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An Investigation of Trem-like Transcript 2 Expression

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AN INVESTIGATION OF TREM-LIKE TRANSCRIPT 2 EXPRESSION

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A DISSERTATION

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AN INVESTIGATION OF TREM-LIKE TRANSCRIPT 2 EXPRESSION

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MICROBIOLOGY

ABSTRACT

Acute inflammation is a necessary component for the clearance of pathogens. Cells at the site of infection utilize highly conserved receptors to both recognize microbes and induce the production of pro-inflammatory stimuli. These stimuli set off a chain reaction that includes localized vasodilation, leukocyte infiltration, and tissue destruction, collectively known as inflammation. Tightly controlled, this process is protective, and can compartmentalize the damage to a defined area, but when dysregulated can be catastrophic, inducing chronic inflammation and even death.

Families of innate receptors can modulate inflammatory processes, and in doing so, increase or decrease their severity, as well as tailor responses to various infections. Of note, proteins of the immunoglobulin super family (IgSF) are a large, well characterized group of receptors that regulate processes such as cell adhesion, migration, signaling, and phagocytosis. Recently, a subclass of IgSF proteins, the Triggering Receptor Expressed on Myeloid cells, or TREM, family of proteins has emerged as a group of receptors involved in modulating cellular responses during inflammation.

Of the genes in the TREM locus, four have conserved protein products; TREM-1, TREM-2, Trem-like transcript 1 (TLT1), and Trem-like transcript 2 (TLT2). Study of the biology of these receptors has revealed that members of the TREM family are capable of modulating a variety of immune cell functions. TREM-1 amplifies the immune response to bacterial stimuli by increasing leukocyte production of pro-inflammatory cytokines, as

well as enhancing leukocyte/platelet interactions. TREM-2 is important for dampening inflammatory signals in the central nervous system and in maintaining homeostasis during bone resorption. TLT1 plays a role in augmenting platelet aggregation as well as leukocyte interactions. Less is known concerning the role TLT2 plays during inflammation.

Here, we describe that TLT2 expression is conserved between mouse and man, and is the only TREM member distributed on cells of both the myeloid and lymphoid lineage. This pattern of expression is modulated by inflammatory stimuli, a preserved feature of the TREM family. Specifically, TLT2 is found intracellularly in neutrophils, and is rapidly translocated to the cell surface upon degranulation. These observations add to the growing biology of TLT2, and will be useful in further elucidating its role during immune responses.

Keywords: Trem-like transcript 2, Inflammation, Neutrophil, Myeloid

DEDICATION

I would like to dedicate this work to Mike.
Without your help, love, and support these past ten years, I wouldn't be here.

ACKNOWLEDGEMENTS

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TREM-LIKE TRANSCRIPT 2 EXPRESSION IS MODULATED IN RESPONSE TO INFLAMAMTORY MEDIATORS AND LOCATED IN NEUTROPHIL GRANULES

LIST OF ABBREVIATIONS

Ab	antibody
APC	antigen presenting cell
BMDC	bone marrow-derived dendritic cell
C5a	cleaved complement 5 component
CD	cluster of differentiation
CNS	central nervous system
DAP12	DNAX activating protein
DC	dendritic cell
DIC	disseminated intravascular coagulation
EC	endothelial cell
FMLF	N-formyl-met-leu-phe
GMCSF	granulocyte-macrophage colony-stimulating factor
IFNγ	interferon gamma
IL	interleukin
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibition motif
LPS	lipopolysaccharide
MCP	monocyte chemotactic protein
MHC-II	MHC class II

MMP	matrix metalloproteinase
MPO	myeloperoxidase
NET	neutrophil extracellular trap
NHD	Nasu Hakola disease
NK	natural killer (cell)
OC	osteoclast
PBS	phosphate buffered saline
PLOSL	polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy
PMA	phorbol myristate acetate
ROS	reactive oxygen species
TLT1	Trem-like transcript 1
TLT2	Trem-like transcript 2
TM	transmembrane
TNFα	tumor necrosis factor
TRAP	thrombin receptor activating peptide
TREM	triggering receptor expressed on myeloid cells

INTRODUCTION

Pathogenic Inflammation

The capacity to recognize and eliminate microbial pathogens is critical for the maintenance of tissue homeostasis and the preservation of viability of complex organisms. The vertebrate immune system consists of two highly evolved branches that orchestrate specific responses to a diverse range of unique and ever changing pathogens. During a typical immune response, immunosurveillant phagocytic cells engulf pathogens and secrete soluble mediators, including cytokines and chemokines, which induce inflammatory processes such as vasodilation and cellular activation [1]. In response to these mediators, endothelial cells up-regulate adhesion markers and constrict through the rearrangement of cell junctions which result in a more permeable barrier allowing increased leukocyte diapedesis [2, 3]. Leukocyte activation in the blood leads to an increase in the expression of adhesion molecules, such as integrins, enabling cells to migrate through the endothelium to the inflamed tissue in response to chemokine gradients [4]. Following exit from the bloodstream and entrance into the interstitial space, leukocytes migrate along the extracellular matrix (ECM) and stromal cells in response to a range of microbial and host derived soluble factors. Continued activation by local cells and the inflammatory milieu provide costimulatory signals for migrating leukocytes to induce effector functions leading to the elimination of pathogenic organisms [5, 6]. Macrophages and neutrophils employ a variety of mechanisms to both kill microbes and resolve inflammation. These include the production and release of microbicidal peptides

1

and reactive oxygen species (ROS), which are released into the interstitial space or concentrated within the phagolysosome [7, 8]. Additionally, macrophages are vital for phagocytosis and the removal of dead cell debris, activities that are necessary for the resolution of the inflammatory response [9, 10]. The release of inhibitory mediators, e.g. lipoxins and resolvins, reestablish homeostasis by inhibiting further leukocyte recruitment, inflammatory cytokine production, and by promoting macrophage-dependent phagocytosis of apoptotic cells [11-14].
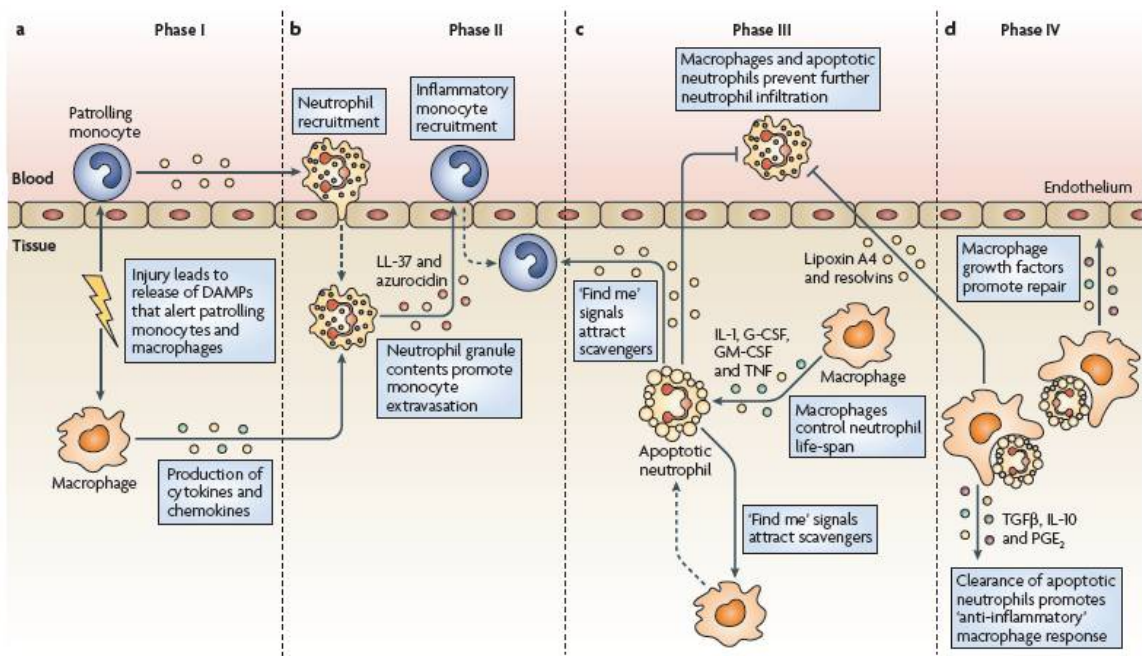
Figure 1. **The Role of Myeloid Cells During Inflammation**. Pathogen recognition by macrophages results in neutrophil and monocyte recruitment from the blood, with subsequent clearing of pathogens and removal of cellular debris, in an effort to restore homeostasis [14].

Microbial Recognition and Cellular Responses

Because recognition of pathogens and modulation of cellular activation are central to immune process, these activities are mediated by numerous classes of innate

receptors which are expressed on many different cell types. The detection and elimination of microbes possesing evolutionarily conserved pathogen associated molecular patterns (PAMPs), is primarily mediated through a set of germ-line encoded receptors called pattern recognition receptors or PRRs [15, 16]. Upon binding of PAMP-containing molecules, PRRs transmit signals that mediate the activation of multiple signaling pathways responsible for initiating processes involved in pathogen clearance [17]. Modulation of both the signals derived through these receptors, and the functional response of cells activated by them, is necessary to ensure a robust antimicrobial response, which is important for preventing microbial outgrowth while also limiting tissue destruction resulting from inflammatory processes. This additional level of fine control of innate immune responses is mediated through additional innate receptor families [18, 19].

The best characterized group of PRRs is the Toll-like receptor (TLR) family, which includes receptors with a primary role in pathogen recognition and cellular activation. TLR1/6, 2/6, 4, and 5 are located on the plasma membrane and are involved in recognizing extracellular pathogens, specifically through interaction with bacterial and fungal products, whereas TLR3, 7/8, and 9 are all expressed on endosomal membranes and specialize in detecting intracellular pathogens, particularly by binding viral proteins and/or microbial DNA/RNA [20, 21]. Activation through TLRs induces a variety of signaling pathways. TLR-mediated activation of NFκB induces the transcription of multiple classical pro-inflammatory soluble mediators, including TNFα, IL-1β, and IL-6. TLR mediated activation is also associated with the upregulation of surface adhesion and costimulatory molecules, including MHC II, CD80/86, and CD11b [22, 23]. This pro-

inflammatory cascade results in a feed-forward activation of signals that function to promote the clearance of pathogens. However, because dysregulation of these processes can lead to tissue destruction, fever, and death, careful modulation of the response to PRR agonists is critical.

The immune system has evolved a complex system of checks and balances which maintain homeostasis and restore the system to baseline following pathogenic insult. One such mechanism relies on the amplification or attenuation of cellular processes induced by activating stimuli. This modulation results from the activity of various innate receptors which regulate cell adhesion, antigen processing and presentation, and cytokine and growth factor signaling [24]. These receptors provide costimulatory and/or inhibitory signals by means of immunoreceptor tyrosine-based signaling motifs, known as ITAMs and ITIMs, respectively [25, 26]. Although some proteins contain cytoplasmic ITAMs or ITIMs, many do not and instead couple with other ITAM or ITIM containing proteins through charged residues positioned within the transmembrane region. Common pairing subunits in leukocytes are the DNAX activating protein 12 (DAP12) and the FcRγ chain. Both of these proteins lack an extracellular domain, but contain an ITAM and can pair with a multitude of receptors to mediate various cellular processes [27]. Some receptors that pair with DAP12 are members of the immunoglobulin super family (IgSF), and include the CD200, CD300, and TREM families of proteins. Members of the CD300 family have been shown to modulate inflammatory cytokine production and phagocytosis in myeloid cells following responses to TLR stimulation, whereas CD200 functions to dampen the responses of myeloid cells to infection and inflammation [28-30].

