INTERROGATING THE ROLE OF TRANSMEMBRANE TNF (tmTNF) IN TLR2 OR TLR4 ACTIVATED MACROPHAGES DURING STAPHYLOCOCCUS AUREUS INFECTION

by

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ABSTRACT

Tumor Necrosis Factor alpha (TNF) is a cytokine involved in diverse cellular processes such as cell survival, proliferation, differentiation, immune defense, and cell death. TNF produced during an immune response is typically associated with the generation and propagation of inflammation. This association with inflammation is particularly strong for soluble TNF (sTNF), one of two bioactive TNF protein forms, but not transmembrane TNF (tmTNF), the second bioactive TNF protein form. Typically, tmTNF is more closely associated with anti-inflammatory or proliferative responses. As a result, much less is known about the role and extent to which tmTNF participates in host immune responses, particularly as it pertains to host defense. During infection, macrophages are known to secrete large amounts of TNF in response to pathogen detection via Toll-Like-Receptor (TLR) stimulation.

In this dissertation research, we investigated the role of sTNF and tmTNF on macrophage priming following TLR2 or TLR4 stimulation and how this priming influenced macrophage ability to clear a subsequent *Staphylococcus aureus* infection. *In vitro* cell culture assays demonstrated that TLR stimulated WT bone marrow derived macrophages (BMDMs) became significantly more efficient at clearing a subsequent *S. aureus* infection. Transcriptomic and protein analysis revealed upregulation of both sTNF and tmTNF following TLR stimulation. We demonstrated that depletion of sTNF, but not tmTNF, during TLR stimulation did not influence BMDM priming which led to improved clearance of a subsequent bacterial infection. Conversely, supplementing exogenous sTNF to TLR2 stimulated TNF-/- BMDMs prior to infection did not improve *S. aureus* clearance. This indicated that sTNF signaling was not required for BMDM priming. Further, this suggested that TLR-induced tmTNF priming could contribute to improved *S. aureus* clearance. TmTNF preferentially signals through TNFR2. Indeed, blocking TNFR2 following TLR2 stimulation led to increased overall *S. aureus* bacterial burden. To mechanistically elucidate how tmTNF could be priming BMDMs prior to *S. aureus* infection, we investigated the potential of tmTNF reverse signaling. These studies provide valuable insight into the differential involvement of sTNF and tmTNF signaling following TLR stimulation of BMDMs which has implications for downstream response to infection.

INTRODUCTION

The Macrophage Immune Cell

Macrophages are professional innate immune cells that specialize in the phagocytosis of pathogens and debris (Acharya et al., 2020). During embryogenesis, the fetal yolk sac gives rise to the first macrophages until the fetal liver is developed and hematopoiesis can take place (Epelman et al., 2014; Wynn et al., 2013). At the later stages of fetal development, the formation of bone causes fetal liver hematopoiesis to decline until it is eventually replaced by bone marrow hematopoiesis (Epelman et al., 2014; Wynn et al., 2013). Macrophages originating from the fetal yolk sac are believed to give rise to tissue resident macrophages, which take on highly specialized phenotypes tailored to their specific tissue environment such as microglia, Langerhans, and Kupffer cells (Davies et al., 2013; Epelman et al., 2014; Wynn et al., 2013). Following development of bone and the establishment of bone marrow hematopoiesis, circulating blood monocytes become the primary source of new macrophages; however, new macrophages are also derived from existing tissue resident macrophages and bone marrow derived myeloid progenitor cells (Davies et al., 2013; Epelman et al., 2014; Wynn et al., 2013).

In normal physiologic conditions, macrophages function as sentinels and mediate critical functions necessary for maintaining homeostasis, such as clearance of cellular debris and mineral recycling (Acharya et al., 2020; Kovtunovych et al., 2010; Soe-Lin et al., 2009). During infection, macrophages play a pivotal role in host defense by initiating pro-inflammatory responses, phagocytosing/killing pathogens, and antigen presentation (Acharya et al., 2020; Castrillo et al., 2001; Hardbower et al., 2016). Because of this, macrophages display a high level of plasticity which is necessary to rapidly respond to shifts in their environment. *In vivo*,

macrophages have been characterized to exist within a spectrum and can fluidly transition between either inflammatory M1 macrophages or anti-inflammatory M2 macrophages (Muñoz-Rojas et al., 2021; Vogel et al., 2014) . During infection, the inflammatory M1 macrophage is the predominant macrophage sub-type because it is optimized for host defense (Benoit et al., 2008). M1 polarized macrophages secrete high levels of nitric oxide, reactive oxygen species, and pro-inflammatory cytokines (T. Yu et al., 2016). One of the stimuli that influences this M1 sub-type transition is ligation of a pathogen associated molecular pattern (PAMP) to specialized receptors called pathogen recognition receptors (PRRs) (Benoit et al., 2008). One of the major classes of PRRs that macrophages are equipped with is a family of receptors known as Toll-Like Receptors (TLRs). TLRs are evolutionary conserved proteins which function to recognize pathogen associated molecular patterns (PAMPs) or endogenous host molecules associated with damage, called damage associated molecular patterns (DAMPs) (L. Yu, Wang, et al., 2010). TLR recognition of either PAMPs or DAMPs on macrophages initiates intracellular signaling which leads to pro-inflammatory responses (Benoit et al., 2008; L. Yu, Wang, et al., 2010). Macrophage induction of pro-inflammatory responses through TLRs is critical for activation of the immune system and initiation of host defense mechanisms.

