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**Investigating the role of the transcription factor Ror α in macrophages in
the context of development, inflammation and circadian rhythms.**

A thesis submitted to Trinity College Dublin for the degree of
Doctor of Philosophy

by

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Under the supervision of Professor Padraic Fallon

School of Medicine

2016



**Molecular
Medicine
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Declaration of Authorship

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Summary

Macrophage subsets in tissue sites such as the peritoneal cavity are extremely heterogeneous in their phenotype and functions. These peritoneal macrophage subsets can be of dual origin: monocyte-derived or tissue-resident embryo-derived. Transcription factors are key to every step of the generation of both monocyte-derived and tissue-resident embryo-derived macrophages. Retinoic-acid receptor-related orphan receptor alpha (*Rora*) is a transcription factor that has a role in a number of different processes within the body including cell development, inflammation and circadian rhythms. In terms of cell development it is known that *Rora* has a role in the development of immune cells ILC2s and Th17 cells. In this thesis the role that *Rora* has in the regulation of macrophage development, in particular in the development of monocyte-derived macrophages or tissue-resident macrophages in the peritoneal cavity was investigated. *Rora*^{sg/sg} mice contain a naturally occurring mutation in *Rora* and were used as to investigate *Rora* function in this thesis.

Based on differential expression of CD11b, F4/80, Tim4 and MHCII four peritoneal populations were identified in the steady state in the peritoneal cavity including a tissue-resident macrophage population and a blood monocyte-derived macrophage population. Levels of the blood monocyte-derived peritoneal macrophage population are decreased in *Rora*^{sg/sg} mice, as are the levels of Ly6C^{Low} blood monocytes, while the levels of Ly6C^{Low} bone marrow monocytes are increased. These results appear to suggest two things: first, that in the steady state blood monocyte-derived peritoneal macrophages may be derived from Ly6C^{Low} blood monocytes, which in turn are derived from Ly6C^{Low} bone marrow monocytes. Secondly, the data suggests that *Rora* begins its role in macrophage development at or before the Ly6C^{Low} bone marrow monocyte stage of development. *Rora*^{fl/sg} *LysMCre* mice were generated to test if these observations were due to *Rora* acting intrinsically in macrophages. In *Rora*^{fl/sg} *LysMCre* mice *Rora* is deleted only in myeloid cells as a result of Cre-Lox recombination. Results in *Rora*^{fl/sg} *LysMCre* mice appear to suggest that the alterations in peritoneal macrophages seen in *Rora*^{sg/sg} mice are due to *Rora* acting extrinsically of myeloid cells as opposed to acting intrinsically.

Rora has also been shown to have a role in inflammatory responses. Macrophages are one of the primary responders in inflammation therefore the role *Rora* has in inflammation in the context of macrophages was investigated in this thesis. It is presented here that

Rora^{sg/sg} mice are less susceptible to LPS-induced endotoxemia compared to controls. The response to LPS in *Rora*^{sg/sg} mice was characterised by decreased serum levels of the pro-inflammatory cytokine IL-1 β compared to controls. In terms of macrophage responses to LPS in the absence of functional *Rora* peritoneal macrophages from *Rora*^{sg/sg} mice also produced decreased levels of IL-1 β compared to control macrophages. It was also shown in WT mice that the blood monocyte-derived macrophage peritoneal population, that is decreased in *Rora*^{sg/sg} mice, produces pro-inflammatory cytokines in response to LPS stimulation highlighting that it is an important responder to inflammation and host responses. Taken together the data highlights a role for *Rora* in inflammatory responses in macrophages and thus *Rora* could represent a target for anti-inflammatory therapies.

Rora is a molecular component of the circadian clock. There has recently been increased interest in the concept of ‘anticipatory inflammation’. This is where circadian oscillations can potentially regulate inflammatory responses by controlling rhythmic trafficking of inflammatory monocytes and other immune cells. The circadian oscillations of the four peritoneal populations were investigated over 24 hours in this thesis. Levels of the blood monocyte-derived peritoneal macrophage population and tissue-resident peritoneal macrophage population displayed a diurnal rhythm with levels of both being highest during the mouse active phase. It was also found that peritoneal macrophages from WT mice express *Rora*, along with *Bmal1*. Future work would elucidate if there is a link between diurnal expression of *Rora* and circadian oscillations of peritoneal macrophages.

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Chapter 1

General Introduction

1.1 The immune system

The immune system is composed of layered cellular defences capable of fighting and defending the body against invading microbes. The immune system is composed of two arms - the innate and the adaptive systems. Both systems have cellular components known as immune cells. The innate system is the first line of defence and is present in all classes of plant and animal life. Adaptive immunity is only found in vertebrates and provides the second line of defences against invading microbes. The innate and adaptive immune systems work in tandem to fight and clear any invading microbe as well as regulate normal physiological cell functions.

1.1.2 Origin of murine immune cells

Murine immune cells are primarily derived from multipotent haematopoietic stem cells (HSCs) that are in the bone marrow. Multipotent HSCs have the property of self-renewal and have the potential to give rise to any cell of the immune system through distinct differentiation pathways in response to internal and external cues. The contemporary model of haematopoiesis hypothesises that through a hierarchical process of cell fate decision and lineage commitment distinct cell types of the immune system are created. The model assumes that the decision for differentiation into the myeloid/lymphoid or megakaryocyte/erythrocyte lineages occurs early in haematopoiesis. HSCs give rise to stem cells of more limited potential, namely the common myeloid progenitor (CMP) or the common lymphoid progenitor (CLP) (**Figure 1.1**). The CLP has the differentiation potential for all types of lymphoid cells including T cells, B cells and Natural Killer (NK) cells. The CMP gives rise to the granulocyte-monocyte progenitor (GMP) and the megakaryocyte-erythrocyte progenitor (MEP). The GMP can further differentiate into myeloid cells including granulocytes, dendritic cells and macrophages. The MEP can differentiate into other components of the blood including megakaryocytes and erythrocytes.

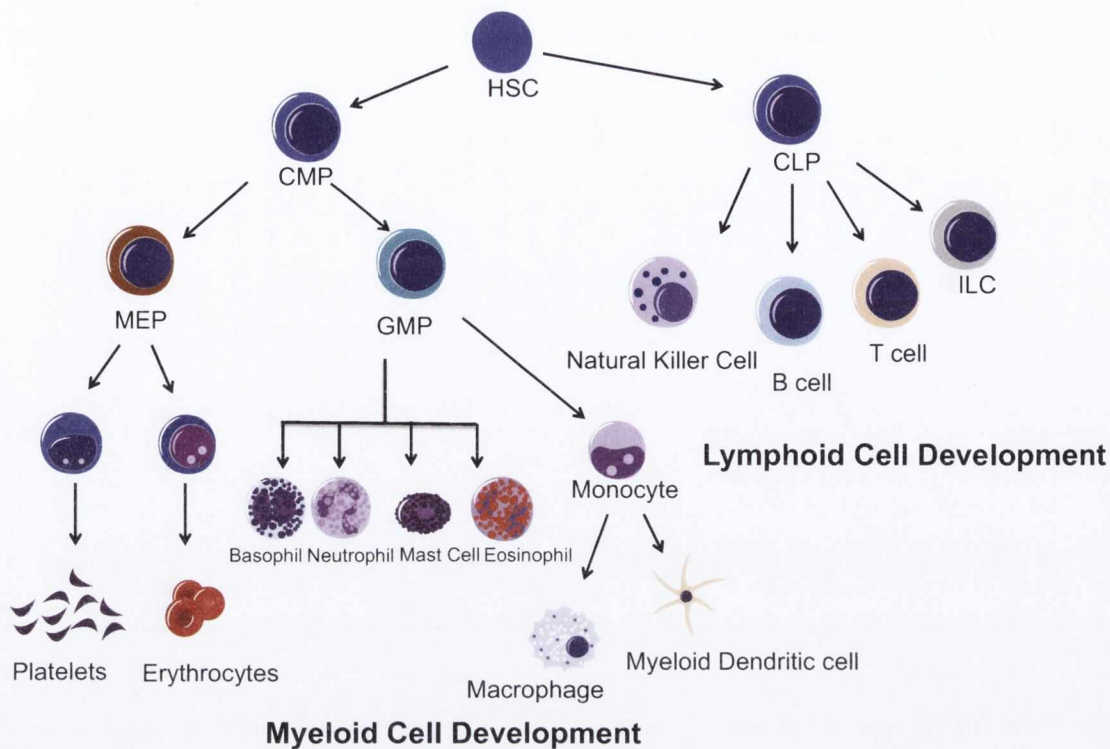


Figure 1.1 Overview of the murine haematopoietic system. All haematopoietic cells are derived from the HSC. The CMP is the progenitor cell for myeloid cells and the CLP is the progenitor cell for lymphoid cells. HSC- Haematopoietic stem cell, CMP- Common myeloid progenitor, CLP- common lymphocyte progenitor, MEP- megakaryocyte-erythrocyte progenitor, GMP- granulocyte monocyte progenitor, ILC- Innate lymphoid cell.

1.2 Innate immune system

The innate immune system is the first line of defence against pathogens. The core elements of the innate immune system have evolved over millions of years. Components of the innate system are found in all multi-cellular organisms and are expressed in humans as conserved homologs that are also present in other vertebrates and some insects (O'Neill et al., 2013). Although in the past the innate immune system has been considered to be crude or unsophisticated, it is now clear that it provides a high level of protection and in fact the majority of pathogens are eliminated by the innate immune system (O'Neill et al., 2013). The innate immune system is composed of two parts: firstly, the anatomical and physiological barriers and, secondly, the cellular component.

1.2.1 Anatomical and physiological barriers

The anatomical and physiological barriers of the innate immune system include intact skin, mucous clearance mechanisms, low stomach pH and secretory bacteriolytic lysozymes in tears and saliva. The skin is the body's largest organ. Across the 1.8m^2 skin surface, around 1 million bacteria reside per square centimetre giving a total of 10^{10} bacteria covering an average human (Belkaid and Segre, 2014). The majority of these bacteria are commensal, meaning that under normal physiological and immunological conditions they are non-pathogenic and survive on the skin. However, the skin also has features that keep these bacteria under control. For example, the skin surface is acidic, low in moisture, cool, coated in salt-laden sweat and replete with antimicrobial components such as antimicrobial peptides, nitric acid, free fatty acids and immunoglobulin (Belkaid and Segre, 2014).

Aside from the skin other sites such as the respiratory tract, digestive tract and reproductive system are also exposed to the external environment and thus also have protective features. The epithelial cells that cover these sites are coated with mucus that serves as a primary defence against pathogens. Mucus provides an important innate immune function by trapping and removing pathogens through mucociliary clearance; mucus also has antiprotease and antimicrobial activities. The gastric acid produced by the stomach is also an important barrier function in the digestive tract. The pH of the gastric environment varies depending on the host species, however it is generally very low. This low pH inactivates and kills the majority of ingested microbes (Brenneman et al., 2014).

The majority of the time these anatomical and physiological barriers are effective in keeping microbes under control and preventing them entering the internal body, however, for various reasons microbes can break through these barriers. When this occurs the cells of the innate immune system are the initial responders and these cells take over the immune defence.

1.2.2 Innate immune cells

Innate immune cells were first observed in 1774 when John Hunter, a Scottish surgeon, first recognised leukocytes at the site of inflammation. The functional analysis of innate immune cells is acknowledged as starting with Ilya Metchnikoff in 1882, who observed the engulfment of particulate dyes and fungal spores by cells (Metchnikoff, 1892). These cells were to become known as phagocytes. Over time different innate immune cells were

identified and characterised including macrophages, dendritic cells, neutrophils, eosinophils, mast cells and basophils. These cells are part of the myeloid lineage.

As innate immune cells play an important role in initiating an immune response, it is critically important that these cells can distinguish between self and non-self molecules. It would be detrimental if these cells initiated responses to naturally occurring molecules from our own bodies; indeed this is often the case in diseases such as autoimmune diseases. Innate immune cells achieve this through receptors that recognise molecular patterns found only in pathogens.

Pathogen-associated molecular patterns

In 1989, Charles A Janeway Jr. proposed that microbial pathogens could be recognised by pattern-recognition receptors (PRRs) and that these receptors recognised microbial components that he termed pathogen-associated molecular patterns (PAMPs) (Janeway, 1989). This proposal was shown to be indeed correct with the identification of Toll-like receptor (TLR) 4, the mammalian homologue of *Drosophila melanogaster* Toll protein (Medzhitov et al., 1997). Medzhitov et al. (1997) showed that activation of TLR4 triggered the expression of costimulatory molecules for T cell activation and the production of inflammatory cytokines through the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B). It was then shown by the group led by Bruce Beutler that TLR4 recognises lipopolysaccharide (LPS), a component found in the outer membrane of gram-negative bacteria-work for which the Nobel Prize was awarded (Poltorak et al., 1998). Various other PRRs aside from TLRs have been identified including nucleotide-binding oligomerisation domain protein (NOD) -like receptors (NLRs), retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) and C-type lectin receptors (CLRs) (O'Neill et al., 2013). These PRRs are distinguishable by the PAMPs they recognise.

Ten TLRs have been identified in humans and thirteen in mice. TLRs are type I transmembrane glycoproteins, TLR5, TLR11, TLR4, and the heterodimers of TLR2–TLR1 or TLR2–TLR6 bind to their respective ligands at the cell surface, whereas TLR3, TLR7–TLR8, TLR9 and TLR13 localise to the endosomes (O'Neill et al., 2013). TLRs recognise a variety of ligands including lipoproteins and lipopeptides (TLR2), double stranded RNA

(TLR3), flagellin (TLR5), single-stranded DNA (TLR7), CpG DNA (TLR9) and profilin (TLR11) (Blander and Sander, 2012).

NLRs are cytosolic proteins and are pivotal sensors of infection and stress in intracellular compartments (Shaw et al., 2010). Nucleotide-binding oligomerisation domain protein 1 (NOD1) and NOD2 recognise bacterial peptidoglycan fragments and activate NF- κ B signalling. Several NLRs including NOD-, LRR- and pyrin domain-containing (NLRP) proteins, NOD-, LRR- and CARD-containing (NLRC) proteins and neuronal apoptosis inhibitory proteins (NAIPs) form complexes termed inflammasomes, which activate caspase 1 leading to the cleavage of interleukin (IL)-1 β and IL-18 from their pro forms to active forms (Shaw et al., 2010).

There are three known RLRs: retinoic acid-inducible gene I (RIG-I), melanoma differentiation-associated gene 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2). RLRs are cytosolic sensors of viral nucleic acids. RIG-I and MDA5 contain an amino-terminal caspase-recruitment domain (CARD), which binds to IFN β -promoter stimulator 1 (IPS1) at the mitochondria to induce type I IFN production and initiate the anti-viral response (Loo and Gale, 2011). RIG-I binds to 3'-triphosphate single-stranded RNA and MDA5 is thought to bind to double-stranded RNA. LGP2 is thought to act as a regulator of RLR activity (Blander and Sander, 2012).

The CLRs are a large superfamily of proteins that possess one or more C-type lectin domains and one or more immune-receptor tyrosine-based activation motifs (ITAMs). They recognise a wide range of ligands including carbohydrate ligands. CLRs are found primarily on myeloid cells including macrophages and dendritic cells. Following activation, they signal through the kinase SYK and CARD9 to promote the production of pro-inflammatory cytokines and reactive oxygen species (Blander and Sander, 2012). Based on molecular structure CLRs can be divided into two membrane bound groups. Type I CLRs including DEC-205 (CD205) and macrophage mannose receptor (MMR or CD206). Type II transmembrane CLRs including Dectin-1, Dectin-2 and Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN or CD209) (Blander and Sander, 2012).

Antigen presentation

Innate immune cells recognise, respond to and contribute to the elimination of invading microbes. Macrophages, dendritic cells and neutrophils can act as antigen presenting cells (APCs). APCs are continuously surveying the microenvironment for signs of infection or stress. When APCs encounter antigenic materials they engulf them, process them and present the resulting fragments to antigen-specific T cells in major histocompatibility complexes (MHC). APCs capture materials through different mechanisms including endocytosis, pinocytosis and phagocytosis. Phagocytosis is the engulfment of particles by a process of actin remodelling to form a cup around the particle that closes to form a phagosome. Phagosomes mature into phagolysosomes when they fuse with a lysosome containing hydrolytic enzymes. Particles are broken down in these phagolysosomes and are complexed with MHCs. Peptides originating from intracellular sources are displayed on the surface of APCs by MHC class I (MHCI) molecules and peptides originating from extracellular sources are displayed by MHC class II (MHCII) molecules. In general, MHCI molecules interact with CD8⁺ T cells and MHCII molecules interact with CD4⁺ cells, however, there can be cross over in these two systems (Neefjes et al., 2011).

1.3 Myeloid cells

1.3.1 Macrophages

Macrophages are the ‘eaters’ of the immune system. One of their primary roles in immunity is to phagocytise microbes, apoptotic cells and cellular debris. Through both the release of immune effector molecules in response to pathogens and also through their role as APCs, macrophages are able to instruct other immune cells and orchestrate the immune response. Macrophages reside in every tissue of the body albeit in different guises for example microglia in the brain, Kupffer cells in the liver, Langerhans cells in the skin, and osteoclasts in the bone (Davies and Taylor, 2015). Macrophages are extremely plastic and their phenotype is dependent on cues they receive from the microenvironment. Through their ability to clear pathogens and signal to other immune cells macrophages have a central role in protecting the host. Macrophages are discussed in greater detail in **Section 1.6**.

1.3.2 Dendritic cells

Dendritic cells (DCs), like macrophages, are professional APCs. In fact they are considered to have a more important role in antigen presentation than macrophages. DCs were initially described by Ralph Steinman and Zanvil Cohn in the early 1970s; who identified a population of haematopoietic cells in the mouse spleen that excelled at antigen presentation and T cell stimulation (Steinman and Cohn, 1973, 1974). DCs reside in most non-lymphoid tissues, where they act as immune sentinels continuously surveying and patrolling the tissue for pathogens. DCs are generally identified predominantly based on phenotypic and functional properties, namely their stellate morphology, their high expression of MHCII and of the integrin CD11c, as well as their superior capacity to migrate from non-lymphoid to lymphoid organs and stimulate naïve T cells (Poltorak and Schraml, 2015). Recent studies in mouse and human indicate that DCs, macrophages, and monocytes have unique ontogenetic properties and thus can be generally considered as distinct cell lineages (Poltorak and Schraml, 2015).

1.3.3 Neutrophils

Neutrophils are polymorphonuclear leukocytes that are continuously generated in the bone marrow from myeloid precursors. Daily cell production of neutrophils can reach up to 2×10^{11} in humans (Bardoel et al., 2014). Neutrophils are released in a mature form from the bone marrow and in mice neutrophils constitute about 10-25% of circulating leukocytes (Kolaczowska and Kubes, 2013). Neutrophils have short half-lives in the circulation, estimated to be about 6-8 hours in mice (Summers et al., 2010). Neutrophil maturation involves granulocyte-macrophage colony stimulating factor (GM-CSF) which is produced in response to IL-17 by neutrophil regulatory T cells (mainly $\gamma\delta$ - and natural killer T cells) (Kolaczowska and Kubes, 2013). Production of IL-17a is controlled by IL-23 released by macrophages and DCs. Neutrophils are typically the first cells recruited to the site of inflammation. As the inflammatory response develops, neutrophils die by apoptosis and are removed by macrophages and DCs.

Neutrophils have three different types of granules that are filled with pro-inflammatory proteins: i. azurophilic granules, filled with myeloperoxidase (MPO); ii. specific granules, which contain lactoferrin; iii. gelatinase granules that contain metalloproteinase 9 (MMP9) (Kolaczowska and Kubes, 2013). When neutrophils encounter pathogens, they

phagocytise them and encapsulate the pathogen within phagosomes. The pathogen is killed through the use of reactive species and the fusion of the phagosome with granules containing antibacterial proteins. Highly activated neutrophils can also kill extracellular pathogens by releasing neutrophil extracellular traps (NETs) (Brinkmann and Zychlinsky, 2012). These NETs are composed of core DNA elements, with proteins, enzymes and histones attached that trap pathogens. The pathogens trapped in NETs are killed by oxidative and non-oxidative mechanisms (Brinkmann and Zychlinsky, 2012).

Neutrophils are recruited from the circulation to an infection site in response to microbial molecules and cytokines produced by tissue-resident cells, including IL-1 β , IL-6, and tumour necrosis factor (TNF) α , or chemokines, such as IL-8, CXCL1, CXCL2 and CXCL5. Neutrophils are the first cells to arrive at an inflammatory site, and they do that in massive numbers. Regardless of the cue, neutrophils in the circulation first recognise signals in the endothelium close to an inflammatory site and, after rolling on the endothelium, extravasate into the tissue (Bardoel et al., 2014).

1.3.4 Eosinophils

Eosinophils are granulocytes first described by Paul Erlich in 1879. They develop in the bone marrow from progenitor cells and are released into the circulation in a mature form. Eosinophils spend a short time in the circulation, with a half-life of about 18 hours, after which they migrate to the thymus and gastro-intestinal tract where they reside under homeostatic conditions. Indeed, eosinophils typically reside in tissues that interact with the external environment. During helminth infection or in allergic diseases eosinophils are rapidly released within 1 hour of stimulation, the rate of eosinophilopoiesis increases and their survival in tissue is enhanced. The development, activation and survival of eosinophils is promoted by the cytokine IL-5. IL-5 is produced primarily by Th2 cells and innate lymphoid cells type 2 (ILC2s) (Neill et al., 2010), and in smaller amounts by natural killer (NK) cells, mast cells NK T cells and also by eosinophils themselves (Rosenberg et al., 2013). IL-5 functions synergistically with the Th2 cytokines IL-4 and IL-13, and with the eosinophil chemoattractants CC-chemokine ligand 11 (CCL11), CCL24 and CCL26 (also known as eotaxin, eotaxin 2 and eotaxin 3, respectively) to promote eosinophil activation and recruitment into tissues during acute inflammatory responses (Rosenberg et al., 2013). The main receptors expressed by eosinophils include IL-5Ra, CC-chemokine

receptor 3 (CCR3) and sialic acid binding immunoglobulin-like F (Siglec-F) (Rosenberg et al., 2013).

Eosinophils are distinguished by their populations of cytoplasmic crystalloid secondary granules. Human eosinophil granules contain preformed cytotoxic and pro-inflammatory proteins such as eosinophil peroxidase (EPO), major basic protein (MBP) and eosinophil associated RNases (EARs) (Rosenberg et al., 2013). When activated eosinophils release the contents of these granules by piecemeal degranulation. The potent cytotoxic proteins kill the pathogen and the pro-inflammatory proteins alert other aspects of the immune system. Eosinophils are generally thought to defend against relatively large organisms such as parasitic helminths, although recent data from animal models suggests this might not be as essential as once thought (Rosenberg et al., 2013).

Through clinical and animal studies eosinophils have also been implicated in the pathogenesis of a number of allergic diseases such as asthma, including roles in airway hyper reactivity, elevated mucus production and airway remodelling (Fulkerson and Rothenberg, 2013). Recently a role for eosinophils as crucial effectors of the IL-23-GM-CSF axis in colitis has been identified (Griseri et al., 2015). Griseri and colleagues (2015) observed that chronic intestinal inflammation was characterised by increased bone marrow eosinophilopoiesis and accumulation of activated intestinal eosinophils. IL-5 blockade or eosinophil depletion ameliorated colitis, implicating eosinophils in disease pathogenesis. GM-CSF was a potent activator of eosinophil effector functions and intestinal accumulation; GM-CSF-activated eosinophils promoted IL-23 driven colitis with an increase in inflammatory cytokines TNF and IL-13 (Griseri et al., 2015).

1.3.5 Mast cells

Mast cells are granulocytes first described in 1878 by Paul Ehrlich in his doctoral thesis (Beaven, 2010). Mast cells are released from the bone marrow in an immature form and have a lifespan of months to years (Amin, 2012). They are found in the skin and all mucosal tissues at homeostasis. Mast cells are activated through the high-affinity receptor for IgE (FcRI) or other surface receptors and upon activation they release pre-formed mediators including the vasodilator histamine as well as proteases, synthesised lipid mediators, cytokines and chemokines that play various roles in the immune response (Moon et al., 2014). Mast cells play a role in T helper 2 inflammatory and immediate

allergic responses (Otsuka and Kabashima, 2015), with the activation of mast cells being strongly linked to the pathophysiology of anaphylaxis and other acute allergic reactions (Metcalf et al., 2009). Mast cells are also effector cells in other allergic diseases such as hay fever and asthma (Beaven, 2010). Aside from their role in allergic responses it is becoming more apparent that mast cells are also important in host defence, homeostatic responses and immunoregulation (Beaven, 2010).

1.3.6 Basophils

Like mast cells, Paul Ehrlich also first described basophils in 1878. Basophils represent a rare cell population, constituting less than 1% of blood leukocytes under steady-state conditions (Stone et al., 2010) and are mobilised to the blood, spleen, lungs, and liver in response to inflammatory signals that rapidly induce basophil expansion in the bone marrow (Otsuka and Kabashima, 2015). Basophils express several of the same effector molecules as mast cells, including mast cell-associated proteases, vasodilating substances, lipid mediators, various cytokines and pro-inflammatory chemokines (Otsuka and Kabashima, 2015). Many of these effector molecules are rapidly released in response to the activation of FcRI and other surface receptors that are expressed on mast cells and basophils (Otsuka and Kabashima, 2015). As with mast cells, basophils are associated with T helper 2 immune responses contributing to protection against helminths and ticks and also play a pivotal role during IgE-mediated chronic allergic inflammation of the skin and are implicated in the late-phase response of allergic asthma (Otsuka and Kabashima, 2015).

1.4 The adaptive immune system

It is clear that the innate immune system is well equipped to defend against the majority of pathogens. However, it is possible that a pathogen can evade or overcome the innate defences. When this happens the pathogen encounters the adaptive immune system. As discussed previously, the innate immune system is evolutionary ancient and is found throughout the animal kingdom. The adaptive immune system, however, is a more recent development. It arose after the innate immune system, approximately 500 million years ago (Flajnik and Kasahara, 2010). Like the innate immune system the adaptive immune system has a cellular component: B (Bursa-derived) cells and T (Thymus-derived) cells. Unlike innate immune cells, which possess PRRs that recognise conserved PAMPs found in pathogens, the adaptive immune cells have receptors that recognise very specific

antigens. These receptors are generated by the mechanism of gene recombination. The adaptive immune system also provides long lasting protection against pathogens through memory cells.

1.5 Lymphoid cells

1.5.1 B cells

B cells are a population of cells that express clonally diverse cell surface immunoglobulin (Ig) receptors that recognise specific antigenic epitopes. B cells were first identified and characterised in the 1960's and 1970's. B cells and T cells were identified simultaneously by Max Cooper and Robert Good in 1965 (Cooper et al., 1965). Cooper and Good showed that the cells formed in chicken bursa of fabricus were responsible for the production of antibodies and that cells that required an intact thymus for manifestation of delayed-type hypersensitivity were functionally independent (Cooper et al., 1965). B cells are the cells formed in the chicken bursa of fabricus. In humans and mice B cells are generated from precursor cells in the bone marrow or fetal liver. HSCs are the original progenitor cell and differentiate into CLP cell cells (**Figure 1.1**). The CLP differentiates through a number of intermediate steps into an immature B cell. These initial stages of B cell development involve stepwise rearrangement of genes encoding the B cell receptor (BCR) and changes in the expression of cell surface receptors. The BCR is composed of two heavy (H) chains and two light (L) chains. Rearrangement of the Ig locus involves combinatorial rearrangement of the V, D and J gene segments in the H chain locus and the V and J gene segments in the L chain locus (Brack et al., 1978).

The immature B cell leaves the bone marrow and with subsequent functional maturation occurring in the secondary lymphoid tissues including lymph nodes and the spleen. The mechanism of central tolerance ensures that B cells that recognise 'self' antigens are removed from the system. B cell activation requires two distinct signals and results in the B cell differentiation into memory B cells or plasma cells. The first signal is antigen binding the BCR. The antigen is then internalised by endocytosis, digested and complexed with MHCII molecules that are then displayed on the B cell surface. The second signal occurs by either T cell independent or T cell dependent mechanisms. The MHCII complex on the B cell enables it to interact with the T cell receptor (TCR) on CD4⁺ T cells and thereby activate them. The activated T cell then provides a second activation signal to the

B cell. Alternatively B cells are activated independently of T cells, the second activation signal is provided by antigens such as bacterial wall components like LPS (Xu et al., 2008). Upon activation B cells proliferate and form germinal centres where they differentiate into plasma cells or memory B cells. The primary function of plasma cells is to secrete antibodies containing unique antigen-binding regions joined to the constant Ig isotope-defining region. Memory B cells form in the germinal centres, these cells are long lived and can rapidly proliferate and differentiate into plasma cells following re-exposure to the initial antigen (Kurosaki et al., 2015). Antibodies perform various roles in the immune response including coating pathogens for phagocytosis by macrophages (opsonisation), coating for complement activation and neutralizing bacterial toxins

1.5.2 T cells

T cells originate in the bone marrow and mature in the thymus (Cooper et al., 1965). T cells populating the peripheral blood or secondary lymphoid organs were distinguished early on by the mutually exclusive expression of either CD4 or CD8 based on the use of particular monoclonal antibodies (Koretzky, 2010). CD4⁺ T cells respond to antigen (Ag) presented by MHCII proteins and CD8⁺ T cells respond to Ag presented by MHCI proteins (Koretzky, 2010). T cells have a T cell receptor (TCR) on their surfaces. T cell activation requires two signals. The first signal occurs when the TCR interacts with Ag/MHC complexes on the surfaces of APC's. The second signal, the costimulatory signal, is provided through the interaction between co-stimulatory molecules found on the surfaces of the APC and T cell. The best-characterised costimulatory molecule expressed on T cells is CD28, which interacts with CD80 and CD86 on APCs.

When activated the CD4⁺ T cell can differentiate into a number of different helper (Th) subsets including Th1, Th2, Th9, Th17, T22 and T Follicular. Th1 and Th2 were the first subsets characterised due to the differences in their cytokine expression pattern (Mosmann and Coffman, 1989). Since then other subsets including Th9, Th17, Th22, T Follicular Helper cells have been characterised (Bluestone et al., 2009). Each T cell subset has a different role in the immune system, for example Th2 cells are primarily involved in responses to parasite infections through the release of the cytokine IL-4 and to a lesser degree IL-5, IL-13 and IL-9 (Fallon et al., 2002). Th1 cells produce interferon-gamma IFN γ , IL-2, and tumour necrosis factor (TNF) β , which activate macrophages and evoke cell-mediated immunity and phagocyte-dependent protective responses (Romagnani, 2000).

CD8⁺ T cells are also known as cytotoxic T cells (CTLs). CTLs are crucial in protecting against primary infection with non- or poorly cytopathic viruses and other intracellular pathogens. When activated by APCs, naïve CD8⁺ T cells undergo clonal expansion, migrate to sites of infection, and kill infected target cells via the secretion of perforin and granzymes or Fas ligand-Fas interactions (Schürch et al., 2014). In addition, cytokines secreted by activated CTLs regulate the immune response and have direct antiviral effector functions. For example, IFN γ is involved in the clearance of viral infections such as lymphocytic choriomeningitis virus (LCMV) and mouse hepatitis virus (Schürch et al., 2014).

1.6 Macrophage classification

The macrophage was first described by Russian bacteriologist Ilya Metchnikov in 1884 (Metchnikoff, 1892). Macrophages are part of the innate immune system and have a variety of roles within the immune system, including phagocytic clearance of infected or dying cells, acting as APCs to activate adaptive immunity and also maintaining tissue homeostasis. Macrophages *in vivo* are the most plastic cells of the haematopoietic system and are found in all tissues where they show huge functional diversity (Wynn et al., 2013). Indeed gene expression analysis of tissue macrophages by the Immunological Genome (ImmGen) project revealed considerable differences between macrophage populations in different tissue (Gautier et al., 2012). Macrophages studied *in vitro* also display a level of heterogeneity depending on the culturing mechanisms and activators added to them.

1.6.1 Classically Activated Macrophages (CAM)

As with the lymphocyte system, a dichotomy has been long been proposed for macrophage activation: classic vs. alternative, also M1 and M2, respectively (Martinez and Gordon, 2014). This classification is based on the differential responses of macrophages to stimulants such as cytokines and endotoxins. The term ‘macrophage activation’ was introduced by Mackaness in the 1960s in an infection context to describe the enhanced antigen-dependent non-specific, activity of macrophages towards *Listeria* upon secondary exposure to the pathogens (Mackaness, 1962). Nathan and colleagues (1983) showed that the Th1 cytokine IFN γ produced by antigen-activated lymphocytes was responsible for this enhanced microbial activity. In the study IFN γ treated macrophages produced increased

levels of hydrogen peroxide (H_2O_2) compared to untreated cells and also enhanced their ability to kill toxoplasma (Nathan et al., 1983). Around the same time other groups further showed that $IFN\gamma$ could enhance macrophage cytotoxic and antitumoral properties (Celada et al., 1984; Pace et al., 1983). In the years following, these activated macrophages were to be generally referred to as 'Classically Activated Macrophages' (CAM).

1.6.2 Alternatively Activated Macrophages (AAM)

Following on from these studies Stein et al. (1992) found that the MMR (CD206) expression is upregulated in macrophages treated with the Th2 cytokine, IL-4. CD206 is an important phagocytic receptor that binds and ingests surface mannose residues and soluble mannose containing glycoproteins. At the time it was known that CD206 was expressed on resident and elicited peritoneal macrophages and alveolar macrophages but not $IFN\gamma$ activated macrophages. It was also known that IL-4 induced the expression of MHCII antigen and inhibited the expression of pro-inflammatory cytokine genes such as IL-1, TNF and IL-8. The observation of enhanced CD206 activity in IL-4 treated macrophages, combined with reduced pro-inflammatory cytokine production led Stein and colleagues to propose that IL-4 induced an alternative activated phenotype in macrophages (Stein et al., 1992). This activation state was very different from the $IFN\gamma$ activated macrophages. Doyle et al. (1994) showed two years later that another Th2 cytokine IL-13, like IL-4 altered the activation state of macrophages. In the years following these activated macrophages were to be called Alternatively Activated Macrophages (AAM).

1.6.3 M1 and M2 macrophages

Initial characterisation

The classification terms M1 and M2 were first proposed by Mills and colleagues in 2000 to explain distinct metabolic profiles of stimulated macrophages (Mills et al., 2000). Mills and colleagues were investigating the factors that regulate macrophage arginine metabolism when they found evidence that macrophages from Th1 and Th2 backgrounds differed in their ability to be activated and also in their responses to the same stimuli. T cells from a Th1-biased mouse strain have a tendency to produce Th1 cytokines such as $IFN\gamma$ whereas T cells from a Th2-biased strain have a tendency to produce Th2 cytokines such as IL-4, IL-5 and IL-10 (Mills et al., 2000). Prototypical Th1 mouse strains include C57BL/6 and B10D2, whereas prototypical Th2 strains include BALB/c and DBA/2.

Mills et al. (2000) found that macrophages from Th1-biased mouse strains were more easily activated to produce inducible nitric oxide synthase (iNos), which uses L-arginine as a substrate to produce NO than macrophages from Th2-biased strain. Also in response to IFN γ and LPS macrophages from Th2-biased mouse strain did not produce NO but increased the production of the enzyme arginase I (Arg-1), which sequesters L-arginine from iNOS and results in the production of ornithine and downstream polyamines and L-proline, whereas macrophages from a Th1-biased strains did not (Mills et al., 2000). Mills et al. (2000) also showed that show that macrophages from Th1 and Th2 strains differentially influenced whether Th1, Th2, or other immune response occurred. They proposed that these different macrophage responses be termed M1 and M2. The M1-M2 terminology has prevailed because it is a simple and useful way to describe the two major and opposing activities of macrophages.

M1 macrophage gene expression

M1 macrophages are considered to be pro-inflammatory, inhibit cell proliferation and cause tissue damage. M1 macrophages are characterised by increased expression of iNos. iNos is a homodimeric enzyme that converts L-arginine and oxygen into L-citrulline and NO in a complex oxidoreductase reaction. A characteristic feature of iNos is its lack of expression in strictly resting cells, instead, it is induced by immunological stimuli in a calcium-independent manner (Bogdan, 2015). NO has antimicrobial action against a variety of pathogens, including viruses, bacteria, fungi, and protozoa. The endothelium of blood vessels uses NO to signal to surrounding smooth muscles to induce vasodilation and increase blood flow.

M2 macrophage gene expression

M2 macrophages are considered to promote cell proliferation and tissue repair. M2 macrophages are characterised by increased expression of Arg-1, Ym1 (chitinase 3-like; Chi3-l3), resistin-like molecule α (Relm α ; Retnla/Fizz1) and retinol dehydrogenase 2 (Raldh2) (Broadhurst et al., 2012; Loke et al., 2002). As discussed above Arg-1 is upregulated in M2 macrophages. Arg-1 is a cytosolic enzyme that functions in nitrogen elimination by catalysing arginine hydrolysis to urea and ornithine (Pesce et al., 2009a). The production of urea removes excess nitrogen from the body, while ornithine can be used to generate polyamines, glutamate, and proline, the latter of which is critical to the

synthesis of collagen (Pesce et al., 2009a). Ym1 is a chitinase-like protein that is a catalytically inactive member of the extended family of mammalian chitinases. Ym1 expression has been closely associated with helminth infections and Th2 responses; however, its function in immunity has remained unclear. Recently Ym1 and Ym2 were shown to induce the accumulation of neutrophils through the expansion of $\gamma\delta$ T cell populations that produced IL-17 (Sutherland et al., 2014). Also the recruitment of neutrophils observed during the migration of *Nippostrongylus brasiliensis* through the lungs was mediated primarily by Ym1 in an IL-17-dependent manner.

Relm α is a member of a family of cysteine-rich secreted proteins, referred to as 'resistin-like molecules' that increase in expression during allergic reactions and following infection with a variety of metazoan parasites. Relm α was originally hypothesised to function as an effector molecule during helminth-induced Th2-type immune responses. However, studies suggest that Relm α primarily functions as a regulatory molecule during helminth infection (Pesce et al., 2009b). Retinol dehydrogenase (Raldh) enzymes are involved in retinoic acid synthesis from Vitamin A. Raldh enzymes are highly expressed by M2 macrophages recruited to liver granulomas during *S. mansoni* infection, and Raldh2 expression in macrophages was induced by activation with IL-4 *in vitro* and *in vivo* (Broadhurst et al., 2012).

Metabolic differences between M1 and M2 macrophages

Gene expression profiles are dramatically altered in macrophages that are activated by PAMPs, such as LPS. In addition to the alterations in gene expression, activated macrophages also undergo profound metabolic changes (O'Neill and Pearce, 2015). Activation of resting macrophages with a range of stimuli induces a metabolic switch in the activated macrophages. Indeed M1 and M2 macrophages have distinct metabolic phenotypes (Galvan-Pena and Neill, 2014). The Warburg effect is the observation that the metabolic profile of most cancer cells switches from oxidative phosphorylation (OXPHOS) to aerobic glycolysis (Warburg et al., 1927). In contrast resting cells have a comparatively low rate of glycolysis followed by oxidation of pyruvate in mitochondria (Warburg et al., 1927).

A phenomenon similar to the Warburg effect is observed in activated M1 and M2 macrophages; M1 macrophages rely on aerobic glycolysis, while M2 macrophages use

fatty acid oxidation (FAO) to fuel mitochondrial OXPHOS (Huang et al., 2014). In M1 macrophages, aerobic glycolysis is induced upon activation, which involves an increase in glucose uptake as well as the conversion of pyruvate to lactate, the pentose phosphate pathway is also induced following classical activation (Galvan-Pena and Neill, 2014). This pathway is key for the generation of NADPH for the NADPH oxidase, which is important for ROS production, but also for NO synthesis. These metabolic events can provide the macrophage with rapid energy and reducing equivalents, which are required for bactericidal activity. M2 macrophages on the other hand obtain much of their energy from FAO and oxidative metabolism, which can be sustained for longer periods of time reflecting their functions in homeostasis and tissue repair (Galvan-Pena and Neill, 2014).

One of the most distinguishable metabolic differences between M1 and M2 macrophages is the metabolism of arginine (Galvan-Pena and Neill, 2014). As discussed above iNos is upregulated in M1 macrophages, resulting in the catabolism of arginine to citrulline and NO, the latter playing key role in the intracellular killing of pathogens (Mills et al., 2000). In an M2 macrophage on the other hand, Arg-1 is induced, which results in the production of urea, polyamines, and ornithine, which are important for the wound healing actions of this macrophage population (Mills et al., 2000).

1.6.4 M1, M2a, M2b, and M2c classification system

In their paper Mills et al. (2000) acknowledge that the terms M1 and M2 are most likely over simplistic when describing macrophage phenotypes. Indeed it was apparent that other cytokines and factors such as IL-10, TGF β and glucocorticoids appeared to elicit similar phenotypes in macrophages but did not clearly fit into the Th1/ Th2 and M1/M2 context. Mantovani addressed this by introducing and describing other subtypes: M1, M2a, M2b and M2c (Mantovani et al., 2004). Like previous classifications M1 macrophages in this model are induced by IFN γ or LPS and are characterised by high capacity to present antigen; high IL-12 and IL-23 production and consequent activation of a polarised type I response; and high production of toxic intermediates such as NO. The generic use of M2 is based on the subsets generally being involved in type 2 responses, tissue remodelling and immunoregulation and on selected functional properties such as being IL-12 low. The subsets differ by the polarizing agent and the cytokines that they produce (**Figure 1.2**). In this classification M2a (activation) cells are induced by IL-4 and IL-13, promote Th2 responses and are important in the killing and encapsulation of parasites. M2b cells

induced by Immune Complexes (IC) and agonists of TLRs and are also important in type 2 responses and also immunoregulation. M2c cells are induced by IL-10 and glucocorticoid hormones (Mantovani et al., 2004), and are important in immunoregulation and matrix deposition and tissue remodelling.

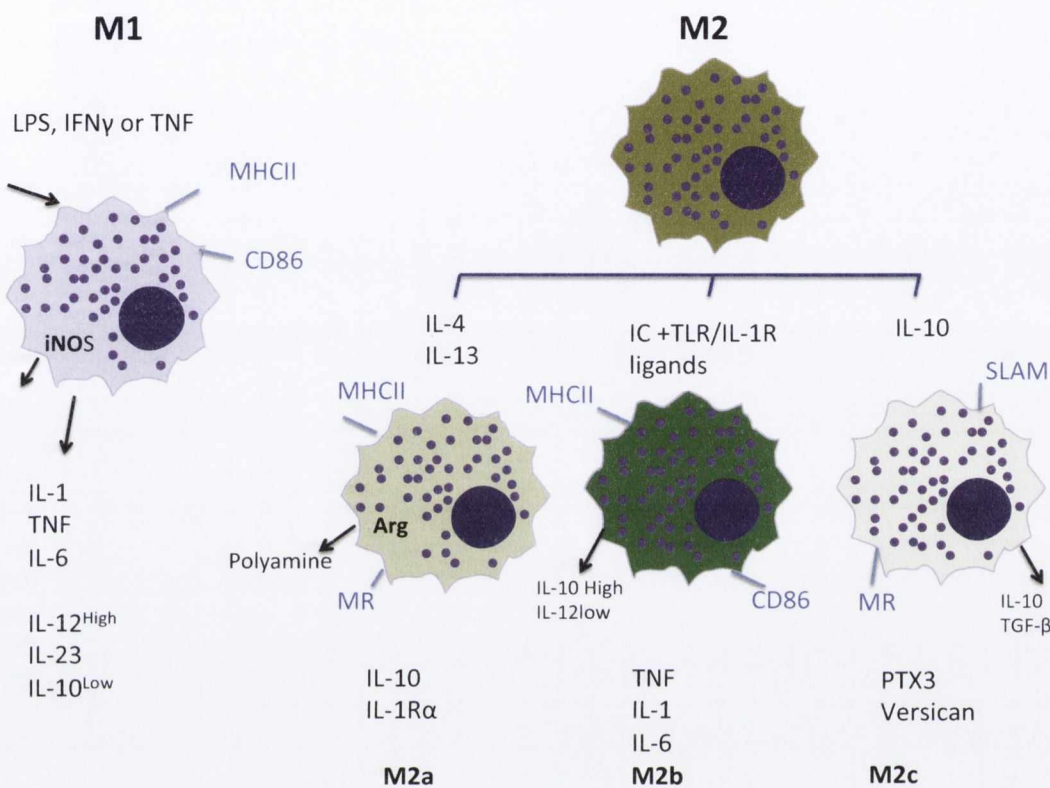


Figure 1.2 Inducers, characteristics and products of different polarised macrophage populations. Mantovani et al. (2000) devised a classification system for polarised macrophages. M1 macrophages are induced by LPS, IFN γ or TNF, and produce pro-inflammatory cytokines. M2 macrophages are divided into three groups depending on the polarizing inducers. M2 macrophages are in general more prone to immunoregulatory and protumoral activities. IC- Immune complexes, MR- Mannose receptor. Adapted from (Martinez and Gordon, 2014).

1.6.5 Granulocyte Macrophage Colony Stimulating Factor (GM-CSF) and Macrophage Colony Stimulating Factor (M-CSF)

GM-CSF and M-CSF were originally defined as haemopoietic growth factors; however, they can both act on macrophage populations and are implicated in host defence and inflammation. The biology of these CSFs are quite distinct with different tissue distribution

in the steady state, different structures, and different receptors (Fleetwood et al., 2009). The addition of M-CSF to murine bone marrow results in the generation of highly purified macrophages called bone marrow-derived macrophages (BMDMs), which are widely used to study macrophage biology (Falk, 1988; Hamilton et al., 1980). Conversely, with a similar protocol, GM-CSF can generate a population that have macrophage and DC properties (Inaba et al., 1992; Jackson et al., 2002). Furthermore, studies have shown that BMDMs grown in GM-CSF have characteristics of M1 macrophages and BMDMs grown in M-CSF have characteristics of M2 macrophages (Fleetwood et al., 2007). Significant differences were documented in the transcriptomes of macrophage populations primarily generated using M-CSF or GM-CSF, with and without exogenous perturbation (Fleetwood et al., 2007).

1.6.6 Current classifications

When Mantovani described the M2a, M2b and M2c classification system (Mantovani et al., 2004), it was acknowledged that it is more of a useful conceptual framework and that fully polarised M1 and M2 cells most likely represent cells on the extreme of a continuum. Indeed in the time since these classifications were first described it has become increasingly apparent that macrophages are extremely plastic cells and do not just fall in to one category. Added to this, the terms AAM/CAM and M1/M2 are used interchangeably in the literature leading to some confusion as to what macrophage type is being described. Based on these issues there has been a call from macrophages researchers to introduce a new clearer classification of macrophages. Murray et al. (2014) describe a set of standards encompassing three principles—the source of macrophages, definition of the activators, and a consensus collection of markers to describe macrophage activation—with the goal of unifying experimental standards for diverse experimental scenarios. Therefore, newer and broader characterisation of subtypes based on the activation of the macrophages under experimental conditions has been proposed (Murray et al., 2014) (**Figure 1.3**). Although this model is useful for classifying *in vitro* activated macrophages it is not able to fully account for *in vivo* macrophage populations. Macrophage phenotype *in vivo* depends on a number of factors including the tissue site and the microenvironment at this site therefore a new way of looking at macrophage activation is to consider a multidimensional model of activation, which integrates the signals to which macrophages are exposed in their specific microenvironments (Ginhoux et al., 2015).

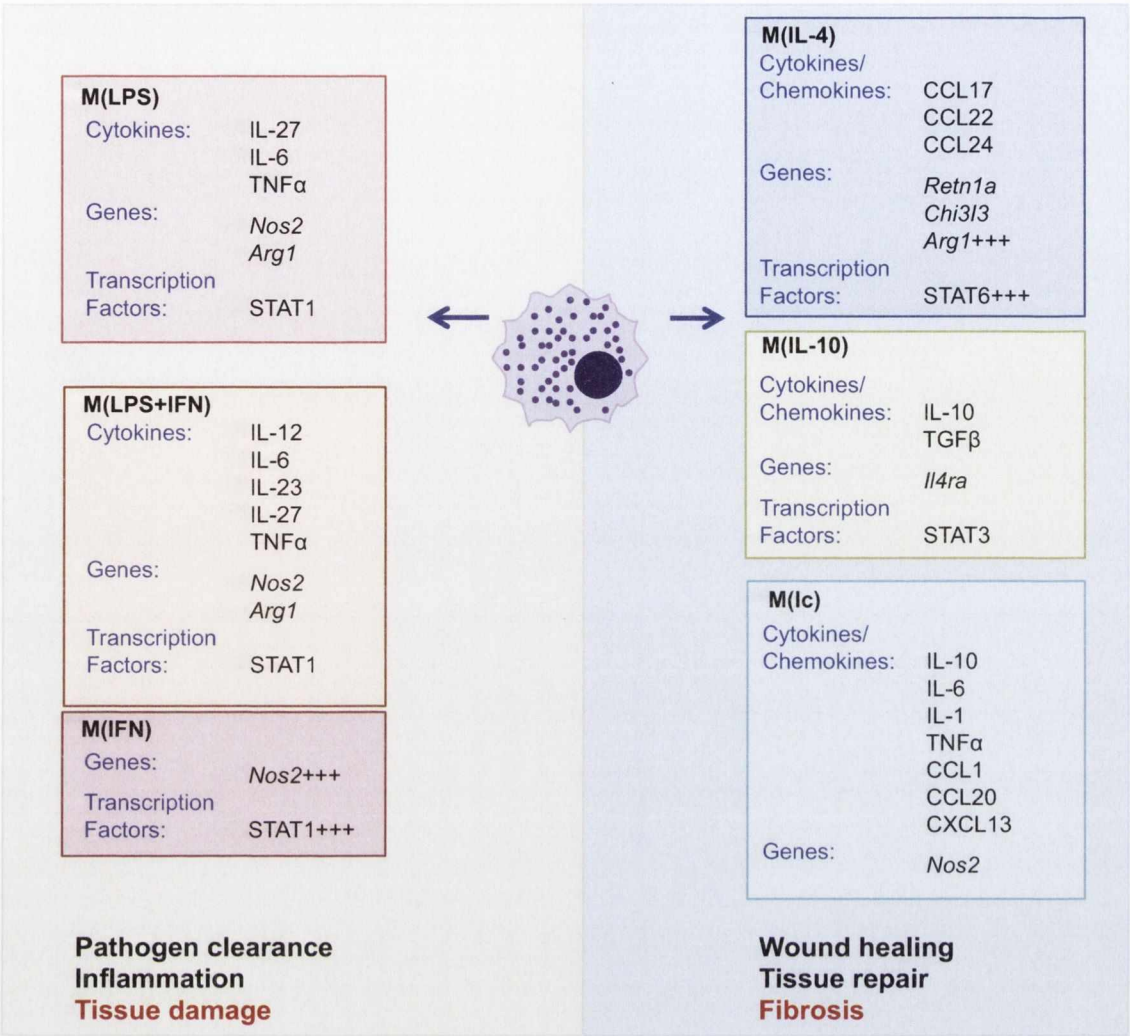


Figure 1.3 Macrophage subtypes can be described by the experimental stimulus used to activate the cells. Although there are multiple macrophage subtypes, these can broadly be split into two groups associated with inflammation and pathogen clearance or wound healing and tissue repair. Macrophages activated with LPS, IFN γ , or a combination of the two (LPS + IFN γ) are associated with increased expression of *Nos2* and production of pro-inflammatory cytokines. Conversely, activation with IL-4 and IL-10 increases expression of IL-10, TGF β , and *Arg1*, while activation with Ic gives a phenotype similar to that elicited by LPS. However, activation by IL-4, IL-10, and immune complexes is associated with tissue repair and wound healing. Taken from Hams, Bermingham and Fallon 2015 (Hams et al., 2015a).

1.7 Bone marrow macrophage progenitor cells

Mouse tissue macrophages can originate from precursors in the bone marrow postnatally (**Section 1.1.2**). **Figure 1.4** outlines the differentiation process for blood monocyte-derived macrophages in the peritoneal cavity. This model was originally established by van Furth's mononuclear phagocytic system (MPS) concept (Van Furth and Cohn, 1968). The long-term repopulating HSC population can self-renew *in vivo*, which supports haematopoiesis for a lifetime. HSCs were first isolated in Stanford University by the Weissman group in 1988. While mature leukocytes are produced at a rate of more than one million cells per second in the adult human, most of the HSCs from which they are derived cycle very infrequently and primarily reside in the G₀ phase of the cell cycle under homeostatic conditions (Seita and Weissman, 2010). Further downstream, HSCs advance to oligopotent progenitors the CLP and the CMP (**Figure 1.4**).

Collectively these oligopotent progenitors then give rise to all the lineage-committed effector cells of the haematopoietic system. The CMP is the progenitor cell for myeloid cells including monocytes and macrophages. In the current model the CMP differentiates into the GMP in the bone marrow, followed by the monocyte dendritic progenitor (MDP) (**Figure 1.4**). MDP have lost the ability to generate granulocytes and gives rise to two monocyte subsets- Ly6C^{High} and Ly6C^{Low} monocytes (**Section 1.8**). The monocytes enter the circulation and subsequently infiltrate the peritoneal cavity in response to a stimulus where they differentiate into macrophages (**Figure 1.4**).

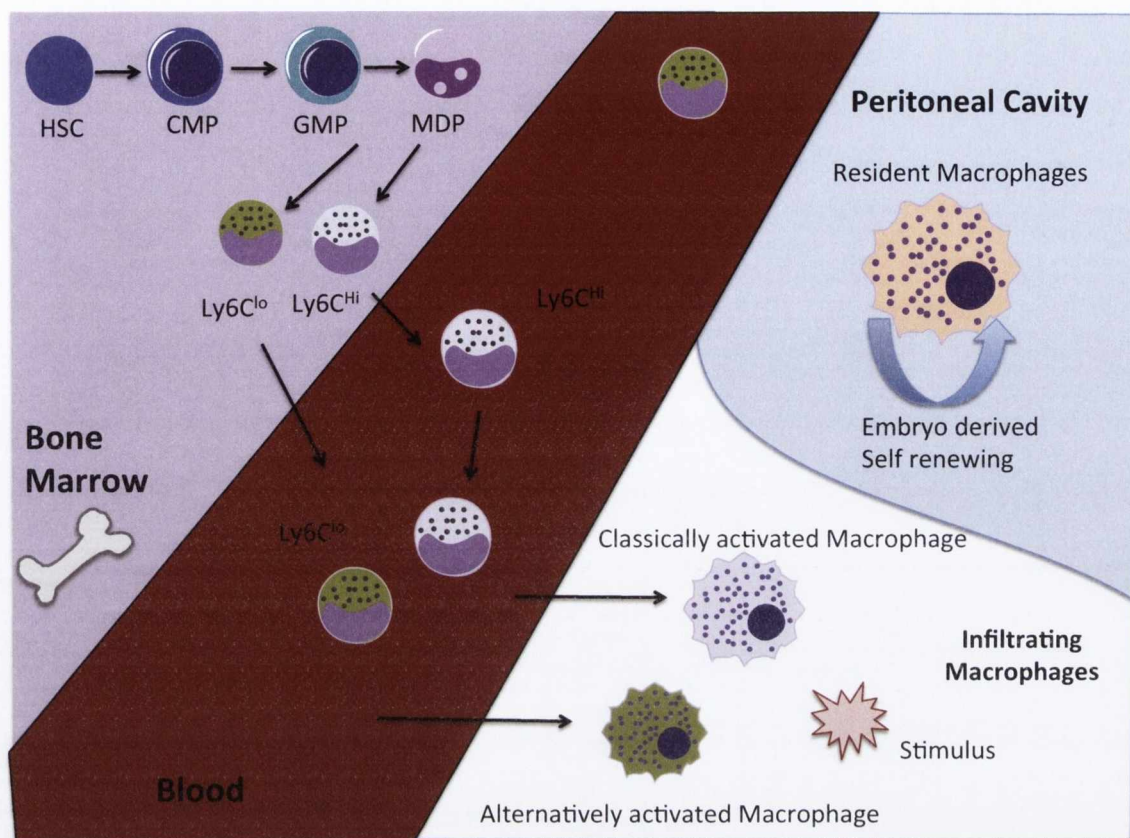


Figure 1.4 Schematic of the differentiation of bone marrow progenitor cells to infiltrating blood monocyte-derived peritoneal macrophages. Through a number of differentiation steps bone marrow progenitor steps give rise to monocytes that enter the circulation. These monocytes infiltrate the peritoneal in response to a stimulus and give rise to macrophages. Tissue-resident macrophages are of embryonic origin and can self renew, this macrophage population is seeded in the peritoneal cavity during embryogenesis.

The macrophage progenitor cells in the bone marrow can be identified by their cell surface marker expression. Table 1.1 outlines the commonly used surface markers for identification of macrophage progenitor cells in the bone marrow. There is no single defined panel of markers used to identify progenitor cells rather different researchers use a combination of haematopoietic markers. Some of the markers used include CD34, CD38, Thy1, CD135, CD105, CD45, FcγRII/III, Sca-1 and c-Kit. Haematopoietic progenitor cells are negative for the markers that are used for detection of lineage commitment, and are, thus, called Lin⁻. Lineage markers include CD3, CD4, CD11b, CD11c, CD19, Ly6G and MHCII. HSCs have been highly enriched as Thy-1^{Low}, Sca-1⁺, Lin⁻, c-kit⁺ cells or CD34⁻, Sca-1⁺, Lin⁻, c-kit⁺ stem cells using combinations of 10-12 markers (Kiel et al., 2005). An

alternative method for identifying progenitor cells uses a signature of signalling lymphocyte activation molecule (SLAM) family of cell surface molecules. Cell surface receptors of the SLAM family, including CD150, CD244, and CD48 are differentially expressed among functionally distinct progenitors (Kiel et al., 2005).

Name	Multipotent
HSC	Lin ⁻ c-kit ⁺ Sca-1 ⁺
	Oligopotent
CLP	Lin ⁻ IL7 α ⁺ Sca-1 ⁺ c-Kit ⁺ CD135 ⁺
CMP	Lin ⁻ Thy1 ⁻ IL7 α ⁻ Sca-1 ⁻ c-Kit ⁺ FcyRII/III ⁺
MDP	Lin ⁻ Sca-1 ⁻ c-Kit ^{High} CD135 ⁺ CD115 ⁺
MEP	Lin ⁻ Thy1 ⁻ IL7 α ⁻ Sca-1 ⁻ c-Kit ⁺
GMP	Lin ⁻ Thy1 ⁻ IL7 α ⁻ Sca-1 ⁻ c-Kit ⁺ FcyRII/III ^{Int}

Table 1.1 Overview of cell surface markers used to identify bone marrow progenitor cells. Lin⁻: Lineage negative. Lin⁻: CD3, CD4, CD11b, CD11c, CD19, Ly6G, MHCII.

Of note, linear developmental schemes as shown in **Figure 1.1** and **Figure 1.4** and outlined in **Table 1.1** should be interpreted with caution, as the populations described have often been historically defined on the basis of the expression of a limited panel of surface markers. It now seems more likely that these populations are themselves heterogeneous and are part of a developmental continuum that is subject to ‘graded commitment’. Also of note is that the MPS model of macrophage development can only account for a small percentage of tissue macrophages. The majority of tissue-resident macrophages are in fact seeded during embryonic development; this is discussed in more detail in **Section 1.9**.

1.8 Monocytes

1.8.1 Murine monocyte subsets

MDPs differentiate into monocytes in primary lymphoid organs including the bone marrow; these monocytes then leave the bone marrow and enter the blood circulation (**Figure 1.4**). Monocytes are a conserved population of leukocytes that are present in all vertebrates. In mice and humans, monocytes represent 4% and 10%, respectively, of the nucleated cells in the blood, with considerable pools in the spleen and lungs that can be

mobilised on demand (Ginhoux and Jung, 2014). Differentiation of two monocyte subsets in mice was first achieved after the observation that monocytes could be subdivided according to their expression of C-C chemokine receptor type 2 (CCR2), CD62L (also known as L-selectin) and CX3C-chemokine receptor 1 (Geissmann et al., 2003) (CX3CR1; measured by expression of green fluorescent protein (GFP) in cells from mice in which GFP had been 'knocked-in' to one of the *Cx3cr1* alleles (Jung et al., 2000)) (Geissmann et al., 2003).

CCR2 regulates monocyte recruitment from blood into many non-lymphoid tissues and also inflamed tissue through CC chemokine monocyte chemotactic protein (MCP-1/CCL2) (Palframan et al., 2001). CD62L acts as a homing receptor for lymphocytes to enter secondary lymphoid tissues through high endothelial venules. Ligands expressed on endothelial cells will bind to lymphocytes expressing L-selectin, slowing lymphocyte trafficking through the blood, and facilitating entry into a secondary lymphoid organ at that point (Arbones et al., 1994). It is important in homeostasis and inflammation. CX₃CR1 is widely expressed in the mononuclear phagocyte system and is the receptor for CX₃C chemokine fractalkine (FKN; neurotactin). Fractalkine is a transmembrane protein and chemokine involved in leukocyte adhesion and migration.

One monocyte subset expresses CCR2, CD62L and moderate amounts of CX₃CR1, whereas the second subset does not express CCR2 or CD62L but expressed higher amounts of CX₃CR1 (Gordon and Taylor, 2005). In addition, Ly6C (part of Gr-1) was identified as a marker found on CCR2⁺, CD62L⁺, CX₃CR1^{Intermediate (Int)} monocytes (Geissmann et al., 2003) (Table 1.2). Ly6C is a glycoprotein encoded by the *ly6* locus. It is expressed by CD8⁺ T cells and also by haematopoietic cells. Despite many reports on Ly6C expression the functional characteristics of Ly6C are not fully known. In the circulation CCR2⁺, CD62L⁺, Ly6C^{High}, CX₃CR1^{Low} monocytes (herein called Ly6C^{High} monocytes) have a short half life of 20 hours whereas CCR2⁻, CD62L⁻, Ly6C^{Low}, CX₃CR1^{High} monocytes (herein called Ly6C^{Low} monocytes) have a half live of 5 days (Ginhoux and Jung, 2014). The development and survival of both monocyte subsets in mice is dependant on M-CSF, also known as CSF1, which acts through the receptor M-CSF, also known as CD115 and CSF1R. Mice deficient in M-CSF or M-CSFR exhibit monocytopenia (Ginhoux and Jung, 2014).

Name	Markers	Function
Ly6C ^{High} Monocyte	CCR2 ⁺ , CX ₃ CR1 ^{Int} , CD45 ⁺ , CD115 ⁺ , Ly6G ⁻ , Ly6C ^{High}	Precursors of peripheral mononuclear phagocytes
Ly6C ^{Low} Monocyte	CCR2 ⁻ , CX ₃ CR1 ⁺ , CD45 ⁺ , CD115 ⁺ , Ly6G ⁻ , Ly6C ^{Low}	Reside in lumen and survey endothelial integrity

Table 1.2 Surface markers and function of monocytes. Monocytes can be divided into Ly6C^{High} and Ly6C^{Low} monocytes based on cell surface marker expression and functions.

1.8.2 Functional characterisation

To functionally characterise the two subsets of monocytes Geissman et al. (2003) adoptively transferred GFP-expressing monocytes from the Cx3cr1-knock-in mice and studied cell fate and function in naive and immunologically challenged recipient mice. They observed that the Ly6C^{High} monocytes were short-lived after adoptive transfer and difficult to detect in the tissues of naive recipients, however, these cells were rapidly recruited to sites of experimentally induced inflammation (Geissmann et al., 2003). The expression of CCR2 and the ability to migrate towards MCP-1 is consistent with the role of this chemokine and its receptor in the recruitment of monocytes to inflammatory sites, therefore the subset of monocytes that expresses CCR2 in mice is known as the ‘inflammatory’ subset (Gordon and Taylor, 2005). After migration into the inflamed site, the inflammatory monocytes upregulate their expression of DC marker CD11c as well MHCII expression.

Based on these observations and other studies, Ly6C^{High} monocytes were considered an intermediate between bone marrow progenitors and tissue macrophages. However, with the recent recognition that the majority of tissue-resident macrophages originate from the yolk sac (YS) early in embryogenesis (Yona et al., 2013), it is now commonly accepted that monocytes are not the primary source of tissue macrophages (discussed in more detail in **Section 1.9**). It is now known that monocytes can develop into a wide range of

terminally differentiated cells and perform versatile functions during infection, tumour formation and in the setting of chronic inflammation (Xiong and Pamer, 2015). The diverse role of monocyte-derived cells includes production of ROS, NO, secreting cytokines, transporting antigens and priming/polarizing T cells following infection with different microbial pathogens. The function of recruited monocytes is dependent on the context of infection, the site of infection and the microenvironment. In other settings, such as chronic inflammation or in tumours, monocytes differentiate into so-called myeloid-derived-suppressive cells (MDSC) or macrophages and produce a complex spectrum of cytokines and growth factors, such as IL-10, Arginase, TGF β and M-CSF, that can suppress immune responses and promote tumour growth (Xiong and Pamer, 2015).

In terms of infection it has recently been shown that following infection with *Citrobacter rodentium* CCR2⁺ monocytes are recruited to the intestine and give rise to CD11c⁺ CD11b⁺ F4/80⁺ CD103⁻ intestinal macrophages within the lamina propria (Seo et al., 2015). Unlike resident intestinal macrophages, de novo differentiated macrophages are phenotypically pro-inflammatory and produce robust amounts of IL-1 β through the non-canonical caspase-11 inflammasome (Seo et al., 2015). Furthermore intestinal macrophages from infected mice elicited the activation of ROR γ t⁺ ILC3 in an IL-1 β -dependent manner. In terms of autoimmunity CCR2⁺ monocytes and CCL2 have been implicated in experimental autoimmune encephalomyelitis (EAE) progression (Saederup et al., 2010). More recently it has been shown that the deletion of the GM-CSF receptor *Csf2rb* in CCR2⁺ Ly6C^{High} monocytes reflects the EAE resistance seen in complete *Csf2rb*-deficient mice. High-dimensional analysis of tissue-infiltrating monocyte-derived DCs revealed that GM-CSF initiates a combination of inflammatory mechanisms (Croxford et al., 2015).

In terms of Ly6C^{Low} monocytes, Geissman et al. (2003) observed that this population persisted longer in recipient mice and were found in sites including the blood, lungs, liver, spleen and brain for up to several days after transfer. This population was later shown to patrol blood vessels where they may be responsible for removing cellular debris from the lumen of the vasculature (Auffray et al., 2007). Following on from this *Nr4a1*-dependent Ly6C^{Low} monocytes have been shown to be enriched within capillaries and scavenge microparticles from their luminal side in a steady state (Carlin et al., 2013). In the kidney cortex, perturbation of homeostasis by a TLR7-dependent nucleic acid “danger” signal, triggers Gai-dependent intravascular retention of Ly6C^{Low} monocytes by the endothelium.

These monocytes then recruit neutrophils in a TLR7-dependent manner to mediate focal necrosis of endothelial cells, whereas the monocytes remove cellular debris (Carlin et al., 2013).

1.8.3 Development of monocyte subsets

Although both Ly6C^{High} and Ly6C^{Low} monocyte subsets originate in the bone marrow the developmental relationship between both subsets has not been fully elucidated. It was shown that the transcription factor NR4A1 (Nur77) can regulate the differentiation of Ly6C^{Low} monocytes from MDPs in the bone marrow (Hanna et al., 2011), suggesting Ly6C^{High} and Ly6C^{Low} monocytes represent separate lineages. However, accumulating evidence suggests that, in the steady state, blood monocyte subsets represent stages in a developmental sequence with Ly6C^{High} monocytes differentiating, most likely in the circulation, into Ly6C^{Low} cells. Fate mapping studies using mice that harbour genes encoding conditional or constitutive active Cre recombinase in their CX3CR1 loci showed that Ly6C^{High} monocytes constitute obligatory steady-state precursors of blood-resident Ly6C^{Low} cells, and that the abundance of Ly6C^{High} blood monocytes dynamically controls the circulation lifespan of their progeny (Yona et al., 2013).

Exploring the origin of M2 macrophages that accumulate around liver granulomas in *Schistosoma mansoni* infection, Girgis et al. (2014) observed that monocytes crawling in the sinusoidal vessels of infected mice comprised of both Ly6C^{High} and Ly6C^{Low} cells. The authors suggest two possibilities for their results, first that Ly6C^{High} monocytes are recruited to the liver sinusoids and then differentiate into Ly6C^{Low} monocytes with patrolling behaviour and then subsequently into M2 macrophages in the tissues. Another possibility suggested is that Ly6C^{High} monocytes directly differentiate into M2 macrophages, and the accumulation of Ly6C^{High} and Ly6C^{Low} monocytes in the liver sinusoids has distinct functions.

The exact pathways involved in monocyte differentiation remain to be fully determined. At present results indicate that the process is very dynamic and may be dependant on a number of different factors.

1.8.4 Human monocytes

Current knowledge defines three major monocyte subsets in human peripheral blood based on the expression patterns of CD16 (FcγRIII) and the LPS-receptor CD14: classical CD14⁺⁺CD16⁻, intermediate CD14⁺⁺CD16⁺ and non-classical CD14⁺CD16⁺⁺ monocytes (Ziegler-Heitbrock et al., 2010). Conventionally, CD14 and CD16 expression levels on human monocytes are represented by +/- signs not High/Low as used to describe Ly6C^{High/Low} mouse monocytes. These subsets differ essentially in their chemokine receptor expression, phagocytic activity and tissue distribution during inflammation or steady-state conditions. Classical monocytes are the most prevalent monocyte subset in human blood and, similarly to mouse Ly6C^{High} monocytes, these cells express CCR2 (Shi and Pamer, 2011). Human classical monocytes express high levels CCR2 and CD62L, and low levels of CX₃CR1. They are phagocytic, exhibit high peroxidase activity, and produce high levels of IL-10 and low levels of TNFα in response to LPS (Yang et al., 2014). In terms of *in vivo* patrolling the non-classical population is similar to the mouse Ly6C^{Low} monocyte population. However, it is important to note that although comparative array profiling has been used as evidence to support orthology between the human and mouse subsets (CD14^{High} = Ly6C^{High}), many genes show species-specific expression profiles (Ingersoll and Spanbroek, 2010).

It is still unknown if intermediate monocytes have a discrete, biologically roles or whether they are intermediates in a continuous differentiation from classical into non-classical monocytes. It is known that the subset has low peroxidase activity but a higher capacity to produce IL-1β and TNFα in response to LPS. Both classical and intermediate monocytes invade tissues during inflammation through CCR2-CCL2 interaction and/or CCR5-CCL5 in a Very Late Activation Antigen-1 (VLA1)/VCAM1 dependant manner (Yang et al., 2014). Human non-classical monocytes patrol vessel walls and invade through the interaction between CX₃CR1/CCL3 via the Leu-CAM family integrin lymphocyte functional antigen-1 (LFA-1)/ICAM1-dependent manner. This population releases IL-1β and TNFα in response to RNA and DNA particles (Yang et al., 2014).

1.9 Tissue-resident macrophages

In 1968, Ralf van Furth, Zanvil Cohn and colleagues formulated the MPS, which stated that the origin of all macrophages was terminal differentiation from blood monocytes. Even at this time there was significant evidence to the contrary including evidence of macrophage proliferation in the tissues, the perseverance of tissue macrophages and the presence of macrophage populations in the YS before primitive haematopoiesis (Davies et al., 2013). However, due to the technological constraints of experiments, the MPS became the prevailing model for macrophage origin (Davies and Taylor, 2015). However, by 2000, accumulated evidence led to the conclusion that embryonic phagocytes developed in both the YS and liver, indicating that they were of a separate lineage, and highlighted the possibility of their persistence into adulthood (Lichanska and Hume, 2000).

Two different types of embryonic haematopoiesis are found in vertebrates: primitive haematopoiesis and definitive haematopoiesis. Primitive haematopoiesis is characterised by the emergence of progenitor cells with limited potential for development into erythrocytes and macrophages and that can be found in the extra-embryonic yolk-sac blood islands in mice (Palis et al., 1999). Definitive haematopoiesis generates enucleated erythrocytes, various kinds of myeloid (including macrophages) and lymphoid cells, and long-term reconstituting HSC (Minehata et al., 2002). These HSCs are responsible for the maintenance of haematopoiesis, including monocytes and macrophages, throughout adult life (De Kleer et al., 2014). Over the past number of years, cumulative evidence indicates that adult tissue macrophages do not only originate from adult MPS haematopoiesis, but can also arise from embryonic primitive and definitive haematopoiesis.

In the current model three waves of progenitor cells are associated with myeloid development and maintenance in the mouse (Perdiguerro and Geissmann, 2015) (**Figure 1.5**). Primitive haematopoiesis is the source of the first haematopoietic cells detected in the developing embryo. These progenitor cells are thought to arise directly from the posterior plate mesoderm, and clonal analysis of the blood islands has revealed that the endothelial cells lining the blood islands and the haematopoietic cells within do not share a common origin (Ueno and Weissman, 2006).

The first definitive progenitor cells known as erythromyeloid progenitors (EMPs) emerge in the yolk sac of mouse embryos at E8.25. EMPs emerge from the yolk-sac hemogenic

endothelium in a Runx1-dependant endothelial-to-haematopoietic transition (Chen et al., 2009). They are generated from Tie2⁺ yolk-sac ancestors from E7.5 to E10.5 (Gomez Perdiguero et al., 2014). The number of EMPs in the yolk sac peaks between E9.5 and E10.5, and they seed the fetal liver as soon as E9. EMPs expand in the fetal liver and differentiate into erythrocytes, megakaryocytes, macrophages, monocytes, granulocytes and mast cells (Gomez Perdiguero et al., 2014). Yolk-sac EMPs express the gene encoding the transcription factor c-Myb, but their commitment and differentiation into the myeloid fate is unaltered in c-Myb-deficient embryos, although their erythroid potential is blocked (Perdiguero and Geissmann, 2015).

Research with fate mapping models suggest that yolk-sac EMPs are major contributors to fetal and adult resident macrophages (**Figure 1.5**) and EMP-derived erythrocytes, monocytes, granulocytes and mast cells are ultimately replaced by HSC-derived myeloid cells in the late embryo, while EMP-derived macrophages persist all through fetal and post-natal development as tissue-resident macrophages (Gomez Perdiguero et al., 2014; Schulz et al., 2012). It has recently been speculated that a first wave of c-Myb-independent EMPs gives rise to microglia and a second wave of c-Myb-dependent EMPs gives rise to other macrophages (Hoeffel et al., 2015). However, there are no data available to suggest the existence of two genetically distinct populations of EMPs, and this hypothesis is not in accordance with the persistence of fetal resident macrophages in all c-Myb-deficient embryonic tissues (Perdiguero and Geissmann, 2015).

HSCs are also definitive progenitor cells that emerge from the hemogenic endothelium of the aorto-gonado-mesonephros (AGM) region (starting at E10.5 in mice) (Bertrand et al., 2010; Kissa and Herbomel, 2010) and migrate to the fetal liver, where they expand and differentiate from E12.5 until definitive haematopoiesis begins to shift to the bone marrow. HSCs colonise the embryonic bone marrow at E15, and active haematopoiesis starts there at E17 (Kieusseian et al., 2012). Fetal and adult HSCs require c-Myb for their self-renewal and maintenance, and loss of c-Myb expression leads to rapid HSC-derived haematopoiesis failure (Schulz et al., 2012). Depending on the age of the organism and environmental challenges, HSC-derived cells can contribute to adult tissue-resident populations.

Therefore tissue-resident macrophages of EMP origin are considered to be seeded in tissue during embryonic development and persist throughout adulthood through self-renewal. In

addition adults HSC-derived cells can contribute to adult tissue-resident macrophages populations. The sources of resident macrophages varies by tissue, with some tissues being populated mainly by embryonic-seeded macrophages for example, Langerhans cells in the skin and microglia in the brain and other tissues having mainly HSC-derived macrophages for example, the intestine (Okabe and Medzhitov, 2015).

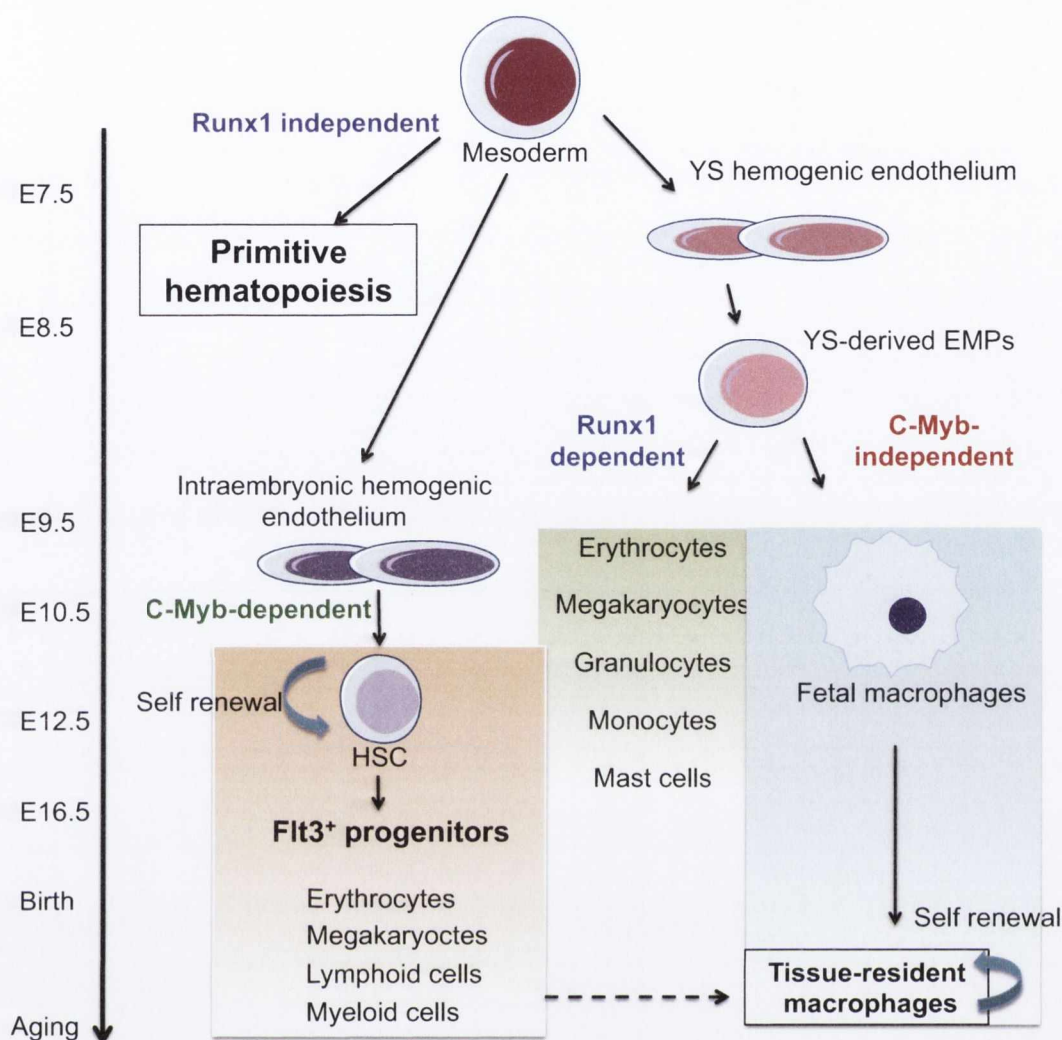


Figure 1.5 Myeloid development and maintenance in the embryo and adult mouse. Primitive progenitor cells first appear in the blood islands in the YS and are not dependent on Runx1 and c-Myb. During definitive haematopoiesis, HSCs and EMPs arise from distinct hemogenic endothelial cells through a Runx1-dependent endothelial-to-haematopoietic transition. EMP-derived haematopoiesis gives rises to erythrocytes and short-lived myeloid cells (monocytes, granulocytes and mast cells) that are replaced by HSC-derived cells later in gestation. During fetal development EMP-derived macrophages colonise all tissues where they specialise to their resident tissue after birth and can persist throughout adult life through self-renewal. Depending on the age of the organism and environmental challenges, HSC-derived cells can contribute to adult tissue-resident populations. YS- yolk-sac. Adapted from Periguero and Geissman (2015).

1.10 Peritoneal macrophages

1.10.1 The peritoneal cavity

The peritoneal cavity is a membrane-bound, fluid filled abdominal cavity of mammals that contains organs such as the liver, kidney, spleen and most of the gastrointestinal tract. Immune cells found in the peritoneal cavity include macrophages, T cells, B cells, NK cells, mast cells, and dendritic cells (Ghosn et al., 2010). This heterogeneous population of cells is involved in both innate and adaptive immunity. Macrophages and B cells are the predominant cell types of the peritoneal cavity (Ghosn et al., 2010). Peritoneal macrophages play key roles in the control of infections and inflammatory pathologies, as well as in the maintenance of immune response robustness (Cassado et al., 2015). Much of the current fundamental macrophage knowledge comes from analysis of macrophages taken from peritoneal cavity of mice. Like macrophage populations at other sites of the body peritoneal macrophages display a great degree of heterogeneity.

1.10.2 Peritoneal macrophage canonical surface markers

Mature macrophages can be characterised by the expression of certain cell surface markers. The surface markers of the macrophage regulate a range of functions, including differentiation, growth and survival, adhesion, migration, phagocytosis, activation, and cytotoxicity. The cell surface marker profile of a macrophage is dependant on the tissue site and microenvironment it resides in. For many years populations of peritoneal macrophages were primarily identified by their expression of the canonical macrophage markers CD11b and F4/80.

CD11b, also known as integrin alpha M (ITGAM), is a 170KDa glycoprotein that is a member of the integrin family. CD11b forms a heterodimeric Integrin $\alpha_M\beta_2$ molecule, also known as macrophage-1 antigen (Mac-1) or complement receptor 3 (CR3). The second subunit of $\alpha_M\beta_2$ is common integrin B2 subunit known as CD18. Integrin $\alpha_M\beta_2$ is expressed in monocytes, granulocytes, macrophages, B cells and natural killer cells and has been implicated in diverse responses of these cells, including phagocytosis, cell-mediated killing, chemotaxis, and cellular activation (Solovjov et al., 2005). Integrin $\alpha_M\beta_2$ serves as a PRR to recognise PAMPs and damage-associated molecular patterns (DAMPs), such as LPS, aggregated β -amyloid, damage-associated alarmin HMGB1 and extracellular Double-Stranded RNA (Zhou et al., 2012).

F4/80 is a 160kDa glycoprotein, also known as Ly71 and EMR1 and is part of the EGF-TM7 family. F4/80 shows 68% homology to its human orthologue EMR1. The rat monoclonal antibody (mAb) F4/80 was first isolated by John Austyn and Siamon Gordon in 1981 (Austyn and Gordon, 1981). Since then F4/80 has become widely used as a mouse macrophage antigen marker, expressed during development and throughout the adult life, constitutively and in a range of inflammatory, infectious, immune and other disease models (Gordon et al., 2011). Unlike the Integrin $\alpha M\beta_2$ antigen, F4/80 is absent on neutrophils and it is dim or absent on selected macrophage populations such as splenic marginal zone macrophages and osteoclasts. In the adult, F4/80 is dim on both resident and inflammatory monocyte subsets in the steady state. It is highly expressed on Kupffer cells, spleen and adrenal glands, as well as on microglia and Langerhans cells. The exact function of F4/80 on macrophages is still unclear as F4/80 knock-out animals show normal development of tissue macrophage populations and normal responses to infection with *Listeria* (Gordon et al., 2011).

1.10.3 Peritoneal macrophage subsets

LPM and SPM subsets

Based on CD11b and F4/80 expression two subsets of peritoneal macrophages were initially identified: small peritoneal macrophages (SPM) and large peritoneal macrophages (LPM) (Ghosn et al., 2010). These subsets differ based on size and expression certain surface markers. LPM is the most abundant macrophage subset (approximately 90% of peritoneal macrophages) in unstimulated mice; SPM accounts for the remaining 10% of peritoneal macrophages (Ghosn et al., 2010). LPM expresses three times as much CD11b and 80 times as much F4/80 as SPM. They also differ in expression of MHCII and Gr-1, with SPM having increased MHCII expression and decreased Gr-1 expression compared to LPM. Notably cell surface markers CD40, CD80, CD86, CD11c and TLR4 are all expressed at higher levels on LPMs than SPMs. Ghosn et al. (2010) observed that dendritic cells in the peritoneal cavity are smaller than either SPMs or LPMs and do not express CD11b and F4/80 at the levels found on SPMs and LPMs.

Ghosn et al. (2010) observed that LPM and SPM differ in responses to inflammatory stimuli, although observations differ when conducted *in vitro* or *in vivo*. *In vitro*, LPS

induces LPMs to produce high levels of intracellular NO but fails to induce NO production by SPM. In contrast, *in vivo* LPS stimulates NO production by both LPMs and SPMs, and in fact stimulates more NO production by SPMs than by LPMs. By day 2 post LPS injection (10 µg) there is a shift in the LPM: SPM ratio, with a higher ratio of SPM present in the peritoneal on day 2. LPS stimulation also induced an influx of cells that initially express the phenotype of the circulating monocytes in blood. Over time, the phenotype expressed by this large population of “monocytes” shifts toward the typical SPM phenotype. Ghosn et al. (2010) concluded that LPM differentiation does not account for this observed increase in SPM frequency and hypothesise that infiltrating monocytes are the source of increased SPM frequency.

Thioglycollate medium is a multi-purpose enriched differential medium designed to test the aerotolerance of bacteria and it is routinely used for sterility testing. Aside from its use in microbiology it is also frequently used as an immunological technique to enrich for activated macrophages in the peritoneal cavity. Thioglycollate is a sterile eliciting agent that increases monocyte migration into the peritoneal cavity, and therefore increases macrophage yield. Thioglycollate elicited macrophages are usually isolated on day 4 post injection (Zhang et al., 2008). Ghosn et al. (2010) observed that, similar to LPS stimulation, thioglycollate stimulation dramatically changes the LPM: SPM ratio, a higher ratio of SPMs is present in the peritoneal cavity on day 4. It is hypothesised that the LPMs die due to thioglycollate or they are forced to leave the peritoneal cavity. Again, similar to LPS stimulation, a small monocyte population (Ly6C^{High}) population is also observed on day 1, which gradually expresses the typical SPM phenotype. Based on their results, Ghosn et al. (2010) concluded that the SPM population is most likely of Ly6C^{High} monocyte origin; however, the origin of LPM was not clear. In the years since this research was conducted, it has been recognised that macrophages found in many tissues can be monocyte-derived or tissue-derived (**Section 1.9**). It is therefore possible that the LPM population is in fact a tissue-resident population.

Dual Origin of Peritoneal Macrophages

Using fate-mapping techniques to explore the murine monocyte and macrophage compartment, Yona et al. (2013) took advantage of *Cx3cr1^{GFP}* mice and *Cx3cr1^{cre}:R26-yfp* mice. GFP expression acts as a direct reporter for current CX₃CR1 expression in *Cx3cr1^{GFP}* mice. In *Cx3cr1^{cre}:R26-yfp* mice YFP expression acts as a direct reporter for current and

previous CX₃CR1 expression. It was found that the majority of peritoneal macrophages did not currently express CX₃CR1 but had expressed CX₃CR1 at some point in their lineage, indicating that these macrophages originated from a CX₃CR1⁺ precursor. Therefore peritoneal macrophages, are primarily established prior to birth and maintain themselves subsequently during adulthood independent of replenishment by blood monocytes (Yona et al., 2013). While this observation is true for the steady state, such dynamics change when a stimulus is encountered. In responses to certain stimuli blood monocytes are recruited the peritoneal cavity.

The dual origin of peritoneal macrophages was also highlighted by Jenkins and colleagues (2011) who explored M2 macrophage expansion and recruitment during Th2-associated inflammation. Th2 inflammation can be driven by helminth infection or allergy and represents a distinct challenge to the immune system that is characterised by the recruitment of cells that produce the cytokine IL-4, including eosinophils, basophils, Th2 cells and the accumulation of large numbers of macrophages in the affected tissue (Jenkins et al., 2011). These macrophages are polarised towards an M2 or AAM phenotype mainly driven by IL-4 and IL-13 and characterised by expression of Arg-1, RELM α , and Ym1.

Jenkins et al. (2011) showed that in the pleural cavity of mice, promotion of Th2 inflammation using infection with the rodent filarial nematode *Litomosoides sigmodontis* induced the expansion and proliferation of resident tissue M2 macrophages, which was independent of monocytes. Concurrently they showed that thioglycollate induced the expansion and proliferation of monocyte-derived M2 macrophages. Bone marrow chimeras in which the peritoneal and pleural cavity cells were shielded from irradiation, and thus cells present remained of host origin, allowed them to further distinguish M2 macrophages of monocyte vs. resident origins. Apart from parasitic infection, IL-4 is also driver of M2 macrophage polarisation. Jenkins et al. (2011) observed that in the peritoneal cavity, IL-4 induced the proliferation and expansion of resident peritoneal M2 macrophages, whereas delivery of IL-4 plus thioglycollate caused recruitment and proliferation of blood monocyte-derived AAMs. Overall this research highlighted that M2 macrophage expansion and proliferation in the peritoneal cavity could be induced, using different methods, from two sources: monocyte-derived macrophages and tissue-derived macrophages.

Gundra et al. (2013) brought this research a step further by showing that the M2 macrophages derived from the two sources are phenotypically and functionally distinct. By transcriptional profiling analysis, using the IL-4/thioglycollate protocol developed by Jenkins et al. (2011), they showed that monocyte-derived macrophages and tissue-derived macrophages expressed high levels of M2 macrophages markers Arg1, YM-1, and Relm α ; however, only monocyte-derived M2 macrophages up-regulated Raldh2 and PD-L2. Monocyte-derived M2 macrophages were also CX₃CR1-GFP^{High} and expressed CD206, whereas tissue-derived AAMs were CX₃CR1-GFP and CD206 negative. Furthermore, monocyte-derived M2 macrophages had high levels of aldehyde dehydrogenase activity and promoted the differentiation of FoxP3 cells from naive CD4 cells via production of retinoic acid. In contrast, tissue-derived M2 macrophages expressed high levels of uncoupling protein 1 (UCP.1) (Gundra et al., 2014).

Tim4⁺ Macrophage Subset

Recently, T cell/transmembrane, Ig, and mucin (TIM)-4 was identified as a marker for tissue-resident macrophages in the peritoneal cavity (Thornley et al., 2014). TIM-4 recognises phosphatidylserine (PtdSer), a specific marker of apoptosis. TIM-4 is expressed primarily on APCs, including dendritic cells, macrophages CD169⁺ marginal metallophilic macrophages, and also on peritoneal B-1 B cells. TIM-4-expressing phagocytic cells efficiently engulf and clear PtdSer expressing, Ag-specific T cells (Albacker et al., 2010). Thornley et al. (2014) were primarily interested in a tissue-resident macrophage subset in the skin (CD45⁺CD11b⁺F4/80⁺Ly6C⁻CD169⁺TIM-4⁺).

It was shown that this population is a major subset of tissue-resident macrophages in the skin, homes to draining lymph nodes following oxidative stress, exhibits an immunoregulatory phenotype, favours preferential induction of antigen-stimulated Tregs, and is highly susceptible to apoptosis (Thornley et al., 2014). In terms of peritoneal macrophages they showed that TIM-4 is expressed on CD11b⁺ F4/80⁺ macrophages. Ablation of TIM-4 rendered peritoneal macrophages resistant to H₂O₂-induced programmed cell death. Thus TIM-4 expressing macrophages may be more susceptible to apoptosis by oxidative stress (Thornley et al., 2014).

1.11 Transcription factors

Transcription factors (TFs) are proteins that bind to DNA sequences in enhancer or promoter regions to induce or repress gene expression. TFs are critical for cellular functions and are found in all branches of the tree of life (bacteria, archaea and eukaryotes). It is predicted that humans have between 1500 to 2000 TFs, making up about 7% of the human proteome (Vaquerizas et al., 2009). Retinoic acid orphan receptor alpha (Ror α) the focus of this thesis is a transcription factor and is discussed in more detail in **Section 1.15**. TFs coordinate many important biological processes such as cell differentiation and development in response to a wide range of stimuli. TFs contain a DNA binding domain (DBD) that enables the TF to bind to specific DNA sequences thereby controlling the rate of transcription of DNA to messenger RNA (mRNA). TFs perform this function alone or complexed with other proteins by recruiting RNA polymerase to specific genes. Successful transcription requires other proteins including co-factors, histones and chromatin remodelling proteins.

1.12 Myeloid lineage differentiation TFs

As discussed in **Section 1.1.2**, the HSC in the bone marrow is the original precursor cell for immune cells. HSCs commit early in the differentiation process to either the CLP or CMP lineage (**Figure 1.1**). The CMP is the progenitor cell for myeloid cells. Through a hierarchical process of cell fate decision and lineage commitment myeloid cells are created from the CMP. The initial steps of myeloid cell differentiation in the bone marrow are determined by cytokine signals that are provided mainly by stromal cells (De Kleer et al., 2014). TFs are key in this differentiation process. Lineage specification at differentiation branch points is dictated by the activation and interaction of a number of lineage specific TFs including PU.1, C/EBPs and GATA-binding proteins (GATAs) (**Figure 1.6**).

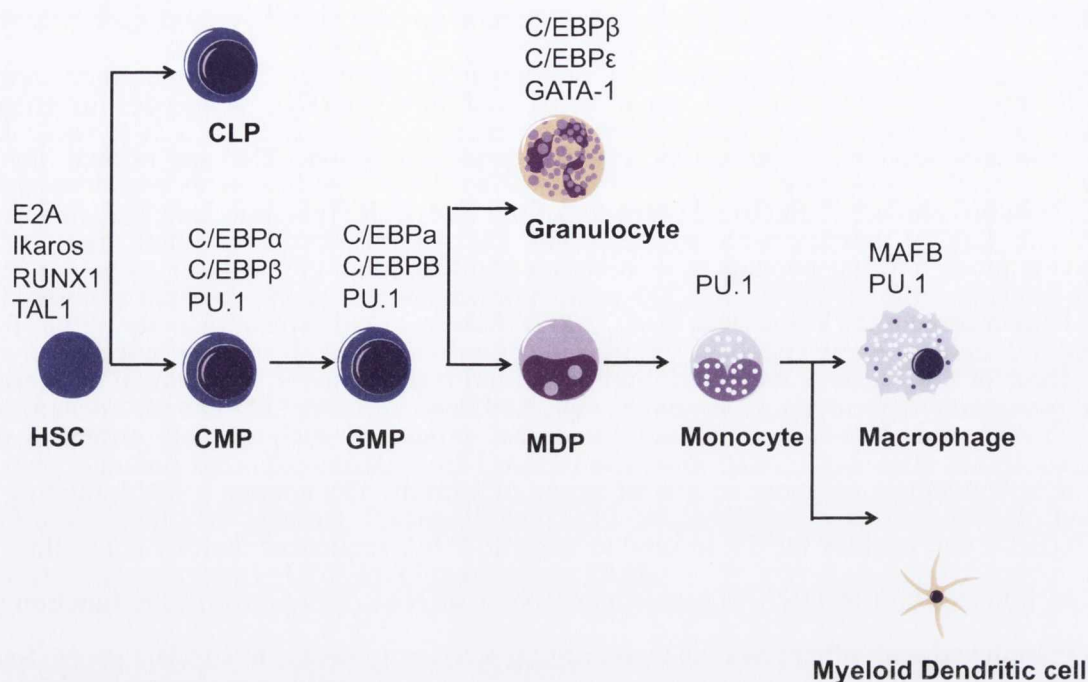


Figure 1.6 Transcription factors important in the myeloid cell differentiation process. HSCs differentiate to macrophages through a number of progenitor cells. Each step of this process is controlled by TFs.

1.12.1 PU.1

PU.1 is expressed in both myeloid and lymphoid cells. PU.1 is a member of the E-twenty six (Ets) family of transcription factors and is encoded by the SPI1 gene. It is essential for embryonic lymphoid and myeloid development. A number of PU.1 null and mutant mouse lines have been generated and display varied phenotypes depending on the nature of the PU.1 defect, highlighting the versatility of this transcription factor in promoting or preventing differentiation depending on the haematopoietic lineage. PU.1 knockout mice succumb to death during the neonatal stage and have a lack of myeloid cells and T and B cells. The self-renewal capacity of erythroid progenitor cells are also altered in the spleen of PU.1 null mutants (Back et al., 2004).

