 ($p>0.05$) or TLR4 ($p>0.05$) was seen on peritoneal macrophages from male versus female mice (Scotland *et al.*, 2011). Similarly, no sex-differences were observed in the expression of either receptor on classical monocytes ($p>0.05$), non-classical monocytes ($p>0.05$), and B cells ($p>0.05$), from any of the compartments sampled. T cell TLR2 and TLR4 expression were similar between the sexes, apart from in the peritoneal cavity where female T cells had more TLR2 than males ($p<0.05$) (Figure 3.4F).

Neutrophils displayed some small but significant sex-differences in TLR expression. Those from male BM had both higher TLR2 ($p<0.001$) and TLR4 ($p<0.05$) compared to neutrophils from female BM. TLR2 and TLR4 expression on circulating neutrophils were however similar between the sexes ($p>0.05$). Aside from the aforementioned differences, male and female leukocytes from blood, peritoneum, spleen and BM showed comparable TLR2 and TLR4 expression levels. Of note however, splenic classical monocytes displayed a trend towards higher expression in male versus female mice ($p=0.076$).

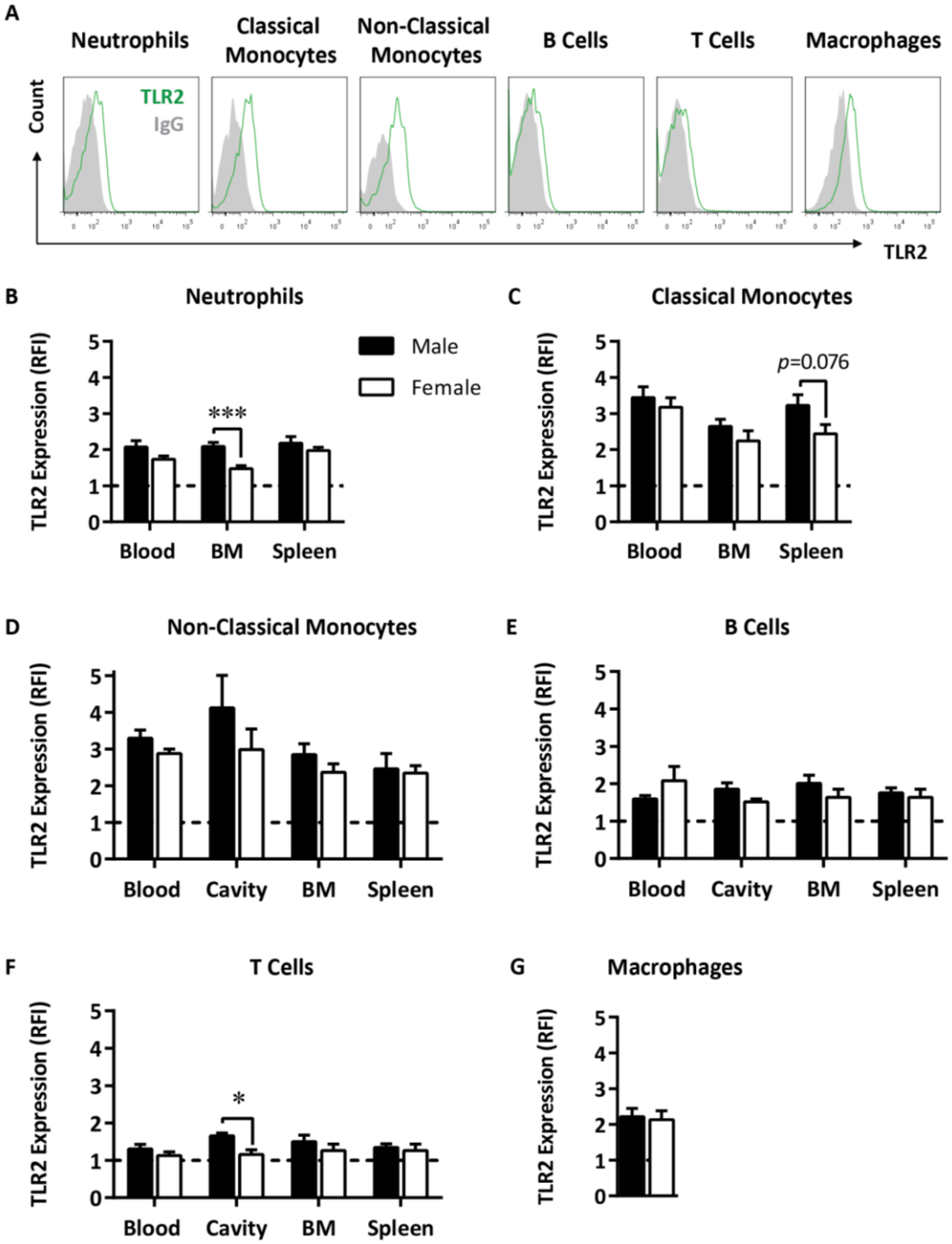


Figure 3.4. Basal leukocyte TLR2 expression profile. Surface TLR2 was assessed on murine leukocyte subsets. (A) Representative histograms showing TLR2 expression (green) and an IgG control (grey fill). Quantification of TLR expression on male (■) and female (□) (B) neutrophils, (C) classical monocytes, (D) non-classical monocytes, (E) $B220^+$ B cells, and (F) $CD3^+$ T cells from blood, BM, spleen, and peritoneal cavity (where present), and (G) peritoneal macrophages. TLR2 was expressed as relative fluorescence intensity (RFI) to isotype control antibody. Differences in expression between males and females were assessed using Student's *t*-test and significance indicated by * ($p<0.05$), and *** ($p<0.001$). Data shown as mean ± SEM of n experiments: (B-D) n=12/14 (blood), n=3/5 (cavity), n=8 (BM), n=5 (spleen); (E-F) n=6/4 (blood), n=4 (cavity), n=5 (BM), n=5 (spleen); (G) n=16/19.

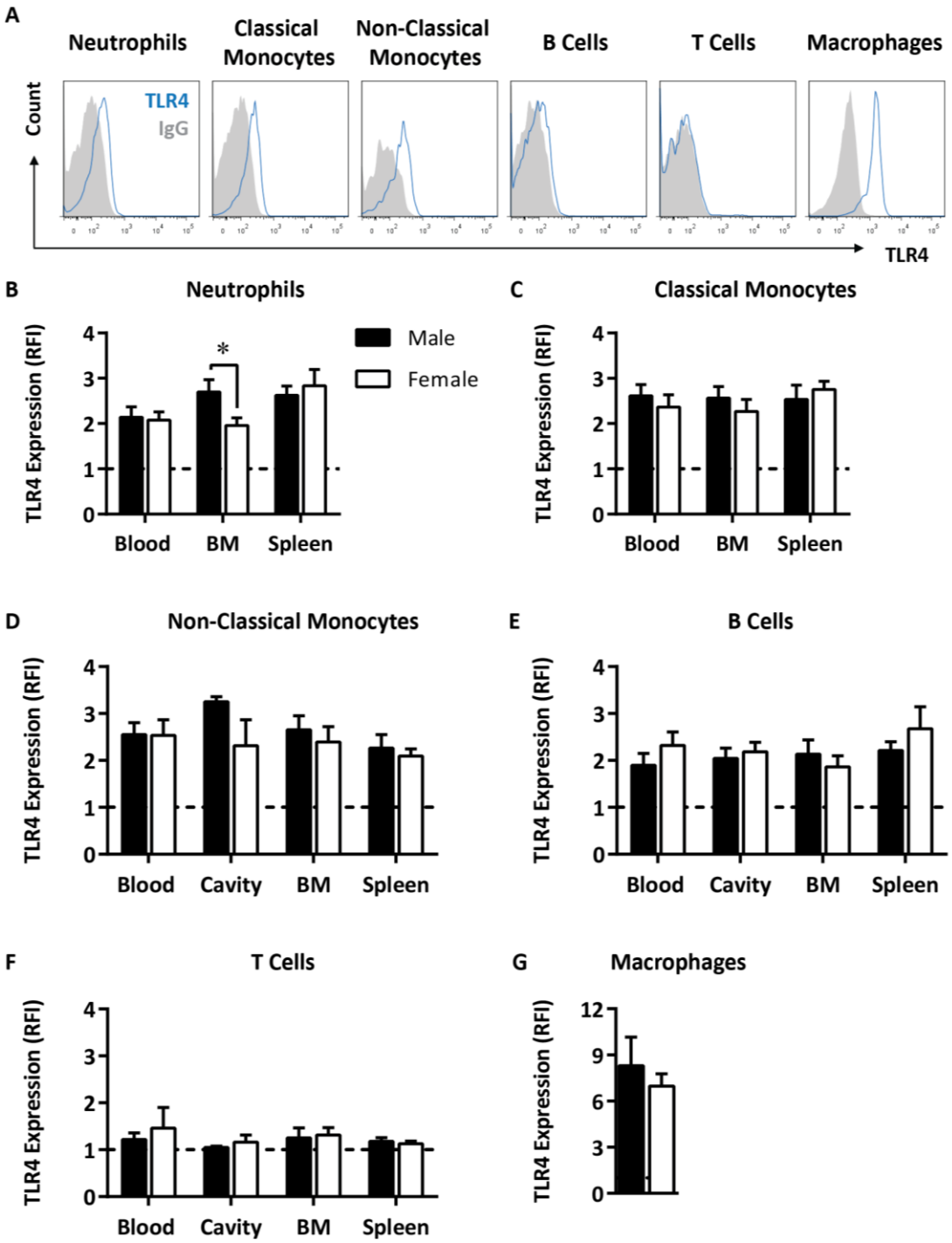


Figure 3.5. Basal leukocyte TLR4 expression profile. Surface TLR4 was assessed on murine leukocyte subsets. (A) Representative histograms showing TLR4 expression (blue) and an IgG control (grey fill). Surface TLR4 was assessed on male (■) and female (□) (B) neutrophils, (C) classical monocytes, (D) non-classical monocytes, (E) $B220^+$ B cells, and (F) $CD3^+$ T cells from blood, BM, spleen, and peritoneal cavity (where present), and (G) peritoneal macrophages. TLR4 was expressed as relative fluorescence intensity (RFI) to isotype control antibody. Differences in expression between males and females were assessed using Student's *t*-test and significance indicated by * (p<0.05). Data presented as mean ± SEM. Data shown as mean ± SEM of n experiments. (B-D) n=12/14 (blood), n=3/5 (cavity), n=8 (BM), n=5 (spleen); (E-F) n=6/4 (blood), n=4 (cavity), n=5 (BM), n=5 (spleen); (G) n=10/12.

3.4. Discussion

Sexual dimorphisms in basal peritoneal macrophage number and phenotype have been implicated in the differential responses to inflammatory stimuli in mice. Little attention has however been paid to the phenotype of other leukocyte types and the source of such cells. In this chapter, an extensive comparison of resting male and female murine leukocyte numbers from diverse pools was conducted using flow cytometry. This chapter provides clear evidence of sex differences in specific leukocytes pools (Table 3.1). Females exhibited greater numbers of resident peritoneal leukocytes, more specifically macrophages and B cells, than age-matched males, confirming recently reported observations (Scotland *et al.*, 2011). Contrary to previous reports, no sex-difference in numbers of $CD3^+$ T cells was seen. A possible explanation may lie with antibody sensitivity: Whilst all other leukocyte markers were the same as for the published sex-differences (Scotland *et al.*, 2011), T cell anti-CD3 antibody clone KT3 was used compared to the 17A2 clone in this study. In addition, males surprisingly had greater numbers of circulating neutrophils under resting conditions than females. This is in agreement with a haematological survey of 11 inbred mouse strains that also showed 8 week C57BL/6 male mice had 2-fold more blood neutrophils than aged-matched females (Jackson, 2014).

Most notably, male mice had significantly larger stores of splenic neutrophils and monocytes than females. This is the first report of such a difference and is compounded by similar lymphocyte numbers and spleen masses. This demonstrates firstly that the spleen may represent a pool of mature innate immune cells that may be mobilised and utilised in infection or tissue damage, and secondly that this may be more greatly exploited by male mice due to enhanced size of the store. Recent studies have demonstrated that the spleen contains

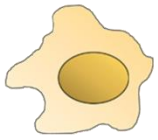
	Neutrophil	Classical Monocyte	Non-Classical Monocyte	B Cell	T Cell	Peritoneal Macrophage
						
Blood	♂ > ♀	♂ = ♀	♂ = ♀	♂ = ♀	♂ = ♀	-
Peritoneum	-	♂ = ♀	♂ = ♀	♂ < ♀	♂ = ♀	♂ < ♀
BM	♂ = ♀	♂ = ♀	♂ = ♀	♂ = ♀	♂ = ♀	-
Spleen	♂ > ♀	♂ > ♀	♂ > ♀	♂ = ♀	♂ = ♀	-

Table 3.1. Summary of sex-differences in leukocyte subset numbers in naïve mice. Sex differences are represented by '<' or '>'. Hyphen indicated not applicable for this leukocyte source. ♂, male; ♀, female.

proliferating myeloid cell progenitors that give rise to their progeny in a process coined extramedullary haematopoiesis (Robbins *et al.*, 2012). Compared to WT controls atherosclerotic-prone ApoE-/- mice were found to have more granulocyte/macrophage colonies and more haematopoietic stem cells in their spleens, indicative of myeloid cell proliferation. It would be useful to characterise the nature of the male and female splenic neutrophils and in particular monocytes, and in addition the myeloid cell progenitors, to assess whether the male spleen indeed possesses more of these cells, and whether they are in a greater state of proliferation compared to females.

This chapter also sought to explore the basal pattern recognition receptor leukocyte phenotype, as female murine resident macrophages have been shown to have greater TLR expression compared to males. Despite remarkable interest in the functional role of TLRs in inflammation and immunity, the expression profile of TLR protein is incompletely characterised. In order to explore the extent of dimorphism in the expression of TLRs, flow cytometry was employed to investigate receptor expression on the above-mentioned leukocyte subsets. Cells were sampled from various leukocyte pools to assess whether location and storage environments affected TLR phenotype. Focus was kept to TLR2 and TLR4, as these are the best characterised and have previously displayed sexual-dimorphisms in macrophages. The present study failed to detect a sex-difference in the expression of TLR2 and TLR4 on resident macrophages. A possible explanation again lies in antibody selection. Anti-TLR2 clone T2.5 and anti-TLR4 clone MTS510 were selected after preliminary testing based on their preferential RFI versus other tested antibodies (Appendix 2). Previously observed sex-differences were seen with anti-TLR2 clone 6C2 and anti-TLR4 clone UT41. The notoriety of TLR antibodies as being poor is one explanation for the lack of protein data in the literature and the majority of expression data being message-based, and likewise for difficulty in replicating such results as in this chapter. Furthermore, TLR expression was presented as relative fluorescence intensity (RFI), a more stringent measure to take into account non-specific binding of IgG and changes to cytometer settings. This is in comparison to previously shown TLR sex-differences that were presented as median fluorescence intensity (MFI), the crude readout of the test antibody only.

Additionally, in the study that reported female mouse macrophages to have greater TLR2 and TLR4 expression, peritoneal cells were collected in media without EDTA, unlike in this study where buffer contained EDTA. The study in question consequently recovered 1.5-fold fewer cells from the cavity than obtained in this thesis, leaving scope to speculate the most adherent macrophages may not have been recovered, excluding their TLR profile from the analysis. Such

macrophage subsets differing in TLR expression is not inconceivable due to the known M1 and M2 phenotypes. Variance in pattern recognition receptor expression in males and females is however a debated subject with no clear answers, in particular at the basal level.

This study found male BM neutrophils have a small but significantly increased expression of TLR2 and TLR4 compared to females. Sex-differences in relation to neutrophil TLRs have not previously been reported. Interestingly, circulating neutrophil TLR2 and TLR4 is similar between the sexes. This may suggest differences in the stores are an indication of different maturation or activation states, or conversely may suggest male neutrophils are more 'poised' for PAMP and DAMP detection in proximity to the BM. Sexual dimorphism in neutrophil TLR expression in this compartment, remote from the site of inflammation leaves scope for the exploration of potential function of these receptors in leukocyte trafficking. The consequence of this observation will be explored in future chapters by monitoring leukocyte subset expression temporally in an acute inflammatory model.

This chapter has also shown levels of TLR2 and TLR4 were similar between the murine classical and non-classical monocyte subsets. This suggests detection of respective ligands is not biased to a particular subset, as would have been likely based on the inflammatory function of classical monocytes. Indeed, some studies show both subsets are able to be activated by TLR ligands, more specifically human $CD16^-$ classical preferentially sense surface TLR ligands (including TLR2 and TLR4), and $CD16^+$ non-classical monocytes sense more so via intracellular TLRs (Strauss-Ayali *et al.*, 2007; Cros *et al.*, 2010; Saha *et al.*, 2011). This finding is contrary to the implications of this investigation. In support however is a report on human monocytes that shows no difference in TLR2 or TLR4 protein between $CD16^-$ classical, and $CD16^+$ non-classical subsets (Aguilar-Ruiz *et al.*, 2011). No reports of differential monocyte TLR2 and TLR4 expression between males and females are present in the literature. Similar expression of both receptors on monocyte subsets between the sexes was observed in all compartments sampled.

Key findings:

- Sexual dimorphisms are present in the magnitude of certain murine leukocyte pools, specifically the spleen and peritoneum.
- Basal pathogen sensing capabilities of leukocytes, from various localisations, are on the whole not different between the sexes.
- Male BM neutrophils express more TLR2 and TLR4 than those from females.

Chapter 4: Sex-differences in leukocyte recruitment in zymosan peritonitis

4.1. Introduction

Recruitment of specific leukocytes is considered to underlie the pathogenesis of many diseases, i.e. monocyte infiltration in atherosclerosis or neutrophil recruitment in ischaemia/reperfusion injuries. Sex-differences in leukocyte recruitment in acute inflammation have previously been demonstrated in rodents with female mice recruiting significantly less leukocytes to the peritoneum in response to both GBS bacterial-induced peritonitis and zymosan-induced peritonitis. The latter was shown to be attributed to lesser neutrophil infiltration as lymphocyte and macrophage numbers were similar (Scotland *et al.*, 2011). Correspondingly, in a rat model of abdominal aortic aneurysms, females accumulated significantly less neutrophils than males (Sinha *et al.*, 2006). Whether this extends to other leukocyte subsets, such as the monocyte subsets, is currently unknown.

The mechanisms of sex-differences in leukocyte recruitment are also unclear. Dimorphisms in upstream events in the inflammatory response are not well documented, and to date no studies have shown whether sex-difference in tissue recruitment extends into the circulation. Similar circulating leukocytes in males and females but fewer recruited cells may be suggestive of a sex-difference in transendothelial migration mechanics, i.e. rolling, activation, adhesion, crawling or transmigration. Male hypercholersterolemic rabbits demonstrated more monocyte adhesion and subendothelial migration, and in addition exhibited higher endothelial VCAM-1 expression than females (Nathan *et al.*, 1999). Beyond this study however, sex differences in such processes have been poorly reported in the literature.

Therefore, to understand the effect of male or female sex on the recruitment of leukocytes in acute inflammation, a murine zymosan induced peritonitis model of acute inflammation was employed. Murine zymosan peritonitis was first described as a suitable model of acute inflammation in 1985, and was characterised by vascular changes and mediator production leading to leukocyte accumulation at the inflammatory focus (Doherty *et al.*, 1985). This model was firstly chosen as zymosan is known to induce inflammatory pathways that result in the accumulation of both neutrophils and monocytes, allowing the study of the trafficking of both cell types (Getting *et al.*, 1997). Secondly, due to a previously published zymosan dose-response that validated a 1mg dose (i.p.) to elicit transient inflammation characterised by

neutrophil clearance, whereas a 10mg dose causes persistent tissue neutrophils, systemic inflammation and impaired restoration of tissue homeostasis, despite eventually resolving (Navarro-Xavier *et al.*, 2010). Owing to an interest of this study being the potential of infiltrating leukocytes to be damaging rather than protective in an acute setting, the 1mg dose was selected. In addition, due to the observation in the previous chapter that male murine BM neutrophils were higher in TLR2 than those from females, the selection of zymosan as a TLR2 ligand was appropriate to further investigate the temporal expression profile of this receptor in inflammation. Finally, zymosan previously produced an inflammatory reaction that was dimorphic between males and females, providing scope for this study to investigate sex-differences further (Scotland *et al.*, 2011).

4.2. Aims

Although a sex-bias in many human diseases is hypothesised to be driven by differences in leukocyte recruitment, there is a lack of evidence in the literature to support this, likely due to the nature of obtaining such measurements. A robust understanding of the mechanisms of sex-differences in leukocyte recruitment in inflammation in rodents is therefore essential in understanding human disease and designing new, more targeted therapies. Consequently, this chapter has the following specific aims:

- Confirm and further identify sex-differences in leukocyte subset recruitment in the zymosan peritonitis model of acute inflammation.
- Investigate the effect of inflammation on circulating leukocyte numbers to advance understanding of the dynamics of this acute inflammatory reaction.

4.3. Results

4.3.1. Neutrophils infiltrate and are cleared in murine zymosan peritonitis

Leukocyte infiltration to the peritoneal cavity in response to 1mg intraperitoneal zymosan was assessed in male and female mice. By 3h, peritoneal leukocytes increased 3-4-fold in number (p<0.001), significantly more so in male versus female mice (p<0.05) (Figure 4.1A). Leukocyte numbers showed a trend to reduction over time, but remained elevated through 96h post induction of inflammation. To confirm the resolving nature of the innate inflammatory response to zymosan, neutrophil numbers in the peritoneal cavity of male and female mice were monitored at various time points until clearance. Zymosan induced rapid and significant (p<0.001) influx of neutrophils into the murine peritoneal cavity peaking to a significantly (p<0.05) extent in male compared to female mice at 3h post induction of inflammation (Figure 4.1B). In line with previous reports neutrophils were virtually absent from the cavity of both sexes by 96h, indicating the transient nature of the 1mg zymosan dose as previously seen (Navarro-Xavier *et al.*, 2010).

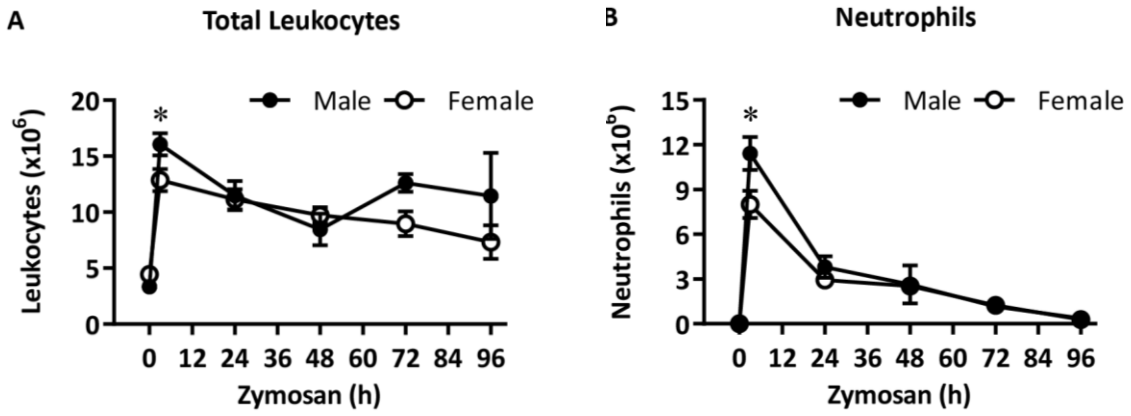


Figure 4.1. Immune cell recruitment in murine zymosan peritonitis. Male (●) and female (○) mice were treated with zymosan (1mg, i.p.) for the indicated time points. (A) Total leukocytes and (B) neutrophils recovered from the peritoneal cavity. Data are shown as mean ± SEM of at least 14 (0h), 16 (3h), 10 (24h), and 3 (48h, 72h, 96h) mice. Sex differences in the number of cells in the peritoneal cavity at each time point was determined by Student's *t*-test and significance indicated by * (p<0.05).

4.3.2. Sex-differences in leukocyte recruitment following zymosan peritonitis

Intraperitoneal zymosan induced accumulation of neutrophils and classical monocytes in the cavity after 3h. Male mice recruited 11.4±1.12 x10^6 neutrophils at this time, significantly more than 8.0±0.91 x10^6 in females (p<0.05) (Figure 4.2A). Similarly, males amassed significantly more (p<0.01) classical monocytes at the same time point than females (2.7±0.35 x10^5 vs 1.52±0.21 x10^5, Figure 4.2B). Numbers of cavity non-classical monocytes decreased during peritonitis 2.5-fold, to the same extent in both sexes (Figure 4.2C). Despite females having more resident peritoneal macrophages than males under basal conditions as described in Chapter 3, administration of zymosan caused clearance of over 95% of macrophages by 3h in both sexes (Figure 4.2D). Like macrophages, peritoneal $B220^+$ B cells were more numerous in the naïve female cavity. 3h after zymosan administration male cavity B cells increased 2-fold (5.4±0.63 x10^5 to 11.4±1.64 x10^5) (p<0.05) whilst no change in female B cell numbers was seen in the inflammation (p>0.05) (Figure 4.2E). $CD3^+$ T cell numbers were similar between the sexes (p>0.05) and unchanged in response to zymosan at the early time point of 3h (p>0.05) (Figure 4.2F).

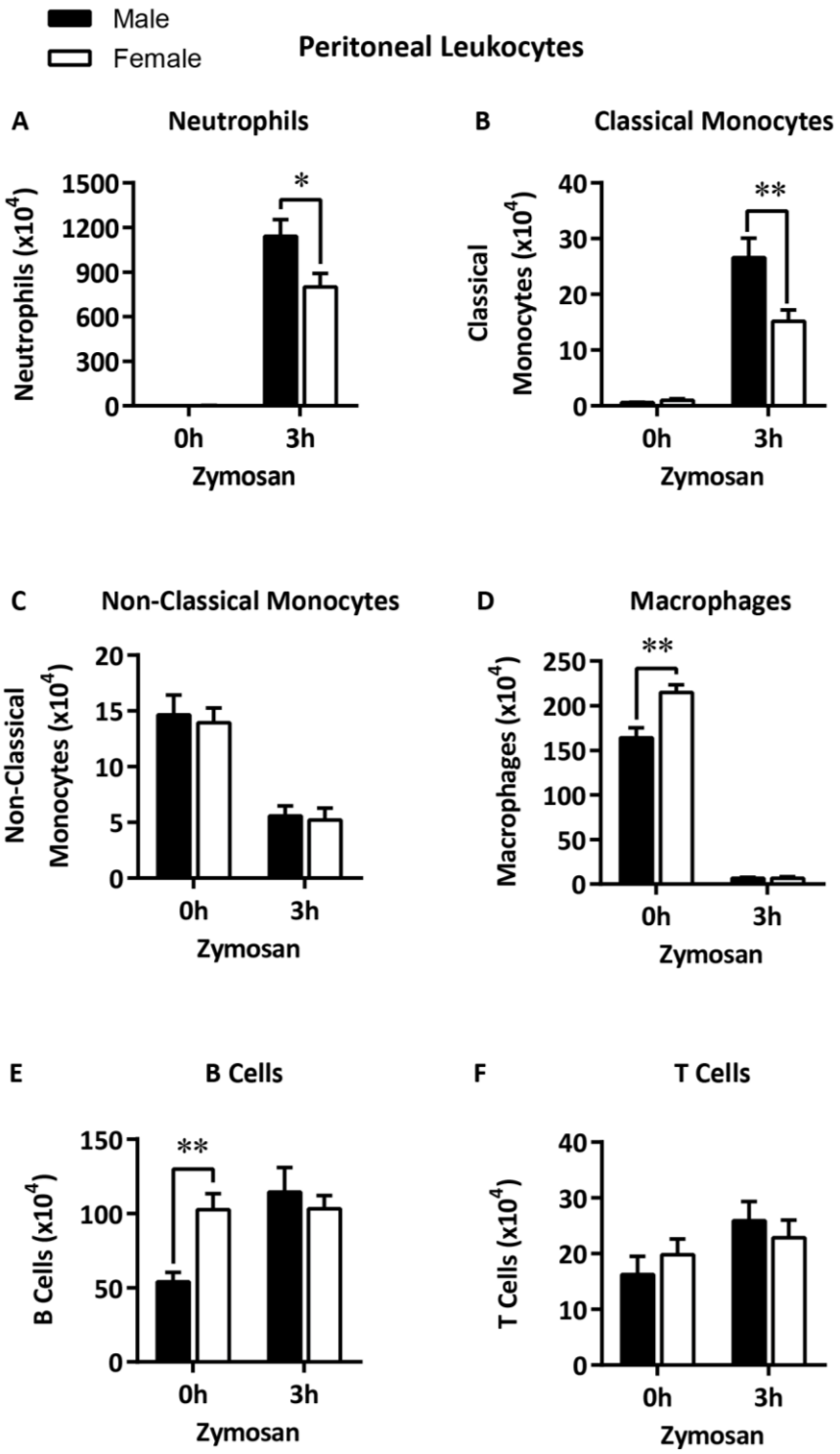


Figure 4.2. Leukocyte subset recruitment in zymosan peritonitis. Male (■) and female (□) mice were treated with or without zymosan (1mg, i.p.) for 3h. Flow cytometry was used to determine the numbers of (A) neutrophils, (B) classical monocytes, (C) non-classical monocytes, (D) macrophages, (E) $B220^+$ B cells, and (F) $CD3^+$ T cells in the peritoneal cavity. Data displayed as mean ± SEM of n mice where n≥14 (neutrophils), n≥10 (classical and non-classical monocytes), n≥9 (macrophages), n≥7 (B cells), and n≥9 (T cells). Comparison of cell numbers at specific time points in female versus male mice was achieved using Student's *t*-test, with significant differences indicated by * (p<0.05), ** (p<0.01).

4.3.3. Sex-differences in circulating leukocytes following zymosan peritonitis

Significant accumulation of neutrophils (p<0.001) and classical monocytes (p<0.001) was seen in the circulation of male and female mice treated with zymosan (1mg, i.p.), peaking at 3h. The 3h peak of neutrophilia was significantly (p<0.01) greater in males (2.7±0.26 x10^6/ml) compared to females (1.6±0.20 x10^6/ml, Figure 4.3A). Equally, classical monocytosis at 3h was significantly (p<0.001) more so in male mice (14.3±1.56 x10^5/ml) versus female mice (6.5±1.21 x10^5/ml, Figure 4.3B). Circulating numbers of non-classical monocytes did not significantly change throughout the peritonitis in either sex (p>0.05). At 3h post zymosan however, a trend toward differential non-classical monocyte regulation resulted in significantly (p<0.01) more cells in the male than female circulation (2.0±0.22 x10^6/ml vs 1.1±0.13 x10^5/ml, Figure 4.3C). By 24h after the induction of peritonitis, blood neutrophils, classical monocytes, and non-classical monocytes had returned to near basal (0h) levels and were no longer different in number between the sexes (p>0.05).

Circulating B cells declined in number 3h after zymosan administration, significantly in male mice (p<0.05) and with a trend toward so in female mice (Figure 4.3D). After 24h, the numbers of B cells in both sexes recovered, with female numbers returning to basal levels, and males elevated beyond the naïve state (p<0.01). This exaggerated response meant at 24h male mice had significantly augmented circulating B cells versus females (1.6±0.18 x10^6/ml vs 1.1±0.69 x10^6/ml) (p<0.05). No significant difference in the number of $CD3^+$ T cells in the blood was seen up to 24h post zymosan (p>0.05) (Figure 4.3E). Furthermore, male and female mice had similar numbers of T cells at the time points analysed (p>0.05).