Host Defense Via Surface TLRs

The discovery of TLRs was a pivotal turning point in the field of immunology. Prior to the discoveries that led to the elucidation of TLRs, the innate immune system was largely unknown and was mostly considered the rudimentary system which activated the more refined adaptive immune response (Weiss & O'Neill, 2022). The characterization of TLRs opened the

field of innate immunology and has led to research that expanded our understanding of how host defense is initiated.

TLRs are germline-encoded transmembrane PRRs that contain leucine rich repeats in their ectodomain, a single helix transmembrane domain, and a cytoplasmic Toll IL-1 Receptor (TIR) signaling domain (Figure 1) (Asami & Shimizu, 2021; M. S. Jin & Lee, 2008). Although originally discovered in Drosophila, TLRs were among one of the first classes of mammalian PRRs to be discovered and are ubiquitously expressed. To date, 10 human and 12 murine TLRs have been characterized, with humans expressing TLR1-10 and mice expressing TLR1-9 and TLR11-13 (Asami & Shimizu, 2021; M. S. Jin & Lee, 2008). These TLRs are important for the recognition of distinct PAMPs and subsequent initiation of appropriate signaling cascades to mount immunological host defense (Asami & Shimizu, 2021; M. S. Jin & Lee, 2008). Upon PAMP recognition, TLRs either homo or hetero-dimerize, which is essential for full receptor activation (Asami & Shimizu, 2021; Botos et al., 2011). The majority of TLRs favor homodimerization for signaling; however, TLR1 and TLR6 require heterodimerization with TLR2 to signal (Asami & Shimizu, 2021; Botos et al., 2011; Farhat et al., 2008; Sandor et al., 2003). This dimerization promiscuity with TLR2 is believed to broaden the range of PAMPs and DAMPs (tri-acylated lipopeptides, di-acylated lipopeptides, peptidoglycan, lipopolysaccharide, and heat shock proteins, etc.,) that TLR2 recognizes (Farhat et al., 2008; Parker et al., 2004; Triantafilou et al., 2006).

Broadly, TLR2/1 and TLR10 recognize triacylated lipoproteins (Guan et al., 2010; M. S. Jin et al., 2007). TLR2/6 recognizes diacylated lipoproteins and TLR4 recognizes lipopolysaccharide (Poltorak et al., 1998; Takeuchi et al., 2001). TLR3 recognizes double

stranded RNA, TLR7 and TLR8 recognize single stranded RNA, TLR13 recognizes bacterial ribosomal RNA, and TLR9 recognizes CpG DNA (Alexopoulou et al., 2001; Heil et al., 2004; Hemmi et al., 2000; Oldenburg et al., 2012). TLR12 recognizes profilin, while TLR5 and TLR11 recognize flagellin (Andrade et al., 2013; Hayashi et al., 2001). This ligand specificity that TLRs display is complemented by their spatial regulation and is a prime example of when form fits function. TLRs can be sub-divided into two groups: cell surface TLRs which primarily encounter their ligand and induce signaling from the plasma membrane or endosomal TLRs which primarily encounter their ligand and induce signaling from within endosomal compartments (T. Duan et al., 2022; Mandraju et al., 2014). Endosomal TLRs include TLR3, TLR7-9, and TLR11-13 (T. Duan et al., 2022; Mandraju et al., 2014). The large majority of these endosomal TLRs recognize nucleic acids from bacteria or viruses (T. Duan et al., 2022; Mandraju et al., 2014). Thus, their localization to the endosome increases the likelihood of nucleic acid ligation and early initiation of host defense responses. Cell surface TLRs include TLR1, TLR2, TLR4-6, and TLR10 (T. Duan et al., 2022; Mandraju et al., 2014). The large majority of these cell surface TLRs recognize proteins associated with the microbial membrane (T. Duan et al., 2022; Mandraju et al., 2014). Thus, their localization to the cell surface optimizes the likelihood of interaction and detection of microbial membrane associated proteins to initiate early host defense responses.