The TREM Family

A more recently studied class of immunomodulatory receptors, the Triggering Receptor Expressed on Myeloid cells (TREM) family, have been shown to modulate cellular responses to pathogenic molecules [31, 32]. There are four proteins in this family whose expression are conserved across species and are evolutionarily conserved within the class Mammalia: TREM-1, TREM-2, Trem-like transcript 1 (TLT1), and Trem-like transcript 2 (TLT2).
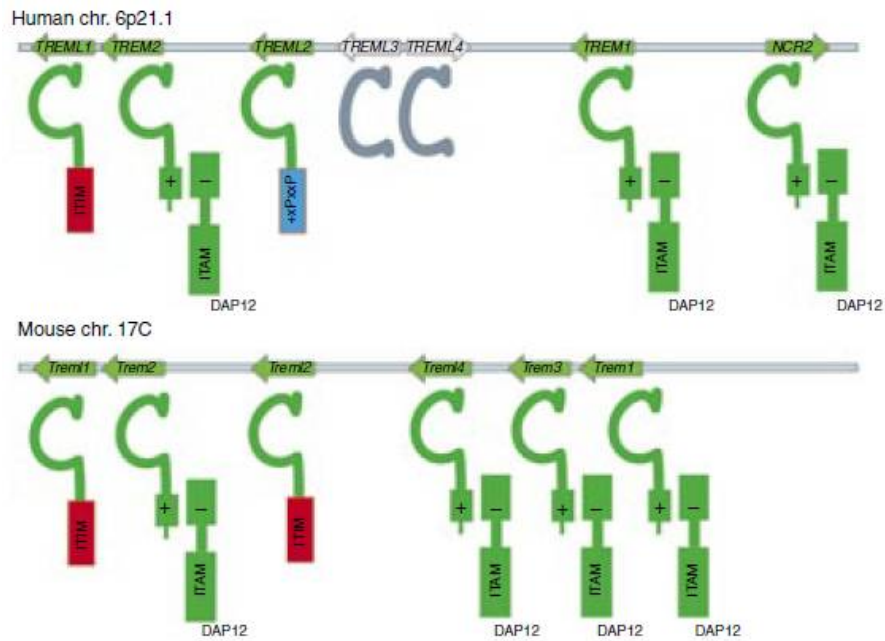
Figure 2. **TREM Locus**. Of the TREM members, 4 have been identified as conserved genes, and corresponding proteins: TREM-1, TREM-2. TLT1, and TLT2 [31].

All members of the TREM locus have a conserved extracellular region, containing a distal single variable type Ig-like domain, followed by a serine/threonine rich stalk located near the plasma membrane [33]. The transmembrane (TM) and cytoplasmic regions of TREM family members are far less conserved. TREM-1 and

TREM-2 have a minimal cytoplasmic tail lacking any known signaling motif, yet both contain a positively charged residue within the TM region. This is necessary for pairing with proteins capable of initiating signal transduction. Both TREM-1 and TREM-2 mediate their functions through pairing with DAP12, an adapter protein containing both a negatively charged TM residue and a cytoplasmic ITAM [34]. DAP12 is known to associate with other receptors similar to the TREMs, such as the lectins MDL-1 and SIRP-β, and is required for signal transduction through these receptors [35]. In contrast to TREM-1 and TREM-2, TLT1 and TLT2 do not utilize DAP12 for their function, as neither contain charged TM residues. Alternatively, TLT1 and TLT2 have short cytoplasmic tails [33].

TREM-1. The most well characterized member of the TREM family, TREM-1, is constitutively expressed on monocytes, neutrophils and alveolar macrophages, but its expression is upregulated following exposure to pathogenic stimuli such as LPS, gram negative bacteria, and fungi [36]. Agonistic monoclonal antibodies (mAb) have been vital for elucidating the role TREM-1 plays in modulating the immune response. Ligation of TREM-1 by mAb alone results in IL-8 and myeloperoxidase (MPO) release from neutrophils and IL-8, TNFα and monocyte chemotractant protein (MCP) release from monocytes. Additionally, priming of cells with LPS results in an enhanced release of these mediators, indicating that ligation of TREM-1 synergistically enhances TLR-mediated cellular responses. TREM-1 binding was also shown to upregulate integrins (CD49e, CD29, CD11c) and costimulatory molecules (CD40, CD54, CD86), thus lowering the activation threshold of the cell [36]. This augmentation of pro-inflammatory

responses *in vitro* lends credence to the hypothesis that TREM-1 plays an important role in bacterial clearance *in vivo*.

During acute inflammation caused by infection, early amplification of pro-inflammatory responses has been shown to be beneficial, whereas amplification during the late stage of infection, i.e. sepsis, has proven deleterious, leading to organ failure and death [37, 38]. Accordingly, *in vivo* ligation of TREM-1 in an acute infectious model with *S. pneumoniae* results in a rapid potentiation of the immune response. Six hours after infection, mice treated with mAb had a two-fold increase in message levels of TNFα, IL-6, and MIP2, and by 48 hours, TREM-1 mAb treated mice exhibited a lower bacterial burden and increased survival over those treated with control mAb [39]. Alternatively, the administration of TREM-1 mAb during a murine model of LPS induced sepsis increased lethality from 50% to 100% [40]. Therefore, whereas activation of TREM-1 during an acute infection can enhance bacterial clearance in a pro-survival manner, TREM-1 ligation during sepsis causes excessive inflammation and increased mortality.

From these data, one would hypothesize that interference with TREM-1 signaling would cause a reduction in pro-inflammatory processes. Notably, siRNA silencing of TREM-1 in a mouse model of endotoxic shock mediated a decrease in TNFα, IL-6, and IL-1β cytokine levels, and increased survival [40]. Moreover, blockade of endogenous TREM-1 signaling using a recombinant TREM-1 fusion protein (rTREM-1) in multiple models of sepsis resulted in a similar reduction of septic shock induced death [41]. Lastly, treatment of mice with LP17, a small peptide inhibitor of TREM-1, conferred protection against sepsis-induced death in a model of cecal ligation and puncture (CLP)

[42]. These experiments show that inhibiting TREM-1 function during polymicrobial sepsis dampens excessive inflammation resulting in increased survival rates. Conversely, complete abrogation of TREM-1 signaling diminishes protection during bacterial challenge. *TREM-1*^{-/-} mice challenged intratracheally with *P. areuginosa* exhibited higher bacterial burdens, increased inflammatory cytokines, and less histological inflammation compared to their wild type counterparts, ultimately leading to an increase in morbidity. Upon further study, it was found that loss of TREM-1 inhibited neutrophils from crossing the epithelial barrier from the bronchioles into the alveolar space [43]. These data demonstrate that TREM-1 plays an important role in the balance between immune function and immunopathology.

The functional synergy observed between TLR and TREM signaling suggests that there is most likely a point, if not multiple points, at which their respective signaling pathways intersect [44]. Concomitant activation of neutrophils by LPS and an anti-TREM-1 mAb results in more rapid and increased PI3K/PLCγ mediated phosphorylation than does activation with either stimulus alone, suggesting that TREM-1 signaling enhances cellular activation in a calcium-dependent manner [45]. In addition, LPS activation of macrophages in which TREM-1 expression has been silences using siRNA showed diminished message levels of effector cytokines (IL-10, IL-1β), adapter molecules (MyD88), and transcriptional regulators (IκBα), again suggesting intersections between the TREM-1 and TLR-4 signaling pathways [46]. Of note, LPS or TREM-1 mAb induced colocalization of TREM-1 and TLR-4 in lipid rafts, further supporting the possibility that TREM-1 signaling drives TLR-4 into lipid rafts, thereby enhancing the stability of TLR-4 dependent signaling processes [47]. In effect, the interplay between

TLR and TREM-1 signaling components further indicates the important role TREM-1 plays in amplifying myeloid responses to LPS stimulation, and that regulating its function is important for surviving bacterial sepsis.

TREM-2. Like TREM-1, TREM-2 is also expressed on myeloid cells, but interestingly its expression is limited to dendritic cells (DCs) and macrophages. Unfortunately, impaired TREM-2 function manifests as Nasu-Hakola disease (NHD), or polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy (PLOSL). Mutations in or the deletion of TREM-2 and/or its paired receptor DAP12 cause multiple bone cysts, often accompanied with a dementia-like neurodegeneration - resulting in premature death usually during the fourth decade of life [48]. Due to this severe phenotype, characterization of TREM-2 has focused on its expression in tissue-specific macrophages, specifically microglia of the central nervous system (CNS) and osteoclasts (OCs) in bone.

Interestingly, TREM-2 expression appears to be regulated in the opposite manner of TREM-1. Using macrophage and microglial cell lines, Piccio et. al. [49] have shown that TREM-2 protein is expressed on the surface, but is quickly downregulated in response to LPS stimulation. This rapid loss suggested that TREM-2 was either shed from the cell surface or internalized; the latter appeared to be the case as intracellular stored pools of TREM-2 were detected in microglia, and are shuttled to and from the plasma membrane by exocytosis and membrane recycling [48]. Other groups have demonstrated that DCs and macrophages derived from either bone marrow or monocytes rapidly decrease TREM-2 surface expression in response to TLR stimulation [50-52].

Functionally, TREM-2 appears to modulate inflammatory cytokine production and phagocytosis in macrophages. Lentiviral-mediated over-expression of TREM-2 resulted in enhanced microglial phagocytosis of apoptotic neurons, and greater inhibition of TNFα, IL-1β, and NOS2 transcription. Conversely, silencing of TREM-2 using shRNA increased these cytokine transcript levels and decreased phagocytosis [50]. Further analysis revealed that crosslinking TREM-2 on microglia potentiates phagocytosis through ERK-dependent polarization and reorganization of actin [53]. Furthermore, *TREM-2$^{-/-}$* mice had attenuated cytokine transcript production by microglia in response to ischemic brain injury as compared to control mice [54]. Finally, in a murine model of multiple sclerosis, mice treated with an antagonsitic TREM-2 mAb displayed exacerbated disease with a greater leukocyte infiltration and increased inflammation [49]. Collectively, these data suggest that TREM-2 plays an important role in the ability of microglia to control inflammation in the CNS.

Less is known regarding TREM-2 function in dendritic cells. TREM-2 ligation on immature DCs induces maturation, as demonstrated by ERK-dependent upregulation of CCR7, MHC II, and CD80/86 [51]. Additionally, *TREM-2$^{-/-}$* mature bone marrow-derived DCs produce significantly higher levels of the inflammatory cytokines TNFα, and IL-6 upon stimulation with TLR ligands, and exhibit increased maturation and antigen presenting capability than their wild type counterparts [55]. These data imply that TREM-2 has an active role in negatively modulating inflammatory responses in both microglia and DCs.

TLT1. The third of the four conserved TREM family members is TREM-like transcript 1, or TLT1. As aforementioned, TLT1 does not associate with the DAP12 signaling subunit as it posseses a cytoplasmic tail containing both a classical and non-classical ITIM motif. Biochemical analysis has revealed that residue Y281 in its classical ITIM is necessary for interactions with SHP2 after phosphorylation, implying that TLT1 has the potential to inhibit cellular processes [56]. Further characterization of its expression established that TLT1 is uniquely expressed within the α-granules of platelets and megakaryocytes, with little to no expression on the surface of resting platelets. Upon activation with TRAP (thrombin receptor agonist peptide), TLT1 is rapidly translocated to the platelet surface and colocalizes with P-selectin (CD62). In addition, mAb-mediated crosslinking of TLT1 and FcεR1 results in enhanced, rather than diminished, calcium signaling [57]. Although TLT1 exhibits classical inhibitory motifs and interactions, it may instead play an activating role in platelet biology.

