2011

Determining the functional role of TREM-like transcript 2 in the innate immune response

Matthew Martin Halpert
University of Alabama at Birmingham

DETERMINING THE FUNCTIONAL ROLE OF TREM-LIKE TRANSCRIPT 2 IN THE INNATE IMMUNE RESPONSE

by

MATTHEW M. HALPERT

DR. LOUIS B. JUSTEMENT, COMMITTEE CHAIR
DR. DAVID E. BRILES
DR. SUZANNE M. MICHALEK
DR. CHANDER RAMAN
DR. CHAD STEELE

A DISSERTATION

Submitted to the graduate faculty of The University of Alabama at Birmingham,
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

BIRMINGHAM, ALABAMA

2011

DETERMINING THE FUNCTIONAL ROLE OF TREM-LIKE TRANSCRIPT 2 IN THE INNATE IMMUNE RESPONSE

MATTHEW M. HALPERT

MICROBIOLOGY

TLT2 is one of four receptors conserved between mouse and human within the TREM locus, and is expressed on B cells, macrophages, and neutrophils. TLT2 ligation on murine macrophages induces the production of chemokines and growth factors, as evidenced by ex vivo treatment with anti-TLT2 mAbs. This treatment did not lead to the upregulation of activation markers such as CD69 or costimulatory molecules such as CD80 and CD86, indicating a specific response following TLT2 ligation. This is recapitulated in vivo following injection of anti-TLT2 mAbs resulting in the production of chemokines and growth factors, which ultimately lead to enhanced neutrophil recruitment. Ligation of TLT2 on murine neutrophils results in an enhanced respiratory burst, as well as potentiation of degranulation and chemotaxis in response to agonists that bind G protein-coupled receptors (GPCRs), including FMLP, KC, MIP-2, IL-8, and C5a. However, the neutrophil responses to GM-CSF, LPS, and FcR ligation were unaltered, suggesting that TLT2 specifically potentiates the neutrophil response to signals derived from GPCRs. Administration of anti-TLT2 mAb results in enhanced neutrophil accumulation in response to nonspecific inflammatory mediators in vivo, and competitive adoptive transfer experiments demonstrate enhanced recruitment of anti-TLT2 mAb treated neutrophils compared to control neutrophils in response to lung inflammation. These results demonstrate that TLT2 regulates an intricate feed-forward loop, wherein it

is important for driving the production of factors that recruit neutrophils, as well as enhancing the response of neutrophils towards chemokines and bacterial products that signal via GPCRs.

Keywords: TLT2, TREM, Neutrophils, Macrophages, GPCR, Inflammation

DEDICATION

I would like to dedicate this work to my close family and friends who have supported my desire to remain a student for as long as possible. My parents (Art and Lisa), who continually fed my passion to learn, were instrumental in my development as both a person and as a scientist. Without their encouraging guidance, it is unlikely that I would have taken my education as far as I have been able to. My younger siblings (Corinne and Jason), who have always looked up to me, drove me to be as positive a role model as possible, and therefore are also partially responsible for the internal motivation and drive that pushed me to reach this point. And of course my wonderful wife, Stephanie, who followed me across the country with nary a complaint so that I could pursue my educational and vocational goals. Without her support, this simply would not have been possible. I thank all of you for allowing me to accomplish this personal goal of mine.

ACKNOWLEDGEMENTS

I would like to acknowledge Kimberly Thomas and Dr. Rodney G. King for being instrumental in the work accomplished on the TLT-2 project. With their hands-on help in the lab and intellectual acumen throughout, this project has been very successful and has pushed our understanding of innate immunity and the TREM family to new heights. They deserve much of the credit for the work published here.

I want to thank the Microbiology department at the University of Alabama at Birmingham for providing an intelligent and collaborative environment conducive to high quality, productive science. More specifically, I would like to acknowledge Dr. Chander Raman, Dr. David Briles, and Dr. Suzanne Michalek for serving on my wonderful dissertation committee. Their input over the years has helped shape this project into the success it has become. I am also grateful to have had Dr. Chad Steele on my committee, as he put forth his intellect, time, and resources that were fundamental to the TLT-2 publications. He and his lab were instrumental in data shown here.

And then finally I must acknowledge my own mentor, Dr. Louis B. Justement, for providing the right balance of freedom and direction over my years at the University of Alabama at Birmingham. His mentorship is one of the primary reasons I have succeeded up to this point, and why I believe I can succeed in the future.

TABLE OF CONTENTS

Page

LIST OF FIGURES

Figures *Page*

TLT2 POTENTIATES NEUTROPHIL ANTIBACTERIAL ACTIVITY AND CHEMOTAXIS IN RESPONSE TO G PROTEIN-COUPLED RECEPTOR-MEDIATED SIGNALING

TLT2 PROMOTES MACROPHAGES TO SECRETE CHEMOKINES AND GROWTH FACTORS WHICH RAPIDLY RECRUIT NEUTROPHILS TO SITES OF INFLAMMATION

LIST OF ABBREVIATIONS

ALI	Acute Lung Injury
BAL	Bronchoalveolar Lavage
BCL-10	B-Cell Lymphoma 10
CARD9	Caspase Recruitment Domain 9
EAE	Experimental Autoimmune Encephalomyelitis
FcR	Fc Receptor
GP	Glycoprotein
GPCR	G-Protein Coupled Receptor
IBD	Inflammatory Bowel Disease
ITAM	Immunoreceptor Tyrosine-based Activation Motif
ITIM	Immunoreceptor Tyrosine-based Inhibitory Motif
MMP	Matrix Metalloproteinase
NETs	Neutrophil Extracellular Traps
NLR	Nod-like Receptor
PRR	Pattern Recognition Receptor
ROS	Reactive Oxygen Species
TLR	Toll-like Receptor
TLT-1	TREM-like Transcript 1
TLT-2	TREM-like Transcript 2
TREM	Triggering Receptor Expressed on Myeloid Cells

INTRODUCTION

The innate immune system, comprised in part of tissue macrophages and neutrophils, is responsible for protecting the host on a day to day basis from the overwhelmingly massive and diverse bacterial milieu constantly encountered. An inflammatory event and pathogenic challenge will elicit a response from the damaged endothelium and surrounding area to release cytokines that alert the immune system to the threat. Neutrophils and macrophages will be among the first responders, as they survey the area and phagocytose invading pathogens. As they move through this initial response, they will also secrete cytokines to recruit additional immune cells. Macrophages will utilize reactive oxygen species (ROS) and enzymes to break down and process the engulfed bacteria, and will present antigen on their surface along with MHC II and other co-stimulatory molecules to aid in the induction of the adaptive immune response. Meanwhile neutrophils, the most prevalent circulating white blood cell, will follow bacterial products, complement components, tissue factors, and chemokine gradients to migrate to the damaged area where they can exert a variety of anti-microbial functions to aid in resolution of the infection. This repertoire includes ROS production, the release of preformed enzymes, the expulsion of DNA-composed neutrophil extracellular traps (NETs), as well as the basic and sometimes underrated phagocytic function. Importantly, one of the greatest strengths of the neutrophil is in the sheer overwhelming number of neutrophils that respond, which tend to overtake the pathogen largely due to the advantage they possess in number. Once the infection is resolved, the

immune system has devised elegant methods to terminate the response without excessive damage to the surrounding tissue, and thus the host is protected.

However, macrophages and neutrophils express receptors that are involved in mediating a wide range of functional responses in addition to responding to chemokine gradients, and in fact have surveillance systems that allow them to directly identify the presence of pathogens (e.g. Toll-Like Receptors, NOD-Like Receptors, Mannose Receptors, etc). When these receptors bind their ligands, the cell is alerted to the surrounding threat, and can begin to respond accordingly. These cells also eloquently express receptors which can be used to modulate the cellular response in either an amplifying or attenuating method, thus providing the ability to fine-tune the response in a quantitative way (e.g. TREM-1, TREM-2). The TREM family has recently been identified as a group of surface expressed receptors which excel at modulating the immune response on several innate cell types, including macrophages and neutrophils. Given how important both the efficacy and regulation of the innate immune response are, the TREM family has recently been shown to be important and is being studied to determine how these receptors may function in controlling the host immune response (Mosser and Edwards, 2008; Nathan, 2006).

CONSERVED RECEPTORS OF THE TREM FAMILY

The Triggering Receptor Expressed on Myeloid cells (TREM) family of transmembrane receptors has attracted increasing attention as important modulators of innate immunity since the first family member, TREM-1, was characterized in the year 2000 (Bouchon et al., 2000). The TREM gene cluster on mouse chromosome 17c3 and

human chromosome 6p21.1 share four direct homologues, TREM-1, TREM-2, TREM-like transcript 1 (TLT-1), and TREM-like transcript 2 (TLT-2), which are expressed only on innate cell types with the exception of TLT-2 (Bouchon et al., 2000; Daws et al., 2001; King et al., 2006; Washington et al., 2002). In general, this family has emerged as important regulators of innate immunity displaying the ability to both potentiate and attenuate immune responses.

TREM family members have low sequence homology to one another (≈30%), but share considerable structural similarities as they all contain a single extracellular Ig-like domain, one transmembrane region, and a relatively short cytoplasmic tail. Most of the TREM family members (TREM-1 and -2 in human; TREM-1, -2, -3, and TLT-4 in mouse) have a positively charged residue within their single-spanning hydrophobic region that allow them to interact with the aspartate residue of the innate adaptor protein DAP12/TYROBP (Ford and McVicar, 2009; Klesney-Tait et al., 2006). TLT-1 is the only family member to signal via its own cytoplasmic tail through an ITIM motif, and does not require DAP12 (Washington et al., 2004). TLT-2 however has no such charged residue and thus does not interact with DAP12 or other membrane bound adaptor proteins, nor does it contain motifs that appear to be involved in tyrosine phosphorylation-based signaling. Moreover, the cytoplasmic region is not highly conserved between mouse and human, and the best candidate for mediating signals through this receptor may be a pseudo proline-rich segment that may allow this receptor to hydrophobically interact with SH3- or WW-domain containing proteins. Additionally, it is possible that these prolines could confer a tertiary structure that allows interaction with other intracellular signaling proteins, though to date no such interacting proteins

have been identified (King et al., 2006). Therefore, TLT-2 may signal via a novel mechanism which requires further in-depth study.

The ITAM of DAP12 is phosphorylated by Src family kinases following binding to an activated membrane-bound receptor, which usually leads to canonical cellular activation via membrane proximal signals through phosphatidylinositol-3-OH kinase (PI3K), phospholipase –Cγ (PLCγ), SLP-76, Vav, Grb2, SOS, c-Cbl and downstream targets including Akt (PKB), protein kinase C (PKC), and mitogen-activated protein kinases (MAPKs) (Aoki et al., 2003; Aoki et al., 2004; Daws et al., 2001; Humphrey et al., 2004; Tessarz and Cerwenka, 2008). Therefore, it is of great interest to determine how TREM-1 and TREM-2 seemingly exert contradictory effects on innate immune cell activation (discussed below) when both recruit DAP12 following activation. Several recent TREM studies have been devoted to solving this perplexing question, and will be highlighted in this review.

TREM-1 OVERVIEW

TREM-1, initially characterized by Colonna and colleagues (Bouchon et al., 2000) is expressed on $CD14^+$ monocytes, neutrophils, and macrophages (although baseline expression varies between subtypes) and is upregulated in response to lipopolysaccharide (LPS) and other microbial products as has been shown *in vitro* by flow cytometer analysis (Bouchon et al., 2000). Although LPS may directly lead to TREM-1 upregulation, it is also possible that the observed result is through an indirect, auto/para-crine action as LPS will drive production of TNFα and COX-2 which have been shown to lead to TREM-1 upregulation (Laskin et al., 2010). In contrast,

environmental cues can push LPS to drive production of IL-10, which abrogates TREM-1 upregulation after TLR stimulation (Bleharski et al., 2003). *In vivo*, TREM-1 has been shown to be upregulated using immunohistochemical analysis of bacterial-induced lesions, but not in non-infectious inflammatory-induced conditions such as vasculitis (Bouchon et al., 2001a). Interestingly, TREM-1 is not expressed on resting intestinal macrophages, and is not induced even after TNFα stimulation, showing specification within cell-type subpopulations (Schenk et al., 2005).

Crosslinking TREM-1 using mAbs has been shown to lead to modest cellular activation (e.g. degranulation, respiratory burst, phagocytosis, calcium mobilization, and cytokine release), but its greater effect is observed when synergistic signals that are driven through Pattern Recognition Receptors (PRRs) such as the TLRs (e.g. TLR4) and NLRs are provided (Bouchon et al., 2000; Bouchon et al., 2001a). As an example, both neutrophils and monocytes secrete significantly more cytokine (IL-8 via neutrophils, IL-8, MCP-1, and TNFα via monocytes) when both TREM-1 and LPS are added compared to either stimulus applied alone (Bouchon et al., 2001a). Thus, TREM-1 is an important amplifier of the innate immune response, effectively lowering the threshold of pathogen detection. TREM-1 and TLR4 share certain downstream signaling effects, but the pathways proximal to the membrane are dissimilar, leaving questions as to the exact nature of their interaction and how they synergistically work together to amplify cellular responses. The fact that prolonged PRR signaling leads to upregulation of TREM-1 on the surface is likely to contribute to enhanced responses, and it has also been shown that stimulation through either TREM-1 or TLR4 leads to colocalization of the receptors in lipid rafts, which could enhance membrane proximal synergy. Additionally, it has also

been shown that both MyD88 and DAP12 can lead to NF-κB activation through PI3K, which may be an important convergent point between TREM-1 and TLR signaling (Klesney-Tait and Colonna, 2007). In 2007, Ornatowaska and colleagues showed that silencing TREM-1 on RAW 264.7 macrophages with shRNAi lead to a severe decrease in the transcription of proteins fundamental to TLR4 signaling, such as MyD88 and CD14, following LPS treatment. Given the wide spread role of MyD88, it is possible that this mediator is the convergent point for TREM-1 signaling and a myriad of other PRR signals, but more in depth analysis will be needed.

Additional intracellular analysis after TREM-1 ligation has shown an increase in nuclear levels of NF-κB activity, which can play a role in the transcription of inflammatory agents such as IL-1β, IL-2, IL-12p40, and TNFα. Recent bone marrow-derived dendritic cell (BMDC) data has shown that Caspase Recruitment Domain 9 (CARD9) associated with B Cell Lymphoma 10 (BCL-10) after TREM-1 ligation to mediate NF-κB activation and cytokine production, and that knocking out either CARD9 or BCL-10 in this system lead to a significant depression in the secretion of the aforementioned cytokines. The signaling mediators upstream of the CARD9/BCL-10 complex that link TREM-1 signaling to NF-κB activation are still largely unknown (Hara et al., 2007). For monocytes, administration of agonistic TREM-1 mAbs lead to an increase in expression of antigen presenting molecules such as MHC II and CD86, and enhanced T cell activation *in vitro* (Bleharski et al., 2003). Therefore, while TREM-1 is mostly thought of as a regulator of the innate immune response, it can have indirect effects on enhancing the adaptive immune response as well.

Although *in vitro* data point towards TREM-1 playing a role in enhancing inflammation, a considerable amount of *in vivo* work has been performed which also solidifies the role of TREM-1 in potentiation of inflammation associated with infection. Administration of a TREM-1/IgG1 Fc fusion protein *in vivo* provided support that TREM-1 is directly involved in enhancing the inflammatory response, because abrogation of membrane-bound TREM-1 signals on cells with this protein lead to a reduced inflammatory response and better survival in models of murine endotoxemia and septic peritonitis (Gibot et al., 2006a). Conversely, addition of an agonistic TREM-1 mAb in models of LPS-induced septic shock doubled the mortality rate (Gibot et al., 2004b). Although blocking TREM-1 signals protected mice from experimentally-induced sepsis, complete silencing of TREM-1 increases the mortality rate in animals given a bacterial challenge, indicating an important role for TREM-1 in bacterial clearance and survival (Gibot et al., 2007b). Therefore, there appears to be a critical balancing act between TREM-1-mediated signaling to enhance the inflammatory response for effective bacterial clearance and that leading to a septic, fatal outcome. Further studies are needed to identify when this tipping point occurs, and how this could be used to therapeutic advantage.

An animal model of LPS-induced shock was employed in which soluble TREM-1 ectodomain (sTREM-1) was injected hours after LPS challenge to compete for the TREM-1 endogenous ligand. The sTREM-1 treated mice experienced lower circulating cytokine concentrations and better survival, indicating that TREM-1 does indeed play a role in potentiating septic shock (Zanzinger et al., 2009). Additionally, a cecal ligation model was used to test the ability of sTREM-1 to attenuate the inflammatory response

against a bacterial infection, demonstrating once again that TREM-1 plays a role in amplifying the innate immune response *in vivo* (Gibot et al., 2006b). Although a TREM-$1^{-/-}$ mouse does not yet exist, $DAP12^{-/-}$ mice exhibited an attenuated inflammatory response to septic shock whereas bacterial control was still possible and effective (Hamerman et al., 2006). Although studies of the $DAP12^{-/-}$ mouse potentially point towards the important role that TREM-1 plays during inflammation, it is not conclusive as DAP12 mediates signaling via several membrane receptors.

Naturally occurring sTREM-1 was found in the supernatants following phagocyte treatment with LPS, in the plasma following endotoxin treatment of mice, in humans treated with LPS as well as septic patients, and in the bronchoalveolar lavage (BAL) of pneumonia patients (Gibot et al., 2007a; Gibot et al., 2004a; Knapp et al., 2004; Mahdy et al., 2006). Despite controversy early on, it has now been well established that this soluble form of TREM-1 is due to shedding of the membrane bound receptor by matrix metalloproteinases (MMPs), as blocking MMP activity but not other proteases, greatly decreased the amount of sTREM-1 present after LPS treatment. Expectedly, blocking MMP activity allowed monocytes and neutrophils to retain normal TREM-1 expression throughout treatment (Gomez-Pina et al., 2007). Despite the overwhelming evidence that supports shedding as the most likely source of sTREM-1, it is still possible that sTREM-1 results from alternative splicing. There has indeed been a TREM-1 alternative mRNA splice variant identified in human monocytes and $CD34^{+}$ bone marrow cells which lacks sequences for the transmembrane and cytoplasmic regions if translated, and this could therefore be the source of sTREM-1 (Begum et al., 2004; Gingras et al., 2002). However,

the predicted mass for this variant is 17.5 kDa, which is approximately 10 kDa lighter than the sTREM-1 (27 kDa) found in patients.

Although it is not completely known exactly why TREM-1 is shed, it is hypothesized that it serves as a negative feedback loop and competes for the endogenous ligand to prevent excessive inflammation. sTREM-1 has become a reliable diagnostic marker given that it is detected at high concentrations in patients with bacterial infections, and in fact has become the most accurate marker at predicting the presence of bacterial or fungal infection in BAL fluid with 98% sensitivity and 90% specificity (Gibot and Cravoisy, 2004).

TREM-1 has also been studied as a potential culprit in disease pathology defined by excessive inflammation and cytokine production such as hemorrhagic fever and gout (Akahoshi, 2008; Mohamadzadeh et al., 2006; Murakami and Kohsaka, 2009). Marburg and Ebola filoviruses are thought to progress through disease states towards mortality as a result of dysregulated innate immune functions, even after successful initial clearance of viral particles, and it is thought that this excessive cytokine production is the cause of the fatal pathology of these diseases. Additionally, the peptide LP17 (comprising the CDR3 of the extracellular domain of TREM-1) has successfully been used to inhibit TREM-1 signaling on human neutrophils during filoviral challenge, most likely either due to blocking interaction with the endogenous ligand or impairing TREM-1 dimerization (Mohamadzadeh et al., 2006). Monocytes cultured with LPS (with or without anti-TREM-1) produced less IL-1β and TNFα when LP17 was also administered, and this anti-inflammatory effect also occurs *in vivo* as either pre-treatment of mice with a single dose of LP17 prior to, or treatment of mice 4 h after a lethal dose of LPS

conferred a survival advantage compared to control mice (Gibot et al., 2009; Schenk et al., 2007). Moreover, a protective effect of using LP17 to attenuate TREM-1 signaling has been shown in several disease models, including *P. aeroginosa* and *B. pseudomallei* pneumonia (Wiersinga et al., 2007).

In addition to sepsis and bacterial disease, TREM-1 has also recently been linked to inflammatory bowel disease (IBD), placing TREM-1 as a potential player in the auto-immune category. Although resting intestinal macrophages predominately do not express TREM-1, TREM-1 expressing macrophages were found in the lamina propria of IBD patients. Ligation of TREM-1 on these macrophages leads to enhanced production of TNFα, IL-6, IL-8, and MCP-1, which are several of the proinflammatory mediators responsible for IBD pathology. Additionally, TREM-1 mRNA is significantly increased in the colon of mice after induction of experimental colitis, indicating a role for TREM-1 in IBD. Treatment with LP17 also conferred protection and survival in this model, decreasing severity even after establishment of colitis (Schenk et al., 2007).

Only putative ligands have been described for TREM-1, and the specific identification of the TREM-1 ligand remains largely unresolved. It has been hypothesized that there may be an exogenous TREM-1 ligand expressed as a glycoprotein (GP) on the surface of filoviruses such as Marburg and Ebola virus. This theory is supported by evidence that LP17 treatment attenuated proinflammatory cytokine production from neutrophils cultured with these viruses and that TREM-1 directly binds to GP-expressing cells (Mohamadzadeh et al., 2006). However, in light of the role of TREM-1 in sepsis and IBD, as well as the protective effects of LP17 and sTREM-1 in these diseases, an endogenous ligand is most likely present. Through mouse studies, a putative ligand has

been shown to be expressed on activated granulocytes and monocytes, which also express the TREM-1 receptor. Therefore, it has been proposed that interaction between TREM-1 and its ligand may occur both in *trans* and in *cis*, and that this may have different functional effects (Zanzinger et al., 2009). However, no interaction or functional consequence has been identified yet, and this hypothesis requires more study. Perhaps the best characterized putative TREM-1 ligand is found on human platelets, and may be the main source of ligand during infection. Use of several recombinant TREM-1 proteins displayed ligand expression on platelets, and that co-treatment with LP17 reduced staining with these proteins. Direct binding of neutrophils or monocytes with platelets was increased when treated with LPS or thrombin, and that this binding was dependent on CD18 and PSGL-1, but not TREM-1. While TREM-1 was not necessary for binding to platelets, co-culture of neutrophils or monocytes with platelets enhanced their anti-microbial repertoire (degranulation, respiratory burst, IL-8 production) after LPS treatment, and that this enhancement was completely abolished by the addition of sTREM-1 or anti-TREM-1. However, TREM-1L expression has not been found on murine platelets after intravenous LPS injection, and the identity of the natural ligand is still largely unknown (Haselmayer et al., 2007).

TREM-2 OVERVIEW

TREM-2 was first characterized shortly after the initial TREM-1 studies, and was originally identified through screening of macrophage cDNA libraries for proteins that interact with DAP12 (Daws et al., 2001). TREM-2 expression has since been found on a variety of cell types, including dendritic cells, macrophages, microglia, osteoclasts, and

even non-myeloid cells such as endothelial cells, and appears to have both immune and non-immune functions (Molloy, 2009). The non-immune function is demonstrated by individuals with Nasu-Hakola disease, a disease described by bone cysts and demyelinating lesions in the central nervous system (CNS) which lead to fatal presenile dementia. A majority of patients were originally found to have DAP12 deficiencies, though it was later discovered that patients with TREM-2 genetic defects suffered from the same disorder. Indeed, osteoclast differentiation in patients with TREM-2 genetic defects was severely arrested, which lead to large aggregates of immature osteoclasts and impaired bone resorptive activity (Cella et al., 2003; Turnbull et al., 2006). Additionally, TREM-2 ligation on bone marrow osteoclast precursors with mAbs enhanced the formation of osteoclasts *in vitro* (Humphrey et al., 2006).

In the immune system, it has generally been accepted that TREM-2 is a negative regulator of inflammation, as it exerts an anti-inflammatory effect on microglia and macrophages. However, it does appear to exert an activating role on dendritic cells where it induces the upregulation of the chemokine receptor CCR7 which plays a role in maturation and survival, which was supported by the observation that blockade of TREM-2 inhibited DC-cell mediated activation of NK cells. Additionally, *in vitro* cross-linking of TREM-2 lead to partial maturation of dendritic cells without additional stimulus (Bouchon et al., 2001b). Whether or not TREM-2 on dendritic cells is required for maturation remains to be determined, as does its role during inflammatory events.

Although originally identified via macrophage cDNA libraries, more extensive expression studies have now revealed that TREM-2 is expressed by recently differentiated macrophages that have been recruited to tissue or alternatively activated

peritoneal macrophages as evidenced by TREM-2 upregulation after macrophage treatment with IL-4. It is not highly expressed on myeloid progenitors, circulating cells, or tissue-resident macrophages. Concordantly with its role as an anti-inflammatory regulator, alveolar macrophages constitutively express the receptor and type II inflammation induces upregulation of TREM-2 *in vivo* during pulmonary inflammation resulting from allergens (Turnbull et al., 2006). Further supporting the claim that TREM-2 exerts an anti-inflammatory action on these cells, knockdown experiments using TREM-2 shRNA lead to doubling the amount of TNFα produced after CpG or Zymosan treatment, and this effect was specific to TREM-2 shRNA (Hamerman et al., 2006). An identical effect was observed in DAP12 deficient macrophages, and the TNFα production by these cells was inhibited by introduction of a TREM-2/DAP12 chimera. Further studies demonstrated that $DAP12^{-/-}$ macrophages unexpectedly secrete more inflammatory cytokines than wild-type macrophages when treated with TLR agonists, and that this effect was similarly observed in $TREM\text{-}2^{-/-}$ macrophages (Hamerman et al., 2005).

Microglia also express TREM-2, and it was shown that overexpression of TREM-2 lead to an increased ability to phagocytose apoptotic neurons, whereas knockdown decreased baseline rates of phagocytosis (Takahashi et al., 2005). Additionally, overexpression of TREM-2 lead to reduced production of TNFα and iNOS mRNA after co-culture with apoptotic neurons, and expectedly receptor knockdown lead to a higher production of these proinflammatory effectors (Frank et al., 2008). Therefore, it appears that although TREM-2 mediates activating signals to the microglia to enhance their

phagocytic function, it simultaneously negatively regulates inflammatory responses, as has been shown in macrophages.

Unactivated microglia in the spinal cord and CNS express TREM-2, and upregulate its expression during both the early and chronic phases of experimental autoimmune encephalomyelitis (EAE). Experiments testing blockade of TREM-2 during EAE showed an exacerbation of the disease state resulting in more inflammatory cell infiltrates and demyelination in the brain parenchyma, again highlighting a potential anti-inflammatory role for TREM-2 on microglia (Melchior et al., 2006).

Several putative ligands for TREM-2 have been described, all with varying degrees of evidence. It was originally reported that TREM-2 can bind multiple anionic ligands expressed on bacterial and mammalian cells, as use of a TREM-2:Fc fusion protein successfully showed that both Gram-negative and Gram-positive bacteria can bind macrophages via TREM-2. Fluorescently labeled *E. coli* and *S. aureus* bind specifically to TREM-2 transfected cells, and this binding is blocked by the addition of LPS, LTA, and peptidoglycan (Daws et al., 2003). Subsequently, it has been demonstrated that macrophages themselves express a putative TREM-2 ligand because they are able to bind TREM-2:Fc fusion proteins. Additionally, $F(ab)_2$ fragments of TREM-2 mAbs block binding of the fusion protein to macrophages, indicating a specific interaction between the TREM-2:Fc fusion protein and the ligand-expressing macrophages (Hamerman et al., 2006). These studies support the possibility that signals delivered via *cis* interactions may mediate tonic signals via TREM-2 that may play an important role in inducing a constitutive anti-inflammatory state in the macrophage.

More recently, the search for TREM-2 ligands has shifted to the CNS where neurons undergoing apoptosis appear to express a putative TREM-2 ligand that aids in their phagocytosis by microglia. This is particularly important from a biological perspective because signaling through TREM-2 has been shown to increase microglia phagocytic activity without eliciting inflammation, something that could be fatal in the CNS. TREM-2:Fc fusion proteins were observed to stain cortical and dopamine neurons as well as Neuro2A cells, and this staining was increased in conjunction with the onset of neuronal apoptosis. More importantly, apoptotic neurons stimulated microglia through TREM-2 signaling and this effect was blocked by administration of TREM-2 mAbs. It was also shown that one TREM-2 mAb (though not all) could reduce microglia-dependent engulfment of neurons, indicating that a potentially very specific and important functional site exists within the extracellular domain. Transfecting TREM-2 into Chinese hamster ovary cells conferred phagocytic ability to these normally non-phagocytic cells, which indicates that TREM-2 is sufficient for phagocytosis of apoptosing neuronal cells (Hsieh et al., 2009). These studies have lead several groups to explore either how TREM-2 could be used to restrain activated microglia, which are characteristic of several neuroinflammatory disorders, or to stimulate microglia to police the CNS by removing apoptotic cells without provoking an overwhelming inflammatory response.

TREM-1 vs. TREM-2: DIVERGING ROLES IN BIOLOGY

Of interest are the divergent roles of TREM-1 and TREM-2 in the innate immune response, especially in light of the fact that both bind to the adaptor protein DAP12, yet

seem to exert opposite effects on the inflammatory response. However, there have been several recent studies that are beginning to shed light on how this may occur.