While each of these TLRs are important for PAMP detection and initiation of host immune defense, TLR2 and TLR4 recognize the broadest range of ligands and are the best-characterized (Kielian, 2009). TLR4 was the first fully characterized TLR, and it is critical for mediating host responses against LPS (Poltorak et al., 1998; Rock et al., 1998). Following

closely behind, TLR2 was discovered to respond to an array of bacterial lipoproteins. Recognition of triacylated lipoproteins is dependent on TLR2 dimerization with TLR1, and recognition of diacylated lipoproteins is dependent on TLR2 dimerization with TLR6 (M. S. Jin & Lee, 2008; Shukla et al., 2018). Although TLR2 and TLR4 recognize distinct PAMPs, they share commonalities which showcase the redundancy and compensatory mechanisms built into immune signaling. Both TLR2 and TLR4 recruit the same signaling adaptor proteins to their TIR domains, and they both activate the same major signaling pathways (Asami & Shimizu, 2021; M. S. Jin & Lee, 2008). The two signaling adaptor proteins utilized by both TLR2 and TLR4 are Myeloid differentiation primary-response protein 88 (MyD88) and MyD88-adaptor-like-protein (MAL; TIRAP). MyD88 and MAL are essential adaptor proteins that are required for TLR2 and TLR4 signal transduction (Asami & Shimizu, 2021; M. S. Jin & Lee, 2008). Together, MyD88 and MAL facilitate induction of several intracellular signaling pathways that lead to the induction of inflammatory responses (Figure 2). These pathways include nuclear factor-kB (NFkB) and mitogen activated protein kinase (MAPK) (Kawasaki & Kawai, 2014).

The NFkB signaling pathway is a prototypical pro-inflammatory pathway that is responsible for mediating expression of various inflammatory cytokines such as TNF, IL-6, IL-12, and IL-1β (Kawasaki & Kawai, 2014; G. Zhang & Ghosh, 2001). In comparison to NFkB, the MAPK signaling pathway displays higher diversity in the cellular programs it mediates. In addition to mediating inflammation, MAPK also plays a role in mediating cellular proliferation, differentiation, metabolism, stress responses, and apoptosis (Cargnello & Roux, 2011). In macrophages, activation of NFkB is critical for M1 macrophage polarization (Wu et al., 2022). Comparatively, the role of MAPK in response to TLR activation is less defined; however, it has

been shown that inhibition of MAPK correlates with a decrease in inflammatory cytokine and nitric oxide production in TLR activated HD11 cells, an avian macrophage cell line (Peroval et al., 2013). Additionally, several studies indicate that MAPK plays a role in regulating TLR induced cytokine transcription (Kuriakose et al., 2019; Peroval et al., 2013; Zou & Shankar, 2015). Together, these studies implicate a role for MAPK signaling in the transcriptional regulation and amplification of existing inflammatory signals which drive macrophage defense against invading pathogens. Despite the variation in outcomes that exist upon induction of either NFkB or MAPK signaling pathways, they both mediate a critical function that is essential for M1 macrophage activation and host defense. This critical function is the regulation and expression of pro-inflammatory cytokines (Peroval et al., 2013; G. Zhang & Ghosh, 2001).

The induction of pro-inflammatory cytokines is crucial for activating both the innate and adaptive immune system. Locally, pro-inflammatory cytokines activate nearby immune cells and polarize macrophages to adopt an M1 phenotype which is optimized for host defense. Distally, pro-inflammatory cytokines activate and recruit additional immune cells to sites of infection (Berger et al., 2016; Driscoll et al., 1997). In addition to the activation and recruitment of immune cells, pro-inflammatory cytokines also mediate the upregulation of antigen presentation and co-stimulatory molecules on the cell surface of innate immune cells, such as M1 polarized macrophages, natural killer cells, and dendritic cells (BLANCO et al., 2008; Z. Duan & Luo, 2021; Zwirner & Domaica, 2010). Although strong initial pro-inflammatory responses are essential for immune activation and defense, uncontrolled inflammation damages native host structures and can be detrimental (Cicchese et al., 2018). Thus, in addition to a strong initial pro-inflammatory response, an anti-inflammatory response is required to limit inflammatory damage

to host structures and eventually stop the inflammatory response upon infection resolution (Cicchese et al., 2018). One of the most potent pro-inflammatory cytokines responsible for mediating inflammation is tumor necrosis factor (TNF) and one of the most potent anti-inflammatory cytokines responsible for reducing TNF mediated inflammation is interleukin 10 (IL-10) (Armstrong et al., 1996; Chen et al., 2023).

TNF and TNF Receptors

The characterization of TNF in the late 1970s was built upon decades of cancer research that identified a factor which caused tumor regression (E. F. McCarthy, 2006; Nauts & McLaren, 1990). The initial cancer research that led to the discovery and characterization of TNF was pioneered by William B. Coley, a bone sarcoma surgeon (E. F. McCarthy, 2006; Nauts & McLaren, 1990). Coley discovered that injection of bacterial preparations to patients diagnosed with inoperable cancer led to tumor regression in these patients. These bacterial preparations would eventually come to be known as “Coley’s Toxins” (E. F. McCarthy, 2006; Nauts & McLaren, 1990). Based on Coley’s research, a number of *in vivo* mouse experiments were published which characterized “hemorrhagic necrosis” of tumors (Carswell et al., 1975; O’MALLEY et al., 2009; Shear & Perrault, 1944). This tumor necrosis was believed to be induced by endotoxin (Carswell et al., 1975; O’MALLEY et al., 2009; Shear & Perrault, 1944), which led to the adoption of the term “tumor necrotizing factor”. Seminal work published in 1975 by Llyod Old identified that the tumor necrotizing factor which caused tumor regression was not bacterial in nature, but rather a host-derived molecule (Carswell et al., 1975). Shortly following Old’s discovery, TNF was isolated and purified from serum, providing definitive evidence that TNF was a host derived protein which was capable of causing tumor regression via

