TPL2 (MAP3K8) REGULATES THE MIGRATION, DIFFERENTIATION, AND FUNCTION OF CRITICAL INNATE IMMUNE CELLS DURING THE INFLAMMATORY RESPONSE

by

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(Under the Direction of Wendy Watford)

ABSTRACT

The protein kinase Tpl2 (MAP3K8) regulates innate inflammatory responses and is being actively pursued for therapeutic inhibition during chronic autoimmunity. Herein, we addressed the contribution of Tpl2 to pro-inflammatory responses of NK cells and macrophages. Despite the Tpl2-dependent regulation of IFNγ secretion by $CD4^+$ T-cells, NK cell IFNγ production, STAT4 expression, and expression of cytotoxic machinery occurred independently of Tpl2. In contrast, *tpl2*$^{-/-}$ macrophages were functionally defective, as they displayed impaired chemokine and chemokine receptor expression following LPS stimulation and were defective in migrating *in vivo* to inflamed tissues. *Tpl2*$^{-/-}$ macrophages were also impaired in their differentiation towards a pro-inflammatory phenotype (M1) while conversely displaying an enhanced anti-inflammatory phenotype (M2). Overall, this work provides additional support for targeting Tpl2, through its effects on macrophage recruitment and differentiation, for the treatment of autoimmunity.

INDEX WORDS: autoimmunity, inflammation, cytokines, chemokines, cellular trafficking, NK cells, macrophage classical activation, macrophage alternative activation

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CHAPTER 1

INTRODUCTION

The innate immune system is an ancient defense mechanism that has evolved to provide host protection against foreign pathogens by engaging a variety of cell types to elicit rapid and diverse antimicrobial responses. These responses are often characterized by the production of critical inflammatory mediators including chemokines and cytokines that regulate the recruitment and function of these innate cell types. Recently, much attention has focused on a particular kinase, known as tumor progression locus 2 (Tpl2, aka Map3k8), that transduces inflammatory signals through various receptors, including Toll-like receptors, cytokine receptors, death receptors and G-protein-coupled receptors. Intervention in these pathways could have significant benefits in the treatment of autoimmune diseases and other inflammatory disorders. Therefore, the overall goal of this work is to determine how Tpl2 might regulate innate immune cell trafficking, differentiation, and functions during the innate inflammatory response. The first aim is to determine whether Tpl2 regulates NK cell functions, including IFNγ secretion. The second aim in this study is to determine Tpl2's role in regulating macrophage trafficking to inflamed tissues. The third and final aim is to determine how Tpl2 controls macrophage differentiation between proinflammatory M1 and anti-inflammatory M2 subtypes. The work presented herein should help to expand our current understanding of how Tpl2 regulates acute inflammation. Furthermore, it will provide valuable information about the potential effects of Tpl2 inhibition clinically.

CHAPTER 2

LITERATURE REVIEW

Tpl2 Overview

Discovery of Tpl2

Tumor progression locus (Tpl2) is a member of the mitogen activated protein kinase (MAPK) family of serine/threonine kinases. The *tpl2* gene is the rat/murine homolog of the cancer Osaka thyroid (*cot*) gene that was identified as an oncogene in human thyroid carcinoma cell lines in 1991 (1). Tpl2 is widely expressed in both hematopoietic and nonhematopoietic cell lineages. Early functional studies demonstrated that Tpl2 was a potent inducer of experimental oncogenesis in mice and rats, and that its kinase activity is enhanced following C-terminal truncation of the protein (2, 3). Overexpression of Tpl2 in cell lines linked the kinase with several important biochemical pathways. For example, Tpl2 overexpression in Jurkat T cells led to the activation of nuclear factor of activated T cells (NFAT) and the production of interleukin-2 (IL-2) (4), both of which are potent mediators of T-cell function and proliferation. Additionally, the overexpression of Tpl2 was shown to be critical for regulating the activation and downstream functions of nuclear factor kappa B (NF-κB) (5). The NF-κB protein complex is critically involved in TLR-dependent signaling pathways, and limited proteolysis of NF-κB proteins leads to their translocation to the nucleus and transcriptional initiation of inflammatory mediators, such

as cytokines and chemokines (6). This evidence led to further exploration into Tpl2's possible involvement in pro-inflammatory responses, especially those involving NF-κB, and particularly Tpl2's ability to influence innate signaling mechanisms following cellular response to foreign agonists.

Tpl2 activation is currently understood to be induced downstream of various innate cellular receptors such as growth factors, cytokine receptors, death receptors and toll-like receptors. A more detailed discussion of Tpl2's relationship to NF-κB signaling mechanisms will be addressed later in this review.

Toll-like receptor function

Toll-like receptors (TLRs) are part of the pattern recognition receptor (PRR) family and are responsible for recognizing many pathogen-associated molecular patterns (PAMPs) (7). TLR signaling is a major pathway for the induction of the innate immune response, and these innate immune receptors are generally activated via exogenous pathogen ligands. Different TLRs responsible for recognizing distinct PAMPs are expressed on the cell surface or within intracellular (endosomal) compartments of various innate immune cell types, most notably macrophages, dendritic cells, B cells, and neutrophils. TLR ligation initiates the active recruitment of adaptor protein complexes and kinases that assist in TLR signaling. MyD88, a major adaptor molecule in innate signaling, is recruited to all TLRs, except TLR3 (7). In conjunction with MyD88, vital molecules belonging to the IL-1 receptor associated kinase family (IRAK1, 2 and 4) and the E3 ubiquitin ligase, TNF receptor associated factor 6 (TRAF6), are also recruited and play roles in TLR pathway activation. Additionally, the activation of other

IL-1 signaling pathway molecules such as the MAP kinase kinase kinase Map3k7 (aka TAK1) can lead to downstream activation of important innate complexes including NF-κB and MAPKs like Tpl2 (8). As a result, Tpl2 is emerging as a kinase of interest, primarily for its role in influencing the TLR-dependent transcription of important innate inflammatory mediators.

A major toll-like receptor, TLR4, is expressed on the surface of innate cells such as monocytes and dendritic cells that can uniquely recognize several ligands including bacterial lipopolysaccharide (LPS) during infection (9). In a landmark study, Tsichlis *el al*. demonstrated that *tpl2*$^{-/-}$ mice were resistant to LPS-induced endotoxic shock (10). This resistant phenotype was due to impaired secretion of the potent inflammatory cytokine tumor necrosis factor-α (TNF-α) and suggested for the first time that Tpl2 is required for mounting a proper innate pro-inflammatory response following TLR-dependent detection of foreign PAMPs. With this relationship between Tpl2 and TLR signaling now firmly established, a number of studies have sought to delineate the mechanisms involved in the regulation of TLR signaling by Tpl2. The following section outlines important discoveries related to Tpl2's function in TLR signaling.

LPS-induced signaling mechanisms

The NF-κB signaling pathway is often associated with TLR and MyD88-dependent inflammatory signaling in many innate immune cells and has been shown to be critical for regulating the transcription of various immune mediators, including immunoreceptors, cytokines, and chemokines in innate cells during inflammation (6). Further research into the LPS-induced activation of Tpl2 showed that, in a basal state, Tpl2 is directly complexed to the NF-κB inhibitory protein termed NF-κB1 p105. Upon triggering by inflammatory agonists such as

bacterial LPS, NFκB1 p105 undergoes limited proteolysis to yield p50 which releases Tpl2 from inhibition so that it may activate downstream signaling leading to the production of immune mediators (11). Following LPS stimulation in monocytic cell lines and bone marrow macrophages, Tpl2 activates the MAP kinase MEK which, in turn, activates the extracellular signal related kinases 1 and 2 (ERK-1/2) (11). ERK-1/2 induction has been shown to be critical for the transcription and production of the pro-inflammatory cytokines IL-1β and TNF-α in monocytic cell lines and has also been implicated in prominent inflammatory diseases such as cystic fibrosis (12, 13). Interestingly, ERK has also been implicated in anti-inflammatory functions, as the overexpression of ERK was shown to suppress NF-κB activation and control inflammatory macrophage functions in human umbilical vein endothelial cells (14)

As mentioned earlier, activation of this Tpl2/MEK/ERK pathway occurs following NF-κB1 p105 proteolysis (11). However, in contrast to the Tpl2-dependent direct activation of MEK/ERK, Tpl2 is no longer considered a major direct activator of NF-κB in response to TLR ligands. In fact, *tpl2*$^{-/-}$ mice display efficient NF-κB activation and p105 proteolysis following LPS stimulation (10), likely because only a small portion of NF-κB p105 in the cells is constitutively associated with Tpl2 (15). Additionally, this finding could also be explained by studies showing that inflammatory activation following LPS stimulation can also occur in MyD88-independent pathways (16). However, it is still possible that direct activation of NF-κB by Tpl2 may still occur and act in a selective fashion, specific to the extracellular stimulus. An illustration of the pathway involving TLR4 signaling, MyD88, NF-κB, and MAPKs such as Tpl2 is illustrated in Figure 2.1.

Tpl2 is now appreciated to be activated by other stimuli, including cytokines. Additionally, the serine/threonine kinase Akt, which is implicated in NF-κB induction (17), physically interacts with and phosphorylates Tpl2 at the serine residue S400 in T-cell lines (18).

Tpl2 and cytokine signaling

In addition to Tpl2's role during TLR-signaling mechanisms, Tpl2 can also transduce innate cytokine signaling. Following stimulation with TNF, the tyrosine kinase Syk activates Tpl2 in an NF-κB dependent manner (19). The major innate cytokine interleukin-1β (IL-1β) has been shown to both trigger Tpl2 activation via TLR-ligation (20) as well as be induced by Tpl2 during bacterial infections (21). IL-1β belongs to the IL-1 superfamily of cytokines that includes its primary homolog, IL-18 (22). Both IL-1β and IL-18 are potent inducers of the pro-inflammatory response (23, 24), and signaling for these cytokines occurs via the IL-1 receptor (IL-1R) and IL-18 receptor (IL-18R), respectively (22). Interestingly, the IL-1R/IL-18R signaling pathways are comprised of many of the same signaling molecules found within the TLR signaling pathways mentioned earlier, including MyD88, IRAK, and TRAF6 which has led to the creation of the TLR/IL-1R superfamily (22). Both TLR and IL-1R/IL-18R signaling pathways converge to promote the activation of MAPKs (such as Tpl2) leading to downstream transcription of innate immune mediators.

Activation of Tpl2 has also been shown to be induced by the pro-inflammatory cytokine IL-12 during *Toxoplasma gondii* infection, and Tpl2 genetic ablation caused mice to become more susceptible to this parasite (25). The major consequence of Tpl2 deficiency in this model was a significant impairment in IFNγ secretion during infection. IL-12 is a major driver and

activator of $CD4^+$ T cell differentiation (26), and loss of Tpl2 disrupted IL-12-induced IFNγ secretion by T cells in response to *T. gondii*. This study implicated Tpl2 in the regulation of IFNγ secretion and $CD4^+$ T helper 1 (Th1) cell development. The coordinated efforts between TLR signaling and IFNγ production are critical to innate immune activation, and the role of Tpl2 in both of these pathways is becoming better understood.

Tpl2's future

The discovery of Tpl2's role in innate inflammation has led to recent efforts to generate a specific Tpl2 kinase inhibitor with hopes that it could be used for the treatment of TNF-associated autoimmune disorders such as rheumatoid arthritis (27, 28). A number of features make Tpl2 especially well-suited for targeting. Although widely expressed among hematopoietic cells, Tpl2 appears to have more restricted, inflammation-related signaling roles compared to other kinases (8). Furthermore, Tpl2 shows low homology to other kinases, is not inhibited by the nonspecific kinase inhibitor staurosporine and is the only known human kinase to have a proline instead of a glycine in its ATP binding region, all of which make it an attractive drug target for selective inhibition (3, 4, 7). Therefore, targeting Tpl2 is anticipated to avoid problems encountered when targeting more ubiquitously expressed or, broadly acting components of the MAPK pathway, such as p38, ERK and JNK, where inhibition may be too global or result in major off-target effects (9). Although development of Tpl2 small molecule inhibitors holds promise, it must be approached with caution until a more complete understanding of Tpl2's functional roles during inflammation is reached. For example, direct clinical inhibition of Tpl2's pro-inflammatory kinase activity could lead to the exacerbation of

opposing anti-inflammatory responses, such as Th2-associated allergic asthma, or conversely could render patients vulnerable to certain infectious diseases. For these reasons, it is important to elucidate the direct roles Tpl2 plays in innate immune cell function during various inflammatory responses.

The natural killer (NK) cell and innate immunity

NK cell biology

The natural killer (NK) cell was originally described as an innate cytolytic effector lymphocyte distinct from cytotoxic T cells. This distinction resulted from the observation that NK cells exert their killing functions directly on targeted cells such as cancer cells without the need for prior antigen stimulation (29). However, it is now recognized that NK cells, like T and B cells, can even mediate immunological memory in response to prior encounter with the same antigen (30). As a part of the host response, the NK cell is a uniquely versatile immune mediator that can directly lyse targeted cells, present antigens, and secrete inflammatory cytokines. An illustration of the various NK cell-mediated effector functions is shown in Figure 2.2. The NK cell is found systemically in various organ sites of mammalian hosts including the blood, liver, spleen, lung, and intestines and active circulation of NK cells among these sites has been shown to be important for mounting sufficient innate immune responses upon activation (31-33).

NK cell-mediated cytolysis

The cytolytic capabilities of NK cells during systemic inflammation are characterized by the release of specialized lytic granules that induce various killing functions. One of the initial components of this NK-mediated cytolysis is the release of the membrane pore-forming protein known as perforin. Perforin polymerizes upon synapsis with target cells, permeabilizes the cell membrane, and allows for the release of cytolytic granules into the cytosol (34). One component of NK cytolysis that enters the target cell cytosol via this perforin-dependent mechanism is granzyme B. Granzyme B can act to induce target cell death via direct activation of intracellular caspase signaling which leads to cellular apoptosis. Additionally, NK cell release of granzyme B can induce further outer membrane permeabilization and loss of cellular integrity of the targeted host cell (35). The importance of this NK cell effector mechanism is substantiated by evidence linking granzyme B production to important inflammatory processes like defense against viral infections and the exacerbation of inflammation in autoimmune conditions such as rheumatoid arthritis (36).

Cytokine signaling and IFNγ production in NK cells

The transcriptional regulation of cellular inflammatory mechanisms observed in NK cells has been shown to be controlled, at least in part, by various cytokines including IL-2, IL-12, and IL-18 (37). Furthermore, detection of these cytokines by NK cell surface receptors also leads to phosphorylation of signal transducer and activators of transcription (STATs). Among these, STAT4 (activated by IL-12) and STAT5 a/b (activated by IL-2) have been implicated in the

induction of NK cell pro-inflammatory responses and thus are vital to elucidating the mechanisms of NK cell activation and function (38).

Originally described as a novel protein responsible for inducing T cell proliferation, interleukin-2 (IL-2) was the first interleukin to be fully characterized in regards to the host immune response (39). T-cell functions such as proliferation, memory, and regulatory responses, were shown to be influenced by IL-2. Interestingly, the generation of cytotoxic T-cells was also shown to act via an IL-2-dependent mechanism (40). These findings pushed the progress of IL-2 research to ask whether this cytokine could also influence the killing capacity of other primary immune cells such as NK cells. Indeed, early studies focusing on NK cell cytotoxicity *in vitro* found that exogenous IL-2 increased NK cell-mediated killing (41). These findings along with others outlining the expression of the IL-2R on the surface of circulating NK cells (42) provided a clear picture of the strong interaction between IL-2 and NK cell functionality.

Subsequent studies have demonstrated the importance of additional cytokines to NK cell functions. IL-12, a prominent pro-inflammatory cytokine, has been shown to enhance the production of IFNγ by NK cells while also boosting the cytotoxic activity of these cells (43, 44). During viral infections, IL-12 is necessary for NK-cell mediated IFNγ production and host protection (45). In addition to NK cells, IL-12 also promotes T-cell differentiation towards a Th1 pro-inflammatory subset and further promotes T cell cytotoxic killing similar to NK cell function (43, 46)

IL-18, another pro-inflammatory cytokine, is released by activated macrophages in response to bacterial pathogens in a manner similar to IL-12 and has been shown to enhance the effector functions of NK cells, including cytotoxicity and IFNγ production (47). As mentioned

previously, IL-18 signals through IL-18R in a MyD88-dependent fashion similar to IL-1 and may potentially act in a Tpl2-dependent manner. In addition, IL-18 interaction may lead to up-regulated expression of the Fas ligand (FasL) on the surface of activated NK cells, a ligand used to recognize target cells expressing the Fas receptor when marked for cell death (48). Interestingly, the combined effect of IL-12 and IL-18 stimulation of NK cells is to synergistically boost IFNγ production (49).

Following activation via exogenous signals, an important NK cell effector function is to produce significant levels of the pro-inflammatory cytokine IFNγ. IFNγ is widely involved in conferring host defense by enhancing the microbicidal actions of macrophages and cell-mediated immunity (50, 51). One of the critical steps in priming NK cells to produce high levels of IFNγ is the phosphorylation of members of the Janus kinase (JAK) family (52), followed by activation of the transcription factor STAT4 (37). An illustration of the coordination of the signaling pathways involving IL-12, IL-18, and STAT4 that leads to IFNγ production by NK cells during the inflammatory response is illustrated in Figure 2.3.

Innate cell trafficking during inflammation

Innate cell trafficking overview

The initial, rapid inflammatory response to foreign pathogens is a hallmark of innate immunity, and one of the key processes characterizing this response is cell trafficking to distal sites of injury or invasion. The first and usually most abundant innate cell to arrive is the neutrophil. This hematopoietically-derived cell generally arrives within the first 3-4 hours of

inflammatory insult and circulates along postcapillary venules before entering various tissue sites (53). Later on, other specialized innate cells such as macrophages and eosinophils arrive to the inflamed tissue in response to locally produced chemokines and cytokines. The biology and mechanisms of innate trafficking of both macrophages and eosinophils are described below.

Recruitment of monocytes/macrophages

Key regulators of innate immune response are monocytoid cells, or monocytes. These long lived cells are recruited from the bone marrow and are characterized by their ability to differentiate into certain subsets of dendritic cells (DCs) and macrophages. The roles of both of these cells are diverse and span both innate and adaptive arms of the immune system. DCs are considered the superior antigen presenting cell, capable of presenting MHC class I- and class II-restricted peptides to $CD8^+$ and $CD4^+$ T cells, respectively. However, macrophages also initiate antigen presentation to T cells and can further influence the nature of the adaptive immune response depending upon their cytokine secretion profiles (54). Macrophages, for example, promote a pro-inflammatory response upon stimulation via IL-12 secretion which drives a T helper 1 $CD4^+$ T cell phenotype associated with the secretion of IFNγ and protection against intracellular pathogens (55). In contrast, the macrophage can also drive anti-inflammatory immune regulation via active IL-10 secretion which can lead to enhanced regulatory T cell functions (56). This T cell subset, commonly referred to as “T-regs”, are important mediators of immunosuppression via regulation of tolerance to self-antigens (57) and control of autoimmune disorders such as inflammatory bowel disease (58). For these studies we focused on macrophages as primary innate cells due to their strict requirement for Tpl2 in TLR signaling

pathways, ease of culture, outgrowth from bone marrow, and their ability to efficiently migrate to sites of inflammation.

A key process during a robust inflammatory response is the coordination of signals between resident cells at the site of injury or infection and circulating leukocytes (i.e. monocytes). One of the major mechanisms coordinating cellular recruitment is chemokine signaling. Chemokines are small chemotactic proteins that are released in response to inflammation due to injury or infection at a tissue site. These proteins bind to corresponding G protein-coupled receptors expressed on the surface of target cells and can act as homing signals for circulating leukocytes that express the necessary receptors. Generation of a concentration gradient of chemokine levels at sites of inflammation initiates the adhesion and rolling process of effector cells across endothelial membranes that leads to migration to inflamed tissue sites (59).

Innate chemokines and chemokine receptors

Bacterial endotoxin induced expression of inflammatory chemokines is dependent on resident cell recognition of LPS by cell surface pattern recognition receptors such as TLRs. Additionally, the release of these chemotactic molecules by tissue-resident cells such as macrophages is regulated in part by NF-κB and MAP kinase activation (60). Therefore, Tpl2-dependent regulation of chemokine or chemokine receptor expression following ligation of PAMPs such as LPS is an area of great interest.

The family of monocyte chemotactic proteins (MCPs) is currently divided into two sub-clusters containing various chemokine ligands that are recognized by one or more corresponding chemokine receptor(s) expressed on the surface of macrophages. The first major MCP to be

discovered was MCP-1, also known as CCL2. This major inflammatory chemokine interacts primarily with surface-expressed G-protein coupled receptors such as CCR2 (as well as CCR4) that are found on the surface of activated or migrating leukocytes such as macrophages (61). CCL2 has been implicated in many inflammatory immune responses, notably as a potent monocyte recruiting factor at sites of infection or inflammation (62). Additionally, MCP-3, also known as CCL7, has been implicated in monocyte recruitment to inflamed tissues through interaction with chemokine receptors CCR1 and CCR2 on the surface of activated leukocytes (63). Taken together, the inflammatory contributions of both CCL2 and CCL7 are critical to our understanding of innate cell trafficking.

In addition to the MCPs, members of the CXC family of chemokines play important roles in inflammation. In particular, two CXC chemokines, CXCL2 and CXCL3 and their shared murine homologue, macrophage inflammatory protein-2 (MIP-2), regulate macrophage migration and recruitment to inflamed tissue sites (64). Further, their expression has been shown to be up-regulated during metastatic cancers such as breast cancer (65). The latter finding is interesting considering the previously discussed physiological influence of Tpl2 on oncogenesis. Thus, determining the extent to which Tpl2 influences CXC chemokine expression would be valuable for furthering research into monocyte migration as well as cancer metastasis.

The chemokine receptor CCR1 is expressed on various immune cells including monocytes, neutrophils, and T cells and is critical for the proper recruitment of these cells to sites of inflammation. CCR1 recognizes multiple chemokine ligands, yet its two major monocyte chemotactic ligands are MIP-1α (macrophage inflammatory protein-1α) and RANTES (regulated and normal T cell expressed and secreted) (66). Mice lacking CCR1 expression were shown to

be more susceptible to *Toxoplasma gondii* infection (67). Importantly, targeting of CCR1 for inhibition has become a new area of research for the treatment of inflammatory and autoimmune diseases such as rheumatoid arthritis (68).

CCR5 is another member of the MCP receptor family, and like CCR1, two of its major chemokine ligands are MIP-1α and RANTES. CCR5 is expressed on the surface of various immune cells including macrophages and is a key regulator of chemokine-dependent migration of circulating leukocytes following an inflammatory stimulus. Interestingly, CCR5 also binds HIV (human immunodeficiency virus) gp120. Gp120 binding to CCR5 is essential for viral entry into infected cells (69). This observation has boosted research into developing CCR5 antagonists to prevent HIV target cell entry and has led to an increased interest in the diverse functions of CCR5 expression by macrophages during infection. Use of CCR5 antagonists to block HIV-1 binding should be approached with caution as CCR5-deficient mice have defective immune responses to bacterial and viral pathogens (70, 71).

Recruitment of eosinophils

Eosinophils are another type of leukocyte involved in innate cell trafficking and responses to inflammatory stimuli. Eosinophils are found in numerous tissue sites including peripheral blood, the lower gastrointestinal (GI) tract, thymus, and spleen, where they have been shown to contribute to pathogenesis in various Th2-associated conditions such as eczema, asthma, and also in inflammatory bowel disease. The chemotaxis of circulating eosinophils to sites of inflammation depends largely on signaling via chemokine ligands such as RANTES and other MCPs in manners similar to macrophage chemotaxis (72). Additionally, the

chemoattractant eotaxin has been implicated in eosinophil-specific chemotaxis in lungs (73). Eosinophils also respond to cytokine-directed signaling fromTh2 cytokines such as IL-4, IL-5, and IL-13 secreted by T cells during allergic inflammation or parasitic infection. Circulating eosinophils express surface receptors for the chemotactic and activating cytokine IL-5, which was originally described as an eosinophil colony stimulating factor (74). Finally, in addition to chemokine and Th2 cytokine-mediated trafficking of eosinophils, the helminth-derived molecule chitin has also been implicated in recruiting eosinophils to sites of infection (75). Chitin is a polymer also found in insects. In helminth parasites chitin is thought to provide structural rigidity. Ongoing research into immune responses against chitin during parasitic infection has provided evidence for this molecule's role in eosinophil-mediated innate immunity.

Eosinophil recruitment and expansion is a hallmark of helminth infection. Helminths comprise a wide variety of parasitic worms that induce robust innate immune responses in the host at sites of infection such as the GI tract. Inflammatory responses to these infections are characterized by heavy Th2 bias, leading to the recruitment of many innate immune mediators such as monocytes/macrophages, mast cells, and eosinophils. The presence of helminth induced T-cell derived cytokines such as IL-4 and IL-5 drives eosinophils to further induce anti-parasitic functions including eosinophil aggregation around the parasite, degranulation of toxic contents, and mediation of cytotoxic killing by other effector cells such as T cells and macrophages (76).

The recruitment of macrophages and eosinophils during the inflammatory response is a critical process and the functional mechanisms behind the chemotaxis and migration of these cells, including the fully defined roles of cellular signaling molecules following the activation of TLRs or cytokine receptors, are still being elucidated. This work aims to assess the contributions

of the protein kinase Tpl2 on macrophage and eosinophil recruitment and how it may regulate the functional characteristics of these migratory cells described above.

Macrophage differentiation: The M1 vs. M2 subtype

Macrophage polarization during inflammation

The functional capabilities of macrophages are numerous and contribute to shaping immune responses. Therefore, an important area of research has begun to focus on how polarization and ultimately differentiation of these innate cells is achieved. In regards to the innate immune response, macrophages may respond with at least two major opposing inflammatory states within the host: pro-inflammatory and anti-inflammatory. Different pathogenic and environmental stimuli can readily induce these two antagonistic responses in the host, and an illustration of them is shown in Figure 2.4. For this reason, the polarization of macrophages is generally thought to be a plastic process that toggles between the two major subtypes described below.

M1 macrophage induction

M1 (or classically activated) macrophages represent a major subtype of macrophages that function primarily in the pro-inflammatory response. Induction of the M1 macrophage depends on various cellular signals from neighboring immune cells as well as foreign pathogens at sites of infection or injury. The major Th1 inflammatory cytokines IFNγ and TNF-α are produced at high levels by activated lymphocytes such as T cells or NK cells during the pro-inflammatory

response, and these cellular signals can directly polarize resident macrophages towards the M1 phenotype to promote cellular killing and pathogen clearance (77). Additionally, microbial triggers and PAMPs, such as bacterial LPS, also play major roles in inducing the M1 macrophage phenotype through PRR-dependent signaling (78). The functional output of M1 macrophages is the stimulus-dependent gene expression of cellular effector mechanisms that aim to promote cytotoxicity as well as proper pathogen clearance. For example, in response to a robust pro-inflammatory signal, M1 macrophages express NOS2/iNOS, major histocompatibility complex class II (MHC class II), IL-12 and TNF.

NOS2/iNOS (inducible nitric oxide synthase) is the inducible gene that encodes for the enzyme that catalyzes the production of nitric oxide (NO) from L-arginine by M1 macrophages (79). iNOS is primarily characterized by its ability to induce an antimicrobial oxidative burst by activated macrophages. NO secretion by macrophages during the inflammatory response has been implicated in immune responses during bacterial infections like *Mycobacterium tuberculosis* (80) as well as in the exacerbation of chronic inflammatory conditions such as arthritis (81). Like the M1 macrophage itself, iNOS expression is up-regulated following pro-inflammatory activation with external stimuli such as IFNγ and microbial triggers like LPS. For this reason iNOS has become a valuable cellular marker of the induction and functional status of M1 macrophages during pro-inflammatory disease states.