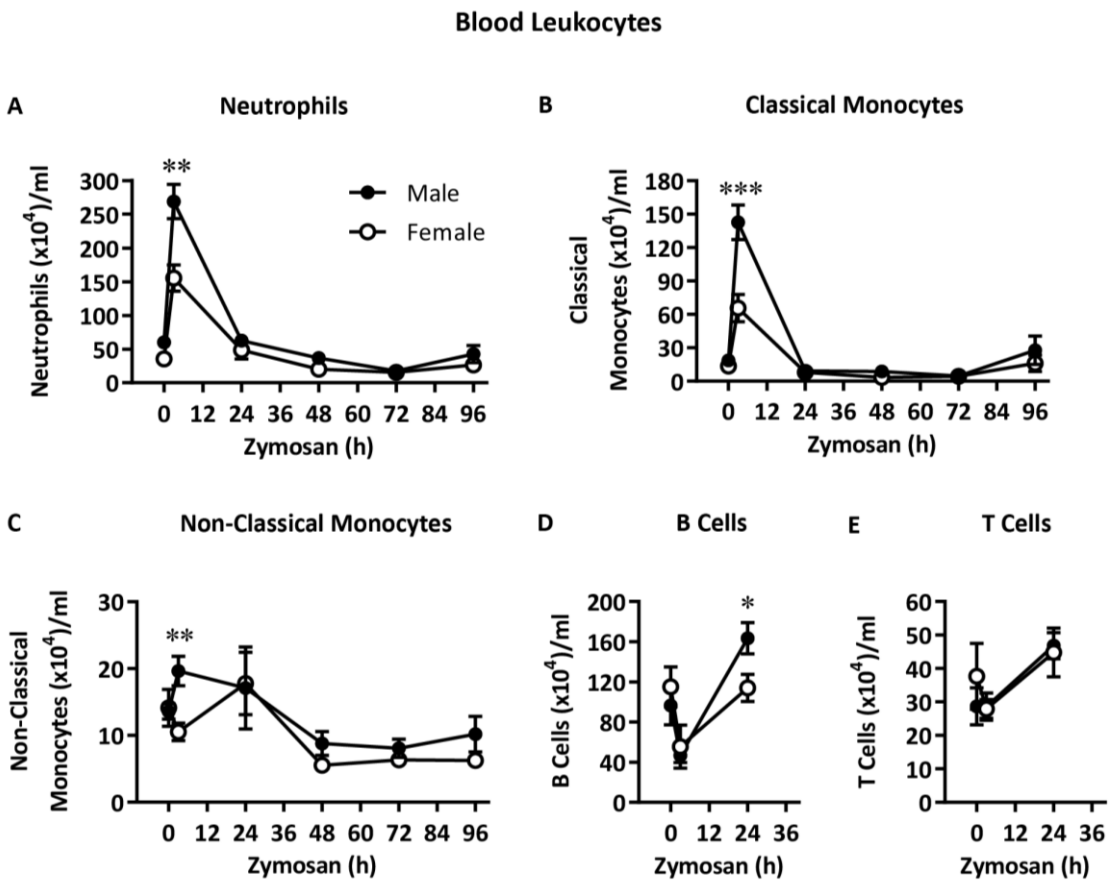


Figure 4.3. Circulating leukocyte subsets in zymosan peritonitis. Zymosan (1mg, i.p.) was administered to male (●) and female (○) mice to induce peritonitis. Numbers of (A) neutrophils, (B) classical monocytes and (C) non-classical monocytes in the blood were determined at 0h (n≥13), 3h (n≥15), 24h (n≥10 (neutrophils); n≥4 (monocytes)), 48h (n=3), 72h (n=3), and 96h (n=3). (D) Numbers of $B220^+$ B cells and (E) $CD3^+$ T cells were determined at 0h (n≥7), 3h (n≥15) and 24h (n≥7). Data is presented as mean ± SEM. Sex-differences at each time point were determined by Student's *t*-test and significance indicated by * (p<0.05), ** (p<0.01), and *** (p<0.001).

4.4. Discussion

The results in this chapter provide evidence for sexual dimorphisms in the size of the inflammatory response in mice. To understand whether sex-differences in leukocyte recruitment are reproducible and extend beyond that of neutrophils, the effect of sex was examined in the murine zymosan peritonitis model of acute inflammation. Indeed, neutrophil accumulation in the peritoneal cavity at 3h post-zymosan was greater in male compared to female mice (Figure 4.4). Most strikingly, male mice recruited significantly more classical monocytes at this time. Interestingly, although monocyte infiltration was previously considered to follow that of neutrophils (Soehnlein *et al.*, 2010), this model demonstrates the ability of classical monocytes and neutrophils to extravasate at the same time, although neutrophils continued to be the principle cell type. Furthermore, while a reduction in the number of recruited neutrophils may be suggested to underlie the reduction in the number of recruited classical monocytes seen in female mice, no correlation exists between the recruitment of these cell types (Appendix 3). To fully disprove this theory however, neutrophil depletion studies could be carried out as have previously been described for thioglycollate peritonitis (Henderson *et al.*, 2003).

Sex-differences in the recruitment of neutrophils and classical monocytes are equally reflected in the blood, with males exhibiting greater numbers of both leukocyte subsets 3h after induction of peritonitis compared to females (Figure 4.4). The neutrophilia and classical monocytosis was, however, transient as by 24h circulating numbers of both cell types returned to basal levels. This is in line with the predicted half-life of a circulating neutrophil of 6-8h (Summers *et al.*, 2010). A sex-difference in the recruitment of neutrophils and classical monocytes therefore appears to be upstream of such disparities already present in the circulation at that time. It is furthermore suggestive that male and female leukocyte adhesion and transendothelial migration mechanisms and kinetics are similar, and a difference in the female cells is not the reason for fewer leukocytes in the peritoneum in response to i.p. zymosan. Nevertheless, to fully prove this hypothesis, evaluation of such kinetics would have to be assessed either *in vivo* using intravital microscopy of the mesentery for example, or *ex vivo* with endothelial monolayers.

Consistent with the literature, resident peritoneal macrophages diminished in number by 3h after induction of peritonitis (Navarro-Xavier *et al.*, 2010). The fate of these macrophages was not commented on in the previous report however it is conceivable that after PAMP recognition, cellular activation, cytokine and chemokine production, and zymosan phagocytosis, these cells undergo apoptosis, accounting for their clearance. Non-classical

monocytes similarly reduce during inflammation, approximately halving in number after 3h. The fate of these cells is less clear and not supported by evidence in the literature. A small increase in circulating non-classical monocytes in male mice is seen at this time however a 'reverse' abluminal to luminal migration in such a model is unlikely and not previously reported. Furthermore, such a phenomenon is not reflected in the female that instead sees a trend toward reduced circulating non-classical monocytes. In fact, the origin of monocyte

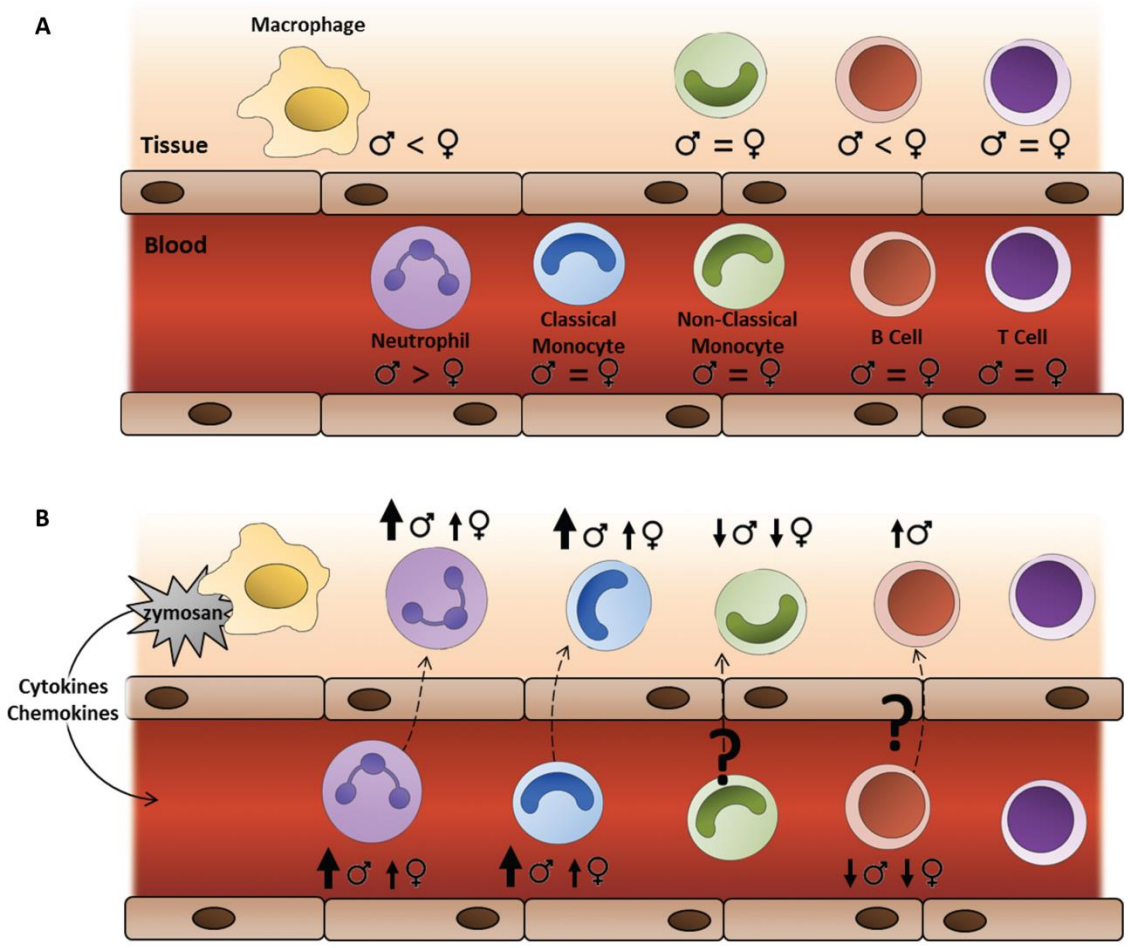


Figure 4.4. Summary of sex-differences in leukocyte recruitment in zymosan peritonitis. (A) Summary of basal circulating (blood) and peritoneal cavity (tissue) neutrophils, classical monocytes, non-classical monocytes, B cells, and T cells in male (♂) and female (♀) mice. Negligible numbers of neutrophils and classical monocytes were present in the naïve cavity. Sex-differences are indicated by '>' or '<'; no difference is indicated by '='. (B) Summary of leukocyte accumulation in the blood and tissue in response to zymosan (1mg, i.p.). Arrows indicate either increase (↑) or decreased (↓) numbers after 3h in male and female mice. Larger arrows indicate greater increase in cells versus the other sex. Broken arrows depict the likely direction of leukocyte movement, where '?' designates uncertainty in this. No arrows signify no suggestion of trafficking or sex-differences.

subsets is highly debated. Some argue that classical monocytes can give rise to non-classical monocytes, doing so by downregulating the Ly6C antigen (Varol *et al.*, 2007). There is, however, no evidence of the reverse of this differentiation occurring, thus a shuttling between monocyte phenotypes is unlikely to explain the observation in this study of increased classical monocytes and decreased non-classical monocytes in peritonitis. It is therefore most plausible and likely to say classical monocytes are a distinct population that extravasate from the circulation to the tissue in zymosan peritonitis.

As expected, T cell numbers in the cavity do not change at the onset of peritonitis in either sex. Conversely and as elucidated in the previous chapter, naïve female mice possess more peritoneal B cells than males. During the onset phase of zymosan peritonitis this sex-difference is lost due to increased numbers of B cells in the male cavity. This is suggestive of either recruitment or local proliferation. Although the increase in male cavity B cells is mirrored by a reduction in the circulation, potentially signifying cell recruitment, a reduction in circulating B cells is equally observed in the female mouse despite no change in peritoneal B cell number during the inflammation. While B cells are generally considered to proliferate in the germinal centres, those present in the peritoneal (and pleural) cavity are not 'normal' B-2 B cells but mostly B-1 B cells (Kantor *et al.*, 1993). B-1 cells differ from B-2 cells as firstly despite being the primary producers of natural antibodies, they do not develop into memory B cells. They also differ on their ability to self-renew from current peritoneal B-1 cells, compared to the B-2 subset which are replenished from BM B cells (Hayakawa *et al.*, 1986). This means they proliferate to replace older cells (Baumgarth, 2011). Whether the extra B cells in the male peritoneum after 3h zymosan are due to proliferation is unknown, however additional experiments using CFSE could address this.

Although 3h would be considered very early for the adaptive immune response, B-1 cells are not regarded as members of the adaptive immune system, but rather the humoral immune system. Quick responses of these cells have previously been documented whereby peritoneal B-1 cells rapidly migrated to the spleen and differentiated into plasma cells in response to LPS (Tung *et al.*, 2007). It is conceivable that the relative protection female mice see in zymosan peritonitis may be attributed to the B-1 cells in the peritoneal cavity. The reasons for this may lie in the capacity of B-1 cells to secrete IgM in greater quantities than IgG (Baumgarth, 2011). Certain secreted IgM antibodies have been shown to be specific for apoptotic cells, and as such may promote clearance of activated macrophages that have phagocytosed zymosan. It is plausible that as females are already known to have more IgM (Bouman *et al.*, 2005) potentially due to greater numbers of B-1 cells, thereby increasing clearance of apoptotic

macrophages and reducing the need for such an intense innate immune cell influx. To investigate such hypotheses it would first be essential to determine the relative quantities of B-1 cells in the naïve and inflamed peritoneum of male and female using specific cell markers beyond the pan-marker of B220. Furthermore, peritoneal and circulating IgM levels could also be compared between the sexes.

This chapter has shown differences in the leukocyte responses of male and female mice to an acute inflammatory stimulus. More specifically, it shows differences in the circulating and subsequently recruited number of neutrophils and classical monocytes. In order to fully dissect the mechanisms of sex-differences in circulating and subsequently recruited neutrophils and classical monocytes in the zymosan peritonitis model, the main leukocyte storage pools must be investigated.

Key findings:

- 1mg zymosan (i.p.) induced rapid accumulation of neutrophils in the peritoneal cavity of male and female mice by 3h after administration. These neutrophils were cleared by 96h.
- Classical monocytes also entered the cavity 3h after induction of zymosan peritonitis.
- Male mice recruit more neutrophils and classical monocytes to the peritoneal cavity at an early, onset, 3h time point after the induction of inflammation in the zymosan peritonitis model.
- Male mice have greater accumulation of circulating neutrophils and classical monocytes at the 3h time point post zymosan administration.
- Male mice have increased peritoneal B cell numbers 3h after induction of inflammation, whilst females show no change in cell numbers.

Chapter 5: Temporal regulation of leukocyte TLR expression in zymosan peritonitis

5.1. Introduction

Since the discovery of *Drosophila* Toll over 20 years ago, mammalian isoforms TLR2 and TLR4 have been arguably the most studied. Their role in the initiation of the inflammatory response is extremely important as TLR knockout mice are highly susceptible to infection and are unable to mount a proper immune response. This is exemplified by TLR2 deficient mice, which have impaired survival in response to Gram-positive bacterial infection (Takeuchi *et al.*, 2000). In addition, TLR4-mutant C3H/HeJ mice, characterised by a point mutation in the TLR4 cytoplasmic region, and C57BL/10ScNJ mice, characterised by a null mutation of *Tlr4*, are highly susceptible to Gram-negative bacterial infections with reduced and delayed chemokine production (Poltorak *et al.*, 1998; Hoshino *et al.*, 1999). Not surprisingly, compounds were developed to target these receptors and initially proved highly efficacious in certain inflammatory conditions. Despite the many that have been synthesised, no compound that targets either receptor has reached the clinic owing to failure in late stage trials. The reasons for these failures remain unclear, however as the precise role of TLRs in the inflammatory response beyond that of PAMP/DAMP recognition is not well characterised, it is unsurprising these drugs failed. Moreover, the exact cellular targets of such compounds are also not evident, and whether they target tissue macrophage, EC, neutrophil, or monocyte TLRs, for example, is unclear. Indeed, these drugs could potentially be targeting TLRs on any cell type, at any location.

What has been reported, however, is that TLR-targeting compounds dampen leukocyte recruitment responses (Arslan *et al.*, 2010b). It is therefore essential to fully understand the expression profile of TLR2 and TLR4 on leukocyte subsets during a course of inflammation. This may provide evidence for modulation of receptor expression during leukocyte recruitment, and furthermore provide potential time points at which TLR-targeted therapies may be better administered to limit uncontrolled leukocyte recruitment and subsequent tissue damage. In addition, as females have been demonstrated to have relative protection versus males in inflammatory disorders, it would be useful to study the temporal expression profile of both receptors to ascertain a comparative profile for both sexes. Indeed, identification of sex-differences in leukocyte TLR2 and TLR4 regulation during inflammation may provide more sex-specific methods of targeting inflammatory disorders pharmacologically.

Previous chapters, in agreement with the literature, have demonstrated that leukocyte subsets express cell surface TLR2 and TLR4. ECs are likewise thought to express TLR2 and TLR4 although there is less evidence in the literature, particularly for expression of TLRs at the protein level (Mullick *et al.*, 2008; Andonegui *et al.*, 2009). Leukocytes also express a plethora of cell adhesion molecules and receptors that aid the transmigration from the luminal to the abluminal side of the vessel by binding to ligands expressed on the endothelium (Nourshargh *et al.*, 2010). L-selectin is one such example, binding to endothelial PSGL-1 to promote leukocyte rolling then subsequently being shed during TEM (Ley *et al.*, 2007). This study also therefore hypothesised that TLRs may play a role more directly in leukocyte recruitment, interacting with the endothelium to potentially aid TEM and in doing so may exhibit modulated receptor expression.

5.2. Aims

Sex-differences in basal leukocyte subset TLR2 and TLR4 expression have been described already in this thesis, with male BM leukocytes expressing more TLR2 and TLR4 than females, and male peritoneal T cells also expressing more TLR2. Dimorphisms in the temporal regulation of leukocyte TLRs following acute inflammation may represent a novel mechanism underlying sex-differences in leukocyte recruitment and inflammatory disease. To further determine whether this may provide a potential mechanism, the following specific aims were addressed:

- Investigate the temporal expression profile of TLR2 and TLR4 on the surface of circulating leukocyte subsets following acute inflammation in the mouse zymosan peritonitis model.
- Investigate the temporal expression profile of TLR2 and TLR4 on the surface of tissue resident and recruited leukocyte subsets in the murine peritoneal cavity following zymosan peritonitis.
- Compare the circulating and peritoneal leukocyte TLR2 and TLR4 expression profiles in male and female mice following zymosan peritonitis.
- Compare TLR expression on neutrophils and classical monocytes in the circulation to those already recruited to the peritoneum to gain insight into any changes during TEM and therefore any more direct involvement of leukocyte TLRs in this process.

5.3. Results

5.3.1. Temporal regulation of circulating leukocyte TLR2 and TLR4 in zymosan peritonitis

Flow cytometry was used to measure expression of TLR2 and TLR4 on the surface of blood leukocyte subsets from male and female mice treated with zymosan (1mg, i.p.) to induce peritonitis. As determined in previous chapters, expression of both receptors on the specific leukocyte subsets was similar in male and female naïve mice (p>0.05). Female blood neutrophil TLR2 was significantly (p<0.05) higher on cells present after the peak of neutrophilia at 24h (2.4±0.12) versus naïve and peak (3h) inflamed states (1.7±0.09 and 1.5±0.08 respectively) (Figure 5.1A). Towards the resolution phase (>72h) of the peritonitis, TLR2 expression returned to basal levels. No significant change in male neutrophil TLR2 was observed throughout the inflammation (p>0.05).

Circulating classical monocyte TLR2 levels were similar in the course of inflammation in both male and female mice (p>0.05) (Figure 5.1B). Like neutrophils, female non-classical monocytes were transiently elevated in TLR2 (p<0.05) at 24h versus control (3.9±0.33 versus 2.9±0.12) yet those cells present afterwards showed similar expression to control conditions (p>0.05) (Figure 5.1C). Again, male non-classical monocyte TLR2 was similar in inflammation to unstimulated (0h) conditions (p>0.05). $B220^+$ B cells and $CD3^+$ T cells had low and unchanged TLR2 (p>0.05) and TLR4 (p>0.05) expression in inflammation (Figure 5.1D-E).

Expression of circulating murine leukocyte TLR4 was also examined. TLR4 expression was transiently higher on neutrophils at 24h compared to those at 0h in both male (24h: 3.2±0.29; 0h: 2.1±0.23) (p<0.05) and female (24h: 3.2±0.26; 0h: 2.1±0.18) (p<0.001) mice (Figure 5.2A). Aside from this, neutrophil TLR4 was similar throughout the time course of zymosan peritonitis in both sexes (p>0.05). By the same pattern, classical monocytes also transiently show higher TLR4 expression at 24h compared to control in both male (24h: 4.4±0.56; 0h: 2.6±0.25) (p<0.01) and female (24h: 3.8±0.39; 0h: 2.4±0.27) (p<0.01) mice (Figure 5.2B). At all other time points however, TLR4 expression did not differ from control in either sex (p>0.05). No significant changes in non-classical monocyte (p>0.05), $B220^+$ B cell (p>0.05), or $CD3^+$ T cell (p>0.05) TLR4 expression were observed at all times sampled following induction of inflammation (Figure 5.2C-E).

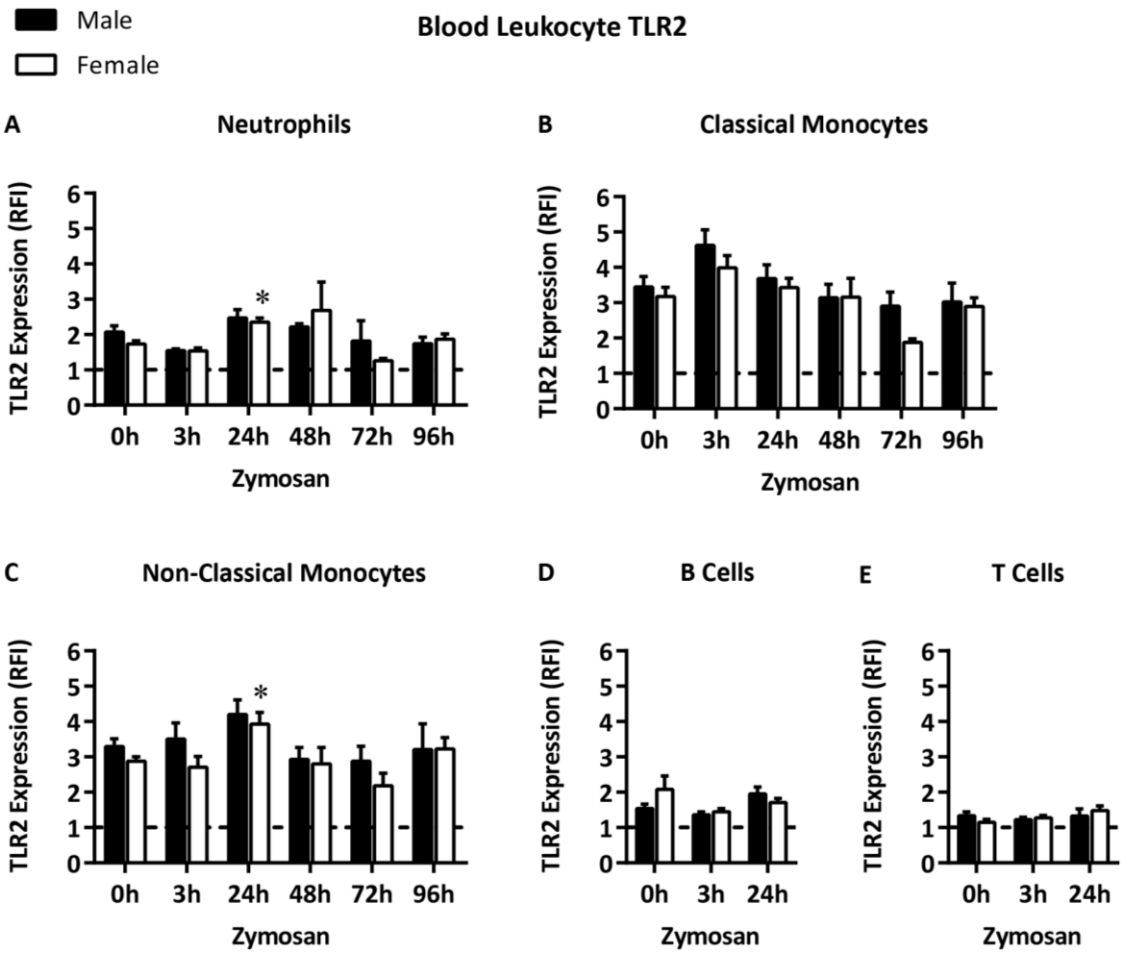


Figure 5.1. Temporal expression profile of TLR2 on circulating leukocyte subsets during zymosan peritonitis. Male (■) and female (□) mice were injected with 1mg zymosan i.p. to induce peritonitis. Surface TLR2 was assessed on (A) neutrophils, (B) classical monocytes, (C) non-classical monocytes, (D) $B220^+$ B cells, and (E) $CD3^+$ T cells in the blood at the indicated time points after induction of inflammation. TLR2 expression was measured as relative fluorescence intensity (RFI) to an isotype control antibody. Data shown is the mean ± SEM of neutrophils and monocyte subsets: n≥12 mice (0h), n≥13 (3h), n≥9 (24h), n=3 (48h), n=2 (72h), n=3 (96h); B and T cells: n≥5 (0h), n≥6 (3h), n≥5 (24h). Temporal changes in expression versus naïve conditions were determined by 1-way ANOVA with Bonferroni's post-test for multiple comparisons. Significant differences in males were indicated by # (p<0.05), and in females by * (p<0.05).

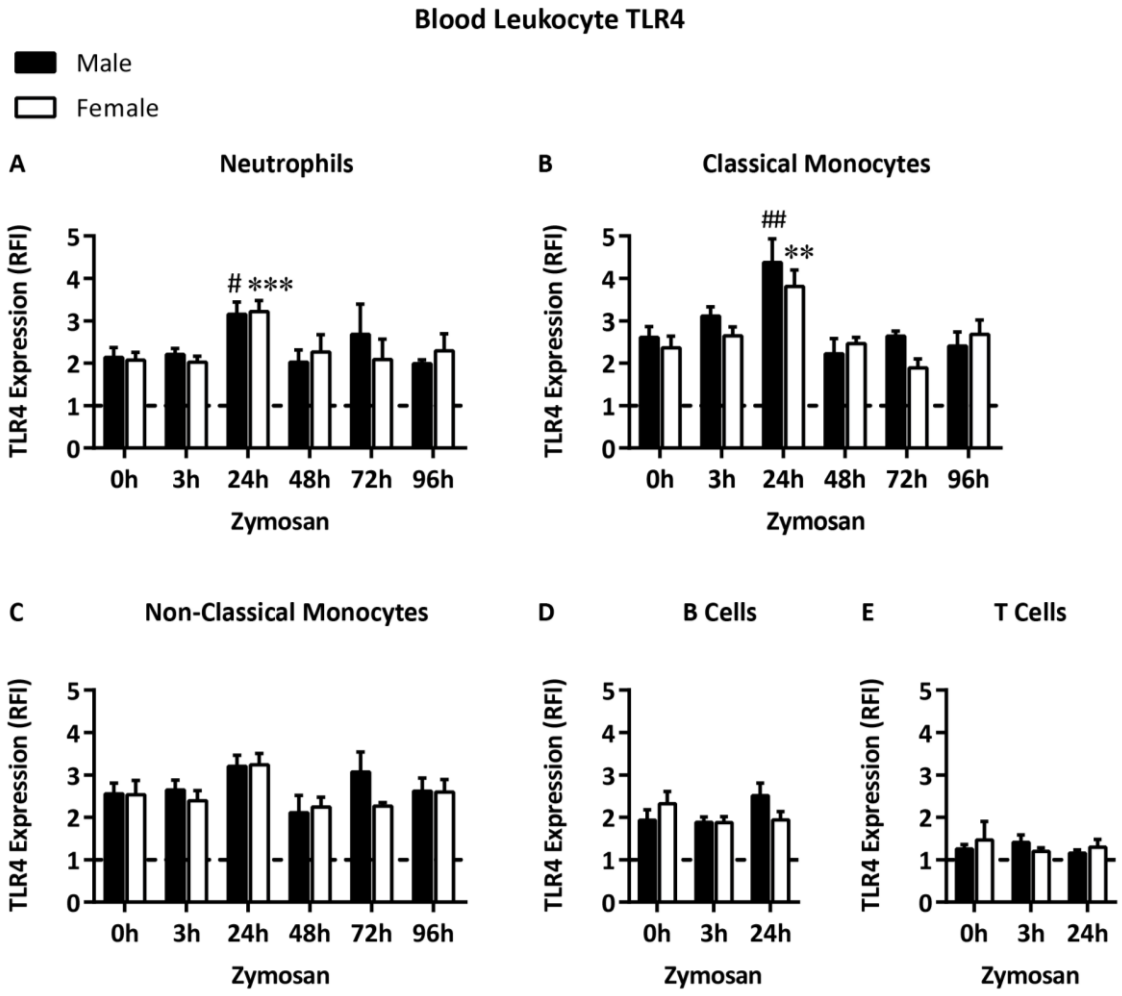


Figure 5.2. Temporal expression profile of TLR4 on circulating leukocyte subsets during zymosan peritonitis. Male (■) and female (□) mice were treated with zymosan (1mg, i.p.) for the indicated times to induce peritonitis. Cell surface TLR4 was evaluated on (A) neutrophils, (B) classical monocytes, (C) non-classical monocytes, (D) $B220^+$ B cells, and (E) $CD3^+$ T cells in the circulation at the specified time points post induction of zymosan peritonitis. TLR4 expression was measured as relative fluorescence intensity (RFI) to isotype control antibody. Data displayed as mean ± SEM of neutrophils and monocyte subsets: n≥12 mice (0h), n≥13 (3h), n≥9 (24h), n=3 (48h), n=2 (72h), n=3 (96h); B and T cells: n≥5 (0h), n≥6 (3h, 24h). Changes in expression versus naïve (0h) conditions were determined by 1-way ANOVA with Bonferroni's post-test for multiple comparisons. Significant differences in males were indicated by # (p<0.05), ## (p<0.01) and in females by ** (p<0.01), and *** (p<0.001).

5.3.2. Temporal regulation of peritoneal leukocyte TLR2 and TLR4 in zymosan peritonitis

In contrast to accumulated neutrophils in the peritoneal cavity at 3h, those present at 24h post-zymosan expressed significantly (p<0.001) higher TLR2 in both male (3h: 1.8±0.08; 24h: 3.2±0.30) and female mice (3h: 1.5±0.07; 24h: 3.2±0.33) (Figure 5.3A). Despite female neutrophils at 24h still having elevated TLR2 (p<0.05), this observation was transient as both male and female neutrophils analysed at all other later time points did not differ in their expression of TLR2 to those first recruited at 3h (p>0.05). Not enough neutrophils or classical monocytes were present in the naïve peritoneum to determine TLR2 expression (Figure 5.3B).

No significant change in TLR2 expression was evident on non-classical monocytes at 3h versus 0h in either sex however a trend towards increased TLR2 on female cells in inflammation was seen (p=0.068) (Figure 5.3C). No modulation of TLR2 levels on $B220^+$ B cells (p>0.05) or $CD3^+$ T cells (p>0.05) was seen during the inflammation (Figure 5.3D-E).

Comparable to TLR2, TLR4 was transiently higher on neutrophils at 24h in the cavity of both male (3h: 2.2±0.17; 24h: 3.4±0.27) (p<0.01) and female (3h: 1.8±0.14; 24h: 3.4±0.27) (p<0.001) mice (Figure 5.4A). This subsided by 48h in males whereby expression resembled that of the neutrophils at 3h, however TLR4 remained elevated on female neutrophils throughout the inflammation until 96h. As with TLR2, not enough classical monocytes were present in the naïve cavity to accurately determine TLR4 expression, and thus it's change in inflammation (Figure 5.4B).

Expression of non-classical monocyte TLR4 was significantly (p<0.001) lower on those cells present in the male, but not female, peritoneum at 3h post-zymosan versus 0h (3.3±0.11 versus 1.5±0.11) (Figure 5.4C). Peritoneal $B220^+$ B cells from female mice showed a small but significant (p<0.05) reduction in TLR4 expression in response to zymosan (0h: 2.2±0.20; 3h: 1.6±0.14; 24h: 1.5±0.09), whilst those from males did not differ from naïve controls (p>0.05) (Figure 5.4D). No change in $CD3^+$ T cell TLR4 with inflammation was apparent (p>0.05) (Figure 5.4E).