TREM-1 vs. TREM-2: Differential Expression

Several studies have shown that TREM-1 mRNA and surface expression are increased after TLR treatment of cells, whereas TREM-2 mRNA and protein expression are typically decreased. Additionally, mycobacterial infection leads to upregulation of TREM-1, although TREM-2 expression was not altered under these experimental conditions (Aoki et al., 2004; Bleharski et al., 2003). $TLR4^{-/-}$ mice do not upregulate TREM-1 after exposure to LPS, indicating that upregulation is a direct result of TLR4 signaling (Zheng et al., 2010). The same has been found for multiple TLRs. Therefore, the different DAP12-mediated outcomes may be tightly regulated at the membrane based on the relative expression levels of TREM-1 vs. TREM-2.

A recent example supporting this idea comes from a study of Vasoactive Intestinal Peptide (VIP), which is an abundant, anti-inflammatory neuropeptide in the lung. VIP can protect the mouse from LPS-induced acute lung injury (ALI), and appears to do so through its control of TREM-1 and TREM-2 expression. The lung tissue from LPS treated mice exhibited higher levels of TREM-1 mRNA than control mice, whereas concurrently TREM-2 mRNA was decreased. TREM-1 was only expressed on stimulated macrophages, whereas TREM-2 mRNA was detected in several cell types, including fibroblasts, endothelial cells, and macrophages. The ratio of TREM-1 mRNA to TREM-2 mRNA increased in ALI mice, potentiating inflammation and a septic outcome. However, administration of VIP inhibited upregulation of TREM-1 mRNA in a time and

dose dependent manner in the lung of ALI mice, whereas TREM-2 mRNA increased, effectively reversing the TREM-1:TREM-2 ratio seen in LPS treated animals (Sun et al., 2011). Therefore, controlling the TREM-1:TREM-2 ratio may explain how both can signal through DAP12 in normal immunity without canceling each other out.

However, although TREM-1 and TREM-2 expression patterns and availability may change based on environmental cues, this still does not explain how signaling through DAP12 can mediate different functional outcomes. Current studies examining the signaling mechanisms associated with TREM-mediated stimulation may help to explain this paradox.

TREM-1 vs. TREM-2: Differential Signaling

Recently, studies examining TREM-mediated signaling have focused on the adaptor proteins Linker for Activation of T cells (LAT) and Linker for Activation of B cells (LAB; NTAL), as both have been found to be recruited to the membrane after TREM ligation. Monocytes, which express high levels of LAT and low baseline levels of LAB, express TREM-1 and promote a proinflammatory environment after TREM-1 ligation, especially in concert with TLR stimulation. This may occur through the recruitment of DAP12, Syk, and LAT by TREM-1, the latter of which activates PLCγ, leading to increased calcium influx. Signaling through TREM-2 leads to recruitment of DAP12, Syk, and LAB, the latter of which can compete with LAT for similar signaling effectors. Importantly, LAB does not activate PLCγ directly and therefore may not be able to transduce the same kind of stimulatory signals as LAT (Orr and McVicar, 2011; Whittaker et al., 2010). This “competition model” may help to explain how signaling

through TREM-1, LAT and PLCγ can lead to strong stimulatory responses whereas signals driven through TREM-2 and LAB elicit no such response. This positions DAP12 as a mediator in both instances, but not the driving force for the functional outcomes.

However, this raises interesting questions as to how TREM-2 exerts a similar anti-inflammatory effect on differentiated macrophages. As monocytes leave the blood to enter tissues and differentiate into macrophages, TREM-1 expression decreases whereas TREM-2 expression is elevated. Concurrently, LAT expression significantly decreased during this differentiation process whereas an increase in LAB expression makes it the predominant adaptor protein. Because LAB is the prevalent adaptor protein recruited after Syk activation, and because LAT is still not utilized even in $LAB^{-/-}$ macrophages, the competition model doesn't seem to fit for this cell type. However, once Syk phosphorylates LAB, several proteins can be recruited, including the E3-ubiquitin ligase c-Cbl, which can actually ubiquitinate Syk and LAB, leading to their degradation and the negative regulation of such signaling (Dangelmaier et al., 2005). Importantly, it has now been shown that c-Cbl can ubiquitinate MyD88 after CD11b activation, and thus CD11b activation negatively regulates TLR signaling, as MyD88 is a major membrane-proximal TLR signaling protein (Han et al., 2010). Therefore, TREM-2 activation could lead to the recruitment and activation of Syk, LAB, and c-Cbl, the latter of which can ubiquitinate MyD88, promoting its degradation and ultimately downregulate proinflammatory signals being sent via TLRs. It has been shown that recruitment of LAB is dependent on DAP12 being present, and this may be the crucial role DAP12 plays in TREM-2 biology.

Given that macrophages and neutrophils express high levels of LAB but not LAT, the more pressing question may not be how TREM-2 operates, but rather how TREM-1 is

able to elicit a proinflammatory response to TLR exposure. Importantly, it has been reported that macrophages may express a constitutive TREM-2 ligand that could theoretically send constant signals through TREM-2 via a *cis* interaction. It is therefore possible that the cell may be continually receiving inhibitory signals through TREM-2, which may put the onus on TREM-1 to upregulate and recruit DAP12 away from TREM-2 in the context of an inflammatory event that may require macrophage activation. When recruited to TREM-1 or TREM-2, DAP12 may continuously send identical activating signals which are only overcome by LAB and c-Cbl that are specifically recruited to TREM-2. Indeed, TREM-1 is upregulated whereas TREM-2 expression is decreased during certain macrophage activation events, possibly freeing up DAP12 stimulatory signals in the absence of c-Cbl inhibitory events. This may explain how TREM-1 can activate macrophages without the use of LAT.

Another possibility could be that because TREM-1 ligation has been shown to lead to the increased production of TLR-signaling proteins such as MyD88 and CD14, that the upregulation of these proteins may lead to an enhancement of cellular activation (Ornatowska et al., 2007). It is not known exactly how this upregulation occurs, and this may be independent of LAT, LAB and DAP12 signaling, thus giving TREM-1 a potential role that TREM-2 does not possess. Indeed, this may be how TREM-1 enhances activation of neutrophils treated with TLR ligands, as neutrophils do not possess LAT or the DAP12-recruiting TREM-2.

Finally, it has also been demonstrated that DAP12 signaling can lead to the activation of PI3K, a kinase typically thought of as a proinflammatory player. Thus, TREM-1 may simply skew signals to run through DAP12, PI3K, and subsequent

downstream cellular activation. However, PI3K has also been shown to phosphorylate and activate the inhibitory Apoptosis Signal-Regulating kinase (ASK), giving PI3K a potential inhibitory role (Fukao and Koyasu, 2003). Perhaps TREM-2 provides cues to skew DAP12 and PI3K signaling towards this kinase and subsequent downstream inhibitory events.

TREM-1 vs. TREM-2: Sink Theory

Although significant evidence is beginning to mount for expression patterns and differential signaling being the predominant means of alternative outcomes for TREM-1 and TREM-2 signaling, it is still possible that the answer lies simply in the extracellular region of these receptors. It has been shown that TREM-2 can possibly bind a wide range of anionic ligands, such as LPS, LTA, peptidoglycan, and potentially other TLR agonists without transducing any signals. In this way, TREM-2 may act as a quiescent sink that recruits available ligand away from the TLRs, effectively inhibiting the macrophage TLR response to some degree, which is only overcome when TREM-1 signals lead to the increased production of TLR effectors. TREM-2 knockdown and knockout studies have revealed an increase in proinflammatory cytokine production, which could be explained by both a defect in the inhibitory intracellular signaling pathway initiated by TREM-2, or by removal of this quiescent sink (Hamerman et al., 2006; Melchior et al., 2010). Further studies are needed to determine exactly how TREM-1 and TREM-2 elicit their divergent responses.

TLT-1 OVERVIEW

TLT-1 is, to date, the only TREM family member that possesses signaling motifs, as there is a proline rich region and an inhibitory ITIM sequence within its cytoplasmic tail which interacts with the phosphatase SHP-1. There is no evidence that TLT-1 interacts with DAP12 as other TREM family members have been shown to do (Washington et al., 2002). TLT-1 has predominately been studied on platelets where it is constitutively expressed and upregulated upon platelet activation. Therefore, although once assumed to play the inhibitory role that TREM-2 has ultimately been found to exert, it has now been shown that TLT-1 plays a predominant role in platelet aggregation and vascular homeostasis. Use of mAbs against TLT-1 was shown to block thrombin-induced platelet aggregation which could be overcome by increasing the thrombin concentration (Washington et al., 2004).

More recently, a soluble form of TLT-1 (sTLT-1) has been identified and has come under scrutiny as it may play a similar diagnostic role as sTREM-1. Septic patients were shown to have high serum levels of sTLT-1 when compared to healthy controls, and this correlated with disseminated intravascular coagulation (Gattis et al., 2006). Further *in vitro* studies revealed that addition of sTLT-1 to platelets augmented aggregation, and conversely platelets from TLT-$1^{-/-}$ mice failed to coagulate in a timely manner, leading to excessive bleeding and higher risk of fatality (Giomarelli et al., 2007). Interestingly, TLT-$1^{-/-}$ mice had higher levels of plasma TNFα after LPS treatment, and were more likely to suffer hemorrhage after vascular injury (Washington et al., 2009). These data indicate that TLT-1 not only plays a role in platelet aggregation, possibly through stimulation of actin polymerization, but also in dampening the inflammatory response.

TLT-2 OVERVIEW

TLT-2 is distinctly unique from other known TREM family members in at least two different ways. First, TLT-2 is expressed on neutrophils, macrophages, human monocytes and B cells, making it the only TREM family member to be expressed on cells of the lymphoid lineage. TLT-2 is expressed on B cells as early as B220, and is most highly expressed on B1b cells. In fact, there is a gradation of TLT-2 expression on B lymphocyte subpopulations, with TLT-2 expression higher on B1 and marginal zone B cells followed by transitional and finally follicular B cells. The expression of TLT-2 appears to be based only on subtype, as inflammatory stimuli such as LPS did not alter TLT-2 expression levels (King et al., 2006).

Secondly, unlike other TREM proteins, TLT-2 does not have a charged residue in its transmembrane region to allow for interaction with DAP12 (like TREM-1 and TREM-2), nor does it have an ITAM or ITIM within its cytoplasmic tail (like TLT-1). TLT-2 has not been shown to interact with any adaptor protein and therefore its signaling mechanism is still completely unresolved. Additionally, the cytoplasmic tail is not highly conserved between human and mouse, raising further questions as to how this transmembrane receptor signals to the cell. Analysis of the cytoplasmic region revealed a conserved pseudo-proline rich region, which may allow for its interaction with SH3- or WW-domain containing proteins. In addition, it is possible that these prolines confer a tertiary structure that allows for TLT-2 to interact with an effector protein through hydrophobic interactions, although this is only speculative at this point.

Neutrophils uniformly express TLT-2, and like macrophages, upregulate TLT-2 along with CD11b in response to inflammatory stimuli. Intravenous or intraperitoneal

injection of LPS lead to an increase in both activation markers, as did injection of SEB, presumably mediated through the production of proinflammatory cytokines. Additionally, excessive stimulation with either anti-TLT-2 mAb or an inflammatory agent such as LPS lead to internalization of the TLT-2 receptor, as evidenced by the late intracellular presence of fluorescently labeled TLT-2. Although other TREM family members have been shown to be shed from the surface and are present in a soluble form, this has yet to be shown for TLT-2.

Recently, the controversial claim that B7-H3 is an endogenous ligand for TLT-2 expressed on $CD8^+$ T cells has raised questions regarding the role of TLT-2 in the immune system (Hashiguchi et al., 2008; Kobori et al., 2010). Importantly, there is little evidence to support the conclusion that TLT-2 is expressed on $CD4^+$ and $CD8^+$ T cells, regardless of their activation state (Leitner et al., 2009). Secondly, our lab has shown that TLT-2 appears to synergistically enhance the response to G protein-coupled receptor (GPCR) signaling, and perhaps more importantly, does not appear to alter phosphotyrosine-based signaling processes. This evidence does not support a role for TLT-2 in mediating co-stimulation of T cells after TCR-dependent signals are delivered. However, this controversy will most likely remain until a more suitable TLT-2 ligand candidate is identified.

Unlike the other conserved TREM family members, the functional role of TLT-2 has not been well characterized. Determination of the functional consequence of TLT-2 ligation will not only determine the role of this receptor in innate/adaptive immunity, but will also identify the specific signaling effectors as well as potential ligands that trigger signaling, and has been the focus of our laboratory. Although the role of TLT-2 on B

cells is of great interest, particularly because it is the only TREM locus receptor expressed on cells of the lymphoid lineage, no conclusive evidence regarding its function has been elucidated to date. However, we have now convincingly demonstrated functional roles for TLT-2 on both neutrophils and macrophages, and how their interplay may be crucial to host defense.

TLT-2 potentiates multiple neutrophil functions, including the respiratory burst, degranulation, and chemotaxis, and does so only in a synergistic fashion with appropriate secondary stimuli. Using a chemiluminescence system, it was shown that TLT-2 ligation increases the primary phase of the respiratory burst by 25-45% in response to the bacterial peptide FMLP. Transwell assays have been used to determine the effect that anti-TLT-2 mAb has on murine neutrophil chemotaxis, and *in vitro* data now demonstrate that TLT-2 ligation increases migration 1.5-2.5 times in response to different chemotactic stimuli, such as FMLP, C5a, IL-8, KC and MIP-2. Importantly, anti-TLT-2 mAb treatment potentiates chemotaxis in response to agonists that signal via GPCRs, but not in response to agonists that signal via tyrosine kinase-dependent pathways (e.g. GM-CSF). This potentiation is conserved both *in vitro* (e.g. transwell assays) and *in vivo*, as evidenced by both intravenous injection of anti-TLT-2 mAb in conjunction with a non-specific inflammatory event as well as adoptively transferred, anti-TLT-2 mAb-treated neutrophils into an animal with experimentally induced lung inflammation. TLT-2 ligation does not enhance FcR mediated phagocytosis, again indicating the importance of a secondary stimulus that signals through Gαi-coupled GPCRs. Additionally, TLT-2 works via a mechanism different from classical neutrophil primers such as LPS, as supported by the observation that there is no kinetic shift or decrease in the threshold of

sensitivity after anti-TLT-2 mAb treatment, both of which typically result from exposure to prototypical primers such as LPS. These data suggest that TLT-2 ligation effectively potentiates multiple neutrophil functional responses, and perhaps does so through a novel mechanism (Halpert et al., 2011).

As monocytes differentiate into macrophages TLT-2 expression is induced, and can subsequently be upregulated in an inflammatory environment as demonstrated by treatment with thioglycollate. Additionally, we have shown that TLT-2 ligation on macrophages can induce the secretion of several chemokines and growth factors *ex vivo*, the most notable being MIP-2, KC, and G-CSF. Importantly, this response occurs in the absence of a secondary stimulus, indicating that TLT-2 ligation is sufficient to drive the production of soluble factors by macrophages. Of note is that TLT-2 ligation does not activate the macrophage in a generalized manner, as evidenced by a lack of upregulated CD69, CD80, and CD86 expression after treatment, as well as the fact that phagocytosis of Zymosan is not affected by TLT-2 ligation. A similar upregulation of these chemokines and growth factors is seen within hours after intravenous, intraperitoneal, or intratracheal injection of anti-TLT-2 mAb, and the ensuing infiltration of neutrophils is rapid and directed to the site of injection. Given that the predominant factors produced by anti-TLT-2-treated macrophages signal through GPCRs, it is not a surprise to observe that neutrophils in the anti-TLT-2-treated mouse expressed higher levels of CD11b than neutrophils recruited in FMLP- or LPS-treated mice. Therefore, it appears that TLT-2 may play an important role in both the initiation and enhancement of neutrophil recruitment and activity during acute inflammation. Additionally, this is the first evidence

to link the TREM and GPCR receptor families, expanding the reach the TREM receptors have on modulating the innate immune response.

TLT2 POTENTIATES NEUTROPHIL ANTIBACTERIAL ACTIVITY AND CHEMOTAXIS IN RESPONSE TO G PROTEIN-COUPLED RECEPTOR-MEDIATED SIGNALING

by

MATTHEW M. HALPERT, KIMBERLY A. THOMAS, R. GLENN KING, AND LOUIS B. JUSTEMENT

Journal of Immunology, **187**: 2346-2355

Abstract

Receptors encoded within the *Trem* locus have been shown to play an important role in modulating the cellular response to PRR signaling. TLT2 is a member of the *Trem* locus that is conserved in mouse and human. TLT2 exhibits a unique expression pattern in that it is expressed on cells of the myeloid and lymphoid lineage, suggesting that it plays a role in both innate and adaptive immunity. Here, studies reveal that TLT2 plays an important role in potentiating neutrophil antibacterial activity and chemotaxis. TLT2 ligation enhances the neutrophil response to the formylated peptide FMLP leading to increased ROS production, degranulation and chemotaxis. Moreover, TLT2 has the ability to specifically potentiate neutrophil activation and chemotaxis in response to a range of agonists that bind to G protein-coupled receptors, as it does not potentiate the response of cells to growth factor receptor-, Fc receptor- or TLR-mediated signaling. Finally, TLT2 ligation potentiates the recruitment of neutrophils to sites of inflammation in vivo. These findings reveal a novel functional role for TLT2 that involves potentiation of neutrophil responses to G protein-coupled receptor signaling. Thus TLT2 appears to play an important role in enhancing the innate immune response via a novel molecular mechanism.

Introduction

Cells of the innate immune system rely on a range of receptors to detect and respond to pathogens. The direct recognition of pathogens or products of cellular stress by pattern recognition receptors (PRRs) such as the toll-like receptors and the nod-like receptors are well characterized (1, 2). Additionally, other families of receptors serve to modulate the response of immune cells to signals derived from PRRs including those encoded within the triggering receptor expressed on myeloid cells (*Trem*) locus (3). Receptors encoded within the *Trem* locus are expressed by a wide range of innate immune cells including neutrophils, monocytes, macrophages, microglia, osteoclasts and dendritic cells, as well as megakaryocytes and platelets (4). TREM-like Transcript 2 (TLT2) is a type 1 transmembrane receptor, with a single extracellular immunoglobulin domain followed by a serine/threonine-rich membrane-proximal segment, and a short cytoplasmic tail following a single transmembrane domain. Unlike other TREM family members, murine TLT2 does not possess an obvious signaling motif within its 39 amino acid cytoplasmic tail, nor does it possess charged amino acids in the transmembrane domain, suggesting that it does not interact with prominent adaptor proteins such as DAP12, FcRγ, or TIRAP. In mice, TLT2 has been shown to be expressed on B cells, macrophages, and neutrophils and thus is the only member of the TREM locus to be expressed by cells of both the innate and adaptive immune systems (5).

TREM-1, the most well characterized TREM protein, is the only TREM family member other than TLT2 that is expressed on neutrophils. TREM-1 ligation mediates increased production of inflammatory cytokines, chemokines, lactoferrin, reactive oxygen species (ROS) and myeloperoxidase (MPO) (6). Functional studies have found

that TREM-1 cross-linking induces only a modest increase in specific antibacterial activities, whereas it predominately acts in a synergistic fashion to amplify the inflammatory response in conjunction with toll-like receptor and nod-like receptor signaling (7, 8). Ligation of TREM-1 in concert with LPS stimulation potentiates cytokine secretion more than either stimulus alone. Thus, TREM-1 modulates immune cell function by effectively amplifying the magnitude of the response to signals delivered via PRRs. TREM-2 is expressed on a variety of cell types, including monocytes, macrophages, osteoclasts, bone marrow-derived dendritic cells, immature dendritic cells, and microglia (9, 10). TREM-2 has recently emerged as an important negative regulator of autoimmunity and appears to exert an anti-inflammatory activity on microglia and macrophages. Thus, TREM family members appear to predominantly function as modulatory proteins that control innate immune cell function in response to other stimuli.

TLT2, like TREM-1, is highly expressed on neutrophils and its expression is upregulated in response to inflammatory conditions in vivo (5). However, the functional role of this receptor on cells of the innate immune system has not been described. Because the TREM-1 and TREM-2 receptors have been shown to modulate the functional response of cells to signals derived from pattern recognition receptors, it was of interest to determine if engagement of the TLT2 receptor would also modulate neutrophil function in response to signals derived from receptors involved in the induction of, or response to, inflammatory conditions. In this study, agonistic mAbs against TLT2 were used to demonstrate that ligation of TLT2 on neutrophils potentiates the respiratory burst and degranulation in response to FMLP and C5a, as well as the chemotactic response to several chemokines. The chemokines MIP-2 and KC, the

complement component C5a, and the bacterial product FMLP all signal via G protein-coupled receptors (GPCR). These receptors contain seven membrane-spanning helices that undergo conformational changes upon ligand binding, which in turn transduces a signal via activation of heterotrimeric G-proteins. GPCRs make up a large family of proteins with broad functions in biological systems. In the context of innate immunity, signals delivered via GPCRs influence many aspects of neutrophil function including promoting the production of reactive oxygen species and the release of antimicrobial mediators, as well as chemotaxis (11). The finding that TLT2 regulates neutrophil function by potentiating the response to agonists that signal via GPCR-dependent pathways represents a novel mechanism whereby antibacterial responses and recruitment of phagocytic cells to sites of infection and inflammation are enhanced.

Materials and Methods

Isolation of mouse neutrophils

C57BL/6 mice 8–10 wk of age were used for isolation of bone marrow cells from the tibias and femurs. All mice were housed in specific pathogen-free conditions in University of Alabama at Birmingham animal facilities, and all procedures were approved by an institutional review committee. The bone marrow was passed through 25 and 20 gauge needles (Becton Dickinson, Franklin Lakes, NJ) to generate a single cell suspension in 1X HBSS (136 mM NaCl, 5.55 mM Glucose, 5.36 mM KCl, 4.16 mM $NaHCO_3$, 1.66 mM KH_2PO_4, 0.338 mM Na_2HPO_4, pH 7.2). Red blood cells were lysed by incubation in AKC (0.15 M NH_4Cl, 12 mM $NaHCO_3$, .1 mM EDTA, pH 7.2) for 5 min on ice, leukocytes were then separated by density sedimentation using a Percoll (Amersham, Piscataway, NJ) gradient (60%/80%) in 1X HBSS centrifuged at room temperature at 1500 RPM for 25 min. The cells at the 60/80 interface, which was comprised of >95% neutrophils as assayed by flow cytometry were collected, counted with a hemocytometer, and resuspended in media at appropriate concentrations. The media used in all experiments was RPMI-1640, supplemented with pen/strep, sodium pyruvate, mercaptoethanol, L-glutamine, and 5% FBS. Anti-CD11b-FITC (BD Pharmingen, San Diego, CA), anti-Gr-1-PE and anti-Gr-1-APC mAbs (Southern Biotech, Birmingham, AL) were used to determine the purity of isolated neutrophils.

Respiratory burst assay

The αTLT2 mAbs 1H4 and 1C5 were generated as previously described (5) and were prepared under LPS-free conditions. The antibody preparations used in these studies were subjected to the limulus amebocyte lysate test, and were demonstrated to contain no detectable endotoxin (limit of detection is 0.03 EU/mL). Where indicated, antibodies were biotinylated using EZ-Link NHS-LC-biotin (Pierce, Rockford, IL). Following isolation, $1x10^6$ purified neutrophils in 100 µl of RPMI-1640 were incubated with αTLT2 mAb, isotype control mAb, LPS, or were left untreated for the specified times. Extracellular ROS scavengers (2000 units/ml catalase, Worthington, Lakewood, NJ) and 50 units/ml superoxide dismutase (Sigma; St. Louis, MO) and 10% luminol (final concentration 5 µM) were added to the samples. After a 5 min equilibration period at 37°C, FMLP or GM-SCF (Calbiochem, Darmstadt, Germany) was added and the respiratory burst response was monitored using an Envision Multi-label Plate reader (Perkin Elmer, Waltham, MA) in the ultrasensitive luminescence mode.

Neutrophil degranulation assay

A total of $1x10^6$ neutrophils per sample in 1 ml of RPMI-1640 were incubated with the appropriate αTLT2 mAb, isotype control mAb or medium alone as indicated for 10 min at 37°C. Following this incubation the FMLP agonist, WKYMVm (Calbiochem) C5a, GM-CSF or the TLR agonists LPS (Sigma), monophosphoryl lipid A (MPLA) (Dr. John Kearney, Microbiology, UAB), Poly(I:C) (Invitrogen), flagellin (Dr. Charles Elson, Medicine, UAB), imiquimod (Invitrogen) and CpG-ODN (Invitrogen) were added at the indicated concentrations for the indicated times. After stimulation, all reactions were

terminated by addition of ice cold 1X PBS. To examine the effect of secondary cross-linking of TLT2, 1 µg of biotinylated αTLT2 mAb was preincubated with varying concentrations (2-40 µg) of streptavidin (Pierce), which was then added to the neutrophil preparations for 10 min prior to the addition of triggering stimuli. After the indicated period of time, the neutrophils were then stained for the surface markers CD11b and Gr-1. Degranulation was measured by a specific increase in the cell surface expression of CD11b.

Phagocytosis assay

A total of $5x10^6$ 2.0 micron biotinylated polystyrene beads (Polysciences, Inc, Warrington, PA) were incubated with a 1:100 dilution of streptavidin-FITC (Biosource, Carlsbad, CA) in 100 µl PBS for 15 min at room temperature in the dark. The beads were then washed 3 times with 1X PBS. Subsequently, 50% of the FITC-labeled beads were incubated at RT with mouse serum containing anti-FITC antibodies for 30 min in the dark. The FITC-specific antiserum was generated by immunizing C57BL/6 mice with FITC conjugated protein antigen, after which serum was harvested on day 10. After opsonization with the antiserum, the beads were washed 3 times with 1X PBS and warmed to 37°C prior to addition to neutrophils. Neutrophils ($1x10^5$ cells/sample) in 500 µl RPMI-1640 were preincubated with the indicated stimuli for 10 min. $1x10^6$ fluorescent beads were then introduced to the cell suspensions followed by a 15 min incubation. Trypan blue was added to quench extracellular FITC fluorescence and phagocytosis of the beads was assayed by flow cytometry.

Chemotaxis assay

Neutrophil chemotaxis was assayed using 3.0 micron, 6.5mm transwell inserts (Costar, Corning, NY) placed in 24-well cell culture plates. $1x10^6$ neutrophils were placed in 100 µl of RPMI-1640 in the upper chamber and the chemotactic factors, FMLP, MIP-2, KC, IL-8, C5a, or GM-CSF were added at the indicated concentrations to 600 µl in the bottom chamber in 37°C RPMI-1640. The murine recombinant proteins KC, MIP-2, GM-CSF and human recombinant IL-8 were obtained from PeproTech (Rocky Hill, NJ), and murine C5a was obtained from eBioscience (San Diego, CA). αTLT2 or isotype control mAb was added to the upper chamber with the neutrophils at specified concentrations, and the samples were incubated for 60 min at 37°C in a humidity controlled incubator under 5% CO_2. Migrating neutrophils were removed from the bottom chamber and counted. The normalized chemotactic index was calculated by dividing the number of cells migrating in samples containing αTLT2 or isotype control mAbs by the number migrating in response to the chemotactic agent alone.

Ear model of inflammation

To induce inflammation in the ear, 20 µl of 2% croton oil (Sigma, St. Louis, MO) in acetone was applied to both sides of the pinna of one ear, whereas acetone alone was applied to the control ear. Either 100 µg of αTLT2 mAb or 1X PBS alone was injected intravenously (IV) into mice 30 min prior to the application of croton oil. Four hours following the application of croton oil, 4 mm biopsies were obtained from the proximal portion of the ear, mechanically homogenized with a Tissue Tearor homogenizer (Biospec Products, Bartlesville, OK) , and assayed for MPO activity. MPO levels were

determined by the addition of the chromogenic substrate 3,3´,5,5´-tetramethylbenzidine (TMB) (Sigma) to 100 µl of the ear lysate. The absorbance at 450 nm was then measured using a Vmax plate reader (Molecular Devices, Sunnyvale, CA). These samples were assayed with a standard curve of known concentrations of the enzyme HRP. Comparing the MPO activity present in specific numbers of purified neutrophils to this standard curve allowed for the estimation of the number of neutrophils present in the biopsy samples. To examine the effect of administration of aTLT2 mAb on the recruitment of cells and architecture of the ear, mice were sacrificed and the ears were removed, fixed overnight in 10% NBF, paraffin embedded, and 8 micron thick sections prepared. The sections were treated to remove the paraffin and rehydrated, after which they were stained with H&E (Harris Hematoxylin and Eosin, Sigma). The stained sections were subsequently dehydrated, cleared, and mounted with Permount (Fisher #SP15). The slides were examined with a Zeiss AX10 microscope using a 20x objective and pictures were taken using a Zeiss AxioCam MRC.

Lung model of inflammation and adoptive transfer of neutrophils

To induce inflammation in the lung 5 µg of LPS was introduced intratracheally. Bone marrow neutrophils were isolated from CD45.1 donor mice as previously described. Once isolated, the neutrophils were divided equally into 3 groups of $3x10^7$ neutrophils and subsequently incubated for 10 min in 500 µl of 1X PBS containing either 5 µM CSFE, 5 µM Cell Tracker CMPTX Red or without fluorescent additives. CSFE and CMTPX cell permeable tracking dyes were obtained from Invitrogen (Carlsbad, CA). Each group of neutrophils was then subjected to the indicated treatment for 15 min at

37°C, followed by 3 washes in prewarmed 1X PBS and resuspended in 100 µl. After loading, the cells were mixed in a 1:1:1 ratio and approximately $3x10^7$ total neutrophils in 100 µl were injected IV into CD45.2 mice 2 h after the initiation of lung inflammation. After an additional 4 h, mice were sacrificed and cells were isolated from lungs by bronchoalveolar lavage. The isolated cell suspensions were stained with anti-CD45.1-APC (eBioscience). Flow cytometry was preformed to indentify CD45.1 expressing cells and neutrophils subjected to the indicated treatment conditions were then resolved based on of CSFE or CMTPX fluorescence, or the absence of fluorescent labeling.