Increased TLT1 expression on the surface of activated platelets suggests it may have a plausible role in modulating coagulation. Indeed, blockade of TLT1 by the addition of single chain mAbs in a platelet aggregation assay, caused a decrease in thrombin induced platelet aggregation [58]. Characterization of TLT1 signaling pathways through coimmunopreciptiation studies defined cytoskeletal proteins of the ezrin-radixin-moesin (ERM) family as intracellular binding partners, thus elucidating a part of the mechanism by which TLT1 augments platelet aggregation. Fittingly, platelets from *TLT1*$^{-/-}$ mice aggregate less efficiently when activated by thrombin, and these mice have impaired clotting, as demonstrated by doubled bleeding times over wild type-control

mice [59]. It would appear that TLT1 is an important component of the coagulation process, specifically mediating its effects through actin-dependent aggregation.

TLT2. The last conserved, and possibly most unique, TREM family member is TREM-like transcript 2, or TLT2. As other TREMs are distinctly expressed on specific sets of myeloid cells, it is of note that TLT2 is expressed on cells of both the myeloid and lymphoid lineage, including neutrophils, monocytes, macrophages, and T and B cells [60-62]. Early characterization delineated TLT2 as the first of the TREM family to be expressed on cells of the lymphoid lineage. TLT2 was found not only on resting neutrophils and macrophages, but on B cells as well. TLT2 can be detected on B cells as early in development as B220, and its expression is maintained throughout development and differentiation. Unlike other TREM members, TLT2 on the B cell surface was not altered in response to TLR stimulation. Non-lymphoid expression of TLT2 extends to alveolar and peritoneal macrophages, as well as developing and circulating neutrophils. Of note, *in vivo* administration of LPS increased surface levels of TLT2 on both neutrophils and peritoneal macrophages [61]. Hashiguchi et. al. reported that TLT2 is expressed by resting CD8$^+$ T cells, as well as activated CD4$^+$ and CD8$^+$ T cells [60]. Recently, TLT2 expression was described in the human immune system. TLT2 was measured on resting B cell and monocyte subsets of peripheral blood leukocytes (PBLs), as well as on CD3$^+$ T cells upon activation with phytohaemagglutinin (PHA) [62]. As such, like other TREMs, TLT2 is expressed on myeloid cells, but uniquely, extends to the lymphoid lineage.

Recent functional analysis of TLT2 has documented multiple roles for it in the immune system. Early reports indicated that TLT2 expressed on T cells is an *in vitro* binding partner for B7H3, a costimulatory molecule present on antigen presenting cells (APCs). Furthermore, this binding interaction enhanced T cell proliferation and IFNγ production [60]. Contradictory work has been published demonstrating that TLT2 and B7-H3 do not interact, and that TLT2 does not enhance T cell responses [63]. In this regard, the function of TLT2 on T cells has yet to be fully determined. Alternatively, TLT2 function in myeloid cells appears to modulate processes involved in pathogen clearance. Ligation of TLT2 in neutrophils enhances degranulation, ROS production, and chemotaxis in response to $G\alpha_i$ GPCR agonists, but does not alter phagocytosis [64]. Meanwhile, TLT2 has been shown to modulate macrophage-dependent efferocytosis, as TLR3 activation of macrophages not only upregulates TLT2 expression, but binding of TLT2 on the surface of TLR3 activated macrophages augments engulfment of apoptotic cells. Also of note, overexpression of TLT2 *in vitro* led to an increase in specific phagocytosis of apoptotic cells [65]. Functionally, TLT2 appears to play a role in enhancing responses of myeloid cells during bacterial infections and resolution of inflammation.

Soluble TREMs

A common mechanism of regulating surface receptor expression is protein shedding. During this process a receptor can be cleaved from the membrane and released thereby inhibiting further transduction of intracellular signals. This mechanism of releasing a soluble functional ectodomain is widespread and used abundantly in the

immune system as a method to regulate inflammation [66]. A good example of this process is the shedding of the TNFα receptor from cells to stop pro-inflammatory signals mediated by TNFα [67]. Recent studies have discovered soluble versions of three of the conserved TREM proteins, indicating that soluble isoforms or receptor shedding may be a conserved method of TREM molecule regulation during immune responses.

As TREM-1 ligation is important for enhancing the early immune response to bacterial infection, its activation during the later stages of infection or sepsis is deadly. Thus, it would be rational to hypothesize that a mechanism exists to rapidly regulate its expression and/or activation. Administration of a recombinant form of TREM-1, rTREM-1, as a decoy receptor during murine sepsis showed that blockade of TREM-1 signaling increased survival [41]. These data, along with the detection of endogenous soluble versions of TREM-1, sTREM-1, in the serum of both mice and humans in response to infection [68, 69], lends credence to the theory that soluble forms of TREM regulate the function of their membrane bound counterparts.

Two established origins of sTREM-1 have been documented in the literature. Evidence of TREM-1 receptor shedding was identified as protease inhibitor pretreatment of LPS activated monocytes reduced levels of sTREM-1 in culture supernatants [42, 70]. Alternatively, multiple TREM-1 transcripts have been found in monocytes, and treatment of LPS-stimulated neutrophils with cycloheximide abolished the detection of sTREM-1 in supernantants. These studies imply that sTREM-1 may also be generated by alternative splicing [71, 72].

The identification of sTREM-1 has provided a valuable clinical tool for the detection of inflammation and sepsis. Many reports have documented elevated levels of

sTREM-1 in patients with a variety of diseases [73-75]. Interestingly, there are multiple reports correlating serum sTREM-1 concentration and the severity of sepsis. Su et. al. [76] showed that the detection and presence of sTREM-1 served as a valuable biomarker for determining the severity of sepsis and its prognosis.

Limited data are available concerning the existence of a soluble form of TREM-2. *In vitro* analysis demonstrated that microglia specifically express an alternative splice form of TREM-2, dubbed svTREM-2b, which lacks a TM region [77]. Recent reports of a soluble form of TREM-2, or sTREM-2, identified in the cerebro-spinal fluid (CSF) of multiple sclerosis patients as well as other inflammatory neurodegenerative disease patients, mimic the positive correlation of sTREM and disease as demonstrated with TREM-1. It remains unclear whether the predicted sv-TREM-2b isoform and the physiological sTREM-2 variant are one and the same. It is reasonable to hypothesize that sTREM-2 may affect ligand binding to membrane bound TREM-2, thereby inhibiting its known role of downmodulating inflammatory responses in the CNS [78].

Recently, a soluble form of TLT1, sTLT1, was detected in the serum of healthy humans and mice, and in the supernatants of thrombin-activated human and murine platelets [79]. Stimulation of platelets with sTLT1 increased actin polymerization and filipodia formation *in vitro* [69]. Due to increased platelet aggregation, it was pertinent to discern whether sTLT1 would be expressed in late stage sepsis, when coagulation leads to advanced organ failure [80]. As predicted, sTLT1 concentrations were extremely high in non-surviving septic patients, and sTLT1 levels positively correlated with increased disseminated intravascular coagulation (DIC) scores [81]. Further study is necessary to understand the exact role of sTLT1 in regulating membrane-bound TLT1 function.

To date, there are no predicted or known soluble forms of TLT2. However, as this seems to be a conserved element of TREM proteins, it seems likely that a soluble version does exist. The identification of soluble TREMs makes a strong case for the self-regulation of TREM-dependent function during the immune response.

TREM Ligands and Heterotypic Interactions

To better understand the role of TREM molecules during the immune response, it is necessary to identify their natural ligands. To date, there are reports of multiple putative binding partners for TREM proteins. Their extracellular domains and Ig structure are highly pleiomorphic and could allow for binding of various epitopes, which lends support to the variety of ligands identified, both endogenous and exogenous in nature.

Most work concerning the ligand of TREM-1 comes from studies of TREM-1 function during sepsis. Use of rTREM-1 to block membrane bound TREM-1 signaling resulted in better survival rates for mice undergoing endotoxic shock [41]. These results implied that TREM-1 most likely bound an endogenous ligand. Shortly thereafter it was shown that rTREM-1 binds to platelets in a specific manner, and that coincubation of platelets and LPS-activated neutrophils enhanced ROS and cytokine production in a TREM-1 specific manner. These findings indicated that the natural ligand for TREM-1 is present on the surface of platelets [82]; however what this interacting partner is has yet to be elucidated. To date, there is only one report of an exogenous ligand for TREM-1; the addition of sTREM-1 to neutrophils prior to activation with filoviruses inhibited neutrophil responses in a TREM-1 specific manner [83]. Therefore, it is likely that both endogenous and exogenous ligands exist for TREM-1.

Early work determining TREM-2 ligands elegantly showed that TREM-2 bound cell wall components of gram negative bacteria through recognition of cell wall components including LPS, LTA, and peptidoglycan, and that these interactions could be inhibited by the addition of negatively charged anionic carbohydrates such as dextran sulfate. Additionally, TREM-2 binding of astrocytoma cells was also abolished upon pretreatment with dextran sulfate [84]. Ultimately though, the search for TREM-2 ligands has been highly influenced by knowing that its absence causes NHD. Within this context, many have looked for endogenous ligands in both the brain and bone in hopes of understanding how the loss of TREM-2/DAP12 interactions with ligand lead to this debilitating disease. Takegahara et. al. [85] demonstrated that TREM-2/DAP12 interactions with Plexin A-1 enhanced dendritic cell activation, and that *Plexin A1$^{-/-}$* mice exhibited a very similar phenotype to TREM-2/DAP12 deficient mice. This evidence of a multimeric complex and a cis-interacting ligand for TREM-2 suggests that to determine the natural endogenous trans-interacting ligand of TREM-2, it may be necessary to look for interactions with co-receptors as well. The proclivity of TREM-2 to bind anionic substrates, as well as its documented trans interactions with other proteins, has complicated the search for TREM-2 ligands, but indicates it may have multiple ligands.

Ligand identification for TLT1 has been more straightforward than for other TREMs. The knowledge that mAb binding of TLT1 on washed platelets abrogated aggregation implied that the ligand for TLT1 was on platelets. To discern the interacting partner, platelet lysates were run over recombinant TLT1 (rTLT1) columns, extensively washed, and eluted proteins were identified by mass spectrometry. Fibrinogen was found to interact with membrane bound TLT1 and to stabilize platelet aggregates, whereas

rTLT1 also bound fibrinogen on the platelet surface, yielding both cis and trans-acting endogenous ligands for TLT1 [81].

As mentioned previously, TLT2 was predicted to serve as a binding partner for B7H3, an APC costimulatory molecule, revealing a possible endogenous TLT2 ligand. Conflicting accounts of whether TLT2 actually binds B7-H3 make it difficult to say with certainty that the ligand has been found [60, 63]. Alternatively, another putative ligand for TLT2 is phosphatidyl serine (PS). TLT2 was shown to specifically bind PS, which is found on apoptotic outer cell membranes, as opposed to phosphatidyl choline (PC), present on the membrane of live cells. Also, the addition of anti-TLT2 mAbs or annexin blocked TLT2 binding to PS in a solid phase ELISA assay, further demonstrating that one of the endogenous ligands for TLT2 is PS [65]. As the ectodomain of TLT2 contains a positively charged loop, it is possible that TLT2 binds anionic molecules like TREM-2 does, but this has yet to be confirmed [61]. Therefore, the exogenous ligand for TLT2 remains to be confirmed, although PS appears to be a putative endogenous ligand.