M2 macrophage induction

The other major subtype of polarized macrophages is termed the M2 (alternatively activated) macrophage. The M2 subtype is further divided into M2a, M2b, and M2c subtypes

based on the nature of external stimuli driving polarization as well as specific effector functions. M2a polarization is associated with induction by the primary Th2/anti-inflammatory cytokines IL-4 and IL-13, and M2a macrophages are generally responsible for promoting fibrosis and anti-inflammatory immune responses (82, 83). M2b macrophages are activated via ligands of the TLR/IL-1R pathway and perform dual roles by mediating macrophage regulatory functions including high IL-10 production as well as production of common M1 cytokines like IL-1 and TNF-α (84). Lastly, M2c macrophages are specialized regulatory macrophages primarily induced by IL-10 and TGF-β to promote immunosuppression (85). In general, M2 macrophages are critical during helminth infection, as they are major players in anti-parasitic responses such as granuloma formation and worm expulsion in conjunction with eosinophils and mast cells (86). In addition to anti-parasite functions, M2 macrophages also mediate fibrosis in inflamed tissues and promote tissue remodeling via interactions with prominent angiogenic cytokines such as TGFβ (87).

Infection with the helminth parasite *Schistosoma mansoni*

A good murine model for assessing M2 macrophage polarization *in vivo* during a parasitic infection is the intestinal helminth parasite, *Schistosoma mansoni*. This organism is the major agent of intestinal schistosomiasis. Schistosomes are trematodes that cause a chronic intestinal infection with high prevalence in many developing nations where water quality and sanitation are below standard (88). Infected individuals pass parasite eggs in urine or feces into fresh water. The larval miracidia hatches from the egg and finds the appropriate snail intermediated host, where asexual multiplication of the parasite takes place. Eventually, the

infective cercariae stage of the parasite emerges from snails and enters the water where they can infect the definitive host (humans) via skin surface entry. Maturation of the parasite in humans to adult worms (in organs sites such as the liver and GI tract) leads to the development of advanced stages of the infection. An illustration of the *Schistosoma mansoni* life cycle is depicted in Figure 2.5.

Disease due to schistosome infection is characterized by the induction of a robust Th2-type immune response, triggered by egg-associated antigens. Host responses to maturing *S. mansoni* parasites are initially characterized by strong pro-inflammatory responses including nitric oxide production along with the secretion of major Th1 cytokines like IFNγ and TNF. This early pro-inflammatory response begins to switch to Th2-type coincident by week 7 of infection, in response to schistosome eggs. This in turn leads to the activation and recruitment of innate mediators such as eosinophils and alternatively activated macrophages (89). A timeline of the immune response during *S. mansoni* infection is illustrated in Figure 2.6

M2 macrophage markers

Like the M1 subtype, M2 macrophage identification in experimental settings is largely based on the expression of multiple proteins unique to the alternatively activated phenotype, including Ym1, Retnla, Fizz1, mannose receptor and IL-4Rα.

Ym1 is a novel murine macrophage protein initially discovered as a secreted molecule from activated peritoneal macrophages following natural infection with the helminth parasite *Trichinella spiralis* (90). Induction of Ym1 gene expression is linked to both IL-4 and IL-13 signaling, leading to induction of alternative M2 macrophages. While the precise function of

Ym1 is still not understood, it shares homology to other mammalian chitinase-like proteins that exhibit strong anti-parasitic immune functions (91).

Retnla (resistin-like molecule) is a cysteine-rich protein that is secreted by M2 macrophages following stimulation with Th2 polarizing cytokines such as IL-4 and IL-13. Secretion of this protein by macrophages has been shown to assist in granuloma formation during parasitic infections with organisms like *Schistosoma mansoni* (92). This M2 protein is also referred to as Fizz1 (found in inflammatory zone) after studies pointed to a significant increase in the expression of the molecule in the bronchial epithelial cells of mice following allergic airway inflammation (93).

Finally, Arg1, the gene encoding for the cytosolic enzyme arginase, has also emerged as a reliable M2 macrophage marker since expression of Arg1 is highly induced following activation with the Th2 cytokines IL-4 and IL-13. Arg1 was first found to be expressed in the liver of mammalian hosts to assist in metabolic arginine hydrolysis (94). Recent studies have observed increased expression of Arg1 during infection with intestinal parasites such as *Schistosoma mansoni* and have proposed roles for the enzyme in wound healing and tissue fibrosis (95).

The balance between M1 and M2 macrophages is becoming better understood in the context of inflammation, and more recent studies have begun to look at possible mechanisms driving this physiological shift in the innate immune response. This work aims to assess Tpl2's possible role in regulating macrophage polarization which could lead to a better overall understanding of how inflammatory cells mediate differing responses to specific pathogenic stimuli.

Figure 2.1 TLR4 signaling pathway

Following ligation with pathogen associated molecular patterns (PAMPs) such as bacterial LPS, toll-like receptors like TLR4 initiate inflammatory signaling cascades by recruiting the vital adaptor molecule MyD88. Additional recruitment of and complex with IL-1R associated kinases (IRAK1, 2, and 4) and TNF receptor associated kinases (TRAF6) begins signal transduction through initial MAP kinases including TAK1. TAK1 signaling activates the IKKβ protein complex as well as other MAP kinases such as Tpl2 (MAP3K8). Proteolysis of the NF-κB p105 inhibitory subunit by IKKβ from inactive Tpl2 leads to the downstream signaling by active Tpl2 through MEK and ERK protein complexes and further activation of the nuclear transcription factor AP-1. AP-1 nuclear translocation along with NF-κB activation within the nucleus leads to the further production of critical inflammatory immune mediators such as cytokines and chemokines by the host cell

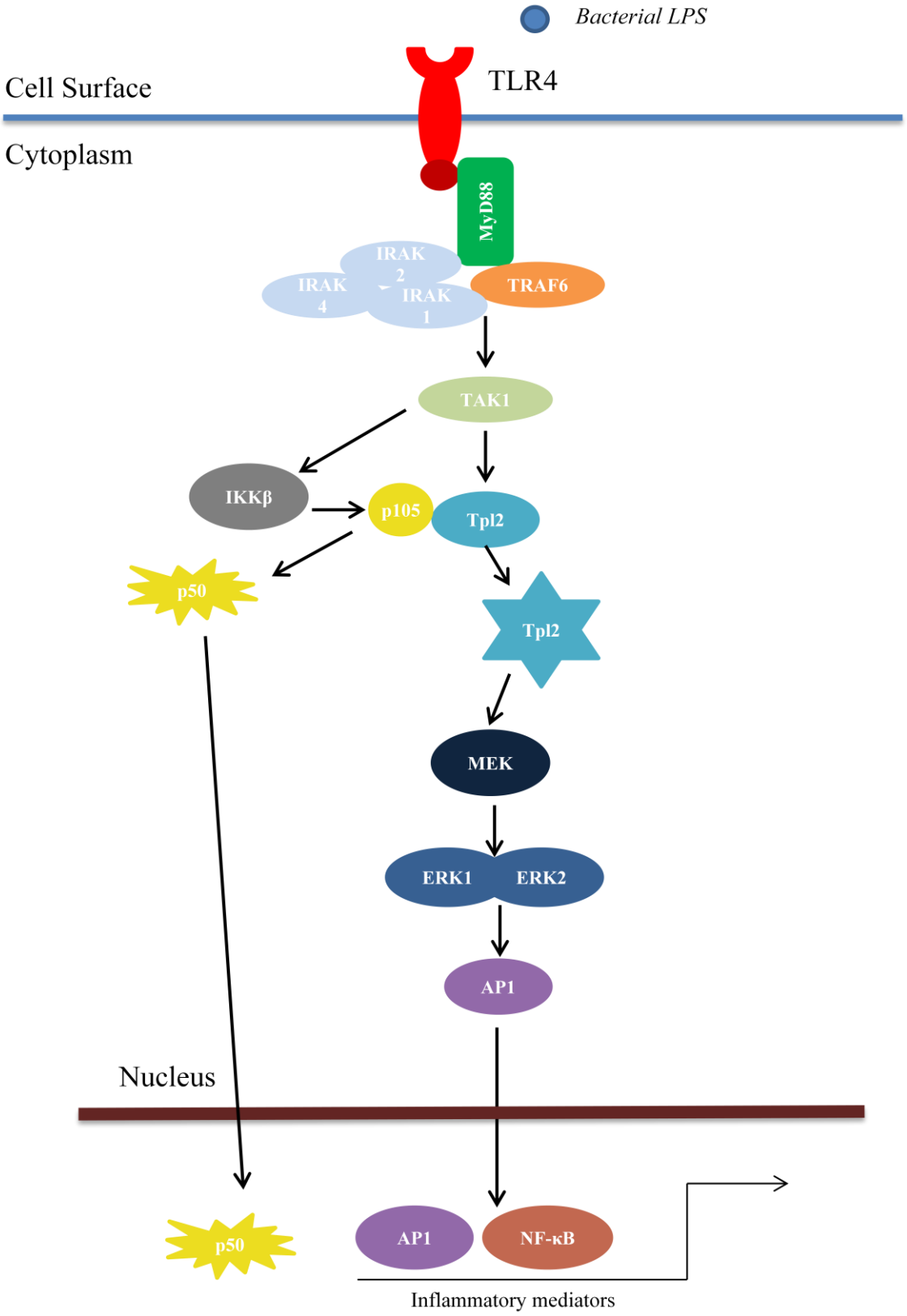

Bacterial LPS
Cell Surface
TLR4
Cytoplasm
MyD88
IRAK 4
IRAK 2
IRAK 1
TRAF6
TAK1
IKKβ
p105
Tpl2
p50
Tpl2
MEK
ERK1
ERK2
AP1
Nucleus
p50
AP1
NF-κB
Inflammatory mediators

Figure 2.2 NK cell effector functions

NK cell effector functions are commonly induced following cytokine signal activation through various cytokine and cytokine receptor interactions including IL-2, IL-12, and IL-18. Cytotoxic effector functions of activated NK cells are characterized by the release of pore-forming proteins such as perforin that permeabilize targeted host cell membranes. Subsequent production of apoptosis-inducing components such as granzyme B in the host cell cytosol also occurs. Additionally, targeted host cell destruction by NK cells can occur by ligation of the death receptor (FasL) with the targeted host cell death receptor (FasR), as well as by the production of the pro-inflammatory cytokine IFNγ to drive Th1-dependent killing of targeted or infected host cells.

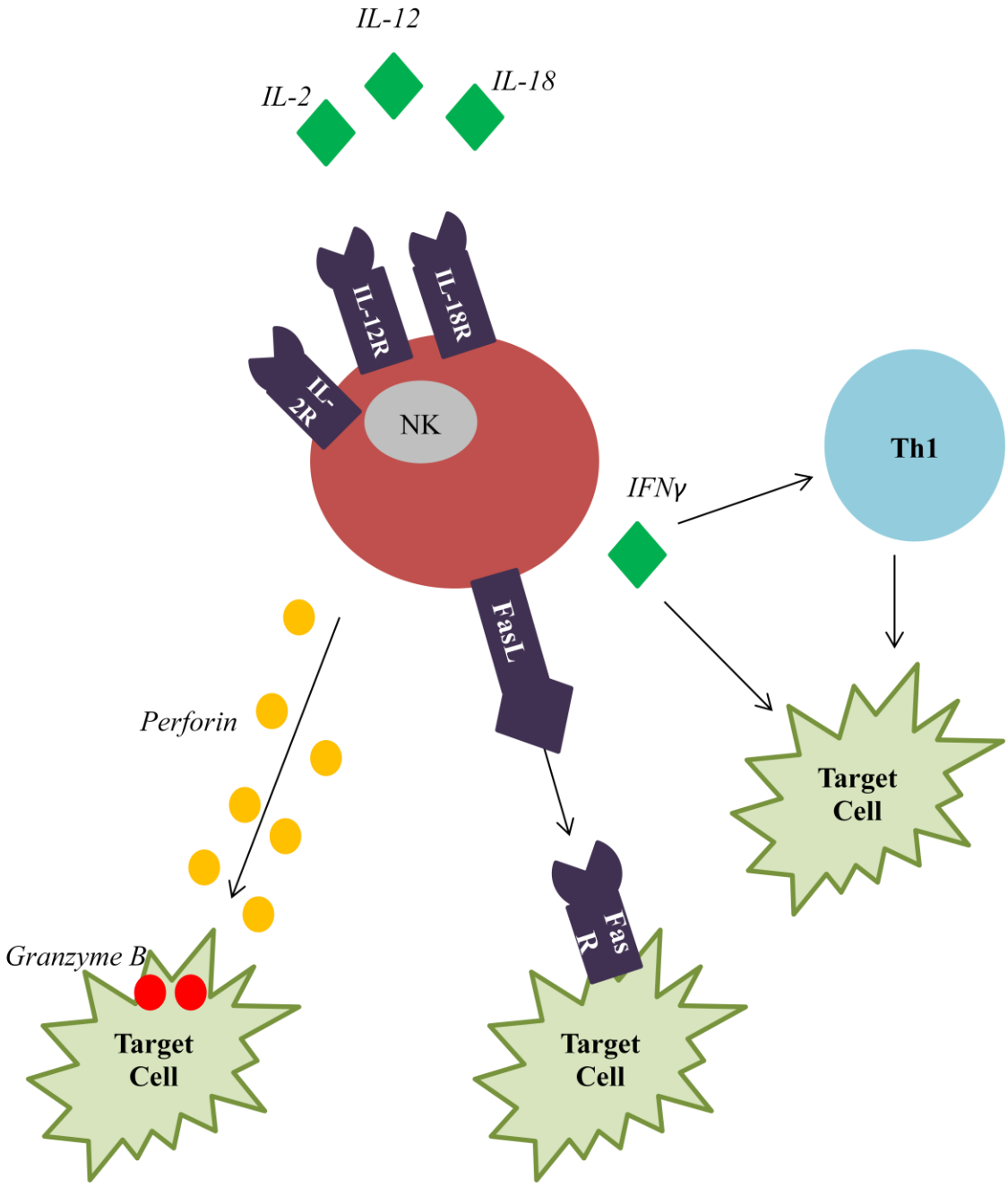

IL-12
IL-2
IL-18
IL-12R
IL-18R
IL-2R
NK
Th1
IFNγ
FasL
Perforin
Target Cell
Granzyme B
Fas R
Target Cell
Target Cell

Figure 2.3 IL-12/IL-18/STAT4 signaling pathways leading to IFNγ production

NK cell development and activation is regulated in part by cell surface receptor signaling involving the inflammatory cytokines IL-12 and IL-18. The IL-12R is expressed on the surface of resting NK cells and signal transduction is initiated following ligation with exogenous IL-12 in the extracellular environment. This signaling pathway is mediated by critical mitogen activated kinases (MAPK) such as the Janus activated kinase-2 (JAK2) that help to translocate signals to the NK cell nucleus. The nuclear transcription factor STAT4 is critical for IL-12 signaling in NK cells and activation of STAT4 leads to the transcription and production of the pro-inflammatory cytokine IFNγ which can promote direct cell killing as well as Th1 T-cell differentiation. Additionally, ligation of the extracellular cytokine IL-18 to the NK cell IL-18 surface receptor (IL-18R) acts synergistically with IL-12R signaling by mediating the transcription and production of IFNγ via an NF-κB dependent pathway following inflammatory activation.

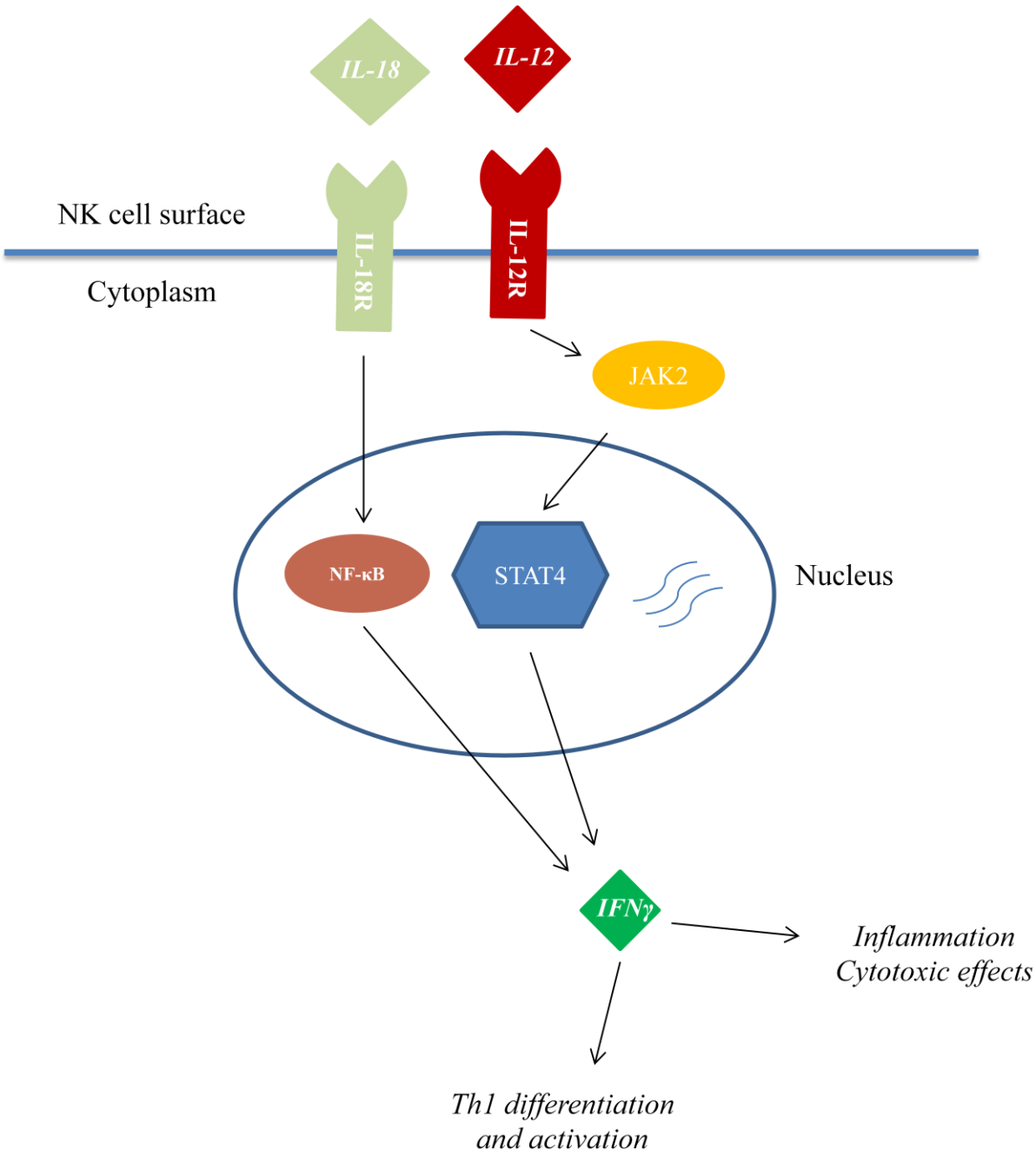

IL-18
IL-12
NK cell surface
Cytoplasm
IL-18R
IL-12R
JAK2
NF-κB
STAT4
Nucleus
IFNγ
Inflammation
Cytotoxic effects
Th1 differentiation
and activation

Figure 2.4 Macrophage polarization during the inflammatory response

During specific inflammatory immune responses, macrophage polarization between two major subtypes is induced by specific stimuli and is characterized by differing effector responses. Classically activated (M1) macrophages are induced following activation by bacterial PAMPs such as LPS and via Th-1 mediated induction through the pro-inflammatory cytokine IFNγ. The M1 macrophage subset is characterized by its ability to mediate pro-inflammatory, cell killing mechanisms through the production of various effector molecules including iNOS, MHC class II, TNF, and IL-12. In contrast, the alternatively activated M2 subset of macrophages is commonly observed following Th2 dependent inflammatory signals via IL-4, IL-5, and IL-13. Additionally, M2 macrophages are also induced following parasitic infections with pathogens including helminths. The M2 macrophage is described as an anti-inflammatory, anti-parasitic immune effector characterized by expression of the specific M2 proteins Ym1, Fizz1, Arg1, mannose receptor and IL-4Rα and is also responsible for promoting tissue remodeling and fibrosis during certain inflammatory conditions.

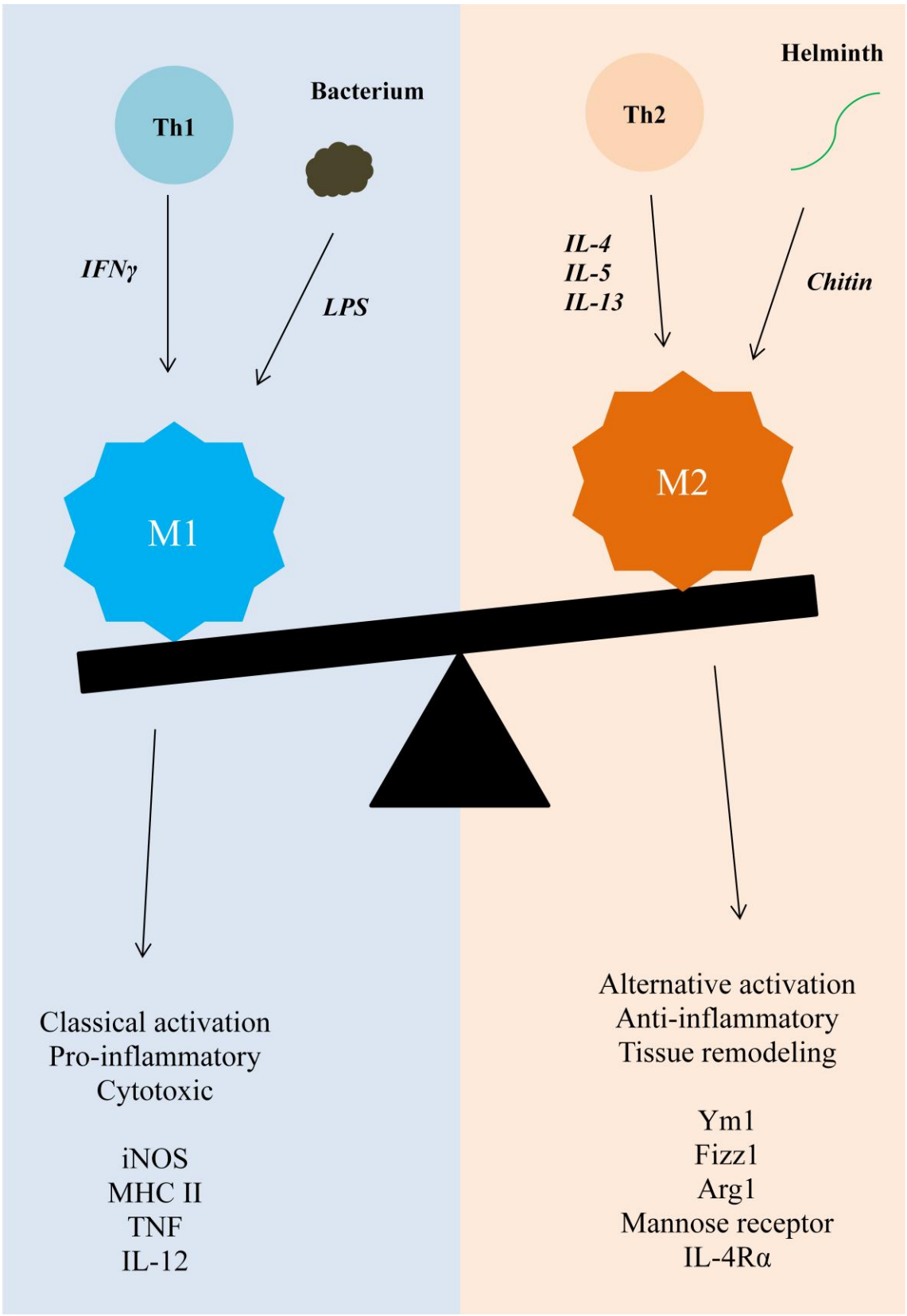

Th1
Bacterium
IFNγ
LPS
M1
Classical activation
Pro-inflammatory
Cytotoxic
iNOS
MHC II
TNF
IL-12
Helminth
Th2
IL-4
IL-5
IL-13
Chitin
M2
Alternative activation
Anti-inflammatory
Tissue remodeling
Ym1
Fizz1
Arg1
Mannose receptor
IL-4Rα

Figure 2.5 *Schistosoma mansoni* life cycle

Eggs are initially deposited in the urine or feces of infected vertebrate hosts into areas of wet soil or ground water. Under optimal growth conditions, eggs can develop into miracidia and infect the snail intermediate host where they proceed to the infective cercariae stage. Developed cercariae exit the snail host and enter water supplies where they can swim and infect the definitive vertebrate host (primarily humans) directly through the skin. Once inside, cercariae mature into adult worms that can spread systemically to multiple tissue sites but are primarily found in the small and large intestine during advanced infection. Once mature, adult *S. mansoni* worms begin to lay eggs inside the host that are eventually excreted in the host urine or feces.