Flow cytometry

Cells were washed 3 times with FACS buffer (1X PBS, 0.01% NaN_3^+, 0.5% FBS) and then incubated with the appropriate fluorescent Ab mixture in 96-well microtiter plates for 15 min on ice. After this incubation, the cells were again washed 3 times with FACS buffer. When necessary, samples were incubated with secondary antibodies for an additional 15 min on ice. Samples were analyzed immediately following labeling on either a FACScan, FACSCalibur or LSR II flow cytometer (BD Biosciences), and the resultant data were analyzed using FlowJo (Tree Star, Ashland OR.

Statistical analysis

Statistical analyses were computed via the student two-tailed t-test. The CD45.1 adoptive transfer experiment was analyzed with the one-way anova. The p-value of significant differences is reported, with a p-value < 0.05 considered statistically significant.

Results

TLT2 ligation potentiates the functional response of neutrophils to FMLP.

Previous studies have demonstrated that TLT2 is constitutively expressed by both macrophages and neutrophils (5). Under experimental inflammatory conditions, including administration of thioglycolate, LPS, or staphylococcal enterotoxin B in vivo, responding macrophages and neutrophils exhibit significant upregulation of TLT2 on their surface (5). This rapid upregulation of TLT2 by neutrophils suggests that TLT2 may play a role in regulation of neutrophil function at sites of inflammation. A principle function of neutrophils is to respond to bacterial pathogens; therefore experiments were performed to determine if ligation of TLT2 with mAb enhances the antimicrobial activity of neutrophils by inducing ROS generation. To determine the effect that engagement of TLT2 has on ROS production, neutrophils were isolated from murine bone marrow and were analyzed using a chemiluminescence assay to measure the generation of ROS. As seen in Figure 1A, and Supplemental Figure 1, ligation of TLT2 with either of the mAbs 1H4 or 1C5 alone does not induce a respiratory burst response.

Neutrophils generate ROS in response to a variety of extracellular stimuli including inflammatory cytokines and growth factors, as well as bacterial products such as LPS and formylated peptides derived from prokaryotic pathogens. FMLP is a short, formylated bacterial peptide that is released during degradation of the bacterial membrane and is a strong chemoattractant for phagocytic cells such as neutrophils (11). The response of innate immune cells to formylated peptides is mediated by binding to the FMLP receptor, a GPCR, resulting in activation of phospholipase C, protein kinase C, and calcium mobilization (12). Experiments were performed to determine if

preincubation of neutrophils with αTLT2 mAb exerts a priming effect resulting in a change in the kinetics or magnitude of the respiratory burst elicited in response to FMLP. Preincubation with αTLT2 mAb prior to stimulation with FMLP resulted in a substantial increase in ROS production compared to neutrophils incubated with FMLP alone (Fig1, S1). In contrast, preincubation of neutrophils with isotype control mAb had no effect on ROS production (Fig. S1) or on the subsequent response following addition of FMLP, regardless of the concentration of isotype control antibody used (Fig. 1). The potentiation of ROS production occurs over a broad range of αTLT2 mAb concentrations (Fig. 1A). When the potentiation of FMLP induced ROS generation induced by αTLT2 mAb pretreatment is compared to that induced by pretreatment with LPS, a prototypic priming agent, the effects, although similar in magnitude, differ in their kinetics (i.e. initiation and duration). The priming effect of LPS is very rapid, as seen in Figure 1B (upper panel). After a 15 min preincubation with LPS, the kinetics of the response to FMLP are shifted, and the overall magnitude of the response is substantially increased, compared to treatment of cells with FMLP alone. In contrast, preincubation with αTLT2 mAb for 15 min fails to significantly alter ROS production induced by FMLP. However, after a 45 min preincubation with αTLT2 mAb, ROS production in response to FMLP is similar to that observed with LPS pretreatment in terms of magnitude. Whereas cells pretreated with LPS exhibited an accelerated, transient potentiation of ROS production, TLT2 ligation did not accelerate the kinetics of the response, but was observed to potentiate ROS production for a prolonged period of time. Finally, pretreatment of neutrophils for 2 h with αTLT2 mAb resulted in a slight shift in the kinetics of the response similar to that observed with LPS treatment, as well as a prolongation of the potentiated response (Fig.

1B). Potentiation of ROS production can be further enhanced by secondary cross-linking as demonstrated by treatment of neutrophils with biotinylated 1C5 in the presence of streptavidin, which results in a further enhancement of ROS production in response to FMLP at all time points assayed (Fig. 1B). This observation suggests that cross-linking of TLT2 may be important for eliciting an enhanced response to FMLP. In summary, whereas ligation of TLT2 alone does not induce the generation of ROS by neutrophils, cross-linking of this receptor serves to potentiate ROS production in response to FMLP. Although ROS generation was similar for neutrophils primed either with αTLT2 mAb or LPS in terms of magnitude, significant differences were observed in the kinetics of ROS production. TLT2 ligation primarily acts to enhance the magnitude of ROS production over an extended period of time, but does not alter the kinetics of the response. In contrast, LPS accelerates the kinetics and increases the magnitude of the response in a transient manner. These data suggest that the mechanism by which TLT2 ligation potentiates ROS production is different from that associated with the priming effect of LPS.

In addition to FMLP, other stimuli, including growth factors induce a respiratory burst response in neutrophils. Therefore, experiments were performed to determine if the significant enhancement in ROS generation induced by TLT2 ligation in response to FMLP would be observed in response to other agonists. The GM-CSF receptor (also known as CD116) is expressed on several cell types, including mature neutrophils. GM-CSF, like FMLP, has been shown to elicit ROS production by neutrophils; however the GM-CSF receptor mediates a phosphotyrosine-based signal leading to ROS production

(13). As seen in Figure 1C, unlike the response to FMLP, ROS generation by neutrophils in response to GM-CSF was not potentiated by ligation of TLT2.

In addition to promoting the generation of ROS, FMLP is a potent chemotactic agent for neutrophils (11). Because ligation of TLT2 was observed to potentiate ROS production in response to FMLP, experiments were performed to determine if the chemotactic response of neutrophils to FMLP is also potentiated. Medium containing varied concentrations of FMLP receptor agonist were placed in the bottom chamber of a 3μm transwell device and neutrophils were placed in the upper chamber. Anti-TLT2 or isotype control mAb, or medium alone was added to the neutrophils just prior their addition to the upper chamber and after a 1 h incubation at 37° C, cells that migrated to the lower chamber were collected and counted. As shown in Figure 2A, inclusion of αTLT2 mAb in these assays resulted in a substantial increase in the number of cells migrating through the transwell in response to FMLP. Of note, the concentration of αTLT2 mAb that maximally potentiates the chemotactic response of neutrophils is the same as that which provides maximal potentiation of ROS production. To rule out the possibility that Fc receptor-mediated signaling generated by the binding of intact antibodies to neutrophils was responsible for these observed effects, $F(ab')_2$ fragments of the 1H4 mAb were generated and used in these assays (Fig. 2B). The observed potentiation of cell migration in response to FMLP was identical whether cells were treated with $F(ab')_2$ fragments or intact 1H4, suggesting that signals delivered via TLT2 were responsible for the observed effects on ROS production and cell migration.

Additional experiments were performed to determine if secondary cross-linking would further enhance this effect using biotinylated 1C5, either alone or in the presence

of streptavidin. As seen in Figure 2C, the inclusion of streptavidin alone has no effect on cell migration in response to FMLP, whereas the inclusion of biotinylated 1C5 mAb alone results in a nearly two-fold increase in the number of migrating cells. As seen in ROS assays, the inclusion of streptavidin and biotinylated 1C5 mAb results in a further enhancement, suggesting again that secondary cross-linking of TLT2 is responsible for this effect.

A possible mechanism by which TLT2 ligation potentiates the response to FMLP is by decreasing the neutrophil threshold of sensitivity for agonist binding to the FMLP receptor. If this were true, then one would expect to see a shift in the dose response to FMLP in neutrophils on which TLT2 has been ligated. To determine if this is the case, neutrophil migration was assayed over a wide range of FMLP concentrations in the presence or absence of αTLT2 mAb. As seen in Figure 2D, the inclusion of an optimal concentration of αTLT2 mAb potentiates neutrophil migration over a wide range of FMLP concentrations. However, TLT2 ligation does not result in a shift in the dose response to FMLP. Indeed, the observed potentiation in the number of migrating neutrophils treated with αTLT2 mAb is proportional regardless of the FMLP concentration as the ligation of TLT2 resulted in a chemotactic index (~1.7) that is nearly identical for all concentrations of FMLP tested (Fig. 2E). In contrast, inclusion of LPS shifts the chemotactic response curve to FMLP, suggesting that it lowers the threshold of sensitivity to this chemoattractant (Fig. 2D). Collectively, these results suggest a potential role for TLT2 in amplifying the cellular response to FMLP, as opposed to altering the threshold of sensitivity of the FMLP receptor. This suggests that TLT2 ligation could amplify the magnitude of the signal delivered via the FMLP receptor and/or prolong the

duration of that signal thereby enhancing the cellular functional response. Alternatively, TLT2 ligation could in theory activate a parallel signaling pathway that amplifies the functional response of the neutrophil. Although, if this were true, one would expect that ligation of TLT2 alone would drive ROS production and chemotaxis in the absence of FMLP triggering, which was not observed. These data further support the conclusion that the mechanism by which TLT2 modifies the response to GPCR-mediated signaling is distinct from that of LPS.

Ligation of TLT2 specifically potentiates the neutrophil response to GPCR-mediated signaling.

Besides triggering both ROS production and neutrophil migration, FMLP and GM-CSF induce degranulation of murine neutrophils. The release of specific granules in response to these stimuli has been shown to mediate the rapid expression of the integrin CD11b resulting in an increase in cell adhesion and is thought to be a mechanism to promote transmigration of neutrophils into inflamed tissues (14). Because the rapid upregulation of CD11b is associated with cellular activation, experiments were performed to determine if TLT2 ligation alone is sufficient to induce the upregulation of CD11b, or if ligation of the TLT2 receptor potentiates upregulation of CD11b induced by FMLP or the complement component C5a, which bind to GPCRs, versus GM-CSF or TLR agonists, which activate distinct signaling pathways that do not utilize heterotrimeric G-proteins.

As seen in Figure 3A, ligation of TLT2 alone has no effect on CD11b expression on purified neutrophils, regardless of the mAb concentration used, compared to the

FMLP receptor agonist WKYMVm, which induced CD11b expression in a dose dependent manner (Fig. 3B). However, when neutrophils were preincubated with 100ng/mL of 1H4 mAb and were subsequently stimulated with 1 nM WKYMVm, a concentration that does not significantly alter CD11b expression, a synergistic upregulation of CD11b was observed (Fig. 3C). As was seen in the ROS assay, the potentiation of CD11b expression in response to FMLP following TLT2 ligation was further enhanced when neutrophils were preincubated in the presence of biotinylated 1C5 mAb plus streptavidin as a secondary cross-linking agent (Fig. 3D-F). Exposure to C5a also results in neutrophil degranulation, and like FMLP, activation of cells by C5a is the result of signal transduction mediated by the C5a receptor, a GPCR. Therefore experiments were performed to determine if TLT2 ligation would potentiate the cellular response to C5a. As seen in Figure 3G, preincubation of neutrophils with anti-TLT2 mAb potentiates degranulation in response to C5a resulting in the upregulation of CD11b. In contrast, ligation of TLT2 does not affect CD11b upregulation when used in conjunction with GM-CSF (Fig. 3H, I). Pretreatment of neutrophils with isotype control mAb alone or in conjunction with any of the agonists tested did not alter CD11b expression (data not shown).

To determine if ligation of TLT2 potentiates the ability of TLR agonists to induce degranulation of neutrophils, experiments were performed in which neutrophils were preincubated in the presence of αTLT2 or isotype control mAb followed by addition of TLR agonists, including LPS, poly(I:C), MPLA, flagellin, imiquinod and CpG-ODN, which bind to TLR2, 3, 4, 5, 7/8 and 9, respectively. Neutrophil degranulation in response to these TLR agonists was not affected by preincubation of neutrophils with

αTLT2 or isotype control mAb (data not shown). A second assay was performed to monitor the effect that TLT2 ligation has on the ability of TLR agonists to prime neutrophil degranulation in response to low concentrations of GM-CSF. TLT2 ligation had no effect on the priming activity of any of the TLR agonists examined (data not shown).

The fact that TLT2 ligation specifically potentiates the response of neutrophils to FMLP, a bacterial component, and is upregulated on neutrophils at sites of inflammation suggests that it may play an important role in the innate immune response to bacterial infection. Phagocytosis of bacteria by neutrophils is mediated by multiple receptors including integrins, lectins, and scavenger receptors (15). However, the phagocytosis of bacteria by neutrophils is greatly enhanced in the presence of opsonizing antibodies, which promote engulfment of particles via Fc receptors (FcR). Thus experiments were performed to determine if the FcR-dependent phagocytic activity of neutrophils is enhanced by ligation of TLT2. To experimentally determine the effect of TLT2 ligation on the engulfment of opsonized particles, two-micron polystyrene beads were conjugated to FITC to facilitate visualization, and then incubated with purified neutrophils either alone or following opsonization with polyclonal αFITC antibody. After 15 min, trypan blue was added to quench extracellular FITC fluorescence and phagocytosis was assayed by flow cytometry. As seen in Supplemental Figure 2, ligation of TLT2 had no measurable effect on the percentage of neutrophils that phagocytosed beads via a FcR-dependent mechanism, as αTLT2 mAb pretreated samples were identical to non-pretreated controls, regardless of whether intact mAb, $F(ab')_2$ fragments, or secondary cross-linking was used.

TLT2 ligation potentiates the functional response of neutrophils to FMLP and C5a, including cell migration, ROS production, and upregulation of CD11b, but exhibits no effect on neutrophil functional responses to TLR agonists, GM-CSF or FcR-mediated activation. Given the important role that GPCRs play in regulating neutrophil migration in vivo, it was of interest to determine if TLT2 ligation potentiates the migration of these cells in response to other chemokines. Chemotaxis of purified neutrophils was assayed in response to a variety of chemoattractants including the murine chemokines, KC and MIP-2, their human homologue IL-8, as well as the activated complement component C5a. In this assay, TLT2 ligation in the absence of chemoattractant has no measurable effect. However, in the presence of chemokine, αTLT2 mAb resulted in a 1.7- to 2.0-fold increase in the number of cells that migrate in response to these chemoattractants (Fig. 4A, B). Importantly this effect is not observed in response to GM-CSF, which is the only member of this panel of chemoattractants that does not signal via a GPCR. Treatment with isotype control mAb had no effect on neutrophil chemotaxis nor did it potentiate migration in response to any of the chemoattractants tested (Fig. 2 and data not shown). Interestingly, although the percentages of cells migrating in these assays varied depending on the chemoattractant utilized, the magnitude of the effect associated with TLT2 ligation was constant. This demonstrates that TLT2 ligation potentiates the response of neutrophils to multiple GPCRs, as the effect of TLT2 ligation is similar whether neutrophils are responding to signals delivered via the FMLP receptor, CXCR2, or the C5a receptor.

TLT2 ligation promotes neutrophil recruitment to sites of inflammation in vivo.

Because ligation of TLT2 potentiates the chemotactic response of neutrophils to agonists that bind GPCRs, and given the importance of these receptors in controlling the migration and recruitment of neutrophils, it was of interest to determine if TLT2 ligation potentiates neutrophil recruitment to sites of experimentally induced inflammation in vivo. Croton oil (2% in acetone) was used to induce a nonspecific inflammatory response in the ear. Thirty minutes following i.v. administration of either 1H4 mAb in the experimental group or saline in control mice, croton oil was applied to the pinna of one ear while acetone alone was applied to the other. After 4 h the mice were sacrificed and a 4mm biopsy was removed from both ears. These biopsies were mechanically dissociated and the resulting lysate was analyzed for the presence of MPO (Fig. 5A) using the colorimetric substrate tetramethylbenzidine (TMB) (16). Assaying the MPO activity present in these samples against standard curves of horse radish peroxidase (HRP) facilitates the estimation of the number of neutrophils present in these samples. As seen in Figure 5B, administration of 1H4 mAb resulted in a two-fold increase in the number of neutrophils present in the inflamed ears, whereas there was no significant difference in the number of neutrophils present in samples derived from non-inflamed ears. To further examine the effect that administration of αTLT2 mAb in vivo has on neutrophil recruitment and the inflammatory process in control and croton oil-treated ears, H&E staining of sections taken from the ear was performed to assess the cellular infiltrate as well as to examine the architecture of the ear. As seen in Figure 5C, administration of αTLT2 mAb was observed to increase the recruitment of neutrophils to croton oil-treated ears in comparison to mice that received a sham injection of saline. The cellular influx

consisted of primarily neutrophils in both treated and control animals based on examination of the sections at high magnification (data not shown). Administration of αTLT2 mAb had no effect on the recruitment of neutrophils to the control ear (Fig. 5C) and as demonstrated by an analysis of MPO activity (Fig. 5A). Finally, examination of the architecture of the ear did not reveal any significant differences at 3 h between the control and αTLT2-treated animals for either the control or croton oil-treated ear. These data support the conclusion that TLT2 engagement in vivo results in an enhanced chemotactic response to signals generated during inflammation thereby increasing the number of neutrophils accumulating in the inflamed tissue.

Because TLT2 is broadly expressed by immune cells, including macrophages, neutrophils, and B lymphocytes, it is difficult to determine if enhanced recruitment of neutrophils following administration of αTLT2 mAb is the result of a direct effect of TLT2 ligation on neutrophils or due to an indirect effect resulting from TLT2 ligation on other cell types. In an effort to determine if the observed enhancement of neutrophil accumulation at sites of inflammation is a direct effect of TLT2 ligation on responding neutrophils in vivo, a series of adoptive transfer experiments were performed in which neutrophils were isolated from the bone marrow of CD45.1 C57Bl/6 mice and labeled with either CFSE or CMPTX, or left unlabeled. These cells were then treated either with isotype control antibodies, $F(ab')_2$ fragments of 1H4 αTLT2 mAb or intact 1H4. An equal mixture of treated cells was then adoptively transferred into CD45.2 C57Bl/6 recipient mice, which had been challenged intratracheally with 5 μg of LPS to induce inflammation in the lung 2 h prior to adoptive transfer. Four hours after adoptive transfer of treated neutrophils, the recipient mice were sacrificed, and cells present in the lung

were isolated by lavage, stained with the appropriate antibodies, and analyzed by flow cytometry. Adoptively transferred neutrophils were discriminated by the expression of CD45.1, and these cells were then analyzed based on the dye they were loaded with; green, red, or unlabeled, allowing for the identification of these populations present in the lungs of recipient mice (Fig. 6A). As seen in Figure 8B, neither pretreatment with isotype control antibody nor dye loading affected the recruitment of adoptively transferred neutrophils into the inflamed lung. Pretreatment with αTLT2 mAb, however, increased the frequency of treated neutrophils recovered in comparison to untreated or isotype control cells, regardless of the labeling conditions (Fig. 6B). The difference in frequencies between controls and αTLT2 mAb treated neutrophils results in approximately a two-fold increase in the absolute number of αTLT2 treated cells isolated from the lungs of the recipient mice (Fig. 6C). The observed increase in the number of neutrophils that migrated occurs whether neutrophils were treated with intact 1H4 mAb or $F(ab')_2$ fragments of this mAb. As these experiments utilized pooled neutrophils isolated from the same group of donor animals and represent a specific enhancement in the recruitment of αTLT2 mAb treated neutrophils compared to control cells within the same recipient animal, these data strongly support the conclusion that ligation of TLT2 potentiates the chemotactic response of neutrophils to chemokines in vivo via a direct effect on the neutrophil itself.

Discussion

The *Trem* locus encodes several receptors expressed on myeloid lineage cells that function to modulate the response of cells to ligands for PRRs. In the case of TREM-1, ligation potentiates the systemic inflammatory response following stimulation of cells via TLRs and in some instances can exert a direct effect on macrophages leading to the production of inflammatory cytokines (7, 8, 10, 17). In contrast, TREM-2, which is expressed on a range of cell types, including macrophages has been shown to exert an inhibitory effect on TLR-mediated signaling resulting in attenuation of activation and cytokine production (9, 18). TLT2 is expressed on cells of the myeloid lineage, including neutrophils and macrophages, and is upregulated in response to inflammatory conditions in vivo (5). Based on these observations, it was of interest to determine if TLT2, like other members of the TREM family, modulates the functional response of myeloid lineage cells to signals derived from PRRs. This study demonstrates that TLT2 ligation potentiates ROS production, degranulation and chemotaxis in response to numerous GPCR agonists, including FMLP, C5a, MIP-2, KC and IL-8. Importantly, TLT2 ligation was not observed to potentiate the cellular response to GM-CSF receptor or FcR-mediated signaling, which involve reversible protein tyrosine phosphorylation. Moreover, TLT2 ligation did not potentiate neutrophil degranulation in response to TLR. Thus, TLT2 appears to mediate signaling processes that selectively potentiate the response to GPCRs. Importantly, ligation of TLT2 was not observed to decrease the threshold of sensitivity to GPCR agonists, supporting the conclusion that may act by amplifying or prolonging the GPCR signal, which in turn potentiates the functional response of the cell. Alternatively, because studies have yet to be performed

demonstrating that TLT2 ligation affects the qualitative or quantitative nature of the biochemical signals associated with GPCRs, it is formally possible that TLT2 initiates a parallel signaling pathway that amplifies the functional response to GPCRs without directly intersecting those pathways. Regardless, the role of TLT2 as a receptor that potentiates the response of neutrophils to GPCR-mediated signals is comparable to the functions of TREM-1 and TREM-2 which modulate TLR-mediated signaling.

As mentioned above, the mechanism by which TLT2 potentiates the response to GPCR signaling is currently not known. TLT2 does not possess charged transmembrane residues like TREM-1 and TREM-2 that would promote the interaction with transmembrane signaling effectors such as DAP12. Unlike TLT1, TLT2 does not contain an ITAM or ITIM in its cytoplasmic domain, which have the ability to recruit SH2 domain-containing effector proteins to mediate signal transduction (4, 19). Indeed, the cytoplasmic tail of human and mouse TLT2 are not highly conserved and there are no apparent signaling motifs that would, a priori, be predicted to be important for potentiating GPCR-mediated signaling. The TLT2 cytoplasmic domains from human and mouse are proline-rich and it is possible that this could confer the ability to interact with WW or SH3 domain-containing proteins. Alternatively, the proline residues may play a role in providing tertiary structure to the cytoplasmic domain that is important for mediating interactions with key effector proteins. Regardless, it is clear that ligation of TLT2 does not potentiate the cellular response to signals delivered via the GM-CSF receptor, which is a receptor protein tyrosine kinase, or Fc receptors, which also engage protein tyrosine kinases. Moreover, studies do not support the conclusion that TLT2 ligation modulates the response to TLR signaling, as is the case for TREM-1 and TREM-

2. Therefore, it appears that TLT2 selectively modulates the cellular response to GPCR-mediated signaling. However, studies have yet to determine if this is true for receptors coupled to a wide range of heterotrimeric G proteins as the receptors examined to date engage G proteins of the $G\alpha_i$ subclass. Whether TLT2 can modulate the response to signaling via receptors coupled to other subclasses of heterotrimeric G proteins, such as $G\alpha_s$ or $G\alpha_q$, remains to be determined.

An equally important question that remains to be determined concerns the identity of the ligand for TLT2. Previous studies have suggested that TLT2 binds to B7-H3 and that TLT2 plays a role in modulating the function of $CD8^+$ T cells in response to engaging its ligand (20). However, it is unlikely that this is the case. First, studies by our group have consistently failed to detect expression of TLT2 on cells of the T lineage (5). Regardless of whether $CD4^+$ or $CD8^+$ T cells are examined in their resting or activated state, TLT2 expression is not observed using two anti-TLT2 mAbs that bind to distinct epitopes. Secondly, it has been reported by an independent group that TLT2 does not bind B7-H3 (21). In conclusion, the identity of the physiological ligand for TLT2 remains to be determined. Currently, little is known regarding the ligands for other TREM family receptors. Studies using recombinant TREM-1 fusion proteins have detected putative ligands on the surface of neutrophils and platelets as well as on necrotic cells and in the serum of patients with sepsis, but their identity has not been determined (22-24). Similarly, TREM-2 fusion proteins have detected putative ligands on the surface of peritoneal and bone marrow-derived macrophages (18). More recently, TREM-2 has been shown to exist in a complex with Plexin-A1, which binds to Sema6D (25). Thus DAP12-mediated signaling can be initiated by virtue of the TREM-2:Plexin-A1

interaction via binding of Plexin-A1 to Sema6D. This observation explains the phenotypic consequences of a genetic loss of the TREM-2 receptor and DAP12 and demonstrates that at least one member of the *Trem* locus is responsible for mediating signals in response to a ligand that binds to a distinct, associated receptor (25). Because TLT2 is expressed on both neutrophils and macrophages, which are critical effectors of the innate immune response against bacteria, and its expression is increased on these cells in response to inflammation, one might predict that the ligand for TLT2 could be derived from infectious organisms, or be generated as a byproduct of the innate immune response against pathogens (e.g. an acute phase protein). This concept is also supported by the fact that TLT2 is most highly expressed on marginal zone and B1 B cell subpopulations, which are involved in mounting the natural/innate humoral immune response against bacterial pathogens (5).

In summary, this study demonstrates that TLT2 plays an important role in potentiating the neutrophil response to GPCR agonists leading to enhanced ROS production, degranulation and chemotaxis. Importantly, TLT2 does not appear to potentiate the cellular response to other types of receptors. Because TLT2 is upregulated on myeloid lineage cells in response to inflammatory conditions in vivo, and plays a role in potentiating the cellular response against agonists derived from pathogens or generated during the innate immune response, it is clear that this member of the TREM locus is important in potentiating the anti-bacterial innate immune response via a novel mechanism that involves the potentiation of the response to GPCR signaling.

Acknowledgements

We thank Dr. Lynn Rasmussen at Southern Research Institute for generously allowing the use of their Envision Multi-label Plate reader for the Respiratory Burst assay.

References

1. Takeda, K., and S. Akira. 2005. Toll-like receptors in innate immunity. *Int Immunol. 17:1*.

2. Chen, G., M. H. Shaw, Y. G. Kim, and G. Nunez. 2009. NOD-like receptors: role in innate immunity and inflammatory disease. *Annu Rev Pathol. 4:365*.

3. Klesney-Tait, J., I. R. Turnbull, and M. Colonna. 2006. The TREM receptor family and signal integration. *Nat Immunol. 7:1266*.

4. Ford, J. W., and D. W. McVicar. 2009. TREM and TREM-like receptors in inflammation and disease. *Curr Opin Immunol. 21:38*.

5. King, R. G., B. R. Herrin, and L. B. Justement. 2006. Trem-like transcript 2 is expressed on cells of the myeloid/granuloid and B lymphoid lineage and is up-regulated in response to inflammation. *J Immunol. 176:6012*.

6. Bouchon, A., J. Dietrich, and M. Colonna. 2000. Cutting edge: inflammatory responses can be triggered by TREM-1, a novel receptor expressed on neutrophils and monocytes. *J Immunol. 164:4991*.

7. Netea, M. G., T. Azam, G. Ferwerda, S. E. Girardin, S. H. Kim, and C. A. Dinarello. 2006. Triggering receptor expressed on myeloid cells-1 (TREM-1) amplifies the signals induced by the NACHT-LRR (NLR) pattern recognition receptors. *J Leukoc Biol. 80:1454*.

8. Bouchon, A., F. Facchetti, M. A. Weigand, and M. Colonna. 2001. TREM-1 amplifies inflammation and is a crucial mediator of septic shock. *Nature. 410:1103*.

9. Turnbull, I. R., S. Gilfillan, M. Cella, T. Aoshi, M. Miller, L. Piccio, M. Hernandez, and M. Colonna. 2006. Cutting edge: TREM-2 attenuates macrophage activation. *J Immunol. 177:3520*.

10. Sharif, O., and S. Knapp. 2008. From expression to signaling: roles of TREM-1 and TREM-2 in innate immunity and bacterial infection. *Immunobiology. 213:701*.

11. Rabiet, M. J., E. Huet, and F. Boulay. 2007. The N-formyl peptide receptors and the anaphylatoxin C5a receptors: an overview. *Biochimie. 89:1089*.

12. Hyduk, S. J., J. R. Chan, S. T. Duffy, M. Chen, M. D. Peterson, T. K. Waddell, G. C. Digby, K. Szaszi, A. Kapus, and M. I. Cybulsky. 2007. Phospholipase C, calcium, and calmodulin are critical for alpha4beta1 integrin affinity up-regulation and monocyte arrest triggered by chemoattractants. *Blood. 109:176*.