Recently, interesting reports of TREM family crosstalk have been published, mostly revolving around TLT1 isoforms and their function in two separate physiological processes. Derive et. al. [86] demonstrated that activated neutrophils secrete a TREM-1 ligand, and rTLT1 competitively inhibits rTREM-1 binding to this counterpart. As the TREM-1 ligand is also expressed on platelets, rTLT1 and rTREM-1 competitively inhibit each other's binding to washed platelets. Currently, there is no data on whether TREM-1 also binds fibrinogen like TLT1. Additionally, rTLT1 diminished the effects of TREM-1 and LPS synergistic activation of monocytes by reducing cytokine levels and intracellular

phosphorylation [86]. This interaction between TLT1 and TREM-1 appears to be another way the TREM proteins self-regulate during inflammation.

Interestingly, a shortened transcript of TLT1, TLT1-s, has been discovered in osteoclasts, and this form can inhibit TREM-2 signaling during osteoclastogenesis. Silencing of TLT1-s enhanced calcium signaling and augmented osteoclastogenesis of pre-osteoclasts in a TREM-2 dependent manner [87]. This TREM family crosstalk, as well as reports of multiple endogenous and exogenous ligands, suggests that there may not be one designated ligand for these proteins, but in fact TREM family members may have multiple binding partners, reflecting their modulation of a variety of cellular functions.

Table 1. TREM Family Properties

TREM MEMBER	SPECIES	EXPRESSION	SOLUBLE FORM	KNOCK OUT MOUSE
TREM-1	Mouse Human	Monocytes Neutrophils	Yes	Yes
TREM-2	Mouse Human	Macrophages Dendritic Cells	Yes	Yes
TLT1	Mouse Human	Platelets	Yes	Yes
TLT2	Mouse	B cells Neutrophils Macrophages	Unknown	No

Summary

In closing, the TREM family is emerging as a group of proteins involved in modulating various aspects of the immune response by both activating and inhibiting processes such as cytokine production, cell migration, and phagocytosis. Soluble versions of these molecules may be useful biomarkers of inflammation, as they have been detected

in the fluids of patients with inflammatory diseases. The existence of both endogenous and exogenous ligands for these molecules may facilitate TREM involvement in a variety of physiological processes. Lastly, among TREM family members, little is known regarding TLT2 biology, which is the focus of this thesis.

TREM-LIKE TRANSCRIPT 2 EXPRESSION IS MODULATED IN RESPONSE TO INFLAMAMTORY MEDIATORS AND STORED IN NEUTROPHIL GRANULES

by

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ABSTRACT

The TREM gene cluster encodes a family of proteins that are emerging as a highly interactive set of molecules involved in modulating the immune response. Currently, of the four conserved members, few studies exist on TLT2 expression and function. Our group has previously characterized TLT2 expression within the murine immune compartment, demonstrating that ligation of TLT2 potentiates neutrophil chemotaxis. In this study, experiments were performed to determine if TLT2 expression is conserved across species. Indeed, human TLT2 is expressed on cells of both the myeloid and lymphoid lineage, similarly to murine TLT2. Additionally, consistent with other TREM family members, its expression is modulated in response to inflammatory mediators. Furthermore, neutrophil expression of TLT2 is predominantly localized to intracellular pools, specifically secretory vesicles and primary granules; in contrast to other primary granule proteins it is not expelled on neutrophil extracellular traps. In summary, these data establish that TLT2 expression is conserved between mouse and human immune compartments, and through localization studies, provide novel insights into TLT2 biology.

ABBREVIATIONS

1C5	anti TLT2 monoclonal antibody
DAP12	DNAX activating protein 12
DIC	disseminated intravascular coagulation
FMLF	n-formyl met-leu-phe
IgSF	immunoglobulin super-family
LPS	lipopolysaccharide
mAb	monoclonal antibody
MPO	myeloperoxidase
NET	neutrophil extracellular trap
PAMP	pathogen associated molecular pattern
PBL	peripheral blood leukocytes
PBMC	peripheral blood mononuclear cells
PMN	polymorphonuclear cell
PRR	pattern recognition receptor
TLR	Toll-like receptor
TLT1	TREM-like transcript 1
TLT2	TREM-like transcript 2
TREM	Triggering Receptor Expressed on Myeloid cells

INTRODUCTION

Initiation of an acute inflammatory response plays a critical role in clearance of infectious organisms by the immune system. Cells of the body use an innate germ-line encoded set of receptors, known as pattern recognition receptors (PRR), to distinguish self from non-self through their affinity for evolutionarily conserved pathogen associated molecular patterns (PAMP) [1, 2]. Upon recognition and binding of PAMPs, PRRs mediate signals that induce cellular changes to enhance processes required for microbial clearance. This includes the activation of pro-inflammatory transcription factors, which may turn on genes encoding soluble mediators as well as costimulatory and effector molecules [3]. Collectively, these alterations in the cell are the beginning of the inflammatory response, during which leukocytes are activated, recruited to sites of infection, and subsequently eliminate the infection by killing and clearing microbial pathogens.

Cell to cell communication and the modulation of effector functions are both necessary for regulating inflammation and are mediated through another group of innate receptors belonging to the immunoglobulin super-family (IgSF) [4-6]. A more recently studied subclass of IgSF receptors, the Triggering Receptor on Myeloid cells (TREM) family, have been shown to modulate cellular responses to pathogenic stimuli as well as to enable cellular crosstalk [7, 8]. There are four conserved members of the TREM locus: TREM-1, TREM-2, TREM-like transcript 1 (TLT1), and TREM-like transcript 2 (TLT2) [9]. In the past decade, many groups have attempted to characterize their function and what role they play during the immune response. The two initially identifed members, TREM-1 and TREM-2, have minimal cytoplasmic tails, and thereby mediate their

functions through pairing with DAP12 in the plasma membrane [10, 11]. TREM-1 is expressed on monocytes and neutrophils, and its expression is upregulated in response to inflammatory stimuli, such as LPS. Ligation of TREM-1 results in synergistic amplification of TLR signaling and cytokine production [10]. Whereas TREM-1 potentiates the inflammatory response on neutrophils and monocytes, TREM-2 activation appears to be involved in dampening inflammatory responses of macrophages and dendritic cells. Upon activation by microbial components, TREM-2 expression is decreased, and activation of TREM-2 attenuates TLR-induced cytokine production [12, 13].

The TREM-like molecules, TLT1 and TLT2, are unique as they do not couple with DAP12 at the membrane, but transduce signals by means of specific motifs in their short cytoplasmic tails [9, 14, 15]. TLT1 is stored in the alpha granules of platelets and megakaryocytes and its expression is upregulated in response to platelet activation, where it plays a role in potentiating clotting through binding of fibrinogen and enhancing cytoskeletal rearrangement [16]. The least well characterized TREM family member is TREM-like transcript 2, or TLT2. Our group and others have demonstrated that it is the only member of the TREM family expressed on cells of both myeloid and lymphoid lineages, and TLT2 expression is augmented *in vivo* in response to inflammatory stimuli [17, 18].

As TREM-1, TREM-2, and TLT1 expression are conserved between mouse and man, it was of interest to identify whether TLT2 expression was also conserved between species. We found that not only is its expression conserved, but its modulation in response to inflammatory stimuli is as well. TLT2 is the only TREM to be expressed on

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cells of both the lymphoid and myeloid lineage, and is therefore unique as it appears to be the most promiscuously expressed TREM. Of further note, TLT2 is located within multiple cellular compartments in human neutrophils, including the plasma membrane, as well as in both secretory vesicles and primary granules. Lastly, although TLT2 is stored in late stage effector granules, it is not extruded on DNA during netosis like other granule proteins are such as MPO [19-21].

MATERIALS AND METHODS

Patient samples

Venous blood was isolated from healthy volunteers according to Institutional Review Board (IRB) requirements.

Reagents

Recombinant human IL-8 (#200-08) was purchased from Peprotech (San Diego, CA), and recombinant human C5a (#2037-C5-025/CF), IFNγ (#285-IF-100/CF), and GM-CSF (#215-GM-010/CF) were purchased from R&D Systems (Minneapolis, MN). Polymorphprep (#1114683) was purchased from Axis-Shield (Oslo, Norway) and used per the manufacturer's protocol. DAPI (#D9564-10MG), LPS (#L5293-2ML), FMLF (#F3506-5MG), and Cytochalasin B (#C27-43) were purchased from Sigma (St. Louis, MO). Phosphate buffered saline (PBS, #10010-023), Hank's balanced salt solution (HBSS, #14025-092), RPMI 1640 media (RPMI, #11875-093), ACK Lysis Buffer (ACK, #A10492-01), and fetal calf serum (FCS, #10437-028) were purchased from LifeTechnologies (Carlsbad, CA). Magnetic cell separation buffer (MACS buffer) was prepared from PBS supplemented with 2% FCS and 1mM EDTA and then sterile filtered.

Antibodies

The anti-TLT2 monoclonal antibody (mAb) 1C5 was generated as previously described [17]. Both 1C5 and isotype control (anti KLH, rat IgG1) mAbs were tested for endotoxin (Endotoxin kit, #88282, Thermo Pierce, Rockford, IL), and shown to have less than 0.01 endotoxin units (EU)/mL. All antibodies were titrated for optimal staining. CD19-PECy7

(#561379) was obtained from BD (San Diego, CA). The following antibodies for flow cytometry were obtained from BioLegend (San Diego, CA): CD11b-PE (#301306), CD86-A488 (#305414), HLADR-PB (#307624), CD3-APCCy7 (#300318), CD14-PerCPCy5.5 (#325621), CD63-PB (#353012), CD66b-PerCPCy5.5 (#305108). The following antibodies were also obtained from BioLegend in purified form and conjugated to Alexa-488 using the monoclonal antibody conjugation kit (#A201-81) from LifeTechnologies: CD35 (#333402), CD63 (#353013), CD66b (#305102). Myeloperoxidase (MPO) mAb was purchased from AbDSerotec (#0400-0002, Raleigh, NC), and an A555-conjugated goat anti-mouse IgG2b secondary antibody from LifeTechnologies (#A-21147).

Flow cytometry

All assays requiring flow cytometry followed the same protocol unless otherwise noted. Washes were performed with FACS buffer (PBS supplemented with 2% FCS and then sterile filtered) and centrifuged at 400g at 4 °C for 5 min. Endogenous Fc binding was blocked using Human TruStain FcX (#422302, BioLegend) at a 1:50 dilution in FACS buffer for 15 min on ice, before antibody incubations were performed in FACS buffer on ice for an additional 30 min. Data were acquired using a BD LSR II and analyzed with FlowJo software (TreeStar, Ashland, OR).

Cell isolation

For neutrophil isolation, whole blood was layered 1:1 over polymorph prep and centrifuged at 550g at 21 °C for 35 min with no brake. Both the upper band containing

peripheral blood mononuclear cells (PBMCs) and the lower band containing polymorphonuclear cells (PMNs) were removed and washed once in HBSS. Residual erythrocytes were lysed in 3 mL of ACK and cells were washed and resuspended in RPMI. For peripheral blood leukocyte (PBL) isolation, whole blood was mixed in 2:1 volumetric ratio with 6% sodium dextran in 0.9% NaCl, inverted 15-20 times, and left for 40 min to sediment erythrocytes. The straw colored upper layer was removed, diluted with PBS and centrifuged to pellet leukocytes. Residual erythrocytes were lysed as above, and cells were washed and resuspended in RPMI to a working concentration. Monocytes were sorted from PBMCs using $CD14^+$ magnetic beads (#130-050-251, Miltenyi, Auburn, CA), washed and resuspended to a working concentration in RPMI.

Phenotypic analysis

Human PBLs were washed once in FACS buffer and resuspended to a concentration of 1×10^6/mL in FACS buffer. Aliquots of 100 μL were plated in 96-well round bottom plates, blocked with Human TruStain FcX, then stained with 1C5-A647, CD16-A488, CD13-PE, CD14-PerCP Cy5.5, CD19-PECy7, and CD3-APCCy7, washed, and analyzed by flow cytometry.