To explore the role of PU.1 in haematopoietic lineages further, studies have been performed using reporter systems in which GFP has been knocked into the PU.1 locus to allow its expression pattern to be tracked throughout haematopoietic differentiation (Mak et al., 2011). While a low level of PU.1 is detected in HSCs, its expression increases as

these cells become MPPs and subsequently develop into CMPs and CLPs. At this stage, the level of PU.1 expression is decisive in permitting progression towards either the myeloid or lymphoid lineage (Spooner et al., 2009). Culturing PU.1+/- fetal liver progenitor cells in cytokines that favours lymphopoiesis predominantly generates pro-B cells, while overexpression of PU.1 in these cells dramatically biases commitment towards the myeloid lineage and results in an increase in macrophage production (DeKoter and Singh, 2000). Therefore, high PU.1 expression promotes macrophage generation, while a lower level is important for B cell development. Consistent with this, PU.1 levels have been estimated to be approximately eightfold higher in macrophages compared to B cells (DeKoter and Singh, 2000).

Binding sites for PU.1 are found on almost all myeloid-specific promoters. Notable target genes includes receptors for the cytokines, M-CSF and GM-CSF (Hohaus et al., 1995; Smith et al., 1996) and the characteristic antigens CD11b/CD18 (Rosmarin et al., 1995) and transcription factors c-Jun and JunB (Steidt 2006).

Alterations in PU.1 have been described in acute myeloid leukaemia (AML). AML is an aggressive, clonal myeloid neoplasm with maturation arrest of myelopoiesis, leading to an accumulation of myeloblasts in bone marrow and/or blood. According to the current WHO Classification, myeloblasts must comprise at least 20% of nucleated cells in bone marrow or blood to establish a diagnosis of AML (Hasserjian, 2013). Mice carrying hypomorphic PU.1 alleles that reduce PU.1 expression to 20% of its normal levels were reported to develop AML unlike mice carrying homo- or heterozygous deletions (Rosenbauer et al., 2004).

1.12.2 C/EBPs

CCAAT/enhancer binding proteins (C/EBP) contain a basic region-leucine zipper transcription factor that recognises CCAAT motifs in the promoters of target genes. C/EBP α , C/EBP β , C/EBP ϵ are involved in myeloid haematopoiesis. C/EBP α is a master regulator of myeloid differentiation that drives haematopoiesis towards the granulocytic lineage; C/EBP α is the predominant C/EBP protein in immature myeloid cells. New-born C/EBP α null mice lack the granulocytes neutrophils and eosinophils, but retain monocytes (Zhang et al., 1997). More recently it was shown using Cre-Lox mice that marrow from adult C/EBP $\alpha^{fl/fl}$ *Mx1-Cre* mice exposed to pIpC to induce *Cebpa* gene deletion have

reduced numbers of GMP, leading to impairment of both granulopoiesis and monopoiesis, with increased numbers of preceding CMP (Zhang et al., 2004). Higher levels of C/EBP α are required for granulocyte and lower levels for monocyte lineage specification, and this may be facilitated by increased *Cebpa* gene expression in granulocyte compared with monocyte progenitors (Ma et al., 2014).

C/EBP α functions are frequently disrupted in particular subtypes of AML, thereby contributing to the differentiation arrest seen in AML blast cells. It is dysregulated in over half of patients with AML. C/EBP α expression is suppressed as a result of common leukaemia-associated genetic and epigenetic alterations (Paz-Priel and Friedman, 2011). In addition 10-15% of patients with AML are characterised by mutations of the CEBPA gene. C/EBP β is expressed from the metamyelocyte stage and on throughout maturation. C/EBP β -deficient mice display normal granulocyte differentiation and steady-state levels of neutrophils but progenitor cells are unable to produce neutrophils in response to exposure to GM-CSF and G-CSF increasing susceptibility to infection (Akagi et al., 2008). C/EBP β -deficient neutrophils also displayed enhanced apoptosis compared with wild-type (WT) neutrophils. C/EBP ϵ is expressed at the promyelocyte to myelocyte stage. C/EBP ϵ -null mice lack mature functional granulocytes, both neutrophils and eosinophils, and the mice die by 3 to 5 months of age because of opportunistic infections (Bedi et al., 2009).

1.12.3 GATA family

The GATA family of nuclear regulatory proteins serves as a prototype for the action of lineage-restricted transcription factors. GATA factors constitute a family of evolutionarily conserved proteins that play crucial roles in the development and differentiation of eukaryotic organisms. They owe their name to their ability to bind the consensus DNA sequence (A/T)GATA(A/G) through two highly conserved zinc finger (ZnF) domains.

GATA1, *GATA2* and *GATA3* are expressed principally in haematopoietic cell lineages. *GATA1* is present in the more differentiated cells of the erythroid, megakaryocytic, eosinophilic and mast lineages, and is an essential transcription factor for the maturation of erythroid and megakaryocytic cells. *GATA2* expression has a broad distribution among haematopoietic cells, with a particularly prominent expression in early progenitors, as well as in megakaryocytes and in mast cell lineages

1.13 Peritoneal macrophage differentiation TFs

Macrophage subsets are defined not only by their phenotype, but also by their expression of TFs required for specific functions. Macrophages differ in phenotype and function dependent on the tissue in which they reside. Several examples of TFs that dictate tissue-specific transcription programs in macrophages have been reported, and the deletion of these TFs resulted in the ablation of particular tissue macrophage subsets suggesting their involvement in the differentiation of the corresponding macrophage populations. Peritoneal tissue macrophage subsets, like macrophage subsets in other tissues, are quite heterogeneous. They can have dual origins: monocyte-derived macrophages and tissue-resident macrophages (**Section 1.9**). It is increasingly apparent that distinct transcriptional master regulators control the development of tissue-resident macrophages compared to monocyte-derived macrophages (Gautier et al., 2012). Here a number of these transcription regulators are discussed.

1.13.1 Gata6

In 2012 the immunological genome consortium identified the zinc finger transcription factor Gata-binding protein 6 (Gata6) as being selectively expressed in resident peritoneal macrophages (Gautier et al., 2012). The role of Gata6 in peritoneal macrophage subsets was explored by two groups, each using a conditional knockout (KO) mouse strain, in which the Gata6 gene is deleted specifically in myeloid cells (*Gata6*-KO^{mye} mice or Mac-*Gata6* KO) (Okabe and Medzhitov., 2014; Rosas et al., 2014). Both studies found that the deletion of Gata6 in myeloid cells resulted in gross phenotypic changes in the CD11b^{High} F4/80^{High} tissue-resident macrophages. However, there were still tissue macrophages present, albeit in reduced numbers. The population considered to be the monocyte-derived macrophages was unaltered. *In vivo* lentiviral manipulation of Gata6 in adult tissue-resident macrophages resulted in similar phenotype changes, indicating that Gata6 is required for cell phenotype in the adult (Rosas et al., 2014). Transcriptome studies, which compared tissue- resident macrophages from *Gata6*-KO^{mye} mice with WT mice, identified a diverse range of genes altered in the absence of Gata6. Crucially, a large number of these genes were tissue-resident macrophages selective, so it was concluded that Gata6 is in part responsible for the tissue- resident macrophages specific gene profile.

Okabe and Medzhitov (2014) identified that vitamin A-derived retinoic acid drives the expression of Gata6 in tissue-resident macrophages; the source of this retinoic acid is the peritoneal omental tissue. Peritoneal tissue-resident macrophages proliferate at a low level to maintain homeostasis, but go through a burst in proliferation to recover lost numbers during the resolution from inflammation. Rosas et al. (2014) identified alterations in genes linked to proliferation in *Gata6*-KO^{mye} mice tissue-resident macrophages. The proportion of *Gata6*-KO^{mye} mice macrophages proliferating was higher than controls, but nuclear polyploidy was also enhanced. *Gata6*-KO^{mye} mice tissue-resident macrophages failed to recover their numbers after acute inflammation and those cells still present were deficient in their proliferative response. Therefore, Gata6 contributes toward tissue-resident macrophages persistence by regulating normal proliferative responses (Rosas et al., 2014).

1.13.2 C/EBPβ

Studies with mice deficient in C/EBPβ (*C/ebpβ*^{-/-}) mice showed that the peritoneal cavity of these mice did not contain LPMs and exhibited increased numbers of SPMs (Cain et al., 2013). Interestingly, adoptively transferred SPMs differentiated into LPMs in *C/ebpβ*^{-/-} mice. However, in control mice that have normal numbers of LPMs, only a small frequency of transferred SPMs acquired the phenotype of LPMs. Based on these results, the authors proposed that under physiological conditions, SPMs appear to contribute in only a small way to generate LPMs, but that SPMs may be involved in the maintenance of LPMs when LPM numbers have been greatly reduced (Cain et al., 2013). Of note the authors do not make reference or inferences on the origin of the LPM and SPM populations. Therefore in terms of LPM and SPM classification, C/EBPβ appears to be important for LPM development.

1.13.3 TSC1

Zhu et al., 2014 recently showed that tuberous sclerosis complex 1 (TSC1) is a critical regulator of M1 and M2 phenotypes of peritoneal macrophages. Mice with myeloid-specific deletion of TSC1 exhibited enhanced M1 response and spontaneously develop M1-related inflammatory disorders. However, TSC1-deficient mice are highly resistant to M2-polarised allergic asthma.

1.14 Macrophage responses to LPS

1.14.1 LPS/TLR4 pathway

Macrophages are of primary importance in the recognition and response to LPS. LPS (also known as endotoxin) is a large molecule consisting of a lipid and polysaccharide composed of O-antigen, it is found in the outer membrane of gram-negative bacteria. Activation of macrophages by bacterial LPS induces transcription of genes that encode for pro-inflammatory regulators of the immune response (**Figure 1.7**). At physiological levels LPS stimulation occurs through a series of interactions with several proteins. LPS initially binds to the serum protein LPS-binding protein LBP (Hambleton et al., 1996). LBP is a soluble shuttle protein that facilitates the interaction between LPS and CD14. CD14 lacks a transmembrane domain and is unable to initiate signals on its own but facilitates the transfer of LPS to the TLR4/MD-2 complex. Using the C3H/HeJ mouse strain which is known to have a defective response to LPS, Beutler's group demonstrated that TLR4 is an important sensor for LPS (Poltorak et al., 1998) (**Section 1.2.2**).

Downstream signalling of the TLR4 receptor complex in response to LPS is largely mediated via the recruitment of adapter proteins, including myeloid differentiation factor 88 (MyD88), MyD88 adapter-like protein (MAL), TRIF-containing adapter molecule (TRIF, also known as TICAM-1), and TRIF-related adapter molecule (TRAM). Thereby, MAL-dependent recruitment of MyD88 orchestrates production of inflammatory cytokines in response to LPS-treatment (Kawai et al., 1999). This requires caspase 1 dependent processing of MAL (Miggin et al., 2007) and involves the activation of the $\text{I}\kappa\text{B}/\text{NF}\kappa\text{B}$ pathway as well as of the p38^{MAPK} and the c-jun N terminal kinase (JNK) members of the mitogen activated protein kinase (MAPK) family (Bode et al., 2012). There are four well-characterised subfamilies of MAPKs- ERK1/2, ERK5, JNKs and p38s. The MAPKs are activated upon dual phosphorylation of tyrosine and threonine residues in a conserved Thr-*Xaa*-Tyr motif (where *Xaa* is any amino-acid) in the activation loop of kinase subdomain VIII (Cuenda and Rousseau, 2007).

In addition to the early MyD88-dependent signals TLR4 triggers a delayed MyD88-independent (Kawai et al., 2001), TRIF-dependent signal transduction (Yamamoto et al., 2002) via TRAF family member associated NF- κ B-activator binding kinase (TBK)1-mediated activation of the IFN response factor (IRF)3 and late activation of NF- κ B (Bode

et al., 2012). This delayed part of TLR4 signalling requires dynamin-dependent internalisation of TLR4 and subsequent recruitment of TRAM which initiates TRIF-dependent pathways enabling TLR4 to trigger the release of type I interferons (Kagan et al., 2008). In short through the two TLR4 signalling pathways described here, LPS stimulation of macrophages culminates in the production of cytokines, chemokines and other communication signals important for the coordination of the inflammatory response.

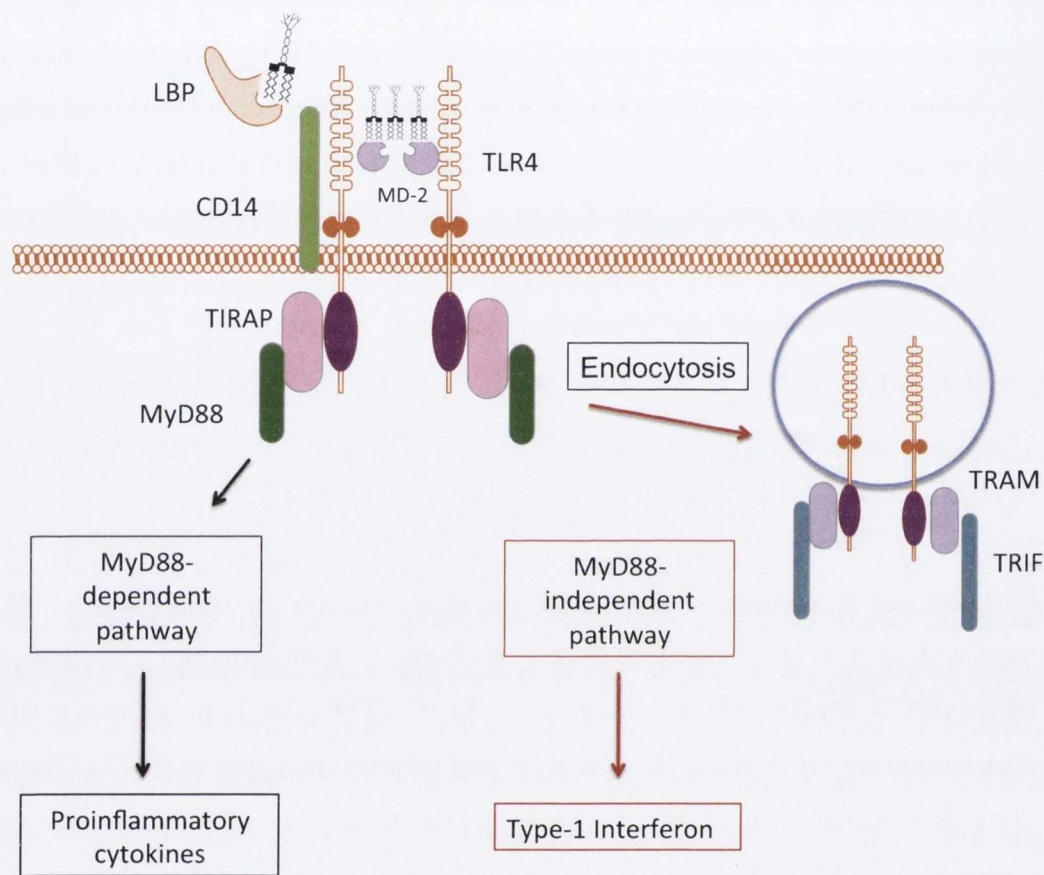


Figure 1.7 Activation of TLR4 by LPS. The LBP facilitates transfer of LPS monomers to CD14 and CD14 subsequently transfers LPS to the TLR4/MD-2 complex. Dimerisation of the receptor complex induces the assembly of TIRAP, MyD88, and IRAK kinases in a myddosome at the TIR domain of TLR4 inducing a signalling pathway that leads to the production of pro-inflammatory cytokines. After endocytosis, TRAM and TRIF associate with TLR4 triggering a signalling pathway that controls production of type I interferons. Adapted from (Płóciennikowska et al., 2015).

1.14.2 Sepsis

Sepsis is a complex process that is thought to develop when the initial host response against infection becomes inappropriately amplified, and dysregulated, thus becoming a

harmful host response. Cytokines regulate a variety of inflammatory responses, including the migration of immune cells to the site of infection, which is important for containing a localised infection and preventing it from becoming systemic. However, a dysregulated cytokine release may lead to endothelial dysfunction, characterised by vasodilation and an increase in capillary permeability. The resulting leakage is clinically associated with hypotension, macromolecular extravasation, haemoconcentration and edema (Schulte et al., 2013). The dysfunctional epithelial barriers enable pathogens to further invade the host organism, to disturb regulatory mechanisms to cause remote organ dysfunctions and in some cases death. More recently, it has become evident that pro-inflammatory and anti-inflammatory pathways are often competing in sepsis with recent studies showing that both pro-inflammatory and anti-inflammatory responses occur early and simultaneously in sepsis, although the initial effect of these competing processes is typically manifested by an early, dominant, hyper-inflammatory phase characterised by shock, fever and hyper-metabolism (Hotchkiss et al., 2013). Further research into the complex interplay of pro-inflammatory and anti-inflammatory processes in sepsis is required to fully elucidate sepsis pathogenesis.

1.14.3 Inflammatory cytokines

Stimulation of macrophages with LPS elicits a variety of different signalling events that culminate in the activation of macrophage effector functions including changes in gene expression and enhanced production of inflammatory cytokines including IFN γ , IL-1 β , TNF α , IL-6 and IL-12 (Bode et al., 2012). IFN γ was originally identified due to its anti-viral activity however; its immunoregulatory role in a range of pathologies is now evident. IFN γ is primarily produced by activated NK cells, Th1, and CD8⁺ cytotoxic T cells (Boehm et al., 1997). The neutralisation of IFN γ or IFN γ receptor makes mice more resistant to an LPS-induced shock (Car et al., 1994; Heinzel, 1990).

IL-1 β and TNF α are among the most extensively studied cytokines in sepsis pathophysiology (Schulte et al., 2013). Both are powerful pro-inflammatory cytokines that have been implicated in a large number of non-infectious and infectious inflammatory diseases including sepsis (Schulte et al., 2013). IL-1 β and TNF α are early regulators of the immune response and are released in response to invasive, infectious, or inflammatory stimuli. Both IL-1 β and TNF α are primarily released by macrophages with release beginning as early as 30 minutes after the inciting event, following gene transcription and

RNA translation (Schulte et al., 2013). Other immune cells such as fibroblasts can also produce TNF α . The administration of bacterial endotoxin results in the production and release of IL-1 β and TNF α into the systemic circulation, with peak concentrations detected 60–90 minutes after LPS administration (Schulte et al., 2013). IL-1 β and TNF α can act together to induce a shock-like state characterised by vascular permeability, severe pulmonary edema, and haemorrhage.

IL-6 is produced by a wide variety of cells including macrophages, DCs, lymphocytes, endothelial cells, fibroblasts, and smooth muscle cells in response to LPS, IL-1, and TNF α (Schulte et al., 2013). IL-6 has a variety of effects, including the activation of B and T cells and the coagulation system, and the modulation of haematopoiesis. A key function of IL-6 is the induction of fever (Celia et al., 1996) and the mediation of the acute phase response (Kopf et al., 1994), a systemic reaction to an inflammatory stimulus that is characterised by fever, leukocytosis, and the release of hepatic acute phase proteins. However, studies in dogs have shown that unlike TNF α and IL-1 β , IL-6 is unable to produce a sepsis-like state (Preiser et al., 1991).

IL-12 is structurally related to the IL-6 cytokine family with monocytes macrophages, neutrophils and DCs being the major sources of IL-12. IL-12 regulates innate immune responses and promotes the development of a type 1 adaptive immune response (Schulte et al., 2013). Upon release, IL-12 induces IFN γ production in T cells and NK cells, which then directly activates macrophages enhancing their bactericidal activity and increasing Th1 cytokine production (Bonecchi et al., 1998). The role of IL-12 in sepsis is somewhat controversial. Increased plasma IL-12 concentrations have been measured in animals following the administration of LPS or *Escherichia coli*, and after polymicrobial sepsis induced by CLP (Schulte et al., 2013). Some studies have shown that immunoneutralisation or genetic deletion of IL-12 resulted in an increased mortality of mice undergoing CLP (Steinhauser et al., 1999), however, another study found that an increase in LPS-induced mortality was observed in mice transiently overexpressing IL-12 and the neutralisation of IL-12 improved survival following LPS challenge (Zisman et al., 1997).

As mentioned above macrophages are one of the first cells recruited post LPS injection. When activated, macrophages release inflammatory mediators such as TNF α , IL-6, IL-1, Nitric Oxide (NO) and reactive oxygen species (**Figure 1.8**). These inflammatory

mediators induce the production of O_2^- , H_2O_2 and other microbicidal products that help resolve inflammation but can also cause extensive tissue damage. Following LPS injection an increase in SPM ($F4/80^{Int} CD11b^+$) is seen in the peritoneal cavity, however, concurrently the LPM ($F4/80^+ CD11b^+$) macrophage population appears to disappear (Ghosn et al., 2010). Recovery of activated resident macrophages in the peritoneal cavity is initially low and has been characterised as a 'disappearance reaction'. This reason for this disappearance is not known but could possibly be due to increased tissue adherence, emigration through draining lymphatics and even cell death (Davies et al., 2013).

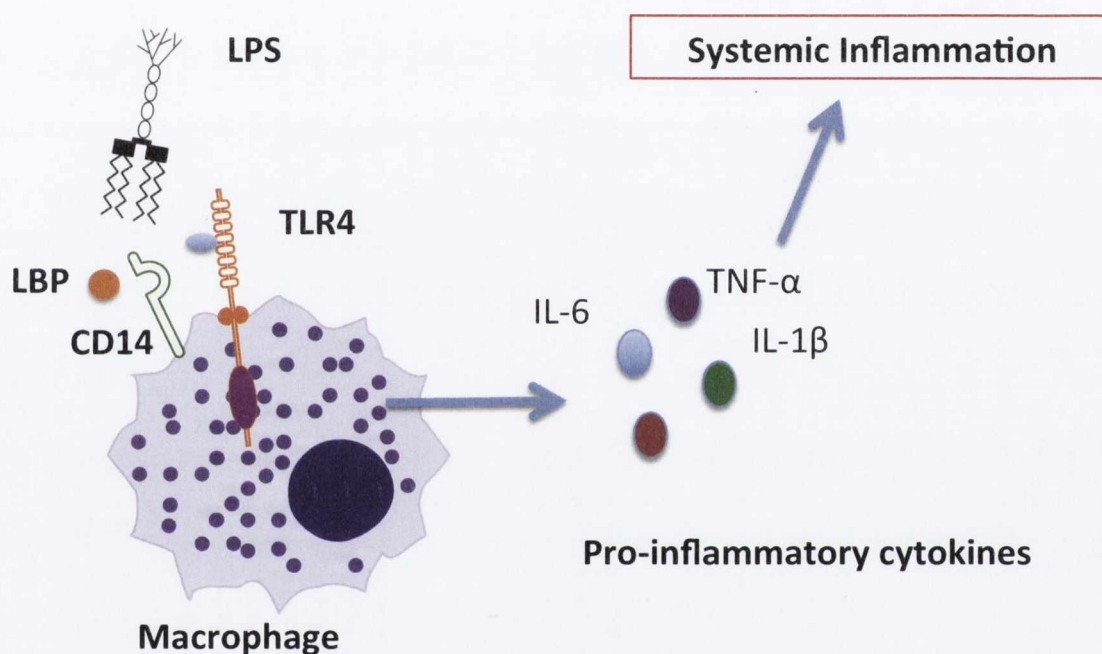


Figure 1.8 LPS activate macrophages through TLR4. LPS activated macrophages produce pro-inflammatory cytokines that in turn leads to systemic inflammation.

1.15 Retinoic acid receptor-related orphan receptor alpha (*Rora*)

1.15.1 Background and structure

Rora is a transcription factor that is a member of the nuclear hormone receptor (NHR) superfamily along with *Rorβ* and *Rory* (Giguere et al., 1994). *RORα* was identified in the 1990s based on sequence similarities to the retinoic acid receptor (RAR) and the retinoid X receptor (RXR), which resulted in the name 'retinoic acid receptor-related orphan receptor alpha' (Giguere et al., 1994). NHRs are generally classified as ligand-regulated transcription factors as many members of the nuclear receptor superfamily serve as

receptors for physiological ligands, including steroid hormones, lipids and fatty acids. However, *Rora* is still considered an orphan receptor because the identification of its endogenous ligands has been controversial. Human ROR α has been found to bind cholesterol (Kallen et al., 2002) (**Figure 1.9A**). Other cholesterol metabolites, including cholesterol-3-O-sulphate, have also been found to bind to the LBD of ROR α (Kallen et al., 2004). More recent studies have revealed that several oxysterols are high-affinity endogenous modulators of ROR α activity, oxysterol ligands bind directly to the LBD of ROR α and modulate the interaction of co-regulators (Wang et al., 2010a, 2010b).

Rora has a typical nuclear receptor structure including a DNA binding domain linked by a hinge region to the ligand-binding domain that modulates its transcriptional activity (**Figure 1.9B**). There are 4 isoforms of *Rora* in mice, α 1- α 4. *Rora* binds as a monomer to ROR-responsive elements (ROREs) that are found in target gene promoter regions, unlike the majority of NHRs that bind as dimers (Zhang et al., 2014). RORE is composed of 5-bp AT-rich sequence preceding the half-core (A/G)GGTCA motif (Giguere et al., 1994). When bound to this RORE within the promoter of a target gene, ROR α constitutively recruit coactivators, which lead to the transcriptional activation of their target genes.

A



B

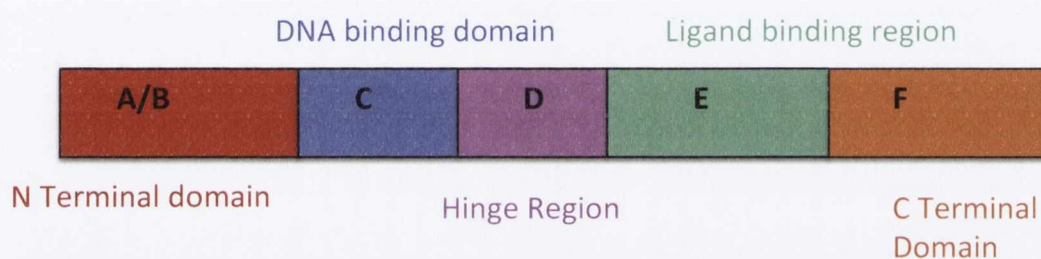


Figure 1.9 Structure of retinoic acid receptor-related orphan receptor alpha ($ROR\alpha$).

A Crystal structure of human $ROR\alpha$ Ligand Binding Domain, (residues 304-556) in complex with cholesterol (PDB 1N83) (Kallen et al., 2002). **B** Schematic $ROR\alpha$ structure. $ROR\alpha$ has a DNA binding domain linked through a ligand-binding domain through a hinge region. The transcriptional activity is in the ligand-binding domain.

1.15.2 $ROR\alpha$ expression

$ROR\alpha$ is widely expressed in a variety of tissues including cerebellar Purkinje cells, thymus, liver, skeletal muscle, lung, skin, adipose tissue and kidney (**Figure 1.10**) (Kojetin and Burris, 2014). It has also been reported that $ROR\alpha$ is expressed in both BMDMs and peritoneal macrophages. $ROR\alpha$ expression increases over 24 hours in BMDMs stimulated with LPS. Peritoneal macrophages stimulated with both LPS and thioglycollate express $ROR\alpha$ at 0 hours and 1 hour post stimulation, but expression is decreased by 7 hours (**Figure 1.10**).

1.15.3 *Rora* natural mutant mouse (*Rora*^{sg/sg} mice)

The *staggerer* mutation arose spontaneously in a stock of obese mice and was recognised by phenotypes characteristic of cerebellar lesions (Gold et al., 2007). *Staggerer* caught to the attention of Richard Sidman during one of his visits to the Jackson Laboratory (Gold et al., 2007). Homozygotes have an ataxic gait, hypotonia, smaller size compared to littermates and a short lifespan (approximately 4 weeks). After 35 years of active study, the *staggerer* mutation was identified by positional cloning as a putative null allele of *Rora* (Hamilton et al., 1996). Mice homozygous for the *staggerer* mutation became known as *Rora*^{sg/sg} mice. The mutant *Rora* allele contains a frameshift at amino-acid 273 in the $\alpha 1$ isoform leading to a truncation 27 residues later. The sequence deleted corresponds to a 122-bp exon removed by a 6.5-kb genomic deletion encoding part of the ligand binding domain (Hamilton et al., 1996).

The allele shift and resulting introduction of a premature stop codon suggests that the *Rora* protein is truncated in *Rora*^{sg/sg} mice. Northern blot experiments show ≥ 10 -fold reduction in *Rora* message abundance relative to *Gapdh*, consistent with degradation of the mutant RNA through the nonsense-mediated decay (NMD) pathway (Hamilton et al., 1996). These results suggest that *Rora*^{sg/sg} mice contain a null allele. Independent *Rora*-null mice created by gene targeting produced essentially identical phenotypes to *Rora*^{sg/sg} mice (Dussault et al., 1998).

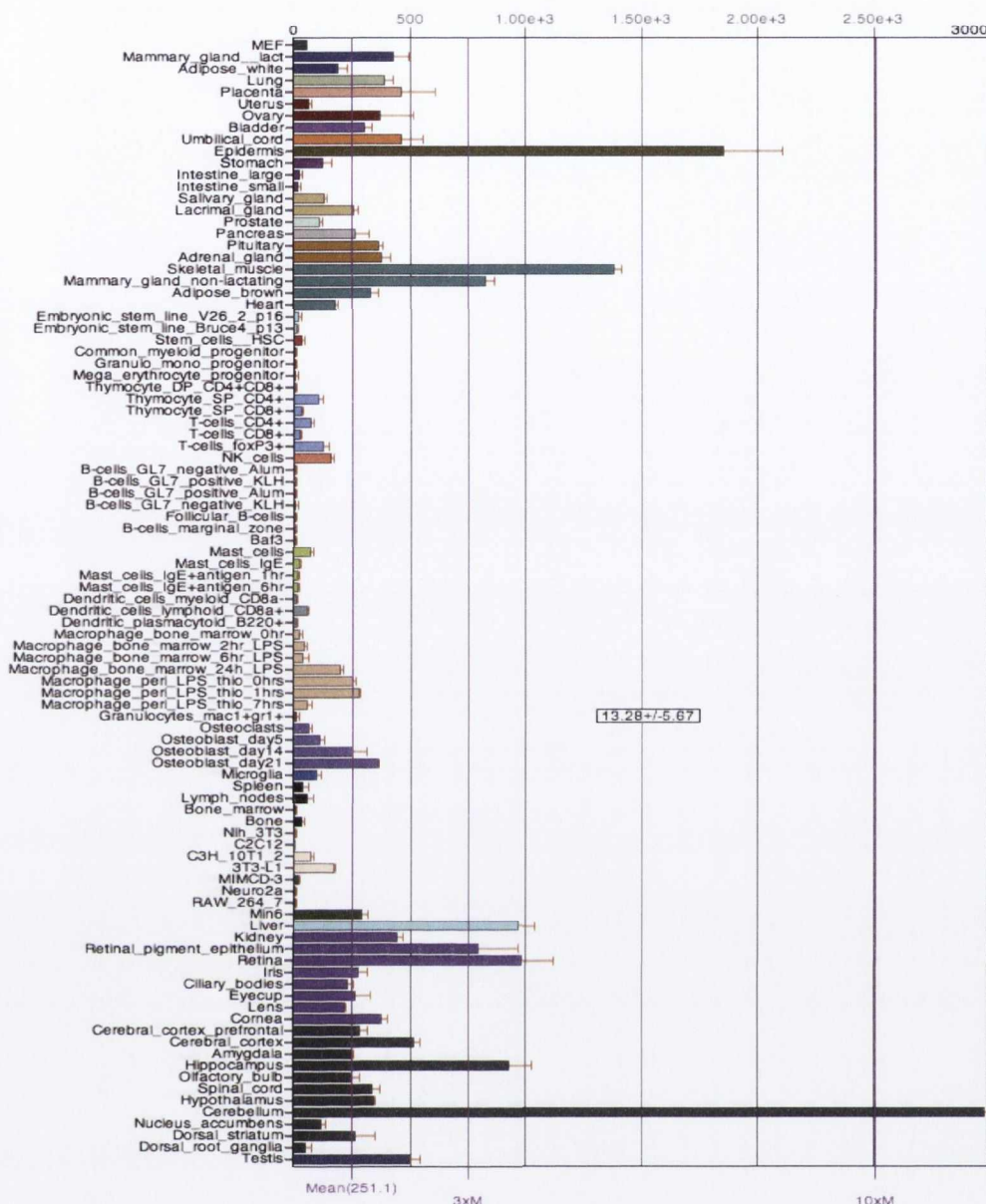


Figure 1.10 Expression of *Rora* in mouse tissue and cells. (<http://biogps.org/#goto=genereport&id=19883>). *Rora* is highly expressed in the cerebellum, liver and epidermis. It is also found in basal and LPS/thioglycollate stimulated BMDMs and peritoneal macrophages.

1.15.4 *Rora* in cerebellum development

Before the mutation in *staggerer* mice was identified as being in *Rora*, it was known that *staggerer* mice had cerebellum developmental issues. Indeed it was through the research on their cerebellum development that Hamilton et al. (1996) first identified a mutation in *Rora* of *staggerer* mice as being the cause of the developmental issues. The cerebellum in

Rora^{sg/sg} mice is dramatically smaller than controls, containing fewer of each principle cell type. The loss of granule cells is the earliest overt phenotypic difference in the cerebellum of *Rora*^{sg/sg} mice. The number of granule cells is somewhat reduced at birth and becomes more pronounced in the early postnatal weeks (Yoon, 1972). Within the developing cerebellum, *Rora* is highly expressed in newly post-mitotic Purkinje cells as early as embryonic day E12.5 and later expressed at lower levels in GABAergic interneurons in the molecular layer as well (Vogel et al., 2000). Mutant Purkinje neurons fail to differentiate fully or properly and are unable to support afferent neurons, including cerebellar granule neurons and climbing fibre projections from the inferior olivary nucleus in the brainstem (Hamilton et al., 1996). In more recent years *Rora* has been linked to a number of neurological disorders including autism spectrum disorders (Nguyen et al., 2010; Sarachana and Hu, 2013).

1.15.5 *Rora* in immunity

Like many other TFs *Rora* has a number of known roles in the immune system. It was found that *Rora* is a negative regulator of the inflammatory response (Delerive et al., 2001). *Rora* is associated with the upregulation of nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor, alpha (I κ B α), the major inhibitory protein of the nuclear factor kappa beta (NF- κ B) signalling pathway. The NF- κ B signalling pathway controls a large number of physiological processes such as immune and inflammatory responses, cellular growth and apoptosis. When p50 and p65 enter the nucleus, they upregulate the expression of a range of genes; which in turn leads to the production of pro-inflammatory cytokines and other inflammatory processes. I κ Bs inhibit the translocation of p50 and p65 into the nucleus thus leading to a decrease in the expression of these pro-inflammatory genes. *Rora* has a role in this process as it upregulates I κ B α , thus inhibiting p50 and p65 translocation and gene expression (Delerive et al., 2001).

Rora is also important in Th17 cell differentiation (Yang et al., 2008). Th17 cells highly express *Rora* that is induced by TGF β and IL-6 in a STAT-3 dependent manner (Yang et al., 2008). Over-expression of *Rora* promotes Th17 differentiation and significantly upregulates IL-17 and IL-17F expression. *Rora* deficiency results in reduced IL-17 expression *in vitro* and *in vivo*. Double deficiencies in *Rora* and *ROR γ* impair Th17 generation and completely protect mice against experimental autoimmune encephalomyelitis (EAE) which is a model for human multiple sclerosis. More recently

(Solt et al., 2011) showed that high-affinity synthetic ligand SR1001, specific for both ROR α and ROR γ t was able to inhibit Th17 differentiation and function as demonstrated by inhibition of IL-17A gene expression and protein production.

Recently *Rora* has been shown to be critical for the development of innate lymphocyte type 2 (ILC2) (Wong et al., 2012). There are three groups of ILC cells, type 1 (ILC1), (ILC2) and (ILC3). They differ by the cytokines that differentiate them, the cytokines they produce and the transcription factors involved in their development (**Figure 1.11**). ILC cells have important roles in the innate response, regulation of homeostasis and inflammation, and interplay with adaptive immunity. ILC2s produce type 2 cytokines IL-4, IL-5, IL-9, IL-13 and amphiregulin. ILC cells are relatively rare in the systemic circulation of naïve mice in comparison to other haematopoietic cells but their numbers expand in response to IL-25, IL-33, TSLP or during helminth infection (Fallon et al., 2006; Neill et al., 2010). ILC2s have been implicated in mouse models of asthma (Hams and Fallon, 2012) and *Rora* has been associated with asthma in a genome wide association study (Moffatt et al., 2010).

More recently it has been shown that ILC2 cells can be grouped into two distinct subsets- natural ILC2 (nILC2) and inflammatory (iILC2) (Huang et al., 2015). nILC2 cells reside in barrier tissues and primarily respond to IL-33. nILC2 cells play critical roles in immune protection and tissue repair. iILC2 cells are not present in peripheral tissues in the steady state but can be elicited at many sites by IL-25 treatment or helminth infection. IL-25-elicited iILC2 cells can be mobilised by distinct types of infections to develop into nILC2-like or ILC3-like cells, functioning in corresponding immune responses (Huang et al., 2015). It is unknown at present the role *Rora* has in each of these subsets, however it is known that iILC2s express both Gata-3 and Ror γ t.

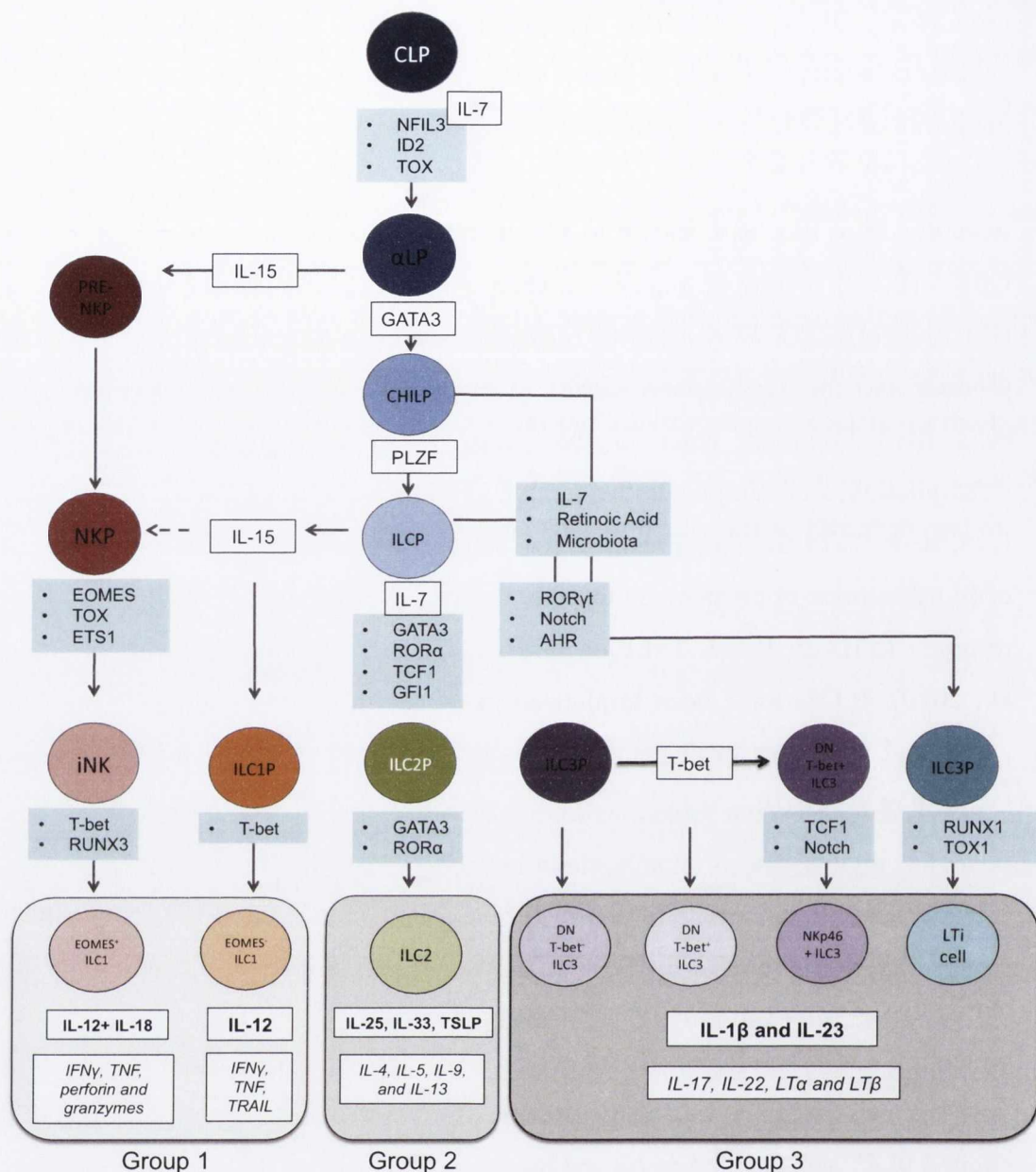


Figure 1.11 *Rora* is required for ILC2 development. ILCs differentiate from HSC via CLP. IL-7 and the transcription factors inhibitor of DNA binding 2 (ID2) and nuclear factor IL-3 induced (NFIL3) promote the differentiation of common precursors of ILC-restricted progenitors. *Rora* is required for the development of ILCP2 cells from ILCP cells and the development of ILC2 cells from ILCP2 cells. Major ILC-activating cytokines are in bold and cytokines produced by cell types are indicated in italics. The dashed arrow and dashed cell outlines indicate hypothetical pathways and precursors. AHR, aryl hydrocarbon receptor; DN, double negative for CD4 and natural cytotoxicity receptors (NCRs); GFI1, growth factor independent 1; LTi, lymphoid tissue-inducer; TCF1, T cell factor 1. Adapted from (Serafini et al., 2015).

Of interest, a group exploring aberrant transcriptional regulation in AML pathophysiology found that *Rora* is implicated in myeloid differentiation. Chang et al. (2008) used an integrated approach to construct a mammalian genetic network by combining the analysis of gene expression profiling data and the identification of TF binding sites (Chang et al., 2008). Using these approaches, they first constructed the genetic network underlying normal myeloid differentiation. In this network, multiple transcriptional regulatory cascades converge on *Rora*, indicating a novel function in modulating myeloid cell development (Chang et al., 2008). Thus *Rora* may represent a TF that is important in macrophage development.

1.15.6 *Rora* in metabolism

Rora can be detected in many metabolic tissues including liver, kidney, adipose tissue and is highly expressed in skeletal muscle (**Figure 1.10**)(Fitzsimmons et al., 2012). The ligand for *RORα* has been questioned for many years; recent X-ray crystallography analysis of *RORα* revealed that cholesterol and several derivatives potentially regulate the activity and function of *RORα*, Kallen and colleagues (2002) identified cholesterol in the ligand-binding domain for *RORα*. (Kallen et al., 2002) (**Figure 1.9A**).

In mouse skeletal muscle, *Rora* influences genes associated with lipid and carbohydrate metabolism, Liver X Receptor (LXR) nuclear receptor signalling and Akt and AMPK signalling, for example, *Akt2* (Raichur et al., 2010). *Rora*^{sg/sg} mice display hypoalphalipoproteinemia, dyslipidemia (decreased serum triglycerides and HDL-cholesterol), susceptibility to atherosclerosis (Mamontova et al., 1998), reduced adiposity and resistance to diet-induced obesity (Lau et al., 2008) and improved insulin susceptibility and glucose uptake (Lau et al., 2011).

1.16 Circadian rhythm and immunity

1.16.1 The circadian clock

Rora is a key component of the circadian clock and thus has a role in circadian rhythm (Sato et al., 2004). Organisms possess the ability to tell the time of day to anticipate and respond to changes in the external environment imposed by the light/dark cycle. Circadian rhythms are the daily changes or oscillations in physiology that occur in our bodies to accommodate the daily rhythm of activity, feeding and rest (Curtis et al., 2014). Circadian rhythms are due to numerous genes whose expression peak and trough approximately every 12 hours apart to undergo a full cycle within 24 hours. Humans and mice have a rest and an active phase over 24 hours. Mice in animal units are kept at 12:12 hour light/dark cycle. Mice are nocturnal and as such their active period is when the lights are off, the opposite is true for humans who have their active phase during light hours. A zeitgeber (ZT) is any external or environmental cue that entrains, or synchronises, an organism's circadian rhythm to a 24-hour cycle. In the animal unit the ZT is light, the time at which the light comes on is known as ZT0 and ZT12 is when the lights go off (**Figure 1.12**).

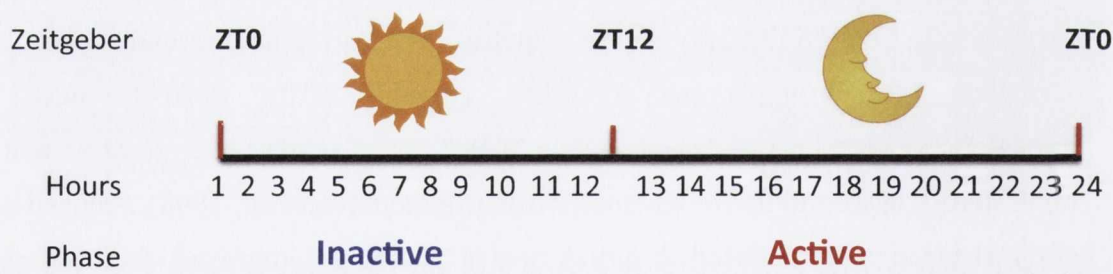


Figure 1.12 The light/dark cycle in the animal facility. There is a 12:12 light dark cycle. During the dark cycle the mice are active and during the light phase they are inactive. ZT- Zeitgeber.

The genes and processes involved in circadian rhythm are collectively known as the circadian clock. The central circadian clock is located within the suprachiasmatic nucleus in the brain; external light detected by the eye is transmitted to the central clock through the retinothalamic tract (Curtis et al., 2014) (**Figure 1.13A**). The components of the circadian clock are illustrated in **Figure 1.13B**. In its simplest form the clock is a

transcription-transcription oscillator loop. In the nucleus BMAL1 (also known as ARNTL) and CLOCK bind E-box sites in the promoter regions of repressors Period (PER) and Cryptochrome (CRY) (**Figure 1.13B**) (Curtis et al., 2014). Proteins PER and CRY leave for the cytoplasm but return to the nucleus to interfere with the BMAL1/CLOCK complex, inhibiting the function of BMAL1 and CLOCK. As PER and CRY are degraded the repression of the BMAL1/CLOCK complex is relieved, restoring their function and the cycle can begin again. Another loop of the clock involves transcription factors Rora (Sato et al., 2004) and Rev-erba. (Preitner et al., 2002) The BMAL1/CLOCK complex binds to the E-box on Rora and Rev-erba promoting their expression, RORa in turn promotes the expression of BMAL1 whereas REV-EVRBa represses BMAL1 expression. The third loop is comprised of D-box binding protein regulated through its E-box and the repressor nuclear factor interleukin 3 (NFIL3), these two factors then regulate the expression of D-box genes including *Per* (Curtis et al., 2014).

An important finding was that the circadian clock also exists in peripheral cells of the body (Reppert and Weaver, 2002). The circadian clock has been found in blood mononuclear cells (Boivin et al., 2003) including B cells (Silver et al., 2012), T cells (Bollinger et al., 2011) and macrophages (Hayashi et al., 2007; Keller et al., 2009). Keller et al. (2009) found that spleen, lymph nodes, and peritoneal macrophages of mice contain intrinsic circadian clockworks that operate autonomously even *ex vivo*. They found that these peripheral clocks regulate circadian responses to inflammatory stimuli; spleen cells stimulated with LPS at different circadian times display altered TNF α and IL-6 secretion and this process is not driven by systemic glucocorticoid variations. They also found that >8% of the macrophage transcriptome oscillates in a circadian fashion and when they analysed genes involved in the TLR4 pathway they found circadian control at every level.

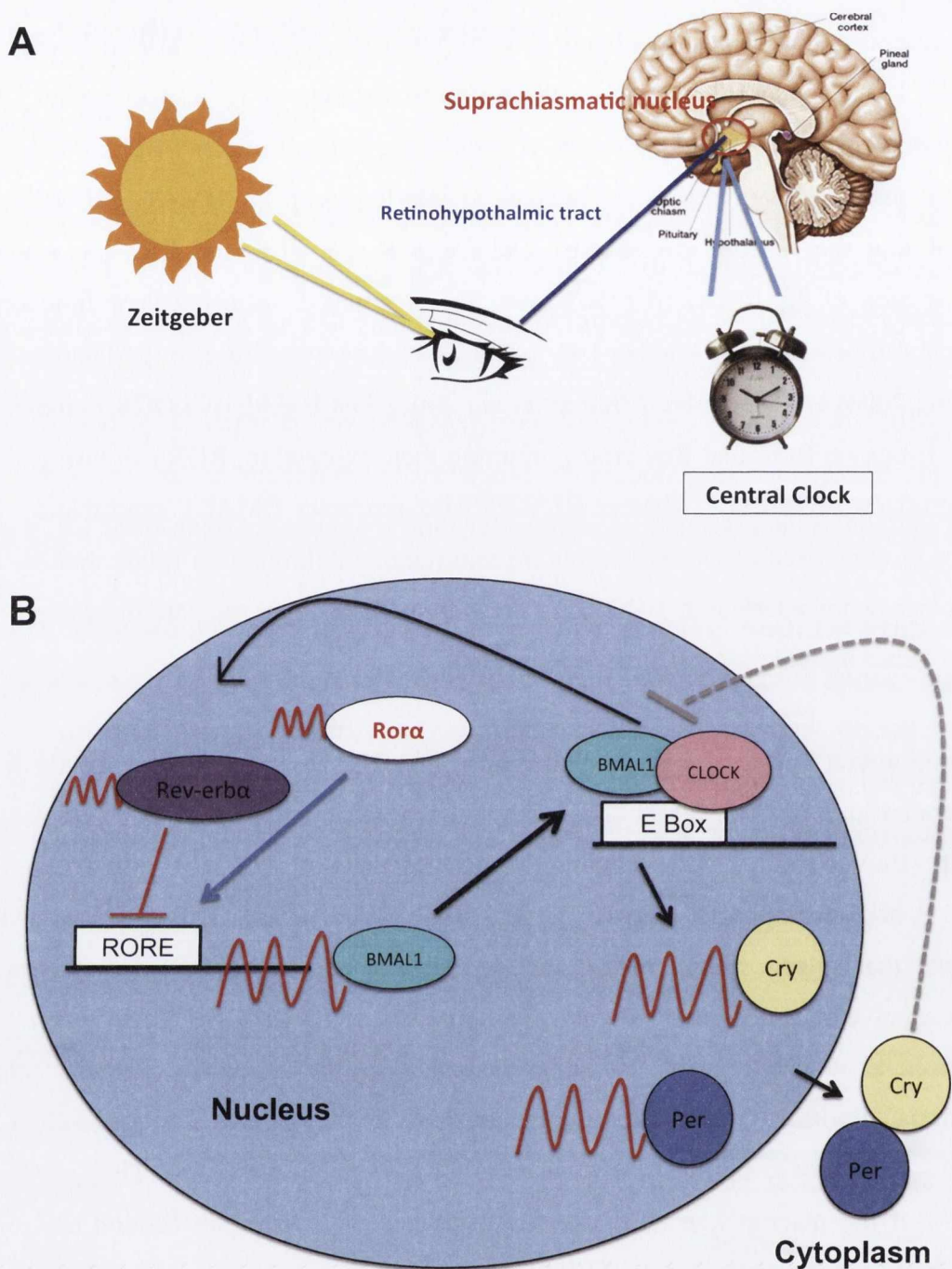


Figure 1.13 *Rorα* is an important component of the circadian clock. **A.** Light signal is sensed by the central clock in the suprachiasmatic nucleus located in the brain. **B** The molecular clock components. The clock consists of different negative feedback loops. *Rorα* is important in promoting *Bmal* expression.

1.16.2 Circadian rhythm and inflammation

Time dependent altered responses to bacterial component LPS were first observed in the 1960s (Halberg et al., 1960), this study showed enhanced lethality to LPS towards the end

of the resting phase. The reason for this difference is not fully understood but difference in cell number and cytokine responses over the 24-hour time-period could explain this observation. Scheiermann et al. (2012) found that leukocytes in the mouse circulation have circadian variability reaching the highest levels at ZT5 and are recruited to tissues at ZT13. The induction of pro-inflammatory cytokines is far greater when mice are challenged with LPS at ZT12 versus ZT0 (Gibbs et al., 2012). This includes a greater induction of IL-6, IL-12p40, CCL2, and CCL5 from macrophages challenged at ZT12 compared to ZT0 (Gibbs et al., 2012). Gibbs and colleagues highlighted a role for the specific clock proteins in the immune response. Disruption of the circadian clockwork in macrophages by conditional targeting of *Bmal1* removed all temporal gating of endotoxin-induced cytokine response in cultured cells and *in vivo*. Loss of circadian gating was coincident with suppressed *Rev-erba* expression, indeed circadian gating of endotoxin response was lost in *Rev-erba*^{-/-} mice and in cultured macrophages from *Rev-erba*^{-/-} mice, despite maintenance of circadian rhythmicity within these cells (Gibbs et al., 2012).

Interestingly chronic disruption of the molecular clock in mice via jet lag leads to enhanced LPS-induced sepsis. Endotoxemic shock induced by LPS was magnified, leading to hypothermia and death after four consecutive weekly 6 hour phase advances of the light/dark schedule, with 89% mortality compared with 21% in unshifted control mice (Castanon-Cervantes et al., 2010). Castanon-Cervantes and colleagues observed altered or abolished rhythms in the expression of clock genes *Per* and *Bmal1* in the central clock, liver, thymus, and peritoneal macrophages in mice after chronic jet lag. Peritoneal macrophages harvested from these mice produce a greater amount of IL-6 in response to LPS indicating that these cells are a target for jet lag (Castanon-Cervantes et al., 2010).

In terms of host clearance of infection Bellet et al. (2013) found that responses to intragastric infection with *Salmonella enterica* serovar typhimurium of WT mice at different circadian times results in different degrees of intestinal inflammation, bacterial colonisation change dependent on time of infection and of sacrifice. The authors found that the antimicrobial peptide lipocalin-2, to which *Salmonella* is resistant, is higher during the day than at night and this suppresses resident microbiota, allowing for the higher growth of *Salmonella* during the day compared to the night (Bellet et al., 2013). Chawla and colleagues focused on Ly6C^{High} blood monocyte responses to *Listeria monocytogenes* infection (Nguyen et al., 2013). The authors observed a diurnal variation in basal numbers of Ly6C^{High} blood monocytes in blood and spleen, and enhanced recruitment of these

monocytes to the inflamed peritoneum at ZT8 compared to ZT0. Improved bacterial clearance was also observed at ZT8 compared to ZT0 and may be due to higher recruitment of Ly6C^{High} blood monocytes and higher production of IL-1 β , IL-6, TNF α , IFN γ and CCL2. The authors also found that these diurnal responses were entirely dependent on BMAL1 expression in the myeloid cells (Nguyen et al., 2013).

Recently more insight has been gained into the molecular mechanism underlying differential temporal responses to LPS. Curtis et al. (2015) found that the clock in myeloid cells plays a role in LPS-induced sepsis by altering NF- κ B activity and the induction of the microRNA miR-155. It was observed that LPS causes the repression of BMAL1 via the targeting of miR-155. *Bmal1* has two miR-155-binding sites in its 3'-UTR, and, in response to LPS, miR-155 binds to these two target sites, leading to suppression of *Bmal1* mRNA and protein in mice and humans (Curtis et al., 2015). Therefore the lack of miR-155 has profound effects on circadian function and circadian induction of cytokines by LPS. Thus, the molecular clock is using miR-155 as an important regulatory component to control inflammation variably across the circadian day in myeloid cells (Curtis et al., 2015).

All of these results suggest that the immune system is partitioned into two states over 24 hours by the circadian system. The first state is that seen in the active phase, with greater immune cell numbers in the circulation and tissues along with increased sensitivity of immune cells to pathogens being observed. Some have termed this state as anticipatory inflammation (Ginhoux and Jung, 2014; Nguyen et al., 2013). The second state is seen when the animal is resting and the risk of injury or infection is lessened, this may be a time for rest and inflammatory resolution (Curtis et al., 2014).

1.17 Thesis objectives

Macrophages are remarkably versatile in their ability to recognise and respond to a wide range of stimuli, expressing a variety of surface and intracellular receptors, multiple signal transduction pathways and complex, adaptable arrays of gene expression. Macrophage subsets in tissue sites, such as the peritoneal cavity, are extremely heterogeneous in their phenotype and functions. These peritoneal macrophage subsets can also be of dual origin: monocyte-derived or tissue-resident embryo-derived. Transcription factors are key to every step of the generation of both monocyte-derived and tissue-resident embryo-derived macrophages. The transcription factor *Rora* is known to have a role in immunity and the development of immune cells such as ILC2s and Th17 cells. Thus, the first objective in this thesis was to explore if *Rora* has a role in the regulation of macrophage development, in particular in the development of monocyte-derived macrophages or tissue-resident macrophages in the peritoneal cavity. *Rora* is also known to have a role in inflammation. As macrophages are one of the primary responders in inflammation the next objective in this thesis was to explore the role *Rora* has in inflammation in the context of macrophages.

Rora is a molecular component of the circadian clock. There has recently been increased interest in the concept of ‘anticipatory inflammation’. This is where circadian oscillations can potentially regulate inflammatory responses by controlling rhythmic trafficking of inflammatory monocytes and other immune cells. Thus the third objective of this thesis was to determine if *Rora* contributes to the regulation of circadian oscillations of macrophages. And furthermore, determine if *Rora* could also potentially contribute to inflammation responses through its role in regulating circadian oscillations of macrophages.

The final objective in this thesis was to generate mice in which *Rora* was conditionally deleted in myeloid cells, using the Cre-Lox recombination technology, and use these mice to further determine the role *Rora* has in macrophage development.

Overall objectives:

- Determine if the transcription factor *Rora* has a role in macrophage development.
- Analyse the role of *Rora* in inflammatory responses to LPS in the context of macrophages.
- Explore if *Rora* has a role in macrophage circadian rhythm and temporal responses to inflammatory stimuli.
- Develop conditional deleter mouse models using Cre-Lox recombination technology to explore the role *Rora* has in myeloid cells.

Chapter 2

Materials and Methods

2.1 Mice

All mice used in the thesis were on a C57BL/6J strain background. Wild-type (WT) C57BL/6J strain mice were from Jackson Laboratories (JAX) and supplied by Charles River Laboratories, UK. Mutant and transgenic mice used include: *B6.C3(Cg)-Rora^{sg/J}* mice (JAX Strain Number: 002651) (Hamilton et al., 1996), *Rora^{fl/fl}* mice (Taconic/Lexicon Pharmaceuticals NY, US), *FVB/N-Tg(ACTB-cre)2Mrt/J* mice (JAX Strain Number: 003376; kindly provided by Dr Adrien Kissenpfennig, Queens University Belfast, UK) (Lewandowski et al., 1997), *B6.129P2-Lyz2^{tm1(cre)Ifo}/J* mice (JAX Strain Number: 0047811; kindly provided by Dr Adrien Kissenpfennig, Queens University Belfast, UK) (Clausen et al., 1999), *Tlr4^{-/-}* mice (Hoshino et al., 1999) were kindly provided by Shizuo Akira, *B6.129P(Cg)-Ptprc^aCx3cr1^{tm1/litt}/J* mice (JAX Strain Number: 008451) (Jung et al., 2000) and *B6.SJL-Ptprc^aPepc^b/BoyJ* mice (JAX Strain Number: 002014) (Shen et al., 1985).

All mice were bred in a Specific Pathogen-Free (SPF) facility. Mice were kept in individually ventilated cages (IVC) under positive pressure at temperatures between 18°C-23°C and humidity between 40%-70%. The mice were kept under a 12:12 hour light/dark cycle. Lights turned on at 7am and turned off at 7pm. When studies on mice were undertaken during the dark phase of the 12:12 light/dark cycle a red light (Paterson, Tipton, UK) was used. Mice were fed an irradiated diet (Harlan, UK) with water and food supplied *ad libitum*. The diet of *Rora^{sg/sg}* mice was supplemented with Nutra-Gel diet (Bio-Serv, ML, US). All animal experiments were performed in compliance with the Health Products Regulatory Authority (HPRA) and approved by the Trinity College Dublin's BioResources ethical review board.

2.1.1 B6.C3(Cg)-Rora^{sg}/J mice

B6.C3(Cg)-Rora^{sg}/J mice contain a naturally occurring mutation in *Rora* (Hamilton et al., 1996) (**Section 1.15.3**). Mice homozygous for the spontaneous staggerer (sg) (*Rora*^{sg/sg} mice) mutation phenotypically display a staggering gait, mild tremor, hypotonia, small size and a short lifespan of approximately 4 weeks. The *Rora* gene in *Rora*^{sg/sg} mice contains a frameshift at amino-acid 273 in the $\alpha 1$ isoform leading to a truncation 27 residues later. The sequence deleted corresponds to a 122 base pair (bp) exon removed by a 6.5 kilo base (kb) genomic deletion encoding part of the ligand binding domain (Hamilton et al., 1996). The primers used for genotyping of the *Rora*^{sg/sg} mutation are outlined in Table 2.1. Details of the polymerase chain reaction (PCR) cycle conditions used in the genotyping protocol are detailed in Appendix I. All PCR primers used herein were purchased from Sigma (UK). A representative agarose gel displaying products of a PCR demonstrating the WT (+/+), Heterozygous (Het; +/-) and homozygous (Hom; -/-) *Rora* mutant mice are shown in **Figure 2.1**. A 318-bp band is amplified in WT mice, a 318-bp band and 450-bp band is amplified in Het mice and a 450-bp band is amplified in Hom mice (**Figure 2.1**).

Primer	Primer Sequence	Band (bp)
1233	TCTCCCTTCTCAGTCCTGACA	WT: 318
1234	TATATTCCACCACACGGCAA	
1235	GATTGAAAGCTGACTCGTTCC	Mutant: 450
1236	CGTTTGGCAAACCTCCACC	

Table 2.1 Primer sequences and predicted size of PCR product used in genotyping *Rora*^{sg/sg} mice. bp- base-pair

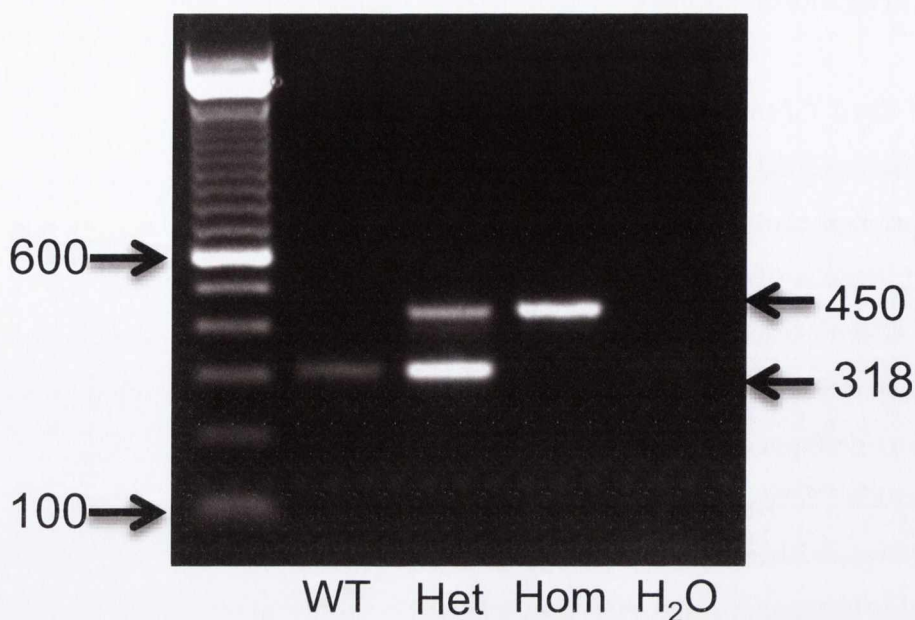
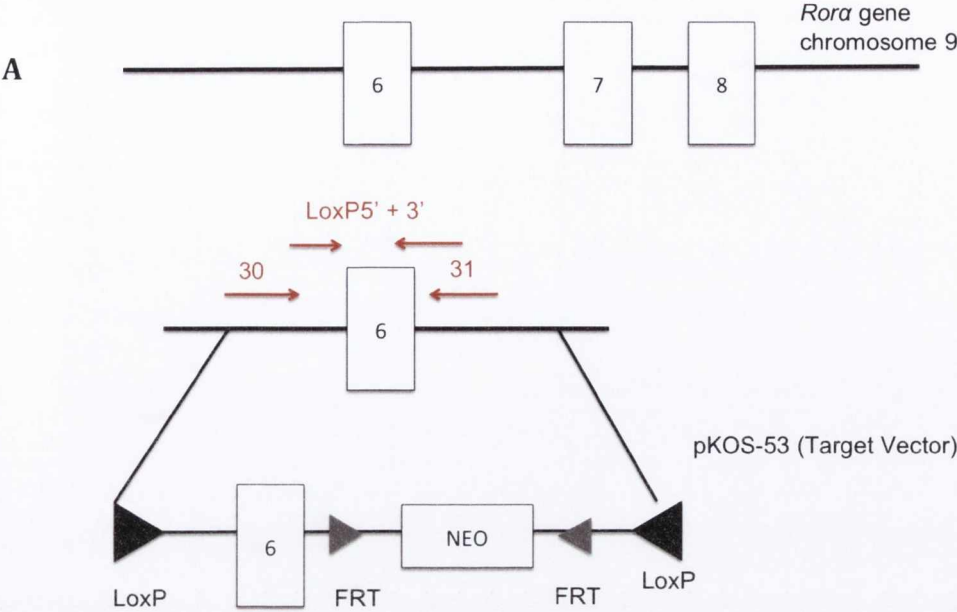


Figure 2.1 Agarose gel with PCR products for genotyping of *Rora*^{sg/sg} mice. 1.5% agarose gel used. PCR products from WT (+/+), Het (+/-) and Hom (-/-) mice are shown. WT band: 318-bp, Mutant band: 450-bp, H₂O: PCR reaction mixture minus gDNA template.

2.1.2 *Rora*^{fl/fl} mice

Conditional *Rora*^{fl/fl} mice were generated by Taconic. A target vector (pKOS-53) was inserted into Exon 6 of *Rora* on chromosome 9 (**Figure 2.2A**). The target vector contains a genomic sequence flanked either side by Locus of X-over of P1 (LoxP) sites. LoxP sites are specific 34-bp sequences consisting of an 8-bp core sequence where recombination takes places and two flanking 13-bp inverted repeats (**Figure 2.2B**). Recombination of these LoxP sites occurs when the Cyclisation Recombination (Cre) recombinase enzyme is present. Recombination results in the excision of the floxed genomic sequence (**Figure 2.2C**). The target vector also contains a pLF neomycin (pLFneo) selection cassette used during transgenic mouse development.



B ATAACTTCGTATAGCATACATTATACGAAGTTAT

C GGTGGATGACACCGGCAGAAGAGTATTATTGGCTCCTAAAATAACGGG
 CTGTTTCATGGTCGTGATCTCCTGTTTTCTCCCCAGAACACCTTGCCCAGA
 ACATATCCAAATCCCACCTGGAAACCTGCCAGTACTTGCGGGAAGAGCT
 CCAGCAGATAACGTGGCAGACCTTCCTGCAGGAGGAGATTGAAAATA
 CCAGAACAAGGTAGAGTTGGAGGACTTTCTCTTCCCACCGAGGCAACAT
 TGGGTGGGGGTGGGAGAAGGGCATTCAAGAGTCCAGACAGAC.

Figure 2.2 Targeting strategy in *Rora*^{fl/fl} mice. **A** Target vector in *Rora*^{fl/fl} mice. Primers (in red) are specific for sequences within Exon 6. **B** The 34-base LoxP sequence containing an 8-bp core sequence (underlined), where recombination takes place, and two flanking 13-bp inverted repeats. **C** Genomic sequence in *Rora* flanked by LoxP sites. Neo: Neomycin, FRT: FLP recognition target.

Two PCR reactions are required for genotyping of *Rora*^{fl/fl} mice. Table 2.2 indicates the primers used in each reaction and the predicted size of the PCR products. Details of the cycle conditions used in the genotyping protocol are detailed in Appendix I. Detailed information on the genetic location of the complimentary sequences for primers used in both PCR reactions within *Rora* can be found in Appendix II. LoxP5' and LoxP3' primers are used to differentiate between WT mice and *Rora*^{fl/fl} mice. The LoxP5' and LoxP3'

primers are complimentary to the same sequences found in the *Rora* gene of WT mice and *Rora*^{fl/fl} mice. In *Rora*^{fl/fl} mice the LoxP5' primer is complimentary to a sequence located outside the floxed region of *Rora* and the Loxp3 primer is complimentary to a sequence located inside the floxed region of *Rora* (Appendix II). The predicted PCR product in *Rora*^{fl/fl} mice is larger (394-bp) than the predicted PCR product in WT mice (323-bp) due to the presence of extra nucleotide sequences associated with the LoxP sites in *Rora*^{fl/fl} mice.

The 30 and 31 primers are used to indicate if the floxed gene sequence in *Rora* has been excised by Cre recombination. The 30 and 31 primers are complimentary to the same sequences found in the *Rora* gene of WT mice and *Rora*^{fl/fl} mice (Appendix II). The 30 and 31 primers are complimentary to sequences located before and after the floxed region in *Rora*^{fl/fl} mice. When recombination occurs the sequence detailed in **Figure 2.2C** is deleted in *Rora* in *Rora*^{fl/fl} and the nucleotide sequence between the 30 and 31 primers sites shortens thus leading to a smaller predicted PCR product (408-bp) in *Rora*^{fl/fl} mice in which the sequence is excised compared to the predicted PCR product (596-bp) in WT mice and in *Rora*^{fl/fl} mice in which the sequence is not excised. Representative agarose gels of products from both PCR reactions are visualised in **Figure 2.3**. Bands were visualised as per **Section 2.2**.

Primer	Primer Sequence	Band (bp)
LoxP5'	TCTGAATCCACCATACTTCC	WT- 323
LoxP3'	AGGTCTGCCACGTTATCTG	Targeted- 394
30	CAAGTCTCTGAATCCACCATAC	WT- 596
31	CAACTGGCACAAAGGAAGGAC	Cre-excised- 408

Table 2.2 Primer sequences and predicted size of PCR product used in genotyping *Rora*^{fl/fl} mice. bp- base-pair

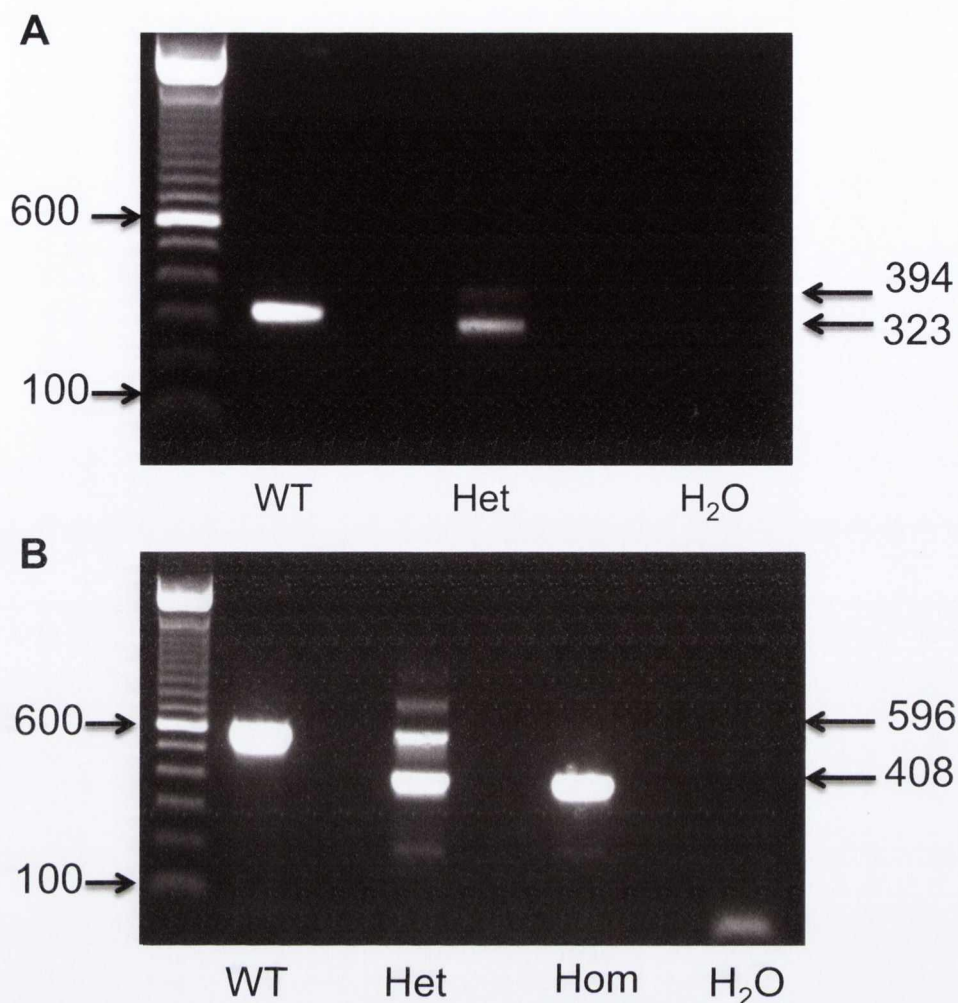


Figure 2.3 Agarose gel with PCR products for genotyping *Rora*^{fl/fl} mice. 1.5% agarose gel used. **A** Genotyping for LoxP sites. WT band: 323-bp, Mutant band: 394-bp H₂O: reaction minus gDNA. **B** Genotyping for LoxP site excision. WT band: 596-bp, Mutant band: 408, H₂O: Reaction minus gDNA.

Custom primers were designed that were complimentary to the floxed sequence in *Rora* in *Rora*^{fl/fl} mice (**Figure 2.2.C**). Table 2.3 indicates the primers used in each reaction and the predicted size of the PCR products. Details of the cycle conditions used in the genotyping protocol are detailed in Appendix I.

Primer	Primer Sequence	DNA Band (bp)
1	GTGGATGACACCGGCAGAA	Floxed-127
2	GCAGGTTTCCAGGTGGGATT	Excised- No band

Table 2.3 Primer sequences complimentary to the floxed sequence in *Rora* in *Rora*^{fl/fl} mice. bp- base-pair.

2.1.3 FVB/N-Tg(ACTB-cre)2Mrt/J mice

In *FVB/N-Tg(ACTB-cre)2Mrt/J (ACTB-cre)* mice the Cre recombinase enzyme is expressed under the control of the human beta-actin gene promoter (Lewandoski, 1997). Beta-actin is ubiquitously expressed in cells and Cre recombinase activity is detected in all cells of the embryo by the blastocyst stage of development (Bunnell, 2011). The Cre recombinase enzyme is a DNA recombinase enzyme that is both necessary and sufficient for sequence-specific recombination in bacteriophage P1. It belongs to the integrase family of site-specific recombinases. It is a 38 kDa protein that catalyses the recombination between two of the LoxP sites (Lakso et al., 1992). The primers used in the standard PCR genotyping protocol do not distinguish between Het and Hom mice (Table 2.4).

Primer	Primer Sequence	DNA Band (bp)
1084	GCGGTCTGGCAGTAAAACTATC	Internal +ve-100
1085	GTGAAACAGCATTGCTGTCACTT	
7338	CTAGGCCACAGAATTGAAAGATCT	Transgene- 324
7339	GTAGGTGGAGGAAATTCTAGCATCATCC	

Table 2.4 Primer sequences and predicted size of PCR product used in genotyping *ACTB-cre* mice. bp-base pair.

2.1.4 B6.129P2-Lyz2^{ml(cre)lfo}/J mice

In *B6.129P2-Lyz2^{ml(cre)lfo}/J (LysMCre)* mice the Cre recombinase is inserted into the lysozyme 2 (Lyz2) gene, both abolishing endogenous Lyz2 gene function and placing NLS-Cre expression under the control of the endogenous Lyz2 promoter/enhancer

elements (Clausen et al., 1999). *Lyz2* is expressed in cells of the myeloid system including monocytes and macrophages (Cross et al., 1988). They have been shown to have a deletion efficiency of 83-98% in mature macrophages (Clausen, 1999). The standard PCR genotyping protocol can distinguish between Het and Hom mice (Table 2.5).

Primer	Primer Sequence	DNA Band (bp)
Lys1	CCCAGAAATGCCAGATTACG	WT- 350
Lys2	CTTGGGCTGCCAGAATTTCTC	
Lys1	CCCAGAAATGCCAGATTACG	Mutant- ~ 700
Cre8	TTACAGTCGGCCAGGCTGAC	

Table 2.5 Primer sequences and predicted size of PCR product used in genotyping *LysMCre* mice. bp- base-pair.

2.1.5 *Rora*^{fl/fl} *ACTB-cre* mice

Using the Cre-Lox system (**Section 1.18**) *Rora* was ubiquitously deleted in beta-actin expressing mouse cells. To achieve this *Rora*^{fl/fl} mice (**Section 2.1.2**) were crossed to *ACTB-cre* mice (**Section 2.1.3**) to generate *Rora*^{fl/fl} *ACTB-cre* mice (**Figure 2.4**).

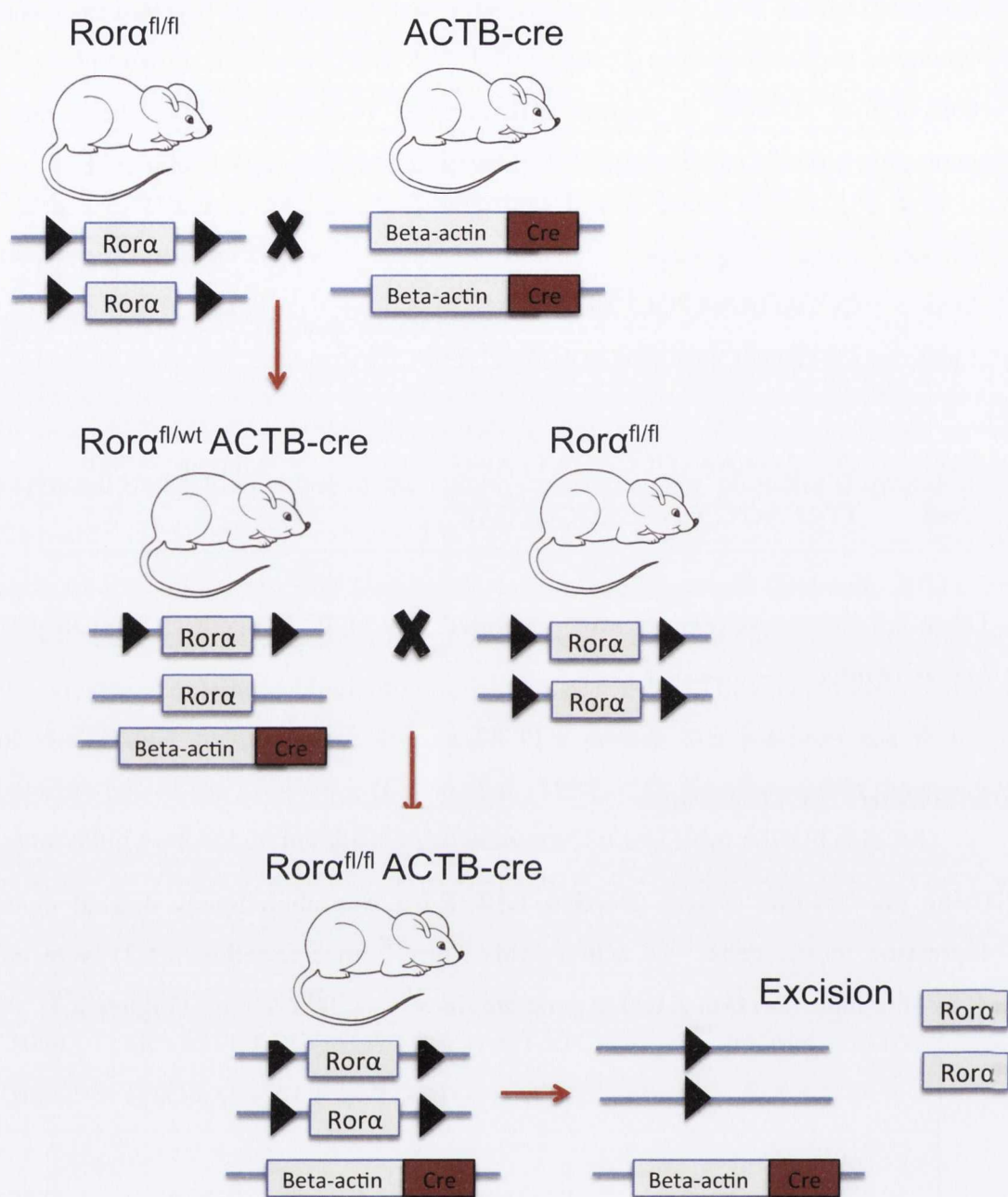


Figure 2.4 Generation of *Rora*^{fl/fl} *ACTB-cre* mice. *Rora*^{fl/fl} mice were crossed to *ACTB-cre* mice. This cross generated a *Rora*^{wt/fl} *ACTB-cre* mouse. The *Rora*^{wt/fl} *ACTB-cre* mouse was then crossed to another *Rora*^{wt/fl} *ACTB-cre* mouse. This cross generated a *Rora*^{fl/fl} *ACTB-cre* mouse. The presence of the Cre enzyme under the control of the human beta-actin promoter results in the excision of the genomic sequence in *Rora* flanked by LoxP sites.

2.1.6 *Rora*^{fl/sg} *ACTB-cre* mice

Rora^{fl/sg} *ACTB-cre* mice were generated by crossing *Rora*^{fl/wt} *ACTB-cre* mice (Section 2.1.5) to *Rora*^{sg/wt} mice (Figure 2.5). This cross generated a *Rora*^{fl/sg} *ACTB-cre* mouse. In this mouse one *Rora* allele contains a germ line mutation. The other *Rora* allele contains a floxed sequence that is excised in the presence of Cre recombinase. Cre recombinase is expressed under the control of the human beta actin promoter.

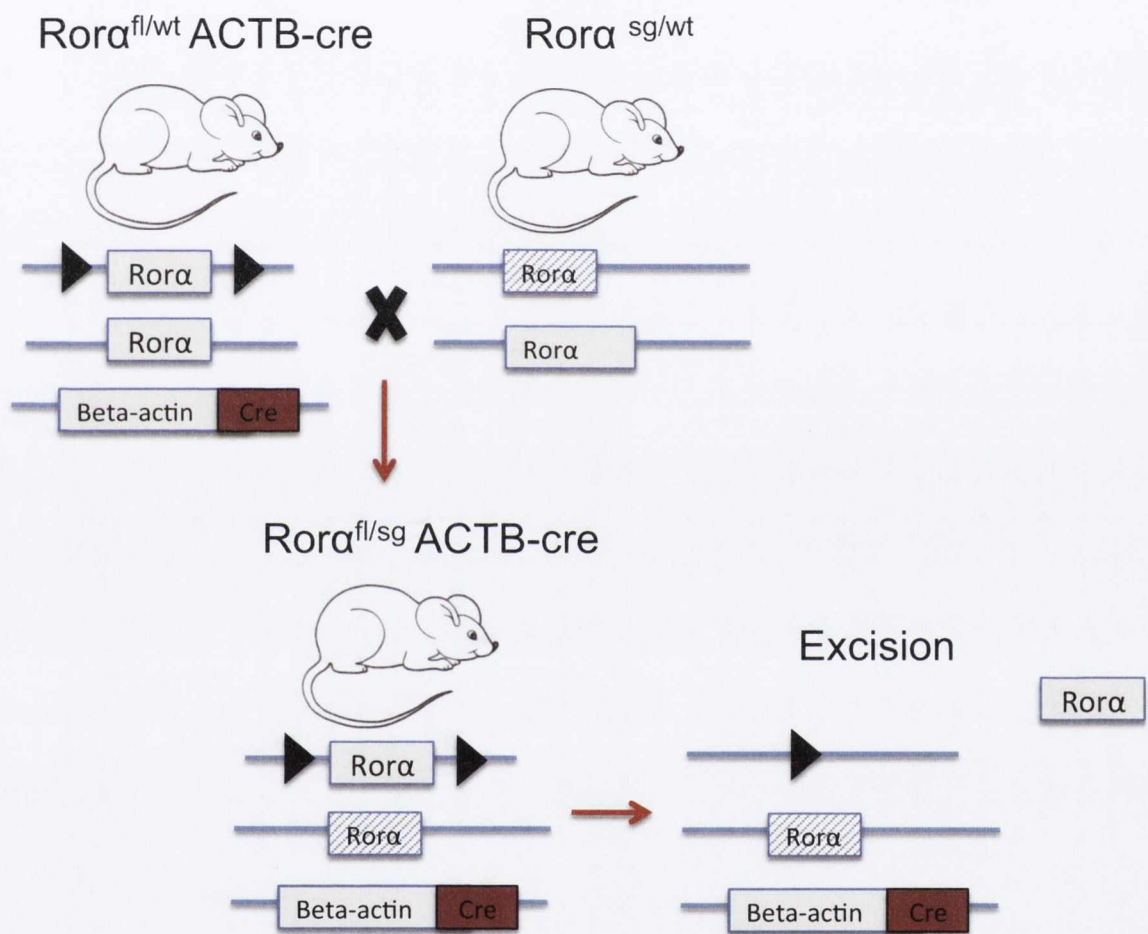


Figure 2.5 Generation of *Rora*^{fl/sg} *ACTB-cre* mice. *Rora*^{sg/wt} mice contain one mutated *Rora* allele (shaded). On the other allele the floxed *Rora* sequence is excised in beta-actin expressing cells.

2.1.7 *Rora*^{fl/fl} *LysMCre* Het mice

Using the Cre-Lox system (Section 1.18) *Rora* was conditionally deleted in lysozyme 2 expressing mouse cells including monocytes and macrophages. To achieve this a *Rora*^{fl/fl}

mouse (Section 2.1.2) was crossed to a *LysMCre Het* mouse (Section 2.1.4) to generate a *Rora^{fl/fl} LysMCre Het* mice (Figure 2.6).

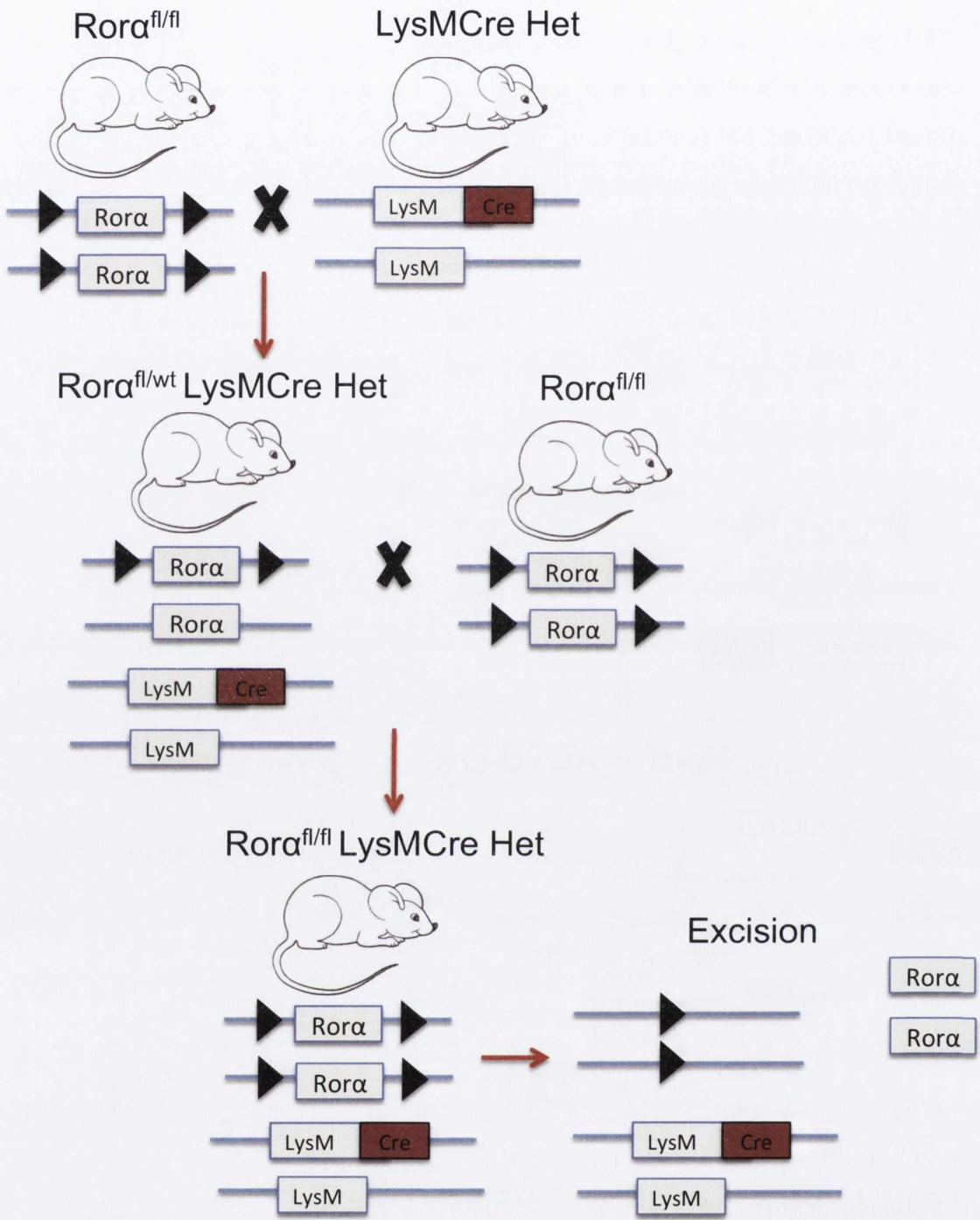


Figure 2.6 Generation of *Rora^{fl/fl} LysMCre Het* mice. A *Rora^{fl/fl}* mouse was crossed to a *LysMCre Het* mouse. This cross generated a *Rora^{wt/fl} LysMCre Het* mouse. This mouse was then crossed to a *Rora^{fl/fl}* mouse. This cross generated a *Rora^{fl/fl} LysMCre Het* mouse, in which *Rora* has been deleted in *Lys2* expressing myeloid cells including monocytes and macrophages.

2.1.8 *Rora*^{fl/sg} *LysMCre* Het mice

Rora^{fl/sg} *LysMCre* Het mice were generated by crossing *Rora*^{fl/fl} *LysMCre* Het mice (Section 2.1.6) to *Rora*^{sg/wt} mice (Figure 2.7). This cross generated a *Rora*^{fl/sg} *LysMCre* Het mouse. In this mouse one *Rora* allele contains a germ line mutation. The other *Rora* allele contains a floxed sequence that is excised in the presence of Cre. Cre is expressed under the control of the *Lys2* promoter; therefore the floxed sequence in *Rora* is excised in myeloid cells.

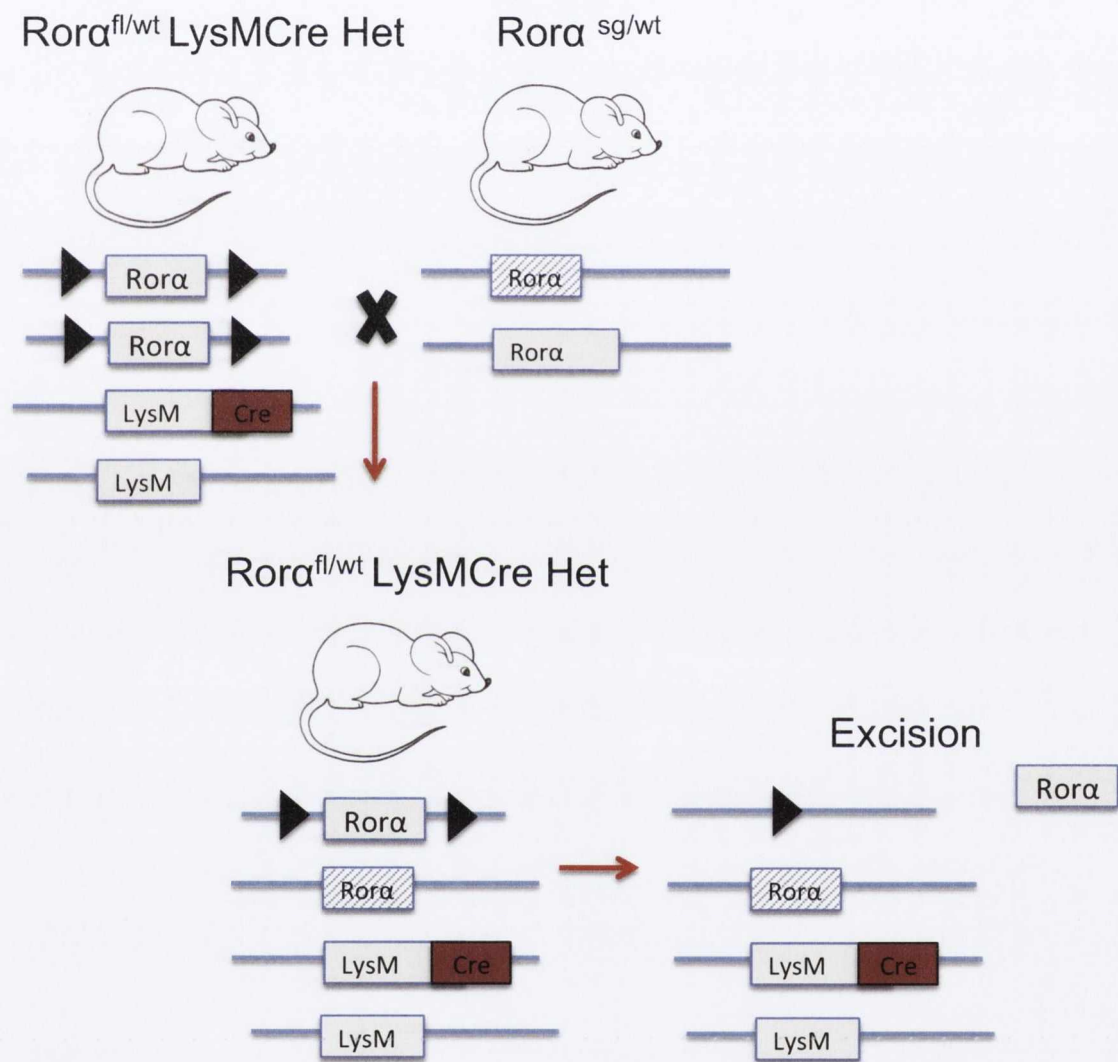


Figure 2.7 Generation of *Rora*^{fl/sg} *LysMCre* mice. *Rora*^{sg/wt} mice contain one mutated *Rora* allele (shaded). On the other allele the floxed *Rora* sequence is excised in *Lys2* expressing cells.

2.1.9 B6.129P(Cg)-*Ptprc*^a *Cx3cr1*^{tm/litt/J} mice (*CX₃CR1*^{gfp/+})

In these mice Enhanced Green Fluorescent Protein (eGFP) is under control of the endogenous *Cx3cr1* locus (Jung et al., 2000). eGFP, but not the endogenous gene, is expressed in monocytes, dendritic cells, NK cells, and brain microglia, mimicking endogenous gene expression. eGFP is derived from the jellyfish *Aequorea victoria*, is a versatile reporter molecule that has found use in many biological applications. When utilised in a transgenic construct, tissue expressing sufficient amounts of eGFP will fluoresce when exposed to a 488 nm light source. eGFP is detected in both Het and Hom mice.