13. Al-Shami, A., W. Mahanna, and P. H. Naccache. 1998. Granulocyte-macrophage colony-stimulating factor-activated signaling pathways in human neutrophils. Selective activation of Jak2, Stat3, and Stat5b. *J Biol Chem. 273:1058*.

14. Borregaard, N. Neutrophils, from marrow to microbes. *Immunity. 33:657*.

15. Underhill, D. M., and A. Ozinsky. 2002. Phagocytosis of microbes: complexity in action. *Annu Rev Immunol. 20:825*.

16. Menegazzi, R., G. Zabucchi, A. Knowles, R. Cramer, and P. Patriarca. 1992. A new, one-step assay on whole cell suspensions for peroxidase secretion by human neutrophils and eosinophils. *J Leukoc Biol. 52:619*.

17. Colonna, M., and F. Facchetti. 2003. TREM-1 (triggering receptor expressed on myeloid cells): a new player in acute inflammatory responses. *J Infect Dis. 187:S397*.

18. Hamerman, J. A., J. R. Jarjoura, M. B. Humphrey, M. C. Nakamura, W. E. Seaman, and L. L. Lanier. 2006. Cutting edge: inhibition of TLR and FcR responses in macrophages by triggering receptor expressed on myeloid cells (TREM)-2 and DAP12. *J Immunol. 177:2051*.

19. Barrow, A. D., E. Astoul, A. Floto, G. Brooke, I. A. Relou, N. S. Jennings, K. G. Smith, W. Ouwehand, R. W. Farndale, D. R. Alexander, and J. Trowsdale. 2004. Cutting edge: TREM-like transcript-1, a platelet immunoreceptor tyrosine-based inhibition motif encoding costimulatory immunoreceptor that enhances, rather than inhibits, calcium signaling via SHP-2. *J Immunol. 172:5838*.

20. Hashiguchi, M., H. Kobori, P. Ritprajak, Y. Kamimura, H. Kozono, and M. Azuma. 2008. Triggering receptor expressed on myeloid cell-like transcript 2 (TLT-2) is a counter-receptor for B7-H3 and enhances T cell responses. *Proc Natl Acad Sci U S A. 105:10495*.

21. Leitner, J., C. Klauser, W. F. Pickl, J. Stockl, O. Majdic, A. F. Bardet, D. P. Kreil, C. Dong, T. Yamazaki, G. Zlabinger, K. Pfistershammer, and P. Steinberger. 2009. B7-H3 is a potent inhibitor of human T-cell activation: No evidence for B7-H3 and TREML2 interaction. *Eur J Immunol. 39:1754*.

22. Wong-Baeza, I., N. Gonzalez-Roldan, E. Ferat-Osorio, N. Esquivel-Callejas, R. Aduna-Vicente, L. Arriaga-Pizano, H. Astudillo-de la Vega, M. A. Villasis-Keever, R. Torres-Gonzalez, I. Estrada-Garcia, C. Lopez-Macias, and A. Isibasi. 2006. Triggering receptor expressed on myeloid cells (TREM-1) is regulated post-transcriptionally and its ligand is present in the sera of some septic patients. *Clin Exp Immunol. 145:448*.

23. Haselmayer, P., L. Grosse-Hovest, P. von Landenberg, H. Schild, and M. P. Radsak. 2007. TREM-1 ligand expression on platelets enhances neutrophil activation. *Blood. 110:1029.*

24. Gibot, S., F. Massin, M. Marcou, V. Taylor, R. Stidwill, P. Wilson, M. Singer, and G. Bellingan. 2007. TREM-1 promotes survival during septic shock in mice. *Eur J Immunol. 37:456*.

25. Takegahara, N., H. Takamatsu, T. Toyofuku, T. Tsujimura, T. Okuno, K. Yukawa, M. Mizui, M. Yamamoto, D. V. Prasad, K. Suzuki, M. Ishii, K. Terai, M. Moriya, Y. Nakatsuji, S. Sakoda, S. Sato, S. Akira, K. Takeda, M. Inui, T. Takai, M. Ikawa, M. Okabe, A. Kumanogoh, and H. Kikutani. 2006. Plexin-A1 and its interaction with DAP12 in immune responses and bone homeostasis. *Nat Cell Biol. 8:615*.

Footnotes

M. M. Halpert was supported by training grants from the HHMI Med into Grad Initiative (Grant 56005705) and a NIH Institutional Training Grant in Immunology (T32 AI 007051-33).

Address correspondence and reprint requests to Dr. Louis B. Justement, Department of Microbiology, University of Alabama at Birmingham, Rm. 502 SHEL, 1825 University Boulevard, Birmingham, AL 35294. E-mail address: lbjust@uab.edu.

Abbreviations used in this paper: GPCR, G protein-coupled receptor; MPO, myeloperoxidase; PRR, pattern recognition receptor; ROS, reactive oxygen species; SA, streptavidin; TLT2, TREM-like transcript 2; TMB, tetramethylbenzidine; TREM, triggering receptor expressed on myeloid cells.

Figure Legends

Figure 1. TLT2 ligation potentiates ROS production by murine neutrophils in response to FMLP. (**A**) Treatment with distinct αTLT2 mAbs (1H4 or 1C5) enhances the production of ROS in a dose dependent manner following the addition of FMLP. Neutrophils ($1x10^6$) were preincubated for 45 min with αTLT2 or isotype control (IC) mAb at the indicated concentrations, and then stimulated with FMLP (1 μM). (**B**) Purified neutrophils were incubated in the presence of αTLT2 mAb or LPS for either 15 min (upper panel) 45 min (middle panel), or 2 h (lower panel) then stimulated with 1 μM FMLP. Alternatively, neutrophils were preincubated for 15, 45 or 120 min with biotinylated 1H4 mAb (1 μg) that had been premixed with SA (20 μg) followed by addition of FMLP. ROS production was monitored at the indicated times. (**C**) TLT2 ligation does not alter ROS generation in response to GM-CSF. Purified neutrophils were incubated in the presence of αTLT2 or isotype control mAb for 45 min followed by stimulation with either FMLP (1 μM) or GM-CSF (1 ng/ml). For (**A**) the maximum ROS production for pretreated samples was normalized to controls that received FMLP alone. For **(C)** the data were normalized to samples that received either FMLP or GM-CSF alone. The absolute values for ROS-dependent chemiluminescence are shown as well. All data represent the average of triplicate samples with mean ± standard deviation shown and are representative of at least three independent experiments. Asterisks denote significance of values for αTLT2 mAb-treated samples compared to the respective control sample.

Figure 2. TLT2 ligation enhances the chemotactic response of neutrophils to FMLP. Purified neutrophils ($1x10^6$) were placed in the upper chamber of a 3 μm transwell device in the presence or absence of the indicated concentration of the αTLT2 mAb 1H4 (**A**) or 100 □g of either intact or $F(ab')_2$ fragments of 1H4 (**B**) or 1 μg of the αTLT2 mAb 1C5 coupled to biotin with or without streptavidin (20 μg) (**C**). For panels **B** and **C**, isotype control indicates samples in which 0.1 or 1 μg/ml of rat mAb was added to the upper chamber, respectively. For panels (**A-C**) 1 μM FMLP was added to the lower chamber for all samples. After 1 h, the cells were harvested from the lower chamber and counted. The number of cells present in the lower chamber for each experimental condition was divided by the number of migrating cells in control samples (FMLP alone) to determine the chemotactic index. The mean ± SD is shown for triplicate samples and the experiments depicted are representative of at least 3 independent experiments. (**D**) TLT2 ligation enhances neutrophil migration in response to a wide range of FMLP concentrations. Neutrophils ($1x10^6$) in medium alone, or containing 100 ng/ml of 1H4 mAb or 2 μg/ml LPS were placed in the upper chambers of transwell devices. The indicated concentrations of FMLP or medium alone were placed in the lower chambers. The percentage of cells migrating into the lower chamber are the average of triplicate samples with the mean ± SD shown, and are representative of at least three independent experiments. (**E**) TLT2 potentiation of FMLP-mediated chemotaxis is proportional across a wide dose response. The chemotactic indices for αTLT2 mAb pretreated versus control neutrophils in response to FMLP are depicted. The values are derived by dividing the % migration data for αTLT2 pretreated samples by the % migration for

neutrophils incubated in medium alone as shown in (**D**). Asterisks denote significance of values for αTLT2 mAb-treated samples compared to the respective control sample.

Figure 3. TLT2 ligation potentiates neutrophil degranulation and upregulation of the activation marker CD11b in response to GPCR-mediated signaling. Purified neutrophils ($1x10^6$) were incubated either with the αTLT2 mAb 1H4 (at the concentrations indicated) or biotinylated 1C5 (1μg/ml). Where indicated, streptavidin (20-40 μg) was included to mediate secondary cross-linking of TLT2. Neutrophils were stimulated with the FMLP agonist WKYMVm (W) at the indicated concentrations (**A-F**), the activated complement component C5a (2 ng/ml) (**G**), or GM-CSF (1 ng/ml) (**H-I**). At the indicated time points the cells were harvested and stained to detect surface expression of CD11b and analyzed by flow cytometry. Filled histograms represent CD11b expression on neutrophils incubated in medium alone. Representative histograms of CD11b expression are shown. All data are representative of at least three independent experiments.

Figure 4. TLT2 ligation results in enhanced migration of neutrophils in response to a variety of chemoattractants *in vitro*. Purified neutrophils were subjected to migration assays using 3 μm transwell filters. Neutrophils ($1x10^6$) were placed in the upper chamber in medium alone (control) or in the presence of the indicated concentration of αTLT2 mAb. Medium alone (control) or containing chemoattractants (FMLP, 1 μM; Mip-2, 5 ng/ml; KC, 5 ng/ml; IL-8, 50-100 ng/ml or GM-CSF, 0.5-10 ng/ml) was placed

in the lower chamber, as indicated. After a 1 h incubation at 37°C, the neutrophils present in the lower chamber were harvested and counted. The percentage of cells present in the lower chamber (**A**) and the chemotactic index (**B**) are shown. The chemotactic index was calculated by dividing the % migration for neutrophils incubated with each chemoattractant in the presence or absence of αTLT2 mAb by the % migration for neutrophils incubated with medium alone. Data represent the average of triplicate samples with the mean ± SD shown.

Figure 5. Administration of αTLT2 mAb results in enhanced accumulation of neutrophils at sites of inflammation *in vivo.* Either 100 μg of the αTLT2 mAb 1H4 or an equivalent volume of PBS was administered to groups of mice via i.v. injection. After 30 min, a solution of 2% Croton Oil in acetone was applied to the pinna of one ear, whereas only acetone was applied to the other (control ear). After 4 h, the mice were sacrificed and a 4 mm biopsy was removed from the center of the ears using a biopsy punch. These samples were homogenized and assayed for MPO activity using the colorimetric substrate TMB. The measured absorbance values are depicted for αTLT2 mAb treated and control mice for both ears (**A**). To determine the number of neutrophils recruited, lysates from known numbers of purified neutrophils were compared to serial dilutions of HRP to develop a standard curve. Using this standard curve for the MPO dependent conversion of TMB, the numbers of neutrophils present in the Croton Oil treated ears for αTLT2 mAb treated and control mice were calculated (**B**). The data presented represent a minimum of 5 mice and the mean ± SD, and significant p values are shown for both (**A**) and (**B**). **(C)** H & E staining of ear sections taken at 3 h from mice

injected with saline (control) or αTLT2 mAb. Representative sections are shown for both control ears that were treated with acetone alone as well as inflamed ears treated with croton oil in acetone. The sections are representative of at least three independent experiments.

Figure 6. TLT2 ligation enhances neutrophil recruitment into sites of inflammation *in vivo*. (**A**) Purified neutrophils from CD45.1 mice were labeled with either CFSE, CMPTX, or left unlabeled. These cells were then incubated in the presence of the αTLT2 mAb as indicated, or an isotype control antibody, or medium alone. After treatment, the purified, labeled neutrophils were mixed at equivalent ratios and $3x10^7$ total cells were adoptively transferred into CD45.2 recipient mice, which had received an intratracheal challenge with 5 μg of LPS to induce inflammation in the lung 30 min prior to adoptive transfer. After 4 h, the recipient mice were sacrificed and the neutrophils present in the lung were isolated by lavage. Donor derived neutrophils were indentified based on the expression of CD45.1, and these cells were analyzed for the presence of the fluorescent dyes, which discriminated the pre-transfer treatment conditions. (**B**) Ligation of TLT2 increases the relative frequency of neutrophils recruited into the lungs of recipient mice. A total of 8 mice are depicted and for each mouse the % PMNs recruited to the lung for each of 3 pre-treatment conditions is shown. The pretreatment condition is coded by the symbol used and the dye used is reflected by the color of the symbol. (**C**) TLT2 ligation causes an absolute increase in the number of neutrophils that migrate into inflamed lungs. The absolute number of neutrophils for each treatment condition was

calculated based on flow cytometric analysis. The mean ± SD for a minimum of 5 mice is shown for each treatment condition.

Figure S1. TLT2 ligation potentiates ROS production by neutrophils in response to FMLP, but not GM-CSF. Neutrophils ($1x10^6$/sample) were pretreated with isotype control or αTLT2 mAb (100ng/ml) for 45min. The cells were then triggered by the addition of FMLP (**A)** or GM-CSF (**B**) and the maximum ROS-dependent chemiluminescence was measured. The data represent the average triplicate samples with the mean ± SD shown.

Figure S2. TLT2 ligation does not increase FcR-mediated phagocytosis. Purified neutrophils ($1x10^6$) were incubated either without stimulus or with 100ng/ml of the intact or $F(ab')_2$ fragment of 1H4, 1µg/ml biotinylated 1C5 plus 20 µg/ml streptavidin, or 2 µg/ml of LPS for 10 min. The cells were washed and $5x10^6$ FITC conjugated 2 µm polystyrene beads were added. The opsonized beads were preincubated with mouse polyclonal αFITC as described. Following a 15 min incubation, the cells were harvested, extracellular FITC fluorescence was quenched with trypan blue and the cells were analyzed by flow cytometry to detect phagocytosis of FITC labeled particles. Representative histograms are depicted in (**A**) and the mean fold increase in the percentage of cells containing phagocytosed beads compared to cells incubated in medium alone with non-opsonized beads is shown in (**B**). The data in (**A**) are

representative of 3 independent experiments and the mean values for fold increase in phagocytosis ± SD in (**B**) were calculated from the three independent experiments.

Figure 1. TLT2 ligation potentiates ROS production by murine neutrophils in response to FMLP.

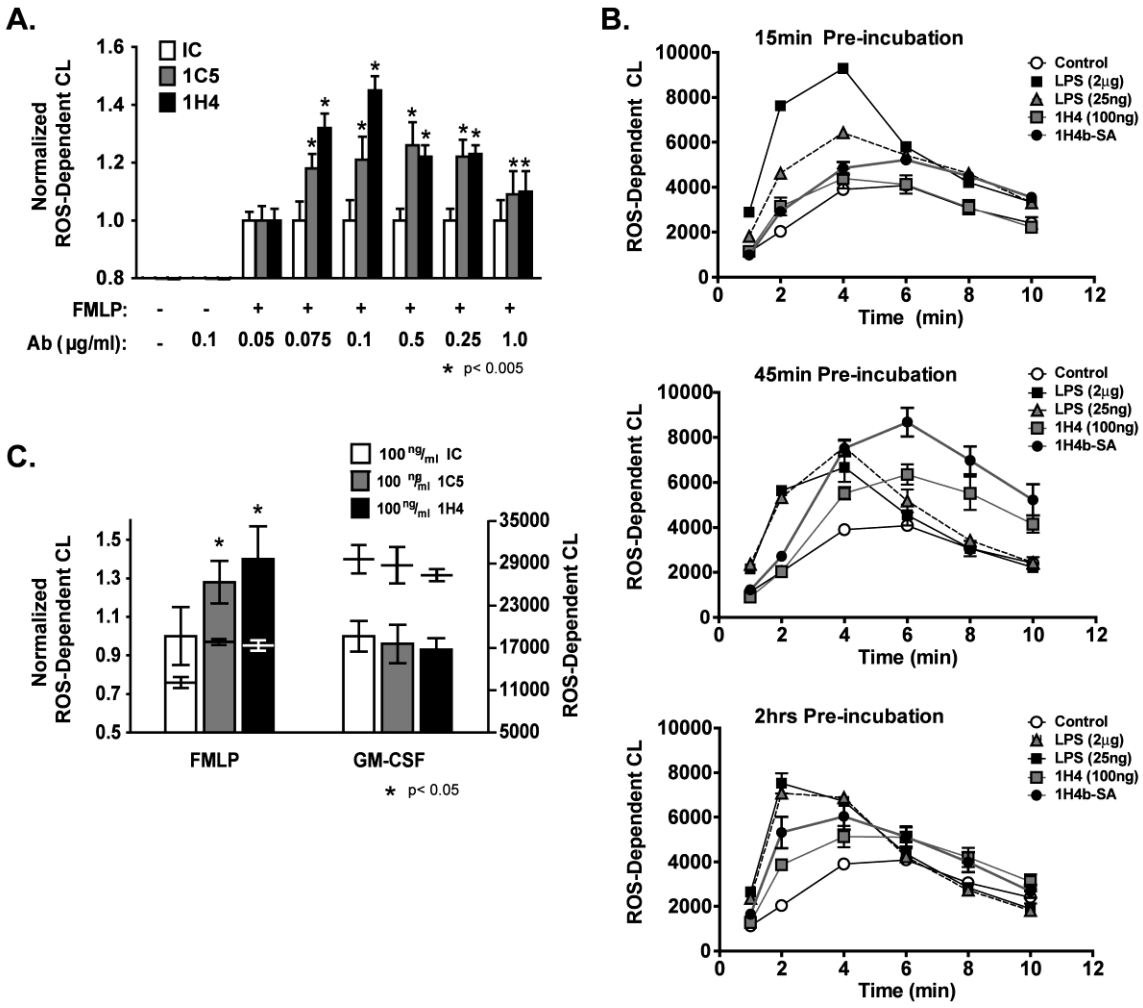


Figure 2. TLT2 ligation enhances the chemotactic response of neutrophils to FMLP.

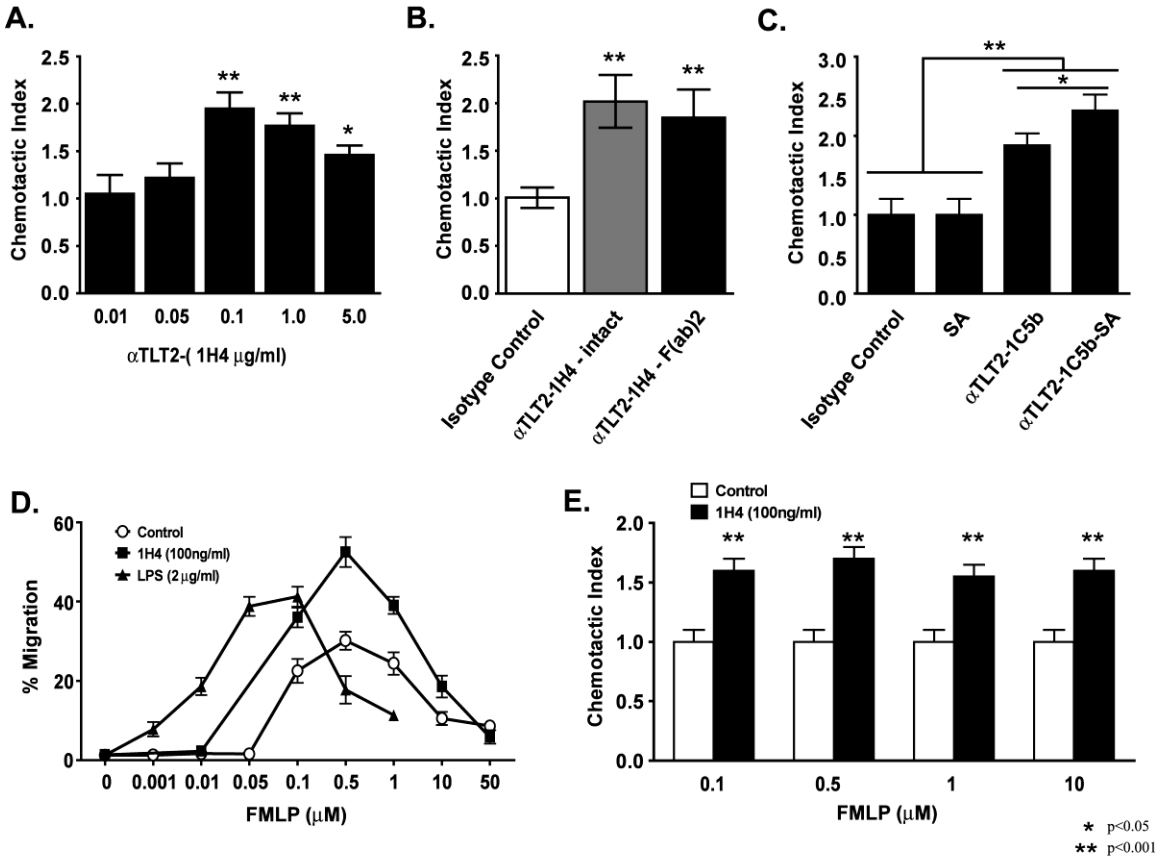


Figure 3. TLT2 ligation potentiates neutrophil degranulation and upregulation of the activation marker CD11b in response to GPCR-mediated signaling.

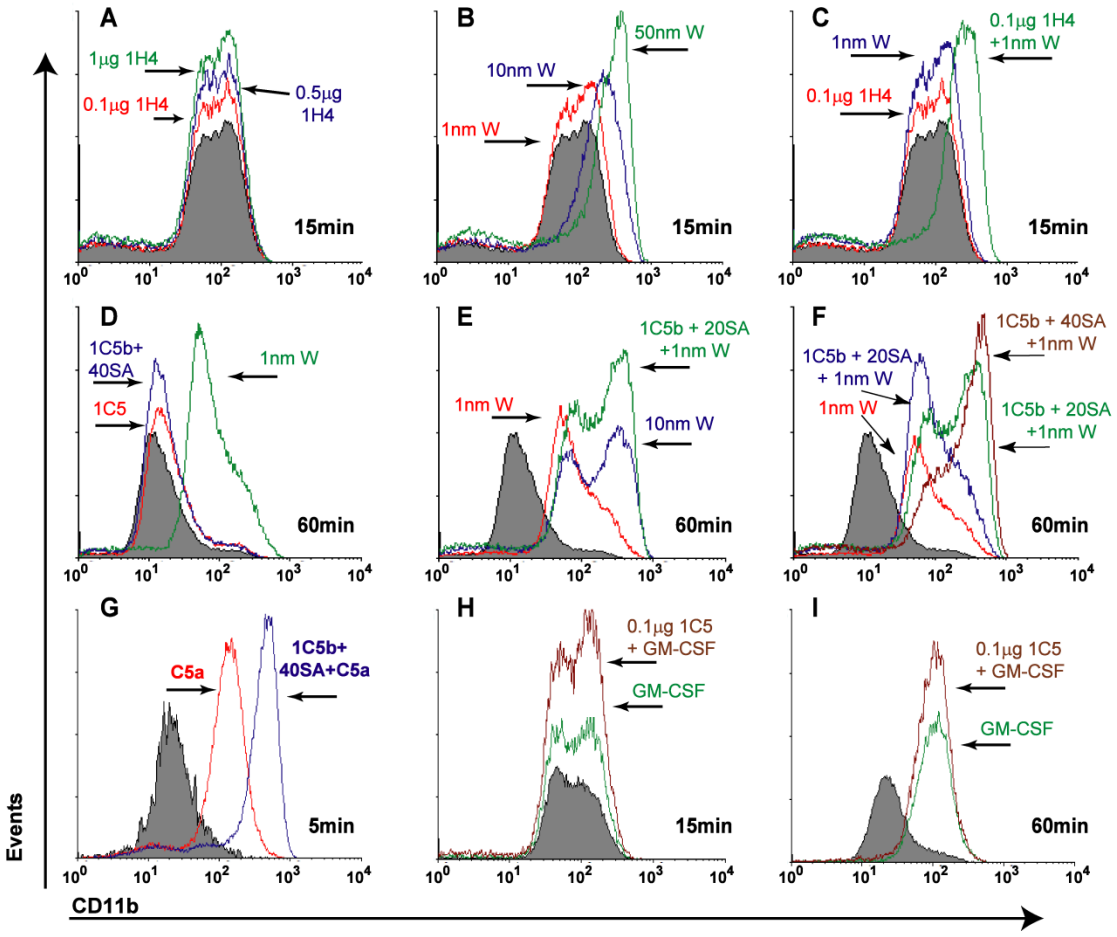


Figure 4. TLT2 ligation results in enhanced migration of neutrophils in response to a variety of chemoattractants *in vitro*.

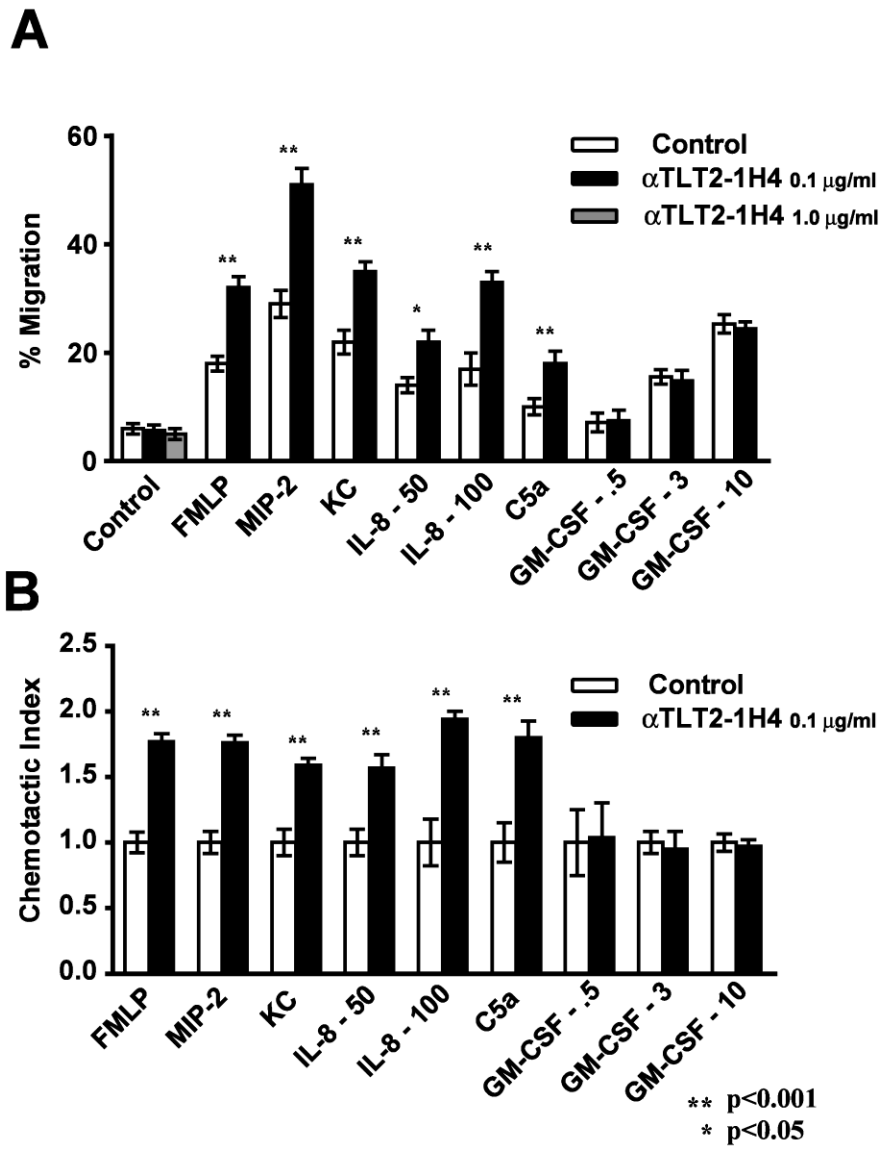


Figure 5. Administration of αTLT2 mAb results in enhanced accumulation of neutrophils at sites of inflammation *in vivo.*

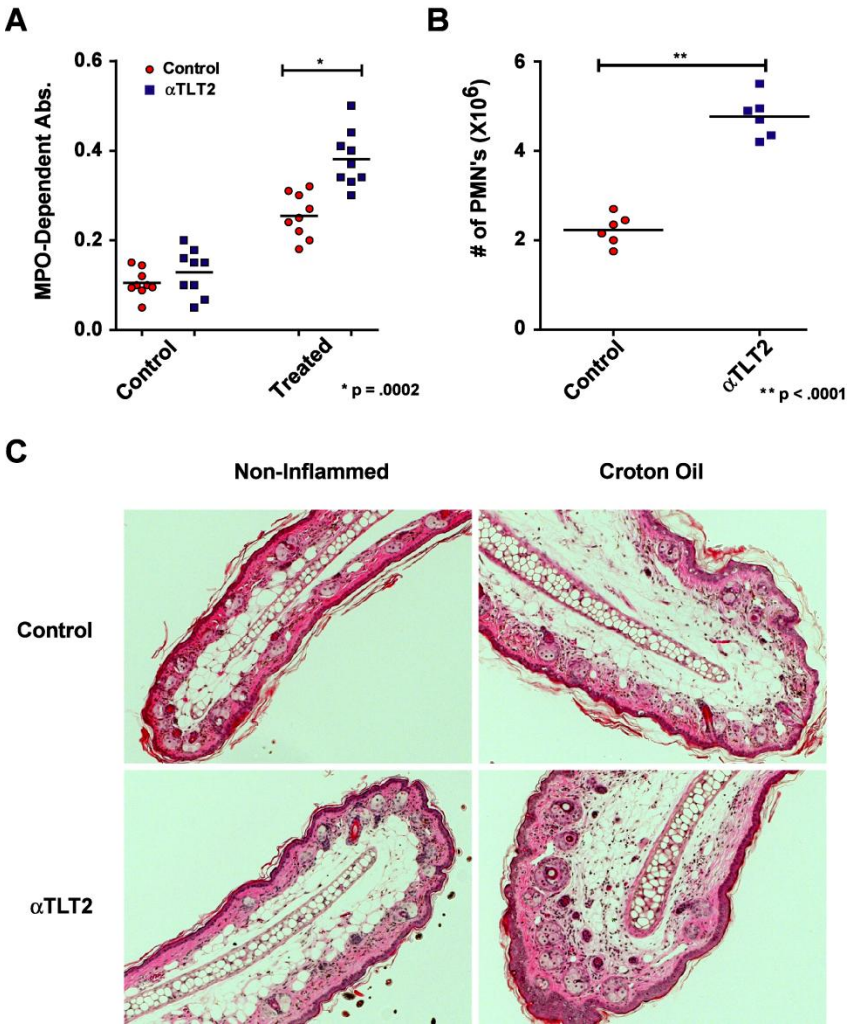


Figure 6. TLT2 ligation enhances neutrophil recruitment into sites of inflammation *in vivo*.