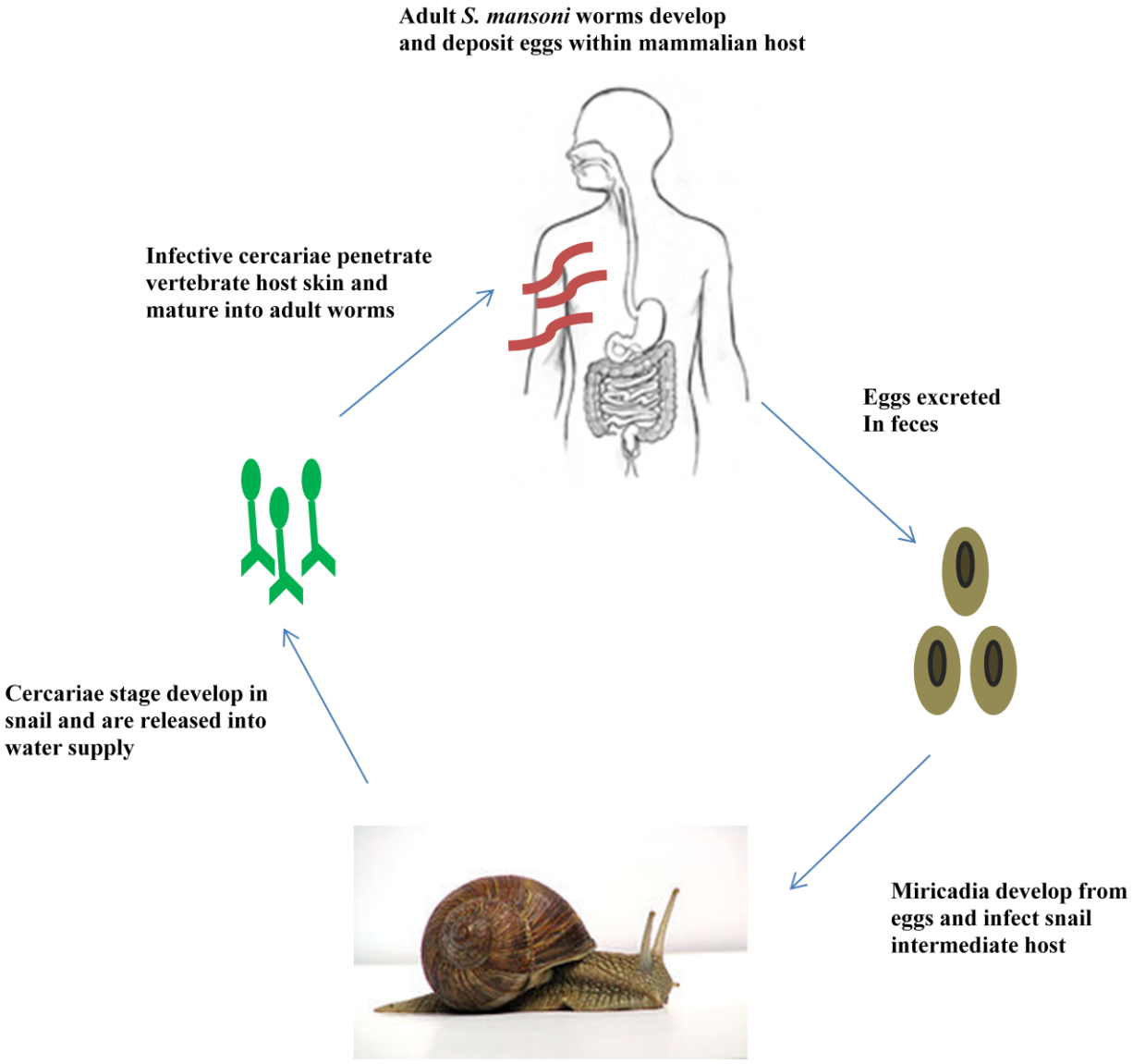
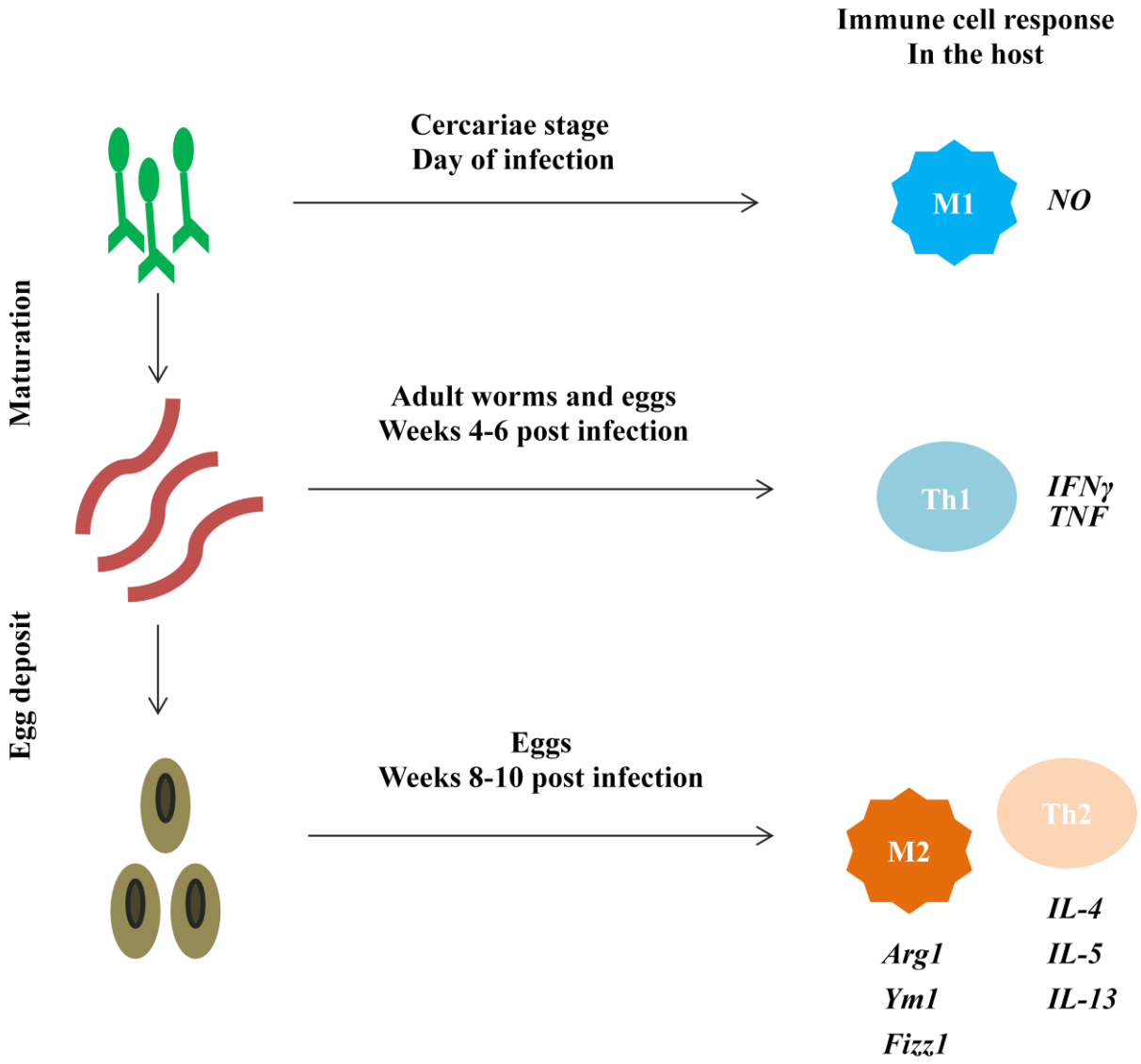

Adult *S. mansoni* worms develop
and deposit eggs within mammalian host
Infective cercariae penetrate
vertebrate host skin and
mature into adult worms
Eggs excreted
In feces
Cercariae stage develop in
snail and are released into
water supply
Miricadia develop from
eggs and infect snail
intermediate host

Figure 2.6 Immune responses to *S. mansoni* life cycle stages in the vertebrate host

During initial infection with infective *S. mansoni* cercariae (from 0-3 weeks post infection), a robust pro-inflammatory immune response develops and is characterized by the induction of pro-inflammatory (M1) macrophages and the release of nitric oxide (NO). Additionally, by week 4-6 post infection as *S. mansoni* worms begin to mature, induction of Th1 T-cells promotes the production of pro-inflammatory cytokines IFNγ and TNF to combat infection. As infection progresses and adult worms begin to lay eggs within the GI tract of infected hosts, a robust Th2 response develops characterized by the induction alternatively activated (M2) macrophages as well as Th2 T-cells. M2 macrophages can be characterized by upregulation of alternative macrophage markers Ym1, Fizz1, and Arg1 while Th2 T-cells actively secrete prominent anti-inflammatory cytokines including IL-4, IL-5, and IL-13 to promote clearance of the parasite via the recruitment of additional innate cells such as eosinophils and mast cells


Immune cell response
In the host
Cercariae stage
Day of infection
M1
NO
Maturation
Adult worms and eggs
Weeks 4-6 post infection
Th1
IFNγ
TNF
Egg deposit
Eggs
Weeks 8-10 post infection
M2
Th2
IL-4
IL-5
IL-13
Arg1
Ym1
Fizz1

CHAPTER 3

MATERIALS AND METHODS

Mice

Wild-type C57BL/6J mice were purchased from The Jackson Laboratory. *Tpl2*$^{-/-}$ mice were a generous gift from Dr. Philip Tsichlis. Breeding colonies of mice were maintained and housed in sterile caging in a specific pathogen-free facility at the University of Georgia. All animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Georgia.

Media and Buffers

Supplemented RPMI

RPMI containing 10% heat-inactivated fetal bovine serum (FBS, Invitrogen), 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine, 15 mM HEPES and 50 μM 2-mercaptoethanol.

Supplemented DMEM

Dulbecco's modified Eagle medium (DMEM) containing 10% heat-inactivated FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 4 mM L-glutamine, and 1000 mg/L glucose was used for macrophage culture *in vitro*.

MACS buffer

1x PBS, 0.5% BSA, and 2 mM ethylenediaminetetraacetic acid (EDTA) filtered

Protein Lysis buffer (10M)

10 mL total volume with DI H_2O containing:

50 mM Tris, 300 mM NaCl, 0.5% Triton X 100, 2 mM EDTA, 0.4 mM Na_3VO_4, 2.5 mM leupeptin, 2.5 mM aprotinin, 2.5 μM NPGB

Western blot running buffer

3-(N-Morpholino) propanesulfonic acid (MOPS) running buffer for Bis-Tris gels (Invitrogen)

FACS staining buffer

Phosphate buffered saline (PBS), 0.1% bovine serum albumin (BSA).

Perm-Wash buffer

PBS, 0.1% BSA, 0.5% saponin

Cell Culture and Purification

Splenocytes and peripheral lymph node cells

Spleens and lymph nodes were removed from mice and placed in 1x PBS in a small culture dish. Organs were dissociated mechanically in 1x PBS over a 70 μM filter using the barrel of a 3 cc

syringe. Cells were centrifuged at 1200 rpm for 10 min at room temperature (RT) and the pellet was re-suspended in MACS buffer prior to $CD4^+$ T-cell or NK cell isolation.

Bone Marrow (BM) isolation and culture of bone marrow derived macrophage (BMDM)

BM was harvested from tibiae and femurs of mice by flushing with supplemented DMEM using a 10 mL syringe and 25-gauge needle. Cells were disaggregated by gentle pipetting and centrifuged at 1200 rpm for 10 min at RT. The cells were then re-suspended in ACK lysing buffer (Invitrogen) for 30 sec to lyse red blood cells. Cells were washed in PBS (20-30 mL) and centrifuged again at 1200 rpm for 10 min. Cell pellet was re-suspended in supplemented DMEM for cell counting. Differentiated macrophages were obtained by culturing BM cells on sterile petri dishes at 2×10^6/mL in supplemented DMEM with the addition of macrophage colony-stimulating factor (M-CSF) (10 ng/mL) for 7-10 days at 37°C, 5% CO_2. Cells were adherence purified on day 7-10 by removing media, washing adherent cells with PBS and then harvesting by incubating in 10 mL cell dissociation buffer (Invitrogen) for 15 min at 37°C. Plates were washed with PBS to collect dislodged cells, and cells were centrifuged at 1200 rpm for 10 min. Cell pellets were re-suspended in supplemented DMEM for cell counting.

Peritoneal exudate cells

Mice were injected intraperitoneally (i.p) with 1 mL of 3% Brewer thioglycollate medium to induce local inflammation and recruit effector cells. After 72 h, mice were sacrificed and the peritoneal cavity was lavaged three times with 3 mL of sterile PBS to collect recruited cells. For *in vivo* activation of thioglycollate recruited cells, mice were additionally injected 24 h prior to

sacrifice with 10 μg lipopolysaccharide (LPS) from *Escherichia coli* (0111:B4) i.p. in order to activate recruited peritoneal cells. Cells were centrifuged at 1200 rpm for 10 min at RT and were re-suspended in supplemented DMEM. 0.5×10^6 or 1×10^6 cells were plated at 0.5×10^6/mL or 1×10^6/mL in 12 well culture plates and allowed to adhere to the wells for 6-8 h. Afterwards, supernatant was removed and fresh media was added to begin stimulation assays.

NK cell isolation

Spleen and lymph node cells were re-suspended in MACS buffer. Cells were incubated with a biotinylated-antibody cocktail (mouse NK Cell Isolation Kit, Miltenyi Biotec) for 10 min at 4°C, followed by incubation with anti-biotin microbeads for 15 min at 4°C to deplete non-NK cells. Magnetically labeled non-NK cells were depleted by magnetic separation through an LS column in a mini-MACS Separator. Unlabeled bulk NK cells that passed through the column were collected and suspended in FACS staining buffer. In some cases, bulk NK cells enriched by magnetic separation were additionally purified by sorting using flow cytometry. For sort purification, enriched NK cells were surface stained with fluorescently labeled antibodies to T-cell markers TCRβ or CD3 as well as NK cell markers DX5 and/or NK1.1 (eBiosciences) in order to isolate pure NK cell populations. Cells were sorted using a Beckman Coulter MoFlo XDP cell sorter, and purified cells were then used for assay stimulation as indicated. To generate lymphocyte activated killer (LAK) cells, magnetically enriched NK cells were cultured in media supplemented with 1000 U/mL of IL-2 for 14d at 37°C, 5% CO_2 to allow for NK cell proliferation and activation.

$CD4^+$ T-cell isolation

Spleen and lymph node cells were re-suspended in MACS buffer and purified using magnetic bead separation similar to NK cell purification described above (mouse CD4 T-cell Isolation Kit, Miltenyi Biotec). Unlabeled bulk $CD4^+$ T-cells were surface stained for sorting by flow cytometry with fluorescently labeled antibodies to the markers CD25, CD4, CD44, and CD62L (eBiosciences). Naïve $CD4^+CD25^-CD44^-CD62L^+$ T-cells and memory $CD4^+CD44^+CD62L^-$ T-cells were sorted and used for assay stimulation as indicated.

Cell Stimulation

After isolation, 0.5×10^6 or 1×10^6 mouse NK cells, BMDMs and adherence purified PECs were plated at either 0.5×10^6/mL or 1×10^6/mL in supplemented RPMI or DMEM media. Cells were left unstimulated (as a control) or stimulated with PMA/ionomycin (both at 500 ng/mL), IL-12 (10 ng/mL), IL-18 (10 ng/mL), lipopolysaccharide (LPS) from *Escherichia coli* (0111:B4, 1 μg/mL), IFNγ (10 ng/mL), or IL-4 (40 ng/mL) for various time-points at 37°C, 5% CO_2. For stimulation of naïve $CD4^+$ T-cells, cells were cultured in supplemented RPMI in wells containing 5 μg/mL anti-CD3/CD28 plate-bound antibodies for indicated time points. For the ERK inhibition experiment, media containing naïve $CD4^+$ T-cells was supplemented with U0126 ethanolate ERK inhibitor at 20 μM or a DMSO vehicle control 30 min prior to culture.

Infection with X31 Influenza virus

C57BL/6J and *tpl2*$^{-/-}$ mice were infected with 1×10^6 PFU X31 Influenza virus (or with allantoic fluid as a control) intranasally under sterile conditions. After 3 days, mice were sacrificed and

organs and tissues were processed. Bronchoalveolar lavage fluid (BAL) was collected from the lungs of mice via lavage with 1 mL PBS twice using a sterile catheter tip syringe. Lungs were perfused with 10 mL PBS via the heart muscle perfusion to remove blood from the lung tissue, and whole lungs were digested using 6 mg/mL collagenase. Total lung cells were resuspended in 47% percoll, layered onto 67% percoll, and centrifuged at 2000 rpm for 20 min with the brake off. Lymphocytes at the gradient interface were removed, washed in PBS and enumerated. Additionally, spleens and mediastinal lymph nodes (MLNs) of mice were also collected, and cells were isolated via mechanical dissociation in PBS through a 70 μM filter using the barrel of 3 cc syringe. Cells were centrifuged at 1200 rpm for 10 min at RT, and cell pellets were re-suspended in supplemented RPMI to be counted and used in assay stimulations.

Schistosoma mansoni egg-induced inflammation

S. mansoni eggs were a generous gift from Dr. Donald Harn and were isolated from the livers of mice infected with *S. mansoni* cercariae 8-10 weeks post infection. C57BL/6J and *tpl2*$^{-/-}$ mice were injected i.p. with 5000 eggs suspended in PBS. After 1 week, mice were sacrificed, and peritoneal cells were harvested via lavage as described previously. Cells were centrifuged at 1200 rpm for 10 min at RT and were re-suspended in appropriate media or buffer prior to use in assays.

Cell counting

Live cell counts were determined via dilution of cell suspensions in Trypan Blue followed by either manual counting in a glass hemocytometer slide with an Invertoskop 40 C microscope (Zeiss) or automatic counting using the TC-10^{TM} Automated Cell Counter (Bio-Rad).

Cytokine measurement by ELISA

IFNγ was measured using an ELISA kit according to the manufacturer's instructions (eBioscience, cat. # 88-7314-88). Plates used were Corning Costar 9018 EIA/RIA plates and were coated with 100 μL/well of capture antibody diluted in manufacturer's coating buffer.

RNA isolation

RNA lysates of NK cells, BMDMs, or adherence purified PECs were prepared directly following assay simulation. For adherent cells, media supernatant was removed and wells were washed with cold PBS. Next, cells were directly lysed in culture wells with 350 μL TRK Lysis buffer containing 20 μL 2-mercaptoethanol (Omega Bio-Tek cat # R6834-02). For suspension cells, cells were collected from plates into tubes and centrifuged in the presence of cold PBS. Cell pellets were lysed in 350 μL TRK Lysis buffer. Isolation of RNA was performed using Total RNA Kit I (Omega Bio-Tek cat # R6834-02) according to manufacturer's instructions. RNA was eluted from the columns in 40 μL of DEPC water and stored at -80°C.

cDNA reverse transcription

Total RNA concentration of individual samples was determined using the NanoDrop Spectrophotometer (Thermo Scientific). Conversion of mRNA to cDNA was achieved using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). cDNA reactions were prepared by adding 10 μL 2x RT Master Mix (10x RT buffer, 25x dNTP Mix, 10x RT Random Primers, 20x MultiScribe Reverse Transcriptase, and nuclease-free H_2O) to 10 uL of RNA solution containing between 80-200 ng total RNA, depending on the individual experiment. To initiate reverse transcription, samples were loaded into a thermal cycler set to the following conditions:

1. 25°C for 10:00
2. 37°C for 120:00
3. 85°C for 5:00
4. 4°C for ∞

cDNA samples were stored at -20°C until further use.

Quantitative Real-Time PCR

RT-PCR was performed using the following primer/probe mixes from TaqMan Gene Expression Assays (Applied Biosystems) and 2x TaqMan Universal Master Mix (Applied Biosystems):

Probe	Assay ID (Applied Biosystems)
Map3k8	Mm00432637_m1
Stat4	Mm00448890_m1
Granzyme B	Mm00442834_m1
Perforin	Mm00812512_m1
CCL2	Mm00441242_m1

CCL7	Mm00443113_m1
CXCL2	Mm00436450_m1
CXCL3	Mm01701838_m1
CCR1	Mm00438260_s1
CCR5	Mm01963251_s1
CCR2	Mm00438270_m1
Nos2 (iNOS)	Mm00440502_m1
Chi313 (Ym1)	Mm00657889_mH
Actb (Actin B)	Mm00607939_s1

Following assay preparation of cDNA sample and desired primer/probe mix, qRT-PCR assays were run in MicroAmp Fast Optical 96-Well Reaction Plates (Applied Biosystems) in a StepOnePlus Real-Time PCR System (Applied Biosystems) set to the following thermal cycler conditions:

1. 95°C for 10:00
2. 95°C for 0:15
3. 60°C for 0:60 (steps 2-3 looped 40 times)
4. 50°C for 2:00

Relative expression levels of all genes were calculated by normalizing Ct levels of the target gene to that of an actin B endogenous control as well as normalizing to a control (wild-type or untreated) sample using the ΔΔCt method and Microsoft Excel.

Western blotting

Enriched NK cells were cultured in RPMI media and were left unstimulated or stimulated with IL-12 (10 ng/mL) for 15 min at 37°C, 5% CO_2. Cells were washed with PBS and were lysed in 100 μL protein lysis buffer. Protein concentration was determined using BCA Protein Assay Kit (Pierce cat # 23227) and approximately 15 μg total protein per sample was separated by SDS-PAGE at 100V for 2 hours using NuPAGE 4-12% gradient Bis-Tris gels (Invitrogen, cat.# NP0335BOX). Electrophoresed proteins were transferred onto PVDF membranes (Invitrogen cat # IB401002) at 15V for 10 min using an iBlot Gel Transfer System (Invitrogen). Membranes were blocked in TBS-Tween wash buffer (10X TBS, 0.1% Tween-20, dH_2O) + 5% skim milk for 1 h at RT and were then incubated with rabbit anti-mouse p-STAT4 (ZYMED cat # 71-7900) or rabbit anti-mouse total STAT4 (Santa Cruz cat # sc-486) primary antibodies at 1:1000 in TBS-T + 5% bovine serum albumin (BSA) overnight at 4°C. Membranes were washed with TBS-T for 15 min and were incubated with a horseradish-peroxidase (HRP)-conjugated anti-rabbit IgG secondary antibody (Cell Signaling cat # 7074) in TBS-T + 5% skim milk for 1 h at RT. Membranes were washed again and protein bands were visualized by enhanced chemiluminescent (ECL) detection.

Flow cytometry

Surface staining

0.5-1.0 x 10^6 splenocytes, NK cells, BMDMs, or adherence purified PECs were incubated in FACS staining buffer with an anti-mouse CD16/CD32 non-labeled antibody to block Fc receptor binding at 4°C for 5 min. Cells were subsequently surface stained with fluorescently conjugated

antibodies for 15 min at 4°C in FACS staining buffer. Cells were washed with PBS and resuspended in FACS staining buffer prior to intracellular staining or analysis. Flow acquisition was performed using a BD LSR II flow cytometer from BD Pharmingen.

Intracellular staining

Prior to intracellular staining, cells were surface stained with appropriate antibodies as described above. For intracellular staining of IFNγ, NK cells were stimulated with cytokines for 2 h initially and then for an additional 4 h in the presence of a golgi inhibitor (2 μM monensin). After surface staining, cells were fixed in PBS + 4% formalin for 10 min at RT protected from light. Cells were washed in PBS and resuspended in Perm-Wash buffer (PBS, 0.1% BSA, 0.5 % saponin) containing fluorescently labeled intracellular antibodies for 1 h at 4°C. Cells were washed, resuspended in Perm-Wash buffer, and analyzed using a BD LSR II flow cytometer from BD Pharmingen.

A list of antibodies used for FACS staining is outlined in Table 1.

Statistical Analysis

Data is represented by the mean +/- the standard deviation (SD) or the standard error of the mean (SEM) where indicated. P values were determined by one-tailed Student's t-test, paired Student's t-test or ANOVA with Tukey's post hoc test, where indicated.

Table 3.1 Antibodies used for FACS staining

Surface/Intracellular Molecule	Clone	Supplier
Surface		
F4/80	BM8	eBioscience
CD11b	M1/70	eBioscience
GR1	RB6-8C5	BD Biosciences
CCR1	643854	R&D Systems
CCR5	7A4	eBioscience
Pan-NK cell (CD49b)	DX5	eBioscience
NK1.1	PK136	eBioscience
NKp46	29A1.4	eBioscience
CD45.2	104	eBioscience
CD25	PC61.5	eBioscience
CD44	IM7	eBioscience
CD62L	MEL-14	eBioscience
TCRβ	H57-597	eBioscience
CD3	145-2C11	eBiosceince
Intracellular		
IFNγ	XMG1.2	eBioscience

CHAPTER 4

TPL2 IS DISPENSABLE FOR NK CELL DEVELOPMENT AND FUNCTION

Introduction

The roles for NK cells during the inflammatory response are diverse but little is understood about relationships between Tpl2 protein kinase signaling and NK activation. Evidence showing Tpl2's importance in the maintenance of $CD4^+$ T-cell IFNγ production as well as the induction of STAT4 expression in TCR-stimulated $CD4^+$ T-cells (25) led us to evaluate the possible related role Tpl2 may have in NK cell functionality. The rationale for this line of study is that NK cells (like $CD4^+$ T-cells) are potent producers of IFNγ and rely upon IL-12 dependent STAT4 signaling for IFNγ production.

To assess NK cell development and function in the context of Tpl2 we used a mouse model of Tpl2 genetic ablation and focused on the spleen as the source of NK cells to be studied. In mouse models, the spleen remains the primary organ used for isolating NK cells for *in vitro* work as it includes roughly 3-5% NK cells out of total splenocyte populations and purification of NK cells from this organ is relatively simple. Additionally, we also considered other tissue sites including the lung and respiratory airways when studying NK cells as they are important lymphocytes during respiratory viral infections like influenza. The following chapter compares the phenotypic characteristics of NK cells from the spleens of naïve WT and *tpl2*$^{-/-}$ mice and also evaluates Tpl2's role in NK cell maintenance and function in the lungs of mice following

infection with the respiratory virus, influenza A. These data demonstrate that, contrary to T cells, NK cell development and pro-inflammatory functions occur in a Tpl2-independent manner.

Results

Tpl2 is expressed at high levels in resting NK cells

In order to begin to assess Tpl2's potential role in NK cell activation and function, we first measured mRNA expression of the *map3k8* gene that encodes Tpl2 in freshly isolated NK cells as well as other lymphocyte populations from the spleens of mice. Splenocytes from wild-type mice were isolated, and NK cells were enriched by negative selection through a magnetic column prior to RNA collection. Expression of Tpl2 was basally high in wild-type (WT) resting NK cells. In fact, Tpl2 expression in resting NK cells was significantly higher than both naïve $CD4^+$ T-cells and memory $CD4^+$ T cells isolated from WT mice (Figure 4.1). The high level of Tpl2 expression in NK cells may indicate a preferential role for Tpl2 in these critical immune cells. This result led us to further explore the possible regulatory effects of Tpl2 on NK cell development and activation.

NK cell development and IFNγ production is normal in the spleens of *tpl2*$^{-/-}$ mice

In order to assess the phenotypic characteristics of NK cells in the presence or absence of Tpl2, bulk splenocytes from WT and *tpl2*$^{-/-}$ mice were freshly isolated by mechanical dissociation through a cell strainer, and cells were surface stained for NK cell and T-cell populations. Following flow cytometric analysis, proportions of $DX5^+TCR\beta^-$ NK cells were found to be similar in the spleens of *tpl2*$^{-/-}$ mice compared to WT mice, with WT and *tpl2*$^{-/-}$

spleens containing roughly 2.5% and 3.5% $DX5^{+}TCR\beta^{-}$ NK cells, respectively (Figure 4.2A and Figure 4.2B). These distributions of splenic NK cells in WT and *tpl2*$^{-/-}$ mice both fall within the range of normal physiological NK cell splenic proportions (2-5%) commonly found in experimental murine models. Additionally, analysis of the absolute numbers of NK cells using these gated populations revealed that total NK cell numbers between WT and *tpl2*$^{-/-}$ mice were not significantly different (Figure 4.2B). Together, these findings indicate that there is no significant impairment in NK cell development in the spleens of *tpl2*$^{-/-}$ mice.

In order to begin to characterize Tpl2's role in regulating NK cell activation, we next measured NK cell-dependent IFNγ production following *in vitro* stimulation with the NK-cell activating cytokines IL-12 and IL-18. In order to properly induce NK cell activation, freshly isolated splenocytes were stimulated in the absence or presence of the anti-NK1.1 antibody in addition to the inflammatory cytokines IL-12 and IL-18. NK1.1 is a primary surface marker used to identity NK cells and direct interaction with this molecule via the α-NK1.1 antibody has been shown to induce NK cell proliferation and cytotoxic activity (96). Following cytokine stimulation, levels of NK-specific IFNγ were first measured by intracellular staining via flow cytometry. The proportions of $DX5^{+}TCR\beta^{-}IFN\gamma^{+}$ NK cells were not significantly different between WT and *tpl2*$^{-/-}$ mice (Figure 4.3A and 4.3B) for any of the stimulations tested. Additionally, while a combination of the NK1.1 antibody and cytokine stimulation did increase the proportion of $IFN\gamma^{+}$ NK cells in both WT and *tpl2*$^{-/-}$ mice, there was no significant difference observed between the two groups (Figure 4.3B). As a negative control, STAT4-deficient cells failed to respond normally to IL-12 or IL-12+IL-18 stimulation, underscoring the critical role of this transcription factor in NK-mediated IFNγ production.

