Using the thioglycollate-elicited murine peritonitis model for investigation of macrophage behaviour and its relationship with Irg1 expression

By

Yanyan Li

A thesis

submitted to the Victoria University of Wellington

in fulfilment of the requirements for the degree of

Master of Biomedical Science

Victoria University of Wellington

2016

Abstract

Macrophages play a crucial role in both innate and adaptive immunity. With a wide tissue distribution, macrophages are found in almost all tissues throughout the body. Upon activation, macrophages orchestrate the initiation and resolution of inflammation, promote wound repair and maintain homeostasis in a tissue-specific manner. This study investigates the behaviour of macrophages during sterile inflammation in the peritoneal cavity.

By using a thioglycollate-induced murine model of peritonitis inflammation, the phenotypic and functional alterations of peritoneal immune cells was investigated and analysed over time. A particular emphasis was set on the dynamics of the macrophage lineage cells. In addition, the expression of Irg1 as a marker for macrophage activation during inflammation was investigated. Finally, the effectiveness of the sterile inflammation model in the context of drug discovery was tested by using the atypical antipsychotic drug clozapine.

Phenotypic analysis showed that the number of resident macrophages was decreased after thioglycollate treatment. This effect was accompanied by a strong increase in monocyte-derived macrophages over time. Furthermore, macrophage colony-stimulating factor (CSF-1) appeared to be one of the key factors in recruiting circulating monocytes and promoting the differentiation of recruited monocytes to macrophages in this inflammation model. In addition, the analysis at different timepoints showed that a population of monocytes (Ly6C-) was present in the peritoneal cavity two days after thioglycollate treatment. For the activation signal of macrophages, the production of the two cytokines IL-10 and TNFα was assessed at two time points by intracellular cytokine staining. Although all investigated cell types produced both cytokines, there was little difference between different treatments. Furthermore, Irg1 expression was found decreased in macrophages stimulated with thioglycollate compared to unstimulated macrophages. Finally, the results from this study suggest that circulating monocytes are likely to be the key cell type responding to clozapine treatment based on the cell population dynamics and cytokine production.

Taken together these results indicate that there is a dynamic change during the thioglycollate-elicited peritonitis involving a massive influx of infiltrating circulating leukocytes and proliferation of peritoneal macrophages. Moreover, application of this system for drug discovery may uncover the effects of a test drug in the context of sterile inflammation thus facilitating the development of effective treatments in the future.

Acknowledgement

First and foremost, I would like to thank my supervisor, Prof. Anne La Flamme for giving me the opportunity to do my masters, for her continuous support, patience, enthusiasm and immense knowledge. Her guidance helped me in all the time of research and writing of this thesis. I could not have asked for a better supervisor!

I would like to thank the past and present members of 'Team LAF': Katharina, Nikki, Vimal, Pirooz, Carl, Saskia, Faith, Alex and Even for all the help and support, science talk and the constant supply of delicious food. I would also like to thank Nicole and Matt for sharing their considerable knowledge and experience on qRT-PCR.

I would like to particular thank Katharina, Nikki, Vimal, Pirooz and Saskia. To Katharina for spending a huge amount of time to read and provide insightful comments on my thesis even though you have plenty of your own work to get through. To Nikki for helping to improve my i.p. injections and encouraged me with many lovely message notes. To Vimal and Pirooz, who helped me with a variety of basic lab techniques to get me started on my research. To Saskia for all the helps that made me get through that tough week, and most of all, her priceless friendship.

A special thank for Varun for the valuable troubleshooting and brainstorming you did with me for my PCR experiments. I am also grateful to Dr. Darren for providing laboratory facility for real-time PCR.

I would also like to thank my friends: Philly, Rushi, Farah and Sisi. I would never forget all the chats and beautiful moments I shared with all of you. Thanks also to Patricia for all the help, understanding and support especially when things went wrong.

A big thank to my homestay family. To Jane, who has supported me throughout entire process, both by keeping me harmonious and helping me put pieces together. I am grateful for all the advice, the extreme patience, and the constant supply of delicious food and caring provided.

Finally, I would like to thank my family for their unflagging love and unconditonal support throughout my life and my study, for never doubting the chioced that I have made, and for being proud of me always for my achievements.

Table of Contents

List of Figures

List of Tables

Abbreviations

Abs	Absolute
AEC	Animal Ethics Committee
AGE	Advanced glycation end product
AP-	Activator protein-
APC	Antigen presenting cell
ATCC	American Type Culture Collection
BCR	B cell receptor
BFA	Brefeldin A
BRU	Biomedical research unit
BSA	Bovine serum albumin
CLR	C-type lectin receptor
CSF-	Colony stimulating factor-
CTCM	Complete T cell media
DAMPs	Damage-associated molecular patterns
DC	Dendritic cell
EAE	Experimental autoimmune encephalomyeliti
EDTA	Ethylenediaminetetraacetic acid
FCS	Foetal calf serum
FoxP3	Forkhead box P3
FSC	Forward scatter
g	Gravity
GM-SCF	Granulocyte macrophage colony-stimulating factor
GPCR	G protein-coupled receptor
GPI	Glycosylphosphatidylinisotol
i.p.	Intra peritoneal
IBD	Inflammatory bowel disease
ICS	Intracellular cytokine staining

IFN	Interferon
Ig	Immunoglobulin
IL-	Interleukin
IRAK	IL-1 receptor-associated kinase
Irg1	immune-responsive gene 1
JAK	Janus kinase
LPM	Large peritoneal macrophage
LPS	Lipopolysaccharide
M-CSF	Macrophage colony-stimulating factor
MDP	Muramyl dipeptide
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MIMR	Malaghan Institute of Medical Research
MØ	Macrophage
MPS	Mononuclear phagocyte system
MR	Mannose receptor
MSU	Monosodium urate
mTEC	Medullary epithelial cell
MyD88	Myeloid differentiation primary response gene 88
NF- κ B	Nuclear Factor-Kappa B
NK cell	Natural killer cell
NLRP	Nod-like receptor protein
NO	Nitric oxide
NOD	Nucleotide-binding oligomerization domain-like receptor
NRT	Minus RT controls
PAMPs	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline

PEC	Peritoneal exudates cells
PFA	Paraformaldehyde
PMA	Phorbol myristate acetate
PRR	Pattern recognition receptor
qRT-PCR	Quantitative real-time polymerase chain reaction
RAGE	Receptor for advanced glycation end product
SPM	Small peritoneal macrophage
SR	Scavenger receptor
SSC	Side scatter
STAT	Signal transducer and activator of transcription
TCR	T cell receptor
TGF- β	Transforming growth factor beta
Th	T-helper
TLR	Toll-like receptor
TNF α	Tumor-necrosis factor alpha
TRAF	TNF receptor activated factor
Treg	Regulatory T cell

Chapter 1: General Introduction

1.1 The immune system

The mammalian immune system is a complex network of cells, tissues and organs developed to protect the host from invading foreign microorganisms or other parasites. The principal function of the immune system is to recognize self from non-self. Failure of the immunity to distinguish between self and non-self can cause damage of tissues and might lead to autoimmune diseases. The immune response can be divided into adaptive and innate immune response. Although the mechanisms of pathogen recognition employed by innate and adaptive immune systems differ from each other, they work together to efficiently eliminate pathogens.

1.1.1 Adaptive immune response

Adaptive immune responses are induced upon the recognition of antigenic peptides by the receptors on the surface of specific cells. Those cells further undergo a pattern of clonal expansion and differentiate into fully functional effector cells resulting in a strong and specialized immune response(Janeway Jr & Medzhitov, 2002). In addition, this response can as well develop immunological memory which allows rapid responses upon re-exposure to the same antigenic peptides (Ahmed & Gray, 1996). This defence mechanism can be divided into cell-mediated and humoral response and involves the participation of T and B lymphocytes and antigen presenting cells (APC).

1.1.1.1 T cells and cell-mediated response

T cells can be divided into two distinct subsets based on their function and surface marker expression; CD8+ T cells (cytotoxic T cells) that are responsible for the elimination of damaged or infected host cells through the release of granzymes or FAS-FAS ligand interactions (Hoves, Trapani, & Voskoboinik, 2010) and CD4+ T cells (T- helper(Th) cells) which can further be defined into Th1, Th2, Th17 and regulatory T cell (Treg) subsets (Zhu & Paul, 2010). They are involved in directing other immune cells to the sites of liasions and are essential for B cell antibody class switching (Mosmann, Cherwinski, Bond, Giedlin, & Coffman, 1986). In the peripheral lymphoid tissues, naive T cells respond to antigens that are presented on the surface of APC by major histocompatibility complex (MHC) molecules and subsequently undergo expansion, differentiation, and migration. There are two kinds of MHC molecules. MHC class I, which is found on all nucleated cells, binds peptides from the cytosol and these are recognised by CD8+ T cells (Janeway Jr, Travers, Walport, & Shlomchik, 2001). MHC class II, which is constitutively expressed on professional APCs including dendritic cells (DCs), macrophages (MØ) and B cells (Holling, Schooten, & van Den Elsen, 2004), binds exogenous proteins that were taken up and generated intracellularly by APCs. These are recognised by CD4+ T cells (Robinson & Delvig,

2002; Shen & Rock, 2006). Therefore, the interaction of T cells and APCs are crucial for the cell-mediated response.

1.1.1.2 B cells and humoral response

B cells can be further divided into conventional B cells (B-2 cells) and B-1 cells with different functions and surface phenotypic markers (Hardy & Hayakawa, 2001). The development of B-2 cells is predominant in the adult. Following a stepwise development process, the immature B cells exit the bone marrow and only a small amount of these newly formed B cells are entering lymphoid organs and become long-lived naive mature B cells (Schneider et al., 2001). When activated by antigen, naive B-2 cells which express membrane immunoglobulin (Ig) in the form of IgM and IgD undergo isotype switching, affinity maturation with or without T-helper cells costimulation and ultimately differentiate into memory cells or short-lived plasma cells (Chaplin, 2010; Schneider et al., 2001).

In contrast, B-1 cell development is predominantly in the fetus and infrequent in the adult (Hardy, 2006). They are found primarily in the peritoneal and pleural cavities as well as in the spleen. (Tumang, Hastings, Bai, & Rothstein, 2004). B-1 cells are self-renewed and have less diversity in B-cell receptor, compared to B-2 cells. They are poised to be activated and differentiate into antibody secreting cells with no requirement of the presence of T-helper cells (Allman & Pillai, 2008; Oracki, Walker, Hibbs, Corcoran, & Tarlinton, 2010).

Given that humoral response is mediated by antibodies produced by B cells, it is not surprising that the humoral response takes place in the peritoneal cavity, spleen, blood, intestine as well as other secondary lymphoid sites and that is essential for the host defense against extracellular microorganisms as well as to prevent the spread of intracellular infections (Twigg III, 2005).

1.1.1.3 Immune tolerance

To avoid recognition of self-tissues, both T cells and B cells, function as key players in maintaining immunological tolerance that can be attained by central or peripheral mechanisms. Central tolerance occurs during early T cell differentiation in the thymus or in the bone marrow for B cell development occurs (Manjarrez-Orduño, Quách, & Sanz, 2009). Negative selection processes account for the elimination effects of self-recognising T cells and B cells. T cells which are able to recognise both foreign and self-pepitde-MHC are positively selected and repetitively interact with many self-antigens expressed by medullary epithelial cells (mTEC) and dendritic cells (DC) resulting in the deletion of T cells with a high affinity for self-peptide-MHC (Palmer, 2003; Xing & Hogquist, 2012). However,

in the case of regulatory T cells (Treg) they are survive via clonal diversion regardless of the high affinity for self-peptide-MHC and followed by the induction of the Forkhead box P3 (FoxP3) and ultimately differentiation of Tregs (Xing & Hogquist, 2012). As with T cells, IgM+ immature B cells undergo apoptosis when tightly bound to self-antigens and those nonautoreactive immature B cells are further selected into the peripheral and mature B-cell populations (Pelanda & Torres, 2012).

Although central tolerance is efficient, this process of elimination of self-reactive lymphocytes by negative selection is not completely efficient. Those mature self-reactive lymphocytes that have escaped central tolerance can migrate to the periphery, where they are deleted or inactivated through a variety of mechanisms, including the lack of co-stimulatory signals or other specific surface receptors that lead to T cell or B cell anergy (Appleman & Boussiotis, 2003; Eris et al., 1994). The existence of immune privileged sites that are thought be less immunologically reactive (London & Schwartz, 2012), elimination of activated T cells via FAS-induced apoptosis (Xing & Hogquist, 2012), and the regulation through Treg to suppress the activity of other T cells, collectively called peripheral tolerance.

1.1.2 Innate immune response

Despite the evolution of adaptive immune systems, innate immune system remains an essential means of a first line of defence against invading pathogens. The innate immune system is composed of a physical barrier, such as skin, mucous membranes as well as a wide range of cell types, including macrophages (MØ), eosinophils, neutrophils, dendritic cells (DC), mast cells, basophils, natural killer (NK) cells (Janeway Jr & Medzhitov, 2002). Unlike the slow-acting but antigen-specific adaptive immune response, the innate immune system provides a rapid response to pathogenic challenge, It is referred to as pathogen-associated molecular patterns (PAMPs) that represent conserved structural motifs or patterns and are present by the microbial pathogens, but not by host cells (Medzhitov & Janeway, 2000). PAMPs are sensed by a set of germline-encoded receptors called pattern recognition receptors (PRRs), which include intracellular proteins such as nucleotide-binding oligomerization domain-like receptors (NODs), C-type lectin receptors (CLRs), cell-surface proteins such as scavenger receptors (SRs) and toll-like receptors (TLRs) (Janeway Jr & Medzhitov, 2002). Although the mechanisms underlying the recognition of infectious microbes or non-infectious molecules by PRRs can be very different, the downstream inflammatory response is quite similar (Rock, Latz, Ontiveros, & Kono, 2010). PAMP-PRR binding triggers the activation of the innate cell. This activation can result in recruitment of other cell types or phagocytosis by secretion of cytokines and chemokines and by the induction of transcription of many immune response genes (Medzhitov & Janeway, 2000).

1.1.3 Sterile inflammation

Most of the knowledge about innate immunity comes from the examination of pathogen-induced inflammation. However, inflammation also occurs in response to non-pathogenic stimuli in all tissues and this, so called sterile inflammation, is associated with a number of human diseases. As with the recognition of PAMPs by PRRs, endogenous damage-associated molecular patterns (DAMPs) which are released by stressed, damaged or dying cells can also be sensed by PRRs and in turn trigger the sterile inflammatory response (G. Y. Chen & Nuñez, 2010). Unlike an invading microbe, usually the sterile stimuli are not injurious themselves. Therefore, engaging immune response to those sterile stimuli may not be beneficial to the host. Moreover an unresolved sterile inflammation can result in pathogenic changes in the host and induce acute or chronic diseases (Rock et al., 2010). Alzheimer's disease is an example of sterile inflammatory disease where it is believed that the stimulation of deposition of amyloid peptide aggregates leads to microglia-mediated neurotoxicity in the cortex of the brain (G. Y. Chen & Nuñez, 2010).

1.2 Macrophages

Macrophages play a pivotal role in both innate and adaptive immunity. They were first discovered by the Russian biologist Elie Metchnikoff (Cavaillon, 2011) as amoeboid phagocytic cells in tissues. This discover, demonstrated the cell-mediated immune response for the first time.

1.2.1 Macrophage distribution and derivation

Macrophages are one of the populations of differentiated myeloid cells. Along with the monocytes and dendritic cells, they constitute the mononuclear phagocyte system (Motwani & Gilroy, 2015). With a wide tissue distribution, macrophages are found in almost all tissues throughout the body, such as liver (Kupffer cells), brain (Microglia), skin (Langerhans cells) and peritoneum (Peritoneal macrophages) (S. Gordon, 2003). Upon activation, macrophages orchestrate the initiation and resolution of inflammation, promote wound repair and maintain homeostasis in a tissue-specific manner (Boe, Curtis, Chen, Ippolito, & Kovacs, 2015).

Macrophages can be broadly divided into two classes based on their origin: tissue-resident macrophages and infiltrating macrophages (Hashimoto et al., 2013). The current understanding is that the infiltrating macrophages found in injured tissues are originated from the classical Ly6C+ monocytes while the original of tissue-resident macrophages is still controversial. It has long been thought that tissue-resident macrophages are also derived from blood monocytes, however, with the establishment of different fate mapping techniques, accumulated evidence suggests that the majority of tissue

macrophages under steady-state conditions are more likely to be embryonically derived (Ginhoux et al., 2010; Varol, Mildner, & Jung, 2015; Yona et al., 2013). Interestingly, recent studies using murine macrophages from different tissues, including spleen, lung and the peritoneal cavity have revealed that infiltrating macrophages and tissue-resident macrophages are not only phenotypically but also functionally distinct from one another (dos Anjos Cassado, Lima, & Bortoluci, 2015).

1.2.2 Macrophage functions

Macrophages are multifunctional cells with a range of abilities. Although they exhibit functional heterogeneity, they also share several common features which will be discussed in more detail below.

1.2.2.1 Phagocytosis

Macrophages belong to the family of phagocytic cells and are responsible for the removal of microbes, irritant particles, apoptotic or necrotic cells through the process of endocytosis (Billack, 2006).

Clearance of apoptotic cells is carried out by unstimulated macrophage upon the recognition of certain receptors, such as scavenger receptors, integrins or complement receptors. (Erwig & Henson, 2007) Interestingly, this process usually does not induce inflammation because of non-functional downstream signal transduction and the inhibition of cytokine production (Mosser & Edwards, 2008; Savill, Dransfield, Gregory, & Haslett, 2002). In contrast, removal of necrotic cells is associated with Toll-like receptors (TLRs), intracellular pattern-recognition receptors (PRRs) and Interleukin-1 (IL-1) receptor, by a MyD88-dependent pathway that subsequently results in the activation of macrophages. Activation is defined by the changes in macrophage surface-protein expression and production of immune mediators, such as IL-6 and chemokine, CXCL1 (Akira, Misawa, Satoh, & Saitoh, 2013; Mosser & Edwards, 2008).

Furthermore, in the case of sterile stimuli, *in vitro* studies showed that macrophages are an important cell population in response to MSU, silica, alum, cholesterol crystals and asbestos, resulting in the production of IL-1β (Hornung et al., 2008; Rock et al., 2010). Mature IL-1β requires processing by caspase-1, a cysteine protease being under the control of Nod-like receptor protein complexes (NLRP) also called inflammasomes (Rock et al., 2010; Tschopp, Martinon, & Burns, 2002). Among identified inflammasomes subtypes, NLRP3 is thought to be involved in the recognition of many sterile stimuli (Rock et al., 2010).

1.2.2.2 Antigen presenting cell function

It is well known that macrophages, along with dendritic cells (DCs) and B cells, function as professional antigen presenting cells (APCs) and ultimately interact with antigen-specific T cells (Unanue, 1984). Naive T cells, either CD8+ T cells or CD4+ T cells require at least two signals to initiate their activation and become effector cells. The binding of peptide-major histocompatibility complexes (MHCs) on mature APC surface to T cell receptors serves as the first signal (Alberts et al., 2002b). The second signal is provided by co-stimulatory molecules, such as CD80 and CD86, present on APCs. This is thought to promote the production of interleukin-2 (IL-2) by T cell through the binding to the co-receptor protein CD28 on the surface (Alberts et al., 2002a). Conversely, unlike CD28, cytotoxic T lymphocyte-associated molecule-4 (CTLA-4) shows opposite effects on T cell activation; Binding of CD80 and CD86 to CTLA-4 supress T cell response (Harris & Ronchese, 1999). Upon recognition by PRRs by macrophages, the antigens or other particles are taken up, processed into peptides and presented to T cells by displaying them on MHCs on the surface of Macrophages (Smith-Garvin, Koretzky, & Jordan, 2009).
In addition, for the differentiation into specific subsets, T cells require a third signal that is mediated by the cytokine milieu of the microenvironment. (Curtsinger & Mescher, 2010; Luckheeram, Zhou, Verma, & Xia, 2012). Following the phagocytic process to a range of stimuli, macrophages release a spectrum of cytokines to provide the environmental signals for T cell activation and differentiation. At an inflammatory site, macrophages seem to induce preferentially the development of T-helper 1 (Th1) cells by secreting IL-12 ((Hsieh et al., 1993) while macrophages, participate in anti-inflammatory process or tissue repair, appear to favor the development of Th2 cells by producing IL-10 (Desmedt, Rottiers, Dooms, Fiers, & Grooten, 1998; Goerdt & Orfanos, 1999). Furthermore, activated Th1 cells produce IFN γ that in turn forms a positive feedback loop for macrophages activation by upregulating the expression of MHC class II in macrophages (J. Ma et al., 2003).

1.2.2.3 Other functions

In addition to the above immunological functions, macrophages are also involved in promoting angiogenesis, vascular remodelling as well as organogenesis in fetal development, which has been evidenced by many groups using different animal models (Jones & Ricardo, 2013). For example, experiments performed by Aurora et al. on neonatal mice have shown that macrophages play a role in heart regeneration. This could be due to the secretion of numerous soluble factors by cardiac macrophages (Aurora et al., 2014).

While multifunctional macrophages have many beneficial effects to the host, macrophages also have been shown to be associated with many inflammatory and malignant diseases. These detrimental effects are due at least in part to the uncontrolled or excessive macrophage activation.

1.2.3 Classification of macrophage phenotype

As one of the most plastic cells of the immune system, macrophages undergo differentiation followed by polarized activation in response to various antigens or signals. (Stout & Suttles, 2004). Therefore, in addition to the classification of macrophages described above, polarized macrophages have been classified as "classical" (or M1) or "alternative" (or M2) macrophages based on the basis of their activation (Wynn, Chawla, & Pollard, 2013). M1 macrophages exhibit enhanced microbicidal and tumoricidal functions and are able to secrete high levels of pro-inflammatory cytokines. Unlike M1 macrophages, M2 macrophages are mainly involved in immunosuppression and tissue repair (Hume, 2015). M1 and M2 activations of macrophages are defined by the response to different stimuli (Fernando O Martinez & Gordon, 2014). Interferon (IFNγ), lipopolysaccharide (LPS) and granulocyte macrophage colony-stimulating factor (GM-CSF; also known as CSF-2) are three of the main stimuli that lead to M1 activation (Fernando O Martinez & Gordon, 2014), of which GM-CSF is indispensable for macrophage development *in vivo*. M1 macrophages are at first primed by a low-dose of IFNγ followed by the activation through the exposure to stimuli such as LPS. This subsequently leads to the up-regulation of surface markers such as MHC class II and CD86 (Mosser, 2003). On the other hand, the activation of M2 macrophages is more complex. Currently three subgroups namely M2a, M2b and M2c exist based on the observation from in vitro studies. M2a is induced by cytokines like IL-4 or IL-13, M2b is generated by immune complexes or LPS stimulation and M2c-polarized cells are activated in the presence of IL-10 or Transforming growth factor-β (TGF-β) (Lech & Anders, 2013). In addition, macrophage colony-stimulating factor (M-CSF) or called colony stimulating factor-1 (CSF-1) is also associated with M2 activation (Fernando O Martinez & Gordon, 2014). Since both GM-CSF and M-CSF are likely involved in the polarisation of macrophages, many efforts have been made in understanding the differences between the two lineage determining cytokines. Indeed, a model of "M-CSF resistance" in macrophages proposed by Hamilton in 2008 suggested that GM-CSF and M-CSF can have competing effects on macrophage-lineage populations during inflammatory reactions. This further sheds light on the potential capacity of macrophages to shift between these activation states.

1.2.4 Regulation of macrophage activation

As described above, the ability of macrophages to sense a variety of stimuli, such as microbes and dead cells, is mainly because they express a diverse range of pattern recognition receptors (PRRs), which includes Toll-like receptors (TLRs), lectin receptors, scavenger receptors (SRs) and complement receptors (Plüddemann, Neyen, & Gordon, 2007). For example, alveolar macrophages have a high-levels of PRRs expression, including SR, mannose receptor (MR) and β -glucan specific receptor, Dectin-1 (Taylor et al., 2005). Among them, the most studied family of receptors are the TLRs, which are

characterised by their abilities to sense invasion of microbial pathogens, including bacteria, fungi, protozoa and viruses (Takeda & Akira, 2005). To date, 10 TLRs are identified in humans and 13 are found in mice (Lim & Staudt, 2013), of which TLR1-9 are conserved in both species (Takeda & Akira, 2005). TLRs are located either on the cell surface (TLR1, 2, 4, 5, 6) or to the endosomal compartments (TLR3, 7, 8, 9) (Wagner, 2004). In response to pathogens, TLRs form either homodimers as in the case of TLR3 (Takeuchi et al., 2000) or heterdimers like TLR2 andTLR1 or TLR6 (Ozinsky, Smith, Hume, & Underhill, 2000).

For most TLRs (TLR1, 2, 4-9), TLR-induced signalling is controlled by the myeloid differentiation primary response gene 88 (MyD88) adaptor molecule. After recognition of ligands, MyD88 recruits IL-1 receptor-associated kinase (IRAK)-4, which in turn causes the phosphorylation of IRAK-1 and subsequently the phosphorylation of TNF receptor activated factor (TRAF) 6, leading ultimately to the activation of transcription factors NF-κB and activator protein 1 (AP-1) (Takeda & Akira, 2004) and allow the synthesis of pro-inflammatory cytokines. However, a delayed activation of NF-κB can be observed in MyD88 deficient macrophages in response to TLR4 ligands with no production of inflammatory cytokines (Kawai, Adachi, Ogawa, Takeda, & Akira, 1999). Different studies have demonstrated a MyD88-independent pathway in TLR4 and TLR3 signalling that leads to the activation of STAT1 (Doyle et al., 2002; Takeda & Akira, 2004; Toshchakov et al., 2002).

Sustained activation of macrophages and excessive release of cytokines can be deleterious to the host and contribute to pathogenesis of many immune diseases such as arthritis, experimental autoimmune encephalomyelitis (EAE) and inflammatory bowel disease (IBD) (Wynn et al., 2013). Therefore, multiple mechanisms are involved in the control of macrophage activity. As mentioned above, TLR signalling is essential for the activation of macrophages and it is necessary to have this signalling strongly controlled. Indeed, many negative regulators for TLRs have been identified (Liew, Xu, Brint, & O'Neill, 2005). For example, NOD2 acts as a negative feedback regulator of TLR2. This is supported by the observation that muramyl dipeptide (MDP), a bacterial product which can be recognized by NOD2, suppresses TLR2-ligand-induced Th1-cell responses in wild-type mice but not in NOD2-deficient mice (Liew et al., 2005; Watanabe, Kitani, Murray, & Strober, 2004).

In addition to the negative feedback loop for TLR signalling, the local environmental factors, which include the consumption of critical amino acids (arginine, tryptophan), production of particular hormones (prostaglandinE2) or circulating cytokines (IL-10) (discussed in more detail in section 1.2.5.2), also contribute to the control of macrophage activity (J. Ma et al., 2003; Silberman, Bucknum, Kozlowski, Matlack, & Riggs, 2010).

1.2.5 Macrophage producing cytokines

As previously mentioned, M1 macrophages are characterised by the production of pro-inflammatory cytokines, especially IL-12, along with other cytokines including tumour-necrosis factor (TNF) α , IL-1, IL-6, IL-12, IL-23 and type 1 interferon (Antonios, Yao, Li, Rao, & Goodman, 2013; Fernando O Martinez, Gordon, Locati, & Mantovani, 2006). In contrast, the main cytokine produced by M2 macrophages is IL-10 as well as the production of IL-4, IL-13 and IL-1ra (Antonios et al., 2013; Fernando Oneissi Martinez, Sica, Mantovani, & Locati, 2007). As this thesis will focus mainly on two cytokines, only these two will be discussed in depth.

1.2.5.1 TNF α

Tumour-necrosis factor alpha (TNF α) is a key pro-inflammatory cytokine which plays an important role in the regulation of immune cells by its activation through binding to the two transmembrane receptors on target cells, TNFR1 and TNFR2 (Parameswaran & Patial, 2010). Among those immune cells, macrophages are one of the major synthesizers of TNF α (Flynn et al., 1995; Pfeffer et al., 1993)and also highly affected by TNF α (Ding, Sanchez, Srimal, & Nathan, 1989). The biological functions of macrophages can be regulated by TNF α in many ways. First, It has been shown that the addition of TNF α , together with IFN γ induce the activation of macrophages in part by promoting the Nuclear Factor-Kappa B (NF- κ B) pathway which in turn increase the production of toxic oxygen species and resulting in the production of nitric oxide (NO) (Parameswaran & Patial, 2010; Wesemann & Benveniste, 2003). Second, a number of studies have shown that the autocrine production of TNF α is involved in the long-term survival, FAS-induced apoptosis, differentiation of macrophages(Lombardo, Alvarez-Barrientos, Maroto, Boscá, & Knaus, 2007; Takada, Sung, Sethi, Chaturvedi, & Aggarwal, 2007; Witsell & Schook, 1992), as well as the macrophages proliferation in the presence of macrophage colony-stimulating factor-1 (M-CSF) (Guilbert, Winkler-Lowen, Smith, Branch, & Garcia-Lloret, 1993; Parameswaran & Patial, 2010). Interestingly, in addition to the induction of inflammatory responses, TNF α also exhibits suppressive effects on inflammation, as seen by the inhibition effect on LPS-mediated phagocytosis of apoptotic neutrophils by macrophages (Michlewska, Dransfield, Megson, & Rossi, 2009).

1.2.5.2 IL-10

Interleukin-10 (IL-10) is a crucial immunosuppressive cytokine which is secreted by a wide range of cell types, including almost all the cells of the innate and adaptive system, such as B cells (Fillatreau, Sweenie, McGeachy, Gray, & Anderton, 2002), CD4+ T helper subsets (Th1, Th2, Th9 and Th17) (Jankovic, Kugler, & Sher, 2010; Saraiva & O'Garra, 2010), CD8+ T cells (Maynard & Weaver, 2008), DCs, eosinophils, macrophages (X. Ma et al., 2015).

Binding of IL-10 to IL-10 receptor, which consists of two distinct subunits (Liu, Wei, Ho, de Waal Malefyt, & Moore, 1994), activates the Janus kinase (JAK) - Signal transducer and activator of transcription (STAT) signalling pathway and the activation of STAT transcription factor, in turn, represses the expression of the inflammatory cytokines. For example, Meraz *et al.* have reported that IL-10 is able to inhibit TNF α production in macrophages stimulated with LPS in a JAK1 but not STAT1 dependent manner. (Meraz et al., 1996; Rodig et al., 1998). In addition to STAT1, IL-10 also has been shown to interact with STAT5 in non-macrophages and STAT3 in B cells and macrophages (Finbloom & Winestock, 1995; Weber-Nordt et al., 1996; Wehinger et al., 1996). Using mice with loss-of-function mutation in the STAT3 gene in macrophages and neutrophils, Takeda et al. have reported that these mice exhibited an increase in the production of inflammatory cytokines, such as TNF α , IL-6, in response to LPS and this is largely due to the impairment in IL-10 production by STAT3-deficient macrophages and neutrophils (Takeda et al., 1999).

1.3 The murine peritoneum and the identification of peritoneal immune cells

The peritoneal cavity contains cell subsets similar to those in the lymph node, including macrophages, T cells, B cells, natural killer (NK) cells, mast cells, dendritic cells (DC), eosinophils, of which macrophages and B cells are the most abundant cell types (Ghosn et al., 2010; Misharin, Saber, & Perlman, 2012; Williams, Wagner, Earp, Vilen, & Matsushima, 2010). Since peritoneal macrophages are easily isolated by peritoneal lavage, they are one of the most studied primary macrophages in mice and have been used for various *in vitro* assays to understand the immunbiology of this cell type. It is has been shown that there are two co-existing distinct macrophages subsets which are classified as large peritoneal macrophages (LPM) and small peritoneal macrophages (SPM) according to their size (Ghosn et al., 2010). Furthermore, LPM are the predominant subset under steady state conditions and they are responsible for the maintenance of tissue homeostasis. SPM appear to be a minor subset in the healthy peritoneum but they become the dominant population during an inflammatory response. It is thought that the inflammatory monocytes are recruited to the cavity during infection and subsequently differentiate into SPM, contributing to the production of inflammatory cytokines, such as IL-12, TNF α and nitric oxide (NO) (dos Anjos Cassado et al., 2015).