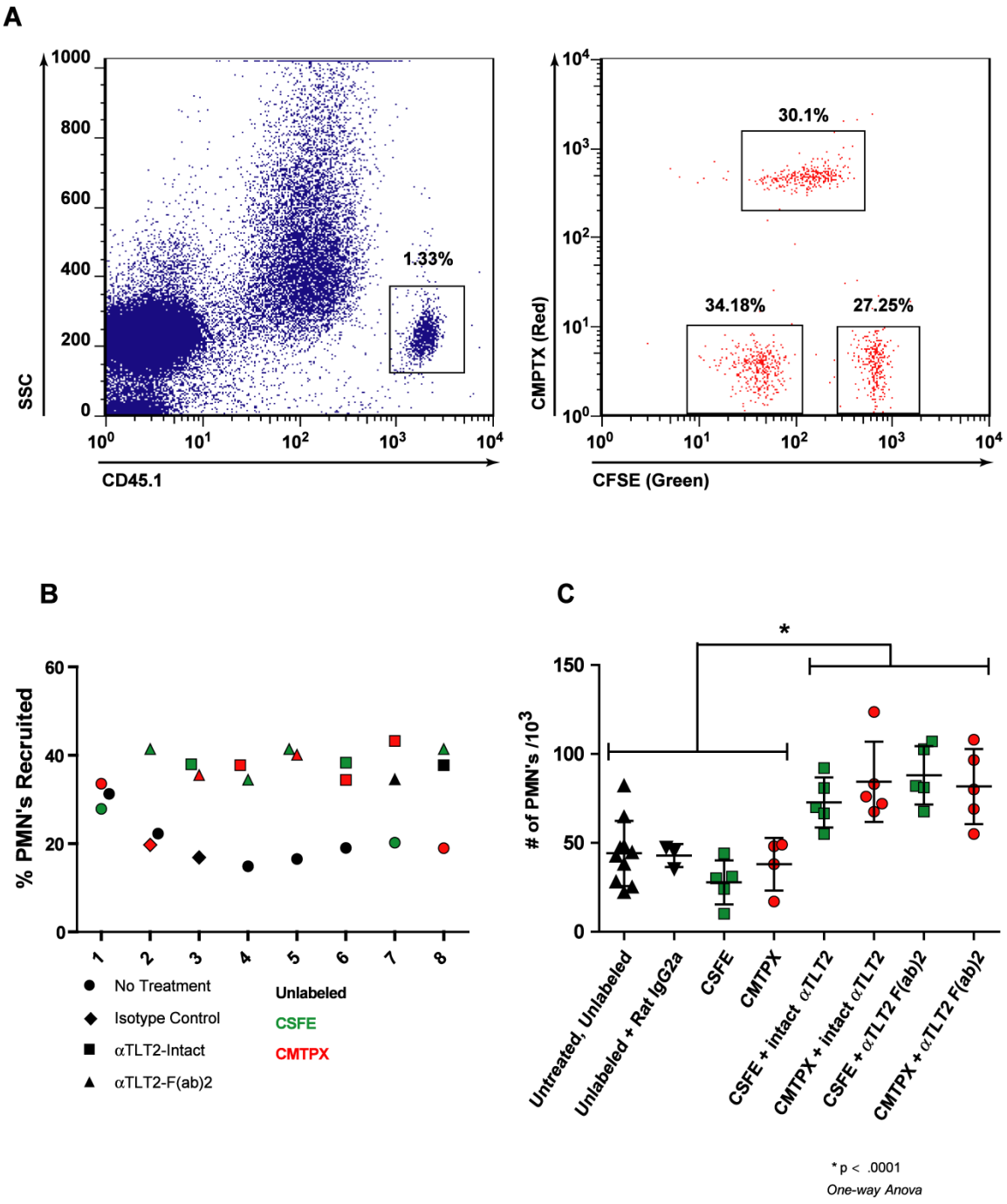


Figure S1. TLT2 ligation potentiates ROS production by neutrophils in response to FMLP, but not GM-CSF.

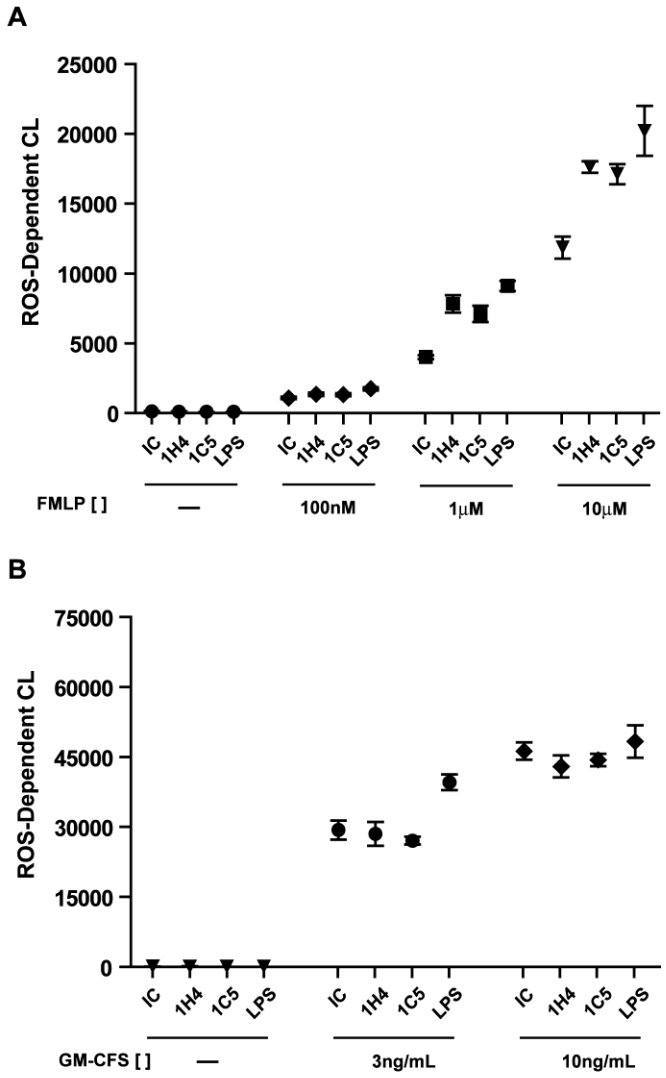
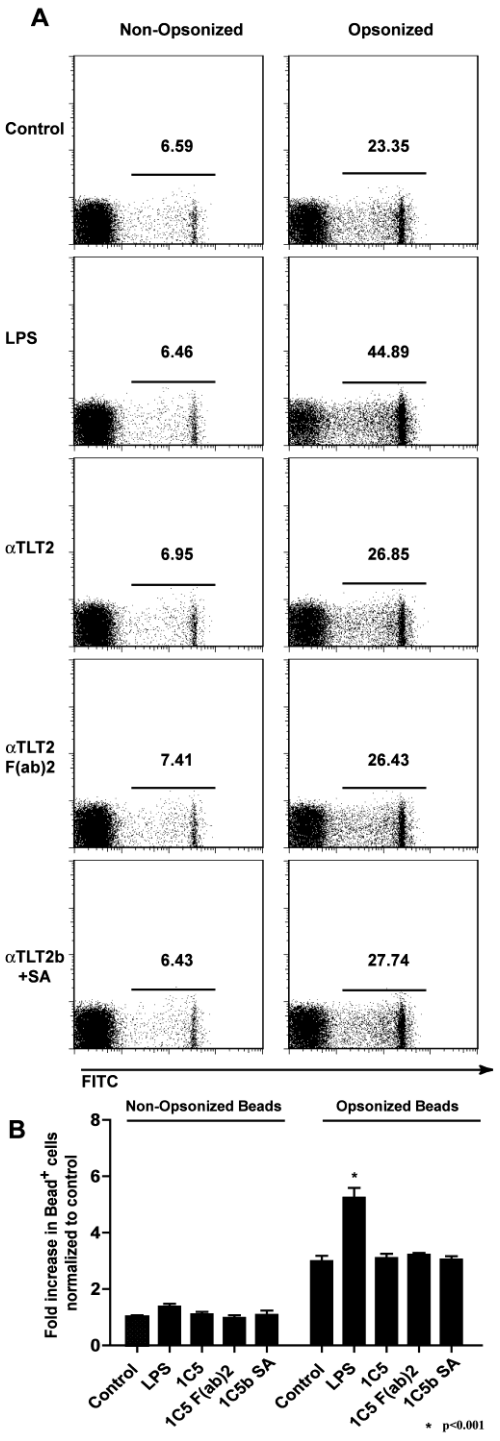


Figure S2. TLT2 ligation does not increase FcR-mediated phagocytosis.



TLT2 PROMOTES MACROPHAGES TO SECRETE CHEMOKINES AND GROWTH FACTORS WHICH RAPIDLY RECRUIT NEUTROPHILS TO SITES OF INFLAMMATION

by

MATTHEW M. HALPERT, CHAD STEELE, R. GLENN KING, AND LOUIS B. JUSTEMENT

Submitted to The Journal of Immunology; Cutting Edge

Format adapted for dissertation

Abstract

TLT2, expressed on neutrophils, macrophages, and B lymphocytes, is the only member of the TREM family to synergize with G-protein coupled receptors (GPCRs) on neutrophils, potentiating a variety of functions including migration. Here we show that ligation of TLT2 on macrophages using anti-TLT2 mAbs induced the production of numerous mediators, including MIP-2, KC, and G-CSF, and did so independent of any secondary stimuli. This phenomenon was recapitulated in vivo following injection of anti-TLT2 mAbs resulting in similar production of chemokines and factors. Ultimately, this lead to enhanced influx of hyperactive neutrophils supporting the conclusion that TLT2 plays an important protective role in normal biology. Thus, TLT2 may be involved in an intricate feed-forward loop wherein it is important for both driving the production of factors that recruit neutrophils to sites of infection/inflammation, as well as enhancing the response of neutrophils towards chemokines and bacterial products that signal via GPCRs.

Introduction

TREM-like transcript 2 (TLT2) is a transmembrane receptor expressed on B cells, macrophages and neutrophils and is one of four conserved triggering receptor expressed on myeloid cells (TREM) members between mice and humans. The other conserved TREM family proteins are TREM-1, TREM-2, and TLT-1, and the TREM family is predominantly involved in modulating the innate immune response [1, 2]. TREM-1 is an activating receptor highly expressed on neutrophils and monocytes, is upregulated following an acute inflammatory event, and amplifies the immune cell's response to pattern recognition receptor (PRR) signaling [3]. TREM-2 is found on a variety of cells including, but not limited to, dendritic cells, monocytes, microglia, and macrophages and has emerged as an important negative regulator of autoimmunity by playing an anti-inflammatory role on these cells when stimulated [4]. TLT-1 has predominately been studied on platelets, and appears to serve a protective role as it dampens the inflammatory response and facilitates platelet aggregation at the site of injury. Accordingly, TLT-$1^{-/-}$ mice are predisposed to hemorrhaging after injury, and septic patients had substantially higher amounts of soluble TLT-1 in their plasma [2]. TLT2 has recently been shown by our lab to have a similar modulatory effect, as it synergistically enhances the migratory response, degranulation status, and respiratory burst of neutrophils towards agonists that signal via G-protein coupled receptors (GPCRs), but differs from TREM-1 in that it does not alter the innate response to PRR agonists [5]. Like other TREM family members, TLT2 has not been shown to exert substantial effect on the neutrophils in the absence of a secondary stimulus, further promoting this family as modulators of innate immunity rather.

In the course of our lab's study on the effects of TLT2 ligation on neutrophil function *in vivo*, it was observed that intravenous injections of anti-TLT2 also lead to extra-neutrophil activity in response to the generalized inflammatory agent 2% croton oil. Application of 2% croton oil-acetone to the pinna of the ear has been shown to induce ear swelling and edema, increase accumulating Myeloperoxidase (MPO) activity within the inflamed tissue, and cause irritant contact dermatitis (ICD) [6]. Oil stimulation leads to rapid upregulation of IL-6, MIP-2, GM-CSF, and TNFα, which can all contribute to the resulting inflammatory symptoms [7]. Intravenous injections (IV) of anti-TLT2 lead to an increase in neutrophil accumulation in the croton-inflamed ear when compared to isotype control IV injections, and in fact it was shown that TLT2 ligation on the neutrophil potentiates neutrophil migratory responses. However, prior the neutrophil recruitment there was an increase quantitatively and categorically in the cytokines produced in the ear and blood, and in fact several of these cytokines were upregulated even in the absence of the triggering stimulus. Therefore, it was imperative to study the global aspect of TLT2 biology as well as to determine the role TLT2 may play on macrophage, one of the initial players in the innate immunity cascade.

In this study, mAbs towards TLT2 were used to determine that in the absence of a secondary stimulus, TLT2 ligation can drive *ex vivo* macrophages to secrete a variety of cytokines and growth factors of which many are important neutrophil chemoattractants (e.g. MIP-2, KC, IL-6, and G-CSF). Importantly, TLT2 drove this "cytokine storm" without upregulating classical macrophage activation markers such as CD69 at early time points or CD80 and CD86 at 24 hrs. This phenomenon was recapitulated *in vivo*, as intratracheal and intraperitoneal injections of anti-TLT2 mAbs induced a similar cytokine

output and the rapid recruitment of neutrophils to the lung and peritoneal cavity, respectively. The neutrophils recruited to the anti-TLT2 injection site were observed to be in a hyperactive state based on CD11b expression when compared to neutrophils recruited to the classical stimulant FMLF, which agrees with previous publications on the functional role of TLT2 on neutrophils [5]. Thus, TLT2 may play a unique and very important role in both initiating and augmenting the innate immune response via multiple avenues.

Results and Discussion

While determining if intravenous injections of anti-TLT2 would lead to increased neutrophil accumulation in the croton oil inflamed ear, it was observed that though the anti-TLT2 treated animals did recruit significantly more neutrophils to the targeted ear there was a statistically significant decrease in ear tissue mass and thickness (**Fig. 1*A*, 1*B***). Due to this outcome, it was of interest to study the potential extra-neutrophil anti-TLT2 activity occurring in the ears and blood of treated mice by assaying the cytokines and growth factors upregulated after stimulation (**Fig. 1*C*, 1*D***). And indeed, it was observed that intravenous injections of anti-TLT2 30 min prior croton oil treatment lead to both quantitative and qualitative differences in the oil-induced cytokine profiles, as seen by the upregulation of MIP-2, KC, G-CSF and MIG in the ears and blood. In light of these anti-TLT2-induced differences towards a croton oil stimulus, it was also of interest to determine which cytokines might be upregulated in the blood after only anti-TLT2 treatment, and anti-TLT2 intravenous injections rapidly lead to the upregulation of several cytokines/growth factors including MIG and G-CSF (**Fig. 1*E***).

Given that the injection of anti-TLT2 mAbs into circulation lead to the upregulation of several cytokines, it was of interest to determine if anti-TLT2 treatment in other, more confined compartments would also lead to cytokine production in the absence of an exogenously added secondary stimulus. Anti-TLT2 treatments were introduced to the mouse either via intratracheal (IT) drips to the lung or intraperitoneal injection (IP), and subsequent cytokine/growth factor production was analyzed within the lung and peritoneal lavages, respectively. While a time course analysis showed upregulation of several cytokines and factors as rapidly as 30 min (data not shown), peak

values of upregulated players common to both the lung and peritoneum showed that anti-TLT2 treatment can lead to the upregulation of a milieu of factors, including MIP-2, KC, IL-6, and G-CSF, and did so between 30 min and 4 hrs after addition (**Fig. 2*A***). As well, increasing the dose of antibody lead to a potentiated increase in cytokine production, further pointing towards a TLT2 specific event (**Fig. 2*B***). Additionally, circulating blood was also analyzed over a 24 hr period for the presence of cytokines and factors after IT and IP injections and compared in an effort to determine potential secondary outcomes which may lead to physiological actions (**Supplementary Fig. 1*A*, 1*B***). While only common cytokines upregulated in the lung and peritoneal cavity after anti-TLT2 treatment are shown in Figs. 2*A* and 2*B*, several other factors were also analyzed for each compartment and largely found to not be affected (**Fig. 2*B*, 2*C***). This included several inflammatory mediators such as TNFα, IL-1, INFγ, IL-12, and GM-CSF. Th2 cytokines IL-4 and IL-13 along with the anti-inflammatory cytokine IL-10 were also not observed to be upregulated after injection, indicating potential granulocyte-centered specificity of TLT2 activity. It is important to note that temporal and spatial limitations of our analysis may have resulted in the failure to detect transient and/or far-reaching cytokines, leading only to the perceived absence of such factors like TNFα and INFγ within these lavage samples. None-the-less, based on our observations, it would seem that anti-TLT2 treatment lead to a relatively granulocyte-focused response without leading to generalized inflammation as the classical stimulus LPS tends to promote (**Supplementary Fig. 2**).

With the observed upregulation of chemoattractants such as MIP-2, KC, and even G-CSF in the lung and peritoneum after anti-TLT2 intratracheal and intraperitoneal injections, respectively, it was crucial to study the resulting cellular infiltrate with a clear

focus on granulocytes given the recent publication discussing TLT2's functional role on this cell type. Over a 24 hr period, only baseline alveolar macrophages and recruited neutrophils appear within the lung, with recruitment occurring as quickly as 2 hrs, peaking at around 6 hrs after injection, and persisting over the entire 24 hr period (**Fig. 3*A***). In contrast, in addition to the rapidly recruited neutrophils, there was a significant increase in residential macrophages, B cells, and $CD4^+$ T cells in the peritoneal cavity at 24 hr after injection (**Fig. 3*B***). However, increases seen at 24 hrs after injection may well be the result of secondary outcomes to anti-TLT2 injection, such as neutrophil recruitment, and may be confounding to the physiological role of TLT2 under normal conditions.

Given that at short time points only neutrophils appeared to be recruited, this cell type was further studied. Since the lung is a highly protected and sensitive area, introduction of almost any foreign substance will lead to irritation and neutrophil recruitment. Though this may be the case, intratracheal injection of anti-TLT2 lead to the rapid and significant recruitment of neutrophils in a dose- and time-dependent manner when compared to the isotype control, and persisted for at least 24 hrs (**Fig. 3*C*, Supplemental Fig. 3**) A similar effect was observed after intraperitoneal injection of antibody, and CD11b analysis of the recruited neutrophils showed them to be in a hyperactive state when compared to FMLF recruited neutrophils (**Fig. 3*D*, and data not shown**). In light of our lab's previous findings that TLT2 ligation in addition to GPCR agonist stimulation lead to a heightened response of the neutrophil, this observation is not surprising.

Because macrophages express TLT2 and are typically present at the tested injection sites of anti-TLT2, it was of interest to determine what, if any, effect TLT2 ligation on this cell type may have. While *in vitro* anti-TLT2 treatment of residential macrophage did not elicit the upregulation of the early activation marker CD69 or the costimulatory molecules CD80 and CD86, it did prompt the secretion of several neutrophil chemoattractants such as MIP-2, KC, IL-6, and G-CSF (**Fig. 4*A*, 4*B***). While the production of IL-6, MIP-2, and KC occurred largely within the first 2 hrs following treatment, the production of G-CSF increased after this time point, possibly to maintain neutrophil efflux from bone marrow into the bloodstream (**Fig. 4*C***).

Our lab has previously shown that TLT2 ligation on neutrophils potentiated the respiratory burst, degranulation, and migration to agonists that signal through the $G_{\alpha i}$ subclass of GPCR's, and that this effect was maintained both *in vitro* and *in vivo*. Importantly, TLT2's functionality on neutrophils works through a mechanism different from the classical primer LPS, and tends to work largely in a synergistic fashion with appropriate triggers. While the ability of TLT2 to potentiate an already established neutrophil response has previously been published, we have now shown that TLT2 possess the ability to also initiate such a response, and do so without the aid of secondary, exogenous stimuli.

The results shown here establish that TLT2 ligation on cell types other than neutrophils can lead to the production of several cytokines and growth factors, most of which are prominent neutrophil chemoattractants (e.g. MIP-2, KC) or stimulants of neutrophil egress from the bone marrow (e.g. G-CSF), and that this production can rapidly lead to the recruitment of neutrophils on which TLT2 ligation can further

potentiate their activity. While we have shown that anti-TLT2 treatment on residential macrophages *in vitro* can lead to the production of the observed chemoattractants following anti-TLT2 injection into the mouse, this does not preclude other cell types from being responsible for the observed *in vivo* cytokine production. In fact, further experiments will need to be carried out to accurately determine if macrophages are one of, if not the sole, cell type responsible for the observed effect. None-the-less, anti-TLT2 treatment did not appear to lead to inflammatory outcomes *in vivo* or *in vitro*, though it did promote a rather large, but controlled, neutrophil response. It is of note that most likely anti-TLT2 moves from the injection site into circulation where it can act on recruited neutrophils to potentiate their migratory response to the anti-TLT2 induced cytokine storm, though without knowing the exact rate of antibody diffusion it is not possible to determine the relative contribution of TLT2 ligation on neutrophils to this physiological outcome. Though questions remain regarding the signaling mechanism responsible for TLT2 activity, it is now apparent that TLT2 can play a role in initiating a neutrophil response that can be augmented through its own activity, thus placing TLT2 in a potentially very prominent role within the innate immune response.

Materials and Methods

In vivo models of inflammation and cytokine analysis

C57BL/6 mice 8–10 wks of age were used in all experiments, and were housed in specific pathogen-free conditions in University of Alabama at Birmingham animal facilities. All procedures were approved by an institutional review committee.

To determine if and how anti-TLT2 altered neutrophil as well as non-neutrophil responses towards a non-specific inflammatory stimulus, intravenous (IV) injection of either PBS, isotype control antibody (control), or 100 µl of 500 µg/ml anti-TLT2 $F(ab)_2$'s preceded the addition of 20 µl of 2% croton oil to the ear by 30 min. 10 µl of 2% croton oil –acetone was applied to each side of one ear pinna while 20 µl of acetone alone was applied to the control ear, and allowed between 30 min and 24 hr before sacrifice by CO_2 inhalation. Immediately after sacrifice, 4 mm ear biopsies were taken from the center of each ear with a biopsy punch, homogenized with a Tissue Tearor homogenizer (Biospec Products; Bartlesville, OK), and the resulting lysate was analyzed for 32 different cytokines/chemokines/growth factors using the Milliplex system (catalog #: mpxmcyto-70k) (Millipore; Billerica, MA) and for Myeloperoxidase (MPO) activity with the 100 µl addition of the chromogenic substrate 3,3´,5,5´-tetramethylbenzidine (TMB) (Sigma; St. Louis, MO). The absorbance at 450 nm was measured using a Vmax plate reader (Molecular Devices, Sunnyvale, CA), and assayed with a standard curve of known concentrations of the enzyme peroxidase (HRP). Comparing this HRP standard to the MPO activity present in a defined concentration of purified neutrophils allowed for the estimation of the number of neutrophils present in each biopsy sample.

To determine the specific *in vivo* effects attributed solely to the anti-TLT2 mAbs, 10 to 100 µg of $F(ab)_2$ portions of the mAbs were injected either via 100µl intravenously (IV), 50 µl intratrachealy (IT) or 300 µl intraperitoneally (IP). Unless otherwise labeled, 50 µg of anti-TLT2 or isotype control antibodies were used in all *in vivo* experiments. Sterile PBS injections were used as a vehicle control. Mice were sacrificed by CO_2 inhalation between 30 min and 24 hr following IV, IT or IP injection, lavaged based on inflammation model and analyzed with the Milliplex system which has a detection range of 3.2 – 10,000 pg/ml. 600 µl of blood from each animal was also collected post-mortem via the left ventricle of the heart using an ultra-fine insulin syringe (1 ml, 31 gauge, 8 mm) (BD; Fanklin Lakes, NJ). The blood was allowed to coagulate at RT, centrifuged at 14,000 rpm for 10 min, and the supernatant was analyzed via the Milliplex system for the presence of cytokines and growth factors.

Neutrophil recruitment to the peritoneal cavity or the Bronchioalveolar lavage (BAL) following IP and IT injections, respectively, were determined by hemacytometer counts and flow cytometry analysis at the times labeled within the figures.

Collection and isolation of resident peritoneal macrophages

To isolate resident peritoneal macrophages, PBS lavages of the peritoneum were collected and subsequently seeded in a 25 cm^2 tissue culture flask for 2 hr in a 37°C, 5% CO_2 incubator. The media used for all *in vitro* macrophage experiments was DMEM, supplemented with pen/strep, sodium pyruvate, mercaptoethanol, L-glutamine, and 10% FBS. Following this incubation period, the media containing non-adherent cells and debris was aspirated out of the flask and the adherent macrophages were lifted and

collected by addition of .5mm EDTA – PBS, pH 8.0 for 10 min at 37°C. The cells collected were > 90% macrophages as determined by flow cytometry.

Ex vivo treatment and cytokine analysis of macrophages

$1x10^5$ macrophages per sample were seeded in a 96-well, flat-bottom microtiter plate in 200 µl of media after a 2 hr incubation at 37°C, 5% CO_2. Following adhesion, 100 µl of media containing either 10 µg/ml αTLT2 $F(ab)_2$'s, 10 µg/ml isotype control, 2 µg/ml LPS or media alone was added and the plate was subsequently incubated at 37°C, 5% CO_2 for up to 4 hr. 90 µl aliquots of the supernatants were collected at 30 min, 2 hr, and 4 hr for Milliplex analysis. Cytokine production was determined with the same method used for the *in vivo* experiments, and the hourly rate of cytokine production was determined by dividing the amount produced in the first 2 hr by the number of incubation hours (2), and the additional amount produced between incubation hours 2 – 4 and again divided by total incubation hours (2).

Analysis of macrophage activation

$1x10^5$ macrophages / 200 µl of media were seeded in a plate or rotated in a microcentrifuge tube at 37°C, 5% CO_2 for indicated times prior analysis. The cells were treated with media alone, 10 µg/ml LPS, 5 µg/ml PMA, or 10 µg/ml of the $F(ab)_2$ portion of either αTLT2 mAb 1H4 or 1C5. At the designated time, reactions were ceased by the addition and subsequent washes with ice cold FACS buffer (1X PBS, 0.01% NaN_3^+, 0.5%

FBS) at 4°C. The macrophages were than analyzed by flow cytometry for activation status and TLT2 expression.

Flow Cytometry

Neutrophils were identified using anti-CD11b-FITC (BD Pharmingen; San Diego, CA), anti-Gr-1-PE (Southern Biotech; Birmingham, AL). Peritoneal macrophage were identified based on forward and side scatter profile and the use of anti-CD11b-APC and anti-F4/80-488 (BioLegend; San Diego, CA). Macrophage activation was assayed using anti-CD80-PE-Cy5.5, anti-CD86-FITC, and anti-CD69-PB (BioLegend; San Diego, CA). B cells were identified with anti-B220-APC-Cy7 (BD Pharmingen; San Diego, CA) and T cells were identified with anti-CD4-Fitc and anti-CD8-PE (Southern Biotech; Birmingham, AL). Monocytes were identified by use of anti-CD115-APC (eBioscience; San Diego, CA). TLT2 expression was determined by the use of biotinylated anti-TLT2 mAbs and streptavidin-647 (Southern Biotech; Birmingham, AL). The anti-TLT-2 mAbs 1H4 and 1C5 were generated as described (King, 2006), and were biotinylated using EZ-Link NHS-LC-biotin (Pierce, Rockford, IL). 7-Aminoactinomycin D (7-AAD) was used as a live-dead cell discriminator (Southern Biotech; Birmingham, AL).

Cells were washed 3 times at 4°C with FACS buffer after a 15 min, 4°C antibody incubation in 96-well microtiter plates and before flow analysis. Samples were analyzed immediately after labeling on either a FACSCalibur or LSR II flow cytometer (BD Biosciences), and analyzed using FlowJo (Tree Star; Ashland Or).

Statistics

Statistical analyses for the ear inflammatory model were computed via the student two-tailed t-test. Significance values for neutrophil recruitment to the lung at 24 hr after IT antibody injection were calculated using the Mann-Whitney test. The p-value of significant differences is reported, with a p-value < 0.05 considered statistically significant.