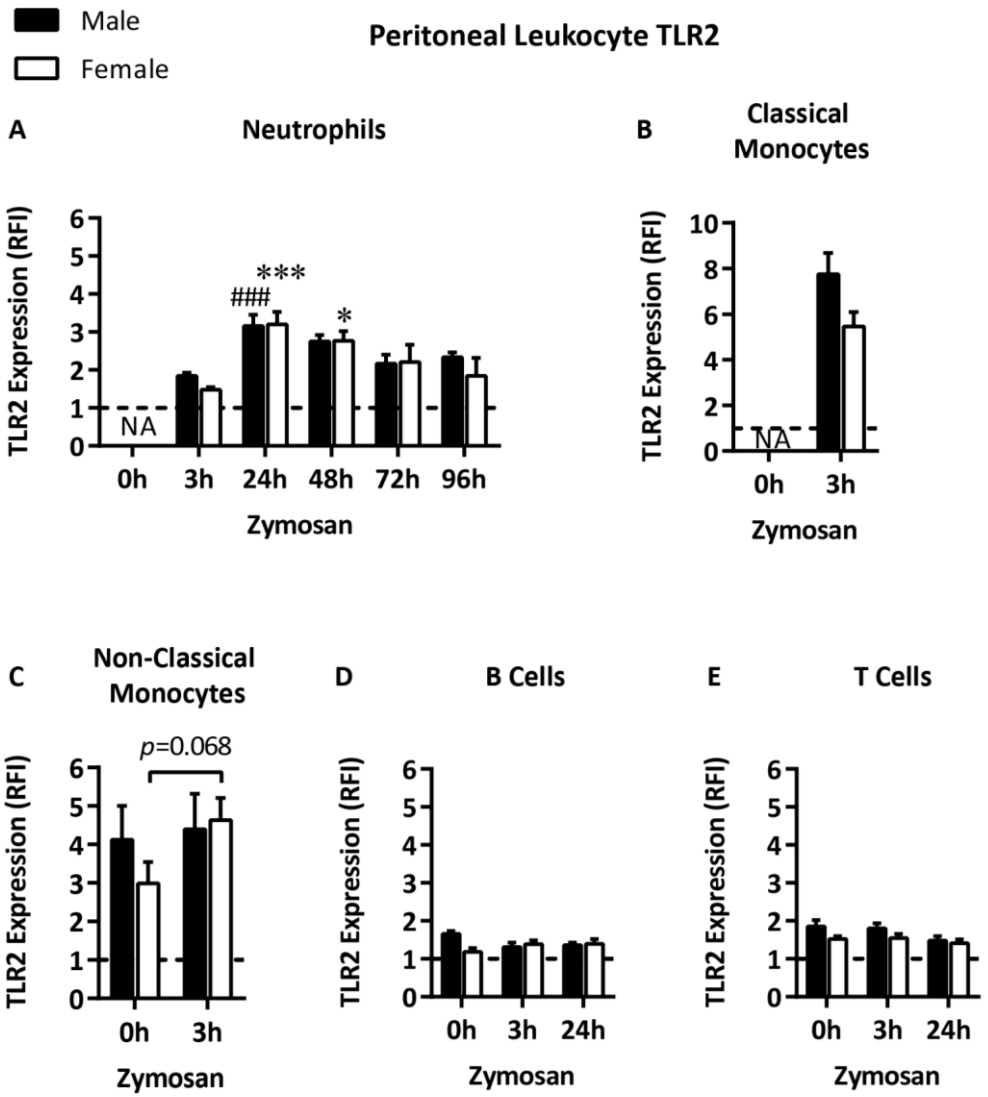


Figure 5.3. Temporal TLR2 expression profile on peritoneal leukocyte subsets in response to zymosan peritonitis. Male (■) and female (□) mice were treated with zymosan (1mg, i.p.) to induce inflammation. Cell surface TLR2 was determined on (A) neutrophils, (B) classical monocytes, (C) non-classical monocytes, (D) $B220^+$ B cells, and (E) $CD3^+$ T cells in the peritoneal cavity at the indicated time points post induction of zymosan peritonitis. TLR2 expression was measured as relative fluorescence intensity (RFI) to isotype control antibody. Changes in expression on non-classical monocytes and lymphocytes (B and T cells) over time versus naïve conditions, and changes on neutrophils over time versus those first present at 3h, were determined by 1-way ANOVA with Bonferroni's post-test for multiple comparisons. Significant differences in males were indicated by ### (p<0.001), and in females by * (p<0.05), and *** (p<0.001). NA, not applicable as cell number was too low to quantify expression. Data shown as mean ± SEM. Neutrophils: n≥15 mice (3h), n≥10 (24h), n=3 (48h, 72h), n=2 (96h); classical monocytes: n≥5 (3h); non-classical monocytes: n≥3 (0h), n≥5 (3h); B and T cells: n≥3 (0h), n≥6 (3h, 24h).

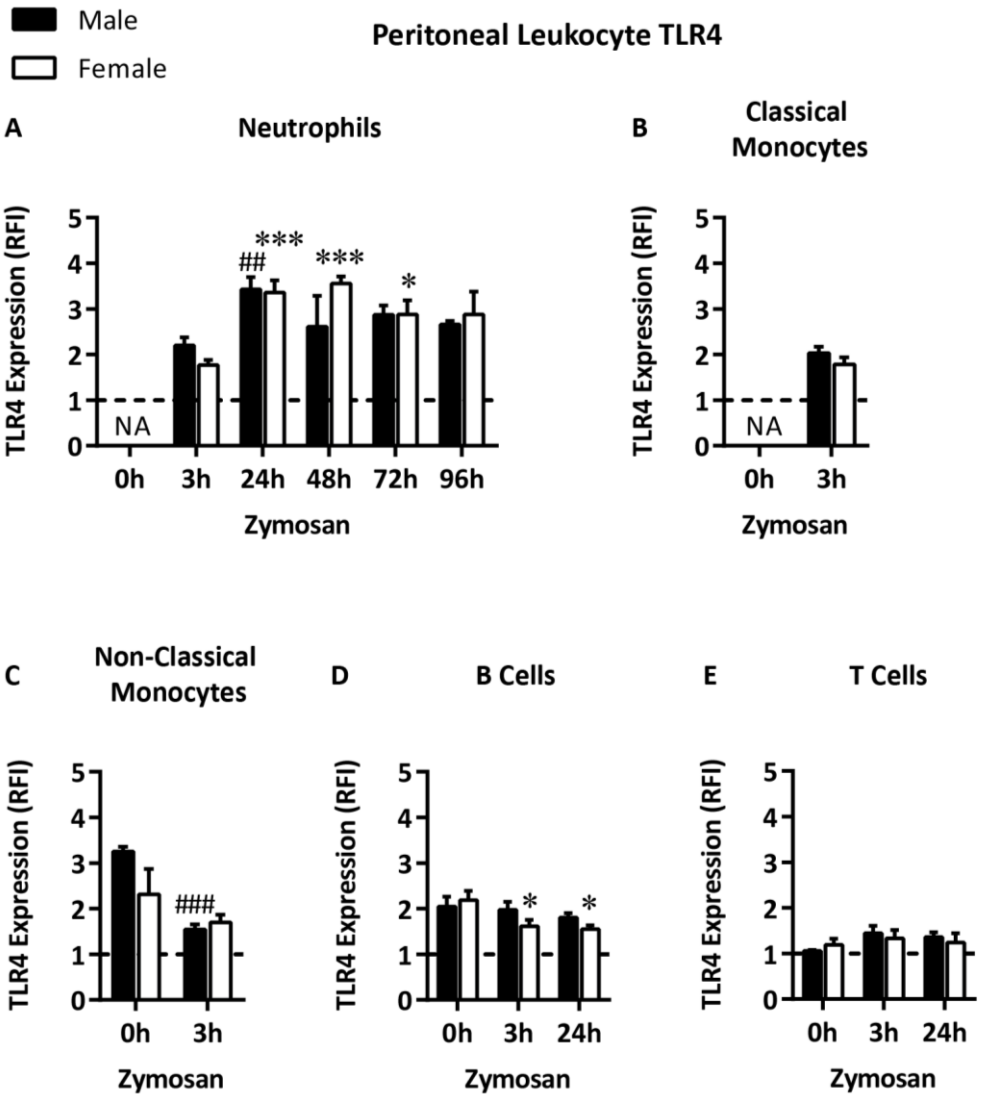


Figure 5.4. Temporal TLR4 expression profile on peritoneal leukocyte subsets in response to zymosan peritonitis. Male (■) and female (□) mice were treated with zymosan (1mg, i.p.) to induce inflammation. TLR4 expression was determined on the surface of peritoneal (A) neutrophils, (B) classical monocytes, (C) non-classical monocytes, (D) $B220^+$ B cells, and (E) $CD3^+$ T cells at the indicated time points after induction of peritonitis. TLR4 was expressed as relative fluorescence intensity (RFI) to isotype control antibody. Changes in expression on non-classical monocytes, B cells and T cells over time versus naïve conditions, and changes on neutrophils over time versus those first present at 3h, were determined by 1-way ANOVA with Bonferroni's post-test for multiple comparisons. Significant differences in males were indicated by ## (p<0.01), ### (p<0.001), and in females by * (p<0.05), and *** (p<0.001). NA, not applicable due to cell number too low to quantify expression. Data shown as mean ± SEM. Neutrophils: n≥14 mice (3h), n≥10 (24h), n=3 (48h, 72h), n=2 (96h); classical monocytes: n≥5 (3h); non-classical monocytes: n≥3 (0h), n≥5 (3h); B and T cells: n≥3 (0h), n≥6 (3h, 24h).

5.3.3. Potential role of TLRs in transendothelial migration

Chapter 4 of this thesis showed that in response to zymosan peritonitis, neutrophils and classical monocytes are recruited to the tissue at a peak of 3h. Both neutrophils and classical monocytes also express TLR2 and TLR4. Changes in TLR2 and TLR4 on leukocytes from the circulation to the tissue may represent a more direct involvement of these receptors in TEM. The rationale being that leukocyte recruitment in inflammation is reduced with TLR antagonist treatment (Arslan *et al.*, 2010b). To gain insight into whether leukocyte TLR2 or TLR4 are more directly involved in TEM, the expression of TLR2 and TLR4 was compared on non-recruited circulating leukocytes versus those already recruited (transmigrated) in response to intraperitoneal zymosan.

TLR2 ($p>0.05$) and TLR4 ($p>0.05$) expression was similar on neutrophils present in the blood (not transmigrated) versus the peritoneal cavity (transmigrated) of male and female mice at 3h post induction of peritontitis (Figure 5.5). Classical monocytes however displayed a trend towards an upregulation of TLR2 on entering the tissue as those cells in the cavity showed a tendancy for higher TLR2 expression than those still in the circulation in both sexes (Figure 5.6A). This was not however significant. No significant differences in classical monocyte TLR4 expression was observed between circulating and recruited monocytes in male or female mice (Figure 5.6B).

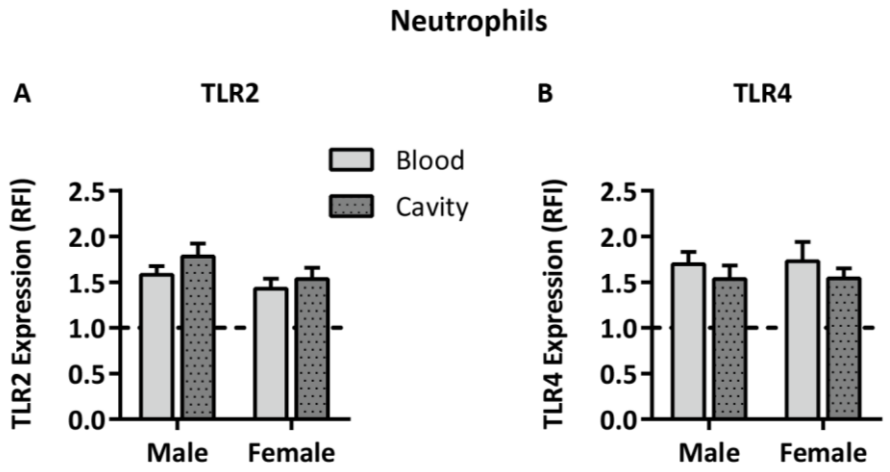


Figure 5.5. Comparison of circulating and recruited neutrophil TLR expression. (A) Effect of transmigration on neutrophil TLR2 and (B) TLR4 expression was evaluated in male and female mice treated with zymosan (1mg, i.p.) for 3h. Cells in the circulation (blood, light grey bars) versus those already recruited (cavity, dark grey bars) at 3h post-zymosan were compared for TLR expression. Expression is quantified as relative fluorescence intensity (RFI) compared to an isotype antibody. Differences in TLR expression between cellular compartments were assessed by Student's *t*-test. Data shown as mean ± SEM of at least 4 (blood), and 6 mice (cavity).

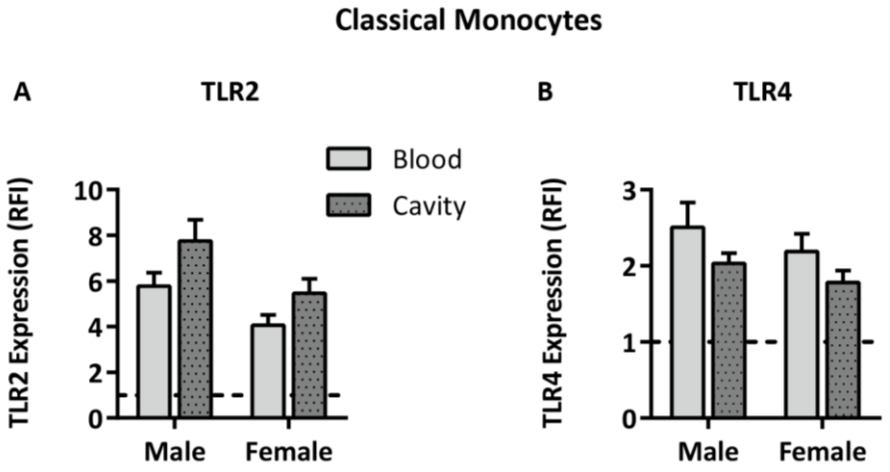


Figure 5.6. Comparison of circulating and recruited classical monocyte TLR expression. (A) Effect of transmigration on classical monocyte TLR2 and (B) TLR4 expression was assessed in male and female mice treated with zymosan (1mg, i.p.) for 3h. Cells in the circulation (blood, light grey bars) compared to those recruited (cavity, dark grey bars) at 3h post-zymosan were compared for TLR expression. TLRs were expressed as relative fluorescence intensity (RFI) to isotype control antibody. Differences in TLR expression between cellular compartments were assessed by Student's *t*-test. Data shown as mean ± SEM of at least 4 (blood), and 5 mice (cavity).

5.4. Discussion

The results in this chapter show that TLR2 and TLR4 expression on certain circulating and tissue leukocyte subsets are temporally modulated in the murine zymosan induced peritonitis model of acute inflammation. Most strikingly, neutrophils in both the circulation and the peritoneal cavity at the post-peak response time of 24h were homogeneously higher in both TLR2 and TLR4 compared to those neutrophils in the respective compartments at earlier time points. This was despite neutrophils being fewer in number at 24h as shown earlier in this thesis. A possible explanation may lie with neutrophil subsets. It has been reported that LPS induces 3 subsets of circulating human neutrophils, distinguishable by nuclear morphology and expression of cell surface markers (Pillay *et al.*, 2012). Indeed, a subset defined as $CD11c^{bright}$/ $CD62L^{dim}$/ $CD11b^{bright}$/ $CD16^{bright}$ was able to suppress T cell proliferation via H_2O_2 release in a Mac-1 dependent manner. Further investigation of both phenotype and function of TLR^{high} neutrophils at 24h may identify possible roles for these cells in dampening of the immune response and resolution of inflammation. Alternatively, as neutrophils are decreasing in number by 24h, it is possible the cells are apoptotic or in a state of stress whereby the expression of many molecules, TLRs included, are upregulated. To determine whether this is the case, further phenotypical analysis of these cells must be carried out. Although in some cases the elevated receptor expression persisted through to 48h after the induction of inflammation, the general trend was transient and TLRs return to basal levels shortly after. Of note, this phenomenon was observed in both male and female mice, and this lack of dimorphism was in line with similar neutrophil numbers in the both the blood and tissue between the sexes at the 24h time point.

Conversely, neutrophils in the peritoneal cavity of male mice at the 3h peak of cell recruitment were significantly higher in TLR2, and showed a trend towards higher TLR4, than those in the female peritoneum. As previously eluded to in this thesis, naïve neutrophils in the circulation of male and female mice have similar TLR2 and TLR4 expression. Neutrophils in the male BM however, express more TLR2 and TLR4 in comparison to females. Thus, it may be fair to speculate that as blood neutrophils would rapidly extravasate in response to zymosan, those cells in the BM and spleen may be mobilised and recruited a little later, and these neutrophils are in fact accumulating in the tissue at 3h, higher in TLR2 and TLR4 in male mice. It is therefore essential to further investigate the BM and spleen as stores of deployable neutrophils.

Another explanation may lie within dimorphisms in the extent of inflammatory cell recruitment. This thesis has already shown male mice recruit more neutrophils than females in

peritonitis. It is therefore tempting to speculate that in addition to more cells, males may also require a somewhat more activated neutrophil phenotype in order to respond effectively to the PAMP. A characteristic of this activation may constitute upregulation of PRRs, including TLR2 and TLR4, however further analysis of these neutrophils phenotype and function would be required to prove such a hypothesis. This is not however the first example of modulation of TLRs in inflammation, for example TLR2 and TLR4 on neutrophils were elevated in human sepsis patients (Härter *et al.*, 2004). Nevertheless, neutrophil TLR2 and TLR4 expression is relatively low, and so the physiological relevance of the modulation to these small degrees remains unknown. *Ex vivo* assays comparing TLR^{high} and TLR^{low} neutrophils would likely provide some insight into this.

Unlike clear patterns of temporal TLR modulation on neutrophils, the remaining circulating leukocyte subsets show some specific but more random changes in TLR2 and TLR4. Circulating male and female classical monocytes have transiently increased expression of TLR4, but not TLR2 at 24h after induction of peritonitis. Similarly, circulating male and female non-classical monocytes have transient increased expression of TLR2, but not TLR4, at 24h after zymosan. It is perhaps possible that these cells represent monocytes released from storage pools such as the BM or spleen, which exhibit a different TLR phenotype. Although under basal conditions monocytes from such stores have similar TLR2 and TLR4 expression to their circulating counterparts, it is possible over 24h of inflammation that reserve pool leukocytes upregulate TLRs by some mechanism. Thus, it would be necessary to investigate the BM and spleen storage pools, not only with regard to trafficking of leukocytes, but also with regard to temporal expression patterns of TLR2 and TLR4.

As perhaps predicted due to their adaptive immune nature, no modulation of circulating or peritoneal cavity lymphocyte TLRs was seen at the time points covered, except for a small, but nonetheless significant reduction in female cavity B cell TLR4 expression. Classical monocytes that had accumulated in the male peritoneal cavity after only 3h were not only more numerous as demonstrated already in this thesis, but also showed a trend towards having more TLR2, but not TLR4, than females. Of note these monocytes had higher expression than circulating counterparts. It could be proposed that if male classical monocytes are higher in TLR2, they may have potential to be involved in the exaggerated response to zymosan male mice demonstrated compared to females. Increased PAMP recognition by male classical monocytes may propagate the inflammatory response and this may even encourage further neutrophil recruitment, although the order of this opposes the accepted paradigm (Soehnlein *et al.*, 2010). An alternative explanation may lie with inherent differences between other male

and female innate immune cells. Female macrophages are more numerous, better at phagocytosis, and have greater NADPH oxidase activity than male equivalents (Scotland *et al.*, 2011). It is possible that female mice clear zymosan earlier than males and it is therefore more necessary for the male classical monocytes with the aforementioned higher TLR2 to indeed have higher receptor expression in order to aid zymosan recognition and pathogen clearance. To explore this hypothesis, the use of labelled zymosan in a similar time course would determine such clearance. Interestingly, male peritoneal non-classical monocytes at 3h after zymosan are lower in TLR4, but not TLR2, with the same trend evident in females. A possible explanation aligns with the reduction in the number of these cells at this time point. It could be that non-classical monocytes, although early in the inflammation, may be differentiating to an M2 macrophage or DC phenotype. Further experiments would be required to determine the nature of these cells.

A further aim of this chapter was to gain insight into whether TLR2 and TLR4 were more directly involved in leukocyte recruitment from the circulation to the tissue. This thesis has already demonstrated neutrophils and classical monocytes to be virtually absent from the peritoneum in naïve mice but to accumulate in the tissue within 3h in response to zymosan peritonitis. Receptor expression was therefore compared on cells remaining in the blood with those already recruited (i.e. transmigrated) with the hypothesis that if TLR2 or TLR4 were directly involved in TEM then the receptor might be shed, internalised, or down regulated on entering the tissue, in a similar fashion to that of L-selectin (Ley *et al.*, 2007). No significant difference in receptor expression between the blood and peritoneum was evident for either neutrophils or classical monocytes. This is suggestive of these receptors not playing a role in the leukocyte-endothelial interaction. To fully exclude this possibility however, the adhesion, rolling, crawling and TEM mechanics should be investigated with intravital microscopy in TLR2 and TLR4 deficient versus WT mice, or alternatively *in vitro* using endothelial monolayers.

Overall, this chapter has shown male and female circulating and recruited neutrophils regulate expression of TLR2 and TLR4 temporally over the time course of the murine zymosan peritonitis model of acute inflammation. Furthermore, experiments suggest no change in the receptors occurs after TEM. Classical monocytes similarly show no change in their TLR2 and TLR4 expression on transmigration. This chapter also shows temporal modulation of these receptors expression is indeed evident on some other leukocyte subsets however no pattern in this regulation is clear.

Key findings:

- Cavity neutrophils transiently show more TLR2 and TLR4 at 24h after induction of peritonitis in both male and female mice.
- Cavity non-classical monocytes showed reduced TLR4, but not TLR2, 3h post-zymosan in male mice, and showed a trend towards this in females.
- Female, but not male, peritoneal B cells after 3 and 24h of zymosan peritonitis had lower TLR4.
- TLR2 and TLR4 expression on neutrophils and classical monocytes was similar on those remaining in the circulating versus those already recruited in response to zymosan peritonitis.

Chapter 6: Investigating sex-differences in tissue cytokine environments in zymosan peritonitis

6.1. Introduction

The typical paradigm suggests that upon PAMP or DAMP recognition by innate immune cell PRRs, cells are activated and inflammatory mediators are released, activating and leading to the recruitment of other inflammatory cells. Such mediators include cytokines (e.g. TNF and IL-1β), chemokines (e.g. CCL2 and CXCL1), and chemoattractants (e.g. LTB_4). Collectively, networks of inflammatory mediators indicate the site of infection, and activate and induce changes in the endothelium, in doing so promoting leukocyte recruitment. The characteristics of this response are specific to the dose and nature of the stimuli and are therefore highly variable. In the same process immune regulatory mediators such as IL-10 and lipoxins are released that control the inflammatory response and ensure the pathogen or damaged tissue is cleared whilst avoiding the dangerous and potentially fatal so-called 'cytokine storm'.

Chapter 4 of this thesis demonstrated sex-differences in leukocyte recruitment in an acute inflammatory model in mice. To investigate these findings and furthermore determine at which stage in the immune response these sex-differences initiate, the cytokine environments of male and female mice during zymosan peritonitis were studied. In addition, the potential capability of male and female murine macrophages to detect zymosan was investigated by assessing the expression of zymosan-associated PRRs TLR2, TLR6, and dectin-1 on the surface of these cells.

Sex-differences in cytokine responses have previously been described in both humans and animals. For example, in healthy adults male monocytes were able to produce more TNF than females (Lefevre *et al.*, 2012), whilst in a disease state, female sepsis patients exhibited more IL-10, and less TNF, than males (Fish, 2008). In rodents, female rats had reduced expression of TNF, IL-1β, and IL-6 after acute myocardial I/R compared to males (Ertan *et al.*, 2007), and furthermore produced less CCL3, CCL5, CCL24, and CCL28 during abdominal aortic aneurysms (Sinha *et al.*, 2006). There are many, sometimes conflicting, reports providing evidence for different inflammatory mediator responses in males and females in inflammation. Differential cytokine responses of male and female mice may indicate reasons for dimorphisms in the inflammatory response to zymosan peritonitis. Should this be the case, the influence of sex

should be considered when proposing pharmacological interventions in inflammatory disorders both at the developmental and clinical stages.

6.2. Aims

This thesis has shown male mice accumulate more neutrophils and classical monocytes in the tissue and circulation in response to zymosan peritonitis. As a result, we sought to dissect whether the mechanisms of such dimorphisms in leukocyte trafficking in inflammation were linked to differences in cytokine environments and responses. Therefore, this chapter has the following specific aims:

- Investigate sex-differences in the expression of zymosan-sensing receptors on peritoneal macrophages.
- Examine sex-differences in peritoneal cytokine and chemokine environments in naïve mice.
- Determine the key cytokines released in the tissue during the early phases of zymosan peritonitis.
- Investigate any sex-differences in cytokine production in the cavity in zymosan peritonitis.

6.3. Results

6.3.1. Peritoneal macrophage zymosan receptor expression

Previous results confirmed in Chapter 3 demonstrate female mice have significantly more resident peritoneal macrophages than male counterparts. To determine any differences in the ability of male and female mice to recognise the PAMP, naïve male and female macrophages were assessed for the cell surface expression of the zymosan receptors TLR2, TLR6, and dectin-1. Levels of TLR2 and the more highly expressed TLR6 were not significantly different on F4/80^{high} macrophages from male and female mice (p>0.05) (Figure 6.1). Conversely, female peritoneal macrophages had a small but significant (p<0.05) increased expression of dectin-1 on their cell surface compared to males (RFI: 4.5±0.17 versus 4.1±0.12, respectively) (Figure 6.1).

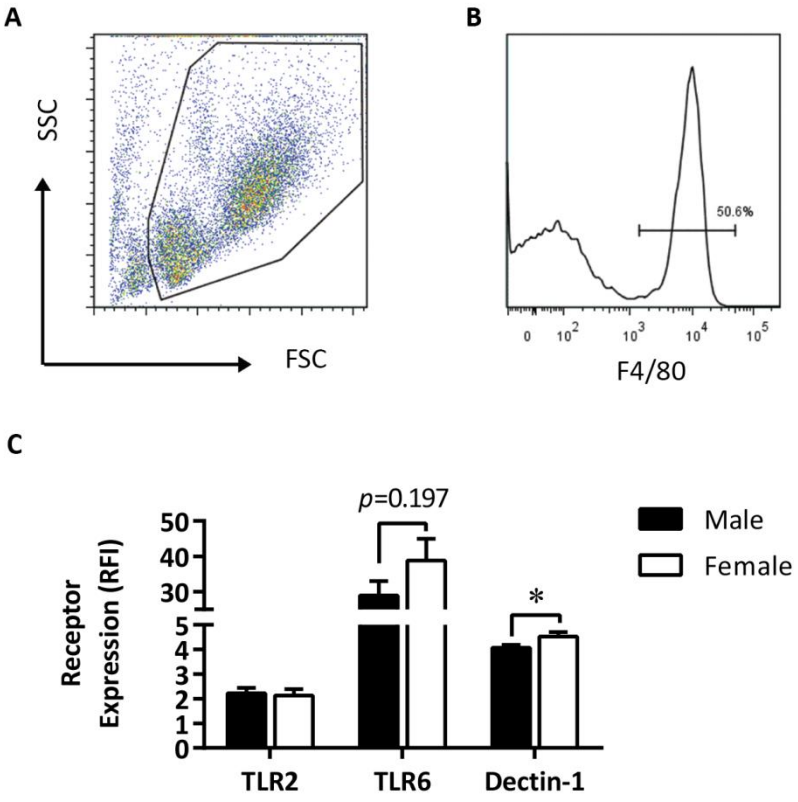


Figure 6.1. Sex-differences in peritoneal macrophage zymosan receptor expression. Expression of TLR2, TLR6, and dectin-1 were assessed on resident peritoneal macrophages isolated from naïve male (■) and female (□) mice. (A) Peritoneal lavage leukocyte gating strategy based on FSC and SSC. (B) Subsequent gating on F4/80^{high} cells to identify macrophages. (C) Expression of TLR2 (n≥18), TLR6 (n≥14), and dectin-1 (n≥11) evaluated as relative fluorescence intensity (RFI) to an isotype antibody. Data is shown as mean ± SEM of n mice. Differences between sexes were determined by Student's *t*-test and significance indicated by * (p<0.05).

6.3.2. Basal peritoneal cytokine environments

The cytokine profile in the resting peritoneal cavity was next investigated using cytokine arrays. Peritoneal fluid was obtained from naïve male and female mice and the protein content of each sample quantified using the BCA protein assay. Peritoneal cell fractions were analysed by flow cytometry and deemed 'naïve' if less than 1% of leukocytes were neutrophils. 4mg of protein was assayed with the Proteome Profiler™ mouse cytokine array (R&D Systems) and male and female membranes were exposed for equal times (3mins) (Figure 6.2A).

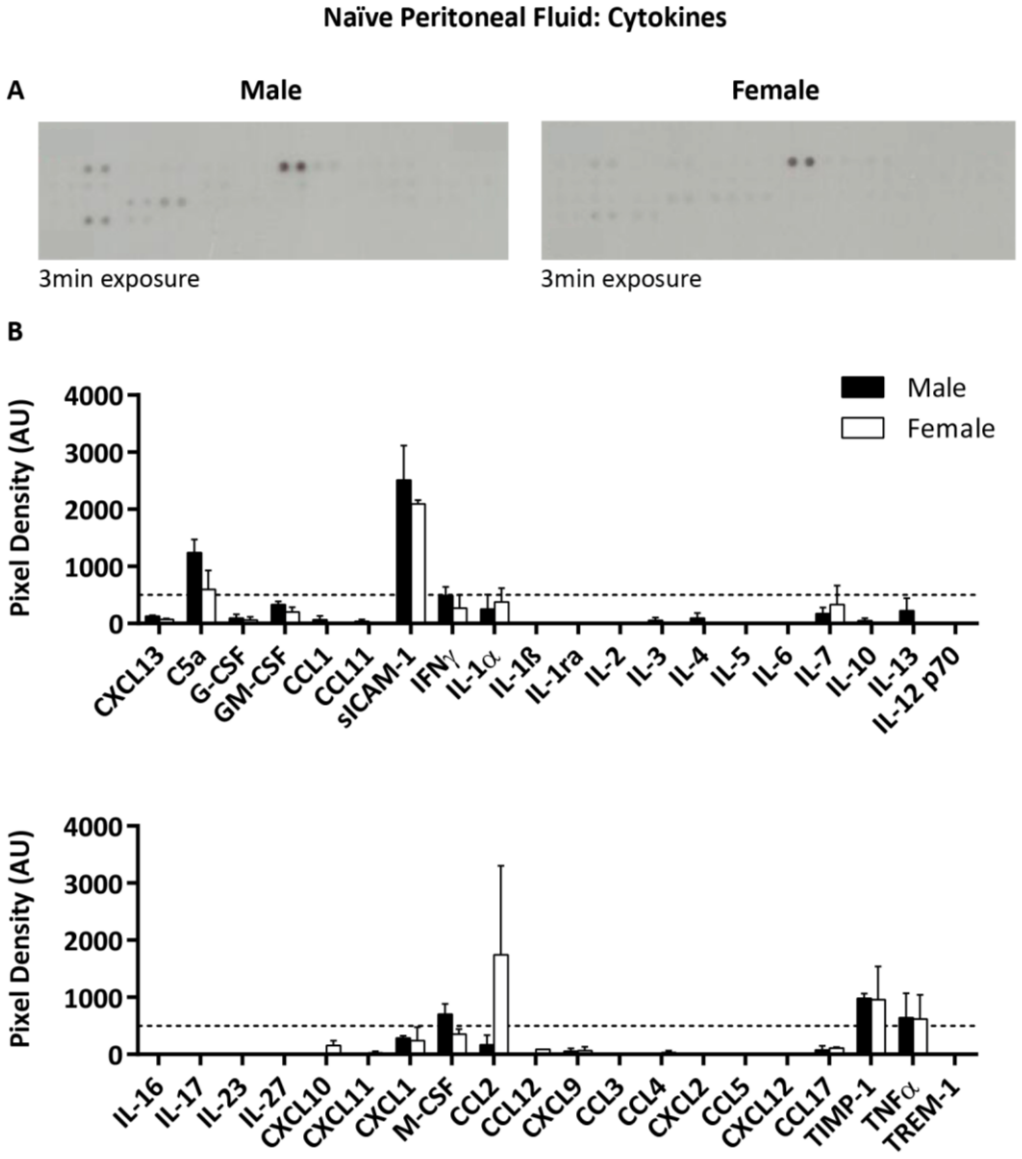


Figure 6.2. Basal peritoneal cavity cytokine environment. Cytokine profile of naïve male (■) and female (□) murine peritoneal lavage supernatants was determined using the Proteome Profiler™ mouse cytokine array. (A) Representative arrays with male and female blots exposed for equal times. (B) Semi-quantification of cytokines by densitometry. Expression represented as pixel density. Data are shown are a mean ± SEM of 2 mice.

Densitometry was employed as a semi-quantitative method to determine cytokine expression, and densities under 500AU were considered below the range of reliable detection. An equivalent profile was also seen when densities were plotted relative to control spots (Control spots are shown in Appendix 4). Soluble ICAM-1 (sICAM-1) was the highest expressed molecule in both male and female peritoneal fluid however overall few cytokines were present in high quantities (Figure 6.2B). Complement component C5a showed a trend towards being greater in male compared to female peritoneal fluid. Conversely, the mean CCL2 was higher in the female versus male cavity. All other cytokines showed similar expression between the peritoneal fluids of untreated male and female mice.

To obtain a full profile of basal mediators in the naïve peritoneum, 4mg of protein from the same samples were assayed with the Proteome Profiler™ mouse chemokine array (R&D Systems). Blots, as shown in Figure 6.3A, were again subjected to densitometry for semi-quantification of chemokine expression. CCL6, chemerin, and CCL9 were in relative abundance in the naïve peritoneal fluid (Figure 6.3B). Overall, of the panel of chemokines studied, levels were similar between males and females.