Both LPM and SPM express the surface markers F4/80 and CD11b but the expression level of markers differs between LPM and SPM; where LPM show F4/80highCD11bhigh phenotype and SPM express only low levels of F4/80 and CD11b (Ghosn et al., 2010). In addition to the different marker expression of F4/80 and CD11b, they also differ in the

expression of other surface markers, such as CD115 (Sasmono et al., 2003), the receptor for CSF-1 and CD14, the co-receptor for TLR4 (dos Anjos Cassado et al., 2011).

As described above, the peritoneal cavity contains a heterogeneous population of immune cells. Therefore, it is necessary to use appropriate combinations of markers to allow the distinguishtion of the different cell types. For example, the expression level of CD11c is similar on many classical DCs but Ly6G is only expressed on neutrophils (Lee, Wang, Parisini, Dascher, & Nigrovic, 2013). In the case of murine circulating monocytes, two subpopulations, namely inflammatory monocytes and residential monocytes, can be distinguished by the level of Ly6C expression in the blood. High levels of Ly6C are expressed on inflammatory monocytes and low Ly6C expression level is on resident monocytes (Auffray et al., 2007). In addition, eosinophils population show a high side scatter (SSC) profile and a low expression of CD11b when analysed by flow cytometry, suggesting SSC profile together with CD11b expression level can be used to identify this cell type (Misharin et al., 2012). The detailed phenotypic profile of LPM and SPM along with the other population of cells in mouse peritoneum is described in table 1.1.

Table 1.1 Cell Surface Markers of Immune Cells in Mouse Peritoneum

Cell types	Subsets	Surface markers
MØ	LPM	F4/80^{high}CD11b^{high}MHC-II^{low}Ly6C^{low}CD14+
	SPM	F4/80$^{int\ to\ low}$CD11b^{int}MHC-II^{high}Ly6C^{low}CD14+
Monocytes	residential monocytes	Ly6C^{low}F4/80+CD115+
	Inflammatory monocytes	Ly6C^{high}F4/80+CD115+
Neutrophils	NA	Ly6G+
Eosinophils	NA	SSC^{high}CD11b^{low}
Classical DCs	NA	CD11c+MHC-II+CD11b-F4/80-
Mast cells	NA	CD117+
B cells	B-1 cells	CD11b^{low}MHC-II+Ly6C-
	B-2 cells	B220+MHC-II+
T cells	CD4+ T cells	CD3+CD4+
	CD8+ T cells	CD3+CD8+
NK cells	NA	NK1.1+/-; CD3+/-; CD8+/-; CD4+/-

(MΦ, macrophage; LPM, large peritoneal macrophages; SPM, small peritoneal macrophages; DCs, dendritic cells; NK cells, natural killer cells; Nature killer MHC, Major histocompatibility complex; SSC, side scatter; NA, not applicable)

1.4 Mouse models in immunology research

The mouse has long been used as a premier mammalian model system for research. They share genes and molecular pathways with humans and in turn mirror human biology well in many respects (Mestas & Hughes, 2004). In immunology research, since its well-defined immune system and a wide range of immunological reagents (Rose, Sakamoto, & Leifer, 2012), the mouse is the experimental model for the majority of immunologists. In addition, using a mouse model also has the advantage of lower cost, strain stability, smaller size and easy breeding. A number of transgenic or knockout mice have been developed since 1980 and have become a major research resource (J. W. Gordon, Scangos, Plotkin, Barbosa, & Ruddle, 1980; Hanahan, Wagner, & Palmiter, 2007). Furthermore, the creation of humanized mice offers a potent tool to study human diseases and biology in mice. For example, a study has used MI(S)TRG mouse strain in which the mice are knocked in four human genes encoding cytokines important for the development of innate immune cell (Rongvaux et al., 2014). Therefore, despite the inevitable biological differences between mouse and human immunology, the utility of a mouse model is powerful in study the human immunity.

1.4.1 Thioglycollate-elicited mouse sterile peritonitis model

Although macrophages in the peritoneal cavity are easily isolated, the number of cells in the unstimulated peritoneal cavity is 1-2x10^6 cells, of which only 40% are macrophages (Misharin et al., 2012). This is because there are not enough cells for the isolation of subsets of cells and for the use *in vivo* or *in vitro* analysis. To overcome the problem of low cell yield, a variety of inflammatory agents such as proteose peptone, sodium caseinate, and thioglycollate have been applied to stimulate the peritoneal cavity resulting in an increased peritoneal macrophages yield (Tsunawaki & Nathan, 1984). Moreover, the elicited macrophages are nonspecific activated and stronger metabolically active than their resident counterparts (Karnovsky & Lazdins, 1978).

A thioglycollate-elicited sterile peritonitis mouse model has the above mentioned advantages of providing large quantities of macrophages and the cells are collected easily. Furthermore, thioglycollate is thought to induce a sterile inflammatory response in the injection site. In addition, because only a small volume of thioglycollate (e.g. 1 mL per mouse) is required to achieve an adequate cell yield, it will not result in overt effects on the behaviour of mice.

Thioglycollate contains abundant protein and reducing sugar that, once injected, will lead to the glycation which is a spontaneous non-enzymatic reaction between reducing sugar and proteins, lipids or nucleic acids in a hyperglycaemic milieu. Through a serious of downstream events, this reactions result in the formation of advanced glycation end products (AGE) (Eris et al., 1994). AGEs are a group of heterogeneous molecules and

thought to contribute to the development of many degenerative disease, such as diabetes, atherosclerosis, cardiovascular diseases (Xie, Méndez, Méndez-Valenzuela, & Aguilar-Hernández, 2013).

AGEs are taken up by several different receptors, of which the receptors for advanced glycation end product (RAGE) are the best-studied ones. RAGE is expressed at a low level on a wide range of immune cells, such as neutrophils, T and B lymphocytes, monocytes, macrophages, dendritic cells (Bierhaus, Stern, & Nawroth, 2006) and can be up-regulated during inflammation (Xie et al., 2013). RAGE also collaborates with TLRs resulting in the amplification of the inflammation in the host (Ibrahim, Armour, Phipps, & Sukkar, 2013).

1.4.2 Immune-responsive gene 1 (Irg1) expression in macrophages

Immune-responsive gene 1 (Irg1) is a highly expressed gene in mammalian macrophages during inflammation (Michelucci et al., 2013). Indeed, several studies have observed the strong up-regulation of Irg1 expression under pro-inflammatory conditions in murine macrophages treated with LPS (Gautam et al., 2011; Michelucci et al., 2013). These results suggest that Irg1 might play an important role in macrophage activation during the immune response. Regarding its biological function, Irg1 has been identified as a gene that codes an enzyme synthesizing the antimicrobial metabolite itaconic acid through the decarboxylation of cis-aconite in macrophages in response to LPS induction (Michelucci et al., 2013). In addition to its contribution to the bactericidal activity of macrophages, Irg1 is also associated in regulating mitochondrial reactive oxygen species (mROS) production in response to LPS as well as in the tumor microenvironment. (Sanderson et al., 2015; Sena & Chandel, 2012). Since emerging evidence has revealed the correlation between Irg1 and macrophages as well as its ability in facilitating immune functions of activated macrophages, further investigation of Irg1 expression in macrophages in response to different stimuli is warranted.

1.5 Objectives of this study

There has been a big focus on the role of macrophages in the inflammatory process, with the aim of facilitating the future development of effective drugs targeting macrophages. However, the diversity in macrophage phenotype and function increases the difficulty in interpretation of these cells types in the human innate immunity. Furthermore, very little is known about macrophage activation during sterile inflammation. Therefore, it is important to set up a reliable mammalian model in order to understand the behaviour of macrophages with a special regard to sterile stimuli induced inflammatory responses. For the therapeutic aspect, it is necessary to identify a possible marker or a potential drug target associated with the activation of macrophages. As described before, Irg1 could

potentially be the biomarker for measuring the activation or deactivation of macrophages and/or become a novel target for the treatment of macrophages associated diseases.

The aim of this thesis is to use mice as an *in vivo* model to study the macrophage behaviour in response to a sterile stimulus by measuring the well-established phenotypic markers of macrophages in the mammalian immune system. In addition, this thesis will also investigate the correlation between Irg1 gene expression and macrophage activation in the context of inflammation.

The overall aims of this thesis are:

1. To develop an informative flow cytometry panel that allows the identification of macrophages and other cell types in the murine peritoneal cavity.

2. To define the alterations of macrophage activation that occurs during thioglycollate-induced peritonitis using the developed panel.

3. To evaluate whether thioglycollate-elicited sterile peritonitis mouse model can be applied to detect macrophages response to the anti-psychotic drug clozapine.

4. To determine if irg1 expression is associated with macrophage activation in response to the sterile inflammatory agent thioglycollate.

Chapter 2: Materials and Methods

2.1 Mice

All animals were housed in the Victoria University of Wellington (VUW) Animal Facility, New Zealand and used between 10-16 weeks of age and both male and female mice were used for these experiments. C57BL/6J mice were bred at the Biomedical Research Unit (BRU) at the Malaghan Institute of Medical Research or in the VUW Animal Facility. Mice were maintained in a temperature-regulated environment with a 12:12 light/dark cycle and were raised in filter-top cages. All animal experiments were approved by the VUW Animal Ethics Committee (AEC) under the license 2014R21 (October, 2014) and carried out according to AEC guidelines.

2.2 *In vivo* techniques

2.2.1 Murine thioglycollate-elicited peritonitis model

The sterile peritonitis was produced by the intraperitoneal (i.p.) injection of 1 mL sterile 4% Brewer-modified thioglycollate (Fort Richard, MO, USA). Control mice were injected i.p. with 1 mL sterile PBS. Mice were sacrificed by carbon dioxide asphyxiation to collect peritoneal exudates cells (PEC).

2.2.2 Oral Clozapine administration

Mice were continuously treated with clozapine (60 mg/kg/day; Douglas Pharmaceuticals Ltd, Auckland, New Zealand) in the drinking water. A 6 mg/mL drug stock was prepared by dissolving 240 mg clozapine in 40 mL of 0.1 M acetic acid solution (Appendix B).

2.3 *Ex vivo* techniques

2.3.1 Preparation of cell suspension from the peritoneal cavity

At different time points, PEC were harvested from thioglycollate-treated or PBS-treated mice by peritoneal lavage. Briefly, after euthanasia, the abdomen of each mouse was soaked with 70% ethanol. The outer skin of the peritoneum was gently opened to expose the intact peritoneal wall. 10 mL of either PBS or FACS buffer was injected into the peritoneal cavity using a 23 gauge needle (BD Biosciences, San Jose, CA, USA) attached to a 10 mL syringe (BD Biosciences, NJ, USA). The peritoneum was gently massaged for 10 seconds and suspension containing PEC was extracted using the same syringe. The collected cell suspension of PEC was transferred to a clean 15 mL Falcon tube (Corning, Tamaulipas, Mexico) and kept on ice. The cells were pelleted by centrifugation at 760 x g for 5 minutes and resuspended in either PBS or FACS buffer. To remove red blood cells, the cell suspension was incubated for 2 minutes with 2 mL of red blood cell lysis buffer

(Sigma, St Louis, MO, USA) at room temperature. Cells were then washed with 8 mL of either PBS or FACS buffer, spun at 760 x g for 5 minutes, and the pellet resuspended in the PBS or FACS buffer as before. Live cell numbers were counted by trypan blue exclusion (Section 2.3.2) and adjusted to the desired concentration for further use.

2.3.2 Trypan blue exclusion

To determine live cell counts, the cell suspension was mixed with 0.4% trypan blue (Sigma) in a 1:10 dilution. 10 µl of typan blue/cell mix was pipetted into a Neubauer hemacytometer (Hawksley, Lancing, UK) and manually counted using a compound microscope (CX41: Olympus, PA, USA).

2.3.3 Cytospin and Giemsa stain

10 µl of cells (0.5-1 x 10^6 cells/mL) were cytocentrifuged using a cytospin centrifuge (Thermo Shandon, Runcorn, UK) at 365.81 x g for 3 minutes on glass slides (LabServ). The cells were fixed using 100% methanol for 30 seconds and followed by staining with 10% Giemsa's solution (Appendix B) for additional 30 seconds. Surplus stain was washed off using ddH_2O. After slides were air dried, they were mounted with DPX (Merck, Darmstadt, Germany) and covered with coverslips. The slides were allowed to air dry before microscopic examination.

2.4 *In vitro* technique

2.4.1 RAW 264.7 cell lines culture

RAW 264.7 cells (American Type Culture Collection (ATCC)) are an adherent mouse leukemic monocyte-macrophage cell line. The cell line was cultured in complete T cell media (CTCM) (Appendix B) in T75 mm^2 tissue culture flasks (BD Biosciences). Cells were incubated in controlled conditions (5% CO2 and 37°C) and passaged twice per week. Briefly, old media was removed from flask and cells were washed with 5 mL of PBS (Appendix B) before adding additional 5ml of PBS. Cells were harvest with a cell scraper, spun down and resuspended in 1 mL of CTCM. Cells were counted using trypan blue dye exclusion staining (Section 2.3.2) and seeded (1 x 10^6 cells total) into fresh 10 mL of CTCM in a new T75 mm^2 flask and incubated at 37°C, 5% CO2 for 3 days. Cell confluency was determined on a daily basis.

2.4.2 RAW 264.7 cell lines stimulation

For stimulation, RAW 264.7 cells were seeded at 7 x 10^5 cells per 5 mL of CTCM in a 60 mm diameter petri dish and treated with 20 U/mL interferon gamma (IFN-γ) for 18 hours followed by addition of 200 ng/mL lipopolysaccharide (LPS; Sigma) or left untreated as a control. At 6 hours after stimulation, cells were then harvested, snap frozen, and stored at -80°C until further use.

2.5 Flow cytometry

After isolation, PEC were plated in a U-bottom 96-well plate (Corning) at 0.5-1 x 10^6 cells/mL. Cells were washed with FACS buffer and incubated with 50 µl of anti-mouse CD16/CD32 antibody for 15 minutes in the dark on ice to block Fc receptors. Cells were washed with 200 µl of FACS buffer and centrifuged at 400 x g for 5 minutes. The supernatant was flicked off and cells were resuspended in residual FACS buffer by gently vortexing the plate. Subsequently, cells were either stained for cell surface markers (Section 2.5.1) or for intracellular proteins in combination with cell surface markers (Section 2.5.2). Antibodies (Appendix A, Table A1) used in the experiments were diluted to previously determine optimal working concentrations. Samples were analysed with a FACS-Canto II (Becton Dickinson, San Jose, CA, USA) equipped with three lasers (488 nm, 640 nm, 405 nm). 5000- 30,000 live events were collected. Unstained samples were used to set voltages for fluorescence channels and single-stained samples were used for colour compensation setup. Data was analysed using FlowJo software version X (Tree Star, Ashland, OR, USA).

2.5.1 Cell surface staining for flow cytometry

After blocking Fc receptors, cells were stained with fluorescently-labeled antibodies (Appendix A, Table A1) for 20 minutes in the dark on ice, followed by two washes with 200 µl of FACS buffer at 400 x g for 5 minutes. When biotinylated secondary antibodies were used, cells were incubated with diluted StreptAvidin for 30 minutes in the dark on ice and washed twice with FACS buffer as described above. Cells were resuspended in FACS buffer and filtered through a strainer to FACS tubes (Corning) before being analysed.

2.5.2 Combined cell surface and intracellular staining for flow cytometry

Cells were first stained with conjugated antibodies for the detection of cell surface antigens as described above (Section 2.5.1), followed by fixation with 100 µl of 1% paraformaldehyde (Appendix B) in the dark for 15 minutes, and subsequently washed twice in FACS buffer. Cells were permeabilized for 10 minutes in 0.1% saponin buffer (Appendix B) allowing the antibodies to penetrate the cell membrane. After centrifugation and discarding of the supernatant, intracellular cytokines were stained with cytokine-specific conjugated antibodies for 30 minutes on ice protected from light. Cells were washed in 150 µl of 0.1% saponin buffer and subsequently washed in 200 µl of FACS buffer, prior to resuspending the pellet in 300 µl of FACS buffer for flow cytometric analysis.

2.6 Quantitative real-time polymerase chain reaction (qRT-PCR)

2.6.1 Total RNA isolation

1-1.5 x 10^6 cells were homogenized in Trizol® (InVitrogen, CA, USA), and total RNA was extracted using the Direct-zol™ RNA kit (Zymo Research, CA, USA) according to the manufacture's instruction. All steps were performed at room temperature. Briefly, Trizol samples were mixed in 1:1 ratio with absolute ethanol (Fisher Scientific, NJ, USA) and added to the Spin Column where RNA binds to the membrane. Genomic DNA was eliminated by incubation with DNase I for 15 minutes. The column with bound RNA was washed with two different buffers with decreasing salt concentration. RNA was eluted 2 times in RNase/DNase-free water and stored at -80°C for further analysis. RNA concentration was determined by measuring absorbance at 260 nm using the NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, DE, USA).

2.6.2 cDNA synthesis

For cDNA reaction at least 250 ng of total RNA in each sample was used as template for cDNA synthesis. First-strand cDNA was synthesized using qScript™ XLT cDNA Supermix (Quanta Biosciences, MD, USA), containing $MgCl_2$, dNTPs, RNase inhibitor, qScript reverse transcriptase, stabilizers, random primers as well as oligo(dT) primer. A final volume of 20 µl of transcription reaction mixture containing total RNA was prepared, following the manufacturer's protocol. Briefly, 250 ng of total RNA and 4 µl of cDNA supermix or RNase/DNase-free water were mixed in 0.2 mL Eppendorf tubes (Axygen, CA, USA) and the final reaction volume was adjusted to 20 µl by adding RNase/DNase-free water. After the solution was briefly centrifuged, the reverse transcription was performed in a PCR instrument (Corbett Research, Sydney, AUS) with the temperature settings detailed in Table 2.1. The cDNA was used immediately for qRT-PCR or stored at -80°C until further use.

Table 1 Reaction Conditions

Cycles	Temperature(°C)	Time
Hold 1	25	5 minutes
Hold 2	50	50 minutes
Hold 3	85	5 minutes
Hold 4	4	∞

2.6.3 qRT-PCR amplification

Quantitative PCR was performed on a CFX96 (Bio-Rad, CA, USA), using TaqMan chemistry. Each reaction mix contained 1 µl of 20x TaqMan® Gene Expression Assay (Applied

Biosystems, CA, USA) (Table 2.2), 10 µl of PerfeCTa® qPCR Toughmix (Quanta Biosciences), 5 µl of RNase/DNase-free water and 4 µl of sample. All samples were assayed in duplicate, with minus Reverse Transcriptase controls (NRT). A water sample was also included in each qRT-PCR experiment, which served as a control for extraneous nucleic acid contamination. The qRT-PCR reaction was performed using the cycling parameters described in Table 2.3. The fluorescence data collected throughout the PCR process was analysed using CFX manager software version 3.0 (Bio-Rad).

Table 2.2 TaqMan® Gene Expression Assay

Gene	Accession number	Product number[1]
Irg1	NM_008392.1	Mm01224532_m1
Hprt	NM_013556.2	Mm03024075_m1
Gapdh	NP_001276655.1	Mm99999915_g1

1. The assays were directly ordered from Applied Biosystems. No sequence information was available.

Table 2.3 qPCR Thermal Cycling Conditions

Temperature(^{o}C)	time	process	cycles
95	30 seconds	polymerase activation	1
95	3 seconds	denature	45
60	30 seconds	anneal/extension	

2.6.4 Agarose electrophoresis

PCR products were checked on a 2% agarose gel for size and purity. The gel was prepared by dissolving 2 g of agarose powder (Bioline, MA,USA) in 50 mL of TAE buffer (Appendix B) and 1.5 µl of ethidium bromide was added in the gel to visualize the qRT-PCR product. Briefly, 5 µl of PCR product was loaded onto the 2% agarose gel and run at 100 volts for 100-120 minutes. The amplicon size was determined when compared to a TrackIt™ 100 base pair DNA ladder (InVitrogen, CA, USA) run in parallel.

2.7 Graphs and statistics

All graphs and statistical analyses were carried out using GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA, USA). For non-parametric comparison of more than two groups a Kruskal-Wallis test was used. For the comparison of 2 variables (e.g. treatment and

time), a 2-way ANOVA with Bonferonni's post-test was. Differences which gave p values of <0.05 were considered significant.

Chapter 3: Flow cytometric approaches for phenotypic characterisation of naïve and thioglycollate-elicited peritoneal cells

3.1 Introduction

As discussed in Chapter 1, the peritoneal cavity has a variety of resident immune cells under steady state conditions including macrophages, eosinophils, B cells and T cells. Among them, macrophages are the most prevalent and can be collected and used to study macrophage functions. In order to gain a better understanding of the function and contribution of peritoneal macrophages during the steady state and inflammation, it is important to have an accurate characterisation of each of the cellular populations present in the peritoneal cavity. However, the precise characterisation of these peritoneal immune cells is difficult, not only because of the overlapping expression of markers in some of cell types, but also the heterogeneity amongst immune cell populations in the peritoneal cavity. Central to the development of our methods was the ability to characterise the specific changes in macrophage populations and their activation state in response to thioglycollate. To achieve this key aim, we developed a myeloid panel to evaluate myeloid subsets with a particular interest in macrophage linage cells, but we also designed a separate antibody panel for the identification of lymphocytes as well. Therefore, the key markers in our myeloid panel include F4/80, CD11b, both of which are the canonical macrophage surface markers, along with Ly6C and CD14, which are mainly used for monocyte detection. Furthermore, to assess the level of macrophage activation, we also measured MHC class II and CD115 expression. The final design of the panels is described in Table 3.1, and the antibodies used for macrophages and other leukocytes are specified in Appendix A Table A1.

By using the antibody combinations rather than relying solely on a single cell specific surface marker, we hope to define individual immune cell subsets that are present in the naive and thioglycollate-elicited peritoneal cavity and investigate how these populations changed during the thioglycollate-induced sterile inflammation.

Table 3.1 Panels for Identifying Myeloid and Lymphoid Cell Populations in the Peritoneal Cavity

Fluorochromes	Myeloid panel	Lymphoid panel
BV421	MHC-II (I-A/I-E)	MHC-II (I-A/I-E)
V500/V510	CD11c (Biotin)	CD117
AlexaFluor 488	CD11b	B220
PE	Ly6C	CD4
PerCP-Cy5.5	F4/80	CD8
PE-Cy7	CD14	NK1.1
APC	Ly6G	CD62L
APC-Cy7	CD115	CD3

(Due to the complexity of the panels and having been limited to the 8 colour flow cytometry, we were not able to use the live/dead stain. Instead, dead cells were defined as events with low FSC and high SSC.)

3.2 Aims

The aim of this chapter is to examine the phenotype of the immune cell compartment present in the steady state peritoneal cavity and define the changes to these populations during inflammation using the thioglycollate-induced peritonitis mouse model.

1. To establish protocols that allow the identification and assessment of mouse peritoneal immune cells using multicolour flow cytometry.

2. To investigate alterations in immune cell subsets during the thioglycollate-induced peritonitis.

3. To investigate the origin and kinetics of peritoneal macrophages in the steady state and responding to thioglycollate.

3.3 Results

3.3.1 Characterisation of the naive peritoneal immune cells

Based on the flow cytometric panels and different phenotype of cell subsets (Table 1.1), we developed our gating strategies for myeloid and lymphoid populations. In the case of myeloid populations as shown in Figure 3.1A, we first gated on the live single cells and subsequently gated to reveal the following subsets: eosinophils ($SSC^{high}CD11b^{low}$), conventional dendritic cells (DCs) (CD11c+MHC-II+CD11b-), neutrophils (CD11b+Ly6G+), macrophages (CD11b+F4/80+CD14+Ly6C-), monocytes (CD11b+CD14+Ly6C+$F4/80^{low}$) and B-1 cells ($CD11b^{low}$ MHC-II+F4/80-). Of these innate immune cells, macrophages were the largest population.

By examining the expression of F4/80 versus MHC-II within the macrophage and

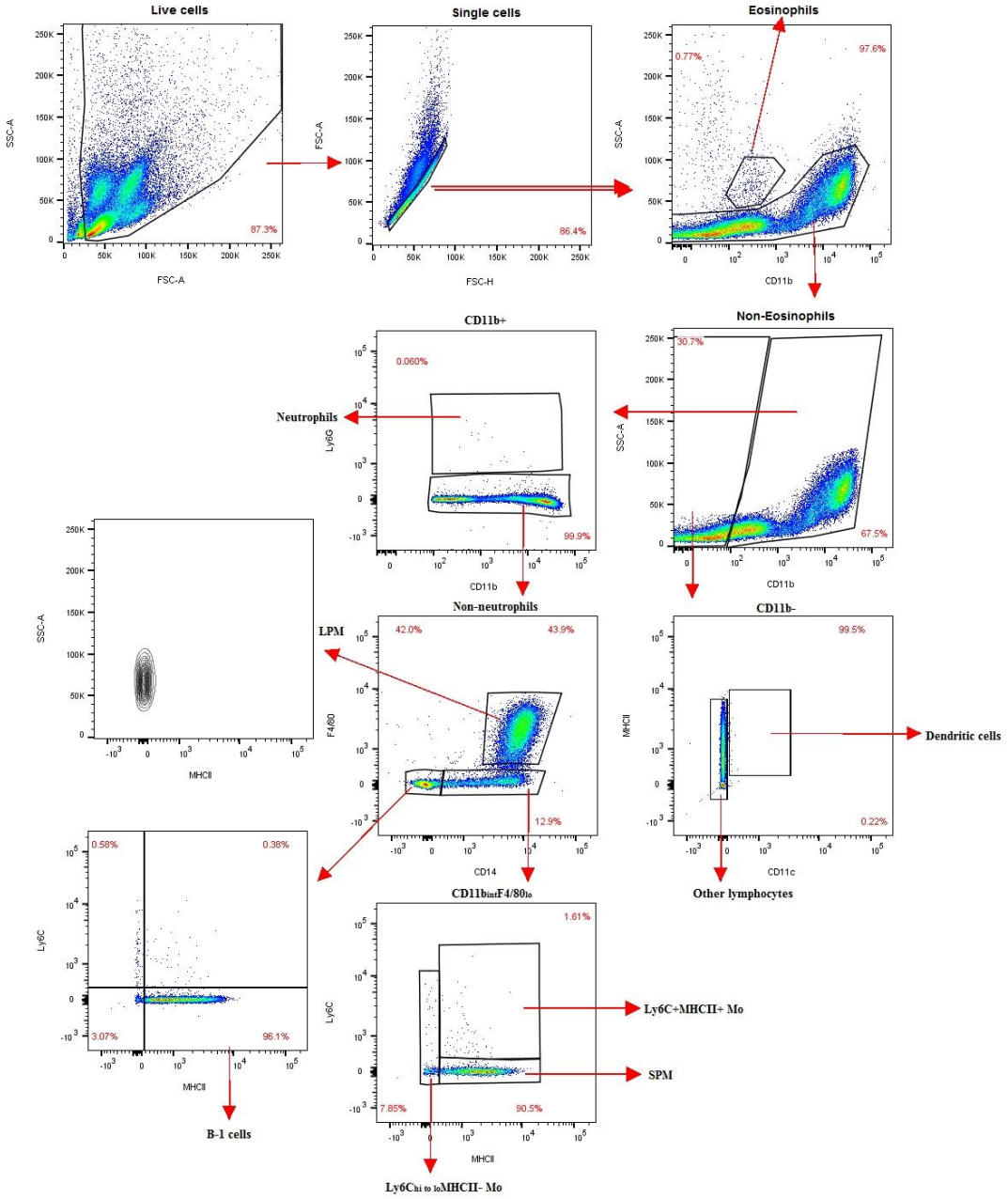
monocyte populations, we found that each of these populations contained two distinct subsets in the naïve peritoneal cavity. The two populations of macrophage were further identified as F4/80^{high}MHC-II$^{low\ to\ neg}$ and F4/80$^{int\ to\ low}$ MHC-II+ cells, which were referred to as large peritoneal macrophages (LPM) and small peritoneal macrophages (SPM) respectively. This observation is consistent with the data from Ghosn *et al*. (Ghosn et al., 2010). On the other hand, a small population of monocytes was distinguishable on the basis of Ly6C expression. In further studies this monocyte population was found to be heterogeneous based upon MHC-II expression. While we developed this panel to define the myeloid populations in the peritoneal cavity, we also found that peritoneal B-1 cells, a unique subset of B cells, could be distinguished by their expression of CD11b. When applied the developed gating strategy to examine the mice with thioglycollate treatment, cell populations as defined above were distinguishable as expected (Figure 3.1B).

In addition to the myeloid cells, we also designed a panel to analyse lymphoid cells, and this panel allowed us to identify CD4+T cells (CD3+CD4+), CD8+T cells (CD3+CD8+), B-2 cells (B220+MHC-II+), as well as NK (NK1.1+CD3-) cells (Figure 3.1C). A previous study has shown that there is also a small population of natural killer T cells present in the normal peritoneal cavity, but despite using the combination of CD3 and NK1.1 in our study, we were unable to distinguish the NKT cells from other lymphocytes (Ghosn et al., 2010). This result could be due to several reasons such as NKT cells only represent a very small population in the peritoneal cavity, or could be due to the high levels of background fluorescence caused by the high autofluorescence of myeloid cell populations that fluoresce in PE-Cy7 channel which was used for NK1.1 or its isotype control and therefore, results in rendering the NKT cells undetectable. Additionally, the detection of CD117, the mast cell growth factor receptor, allowed identification of mast cells, and CD62L was used as an activation marker to determine the activation status of lymphoid cells. By using the combination of myeloid and lymphoid cells panels, we wished to build a more complete picture of the immune system in the peritoneum and help us to understand how resident immune system changes during inflammation.