Figure Legends

Figure 1. Intravenous injection of anti-TLT2 increases neutrophil recruitment towards a non-specific inflammatory event, and qualitatively and quantitatively alters cytokine production. 4 mm biopsies were analyzed at time points between 30 min and 24 hr after croton oil treatment for neutrophil recruitment with the largest recruitment difference between the two groups at 4 hr with a ~2.5x increase in neutrophil recruitment to the croton-inflamed ear in the anti-TLT2-treated mice **(A)**. There was a significant decrease of biopsy mass in the anti-TLT2 treated mice **(B).** Out of the 32 cytokines and factors analyzed, the anti-TLT2 altered cytokines are shown for the ear **(C)** and blood **(D)** after croton treatment, and the blood after only anti-TLT2 injections **(E).** All data represented are the average of at least five samples with mean and standard deviations shown and are representative of at least three independent experiments. (○ = control group, ■ = anti-TLT2 group) *p=0.0001, **p=0.0002

Figure 2. Injection of anti-TLT2 resulted in the rapid upregulation of cytokines and growth factors. Following IT and IP injections of anti-TLT2, the peak values of cytokine upregulation common between models were analyzed with the incubation times resulting in the highest concentrations labeled (● =30 min, ■ =2 hr, ◘ =4 hr) **(A)**. Doubling the injected dose of anti-TLT2 altered the concentration of the cytokines produced, but did not change the profile of upregulated cytokines; a representative graph is shown **(B)**. Heat maps display the panel of 32 cytokines and factors tested for the lung **(C)** and peritoneum **(D)**.

Figure 3. Analysis of the cellular infiltrate following anti-TLT2 treatment revealed neutrophils are rapidly recruited to the site of injection. Analysis of the BAL cellular infiltrate following anti-TLT2 intratracheal injection **(A)** and peritoneal lavage after intraperitoneal injection **(B)** over a 24 hr period. Neutrophils were further analyzed based on anti-TLT2 dose and time dependent recruitment into the lung **(C)** and peritoneal cavity **(D)**. All data represented are the average of at least five samples with mean and standard deviations shown and are representative of at least three independent experiments.

Figure 4. *In vitro* anti-TLT2 treatment on peritoneal macrophages prompted the production of neutrophil chemoattractants independent of secondary stimuli and without generalized activation. A 10 μg/ml dose of anti-TLT2 $F(ab)_2$'s added to $1x10^5$ *ex* vivo peritoneal macrophages / well in a 96-well plate did not lead to the upregulation of the activation marker CD69 in 1 hr or costimulatory molecules CD80 and CD86 after 24 hr as did LPS with or without anti-TLT2 **(A)**. The supernatants of these samples were analyzed for cytokine upregulation with only anti-TLT2-induced changes shown **(B)**. The relative rates of cytokine production from the *ex vivo* treated macrophages were also analyzed, and a representative graph is shown as hourly rates between 0-2 hr and 2-4 hr, as well as fold increase in production over baseline **(C)**. All data represented are the average of triplicate samples with means shown and are representative of at least three independent experiments.

Supplemental Figure 1. Intratracheal and intraperitoneal injections of anti-TLT2 lead to rapid cytokine upregulation in circulation that persisted over time. Following either intratracheal injection or intraperitoneal injection of 10 µg of anti-TLT2 $F(ab)_2$'s, blood was collected and analyzed for cytokine production along with the corresponding lavages. Out of the 32 cytokines analyzed, the common cytokines that were altered in circulation over a 24 hr period as a result of intratracheal **(A)** or intraperitoneal **(B)** injections are shown. All data represented are the average of triplicate samples with means shown and are representative of at least three independent experiments.

Supplemental Figure 2. Injection of the classical TLR4 ligand LPS resulted in the upregulation of different cytokines than anti-TLT2 injections. To compare the anti-TLT2 induced cytokine production to the cytokine response to the classical bacterial challenge LPS, lungs were lavaged and analyzed for cytokine upregulation following 50 µl IT injection of either PBS, 5 µg LPS or 50 µg anti-TLT2 $F(ab)_2$'s. More inflammatory cytokines are upregulated following LPS injection when compared to antibody. PBS did not elicit much of a reaction. Additionally, the anti-TLT2 antibody solution was tested with the limulus test, with the resulting LPS concentration below the level of detection (0.03 EU/ml).

Supplemental Figure 3. Intratracheal injection of anti-TLT2 lead to more neutrophil recruitment than isotype control after 24 hrs. Varying doses of either anti-TLT2 or isotype control were intratrachealy injected into the mouse, and the BAL was analyzed 24 hr later. Regardless of dose, anti-TLT2 lead to a higher recruitment of

neutrophils than isotype control which still persisted after 24 hrs. All data samples are shown. *****p=0.004, ******p=0.03, *******p=0.016

Acknowledgements

We would like to sincerely thank Allison Metz of the Steele lab for being instrumental in the Milliplex-dependent experiments.

References

1. Molloy, E.J., *Triggering Receptor Expressed on Myeloid Cells (TREM) family and the application of its antagonists.* Recent patents on anti-infective drug discovery, 2009. **4**(1): p. 51-6.

2. Washington, A.V., et al., *TREM-like transcript-1 protects against inflammation-associated hemorrhage by facilitating platelet aggregation in mice and humans.* The Journal of clinical investigation, 2009. **119**(6): p. 1489-501.

3. Bouchon, A., et al., *TREM-1 amplifies inflammation and is a crucial mediator of septic shock.* Nature, 2001. **410**(6832): p. 1103-7.

4. Turnbull, I.R., et al., *Cutting edge: TREM-2 attenuates macrophage activation.* Journal of immunology, 2006. **177**(6): p. 3520-4.

5. Halpert, M.M., et al., *TLT2 Potentiates Neutrophil Antibacterial Activity and Chemotaxis in Response to G Protein-Coupled Receptor-Mediated Signaling.* Journal of immunology, 2011.

6. Han, M.H., et al., *Topical application of silymarin reduces chemical-induced irritant contact dermatitis in BALB/c mice.* Int Immunopharmacol, 2007. **7**(13): p. 1651-8.

7. Lange, R.W., et al., *Antioxidants attenuate anthralin-induced skin inflammation in BALB/c mice: role of specific proinflammatory cytokines.* Journal of leukocyte biology, 1998. **64**(2): p. 170-6.

Figure 1. Intravenous injection of anti-TLT2 increases neutrophil recruitment towards a non-specific inflammatory event, and qualitatively and quantitatively alters cytokine production.

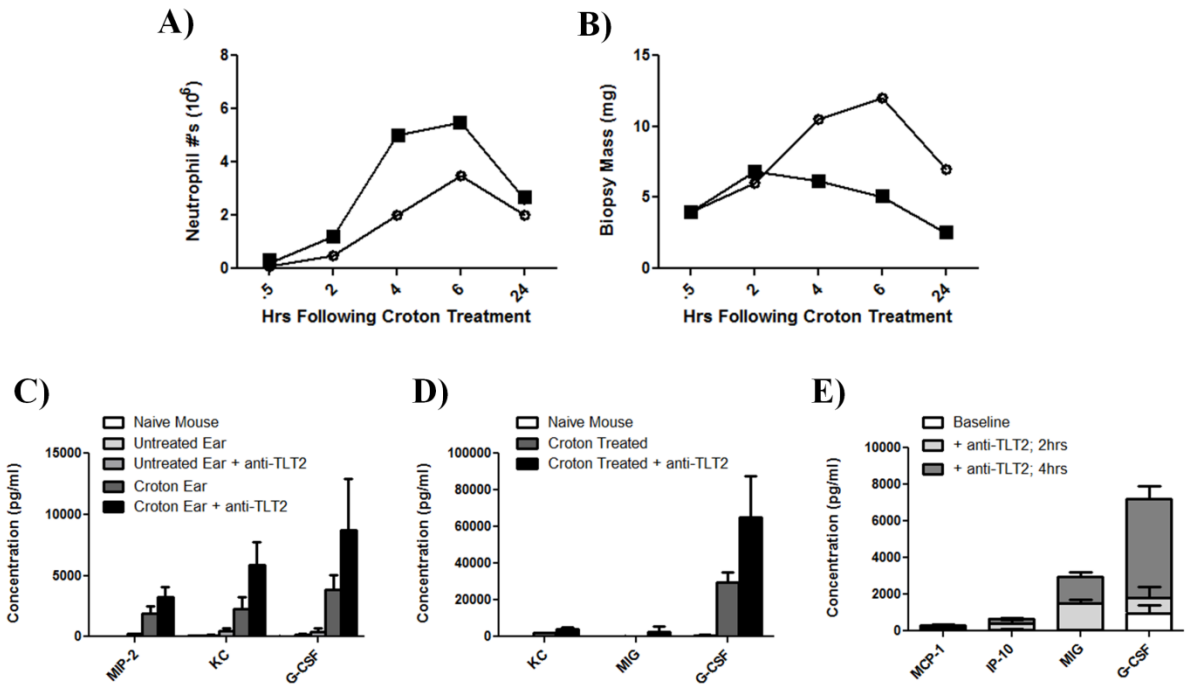


Figure 2. Injection of anti-TLT2 resulted in the rapid upregulation of cytokines and growth factors.

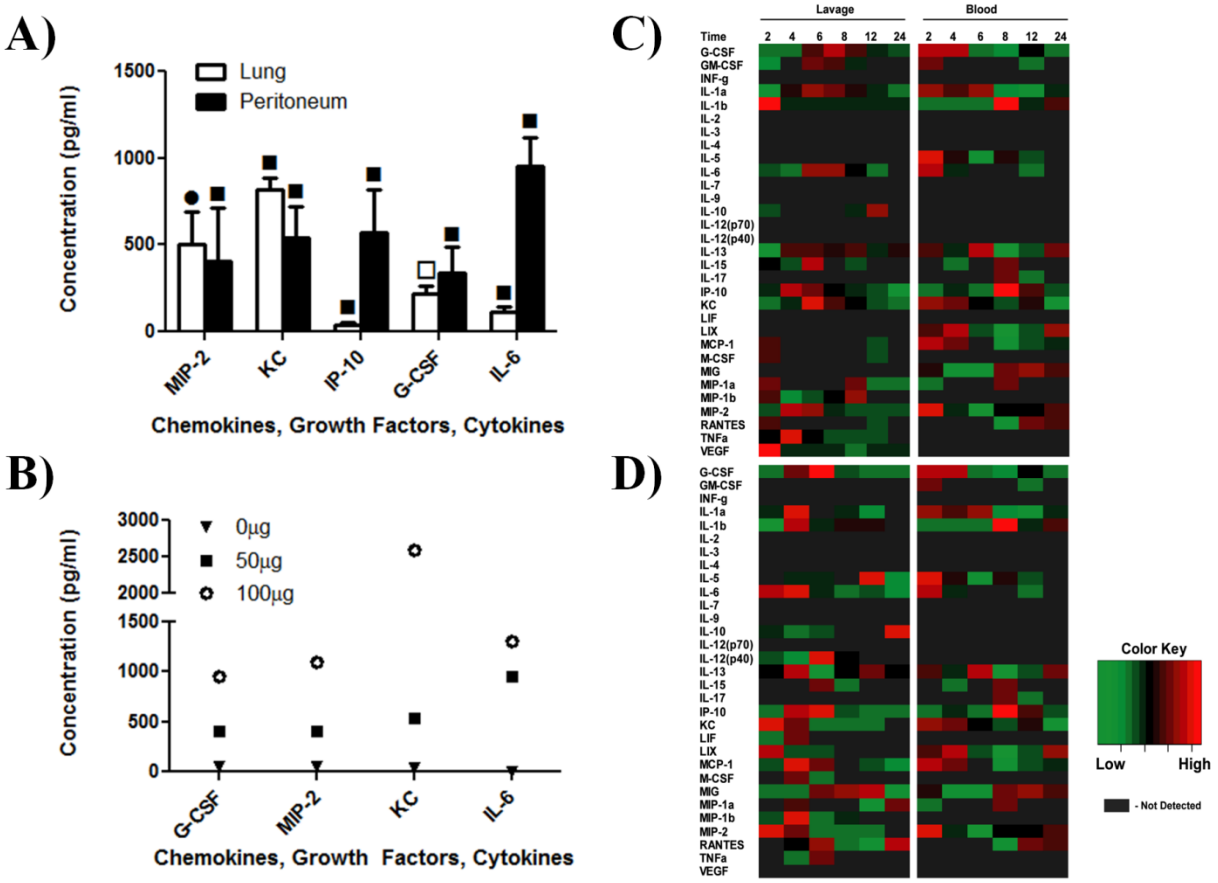


Figure 3. Analysis of the cellular infiltrate following anti-TLT2 treatment revealed neutrophils are rapidly recruited to the site of injection.

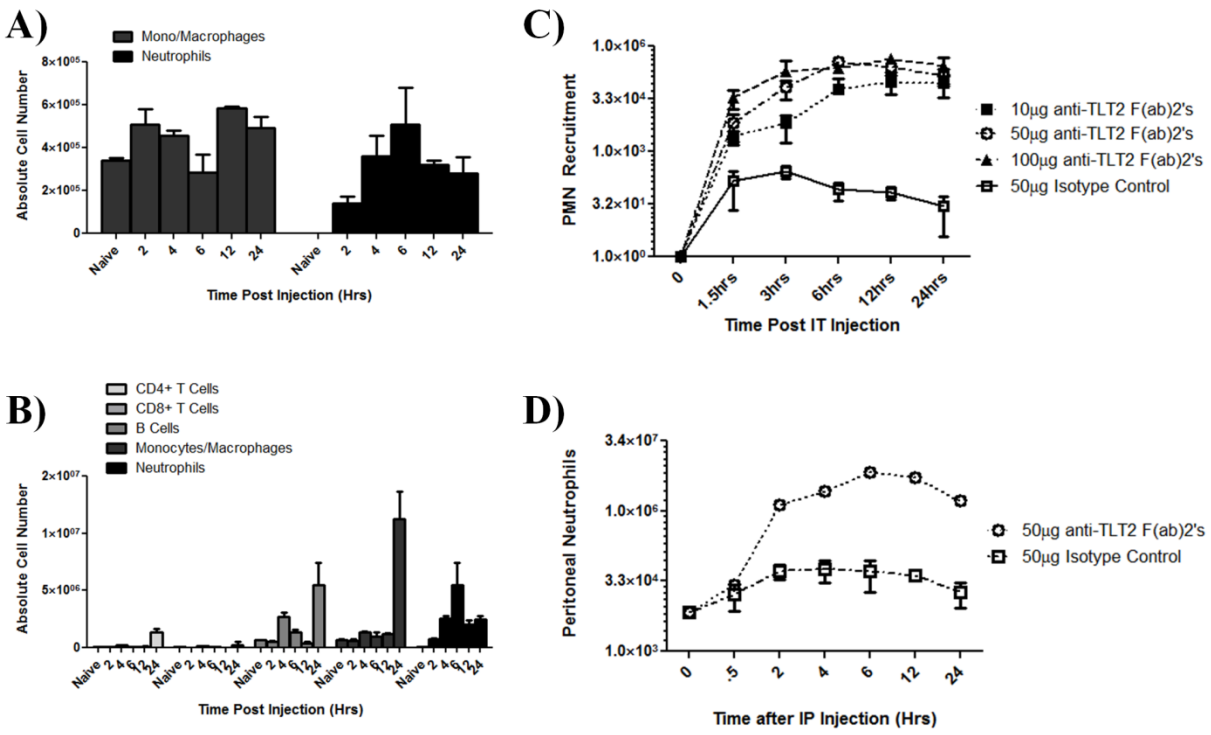
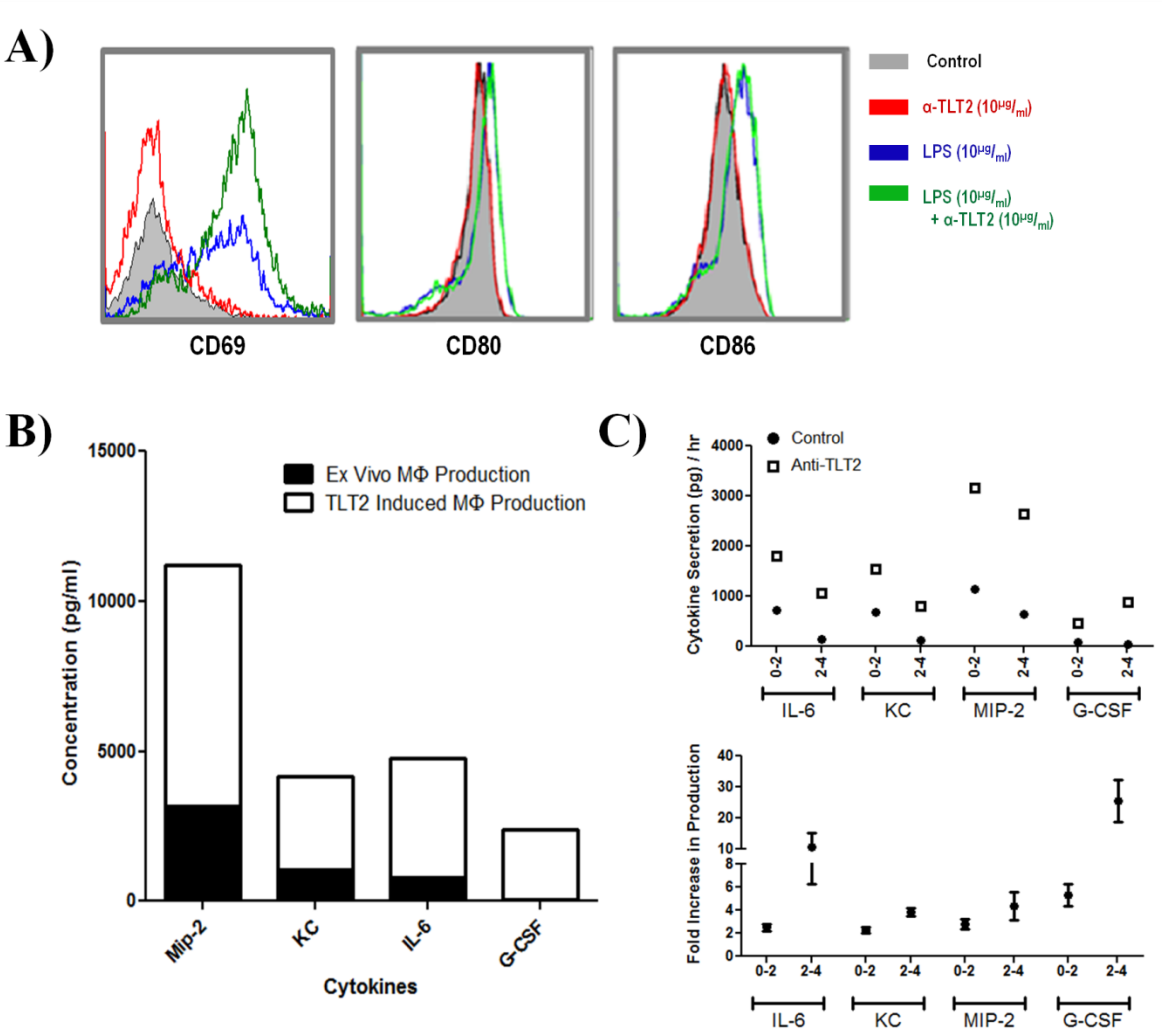
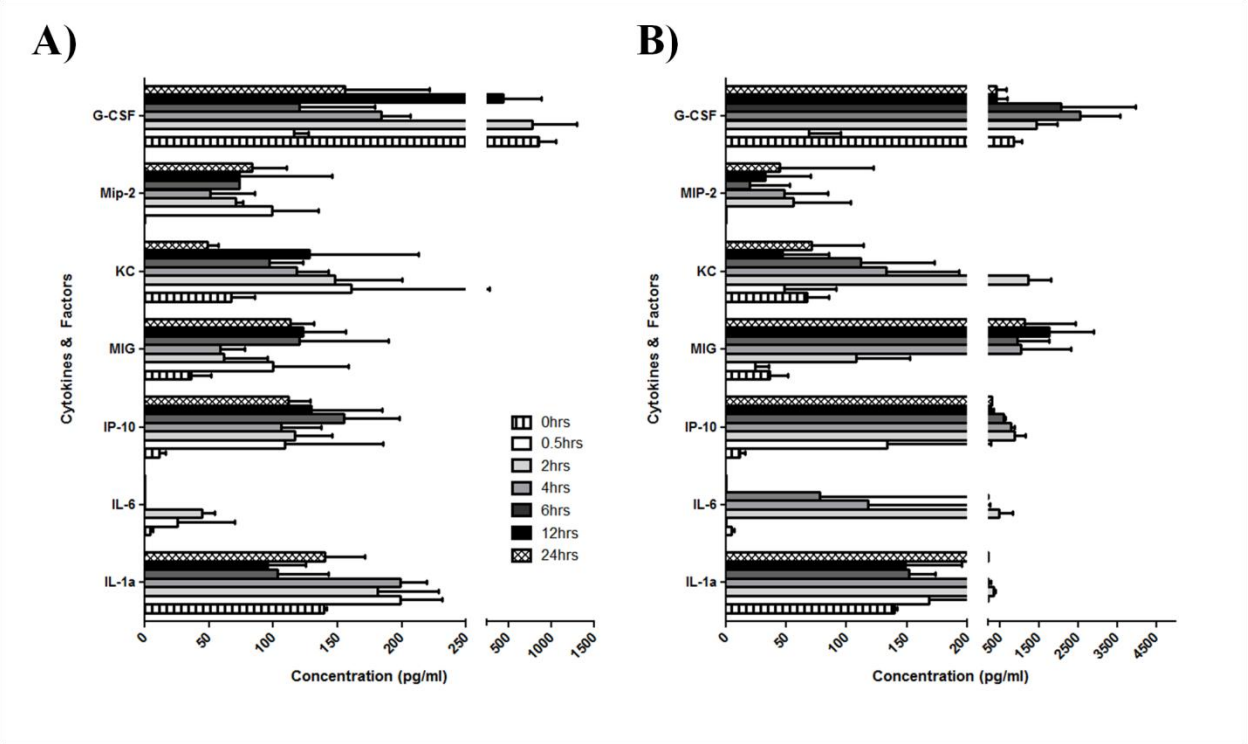


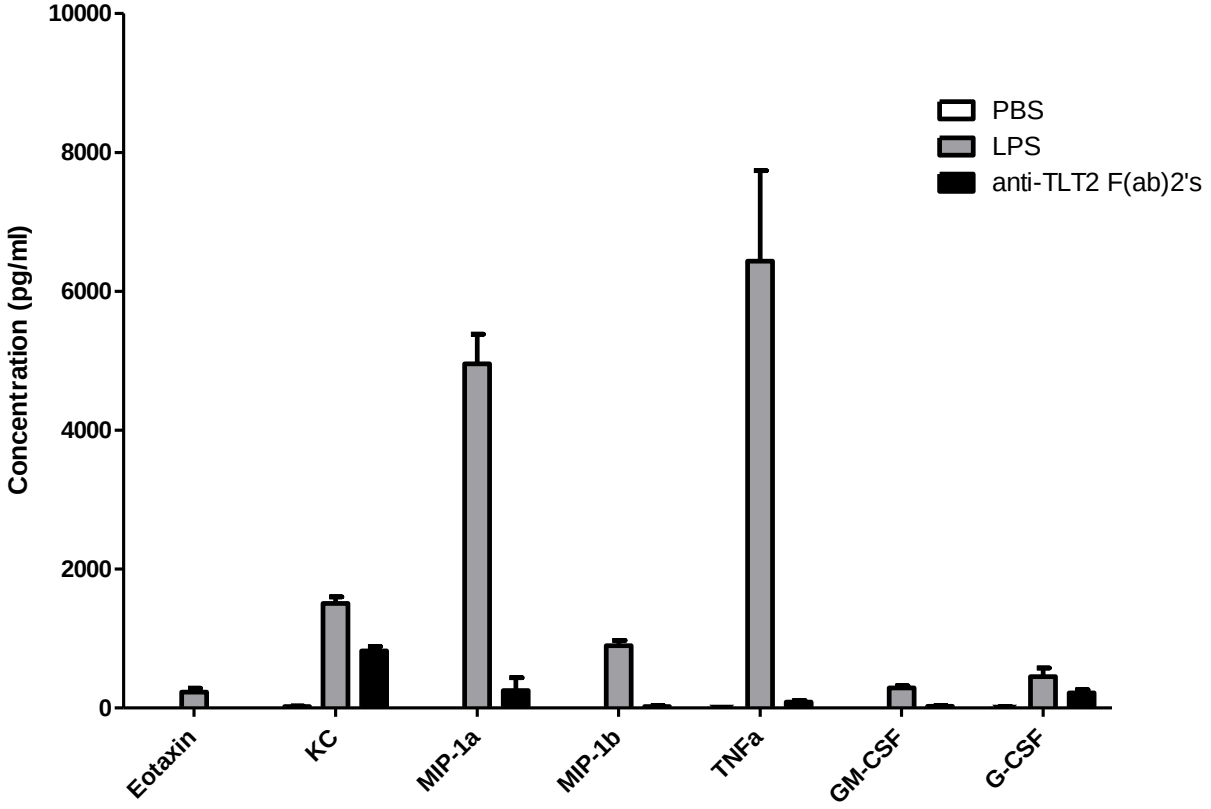
Figure 4. *In vitro* anti-TLT2 treatment on peritoneal macrophages prompted the production of neutrophil chemoattractants independent of secondary stimuli and without generalized activation.



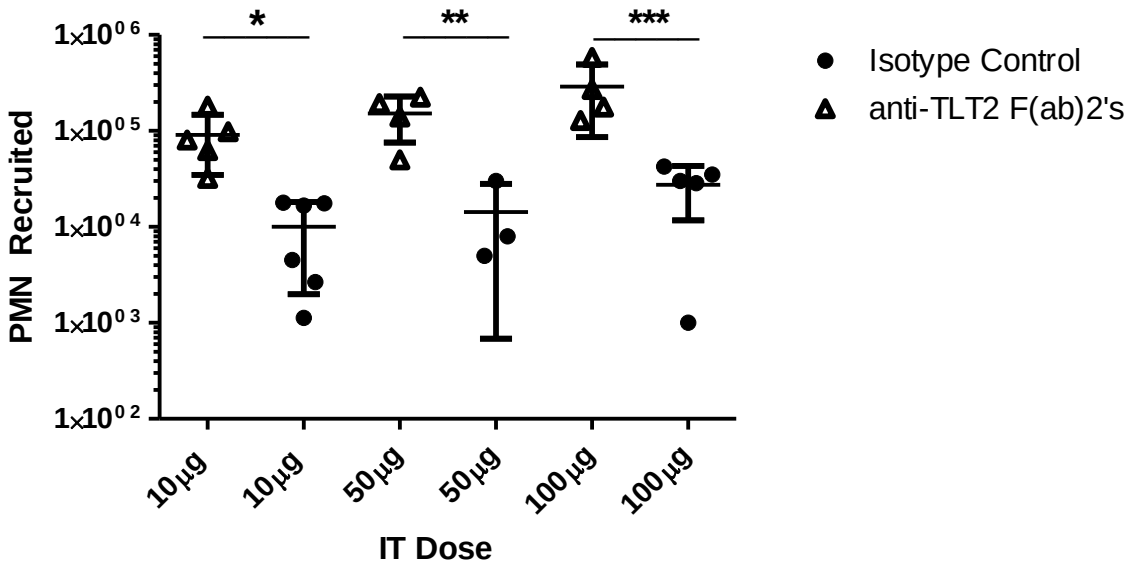
Supplemental Figure 1. Intratracheal and intraperitoneal injections of anti-TLT2 lead to rapid cytokine upregulation in circulation that persisted over time.



Supplemental Figure 2. Injection of the classical TLR4 ligand LPS resulted in the upregulation of different cytokines than anti-TLT2 injections.



Supplemental Figure 3. Intratracheal injection of anti-TLT2 lead to more neutrophil recruitment than isotype control after 24 hrs.



CONCLUSIONS

TLT-2 is a transmembrane receptor which consists of a single Ig domain, a serine-threonine rich region and a single hydrophobic membrane spanning domain, followed by a relatively short cytoplasmic tail. Although the sequence is not overly homologous with other TREM family members (≈30%), structurally they are similar Ig-like transmembrane receptors that possess the above characteristics. Within the TLT-2 cytoplasmic domain is a relatively conserved proline-rich segment, which may allow for interaction with SH3- or WW-domain containing intracellular proteins that transmit signals into the cell. It is also possible that these prolines confer a tertiary structure, which could allow the receptor to interact with proteins that contain complementary structural regions. In this way, TLT-2 differs from the other conserved family members, as they have either a phosphotyrosine-based motif (e.g. the ITIM within TLT-1), or interact with adaptor proteins such as DAP12, LAT, and LAB (e.g. TREM-1 and TREM-2) via charged transmembrane amino acids.

TLT-2 SIGNALING IN RELATION TO THE TREM FAMILY

To date, interacting effector proteins that associate with TLT-2 have not been identified, nor does the sequence of TLT-2 predict which transmembrane adaptor proteins might be involved. Downstream signaling events have not been studied as extensively as the other TREM members, and it is unknown how TLT-2 regulates innate immune cell function.