In order to further examine Tpl2's influence on NK cell IFNγ production, enriched NK cells were sorted to purity via flow cytometry by selecting $DX5^{+}TCR\beta^{-}$ NK cells and were later stimulated with IL-12 or a combination of IL-12 and IL-18 to assess IFNγ secretion by ELISA. The levels of IFNγ in the culture supernatant were not significantly different between WT and *tpl2*$^{-/-}$ mice following stimulation with either IL-12 or IL-12 in conjunction with IL-18 (Figure 4.4). Overall, these results indicate that the distribution of NK cells in the spleen as well as their ability to produce the pro-inflammatory cytokine IFNγ following cytokine stimulation *ex vivo* is not impaired in *tpl2*$^{-/-}$ mice.

STAT4 expression is differentially regulated in $CD4^{+}$ T-cells and NK cells

We have previously shown that IL-12-induced IFNγ production by $CD4^{+}$ T-cells is Tpl2-dependent (25). However, our current findings demonstrate that NK regulation of IFNγ production is Tpl2-independent. Our results strongly suggest that the regulation of IFNγ production is fundamentally different between NK cells and T-cells. Consistent with this, Biron *et al.* (97) have demonstrated that, unlike $CD4^{+}$ T-cells, NK cells express high levels of STAT4 basally, in an unactivated state. We have previously shown that the expression of STAT4, a well characterized transcription factor required for inducing IFNγ production downstream of IL-12 signaling (38), is induced by TCR ligation in a Tpl2-dependent manner in $CD4^{+}$ T-cells (25). Therefore, we reasoned that the effect of Tpl2 on IFNγ expression might act at the regulation of STAT4 expression.

To further investigate Tpl2-dependent regulation of NK cell activation, we assessed the expression levels of the STAT4 in wild-type $CD4^{+}$ T cells and NK cells. We found that naïve

$CD4^+$ T cells from WT mice upregulated STAT4 mRNA expression by day 3 of culture following TCR stimulation with plate-bound anti-CD3 and anti-CD28 antibodies (Figure 4.5B). Additionally, this upregulation was shown to act via an ERK-dependent mechanism, as $CD4^+$ T-cells pre-treated with the MEK/ERK inhibitor U0126 ethanolate failed to induce STAT4 expression by day 3 (Figure 4.5A). When we assessed STAT4 expression in enriched NK cells from the spleens of WT mice, we found that STAT4 is expressed at higher basal levels in resting NK cells than in naïve $CD4^+$ T cells and at similar levels to memory $CD4^+$ T-cells (Figure 4.5B). Therefore, we have shown that STAT4 expression occurs via an ERK-dependent manner in activated $CD4^+$ T-cells, and that STAT4 expression is already basally high in NK cells.

Tpl2$^{-/-}$ NK cells express similar levels of STAT4 compared to WT NK cells following activation

Due to the observed differential regulation of STAT4 between T-cells and NK cells, we next assessed whether Tpl2 is necessary for the high basal STAT4 expression observed in freshly isolated WT NK cells. Splenic NK cells from WT and *tpl2*$^{-/-}$ mice were enriched for NK cell populations with magnetic microbeads, and fresh RNA lysates were analyzed for STAT4 expression via qRT-PCR. We found that relative levels of STAT4 expression in resting NK cells were similar between WT and *tpl2*$^{-/-}$ mice. Furthermore, when we measured STAT4 expression in activated NK cells that had been cultured with the activating cytokine IL-2 (1000 U/mL) for 14 days to shift towards a lymphokine activated killer (LAK) cell phenotype, *tpl2*$^{-/-}$ LAK cells had STAT4 levels comparable to WT LAK cells (Figure 4.6A). To further investigate this, we measured STAT4 phosphorylation in enriched NK cells from WT and *tpl2*$^{-/-}$ mice that were left

unstimulated or stimulated with IL-12 (10 ng/mL) for 15 min at 37°C. Western blots of p-STAT4 expression revealed that *tpl2*$^{-/-}$ NK cells were still capable of phosphorylating STAT4 following inflammatory activation with IL-12, and total STAT4 protein expression was similar between wild-type and *tpl2*$^{-/-}$ mice (Figure 4.6B). Collectively, these findings indicate that neither resting nor activated NK cells require Tpl2 for STAT4 expression or activation, and this explains why IFNγ responses are unaltered in *tpl2*$^{-/-}$ NK cells.

Tpl2$^{-/-}$ NK cells are capable of up-regulating the expression of cytotoxic proteins

We next addressed whether Tpl2 has any effect on NK cell killing functions and cytotoxic activation. A defining characteristic of NK cell activation is induction of a cytotoxic effector state, including the generation of cytotoxic granules and acquisition of pore-forming proteins used to mediate directed cell killing. The pore-forming protein perforin as well as the cell-death inducing cytosolic protein granzyme B are both produced by activated cytotoxic NK cells during inflammatory responses to help mediate cell lysis. In order to assess Tpl2's role in regulating the functional expression of these two NK cell-specific cytotoxic markers, we compared freshly isolated and IL-2 activated LAK cells from WT and *tpl2*$^{-/-}$ mice. Fresh NK cells were enriched by magnetic column separation and sorted by flow cytometry for $DX5^{+}TCR\beta^{-}$ populations. Additionally, enriched NK cells were cultured in RPMI containing 1000 U/mL IL-2 for 14 days in order to differentiate towards a LAK cell state that is characterized by enhanced cytotoxic functionality. Figure 4.7 shows that the expression of granzyme B and perforin were significantly up-regulated in LAK cells compared to fresh NK cells, and this increase was independent of Tpl2. Overall, these results indicate that at a basal

state splenic NK cells as well as LAK cells do not require Tpl2 to regulate expression of the cytotoxic effectors granzyme B and perforin.

NK cell numbers and functions are normal in the lung tissue of *tpl2*$^{-/-}$ mice following influenza infection

As studies with splenic NK cells indicated that Tpl2 is not required for NK cell development and activation, one other approach was to determine what role Tpl2 may have in NK cell function at other organ sites such as the lung during influenza infection. We chose to use an influenza infection model to naturally activate NK cells for two reasons. First, it has been shown that innate immune responses towards influenza infection, in both human and mouse models, are mediated by activated NK cells in the lung (98, 99). Second, there has been recent research into the possible roles that MAP kinases such as Tpl2 may have during widespread viral infections such as influenza and more specifically what immune cell types are being regulated by these kinases during infection (100, 101).

To assess Tpl2's role in NK cell development and function during influenza infection, we infected WT and *tpl2*$^{-/-}$ mice intranasally with 10^6 PFU of the X31 strain of influenza A virus or with allantoic fluid as a negative control. On day 3 of infection, mice were sacrificed, and bronchoalveolar lavage fluid (BAL) and lung tissue were collected. Freshly collected BAL cells and lymphocytes isolated from digested lung tissue were surface stained for NK cell and T cell markers and analyzed by flow cytometry. Proportions of NK1.1$^+$CD3$^-$ NK cells in the BAL were increased in both groups of mice following X31 infection but were similar between WT and *tpl2*$^{-/-}$ mice (Figure 4.8A). However, when factoring in total cell numbers, we observed

significantly more total NK cells in the BAL of *tpl2*$^{-/-}$ mice on D3 of influenza infection than in WT mice (Figure 4.8B). Similarly,, we found that proportions of NK cells in the lung tissue were higher in *tpl2*$^{-/-}$ mice on D3 of infection (Figure 4.8A) but total NK cell numbers were not significantly different in the lung tissue of WT and *tpl2*$^{-/-}$ mice (Figure 4.8B). Interestingly, total NK cell numbers in lung tissue decreased in both groups of mice following influenza challenge possibly indicating migration of these cells from the lung tissue into the BAL during infection (Figure 4.8B). Overall, these results show that Tpl2 is not required for the active recruitment or maintenance of NK cells in the BAL of influenza infected-mice, and furthermore, Tpl2 does not influence total NK cells numbers in the lung tissue of infected mice.

To further test the functional output of influenza-infected NK cells in the context of Tpl2, we re-stimulated lymphocytes isolated from the lung tissue of WT and *tpl2*$^{-/-}$ mice with various stimuli for 6 hours in RPMI media and subsequently measured NK cell IFNγ production by intracellular staining. Following stimulation with the NK specific antibody NK1.1 or with the non-specific mitogens PMA and ionomycin, we observed an increase in the proportions of IFNγ^{+} NK cells in both WT and *tpl2*$^{-/-}$ mice following X31 influenza virus infection. However, there appeared to be only modest differences in the amount of NK-specific IFNγ production between WT versus *tpl2*$^{-/-}$ groups following infection (Figure 4.9). These results suggest that IFNγ production by NK cells in the lung tissues of mice infected with influenza virus is not substantially affected by the loss of Tpl2.

Discussion

The results of this study clearly show that the development and function of NK cells is independent of the inflammatory MAP kinase, Tpl2. In the context of the spleen, the maintenance and cellular function of NK cells was similar between WT and *tpl2*$^{-/-}$ mice and production of the NK inflammatory cytokine IFNγ was also similar following stimulation with prominent NK-activating cytokines IL-12 and IL-18. Furthermore, expression of the cytotoxic proteins perforin and granzyme B in activated NK cells was also unaffected by the loss of Tpl2. Lastly, NK cell maintenance and IFNγ production in the lung tissue of mice following influenza infection was similar between WT and *tpl2*$^{-/-}$ mice. However, absolute numbers of NK cells were increased in the BAL of *tpl2*$^{-/-}$ mice following influenza infection indicating enhanced recruitment of NK cells from the lung tissue to the airways in *tpl2*$^{-/-}$ mice.

These results demonstrate that Tpl2-dependent regulation of STAT4 and IFNγ expression by NK cells is different than what has been observed in CD4^{+} T-cells. Although STAT4 expression in T cells is basally low and requires TCR-driven induction via a Tpl2- and ERK-dependent mechanism ((25) and Figure 4.5A herein), STAT4 expression in NK cells was basally high and Tpl2-independent. High basal expression of STAT4 in NK cells correlates with their immediate responsiveness to IL-12 cytokine stimulation leading to IFNγ secretion, an important aspect of NK cell physiology and innate immune function. Consistent with this, STAT4 phosphorylation and IFNγ secretion in *tpl2*$^{-/-}$ NK cells following IL-12 cytokine stimulation was similar to that observed in WT NK cells. Clearly, there are critical differences in how Tpl2 is involved in the mechanisms of STAT4 induction and homeostasis between NK cells and T-cells during inflammation. One possible explanation is that another MAP3K, such as Raf-1, may

carry out these functions in NK cells. We previously demonstrated that in macrophages, Raf-1 was able to transduce zymosan-driven signals downstream of TLR2 via a Tpl2-independent pathway, while all other TLR ligands required Tpl2 (21).

Our initial finding that wild-type resting NK cells express Tpl2 at high levels, i.e. significantly higher levels than in either naïve or memory $CD4^+$ T-cells, suggests that NK cells likely utilize this kinase to some degree during development or activation. However, regulation of IL-12-induced IFNγ secretion is apparently unaffected by Tpl2 ablation. This led us to question whether other functions of NK cells, such as cytotoxicity, might be regulated by this kinase. However, the acquisition of cytotoxic effector mechanisms, such as expression of perforin and granzyme B, was also shown to occur independently of Tpl2 in LAK cells. One important caveat is that this finding was based on gene expression of these two proteins, which may not correlate precisely with protein expression. In particular, Tpl2 has been shown to alter the post-transcriptional regulation of TNF (10, 102). More recently, Alemany et al. have shown that stability of several pro-inflammatory mRNAs, particularly those with 3' AU-rich elements (AREs), is Tpl2-dependent (103). In addition to measuring protein levels of these effectors, further studies are needed to extrapolate these findings by directly assessing the cytotoxic killing capabilities of *tpl2*$^{-/-}$ NK cells by analyzing their ability to directly lyse target cells. Additionally, evaluation of the expression of NK-specific cytotoxic markers such as CD107a on the surface of *tpl2*$^{-/-}$ NK cells during these killing assays will lead to a better understanding of how Tpl2 may regulate NK-cell cytotoxicity.

Lastly, the finding that absolute NK cell numbers in the BAL of influenza infected mice was higher in the absence of Tpl2 was unexpected due to the finding that development and

functional capacity (i.e. IFNγ production) of NK cells following infection in the lung tissue of *tpl2*$^{-/-}$ mice were similar to WT mice. Of note, increased cellular recruitment in general, and not specifically increased recruitment of NK cells, was responsible for this observation. It is possible that Tpl2 may be playing a measured anti-inflammatory role in this particular case, similar to Tpl2's role in controlling excessive airway inflammation during experimentally induced asthma (104). Increased cellular infiltration into the BAL of influenza-infected *tpl2*$^{-/-}$ mice may ultimately lead to harmful inflammation in the airways. Further studies will need to characterize the mechanisms responsible for enhanced cellular recruitment to the airways. Certain cytokines (IL-1β, IL-6, IL-18 and TNF) and chemokines (RANTES, MIP-1α, MCP-1, MCP-3, and IP-10) are known to be particularly important for cellular recruitment and activation during influenza infection (105). Additional studies assessing the expression of these various chemokines in the BAL fluid early after influenza infection will undoubtedly shed light on the mechanism(s) for enhanced recruitment of immune cells in influenza-infected *tpl2*$^{-/-}$ mice.

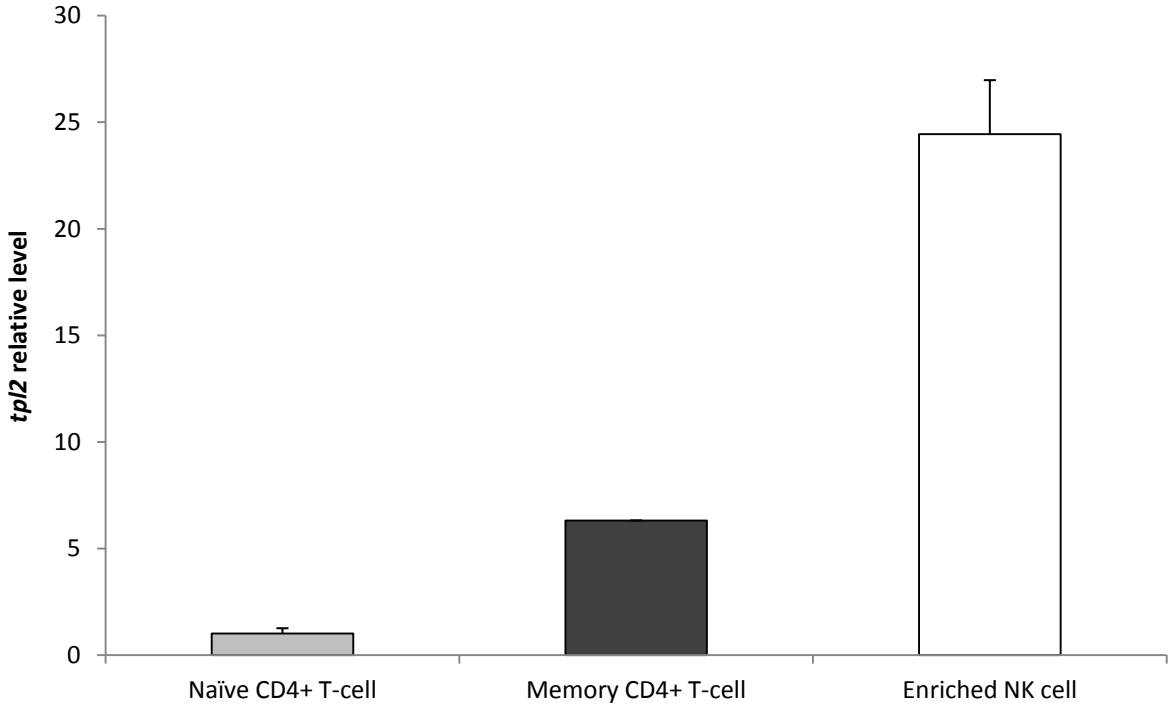


Figure 4.1 Tpl2/MAP3K8 is expressed in lymphocytes including NK cells

NK cells and naïve and memory $CD4^+$ T-cells from the spleens of WT mice were purified using magnetic cell sorting and sorting by flow cytometry, respectively. All cells were lysed immediately after purification and were analyzed for MAP3K8 expression by qRT-PCR relative to an actin control. Results are representative of 2 independent experiments.

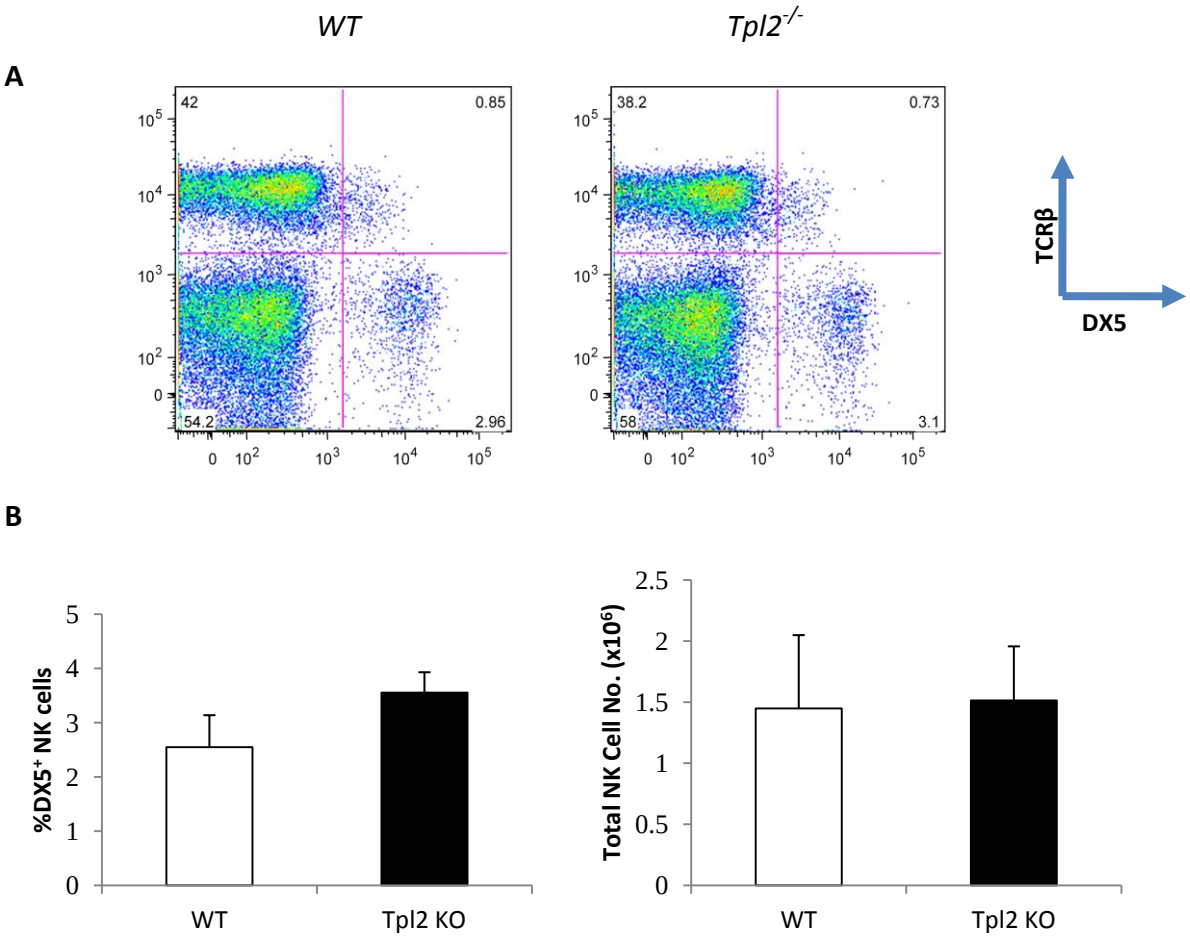


Figure 4.2 NK cell numbers are similar in the spleens of WT and *tpl2*$^{-/-}$ mice

Fresh splenocytes were harvested and purified from WT and *tpl2*$^{-/-}$ mice and were surface stained for NK and T-cell markers DX5 and TCRβ, respectively. **(A)** Representative FACS plots showing proportions of DX5^{+}TCRβ^{-} NK cells in the spleens of WT and *tpl2*$^{-/-}$ mice. Results are representative of 2 independent experiments **(B)** Proportions (left) and total numbers (right) of DX5^{+} NK cells from WT and *tpl2*$^{-/-}$ mice. Results are average of 2 independent experiments and error bars represent standard deviation.

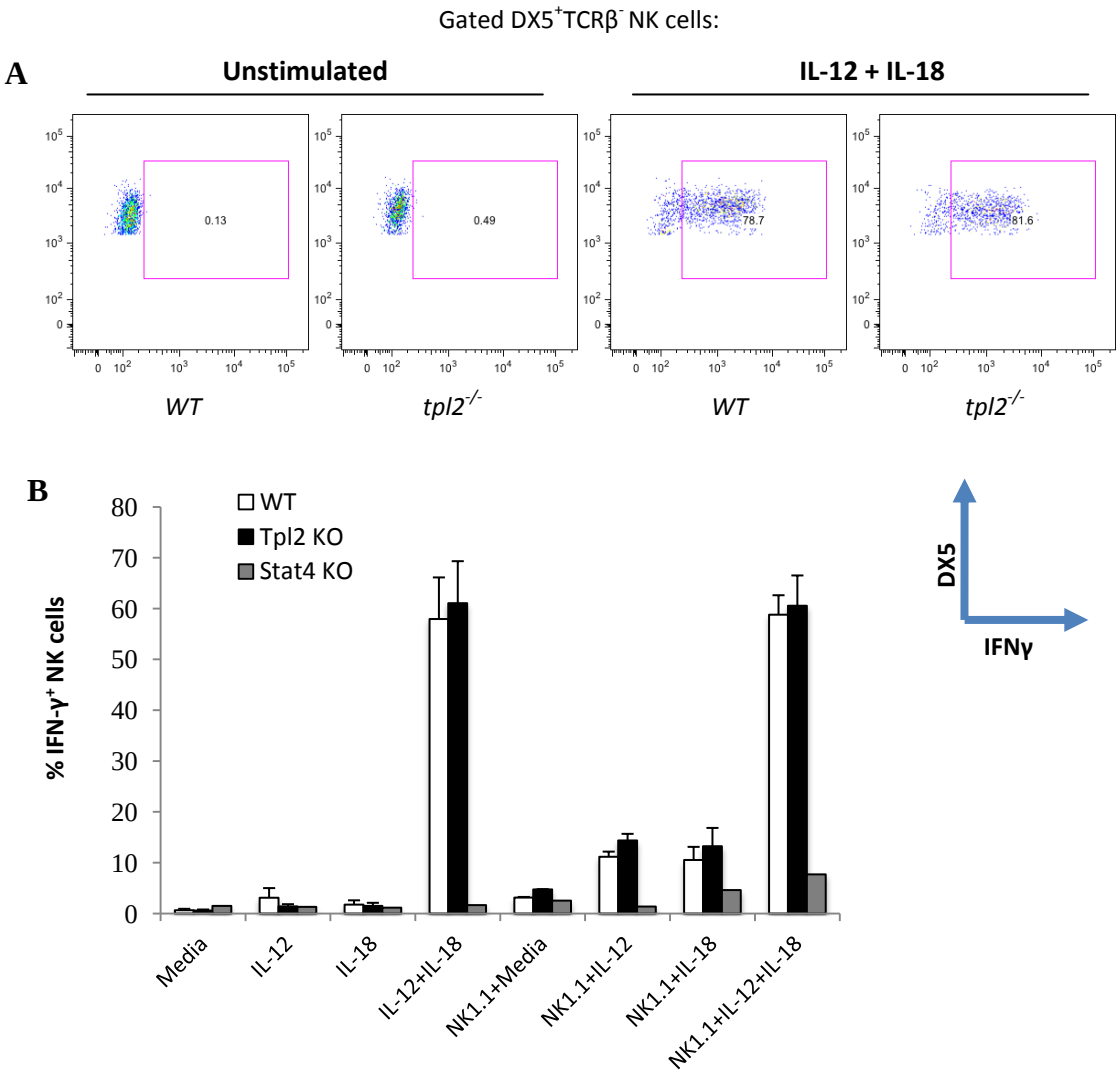


Figure 4.3 Proportions of IFNγ$^+$ NK cells are similar in WT and *tpl2*$^{-/-}$ mice

Bulk splenocytes from WT or *tpl2*$^{-/-}$ mice were stimulated with indicated cytokines and were surface stained for DX5 and TCRβ as shown in Figure 4.2 and were then stained for intracellular IFNγ. **(A)** Representative FACS plot of DX5$^+$TCRβ$^-$IFNγ$^+$ NK cells following stimulation with media alone or with IL-12+IL-18 for 6 h. **(B)** Average proportions of DX5$^+$ TCRβ$^-$IFNγ$^+$ NK cells under all stimulus conditions in WT, *tpl2*$^{-/-}$, and *STAT4*$^{-/-}$ mice. Results are average of 4 independent experiments and error bars represent SEM.

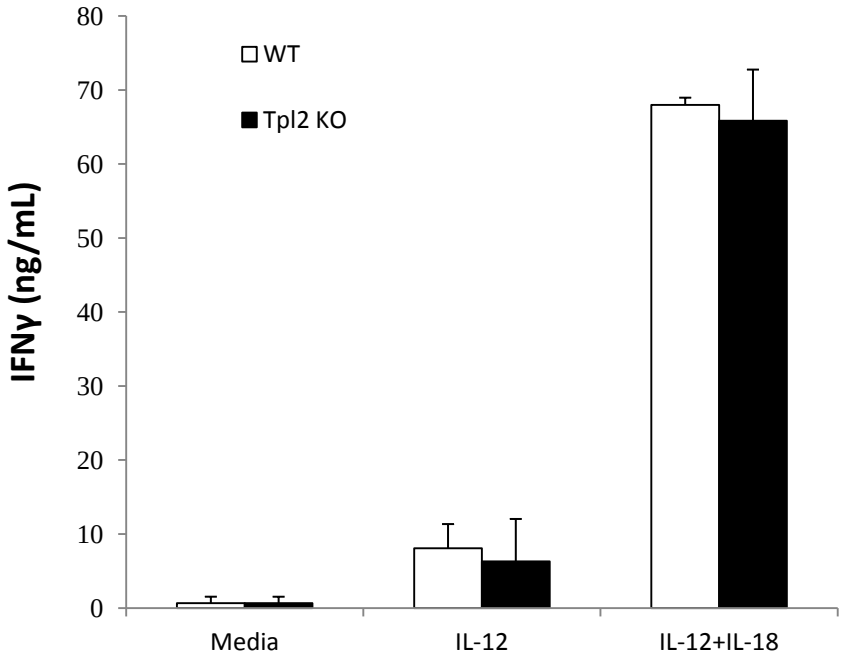


Figure 4.4 NK cell IFNγ secretion is not dependent on Tpl2

Bulk splenic NK cells enriched by magnetic sorting were further purified by flow cytometry sorting for $DX5^{+}TCR\beta^{-}$ NK cells and were stimulated with IL-12 or IL-12+IL-18 for 24 h. Culture supernatants were analyzed for IFNγ levels by ELISA. Results represent averages of 2 independent experiments and error bars represent standard deviation.