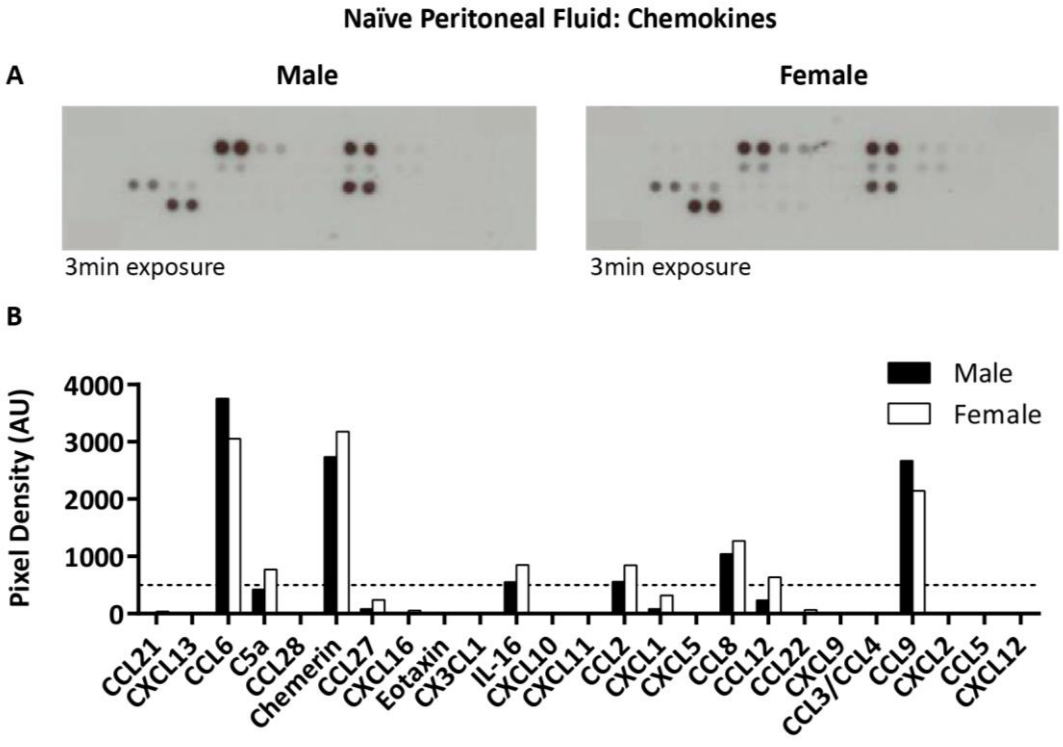


Figure 6.3. Basal peritoneal cavity chemokine environment. Chemokine profile of naïve male (■) and female (□) murine peritoneal lavage supernatants was determined using the Proteome Profiler™ mouse chemokine array. (A) Male and female arrays (n=1) as exposed for equal times. (B) Semi-quantification of cytokines by densitometry. Expression represented as pixel density.

6.3.3. Zymosan-mediated cytokine environments

Having established comparable basal peritoneal cytokine environments in male and female mice, the inflammatory cytokine profile was next investigated. Mice were treated with zymosan (1mg, i.p.) for 3h, the time point at which dimorphisms in the accumulation of leukocytes in the blood and tissue were observed. Peritoneal lavage yielded fluid for the assessment of cytokines, and cells for the assessment of inflammation as determined by neutrophil accumulation. Peritoneal conditions were evaluated as 'inflamed' to a degree

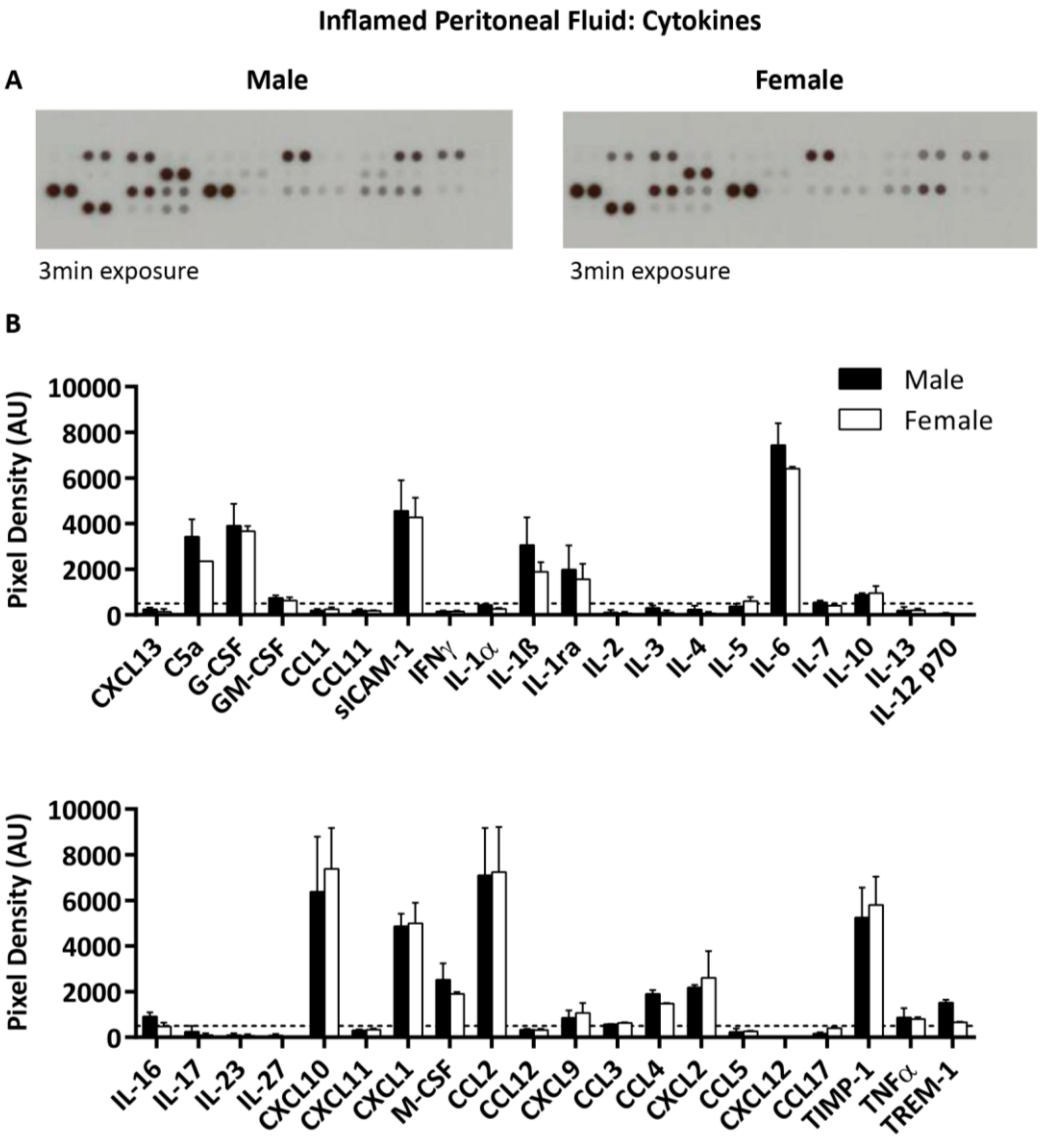


Figure 6.4. Peak inflammation peritoneal cytokine profile. Male (■) and female (□) mice were treated with zymosan (1mg, i.p.) for 3h and the cytokine content of the peritoneal lavage supernatants were determined by Proteome Profiler™ mouse cytokine array. (A) Representative arrays. (B) Cytokine expression as semi-quantified by densitometry. Data shown is mean ± SEM of 2 experiments.

analogous to those in previous chapters if greater than 50% of cells were neutrophils. Protein contents of male and female peritoneal lavage fluid were determined by the BCA protein assay and Proteome Profiler™ cytokine array membranes were subsequently incubated with equal amounts of protein (4mg). In addition, membranes of male and female mice were exposed for the same time (3mins). In comparison to the naïve peritoneal cytokine profiles (Figure 6.2), peritoneal samples from mice after 3h of zymosan peritonitis indicated induced expression of numerous cytokines (Figure 6.4A). Semi-quantification by densitometry revealed the most highly expressed cytokines to be IL-6, CXCL10, CCL2 and CXCL1 (Figure 6.4B). The expression of TIMP-1, sCIAM-1, G-CSF, C5a, IL-1β, M-CSF, CXCL2, IL-1ra, and CCL4 were also induced above the 500AU limit of accurate quantification enforced. No large sex-differences in the expression of the key cytokines were observed, however small trends towards greater levels of C5a, IL-6, TREM-1 and IL-1β in male versus female mice were observed.

By the same means, and to fully establish the inflammatory cytokine profile in early zymosan peritonitis, the Proteome Profiler™ mouse chemokine array was also employed. In a similar

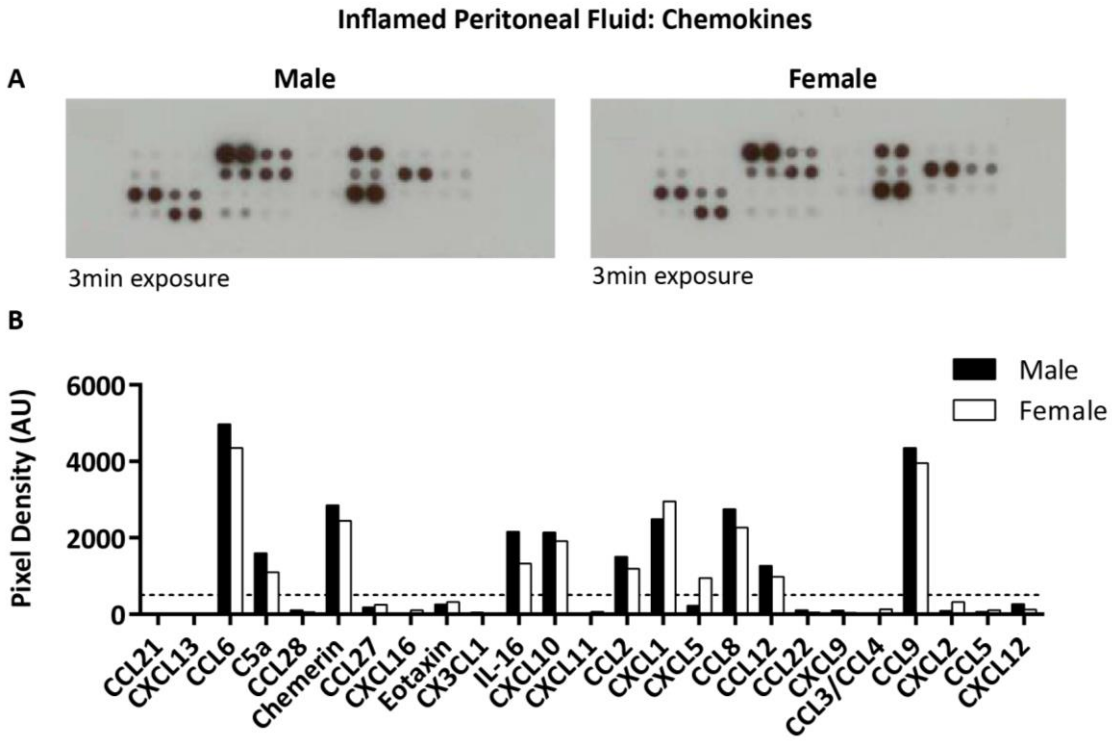


Figure 6.5. Peak inflammation peritoneal chemokine profile. Zymosan (1mg, i.p.) was administered to male (■) and female (□) mice to induce peritonitis. The cytokine content of the peritoneal lavage fluids after 3h of peritonitis were determined by Proteome Profiler™ mouse chemokine array. (A) Representative arrays. (B) Chemokine expression as semi-quantified by densitometry. n=1.

fashion to the cytokine array, there was clear induction of chemokine expression in zymosan-treated mice in comparison to naïve animals (Figure 6.5A). Increased expression in CCL6, CCL9, IL-16, CXCL10, CCL2, CXCL1, C5a, CCL12 and CCL8 were evident (Figure 6.5B). Of note, chemokine levels as measured by array with densitometry were similar between male and female mice.

As peritoneal cavity cytokine and chemokine environments had thus far proved similar between male and female mice, an earlier time point was investigated. Sex-differences in leukocyte recruitment in peritonitis were evident at 3h, therefore it was rationalised that establishment of chemokine gradients would be generated prior to this time point. Male and female mice were therefore given zymosan (1mg, i.p.) and the peritoneal lavage taken after 1h. Lavage supernatants were assessed for protein content using the BCA protein assay and 5.9mg protein were analysed using the Proteome Profiler™ mouse cytokine array (Figure 6.6A). High expression of C5a, sICAM-1, IL-6, CXCL10, CXCL1, M-CSF, CCL2, CXCL2 and TIMP-1, as quantified by densitometry, demonstrated the early-inflamed profile of peritoneal cavity was more abundant in cytokines versus control conditions (Figure 6.6B). Expression of cytokines was similar between male and female mice, with the exception of CCL3, which despite low expression, was 3-fold higher in male mice.

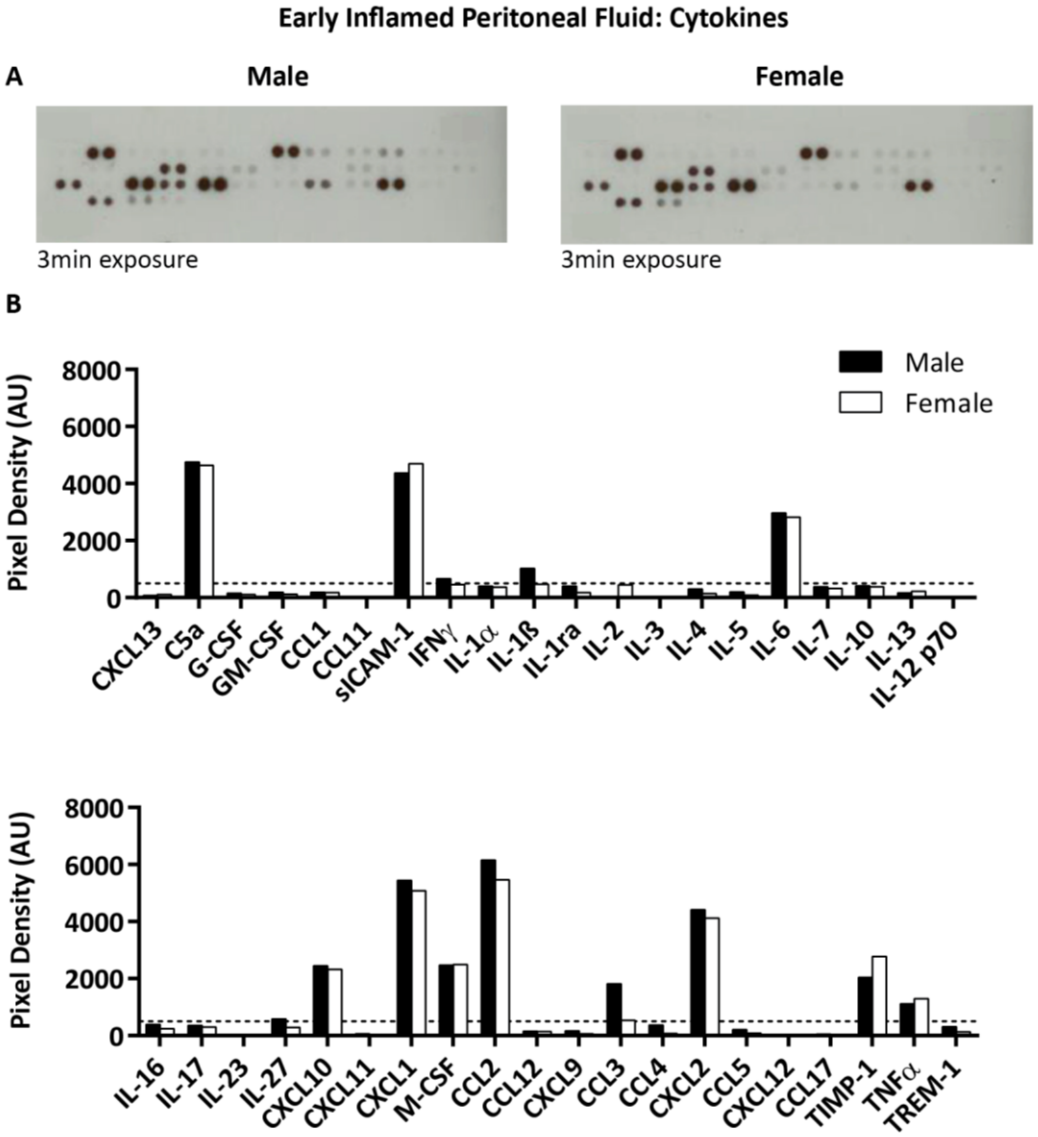


Figure 6.6. Early peritonitis cytokine environment. Male (■) and female (□) mice were treated with intraperitoneal zymosan (1mg, 1h) to induce peritonitis. Peritoneal lavage supernatants were analysed for the cytokine profile using Proteome Profiler™ mouse cytokine array. (A) Male and female membranes, as exposed for equal times. (B) Cytokines expression as determined by densitometry (n=1).

6.4. Discussion

This chapter investigated the initiation stages of the immune response to explore the potential mechanisms that underlie sexual dimorphisms in leukocyte recruitment during peritonitis. Detection of intraperitoneal zymosan occurs through PRRs TLR2, TLR6, and dectin-1 on resident peritoneal macrophages. The TLR2-TLR6 heterodimer is required for the inflammatory response and activation of NFκB. Dectin-1 on the other hand is a C-type lectin family member that despite involvement in induction of classical inflammatory responses is also responsible for zymosan phagocytosis (Gantner *et al.*, 2003). Ligation of this receptor has been shown to enhance TLR-mediated activation of NFκB, but also to trigger the production of ROS. It should be noted however that this is not an exhaustive characterisation of the expression of zymosan receptors as complement receptors CR3 and C5aR have also been implicated in its detection (Ross *et al.*, 1985; Mullaly *et al.*, 2007).

As already demonstrated in Chapter 3, resident peritoneal macrophages from male and female mice exhibit similar cell surface expression of TLR2. This chapter extends this finding to equally show the expression of TLR6 is not significantly different between sexes, indicating dimorphisms in the leukocyte recruitment response is unlikely to stem from differences in zymosan recognition and thus TLR activation. Similar TLR6 expression between the sexes is in line with previously published mRNA data (Scotland *et al.*, 2011). On the other hand, macrophages from female mice show a small but nevertheless significant increase in the expression of the zymosan phagocytosis receptor dectin-1 compared to male macrophages. Although this does not represent a vast difference in cell phenotype between the sexes, it is in line with previous reports of greater uptake of zymosan in female versus male macrophages (Scotland *et al.*, 2011). To fully determine any sex-differences in the ability of the peritoneal cavity to detect zymosan however, it would be useful to also assess the expression of the afformentioned complement receptors C3R and C5aR. In addition it would be of use to investigate the expression of TLR2/TLR6 on other resident peritoneal cells such as mesothelial cells and mast cells to fully dismiss dimorphisms in zymosan recognition.

Having demonstrated similar zymosan-sensing apparatus of peritoneal macrophages, the production of cytokines was next investigated. It was hypothesised that differences in the nature and amount of cytokines and chemokines produced by male and female peritoneal immune cells could account for sex-differences in accumulation of leukocytes both in the tissue and the circulation. Sex-differences in leukocyte recruitment have previously been linked to cytokine production in the literature as more chemokine prodcution in male rats with abdominal aortic aneurysms correlated with more neutrophil accumulation in males, whilst

the opposite was true for equally treated female rats (Sinha *et al.*, 2006). However despite indications for sex-differences in both leukocyte recruitment and cytokine production in the literature, few studies connect the two, leaving a lack of evidence for the underlying cause of dimorphisms in inflammatory diseases.

Under basal conditions sICAM-1, CCL6, chemerin, and CCL9 were most highly expressed in the peritoneal fluid (Table 6.1). sICAM-1 generation is not well defined, however it is proposed both that cell surface ICAM-1 shedding by proteases, and specific sICAM-1 transcription are possible (Witkowska *et al.*, 2004). The exact reason for high sICAM-1 in unstimulated conditions in this chapter is unclear as it is generally associated with pro-inflammatory responses, however nanomolar range concentrations have previous been identified in the serum of healthy volunteers indicating some basal levels are normal (Lawson *et al.*, 2009).

Cytokine	Alternative Name	Receptor/ Target	Chemoattractant	
			Neutrophils	Monocytes
CCL2	MCP-1	CCR2	×	✓
IL-6	-	IL-6Ra + gp130	-	-
CXCL10	IP-10	CXCR3	×	✓
TIMP-1	-	MMP2, MMP9	-	-
CXCL1	KC	CXCR2	✓	×
sICAM-1	CD54	LFA-1, Mac-1, fibrinogen	-	-
C5a	-	C5aR1	✓	✓
CXCL2	MIP-2α	CXCR2	✓	×
CCL6	C10	CCR1	×	✓
CCL9	MIP-1γ/ CCL10	CCR1	×	✓
G-CSF	-	G-CSF-R	-	-
IL-1β	-	IL-1R1	-	-
M-CSF	-	CSF-1R	-	-
Chemerin	RARRES2	CMKLR1	-	-
IL-16	-	CD4	-	-
CCL4	MIP-1β	CCR1, CCR5	×	✓
CCL8	MCP-2	CCR1, CCR2, CCR5	×	✓

Table 6.1. Key cytokines and chemokines produced during early murine zymosan peritonitis. Mice were treated with zymosan (1mg, i.p.) for 3h and the cytokine profile of the peritoneal cavity was assessed by Proteome Profiler™ arrays. Details of the highest expressed cytokines are presented alongside their reported function to act as neutrophil or monocyte chemoattractants. Hyphen (-) indicates the molecule does not directly possess chemokine activity.

One study however showed micromolar concentrations of sICAM-1 inhibit the ICAM-1/LFA-1 interaction *in vitro (Meyer et al., 1995)*, suggesting that in naïve mice high sICAM-1 may have the potential to maintain tissue homeostasis by damping the inflammatory outcomes of such an interaction. ELISA of the peritoneal fluid would be required to fully quantify the levels of sICAM-1 in mice to begin investigating it's function in the naïve peritoneum. CCL6 is expressed in relatively high levels as determined by chemokine array and densitometry. This is in contrast to another study which showed low CCL6 levels (<0.5ng/ml) in the control peritoneal cavity by ELISA (Coelho *et al.*, 2007). The cell surface receptor for CCL6 is CCR1, thus CCL6 is chemotactic for monocytes (LaFleur *et al.*, 2004; Berahovich *et al.*, 2005). Put in context alongside previous descriptions of myeloid cell differentiation (Nardi *et al.*, 2009), it may be possible that via CCR1, CCL6 in the naïve peritoneal cavity is playing a role in monocyte recruitment and differentiation for the repopulation of resident macrophages.

Also found to be present in similar levels to CCL6 was chemerin, known also as retinoic acid receptor responder protein 2 (RARRES2). Chemerin is chemotactic for macrophages and DCs by binding to CMKLR1 (Wittamer *et al.*, 2003) and therefore it may play a role in the homeostasis of the naïve peritoneal cavity. Furthermore, chemerin is highly expressed in white adipose tissue and has been implicated in adipocyte biology (Bozaoglu *et al.*, 2007), therefore it is also possible peritoneal chemerin is not playing a role in immune regulation, but rather is functioning with respect to the peritoneal fat observed in these mice. Also in abundance in the naïve cavity was CCL9, a chemokine constitutively expressed in macrophages and also secreted from follicle-associated epithelium such as those present around Peyer's patches (Zhao *et al.*, 2003). As both macrophages and Peyer's patches are found within the peritoneal cavity, it is unsurprising CCL9 was relatively highly expressed. CCL9 is known to be chemotactic for DCs and monocytes, both of which express the CCL9 receptor CCR1 (Berahovich *et al.*, 2005). It could be speculated that high basal CCL9 is required for recruitment of these cells for repopulation of the cavity with DCs and macrophages. Further experiments would nevertheless need to be carried out to determine such hypotheses on the function of highly expressed basal chemokines.

Importantly, no sex-differences were observed in the peritoneal fluids of male and female naïve mice besides in levels of CCL2. Levels of this chemokine in female mice were heterogenous with one high expressing and one low expressing mouse, therefore more samples would be required to determine whether female mice have more basal CCL2 than males. If this were the case, it may offer explanation to the previous findings in this thesis, and

published reports, that more resident macrophages are present in the female peritoneal cavity (Scotland *et al.*, 2011).

In line with the nature of the inflammatory response, cytokine and chemokine expression was amplified by 3h of zymosan peritonitis in comparison to basal conditions. Cytokines with augmented expression were comprised of classical proinflammatory mediators (i.e. IL-1β, C5a, and IL-6), neutrophil-associated chemokines (i.e. CXCL1 and CXCL2), and monocyte-associated chemokines (i.e. CCL2, CXCL10, CCL8). In addition, growth factors (i.e. G-CSF and M-CSF) and inflammation inhibitors (i.e. TIMP-1 and IL-1ra) were also induced during zymosan peritonitis (Table 6.1). Contrary to other reports, only a small amount of TNF was produced after 3h of zymosan versus control, however arrays such as those used in these studies are only semi-quantitative compared to the ELISAs describing TNF responses in the literature (Gantner *et al.*, 2003). Likewise to the analysis of basal conditions, no clear differences in the cytokine and chemokine responses were evident between the sexes after 3h of zymosan peritonitis. Sex-differences in leukocyte recruitment were observed by 3h, therefore an early 1h peritonitis time point was investaged as establishment of chemotactic gradients would have been required prior to leukocyte recruitment. Peritoneal cytokine expression was indeed already augmented at the 1h early time point compared to control. CCL3 and IL-1β levels appeared greater in male peritoneal fluid versus females however female values were on the border of accurate detection. The levels of all other cytokines were once more not different between male and female mice, although small trends resembling sexual dimorphisms were observed, such as for C5a, IL-16, IL-1β, and TREM-1. It might be suggested that such minor differences in the context of this degree of inflammation (accumulation of approximately 8-11x10^7 neutrophils) is unlikely to account for such disparity in the recruitment of male and female leukocytes.

Key findings:

- Male and female mouse resident peritoneal macrophages exhibit similar levels of zymosan-sensing TLR2/TLR6.
- Dectin-1 expression on female peritoneal macrophages is small but significantly greater than males.
- Zymosan peritonitis induced upregulation of a plethora of cytokines and chemokines as early as 1h, including neutrophil and monocyte chemoattractants.
- No major sex-differences in cytokine and chemokine profile or expression level, as determined by array, were evident in naïve or zymosan-treated mice.

Chapter 7: Mobilisation of bone marrow leukocyte stores

7.1. Introduction

Uncontrolled influx of immune cells such as neutrophils can have dangerous consequences and have been implicated in the pathogenesis of many inflammatory diseases such as sepsis, I/R injury, and arthritis. One of the first steps in the trafficking of immune cells to the site of infection however, is mobilisation of leukocytes from the bone marrow (BM). It represents a large and rapidly releasable source of leukocytes and is therefore highly important. Mobilisation of BM stores can vastly expand the number of circulating leukocytes, thus amplifying the total number of cells available for recruitment. The BM therefore functions not only as a site of haematopoiesis, but also as a readily releasable cellular storage pool.

Chapter 4 of this thesis shows that female mice accumulate fewer neutrophils and classical monocytes in their circulation and tissue during zymosan peritonitis in comparison to male counterparts. Similarly, in certain inflammatory diseases men display more exaggerated responses compared to women. Increasing evidence suggests this may be the result of augmented leukocyte recruitment (Marriott *et al.*, 2006a; Scotland *et al.*, 2011). The mechanism behind this theory and the source of additional leukocytes in males are not however clear. What is clear is that the BM is pivotal as a source of leukocytes for defence from infection, and furthermore much literature is available detailing mechanisms regulating leukocyte production, retention, and release (Borregaard, 2010; Shi *et al.*, 2011a). Whether sex-differences in the mobilisation of these stores in inflammation exist however has not been studied.

BM classical monocytes have recently been shown to emigrate in response to circulating TLR ligands (Shi *et al.*, 2011b). In addition, BM neutrophils and monocytes have been shown in the literature and in this thesis to express cell surface TLRs including TLR2 and TLR4 (Charmoy *et al.*, 2007; Papatriantafyllou, 2011; Thomas *et al.*, 2013). This thesis has already described that neutrophils from the BM of male mice are significantly higher in TLR2 and TLR4 than those from female mice. To complement these findings and further dissect whether this difference in TLR expression plays a role in leukocyte trafficking beyond PAMP and DAMP recognition in the tissue, the expression of BM leukocyte TLRs during the inflammatory reaction of zymosan peritonitis was also investigated.

7.2. Aims

As similar pattern recognition and cytokine responses in male and female mice fail to explain the sex-differences in leukocyte recruitment during zymosan peritonitis, the BM of male and female mice was investigated. This chapter sought to evaluate the TLR characteristics of BM leukocytes and test the hypothesis that differences in the mobilisation of BM leukocyte stores underlie protection from circulating and tissue leukocyte accumulation observed in zymosan peritonitis. Therefore the specific aims were to:

- Investigate sex-differences in mobilisation of BM leukocytes during murine zymosan peritonitis.
- Compare the BM leukocyte temporal TLR2 and TLR4 expression profile during inflammation in male and female mice.

7.3. Results

7.3.1. BM leukocyte mobilisation in murine zymosan peritonitis

Zymosan peritonitis (1mg, i.p.) induced significant trafficking of neutrophils (p<0.001), classical monocytes (p<0.001), B cells (p<0.05) and T cells (p<0.01), with a trend in non-classical monocytes (p=0.051) (Figure 7.1). Neutrophils reduced approximate 2-fold in number in the BM of male and female mice after 3h compared to control conditions (male: 0h: 6.1±0.54 x10^6, 3h: 3.6±0.64 x10^6; female: 0h: 5.2±0.41 x10^6, 3h: 2.7±0.24 x10^6, Figure 7.1A). By the same pattern, classical monocyte numbers in the BM were also reduced around 2-fold by 3h in male and female mice compared to controls (male: 0h: 12.7±1.59 x10^5, 3h: 6.3±0.81 x10^5; female: 0h: 11.0±0.12 x10^5, 3h: 5.6±0.53 x10^5, Figure 7.1B). Non-classical monocytes displayed a small trend to reduced cell numbers in response to zymosan (Figure 7.1C). By 72h, neutrophils and monocytes returned to levels comparable to basal conditions. 3h of zymosan peritonitis induced a trend towards a transient increase in BM B220$^+$ B cell numbers in both sexes (Figure 7.1D). Zymosan did not affect BM CD3$^+$ T cell numbers in male mice however and increase in cell numbers at 3h was seen in the female BM, resulting in more cells in the female versus the male BM at this early time point (4.1±0.44 x10^5 vs 2.4±0.41 x10^5, Figure 7.1E). By 24h, female T cell numbers decreased to that of control.

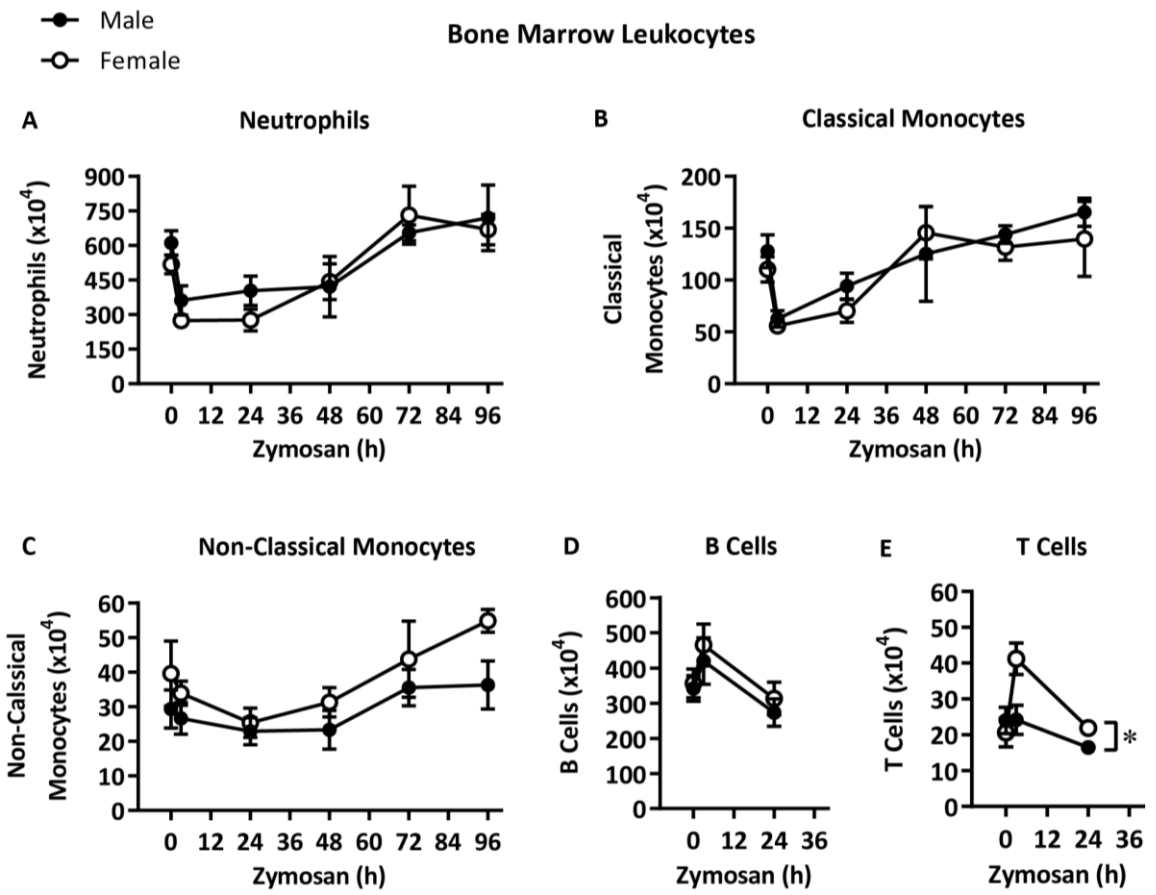


Figure 7.1. Leukocyte mobilisation from the murine BM during peritonitis. Zymosan (1mg, i.p.) was administered to male (●) and female (○) mice to induce peritonitis. (A) Numbers of neutrophils, (B) classical monocytes, (C) non-classical monocytes, (D) $B220^+$ B cells and (E) $CD3^+$ T cells were counted from the right femur bone marrow at the indicated time points. Data are presented as the mean ± SEM of n mice. For neutrophils and monocytes: n≥8 (0h), n=13 (3h), n=9 (24h), n=3 (48-96h); B and T cells: n≥9 (0h), n≥10 (3h), n≥5 (24h). Male and female mobilisation was compared by 2-way ANOVA with Bonferroni's post-test, and significance indicated by * (p<0.05).