Recently in the field of TREM biology, effort has been devoted to determine how TREM-1 and TREM-2 exert opposing effects on the cell because both receptors interact with and signal through DAP12, a transmembrane adaptor protein involved in mediating the inflammatory response. It is still not known whether TREM-1 and TREM-2 are actually able to drive distinct signals through DAP12, but it has now become clear that other membrane proximal players are critical for appropriate TREM signaling, and that this is where the two receptors may diverge in terms of the signals they transduce. For example, TREM-1 recruits LAT, an adaptor protein able to activate PLCγ, which can lead to large increases in calcium mobilization and enhancement of cellular activity. On the other hand, TREM-2 recruits LAB, a LAT homologue that is unable to activate PLCγ. In contrast, LAB recruits c-Cbl, an E3-Ubiquitin ligase capable of targeting several membrane proximal players for degradation, including two predominant TREM-2 effectors, Syk and LAB. Perhaps more importantly though is that c-Cbl has now been demonstrated to ubiquitinate membrane proximal MyD88, an extremely important adaptor protein in TLR signaling. Given that neither LAT nor LAB are effectively recruited in the absence of DAP12, it is possible that in this way TREM-1 and TREM-2 do in fact both signal via DAP12, but that their divergent roles are dependent on other effector proteins recruited.

Based on the structural characteristics of the TLT-2 cytoplasmic domain, the mostly likely candidates for TLT-2 interacting proteins would be effector proteins containing an SH3- or WW-domain that could interact with the proline rich motifs in the cytoplasmic tail. Although TREM-1 requires DAP12 to recruit several SH3-domain containing proteins, such as Grb2 and GADS (both of which can recruit LAT/LAB), it is

possible that TLT-2 may recruit these proteins or similar effectors without the aid of transmembrane adaptor proteins. Further intracellular studies will be required to determine which effector proteins are recruited and activated in response to TLT-2 ligation.

TLT-2 LIGAND(S) IN RELATION TO THE TREM FAMILY

A definitive ligand for TLT-2, endogenous or exogenous, has yet to be identified, and controversy in this field may further delay actual answers. Recent publications have indicated that B7-H3 is a ligand for TLT-2 expressed on $CD8^+$ T cells, which functions as a co-stimulatory molecule in concert with TCR ligation. However, our lab has failed to detect significant TLT-2 on T cells, regardless of their activation state, and the conclusion that TLT-2 functions as a co-stimulatory molecule on T cells that binds to a B7 family member does not appear to agree with the functional role ascribed to TLT-2 by our laboratory. Specifically, we have shown that TLT-2 potentiates signals delivered via the $G_{\alpha i}$ subtype of GPCRs (such as the CXCR2 receptor on neutrophils), but does not enhance phosphotyrosine-based signaling (such as through the GM-CSF or FcR receptors). Given that TCR signaling is mediated by tyrosine phosphorylation and not activation of G proteins, this would indicate that TLT-2 functions via a completely different mechanism on T cells than other cell types, which is extremely unlikely, especially given that T cells do not express TLT-2.

Nevertheless, until a more convincing TLT-2 ligand is successfully identified, this controversy will remain. Several putative ligands have been suggested for other TREM receptors, although conclusive identification of physiological ligands has yet to be

reported. It was recently reported that there is a functional TREM-1 ligand expressed on platelets, and that although this TREM-1/TREM-1L interaction is not necessary for platelet-neutrophil aggregation, it is able to enhance the neutrophil inflammatory response to LPS. TREM-2 has several ligands identified through observational analysis, including exogenous anionic TLR agonists. Endogenously, TREM-2:Fc fusion proteins have been able to stain neurons undergoing apoptosis (displaying a potential phagocytic role for TREM-2 on microglia), as well as on macrophages themselves, indicating that macrophages may express both the TREM-2 receptor and ligand, potentially allowing for a continual *cis* interaction. Additionally, it has been shown that TREM-2 can form a complex with Plexin-A1 on dendritic cells, providing a connection between the Plexin-A1 ligand, Semaphorin 6D (Sema6D), to the ITAM containing intracellular adaptor protein DAP12 through TREM-2 (Takegahara et al., 2006). Although Sema6D is not specifically a TREM-2 ligand, in this way it is able to send signals via TREM-2, making it a TREM-2 ligand by complex association.

It is not known whether any other Plexins associate with additional members of the TREM family, however given that TLT-2 does not have an apparent conserved signaling motif, it is plausible that perhaps TLT-2 signals much the same way as TREM-2 does via Plexin-A1. Whereas Plexin-A1 recruits TREM-2 as a signaling mediator, TLT-2 may recruit an as of yet unidentified membrane-bound protein to facilitate its signaling processes. This event may take place in lipid rafts, as colocalization of transmembrane proteins following stimulation often takes place in these regions which are highly enriched in signaling effectors and are thus conducive to complex formation. Upon stimulation, GPCRs, such as CXCR1 and CXCR2 on neutrophils, mobilize to lipid

rafts to limit cross-talk and enhance intracellular signaling cascades (Rose et al., 2004). It has been proposed that this occurs predominately at the leading edge of the migrating granulocyte, and that this may be necessary to mediate the appropriate cellular reaction to environmental cues (Manes and Viola, 2006). In light of the fact that TLT-2 appears to potentiate the response to GPCR signaling, it would be interesting to determine if TLT-2 colocalizes with GPCRs in lipid rafts in response to stimulation, as this may facilitate the ability of TLT-2 to enhance GPCR agonist-dependent signaling. Accumulating in lipid rafts would not be a new feature to the TREM family, as TREM-1 has been shown to move to lipid rafts on neutrophils to interact with TLR4 after either anti-TREM-1 or LPS treatment.

Although the TLT-2 ligand has yet to be identified, our data indicate that TLT-2 ligation alters neutrophil function only in concert with GPCR stimulation, specifically of the $G_{\alpha i}$ subclass. All of the GPCR agonists tested in our assays interact with receptors of this superfamily (e.g. CXCR2, C5aR, FPR), raising the question of whether TLT-2 only synergizes with this class or with the GPCR receptors that associate with other subclasses of G-proteins. Understanding whether the TLT-2 synergistic effect may be restricted would provide insight into how TLT-2 signals to the cell and works in concert with GPCR agonists. Although TLT-2 has been shown to synergize with $G_{\alpha i}$ chemotactic receptors to potentiate neutrophil migration in response to chemotactic agonists, it will be interesting to determine if TLT-2 also synergizes with the A_{2a} adenosine receptor on neutrophils, a $G_{\alpha s}$ GPCR that leads to increased intracellular concentrations of cAMP, phosphorylated PKA, and rapid clearance of cytosolic calcium that has been released from intracellular stores following cellular stimulation. This particular GPCR responds to

adenosine in an inhibitory manner on neutrophils, leading to decreased adhesion, degranulation, superoxide production, and phagocytosis (Chen et al., 2009; Cronstein, 2004). If TLT-2 not only potentiates the response to chemotactic receptors, but to other classes of GPCRs, then one would expect to observe TLT-2 potentiate the inhibitory response to adenosine, thereby effecting more potent inhibition of neutrophil function. This would indicate that TLT-2 regulates a proximal event associated with multiple types of GPCRs, and would demonstrate a ubiquitous action of TLT-2. If TLT-2 only interacts with $G_{\alpha i}$ receptors, than one would expect to observe that TLT-2 ligation has no effect on the response of neutrophils to adenosine. If TLT-2 actually works via a pathway independent of GPCR signaling, but the outcome of such signaling leads to a pro-stimulatory outcome, which only appears to synergize with $G_{\alpha i}$ signals, then it is possible that TLT-2 ligation will counteract the adenosine-dependent signals. Additionally, it has occasionally been reported that $G_{\alpha s}$ signals, which lead to an increase in intracellular cAMP can have a pro-stimulatory effect on neutrophils, although the mechanistic specifics are unknown. For example, the Prostaglandin E2 (PGE2) receptor couples to the $G_{\alpha s}$ protein, but when stimulated on neutrophils results in an increase in IL-6 secretion (Nagasawa et al., 2005; Sugimoto et al., 2005). Again, it would be fascinating to determine if TLT-2 has any effect on this pathway, which involves activation of the inhibitory $G_{\alpha s}$ protein as well as the production of IL-6. Understanding the potential role of TLT-2 in these pathways will help to determine if the fundamental function of TLT-2 is to interact with a specific GPCR subclass leading to selected functional outcomes. Finally, there are also additional classes of GPCRs that need to be studied under the same conditions, such as $G_{\alpha q}$ and $G_{\alpha 12/13}$.

The use of TLT-2:Fc fusion proteins has provided preliminary histological and functional evidence supporting the possibility that an endogenous TLT-2 ligand exists. Staining of sections from croton oil-treated mouse ears as well as the omentum of the inflamed peritoneum with TLT-2:Fc fusion protein was intensified compared to wild type control tissue sections (M. Halpert, data not shown), indicating that the endogenous ligand is not only present, but may be inducibly upregulated by inflammatory events. However, additional data are required to confirm this observation.

Injection of the recombinant TLT-2:Fc fusion protein into either the lung or peritoneal cavity elicited a similar cytokine storm and cellular infiltrate to injection of the anti-TLT-2 mAbs (M. Halpert, data not shown), and leads to the hypothesis that the TLT-2 ligand can serve as a counter-receptor promoting a feed-forward loop that would elicit the production of factors that recruit additional neutrophils. However, if this is the case then one might predict that it could lead to excessive recruitment and inflammation which may lead to tissue damage. Indeed, this may be a factor that is responsible for disease pathology resulting in excessive neutrophil activation associated with diseases, such as Cystic Fibrosis (CF) and Chronic Obstructive Pulmonary Disease (COPD) (Hartl et al., 2007; Hartl et al., 2008; Nadel, 2000). This raises questions as to what might be the switch or negative regulator that attenuates this feed-forward loop. The ability of neutrophils to internalize the TLT-2 receptor in response to prolonged inflammatory conditions may be critical to shutting off this potentially dangerous cycle. By removing the receptor from the surface thereby abrogating the interaction between TLT-2 and its ligand, TLT-2-dependent signals would be attenuated. It has not been shown whether or not surface expression of the ligand is regulated, although internalization or shedding of

the ligand could also constitute a possible mechanism that negatively regulates TLT-2-dependent signaling.

TLT-2 REGULATES NEUTROPHIL MIGRATION AND ANTI-MICROBIAL FUNCTIONS

We have shown that when ligated on neutrophils, TLT-2 synergistically enhances a wide range of anti-microbial functions, including the respiratory burst, degranulation, and migration when in the presence of several GPCR agonists (e.g. MIP-2, KC, IL-8, C5a, and FMLP). Importantly, the nearly 2-fold increase in migration was observed both *in vitro* as well as in two distinctly different *in vivo* models. Without additional stimuli, TLT-2 ligation did not potentiate any of the measured outcomes, and did not alter cell function in response to signals that were mediated by the GM-CSF receptor, Fc receptor, or TLR4. Therefore, evidence suggests that TLT-2 works to enhance signals derived through GPCRs, perhaps specific to the $G_{\alpha i}$ chemotactic receptor subclass, although the mechanism by which it does so is still unknown.

TLT-2 is the only TREM member other than TREM-1 to be expressed by neutrophils, and performs a function not originally ascribed to the TREM family. To date, evidence has not been reported functionally linking any TREM receptor with GPCRs. Nevertheless, TREM-1 has been shown to enhance PRR-mediated signals on neutrophils, potentiating the neutrophil response to bacterial challenge. The chemotactic receptors within the GPCR family make up a relatively large percentage of the receptors expressed on neutrophils, as migration from the bone marrow to the blood and then to sites of infection is one of the earliest and most critical functions a neutrophil performs (Eash et al., 2010). If this ability is absent or impaired, the neutrophil response is hampered, which

confers an advantage to the invading pathogen. Relatively few transmembrane receptors are known that enhance the response to GPCR-derived signals, and this therefore puts TLT-2 and the TREM family into a potentially very important position with respect to host defense. If TREM-1 is responsible for enhancing the neutrophil's response to bacterial challenge, it appears that TLT-2 is important for potentiating the ability of neutrophils to migrate to the site of infection and inflammation. In this way, TLT-2 and TREM-1 work together to provide maximal neutrophil function, and demonstrates that the TREM family are potent modulators of innate immunity.

ADDITIONAL NEUTROPHIL MICROBICIDAL ACTIVITY THAT TLT-2 MAY MODULATE

Although we have shown that TLT-2 potentiates the respiratory burst, degranulation, and migration, this does not preclude it from potentially enhancing other cellular functions typically associated with neutrophils. For example, it has recently become a focal point in neutrophil research to determine how Neutrophil Extracellular Traps (NETs) work, and the specific conditions that stimulate their release. Originally viewed as a suicidal function of the neutrophil, it has been reported that neutrophils can expel mitochondrial DNA or even their own chromosomal DNA, and yet retain anti-microbial function (Brinkmann et al., 2004; Buchanan et al., 2006; Fuchs et al., 2007). Whether or not TLT-2 can potentiate the release of NETs is unknown, nor is it known if TLT-2 possesses the ability to direct NET formation using a specific DNA source.

Neutrophils are also professional phagocytic cells, and although we have demonstrated that TLT-2 does not potentiate the phagocytic response to opsonized polystyrene beads, we have not conclusively shown whether or not TLT-2 has any effect

whatsoever on phagocytic function. Neutrophils are able to phagocytose surrounding bacteria via a number of different mechanisms, and it is possible that in the same way TLT-2 primes the neutrophil for an FMLP-induced respiratory burst, one might observe priming of the FMLP-induced phagocytic response.

It has also been shown that TLT-2 in concert with GPCR agonists will enhance degranulation, as determined by analysis of CD11b expression. However, this is a relatively non-specific study given the fact that neutrophils degranulate in a step-wise fashion as opposed to releasing every type of granule simultaneously. Therefore, one could examine in much more detail the role of TLT-2 in mediating selective degranulation of neutrophils. We propose that TLT-2 ligation primes the neutrophil to degranulate at a faster rate in response to GPCR agonists such as FMLP and C5a, but this does not specifically address whether TLT-2 potentiates the degranulation of specific granule subsets. For example, TLT-2 may only potentiate the mobilization of tertiary (gelatinase) and secondary (lactoferrin) granules without affecting mobilization of the primary, azurophilic granules (myeloperoxidase). If this is the case, one would most likely still observe an enhancement in CD11b expression (given that all types of granules appear to contain this integrin), but under these circumstances, TLT-2 ligation would not enhance release of the granule sets carrying the most potent antimicrobial weapons (e.g. myeloperoxidase, bactericidal/permeability increasing protein, defensins, elastase, and cathepsin G). If this were the case, this would continue to point to TLT-2 being more heavily involved in regulation of migration and extravasation compared to other neutrophil bactericidal actions. Given that the granule subsets can be discriminated by specific identification markers, it is possible to assay whether or not TLT-2 enhances the

mobilization of only certain granules, or if it simply promotes increased degranulation in a more general sense.

In addition to the ability of neutrophils to release factors via degranulation they also posses the ability to secrete cytokines. Originally thought to be limited to secretion of IL-8 (the predominant neutrophil chemoattractant; murine homologue is KC), neutrophils have now been shown to secrete several chemokines and cytokines, including MIP-1, ENA-78, IP-10, MIG, and IL-12, and the list continues to grow. Because TLT-2 ligation was sufficient to drive macrophage-dependent production of several cytokines and growth factors, it is of interest to determine if TLT ligation, alone or in conjunction with GPCR agonists, drives neutrophils to secrete chemokines or cytokines. These studies will facilitate efforts to elucidate the full spectrum of processes that TLT-2 effects in neutrophils, and what long term effects TLT-2 ligation on neutrophils may have on the host.

Finally, despite the fact that we have shown that TLT-2 in concert with GPCR agonists potentiates migration, degranulation, and the respiratory burst response, it has not actually been shown whether or not TLT-2 ligation enhances the overall anti-bacterial activity of the neutrophil. As neutrophils are primarily involved in the clearing and destruction of invading pathogens, this particular aspect is paramount to understanding the role of TLT-2 in neutrophil biology. Because neutrophil recruitment *in vivo* is enhanced in response to anti-TLT-2 mAb treatment, it is logical that the increased presence of neutrophils at sites of inflammation would result in a more rapid clearance of the bacterial invader. It is not known, however, whether or not this was due to potentiation of neutrophil function, or due to increased recruitment of cells. In either case,

it would be of interest to determine if administration of anti-TLT-2 mAb *in vivo* would lead to a more rapid recovery from bacterial challenge. It is also possible that the excessive, prolonged increase in neutrophil recruitment leads to severe tissue damage as seen in Cystic Fibrosis patients, and this may ultimately confound the *in vivo* results. Another potential caveat to this experimental system will be to find a suitable bacterial challenge model that does not by itself elicit the full neutrophil response. If the neutrophil response is maximally stimulated by the pathogenic threat, it will be difficult to determine if TLT-2 ligation makes any difference under these conditions. Therefore, perhaps the best way to answer this particular question is to focus on *in vitro* killing assays that eliminate several of these possible confounders. Determining whether or not TLT-2 ligation actually has an effect on the ability of neutrophils to kill bacteria is critical and needs to be addressed to fully understand the role of TLT-2 on neutrophils.

POTENTIAL ALTERNATIVE ROLES THAT TLT-2 MAY SERVE WITHIN NEUTROPHIL BIOLOGY

Given that TLT-2 appears to interact with GPCRs, a family of receptors involved in more than just anti-bacterial function, such as development and homeostatic control, it is possible that TLT-2 exerts broader, more far reaching effects not yet examined. For example, the use of $CXCR4^{-/-}$ mice demonstrated that CXCR4 is necessary to retain granulocytes in the bone marrow, whereas conversely CXCR2 is required for proper efflux into the bloodstream. Stromal and endothelial cells express MIP-2 and KC, which interact with CXCR2 and direct neutrophils to emigrate out of the bone marrow and into the bloodstream to maintain normal homeostatic neutrophil surveillance in the periphery. Evidence suggests that inflammatory events which lead to the production of G-CSF

ultimately lead to an increase in MIP-2 and KC production within the bone marrow, which results in more rapid neutrophil mobilization into the blood. Given our recent findings related to TLT-2, it is possible that TLT-2 knockout mice may exhibit impaired neutrophil efflux out of the bone marrow both under homeostatic and inflammatory conditions.

TLT-2 AND NEUTROPHILS; FUTURE DIRECTIONS

We have successfully demonstrated a functional role for TLT-2 on neutrophils, in which TLT-2 ligation predominately potentiates the neutrophil response to GPCR agonists. These findings provide a link between the TREM family of innate modulators and the $G_{\alpha i}$ subclass of receptors within the GPCR superfamily. However, we have not elucidated the molecular mechanism by which TLT-2 mediates this effect with respect to how TLT-2 transmits signals into the cell. Determining the signaling pathway that TLT-2 utilizes will provide substantial information that will aid in understanding TLT-2 biology, as well as likely provide intracellular evidence that TLT-2 converges with GPCR signals.

Based on the finding that TLT-2 ligation does not enhance neutrophil function in the absence of a secondary stimulus, as well as the fact that TLT-2 ligation does not shift the kinetics or threshold of sensitivity in the way classical primers typically do, it would seem unlikely that TLT-2 ligation leads to the phosphorylation and stimulation of an activating kinase. Typically, kinases act as “on” switches for intracellular signaling whereas phosphatases function as the “off” switches. If TLT-2 ligation were directly activating a kinase, we would expect to see a more rapid response towards the secondary

stimulus. Because that is not observed, another way to explain the enhanced effect is that TLT-2 ligation delays attenuation of GPCR-mediated signaling.

Based on the finding that TLT-2 enhances the response to GPCR-mediated activation, there are important phosphatases which attenuate GPCR signaling that can be studied in connection with TLT-2. Phosphatase and tensin homologue deleted on chromosome 10 (PTEN) is one of the major negative regulators of GPCR activity, as it tends to operate proximal to the membrane. Activation of the heterotrimeric guanine nucleotide-binding regulatory protein (G-protein) leads to the activation of PI3K, which leads to the synthesis and localization of phosphatidylinositol (3,4,5)-trisphosphate (PIP3) at the leading edge of the activated neutrophil. PIP3 recruits proteins containing plextrin homology (PH) domains to the membrane where they aid in polarization, migration of the neutrophil, as well as superoxide generation. PTEN is a phosphatase that exerts its effect by dephosphorylating PIP3 to PIP2 and subsequently shuts off signals downstream of the activated GPCR, in part by reducing AKT phosphorylation (which has a PH domain specific for PIP_3) and actin polymerization. As would be expected, neutrophils from a myeloid-specific $PTEN^{-/-}$ mouse exhibited enhanced responsiveness towards chemoattractants, such as increased phosphorylation levels of AKT, more actin polymerization, augmented migration in a transwell assay, and increased generation of superoxide. When PTEN is phosphorylated on its C-terminus, it assumes a closed conformation and cannot bind to the plasma membrane or impose its activity on PIP_3. After the tail of PTEN itself is dephosphorylated, it can negatively regulate PI3K activity through its phosphatase activity on PIP_3. Given the molecular mechanism that regulates PTEN and the role that PTEN plays in the neutrophils after GPCR activation, it is

possible that TLT-2 ligation may lead to the phosphorylation of PTEN or a delay in the dephosphorylation of the PTEN tail, either of which would inhibit this negative regulator of GPCR signaling and increase the magnitude of the cellular response. If true, it would also be interesting to determine if TLT-2 ligation exerts this action on PTEN after cellular stimulation by GPCR agonists, or if it occurs as soon as TLT-2 signals are transmitted. If the latter were the case, then TLT-2 ligation alone could in fact have downstream effects without secondary stimuli, but the outcome would only be manifest in the presence of GPCR agonists.

PTEN activity can also be controlled by altering the subcellular localization of this phosphatase; a mechanism that may be equally important for controlling the activation status of PTEN. PI3K, activated in chemoattractant-stimulated neutrophils, is found localized at the leading edge where it synthesizes PIP3 and promotes actin polymerization and movement towards the direction of the stimulus. At the same time, PTEN is restrained to the uropod (the back of the migrating neutrophil). This reciprocal front/back localization of PI3K and PTEN ensure that PIP3 is confined to the leading edge of the cell, and is necessary for maximal neutrophil migratory efficiency. If TLT-2 ligation ultimately alters this intricate subcellular spatial relationship between PI3K and PTEN, it is possible that this could lead to the physiological outcome observed. Although it is not known how TLT-2 could promote this effect, it is possible that it could be through the control of RhoA and/or Cdc42, members of the Rho family of small GTPases. It has been shown that PTEN distribution in neutrophils is dependent on several key residues within the phosphatase that allow it to interact with GTPases, and that blocking Cdc42 or RhoA activity impairs proper alignment of PTEN in the activated cell.

Under normal conditions, PTEN colocalizes with RhoA at the posterior, whereas Cdc42 is active at the leading edge. Interfering with the normal function of either RhoA or Cdc42 could lead to impaired neutrophil migration due to alterations in the distribution of PTEN. Additionally, the effects of RhoA and Cdc42 on PTEN localization could be recapitulated in human embryonic kidney cells (HEKs), providing evidence that these small GTPases are sufficient for proper PTEN localization within the migrating cell. Therefore, TLT-2 could exert its effect on either of these GTPases, giving it indirect control of PTEN activity.

Alternatively, hematopoietic cell-specific SHIP1 has been found to be responsible for a large percentage of the phosphatase activity exerted on PIP3 specifically in neutrophils following cellular stimulation, and in fact dephosphorylates PIP3 to the phosphatidylinositol (3,4)-bisphosphate form of PIP2. This, like phosphatidylinositol (4,5)-bisphosphate, leads to the ultimate attenuation of AKT activity and negative regulation of GPCR signaling in the neutrophil. Indeed, $SHIP1^{-/-}$ neutrophils exhibited increased levels of PIP3, and importantly exhibited severe defects in polarization and cell motility. Furthermore, it has been demonstrated that although $PTEN^{-/-}$ neutrophils display a higher migratory speed, they are not as directed in terms of their pseudopodia. Therefore, the defect in directional sensing and promotion of migratory speed largely canceled each other out as shown in a single-cell micropipette assay, indicating that perhaps PTEN is not the phosphatase most responsible for regulating neutrophil chemotaxis. It has been proposed recently that directional sensing in neutrophils might be regulated by SHIP1, although further studies are required to address this issue. Given the

role that SHIP1 plays in neutrophils, it is possible that TLT-2 ligation interferes with this phosphatase rather than PTEN, conferring the same outcome as previously mentioned.

It is also feasible that TLT-2 has no direct or indirect interaction with PTEN (or PTEN players such as RhoA or Cdc42), SHIP1, or any other phosphatase, but only exerts an effect on other small GTPases that have critical roles in neutrophil biology. For example, GPCR stimulation leads to PI3K activation, which leads to the conversion of Rac2-GDP to the active form, Rac2-GTP. PI3K deficient neutrophils display almost no degranulation, superoxide production, and chemotaxis in response to GPCR agonists. Rac2 deficient mice display an identical phenotype, highlighting the importance of this GTPase for neutrophil activity. Knockout of P-Rex1, a guanine nucleotide exchange factor (GEF) for Rac2, resulted in impaired *in vivo* recruitment of neutrophils, as well as a severely depressed respiratory burst response. However, migratory ability and degranulation were largely unaffected, indicating that P-Rex1 is important but not absolutely required for neutrophil function as Rac2 appears to be. In light of the phenotypic outcome in Rac2 knockouts, this is another possible convergent point for TLT-2 and GPCR signaling. If TLT-2 slows the rate of hydrolysis from Rac2-GTP to the inactive form, Rac2-GDP, it is possible that we would observe a heightened response to GPCR agonists. Conversely, if TLT-2 signaling leads to higher GEF activity, such as P-Rex1, then the rate of Rac2-GDP to Rac2-GTP may be increased, leading to enhanced responsiveness.

In relation to intracellular signaling, it also needs to be determined if TLT-2 has any effect on calcium mobilization because mobilization of intracellular stores of calcium is one of the earliest events to take place in activated neutrophils in response to GPCR

agonists. Calcium acts as a second messenger, and is involved in a variety of neutrophil effector functions, such as degranulation and phagocytosis. Given how important and involved calcium is for neutrophil function, it is possible that this is where TLT-2 converges with other external stimuli. Understanding if TLT-2 ligation has any effect on the concentration or rate of calcium mobilization may help to determine how TLT-2 signals to the cell and how TLT-2 potentiates functional responses following ligation.

It has also been shown that neutrophils upregulate TLT-2 expression on their surface after stimulation, and this most certainly is via the mobilization of neutrophil granules. Unlike other cell types, neutrophils typically do not synthesize proteins as needed, but already have them prepackaged in granules to allow for rapid response. As discussed earlier, neutrophils have three different sets of granules (four if one considers the secretory vesicles, which are the first to be mobilized and are mostly used to add membrane to the leading edge of the responding neutrophil), and it is currently unknown which set(s) of granules store TLT-2. It is possible that, like CD11b, TLT-2 is in all granule subsets, although given the relatively homogenous upregulation of TLT-2 observed; it may be more likely that TLT-2 is stored within only one subset of granules. Because granule subsets are often specifically mobilized after certain stimuli, understanding how TLT-2 is stored may provide insight into the types of external stimuli that ultimately lead to TLT-2 upregulation. Additionally, because TLT-2 can be internalized, it would be interesting to elucidate if the TLT-2 receptor is degraded or recycled to the surface after this event.

TLT-2 DIRECTS THE MACROPHAGE TO RECRUIT NEUTROPHILS

Our lab has shown that macrophages also express TLT-2 and upregulate its expression following thioglycollate treatment. We have also now shown that TLT-2 ligation on resident macrophages stimulates the *ex vivo* secretion of a myriad of cytokines and growth factors, including MIP-2, KC, IL-6, and G-CSF (all of which predominately exert their effects on neutrophils). Importantly, TLT-2 ligation induces the production of soluble factors without stimulating the macrophage in a general sense, as was indicated by the absence of CD69, CD80, and CD86 upregulation after TLT-2 ligation. LPS prompted both cytokine release (although of a more inflammatory nature) and upregulation of these activation markers. This indicates that the role of TLT-2 on macrophages is to prevent an immediate inflammatory response while promoting the production of neutrophil recruiting chemoattractants in high concentrations. This is interesting, as TLT-2 ligation in concert with GPCR agonists on neutrophils appears to play a similar role as TREM-1 ligation in concert with PRR agonists; that is, the synergistic amplification of the neutrophil anti-microbial response. But on macrophages, TLT-2 behaves more similarly to TREM-2, exerting an anti-inflammatory action while stimulating specific cellular functions. Although TREM-2 promotes macrophage phagocytosis without the secretion of proinflammatory cytokines such as TNFα, GM-CSF and IL-1, TLT-2 directs the macrophage to secrete neutrophil recruiting chemoattractants without producing proinflammatory cytokines.

Intravenous, intratracheal, and intraperitoneal injections of anti-TLT-2 mAb leads to the production of a similar set of cytokines (e.g. MIP-2, KC, IL-6, G-CSF) in the respective lavages or blood, and the subsequent rapid recruitment of neutrophils. Based

on the Ly6G (GR-1) profile of recruited neutrophils, it appears that immature neutrophils were also recruited after anti-TLT-2 mAb injection, most likely due to the large increase in the concentration of G-CSF. Additionally, often the recruited neutrophils would exhibit higher CD11b expression than when recruited in response to other stimuli (e.g. FMLP), although this is most likely explained by the synergistic effect mediated by TLT-2 ligation and the addition of GPCR agonists. Although one can hypothesize that the predominant *in vivo* player responding to anti-TLT-2 mAb treatment is the macrophage (because macrophages express TLT-2, are present in the peritoneum and lung, and have been shown to respond to TLT-2 ligation *ex vivo* by producing neutrophil recruiting chemokines), it is not impossible that the effect observed is mediated at least in part by another cell type. It is not known if endothelial or epithelial cells express TLT-2, but these cell types are present at the sites of injection and have stored chemokines (such as MIP-2 and KC) that can be released upon detection of inflammatory stimuli. It is therefore possible that the observed outcome following anti-TLT-2 mAb injections is mediated by a cell type other than macrophages, although one would presume that macrophages were involved to some degree based on *in vitro* data.