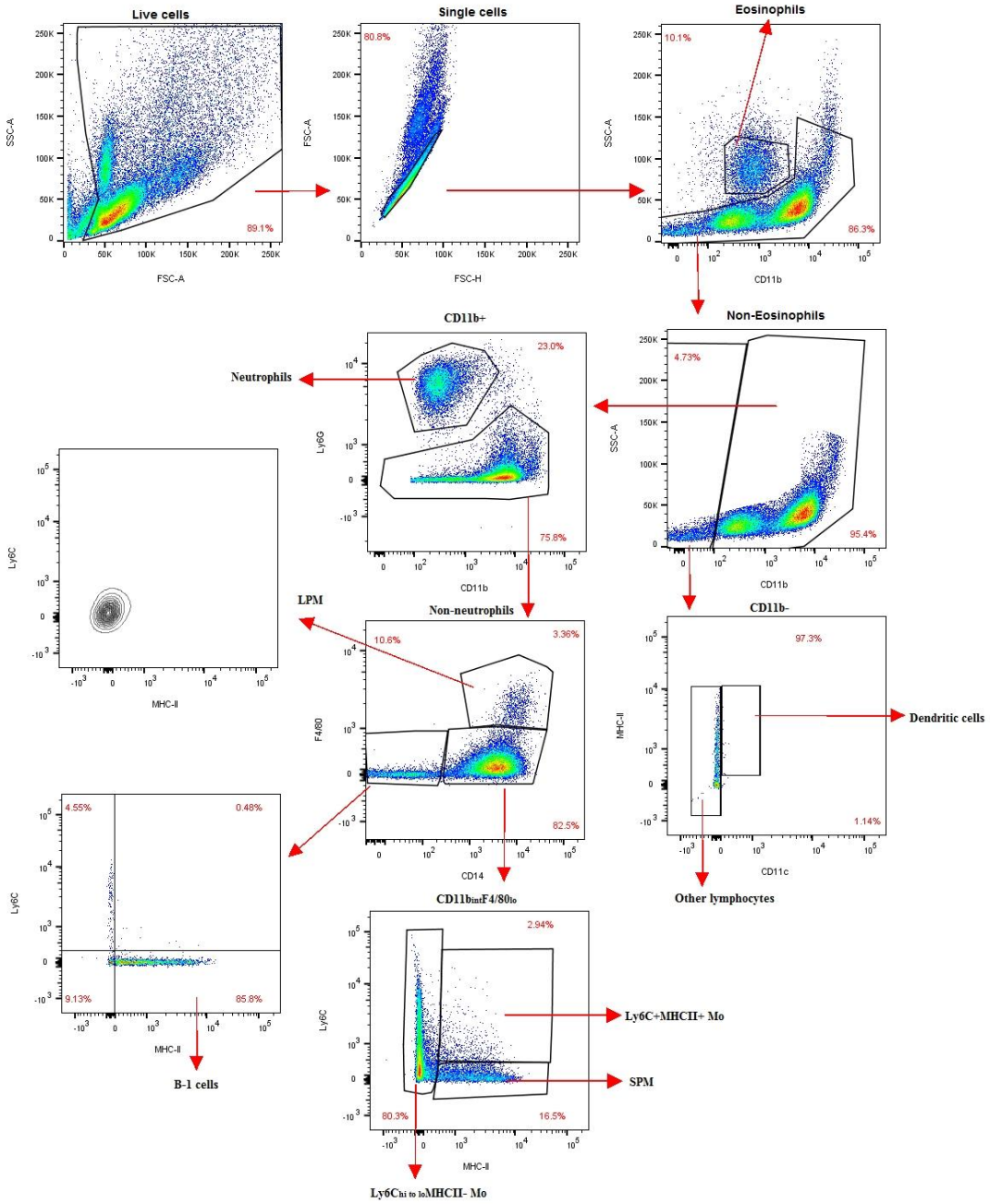
Although the functionality of each cell type has not yet been assessed by using these two gating strategies, the flow cytometric analysis has enabled us to quantify each subset of cells present in the peritoneal cavity. To quantitate peritoneal immune cell subsets, the percentage of a particular cell subset is measured by flow cytometry and the absolute cell count is determined by using the following calculation: *Absolutes (Abs) number of cell of interest = total number of live single cells in each sample (section2.3.2) x percent of cell of interest*. The data collected from two untreated mice showed the presence of a high number of macrophages and B cells in the naïve peritoneal cavity (Figure 3.1D). Although we detected two subsets of macrophages in the steady state peritoneum, the majority of

those cells were identified as LPM displaying high levels of F4/80. They constituted approximately 30% of the total cells, whereas SPM were found to only make up 6-8% of the total cell population. Using both panels, the B cells identified in the naïve peritoneal cavity were separated as two subsets: B-1 cells and B-2 cells. As described in Chapter 1, B-1 cells represent a unique B cell population and are found predominantly in the peritoneal and pleural cavities. B-1 cells were identified as CD11b^{low}MHC-II+ and we found these cells consisted -25% of the peritoneal cells. In contrast with B-1 cells, B-2 cells with the B220+MHC-II+ phenotypes represent about 10% of total peritoneal cell populations. Together, B-1 and B-2 cells are likely to comprise -35% of the total cells as observed in our study. Thus flow cytometric analysis allowed us to analysed the proportions and absolute numbers of immune cell populations in the resting peritoneal cavity and we found that B cells (-35%) and LPM (-30%) are the major cell types, along with SPM (4-7%), T cells (6-8%) and other cell types, including monocytes, eosinohpils, neutrophils, mast cells, DCs contribute to the heterogeneous immune cell populations in the peritoneal cavity.

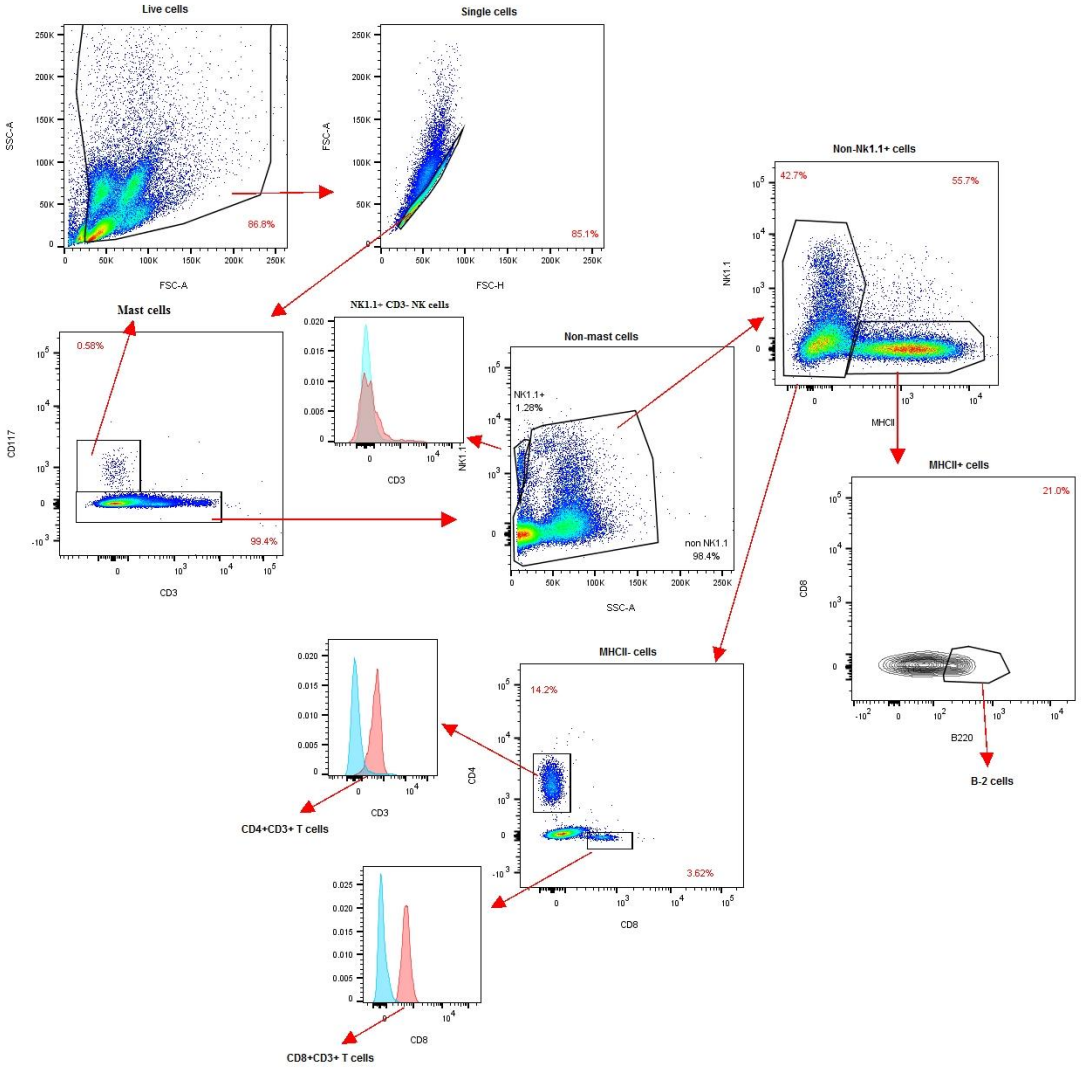
A. Peritoneal exudates cells from naïve mice (Day 0)



B. Peritoneal exudates cells from mice at Day 1 after thioglycollate injection



C. Peritoneal exudates cells from naïve mice (Day 0)



D

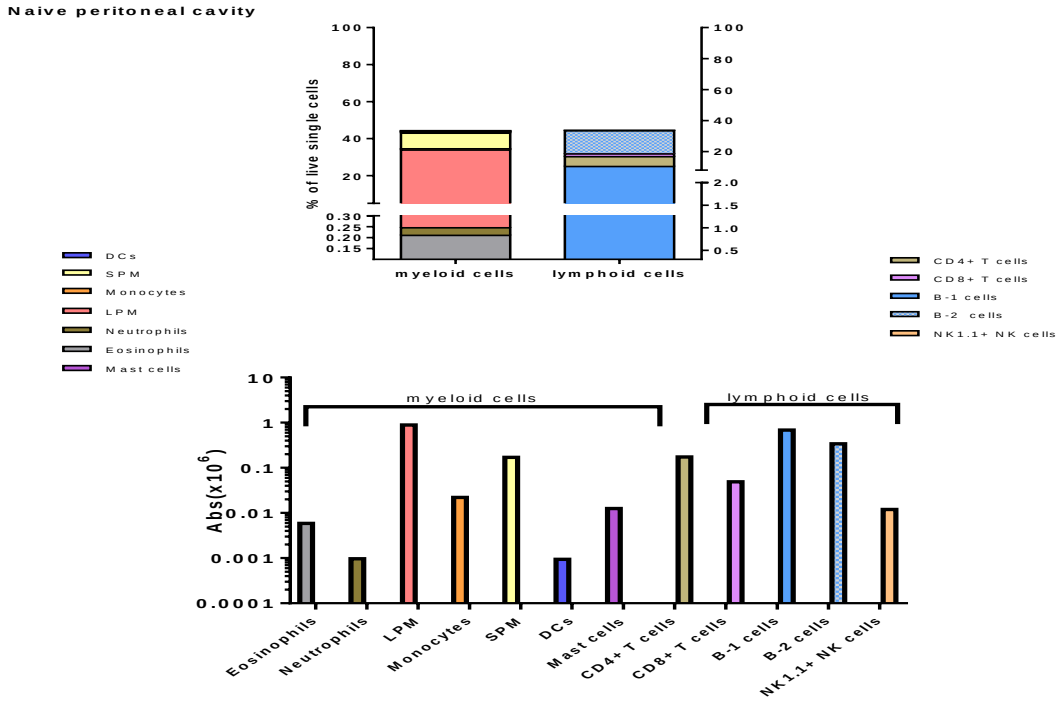


Figure 3.1 Flow cytometric analysis of immune cells in naïve and thioglycollate treated mice peritoneal cavity

A) Gating strategy used to identify peritoneal immune cell populations in naïve mice. Debris and dead cells were excluded on FSA-A versus SSC-A dot plots, and subsequently doublets were excluded on FSC-H versus FSC-A dot plots. Eosinophils were gated out using CD11b versus SSC dot plots. The non-eosinophils populations were further divided by the expression of CD11b, followed by the remove of CD11C+ DCs and Ly6G+ neutrophils. By assessing the expression of F4/80 within the non-neutrophils population, LPM were gated from CD14+ and F4/80^{high} population. F4/80$^{int\ to\ low}$CD14+ cells were divided further into 3 subpopulations based on their Ly6C versus MHC-II properties, which were Ly6C-MHC-II+ SPM, Ly6C+MHC-II+ monocytes and Ly6C+/-MHCII- monocytes. For F4/80- cells, MHCII and Ly6C were used to identify MHCII+Ly6C- B-1 cells. B) Same gating strategy used to identify individual cell populations in mice treated with thioglycollate. PEC were harvested at day 1 after thioglycollate injection. C) Gating strategy used for lymphoid cell population in the peritoneal cavity. Debris, dead cells, doubles were excluded from total cells as described in Figure 3.1A. After gating out mast cells using CD117, MHC-II and CD62L was used in combination with other lineage markers, including CD3, CD4, CD8, B220 and NK1.1 to identify lymphoid cell populations. The comparison of CD3 level (in red) to isotype control (IgG2b-APC-Cy7, in blue) was used to confirm that CD4+ or CD8+ cell populations stained positively for CD3 while NK1.1+ cells stained negatively for CD3. Frequencies of cells in each sub-gate (after debris and doublet exclusion) are expressed as a percentage of live cells. Shown are representative plots from one mouse. D) The percentage and absolute numbers of each cell types identified by flow cytometry in the naïve peritoneal cavity. Data point represents a single experiment with 2 mice.

3.3.2 Thioglycollate-induced alterations on immune cells during peritonitis over time

The yield of macrophages from the unstimulated peritoneal cavity was approximately 1-2x10^6 per mouse, which is also consistent with our results from the previous section(Misharin et al., 2012) To overcome the low yield of macrophages, thioglycollate is widely used for "inducing" peritoneal macrophages, and a number of studies have shown that in addition to macrophages other myeloid cells such as eosinophils, monocytes, dendritic cells accumulate after thioglycollate injection. However, few studies have been performed to investigate the changes on both myeloid cells and lymphoid cells in the peritoneal immune system in mice treated with thioglycollate. Therefore, as a first step, by using the gating strategies shown in Figure 3.1A&C, we examined each peritoneal immune cell population that had been defined in the naïve peritoneal cavity previously to understand the effect of thioglycollate on those cells over a period of time (day 0, day 1, day 2 and day 4). The time points were chosen because previous studies suggested the number of total cells increased dramatically over the first two days and the highest number of peritoneal macrophages was observed 4 days after injection of thioglycollate (Ghosn et al., 2010; Leijh, Van Zwet, Ter Kuile, & Van Furth, 1984). Furthermore, by understanding the changes on immune cells during the thioglycollate-induced peritonitis, we wished to explore potential relationship of macrophages to other leukocytes in the peritoneal cavity.

3.3.2.1 Morphology of cells involved in the thioglycollate-induced sterile peritonitis model

The appearance of the cells in the peritoneal cavity obtained by peritoneal lavage at different time points (day 1, day 2 and day 4) from mice injected with 1 mL 4% sterile thioglycollate or 1 mL sterile PBS are shown in Figure 3.2. Cytospin analysis at each time point revealed significant morphological changes in thioglycollate-elicited macrophages. Those macrophages possessed a large foamy appearance and contained large cytoplasmic vacuoles whereas those in the PBS-treated control mice were much smaller in size and had more condensed chromatin in their nuclei. Firstly, we found that there were comparable numbers of neutrophils present in thioglycollate treated mice or PBS-treaded control mice (Figure 3.2A&B), but the number decreased significantly after day 1 and nearly disappeared at day 4 (Figure 3.2C&D). As a result of thioglycollate stimulation, the number of macrophages reached its peak at day 4 post injection. Indeed, we barely saw other types of immune cells except for large foamy macrophages along with a population of eosinophils which were characterised by the ring-shaped nuclei and the presence of eosinophilic granules. This observation was in accordance with a previous study suggested

that eosinopoihls were the main contaminating cell type in thioglycollate-elicited macrophages (Misharin et al., 2012).

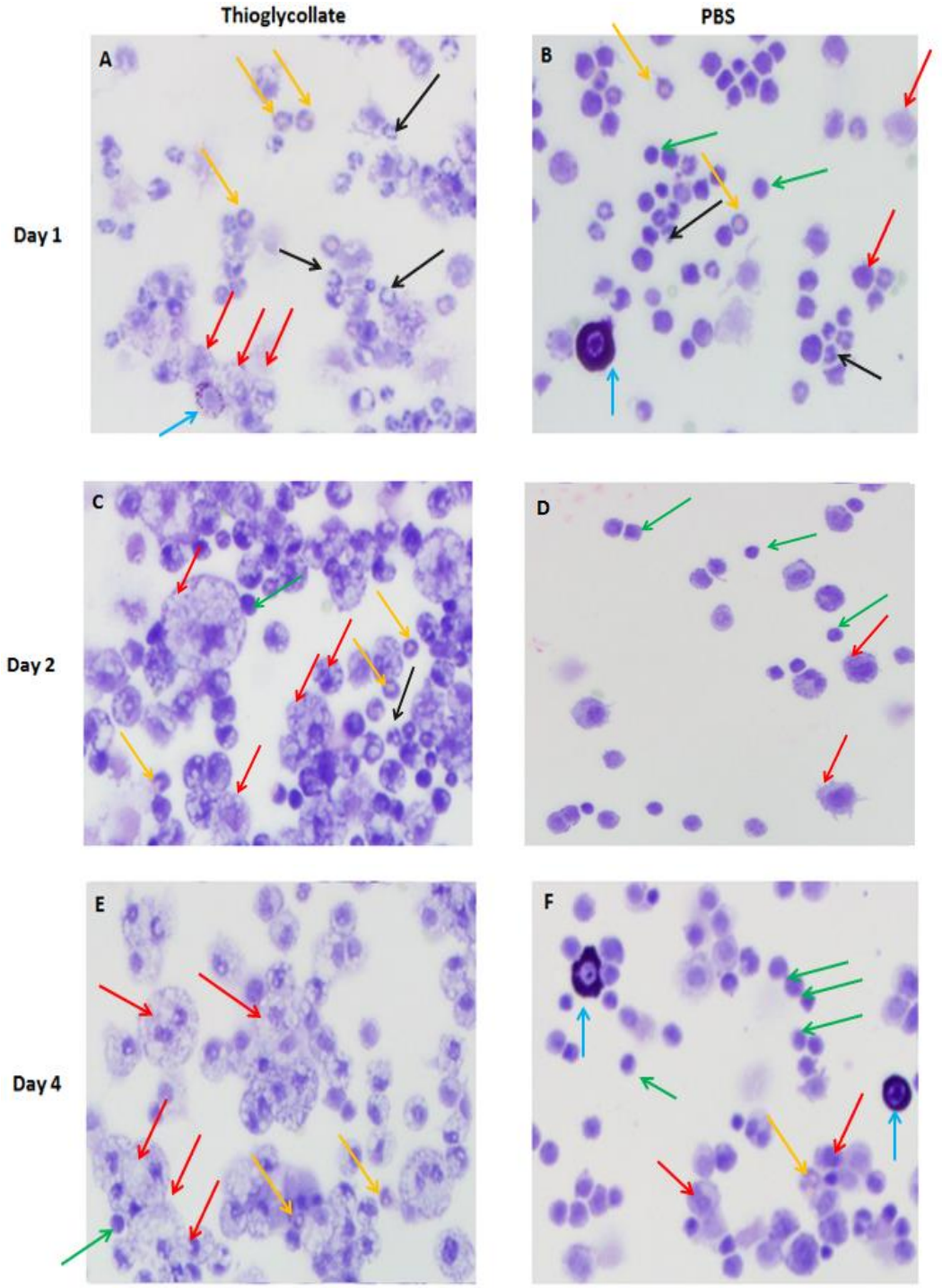


Figure 3.2 Morphological analysis of cytospins of peritoneal exudates cells at the indicated times after injection with thioglycollate or PBS

Peritoneal exudates cells were harvested from mice at day 1(A, B), day 2(C, D) or day 4 (E, F) after i.p. either thioglycollate or PBS. The cell suspension was analysed on cytospins stained with 10% Giemsa. Cell suspension at indicated time points contained different type of immune cells including macrophages/monocytes (red arrows), eosinophils (yellow arrows), neutrophils (black

arrows), mast cells (blue arrows) and lymphocytes (green arrows). Results are representative of three independent experiments. Final magnification x200.

3.3.2.2 Thioglycollate-induced alterations on macrophages and monocytes in the peritoneal cavity

As shown in figure 3.1A&C, there were two macrophage subsets in the naïve peritoneal cavity with the LPM being the most abundant macrophage subset. Intriguingly, examining the changes in macrophage populations at each time point revealed that the proportion of SPM increased dramatically two days after thoiglycollate injection and reached the greatest number at day 4 time point, with an increase of 200% whereas LPM disappeared very quickly by 1 day after injection. Thereafter, they began to increase gradually but the number of LPM did not recover to normal (day 0) (Figure 3.3A&B). Surprisingly, although not statistically significant, it was found that LPM in thioglycollate treated mice accumulated with a slightly slower kinetics compared to the mice injected with PBS (Figure 3.3B). Together, these results suggest that thioglycollate treatment induced a large shift in the ratio of SPM and LPM, with SPM being the predominant macrophage subset in the peritoneal cavity after thioglycollate stimulation. Furthermore, the increased number of SPM during this sterile peritonitis model may not contribute to the conversion into LPM as indicated by the fact that the significant increase in the absolute number of SPM was not paralleled by the recovery of the number of LPM.

As we have identified two subsets of monocytes in the naïve peritoneal cavity, which were phenotypically similar to SPM, we next examined the population dynamic of monocyte subsets. Firstly, we noted that the phenotype of Ly6C+/-MHC-II- monocyte, which we defined in the naïve peritoneal cavity, changed during the thioglycollate-induced inflammation. We observed that thioglycollate injection induced a massive influx of Ly6C+MHC-II- monocytes (inflammatory monocytes) with the absolute number increasing to approximately 5.5 x 10^6 per mouse by day 1. The cell number remained high for the next two days and gradually reached the peak at day 4. In contrast, the PBS control mice had a far lower cell number of Ly6C+MHC-II- monocytes suggesting thioglycollate was able to induce the recruitment of blood monocytes into the inflamed peritoneal cavity (Figure 3.3C). On the other hand, there was also a significant increase in the number of Ly6C+MHC-II+ monocytes observed at day 1 compared to the control mice, but it fell rapidly to baseline by 2 days (Figure 3.3D). Despite the striking difference in cell number of Ly6C+MHC-II- monocytes between mice injected with thioglycollate and PBS, a similar trend in the monocyte subsets was observed, which shifted with time from Ly6C+MHC-II- monocytes toward either Ly6C-MHC-II- monocytes or Ly6C-MHC-II+ SPM (Figure 3.4). It is also noteworthy that we could barely detect Ly6C-MHC-II- monocytes on day 1 but from day 1 onwards, a comparable numbers of Ly6C- monocytes could be observed within the

MHC-II- compartment and this population continually expanded to become the predominant monocyte subset in the peritoneal cavity during inflammation.

The infiltration of inflammatory Ly6C+ monocytes to the inflamed site during inflammation has been well established, and they are considered as the origin of infiltrating macrophages, in this case, SPM whereas little is known about Ly6C- monocytes. In order to further investigate the relationship between peritoneal macrophage subsets and monocyte subsets under inflammatory conditions. We subdivided the Ly6C+/-MHC-II- monocyte population into Ly6C+MHC-II- monocytes and Ly6C-MHC-II- monocytes and looked at their changes during the course of thioglycollate-induced peritonitis. Firstly, we assessed the cell expansion over time by comparing cell numbers of the three individual monocyte subsets (Figure 3.5). We found that there was a great number of Ly6C-MHC-II- monocytes at day 2 but not day 1 after thioglycollate injection and the number of this cell population was approximately 10 times greater than that in control mice. Moreover, those cells also experienced a slight increase on day 4 in the thioglycollate treated mice (Figure 3.5B). A similar pattern of change in the Ly6C-MHC-II- monocyte subset was observed in mice with PBS injection (Figure 3.5B). In contrast, the other two monocyte subsets decreased dramatically from day 1 onwards and dropped to approximately the equal number of those in PBS-treated control mice (Figure 3.5A&C).

Taken together, these findings suggest the infiltrating Ly6C+MHC-II- monocytes are likely to be the origin of the Ly6C-MHCII- monocytes because the number of Ly6C+ monocytes decreased with time and this was paralleled by an increase in the number of Ly6C- monocytes (Figure 3.5A&B). On the other hand, considering the remarkable number of Ly6C- monocytes present on day 2, the possibility exists that there might be a new wave of Ly6C- monocytes recruited into the injured site at a late stage during inflammation. Furthermore, studies have shown that Ly6C+ inflammatory monocytes differentiate into inflammatory macrophages during the early phase of inflammation (dos Anjos Cassado et al., 2015; Geissmann et al., 2010).Therefore, it would be interesting to further investigate the relationship between Ly6C- monocyte subset and macrophages during different stages of inflammation.

As shown previously, a significant decrease was only seen in LPM 1 day post thioglycollate injection, indicating that thioglycollate had little effect on inducing the expansion of LPM in the peritoneal cavity. Interestingly, we also found that the recovery of this resident macrophage population was slower in thioglycollated-injected mice compared to PBS-treated control mice over time (Figure 3.3B). In order to investigate the potential mechanism associated with this phenomenon, we examined the expression of CD115. CD115 is the receptor of CSF-1, which is known to drive the differentiation of monocytes

and the proliferation of macrophages. Additionally, we also assessed the expression of MHC-II to further study if thioglycollate had effect on altering the activation of macrophages. As shown in Figure 3.6, overall thioglycollate treatment induced a strong up-regulation in CD115 expression (CSF-1R) compared to PBS-treated control mice, which maintained approximately the same expression level over time. At day 1 after injection, CD115 expression was lower in thioglycollate injected mice than that in control mice. By 24 hours post injection, up-regulation of CD115 expression was observed on all monocyte and macrophage subsets in the mice injected with thioglycollate and was higher compared to PBS-treated control mice. Moreover, the expression of CD115 in thioglycollate treated mice steadily increased with time. However, due to the considerable biological variability in the individual mice, the increase in the expression of CD115 was only statistically significant in LPM at day 4. Our finding in the up-regulation of CD115 on surface of monocytes and macrophages induced by thioglycollate would somewhat explain why the recovery of the LPM population was slower during this peritonitis model. This could be in part due to the increased recruitment of monocytes from blood and the increased level of CSF-1R on those monocytes upon the stimulation of thioglycollate. Together these events would lead to a increase in the clearance of surrounding CSF-1 by those CD115 highly expressed mature monocytes and ultimately results in a decrease in the proliferation of LPM in the peritoneal cavity (Italiani & Boraschi, 2015).

As described in Chapter 1, macrophages function as professional APCs and interact with T cells by presenting antigens on MHC class II molecules on cell surfaces. Although i.p. injection of thioglycollate was known to induce a sterile inflammatory condition, the population shift in SPM and LPM induced by thioglycollate has been shown similar to that induced by LPS (Ghosn et al., 2010). Therefore, we investigated the effect of thioglycollate on altering the activity of macrophages (SPM) as well as Ly6C+MHC-II+ monocytes by measuring the expression of MHC-II. However, MHC-II expression level showed a decrease in both cells in all mice at day 1 post injection, with lower MHC-II expression was found in thioglycollate treated mice and the level of MHC-II remained nearly constant over time (Figure 3.7). This result may suggest that although injection with thioglycollate in mouse peritoneum is able to induce the shift in the immune cell populations that resemble the effects of LPS, this sterile inflammation model is not likely to promote inflammation through common mechanisms that used by the immune system to kill microbes.

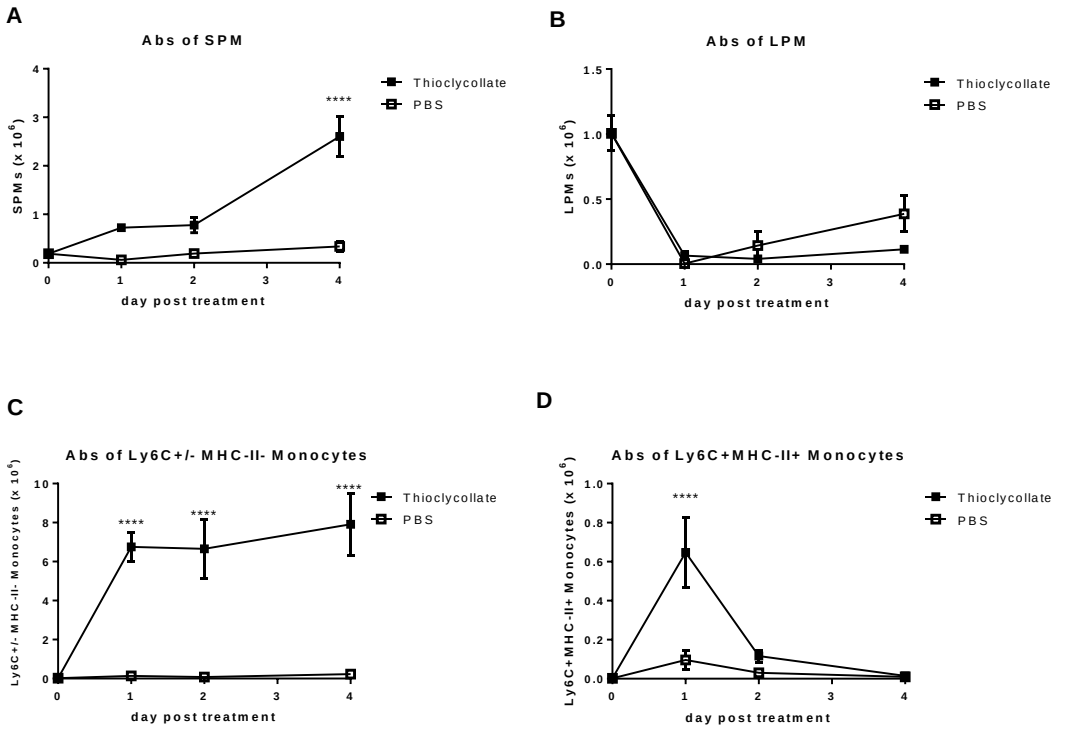


Figure 3.3 Intraperitoneal injection of thioglycollate-induced significant alterations in terms of the number of SPM and monocytes but not LPM in the peritoneal cavity over time

Course of absolute numbers of peritoneal macrophage subsets(SPM(A), LPM(B)) and monocyte subsets (Ly6C+/-MHC-II- Monocytes(C), Ly6C+MHC-II+ Monocytes(D))after i.p. injection of 1 mL 4% sterile thioglycollate or 1 mL sterile PBS. Shown are the means ± SEM of values from individual mice from 2 (n=2-9/timepoint) experiments. ****p<0.0001: thioglycollate-treated compared to PBS-treated control mice by 2-way ANOVA with Bonferronis Multiple Comparison post-test. SPM, small peritoneal macrophages; LPM, large peritoneal macrophages.

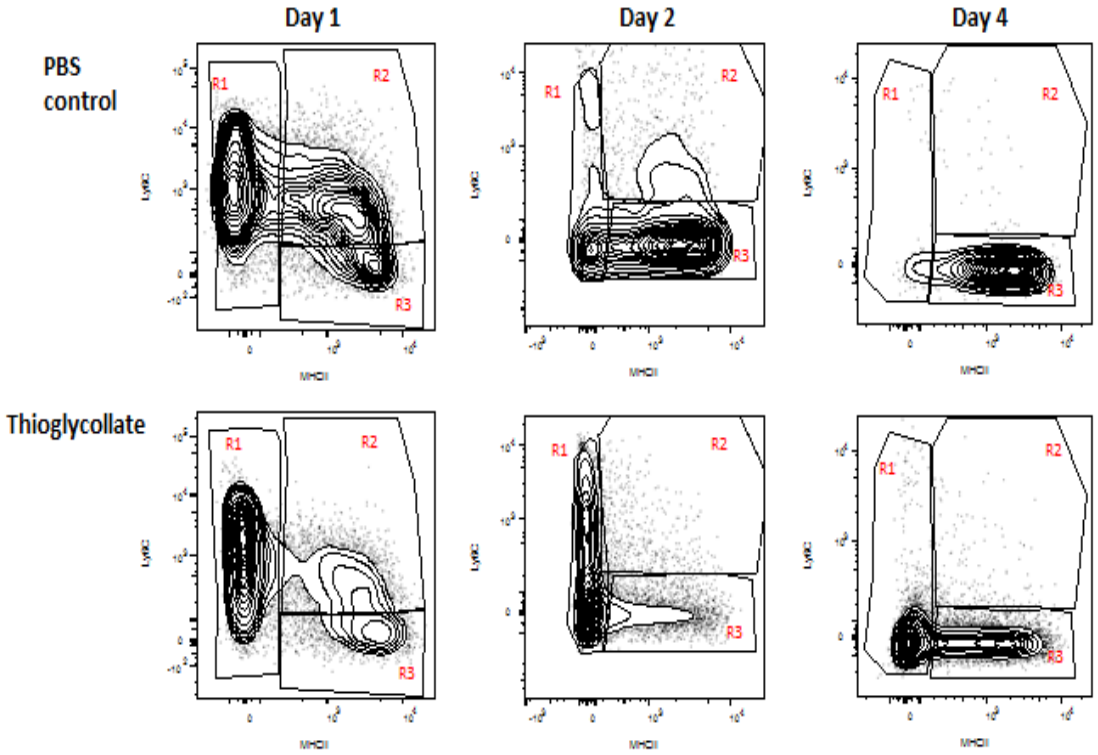


Figure 3.4 Flow cytometric analysis shows the changes in monocytes and SPM populations during the course of thioglycollate-induced peritonitis

F4/80$^{int\ to\ lo}$CD11b+ cells from three time points (day1, day2 and day 4) were analysed on MHC-II versus Ly6C. Results are representative of three independent experiments. Contour plots are shown. Top panel: Control mice injection with PBS; Bottom panel: mice i.p. injection with thioglycollate. R1, Ly6C+MHC-II- monocytes; R2, Ly6C+MHC-II+ monocytes; R3, Ly6C- MHC-II+ SPM.