7.3.2. The CXCR4/CXCL12 axis

Sex-differences in the accumulation of leukocytes in the circulation and tissue during peritonitis were not explained by the hypothesis of differential mobilisation of BM stores. To confirm these findings, the expression of CXCR4 on neutrophils was assessed between the sexes with the rationale that high CXCR4 expression is indicative of homing back to the BM, whilst low CXCR4 suggests mobilisation. Very low (RFI<2) and unchanging expression of CXCR4 was seen on neutrophils in the male and female mouse circulation throughout zymosan peritonitis until 96h (Figure 7.2A). CXCR4 expression on neutrophils in the BM was both higher and more variable in response to zymosan (Figure 7.2B). Lowest expression was observed at 3h, whilst highest was seen at 24h post-zymosan. By 72h CXCR4 had returned to basal levels. Under basal conditions, a trend towards higher CXCR4 expression on male compared to female BM neutrophils was seen.

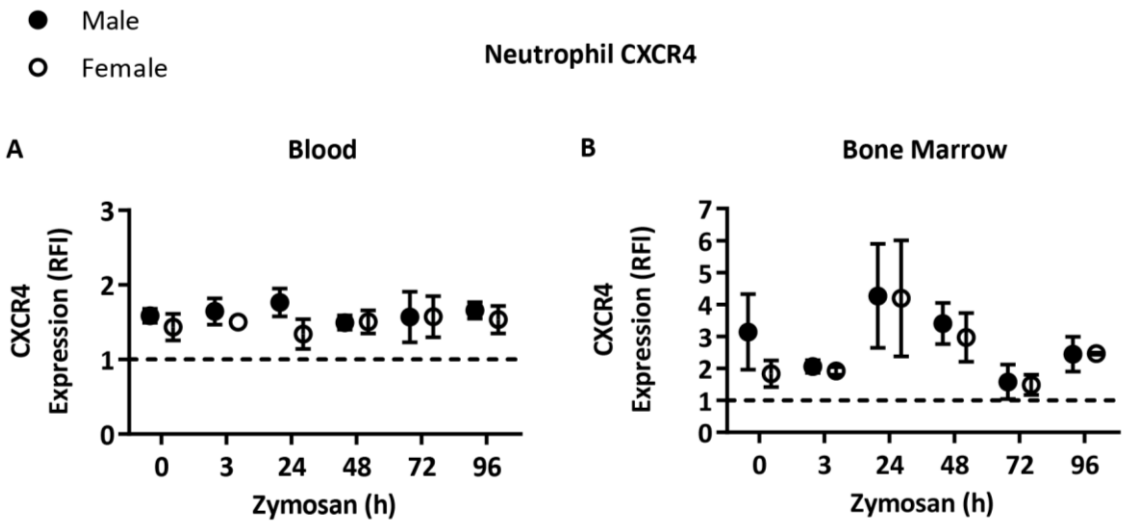


Figure 7.2. Neutrophil CXCR4 expression. Zymosan (1mg, i.p.) was administered to male (●) and female (○) mice to induce peritonitis. The expression of CXCR4 on neutrophils isolated from (A) blood and (B) BM at 0h, 3h, 24h, 48h, 72h, and 96h was evaluated. CXCR4 was expressed at relative fluorescence intensity (RFI) to isotype antibody. Data is expressed as mean ± SEM of 3 (0h) or 2 mice (3-96h).

7.3.3. Temporal expression profile of BM leukocyte TLR2 and TLR4 in murine zymosan peritonitis

In response to zymosan peritonitis, female BM neutrophils had significantly (p<0.01) increased expression of TLR2 by 24h (0h: 1.5±0.08; 24h: 2.1±0.15) that returned to levels equivalent to control by 48h (Figure 7.3A). Male mouse neutrophils showed a trend toward this. No significant changes in the expression of TLR2 on BM classical- and non-classical monocytes, B cells, or T cells was observed at the time points studied in response to zymosan (Figure 7.3B-E). Both monocyte subsets however showed a similar, albeit not significant, pattern to that described for neutrophils with a trend to increased TLR2 expression at 24h that returned to basal levels by 48h.

The expression profile of female BM neutrophil TLR4 during zymosan peritonitis was analogous to that of TLR2, increasing significantly (p<0.001) at 24h (0h: 2.0±0.17; 24h: 3.5±0.29) and returning to control levels by 48h (Figure 7.4A). Although not significant, a trend toward increased BM neutrophil TLR4 was also evident in male mice. Female BM classical monocyte TLR4 showed the same pattern, with a significant but transient increase in receptor expression at 24h (0h: 2.3±0.26; 24h: 3.8±0.42) that again returned to levels comparable to control by 48h (Figure 7.4B). No significant differences in the expression of TLR4 on male BM classical monocytes was seen however a trend towards the transient increased at 24h was observed. The expression of TLR4 on non-classical monocytes, B cells, and T cells in the BM was similar throughout the peritonitis (Figure 7.4C-E).

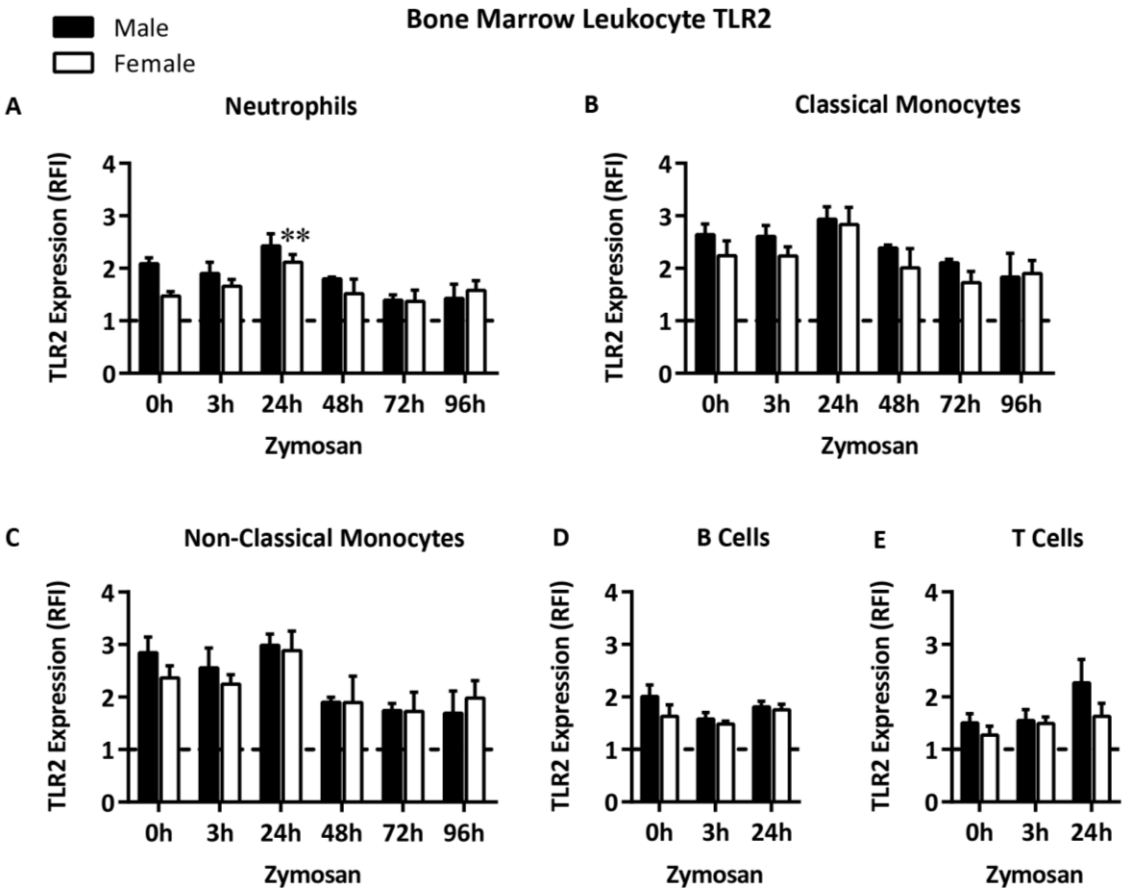


Figure 7.3. Temporal expression profile of BM leukocyte TLR2 during zymosan peritonitis. BM leukocytes were isolated at the indicated time points from male (■) and female (□) mice treated with zymosan (1mg, i.p.) to induce peritonitis. TLR2 expression was assessed on the surface of (A) neutrophils, (B) classical monocytes, (C) non-classical monocytes, (D) $B220^+$ B cells, and (E) $CD3^+$ T cells. TLR2 was expressed as relative fluorescence intensity (RFI) to isotype control antibody. Change in expression versus control (0h) conditions was determined by 1-way ANOVA with Bonferroni's post-test for multiple comparisons and significant differences in males were indicated by # (p<0.05) and in females by ** (p<0.01). Data are presented as mean ± SEM of n mice where for neutrophils and monocytes n=8 (0h), n≥8 (3h), n=9 (24h), n=3 (48h, 72h, 96h), and for B and T cells n≥5 (0h, 3h, 24h).

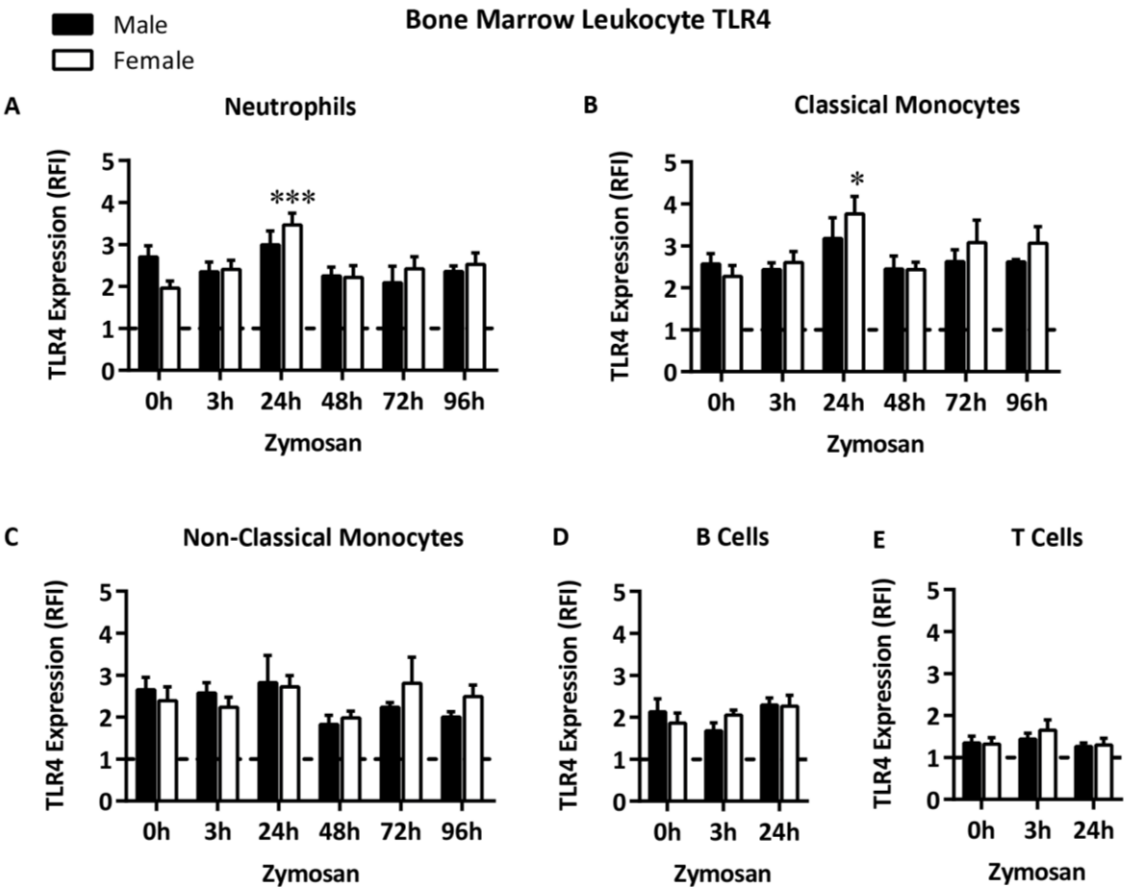


Figure 7.4. Temporal profile of BM leukocyte TLR4 expression during zymosan peritonitis. Male (■) and female (□) mice were treated with zymosan (1mg, i.p.). Cell surface TLR4 was evaluated on (A) neutrophils, (B) classical monocytes, (C) non-classical monocytes, (D) $B220^+$ B cells, and (E) $CD3^+$ T cells isolated from the BM at the indicated times after induction of inflammation. TLR4 was expressed as relative fluorescence intensity (RFI) to isotype control antibody. Changes in expression over time versus under control conditions (0h) were determined by 1-way ANOVA followed by Bonferroni's post-test for multiple comparisons. Significant differences in males were indicated by # (p<0.05) and in females by * (p<0.05), and *** (p<0.001). Data are presented as mean ± SEM of n mice where for neutrophils and monocytes n=8 (0h), n≥8 (3h), n=9 (24h), n=3 (48h, 72h, 96h), and for B and T cells n≥5 (0h, 3h, 24h).

7.4. Discussion

The BM represents the site of both production and storage of many innate immune cells. For example it contains a large storage pool of mature neutrophils, termed the BM reserve (Furze *et al.*, 2008). The rate of the release of these cells has been demonstrated as a major determinant of the number of circulating neutrophils (Martin *et al.*, 2003a). The results in the chapter show clearly that the murine BM represents a reserve of mature leukocytes. In response to zymosan peritonitis, a rapid, significant reduction in the number of both neutrophils and classical monocytes is indicative of their mobilisation (Figure 7.5). Indeed, at 3h post induction of inflammation when BM leukocytes are at their lowest, circulating counterparts consequently peak in number. Previous studies have shown that zymosan

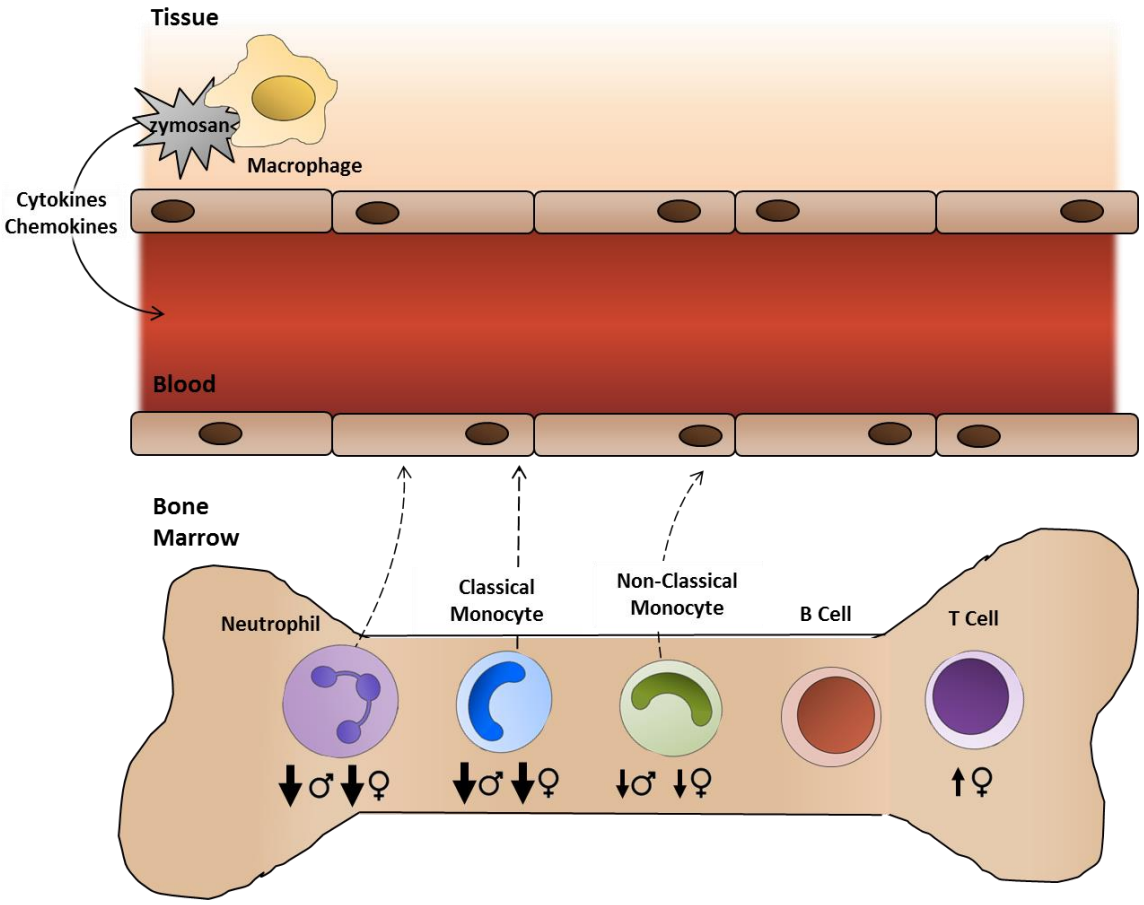


Figure 7.5. Summary of leukocyte trafficking in murine bone marrow during zymosan peritonitis. Summary of neutrophils, classical-monocytes, non-classical monocytes, B cells, and T cells in bone marrow of male (♂) and female (♀) mice treated with zymosan to induce peritonitis. Solid arrows indicate either increase (↑) or decreased (↓) numbers after 3h in male and female mice. Larger arrows indicate greater increase or decrease in cell number. Broken arrows represent likely trafficking between compartments. No arrows signify no change in cell number or no suggestion of trafficking.

induces mobilisation of neutrophils from the BM at a similar time point and to a similar extent than described in this chapter (Takeshita *et al.*, 2004). No description of the mobilisation of BM classical monocytes has however been reported for this reaction. Mobilisation of neutrophils and classical monocytes from the BM in response to zymosan peritonitis was evident to the same extent in male and female mice.

Measurement of mobilisation by this method may be deemed crude by some and potentially even masking any sex-differences, however at present it represents the standard approach in studying such responses of BM leukocytes. More recently, a more accurately quantifiable method of mobilisation was developed whereby femoral artery and vein cannulation *in situ* allows perfusion of the hind limb vasculature and thus collection of exiting cells (Pitchford *et al.*, 2010). Such a method could be utilised to confirm the mobilisation findings in this thesis, and furthermore confirm similar mobilisation in male and female mice.

To confirm the lack of sexual dimorphism in the BM leukocyte responses to zymosan, neutrophil retention signal was studied. It is well documented that CXCL12 (SDF-1α), produced constitutively in the BM by stromal cells, acts via neutrophil CXCR4, creating a mechanism of neutrophil retention in the BM (Eash *et al.*, 2010). During inflammation BM neutrophil CXCR4 is downregulated and CXCR2 is upregulated causing the cells to lose their CXCL12 retention signal, and become chemotactic towards circulating CXCR2 ligands (i.e. CXCL2) (Furze *et al.*, 2008). Moreover, senescent circulating neutrophils are considered to upregulate CXCR4 and in doing so acquire the ability to migrate towards CXCL12 and thus home back to the BM for clearance (Martin *et al.*, 2003a; Rankin, 2010). In line with previous reports, neutrophils from the blood of male and female mice were consistently low in CXCR4 on their cell surface (Martin *et al.*, 2003a). In contrast, expression of CXCR4 on BM neutrophils was higher than those in the circulation. Interestingly, expression was highest at 24h perhaps suggestive of aged neutrophils returning to the BM after peaking in number earlier in the circulation. After 24h, BM neutrophil CXCR4 expression declined as the inflammation reached resolution. No sex-differences in CXCR4 expression were observed during zymosan peritonitis, in particular at 3h when male mice had more leukocytes both in their circulation and tissue compared to female mice. Under naïve conditions however, males showed a trend towards greater CXCR4 expression on BM neutrophils, contrary to possible hypothesis than a 'weaker' BM CXCR4/CXCL12 retention axis may result in greater release of neutrophils in inflammation. Low n numbers however mean direct conclusions are not possible, nevertheless no sex-differences in CXCR4 expression back up similar mobilisation profiles in male and female mice in response

to zymosan. It would also be useful to evaluate monocyte CCR2 expression between the sexes to confirm no differences in the CCL2/CCL7/CCR2 axis.

This chapter also continued the investigation of a role of leukocyte TLRs in trafficking beyond local PAMP and DAMP recognition. As demonstrated in Chapter 3, male BM neutrophils are significantly higher in TLR2 and TLR4 than female equivalents. Such differences in PPRs may indicate a role for TLRs in the mobilisation and migration of leukocytes from the BM to the site of inflammatory insult. Temporal analysis of leukocyte subset TLRs during zymosan peritonitis indicated little change in the expression of TLR2 and TLR4 on BM leukocytes. Exceptions include neutrophils, specifically at 24h after induction of peritonitis, which show higher expression of both receptors significantly in females and with a trend in males. In addition, classical monocyte TLR4 is also higher at 24h significantly in females and with a trend in males. The reasons for this modulation of TLR expression solely at this time point are unclear. Alternatively, due to a reduced size of the neutrophil and classical monocyte reserve pools at this time point, it is plausible that these remaining cells would require a heightened capacity to respond to pathogens or damaged tissue whilst newer leukocytes reach maturation to replenish the pool. It is possible also that BM mobilisation occurs by numerous methods with initial disruption of retention signals triggering a first wave (i.e. at 3h), and by 24h a large enough chemokine gradient would be established in the plasma to further draw leukocytes from their remote stores in a second wave. These later cells may be in a more activated state than early in the inflammation and may therefore have upregulated TLRs. Although such large chemotactic gradients are reported to occur (Jia *et al.*, 2008), no evidence for release of BM leukocytes at this time point is evident in the circulation. It should also be noted that elevations in TLR expression are only small and with this in mind the biological relevance of such a small alteration in expression may be questioned.

A final explanation for temporal differences in TLR expression amongst neutrophils only in zymosan peritonitis may lie within different subsets. A subset of neutrophils identified as $CD11c^{bright}CD62L^{dim}CD11b^{bright}CD16^{bright}$ in the circulation has been shown to suppress T cell proliferation in vivo (Pillay *et al.*, 2012). It is conceivable the origin of such cells is indeed the BM, and by 24h post induction of inflammation it is again plausible T cell proliferation would be favourable. These studies were however described in humans therefore further analysis of these cells would be required. For example, investigations into hydrogen peroxide release, nuclear morphology, and integrin Mac-1 expression, all of which were either distinct or required for suppression of human T cells by the above-mentioned neutrophils.

Overall, it is clear that murine zymosan peritonitis induces mobilisation of BM leukocyte stores, most prominently those of neutrophils and classical monocytes. Sexual dimorphisms in leukocyte accumulation in the blood and peritoneal cavity are not however explained by differential utilisation of BM stores as mobilisation is equivalent in male and female mice. This is further backed up by similar expression of CXCR4 between the sexes. Additionally, BM leukocyte TLR2 and TLR4 overall demonstrated limited modulation of expression, further suggestive of a redundant role for them in the BM as one might expect.

Key findings:

- Zymosan peritonitis induces mobilisation of neutrophils and classical monocytes from the mouse BM by 3h.
- The degree and temporal profile of neutrophil and classical monocyte BM mobilisation is similar in male and female mice.
- Similar mobilisation profiles between male and female mice were consolidated with similar neutrophils CXCR4 expression during zymosan peritonitis.
- Female mouse BM neutrophils have significantly greater expression of TLR2 and TLR4 on neutrophils at 24h post induction of peritonitis versus resting conditions. Male mouse neutrophils show the same trend.
- No other major changes in the expression of TLR2 and TLR4 on BM leukocytes were evident in response to zymosan.

Chapter 8: The spleen as a leukocyte storage pool

8.1. Introductions

The spleen is considered a multifunctional organ with roles in blood filtration, removal of old erythrocytes, metabolism of senescent cells and haemoglobin, as well as in antibody synthesis (Bronte *et al.*, 2013). It consists of mainly B and T lymphocytes, and specialised splenic macrophages. Despite its multiple complex functions, the exact importance of the spleen is unclear, as both human and rodents who have undergone splenectomy are able to live a normal life (Robinette *et al.*, 1977; Higashijima *et al.*, 2009). The main risk of splenectomy is infection, however such a risk is routinely controlled by regular low-dose antibiotics (Mebius *et al.*, 2005).

More recently, alternative roles for the spleen have been proposed. The BM is well accepted, and also demonstrated in Chapter 3 of this thesis, to represent a large leukocyte storage pool (Ma *et al.*, 1999). The spleen of mice has recently also been shown to have a similar role (Swirski *et al.*, 2009). It was reported to exhibit a population of *bone fide* monocytes, undifferentiated and analogous to those in the circulation. In accordance with these previous reports, Chapter 3 also showed the presence of both classical and non-classical monocytes in the resting spleen. In response to MI, Ly6C(Gr1)^{high} classical monocytes are reported to rapidly exit the spleen, and not the BM, and migrate to the damaged myocardium. Accordingly, splenic classical, but not non-classical, monocytes enter the circulation and accumulate in lesions in atherosclerosis-prone mice (Robbins *et al.*, 2012). It is however unclear whether this occurs in diverse inflammatory models. Of note, the spleen did not contribute neutrophils in the former model despite these cells being present in the injured tissue, thus the BM is the likely primary site for neutrophil mobilisation in MI.

Characterisation of the role of the spleen in disease is poor, meaning its role as a leukocyte reservoir is unclear. It is essential more studies are done to establish its function in this context. Chapter 3 showed that neutrophils are present in the resting spleen. More recent studies have also demonstrated splenic neutrophils were found in the B cell area and exhibited specific functions in the context of splenic B cells (Puga *et al.*, 2012). The existence and proportionality of neutrophils, and neutrophil subsets with specific functions, is not well documented. Overall, it is clear that great naivety still exists with regards to the function of the spleen, and more specifically with its role in the immune response and inflammation.

Blood flow through the highly organised splenic structures, each of which contains specific sets of immune cells, suggests the spleen has an important role in immune surveillance. Marginal zone and marginal zone metallophillic macrophages are two splenic macrophage subsets possessing specific sets of PRRs that are capable of trapping circulating pathogens (Mebius *et al.*, 2005; Bronte *et al.*, 2013). The role of other splenic innate immune cells, such as monocytes and neutrophils, in the context of PAMP and DAMP recognition, is poorly documented, as is the TLR expression profile of splenic leukocytes.

The presence of mature monocytes and neutrophils has therefore been established in the spleen, however to date no reports of sex-differences exist. Previous results in this thesis have demonstrated similar basal leukocyte numbers in the BM of male and female mice. In addition, no sex-differences in their BM mobilisation profiles were evident in response to zymosan peritonitis. In order to explain the sexual dimorphism in leukocyte accumulation in the blood and tissue during peritonitis the spleen was investigated as a source of leukocytes. More specifically, a source of leukocytes this thesis has already demonstrated to be different in male and female mice. Understanding the role of the spleen in male and female mice in inflammation is crucial to full understanding of sex-differences in the murine zymosan peritonitis model.

8.2. Aims

Sex-differences in leukocyte trafficking in zymosan peritonitis are evident early in the inflammatory reaction. To establish the source of the additional cells male mice possess in response to zymosan, the spleen was investigated as significantly more neutrophils and monocytes were found in the male resting spleen (Chapter 3). In addition the effect of inflammation on innate immune cell TLRs is unclear and will be investigated to finalise the temporal characterisation of leukocyte subset TLR expression in different compartments. The following specific aims were therefore addressed in this chapter:

- Investigate changes in leukocyte subset numbers in the spleen during murine zymosan peritonitis.
- Examine sex-differences in leukocyte trafficking in the mouse spleen in response to zymosan.
- Determine the influence of zymosan peritonitis on splenic leukocyte TLR2 and TLR4 expression in male and female mice.
- Explore possible mechanisms for sex-differences in basal splenic neutrophil and monocyte numbers.

8.3. Results

8.3.1. Sex-differences in splenic leukocyte trafficking

As previously described in Chapter 3 of this thesis, naïve male mouse spleens had significantly more neutrophils, classical and non-classical monocytes than female mice. No sex-differences in the numbers of splenic lymphocytes were reported. In response to zymosan peritonitis, at the time point of 3h when peak neutrophilia and leukocyte recruitment were observed, the number of neutrophils in the male spleen were not different to control conditions (p>0.05) (Figure 8.1A). Conversely, female mice had significantly (p<0.01) more neutrophils in their spleens compared to controls (3h: 5.1±1.72 x10^6; 0h: 1.9±0.20x10^6, Figure 8.1A). Zymosan peritonitis induced a significant (p<0.05) reduction in the numbers of classical monocytes in the male spleen (3h: 0.7±0.14 x10^6; 0h: 1.6±0.25x10^6, Figure 8.1B). No change in the number of these cells in response to zymosan was seen in females (p>0.05). By 3h of peritonitis, male and female mice therefore had similar numbers of splenic neutrophils and monocytes. No change in female splenic non-classical monocytes was evident in response to zymosan (Figure 8.1C). During this early phase of inflammation, no changes in the numbers of $CD3^+$ T cells or $B220^+$ B cells were observed in the spleens of male or female mice compared to control (Figure 8.1D-E).

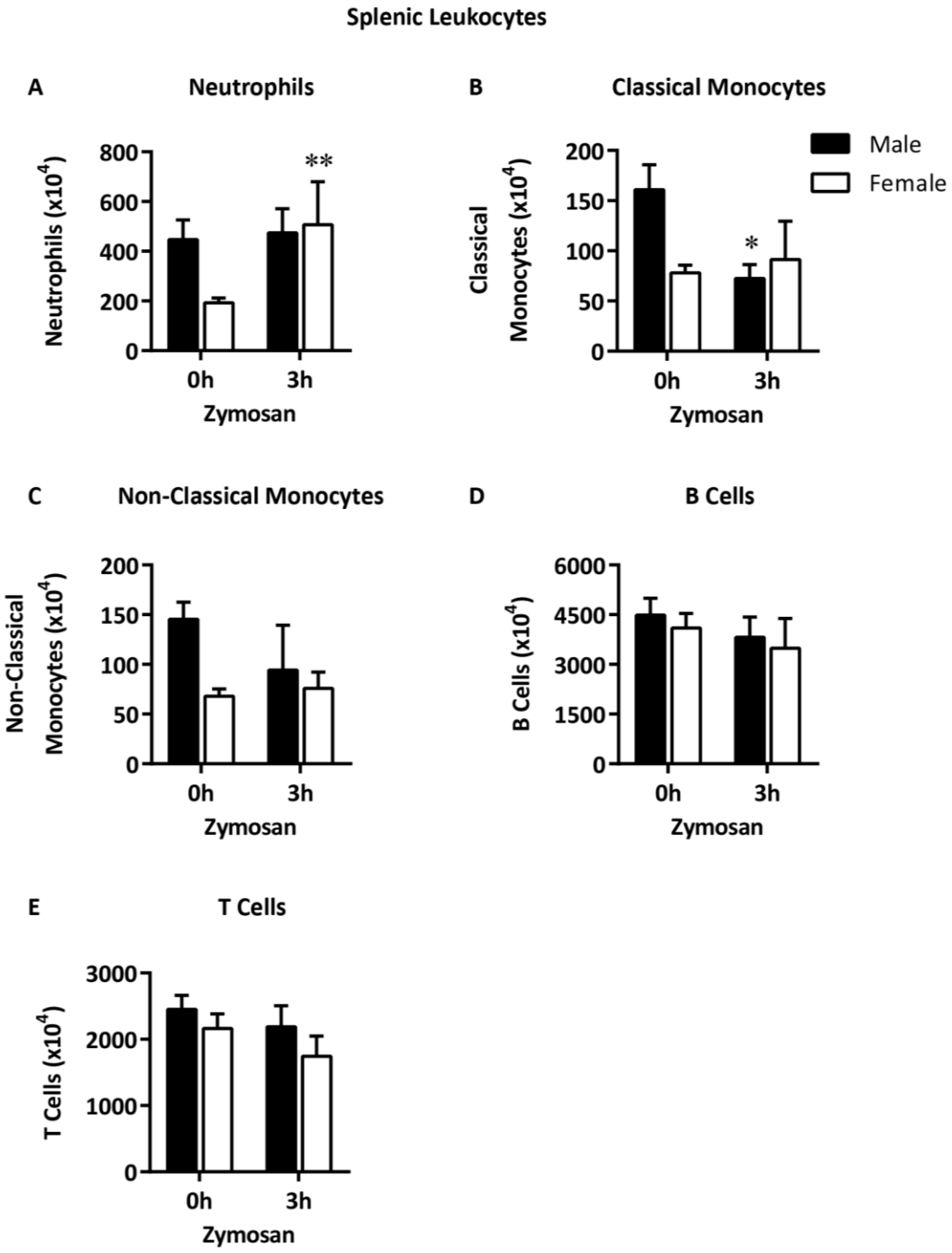


Figure 8.1. Splenic leukocyte subset trafficking during zymosan peritonitis. Male (■) and female (□) mice were treated with or without zymosan (1mg, i.p.) for 3h. The number of (A) neutrophils, (B) classical monocytes, (C) non-classical monocytes, (D) $B220^+$ B cells and (E) $CD3^+$ T cells in the spleen were assessed for change in leukocyte number in inflammation. Change in the number of splenic leukocytes after 3h zymosan compared to control (0h) conditions was assessed using Student's *t*-test and significance indicated by * (p<0.05) and ** (p<0.01). Data are expressed as mean ± SEM of n mice where for 0h n≥11 (males), n≥14 (female), and for 3h n≥5.