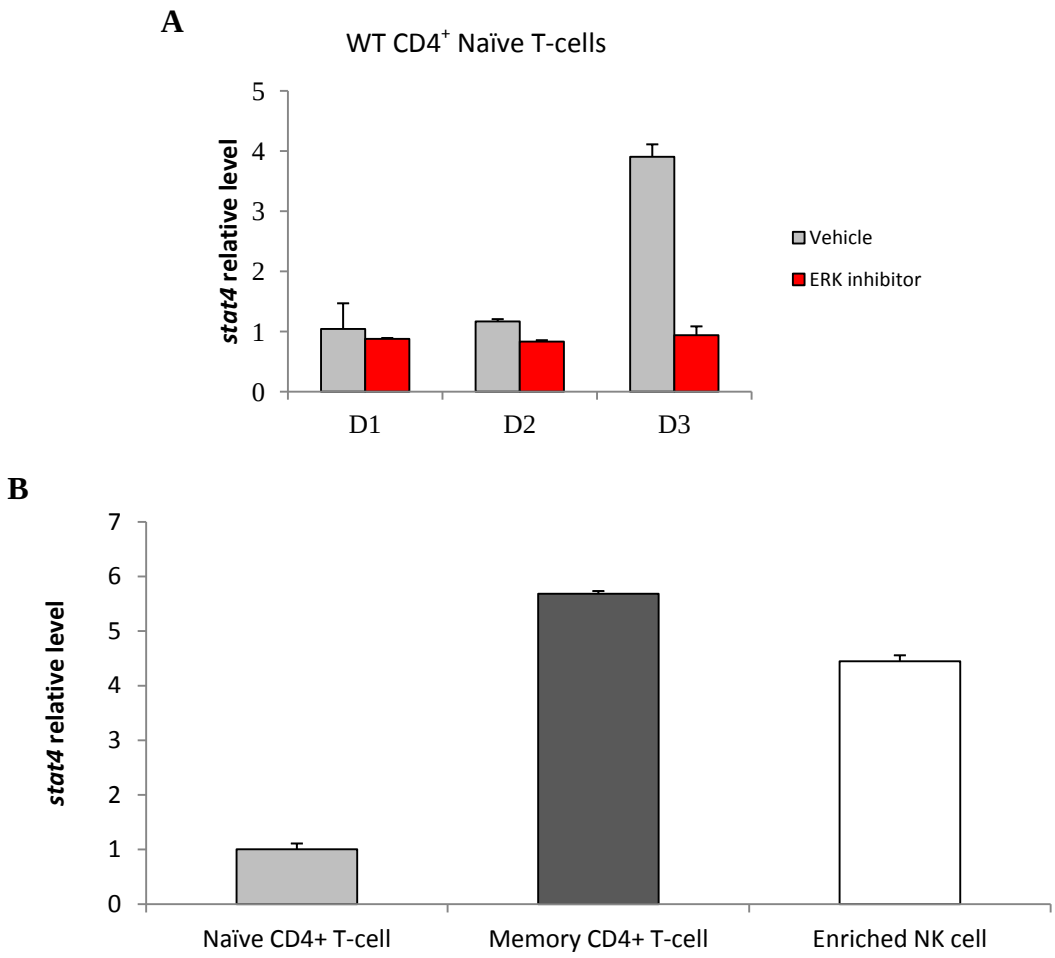


Figure 4.5 STAT4 is differentially regulated by CD4$^+$ T cells and NK cells

(A) Naïve CD4$^+$ T-cells from the spleens of WT mice were purified and sorted by flow cytometry and were pre-incubated with or without the U0126 ethanolate ERK inhibitor (20 μM) 30 min prior to culture in media containing plate-bound CD3/CD28 antibodies for 3 days. **(B)** Naïve and memory CD4$^+$ T-cells and enriched NK cells from the spleens of WT mice were purified as described in Figure 4.1. All cells from **(A)** and **(B)** were lysed and analyzed for STAT4 expression by qRT-PCR relative to an actin control. Results are representative of 1 experiment.

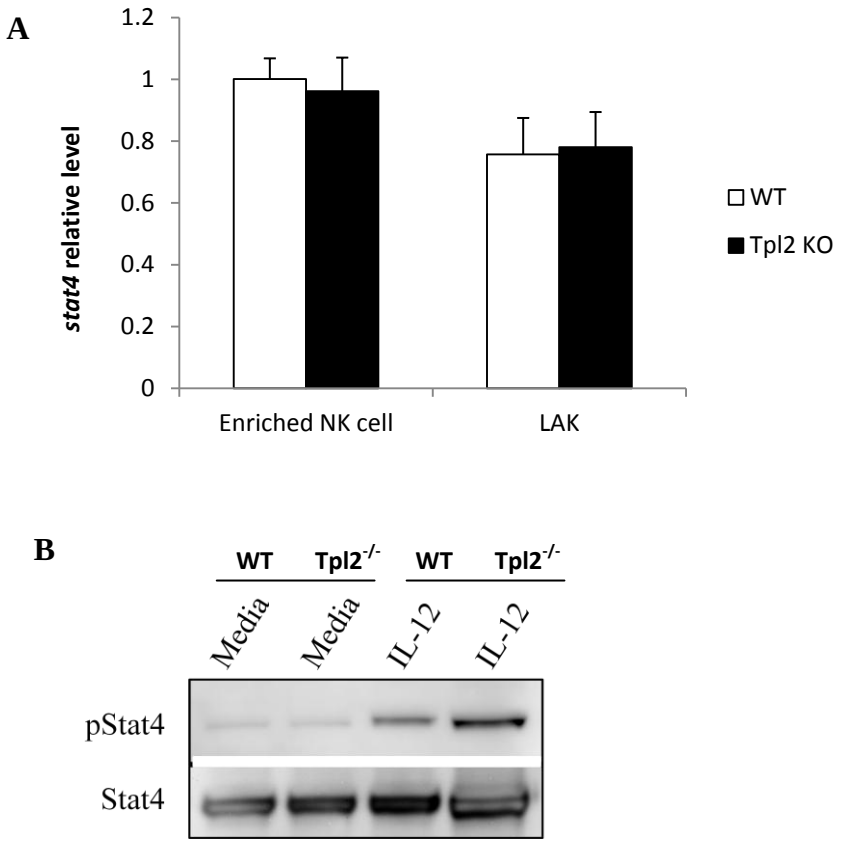


Figure 4.6 *Tpl2*$^{-/-}$ NK cells are capable of maintaining STAT4 expression following activation

(A) Bulk splenocytes from WT or *tpl2*$^{-/-}$ mice were enriched for NK cells by magnetic bead sorting. Some enriched NK cells were freshly lysed for RNA content while other enriched NK cells were placed in culture with 1000 U/mL IL-2 for 14 days to induce lymphokine activated killer (LAK) cell development prior to cell lysis. RNA lysates were analyzed for STAT4 expression by qRT-PCR relative to an actin control. **(B)** Protein lysates were obtained from enriched WT or *tpl2*$^{-/-}$ NK cells that were left unstimulated or stimulated with IL-12 (10 ng/mL) for 15 min at 37°C. p-STAT4 and total STAT4 protein expression was measured by western blotting. Results are representative of 2 experiments.

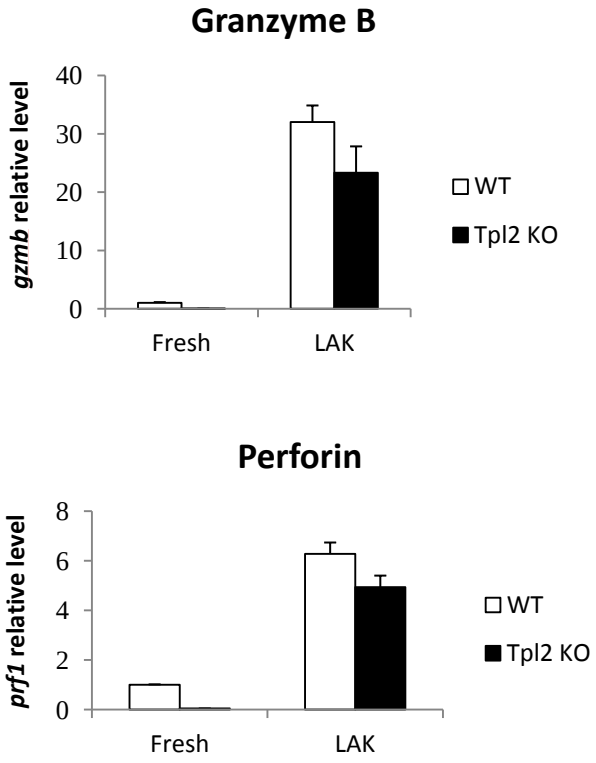


Figure 4.7 *Tpl2*$^{-/-}$ NK cells are capable of up-regulating mRNA expression for cytotoxic components following activation

Bulk splenocytes from WT or *tpl2*$^{-/-}$ mice were enriched for NK cells by magnetic bead selection and further sorted by flow cytometry for DX5^{+}TCRβ^{-} NK cells. Some enriched, sorted NK cells were freshly lysed while other enriched NK cells were placed in culture with 1000 U/mL IL-2 for 14 days to induce lymphokine activated killer (LAK) cell development prior to cell lysis. Cell lysates were then analyzed for mRNA expression by qRT-PCR relative to an actin control. Results are representative of 1 experiment (will soon be 2 experiments).

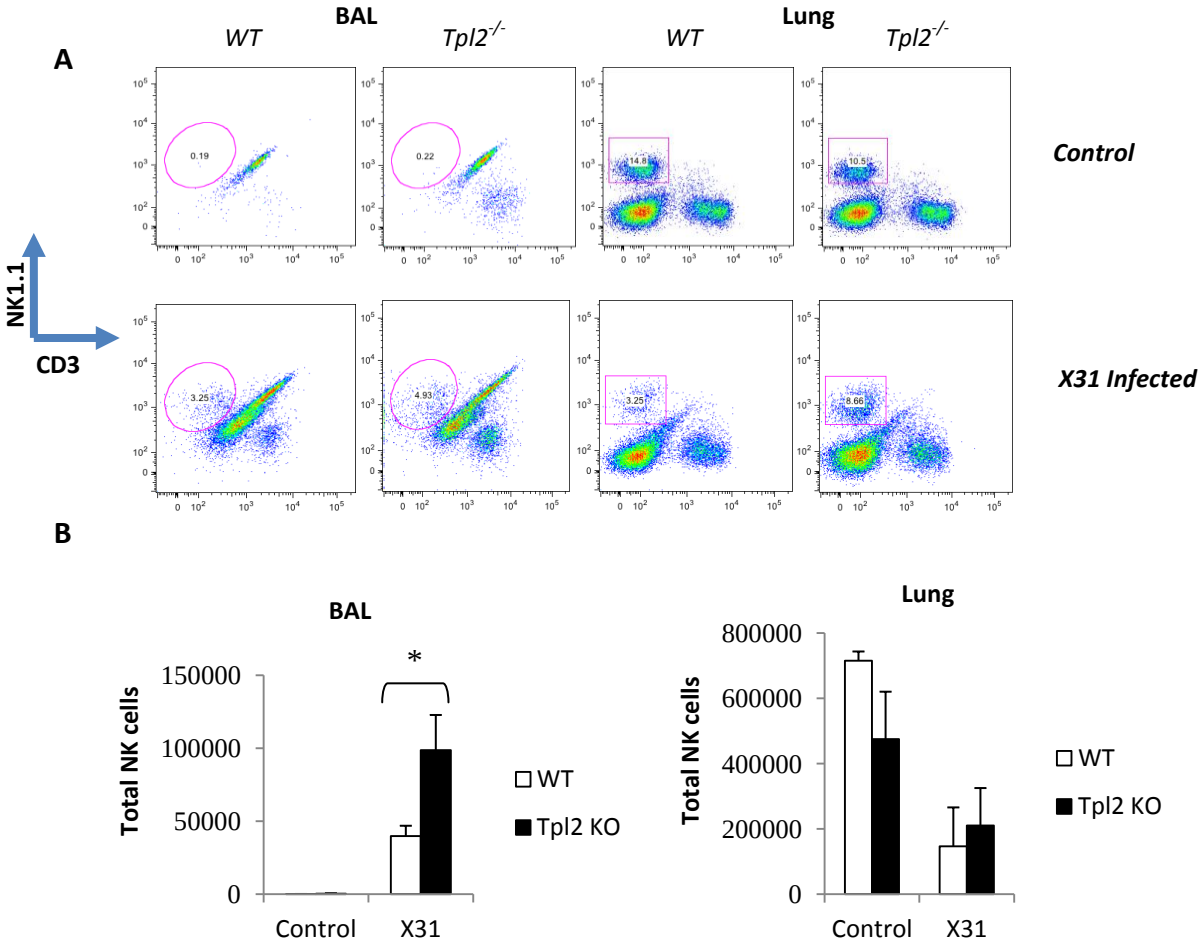


Figure 4.8 NK cell distribution in the BAL but not the lung tissue is altered in *tpl2*$^{-/-}$ mice following influenza infection

WT and *tpl2*$^{-/-}$ were infected intranasally with 10^6 PFU X31 Influenza virus or allantoic fluid as a negative control. After 3 days, mice were sacrificed and bronchoalveolar lavage fluid (BAL) and lung epithelial cells were collected, and NK cell populations were analyzed. **(A)** Representative FACS plots showing NK1.1$^+$CD3$^-$ NK cells in the BAL and lung tissue. **(B)** Total numbers of NK cells in the BAL and lungs of WT and *tpl2*$^{-/-}$ mice. Results represent 2 (control) or 4 (X31 infected) mice per group. Error bars represent standard deviation. Student's t-test, *$p < 0.01$

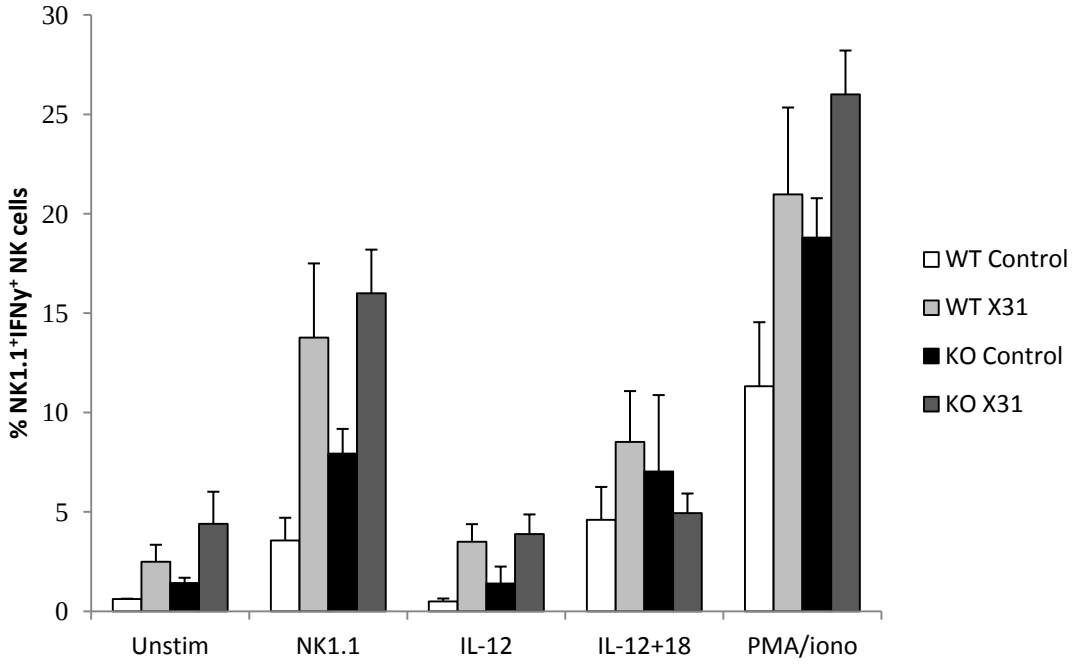


Figure 4.9 Proportions of IFNγ$^+$ NK cells in the lung tissue are similar between WT and *tpl2*$^{-/-}$ mice following influenza infection

WT and *tpl2*$^{-/-}$ were infected intranasally with 10^6 PFU X31 Influenza virus or allantoic fluid as a negative control. After 3 days, mice were sacrificed and lung tissue was digested and epithelial cells were isolated by percoll gradient purification. Cells were further activated with indicated stimuli for 6 h in media and later stained for the NK and T-cell surface markers as well as for intracellular IFNγ. Proportions of NK1.1$^+$CD3$^-$ IFNγ$^+$ NK cells are shown and represent 2 (control) or 4 (X31 infected) mice per group. Error bars represent standard deviation and statistical differences between WT and *tpl2*$^{-/-}$ groups were analyzed by One-way ANOVA with Tukey's post hoc test.

CHAPTER 5

TPL2 PROMOTES MACROPHAGE CHEMOKINE/CHEMOKINE RECEPTOR EXPRESSION AND MACROPHAGE MIGRATION DURING INFLAMMATION

Introduction

The functional migration of innate immune cells such as macrophages is regulated, in part, by the coordination of signals between locally secreted chemokines and the corresponding chemokine receptors present on the surface of these activated leukocytes. The expression of genes encoding chemokine or chemokine receptors during the inflammatory response is becoming better understood, yet the precise molecular signaling pathways regulating these remain unclear. Tpl2 has been shown to regulate inflammatory cytokine secretion, but its contribution to macrophage chemokine expression and cellular recruitment are less well understood. The active recruitment of macrophages to sites of inflammation or injury is vital to mounting antimicrobial responses at sites of infection or injury. However, exacerbated infiltration of macrophages during autoimmune disorders such as inflammatory bowel disease (IBD) or rheumatoid arthritis can cause deleterious inflammatory effects. Clinical manipulation of the migration of macrophages is a promising, yet complex, area of research that requires a better understanding of the regulatory mechanisms involved.

This chapter aims to determine what role Tpl2 plays in the expression of macrophage chemokines and chemokine receptors. The rationale for initiating this study came from a recent

report outlining the requirement of Tpl2 for the efficient recruitment of neutrophils to a peripheral site of inflammation in response to the TLR2 agonist, zymosan in mice (106). Here, we extend these findings to macrophages and additional stimuli by assessing their chemokine/chemokine receptor expression following stimulation with LPS. Additionally, we investigated what effects Tpl2 deficiency may have on the functional migration of macrophages *in vivo* during the inflammatory response. These studies demonstrate that Tpl2 is required for the expression of a multitude of chemokines and chemokine receptors in response to LPS. Furthermore, the functional consequence of Tpl2 ablation was impaired macrophage recruitment to tissue sites of inflammation. These findings provide additional support for therapeutic inhibition of Tpl2 in chronic autoimmune diseases, as it may block the continued accumulation of inflammatory infiltrates in affected tissues.

Results

Chemokine and chemokine receptor gene expression is impaired in *tpl2*$^{-/-}$ macrophages

In order to gain a global view of Tpl2's early innate functions in macrophages, we analyzed gene expression profiles by microarray in WT versus *tpl2*$^{-/-}$ BMDMs stimulated with LPS. Among the most highly changed genes were a number of chemokines and chemokine receptors, some represented multiple times in the array (Figure 5.1). Genes dramatically reduced in *tpl2*$^{-/-}$ macrophages were *ccl2*, *ccl7*, *ccr1*, and *ccr5* (Figure 5.1). To validate the array findings, we cultured BMDMs from WT and *tpl2*$^{-/-}$ mice for 7 days and stimulated the cells in media with or without bacterial LPS for 4 h. The mRNA expression levels of multiple chemokines and chemokine receptors were then measured via qRT-PCR. In agreement with our

microarray data, mRNA expression for chemokines CCL2, CCL7, and CXCL3 were reduced in *tpl2*$^{-/-}$ BMDMs, and the expression of the chemokine CXCL2 was significantly reduced in the absence of Tpl2 (Figure 5.2). Additionally, we also measured expression of chemokine receptor genes and found that both CCR1 and CCR5 were significantly impaired in *tpl2*$^{-/-}$ cells following LPS stimulation (Figure 5.2). Further analysis of CCR1 and CCR5 mRNA expression following LPS stimulation for shorter time points revealed that expression of these two chemokine receptors was actively repressed in *tpl2*$^{-/-}$ macrophages by 2 h post stimulation and remained consistently lower than levels in WT BMDMs at 2.5 and 3 hours post stimulation (Figure 5.3). Overall, these results indicate that the absence of Tpl2 reduces expression of both chemokine and chemokine receptor gene expression in BMDMs following LPS stimulation.

Chemokine receptor protein expression is impaired in *tpl2*$^{-/-}$ macrophages

In order to further define the role of Tpl2 in the regulation of chemokine receptor expression we next measured the surface expression of CCR1, a chemokine receptor implicated in macrophage migration and function during inflammation. BMDM from WT and *tpl2*$^{-/-}$ mice were stimulated with bacterial LPS for 24 h to induce CCR1 protein expression on the cell surface. Following stimulation, cells were harvested and stained with the primary macrophage surface antibodies F4/80 and CD11b as well as an antibody for CCR1. F4/80^{+}CD11b^{+} macrophages from WT and *tpl2*$^{-/-}$ mice both displayed specific CCR1 surface expression relative to the isotype control (Figure 5.4A). LPS stimulation further upregulated CCR1 protein expression, but was significantly impaired in *tpl2*$^{-/-}$ macrophages. This was confirmed by the overall lower median fluorescence intensity (MFI) in *tpl2*$^{-/-}$ macrophages (Figure 5.4B). Similar

experiments were performed for CCR5, but specific surface staining was not detectable using the available antibody (data not shown). Collectively, these results indicate that protein expression, in addition to gene expression, of the chemokine receptor CCR1 is impaired in *tpl2*$^{-/}$ macrophages following LPS stimulation and this may have an overall impact on macrophage migration during inflammatory responses.

Recruitment of primary macrophages to the peritoneal cavity is impaired in *tpl2*$^{-/-}$ mice

We next sought to determine whether functional macrophage migration was altered due to changes in chemokine/chemokine receptor expression by Tpl2 ablation in an *in vivo* setting. To do this, WT or *tpl2*$^{-/-}$ mice were injected intraperitoneally (i.p.) with 1 mL of 3% Brewer's thioglycollate medium or 1 mL PBS as a control. Thioglycollate medium is a potent inducer of local sterile inflammation at the site of injection and serves to actively recruit effector cells to the inflamed site. Cells were allowed to actively migrate to the peritoneal cavity for 72 h before mice were sacrificed, and peritoneal exudate cells (PECs) were lavaged from the cavity with sterile PBS. Following thioglycollate injection, total peritoneal cells recovered were significantly increased in both groups of mice compared to the PBS controls, yet there was a significant reduction in the number of total cells recovered from *tpl2*$^{-/-}$ mice (Figure 5.5A). Additional characterization of the thioglycollate-recruited cells via flow cytometry staining with F4/80 and CD11b revealed a similar proportion of F4/80^{+}CD11b^{+} primary macrophages recruited to the peritoneal cavity of WT and *tpl2*$^{-/-}$ mice (Figure 5.5B). However, total numbers of primary macrophages were significantly reduced in *tpl2*$^{-/-}$ mice, indicating an impairment in

the ability of these mice to efficiently recruit macrophages directly to a site of inflammation (Figure 5.5C).

To further analyze the migratory impairment of these macrophages, thioglycollate-elicited primary peritoneal cells were cultured in media for 6-8 h to allow for adherence purification of primary macrophages. Cells were then stimulated with LPS for 4 h, and the gene expression of various chemokines and chemokine receptors was analyzed via qRT-PCR. The results showed that primary macrophages from *tpl2*$^{-/-}$ mice also displayed decreased gene expression of the chemokines CCL2 and CCL7 as well as the chemokine receptors CCR1 and CCR5 following activation with LPS (Figure 5.6). These results provide an *in vivo* correlate for the impaired chemokine and chemokine receptor expression observed in *tpl2*$^{-/-}$ BMDM following *in vitro* stimulation with LPS.

Discussion

This study has demonstrated that the expression of macrophage chemokines and chemokine receptors in response to inflammatory stimuli are induced via a Tpl2-dependent mechanism. Specifically, we have demonstrated that expression of the chemokine ligands CCL2, CCL7, CXCL2, and CXCL3 as well as the chemokine receptors CCR1 and CCR5 are reduced in *tpl2*$^{-/-}$ macrophages stimulated with the TLR4 ligand LPS. To correlate these *in vitro* chemokine impairments with a possible *in vivo* functional impairment in macrophage migration, we demonstrated that *tpl2*$^{-/-}$ mice were defective in the active recruitment of primary peritoneal macrophages to the peritoneal cavity following thioglycollate-induced inflammation and that the

expression of chemokine ligands CCL2 and CCL7 as well as the receptors CCR1 and CCR5 were impaired in *tpl2*$^{-/-}$ peritoneal macrophages isolated *ex vivo*.

The macrophage chemokine ligands and receptors we identified as regulated by Tpl2 following inflammatory stimulation, are all notable for their previously reported roles in macrophage migration to inflamed tissue sites. The observed impairment in the chemokine ligands CCL2 and CCL7 in *tpl2*$^{-/-}$ macrophages following either LPS stimulation *in vitro* or thioglycollate administration *in vivo* is particularly interesting, as these chemokines are two of the most important monocyte chemotactic proteins (MCPs) upregulated during the inflammatory response (63, 64). Additionally, the finding that expression of chemokines belonging to the CXC family, including CXCL2 and CXCL3, were impaired in *tpl2*$^{-/-}$ macrophages is also noteworthy due to reports of their upregulation during metastatic cancers such as breast cancer (65). Lastly, the reduction in CCR1 and CCR5 expression in *tpl2*$^{-/-}$ macrophages is also relevant as both receptors are critical for active macrophage migration during inflammation (66, 69). CCR1 on the surface of macrophages along with its chemokine ligand CCL7 were both coordinately downregulated in macrophages in the absence of Tpl2. These combined defects likely contributed to the observed defective *in vivo* recruitment of these macrophages following thioglycollate injection in our mouse model.

Future studies are needed to determine which chemokines and chemokine receptors are central to impaired migration of Tpl2-deficient macrophages *in vivo*. For example, determining whether the recruitment defect is indeed intrinsic to macrophage CCR1 expression will require the adoptive transfer of either WT or *tpl2*$^{-/-}$ macrophages into WT thioglycollate-treated mice in order to assess the ability of *tpl2*$^{-/-}$ cells to migrate within an otherwise normal inflammatory

setting. Alternatively, selective blockade of individual chemokines or chemokine receptors with antibody administration *in vivo* may help clarify which factors are dominant. Experiments involving *in vitro* transwell chemotaxis assays could be performed to determine the capacity of *tpl2*$^{-/-}$ macrophages to migrate directly towards specific chemotactic ligands.

During the course of this study, a report by Bandow *et al.* demonstrated impaired expression of various chemokine ligands including CCL2, CCL7, CXCL2, and CXCL3 in *tpl2*$^{-/-}$ BMDMs following LPS stimulation, and this was shown to act via a MyD88-dependent mechanism (107). Importantly, however, the study by Bandow *et al.* did not address Tpl2's effects on chemokine receptor expression or how these chemokine/chemokine receptor impairments affected active macrophage migration *in vivo*. Our data support and significantly extend the findings of Bandow *et al.* Our study uniquely shows that Tpl2 is required for TLR-dependent upregulation of CCR1 and CCR5 in macrophages, two chemokine receptors shown to play critical roles in mediating macrophage migration during various inflammatory responses. Furthermore, defective functional migration of *tpl2*$^{-/-}$ macrophages was demonstrated in our mouse model of peritoneal inflammation induced by thioglycollate treatment.

The current data suggest that Tpl2 inhibition (which can lead to selective downregulation of macrophage chemokine receptors such as CCR1) may be an attractive alternative treatment for a variety of autoimmune diseases associated with increased inflammatory cellular infiltration. The selective inhibition of CCR1 has been explored for the treatment of specific autoimmune diseases like rheumatoid arthritis, where CCR1 inhibition correlated with reductions in cellular infiltration and inflammation in the joints of arthritis-induced mice (68). Additionally, CCR1 inhibition has also been investigated in studies for the treatment of tumor-associated diseases

such as multiple myeloma where results showed that therapeutic inhibition of CCR1 resulted in decreased tumor burden in affected mice (108). These findings support the idea that CCR1 inhibition may be a potential anti-inflammatory treatment for certain diseases. Furthermore, CCR1 antagonism may be enhanced by the selective inhibition of Tpl2, since our findings have suggested that this kinase is responsible for regulating CCR1 expression in macrophage populations. However, the physiological effect of Tpl2 ablation (or clinical inhibition) could significantly impair the host's ability to respond to a variety of bacterial, parasitic and viral infections as well as weaken host responses to cancers if inhibition is too robust.