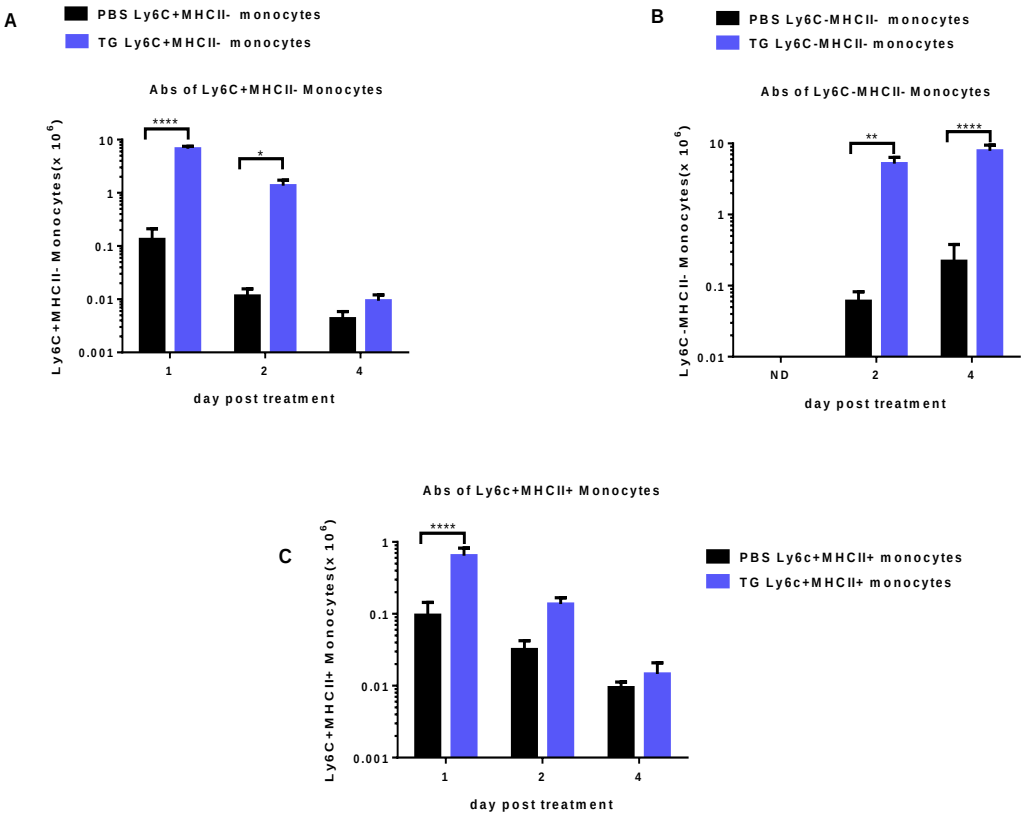


Figure 3.5 Dynamics of the individual monocyte subsets during the course of thioglycollate-induced peritonitis

Course of absolute number of three individual monocyte subsets (Ly6C+MHC-II-(A); Ly6C-MHC-II-(B); Ly6C+MHC-II+(C)) after i.p. injection of 1 mL 4% sterile thioglycollate or 1 mL sterile PBS. Shown are the means ± SEM of values from individual mice from 2 (n=2-9/timepoint) experiments. *p<0.05, **p<0.01 and ****p<0.0001: thioglycollate-treated compared to PBS-treated control mice by 2-way ANOVA with Bonferronis Multiple Comparison post-test. ND, not detected.

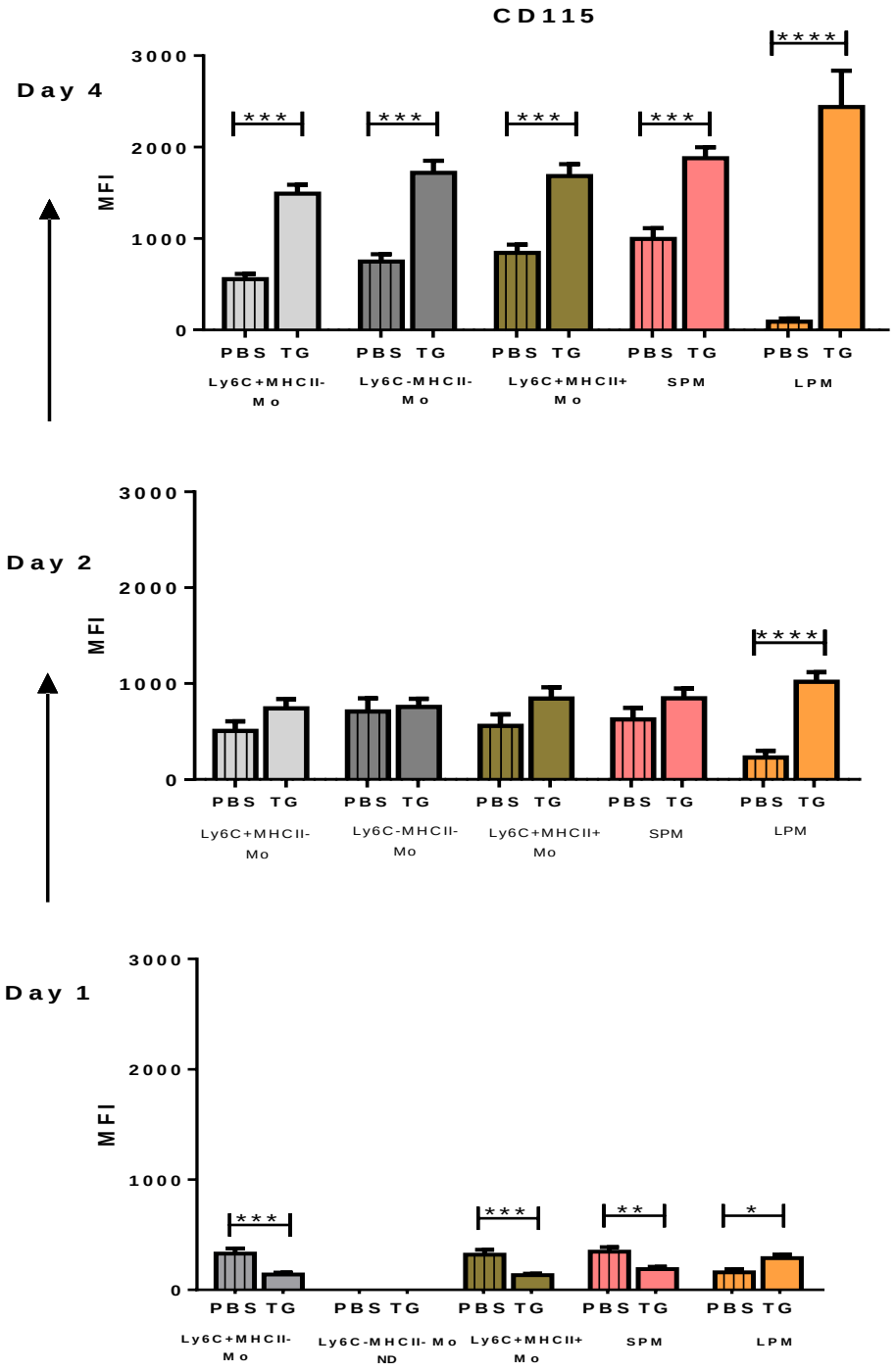


Figure 3.6 Intraperitoneal injection of thioglycollate increased CD115 expression in macrophage and monocytes over time

CD115 expression on Ly6C+MHC-II- monocytes, Ly6C-MHC-II- monocytes, Ly6C+MHC-II+ monocytes, SPM and LPM from the peritoneal cavity at day 1, day 2 and day 4 post injection. Shown are the means ± SEM of values from individual mice from 2 (n=2-9/timepoint) experiments. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001: thioglycollate-treated compared to PBS-treated control mice by 2-way ANOVA with Bonferronis Multiple Comparison post-test. Mo, monocytes; ND, not detected.

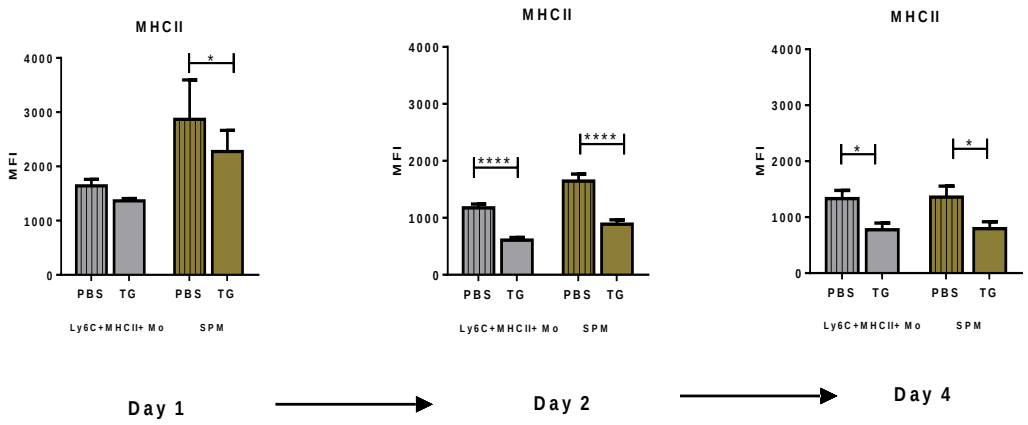


Figure 3.7 Intraperitoneal injection of thioglycollate decreased the expression of MHC-II in SPM and Ly6C+MHC-II+ monocytes over time

MHC-II expression on SPM and Ly6C+MHC-II+ monocytes from the peritoneal cavity was measured at day 1, day 2 and day 4 after injection. Shown are the means ± SEM of values from individual mice from 2 (n=2-9/timepoint) experiments. *p<0.05, ****p<0.0001: thioglycollate-treated compared to PBS-treated control mice by 2-way ANOVA with Bonferronis Multiple Comparison post-test. Mo, monocytes; ND, not detected.

3.3.2.3 Thioglycollate-induced alterations on neutrophils, eosinophils and mast cells in the peritoneal cavity

As shown in Section 3.3.2.2, thioglycollate had major effects on recruiting inflammatory monocytes from blood to the peritoneal cavity, and the population of SPM and Ly6C-MHC-II- monocytes increased significantly over time, reaching the highest number of macrophage like cells at day 4. In order to fully study the effect of thioglycollate, we further investigated the remaining myeloid cell types including eosinophils, neutrophils, dendritic cells and mast cells during the thioglycollate-induced inflammation.

Thioglycollate injection led to a large influx of neutrophils and eosinophils at day 1, with the absolute number being over 6 x 10^6 and 2.4 x 10^6 respectively (Figure 3.8A&B). However, the following day, the number of neutrophils dropped to almost zero whereas only a small reduction in the number of eosinophils was observed at day 2. Thereafter, the number of eosinophils began to increase gradually and remained relatively high in comparison to the absolute numbers of other myeloid cells.

Mast cells were found consistently increased following thioglycollate injection, suggesting the maturation of mast cells may be associated with thioglycollate treatment and it is likely that mast cells play a role in facilitating the recruitment of monocytes and neutrophils by releasing granule compounds and cytokines (Figure 3.8C). In terms of dendritic cells, although we observed changes in the cell number during the course of inflammation, the absolute numbers of dendritic cells were very small (Figure 3.8D). Given that, we did not investigate further on dendritic cells.

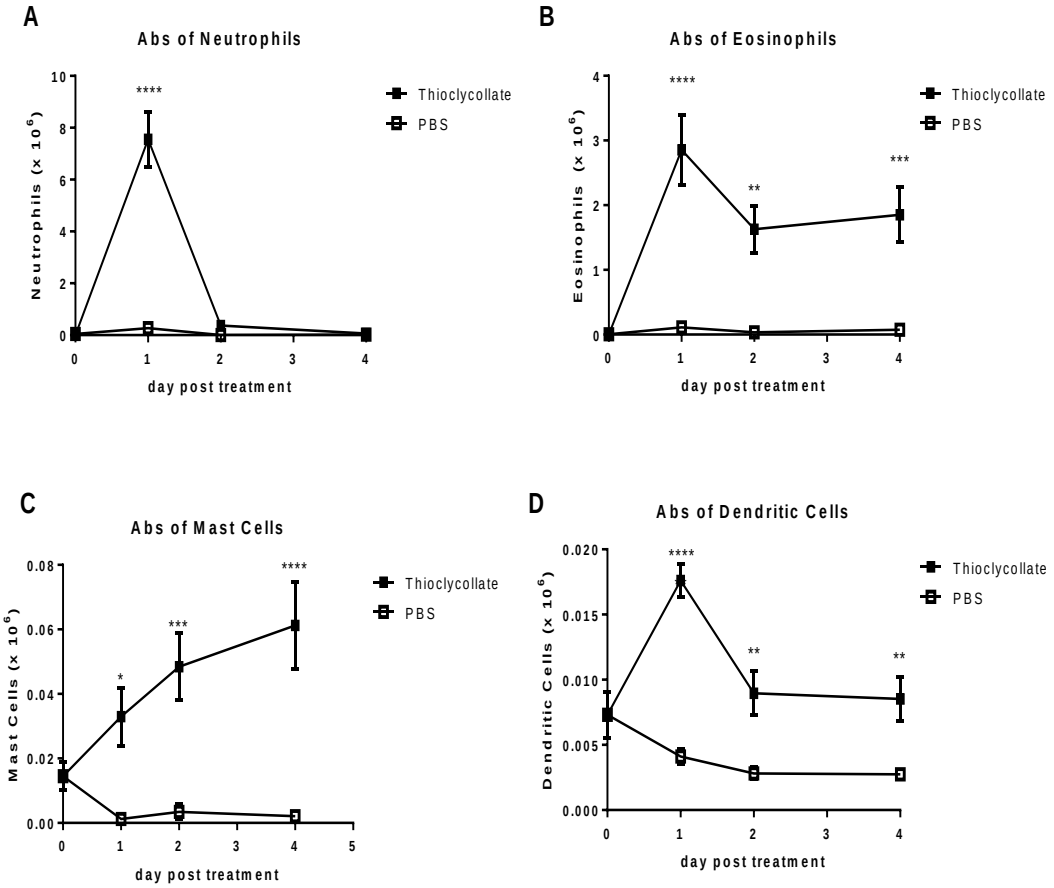


Figure 3.8 Intraperitoneal injection of thioglycollate-induced neutrophils, eosinophils, mast cells and dendritic cells recruiting to the peritoneal cavity

Absolute numbers of A) neutrophils, B) eosinophils, C) mast cells and D) dendritic cells at the indicated time points during thiogolycollate-induced peritonitis. Shown are the means ± SEM of values from individual mice from 2 (n=2-9/timepoint) experiments. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001: thioglycollate-treated compared to PBS-treated control mice by 2-way ANOVA with Bonferronis Multiple Comparison post-test.

3.3.2.4 The effects of thioglycollate on lymphoid cells in the peritoneal cavity

In this section, we investigated the effects of thioglycollate on lymphoid cells during sterile peritonitis. The results showed a similar pattern of changes in T cells and B cells in absolute numbers following the injection of either thoiglycollate or PBS. The cell numbers had a progressive decrease within the first two days post injection followed by a slow increase from day 2 to day 4 and the number of those cells approximately returned to basal level (Figure 3.9A-D). Furthermore, we found that the population of B-1 cells was slightly greater in thioglycollate treated mice than that in the control mice (Figure 3.9C). However, this increase did not reach statistical significance. It should be noted that B-1 cells are the unique B cell subsets with distinct innate-like functions and are found predominately in the peritoneal cavity. Further investigation is required to determine if the increased number of B-1 cells is associated with the stimulation of thioglycollate,

In addition, the number of NK1.1+ NK cells significantly increased in thioglycollate treated mice over time (Figure 3.9E). However, it is worth mentioning that due to limitations in the number of surface markers that we could add into our lymphoid panel, the NK1.1+ NK cell population may contain both NK cells and NKT cells. To further investigate the fate of those cells separately, a flow panel that is more specific to these lymphocyte populations is necessary.

Additionally, we also assessed the expression of CD62L, which is the marker for naïve T cells. However, we found that the levels of CD62L expressing by T cells were variable in individual mice and no distinct pattern could be detected.

Overall, these findings indicate that despite the massive recruitment of monocytes and the large increase of the number of SPM, thioglycollate has little effect on lymphocytes during the observed time period.

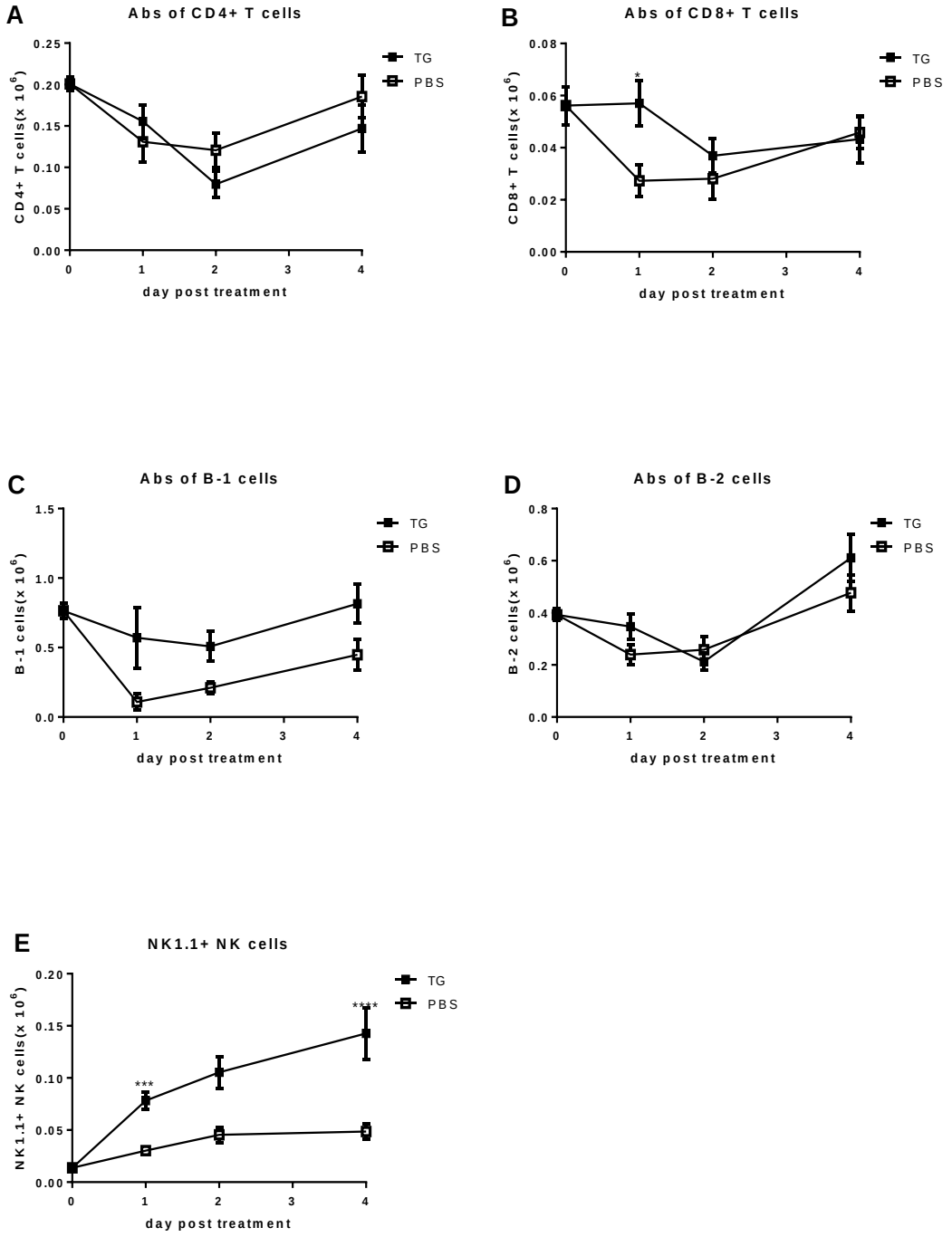


Figure 3.9 Thioglycollate treatment had little effect on lymphocytes in the peritoneal cavity
Absolute numbers of A) CD4+ T cells, B) CD8+ T cells, C) B-1 cells, D) B-2 cells and E) NK1.1 NK cells at the indicated time points during thiogolycollate induced peritonitis. Shown are the means ± SEM of values from individual mice from 2 (n=2-9/timepoint) experiments. *p<0.05, ****p<0.0001: thioglycollate-treated compared to PBS-treated control mice by 2-way ANOVA with Bonferronis Multiple Comparison post-test.

3.4 Discussion

In this chapter, we first developed informative flow panels with the focus on peritoneal macrophage-like cells. To devise a myeloid panel, we used a combination of commonly used macrophage markers (F4/80 and CD11b), an activation marker (MHC-II) and proliferation marker (CD115) to not only identify peritoneal macrophages but also investigate the activation of macrophages. Indeed, along with other markers including CD14, Ly6C, Ly6G and CD11c, we successfully characterised the populations of myeloid cells present in naive peritoneum using multi-parameter flow cytometry. The flow analysis revealed that the cell populations in the naive peritoneal cavity were remarkably heterogeneous and among them, macrophages and B-1 cells were the most abundant cell populations in healthy peritoneal cavity. We also found that peritoneal macrophages were comprised of two distinct subsets, with LPM being the dominant population. The observation from our experiments is in accordance with previous studies (Ghosn et al., 2010; Misharin et al., 2012). This further confirmed that the myeloid panel and strategy we developed was reliable and was able to distinguish different cell populations accurately. Together with the panel for lymphocytes, these panels provided us a fuller picture of macrophages and other immune cells in the peritoneal cavity.

We then applied the panels to examine the alterations in peritoneal immune cells during the thioglycollate-induced peritonitis. This model is a sterile inflammation model, and it has been widely used to isolate peritoneal macrophages and measure the functions of macrophages *in vitro*. Our study attempted to investigate macrophage behavioural changes under this sterile inflammation condition *in vivo* and to explore the potential relationship between aberrant macrophage activity and many human inflammatory diseases, such as inflammatory bowel disease, atherosclerosis and rheumatoid arthritis. We observed visualized immunopheontypic changes in macrophages but not in any other types of immune cells during the course of the thioglycollate-induced peritonitis suggesting that thioglycollate has direct effects on macrophages, although the mechanisms and reasons still remain elusive. Unsurprisingly, our results showed that upon the stimulation with thioglycollate, there were massive increases in both monocyte and macrophage populations. The flow data also revealed that inflammatory monocytes displaying a Ly6C+MHC-II- phenotype were recruited from blood to the peritoneal cavity and it is likely that they continually converted to F4/80+MHC-II+Ly6C-SPM after recruitment. Several studies have shown that it is the inflammatory monocytes that give rise to SPM during inflammation by using confocal microscopy (Ghosn et al., 2010; Okabe & Medzhitov, 2014).

Interestingly, at day 2 we observed a monocyte subset with a Ly6C-MHC-II- phenotype

which was not seen at day 1 after i.p. injection. This subset of monocytes is referred to as Ly6C- monocytes or termed patrolling monocytes. To date, the role of Ly6C- monocytes and their relationship to other immune cells during inflammation is still unclear. As shown in Figure 3.4 and 3.5, our data revealed the dynamics of two monocyte subsets during the course of inflammation. A large number of Ly6C+ inflammatory monocytes first infiltrated into the peritoneal cavity on day 1 after injection followed by a decrease afterwards whereas Ly6C- monocytes were only found at day 2 and continually increased by day 4. This leads to questions concerning the origin of the Ly6C- monocytes, which accumulated in the course of thioglycollate-induced inflammation. Studies of myocardial infarction disease model have shown the two subsets of monocytes are recruited at different stages of infraction(Nahrendorf, Pittet, & Swirski, 2010; Nahrendorf et al., 2007). One other study also used the thioglycollate-induced peritonitis model with L-selectin-deficient mice and reported that monocyte recruitment was impaired during the course of inflammation(Tedder, Steeber, & Pizcueta, 1995). Interestingly, another study using adoptive transfer mouse model investigated the turnover of Ly6C+ and Ly6C- monocytes during parasite-induced liver inflammation and found that Ly6C+ monocytes differentiate mainly into macrophages but also into Ly6C- monocytes in the infected liver (Okabe & Medzhitov, 2014). Taken together, it is likely that the Ly6C- monocytes present in the peritoneum from day 2 onwards are derived from either the conversion of LyC6+ monocytes or blood circulating Ly6C- monocytes which are recruited at a later stage during the course of inflammation. However, it must be noted that there might be different mechanism involved in monocytes recruitment in the context of infection and sterile inflammation.

On the other hand, LPM, the previously dominant macrophage population in the normal peritoneal cavity, disappeared in response to thioglycollate at day 1. This suggests that thioglycollate is not able to induce an increase of LPM. In addition to the decrease in the LPM population at day 1, we also found a lower recovery rate of LPM in thioglycollate treated mice from day 2 to day 4. A previous study reported that the origin of LPM was more likely to be embryonically derived and appeared to be maintained by self-renewal (Yona et al., 2013). These findings raise the question of why injection with thioglycollate has a slight suppressive effect on the development of LPM.

Our result investigating CD115 expression level in macrophages and monocytes may somewhat explain this question. The differentiation of mononuclear phagocytes required CSF-1 which is produced by various of cell types, such as stromal cells (Hamilton, 2008). CD115 is the receptor for CSF-1 and can be found on the surfaces of mature monocytes and macrophages. There is a negative feedback loop between CSF-1 and CSF-1R that controls the proliferation of monocytes and macrophages (Italiani & Boraschi, 2015). We

hypothesise that in this model, the large number of mononuclear phagocytes being recruited to the peritoneal cavity in response to thioglycollate results in the decrease of CSF-1 level in the microenvironment, which in turn suppresses the proliferation of self-renewing LPM. Indeed, one study has reported that intestinal macrophages were depleted after administration of an anti-CD115 antibody (MacDonald et al., 2010). However, in order to verify whether this hypothesis is correct in our model, additional work is warranted.

Along with the recruitment of monocytes, an intense influx of neutrophils was also observed in the peritoneal cavity after injection with thioglycollate and was followed by a dramatic reduction in the numbers, almost to zero. Although neutrophils act as the first line of defense and are recruited at a very early stage during the inflammation, it is surprising that there was a large influx of neutrophils into the peritoneal cavity after the initiation of thioglycollate-induced inflammation. Some studies have also shown that neutrophils are one of the major cell types infiltrating into inflammatory sites in other sterile inflammation models, such as in the MSU-induced inflammation or silica-induced acute peritonitis (Kono, Orlowski, Patel, & Rock, 2012; Shaw, Steiger, Liu, Hamilton, & Harper, 2014). Additionally, it is likely that mast cells are involved in the cell recruitment as well. Indeed, Ajuebor and colleague has shown that the removal of mast cells in LPS-induced inflammation model inhibited the accumulation of monocytes and neutrophils within the inflammatory site (Ajuebor et al., 1999). Furthermore, mast cells play an important role in immune regulation via the interaction with other resident immune cells, such as macrophages during inflammation. Moreover, they may involve in the activation of mast cells via IgM production. However, their contribution to during sterile inflammation remains poorly understood. It would be interesting to study further about neutrophils and mast cells and explore their contributions during the thioglycollate-induced sterile peritonitis model. Furthermore, this observed intense influx of neutrophils also implicated the high phagocytic ability of thioglycollate-induced macrophage populations, which is composed mainly of SPM. *In vitro* studies have shown that SPM appear to exhibit M1 characteristics in response to inflammatory stimuli while LPM display a M2 profile. However, when examining the expression of MHC-II in SPM over time, we could not find the up-regulation of MHC-II expression. Taken together, it is likely that the polarisation of macrophages induced by thioglycollate cannot be simply accommodated in the M1/M2 framework. Therefore, further investigation would be required to assess the functional properties of SPM during the stimulation of thioglycollate.

Additionally, we also found that peritoneal exudates cells obtained from each time point comprised comparable numbers of eosinophils. However few studies have been done to

investigate the functional role of eosinophils and their relationship with other immune cells in the thioglycollate-elicited peritonitis. Therefore, further investigation into the effects of eosinophils is warranted.

Although the absolute number of lymphocyte populations such as CD4+T cells, CD8+ T cells and B-2 cells in the thioglycollate-induced mice were similar to those in control mice, we did find a difference in B-1 cell numbers between treated and control mice with the B-1 cell population became higher after thioglycollate. However, this increase did not reach significance, which may be due in part to the small sample size examined in our study. It would still be interesting to investigate if the reduction in LPM number contributes to the increase of B-1 cell population. A recent study has reported that LPM contribute to the production of IgA by B-1 cell (Okabe & Medzhitov, 2014). Based on this fact, it is possible to investigate functions of B-1 cells in the context of LPM by using the thioglycollate-elicited peritonitis model. An advantage of using this model would be the kinetic of LPM during the course of inflammation, which is the dominated cell population in peritoneum in the steady state and began to disappear in response to thioglycollate administration.

In summary, the thioglycollate-induced mouse peritonitis is a powerful model in terms of monocyte and neutrophil recruitment. Moreover, the recruitment of monocytes appears to be stepwise, with Ly6C+ monocytes first migrate to the peritoneum followed by the infiltration of Ly6C- monocytes. This system is an effective model for assessing dynamics of innate immune cells over the course of inflammation. In addition, it can also be utilized to investigate the nature of cells during sterile inflammation and aid our understanding in certain inflammatory diseases that are caused by non-infectious stimuli.

Chapter 4: Functional analysis of myeloid cells during the thioglycollate-induced peritonitis

4.1 Introduction

In Chapter 3, the individual myeloid cell populations were phenotypically characterised in the healthy peritoneal cavity and at various time points post thioglycollate injection. Using this model of inflammation, we found that a large number of blood monocytes were recruited day one after thioglycollate injection, and, the number of eosinophils increased. The ratio of SPM and LPM as well as the change in the proportion of Ly6C+ and Ly6C- monocyte subsets also changed during the course of inflammation. These results could reveal the presence of a large number of pro-inflammatory innate cells after the initiation of inflammation followed by differentiation of the recruited monocytes and SPM, which was revealed by phenotypic characterization of these populations by flow cytometry. While the results in Chapter 3 reveal distinct phenotypic changes, the effect of thioglycollate-induced inflammation on the functional properties of these cells needs to be determined. Therefore, the current chapter will assess changes in the functionality of different cell populations over time following stimulation, with a particular interest in the cells that have been shown highly affected by thioglycollate.

As described in Chapter 1, macrophages are highly plastic innate cells. They can induce polarized activation in response to a number of different stimuli, which are classified into classically activated macrophages (M1) and alternatively activated macrophages (M2)(Wynn et al., 2013). M1 macrophages can be characterised by the production of pro-inflammatory cytokines, such as TNFα, IL-6, and IL-12, while M2 macrophages are characterised by the expression of several anti-inflammatory cytokines, including IL-10 and TGF-β (Lech & Anders, 2013). In the present study, thioglycollate was used as a sterile stimulus to trigger the activation of peritoneal macrophages. The advanced glycation end products (AGE) in thioglycollate are thought to be the major inflammation inducers in this sterile peritonitis model (Neeper et al., 1992). In a recent study, Xian Jin and colleagues have shown that bone marrow-derived macrophages (BMDMs) produced high levels of TNFα in a dose-dependent manner after AGE stimulation partly via the RAGE/NF-κB pathway, suggesting that AGEs are capable of promoting macrophages polarization into M1 phenotype (Jin et al., 2015).