cytotoxic activation of macrophages and neutrophils (Aggarwal et al., 1985; Shalaby et al., 1985).

TNF is a pleiotropic cytokine that can exert its effects both locally and systemically. Following transcription and translation, TNF is shuttled through the secretory pathway and eventually makes its way to the cell membrane of activated immune cells where it exists as a 26kDa precursor protein known as transmembrane TNF (tmTNF) (Josephs et al., 2018; Shurety et al., 2000; Su et al., 2022). Following this, the mature 17kDa protein form of TNF, known as soluble TNF (sTNF), is released from the cell membrane upon cleavage by A Disintegrin and Metalloprotease 17 (ADAM17; TACE). TNF exists as either tmTNF or sTNF, both of which are functional proteins that have distinct receptors and signaling outcomes (Shurety et al., 2000; Su et al., 2022). Although there is a high degree of overlapping elements and crosstalk between tmTNF and sTNF, they are believed to regulate and mitigate each other's outcomes. Broadly, tmTNF is believed to induce anti-inflammatory responses via ligation to tumor necrosis factor receptor II (TNFR2) while sTNF is believed to induce pro-inflammatory responses via ligation to tumor necrosis factor receptor I (TNFR1) (Horiuchi et al., 2010). Although TNFR2 is the primary receptor for tmTNF, it is important to note that tmTNF can also bind and signal through TNFR1; however, this interaction is believed to be less stable and more transient (Grell et al., 1995, 1998). Conversely, sTNF is unable to induce signaling through TNFR2, despite being capable of binding this receptor (Grell et al., 1995, 1998).

TNFR1 and TNFR2 belong to a group of receptors known as tumor necrosis receptor superfamily (TNFRSF) which is a superfamily of cytokine receptors characterized by recurring cysteine rich amino acid motifs in their ectodomains (Kucka & Wajant, 2021). This superfamily

of receptors can be further sub-divided into two groups, receptors that are death receptors due to the presence of a death domain (DD) and receptors that do not contain a death domain (Fiedler et al., 2023; Kucka & Wajant, 2021). TNFR1 falls into the former group of receptors and harbors a DD in its cytoplasmic tail. TNFR2 falls into the latter group and does not contain a DD (Fiedler et al., 2023; Kucka & Wajant, 2021). The presence of a DD on the cytoplasmic tail of TNFR1 links this receptor to cytotoxic signaling which allows apoptosis and necroptosis to be directly mediated through this receptor. In addition to regulation of cytotoxic signaling, sTNF signaling through TNFR1 mediates inflammatory signaling through MAPK and NFkB activation (Fiedler et al., 2023; Kucka & Wajant, 2021; Tseng et al., 2018). Conversely, TNFR2 does not contain a DD and therefore lacks the ability to directly induce cytotoxic signaling pathways. Instead, the cytoplasmic tail of TNFR2 contains a short amino acid motif that recruits the signaling adaptor protein TNF receptor associated factor 2 (TRAF2) and its associated proteins cellular inhibitor of apoptosis protein 1 and cellular inhibitor of apoptosis protein 2 (cIAP1, cIAP2). This allows for the activation of kinases that subsequently phosphorylate and activate MAPK, NFkB, and non-canonical NFkB (Tseng et al., 2018).

In response to TNFR1 ligation of either sTNF or tmTNF, the DD containing cytoplasmic proteins TNFR1-associated death domain (TRADD) and receptor interacting protein kinase-1 (RIPK1) are recruited to the DD present on the cytoplasmic tail of TNFR1 (X. Li et al., 2020). Subsequently, TRADD and RIPK1 recruit TRAF2 and cIAP1/2 proteins to form the core of the TNFR1 signaling complex (complex I). After assembly of complex I, the ubiquitination status of RIPK1 largely determines the intracellular signaling fate (Holbrook et al., 2019; X. Li et al., 2020). Polyubiquitination of RIPK1 drives intracellular signaling towards NFkB and MAPK