8.3.2. Splenic leukocyte TLR2 and TLR4 expression in zymosan peritonitis

Murine splenic leukocytes were shown in Chapter 3 to express TLR2 and TLR4. The influence of inflammation on the expression of TLRs on splenic leukocytes was assessed in the zymosan peritonitis model, using the 3h time point at which peak leukocyte accumulation responses in the periphery were observed. No sex-differences or modulation of TLR2 on neutrophils, non-classical monocytes, B cells, or T cells was detected in response to intraperitoneal zymosan (Figure 8.2). On the other hand, female classical monocytes at 3h showed trends toward higher TLR2 than those at 0h (3h: 3.6±0.28; 0h: 2.4±0.25, Figure 8.2B). As similar trend was also observed in male mice.

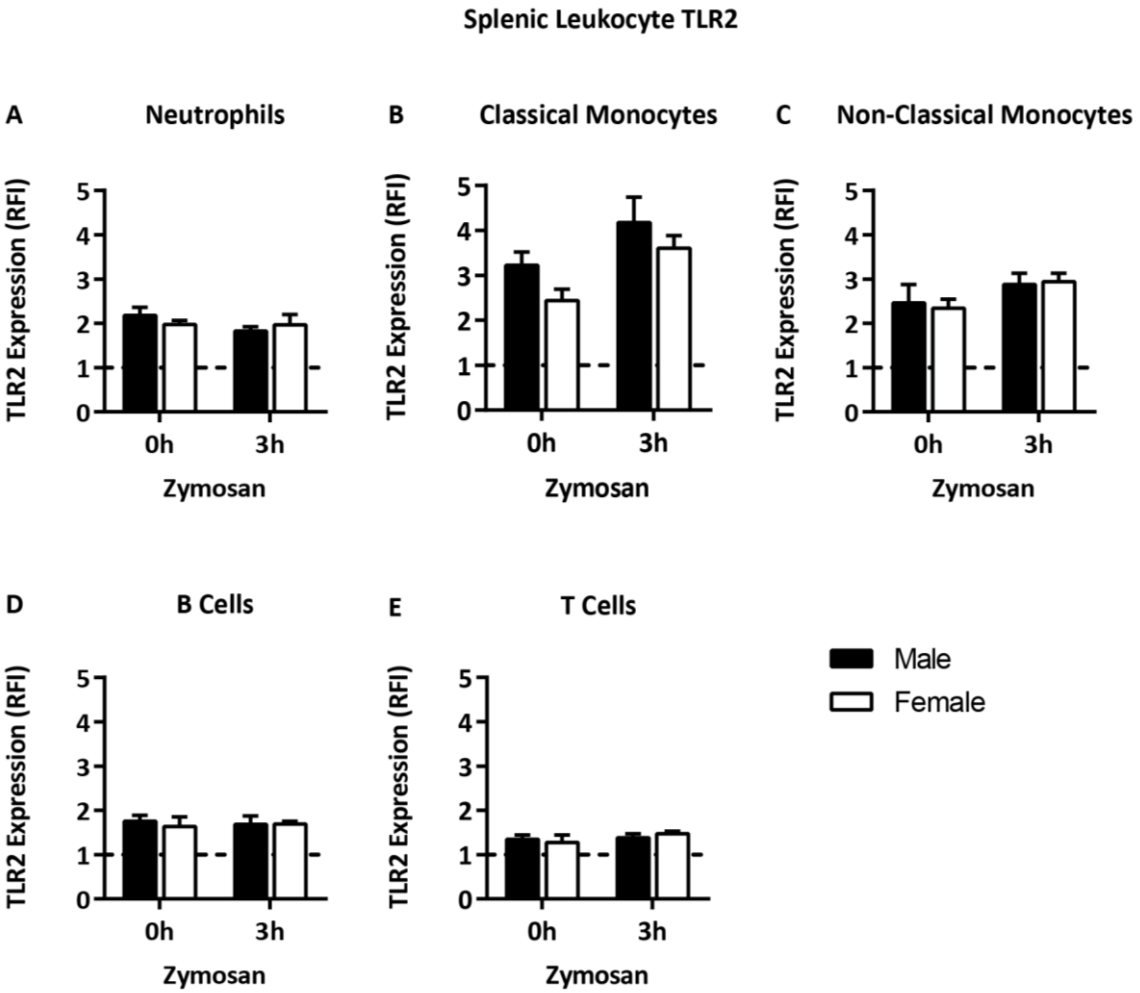


Figure 8.2. Splenic leukocyte TLR2 expression in early zymosan peritonitis. TLR2 expression was assessed on (A) neutrophils, (B) classical monocytes, (C) non-classical monocytes, (D) $B220^+$ B cells, and (E) $CD3^+$ T cells from the spleen of male (■) and female (□) mice treated with or without zymosan (1mg, i.p.) for 3h to induce peritonitis. Receptor expression was displayed as relative fluorescence intensity (RFI) to isotype antibody. Alterations in TLR2 expression with inflammation (3h) versus control conditions in male and female mice were determined by 1-way ANOVA with Bonferroni's post-test and significant differences indicated by * (p<0.05). Data are displayed as mean ± SEM (n≥5).

Similar to the observations with TLR2, there was no evidence of modulation of the receptor in response to zymosan peritonitis in any of the leukocyte subsets (Figure 8.3).

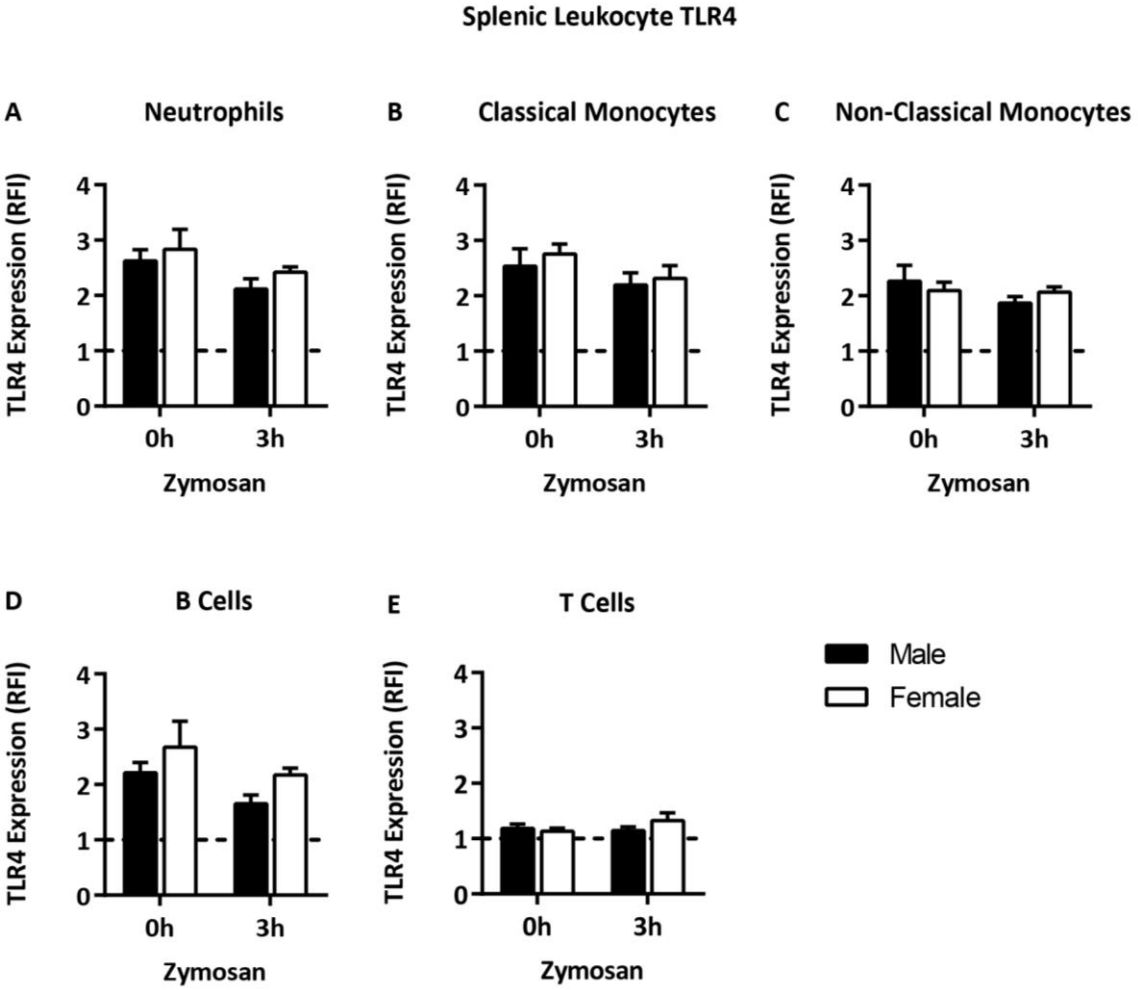


Figure 8.3 Splenic leukocyte TLR4 expression in early zymosan peritonitis. TLR4 expression was assessed on the surface of (A) neutrophils, (B) classical monocytes, (C) non-classical monocytes, (D) $B220^+$ B cells, and (E) $CD3^+$ T cells from the spleen of male (■) and female (□) mice treated with or without zymosan (1mg, i.p.) for 3h to induce peritonitis. Receptor expression was displayed as relative fluorescence intensity (RFI) to isotype antibody. Changes in TLR4 expression in male and female mice with inflammation were determined by S1-way ANOVA with Bonferroni's post-test with significant differences indicated by * (p<0.05). Data are displayed as mean ± SEM (n≥5).

8.3.3. Basal splenic cytokine environments

To investigate potential mechanisms for the sex-differences in basal splenic leukocyte neutrophil and monocyte numbers, the naïve splenic cytokine/chemokine environment was assessed with the rationale that a greater chemotactic ambience in the male spleen would result in a larger storage pool of certain leukocyte subsets. The environment of the resting spleen was studied using cytokine arrays. Spleen lysate supernatants were obtained from naïve male and female mice and the protein contents of each were quantified using the BCA protein assay. 1.5mg of protein was assayed with the Proteome Profiler™ mouse cytokine array (R&D systems) and the membranes with male and female samples were exposed for 3mins (Figure 8.4A). Control reference spots confirmed successful assay (Appendix 4). Cytokine expression was semi-quantified using densitometry and densities under 500AU were deemed below the range of reliable quantification. Of those molecules analysed, CCL5, CXCL9, sICAM-1, IL-1ra, CXCL10, and IL-1α were present in the highest levels in the naïve spleen (Figure 8.4B). Key monocyte and neutrophil associated chemokines such as CCL2, CXCL1, CXCL2, and CCL3 were lowly expressed. Of note, similar cytokine/chemokine expression profiles were observed in spleens from male and female mice.

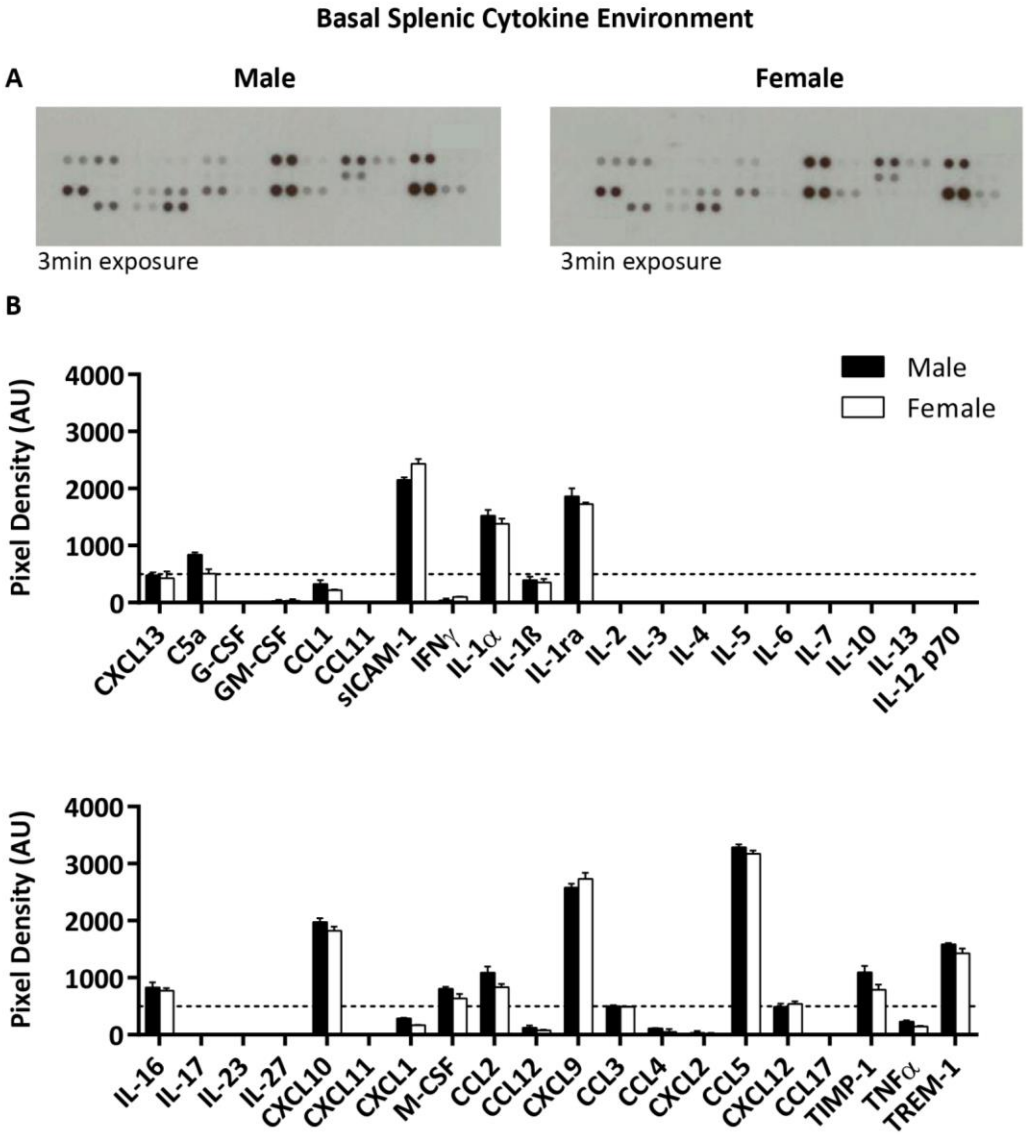


Figure 8.4. Naïve splenic cytokine environment. Cytokine profile of naïve male (■) and female (□) murine spleens was determined using the Proteome Profiler™ mouse cytokine array. (A) Representative arrays with male and female blots exposed for equal times. (B) Semi-quantification of cytokines by densitometry. Expression represented as pixel density. Data are shown are a mean ± SEM of 2 mice.

8.3.4. Expression profile of leukocyte chemokine receptors in the spleen

Assessment of the splenic chemokine environment by array revealed similar profiles for male and female mice. In addition it revealed the presence of those molecules with neutrophil or monocyte chemotactic capabilities such as CCL2, CCL3, CCL5, C5a, and CXCL12, which were present in vastly ranging relative quantities, but to similar extents in both sexes. The corresponding receptors on leukocyte subsets from the spleens of male and female mice were therefore assessed to investigate the observed sexual dimorphisms in the composition of the leukocyte splenic reservoir.

CXCL1 and CXCL2 are potent neutrophil-attracting chemokines that exert their functions via CXCR2. Correspondingly, CXCR2 was highly expressed on the splenic neutrophil cell surface,

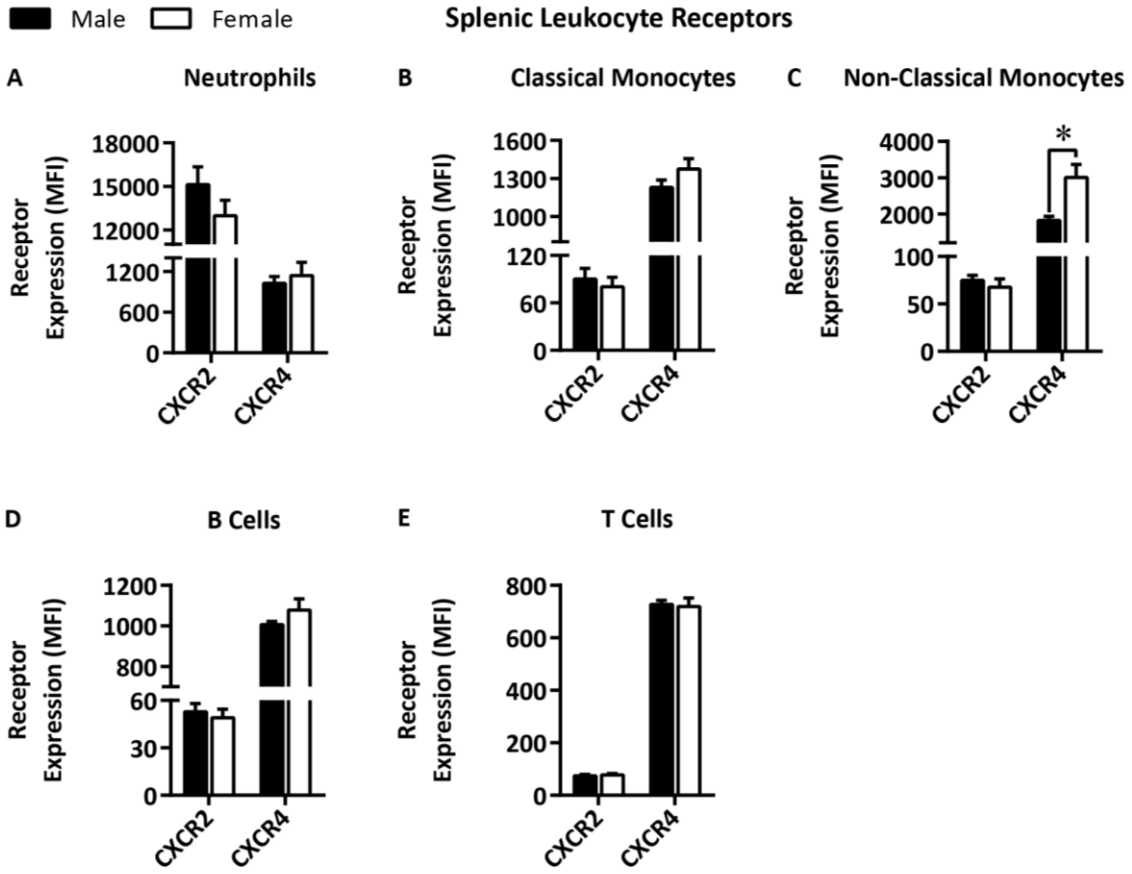


Figure 8.5. Basal splenic leukocyte CXC chemokine receptor expression. Leukocytes were isolated from the spleens of male (■) and female (□) naïve mice. The expression of CXCR2 and CXCR4 was assessed on the cell surface of (A) neutrophils, (B) classical monocytes, (C) non-classical monocyte, (D) $B220^+$ B cells, and (E) $CD3^+$ T cells. Receptor expression is displayed as median fluorescence intensity (MFI). Data are displayed as the mean ± SEM (n=6). Differences in receptor expression were determined by Student's *t*-test and significance indicated by * ($p<0.05$).

and expressed less on the surface of the other leukocyte subsets (Figure 8.5). No significant difference between the expression of CXCR2 on the surface of male and female splenic leukocytes was observed (p>0.05). CXCL12 on the other hand acts on CXCR4 on neutrophils, but also on monocytes, to retain these cells in the BM. CXCR4 was expressed in similar levels on the surface of neutrophils, monocyte subsets, and B cells from the spleen (Figure 8.5). Non-classical monocytes from the female spleen however, had significantly (p<0.05) higher levels of CXCR4 than those from the male spleen (female MFI: 3003±359.6; male MFI: 1821±108.9, Figure 8.5C).

Chemokines CCL2, CCL3, and CCL5 were all detectable, albeit at low levels, in the mouse spleen as determined by cytokine array. CC chemokines are more promiscuous than those containing the CXC motif and exert their effects through binding to a selection of CC chemokine receptors. CCR1, CCR2, CCR3 and CCR5 represent key monocyte chemokine receptors that are associated with the above ligands. CCR1 represents a receptor for an array of ligands primarily chemotactic for monocytes but in some cases for neutrophils also. Consequently, CCR1 was most highly expressed on the monocyte subsets and neutrophils, but was also expressed on T cells, in line with CCL5 also a T cell chemoattractant, and B cells (Figure 8.6). CCR2, the receptor responsible for the chemotactic properties of CCL2 for classical monocytes, are well documented, and it is known not to attract neutrophils. In the spleen, CCR2 was found to be most highly expressed on classical monocytes, followed by the non-classical subset, with very low expression on neutrophils and lymphocytes (Figure 8.6). CCR3 is the receptor for chemokines including CCL5, CCL7, CCL11 and CCL13, and CCR5, the receptor for chemokines including CCL3, CCL4 and CCL5, showed low expression on all splenic leukocyte subsets studied (Figure 8.6). Notably, no sex-differences in the expression of CCR1, CCR2, CCR3, or CCR5 were evident on splenic leukocyte subsets with the exception of non-classical monocyte CCR2 which was more greatly expressed on male (MFI: 20.6±1.01 $x10^3$) versus female cells (MFI: 13.0±1.69 $x10^3$) (p<0.01) (Figure 8.6C).

Finally addressed was the complement component C5a receptor, C5aR. Although not its primary function, C5a also represents an effective chemoattractant for neutrophils and monocytes via C5aR. C5aR was highly expressed on splenic neutrophils, with moderate monocytic expression and low lymphocyte expression. Sex-differences were again evident on non-classical monocytes with female cells exhibiting significantly (p<0.01) higher expression of C5aR versus male cells (MFI: 4.6±0.35 $x10^3$ versus 2.8±0.34 $x10^3$, Figure 8.6C). All other splenic leukocyte subsets demonstrated similar expression levels between the sexes.

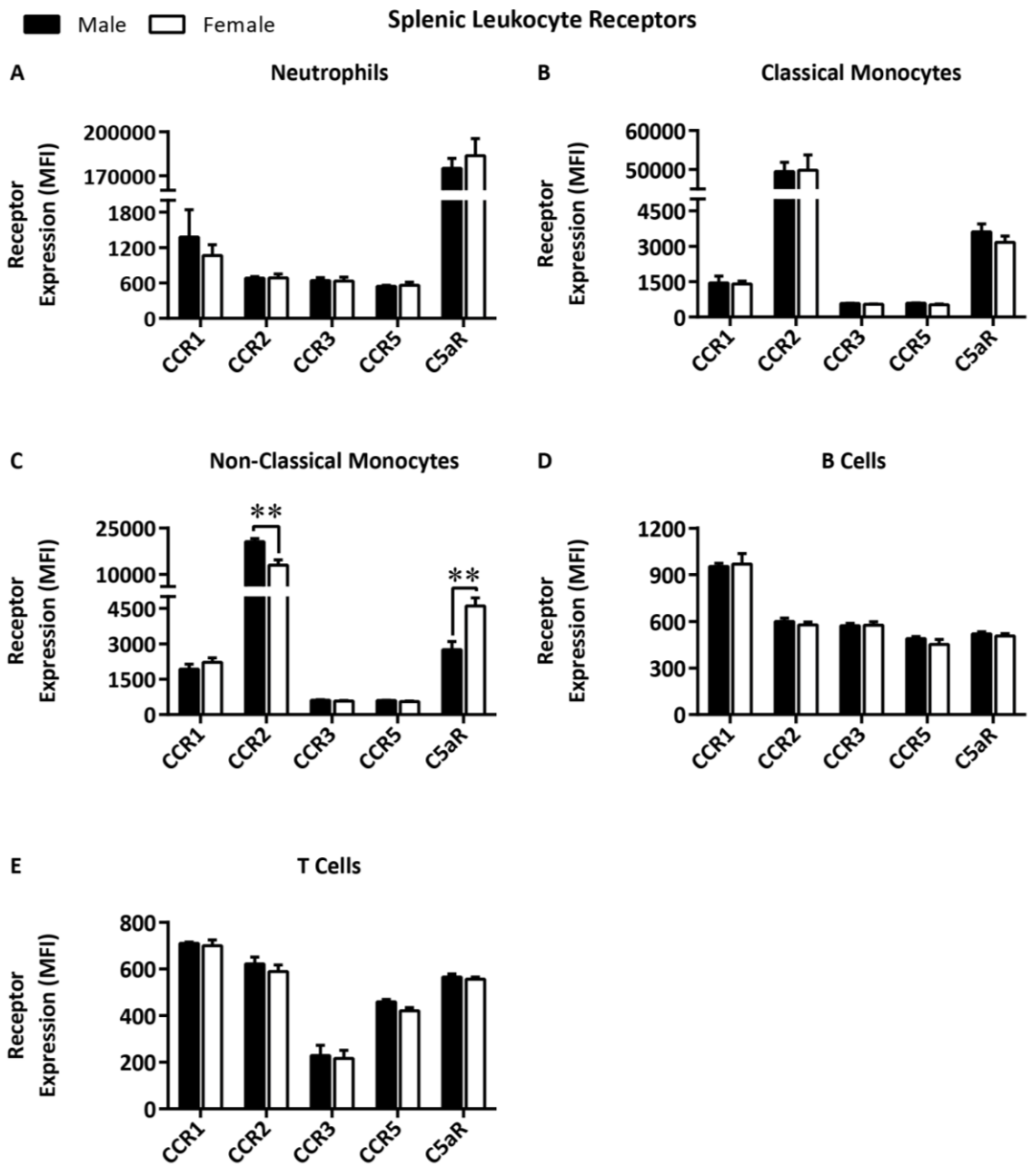


Figure 8.6. Basal splenic leukocyte CC chemokine and complement receptor expression. Leukocytes were isolated from the spleens of male (■) and female (□) untreated mice. Cell surface expression of CCR1, CCR2, CCR3, CCR5, and C5aR was assessed on (A) neutrophils, (B) classical monocytes, (C) non-classical monocyte, (D) $B220^+$ B cells, and (E) $CD3^+$ T cells. Receptor expression is displayed as median fluorescence intensity (MFI). Data are displayed as the mean ± SEM (n=6). Differences in receptor expression were determined by Student's *t*-test and significance indicated by ** ($p<0.01$).

8.4. Discussion

This chapter demonstrates differential trafficking of leukocyte subsets to and from the murine spleen in the zymosan peritonitis model of acute inflammation. Chapter 4 of this thesis described male mice to accumulate more neutrophils and classical monocytes in the circulation during peritonitis, and furthermore recruit more of these cells to the peritoneal cavity peaking at 3h after zymosan administration. To address the mechanism of such dimorphisms, the leukocyte storage pools were investigated with the hypothesis that male mice utilised these reserves differentially to female mice. Investigations into the BM however yielded similar mobilisation responses in male and female mice. This chapter therefore evaluated the spleen as another leukocyte storage pool.

It has already been demonstrated in Chapter 3 of this thesis that the spleen indeed represents a pool of neutrophils and monocytes in addition to the well characterised B and T cells. Furthermore, sex-differences in basal numbers were observed with male mice possessing significantly more neutrophils, classical and non-classical monocytes compared to females. In response to zymosan peritonitis, classical monocytes were found to mobilise from the spleen of male, but not female mice (Figure 8.7). Mobilisation of splenic classical monocytes in inflammation is in line with a previous study that demonstrated such cells exited the spleen in response to myocardial I/R in mice (Swirski *et al.*, 2009). The function and fate of mobilised splenic classical monocytes in male mouse peritonitis is not however clear. Indeed, mobilisation of splenic monocytes has yet to be reported in response to infection. It could be hypothesised that these cells represent the additional classical monocytes that accumulate in the male circulation compared to females, and those cells that also are subsequently recruited to the tissue in zymosan peritonitis. Whether they are actually recruited to the tissue is uncertain in this model. Transplantation of spleens from $CX_3CR1^{+/gfp}$ to WT mice however would definitively address this hypothesis. Alternatively, a failure to observe sex-differences in circulating and recruited classical monocyte numbers after splenectomy would also be highly suggestive of these additional cells originating in the spleen.

In a similar fashion to the classical subset, non-classical monocytes were not mobilised from the spleens of female mice by 3h post zymosan. A wide spread of data for male mouse non-classical monocytes responses made conclusions difficult. Although total monocytes have been shown to exit the spleen and the classical subset shown to accumulate in the tissue in myocardial I/R as described above, the topic of splenic non-classical monocyte mobilisation remains unclear as the exiting monocytes were not studied in subsets (Swirski *et al.*, 2009; Ingersoll *et al.*, 2011). Mobilisation of non-classical monocytes at the 3h time point would

however be contrary to the two accepted phases of these cells, firstly as patrolling cells that extravasate within 1h, and secondly later in the inflammation in tissue remodelling and wound repair (Auffray *et al.*, 2009a).

Unlike classical monocytes, neutrophils were not mobilised from the spleens of male or female mice during early zymosan peritonitis. This is in line with the lack of reports of splenic neutrophil mobilisation in inflammation. In female mice however, neutrophils traffic to the spleen, a phenomenon that was not observed in males (Figure 8.7). Accumulation of neutrophils in the spleen in inflammation has been previously described in response to viral infection (Norris *et al.*, 2013). In addition, homing to the spleen (as well as the BM and liver) is a well described process for the uptake and removal of senescent neutrophils (Summers *et al.*, 2010), and may suggest that male and female neutrophils early in zymosan peritonitis are at different stages of maturity. Another possible explanation for the observations described may

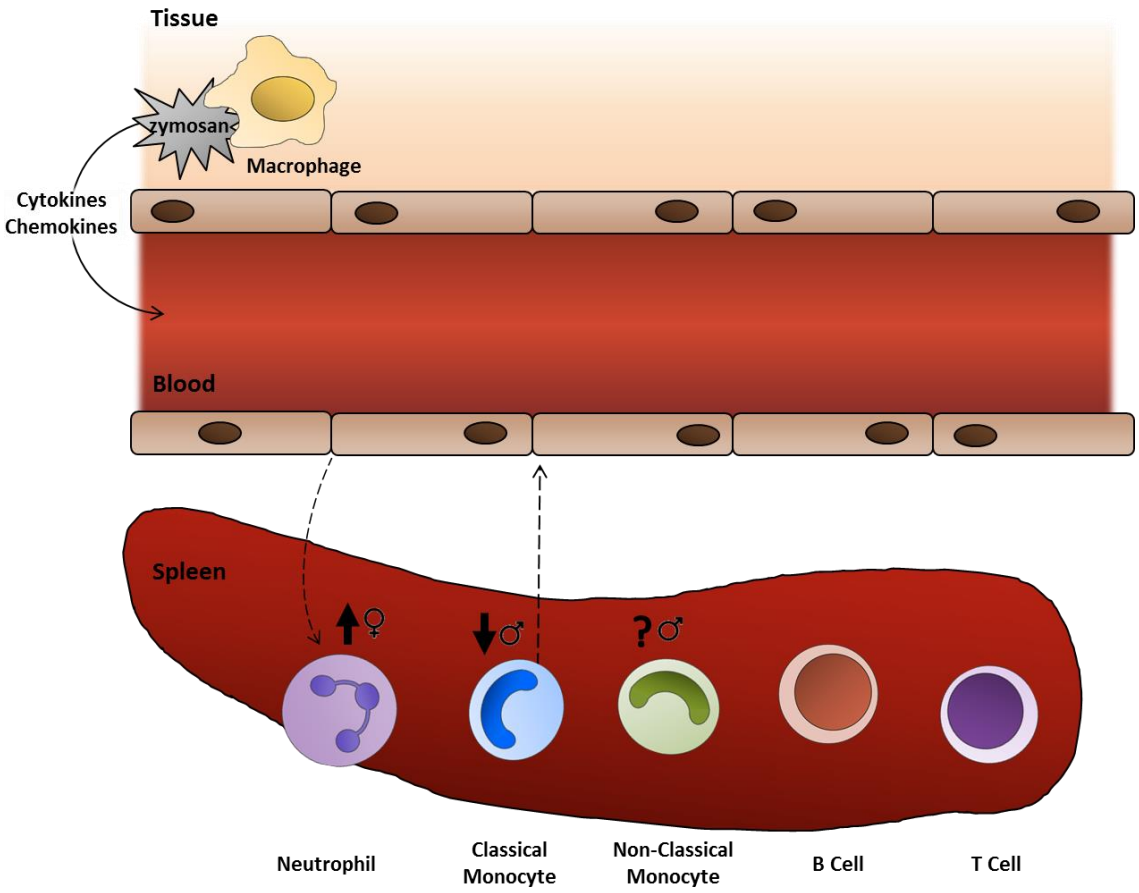


Figure 8.7. Summary of splenic leukocyte trafficking during zymosan peritonitis. Administration of intraperitoneal zymosan and subsequent detection by resident macrophages in male (♂) and female (♀) mice induces leukocyte trafficking to and from the spleen. Solid arrows indicate either increase (↑) or decreased (↓) numbers after 3h of peritonitis. Broken arrows represent likely direction of trafficking between compartments.

lie within the recently described B cell-helper neutrophils (Puga *et al.*, 2012). Neutrophils identified around the specialised B cell area marginal zone in the spleen of mice were able to induce various functions in B cells including immunoglobulin class switching, somatic hypermutation, and antibody production. Furthermore, neutropenic patients had fewer and hypomutated marginal zone B cells, identifying a role for these neutrophils in humans also. This study is consistent with an increasing number of other works that show neutrophils not only act in innate immunity, but can also do so in the context of adaptive immunity. It is conceivable that the homing of neutrophils specifically to the female spleen is indeed inducing B cell responses that dampen the immune response, or at least initiate early adaptive responses that account for a less severe reaction in female mice. Analysis of the localisation and extent of peri-marginal zone neutrophils in sectioned spleens of naïve and zymosan-treated male and female mice could test such a hypothesis.