The previous chapter has demonstrated that, aside from Ly6C+ monocytes, neutrophils seems to be the main recruited leukocytes during the initial phases of thioglycollate-induced inflammatory response. Given that macrophages are one of the professional

phagocytes and are responsible for the clearance of apoptotic cells, it is likely that macrophages may contribute to the uptake of apoptotic neutrophils and that they mediate resolution of inflammation in the peritoneal through anti-inflammatory molecules. Among anti-inflammatory cytokines, IL-10 is thought to be one of the most critical inhibitory cytokines in the regulation of immune response, and the anti-inflammatory activity of IL-10 is mediated in part through the inhibition of NF-κB signalling (Driessler, Venstrom, Sabat, Asadullah, & Schottelius, 2004). Therefore, the expression of TNFα and IL-10 was measured as indicators of pro- or anti- inflammatory cytokine responses in the cell populations.

In addition to assess the production of cytokines in defined cell populations, the expression level of immune-responsive gene 1 (Irg1) in the peritoneal macrophages in response to thioglycollate was investigated. Irg1 is highly expressed in macrophages during LPS-induced inflammation, and it is involved in regulating mROS production in macrophages in response to LPS (Michelucci et al., 2013; Sena & Chandel, 2012). These findings suggest that Irg1 expression could be a potential measurable indicator of macrophage activity during microbe-associated inflammation. Although the model used in this study is to induce a sterile inflammatory response, which is defined as occurring in the absence of infectious stimuli, it is important to note that many DAMPs can also be sensed by PRRs. The cross-reactive nature of these receptors indicates that pathogen-induced inflammation and sterile inflammation are not mutually exclusive (G. Y. Chen & Nuñez, 2010). By measuring the mRNA expression changes of Irg1 in the sterile inflammation model, it may shed light on the mechanism underlying the effects of thioglycollate on peritoneal macrophages.

4.2 Aims

The aim of this chapter is to examine the ability of the cells of interest to produce TNFα and IL-10 during the course of inflammation and measure Irg1 mRNA expression in macrophages in response to thioglycollate. By doing these, it can be distinguished whether the changes in the functional capacity of peritoneal immune cells and the activation of macrophages are via similar mechanisms for the defense against invading microbes.

1. To measure the *ex vivo* TNFα and IL-10 production in the cells of interest in response to thiogycollate by flow cytometry.

2. To determine if thioglycollate induces Irg1 gene expression in peritoneal macrophages by qRT-PCR.

4.3 Results

4.3.1 Thioglycollate did not induce significant alterations in TNFα and IL-10 production by F4/80+ cells and eosinophils

In order to assess TNFα and IL-10 expression in F4/80+ cell populations as well as eosinophils, an adapted Intracellular Cytokine Staining (ICS) protocol was used for the detection of intracellular cytokines. For all ICS experiments, PEC were first stained for surface molecules. This was followed by the ICS protocol without the additional *in vitro* stimulation step in which appropriate reagents such as phorbol myristate acetate (PMA) and ionomycin are used to increase the cytokine levels in an attempt to allow accumulation of cytokines to levels detectable by flow cytometry. Additionally, during the *in vitro* stimulation step, a Golgi transport inhibitor such as brefeldin A (BFA) or monensin is commonly added to the culture to retain the cytokines within the cell. For these experiments, ICS was performed without treating cells with BFA in an attempt to minimize sample loss due to the strongly adherent property of macrophages. Furthermore, it was believed that this *ex vivo* cytokine analysis might be a better indicator of *in vivo* cytokine production. Considering that in the absence of additional stimuli and a Golgi transport inhibitor cytokine production may be low, cytokine production was assessed based on MFI value. Together, by using this adapted protocol, the spontaneous cytokine levels in each cell populations during the steady state and in response to thioglycollate could be measured.

To measure TNFα and IL-10 simultaneously, a flow cytometry panel based on the previous myeloid panel was developed. However, due to the limited number of channels for flow cytometry, the individual monocytes subsets and SPM could not be resolved. Instead, cytokine expression in the F4/80$^{int\text{-}lo}$ compartment was measured, which contains SPM and monocytes subsets. Further investigation into cytokine profiles of each monocyte subset as well as SPM is warranted.

Using the gating strategy shown in Figure 4.1, PEC were analysed 2 and 4 days after injection with thioglycollate or PBS. Untreated mice were used for day 0. TNFα and IL-10 levels were measured in LPM, SPM-like cells and eosinophils isolated from mice injected with thioglycollate or PBS. Firstly, it could be seen that in all three cell populations from untreated mice (i.e. steady state) only low levels of TNFα and IL-10 could be detected (Figure 4.2 Day 0). By day 2 after injection, cell populations from all mice expressed a higher intracellular level of IL-10 compared to cells from day 0 mice (Figure 4.2A-C). Interestingly, there was no difference in IL-10 expression in cells from thioglycollate treated mice compared to PBS-treated mice (Figure 4.2A-C). In contrast to the day 2 time point, the expression of IL-10 returned to baseline by day 4 in all mice, While there was a

slight decrease in IL-10 in each of the cell types from mice treated with thioglycollate compared to those from PBS-treated mice, these changes did not reach statistical significance possibly due to the small sample size used in this experiment (Figure 4.2A-C).

Similarly, TNFα production slightly increased at day 2 and returned to baseline level at day 4. Like IL-10, no alteration in the production of TNFα in cells from mice stimulated with thioglycollate compared to control mice could be seen (Figure 4.2D-F). Together, these data revealed that thioglycollate did not appear to specifically induce IL-10 or TNFα production. Instead the injection itself appears to induce IL-10 and TNFα in macrophages, monocytes and enosinophils by day 2 post injection. Even though the IL-10 levels at day 2 in treated mice were not significantly higher than uninjected mice the elevated levels may still have biological relevance. In contrast, since the expression levels of TNFα showed no change between treated and uninjected control mice, this data suggests that thioglycollate has little or no effect on promoting M1 polarization.

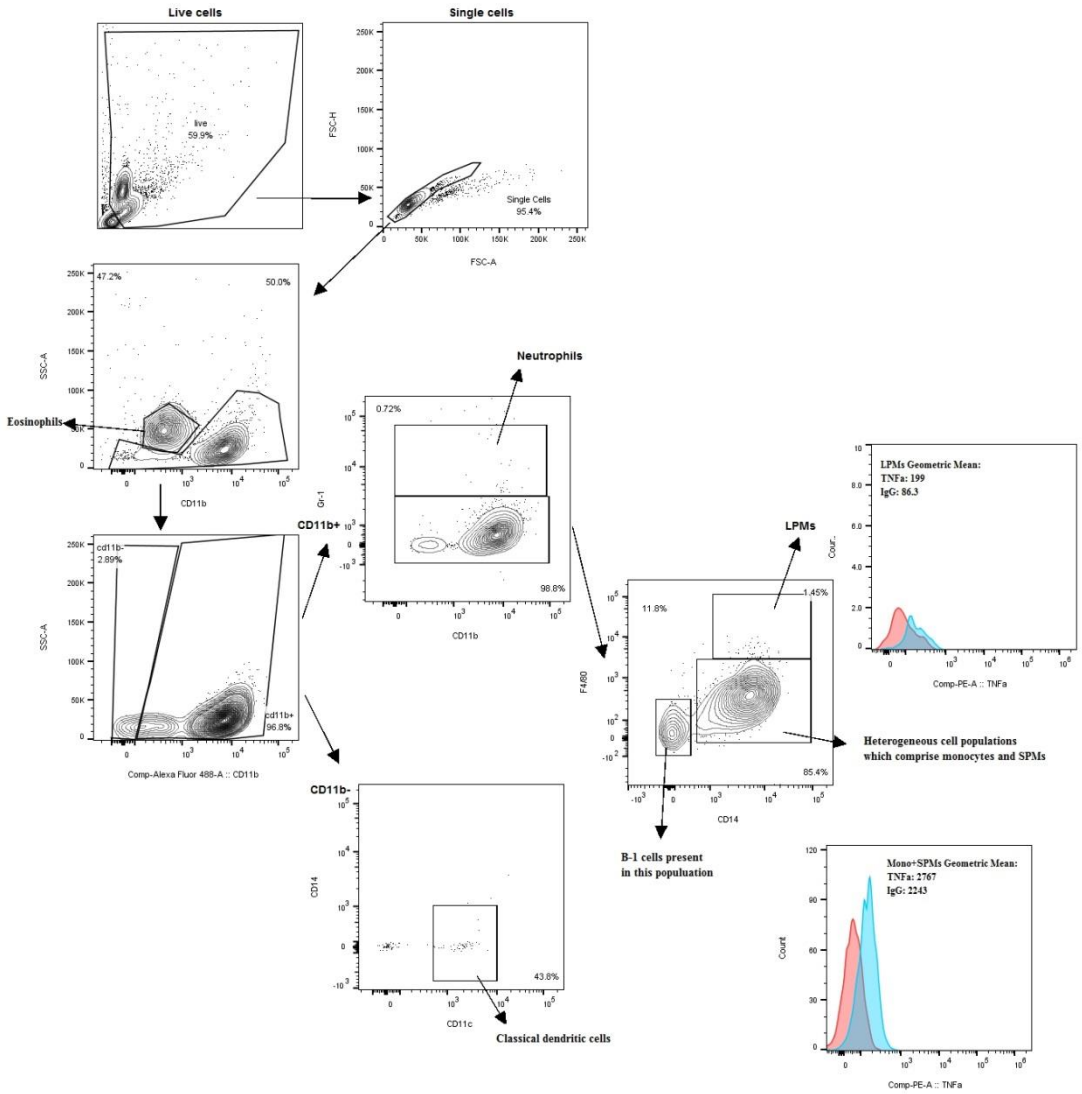


Figure 4.1 Optimised myeloid cells intracellular TNFα and IL-10 gating strategy

First, dead cells and doublets were gated out and single cells were selected using similar strategy described in Figure 3.1. After eosinophils were gated using CD11b vs SSC, cell populations were divided into CD11b+ or CD11b-. Followed by the removal of neutrophils based on the high expression levels of Gr-1, the remaining CD11b+ cells were then selected for comparing different expression levels on F4/80 over CD14. LPM were gated from CD14+ and F4/80^{high} population. All F4/80$^{int\ to\ low}$ cells were gated on as a heterogeneous population of SPM and monocytes. The expression of TNFα and IL-10 was assessed for LPM, heterogeneous populations that comprised SPM and monocytes and eosinophils by subtracting the geometric mean of isotype control (IgG-PE, in red and IgG-BV421, not shown) from the geometric mean of TNFα (in blue) and IL-10 (not shown), respectively. Shown are representative plots from 1 mouse treated with thioglycollate.

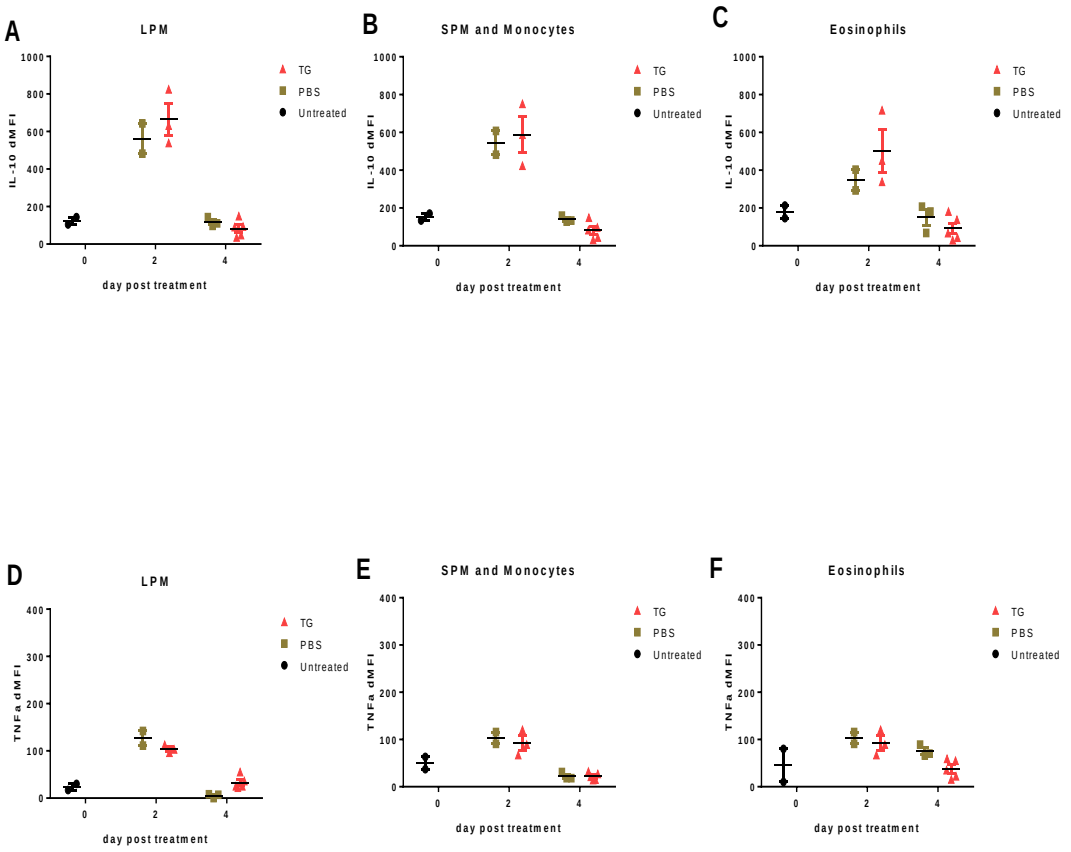


Figure 4.2 Thioglycollate stimulation did not significantly alter the TNFα and IL-10 levels in macrophages, monocytes and enosinophils

Peritoneal exudates cells were harvested from mice at day 0, day 2 and day4 post i.p. injection with either thioglycollate or PBS. Using gating strategy in Figure 4.1, (A&D) LPM, (B&E) SPM-like cells which including a proportion of monocyte populations and (C&F) enosinophils were identified and assessed for intracellular cytokines with flow cytometry. Cytokine expression levels are determined as geometric mean fluorescence intensity difference (dMFI) between anti-TNFα and anti-IL-10 and isotype control. Shown are the means ± SEM of values from individual mice (n = 2-5/group) from 2 experiments. Difference between thioglycollate treated versus untreated or PBS was not statistical significance P>0.05 determined by Kruskal-Wallis test.

4.3.2 Thioglycollate did not affect Irg1 expression in the thioglycollate-elicited peritoneal macrophages

In order to determine whether thioglycollate had an effect on M1 polarisation, Irg1 gene expression after thioglycollate stimulation was analysed since Irg1 was reported to be highly expressed in macrophages upon stimulation with microbial products (Michelucci et al., 2013).

In order to validate the induction of Irg1 expression in macrophages during infectious inflammation, RAW264.7 cells were stimulated with LPS as per methods (Section 2.4.2) and qRT-PCR was performed for Irg1 expression. In line with previous studies (Basler, Jeckstadt, Valentin-Weigand, & Goethe, 2006; Michelucci et al., 2013), there was a significant increase in Irg1 expression by cells with LPS stimulation in comparison to cells cultured in the absence of LPS (Figure 4.3). Although commercially available Taqman gene expression assays were used for qRT-PCR, we experienced problems with getting consistent results from GAPDH expression. qRT-PCR for GAPDH resulted in a band from the NRT negative control, equal in size and intensity to the band from the experimental reaction (Appendix A, Figure A1). It was very hard to prove there was genomic DNA contamination in the sample, because the assessment of total RNA by 1% agarose gel did not show any genomic DNA contamination and the extracted total RNA was processed with DNase treatment to eliminate the trace of genomic DNA before being used for cDNA synthesis. In order to resolve this issue, we tested several commonly used housekeeping genes such as S18, cyclophlin A, HPRT and found that HPRT expression was the most stable and consistent among all samples and no positive signals were observed in NRT samples.

By using the optimised PCR protocol, Irg1 expression was measured at day 2 post i.p. injection with thioglycollate because the highest expression level of cytokines was observed at this time point. In addition, preliminary results from PEC harvested at day 4 post-treatment also showed a decrease in Irg1 expression in treated mice compared to control mice (Appendix A, Figure A2). Although GAPDH was used as control gene in that experiment, no positive signals were found in NRT samples. Similar results were obtained from PEC harvested at day 2 time point showing that Irg1 showed a small decrease in treated mice compared to their control counterparts. Taken together these data suggests that thioglycollate is not involved in classical macrophage polarization (Figure 4.4).

However, this result should be interpreted cautiously for several reasons. First, due to low yield of RNA from PEC isolated from PBS treated mice, pooled mRNA from all mice and not from each individual PEC samples were used. It has been reported that pooled samples do not always replicate the population variations and instead, it is likely to reduce overall variability (Kendziorski, Irizarry, Chen, Haag, & Gould, 2005). Therefore it is

possible that the Irg1 expression in the PBS group may be more variable than shown. Second, the shown results are from a limited number of animals and given that there was large biological variability between the individual mice, further investigation is required to confirm these results. However, even with this large variability, Irg1 expression in all the PEC samples from the thioglycollate-treated group was well below that of the PBS group suggesting that thioglycollate certainly did not lead to an increased expression.

A

RAW 264.7	Irg1 Average C_T	HPRT Average C_T	ΔC_T C_TIrg1-HPRT (mean±SD)	$\Delta\Delta C_T$ ΔC_TLPS+ - ΔC_T LPS- (mean±SD)	Fold difference in Irg1 relative to LPS- control $2^{-\Delta\Delta CT}$
LPS-	34.27±0.71	34.51±0.63	-0.24±0.95	0.00±0.95	1.00(0.51-1.93)
LPS+	31.49±0.17	35.82±0.21	-4.33±0.27	-4.09±0.27	17.03(14.12-20.53)

B

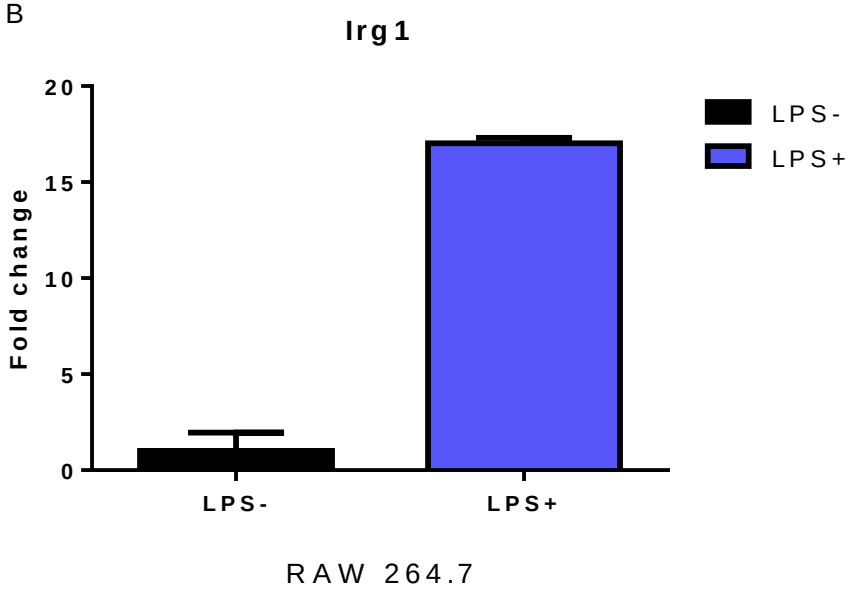


Figure 4.3 Fold change expression of Irg1 in RAW 264. 7 cells treated with LPS, calculated by ΔΔCT method

A) Irg1 was normalised to the housekeeping gene (HPRT) and fold difference express the amount of Irg1 RNA relative to cells treated in the absence of LPS. B) Shown are the means ± SD of 2 RNA samples from 1 experiment.

A

Sample	Irg1 Average C_T	HPRT Average C_T	ΔC_T C_TIrg1-HPRT (mean±SD)	$\Delta\ \Delta C_T$ ΔC_TThioglycollate - ΔC_TPBS (mean±SD)	Fold difference in Irg1 relative to PBS control $2^{-\Delta\Delta CT}$
PBS(pooled RNA)	26.53±0.21	23.63±0.20	2.90±0.21	0.00±0.21	1.00(0.86-1.16)
Thioglycollate A	30.54±0.36	24.75±0.00	5.79±0.36	2.89±0.36	0.13(0.11-0.17)
Thioglycollate B	36.39±1.27	31.52±0.14	4.87±1.28	1.97±1.28	0.25(0.06-0.62)
Thioglycollate C	33.39±0,23	24.75±0.65	8.64±0.67	5.74±0.67	0.02(0.01-0.03)

B

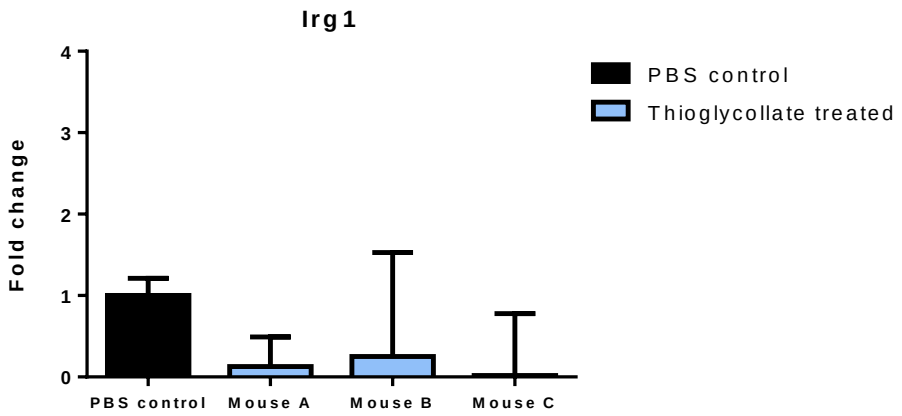


Figure 4.4 Fold change expression of Irg1 at day 2 after thioglycollate treatment, calculated by ΔΔCT method

Since commercially available Taqman Gene Expression Assays were used for both target gene (Irg1) and housekeeping gene (HPRT) which have 100% amplification efficiency, efficiency measurement is not necessary.

A) Irg1 was normalised to the housekeeping gene (HPRT) and fold difference express the amount of Irg1 RNA relative to the PBS control sample. Fold change relative to control was calculated by ΔΔCT method B) Shown are the means ± SD of 4 RNA samples from 1 experiment.

4.4 Discussion

In Chapter 4, the functional changes in thioglycollate-induced immune cells recruited into the peritoneal cavity were investigated to determine whether the phenotypic differentiation of macrophages was accompanied by alterations in their functional activity. First, TNFα and IL-10 production by the cells was measured, which are representative of pro-inflammatory and anti-inflammatory cytokines, respectively. In addition, the activity of macrophages upon the exposure to thioglycollate, a sterile stimulus was studied. In general, macrophages can be polarized into M1 macrophages and M2 macrophages depending on the stimuli. Given LPS will induced M1 macrophages polarization leading to the release of pro-inflammatory mediators, such as TNFα. In contrast, IL-10 is the major cytokine produced by M2 macrophages. Using an adapted ICS protocol, the levels of these two cytokine in LPM, SPM-like cell population and eosinophils were analysed at two time points (day 2 and day 4). The ICS results showed that mice treated with thioglycollate or PBS showed a similar pattern of TNFα and IL-10 production over time. Both conditions increased cytokine production at day 2 post injection and the value dropped back to baseline level at day 4. No difference in TNFα expression level between PBS control and treated mice could be found. In contrast to TNFα expression, the IL-10 MFI was slightly higher in treated mice in all analysed cell populations although it did not reach significance. This suggests that thioglycollate might enhance IL-10 expression by the analysed cells during the early stage of inflammation. On the other hand, it could as well be explained by the massive increase in the number of infiltrating cells leading to increased number of cells individually producing a low amount of IL-10. However, as the results are from a single experiment only and the difference between the treatment groups was not statistically significant, further work is required to confirm the observation. Moreover, in order to gain a better understanding of cytokine changes over the course of inflammation, it would be valuable to include day 1 time point into investigation as well. Given the large recruitment of cells by day 1, it is possible that thioglycollate may exert greater effects on the function of resident and infiltrating immune cells at this time point.

Irg1 gene expression in macrophages during the inflammation was investigated to complement the ICS results and to investigate the activity of macrophages in response to the sterile peritonitis model. This is particularly interesting over the time period when recruited monocytes begin to differentiating into SPM. Irg1 is a LPS-inducible gene but also plays a role in the negative regulation of M1 macrophages activation via increasing ROS production (Y. Li et al., 2013). A study has reported that blocking the ROS generation affects the polarization to M2 macrophages. The qRT-PCR results in the present study showed an unchanged or even a slightly reduced expression of Irg 1 mRNA in cells from thioglycollate treated mice. This reduction may be due to the large proportion of other

cell types in the thioglycollate-treated PEC samples resulting in a proportional reduction in Irg1 mRNA level. Using cell sorting to isolate the specific cell populations may resolve this issue to some extent. Additionally, it would be interested to look at the ROS production changes between treated and uninjected control mice since a decrease in Irg1 expression was observed at day 2 as well as day 4 after thioglycollate stimulation. Given that the recovery rate of LPM was lower in treated mice as shown in Chapter 3, one may expect a lower ROS production in treated mice.

Furthermore, it was shown in Chapter 3 that CSF-1R expression increased over the course of inflammation, possibly due to the negative feedback loop of CSF-1R in control of LPM proliferation. Ultimately, this may contribute to the low recovery rate of LPM as well. Unlike LPM, the recruited monocytes consistently give rise to SPM, but in the absence of microbe, SPM are not activated as indicated through the expression of MHC-II, which shows no difference between treated and uninjected control mice. This would to some extent explain the observation of a lower TNFα production in mice treated with thioglycollate. Although further investigation is required, a dynamic thioglycollate-induced peritonitis model incorporating the preliminary data can be proposed (Figure 4.5). It is worthwhile to note that this model does not include dendritic cells, further investigation on this cell is necessary.

The stages of the thioglycollate induced model of sterile peritonitis are shown in Figure 4.5. The 9 stages are as follows. **1)** The uptake of AGEs (major inflammatory inducer in thioglycollate) by macrophages increases the CSF-1 and chemokine production, which attracts inflammatory monocytes into the peritoneal cavity. Tissue resident mast cells are activated upon stimulation results in degranulation and release of chemokines. Aside from interacting with LPMs, mast cells appear to crosstalk with B-1 cells as well. IgM production by B-1 cells involves in the activation of mast cells. Furthermore, endothelial cells have also been found to express RAGE, the receptor for AGEs as well and mediate the adhesion of neutrophils and monocytes via the interaction of RAGE-Mac-1 (Chuah, Basir, Talib, Tie, & Nordin, 2013). **2)** A massive influx of Ly6C+ inflammatory monocytes and neutrophils migrate to the inflamed peritoneum by day 1 post treatment. **3)** LPM migrate to the omentum at the early stage of inflammation. In the case of LPS stimulation, this migration is depend on retinoic acid and GATA-6 (dos Anjos Cassado et al., 2015) **4)** Recruited monocytes consistently differentiate into SPM while the large number of neutrophils undergo apoptosis due to its short half-life. The apoptotic cells are taken up by phagocytes. **5-6**) At day 2, Ly6C- monocytes begin to be recruited into the inflammatory site attracted by different chemokines released from epithelial cells or cells in the peritoneum. Alternatively, Ly6C+ cells may differentiate into Ly6C- monocytes during the course of inflammation. **7)** The monocyte-derived SPM are not functionally activated due

to the absence of microbes. At this time point, IL-10 expression is unregulated at least in macrophages and monocytes as well as eosinophils. The increased level of IL-10 may hamper the production of TNFα by those cells. Due to the increase of CSF-1R on monocytes and SPM, the number of LPM recovered from omentum is affected. In addition to CSF-1, the reduction of Irg1 expression may has a negative role in LPM recovery via ROS production. **8)** A relatively large population of eosinophils with a slightly higher IL-10 expression level can be found at day 2. They may crosstalk with Th2 cells. Therefore, further investigation into the role of eosinophils involved in this model is warranted. **9**) At the late stage of inflammation, IL-10 and TNFα levels return to baseline levels. SPM and Ly6C- monocytes are the dominate cell pollutions in the peritoneal cavity along with a proportion of eosinophils and B-1 cells and mast cells.

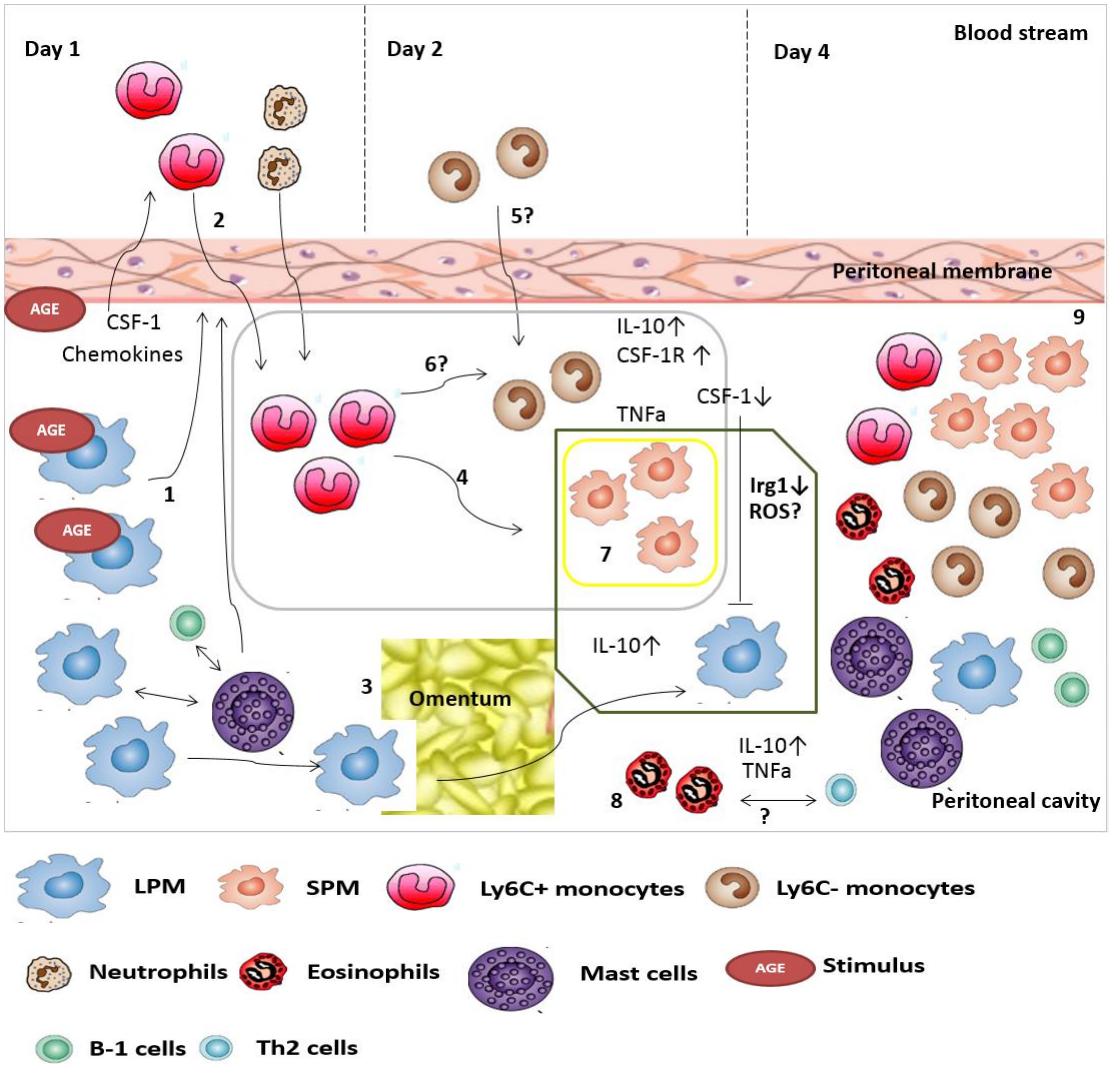


Figure 4.5 The proposed thioglycollate-elicited sterile peritonitis model

Chapter 5: The use of thioglycollate-elicited peritonitis model for drug discovery

5.1 Introduction

As shown in Chapters 3 & 4, thioglycollate treatment induced a significant recruitment of immune cells into the peritoneal cavity although it did not show a significant effect on ex vivo cytokine production or Irg1 expression. In particular, as indicated by the TNFα level, this system did not appear to induce a strong inflammation response. Because the large number of innate immune cells recruited to the inflammatory site that provides an enriched immune cell microenvironment, this characteristic may be used to test how a drug may interfere with immune cell recruitment and reveal how the immune system is changed by a test drug *in vivo*. Moreover, this system is particularly appropriate for understanding macrophage or monocyte activity in the context of test drugs.