2.1.10 *Rora*^{sg/sg} *CX₃CR1*^{gfp/+} mice

To generate *Rora*^{sg/sg} *CX₃CR1*^{gfp/+} mice *Rora*^{sg/wt} mice were crossed to *CX₃CR1*^{gfp/gfp} mice to generate *Rora*^{sg/wt} *CX₃CR1*^{gfp/+} mice. *Rora*^{sg/wt} *CX₃CR1*^{gfp/+} mice were then crossed to *Rora*^{sg/wt} mice to generate *Rora*^{sg/sg} *CX₃CR1*^{gfp/+} mice.

2.1.11 *Tlr4*^{-/-} mice

Tlr4^{-/-} mice were originally generated by Hoshino *et al.* (1999). The murine TLR4 genomic clone was screened from the 129/SvJ mouse genomic library (Stratagene, La Jolla, CA). A targeting vector was designed to replace a 2.54-kbp genomic fragment with neomycin resistance gene (*neo*) from pMC1-neo-poly(A) (Stratagene). A herpes simplex virus-thymidine kinase cassette (*HSV-TK*) was inserted into the 3' end of the vector. The resultant targeting vector was electroporated into E14.1 ES cells (Hoshino et al., 1999).

2.1.12 B6.SJL-*Ptprc*^a *Pepc*^{b/} *BoyJ* mice

B6.SJL-*Ptprc*^a *Pepc*^{b/} *BoyJ* mice carry the differential *Ptprc*^a pan leukocyte marker commonly known as CD45.1 or Ly5.1. Wild-type C57BL/6 inbred mice express the *Ptprc*^b (CD45.2 or Ly5.2) allele. The *Ptprc*^a allele and closely linked *Pepc*^b allele were serially backcrossed from SJL/J onto C57BL/6 with selection at each generation for *Ptprc*^a by serotyping (Shen et al., 1985).

2.2 Agarose gel electrophoresis

A 1.5% agarose gel was prepared by mixing 1.5 g of agarose (Sigma, UK) in Tris-borate ethylenediaminetetraacetic acid (TBE) (Appendix III), followed by boiling the mixture for 2 minutes to dissolve the agarose. Following cooling, 1.5 µl of ethidium bromide (EtBr) was added to visualise bands under UV light. The gel was then placed in a gel rig (Bio-Rad) containing 1X TBE buffer (Appendix III). DNA ladder (size: 100 bp-1500 bp; Invitrogen, UK) and 7 µl of the sample were run on the gel for 45 minutes at 100 volts (v) using a PowerPac Universal Power Supply (Bio-Rad). DNA bands were visualised under UV light using the Fusion FX (Vilber Lourmat, Germany).

2.3 Mouse tissue and cell isolation and culture

2.3.1 Isolation of peritoneal macrophages

Peritoneal macrophages were isolated from mice by lavage as described (Hams et al., 2011). The peritoneal cavity was lavaged with 5 ml ice cold 1X PBS (Gibco Invitrogen). The resulting peritoneal cells were washed in Roswell Park Memorial Institute (RPMI)-1640 (Invitrogen Life Technologies, US) and resuspended in RPMI-1640. Peritoneal cells at a density of 1×10^6 cells/ml were incubated at 37°C and 5% CO₂ in RPMI-1640 media for 2 hours in T25 flasks (Falcon, Corning, NY, US) and any non-adherent cells were removed by washing with 1X PBS.

2.3.2 Isolation and culture of bone marrow-derived macrophages (BMDMs)

Bone marrow cells from mice were isolated by standard protocol as described (Aviello et al., 2014). Mouse femurs were obtained by removing the skin, followed by carefully cutting into the pelvis and finally removing the foot attached to the tibia. Muscle and fat were removed from the bone using a scissors and tweezers. The bones were flushed with media using a 27 gauge needle and a 1 ml syringe. Single cell suspensions were prepared by passing bone marrow cells through a 70 µm cell strainer (Falcon, Corning), erythrocytes were removed by lysis (1X Pharm Lyse Buffer; BD) (2 ml, 7 minutes), washed and resuspended in culture media (Appendix III). Bone marrow cells were incubated in T25 flasks at a density of 1×10^6 cells / ml, at 37°C and 5% CO₂ for 7 days with either 10 ng/ml Macrophage Colony Stimulating Factor (M-CSF; R&D Systems, MN, US) or 1 ng/ml Granulocyte Macrophage Colony Stimulating Factor (GM-CSF; Miltenyi Biotec,

UK). Stimulating factor was added to cell cultures on days 0, 3 and 5. Culture media was replenished on days 3 and 5.

2.3.3 Blood and serum collection

Blood was collected by cheek bleeding. For cheek bleeding the cheek was pierced with a 4 mm lancet (Braintree, MA, US) and collected into either a Vacuette EDTA-coated blood collection tube (VWR, US) for analysis by flow cytometry or a 1.5 ml eppendorf for serum collection. Mice were terminated by cervical dislocation. For cell isolation, blood was lysed in 10 ml lysis buffer (1X Pharm Lyse) for 10 minutes, washed in RPMI-1640 media and resuspended in RPMI-1640 media. For serum collection eppendorf tubes containing blood were allowed to clot at room temperature before being placed at 4°C for 1 hour. Eppendorf tubes were then centrifuged at 2,000 g for 10 minutes, the serum was then collected and stored at -20°C.

2.3.4 Brain and liver tissue isolation

Unperfused brain and liver tissue was aseptically removed from a euthanised mouse. Tissue was snap frozen in liquid Nitrogen.

2.3.5 Spleen isolation and single cell suspension

The whole spleen was aseptically removed from a euthanised mouse. Single cell suspensions were prepared from the spleen by passing through a 70 µm cell strainer (Falcon, Corning). Spleen cells were washed in RPMI-1640 media, lysed in 2 ml lysis buffer (1X Pharm Lyse) for 5 minutes, washed in 10 ml RPMI-1640 media and resuspended in RPMI-1640 media (Invitrogen).

2.4 Polarisation of BMDMs towards M1 and M2 phenotype

Bone marrow cells were cultured as outlined in **Section 2.3.2**. BMDMs were polarised towards an M1 or M2 phenotype as described (Hams et al., 2011). On day 7 media was removed from cultures and cells were washed 3 X with RPMI-1640 media. Cells were cultured in RPMI-1640 media with ultra-pure LPS (100 ng/ml; InvivoGen, CA, US) + IFN γ (10 ng/ml; R&D) for 24 hours at 37°C and 5% CO $_2$ to polarise towards an M1

phenotype; and cultured with IL-4 (10 ng/ml; R&D) + IL-13 (10 ng/ml; R&D) to polarise towards an M2 phenotype.

Studies have shown that BMDMs cultured in GM-CSF (GM-CSF BMDMs) have characteristics of M1 macrophages and BMDMs cultured in M-CSF (M-CSF BMDMs) have characteristics of M2 macrophages (Fleetwood et al., 2009) (**Section 1.6.5**). To test this here cells were cultured as in **Section 2.3.2**. BMDMs were polarised towards an M1 or M2 phenotype as described above.

Protein markers associated with M1 macrophages include iNos, programmed death ligand-1 (PD-L1) and MHCII (**Section 1.6.3**). Increased *iNos* gene transcript expression was also used as a marker for M1 polarisation. PD-L1 is a ligand for PD-1, a costimulatory molecule that plays an inhibitory role in regulating T cell activation. Studies have shown that PD-L1 is highly expressed on inflammatory macrophages both *in vivo* and *in vitro* (Loke and Allison, 2003; Parsa et al., 2012). Increased expression of these proteins indicated that BMDMs were polarised towards an M1 phenotype. Protein markers associated with M2 macrophages include Relm α , CD206 and PD-L2. Increased expression of these proteins indicated that BMDMs were polarised towards an M2 phenotype. Increased *Arg-1* gene transcript expression was used as a marker for M2 polarisation (**Section 1.6.3**).

In agreement with previous studies, it was confirmed that GM-CSF generated BMDMs had increased expression of protein markers associated with M1 macrophages compared to M-CSF generated BMDMs (**Figure 2.8A**). Conversely, M-CSF BMDMs displayed increased expression protein markers associated with M2 macrophages compared to GM-CSF BMDMs (**Figure 2.8B**). GM-CSF BMDMs also displayed higher gene expression of M1 macrophage marker *iNos* and M-CSF BMDMs displayed higher gene expression of M2 macrophage marker *Arg-1* (**Figure 2.9**). Relative expression shown was based on comparison to unstimulated BMDMs.

In line with convention M-CSF was chosen as the growth factor used in culturing BMDMs in this thesis. It was acknowledged in analysis that BMDMs would have characteristics of M2 macrophages

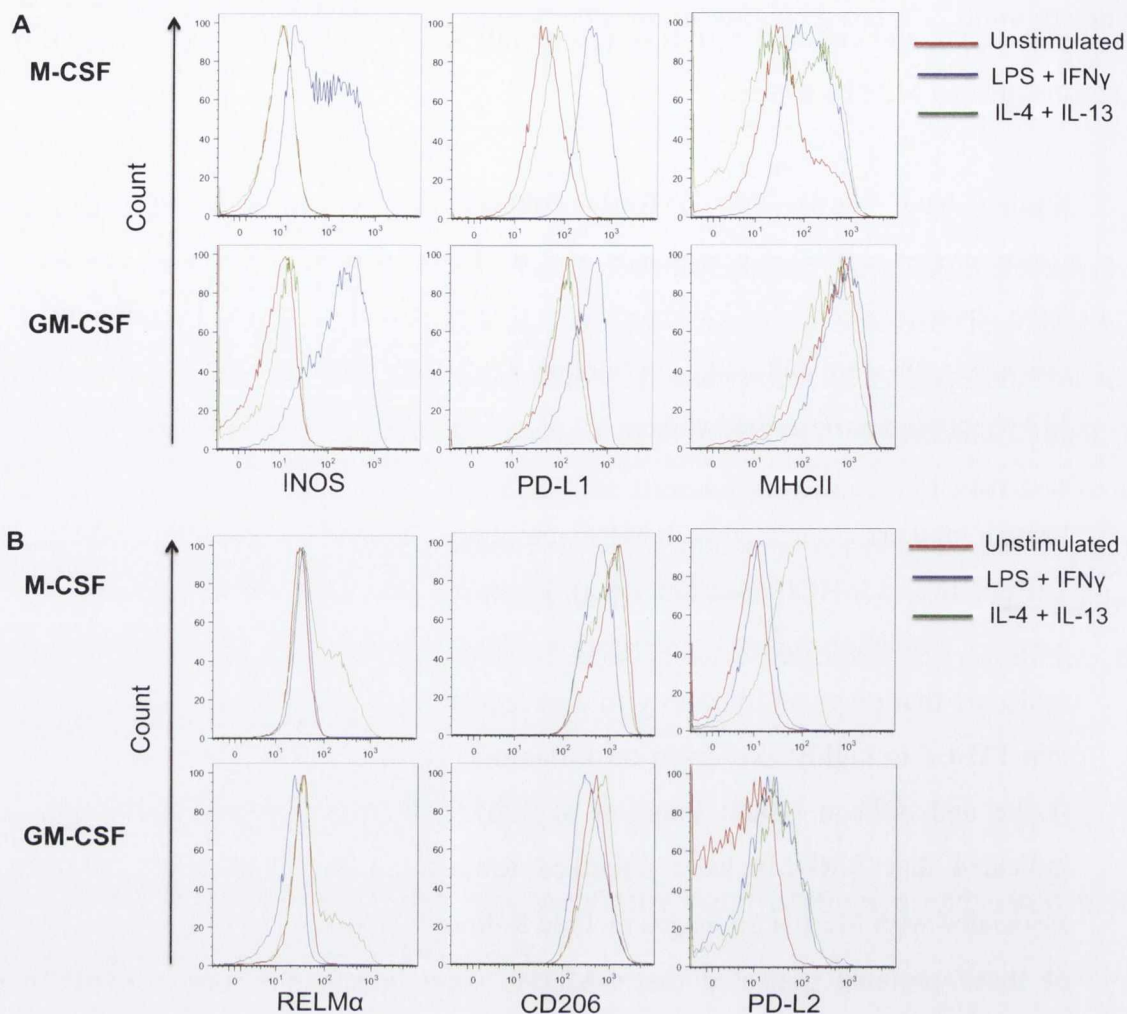


Figure 2.8 Protein expression on polarised BMDMs cultured in M-CSF or GM-CSF. Polarised BMDMs were cultured in either M-CSF or GM-CSF. **A** Representative flow cytometry plots of expression of M1 markers (iNos, PD-L1 and MHCII) (pre-gate: CD11b⁺, F4/80⁺). **B** Representative flow cytometry plots of expression of M2 markers (Relma, CD206 and PD-L2) (pre-gate: CD11b⁺, F4/80⁺).

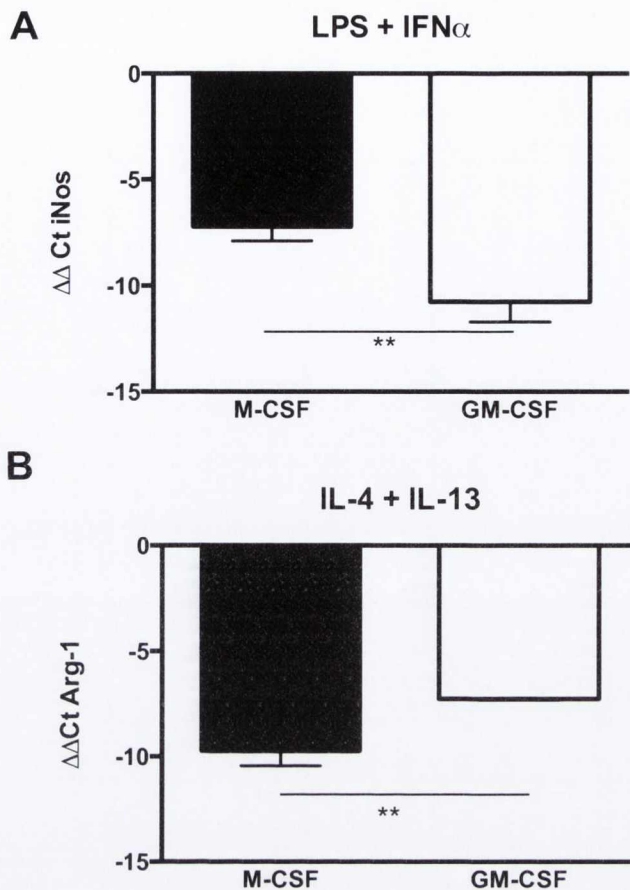


Figure 2.9 Gene expression in polarised BMDMs cultured in M-CSF and GM-CSF. Comparison of mRNA expression of (A) M1 marker *iNos* and (B) M2 marker *Arg1* in polarised BMDMs cultured in M-CSF or GM-CSF. $\Delta\Delta\text{Ct} = \Delta\text{Ct Stimulated (Ct Target gene - Ct Gapdh)} - \Delta\text{Ct unstimulated (Ct Target gene - Ct Gapdh)}$. Replicate= 3. All data represent means $\pm$ s.e.m. Differences, indicated as two-tailed *p* values, were considered significant when *p* < 0.05 as assessed by unpaired Student *t* test.

2.5 Nippostrongylus brasiliensis culture and infection

Nippostrongylus brasiliensis is an important parasite in biomedical research as it is a potent activator of both mucosal and systemic Th2 type immune responses (Fallon et al., 2006). It provides an acute model of a gastrointestinal parasite infection. The parasitic larvae migrate through the tissues to the lung, are coughed up and swallowed and reach the intestine in 3-4 days. In the intestine the worms develop into adults that mate and produce eggs that are excreted in the faeces (Camberis, 2003).

Infection and egg culture

A 150-200 g female Wistar rat was sub-cutaneously (SC) infected with 5,000 to 7,000 L3 *N. brasiliensis* in tap water. The rat was placed in a cage with a grid as the base to allow faeces to fall on to damp iso-pads placed beneath the grid. From day 4 through to day 8, rat faeces present on the iso-pads were collected. Faeces were then mixed with an equal amount of activated charcoal (Sigma). The faecal mixture was incubated on circular Whatman filter paper (No 54; GE Healthcare, UK) placed on damp cotton wool in petri-dishes at 21-26 °C in a damp and well sealed plastic containers. Eggs hatched at >5 days and larvae molted and migrated to the edges of the filter paper (**Figure 2.10**).

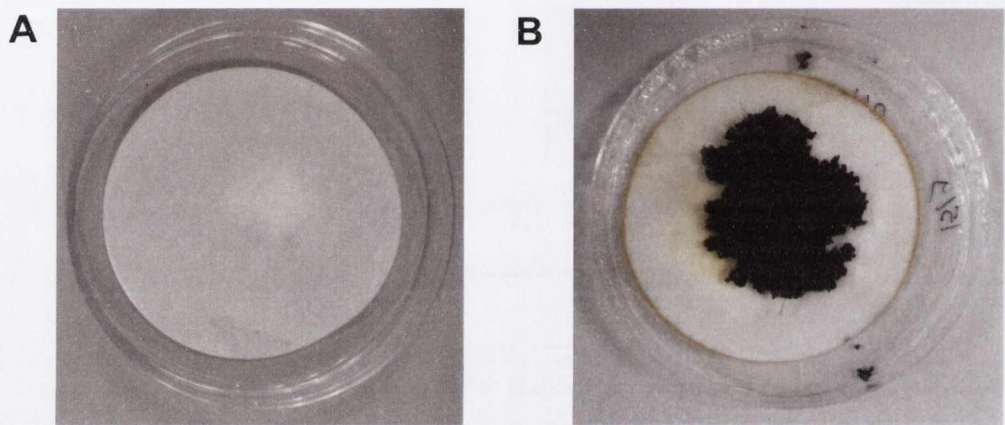


Figure 2.10 Filter paper before and after addition of faecal mixture. **A** Filter paper minus faecal mixture. **B** Filter paper at day 6 post addition of faecal mixture. Larvae have molted and migrated to the edges of the filter paper (brown fringe).

N. brasiliensis L3 Larvae recovery and counting

To recover *N. brasiliensis* L3 larvae a petri-dish was filled with tap water, placed under a dissection microscope and the light turned on to heat the water slightly (**Figure 2.11**). The edge of the filter paper containing the faeces was cut off and placed in the petri-dish of water. Larvae moved towards the centre of the petri-dish and were collected after 10 minutes. The volume was corrected to 3ml with tap water.

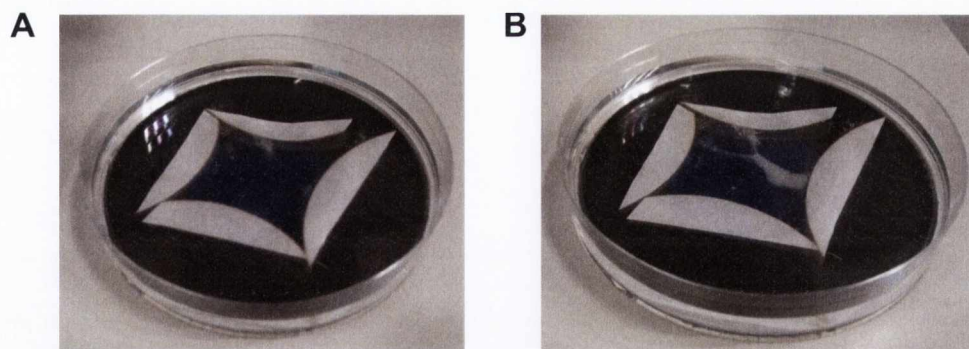


Figure 2.11 Larvae migration. **A** The edge of the filter paper containing the faeces was cut off and placed in the petri-dish of water. **B** Larvae (white) moved towards the centre of the petri-dish and were collected after 10 minutes.

A 1:20 dilution of the collected larvae was performed. Five 50 μ l aliquots were pipetted onto a petri-dish and the number of live larvae counted. The counts were averaged, multiplied by 20 (for counting dilution) and then multiplied by 60 (for sample dilution if 3 ml starting volume). Calculations were made taking into account 10% variation in counts.

Infection

Mice were injected SC with 500 L3 *N brasiliensis* larvae per mouse and sacrificed at day 6.

2.6 Positive selection of peritoneal macrophages

Peritoneal macrophages were isolated from peritoneal exudate cells using Magnetic Activated Cell Sorting (MACS; autoMACS Pro Separator, Miltenyi Biotec, UK). Peritoneal cells were collected (**Section 2.3.1**), washed in 2% FCS/PBS buffer and pelleted by centrifugation at 2,000 g for 5 minutes. The supernatant was aspirated and the pellet resuspended in 100 μ l 2% FCS/PBS buffer and 10 μ l primary APC-conjugated anti-F4/80 mAb (clone number; eBioscience; Appendix IV) was added per 1×10^7 cells. This mix was left at 4°C for 10 minutes and then washed with buffer by centrifugation at 2,000 g for 5 minutes. The pellet was resuspended in 90 μ l of 2% FCS/PBS buffer and 10 μ l of Anti-APC Microbeads (Miltenyi Biotec) per 1×10^7 cells and left at 4°C for 15 minutes. Cells were washed in 2% FCS/PBS buffer, centrifuged as before and resuspended in 500 μ l 2% FCS/PBS buffer. Cells were then separated using the autoMACS Pro Separator using positive selection based on F4/80 expression. F4/80 positive cells constituted ~85% of

peritoneal cells following positive selection using the autoMACS Pro Separator (**Figure 2.12**). F4/80 positive cells were stored at -20°C.

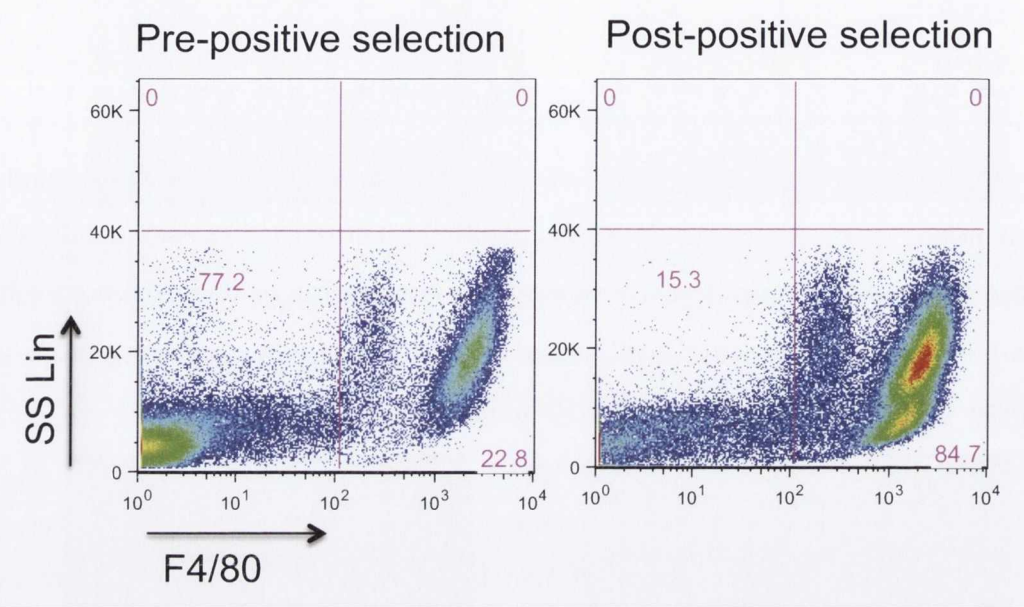


Figure 2.12 Positive selection of F4/80 expressing peritoneal cells. F4/80 cells were positively selected from peritoneal cells using the autoMACS Pro Separator. Flow plots are representative of the percentage of F4/80 positive cells present pre- and post- positive selection. F4/80 positive cells constituted 84.7% of the post positively selected cells.

2.7 Cell Counting

Cells in media were diluted in Trypan Blue Solution (Fluka, Sigma) and viable cells (clear) were counted on a light microscope using a haemocytometer (KOVA International, CA, USA). The total number of cells was calculated from the following formula:

$$\text{Average cell count in one quadrant} \times 10^4 \times \text{Tryptan Blue dilution} \times \text{ml of cells} = \text{Total cell count.}$$

2.8 Protein extraction

Cells were isolated and cultured as described above (**Section 2.3.1-2.3.5**). Whole cell extracts were prepared following cell lysis, as described (Hams et al., 2011). Cells were lysed in RIPA buffer (Appendix III) plus 1 mM Complete Protease-Inhibitor Cocktail (Roche, Switzerland) for 30 minutes on ice. RIPA Buffer is non-ionic and enables efficient

cell lysis and protein solubilisation while avoiding protein degradation and interference with the protein immunoreactivity and biological activity (Harow and Lane, 2006). Protein concentrations of the samples were then quantified (**Section 2.8.1**).

2.8.1 Protein quantification using the bicinchoninic acid (BCA) assay

Protein quantification was performed using the BCA Protein Assay Reagent kit (Thermo Scientific). The BCA Protein Assay combines the reduction of Cu^{2+} to the cuprous cation (Cu^{1+}) by protein in an alkaline medium with the highly sensitive and selective colorimetric detection of Cu^{1+} by bicinchoninic acid. The peptide bonds in the protein sample reduce Cu^{2+} ions, in a temperature dependent reaction, from the copper solution to Cu^{1+} . The amount of Cu^{2+} reduced is proportional to the amount of protein present in the solution. Next, two molecules of BCA chelate with each Cu^{1+} ion, forming a purple-coloured product that strongly absorbs light at a wavelength of 562 nm.

Standards were prepared from a stock solution of 2 mg/ml BSA in sterile PBS and ranged from 125 $\mu\text{g/ml}$ to 2000 $\mu\text{g/ml}$. Standards and replicates were added to a 96-well high binding plate in a volume of 10 μl and 200 μl of the BCA solution was added to each well. The plate was incubated for 30 minutes at 37°C to allow the reaction to develop. Absorbance was measured at 562 nm. The concentration of the protein was measured according to the standard curve of the BSA standards.

2.9 Western Immunoblotting

Following protein quantification using the BCA assay (**Section 2.8.1**), protein was denatured in loading buffer (Appendix III) by boiling at 100°C for 5 minutes. Cell extracts were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% polyacrylamide gels in running buffer (Appendix III). Electrophoresis was performed at 100V for 2 hours using the Mini-PROTEAN Tetra Vertical Electrophoresis Cell (Bio-Rad, CA, USA). PageRuler Plus Prestained ladder (Thermo Scientific) was used to determine molecular weight. Following separation, the proteins were subsequently transferred electrophoretically, at 365-450 mA, for 1 hour 10 minutes onto a polyvinylidene fluoride membrane (PVDF) (Millipore, UK) in transfer buffer (Appendix III). Protein transfer was examined using Ponceau Solution (1% w/v, 5% Acetic acid; Sigma). Ponceau Solution staining was reversed by washing the membrane 3 x 15 minutes

in distilled H₂O (dH₂O). Non-specific binding was blocked by incubation with PBS-Tween (Appendix III) containing 5% (w/v) skimmed milk powder for 1 hour at room temperature (rt). The membranes were then washed 3 x 30 minutes in PBS-Tween.

Membranes were probed with primary antibodies (Appendix IV) overnight (4°C) and washed as above. The membranes were then washed in PBS-Tween and incubated with secondary antibody conjugated to Horseradish Peroxidase (HRP) (Appendix IV) for 1 hour at rt. Following washing immunoreactive detection was made using the Enhanced Chemiluminescence (ECL) kit (Thermo Scientific). HRP catalyses the conversion of the ECL substrate into a sensitised reagent in the vicinity of the molecule of interest, which on further oxidation by hydrogen peroxide; produces a triplet (excited) carbonyl group, which emits light when it decays to the single carbonyl. Membranes were exposed to UV light and protein bands visualised using the ChemiDoc MP system and Image Lab Software (Bio-Rad).

Membranes were stripped using Re-Blot Plus Western Blot Mild Antibody Stripping Solution (Millipore) to allow for re-probing. Membranes were incubated in 20 ml of 1X Re-Blot solution for 20 minutes at rt.

2.10 RNA Isolation and complimentary DNA (cDNA) synthesis

RNA was isolated from cells using the RNeasy kit (QIAGEN, Crawley, UK) as described (Hams et al., 2011). Isolated and cultured cells were lysed using RLT lysis buffer plus β -mercaptoethanol (1 ml/ 100ml). Brain and liver tissue was homogenised using an Ultra-Turrax homogeniser (IRK-WERKE, Germany) in RLT lysis buffer plus β -mercaptoethanol. Through a series of wash steps RNA was bound to an RNeasy spin column membrane and contaminants are washed away, before RNA was eluted into an eppendorf with 30 μ l of RNase-free water and stored at -80°C. RNA purity was measured using a Nanodrop ND 1000 Spectrophotometer (Thermo Scientific). A measurement of 260 nm / 280 nm ratio of 2 was considered to represent pure RNA. RNA purity was also analysed by running RNA (1 ng) on a 1.5% agarose gel (Section 2.2)(Figure 2.13).

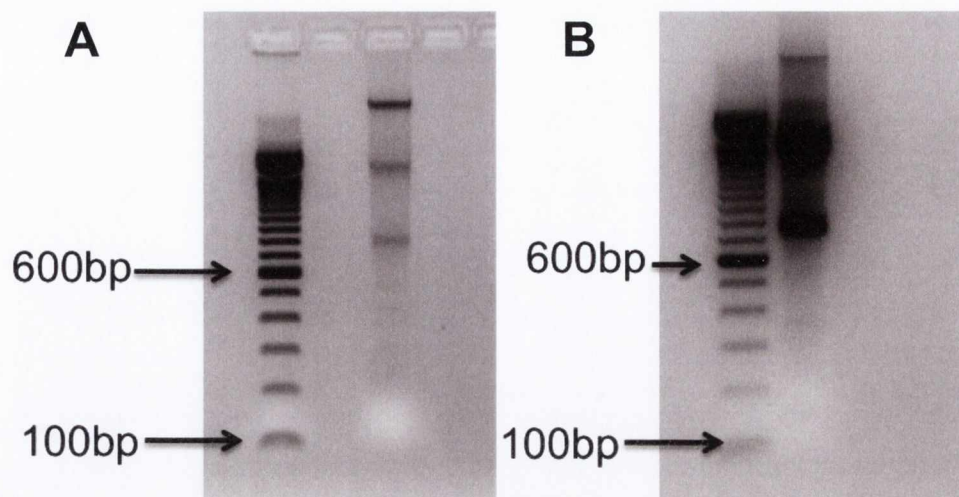


Figure 2.13 RNA purity was determined by agarose gel electrophoresis. RNA (1 ng) mixed with DNA binding solution added ran on a 1.5% agarose gel, visualised as describes in **Section 2.2**. **A** RNA from peritoneal macrophages from WT mice. **B** RNA from BMDMs from WT mice.

RNA was reverse transcribed using the Quantitect reverse transcription kit incorporating a genomic DNA (gDNA) elimination step (QIAGEN). The purified RNA sample (100 ng) was briefly incubated in gDNA Wipeout Buffer at 42°C for 2 minutes to effectively remove contaminating genomic DNA. The RNA sample was reverse transcribed (RT) using a master mix prepared from Quantiscript Reverse Transcriptase, Quantiscript RT Buffer, and RT Primer Mix. The entire reaction took place at 42°C and is then inactivated at 95°C.

2.11 Quantitative Real-Time PCR (qRT-PCR)

qRT-PCR was performed using the TaqMan Fast Universal Master Mix (Applied Biosystems). This allows for the detection and quantification of amplified DNA. The reaction mix (18 µl) containing the specific primers of the target gene and the endogenous control gene *Gapdh* was pipetted per well of a FrameStar 96 well PCR plate, followed by the addition of 2 µl of cDNA sample per well. The plate was sealed with adhesive film (Applied Biosystems) and centrifuged at 2,000 g for 10 seconds. The plate was read by the ABI NextStep detection system (Applied Biosystems) and run according to the following PCR cycle conditions: 95°C for 2 minutes to allow for activation, 40 cycles of 95°C for 10

seconds to allow for amplification and 60°C for 30 seconds. Specific gene expression was normalised to murine *Gapdh* required to eliminate variations in cDNA concentrations. The TaqMan gene expression assay kits used are detailed in Appendix V.

2.11.1 Designing of custom *Rora* primers

The genomic sequence deleted in *Rora*^{sg/sg} mice (**Section 2.1.1**) was inputted into Primer-Blast (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>). Primers based on this output were ordered from Sigma for use in semi-quantitative PCR (sqPCR). The genotyping protocol used in sqPCR is outlined in Appendix I. A custom TaqMan gene assay kit was ordered from Life Technologies that contained the following primer and probe sequences:

Forward TTGCCCAGAACATATCCAAA

Reverse GGAAGGTTCTGCCACGTTAT

Probe TGCTGGAGCTCTTCCCGCAA FAM-MGB

2.12 V-PLEX Plus Proinflammatory Panel 1 (mouse) Kit

Pro-inflammatory cytokine levels were measured using the V-PLEX Plus Proinflammatory Panel 1 (mouse) Kit (MESO Scale Discovery, Maryland, USA). The Proinflammatory Panel 1 (mouse) Kit provides assay-specific components for the quantitative determination of IFN γ , IL-1 β , IL-2, IL-4, IL-5, IL-6, KC/GRO, IL-10, IL-12p70, and TNF α .

The kit was used in accordance with manufacturing instructions. Samples were diluted using Diluent 41 and 50 μ l of the diluted samples were added per well. Serum samples were diluted 1:2 and supernatant samples were diluted 1:5. Calibrator samples were made according to instructions and added to the plate. Samples were incubated on a plate shaker for 2 hours at rt. Following incubation each well was washed 3 X with 200 μ l PBS-Tween. The detection antibody mixture was made using Diluent 45 according to kit instructions. The detection antibody mixture (50 μ l) was added to each well and the plate was incubated on a plate shaker for 2 hours at rt. Following incubation each well was washed 3 X with 200 μ l PBS-Tween. Read buffer (1X) (50 μ l) was added to each well and the plate was read on a MESO Scale Discovery SI6000.

2.13 Flow-cytometric staining and analysis

Surface marker expression was assessed by flow cytometry as described (Hams et al., 2011). Data were collected on a CyAn ADP (Beckman Coulter, Germany). The CyAn ADP has 3 lasers (405, 488 & 633 nm) and 9 fluorescence parameters. Cell viability was assessed by incubation in the LIVE/DEAD Fixable Aqua Dead Cell Stain kit (Invitrogen) (1:1000 dilution in PBS) for 30 minutes in the dark at 4°C, followed by a single wash in 1X PBS. For cell surface staining cells were incubated for 20 minutes at 4°C with 1:100 dilution of Fc block (BD Bioscience) and 1:200 dilution of flouochrome antibodies, followed by a single wash in FACS buffer and resuspended in FACS buffer (Appendix III). The antibodies used are detailed in Appendix IV. Data were analysed using FlowJo software (Tree Star, OR, US).

2.14 Aldehyde dehydrogenase activity detection

The ALDEFLUOR™ reagent system (Stemcell Technologies, Canada) was used to measure aldehyde dehydrogenase (ALDH) cellular activity. ALDH is an enzyme that catalyses the oxidation of aldehydes to carboxylic acids. Cells expressing high levels of ALDH become brightly fluorescent (ALDH^{br}) and can be identified and enumerated using a flow cytometer.

Peritoneal cells were prepared as described (**Section 2.3.1**). To determine the optimal cell sample concentration to use cell samples were adjusted to 0.5×10^6 cells/ml, 1×10^6 cells/ml and 2×10^6 cells/ml using ALDEFLUOR Assay Buffer (**Figure 2.14**). To the cell sample, 5 µl of ALDEFLUOR Reagent was added (Test). Immediately following this 500 µl of the cell sample was added to an eppendorf tube containing 5 µl ALDEFLUOR DEAB Reagent (Control). The Test and Control cell samples were then incubated at 37°C for 30 minutes. Following incubation cells were washed in ALDEFLUOR Assay Buffer. Where indicated surface staining was performed following washing, staining was performed in ALDEFLUOR Assay Buffer.

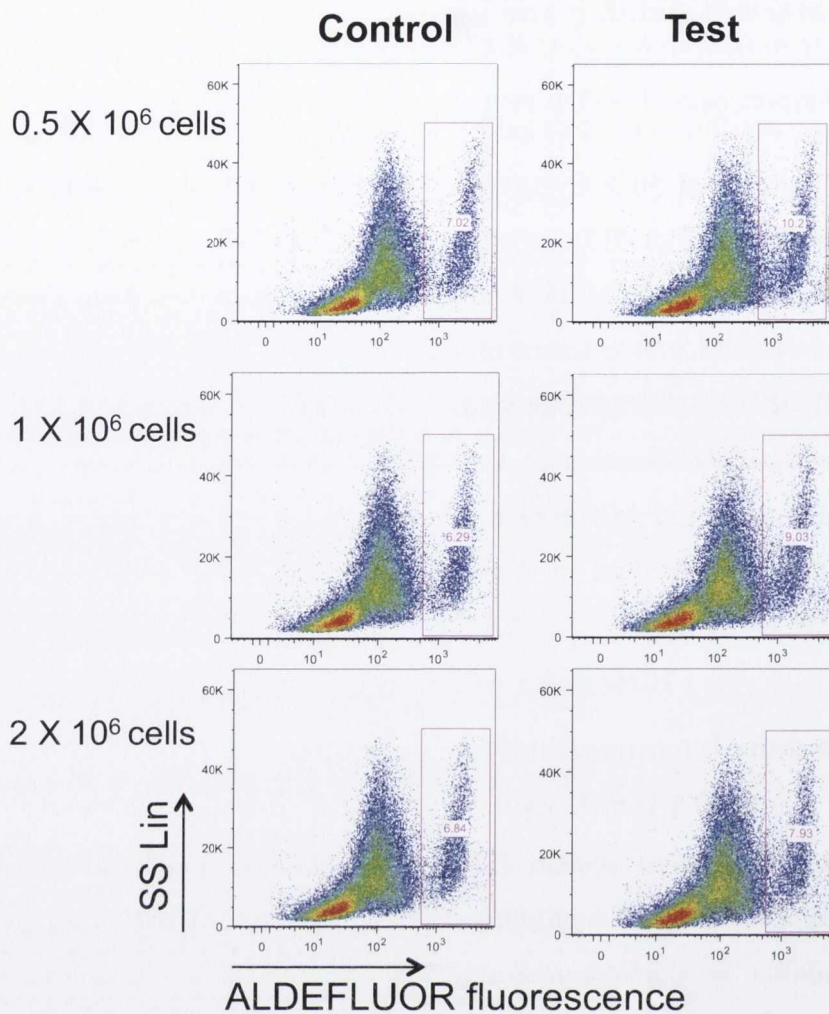


Figure 2.14 Optimising cell sample concentration for Aldefluor Assay. All viable and intact cells are fluorescent after exposure to the activated ALDEFLUOR™ Reagent. Samples exposed to ALDEFLUOR™ in the presence of the ALDH inhibitor, DEAB, is the only appropriate negative control for the assay. The cell concentration 0.5×10^6 has the strongest fluorescence intensity of ALDH^{Br} cells and the highest signal to background ratio.

2.15 Intracellular staining

For intracellular staining of proteins the FoxP3/Transcription Factor Staining Buffer Set (eBioscience) was used. Fixation/Permeabilisation Concentrate (4X) was diluted to 1X concentration using the Fixation/Permeabilisation Diluent. Permeabilisation buffer (10X) was diluted using dH₂O. Surface staining was performed on cells as described (Section 2.13). Following this cells were fixed in the 1X Fixation/Permeabilisation buffer for 20 minutes at 4°C. Cells were then washed in 1X Permeabilisation Buffer and stained for

antibodies in Permeabilisation Buffer for 20 minutes at 4°C. Cells were then washed in FACS buffer and resuspended in FACS buffer.

2.16 Intracellular staining for Relm α and iNos.

For intracellular staining of Relm α and iNos, as described previously (Huang et al., 2014), cells were first fixed in Fixation/Permeabilisation buffer as above (**Section 2.13**). Cells were then washed in Permeabilisation Buffer and stained for 20 minutes at 4°C with rabbit anti-Relm α (PeproTech) and mouse anti-iNos (Santa Cruz Biotechnology) in Permeabilisation Buffer. Cells were washed in Permeabilisation Buffer followed by incubation with FITC-conjugated antibody to rabbit IgG (711-096-152; Jackson ImmunoResearch) or PE-conjugated antibody to mouse IgG (A85-1; BD Biosciences) in the Permeabilisation Buffer. Cells were then washed in FACS buffer and resuspended in FACS buffer.

Antibody specificity was tested using *in vitro* and *in vivo* methods. To test the specificity of antibody staining for Relm α and iNos *in vitro* BMDMs from WT mice were cultured for 7 days and stimulated with either IL-4 + IL-13 or LPS + IFN γ for 24 hours to polarised towards an M1 and M2 phenotype respectively (**Section 2.4**) (**Figure 2.15**). There was increased iNos expression on BMDMs polarised towards an M1 phenotype compared to unstimulated cells. Conversely there was increased Relm α expression on BMDMs polarised towards an M2 phenotype compared to unstimulated cells.

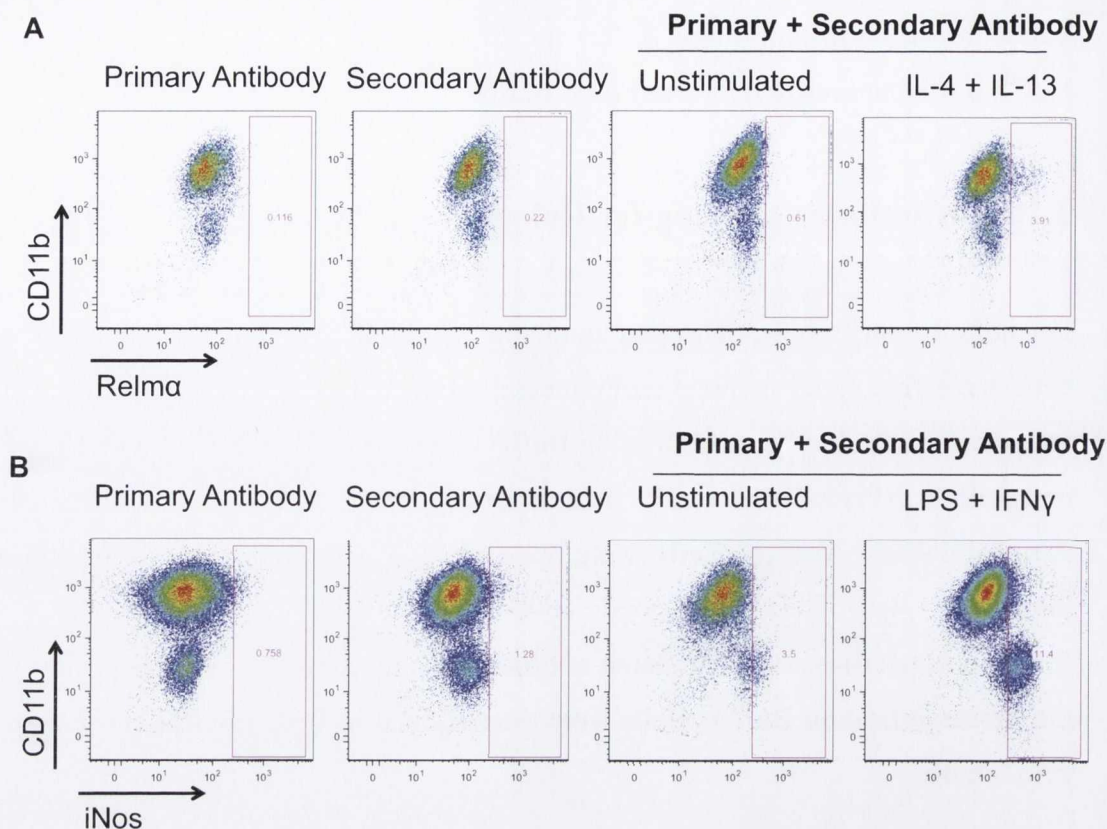


Figure 2.15 Testing Relma and iNos antibody specificity *in vitro*. **A** Relma expression on BMDMs from WT mice. Primary Relma antibody and secondary anti- Relma FITC antibodies were tested individually (for controls) and in combination on CD11b⁺ polarised BMDMs from WT mice. **B** iNos expression on CD11b⁺ BMDMs from WT mice. Primary iNos antibody and secondary anti-iNos PE antibodies were tested individually (for controls) and in combination on polarised BMDMs from WT mice.

Relma and iNos antibody specificity *in vivo* was also tested (**Figure 2.16**). *N. brasiliensis* infection was used to induce the proliferation of M2 macrophages in the peritoneal cavity of WT mice (**Section 2.5**). Peritoneal cells were isolated on day 6 of *N. brasiliensis* infection. LPS (10 mg/kg) was used to induce M1 macrophages in the peritoneal cavity (**Section 2.15**) (**Figure 2.16**). Peritoneal cells were isolated 3 hours post LPS treatment. Relma expression was increased in the peritoneal cavity of WT mice infected with *N. brasiliensis* (day 6) compared to unstimulated mice. However iNos expression did not increase in response to LPS compared to unstimulated mice.

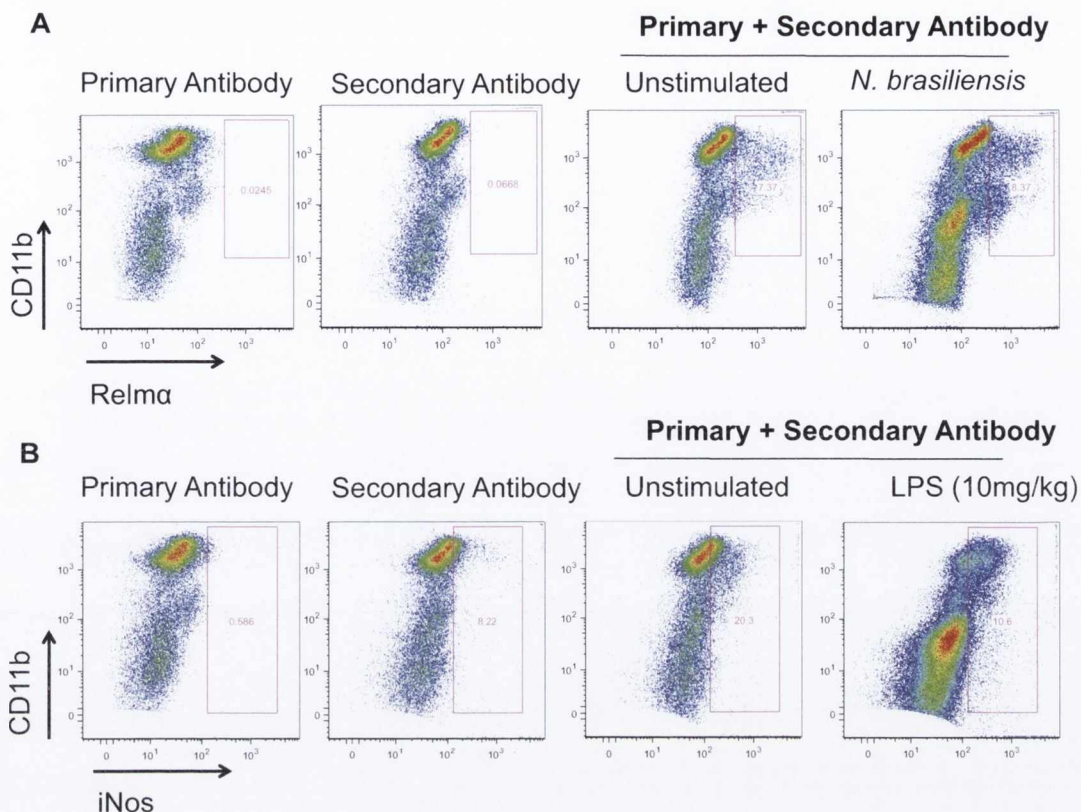


Figure 2.16 Testing Relma and iNos antibody specificity *in vivo*. **A** Relma expression on BMDMs from WT mice. Primary Relma antibody and secondary anti-Relma FITC antibodies were tested individually (for controls) and in combination on stimulated CD11b⁺ peritoneal cells from WT mice. **B** iNos expression on BMDMs from WT mice. Primary iNos antibody and secondary anti-iNos PE antibodies were tested individually (for controls) and in combination on stimulated CD11b⁺ peritoneal cells from WT mice.

2.17 Thioglycollate-elicited peritoneal macrophages

Thioglycollate-elicited peritoneal macrophages were introduced in 1964 by Gallily and colleagues (Gallily et al., 1964) as a modification of Barski's method and have become a common tool for eliciting peritoneal macrophages. Non-enzymatic reactions between proteins and reducing sugars in thioglycollate broth lead to formation of advanced glycation end-products (Misharin et al., 2012). These end products are recognised by members of the PRR family, such as receptor for advanced glycation end-products and the macrophage scavenger receptor. Following thioglycollate injection into the peritoneal cavity of mice, the total number of cells in the peritoneal cavity increases from an average of $0.5-1 \times 10^6$ cells to an average of $3-4 \times 10^6$ cells over 4 days, as illustrated in the SS Lin

v FS Lin flow cytometry plot (**Figure 2.17A**). The reason for this cellular increase is that thioglycollate treatment results in sterile peritonitis that attracts increased numbers of infiltrating cells. These cells include monocytes that upon entrance into peritoneal cavity differentiate into blood monocyte-derived macrophages (**Figure 2.17B**). Macrophage numbers gradually increases and reaches a peak at 72 hours (Hoover-Plow et al., 2008). Levels of eosinophils in the peritoneal cavity are also increased greatly following thioglycollate treatment (**Figure 2.17C**) (Misharin et al., 2012). Levels of neutrophils reach a peak at 6 hours but decline rapidly after this time (Hoover-Plow et al., 2008), as evident on day 4 (**Figure 2.17D**).

Thioglycollate (4%) was prepared by adding 4.05 g thioglycollate medium (T9032, Sigma) to 100 ml dH₂O (Gibco). The solution was boiled and stirred until completely dissolved. It was then autoclaved (121°C, 10 minutes) and stored in the dark at 4°C until use. Thioglycollate (800 µl) was injected intra peritoneal (I.P) and mice were sacrificed on day 4 as described (Jenkins et al., 2011).

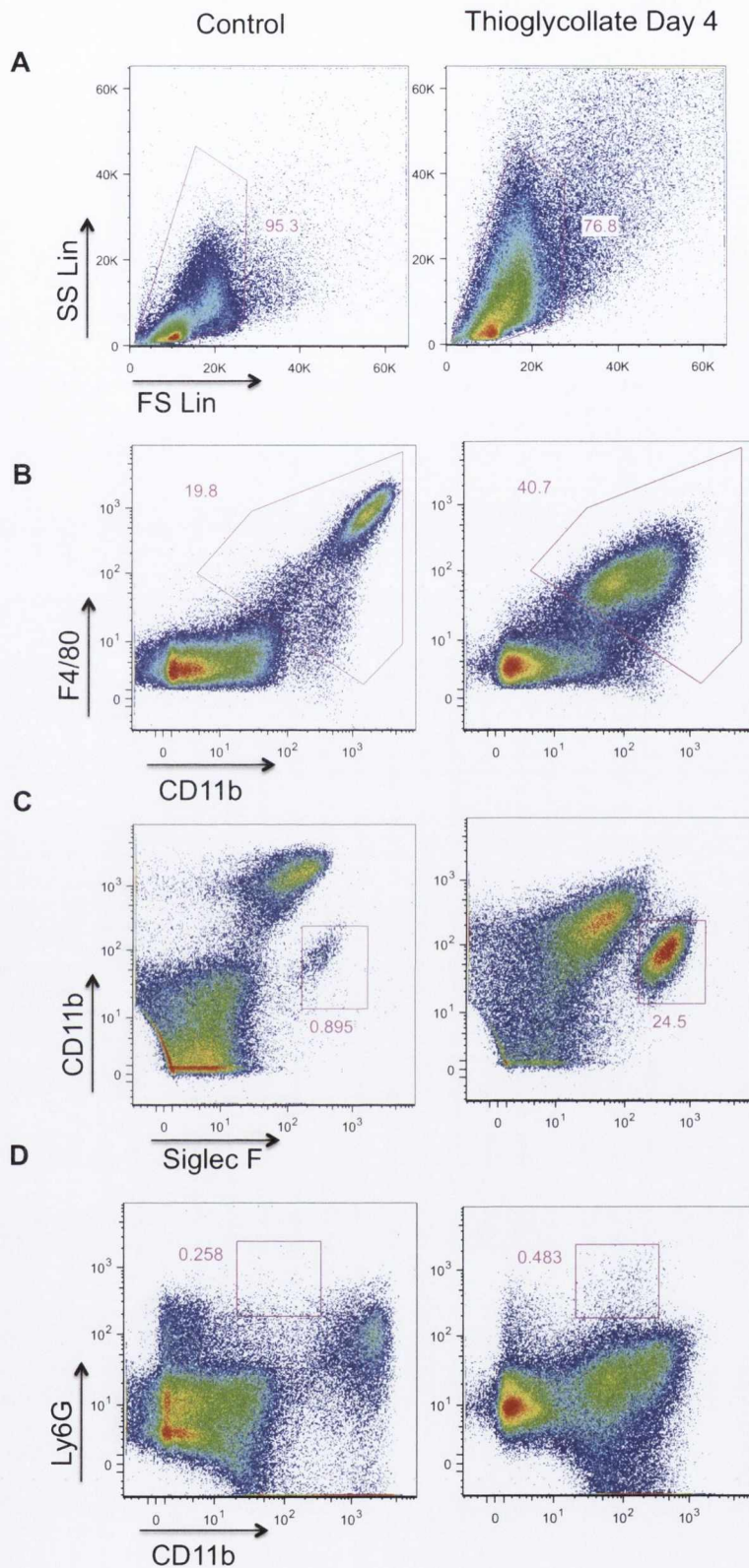


Figure 2.17 Thioglycollate treatment leads to an increase in total peritoneal cells. Mice were treated with 800 μ l 4 % thioglycollate. Representative flow cytometry plots of total peritoneal cells. **A** FS Lin v SS Lin **B** Macrophages (CD11b⁺, F4/80⁺). **C** Eosinophils (CD11b⁺ Siglec-F⁺). **D** Neutrophils (CD11b⁺, Ly6G⁺) on day 0 and day 4 post thioglycollate treatment.

2.18 Inducing M2 macrophages *in vivo* within the peritoneal cavity

To induce *in vivo* M2 polarisation and proliferation in the peritoneal cavity, WT mice were treated with IL-4/anti-IL-4 mAb complexes (IL-4c) prepared as previously described (Jenkins et al., 2011) (**Figure 2.18**). Treatment of mice with a complex of IL-4 and anti-IL-4 mAb results in the slow continuous release of cytokine. IL-4c consisted of a 2:1 molar ratio of recombinant mouse IL-4 (Peprotech, London) and anti-IL-4 mAb (clone 11B11; BioXcell, NH). Mice were injected I.P. with IL-4c containing 5 µg of IL-4 and 25 µg of 11B11, or PBS vehicle control on days 0 and 2. For co-administration experiments, mice were given thioglycollate by I.P injection (800 µl) on day 0 immediately prior to the first dose of IL-4c. Mice were sacrificed on day 4.

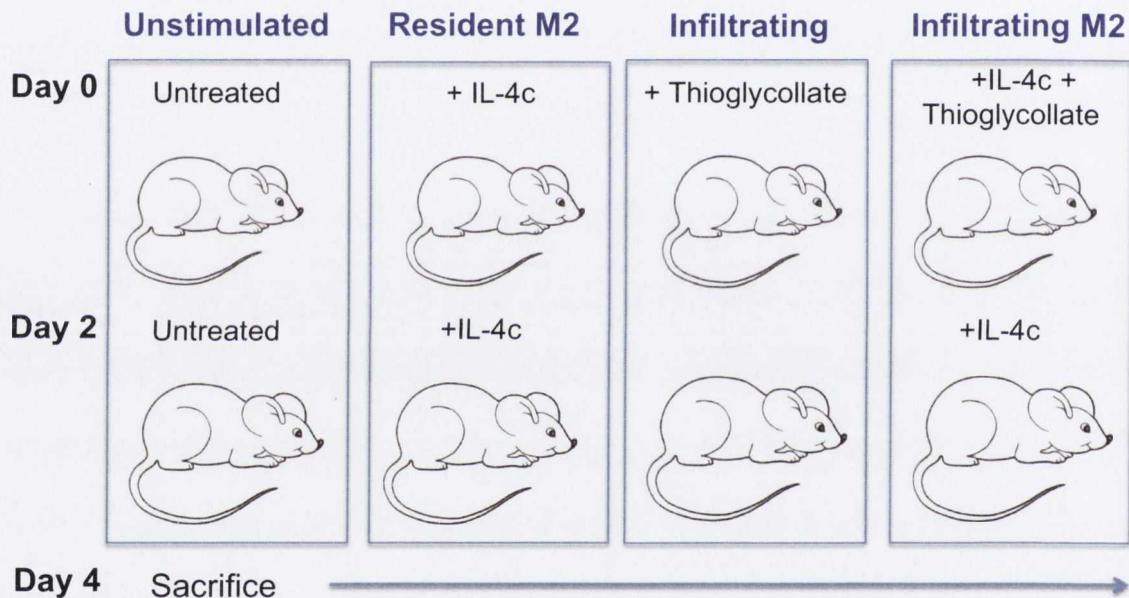


Figure 2.18 Schematic of the protocol used to induce M2 proliferation in the peritoneal cavity. WT mice were treated with IL-4c alone on day 0 and day 2 to induce proliferation of resident peritoneal M2 and thioglycollate and IL-4c on day 0 and day 2 to induce the proliferation of blood monocyte-derived M2. Thioglycollate treatment on day 0 induced the recruitment of infiltrating cells. Untreated and thioglycollate-treated WT mice were used as controls.

2.19 Cell sorting

Whole peritoneal cells were isolated from mice as described in **Section 2.3.1**. Cells were stained for flow cytometry as described in **Section 2.13**. For the isolation of peritoneal macrophage populations the following antibodies were used in the flow cytometry antibody panel- F4/80 FITC, Tim4 PE, MHCII V450 and CD11b APC. Peritoneal cells were sorted into three populations by Dr Barry Moran using the BD FACS Aria Fusion Cell Sorter. Peritoneal cells were sorted into three populations- Tim4⁺, Tim4⁻ MHCII⁺ and Tim4⁻ MHCII⁻ populations.

2.20 *In vitro* LPS stimulation peritoneal macrophages

Peritoneal cells were isolated as described (**Section 2.1.3**). Cells were seeded in 96-well plate at a cell density of 0.1×10^6 cells and incubated for 2 hours at 37°C. Cells were washed 3 X with RPMI-1640 media. Adherent cells were then incubated with ultra-pure LPS (100 ng; Invivogen) for 0, 6 and 24 hours at 37°C. For cytokine analysis media was removed from each well and stored at -20°C.

2.21 *In vitro* LPS stimulation BMDMs

BMDMs were prepared and cultured as described (**Section 2.1.4**). On day 7 of culture BMDMs were washed 3 X with RPMI-1640 and removed from the culture flask surface using ice-cold PBS and a cell scraper. In a 6-well plate or 96-well plate 1×10^6 cells/ml or 0.3×10^6 cells/ml respectively were seeded and left for 24 hours. Cells were washed 3 X with RPMI-1640 followed by the addition of ultra-pure LPS (100 ng/ml; Invivogen).

2.21.1 Protein Expression

Expression of Phospho-p38 and Phospho-SAPK/JNK was measured in BMDMs cells that were incubated with ultra-pure LPS (100 ng/ml) (Invivogen) for 0, 10, 30, 45, 60, 90 minutes. BMDMs were removed by dry lysis. In brief cells were washed 3 X with RPMI-1640 media, allowed to dry, followed by the addition of 60 µl of 5X sample buffer (10 % Glycerol, 0.02 % SDS, 0.02 % Bromphenol Blue, 0.125 M Tris pH 6.8 and dH₂O). Cells in the 5X sample buffer were removed using a cell scraper. Sample denaturation by heating to

100°C (5 minutes) was performed immediately. Immunoblot analysis was performed as described (**Section 2.9**).

2.22 LPS stimulation of sorted peritoneal populations

Peritoneal populations were sorted as described in **Section 2.19**. The peritoneal populations were seeded into a 96 well plate and stimulated with ultra-pure LPS (100ng/ml) in RPMI-1640 media for 3 hours.

2.23 LPS induced-endotoxemia model

The endotoxin LPS, a component of the outer membrane of Gram-negative bacteria, is involved in the pathogenesis of sepsis. In mice LPS administration induces systemic inflammation that mimics aspects of the initial clinical features of sepsis in man (**Section 1.14**). An LPS injection model has been widely used for sepsis research (Doi et al., 2009). Here the LPS-induced endotoxemia model was performed, as described (Saunders et al., 2010).

To measure body temperatures mice were implanted subcutaneously with a temperature transponder (IPTT-300, Bio Medic Data Systems) 24 hours prior to LPS injection as described (Hams et al., 2011; Saunders et al., 2010). These temperature chips were read using the SP-6004 Smart Probe (Bio Medica Data Systems).

Mice were injected I.P with 10 mg/kg ultrapure LPS-EB (Invivogen; **Section 2.22**). To eliminate batch variation five batches of ultrapure LPS were reconstituted in PBS and combined. The protocols for visually scoring mice and recording temperatures used in the model were used as described (Saunders et al., 2010). These are outlined below:

Visual scoring protocol:

- Each animal is removed from the cage and scored individually.
- Animals are scored at 1, 3, 6, 9, 12, 24, 36 and 48 hours post-induction.
 - Score 1 – Animals demonstrate piloerection. All other behaviour seems normal.
 - Score 2 – Animals demonstrate piloerection and are huddled, slight diarrhoea. Still responding to stimuli.
 - Score 3 – Animals demonstrate slower responses and are passive when handled, worsening diarrhoea.
 - Score 4 – Animals demonstrate a lack of curiosity, little response to stimuli and impaired self-righting reflex. Mice are humanely euthanised.
 - Score 5- Death

Body temperature is monitored in mice at 1, 3, 6, 9, 12, 24, 36 and 48 hours post-induction alongside visual scoring. Body temperature is monitored by placing the temperature probe over the temperature transponder that is SC implanted in the mice.

Any animal with a body temperature below 30°C were humanely killed and removed from the study.

2.24 Determining the LPS concentration to use in the LPS-induced endotoxemia model.

WT mice (male, 8 weeks of age) were injected with either PBS, 10 mg/kg, 20 mg/kg or 30 mg/kg of the combined ultrapure LPS. Survival and sepsis scores were recorded in each group over 48 hours. All concentrations of LPS resulted in responses as indicated by survival percentages (**Figure 2.19A**) and LPS-induced sepsis scores (**Figure 2.19B**). The LPS concentration 10 mg/kg was chosen to use in the model as it was the lowest concentration that resulted in decreased survival and increased sepsis scores in mice compared to control mice. The LPS dose of 10 mg/kg is in line with previously published data (Hams et al., 2011; Saunders et al., 2010).

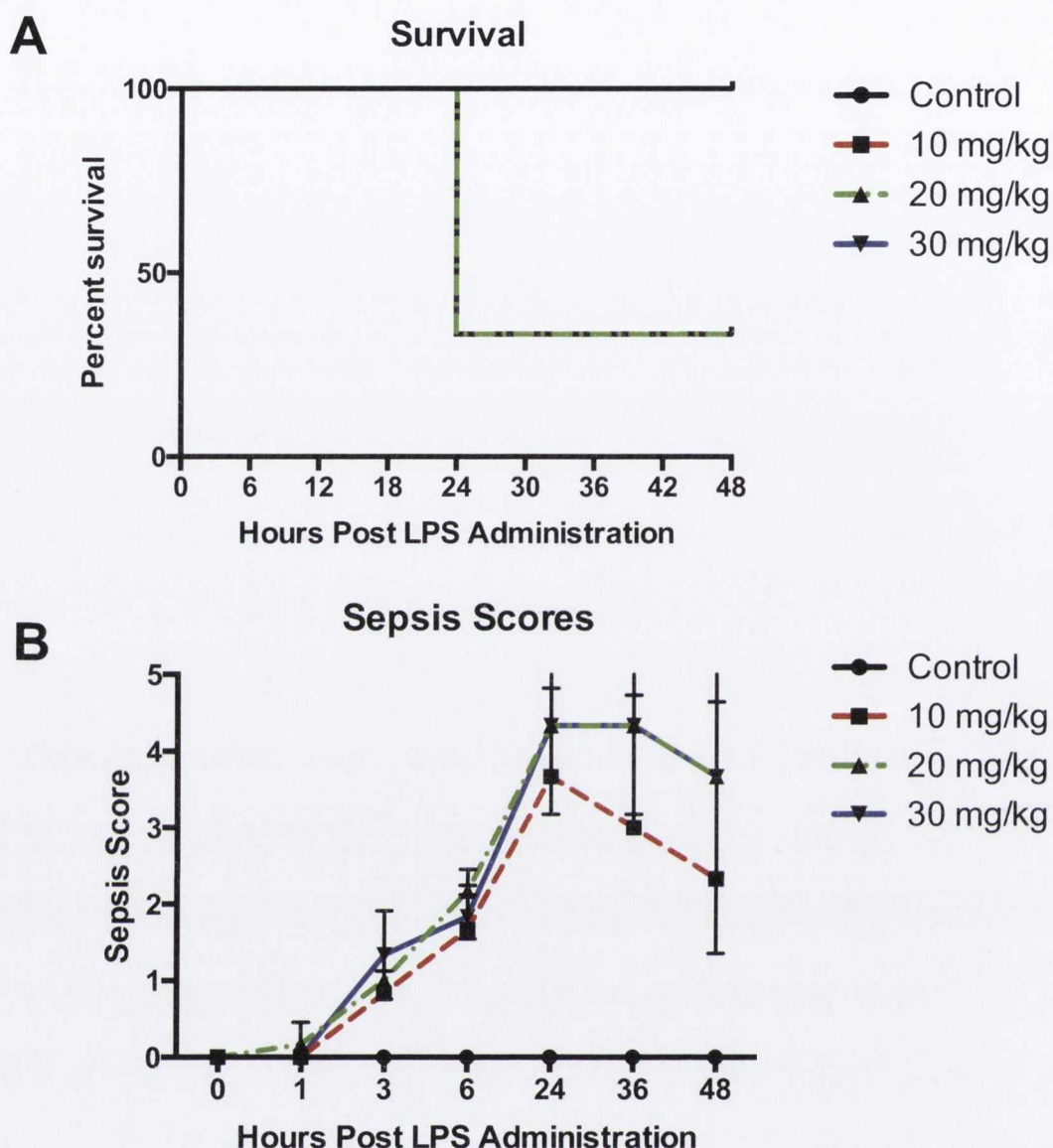


Figure 2.19 Determining the LPS concentration to use in the LPS induced endotoxemia model. WT mice were injected with 10 mg/kg, 20 mg/kg or 30 mg/kg ultrapure LPS. **A** Percentage survival of each group. Kaplan-Meier method was used to evaluate survival. **B** Sepsis scores of each group. All data represent means $\pm$ s.e.m. Differences, indicated as two-tailed p values. $n=3$.

2.25 Generation of Bone Marrow (BM) chimeras.

Chimeric mice were generated by total body X-ray irradiation followed by transfer of bone marrow cells as described (Aviello et al., 2014). Briefly recipient WT mice were lethally

irradiated with 5 Gray (Gy) followed by 4 Gy 3 hours later using the RS225 Cabinet Irradiator (Gulmay Medical, US). BM from donor *Rora*^{sg/sg} mice and WT mice was isolated as described in **Section 2.3.2**. Recipient WT mice were reconstituted intra-orbitally with BM cells (1×10^7) from either *Rora*^{sg/sg} mice or WT mice. To determine reconstitution efficiency *B6.SJL-Ptprc^a Pepc^b/BoyJ* mice were used (**Section 2.1.11**). *B6.SJL-Ptprc^a Pepc^b/BoyJ* express the marker CD45.1 on their cell surface, unlike other mouse strains that express CD45.2. Reconstitution efficiency was assessed by measuring the cell surface expression of CD45.1 and CD45.2 by flow cytometry after 8 weeks of reconstitution.

2.26 Statistical analysis

Statistical analysis was performed using GraphPad Prism (Version 6.0). Results are presented as mean ± standard error mean (s.e.m) or mean ± standard deviation (s.d). s.e.m was chosen to represent the variance between experimental groups that have different n numbers. It takes into account both the value of the s.d and the sample size. Differences, indicated as two-tailed *p* values, were considered significant when *p* < 0.05 as assessed by unpaired Student *t* test with Welch correction applied as necessary. The Kaplan-Meier method was used to evaluate survival. Table 2.6 defines the statistical symbols used.

P value	Wording	Summary
< 0.0001	Extremely significant	****
0.0001 to 0.001	Extremely significant	***
0.001 to 0.01	Very significant	**
0.01 to 0.05	Significant	*
≥ 0.05	Not significant	NS

Table 2.6 Explanation of GraphPad Prism statistical symbols.

Chapter 3

Determining the role of the transcription factor Rora in macrophage development.

3.1 Introduction

Macrophages are extremely heterogeneous cells that assume specific phenotypes and have distinct functions depending on the tissue in which they reside; for example alveolar macrophages differ in phenotype to other populations including splenic and peritoneal macrophages. Even within tissues different subsets of macrophages exist with distinct phenotypes and functions. An example of this is observed in the spleen where different macrophage subsets are found in the white pulp, marginal zone and red pulp. Such heterogeneity predicts that discrete transcriptional controls exist in distinct macrophage populations that determine both their specific phenotypes and tissue-specific functions.

These transcriptional controls are relatively well defined in other fields of immunity, such as T-cell subsets; however, the transcriptional basis for producing tissue macrophage subsets remains unknown. There have been notable studies on tissue niche-specific transcriptional control of macrophage populations in the past, such as in the spleen. The haem-induced transcription factor Spi-c is required for the presence of the tissue-resident red pulp macrophages in the spleen that function to engulf aging red blood cells and recycle haem-associated iron (Kohyama et al., 2009), whereas LXR α is necessary for all marginal zone macrophages (A-Gonzalez et al., 2013). However, in other tissue macrophage populations the transcriptional controls involved in function is not as clear.

In addition to heterogeneity in tissue macrophage phenotypes and functions, macrophage populations can also differ in their origin. In recent years a complex scenario to describe macrophage origins has been developed to replace the simplistic traditional view that myeloid precursors in the bone marrow give rise to blood monocytes that in turn giving rise to tissue macrophages (**Section 1.9**). Tissue-resident macrophages from the brain, lung, spleen, liver and peritoneal cavity are not primarily differentiated from monocytes, instead they can be derived from an embryonic precursor and are further maintained by self-renewal (Yona et al., 2013). In addition to embryo-derived tissue-resident macrophages, infiltrating monocytes originating in the bone marrow can enter tissue from the blood in a steady state or in response to inflammation, where they differentiate into blood monocyte-derived macrophages. It has recently been shown that these tissue-resident macrophages and blood monocyte-derived macrophages are phenotypically and functionally distinct (Gundra et al., 2014).

It is clear that tissue macrophages can have different origins, developing through two distinct pathways. However, the factors controlling these processes remain ill defined; it is believed that discrete transcriptional controls govern both pathways. Studies in gene-knockout (KO) mice have previously identified and characterised transcription factors that operate at discrete stages of monocyte-derived macrophage differentiation such as PU.1 and C/EBPs (**Section 1.12**). The transcription factors involved in the differentiation of tissue-derived macrophages are not as well defined. Early studies found that Myb was required for the development of haematopoietic stem cells (HSCs) and all CD11b^{high} monocytes and macrophages, but was dispensable for yolk sac (YS) macrophages and for the development of YS-derived F4/80^{bright} macrophages in several tissues including the microglia, liver Kupffer cells and epidermal Langerhans cells; cell populations that can persist in adult mice independently of HSCs (Schulz et al., 2012).

More recently *Gata6* has been shown to have a central-role in promoting the tissue-dependent characteristics of resident peritoneal macrophages (Okabe and Medzhitov, 2014; Rosas et al., 2014). In these two studies mice were generated with *Gata6* selectively deleted in macrophages, which resulted in a specific reduction in the number of resident peritoneal macrophages and a reduction in F4/80 expression in the remaining resident peritoneal macrophages. Okabe and Medzhitov (2014) also showed that *Gata6* expression was restricted to the classic F4/80^{High} CD11b^{High} peritoneal resident macrophages and that most other peritoneal macrophages (F4/80^{Low} MHCII^{High}) were monocyte-derived macrophages.

Retinoic acid-receptor-related orphan receptor (*Rora*) is a nuclear transcription factor known to have a role in brain development, circadian rhythm and immunity (**Section 1.15**). In terms of immunity, *Rora* has been shown to be required for Th17 lineage differentiation (Yang et al., 2008). More recently it was found that *Rora* is required for the development of Type-2 Innate Lymphoid Cells (ILC2s) (Wong et al., 2012). *Rora* has been shown to be expressed in macrophages (**Figure 1.8**) therefore it was considered that *Rora* could also play a role in the development of macrophages.

3.2 Chapter objectives

As it is known that transcription factors are key controllers of macrophage differentiation and that the transcription factor *Rora* plays a role in the differentiation of Th17 cells and ILC2s, it was decided to explore if *Rora* also has a role in the differentiation of macrophages. In particular, does *Rora* play a role in the development of tissue-resident macrophages or blood monocyte-derived macrophages in the peritoneal cavity. To achieve this *Rora*^{sg/sg} mice were used, which harbour a spontaneous mutation in the *Rora* gene leading to the introduction of a premature stop codon. The aims of this chapter are-

1. To investigate *Rora* gene and protein expression in mouse macrophages.
2. To assess if *Rora* has a role in the differentiation of tissue-resident macrophages or blood monocyte-derived peritoneal macrophages.

3.3 Results

3.3.1 *Rora* natural mutant mice *Rora*^{sg/sg} mice.

Mice homozygous for the staggerer spontaneous mutation (*Rora*^{sg/sg} mice) (Section 1.15.3) display a characteristic phenotype of a staggering gait, mild tremor, hypotonia and small size compared to wild-type (WT; *Rora*^{wt/wt}) littermates (Figure 3.1). *Rora*^{sg/sg} mice are stunted and as a result fail to thrive and succumb to death by 3-4 weeks of age; in comparison, Het (*Rora*^{wt/sg}) littermates have a normal lifespan. To extend their lifespan and improve their condition the conventional normal rodent chow (Harlan 18% protein Rodent Diet) used in our facility was supplemented with a liquidised Nutra-Gel diet. Nutra-Gel diet is a commercial nutritionally complete purified gel diet; it primarily differs from the normal chow diet in terms of the percentage of moisture content (Figure 3.2A). The lifespan of *Rora*^{sg/sg} mice was significantly extended ($p < 0.0008$) by up to 2 weeks through the use of the Nutra-Gel diet (Figure 3.2B). The modified diet improved the condition of *Rora*^{sg/sg} mice increasing numbers of mice available for further experiments.

Rora^{wt/wt}

Rora^{sg/sg}



Figure 3.1 Gross appearance of *Rora*^{wt/wt} mice and *Rora*^{sg/sg} mice. *Rora*^{sg/sg} mice are smaller in size compared to age-matched *Rora*^{wt/wt} littermates. The photograph is representative of male littermates aged 4 weeks old.

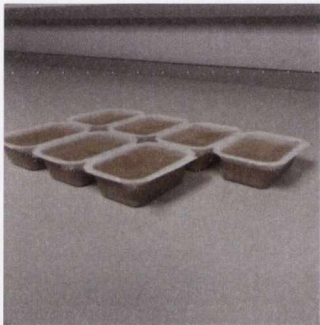
A Harlan Teklad Global 18%

Protein	18.6%
Fat	6.2%
Fibre	3.5%
Ash	5.3%
Carbohydrate	44.2%
Calories	3.1 kcal/g



Nutra-Gel Diet

Protein	9.5%
Fat	3.3%
Fiber	1.9%
Ash	0.9%
Moisture	<70%
Carbohydrate	22.2%
Calories	1.57 kcal/g



B

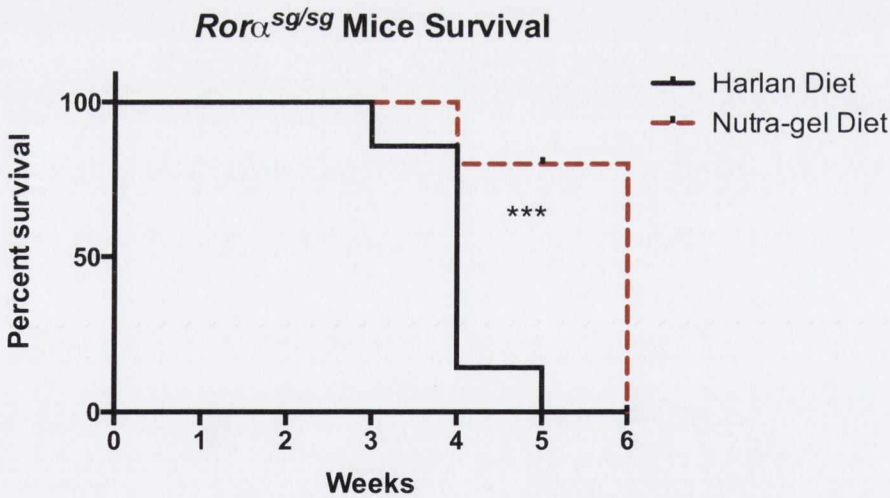


Figure 3.2 The percentage survival of *Rorα^{sg/sg}* mice is extended through the use of the Nutra-Gel diet. **A.** Nutritional composition of normal chow and Nutra-Gel diets. **B.** *Rorα^{sg/sg}* mice survival is extended through the use of Nutra-Gel diet compared to the Harlan diet. Kaplan-Meier method was used to evaluate survival. *** $p < 0.0001$ to 0.001 . *Rorα^{sg/sg}* mice $n=6$ per group.

3.3.1.1 ILC2s fail to expand in *Rora*^{sg/sg} mice following infection with *Nippostrongylus brasiliensis*.

Infection with the helminth *N. brasiliensis* promotes Th2 and ILC2 responses in mice (Neill et al., 2010) (**Section 2.5**). It has been shown that ILC2s fail to expand in *Rora*^{sg/sg} mice following infection with *N. brasiliensis* (Wong et al., 2012). We wanted to confirm this observation ourselves in *Rora*^{sg/sg} mice. ILC2s are present in the mesenteric lymph nodes (MLNs) of naïve mice where they represent less than 0.2% of cells but are expanded in MLNs during helminth infection (Neill et al., 2010). To explore ILC2 expansion in the absence of functional *Rora*, WT mice and *Rora*^{sg/sg} mice were infected with 500 *N. brasiliensis* L3 (**Section 2.5**) and sacrificed 6 days post infection.

ILC2s can be identified by a combination of several cell surface markers, including T1/ST2 (IL-33R), IL-25R, ICOS, c-kit, Sca-1, KLRG-1 and IL-7R α (Yagi et al., 2014). Here ILC2s in the MLNs were identified as Lin⁻ (CD3, CD19, CD11b, CD11c, Gr-1, CD4), Sca-1⁺, ST2⁺, ICOS⁺, and KLRG-1⁺. Analysis by flow cytometry showed that following 6 days of *N. brasiliensis* infection ILC2s were expanded in the MLNs of WT mice, but not in the MLNs of *Rora*^{sg/sg} mice (**Figure 3.3**). This confirmed that *Rora* is required for ILC2 expansion following infection with *N. brasiliensis* as reported by Wong and colleagues (2012).

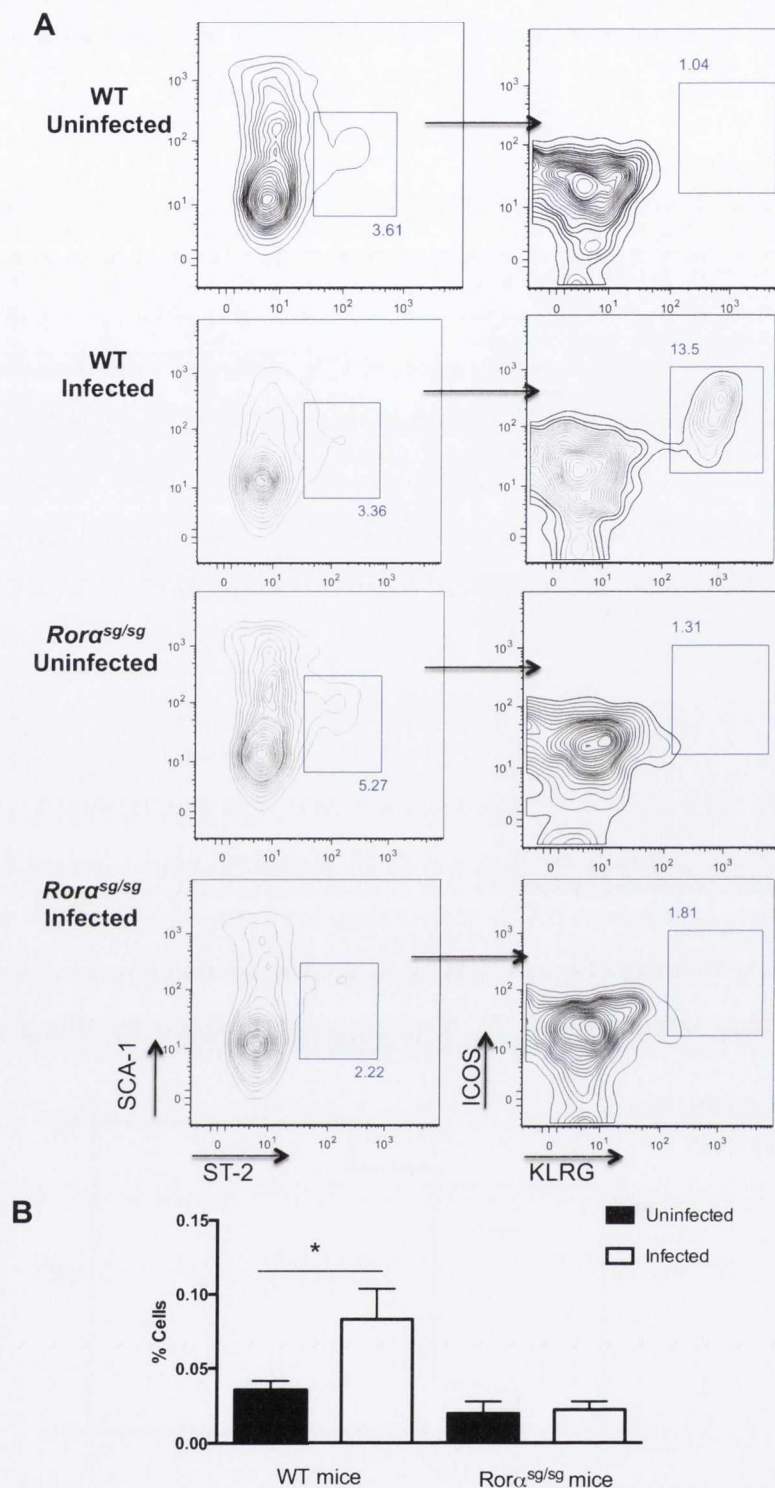


Figure 3.3 ILC2s are not expanded in the mesenteric lymph nodes (MLNs) of *Rora*^{sg/sg} mice after *N. brasiliensis* infection. **A** Representative flow plots of ILC2 gating (Lin⁻ (CD3, CD19, CD11b, CD11c, Gr-1, CD4) ST2⁺, Sca-1⁺, ICOS⁺ and KLRG1⁺) in MLNs following infection with 500 *N. brasiliensis* L3. **B** Quantification of ILC2 numbers in MLNs. All data represent means $\pm$ s.e.m. Differences, indicated as two-tailed *p* values, as assessed by unpaired Student *t* test. * *p* < 0.01 to 0.05. WT mice n=4 per group. *Rora*^{sg/sg} mice n=3 per group.

3.3.2 *Rora* gene is expressed in bone marrow-derived macrophages (BMDMs) and peritoneal macrophages from WT mice.

High throughput gene expression profiling (<http://biogps.org/#goto=genereport&id=19883>) has shown that *Rora* is expressed in unstimulated bone marrow derived macrophages (BMDMs) and peritoneal macrophages from WT mice (**Figure 1.8**). To confirm these observations, BMDMs and peritoneal macrophages were isolated from WT mice and cultured *in vitro* (**Section 2.3**). RNA was isolated from these macrophages populations and *Rora* expression was explored by qRT-PCR (**Section 2.11**) using a commercially available *Rora* Taqman Gene Expression Assay (Wong et al., 2012). *Rora* is known to be highly expressed in the mouse cerebellum (**Figure 1.8**), where it plays a role in the differentiation of Purkinje neurons (Gold et al., 2007) (**Section 1.15.4**), therefore RNA was isolated from whole brain from WT mice and used as a positive control for *Rora* expression. Analysis showed that *Rora* expression was detected in unstimulated BMDMs and peritoneal macrophages from WT mice (**Figure 3.4A**).

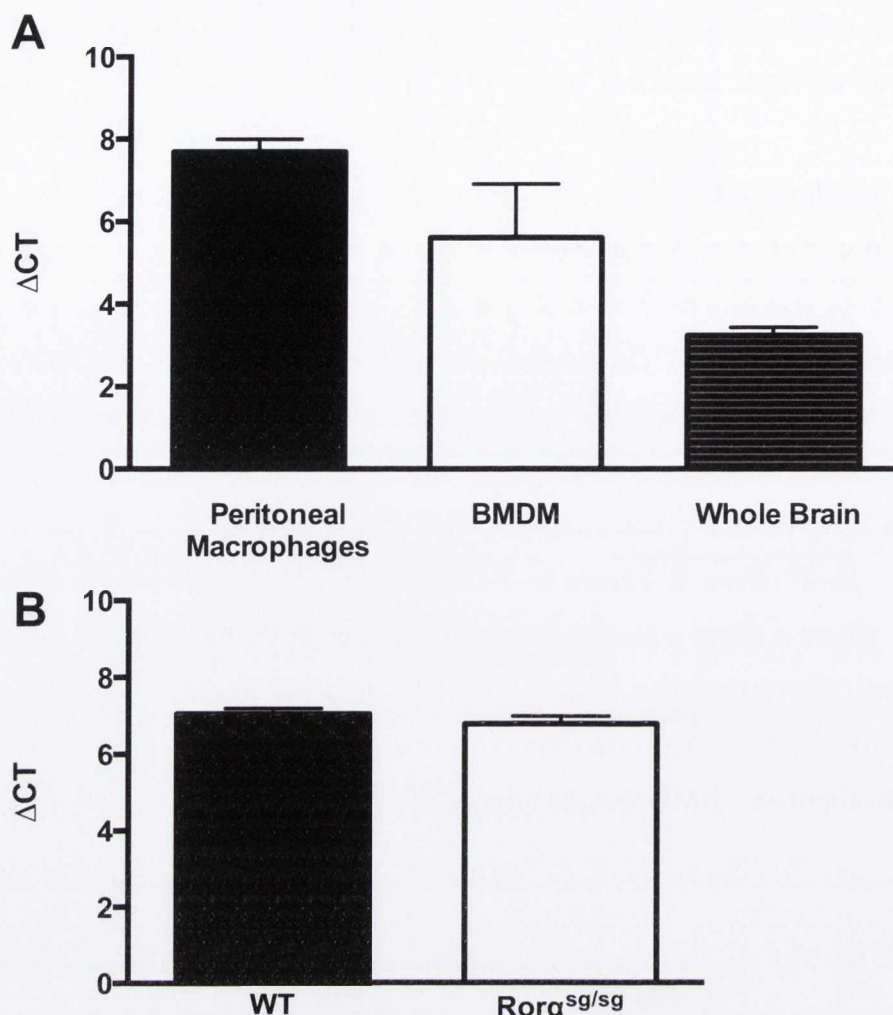


Figure 3.4 *Rora* expression in macrophages from WT mice and *Rora*^{sg/sf} mice using a commercial *Rora* Gene Taqman Assay. **A** *Rora* expression in peritoneal, BMDMs and whole brain from WT mice. **B** *Rora* expression in BMDMs from WT mice and *Rora*^{sg/sf} mice. $\Delta Ct = Ct \text{ target gene} - Ct \text{ Gapdh}$. All data represent means $\pm$ s.e.m. Differences, indicated as two-tailed *p* values, as assessed by unpaired Student *t* test. Gene expression was normalised to *Gapdh*. WT mice *n*= 4, *Rora*^{sg/sf} mice *n*=3

To test if the commercial *Rora* Taqman Gene Expression Assay, as used on BMDMs from WT mice in **Figure 3.5A**, also amplifies the DNA sequence in the mutated *Rora* gene, BMDMs were isolated and cultured from WT mice and *Rora*^{sg/sf} mice (**Section 2.3**). Gene expression analysis by qRT-PCR revealed that *Rora* expression is detected in BMDMs from both WT mice and *Rora*^{sg/sf} mice (**Figure 3.5B**). Analysis of the genomic sequence amplified by the commercially available *Rora* Taqman Gene Expression Assay (**Section 2.11**) revealed that the genomic sequence amplified in the commercial assay is located upstream of the deletion break-point that occurs in *Rora* in *Rora*^{sg/sf} mice (**Figure 3.6**). The primers in the commercial kit would therefore recognise and amplify this DNA

sequence in both WT mice and *Rora*^{sg/sg} mice, as the DNA sequence remains intact in *Rora*^{sg/sg} mice.

To ensure that the WT and mutated *Rora* genes could be distinguished by qRT-PCR, a custom Taqman Gene Expression Assay was designed. First, primers were designed for use in semi-quantitative PCR (sqPCR) that were specific to a genomic sequence deleted in *Rora* in *Rora*^{sg/sg} mice (**Section 2.11.1**) (**Figure 3.5**). Using these custom designed primers a product is amplified by sqPCR in the BMDM sample from WT mice. However, no amplified product is present in the BMDM sample from *Rora*^{sg/sg} mice (**Figure 3.6A**). This result indicates that the custom designed primers are specific for the sequence deleted in *Rora* in *Rora*^{sg/sg} mice.

Using the primer sequences verified above by sqPCR, a custom Taqman Gene Expression Assay was designed for use in qRT-PCR (**Section 2.11.1**). Using this custom Taqman Gene Expression Assay it was found that amplification was detected in the BMDM sample from WT mice but was not detected in the BMDM sample from *Rora*^{sg/sg} mice (**Figure 3.6B**). This result indicated that the custom gene expression assay could differentiate between the WT and mutated *Rora* gene found in *Rora*^{sg/sg} mice, thus confirming that *Rora* is mutated in macrophages from *Rora*^{sg/sg} mice. The optimisation of a specific qRT-PCR assay for *Rora* was important for validating results from subsequent experiments using macrophages from *Rora*^{sg/sg} mice.

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 agcttgtagccgaggtgcagaagcaccggatgcagcagcagcagcagagaccaccagca
 gcagcctggggaggcggagccgctgacgcccacctacaacatctcagccaatgggctgac
 ggaactgcatgatgacctcagcacctatatggatgggcacacccccgagggcagcaaggc
 cgactcagccgtcagcagcttctacctggacatccagccctccccagaccagtcgggattgg
 acatcaatgggatcaaaccgaaccatattgtgactacacaccagcatctggcttctccct
 actgttccttcaccaacggagagacttcccccaaccgtgtccatggcagaactag****aaca
 cct**tgcccagaacatatccaaatcccacctggaaacctgccagtacttgcgggaagagct**
 ccagcagataacgtggcagac**ccttctgcaggaggagatt**gaaaactaccagaacaagc
 agagagaggtgatgtggcagctgtgtgccatcaagattacagaagctatccagtatgtggtg
 gagtttgccaaacgcattgatggatttatggagctgtgtcaaatgatcaattgtgcttctaaa
 agcaggctcgtagaggtggtgtttattaggatgtgccgtgcctttagctctcagaacaacac
 cgtgtactttgacgggaagtatgcgagccccgatgtcttcaagtccctaggtgtgaagacttc
 atcagctttgtgtttgaatttgggaagagtttgtgttctatgcacctgaccgaagacgaaatcgc
 gttattttctgcattcgtactgatgtcagcggatcgtcgtggttcaggaaaaggtaaaaatag
 aaaagctgcaacagaaaattcagctggcccttcagcacgtcctacagaagaaccaccgaga
 agatggaattctaaccaagctaatatgcaaggtgtctacgttaagagccctatgtggacgaca
 tacggaaaagctaattggcatttaaagcaatataccagacattgtgcgactccattttcctccat
 tatacaaggaattgttcacttcagaatttgagccagccatgcaaactgatgggtaaatgtcgcg
 cccgagcac.....

Figure 3.5 Rora mRNA sequence. NCBI Reference Sequence: NM_013646.2. The sequence in red represents the region amplified by the commercially available Rora Taqman Gene Expression Assay. The asterix (****) represents the point that the deletion break-point occurs in *Rora^{sg/sg}* mice. The sequence in blue represents the sequence of the forward primer of the custom Rora Taqman Gene Expression Assay. The sequence in blue and underlined represents the sequence of the reverse primer of the custom Rora Taqman Gene Expression Assay.

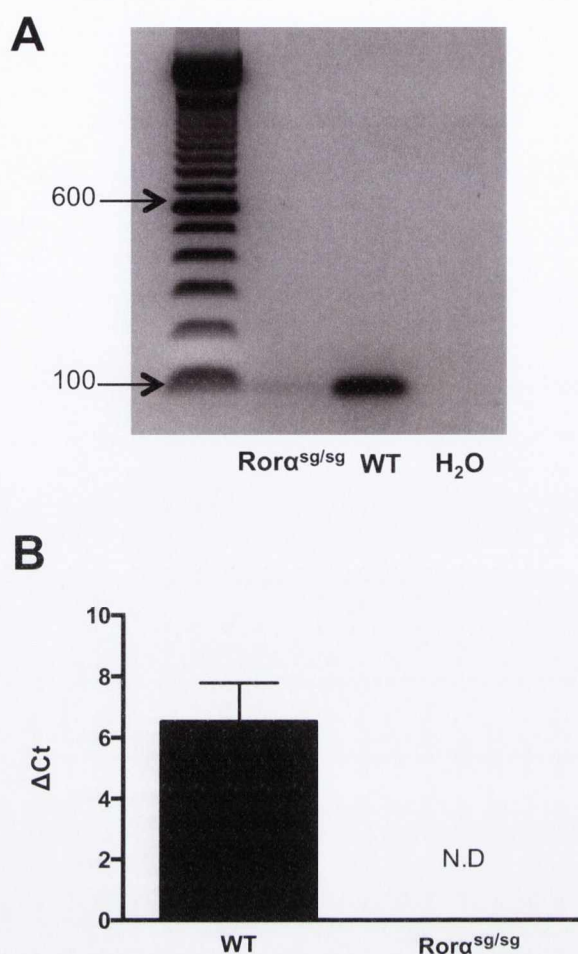


Figure 3.6 *Rora* expression in macrophages from WT mice and *Rora*^{sg/sg} mice using a custom designed *Rora* Gene Taqman Gene Expression Assay. **A** Agarose gel showing sqPCR band amplified in BMDM samples from WT mice and *Rora*^{sg/sg} mice using a custom designed *Rora* Gene Taqman Gene Expression Assay. **B** *Rora* expression in BMDM samples from WT mice and *Rora*^{sg/sg} mice using a custom designed *Rora* Gene Taqman Gene Expression Assay. $\Delta\text{Ct} = \text{Ct target gene} - \text{Ct Gapdh}$. All data represent means $\pm$ s.e.m. Differences, indicated as two-tailed *p* values, as assessed by unpaired Student *t* test. Gene expression was normalised to *Gapdh*. ND= Not detected. WT mice *n*=4, *Rora*^{sg/sg} mice *n*=3

3.3.3 *Rora* protein is detected in *Rora*^{sg/sg} mice.

Having established that the genomic sequence upstream of the deletion breakpoint in the *Rora* gene in *Rora*^{sg/sg} mice remains intact, it was predicted that a truncated *Rora* protein could be made in *Rora*^{sg/sg} mice. Indeed others have also suggested that a truncated protein is translated in *Rora*^{sg/sg} mice (Dussault et al., 1998). The deletion in the *Rora* gene in

Rora^{sg/sg} mice occurs in the sequence encoding the ligand-binding domain (LBD) leading to an alteration of the amino acid sequence and the introduction of a premature stop codon (**Figure 3.7**). Therefore, it was postulated that a truncated *Rora* protein would contain the DNA binding domain (DBD) at the N terminus and also the hinge region but have a truncated C terminus LBD.