To investigate the mechanism behind the sex-differences in basal splenic neutrophil and monocyte numbers, the splenic chemokine environments of male and female mice were assessed with the rationale that different splenic environments account for different immune cell reserve pools. Cytokine arrays revealed a comparable cytokine/chemokine profile in male and female spleens. In line with the function of the spleen primarily as a lymphoid organ, CCL5, CXCL9 and CXCL10 all possessing T cell chemotactic abilities, were found in high relative quantities. sICAM-1 and IL-1ra were also detected in moderate relative quantities. High sICAM-1 signal is likely derived from residues of endothelium or leukocytes, both of which are abundant in the spleen and are known to be ICAM-1 expressing (Dennig *et al.*, 1994; Lin *et al.*, 2000). The role of either the soluble or membrane bound ICAM-1 in the spleen is not however well documented. It is furthermore probable that IL-1ra, as a natural inhibitor of IL-1R, is abundant in order to dampen the immune response due to the concentrated nature of immune cells in the spleen. Nevertheless, the cytokine/chemokine profiles of the male and female mouse spleen were similar, indicating the splenic environment is unlikely to underlie sex-differences in basal leukocyte subsets. The use of such arrays however is not quantitative and to fully define differences in the male and female splenic chemokines ELISAs of specific key molecules should be carried out.

As assessment of basal splenic cytokine/chemokine environments elucidated no explanation for the observed sex-differences, expression of splenic leukocyte receptors were investigated. Molecules expressed in the spleen with neutrophil or monocyte chemotactic properties were identified from the arrays with male and female splenic leukocytes assessed for the expression of the corresponding receptors (i.e. CCR1, CCR2, CCR3, CCR5), and in addition for receptors

known to be involved in the chemotaxis of these cells (i.e. CXCR2, CXCR4). Neutrophils from male and female mouse spleens expressed similarly high levels of CXCR2, whilst all other leukocyte subsets showed very low receptor expression in both sexes. Despite high neutrophil CXCR2, only negligible levels of its ligands CXCL1 and CXCL2 were detected in the array. Investigations into other CXCR2 ligands such as CXCL3, CXCL5, CXCL6, and CXCL7 may reveal retention axes, however from this data it is unlikely that neutrophils are retained in the spleen via the actions of CXCL1 and CXCL2. In addition, the expression of CXCR2 on splenic neutrophils suggests that despite their lack of mobilisation in response to zymosan peritonitis, they may exit the spleen should the chemotactic signal from CXCR2 ligands be strong enough.

In the BM, neutrophils and classical monocytes are retained via the CXCL12/CXCR4 axis, with CXCL12 expressed by BM stromal cells in high amounts under basal conditions (Ma *et al.*, 1999). Others have demonstrated that CXCL12 is expressed in the red pulp of the spleen (Puga *et al.*, 2012), making a similar axis plausible in the context of the spleen. Neutrophils, monocytes, and B cells displayed similar levels of CXCR4. No difference in expression levels between the sexes was observed on neutrophils, classical monocytes, B or T cells. Female non-classical monocytes however had significantly elevated CXCR4 expression versus male cells. This contradicts the sex-differences in basal splenic non-classical monocyte numbers whereby male mice exhibit more of these cells compared to females. Added to similar expression levels in neutrophils and classical monocytes, it could be that a stronger retention axis that overrides that of CXCL12/CXCR4 exists in the spleen and is responsible for differential leukocyte subset numbers in uninflamed conditions.

CC-chemokine receptors, although generally not associated with neutrophil chemotaxis, showed similar expression between neutrophils, monocytes and lymphocytes of CCR1, CCR3 and CCR5. CCR2 however, shown to be important for classical monocyte trafficking to the spleen in bacterial infection (Serbina *et al.*, 2006) and one of the most potent monocyte chemoattractants (Boring *et al.*, 1997), was very highly expressed on classical monocytes, and was also expressed in relatively high levels on the non-classical subset. The expression of the latter is a debated subject, with some describing non-classical monocytes as $CCR2^{-}$ (Geissmann *et al.*, 2010) and others describing them as $CCR2^{low}$ (Shi *et al.*, 2011a). This chapter demonstrates in the context of the spleen, these cells indeed express the CCR2 receptor. Further analysis of splenic leukocyte subset receptors yielded similar expression of CCR1, CCR2, CCR3 and CCR5 between the sexes. The exception from this rule however was non-classical monocytes that indeed displayed sexual dimorphisms with respect to chemokine receptors. In addition to higher CXCR4 expression in females, non-classical monocytes were

also higher in C5aR in females, and higher in CCR2 in males. The reasons for sex-differences in the expression of these receptors are unclear. Although not highly expressed as demonstrated in the cytokine array, C5a represents an effective chemoattractant for neutrophils and monocytes via C5aR and so was analysed in addition to the classic chemokine receptors. With the exception of the aforementioned differences in non-classical monocytes, the expression of C5aR was comparable between male and female mice.

As described in Chapter 3, splenic neutrophils, monocytes, $B220^+$ B cells and $CD3^+$ T cells express TLR2 and TLR4, albeit in particularly low levels on T cells. The temporal assessment of these receptors on leukocytes in different compartments during inflammation was analysed in the spleen. Expression was assessed at the early time point in the zymosan peritonitis model in male and female mice with the hypothesis that sex-differences in remote leukocyte TLRs may also play a role in cellular mobilisation. This thesis has already demonstrated no sex-differences in the TLR2 and TLR4 expression on neutrophils, classical monocytes, non-classical monocytes, B cells and T cells. During early (3h) zymosan peritonitis, when leukocyte mobilisation and tissue accumulation peaked, similar expression was also seen on the said male and female leukocyte subsets. The only exception being at this time point, female splenic $B220^+$ B cells had a trend towards more TLR4 than males. Owing to the spleen being a complex organ for B cells, with both marginal zone and follicular types as well as plasmablasts and plasma cells (Mebius *et al.*, 2005), it is difficult to draw conclusions on this modulation and the inclusion of more markers to differentiate B cell type would be required. Moreover, in response to 3h of zymosan peritonitis little modulation of either TLR2 or TLR4 was observed, apart from a trend towards an increase in the expression of classical monocyte TLR2 in female mice. The reasons for this change are unclear however it represents only a small upregulation of the receptor and therefore the likelihood of any biological consequences may be questioned.

Overall, this chapter extends upon the sexual dimorphisms in basal splenic leukocyte subset pools and outlines key differences in the trafficking of these leukocytes to and from the spleen in the zymosan peritonitis model. Both an accumulation of female neutrophils and a mobilisation of male classical monocytes logically address the sex-differences in leukocyte responses in inflammation with greater accumulation of neutrophils and classical monocytes in the male blood and tissue. Whether the mobilised classical monocytes represent cells that contribute to pathogen clearance, or host tissue damage is at present unclear. Furthermore, whether additional neutrophils in the female spleen contribute to more efficient resolution of inflammation also remains open for further investigation. Little modulation of splenic

leukocyte TLR2 and TLR4, and overall similar expression between male and females, suggests these receptors have little involvement in the responses to zymosan at the time point studied. Nevertheless, the mechanisms behind sex-differences under naïve conditions continue to remain uncertain as similar splenic cytokine/chemokine profiles were observed between the sexes, in addition to similar expression of the key neutrophil and monocyte chemokine receptors on the splenic leukocytes. Although a long and diverse path exists between the zymosan peritonitis inflammatory model and human disease, improving knowledge of the role of the spleen may provide new targets for the treatment of inflammatory disorders.

Key findings:

- Neutrophils traffic to the spleen in female, but not male, mice early during zymosan peritonitis.
- Classical monocytes exit the male, but not female, spleen during zymosan peritonitis.
- Similar basal splenic cytokine/chemokine profiles exist between the sexes.
- No difference was seen in the expression of CXCR2, CXCR4, CCR1, CCR2, CCR3, CCR5, and C5aR on male and female murine splenic neutrophils, classical monocytes, $B220^+$ B cells, and $CD3^+$ T cells.
- Male mouse splenic non-classical monocytes had greater CCR2 expression, and reduced C5aR and CXCR4 expression compared to females.

Chapter 9: General Discussion

9.1. Project overview

Considerable sex-differences exist in both the prevalence and severity of immune diseases in humans. Whilst men are more prone to diseases with an inflammatory nature, they are relatively protected in autoimmune diseases (Whitacre, 2001). This disparity is most evident in conditions involving excess or uncontrolled activation and recruitment of leukocytes, for example I/R injuries and infection (Luster *et al.*, 2005). Such diseases also represent the indications of new TLR2 and TLR4 targeted therapies (Ehrentraut *et al.*, 2011b; Arslan *et al.*, 2012). The precise mechanisms underlying sexual dimorphisms in inflammatory diseases are not well understood. More recently disparities in the phenotype of innate immune cells have been reported between the sexes (Scotland *et al.*, 2011). Female mouse tissue leukocytes were shown to be both more numerous and more abundant in TLR2 and TLR4, resulting in a more efficient, and less severe, response to inflammagens. This study therefore aimed to comprehensively analyse the temporal profile of leukocyte recruitment and TLR expression in male and female mice during the zymosan peritonitis model of acute inflammation. The findings of this work contribute to our understanding of sex-differences both in the innate immune cell storage pools, and the leukocyte trafficking responses during acute inflammation. This thesis has demonstrated for the first time:

- Greater basal neutrophil and monocyte stores in the male mouse spleen.
- Higher basal TLR2 and TLR4 expression on male BM neutrophils.
- Sex-differences in the trafficking of neutrophils and classical-monocytes during zymosan peritonitis.
- Limited regulation of TLR2 and TLR4 expression on leukocyte subsets during zymosan peritonitis.

9.1.1. Sex-differences in basal splenic leukocyte pools

Due to previous reports of female mice having more resident peritoneal and pleural cavity leukocytes compared to aged-matched male counterparts (Scotland *et al.*, 2011), initial studies investigated sex-differences in leukocyte subset compositions in other leukocyte containing pools. These experiments not only showed greater numbers of macrophages and B cells occupying the naïve female mouse peritoneum, a finding reflected in the literature, but also

unveiled the murine spleen as a dimorphic pool of leukocytes. The results revealed male mice to have larger numbers of neutrophils, classical, and non-classical monocytes compared to female counterparts. No differences in the numbers of lymphocytes were observed. This is the first description of such differences in the murine spleen composition.

Monocytes that enter tissues are considered to differentiate into macrophages. Indeed 3 specific splenic macrophage subsets exist (red-pulp, marginal zone, and metallophilic), all of which are monocyte-derived (Mebius *et al.*, 2005). More recently however, the spleen has been shown to act as a reservoir for mature, undifferentiated monocytes in both humans and mice (Swirski *et al.*, 2009; van der Laan *et al.*, 2014). Correspondingly, the splenic monocytes described in this study indeed appear undifferentiated, resembling those in the circulation both with regards to CD115 and CX_3CR1 expression, and also in terms of their FSC/SSC (size/complexity) profile. It can therefore be concluded that monocytes present in the spleens in this study are indeed *bona fide* monocytes available for release should the body require them.

The spleen functioning as a reservoir for neutrophils is more controversial. Neutrophils were considered not to reside in the spleen in the absence of infection however recently a new subset, termed 'B-helper' neutrophils was reported (Puga *et al.*, 2012). These neutrophils specifically colonised peri-marginal zone areas of the spleen under resting conditions, forming NET-like structures to interact with B cells. The consequences of such interactions caused B cell activation and stimulated Ig class switching, somatic hypermutation, and antibody production. Notably, these neutrophils were phenotypically distinct from circulating counterparts. No difference in expression of Gr1 or FSC/SSC profile was evident between those neutrophils in the spleen and circulation in the experiments in this thesis. Whether the neutrophils described in this thesis are of the B-helper subset remain unknown and further studies would be required to define both their phenotype and function. Should the splenic neutrophils prove analogous to those in the circulation, these cells may represent a readily releasable pool that are utilised in response to specific infectious stimuli (although not in response zymosan, as shown in this thesis). Conversely, should they be identified as B-helper neutrophils, this could have substantial consequences in the development of potential therapies. Vaccines, for example, could be developed that increase splenic B-helper neutrophil number and function, thus stimulating B cells to produce more antibodies. The fact that male mice potentially have more of these neutrophils means male and females would likely respond differently to such a therapy.

The implications of male mice having more neutrophils and monocytes than female mice provide wide scope for extrapolation into the worlds of immune disorders and therapies. However before extensive conclusions and implications can be made, similar studies in humans must be performed. Although the overall structure of the human spleen is similar to that of the mouse, differences exist in the white pulp (Mebius *et al.*, 2005). More specifically, whilst mice have a simpler single marginal zone surrounding the T-cell area and B cell follicles of the white pulp, humans have an inner and outer marginal zone which itself is surrounded by a larger perifollicular zone (Figure 9.1). Very recently the presence of neutrophils in the resting human spleen was described (Nagelkerke *et al.*, 2014). These neutrophils were however comparable to those in the circulation and exhibited none of the B-helper characteristics or functions that were described in mice. Whether B-helper neutrophils are absent in all humans and thus are mouse-specific is still unclear. Spleen samples used in the described study were excess tissue from healthy organ transplant donors. If the donors were living or deceased however, is not clear due to ambiguity in the methods. Although it is very plausible B-helper neutrophils are not present in humans, the nature of the samples and donors mean it is still possible these cells exist in the human spleen. Furthermore, if those neutrophils reported in the human spleen are not of a B-helper nature, the question still remains as to their function. Experiments to both investigate the localisation and phenotype of human splenic neutrophils may reveal some indications with regards to their function.

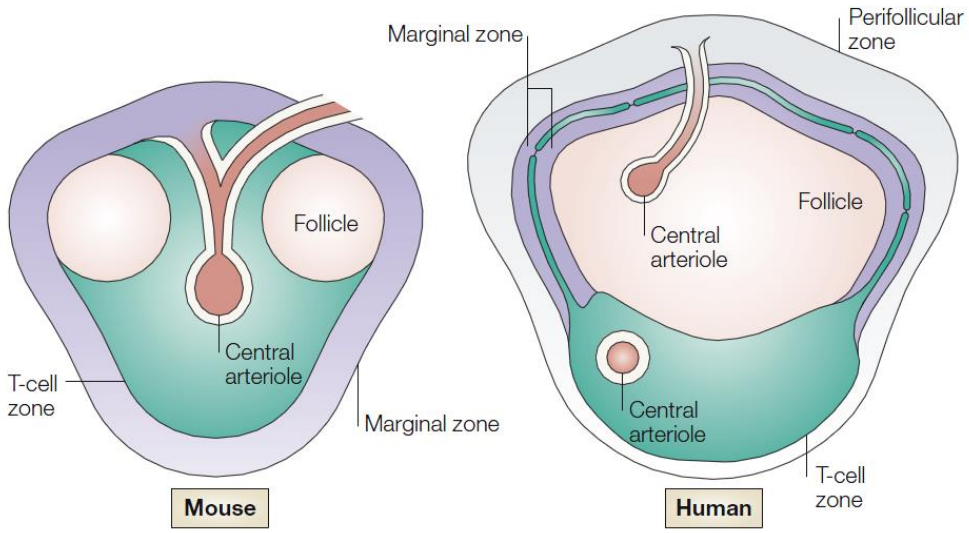


Figure 9.1. Structural differences between mouse and human splenic white pulp. The marginal zone surrounds the splenic white pulp. Whilst mice have a single marginal zone, humans have both an inner and outer zone which itself is surrounded by the perifollicular zone. Human and mice also have differences in the location of terminating blood vessels. (Reproduced from Puga *et al*., 2005).

Interestingly, female mice had comparable spleen sizes and total leukocytes to aged-matched males, suggesting a difference in body size does not affect this key lymphoid organ. In agreement with these findings, spleens from men and women have also been shown to be similar in size (Machalek *et al.*, 1998). This suggests, in addition to similar splenic lymphocyte numbers between the sexes, that female mice do not have fewer neutrophils and monocytes because they are smaller than aged-matched male comparisons. With the aim to elucidate the mechanism by which different leukocytes were present in the male and female spleen, initial investigations examined potential retention axes. No differences in either cell surface chemokine receptors or splenic chemokines themselves were evident between the sexes. Although not every potential axis was examined, key ones known to exist for other storage pools (i.e. CXCL12/CXCR4, CCL2/CCR2) were studied but did not reveal any potential mechanisms to explain the observed sex-differences. As already discussed, the functions of neutrophils and monocytes in the naïve spleen are yet to be elucidated. Further investigation into the functions of these cells may provide insight into the prominent chemokine-chemokine receptor interactions by which they are retained and released from the spleen. Studies of the localisation of neutrophils and monocyte subsets within the spleen could be important to shed light on their functions.

Overall the sex-differences identified in the splenic leukocyte pool may have important translational implications. However the significance of such a finding can only be established and utilised if similar dimorphisms are identified in humans. As yet, no studies have evaluated the leukocyte compositions of the spleens of men and women. Conducting such studies may well reveal the spleen as a contributing factor to inflammatory or autoimmune diseases that exhibit disproportion in their prevalence between the sexes. As such, this difference could be exploited to develop new therapeutics to treat such diseases in a more personalised manner.

9.1.2. Differential trafficking of neutrophils and classical monocytes in male and female mice during zymosan peritonitis

Previous studies have reported reduced severity and neutrophil recruitment in peritonitis in female mice (Scotland *et al.*, 2011). To investigate the influence of sex on the recruitment of other leukocyte subsets, i.e. monocytes, the zymosan peritonitis model of acute inflammation was employed. Male mice recruited significantly more neutrophils and classical monocytes to the peritoneal cavity 3h after induction of peritonitis. Interestingly, this data shows neutrophils and classical monocytes are able to extravasate at the same time. This suggests that although neutrophil-mediated monocyte recruitment has previously been described (Soehnlein *et al.*,

2009a), neutrophils and classical monocytes can also be recruited simultaneously, likely via chemotactic gradients established by activated macrophages. The ability of classical monocytes to be recruited independently of neutrophils has also previously been documented (Henderson *et al.*, 2003). The temporal involvement of non-classical monocytes in zymosan peritonitis is not as clear. In the dermis, non-classical monocytes reportedly patrol the endothelium and extravasate within 1h upon inflammagen application. The data in this thesis does not support such trafficking as by 3h peritoneal non-classical monocyte numbers are lower than control conditions. To fully establish the time at which non-classical monocytes play a role in the response to zymosan, an earlier and later time point should be analysed.

Also of note is the rapid loss of resident peritoneal macrophages, with almost complete clearance after 3h of zymosan peritonitis. The fate of these macrophages is not clear, but is in agreement with previous reports (Navarro-Xavier *et al.*, 2010). It is most plausible that after the detection and phagocytosis of zymosan, macrophages undergo apoptosis and were therefore cleared. In line with the current paradigm, it is likely that infiltrating classical monocytes differentiate to repopulate macrophage populations, although this currently remains speculative in the case of zymosan peritonitis (Randolph, 2011).

Sexual dimorphism in cavity leukocyte accumulation detailed in this thesis also highlights the possibility that male or female sex may influence the ability of leukocytes to transmigrate. To determine if this was the case, the influence of peritonitis on the circulating leukocyte number was evaluated. For the first time males were shown to also have greater numbers of neutrophils and classical monocytes in their circulation at the 3h time point. Since greater leukocyte numbers were evident upstream of the transmigration process, differences in the ability of male and female neutrophils and classical monocytes to transmigrate was deemed an unlikely explanation for the dimorphism in accumulated cavity immune cells. Indeed, previous studies have shown both differences in tissue accumulation and blood accumulation (Hannawa *et al.*, 2006; Scotland *et al.*, 2011), but few have linked the two. Little evidence exists to support differential transmigration with surprisingly no *in vivo* reports to date detailing integrin and adhesion molecule differences between the sexes in the context of neutrophils, monocytes or ECs.

To determine the source of the additional circulating and peritoneal cells male mice accumulate during zymosan peritonitis, major leukocyte storage pools were studied. Neutrophils and classical monocytes exited the BM by 3h to equivalent extents in both sexes, demonstrating the importance of the BM storage pool in contributing leukocytes to the response to the peritonitis. Although the BM has long been established as the origin of

monocytes from the common monocyte-macrophage-DC precursor (MDP), the development of the subsets is a debated topic (Saha *et al.*, 2011). The literature contains evidence for both subsets deriving independently from a common precursor, but also for the non-classical ($Ly6C^-$) deriving from classical ($Ly6C^+$) subset (Varol *et al.*, 2007; Auffray *et al.*, 2009a; Geissmann *et al.*, 2010). The data generated in this thesis demonstrates the presence of both monocyte subsets in the BM, however classical monocytes were approximately 3-fold more numerous than non-classical. Such a difference in numbers was not evident in the circulation, however from the data obtained it is unfortunately not possible to comment on, or contribute to the knowledge of, monocyte subset development beyond that both subsets were indeed present in the resting BM and circulation.

In contrast to the BM, the splenic leukocyte storage pool demonstrated evidence of sexual dimorphisms. This study shows for the first time that neutrophils traffic to the spleen of female mice in zymosan peritonitis, a phenomenon not observed in males. Conversely, male, but not female, mice demonstrate a release of their splenic classical monocyte pool. Homing of neutrophils to the spleen in females, coupled with a release of the additional splenic classical monocytes in males, produces the gross outcome of more neutrophils and classical monocytes in the male circulation. This is in agreement with the data in this thesis and produces a logical model for sex-differences in leukocyte trafficking in early zymosan peritonitis (Figure 9.2). This represents the first report of differential trafficking of leukocyte subsets in the spleen between male and females.

The relative contribution of the spleen to the peritonitis can be demonstrated by comparing cell numbers to that of the BM. Due to the closed system nature of the inflammation, an '*in silico*' model can essentially be developed. Changes in numbers of neutrophils and classical monocytes are demonstrated in Table 9.1. By these means, it is clear that despite the spleen being a dimorphic pool of these cells, the BM is by far the biggest contributor in terms of mobilised cells. Indeed, these cells are released in excess with a large number unaccounted for in the compartments analysed (blood, spleen, peritoneal cavity). Whilst this model assumes equal BM leukocyte mobilisation from primarily the long bones (Boggs, 1984), it is highly possible that these are heterogeneous depending on the bone and proximity to the stimulus, therefore the total BM release may represent an over estimation. Other explanations for the large number of additional cells that are not accounted for by the BM may be trapping in other organs, such as the lung and liver, as is known to occur in other inflammatory diseases (Woodfin *et al.*, 2011). Furthermore, there is likely to be a large number of both neutrophils and classical monocytes that are in various stages of the leukocyte adhesion cascade that are

unlikely to have been successfully isolated, be it rolling, crawling, or undergoing transendothelial migration throughout the highly vascular mesentery. Therefore whilst the spleen appears the minority contributor to the overall mobilised classical monocytes, further studies must be carried out to determine whether the recruited cells do in fact originate from the spleen. It is plausible that the phenotype, function, or primed status of splenic classical monocytes differ from that of the BM and that whilst they are fewer in number, they are better equipped to traffic to a site of inflammatory insult and remove the pathogen or damaged tissue.

	Neutrophils		**Classical Monocytes**	
	Male	Female	Male	Female
Total Blood*	$+3.2x10^6$	$+1.4x10^6$	$+1.8x10^6$	$+0.64x10^6$
Peritoneal Cavity	$+11.4x10^6$	$+8.0x10^6$	$+0.27x10^6$	$+0.15x10^6$
Spleen	-	$+3.2x10^6$	$+0.90x10^6$	-
Total BM$^{\$}$	$-20x10^6$	$-20x10^6$	$-5.2x10^6$	$-4.3x10^6$
Surplus	$+5.4x10^6$	$+7.4x10^6$	$+2.2x10^6$	$+3.5x10^6$

Table 9.1 Summary of changes in neutrophil and classical monocyte numbers in murine zymosan peritonitis. Neutrophils and classical monocytes were isolated from the blood, peritoneal cavity, spleen, and femur of male and female mice treated with or without zymosan (1mg i.p., 3h). Figures in the table indicate the number of leukocytes that have increased (+) or decreased (-) during the inflammation. *Total blood is values adjusted for total blood volume assuming 1.5ml for males and 1.2ml for females. $^{\$}$Total BM assumes 8 long bones, therefore femur leukocyte number x8. Surplus indicates the number of cells released from the BM but not accounted for in the other compartment studied.

The mechanisms by which neutrophils home to the spleen and classical monocytes exit the spleen are unclear. In a mouse model of MI, classical monocytes were shown to leave the spleen and accumulate in the circulation and myocardium in a process dependent on angiotensin II and the monocyte angiotensin II receptor 1 (AT-1) (Swirski *et al.*, 2009). Whether angiotensin II may firstly play a role in classical monocyte trafficking in zymosan peritonitis, and secondly account for the sex-differences observed with regards to monocytes, is not clear. No reports of differences in angiotensin II concentrations or activities in men and women exist in the literature, however the AT-2 receptor is located on the X chromosome (Fish, 2008). During investigations of angiotensin II-dependent splenic monocyte egress in MI, only the AT-1 was studied leaving scope to evaluate the role of AT-2 also to explain sex-differences in classical monocyte trafficking identified in this thesis.

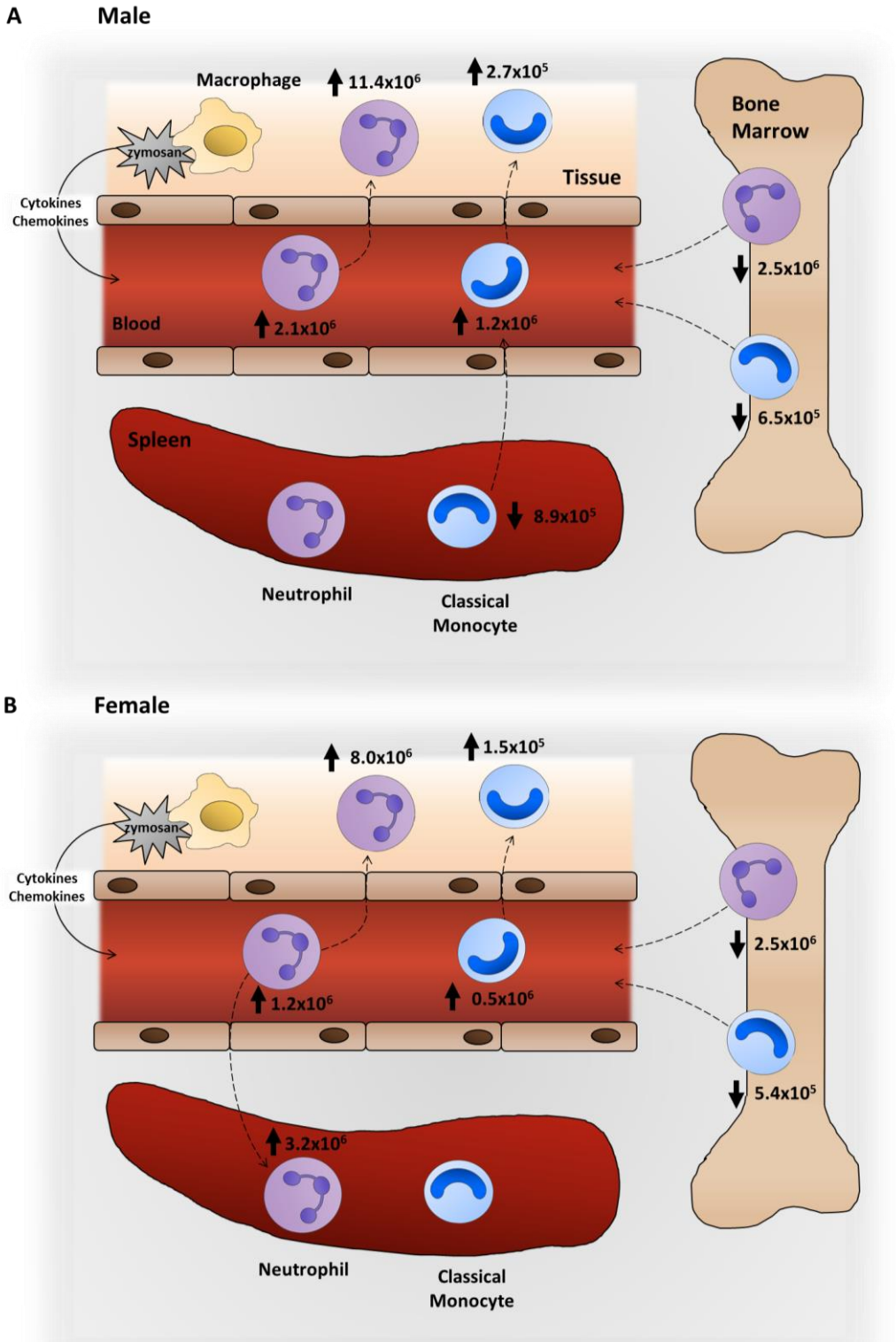


Figure 9.2. Summary of sex-differences in neutrophil and classical monocyte trafficking in zymosan peritonitis. Administration of zymosan to the peritoneal cavity induced differential trafficking of neutrophils and classical monocytes in (A) male and (B) female mice after 3h.. Neutrophils and classical monocytes egressed from the bone marrow and accumulated in the circulation (blood) and peritoneum (tissue). Both cell types trafficked differently to or from the spleen in male and females. Block arrows indicate increase (↑) or decrease (↓) in the indicated cell numbers in the specified compartment. Dashed lines denote the direction of movement between compartments after 3h of zymosan peritonitis.

Paradoxically, despite male mice mounting a more exaggerated response to zymosan peritonitis at the peak 3h time point, neutrophils in both the circulation and peritoneal cavity by 24h were similar in number between the sexes. This data is highly robust as sex-differences still prevail should the data be presented as percentage leukocytes, subset numbers versus body weight and versus organ (BM, spleen) weight (not shown). In essence, males have a transiently enlarged response however still resolve the inflammation at the same rate and time as female mice. The reasons for this are unclear both from the literature and this study. It would be fair to speculate that more peritoneal macrophages, with greater phagocytic and ROS-producing capabilities (Scotland *et al.*, 2011) are able to clear the zymosan quicker, thereby less neutrophils and monocytes are required to help, meaning fewer are recruited. On the other hand male neutrophils and monocytes appear efficacious enough to remove the inflammagen but just require a greater workforce to do so. Of note, as the inflammation appears to resolve on a similar time scale between male and female mice, it would suggest the lifespan and clearance of the neutrophils is not different between the sexes. Therefore, it does not appear that male mice are unable to deal with the infection, however it is plausible that if given a stronger inflammagen the dimorphisms in the inflammatory response may become more prominent and begin to affect the outcome of the animal. Also not to be overlooked in the resolution of the peritonitis is the influence of the spleen. In female mice neutrophils accumulate in the spleen. The outcome of this clear dimorphism is one that should not be ignored. Whilst it is difficult to draw the functional consequence of this phenomenon from the data obtained in this study, it could be that at this early 3h time point after inflammation, activated neutrophils home to the female spleen and induce the adaptive immune response, thereby promoting the dampening of responses and resolution, something that does not occur in the male.

An obvious factor that has not been discussed thus far is the influence of sex hormones on the inflammatory response to zymosan. Despite being considered, female mice in the study were not selected at specific times in their oestrous cycle (referred to as menstrual cycle in humans). The murine oestrous cycle occurs every 4-5days (Caligioni, 2009). It was therefore reasoned that due to large n numbers over a period of 2 years, all stages of the cycle should be represented in this study. During certain phases of the murine oestrous cycle, i.e. pre-ovulation, plasma 17β-oestradiol is high and may be further protective in inflammation (Caligioni, 2009), however the experiments in this thesis are sure to fall over all phases of the cycle yet still produce damped inflammatory responses versus males during zymosan peritonitis. Therefore, when designing human studies whereby oestrogens may influence the

results, it is essential to choose the correct phase of the menstrual cycle, make records of such phases, and interpret results accordingly to obtain the correct model. Alternatively, larger cohorts must be used representing all phases of the longer, 28-day, human female menstrual cycle to abolish any skewing of results due to varying hormonal levels.

