**ROLE OF THE SERINE-THREONINE KINASE TPL2 IN TH17 RESPONSES IN INFECTIOUS AND AUTOIMMUNE DISEASES**

**by**

**NICOLE V. ACUFF**

**(Under the Direction of Wendy T. Watford)**

## ABSTRACT

Tpl2 is a serine threonine kinase that regulates inflammatory pathways of the immune system. Small molecule inhibitors of Tpl2 are being developed as possible therapeutics for autoimmune diseases in which Th1 and Th17 cells contribute to pathology. A more complete understanding of how Tpl2 regulates Th17 responses during autoimmune and infectious diseases may provide valuable information about the potential benefits versus risks associated with Tpl2 inhibition. We show that Tpl2 promotes Th17 differentiation and effector functions at several stages. *In vitro*, IL-17A expression by *Tpl2*$^{-/-}$ CD4 T cells was dependent upon TGF-β and inhibited by FoxP3 expression. In a T cell transfer model of colitis, *Tpl2*$^{-/-}$ CD4 T cells were capable of differentiating into Th17 cells expressing IL-17A, but were impaired in Th1 differentiation and IFNγ production. *Tpl2*$^{-/-}$ CD4 T cells also showed enhanced lymphopenia-induced proliferation, which compensated for reduced IFNγ in driving similar overall pathology scores. A Tpl2-dependent role for CD4 T cell differentiation into Th1 and Th17 cells expressing

IFNγ and IL-17A was observed in *Tpl2*$^{-/-}$ mice infected with *Citrobacter rodentium*, although adoptive transfer of CD4 T cells into *Rag1*$^{-/-}$ mice prior to infection confirmed that the T cell defect was not responsible for increased bacterial burdens observed in *Tpl2*$^{-/-}$ mice. Despite enhanced bacterial burdens, *Tpl2*$^{-/-}$ mice experienced reduced pathology and inflammatory neutrophil infiltrate into the intestines during *C. rodentium* infection. Neutrophil egress from the bone marrow during homeostasis and inflammation-induced neutrophil recruitment from the blood to inflammatory sites was reduced in *Tpl2*$^{-/-}$ mice due to neutrophil-extrinsic defects in inflammatory chemokine and cytokine production. Furthermore, *Tpl2*$^{-/-}$ neutrophils showed impaired antimicrobial functions, including cytokine secretion and superoxide production, compared to wild type neutrophils. Consequently, *Tpl2*$^{-/-}$ and *Rag1*$^{-/-}$*Tpl2*$^{-/-}$ mice developed greater bacterial burdens and disseminated infection to the Gram-negative and Gram-positive extracellular bacteria, *Citrobacter rodentium* and *Staphylococcus xylosus*. Overall, the role for Tpl2 in driving an inflammatory response was model-specific, confirming a cell-type and stimulus-specific role for Tpl2. These data suggest that Tpl2 inhibitors would be beneficial in preventing inflammatory cell infiltrates and immune pathology during autoimmunity but caution that chronic administration may predispose patients to extracellular bacterial infections.

INDEX WORDS: Inflammation, CD4 T cell, Th17 cell, Neutrophil, Inflammatory bowel disease, *Citrobacter rodentium*, *Staphylococcus xylosus*

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# CHAPTER 1

# INTRODUCTION AND LITERATURE REVIEW

Innate and adaptive immunity are the two major components of the host immune response against invading pathogens. The innate immune system recognizes highly conserved molecules called pathogen associated molecular patterns (PAMPs) or damage associated molecular patterns (DAMPs) via pattern recognition receptors (PRRs). In innate immunity, PRRs recognize DAMPs and PAMPs with broad specificity, whereas the adaptive immune system recognizes a wide range of antigens via somatically rearranged antigen receptors with exquisitely narrow specificities. The adaptive immune response is initiated by antigen presentation on self-restricted major histocompatibility complex (MHC) molecules displayed on innate antigen presenting cells (APCs). APCs present antigen to T cells on either MHC I or MHC II molecules on their cell surface, with most cell types expressing MHC I whereas expression of MHC II is restricted to APCs. In addition to antigen presentation in the context of MHC molecules, T cell activation requires expression of costimulatory molecules on APCs and is also influenced by cytokines. MHC class I presentation activates $CD8^+$ cytotoxic T cells (CTLs) that are able to lyse infected cells. Antigen presentation on MHC II induces differentiation of $CD4^+$ T cells into a variety of effector lineages, including T helper 1 (Th1), Th2, Th17, and regulatory T (Treg) cells.

T helper lineages are induced by different environmental stimuli, and each has specific effector functions tailored to eradication of different classes of pathogens (reviewed in (1)) (Figure 1.1). Th1 cell differentiation occurs in the presence of IL-12 and IFNγ and leads to the secretion of IFNγ and TNF. These cells are important in clearance of microorganisms that replicate within

host cells, including intracellular bacteria, parasites and viruses, and their dysregulation has been associated with the development of autoimmune diseases. Th17 cell differentiation occurs in the presence of IL-6, IL-23, and transforming growth factor beta (TGF-β), and the secretion of IL-17A by these cells assists in the clearance of extracellular bacteria and fungi. Like Th1 cells, dysregulation of Th17 cells is associated with the development of autoimmune diseases (reviewed in (2)). Th2 cells differentiate in the presence of IL-4 and secrete the cytokines IL-4, IL-5, and IL-13. These cytokines are associated with humoral immunity, development of allergy response, and clearance of helminths. Regulatory T cells are induced in the presence of IL-2 and TGF-β and reduce inflammation produced by other T helper subtypes through production of anti-inflammatory cytokines TGF-β and IL-10 as well as through contact dependent mechanisms. Despite this apparent specialization of immune responses, helper T cells are plastic and can convert between the different subsets depending upon environmental cues (3-5). The focus of this introduction will examine the role of Tpl2 in Th17 cell differentiation and effector functions.

**TUMOR PROGRESSION LOCUS 2 (TPL2)**

Tumor progression locus 2, Tpl2 (also known as MAP3K8 and Cot), is a serine-threonine protein kinase originally described as an oncogene because its C-terminal truncation promoted tumor growth in rodents (6, 7). The C-terminal truncation in Tpl2 was shown to increase its half-life and kinase specific activity, which endowed it with oncogenic properties (8). However, Tpl2 has been shown to have both tumor suppressing and pro-oncogenic functions (reviewed in (9)).

Tpl2 is found in diverse tissues including the spleen, thymus, liver, lung, and adipose (6, 10-12). Tpl2 is expressed as two distinct isoforms of 52 and 58 kDa in both innate and adaptive immune cells (13). In macrophages, Tpl2 is constitutively expressed and held in an inactive state by the NF-κB subunit p105 (14, 15); however, a majority of NF-κB p105 subunits are not associated

with Tpl2. Direct interaction of the death domain of p105 with the kinase domain of Tpl2 prevents the MEK kinase activity of Tpl2 (16, 17). A20-binding inhibitor of NF-κB 2 (ABIN-2) associates with both Tpl2 and NF-κB p105 to form a stable ternary complex (18-21). In addition to p105 and ABIN2, the p38 MAPK family members p38γ and p38δ indirectly regulate Tpl2 stability by regulating steady state levels of ABIN-2 (22). Tpl2 can be activated by toll-like receptors (23-25), cytokines (10, 26), antigen receptors (27, 28), G protein-coupled receptors (29) or FcγR signals (30). Upon activation and phosphorylation at Thr290 and Ser400 (31-35), Tpl2 dissociates from p105 and ABIN-2, and is subsequently degraded by the proteosome (14, 15). Thr290 is believed to be autophosphorylated, whereas Akt (36) and IκB kinase 2 directly phosphorylate Ser400 to activate Tpl2 (31, 35, 37). Many of Tpl2's downstream functions have been attributed to its activation of the MEK/ERK pathway (reviewed in (11)); however, Tpl2 can activate additional pathways in a cell type- and stimulus-specific manner (10, 38-40).

Tpl2 promotes inflammation induced by cytokines (24, 25, 28, 41), chemokines (42, 43) and other inflammatory mediators (44). Initial characterization of Tpl2-deficient mice identified major defects in the induction of proinflammatory cytokines, particularly TNFα, by antigen presenting cells that conveyed resistance to endotoxin-induced shock in Tpl2-deficient mice (24). In macrophages, Tpl2 is required for the secretion of TNF through the ERK-dependent nuclear-cytoplasmic transport of TNF mRNA (24) as well as the ERK-dependent maturation of the TNF protein by TNFα converting enzyme (TACE) (45). However, TNF production by BMDCs is only partially dependent on Tpl2, indicating that Tpl2 regulates TNF production in a cell type-specific manner (25). Additionally, Tpl2 promotes production of IL-1β and IL-10 but inhibits secretion of IFNβ in both macrophages and dendritic cells (25, 46). Therefore Tpl2 has both pro-inflammatory and anti-inflammatory functions in innate cells.

In addition to its regulation of innate cell responses, Tpl2 also influences the differentiation of CD4 helper T cell lineages. We have previously shown that Tpl2 is required for *in vitro* and *in vivo* Th1 differentiation and IFNγ production from $CD4^+$ T cells in a cell intrinsic manner (28). Therefore, *Tpl2*$^{-/-}$ mice experience greater susceptibility and infectious burden in response to the protozoan parasite, *Toxoplasma gondii* (28), or the intracellular bacteria, *Listeria monocytogenes* (25) and *Mycobacterium tuberculosis* (47). Additionally, *Tpl2*$^{-/-}$ mice also experience greater susceptibility to group B streptococcal diseases and influenza infection, due to defects in other arms of the immune response (48, 49). In contrast to the above studies, experiments using an independently generated *Tpl2*$^{-/-}$ mice strain showed an enhanced Th1 response and IL-12 production following infection with *Leishmania major* (50). Conflicting roles for Tpl2 in Th1 cell differentiation and response to infection could be due to (a.) differences in the knockout mouse strains or (b.) differences in host responses elicited against the two parasites. In contrast to Th1 cell differentiation, Tpl2 inhibits T helper 2 (Th2) cell responses during OVA-induced allergic asthma in mice, with *Tpl2*$^{-/-}$ mice experiencing enhanced lung inflammation and expressing elevated levels of IL-4 and IL-5 (51).

Despite these T helper cell defects, there is little information regarding the regulation of Th17 differentiation by Tpl2. A recent study demonstrated that Tpl2 ablation protects mice during experimental autoimmune encephalomyelitis (EAE) due to Tpl2 functions in CNS-resident microglia and astrocytes (52). In addition, Kakimoto *et al.* demonstrated that IL-23p19, a component of the heterodimeric Th17-inducing cytokine, IL-23, requires Tpl2 for its transcription by macrophages in response to LPS (53). Therefore, we hypothesize that Tpl2 will be important in driving Th17 differentiation and inflammation in models of Th17-associated colitis in which IL-23 and IL-23R have been clearly implicated (54, 55). Understanding how

Tpl2 regulates Th17 cell differentiation may provide valuable information about the range of potential benefits of Tpl2 inhibition in various disease settings.

**TH17 CELLS**

Th17 cells were first described in 2005 as a distinct lineage of $CD4^+$ T cells that produce IL-17A (56, 57). However, γδ T cells (58), type 3 innate lymphoid cells (ILC3) (59), lymphoid tissue inducer-like cells (LTi) (60), neutrophils (61, 62), and CD8 T cells (63, 64) are also capable of IL-17A production. Th17 differentiation occurs in the presence of at least two cytokines (65). Activation of dendritic cells and other innate immune cells, such as during infection with extracellular bacterial pathogens or fungi, induces their production and secretion of IL-6, IL-1, and IL-23 (a heterodimer composed of IL-23p19 and IL-12p40 (66)). Traditionally, Th17 cells have been induced *in vitro* using the cytokines IL-6 and TGF-β (67-69). These cytokines, combined with presentation of antigen peptides on MHCII, activate CD4 T cells and polarize them towards a Th17 phenotype. Upon differentiation, Th17 cells secrete a variety of effector cytokines including IL-17A, IL-17F, IL-21, and IL-22 (70-74). One of the main downstream effects of Th17 cytokine production is the induction of inflammation through neutrophil recruitment (74, 75) and clearance of extracellular bacterial and fungal infections: including *Klebsiella pneumoniae*, *Citrobacter rodentium*, *Staphylococcus aureus*, and *Candida albicans* (reviewed in (76)). However dysregulated Th17 responses are also implicated in the development of autoimmune diseases: including rheumatoid arthritis, multiple sclerosis, and colitis (reviewed in (77)).

Th17 cell differentiation from a naïve CD4 T cell begins with IL-6 activation of signal transducer and activator of transcription 3 (STAT3) and expression of IL-21 (78-80). IL-21 then functions in an autocrine fashion to further polarize Th17 cells (65). Together IL-6 and IL-21

activate STAT3, which induces expression of IL-23 receptor (IL-23R) on the surface of the cell, whose expression is further enhanced by IL-23 signaling (79, 81, 82) and allows for enhanced polarization of Th17 cells. IL-1 and IL-23 can act in combination on γδT cells to induce production of IL-17A and IL-21, which assist in further polarization of Th17 cells (58). The primary Th17 transcription factors of Th17 cells are RAR-related orphan nuclear receptor gamma t (RORγt) and RORα (83, 84). Inhibition of RORγt and RORα directly prevents Th17 differentiation and IL-17A production (85). Th17 cell differentiation also utilizes the transcription factors basic leucine zipper transcription factor, ATF-like (BATF) (86), c-MAF (87), interferon regulatory factor 4 (IRF4) (88) and aryl hydrocarbon receptor (AhR) (89). In addition to promoting Th17 differentiation, IL-6 and IL-21 also induce the transcription of suppressor of cytokine signaling 3 (SOCS3), which negatively regulates Th17 differentiation through inhibition of STAT3 (68) in a negative feedback loop.

Several groups have investigated the requirement for TGF-β in Th17 differentiation. TGF-β is expressed ubiquitously and is present at functionally significant concentrations in various tissues and systemically in the blood. Mean TGF-β plasma concentrations are approximately 4 ng/ml but vary by individual (90). Ubiquitous expression of TGF-β suggests that Th17 cells that differentiate *in vivo* are likely to be influenced by local TGF-β production. Mice with impaired in TGF-β responsiveness, TGF-β secretion, or FoxP3 expression develop patent autoimmunity characterized by lymphoproliferation, cellular activation and pro-inflammatory cytokine secretion (91-95). Because of their similar utilization of TGF-β, Th17 cells and regulatory T cells (Tregs) are intimately related, and both cell types can express the TGF-β-induced transcription factor FoxP3 and secrete IL-10 (96-99). TGF-β significantly enhances Th17 differentiation at low concentrations (100) through the inhibition of the negative regulator SOCS3 (68).

Importantly, Littman *et al.* demonstrated that TGF-β, while required for optimal Th17 differentiation *in vitro*, could also suppress Th17 differentiation at high concentrations. This was due to excessive activation of FoxP3, which they showed interacts directly with RORγt to antagonize IL-17A transcription (100). Alternatively, elevated concentrations of IL-6 can inhibit T cell differentiation in $FoxP3^+$ Tregs (101). Therefore, the relative expression of FoxP3 helps to direct T cells towards a Treg versus Th17 fate.

We have previously demonstrated that Th17 cells can differentiate *in vivo* and *in vitro* in the absence of TGF-β (82). In the absence of TGF-β, IL-6 and IL-23 induce and expand Th17 cells (70, 82, 102, 103), and consequently, IL-23 is associated with several autoimmune disorders (70, 104, 105). IL-23 amplifies the Th17 response by inducing the secretion of IFNγ and GM-CSF by Th17 cells, which further enhances IL-23 production by APCs, Th17 polarization, and pathogenicity (70, 99, 106, 107). Importantly, Th17 cells induced in the absence of TGF-β are more pathogenic and inflammatory *in vivo* than those driven by IL-6 and TGF-β. This is due to the fact that Th17 cells driven by IL-6 and TGF-β also co-express the anti-inflammatory cytokine IL-10, but not IL-21 or IL-22, under the control of c-MAF (67, 87, 108, 109). In contrast, Th17 cells induced by IL-6 and IL-23 co-express IL-22 and IL-17, but not IL-10 (110, 111). IL-10 is an anti-inflammatory cytokine belonging to the IL-10 family of cytokines, including the Th17 effector cytokine IL-22 (112). Both innate and adaptive immune cells secrete IL-22: including CD4 T cells, innate lymphoid cells (ILCs) and NK cells (60, 110, 113). IL-22 signals through IL-22R (114, 115) and has been shown to have both pro-inflammatory and anti-inflammatory functions. Within the mucosal epithelium, IL-22 has a variety of functions, including goblet cell activation and mucus production, epithelial cell proliferation, and epithelial cell production of antimicrobial peptides and IL-18 (73, 116-119). The importance of these functions will be

discussed later in more detail.

IL-17A and IL-17F signal through a heterodimeric receptor composed of IL-17RA and IL-17RC (74, 120), and IL-22 signals through a heterodimeric receptor composed of IL-22R and IL-10Rβ (121) (Figure 1.2). The IL-17 receptor proximal signaling complex is made up of IL-17R, Act1, and tumor necrosis factor receptor (TNFR)-associated factor 6 (TRAF6) (reviewed in (122)). The IL-17A signaling cascade is similar to MyD88-dependent TLR signaling, both of which utilize TRAF6 and TGF-β activated kinase 1 (TAK1) (123). Complex formation is inhibited by TRAF3, which binds directly to IL-17R, or TRAF4, which competitively binds Act1 (124, 125). Act1, an E3 ubiquitin ligase, directly interacts with the cytoplasmic tail of the IL-17R and recruits TAK1 and TRAF6 to the signaling complex (126-128). Downstream of the signaling complex, IL-17 signals through NF-κB, MAPKs, and C/EBPs allowing for expression of inflammatory cytokines, chemokines, and matrix metallopreoteinases (MMPs) including KC, GM-CSF, and IL-6 (reviewed in (129, 130)). Alternatively, IL-22 signals through MAPKs as well as JAK/STAT pathways (121). Signaling can be inhibited by TRAF6-dependent phosphorylation of Act1 (131) or through activation of C/EBPs by IL-17, which induces dual phosphorylation of C/EBP and inhibits expression of proinflammatory genes (132). Additionally, Act1 ubiquitinates TRAF6, which then autoubiquitinates, resulting in proteosomal degradation (128). It was recently shown that Tpl2 is phosphorylated by IL-17A in a mouse skin epidermal cell line, which leads to the activation of MEK/ERK, JNK/c-Jun and AP-1-induced cellular transformation (133).

**NEUTROPHILS**

Neutrophils, also known as polymorphonuclear granulocytes, are abundantly recruited during Th17-associated immune responses and are critical for the eradication of extracellular pathogens

(74, 75). Neutrophils are one of the most abundant leukocytes within mouse peripheral blood, following lymphocytes. Their development from progenitors proceeds through several stages within the bone marrow before they are released into the circulation (134). Neutrophils are derived from hematopoietic stem cells (HSC) that differentiate into granulocyte/monocyte progenitors (GMP). From this stage, GMP can then terminally differentiate into neutrophils, macrophages or dendritic cells. GMP differentiate into neutrophils in the presence of G-CSF (reviewed in (135)). The cytoplasm of neutrophils contains different characteristic primary (azurophilic) and secondary (specific) granules depending on their stage of development. Examples of granules include myeloperoxidase (MPO), elastase, lactoferrin, lysozyme, collagenase, and defensins (136, 137). As neutrophils develop, their ability to release granules increases (138). Mature neutrophils contain a segmented nucleus with three to five lobules as well as gelatinase granules within their cytoplasm (139).

Mature neutrophils with segmented nuclei egress from the bone marrow into the blood where they can be recruited to sites of inflammation. The presence of immature neutrophils within the circulation indicates an ongoing infection within the host. G-CSF is thought to positively regulate neutrophil mobilization from the bone marrow into the circulation (140), whereas CXCR4 expression on the surface of neutrophils is thought to negatively regulate neutrophil egress from the bone marrow into the circulation (141, 142). Once in circulation, neutrophils are short-lived, surviving only about 6 hours, which necessitates a constant turn-over within the bone marrow. Upregulation of CXCR4 on circulating neutrophils regulates their ability to home back to the bone marrow for clearance (143).

Neutrophils are the first cell type recruited to sites of inflammation from the circulation. Innate production of TNF and IL-1 induces migration of neutrophils. Human neutrophils are recruited

to sites of inflammation through upregulation of CXCR1 and CXCR2, which primarily bind CXCL8 (IL-8) (144). In mice, which do not produce IL-8, neutrophils upregulate the chemokine receptors CXCR2 and CXCR4 that bind CXCL1 (KC), CXCL2, CXCL15, and CXCL12 (142, 145-147). Once recruited to the site of inflammation, L-selectin expressed on the surface of neutrophils binds P-selectin and E-selectins on endothelial surfaces (148, 149). CD44 expression on the surface of neutrophils is also capable of functioning as an E-selectin within the circulation (150). Strong adhesion to endothelial cells occurs when expression of integrins, such as LFA-1 and Mac-1 (CD11b/CD18), on neutrophils are upregulated while expression of L-selectin decreases (147, 151). LFA-1 and Mac-1 bind ICAM-1 expressed on the endothelial cell surface and then neutrophils migrate between cells of the endothelium and into the tissue.

The main goal of neutrophil antimicrobial functions is to promote an inflammatory environment and kill microbes. Neutrophil-mediated killing is a multistep process (reviewed in (152)) that includes cytokine secretion, phagocytosis, degranulation, respiratory burst and release of neutrophil extracellular traps (NETs). Tpl2 has already been shown to influence macrophage phagocytosis through FcγR (30) and likely promotes FcγR-mediated uptake by neutrophils as well. After phagocytosis, neutrophils produce reactive oxygen species (ROS), reactive nitrogen species (RNS), and antibacterial proteins including defensins, lactoferrin, lysozyme and neutrophil elastase (reviewed in (152, 153)). Superoxide production is a major component of neutrophil antimicrobial functions. Superoxide is generated through activation and formation of the NADPH oxidase complex, which is composed of five subunits: two membrane bound subunits ($p22^{phox}$ and $gp91^{phox}$) that form cytochrome b558 and three cytoplasmic subunits ($p40^{phox}$, $p47^{phox}$ and $p67^{phox}$) (154). Upon activation, the three cytoplasmic subunits are recruited to the cell membrane, generating the active NADPH oxidase and allowing for production of

superoxide (154). Phosphorylation of the p47 subunit is required for complex formation and can be induced by TNF, GM-CSF, fMLP, and PMA among others (155-158). Similar to Th17 cells, neutrophils are also capable of secreting IL-17A and IL-22 (61, 62, 159), of which IL-17A can signal through IL-17RC and activate neutrophils in an autocrine manner leading to superoxide production (62). Mice deficient in superoxide formation, such as p47-deficient mice, have increased susceptibility to infection and are commonly used as a model for chronic granulomatous disease (CGD) (160-163). Patients, especially children, with CGD experience recurrent fungal and bacterial infections not normally present in a healthy individual including *Staphylococcus aureus, Escherichia coli, Pseudomonas,* and *Candida* due to defects in phagocyte superoxide production (164). Neutrophils are also capable of producing neutrophil extracellular traps (NETs), through a process termed NETosis, which traps and kills extracellular bacteria and fungi (165-170). Despite several mechanisms of microbial clearance, some bacteria and fungi, such as *Staphylococcus aureus* and *Candida albicans,* have various mechanisms to avoid clearance by neutrophils (171-173). In addition, over-activation of neutrophils can lead to damage of normal tissue through release of ROS, RNS, and enzymes into the environment.

**INTESTINAL IMMUNITY**

The digestive tract is composed of the large and small intestine. The small intestine is further divided into the duodenum, jejunum and ileum, and the large intestine is divided into the cecum, colon and rectum. In the steady state, there are Th17 cells within the small intestine that contribute to maintenance of homeostasis and are antigen specific (174). These cells require colonization of the gut with segmented filamentous bacteria (175) and retinoic acid (176). When the intestinal environment changes, inflammation can occur. Inflammation of the colon is known as colitis and is primarily caused by inflammatory bowel disease (IBD) or infection. As has been

mentioned previously, IL-6 and IL-21 upregulate expression of IL-23R on the cell surface (81, 82) which allows for polarization by IL-23 towards a pathogenic Th17 phenotype (70). In 2006 it was shown in a genome-wide association study that single nucleotide polymorphisms in the human IL-23R gene was associated with the development of IBD and firmly established IL-23 as a major contributor of intestinal inflammation (54, 55).

The intestinal barrier is a single layer of columnar epithelial cells on a basement membrane held together by proteins forming tight junctions, such as claudins (177). Within the intestines of mice, claudins 2, 3, 7 and 15 are the most highly expressed whereas other claudins are more regionally expressed (reviewed in (178)). Claudins 2 and 5 require signaling through MEK/ERK for protein expression on epithelial cell lines (177, 179), and gene expression of claudins 1 and 2 can be upregulated by IL-17A stimulation (177). Protein expression of claudin 7 on epithelial cells alternatively requires signaling through p38, and not MEK or JNK (180). Additionally, lymphocytes are required to maintain the intestinal barrier by promoting epithelial cell differentiation (181). Therefore, *Rag*$^{-/-}$, CD4-deficient or B-cell deficient (μMT) mice have greater intestinal permeability, which can be rescued by adoptive transfer of lymphocytes (181, 182).

Antigens are primarily delivered to immune cells from the lumen through phagocytosis by M cells along the epithelium. M cells are capable of transporting bacteria, viruses and microbial products out of the lumen; however M cells do not present antigen to T cells (183). Immune cells can also sample antigens in the lumen. For example, intestinal dendritic cells extend dendrites between adjacent cells of the intestinal epithelium to directly sample antigen (184). Dendritic cells are capable of sampling the lumen without impairing the integrity of the intestinal barrier through expression of occludin, claudin 1 and zonula occludens 1, which allows for formation of

tight junctions with epithelial cells (184). Another function of the epithelium is mucus production, which is accomplished by a specific cell type known as a goblet cell. Goblet cells produce mucins, which are glycoproteins that form a viscous mucus when secreted and combined with extracellular secretions. Mucus forms a barrier between the extracellular space of the lumen and the intestine. Cytokines IL-1, IL-4, IL-6, IL-13, TNF, and neutrophil products can induce production of mucus by goblet cells (185, 186). Epithelial cells also produce defensins, antimicrobial molecules that form pores in microbial cell membranes (reviewed in (187)). Neutrophils, NK cells, and CD8 T cells can also produce defensins. Paneth cells within crypts of the small intestine produce primarily α-defensins, also known as crypticidins, whereas cells within the large intestine produce β-defensins, which can be either constitutively activated or induced by IL-1 or specific bacteria (188).

Below M cells are Peyer's patches, which are areas rich in lymphocytes and innate immune cells (189), and below the epithelial barrier is the lamina propria containing lymphocytes, dendritic cells, macrophages and mast cells. Together these cells make up the gut-associated lymphoid tissue (GALT) (Figure 1.3). Within the lamina propria, dendritic cells and macrophages produce the anti-inflammatory cytokine IL-10 and are less sensitive to TLR4 ligands (190, 191), which contributes to tolerance and immune homeostasis. The human intestinal immune system is composed of approximately $5x10^{10}$ lymphocytes or 10.8% of the body's total lymphocytes, with more than half found within the lamina propria (reviewed in (192)). A majority of the lymphocytes within the lamina propria are CD4 T cells versus CD8 T cells (193). During inflammation and infection of the intestine, the primary adaptive immune responses are by Th17 and B cells; however, there are basal amounts of Th17 cells and IgA within the intestine during steady state (174, 175, 194-197). During acute infection, such as with influenza or sepsis,

additional Th17 cells migrate to the small intestine, with some migrating to the large intestine (198). Th17-associated cytokines IL-17A and IL-22 assist in maintenance of the epithelial barrier by inducing production of defensins (73). Once within the small intestine, inflammatory Th17 cells are controlled through either elimination via translocation into the lumen or are converted into IL-10 expressing Th17 cells (198).