Myeloid cell activation

Neutrophils were resuspended in RPMI to a concentration of 1×10^6/mL, and 100 μL aliquots were placed in 96-well plates and rested in a 37 °C 5% CO_2 incubator for 15 min. Various activating factors were diluted in RPMI to a 2x working concentration, then added to cells in 100 μL aliquots for the indicated times. To stop activation, the cells

were placed on ice for 10 min and centrifuged at 400g at 4 °C for 10 min. Cells were blocked with Human TruStain FcX, then stained with 1C5-A647, CD11b-PE, and CD14-PerCPCy5.5, washed, and analyzed by flow cytometry.

Granule localization

Isolated PMNs were resuspended to 5×10^5/mL in PBS, fixed in 4% paraformaldehyde (PFA) for 10 min at RT, rinsed with PBS, and permeabilized with 0.5% saponin for 10 min at RT. After permeabilization, cells were stained using the aforementioned flow cytometry protocol, but with washes/stains performed in 0.2% saponin. Briefly, cells were blocked with Human TruStain FcX, then stained with 1C5-A647 and one of the following: CD35-A488, CD63-A488, or CD66b-A488. Stained cells were washed once in PBS and cytospun onto slides at 350 rpm at RT for 3 min with medium acceleration. Slides were rinsed in PBS, then incubated for 5 min with DAPI (1 μg/mL), washed in PBS, and mounted with coverslips using Prolong Gold Antifade (#P36930, LifeTechnologies). Slides were imaged using a Nikon Eclipse Ti microscope and NIS Elements software.

Degranulation assays

Neutrophils were resuspended to 1×10^6/mL in RPMI, and treated with Cytochalasin B (Cyto B, 5 μg/mL) or vehicle (DMSO) for 10 min in a 37 °C 5% CO_2 incubator, before addition of FMLF (100 nM) or LPS (100 ng/mL) for 30 or 60 min. The reaction was stopped by placing cells on ice and diluting with ice cold PBS. Cells were blocked as

above and stained with 1C5-A647, CD66b-PerCPCy5.5, CD63-PB, and CD11b-PE, washed, and analyzed by flow cytometry.

NET induction

Neutrophils were resuspended to 1×10^6/mL in RPMI and a 200 µL aliquot of suspension was added to each well of a chamber slide (#PEZGS0816, Millipore, Billerica, MA), and subsequently rested for 30 min in a 37 °C 5% CO_2 incubator to allow time for adherence. Supernatants were carefully removed and replaced with RPMI containing vehicle (DMSO) or 100 nM PMA and incubated for 4 h in a 37 °C 5% CO_2 incubator. After stimulation, supernatants were removed and cells were washed three times in PBS so as not to disturb NET formation, and then fixed in 4% PFA for 10 min at RT. Slides were blocked with 2.5% FCS in PBS for 10 min at RT, then incubated with Human TruStain FcX for 15 min at RT. Slides were incubated with MPO primary antibody (1:250) for 30 min, washed, then incubated with secondary (goat anti-mouse IgG2b A555) and either isotype control (rat IgG1 A488) or 1C5-A488 for an additional 30 min. Slides were rinsed in PBS, incubated for 5 min with DAPI (1 µg/mL), washed, mounted with coverslips using Prolong Gold Antifade and imaged using a Nikon Eclipse Ti microscope and NIS Elements software.

Statistical analysis

Statistical significance was determined by one-way ANOVA or the unpaired students' t-test using Prism software (Graph Pad, La Jolla CA). Statistical significance is denoted as * $p < 0.05$.

RESULTS

TLT2 expression on circulating human leukocytes

Our group has previously described murine TLT2 expression as the only TREM member found on cells of both the myeloid and lymphoid lineages [17]. To ascertain whether TLT2 expression is conserved between mice and humans, resting PBLs were isolated from venous blood (n=10 donors) and analyzed for surface TLT2 by flow cytometry. Side scatter vs. TLT2 showed a distinct pattern of TLT2 expression (Fig. 1). When each subpopulation is gated and stained with lineage markers, TLT2 is most highly expressed on B cells ($CD19^+$), followed by monocytes ($CD14^+$) and granulocytes (high side scatter, $CD14^{low}$), but with little to no expression on T cells or NK cells. This same expression profile is observed in the murine immune compartment, revealing that TLT2 expression is conserved between species.

TLT2 expression is upregulated on myeloid cells in response to TLR ligation

Other groups have shown that TREM protein expression is modulated in response to inflammatory stimuli. TREM-1 is upregulated on monocytes and neutrophils in response to LPS [10], whereas TLT1 expression is also increased on platelets activated with LPS [16]. To determine if surface TLT2 is altered in response to LPS, neutrophils were activated with LPS (0-1000 ng/mL) for 30 min. Stimulation with LPS results in a rapid increase of TLT2 on the surface of neutrophils (Fig. 2B). These data mimic *in vivo* modulation of TLT2 expression on murine neutrophils after LPS administration, supporting the conclusion that myeloid upregulation of TLT2 is a conserved aspect of TLT2 biology.

Modulation of TLT2 expression by inflammatory mediators

It was of interest to determine if other common soluble mediators involved in inflammation could also alter neutrophil TLT2 expression. As PMNs respond to many different classes of molecules, including growth factors (e.g. GM-CSF), chemokines (e.g. C5a & IL-8), bacterial products (e.g. LPS & FMLF), and cytokines (e.g. IFNγ), it was of interest to determine if TLT2 expression could be modulated in response to any of these factors. All stimuli, except IFNγ, caused immediate and sustained activation as demonstrated by CD11b upregulation (Fig. 3A). Notably, FMLF and LPS resulted in the only significant increase in TLT2 expression, whereas chemokines C5a and IL8 and the growth factor GM-CSF induced a minimal effect on its expression (Fig. 3B). These data suggest neutrophils upregulate TLT2 surface expression primarily in response to bacterial products as opposed to other inflammatory mediators.

TLT2 is stored in intracellular pools

Other TREM proteins have been shown to be stored in vesicles and their surface expression modulated through movement of these stores upon cellular activation. Specifically, TLT1 is located in the α-granules of platelets and megakaryocytes, and is translocated to the surface upon activation by bacterial peptides and clotting factors [16]. Prada et. al. demonstrated that TREM-2 is stored in vesicles within microglia, and is consistently shuttled back and forth from the plasma membrane in response to activating stimuli [22]. As TLT2 expression is rapidly increased on neutrophils in response to stimulation with bacterial products (Figs. 2 and 3), we hypothesized that it may be stored

in granules for immediate transport to the cell surface in response to environmental changes. Indeed, on resting PMNs, TLT2 stains in a punctate manner, reminiscent of a granular pattern (Fig. 4). In order to determine which vesicles TLT2 may be stored in, neutrophils were co-stained with markers specific for different types of granules. TLT2 does not completely colocalize with any granule set, as seen with other markers [23-25]. There is a small percentage of overlap with CD35, or complement receptor 1 (CR1), a marker of secretory vesicles, as well as CD63, or tetraspannin LAMP-3, a membrane component of primary granules (Fig. 4B–C). Conversely, TLT2 and CD66b, a marker of secondary and tertiary granules, do not colocalize at all, but are positioned at separate subcellular compartments (Fig. 4A).

In order to determine if TLT2 is predominantly stored in intracellular pools, neutrophils were activated with LPS, FMLF, or FMLF + Cytochalasin B, a known actin depolymerizing agent which induces complete degranulation. We have shown that neutrophil activation with LPS increases surface TLT2 expression two-fold (Figs. 2 and 3). FMLF + Cyto B-induced neutrophil exocytosis results in a 25-fold increase in surface TLT2, which mimics CD63 release (Fig. 5). This enhanced TLT2 expression after complete degranulation is consistent with TLT2 storage within the primary granules.

TLT2 is not a component of NETs

Upon activation with pathogenic stimuli, neutrophils expel their DNA as an anti-microbial weapon, whereby microbicidal contents contained within the primary granules (e.g. MPO, defensins, cationic peptides) are strewn across the stretched out DNA creating

neutrophil extracellular traps (NETs). This process, commonly known as netosis, is a recently discovered neutrophil-specific method of killing microbes [19, 20]. As it appears that TLT2 is stored in primary granules (Figs. 4 and 5), experiments were performed to determine if TLT2 is extruded on NETs, to possibly serve some antimicrobial function. Neutrophils were activated with the classic NET inducer, PMA, and stained for TLT2 and MPO, an antimicrobial enzyme stored in primary granules that is thrown out and coats the surface of NETs. Surprisingly, unlike MPO (red), upon netosis TLT2 (green) is not strewn across DNA (DAPI, blue) (Fig. 6). Of note, TLT2 maintains a punctate staining pattern, suggesting that it is retained in the cytosolic remnants of the cell (Fig. 6).

DISCUSSION

These data further characterize TLT2 expression in humans and demonstrate that its expression is indeed conserved across species. It was shown that resting human B cells, monocytes, and neutrophils basally express cell surface TLT2, but when activated by inflammatory mediators, neutrophils upregulate TLT2. Furthermore, large intracellular pools of TLT2 can be identified in neutrophils, and are localized to three distince vesicular compartments within PMNs. Lastly, although it is largely stored in primary granules, unlike other primary granule proteins TLT2 is not extruded on DNA upon netosis.

As murine TLT2 is the only TREM member documented on non-myeloid cells, and the profiles of TREM members are conserved, it was important to see if this expression pattern would be maintained across species. Indeed, human TLT2 is also expressed on B cells as well as myeloid cells (Fig. 1). Of note, B cells exhibit the highest TLT2 surface expression levels; this same profile is observed upon staining of mouse leukocytes for TLT2. Contrary to reports of TLT2 expression on T cells, to previous reports by our group concerning murine T cells, our monoclonal antibody does not detect its expressions on the surface of CD3$^+$ T cells. Another conserved aspect of TREM biology is its modulation following exposure to microbial products. We have now found evidence of this across species for all conserved TREM members. Specifically, surface TLT2 on neutrophils is upregulated two-fold in response to LPS activation, similar to that demonstrated for mice *in vivo*. Human monocytes also induce TLT2 in response to stimulation with LPS, as is evident for TREM-1 expression upon activation. This increase

in TLT2 on myeloid cells could be indicative of a proinflammatory function, as increases in TREM-1 and TLT1 expression amplify the immune response [15, 26].

Interestingly, the surface expression of TLT2 on neutrophils appears limited, but remarkably we have identified large intracellular pools of TLT2 within PMNs (Fig. 4). These stores of TLT2 appear to colocalize with CD35 and CD63, markers of secretory vesicles and primary granules, respectively. TLT2 is not stored in secondary or tertiary granules as evidenced by the lack of overlap between CD66b and TLT2. Secretory vesicles are highly specialized for rapid transport to the cell surface to deliver proteins important for opsonization as well as cell adhesion and migration to prime neutrophils for rolling along endothelium [27, 28]. Our group has previously demonstrated that TLT2 ligation enhances murine neutrophil chemotaxis towards various chemotactic factors, including IL8 and C5a [29]; the storage of TLT2 in secretory vesicles bolsters this observation, as neutrophil responses to chemokines are an integral part of leukocyte adhesion and migration through the endothelium.

TLT2 also colocalizes in primary granules (Fig. 4), which can be segregated into two subsets according to the composition of defensins [30]. Primary granules do not typically exocytose to the plasma membrane, but instead fuse to the phagolysosome where they empty their microbicidal contents [31]. Alternatively, β_2 integrin ligation has been shown to induce primary granule exocytosis, releasing defensins into the extracellular space to activate macrophages and enhance phagocytosis [32]. Additionally, primary granule exocytosis and defensin release have been reported to occur during microbial infection and also upon chemokine binding [33]. It seems worthwhile to determine if TLT2 and defensins are stored in the same granules, because exocytosis of

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these granules to the surface would increase TLT2 expression, facilitating its interaction with putative ligands to trigger signal transduction, and possibly allow for enhanced migration.