In conclusion, this chapter outlines a previously unreported requirement for Tpl2 by macrophages during the expression of inflammatory chemokine receptors and migration *in vivo* which may be critical for innate immune responses during inflammation. Findings reported here should bolster clinical research investigating how Tpl2 inhibition may help to complement the selective inhibition of macrophage chemokines or chemokine receptors for the treatment of inflammatory disorders.

Decreased expression in *tpl2*$^{-/-}$ BMDMs

Probe Set ID	Gene Symbol	Fold change WT LPS/KO LPS
1419610_at	Ccr1	31.6
1421228_at	Ccl7	14.7
1419609_at	Ccr1	10.7
1422259_a_at	Ccr5	8.9
1422260_x_at	Ccr5	5.7
1424727_at	Ccr5	5.2
1420380_at	Ccl2	4.9
1438148_at	Cxcl3	2.7

Figure 5.1 Chemokine and chemokine receptor expression are regulated by Tpl2

Bone marrow derived macrophages (BMDMs) from WT and *tpl2*$^{-/-}$ mice were stimulated with LPS (1 μg/mL) for 4 h, and mRNA expression was analyzed by gene chip microarray. Data are presented as the fold change in gene expression in WT macrophages compared to *tpl2*$^{-/-}$ macrophages.

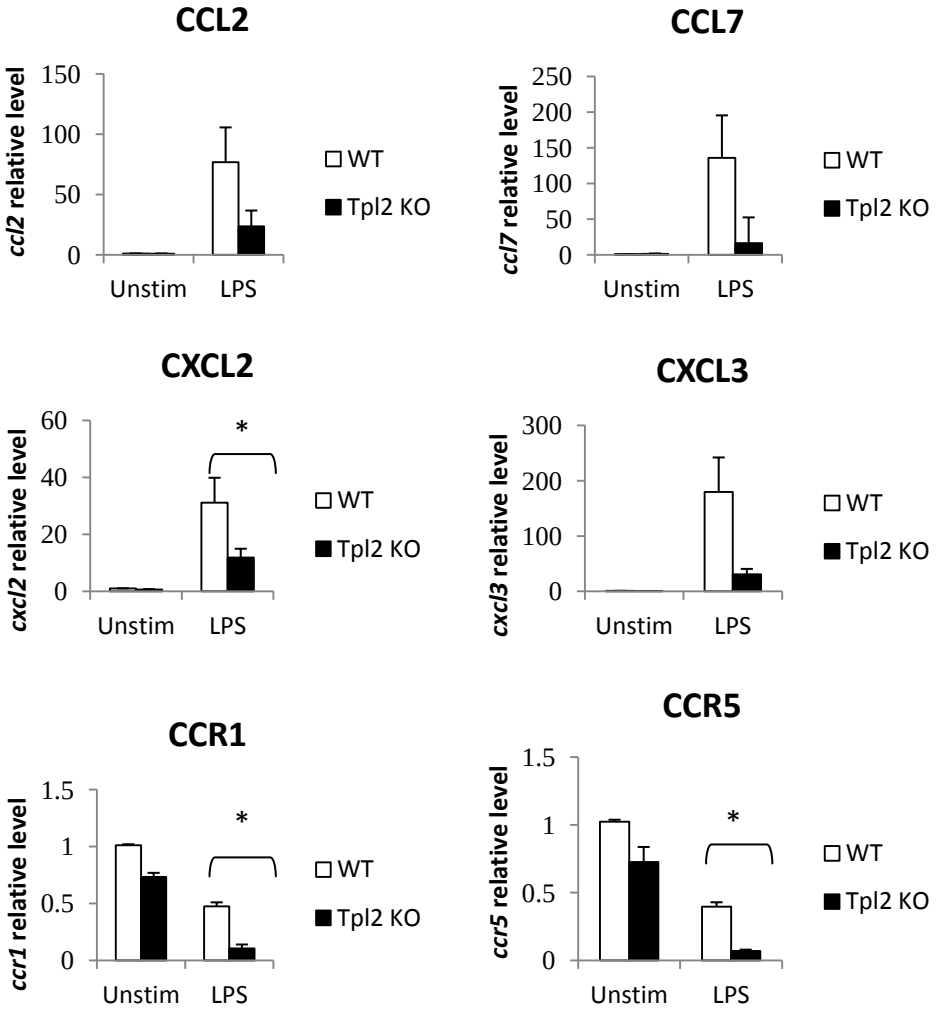


Figure 5.2 Chemokine and chemokine receptor expression profiles are impaired in *tpl2*$^{-/-}$ BMDMs

BMDMs from WT and *tpl2*$^{-/-}$ mice were stimulated in media with LPS for 4h and cells were lysed and analyzed for mRNA expression of indicated chemokine/chemokine receptor gene targets by qRT-PCR relative to an actin control. Results are average of 4 independent experiments for CCL2 and CCL7 and of 3 independent experiments for all other targets. Error bars represent SEM. Paired student's t-test, *$p < 0.05$

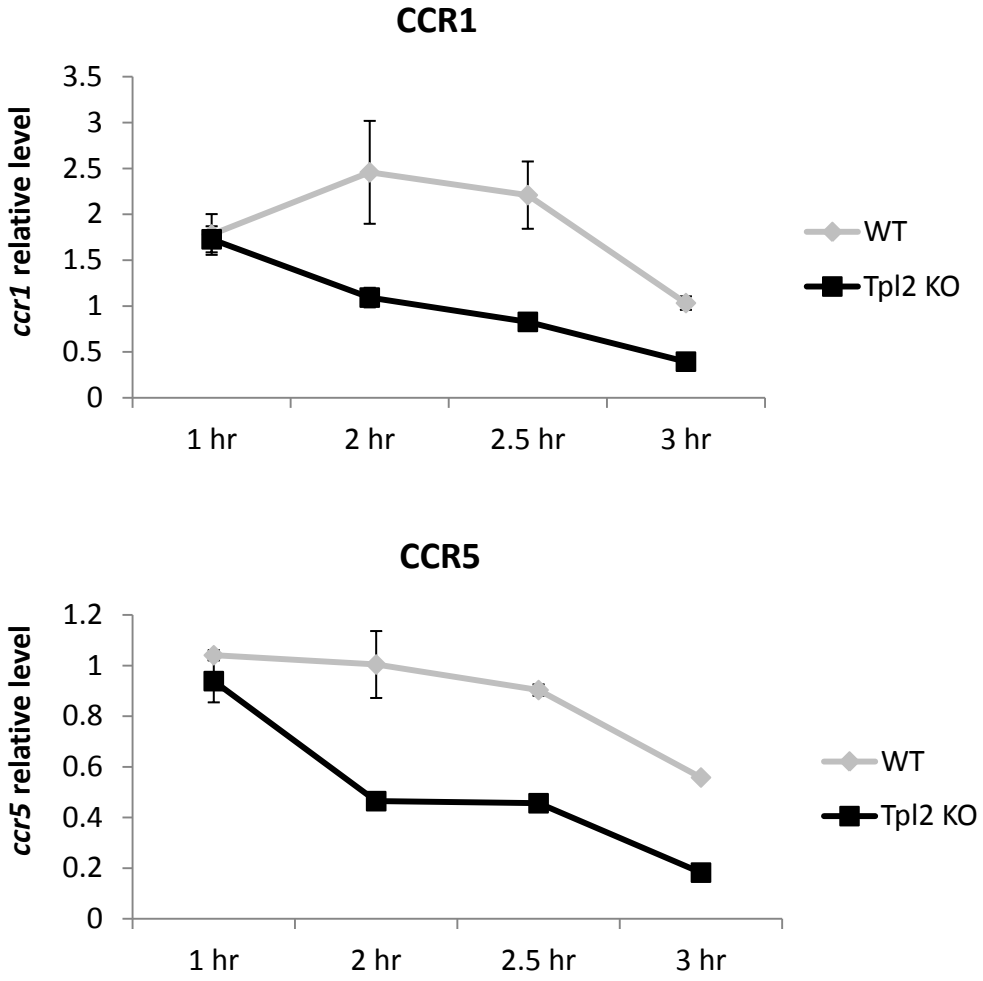


Figure 5.3 Chemokine receptor expression is impaired in *tpl2*$^{-/-}$ BMDMs at early time points following LPS stimulation

BMDMs from WT and *tpl2*$^{-/-}$ mice were stimulated in media with LPS (1μg/mL) for indicated time points and cell lysates were analyzed for mRNA expression of CCR1 and CCR5 by qRT-PCR relative to an actin control. Expression levels at each time point are relative to unstimulated condition (media only). Results are representative of 1 experiment.

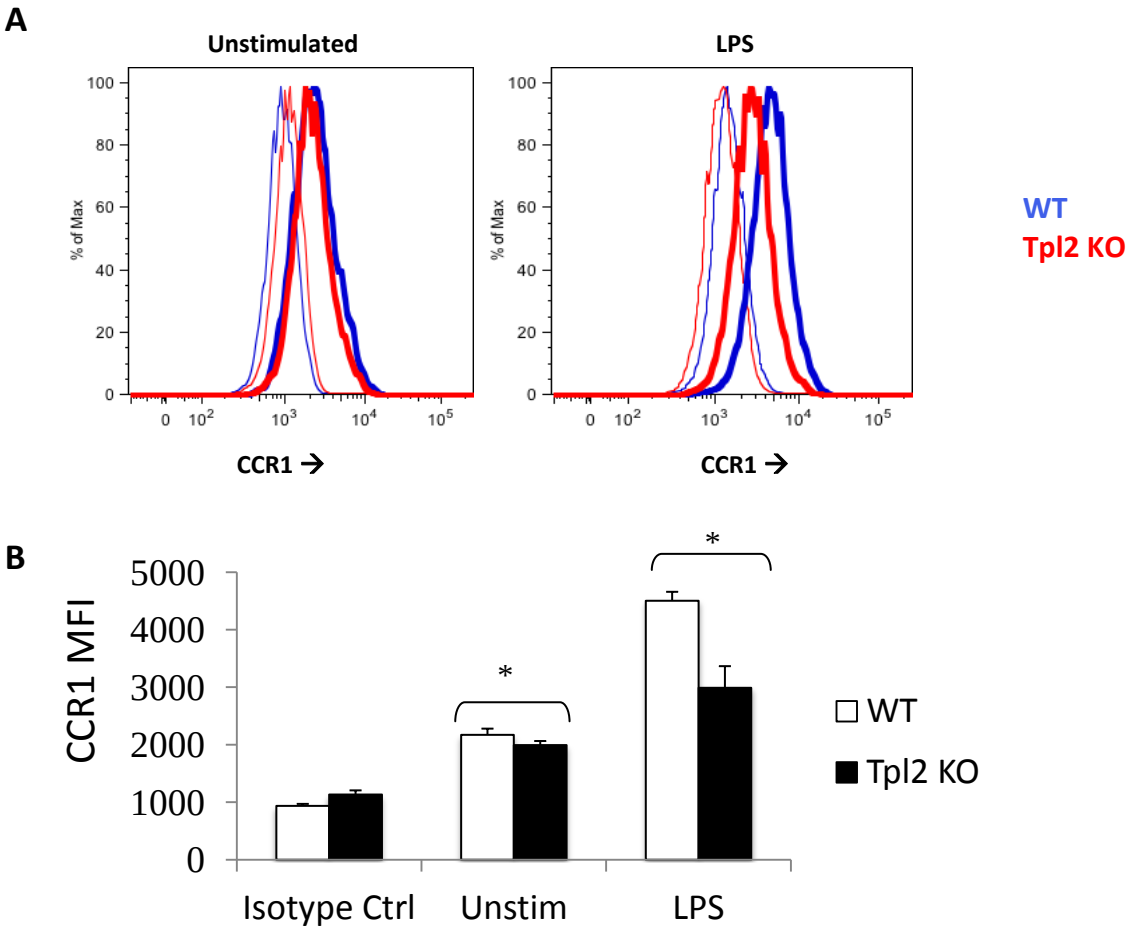


Figure 5.4 CCR1 cell surface protein expression is impaired in *tpl2*$^{-/-}$ BMDMs

BMDMs from WT and *tpl2*$^{-/-}$ mice were stimulated in media with LPS (1 µg/mL) for 24 h, and cells were surface stained with F4/80, CD11b, and CCR1 antibodies to detect CCR1^{+} macrophage populations. **(A)** Representative flow cytometric histograms showing the median fluorescence intensity (MFI) of the FITC-conjugated CCR1 antibody in F4/80^{+}CD11b^{+} macrophages following LPS stimulation. **(B)** Average MFI of CCR1 in F4/80^{+}CD11b^{+} macrophages following LPS stimulation. Results are average of 2 independent experiments and error bars represent standard deviation. Student's t-test, *$p < 0.01$

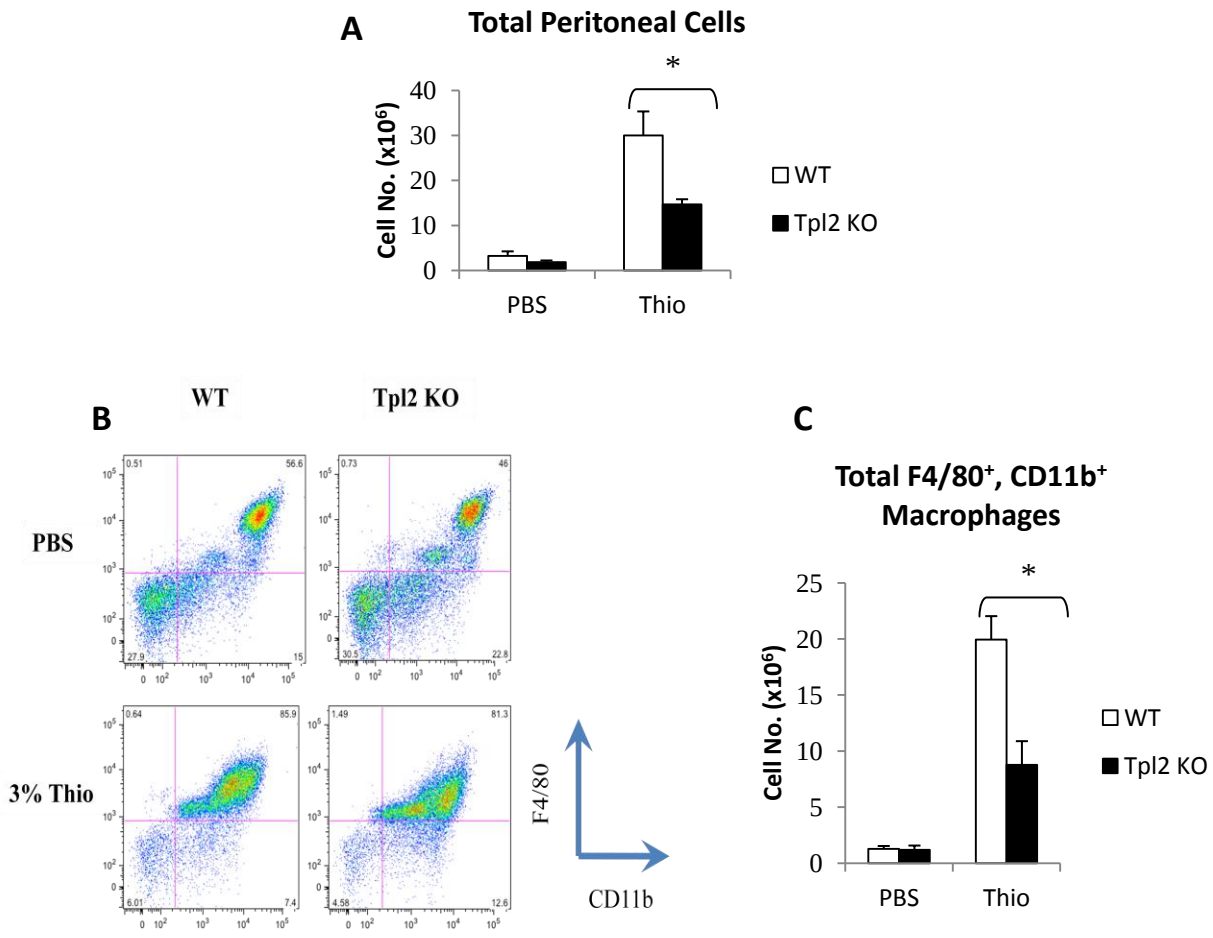


Figure 5.5 Thioglycollate-elicited recruitment of total cells including primary macrophages is impaired in *tpl2*$^{-/-}$ mice

WT and *tpl2*$^{-/-}$ mice were injected i.p. with PBS (control) or 3% thioglycollate medium to induce sterile inflammation and macrophage recruitment. Mice were sacrificed 72 h later and peritoneal exudate cells (PECs) were obtained and analyzed. **(A)** Total numbers of PECs obtained from mice 72 h post injection. **(B)** Representative flow cytometric plots showing F4/80^{+}CD11b^{+} macrophages following recruitment. **(C)** Absolute numbers of recruited macrophages from the peritoneal cavity. Results are averages of 3 independent experiments, and error bars represent SEM. Student's t-test, *$p < 0.05$

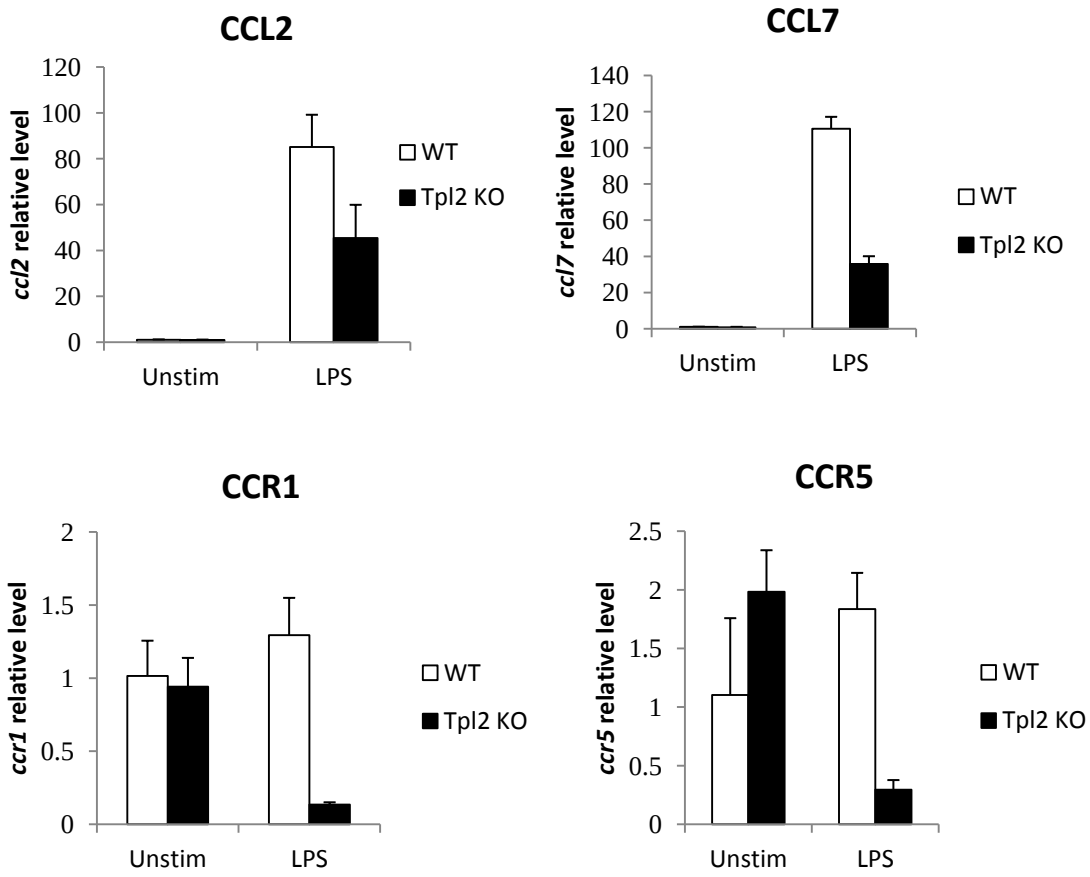


Figure 5.6 Chemokine and chemokine receptor expression is impaired in recruited *tpl2*$^{-/-}$ primary peritoneal macrophages

PECs were harvested from WT or *tpl2*$^{-/-}$ mice 72 h after i.p. injection with 3% thioglycollate medium, macrophages were adherence purified and stimulated with LPS (1μg/mL) for 4 h. Following stimulation, cells were lysed and analyzed for mRNA expression of chemokine and chemokine receptors by qRT-PCR relative to an actin control. Results are representative of 1 experiment.

CHAPTER 6

TPL2 REGULATES MACROPHAGE DIFFERENTIATION BETWEEN CLASSICAL (M1) AND ALTERNATIVE (M2) PHENOTYPES

Introduction

The previous chapter has provided insight into how the loss of Tpl2 in macrophage populations can result in impaired expression of chemokines and chemokine receptors correlating with impaired migration to inflamed tissues. To further analyze macrophage functions in the context of Tpl2, we examined whether the ability of macrophages to polarize towards M1 or M2 subsets is regulated by Tpl2 following inflammatory stimulation. Macrophage subsets implicated in the innate inflammatory response are the classical (M1) and for anti-inflammatory, alternatively activated (M2) macrophages. Polarization towards the M1 macrophage phenotype (via bacterial LPS and Th1 cytokines such as IFNγ) represents a classical, pro-inflammatory macrophage response that can be evaluated by the expression of M1-specific markers such as inducible nitric oxide synthase (iNOS) indicating the production of reactive oxygen intermediates including nitric oxide (NO) at the site of inflammation. In contrast, M2 macrophage polarization (via Th2 cytokines including IL-4 as well as infection with helminth parasites like *Schistosoma mansoni*) represents an alternative, anti-parasitic macrophage response characterized by expression of specific M2 markers Ym1, Fizz1, and Arg1. Understanding the factors and signaling pathways that regulate this balance between M1

and M2 subtypes is an active area of research. This chapter aims to determine whether induction of M1 and/or M2 macrophages is regulated by Tpl2 following stimulation with subtype-specific agonists. Our preliminary findings demonstrate that Tpl2 is required for M1 macrophage generation, as assessed by *inos* gene expression, and Tpl2 suppresses M2 macrophage differentiation, as assessed by *ym1* gene expression.

Results

Tpl2 promotes expression of the classical macrophage marker iNOS during *in vitro* and *in vivo* inflammatory settings

Macrophages from either the bone marrow (BMDM) or peritoneal cavity (PEC) of WT or *tpl2*$^{-/-}$ mice were isolated in supplemented DMEM media. To induce a classical inflammatory response, cells were stimulated *in vitro* with either bacterial LPS alone or in combination with the pro-inflammatory cytokine IFNγ for 24 h. To characterize M1 macrophage induction, expression levels of the M1 marker inducible nitric oxide synthase (iNOS) were measured in stimulated cells via qRT-PCR. Following stimulation with LPS alone, *tpl2*$^{-/-}$ BMDMs displayed higher levels of iNOS mRNA than WT macrophages, while expression in PECs showed no difference (Figure 6.1). This result, although initially unexpected, was consistent with a recently published report by Lopez-Pelaez *et al.* in 2011 that also showed *tpl2*-/- deficient BMDM have increased levels of iNOS mRNA following LPS stimulation (109). However, the prototypical induction of the M1 phenotype utilizes a combination of both LPS and IFNγ. Under these optimal M1-inducing conditions, we observed that *tpl2*$^{-/-}$ macrophages from the bone marrow and peritoneal cavity showed significantly reduced iNOS expression compared to WT

macrophages (Figure 6.1). Additionally, mRNA expression of the major histocompatibility complex II (MHC II, another prominent M1 marker) by 48 h post stimulation with LPS and IFNγ was also impaired in *tpl2*$^{-/-}$ macrophages (data not shown). These results indicate that under a more robust and perhaps more physiologically relevant response involving pro-inflammatory cytokine activation, *tpl2*$^{-/-}$ macrophages were impaired in their ability to polarize towards the M1 phenotype.

In fact, when we assessed M1 induction in an *in vivo* setting using a combination of thioglycollate-mediated recruitment and LPS activation *in vivo* by injecting both substances directly i.p. into WT or *tpl2*$^{-/-}$ mice, we found that peritoneal macrophages obtained from *tpl2*$^{-/-}$ mice 1 day post LPS injection displayed impaired expression of iNOS compared to WT mice (Figure 6.2). This finding indicates that *in vivo* classical activation of peritoneal macrophages may require Tpl2 to induce expression of the M1 marker iNOS. To determine whether this defect in M1 induction in the *tpl2*$^{-/-}$ mice was due to impaired IFNγ signaling, we also measured activation of the IFNγ signal transducer STAT1 in macrophages following IFNγ stimulation *in vitro*. We observed no differences in STAT1 activation by Western blot between WT and *tpl2*$^{-/-}$ macrophages, indicating that these cells are capable of responding efficiently to IFNγ (data not shown).

Expression of the alternative macrophage marker Ym1 is enhanced in *tpl2*$^{-/-}$ macrophages following IL-4 stimulation

Following the observations that Tpl2 is required for polarizing macrophages towards a classically activated phenotype, we next assessed whether Tpl2 could conversely inhibit

macrophage polarization to the opposing, alternative M2 phenotype. To test this, we first isolated BMDMs and PECs from WT and *tpl2*$^{-/-}$ mice and stimulated the cells in culture with the Th2 cytokine IL-4 for 24 h to induce M2 polarization. To evaluate M2 induction, stimulated macrophages were analyzed for mRNA expression of the M2 marker Ym1. *Tpl2*$^{-/-}$ BMDMs expressed markedly higher levels of Ym1 relative to WT BMDMs following IL-4 stimulation, indicating a possible inhibitory role of Tpl2 in the context of alternative macrophage induction (Figure 6.3). Additionally, *tpl2*$^{-/-}$ PECs also showed slightly higher levels of Ym1 expression compared to WT PECs, although the difference was much less substantial than seen with BMDMs (Figure 6.3). These results show that Tpl2 plays a role in regulating expression of the M2 marker Ym1 and furthermore may inhibit macrophage polarization to an alternative phenotype during Th2 inflammatory conditions such as parasitic infection.

Tpl2$^{-/-}$ mice display altered macrophage and eosinophil phenotypes during *Schistosoma mansoni* egg-induced inflammation

In order to further characterize Tpl2's possible effects on M2 macropahge polarization, we employed an *in vivo* model of alternative activation which would mimic a natural Th2 inflammatory response in a mammalian host. The model we chose to use was intraperitoneal injection of mice with *Schistosoma mansoni* eggs. As shown in Figure 2.6, anti-inflammatory immune responses consisting of robust Th2 and M2 activation occur in mammalian hosts after egg deposition during *S. mansoni* infection. This intestinal helminthic parasite is widely used in experimental murine models and has helped to delineate the factors that contribute to alternative macrophage development and functions *in vivo*. WT and *tpl2*$^{-/-}$ mice were injected i.p. with 5 x

10^3 *S. mansoni* eggs. One week later, mice were sacrificed, and peritoneal cavities were lavaged with sterile PBS. Peritoneal cells were surface stained for $F4/80^+CD11b^+$ macrophages as well as for $CD11b^{int}Siglec\text{-}F^+$ eosinophils. Following *S. mansoni* egg injection, there was a trend towards a decrease in both the proportion and absolute numbers of $F4/80^+CD11b^+$ peritoneal macrophages in *tpl2*$^{-/-}$ mice indicating a defect in the recruitment of these cells to the site of egg injection (Figure 6.4A and 6.5A). Eosinophilia is often associated with Th2 immune responses such as allergic asthma and helminth infections, due to the abundant expression of IL-5 in these conditions. Therefore, we quantified eosinophils present in the peritoneal cavity following egg administration. Interestingly, proportions of eosinophils in the peritoneal cavity following *S. mansoni* infection were increased in *tpl2*$^{-/-}$ mice compared to WT mice (Figure 6.4B). However, absolute numbers of eosinophils in the peritoneal cavity following infection were not significantly different between WT and *tpl2*$^{-/-}$ mice (Figure 6.5B).