In this chapter, the thioglycollate-elicted peritonitis model was applied to test the effect of clozapine treatment in this dynamic biological network. Clozapine is an antipsychotic drug with anti-inflammatory properties. While it is mainly used for schizophrenia, its anti-inflammatory and neuroprotective effects are attracting more and more attention. Moreover, the key cell type affected by clozapine appears to be resident macrophages. Indeed, clozapine is found to elicit anti-inflammatory effects and is associated with protection against inflammation-related neurodegeneration in LPS-induced disease models or animal models of multiple sclerosis, experimental autoimmune encephalomyelitis (EAE) (Hu et al., 2012; O'Sullivan et al., 2014). Therefore, clozapine is a good candidate to test the thioglycollate-elicited peritonitis model.

5.2 Aims

The aim of this chapter was to examine the effect of clozapine treatment in the murine peritoneal inflammation system induced by thioglycollate. Cell recruitment and cytokine production was measured in an attempt to gain a better understanding of how clozapine treatment alters the immune cells during inflammation as well as the potential mechanism underlying the anti-inflammatory effects of clozapine.

1. To elucidate how thioglycollate-induced cell recruitment is altered by clozapine administration.

2. To determine if the production of TNFα and IL-10 in cells of interest is altered by clozapine treatment during the thioglycollate-elicted peritonitis.

5.3 Results

5.3.1 Clozapine treatment decreased thioglycollate-induced cell recruitment

In order to examine the effect of clozapine on cells both in the steady state and inflammation conditions, day 4 was chosen for assessment. Control mice were not given any injection.

Using the gating strategy from Chapter 3, the myeloid cell populations were analyzed. As shown in Figure 5.1, the numbers of SPM and Ly6C+MHC-II+ monocytes at day 4 were reduced in the mice treated with clozapine in response to thioglycollate compared to mice treated with thioglycollate alone (Figure 5.1B&F). These results suggest that while clozapine did not alter the recruitment of Ly6C+MHC-II- monocytes into the peritoneum, it may suppress the further activation and maturation steps following recruitment to the peritoneal cavity (Figure 5.1B, E&F). We also found that, as opposed to SPM like cells, there was a decrease in the number of LPM in clozapine treated mice compared to that in uninjected mice (Figure 5.1A). Although statistically non-significant, this is similar to the effect seen on thioglycollate treated mice (Figure 5.1A). Additionaly, there were no difference observed between clozapine & thioglycollate-treated mice and mice treated with thioglycollate alone with respect to the Ly6C-MHC-II- cells, although the accumulation of Ly6C-MHC-II- cells surpassed that in both uninjected mice groups (Figure 5.1F).

In contrast to the change in SPM and Ly6C+MHC-II+ monocytes, there was a similar number of neutrophils and mast cells in clozapine & thioglycollate-treated mice compared to mice treated with thioglycollate alone (Figure 5.1H&I). However, although clozapine did not alter the number of mast cells in thioglycollate treated animals, mast cell numbers were increased in mice treated with clozapine alone compared to untreated mice (Figure 5.1H&I). On the other hand, analysis of eosinophils also showed a decrease cell number in clozapine & thioglycollat-treated mice (Figure 5.1C). Similarly, Ly6C+MHC-II+ monocytes, defined as inflammatory monocytes, were found decreased in clozapine treated mice compare to naive mice while the opposite pattern was seen in mice administered thioglycollate (Figure 5.1E). Although Ly6C+MHC-II+ monocytes only represent a very small proportion of cells in the peritoneal cavity, it may shed light on the mechanism underlying the effect of clozapine on reducing the infiltrating cells. It might have influence on blood monocytes that leads to the reduced monocytes migration.

Additionally, clozapine alone slightly increased the number of dendritic cells compared to naive mice although the cell number in mice with clozapine in combination with thioglycollate slightly reduced (Figure 5.1H). However, the absolute number of dendritic

cells represents a very small population of immune cells in the peritoneal cavity. The changes in dendritic cell number may have less overall effect on cell populations. All observed alterations did not reach statistical significance, which is in part due to the small sample size. The data presented in Figure 5.1 is from one experiment, and therefore careful considerations must be made in drawing strong conclusions.

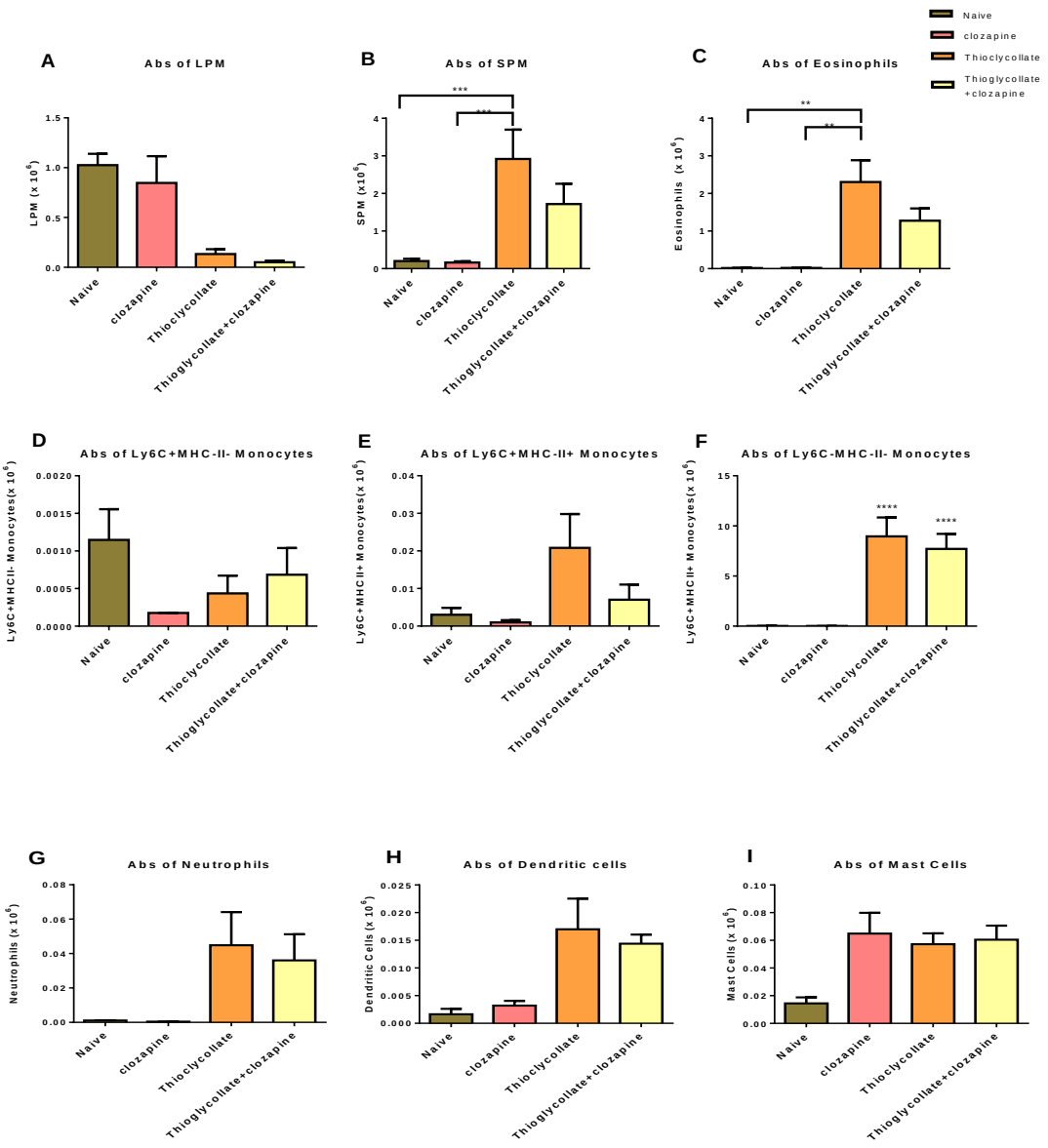


Figure 5.1 Clozapine treatment reduced thioglycollate-induced cell recruitment

Male C57BL/6 mice in treatment group received 60mg/kg/day clozapine in their drinking water for a maximum of 2 days to make sure the mice had taken clozapine while mice in control group received vehicle drinking water. Two days later, thoiglycollate was injected i.p. into mice that had received clozapine or vehicle treatment. Following injection, mice continued to receive clozapine or vehicle drinking water for a further 4 days, after which they were sacrificed and PECs were harvested and analysed for extracellular staining by flow cytometry. The absolute numbers of A) large peritoneal macrophages, B) small peritoneal macrophages, C) eosinophils, D-F) monocytes subsets, G) neutrophils, H) dendritic cells and I) mast cells in mice within each treatment groups are depicted. Shown are the means ± SEM of values from individual mice (n = 2-3/group) from 1 experiment. **p<0.01, ***p<0.001, ****p<0.0001: Difference between clozapine treated alone, thioglycollate treated alone or treated with clozapine and thioglycollate versus PBS alone control mice by 2-way ANOVA with Bonferronis Multiple Comparison post-test.

5.3.2 Clozapine treatment induced alterations on lymphoid cell populations in the thioglycollate-induced peritonitis

The previous experiment showed that clozapine treatment reduced the thioglycollate-induced cell recruitment of specific myeloid populations. In order to gain a better understanding of the effect of clozapine treatment in this model, the alteration of lymphoid cells were assessed. Treatment with clozapine alone decreased both CD4+ and CD8+ T cells (Figure 5.2A&B). This effect was found as well in mice treated with thioglycollate alone. Moreover, mice given thioglycollate and clozapine in combination showed no difference in T cells numbers in comparison with the two single treatments. This suggests that clozapine treatment induces a decrease in T cell populations but this effect was not enhanced in the inflammation conditions. However, it should be noted that results from this experiment vary from the data obtained from time course experiments where higher T cell numbers were observed at day 4 after treatment. One explanation for this might be due to the biological variability in each individual mouse. Therefore, to confirm this result, replication is required.

Unlike the changes in the T cells population, the absolute number of B cells in mice stimulated with thioglycollate alone almost returned to basal level in comparison to their uninjected control (Figure 5.2C&D). However, it seems that B-1 cells are more responsive to clozapine treatment as the number of B-1 cells reduced by half in mice treated with clozapine alone. Interestingly, the decrease of B-1 cells was lower in mice treated with thioglycollate and clozapine in combination, suggesting the population of B-1 cells was enhanced in the context of thioglycollate-elicited sterile peritonitis. In contrast to B-1 cells, mice given both treatments had the lowest cell number of B-2 cells compared to other treatment groups. This data might reveal the innate-like nature of B-1 cells. It would be interested to further study the role of B-1 cells involved in the inflammation response and its relationship with other resident immune cells in the peritoneal cavity.

Finally, there was an increase of NK cells after stimulation with thioglycollate alone, and a similar increase was seen in mice treated with thioglycollate and clozapine in combination (Figure 5.2E). Together these results suggest that clozapine alone or during thioglycollate-mediated inflammation may subtly alter lymphoid as well as myeloid immune subsets in the peritoneal cavity.

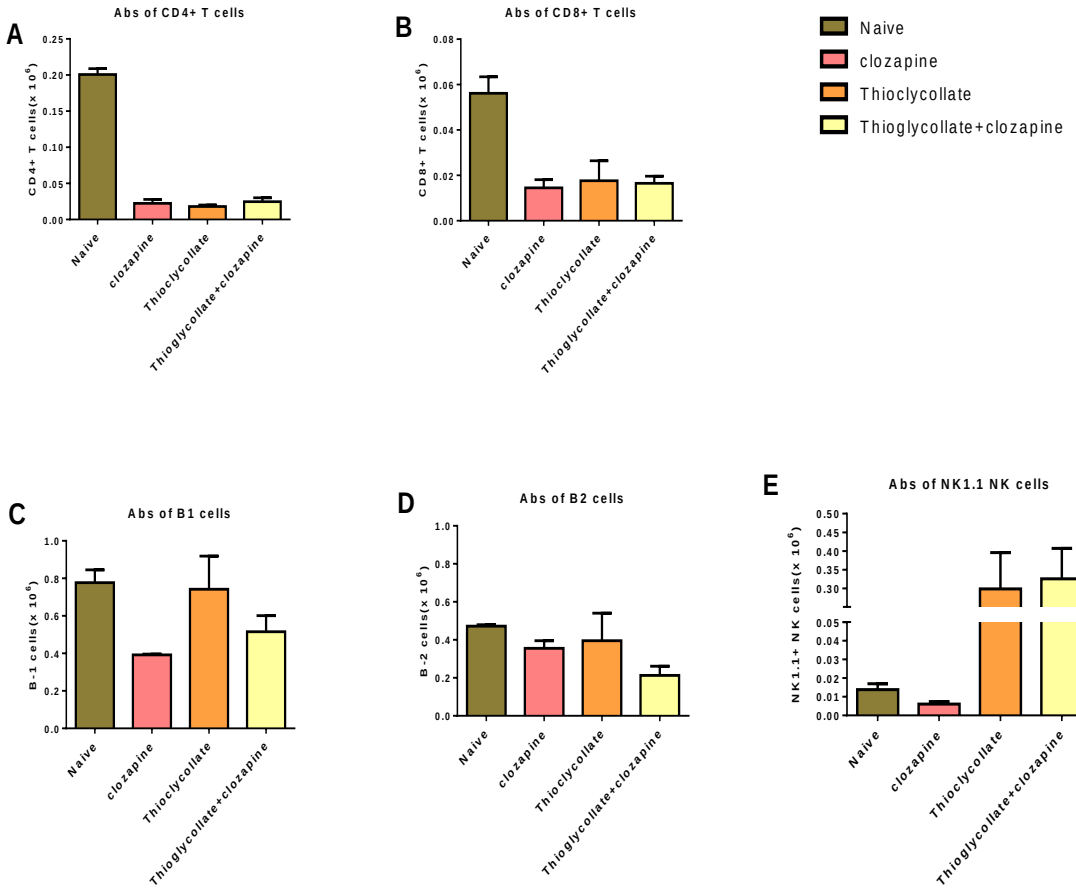


Figure 5.2 Clozapine treatment induced alterations on lymphoid cell populations in the naive as well as the thioglycollate-induced peritonitis

Peritoneal exudates cells were obtained from the same experiment conditions as described in Figure 5.1. The absolute numbers of A&B) T cells, C&D) B cells, E) NK cells in mice within each treatment groups are depicted. Shown are the means ± SEM of values from individual mice (n = 2-3/group) from 1 experiments. Difference within each group was not statistical significance P>0.05 determined by 2-way ANOVA with Bonferronis Multiple Comparison post-test.

5.3.3 Clozapine treatment induced CD115 expression on monocytes subsets and SPM in the peritoneal cavity during steady state conditions

Myeloid cells such as macrophages and monocytes appeared to be the key cell types that respond to clozapine, as indicated by the greatest effects on cell numbers in the thioglycollate-induced inflammation model. Therefore, to assess if clozapine altered the activation state of these cell populations, CD115 and MHC-II expression levels were assessed on macrophage and monocytes subsets. In comparison to naive mice, the expression of CD115 in mice treated with clozapine alone showed an increase on all subsets except for Ly6C+MHC-II+ monocytes (Figure 5.3A). This suggests that clozapine treatment is able to up-regulate CSF-1R on the surface of monocytes and SPM. This data further supports previous findings that clozapine treatment had a suppressive effect on the cell recruitment induced by thioglycollate, which could be in part due to the decreased level of CSF-1 in the peritoneal cavity. Moreover, Ly6C+MHC-II- monocyte population showed a higher level of CD115 4 days after thioglycollate injection. This might be due to the fact that this monocyte subset showed an increase in cell number in clozapine treated mice at day 4 upon thioglycollate stimulation. Together, these findings indicate that clozapine has an effect on inducing CSF-1R expression. The key cell type responding to clozapine is likely to be circulating Ly6C+ monocytes. Even though this difference was not significantly higher than naive mice, the elevated levels may still have biological relevance and reveal the effect of clozapine on monocytes.

In contrast to CD115, clozapine did not change MHC-II expression on Ly6C+MHC-II+ monocytes or SPM between uninjected mice groups. Similarly, in comparing the two thioglycollate treated mice groups, there was no difference in MHC-II expression between the mice administrated with or without clozapine (Figure 5.3B). Additionally, the difference observed between thioglycollated treated and those without thioglycollate treatment was similar to what was seen from the time course experiment described in Chapter 3 (Figure 3.6). Altogether, administration of clozapine did not affect the expression of MHC-II on those two cell types. On the other hand, thioglycollate was able to reduce the level of MHC-II, but only on SPM.

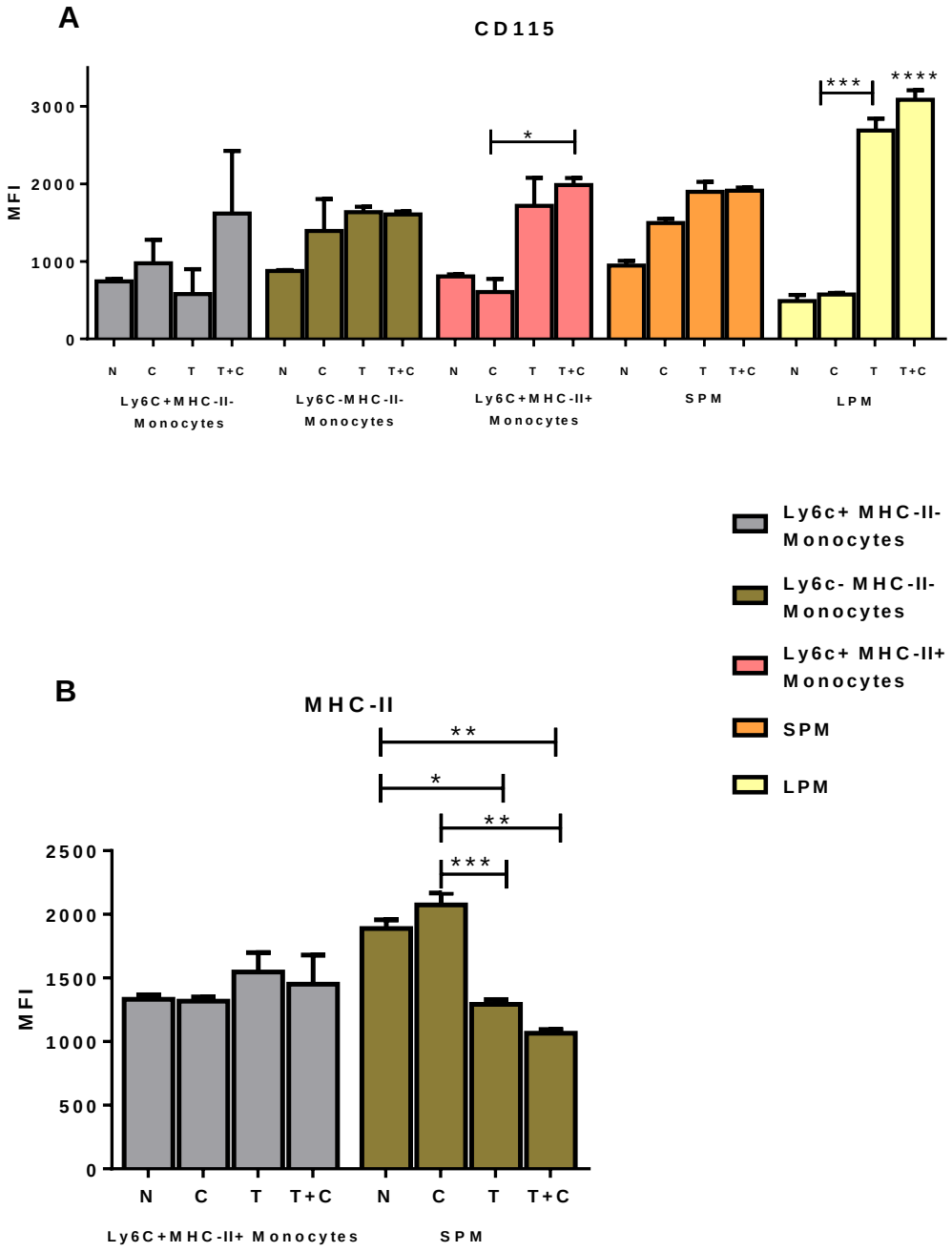


Figure 5.3 Clozapine treatment induced CD115 expression on monocytes subsets and SPM in the peritoneal cavity during steady state conditions

PEC were obtained at day 4 from mice i.p injected with thioglycollate. A) CD115 expression on Ly6C+MHC-II- monocytes, Ly6C-MHC-II- monocytes, Ly6C+MHC-II+ monocytes, SPM and LPM in mice with or without clozapine treatment, B) MHC-II expression on SPM and Ly6C+MHC-II+ monocytes in mice treated with or without clozapine. Shown are the means ± SEM of values from individual mice from 1 (n=2-3/group) experiment. *p<0.5, **p<0.01, ***p<0.001: 2-way ANOVA with Bonferronis Multiple Comparison post-test. N, untreated, C, clozapine treatment, T, thioglycollate treated, T+C, clozapine and thioglycollate treated.

5.3.4 Clozapine treatment enhanced TNFα and IL-10 production by F4/80+ cells and eosinophils in the peritoneal cavity

As shown in chapter 4, thioglycollate is not only capable of inducing a flux of peripheral cells into inflammatory site, but also alters the cytokine profiles in peritoneal cells with relatively significant differences in IL-10 production in all observed cell types at day 2 post i.p. with thioglycollate. In order to investigate the effect of clozapine treatment on cytokine production during the course of inflammation, IL-10 and TNFα expression were measured by ICS. Male C57BL/6 mice were given clozapine treatment or vehicle water for 3 days before treatment followed by i.p. injection with either 4% of 1 mL thioglycollate or 1 mL of PBS. PEC from day 2 and day 4 after injection were harvested and assessed for ICS by flow cytometry.

Overall IL-10 expression level was higher at day 2 than day 4 in mice that received the same treatments (Figure 5.4A-C). Interestingly, mice treated with clozapine alone showed the highest IL-10 in SPM-like cells and eosinophils but not in resident LPM at day 2 time point. These results suggest clozapine treatment mainly affects the recruited immune cells not resident peritoneal macrophages. However, the addition of thioglycollate had a suppressive effect on IL-10 expression in clozapine treated mice. Given that Ly6C+ monocytes gave rise to SPM after their migration to the peritoneal cavity which results in a shift of the proportion of Ly6C+ monocytes and SPM in mice injected with thioglycollate in comparison to mice treated with clozapine alone, it is reasonable to presume that Ly6C+ monocytes are the major contributors to the elevation of IL-10 expression at day 2 after treatment. However, further investigation into the effect of clozapine on monocytes is required.

As IL-10 has an inhibitory role on TNFα production, one may expect that TNFα expression level would be decreased in mice with clozapine treatment. However, the highest TNFα level was detected in SPM-like cell populations in mice treated with clozapine alone (Figure 5.4E). One explanation is that the increased production of TNFα appears in a CSF-R dependent manner. Several *in vitro* studies have reported that CSF-1 act as a subsequent activation stimulus to enhance TNFα production by thioglycollate-elicited peritoneal macrophages upon LPS stimulation (Evans, Shultz, Dranoff, Fuller, & Kamdar, 1998; Kamdar, Fuller, Nishikawa, & Evans, 1997). Interestingly, a similar pattern of TNFα production was seen in eosinophils in mice treated with clozapine alone where both, TNFα and IL-10 expression, were significantly increased at day 2 after PBS injection (Figure 5.4F). Since activation of eosinophils is associated with Th2 cells, further studies into those two cell types would be necessary.

Furthermore, in contrast to SPM-like cells, the level of TNFα expressed by LPM did not

show difference within the treatment groups, although there was a slightly increase at day 2 in mice in each treatment group compared to untreated mice with PBS injection only (Figure 5.4G). This finding further supports that clozapine treatment appear not to have a directly effect on resident macrophages.

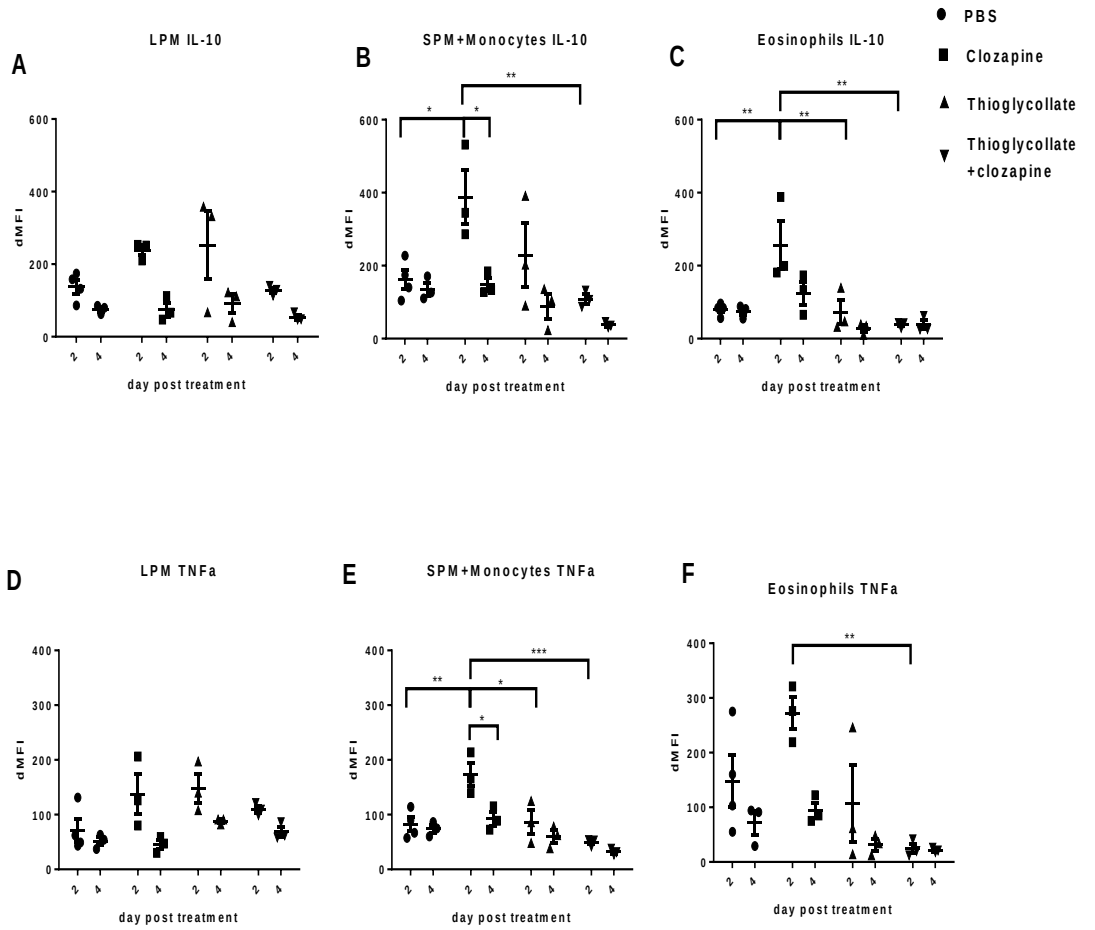


Figure 5.4 Clozapine treatment enhanced TNFα and IL-10 production by F4/80+ cells and eosinophils in the peritoneal cavity

Male C57BL/6 mice in treatment group received 60mg/kg/day clozapine in their drinking water for a maximum of 2 days to make sure the mice had taken clozapine while mice in control group received vehicle drinking water. Peritoneal exudates cells were obtained at day 2 and day 4 from mice post injection with either thioglycollate or PBS. IL-10 and TNFα expressed by A&D) large peritoneal macrophages (LPM), B&E) small peritoneal macrophages-like cells (SPM) which including a proportion of monocyte populations and C&F) enosinophils were assessed for intracellular cytokines with flow cytometry. Cytokine expression levels were determined as geometric mean fluorescence intensity difference (dMFI) between anti-TNFα and anti-IL-10 and isotype control. Shown are the means ± SEM of values from individual mice (n = 3-4/group) from 2 experiments. **p<0.01, ***p<0.001: Difference between clozapine treated alone, thioglycollate treated alone or treated with clozapine and thioglycollate versus PBS alone control mice by2-way ANOVA with Bonferronis Multiple Comparison post-test.

5.3.5 Clozapine treatment did not affect Irg1 expression in the peritoneal macrophages

Our ICS experiments showed that clozapine induced high levels of TNFα in SPM like cells in the absence of thioglycollate suggesting clozapine may induce the activation of SPM during inflammation. To determine the effect of clozapine on SPM activation, Irg1 expression was measured per method described in Chapter 4.

However, similar to the results obtained in thioglycollate treated mice, clozapine treatment alone did not induce Irg1 expression in macrophages implicating that the enhanced TNFα level as seen in mice with clozapine treatment alone is most likely due to the infiltrating monocytes (Figure 5.5). Furthermore, the observed reduced level of Irg1 may be associated with the elevated IL-10 levels at day 2 time point. This finding correlates with a previous study demonstrating that a direct suppressive effect of IL-10 on Irg1 upon LPS stimulation(Gautam et al., 2011).

A

Sample	Irg1 Average C_T	HPRT Average C_T	ΔC_T C_TIrg1-HPRT (mean±SD)	$\Delta\Delta C_T$ ΔC_Tclozapine - ΔC_TPBS (mean±SD)	Fold difference in Irg1 relative to PBS control $2^{-\Delta\Delta CT}$
PBS(pooled RNA)	26.53±0.21	23.63±0.20	2.90±0.21	0.00±0.21	1.00(0.86-1.16)
Clozapine(pooled RNA)	34.98±0.94	26.38±0.17	8.60±0.96	5.70±0.96	0.02(0.01-0.04)

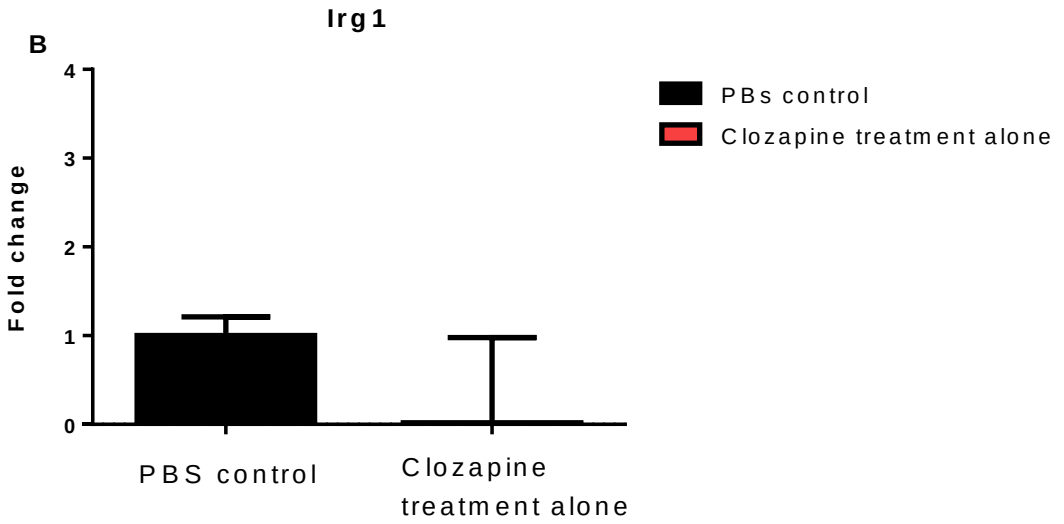


Figure 5.5 Fold change expression of Irg1 at day 2 after clozapine treatment, calculated by ΔΔCT method

A) Irg1 was normalised to the housekeeping gene (HPRT) and fold difference express the amount of Irg1 RNA relative to the PBS control sample. Fold change relative to control was calculated by ΔΔCT method B) Shown are the means ± SD of 2 RNA pooled samples from 1 experiment.