Upon migration into infected tissue, neutrophils kill microbes, traditionally via phagocytosis and the secretion of antimicrobial peptides. A third mechanism of killing microbes, termed netosis, has been demonstrated in the past decade. During this process, neutrophils expel DNA and primary granule components, creating a neutrophil extracellular trap or NET, which ensnares microbes and kills them [20]. As TLT2 is stored in primary granules, it was of interest to see if it would be extruded on DNA similarly to other granule proteins such as MPO and elastase. PMA-induced netosis showed that TLT2 is, in fact, not expelled onto NETs, but maintains a punctate staining pattern within the cell (Fig. 6). Another primary granule membrane protein, tetraspannin CD63 is also not expelled onto NETs [34, 35]. Indeed, upon neutrophil degranulation, we show that TLT2 expression matches that of CD63 (Fig. 5), indicating that these two proteins are colocalized.

These data, along with other reports of TREM function, provide novel insight into TLT2 biology. As other TREM family members have been shown to interact in multi-protein complexes involved in modulating immune responses, it is likely that TLT2 may do the same. Subcellular localization in regions dense with other receptors involved in cell-cell interactions and phagocytosis allows for a multitude of putative TLT2 interactions with other proteins. Of note, TLT2 has two putative N-linked glycosylation sites present in its ectodomain and glycosylation is a known mechanism for enhancing and stabilizing molecular interactions. Additionally, published observations regarding

TLT2-mediated potentiation of chemotaxis and phagocytosis lend credence to the theory that TLT2 upregulation following pathogenic stimulation augments the inflammatory response through interactions with an as yet to be determined endogenous ligand.

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AUTHOR CONTRIBUTIONS

K.A.T., R.G.K., and L.B.J. designed research; K.A.T., C.S., and T.S. performed research; K.A.T. and L.B.J. analyzed data; K.A.T. and L.B.J. wrote the paper.