activation while non-ubiquitinated RIPK1 dissociates from TRADD to form a complex with Fas-associated death domain (FADD) and caspase 8 (complex II) to initiate cell death pathways (Holbrook et al., 2019; X. Li et al., 2020). Polyubiquitylation of RIPK1 creates docking sites for linear Ub chain assembly complex (LUBAC). LUBAC further ubiquitinates RIPK1, which leads to the recruitment of proteins that make up the TAK1 signaling complex (X. Li et al., 2020). The TAK1 signaling complex consists of transforming growth factor-beta activated kinase (TAK1), TAK1-binding protein-2 (TAB2), and TAK1-binding protein 3 (TAB3). This TAK1 complex phosphorylates inhibitor of kappa B kinases (IKK) complex and mitogen-activated protein kinase kinase (MAPKKs), which results in a cascade of protein phosphorylations that culminate in translocation of NFkB (RelA and p65) and Activator Protein-1 (AP-1) transcription factors into the nucleus to induce pro-inflammatory gene expression (Figure 3) (Holbrook et al., 2019; Morton et al., 2019; Yuasa et al., 1998).

Conversely, TNFR2 does not contain a DD and therefore lacks the ability to directly induce cytotoxic signaling pathways. Instead, the cytoplasmic tail of TNFR2 contains a short amino acid motif that recruits the signaling adaptor protein TNF receptor associated factor 2 (TRAF2) and its associated proteins cellular inhibitor of apoptosis protein 1 and cellular inhibitor of apoptosis protein 2 (cIAP1, cIAP2). This allows for the activation of kinases that subsequently phosphorylate and activate MAPK, NFkB, and non-canonical NFkB (Tseng et al., 2018)

The intracellular signaling events that occur in response to TNFR2 ligation of tmTNF are less characterized; however, it is accepted that signaling through this receptor skews cellular responses towards proliferative and anti-apoptotic pathways via activation of both the classical NFkB and non-canonical NFkB pathways (Fu et al., 2021; Naserian et al., 2020; Thommesen &

Laegreid, 2005). Similar to TNFR1, ligation of tmTNF to TNFR2 results in recruitment of the signaling adaptor proteins TRAF2 and cIAP1/2. Unlike TNFR1, however, TRAF2 directly interacts with the cytoplasmic domain of TNFR2 (Choudhary et al., 2013; L. Zhang et al., 2011). Subsequently, TRAF2 recruits the adaptor proteins TRAF1 and TRAF3. Recruitment of TRAF2, TRAF1, TRAF3, and cIAP1/2 to the cytoplasmic domain of TNFR2 lifts a critical suppression signal that allows non-canonical NFkB signaling to occur (Choudhary et al., 2013; L. Zhang et al., 2011). Under homeostatic conditions (unstimulated cells) TRAF2, TRAF3, and cIAP1/2 constitutively ubiquitinate the cytosolic protein NFkB-inducing kinase (NIK) which targets it for proteasomal degradation (Choudhary et al., 2013; L. Zhang et al., 2011). However, recruitment of TRAF2, TRAF3, and cIAP1/2 to TNFR2 prevents constitutive ubiquitination of NIK, which allows it to accumulate in the cytosol and activate IKKα. Together, NIK and IKKα phosphorylate p100 which has an inhibitory function similar to the IkB proteins. Phosphorylation of p100 leads to the proteasomal degradation of ankyrin repeats located at the c-terminal which releases the transcription factors p52 and RelB (Choudhary et al., 2013; J. Liu et al., 2012; L. Zhang et al., 2011). Subsequently, released NFkB transcription factors RelB and p52 translocate to the nucleus to induce gene transcription (Figure 4) (Choudhary et al., 2013; J. Liu et al., 2012; L. Zhang et al., 2011). Signaling through non-canonical NFkB has been shown to induce transcription of genes implicated in cellular proliferation, secondary lymphoid development, and anti-apoptotic processes (Claudio et al., 2006; Yilmaz, 2003). As a result, aberrant non-canonical NFkB pathway activation has been implicated in some autoimmune disorders (Choudhary et al., 2011; Sanz et al., 2010) and has been shown to be a target for some pathogens (J. Jin et al., 2014; P. Liu

et al., 2008; Manches et al., 2012); however, its specific role during active bacterial infection remains poorly defined.

Although TNFR1 and TNFR2 have distinct signaling mechanisms and outcomes, there are apparent redundancies and overlap in the signaling adaptor proteins that are recruited to each receptor. Both TNFR1 and TNFR2 recruit TRAF2 and cIAP1/2, and both of these signaling adaptor proteins are required for signaling through either NFkB, non-canonical NFkB, or MAPK to occur (Choudhary et al., 2013; Tseng et al., 2018; L. Zhang et al., 2011). Because of this shared signaling adaptor protein recruitment, it is believed that TNFR1 and TNFR2 have a high degree of crosstalk, allowing them to co-regulate one another's signaling. Evidence of crosstalk between TNFR1 and TNFR2 is supported by numerous *in vitro* cell line-based studies which have demonstrated TNFR2 mediated apoptosis, despite TNFR2's lack of a DD (Bigda et al., 1994; Medvedev et al., 1994; Siegmund et al., 2016). Selective stimulation of TNFR2 was shown to deplete cytosolic TRAF2 and cIAP1/2 which was correlated with attenuation of TNFR1-mediated NFkB and c-Jun activation as well as an increase in TNFR1/RIPK1/Caspase 8/FADD mediated apoptosis (Fotin-Mleczek et al., 2002; Siegmund et al., 2016). To add to this signaling complexity, studies using either TNFR1 or TNFR2 deficient mice have shown that the absence of one receptor does not ablate signaling of the other receptor (Stillie & Stadnyk, 2009; Tacchini-Cottier et al., 1998; Yang et al., 2019) . This suggests two possibilities: 1.) crosstalk between TNFR1 and TNFR2 is not required for regulation of signaling through individual receptors or 2.) compensatory signaling mechanisms are activated to compensate for the lack of signaling input from the deficient receptor. Given the intricacy of intracellular signaling, the