**MODELS OF COLITIS**

Patients with unregulated inflammation of the intestine, or IBD, experience symptoms such as abdominal pain, vomiting, diarrhea, cramps and weight loss. The two main forms of IBD include Crohn's disease (CD) and ulcerative colitis (UC) (reviewed in (199, 200)). During inflamed states of disease, patients with CD or UC have increased proportions of IL-17A and IFNγ within their lamina propria (201), but CD4 T cells within the lamina propria of patients with UC are capable of secreting more IL-17A upon stimulation with IL-23 compared to patients with CD (202). Crohn's disease causes patchy areas of lesions in both the large and small intestines. Associated inflammation goes deep into the intestinal barrier. Stenosis of the bowels can occur and lead to blockage of the intestinal tract. Additionally, patients with CD show reduced ability to produce defensins within their intestines. Complications of CD include nutrient deficiencies and formation of ulcers, which can turn into fistulas. Unlike CD, ulcerative colitis primarily affects the large intestine. Associated inflammation is continuous throughout the colon but remains in the mucosal regions of the intestines. The primary complication of UC is colon cancer. Treatment of IBD is non-specific. Patients are treated with anti-inflammatory and immunosuppressive medications as well as antibiotics. However, approximately 25-75% of patients require surgery, with some patients requiring multiple surgeries.

Several models exist for studying IBD in mice, but each model has limitations in replicating

human disease (203-205). These models include certain knockout or transgenic mouse strains that spontaneously develop colitis, such as $TNF^{\Delta ARE}$ or IL-$10^{-/-}$ mice (206-211), a T cell transfer model of colitis (212), chemically induced colitis with dextran sulfate sodium (DSS) (213-216), and infection induced colitis with *Citrobacter rodentium* (217, 218). $TNF^{\Delta ARE}$ mice have a deletion in the AU-rich element (ARE) within the 3' untranslated region (UTR) of the TNF mRNA that inhibits its posttranscriptional regulation. Increase stability of TNF mRNA leads to TNF accumulation in the mouse and spontaneous development of IBD, which fully develops by 8-10 weeks of age. Tpl2-deficient mice crossed with $TNF^{\Delta ARE}$ mice showed delayed onset and attenuated progression of IBD, indicating the importance of Tpl2 in TNF signaling (219). Alternatively, in one chemically induced model of colitis, DSS is administered to mice in drinking water. DSS damages intestinal epithelial cells and therefore alters barrier function of the intestines leading to hematochezia, body weight loss, shortening of the intestine, mucosal ulcers, and infiltration of neutrophils (213-216). In this model, Tpl2-deficient mice experienced mild colitis with reduced production of inflammatory cytokines IL-1α, IL-1β, IL-6, and IL-17 as well as reduced production of the anti-inflammatory cytokine IL-10 (220). However, neither of these models directly assessed the affects of Tpl2 on CD4 T cells during development of colitis. Since Tpl2 is important in regulation of helper T cell differentiation, we hypothesize that it will influence the development of colitis. We therefore utilized the T cell transfer model and *C. rodentium* model of colitis to examine the role of Tpl2 in Th1/17 cell mediated inflammation and clearance of infection.

**T CELL TRANSFER MODEL OF COLITIS (CD45RB)**

CD45RB is a 220 kDa glycoprotein expressed on the surface of several immune cells, including B cells, naïve T cells, thymocytes, macrophages, and dendritic cells. Within the peripheral blood

of patients suffering from UC or CD, CD4 T cells express elevated levels of CD45RB (221). Upon stimulation, CD45RBhi cells secrete elevated concentrations of TNF and reduced concentrations of IL-10 and IL-4 (221), classifying these cells as proinflammatory. The CD45RB model of colitis leads to inflammation of both the large and small intestines (222) and is therefore an effective model for studying the effect of T cells on the development of IBD. Another benefit of using the CD45RB model versus a chemically-induced model of colitis is that it most closely resembles the alterations in transcriptional activity of the intestine during IBD in humans (223).

In the T cell transfer model of colitis, $CD4^+CD25^-CD45RB^{hi}$ cells are adoptively transferred into *Rag*$^{-/-}$ mice. *Rag*$^{-/-}$ mice are unable to undergo V(D)J recombination and therefore do not have mature T cells or B cells (224, 225). When CD4 T cells are transferred into a lymphopenic environment, homeostatic proliferation of the transferred CD4 T cells occurs (226, 227). Because $CD25^+$ regulatory T cells are excluded from the adoptive transfer, *Rag*$^{-/-}$ mice do not develop peripheral tolerance characterized by $FoxP3^+$ Tregs (228). Consequently, increasing numbers of CD4 T cells within the Rag-deficient mouse respond to intestinal antigens associated with the commensal flora and drive an unchecked inflammatory response within the small and large intestine leading to measurable weight loss and diarrhea (205, 212, 222). TNF, IFNγ, IL-17A, IL-22, and IL-23 are known to be important in driving inflammation in this model (229-233), whereas IL-10 plays a more protective role (234, 235). Therefore, treatment with neutralizing IFNγ or TNF antibodies or with IL-10 can reduce or delay onset of colitis (231). Interestingly, pretreatment of *Rag*$^{-/-}$ mice with IL-4 and IL-13 or alternatively activated macrophages boosts Th17 and Treg cell differentiation within the host (236), but addition of $CD4^+CD25^+$ Tregs can restore the epithelial barrier and reduce inflammatory cell infiltrates seen during colitis (237).

## CITROBACTER RODENTIUM

*Citrobacter rodentium* is a non-motile gram-negative rod belonging to the family *Enterobacteriaceae*. It was previously known as *Citrobacter freundii* biotype 4280 or *Citrobacter* genomespecies 9. *C. rodentium* is a natural mouse and gerbil pathogen (238, 239) but is unable to colonize the intestine and cause colonic hyperplasia in hamsters or rats (240). *C. rodentium* is the causative agent of transmissible murine colonic hyperplasia (TMCH) (241, 242). TMCH is characterized by severe mucosal hyperplasia at 2-3 weeks post infection followed by regression of hyperplasia and clearance of bacteria (243, 244). Severity of colonic hyperplasia varies with the mouse strain used (240, 245), TLR activation (246-250), and NF-κB activity (251, 252). Symptoms of infection include slowed growth, ruffled fur, soft feces, rectal prolapse, and mortality (218, 243). However, symptoms are more easily detected in young mice and can be easily overlooked in adult mice. Similar to enteropathogenic *Escherichia coli* (EPEC), *C. rodentium* produces attaching and effacing lesions in the large intestine (253, 254) and is commonly used as a murine model for human EPEC and EHEC (241, 255).

Disease is transmitted via the fecal-oral route. The mode of transmission allows for easy testing of Koch's postulates. This can be seen in early experiments where germ-free mice were treated with unfiltered colonic suspensions from infected mice, and *C. rodentium* could be isolated from the newly infected mice (244). Before the bacterium was identified, *C. rodentium* was simply maintained by mouse-to-mouse passages of colonic suspensions (244). Mice can be clinically induced through either oral gavage or 'naturally' by gavage of a single mouse in a cage and allowing time for coprophagy by cagemates. It has been shown that 'natural infection' leads to a hyperinfectious state whereby a lower dose is sufficient for infection of cagemates, and the bacterium no longer requires primary colonization of the cecum (256, 257). Upon infection,

*Citrobacter rodentium* colonizes the large intestine, primarily the cecum and distal portion of the colon (257). During the first week of infection, *C. rodentium* overwhelms all other aerobic and anaerobic microflora and becomes the primary bacterium found within the large intestine (240). *C. rodentium* can also disseminate out of the intestines and be found in the nasopharynx, lung, heart, liver, and spleen (244).

The innate immune response generated during early infection is associated with recruitment of macrophages, neutrophils, B cells, NK cells and innate lymphoid cells to the colon, as well as innate production of IL-17A, IL-22, cytokines that assist in Th1/Th17 differentiation, anti-inflammatory cytokines such as IL-10, and antibodies (258-267). During induction of an adaptive immune response, bacterial association with the lamina propria induces a strong Th1 and Th17 response accompanied by expression of IL-12, IFNγ, TNF, IL-17A, and IL-22 (268-270). CD4 T cells assist in localization of the bacterium within the lumen and prevent *C. rodentium* from penetrating deep into the crypts (260), and both CD4 T cells and B cells prevents dissemination of *C. rodentium* through maintenance of the epithelial barrier (182). Pathology induced by infection is associated with IL-12, IFNγ, and TNF production by Th1 cells (269, 271), whereas clearance requires the function of both CD4 T cells and B cells (261, 272) and is facilitated by IL-17A and IL-22 production by Th17 cells (273). Additional recruitment of Th17 cells to the intestine occurs through the CCR6-CCL20 axis (274). Interestingly, the generation of a Th17 response during infection requires the presence of Tregs that preferentially consume local IL-2 and allow for IL-17A production (275). Conversely, Th17 cell generation during infection with *C. rodentium* is inhibited by IL-27 (265), whose expression is upregulated in Tpl2-deficient mice infected with *Listeria monocytogenes* (25).

Because *C. rodentium* induces a strong CD4 T cell and B cell response required for clearance (261, 272), absence of these cells within certain knockout strains predisposes them to more severe infection. Absence of B cells and antibody production is associated with significantly delayed *C. rodentium* clearance as well as enhanced fecal burden over time (261, 262). In contrast, T cell-deficient mice are not able to clear *C. rodentium* and quickly succumb to infection (182, 276). Passive transfer of serum from infected wild-type mice can protect *Cd4*$^{-/-}$ mice against mortality (182). Death in all strains is associated with dehydration and impaired intestinal ion transport associated with diarrhea (277). However, mice capable of bacterial clearance, such as C57BL/6, are protected from re-challenge through development of an effective adaptive immune response (261).

**TREATMENT OF AUTOIMMUNE DISEASES**

Treatments for autoimmune diseases are generally nonspecific with the use of anti-inflammatory steroids or immunosuppressive therapies. Some drugs prevent the initial costimulation event required to activate naïve T cells and can therefore create a more tolerogenic environment (278, 279). For example, abatacept competitively binds CD80/CD86 expressed on the surface of antigen presenting cells and prevents binding to CD28 expressed on T cells (280, 281). Alternatively, inhibition of inflammatory cytokines generated during autoimmune diseases can be utilized, such as anti-TNF (279, 282-285) and anti-IL-6 (278, 286). A newer 'headline grabbing' approach to autoimmune disease is treatment of IBD patients with helminths, including: *Trichuris suis* and *Necator americanus* (reviewed in (287)). However, these immunosuppressive therapies do not work in all patients and several therapies make patients more susceptible to infection. Therefore, there is a need for the continued development of more specific treatments for autoimmune diseases.

Because Tpl2 promotes TNF secretion and signaling, Tpl2 is being investigated as an alternative therapeutic target for treating autoimmune diseases exacerbated by TNF, such as rheumatoid arthritis (288-290). Tpl2 is thought to make a desirable target for small molecule inhibitors because Tpl2 shows low homology to other kinases and is the only known human kinase to have a proline instead of a glycine in its ATP binding region (291-293). Because of Tpl2's low homology to other kinases, stauropsorin is an ineffective inhibitor (294). Development of a Tpl2 inhibitor has been hindered by the lack of a Tpl2 crystal structure. Despite this, several classes of Tpl2 inhibitors have been identified and have shown selectivity and efficacy in Tpl2 inhibition *in vivo*. To determine functionality, possible inhibitors were screened for selectivity over other kinases and for ability to block TNF production (289, 290, 294-296). Recently indazoles have also been described as Tpl2 inhibitors (297). Because of the importance of Tpl2 in driving Th1 cell differentiation (28) and possibly Th17 cell differentiation, Tpl2 inhibitors may be beneficial in treating Th1/Th17 associated autoimmune diseases; however, these patients may then be more susceptible to infections cleared by Th1/Th17 cells.

**CONCLUSION**

Autoimmune diseases are approaching epidemic levels, estimated to affect 5-8% of the population. A number of autoimmune diseases are believed to be driven by autoreactive T cells. T helper 1 (Th1) cells have long been implicated in autoimmunity, but more recently T helper 17 cells (Th17) have also been shown to significantly contribute. Despite their roles in driving inflammation and pathology in autoimmune diseases, Th17 cells are essential for the clearance of extracellular bacteria and fungal infections. Th17 cells secrete the cytokines IL-17A, IL-17F, IL-21, and IL-22 (56, 57, 70-73), which collectively promote the recruitment of neutrophils that eradicate the pathogen. Because of their importance in both host immunity and the development

of autoimmune diseases, a better understanding of the pathways and molecules that regulate Th17 immune responses is needed. One molecule gaining interest as a therapeutic target against autoimmune disease is the serine-threonine kinase, Tpl2, which promotes expression of proinflammatory mediators through activation of the ERK pathway. Tumor progression locus 2 (Tpl2), also designated Cot or MAP3K8, is a serine-threonine MAP kinase with critical functions in regulating innate and adaptive immune responses. Previous studies have demonstrated the importance of Tpl2 in the secretion of IFN-γ (28), TNF-α (24) and IL-1β (25) and in host defense against the Th1-type pathogens *Toxoplasma gondii, Listeria monocytogenes, and Mycobacterium tuberculosis*. However, the effects of Tpl2 on Th17 responses and clearance of extracellular bacteria have not been well studied. Understanding how Tpl2 regulates Th17 cell differentiation may provide valuable information about the range of potential benefits of Tpl2 inhibition in various disease settings. The objective of these studies is to determine how Tpl2 regulates Th17 differentiation and how this affects the development of autoimmunity and clearance of extracellular bacteria. The central hypothesis of our study is that Tpl2 is required for Th17 differentiation and host defense against extracellular pathogens but may also predispose the host to autoimmunity. The rationale for this study is that these experiments will not only provide valuable information about how Tpl2 influences CD4 T cell differentiation but will also help to anticipate any potential undesired consequences of Tpl2 inhibition in patient populations, including whether they may be predisposed to common bacterial infections such as *Escherichia coli*.

## FIGURES

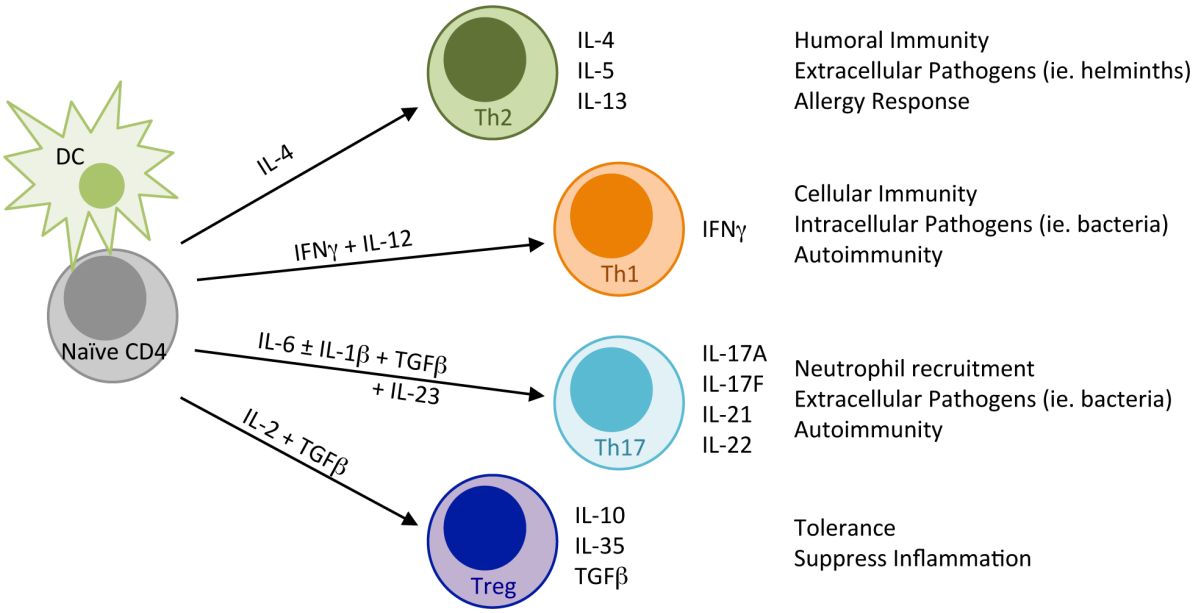



**Figure 1.1: Summary of T helper cell lineages**.

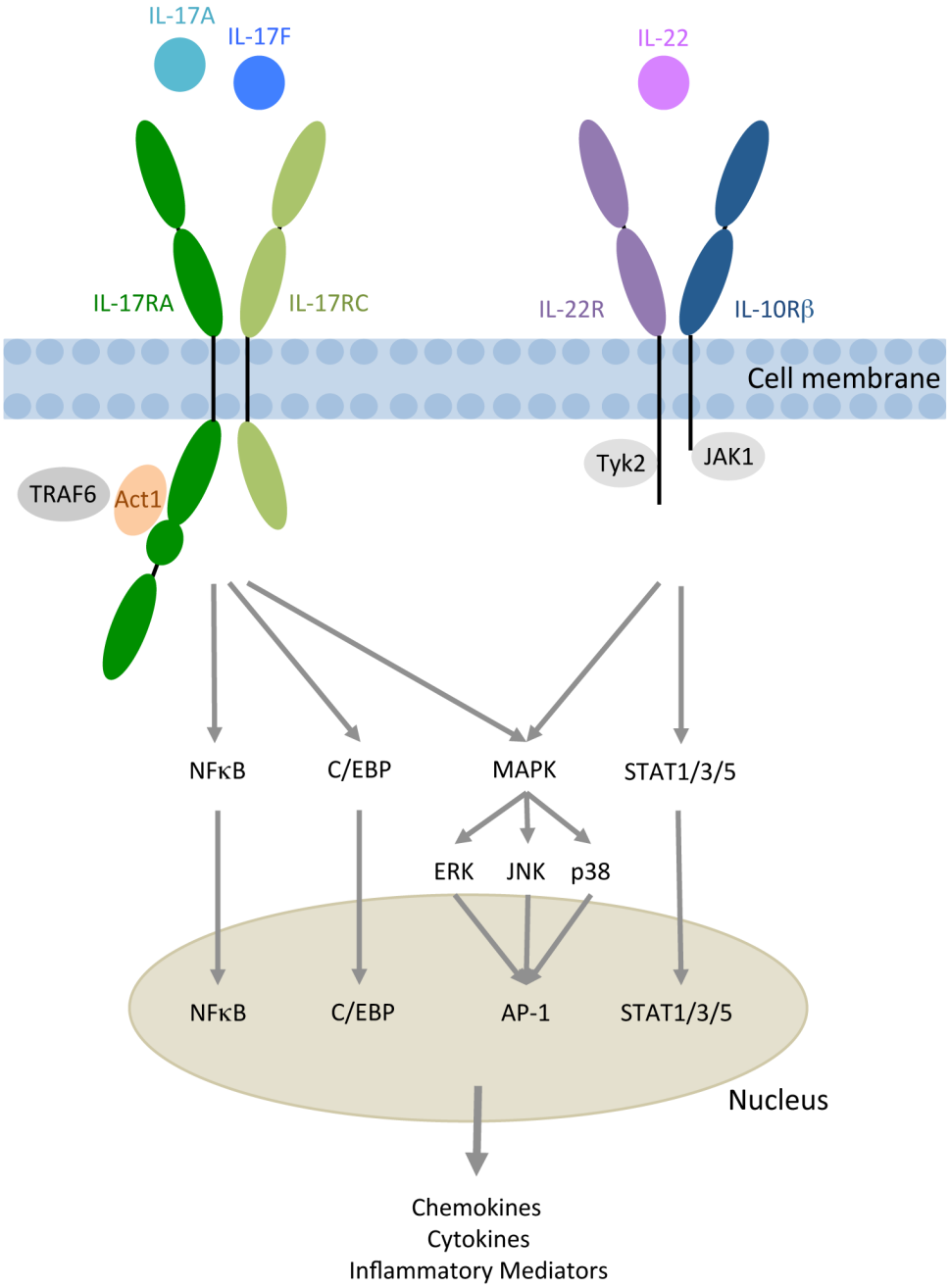



**Figure 1.2: IL-17A and IL-22 receptor signaling.**

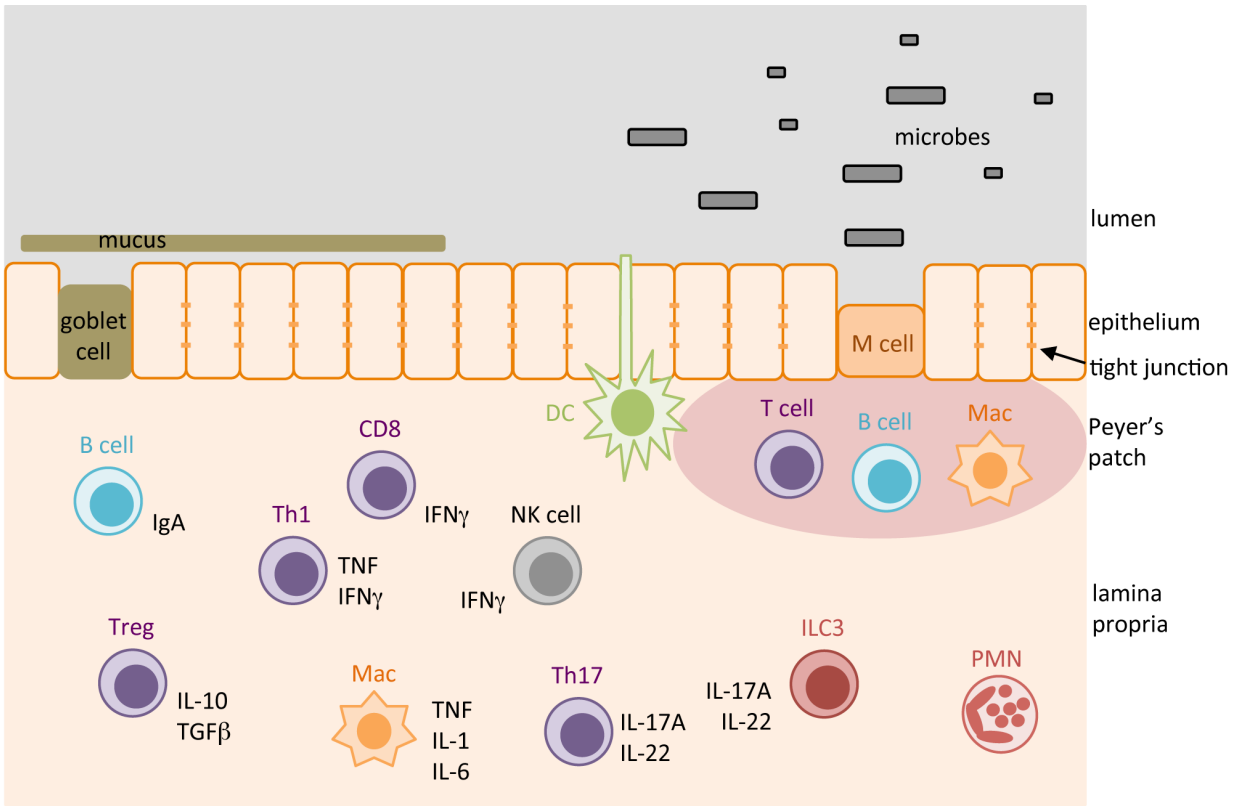



**Figure 1.3: Summary of intestinal immunity.**

# CHAPTER 2

# TUMOR PROGRESSION LOCUS 2 DIFFERENTIALLY REGULATES IFNγ AND IL-17 PRODUCTION BY EFFECTOR $CD4^+$ T CELLS IN A T CELL TRANSFER MODEL OF COLITIS [1]

[1] Acuff, N.V., X. Li, R. Kirkland, T. Nagy and W.T. Watford. 2015. *PLoS ONE*. Open Access. 10(3):e0119885.

**ABSTRACT**

Autoimmune diseases are approaching epidemic levels, estimated to affect 5-8% of the population. A number of autoimmune diseases are believed to be driven by autoreactive T cells, specifically by T helper 1 (Th1) cells and T helper 17 (Th17) cells. One molecule gaining interest as a therapeutic target is the serine-threonine kinase, Tpl2, which promotes expression of proinflammatory mediators. We previously demonstrated that Tpl2 regulates Th1 differentiation, secretion of the inflammatory cytokine IFNγ, and host defense against the intracellular parasite *Toxoplasma gondii*. The goal of this study was to determine whether Tpl2 also regulates Th1 or Th17 differentiation *in vivo* in a model of colitis associated with mixed Th1/Th17 pathology. *In vitro, Tpl2*$^{-/-}$ naïve CD4 T cells were significantly impaired in IL-17A secretion under traditional Th17 inducing conditions. Reduced IL-17A secretion correlated with increased expression of FoxP3, a transcription factor known to antagonize RORγt function. In a murine T cell transfer model of colitis, transfer of *Tpl2*$^{-/-}$ T cells resulted in reduced proportions of CD4 T cells expressing IFNγ, but not IL-17A, compared to that induced by wild type T cells. Further studies revealed that IL-17A differentiation induced by IL-6 and IL-23, cytokines implicated in driving Th17 differentiation *in vivo*, was unaffected by Tpl2 deficiency. Collectively, these results implicate Tpl2 in TGF-β-induced FoxP3 expression. Additionally, they underscore the contribution of Tpl2 to Th1 immunopathology specifically, which suggests that Tpl2 inhibitors may selectively target Th1-based inflammation.

## INTRODUCTION

Tumor progression locus 2, Tpl2 (also known as MAP3K8), is a serine-threonine protein kinase originally described as an oncogene, because its C-terminal truncation promoted tumor growth (6). Tpl2 is expressed in both innate and adaptive immune cells in diverse tissues, including the spleen, thymus, liver, and lung (6, 10, 11). Activated by toll-like receptors, cytokines, antigen receptors and G protein-coupled receptors (10, 23-29), Tpl2 enhances inflammation by promoting expression of cytokines, chemokines and other inflammatory mediators (24, 25, 28, 41-44). Many of Tpl2's functions have been attributed to its activation of the MEK/ERK pathway (reviewed in (11)). Initial characterization of *Tpl2*$^{-/-}$ mice identified major defects in the induction of proinflammatory cytokines, particularly TNFα, by antigen presenting cells that conveyed resistance to endotoxin-induced shock (24). Because it promotes inflammatory mediators, Tpl2 is being investigated as a therapeutic target for treating autoimmune diseases (288-290).

We previously demonstrated that Tpl2 promotes Th1 differentiation and IFNγ production in response to the intracellular parasite, *Toxoplasma gondii* (28), inhibits T helper 2 (Th2) cell responses during OVA-induced allergic asthma in mice (51) and promotes T helper 17 (Th17) cell secretion of IL-17A *in vitro* (28). Th17 cells are a distinct lineage of CD4 T cells that produce IL-17A, IL-17F, IL-21, and IL-22 (56, 57, 70-73). Together, Th17 effector cytokines are required for the clearance of extracellular bacterial and fungal infections, but dysregulated Th17 responses have also been implicated in the development of autoimmune diseases, including multiple sclerosis, rheumatoid arthritis and inflammatory bowel diseases (reviewed in (76)). The importance of Tpl2 in Th17 cell differentiation *in vivo* has not been extensively studied, but Tpl2 is dispensable for driving Th17 differentiation in experimental autoimmune encephalomyelitis

(EAE) (38, 52).

In this study, we addressed whether Tpl2 contributes to the development of colitis, an alternative autoimmune disease, in a T cell specific manner. The importance of Tpl2 in certain aspects of inflammatory bowel diseases (IBD), a complex spectrum of autoimmune diseases of the small intestine and colon, has been studied previously. For example, $TNF^{\Delta ARE}$ mice that express a stabilized TNF transcript and spontaneously develop colitis, showed delayed onset and attenuated progression of IBD when crossed onto the *Tpl2*$^{-/-}$ background (219). Because colitis in $TNF^{\Delta ARE}$ mice is due to accumulation of TNF, these results indicate the importance of Tpl2 in transducing TNF signals. Additionally, in a chemically induced model of colitis, dextran sulfate sodium (DSS) damages intestinal epithelial cells and therefore alters barrier function of the intestines, leading to hematochezia, body weight loss, shortening of the intestine, mucosal ulcers, and infiltration of neutrophils. In this innate immune model of colitis, *Tpl2*$^{-/-}$ mice experienced milder colitis compared to wild type mice with reduced production of inflammatory cytokines IL-1α, IL-1β, IL-6, and IL-17, as well as reduced production of the anti-inflammatory cytokine IL-10 (220). Despite multiple lines of evidence for Tpl2 in various aspects of colitis development, a T cell-intrinsic function for Tpl2 during colitis has not yet been explored.