Expression of the alternative macrophage marker Ym1 is enhanced in *tpl2*$^{-/-}$ peritoneal macrophages during *S. mansoni* egg injection

To further characterize Tpl2's possible role in the induction of M2 macrophages during an *in vivo* inflammatory response towards *S. mansoni* eggs, we measured the gene expression of the M2 marker Ym1 via qRT-PCR in the peritoneal macrophages recovered one week after egg injection. Peritoneal cells were recovered from uninfected (PBS) and infected mice and were additionally adherence purified in supplemented DMEM media at 37°C, 5% CO_2 for 6-8 h to isolate peritoneal macrophages. We observed an increase in Ym1 expression in both WT and *tpl2*$^{-/-}$ peritoneal macrophages, but there was a trend towards increased Ym1 expression in *tpl2*$^{-/-}$

compared to WT peritoneal macrophages (Figure 6.6). This finding correlates with our previous *in vitro* results showing enhanced Ym1 expression in *tpl2*$^{-/-}$ bone marrow derived macrophages following IL-4 stimulation (Figure 6.3). Collectively, these results indicate that Th2 inflammation promotes macrophage-specific expression of the M2 marker Ym1, and this expression is enhanced in the absence of Tpl2.

Discussion

These findings provide valuable insight into the possible regulatory effects Tpl2 may have on macrophage polarization during the inflammatory response. Specifically, they posit Tpl2 as a regulatory kinase capable of mediating innate cell responses depending on environmental cues. While this study provides valuable insights into the regulation of macrophage differentiation by Tpl2, several limitations exist and necessitate future studies. One important caveat of this study is that it relies on transcriptional regulation of key markers of M1 versus M2 differentiation. Future studies evaluating protein expression of these various markers are clearly warranted.

Another unanswered question is whether the effect of Tpl2 on macrophage phenotype *in vivo* is macrophage cell intrinsic or extrinsic. Adoptive transfer of *tpl2*$^{-/-}$ macrophages into WT recipients during egg-induced Th2 inflammation would help to address this important issue. Due to the fact that *in vitro* experiments recapitulate the *in vivo* experiments, it is highly likely that Tpl2 functions in macrophages in a cell autonomous way. In support of this, abundant evidence exists supporting major functions of Tpl2 in macrophages, particularly in inflammatory conditions, such as TLR signaling (10, 21, 102). What is unclear is whether Tpl2 may also

function directly downstream of IL-4 in this cell type, or alternatively, whether effects of Tpl2 on M2 differentiation are indirect. Future experiments will address Tpl2 kinase activation by IL-4 as well as IL-4 signal transduction leading to STAT6 phosphorylation in WT versus *tpl2*$^{-/-}$ macrophages.

In this regard, a recent study has sought to clarify the signaling pathways regulated by Tpl2 under inflammatory conditions. Lopez-Pelaez *et al.* reported that Tpl2 repressed iNOS expression in BMDMs following LPS stimulation, and this was associated with activation of the critical PI3K-Akt-mTOR-p70 S6k pathway (109). Future studies will need to assess how Tpl2 regulates macrophage polarization on a biochemical level following these specific pro-inflammatory stimulations as well as during IL-4 driven Th2 responses to address whether Tpl2 differentially regulates alternative macrophage induction by utilizing pathways such as the PI3K pathway.

Finally, Th2-associated cytokine levels need to be quantified in the peritoneal cavity during egg-induced Th2 inflammation. This could help to determine whether an underlying Th2 bias previously observed in *tpl2*$^{-/-}$ mice might also contribute, in part, to M2 macrophage differentiation observed *in vivo* (104). In particular, IL-5, IL-13, IL-25 and IL-33 all contribute significantly to Th2 responses and M2 macrophage differentiation and should be evaluated in fluid recovered from the peritoneal cavity (83, 110-112).

In terms of clinical therapies involving Tpl2 inhibition, the data presented here indicates the possibility of exacerbated Th2 or M2 macrophage responses in individuals where Tpl2 kinase activity is disrupted. While the absence of Tpl2 as a result of clinical inhibition may lead to enhanced susceptibility to helminth infections (such as *S. mansoni* infection), this is not an

immediate concern as there are few if any helminth parasites endemic in the United States and other developed nations. In the US, a more immediate concern of Tpl2 clinical inhibition would be the potential exacerbation of Th2-induced allergic asthma conditions, which have already been shown to be enhanced in the absence of Tpl2 in mouse models (104). For this reason it is critical that future studies outline the overall risks versus benefits of clinical Tpl2 inhibition in the context of macrophage polarization and Th2-associated immune responses. Understanding the mechanisms by which Tpl2 causes these cell type-specific phenotypic switches should help to reduce the chances of harmful inflammatory side effects in patients receiving this treatment.

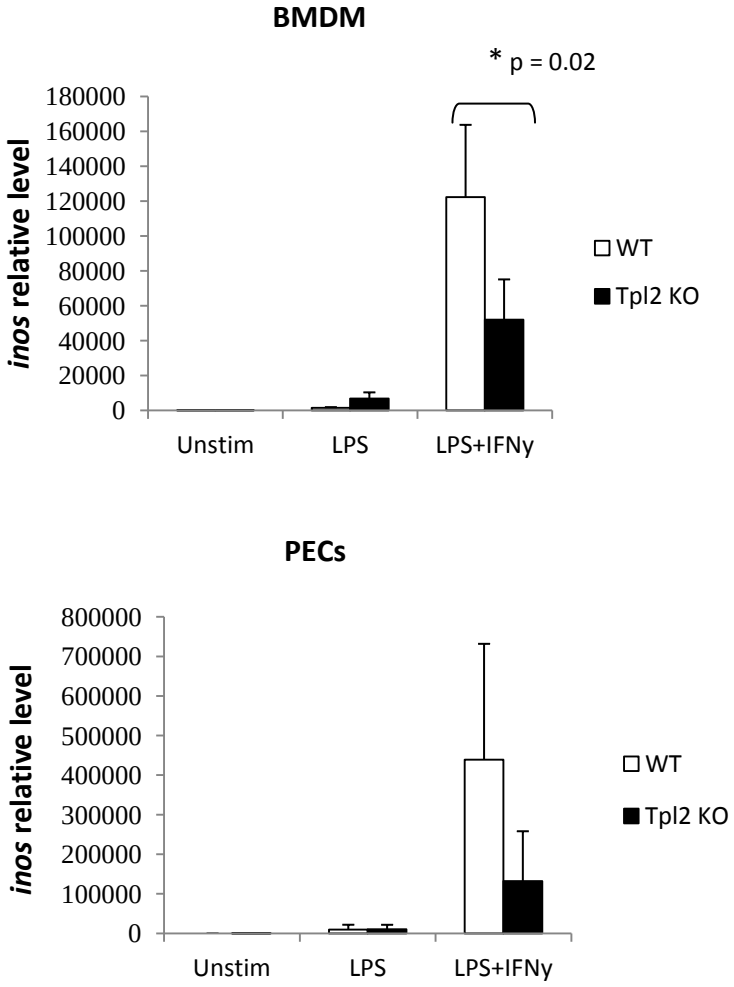


Figure 6.1 Tpl2 promotes expression of the classical macrophage marker iNOS following *in vitro* stimulation

Bone marrow derived macrophages and peritoneal macrophages were obtained from WT and *tpl2*$^{-/-}$ mice and were stimulated in media with LPS (1µg/mL) alone or with LPS and IFNγ (10 ng/mL) for 24 h. Cells were lysed and analyzed for iNOS mRNA expression by qRT-PCR relative to an actin control. Results are average of 3 independent experiments for BMDMs and 2 independent experiments for PECs. Error bars represent SEM (BMDMs) or standard deviation (PECs). Paired student's t-test for p-value.

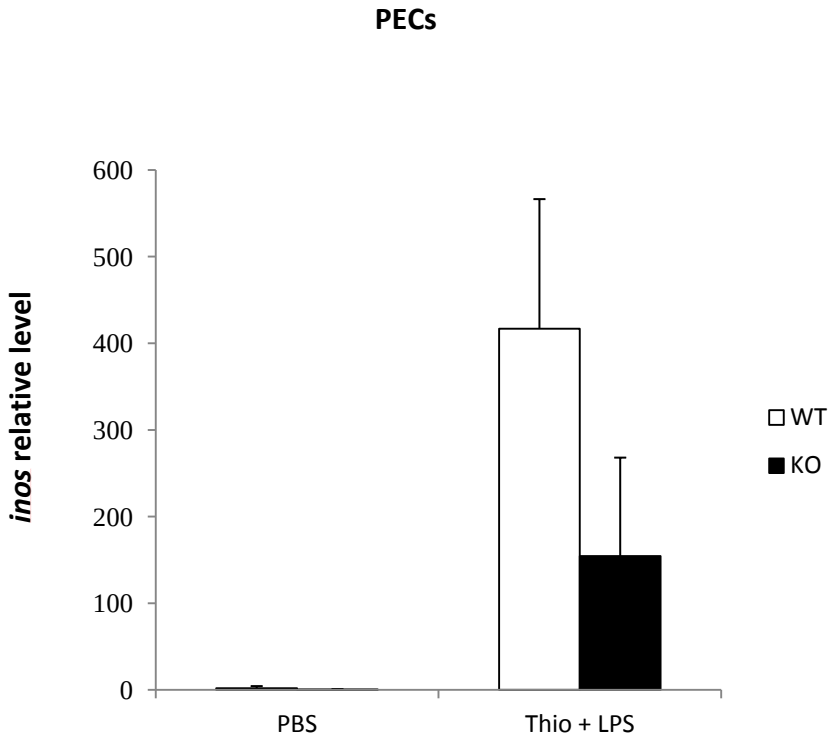


Figure 6.2 Tpl2 promotes expression of the classical macrophage marker iNOS following *in vivo* LPS administration

WT or *tpl2*$^{-/-}$ mice were given i.p. injections of PBS (control) or 3% thioglycollate to recruit peritoneal macrophages. Additionally, thioglycollate-injected mice were also injected with 10 μg LPS i.p. 24 h prior to sacrifice to activate recruited cells. After 72 h post initial injections, PECs were analyzed for iNOS mRNA expression by qRT-PCR relative to an actin control. Results are representative of 1 experiment and error bars represent standard deviation.

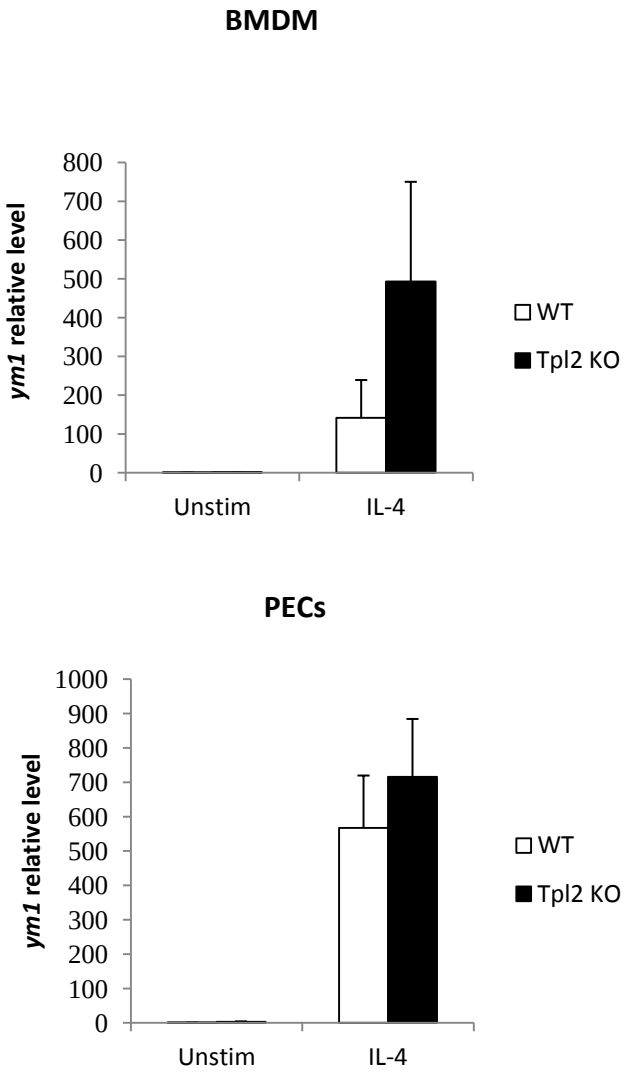


Figure 6.3 Expression of alternative macrophage marker Ym1 is enhanced in *tpl2*$^{-/-}$ macrophages following IL-4 stimulation

Bone marrow derived macrophages and peritoneal macrophages were obtained from WT and *tpl2*$^{-/-}$ mice and were stimulated with IL-4 (40 ng/mL) for 24 h. Cells were lysed and analyzed for Ym1 mRNA expression by qRT-PCR relative to an actin control. Results are average of 3 independent experiments and error bars represent SEM. Paired student's t-test for p-value.

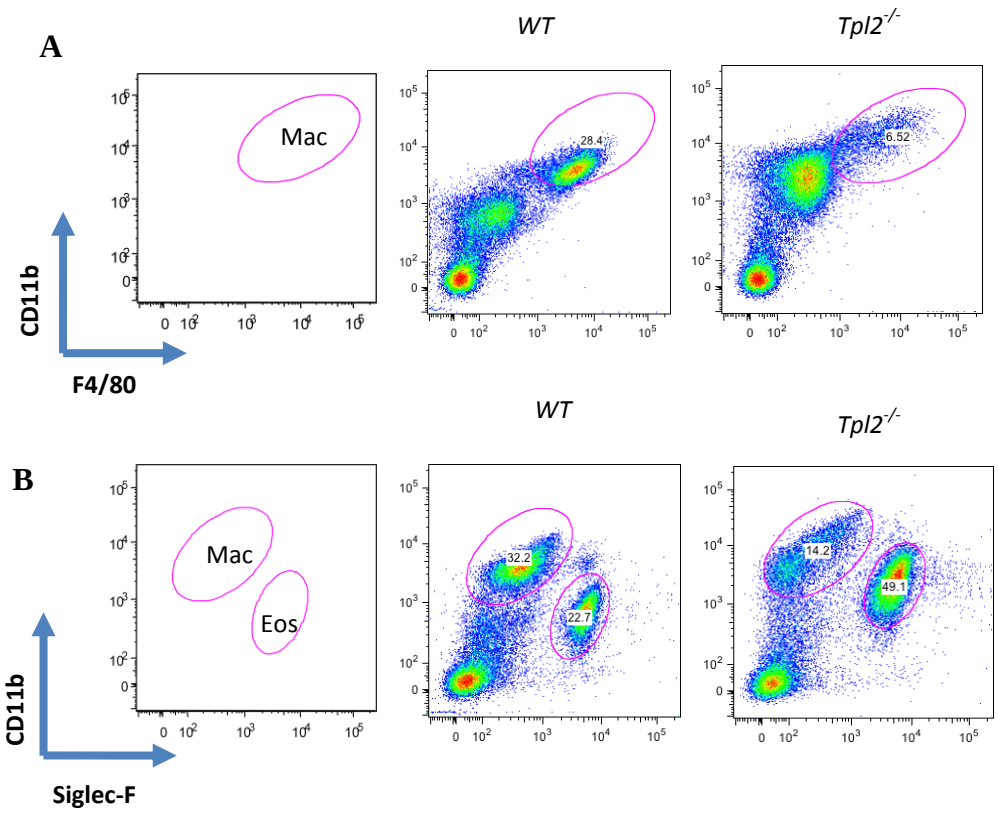


Figure 6.4 Increased proportions of eosinophils and decreased proportions of macrophages in *tpl2*$^{-/-}$ mice following *S. mansoni* egg injection

WT and *tpl2*$^{-/-}$ mice were injected i.p. with 5 x 10^3 *S. mansoni* eggs or PBS, 1 week later peritoneal cells were collected and surface stained for macrophage and eosinophil populations. **(A)** Representative FACS plots of F4/80^{+}CD11b^{+} macrophage populations following egg injection. Results are representative of 3 independent experiments. **(B)** Representative FACS plots of CD11b^{int}Siglec-F^{+} eosinophils following egg injection. Results are representative of 2 independent experiments.

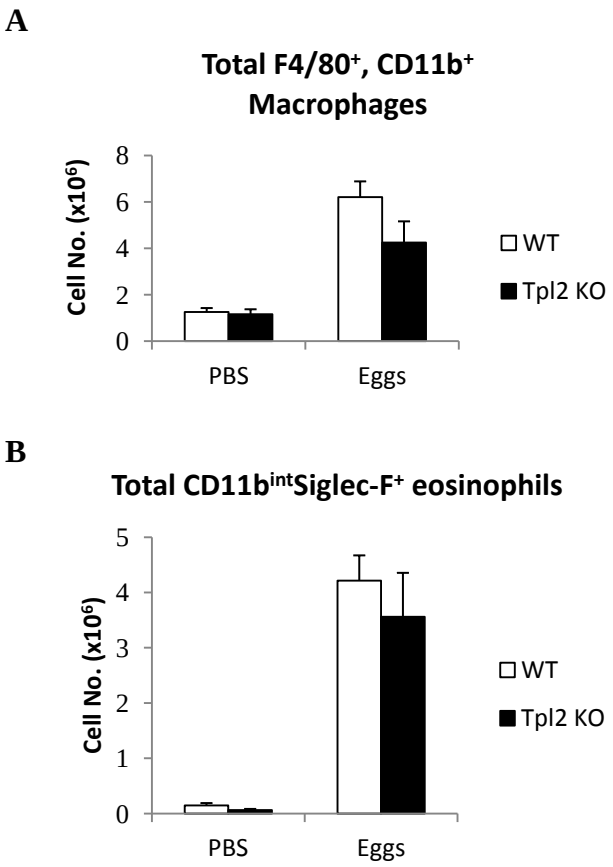


Figure 6.5 Decreased numbers of total peritoneal macrophages but similar total eosinophil numbers between WT and *tpl2*$^{-/-}$ mice following *S. mansoni* egg injection

WT and *tpl2*$^{-/-}$ mice were injected i.p. with 5 x 10^3 *S. mansoni* eggs or PBS and after 1 week peritoneal cells were collected and surface stained for macrophage and eosinophil populations. **(A)** Total F4/80$^+$, CD11b$^+$ macrophages from the peritoneum. Results are average of 3 independent experiments and error bars represent SEM. Paired student's t-test for p-value. PBS (N=10 mice per group), Eggs (N=13 mice for WT and N=10 mice for Tpl2 KO). **(B)** Total CD11b^{int}Siglec-F$^+$ eosinophils from the peritoneum. Results are average of 2 independent experiments and error bars represent SEM. Paired student's t-test for p-value. PBS (N=7 mice per group), Eggs (N=8 mice per group).

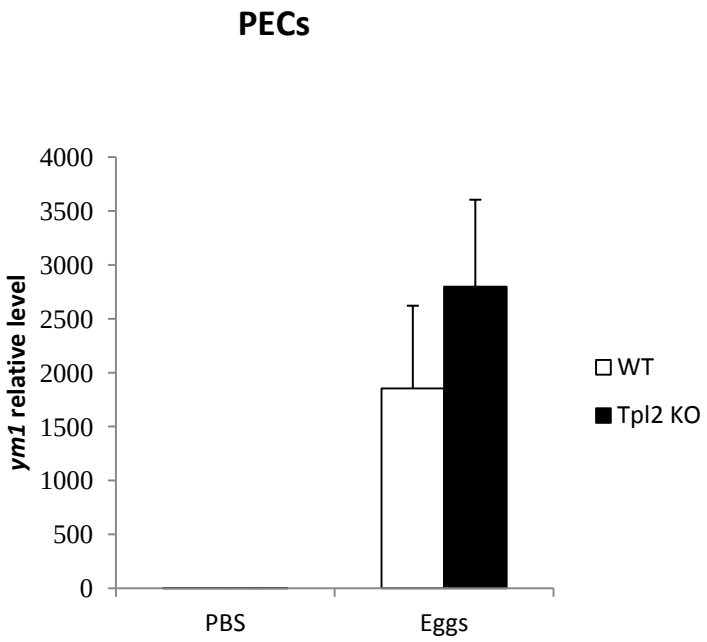


Figure 6.6 Expression of alternative macrophage marker Ym1 is enhanced in *tpl2*$^{-/-}$ peritoneal macrophages following *S. mansoni* egg injection

Peritoneal cells were obtained from WT and *tpl2*$^{-/-}$ mice 1 week after intraperitoneal *S. mansoni* egg injection and peritoneal macrophages were isolated by adherence purification for 6-8 hours in supplemented DMEM media at 37°C, 5% CO_2. Adherent macrophages were lysed and analyzed for mRNA expression of various alternative macrophage markers by qRT-PCR relative to an actin control. Results are average of 4 independent experiments and error bars represent SEM. Paired student's t-test for p-value.

CHAPTER 7

CONCLUSIONS AND FUTURE DIRECTIONS

The results and findings outlined in this work have given new insight into the roles that the MAP kinase Tpl2 plays in regulating the development and function of critical innate immune cells. Based on previous studies outlining Tpl2's role in regulating $CD4^+$ T-cell function and development during the inflammatory response (25), we wanted to address whether Tpl2 also affected the development and/or function of critical innate immune cells during this response. Our results clearly showed that Tpl2 is dispensable for NK cell development and function in the spleens of mice. Additionally, NK cell maintenance and function in other sites such as the lung tissue during site-specific inflammation following influenza infection also did not require Tpl2. However, in regards to macrophage development and function, we showed that Tpl2 was critically important for regulating the function of these innate cells as there was impaired expression of chemokine ligands and chemokine receptors in *tpl2*$^{-/-}$ macrophages following activation with LPS. We further demonstrated that the impaired expression of these critical chemokines and chemokine receptors in the absence of Tpl2 affected the ability of these macrophages to efficiently migrate *in vivo* to sites of local inflammation. In continuing with the focus on macrophages, we also assessed whether Tpl2 was responsible for regulating the polarization of these activated cells towards distinct M1 or M2 phenotypes. While our results were not entirely conclusive, we did observe a reproducible reduction in M1 macrophage polarization (via iNOS gene expression) and enhanced M2 macrophage polarization (via Ym1

gene expression) from *tpl2*$^{-/-}$ mice following pro-inflammatory or anti-inflammatory stimulation, respectively. Consistent with our previously reported exacerbation of Th2-driven allergic asthma in *tpl2*$^{-/-}$ mice, these results indicate that Tpl2 deficiency may exacerbate M2 proliferation. Therefore, we also looked at the effect of Tpl2-dependent M2 polarization in response to *Schistosoma mansoni* eggs and observed impaired macrophage recruitment to the peritoneal site of egg injection in *tpl2*$^{-/-}$ mice coincident with increased proportions of eosinophils in the peritoneal cavity of *tpl2*$^{-/-}$ mice. Additionally, expression of the M2 marker Ym1 was enhanced in peritoneal macrophages from *tpl2*$^{-/-}$ mice, possibly indicating an M2 macrophage bias in the absence of Tpl2.

Collectively the results of this study provide insights into the diverse roles that Tpl2 has in the regulation of critical innate cells such as NK cells and macrophages during robust inflammatory and anti-inflammatory responses. The differences in Tpl2-dependent regulation between these two cells types suggest that, while this kinase is broadly expressed, its functional contributions can differ greatly in a cell type- and stimulus-specific fashion. It is our hope that future work regarding Tpl2's influence on innate cell function and development will use the findings presented here as a rationale for investigating more precise roles for Tpl2 in this regulatory process. For example, future studies interrogating additional functions of *tpl2*$^{-/-}$ NK cells such as cytotoxic killing capacity in the spleen and lungs at resting states or during pathogen infection will help to more definitively conclude whether NK cells utilize Tpl2 for their cytotoxic killing abilities. Additionally, our studies on the role of Tpl2 and macrophage function and polarization are expected to influence future research, as the functional impairments observed in these cells in the absence of Tpl2 may be utilized in the development of therapies

towards autoimmune inflammation. Using Tpl2 blockade to downregulate macrophage chemokine/chemokine receptor expression and functional migration represents a novel immune therapy to curtail excessive inflammation during chronic conditions such as rheumatoid arthritis and inflammatory bowel disease. Furthermore, the preliminary findings that Tpl2 ablation promotes M2 macrophages, associated with wound-healing responses, at the expense of pro-inflammatory M1 macrophages provides an additional mechanism for dampening inflammation via Tpl2 inhibition. Furthermore, understanding precisely how Tpl2 influences macrophage differentiation may ultimately allow for specific retooling of the macrophage inflammatory responses during certain pro-inflammatory situations. While Tpl2 inhibition appears to be a promising treatment for chronic or advanced autoimmune disease, modulation of Tpl2 function should be approached cautiously as we also predict that Tpl2 blockade may lead to exacerbated Th2 responses, such as allergic asthma. The possibility that Tpl2 may regulate M1/M2 balance is still an ongoing area of investigation that will need to involve studies looking at protein expression of specific macrophage markers as well as the macrophage-specific effects on certain tissues (i.e. necrosis or fibrosis) in *tpl2*$^{-/-}$ mice following M1 or M2 responses. Overall, future studies that can elucidate the precise mechanism behind Tpl2-dependent regulation of macrophage polarization should help in determining the clinical outcomes associated with therapeutic manipulation of this inflammatory axis.