latter possibility is more likely; however, more studies characterizing the role of TNFR1 and TNFR2 in the context of infection is needed.

Transmembrane TNF Reverse Signaling

Despite the many shared similarities between TNFR1, TNFR2, sTNF, and tmTNF, there exists an intriguing difference which distinguishes TNFR2/tmTNF ligand receptor interactions from TNFR1/sTNF ligand receptor interactions. This distinguishing feature is referred to as "reverse" signaling, in which a receptor (TNFR2 or TNFR1) binding to a membrane-bound ligand (tmTNF) induces intracellular signaling in the cell bearing the ligand (Diallo et al., 2021; Pallai et al., 2016; Qu et al., 2017). This is in contrast to canonical "forward" signaling in which a soluble ligand (sTNF) binding to a receptor (TNFR1) induces intracellular signaling in the cell that bears the receptor (Qu et al., 2017). This ability for membrane-bound ligands to exhibit receptor-like behavior and transmit positive or negative feedback signals bi-directionally is not exclusive to tmTNF. Reverse signaling, in addition to canonical forward signaling, has been described for three major classes of membrane bound ligands, which include Type I transmembrane proteins, Type II transmembrane proteins, and GPI-anchored proteins (Ross et al., 2016; Sun & Fink, 2007).

Type I transmembrane proteins shown to have this ability for bi-directional signaling include ephrin ligands, neuregulin ligands, semaphorin ligands, and a novel membrane IL-15 isoform expressed in human renal cancer cells (Bao et al., 2003; Khawam et al., 2009; Xu & Henkemeyer, 2012; L. Yu, Zhou, et al., 2010). All of these Type I transmembrane proteins, with the exception of IL-15 which typically plays a role promoting inflammation, are involved in axonal or nervous system development and signaling (Bao et al., 2003; Khawam et al., 2009; Xu

& Henkemeyer, 2012; L. Yu, Zhou, et al., 2010). In addition to Type I transmembrane proteins, there is a small body of literature which indicates that GPI-anchored proteins are capable of bi-directional signaling, most of which are involved in nervous system regulation (Coate et al., 2009; Konstantinova et al., 2007; Lim et al., 2008). Lastly, Type II transmembrane proteins which have been shown to signal bi-directionally come largely from the TNF superfamily of ligands (TNFSF) and include, but are not limited to TNFSF14, TNFSF18, TNFSF13B, TNFSF13, and tmTNF (W.-H. Lee et al., 2019; Ross et al., 2016). These TNFSF ligands are predominantly involved in regulation of the immune system (W.-H. Lee et al., 2019; Ross et al., 2016).

While these classes of membrane bound ligands have been described to have the ability to signal bi-directionally with distinct outcomes in the context of forward signaling as a ligand or reverse signaling as a receptor, the mechanisms and purpose of reverse signaling have yet to be fully characterized. The primary hypothesis addressing the function of reverse signaling centers around the idea that reverse signaling may function as a counterbalance to the forward signaling mediated by ligand receptor interactions and that this bidirectional signaling increases the overall plasticity of intracellular signaling (Coate et al., 2009; Eissner et al., 2004; Konstantinova et al., 2007). Mechanistically, the majority of TNFSF proteins that have been shown to participate in bi-directional signaling contain casein kinase I (CK1) binding sites in their cytoplasmic tails (Ross et al., 2016; Watts, 1999) . The presence of conserved CK1 binding motifs in the cytoplasmic tails of TNFSF proteins across species suggests that this kinase may be involved in mediating reverse signaling (Watts, 1999); however, more experiments are needed to confirm this hypothesis.