We first confirmed that *Tpl2*$^{-/-}$ T cells are impaired *in vitro* in the production of IL-17A under the classical Th17 polarizing conditions of IL-6 and TGF-β, and this impairment was associated with elevated expression of FoxP3. In a T cell transfer model of colitis, Tpl2 ablation within the transferred T cell population reduced the proportion of CD4 T cells expressing IFNγ without altering IL-17 expression. Notably, Tpl2 ablation also increased CD4 T cell accumulation in Rag1-deficient recipients *in vivo.* The discrepancy between Tpl2's regulation of IL-17 production *in vitro* versus *in vivo* was clarified by the finding that IL-17A production was

restored to wild type levels in *Tpl2*$^{-/-}$ Th17 cells when the TGF-β concentration was reduced, neutralizing IL-2 antibody was added, or when Th17 cells were alternatively induced by IL-6 and IL-23, all of which failed to induce FoxP3 expression. This study defined a TGF-β- and FoxP3-restricted defect in IL-17A secretion by *Tpl2*$^{-/-}$ T cells. Overall, these findings demonstrate that Tpl2 is dispensable for Th17 differentiation during a T cell transfer model of colitis where IL-6 and IL-23 have a dominant role but underscore the contribution of Tpl2 to Th1 differentiation in this model.

## MATERIALS AND METHODS

### *Ethics Statement*

All experiments involving mice were performed according to the University of Georgia guidelines for laboratory animals and were approved by the UGA Institutional Animal Care and Use Committee. The internal IACUC approval number currently is A2012 06-002-Y3-A9.

### *Mice*

Wild type (C57BL/6) mice were obtained from the Jackson Laboratory (Bar Harbor, Maine). *Tpl2*$^{-/-}$ mice backcrossed onto the C57BL/6 genetic background were kindly provided by Dr. Philip Tsichlis (Tufts University) and Thomas Jefferson University, where the mice were generated. OT-II mice were obtained from the National Institute of Health (NIH), and *Rag1*$^{-/-}$ mice were purchased from Jackson Laboratories. Animals were used at six to twelve weeks of age, and were age- and sex-matched for individual experiments. Animals were bred within the same facility and maintained in sterile microisolator cages.

### *Cell sorting*

Wild type or *Tpl2*$^{-/-}$ cells from spleens and lymph nodes were disaggregated by pressing through a 70 μm filter, and CD4 T cells were column purified by negative selection using a CD4$^{+}$ T cell

isolation kit according to manufacturer's guidelines (Miltenyi Biotech, Auburn, CA). CD4 T cells were stained for 15 min at 4°C in PBS + 0.5% FBS (Life Technologies, Carlsbad, CA) using anti-mouse antibodies purchased from eBioscience (San Diego, CA): CD16/CD32 (93), CD4 (RM4-5), TCRβ (H57-597), CD25 (PC61.5), CD44 (IM7), CD62L (MGL-14), and CD45RB (C363.16A). Live cells were first gated by excluding propidium iodide positive ($PI^+$) cells and then sorted for naïve effectors ($CD4^+CD25^-CD62L^+CD44^-$ or $CD4^+CD25^-CD45RB^{hi}$) using a Beckman Coulter MoFlo XDP cell sorter.

*Cell culture*

Sorted naïve CD4 T cells ($CD4^+CD25^-CD62L^+CD44^-$) were enumerated and plated at a concentration of $1x10^6$ cells/ml in cell culture wells with immobilized anti-CD3 and anti-CD28 (5 μg/ml each). Cells were cultured at 37°C and 5% $CO_2$ in complete RPMI (RPMI 1640 containing 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine (Life Technologies), 0.01 M HEPES (Fisher Scientific, Waltham, MA), and 50 μM 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO)). Th17 differentiation was induced in the presence of 10 ng/ml IL-6, 5 ng/ml TGF-β, and 10 μg/ml of both anti-IL-4 and anti-IFNγ (BD Biosciences, San Jose, CA) for 3 days, unless otherwise indicated. Additionally, 10 ng/ml IL-1β or 5 μg/ml anti-IL-2 (BD Biosciences) were used in certain polarizing conditions where indicated. Alternatively, for some experiments, Th17 differentiation was induced in the presence of 10 ng/ml IL-6 and 10 ng/ml IL-23 for 3 days in the presence or absence of decreasing concentrations of TGF-β.

Bone marrow-derived dendritic cells (BMDCs) were generated by culture of bone marrow cells from femurs and tibiae of mice. Briefly, bone marrow cells ($2x10^6$ cells/ml) were cultured at 37°C and 5% $CO_2$ in complete RPMI supplemented with 40 ng/ml GM-CSF (PeproTech, Rocky Hill, NJ). On days 3 and 5, fresh medium equal to half of the initial volume of the culture

containing 40 ng/ml GM-CSF was added. On day 7, non-adherent cells were collected, incubated with anti-mouse CD11c labeled microbeads, and $CD11c^+$ cells were column purified by positive selection according to manufacturer's guidelines (Miltenyi Biotech).

For co-culture experiments, $10^4$ wild type BMDCs were incubated with a 10-fold excess of naïve OT-II CD4 T cells ($10^5$) which express a transgenic TCR specific for $OVA_{323–339}$ (Peptides International, Louisville, KY). Cells were cultured under neutral (media alone) or Th17 (IL-6 + TGF-β) conditions in 96-well microtiter plates in a volume of 200 μL for 3 days.

*Cytokine Measurements*

IL-17A expression, as determined by intracellular staining followed by flow cytometry, was the primary measure of Th17 development. Prior to cell staining, cells were stimulated 4 hours at 37°C with 50 ng/ml PMA (Sigma-Aldrich), 0.5 μg/ml ionomycin (Sigma-Aldrich), and golgi transport inhibitor (BD Biosciences) according to manufacturer's specifications. The following anti-mouse monoclonal antibodies used were from eBiosciences: CD16/CD32 (93), CD4 (RM4-5), IL-17A (eBio17B7), FoxP3 (FJK-16s), and IFNγ (XMG1.2). Prior to intracellular staining, cells were fixed in either 4% formalin or fixation/permeabilization buffer (eBioscience) and subsequently washed and stained in permeabilization buffer (eBioscience). Samples were run on a BD LSRII flow cytometer and analyzed using FlowJo software (Tree Star, Inc., Ashland, OR). IL-17A, IL-17F, IL-22, IL-2 and IFNγ proteins were measured in supernatants by ELISA (eBiosciences) or Th1/2/17 cytokine bead array (BD Biosciences) according to manufacturer's guidelines. RNA was isolated from cell pellets on day 3 (unless otherwise indicated) or colon tissue using EZRNA extraction kit (Omega Bio-Tek, Norcross, GA) and converted to cDNA by high capacity cDNA reverse transcription kit (Life Technologies). Relative expression levels of *Il17a, Il17f, Il21, Il22, Rorc, Rorα, Irf4,* and *Foxp3* were measured using SensiFAST Probe Hi-

ROX kit (Bioline, Taunton, MA) and specific TaqMan probes (Applied Biosystems, Grand Island, NY). Samples were run on a StepOnePlus qPCR machine (Applied Biosystems). Results given are relative to actin control and wild type Th0 conditions ($\Delta\Delta C_T$). In some cases, wild type Th0 conditions were assigned a $C_T$ value of 40 when no amplification occurred within 40 cycles.

*T cell transfer of colitis*

Rag1-deficient mice were injected i.p. with approximately $3x10^5$ wild type or *Tpl2*$^{-/-}$ naïve T cells ($CD4^+CD25^-CD45RB^{hi}$). Mice were weighed prior to injection and weekly thereafter. Blood was collected at 3, 6 and 8 weeks from the tail vein or by terminal cardiac puncture, and serum cytokines were quantified by Th1/Th2/Th17 cytokine bead array (BD Biosciences). Spleen and mesenteric lymph nodes were isolated and counted. Cells were restimulated *ex vivo* for 4 hours with PMA, ionomycin, and Golgi Plug (BD Biosciences) at a concentration of 1-$2x10^6$ cells/ml and stained similarly to *in vitro* cultures.

*Pathology Scoring*

Colonic sections from mice were collected and fixed in 10% neutral buffered formalin for 24 h at room temperature. Complete cross sections of formalin-fixed intestinal sections were placed in cassettes, embedded in paraffin, sectioned at 4 μm thickness, mounted on glass slides, and stained with hematoxylin and eosin (H&E). Histological sections were evaluated by a veterinary pathologist (TN) and scored according to the following criteria: (A) Distribution of the inflammation: 0=None, 1=Focal, 2=Multifocal, 3=Diffuse; (B) Degree of inflammation: 0=None, 1=Mild, 2=Moderate, 3=Severe; (C) Extent of erosion and/or ulceration: 0=None, 1=Superficial (lamina propria only), 2: Moderate (extends to the submucosa), 3: Severe (transmural) and then pooled to calculate total pathology score.

*Western Blotting*

Cell pellets were washed in cold PBS and lysed in protein lysis buffer ($dH_2O$, 0.05 M Tris, 0.3 M NaCl, 0.5% TTX 100, 2 mM EDTA, 0.4 mM $Na_3VO_4$, 2.5 mM Leupeptin, 2.5 mM Aprotinin, 2.5 mM 4-Nitrophenyl 4-guanidinobenzoate hydrochloride (NPGB)). Protein concentration was measured using a BCA protein assay (Thermo Scientific, Suwanee, GA). Twelve micrograms of total protein were separated on a 4-12% Bis-Tris gel (Life Technologies) and probed with antibodies for phospho-STAT3 (Ser727), phospho-STAT3 (Tyr705) and total STAT3 (Cell Signaling Technology, Danvers, MA).

*Statistics*

*P* values were derived by paired or unpaired two-tailed Student's t-test using Prism software, unless otherwise indicated. Differences were considered statistically significant if $p \leq 0.05$.

**RESULTS**

**Tpl2 promotes Th17 development in vitro**

We previously demonstrated that Th17 differentiation was impaired in *Tpl2*$^{-/-}$ T cells (28). To further confirm the importance of Tpl2 in Th17 differentiation *in vitro*, we stimulated wild type and *Tpl2*$^{-/-}$ naïve CD4 T cells in the presence of IL-6 and TGF-β. On day 3 of culture, *Tpl2*$^{-/-}$ CD4 T cells secreted significantly less IL-17A and IL-17F relative to wild type cells (Figure 2.1A-B). Transcription of *Il17a* was also reduced in *Tpl2*$^{-/-}$ cells. No difference in transcription of *Il17f* or *Il21* was detected (Figure 2.1C). Enhanced differentiation of wild type Th17 cells, as seen by increased production of IL-17A, was accomplished through addition of IL-1β to the cultures (298, 299). Even with the addition of IL-1β, IL-17A production was still reduced in *Tpl2*$^{-/-}$ cells relative to wild type cells (Figure 2.1A-B). Therefore, Tpl2 was required for optimal IL-17A production in CD4 T cells stimulated with IL-6 and TGF-β, and this defect could not be

overcome by addition of exogenous IL-1β.

To confirm our results under more physiological conditions, we used a co-culture system to stimulate T cells with antigen presented in the context of MHC class II molecules by dendritic cells. We cultured either *Tpl2*$^{+/+}$ OT-II T cells or *Tpl2*$^{-/-}$ OT-II T cells expressing an OVA-specific transgenic TCR in the presence of their cognate antigen, ovalbumin peptide, with wild type BMDCs and the Th17 polarizing cytokines, IL-6 and TGF-β. Importantly, at all concentrations of OVA, *Tpl2*$^{-/-}$ Th17 cultures displayed reduced proportions of IL-17A positive cells (Figure 2.1D). These results indicate that there is a T cell-intrinsic defect in the ability of *Tpl2*$^{-/-}$ Th17 cells to produce IL-17A.

**Tpl2 promotes Th1, but not Th17, differentiation in a CD45RB T cell transfer model of colitis**

Having characterized a Tpl2-dependent defect in IL-17A expression, along with the previously identified IFNγ defect, we next examined the capacity of *Tpl2*$^{-/-}$ T cells to induce disease in a T cell transfer model of colitis associated with a mixed Th1 and Th17 inflammatory response. In this model, naïve CD4 effector T cells ($CD4^{+}CD25^{-}CD45RB^{hi}$) adoptively transferred into Rag1-deficient mice undergo lymphopenia-induced expansion and cause intestinal inflammation that recapitulates human IBD (212). Proliferating CD4 T cells respond to intestinal antigens to drive inflammation within the small and large intestines, characterized by increases in TNF, IFNγ, IL-17A, and IL-23, leading to weight loss and diarrhea (212, 222, 226, 227, 230-232). When colitis was induced in Rag1-deficient mice, recipients of either wild type or *Tpl2*$^{-/-}$ naïve CD4 T cells experienced similar weight loss kinetics (Figure 2.2A). As disease progressed, we observed increases in circulating TNF and IFNγ that waned at later time points once inflammation established within the intestine (Figure 2.2B). Tpl2 ablation had no significant effect on the

levels of circulating TNF at any time point but modestly reduced circulating IFNγ (Figure 2.2B). Colitic mice were euthanized, and colons were scored for inflammation. Similar total pathology scores were observed between recipients of either wild type or *Tpl2*$^{-/-}$ cells (Figure 2.2C).

Because of our previous identification of Tpl2 as a promoter of IFNγ secretion and Th1 differentiation (28), we hypothesized that Tpl2 ablation within the transferred T cell population would limit disease. Therefore, the nearly normal circulating IFNγ levels and colitis pathology were surprising. Since both Th1 and Th17 cells are associated with colitis, we investigated whether Tpl2 altered the proportions of IFNγ or IL-17A positive CD4 T cells within the spleen and mesenteric lymph nodes (MLN). Recipients of *Tpl2*$^{-/-}$ cells had reduced proportions of CD4$^{+}$IFNγ$^{+}$ cells, but not CD4$^{+}$IL-17A$^{+}$ cells (Figure 2.2D). However, recipients of *Tpl2*$^{-/-}$ CD4 T cells also had more total cells and CD4 T cells within their spleens and MLN compared to recipients of wild type cells (Figure 2.2E). Therefore, despite reduced proportions of CD4$^{+}$IFNγ$^{+}$ T cells within recipients of *Tpl2*$^{-/-}$ T cells, their absolute number was either unchanged or increased (depending on the organ) relative to recipients of wild type T cells (Figure 2.2F).

**Tpl2 deficiency promotes FoxP3-mediated antagonism of IL-17A expression**

In order to reconcile the conflicting requirements for Tpl2 in IL-17 production *in vitro* and *in vivo*, we next investigated the mechanism for reduced IL-17A production in *Tpl2*$^{-/-}$ T cells *in vitro* by determining whether reduced IL-17A production correlated with decreased levels of Th17-associated transcription factors. Th17-associated transcription factors include RORα, RORγt, BATF, IRF4, and STAT3 (84, 86, 88, 300), but Th17 cell differentiation is negatively regulated by the transcription factor FoxP3 (100). Surprisingly, in *Tpl2*$^{-/-}$ T cells, there was no impairment in *Rorc, Rorα, or Irf4* expression or STAT3 activation (Supplementary Figure 2.5). In the absence of impaired expression or activation of transcription factors that induce IL-17A

transcription, we reasoned that a negative regulatory mechanism was constraining IL-17A secretion in *Tpl2*$^{-/-}$ T cells. To examine whether FoxP3 expression was altered in *Tpl2*$^{-/-}$ T cells, we performed real-time PCR analysis of wild type and *Tpl2*$^{-/-}$ T cells cultured under Th17 conditions. *Tpl2*$^{-/-}$ Th17 cells expressed significantly elevated levels of FoxP3 relative to wild type Th17 cells (Figure 2.3A). Additionally, in our co-culture system, *Tpl2*$^{-/-}$ Th17 cultures displayed higher proportions of FoxP3 positive cells compared to wild type (Figure 2.3B) indicating there is a T cell-intrinsic defect in the ability of *Tpl2*$^{-/-}$ Th17 cells to produce IL-17A, which correlates with increased FoxP3 expression.

**Th17 differentiation is Tpl2-independent under conditions that fail to induce FoxP3**

We next addressed whether Tpl2 was similarly dispensable for Th17 differentiation under alternative Th17-inducing conditions, some of which might more closely recapitulate the conditions present in the colitis model. First, we assessed the effect of IL-2, which inhibits Th17 differentiation (301) and promotes Treg cell differentiation (302), on Th17 differentiation of *Tpl2*$^{-/-}$ T cells. We added anti-IL-2 to cultures to neutralize its effects, and as expected, IL-2 neutralization reduced FoxP3 expression to below basal levels observed in Th0 conditions (Figure 2.4C). Additionally, IL-2 neutralization significantly increased IL-17A differentiation, boosting the proportion of wild type IL-17A-expressing T cells from approximately 15% to 60% (Figure 2.4A). Anti-IL-2 also greatly enhanced IL-17A expression and secretion (Figure 2.4B-C). Notably, upon IL-2 neutralization, *Tpl2*$^{-/-}$ CD4$^{+}$ T cells acquired the ability to produce IL-17A at wild type levels (Figure 2.4A-C).

Because IL-2 neutralization reversed the phenotype, we investigated whether IL-2 secretion is increased in *Tpl2*$^{-/-}$ T cells. However, we observed no differences in IL-2 secretion between wild type and *Tpl2*$^{-/-}$ Th0 or Th17 cultures (Supplementary Figure 2.6A). Interestingly, IL-2 was

nearly absent at this time in Th17 cultures (Supplementary Figure 2.6A). We next performed new experiments to observe IL-2 secretion over a time course under Th17 conditions. At days 1 and 2 of culture, wild type and *Tpl2*$^{-/-}$ T cells secreted similar levels of IL-2 (Supplementary Figure 2.6B). However, IL-2 was consumed within Th17 cultures by day 3, which matched the results from our initial day 3 cultures (Supplementary Figure 2.6). Normal IL-2 secretion by *Tpl2*$^{-/-}$ T cells is consistent with a previous report (24). These findings suggest that IL-2 secretion does not underlie the Th17 defect. Instead, they indicate that autocrine IL-2 permits TGF-β-induced FoxP3 expression, which is amplified in *Tpl2*$^{-/-}$ Th17 cells and suppress IL-17A expression.

In the absence of TGF-β, IL-6 and IL-23 have been shown to induce and expand Th17 cells that are more pathogenic and inflammatory *in vivo* than those driven by IL-6 and TGF-β, as they do not express FoxP3 (70, 82, 103). We therefore cultured wild type and *Tpl2*$^{-/-}$ naïve T cells with IL-6 and IL-23. As expected, expression of *Foxp3* was extremely low in both wild type and *Tpl2*$^{-/-}$ Th17 cells cultured in this way (Figure 2.4B-C). Under these conditions, *Tpl2*$^{-/-}$ Th17 cells produced wild type levels of IL-17A as seen by flow cytometry, ELISA, and RT-PCR (Figure 2.5A-C). We also observed secretion and expression of IL-22 in Th17 cells cultured with IL-6 and IL-23 (73, 103), which was similar between wild type and *Tpl2*$^{-/-}$ cells (Figure 2.4B-C). No significant IFNγ secretion was observed under these conditions (data not shown).

**The regulation of IL-17A and FoxP3 by Tpl2 is TGF-β-dependent**

To confirm the importance of TGF-β-induced FoxP3 transcription in the reduction of IL-17A expression in Tpl2$^{-/-}$ T cells, we titrated TGF-β from Th17 conditions. For these studies, Th17 cells were induced by IL-6 and IL-23 in the presence of decreasing concentrations of TGF-β, and we assessed the effects on both IL-17A and FoxP3 induction. At high TGF-β concentrations,

Tpl2$^{-/-}$ CD4 T cells expressed higher proportions of FoxP3 and reduced proportions of IL-17A compared to wild type cells (Figure 2.4D-E). Littman et al. demonstrated that TGF-β, while required for optimal Th17 differentiation in vitro, could also suppress Th17 differentiation at high concentrations (100). With reduced concentrations of TGF-β, proportions of IL-17A positive cells increased, proportions of FoxP3 positive cells dropped and both FoxP3 and IL-17A expression in Tpl2$^{-/-}$ cells normalized to wild type levels (Figure 2.4E). Collectively, these findings demonstrate that there is no obligate requirement for Tpl2 in driving IL-17 or IL-22 secretion by CD4 T cells, but suggests instead that Tpl2 promotes TGF-β/FoxP3-mediated suppression of Th17 responses.

**DISCUSSION**

Herein, we describe a T cell-intrinsic defect in IL-17A production by *Tpl2*$^{-/-}$ Th17 cells driven by IL-6 and TGF-β. This defect in IL-17A production did not correlate with impaired expression of Th17 associated transcription factors RORα, RORγt, or IRF4 but was instead associated with increased levels of FoxP3. In the absence of FoxP3 induction, as seen with addition of anti-IL-2 to Th17 conditions or by alternatively differentiating Th17 cells with IL-6 and IL-23, there was no defect in IL-17A production by *Tpl2*$^{-/-}$ Th17 cells. Furthermore, titration of TGF-β revealed that increased FoxP3 expression and decreased IL-17A expression in *Tpl2*$^{-/-}$ cells were a direct consequence of TGF-β signaling. These findings demonstrate that Tpl2 normally constrains TGF-β-driven FoxP3 transcription, which allows for increased production of IL-17A.

A T cell transfer model of colitis was employed to directly assess the T cell-intrinsic functions of Tpl2 *in vivo* in driving autoimmune disease characterized by a mixed Th1 and Th17 pathology. Although Tpl2 promotes Th17 differentiation *in vitro*, Tpl2 did not alter Th17 differentiation in this model of colitis. There are several possible explanations for this. First, although it is standard

practice to use TGF-β and IL-6 to drive Th17 differentiation *in vitro*, it has been well established that other factors are also important for Th17 development both *in vitro* and *in vivo*, such as IL-6 and IL-23. Th17 cells generated in this manner are more pathologic in a murine experimental autoimmune encephalomyelitis (EAE) model (82). IL-23 is also required for disease development in the T cell transfer model of colitis (230). The fact that Th17 differentiation induced by IL-6 and IL-23 was unaltered in *Tpl2*$^{-/-}$ T cells *in vitro* likely explains the normal Th17 differentiation in colitic recipients of *Tpl2*$^{-/-}$ effector cells. It remains possible that Th17 differentiation may be regulated by Tpl2 *in vivo* in a context-dependent manner where TGF-β concentrations are locally high, as at mucosal sites.

Interestingly, Tpl2 deficiency enhanced the lymphopenia-induced accumulation of transferred effector CD4 T cells in this colitis model. Lymphopenia-induced rapid proliferation occurs independently of IL-7 cytokine signals, but is thought to rely instead upon the TCR signal strength to available ligands within the lymphopenic host (227). This raises the interesting possibility of either increased TCR signal strength within *Tpl2*$^{-/-}$ CD4 T cells that drives T cell proliferation or altered cell cycle progression in the absence of Tpl2. In this regard, Tpl2 ablation has been demonstrated to promote CD8 T cell proliferation in response to antigen stimulation (27). In addition to T cells, *Tpl2*$^{-/-}$ colonic epithelial cells also proliferate at a higher rate with reduced apoptosis compared to wild type cells during DSS colitis, leading to tumor development (303). Because increased cell survival has also been noted, we cannot exclude the possibility that increased accumulation of *Tpl2*$^{-/-}$ CD4 T cells may result from reduced apoptosis. Further studies are required to determine how Tpl2 affects CD4 T cell proliferation and survival *in vivo*. Despite significantly increased accumulation of *Tpl2*$^{-/-}$ effector cells within the lymphoid organs, recipients of *Tpl2*$^{-/-}$ T cells were no more susceptible to the development of colitis than recipients

of wild type T cells. This was due to the impaired capacity of *Tpl2*$^{-/-}$ T cells to produce IFNγ. This finding is consistent with our previous work establishing Tpl2 as an important positive regulator of Th1 differentiation and IFNγ secretion (28).

The increased TGF-β-induced FoxP3 expression observed in *Tpl2*$^{-/-}$ T cells may have broader implications. In addition to its induction of Th17 cells, TGF-β is critical for the development and function of FoxP3$^{+}$ immunosuppressive Tregs. Indeed, mice with mutations in TGF-β responsiveness, TGF-β secretion, or FoxP3 expression develop patent autoimmunity characterized by lymphoproliferation, cellular activation and pro-inflammatory cytokine secretion (91-95). The observation that FoxP3 expression is increased in *Tpl2*$^{-/-}$ Th17 cells raises the possibility that FoxP3$^{+}$ Tregs might also be increased in *Tpl2*$^{-/-}$ mice, further promoting the development of an immunosuppressive environment. If this is a generalized phenomenon, then Tpl2 inhibition would be expected to reduce severity of a range of autoimmune diseases where a more regulatory environment is desired. Ongoing studies are exploring this possibility.

Overall, our findings underscore the importance of Tpl2 in driving the development of the pro-inflammatory Th1 lineage. It further provides new insights into the regulation of TGF-β-induced FoxP3 expression as well as lymphopenia-induced expansion of T cells by Tpl2. These findings support the use of Tpl2 inhibitors for the targeted treatment of Th1-driven autoimmune diseases, such as diabetes and colitis (232, 304, 305) but suggest that Tpl2 inhibitors may have more limited utility in treating Th17-mediated diseases. Further studies are needed to fully elucidate the effects of Tpl2 in specific autoimmune disease settings on a case-by-case basis, as the specific cytokine milieu will differentially engage the Tpl2 kinase.

**ACKNOWLEDGEMENTS**

We thank Julie Nelson in the Center for Tropical and Emerging Global Diseases Flow Cytometry Core Facility for flow sorting and UGA's Veterinary Medicine Central Animal Facility for animal care.

## FIGURES

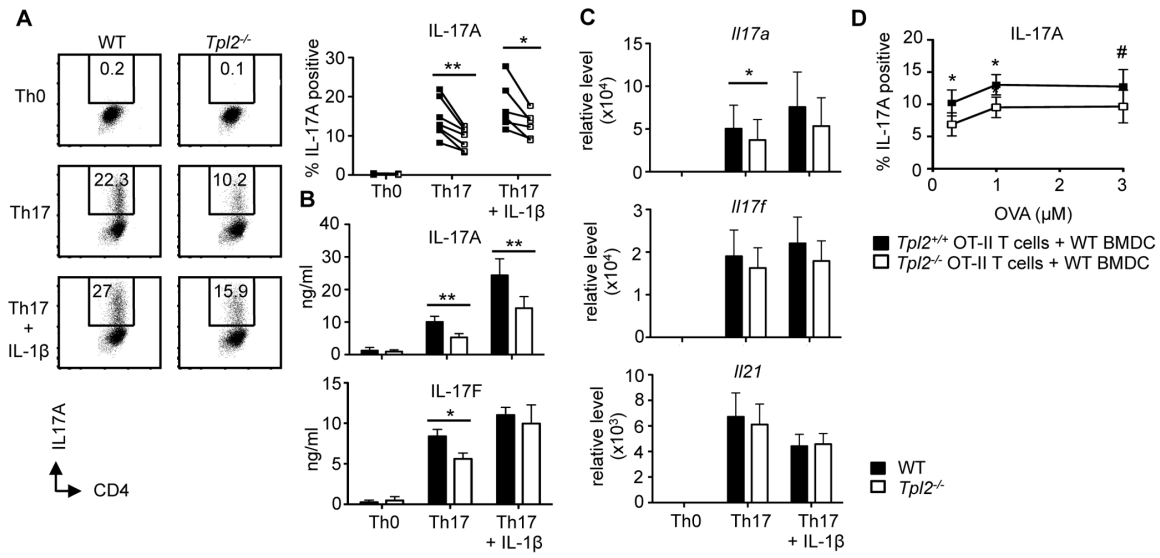



**Figure 2.1**: **Tpl2 promotes Th17 development *in vitro* independently of IL-1β.** Naïve T cells were cultured under Th17 polarizing conditions for 3 days +/- 10 ng/ml IL-1β. (A) IL-17A levels by flow cytometry in Th0 and Th17 conditions. Cells were gated on $CD4^+$ cells. Connected symbols represent individual experiments. (B) IL-17A and IL-17F secretion by ELISA. (C) On day 3 of culture, *Il17a*, *Il17f,* and *Il21* expression levels by RT-PCR. (A-C) N≥6 experiments. (D) $Tpl2^{+/+}$ OT-II and $Tpl2^{-/-}$ OT-II naïve T cells were cultured for 3 days with wild type $CD11c^+$ BMDCs, OVA peptide, and polarizing cytokines (IL-6 + TGF-β). IL-17A was measured by flow cytometry. Cells were gated on $CD4^+$ cells. N=3 experiments except where noted. #N=2. Error bars represent means ± sem. *p<0.05, **p<0.005.