REFERENCES

1. Miyoshi J, Higashi T, Mukai H, Ohuchi T, Kakunaga T. 1991. Structure and transforming potential of the human cot oncogene encoding a putative protein kinase. *Mol Cell Biol* 11: 4088-96
2. Gandara ML, Lopez P, Hernando R, Castano JG, Alemany S. 2003. The COOH-terminal domain of wild-type Cot regulates its stability and kinase specific activity. *Mol Cell Biol* 23: 7377-90
3. Patriotis C, Makris A, Bear SE, Tsichlis PN. 1993. Tumor progression locus 2 (Tpl-2) encodes a protein kinase involved in the progression of rodent T-cell lymphomas and in T-cell activation. *Proc Natl Acad Sci U S A* 90: 2251-5
4. Tsatsanis C, Patriotis C, Bear SE, Tsichlis PN. 1998. The Tpl-2 protooncoprotein activates the nuclear factor of activated T cells and induces interleukin 2 expression in T cell lines. *Proceedings of the National Academy of Sciences* 95: 3827-32
5. Tsatsanis C, Patriotis C, Tsichlis PN. 1998. Tpl-2 induces IL-2 expression in T-cell lines by triggering multiple signaling pathways that activate NFAT and NF-kappaB. *Oncogene* 17: 2609-18
6. Gilmore TD, Wolenski FS. 2012. NF-κB: where did it come from and why? *Immunological Reviews* 246: 14-35
7. Akira S, Takeda K. 2004. Toll-like receptor signalling. *Nat Rev Immunol* 4: 499-511
8. Ninomiya-Tsuji J, Kishimoto K, Hiyama A, Inoue J-i, Cao Z, Matsumoto K. 1999. The kinase TAK1 can activate the NIK-I[kappa]B as well as the MAP kinase cascade in the IL-1 signalling pathway. *Nature* 398: 252-6
9. Chow JC, Young DW, Golenbock DT, Christ WJ, Gusovsky F. 1999. Toll-like Receptor-4 Mediates Lipopolysaccharide-induced Signal Transduction. *Journal of Biological Chemistry* 274: 10689-92
10. Dumitru CD, Ceci JD, Tsatsanis C, Kontoyiannis D, Stamatakis K, Lin JH, Patriotis C, Jenkins NA, Copeland NG, Kollias G, Tsichlis PN. 2000. TNF-alpha induction by LPS is regulated posttranscriptionally via a Tpl2/ERK-dependent pathway. *Cell* 103: 1071-83
11. Beinke S, Robinson MJ, Hugunin M, Ley SC. 2004. Lipopolysaccharide activation of the TPL-2/MEK/extracellular signal-regulated kinase mitogen-activated protein kinase cascade is regulated by IkappaB kinase-induced proteolysis of NF-kappaB1 p105. *Mol Cell Biol* 24: 9658-67
12. Kurosawa M, Numazawa S, Tani Y, Yoshida T. 2000. ERK signaling mediates the induction of inflammatory cytokines by bufalin in human monocytic cells. *American Journal of Physiology - Cell Physiology* 278: C500-C8
13. Verhaeghe C, Remouchamps C, Hennuy B, Vanderplasschen A, Chariot A, Tabruyn SP, Oury C, Bours V. 2007. Role of IKK and ERK pathways in intrinsic inflammation of cystic fibrosis airways. *Biochem Pharmacol* 73: 1982-94

14. Maeng Y-S, Min J-K, Kim JH, Yamagishi A, Mochizuki N, Kwon J-Y, Park Y-W, Kim Y-M, Kwon Y-G. 2006. ERK is an anti-inflammatory signal that suppresses expression of NF-κB-dependent inflammatory genes by inhibiting IKK activity in endothelial cells. *Cellular Signalling* 18: 994-1005
15. Beinke S, Deka J, Lang V, Belich MP, Walker PA, Howell S, Smerdon SJ, Gamblin SJ, Ley SC. 2003. NF-κB1 p105 Negatively Regulates TPL-2 MEK Kinase Activity. *Mol Cell Biol* 23: 4739-52
16. Kawai T, Takeuchi O, Fujita T, Inoue J-i, Mühlradt PF, Sato S, Hoshino K, Akira S. 2001. Lipopolysaccharide Stimulates the MyD88-Independent Pathway and Results in Activation of IFN-Regulatory Factor 3 and the Expression of a Subset of Lipopolysaccharide-Inducible Genes. *The Journal of Immunology* 167: 5887-94
17. Kane LP, Shapiro VS, Stokoe D, Weiss A. 1999. Induction of NF-κB by the Akt/PKB kinase. *Current Biology* 9: 601-S1
18. Kane LP, Mollenauer MN, Xu Z, Turck CW, Weiss A. 2002. Akt-Dependent Phosphorylation Specifically Regulates Cot Induction of NF-κB-Dependent Transcription. *Mol Cell Biol* 22: 5962-74
19. Eliopoulos AG, Das S, Tsichlis PN. 2006. The tyrosine kinase Syk regulates TPL2 activation signals. *J Biol Chem* 281: 1371-80
20. Handoyo H, Stafford MJ, McManus E, Baltzis D, Peggie M, Cohen P. 2009. IRAK1-independent pathways required for the interleukin-1-stimulated activation of the Tpl2 catalytic subunit and its dissociation from ABIN2. *Biochem J* 424: 109-18
21. Mielke LA, Elkins KL, Wei L, Starr R, Tsichlis PN, O'Shea JJ, Watford WT. 2009. Tumor progression locus 2 (Map3k8) is critical for host defense against Listeria monocytogenes and IL-1 beta production. *J Immunol* 183: 7984-93
22. O'Neill LA, Greene C. 1998. Signal transduction pathways activated by the IL-1 receptor family: ancient signaling machinery in mammals, insects, and plants. *Journal of Leukocyte Biology* 63: 650-7
23. Krelin Y, Voronov E, Dotan S, Elkabets M, Reich E, Fogel M, Huszar M, Iwakura Y, Segal S, Dinarello CA, Apte RN. 2007. Interleukin-1β–Driven Inflammation Promotes the Development and Invasiveness of Chemical Carcinogen–Induced Tumors. *Cancer Res* 67: 1062-71
24. Joosten LAB, Smeets RL, Koenders MI, van den Bersselaar LAM, Helsen MMA, Oppers-Walgreen B, Lubberts E, Iwakura Y, van de Loo FAJ, van den Berg WB. 2004. Interleukin-18 Promotes Joint Inflammation and Induces Interleukin-1-Driven Cartilage Destruction. *Am J Pathol* 165: 959-67
25. Watford WT, Hissong BD, Durant LR, Yamane H, Muul LM, Kanno Y, Tato CM, Ramos HL, Berger AE, Mielke L, Pesu M, Solomon B, Frucht DM, Paul WE, Sher A, Jankovic D, Tsichlis PN, O'Shea JJ. 2008. Tpl2 kinase regulates T cell interferon-gamma production and host resistance to Toxoplasma gondii. *J Exp Med* 205: 2803-12
26. O'Shea JJ, Paul WE. 2002. Regulation of T(H)1 differentiation--controlling the controllers. *Nat Immunol* 3: 506-8
27. Hall JP, Kurdi Y, Hsu S, Cuozzo J, Liu J, Telliez J-B, Seidl KJ, Winkler A, Hu Y, Green N, Askew GR, Tam S, Clark JD, Lin L-L. 2007. Pharmacologic Inhibition of Tpl2 Blocks

Inflammatory Responses in Primary Human Monocytes, Synoviocytes, and Blood. *Journal of Biological Chemistry* 282: 33295-304

28. George D, Salmeron A. 2009. Cot/Tpl-2 protein kinase as a target for the treatment of inflammatory disease. *Current Topics In Medicinal Chemistry* 9: 611-22
29. Kiessling R, Klein E, Pross H, Wigzell H. 1975. „Natural” killer cells in the mouse. II. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Characteristics of the killer cell. *European Journal of Immunology* 5: 117-21
30. Sun JC, Beilke JN, Lanier LL. 2009. Adaptive immune features of natural killer cells. *Nature* 457: 557-61
31. Bekiaris V, Timoshenko O, Hou TZ, Toellner K, Shakib S, Gaspal F, McConnell FM, Parnell SM, Withers D, Buckley CD, Sweet C, Yokoyama WM, Anderson G, Lane PJL. 2008. Ly49H+ NK Cells Migrate to and Protect Splenic White Pulp Stroma from Murine Cytomegalovirus Infection. *The Journal of Immunology* 180: 6768-76
32. Claus M, Greil J, Watzl C. 2009. Comprehensive analysis of NK cell function in whole blood samples. *Journal of Immunological Methods* 341: 154-64
33. Yadav PK, Chen C, Liu Z. 2011. Potential Role of NK Cells in the Pathogenesis of Inflammatory Bowel Disease. *Journal of Biomedicine and Biotechnology* 2011
34. Voskoboinik I, Dunstone MA, Baran K, Whisstock JC, Trapani JA. 2010. Perforin: structure, function, and role in human immunopathology. *Immunological Reviews* 235: 35-54
35. Ko Y-H, Park S, Jin H, Woo H, Lee H, Park C, Kim K. 2007. Granzyme B leakage-induced apoptosis is a crucial mechanism of cell death in nasal-type NK//T-cell lymphoma. *Lab Invest* 87: 241-50
36. Chowdhury D, Lieberman J. 2008. Death by a thousand cuts: granzyme pathways of programmed cell death. *Annu Rev Immunol* 26: 389-420
37. Zwirner NW, Domaica CI. 2010. Cytokine regulation of natural killer cell effector functions. *BioFactors* 36: 274-88
38. Kaplan M. 2005. STAT4. *Immunologic Research* 31: 231-41
39. Kundig T, Schorle H, Bachmann M, Hengartner H, Zinkernagel R, Horak I. 1993. Immune responses in interleukin-2-deficient mice. *Science* 262: 1059-61
40. Andrus L, Granelli-Piperno A, Reich E. 1984. Cytotoxic T cells both produce and respond to interleukin 2. *J Exp Med* 159: 647-52
41. Henney CS, Kuribayashi K, Kern DE, Gillis S. 1981. Interleukin-2 augments natural killer cell activity. *Nature* 291: 335-8
42. Ben Aribia MH, Moiré N, Métivier D, Vaquero C, Lantz O, Olive D, Charpentier B, Senik A. 1989. IL-2 receptors on circulating natural killer cells and T lymphocytes. Similarity in number and affinity but difference in transmission of the proliferation signal. *The Journal of Immunology* 142: 490-9
43. Bacellar O, Brodskyn C, Guerreiro J, Barral-Netto M, Costa CH, Coffman RL, Johnson WD, Carvalho EM. 1996. Interleukin-12 restores interferon-gamma production and cytotoxic responses in visceral leishmaniasis. *J Infect Dis* 173: 1515-8
44. Ye J, Ortaldo JR, Conlon K, Winkler-Pickett R, Young HA. 1995. Cellular and molecular mechanisms of IFN-gamma production induced by IL-2 and IL-12 in a human NK cell line. *J Leukoc Biol* 58: 225-33

45. Orange JS, Biron CA. 1996. Characterization of early IL-12, IFN-alphabeta, and TNF effects on antiviral state and NK cell responses during murine cytomegalovirus infection. *The Journal of Immunology* 156: 4746-56
46. Athie-Morales V, Smits HH, Cantrell DA, Hilkens CMU. 2004. Sustained IL-12 Signaling Is Required for Th1 Development. *The Journal of Immunology* 172: 61-9
47. Chaix J, Tessmer MS, Hoebe K, Fuséri N, Ryffel B, Dalod M, Alexopoulou L, Beutler B, Brossay L, Vivier E, Walzer T. 2008. Cutting Edge: Priming of NK Cells by IL-18. *The Journal of Immunology* 181: 1627-31
48. Hyodo Y, Matsui K, Hayashi N, Tsutsui H, Kashiwamura S-i, Yamauchi H, Hiroishi K, Takeda K, Tagawa Y-i, Iwakura Y, Kayagaki N, Kurimoto M, Okamura H, Hada T, Yagita H, Akira S, Nakanishi2 K, Higashino K. 1999. IL-18 Up-Regulates Perforin-Mediated NK Activity Without Increasing Perforin Messenger RNA Expression by Binding to Constitutively Expressed IL-18 Receptor. *The Journal of Immunology* 162: 1662-8
49. Lauwerys BR, Renauld JC, Houssiau FA. 1999. Synergistic proliferation and activation of natural killer cells by interleukin 12 and interleukin 18. *Cytokine* 11: 822-30
50. Thirion G, Coutelier J-P. 2009. Production of protective gamma interferon by natural killer cells during early mouse hepatitis virus infection. *Journal of General Virology* 90: 442-7
51. Dunn PL, North RJ. 1991. Early gamma interferon production by natural killer cells is important in defense against murine listeriosis. *Infect Immun* 59: 2892-900
52. Rawlings JS, Rosler KM, Harrison DA. 2004. The JAK/STAT signaling pathway. *Journal of Cell Science* 117: 1281-3
53. Kobayashi SD, Voyich JM, Burlak C, DeLeo FR. 2005. Neutrophils in the innate immune response. *Arch Immunol Ther Exp (Warsz)* 53: 505-17
54. Burgdorf S, Kautz A, Böhnert V, Knolle PA, Kurts C. 2007. Distinct Pathways of Antigen Uptake and Intracellular Routing in CD4 and CD8 T Cell Activation. *Science* 316: 612-6
55. Watford WT, Moriguchi M, Morinobu A, O'Shea JJ. 2003. The biology of IL-12: coordinating innate and adaptive immune responses. *Cytokine Growth Factor Rev* 14: 361-8
56. Cao Q, Wang Y, Zheng D, Sun Y, Wang Y, Lee VWS, Zheng G, Tan TK, Ince J, Alexander SI, Harris DCH. 2010. IL-10/TGF-β–Modified Macrophages Induce Regulatory T Cells and Protect against Adriamycin Nephrosis. *Journal of the American Society of Nephrology* 21: 933-42
57. Seltrecht N, Mpofu N, Hardtke-Wolenski M, Noyan F, Manns MP, Jaeckel E. 2012. Antigen-specific regulatory T cells can induce tolerance to immunogenic grafts without the need for chronic immunosuppression. *Xenotransplantation* 19: 19-
58. Uraushihara K, Kanai T, Ko K, Totsuka T, Makita S, Iiyama R, Nakamura T, Watanabe M. 2003. Regulation of Murine Inflammatory Bowel Disease by CD25+ and CD25− CD4+ Glucocorticoid-Induced TNF Receptor Family-Related Gene+ Regulatory T Cells. *The Journal of Immunology* 171: 708-16
59. Mortier A, Van Damme J, Proost P. 2012. Overview of the mechanisms regulating chemokine activity and availability. *Immunol Lett* 145: 2-9

60. Sun J, Ramnath RD, Zhi L, Tamizhselvi R, Bhatia M. 2008. Substance P enhances NF-κB transactivation and chemokine response in murine macrophages via ERK1/2 and p38 MAPK signaling pathways. *American Journal of Physiology - Cell Physiology* 294: C1586-C96
61. Tylaska LA, Boring L, Weng W, Aiello R, Charo IF, Rollins BJ, Gladue RP. 2002. CCR2 REGULATES THE LEVEL OF MCP-1/CCL2 IN VITRO AND AT INFLAMMATORY SITES AND CONTROLS T CELL ACTIVATION IN RESPONSE TO ALLOANTIGEN. *Cytokine* 18: 184-90
62. Jia T, Serbina NV, Brandl K, Zhong MX, Leiner IM, Charo IF, Pamer EG. 2008. Additive Roles for MCP-1 and MCP-3 in CCR2-Mediated Recruitment of Inflammatory Monocytes during Listeria monocytogenes Infection. *The Journal of Immunology* 180: 6846-53
63. Tsou C-L, Peters W, Si Y, Slaymaker S, Aslanian AM, Weisberg SP, Mack M, Charo IF. 2007. Critical roles for CCR2 and MCP-3 in monocyte mobilization from bone marrow and recruitment to inflammatory sites. *J Clin Invest* 117: 902-9
64. Tanimoto N, Terasawa M, Nakamura M, Kegai D, Aoshima N, Kobayashi Y, Nagata K. 2007. Involvement of KC, MIP-2, and MCP-1 in leukocyte infiltration following injection of necrotic cells into the peritoneal cavity. *Biochem Biophys Res Commun* 361: 533-6
65. Bièche I, Chavey C, Andrieu C, Busson M, Vacher S, Le Corre L, Guinebretière J-M, Burlinchon S, Lidereau R, Lazennec G. 2007. CXC chemokines located in the 4q21 region are up-regulated in breast cancer. *Endocrine-Related Cancer* 14: 1039-52
66. Richardson RM, Pridgen BC, Haribabu B, Snyderman R. 2000. Regulation of the Human Chemokine Receptor CCR1. *Journal of Biological Chemistry* 275: 9201-8
67. Khan IA, Murphy PM, Casciotti L, Schwartzman JD, Collins J, Gao J-L, Yeaman GR. 2001. Mice Lacking the Chemokine Receptor CCR1 Show Increased Susceptibility to Toxoplasma gondii Infection. *The Journal of Immunology* 166: 1930-7
68. Amat M, Benjamim CF, Williams LM, Prats N, Terricabras E, Beleta J, Kunkel SL, Godessart N. 2006. Pharmacological blockade of CCR1 ameliorates murine arthritis and alters cytokine networks in vivo. *British Journal of Pharmacology* 149: 666-75
69. Blanpain C, Libert F, Vassart G, Parmentier M. 2002. CCR5 and HIV infection. *Receptors Channels* 8: 19-31
70. Scott Algood HM, Flynn JL. 2004. CCR5-Deficient Mice Control Mycobacterium tuberculosis Infection despite Increased Pulmonary Lymphocytic Infiltration. *The Journal of Immunology* 173: 3287-96
71. Thapa M, Kuziel WA, Carr DJ. 2007. Susceptibility of CCR5-deficient mice to genital herpes simplex virus type 2 is linked to NK cell mobilization. *J Virol* 81: 3704-13
72. Hogan S, Rothenberg M, Forbes E, Smart V, Matthaei K, Foster P. 2004. Chemokines in eosinophil-associated gastrointestinal disorders. *Current Allergy and Asthma Reports* 4: 74-82
73. Yang M, Hogan SP, Mahalingam S, Pope SM, Zimmermann N, Fulkerson P, Dent LA, Young IG, Matthaei KI, Rothenberg ME, Foster PS. 2003. Eotaxin-2 and IL-5 cooperate in the lung to regulate IL-13 production and airway eosinophilia and hyperreactivity. *J Allergy Clin Immunol* 112: 935-43

74. Yamaguchi Y, Suda T, Suda J, Eguchi M, Miura Y, Harada N, Tominaga A, Takatsu K. 1988. Purified interleukin 5 supports the terminal differentiation and proliferation of murine eosinophilic precursors. *J Exp Med* 167: 43-56
75. Reese TA, Liang H-E, Tager AM, Luster AD, Van Rooijen N, Voehringer D, Locksley RM. 2007. Chitin induces accumulation in tissue of innate immune cells associated with allergy. *Nature* 447: 92-6
76. Behm CA, Ovington KS. 2000. The Role of Eosinophils in Parasitic Helminth Infections: Insights from Genetically Modified Mice. *Parasitology Today* 16: 202-9
77. Biswas SK, Mantovani A. 2010. Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm. *Nat Immunol* 11: 889-96
78. Benoit M, Desnues B, Mege J-L. 2008. Macrophage Polarization in Bacterial Infections. *The Journal of Immunology* 181: 3733-9
79. Qidwai T, Jamal F. 2010. Inducible Nitric Oxide Synthase (iNOS) Gene Polymorphism and Disease Prevalence. *Scandinavian Journal of Immunology* 72: 375-87
80. Herbst S, Schaible UE, Schneider BE. 2011. Interferon Gamma Activated Macrophages Kill Mycobacteria by Nitric Oxide Induced Apoptosis. *PLoS ONE* 6: e19105
81. McCartney-Francis N, Allen JB, Mizel DE, Albina JE, Xie QW, Nathan CF, Wahl SM. 1993. Suppression of arthritis by an inhibitor of nitric oxide synthase. *J Exp Med* 178: 749-54
82. Pechkovsky DV, Prasse A, Kollert F, Engel KMY, Dentler J, Luttmann W, Friedrich K, Müller-Quernheim J, Zissel G. 2010. Alternatively activated alveolar macrophages in pulmonary fibrosis—mediator production and intracellular signal transduction. *Clinical Immunology* 137: 89-101
83. Nelson MP, Christmann BS, Werner JL, Metz AE, Trevor JL, Lowell CA, Steele C. 2011. IL-33 and M2a Alveolar Macrophages Promote Lung Defense against the Atypical Fungal Pathogen Pneumocystis murina. *The Journal of Immunology* 186: 2372-81
84. Edwards JP, Zhang X, Frauwirth KA, Mosser DM. 2006. Biochemical and functional characterization of three activated macrophage populations. *J Leukoc Biol* 80: 1298-307
85. Zizzo G, Hilliard BA, Monestier M, Cohen PL. 2012. Efficient Clearance of Early Apoptotic Cells by Human Macrophages Requires M2c Polarization and MerTK Induction. *The Journal of Immunology*
86. Kreider T, Anthony RM, Urban JF, Jr., Gause WC. 2007. Alternatively activated macrophages in helminth infections. *Curr Opin Immunol* 19: 448-53
87. Gong D, Shi W, Yi S-j, Chen H, Groffen J, Heisterkamp N. 2012. TGFbeta signaling plays a critical role in promoting alternative macrophage activation. *BMC Immunology* 13: 31
88. Ross AGP, Bartley PB, Sleigh AC, Olds GR, Li Y, Williams GM, McManus DP. 2002. Schistosomiasis. *New England Journal of Medicine* 346: 1212-20
89. de Jesus AR, Silva A, Santana LB, Magalhães A, de Jesus AA, de Almeida RP, Rêgo MAV, Burattini MN, Pearce EJ, Carvalho EM. 2002. Clinical and Immunologic Evaluation of 31 Patients with Acute Schistosomiasis mansoni. *Journal of Infectious Diseases* 185: 98-105

90. Chang NC, Hung SI, Hwa KY, Kato I, Chen JE, Liu CH, Chang AC. 2001. A macrophage protein, Ym1, transiently expressed during inflammation is a novel mammalian lectin. *J Biol Chem* 276: 17497-506
91. Cai Y, Kumar RK, Zhou J, Foster PS, Webb DC. 2009. Ym1/2 Promotes Th2 Cytokine Expression by Inhibiting 12/15(S)-Lipoxygenase: Identification of a Novel Pathway for Regulating Allergic Inflammation. *The Journal of Immunology* 182: 5393-9
92. Pesce JT, Ramalingam TR, Wilson MS, Mentink-Kane MM, Thompson RW, Cheever AW, Urban JF, Jr., Wynn TA. 2009. Retnla (Relmα/Fizz1) Suppresses Helminth-Induced Th2-Type Immunity. *PLoS Pathog* 5: e1000393
93. Holcomb IN, Kabakoff RC, Chan B, Baker TW, Gurney A, Henzel W, Nelson C, Lowman HB, Wright BD, Skelton NJ, Frantz GD, Tumas DB, Peale FV, Jr., Shelton DL, Hebert CC. 2000. FIZZ1, a novel cysteine-rich secreted protein associated with pulmonary inflammation, defines a new gene family. *EMBO J* 19: 4046-55
94. Yu H, Yoo PK, Aguirre CC, Tsoa RW, Kern RM, Grody WW, Cederbaum SD, Iyer RK. 2003. Widespread Expression of Arginase I in Mouse Tissues: Biochemical and Physiological Implications. *Journal of Histochemistry & Cytochemistry* 51: 1151-60
95. Pesce JT, Ramalingam TR, Mentink-Kane MM, Wilson MS, El Kasmi KC, Smith AM, Thompson RW, Cheever AW, Murray PJ, Wynn TA. 2009. Arginase-1–Expressing Macrophages Suppress Th2 Cytokine–Driven Inflammation and Fibrosis. *PLoS Pathog* 5: e1000371
96. Reichlin A, Yokoyama WM. 1998. Natural killer cell proliferation induced by anti-NK1.1 and IL-2. *Immunology And Cell Biology* 76: 143-52
97. Miyagi T, Gil MP, Wang X, Louten J, Chu W-M, Biron CA. 2007. High basal STAT4 balanced by STAT1 induction to control type 1 interferon effects in natural killer cells. *J Exp Med* 204: 2383-96
98. Mao H, Tu W, Qin G, Law HKW, Sia SF, Chan P-L, Liu Y, Lam K-T, Zheng J, Peiris M, Lau Y-L. 2009. Influenza Virus Directly Infects Human Natural Killer Cells and Induces Cell Apoptosis. *J Virol* 83: 9215-22
99. Stein-Streilein J, Bennett M, Mann D, Kumar V. 1983. Natural killer cells in mouse lung: surface phenotype, target preference, and response to local influenza virus infection. *The Journal of Immunology* 131: 2699-704
100. Mizumura K, Hashimoto S, Maruoka S, Gon Y, Kitamura N, Matsumoto K, Hayashi S, Shimizu K, Horie T. 2003. Role of mitogen-activated protein kinases in influenza virus induction of prostaglandin E2 from arachidonic acid in bronchial epithelial cells. *Clinical & Experimental Allergy* 33: 1244-51
101. Gao W, Sun W, Qu B, Cardona CJ, Powell K, Wegner M, Shi Y, Xing Z. 2012. Distinct Regulation of Host Responses by ERK and JNK MAP Kinases in Swine Macrophages Infected with Pandemic (H1N1) 2009 Influenza Virus. *PLoS ONE* 7: e30328
102. Rousseau S, Papoutsopoulou M, Symons A, Cook D, Lucocq JM, Prescott AR, O'Garra A, Ley SC, Cohen P. 2008. TPL2-mediated activation of ERK1 and ERK2 regulates the processing of pre-TNFα in LPS-stimulated macrophages. *Journal of Cell Science* 121: 149-54

103. López-Pelaéz M, Fumagalli S, Sanz C, Herrero C, Guerra S, Fernandez M, Alemany S. 2012. Cot/tpl2-MKK1/2-Erk1/2 controls mTORC1-mediated mRNA translation in Toll-like receptor–activated macrophages. *Molecular Biology of the Cell* 23: 2982-92
104. Watford WT, Wang CC, Tsatsanis C, Mielke LA, Eliopoulos AG, Daskalakis C, Charles N, Odom S, Rivera J, O'Shea J, Tsichlis PN. 2010. Ablation of tumor progression locus 2 promotes a type 2 Th cell response in Ovalbumin-immunized mice. *J Immunol* 184: 105-13
105. Julkunen I, Sareneva T, Pirhonen J, Ronni T, Melén K, Matikainen S. 2001. Molecular pathogenesis of influenza A virus infection and virus-induced regulation of cytokine gene expression. *Cytokine & Growth Factor Reviews* 12: 171-80
106. Soria-Castro I, Krzyzanowska A, Pelaéz ML, Regadera J, Ferrer G, Montoliu L, Rodríguez-Ramos R, Fernández M, Alemany S. 2010. Cot/tpl2 (MAP3K8) Mediates Myeloperoxidase Activity and Hypernociception following Peripheral Inflammation. *Journal of Biological Chemistry* 285: 33805-15
107. Bandow K, Kusuyama J, Shamoto M, Kakimoto K, Ohnishi T, Matsuguchi T. 2012. LPS-induced chemokine expression in both MyD88-dependent and -independent manners is regulated by Cot/Tpl2-ERK axis in macrophages. *FEBS Letters* 586: 1540-6
108. Dairaghi DJ, Oyajobi BO, Gupta A, McCluskey B, Miao S, Powers JP, Seitz LC, Wang Y, Zeng Y, Zhang P, Schall TJ, Jaen JC. 2012. CCR1 blockade reduces tumor burden and osteolysis in vivo in a mouse model of myeloma bone disease. *Blood*
109. López-Peláez M, Soria-Castro I, Boscá L, Fernández M, Alemany S. 2011. Cot/tpl2 activity is required for TLR-induced activation of the Akt p70 S6k pathway in macrophages: Implications for NO synthase 2 expression. *European Journal of Immunology* 41: 1733-41
110. Upadhyaya B, Yin Y, Hill BJ, Douek DC, Prussin C. 2011. Hierarchical IL-5 Expression Defines a Subpopulation of Highly Differentiated Human Th2 Cells. *The Journal of Immunology* 187: 3111-20
111. Sinha P, Clements VK, Ostrand-Rosenberg S. 2005. Interleukin-13–regulated M2 Macrophages in Combination with Myeloid Suppressor Cells Block Immune Surveillance against Metastasis. *Cancer Res* 65: 11743-51
112. Cao Q, Wang C, Zheng D, Wang Y, Lee VWS, Wang YM, Zheng G, Tan TK, Yu D, Alexander SI, Harris DCH, Wang Y. 2011. IL-25 Induces M2 Macrophages and Reduces Renal Injury in Proteinuric Kidney Disease. *Journal of the American Society of Nephrology* 22: 1229-39