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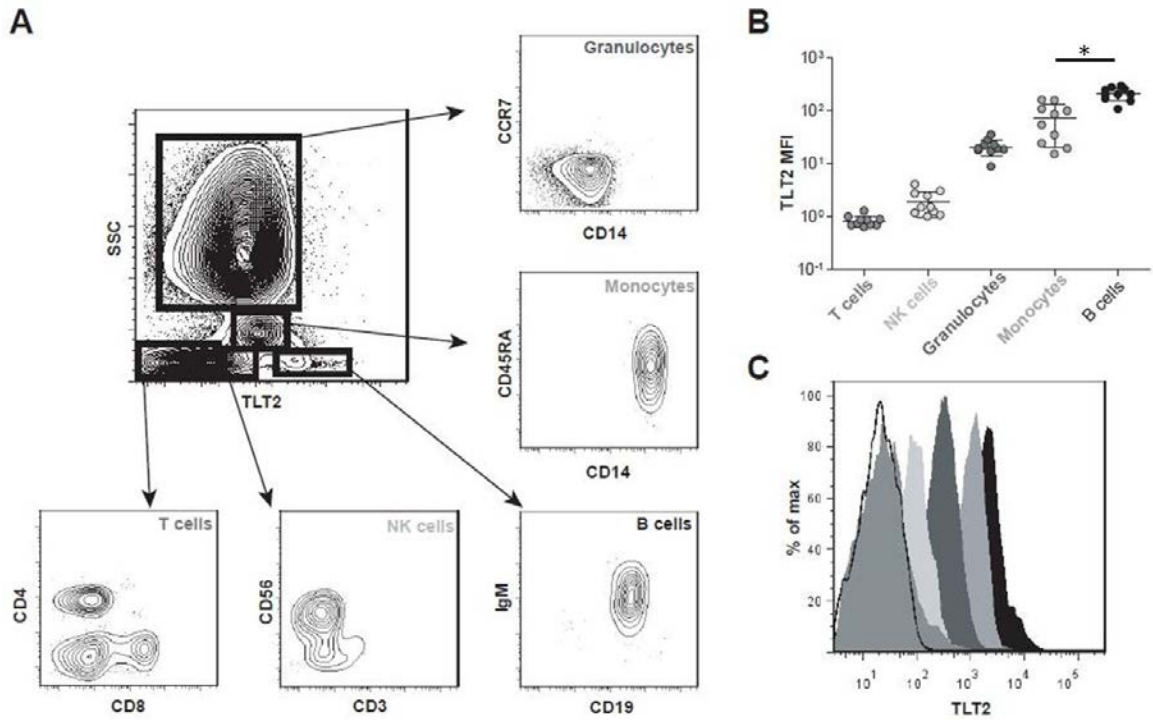
Fig. 1. Distinctive pattern of TLT2 expressed by human peripheral blood leukocytes. Peripheral blood leukocytes were isolated by sodium dextran sedimentation, washed, and stained with CD19, CD14, CD56, CD4, CD8, and 1C5. Cells were analyzed using a BD LSR II flow cytometer and FlowJo software. A) A representative scatter plot showing populations staining positive for TLT2 are uniformly specific. B) TLT2 expression on PBLs quantified and normalized to unstained controls. C) A representative histogram showing incrementally increased expression of TLT2 on specific cell subsets. Data shown is from healthy donors, n=10, *p<0.05.
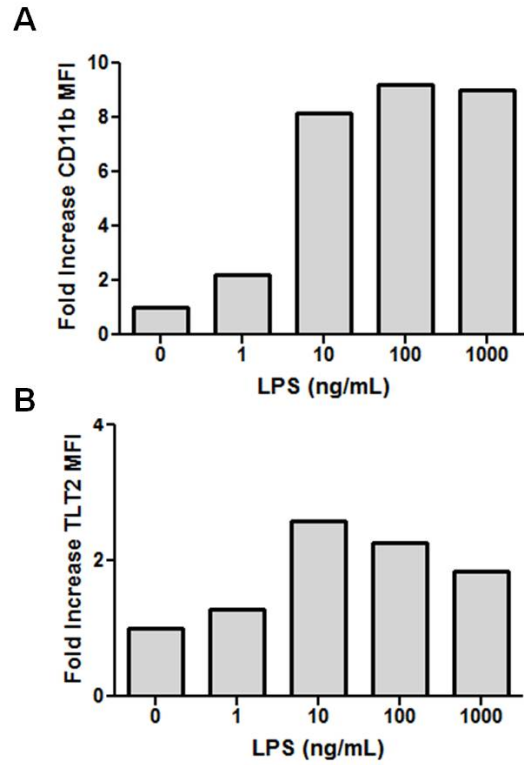
Fig. 2. TLR stimulation upregulates TLT2 expression on neutrophils. Neutrophils were isolated and stimulated with LPS for 30 min and then stained for expression of the activation marker CD11b (A) and TLT2 (B). Data are representative of 3 donors.
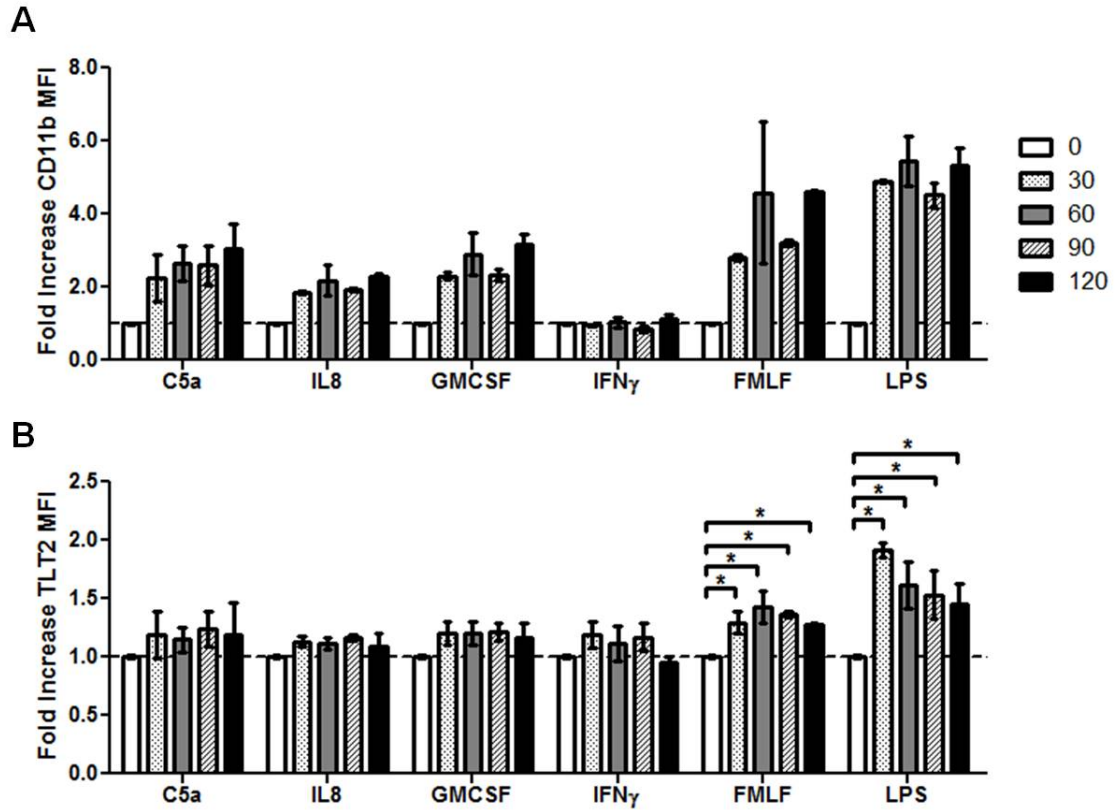
Fig. 3. Surface TLT2 is modulated by inflammatory mediators. Isolated neutrophils were stimulated with C5a (50 ng/mL), IL-8 (100 ng/mL), GM-CSF (50 ng/mL), IFNγ (1000 u/mL), FMLF (100 nM), or LPS (100 ng/mL) for 30, 60, 90, or 120 minutes. Cells were then stained with CD11b-PE and 1C5-A647 (TLT2). The mean fluorescence intensity (MFI) of (A) CD11b or (B) TLT2 was determined with FlowJo software, and activated samples were normalized to unstimulated samples. Data are from healthy donors, n=6, *p<0.05.
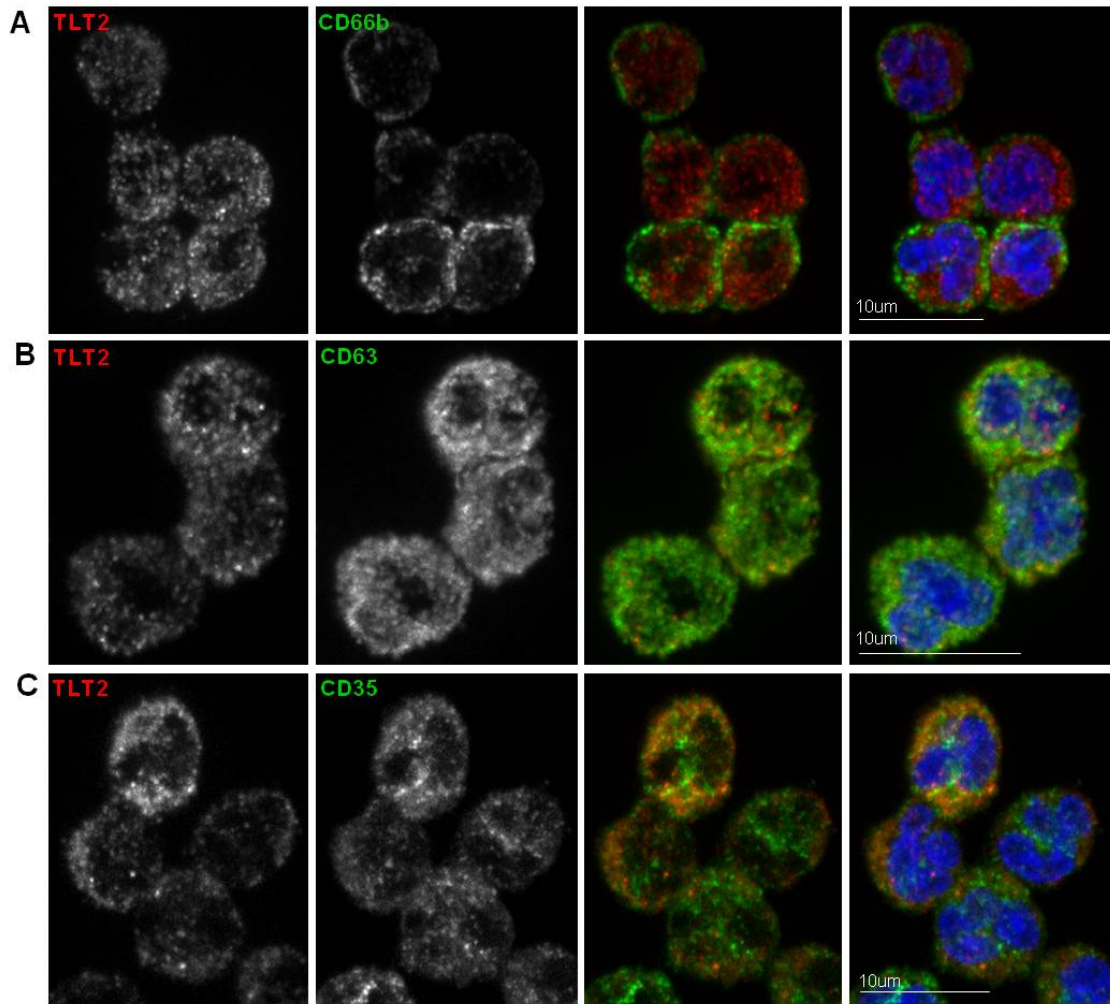
Fig. 4. TLT2 is found in intracellular pools in neutrophils. Resting neutrophils were fixed, permeabilized, and stained with DAPI, 1C5-A647 (TLT2), and granule specific markers (A) CD66b-A488 (secondary and tertiary), (B) CD63-A488 (primary/azurophilic), and (C) CD35-A488 (secretory vesicles). Green designates granules, red designates TLT2, and yellow indicates a colocalization of markers. Images shown are representative of 3 different donors. Scale bars are 10 μm.
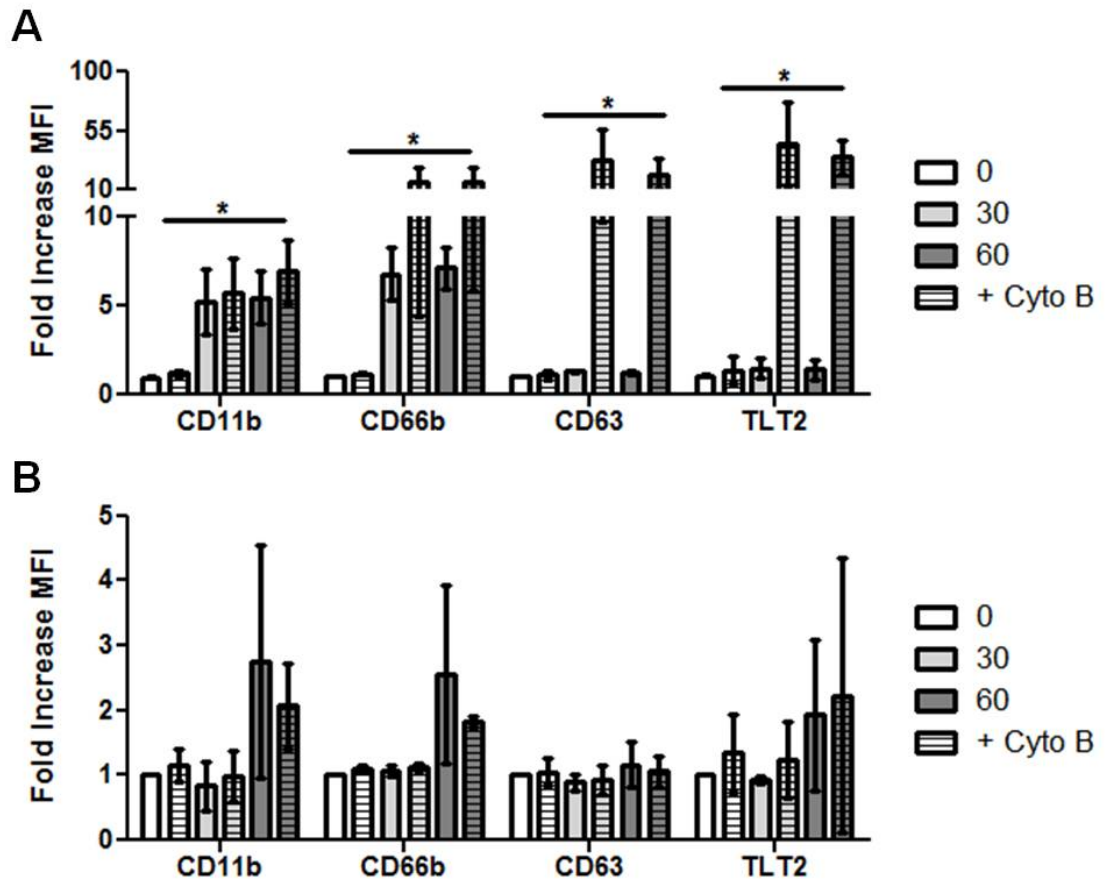
Fig. 5. Neutrophil degranulation results in the tranlsocation of TLT2 to the cell surface. Neutrophils were treated with (A) FMLF (100 nM) alone or (B) FMLF + Cyto B (5 μM) to induce full degranulation. Activated cells were stained with 1C5 (TLT2), CD66b (secondary and tertiary granule marker), CD63 (primary granule marker), and CD11b (activation and secretory vesicle marker). Data are from healthy donors, n=3, *p<0.05.
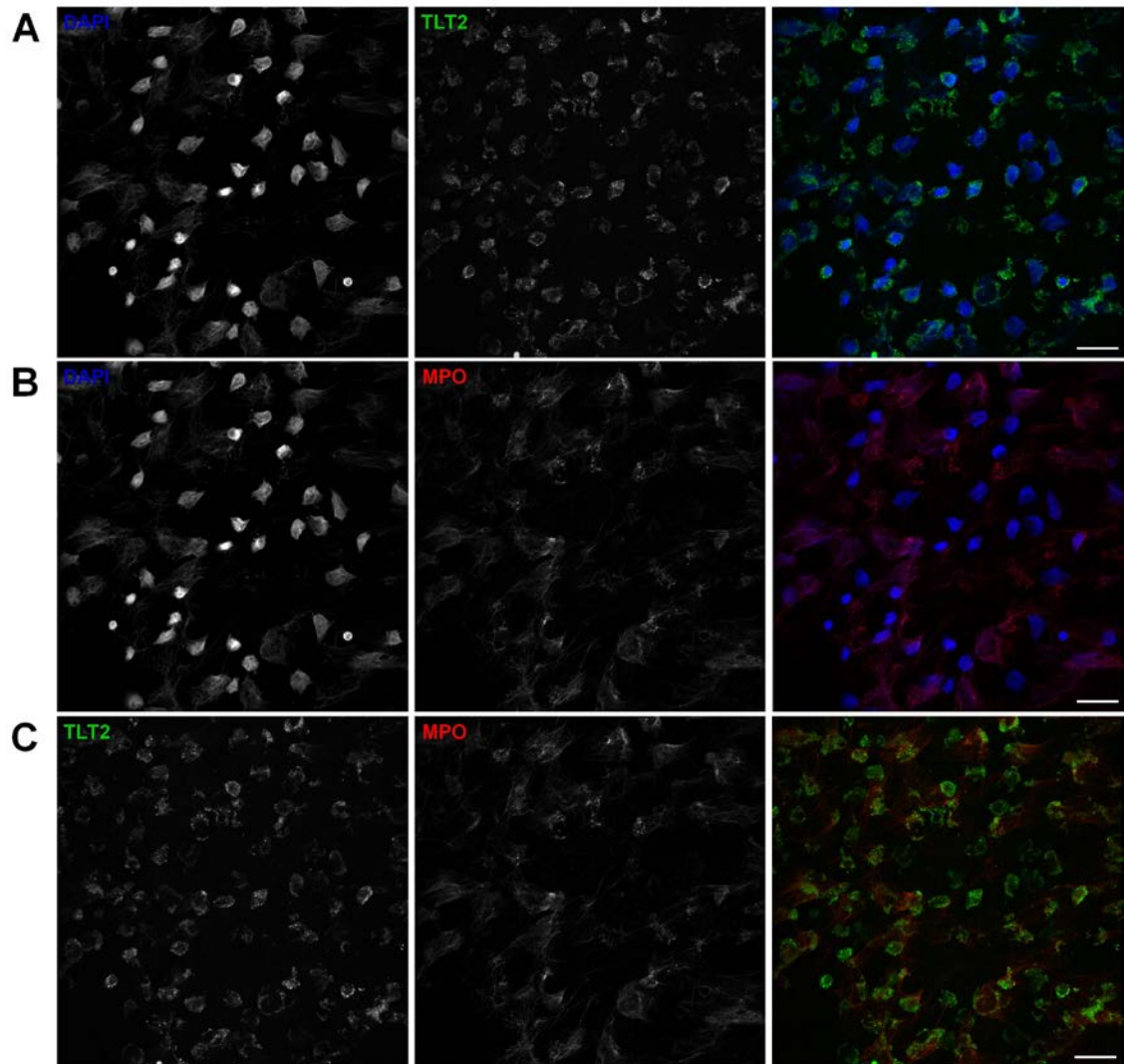
Fig. 6. TLT2 is not found in NETs. Neutrophils were treated with PMA (100 nM) for 4 hours to induce NET formation. Cells were fixed and stained for MPO (red), DAPI (blue), and an isotype control or TLT2 (green). Scale bar is 20 μm. Image is representative of three different donors.

DISCUSSION

New Findings in TLT2 Biology

The goal of this dissertation was to begin to elucidate the role of TLT2 in immunity, and how the function of this receptor relates to the greater field of TREM biology. TLT2 expression was shown to be conserved across species, with minor differences. In the myeloid compartment, human TLT2 is expressed on neutrophils, macrophages, and monocytes. Of note, TLT2 expression is most abundant on B cells in both mice and humans. Upon further evaluation, TLT2 surface levels on neutrophils are modulated in response to cellular activation with bacterial peptides, to a greater degree than cytokines or growth factors. Intriguingly, within neutrophils the majority of TLT2 protein is stored in intracellular pools, and upon complete degranulation, this expression pattern mimics that of the primary granule marker CD63. Unlike other primary granule components such as myeloperoxidase (MPO), TLT2 was not expressed on neutrophil extracellular traps or NETs [88].