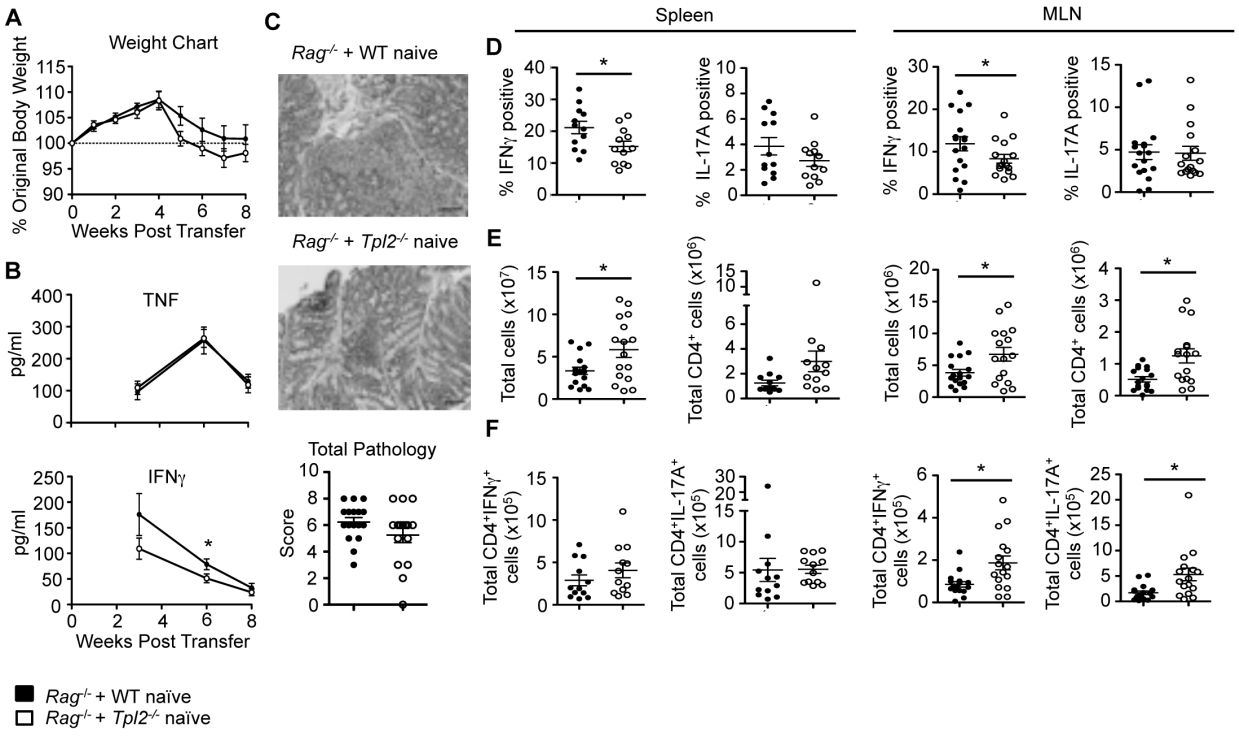



**Figure 2.2: Tpl2 promotes Th1, but not Th17, differentiation in a CD45RB T cell transfer model of colitis.** Wild type or *Tpl2*$^{-/-}$ naïve T cells (CD45RB$^{hi}$CD25$^{-}$CD4$^{+}$) were injected i.p. into female Rag-deficient mice. (A) Weight loss curves as a percentage of original body weight. (B) Measure of serum cytokine levels in colitic mice. Significance was measured by one-tailed Student's t-test. (C) Representative histologic images are shown along with scoring for total pathology in the colon. Pathology scores were evaluated using Mann-Whitney U test. (D) Proportions of IFNγ and IL-17A in the spleen and mesenteric lymph nodes as measured by intracellular staining and flow cytometry. Cells were gated on CD4$^{+}$TCRβ$^{+}$ cells. Significance was measured by one-tailed Student's t-test. (E) Total cells and CD4$^{+}$TCRβ$^{+}$ cells in the spleen and mesenteric lymph nodes. (F) Total CD4$^{+}$IFNγ$^{+}$ and CD4$^{+}$IL-17A$^{+}$ cells in the spleen and mesenteric lymph nodes. N≥12. Pooled from 3 independent experiments. Error bars represent means ± sem. *p<0.05.

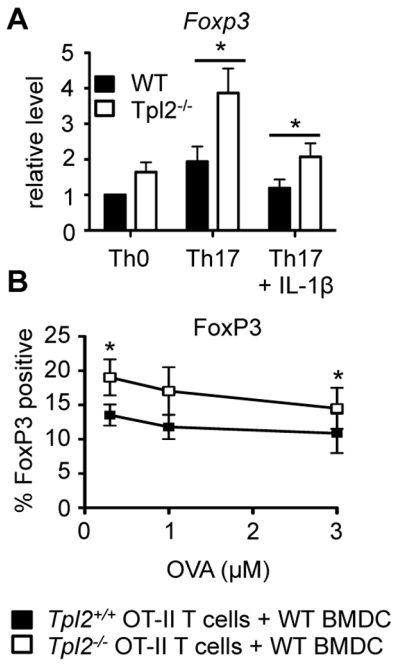



**Figure 2.3: *Tpl2*$^{-/-}$ T cells express increased levels of the FoxP3 transcription factor.** Naïve T cells were cultured under Th17 polarizing conditions for 3 days +/- 10 ng/ml IL-1β. (A) *Foxp3* expression was measured by RT-PCR. (B) *Tpl2*$^{+/+}$ OT-II and *Tpl2*$^{-/-}$ OT-II naïve T cells were cultured for 3 days with wild type CD11c$^{+}$ BMDCs, OVA peptide, and polarizing cytokines (IL-6 + TGF-β). FoxP3 levels were measured by flow cytometry. Cells were gated on CD4$^{+}$ cells. N=3 experiments. Error bars represent means ± sem. *$p<0.05$.

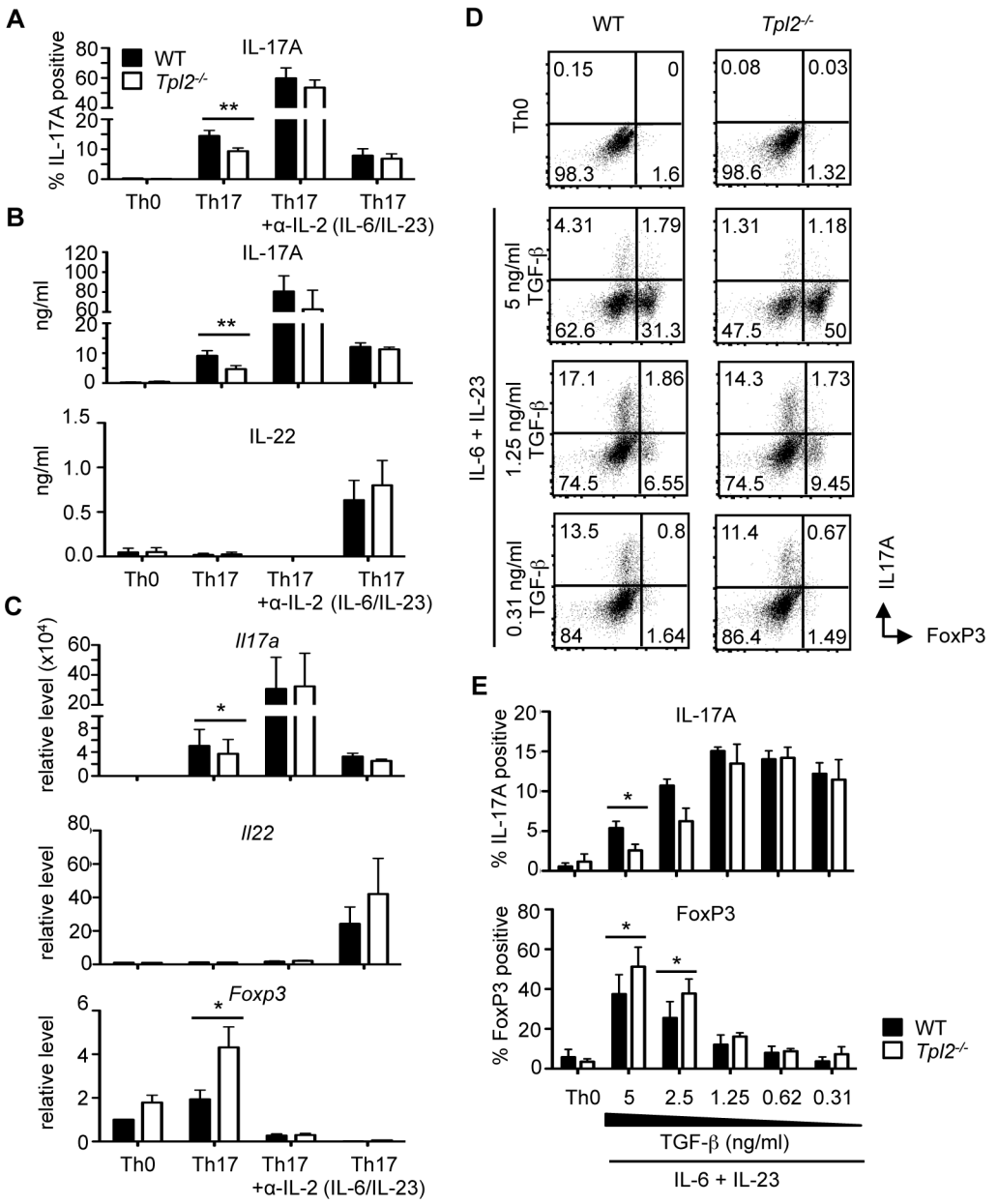
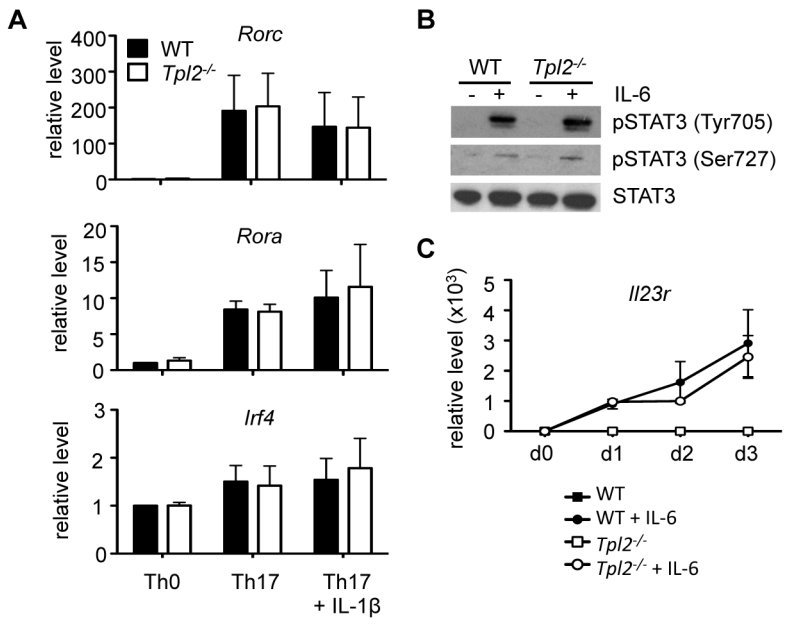
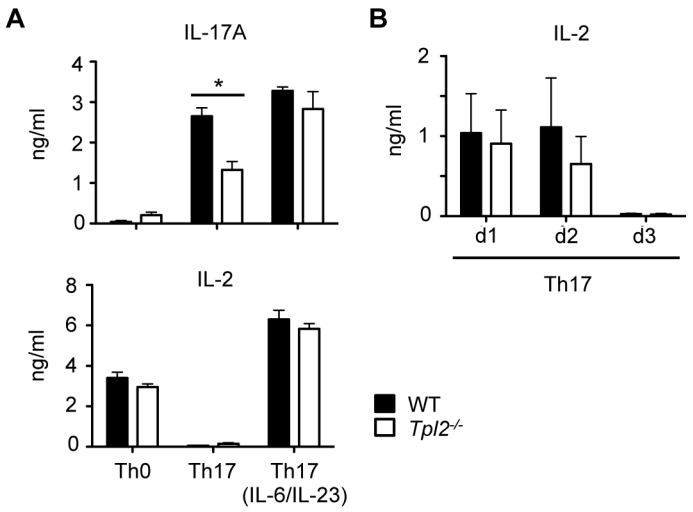



**Figure 2.4**: **The differential regulation of IL-17A and FoxP3 by Tpl2 is TGF-β-dependent.** Naïve T cells were cultured under Th17 polarizing conditions for 3 days +/- 5 μg/ml anti-IL-2 or with IL-6 and IL-23. (A) IL-17A levels were measured by intracellular staining and flow cytometry. Cells were gated on $CD4^+$ cells. (B) IL-17A, IL-22, and IL-10 secretion was measured by ELISA. (C) *Il17a, Il22* and *Foxp3* expression was quantified by RT-PCR. (A-C) Pooled Th0 and Th17 results depicted in Figures 2.1 and 2.2 are shown again for comparison.

N≥3. (D) Representative flow plots of $CD4^+$ cells gating on IL-17A and FoxP3 in Th0 and IL-6 + IL-23 + TGF-β (various concentrations) conditions. (E) Naïve T cells were cultured for 3 days with IL-6, IL-23, and varying concentrations of TGF-β. Proportions of IL-17A and FoxP3 positive cells were measured by intracellular staining and flow cytometry. Cells were gated on $CD4^+$ cells. N=3. Error bars represent means ± sem. *$p<0.05$, **$p<0.005$.




**Supplementary Figure 2.5: Tpl2 does not regulate Th17 transcription factor expression or activation**. Naïve T cells were cultured under Th17 polarizing conditions for 3 days +/- 10 ng/ml IL-1β. (A) *Rorc*, *Rora* and *Irf4* expression was measured by RT-PCR on day 3 of culture. N≥6 experiments. (B) Th0 cells cultured for 3 days were expanded an additional 4 days with IL-2 (40 IU/ml) prior to stimulation with IL-6 (10 ng/ml) for 30 minutes at 37°C. Whole cell lysates were immunoblotted for phosphorylated STAT3 (pSTAT3) and total STAT3 (STAT3). N=2 experiments. (C) *Il23r* expression by RT-PCR of T cells cultured for up to 3 days +/- 10 ng/mL IL-6. Expression levels are relative to wild type day 0. N=2 experiments.




**Supplementary Figure 2.6: Tpl2 does not regulate IL-2 secretion in Th0 or Th17 cells**. Naïve CD4 T cells were cultured under Th17 polarizing conditions or with IL-6 and IL-23 up to 3 days. (A) On day 3, supernatants were collected and analyzed for IL-17A and IL-2 secretion by ELISA. Data shown are representative of 4 independent experiments. (B) On days 1 through 3, supernatants were collected from Th17 cultures and analyzed for IL-2 secretion by ELISA. N≥4. Error bars represent means ± SE. *$p<0.05$

# CHAPTER 3

# TPL2 PROMOTES TH1/TH17 DIFFERENTIATION AND NEUTROPHIL ACCUMULATION AND PROTECTS AGAINST DISSEMINATION DURING *CITROBACTER DODENTIUM* INFECTION[2]

[2] Acuff, N.V, X. Li, T. Nagy and W.T. Watford. To be submitted to *Infection and Immunity*

**ABSTRACT**

Tumor progression locus 2 (Tpl2) is a serine-threonine kinase that regulates Th1 differentiation, secretion of the inflammatory cytokine IFNγ, and host defense against the intracellular pathogens *Toxoplasma gondii, Listeria monocytogenes*, and *Mycobacterium tuberculosis*. However, there is relatively little known about the contribution of Tpl2 to Th17 differentiation and immune cell function during infection with an extracellular pathogen. The goal of this study was to determine whether Tpl2 influences the immune response generated to the extracellular bacterium *Citrobacter rodentium*, which induces a mixed Th1/Th17 response. During peak infection with *C. rodentium*, *Tpl2*$^{-/-}$ mice experienced greater bacterial burdens with greater dissemination but ultimately cleared the bacteria within three weeks post infection similar to wild type mice. *Tpl2*$^{-/-}$ mice also recruited fewer neutrophils to the colon during peak infection, which correlated with enhanced dissemination of the bacteria into the liver and spleen and increased peak bacterial burdens. Within mixed bone marrow chimeras, Tpl2 was shown to play a T-cell intrinsic role in promoting both IFNγ and IL-17A, but not IL-22, production during infection with *C. rodentium*. However, upon CD4 T cell transfer into *Rag*$^{-/-}$ mice, recipients of *Tpl2*$^{-/-}$ CD4 cells were equally protective against dissemination of bacteria and mortality. These data indicate that enhanced bacterial burdens within *Tpl2*$^{-/-}$ mice are not caused by impairments in CD4 T cell function but result from defects in innate immune cell recruitment and function.

## INTRODUCTION

*Citrobacter rodentium* is a non-motile gram-negative rod that is a natural mouse and gerbil pathogen (238, 239). Upon infection, *C. rodentium* colonizes the large intestine, primarily the cecum and distal portion of the colon (257), and forms a close association with the epithelium and lamina propria that results in attaching and effacing lesions in the large intestine (253, 254) similar to human *Escherichia coli* infection. However, *C. rodentium* can disseminate out of the intestines and be found in the nasopharynx, lung, heart, liver, and spleen (244). Early innate responses to *C. rodentium* are associated with recruitment and antimicrobial functions of neutrophils, macrophages, NK cells and innate lymphoid cells (250, 258, 263, 264, 267, 306). Neutrophils secrete IL-17A and IL-22, promote the production of antimicrobial defensins by epithelial cells, and protect against development of diarrhea (159, 306). Bacterial association with the lamina propria of the large intestine subsequently induces a mixed Th1 and Th17 response associated with IL-12, IFNγ, TNF, IL-17A, and IL-22 expression (268-270, 273). Clearance of the bacteria occurs within three weeks in a wild type host and is dependent upon both CD4 T cell and B cell functions (261, 272).

Tumor progression locus 2, Tpl2 (also known as MAP3K8), is a serine-threonine protein kinase that is expressed in both innate and adaptive immune cells. The role of Tpl2 in promoting an inflammatory immune response has been extensively studied in macrophages and dendritic cells (24, 25). Tpl2 has also been shown to promote Th1 cell differentiation and production of IFNγ (28). Therefore, *Tpl2*$^{-/-}$ mice experience greater susceptibility and infectious burden in response to the protozoan parasite *Toxoplasma gondii* (28), or the intracellular bacteria *Listeria monocytogenes* (25) and *Mycobacterium tuberculosis* (47). However, *Tpl2*$^{-/-}$ mice are resistant to endotoxin-induced septic shock due to reduced production of TNF (24). Because Tpl2 promotes

TNF processing and secretion (24, 45), it is being investigated as a therapeutic target for treating autoimmune diseases, especially those exacerbated by TNF, such as rheumatoid arthritis (288-290).

Th17 cells are a distinct lineage of $CD4^+$ T cells that produce IL-17A IL-17F, IL-21, and IL-22 (56, 57, 70-73), with one of their main downstream functions being recruitment of neutrophils to assist in clearance of microbes (307-309). Together, Th17 effector cytokines are required for the clearance of extracellular bacterial and fungal infections, including *Citrobacter rodentium*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, and *Candida albicans,* while also contributing to inflammation associated with autoimmune diseases (reviewed in (76)). We have recently shown that Tpl2 promotes Th17 cell differentiation and secretion of IL-17A, but not IL-22, *in vitro* (107). However, Tpl2 has little impact on Th17 cell production of IL-17A *in vivo* during MOG-induced EAE (52) or in a T cell transfer model of colitis (107). It has yet to be investigated whether Tpl2 influences Th17 cell differentiation, IL-17A production or neutrophil accumulation during extracellular bacterial or fungal infections. Understanding how Tpl2 regulates Th17 responses during infection may provide valuable information about the range of potential benefits or risks associated with Tpl2 inhibition in various disease settings.

Upon infection with *C. rodentium, Tpl2*$^{-/-}$ mice experienced greater bacterial burdens during peak infection, but were capable of clearing the bacteria within three weeks similar to wild type mice. Infection within *Tpl2*$^{-/-}$ mice was not confined to the intestines and was also detected within the liver and spleen of infected mice. Eleven days post infection, lymphocytes within the lamina propria expressed IL-17A, IL-22, and IFNγ with *Tpl2*$^{-/-}$ CD4 T cells trending towards reduced proportions of IL-17A-, IL-22-, and IFNγ-positive cells. This defect was confirmed to be T cell-intrinsic as *Tpl2*$^{-/-}$ CD4 T cells within mixed bone marrow chimeras were less likely to

differentiate into Th1 and Th17 cells expressing IL-17A and IFNγ, respectively. Despite this T cell-intrinsic defect, *Tpl2*$^{-/-}$ CD4 T cells transferred into *Rag*$^{-/-}$ mice were as equally protective as wild type CD4 T cells and protected against death and bacterial dissemination, suggesting critical T cell-extrinsic functions for Tpl2 in protection against *C. rodentium* infection. Interestingly, the colons of *Tpl2*$^{-/-}$ mice had reduced inflammation and neutrophil infiltrates relative to wild type mice, indicating that impaired neutrophil recruitment and function may contribute to enhanced bacterial burdens within *Tpl2*$^{-/-}$ mice. Overall, our findings confirm the importance of Tpl2 in driving the development of the pro-inflammatory Th1 lineage and neutrophil recruitment as well as promoting IL-17A expression during infection with extracellular bacteria.

**MATERIALS AND METHODS**

*Mice*

Wild type (C57BL/6) and *Rag1*$^{-/-}$ mice were obtained from the Jackson Laboratory. Tpl2-deficient mice backcrossed more than ten generations onto the C57BL/6 genetic background were kindly provided by Dr. Philip Tsichlis and Thomas Jefferson University. B6-Ly5.1/Cr (CD45.1$^{+}$) mice were obtained from Charles River Laboratories and were intercrossed with wild type mice to generate heterozygous CD45.1/CD45.2 mice at the University of Georgia. *Rag1*$^{-/-}$ mice were crossed with *Tpl2*$^{-/-}$ mice to generate *Rag1*$^{-/-}$*Tpl2*$^{-/-}$ mice at the University of Georgia. *Tpl2*$^{+/-}$ matings generated *Tpl2*$^{+/+}$, *Tpl2*$^{+/-}$ and *Tpl2*$^{-/-}$ mice used for infections. Animals were used at six to twelve weeks of age, and were age- and sex-matched for individual experiments. All experiments involving mice were performed according to the University of Georgia guidelines for laboratory animals and were approved by the UGA Institutional Animal Care and Use Committee.

*Adoptive transfer and bone marrow chimeras*

Wild type or *Tpl2*$^{-/-}$ cells from spleens and lymph nodes were disaggregated by pressing through a 70 μm filter, and CD4 T cells were purified by negative selection using a CD4 T cell isolation kit (Stemcell Technologies, Vancouver, Canada) according to the manufacturer's guidelines. 2-3x10$^6$ CD4 T cells were transferred into *Rag1*$^{-/-}$ mice i.v. Two to three weeks post transfer, reconstitution of CD4 T cells was confirmed by tail bleed and flow cytometry. CD45.1$^+$ mice were lethally irradiated with a dose of 1100 rad. The following day, mice were reconstituted with bone marrow from CD45.1$^+$CD45.2$^+$ (WT) and *Tpl2*$^{-/-}$ mice. Bone marrow was isolated from femurs and tibiae of naïve mice. T cells were depleted by positive selection using CD3 biotin (145-2C11) (eBioscience), anti-biotin microbeads (Miltenyi Biotech, Auburn, CA), and collection of the negative fraction using an AutoMACS (Miltenyi Biotech) according to manufacturer's guidelines. Cells were counted, and 3-4x10$^6$ mixed bone marrow cells were injected into CD45.1$^+$ mice i.v.

*Citrobacter rodentium infection and burden quantification*

The *C. rodentium* strain used was a luminescent strain (ICC180) kindly provided by Gad Frankel at Imperial College, London UK (310). Mice were inoculated with a low dose (1–2 × 10$^7$ CFU) or high dose (1–2 × 10$^9$ CFU) in a total volume of 200 μl via gastric gavage. The dose was confirmed by retrospective plating on LB agar plates. For quantification of bacterial burden, feces were diluted in 100 μl PBS per 0.01 g feces, spleens were homogenized in 1 ml PBS, livers were homogenized in 2 ml PBS, and 100 μl of blood was immediately diluted in 900 μl PBS followed by serial dilutions and plating on LB agar in triplicate. Plates were imaged for luminescent colonies using an IVIS Imager (Perkin Elmer) and counted to determine CFU/g feces, CFU/spleen, CFU/liver, and CFU/ml blood. Limit of detection was set at 10$^3$ CFU/g feces,

500 CFU/ml blood, 50 CFU/liver, and 25 CFU/spleen. For imaging, mice were anesthetized using either TBE or isoflurane and imaged for 1 minute using an IVIS Lumina Imager (Perkin Elmer) to collect luminescence data.

*Intraepithelial Cells and Lamina propria lymphocytes (LPL)*

LPLs were purified from colons of mice as previously described (311, 312). For isolation of intraepithelial cells, colons were cut into fragments and washed 3 times with RPMI 1640 medium containing 5% FCS (Invitrogen) and 5 mM EDTA (Fisher Scientific) for 15 min at 37°C in a shaking incubator. For LPLs, tissue was further subjected to two digestions with 0.5 mg/mL collagenase (Sigma-Aldrich) and 0.1 mg/mL DNase (Roche) in RPMI containing 5% FCS and 15 mM Hepes (Invitrogen) with continuous shaking at 37° for 20 min. Supernatants from each digestion were passed through a 70 μm cell strainer. Lymphocytes were enriched by Percoll (GE healthcare) gradient purification using a 30/45/70% gradient and collecting the cells at the 45/70% interface. Cells were stimulated 4 hours at 37°C with 50 ng/ml PMA (Sigma-Aldrich), 0.5 μg/ml ionomycin (Sigma-Aldrich), and golgi transport inhibitor (BD Biosciences) according to manufacturer's specifications. The following anti-mouse monoclonal antibodies used were from eBiosciences: CD16/CD32 (93), CD45.1 (A20), CD45.2 (104), CD4 (RM4-5), TCRβ (H57-597), Gr-1 (RB6-8C5), CD11b (M1/70), IL-17A (eBio17B7), IFNγ (XMG1.2), and IL-22 (1H8PWSR). Samples were run on a BD LSRII flow cytometer and analyzed using FlowJo software (Tree Star, Inc., Ashland, OR).

*Pathology*

Colonic sections from mice were collected and fixed in 10% neutral buffered formalin for 24 h at room temperature. Complete cross sections of formalin-fixed intestinal sections were placed in cassettes, embedded in paraffin, sectioned at 4 μm thickness, mounted on glass slides, and

stained with H&E. Histological sections were evaluated by a veterinary pathologist (TN) and scored according to the following criteria: Distribution of the inflammation: 0=None, 1=Focal, 2=Multifocal, 3=Diffuse; Degree of inflammation: 0=None, 1=Mild, 2=Moderate, 3=Severe; Extent of erosion and/or ulceration: 0=None, 1=Superficial (lamina propria only), 2: Moderate (extends to the submucosa), 3: Severe (transmural). Scores were summed to give a total pathology score.

*Statistics*

*P* values were derived by paired or unpaired student's t-test, log-rank test, or two-way ANOVA using Prism software. Differences were considered statistically significant if $p \leq 0.05$.