Although many facets of tmTNF reverse signaling remain elusive, research using *in vivo* and *in vitro* murine models demonstrate that one of the major outcomes of tmTNF reverse signaling is LPS tolerance (Diallo et al., 2021; Eissner et al., 2000; Krasselt et al., 2022; Pallai et al., 2016). However, the mechanism through which LPS tolerance is achieved is controversial due to conflicting results between these studies. Some groups have shown that the anti-inflammatory cytokine Interleukin 10 (IL-10) is a major by-product of reverse signaling. Due to this, these groups hypothesized that IL-10 suppresses TNF production, which in turn mitigates inflammatory signaling (Diallo et al., 2021; Krasselt et al., 2022). Others have shown that tmTNF reverse signaling led to an overall cytokine suppressive environment, which included down-regulation of IL-10. Broadly, they hypothesized that tmTNF reverse signaling may confer LPS resistance via global down-regulation of LPS associated cytokines (Eissner et al., 2000; Pallai et al., 2016). While each of these studies sought to characterize tmTNF reverse signaling, the experimental design for these studies contained nuanced differences. Although it is tempting to overlook these slight differences and focus primarily on the major research findings, it is important to keep in mind the influence that cellular context has on signaling outcomes. Eissner *et al*., explored tmTNF reverse signaling in the context of human endothelial cell interaction with human monocytes/macrophages and found that tmTNF reverse signaling in monocytes induced by TNFR1 on epithelial cells mediated monocyte/macrophage secretion of soluble apoptotic factors and downregulated LPS associated cytokine production. Diallo *et al*., assessed tmTNF reverse signaling in the context of murine macrophage polarization using primary BMDMs derived from wild type and triple transgenic mice: TNFR1-/-, TNFR2-/-, and tmTNF+/+. Diallo *et al.,* found that tmTNF reverse signaling dampened classical M1 macrophage markers and

favored upregulation of M2 macrophage phenotypes, which included upregulation of IL-10 (Diallo et al., 2021). Krasselt *et al.,* assessed the relationship between the level of IL-10 in human patients who responded to anti-TNF treatment for rheumatoid arthritis before and after anti-TNF treatment. Their baseline control included IL-10 concentrations induced via tmTNF reverse signaling in blood monocytes. This was achieved by crosslinking TNFR receptor to tmTNF. They found that patients who had higher baseline concentrations of IL-10 responded better to subsequent anti-TNF therapies (Krasselt et al., 2022).

Although these studies implicate tmTNF reverse signaling in regulating subsequent IL-10 production, IL-10 regulation outcomes varied from study to study depending on the cell type(s)/species used and the mode of tmTNF reverse signal induction. Specifically, Eissener *et al.,* found that IL-10 was downregulated in their system (Eissner et al., 2000), Diallo *et al.,* found an initial early peak of IL-10 that correlated with inhibition of IL-6 and IL-12 (Diallo et al., 2021), whereas Krasselt *et al.,* found that IL-10 production could be used as a predictor for rheumatoid arthritis patient response to anti-TNF therapeutics (Krasselt et al., 2022). These varied and sometimes conflicting results highlight the complexity and plasticity of intracellular signaling and the role in which cellular context and signal integration influences downstream signaling outcomes. As such, future studies assessing the role of tmTNF reverse signaling should take cellular context and disease state into account when correlating reverse signaling to phenotypes and outcomes.

In summary, signaling induced by TNF is tightly regulated and can have opposing effects such as pro-inflammatory or anti-inflammatory processes, or controlled cell death vs. inflammatory cell death (Ardestani et al., 2013; Bigda et al., 1994; Ebach et al., 2005; Siegmund

et al., 2016) . The current body of literature suggests that these opposing effects are dependent on the cellular context which in turn regulates signaling at all levels. This regulation encompasses the ligand (i.e. bioactive form of TNF), the extracellular receptor, the recruited cytoplasmic proteins, and the transcriptional expression and production of proteins which subsequently feedback into the signaling loop to perpetuate or attenuate signaling (Ardestani et al., 2013; Choudhary et al., 2013; Ebach et al., 2005; Fu et al., 2021; Holbrook et al., 2019; X. Li et al., 2020; J. Liu et al., 2012; Thommesen & Laegreid, 2005; Tseng et al., 2018; Yuasa et al., 1998; L. Zhang et al., 2011) . One of the most clinically relevant and well-studied pathologies involving context dependent dysregulation of TNF signaling is autoimmunity, specifically rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), and inflammatory bowel disease (IBD) (Edwards et al., 1996; Levin et al., 2016; Matsuno et al., 2002). Broadly, these autoimmune disorders are characterized by aberrant immune autoreactivity to native host structures which is accompanied by constitutive production of TNF, leading to chronic inflammation (Edwards et al., 1996; Gordon et al., 1989; Levin et al., 2016; Ma & Xu, 2013; Matsuno et al., 2002). Interestingly, conflicting results have indicated that blocking TNF contributes to the generation of autoantibodies, while other studies have shown that blocking TNF can ameliorate autoimmune disease (Arends et al., 2010; De Bandt, 2019; Edwards et al., 1996; Gordon et al., 1989; Levin et al., 2016; Ma & Xu, 2013; Matsuno et al., 2002). Additionally, many anti-TNF therapies used to manage SLE, RA, or IBD have shown that inhibition of TNF leads to “paradoxical effects” which has been defined as an exacerbation of the inflammatory disease, despite concurrent anti-TNF treatment (Aringer & Smolen, 2008; Toussirot et al., 2012). Furthermore, the varying efficacies of anti-TNF therapeutics highlight the complex intersection between TNF,