Differences such as those in the menstrual cycle and spleen structure between mice and humans raised the perpetually important question: Are mice a good model? Although there are obvious distinct differences between humans and mice, 99% of murine genes have human analogues (Gunter *et al.*, 2002). Basic science studies such as those in this thesis would be unable to be conducted in humans, and therefore mice do resemble the next best feasible model. This thesis uses zymosan peritonitis as a model of acute inflammation to investigate sex-differences. To replicate a real human situation is only possible up to a point in murine models, with humans subjected to vast amounts of environmental factors that cannot be replicated in mice and which will be sure to influence disease progression. Indeed, few humans develop infectious peritonitis, however humans are susceptible to acute infections and these are reported to exhibit a sex disparity. What the use of mice in this study has facilitated is identification of sex-differences in specific compartments that can then be translated into more targeted, specific, and therefore less invasive and costly, studies in humans. If the hypothesis has foundations and the model is appropriate, mice can indeed represent a good model for basic science. Their relevance to other human-specific disease models is however outside the scope of this thesis.

These findings could have important implications with respect to human health and disease. Firstly, the spleen is a poorly explored organ in inflammatory conditions. Further research into its role in human disease may present it as a candidate for therapeutic intervention, using both antagonists to dampen an overwhelming leukocyte recruitment response, and agonists to improve such a response as may be desirable in immunocompromised patients. Secondly, as sexual dimorphisms exist in both the prevalence and severity of many immune related conditions, the spleen could prove a novel therapeutic target for personalised medicines. Should sexual dimorphisms in the spleen leukocyte pool and spleen leukocyte trafficking be unveiled in humans also, targeting these for disease prevention and treatment, respectively, is likely to prove beneficial.

These data also have highly important implications for basic research. Few labs consider the sex of animals when conducting experiments, and a huge number of scientific papers fail to disclose such information in the materials and methods. Whilst it is possible that many *in vivo* models show no sex-bias, the data from this thesis validates clearly that sex should be an

important consideration with regards to experimental design and data interpretation. In addition, it is well established that many immune system disorders have a sex bias in humans. It is therefore important to select the correct sex (or investigate both sexes), when developing *in vivo* models of such inflammatory conditions. This again highlights the importance of correct experimental design and the consideration of sex is likely to in fact reduce the number of animals used, in line with the National Centre for the 3Rs, as studies would have a greater chance of success and a lesser chance of failure. Furthermore, the use of a mixture of male and female animals in inflammatory models could essentially mask any differences and may provide an explanation for failed studies. Again, consideration and selection of the correct experimental model could save animals by improving a positive study outcome and therefore reduce the chances of having to repeat studies.

9.1.3. Elevated TLR2 and TLR4 expression on basal BM neutrophils in male mice

Sex-differences in TLR expression have previously been identified in resident peritoneal macrophages (Scotland *et al.*, 2011). To explore the extent of this male-female difference, this thesis comprehensively compared basal TLR expression of various leukocyte subsets in multiple leukocyte-containing compartments. The focus was placed on TLR2 and TLR4 as these receptors were identified both as sexually dimorphic on macrophages in the above mentioned study, and as therapeutic targets in inflammatory diseases with a leukocyte recruitment pathogenesis (Shimamoto *et al.*, 2006; Arslan *et al.*, 2010b). Whether such dimorphisms exist on other leukocytes and elsewhere in the body was assessed in this thesis. Male mouse BM neutrophils were significantly higher in cell surface TLR2 and TLR4 than neutrophils from female BM. This represents the first report of sex-differences in leukocytes TLRs in the BM. No sex-differences in TLR2 or TLR4 expression were observed on neutrophils from other sources, or on macrophages, classical monocytes, non-classical monocytes, B cells or T cells obtained from the blood, peritoneum, BM or spleen. Sex-difference in leukocyte TLR2 and TLR4 expression at sites remote from the initial stimuli could suggest involvement of these receptors in the dimorphic inflammatory response.

Blood neutrophils are known to express TLRs and respond to both TLR2 and TLR4 activators (Sabroe *et al.*, 2003; Prince *et al.*, 2011). Surprisingly, the expression of TLR2 and TLR4 protein on BM neutrophils is poorly documented with only one study to date showing protein in mice by Western blot (Zhang *et al.*, 2005). Therefore, this thesis provides the first characterisation of cell-surface TLR2 and TLR4 protein on BM neutrophils, and furthermore is the first report of sex-differences in this expression. Sex-differences in BM neutrophil TLR2 and TLR4 expression

were initially hypothesised to influence the mobilisation of BM neutrophils and therefore the accumulation of these cells in the circulation and peritoneal cavity during zymosan peritonitis. Investigations into the BM however yielded similar mobilisation profiles in male and female mice, opposing a role for these receptors in release of BM stores. The purpose and function of TLR^{high} BM neutrophils in male mice remains unclear. Various speculations into neutrophil subsets, maturation, and activation states could however be made.

Neutrophils are produced and mature in the BM during the process of granulopoiesis, mediated by G-CSF (Kolaczkowska *et al.*, 2013). It is likely that granulocytes develop expression of TLRs on their cell surface only towards the point of maturity, as only mature neutrophils would be fully equipped to clear PAMPs and DAMPs. This is, however, purely speculative as there is a little evidence in the literature detailing the time at which TLRs become expressed. Although it has not been studied, sex-differences in the rate at which granulopoiesis occurs and neutrophils mature may exist, therefore it is possible that the dimorphisms observed in BM neutrophil TLR expression are in fact a reflection of different states of maturity. By the same token, different TLR expression may also be the result of sex-differences in neutrophil senescence. Whilst one group reported that male neutrophils undergo more spontaneous apoptosis, this was not in the context of the BM, and neutrophils were aged for 24h only (Molloy *et al.*, 2003). Therefore whether senescent neutrophils firstly express different levels of TLRs, and secondly, age and home back to the BM differently in males and females is currently unknown but may provide some explanations for the sex-differences observed.

The existence of neutrophil subsets in the BM combined with differences in such subsets between the sexes may provide another explanation for the sex-differences in BM neutrophil TLR2 and TLR4 described in this thesis. Three subsets of circulating neutrophils, distinguishable by cytokine production, macrophage activation, and surface antigen expression have previously been described in mice (Tsuda *et al.*, 2004). Subsets were also distinguishable by TLR5, TLR7, TLR8, and TLR9 expression however all 3 neutrophils also expressed mRNA for both TLR2 and TLR4. As the sex of the mice used in this study was not disclosed, it is possible that differences in TLR2 and TLR4 between the neutrophil subsets exist if sex is taken into consideration. Whether mature neutrophil subsets are present in the BM is not clear but may provide explanation for the sex-differences seen if such subsets themselves prove dimorphic.

It is also possible that differences in the activation states of BM neutrophils occur between male and female mice. As TLR expression has been shown to alter in certain inflammatory conditions, it is possible that sex-differences in neutrophil activation status underlie differences in TLR2 and TLR4 expression. Under basal conditions in the BM, one of the most

sterile organs, this theory is least plausible. In reality, the consequences of the TLR2 and TLR4 expression difference to the function of male and female BM neutrophils should first be assessed before dissecting the cause. Male mice express approximately 25-30% more TLR2 and TLR4 on BM neutrophils than female mice. If this is enough to influence inflammatory responses between the sexes must be determined. Surprisingly, despite this difference in the TLR expression in the BM, circulating and splenic neutrophils have similar expression in males and females. This specificity to the BM store of neutrophils suggests that TLRs may play role in the egress of the neutrophils into circulation, but also that sex-differences are likely to be a consequence of the above mentioned factors (i.e. maturation, senescence, subsets), rather than peripheral factors.

9.1.4. Stable TLR2 and TLR4 expression profile of leukocyte subsets in male and female mouse zymosan peritonitis

The temporal expression profile of leukocyte TLR2 and TLR4 was assessed during the zymosan peritonitis model of acute inflammation in male and female mice. The expression of these receptors was studied on blood, peritoneal, BM, and splenic neutrophils, classical monocytes, non-classical monocytes, B cells, and T cells. Overall, little change in receptor expression was evident on any of the leukocyte subsets throughout the inflammation. Modulation of TLR expression has however previously been reported in a number of inflammatory disease (Härter *et al.*, 2004; Ashida *et al.*, 2005; Kashiwagi *et al.*, 2012). Changes in TLR expression has therefore been implicated as a compounding factor in the pathogenesis of certain inflammatory conditions, with increased TLR expression proposed to exaggerate the inflammation (Arslan *et al.*, 2010a; Reilly *et al.*, 2013).

Although the data in this thesis present some small modulations of TLRs, e.g. increased blood neutrophil TLR4 and cavity neutrophil TLR2 and TLR4 at 24h, the relative expression levels, particularly on neutrophils, remained low. Once activated and signalling cascades are initiated, the functional consequence of a cell possessing marginally more TLRs is questionable. Whether these small but significant TLR upregulations in fact effect neutrophil function would need to be tested, however overall TLR2 or TLR4 expression remained stable during the peritonitis. Low expression of TLR2 and TLR4 on certain leukocyte subsets, including neutrophils was consistent in this study. The use of TLR antibodies may account for the low RFIs and expression levels obtained as they are renowned for being poor. This also provides explanation for the lack of TLR protein expression data, compared to the abundance of functional TLR data in the

literature. Antibody quality therefore continues to be a challenge in the quantification of proteins.

Eritoran, a synthetic TLR4 antagonist, and OPN-305, a fully humanised monoclonal antibody targeting TLR2, have reached clinical trials for conditions with leukocyte recruitment underlying their pathogenesis (sepsis and renal transplant (renal I/R), respectively) (Tidswell *et al.*, 2010; Reilly *et al.*, 2013). Eritoran, after proving beneficial in pre-clinical and Phase II clinical trials, failed in a Phase III trial of severe sepsis (Opal *et al.*, 2013). The mechanisms by which both molecules work are not completely clear, and whether such molecules are targeting leukocyte, endothelial, or tissue TLRs is also unknown. It is likely such drugs elicit their beneficial effects via blockade of leukocyte or even EC TLRs, preventing activation of these cells and thereby limiting leukocyte transendothelial migration and recruitment, and their damaging potential.

As previously discussed in this thesis, sex imbalances are evident in the prevalence of inflammatory disorders where aberrant leukocyte recruitment underlies the pathogenesis. Sex-differences in leukocyte TLR expression have previously been proposed to underlie the dimorphisms seen in these inflammatory conditions (Scotland *et al.*, 2011). As already alluded to, there is also evidence of modulation of TLR expression in inflammation. It could be hypothesised that men and women may differentially modulate expression of TLRs and in doing so affect the extent of the inflammatory reaction. The results in this thesis demonstrate that men and women have similar leukocyte TLR2 and TLR4 expression throughout zymosan peritonitis, and despite some small modulations in receptor expression, this was evident in both sexes. Overall this study provides evidence against 'personalised' TLR-targeting medicines and supports the equal treatment of male and female patients with TLR2- and TLR4-targetting therapies.

9.2. Future directions

9.2.1. Validation of TLR expression and cytokine environment data

The data demonstrate that expression of TLR2 and TLR4 were overall similar on leukocyte subsets from male and female mice under both basal and inflamed conditions. In contrast, in a number of conditions sex-differences were evident either significantly or with a trend. Previous studies have shown female murine resident peritoneal leukocytes have more TLR2, TLR3 and TLR4 mRNA compared to males (Scotland *et al.*, 2011). It would therefore be useful

to validate the data in this thesis using qPCR. Such experiments will also leave scope to investigate the expression of the other TLRs. Evaluation of TLR message may also yield information on the life span and cycling of TLRs to and from the membrane, and may provide potential indications for mechanisms of the trends in expression changes observed. Similarly, to further our understanding of the cycling of TLRs, cells could be permeabilised prior to antibody labelling and analysed by flow cytometry for both cell surface and intracellular TLR2 and TLR4. Of note, whilst TLR localisation could also be investigated by confocal microscopy, TLR antibodies were found not to be amenable to such a technique as demonstrated in Appendix 5.

Proteome Profiler™ arrays revealed no differences in the expression of cytokines and chemokines in the peritoneal cavity or spleen of male and female mice. Limitations of this technique however mean it is not fully quantitative. It would therefore be beneficial to repeat these experiments using either ELISA or a LI-COR system to detect an infrared dye as a quantitative alternative to HRP chemiluminescence. Problems with signal saturation would also be avoided by using these techniques.

9.2.2. Investigation into the mechanisms of sex-differences in basal splenic leukocyte subset numbers

The data show clearly that male mice have significantly larger stores of neutrophils and monocyte subsets in their spleens compared to aged-matched females. Investigations into both the splenic chemokine environments and splenic leukocyte chemokine receptors yielded similar results from male and female mice. Sectioning of the LysM-eGFP and $CX_3CR1^{+/gfp}$ spleens and analysis by confocal microscopy may provide some insight into differential localisation of neutrophils and monocytes, respectively, in the spleens of male and female mice. This may also provide indications to the potential functions of these cells in the spleen.

In reality however, it is unlikely that one molecule or receptor will be responsible for such difference. To begin dissecting the precise mechanisms by which the sex-difference in splenic leukocyte numbers occur, it would first be useful to assess whether it is inherent properties and characteristics of the leukocyte, or whether it is the properties and environment of the body and spleen which are different between males and females and underpinning this observation. Future studies should address these questions using leukocyte transfer experiments. Analysis of the accumulation of transferred labelled male and female donor leukocytes in the spleens of male and female recipients will shed light on this question. Equivalent splenic accumulation of male and female transferred leukocytes would suggest it is

properties of the spleen itself that affects the size of the leukocyte pool. Conversely, differing numbers of male and female transferred leukocytes homing to the spleen would suggest the phenotypes of these cells are heterogeneous.

Appendix 6 shows preliminary leukocyte transfer studies that begin to address this future work. Should the trends demonstrated in Appendix 5 prevail, it is likely that differences between the male and female leukocytes underlie dimorphisms in basal spleen number. To further investigate the differences between male and female leukocyte subsets, splenic neutrophils and monocyte subsets could be isolated and subjected to gene expression and proteomic studies. By these means, genes that are differentially expressed in male and female neutrophils and monocytes can be identified and selected for further investigation.

9.2.3. Validation of the role of the spleen in sex-differences in neutrophil and classical monocyte trafficking in zymosan peritonitis

This study demonstrates that with regards to the murine spleen, zymosan peritonitis induces the homing of female neutrophils and mobilisation of male classical monocytes. The overall result of such trafficking is likely to represent explanation for the elevation of neutrophils and classical monocytes in the male mouse circulation. Evaluating the leukocyte trafficking responses in male and female splenectomised mice during zymosan peritonitis would validate the role of the spleen in this inflammatory reaction. The removal of the spleen would result in an elimination of the site of proposed neutrophil homing in female mice, whilst also eliminating the likely source of the additional classical monocytes released in male mice. It would be hypothesised that sex-differences seen with regards to circulating and recruited neutrophils and classical monocytes during early zymosan peritonitis would be no longer evident in splenectomised mice.

9.2.4. Investigation into the role of ovarian sex hormones in the dampened female responses during zymosan peritonitis

The influence of hormones has not been studied in this comprehensive analysis of sex-differences in the trafficking of leukocyte subsets in acute inflammation. Previous studies report that in response to bacterial peritonitis, greater leukocyte recruitment is evident in ovariectomised (OVX) female mice with 2-fold reduction in serum 17β-oestradiol versus sham controls (Scotland *et al.*, 2011). Future experiments could use OVX mice to assess the influence of ovarian sex hormones on the sexually dimorphic observations in the study (i.e. accumulation of neutrophils and classical monocytes in the blood and tissue, and the basal

splenic leukocyte numbers). Furthermore, microarray analysis of male and female neutrophils and monocyte subsets treated with or without 17β-oestradiol ex vivo would improve our understand of the regulatory effects sex hormones have on immune cells, and identify candidate genes for activation or blockade in future leukocyte trafficking experiments.

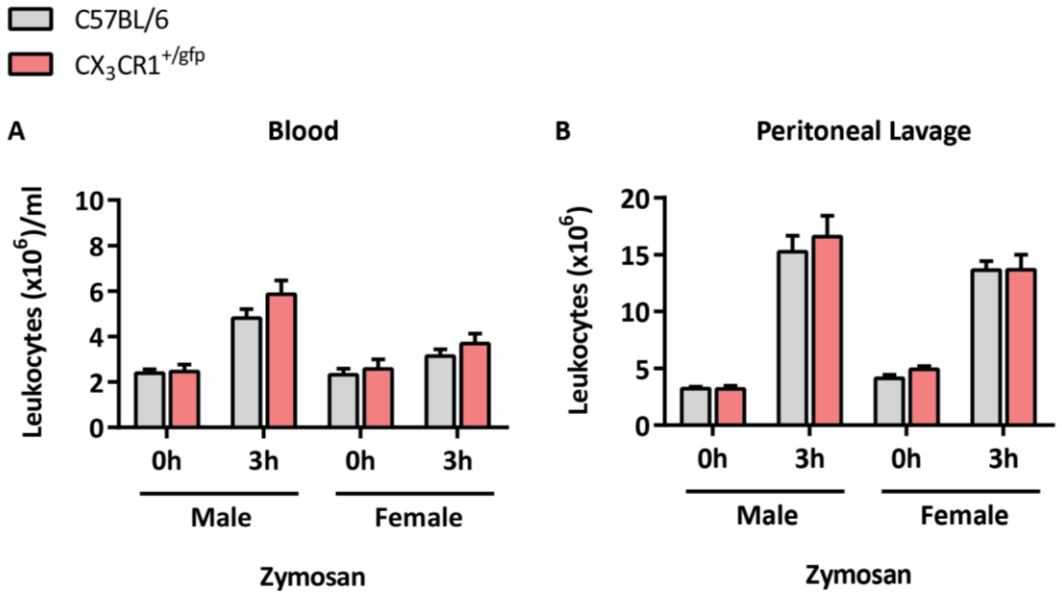
9.3. Concluding remarks

This study has demonstrated for the first time a role for the murine spleen in sexually dimorphic responses to acute inflammation. It was found that the resting male spleen contained a significantly greater pool of neutrophils, classical monocytes, and non-classical monocytes than that of the female. During early zymosan peritonitis, male mice accumulated more neutrophils and classical monocytes in their circulation, and furthermore had greater recruitment of both cell types to the peritoneal cavity. These were in parallel with a differential leukocyte trafficking about the spleen between the sexes with male mice releasing splenic classical monocytes, and neutrophils homing to the female spleen. Similar TLR expression between the sexes and during the inflammation was observed, suggesting differences in TLRs do not underlie the dimorphisms reported.

Collectively these findings demonstrate that sex-differences in the inflammatory response exist, and highlight the importance of considering sex in study design, inflammatory model selection, and data interpretation. These findings also propose the spleen as a key orchestrator in the dimorphic inflammatory response in mice. This suggests the spleen as a potentially novel therapeutic target for inflammatory diseases with leukocyte recruitment as a contributor to the pathogenesis. Should the mechanisms of differential trafficking in males and females be defined in future studies, the spleen represents an exciting novel target for personalised, sex-specific medicine development.

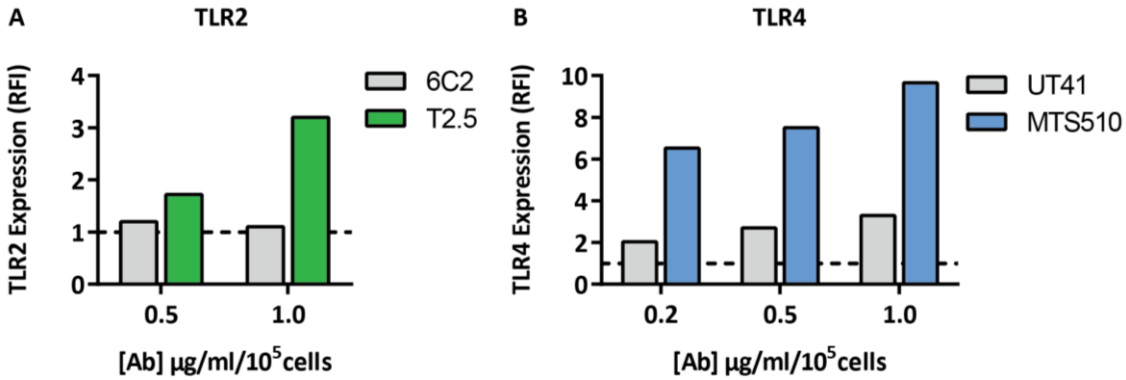
Appendices

Appendix 1



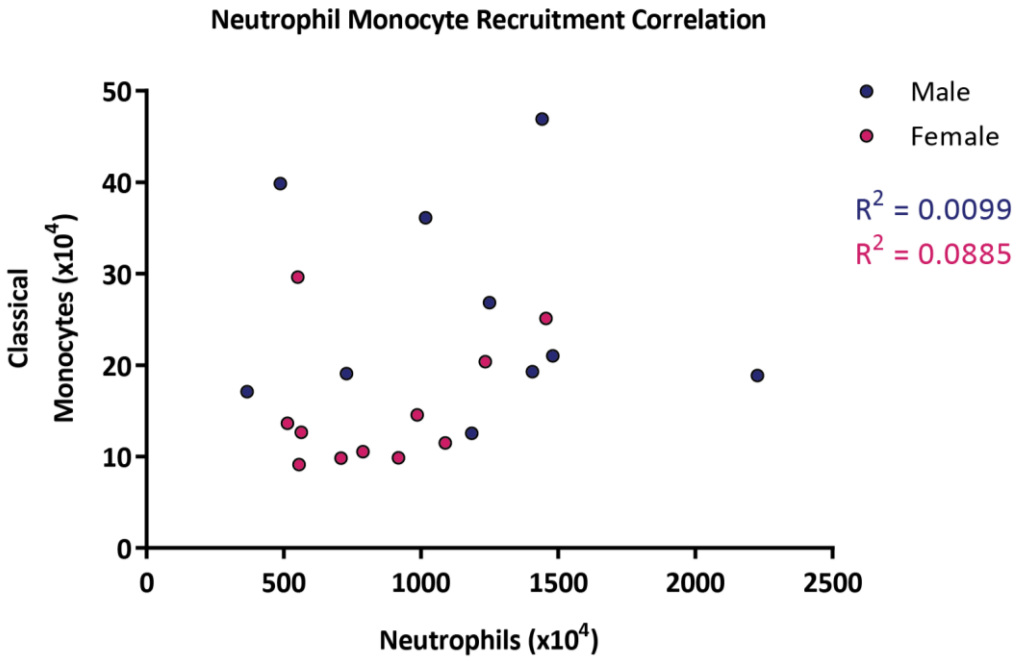
Appendix 1. Assessment of the leukocyte recruitment profile of $CX_3CR1^{+/gfp}$ mice. Male and female C57BL/6 (WT) and $CX_3CR1^{+/gfp}$ mice were treated with or without zymosan (1mg, i.p.) for 3h. Leukocytes were isolated from the (A) blood and (B) peritoneal cavity of mice and counted. Differences in total leukocytes at each time point were compared between both genotypes of mice by Student's *t*-test. Data is displayed at mean ± SEM of at least 7 mice.

Appendix 2



Appendix 2. Characterisation of TLR antibodies. Concentration-response graphs of ex-vivo labelling of murine peritoneal lavage macrophages. (A) Anti-TLR2 antibodies clone 6C2 (grey) and T2.5 (green) and (B) anti-TLR4 antibodies clone UT41 (grey) and MTS510 (blue). TLR expression was measured as relative fluorescence intensity (RFI) to an isotype control antibody.

Appendix 3



Appendix 3. Recruited neutrophil and classical monocyte correlation. Male (blue) and female (pink) mice were treated with zymosan (1mg, i.p.) for 3h to induce peritonitis. Correlation of the number of neutrophils and classical monocytes recruited to the cavity by 3h. n=11.

Appendix 4

		Male	Female
Cytokine array (Lavage)	Naïve		
	Zymosan (3h)		
	Zymosan (1h)		
Chemokine array (Lavage)	Naïve		
	Zymosan (3h)		
Cytokine array (Spleen)	Naïve		

Appendix 4. Proteome Profiler™ array reference spots. Duplicate reference control spots from Proteome Profiler™ mouse cytokine and chemokine arrays. Spots are in triplicate per membrane. Shown are from representative membranes of each condition. Male and female membranes were always exposed for equal times.

Appendix 5

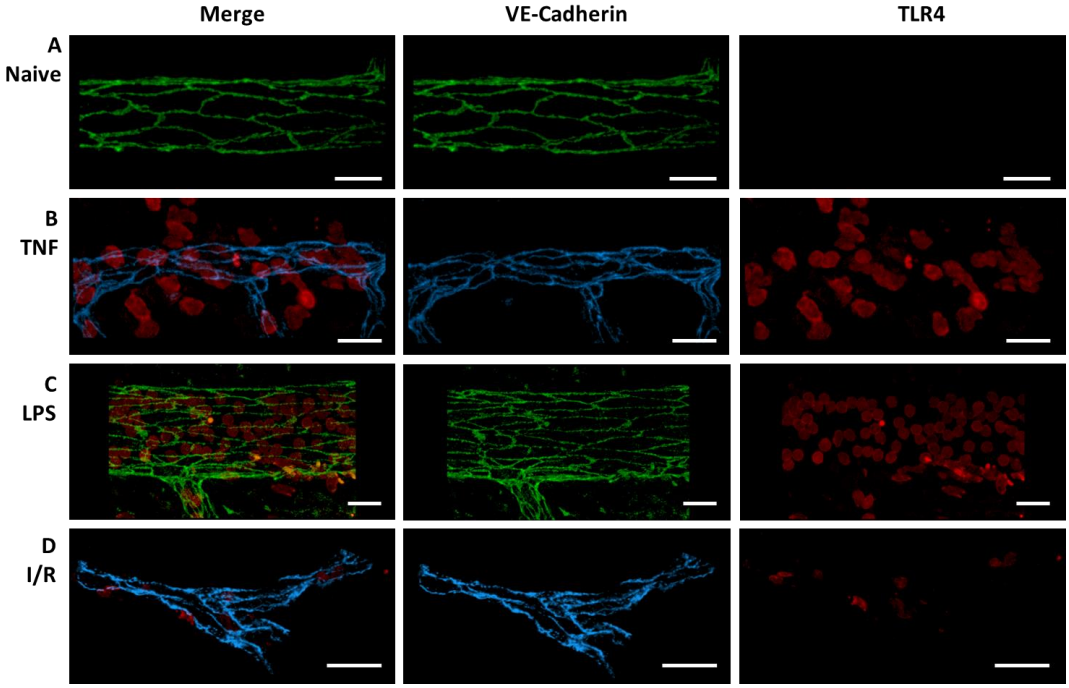
Study rationale: Some studies have demonstrated the expression of TLR4 protein in primary ECs by Western blot, however there is currently no clear evidence for the presence of the receptor on whole tissue blood vessels. Detection of EC TLR4, and evidence of modulation in inflammation may provide a link to aberrant leukocyte recruitment in inflammatory diseases.

Aim: Investigate the expression of TLR4 in the murine microvasculature in basal and inflamed conditions.

Experimental approach: Mice were treated ± intrascrotal (i.s.) TNF (300ng) or LPS (300ng) for 4h, or were exposed to 30min ischaemia of the cremaster circulation with 2h reperfusion (I/R). Cremasters were dissected, fixed, permeabilised and stained with fluorescently labelled antibodies to VE-Cadherin and TLR4 (clone MTS510). Whole mount tissues were imaged for TLR4 expression using the Zeiss LSM 5 Pascal laser-scanning microscope with a 63x objective. Other techniques for employed for EC TLR4 analysis included cell culture and Western blot.

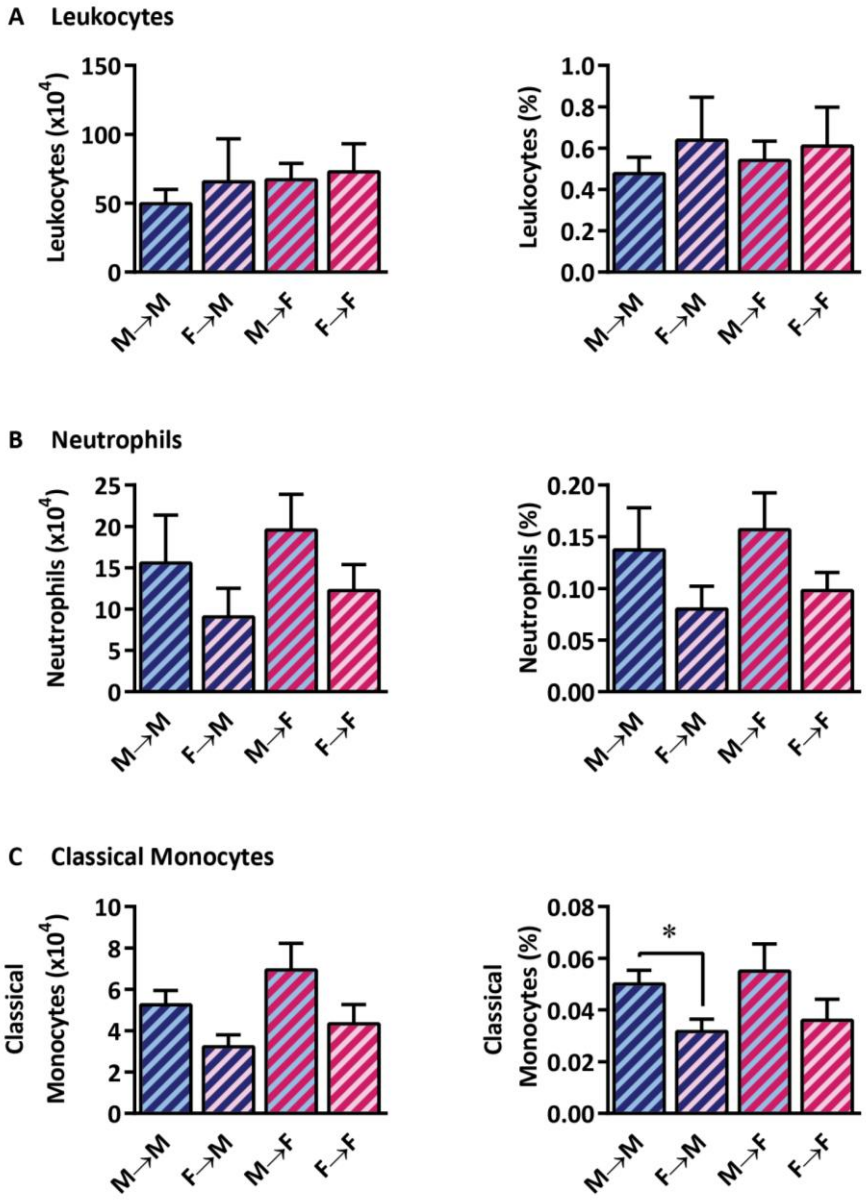
Results: Anti-TLR4 antibody labelled infiltrating leukocytes in mouse cremasters stimulated with TNF, LPS, or I/R injury, however was unable to detect TLR4 on the vasculature.

Conclusions: MTS510 anti-TLR4 detected leukocyte, but not vascular TLR4. A detailed description of this work is however beyond the scope of this Thesis.



Appendix 5. Staining of mouse cremaster muscle with anti-TLR4 antibody. Male mice were (A) naïve, or treated with (B) TNF or (C) LPS (300ng, i.s., 4h), or (D) were exposed to 30min cremaster ischaemia followed by 2h reperfusion. Cremasters were then excised, fixed, permeabilised and stained with Alexa Fluor® 555 conjugated anti-TLR4 (red), and Alexa Fluor®488 or 647 conjugated anti-VE-Cadherin. Scale bars, 20µm.

Appendix 6



Appendix 6. Trafficking of donor transfer leukocytes to recipient spleens. Leukocytes were isolated from the BM of donor male and female C57BL/6 mice and cells were labelled ex vivo with CellTracker™ Orange and CellTracker™ Violet dyes (2µM, Life Technologies), respectively. $10x10^6$ labelled donor leukocytes ($5x10^6$ male + $5x10^6$ female) were transferred (i.v.) into recipient male or female mice. After 4h recipient mice were culled by Schedule 1 (cervical dislocation) and spleen leukocytes harvested. Leukocytes were counted on a hemocytometer and labelled with fluorescently conjugated antibodies to CD115 and Gr1. Samples were analysed by flow cytometry. (A) Donor male cells in the recipient spleen were identified as CellTracker™ Orange$^+$ and donor female cells as CellTracker™ Violet$^+$. (B) Presence of donor neutrophils were identified by subsequent gating on Gr1^{high} cells. (C) Presence of donor classical monocytes were identified by subsequent gating on CD115$^+$Gr1$^+$ cells. Data are mean ± SEM, n=7/6/6/5. Differences in the accumulation of donor leukocyte subsets in recipient spleens were determined by 1-way ANOVA with Bonferroni's post-test for multiple comparisons. Significance was indicated by * (p<0.05).

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