WT mice

mesapaapdpaasepgssgseaaagsretpltqdtgrkseapgagrrqsyasssrgisvtkkt
htsqieiipckicgdkssgihygvitcegcgkffrrsqqsnatyscprqknclidrtsnrcqhc
rlqkclavgmsrdavkfgrmskkqrdsl yaevqkhrmqqqqrdhqqqpgeaepltptyn
isangltelhddlstymdghtpegskadsavssfyldiqpspdqsgldingikpepicdytp
asgffpysfntgetsptvsmalehlaqniskshletcqlreelqqitwqtflqeeienyqn
kqrevmwqlcaikiteaiqyvvefakridgfmelcqndqivllkagslevvfirncrafdsq
nntvyfdgkyaspdvfksgcedfisfvfegkslcsmlhtedeialfsafvlmsadrswlqe
kvkiekllqqikqlalqhvllqknhredgiltclickvstlralcgrhteklmafkaiypdivrlhf
pplykelftsefepamqidg

***Rora*^{sg/sg} mice**

mesapaapdpaasepgssgseaaagsretpltqdtgrkseapgagrrqsyasssrgisvtkkt
htsqieiipckicgdkssgihygvitcegcgkffrrsqqsnatyscprqknclidrtsnrcqhc
rlqkclavgmsrdavkfgrmskkqrdsl yaevqkhrmqqqqrdhqqqpgeaepltptyn
isangltelhddlstymdghtpegskadsavssfyldiqpspdqsgldingikpepicdytp
asgffpysfntgetsptvsmalehlaqniskshletcqlreelqqitwqtflqeeienyqn
kergdvaavchqdyrsypvcggvcqth*

Figure 3.7 *Rora* amino acid sequence in WT mice and *Rora*^{sg/sg} mice. NCBI Reference Sequence: NP_038674.1. The underlined sequence represents the ligand-binding domain (LBD) in *Rora* that is altered in *Rora*^{sg/sg} mice as a result of the upstream genomic deletion in *Rora*^{sg/sg} mice. The amino acid sequence highlighted in red is deleted in *Rora*^{sg/sg} mice. The amino acid sequence in bold represents the sequence changed as a result of the upstream genomic deletion in *Rora*^{sg/sg} mice. The asterix (*) represents a stop codon.

To explore the possibility that a truncated *Rora* protein is made in *Rora*^{sg/sg} mice, a commercial polyclonal antibody (Santa Cruz Biotechnology) raised against an epitope located in the C-terminus of *Rora* was chosen as the C-terminus is the region predicted to be truncated in *Rora*^{sg/sg} (**Section 2.9**). As mentioned previously *Rora* is highly expressed

in the brain, therefore it was decided to first test the specificity of the Ror α antibody on whole brain lysate. Brain tissue was isolated from WT mice and Ror $\alpha^{sg/sg}$ mice and a protein extract prepared; following this a western immunoblot was performed for detection of Ror α protein (**Section 2.9**). The antibody raised against an epitope located in the C-terminus detected a protein at around 60 kDa in whole brain lysate from both WT mice and Ror $\alpha^{sg/sg}$ mice (**Figure 3.8A**). The predicted molecular weight of Ror α using the N-terminus antibody is 67 KDa.

To ensure that the commercial Ror α antibody used was indeed specific for Ror α , an immunoblot for Ror α was performed on whole liver lysate prepared from WT mice fed *ad libitum* and fasted WT mice. It has been reported that Ror α levels are increased in the livers of fasted mice compared to mice fed *ad libitum*, due to cAMP activated protein kinase A-mediate phosphorylation and stabilisation of the protein (Pathak et al., 2013). It was hypothesised that if the commercial antibody is specific for Ror α it would detect this increased Ror α protein expression in the liver lysate from fasted mice. To test this hypothesis WT mice were fed mice fed *ad libitum* or fasted for 12 hours (8pm to 8am), sacrificed and their livers were harvested. Results showed that Ror α protein expression was increased in livers from fasted mice compared to mice fed *ad libitum* (**Figure 3.8B**).

This result indicates that the antibody raised against an epitope located in the C-terminus of Ror α is specific for Ror α ; therefore, indicating that the protein being detected in brain lysate from WT mice and Ror $\alpha^{sg/sg}$ mice (**Figure 3.8A**) is indeed Ror α . Exact details on the C-terminus epitope recognised by the antibody are not available but these results suggest that the antibody binds to a site that is present in both WT and truncated Ror α protein forms.

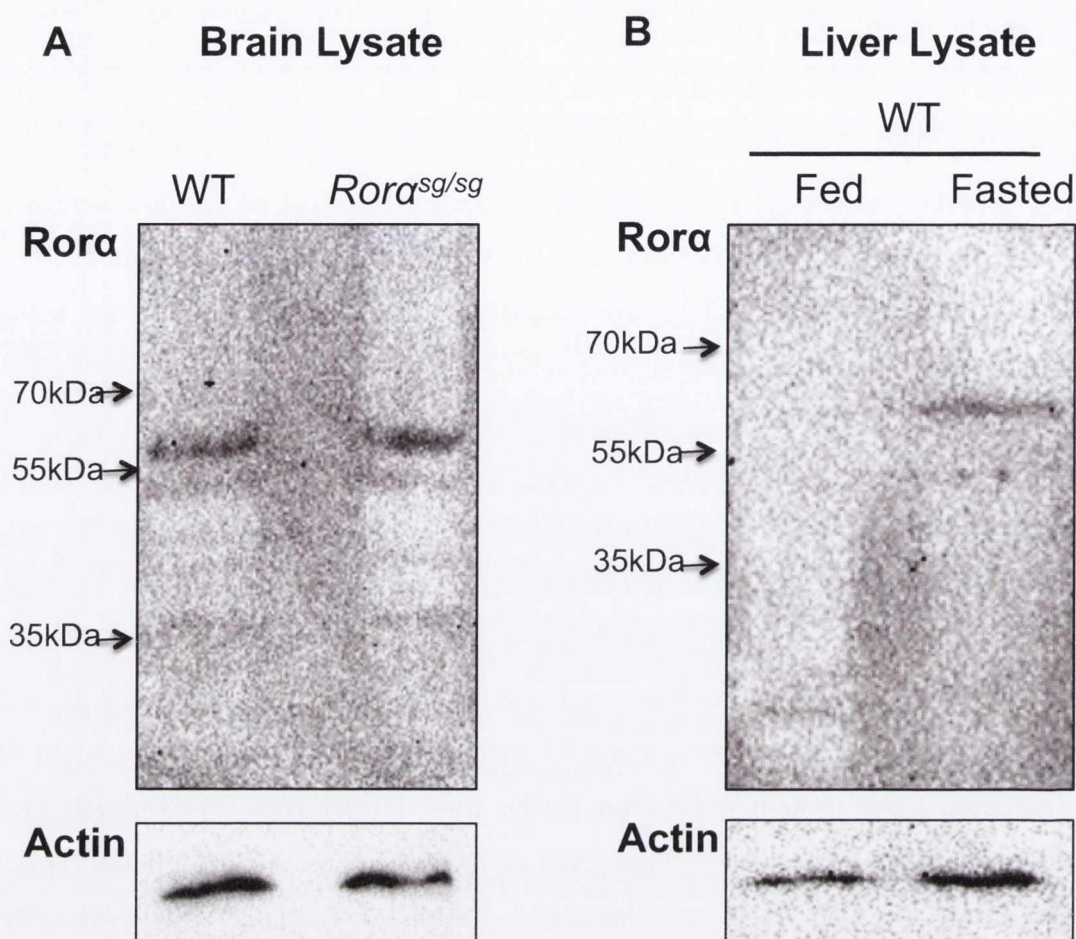


Figure 3.8 *Rora* protein is detected in brain lysate from WT mice and *Rora*^{sg/sg} mice. **A** *Rora* was detected in the whole brain lysate from WT mice and *Rora*^{sg/sg} mice. The predicted molecular weight of *Rora* is 67KDa. **B** Mice were fed *ad libitum* or fasted for 12 hours (8pm to 8am) and then sacrificed. *Rora* expression was induced in the liver lysate of fasted WT mice compared to mice fed *ad libitum*.

3.3.4 BMDMs can be polarised towards an M1 and M2 phenotype *in vitro* in the absence of functional *Rora*.

Having established that *Rora* is expressed in macrophages from WT mice it was next determined if *Rora* has a role in the polarisation of macrophages towards an M1 and M2 phenotype *in vitro*. Macrophages have historically been classified as being either M1 or M2 depending on their protein and gene expression profiles (Section 1.6.3). In the standard protocol used for polarizing macrophages, BM cells are cultured in the growth factor M-CSF to expand BMDMs followed by polarisation with an M1 or M2 stimulus (Section 2.4). Here, the addition of LPS + IFN γ was used to polarise BMDMs towards an

M1 phenotype, with the addition of IL-4 + IL-13 was used to polarise towards an M2 phenotype (**Section 2.4**).

To investigate if *Rora* has a role in the polarisation of BMDMs towards an M1 and M2 phenotype *in vitro*, BMDMs from WT mice and *Rora*^{sg/sg} mice were cultured and polarised as described (**Section 2.4**). Polarisation was measured in terms of increased expression of intracellular or surface M1 or M2 associated proteins (iNos, PD-L1, MHCII, Relma, CD206, PD-L2) or M1 or M2 genes (*iNos* and *Arg1*, respectively) (**Section 1.6.3**). Analysis by flow cytometry and qRT-PCR confirmed the M1 and M2 polarisation of BMDMs from WT mice in terms of protein markers (**Figure 3.9**) and transcript markers (**Figure 3.10**). M1 and M2 polarisation of BMDMs from *Rora*^{sg/sg} mice were comparable to WT cells in terms of protein markers (**Figure 3.9**) and transcript markers (**Figure 3.10**). These results show that M1 and M2 polarisation can occur *in vitro* in the absence of functional *Rora*. Therefore *Rora* does not have a role in the polarisation of BMDMs towards an M1 and M2 phenotype in the *in vitro* cell culture conditions used herein.

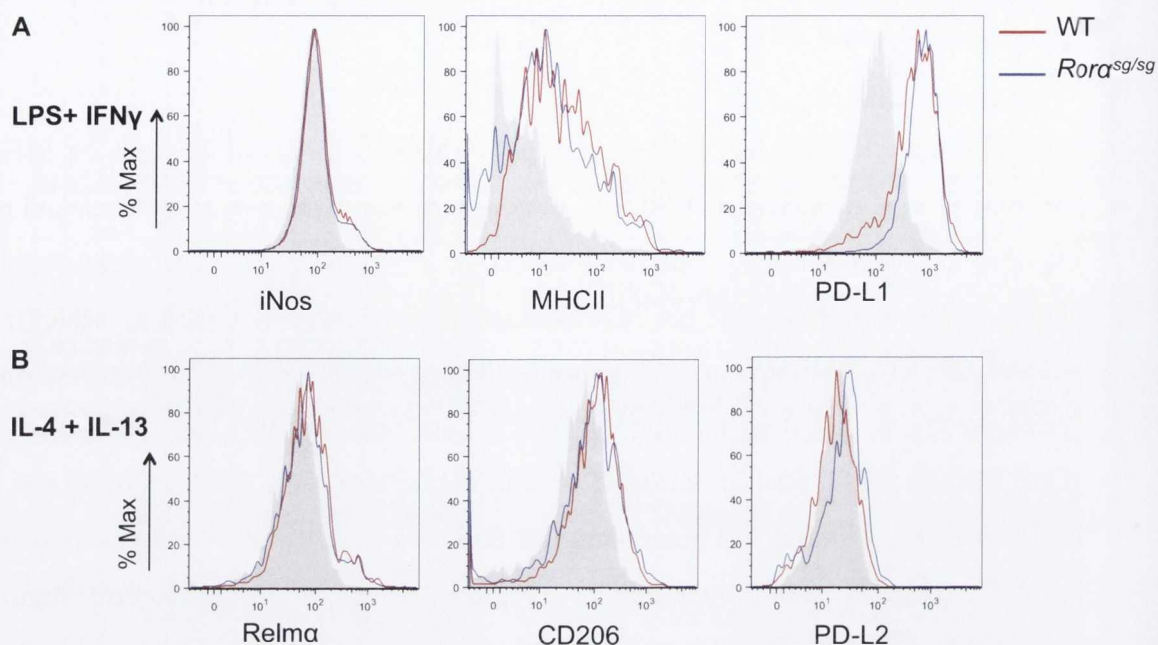


Figure 3.9 Polarisation of BMDMs towards an M1 and M2 phenotype *in vitro* occurs in the absence of functional *Rora*. Polarised BMDMs from WT mice and *Rora*^{sg/sg} mice (pre-gate: CD11b⁺, F4/80⁺). **A** Representative flow cytometry plots of expression of M1 markers (iNos, PD-L1 and MHCII) in BMDMs stimulated with LPS+IFN γ **B** M2 markers (Relma, CD206 and PD-L2), in BMDMs stimulated with IL-4+IL-13. Data is representative of three independent experiments.

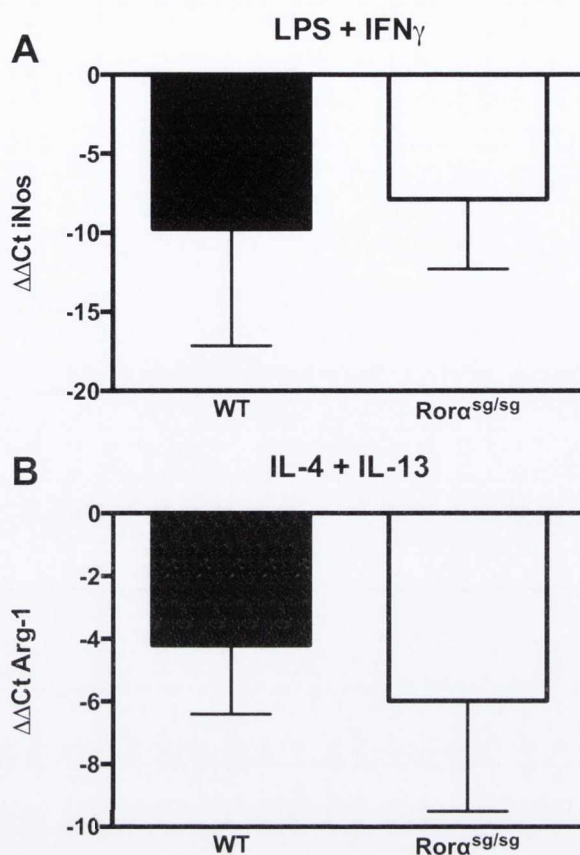


Figure 3.10 Gene expression in polarised BMDMs from WT mice and $Rora^{sg/sg}$ mice. Comparison of mRNA expression of prototypical (A) M1 markers *iNos* and (B) M2 marker *Arg-1* in BMDMs from WT mice and $Rora^{sg/sg}$ mice following *in vitro* polarisation. $\Delta\Delta\text{Ct} = \Delta\text{Ct stimulated (Ct target gene - Ct Gapdh)} - \Delta\text{Ct unstimulated (Ct target gene - Ct Gapdh)}$. All data represent means $\pm$ s.e.m. from three independent experiments. Differences, indicated as two-tailed *p* values, as assessed by unpaired Student *t* test. WT mice *n*=3, $Rora^{sg/sg}$ mice *n*=3.

3.3.5 The absence of functional *Rora* alters peritoneal macrophage populations *in vivo*.

In recognition that *in vitro* generated macrophages represent extremes on a continuum of macrophage phenotypes, the next step taken was to explore the role *Rora* has in macrophages *in vivo*. The peritoneal cavity in mice is the preferred site for the collection of macrophages, due to the presence of a high number of naïve macrophages and the ease of access to this site (Zhang et al., 2008). As such the peritoneal cavity was chosen as the site of macrophage isolation in this study. Two peritoneal macrophage populations have

previously been identified and characterised in mice; the large peritoneal macrophage (LPM) and the small peritoneal macrophage (SPM) (Ghosn et al., 2010) (**Section 1.10.3**).

The two populations were initially defined by their differential expression of the prototypical macrophage markers CD11b and F4/80; LPMs express higher levels of both CD11b and F4/80 compared to SPMs. Phenotypic profiling by flow cytometry revealed that the two populations differentially expressed a number of other macrophage markers including Gr-1, CD80, CD86, CD11c and MHCII (Ghosn et al., 2010). Ghosn et al (2012) speculated on the origin of these two populations and considered the SPM to be of monocyte origin, however, the origin of LPMs was unclear. Their study was completed before it was shown that macrophages, including peritoneal macrophages, can originate from the adult bone marrow, monocyte-derived macrophages, or be of embryonic origin, tissue-resident macrophages (Yona et al., 2013) (**Section 1.9-1.10**).

With this in mind, it was decided to characterise peritoneal macrophages based on their origin, i.e. being either blood monocyte-derived or tissue-resident macrophages. As such the antibody panel used here included cell surface markers that could identify both these populations including CD11b, F4/80, MHCII and Tim4. This antibody panel is based on the gating strategy used by Rosas and colleagues in their 2014 paper that identifies the transcription factor Gata6 as having a role in tissue-resident peritoneal macrophages. To identify peritoneal macrophage populations cells were isolated from the peritoneal cavities of naive WT mice and characterised by flow cytometry. Four peritoneal populations were identified in WT mice following pre-gating on CD11b and F4/80 expressing cells and then on the basis of Tim4 and MHCII expression. These four populations were: Tim4⁺ MHCII⁻, Tim4⁺ MHCII⁺, Tim4⁻ MHCII⁺ and Tim4⁻ MHCII⁻ populations (**Figure 3.11A**).

Having identified these four peritoneal populations in WT mice, it was next determined if any of these populations were altered in the absence of functional *Rora*. To achieve this, cells were isolated from the peritoneal cavities of naive *Rora*^{sg/sg} mice and characterised by flow cytometry. Analysis by flow cytometry revealed that *Rora*^{sg/sg} mice had statistically significantly decreased levels ($p < 0.037$ and $p < 0.0006$) of the Tim4⁻ MHCII⁺ and Tim4⁻ MHCII⁻ peritoneal populations compared to WT mice (**Figure 3.12**). Levels of the Tim4⁺ MHCII⁻ and Tim4⁺ MHCII⁺ peritoneal populations were comparable between the two groups (**Figure 3.12**).

Rosas and colleagues (2014) considered the Tim4⁺ MHCII⁻ population to be tissue-resident macrophages, the Tim4⁻ MHCII⁺ population as being monocyte-derived macrophages and the Tim4⁻ MHCII⁻ population as being eosinophils. Rosas and colleagues did not identify the Tim4⁺ MHCII⁺ peritoneal population. Based on this classification these results suggest that blood monocyte-derived macrophages and eosinophils are decreased in the peritoneal cavity in the absence of functional *Rora*.

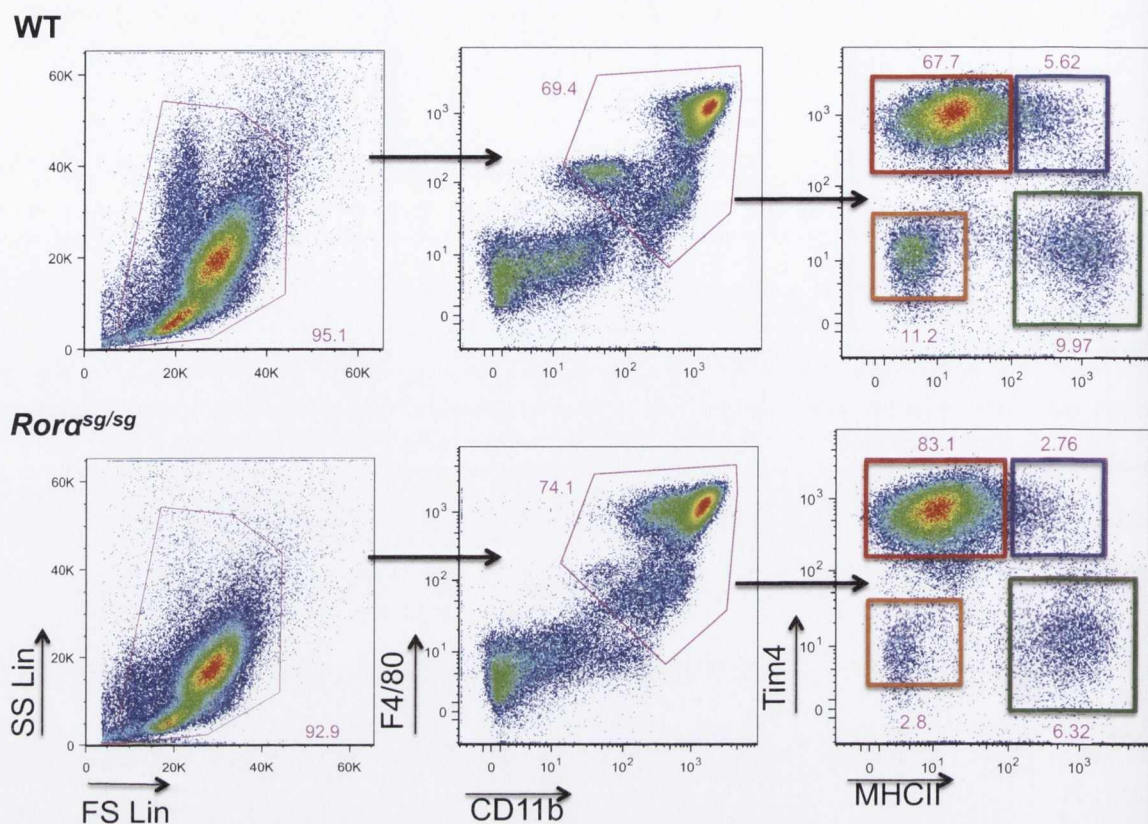


Figure 3.11 Representative flow cytometry plots of the gating strategy used in WT mice and *Rora^{sg/sq}* mice for identifying peritoneal populations. Based on CD11b, F4/80, MHCII and Tim4 expression, percentages indicate typical proportions of the cell types of all peritoneal cells. Doublet exclusion was also performed.

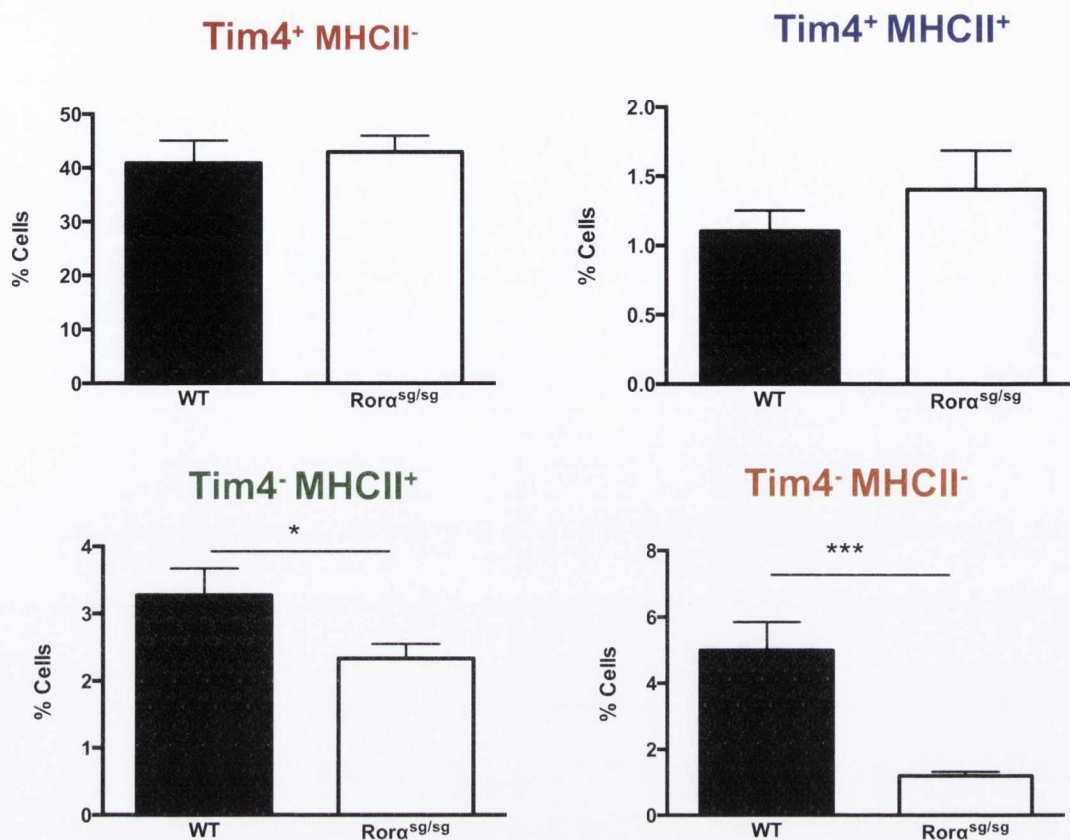


Figure 3.12 Quantification of peritoneal populations in WT mice and *Rora*^{sg/sg} mice analysed by flow cytometry in Figure 3.11. All data represent means $\pm$ s.e.m. Differences, indicated as two-tailed *p* values, as assessed by unpaired Student *t* test. * $p < 0.01$ to 0.05 , *** $p < 0.0001$ to 0.001 . WT mice $n=15$, *Rora*^{sg/sg} mice $n=12$.

It is worth noting that due to the decreased size of *Rora*^{sg/sg} mice compared to WT mice there is a significant decrease ($p < 0.0001$) in the number of peritoneal cells isolated per 1 ml from the peritoneal cavity of *Rora*^{sg/sg} mice compared to WT mice (**Figure 3.13**). This in turn leads to a significant decrease in cell numbers for each of the four peritoneal populations isolated from *Rora*^{sg/sg} mice compared to WT mice (**Figure 3.13**). Although the number of cells in each of the four peritoneal populations are decreased in *Rora*^{sg/sg} mice it is interesting that the levels of the Tim4⁺ MHCII⁻ and Tim4⁺ MHCII⁺ peritoneal populations are the same in *Rora*^{sg/sg} mice compared to WT mice when looking at percentage of total cells (**Figure 3.12**). This indicates that although decreased cell numbers are isolated from *Rora*^{sg/sg} mice the number of cells in each peritoneal population is relative to total overall cell number. This indicates that the decrease in the Tim4⁻ MHCII⁺ and Tim⁻ MHCII⁻ populations is due to the absence of *Rora* and not just due to there being decreased cell numbers isolated from *Rora*^{sg/sg} mice compared to WT mice.

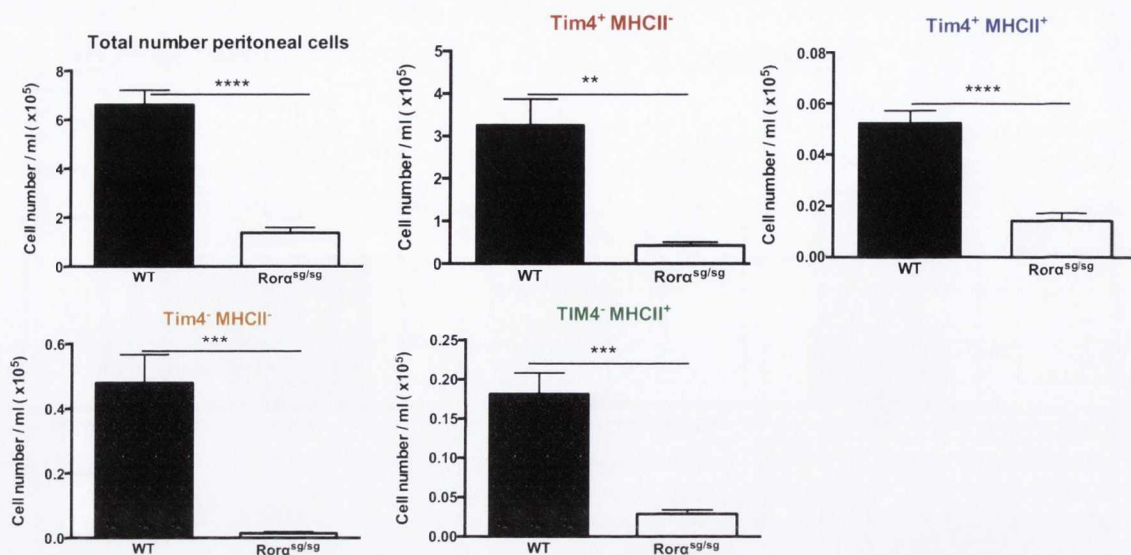


Figure 3.13 Decreased number of cells are isolated from *Rora*^{sg/sg} mice compared to WT mice. Quantification of number of cells/ml in each peritoneal populations in WT mice and *Rora*^{sg/sg} mice, as analysed by flow cytometry. All data represent means $\pm$ s.e.m. Differences, indicated as two-tailed *p* values, as assessed by unpaired Student *t* test. ** $p < 0.001$ to 0.01 ***, $p < 0.0001$ to 0.001 , **** $p < 0.0001$. WT mice $n=9$, *Rora*^{sg/sg} mice $n=6$.

3.3.6 The Tim4⁻ MHCII⁺ peritoneal macrophage population expresses markers of infiltration.

Having identified that *Rora*^{sg/sg} mice have decreased levels of two of the peritoneal populations identified by CD11b, F4/80, Tim4 and MHCII expression (**Figure 3.12**), including a possible blood monocyte-derived population, it was important to characterise these cell populations further. Therefore the expression levels of an array of protein markers were analysed by flow cytometry on the four populations identified in **Figure 3.11**. Tissue-resident peritoneal macrophages are classically considered to have an M2 phenotype and blood monocyte-derived macrophages are considered to have an M1 phenotype in the steady state (Davies et al., 2013). We therefore explored the expression of a selection of M1 and M2 associated markers on each of the four populations identified.

Analysis by flow cytometry revealed that the possible blood monocyte-derived Tim4⁻ MHCII⁺ peritoneal population expressed M2 macrophage associated markers Relm α and CD206 (**Figure 3.14A**). Interestingly, the Tim4⁻ MHCII⁺ peritoneal population also expressed markers of M1 macrophage associated markers including infiltration markers

CCR2 and CX₃CR1 (**Figure 3.14B**). These results indicate that the Tim4⁺ MHCII⁺ peritoneal population expresses both M1 and M2 macrophage associated cell surface markers suggesting that this population is an infiltrating population that also has an M2-like phenotype in the peritoneal cavity.

The possible tissue-resident macrophage Tim4⁺ MHCII⁺ peritoneal population identified in **Figure 3.11** expressed M2 macrophage markers PD-L2, CD80 and CD86 but did not express M1 macrophage markers (**Figure 3.14**). The expression of M2 markers and the lack of expression of M1 macrophage markers and infiltration markers indicate that the Tim4⁺ MHCII⁺ peritoneal population is indeed a tissue-resident macrophage population that has an M2-like phenotype. The Tim4⁺ MHCII⁺ peritoneal population expressed M1 and M2 macrophage markers including CD206, PD-L2, CD80 and CD86 and CCR2 and CX₃CR1. The Tim4⁺ MHCII⁺ peritoneal population also expressed CD11c and CD103 (**Figure 3.14**). The expression of CD64, the high-affinity IgG receptor FcγR, has been used to distinguish macrophages from DCs in the lamina propria of the large and small intestine and the MLNs of mice (Tamoutounour et al., 2012). In the peritoneal cavity the Tim4⁺ MHCII⁺ peritoneal population expresses CD64. Taken together these results suggest that the Tim4⁺ MHCII⁺ peritoneal population represents a mixed dendritic population.

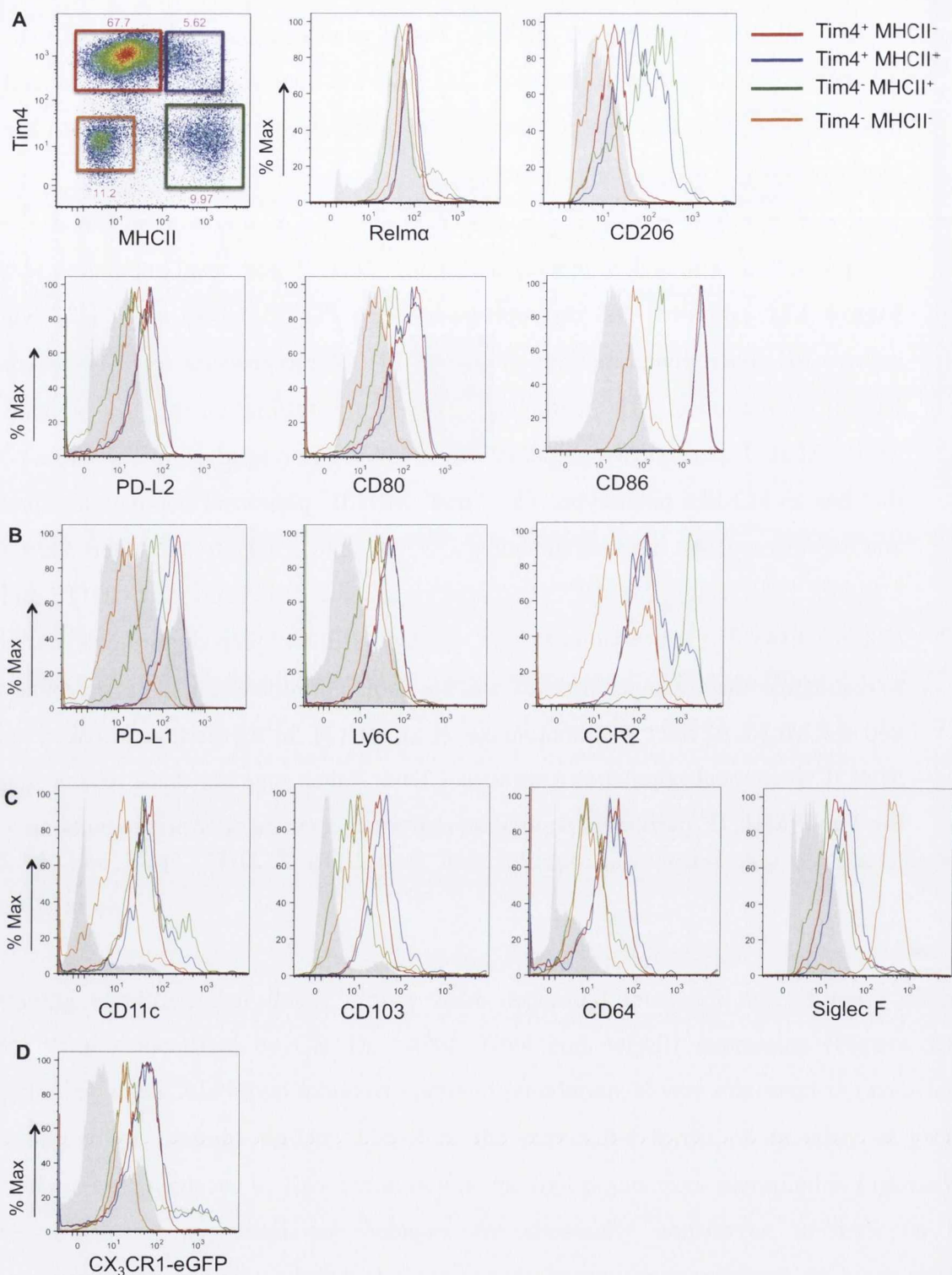


Figure 3.14 The Tim4⁻ MHCII⁺ peritoneal population expresses markers of M2 macrophages and markers of infiltration. Cell surface marker expression on each of the four peritoneal populations identified by Tim4 and MHCII expression (pre-gate CD11b⁺, F4/80⁺). **A** M2 associated marker expression. **B** M1 associated marker expression. **C** Other markers associated with macrophages/dendritic cells and eosinophils. **D** CX₃CR1 expression as measured by eGFP fluorescence in CX₃CR1-eGFP mice.

Monocyte derived-macrophages have been shown to have high levels of aldehyde dehydrogenase (ALDH) activity, with ALDH activity a potential marker for blood monocyte derived-macrophages (Gundra et al., 2014). ALDH enzymes regulate the production of retinoic acid with important implications for immune regulation. The ALDEFLUOR™ reagent system (**Section 2.14**) was used to measure ALDH activity in the peritoneal cell populations identified by Tim4 and MHCII expression (**Figure 3.15A**). ALDH activity was highest in the Tim4⁺ MHCII⁻ peritoneal population followed by the Tim4⁺ MHCII⁺ and Tim4⁻ MHCII⁺ peritoneal populations (**Figure 3.15B**). ALDH activity was the lowest in the Tim4⁻ MHCII⁻ peritoneal population (**Figure 3.15B**). These results suggest that under basal conditions ALDH activity is the highest in tissue-resident peritoneal macrophages.

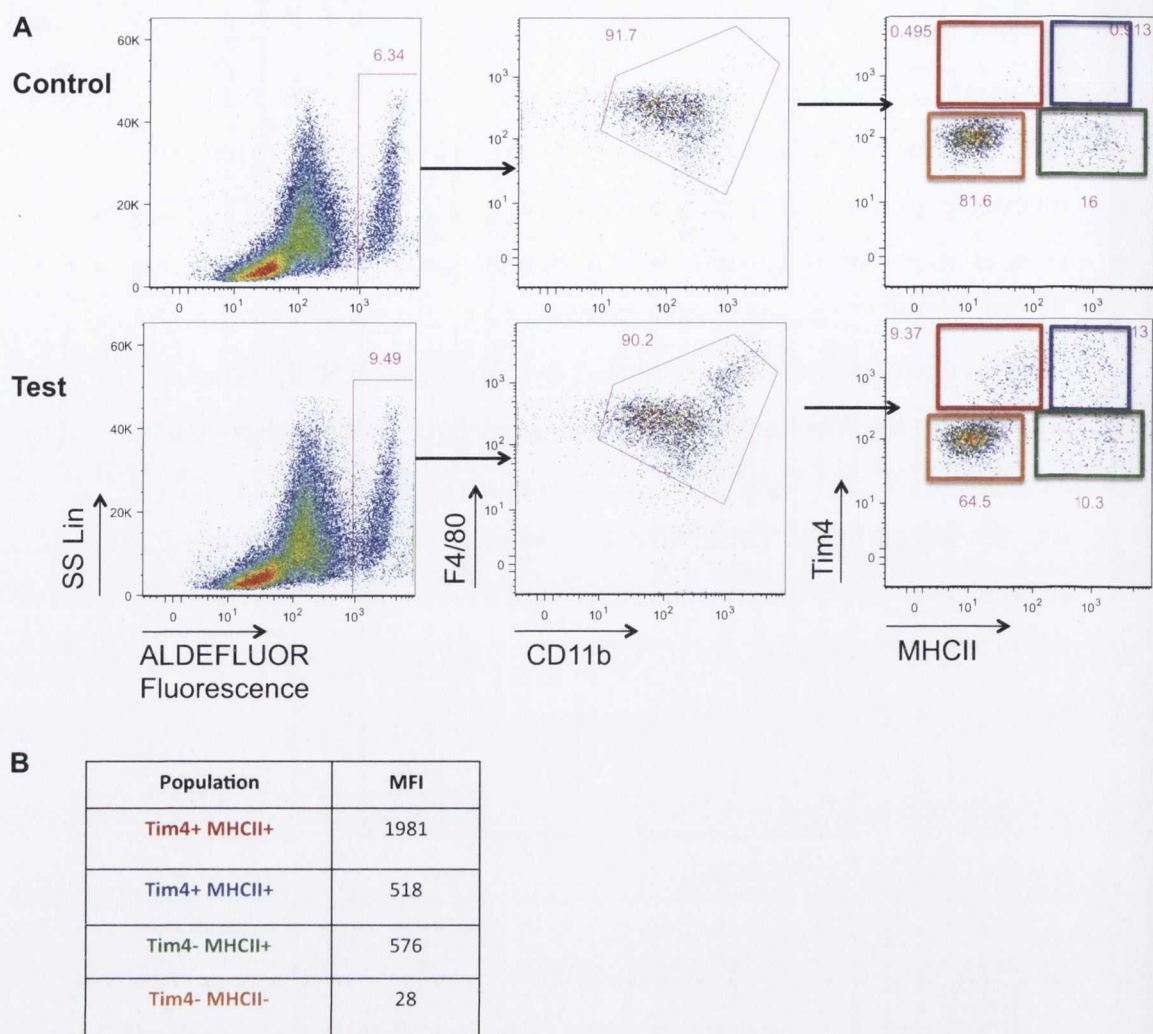


Figure 3.15 ALDH activity in peritoneal populations. **A** Representative flow cytometry plots of gating strategy used for identifying ALDH activity in peritoneal populations. Control: Peritoneal cells exposed to ALDEFLUORTM in the presence of the ALDH inhibitor, DEAB. Test: Peritoneal cells exposed to ALDEFLUORTM (Section 2.14). **B** MFI values for each identified peritoneal population. MFI: mean fluorescent intensity. MFI calculated: MFI test-MFI test.

3.3.7 Eosinophil and neutrophil levels in the peritoneal cavity are altered in the absence of functional *Rora*.

Rosas et al. (2014) suggested that the Tim4⁻ MHCII⁻ peritoneal population in the peritoneal cavity contains eosinophils. Eosinophils are commonly found in the peritoneal cavity and together with neutrophils, monocytes and mast cells they account for 2–3% of the total cells (Ghosn et al., 2010; Misharin et al., 2012). Eosinophils typically express the marker

Siglec-F (Rosenberg et al., 2013). Siglec-F is a cell surface lectin belonging to the Ig superfamily that binds glycoconjugates containing sialic acids that are commonly found on various cell types. To confirm that the Tim4⁻ MHCII⁻ peritoneal cell population contains eosinophils Siglec-F expression was analysed on Tim4⁻ MHCII⁻ peritoneal cells. Analysis by flow cytometry revealed that the Tim4⁻ MHCII⁻ peritoneal cell population contained cells that expressed higher levels of Siglec-F compared to the other cell populations (**Figure 3.14C**), thus indicating that they are eosinophils.

To confirm this further, the position the Tim4⁻ MHCII⁻ Siglec-F⁺ cells are located on a FS Lin v SS Lin flow cytometry plot was explored. Eosinophils are granulocytes and as such have high granularity and therefore produce a characteristic high side scatter (SS^{High}) and an intermediate forward scatter (FS^{Int}). Using the back-gating technique in FlowJo, it was found that the Tim4⁻ MHCII⁻ Siglec-F⁺ peritoneal cells identified in were SS^{High} FS^{Int} on the FS Lin v SS Lin flow cytometry plot (**Figure 3.16A**). To validate that these SS^{High} FS^{Int} cells are eosinophils, the expression of CCR3 was explored. Along with Siglec-F, CCR3 is highly expressed by eosinophils and is important for their migration to sites of inflammation (Humbles et al., 2002). It was found that SS^{High} FS^{Int} cells expressed both CCR3 and Siglec-F (**Figure 3.16B**). This confirmed that the Tim4⁻ MHCII⁻ peritoneal population contains eosinophils.

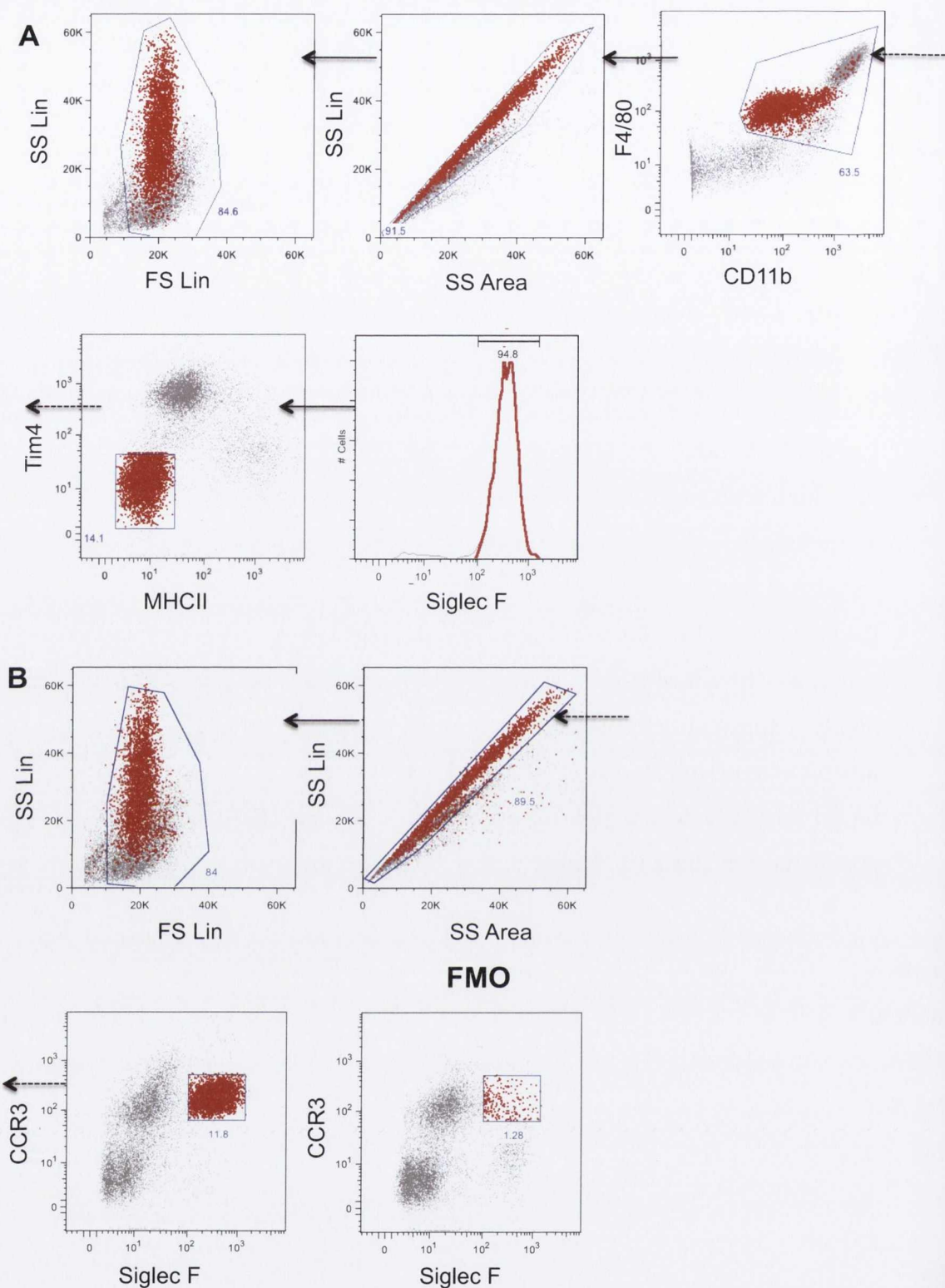


Figure 3.16 The $\text{Tim4}^- \text{MHCII}^- \text{Siglec-F}^+$ peritoneal population contains eosinophils. **A** Back-gating of the $\text{Tim4}^- \text{MHCII}^- \text{Siglec-F}^+$ peritoneal population. **B** Back-gating of Siglec-F^+ and CCR3^+ peritoneal cells.

The Tim4⁺ MHCII⁺ peritoneal population is decreased in *Rora*^{sg/sg} mice compared to WT mice (**Figure 3.12**). To explore eosinophil levels in these groups in a more detailed way, Siglec-F and CD11b were used as cell surface markers to identify eosinophils (Siglec-F⁺ CD11b^{Int}) by flow cytometry (**Figure 3.17A**). Results confirmed that by this gating strategy eosinophils were significantly ($p < 0.0001$) decreased in *Rora*^{sg/sg} mice compared to WT mice (**Figure 3.17A**).

The finding that eosinophils were decreased in *Rora*^{sg/sg} mice compared to WT mice led to the examination of the neutrophil population in *Rora*^{sg/sg} mice and WT mice. This decision was made based Satoh et al. (2013) reporting that in the absence of Trib1, a pseudokinase protein, mice displayed decreased levels of F4/80⁺ CD206⁺ tissue-resident macrophages and eosinophils but also increased levels of neutrophils. Neutrophil levels are low in the unstimulated peritoneal cavity of mice with levels increasing during inflammation (Kolaczowska and Kubes, 2013). Neutrophils are identified by their expression of the cell surface marker Ly6G. Ly6G is a glycosylphosphatidylinositol (GPI)-anchored protein that may have a role as a modulator of neutrophil migration to sites of inflammation via a 2-integrin-dependent mechanism (Wang et al., 2012). Neutrophils were identified as being Ly6G⁺ CD11b^{Int} by flow cytometry (**Figure 3.17A**). Results showed that neutrophil levels were significantly increased ($p < 0.0006$) in the peritoneal cavity of *Rora*^{sg/sg} mice as compared to WT mice (**Figure 3.17B**). These results indicate that *Rora* may have a role in the development of myeloid cells aside from macrophages.

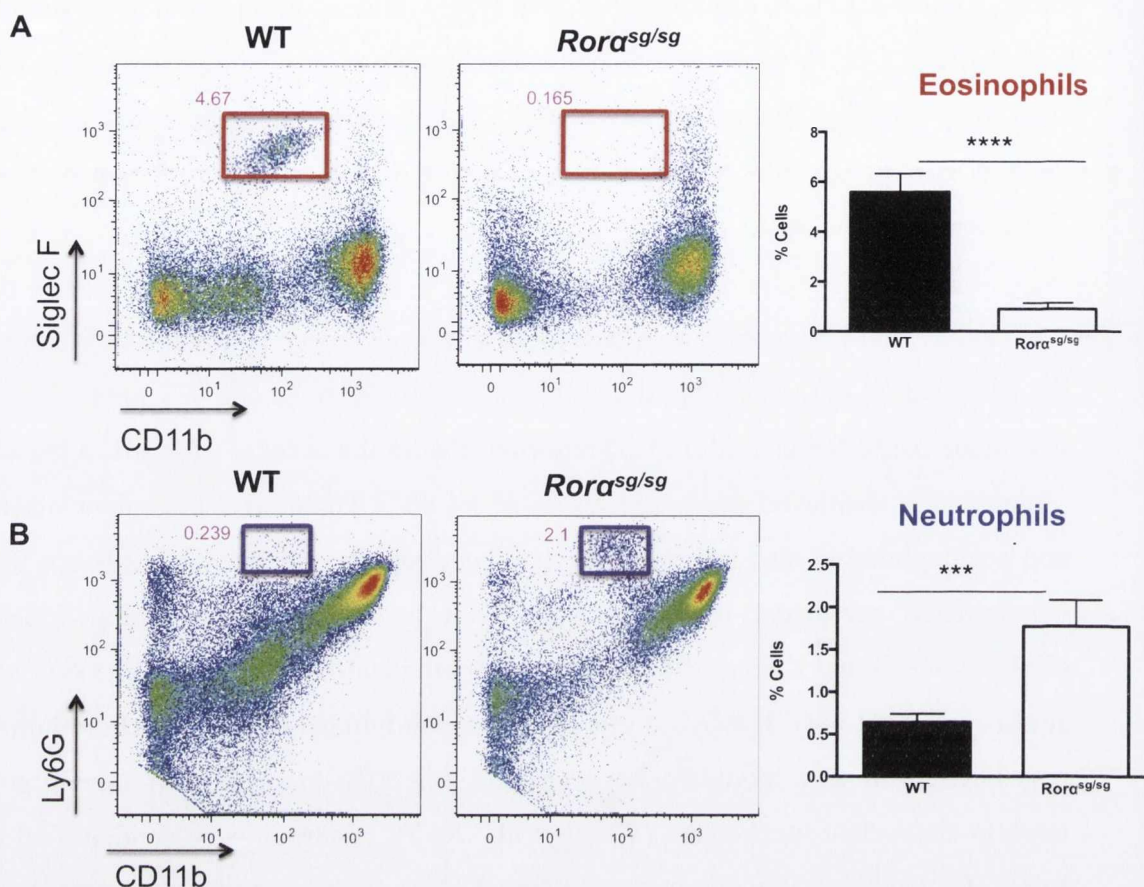


Figure 3.17 Eosinophil and neutrophil levels are altered in the absence of functional *Rora*. **A** Representative flow cytometry plots of gating strategy used for eosinophils (Siglec-F⁺, CD11b^{Int}). **B** Representative flow cytometry plots of gating strategy used for neutrophils (Ly6G⁺, CD11b^{Int}). All data represent means $\pm$ s.e.m. Differences, indicated as two-tailed *p* values, as assessed by unpaired Student *t* test. *** *p* < 0.0001 to 0.001, **** *p* < 0.0001. Numbers on flow cytometry plot reflect percentage of total cells. WT mice *n*=12, *Rora^{sg/sg}* mice *n*=10.

3.3.8 The Tim4⁺ MHCII⁺ peritoneal population represents a blood monocyte-derived macrophage population.

The Tim4⁺ MHCII⁺ peritoneal population that is decreased in *Rora^{sg/sg}* mice compared to WT mice (**Figure 3.12**) expresses M2 macrophage markers (Relm α and CD206) and also markers of infiltration (CCR2 and CX₃CR1) (**Figure 3.14**), thus indicating that the population may be a blood monocyte-derived macrophage population that has M2-like characteristics. It was hypothesised that the Tim4⁺ MHCII⁺ peritoneal population would be recruited to the peritoneal cavity of mice treated with stimuli to induce the accumulation of

monocytes. In the context of M2 stimulation, once in the peritoneal cavity the monocytes would be polarised towards an M2 phenotype. Conversely, this blood monocyte-derived population would not be expanded by stimuli that induce the proliferation and polarisation of tissue-resident macrophages.

To test this hypothesis a method developed by Jenkins et al. (2011) was utilised to expand blood monocyte-derived macrophages and tissue-resident macrophages while also polarizing them towards an M2 phenotype. This method is described in more detail in **Section 2.18**. Briefly WT mice were administered an IL-4/anti-IL-4 mAb complex (IL-4c), which results in the slow continuous release of the cytokine. Mice were injected i.p. with IL-4c or PBS, as a vehicle control, on days 0 and 2. For co-administration experiments, mice were given thioglycollate by i.p injection (800 µl) on day 0, immediately prior to the first dose of IL-4c. Mice were sacrificed on day 4 and peritoneal cells recovered by lavage.

Gundra and colleagues (2014) recently used this method to demonstrate that within the peritoneum there is an expansion of blood monocyte-derived M2 macrophages that are phenotypically and functionally distinct to the tissue-resident M2 macrophages. Flow cytometry analysis revealed that monocyte-derived M2 macrophages, induced by thioglycollate + IL-4c, were CX₃CR1-GFP^{high} and expressed increased levels of PD-L2, CD206, and MHCII, whereas tissue-derived M2 macrophages, induced by IL-4c, were CX₃CR1-GFP^{Lo} and PD-L2, CD206, and MHCII negative (Gundra et al., 2014). To confirm that the method used here successfully recruited/expanded and polarised both monocyte-derived M2 macrophages and tissue-resident M2 macrophages the expression of these cell surface markers was explored in the treated groups. Results showed increased expression of PD-L2, CD206 and MHCII on monocyte-derived M2 macrophages (CD11b⁺) compared to tissue-resident M2 macrophages (CD11b⁺) (**Figure 3.18A-B**). These findings are in-line with the results found in Gundra et al. (2014) indicating that the method worked successfully.

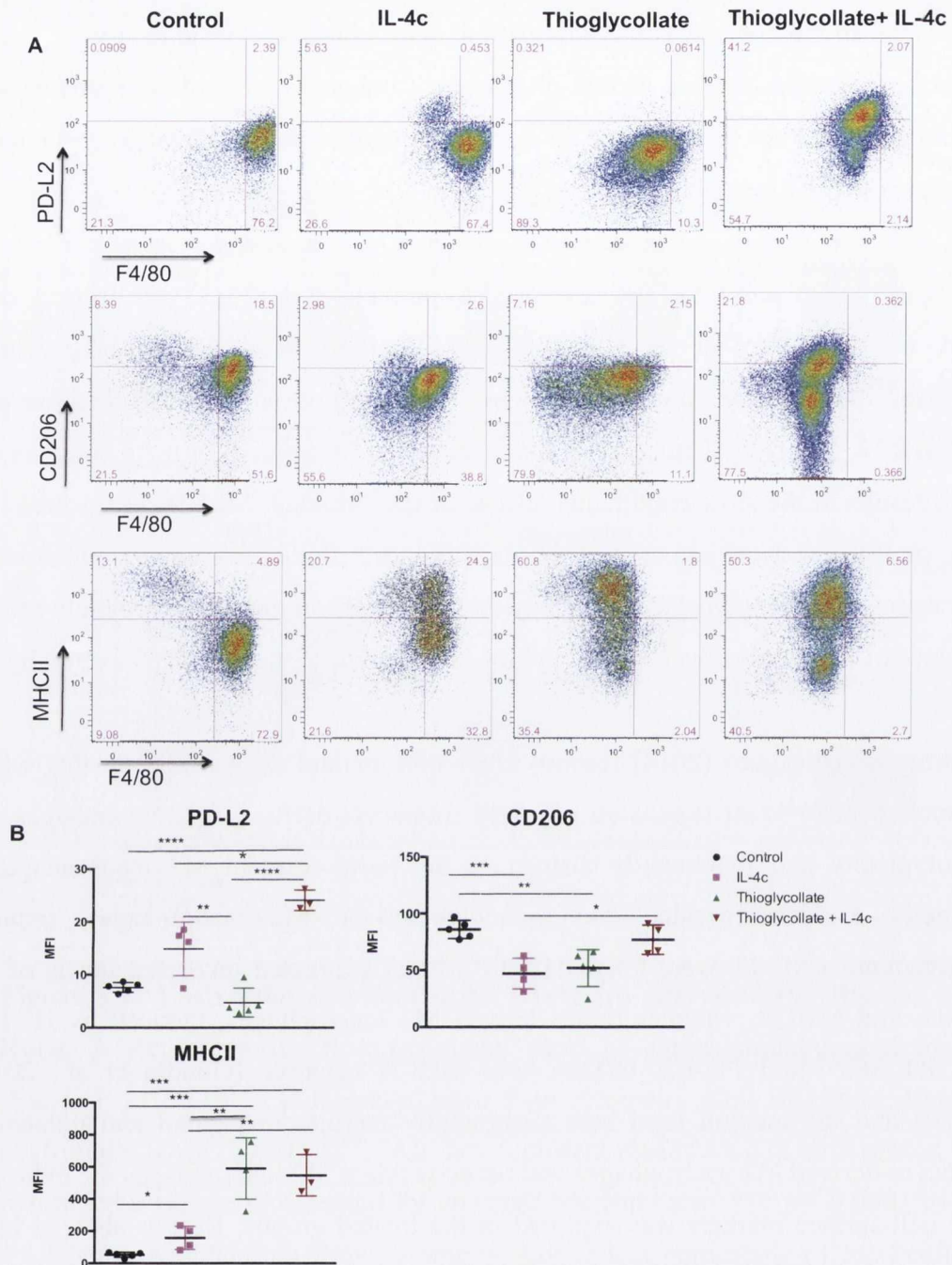


Figure 3.18 Blood monocyte-derived M2 macrophages and tissue-resident M2 macrophages are phenotypically distinct in the peritoneal cavity of WT mice. WT mice were injected with IL-4c-alone (IL-4c), thioglycollate-alone, thioglycollate and IL-4c or PBS (Control). **A** Representative flow cytometry plots of PD-L2, CD206 and MHCII expression on peritoneal macrophages from each group (Pre-gate CD11b⁺ cells). **B** Graphs depict the geometric median fluorescent intensity (MFI) of PD-L2, CD206 and MHCII, gated on CD11b⁺ cells from the peritoneal cavity of individual mice. MFI= MFI of the stained cells - MFI of fluorescence-minus-one control. All data represent means $\pm$ s.e.m. Differences, indicated as two-tailed *p* values, as assessed by unpaired Student *t* test. * *p* < 0.01 to 0.05, ** *p* < 0.001 to 0.01, *** *p* < 0.0001 to 0.001, **** *p* < 0.0001.

Having established that using the IL-4c/Thioglycollate method resulted in the recruitment/expansion and polarisation of both blood monocyte-derived and tissue-resident peritoneal macrophage populations (**Figure 3.18**), the expansion of the Tim4⁻ MHCII⁺ peritoneal population was explored in treated mice. It was hypothesised that if the Tim4⁻ MHCII⁺ peritoneal population represented a blood monocyte-derived macrophage population its levels would be expanded following treatment with thioglycollate + IL-4c. Indeed analysis by flow cytometry revealed that levels of the Tim4⁻ MHCII⁺ peritoneal population were significantly increased ($p < 0.0001$) in the group treated with thioglycollate and IL-4c compared to the group treated with IL-4c alone (**Figure 3.19A-B**). This indicates that the Tim4⁻ MHCII⁺ peritoneal population is a blood monocyte-derived macrophage population that infiltrates into the peritoneal cavity following thioglycollate stimulation and takes on an M2-like phenotype in the presence of IL-4c.

Interestingly in mice treated with IL-4-alone the levels of the Tim4⁺ MHCII⁻ peritoneal population were significantly increased ($p < 0.004$ and $p < 0.0051$ respectively) compared to control mice and mice treated with IL-4c + Thioglycollate (**Figure 3.19A-B**). This result indicates that, as the expression of M2 cell surface markers suggested, the Tim4⁺ MHCII⁻ peritoneal population represents tissue-resident macrophages.

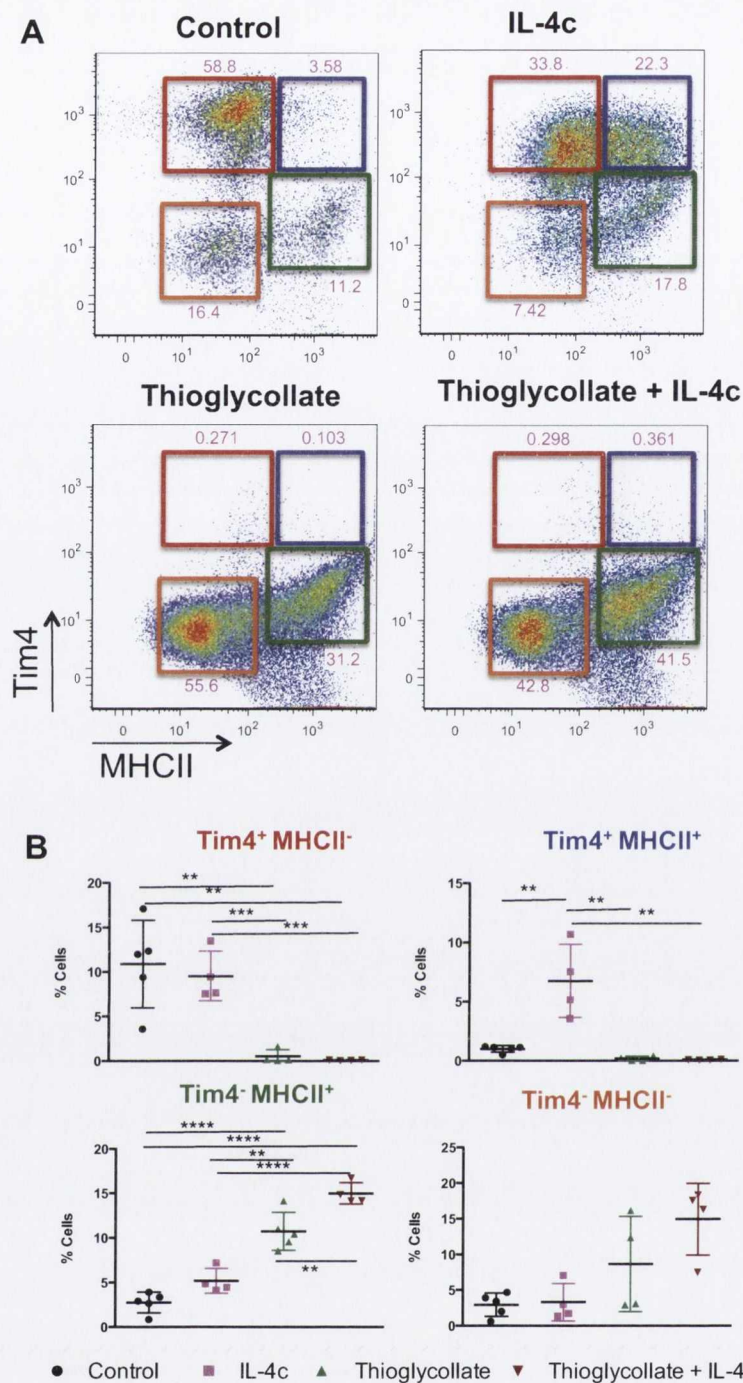


Figure 3.19 The Tim4⁻ MHCII⁺ peritoneal population is increased in WT mice treated with thioglycollate and IL-4c. **A** Representative flow cytometry plots of the four peritoneal populations identified by Tim4 and MHCII gating in control, IL-4c, thioglycollate and thioglycollate + IL-4c treated WT mice. (Pre-gate- CD11b⁺, F4/80⁺ cells). **B** Graphs representing the percentage of each peritoneal population identified in (A) in each treated group. All data represent means $\pm$ s.e.m. Differences, indicated as two-tailed *p* values, as assessed by unpaired Student *t* test. ***p* < 0.001 to 0.01, ****p* < 0.0001 to 0.001, *****p* < 0.0001.

Overall these results indicate that the Tim4⁻ MHCII⁺ peritoneal population represents a blood monocyte-derived population and the Tim4⁺ MHCII⁻ peritoneal population represents a tissue-resident macrophage population.

3.3.9 Macrophage populations are altered in other macrophage rich tissues.

Having shown that blood monocyte-derived macrophages are altered in the peritoneal cavity of *Rora*^{sg/sg} mice other macrophage rich tissues were examined. The lung and spleen are both macrophage rich tissues (Cassado et al., 2015). Similar, to peritoneal macrophages, alveolar and splenic macrophages were until recently considered to all be of blood monocyte-derived (Yona et al., 2013). However, fate mapping studies showed that these macrophages are in fact primarily of embryonic origin and are seeded in tissue before birth (Yona et al., 2013).

To characterise pulmonary alveolar macrophages in *Rora*^{sg/sg} mice and WT mice an adapted gating strategy outlined by Guillems and colleagues (2013) was used. Among tissue-resident macrophages, alveolar macrophages have a distinctive phenotype in that they are highly autofluorescent, express low levels of CD11b and high levels of CD11c and Siglec-F (Guillems et al., 2013). As such, tissue-resident macrophages were identified as CD11c⁺, Siglec-F⁺ and monocyte-derived alveolar macrophages were identified as CD11c^{Low} Siglec-F^{Low} following gating on CD11b and F4/80 expressing cells and the exclusion of neutrophils and eosinophils (**Figure 3.20**). The CD11c⁺ Siglec-F⁺ population was statistically significantly increased ($p < 0.0013$) in *Rora*^{sg/sg} mice compared to WT mice (**Figure 3.21**). Levels of the CD11c^{Low} Siglec-F^{Low} population were unchanged between the two groups, as were levels of neutrophils and eosinophils (**Figure 3.21**). Therefore these results indicate that resident alveolar macrophages are increased in the lung of *Rora*^{sg/sg} mice but blood monocyte-derived alveolar macrophage levels are unchanged. This result is in contrast with the peritoneal cavity of *Rora*^{sg/sg} mice, however, alterations are still seen in tissue macrophage populations. It may be possible that the role that *Rora* plays in macrophages varies between tissue sites.

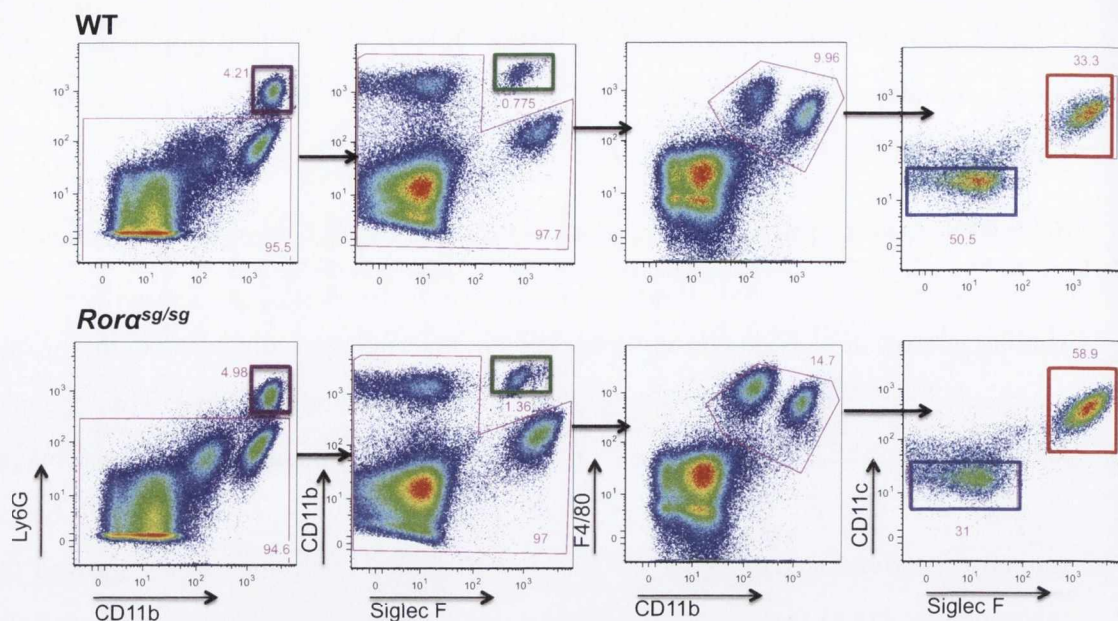


Figure 3.20 Representative flow cytometry plots of the gating strategy used in WT mice and *Rora*^{sg/sg} mice for identifying lung populations. Flow cytometry plot of the gating strategy used for identifying alveolar macrophages in whole lung tissue from WT mice and *Rora*^{sg/sg} mice. The CD11c⁺ Siglec-F⁺ population (Ly6G⁻, CD11b⁺, F4/80⁺, CD11c⁺, Siglec-F⁺) was taken as resident alveolar macrophages. The CD11c^{Low} Siglec-F^{Low} population (Ly6G⁻, CD11b⁺, F4/80⁺, CD11c^{Low}, Siglec-F^{Low}) was taken as monocyte-derived alveolar macrophages. This gating strategy is adapted from Guilliams et al. (2013). Neutrophils (Ly6G^{High}, CD11b⁺) and eosinophils (Siglec-F^{High}, CD11b⁺) were also identified by this gating strategy. Numbers on flow cytometry plot reflect percentage of total cells.

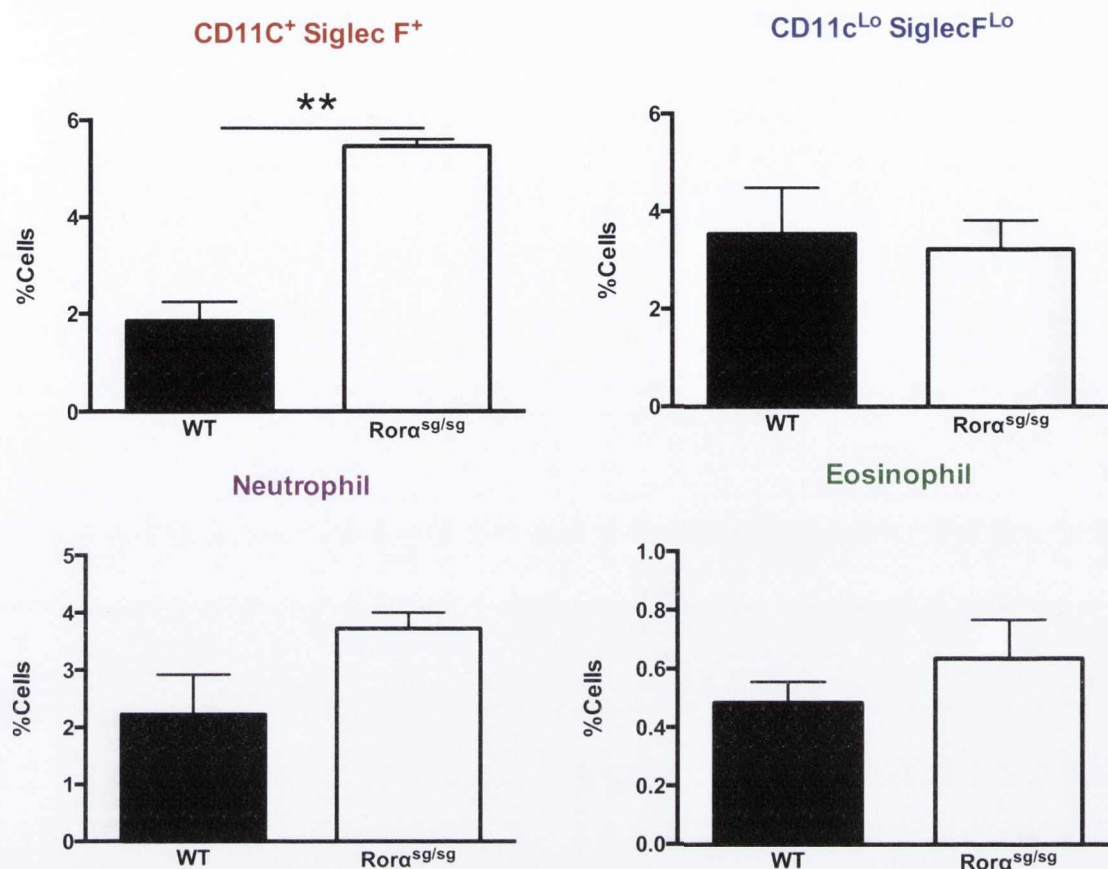


Figure 3.21 Lung tissue-resident macrophages are increased in the absence of functional *Rora*. Quantification of alveolar macrophage populations in WT mice and *Rora*^{sg/sg} mice identified in **Figure 3.20**. All data represent means $\pm$ s.e.m. Differences, indicated as two-tailed *p* values, as assessed by unpaired Student *t* test. ** *p* < 0.001 to 0.01. WT mice *n*=4, *Rora*^{sg/sg} mice *n*=3.

3.3.10 Blood monocyte levels are altered in the absence of functional *Rora*.

Having confirmed in WT mice that the Tim4⁺ MHCII⁺ peritoneal population that is decreased in *Rora*^{sg/sg} mice (**Figure 3.12**) is blood monocyte-derived (**Figure 3.19**) it was next explored if blood monocyte levels are also altered between WT mice and *Rora*^{sg/sg} mice. It was hypothesised that levels of the Tim4⁺ MHCII⁺ blood monocyte-derived peritoneal population were decreased in *Rora*^{sg/sg} mice because the precursor blood monocytes were decreased in the blood. Blood monocytes are classically divided into two subgroups: Ly6C^{High} and Ly6C^{Low} blood monocytes (**Section 1.8**). To explore if either of these blood monocyte subgroups were decreased in the absence of functional *Rora* blood was isolated from WT mice and *Rora*^{sg/sg} mice and analysed by flow cytometry.

The antibody panel used to identify blood monocytes by flow cytometry included: CD45, CD115, Ly6G, Ly6C and is based on a recent paper by Nguyen and colleagues (2013). Using this panel of markers Ly6C^{High} blood monocytes are identified as CD45⁺, CD115⁺, Ly6G⁻ and Ly6C^{High} and Ly6C^{Low} blood monocytes are identified as CD45⁺, CD115⁺, Ly6G⁻ and Ly6C^{Low} (**Figure 3.22A**). By this gating strategy neutrophils (CD45⁺, CD115⁻ and Ly6G⁺) can also be identified. Analysis revealed that the levels of Ly6C^{Low} blood monocytes were significantly decreased ($p < 0.0003$) in *Rora*^{sg/sg} mice compared to WT mice (**Figure 3.22B**). Levels of Ly6C^{High} monocytes were unchanged between the two groups. The levels of neutrophils (CD45⁺, Ly6G⁺, CD115⁻) were significantly increased ($p < 0.036$) in blood of *Rora*^{sg/sg} mice compared to WT mice (**Figure 3.22B**).

These results indicate that levels of the Ly6C^{Low} blood monocytes are decreased in the absence of functional *Rora*. Suggesting that Ly6C^{Low} blood monocytes may be the precursor for blood monocyte-derived macrophages, also decreased in the absence of functional *Rora*.

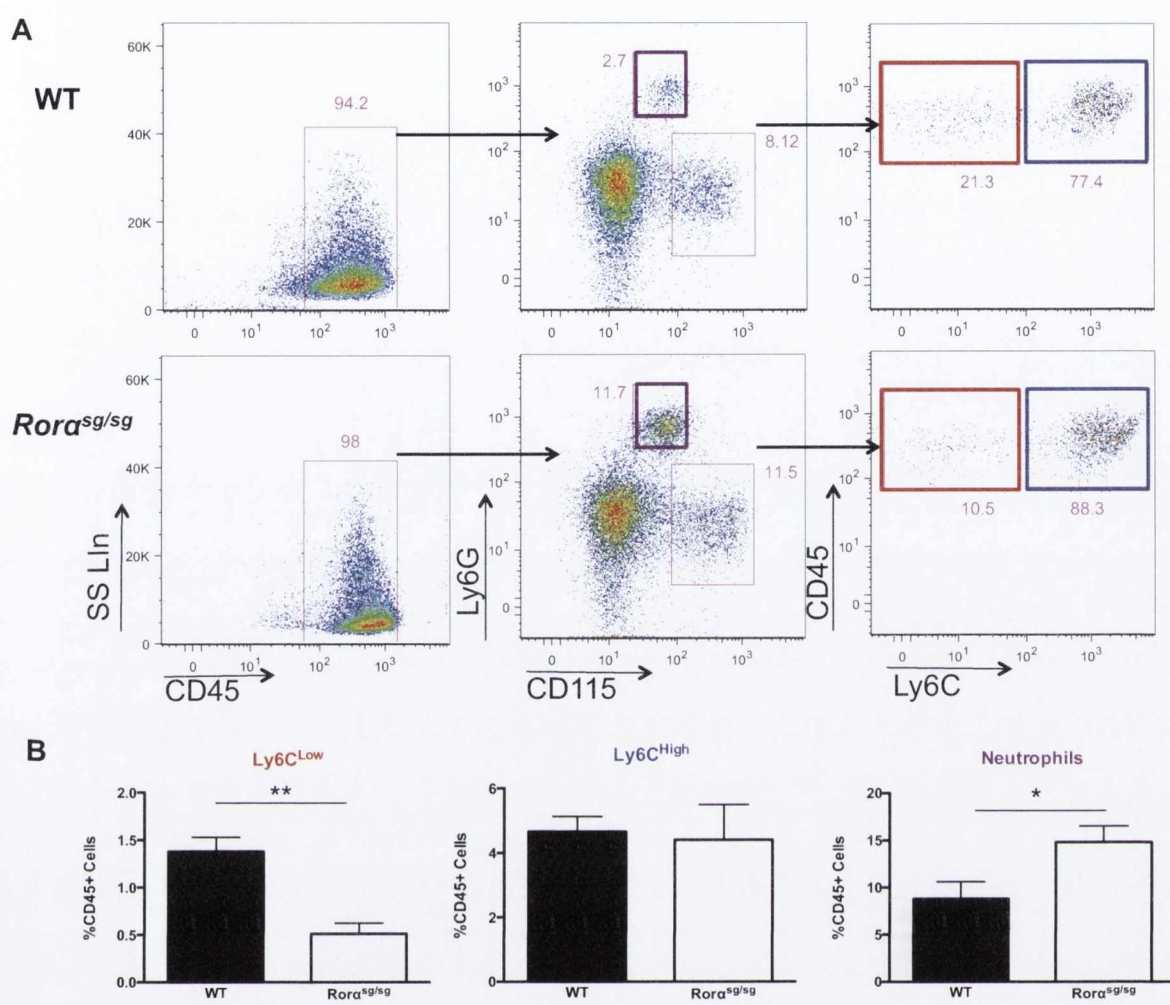


Figure 3.22 Ly6C^{Low} blood monocyte levels are altered in the absence of functional *Rora*. **A** Representative flow cytometry plot of gating strategy used for identifying Ly6C^{High} (CD45⁺, Ly6G⁻, CD115⁺, Ly6C^{High}) and Ly6C^{Low} blood monocytes (CD45⁺, Ly6G⁻, CD115⁺ Ly6C^{Low}). The gating strategy taken from Nguyen et al., (2013). Neutrophils (CD45⁺, Ly6G⁺, CD115⁻) were also identified by this gating strategy. **B** Quantification of blood monocyte populations (percentage of CD45⁺ cells) analysed by flow cytometry in (A) above. All data represent means $\pm$ s.e.m. Differences, indicated as two-tailed *p* values, as assessed by unpaired Student *t* test. * *p* < 0.01 to 0.05, ** *p* < 0.001 to 0.01. Numbers on flow cytometry plot reflect percentage of total cells. WT mice n=16, *Rora^{sg/sg}* mice n=12.

CX₃CR1 is a marker that is used to identify the two different blood monocyte subsets (Geissmann et al., 2003). More recently CX₃CR1 expression has been used in fate mapping studies to determine origin and relationship between murine monocytes and

macrophages (Yona et al., 2013). It is known that Ly6C^{High} blood monocytes express low levels of CX₃CR1 whereas Ly6C^{Low} blood monocytes express high levels of CX₃CR1 (**Section 1.8**). As it was observed that blood monocyte and blood monocyte-derived peritoneal macrophage levels were altered in the absence of functional *Rora* we wanted to further determine the relationships between blood monocytes and peritoneal macrophages by using CX₃CR1 expression. To achieve this *Rora*^{sg/sg} mice were crossed to CX₃CR1 mice to generate dual *Rora*^{sg/sg} CX₃CR1^{gfp/+} mice (**Section 2.1.10**). However, the cross did not appear to be successful as no *Rora*^{sg/sg} CX₃CR1^{gfp/+} mice were generated after 4 litters as determined by genotyping.

One possible explanation as to why no *Rora*^{sg/sg} CX₃CR1^{gfp/+} mice were generated is that both genes are found in close proximity on the same chromosome. The CX₃CR1 maps to chromosome 9 ~ 120 Mbps and *Rora* maps to chromosome 9 ~ 68 Mbps. Genetic linkage is the tendency of alleles that are located in close proximity on a chromosome to be inherited together during the meiosis phase of sexual reproduction. Genetic linkage results in the production of increased offspring that have the same combination of alleles that were in the original parents (parental offspring). Examination of the offspring produced in the cross to generate *Rora*^{sg/sg} CX₃CR1^{gfp/+} mice described in **Section 2.1.10** indicated that the majority of the offspring are indeed parental indicating *Rora* and CX₃CR1 are genetically linked. This means that the generation of *Rora*^{sg/sg} CX₃CR1^{gfp/+} mice would be extremely rare.

3.3.11 Macrophage progenitor cells are altered in the absence of functional *Rora*.

Blood monocytes are part of the myeloid lineage and originate in the bone marrow of mice (**Figure 1.1**). Transcription factors are essential to the haematopoietic differentiation process (**Figure 1.12**). Lineage specification at differentiation branch points is dictated by the activation and interaction of a number of lineage specific transcription factors including PU.1, C/EBPs and GATA-1. It was hypothesised that *Rora* could also have a role in one of the differentiation branch points that effects levels of Ly6C^{Low} blood monocytes and, consequently, also the downstream Tim4⁺ MHCII⁺ peritoneal macrophage population. This could account for the decreased levels of Ly6C^{Low} blood monocytes seen in *Rora*^{sg/sg} mice compared to WT mice.

To explore this possibility further, myeloid progenitor cells in the bone marrow of WT mice and *Rora*^{sg/sg} mice were phenotyped by flow cytometry. Bone marrow myeloid

progenitor cells can be identified by their cell surface marker expression (**Section 1.12**). There is no one defined panel of markers used to identify progenitor cells rather different researchers use a combination of haematopoietic markers (Table 1.1). One of the earliest bone marrow progenitor cells is the LSK (Lin⁻, IL-7Rα⁻, Sca-1⁺ and c-Kit⁺) (**Figure 3.23A**), which differentiates into either common lymphoid progenitor (CLP, i.e. Lin⁻, IL-7Rα⁺, Sca-1⁻, c-kit⁺) (**Figure 3.23A**), or common myeloid progenitor (CMP) cells (Lin⁻, Sca-1⁻, c-kit⁺, FcII/III⁺) (**Figure 3.24A**). CMP cells in turn give rise to all cells of the myeloid lineage including granulocyte macrophage progenitor cells (GMP) (Lin⁻, Sca-1⁻, c-Kit⁺, FcII/III^{Int}) (**Figure 3.24A**). Levels of LSK cells are unchanged between WT mice and *Rora*^{sg/sg} mice (**Figure 3.23B**) as are levels of CLP (**Figure 3.23B**), CMP (**Figure 3.24B**) and GMP cells (**Figure 3.23B**). These results indicate that *Rora* does not play a role within the generation of these progenitor cells.

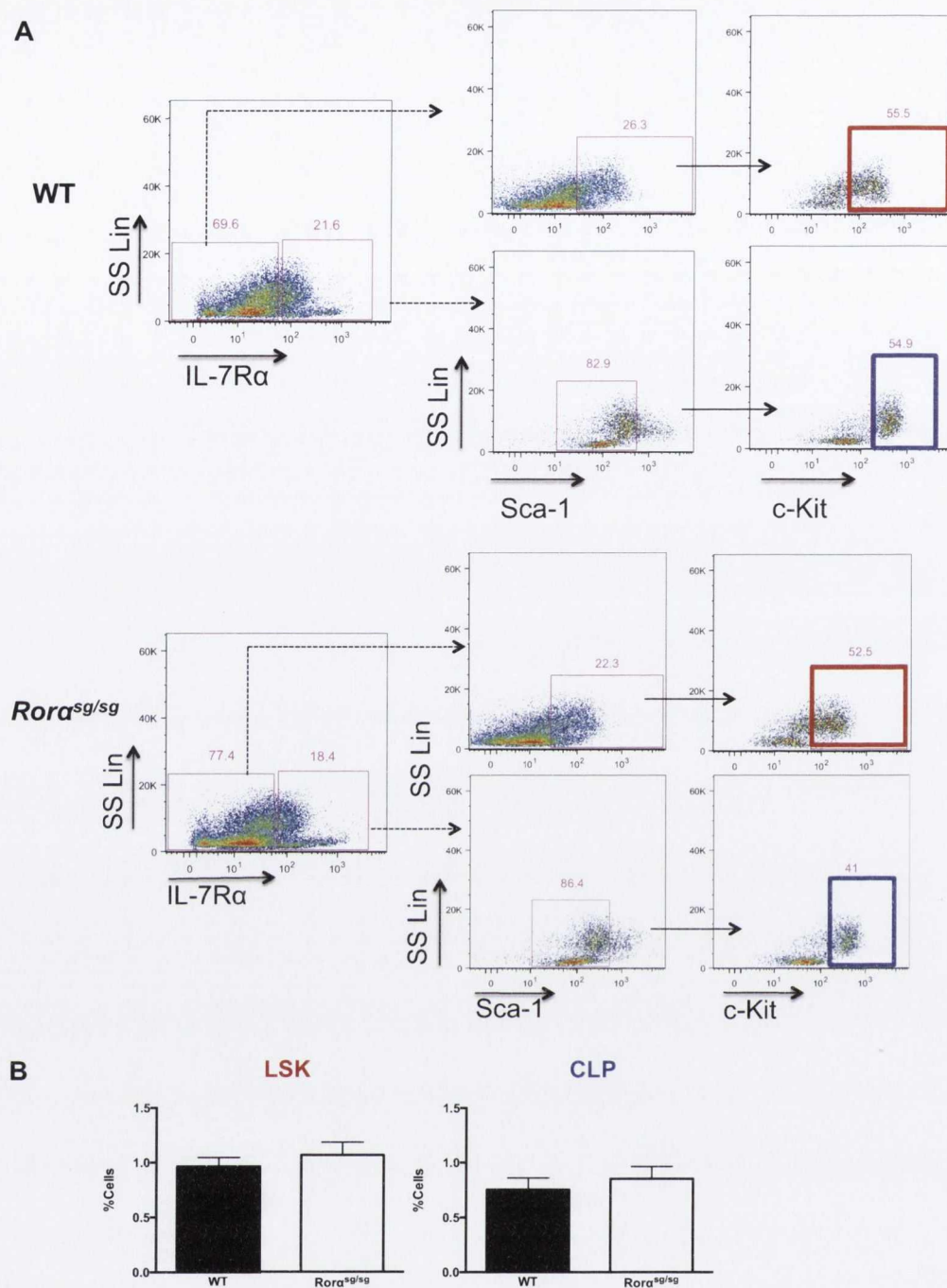


Figure 3.23 Levels of LSK and CLP cells in the bone marrow are unchanged in the absence of functional *Rora*. **A** Representative gating strategy used for identifying LSK cells (Lin⁻, IL-7Rα⁻, Sca-1⁺ and c-Kit⁺) and CLP cells (Lin⁻, IL-7Rα⁺, Sca-1⁻, c-kit⁺) (Lin⁻CD3⁻, CD4⁻, CD11b⁻, CD11c⁻, CD19⁻, Gr-1⁻). **B** Quantification of LSK and CLP populations in WT mice and *Rora^{sg/sg}* mice analysed by flow cytometry in (A) above. All data represent means ± s.e.m. Differences, indicated as two-tailed *p* values, as assessed by unpaired Student *t* test. LSK WT mice n=15, *Rora^{sg/sg}* mice n=12. CLP WT mice n=11, *Rora^{sg/sg}* mice n=11.

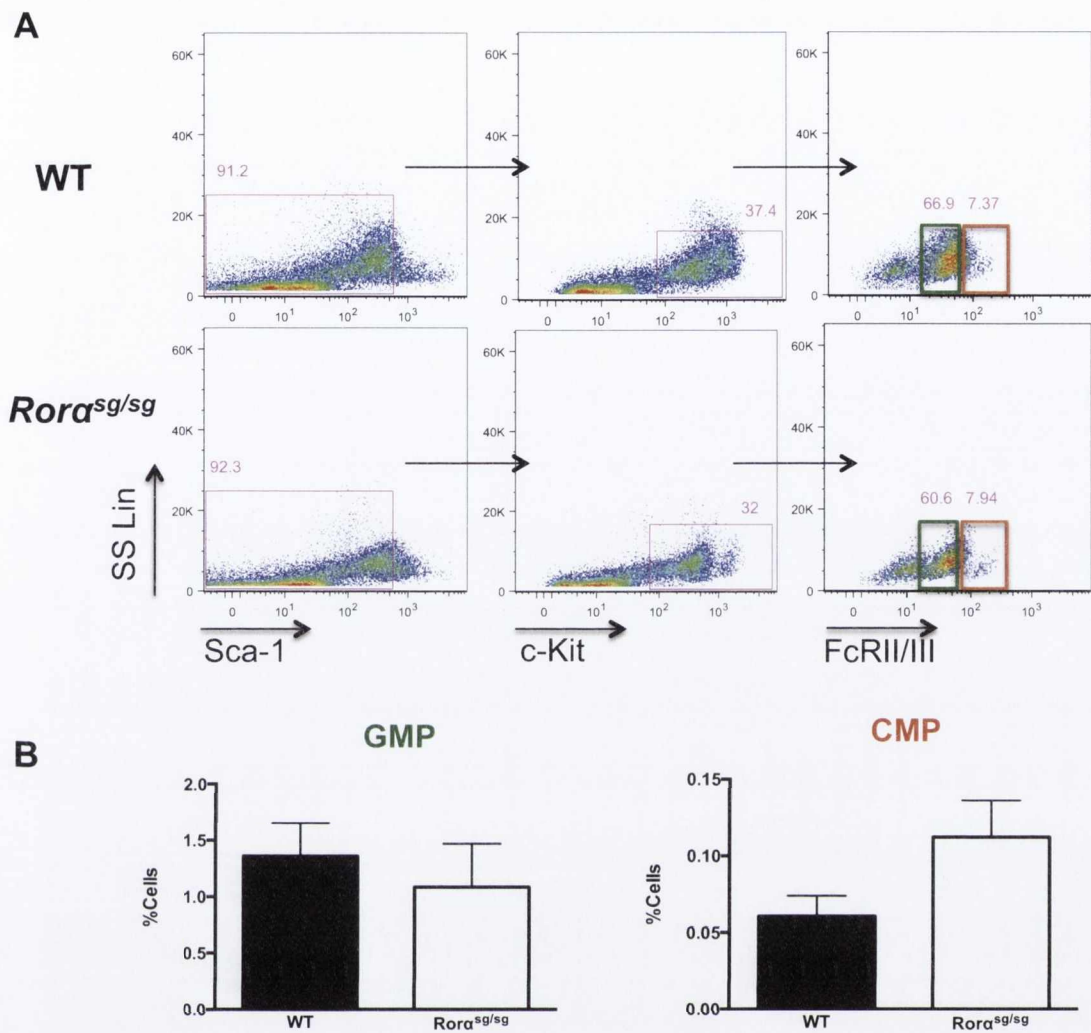


Figure 3.24 Levels of common myeloid progenitor (CMP) cells and granulocyte macrophage progenitor (GMP) cells in the bone marrow are unchanged in the absence of functional *Rora*. **A** Representative gating strategy used for identifying common myeloid progenitor (CMP) cells (Lin⁻, Sca-1⁻, c-kit⁺, FcII/III⁺) and granulocyte macrophage progenitor (GMP) cells (Lin⁻, Sca-1⁻, c-Kit⁺, FcII/III^{Int}) (Lin⁻: MHCII⁻, IL-7R α ⁻, CD3⁻, CD4⁻, CD11b⁻, CD11c⁻, CD19⁻, Gr-1⁻). Gating strategy adapted from Satoh et al. (2013). **B** Quantification of the CMP and GMP populations (percentage of total cells) in WT mice and *Rora^{sg/sg}* mice analysed by flow cytometry in (A) above. All data represent means $\pm$ s.e.m. Differences, indicated as two-tailed *p* values, as assessed by unpaired Student *t* test. Numbers on flow cytometry plot reflect percentage of total cells. WT mice *n*=9, *Rora^{sg/sg}* mice *n*=7.

GMP cells differentiate into monocyte dendritic progenitor (MDP) cells (Lin⁻, c-Kit^{Int}, CD115⁺) (**Figure 3.25A**). MDP cells have lost the ability to generate granulocytes and gives rise to the two monocyte subsets, Ly6C^{Low} (CD11b⁺, Ly6G⁻, Ly6C^{Low}) or Ly6C^{High} (CD11b⁺, Ly6G⁻, Ly6C^{High}) bone marrow monocytes (**Figure 3.25B**)(Section 1.8). In bone

marrow of *Rora*^{sg/sg} mice levels of the MDP cells were unchanged between the two groups (**Figure 3.25C**). The levels of Ly6C^{Low} monocytes were statistically significantly increased compared to WT mice ($p < 0.05$) (**Figure 3.25C**). However, the levels of Ly6C^{High} blood monocytes were unchanged between the two groups (**Figure 3.25C**). These results indicate that *Rora* plays a role in the myeloid lineage differentiation process with its role occurring in the Ly6C^{Low} bone marrow progenitor monocyte.