**RESULTS**

**Tpl2-deficient mice have higher bacterial burdens during peak infection with *Citrobacter rodentium*.**

Wild type and *Tpl2*$^{-/-}$ mice were infected with $2x10^9$ CFU *Citrobacter rodentium* ICC180. Bioluminescent images from the gastrointestinal region were collected throughout infection until clearance of the bacteria 21 days post infection (dpi). Infection was confirmed by measuring fecal burdens of *C. rodentium*. Beginning at 7 dpi, *Tpl2*$^{-/-}$ mice had significantly higher bacterial burdens than wild type mice as detected by changes in luminescence (Figure 3.1A-B). This trend continued until approximately 12-14 dpi, at which point both wild type and *Tpl2*$^{-/-}$ mice similarly cleared the bacteria (Figure 3.1).

**Tpl2 is required for maintenance of the intestinal barrier**

Because *Tpl2*$^{-/-}$ mice showed greater luminescence at 7-12 dpi (Figure 3.1B) but no differences in fecal burden until 12 dpi (Figure 3.1C), and because *C. rodentium* is known to disseminate out of the intestines (244), we hypothesized that greater bacterial dissemination was occurring in

*Tpl2*$^{-/-}$ mice. As expected, *Tpl2*$^{-/-}$ mice had greater bacterial burdens within the liver and spleen at 8 and 11 dpi (Figure 3.2). Pinpoint lesions were also more frequently observed on the livers of *Tpl2*$^{-/-}$ mice (Figure 3.2A). Combined, *Tpl2*$^{-/-}$ mice not only showed greater bacterial burdens compared to wild type mice in the intestines (Figure 3.1), but also dissemination to other organs, including the liver and spleen (Figure 3.2).

**Tpl2 enhances Th1 and Th17 responses to *C. rodentium* via a T cell-intrinsic mechanism.**

Due to elevated bacterial burdens within *Tpl2*$^{-/-}$ mice by luminescence at 8-12 dpi (Figure 3.1B) and greater fecal burdens at 12-14 dpi (Figure 3.1C), we hypothesized there may be a Tpl2-dependent defect in the adaptive immune response. Because of the overlap between elevated luminescence and fecal burdens at 12 dpi, generation of adaptive immune responses was evaluated at this time. As expected, lymphocytes within the lamina propria expressed IL-17A, IL-22, and IFNγ (268-270, 273). Compared to wild type mice, *Tpl2*$^{-/-}$ mice consistently trended towards reduced induction of Th1 and Th17 cells expressing IL17A, IL22, and IFNγ (Figure 3.3A-B). To determine whether Tpl2 influences T helper cell polarization *in vivo* via a T cell-intrinsic mechanism, mixed bone marrow chimeras were generated and similarly infected with *C. rodentium*. Within mixed bone marrow chimeras, a significantly lower proportion of *Tpl2*$^{-/-}$ T cells (CD45.2$^{+}$) expressed IL-17A and IFNγ compared to wild type T cells (CD45.1/2$^{+}$) within the same host (Figure 3.3C). However, *Tpl2*$^{-/-}$ T cells were similarly able to express IL-22 (Figure 3.3C). These data confirm the role of Tpl2 in Th1 cell differentiation and IFNγ expression as well as Th17 cell differentiation and expression of IL-17A, but not IL-22, in response to *C. rodentium* infection.

### *Tpl2*$^{-/-}$ CD4 T cells adoptively transferred into *Rag1*$^{-/-}$ mice are protective against *C. rodentium*

To determine whether defects in Th1 and Th17 cell differentiation within the large intestines influenced disease outcome, wild type or *Tpl2*$^{-/-}$ CD4 T cells were adoptively transferred into *Rag1*$^{-/-}$ mice. Approximately 2-3 weeks post-transfer, mice were infected with a low dose of *C. rodentium.* Because *Rag*$^{-/-}$ mice are deficient in T cells and B cells (224), they are incapable of clearing the bacteria and quickly succumb to infection (276). Compared to *Rag1*$^{-/-}$ mice, recipients of either wild type or *Tpl2*$^{-/-}$ CD4 T cells survived up to 21 dpi (Figure 3.4A) despite similarly elevated bacterial burdens (Figure 3.4B). Localization of *C. rodentium* was visualized using luminescent images taken throughout the time course (Figure 3.4C) and dissemination was quantified at time of death. Notably, transfer of either wild type (181) or *Tpl2*$^{-/-}$ CD4 T cells into *Rag1*$^{-/-}$ mice restored intestinal barrier function to *Rag1*$^{-/-}$ mice. Even though all *Rag1*$^{-/-}$ mice experienced similar bacterial burdens throughout infection, independent of adoptive transfer (Figure 3.4B), *Rag1*$^{-/-}$ mice that received either wild type or *Tpl2*$^{-/-}$ CD4 T cells also had similarly reduced dissemination of *C. rodentium* into the blood, liver, and spleen (Figure 3.4D). For most *Rag1*$^{-/-}$ mice that received CD4 T cells, *C. rodentium* was no longer detected within the circulation (Figure 3.4D). These results indicate that *Tpl2*$^{-/-}$ CD4 T cells are as protective as wild type CD4 T cells upon transfer into *Rag1*$^{-/-}$ mice and that Tpl2 primarily functions in a CD4 T cell-extrinsic manner to influence bacterial burdens and dissemination during *C. rodentium* infection.

### Tpl2 promotes neutrophil accumulation within the colon during *C. rodentium* infection

Because Tpl2 within CD4 T cells did not influence total bacterial burdens experienced by *Rag1*$^{-/-}$ mice (Figure 3.4B), we next investigated innate immune responses generated during peak

infection. *C. rodentium* infection induces neutrophil recruitment into the large intestines (250, 306), which not only phagocytose and kill bacteria (250, 306), but also contribute to inflammation and pathology (249). A comparison of pathology between wild type and *Tpl2*$^{-/-}$ mice showed reduced total inflammation within the large intestines of *Tpl2*$^{-/-}$ mice (Figure 3.5A-B) despite increased bacterial burdens at 8 and 11 dpi. Accordingly, an evaluation of neutrophil recruitment at the same time points showed reduced proportions of neutrophils recruited to the epithelial layer of the colons of *Tpl2*$^{-/-}$ mice during infection (Figure 3.5C), consistent with reduced pathology scores.

**DISCUSSION**

Understanding how Tpl2 regulates Th17 responses during infection may provide valuable information about the range of potential benefits or risks associated with Tpl2 inhibition in various disease settings. In this study, we investigated the role of Tpl2 during host defense against the gram-negative extracellular bacterium, *Citrobacter rodentium,* which induces a mixed Th1/Th17 response for protection. *Tpl2*$^{-/-}$ mice experienced greater bacterial burdens during peak infection with *C. rodentium* as detected by luminescence and fecal burdens. Interestingly, the colons of *Tpl2*$^{-/-}$ mice had reduced inflammation and neutrophil infiltrates relative to wild type mice, indicating that impaired neutrophil recruitment and function may contribute to enhanced bacterial burdens within *Tpl2*$^{-/-}$ mice. Despite greater bacterial burdens and dissemination, *Tpl2*$^{-/-}$ mice were equally capable of clearing the bacteria within three weeks similar to wild type mice. Evaluation of *Tpl2*$^{-/-}$ CD4 T cells within the lamina propria of mixed bone marrow chimeras indicated a T cell-intrinsic role for Tpl2 in IL-17A and IFNγ expression by CD4 T cells. However, *Tpl2*$^{-/-}$ CD4 T cells transferred into *Rag*$^{-/-}$ mice were as equally protective as wild type CD4 T cells against death and bacterial dissemination, suggesting critical

T cell-extrinsic functions for Tpl2 in protection against *C. rodentium* infection.

Tpl2 has previously been shown to promote Th1 cell differentiation and production of IFNγ *in vitro* and *in vivo* (28), as well as Th17 cell differentiation and secretion of IL-17A, but not IL-22, *in vitro* (107). Accordingly, *Tpl2*$^{-/-}$ mice are more susceptible to the Th1-inducing intracellular pathogens *Toxoplasma gondii* (28), *Listeria monocytogenes* (25) and *Mycobacterium tuberculosis* (47). *M. tuberculosis* is known to induce a mixed Th1/Th17 response (reviewed in (313)), but whether Tpl2 impacted Th17 cell differentiation in this model was not investigated. Consistent with our previous reports of Tpl2 regulating Th1 and Th17 cell differentiation, *Tpl2*$^{-/-}$ CD4 T cells within the lamina propria were less likely to differentiate into Th1 and Th17 cells expressing IFNγ and IL-17A, respectively, during infection with *C. rodentium*. In contrast, Tpl2 did not influence CD4 T cell expression of the Th17-associated cytokine IL-22 during infection. These findings are similar to those observed in Th17 cells cultured *in vitro* in which IL-17A expression was significantly reduced but the expression of IL-22 or the Th17-associated transcription factors RORα, RORγt and IRF4 were unaffected (107), suggesting a specific defect in IL-17A expression rather than a global defect in Th17 cell differentiation.

Greater dissemination of *C. rodentium* into organs of *Tpl2*$^{-/-}$ mice indicates that Tpl2 regulates permeability of the intestinal barrier. Lymphocytes within the lamina propria are known to assist in maintenance of the intestinal barrier by promoting epithelial cell differentiation (181); therefore, CD4- and B cell-deficient mice display reduced ability to limit dissemination of *C. rodentium* (182). However, because *Rag1*$^{-/-}$ mice that received either wild type or *Tpl2*$^{-/-}$ CD4 T cells experienced similar levels of dissemination into the liver and spleen, Tpl2 within CD4 T cells does not influence intestinal permeability. Absence of B cells and antibody production during infection with *C. rodentium* is associated with significantly delayed clearance as well as

enhanced fecal burden over time (261, 262). B cell proliferation, activation, and secretion of antibodies is initialized through crosslinking CD40 expressed on the surface of B cells (reviewed in (314)), which signals via Tpl2 to activate ERK and promote class switching to IgE (26). However, Tpl2 ablation does not impair B cell activation, proliferation or secretion of IgG1 (26). Therefore, although B cells do not appear to influence bacterial clearance due to the ability of *Tpl2*$^{-/-}$ mice to clear infection similar to wild type mice, we cannot exclude the possibility that Tpl2-deficient B cells contribute to higher bacterial burdens and dissemination experienced at two weeks post infection.

Prior to induction of an adaptive immune response, innate cells within the intestines respond to PAMPs expressed by *C. rodentium*, including LPS and lipoproteins that activate TLR2 (315) and TLR4 (248) leading to downstream signaling through MyD88. TLR2-, TLR4- and MyD88-deficient mice infected with *C. rodentium* have significant mortality associated with epithelial barrier damage and reduced barrier function (248-250, 315). Because Tpl2 is activated downstream of TLR2 and TLR4, among others (23-25, 316), we would expect severe pathology and reduced barrier function in *Tpl2*$^{-/-}$ mice. However, *Tpl2*$^{-/-}$ mice did not have more severe pathology compared to wild type mice, but did have enhanced bacterial dissemination that may be due to impaired intestinal integrity. Claudins are known to play a significant role in maintaining intestinal integrity through formation of tight junctions. As an example of some claudins, claudin 2 and 5 require signaling through MEK/ERK for protein expression on epithelial cell lines (177, 179) and gene expression of claudin 1 and 2 can be upregulated by IL-17A stimulation (177). Claudin 7 alternatively requires signaling through p38, and not MEK or JNK, for protein expression in epithelial cells (180). Therefore, because Tpl2 signals upstream of the MEK/ERK pathway (11), contributes to IL-17A production *in vitro* (107) and *in vivo*

(herein), as well as IL-17R signaling through p38 (38), it is possible that Tpl2 regulates the expression of various claudins on the surface of epithelial cells. Therefore, Tpl2 may normally promote integrity of the intestinal barrier, as well as other epithelial barriers, by inducing the expression of tight junction proteins.

Even with greater bacterial burdens, *Tpl2*$^{-/-}$ mice experienced reduced pathology and neutrophil recruitment to the large intestine. These results are consistent with the findings that Tpl2 regulates the expression of inflammatory cytokines and chemokines and the recruitment of neutrophils to the site of inflammation (38, 42, 317, 318). Reduced pathology within *Tpl2*$^{-/-}$ mice may also be due to greater dissemination, which would induce a more diffuse and less localized inflammatory response within the host. Additionally, because reduced pathology is observed during the adaptive phase of the immune response, defects in neutrophil recruitment within *Tpl2*$^{-/-}$ mice may be an indirect result of reduced TNF production and/or signaling in *Tpl2*$^{-/-}$ mice in response to LPS (24) as well as reduced IL-17A production. IL-17A and TNF in combination enhance neutrophil recruitment through elevated secretion of CXCL1, CXCL2, CXCL8, GM-CSF and G-CSF (309, 319, 320). Similar to *Tpl2*$^{-/-}$ mice, *Cxcr2*$^{-/-}$ mice, which are deficient in neutrophil recruitment, experience elevated fecal burdens two weeks post infection with *C. rodentium* as well as greater dissemination into the liver and spleen (306). These data suggest that the observed defects in neutrophil recruitment into the large intestine of *Tpl2*$^{-/-}$ mice may explain both the greater bacterial burdens as well as increased dissemination in *Tpl2*$^{-/-}$ mice.

Overall, our findings underscore the importance of Tpl2 in driving the development of the pro-inflammatory Th1 lineage as well as promoting IL-17A expression. These results also confirm the importance of Tpl2 in promoting neutrophil recruitment and development of pathology

during inflammation. Furthermore, it highlights the important role of Tpl2 in maintenance of epithelial barriers and prevention of bacterial dissemination.

**ACKNOWLEDGEMENTS**

We thank Monica LaGatta for excellent technical support and maintenance of our mouse colony as well as UGA's Veterinary Medicine Central Animal Facility for animal care.

## FIGURES

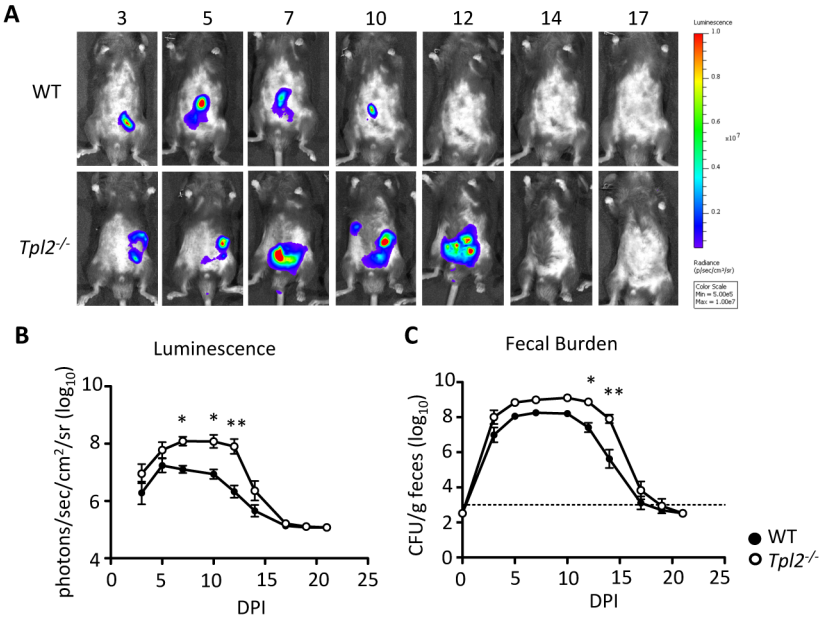



**Figure 3.1- *Tpl2*$^{-/-}$ mice have greater bacterial during peak infection.** Wild type and *Tpl2*$^{-/-}$ mice were gavaged with 2x10$^{9}$ CFU *Citrobacter rodentium* (ICC180). Bioluminescent images from the gastrointestinal region are displayed as pseudocolor images, with variations in color representing the light intensity at a given location. Red represents the most intense light emission, while purple corresponds to the weakest signal. (A) Representative and (B) pooled luminescence data of wild type and *Tpl2*$^{-/-}$ mice from 3 – 21 dpi are shown. (C) Fecal burden was quantified 0 – 21 dpi. Dashed line represents limit of detection. When no fecal burden was detected, a value of 3x10$^{2}$ CFU/g was assigned. N=8. Data are pooled from 2 independent experiments. Error bars represent means ± sem. *p<0.05, **p<0.005.

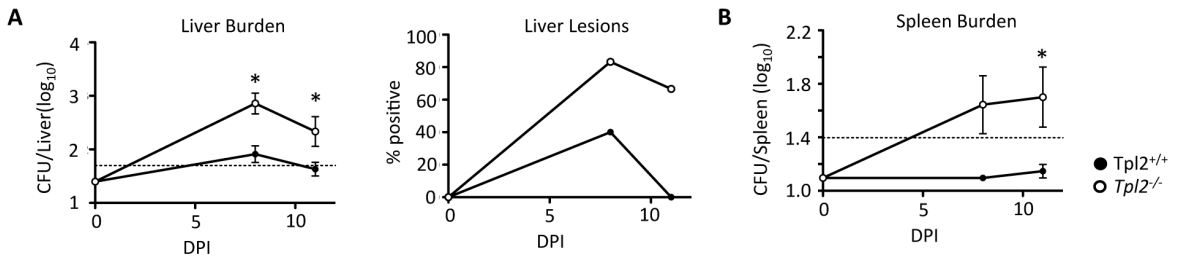



**Figure 3.2- *Tpl2*$^{-/-}$ mice have greater bacterial dissemination during peak infection.** Wild type and *Tpl2*$^{-/-}$ mice were gavaged with 2x10$^{9}$ CFU *Citrobacter rodentium* (ICC180). Mice were euthanized 8 or 11 dpi. (A-B) Liver and spleens were collected, assessed for lesions, and homogenized to measure bacterial burden. Dashed line represents limit of detection. N≥4. Data pooled from 3 independent experiments. *p<0.05.

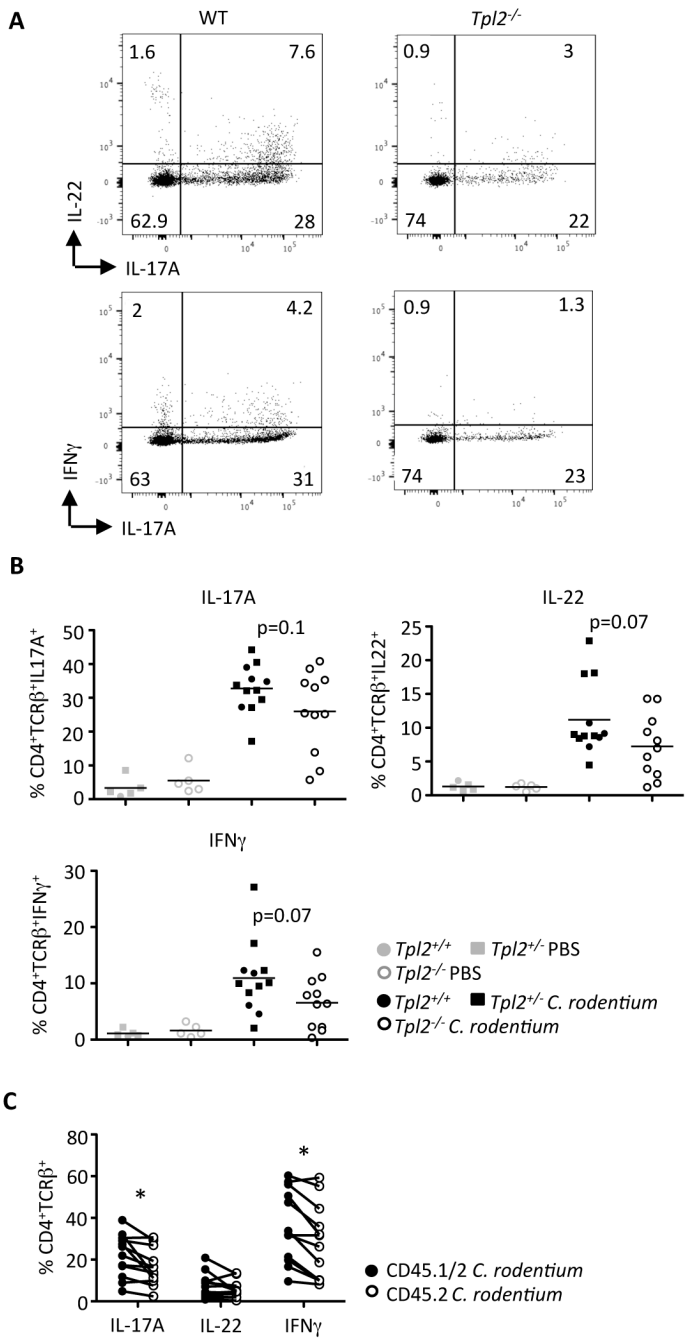



**Figure 3.3- Tpl2 promotes a mixed Th1/17 response.** (A-B) Wild type (*Tpl2*$^{+/+}$ and *Tpl2*$^{+/-}$) and *Tpl2*$^{-/-}$ mice or (D) mixed bone marrow chimeras were gavaged with 2x10$^{9}$ CFU *Citrobacter rodentium* (ICC180). (A) Representative plots of LPLs isolated 11 dpi and stained for (A-B) CD4$^{+}$TCRβ$^{+}$ or (C-D) CD45.1$^{+}$CD45.2$^{+}$ wild type or CD45.1$^{-}$CD45.2$^{+}$ *Tpl2*$^{-/-}$ CD4$^{+}$TCRβ$^{+}$ cells

expressing IL-17A, IL-22, or IFNγ. (B) Lines represent mean. N≥5. (C) Lines represent data from a single host. N=12. Data pooled from 2 or more independent experiments. *$p<0.05$.

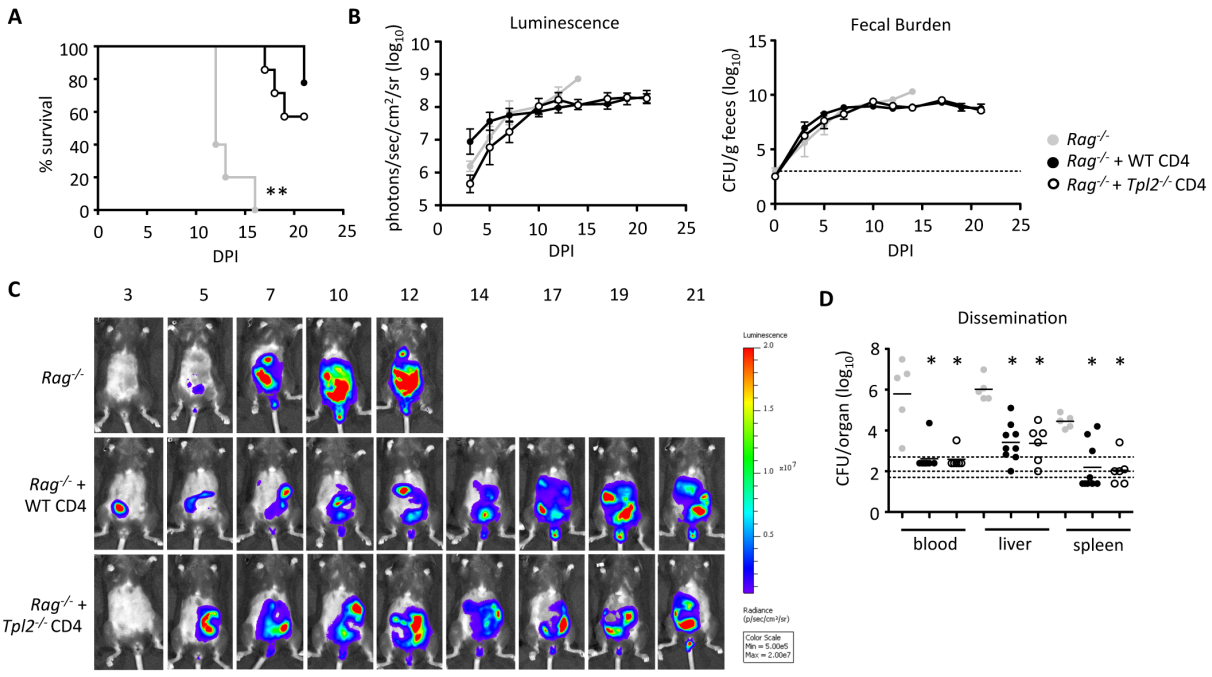



**Figure 3.4- *Tpl2*$^{-/-}$ CD4 cells are equally protective once transferred into *Rag*$^{-/-}$ mice.** *Rag*$^{-/-}$ mice receiving wild type CD4, *Tpl2*$^{-/-}$ CD4, or no CD4 cells were infected with low dose *Citrobacter rodentium*. (A) Body weights were recorded and mice exhibiting severe signs of disease, including more than 20% weight loss were euthanized. (B) Pooled luminescence data and fecal burden, plus (C) representative images from 3 – 21 dpi are shown. Dashed lines represents limit of detection. Error bars represent means ± sem. (D) Dissemination into the blood, liver, and spleen was quantified at time of death. Lines represents mean. Dashed lines represents limit of detection for blood (top), liver (middle), and spleen (lower). N≥5. Data are pooled from 2 independent experiments. *p<0.05, **p<0.005 comparing *Rag*$^{-/-}$ ± CD4 cells.

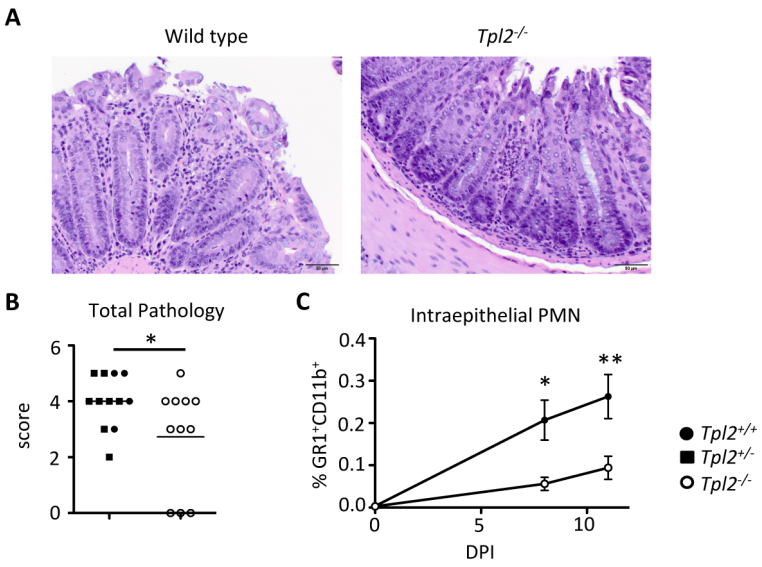



**Figure 3.5- *Tpl2*$^{-/-}$ mice have reduced neutrophil recruitment to the colon during the adaptive immune response.** Wild type and *Tpl2*$^{-/-}$ mice were gavaged with 2x10$^{9}$ CFU *Citrobacter rodentium* (ICC180). Mice were euthanized 8 or 11 dpi. Colons were isolated and (B) scored for pathology at 11 dpi and (C) the proportion of neutrophils within the epithelial barrier were determined by flow cytometry staining for Gr-1 and CD11b at 8 and 11 dpi. (A) Representative images are given. 200x original magnification, scale bar=50µm. Lines represent mean. N≥4. Data pooled from 3 independent experiments. *p<0.05, ** p<0.005

# CHAPTER 4

# TPL2 PROMOTES NEUTROPHIL TRAFFICKING, OXIDATIVE BURST AND BACTERIAL KILLING [3]

[3] Acuff, N.V, J. Elmore, B. Rada and W.T. Watford. Submitted to the *Journal of Leukocyte Biology* (under review)

**ABSTRACT**

Tumor progression locus 2 (Tpl2) is a serine/threonine kinase that promotes inflammatory cytokine production by activating the MEK/ERK pathway. Tpl2 has been shown to be important for eliciting the inflammatory properties of macrophages, however there is relatively little known about Tpl2's contribution to neutrophil effector functions. This is an important consideration, since neutrophils provide the first line of defense against infection in the innate immune system. We found that Tpl2 is expressed in both human and murine neutrophils, suggesting a potential function for Tpl2 within this lineage. Despite significantly higher proportions of bone marrow neutrophils in *Tpl2*$^{-/-}$ mice compared to wild type mice, *Tpl2*$^{-/-}$ mice have significantly reduced proportions of circulating neutrophils due to reduced circulating levels of G-CSF. In response to infection, neutrophils secrete inflammatory cytokines and produce reactive oxygen species (ROS), which promote bacterial killing. Tpl2 ablation impaired neutrophil TNF production in response to LPS stimulation, superoxide generation in response to the chemotactic peptide formyl-methionyl-leucyl-phenylalanine (fMLP), and killing of the extracellular bacterium, *Citrobacter rodentium*. These results implicate Tpl2 in the regulation of multiple neutrophil antimicrobial pathways, including inflammatory cytokine secretion and oxidative burst. Furthermore, they indicate that Tpl2 functions early during infection to bolster neutrophil-mediated innate immunity against extracellular bacteria.