Due to the relative paucity of information pertaining to TLT-2, there are numerous questions that remain concerning the biological role of TLT-2 on the macrophage. Because many functions of these two innate immune players tend to overlap, it would be of interest to determine if the observed effects of TLT-2 ligation on neutrophils are also conserved on macrophages (e.g. degranulation, respiratory burst, migration, and phagocytosis). It is also of interest whether or not a secondary stimulus is needed following TLT-2 ligation on macrophages (as it is for the neutrophil), given that

TLT-2 ligation alone was sufficient to drive cytokine production by these cells. It also is not known whether TLT-2 synergizes with GPCRs on macrophages as it does on the neutrophil, though one would hypothesize that this is a conserved mechanism. Additionally, macrophages are more involved as professional antigen presenting cells than neutrophils, which process and present antigen to T cells after engulfing an invading pathogen. Simultaneously, they provide co-stimulatory signals, such as CD80 and CD86, which help drive T cell proliferation and activation. Although we have shown that TLT-2 ligation alone does not drive up expression of these costimulatory molecules, it has not been determined whether or not TLT-2 ligation in concert with peptide processing and presentation leads to enhanced activation. Moreover, it would be interesting to determine if TLT-2 has any role in altering the rate or efficiency of peptide presentation as that is one of the main functions of the tissue macrophage.

It would also be interesting to determine if TLT-2 ligation exerts any effect on macrophage differentiation into either M1 or M2 subclasses, something that could be determined via the presence of either iNos (M1) or Arginase1 (M2). Typically, macrophages are driven to the M1 lineage in the presence of classical proinflammatory mediators, such as GM-CSF, TNFα, and LPS and are then characterized by being IL-12^{high}, IL-23^{high}, IL-10^{low}. They also produce a considerable amount of reactive oxygen species, nitrogen intermediates, and inflammatory cytokines, and provide a milieu appropriate for a Th1 microenvironment. Given that TLT-2 does not appear to be driving a proinflammatory environment based on the inability to detect INFγ, TNFα, GM-CSF in our system, or the fact that we see no evidence of IL-12 being produced by TLT-2-

stimulated macrophages, it would not seem that TLT-2 is promoting an aggressive M1 response.

There are several forms of M2 macrophages, because in many ways any macrophage not classically pushed towards the M1 lineage tends to be dubbed a M2-macrophage. M2 macrophages appear to be more anti-inflammatory by nature, and are classically driven down this path via IL-4 and IL-13 cytokines. Their phenotype is IL-12^{low}, IL-23^{low}, IL-10^{high}, and they have high levels of scavenger, mannose, and galactose-type receptors. M2's contain arginase activity, unlike M1's, and also express the IL-1 receptor antagonist on their surface. As expected, M2 macrophages tend to promote a microenvironment suitable for a Th2 response. To date, the effect that TLT-2 ligation may have on macrophage differentiation *in vivo* and *in vitro* has not been looked at, and no conclusions can yet be drawn as to its possible role. However, understanding if TLT-2 is involved in driving macrophage differentiation may help us to understand the biological role of TLT-2 as it relates to inflammation and the innate immune response.

THERAPEUTIC POTENTIAL

In light of data we have presented, it is possible that modulation of TLT-2 function could be utilized therapeutically to treat a wide variety of disorders. People that are neutropenic (often chemotherapy patients) are prescribed G-CSF injections to counteract poor white cell production via the bone marrow. Chemotherapy patients are more susceptible to opportunistic infections due to the fact that their cancer treatment damages their bone marrow, and injections of the G-CSF hormone lessen the duration and severity of the neutropenia. Because injection of anti-TLT-2 mAb has been shown to

both stimulate production of neutrophils from the bone marrow and to increase their anti-microbial response, perhaps this would make for a suitable therapy. Rather than passively provide G-CSF, stimulating the host cells to produce this hormone may actually aid in the patient's recovery.

However, more to the therapeutic potential of TLT-2, is the fact that a majority of the best selling drugs target GPCRs, and in fact roughly 40% of all prescribed pharmaceuticals target this receptor superfamily. Indeed, given the amount of research devoted to understanding GPCRs, it seems that this family of receptors will continue to be a focus in the pharmaceutical world. If TLT-2 ligation can specifically and transiently enhance the activity of these drugs in the way it has enhanced the activity towards other GPCR agonists, then this may significantly alter the way drugs are prescribed in a very general sense. The pendulum between optimal drug dosage and side effects is one that can be tricky to balance and ultimately quite limiting when trying to help a patient. Doctors must prescribe a dosage that they believe will have the desired effect without an unbearable amount of pain (or fatality) due to side effects of the drug at such high doses. It seems that administration of anti-TLT-2 mAb in the absence of other pharmaceuticals may have a relatively minor effect, such as exerting a low proinflammatory index while predominately recruiting short lived neutrophils into the bloodstream which remain in a quiescent, surveillance state until a pathogenic threat is detected. Addition of GPCR targeting drugs would now give anti-TLT-2 mAb a target to synergize with and enhance the cellular response, effectively lowering the threshold needed for this particular drug. Therefore, drugs that lead to particularly bad side effects at high doses could be administered in lower concentrations along with a suitable dose of anti-TLT-2 mAb, and

provide a similar positive outcome as before. Alternatively, because many of the GPCR-targeted drugs exert an antagonistic effect, it would also be beneficial to develop a blocking antibody to TLT-2, which could potentially lead to administration of a lower dose of drug. Further studies are required to determine if this pharmaceutical pathway is viable for TLT-2 biology, but if possible, could reshape the way pharmaceutical drugs are prescribed.

REFERENCES

Akahoshi, T. 2008. [Pathological mechanisms of gouty arthritis]. *Nippon Rinsho* 66:705-710.

Aoki, N., S. Kimura, and Z. Xing. 2003. Role of DAP12 in innate and adaptive immune responses. *Curr Pharm Des* 9:7-10.

Aoki, N., A. Zganiacz, P. Margetts, and Z. Xing. 2004. Differential regulation of DAP12 and molecules associated with DAP12 during host responses to mycobacterial infection. *Infect Immun* 72:2477-2483.

Begum, N.A., K. Ishii, M. Kurita-Taniguchi, M. Tanabe, M. Kobayashi, Y. Moriwaki, M. Matsumoto, Y. Fukumori, I. Azuma, K. Toyoshima, and T. Seya. 2004. Mycobacterium bovis BCG cell wall-specific differentially expressed genes identified by differential display and cDNA subtraction in human macrophages. *Infect Immun* 72:937-948.

Bleharski, J.R., V. Kiessler, C. Buonsanti, P.A. Sieling, S. Stenger, M. Colonna, and R.L. Modlin. 2003. A role for triggering receptor expressed on myeloid cells-1 in host defense during the early-induced and adaptive phases of the immune response. *J Immunol* 170:3812-3818.

Bouchon, A., J. Dietrich, and M. Colonna. 2000. Cutting edge: inflammatory responses can be triggered by TREM-1, a novel receptor expressed on neutrophils and monocytes. *J Immunol* 164:4991-4995.

Bouchon, A., F. Facchetti, M.A. Weigand, and M. Colonna. 2001a. TREM-1 amplifies inflammation and is a crucial mediator of septic shock. *Nature* 410:1103-1107.

Bouchon, A., C. Hernandez-Munain, M. Cella, and M. Colonna. 2001b. A DAP12-mediated pathway regulates expression of CC chemokine receptor 7 and maturation of human dendritic cells. *J Exp Med* 194:1111-1122.

Brinkmann, V., U. Reichard, C. Goosmann, B. Fauler, Y. Uhlemann, D.S. Weiss, Y. Weinrauch, and A. Zychlinsky. 2004. Neutrophil extracellular traps kill bacteria. *Science* 303:1532-1535.

Buchanan, J.T., A.J. Simpson, R.K. Aziz, G.Y. Liu, S.A. Kristian, M. Kotb, J. Feramisco, and V. Nizet. 2006. DNase expression allows the pathogen group A Streptococcus to escape killing in neutrophil extracellular traps. *Curr Biol* 16:396-400.

Cella, M., C. Buonsanti, C. Strader, T. Kondo, A. Salmaggi, and M. Colonna. 2003. Impaired differentiation of osteoclasts in TREM-2-deficient individuals. *J Exp Med* 198:645-651.

Chen, C.M., O. Penuelas, K. Quinn, K.C. Cheng, C.F. Li, H. Zhang, and A.S. Slutsky. 2009. Protective effects of adenosine A2A receptor agonist in ventilator-induced lung injury in rats. *Crit Care Med* 37:2235-2241.

Cronstein, B.N. 2004. Adenosine receptors and wound healing. *ScientificWorldJournal* 4:1-8.

Dangelmaier, C.A., P.G. Quinter, J. Jin, A.Y. Tsygankov, S.P. Kunapuli, and J.L. Daniel. 2005. Rapid ubiquitination of Syk following GPVI activation in platelets. *Blood* 105:3918-3924.

Daws, M.R., L.L. Lanier, W.E. Seaman, and J.C. Ryan. 2001. Cloning and characterization of a novel mouse myeloid DAP12-associated receptor family. *Eur J Immunol* 31:783-791.

Daws, M.R., P.M. Sullam, E.C. Niemi, T.T. Chen, N.K. Tchao, and W.E. Seaman. 2003. Pattern recognition by TREM-2: binding of anionic ligands. *J Immunol* 171:594-599.

Eash, K.J., A.M. Greenbaum, P.K. Gopalan, and D.C. Link. 2010. CXCR2 and CXCR4 antagonistically regulate neutrophil trafficking from murine bone marrow. *J Clin Invest* 120:2423-2431.

Ford, J.W., and D.W. McVicar. 2009. TREM and TREM-like receptors in inflammation and disease. *Curr Opin Immunol* 21:38-46.

Frank, S., G.J. Burbach, M. Bonin, M. Walter, W. Streit, I. Bechmann, and T. Deller. 2008. TREM2 is upregulated in amyloid plaque-associated microglia in aged APP23 transgenic mice. *Glia* 56:1438-1447.

Fuchs, T.A., U. Abed, C. Goosmann, R. Hurwitz, I. Schulze, V. Wahn, Y. Weinrauch, V. Brinkmann, and A. Zychlinsky. 2007. Novel cell death program leads to neutrophil extracellular traps. *J Cell Biol* 176:231-241.

Fukao, T., and S. Koyasu. 2003. PI3K and negative regulation of TLR signaling. *Trends Immunol* 24:358-363.

Gattis, J.L., A.V. Washington, M.M. Chisholm, L. Quigley, A. Szyk, D.W. McVicar, and J. Lubkowski. 2006. The structure of the extracellular domain of triggering receptor expressed on myeloid cells like transcript-1 and evidence for a naturally occurring soluble fragment. *J Biol Chem* 281:13396-13403.

Gibot, S., C. Alauzet, F. Massin, N. Sennoune, G.C. Faure, M.C. Bene, A. Lozniewski, P.E. Bollaert, and B. Levy. 2006a. Modulation of the triggering receptor expressed on myeloid cells-1 pathway during pneumonia in rats. *J Infect Dis* 194:975-983.

Gibot, S., C. Buonsanti, F. Massin, M. Romano, M.N. Kolopp-Sarda, F. Benigni, G.C. Faure, M.C. Bene, P. Panina-Bordignon, N. Passini, and B. Levy. 2006b. Modulation of the triggering receptor expressed on the myeloid cell type 1 pathway in murine septic shock. *Infect Immun* 74:2823-2830.

Gibot, S., and A. Cravoisy. 2004. Soluble form of the triggering receptor expressed on myeloid cells-1 as a marker of microbial infection. *Clin Med Res* 2:181-187.

Gibot, S., A. Cravoisy, R. Dupays, D. Barraud, L. Nace, B. Levy, and P.E. Bollaert. 2007a. Combined measurement of procalcitonin and soluble TREM-1 in the diagnosis of nosocomial sepsis. *Scand J Infect Dis* 39:604-608.

Gibot, S., A. Cravoisy, B. Levy, M.C. Bene, G. Faure, and P.E. Bollaert. 2004a. Soluble triggering receptor expressed on myeloid cells and the diagnosis of pneumonia. *N Engl J Med* 350:451-458.

Gibot, S., M.N. Kolopp-Sarda, M.C. Bene, P.E. Bollaert, A. Lozniewski, F. Mory, B. Levy, and G.C. Faure. 2004b. A soluble form of the triggering receptor expressed on myeloid cells-1 modulates the inflammatory response in murine sepsis. *J Exp Med* 200:1419-1426.

Gibot, S., F. Massin, C. Alauzet, M. Derive, C. Montemont, S. Collin, S. Fremont, and B. Levy. 2009. Effects of the TREM 1 pathway modulation during hemorrhagic shock in rats. *Shock* 32:633-637.

Gibot, S., F. Massin, M. Marcou, V. Taylor, R. Stidwill, P. Wilson, M. Singer, and G. Bellingan. 2007b. TREM-1 promotes survival during septic shock in mice. *Eur J Immunol* 37:456-466.

Gingras, M.C., H. Lapillonne, and J.F. Margolin. 2002. TREM-1, MDL-1, and DAP12 expression is associated with a mature stage of myeloid development. *Mol Immunol* 38:817-824.

Giomarelli, B., V.A. Washington, M.M. Chisholm, L. Quigley, J.B. McMahon, T. Mori, and D.W. McVicar. 2007. Inhibition of thrombin-induced platelet aggregation using human single-chain Fv antibodies specific for TREM-like transcript-1. *Thromb Haemost* 97:955-963.

Gomez-Pina, V., A. Soares-Schanoski, A. Rodriguez-Rojas, C. Del Fresno, F. Garcia, M.T. Vallejo-Cremades, I. Fernandez-Ruiz, F. Arnalich, P. Fuentes-Prior, and E. Lopez-Collazo. 2007. Metalloproteinases shed TREM-1 ectodomain from lipopolysaccharide-stimulated human monocytes. *J Immunol* 179:4065-4073.

Halpert, MM., Thomas, KA., King, RG., and L.B. Justement. 2011. TLT2 potentiates neutrophil antibacterial activity and chemotaxis in response to G protein-coupled receptor-mediated signaling. *J Immunol* 187:2346-2355.

Hamerman, J.A., J.R. Jarjoura, M.B. Humphrey, M.C. Nakamura, W.E. Seaman, and L.L. Lanier. 2006. Cutting edge: inhibition of TLR and FcR responses in macrophages by triggering receptor expressed on myeloid cells (TREM)-2 and DAP12. *J Immunol* 177:2051-2055.

Hamerman, J.A., N.K. Tchao, C.A. Lowell, and L.L. Lanier. 2005. Enhanced Toll-like receptor responses in the absence of signaling adaptor DAP12. *Nat Immunol* 6:579-586.

Han, C., J. Jin, S. Xu, H. Liu, N. Li, and X. Cao. 2010. Integrin CD11b negatively regulates TLR-triggered inflammatory responses by activating Syk and promoting degradation of MyD88 and TRIF via Cbl-b. *Nat Immunol* 11:734-742.

Hara, H., C. Ishihara, A. Takeuchi, T. Imanishi, L. Xue, S.W. Morris, M. Inui, T. Takai, A. Shibuya, S. Saijo, Y. Iwakura, N. Ohno, H. Koseki, H. Yoshida, J.M. Penninger, and T. Saito. 2007. The

adaptor protein CARD9 is essential for the activation of myeloid cells through ITAM-associated and Toll-like receptors. *Nat Immunol* 8:619-629.

Hartl, D., P. Latzin, P. Hordijk, V. Marcos, C. Rudolph, M. Woischnik, S. Krauss-Etschmann, B. Koller, D. Reinhardt, A.A. Roscher, D. Roos, and M. Griese. 2007. Cleavage of CXCR1 on neutrophils disables bacterial killing in cystic fibrosis lung disease. *Nat Med* 13:1423-1430.

Hartl, D., N. Lehmann, F. Hoffmann, A. Jansson, A. Hector, G. Notheis, D. Roos, B.H. Belohradsky, and U. Wintergerst. 2008. Dysregulation of innate immune receptors on neutrophils in chronic granulomatous disease. *J Allergy Clin Immunol* 121:375-382 e379.

Haselmayer, P., L. Grosse-Hovest, P. von Landenberg, H. Schild, and M.P. Radsak. 2007. TREM-1 ligand expression on platelets enhances neutrophil activation. *Blood* 110:1029-1035.

Hashiguchi, M., H. Kobori, P. Ritprajak, Y. Kamimura, H. Kozono, and M. Azuma. 2008. Triggering receptor expressed on myeloid cell-like transcript 2 (TLT-2) is a counter-receptor for B7-H3 and enhances T cell responses. *Proc Natl Acad Sci U S A* 105:10495-10500.

Hsieh, C.L., M. Koike, S.C. Spusta, E.C. Niemi, M. Yenari, M.C. Nakamura, and W.E. Seaman. 2009. A role for TREM2 ligands in the phagocytosis of apoptotic neuronal cells by microglia. *J Neurochem* 109:1144-1156.

Humphrey, M.B., M.R. Daws, S.C. Spusta, E.C. Niemi, J.A. Torchia, L.L. Lanier, W.E. Seaman, and M.C. Nakamura. 2006. TREM2, a DAP12-associated receptor, regulates osteoclast differentiation and function. *J Bone Miner Res* 21:237-245.

Humphrey, M.B., K. Ogasawara, W. Yao, S.C. Spusta, M.R. Daws, N.E. Lane, L.L. Lanier, and M.C. Nakamura. 2004. The signaling adapter protein DAP12 regulates multinucleation during osteoclast development. *J Bone Miner Res* 19:224-234.

King, R.G., B.R. Herrin, and L.B. Justement. 2006. Trem-like transcript 2 is expressed on cells of the myeloid/granuloid and B lymphoid lineage and is up-regulated in response to inflammation. *J Immunol* 176:6012-6021.

Klesney-Tait, J., and M. Colonna. 2007. Uncovering the TREM-1-TLR connection. *Am J Physiol Lung Cell Mol Physiol* 293:L1374-1376.

Klesney-Tait, J., I.R. Turnbull, and M. Colonna. 2006. The TREM receptor family and signal integration. *Nat Immunol* 7:1266-1273.

Knapp, S., S. Gibot, A. de Vos, H.H. Versteeg, M. Colonna, and T. van der Poll. 2004. Cutting edge: expression patterns of surface and soluble triggering receptor expressed on myeloid cells-1 in human endotoxemia. *J Immunol* 173:7131-7134.

Kobori, H., M. Hashiguchi, J. Piao, M. Kato, P. Ritprajak, and M. Azuma. 2010. Enhancement of effector CD8+ T-cell function by tumour-associated B7-H3 and modulation of its counter-receptor triggering receptor expressed on myeloid cell-like transcript 2 at tumour sites. *Immunology* 130:363-373.

Laskin, D.L., L. Chen, P.A. Hankey, and J.D. Laskin. 2010. Role of STK in mouse liver macrophage and endothelial cell responsiveness during acute endotoxemia. *J Leukoc Biol* 88:373-382.

Leitner, J., C. Klauser, W.F. Pickl, J. Stockl, O. Majdic, A.F. Bardet, D.P. Kreil, C. Dong, T. Yamazaki, G. Zlabinger, K. Pfistershammer, and P. Steinberger. 2009. B7-H3 is a potent inhibitor of human T-cell activation: No evidence for B7-H3 and TREML2 interaction. *Eur J Immunol* 39:1754-1764.

Mahdy, A.M., D.A. Lowes, H.F. Galley, J.E. Bruce, and N.R. Webster. 2006. Production of soluble triggering receptor expressed on myeloid cells by lipopolysaccharide-stimulated human neutrophils involves de novo protein synthesis. *Clin Vaccine Immunol* 13:492-495.

Manes, S., and A. Viola. 2006. Lipid rafts in lymphocyte activation and migration. *Mol Membr Biol* 23:59-69.

Melchior, B., A.E. Garcia, B.K. Hsiung, K.M. Lo, J.M. Doose, J.C. Thrash, A.K. Stalder, M. Staufenbiel, H. Neumann, and M.J. Carson. 2010. Dual induction of TREM2 and tolerance-related transcript, Tmem176b, in amyloid transgenic mice: implications for vaccine-based therapies for Alzheimer's disease. *ASN Neuro* 2:e00037.

Melchior, B., S.S. Puntambekar, and M.J. Carson. 2006. Microglia and the control of autoreactive T cell responses. *Neurochem Int* 49:145-153.

Mohamadzadeh, M., S.S. Coberley, G.G. Olinger, W.V. Kalina, G. Ruthel, C.L. Fuller, D.L. Swenson, W.D. Pratt, D.B. Kuhns, and A.L. Schmaljohn. 2006. Activation of triggering receptor expressed on myeloid cells-1 on human neutrophils by marburg and ebola viruses. *J Virol* 80:7235-7244.

Molloy, E.J. 2009. Triggering Receptor Expressed on Myeloid Cells (TREM) family and the application of its antagonists. *Recent Pat Antiinfect Drug Discov* 4:51-56.

Mosser, D.M., and J.P. Edwards. 2008. Exploring the full spectrum of macrophage activation. *Nat Rev Immunol* 8:958-969.

Murakami, Y., and H. Kohsaka. 2009. [Triggering receptor expressed on myeloid cells-1 as an inflammation amplifier]. *Nihon Rinsho Meneki Gakkai Kaishi* 32:242-248.

Nadel, J.A. 2000. Role of neutrophil elastase in hypersecretion during COPD exacerbations, and proposed therapies. *Chest* 117:386S-389S.

Nagasawa, S.Y., N. Takuwa, N. Sugimoto, H. Mabuchi, and Y. Takuwa. 2005. Inhibition of Rac activation as a mechanism for negative regulation of actin cytoskeletal reorganization and cell motility by cAMP. *Biochem J* 385:737-744.

Nathan, C. 2006. Neutrophils and immunity: challenges and opportunities. *Nat Rev Immunol* 6:173-182.

Ornatowska, M., A.C. Azim, X. Wang, J.W. Christman, L. Xiao, M. Joo, and R.T. Sadikot. 2007. Functional genomics of silencing TREM-1 on TLR4 signaling in macrophages. *Am J Physiol Lung Cell Mol Physiol* 293:L1377-1384.

Orr, S.J., and D.W. McVicar. 2011. LAB/NTAL/Lat2: a force to be reckoned with in all leukocytes? *J Leukoc Biol* 89:11-19.

Rose, J.J., J.F. Foley, P.M. Murphy, and S. Venkatesan. 2004. On the mechanism and significance of ligand-induced internalization of human neutrophil chemokine receptors CXCR1 and CXCR2. *J Biol Chem* 279:24372-24386.

Schenk, M., A. Bouchon, S. Birrer, M. Colonna, and C. Mueller. 2005. Macrophages expressing triggering receptor expressed on myeloid cells-1 are underrepresented in the human intestine. *J Immunol* 174:517-524.

Schenk, M., A. Bouchon, F. Seibold, and C. Mueller. 2007. TREM-1--expressing intestinal macrophages crucially amplify chronic inflammation in experimental colitis and inflammatory bowel diseases. *J Clin Invest* 117:3097-3106.

Sugimoto, Y., Y. Fukada, D. Mori, S. Tanaka, H. Yamane, Y. Okuno, K. Deai, S. Tsuchiya, G. Tsujimoto, and A. Ichikawa. 2005. Prostaglandin E2 stimulates granulocyte colony-stimulating factor production via the prostanoid EP2 receptor in mouse peritoneal neutrophils. *J Immunol* 175:2606-2612.

Sun, G.Y., C.X. Guan, Y. Zhou, Y.P. Liu, S.F. Li, H.F. Zhou, C.Y. Tang, and X. Fang. 2011. Vasoactive intestinal peptide re-balances TREM-1/TREM-2 ratio in acute lung injury. *Regul Pept* 167:56-64.

Takahashi, K., C.D. Rochford, and H. Neumann. 2005. Clearance of apoptotic neurons without inflammation by microglial triggering receptor expressed on myeloid cells-2. *J Exp Med* 201:647-657.

Takegahara, N., H. Takamatsu, T. Toyofuku, T. Tsujimura, T. Okuno, K. Yukawa, M. Mizui, M. Yamamoto, D.V. Prasad, K. Suzuki, M. Ishii, K. Terai, M. Moriya, Y. Nakatsuji, S. Sakoda, S. Sato, S. Akira, K. Takeda, M. Inui, T. Takai, M. Ikawa, M. Okabe, A. Kumanogoh, and H. Kikutani. 2006. Plexin-A1 and its interaction with DAP12 in immune responses and bone homeostasis. *Nat Cell Biol* 8:615-622.

Tessarz, A.S., and A. Cerwenka. 2008. The TREM-1/DAP12 pathway. *Immunol Lett* 116:111-116.

Turnbull, I.R., S. Gilfillan, M. Cella, T. Aoshi, M. Miller, L. Piccio, M. Hernandez, and M. Colonna. 2006. Cutting edge: TREM-2 attenuates macrophage activation. *J Immunol* 177:3520-3524.

Washington, A.V., S. Gibot, I. Acevedo, J. Gattis, L. Quigley, R. Feltz, A. De La Mota, R.L. Schubert, J. Gomez-Rodriguez, J. Cheng, A. Dutra, E. Pak, O. Chertov, L. Rivera, J. Morales, J. Lubkowski, R. Hunter, P.L. Schwartzberg, and D.W. McVicar. 2009. TREM-like transcript-1 protects against inflammation-associated hemorrhage by facilitating platelet aggregation in mice and humans. *J Clin Invest* 119:1489-1501.

Washington, A.V., L. Quigley, and D.W. McVicar. 2002. Initial characterization of TREM-like transcript (TLT)-1: a putative inhibitory receptor within the TREM cluster. *Blood* 100:3822-3824.

Washington, A.V., R.L. Schubert, L. Quigley, T. Disipio, R. Feltz, E.H. Cho, and D.W. McVicar. 2004. A TREM family member, TLT-1, is found exclusively in the alpha-granules of megakaryocytes and platelets. *Blood* 104:1042-1047.

Whittaker, G.C., S.J. Orr, L. Quigley, L. Hughes, I.M. Francischetti, W. Zhang, and D.W. McVicar. 2010. The linker for activation of B cells (LAB)/non-T cell activation linker (NTAL) regulates triggering receptor expressed on myeloid cells (TREM)-2 signaling and macrophage inflammatory responses independently of the linker for activation of T cells. *J Biol Chem* 285:2976-2985.

Wiersinga, W.J., C.T. Veer, C.W. Wieland, S. Gibot, B. Hooibrink, N.P. Day, S.J. Peacock, and T. van der Poll. 2007. Expression profile and function of triggering receptor expressed on myeloid cells-1 during melioidosis. *J Infect Dis* 196:1707-1716.

Zanzinger, K., C. Schellack, N. Nausch, and A. Cerwenka. 2009. Regulation of triggering receptor expressed on myeloid cells 1 expression on mouse inflammatory monocytes. *Immunology* 128:185-195.

Zheng, H., C.A. Heiderscheidt, M. Joo, X. Gao, N. Knezevic, D. Mehta, and R.T. Sadikot. 2010. MYD88-dependent and -independent activation of TREM-1 via specific TLR ligands. *Eur J Immunol* 40:162-171.

APPENDIX

IACUC APPROVAL

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

NOTICE OF APPROVAL

DATE: September 20, 2010

TO: Justement, Louis B.
SHEL-520D 2182
934-1429

FROM: Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee

SUBJECT: Title: Regulation of B Lymphocyte Survival and Differentiation by HSH2
Sponsor: NIH
Animal Project Number: 100907939

On September 20, 2010, the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) reviewed the animal use proposed in the above referenced application. It approved the use of the following species and numbers of animals:

Species	Use Category	Number in Category
Mice	B	250
Mice	A	1300

Animal use is scheduled for review one year from September 2010. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 100907939 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at 934-7692.

Institutional Animal Care and Use Committee
CH19 Suite 403
933 19th Street South
205.934.7692
FAX 205.934.1188

Mailing Address:
CH19 Suite 403
1530 3RD AVE S
BIRMINGHAM AL 35294-0019

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

MEMORANDUM

DATE: September 20, 2010

TO: Justement, Louis B.
SHEL-520D 2182
934-1429

FROM: Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee

SUBJECT: **NOTICE OF APPROVAL - Please forward this notice to the appropriate granting agency.**

The following application was reviewed and approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) on September 20, 2010.

Title: Regulation of B Lymphocyte Survival and Differentiation by HSH2

Sponsor: NIH

This institution has an Animal Welfare Assurance on file with the Office for Protection from Research Risks (Assurance Number A3255-01) and is registered as a Research Facility with the United States Department of Agriculture. The animal care and use program is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC International)

Institutional Animal Care and Use Committee
CH19 Suite 403
933 19th Street South
205.934.7692
FAX 205.934.1188

Mailing Address:
CH19 Suite 403
1530 3RD AVE S
BIRMINGHAM AL 35294-0019