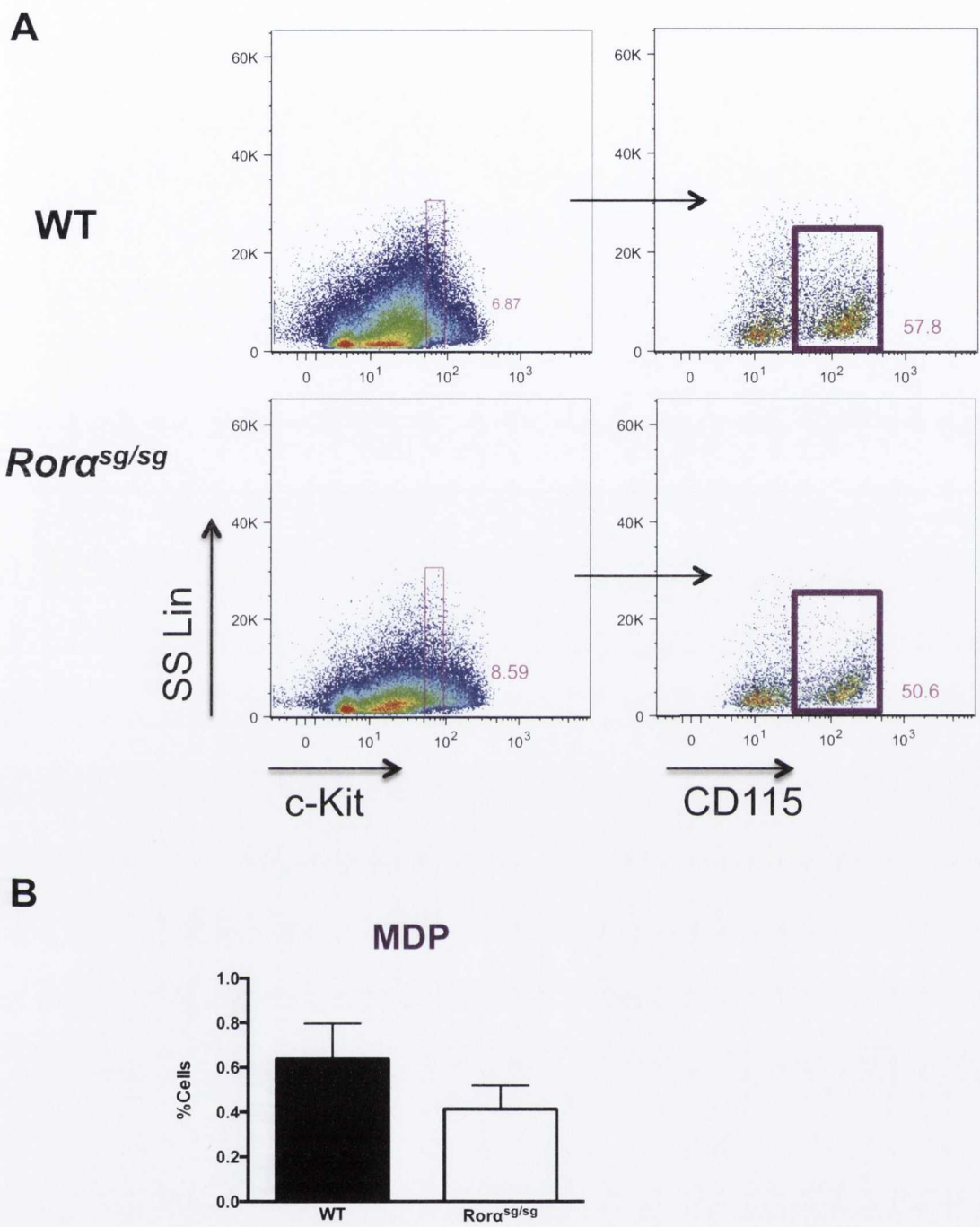


Figure 3.25 The levels of MDP cells in the bone marrow are unchanged in the absence of functional *Rora*. **A** Representative gating strategy used for identifying monocyte dendritic progenitor (MDP) cells (Lin⁻, Sca-1⁻, c-kit^{Int}, CD115⁺) (Lin⁻: MHCII⁻, IL-7Rα⁻, CD3⁻, CD4⁻, CD11b⁻, CD11c⁻, CD19⁻, Gr-1⁻). **B** Quantification of the MDP (percentage of total cells) in WT mice and *Rora*^{sg/sg} mice analysed by flow cytometry in (A) above. All data represent means ± s.e.m. Differences, indicated as two-tailed *p* values, as assessed by unpaired Student *t* test. Numbers on flow cytometry plot reflect percentage of total cells. MDP WT mice n=7, *Rora*^{sg/sg} mice n=5.

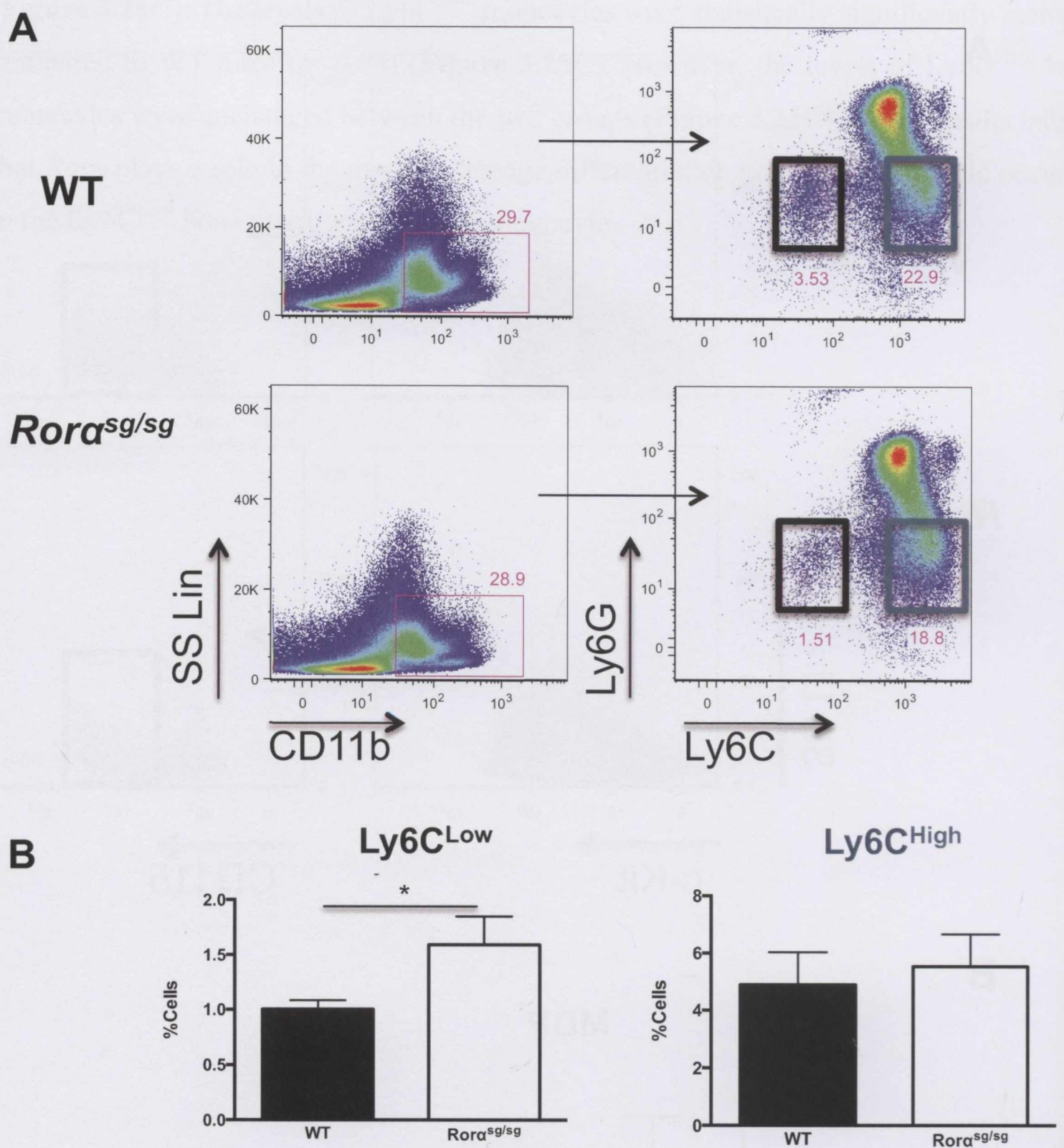


Figure 3.26 The levels of Ly6C^{Low} bone marrow monocytes are increased in the absence of functional *Rora*. **A** Representative gating strategy used for identifying Ly6C^{Low} (Lin⁻, CD11b⁺, Ly6G⁻, Ly6C^{Low}) and Ly6C^{High} (Lin⁻, CD11b⁺, Ly6G⁻, Ly6C^{High}) bone marrow monocytes (Lin⁻: CD3⁻, CD4⁻, CD19⁻). **B** Quantification of Ly6C^{Low} and Ly6C^{High} bone marrow monocytes (percentage of total cells) in WT mice and *Rora*^{sg/sg} mice analysed by flow cytometry in (A) above. All data represent means $\pm$ s.e.m. Differences, indicated as two-tailed *p* values, as assessed by unpaired Student *t* test. * *p* < 0.01 to 0.05. Numbers on flow cytometry plot reflect percentage of total cells. Bone marrow monocytes WT mice *n*=5, *Rora*^{sg/sg} mice *n*=5.

3.3.12 Ly6C^{Low} monocyte levels are decreased in the spleen.

The bone marrow is traditionally seen as the primary reservoir for monocytes. However, in 2010, Swirski and colleagues showed that undifferentiated monocytes, both Ly6C^{High} and Ly6C^{Low} monocytes, also reside in the spleen in greater numbers than their equivalents in the circulation. They also showed that in response to ischemic myocardial injury, splenic monocytes increase their motility, exit the spleen, accumulate in injured tissue, and also participate in wound healing (Swirski et al., 2010). The spleen also contains cMoP and MDP populations with the same surface phenotype and similar proliferative activity as that of their bone marrow counterparts (Hettinger et al., 2013). As the spleen is a reservoir for macrophage progenitor and having identified an alteration in levels of Ly6C^{Low} monocytes in the bone marrow (**Figure 3.26**) of *Rora*^{sg/sg} mice, the levels of splenic monocytes were explored in these mice to see if this population is also altered in the absence of functional *Rora*.

Using the gating strategy described by Swirski et al. (2010), Ly6C^{Low} (CD11b⁺, Ly6G⁻, F4/80⁻, Ly6C^{Low}), Ly6C^{High} monocytes (CD11b⁺, Ly6G⁻, F4/80⁻, Ly6C^{High}), splenic macrophages (CD11b⁺, Ly6G⁻, F4/80⁺, Ly6C^{Low}) and neutrophils (CD11b⁺, Ly6G⁺) were identified in the spleen (**Figure 3.27A**). The levels of Ly6C^{Low} splenic monocytes were statistically significantly ($p < 0.0008$) decreased in *Rora*^{sg/sg} mice compared to WT mice (**Figure 3.27B**). The levels of Ly6C^{High} monocytes, splenic macrophages and neutrophils were unchanged between the two groups (**Figure 3.27B**). This decrease in Ly6C^{Low} splenic monocytes reflects what is observed in Ly6C^{High} blood monocytes in *Rora*^{sg/sg} mice.

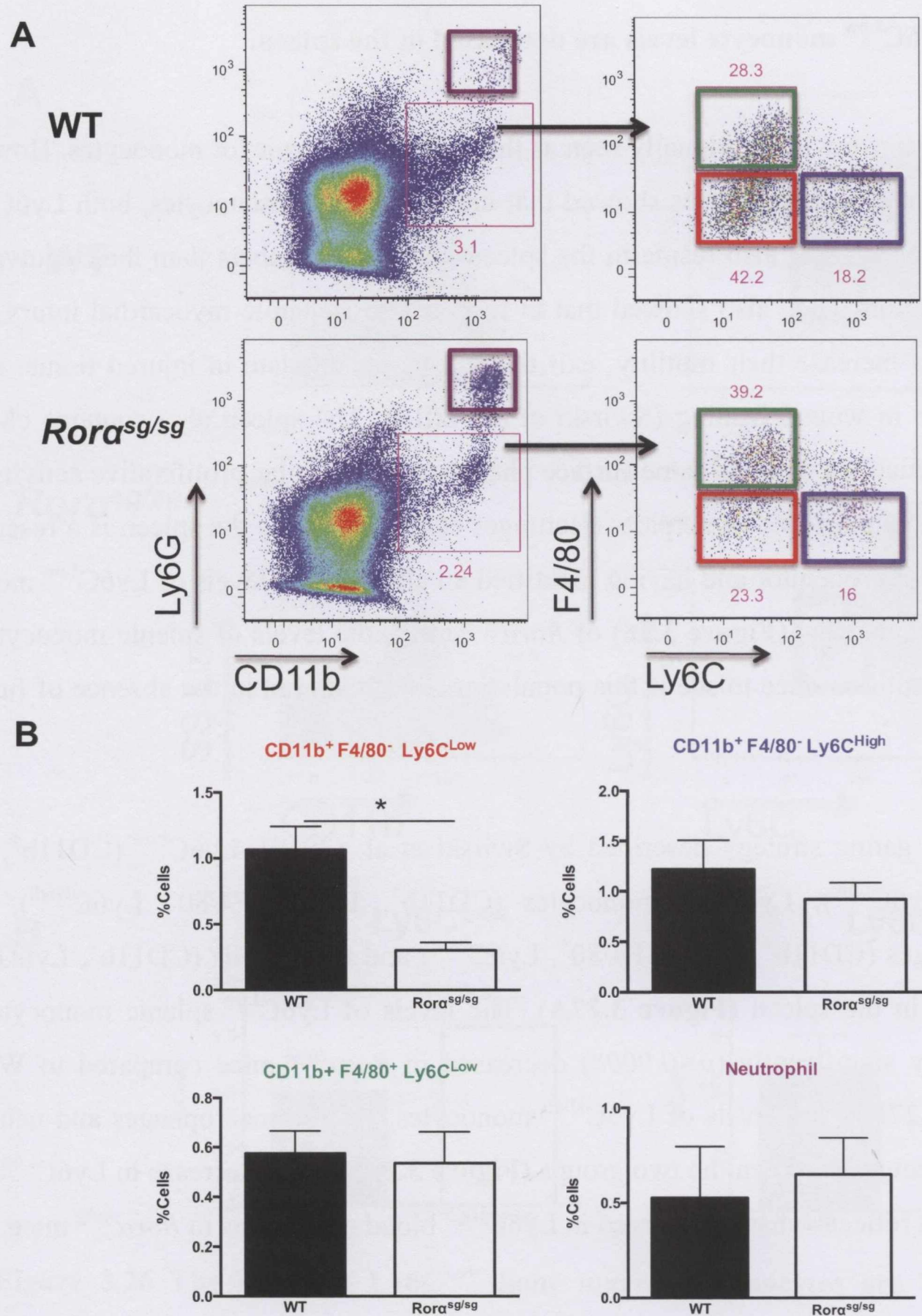


Figure 3.27 The levels of Ly6C^{Low} monocytes are altered in the spleen. **A** Representative flow cytometry plots of gating strategy used for identifying Ly6C^{Low} monocytes (CD11b⁺, Ly6G⁻, F4/80⁻, Ly6C^{Low}), Ly6C^{High} monocytes (CD11b⁺, Ly6G⁻, F4/80⁻, Ly6C^{High}), macrophages (CD11b⁺, Ly6G⁻, F4/80⁺, Ly6C^{Low}) and neutrophils (CD11b⁺, Ly6G⁺). Strategy based on Swirski et al. (2010). **B** Quantification of the Ly6C^{Low}, Ly6C^{High} monocytes, macrophages and neutrophils (percentage of total cells) in WT mice and *Rora^{sg/sg}* mice analysed by flow cytometry in (A) above. All data represent means $\pm$ s.e.m. Differences, indicated as two-tailed *p* values, as assessed by unpaired Student *t* test. * *p* < 0.01 to 0.05. WT mice *n*=5, *Rora^{sg/sg}* mice *n*=7.

3.3.13 Mice reconstituted with bone marrow from *Rora*^{sg/sg} mice have altered levels of blood monocytes.

Having shown that *Rora* may have a role in the differentiation of Ly6C^{Low} bone marrow monocytes (**Figure 3.26**), it was next explored whether this alteration in the bone marrow accounted for the alterations observed in the levels blood monocyte-derived peritoneal macrophages (**Figure 3.12**) and Ly6C^{Low} blood monocytes (**Figure 3.22**) in *Rora*^{sg/sg} mice. To achieve this *bone marrow* (BM) *chimeric* mice were created. Recipient WT mice were irradiated, thus eradicating their BM and all BM-derived cells (haematopoietic cells) and were reconstituted with BM from donor *Rora*^{sg/sg} mice (*Rora*^{sg/sg}→WT) or WT mice (WT→WT) (**Section 2.23**). To check the efficiency of irradiation and BM reconstitution WT mice (CD45.2) were reconstituted with BM from *B6.SJL-Ptprc^a Pepc^b/BoyJ* mice (CD45.1) (**Section 2.1.12**) (CD45.1→CD45.2) and *B6.SJL-Ptprc^a Pepc^b/BoyJ* mice were reconstituted with BM from WT mice (CD45.2) (CD45.2→CD45.1 mice). Analysis by flow cytometry 8 weeks post BM transfer revealed that the reconstitution efficiency in CD45.1→CD45.2 mice was about 69.4% in the blood and 68.2% in CD45.2→CD45.1 mice, meaning that approximately 70% of cells in the blood of recipient mice were from donor cells (**Figure 3.28**).

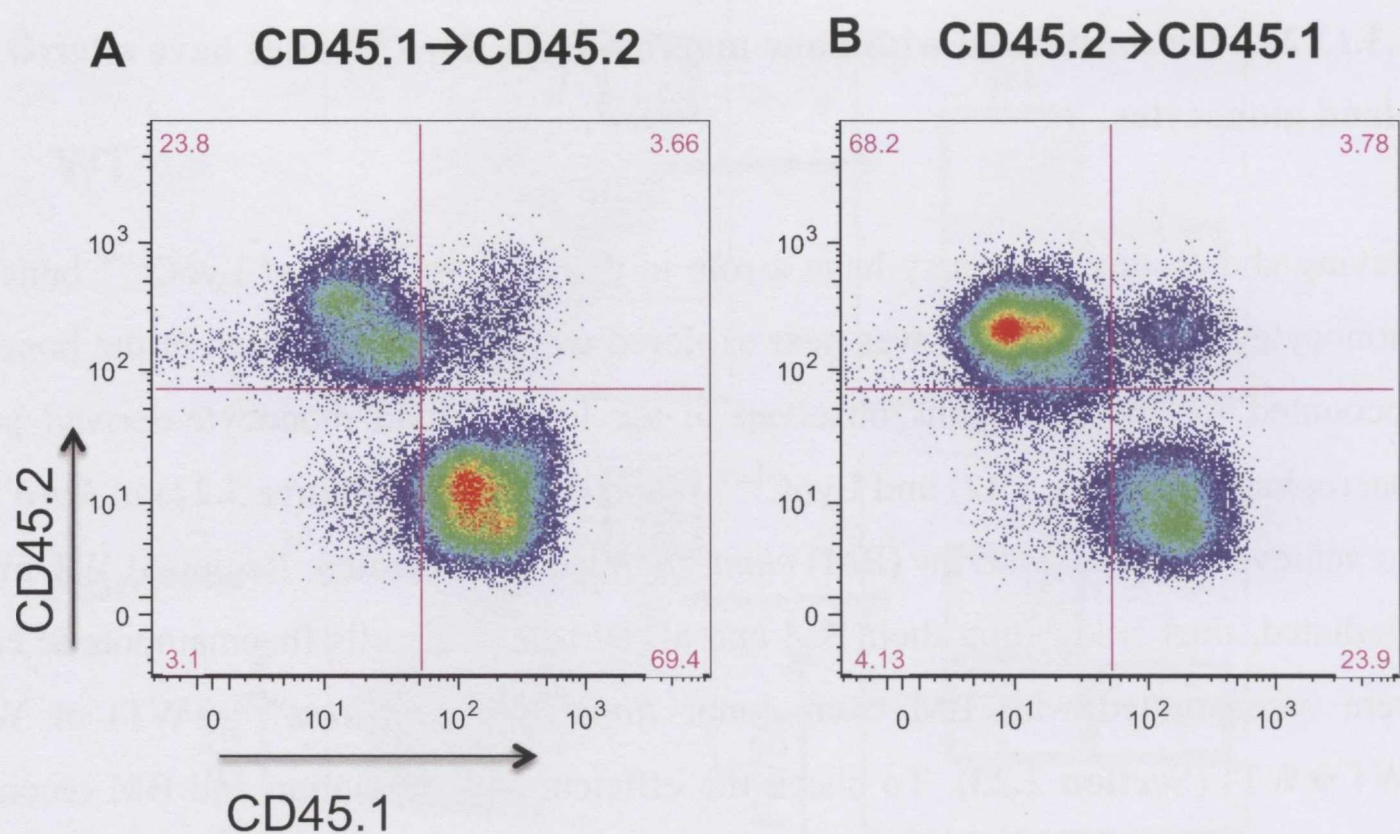
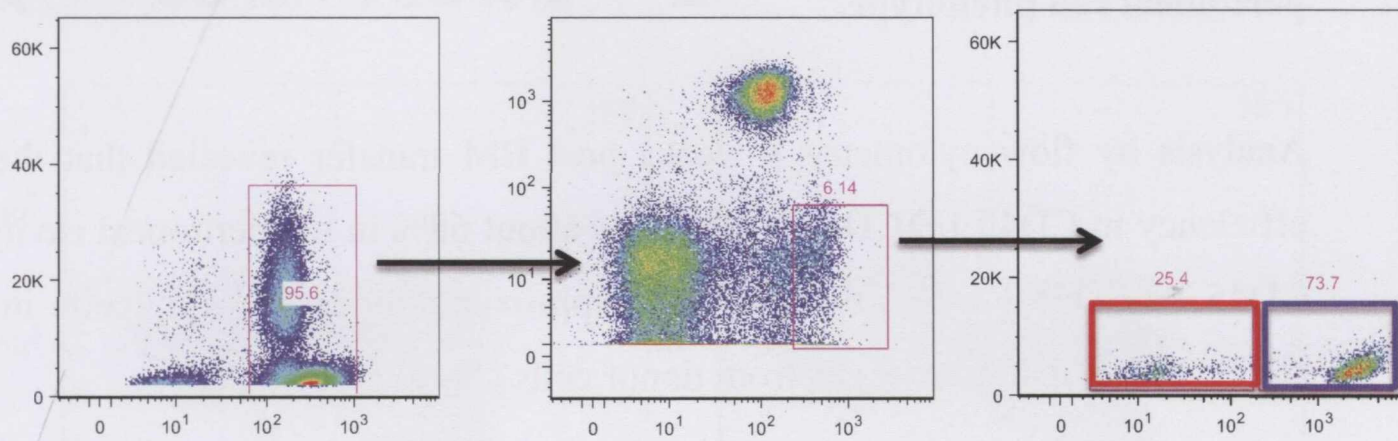


Figure 3.28 Reconstitution efficiency of bone marrow chimeras in the blood. **A** WT mice (CD45.2) were irradiated and reconstituted with bone marrow from Pep Boys (CD45.1). **B** Pep Boys (CD45.1) were irradiated and reconstituted with bone marrow from WT mice (CD45.2).

Following reconstitution for 8 weeks levels of basal $\text{Ly6C}^{\text{High}}$ and Ly6C^{Low} blood monocytes, identified in **Figure 3.22**, were explored by flow cytometry in the blood of $\text{Rora}^{\text{sg/sg}} \rightarrow \text{WT}$ mice and $\text{WT} \rightarrow \text{WT}$ mice (**Figure 3.29A**). Analysis showed that levels of the $\text{Ly6C}^{\text{High}}$ blood monocyte were statistically significantly decreased ($p < 0.05$) in $\text{Rora}^{\text{sg/sg}} \rightarrow \text{WT}$ mice compared to $\text{WT} \rightarrow \text{WT}$ mice (**Figure 3.29B**). Levels of the Ly6C^{Low} blood monocyte were unchanged between the two groups (**Figure 3.29B**). This result is in contradiction to the result observed in $\text{Rora}^{\text{sg/sg}}$ mice, in which levels of the Ly6C^{Low} blood monocytes were decreased compared to WT mice.

A WT → WT



B *Rora*^{sg/sg} → WT

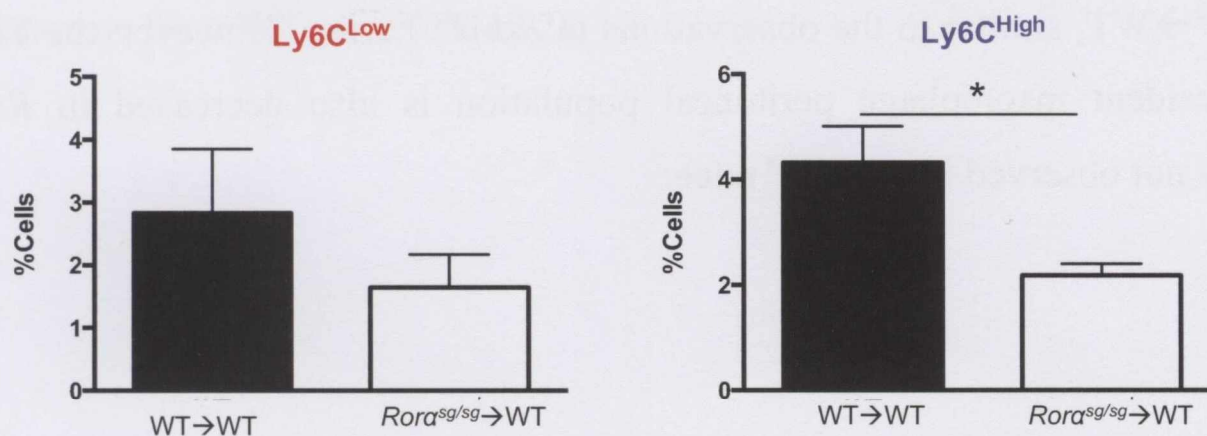
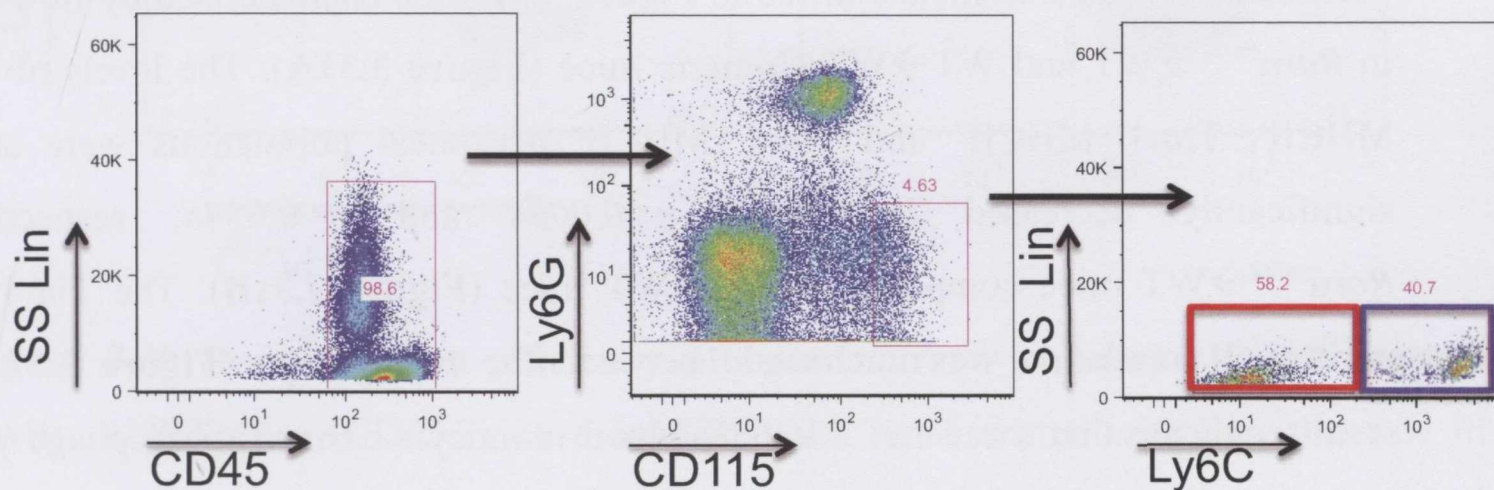


Figure 3.29 Blood monocyte levels are altered in mice reconstituted with bone marrow from *Rora*^{sg/sg} mice. **A** Representative flow cytometry plots of the gating strategy used in WT → WT mice and *Rora*^{sg/sg} → WT mice for identifying Ly6C^{Low} (CD45⁺, Ly6G⁻, CD115⁺, Ly6C^{Low}) and Ly6C^{High} (CD45⁺, Ly6G⁻, CD115⁺, Ly6C^{High}) blood monocytes. Percentages indicate typical proportions of the cell types of all peritoneal cells. **B** Quantification of blood monocyte populations (percentage of CD45⁺ cells) in WT → WT mice and *Rora*^{sg/sg} → WT mice analysed by flow cytometry in (A) above. All data represent means ± s.e.m. Differences, indicated as two-tailed *p* values, as assessed by unpaired Student *t* test. * *p* < 0.01 to 0.05. WT mice *n* = 5, *Rora*^{sg/sg} mice *n* = 5.

3.3.14 Mice reconstituted with bone marrow from *Rora*^{sg/sg} mice have an altered peritoneal cell phenotype.

Analysis by flow cytometry 8 weeks post BM transfer revealed that the reconstitution efficiency in CD45.1→CD45.2 mice was about 68% in the peritoneal cavity and 62.5% in CD45.2→CD45.1 mice, meaning that approximately 60-70% of cells in the peritoneal cavity of recipient mice were from donor cells (**Figure 3.30**).

Peritoneal cell populations identified in **Figure 3.11** were characterised by flow cytometry in *Rora*^{sg/sg}→WT and WT→WT chimeric mice (**Figure 3.31A**). The levels of the Tim4⁺ MHCII⁻, Tim4⁻ MHCII⁺ and Tim4⁻ MHCII⁻ peritoneal populations were statistically significantly decreased ($p < 0.0169$, $p < 0.0034$ and $p < 0.0348$, respectively) in *Rora*^{sg/sg}→WT mice compared to WT→WT mice (**Figure 3.31B**). The Tim4⁺ MHCII⁺ peritoneal population was unchanged between the two groups (**Figure 3.31B**). These results indicate that the Tim4⁻ MHCII⁺ blood monocyte-derived macrophage population and the Tim4⁻ MHCII⁻ eosinophil peritoneal population are both decreased in *Rora*^{sg/sg}→WT, similar to the observations in *Rora*^{sg/sg} mice. However, the Tim4⁺ MHCII⁺ tissue-resident macrophage peritoneal population is also decreased in *Rora*^{sg/sg}→WT, which is not observed in *Rora*^{sg/sg} mice.

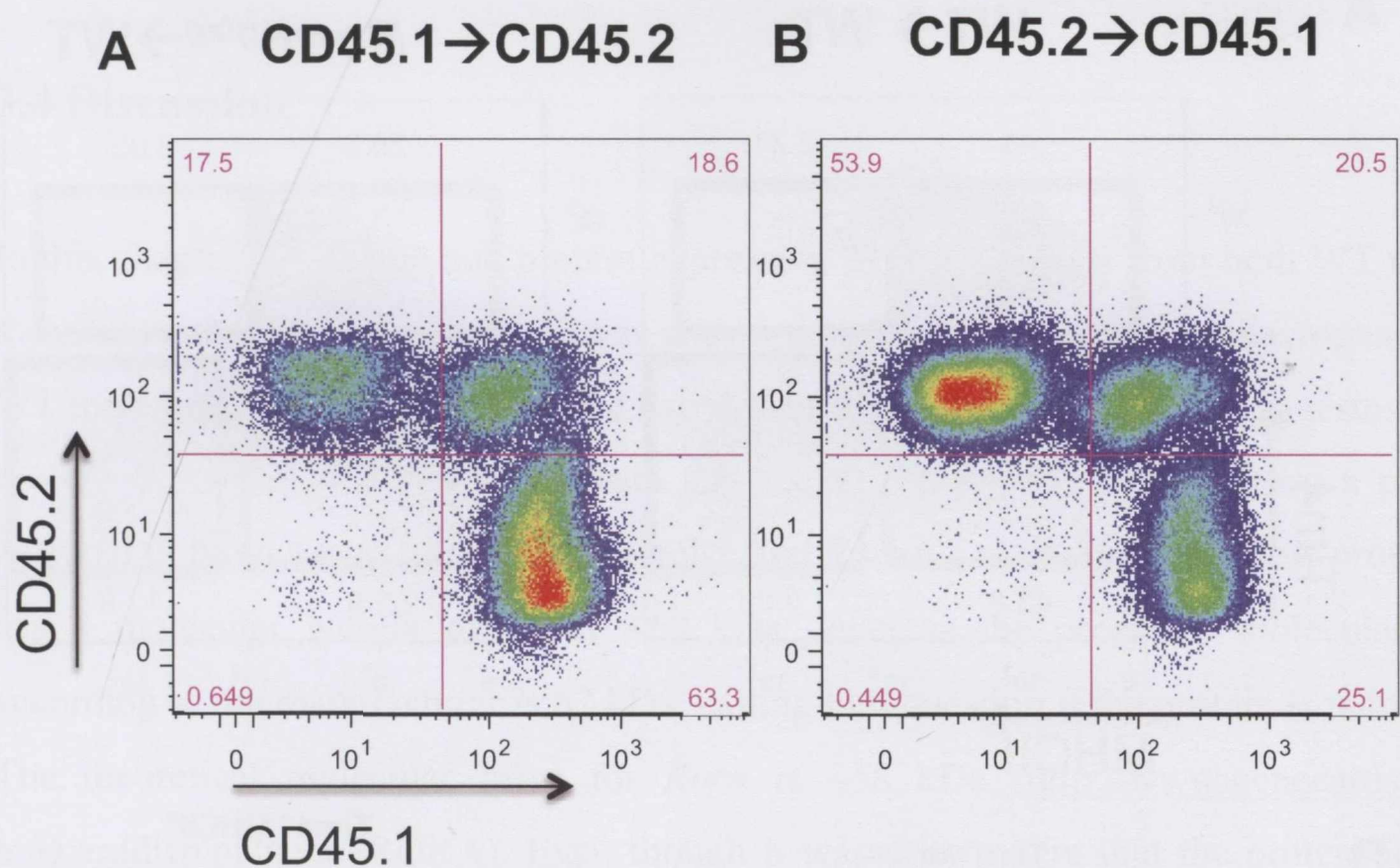


Figure 3.30 Reconstitution efficiency of bone marrow chimeras in the peritoneal cavity. **A** WT mice (CD45.2) were irradiated and reconstituted with bone marrow from Pep Boys (CD45.1) mice. **B** Pep Boys (CD45.1) were irradiated and reconstituted with bone marrow from WT mice (CD45.2).

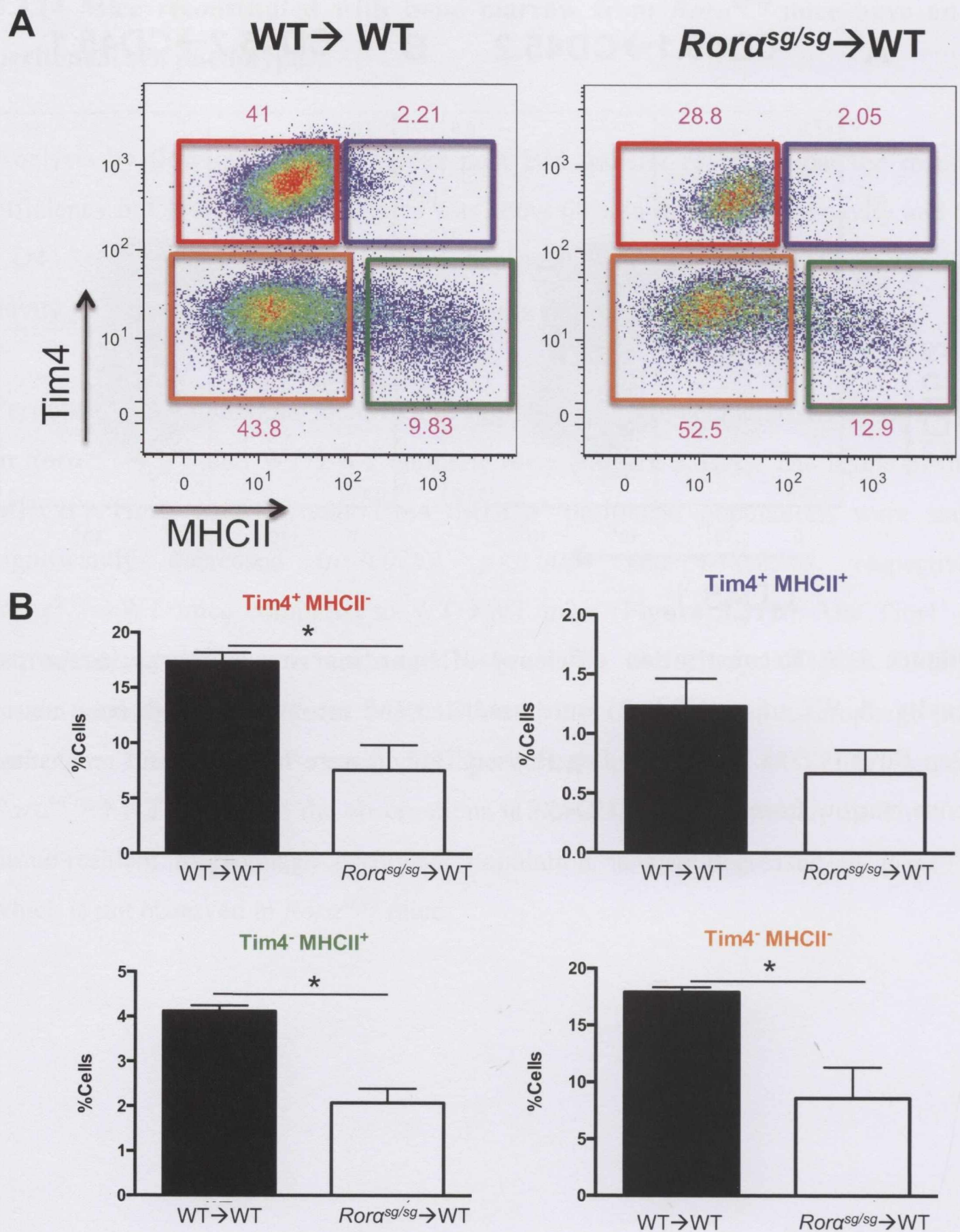


Figure 3.31 Peritoneal populations are altered in mice reconstituted with bone marrow from *Rora*^{sg/sg} mice. **A** Representative flow cytometry plots of the gating strategy used in WT → WT mice and *Rora*^{sg/sg} → WT mice for identifying peritoneal populations based on MHCII and Tim4 expression. Percentages indicate typical proportions of the cell types of all peritoneal cells. **B** Quantification of peritoneal populations in WT → WT mice and *Rora*^{sg/sg} → WT mice analysed by flow cytometry in (A) above. All data represent means ± s.e.m. Differences, indicated as two-tailed *p* values, as assessed by unpaired Student *t* test. * *p* < 0.01 to 0.05. WT mice *n* = 3, *Rora*^{sg/sg} mice *n* = 4.

3.4 Discussion

In this chapter *Rora* gene and protein expression in macrophages from both WT mice and *Rora*^{sg/sg} mice was investigated. It was shown that *Rora* is expressed in macrophages from WT mice and also that *Rora*^{sg/sg} mice express a truncated *Rora* transcript. In terms of *Rora* protein expression the results indicate that using an antibody against *Rora* a protein is detected in brain tissue from both WT mice and *Rora*^{sg/sg} mice. However, this protein band has a molecular weight of about ~60 kDa, whereas the predicted molecular weight according to the manufacturer is 67 kDa, calling into question if the protein is indeed *Rora*. The theoretical molecular mass for *Rora* is ~58 kDa (<http://www.genecards.org/cgi-bin/carddisp.pl?gene=RORA>). Even though it was shown here that the protein identified using the commercial antibody was inducible in liver extract from fasted WT mice, as was previously shown for *Rora* by Pathak and colleagues, it was important to try and verify that the protein band was *Rora*. Analysis by mass spectrometry was performed on the band to try and identify the protein present however the results were inconsistent. Two other commercial antibodies against *Rora* were tested in this thesis, however a high level of unspecific binding was detected using both antibodies.

A review of the literature reveals that another study using the same commercial antibody detected a protein band with a molecular weight of ~58 kDa in mouse brain extracts (Ino, 2004). This study also demonstrated the localisation of *Rora* mRNA and protein by immunohistochemistry to regions within the mouse brain known to express *Rora* suggesting further that the protein band identified by western blot using the antibody is *Rora* (Ino, 2004). Therefore the predicted molecular weight according to the manufacturer of 67 kDa may in fact be incorrect. Taken together the results suggest that the protein bands observed in this thesis at 60k Da is *Rora* and therefore a protein containing the epitope recognised by the commercial antibody is present in brain lysate from both WT mice and *Rora*^{sg/sg} mice.

It was also identified in this chapter that *Rora* has a role in the development of blood monocyte-derived macrophages in the peritoneal cavity. The blood monocyte-derived peritoneal macrophage population was identified based on the expression of CD11b, F4/80, Tim4 and MHCII (CD11b⁺, F4/80⁺, Tim4⁻ and MHCII⁺). This antibody surface marker panel is based on the gating strategy used in a recent paper that identifies the transcription

factor Gata6 as having a role in tissue-resident peritoneal macrophages (Rosas et al., 2014). Rosas et al. (2014) used Tim4 as a marker for tissue-resident macrophages. Others, including Thornley et al. (2014) have also shown that Tim4 is a marker for tissue-resident macrophages in the skin and the peritoneal cavity. Tim4 is primarily expressed on APCs where it recognises phosphatidylserine (PS), a specific marker of apoptosis. MHCII was also used in the panel as this has been shown to be a marker for monocyte-derived macrophages (Ghosh et al., 2010). Using this antibody panel four CD11b⁺ F4/80⁺ peritoneal populations were identified in WT mice. Rosas et al (2014) considered the Tim4⁺ MHCII⁻ population to be tissue-resident macrophages, the Tim4⁻ MHCII⁺ population as being monocyte-derived macrophages and the Tim4⁻ MHCII⁻ population as being eosinophils. They did not identify the Tim4⁺ MHCII⁺ population.

The Tim4⁻ MHCII⁻ and Tim4⁻ MHCII⁺ peritoneal populations were of interest as levels of both populations were decreased in *Rora*^{sg/sg} mice. The high expression of Siglec-F on the Tim4⁻ MHCII⁻ indicated that this population contained eosinophils; indeed this was confirmed as the population also expressed high levels of the eosinophil marker CCR3. Although an alteration in levels of eosinophils in the absence of functional *Rora* is of considerable interest in its own right, the focus of this thesis was the alteration in the Tim4⁻ MHCII⁺ peritoneal population. Surface marker expression of CCR2 and CX₃CR1 suggested that the Tim4⁻ MHCII⁺ peritoneal population was an infiltrating population as suggested by Rosas and colleagues. To confirm that was indeed a blood monocyte-derived population WT mice were treated with thioglycollate and IL-4c, a combination that has been shown to induce the recruitment and polarisation of monocyte-derived M2 macrophages in the peritoneum. Levels of the Tim4⁻ MHCII⁺ peritoneal population were increased in mice treated with thioglycollate and IL-4c. Interestingly, in naive WT mice the Tim4⁻ MHCII⁺ peritoneal population also expresses markers associated with M2 macrophages including Relm α and CD206. This suggests that in the steady state blood monocytes enter the peritoneal cavity and then differentiate into a macrophage population that has an M2-like phenotype.

Convention states that Ly6C^{High} blood monocytes are the precursors to blood monocyte-derived macrophages and express CCR2, which is expressed on the Tim4⁻ MHCII⁺ peritoneal population. Therefore it was expected that levels of the Ly6C^{High} blood monocytes would be decreased in *Rora*^{sg/sg} mice. However, here we found that levels of the Ly6C^{Low} blood monocyte population were decreased in *Rora*^{sg/sg} mice. It is important to

highlight that the dynamics of blood monocytes has come under debate in recent years. A report in 2014 showed that Ly6C^{Low} monocytes are crucial for the initiation of arthritis and they give rise to inflammatory macrophages during arthritis. However, these macrophages change their phenotype from M1 to M2 in situ during disease progression, thus contributing to disease resolution (Misharin et al., 2014). Another group found that M2 macrophages in liver granulomas of *Schistosoma mansoni* infected mice arise predominantly from recruited Ly6C^{High} monocytes, but that these cells may transition through a Ly6C^{Low} state (Girgis et al., 2014). Indeed it has been shown that Ly6C^{High} monocytes can also differentiate into Ly6C^{Low} cells in the bone marrow and blood under steady state conditions (Yona et al., 2013). These studies and others highlight that the developmental relationship between Ly6C^{High} and Ly6C^{Low} monocytes has not been fully elucidated. The results in this thesis would indicate that in the steady state Ly6C^{Low} blood monocytes are the primary precursors for the Tim4⁻ MHCII⁺ peritoneal monocyte-derived macrophage population. However, Ly6C^{High} blood monocytes may also be contributors; this requires further investigation.

Levels of myeloid progenitor cells were explored in the bone marrow and levels of Ly6C^{Low} bone marrow monocytes were observed to be increased in *Rora*^{sg/sg} mice compared to WT mice, while levels of Ly6C^{High} bone marrow monocytes were unchanged. This result is of interest as it shows it reflects the fact that there is also an alteration in Ly6C^{Low} blood monocyte levels and Ly6C^{Low} splenic monocyte levels in *Rora*^{sg/sg} mice. Taken together these results suggest that in the absence of functional *Rora*, Ly6C^{Low} bone marrow monocytes are accumulating in the bone marrow and not transitioning into the blood, therefore there is lack of Ly6C^{Low} blood monocytes in the circulation. The reason for this alteration is unclear although there are a number of possibilities. The first of these is that *Rora* is involved in regulating the development of Ly6C^{Low} bone marrow monocytes and when *Rora* is absent dysregulation occurs and an altered Ly6C^{Low} bone marrow monocyte population develops that does not have the capacity to transition into the blood. The lineage differentiation point at which *Rora* may act is unclear. In the conventional model Ly6C^{Low} bone marrow monocytes differentiate from MDP cells. However, as discussed above research shows that Ly6C^{High} bone marrow monocytes can also differentiate into Ly6C^{Low} bone marrow monocytes.

Rora^{sg/sg} BM chimeras were generated (*Rora*^{sg/sg} → WT mice) to explore the possibility that the accumulation of Ly6C^{Low} bone marrow monocytes in *Rora*^{sg/sg} mice was due to *Rora*

having an intrinsic role in their development. It was hypothesised that if *Rora* had an intrinsic role in the development of Ly6C^{Low} bone marrow monocytes, this defect would be present in the donor bone marrow cells used to reconstitute donor WT mice and therefore the alterations in Ly6C^{Low} monocytes and the Tim4⁺ MHCII⁺ peritoneal macrophage population seen in *Rora*^{sg/sg} mice would also be seen in *Rora*^{sg/sg} → WT mice. However, the interpretation of results from these BM chimera mice is difficult as chimerism was only 70:30, indicating that the irradiation of recipient mice was not very efficient. Therefore it would be beneficial to re-examine the irradiation protocol and when 90% chimerism is achieved, repeat the BM chimera experiment.

An alternative to using full BM chimeras is to use mixed BM chimeras to test the hypothesis that *Rora* is important for maintenance of Ly6C^{Low} monocytes. In this experiment recipient WT mice would be reconstituted with BM from *Rora*^{sg/sg} mice (CD45.2) mixed with BM from WT Pepboys (CD45.1) at an equal 50:50 ratio. By examining the expression of CD45.1 and CD45.2 in the Ly6C^{Low} monocyte population it would be possible to determine what ratio of this population is derived from BM from *Rora*^{sg/sg} mice compared to BM from WT Pepboys. If the ratio is not 1:1 and the CD45.2 is underrepresented this would indicate that *Rora* is important for maintenance of Ly6C^{Low} monocytes.

The second possibility is that the Ly6C^{Low} bone marrow monocyte population is functionally comparable to WT mice but the signals for egress from the bone marrow into the blood is defective in the absence of functional *Rora*. It is known that Ly6C^{High} bone marrow monocytes but not Ly6C^{Low} bone marrow monocytes require CCR2 for their transition into the blood (Serbina and Pamer, 2006). However, it has been shown that both Ly6C^{High} bone marrow monocytes and Ly6C^{Low} blood monocyte levels are altered in mice administered the CCR2 antibody MC21 (Yona et al., 2013). The authors suggest that this is due to the precursor/production relationship between the two populations, however, it may also suggest that Ly6C^{Low} blood monocytes can respond to CCL2. Furthermore it has been shown that *Rora* binds to a RORE present in the promoter region of *Ccl2*, promoting the expression of CCL2 (Sato et al., 2014). Therefore in the absence of functional *Rora* levels of CCL2 would theoretically be decreased, which could contribute to the build up of Ly6C^{Low} bone marrow monocytes. However, in this case levels of Ly6C^{High} blood monocytes would also be altered, which is not observed here.

In addition to CCL2 other cytokines such as CXCL12 and CXCL3 are important in monocyte egress from the bone marrow. Monocytes in the bone marrow express CXCR4 the receptor for CXCL12, whose activation immobilizes cells in the bone marrow, and are in contact with stromal cells expressing CXCL12. During the inflammatory response, CCL2 is released and activates CCR2 on neighbouring monocytes. The activation of CCR2 attenuates an anchoring signalling provided by CXCR4 expressed by the same cell and mobilizes bone marrow monocytes to the blood stream. Inhibiting CXCR4 by a specific receptor antagonist in mice causes CCR2-expressing cells to exit the bone marrow in absence of inflammatory insults (Jung et al., 2015).

A chemotaxis assay could be developed to investigate the possibility that the egress of Ly6C^{Low} bone marrow monocytes in $\text{Rora}^{\text{sg/sg}}$ mice is altered compared to WT mice. In this assay Ly6C^{Low} bone marrow monocytes would be isolated from WT mice and $\text{Rora}^{\text{sg/sg}}$ mice and the migration of both these populations towards a chemokine could be determined. A variety of different chemokines important in bone marrow monocyte egress could be used including CCL2, CCL7 and CXCL12. If significantly decreased numbers of cells from $\text{Rora}^{\text{sg/sg}}$ mice migrate towards the chemokine compared to cells from WT mice then this indicates that Ly6C^{Low} bone marrow monocytes from $\text{Rora}^{\text{sg/sg}}$ mice have a defect in migration. This could in turn explain why there are increased levels of Ly6C^{Low} bone marrow monocytes in $\text{Rora}^{\text{sg/sg}}$ mice compared to WT mice. The defect could be due to an alteration in the corresponding receptor for the chemokine. Alternatively if no difference is observed in cell migration between $\text{Rora}^{\text{sg/sg}}$ mice and WT mice it is also possible that the *in vivo* levels of the chemokines could be altered between the two groups. It would then be worth investigating *in vivo* levels of each chemokine in both $\text{Rora}^{\text{sg/sg}}$ mice and WT mice.

It is also possible that the decrease in Ly6C^{Low} blood monocytes in $\text{Rora}^{\text{sg/sg}}$ mice is not due to a defect in egress but rather is due to a defect in homeostatic persistence of this population in the periphery. Therefore Ly6C^{Low} blood monocytes enter the blood stream normally but do not persist or survive. To test this bromodeoxyuridine (BrdU) labelling could be used to track Ly6C^{Low} monocyte survival in WT mice and $\text{Rora}^{\text{sg/sg}}$ mice. Cells in mitosis incorporate BrdU into replicating DNA thus representing a convenient means of studying the transit time of cells in the bone marrow and their subsequent trafficking in peripheral blood. Furthermore, the levels of apoptosis in peripheral monocytes in WT mice and $\text{Rora}^{\text{sg/sg}}$ mice could also be potentially investigated. Ly6C^{Low} blood monocytes could be isolated from WT mice and $\text{Rora}^{\text{sg/sg}}$ mice and stained with conjugated Annexin V to

detect translocated phosphatidylserine and thus determine levels of cell death in the monocyte population from the two groups. Any differences in apoptosis between monocytes from WT mice and *Rora*^{sg/sg} mice could then be investigated further.

In summary levels of the Tim4⁻ MHCII⁺ peritoneal monocyte-derived macrophage population and Ly6C^{Low} blood monocytes are decreased and levels of Ly6C^{Low} bone marrow monocytes are increased in the absence of functional *Rora*. Taken together, the results suggest that *Rora* has a role in the development of Tim4⁻ MHCII⁺ peritoneal monocyte-derived macrophages and Ly6C^{Low} blood monocytes and that this role begins at the Ly6C^{Low} bone marrow monocytes lineage differentiation stage. It is unclear at present if *Rora* has an intrinsic or extrinsic role at the Ly6C^{Low} bone marrow monocytes lineage differentiation stage; further research to establish this is needed.

Chapter 4

*Determining the role of Rora in
inflammation in the context of
macrophages.*

4.1 Introduction

Inflammation is the immediate response of our tissues to danger such as microbial invasion, injury, or a serious malfunction of our body's internal workings. Whereas all cells can respond to danger, innate immune cells including macrophages are primary responders through their expression of PRRs (**Section 1.2.2**). TLR4 is one of the most extensively studied PRR in mice. TLR4 is a type I transmembrane receptor protein that recognises LPS (Poltorak et al., 1998). Stimulation of macrophages with LPS elicits a variety of different signalling events that culminate in the activation of macrophage effector functions including changes in gene expression and enhanced production of inflammatory cytokines including cytokines such as IFN γ , IL-1 β , IL-6, IL-12 and TNF α (Bode et al., 2012) (**Section 1.14**). Sepsis is a complex inflammatory process that is believed to develop when the initial host response against infection described above becomes inappropriately amplified and dysregulated, thus becoming a harmful host response (**Section 1.17.2**).

Rora has been shown to have a role in inflammation and sepsis. It has been shown that ectopic expression of ROR α inhibits TNF α -induced IL-6, IL-8 and COX-2 expression in human primary smooth-muscle cells (Delerive et al., 2001). ROR α negatively interferes with the NF- κ B signalling pathway by reducing p65 translocation. This action of ROR α on NF- κ B is associated with the induction of I κ B α , the major inhibitory protein of the NF- κ B signalling pathway, whose expression was found to be transcriptionally upregulated by ROR α via a ROR response element in the I κ B α promoter (Delerive et al., 2001). Furthermore *Rora* has been shown to have a role in the induction of airway inflammation in response to LPS challenge. Stapleton and colleagues showed that *Rora*^{sg/sg} mice displayed a higher degree of LPS-induced inflammation than WT mice and that this increased susceptibility correlated with a higher number of total cells and neutrophils in bronchoalveolar lavage (BAL) fluids from LPS-treated *Rora*^{sg/sg} mice. In addition, IL-1 β , IL-6, and CXCL2 were elevated in BAL fluids from LPS-treated *Rora*^{sg/sg} mice compared to WT mice (Stapleton et al., 2005).

Of interest, *Rora* appears to have a role in macrophage responses to LPS. Kopmels and colleagues showed that macrophages from *Rora*^{sg/sg} mice hyper-expressed IL-1 β mRNA when stimulated with LPS, even for periods as short as 15 min. This hyper-inducibility of macrophages from *Rora*^{sg/sg} mice was observed even when very low doses of LPS were

used (0.01 µg /ml) and reached its maximum when 5 µg/ml LPS was used (Kopmels et al., 1992). Hyper-expression of TNFα and IL-1α mRNAs was also detected in LPS-stimulated BMDMs of mutant mice. Macrophages from *Rora* knock-out mice (*Rora*^{-/-} mice) were also shown to produce elevated levels of TNFα and IL-6 in response to LPS stimulation (Dzhagalov et al., 2004). These results indicated that *Rora* plays a role in enhancing inflammatory responses in macrophages.

Rora has recently been shown to have a role in the initiation of the innate immune response against inflammation in a mouse model of sepsis. Garcia and colleagues showed that NF-κB and inflammasome NLRP3 interact in sepsis induced by cecal ligation and puncture (CLP), leading to pro-inflammatory and pro-oxidant status in heart tissue of septic C57BL/6J mice (Garcia et al., 2015). They showed that melatonin administration blunts NF-κB transcriptional activity through a sirtuin1-dependent NF-κB deacetylation in septic mice. Melatonin also decreased the NF-κB-dependent pro-inflammatory response and restored redox balance and mitochondrial homeostasis, thus inhibiting the NLRP3 inflammasome. Interestingly, the inhibition of NF-κB by melatonin, but not that of NLRP3, was blunted in *Rora*^{sg/sg} mice, indicating that functional *Rora* is necessary for the initiation of the innate immune response against inflammation (Garcia et al., 2015).

In their paper Garcia and colleagues (2015) used CLP to induce sepsis in mice. In this method the cecum is exteriorised through a small abdominal incision, a suture ligature is placed distal to the ileocolic junction, and a needle is used to perforate the viscus (Nemzek et al., 2008). Another widely used model of inducing sepsis in mice is through a single injection of LPS (LPS-induced endotoxemia). After LPS administration, the onset of systemic clinical signs, including reduced motor activity, shivering, lethargy, and piloerection, occurs quickly and progresses rapidly. In general, LPS administration also results in hypothermia, most likely due to the large surface-area-to-volume ratio of these small animals (Nemzek et al., 2008). Complete blood counts in septic mice reveal decreased total white blood cell numbers, with notable reductions of lymphocytes and neutrophils (Remick et al., 2000). Injection of high-dose LPS also induces a very rapid, but transient, increase in systemic inflammatory cytokine levels with levels reaching a peak between 1.5 to 4.5 hours (Buras et al., 2005; Remick et al., 2000). The LPS-induced endotoxemia model is used in this study (**Section 2.21**).

Aside from its role in inflammation *Rora* is also known to be a molecular component of the circadian clock (**Section 1.17**). Circadian clocks coordinate physiology and behaviour within the 24 hour day to provide temporal homeostasis with the external environment (Partch et al., 2014). Circadian rhythms are emerging as important regulators of specific immune functions, possibly allowing the host to anticipate and respond to microbial threats more effectively (Scheiermann et al., 2013). A number of studies have shown the importance of these circadian oscillations in macrophage inflammatory responses (**Section 1.16**). More recently the term ‘anticipatory inflammation’ has been coined, this is where circadian oscillations can potentially regulate inflammatory responses by modulating rhythmic expression of inflammatory genes in tissue macrophages or by controlling rhythmic trafficking of inflammatory monocytes (Ginhoux and Jung, 2014). These circadian oscillations in immune cells are under direct control of genes of the circadian clock, for instance Nguyen and colleagues (2013) showed that *Bmal1* controls the circadian oscillation of Ly6C^{High} blood monocytes.

4.2 Chapter Objectives

Rora has been shown to have a role in inflammatory responses as discussed in **Section 4.1**. Here we first wanted to determine ourselves if the absence of functional *Rora* results in altered responses to LPS. Results in Chapter 3 showed that in the absence of functional *Rora* levels of blood monocyte-derived peritoneal macrophages are decreased. We therefore wanted to determine if this macrophage population is involved in inflammatory responses and if inflammatory responses are altered when this population is decreased due to the absence of functional *Rora*. Alongside this, there has recently been increased interest in the concept of ‘anticipatory inflammation’ as discussed above. Thus the second overall objective of this chapter was to determine if *Rora* contributes to the regulation of circadian oscillations of peritoneal macrophage populations. And furthermore, determine if *Rora* could also potentially contribute to inflammatory responses through its role in regulating these circadian oscillations of peritoneal macrophage populations.

1. To investigate if *Rora* has a role in inflammatory responses to LPS *in vivo*.
2. To determine if macrophage responses to LPS are altered in the absence of *Rora* *in vitro*.
3. To explore if *Rora* has a role in the circadian oscillations of peritoneal macrophages *in vivo*.
4. To investigate if the circadian oscillations of peritoneal macrophages alter diurnal responses to an LPS.

4.3 Results

4.3.1 *Rora*^{sg/sg} mice are less susceptible to LPS-induced endotoxemia compared to WT mice.

To determine if responses to the inflammatory stimulus LPS *in vivo* are altered in the absence of *Rora*, WT mice and *Rora*^{sg/sg} mice were injected intra-peritoneal (I.P) with LPS (10 mg/kg). Mice were then monitored for 48 hours as outlined in **Section 2.21**. Sepsis scores and body temperature were measured over this time period. The percentage survival of WT mice decreased by 6 hours post LPS administration and was 0% by 48 hours (**Figure 4.1A**). The percentage survival of *Rora*^{sg/sg} mice also began to decrease by 6 hours post LPS administration, however, percentage survival was significantly higher 66% ($p < 0.0123$) compared to WT mice at 48 hours (**Figure 4.1A**). Sepsis scores were recorded 1-hour post LPS administration in WT mice, increasing to the maximum score of 5 by 12 hours (**Figure 4.1B**). Sepsis scores were also recorded 1-hour post LPS administration in *Rora*^{sg/sg} mice, however, in comparison to WT mice *Rora*^{sg/sg} mice had significantly decreased sepsis scores ($p < 0.0075$) post LPS administration as calculated by area under the curve (AUC) (**Figure 4.1B**).

Hypothermia is associated with LPS-induced endotoxemia (Nemzek et al., 2008), therefore changes in body temperature were monitored in both groups. The body temperatures of WT mice decreased following LPS administration and were at the lowest levels 12 hours post LPS administration (**Figure 4.1C**). The body temperatures of *Rora*^{sg/sg} mice also decreased following LPS administration and were at the lowest levels 12 hours post LPS administration (**Figure 4.1C**). However, the body temperatures of *Rora*^{sg/sg} mice were significantly higher ($p < 0.024$) than the body temperatures of WT mice post LPS administration as calculated by AUC (**Figure 4.1C**), and after 12 hours the body temperatures of *Rora*^{sg/sg} mice began to increase. These results indicate that in comparison to WT mice, *Rora*^{sg/sg} mice have increased survival in the model of LPS-induced endotoxemia.

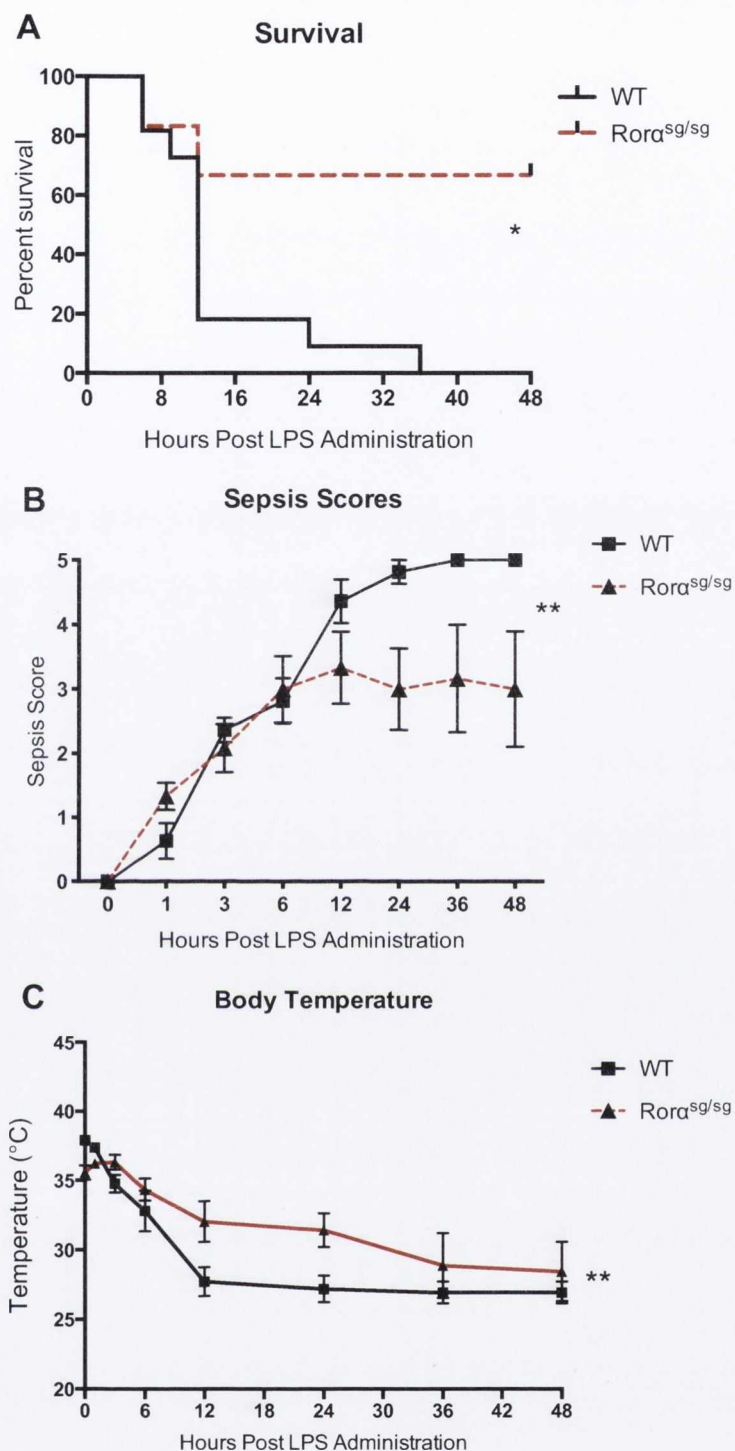


Figure 4.1 $Rora^{sg/sg}$ mice are less susceptible to LPS-induced endotoxemia compared to WT mice. WT mice and $Rora^{sg/sg}$ mice were administered LPS I.P (10 mg/kg). **A** Percentage survival. **B** Sepsis scores. **C** Body temperatures. The Kaplan-Meier method was used to evaluate statistical difference in survival between groups. Data represent means $\pm$ s.e.m. Sepsis scores and body temperature analysed by area under curve (AUC). Differences in AUC values indicated as two-tailed p values, as assessed by unpaired Student t test. * $p < 0.01$ to 0.05 , ** $p < 0.001$ to 0.01 . Survival and sepsis scores WT mice $n=11$, $Rora^{sg/sg}$ mice $n=6$. Body temperature WT mice $n=8$, $Rora^{sg/sg}$ mice $n=4$.

4.3.2 Serum cytokine levels are altered in *Rora*^{sg/sg} mice compared to WT mice following administration of LPS.

Injection of mice with LPS induces a very rapid, but transient, increase in systemic cytokine levels (Buras et al., 2005). Cytokine levels were explored in the serum of WT mice and *Rora*^{sg/sg} mice 3 hours post LPS administration (10 mg/kg) (**Section 2.12**). Three hours was chosen as it is between 1.5 and 4.5 hours, a time period that has been described as the peak cytokine production time post LPS administration (Remick et al., 2000). Results showed that LPS administration significantly increased serum levels of the cytokines IL-1 β , IL-2, IL-6, IL-10 and IL-12-p70 3 hours post LPS administration in both WT mice and *Rora*^{sg/sg} mice compared to controls (**Figure 4.2**). However, the serum levels of IL-1 β and IL-5 induced were significantly reduced in *Rora*^{sg/sg} mice when compared to WT mice ($p < 0.036$ and $p < 0.0089$ respectively) (**Figure 4.2**). Interestingly, the levels of IL-1 β and IL-5 were also significantly decreased ($p < 0.0367$ and $p < 0.0089$ respectively) in control *Rora*^{sg/sg} mice compared to control WT mice (**Figure 4.2**). The levels of serum cytokines IFN γ , TNF α IL-2, IL-6 and IL-10 were statistically unchanged between the two groups.

These results suggest that inflammatory cytokines are being produced in response to LPS in the absence of *Rora*. However, IL- β and IL-5 are being produced at decreased levels as compared to cytokine levels produced in WT mice in response to LPS.

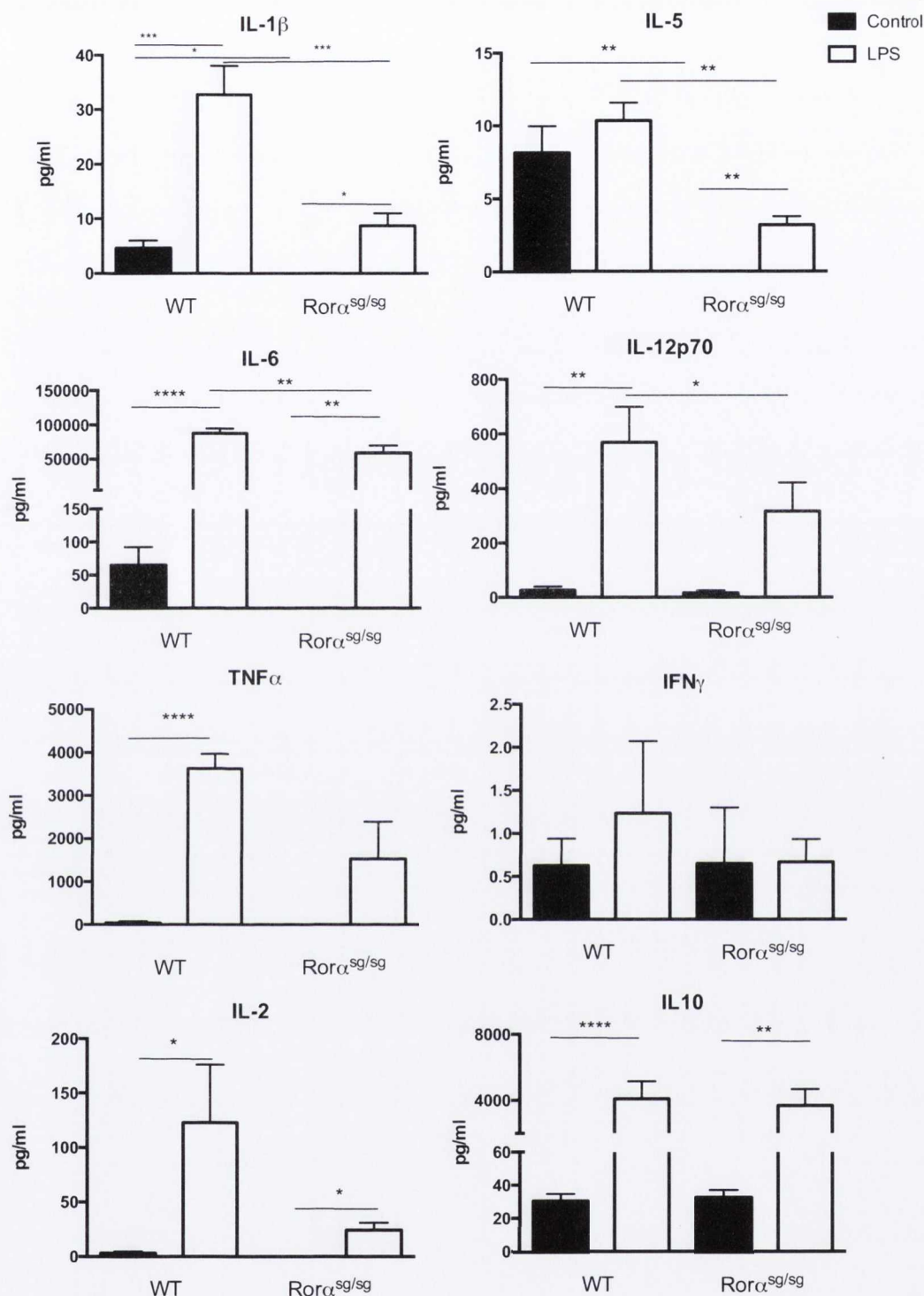


Figure 4.2 Serum cytokine levels are altered in *Rora^{sg/sg}* mice compared to WT mice following LPS administration. Serum cytokine levels were measured 3 hours post LPS administration. All data represent means $\pm$ s.e.m. Differences, indicated as two-tailed *p* values, as assessed by unpaired Student *t* test. * *p* < 0.01 to 0.05, ** *p* < 0.001 to 0.01, *** *p* < 0.0001 to 0.001, **** *p* < 0.0001. WT mice control *n*=7, WT mice LPS *n*=4, *Rora^{sg/sg}* mice control *n*=4, *Rora^{sg/sg}* mice LPS *n*=7. TNF α WT mice control *n*=4, WT mice LPS *n*=4, *Rora^{sg/sg}* mice control *n*=3, *Rora^{sg/sg}* mice LPS *n*=3.

4.3.3 Levels of inflammatory cytokines are altered in the peritoneal cavity of *Rora*^{sg/sg} mice in response to LPS compared to WT mice.

In the LPS-induced endotoxemia model LPS is injected directly into the peritoneal cavity. Therefore in this model cells present in the peritoneal cavity are the first responders to LPS. Among the first responder immune cells are macrophages which represent an estimated 30% of total live cells in the peritoneal cavity (Ray and Dittel, 2010). We have previously shown that *Rora*^{sg/sg} mice display an altered macrophage immunophenotype in the peritoneal cavity (**Figure 3.11**) therefore it was decided to analyse if peritoneal cells, which would include peritoneal macrophages, have an altered inflammatory response to LPS *in vivo* in *Rora*^{sg/sg} mice. To achieve this LPS (10 mg/kg) was administered I.P to WT mice and *Rora*^{sg/sg} mice and peritoneal cells were isolated in PBS 3 hours post LPS administration. Results showed that IL-1 β levels were significantly higher ($p < 0.0175$) in the peritoneal cavity of WT mice compared to IL-1 β levels in the peritoneal cavity of *Rora*^{sg/sg} mice 3 hours post LPS administration (**Figure 4.3**). These results indicate that in the absence of *Rora* IL-1 β levels are decreased in the peritoneal cavity following LPS administration.

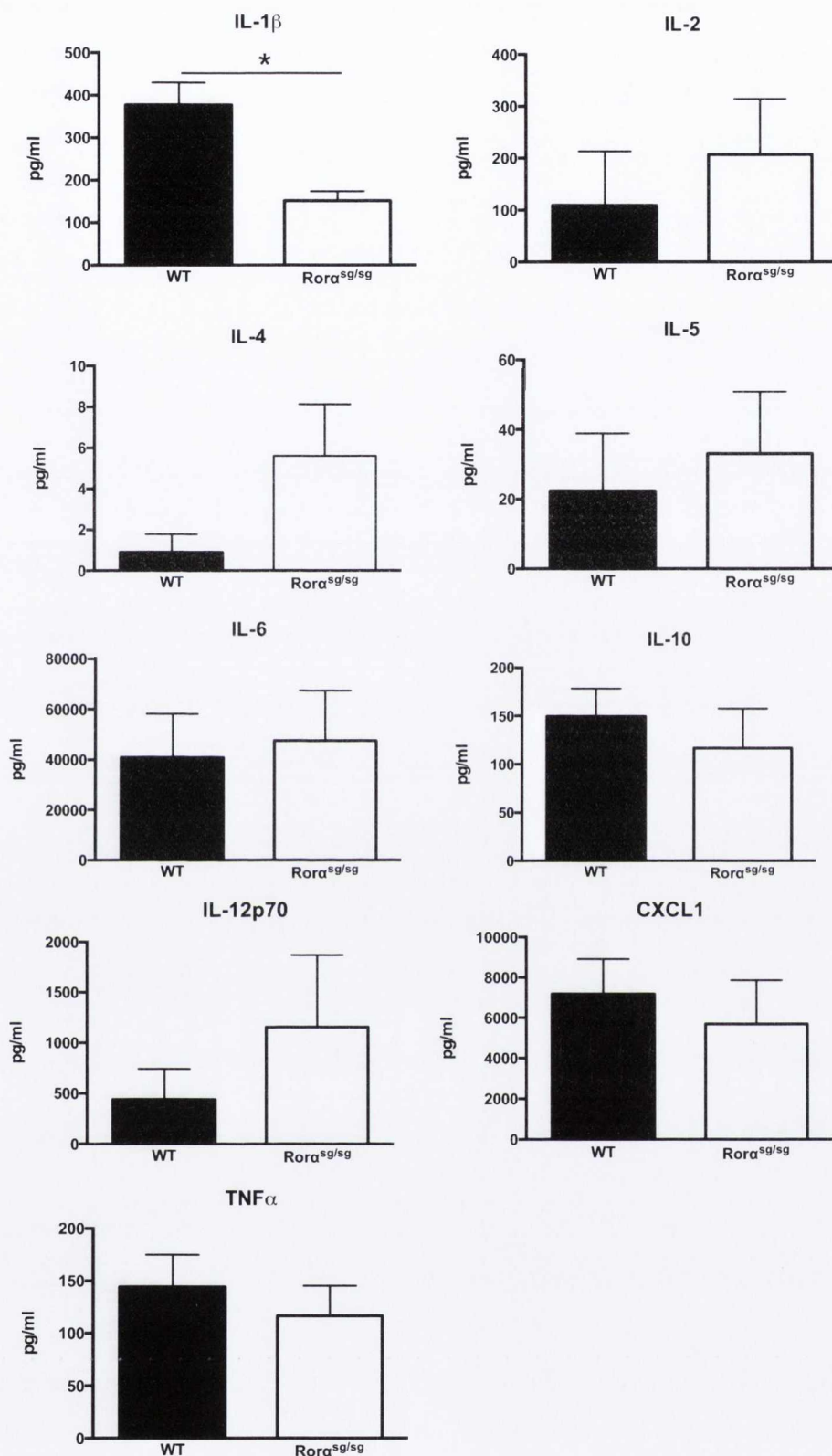


Figure 4.3 Peritoneal cells in *Rora^{sg/sf}* mice have altered cytokine production in response to LPS compared to peritoneal cells in WT mice. Cytokine levels were measured 3 hours post LPS administration. All data represent means $\pm$ s.e.m. Differences, indicated as two-tailed p values, as assessed by unpaired Student t test. * $p < 0.01$ to 0.05 . WT mice $n=4$, *Rora^{sg/sf}* mice $n=3$.

4.3.4 Peritoneal macrophages from *Rora*^{sg/sg} mice produce altered levels of inflammatory cytokines in response to LPS *in vitro* compared to WT mice.

In addition to macrophages the peritoneal cavity contains a number of other immune cell populations including B-cells, T-cells, DCs, eosinophils, neutrophils, mast cells, natural killer cells and invariant natural killer cells (Ghosn et al., 2010). In addition to these immune cells the peritoneal cavity is lined by a monolayer of mesothelial cells that are known to express pro-inflammatory cytokines in response to LPS (Kato et al., 2004; Park et al., 2007). Therefore although macrophages are primary responders to LPS other peritoneal cell populations and indeed mesothelial cells could potentially produce cytokines in response to LPS. Thus other cells could account for the differences in IL-1 β production observed between peritoneal cells from WT mice and *Rora*^{sg/sg} mice in response to LPS (**Figure 4.3**). To analyse the levels of cytokine produced specifically by peritoneal macrophages in response to LPS, peritoneal macrophages were isolated from WT mice and *Rora*^{sg/sg} mice (**Section 2.3.1**) and cultured *in vitro* with LPS (100 ng/ml) for 6 hours and 24 hours using a standard protocol (Mosser et al., 2008)(**Section 2.19**).

Results showed that peritoneal macrophages from WT mice stimulated with LPS, increased production of pro-inflammatory cytokines over 24 hours (**Figure 4.4**). Over 24 hours peritoneal macrophages from *Rora*^{sg/sg} mice stimulated with LPS also increased production of pro-inflammatory cytokines (**Figure 4.4**), however, peritoneal macrophages from *Rora*^{sg/sg} mice produced significantly decreased levels of IL-1 β , IL-6, IL-10 and CXCL1 ($p < 0.0327$, $p < 0.013$, $p < 0.0073$ and $p < 0.036$) when stimulated with LPS for 6 hours (**Figure 4.4**). Following stimulation with LPS for 24 hours peritoneal macrophages from *Rora*^{sg/sg} mice produced significantly decreased levels of IL-1 β ($p < 0.0128$) compared to peritoneal macrophages from WT mice (**Figure 4.4**).

These results indicate that peritoneal macrophages have altered cytokine responses to LPS *in vitro* in the absence of *Rora*. In particular IL-1 β cytokine production is decreased in the absence of *Rora* following 6 hours and 24 hours stimulation with LPS.

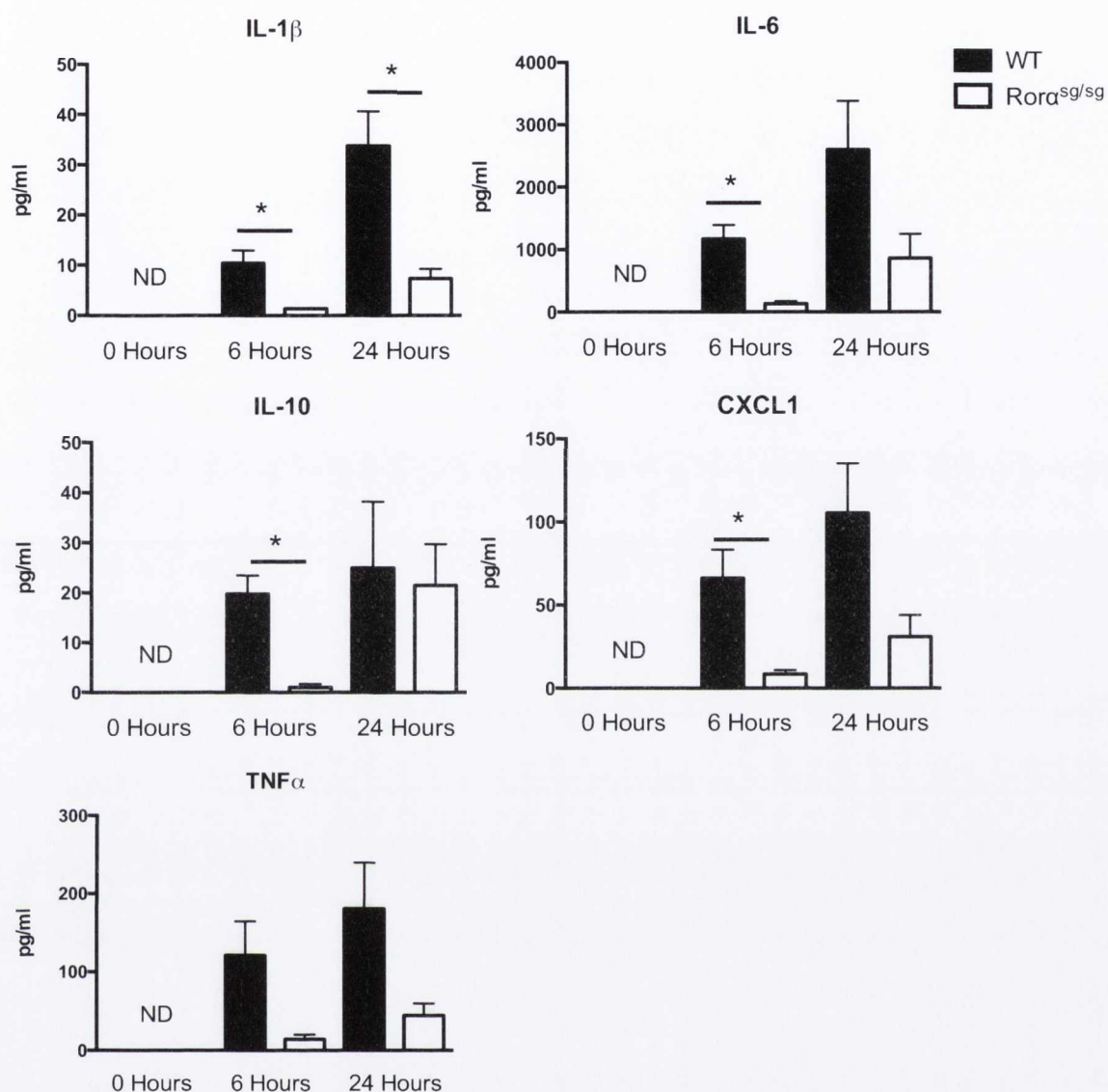


Figure 4.4 Peritoneal macrophages from *Rora*^{sg/sg} mice produce decreased levels of inflammatory cytokines in response to LPS *in vitro* compared to peritoneal macrophages from WT mice. Peritoneal macrophages were stimulated with LPS (100 ng/ml) and cytokine levels measured in supernatants at 6 and 24 hours. All data represent means $\pm$ s.e.m. Differences, indicated as two-tailed *p* values, as assessed by unpaired Student *t* test. * *p* < 0.01 to 0.05. Replicates WT mice *n*=6, replicates *Rora*^{sg/sg} mice *n*=6.

4.3.5 Peritoneal populations in WT mice display differential expression of TLR4.

Results in **Section 4.3.4** indicate that peritoneal macrophages stimulated with LPS *in vitro* increase their inflammatory cytokine production. The four peritoneal populations identified by CD11b, F4/80, Tim4 and MHCII expression in Chapter 3 may be among these populations that respond to LPS. LPS binds to TLR4 in complex with MD2 on cells and cytokine production is the end product of the signalling cascade initiated by LPS/TLR4 binding (**Section 1.14**). It has also been shown that TLR4 expression levels determine the degree of LPS responses in a variety of cells including macrophages (Kalis et al., 2003). Therefore, the level of TLR4 expression on each of the four peritoneal populations was determined to establish if all four peritoneal populations express TLR4 and can therefore respond to LPS. In chapter 3 it was also established that in the absence of functional *Rora* levels of the Tim4⁻ MHCII⁺ (blood monocyte-derived macrophages) and Tim4⁻ MHCII⁻ (eosinophils) peritoneal populations are decreased. It was of interest therefore to determine if these two populations express TLR4 in WT mice and could therefore be responsive to LPS.

It was first determined if the TLR4 antibody used was specific for TLR4 using *Tlr4*^{-/-} mice as a negative control for flow cytometry staining. Peritoneal cells from WT mice and *Tlr4*^{-/-} mice were isolated and stained with an antibody panel including the TLR4 antibody. Results confirmed that no TLR4 staining was observed in *Tlr4*^{-/-} mice while in WT mice TLR4 staining was observed (**Figure 4.5**), indicating that the TLR4 antibody is specific for TLR4.

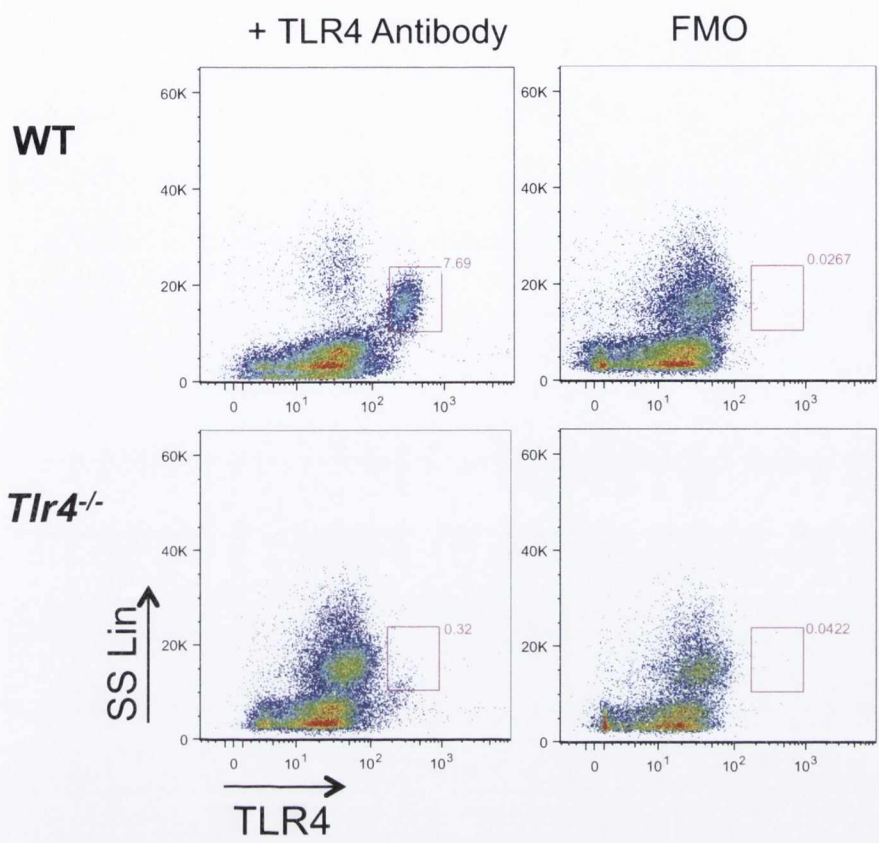


Figure 4.5 The TLR4 antibody is specific for TLR4. Peritoneal cells were isolated from WT mice and *Tlr4*^{-/-} mice and stained with the TLR4 antibody. *Tlr4*^{-/-} mice lack expression of TLR4 and were used as a negative control. The fluorescence minus one (FMO) antibody panel contained CD11b (PE-Cy7), F4/80 (V450), MHCII (FITC), Tim4 (PE), Ly6G (APC-Cy7) minus TLR4 (APC). The FMO control ensures that the any spread of the fluorochromes into the channel of interest is properly identified. Percentages indicate typical proportions of the cell types of all peritoneal cells.

Once the specificity of the TLR4 antibody was confirmed TLR4 expression on the four CD11b F4/80 expressing peritoneal populations identified (**Figure 4.6A**) was explored. Analysis of TLR4 MFI values revealed that TLR4 is expressed on all four peritoneal populations (**Figure 4.6B**). TLR4 was expressed at significantly higher levels on the Tim4⁺ MHCII⁺ peritoneal population compared to the Tim4⁺ MHCII⁻ population ($p < 0.0024$), the Tim4⁻ MHCII⁺ population ($p < 0.0002$), and the Tim4⁻ MHCII⁻ population ($p < 0.0006$) (**Figure 4.6B**). The Tim4⁺ MHCII⁻ population expressed significantly higher levels of TLR4 than the Tim4⁻ MHCII⁺ population ($p < 0.0029$), and the Tim4⁻ MHCII⁻ population ($p < 0.0023$) (**Figure 4.6B**). And the Tim4⁻ MHCII⁺ population expressed

significantly higher levels of TLR4 than the Tim4⁺ MHCII⁺ population ($p<0.01$) (**Figure 4.6B**).

These results indicate that all four populations express TLR4 indicating that they can respond to stimulation with LPS. TLR4 expression was the highest on the Tim4⁺ MHCII⁺ population suggesting that LPS responses would be highest in this population, followed by the Tim4⁺ MHCII⁻ population. The populations of interest, the Tim4⁻ MHCII⁺ population and the Tim4⁻ MHCII⁻ population expressed the two lowest levels of TLR4.

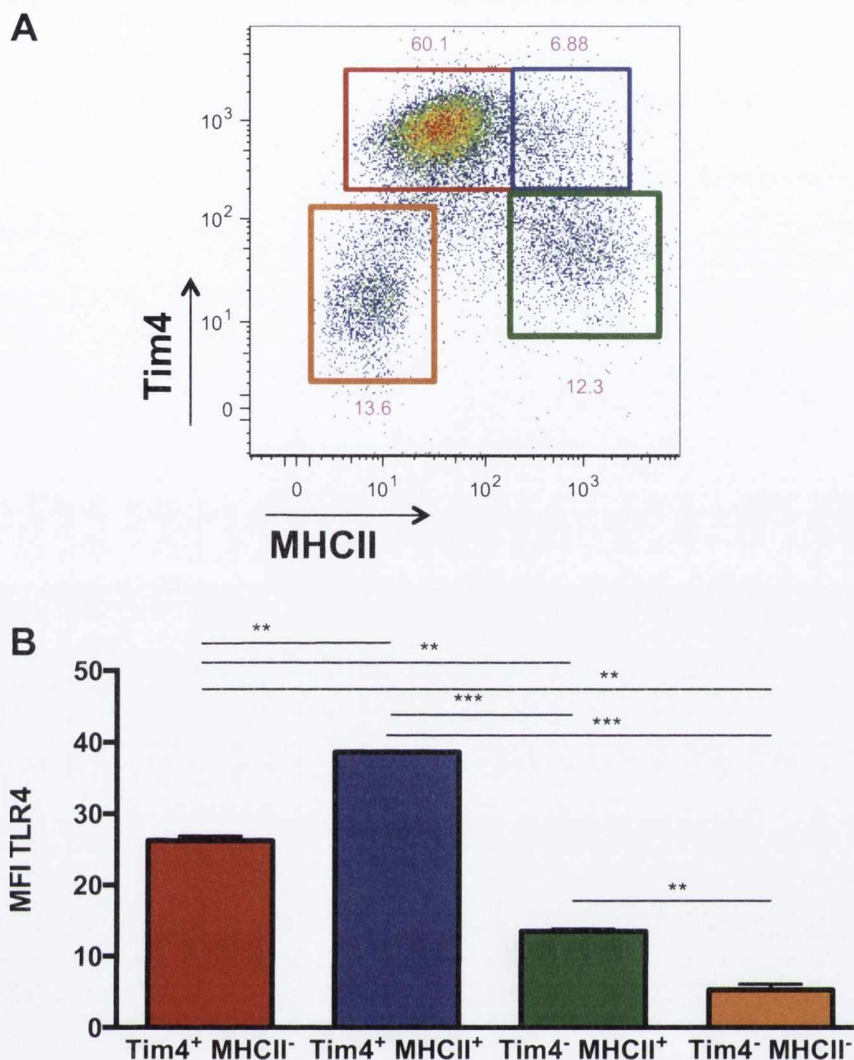


Figure 4.6 TLR4 expression on CD11b F4/80 expressing peritoneal cells from WT mice. **A** Representative flow cytometry plots of the gating strategy used in WT mice for identifying peritoneal populations based on MHCII and Tim4 expression. Percentages indicate typical proportions of the cell types of all peritoneal cells. (Pre-gate: CD11b⁺, F4/80⁺). **B** Graphs depicting the geometric MFI of TLR4 gated on the peritoneal populations. MFI = MFI value of cells stained with the full antibody panel - MFI value of fluorescence-minus-one control. All data represent means $\pm$ s.e.m. Differences, indicated as two-tailed p values, as assessed by unpaired Student t test. ** $p < 0.001$ to 0.01 , *** $p < 0.0001$ to 0.001 . $n=4$.

4.3.6 Peritoneal populations from WT mice have differential cytokine responses to LPS.

Having determined that all four peritoneal populations express TLR4 to varying levels the next step was to explore pro-inflammatory cytokine responses to LPS stimulation in each

population. To achieve this three CD11b⁺ F4/80⁺ peritoneal populations were isolated based on expression of Tim4 and MHCII from WT mice using a cell sorter (**Section 2.19**). These three peritoneal populations were Tim4⁺ and the Tim4⁺ MHCII⁺ and Tim4⁺ MHCII⁻ populations. The Tim4⁺ MHCII⁻ and Tim4⁺ MHCII⁺ populations were combined into one Tim4⁺ population as it was not possible to isolate enough cells of the Tim4⁺ MHCII⁻ population required for the experiments. Following isolation the three peritoneal populations were cultured with LPS (100 ng/ml) *in vitro* for 3 hours (**Section 2.19**) and culture media collected for cytokine production analysis by ELISA.

Analysis of the preliminary data revealed that following LPS stimulation both the Tim4⁺ and Tim4⁺ MHCII⁻ peritoneal populations produced significantly increased levels of IL-1 β compared to untreated populations ($p < 0.0058$ and $p < 0.0043$ respectively) (**Figure 4.7A**). The Tim4⁺ MHCII⁺ peritoneal population also increased IL-1 β production following LPS stimulation however not to significant levels (**Figure 4.7A**). The Tim4⁺ MHCII⁻ peritoneal population appeared to produce the highest levels IL-1 β following LPS stimulation. In terms of IL-6 production both the Tim4⁺ and Tim4⁺ MHCII⁻ peritoneal populations produced significantly increased levels of IL-6 following LPS stimulation compared to untreated populations ($p < 0.0017$ and $p < 0.0445$) (**Figure 4.7B**). The Tim4⁺ MHCII⁻ peritoneal population produces the highest levels of IL-6 following LPS stimulation. Indeed the Tim4⁺ MHCII⁻ peritoneal population produces significantly increased levels ($p < 0.0144$) of IL-6 compared to the Tim4⁺ peritoneal population following LPS stimulation (**Figure 4.7B**).

TNF α production was significantly increased in the Tim4⁺ peritoneal population following LPS stimulation (**Figure 4.7C**). TNF α production was also increased but not to significant levels in the Tim4⁺ MHCII⁺ and Tim4⁺ MHCII⁻ peritoneal populations following LPS stimulation, indeed the Tim4⁺ MHCII⁻ peritoneal population appears to produce the highest levels of TNF α (**Figure 4.7C**). In response to LPS stimulation IL-10 is increased in all three peritoneal populations however not to significant levels (**Figure 4.7D**). The Tim4⁺ and Tim4⁺ MHCII⁺ peritoneal populations produced similar levels of IL-10 following LPS stimulation. The Tim4⁺ peritoneal population produced significantly increased ($p < 0.0185$) levels of IL-10 compared to the Tim4⁺ MHCII⁻ population following LPS stimulation (**Figure 4.7D**).

Taken together these results indicate that all three of the peritoneal populations can produce inflammatory cytokines in response to LPS stimulation. The Tim4⁺ MHCII⁺ peritoneal population the highest levels of IL-6 in response to LPS stimulation compared to the other two peritoneal populations. The Tim4⁺ MHCII⁺ peritoneal population produces the highest levels of IL-1 β and TNF α in response to LPS stimulation compared to the other two peritoneal populations and conversely produces the lowest levels of IL-10. These results indicate that the Tim4⁺ MHCII⁺ population and the Tim4⁺ MHCII⁺ population, decreased in the absence of functional *Rora*, are important responder to LPS.