## INTRODUCTION

Tumor progression locus 2, Tpl2 (also known as MAP3K8), is a serine-threonine protein kinase originally described as an oncogene, because its C-terminal truncation promoted tumors in rodents (6). Tpl2 is expressed in both innate and adaptive immune cells and in diverse tissues including the spleen, thymus, liver, lung, and intestines (6, 10, 11). Tpl2 promotes inflammation by inducing cytokines (24, 25, 28, 41), chemokines (42, 43) and other inflammatory mediators (44). Many of Tpl2's functions have been attributed to its activation of the MEK/ERK pathway (reviewed in (11)), but Tpl2 also influences other cellular pathways as well, including p38, JNK, Akt and mTOR in a context-dependent manner (10, 38, 39). In macrophages, Tpl2 is required for the processing and secretion of TNF (24, 45). Initial characterization of *Tpl2*$^{-/-}$ mice identified major defects in the induction of proinflammatory cytokines, particularly TNF, by antigen presenting cells that conveyed resistance to endotoxin-induced septic shock in *Tpl2*$^{-/-}$ mice (24). Because it promotes TNF secretion, Tpl2 is being investigated as a therapeutic target for treating autoimmune diseases, especially those exacerbated by TNF, such as rheumatoid arthritis (288-290). However, little is known about how Tpl2 regulates neutrophil functions.

Neutrophils are the first cell type recruited to sites of inflammation from the circulation. It has been shown previously that *Tpl2*$^{-/-}$ mice treated with zymosan, acetaminophen, or caerulein show decreased neutrophil recruitment associated with peripheral inflammation (42, 317, 318). However, reduced recruitment was attributed to impaired expression of inflammatory chemokines at the tissue sites, and the functionality of *Tpl2*$^{-/-}$ neutrophils was not investigated in these studies. Once within inflamed tissue, the primary function of neutrophils is the release of granules and phagocytosis of microbes and infected cells. Neutrophils kill and degrade microbes within the neutrophil phagolysosome through production of reactive oxygen species (ROS) by

NAPDH oxidase, reactive nitrogen species (RNS) and proteolytic enzymes, such as lysozyme and neutrophil elastase (reviewed in (153)). Overactivation of neutrophils can lead to damage of normal tissue through release of ROS, RNS, and enzymes. Downstream signaling through ERK assists in neutrophil development and differentiation from common progenitor cells (321) and in neutrophil antimicrobial functions (322, 323). Because Tpl2 regulates ERK function in a cell type- and stimulus-specific manner (10), we hypothesize that Tpl2 may contribute to neutrophil development and/or antimicrobial functions.

We first confirmed the expression of Tpl2 within neutrophils. In *Tpl2*$^{-/-}$ mice, we observed greater accumulation of neutrophils within the bone marrow and reduced proportions of circulating neutrophils. This difference correlated with reduced neutrophil recruitment in response to thioglycollate. However, *Tpl2*$^{-/-}$ neutrophils within the circulation expressed similar levels of the chemokine receptors and selectins CXCR2, CXCR4, CD44 and CD62L, and Tpl2 primarily regulated neutrophil recruitment in a cell-extrinsic manner. However, upon *ex vivo* stimulation, reduced TNF secretion, superoxide production and bacterial killing was observed in *Tpl2*$^{-/-}$ neutrophils due to cell-intrinsic functions of Tpl2. Combined, these data indicate a requirement for Tpl2 in neutrophil oxidative burst and bacterial killing that is compounded within a *Tpl2*$^{-/-}$ mouse that also experiences reduced neutrophil recruitment. These findings further support the use of Tpl2 inhibitors as an alternative therapeutic approach against autoimmune diseases exacerbated by TNF production, but cautions that Tpl2 inhibitors may also predispose patients to bacterial infections that require neutrophil function for clearance.

## MATERIALS AND METHODS

*Mice*

Wild type (C57BL/6) were obtained from the Jackson Laboratory, *Ptprc* (B6-Ly5.1/Cr) were obtained from Charles River Laboratories, and both were intercrossed to generate heterozygous CD45.1/CD45.2 mice at the University of Georgia. *Tpl2*$^{-/-}$ mice backcrossed more than ten generations onto the C57BL/6 genetic background were kindly provided by Dr. Philip Tsichlis and Thomas Jefferson University. Animals were used at six to twelve weeks of age, and were age- and sex-matched for individual experiments. All experiments involving mice were performed according to the University of Georgia guidelines for laboratory animals and were approved by the UGA Institutional Animal Care and Use Committee.

*Human subjects*

Peripheral blood collection from anonymous healthy donors has been approved by The Institutional Review Board of the University of Georgia (UGA# 2012-10769-06). Enrolled adult volunteers were non-pregnant and heavier than 110 pounds without any infectious disease complication (based on self-report). All subjects provided informed consent. The studies were performed following the guidelines of the World Medical Association's Declaration of Helsinki.

*Neutrophil Isolation*

Human neutrophil granulocytes were obtained from the peripheral blood of healthy volunteers as described (324). Briefly, red blood cells were removed by dextran sedimentation of the anticoagulant-treated blood, and neutrophils were separated using multistep Percoll gradient centrifugation. In mice, bone marrow was isolated from femurs and tibiae of naïve mice, and peritoneal cells were collected 4 hours post i.p. injection with 3% thioglycollate. Bone marrow or peritoneal cells were treated with anti-Gr-1 biotinylated antibody (RB6-8C5) (eBioscience) and

then labeled with anti-biotin microbeads (Miltenyi Biotech, Auburn, CA). Gr-1$^+$ cells were purified by positive selection using an AutoMACS (Miltenyi Biotech) according to the manufacturer's guidelines. Alternatively, neutrophils were purified by negative selection using a neutrophil enrichment kit (Stemcell Technologies, Vancouver, Canada) according to manufacturer's guidelines.

*Cell Isolation and Cell Sorting*

Wild type spleens were disaggregated by pressing through a 70 μm filter, and red blood cells were lysed using Ack lysis buffer. NK cells were column purified by negative selection using an NK cell isolation kit according to manufacturer's guidelines (Miltenyi Biotech). For isolation of lymphocyte populations, whole splenocytes were stained for 15 min at 4°C in PBS + 0.5% FBS (Life Technologies, Carlsbad, CA) using anti-mouse antibodies purchased from eBioscience (San Diego, CA): CD16/CD32 (93), CD4 (RM4-5), CD8 (53-6.7), CD25 (PC61.5), CD44 (IM7) and CD62L (MGL-14) and CD19 (eBio1D3). Live cells were first gated by excluding propidium iodide positive (PI$^+$) cells and then sorted for naïve CD4 cells (CD4$^+$CD25$^-$CD62L$^+$CD44$^-$), naïve CD8 cells (CD8$^+$CD25$^-$CD62L$^+$CD44$^-$) or B cells (CD19$^+$) using a Beckman Coulter MoFlo XDP cell sorter. Peritoneal macrophages were collected by peritoneal lavage and were adherence purified for 6 hours at 37°C and 5% $CO_2$ in complete RPMI [RPMI 1640 containing 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine (Life Technologies), 0.01 M HEPES (Fisher Scientific, Waltham, MA), and 50 μM 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO)]. Human airway epithelial cell lines A549 and NCI-H292 were obtained from ATCC (Manassas, VA), and Cdk4/hTERT were kindly provided by Dr. John Minna at the University of Texas Southwestern Medical Center in Dallas, Texas (325). NCI-H292 cells were

maintained in complete RPMI medium as described (326). The Cdk4/hTERT human bronchial epithelial cell line was cultured and differentiated as described (327).

*Adoptive transfer and bone marrow chimeras*

Purified bone marrow neutrophils from C57BL6 CD45.1$^+$ and *Tpl2*$^{-/-}$ CD45.2$^+$ mice were labeled with 2.5 μM CFSE for 8 minutes at room temperature. Cells were counted, combined at a 1:1 (WT CD45.1$^+$:*Tpl2*$^{-/-}$ CD45.2$^+$) ratio, and 1-3x10$^6$ cells were injected into CD45.2$^+$ mice i.v. For generation of bone marrow chimeras, CD45.1$^+$CD45.2$^+$ mice were lethally irradiated with 1100 rad. The following day, mice were reconstituted with a mixture of bone marrow from CD45.1$^+$ and *Tpl2*$^{-/-}$ mice. Bone marrow was isolated from femurs and tibiae of naïve mice and red blood cells were lysed. A mixture of 3-4 x10$^6$ bone marrow cells were injected i.v into each recipient.

*Western Blotting*

Bone marrow neutrophils were lysed in protein lysis buffer ($dH_2O$; 100mM HEPES pH 7.5; 20% glycerol; 2% Triton X-100; 3 mM $MgCl_2$; 2 mM EDTA; 1 ng/ml Leupeptin, Aprotinin and Pepstatin; 1 nM PMSF; protease inhibitor tablet [Roche, Switzerland]). Protein concentration was measured using a BCA protein assay (Thermo Scientific, Suwanee, GA). Twenty micrograms of total protein were separated on a 4-12% Bis-Tris gel (Life Technologies) and probed with antibodies for Tpl2, β-Actin (Santa Cruz Biotechnology, Santa Cruz, CA), and GAPDH (GeneTex, Irvine, CA).

*RNA Isolation and RT-PCR*

RNA was isolated from cells using EZRNA extraction kit (Omega Bio-Tek, Norcross, GA) and converted to cDNA by high capacity cDNA reverse transcription kit (Life Technologies). Relative expression levels of *Tpl2* were measured using SensiFAST Probe Hi-ROX kit (Bioline, Taunton, MA) and predesigned TaqMan probe and primer sets (Applied Biosystems, Grand

Island, NY). Samples were run on a StepOnePlus qPCR machine (Applied Biosystems). Results given are relative to neutrophils (mouse samples) or Cdk4/hTERT (human samples) and an actin housekeeping gene using the $\Delta\Delta C_T$ method.

*Murine Blood Collection*

Blood was collected by tail bleed or terminal cardiac puncture into microvette tubes containing EDTA (Starstedt, Germany). Proportions of white blood cells were determined by CBC analysis. Surface staining of cells used the following anti-mouse monoclonal antibodies from eBiosciences: CD16/CD32 (93), CD4 (RM4-5), B220 (RA3-6B2), Gr-1 (RB6-8C5), CD11b (M1/70), CD44 (IM7), CD62L (MEL-14), CD45.1 (A20), and CD45.2 (104) or from BioLegend: CXCR2 (SA044G4) and CXCR4 (L276F12). Samples were run on a BD LSRII flow cytometer and analyzed using FlowJo software (Tree Star, Inc., Ashland, OR).

*Measurement of superoxide production and bacterial killing*

Superoxide production in adherent neutrophils was measured by Diogenes superoxide detection chemiluminescence kit (National Diagnostics, Atlanta, GA). A total of 50,000 neutrophils/well were allowed to adhere to 96-well white plates (15 min, 37°C, HBSS). Cells were stimulated by addition of fMLP (100 nM), PMA (1 μM), *C. rodentium* (MOI 1, 2, and 5), or were left unstimulated. Chemiluminescence was measured by Varioskan Flash luminometer (Thermo Scientific, Logan, UT) for 30 min. Alternatively, 100,000 bone marrow neutrophils were combined with increasing concentrations of luminescent *C. rodentium* (ICC180, kindly provided by Dr. Gad Frankel at Imperial College, London UK (310) that had been opsonized with murine serum 15 minutes at 37°C. Luminescence was measured by Varioskan Flash luminometer (Thermo Scientific, Logan, UT) for 3 hours (37°C, HBSS, 100 mM HEPES, 5 mM glucose and 1% mouse serum).

*Cytokine Measurements*

A total of 250,000 neutrophils/well were allowed to adhere to 96-well plates. Cells were stimulated for 24 hr with 100 nM fMLP or 100 ng/mL LPS at 37°C in media containing HBSS, 100 mM HEPES, 5 mM glucose and 1% mouse serum. TNF concentrations were measured by ELISA (eBiosciences) according to manufacturer's guidelines. Serum concentrations of G-CSF were measured by ELISA (RayBiotech, Norcross, GA) according to the manufacturer's guidelines.

*Statistics*

*P* values were derived by two-tailed paired or unpaired student's *t*-test using Prism software, unless otherwise indicated. Differences were considered statistically significant if $p \leq 0.05$.

**RESULTS**

**Tpl2 is expressed within mouse and human neutrophils**

The importance of Tpl2 in innate immune cells, such as macrophages and dendritic cells, and adaptive immune cells, such as CD4 T cells, has been extensively studied. However, how Tpl2 influences neutrophil function has yet to be investigated. First, we evaluated the expression of Tpl2 in neutrophils relative to other innate or adaptive immune cells. Tpl2 is expressed as two distinct isoforms of 52 and 58 kDa in both innate and adaptive immune cells (13). In murine cells, Tpl2 is more highly expressed in neutrophils compared to naïve CD4 T cells, but not as highly expressed as in naïve peritoneal macrophages, B cells or NK cells (Figure 4.1A-B). Notably, the p52 isoform appears to be the dominant isoform expressed in neutrophils compared to other cell types analyzed (Figure 4.1A). Tpl2 has previously been shown to influence cytokine secretion of IL-6 and IL-8 (CXCL8) within the human bronchial airway epithelial cell line BEAS-2B (328). Accordingly, Tpl2 is expressed within the human lung epithelial cell lines

Cdk4/hTERT, NCI-H292, and A549 (Figure 4.1C), but Tpl2 expression is even higher in primary human neutrophils (Figure 4.1C). These results indicate that Tpl2 is expressed in neutrophils at physiologically relevant levels where it may contribute to neutrophil antimicrobial functions.

### *Tpl2*$^{-/-}$ mice have greater accumulation of neutrophils within the bone marrow

Neutrophil development from progenitors proceeds through several stages within the bone marrow before being released into the circulation (134). We therefore investigated whether Tpl2 contributes to neutrophil development within the bone marrow. Whole bone marrow from *Tpl2*$^{-/-}$ mice had higher proportions of both granulocytes (as determined by forward scatter and side scatter) and Gr-1 positive neutrophils (Figure 4.2A-B). One factor that has been shown to regulate neutrophil proportions within the bone marrow is the microbiota (329, 330). However, *Tpl2*$^{-/-}$ mice had similarly elevated proportions of bone marrow neutrophils compared to their wild type littermates (Figure 4.2C), confirming a role for Tpl2 in the accumulation of bone marrow neutrophils independent of the microbiota. Once neutrophils mature in the bone marrow, they are released into the circulation. Despite elevated proportions of neutrophils within the bone marrow, *Tpl2*$^{-/-}$ mice have reduced proportions of neutrophils within the blood, with a commensurate increase in the proportions of lymphocytes (Figure 4.2D). However, Tpl2 only directly impacts neutrophil numbers within the blood (Figure 4.2E), which in turn indirectly influences lymphocyte proportions. To prevent underlying differences in how microbiota may influence neutrophil function, all remaining experiments were conducted using wild type and *Tpl2*$^{-/-}$ mice bred from heterozygous matings.

Within *Tpl2*$^{-/-}$ mice we observed greater accumulation of neutrophils within the bone marrow and reduced circulating neutrophils, indicating defects in neutrophil egress from the bone marrow.

Tpl2 has previously been shown to regulate chemokine receptor expression on macrophages, including CCR1 (331). Because CXCR4 is thought to negatively regulate neutrophil egress from the bone marrow into the circulation (141, 142), we hypothesized that CXCR4 expression on the surface of *Tpl2*$^{-/-}$ bone marrow neutrophils may be altered. However, wild type and *Tpl2*$^{-/-}$ neutrophils within the bone marrow expressed similar levels of CXCR4 (Figure 4.3A). Alternatively, production of G-CSF is also thought to enhance neutrophil mobilization from the bone marrow into the circulation (140). Consistently, naïve *Tpl2*$^{-/-}$ mice trended towards less G-CSF within their sera (Figure 4.3C), suggesting that Tpl2 enhances neutrophil egress from the bone marrow into the circulation by promoting systemic G-CSF expression.

**Neutrophil recruitment is impaired in *Tpl2*$^{-/-}$ mice due to a neutrophil-extrinsic defect**

Tpl2 has been shown to be required for neutrophil recruitment in response to treatment with zymosan (42), acetaminophen (317), and caerulein (318), as well as during $MOG_{35-55}$-induced experimental autoimmune encephalomyelitis (EAE) (38). Decreased recruitment was attributed to reduced chemotactic factors generated at the site of inflammation within *Tpl2*$^{-/-}$ mice (38, 42, 317, 318). Consistent with these findings, 3% thioglycollate, which elicits a sterile inflammatory response, recruited 10-20% less neutrophils into the peritoneal cavity of *Tpl2*$^{-/-}$ mice compared to wild type mice (Figure 4.4A). These results confirm the importance of Tpl2 in recruitment of neutrophils within the host. However, it is still unclear whether Tpl2 influences neutrophil recruitment in a neutrophil-intrinsic manner.

To determine the role of Tpl2 in neutrophil recruitment, we utilized two models to measure the cell-instrinsic role of Tpl2 during neutrophil recruitment within a wild type host. In the first model, bone marrow neutrophils were isolated from congenic CD45.1$^{+}$ C57BL/6 and CD45.2$^{+}$ *Tpl2*$^{-/-}$ mice, combined at a 1:1 ratio, and labeled with CFSE. Neutrophils were injected i.v. into

wild type (C57BL/6) mice, and the recipients were immediately treated with 1 ml of 3% thioglycollate i.p. Four hours post treatment, mice were euthanized, and peritoneal cells were collected. Similar proportions of both $CD45.1^+$ wild type and $CD45.1^-$ *Tpl2*$^{-/-}$ neutrophils were recruited into the peritoneal cavity (Figure 4.4B). Unimpaired recruitment of *Tpl2*$^{-/-}$ neutrophils within the circulation is consistent with normal levels of neutrophil chemokine receptors CXCR2 and CXCR4 (142, 145-147), E-selectin CD44 (150), and L-selectin CD62L (Figure 4.3B). In the second model, mixed bone marrow chimeras were generated and treated with either 3% thioglycollate or *C. rodentium*. Eight weeks post transfer, CD45.1/CD45.2 heterozygous recipient mice contained approximately a 1:1 (wild type $CD45.1^+$:*Tpl2*$^{-/-}$ $CD45.2^+$) neutrophil ratio within the blood (Figure 4.4C). Contrary to expectations, after treatment with 3% thioglycollate, both the blood and peritoneum unexpectedly showed elevated proportions of *Tpl2*$^{-/-}$ neutrophils compared to wild type $CD45.1^+$ neutrophils (Figure 4.4C). These two models have confirmed that recruitment is not impaired in Tpl2-deficient neutrophils. Therefore, Tpl2 predominately regulates neutrophil recruitment via a neutrophil-extrinsic mechanism, since *Tpl2*$^{-/-}$ mice have impaired neutrophil recruitment (Figure 4.4A), even though *Tpl2*$^{-/-}$ neutrophils are capable of being recruited within a wild type host (Figure 4.4B-C).

**Tpl2 promotes TNF secretion, respiratory burst and bacterial killing**

Once within an inflamed tissue, recruited neutrophils produce inflammatory cytokines, phagocytose microbes, generate superoxide, and form neutrophil extracellular traps (NETs) to control infection. To determine how Tpl2 influences neutrophil antimicrobial functions, neutrophils were isolated from the bone marrow of wild type and *Tpl2*$^{-/-}$ mice and stimulated *ex vivo* with the chemotactic peptide f-Met-Leu-Phe (fMLP), LPS, or phorbol myristate acetate (PMA). Following stimulation with LPS, *Tpl2*$^{-/-}$ neutrophils secreted significantly less TNF

compared to wild type neutrophils (Figure 4.5A). fMLP induced significant superoxide production within wild type neutrophils, which was dramatically diminished in *Tpl2*$^{-/-}$ neutrophils (Figure 4.5B-C). Defects in superoxide generation by *Tpl2*$^{-/-}$ neutrophils in response to fMLP is consistent with defects in superoxide generation we previously observed in *Tpl2*$^{-/-}$ macrophages in response to TLR ligands (316) suggesting a fundamental defect in NADPH oxidase activity in *Tpl2*$^{-/-}$ innate immune cells in response to physiological stimuli. However, both *Tpl2*$^{-/-}$ neutrophils (Figure 4.5B-C) and macrophages (316) are capable of producing superoxide in response to PMA stimulation, indicating a stimulus-specific defect in superoxide production within *Tpl2*$^{-/-}$ neutrophils.

Based on the observation that *Tpl2*$^{-/-}$ neutrophils are impaired in TNF and superoxide production in response to bacterial ligands, we hypothesized that they would be impaired in bacterial killing. *Citrobacter rodentium* is a non-motile gram-negative rod that is a natural mouse and gerbil pathogen (238, 239) and is capable of being killed by murine neutrophils *in vitro* (250, 306). Wild type or *Tpl2*$^{-/-}$ neutrophils were incubated with a luminescent strain of *C. rodentium* at a low dose of infection (MOI 1, 2, and 5), and bacterial persistence was monitored over three hours. *Tpl2*$^{-/-}$ bone marrow neutrophils were less capable of killing *C. rodentium* compared with wild type neutrophils, and all neutrophils were overwhelmed at the highest MOI (Figure 4.6). These data indicate basal defects in the ability of *Tpl2*$^{-/-}$ neutrophils to kill bacteria, despite similar expression of the activation marker CD11b (Figure 4.3A).

**DISCUSSION**

In this study, we sought to explore the functional consequences of Tpl2 ablation on neutrophil development and function. While previous reports have demonstrated reduced neutrophil recruitment to inflammatory sites in *Tpl2*$^{-/-}$ mice (38, 42, 317, 318), this study is the first to

document a defect in neutrophil homeostasis and bone marrow egress in naïve *Tpl2*$^{-/-}$ mice. Greater accumulation of neutrophils within the bone marrow and reduced proportions of circulating neutrophils was independent of bone marrow progenitors or neutrophil expression of the chemokine receptors and selectins CXCR2, CXCR4, CD44 and CD62L but correlated with a trend towards reduced circulating G-CSF concentrations. Furthermore, this study also established important cell-intrinsic roles for Tpl2 in promoting neutrophil antimicrobial functions, including TNF secretion, superoxide production, and bacterial killing.

Within the circulation, *Tpl2*$^{-/-}$ neutrophils express similar levels of chemokine receptors to wild type neutrophils and are equally capable of being recruited in response to thioglycollate within a wild type host. However, within a *Tpl2*$^{-/-}$ host, neutrophil recruitment to sites of inflammation is impaired (38, 42, 317, 318). These data are consistent with the observation that *Tpl2*$^{-/-}$ mice produce less neutrophil chemoattractant cytokines and chemokines, including G-CSF (42, 220) KC and CXCL2 (332-334) during inflammatory responses. KC and CXCL2 are known to bind CXCR2 expressed on the surface of neutrophils (306, 335, 336). Altogether, these data indicate that Tpl2-dependent neutrophil recruitment occurs via neutrophil-extrinsic mechanisms that result in defects in local chemokine production in *Tpl2*$^{-/-}$ mice. Furthermore, a basal defect in egress of mature neutrophils from the bone marrow and into the circulation within *Tpl2*$^{-/-}$ hosts further impedes neutrophil recruitment during inflammation.

Neutrophils utilize various antimicrobial functions shared by other innate immune cells, such as phagocytosis of microbes, superoxide production, and cytokine secretion. Signaling through FcγR has been shown to activate Tpl2 and ERK in macrophages, allowing for secretion of TNF, IL-6, and IL-10 and for phagocytosis, all of which are reduced by Tpl2 inhibition (30). Superoxide production is a major component of neutrophil antimicrobial functions. Superoxide is

generated through activation and formation of the NADPH oxidase complex, which is composed of five subunits: two membrane bound subunits ($p22^{phox}$ and $gp91^{phox}$) and three cytoplasmic subunits ($p40^{phox}$, $p47^{phox}$ and $p67^{phox}$) (154). Upon activation, the three cytoplasmic subunits are recruited to the cell membrane, generating the active NADPH oxidase and allowing for production of superoxide (154). We hypothesize that defects in superoxide production in response to fMLP by *Tpl2*$^{-/-}$ neutrophils is due to defects in the NADPH oxidase. However, this defect is stimulus-specific and can be overcome in response to PMA. fMLP is known to activate the MEK/ERK signaling pathway leading to down-stream superoxide production (337) whereas PMA instead utilizes the PKC signaling pathway (338). The observed impairment in fMLP-induced oxidative burst is consistent with the known role for Tpl2 in activation of the MEK/ERK pathway (reviewed in (11)).

The main goal of neutrophil antimicrobial functions is to promote an inflammatory environment and kill microbes. It has previously been shown that murine neutrophils are capable of killing the bacterial pathogen *C. rodentium in vitro* (250, 306). In the current study, *Tpl2*$^{-/-}$ neutrophils were significantly impaired in their antimicrobial functions. These data implicate Tpl2 in bacterial killing *in vitro,* but neutrophil-mediated killing is a multistep process (reviewed in (152)) that includes phagocytosis, degranulation, respiratory burst and release of neutrophil extracellular traps (NETs). Tpl2 has already been shown to influence macrophage phagocytosis through FcγR (30) and likely promotes FcγR-mediated uptake by neutrophils as well. After phagocytosis, neutrophils produce reactive oxygen species through NADPH oxidase as mentioned above and antibacterial proteins including defensins, lactoferrin, and lysozyme (reviewed in (152)). Therefore, a combination of defects in both phagocytosis and oxidative burst likely underlie the reduced antimicrobial activity of *Tpl2*$^{-/-}$ neutrophils.

Overall, our findings underscore the importance of Tpl2 in driving innate immune cell inflammatory and antimicrobial properties. These findings further support the use of Tpl2 inhibitors as an alternative therapeutic approach against rheumatoid arthritis to combat overproduction of TNF (288-290) as well as limit neutrophil recruitment. Besides being used as an infectious model for inflammatory bowel disease (205), *C. rodentium* is also a model for intestinal bacterial pathogens that cause attaching and effacing lesions, similar to *Escherichia coli* infection (253, 254). Therefore, while inhibition of Tpl2 may benefit patients who suffer from autoimmune diseases potentiated by inflammatory neutrophils, Tpl2 inhibitors taken chronically may also predispose patients to commensal bacterial infections that require neutrophil function for clearance.

**ACKNOWLEDGEMENTS**

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## FIGURES

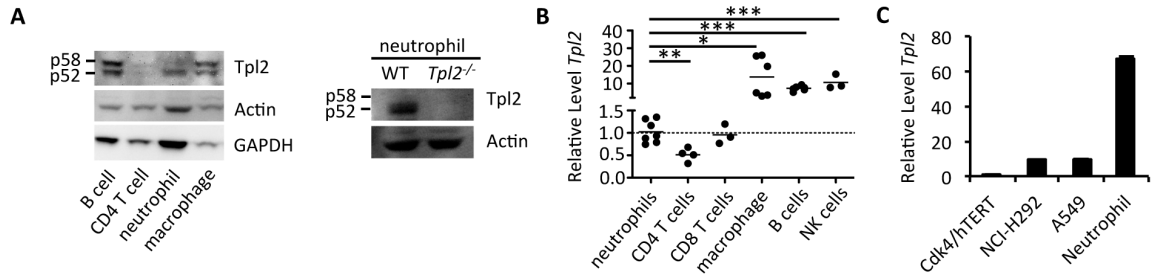


**Figure 4.1- Tpl2 is expressed within neutrophils.** (A) Naïve CD4 cells, B cells and peritoneal macrophages were isolated from wild type mice. Neutrophils were isolated from wild type and *Tpl2*$^{-/-}$ mice. Twenty micrograms of protein were immunoblotted for Tpl2, actin, and GAPDH. (B) Naïve CD4 cells, CD8 cells, B cells, NK cells, peritoneal macrophages and neutrophils were isolated from wild type mice and analyzed for their relative expression of *Tpl2* by RT-PCR. N≥3. Lines represent means. (C) Human cell lines and primary neutrophils were analyzed for their relative expression of *Tpl2* by real-time PCR. N=2 donors. Error bars represent means ± SE.