5.4 Discussion

Clozapine is an effective antipsychotic drug that used in patient for the treatment of resistant schizophrenia. However, due to the risk of many adverse effects, such as agranulocytosis, myocarditis and type-2 diabetes, it is not considered as a first-line antipsychotic drug (Henderson et al., 2000; Krupp & Barnes, 1989). While evidence is emerging that clozapine is involved in immunomodulation, however, they concern mainly the effect of clozapine in the context of non-sterile inflammation disease model and restrict to serum cytokine levels. Given that those adverse effects are associated with clozapine treatment, may suggest the potential role of clozapine involve in autoimmune process. Therefore, it would be beneficial to investigate clozapine effect during the course of sterile inflammation.

In this chapter, the effect of clozapine treatment during sterile inflammation was assessed by using the thioglycollate-elicited sterile peritonitis model. We found that clozapine treatment slightly reduced the number of SPM and Ly6C+MHC-II+ monocytes at day 4 after thioglycollate injection, indicating the potential suppressive effect of clozapine on cell recruitment during thioglycollate mediated inflammation. Several possible reasons could account for this suppressive effect of clozapine treatment in thioglycollate-elicited inflammation model. First, clozapine treatment might decrease the number of resident macrophages (LPM) in the peritoneal cavity in the stead state. This would result in a decreased efficiency of phagocytosis of AGEs after thioglycollate treatment, leading to the reduction in monocytes recruitments. Second, in contrast to other subsets of monocytes as we defined in Chapter 3 during peritonitis, only the Ly6C+MHC-II- monocyte subset experienced a modest decrease in the presence of clozapine in uninjected mice. This suggests clozapine treatment could reduce infiltrating inflammatory monocytes. Moreover, although no difference was observed in the number of Ly6C+MHC-II- cells 4 days after thioglycollate injection between clozapine treated and untreated mice, as shown in Figure 5.3A, CSF-1R expression was up regulated on Ly6C+MHC-II- monocytes in clozapine treated mice and a even higher level of expression of CSF-1R was detected in mice treated with clozapine and thioglycollate in combination. These results suggest that CSF-1 may not only be involved in the differentiation of monocytes and the proliferation of resident macrophage but also that CSF-1 is responsible for monocyte recruitment. Indeed, Zory and colleagues found that monocyte migration was decreased using CSF-1 neutralizing antibody in vitro. Furthermore, they found that monocyte migration was decreased in Csf1 deficient mice treated with thioglycollate (Shaposhnik, Wang, & Lusis, 2010). The other reason for the reduction in these cells may be associated with the involvement of mast cells, as an increased number of mature mast cells is found in the

mice treated with clozapine alone. However, pervious work has demonstrated that serum IgG but not serum IgM or IgA levels were significantly elevated in patient after 6 weeks of clozapine treatment (Hinze-Selch et al., 1998). Because natural IgM-secreting B-1 cells are enriched in the peritoneal cavity and both B cell populations are reduced in thiglyocollat mediated inflammation in the presence of clozapine. Further investigation into whether clozapine treatment alters the humoral immunity has to be carried out. These will help to elucidate the underlying mechanisms involved in driving the maturation of mast cells and its relationship to B cells during the thioglycollate-induced peritonitis.

Additionally, aside from the decreased number of B cell subsets observed in this study, our findings also has shown that clozapine treatment in healthy mice results in the decreased number of CD4+ and CD8+ T cells compared to their naïve counterparts. Given that clozapine acts as an antagonist of the 5-HT receptors which are widely expressed on the surface of T cells (Yin, Albert, Tretiakova, & Jameson, 2006), it is likely that clozapine exert immunomodulatory effects on T cells through inhibiting activation of 5-HT receptors. However, to reveal the mechanism of clozapine mediated inhibition on T cells, further investigation is required.

With respect to cytokine production, increased production of pro-inflammatory cytokine TNFα was detected by eosinophils and SPM like cells at day 2 time point compared to navie untreated mice was observed suggesting clozapine may play a beneficial and detrimental role during inflammation. In support of our result, one *in vitro* study using RAW 264.7 cells has reported that serveral pro-inflammatory cytokines like GM-CSF, IL-1b, IL-12p70, IL-13, TNFα are detectable in RAW 264.7 cell culture supernatants after clozapine treatment. Moreover among these cytokines, the production of GM-CSF, IL-1β, IL-12p70 are significantly increased following clozapine treatment in a dose-dependent manner (Contreras-Shannon et al., 2013). The increase of IL-1β in their findings is of our particular interest, because IL-1β is a known protein that is activated by the inflammasome through a two-step signalling process that involves the participants of various receptors leading to the activation of NF-κB that can trigger the activation of inflammasome. Activated Inflammasome can trigger an inflammatory response leading to the secretion of IL-1β in response to ROS that is one of the key mediators produced by M1 macrophages. Interestingly, we did not find the increase of Irg1 expression in clozapine treated healthy mice. On the contrary, the expression of Irg1 was significantly lower than PBS control mice. This result suggests that SPM are inactivated at day 2 post clozapine treatment and further provides the evidence that clozapine treatment shapes infiltrating monocytes towards a “pro-inflammatory” state during the early stage of sterile inflammation.

As with the production of IL-10 in each investigated cell type, similar to TNFα production, an elevated IL-10 production by eosinophils and SPM like cells was observed in PBS injected mice 2 days following administration with clozapine. In line with these results, several previous studies showed that treatment with clozapine alters the immune function of cells by increasing the anti-inflammatory cytokine IL-10 production both *in vitro* and *in vivo* (Al-Amin, Uddin, & Reza, 2013; M.-L. Chen et al., 2012). Additionally, the clozapine-induced increase in IL-10 and TNFα productions were not observed at day 4 in all groups, suggesting the resolution of inflammation involves in the restoration in the balance of pro-and anti-inflammatory cytokines rather than inducing a directional shift in the number of resident immune cells.

Therefore, despite only small differences detected between thioglycollate treated mice and mice with clozapine & thioglycollate treatment, taken together, results from clozapine treatment interfering with this model indicate that clozapine plays a complex role in immunomodulation during thioglycollate-elicited sterile inflammation. Furthermore, this study provides evidence suggesting that the potential key cell type in response to clozapine is blood monocytes.

In addition, future work should be conducted based on the results from this study. However, it is important to note that this study was mainly focused on investigating the fate of macrophages during the thioglycollate-elicited inflammation model. There were limitations when using the designed panel and adapted ICS protocol to study other immune cell types, such as NK cells, dendritic cells and T cells. However, data from this study can shed light on alterations and correlation of those cell types as well as the different cytokine microenvironment. For instance, Th2 proliferation is associated with M2 macrophage activation and high level of IL-10 and reduced IL-12 levels promote the development of Th2 cells (Le Gros, Ben-Sasson, Seder, Finkelman, & Paul, 1990).

On the other hand, only CD115 and MHC-II expression level were assessed to study the activation of macrophages in this study. Measurement of other phenotypic markers, such as CD11c, F4/80 should be included in future studies. Indeed, a previous study indicated that M1-like adipose tissue macrophages appear to express CD11c surface marker (P. Li et al., 2010). Similarly, by assessing other cytokines candidates, including IL-4, IL-12, IL-1, IL-6, TGF-β, a better understanding of the cells environment in response to test drugs in the context of thioglycollate-mediated inflammation could be provided.

Taken together, it can be concluded that the thioglycollate-elicited murine peritonitis model is a useful tool for the assessment of a test drugs and if they interfere with the inflammatory processes, phenotypically and functionally.

Chapter 6: General Discussion

6.1 Introduction

The murine peritoneal immune system is mainly composed of resident macrophages and peritoneal unique B-1 cells along with a small proportion of other immune cells in the steady state. Resident macrophages play a crucial role not only in responding to various pathologic stimuli but also for the maintenance of tissue homeostasis (dos Anjos Cassado et al., 2015). Therefore, abnormalities in peritoneal macrophage function may enhance the susceptibility to peritonitis. Thioglycollate-elicited murine peritonitis has long been used to investigate the functionalities of macrophages *in vitro* and *in vivo(Ghosn et al., 2010; Tsunawaki & Nathan, 1984)*. This mode is also utilized as an approach for compounds and new drug testing in the context of inflammation. However, very little is known about the underlying mechanism of thioglycollate- induced sterile peritonitis as well as the cell population dynamics in the peritoneal cavity, in particular macrophages, during the course of inflammation. Moreover, sterile inflammation appears to be associated with auto-inflammatory disorders (Rock et al., 2010). Therefore, understanding the mechanism of this model system may benefit the development of drugs which target chronic inflammatory diseases.

This study, investigated in detail the cell population changes as well as the alterations of specific cytokines over time during the thioglycollate-induced peritonitis, with a particular emphasis on macrophage like cells. The results obtained in this study extend the current understanding of this model. Based on these findings, a stepwise process of thioclycollate-induced peritonitis has been summarized and proposed (Figure 4.3).

6.2 Effects of thioglycollate on peripheral leukocytes recruitment and heterogeneity of monocytes subsets

Infiltration of circulating neutrophils and monocytes into the site of injury is an integral part of the inflammatory process. In this study, it was shown that thioglycollate administration induced the recruitment of peripheral blood neutrophils and monocytes into the peritoneal cavity during the initiation of the inflammation. While aged neutrophils undergo spontaneous apoptosis following recruitment, monocytes begin to differentiate into small peritoneal macrophages (SPM). Furthermore, on the basis of Ly6C expression, a monocyte marker, the results have revealed the heterogeneity in the monocyte population during the course of inflammation. This includes Ly6C+MHC-II- inflammatory monocytes, Ly6C+MHC-II+ differentiating monocytes and Ly6C-MHC-II- patrolling monocytes. The exact relationship between Ly6C+ and Ly6C- monocytes during

inflammation is complicated by contradictory observations from different studies. One study employing a mouse model of myocardial infarction, a circumstance of sterile inflammation, has demonstrated the stepwise recruitment of the two monocytes subsets, Ly6C+ and Ly6C-, with the Ly6C-monocytes being recruited at a later phase (Nahrendorf et al., 2007). Results from an adoptive transfer study indicates that Ly6C+ monocytes serve as precursors to Ly6C- cells during a parasite-induced liver inflammation (Okabe & Medzhitov, 2014). In this regards, Ly6C+ monocytes appear to exhibit a shorter half-life of 8 hours compared to 5-7 days for Ly6C- monocytes (Italiani & Boraschi, 2015). These different observations may be due to differences in the inflammatory conditions. Indeed, in a model of *Listeria monocytogenes* infection, Ly6C- monocytes have been reported to infiltrate the infected site earlier than Ly6C+ monocytes and Ly6C- monocytes have been associate with the induction of an early inflammatory response (Auffray et al., 2007).

These findings suggest that Ly6C-monocytes might be mediating sterile inflammation. In general, they are thought to be involved in the removal of debris, tissue repair and promoting the resolution of inflammation. This function is similar to the function displayed by M2 macrophages. Indeed, several studies found that LyC6-monocytes are able to differentiate into resident macrophages in lung (Jakubzick et al., 2008; Landsman, Varol, & Jung, 2007), suggesting their special relationship with M2 macrophages. However it has also been shown that this differentiation ability of Ly6C- monocytes to M2 macrophages may be specific for tissues or stimulus. Furthermore, although in human, CD14+CD16++ monocytes, which are similar to mouse Ly6C- monocytes, only represent a small cell population in blood (Shi & Pamer, 2011), evidence has shown that they may have negative effect on autoimmune disease including atherosclerosis, myocardial infraction, neurological disease, arthritis and lupus nephritis (Thomas, Tacke, Hedrick, & Hanna, 2015). Therefore, nonclassical monocytes (Ly6C- monocytes in mouse and CD14+CD16++ monocytes in human) could be potential therapeutic targets for many human autoimmune diseases.

6.3 CSF-1 plays an important role in leukocytes recruitment and in the proliferation and differentiation of the macrophage lineage

When resident macrophages encounter an inflammatory inducer such as advanced glycation end products (AGE), these molecules are ingested by the cells, which result in lytic cell death and the release of inflammatory mediators and chemokines. One of the possible candidates contributing to the macrophages recruitment is CSF-1. CSF-1 is detected being produced constitutively by several cell populations, such as endothelial cells, stromal cells and osteoblasts *in vitro* (Metcalf & Nicola, 1995). *In vivo*, macrophages

can produce CSF-1 upon stimulation. Stimulated macrophages are able to activate neighboring non-hematopoietic cells to produce CSF-1 by secreting cytokines such as IL-1 and TNFα (Hamilton, 2008). Furthermore, evidence has shown that increased levels of CSF-1 can enhance the movement of monocyte from the bone marrow to the blood, suggesting the trafficking effect of CSF-1 (Hamilton, 2008). In line with these findings, the expression of CD115, the receptor for CSF-1, was constitutively increased on the surface of macrophages and monocytes during the course of inflammation. This indicates that the elevated level of CSF-1 after thioglycollate treatment can drive the recruitment of monocytes resulting in an increased differentiation of SPM.

Interestingly, clozapine treatment in normal mice appears to have the same effect on driving the expression of CD115 on Ly6C+ and Ly6C- monocytes as shown in this thesis. This implicates that clozapine-induced increase of the expression level of CD115 on circulating monocytes results in a reduction of CSF-1 level in microenvironment. This fact might explain the anti-inflammatory activity by a decreased number of macrophages in inflammatory lesions. Although additional investigation is required to verify this effect of clozapine, however, accumulating evidence support the idea that CSF-1 depletion might attenuate certain disease severity, such as arthritis, lung fibrosis and atherosclerosis (Hamilton, 2008). Therefore, therapies which can reduce or block CSF-1 will have remarkable benefit for inflammation or autoimmune conditions.

6.4 Dynamics and features of LPM and SPM during peritonitis

Peritoneal resident macrophages (LPM) contribute to the initiation of thioglycollate-induced inflammation (Hussain & Stohlman, 2012). Following the recruitment of monocytes and neutrophils, LPM are reduced and the cell number slowly recovers during the course of inflammation. In contrast, monocytes continually differentiate into SPM and therefore, SPM become the predominant cell population in the course of inflammation. The shift of LPM to SPM is observed in LPS-induced inflammation as well (Ghosn et al., 2010), suggesting it might be the way of peritoneal immune system against either invading pathogens or sterile stimuli. Under the conditions of pathogen-induced inflammation, SPM appear to be activated and to produce high pro-inflammatory mediators, including TNFα, NO, ROS and IL-12 (dos Anjos Cassado et al., 2015). However, in the model used in this study, no production of TNFα was detected. Furthermore, primed SPM could be activated to secrete significant levels of pro-inflammatory mediators by encountering a second stimulus, such as LPS (Hamilton, 1993). Pervious study has shown that the upregulation of Irg1 expression in LPS stimulated mouse macrophages result in the suppression of the pro-inflammatory cytokines production (Y. Li et al., 2013). The authors found that this suppressive activity of Irg1 was achieved by increasing A20 expression via

increased ROS production (Y. Li et al., 2013). The data presented here showed that Irg1 expression is down-regulated in the absence of pathogens. However, it would be interesting to investigate the relationship within Irg1 and ROS in primed SPM, which may shed light on the mechanisms of hyperinflammation or autoimmune diseases, in particular since evidence has shown that Irg1 is significantly up-regulated in EAE spinal cords and in the peripheral blood mononuclear cells of septic patients (Y. Li et al., 2013; Spach et al., 2004). In addition to the Irg1 expression, the analysis of cytokines revealed the cytokine production by the macrophages linage as well as by eosinophils during the inflammation. IL-10 and TNFα are produced by all investigated cells. However, it is not known whether SPM themselves are the major source of IL-10 and TNFα, since it was not possible to distinguish monocytes from SPM. Moreover, given that clozapine treatment induced a much higher IL-10 and TNFα levels by SPM-like population, it is likely that but infiltrating monocytes and no SPM are the major IL-10 and TNFα producers. Further studies are required to verify this hypothesis.

6.5 Future directions

Future studies should the investigate role of Ly6C- monocytes and the relationship between Irg1 and primed SPM as well as the different cytokine production in these cell types. Investigations to elucidate whether the alterations induced by thioglycollate or clozapine occurs as well in other tissue or organs such as the spleen or the lymph nodes are also merited.

An interesting finding in this thesis was the increase in the number of mast cells after treatment with either clozapine or thioglycollate. This increase might be associated with an increased permeability of the peritoneal membrane leading to an increased recruitment of circulating leukocytes. Therefore, further investigation of mast cells involved in the initiation and resolution of inflammation will help to gain a better understanding on effector cells recruitment into inflammatory lesions.

In conclusion, the results from this thesis have outlined the cell population dynamics during steril inflammation by using the thioglycollate-induced peritonitis model. It uncovered the relationship between resident macrophages and infiltrating monocytes, and It also demonstrated that this model is useful for testing the effect of drugs in the context of sterile inflammation.

Chapter 7: Appendices

Appendix A: Supplementary tables and figures

Table A1 Monoclonal Antibodies Used to Characterise Peritoneal Macrophages and other Leukocytes

Specificity	Label	Species and Isotype	Manufacturer	Optimal dilution	Clone	Comments
F4/80	PerCP-Cy5.5	Rat IgG2a	eBioscience	1:400	BM8	Pan-MØ marker
CD11b	AF488	Rat IgG2b,k	BD pharmingen	1:400	M1/70	Pan-myeloid integrin
CD115	APC-eFluor780	Rat IgG2a, k	eBioscience	1:1000	AFS98	Receptor for CSF-1
MHC-II(I-A/I-E)	BV421	Rat IgG2b, k	Biolegend	1:400	M5/114.15.2	Activation marker
CD14	PE-Cy7	Rat IgG2b	Biolegend	1:1000	RTK2758	Coreceptor of TLR4
Ly6C	PE	Rat IgG2c	Biolegend	1:800	HK1.4	Monocyte marker
Ly6G	APC	Rat IgG2a,k	Biolegend	1:300	1A8	neutrophil-specific marker
CD11c(Biotin)	V500	Armenian Hamster IgG	BD pharmingen	1:500	HL3	pan-DC marker
CD11c	APC	Armenian Hamster IgG	Biolegend	1:400	N418	
CD3	APC-Cy7	Rat IgG2b, k	Biolegend	1:200	17A2	T-cell co-receptor
CD4	PE	Rat IgG2a	BD pharmingen	1:400	RM4-5	Th cell marker
CD8	PerCP-Cy5.5	Rat IgG2a	eBioscience	1:800	53-6.7	cytotoxic T cell marker
CD62L	APC	Rat IgG2a, k	BD pharmingen	1:400	MEL-14	Homing receptor; naïve T cell marker
B220	FITC	Rat IgG2a	BD pharmingen	1:600	RA3-6B2	B cell marker

NK1.1	PE-Cy7	Mouse IgG2a, k	Biolegend	1:300	PK136	Pan-NK cell marker
CD117	BV510	Rat IgG2b, k	Biolegend	1:300	ACK2	Mast cell growth factor receptor
Gr-1	APC-Cy7	Rat IgG2b,k	BD pharmingen	1:400	RB6-8C5	Recognizing both Ly6G and Ly6C
Isotype controls	BV421	Rat IgG2b, k	Biolegend	1:400 1:100	RTK4530	
	FITC	Rat IgG2a	BD pharmingen	1:400	R35-95	
	AF488	Rat IgG2b,k	BD pharmingen	1:400	RTK4530	
	PE	Rat IgG2a	BD pharmingen	1:400 1:800	R35-95	
		Rat IgG1	BD pharmingen	1:100	RTK2071	
	PerCP-Cy5.5	IgG2a, k	Biolegend	1:400 1:1000	R35-95	
	PE-Cy7	Rat IgG2a	Biolegend	1:1000	RTK2758	
		Mouse IgG2a, k	Biolegend	1:300	MOPC-173	
	APC	Rat IgG2a	Biolegend	1:300 1:400	R35-95	
		Armenian Hamster IgG	Biolegend	1:400	HTK888	
	APC-Cy7	Rat IgG2b, k	BD pharmingen	1:1000 1:400 1:200	R35-38	
	FITC	Rat IgG2a	BD pharmingen	1:600	R35-95	
	APC-eFluor780	Rat IgG2a, k	eBioscience	1:1000	eBR2a	
	BV510	Rat IgG2b, k	Biolegend	1:300	RTK4530	
Intracellular Molecules						
IL-10	BV421	Rat IgG2b, k	Biolegend	1:100	JES5-16E3	
TNFα	PE	Rat IgG1	BD pharmingen	1:100	A85-1	

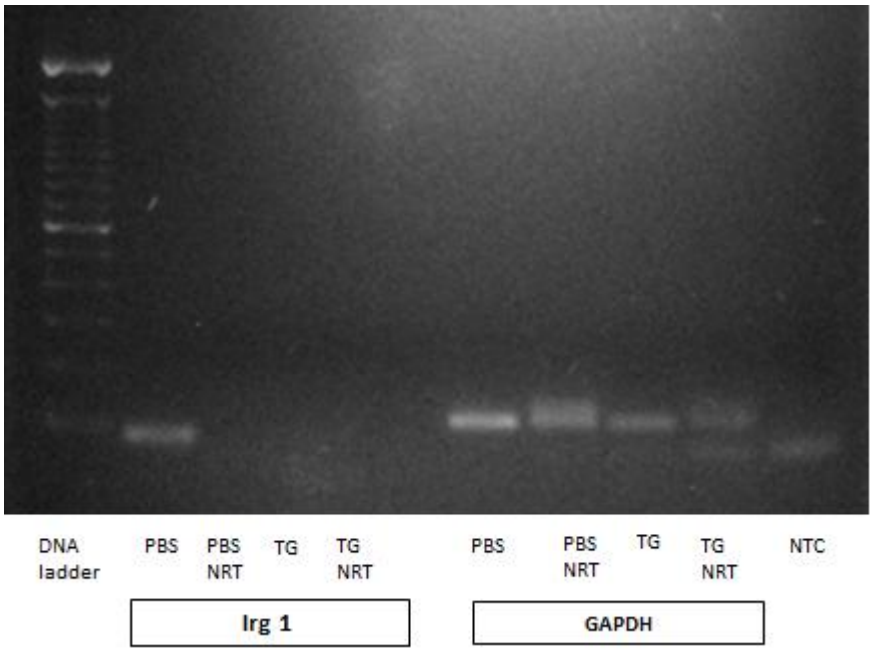


Figure A1 PCR amplification of Irg1 from PBS pooled sample

The figure shows the agarose gel of the qRT-PCR product of Irg1 using cDNA generated from PBS pooled RNA sample as template. In comparison to the DNA ladder (first lane), the Irg1 product were found to be of the correct size (77 base pairs). However, NRT samples for GAPDH were showing positive signals and primer dimers were found in NRT sample as well as water sample. Data shown is from one experiment.

A

Sample	Irg1 Average C_T	GAPDH Average C_T	ΔC_T C_TIrg1-GAPDH (mean±SD)	$\Delta\Delta C_T$ ΔC_TThioglycollate - ΔC_T GAPDH (mean±SD)	Fold difference in Irg1 relative to PBS control $2^{-\Delta\Delta CT}$
PBS (Pooled RNA)	27.49±0.16	19.35±0.08	8.14±0.18	0.00±0.18	1.00(0.88-1.13)
Thioglycollate	29.12±0.11	17.99±0.27	11.13±0.29	2.99±0.29	0.13(0.10-0.15)

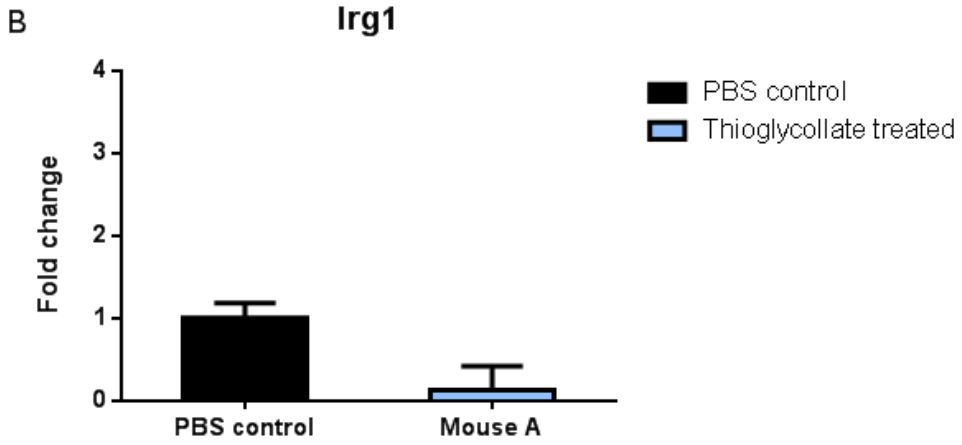


Figure A2 Fold change expression of Irg1 at day 4 after thioglycollate treatment, calculated by ΔΔCT method

A) Irg1 was normalised to the housekeeping gene (GAPDH) and fold difference express the amount of Irg1 RNA relative to the calibrator sample. B) Shown are the means ± SD of 2 RNA samples from 1 experiment.

Appendix B: Buffers and solutions

Phosphate Buffered Saline (PBS) (10x)

NaCl	170 g	Merck
$Na_2HPO_4.12H_2O$	62.32 g	Merck
$Na_2HPO_4.2H_2O$	4.04 g	Merck
ddH_2O	Adjust the volume to 2 L	

PBS (1x)

Dilute the 10X PBS stock to 1X working concentration

0.1 M acetic acid solution

Glacial acetic acid	287 µl	Sigma-Aldrich
ddH_2O	Adjust the volume to 50 mL	

FACS Buffer (v/v)

FCS	2%
Sodium azide	0.1%
1xPBS	97.9%

0.1% Saponin Buffer

Bovine serum albumin (BSA)	0.02 g	ICP biologicals
Saponin	0.02 g	Sigma
Hepes (10 mM)	0.0476 g	Fisher Scientific
Sodium azide	30.768 µl	
ddH_2O	Adjust the volume to 20 mL	

1% Paraformaldeyde (w/v)

Paraformaldehyde	1%	
1x PBS	99%	

Store at 4°C for 1 month, protected from light

Complete T cell media (CTCM) (v/v)

Dulbecco's Modified Eagle Medium	86%	Invitrogen
Foetal calf serum (FCS)	10%	Invitrogen
Penicillin/Streptomycin(100U/mL/10 mg/mL)	1%	Invitrogen
Hepes (1M)	1%	Invitrogen
Non-essential amino acids	1%	Invitrogen
L-glutamine (200 mM)	1%	Invitrogen
β-Mecaptoethanol (55 mM)	0.1%	Invitrogen

10% Giemsa's solution (v/v)

Giemsa	10%	BDH Chemicals
ddH_2O	90%	

TAE Buffer (50x)

Tris base	242 g	Calbiochem
Ethylenediaminetetraacetic acid (EDTA) (0.5M)	100 mL	Invitrogen
Glacial acetic acid	57.1 mL	Sigma-Aldrich
ddH_2O	Adjust the volume to 1 L	

TAE Buffer (1x)

Dilute the 50X TAE stock to 1X working concentration

References

Ahmed, R., & Gray, D. (1996). Immunological memory and protective immunity: understanding their relation. *Science, 272*(5258), 54.

Ajuebor, M. N., Das, A. M., Virág, L., Flower, R. J., Szabó, C., & Perretti, M. (1999). Role of resident peritoneal macrophages and mast cells in chemokine production and neutrophil migration in acute inflammation: evidence for an inhibitory loop involving endogenous IL-10. *The Journal of Immunology, 162*(3), 1685-1691.

Akira, S., Misawa, T., Satoh, T., & Saitoh, T. (2013). Macrophages control innate inflammation. *Diabetes, Obesity and Metabolism, 15*(s3), 10-18.

Al-Amin, M. M., Uddin, M. M. N., & Reza, H. M. (2013). Effects of antipsychotics on the inflammatory response system of patients with schizophrenia in peripheral blood mononuclear cell cultures. *Clinical Psychopharmacology and Neuroscience, 11*(3), 144.

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., & Walter, P. (2002a). Helper T cells and Lymphocyte activation.

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., & Walter, P. (2002b). T cells and MHC proteins.

Allman, D., & Pillai, S. (2008). Peripheral B cell subsets. *Current opinion in immunology, 20*(2), 149-157.

Antonios, J. K., Yao, Z., Li, C., Rao, A. J., & Goodman, S. B. (2013). Macrophage polarization in response to wear particles in vitro. *Cellular & molecular immunology, 10*(6), 471-482.

Appleman, L. J., & Boussiotis, V. A. (2003). T cell anergy and costimulation. *Immunological reviews, 192*(1), 161-180.

Auffray, C., Fogg, D., Garfa, M., Elain, G., Join-Lambert, O., Kayal, S., . . . Geissmann, F. (2007). Monitoring of blood vessels and tissues by a population of monocytes with patrolling behavior. *Science, 317*(5838), 666-670.

Aurora, A. B., Porrello, E. R., Tan, W., Mahmoud, A. I., Hill, J. A., Bassel-Duby, R., . . . Olson, E. N. (2014). Macrophages are required for neonatal heart regeneration. *The Journal of clinical investigation, 124*(3), 1382-1392.

Basler, T., Jeckstadt, S., Valentin-Weigand, P., & Goethe, R. (2006). Mycobacterium paratuberculosis, Mycobacterium smegmatis, and lipopolysaccharide induce different transcriptional and post-transcriptional regulation of the IRG1 gene in murine macrophages. *Journal of leukocyte biology, 79*(3), 628-638.

Bierhaus, A., Stern, D. M., & Nawroth, P. P. (2006). RAGE in inflammation: a new therapeutic target? *Current opinion in investigational drugs (London, England: 2000), 7*(11), 985-991.

Billack, B. (2006). Macrophage activation: role of toll-like receptors, nitric oxide, and nuclear factor kappa B. *American journal of pharmaceutical education, 70*(5), 102.

Boe, D. M., Curtis, B. J., Chen, M. M., Ippolito, J. A., & Kovacs, E. J. (2015). Extracellular traps and macrophages: new roles for the versatile phagocyte. *Journal of leukocyte biology, 97*(6), 1023-1035.

Cavaillon, J.-M. (2011). The historical milestones in the understanding of leukocyte biology initiated by Elie Metchnikoff. *Journal of leukocyte biology, 90*(3), 413-424.

Chaplin, D. D. (2010). Overview of the immune response. *Journal of Allergy and Clinical Immunology, 125*(2), S3-S23.

Chen, G. Y., & Nuñez, G. (2010). Sterile inflammation: sensing and reacting to damage. *Nature Reviews Immunology, 10*(12), 826-837.

Chen, M.-L., Tsai, T.-C., Wang, L.-K., Lin, Y.-Y., Tsai, Y.-M., Lee, M.-C., & Tsai, F.-M. (2012). Clozapine inhibits Th1 cell differentiation and causes the suppression of IFN-γ production in peripheral blood mononuclear cells. *Immunopharmacology and immunotoxicology, 34*(4), 686-694.

Chuah, Y. K., Basir, R., Talib, H., Tie, T. H., & Nordin, N. (2013). Receptor for advanced glycation end products and its involvement in inflammatory diseases. *International journal of inflammation, 2013*.

Contreras-Shannon, V., Heart, D. L., Paredes, R. M., Navaira, E., Catano, G., Maffi, S. K., & Walss-Bass, C. (2013). Clozapine-induced mitochondria alterations and inflammation in brain and insulin-responsive cells. *PloS one, 8*(3), e59012.

Curtsinger, J. M., & Mescher, M. F. (2010). Inflammatory cytokines as a third signal for T cell activation. *Current opinion in immunology, 22*(3), 333-340.