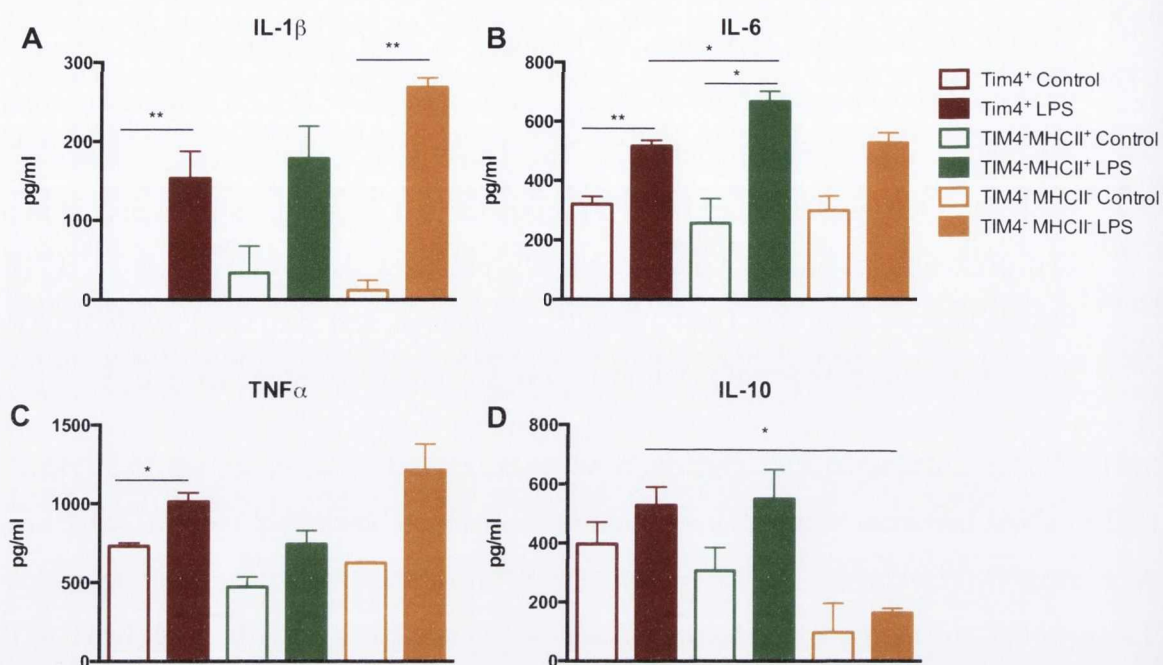


Figure 4.7 Cytokine production by CD11b⁺ F4/80⁺ peritoneal populations in response to LPS stimulation. CD11b⁺ F4/80⁺ peritoneal populations were isolated based on Tim4 and MHCII expression and stimulated *in vitro* with LPS (100 ng/ml) for 3 hours. **A** IL-1β levels **B** IL-6 levels **C** TNFα levels and **D** IL-10 levels. All data represent means ± s.e.m. Differences, indicated as two-tailed *p* values, as assessed by unpaired Student *t* test. * *p* < 0.01 to 0.05, ** *p* < 0.001 to 0.01. Tim4⁺ population control and LPS *n* = 4, all other populations *n* = 2.

4.3.7 Blood monocyte-derived macrophage levels do not increase in the peritoneal cavity of WT mice following LPS administration.

It was shown in **Section 4.3.6** that the Tim4⁺ MHCII⁺ blood monocyte-derived macrophage peritoneal population can produce pro-inflammatory cytokines in response to LPS. Blood monocyte-derived macrophages differentiate from blood monocytes that are recruited to affected tissue during inflammation where they have an important role in responding to the inflammatory stimulus (Dey et al., 2015). As blood monocyte-derived macrophages have an important role in responding to inflammation it was hypothesised that levels of the Tim4⁺ MHCII⁺ blood monocyte-derived peritoneal macrophage population would increase in the peritoneal cavity of WT mice following LPS administration.

To test this hypothesis LPS (10 mg/kg) was administered I.P. to WT mice and levels of the four peritoneal populations were explored 3 hours post administration (**Figure 4.8A**). This time-point was chosen as both physical responses (**Figure 4.1**) and cytokine responses (**Figure 4.2**) were observed by 3 hours post LPS administration. Analysis by flow cytometry revealed that levels of the Tim4⁻ MHCII⁺ blood monocyte-derived macrophage population did not change in the peritoneal cavity 3 hours post LPS administration (**Figure 4.8B**). Notably the Tim4⁺ MHCII⁻ tissue-resident macrophage population significantly decreased ($p < 0.0064$) 3 hours post LPS (**Figure 4.8B**). The ‘disappearance’ of tissue-resident peritoneal macrophages following LPS administration has previously been reported by other groups (Cassado et al., 2015).

These results indicate that increased levels of the Tim4⁻ MHCII⁺ blood monocyte-derived macrophage population in the peritoneal cavity is not a characteristic of the inflammatory response observed 3 hours post LPS administration in WT mice.

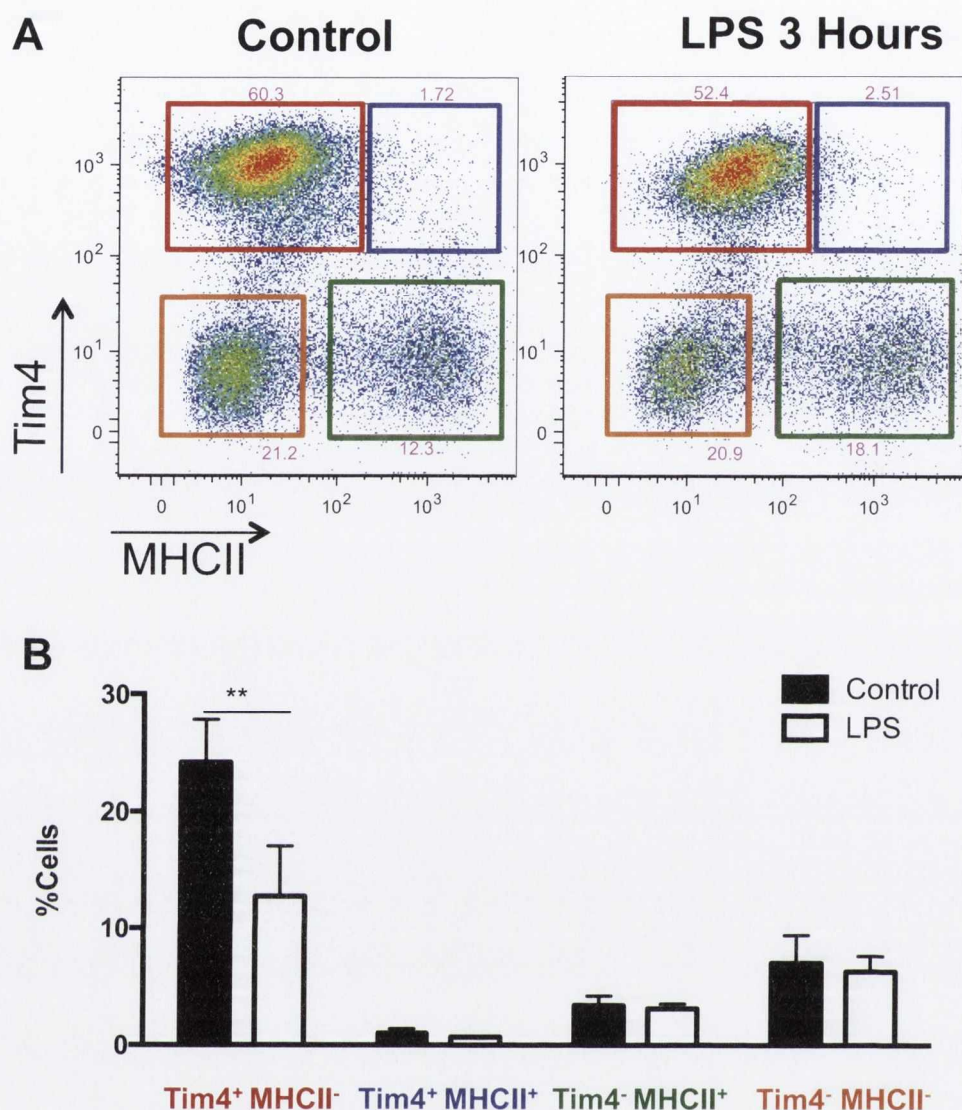


Figure 4.8 Levels of CD11b F4/80 expressing populations in the peritoneal cavity 3 hours post LPS administration. WT mice were administered LPS I.P (10 mg/kg). **A** Representative flow cytometry plots of the gating strategy used in WT mice for identifying peritoneal populations based on MHCII and Tim4 expression. Percentages indicate typical proportions of the cell types of total peritoneal cells. (Pre-gate: CD11b⁺, F4/80⁺). **B** Quantification of peritoneal populations in WT mice analysed by flow cytometry in (A) above. All data represent means $\pm$ s.e.m. Differences, indicated as two-tailed p values, as assessed by unpaired Student t test. ** $p < 0.001$ to 0.01 . $n=4$

4.3.8 The expression of M1 and M2 associated genes in the peritoneal cavity is comparable between WT mice and *Rora*^{sg/sg} mice.

Rora^{sg/sg} mice have decreased levels of blood monocyte-derived peritoneal macrophages compared to WT mice but comparable levels of tissue-resident peritoneal macrophages (**Figure 3.11**). Infiltrating blood monocyte-derived macrophages are classically considered to have an M1 phenotype and tissue-resident peritoneal macrophages are considered to have an M2 phenotype (Dey et al., 2015). It was therefore considered that the peritoneal cavity of unstimulated *Rora*^{sg/sg} mice would have an M2 bias as it contains decreased levels of blood monocyte-derived peritoneal macrophages but normal levels of tissue-resident peritoneal macrophages. This in turn may contribute to the differential cytokine responses observed in LPS stimulated peritoneal cells from WT mice and *Rora*^{sg/sg} mice (**Figure 4.3**).

To test this hypothesis peritoneal cells were isolated from unstimulated WT mice and *Rora*^{sg/sg} mice and the expression of M1 (*iNos*) and M2 (*Arg-1* and *Ym-1*) associated genes (**Section 1.6.3**) were explored by qRT-PCR. Gene expression analysis revealed that the expression levels of M2 associated genes *Arg-1* and *Ym-1* were comparable in peritoneal cells from WT mice and *Rora*^{sg/sg} mice (**Figure 4.9**). The expression of M1 associated gene *iNos* was not detectable in peritoneal cells from either WT mice or *Rora*^{sg/sg} mice (**Figure 4.9**). These results indicate that the expression of M1 and M2 genes is comparable in peritoneal cells from WT mice and *Rora*^{sg/sg} mice suggesting there is no M2 macrophage bias present in *Rora*^{sg/sg} mice.

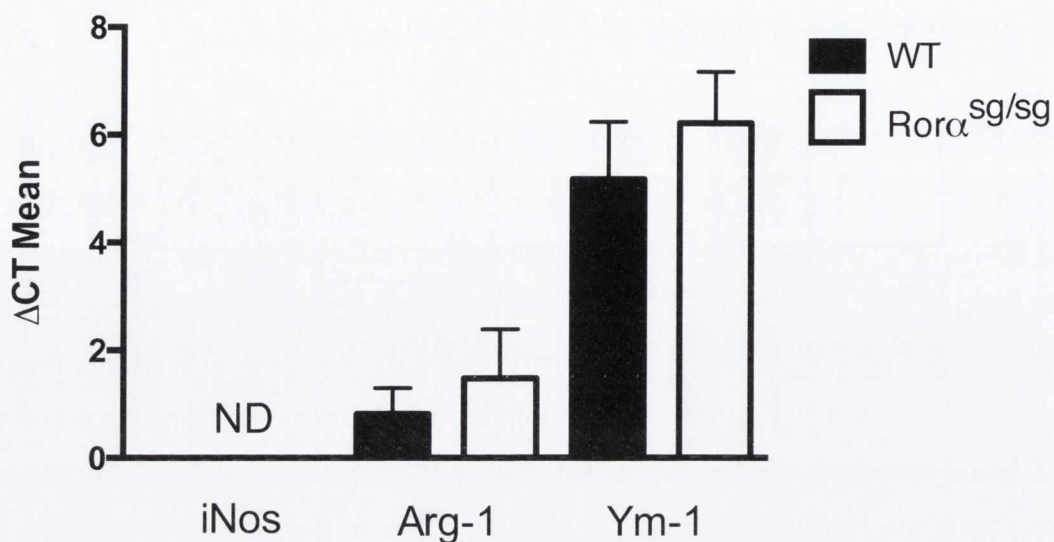


Figure 4.9 Expression of M1 and M2 associated genes in the peritoneal cavity is comparable between WT mice and *Rora*^{sg/sg} mice. Peritoneal cells were isolated from WT mice and *Rora*^{sg/sg} mice and gene expression levels of M1 (*iNos*) and M2 (*Arg-1*, *Ym-1*) associated genes were measured by qRT-PCR. All data represent means $\pm$ s.e.m. Differences, indicated as two-tailed *p* values, as assessed by unpaired Student *t* test. Gene expression was normalised to murine *Gapdh*. ND: Not detected. WT mice *n*=5, *Rora*^{sg/sg} mice *n*=4.

4.3.9 *Rora* expression is not altered in macrophages from WT mice stimulated with LPS *in vitro*.

Gene expression studies have found that *Rora* is expressed in unstimulated BMDMs from WT mice and remains at a constant level in BMDMs stimulated with LPS up until 24 hours when expression is increased (**Figure 1.8**). To explore *Rora* expression in macrophages here, BMDMs from WT mice were cultured and subsequently stimulated with LPS for 3 hours and 24 hours (**Section 2.3.2**). Expression of *Rora* and *iNos* were explored in LPS stimulated BMDMs by qRT-PCR. *Rora* expression was analysed using the custom designed TaqMan gene expression kit described in **Section 2.11.1**. An increase in *iNos* expression was used as a positive indicator that BMDMs were successfully activated by LPS (Mills et al., 2000). Gene expression analysis revealed that *iNos* expression increased in BMDMs following stimulation with LPS for 3 hours and 24 hours as compared to *iNos* expression at 0 hours (**Figure 4.10A**). *Rora* expression remained constant in BMDMs stimulated with LPS for 0 hours, 3 hours and 24 hours (**Figure 4.10A**).

These results indicate that *Rora* expression in BMDMs from WT mice is not altered following LPS stimulation suggesting that *Rora* is not part of the direct inflammatory response pathways that takes place within BMDMs following LPS stimulation.

Murine *Gapdh* was used as a housekeeper gene when performing qRT-PCR. However, recent reports have questioned the use of *Gapdh* as a housekeeper gene in qRT-PCR, as its expression levels appear to change in certain inflammatory conditions (Montero-Melendez and Perretti, 2014). Analysis of the cycle thresholds (CTs) recorded for *Gapdh* revealed that the expression levels of *Gapdh* were similar in BMDMs stimulated with LPS for 0 hours, 3 hours and 24 hours (**Figure 4.10B**) suggesting that *Gapdh* expression remains constant in BMDMs following LPS stimulation.

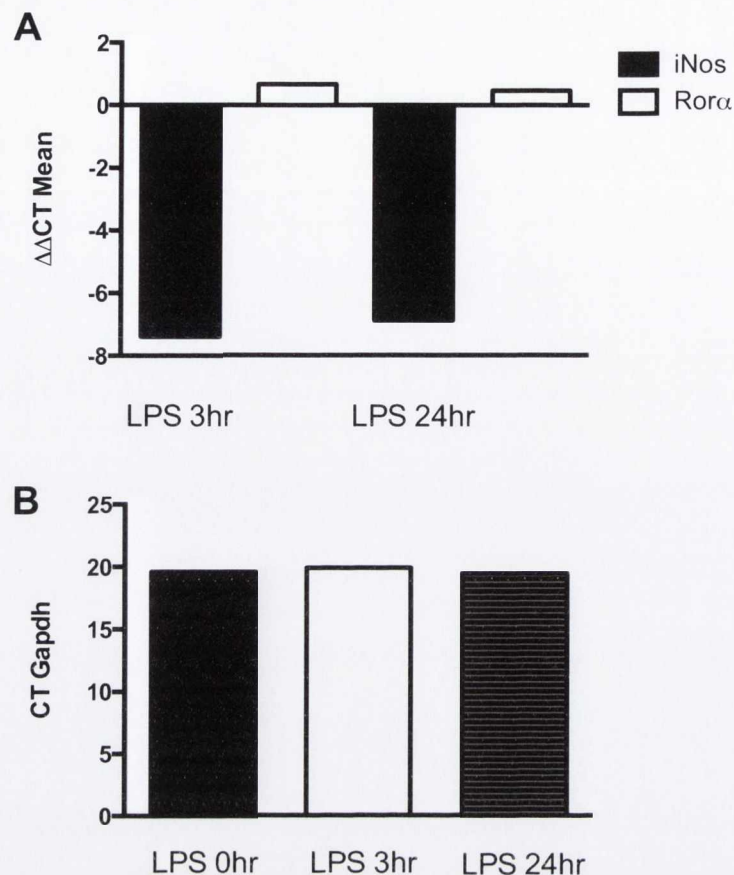


Figure 4.10 *Rora* expression is decreased in macrophages from WT mice following stimulation with LPS *in vitro*. BMDMs from WT mice were stimulated with LPS (100 ng/ml). **A** *Rora* and *iNos* expression as measured by qRT-PCR at 3 and 24 hours. Gene expression levels in BMDMs stimulated with LPS for 3 and 24 hours were normalised to expression levels in unstimulated BMDMs. Gene expression was normalised to murine *Gapdh*. $\Delta\Delta Ct = \Delta Ct$ stimulated (Ct target gene – Ct *Gapdh*) – ΔCt unstimulated (Ct target gene – Ct *Gapdh*) **B** Graph representing CT values for *Gapdh* expression in BMDMs stimulated with LPS for 0, 3 and 24 hours.

4.3.10 The LPS/TLR4 signalling pathway is comparable in macrophages from WT mice and *Rora*^{sg/sg} mice stimulated with LPS.

Cytokine production is the end product of the signalling cascade initiated when LPS binds to TLR4 in complex with MD2 (Section 1.14). There are many proteins involved in this pathway including the downstream p38 and the SAPK/JNK members of the MAPK family. Both p38 and SAPK/JNK are activated upon dual phosphorylation of tyrosine and

threonine residues (Bode et al., 2012). Analysing the level of phosphorylation of these MAPKs can be used as an indicator of activation of the LPS/TLR4 signalling pathway (Kenny et al., 2009). To explore if the difference observed in cytokine production in macrophages response to LPS in *Rora*^{sg/sg} mice and WT mice (**Figure 4.4**) is due to an alteration in the TLR4 signalling pathway, the phosphorylation of these MAPKs was analysed in BMDMs from these two groups. BMDMs were used instead of peritoneal macrophages, as it was technically difficult to obtain adequate peritoneal macrophage numbers from *Rora*^{sg/sg} mice to carry out this experiment.

BMDMs from WT mice and *Rora*^{sg/sg} mice were isolated and cultured as described (**Section 2.3.2**). BMDMs were stimulated with LPS (100 ng/ml) for 0, 10, 30, 45, 60 and 90 minutes followed by protein extraction (**Section 2.20**). In BMDMs from WT mice the levels of phospho-p38 gradually increased from 0 minutes to 90 minutes and was at the highest levels in BMDMs stimulated with LPS for 90 minutes (**Figure 4.11A**). Analysis using ImageJ software, revealed that at each time-point BMDMs from *Rora*^{sg/sg} mice expressed levels of phospho-p38 that were comparable to phospho-p38 levels observed in BMDMs from WT mice (**Figure 4.11A**). Total p38 was used as a loading control for phospho-p38 to calculate relative density (**Figure 4.11B**).

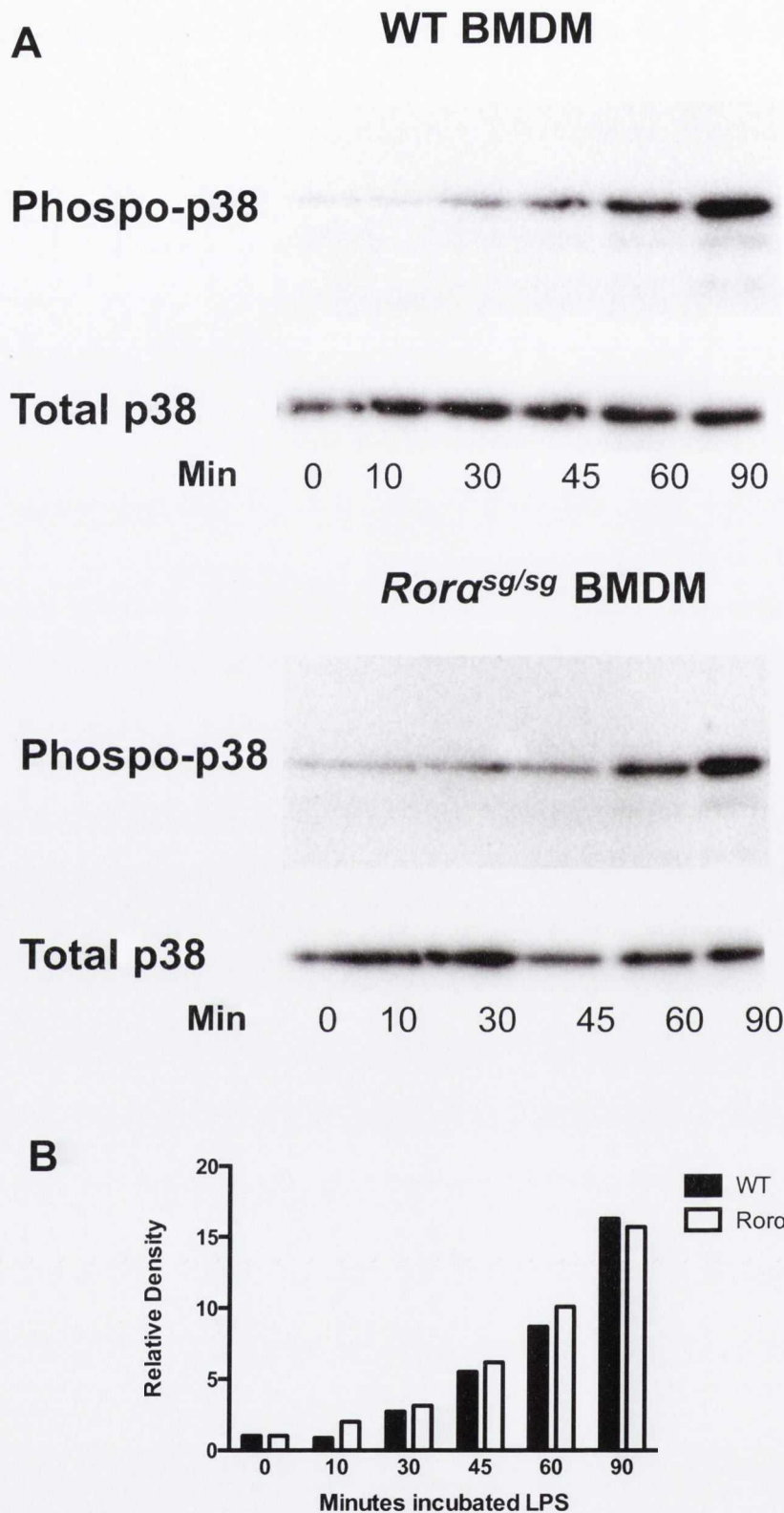


Figure 4.11 Phospho-p38 expression in BMDMs from WT mice stimulated with LPS. BMDMs from WT mice were stimulated with LPS (100 ng/ml) for 0, 10, 30, 45, 60 and 90 minutes. **A** Representative image of phospho-p38 (43 kDa) and total p38 (43 kDa) protein expression. **B** Graphical representation of the density of phospho-p38 bands relative to total p38 bands.

In BMDMs from WT mice the levels of phospho-SAPK/JNK remained constant from 0 minutes to 45 minutes, increasing at 60 minutes and were at the highest levels in BMDMs stimulated with LPS for 90 minutes (**Figure 4.12A**). In BMDMs from *Rora*^{sg/sg} mice the levels of phospho-SAPK/JNK that were comparable to phospho-SAPK/JNK levels observed in BMDMs from WT mice from 0 to 45 minutes (**Figure 4.12A**). However the levels of phospho-SAPK/JNK appears to be decreased in BMDMs from *Rora*^{sg/sg} mice at 60 minutes and 90 minutes compared to BMDMs from WT mice (**Figure 4.12A**). Total SAPK/JNK was used as a loading control for phospho-SAPK/JNK to calculate relative density (**Figure 4.12B**).

Taken together these results indicate that there may be a decrease in SAPK/JNK phosphorylation in BMDMs from *Rora*^{sg/sg} mice compared to WT mice. However, p-38 phosphorylation appears to remain constant in the two groups.

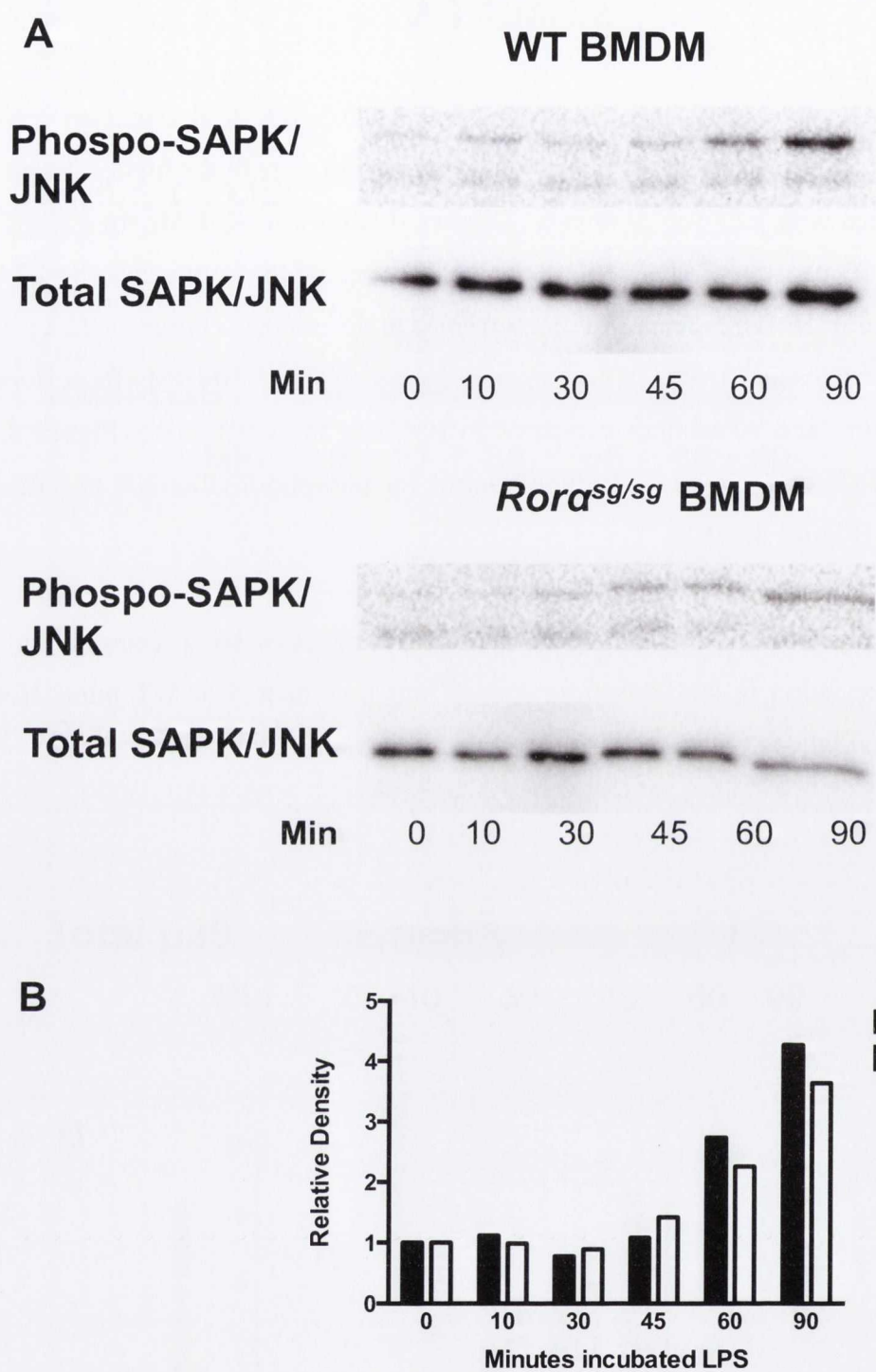


Figure 4.12 Phospho-SAPK/JNK expression in BMDMs from WT mice stimulated with LPS. BMDMs from WT mice were stimulated with LPS (100 ng/ml) for 0, 10, 30, 45, 60 and 90 minutes. **A** Representative image of phospho-SAPK/JNK (46 kDa, 54 kDa) and total JNK (46 kDa, 54 kDa) protein expression. **B** Graphical representation of the density of phospho-SAPK/JNK bands relative to total JNK bands.

4.3.11 F4/80⁺ peritoneal cells in WT mice display diurnal expression of *Rora*.

It is clear that *Rora* plays a role in a variety of macrophage functions, including a role in the development of blood monocyte-derived macrophages (**Figure 3.11**) and also a role in peritoneal macrophage cytokine responses to LPS (**Figure 4.4**). Interestingly, *Rora* could also potentially contribute to inflammatory responses through its role in the circadian clock. *Rora* induces the expression of *Bmal1* in the circadian clock, and in turn *Bmal1* in complex with *Clock* drives the expression of *Rora* (**Figure 4.13**). As discussed in **Section 4.1**, there has recently been increased interest in the concept of ‘anticipatory inflammation’. This is where circadian oscillations can potentially regulate inflammatory responses by controlling rhythmic trafficking of inflammatory monocytes and other immune cells (Nguyen et al., 2013) (**Section 1.17.2**).

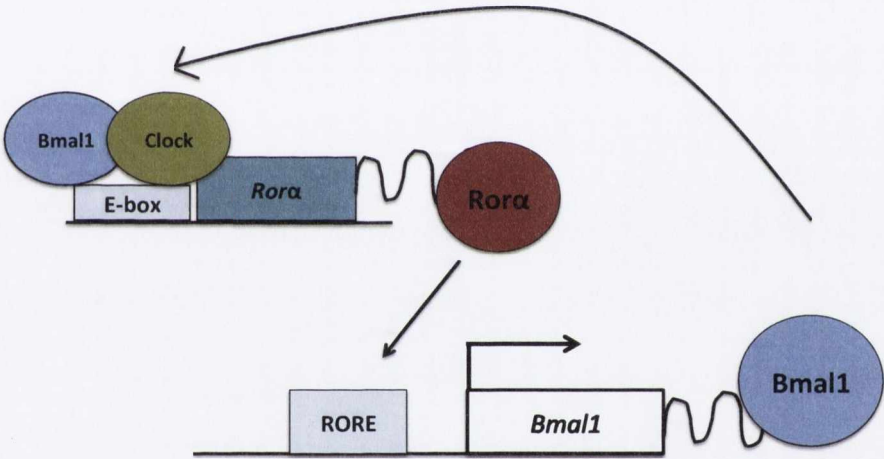


Figure 4.13 *Rora* and *Bmal1* interaction in the circadian clock. *Rora* binds to a retinoic acid-related orphan receptor response element (RORE) located in the promoter of *Bmal1* inducing its expression. In turn *Bmal1* forms a heterodimer complex with another circadian clock protein *Clock*. The *Bmal1*: *Clock* complex then binds to an E-box located in the promoter of *Rora*, inducing *Rora* expression.

Based on the study by Nguyen and colleagues, and having previously shown *Rora* contributes to the development of blood monocyte-derived macrophages (**Figure 3.11**), we hypothesised that *Rora* could also contribute to the regulation of circadian oscillations of macrophages. Circadian rhythms can be maintained independently of any external cue. The alignment of an organism’s endogenous circadian rhythm to an external rhythm is called entrainment. Light patterns represent the principal environmental cue or zeitgeber (ZT) to align the daily oscillations of the organism. Light anchors the organism in its geophysical

time by setting the rest–activity cycle. Mice have an active phase and a rest phase (**Section 1.17.1**). Their rest phase is from ZT0 to ZT12, equating to 7am to 7pm and their active phase is from ZT12 to ZT23, which is 7pm to 7pm (**Figure 4.14**). ZT0 (7am) is when the lights are turned on in the animal unit, and ZT12 (7pm) is when the lights are turned off.

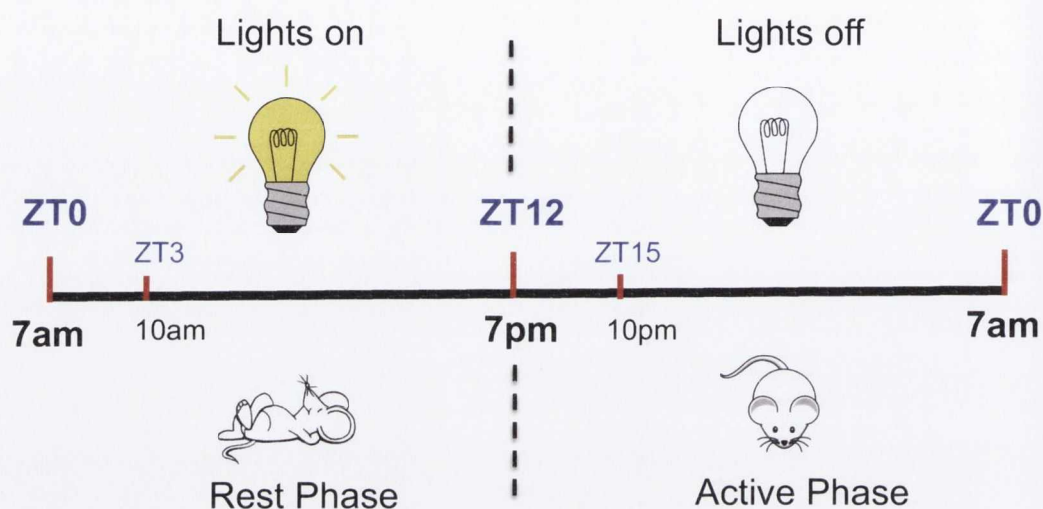


Figure 4.14 Light schedule used in the animal facility. Lights are turned on at 7am (ZT0) and turned off 12 hours later at 7pm (ZT12). The mouse rest phase occurs during the period that the lights are turned on. The mouse active phase occurs when the lights are turned off.

To explore if peritoneal macrophages displayed diurnal expression of *Rora* *Bmal1*, F4/80⁺ peritoneal cells were isolated from WT mice every 4 hours over a 24-hour period and *Rora* and *Bmal1* mRNA expression was analysed by qRT-PCR. *Bmal1* expression was used as a control to indicate rhythmic expression over 24 hours as it has been shown that rhythmically expressed in a circadian manner in peritoneal macrophages (Keller et al., 2009). *Bmal1* is also one of the most important components of the circadian clock is considered the central mediator on the circadian control of the immune system (Curtis et al., 2014). The results showed that *Rora* expression displays a diurnal rhythm in peritoneal macrophages, with expression levels reaching the highest at ZT11 and ZT15 during the active phase (**Figure 4.15A**). *Bmal1* expression also displayed a diurnal rhythm, however, counter to *Rora*, *Bmal1* expression levels were significantly lower than *Rora* expression levels at ZT11 ($p < 0.0077$) and ZT15 ($p < 0.027$). These results show that *Rora* is rhythmically expressed in a circadian manner in peritoneal macrophages with highest levels of expression observed during the active phase compared to the rest phase.

It is possible that the variation observed in *Rora* and *Bmal1* expression over 24 hours could be a result of variation in the housekeeper gene *Gapdh* used here to normalise gene expression in qRT-PCR. To rule out this possibility *Gapdh* CT values were analysed. The results showed that the CT values of *Gapdh* remain relatively constant over 24 hours (**Figure 4.15B**).

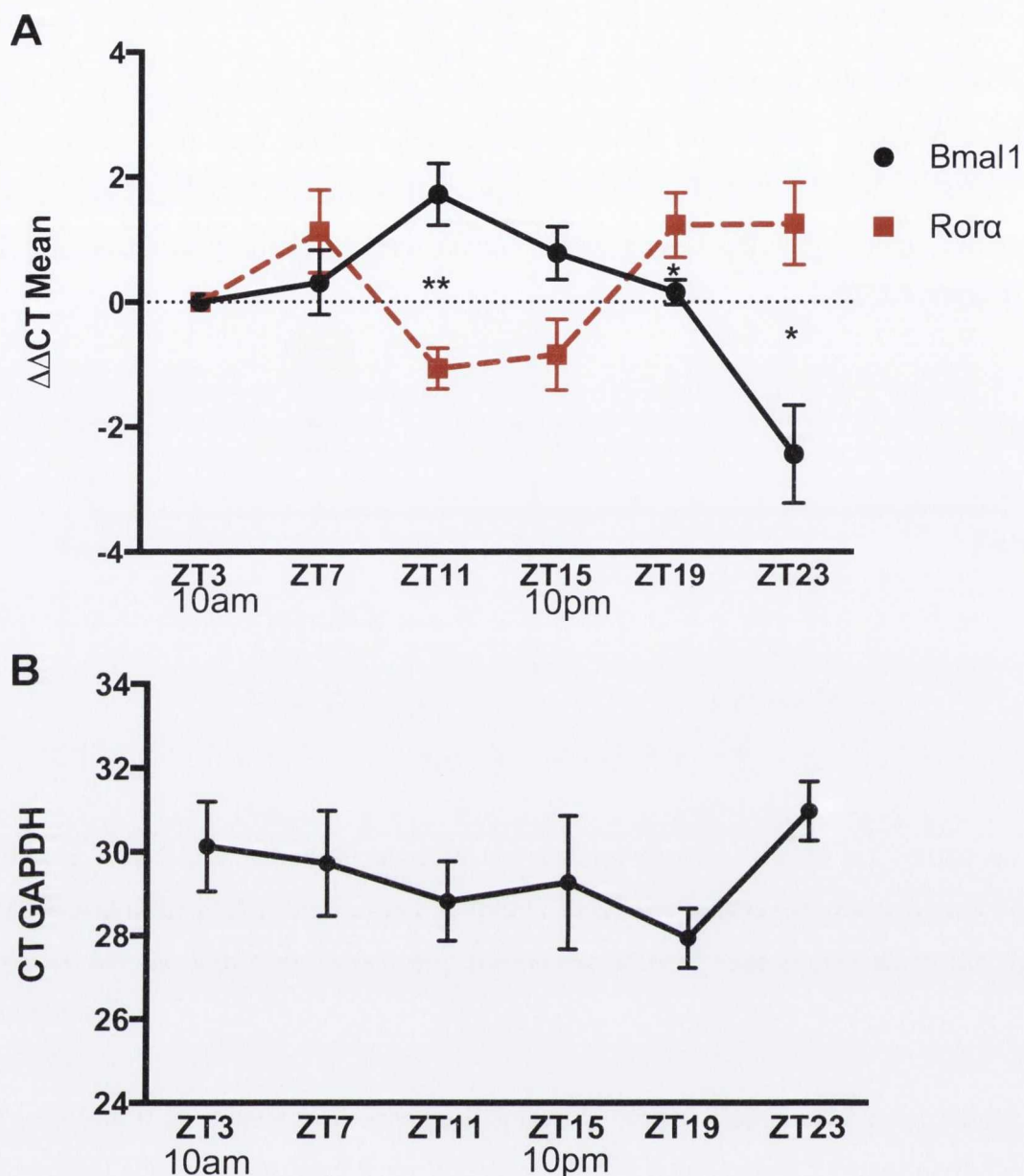


Figure 4.15 *Rora* and *Bmal1* expression in F4/80⁺ peritoneal cells from WT mice. F4/80⁺ peritoneal cells from WT mice were isolated every 4 hours by positive selection. **A** *Rora* and *Bmal* expression levels over 24 hours were explored by qRT-PCR. Gene expression was normalised to murine *Gapdh*. **B** CT values for *Gapdh* over 24 hours were explored by qRT-PCR. All data represent means $\pm$ s.e.m. Differences, indicated as two-tailed *p* values, as assessed by unpaired Student *t* test. * $p < 0.01$ to 0.05 , ** $p < 0.001$ to 0.01 . $n=4$ or 5 .

4.3.12 Levels of peritoneal macrophage populations increase in WT mice during the active phase compared to the rest phase.

It has been shown by others that the rhythmic expression of the circadian clock component *Bmal1* correlates with cyclic trafficking of monocytes in basal conditions (Nguyen et al., 2013). Having observed that *Rora* and *Bmal1* are rhythmically expressed in a circadian manner in peritoneal macrophages (**Figure 4.15A**) it was hypothesised that this rhythmic expression could correlate with rhythmic oscillations in the levels of peritoneal macrophages.

To address this hypothesis levels of CD11b F4/80 expressing peritoneal populations in WT mice were analysed by flow cytometry every 4 hours over a 24-hour time period. Results showed that the levels of the $\text{Tim4}^- \text{MHCII}^+$ blood monocyte-derived peritoneal macrophage population displayed a diurnal rhythm, increasing during the rest phase and remaining increased through the active phase before decreasing again at ZT3 (**Figure 4.16**). Levels of the $\text{Tim4}^- \text{MHCII}^+$ blood monocyte-derived peritoneal macrophage population were significantly increased at ZT11 ($p < 0.014$), ZT15 ($p < 0.015$), ZT19 ($p < 0.0140$) and ZT23 ($p < 0.0047$) compared to levels at ZT3 (**Figure 4.16**). The $\text{Tim4}^+ \text{MHCII}^-$ tissue-resident peritoneal macrophage population also displayed a diurnal rhythm, with the lowest levels observed during the rest phase before increasing to the highest levels during the active phase (**Figure 4.16**). Levels of the $\text{Tim4}^+ \text{MHCII}^-$ tissue-resident peritoneal macrophage population were significantly increased at ZT19 ($p < 0.008$) and ZT23 ($p < 0.0384$) compared to levels at ZT3 (**Figure 4.16**). The $\text{Tim4}^+ \text{MHCII}^+$ and $\text{Tim4}^- \text{MHCII}^-$ peritoneal populations do not display a diurnal rhythm (**Figure 4.16**). These results suggest that the rhythmic expression of *Rora* and *Bmal1* in a circadian manner in peritoneal macrophages correlates with rhythmic oscillations in the levels of both blood monocyte-derived peritoneal macrophages and tissue-resident peritoneal macrophages.

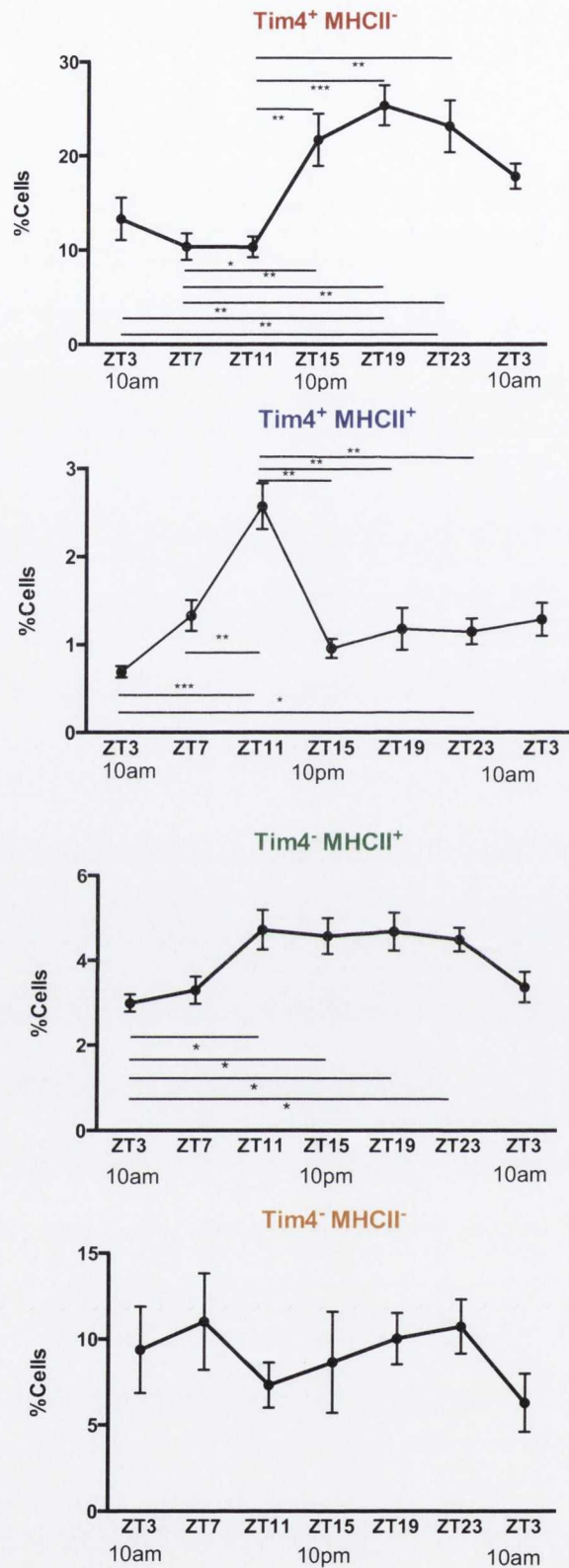


Figure 4.16 Peritoneal macrophage populations display a diurnal rhythm. Graphs depicting levels of peritoneal populations based on MHCII and Tim4 expression in WT mice over 24 hours. (Pre-gate: CD11b⁺, F4/80⁺). All data represent means $\pm$ s.e.m. Differences, indicated as two-tailed p values, as assessed by unpaired Student t test. * $p < 0.01$ to 0.05 , ** $p < 0.001$ to 0.01 . n=4

4.3.13 Switching the light cycle in the animal unit alters the diurnal rhythm of peritoneal macrophage populations.

Circadian rhythms can be entrained to the external cue of light; this means that rhythms can be changed by altering the light cycle (Curtis et al., 2014). To test if the diurnal rhythms observed in peritoneal macrophage populations in WT mice (**Figure 4.16**) represent true rhythms, the light cycle in the animal unit was reversed. In the new light cycle lights came on at 10pm as opposed to 10am (ZT3) and were turned off at 10am as opposed to 10pm (ZT12). If the diurnal rhythms observed in **Figure 4.16** represent true rhythms it was hypothesised that the same rhythms would be observed from ZT3- ZT23 in peritoneal populations in WT mice kept in the new light cycle, regardless of the time of day.

To test this hypothesis, levels of CD11b F4/80 expressing peritoneal populations in WT mice were analysed by flow cytometry every 4 hours over a 24-hour time period. Results showed that the diurnal rhythms observed in levels of the Tim4⁺ MHCII⁻ tissue-resident peritoneal macrophage population and the Tim4⁻ MHCII⁺ blood monocyte-derived peritoneal macrophage population in **Figure 4.16** were not comparable to the rhythms observed in the new light cycle (**Figure 4.17**).

Levels of the Tim4⁺ MHCII⁻ tissue-resident peritoneal macrophage population were at the highest during the rest phase, decreasing during the active phase before increasing again at the end of the active phase (**Figure 4.17**). Levels were significantly higher at ZT3 compared to at ZT15 ($p<0.035$) and ZT19 ($p<0.007$), and significantly higher at ZT7 compared to ZT19 ($p<0.0083$) (**Figure 4.17**). Levels of the Tim4⁻ MHCII⁺ blood monocyte-derived peritoneal macrophage population were stable over 24 hours however levels were significantly higher at ZT15 ($p<0.0109$) compared to at ZT7 (**Figure 4.17**).

Therefore, although levels of the Tim4⁺ MHCII⁻ tissue-resident peritoneal macrophage population and the Tim4⁻ MHCII⁺ blood monocyte-derived peritoneal macrophage population varied over 24 hours in the new light cycle, these rhythms did not mirror the rhythms observed in **Figure 4.16**.

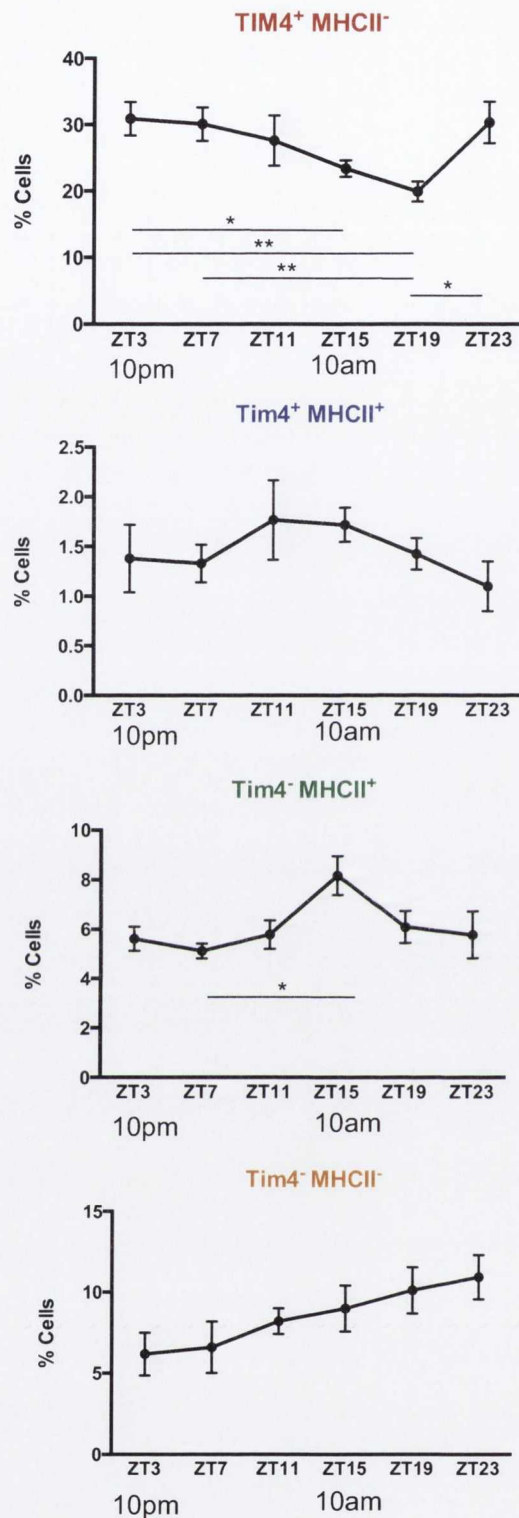


Figure 4.17 Levels of peritoneal populations following light switch in the animal unit. Graphs depicting levels of peritoneal macrophage populations based on MHCII and Tim4 expression in WT mice over 24 hours (pre-gate: CD11b⁺, F4/80⁺) following light switch. All data represent means $\pm$ s.e.m. Differences, indicated as two-tailed p values, as assessed by unpaired Student t test. * $p < 0.01$ to 0.05 , ** $p < 0.001$ to 0.01 . $n=4$

4.3.14 WT mice are more susceptible to LPS-induced endotoxemia when LPS is administered during the active phase.

Halberg and colleagues (1960) first described dramatic diurnal variations in survival rate in an endotoxic shock mouse model (**Section 1.17.2**). In their study LPS administered towards the end of the resting phase was associated with enhanced lethality in WT mice. Other studies have since confirmed and expanded on this observation. Suggested reasons for the diurnal variations in survival rates include increased production of cytokines in response to inflammation during the active phase and also increased immune cell numbers in the circulation and in tissue during the active phase (Curtis et al., 2014). Having shown that blood monocyte-derived and tissue-resident macrophage populations were increased in the peritoneal cavity during the active phase (**Figure 4.15**) and knowing both populations express TLR4 (**Figure 4.11**) we were interested in confirming that mice have decreased survival when administered LPS during the active phase. To achieve this WT mice were administered LPS at ZT3 (10am, rest phase) or ZT15 (10pm, active phase) and monitored for 48 hours.

Results showed that mice administered LPS at ZT15 had significantly decreased percentage survival ($p < 0.0093$) compared to mice administered LPS at ZT3 (**Figure 4.18A**). In mice administered LPS at ZT15 (LPS ZT15) percentage survival began to decrease at 12 hours post LPS administration and percentage survival was 25% after 48 hours (**Figure 4.18A**). In comparison, in mice administered LPS at ZT3 (LPS ZT3) the percentage survival began to decrease 36 hours post LPS administration and percentage survival was 87.5% after 48 hours (**Figure 4.18A**). In terms of sepsis scores LPS ZT15 mice had significantly increased ($p < 0.0058$) scores compared to LPS ZT3 mice as analysed by AUC (**Figure 4.18B**). Additionally LPS ZT15 mice had significantly decreased ($p < 0.0078$) body temperature compared to LPS ZT3 mice as analysed by AUC (**Figure 4.18C**).

These results confirm that WT mice administered LPS during their active phase have decreased survival in the LPS-induced endotoxemia model compared to WT mice administered LPS during their active phase.

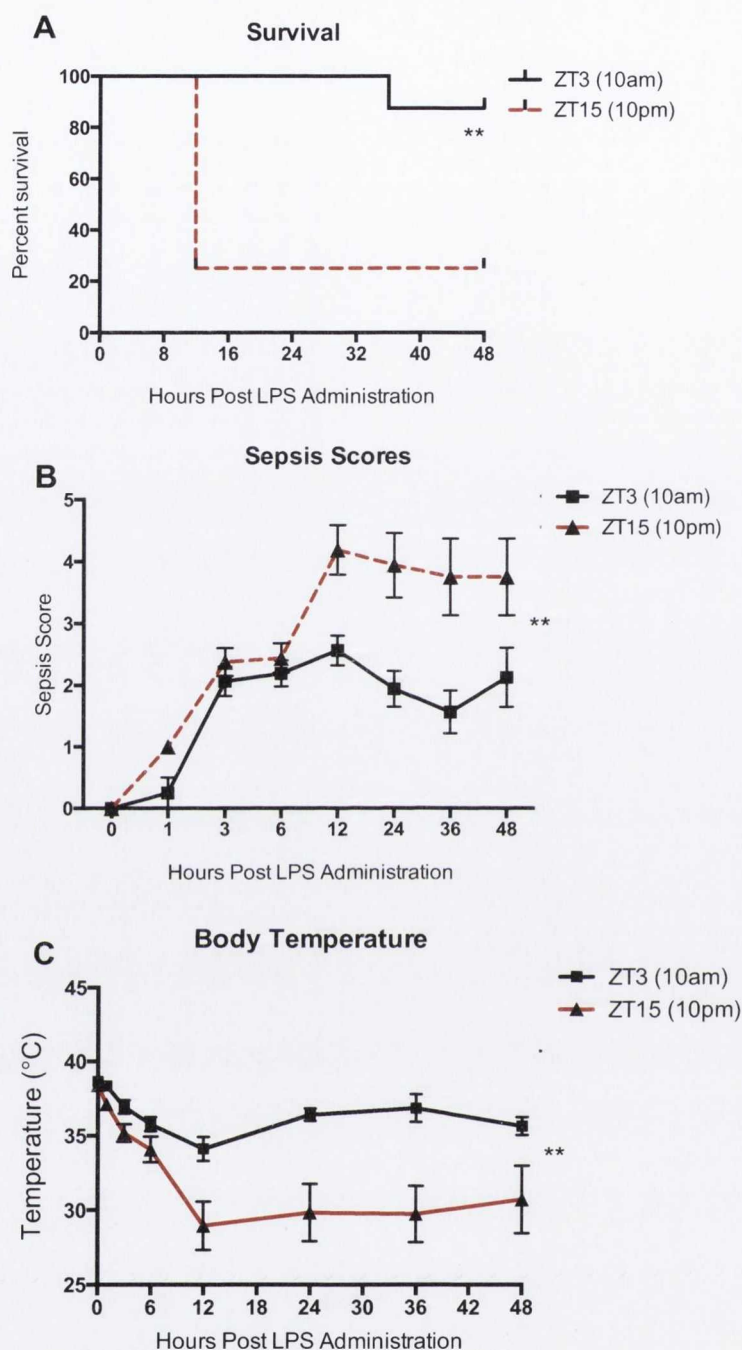


Figure 4.18 WT mice have increased susceptibility to LPS induced endotoxemia when administered LPS during the active phase. WT mice were administered LPS (10 mg/kg) at ZT3 (10am, rest phase) or ZT15 (10pm, active phase). **A** Percentage survival. **B** Sepsis scores. **C** Body temperature. The Kaplan-Meier method was used to evaluate survival. Sepsis scores and body temperature analysed by area under curve (AUC). Differences in AUC values indicated as two-tailed p values, as assessed by unpaired Student t test. $**p < 0.001$ to 0.01 . $n=6$

4.3.15 WT mice administered LPS during the active phase have an altered serum cytokine profile.

Serum cytokine levels were measured from WT mice administered LPS during their rest phase (ZT3) and during their active phase (ZT15) to explore if a difference in cytokine production in response to LPS could account for the differential temporal responses to LPS (**Figure 4.18**). Cytokine analysis showed that the cytokines IL-10, CXCL1, IL-1 β , IL-6, IL-12p70 and TNF α were increased in the serum of LPS ZT3 mice and LPS ZT15 mice in response to LPS (**Figure 4.19**). Interestingly LPS ZT15 mice had significantly increased serum levels of IL-10 ($p < 0.013$) compared LPS ZT3 mice (**Figure 4.19**). Serum levels of CXCL1, IL-1 β , IL-2, IL-5, IL-6, IL-12p70, and TNF α were similar between LPS ZT15 mice and LPS ZT3 mice (**Figure 4.19**).

These results suggest that increased IL-10 production is associated with the decreased survival observed in mice administered LPS during their active phase.

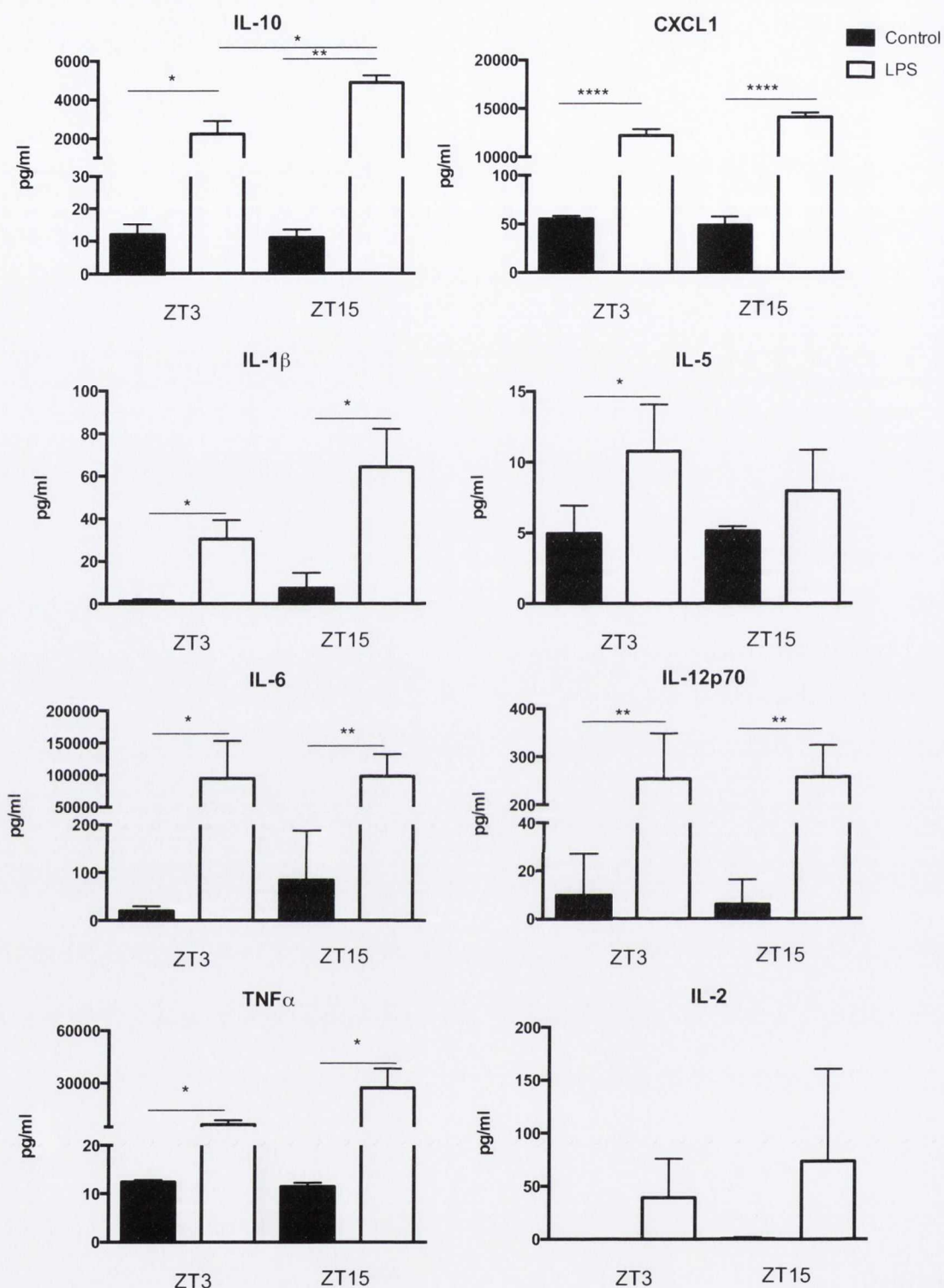


Figure 4.19 Serum cytokine levels are altered in WT mice administered LPS during the active phase compared to the rest phase. Serum was isolated from WT mice administered LPS at either ZT3 (rest phase) or ZT15 (active phase). All data represent means $\pm$ s.e.m. Differences, indicated as two-tailed p values, as assessed by unpaired Student t test. * $p < 0.01$ to 0.05 , ** $p < 0.001$ to 0.01 , **** $p < 0.0001$. WT mice control $n=3$, WT mice LPS $n=4$.

4.4 Discussion

A number of different studies have highlighted a role for *Rora* in inflammatory responses (**Section 4.1**). The majority of these studies indicate that *Rora* has a role in keeping inflammatory responses under control and in the absence of *Rora* inflammatory responses are exacerbated (Delerive et al., 2001; Stapleton et al., 2005). Here we have used LPS-induced endotoxemia to model sepsis in both WT mice and *Rora*^{sg/sg} mice. The results show that both groups display physical and behavioural characteristics of LPS-induced endotoxemia following LPS administration. However, *Rora*^{sg/sg} mice have lower sepsis scores, better body temperature recovery and increased percentage survival compared to WT mice. These results suggest that *Rora* does not have a role in keeping inflammatory responses under control but in fact contributes to the dysregulated inflammatory responses that are seen in endotoxemia.

Injection of high-dose LPS induces a very rapid increase in systemic cytokine levels (Buras et al., 2005) (**Section 1.14.3**). Cytokines regulate a variety of important inflammatory responses, however, dysregulated cytokine release may lead to processes associated with sepsis (Rivers et al., 2001). Here we found that IL-1 β levels were decreased and IL-5 levels were increased in the serum of LPS treated *Rora*^{sg/sg} mice compared to LPS treated WT mice (**Section 4.3.2**). IL-1 β is very important in early immune responses and the injection of IL-1 β into experimental animals causes a syndrome that is largely indistinguishable from septic shock (**Section 1.14.3**). A decrease in serum IL-1 β levels in *Rora*^{sg/sg} mice compared to WT mice could therefore account for their increased survival in the LPS-induced endotoxemia model. Of note, IL-1 β levels are also decreased in the peritoneal cavity of LPS treated *Rora*^{sg/sg} mice compared to WT mice. Peritoneal cells are the first responders to LPS in the LPS-induced endotoxemia model, therefore indicating that IL-1 β levels are decreased in *Rora*^{sg/sg} mice from the early stages of the inflammatory response.

IL-1 β is released primarily from activated macrophages within 30 minutes after the inciting event, following gene transcription and RNA translation (Schulte et al., 2013). We wanted to explore if the decrease in IL-1 β levels in the peritoneal cavity of *Rora*^{sg/sg} mice following LPS administration is also seen in peritoneal macrophages from these mice. Indeed, peritoneal macrophages from *Rora*^{sg/sg} mice also produced decreased levels of IL-

1 β when stimulated with LPS *in vitro* for 6 and 24 hours. Peritoneal macrophages from *Rora*^{sg/sg} mice also produced decreased levels of IL-6, IL-10, CXCL1 and TNF α compared to WT mice when stimulated with LPS for 6 hours. These results suggest that in the absence of *Rora* peritoneal macrophages produce decreased inflammatory cytokines, in particular IL-1 β , in response to LPS. There may be a number of different reasons for this.

The first explanation could be that *Rora* has an indirect role in macrophage responses to LPS through its role in the development of the Tim4⁻ MHCII⁺ blood monocyte-derived macrophage population in the peritoneal cavity as discussed in Chapter 3. *Rora*^{sg/sg} mice have decreased levels of blood monocyte-derived peritoneal macrophages compared to WT mice. Cell populations present in the peritoneal cavity when LPS is injected I.P will be the first responders to LPS. The peritoneal macrophages stimulated with LPS *in vitro* in **Section 4.3.4** represents a mixed population that would include the blood monocyte-derived macrophage population therefore it is difficult to establish how this population alone responds to LPS. Stimulation of the blood monocyte-derived macrophage population individually revealed that it can respond to LPS, indeed it produced the highest levels of IL-6 in response to LPS stimulation; Thus indicating that this cell is involved in inflammatory responses. Therefore it is possible that as basal levels of this blood monocyte-derived macrophage population are decreased in the peritoneal cavity of *Rora*^{sg/sg} mice, cytokine production in response to LPS could also be decreased in *Rora*^{sg/sg} mice as a consequence.

Interestingly, although the Tim4⁻ MHCII⁻ peritoneal population expressed the lowest levels of TLR4 of all four peritoneal populations this population produced the highest levels of IL-1 β and TNF α and the lowest levels of IL-10 in response to LPS stimulation. This population has been shown to contain eosinophils in Chapter 3 and is also decreased in the peritoneal cavity of *Rora*^{sg/sg} mice. It is possible that cytokine production in response to LPS could be decreased in *Rora*^{sg/sg} mice as a consequence of the decreased levels of this eosinophil containing population. Further investigation is needed to test this hypothesis in *Rora*^{sg/sg} mice. The next steps would be to isolate the blood monocyte-derived and eosinophil containing peritoneal populations from *Rora*^{sg/sg} mice and stimulate with LPS and analysis pro-inflammatory cytokine production.

Another possibility related to this first possibility is that as *Rora*^{sg/sg} mice have decreased basal levels of the blood monocyte-derived macrophage population, decreased levels of

this population would be recruited to the peritoneal cavity following LPS administration and that this could account for the decreased cytokine levels observed in *Rora*^{sg/sg} mice compared to WT mice. Blood monocyte-derived macrophages differentiate from blood monocytes that are recruited to affected tissue during inflammation where they have an important role in responding to the inflammatory stimulus including cytokine production. We hypothesised that as this population is an infiltrating population and it also has an important role in inflammatory responses its levels would increase in the peritoneal cavity following LPS administration. To test this hypothesis we first analysed the blood monocyte-derived macrophage population in the peritoneal cavity of WT mice 3 hours post LPS stimulation and found that contrary to our hypothesis there was no increase in the levels of the population. This suggests that an increase in the numbers of blood monocyte-derived peritoneal macrophages is not associated with early responses to LPS and therefore is most likely not a factor in differential responses to LPS between WT mice and as *Rora*^{sg/sg} mice.

Another possibility is that *Rora* could have a direct role in macrophage responses to LPS through interacting with the LPS/TLR4 signalling pathway. It is possible that in the absence of *Rora* the LPS/TLR4 signalling pathway is altered and thus cytokine production is also altered. As discussed, LPS activates the NF- κ B signalling pathway through interaction with TLR4 and the phosphorylation of the MAPKs p38 (phospho-p38) and JNK (phospho-SAPK/JNK) are steps in this pathway. Preliminary immunoblot analysis revealed that the levels of phospho-p38 between WT mice and *Rora*^{sg/sg} mice. However levels of phospho-SAPK/JNK appear to be decreased in BMDMs from *Rora*^{sg/sg} mice compared to WT mice. This result hints at *Rora* having a role within the TLR4/LPS signalling pathway however further analysis is needed to verify this result.

In addition to reinvestigating MAPK's phosphorylation, the activation of the downstream NF- κ B transcription factors could also be explored in macrophages from WT mice and *Rora*^{sg/sg} mice following stimulation with LPS. The transcription factor NF- κ B, is critical for inducible expression of many genes involved in immunity and exists in the cytosol of resting cells bound to inhibitory IKB proteins. Stimulation with specific inducers, such as LPS, activates an IKB kinase (IKK) complex that phosphorylates IKB, triggering its degradation by the proteasome and allowing free NF- κ B to translocate to the nucleus and activate gene expression; which in turn leads to the production of pro-inflammatory cytokines and other inflammatory processes. There are five members of the NF- κ B family

of transcription factors which can form different dimer combinations, but a heterodimer between p50 and the p65 subunit is the most frequently detected form. It is possible to explore NF- κ B activation by investigating the translocation of p50 and p65 into the nucleus from the cytosol by immunoblot. It is also possible to explore the degradation of the I κ Bs by immunoblot. Therefore it would be worth investigating these factors in both WT mice and *Rora*^{sg/sg} mice to determine if there are any differences between the two groups.

Another possibility to explain the difference in responses to LPS between WT mice and *Rora*^{sg/sg} mice could be endotoxin tolerance. LPS challenge is known to induce a refractory state in cells, whereupon subsequent challenge, even with a high dose of LPS, is characterized by less robust induction of pro-inflammatory cytokines and increased production of anti-inflammatory cytokines, a state known as endotoxin tolerance (Morris et al., 2015). This refractory state is associated with the innate immune system and, in particular, with monocytes and macrophages, which act as the main participants. It is possible that the decreased susceptibility to LPS-induced endotoxemia observed in *Rora*^{sg/sg} mice compared to WT mice is due to endotoxin tolerance. Macrophages from *Rora*^{sg/sg} mice may be altered in a way that gives them a phenotype similar to that found in endotoxin tolerance. One way to initially test this possibility is to isolate macrophages from both WT mice and *Rora*^{sg/sg} mice and stimulate them twice with LPS. You could then explore cytokine levels produced by both populations and determine if macrophages from WT mice and *Rora*^{sg/sg} mice decreased their production of inflammatory cytokines. If macrophages from *Rora*^{sg/sg} mice did not decrease production following the second stimulation of LPS this could indicate that these macrophages are already endotoxin tolerant before any LPS treatment.

To investigate endotoxin tolerance in more detail the mechanisms thought to be responsible for the tolerance could be investigated in macrophages from both WT mice and *Rora*^{sg/sg} mice. The exact mechanisms of endotoxin tolerance are not understood but in human systems it has consistently been linked with particular regulatory events including the deficient recruitment of the adapter MyD88 to TLR4, decreased IL-1 receptor-associated kinase (IRAK)4–MyD88 association, deficient IRAK1 activation, upregulation of negative regulators such as IRAK-M, SOCS-1, TOLLIP and SHIP-1 (Fjell et al., 2015). Furthermore, a variation in the composition of NF- κ B subunits favoring RelB (Yoza et al., 2006) and p50 (Porta et al., 2009), as well as the presence of PPAR γ (Von Knethen A and

Brüne, 2001) have also been found to play an important role in the development of endotoxin tolerance in humans . Foster and colleagues showed that these responses could mainly be attributed to TLR-induced chromatin modifications (Foster et al., 2007). All of these mechanisms could be explored in macrophages from both WT mice and *Rora*^{sg/sg} mice to determine if any of the mechanisms are already occurring in macrophages from *Rora*^{sg/sg} mice before LPS stimulation thus indicating a state of endotoxin tolerance pre-LPS stimulation.

As discussed, decreased levels of a number of pro-inflammatory cytokines including IL-1 β were produced by peritoneal macrophages from *Rora*^{sg/sg} mice compared to WT mice in response to LPS. Interestingly, decreased levels of only IL-1 β were also observed in the serum and the peritoneal cavity of *Rora*^{sg/sg} mice following LPS administration. These results suggest that *Rora* has a role in the process of IL-1 β production. Several mechanisms control the production and activity of IL-1 β , including the processing of the inactive IL-1 β precursor form into the bioactive IL-1 β (Wilson et al., 1994), and the release from secretory lysosomes through K⁺-dependent mechanisms. Processing of bioactive IL-1 β depends on activation of caspase-1 by inflammasomes (Martinon et al., 2002). Work has been carried out within our lab since the completion of this thesis exploring the processing of IL-1 β by the inflammasome in macrophages from WT mice and *Rora*^{sg/sg} mice. Early results indicate that there is a difference in IL-1 β and caspase-1 cleavage in the absence of functional *Rora*; work is on going to determine the reasons behind these observations.

With the recent interest in ‘anticipatory inflammation’ a term coined to describe *Bmal1* control of Ly6C^{High} blood monocytes levels and the link between increased Ly6C^{High} blood monocyte levels during the active phase and inflammatory responses to *Listeria*, we began to question if *Rora* could also have a similar role in circadian oscillations of peritoneal macrophages. Having shown that *Rora* has a role in the development of blood monocyte-derived peritoneal macrophages we hypothesised that this population could also potentially display a diurnal rhythm.

Having shown that *Rora* and *Bmal1* were expressed in a circadian manner in peritoneal macrophages we next showed that levels of the blood monocyte-derived peritoneal macrophage population were increased during the active phase compared to the rest phase. Interestingly the tissue-resident peritoneal macrophage population also displayed a similar

diurnal rhythm. Although we have shown that *Rora* is expressed in a circadian manner in peritoneal macrophages and that levels of peritoneal macrophage populations display a circadian rhythm, it is not known to what extent *Rora* contributes to the regulation of these rhythms. To investigate this further peritoneal macrophage population rhythm needs to be investigated in the absence of *Rora* over 24 hours.

To confirm that these diurnal rhythms represent true rhythms mice were kept in a reversed light cycle, if the rhythms were true then the same rhythms would be seen in the reversed light cycle. However, this was not observed for either the blood monocyte-derived peritoneal macrophage population or the tissue-resident peritoneal macrophage population. This suggests that the rhythms in these populations observed in the normal light cycle are not true circadian rhythms. One possible issue with the reversed light cycle experiment is that mice were kept at the reversed light cycle for 3 weeks before peritoneal macrophage populations were analysed. Therefore, it may be possible that mice need to be kept longer under the new light cycle for circadian rhythms to be properly entrained to the light change.

Inflammatory responses are more pronounced in mice during their active phase. As peritoneal macrophage population levels were at the highest during the active phase we considered that this could contribute to effect responses to LPS. We confirmed here that WT mice administered LPS during their active phase have decreased survival compared to mice administered LPS during the rest phase. Interestingly mice administered LPS during their active phase have increased serum IL-10 levels compared to mice administered LPS during the rest phase. IL-10 is considered to have primarily anti-inflammatory functions and has been shown to have a protective role in sepsis (Schulte et al., 2013); therefore it is surprising that it is increased in the group that has decreased percentage survival. However, some studies have reported an increase in IL-10 levels, along with macrophage migration factor, in patients that had a rapidly fatal outcome as a result of sepsis (death within 48 hours) (Chuang et al., 2014). Another group found that IL-10 levels were 3.3-fold higher, TNF α were 4.7-fold, IL-8 were 4.6-fold and IL-1ra were 1.5-fold higher in non-survivors of severe sepsis compared with the survivors (Surbatovic et al., 2015). Therefore, high levels of IL-10 are not always associated with protection in sepsis. The source of serum IL-10 in mice administered LPS during their active phase remains to be determined. Having confirmed that mice have decreased survival in LPS-induced endotoxemia when LPS is administered during the active phase, the next step is to determine if increased levels of

peritoneal macrophage populations contribute to this and also determine if *Rora* has a role in the process.

In summary *Rora*^{sg/sg} mice are less susceptible to LPS-induced endotoxemia compared to WT mice and peritoneal macrophages from *Rora*^{sg/sg} mice produce decreased levels of inflammatory cytokines in response to LPS as compared to macrophages from WT mice. It was found that the blood monocyte-derived peritoneal macrophage population, decreased in *Rora*^{sg/sg} mice compared to WT mice, can produce inflammatory cytokines in response to LPS. A role for the eosinophil containing peritoneal population in inflammatory responses to LPS is also suggested in the results. Taken together the data suggests that the decreased basal levels of the blood monocyte-derived peritoneal macrophages and also the eosinophil containing population in *Rora*^{sg/sg} mice compared to WT mice may account for differential responses to LPS. Further investigation is needed to confirm this hypothesis.

In terms of circadian rhythm it was found that *Rora* is rhythmically expressed in a circadian manner in peritoneal macrophages from WT mice with highest levels of expression observed during the active phase compared to the rest phase. It was also observed that levels of blood monocyte-derived peritoneal macrophage and tissue-resident peritoneal macrophage populations display rhythmic oscillations in the peritoneal cavity with the highest levels of both populations observed during the active phase. However, reversing the light cycle of the animal unit did not result in the reversal of the rhythmic oscillations of the blood monocyte-derived peritoneal macrophage and tissue-resident peritoneal macrophage populations in WT mice. It was confirmed that WT mice administered LPS during their active phase have decreased survival in the LPS-induced endotoxemia model compared to WT mice administered LPS during their active phase. More research is needed to establish the exact reason for this differential temporal response to LPS in WT mice and if the circadian oscillations of the blood monocyte-derived peritoneal macrophage population, known now to be responsive to LPS, is linked to this phenomenon and also establish the role *Rora* plays within these circadian oscillations.

Chapter 5

*Determining the role of Rora in
macrophages using Rora conditional
deletor mice.*

5.1 Introduction

Cre-Lox recombination is a type of site-specific recombination developed by Brian Sauer in the 1990s (Sauer, 1998). The procedure takes advantage of the basic research performed on the bacteriophage called P1. In this virus, there is an enzyme called Cre recombinase and particular DNA sequences called LoxP sites. Cre recombinase is a 38-kDa monomeric protein, its role in the P1 life cycle is believed to include cyclisation of the linear genome following infection of a host cell and resolution of P1 dimers generated by homologous recombination to promote stable segregation of the P1 chromosome to daughter cells (Ghosh and Duyne, 2002). The LoxP sites work in pairs and they flank a segment of genomic DNA called a target. When cells that have LoxP sites in their genome express Cre recombinase, a recombination event can occur between the LoxP sites. Cre recombinase proteins bind to the first and last 13 bp regions of a lox site forming a dimer (Sauer, 1998). This dimer then binds to a dimer on another Lox site to form a tetramer. Lox sites are directional and the two sites joined by the tetramer are parallel in orientation. The double stranded DNA is cut at both *loxP* sites by the Cre recombinase. The strands are then rejoined with DNA ligase. The result of recombination depends on the orientation of the LoxP sites, inverted LoxP sites will cause an inversion of the intervening DNA, while a direct repeat of LoxP sites will cause a deletion event.

Cre-Lox recombination technology can be used to carry out deletions, insertions, translocations and inversions at specific DNA sites. The technology is frequently used to generate transgenic mice. Typically a mouse strain will be generated that has a target DNA sequence flanked by two LoxP sites (floxed mouse). This floxed mouse strain is then crossed with a mouse strain that expresses the cre enzyme under a specific promoter, for example the actin-beta promoter. The mice generated from this cross will possess LoxP sites and will also express Cre recombinase, thus leading to recombination and the alteration of the target DNA sequence. In this thesis *Rora*^{fl/fl} *ACTB-cre* mice and *Rora*^{fl/fl} *LysMCre* mice were generated, which utilise Cre-Lox recombination technology.

Rora^{sg/sg} mice were used in Chapter 3 and Chapter 4 to determine the role *Rora* has in the development of peritoneal macrophages and in macrophage inflammatory responses. *Rora*^{sg/sg} mice contain a spontaneous mutation in *Rora* meaning the gene is mutated in every cell thus making it difficult to determine the role *Rora* has specifically in

macrophages. For this reason $Rora^{fl/fl}$ mice, generated by Taconic, were obtained for this project (**Section 2.1.2**). In $Rora^{fl/fl}$ mice a sequence within exon 6 of *Rora* is flanked by two LoxP sites (Appendix II); when Cre recombinase is present recombination of the LoxP sites occurs resulting in the excision of the floxed sequence in *Rora*. Cre recombinase is expressed under the control of specific promoters in mouse cells. In this Chapter two strains of mice expressing Cre recombinase were used: *ACTB-cre* mice and *LysMCre* mice (**Section 2.1.3** and **Section 2.1.4**). Cre recombinase is expressed under the control of the human beta-actin gene promoter in *ACTB-cre* mice (Lewandoski, 1997). Cre recombinase activity in *ACTB-cre* mice is detected in all cells of the embryo by the blastocyst stage of development (Bunnell, 2011). In *LysMCre* mice the Cre enzyme is inserted into the *lysozyme 2* (*Lyz2*) gene, both abolishing endogenous *Lyz2* gene function and placing Cre recombinase expression under the control of the endogenous *Lyz2* promoter/enhancer elements (Clausen et al., 1999).

Both *ACTB-cre* mice and *LysMCre* mice were crossed to $Rora^{fl/fl}$ mice to generate $Rora^{fl/fl}$ *ACTB-cre* mice and $Rora^{fl/fl}$ *LysMCre* mice (**Section 2.1.5** and **Section 2.1.7**). As beta-actin is ubiquitously expressed in $Rora^{fl/fl}$ *ACTB-cre* mice the floxed sequence in *Rora* is excised in every cell. In $Rora^{fl/fl}$ *LysMCre* mice Cre recombinase is expressed under the control of *Lyz2* which is expressed in cells of the myeloid system (Cross et al., 1988), therefore in $Rora^{fl/fl}$ *LysMCre* mice the floxed sequence in *Rora* is excised in cells such as macrophages, monocytes, eosinophils and neutrophils.

5.2 Chapter Objectives

Results in Chapter 3 using *Rora*^{sg/sg} mice indicate that *Rora* may contribute to the development of blood monocyte-derived peritoneal macrophages through a mechanism involving Ly6C^{Low} bone marrow monocytes. Furthermore, results in Chapter 4 using *Rora*^{sg/sg} mice indicate that *Rora* may contribute to inflammatory responses to LPS with a particular role for macrophage inflammatory responses to LPS. Using mice in which *Rora* is deleted only in cells of the myeloid cell lineage we wanted to determine if the observations made in Chapter 3 and Chapter 4 are a consequence of *Rora* acting intrinsically within macrophages or are a consequence of an extrinsic and systemic role *Rora* has in other cell types.

1. Verify that Cre-Lox recombination occurs in *Rora*^{fl/fl} mice in the presence of Cre recombinase.
2. Analyse if *Rora* contributes intrinsically to the development of blood monocyte-derived peritoneal macrophages *in vivo*.
3. Determine if inflammatory responses to LPS are altered *in vivo* when *Rora* is absent in myeloid cells.

5.3 Results

5.3.1 Cre-Lox recombination results in the excision of *Rora* in beta-actin expressing cells in *Rora^{fl/fl} ACTB-cre* mice.

Rora^{fl/fl} ACTB-cre mice were generated to verify that that Cre-Lox recombination occurred successfully in *Rora^{fl/fl}* mice in the presence of Cre recombinase (**Section 2.1.5**). As Cre recombinase is expressed under the control of the ubiquitously expressed beta-actin promoter in *ACTB-cre* mice then the floxed sequence in *Rora* would be excised in every cell in *Rora^{fl/fl} ACTB-cre* mice. *Rora^{sg/sg}* mice were used as a positive control for ubiquitous loss of functional *Rora* as these mice harbour a spontaneous mutation in *Rora* in every cell (**Section 1.15.3**). *Rora^{sg/sg}* mice display obvious physical *phenotypes* including ataxia and small size compared to littermate controls (**Figure 3.1**) These physical phenotypes are associated with cerebellar degeneration that occurs in Purkinje cells due to the absence of functional *Rora* (Dussault et al., 1998). It was anticipated that if the floxed sequence in *Rora* is excised in every cell in *Rora^{fl/fl} ACTB-cre* mice, including in Purkinje cells, then *Rora^{fl/fl} ACTB-cre* mice would display an altered physical phenotype compared to controls as observed in *Rora^{sg/sg}* mice.

Indeed, *Rora^{fl/fl} ACTB-cre* mice display ataxia and are small in size in comparison to control *Rora^{fl/fl}* mice (**Figure 5.1**). This result suggests that the floxed sequence in *Rora* is being excised in the presence of Cre recombinase in every cell in *Rora^{fl/fl} ACTB-cre* mice.



Rora^{fl/fl} *Rora^{fl/fl} ACTB-cre*

Figure 5.1 Gross appearance of *Rora^{fl/fl}* mice and *Rora^{fl/fl} ACTB-cre* mice. *Rora^{fl/fl} ACTB-cre* mice are smaller in size compared to *Rora^{fl/fl}* mice. The photograph is representative of 6 week-old males.

Due to a failure to thrive *Rora^{sg/sg}* mice are also characterised by a shortened life span compared to WT littermates (**Section 3.3.1**). Interestingly, although *Rora^{fl/fl} ACTB-cre* mice also have a decreased life span of about 8 weeks compared to *Rora^{fl/fl}* littermates, their life span is significantly extended ($p < 0.0046$) compared to *Rora^{sg/sg}* mice (**Figure 5.2**). Therefore, these results suggest that although both *Rora^{fl/fl} ACTB-cre* mice and *Rora^{sg/sg}* mice display a similar altered physical phenotype compared to controls there may be physiological differences between the two groups. Thus suggesting that the loss of functional *Rora* due to the excision of the floxed sequence in *Rora* in *Rora^{fl/fl} ACTB-cre*

mice is not identical to the loss of functional *Rora* due to mutation in *Rora* in *Rora*^{sg/sg} mice and that this difference appears to affect lifespan.

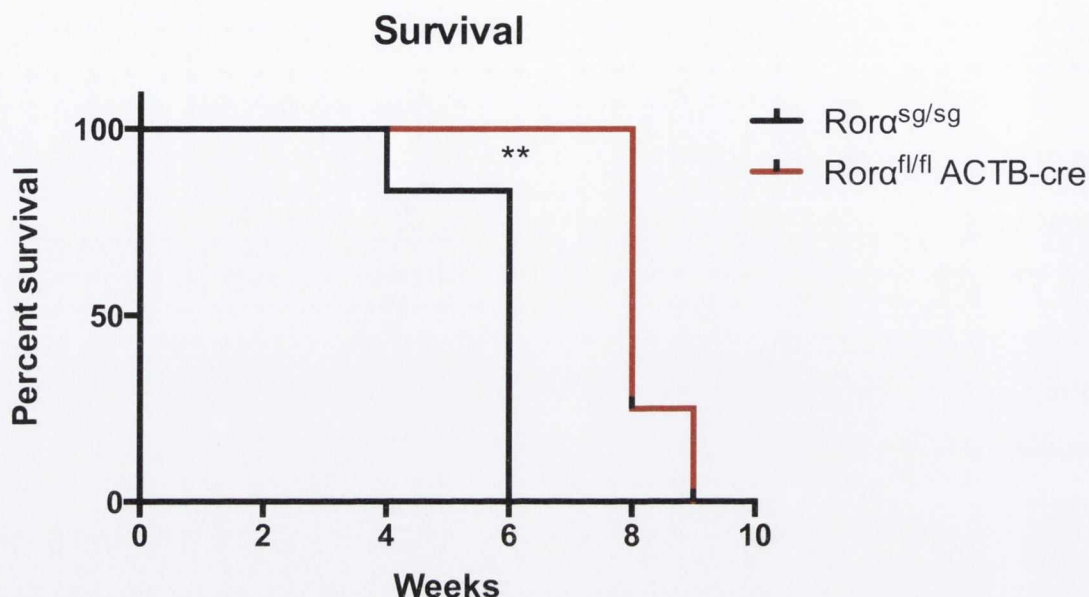


Figure 5.2 *Rora*^{fl/fl} ACTB-cre mice have extended lifespan compared to *Rora*^{sg/sg} mice. Both groups of mice were fed Nutra-Gel diet. The Kaplan-Meier method was used to evaluate survival. . ** $p < 0.001$ to 0.01 . *Rora*^{fl/fl} ACTB-cre mice $n=4$, *Rora*^{sg/sg} mice $n=6$.

5.3.2 The peritoneal immune-phenotype is altered in *Rora*^{fl/fl} ACTB-cre mice.

The results in Chapter 3 using *Rora*^{sg/sg} mice suggest that in the absence of functional *Rora* levels of blood monocyte-derived peritoneal macrophages are altered (**Figure 3.12**). The altered physical phenotype of *Rora*^{fl/fl} ACTB-cre mice compared to control mice appears to verify that the floxed sequence in *Rora* is excised in these mice (**Figure 5.1**) therefore it was next determined if the absence of functional *Rora* due to Cre-Lox recombination in *Rora*^{fl/fl} ACTB-cre mice also resulted in an altered peritoneal macrophage phenotype. *Rora*^{sg/sg} mice have decreased Tim4⁺ MHCII⁺ and Tim4⁺ MHCII⁻ peritoneal populations (**Figure 3.12**). To achieve this, peritoneal cells were isolated from age- and sex-matched *Rora*^{fl/fl} mice and *Rora*^{fl/fl} ACTB-cre mice and flow cytometry was performed. *Rora*^{fl/fl} mice littermates were used as controls for all experiments involving *Rora*^{fl/fl} ACTB-cre mice.

Four peritoneal cell populations were identified based on CD11b, F4/80, Tim4 and MHCII expression in *Rora*^{fl/fl} mice and *Rora*^{fl/fl} ACTB-cre mice (**Figure 5.3 A**). In comparison to

Rora^{fl/fl} mice, *Rora*^{fl/fl} *ACTB-cre* mice have significantly ($p < 0.0139$) increased levels of the Tim4⁺ MHCII⁺ peritoneal population (**Figure 5.3B**). Levels of the Tim4⁺ MHCII⁻ (tissue-resident macrophages) and Tim4⁻ MHCII⁺ (blood monocyte-derived macrophages) peritoneal populations were unchanged between *Rora*^{fl/fl} mice and *Rora*^{fl/fl} *ACTB-cre* mice (**Figure 5.3B**). Therefore, the absence of functional *Rora* in every cell due to Cre-Lox recombination in *Rora*^{fl/fl} *ACTB-cre* mice results in an altered peritoneal macrophage phenotype. However, this altered phenotype is not the same as the altered phenotype observed in *Rora*^{sg/sg} mice.

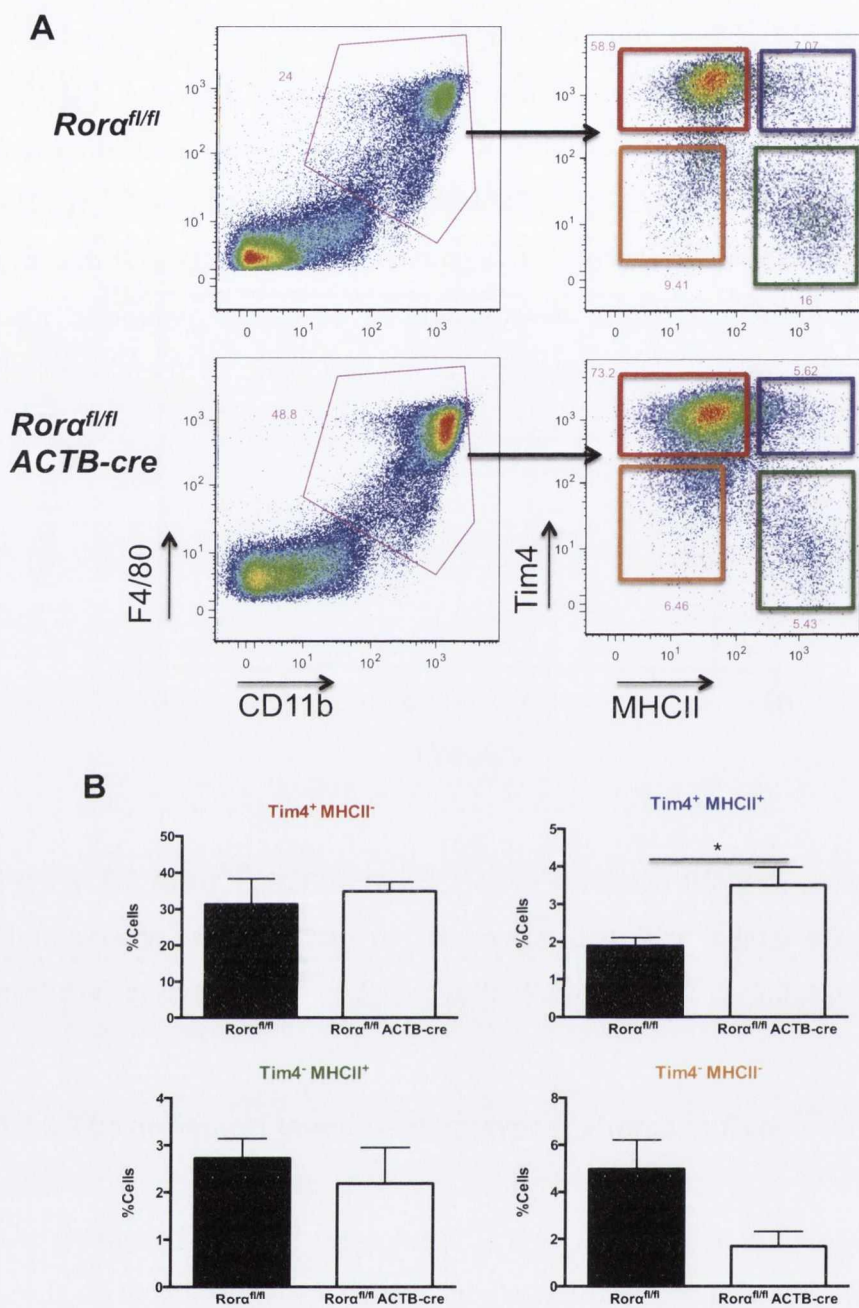


Figure 5.3 Peritoneal macrophage populations are altered in *Rora^{fl/fl} ACTB-cre* mice compared to *Rora^{fl/fl}* mice. **A** Representative flow cytometry plots of the gating strategy used in *Rora^{fl/fl}* mice and *Rora^{fl/fl} ACTB-cre* mice for identifying peritoneal populations based on MHCII and Tim4 expression. Percentages indicate typical proportions of the cell types of all peritoneal cells (Pre-gate CD11b⁺, F4/80⁺). **B** Quantification of peritoneal populations analysed by flow cytometry in (A) above. All data represent means $\pm$ s.e.m. Differences, indicated as two-tailed *p* values, as assessed by unpaired Student *t* test. * *p* < 0.01 to 0.05. *Rora^{fl/fl}* mice *n*=6, *Rora^{fl/fl} ACTB-cre* mice *n*=5.

Levels of the Tim4⁺ MHCII⁺ peritoneal population are decreased in *Rora*^{fl/fl} *ACTB-cre* mice compared to *Rora*^{fl/fl} mice but do not reach significance. The Tim4⁺ MHCII⁺ peritoneal population in WT mice has been shown to contain eosinophils (**Figure 3.16**), which are significantly decreased in the absence of functional *Rora* in *Rora*^{sg/sg} mice (**Figure 3.17**). To explore peritoneal eosinophil levels in more detail *Rora*^{fl/fl} mice and *Rora*^{fl/fl} *ACTB-cre* mice Siglec-F and CD11b were used as cell surface markers to identify eosinophils (Siglec-F⁺ CD11b^{Int}) by flow cytometry. Results confirmed that by this gating strategy peritoneal eosinophils were significantly decreased ($p < 0.0001$) in *Rora*^{fl/fl} *ACTB-cre* mice compared to *Rora*^{fl/fl} mice (**Figure 5.4**). Neutrophils are increased in the peritoneal cavity in the absence of functional *Rora* (**Figure 3.17**) therefore neutrophil levels were also explored in *Rora*^{fl/fl} *ACTB-cre* mice. Analysis revealed that neutrophil levels in *Rora*^{fl/fl} *ACTB-cre* mice were significantly increased ($p < 0.0122$) compared to *Rora*^{fl/fl} mice (**Figure 5.4**). Decreased eosinophil levels and increased neutrophil levels in the peritoneal cavity of *Rora*^{fl/fl} *ACTB-cre* mice compared to controls suggests that the loss of functional *Rora* through Cre-Lox recombination alters levels of these myeloid cells in the peritoneal cavity.