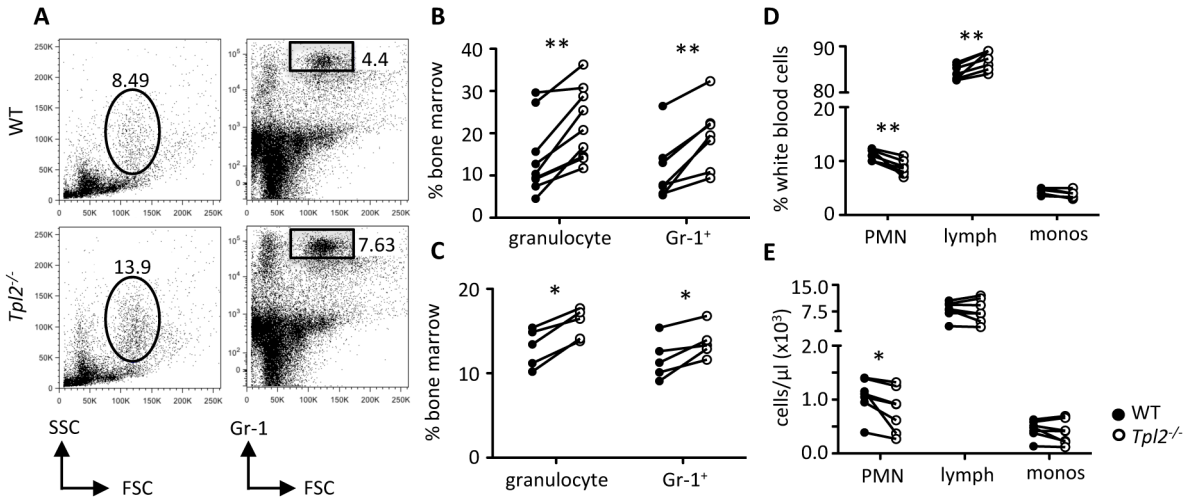


**Figure 4.2- *Tpl2*$^{-/-}$ mice have elevated proportions of neutrophils within the bone marrow and reduced proportions within the blood.** (A-C) Bone marrow was isolated from (A-B) wild type and *Tpl2*$^{-/-}$ mice from homozygous matings or (C) from wild type and *Tpl2*$^{-/-}$ littermates derived from heterozygous matings. Whole bone marrow isolates were stained for Gr-1. Cells were gated on granulocytes or Gr-1$^{+}$ cells. N≥5. Blood was collected from littermates and analyzed for (D) proportions and (E) concentration of white blood cells. Results are shown as a (D) proportion of all WBC and (E) cells/µl. N=6. Connecting lines represent paired experiments. *p<0.05, **p<0.005

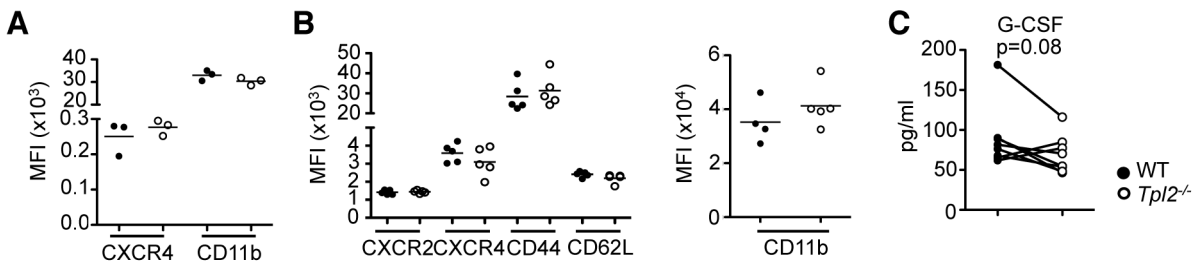


**Figure 4.3- Tpl2 does not influence surface expression of neutrophil chemokine receptors or activation markers.** Wild type and *Tpl2*$^{-/-}$ neutrophils from (A) bone marrow or (B) blood were analyzed for their relative expression of CXCR2, CXCR4, CD44, CD62L, and CD11b. Data are shown as MFI for each mouse. N≥3. Results shown are representative of 2 or more experiments**.** (C) Wild type and *Tpl2*$^{-/-}$ serum concentration of G-CSF was measured. N=7.

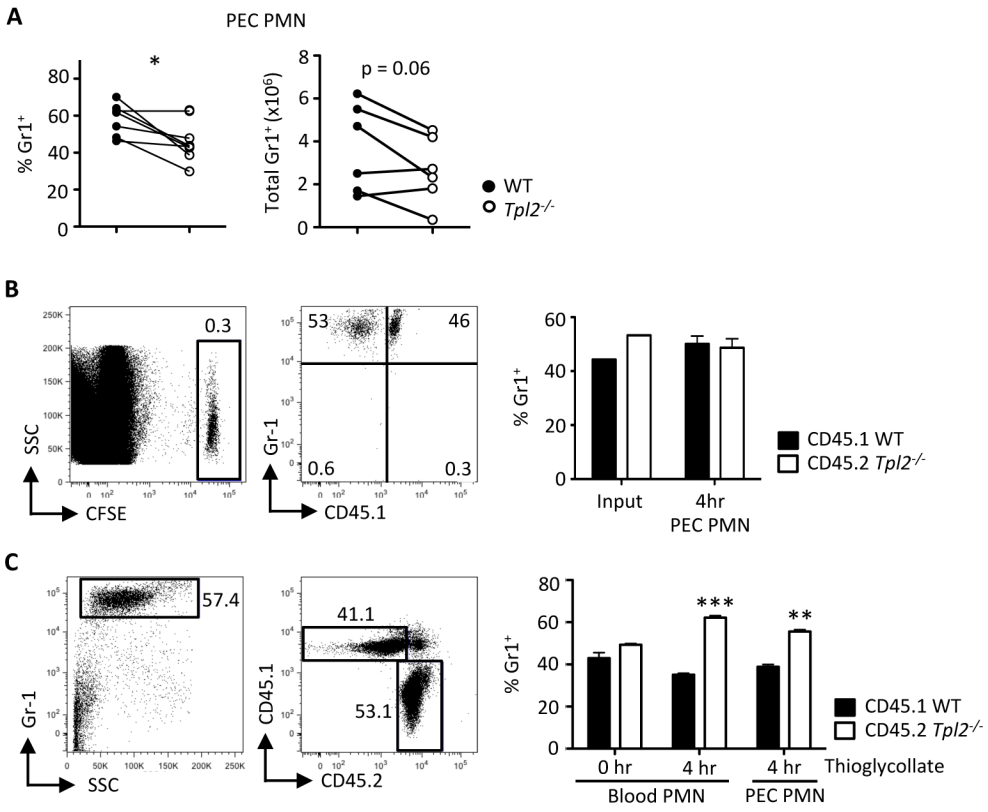


**Figure 4.4- Tpl2 primarily regulates recruitment in a neutrophil-extrinsic manner.** (A) Wild type and *Tpl2*$^{-/-}$ mice were treated i.p. with 1 ml 3% thioglycollate. Four hours later, peritoneal cells were lavaged and stained for Gr-1$^{+}$ neutrophils. N≥7. (B) CD45.1$^{+}$ and CD45.1$^{-}$ *Tpl2*$^{-/-}$ bone marrow neutrophils were stained with CFSE, combined at a 1:1 ratio, and 2 x 10$^{6}$ cells were injected i.v. along with 1 ml 3% thioglycollate i.p into wild type mice. Four hours later, peritoneal exudate cells were stained for Gr-1 and CD45.1. A representative plot of peritoneal exudate cells shows the gating strategy. N=3. Results shown are representative of 2 experiments. (C) Mixed bone marrow chimeras were injected with 1 ml 3% thioglycollate i.p. Peritoneal cells were collected 4 h later and stained for Gr-1, CD45.1, and CD45.2. A representative plot of peritoneal exudate cells shows the gating strategy. N=4. Results shown are representative of 2 experiments. Error bars represent means ± SEM. *p<0.05, **p<0.005, ***p<0.001

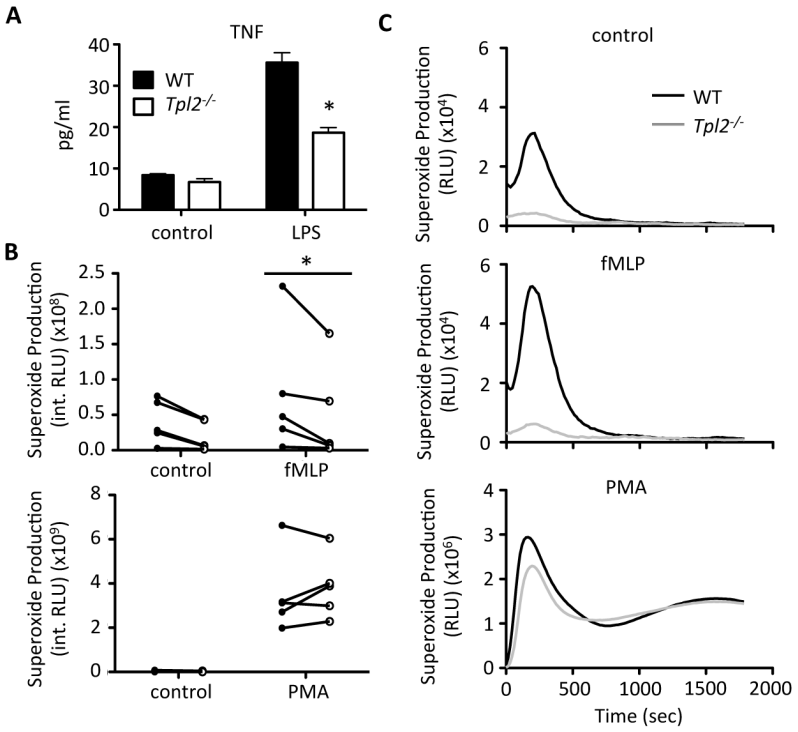


**Figure 4.5- Tpl2 is required for neutrophil antimicrobial functions.** (A) Bone marrow neutrophils were treated with indicated stimuli for 24 hours. Supernatants were collected and analyzed for TNF concentration. N=3. Results shown are representative of 3 independent experiments. Error bars represent means ± SD. (B-C) Bone marrow neutrophils were treated with indicated stimuli for 30 minutes. Superoxide production was measured by a Diogenes-based chemiluminescence assay. Data are shown as (C) kinetics of representative curves (relative luminescence units [RLUs]) or as (B) summarized luminescence integrated for the entire duration of the measurement (int. RLU). N=5. *$p<0.05$

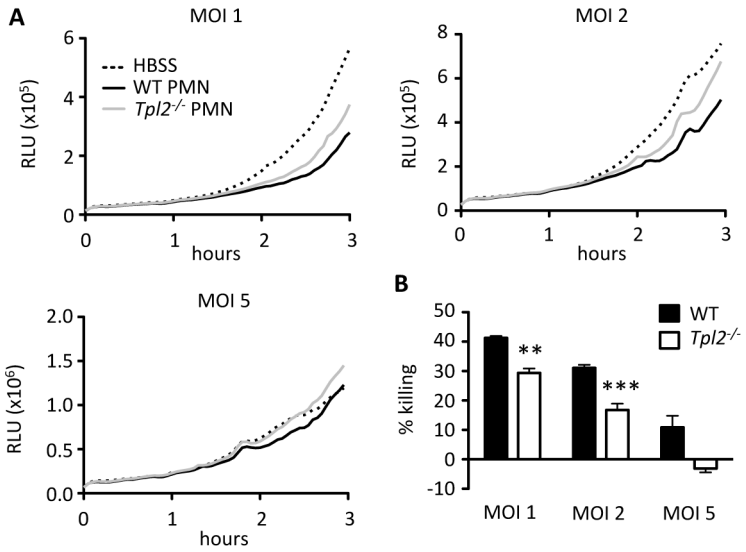


**Figure 4.6- Tpl2 is required for neutrophil killing of *C. rodentium.*** Bone marrow neutrophils were isolated from wild type and *Tpl2*$^{-/-}$ mice. 1-2x10$^5$ neutrophils were combined with opsonized luminescent *C. rodentium* at an MOI of 1, 2, and 5 for 3 hours, and luminescence was measured. Data are shown as (A) kinetics of representative curves and (B) percent killing relative to medium control. N=3. Results shown are representative of 3 experiments. Error bars represent means ± SD. **p<0.005, ***p<0.001

# CHAPTER 5

# SEVERE DERMATITIS IN RAG1$^{-/-}$*TPL2*$^{-/-}$ MICE DUE TO SPONTANEOUS *STAPHYLOCCOUS XYLOSUS* INFECTION [4]

[4] Acuff, N.V, M. LaGatta, T. Nagy and W.T. Watford. Submitted to *Comparative Medicine* (under review)

**ABSTRACT**

*Staphylococcus xylosus* is a commensal bacterium found on the skin and mucosal surfaces of specific pathogen free (SPF) mice. *S. xylosus* is rarely pathogenic, most often causing skin lesions and dermatitis in immunocompromised strains, particularly those with impaired NADPH oxidase function. Herein, we report spontaneous infection with *S. xylosus* in *Rag1*$^{-/-}$*Tpl2*$^{-/-}$ mice. Infection was characterized by the presence of alopecia, crusts, and scaly skin. *S. xylosus* was detected within the feces, skin, lymph nodes and lungs of *Rag1*$^{-/-}$*Tpl2*$^{-/-}$ mice and led to mortality or euthanasia due to humane endpoints. C57BL/6 mice tested culture positive for *S. xylosus* on the skin, and *Rag1*$^{-/-}$ and *Tpl2*$^{-/-}$ mice tested culture positive on the skin and occasionally within the feces. However, *S. xylosus* did not cause clinical symptoms in C57BL/6, *Rag1*$^{-/-}$ or *Tpl2*$^{-/-}$ mice. Infection did not influence relative concentrations of neutrophils or lymphocytes within the circulation of *Rag1*$^{-/-}$*Tpl2*$^{-/-}$ mice, but did enhance concentrations of circulating monocytes compared to *Rag1*$^{-/-}$ mice, which correlated with bacterial dissemination. This case study suggests a role for Tpl2 in innate immune cell superoxide production and also cautions that differences in immune status of genetically engineered mice due to underlying infection with opportunistic pathogens may bias experimental interpretation.

## INTRODUCTION

*Staphylococcus xylosus* is a non-motile coagulase-negative, gram-positive cocci that was first identified in 1975 (339). *Staphylococci* are common bacteria in the environment and have been linked to opportunistic infections in both humans and animals. *S. xylosus* is generally considered a nonpathogenic bacterium commonly used in the production of meat and cheese products (340-345). One reason *Staphylococcal spp.* are useful in food production is due to expression of the MprF gene, which prevents spoiling through production of lantibiotics (346). *S. xylosus* is a commensal bacterium found on the skin of specific pathogen free (SPF) C57BL/6 mice (347). All strains of *S. xylosus* isolated from laboratory animals were found to be susceptible to multiple antibiotics (162). In addition, there have been reports of *S. xylosus* isolated from humans, dairy cows, ewes, gerbils, and poultry (348, 349).

Although large numbers of mice are used in research, there are few reports of spontaneous *S. xylosus* infection. Spontaneous infection with *S. xylosus* has been previously reported in athymic nude mice, characterized by extensive dermatitis on the neck, thorax, and shoulders (350, 351). Significant dermal inflammation and the presence of gram-positive cocci were observed within the lesions (350, 351). This is consistent with the induction of a localized CD4 T cell response after colonization of germ-free C57BL/6 mice with *S. xylosus* (352). Importantly, mouse strains deficient in phagocyte superoxide production also show increased susceptibility to spontaneous *S. xylosus* infection, characterized by dermatitis and abscess formation (161-163, 353, 354). Development of severe dermatitis, morbidity and mortality in these strains suggests that both T cells and superoxide production contribute to host resistance. Superoxide is generated through activation and formation of the NADPH oxidase complex, which is composed of 5 subunits: $p22^{phox}$ and $gp91^{phox}$ (Cybb) on the surface and $p40^{phox}$, $p47^{phox}$, and $p67^{phox}$ within the

cytoplasm. Upon activation, the 5 subunits combine at the cell membrane, generating the active NADPH oxidase, and enable superoxide production. Mice lacking the gp91$^{phox}$ subunit (B6.129S6-*Cybb*$^{tm1Din}$/J) or p47$^{phox}$ subunit (B6p47$^{phox-/-}$HLL and B6.129S2-Ncf1$^{tm1shl}$N14), which are commonly used as models for chronic granulomatous disease, have been reported to develop spontaneous *S. xylosus* infection (161, 350, 353). Of note, infection in superoxide-deficient mice is also characterized by dissemination into other organs, such as the brain, lung, lymph nodes, and bone, whereas *S. xylosus* remains localized to the skin within superoxide-competent mice (161, 162).

In the current report, we describe spontaneous infection with *S. xylosus* as the main cause of morbidity and mortality in *Rag1*$^{-/-}$*Tpl2*$^{-/-}$ mice. Affected *Rag1*$^{-/-}$*Tpl2*$^{-/-}$ mice presented with alopecia, scaly skin, crusts and mortality or euthanasia due to humane endpoints. However, C57BL/6, *Rag1*$^{-/-}$, and *Tpl2*$^{-/-}$ mice did not develop clinical signs of infection, despite all strains being culture positive for *S. xylosus* on the skin. Within *Rag1*$^{-/-}$*Tpl2*$^{-/-}$ mice, *S. xylosus* was additionally detected within the feces and occasionally disseminated to lymph nodes and lung. Dissemination into other organs is indicative of defects in the NADPH oxidase as previously reported (162), which implicates Tpl2 in phagocyte production of superoxide *in vivo*. Furthermore, this case study provides a cautionary note that unanticipated opportunistic infections and altered microbiota of immunocompromised mice may bias experimental interpretations.

## MATERIALS AND METHODS

### *Mice*

Wild type (C57BL/6J) and *Rag1*$^{-/-}$ mice were obtained from the Jackson Laboratory. *Tpl2*$^{-/-}$ mice backcrossed more than ten generations onto the C57BL/6 genetic background were kindly

provided by Dr. Philip Tsichlis and Thomas Jefferson University. *Rag1*$^{-/-}$ mice were crossed with *Tpl2*$^{-/-}$ mice to generate *Rag1*$^{-/-}$*Tpl2*$^{-/-}$ mice. C57BL/6 and *Tpl2*$^{-/-}$ mice were crossed to generate *Tpl2*$^{+/-}$ mice, and wild type and *Tpl2*$^{-/-}$ mice used in this report were bred from heterozygous matings. Animals were bred and maintained in sterile microisolator cages at the University of Georgia using guidelines for laboratory animals that were approved by the UGA Institutional Animal Care and Use Committee.

*Blood and Tissue Collection*

Blood was collected by terminal cardiac puncture into microvette tubes containing EDTA (Starstedt, Germany). Proportions of white blood cells were determined by CBC analysis. Skin, spleen, lymph nodes (mesenteric and inguinal), lung, brain, and fecal samples were collected using aseptic technique and submitted for aerobic bacterial culture of *Staphylococcus xylosus* and pathology at the UGA Veterinary Diagnostic Laboratories. Skin samples were also fixed in 10% neutral buffered formalin for 24 h at room temperature. Complete cross sections of formalin-fixed sections were placed in cassettes, embedded in paraffin, sectioned at 4 μm thickness, mounted on glass slides, and stained with hematoxylin and eosin. Histological sections were evaluated by a veterinary pathologist (TN).

*Statistics*

*P* values were derived by Fischer's exact test or by one-way ANOVA and Tukey's post hoc test using Prism software. Differences were considered statistically significant if $p \leq 0.05$.

**CASE REPORT**

Over a one-year period within our mouse colony, we observed thickened scaly skin in several naive *Rag1*$^{-/-}$*Tpl2*$^{-/-}$ mice that required euthanasia or led to mortality. The skin of *Rag1*$^{-/-}$*Tpl2*$^{-/-}$ mice was characterized by ruffled fur, mild alopecia, scabs, and scattered discrete crusts (Figure

5.1A). Microscopic analysis revealed a markedly hyperplastic epidermis (E) focally covered with a serocellular crust (SC) composed of neutrophils, cellular debris, and bacterial colonies. There is distinct fibrosis (F) beneath the epidermis indicating chronic infection as well as acute hemorrhage between the epidermis and the superficial dermis signifying a severe inflammatory condition (Figure 5.1B). Necropsy and diagnostic testing confirmed that skin lesions were due to spontaneous *S. xylosus* infection, which occurred within three different male cages at different time points over the one-year period. No naïve C57BL/6, *Rag1*$^{-/-}$ or *Tpl2*$^{-/-}$ mice showed clinical signs of infection nor required euthanasia.

After several occurrences of spontaneous *S. xylosus* infection within *Rag1*$^{-/-}$*Tpl2*$^{-/-}$ mice, a cohort of male C57BL/6, *Rag1*$^{-/-}$, *Tpl2*$^{-/-}$, and *Rag1*$^{-/-}$*Tpl2*$^{-/-}$ mice were euthanized and examined for the presence of *S. xylosus* on the skin as well as within the feces, spleen, lymph nodes, lung, and brain. Upon euthanasia, *S. xylosus* was detected on the skin of all mice and within the feces of a single *Rag1*$^{-/-}$, two *Tpl2*$^{-/-}$ and all *Rag1*$^{-/-}$*Tpl2*$^{-/-}$ mice (Table 5.1). Fecal testing of *Rag1*$^{-/-}$*Tpl2*$^{-/-}$ mice also included female cages and confirmed that colonization with *S. xylosus* within *Rag1*$^{-/-}$ *Tpl2*$^{-/-}$ mice was not gender specific, although dermatitis, morbidity and mortality were restricted to males. Aerobic culture of *S. xylosus* also confirmed dissemination to the lymph nodes of one *Rag1*$^{-/-}$*Tpl2*$^{-/-}$ mouse and lung of another (Table 5.1). These data indicate that within immunocompromised *Rag1*$^{-/-}$ mice, the additional absence of Tpl2 predisposes mice to *S. xylosus* infection, indicating the importance of Tpl2 within innate immune cells.

Due to the presence of infection, concentrations of white blood cells within the circulation were measured to evaluate immune responses generated to infection. As expected, *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$ *Tpl2*$^{-/-}$ mice had significantly less circulating lymphocytes compared to C57BL/6 and *Tpl2*$^{-/-}$ mice (224, 225) (Figure 5.2). C57BL/6, *Rag1*$^{-/-}$, *Tpl2*$^{-/-}$, and *Rag1*$^{-/-}$*Tpl2*$^{-/-}$ mice all had similar

concentrations of circulating neutrophils (Figure 5.2). Interestingly, *Rag1*$^{-/-}$ mice had significantly fewer circulating monocytes compared to C57BL/6 and *Tpl2*$^{-/-}$ mice, and *Rag1*$^{-/-}$ *Tpl2*$^{-/-}$ mice had elevated levels of monocytes compared to *Rag1*$^{-/-}$ mice (Figure 5.2). However, *Rag1*$^{-/-}$*Tpl2*$^{-/-}$ mice still had significantly fewer circulating monocytes compared to C57BL/6 and *Tpl2*$^{-/-}$ mice (Figure 5.2). These data indicate that defects in lymphocyte production impacts monocyte concentrations within the circulation. Additionally, elevated monocytes within *Rag1*$^{-/-}$ *Tpl2*$^{-/-}$ mice correlates with the presence of disseminated *S. xylosus* within the host.

**DISCUSSION**

Tumor progression locus 2, Tpl2 (also known as MAP3K8), is a serine-threonine protein kinase that is expressed in both innate and adaptive immune cells. *Tpl2*$^{-/-}$ mice were originally described as being resistant to endotoxin-induced septic shock due to reduced production of TNF (24). Because Tpl2 promotes TNF processing and secretion (24, 45), it is being investigated as a therapeutic target for treating autoimmune diseases, especially those exacerbated by TNF, such as rheumatoid arthritis (288-290). Therefore, the roles for Tpl2 in immune responses during autoimmune and infectious diseases are currently being investigated. During infection, *Tpl2*$^{-/-}$ mice are more susceptible to *Toxoplasma gondii* (28), *Listeria monocytogenes* (25, 47), *Mycobacterium tuberculosis* (47) and influenza (49) compared to wild type mice. Similarly, *Rag1*$^{-/-}$*Tpl2*$^{-/-}$ mice develop greater bacterial burdens in response to *Mycobacterium tuberculosis* and *Listeria monocytogenes* infection compared to *Rag1*$^{-/-}$ mice (47), indicating an important role of Tpl2 in regulating immune responses to infection.

In this case study, we describe spontaneous infection of *Rag1*$^{-/-}$*Tpl2*$^{-/-}$ mice with *Staphylococcus xylosus. S. xylosus* is an environmental contaminant and a common commensal bacterium of barrier surfaces of mammals, including laboratory mice (348). Indeed, it is detected on the skin

of C57BL/6 mice housed under specific pathogen free (SPF) conditions (347), Consistent with this, all mice in this study cultured positive for *S. xylosus* on the skin., and all *Rag1*$^{-/-}$*Tpl2*$^{-/-}$ mice, one *Rag1*$^{-/-}$ mouse and two *Tpl2*$^{-/-}$ mice also contained *S. xylosus* within their feces. Despite detection of *S. xylosus* on the skin and within feces of *Tpl2*$^{-/-}$ and *Rag1*$^{-/-}$ mice, clinical disease of *Tpl2*$^{-/-}$ and *Rag1*$^{-/-}$ mice was never observed. Notably, dermatitis, morbidity and mortality associated with *S. xylosus* infection was only observed in male mice, even though both male and female *Rag1*$^{-/-}$*Tpl2*$^{-/-}$ mice tested culture positive for *S. xylosus*. Spontaneous *S. xylosus* infection in athymic nude mice also occurred with increased frequency in male mice (350), although a second study reported a similar frequency in males and females (351). Additionally, male and female mice deficient in NADPH oxidase showed similar susceptibility to opportunistic infections by *S. xylosus* (25). The fact that dermatitis, morbidity and mortality caused by *S. xylosus* in this study was restricted to male *Rag1*$^{-/-}$*Tpl2*$^{-/-}$ mice, and not females, may represent variability due to the small number of severe dermatitis cases or it may correlate with the increased cage mate aggression generally observed in male mice (355-357). Resultant bite wounds or skin abrasions may facilitate bacterial entry into the skin and establishment of infection.

Although S. xylosus is generally considered a commensal bacterium, it has been reported to cause opportunistic infections in immunocompromised animals. Spontaneous *S. xylosus* infection has previously been reported in athymic nude mice (350, 351), indicating that T cells contribute to protection against spontaneous S. xylosus infection. *Rag1*$^{-/-}$ mice, like athymic nude mice, also lack mature T cells (224, 225, 358) but fail to develop clinical symptoms. The apparent difference in susceptibility to infection between these two strains is currently unclear but may be attributed to specific conditions within different animal facilities. Additionally, because athymic

nude mice lack a protective layer of hair, they may be more susceptible to dermal injuries that predispose to bacterial infections of the skin. In athymic nude mice, *S. xylosus* was never detected within internal organs but was localized to the skin (350). Dissemination into internal organs has only been reported in mice deficient in NADPH oxidase and superoxide production (161, 162, 353). It has previously been reported that Tpl2 is required for superoxide production in macrophages (316) and peritoneal exudate cells (317), which are likely due to defects in formation of the NADPH oxidase complex (316). Therefore, the combined defects in mature T cells and superoxide production within *Rag1*$^{-/-}$*Tpl2*$^{-/-}$ mice is consistent with their increased susceptibility to *S. xylosus* infection.