Structure Based TLT2 Biology

Intracellular Structure. Of the conserved TREMs, TREM-1 and TREM-2 have minimal cytoplasmic tails, and require association with the ITAM containing DAP12 polypeptide to transduce biochemical signals. TLT1 and TLT2 differ in this regard as they do not require pairing with DAP12 to function, but instead contain short cytoplasmic tails with

signaling motifs. TLT1 contains two ITIMs and a proline-rich motif (PRM). The classical ITIM has been show to associate with the protein tyrosine phosphatase SHP-2, which interestingly enhances rather than inhibits calcium signaling. Of note, TLT2 is the only TREM member that lacks any conventional immunomodulatory tyrosine-based motif, but it does possess a cytoplasmic PRM, which is the preferred binding motif for SH3 domain-containing proteins, as well as an endocytic motif YxxV [33].

SH3 domains are commonly found in proteins that regulate cytoskeletal reorganization (Grb2, myosin proteins, Nck) [89], and notably activation of the conserved TREM member, TLT1, increases actin polymerization which stabilizes platelet aggregates [69]. Evidence that ligation of TLT2 enhances phagocytosis [65] could imply that TLT2 may interact with proteins of the cytoskeleton and thus modulate processes dependent on actin rearrangement. Another reported function of TLT2, regulation of GPCR agonist induced responses, could be mediated by the TLT2 intracellular tail as well. GPCR recycling and cellular resensitization are important mechanisms for regulating GPCR signaling [90-92]. Through interactions with the PRM of TLT2, SH3-containing proteins such as the Ras GTPase-activating proteins, or GAPs, could enhance receptor recycling and therefore resensitize cells more rapidly, enhancing the response to GPCR agonists. Lastly, the putative endocytosis motif, could allow for its targeted removal from the membrane of the cell, adding an additional point of regulation of TLT2 expression.

Extracellular Structure. The structural commonality between TREMs exists in the Ig ectodomain and possibly in the ligand binding pocket as TREM-1 and TLT1 were shown

to have very similar structures and could outcompete each other for the same ligand [79]. Daws et. al. reported that TREM-2 was capable of binding to an assortment of intact pathogens (gram positive and negative bacteria, yeast), and binding could be abrogated by coincubation with smaller bacterial products such as LPS, LTA, and peptidoglycan, or even anionic carbohydrates such as dextran sulfate [84]. These data suggest that TREM-2 ligand interactions may be heavily based on charge. Of note, TLT2 amino acid sequence and structure analysis reveals a highly positively charged loop that could influence ligand binding [61]. As TLT2 been shown to bind phosphatidyl serine (PS), a negatively charged phsopholipid, and recombinant TLT2 (rTLT2) binds serum components as well as milk proteins (KAT, unpublished observations), similar to TREM-2, TLT2 appears to be promiscuous with respect to binding anionic polypeptides such as lipids [65].

As previously mentioned, TREM members appear to physically interact with other molecules in both a cis and trans manner. TREM-1 on neutrophils binds platelets through an unknown protein interaction, but also interacts with the neighboring LPS receptor complex, while TREM-2/DAP12 interacts with Plexin A-1 to enhance Sema6D signaling, forming a multimeric complex in conjunction with ligand binding [85]. Notably, TLT1 appears to interact with fibrinogen on platelets in both a cis and trans fashion, and a soluble form of TLT1 can also bind an unknown TREM-1 ligand [86]. These multiple interactions imply that TREMs may play a role in modulating multiple cell processes through interactions with various molecules. This also indicates the difficulty in searching for endogenous TLT2 ligands, as it may require positioning in a multi-protein complex for detecting or binding to its counter ligand.

Lastly, the amino acid sequence of TLT2 has two putative N-linked glycosylation sites located in the extracellular region of the protein [33, 61]. Glycosylation is one way to increase secreted protein stability to prevent degradation but may also provide an added source of cell adhesion through lectin binding [93-95]. If TLT2 is indeed glycosylated, this post-translational modification could be indicative of both a secreted form of TLT2 and a possible cis lectin interaction that facilitates the formation of a protein complex important for ligand binding.

Expression Based TLT2 Biology

As three of the four conserved TREMs are expressed on myeloid cells, we posited that TLT2 would also be expressed on these cells. Similar to TREM-1, TLT2 is expressed on monocytes, neutrophils, and macrophages [61]. Specifically, TLT2 is expressed at low levels on the surface of neutrophils, whereas the majority of the protein is found in intracellular pools. This is very similar to both TLT1 and TREM-2 expression, in that the surface expression of both of these molecules is minimal, but large stores are found within the cell. TLT1 is located in the α-granules of platelets, whereas TREM-2 is stored in vesicles that are shuttled back and forth from the membrane when needed [57, 96]. This compartmentalization of TREM molecules could reflect the need for distinct regulation of TREM expression, possibly due to the constitutive presence of a ligand, or the propensity for highly sensitive responses to ligation.

Interestingly, TLT2 is the only TREM protein expressed on cells of the lymphoid lineage and specifically by B cells in both mouse and man. Also of note, resting surface expression of TLT2 on the cells examined is highest on B cells, but within the B cell

population there is a distinct hierarchy of TLT2 expression that is conserved across species. Innate-like B cells, specifically marginal zone (MZ) and B-1 B cells, as well as naive B cells express the highest levels of TLT2, with decreased expression on follicular B cells [61]. Indeed, memory B cells have decreased TLT2 levels, as antigen experience downregulates its expression three fold (KAT, unpublished observations). This pattern of expression would imply that the function of TLT2 is likely biased towards innate-like as opposed to adaptive immune responses.

Another commonality between TREM family members is altered expression upon cellular activation by bacterial peptides. LPS stimulation results in the upregulation of surface expression of TREM-1 and TLT1 in monocytes/netutrophils and platelets, respectively, whereas TREM-2 expression is decreased on microglia and osteoclasts in response to TLR agonists [36, 59]. TLT2 shares this trait, as activation with LPS increases its surface expression on monocytes, neutrophils and macrophages, but not on B cells. Culturing B cells with LPS, IgM, CD40, or a combination of these stimuli does not alter TLT2 expression. Therefore TLT2 surface expression is modulated differently in lymphoid cells compared to myeloid cells [61].

Differential localization and regulation of TLT2 expression on immune cells makes it difficult to hypothesize the role that TLT2 may play during an immune response. Shared functions between monocytes, neutrophils, and B cells are limited. B cells are mainly involved in the adaptive immune response as their hallmark effector function is antibody production in response to antigen-specific activation. TLT2 may not regulate B cell differentiation into antibody secreting cells directly, but it may regulate chemotaxis which is a critical component of B cell function and thus antibody

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production. Data from our group [64] show no correlation between TLT2 ligation and enhanced neutrophil phagocytosis, but demonstrate that TLT2 binding potentiates neutrophil chemotaxis in response to $G\alpha_i$ GPCR agonists. Conversely, de Freitas et. al. established that ligating TLT2 with a mAb augments macrophage phagocytosis of apoptotic cells [65]. To date, there have been no reports of TLT2 modulating B cell and macrophage chemotaxis. Analysis of the effect of TLT2 ligation on B cell innate-like responses will be important piece for elucidating TLT2 function.

Soluble TREM Biology

Currently, TLT2 is the only conserved TREM member that has not been shown to generate a soluble variant. Evidence for soluble TREMs have been documented by both the presence of alternate splice variants, as well as protease cleaved ectodomains shed from the surface of cells [70, 77]. These isoforms inhibit membrane bound TREM signaling by interfering with TREM ligand binding [86]. To date, there are no predicted alternate splice forms for TLT2, and no physical evidence of proteolytic cleavage, although bioinformatic algorithms report multiple low scoring predicted metalloprotease cleavage sites in the ectodomain of TLT2 (KAT, unpublished observations).

Both sTREM-1 and sTLT1 are detectable in septic patients and sTREM-1 has been found to be a prognostic indicator of sepsis severity in different infections [68, 79, 97]. Similarly, sTREM-2 was found in measurable quantities in the cerebro-spinal fluid (CSF) of patients with multiple sclerosis (MS) as well as other inflammatory neurodegenerative diseases [78]. Discovering a soluble form of TLT2 present in the

context of inflammatory disease would be beneficial for understanding the role of TLT2 in immune responses.

To help discern whether a naturally existing soluble form of TLT2 exists, use of a recombinant ectodomain could aid in determining the function of membrane bound TLT2. Manufactured short peptides of the CDR3 region from both TREM-1 and TLT1 mAB, LP17 and LR17 respectively, were found to bind an endogenous ligand and inhibit membrane TREM signaling. In the case of TREM-1, LP17 administration in a model of endotoxemic sepsis prevented death, whereas LR17 was demonstrated to increase survival during polymicrobial sepsis [42, 86]. To further elucidate the role of TLT2 in the immune system, it may be worthwhile to create a similar truncated peptide from a conserved segment of the CDR3 region from both the mouse and human protein. We have recently created recombinant TLT2 (rTLT2); specifically, the murine ectodomain of TLT2 fused to a murine IgG1[a] allotype heavy chain. Further studies using this rTLT2, or a shortened peptide, could be performed using a variety of inflammatory/infectious mouse models to both possibly identify the endogenous ligand of TLT2 and/or block the function of membrane bound TLT2 during an immune response.

TLT2 and Future Directions

In summary, although TLT2 may be a conserved member of the TREM family, and have structural and phenotypical similarities, it is the most unique TREM representative and much is left to learn regarding its role in immune responses. As in the study of other TREMs, the construction of recombinant TLT2 extracellular domain protein, as well as a $TLT2^{-/-}$ mouse model will prove invaluable for further elucidation of

its function during the immune response, as well as for identification of a physiological ligand. This study has demonstrated that the pattern of TLT2 expression is conserved across species, which confirms that TLT2 is the only member of the TREM locus to be found on cells of lymphoid origin. Additionally, TLT2 levels are modulated in response to inflammatory stimuli, which is a common aspect of TREM biology, substantiating the claim that these receptors play a role in altering the immune response during inflammation. Lastly, similar to other TREMs, the majority of TLT2 protein is stored intracellularly, more specifically, within the secretory vesicles and primary granules of neutrophils. These data add to the growing knowledge of TLT2 expression and function and imply a need for regulation of surface TLT2 expression to control its interaction with constitutively expressed endogenous ligands, and thus its function.

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APPENDIX A

IRB APPROVAL FORM (2013)

Form 4: IRB Approval Form
Identification and Certification of Research
Projects Involving Human Subjects

UAB's Institutional Review Boards for Human Use (IRBs) have an approved Federalwide Assurance with the Office for Human Research Protections (OHRP). The Assurance number is FWA00005960 and it expires on January 24, 2017. The UAB IRBs are also in compliance with 21 CFR Parts 50 and 56.

Principal Investigator:	JUSTEMENT, LOUIS B
Co-Investigator(s):	KING, RODNEY G
	THOMAS, KIMBERLY ANNE
Protocol Number:	**X090424003**
Protocol Title:	*Analysis of TLT2 Expression and Function in the Immune System*

The IRB reviewed and approved the above named project on ⁊3013 . The review was conducted in accordance with UAB's Assurance of Compliance approved by the Department of Health and Human Services. This Project will be subject to Annual continuing review as provided in that Assurance.

This project received EXPEDITED review.

IRB Approval Date: 7-30-13

Date IRB Approval Issued: ⁊3013

Marilyn Doss, M.A.
Vice Chair of the Institutional Review
Board for Human Use (IRB)

Investigators please note:

The IRB approved consent form used in the study must contain the IRB approval date and expiration date.

IRB approval is given for one year unless otherwise noted. For projects subject to annual review research activities may not continue past the one year anniversary of the IRB approval date.

Any modifications in the study methodology, protocol and/or consent form must be submitted for review and approval to the IRB prior to implementation.

Adverse Events and/or unanticipated risks to subjects or others at UAB or other participating institutions must be reported promptly to the IRB.