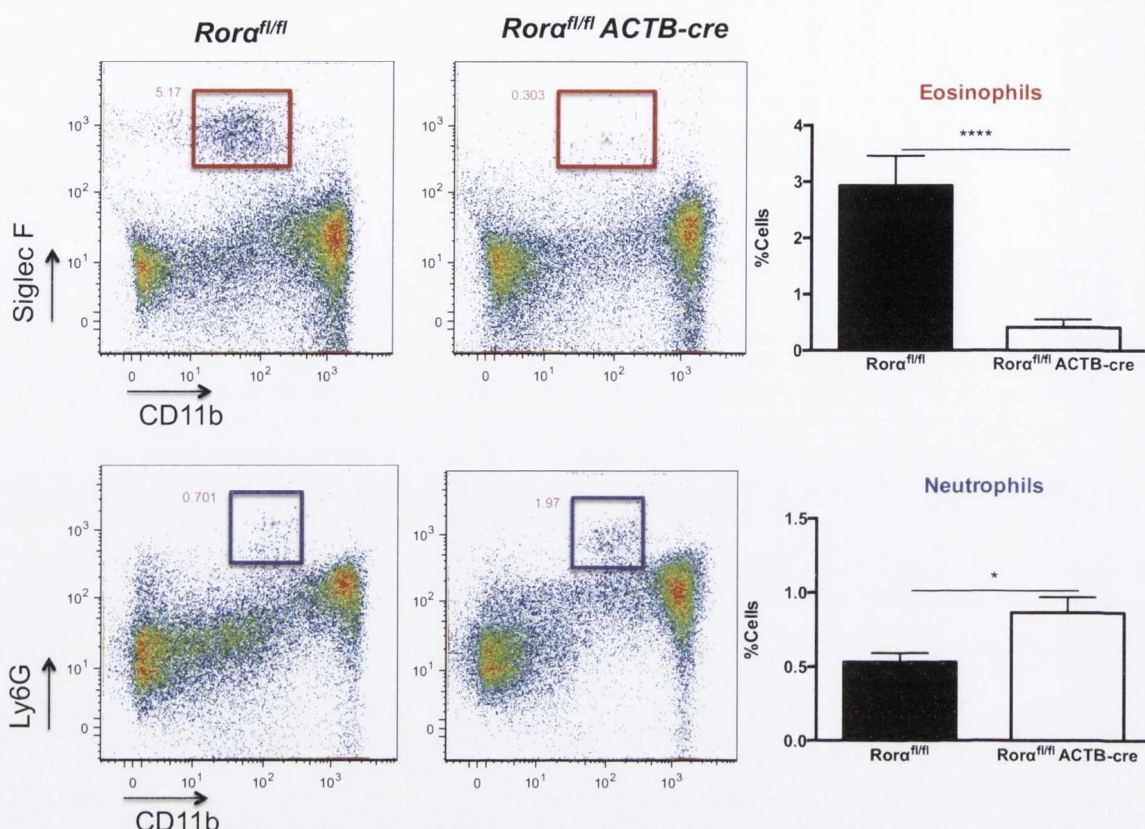


Figure 5.4 Eosinophil and neutrophil levels are altered in the absence of *Rora*. **A** Representative flow cytometry plots of gating strategy used for eosinophils (Siglec-F⁺, CD11b^{Int}) in *Rora^{fl/fl}* mice and *Rora^{fl/fl} ACTB-cre* mice. **B** Representative flow cytometry plots of gating strategy used for neutrophils (Ly6G⁺, CD11b^{Int}). Numbers on flow cytometry plot reflect percentage of total cells. All data represent means $\pm$ s.e.m. Differences, indicated as two-tailed p values, as assessed by unpaired Student t test. * $p < 0.01$ to 0.05 , **** $p < 0.0001$. *Rora^{fl/fl}* mice $n=14$, *Rora^{fl/fl} ACTB-cre* mice $n=17$.

5.3.3 Blood monocyte levels are altered in *Rora^{fl/fl} ACTB-cre* mice compared to *Rora^{fl/fl}* mice.

It was previously shown in Chapter 3 using *Rora^{sg/sg}* mice that levels of Ly6C^{Low} blood monocytes were decreased in the absence of functional *Rora* (Figure 3.22). To determine if the absence of functional *Rora* in *Rora^{fl/fl} ACTB-cre* mice also resulted in altered blood monocyte levels blood was isolated from *Rora^{fl/fl}* mice and *Rora^{fl/fl} ACTB-cre* mice and analysed by flow cytometry. Two blood monocyte populations- Ly6C^{Low} (CD45⁺, Ly6G⁻, CD115⁺, Ly6C^{Low}) and Ly6C^{High} (CD45⁺, Ly6G⁻, CD115⁺, Ly6C^{High}) blood monocytes

were identified in *Rora*^{f/f} mice and *Rora*^{f/f} *ACTB-cre* mice (**Figure 5.5A**). Analysis revealed that *Rora*^{f/f} *ACTB-cre* mice had significantly decreased levels ($p<0.033$) of Ly6C^{Low} blood monocytes compared to *Rora*^{f/f} mice (**Figure 5.5B**). *Rora*^{f/f} *ACTB-cre* mice also had significantly decreased levels ($p<0.0015$) of Ly6C^{High} blood monocytes and significantly increased levels ($p<0.0015$) of neutrophils compared to *Rora*^{f/f} mice (**Figure 5.5B**). Therefore using *Rora*^{f/f} *ACTB-cre* mice it was observed that in the absence of functional *Rora* Ly6C^{Low} blood monocytes are decreased and neutrophil levels are increased. This observation was also made in *Rora*^{sg/sg} mice. Interestingly Ly6C^{High} blood monocytes are also decreased in *Rora*^{f/f} *ACTB-cre* mice, which was not observed in *Rora*^{sg/sg} mice. Therefore, in the absence of functional *Rora* due to Cre-Lox recombination blood monocyte levels are altered.

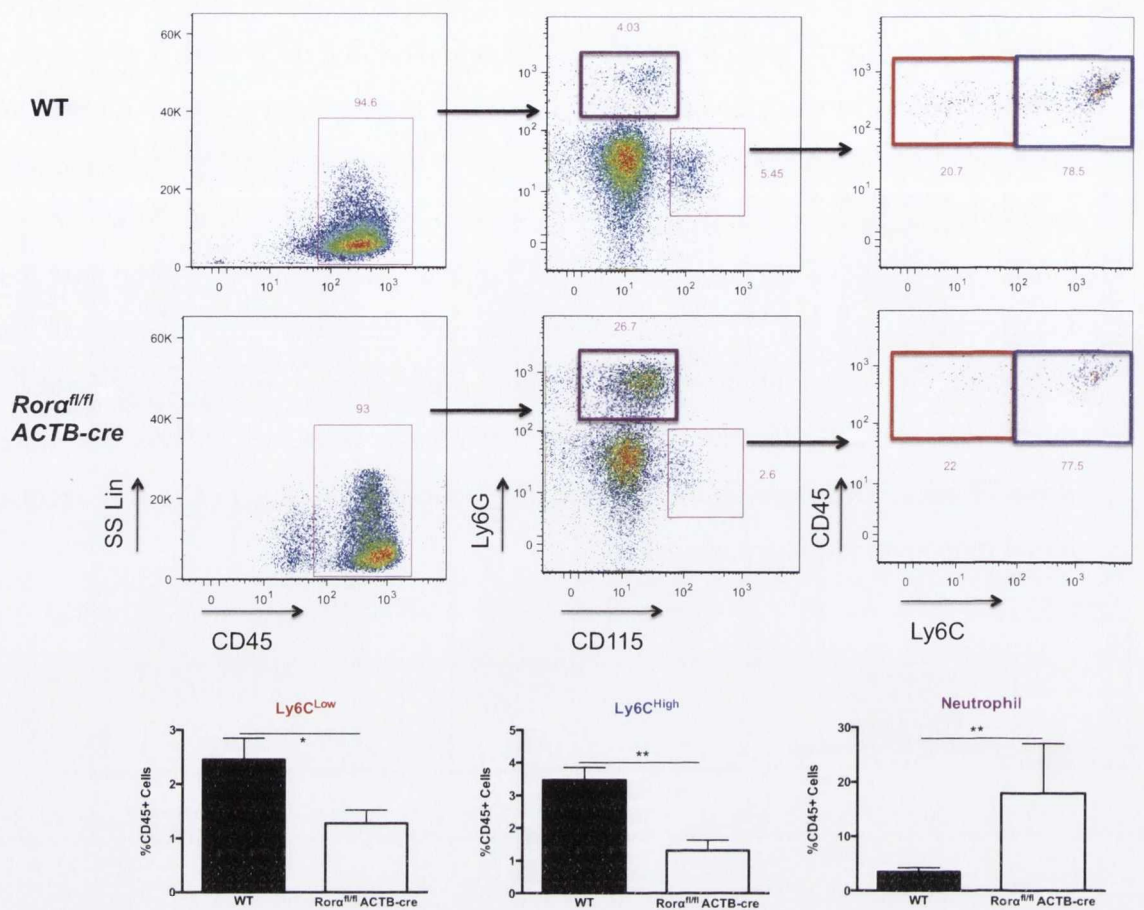
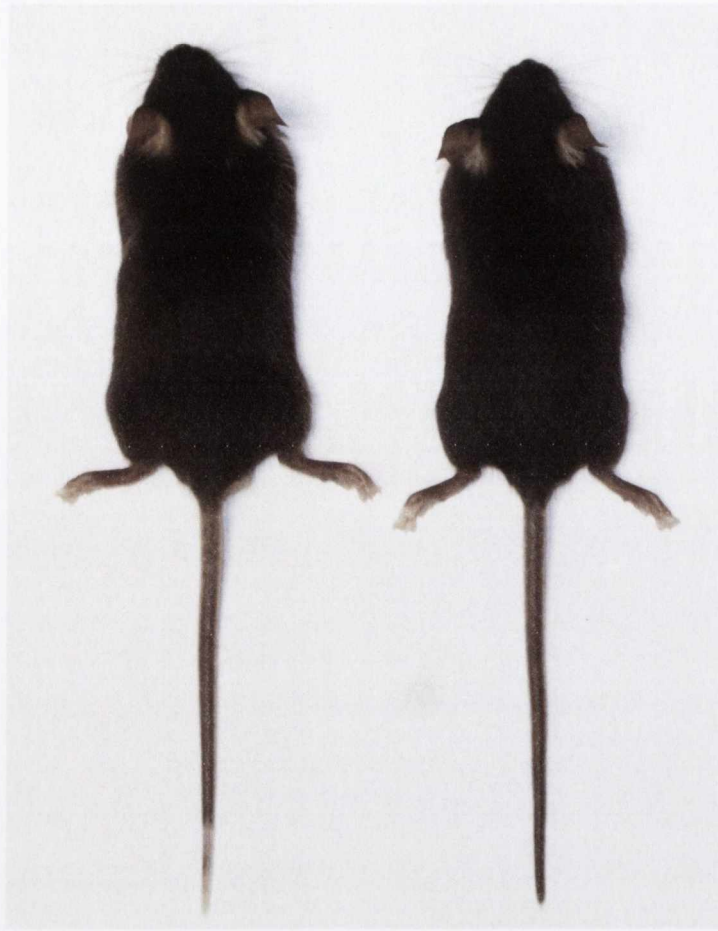


Figure 5.5 Blood monocyte levels are altered in *Rora^{fl/fl} ACTB-cre* mice compared to *Rora^{fl/fl}* mice. Representative flow cytometry plot of gating strategy used for identifying Ly6C^{High} blood monocytes (CD45⁺, Ly6G⁻, CD115⁺, Ly6C^{High}) and Ly6C^{Low} blood monocytes (CD45⁺, Ly6G⁻, CD115⁺ Ly6C^{Low}) in *Rora^{fl/fl}* mice and *Rora^{fl/fl} ACTB-cre* mice. The gating strategy is taken from Nguyen et al., (2013). Neutrophils (CD45⁺, Ly6G⁺, CD115⁻) were also identified by this gating strategy. **B** Quantification of blood monocyte populations (percentage of CD45⁺ cells) in analysed by flow cytometry in (A) above. Numbers on flow cytometry plot reflect percentage of total cells. All data represent means $\pm$ s.e.m. Differences, indicated as two-tailed *p* values, as assessed by unpaired Student *t* test. * *p* < 0.01 to 0.05, ** *p* < 0.001 to 0.01. *Rora^{fl/fl}* mice *n*=7, *Rora^{fl/fl} ACTB-cre* mice *n*=6.

5.3.4 *Rora*^{fl/fl} *LysMCre* mice have an altered peritoneal macrophage phenotype compared to *Rora*^{fl/fl} mice.

Rora^{fl/fl} *LysMCre* mice were generated following verification in *Rora*^{fl/fl} *ACTB-cre* mice that Cre-Lox recombination can occur successfully in *Rora*^{fl/fl} mice in the presence of Cre recombinase (Section 5.3.1). In *Rora*^{fl/fl} *LysMCre* mice Cre recombinase is expressed under the control of the *Lyz2* promoter that is expressed in cells of the myeloid lineage (Section 2.1.7). Therefore, in *Rora*^{fl/fl} *LysMCre* mice the floxed sequence in *Rora* is excised only in myeloid cells including monocytes, macrophages, eosinophils and neutrophils. Unlike *Rora*^{fl/fl} *ACTB-cre* mice, *Rora*^{fl/fl} *LysMCre* mice do not display an altered physical phenotype compared to control *Rora*^{fl/fl} mice (Figure 5.6).

Results in both *Rora*^{fl/fl} *ACTB-cre* mice and *Rora*^{sg/sg} mice suggest that *Rora* has a role in the development of peritoneal macrophage populations. To determine if these observations are a result of *Rora* acting intrinsically in myeloid cells or are due to extrinsic factors outside myeloid cells peritoneal cells were isolated from *Rora*^{fl/fl} *LysMCre* mice and *Rora*^{fl/fl} mice and analysed by flow cytometry. Four peritoneal populations were identified based on CD11b, F4/80, Tim4 and MHCII expression in *Rora*^{fl/fl} mice and *Rora*^{fl/fl} *LysMCre* mice (Figure 5.7A). In comparison to *Rora*^{fl/fl} mice, *Rora*^{fl/fl} *LysMCre* mice had significantly decreased levels ($p<0.0038$) of the Tim4⁺ MHCII⁻ tissue-resident peritoneal macrophage population (Figure 5.7B). Levels of the Tim4⁺ MHCII⁺, Tim4⁻ MHCII⁺ and Tim4⁻ MHCII⁻ peritoneal populations were unchanged between *Rora*^{fl/fl} mice and *Rora*^{fl/fl} *LysMCre* mice (Figure 5.7B).



$Rora^{fl/fl}$ $Rora^{fl/fl} LysMCre$

Figure 5.6 Gross appearance of $Rora^{fl/fl}$ mice and $Rora^{fl/fl} LysMCre$ mice. $Rora^{fl/fl} LysMCre$ mice are similar in size compared to $Rora^{fl/fl}$ mice. The photograph is representative of 6 week-old males.

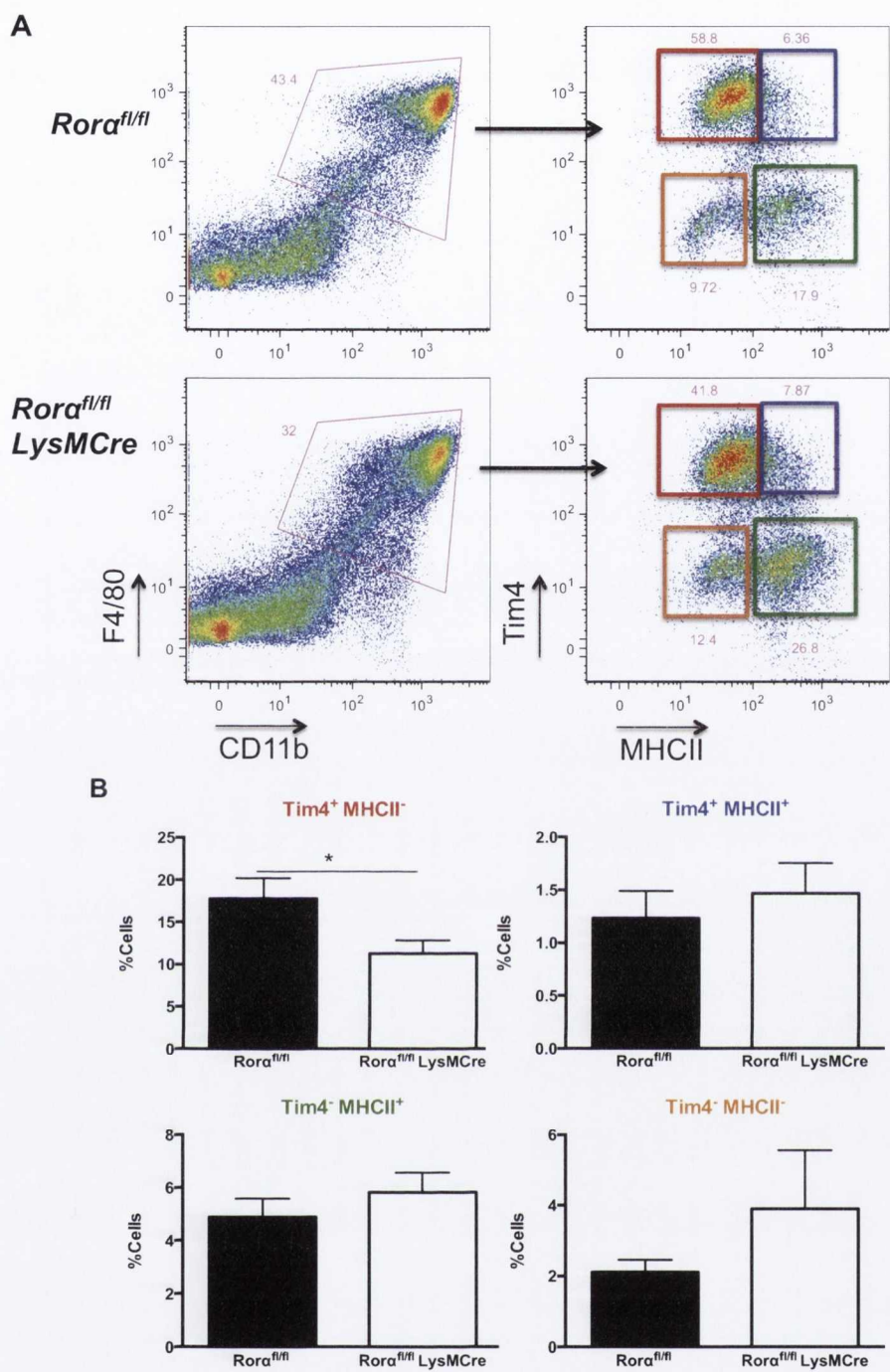


Figure 5.7 Peritoneal macrophage populations are altered in *Rora*^{fl/fl} LysMCre mice compared to *Rora*^{fl/fl} mice. **A** Representative flow cytometry plots of the gating strategy used in *Rora*^{fl/fl} mice and *Rora*^{fl/fl} LysMCre mice for identifying peritoneal populations based on MHCII and Tim4 expression. Percentages indicate typical proportions of the cell types of all peritoneal cells (Pre-gate CD11b⁺, F4/80⁺). **B** Quantification of peritoneal populations analysed by flow cytometry in (A) above. All data represent means $\pm$ s.e.m. Differences, indicated as two-tailed *p* values, as assessed by unpaired Student *t* test. * *p* < 0.01 to 0.05. *Rora*^{fl/fl} mice n=9, *Rora*^{fl/fl} LysMCre mice n= 9.

This finding is in contrast to observations in *Rora*^{fl/fl} *ACTB-cre* mice, which had increased levels of the Tim4⁺ MHCII⁺ peritoneal population, and observations in *Rora*^{sg/sg} mice that had decreased levels of the Tim4⁺ MHCII⁺ blood monocyte-derived and Tim4⁺ MHCII⁺ eosinophil peritoneal populations. The results in *Rora*^{fl/fl} *LysMCre* mice appear to suggest that when *Rora* is absent in only myeloid cells levels of tissue-resident peritoneal macrophage population are decreased compared to controls.

Results in both *Rora*^{fl/fl} *ACTB-cre* mice and *Rora*^{sg/sg} mice suggest that in the absence of functional *Rora* eosinophil levels are decreased and neutrophil levels are increased in the peritoneal cavity compared to controls (**Figure 3.16 and Figure 5.4**). Therefore, eosinophil and neutrophil levels were analysed in the peritoneal cavity of *Rora*^{fl/fl} mice and *Rora*^{fl/fl} *LysMCre* mice to explore if these observations are due to *Rora* acting intrinsically in these cells. Analysis revealed that in the peritoneal cavity eosinophil (CD11b^{Int} Siglec-F⁺) levels and neutrophil (CD11b^{Int} Ly6G⁺) levels were comparable between *Rora*^{fl/fl} mice and *Rora*^{fl/fl} *LysMCre* mice (**Figure 5.8**). This result suggests that *Rora* may not have an intrinsic role in the development of eosinophils and neutrophils.

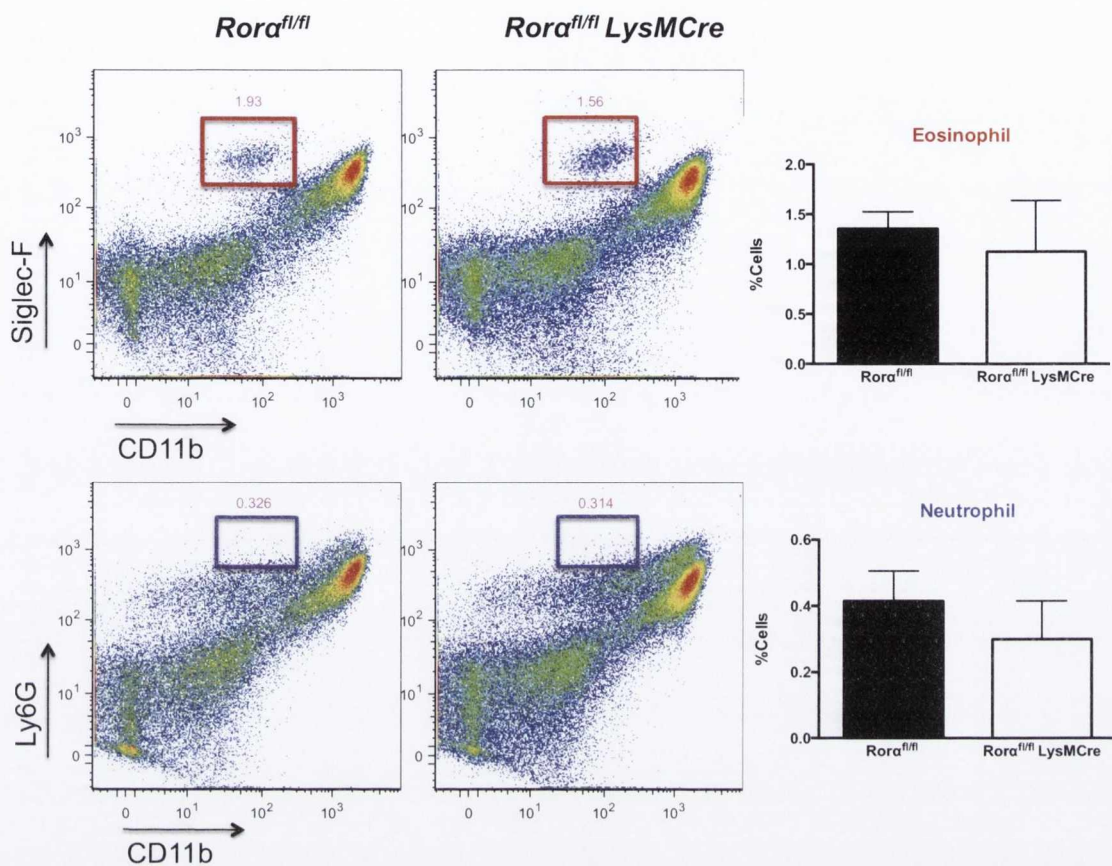


Figure 5.8 Eosinophil and neutrophil levels are unaltered in *Rora^{fl/fl} LysMCre* mice compared to *Rora^{fl/fl}* mice. Representative flow cytometry plots of gating strategy used for eosinophils (Siglec-F⁺, CD11b^{Int}) and for neutrophils (Ly6G⁺, CD11b^{Int}). Graphs depict quantification of eosinophils and neutrophils analysed by flow cytometry. Numbers on flow cytometry plot reflect percentage of total cells. All data represent means $\pm$ s.e.m. Differences, indicated as two-tailed *p* values, as assessed by unpaired Student *t* test. *Rora^{fl/fl}* mice *n*=7, *Rora^{fl/fl} LysMCre* mice *n*=8.

5.3.5 Blood monocyte levels are unaltered in *Rora^{fl/fl} LysMCre* mice compared to *Rora^{fl/fl}* mice.

Observations in *Rora^{fl/fl} ACTB-cre* mice and *Rora^{sg/sg}* mice indicate that in the absence of functional *Rora* blood monocyte levels are altered, in particular Ly6C^{Low} blood monocytes. To explore if this alteration is due to *Rora* acting intrinsically within monocytes, blood was isolated from *Rora^{fl/fl}* mice and *Rora^{fl/fl} LysMCre* mice and analysed by flow cytometry. As in **Section 5.4.3** two blood monocyte subsets were identified in blood from both groups-

Ly6C^{Low} and Ly6C^{High} blood monocytes (**Figure 5.9A**). Analysis revealed that levels of both Ly6C^{Low} blood monocytes and Ly6C^{High} blood monocytes were unchanged in *Rora*^{fl/fl} mice and *Rora*^{fl/fl} *LysMCre* mice (**Figure 5.9B**). Neutrophil levels were also unchanged between the two groups (**Figure 5.9B**).

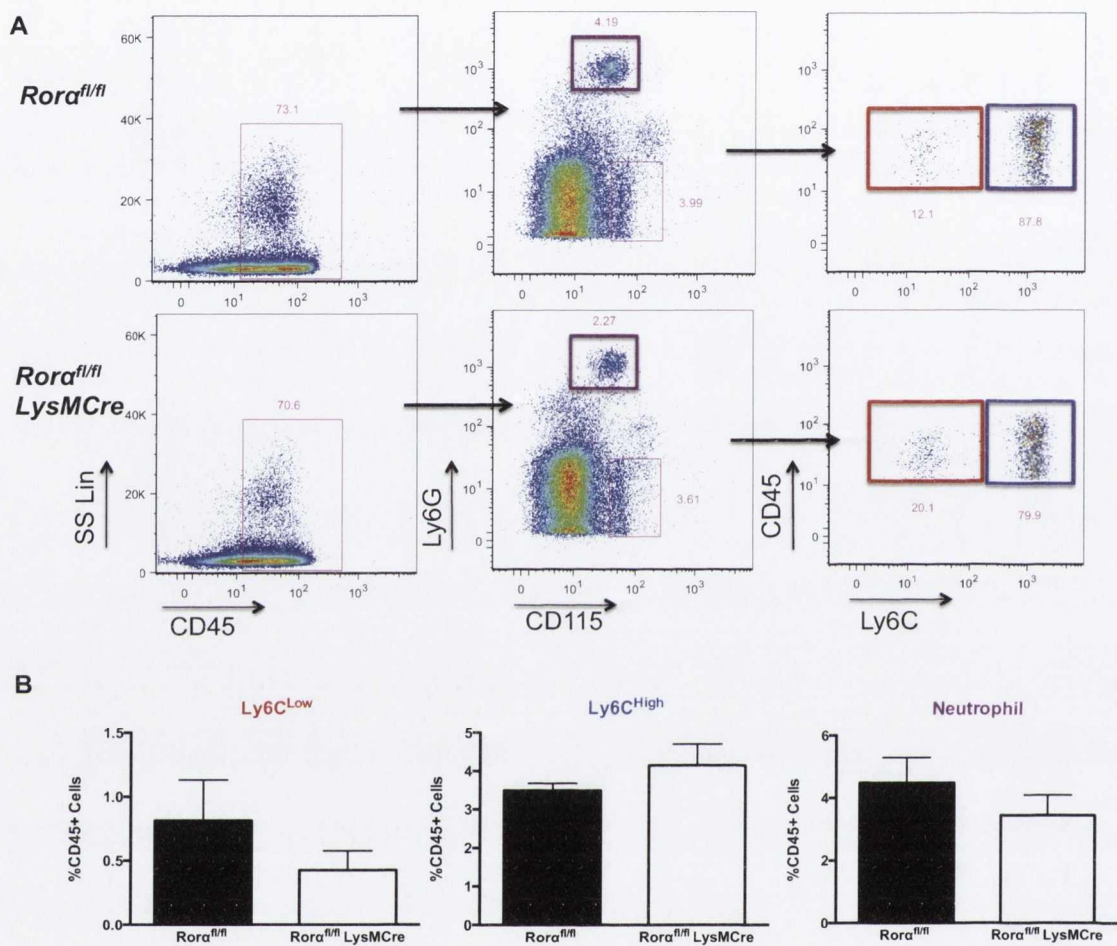


Figure 5.9 Blood monocyte levels are unaltered in *Rora*^{fl/fl} *LysMCre* mice compared to *Rora*^{fl/fl} mice. **A** Representative flow cytometry plot of gating strategy used for identifying Ly6C^{High} blood monocytes (CD45⁺, Ly6G⁻, CD115⁺, Ly6C^{High}) and Ly6C^{Low} blood monocytes (CD45⁺, Ly6G⁻, CD115⁺ Ly6C^{Low}). The gating strategy is taken from Nguyen et al., (2013). Neutrophils (CD45⁺, Ly6G⁺, CD115⁻) were also identified by this gating strategy. **B** Quantification of blood monocyte populations (percentage of CD45⁺ cells) analysed by flow cytometry in (A) above. Numbers on flow cytometry plot reflect percentage of total cells. All data represent means $\pm$ s.e.m. Differences, indicated as two-tailed *p* values, as assessed by unpaired Student *t* test. *Rora*^{fl/fl} mice *n*=4, *Rora*^{fl/fl} *LysMCre* mice *n*=4.

5.3.6 Levels of bone marrow monocytes are unchanged between *Rora*^{fl/fl} mice and *Rora*^{fl/fl} *LysMCre* mice.

In the absence of functional *Rora* levels of Ly6C^{Low} bone marrow monocytes were increased (**Figure 3.27**), therefore it was suggested that *Rora* might have a role in Ly6C^{Low} bone marrow monocyte differentiation. To determine if *Rora* has an intrinsic role in Ly6C^{Low} bone marrow monocyte differentiation, bone marrow was isolated from *Rora*^{fl/fl} mice and *Rora*^{fl/fl} *LysMCre* mice and flow cytometry performed. Two bone marrow monocyte subsets were identified- Ly6C^{High} (CD11b⁺, Ly6G⁻ and Ly6C^{High}) and Ly6C^{Low} (CD11b⁺, Ly6G⁻ and Ly6C^{Low}) bone marrow monocytes (**Figure 5.10A**). Results showed that levels of both Ly6C^{High} and Ly6C^{Low} bone marrow monocytes were unchanged between the *Rora*^{fl/fl} mice and *Rora*^{fl/fl} *LysMCre* mice (**Figure 5.10B**). This result suggests that *Rora* does not have an intrinsic role in in Ly6C^{Low} bone marrow monocyte differentiation.

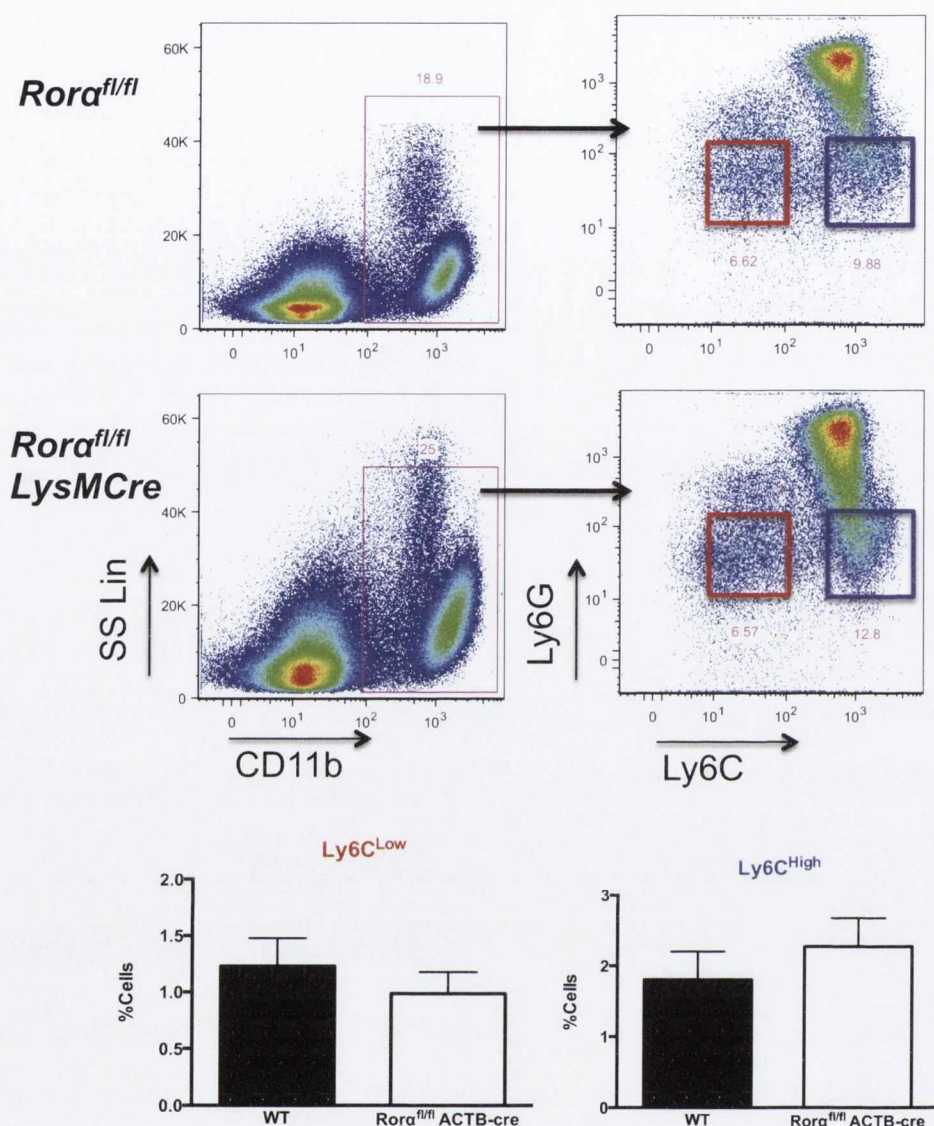


Figure 5.10 Levels of bone marrow monocytes are unaltered in *Rora*^{fl/fl} *LysMCre* mice compared to *Rora*^{fl/fl} mice. **A** Representative gating strategy used for identifying Ly6C^{Low} (Lin⁻, CD11b⁺, Ly6G⁻, Ly6C^{Low}) and Ly6C^{High} (Lin⁻, CD11b⁺, Ly6G⁻, Ly6C^{High}) bone marrow monocytes (Lin⁻: CD3⁻, CD4⁻, CD19⁻). **B** Quantification of Ly6C^{Low} and Ly6C^{High} bone marrow monocyte levels (percentage of total cells) in *Rora*^{fl/fl} mice and *Rora*^{fl/fl} *LysMCre* mice analysed by flow cytometry in (A) above. Numbers on flow cytometry plot reflect percentage of total cells. All data represent means $\pm$ s.e.m. Differences, indicated as two-tailed *p* values, as assessed by unpaired Student *t* test. *Rora*^{fl/fl} mice *n*=4, *Rora*^{fl/fl} *LysMCre* mice *n*=4.

5.3.7 Inflammatory responses to LPS are comparable in *Rora*^{fl/fl} mice and *Rora*^{fl/fl} *LysMCre* mice.

In Chapter 4 it was shown using *Rora*^{sg/sg} mice that in the absence of functional *Rora* mice were less susceptible to LPS-induced endotoxemia (**Figure 4.1**). Macrophages are early innate responders to LPS and are important in the development of LPS-induced endotoxemia (Schulte et al., 2013). To determine if the absence of *Rora* in macrophages contributes to decreased susceptibility to LPS-induced endotoxemia *Rora*^{fl/fl} mice and *Rora*^{fl/fl} *LysMCre* mice were administered LPS i.p. (10 mg/kg). Survival in *Rora*^{fl/fl} mice and *Rora*^{fl/fl} *LysMCre* began to decrease 12 hours post LPS administration, (**Figure 5.11A**), with was no significant difference in percentage survival between *Rora*^{fl/fl} mice and *Rora*^{fl/fl} *LysMCre* mice. In terms of sepsis scores *Rora*^{fl/fl} mice had increasing scores over 12 hours post LPS administration as did *Rora*^{fl/fl} *LysMCre*, however there was significant difference between the two groups as analysed by area under the curve (AUC) (**Figure 5.11B**). Body temperatures of *Rora*^{fl/fl} mice decreased over 24 hours post LPS administration as did body temperatures of *Rora*^{fl/fl} *LysMCre* mice however there was significant difference between the two groups as analysed by AUC (**Figure 5.11C**).

Taken together these results indicate that the absence of functional *Rora* in only myeloid cells including macrophages does not result in increased survival in the LPS-induced endotoxemia model.

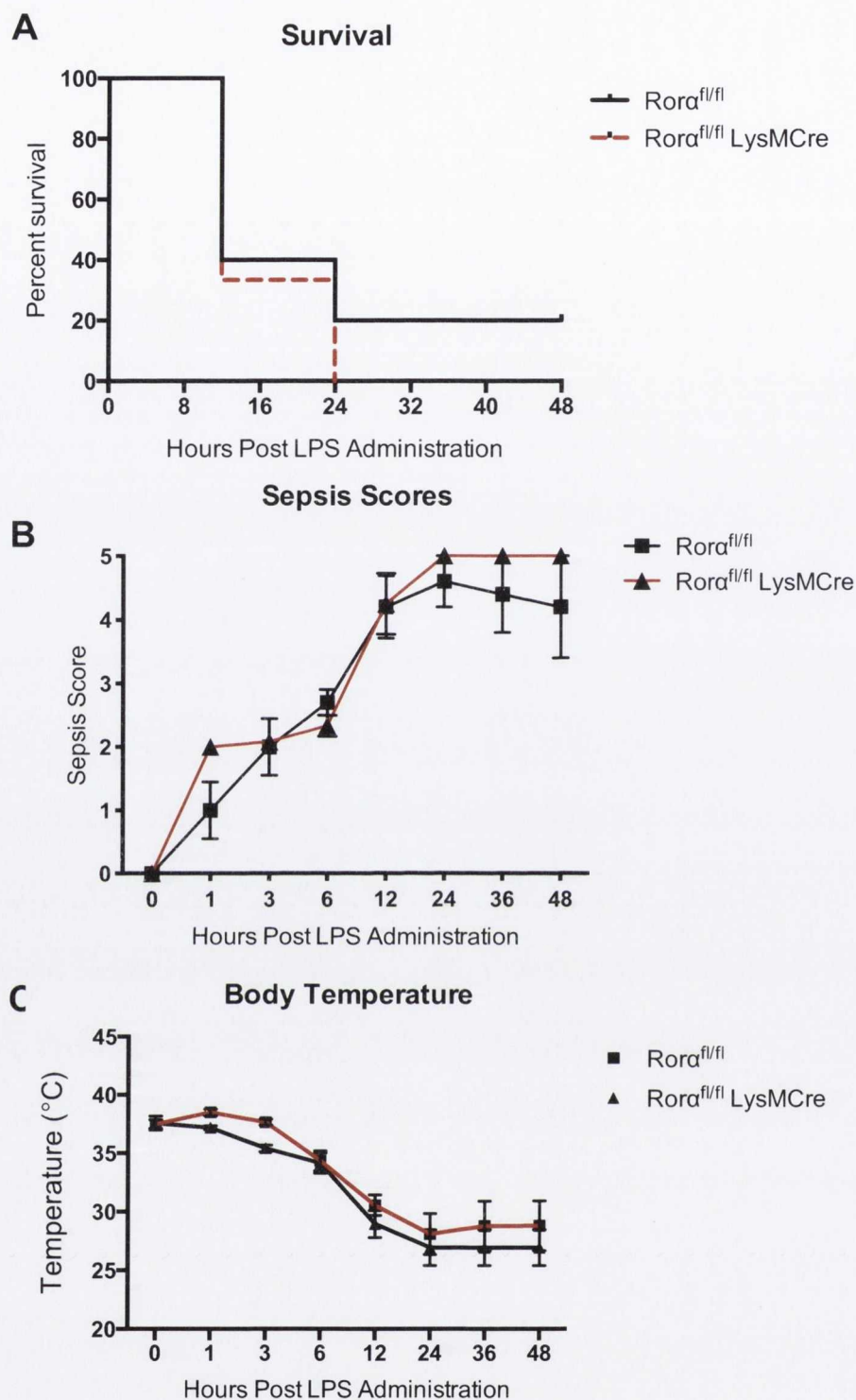


Figure 5.11 $Rora^{fl/fl} LysMCre$ mice are equally susceptible to LPS-induced endotoxemia as $Rora^{fl/fl}$ mice. Mice were administered with LPS I.P (10 mg/kg). **A** Percentage survival. **B** Sepsis scores. **C** Body temperature. The Kaplan-Meier method was used to evaluate statistical difference in survival between groups. Data represent means $\pm$ s.e.m. Sepsis scores and body temperature analysed by area under curve (AUC). Differences in AUC values indicated as two-tailed p values, as assessed by unpaired Student t test. $Rora^{fl/fl}$ mice $n=8$, $Rora^{fl/fl} LysMCre$ mice $n=8$.

5.3.8 *Rora* excision is incomplete in *Rora^{fl/fl} LysMCre* mice

While preparing *Rora^{fl/fl} LysMCre* mice the group were also using the *Rora^{fl/fl}* mice to generate ILC2 deficient mice, based on *Rora* expression in ILC2 (Wong et al., 2012). When generating ILC2 deficient mice it was noted that more efficient ILC2 depletion was achieved with a *Rora^{fl/sg} Il7RaCre* mouse then a *Rora^{fl/fl} Il7RaCre* mouse (Hams et al., 2015b; Oliphant et al., 2014). Thus while it has been documented that the efficiency of excision of floxed sequences in peritoneal cells using LysMCre mice is 80% (Nikolic et al., 2014), a concern was efficiently using the *Rora^{fl/fl}* mice. Indeed, during these studies Wynn and colleagues reported that *LysMCre* mice achieves incomplete deletion of floxed IL-4R α in macrophages (Vannella et al., 2014). It is therefore possible that the results observed in **Figure 5.10** and **Figure 5.11** are thus a result of incomplete excision of the floxed sequence in *Rora*. To test this possibility bone marrow was isolated from *Rora^{fl/fl}* mice, *Rora^{fl/fl} ACTB-cre* mice and *Rora^{fl/fl} LysMCre* mice and cultured for 7 days in M-CSF to generate BMDMs (**Section 2.3.2**). RNA was then isolated from these BMDMs and cDNA was made (**Section 2.10**). Semi-quantitative PCR (sq-PCR) primers were designed that were complimentary to the floxed sequence in *Rora* in *Rora^{fl/fl}* mice (**Section 2.1.2**). If excision was successful then this sequence would not be present therefore amplification would not occur and no PCR product would be observed by gel electrophoresis.

Using these custom designed primers PCR product was observed in the BMDM sample from *Rora^{fl/fl}* mice as expected. However, PCR product was also observed in the BMDM sample from *Rora^{fl/fl} LysMCre* mice (**Figure 5.12**). In contrast, no PCR product was observed in the BMDM sample from *Rora^{fl/fl} ACTB-cre* mice (**Figure 5.12**) confirming that the floxed sequence in *Rora* in *Rora^{fl/fl} ACTB-cre* mice is successfully excised, as indicated by the data discussed in **Section 5.1**. However the results also suggest that the floxed sequence in *Rora* is not completely excised in *Rora* in macrophages from *Rora^{fl/fl} LysMCre* mice.

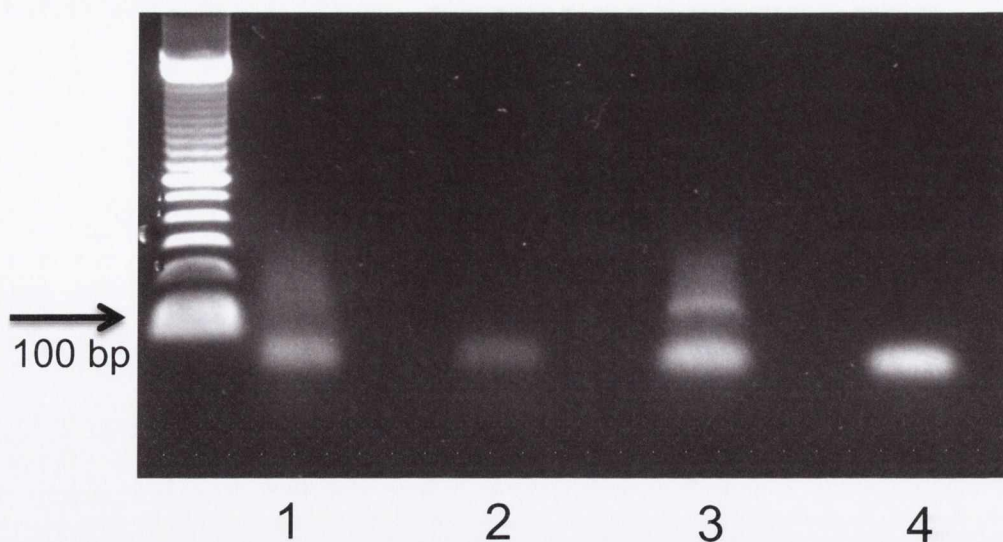
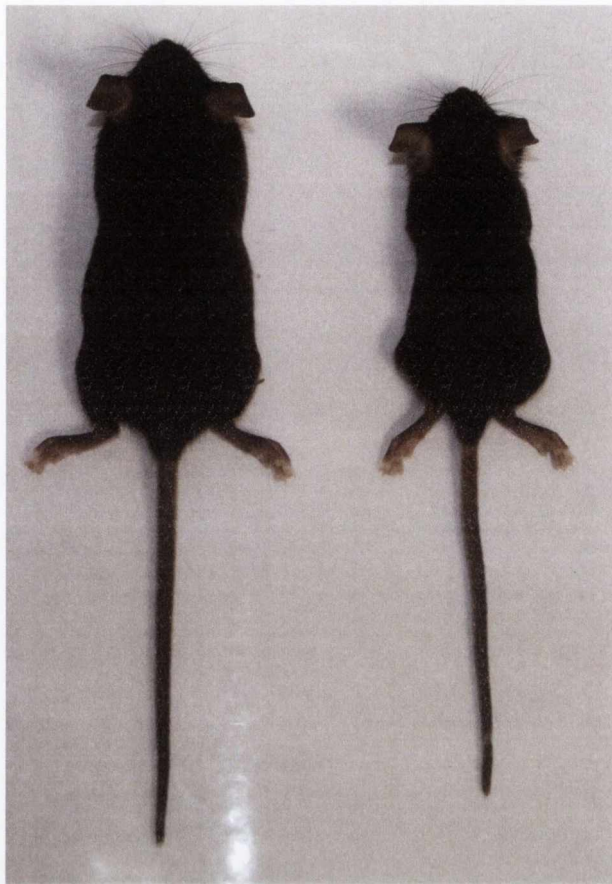


Figure 5.12 *Rora* excision is not complete in *Rora^{fl/fl} LysMCre* mice. Agarose gel with representative semi-quantitative PCR product bands from (1) *Rora^{fl/fl}* mice, (2) *Rora^{fl/fl} ACTB-cre* mice, (3) *Rora^{fl/fl} LysMCre* mice and (4) PCR reaction mixture minus genomic DNA. Predicted PCR product band size - 127 bp. bp- base pair.

5.3.9 Generation of *Rora^{fl/sg}* mice.

To try and improve the efficiency of *Rora* excision using Cre-Lox recombination *Rora^{fl/sg}* mice were generated and crossed to Cre expressing mice; a strategy used with an IL-7RCre mouse (Hams et al., 2015b; Oliphant et al., 2014). In *Rora^{fl/sg}* mice one *Rora* allele contains the floxed sequence and the other *Rora* allele contains the spontaneous mutation. The theory behind using these mice is that efficiency of excision will be improved as there are less Floxed sequences to be excised by Cre as half the *Rora* alleles already contain a mutation (Herbert et al., 2004). *Rora^{fl/sg} ACTB-cre* mice were generated (Section 2.1.7) to test if the Cre-Lox recombination occurs successfully in this cross. It was expected that if the floxed sequence in *Rora* was excised then both alleles of *Rora* would then be altered in every cell and *Rora^{fl/sg} ACTB-cre* mice would have a similar physical phenotype to *Rora^{sg/sg}* mice. Indeed *Rora^{fl/sg} ACTB-cre* mice displayed ataxia and were smaller in size compared to control *Rora^{fl/fl}* mice (Figure 5.13).



$Rora^{fl/fl}$ $Rora^{fl/sg} ACTB-cre$

Figure 5.13 Gross appearance of $Rora^{fl/fl}$ mice and $Rora^{fl/sg} ACTB-cre$ mice. $Rora^{fl/sg} ACTB-cre$ mice are smaller in size compared to $Rora^{fl/fl}$ mice. The photograph is representative of 6 week-old males.

Following confirmation that Cre-Lox recombination was successful in $Rora^{fl/sg} ACTB-cre$ mice $Rora^{fl/sg} LysMCre$ mice were generated (**Section 2.1.8**). In $Rora^{fl/sg} LysMCre$ mice one *Rora* allele contains the floxed sequence and the other *Rora* allele contains the spontaneous mutation. Cre is only expressed in myeloid cells therefore excision of the floxed *Rora* sequence will only occur in cells of the myeloid lineage. Unlike $Rora^{fl/sg} ACTB-cre$ mice, $Rora^{fl/fl} LysMCre$ mice have a similar physical phenotype to control $Rora^{fl/fl}$ mice (**Figure 5.14**).



$Rora^{fl/fl}$ $Rora^{fl/sg} LysMCre$

Figure 5.14 Gross appearance of $Rora^{fl/fl}$ mice and $Rora^{fl/sg} LysMCre$ mice. $Rora^{fl/sg} LysMCre$ mice are similar in size compared to $Rora^{fl/fl}$ mice. The photograph is representative of 6 week-old males.

5.3.10 The peritoneal macrophage phenotype is unaltered in $Rora^{fl/sg} LysMCre$ mice compared to $Rora^{fl/fl}$ mice.

Having generated $Rora^{fl/sg} LysMCre$ mice it was next determined if these mice had an altered peritoneal macrophage phenotype as was observed in $Rora^{sg/sg}$ mice (**Figure 3.12**). To achieve this peritoneal cells were isolated from $Rora^{fl/sg} LysMCre$ mice and $Rora^{fl/fl}$ mice and analysed by flow cytometry. Four peritoneal populations were identified based on CD11b, F4/80, Tim4 and MHCII expression in $Rora^{fl/fl}$ mice and $Rora^{fl/sg} LysMCre$ mice (**Figure 5.15A**). Analysis revealed that levels of the $Tim4^+ MHCII^+$, $Tim4^+ MHCII^-$, $Tim4^- MHCII^+$ and $Tim4^- MHCII^-$ peritoneal populations were unchanged between $Rora^{fl/fl}$

mice and *Rora*^{fl/sg} *LysMCre* mice (**Figure 5.15B**). These results indicate that the levels of peritoneal macrophages are unaltered when *Rora* is absent from only myeloid cells.

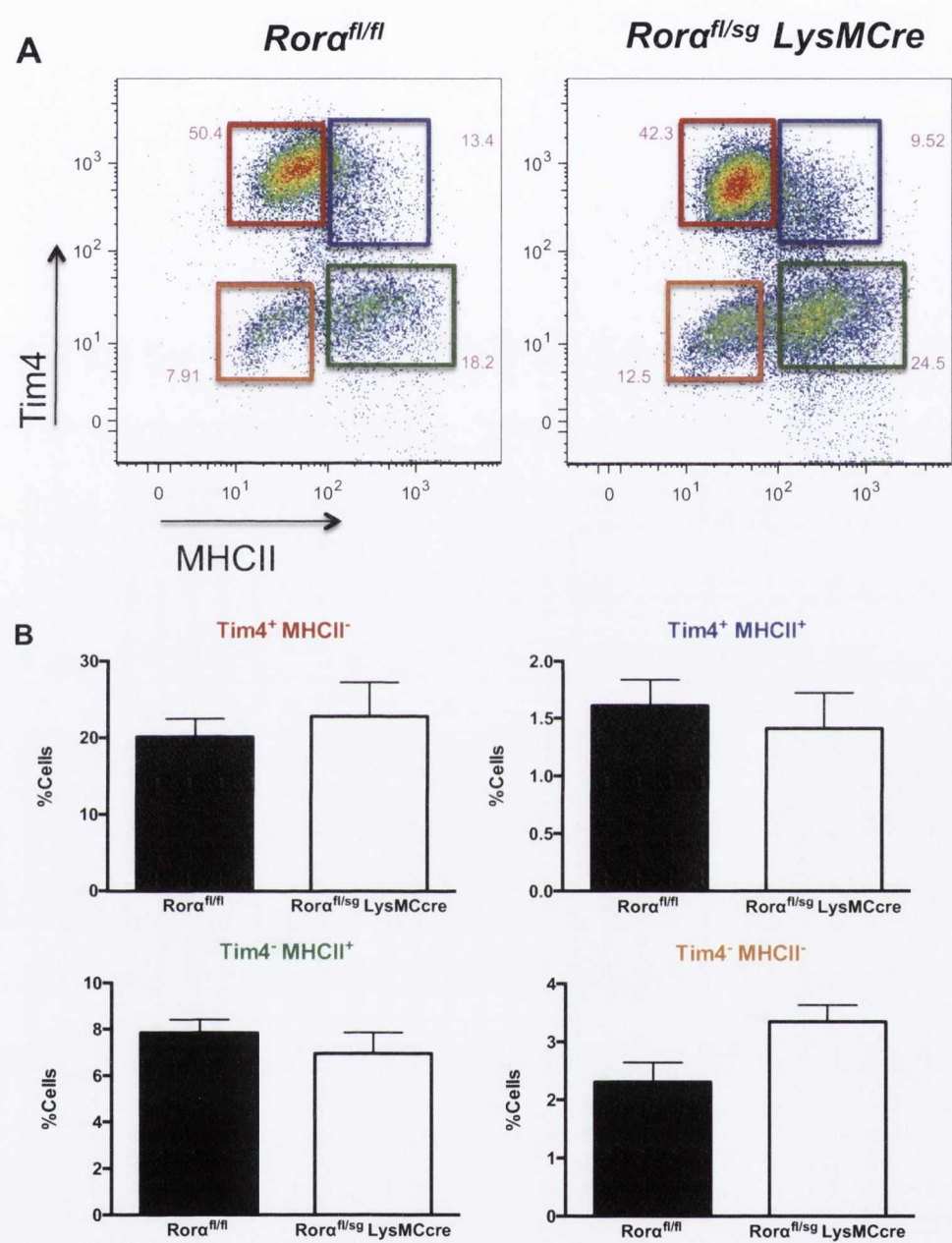


Figure 5.15 Peritoneal macrophage populations are unchanged in *Rora*^{fl/sg} *LysMCre* mice compared to *Rora*^{fl/fl} mice. **A** Representative flow cytometry plots of the gating strategy used in *Rora*^{fl/fl} mice and *Rora*^{fl/sg} *LysMCre* mice for identifying peritoneal populations based on MHCII and Tim4 expression. Percentages indicate typical proportions of the cell types of all peritoneal cells (Pre-gate CD11b⁺, F4/80⁺). **B** Quantification of peritoneal populations analysed by flow cytometry in (A) above. All data represent means ± s.e.m. Differences, indicated as two-tailed *p* values, as assessed by unpaired Student *t* test. *Rora*^{fl/fl} mice n=7, *Rora*^{fl/sg} *LysMCre* mice n= 4.

Eosinophil and neutrophil levels are altered in the peritoneal cavity compared to controls when functional *Rora* is absent in every cell. To explore if these observations are also made in *Rora^{fl/s} LysMCre* mice eosinophil and neutrophil levels were explored by flow cytometry in the peritoneal cavity of *Rora^{fl/fl}* mice and *Rora^{fl/s} LysMCre* mice. Analysis revealed that in the peritoneal cavity eosinophil (CD11b^{Int} Siglec-F⁺) levels and neutrophil (CD11b^{Int} Ly6G⁺) levels were comparable between *Rora^{fl/fl}* mice and *Rora^{fl/s} LysMCre* mice (**Figure 5.16**). These results indicate that the levels of eosinophils and neutrophils in the peritoneal cavity are unaltered when *Rora* is absent from only myeloid cells.

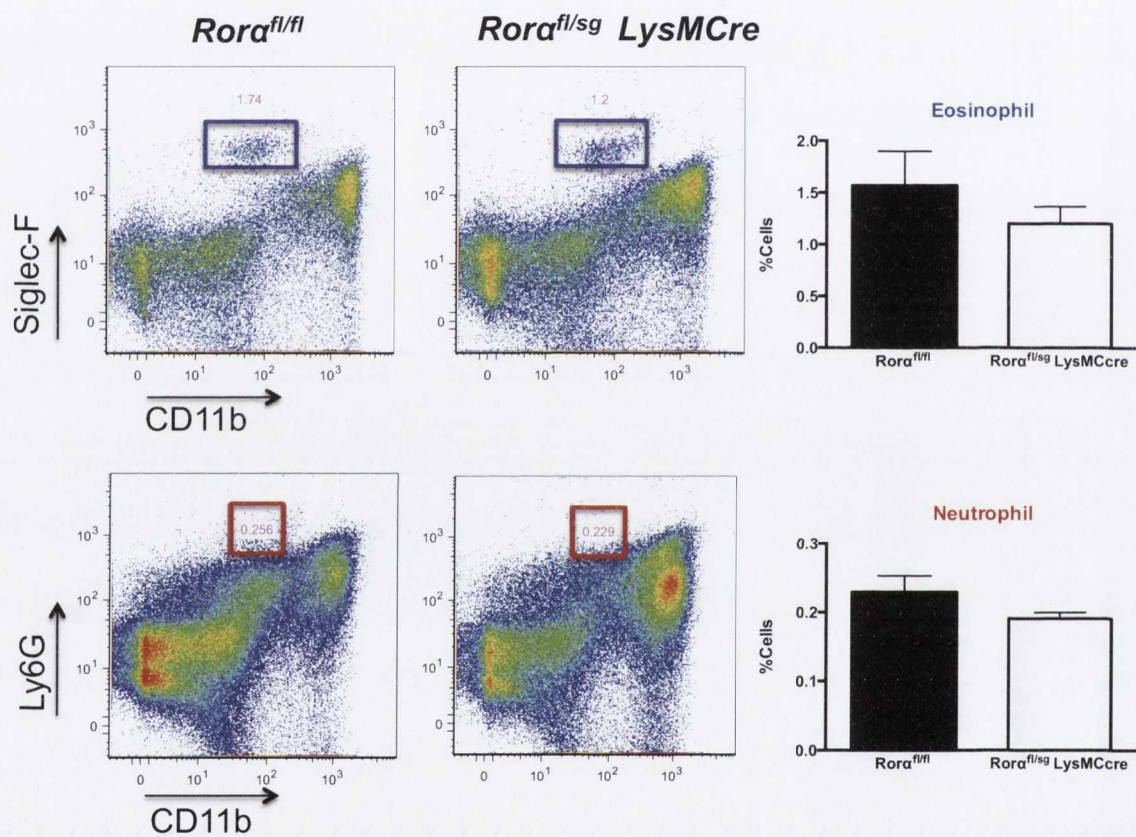


Figure 5.16 Eosinophil and neutrophil levels are unaltered in the peritoneal cavity in *Rora^{fl/s} LysMCre* mice compared to *Rora^{fl/fl}* mice. Representative flow cytometry plots of gating strategy used for eosinophils (Siglec-F⁺, CD11b^{Int}) and neutrophils (Ly6G⁺, CD11b^{Int}). All data represent means $\pm$ s.e.m. Differences, indicated as two-tailed *p* values, were considered significant when *p* < 0.05 as assessed by unpaired Student *t* test. Numbers on flow cytometry plot reflect percentage of total cells. *Rora^{fl/fl}* mice n=7, *Rora^{fl/s} LysMCre* mice n=4.

5.3.11 *Rora*^{fl/s^g} *LysMCre* mice are less susceptible to LPS-induced endotoxemia compared to WT mice.

Using *Rora*^{sg/sg} mice it was shown that in the absence of functional *Rora* mice were less susceptible to LPS-induced endotoxemia (**Figure 4.1**). To determine if the absence of *Rora* in only myeloid cells contributes to decreased susceptibility to LPS-induced endotoxemia WT mice and *Rora*^{fl/s^g} *LysMCre* mice were administered LPS (10 mg/kg) I.P and monitored for 48 hours. Results showed that percentage survival began to decrease in WT mice 6 hours post LPS administration, with survival being 0% at 48 hours (**Figure 5.17A**). Percentage survival began to decrease in *Rora*^{fl/s^g} *LysMCre* mice 24 hours post LPS administration, with survival being 25% at 48 hours. Therefore, *Rora*^{fl/s^g} *LysMCre* mice had significantly increased survival ($p<0.022$) compared to WT mice (**Figure 5.17A**).

Sepsis scores were recorded in WT mice from 1-hour post LPS administration, with scores reaching the highest score of 5 by 36 hours (**Figure 5.17B**). Sepsis scores were also recorded in *Rora*^{fl/s^g} *LysMCre* mice from 1-hour post LPS administration. *Rora*^{fl/s^g} *LysMCre* mice had significantly decreased scores ($p<0.0045$) compared to WT mice post LPS administration as analysed by AUC (**Figure 5.17B**). The body temperatures of WT mice decreased over 24 hours post LPS administration, as did the body temperatures of *Rora*^{fl/s^g} *LysMCre* mice. Body temperatures of *Rora*^{fl/s^g} *LysMCre* mice were significantly higher ($p<0.036$) compared to controls as analysed by AUC (**Figure 5.17C**).

These results indicate that when *Rora* is absent only in myeloid cells mice are less susceptible to LPS-induced endotoxemia suggesting that *Rora* plays a role in myeloid cell inflammatory responses to LPS.

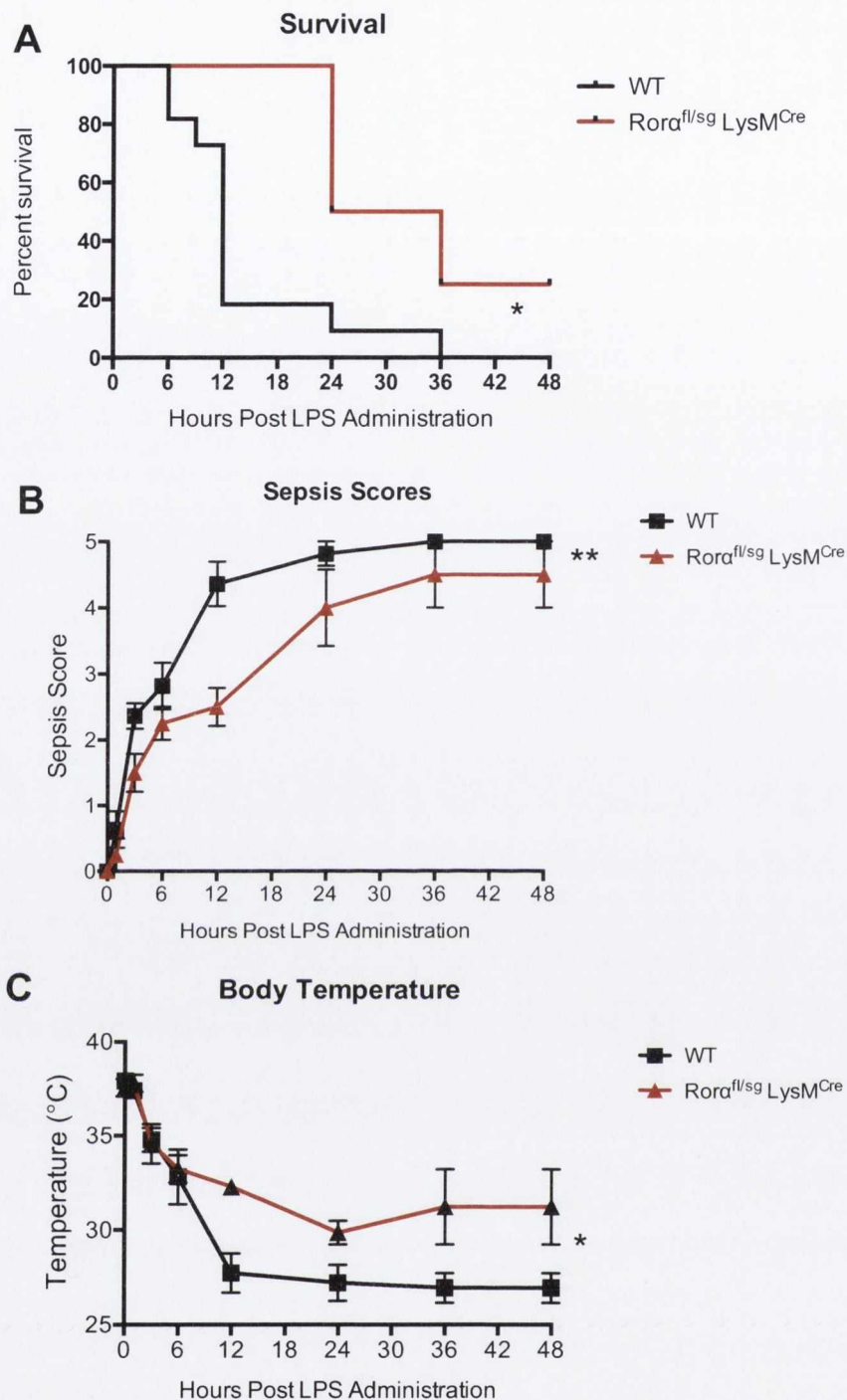


Figure 5.17 $Rora^{fl/sg} LysM^{Cre}$ mice are less susceptible to LPS-induced endotoxemia compared to WT mice. Mice were administered with LPS I.P (10 mg/kg). **A** Percentage survival. **B** Sepsis scores. **C** Body temperature. The Kaplan-Meier method was used to evaluate statistical difference in survival between groups. Data represent means $\pm$ s.e.m. Sepsis scores and body temperature analysed by area under curve (AUC). Differences in AUC values indicated as two-tailed p values, as assessed by unpaired Student t test. * $p < 0.01$ to 0.05 , ** $p < 0.001$ to 0.01 . WT mice $n=8$, $Rora^{fl/sg} LysM^{Cre}$ mice $n=4$.

5.4 Discussion

In previous chapters *Rora*^{sg/sg} mice containing a spontaneous mutation in *Rora* were used to explore the role *Rora* played in the development of peritoneal macrophages and inflammatory responses. Although a very useful mouse model, *Rora* is absent in every cell in *Rora*^{sg/sg} mice making it difficult to determine the role *Rora* plays within only myeloid cells. *LysMCre* mice are used in the literature as the mouse model to delete specific floxed sequences in myeloid cells, therefore *LysMCre* mice were chosen here to conditionally delete a floxed region of *Rora* in myeloid cells. To first try and confirm that the Cre-Lox recombination occurs successfully in *Rora*^{fl/fl} mice these mice were crossed to *ACTB-cre* mice. If the floxed sequence in *Rora* is excised in the presence of Cre recombinase it was hypothesised that *Rora*^{fl/fl} *ACTB-cre* mice would display an altered physical phenotype compared to controls as is observed in *Rora*^{sg/sg} mice compared to controls. Indeed *Rora*^{fl/fl} *ACTB-cre* mice did display a similar small size and ataxia compared to controls as displayed by *Rora*^{sg/sg} mice. Interestingly *Rora*^{fl/fl} *ACTB-cre* mice had significantly increased survival compared to *Rora*^{sg/sg} mice suggesting that although the *Rora*^{fl/fl} *ACTB-cre* mice and *Rora*^{sg/sg} mice display a similar physical phenotype the processes in which *Rora* is altered in these mice (natural mutation v Cre-Lox recombination excision) may result in different physiological effects.

Having shown in Chapter 3 using *Rora*^{sg/sg} mice that *Rora* may have a role in the development of blood monocyte-derived peritoneal macrophages, Ly6C^{Low} blood monocytes and Ly6C^{Low} bone marrow monocytes we wanted to explore if these alterations were also observed when *Rora* is excised by Cre-Lox recombination in *Rora*^{fl/fl} *ACTB-cre* mice. Results showed that there was an alteration in the levels of peritoneal macrophage populations in *Rora*^{fl/fl} *ACTB-cre* mice compared to controls. However, these alterations were not the same as those observed in peritoneal macrophage populations in *Rora*^{sg/sg} mice. Therefore, although *Rora* is non-functional in all cells in both *Rora*^{fl/fl} *ACTB-cre* mice and *Rora*^{sg/sg} mice this does not result in identical alterations in peritoneal macrophage populations in the two groups. In terms of blood monocyte levels *Rora*^{fl/fl} *ACTB-cre* mice have decreased levels of Ly6C^{Low} and Ly6C^{High} blood monocytes and increased levels of neutrophils. These results are similar to the alterations in blood monocytes observed in *Rora*^{sg/sg} mice thus suggesting that the loss of functional *Rora* through Cre-Lox recombination and through a natural mutation results in altered levels of

blood monocytes. Thus, further suggesting that *Rora* has a role in the development of these cells.

The altered physical phenotype of *Rora*^{fl/fl} *ACTB-cre* mice compared to controls indicated that Cre-Lox recombination occurs successfully in these mice, therefore it was next determined if the observations in *Rora*^{sg/sg} mice made in Chapter 3 and Chapter 4 were due to *Rora* acting intrinsically within myeloid cells. To achieve this *LysMCre* mice were crossed to *Rora*^{fl/fl} mice to create *Rora*^{fl/fl} *LysMCre* mice in which the floxed sequence in *Rora* is excised only in myeloid cells. In Chapter 3 it was observed that in the absence of functional *Rora* mice have decreased levels of blood monocyte-derived peritoneal macrophages, Ly6C^{Low} blood monocytes and increased levels of Ly6C^{Low} bone marrow monocytes. To analyse if these observations are a result of *Rora* acting within these myeloid cell populations each of these populations was analysed by flow cytometry in *Rora*^{fl/fl} *LysMCre* mice and compared to control *Rora*^{fl/fl} mice. Analysis revealed that the levels of Ly6C^{Low} blood monocytes and levels of Ly6C^{Low} bone marrow monocytes were comparable between *Rora*^{fl/fl} *LysMCre* mice and control *Rora*^{fl/fl} mice. In terms of peritoneal macrophage populations, *Rora*^{fl/fl} *LysMCre* mice had decreased levels of the Tim4⁺ MHCII⁺ tissue-resident macrophage population compared to controls. This result is in contrast to *Rora*^{sg/sg} mice that have decreased levels of the Tim4⁻ MHCII⁺ blood monocyte-derived macrophage population and Tim4⁻ MHCII⁻ eosinophil containing population.

In Chapter 4 it was observed using *Rora*^{sg/sg} mice that in the absence of functional *Rora* mice are less susceptible to LPS-induced endotoxemia suggesting that *Rora* has a role in inflammatory responses to LPS. To determine if the role *Rora* has in inflammatory responses to LPS is within myeloid cells *Rora*^{fl/fl} *LysMCre* mice were administered LPS and monitored for 48 hours. Results showed that *Rora*^{fl/fl} *LysMCre* mice were comparably susceptible to LPS-induced endotoxemia as control *Rora*^{fl/fl} mice. This result is in contrast to what was observed in *Rora*^{sg/sg} mice, which were less susceptible to LPS-induced endotoxemia as control mice.

There are two explanations as to why *Rora*^{fl/fl} *LysMCre* mice did not display the same alterations in the levels of peritoneal macrophage populations; blood monocytes or bone marrow monocytes compared to control mice, and why *Rora*^{fl/fl} *LysMCre* mice were comparably susceptible to LPS-induced endotoxemia as controls. The first of these is that

Cre-Lox recombination did not occur efficiently in *Rora^{fl/fl} LysMCre* mice and second is that excision is effective but the effects of *Rora* on myeloid cells is due to a role *Rora* has outside of these cells. To test the former hypothesis primers were designed that were complimentary to a sequence located within the floxed region of *Rora*, therefore if recombination occurs successfully this sequence will be excised and the primers will not have a sequence to amplify. BMDMs were chosen as the representative myeloid cell, if excision was successful in *Rora^{fl/fl} LysMCre* mice then there would be no amplification by PCR in the BMDM sample from *Rora^{fl/fl} LysMCre* mice. However, this was not the case and a PCR product was detected in the BMDM sample from *Rora^{fl/fl} LysMCre* mice thus suggesting that excision is incomplete in *Rora^{fl/fl} LysMCre* mice. On the other-hand no PCR product was detected in the BMDM sample from *Rora^{fl/fl} ACTB-cre* mice thus appearing to support the conclusion made, based on the physical phenotype of *Rora^{fl/fl} ACTB-cre* mice, that *Rora* is excised in these mice. It is not possible to quantify the efficiency of excision in *Rora^{fl/fl} LysMCre* mice using sqPCR, however if functional *Rora* is still present in myeloid cells due to incomplete excision then this may account for the lack of differences between *Rora^{fl/fl} LysMCre* mice and *Rora^{fl/fl}* mice. As a result of the incomplete excision of the floxed sequence in *Rora* it was decided to not continue conducting experiments in *Rora^{fl/fl} LysMCre* mice.

To try and improve the efficiency of *Rora* excision using Cre-Lox recombination *Rora^{fl/sg}* mice were generated and crossed to Cre expressing mice. A number of different groups have adapted this method including collaborations with Oliphant and colleagues that used *Rora^{fl/sg} IL7RaCre* mice in their recent paper (Oliphant et al., 2014) as well as studies that I was involved in (Hams et al., 2015b). *Rora^{fl/sg} ACTB-cre* mice were generated to test if Cre-Lox recombination occurs successfully in this cross and indeed *Rora^{fl/sg} ACTB-cre* mice displayed ataxia and were smaller in size compared to control *Rora^{fl/fl}* mice. Following this, *Rora^{fl/sg} LysMCre* mice were generated. As in *Rora^{fl/fl} LysMCre* mice, peritoneal macrophage populations were analysed by flow cytometry in *Rora^{fl/sg} LysMCre* mice to determine if their levels were altered as compared to controls. Analysis revealed that levels of the peritoneal macrophage populations were unchanged compared to controls. These results indicate that the levels of peritoneal macrophages are unaltered when *Rora* is absent from only myeloid cells, suggesting that the altered peritoneal macrophage phenotype observed in *Rora^{sg/sg}* mice is a result of *Rora* acting extrinsically of myeloid cells suggesting that *Rora* has an indirect role in the development of blood monocyte-derived peritoneal macrophages. Table 5.1 outlines the differences in the peritoneal cell

populations investigated in this thesis in *Rora*^{sg/sg} mice, *Rora*^{fl/fl} *ActBCre* mice, *Rora*^{fl/fl} *LysMCre* mice and *Rora*^{fl/sg} *LysMCre* mice as compared to control mice.

	Mouse strain			
	<i>Rora</i> ^{sg/sg}	<i>Rora</i> ^{fl/fl} <i>ActBCre</i>	<i>Rora</i> ^{fl/fl} <i>LysMCre</i>	<i>Rora</i> ^{fl/sg} <i>LysMCre</i>
Cell type				
Tim4 ⁺ MCHII ⁺	Same	Same	Decreased	Same
Tim4 ⁺ MCHII ⁻	Same	Increased	Same	Same
Tim4 ⁻ MCHII ⁺	Decreased	Same	Same	Same
Tim4 ⁻ MCHII ⁻	Decreased	Same	Same	Same
CD11b ⁺ SiglecF ⁺ (Eosinophils)	Decreased	Decreased	Same	Same
CD11b ⁺ Ly6G ⁺ (Neutrophils)	Increased	Increased	Same	Same
Control	WT	<i>Rora</i> ^{fl/fl}	<i>Rora</i> ^{fl/fl}	<i>Rora</i> ^{fl/sg}

Table 5.1 Peritoneal cell populations in the different mouse strains used as compared to control mice. Same- same levels* of population as compared to controls. Increased- increased levels* of population as compared to controls. Decreased- decreased level* of population as compared to controls. *percentage of total cells

To determine if the role *Rora* has in inflammatory responses to LPS is within myeloid cells *Rora*^{fl/sg} *LysMCre* mice were administered LPS and monitored for 48 hours. Results showed that *Rora*^{fl/sg} *LysMCre* mice were less susceptible to LPS-induced endotoxemia as compared to control mice. This result is similar to what was observed in *Rora*^{sg/sg} mice, which are also less susceptible to LPS-induced endotoxemia compared to controls. This result suggests that *Rora* plays a role in inflammatory responses to LPS in myeloid cells. In Chapter 4 it was hypothesised that altered inflammatory responses in *Rora*^{sg/sg} mice was due to the fact that these mice have decreased levels of blood monocyte-derived macrophages in the peritoneal cavity, however, levels of this population are unaltered in *Rora*^{fl/sg} *LysMCre* mice. Therefore suggesting that *Rora* is acting through a different mechanism in *Rora*^{fl/sg} *LysMCre* mice. Further research is needed to determine the exact

role *Rora* has in inflammatory responses to LPS in myeloid cells and the mechanisms involved.

In summary excision of the floxed region in *Rora* by Cre-Lox recombination was shown to be successful using *Rora^{fl/fl} ACTB-cre* mice. Results in *Rora^{fl/fl} ACTB-cre* mice also appeared to confirm observations in Chapter 3 that *Rora* has a role in the development of peritoneal macrophage populations, blood monocytes and eosinophils and neutrophils (Table 5.1). Results using *Rora^{fl/fl} LysMCre* mice suggested that *Rora* did not have an intrinsic role in peritoneal macrophage, blood monocyte or bone marrow monocyte development. However, it was then shown that excision of *Rora* is incomplete in *Rora^{fl/fl} LysMCre* mice. Therefore, no further experiments were carried out on *Rora^{fl/fl} LysMCre* mice instead we switched to using *Rora^{fl/sg} LysMCre* mice. Results in *Rora^{fl/sg} LysMCre* mice suggest that *Rora* does not have an intrinsic role in macrophage development. However, *Rora* does appear to have a role in inflammatory responses, as *Rora^{fl/sg} LysMCre* mice are less susceptible to LPS-induced endotoxemia compared to controls. Further research is needed to elucidate the exact role *Rora* plays in inflammatory responses within macrophages.

Chapter 6

General Discussion

and

Future Directions

In this thesis I have investigated the role that the transcription factor *Rora* has in macrophages in the context of development, inflammatory responses and circadian rhythm. In terms of macrophage development the focus of this thesis was on peritoneal macrophage populations. It is becoming increasingly evident that macrophage populations can have dual origins and there is a growing interest in the transcription factors that are important in the development of these different macrophage populations. Indeed three separate groups have recently identified the transcription factor *Gata6* as having a central role in promoting the tissue-dependent characteristics of tissue-resident peritoneal macrophages (Gautier et al., 2014; Okabe and Medzhitov, 2014; Rosas et al., 2014). Based on these recent publications I investigated if *Rora*, known to be involved in the development of other cells types (Wong et al., 2012; Yang et al., 2008), also has a role in the development of peritoneal macrophages, in particular in the development of blood monocyte-derived and/or tissue-resident peritoneal macrophages.

I identified four peritoneal populations in WT mice based on positive expression of both CD11b and F4/80, and differential expression of Tim4 and MHCII. Analysis by flow cytometry of the expression of a number of different protein markers on each of these populations indicated that the Tim4⁺ MHCII⁻ peritoneal population represented a tissue-resident macrophage population and the Tim4⁻ MHCII⁺ peritoneal population represented a blood monocyte-derived population. These indications were then confirmed as the Tim4⁻ MHCII⁺ peritoneal population was expanded by the treatment of WT mice with Thioglycollate + cIL-4, the combination of which has been shown to induce the expansion of the blood monocyte-derived macrophage population (Gundra et al., 2014). Conversely, the Tim4⁺ MHCII⁻ peritoneal population was expanded by the treatment of WT mice with cIL-4 alone, known to induce the expansion and proliferation of tissue-resident macrophages (Gundra et al., 2014).

Interestingly I observed that levels of the Tim4⁻ MHCII⁺ blood monocyte-derived macrophage peritoneal population were decreased in *Rora*^{sg/sg} mice suggesting that *Rora* has a role in blood monocyte-derived macrophages. Blood monocyte-derived macrophages are derived from progenitor cells originating in the bone marrow therefore I investigated if these progenitor cells are also altered in *Rora*^{sg/sg} mice to determine if, and at what point, *Rora* acts in the developmental pathway. It was found that levels of Ly6C^{Low} blood monocytes and Ly6C^{Low} bone marrow monocytes are decreased in *Rora*^{sg/sg} mice. These results appear to suggest two things: first, that in the steady state blood monocyte-derived

peritoneal macrophages may be derived from Ly6C^{Low} blood monocytes, which in turn are derived from Ly6C^{Low} blood monocytes. Secondly, the data suggests that *Rora* begins its role in macrophage development at or before the Ly6C^{Low} bone marrow monocyte stage of development (Figure 6.1).

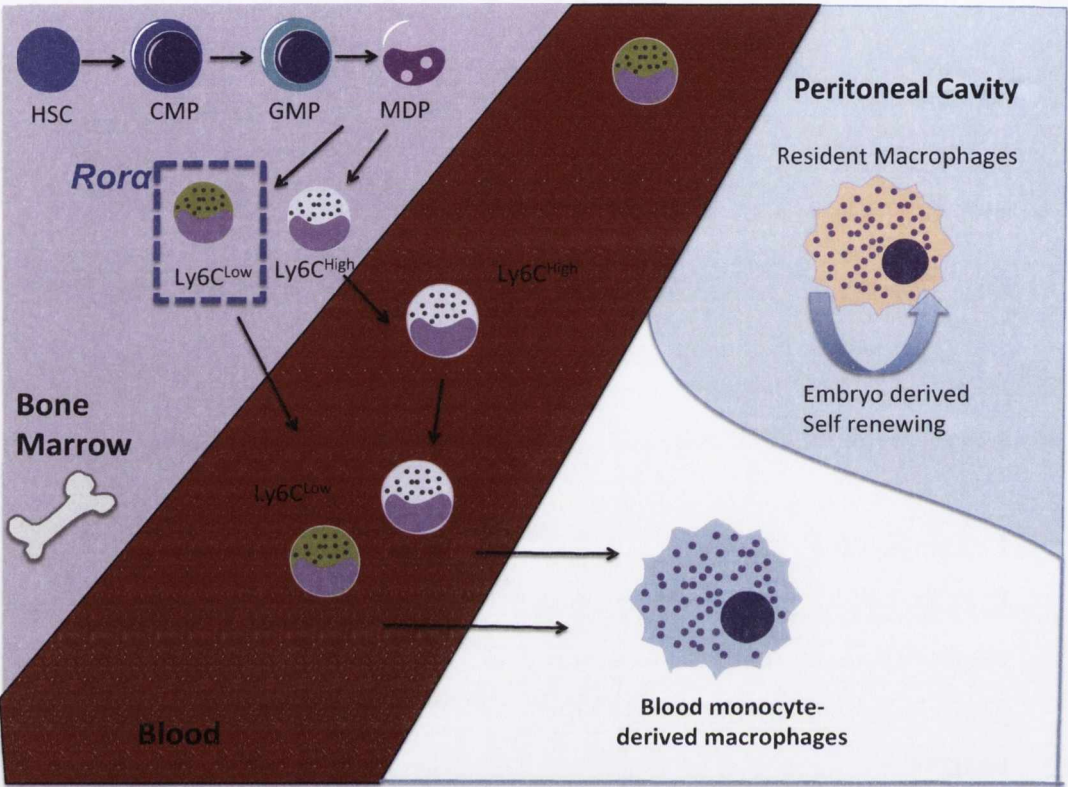


Figure 6.1 Schematic representing the stage in the steady state at which it is hypothesised *Rora* acts in the developmental pathway of blood monocyte-derived macrophages. The data indicates that *Rora* may act at the stage of Ly6C^{Low} bone marrow monocyte development.

Convention states that $\text{Ly6C}^{\text{High}}$ blood monocytes are the precursors to blood monocyte-derived macrophages. However, the exact dynamics of blood monocytes has come under debate in recent years (Yona et al., 2013). For example, a report in 2014 showed that Ly6C^{Low} monocytes are crucial for the initiation of arthritis and they give rise to inflammatory macrophages during arthritis (Misharin et al., 2014). While another group found that M2 macrophages in liver granulomas of *Schistosoma mansoni* infected mice arise predominantly from recruited $\text{Ly6C}^{\text{High}}$ monocytes, but that these cells may transition through a Ly6C^{Low} state (Girgis et al., 2014). Indeed it has been shown that $\text{Ly6C}^{\text{High}}$ monocytes can also differentiate into Ly6C^{Low} cells in the bone marrow and blood under steady state conditions (Yona et al., 2013). It should be noted that there is a disparity

between the models used and sites addressed in these various studies on blood monocytes. Nevertheless, these studies and others highlight that the developmental relationship between Ly6C^{High} and Ly6C^{Low} monocytes has not been fully elucidated. The results in this thesis would possibly indicate that in the steady state Ly6C^{Low} blood monocytes are derived from Ly6C^{Low} bone marrow monocytes, in a process distinct from Ly6C^{High} monocyte development. The data also suggest that Ly6C^{Low} blood monocytes are the primary precursors for the blood monocyte-derived peritoneal macrophage population. However, the dual expression of CCR2 and CX₃CR1 on the blood monocyte-derived peritoneal macrophage population suggests that alongside Ly6C^{Low} blood monocytes, Ly6C^{High} blood monocytes may also be contributors to the blood monocyte-derived peritoneal macrophage population.

The accumulation of Ly6C^{Low} bone marrow monocytes in *Rora*^{sg/sg} mice indicates that *Rora* begins its role in macrophage development at the Ly6C^{Low} bone marrow monocyte stage. *Rora*^{sg/sg} BM chimeras were generated (*Rora*^{sg/sg}→WT mice) to explore the possibility that the accumulation of Ly6C^{Low} bone marrow monocytes in *Rora*^{sg/sg} mice was due to *Rora* having an intrinsic role in their development and thus in the downstream development of blood monocyte-derived peritoneal macrophages. The results indicate that the blood monocyte-derived peritoneal macrophage population was indeed decreased in *Rora*^{sg/sg}→WT mice compared to control WT→WT mice, as was the tissue-resident macrophage peritoneal population and the eosinophil containing peritoneal population. However, even though the same number of cells were used for BM reconstitution in both groups there appears to be an overall decrease in cell numbers in *Rora*^{sg/sg}→WT mice compared to WT→WT mice which may account for these results. The decrease in cell numbers may be as a result of decreased efficiency of bone marrow reconstitution in *Rora*^{sg/sg}→WT mice compared to WT→WT mice, leading to a decrease in the levels of reconstituted cells in *Rora*^{sg/sg}→WT mice. The survival and proliferation of BM cells from *Rora*^{sg/sg} would need to be examined compared to controls in more detail to further elucidate what is occurring in the *Rora*^{sg/sg}→WT mice.

In Chapter 5, *Rora*^{fl/fl} *LysMCre* mice were used to investigate if *Rora* has an intrinsic role in the development of blood monocyte-derived peritoneal macrophages. Cre recombinase is expressed under the control of *Lyz2* which is expressed in cells of the myeloid system in *Rora*^{fl/fl} *LysMCre* mice (Cross et al., 1988), therefore in *Rora*^{fl/fl} *LysMCre* mice the floxed sequence in *Rora* is excised in cells such as macrophages, monocytes, eosinophils and

neutrophils. Using *Rora*^{fl/fl} *LysMCre* mice it was observed that levels of blood monocyte-derived peritoneal macrophages, Ly6C^{Low} blood monocytes and Ly6C^{Low} bone marrow monocytes were unchanged compared to controls. However, further investigation of *Rora*^{fl/fl} *LysMCre* mice revealed that the excision of the floxed sequence in *Rora* was incomplete in these mice therefore calling into question the validity of the results observed in these mice. *Rora*^{fl/sg} *LysMCre* mice were then generated to improve the efficiency of excision of the floxed sequence in *Rora*. If I were to do things differently, I would have started out using *Rora*^{fl/sg} *LysMCre* mice sooner in the project. Investigation of the levels of blood monocyte-derived peritoneal macrophages in *Rora*^{fl/sg} *LysMCre* mice revealed that there was no difference in their levels between *Rora*^{fl/sg} *LysMCre* mice and control mice. These results indicate that the altered peritoneal macrophage phenotype observed in *Rora*^{sg/sg} mice is a result of *Rora* acting extrinsically of myeloid cells suggesting that *Rora* has an indirect role in the development of blood monocyte-derived peritoneal macrophages.

It is possible that *Rora* has an extrinsic role in the process of Ly6C^{Low} bone marrow monocyte transition from the bone marrow into the blood circulation. Indeed *Rora* has a role in CCL2 expression that could effect Ly6C^{Low} bone marrow monocyte transition into the blood and as a consequence effect levels of Ly6C^{Low} blood monocytes and blood monocyte-derived peritoneal macrophages. CCL2 is conventionally associated with the transition of Ly6C^{High} monocytes from the bone marrow, however it has been shown that both Ly6C^{High} bone marrow monocytes and Ly6C^{Low} blood monocyte levels are altered in mice administered the CCR2 antibody MC21 (Yona et al., 2013). It has also been shown that *Rora* binds to a RORE present in the promoter region of *Ccl2*, promoting the expression of CCL2 (Sato et al., 2014). Therefore in the absence of functional *Rora* levels of CCL2 would theoretically be decreased, which could contribute to the build up of Ly6C^{Low} monocytes in the bone marrow. However, in this case levels of Ly6C^{High} blood monocytes would also be altered, which is not observed here.

It is also possible that *Lyz2* expression in *Rora*^{fl/sg} *LysMCre* mice begins after the step at which *Rora* acts in the development of myeloid cells. Therefore functional *Rora* could still be present in *Rora*^{fl/sg} *LysMCre* mice at the stage when it contributes to myeloid cell development. Vannella and colleagues recently investigated a similar concept in *IL-4Ra*^{fl/-} *LysMCre* mice. They observed that different macrophage populations expressed variable levels of *Lyz2*. This in turn resulted in incomplete excision of *IL-4Ra* in macrophage populations in *IL-4Ra*^{fl/-} *LysMCre* mice that expressed low levels of *Lyz2* (Vannella et al.,

2014). Expression levels of Lyz2 in bone marrow progenitor cells would therefore be of interest as it is hypothesised that *Rora* acts at the stage of Ly6C^{Low} bone marrow monocyte development.

Using lysozyme EGFP mice it has been shown that 21.2 % of HSC cells, 39.0% of CMP, 19.5% of MEP, 65.2% of GMP cells and 97% of granulocytes and macrophages express EGFP (Ye et al., 2003). GMP cells are the nearest progenitor to Ly6C^{Low} bone marrow monocytes (MDP cells were not examined). The results from the Ye paper would suggest that 65.2% of GMP cells express lysozyme meaning that about 34.8% of GMP cells do not. This may have an effect on the efficiency of *Rora* excision by Cre recombinase in GMP progenitor cells in *Rora*^{fl/sf} *LysMCre* mice, which in turn may have a downstream effect on the developmental pathway and may explain why myeloid cells are unaltered in *Rora*^{fl/sf} *LysMCre* mice compared to controls. The efficiency of *Rora* excision in GMP progenitor cells needs to be investigated further to establish if this possibility is occurring in *Rora*^{fl/sf} *LysMCre* mice.

Although the focus of this thesis is on the role of *Rora* in macrophage development it is worth noting that the levels of two other myeloid cells- eosinophils and neutrophils, are altered in the peritoneal cavity in the absence of functional *Rora*. Eosinophil levels are decreased and neutrophil levels are increased in *Rora*^{sg/sg} mice, this increase in neutrophil levels is also observed in the blood of *Rora*^{sg/sg} mice. The developmental pathway of eosinophils and neutrophils was not the focus of this thesis and therefore were not investigated further. It was also found that eosinophil and neutrophil levels are unaltered in *Rora*^{fl/sf} *LysMCre* mice compared to controls. However, interestingly it is known that both cell types are derived from the GMP progenitor cell in the bone marrow, the same progenitor cell as bone marrow monocytes. Therefore, as discussed above, the observation that levels of eosinophils and neutrophils are unaltered in *Rora*^{fl/sf} *LysMCre* mice compared to controls may be due to inefficient excision of *Rora* in GMP cells due to lower expression levels of Lyz2.

It is known that *Rora* has a role in inflammatory responses (**Section 4.1**). The majority of these studies indicate that *Rora* has a role in keeping inflammatory responses under control and in the absence of *Rora* inflammatory responses are exacerbated (Delerive et al., 2001; Stapleton et al., 2005). In this thesis I wanted to determine the role *Rora* has in inflammatory responses in the context of macrophages. We observed that *Rora*^{sg/sg} mice

have decreased susceptibility to LPS-induced endotoxemia which was characterised by decreased serum levels of the pro-inflammatory cytokine IL-1 β , hinting that inflammatory responses are impaired in *Rora*^{sg/sg} mice. In terms of macrophage responses to LPS in the absence of functional *Rora* peritoneal macrophages from *Rora*^{sg/sg} mice produce decreased levels of IL-1 β compared to control macrophages. Unlike previously published data which indicate a role for *Rora* in the down regulation of inflammatory responses (Delerive et al., 2001; Stapleton et al., 2005), the results in this thesis suggest that *Rora* in fact contributes to inflammatory responses in response to LPS.

Blood monocyte-derived macrophages have an important role in responding to the inflammatory stimulus (Dey et al., 2015). Knowing that levels of the blood monocyte-derived macrophage population are decreased in the absence of functional *Rora* I wanted to determine if inflammatory responses are altered in *Rora*^{sg/sg} mice due to the decrease in this macrophage population. Given the documented role of blood monocyte-derived macrophages in inflammation it was hypothesised that this population could produce pro-inflammatory cytokines in responses to LPS. Results from WT mice showed that the blood monocyte-derived macrophages produced pro-inflammatory cytokines in response to LPS including IL-1 β . A limitation of the study was that I was unable to perform the same experiment on peritoneal macrophage populations from *Rora*^{sg/sg} mice as a large number of *Rora*^{sg/sg} mice would have been required to acquire the cell numbers needed. Although a large number of harems to generate *Rora*^{sg/sg} mice were consistently breeding it was rare to have more than 4 *Rora*^{sg/sg} mice at the same time. Indeed throughout this thesis the lack of enough *Rora*^{sg/sg} mice at the one time has been a limitation.

As mentioned previously it was shown in Chapter 5 that *Rora*^{fl/sg} *LysMCre* mice do not have altered levels of the blood monocyte-derived peritoneal macrophage population. However, similar to *Rora*^{sg/sg} mice, *Rora*^{fl/sg} *LysMCre* mice are less susceptible to LPS-induced endotoxemia compared to controls. Taken together the data in *Rora*^{fl/sg} *LysMCre* mice indicate that the decrease in the blood monocyte-derived peritoneal macrophage population is not linked to decreased susceptibility to LPS-induced endotoxemia in *Rora*^{fl/sg} *LysMCre* mice, therefore suggesting that *Rora* is involved in another aspect of inflammatory responses to LPS. Cytokine analysis needs to be performed on the serum of *Rora*^{fl/sg} *LysMCre* mice to investigate if similar to *Rora*^{sg/sg} mice IL-1 β is decreased. It also needs to be determined if macrophages from *Rora*^{fl/sg} *LysMCre* mice have altered responses to LPS compared to controls as observed in *Rora*^{sg/sg} mice.