Interestingly, *Rag1*$^{-/-}$*Tpl2*$^{-/-}$ mice had elevated circulating monocytes compared to *Rag1*$^{-/-}$ mice. These results are inconsistent with a previous report in which generation of a microbiota through colonization of germ-free C57BL/6 mice with *S. xylosus,* among others, was not shown to impact relative levels of blood myeloid cells (330). Absolute monocyte/lymphocyte ratios are currently being investigated as methods to predict disease outcome. For example, changes in the monocyte/lymphocyte ratios have been shown to be useful in predicting incidence of tuberculosis (359, 360) and susceptibility to malaria (361). However, there was no significant difference in the absolute monocyte/lymphocyte ratio observed between *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$*Tpl2*$^{-/-}$ mice ($0.24 \pm 0.013$ versus $0.25 \pm 0.011$, respectively). Alternatively, elevated monocytes could indicate systemic infection with *S. xylosus*, as seen with *Staphylococcus aureus* (362).

In conclusion, we describe spontaneous *S. xylosus* infection within a mouse strain not previously identified. Infection within *Rag1*$^{-/-}$*Tpl2*$^{-/-}$ mice correlated with disseminated bacteria and elevated levels of circulating monocytes compared to *Rag1*$^{-/-}$ mice. Overall, these data are an important consideration for future research utilizing *Rag*$^{-/-}$*Tpl2*$^{-/-}$ mice in which underlying *S. xylosus*

infection may alter immune status and obscure experimental interpretations.

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**Table 5.1- Detection of S. *xylosus* in murine organs.**

| | *S. xylosus* | | | | | |
|---|---|---|---|---|---|---|
| Genotype | Feces | Skin | Spleen | LN | Lung | Brain |
| C57BL/6 | 0/3 | 3/3 | 0/3 | 0/3 | 0/3 | 0/3 |
| *Tpl2*$^{-/-}$ | 2/4 | 4/4 | 0/4 | 0/4 | 0/4 | 0/4 |
| *Rag1*$^{-/-}$ | 1/6 | 5/5 | 0/5 | 0/5 | 0/5 | 0/5 |
| *Rag1*$^{-/-}$ *Tpl2*$^{-/-}$ | 10/10 ** | 8/8 | 0/8 | 1/4 | 1/3 | 0/3 |

Feces, skin, spleen, lymph nodes, lung, and brain were collected from age-matched mice and cultured for *S. xylosus*. Data were analyzed by Fischer's exact test relative to C57BL/6 mice.

**$p < 0.005$.

## FIGURES

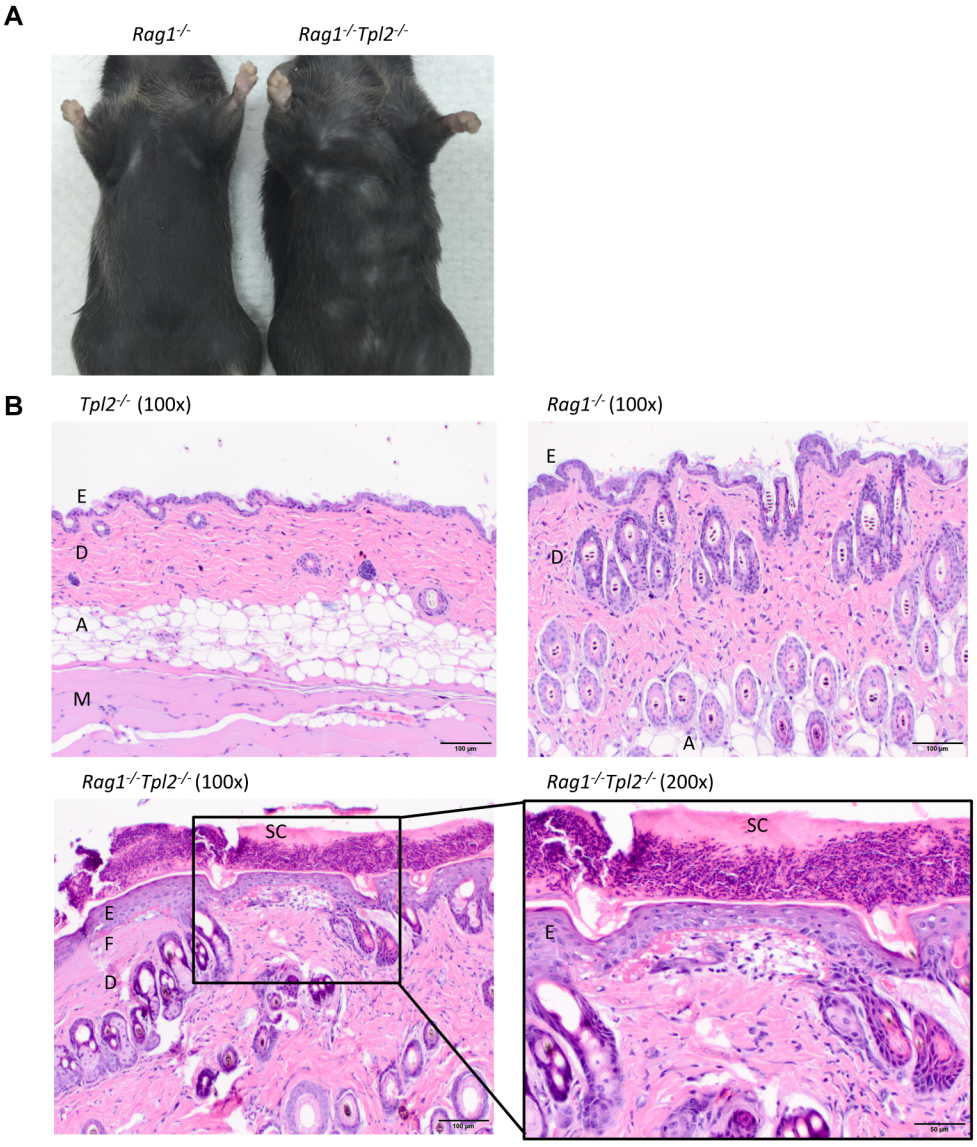


**Figure 5.1- Gross morphology and histology in *Rag*$^{-/-}$*Tpl2*$^{-/-}$ mice.** (A) *Rag*$^{-/-}$*Tpl2*$^{-/-}$ mouse showing ruffled fur, alopecia, crusts and scabbing next to a *Tpl2*$^{-/-}$ mouse. (B) Representative histology from skin of *Rag*$^{-/-}$, *Tpl2*$^{-/-}$, and *Rag*$^{-/-}$*Tpl2*$^{-/-}$ mice. 100x original magnification, scale bar=100µm. Square indicates area at 200x original magnification, scale bar=50µm. SC- serocellular crust, E- epidermis, F- fibrosis, D- dermis, A- adipose, M- cutaneous muscle.

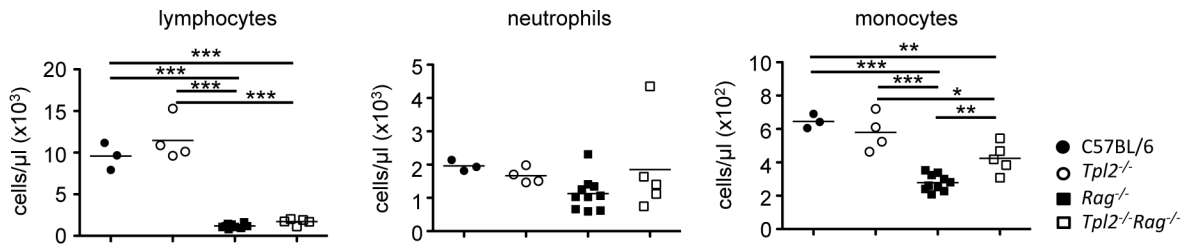


**Figure 5.2- *Rag*$^{-/-}$*Tpl2*$^{-/-}$ mice have elevated numbers of circulating monocytes**. Blood was collected from age-matched mice and analyzed for concentration of white blood cells. Results are shown as a cells/µl. N≥3. Lines represent means. *p<0.05, **p<0.01, ***p<0.001

# CHAPTER 6

# CONCLUSION

Tpl2 is known to influence CD4 T helper cell differentiation into Th1 and Th2 cells; however, the role of Tpl2 in Th17 cell differentiation and effector function has not been well established. The main goal of this study was to determine how Tpl2 is involved in Th17 cell differentiation and disease outcome in both autoimmune and infectious disease settings. We found that Tpl2 regulates Th17 differentiation and effector functions at several stages, which influences disease outcome in a context-dependent manner. First, Tpl2 regulates Th17 cell production of IL-17A in a TGF-β-dependent and FoxP3-restricted manner (Figure 2.4) (107). Second, Tpl2 promoted neutrophil egress from the bone marrow and into the circulation at homeostasis (Figure 4.2) and inflammation-induced recruitment at both innate (Figure 4.4A) (42, 317, 318) and adaptive (Figure 3.5C) (38) time points in a cell-extrinsic manner through defects in inflammatory cytokine and chemokine production. *Tpl2*$^{-/-}$ neutrophils secreted less inflammatory cytokines, produced less superoxide and killed *C. rodentium* less efficiently compared to wild type neutrophils (Figures 4.5-4.6). Combined, the role for Tpl2 in Th17 cell differentiation and disease outcome in autoimmune and infectious models of inflammatory bowel disease was shown to be model-specific (Table 6.1). The following presentation is a summary of the multiple and varied roles of Tpl2 in the context of Th17 associated immunity (Figure 6.1).

## TPL2 IN NEUTROPHIL DEVELOPMENT, RECRUITMENT AND FUNCTIONS

Development of both innate and adaptive immune cells begins in the bone marrow from hematopoietic stem cells (HSC). Neutrophil development from progenitors proceeds through

several stages within the bone marrow before being released into the circulation (Figure 6.1). Within the bone marrow, Tpl2 does not influence the relative proportions of common myeloid progenitors (CMP) or granulocyte/monocyte progenitors (GMP), indicating normal development of neutrophils in *Tpl2*$^{-/-}$ mice (Xin Li and Wendy Watford, unpublished data). Despite normal neutrophil development, we observed higher proportions of Gr-1$^{+}$ neutrophils within the bone marrow and reduced concentrations of neutrophils within the circulation of *Tpl2*$^{-/-}$ mice (Figure 4.2). CXCR4 expression on the surface of neutrophils is thought to negatively regulate neutrophil egress from the bone marrow into the circulation (141, 142); however, *Tpl2*$^{-/-}$ neutrophils expressed similar levels of CXCR4 (Figure 4.3). G-CSF is thought to positively regulate neutrophil mobilization from the bone marrow into the circulation (140), and consistent with this report we observed reduced concentrations of G-CSF within the blood of *Tpl2*$^{-/-}$ mice (Figure 4.3). Therefore, although Tpl2 does not regulate development of neutrophils from progenitors, it does promote neutrophil egress from the bone marrow and into the circulation via chemokine production.

Neutrophils are the first cell type recruited to sites of inflammation from the circulation. Neutrophils are recruited through upregulation of CXCR2 and CXCR4 that bind CXCL1 (KC), CXCL2 (MIP-2), CXCL15, and CXCL12 (142, 145-147) and adhere to the endothelium upon upregulation of P-selectins and E-selectins (148, 149). Both wild type and *Tpl2*$^{-/-}$ neutrophils express similar levels of CXCR2, CXCR4, CD44, and CD62L (Figure 4.3), indicating similar capabilities of being recruited into inflammatory tissues. However, *Tpl2*$^{-/-}$ mice show impaired neutrophil recruitment in response to treatment with zymosan (42), acetaminophen (317), caerulein (318), and thioglycollate (Figure 4.4A), as well as during $MOG_{35-55}$-induced experimental autoimmune encephalomyelitis (EAE) (38) and infection with *Citrobacter*

*rodentium* (Figure 3.5C). Defects in neutrophil recruitment are neutrophil extrinsic, as *Tpl2*$^{-/-}$ neutrophils are capable of being recruited within a wild type host (Figure 4.4B-C). Therefore, defects in inflammatory cytokine and chemokine production, including TNF, IL-1β, G-CSF, KC and CXCL2, by *Tpl2*$^{-/-}$ innate immune cells during inflammatory responses, such as dendritic cells and macrophages (24, 25, 42, 220, 332-334), induces less neutrophil recruitment to sites of inflammation (Figure 6.1).

Once within the tissue, the main goal of neutrophil antimicrobial functions is to promote an inflammatory environment and kill microbes. Neutrophil-mediated killing is a multi-step process (reviewed in (152)) that includes cytokine secretion, phagocytosis, degranulation, respiratory burst and release of neutrophil extracellular traps (NETs) (Figure 6.1). Similar to macrophages and dendritic cells (24, 25), Tpl2 promotes neutrophil secretion of TNF upon stimulation with LPS (Figure 4.5A). Tpl2 has already been shown to influence macrophage phagocytosis through FcγR (30) and may similarly promote neutrophil phagocytosis. After phagocytosis, neutrophils produce reactive oxygen species (ROS), including superoxide, to kill microbes such as *Staphylococcus, Escherichia, Pseudomonas,* and *Candida spp.* (164). Tpl2 promotes superoxide production in both macrophages (316) and neutrophils (Figure 4.5B) in a stimulus-specific manner. *Rag1*$^{-/-}$*Tpl2*$^{-/-}$ mice have enhanced skin inflammation and pathology due to spontaneous infection with the commensal bacterium *Staphylococcus xylosus* (Figure 5.1) as well as bacterial dissemination (Table 5.1), which may be due to reduced superoxide production by *Tpl2*$^{-/-}$ phagocytes through NADPH oxidase (161, 350, 353). Neutrophils are also capable of producing neutrophil extracellular traps (NETs), through a process termed NETosis, which traps and kills extracellular bacteria and fungi (165-170). Because ERK is required for NET formation (322, 323), Tpl2 ablation may also impair the ability of neutrophils to form NETs, although this was

not investigated in this study. However, this study does confirm the importance of Tpl2 in neutrophil-mediated killing of the extracellular bacterium *Citrobacter rodentium* (Figure 4.6).

**REGULATION OF TH17 CELL DIFFERENTIATION BY TPL2**

During the adaptive phase of immune responses, Tpl2 has been shown to influence the differentiation of CD4 helper T cell lineages; however, there is little information regarding the regulation of Th17 differentiation by Tpl2. Traditionally, Th17 cells have been induced *in vitro* using the cytokines IL-6 and TGF-β (67-69). We first confirmed that *Tpl2*$^{-/-}$ T cells are impaired *in vitro* in the production of IL-17A and IL-17F under these classical Th17 polarizing conditions (Figure 2.1); however, there was no impairment in *Rorc, Rorα, or Irf4* expression or STAT3 activation (Supplemental Figure 2.5) (107). Decreased expression of IL-17A correlated with increased FoxP3 expression by *Tpl2*$^{-/-}$ CD4 T cells (Figure 2.3). IL-17A production was restored to wild type levels in *Tpl2*$^{-/-}$ Th17 cells when the TGF-β concentration was reduced, neutralizing IL-2 antibody was added, or when Th17 cells were alternatively induced by IL-6 and IL-23, all of which failed to induce FoxP3 expression (Figure 2.4) (107). Similarly, wild type and *Tpl2*$^{-/-}$ Th17 cells secreted similar concentrations of IL-22 when induced by IL-6 and IL-23 (Figure 2.4A) (107). These data defined a TGF-β- and FoxP3-restricted defect in IL-17A secretion by *Tpl2*$^{-/-}$ T cells, but indicate that Tpl2 does not regulate IL-22 secretion by CD4 T cells (Figure 6.1).

IL-17A and IL-17F signal through a heterodimeric receptor composed of IL-17RA and IL-17RC, and IL-22 signals through a heterodimeric receptor composed of IL-22R and IL-10Rβ (Figure 1.2) (121). Downstream of the signaling complex, IL-17 signals through NF-κB, MAPKs, and C/EBPs whereas IL-22 signals through MAPKs as well as JAK/STAT pathways allowing for expression of inflammatory cytokines, chemokines, and inflammatory mediators (reviewed in

(121, 129, 130)). In a mouse skin epidermal cell line, astrocytes and murine embryonic fibroblasts (MEF), Tpl2 is phosphorylated by IL-17A, which leads to the activation of JNK and p38 (38, 133). Because of similar signaling through MAPKs, Tpl2 may also influence IL-22 receptor signaling as well as IL-17A receptor signaling.

**TPL2 IN TH17-ASSOCIATED INFLAMMATORY BOWEL DISEASE**

Dysregulated Th17 responses are implicated in the development of autoimmune diseases, including inflammatory bowel disease (IBD) (reviewed in (77)). Several models exist for studying IBD in mice, including (A) chemically induced colitis with dextran sulfate sodium (DSS) (213-216), (B) a T cell transfer model of colitis (212), and (C) infection induced colitis with *Citrobacter rodentium* (217, 218). Comparisons of diseases outcome and immune responses generated in models of IBD by *Tpl2*$^{-/-}$ mice are shown in Table 6.1.

A. In a chemically induced model of colitis, dextran sulfate sodium (DSS) damages intestinal epithelial cells and therefore alters barrier function of the intestines, leading to hematochezia, body weight loss, shortening of the intestine, mucosal ulcers, and infiltration of neutrophils (213-216). In this innate immune model of colitis, *Tpl2*$^{-/-}$ mice experienced milder colitis compared to wild type mice with reduced production of inflammatory cytokines IL-1α, IL-1β, IL-6, and IL-17, as well as reduced production of the anti-inflammatory cytokine IL-10 (220). Additionally, *Tpl2*$^{-/-}$ mice trended towards reduced recruitment of neutrophils into the intestines (220).

B. In a T cell transfer model of colitis, $CD4^{+}CD25^{-}CD45RB^{hi}$ cells are adoptively transferred into *Rag*$^{-/-}$ mice. Because immunosuppressive $CD25^{+}$ regulatory T cells are excluded from the adoptive transfer, *Rag*$^{-/-}$ mice do not develop peripheral tolerance characterized by $FoxP3^{+}$ Tregs (228) leading to an unchecked inflammatory response within production of TNF, IFNγ, IL-17A, IL-22, and IL-23 (205, 212, 222, 229-233). Recipients of *Tpl2*$^{-/-}$ cells had reduced proportions of

$CD4^+IFN\gamma^+$ cells, but not $CD4^+IL\text{-}17A^+$ cells, within the spleen and mesenteric lymph nodes (MLN) (Figure 2.2D) (107). However, recipients of *Tpl2*$^{-/-}$ CD4 T cells also had more total cells and CD4 T cells within their spleens and MLN compared to recipients of wild type cells (Figure 2.2E) (107), indicating that Tpl2 deficiency enhanced lymphopenia-induced accumulation of transferred effector CD4 T cells. Therefore, despite reduced proportions of $CD4^+IFN\gamma^+$ T cells within recipients of *Tpl2*$^{-/-}$ T cells, their absolute number was either unchanged or increased relative to recipients of wild type T cells (Figure 2.2F). Consequently, recipients of Tpl2-deficient T cells were no more susceptible to the development of colitis than recipients of wild type T cells as measured by weight loss (Figure 2.2A) and pathology (Figure 2.2C) (107).

C. *Citrobacter rodentium* is a non-motile gram-negative rod that colonizes the large intestine, primarily the cecum and distal portion of the colon (257). During peak infection, *Tpl2*$^{-/-}$ mice had significantly higher bacterial burdens than wild type mice as detected by changes in luminescence (Figure 3.1A-B), yet both wild type and *Tpl2*$^{-/-}$ mice similarly cleared the bacteria by three weeks post infection (Figure 3.1). Because, *C. rodentium* can disseminate out of the intestines and be found in the nasopharynx, lung, heart, liver, and spleen (244), we evaluated bacterial dissemination within *Tpl2*$^{-/-}$ mice. At the same time points in which *Tpl2*$^{-/-}$ mice experienced greater bacterial burdens, *Tpl2*$^{-/-}$ mice also had greater bacterial dissemination within the liver and spleen (Figure 3.2). Evaluation of the CD4 T cell response generated two weeks post infection showed trends towards reduced proportions of IL-17A-, IL-22-, and IFNγ-positive *Tpl2*$^{-/-}$ CD4 T cells within the lamina propria. (Figure 3.3A-B). This was shown to be a T cell-intrinsic defect in IFNγ and IL-17A production by *Tpl2*$^{-/-}$ CD4 T cells (Figure 3.3.C). The colons of *Tpl2*$^{-/-}$ mice also had reduced pathology and neutrophil infiltrates relative to wild type mice (Figure 3.5). Reduced pathology during the adaptive phase of the immune response is consistent

with reduced IFNγ production by *Tpl2*$^{-/-}$ Th1 cells as well as Th17 cell differentiation, which are known to regulate development of pathology (269) and neutrophil recruitment.

**IMPLICATIONS**

Because of its contributions to inflammatory pathways, Tpl2 is being considered as an immunotherapeutic target for treating autoimmune diseases. Many autoimmune diseases, such as rheumatoid arthritis and inflammatory bowel diseases, are characterized by a mixed Th1/Th17 pathology. These studies demonstrate how Tpl2 regulates Th17 responses and disease outcome and provide valuable insights into potential benefits and risks associated with Tpl2 inhibition in patients. Development of IL-17A expressing CD4 T cells *in vivo* was model-specific, with Tpl2 regulating Th17 cell differentiation during infection with *C. rodentium* but not during a T cell transfer model of colitis. Tpl2 was shown to promote IL-17A by negatively regulating FoxP3 expression in Th17 cells. Therefore, differential Th17 cell induction could be due to differences in utilization of TGF-β and FoxP3 between these models. Specifically, the adoptive T cell transfer model excludes the presence of FoxP3$^{+}$ Treg cells, a source of TGF-β, which may explain why no difference was observed in this model. Because Tpl2 ablation led to increased FoxP3 expression by Th17 cells cultured in the presence of TGF-β, inhibition of Tpl2 may also be expected to promote T regulatory cell differentiation, FoxP3 expression, and generation of an immunosuppressive environment.

Consistent within all experimental models was reduced neutrophil recruitment to sites of inflammation, during both innate and adaptive phases of the immune response in *Tpl2*$^{-/-}$ mice. Defects in recruitment were shown to be neutrophil-extrinsic (Figure 4.4) and therefore due to reduced inflammatory chemokines produced at inflammatory sites within *Tpl2*$^{-/-}$ mice (42, 317, 318). Therefore, Tpl2 inhibitors would be predicted to be beneficial in reducing inflammatory

cytokine production and inflammatory cell infiltrates seen in autoimmune diseases, but chronic administration may also predispose patients to bacterial and fungal infections normally cleared by a functional neutrophil response.

*Tpl2*$^{-/-}$ and *Rag1*$^{-/-}$*Tpl2*$^{-/-}$ mice were more susceptible to systemic infection with *Citrobacter rodentium* and *Staphylococcus xylosus*, respectively, indicating that Tpl2 regulates the integrity of the epithelial barrier within the intestines. How Tpl2 regulates intestinal permeability remains unknown, but indicates that chronic Tpl2 inhibition could disrupt the integrity of the intestinal barrier within patients making them more susceptible to opportunistic infections caused by gut microflora.

These data confirm a cell-type and stimulus-specific role for Tpl2 in regulating inflammation. Furthermore, due to model-specific variations in the regulation of immune responses by Tpl2, inhibition of Tpl2 must be thoroughly evaluated in different models of autoimmunity and infection.

**Table 6.1- Tpl2 in Th17-associated inflammatory bowel diseases**

| | | ***Tpl2*$^{-/-}$ mice** | **REF** |
|---|---|---|---|
| **IBD** | DSS colitis | ↓ IL-1α, IL-1β, G-CSF, IL-17A in serum<br>↓ pathology | (220) |
| | T-cell transfer | ↓ % IFNγ$^{+}$CD4$^{+}$ cells in periphery<br>= % IL-17A$^{+}$CD4$^{+}$ cells in periphery<br>↑ CD4$^{+}$ cells in periphery<br>= pathology | (Ch. 2) |
| | *C. rodentium* | ↓ % IL-17A$^{+}$CD4$^{+}$ cells in LPL<br>↓ % IFNγ$^{+}$CD4$^{+}$ cells in LPL<br>↑ burden at peak infection<br>↓ neutrophils in IEL<br>↓ pathology | (Ch. 3) |

## FIGURES

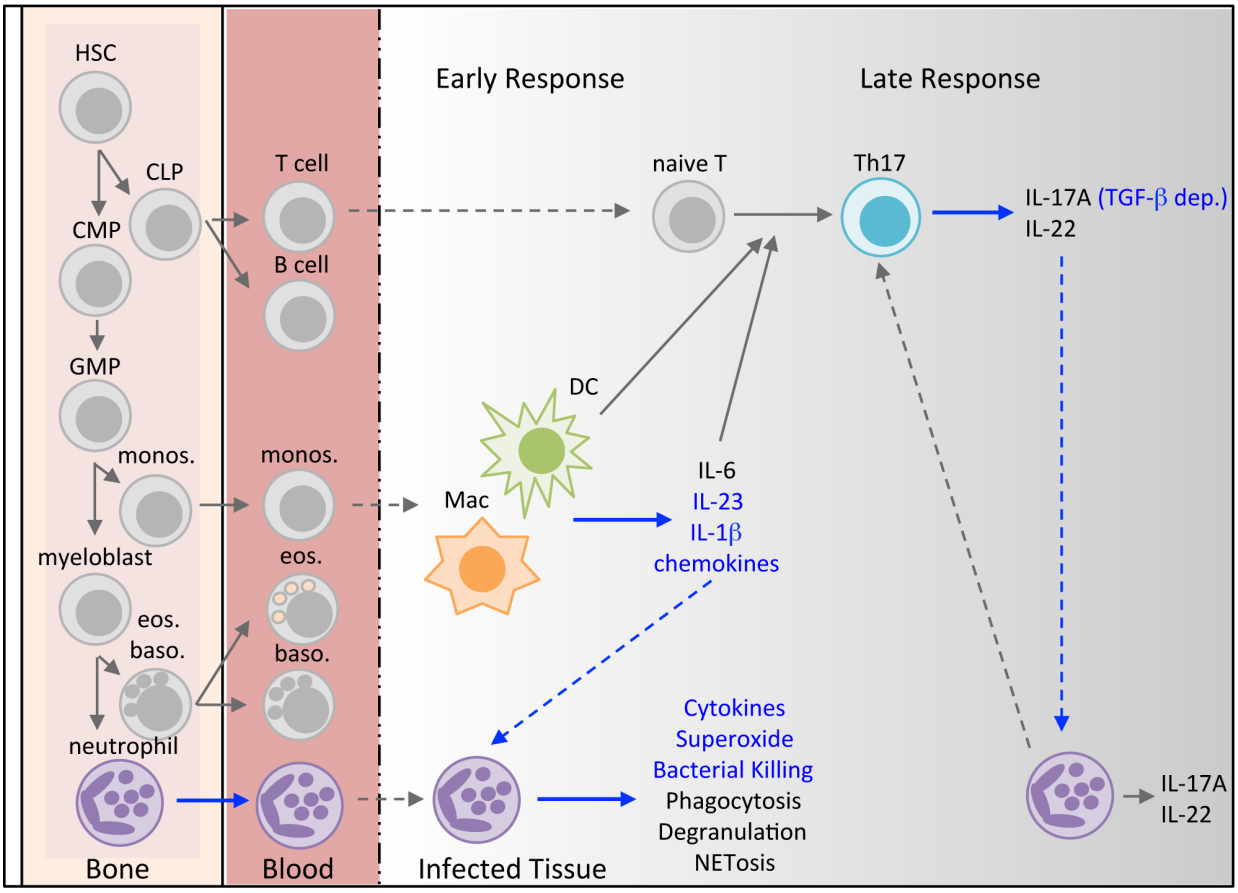


**Figure 6.1: Summary influence Tpl2 on Th17 cell development and neutrophil function.** Solid line represents differentiation or secretion. Dashed lines represent recruitment. Blue lines/words indicate where Tpl2 influences induction of response.

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