Desmedt, M., Rottiers, P., Dooms, H., Fiers, W., & Grooten, J. (1998). Macrophages induce cellular immunity by activating Th1 cell responses and suppressing Th2 cell responses. *The Journal of Immunology, 160*(11), 5300-5308.

Ding, A. H., Sanchez, E., Srimal, S., & Nathan, C. F. (1989). Macrophages rapidly internalize their tumor necrosis factor receptors in response to bacterial lipopolysaccharide. *Journal of Biological Chemistry, 264*(7), 3924-3929.

dos Anjos Cassado, A., de Albuquerque, J. A. T., Sardinha, L. R., de Lima Buzzo, C., Faustino, L., Nascimento, R., . . . Bortoluci, K. R. (2011). Cellular renewal and improvement of local cell effector activity in peritoneal cavity in response to infectious stimuli. *PloS one, 6*(7), e22141.

dos Anjos Cassado, A., Lima, M. R. D. I., & Bortoluci, K. R. (2015). Revisiting mouse peritoneal macrophages: heterogeneity, development, and function. *Frontiers in immunology, 6*.

Doyle, S. E., Vaidya, S. A., O'Connell, R., Dadgostar, H., Dempsey, P. W., Wu, T.-T., . . . Modlin, R. L. (2002). IRF3 mediates a TLR3/TLR4-specific antiviral gene program. *Immunity, 17*(3), 251-263.

Driessler, F., Venstrom, K., Sabat, R., Asadullah, K., & Schottelius, A. (2004). Molecular mechanisms of interleukin‐10‐mediated inhibition of NF‐κB activity: a role for p50. *Clinical & Experimental Immunology, 135*(1), 64-73.

Eris, J. M., Basten, A., Brink, R., Doherty, K., Kehry, M. R., & Hodgkin, P. D. (1994). Anergic self-reactive B cells present self antigen and respond normally to CD40-dependent T-cell signals but are defective in antigen-receptor-mediated functions. *Proceedings of the National Academy of Sciences, 91*(10), 4392-4396.

Erwig, L.-P., & Henson, P. M. (2007). Immunological consequences of apoptotic cell phagocytosis. *The American journal of pathology, 171*(1), 2-8.

Evans, R., Shultz, L. D., Dranoff, G., Fuller, J. A., & Kamdar, S. J. (1998). CSF-1 regulation of Il6 gene expression by murine macrophages: a pivotal role for GM-CSF. *Journal of leukocyte biology, 64*(6), 810-816.

Fillatreau, S., Sweenie, C. H., McGeachy, M. J., Gray, D., & Anderton, S. M. (2002). B cells regulate autoimmunity by provision of IL-10. *Nature immunology, 3*(10), 944-950.

Finbloom, D. S., & Winestock, K. D. (1995). IL-10 induces the tyrosine phosphorylation of tyk2 and Jak1 and the differential assembly of STAT1 alpha and STAT3 complexes in human T cells and monocytes. *The Journal of Immunology, 155*(3), 1079-1090.

Flynn, J. L., Goldstein, M. M., Chan, J., Triebold, K. J., Pfeffer, K., Lowenstein, C. J., . . . Bloom, B. R. (1995). Tumor necrosis factor-α is required in the protective immune response against Mycobacterium tuberculosis in mice. *Immunity, 2*(6), 561-572.

Gautam, A., Dixit, S., Philipp, M. T., Singh, S. R., Morici, L. A., Kaushal, D., & Dennis, V. A. (2011). Interleukin-10 alters effector functions of multiple genes induced by Borrelia burgdorferi in macrophages to regulate Lyme disease inflammation. *Infection and immunity*, IAI. 05451-05411.

Geissmann, F., Manz, M. G., Jung, S., Sieweke, M. H., Merad, M., & Ley, K. (2010). Development of monocytes, macrophages, and dendritic cells. *Science, 327*(5966), 656-661.

Ghosn, E. E. B., Cassado, A. A., Govoni, G. R., Fukuhara, T., Yang, Y., Monack, D. M., . . . Herzenberg, L. A. (2010). Two physically, functionally, and developmentally distinct peritoneal macrophage subsets. *Proceedings of the National Academy of Sciences, 107*(6), 2568-2573.

Ginhoux, F., Greter, M., Leboeuf, M., Nandi, S., See, P., Gokhan, S., . . . Stanley, E. R. (2010). Fate mapping analysis reveals that adult microglia derive from primitive macrophages. *Science, 330*(6005), 841-845.

Goerdt, S., & Orfanos, C. E. (1999). Other functions, other genes: alternative activation of antigen-presenting cells. *Immunity, 10*(2), 137-142.

Gordon, J. W., Scangos, G. A., Plotkin, D. J., Barbosa, J. A., & Ruddle, F. H. (1980). Genetic transformation of mouse embryos by microinjection of purified DNA. *Proceedings of the National Academy of Sciences, 77*(12), 7380-7384.

Gordon, S. (2003). Alternative activation of macrophages. *Nature reviews immunology, 3*(1), 23-35.

Guilbert, L., Winkler-Lowen, B., Smith, A., Branch, D. R., & Garcia-Lloret, M. (1993). Analysis of the synergistic stimulation of mouse macrophage proliferation by macrophage colony-stimulating factor (CSF-1) and tumor necrosis factor alpha (TNF-alpha). *Journal of leukocyte biology, 54*(1), 65-72.

Hamilton, J. A. (1993). Colony stimulating factors, cytokines and monocyte-macrophages-some controversies. *Immunology today, 14*(1), 18-24.

Hamilton, J. A. (2008). Colony-stimulating factors in inflammation and autoimmunity. *Nature Reviews Immunology, 8*(7), 533-544.

Hanahan, D., Wagner, E. F., & Palmiter, R. D. (2007). The origins of oncomice: a history of the first transgenic mice genetically engineered to develop cancer. *Genes & development, 21*(18), 2258-2270.

Hardy, R. R. (2006). B-1 B cell development. *The Journal of Immunology, 177*(5), 2749-2754.

Hardy, R. R., & Hayakawa, K. (2001). B cell development pathways. *Annual review of immunology, 19*(1), 595-621.

Harris, N. L., & Ronchese, F. (1999). The role of B7 costimulation in T-cell immunity. *Immunology and cell biology, 77*(4), 304-311.

Hashimoto, D., Chow, A., Noizat, C., Teo, P., Beasley, M. B., Leboeuf, M., . . . Lucas, D. (2013). Tissue-resident macrophages self-maintain locally throughout adult life with minimal contribution from circulating monocytes. *Immunity, 38*(4), 792-804.

Henderson, D. C., Cagliero, E., Gray, C., Nasrallah, R. A., Hayden, D. L., Schoenfeld, D. A., & Goff, D. C. (2000). Clozapine, diabetes mellitus, weight gain, and lipid abnormalities: a five-year naturalistic study. *American Journal of Psychiatry, 157*(6), 975-981.

Hinze-Selch, D., Becker, E. W., Stein, G. M., Berg, P. A., Mullington, J., Holsboer, F., & Pollmächer, T. (1998). Effects of clozapine on in vitro immune parameters: a longitudinal study in clozapine-treated schizophrenic patients. *Neuropsychopharmacology, 19*(2), 114-122.

Holling, T. M., Schooten, E., & van Den Elsen, P. J. (2004). Function and regulation of MHC class II molecules in T-lymphocytes: of mice and men. *Human immunology, 65*(4), 282-290.

Hornung, V., Bauernfeind, F., Halle, A., Samstad, E. O., Kono, H., Rock, K. L., . . . Latz, E. (2008). Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization. *Nature immunology, 9*(8), 847-856.

Hoves, S., Trapani, J. A., & Voskoboinik, I. (2010). The battlefield of perforin/granzyme cell death pathways. *Journal of leukocyte biology, 87*(2), 237-243.

Hsieh, C.-S., Macatonia, S. E., Tripp, C. S., Wolf, S. F., O'Garra, A., & Murphy, K. M. (1993). Development of TH1 CD4+ T Cells Through IL-12. *Science, 260*, 547.

Hu, X., Zhou, H., Zhang, D., Yang, S., Qian, L., Wu, H.-M., . . . Lu, R.-b. (2012). Clozapine protects dopaminergic neurons from inflammation-induced damage by inhibiting microglial overactivation. *Journal of Neuroimmune Pharmacology, 7*(1), 187-201.

Hume, D. A. (2015). The many alternative faces of macrophage activation. *Frontiers in immunology, 6*.

Hussain, S., & Stohlman, S. A. (2012). Peritoneal macrophage from male and female SJL mice differ in IL-10 expression and macrophage maturation. *Journal of leukocyte biology, 91*(4), 571-579.

Ibrahim, Z. A., Armour, C. L., Phipps, S., & Sukkar, M. B. (2013). RAGE and TLRs: relatives, friends or neighbours? *Molecular immunology, 56*(4), 739-744.

Italiani, P., & Boraschi, D. (2015). From monocytes to M1/M2 macrophages: phenotypical vs. functional differentiation. *M1/M2 Macrophages: The Arginine Fork in the Road to Health and Disease*, 47.

Jakubzick, C., Tacke, F., Ginhoux, F., Wagers, A. J., van Rooijen, N., Mack, M., . . . Randolph, G. J. (2008). Blood monocyte subsets differentially give rise to CD103+ and CD103− pulmonary dendritic cell populations. *The Journal of Immunology, 180*(5), 3019-3027.

Janeway Jr, C. A., & Medzhitov, R. (2002). Innate immune recognition. *Annual review of immunology, 20*(1), 197-216.

Janeway Jr, C. A., Travers, P., Walport, M., & Shlomchik, M. J. (2001). The major histocompatibility complex and its functions.

Jankovic, D., Kugler, D., & Sher, A. (2010). IL-10 production by CD4 effector T cells: a mechanism for self-regulation. Mucosal Immunol. 3: 239–246.

Jin, X., Yao, T., Zhou, Z. e., Zhu, J., Zhang, S., Hu, W., & Shen, C. (2015). Advanced glycation end products enhance macrophages polarization into M1 phenotype through activating RAGE/NF-κB pathway. *BioMed research international, 2015*.

Jones, C. V., & Ricardo, S. D. (2013). Macrophages and CSF-1: implications for development and beyond. *Organogenesis, 9*(4), 249-260.

Kamdar, S. J., Fuller, J. A., Nishikawa, S.-I., & Evans, R. (1997). Priming of Mouse Macrophages with the Macrophage Colony-Stimulating Factor (CSF-1) Induces a Variety of Pathways That Regulate Expression of the Interleukin 6 (Il6) and Granulocyte–Macrophage Colony-Stimulating Factor (Csfgm) Genes. *Experimental cell research, 235*(1), 108-116.

Karnovsky, M. L., & Lazdins, J. K. (1978). Biochemical criteria for activated macrophages. *The Journal of Immunology, 121*(3), 809-813.

Kawai, T., Adachi, O., Ogawa, T., Takeda, K., & Akira, S. (1999). Unresponsiveness of MyD88-deficient mice to endotoxin. *Immunity, 11*(1), 115-122.

Kendziorski, C., Irizarry, R., Chen, K.-S., Haag, J., & Gould, M. (2005). On the utility of pooling biological samples in microarray experiments. *Proceedings of the National Academy of Sciences of the United States of America, 102*(12), 4252-4257.

Kono, H., Orlowski, G. M., Patel, Z., & Rock, K. L. (2012). The IL-1–Dependent Sterile Inflammatory Response Has a Substantial Caspase-1–Independent Component That Requires Cathepsin C. *The Journal of Immunology, 189*(7), 3734-3740.

Krupp, P., & Barnes, P. (1989). Leponex—associated granulocytopenia: a review of the situation. *Psychopharmacology, 99*, S118-S121.

Landsman, L., Varol, C., & Jung, S. (2007). Distinct differentiation potential of blood monocyte subsets in the lung. *The Journal of Immunology, 178*(4), 2000-2007.

Le Gros, G., Ben-Sasson, S. Z., Seder, R., Finkelman, F., & Paul, W. (1990). Generation of interleukin 4 (IL-4)-producing cells in vivo and in vitro: IL-2 and IL-4 are required for in vitro generation of IL-4-producing cells. *The Journal of experimental medicine, 172*(3), 921-929.

Lech, M., & Anders, H.-J. (2013). Macrophages and fibrosis: How resident and infiltrating mononuclear phagocytes orchestrate all phases of tissue injury and repair. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease, 1832*(7), 989-997.

Lee, P. Y., Wang, J.-X., Parisini, E., Dascher, C. C., & Nigrovic, P. A. (2013). Ly6 family proteins in neutrophil biology. *Journal of leukocyte biology, 94*(4), 585-594.

Leijh, P., Van Zwet, T., Ter Kuile, M., & Van Furth, R. (1984). Effect of thioglycolate on phagocytic and microbicidal activities of peritoneal macrophages. *Infection and immunity, 46*(2), 448-452.

Li, P., Lu, M., Nguyen, M. A., Bae, E. J., Chapman, J., Feng, D., . . . Nguyen, A.-K. (2010). Functional heterogeneity of CD11c-positive adipose tissue macrophages in diet-induced obese mice. *Journal of Biological Chemistry, 285*(20), 15333-15345.

Li, Y., Zhang, P., Wang, C., Han, C., Meng, J., Liu, X., . . . Shi, X. (2013). Immune responsive gene 1 (IRG1) promotes endotoxin tolerance by increasing A20 expression in macrophages through reactive oxygen species. *Journal of Biological Chemistry, 288*(23), 16225-16234.

Liew, F. Y., Xu, D., Brint, E. K., & O'Neill, L. A. (2005). Negative regulation of toll-like receptor-mediated immune responses. *Nature Reviews Immunology, 5*(6), 446-458.

Lim, K.-H., & Staudt, L. M. (2013). Toll-like receptor signaling. *Cold Spring Harbor perspectives in biology, 5*(1), a011247.

Liu, Y., Wei, S., Ho, A., de Waal Malefyt, R., & Moore, K. W. (1994). Expression cloning and characterization of a human IL-10 receptor. *The Journal of Immunology, 152*(4), 1821-1829.

Lombardo, E., Alvarez-Barrientos, A., Maroto, B., Boscá, L., & Knaus, U. G. (2007). TLR4-mediated survival of macrophages is MyD88 dependent and requires TNF-α autocrine signalling. *The Journal of Immunology, 178*(6), 3731-3739.

London, A., & Schwartz, M. (2012). The privileged immunity of immune privileged organs: the case of the eye. *Frontiers in immunology, 3*, 296.

Luckheeram, R. V., Zhou, R., Verma, A. D., & Xia, B. (2012). CD4< sup. *Clinical and developmental immunology, 2012*.

Ma, J., Chen, T., Mandelin, J., Ceponis, A., Miller, N., Hukkanen, M., . . . Konttinen, Y. (2003). Regulation of macrophage activation. *Cellular and Molecular Life Sciences CMLS, 60*(11), 2334-2346.

Ma, X., Yan, W., Zheng, H., Du, Q., Zhang, L., Ban, Y., . . . Wei, F. (2015). Regulation of IL-10 and IL-12 production and function in macrophages and dendritic cells. *F1000Research, 4*.

MacDonald, K. P., Palmer, J. S., Cronau, S., Seppanen, E., Olver, S., Raffelt, N. C., . . . Wainwright, B. (2010). An antibody against the colony-stimulating factor 1 receptor depletes the resident subset of monocytes and tissue-and tumor-associated macrophages but does not inhibit inflammation. *Blood, 116*(19), 3955-3963.

Manjarrez-Orduño, N., Quách, T. D., & Sanz, I. (2009). B cells and immunological tolerance. *Journal of Investigative Dermatology, 129*(2), 278-288.

Martinez, F. O., & Gordon, S. (2014). The M1 and M2 paradigm of macrophage activation: time for reassessment. *F1000Prime Rep, 6*(13.10), 12703.

Martinez, F. O., Gordon, S., Locati, M., & Mantovani, A. (2006). Transcriptional profiling of the human monocyte-to-macrophage differentiation and polarization: new molecules and patterns of gene expression. *The Journal of Immunology, 177*(10), 7303-7311.

Martinez, F. O., Sica, A., Mantovani, A., & Locati, M. (2007). Macrophage activation and polarization. *Frontiers in bioscience: a journal and virtual library, 13*, 453-461.

Maynard, C. L., & Weaver, C. T. (2008). Diversity in the contribution of interleukin‐10 to T‐cell‐mediated immune regulation. *Immunological reviews, 226*(1), 219-233.

Medzhitov, R., & Janeway, C. (2000). Innate immune recognition: mechanisms and pathways. *Immunological reviews, 173*(1), 89-97.

Meraz, M. A., White, J. M., Sheehan, K. C., Bach, E. A., Rodig, S. J., Dighe, A. S., . . . Campbell, D. (1996). Targeted disruption of the Stat1 gene in mice reveals unexpected physiologic specificity in the JAK–STAT signaling pathway. *Cell, 84*(3), 431-442.

Mestas, J., & Hughes, C. C. (2004). Of mice and not men: differences between mouse and human immunology. *The Journal of Immunology, 172*(5), 2731-2738.

Metcalf, D., & Nicola, N. (1995). *The hemopoietic colony-stimulating factors: from biology to clinical applications*: Cambridge University Press.

Michelucci, A., Cordes, T., Ghelfi, J., Pailot, A., Reiling, N., Goldmann, O., . . . Rausell, A. (2013). Immune-responsive gene 1 protein links metabolism to immunity by catalyzing itaconic acid production. *Proceedings of the National Academy of Sciences, 110*(19), 7820-7825.

Michlewska, S., Dransfield, I., Megson, I. L., & Rossi, A. G. (2009). Macrophage phagocytosis of apoptotic neutrophils is critically regulated by the opposing actions of pro-inflammatory and anti-inflammatory agents: key role for TNF-α. *The FASEB Journal, 23*(3), 844-854.

Misharin, A. V., Saber, R., & Perlman, H. (2012). Eosinophil contamination of thioglycollate-elicited peritoneal macrophage cultures skews the functional readouts of in vitro assays. *Journal of leukocyte biology, 92*(2), 325-331.

Mosmann, T. R., Cherwinski, H., Bond, M. W., Giedlin, M. A., & Coffman, R. L. (1986). Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *The Journal of Immunology, 136*(7), 2348-2357.

Mosser, D. M. (2003). The many faces of macrophage activation. *Journal of leukocyte biology, 73*(2), 209-212.

Mosser, D. M., & Edwards, J. P. (2008). Exploring the full spectrum of macrophage activation. *Nature Reviews Immunology, 8*(12), 958-969.

Motwani, M. P., & Gilroy, D. W. (2015). *Macrophage development and polarization in chronic inflammation.* Paper presented at the Seminars in immunology.

Nahrendorf, M., Pittet, M. J., & Swirski, F. K. (2010). Monocytes: protagonists of infarct inflammation and repair after myocardial infarction. *Circulation, 121*(22), 2437-2445.

Nahrendorf, M., Swirski, F. K., Aikawa, E., Stangenberg, L., Wurdinger, T., Figueiredo, J.-L., . . . Pittet, M. J. (2007). The healing myocardium sequentially mobilizes two monocyte subsets with divergent and complementary functions. *The Journal of experimental medicine, 204*(12), 3037-3047.

Neeper, M., Schmidt, A., Brett, J., Yan, S., Wang, F., Pan, Y., . . . Shaw, A. (1992). Cloning and expression of a cell surface receptor for advanced glycosylation end products of proteins. *Journal of Biological Chemistry, 267*(21), 14998-15004.

O'Sullivan, D., Green, L., Stone, S., Zareie, P., Kharkrang, M., Fong, D., . . . La Flamme, A. C. (2014). Treatment with the antipsychotic agent, risperidone, reduces disease severity in experimental autoimmune encephalomyelitis. *PloS one, 9*(8), e104430.

Okabe, Y., & Medzhitov, R. (2014). Tissue-specific signals control reversible program of localization and functional polarization of macrophages. *Cell, 157*(4), 832-844.

Oracki, S. A., Walker, J. A., Hibbs, M. L., Corcoran, L. M., & Tarlinton, D. M. (2010). Plasma cell development and survival. *Immunological reviews, 237*(1), 140-159.

Ozinsky, A., Smith, K., Hume, D., & Underhill, D. (2000). Co-operative induction of pro-inflammatory signaling by Toll-like receptors. *Journal of endotoxin research, 6*(5), 393-396.

Palmer, E. (2003). Negative selection—clearing out the bad apples from the T-cell repertoire. *Nature Reviews Immunology, 3*(5), 383-391.

Parameswaran, N., & Patial, S. (2010). Tumor necrosis factor-α signaling in macrophages. *Critical Reviews™ in Eukaryotic Gene Expression, 20*(2).

Pelanda, R., & Torres, R. M. (2012). Central B-cell tolerance: where selection begins. *Cold Spring Harbor perspectives in biology, 4*(4), a007146.

Pfeffer, K., Matsuyama, T., Kündig, T. M., Wakeham, A., Kishihara, K., Shahinian, A., . . . Mak, T. W. (1993). Mice deficient for the 55 kd tumor necrosis factor receptor are resistant to endotoxic shock, yet succumb to L. monocytogenes infection. *Cell, 73*(3), 457-467.

Plüddemann, A., Neyen, C., & Gordon, S. (2007). Macrophage scavenger receptors and host-derived ligands. *Methods, 43*(3), 207-217.

Robinson, J. H., & Delvig, A. A. (2002). Diversity in MHC class II antigen presentation. *Immunology, 105*(3), 252-262.

Rock, K. L., Latz, E., Ontiveros, F., & Kono, H. (2010). The sterile inflammatory response. *Annual review of immunology, 28*, 321.

Rodig, S. J., Meraz, M. A., White, J. M., Lampe, P. A., Riley, J. K., Arthur, C. D., . . . Pennica, D. (1998). Disruption of the Jak1 gene demonstrates obligatory and nonredundant roles of the Jaks in cytokine-induced biologic responses. *Cell, 93*(3), 373-383.

Rongvaux, A., Willinger, T., Martinek, J., Strowig, T., Gearty, S. V., Teichmann, L. L., . . . Palucka, A. K. (2014). Development and function of human innate immune cells in a humanized mouse model. *Nature biotechnology, 32*(4), 364.

Rose, W. A., Sakamoto, K., & Leifer, C. A. (2012). Multifunctional role of dextran sulfate sodium for in vivo modeling of intestinal diseases. *BMC immunology, 13*(1), 41.

Sanderson, L. E., Chien, A.-T., Astin, J. W., Crosier, K. E., Crosier, P. S., & Hall, C. J. (2015). An inducible transgene reports activation of macrophages in live zebrafish larvae. *Developmental & Comparative Immunology, 53*(1), 63-69.

Saraiva, M., & O'Garra, A. (2010). The regulation of IL-10 production by immune cells. *Nature Reviews Immunology, 10*(3), 170-181.

Sasmono, R. T., Oceandy, D., Pollard, J. W., Tong, W., Pavli, P., Wainwright, B. J., . . . Hume, D. A. (2003). A macrophage colony-stimulating factor receptor–green fluorescent protein transgene is expressed throughout the mononuclear phagocyte system of the mouse. *Blood, 101*(3), 1155-1163.

Savill, J., Dransfield, I., Gregory, C., & Haslett, C. (2002). A blast from the past: clearance of apoptotic cells regulates immune responses. *Nature Reviews Immunology, 2*(12), 965-975.

Schneider, P., Takatsuka, H., Wilson, A., Mackay, F., Tardivel, A., Lens, S., . . . Tschopp, J. (2001). Maturation of marginal zone and follicular B cells requires B cell activating factor of the tumor necrosis factor family and is independent of B cell maturation antigen. *The Journal of experimental medicine, 194*(11), 1691-1698.

Sena, L. A., & Chandel, N. S. (2012). Physiological roles of mitochondrial reactive oxygen species. *Molecular cell, 48*(2), 158-167.

Shaposhnik, Z., Wang, X., & Lusis, A. J. (2010). Arterial colony stimulating factor-1 influences atherosclerotic lesions by regulating monocyte migration and apoptosis. *Journal of lipid research, 51*(7), 1962-1970.

Shaw, O. M., Steiger, S., Liu, X., Hamilton, J. A., & Harper, J. L. (2014). Brief Report: Granulocyte‐Macrophage Colony‐Stimulating Factor Drives Monosodium Urate Monohydrate Crystal‐Induced Inflammatory Macrophage Differentiation and NLRP3 Inflammasome Up‐Regulation in an In Vivo Mouse Model. *Arthritis & Rheumatology, 66*(9), 2423-2428.

Shen, L., & Rock, K. L. (2006). Priming of T cells by exogenous antigen cross-presented on MHC class I molecules. *Current opinion in immunology, 18*(1), 85-91.

Shi, C., & Pamer, E. G. (2011). Monocyte recruitment during infection and inflammation. *Nature Reviews Immunology, 11*(11), 762-774.

Silberman, D., Bucknum, A., Kozlowski, M., Matlack, R., & Riggs, J. (2010). Cytokine treatment of macrophage suppression of T cell activation. *Immunobiology, 215*(1), 70-80.

Smith-Garvin, J. E., Koretzky, G. A., & Jordan, M. S. (2009). T cell activation. *Annual review of immunology, 27*, 591.

Spach, K. M., Pedersen, L. B., Nashold, F. E., Kayo, T., Yandell, B. S., Prolla, T. A., & Hayes, C. E. (2004). Gene expression analysis suggests that 1, 25-dihydroxyvitamin D3 reverses experimental autoimmune encephalomyelitis by stimulating inflammatory cell apoptosis. *Physiological genomics, 18*(2), 141-151.

Stout, R. D., & Suttles, J. (2004). Functional plasticity of macrophages: reversible adaptation to changing microenvironments. *Journal of leukocyte biology, 76*(3), 509-513.

Takada, Y., Sung, B., Sethi, G., Chaturvedi, M. M., & Aggarwal, B. B. (2007). Evidence that genetic deletion of the TNF receptor p60 or p80 inhibits Fas mediated apoptosis in macrophages. *Biochemical pharmacology, 74*(7), 1057-1064.

Takeda, K., & Akira, S. (2004). *TLR signaling pathways.* Paper presented at the Seminars in immunology.

Takeda, K., & Akira, S. (2005). Toll-like receptors in innate immunity. *International immunology, 17*(1), 1-14.

Takeda, K., Clausen, B. E., Kaisho, T., Tsujimura, T., Terada, N., Förster, I., & Akira, S. (1999). Enhanced Th1 activity and development of chronic enterocolitis in mice devoid of Stat3 in macrophages and neutrophils. *Immunity, 10*(1), 39-49.

Takeuchi, O., Takeda, K., Hoshino, K., Adachi, O., Ogawa, T., & Akira, S. (2000). Cellular responses to bacterial cell wall components are mediated through MyD88-dependent signaling cascades. *International immunology, 12*(1), 113-117.

Taylor, P. R., Martinez-Pomares, L., Stacey, M., Lin, H.-H., Brown, G. D., & Gordon, S. (2005). Macrophage receptors and immune recognition. *Annu. Rev. Immunol., 23*, 901-944.

Tedder, T. F., Steeber, D. A., & Pizcueta, P. (1995). L-selectin-deficient mice have impaired leukocyte recruitment into inflammatory sites. *The Journal of experimental medicine, 181*(6), 2259-2264.

Thomas, G., Tacke, R., Hedrick, C. C., & Hanna, R. N. (2015). Nonclassical patrolling monocyte function in the vasculature. *Arteriosclerosis, thrombosis, and vascular biology, 35*(6), 1306-1316.

Toshchakov, V., Jones, B. W., Perera, P.-Y., Thomas, K., Cody, M. J., Zhang, S., . . . Fenton, M. J. (2002). TLR4, but not TLR2, mediates IFN-β–induced STAT1α/β-dependent gene expression in macrophages. *Nature immunology, 3*(4), 392-398.

Tschopp, J., Martinon, F., & Burns, K. (2002). The Inflammasome: A Molecular Platform Triggering Activation of Inflammatory Caspases and Processing of proIL-beta: A Molecular Platform Triggering Activation of Inflammatory Caspases and Processing of proIL-beta. *Molecular cell*(10), 417-426.

Tsunawaki, S., & Nathan, C. (1984). Enzymatic basis of macrophage activation. Kinetic analysis of superoxide production in lysates of resident and activated mouse peritoneal macrophages and granulocytes. *Journal of Biological Chemistry, 259*(7), 4305-4312.

Tumang, J. R., Hastings, W. D., Bai, C., & Rothstein, T. L. (2004). Peritoneal and splenic B‐1 cells are separable by phenotypic, functional, and transcriptomic characteristics. *European journal of immunology, 34*(8), 2158-2167.

Twigg III, H. L. (2005). Humoral immune defense (antibodies) recent advances. *Proceedings of the American Thoracic Society, 2*(5), 417-421.

Unanue, E. R. (1984). Antigen-presenting function of the macrophage. *Annual review of immunology, 2*(1), 395-428.

Varol, C., Mildner, A., & Jung, S. (2015). Macrophages: development and tissue specialization. *Annual review of immunology, 33*, 643-675.

Wagner, H. (2004). The immunobiology of the TLR9 subfamily. *Trends in immunology, 25*(7), 381-386.

Watanabe, T., Kitani, A., Murray, P. J., & Strober, W. (2004). NOD2 is a negative regulator of Toll-like receptor 2–mediated T helper type 1 responses. *Nature immunology, 5*(8), 800-808.

Weber-Nordt, R. M., Riley, J. K., Greenlund, A. C., Moore, K. W., Darnell, J. E., & Schreiber, R. D. (1996). Stat3 recruitment by two distinct ligand-induced, tyrosine-phosphorylated docking sites in the interleukin-10 receptor intracellular domain. *Journal of Biological Chemistry, 271*(44), 27954-27961.

Wehinger, J., Gouilleux, F., Groner, B., Finke, J., Mertelsmann, R., & Maria Weber-Nordt, R. (1996). IL‐10 induces DNA binding activity of three STAT proteins (Stat1, Stat3, and Stat5) and their distinct combinatorial assembly in the promoters of selected genes. *FEBS letters, 394*(3), 365-370.

Wesemann, D. R., & Benveniste, E. N. (2003). STAT-1α and IFN-γ as modulators of TNF-α signaling in macrophages: regulation and functional implications of the TNF receptor 1: STAT-1α complex. *The Journal of Immunology, 171*(10), 5313-5319.

Williams, J. C., Wagner, N. J., Earp, H. S., Vilen, B. J., & Matsushima, G. K. (2010). Increased hematopoietic cells in the mertk−/− mouse peritoneal cavity: a result of augmented migration. *The Journal of Immunology, 184*(12), 6637-6648.

Witsell, A. L., & Schook, L. B. (1992). Tumor necrosis factor alpha is an autocrine growth regulator during macrophage differentiation. *Proceedings of the National Academy of Sciences, 89*(10), 4754-4758.

Wynn, T. A., Chawla, A., & Pollard, J. W. (2013). Macrophage biology in development, homeostasis and disease. *Nature, 496*(7446), 445-455.

Xie, J., Méndez, J. D., Méndez-Valenzuela, V., & Aguilar-Hernández, M. M. (2013). Cellular signalling of the receptor for advanced glycation end products (RAGE). *Cellular signalling, 25*(11), 2185-2197.

Xing, Y., & Hogquist, K. A. (2012). T-cell tolerance: central and peripheral. *Cold Spring Harbor perspectives in biology, 4*(6), a006957.

Yin, J., Albert, R. H., Tretiakova, A. P., & Jameson, B. A. (2006). 5-HT 1B receptors play a prominent role in the proliferation of T-lymphocytes. *Journal of neuroimmunology, 181*(1), 68-81.

Yona, S., Kim, K.-W., Wolf, Y., Mildner, A., Varol, D., Breker, M., . . . Misharin, A. (2013). Fate mapping reveals origins and dynamics of monocytes and tissue macrophages under homeostasis. *Immunity, 38*(1), 79-91.

Zhu, J., & Paul, W. E. (2010). Heterogeneity and plasticity of T helper cells. *Cell research, 20*(1), 4-12.