If the decreased production of IL-1 β in the absence of functional *Rora* is not due to decreased levels of the blood monocyte-derived peritoneal macrophage population then it is possible that it is due to a defect in IL-1 β processing. Processing of bioactive IL-1 β depends on the activation of caspase-1 by inflammasomes (Martinon et al., 2002). Work has been carried out within our lab since the completion of this thesis exploring the processing of IL-1 β in macrophages from WT mice and *Rora*^{sg/sg} mice. Early results indicate that there is a difference in IL-1 β and caspase-1 cleavage in the absence of functional *Rora*; work is on going to determine the reasons behind these observations. If I were to do things differently I would have focused on IL-1 β processing first as opposed to examining the LPS/TLR4 signalling pathway.

The role *Rora* has in the circadian clock is well documented. Having shown in *Rora*^{sg/sg} mice that *Rora* is involved in the development of blood monocyte-derived peritoneal macrophages I hypothesised that this population could also potentially display a diurnal rhythm. This idea was formulated based on recent publications investigating the molecular basis of diurnal oscillations of innate immune cells and temporal immune responses to stimuli (Bellet et al., 2013; Curtis et al., 2015; Nguyen et al., 2013). We demonstrated that *Rora* and *Bmal1* are expressed in a circadian manner in peritoneal macrophages and that levels of the blood monocyte-derived peritoneal macrophage population are increased during the active phase compared to the rest phase. The next step would be to determine the exact role that *Rora* has in the regulation of the circadian oscillations of peritoneal macrophage populations. To achieve this peritoneal macrophage population rhythms would need to be analysed over 24 hours in *Rora*^{sg/sg} mice and determine if these rhythms are altered compared to the those observed in Chapter 4 in WT mice. However, this experiment is technically difficult because as mentioned previously it is difficult to obtain sufficient numbers of *Rora*^{sg/sg} mice to power the experiment sufficiently. An alternative option is to use *Rora*^{fl/sg} *LysMCre* mice instead of *Rora*^{sg/sg} mice. In terms of temporal responses to LPS it was confirmed in Chapter 5 that WT mice are more susceptible to LPS during the active phase, the next step would be to investigate if a similar result is observed in the absence of functional *Rora*.

All experiments within this thesis were conducted in mice. Animal models are considered valuable research tools that provide insight into the complex world of human processes and disease however it is also accepted that animal studies are not always directly translatable

to humans. Murine *Rora* is a homologue of human *Rora* and humans also have bone marrow progenitor cells, blood monocytes and peritoneal macrophages albeit not identical to those found in mice. Humans also have a pro-inflammatory response to LPS that can result in sepsis. Our understanding of the exact mechanism of sepsis in humans is always evolving and it is possible that the role *Rora* has in inflammatory responses in mice could be translated to humans and could potentially represent an anti-inflammatory target in humans.

In conclusion, in this thesis I identified four peritoneal populations based on differential expression of CD11b, F4/80, Tim4 and MHCII. I also confirm that the CD11b⁺ F4/80⁺ Tim4⁺ MHCII⁺ peritoneal population represents a tissue-resident macrophage population and the CD11b⁺ F4/80⁺ Tim4⁻ MHCII⁺ peritoneal population represents a blood monocyte-derived macrophage population. I also demonstrate that this population is present in the peritoneal cavity under the steady state suggesting a possible role in tissue homeostasis. I have shown that levels of this blood monocyte-derived peritoneal macrophage population along with Ly6C^{Low} blood monocytes are decreased in the absence of functional *Rora*, while levels of Ly6C^{Low} bone marrow monocytes are increased in the absence of functional *Rora* (**Figure 6.2**). The data appears to suggest that blood monocyte-derived peritoneal macrophages are at least in part derived from Ly6C^{Low} blood monocytes. Further work is needed to confirm this possibility. The use of *Rora*^{fl/sg} *LysMCre* mice, in which *Rora* is deleted only in myeloid cells, appears to suggest that these alterations are due to *Rora* acting extrinsically of myeloid cells as opposed to acting intrinsically. This is unlike the role of other recently characterised transcription factors that have a role intrinsically in peritoneal macrophage development. However, *Rora* could still be important in determining steady state levels of peritoneal macrophages and thus tissue homeostasis and host defences. Further work is needed to determine the extrinsic role *Rora* has that affects levels of Ly6C^{Low} bone marrow monocytes.

In terms of inflammation I have shown in this thesis that *Rora*^{sg/sg} mice are less susceptible to LPS-induced endotoxemia compared to controls (**Figure 6.2**). The response to LPS in *Rora*^{sg/sg} mice was characterised by decreased serum levels of the pro-inflammatory cytokine IL-1 β , hinting that inflammatory responses are impaired in *Rora*^{sg/sg} mice. In terms of macrophage responses to LPS in the absence of functional *Rora* peritoneal macrophages from *Rora*^{sg/sg} mice produce decreased levels of IL-1 β compared to control macrophages. These data suggest that further examination of the process of IL-1 β

processing is needed. It was also shown that the blood monocyte-derived macrophage peritoneal population in WT mice produces pro-inflammatory cytokines in response to LPS stimulation highlighting that it is an important responder to inflammation and host responses. Taken together the data highlights a role for *Rora* in inflammatory responses and thus *Rora* could represent a target for anti-inflammatory therapies.

I have also shown that peritoneal macrophages express *Rora*, along with *Bmal1*, in a diurnal manner. I also show that levels of the blood monocyte-derived peritoneal macrophage population and tissue-resident peritoneal macrophage population display a diurnal rhythm with levels being highest during the mouse active phase (**Figure 6.2**). I also confirm that WT mice are more susceptible to LPS-induced endotoxemia when LPS is administered during the active phase. Further work is needed to elucidate the exact role *Rora* plays in the circadian oscillations of peritoneal macrophage populations and also if these oscillations can account for differential temporal responses to LPS.

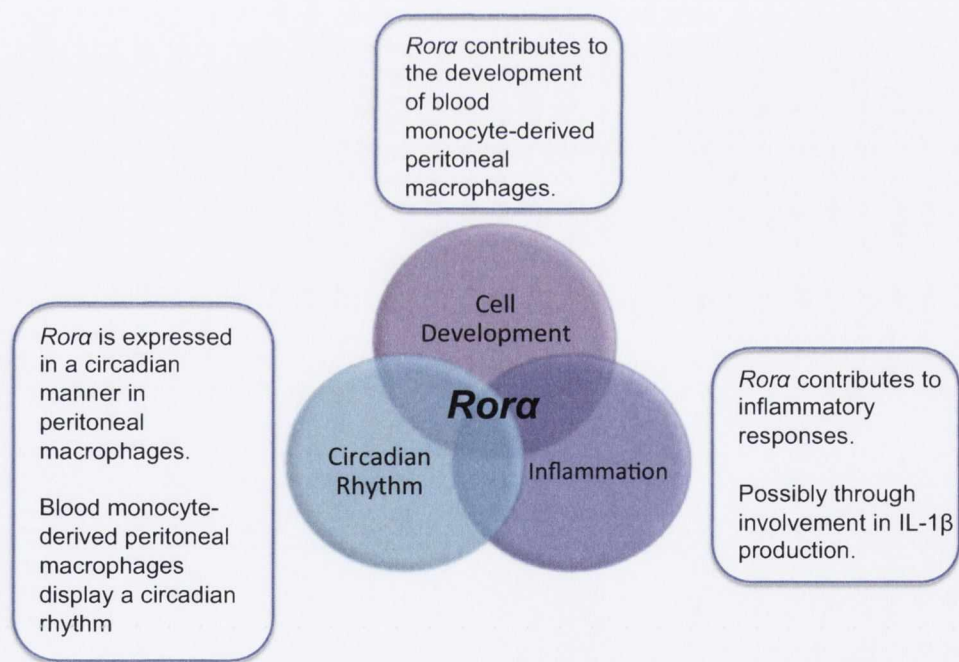


Figure 6.2 Summary of conclusions. *Rora* has a role in macrophage cell development and inflammatory responses.

Appendices

Appendix I

Genotyping protocols

Step	Temp (°C)	Time	Note
1	94	3'	
2	94	30"	
3	54	1'	
4	72	1'	Repeat steps 2-4 for 35 cycles
5	72	2'	
6	10	-	Hold

Conditions for running PCR reaction for genotyping for *Rora*^{sg/sg} mice. Time: ‘: Minute, “: Second.

Step	Temp (°C)	Time	Note
1	94	15"	
2	65	30"	Decrease 1C/cycle
3	72	40"	Go to 1, 10cycles
4	94	15"	
5	55	30"	
6	72	40"	Go to 4. 30 cycles

Conditions for running PCR reaction for genotyping for *Rora*^{fl/fl} mice. Time “: Seconds

Step	Temp (°C)	Time	Note
1	94	3'	
2	94	30 "	
3	63	1'	
4	72	1'	Repeat 2-4 35 Cycles
5	72	2'	
6	10	Hold	

Conditions for running PCR reaction for genotyping for *Rora*^{sg/sg} mice using custom designed primers. Time ‘: minutes, “: seconds

Step	Temp (°C)	Time	Note
1	94	3'	
2	94	30 "	
3	65	1'	
4	72	1'	Repeat 2-4 35 Cycles
5	72	2'	
6	10	Hold	

Conditions for running PCR reaction for genotyping for *Rora*^{fl/fl} mice using custom designed primers. Time ‘: minutes, “: seconds

Appendix II

Rora DNA sequence

WT Mice

CATACTGCAGAAATAAAAAGATGTATGCTCTTGTGCTCACACACAGACATGTG
CACACTTACATAACTTCCTGGCTTTTAAAGACCCACTTTTGTGCAGGACAGCACA
AAAAGCCAAGAAGAGGCTGTTTTTCATTTTTCTGCCTAACCCATGTTTCATCTC
CAGACACGGGCAGAAAGCCTATGACAAGGCCAAGGTCTT**CAAGTCTCTGAAT**
CCACCATACTTCCAGCAGTCTTGTTGGCTTTGCAAAGCCTCTCTCCTCCATCCC
CTGGGGCACAACACAGGCGGTTTCCATCTTCCTTAGCTATACAAAGGAAACC
AAATGTCAGCACATTGCTTTAAGTGAGTTGCTTTTTAAGGTGGATGACACCGG
CAGAAGAGTATTATTGGCTCCTAAAATAACGTGGCTGTTTCATGGTCGTGATCTC
CTGTTTTCTCCCCAGAACACCTTGCCCAGAACATATCCAAATCCCACCTGGAAA
CCTGCCAGTACTTGCGGGAAGAGCT**TCCAGCAGATAACGTGGCAGACCT**TCCT
GCAGGAGGAGATTGAAAACCTACCAGAACAAGGTAGAGTTGGAGGACTTTCTC
TTCCACCGAGGCAACATTGGGTGGGGGTGGGAGAAGGGCATTTCATTCAAGA
GTCCAGACAGACCAAGGCTGACTGTCCTGCATCAGAGCTCAAGTCTCTGATCA
GGATCTTACAGCCAGAGCTTTGTTGTCTTCCTGAGATGCTAGCGTGTGAAAGTT
CAGCTGGAACAAAGTTTATTGTGTTTCGTAA**GTCTTCCTTTGTGCCAGTTG**
GGATAACTGGATCGGGCAGCTTCCGAAAGCCTCCACGAGTGAGGATTTGTCCT
GATGCCACAGAAAAAGATTCTGAGGTCTAAGAATTAAGGAAAGTACTCTCAGC
C....

Rora^{fl/fl} mice

CACAGACATGTGCACACTTACATAACTTCCTGGCTTTTAAAGACCCACTTTTGT
CAGGACAGCACAAAAAGCCAAGAAGAGGCTGTTTTTCATTTTTCTGCCTAAC
CCATGTTTCATCTCCAGACACGGGCAGAAAGCCTATGACAAGGCCAAGGTCTT**C**
AAGTCTCTGAATCCACCATACTTTCCAGCAGTCTTGTTGGCTTTGCAAAGCCTC
TCTCCTCCATCCCCTGGGGCACAACACAGGCGGTTTCCATCTTCCTTAGCTAT
ACAAAGGAAACCAAATGTCAGCACATTGCTTTAAGTGAGTTGCTTTTTAAGGG
CCGCTCTAGAGGCCATAGCGGCCGGCC**ATAACTTCGTATAGCATAACATTATA**
CGAAGTTATGGCGCGCCGTGGATGACACCGGCAGAAAGAGTATTATTGGCTCC

TAAAATAACGTGGCTGTTTCATGGTCGTGATCTCCTGTTTTCTCCCCAGAACACC
TTGCCCAGAACATATCCAAATCCCACCTGGAAACCTGCCAGTACTTGCGGGAA
GAGCTCCAGC**AGATAACGTGGCAGACCT**TCCTGCAGGAGGAGATTGAAAACCT
ACCAGAACAAAGGTAGAGTTGGAGGACTTTCTCTTCCCACCGAGGCAACATTGG
GTGGGGGTGGGAGAAGGGCATCATTCAAGAGTCCAGACAGACGGCGCGCCCT
CGAGCTTTCGGAAGTTCCTATTCGGAAGTTCCTATTCTCTAGAAAGTATAGGAA
CTTCTCGAGATCCGATATCGAATTCCCGCGCCCCCAGCTGGTTCTTTCCGCCTC
AGAAGCCATAGAGCCCACCGCATCCCCAGCATGCCTGCTATTGTCTTCCCAAT
CCTCCCCCTTGCTGTCCTGCCCCACCCCACCCCCAGAATAGAATGACACCTACT
CAGACAATGCGATGCAATTTCTCATTTTATTAGGAAAGGACAGTGGGAGTGG
CACCTTCCAGGGTCAAGGAAGGCACGGGGGAGGGGCAAACAACAGATGGCTG
GCAACTAGAAGGCACAGTCGAGGCTGATCAGCGAGCTCTAGAGAATTGATCCC
CTCAGAAGAACTCGTCAAGAAGGCGATAGAAGGCGATGCGCTGCGAATCGGG
AGCGGCGATACCGTAAAGCACGAGGAAGCGGTCAGCCCATTTCGCCGCCAAGC
TCTTCAGCAATATCACGGGTAGCCAACGCTATGTCCTGATAGCGATCCGCCAC
ACCCAGCCGGCCACAGTCGATGAATCCAGAAAAGCGGCCATTTTCCACCATGA
TATTCGGCAAGCAGGCATCGCCATGGGTACGACGAGATCCTCGCCGTCGGGC
ATGCGCGCCTTGAGCCTGGCGAACAGTTCGGCTGGCGCGAGCCCCTGATGCTC
TTCGTCCAGATCATCCTGATCGACAAGACCGGCTTCCATCCGAGTACGTGCTCG
CTCGATGCGATGTTTCGCTTGGTGGTCGAATGGGCAGGTAGCCGGATCAAGCG
TATGCAGCCGCCGCATTGCATCAGCCATGATGGATACTTTCTCGGCAGGAGCA
AGGTGAGATGACAGGAGATCCTGCCCCGGCACTTCGCCCAATAGCAGCCAGTC
CCTTCCCGCTTCAGTGACAACGTCGAGCACAGCTGCGCAAGGAACGCCCCTCG
TGGCCAGCCACGATAGCCGCGCTGCCTCGTCCTGCAGTTCATTTCAGGGCACCG
GACAGGTCGGTCTTGACAAAAAGAACCGGGCGCCCCTGCGCTGACAGCCGGA
ACACGGCGGCATCAGAGCAGCCGATTGTCTGTTGTGCCCAGTCATAGCCGAAT
AGCCTCTCCCAAGGCGGCCGGAGAACCTGCGTGCAATCCATCTTGTTCAATGG
CCGATCCCATTATGACCTGCAGGTCGAAAGGCCCGGAGATGAGGAAGAGGAG
AACAGCGCGGCAGACGTGCGCTTTTGAAGCGTGCAGAATGCCGGGCTCCGGA
GGACCTTCGCGCCCCGCCCCGCCCCTGAGCCCGCCCCTGAGCCCGCCCCCGGAC
CCACCCCTTCCCAGCCTCTGAGCCCAGAAAGCGAAGGAGCCAAGCTGCTATTG
GCCGCTGCCCCAAAGGCCTACCCGCTTCCATTGCTCAGCGGTGCTGTCCATCTG
CACGAGACTAGTGAGACGTGCTACTTCCATTTGTACGTCCTGCACGACGCGA
GCTGCGGGGCGGGGGGGAACTTCCTGACTAGGGGAGGAGTAGAAGGTGGCGC
GAAGGGGCCACCAAAGAAGGGAGCCGGTTGGCGCTACCGGTGGATGTGGAAT

GTGTGCGAGGCCAGAGGCCACTTGTGTAGCGCCAAGTGCCAGCGGGGCTGCTA
AAGCGCATGCTCCAGACTGCCTTGGGAAAAGCGCCTCCCCTACCCGGTAGGGC
GCGGGAATTCGATATCGAATTCGAGCTCGGTACCCGGGGATCGAAGTTCCTAT
TCGGAAGTTCCTATTCTCTAGAAAGTATAGGAACCTTCTCGACCTGCAGGCATG
CAAGCTGATCCGGCGCGT**ATAACTTCGTATAGCATAACATTATA**CGAAGTTAT
CCTCAGGCCAGCGAGGCCGGTACCCAATTGCCCCTATAGCAAGGCTGACTGTC
CTGCATCAGAGCTCAAGTCTCTGATCAGGATCTTACAGCCAGAGCTTTGTTGTC
TTCCTGAGATGCTAGCGTGTGAAAGTTCAGCTGGAACAAAGTTTATTGTGTTTC
GTAAG**GTCCTTCCTTTGTGCCAGTT**GGGATAACTGGATCGGGCAGCTTCCGAA
AGCCTCCACGAGTGAGGATTTGTCCTGATGCCACAGAAAAAGATTCTGAGGTC
TAAGAATTAAGGAAAGTACTCTCAGCCCCATGCTACATGCTTCTAGATTAACA
AATAGTTGGCTAGAGATTGTGGGCAAAGGTGGGGCTGGTGAGATAGATGGTTC
ACTGGATAAGAGCACTTGCTGCTCTTCC.....

Nucleotide sequences in red represent the sequences recognised by primers LoxP5' and LoxP3'. Nucleotide sequences that are highlighted in grey represent sequences recognised by 30 and 31 primers. Nucleotide sequences that are highlighted in yellow represent LoxP sites. Nucleotide sequences underlined represent an 8-bp core sequence where recombination takes place,

Appendix III

All reagents were obtained from Sigma unless otherwise stated.

Phosphate Buffered Saline (PBS; 10X)

85g Sodium Chloride (NaCl)

12.8g Di-sodium hydrogen orthophosphate (Na_2HPO_4)

1.56g Sodium dihydrogen phosphate (Na_2PO_4)

Add to 1L distilled water (dH_2O) and adjusted to pH 7.6 for 10X solution.

Diluting to 1L of the 10X PBS in 9L dH_2O and adjusting to pH 7.6 prepared a 1X solution.

Tris-borate EDTA (TBE, 10X)

107.8g Tris base

50g Boric acid

7.44g EDTA

Dissolve in 1L dH_2O and adjust to pH 8.3

A 1X solution was prepared by diluting 1L of the 10X TBE in 9L dH_2O

Culture media

RPMI-1640 media (Gibco Invitrogen)

2mM L-Glutamine

10% Fetal Bovine Serum (FBS, Biowest, France)

100 U/ml Penicillin-streptomycin (Gibco Invitrogen)

Protein Extraction

Radio-Immunoprecipitation Assay (RIPA) buffer

10 mM Tris-Cl (pH 8.0)

1 mM EDTA

0.5 mM EGTA
1% Triton X-100
0.1% sodium deoxycholate
0.1% SDS
140 mM NaCl

Loading Buffer

200 mM Tris-Cl (pH 6.8)
400 mM DTT
8% SDS
0.4% bromophenol
40% glycerol

SDS-PAGE

Running buffer (10X)

30.0 g Tris base
144.0 g glycine
10.0 g SDS

For 10X solution dissolve in 1000 ml of dH₂O. For 1X solution add 100ml of 10X solution to 900ml dH₂O.

The pH of the buffer should be 8.3

Transfer buffer (1X)

3.0 g Tris base
14.0 g glycine
200ml Methanol

Adjust volume to 1L with dH₂O. The pH will vary between 8.1 and 8.5

PBS-Tween

100 ml PBS (10X)

900 ml dH₂O

2.5 ml Tween 20.

Flow Cytometry

500 ml PBS (Gibco, Invitrogen)

10 ml FBS

250 µl Sodium Azide (NaN₃)

Appendix IV

Antibodies

Western blot primary antibodies-

Primary Antibody	Clone	Species	Company
Rora (C-16)	Polyclonal	Goat	Santa Cruz Biotechnology, TX, US,
Actin (I-19)	Polyclonal	Goat	Santa Cruz Biotechnology
P38 MAPK	Polyclonal	Rabbit	Cell Signalling Technologies
Phospho-p38 MAPK (Thr180/Tyr182)(12F8)	Polyclonal	Rabbit	Cell Signalling Technologies
SAPK/JNK	Polyclonal	Rabbit	Cell Signalling Technologies
Phospho-SAPK/JNK (Thr183/Tyr185)(81E11)	Polyclonal	Rabbit	Cell Signalling Technologies

Western blot secondary antibodies-

Secondary Antibody	Clonal	Species	Company
Anti-Rabbit Immunoglobulins/HRP	Polyclonal	Goat	Dako
Anti-Goat IgG/HRP	Polyclonal	Donkey	Santa Cruz Biotechnology

Antibody	Fluorochrome	Clone	Company
CCR2	APC	475301	R&D
CD3	APC	17A2	eBioscience
CD4	Percp-Cy5.5	RM4-5	BD Bioscience
CD4	APC	RM4-5	BD Bioscience
CD4	PE	RM4-5	BD Bioscience
CD11b	Percp-Cy5.5	M1/70	eBioscience
CD11b	PeCy7	M1/70	eBioscience
CD11b	APC	M1/70	BD Bioscience
CD11c	FITC	N418	Miltenyi Biotec
CD11c	PeCy7	N418	eBioscience
CD11c	APC	HL3	BD Bioscience
CD19	Percp-Cy5.5	1D3	BD Bioscience
CD19	APC	1D3	BD Bioscience
CD45	PerCP	30F11.1	Miltenyi Biotec
CD45.1	FITC	A20	BD Bioscience
CD45.1	PE	A20	BD Bioscience
CD45.2	eFlour450	104	eBioscience
CD45R (B220)	APC-Vio770	RA3-6B2	Miltenyi Biotec
CD62L	eFlour450	MEL-14	eBioscience
CD64	APC	X54-5/7.1	Biolegend
CD80	PE-CF594	16-10A1	BD Bioscience
CD86	BV4510	GL-1	BD Horizon
CD103	FITC	M290	BD Bioscience
CD103	BV21	M290	BD Bioscience
CD115 (CSF-1R)	PE	AF598	Biolegend
CD117 (c-Kit)	APC	2B8	BD Bioscience
CD117 (c-Kit)	PerCP-eFluor710	2B8	eBioscience
CD127 (IL-7R α)	APC-eFluor780	A7R34	eBioscience
CD135	Brilliant Violet 421	A2F10	Biolegend
CD206	Pe/Cy7	C068C2	Biolegend
CD206	APC	C068C2	Biolegend

F4/80	FITC	BM8	eBioscience
F4/80	APC	BM8	eBioscience
F4/80	eFlour450	BM8	eBioscience
Gr-1	APC	RB6-8C5	BD Bioscience
Gr-1	APC-Cy7	RB6-8C5	BD Bioscience
KLRG1	BV421	2F1	BD Bioscience
ICOS	PE	7E.17G9	BD Bioscience
Ly6C	FITC	HK1.4	Biolegend
Ly6G	APC-Cy7	1A8	Biolegend
MHCII	FITC	NIMR4	eBioscience
MHCII	eFluor450	M5/144.15.2	eBioscience
MHCII	APC-eFlour780	M5/144.15.2	eBioscience
PD-L1	PE	MIH5	eBioscience
PD-L1	APC	10F.96-2	Biolegend
PD-L2	PE	122	eBioscience
PD-L2	FITC	122	eBioscience
PD-L2	APC	MIH37	Miltenyi Biotech
Sca-1 / Ly-6G	Pe/Cy7	D7	BD Bioscience
Siglec F	PE	E50-2440	BD Bioscience
Siglec F	Alexa fluor 647	E50-2440	BD Bioscience
ST2	FITC	DJ8	MD Bioproducts
TIM-4	PE	F31-5G3	Biolegend
TLR4	APC	SA15-21	Biolegend

Appendix V

qRT-PCR Primers

All assays were purchased from Applied Biosystems (Paisley, UK).

Murine gene	Assay code
iNos	(Mm00440485_m1)
Arginase-1	(Mm00475988_m1)
Ym1	(Mm00657889_m1)
Relm α	(Mm00445109_m1)
Arntl	(Mm00500226_m1)
Ror α	(Mm00443103_m1)

References

- A-Gonzalez, N., Guillen, J.A., Gallardo, G., Diaz, M., de la Rosa, J. V, Hernandez, I.H., Casanova-Acebes, M., Lopez, F., Tabraue, C., Beceiro, S., Hong, C., Lara, P.C., Andujar, M., Arai, S., Miyazaki, T., Li, S., Corbi, A.L., Tontonoz, P., Hidalgo, A., et al. (2013). The nuclear receptor LXR α controls the functional specialization of splenic macrophages. *Nat. Immunol.* *14*, 831–839.
- Akagi, T., Saitoh, T., O’Kelly, J., Akira, S., Gombart, A.F., and Koeffler, H.P. (2008). Impaired response to GM-CSF and G-CSF, and enhanced apoptosis in C/EBP 2-deficient hematopoietic cells. *Blood* *111*, 2999–3004.
- Albacker, L.A., Karisola, P., Chang, Y.-J., Umetsu, S.E., Zhou, M., Akbari, O., Kobayashi, N., Baumgarth, N., Freeman, G.J., Umetsu, D.T., and DeKruyff, R.H. (2010). TIM-4, a receptor for phosphatidylserine, controls adaptive immunity by regulating the removal of antigen-specific T cells. *J. Immunol.* *185*, 6839–6849.
- Amin, K. (2012). The role of mast cells in allergic inflammation. *Respir. Med.* *106*, 9–14.
- Auffray, C., Fogg, D., Garfa, M., Elain, G., Olivier, J.L., Kayal, S., Sarnacki, S., Cumano, A., Lauvau, G., and Geissmann, F. (2007). Monitoring of blood vessels and tissues by a population of monocytes with patrolling behaviour. *Science* *317*, 666–671.
- Aviello, G., Corr, S.C., Johnston, D.G.W., O’Neill, L.A., and Fallon, P.G. (2014). MyD88 adaptor-like (Mal) regulates intestinal homeostasis and colitis-associated colorectal cancer in mice. *Am. J. Physiol. Gastrointest. Liver Physiol.* *306*, G769–G778.
- Back, J., Dierich, A., Bronn, C., Kastner, P., and Chan, S. (2004). PU.1 determines the self-renewal capacity of erythroid progenitor cells. *Blood* *103*, 3615–3623.
- Bardoel, B.W., Kenny, E.F., Sollberger, G., and Zychlinsky, A. (2014). The balancing act of neutrophils. *Cell Host Microbe* *15*, 526–536.
- Beaven, M. (2010). Our perception of the mast cell from Paul Ehrlich to now. *Eur. J. Immunol.* *39*, 11–25.
- Bedi, R., Du, J., Sharma, A.K., Gomes, I., and Ackerman, S.J. (2009). Human C/EBP- ϵ activator and repressor isoforms differentially reprogram myeloid lineage commitment and differentiation. *Blood* *113*, 317–327.
- Belkaid, Y., and Segre, J.A. (2014). Dialogue between skin microbiota and immunity. *Science* *346*, 954–960.
- Bellet, M.M., Deriu, E., Liu, J.Z., Grimaldi, B., Blaschitz, C., Zeller, M., Edwards, R.A., Sahar, S., Dandekar, S., Baldi, P., George, M.D., Raffatellu, M., and Sassone-Corsi, P. (2013). Circadian clock regulates the host response to Salmonella. *Proc. Natl. Acad. Sci. U. S. A.* *110*, 9897–9902.
- Bertrand, J.Y., Chi, N.C., Santoso, B., Teng, S., Stainier, D.Y.R., and Traver, D. (2010). Haematopoietic stem cells derive directly from aortic endothelium during development. *Nature* *464*, 108–111.
- Blander, J.M., and Sander, L.E. (2012). Beyond pattern recognition: five immune

checkpoints for scaling the microbial threat. *Nat. Rev. Immunol.* 12, 215–225.

Bluestone, J.A., Mackay, C.R., O'Shea, J.J., and Stockinger, B. (2009). The functional plasticity of T cell subsets. *Nat. Rev. Immunol.* 9, 811–816.

Bode, J.G., Ehrling, C., and Häussinger, D. (2012). The macrophage response towards LPS and its control through the p38 MAPK-STAT3 axis. *Cell. Signal.* 24, 1185–1194.

Boehm, U., Klamp, T., Groot, M., and Howard, J.C. (1997). Cellular responses to interferon-gamma. *Annu. Rev. Immunol.* 15, 749–795.

Bogdan, C. (2015). Nitric oxide synthase in innate and adaptive immunity: an update. *Trends Immunol.* 36, 161–178.

Boivin, D.B., James, F.O., Wu, A., Cho-park, P.F., Xiong, H., and Sun, Z.S. (2003). Circadian clock genes oscillate in human peripheral blood mononuclear cells. *Blood* 102, 4143–4145.

Bollinger, T., Leutz, A., Leliavski, A., Skrum, L., Kovac, J., Bonacina, L., Benedict, C., Lange, T., Westermann, J., Oster, H., and Solbach, W. (2011). Circadian clocks in mouse and human CD4⁺ T cells. *PLoS One* 6, e29801.

Bonecchi, R., Bianchi, G., Bordignon, P.P., D'Ambrosio, D., Lang, R., Borsatti, A., Sozzani, S., Allavena, P., Gray, P.A., Mantovani, A., and Sinigaglia, F. (1998). Differential expression of chemokine receptors and chemotactic responsiveness of type 1 T helper cells (Th1s) and Th2s. *J. Exp. Med.* 187, 129–134.

Brack, C., Hirama, M., Lenhard-Schuller, R., and Tonegawa, S. (1978). A complete immunoglobulin gene is created by somatic recombination. *Cell* 15, 1–14.

Brenneman, K.E., Willingham, C., Kilbourne, J.A., Curtiss, R., and Roland, K.L. (2014). A low gastric pH mouse model to evaluate live attenuated bacterial vaccines. *PLoS One* 9.

Brinkmann, V., and Zychlinsky, A. (2012). Neutrophil extracellular traps: Is immunity the second function of chromatin? *J. Cell Biol.* 198, 773–783.

Broadhurst, M.J., Leung, J.M., Lim, K.C., Girgis, N.M., Gundra, U.M., Fallon, P.G., Premenko-Lanier, M., McKerrow, J.H., McCune, J.M., and Loke, P. (2012). Upregulation of retinal dehydrogenase 2 in alternatively activated macrophages during retinoid-dependent type-2 immunity to helminth infection in mice. *PLoS Pathog.* 8, e1002883.

Bunnell, T.M., Burbach, B.J., Shimizu, Y., and Ervasti, J.M. (2011). β -Actin specifically controls cell growth, migration, and the G-actin pool. *Mol. Biol. Cell* 22, 4047–4058.

Buras, J.A., Holzmann, B., and Sitkovsky, M. (2005). Animal models of sepsis: setting the stage. *Nat. Rev. Drug Discov.* 4, 854–865.

Cain, D.W., O'Koren, E.G., Kan, M.J., Womble, M., Sempowski, G.D., Hopper, K., Gunn, M.D., and Kelsoe, G. (2013). Identification of a tissue-specific, C/EBP β -dependent pathway of differentiation for murine peritoneal macrophages. *J. Immunol.* 191, 4665–4675.

- Camberis, M., Le Gros, G., and Urban, J. (2003). Animal model of *Nippostrongylus brasiliensis* and *Heligmosomoides polygyrus*. *Curr. Protoc. Immunol. Chapter 19*, Unit 19.12.
- Car, B.D., Eng, V.M., Schnyder, B., Ozmen, L., Huang, S., Gallay, P., Heumann, D., Aguet, M., and Ryffel, B. (1994). Interferon gamma receptor deficient mice are resistant to endotoxic shock. *J. Exp. Med.* 179, 1437–1444.
- Carlin, L.M., Stamatiades, E.G., Auffray, C., Hanna, R.N., Glover, L., Vizcay-Barrena, G., Hedrick, C.C., Cook, H.T., Diebold, S., and Geissmann, F. (2013). Nr4a1-dependent Ly6Clow monocytes monitor endothelial cells and orchestrate their disposal. *Cell* 153, 362–375.
- Cassado, A.D.A., D'Império Lima, M.R., and Bortoluci, K.R. (2015). Revisiting mouse peritoneal macrophages: Heterogeneity, development, and function. *Front. Immunol.* 6, 1–9.
- Castanon-Cervantes, O., Wu, M., Ehlen, J.C., Paul, K., Gamble, K.L., Johnson, R.L., Besing, R.C., Menaker, M., Gewirtz, A.T., and Davidson, A.J. (2010). Dysregulation of inflammatory responses by chronic circadian disruption. *J. Immunol.* 185, 5796–5805.
- Celada, A., Gray, P., Rinderknecht, E., and Schreiber, R. (1984). Evidence for a gamma-interferon receptor that regulates macrophage tumoricidal activity. *J. Exp. Med.* 160, 55–74.
- Celia, B.M., Scheidegger, D., Palmer-lehmann, K., Lane, P., Lanzavecchia, A., and Alber, G. (1996). Interleukin (IL)-6 gene expression in the central nervous system is necessary for fever response to lipopolysaccharide or IL-1 β : a study on IL-6-deficient mice. *J. Exp. Med.* 184, 0–5.
- Chang, L.W., Payton, J.E., Yuan, W., Ley, T.J., Nagarajan, R., and Stormo, G.D. (2008). Computational identification of the normal and perturbed genetic networks involved in myeloid differentiation and acute promyelocytic leukemia. *Genome Biol.* 9, R38.
- Chen, M.J., Yokomizo, T., Zeigler, B.M., Dzierzak, E., and Speck, N.A. (2009). Runx1 is required for the endothelial to haematopoietic cell transition but not thereafter. *Nature* 457, 887–891.
- Chuang, T.-Y., Chang, H.-T., Chung, K.-P., Cheng, H.-S., Liu, C.-Y., Liu, Y.-C., Huang, H.-H., Chou, T.-C., Chang, B.-L., Lee, M.-R., Lin, C.-J., Lee, S.-W., Yu, C.-J., and Hsueh, P.-R. (2014). High levels of serum macrophage migration inhibitory factor and interleukin 10 are associated with a rapidly fatal outcome in patients with severe sepsis. *Int. J. Infect. Dis.* 20, 13–17.
- Clausen, B.E., Burkhardt, C., Reith, W., Renkawitz, R., and Förster, I. (1999). Conditional gene targeting in macrophages and granulocytes using LysMcre mice. *Transgenic Res.* 8, 265–277.
- Cooper, M.D., Peterson, R.D.A., and Good, R.A. (1965). Delineation of the thymic and bursal lymphoid systems in the chicken. *Nature* 205, 143–146.
- Cross, M., Mangelsdorf, I., Wedel, A., and Renkawitz, R. (1988). Mouse lysozyme M

- gene: isolation, characterization, and expression studies. *Proc. Natl. Acad. Sci. U. S. A.* 85, 6232–6236.
- Croxford, A.L., Lanzinger, M., Hartmann, F.J., Jung, S., Greter, M., and Becher, B. (2015). The Cytokine GM-CSF Drives the Inflammatory Signature Article The Cytokine GM-CSF Drives the Inflammatory Signature of CCR2 + Monocytes and Licenses Autoimmunity. *Immunity* 43, 502–514.
- Cuenda, A., and Rousseau, S. (2007). p38 MAP-kinases pathway regulation, function and role in human diseases. *Biochim. Biophys. Acta* 1773, 1358–1375.
- Curtis, A.M., Bellet, M.M., Sassone-Corsi, P., and O'Neill, L.A.J. (2014). Circadian clock proteins and immunity. *Immunity* 40, 178–186.
- Curtis, A.M., Fagundes, C.T., Yang, G., Palsson-McDermott, E.M., Wochal, P., McGettrick, A.F., Foley, N.H., Early, J.O., Chen, L., Zhang, H., Xue, C., Geiger, S.S., Hokamp, K., Reilly, M.P., Coogan, A.N., Vigorito, E., FitzGerald, G. a., and O'Neill, L.A.J. (2015). Circadian control of innate immunity in macrophages by miR-155 targeting Bmal1. *PNAS* 112, 7231–7236.
- Davies, L.C., and Taylor, P.R. (2015). Tissue resident macrophages: Then and now. *Immunology* 144, 541–548.
- Davies, L.C., Jenkins, S.J., Allen, J.E., and Taylor, P.R. (2013). Tissue-resident macrophages. *Nat. Immunol.* 14, 986–995.
- Delerive, P., Monté, D., Dubois, G., Trottein, F., Fruchart-Najib, J., Mariani, J., Fruchart, J.C., and Staels, B. (2001). The orphan nuclear receptor ROR alpha is a negative regulator of the inflammatory response. *EMBO Rep.* 2, 42–48.
- Dey, A., Allen, J., and Hankey-Giblin, P.A. (2015). Ontogeny and polarization of macrophages in inflammation: Blood monocytes versus tissue macrophages. *Front. Immunol.* 5, 1–15.
- Doi, K., Leelahavanichkul, A., Yuen, P.S.T., and Star, R.A. (2009). Science in medicine Animal models of sepsis and sepsis-induced kidney injury. *J. Clin. Invest.* 119, 2868–2878.
- Dussault, I., Fawcett, D., Matthyssen, A., Bader, J.A., and Giguère, V. (1998). Orphan nuclear receptor ROR alpha-deficient mice display the cerebellar defects of staggerer. *Mech. Dev.* 70, 147–153.
- Dzhagalov, I., Giguère, V., and He, Y.-W. (2004). Lymphocyte development and function in the absence of retinoic acid-related orphan receptor alpha. *J. Immunol.* 173, 2952–2959.
- Falk, A. (1988). Comparison of bone marrow progenitors Responsive to Granulocyte-Macrophage Colony Stimulating Factor and Macrophage Colony Stimulating Factor-1. *J. Leukoc. Biol.* 43, 148–157.
- Fallon, P.G., Jolin, H.E., Smith, P., Emson, C.L., Townsend, M.J., Fallon, R., Smith, P., and McKenzie, A.N.J. (2002). IL-4 induces characteristic Th2 responses even in the combined absence of IL-5, IL-9, and IL-13. *Immunity* 17, 7–17.

- Fallon, P.G., Ballantyne, S.J., Mangan, N.E., Barlow, J.L., Dasvarma, A., Hewett, D.R., McIlgorm, A., Jolin, H.E., and McKenzie, A.N.J. (2006). Identification of an interleukin (IL)-25-dependent cell population that provides IL-4, IL-5, and IL-13 at the onset of helminth expulsion. *J. Exp. Med.* *203*, 1105–1116.
- Fitzsimmons, R.L., Lau, P., and Muscat, G.E.O. (2012). Retinoid-related orphan receptor alpha and the regulation of lipid homeostasis. *J. Steroid Biochem. Mol. Biol.* *130*, 159–168.
- Fjell, R.E.W., Hancock, O.M., Pena, J., Pistolic, D., Raj, C.D., Pena, O.M., Pistolic, J., Raj, D., Fjell, C.D., and Hancock, R.E.W. (2015). Human Mononuclear Cells State of Alternative Polarization (M2) in Endotoxin Tolerance Represents a Distinctive Endotoxin Tolerance Represents a Distinctive State of Alternative Polarization (M2) in Human Mononuclear Cells. *Southampt. Univ Heal. Serv. Libr.* *186*, 7243–7254.
- Flajnik, M.F., and Kasahara, M. (2010). Origin and evolution of the adaptive immune system: genetic events and selective pressures. *Nat. Rev. Genet.* *11*, 47–59.
- Fleetwood, A.J., Lawrence, T., Hamilton, J.A., and Cook, A.D. (2007). Granulocyte-macrophage colony-stimulating factor (CSF) and macrophage CSF-dependent macrophage phenotypes display differences in cytokine profiles and transcription factor activities: implications for CSF blockade in inflammation. *J. Immunol.* *178*, 5245–5252.
- Fleetwood, A.J., Dinh, H., Cook, A.D., Hertzog, P.J., and Hamilton, J.A. (2009). GM-CSF- and M-CSF-dependent macrophage phenotypes display differential dependence on type I interferon signaling. *J. Leukoc. Biol.* *86*, 411–421.
- Foster, S.L., Hargreaves, D.C., and Medzhitov, R. (2007). Gene-specific control of inflammation by TLR-induced chromatin modifications. *Nature* *447*, 972–978.
- Fulkerson, P.C., and Rothenberg, M.E. (2013). Targeting eosinophils in allergy, inflammation and beyond. *Nat. Rev. Drug Discov.* *12*, 117–129.
- Van Furth, R., and Cohn, Z.A. (1968). The origin and kinetics of mononuclear phagocytes. *J. Exp. Med.* *128*, 415–435.
- Gallily, R., Warwick, A., and Bang, F. (1964). Effect of cortisone on genetic resistance to mouse hepatitis virus in vivo and in vitro. *PNAS* *1564*, 1158–1164.
- Galvan-Pena, S., and Neill, L.A.J. (2014). Metabolic reprogramming in macrophage polarization. *Front. Immunol.* *5*, 420.
- Garcia, J., Volt, H., Venegas, C., Carolina, D., Escames, G., Lopez, L., and Acuna-Castroviejo, D. (2015). Disruption of the NF- κ B/NLRP3 connection by melatonin requires retinoid-related orphan receptor- α and blocks the septic response in mice. *FASEB J.* *29*.
- Gautier, E.L., Shay, T., Miller, J., Greter, M., Jakubzick, C., Ivanov, S., Helft, J., Chow, A., Elpek, K.G., Gordonov, S., Mazloom, A.R., Ma'ayan, A., Chua, W.-J., Hansen, T.H., Turley, S.J., Merad, M., Randolph, G.J., Gautier, E.L., Jakubzick, C., et al. (2012). Gene-expression profiles and transcriptional regulatory pathways that underlie the identity and diversity of mouse tissue macrophages. *Nat. Immunol.* *13*, 1118–1128.

- Gautier, E.L., Ivanov, S., Williams, J.W., Huang, S.C.-C., Marcelin, G., Fairfax, K., Wang, P.L., Francis, J.S., Leone, P., Wilson, D.B., Artyomov, M.N., Pearce, E.J., and Randolph, G.J. (2014). Gata6 regulates aspartoacylase expression in resident peritoneal macrophages and controls their survival. *J. Exp. Med.* *211*, 1525–1531.
- Geissmann, F., Jung, S., and Littman, D.R. (2003). Blood monocytes consist of two principal subsets with distinct migratory properties. *Immunity* *19*, 71–82.
- Ghosh, K., and Duyne, G. Van (2002). Cre–loxP biochemistry. *Methods* *28*, 374–383.
- Ghosn, E.E.B., Cassado, A.A., Govoni, G.R., Fukuhara, T., Yang, Y., Monack, D.M., Bortoluci, K.R., Almeida, S.R., Herzenberg, L.A., and Herzenberg, L.A. (2010). Two physically, functionally, and developmentally distinct peritoneal macrophage subsets. *Proc. Natl. Acad. Sci. U. S. A.* *107*, 2568–2573.
- Gibbs, J.E., Blaikley, J., Beesley, S., Matthews, L., Simpson, K.D., Boyce, S.H., Farrow, S.N., Else, K.J., Singh, D., Ray, D.W., and Loudon, A.S.I. (2012). The nuclear receptor REV-ERB α mediates circadian regulation of innate immunity through selective regulation of inflammatory cytokines. *PNAS* *102*, 2–7.
- Giguere, V., Tini, M., Flock, G., Ong, E., Evans, R.M., and Otulakowski, G. (1994). Isoform-specific amino-terminal domains dictate DNA-binding properties of ROR α , a novel family of orphan hormone nuclear receptors. *Genes Dev.* *8*, 538–553.
- Ginhoux, F., and Jung, S. (2014). Monocytes and macrophages: developmental pathways and tissue homeostasis. *Nat. Rev. Immunol.* *14*, 392–404.
- Ginhoux, F., Schultze, J.L., Murray, P.J., Ochando, J., and Biswas, S.K. (2015). New insights into the multidimensional concept of macrophage ontogeny, activation and function. *Nat. Immunol.* *17*, 34–40.
- Girgis, N.M., Gundra, U.M., Ward, L.N., Cabrera, M., Frevert, U., and Loke, P. (2014). Ly6Chigh monocytes become alternatively activated macrophages in schistosome granulomas with help from CD4⁺ cells. *PLoS Pathog.* *10*, e1004080.
- Gold, D.A., Gent, P.M., and Hamilton, B.A. (2007). ROR α in genetic control of cerebellum development: 50 staggering years. *Brain Res.* *1140*, 19–25.
- Gomez Perdiguero, E., Klapproth, K., Schulz, C., Busch, K., Azzoni, E., Crozet, L., Garner, H., Trouillet, C., de Bruijn, M.F., Geissmann, F., and Rodewald, H.-R. (2014). Tissue-resident macrophages originate from yolk-sac-derived erythro-myeloid progenitors. *Nature* *518*, 547–551.
- Gordon, S., and Taylor, P.R. (2005). Monocyte and macrophage heterogeneity. *Nat. Rev. Immunol.* *5*, 953–964.
- Gordon, S., Hamann, J., Lin, H.H., and Stacey, M. (2011). F4/80 and the related adhesion-GPCRs. *Eur. J. Immunol.* *41*, 2472–2476.
- Griseri, T., Arnold, I.C., Pearson, C., Krausgruber, T., Schiering, C., Franchini, F., Schulthess, J., McKenzie, B.S., Crocker, P.R., and Powrie, F. (2015). Granulocyte

macrophage colony-stimulating factor-activated eosinophils promote Interleukin-23 driven chronic colitis. *Immunity* 43, 187–199.

Guilliams, M., Kleer, I. De, Henri, S., Post, S., Vanhoutte, L., Prijck, S. De, Deswarte, K., Malissen, B., Hammad, H., and Lambrecht, B.N. (2013). Alveolar macrophages develop from fetal monocytes that differentiate into long-lived cells in the first week of life via GM-CSF. *J. Exp. Med.* 210, 1977–1992.

Gundra, U.M., Girgis, N.M., Ruckerl, D., Jenkins, S., Ward, L.N., Kurtz, Z.D., Wiens, K.E., Tang, M.S., Basu-Roy, U., Mansukhani, A., Allen, J.E., and Loke, P. (2014). Alternatively activated macrophages derived from monocytes and tissue macrophages are phenotypically and functionally distinct. *Blood* 123, e110–e122.

Hambleton, J., Weinstein, S.L., Lemt, L., Defrancots, A.L., and Bishop, J.M. (1996). Activation of c-Jun N-terminal kinase in bacterial lipopolysaccharide-stimulated macrophages. *PNAS* 93, 2774–2778.

Hamilton, B., Frankel, W., Kerrebrock, A., Hawkins, T., FitzHugh, W., Kusumi, K., Russell, L., Mueller, K., van Berkel, V., Birren, B., Kruglyak, L., and Lander, E. (1996). Disruption of the nuclear hormone receptor ROR α in staggerer mice. *Nature* 379, 736–739.

Hamilton, J.A., Stanley, E.R., Burgess, A.W., and Shadduck, R.K. (1980). Stimulation of macrophage plasminogen activator activity by colony-stimulating factors. *J. Cell. Physiol.* 103, 435–445.

Hams, E., and Fallon, P.G. (2012). Innate type 2 cells and asthma. *Curr. Opin. Pharmacol.* 12, 503–509.

Hams, E., Saunders, S.P., Cummins, E.P., Connor, A.O., Murtaza, T., Gallagher, W.M., Byrne, A., Campos-torres, A., Paul, M., Jobin, C., Taylor, C.T., and Fallon, P.G. (2011). The hydroxylase inhibitor DMOG attenuates endotoxin shock alternative activation and IL-10 production by B-1 cells. *Shock* 36, 295–302.

Hams, E., Bermingham, R., and Fallon, P. (2015a). Macrophage and innate lymphoid cell interplay in the genesis of fibrosis. *Front. Immunol.* 6, 1–11.

Hams, E., Bermingham, R., Wurlod, F.A., Hogan, A.E., O'Shea, D., Preston, R.J., Rodewald, H.-R., McKenzie, A.N.J., and Fallon, P.G. (2015b). The helminth T2 RNase 1 promotes metabolic homeostasis in an IL-33 and group 2 innate lymphoid cell-dependent mechanism. *FASEB J.* 152–160.

Hanna, R.N., Carlin, L.M., Hubbeling, H.G., Nackiewicz, D., Green, A.M., Punt, J.A., Geissmann, F., and Hedrick, C.C. (2011). The transcription factor NR4A1 (Nur77) controls bone marrow differentiation and the survival of Ly6C⁺ monocytes. *Nat. Immunol.* 12, 778–785.

Hasserjian, R.P. (2013). Acute myeloid leukemia: Advances in diagnosis and classification. *Int. J. Lab. Hematol.* 35, 358–366.

Hayashi, M., Shimba, S., and Tezuka, M. (2007). Characterization of the molecular clock in mouse peritoneal macrophages. *Biol. Pharm. Bull.* 30, 621–626.

- Heinzel, F.P. (1990). The role of IFN-gamma in the pathology of experimental endotoxemia. *J Immunol* 145, 2920–2924.
- Herbert, D.R., Hölscher, C., Mohrs, M., Arendse, B., Schwegmann, A., Radwanska, M., Leeto, M., Kirsch, R., Hall, P., Mossmann, H., Claussen, B., Förster, I., and Brombacher, F. (2004). Alternative macrophage activation is essential for survival during Schistosomiasis and downmodulates T Helper 1 responses and immunopathology. *Immunity* 20, 623–635.
- Hettinger, J., Richards, D.M., Hansson, J., Barra, M.M., Joschko, A.-C., Krijgsveld, J., and Feuerer, M. (2013). Origin of monocytes and macrophages in a committed progenitor. *Nat. Immunol.* 14, 821–830.
- Hoeffel, G., Chen, J., Lavin, Y., Low, D., Almeida, F.F., See, P., Beaudin, A.E., Lum, J., Low, I., Forsberg, E.C., Poidinger, M., Zolezzi, F., Larbi, A., Ng, L.G., Chan, J.K.Y., Greter, M., Becher, B., Samokhvalov, I.M., Merad, M., et al. (2015). C-Myb⁺ Erythro-Myeloid Progenitor-Derived Fetal Monocytes Give Rise to Adult Tissue-Resident Macrophages. *Immunity* 42, 665–678.
- Hohaus, S., Petrovick, M.S., Voso, M.T., Sun, Z., Zhang, D.E., Hohaus, S., Petrovick, M.S., Voso, M.T., Sun, Z., Zhang, D., and Tenen, D.G. (1995). PU.1 (Spi-1) and C/EBP alpha regulate expression of the granulocyte-macrophage colony-stimulating factor receptor alpha gene. *Mol. Cell. Biol.* 1, 5830–5845.
- Hoover-Plow, J., Gong, Y., Shchurin, A., Busuttil, S., Schneeman, T., and Hart, E. (2008). Strain and model dependent differences in inflammatory cell recruitment in mice. *Inflamm Res* 57, 457–463.
- Hoshino, K., Takeuchi, O., Kawai, T., Sanjo, H., Ogawa, T., Takeda, Y., Takeda, K., and Akira, S. (1999). Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. *J. Immunol.* 162, 3749–3752.
- Hotchkiss, R.S., Monneret, G., and Payen, D. (2013). Sepsis-induced immunosuppression: from cellular dysfunctions to immunotherapy. *Nat. Rev. Immunol.* 13, 862–874.
- Huang, S.C.-C., Everts, B., Ivanova, Y., O’Sullivan, D., Nascimento, M., Smith, A.M., Beatty, W., Love-Gregory, L., Lam, W.Y., O’Neill, C.M., Yan, C., Du, H., Abumrad, N.A., Urban, J.F., Artyomov, M.N., Pearce, E.L., and Pearce, E.J. (2014). Cell-intrinsic lysosomal lipolysis is essential for alternative activation of macrophages. *Nat. Immunol.* 15, 1–12.
- Huang, Y., Guo, L., Qiu, J., Chen, X., Hu-Li, J., Siebenlist, U., Williamson, P.R., Urban, J.F., and Paul, W.E. (2015). IL-25-responsive, lineage-negative KLRG1(hi) cells are multipotential “inflammatory” type 2 innate lymphoid cells. *Nat. Immunol.* 16, 161–169.
- Humbles, A.A., Lu, B., Friend, D.S., Okinaga, S., Lora, J., Al-Garawi, A., Martin, T.R., Gerard, N.P., and Gerard, C. (2002). The murine CCR3 receptor regulates both the role of eosinophils and mast cells in allergen-induced airway inflammation and hyperresponsiveness. *Proc. Natl. Acad. Sci. U. S. A.* 99, 1479–1484.

- Inaba, K., Inaba, M., Romani, N., Aya, H., Deguchi, M., Ikehara, S., Muramatsu, S., and Steinman, R.M. (1992). Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J. Exp. Med.* 176, 1693–1702.
- Ingersoll, M., and Spanbroek, R. (2010). Comparison of gene expression profiles between human and mouse monocyte subsets. *Blood* 115, 10–20.
- Ino, H. (2004). Immunohistochemical characterization of the orphan nuclear receptor ROR alpha in the mouse nervous system. *J. Histochem. Cytochem.* 52, 311–323.
- Jackson, S.H., Alicea, C., Owens, J.W., Eigsti, C.L., and Malech, H.L. (2002). Characterization of an early dendritic cell precursor derived from murine lineage-negative hematopoietic progenitor cells. *Exp. Hematol.* 30, 430–439.
- Janeway, C.A. (1989). Approaching the asymptote? Evolution and revolution in immunology. *Cold Spring Harb. Symp. Quant. Biol.* 54, 1–13.
- Jenkins, S.J., Ruckerl, D., Cook, P.C., Jones, L.H., Finkelman, F.D., Rooijen, N. Van, Macdonald, A.S., and Allen, J.E. (2011). Local macrophage proliferation, rather than recruitment from the blood, is a signature of Th2 inflammation. *Science* 332, 1284–1288.
- Jung, H., Mithal, D.S., Park, J.E., and Miller, R.J. (2015). Localized CCR2 activation in the bone marrow niche mobilizes monocytes by desensitizing CXCR4. *PLoS One* 10, 1–13.
- Jung, S., Aliberti, J., Graemmel, P., Jean, M., Kreutzberg, G.W., Sher, A., Dan, R., Sunshine, M.J., and Littman, D.A.N.R. (2000). Analysis of fractalkine receptor CX3CR1 function by targeted deletion and green fluorescent protein reporter gene insertion. *Mol. Cell Biol.* 20, 4106–4114.
- Kagan, J.C., Su, T., Horng, T., Chow, A., Akira, S., and Medzhitov, R. (2008). TRAM couples endocytosis of Toll-like receptor 4 to the induction of interferon-beta. *Nat. Immunol.* 9, 361–368.
- Kalis, C., Kanzler, B., Lembo, A., Poltorak, A., Galanos, C., and Freudenberg, M. a (2003). Toll-like receptor 4 expression levels determine the degree of LPS-susceptibility in mice. *Eur. J. Immunol.* 33, 798–805.
- Kallen, J., Schlaeppli, J.-M., Bitsch, F., Delhon, I., and Fournier, B. (2004). Crystal structure of the human RORalpha Ligand binding domain in complex with cholesterol sulfate at 2.2 Å. *J. Biol. Chem.* 279, 14033–14038.
- Kallen, J.A., Schlaeppli, J.-M., Bitsch, F., Geisse, S., Geiser, M., Delhon, I., and Fournier, B. (2002). X-ray structure of the hRORalpha LBD at 1.63 Å: structural and functional data that cholesterol or a cholesterol derivative is the natural ligand of RORalpha. *Structure* 10, 1697–1707.
- Kato, S., Yuzawa, Y., Tsuboi, N., Maruyama, S., Morita, Y., Matsuguchi, T., and Matsuo, S. (2004). Endotoxin-Induced Chemokine Expression in Murine Peritoneal Mesothelial Cells: The Role of Toll-Like Receptor 4. *J. Am. Soc. Nephrol.* 15, 1289–1299.

- Kawai, T., Adachi, O., Ogawa, T., Takeda, K., and Akira, S. (1999). Unresponsiveness of MyD88-deficient mice to endotoxin. *Immunity* *11*, 115–122.
- Kawai, T., Takeuchi, O., Fujita, T., Inoue, J., Mührladt, P.F., Sato, S., Hoshino, K., and Akira, S. (2001). Lipopolysaccharide stimulates the MyD88-independent pathway and results in activation of IFN-regulatory factor 3 and the expression of a subset of lipopolysaccharide-inducible genes. *J. Immunol.* *167*, 5887–5894.
- Keller, M., Mazuch, J., Abraham, U., Eom, G.D., Herzog, E.D., Volk, H.-D., Kramer, A., and Maier, B. (2009). A circadian clock in macrophages controls inflammatory immune responses. *Proc. Natl. Acad. Sci. U. S. A.* *106*, 21407–21412.
- Kenny, E.F., Talbot, S., Gong, M., Golenbock, D.T., Bryant, C.E., and O'Neill, L.A.J. (2009). MyD88 adaptor-like is not essential for TLR2 signaling and inhibits signaling by TLR3. *J. Immunol.* *183*, 3642–3651.
- Kiel, M.J., Yilmaz, Ö.H., Iwashita, T., Yilmaz, O.H., Terhorst, C., and Morrison, S.J. (2005). SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. *Cell* *121*, 1109–1121.
- Kieusseian, A., de la Grange, P.B., Burlen-Defranoux, O., Godin, I., and Cumano, A. (2012). Immature hematopoietic stem cells undergo maturation in the fetal liver. *Development* *139*, 3521–3530.
- Kissa, K., and Herbomel, P. (2010). Blood stem cells emerge from aortic endothelium by a novel type of cell transition. *Nature* *464*, 112–115.
- De Kleer, I., Willems, F., Lambrecht, B., and Goriely, S. (2014). Ontogeny of myeloid cells. *Front. Immunol.* *5*, 1–11.
- Von Knethen A, a, and Brüne, B. (2001). Delayed activation of PPARgamma by LPS and IFN-gamma attenuates the oxidative burst in macrophages. *FASEB J.* *15*, 535–544.
- Kohyama, M., Ise, W., Edelson, B.T., Wilker, P.R., Hildner, K., Mejia, C., Frazier, W.A., Murphy, T.L., and Murphy, K.M. (2009). Role for Spi-C in the development of red pulp macrophages and splenic iron homeostasis. *Nature* *457*, 318–321.
- Kojetin, D.J., and Burris, T.P. (2014). REV-ERB and ROR nuclear receptors as drug targets. *Nat. Rev. Drug Discov.* *13*, 197–216.
- Kolaczowska, E., and Kubes, P. (2013). Neutrophil recruitment and function in health and inflammation. *Nat. Rev. Immunol.* *13*, 159–175.
- Kopf, M., Baumann, H., Freer, G., Freudenberg, M., Lamers, M., Kishimoto, T., Zinkernagel, R., Bluethmann, H., and Köhler, G. (1994). Impaired immune and acute-phase responses in interleukin-6-deficient mice. *Nature* *368*, 339–342.
- Kopmels, B., Mariani, J., Delhay-Bouchaud, N., Audibert, F., Fradelizi, D., and Wollman, E.E. (1992). Evidence for a hyperexcitability state of staggerer mutant mice macrophages. *J. Neurochem.* *58*, 192–199.
- Koretzky, G.A. (2010). Multiple roles of CD4 and CD8 in T cell activation. *J. Immunol.*

- Kurosaki, T., Kometani, K., and Ise, W. (2015). Memory B cells. *Nat. Rev. Immunol.* 15, 149–159.
- Lakso, M., Sauer, B., Mosinger, B., Lee, E.J., Manning, R.W., Yu, S.H., Mulder, K.L., and Westphal, H. (1992). Targeted oncogene activation by site-specific recombination in transgenic mice. *Proc. Natl. Acad. Sci. U. S. A.* 89, 6232–6236.
- Lau, P., Fitzsimmons, R.L., Raichur, S., Wang, S.-C.M., Lechtken, A., and Muscat, G.E.O. (2008). The orphan nuclear receptor, RORalpha, regulates gene expression that controls lipid metabolism: staggerer (SG/SG) mice are resistant to diet-induced obesity. *J. Biol. Chem.* 283, 18411–18421.
- Lau, P., Fitzsimmons, R.L., Pearen, M.A., Watt, M.J., and Muscat, G.E.O. (2011). Homozygous staggerer (sg/sg) mice display improved insulin sensitivity and enhanced glucose uptake in skeletal muscle. *Diabetologia* 54, 1169–1180.
- Lichanska, A.M., and Hume, D.A. (2000). Origins and functions of phagocytes in the embryo.
- Loke, P., and Allison, J.P. (2003). PD-L1 and PD-L2 are differentially regulated by Th1 and Th2 cells. *Proc. Natl. Acad. Sci. U. S. A.* 100, 5336–5341.
- Loke, P., Nair, M.G., Parkinson, J., Guiliano, D., Blaxter, M., and Allen, J.E. (2002). IL-4 dependent alternatively-activated macrophages have a distinctive in vivo gene expression phenotype. *BMC Immunol.* 3, 7.
- Loo, Y.M., and Gale, M. (2011). Immune signaling by RIG-I-like receptors. *Immunity* 34, 680–692.
- Lu, Y.-C., Yeh, W.-C., and Ohashi, P.S. (2008). LPS/TLR4 signal transduction pathway. *Cytokine* 42, 145–151.
- Ma, O., Hong, S., Guo, H., Ghiaur, G., and Friedman, A.D. (2014). Granulopoiesis requires increased C/EBPα compared to monopoiesis, correlated with elevated Cebpa in immature G-CSF receptor versus M-CSF receptor expressing cells. *PLoS One* 9, 1–14.
- Mackaness, G. (1962). Cellular resistance to infection. *J. Exp. Med.* 381–406.
- Mak, K.S., Funnell, A.P.W., Pearson, R.C.M., and Crossley, M. (2011). PU.1 and haematopoietic cell fate: Dosage matters. *Int. J. Cell Biol.* 2011.
- Mamontova, A., Seguret-Mace, S., Esposito, B., Chanial, C., Bouly, M., Delhaye-Bouchaud, N., Luc, G., Staels, B., Duverger, N., Mariani, J., and Tedgui, A. (1998). Severe atherosclerosis and hypoalphalipoproteinemia in the staggerer mouse, a mutant of the nuclear receptor RORalpha. *Circulation* 98, 2738–2743.
- Mantovani, A., Sica, A., Sozzani, S., Allavena, P., Vecchi, A., and Locati, M. (2004). The chemokine system in diverse forms of macrophage activation and polarization. *Trends Immunol.* 25, 677–686.

- Martinez, F.O., and Gordon, S. (2014). The M1 and M2 paradigm of macrophage activation: time for reassessment. *F1000Prime Rep.* 6, 13.
- Martinon, F., Burns, K., and Tschopp, J. (2002). The Inflammasome: A molecular platform triggering activation of inflammatory caspases and processing of proIL- β . *Mol. Cell* 10, 417–426.
- Medzhitov, R., Preston-Hurlburt, P., and Janeway, C.A. (1997). A human homologue of the *Drosophila* Toll protein signals activation of adaptive immunity. *Nature* 388, 394–397.
- Metcalf, D.D., Peavy, R.D., and Gilfillan, A.M. (2009). Mechanisms of mast cell signaling in anaphylaxis. *J. Allergy Clin. Immunol.* 124, 639–646.
- Metchnikoff, E. (1892). *Lecons sur la pathologies comparee de l'inflammation*. Masson.
- Miggin, S.M., Pålsson-McDermott, E., Dunne, A., Jefferies, C., Pinteaux, E., Banahan, K., Murphy, C., Moynagh, P., Yamamoto, M., Akira, S., Rothwell, N., Golenbock, D., Fitzgerald, K. a, and O'Neill, L.A.J. (2007). NF-kappaB activation by the Toll-IL-1 receptor domain protein MyD88 adapter-like is regulated by caspase-1. *Proc. Natl. Acad. Sci. U. S. A.* 104, 3372–3377.
- Mills, C.D., Kincaid, K., Alt, J.M., Heilman, M.J., and Hill, A.M. (2000). M-1/M-2 macrophages and the Th1/Th2 paradigm. *J. Immunol.* 164, 6166–6173.
- Minehata, K.I., Mukouyama, Y.S., Sekiguchi, T., Hara, T., and Miyajima, A. (2002). Macrophage colony stimulating factor modulates the development of hematopoiesis by stimulating the differentiation of endothelial cells in the AGM region. *Blood* 99, 2360–2368.
- Misharin, A. V, Cuda, C.M., Budinger, G.R.S., Perlman, H., Misharin, A. V, Cuda, C.M., Saber, R., Turner, J.D., Gierut, A.K., and Iii, G.K.H. (2014). Nonclassical Ly6C - monocytes drive the development of inflammatory arthritis in mice. *Cell* 9, 591–604.
- Misharin, A. V., Saber, R., and Perlman, H. (2012). Eosinophil contamination of thioglycollate-elicited peritoneal macrophage cultures skews the functional readouts of in vitro assays. *J. Leukoc. Biol.* 92, 325–331.
- Moffatt, M.F., Gut, I.G., Demenais, F., Strachan, D.P., Bouzigon, E., Heath, S., von Mutius, E., Farrall, M., Lathrop, M., and Cookson, W.O.C.M. (2010). A large-scale, consortium-based genomewide association study of asthma. *New Engl. J. Med. Engl. J. Med.* 363, 1211–1221.
- Montero-Melendez, T., and Perretti, M. (2014). Gapdh gene expression is modulated by inflammatory arthritis and is not suitable for qPCR normalization. *Inflammation* 37, 1059–1069.
- Moon, T.C., Befus, A.D., and Kulka, M. (2014). Mast cell mediators: Their differential release and the secretory pathways involved. *Front. Immunol.* 5, 1–19.
- Morris, M.C., Gilliam, E.A., and Li, L. (2015). Innate immune programming by endotoxin and its pathological consequences. *Front. Immunol.* 6, 1–8.

- Mosmann, T.R., and Coffman, R.L. (1989). TH1 and TH2 cells: Different patterns of lymphokine secretion lead to different functional properties. *Annu. Rev. Immunol.* 7, 145–173.
- Mosser, D.M., Zhang, X., and David M. Mosser and Xia Zhang (2008). Activation of murine macrophages. *Curr. Protoc. Immunol. Chapter 14*, Unit 14.2.
- Murray, P.J., Allen, J.E., Biswas, S.K., Fisher, E.A., Gilroy, D.W., Goerdt, S., Gordon, S., Hamilton, J.A., Ivashkiv, L.B., Lawrence, T., Locati, M., Mantovani, A., Martinez, F.O., Mege, J.L., Mosser, D.M., Natoli, G., Saeij, J.P., Schultze, J.L., Shirey, K., et al. (2014). Macrophage activation and polarization: nomenclature and experimental guidelines. *Immunity* 41, 14–20.
- Nathan, B.Y.C.F., Murray, H.W., Wlebe, I.E., and Rubin, B.Y. (1983). Identification of interferon gamma as the lymphokine that activates human macrophage oxidative metabolism and antimicrobial activity. *J. Exp. Med.* 158, 670–689.
- Neefjes, J., Jongsma, M.L.M., Paul, P., and Bakke, O. (2011). Towards a systems understanding of MHC class I and MHC class II antigen presentation. *Nat. Rev. Immunol.* 11, 823–836.
- Neill, D.R., Wong, S.H., Bellosi, A., Flynn, R.J., Daly, M., Langford, T.K. a, Bucks, C., Kane, C.M., Fallon, P.G., Pannell, R., Jolin, H.E., and McKenzie, A.N.J. (2010). Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity. *Nature* 464, 1367–1370.
- Nemzek, J.A., Hugunin, K.M.S., and Opp, M.R. (2008). Modeling sepsis in the laboratory: Merging sound science with animal well-being. *Comp. Med.* 58, 120–128.
- Nguyen, A., Rauch, T.A., Pfeifer, G.P., and Hu, V.W. (2010). Global methylation profiling of lymphoblastoid cell lines reveals epigenetic contributions to autism spectrum disorders and a novel autism candidate gene, RORA, whose protein product is reduced in autistic brain. *FASEB J.* 24, 3036–3051.
- Nguyen, K.D., Fentress, S.J., Qiu, Y., Yun, K., Cox, J.S., and Chawla, A. (2013). Circadian gene *Bmal1* regulates diurnal oscillations of Ly6C(hi) inflammatory monocytes. *Science* 341, 1483–1488.
- Nikolic, T., Movita, D., Lambers, M.E., de Almeida, C.R., Biesta, P., Kreefft, K., de Bruijn, M.J., Bergen, I., Galjart, N., Boonstra, A., and Hendriks, R. (2014). The DNA-binding factor Ctf critically controls gene expression in macrophages. *Cell. Mol. Immunol.* 11, 58–70.
- O'Neill, L.A.J., and Pearce, E.J. (2015). Immunometabolism governs dendritic cell and macrophage function. *J. Exp. Med.* jem.20151570.
- O'Neill, L.A.J., Golenbock, D., and Bowie, A.G. (2013). The history of Toll-like receptors - redefining innate immunity. *Nat. Rev. Immunol.* 13, 453–460.
- Okabe, Y., and Medzhitov, R. (2014). Tissue-specific signals control reversible program of localization and functional polarization of macrophages. *Cell* 157, 832–844.

- Okabe, Y., and Medzhitov, R. (2015). Tissue biology perspective on macrophages. *Nat. Immunol.* *17*, 9–17.
- Oliphant, C.J., Hwang, Y.Y., Walker, J.A., Salimi, M., Wong, S.H., Brewer, J.M., Englezakis, A., Barlow, J.L., Hams, E., Scanlon, S.T., Ogg, G.S., Fallon, P.G., and McKenzie, A.N.J. (2014). MHCII-mediated dialog between group 2 innate lymphoid cells and CD4⁺ T cells potentiates type 2 immunity and promotes parasitic helminth expulsion. *Immunity* *41*, 283–295.
- Otsuka, A., and Kabashima, K. (2015). Mast cells and basophils in cutaneous immune responses. *Allergy* *70*, 131–140.
- Pace, J.L., Russell, S.W., Schreiber, R.D., Altman, A., and Katz, D.H. (1983). Macrophage activation: priming activity from a T-cell hybridoma is attributable to interferon-gamma. *Proc. Natl. Acad. Sci. U. S. A.* *80*, 3782–3786.
- Palframan, R.T., Jung, S., Cheng, G., Weninger, W., Luo, Y., Dorf, M., Littman, D.R., Rollins, B.J., Zweerink, H., Rot, A., and von Andrian, U.H. (2001). Inflammatory chemokine transport and presentation in HEV: a remote control mechanism for monocyte recruitment to lymph nodes in inflamed tissues. *J. Exp. Med.* *194*, 1361–1373.
- Palis, J., Robertson, S., Kennedy, M., Wall, C., and Keller, G. (1999). Development of erythroid and myeloid progenitors in the yolk sac and embryo proper of the mouse. *Development* *126*, 5073–5084.
- Park, J.-H., Kim, Y.-G., Shaw, M., Kanneganti, T.-D., Fujimoto, Y., Fukase, K., Inohara, N., and Núñez, G. (2007). Nod1/RICK and TLR signaling regulate chemokine and antimicrobial innate immune responses in mesothelial cells. *J. Immunol.* *179*, 514–521.
- Parsa, R., Andresen, P., Gillett, A., Mia, S., Zhang, X.-M., Mayans, S., Holmberg, D., and Harris, R. a (2012). Adoptive transfer of immunomodulatory M2 macrophages prevents type 1 diabetes in NOD mice. *Diabetes* *61*, 2881–2892.
- Partch, C.L., Green, C.B., and Takahashi, J.S. (2014). Molecular architecture of the mammalian circadian clock. *Trends Cell Biol.* *24*, 90–99.
- Pathak, P., Li, T., and Chiang, J.Y.L. (2013). Retinoic acid-related orphan receptor alpha regulates diurnal rhythm and fasting induction of sterol 12alpha-hydroxylase in bile acid synthesis. *J. Biol. Chem.* *288*, 37154–37165.
- Paz-Priel, I., and Friedman, A. (2011). C/EBPa Dysregulation in AML and ALL. *Crit. Rev. Oncol.* *16*, 93–102.
- Perdiguero, E.G., and Geissmann, F. (2015). The development and maintenance of resident macrophages. *Nat. Immunol.* *17*, 2–8.
- Pesce, J.T., Ramalingam, T.R., Mentink-Kane, M.M., Wilson, M.S., Kasmi, K.C.E., Smith, A.M., Thompson, R.W., Cheever, A.W., Murray, P.J., and Wynn, T.A. (2009a). Arginase-1-expressing macrophages suppress Th2 cytokine-driven inflammation and fibrosis. *PLoS Pathog.* *5*.

- Pesce, J.T., Ramalingam, T.R., Wilson, M.S., Mentink-Kane, M.M., Thompson, R.W., Cheever, A.W., Urban, J.F., and Wynn, T.A. (2009b). Retnla (Relmalpha/Fizz1) suppresses helminth-induced Th2- type immunity. *PLoS Pathog.* 5, 1–15.
- Płóciennikowska, A., Hromada-Judycka, A., Borzęcka, K., and Kwiatkowska, K. (2015). Co-operation of TLR4 and raft proteins in LPS-induced pro-inflammatory signaling. *Cell. Mol. Life Sci.* 72, 557–581.
- Poltorak, M.P., and Schraml, B.U. (2015). Fate mapping of dendritic cells. *Front. Immunol.* 6, 1–16.
- Poltorak, A., Xiaolong, H., Smirnova, I., Mu-Ya, L., Van Huffel, C., Du, X., Birdwell, D., Alejos, E., Silva, M., Galanos, C., Freudenberg, M., Ricciardi-Castagnoli, P., Layton, B., and Beutler, B. (1998). Defective LPS Signaling in C3H/HeJ and C57BL/10ScCr Mice: Mutations in Tlr4 Gene. *Science* 282, 2085–2088.
- Porta, C., Rimoldi, M., Raes, G., Brys, L., Ghezzi, P., Di Liberto, D., Dieli, F., Ghisletti, S., Natoli, G., De Baetselier, P., Mantovani, A., and Sica, A. (2009). Tolerance and M2 (alternative) macrophage polarization are related processes orchestrated by p50 nuclear factor kappaB. *Proc. Natl. Acad. Sci. U. S. A.* 106, 14978–14983.
- Preiser, J.C., Schmartz, D., Van der Linden, P., Content, J., Vanden Bussche, P., Buurman, W., Sebald, W., Dupont, E., Pinsky, M.R., and Vincent, J.L. (1991). Interleukin-6 administration has no acute hemodynamic or hematologic effect in the dog. *Cytokine* 3, 1–4.
- Preitner, N., Damiola, F., Zakany, J., Duboule, D., Albrecht, U., and Schibler, U. (2002). The orphan nuclear receptor REV-ERB alpha controls circadian transcription within the positive limb of the mammalian circadian oscillator. *Cell* 110, 251–260.
- Raichur, S., Fitzsimmons, R.L., Myers, S.A., Pearen, M.A., Lau, P., Eriksson, N., Wang, S.M., and Muscat, G.E.O. (2010). Identification and validation of the pathways and functions regulated by the orphan nuclear receptor, ROR alpha1, in skeletal muscle. *Nucleic Acids Res.* 38, 4296–4312.
- Ray, A., and Dittel, B.N. (2010). Isolation of mouse peritoneal cavity cells. *J. Vis. Exp.* 9–11.
- Remick, D.G., Newcomb, D.E., Bolgos, G.L., and Call, D.R. (2000). Comparison of the mortality and inflammatory response of two models of sepsis: lipopolysaccharide vs. cecal ligation and puncture. *Shock* 13, 110–116.
- Reppert, S.M., and Weaver, D.R. (2002). Coordination of circadian timing in mammals. *Nature* 418, 935–941.
- Rivers, E., Nguyen, B., Havstad, S., Ressler, J., Muzzin, A., Knoblich, B., Peterson, E., and Tomlanovich, M. (2001). Early goal-directed therapy in the treatment of severe sepsis and septic shock. *New Engl. J. Med.* 345, 1368–1377.
- Romagnani, S. (2000). T-cell subsets (Th1 versus Th2). *Ann. Allergy. Asthma Immunol.* 85, 9–18;

- Rosas, M., Davies, L.C., Giles, P.J., Liao, C.-T., Kharfan, B., Stone, T.C., O'Donnell, V.B., Fraser, D.J., Jones, S.A., and Taylor, P.R. (2014). The transcription factor Gata6 links tissue macrophage phenotype and proliferative renewal. *Science* 344, 645–648.
- Rosenbauer, F., Wagner, K., Kutok, J.L., Iwasaki, H., Le Beau, M.M., Okuno, Y., Akashi, K., Fiering, S., and Tenen, D.G. (2004). Acute myeloid leukemia induced by graded reduction of a lineage-specific transcription factor, PU.1. *Nat. Genet.* 36, 624–630.
- Rosenberg, H.F., Dyer, K.D., and Foster, P.S. (2013). Eosinophils: changing perspectives in health and disease. *Nat. Rev. Immunol.* 13, 9–22.
- Rosmarin, A.G., Caprio, D., Levy, R., and Simkevich, C. (1995). CD18 (beta 2 leukocyte integrin) promoter requires PU.1 transcription factor for myeloid activity. *Proc. Natl. Acad. Sci. U. S. A.* 92, 801–805.
- Saederup, N., Cardona, A.E., Croft, K., Mizutani, M., Cotleur, A.C., Tsou, C.-L., Ransohoff, R.M., and Charo, I.F. (2010). Selective chemokine receptor usage by central nervous system myeloid cells in CCR2-red fluorescent protein knock-in mice. *PLoS One* 5, e13693.
- Sarachana, T., and Hu, V.W. (2013). Genome-wide identification of transcriptional targets of RORA reveals direct regulation of multiple genes associated with autism spectrum disorder. *Mol. Autism* 4, 14.
- Sato, S., Sakurai, T., Ogasawara, J., Takahashi, M., Izawa, T., Imaizumi, K., Taniguchi, N., Ohno, H., and Kizaki, T. (2014). A circadian clock gene, Rev-erba, modulates the inflammatory function of macrophages through the negative regulation of Ccl2 expression. *J. Immunol.* 192, 407–417.
- Sato, T.K., Panda, S., Miraglia, L.J., Reyes, T.M., Rudic, R.D., McNamara, P., Naik, K.A., FitzGerald, G.A., Kay, S.A., and Hogenesch, J.B. (2004). A functional genomics strategy reveals Rora as a component of the mammalian circadian clock. *Neuron* 43, 527–537.
- Sauer, B. (1998). Inducible gene targeting in mice using the Cre / lox system. *Methods* 392, 381–392.
- Saunders, S.P., Barlow, J.L., Walsh, C.M., Bellsoi, A., Smith, P., McKenzie, A.N.J., and Fallon, P.G. (2010). C-type lectin SIGN-R1 has a role in experimental colitis and responsiveness to lipopolysaccharide. *J. Immunol.* 184, 2627–2637.
- Scheiermann, C., Kunisaki, Y., Lucas, D., Chow, A., Jang, J.-E., Zhang, D., Hashimoto, D., Merad, M., and Frenette, P. (2012). Adrenergic nerves govern circadian leukocyte recruitment to tissues. *Immunity* 37, 290–301.
- Scheiermann, C., Kunisaki, Y., and Frenette, P.S. (2013). Circadian control of the immune system. *Nat.Rev.Immunol.* 13, 190–198.
- Schulte, W., Bernhagen, J., and Bucala, R. (2013). Cytokines in sepsis: Potent immunoregulators and potential therapeutic targets - An updated view. *Mediators Inflamm.* 2013.

- Schulz, C., Perdiguero, E.G., Chorro, L., Szabo-Rogers, H., Cagnard, N., Kierdorf, K., Prinz, M., Wu, B., Jacobsen, S.E.W., Pollard, J.W., Frampton, J., Liu, K.J., and Geissmann, F. (2012). A Lineage of myeloid cells independent of Myb and hematopoietic stem cells. *Science* 336, 86–90.
- Schürch, C.M., Riether, C., and Ochsenbein, A.F. (2014). Cytotoxic CD8⁺ T cells stimulate hematopoietic progenitors by promoting cytokine release from bone marrow mesenchymal stromal cells. *Cell Stem Cell* 14, 460–472.
- Seo, S.-U., Kuffa, P., Kitamoto, S., Nagao-Kitamoto, H., Rousseau, J., Kim, Y.-G., Núñez, G., and Kamada, N. (2015). Intestinal macrophages arising from CCR2(+) monocytes control pathogen infection by activating innate lymphoid cells. *Nat. Commun.* 6, 8010.
- Serafini, N., Vosshenrich, C.A.J., and Di Santo, J.P. (2015). Transcriptional regulation of innate lymphoid cell fate. *Nat. Rev. Immunol.* 15, 415–428.
- Serbina, N. V., and Pamer, E.G. (2006). Monocyte emigration from bone marrow during bacterial infection requires signals mediated by chemokine receptor CCR2. *Nat. Immunol.* 7, 311–317.
- Shaw, P., Lamkanfi, M., and Kanneganti, T.-D. (2010). NOD-like receptor signaling beyond the inflammasome. *Eur. J. Immunol.* 40, 624–627.
- Shen, F.W., Saga, Y., Litman, G., Freeman, G., JTung, J.S., Cantor, H., and Boyse, E.A. (1985). Cloning of Ly-5 cDNA. *PNAS* 82, 7360–7363.
- Shi, C., and Pamer, E.G. (2011). Monocyte recruitment during infection and inflammation. *Nat. Rev. Immunol.* 11, 762–774.
- Silver, A.C., Arjona, A., Hughes, M.E., Nitabach, M.N., and Fikrig, E. (2012). Circadian expression of clock genes in mouse macrophages, dendritic cells, and B cells. *Brain. Behav. Immun.* 26, 407–413.
- Smith, L.T., Hohaus, S., Gonzalez, D.A., Dziennis, S.E., and Tenen, D.G. (1996). PU.1 (Spi-1) and C/EBP alpha regulate the granulocyte colony-stimulating factor receptor promoter in myeloid cells. *Blood* 88, 1234–1247.
- Solovjov, D.A., Pluskota, E., and Plow, E.F. (2005). Distinct roles for the alpha and beta subunits in the functions of integrin alphaMbeta2. *J. Biol. Chem.* 280, 1336–1345.
- Solt, L.A., Kumar, N., Nuhant, P., Wang, Y., Lauer, J.L., Liu, J., Istrate, M.A., Kamenecka, T.M., Roush, W.R., Vidović, D., Schürer, S.C., Xu, J., Wagoner, G., Drew, P.D., Griffin, P.R., and Burris, T.P. (2011). Suppression of TH17 differentiation and autoimmunity by a synthetic ROR ligand. *Nature* 472, 491–494.
- Spooner, C.J., Cheng, J.X., Pujadas, E., Laslo, P., and Singh, H. (2009). A recurrent network involving the transcription factors PU.1 and Gfi1 orchestrates innate and adaptive immune cell fates. *Immunity* 31, 576–586.
- Stapleton, C.M., Jaradat, M., Dixon, D., Kang, H.S., Kim, S.-C., Liao, G., Carey, M.A., Cristiano, J., Moorman, M.P., and Jetten, A.M. (2005). Enhanced susceptibility of

- staggerer (ROR α sg) mice to lipopolysaccharide-induced lung inflammation. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 289, L144–L152.
- Stein, B.M., Keshav, S., Harris, N., and Gordon, S. (1992). Interleukin 4 potently enhances murine macrophage mannose receptor activity: A marker of alternative immunologic macrophage activation. *J. Exp. Med.* 176, 287–292.
- Steinhauser, M.L., Hogaboam, C.M., Lukacs, N.W., Strieter, R.M., and Kunkel, S.L. (1999). Multiple roles for IL-12 in a model of acute septic peritonitis. *J. Immunol.* 162, 5437–5443.
- Steinman, R.M., and Cohn, Z.A. (1973). Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. *J. Exp. Med.* 137, 1142–1162.
- Steinman, R.M., and Cohn, Z.A. (1974). Identification of a novel cell type in peripheral lymphoid organs of mice. II. Functional properties in vitro. *J. Exp. Med.* 139, 380–397.
- Stone, K.D., Prussin, C., and Metcalfe, D.D. (2010). IgE, mast cells, basophils, and eosinophils. *J. Allergy Clin. Immunol.* 125, S73–S80.
- Summers, C., Rankin, S.M., Condliffe, A.M., Singh, N., Peters, M., and Chilvers, E.R. (2010). Neutrophil kinetics in health and disease. *Trends Immunol.* 31, 318–324.
- Surbatovic, M., Popovic, N., Vojvodic, D., Milosevic, I., Acimovic, G., Stojicic, M., Veljovic, M., Jevdjic, J., Djordjevic, D., and Radakovic, S. (2015). Cytokine profile in severe gram-positive and gram-negative abdominal sepsis. *Sci. Rep.* 5, 11355.
- Sutherland, T.E., Logan, N., Rückerl, D., Humbles, A.A., Allan, S.M., Papayannopoulos, V., Stockinger, B., Maizels, R.M., and Allen, J.E. (2014). Chitinase-like proteins promote IL-17-mediated neutrophilia in a tradeoff between nematode killing and host damage. *Nat. Immunol.* 15, 1116–1125.
- Swirski, F.K., Nahrendorf, M., Etzrodt, M., Wildgruber, M., Cortez-Retamozo, V., Panizzi, P., Figueiredo, J.-L., Kohler, R., Chudnovsky, A., Waterman, P., Aikawa, E., Mempel, T., Libby, P., Weissleder, R., and Pittet, M. (2010). Identification of splenic reservoir monocytes and their deployment to inflammatory sites. *Science* 326, 612–617.
- Tamoutounour, S., Henri, S., Lelouard, H., de Bovis, B., de Haar, C., van der Woude, C.J., Woltman, A.M., Rey, Y., Bonnet, D., Sichien, D., Bain, C.C., Mowat, A.M., Reis e Sousa, C., Poulin, L.F., Malissen, B., and Guillemin, M. (2012). CD64 distinguishes macrophages from dendritic cells in the gut and reveals the Th1-inducing role of mesenteric lymph node macrophages during colitis. *Eur. J. Immunol.* 42, 3150–3166.
- Thornley, T.B., Fang, Z., Balasubramanian, S., Larocca, R.A., Gong, W., Gupta, S., Csizmadia, E., Degauque, N., Kim, B.S., Koulmanda, M., Kuchroo, V.K., and Strom, T.B. (2014). Fragile TIM-4 – expressing tissue resident macrophages are migratory and immunoregulatory. *J. Clin. Invest.* 124, 3443–3454.
- Ueno, H., and Weissman, I.L. (2006). Clonal Analysis of Mouse Development Reveals a Polyclonal Origin for Yolk Sac Blood Islands. *Dev. Cell* 11, 519–533.

- Vannella, K.M., Barron, L., Borthwick, L.A., Kindrachuk, K.N., Narasimhan, P.B., Hart, K.M., Thompson, R.W., White, S., Cheever, A.W., Ramalingam, T.R., and Wynn, T.A. (2014). Incomplete deletion of IL-4R α by LysMCre reveals distinct subsets of M2 macrophages controlling inflammation and fibrosis in chronic Schistosomiasis. *PLoS Pathog.* *10*, e1004372.
- Vaquerizas, J.M., Kummerfeld, S.K., Teichmann, S.A., and Luscombe, N.M. (2009). A census of human transcription factors: function, expression and evolution. *Nat. Rev. Genet.* *10*, 252–263.
- Vogel, M.W., Sinclair, M., Qiu, D., and Fan, H. (2000). Purkinje cell fate in staggerer mutants: agenesis versus cell death. *J Neurobiol* *42*, 323–337.
- Wang, J.X., Bair, A.M., King, S.L., Shnyder, R., Huang, Y.F., Shieh, C.C., Soberman, R.J., Fuhlbrigge, R.C., and Nigrovic, P.A. (2012). Ly6G ligation blocks recruitment of neutrophils via a β 2-integrin- dependent mechanism. *Blood* *120*, 1489–1498.
- Wang, Y., Kumar, N., Solt, L.A., Richardson, T.I., Helvering, L.M., Crumbley, C., Garcia-Ordenez, R.D., Stayrook, K.R., Zhang, X., Novick, S., Chalmers, M.J., Griffin, P.R., and Burris, T.P. (2010a). Modulation of retinoic acid receptor-related orphan receptor α and γ activity by 7-oxygenated sterol ligands. *J. Biol. Chem.* *285*, 5013–5025.
- Wang, Y., Kumar, N., Crumbley, C., Griffin, P.R., and Burris, T.P. (2010b). A second class of nuclear receptors for oxysterols: Regulation of ROR α and ROR γ activity by 24S-hydroxycholesterol (cerebrosterol). *Biochim. Biophys. Acta - Mol. Cell Biol. Lipids* *1801*, 917–923.
- Warburg, O., Wind, F., and Negelein, E. (1927). The metabolism of tumors in the body. *J. Gen. Physiol.* *8*, 519–530.
- Wilson, K.P., Black, J.A., Thomson, J.A., Kim, E.E., Griffith, J.P., Navia, M.A., Murcko, M.A., Chambers, S.P., Aldape, R.A., and Raybuck, S.A. (1994). Structure and mechanism of interleukin-1 beta converting enzyme. *Nature* *370*, 270–275.
- Wong, S.H., Walker, J.A., Jolin, H.E., Drynan, L.F., Hams, E., Camelo, A., Barlow, J.L., Neill, D.R., Panova, V., Koch, U., Radtke, F., Hardman, C.S., Hwang, Y.Y., Fallon, P.G., and McKenzie, A.N.J. (2012). Transcription factor ROR α is critical for nuocyte development. *Nat. Immunol.* *13*, 229–236.
- Wynn, T.A., Chawla, A., and Pollard, J.W. (2013). Macrophage biology in development, homeostasis and disease. *Nature* *496*, 445–455.
- Xiong, H., and Pamer, E.G. (2015). Monocytes and infection: Modulator, messenger and effector. *Immunobiology* *220*, 210–214.
- Xu, H., Liew, L.N., Kuo, I.C., Huang, C.H., Goh, D.L.M., and Chua, K.Y. (2008). The modulatory effects of lipopolysaccharide-stimulated B cells on differential T-cell polarization. *Immunology* *125*, 218–228.
- Yagi, R., Zhong, C., Northrup, D., Yu, F., Bouladoux, N., Spencer, S., Hu, G., Barron, L., Sharma, S., Nakayama, T., Belkaid, Y., Zhao, K., and Zhu, J. (2014). The transcription

factor GATA3 is critical for the development of all IL-7R α -expressing innate lymphoid cells. *Immunity* 40, 378–388.

Yamamoto, M., Sato, S., Mori, K., Hoshino, K., Takeuchi, O., Takeda, K., and Akira, S. (2002). Cutting edge: a novel Toll/IL-1 receptor domain-containing adapter that preferentially activates the IFN- β promoter in the Toll-like receptor signaling. *J. Immunol.* 169, 6668–6672.

Yang, J., Zhang, L., Yu, C., Yang, X.-F., and Wang, H. (2014). Monocyte and macrophage differentiation: circulation inflammatory monocyte as biomarker for inflammatory diseases. *Biomark. Res.* 2, 1.

Yang, X.O., Pappu, B., Nurieva, R., Akimzhanov, A., Soon, H., Chung, Y., Ma, L., Shah, B., Panopoulos, A.D., Watowich, S.S., Tian, Q., Jetten, A.M., and Dong, C. (2008). Th17 lineage differentiation is programmed by orphan nuclear receptors ROR α and ROR γ . *Immunity* 28, 29–39.

Ye, M., Iwasaki, H., Laiosa, C. V., Stadtfeld, M., Xie, H., Heck, S., Clausen, B., Akashi, K., and Graf, T. (2003). Hematopoietic stem cells expressing the myeloid lysozyme gene retain long-term, multilineage repopulation potential. *Immunity* 19, 689–699.

Yona, S., Kim, K.W., Wolf, Y., Mildner, A., Varol, D., Breker, M., Strauss-Ayali, D., Viukov, S., Guillemins, M., Misharin, A., Hume, D. a., Perlman, H., Malissen, B., Zelzer, E., and Jung, S. (2013). Fate mapping reveals origins and dynamics of monocytes and tissue macrophages under homeostasis. *Immunity* 38, 79–91.

Yoza, B.K., Hu, J.Y.-Q., Cousart, S.L., Forrest, L.M., and McCall, C.E. (2006). Induction of RelB Participates in Endotoxin Tolerance. *J. Immunol.* 177, 4080–4085.

Zhang, D.E., Zhang, P., Wang, N.D., Hetherington, C.J., Darlington, G.J., and Tenen, D.G. (1997). Absence of granulocyte colony-stimulating factor signaling and neutrophil development in CCAAT enhancer binding protein α -deficient mice. *Proc. Natl. Acad. Sci. U. S. A.* 94, 569–574.

Zhang, P., Iwasaki-Arai, J., Iwasaki, H., Fenyus, M.L., Dayaram, T., Owens, B.M., Shigematsu, H., Levantini, E., Huettner, C.S., Lekstrom-Himes, J. a., Akashi, K., and Tenen, D.G. (2004). Enhancement of hematopoietic stem cell repopulating capacity and self-renewal in the absence of the transcription factor C/EBP β . *Immunity* 21, 853–863.

Zhang, X., Goncalves, R., and Mosser, D. (2008). The isolation and characterization of murine macrophages.

Zhang, Y., Luo, X., Wu, D., and Xu, Y. (2014). ROR nuclear receptors: structures, related diseases, and drug discovery. *Acta Pharmacol. Sin.* 36, 71–87.

Zhou, H., Liao, J., Aloor, J., Nie, H., Wilson, B.C., Fessler, M.B., Gao, H.-M., and Hong, J.-S. (2012). CD11b/CD18 (Mac-1) Is a novel surface receptor for extracellular double-stranded RNA to mediate cellular inflammatory responses. *J. Immunol.* 190, 115–125.

Zhu, L., Yang, T., Li, L., Sun, L., Hou, Y., Hu, X., Zhang, L., Tian, H., Zhao, Q., Peng, J., Zhang, H., Wang, R., Yang, Z., Zhang, L., and Zhao, Y. (2014). TSC1 controls

macrophage polarization to prevent inflammatory disease. *Nat. Commun.* 5, 4696.

Ziegler-Heitbrock, L., Ancuta, P., Crowe, S., Dalod, M., Grau, V., Hart, D.N., Leenen, P.J.M., Liu, Y., Macpherson, G., Randolph, G.J., and Scherberich, J. (2010). Nomenclature of monocytes and dendritic cells in blood. *Blood* 116, 5–7.

Zisman, D.A., Kunkel, S.L., Strieter, R.M., Gauldie, J., Tsai, W.C., Bramson, J., Wilkowski, J.M., Bucknell, K.A., and Standiford, T.J. (1997). Anti-interleukin-12 therapy protects mice in lethal endotoxemia but impairs bacterial clearance in murine *Escherichia coli* peritoneal sepsis. *Shock* 8, 349–356.

Publications

Hams, E., **Bermingham, R.**, and Fallon, P. (2015a). Macrophage and innate lymphoid cell interplay in the genesis of fibrosis. *Front. Immunol.* 6, 1–11.

Hams, E., **Bermingham, R.**, Wurlod, F.A., Hogan, A.E., O'Shea, D., Preston, R.J., Rodewald, H.-R., McKenzie, A.N.J., and Fallon, P.G. (2015b). The helminth T2 RNase 1 promotes metabolic homeostasis in an IL-33 and group 2 innate lymphoid cell-dependent mechanism. *FASEB J.* 152–160.