inflammation, and host factors (Aringer & Smolen, 2008; Ma & Xu, 2013; Toussirot et al., 2012). Within autoimmune treatment populations, a subset of individuals referred to as "primary or secondary failures" has been identified (Gisbert & Chaparro, 2021; Roda et al., 2016; Rubbert-Roth et al., 2019). Primary failures consist of ~10-30% of the treated population and are defined as individuals who do not respond to anti-TNF treatment (Gisbert & Chaparro, 2021; Roda et al., 2016; Rubbert-Roth et al., 2019). Secondary failures are defined as individuals who respond to anti-TNF treatment initially, but subsequently fail to respond to following treatments. Secondary failures are more diverse and appear to be disease dependent; however, in RA models, secondary failures are estimated to account for 30-40% of the treated population (Gisbert & Chaparro, 2021; Roda et al., 2016; Rubbert-Roth et al., 2019). While the definitive causes for these differential outcomes to anti-TNF therapies remains elusive, strong evidence indicates that several factors may orchestrate this complex host response. These factors include physiological disease relevance (i.e. disease stage and individual genetic factors), the intrinsic spatiotemporal regulation of TNF, and the cellular context surrounding TNF production (M. Han et al., 2020; Quach et al., 2022; Yarilina et al., 2008).

One of the most crucial signaling events that is involved in all of these factors centers around the bioactive form of TNF (sTNF or tmTNF) and the receptors (TNFR1 and TNFR2) through which signaling is transduced. Using mechanisms that have yet to be fully defined, the activated macrophage cell integrates signals from its cellular environment to mediate TNF signaling. Upregulation and production of tmTNF, sTNF, TNFR1, and TNFR2 are heavily involved in coordinating the inflammatory response via interactions with one another, which initiate a complex network of intracellular signaling. This intracellular signaling is likely

mediated by signals from both TNFR1 and TNFR2 binding their cognate ligands (tmTNF and/or sTNF). In addition to the induced canonical forward signaling (ligand to receptor), compelling evidence has demonstrated induction of reverse signaling (receptor to ligand) upon TNFR1 or TNFR2 binding tmTNF (Diallo et al., 2021; Pallai et al., 2016); however, there remain many unanswered questions about this phenomenon. What is the purpose/outcome of tmTNF reverse signaling? Do canonical forward signaling and reverse signaling occur simultaneously? Could reverse signaling be a mechanism that mediates macrophage clearance of bacterial infections after PAMP recognition? What is the tmTNF signaling cascade? Addressing these questions could provide insight into the paradoxical effects known to occur with anti-TNF treatment in SLE, RA, and IBD. Furthermore, it could help to uncover unexplored mechanisms that contribute to the differences in anti-TNF therapeutic efficacy that is observed for different inflammatory diseases. Lastly, a deeper understanding of the holistic TNF signaling pathway which encompasses both canonical forward TNF signaling and reverse tmTNF signaling would broaden our understanding of the balance and regulation of inflammation, especially in the context of infection.

Hypothesis

The signaling cascades induced in response to TLR stimulation have been well studied. The individual signaling events within the TLR cascade as well as the overall signaling that initiates pro-inflammatory gene transcription have been extensively characterized. However, the signaling mechanisms of TNF induced in response to TLR2 or TLR4 stimulation is poorly defined, especially in the context of *both bioactive forms* of TNF (sTNF and tmTNF). Furthermore, because TNF is a critical cytokine that is responsible for mediating inflammatory

host defense mechanisms in response to infection, elucidating how both bioactive forms of TNF coordinate responses following TLR stimulation (PAMP recognition) is especially important. Here, we hypothesized that tmTNF produced as a result of TLR stimulation may be critical for macrophage priming, which leads to increased clearance of a subsequent *Staphylococcus aureus* infection by primed macrophages. The primary goals of this research were to 1) characterize both forms of TNF within our cell culture system, 2) determine whether/which bioactive form of TNF is required for macrophage priming, and 3) begin to interrogate potential signaling mechanisms of TNF priming which could lead to improved clearance of a subsequent *S. aureus* infection. To investigate these research goals, we utilized an *in vitro* model of immortalized WT murine bone marrow derived macrophages (BMDMs) and primary TNF-/- murine BMDMs.

In this dissertation, the terms “activation”, “stimulation”, “induction”, and “priming” are used frequently. Activation and stimulation are used interchangeably and refer to the process of ligand binding to cognate TLRs. Induction is used to refer to the initiation of intracellular signaling following TLR ligation. Priming is used to refer to a heightened active state of the macrophage cell resulting from TLR ligation followed by induction of intracellular signaling; whereby the macrophage cell is quicker to respond to a secondary stimulus.

In Chapter 2, we identified that TNF was a robustly induced cytokine in response to TLR2 stimulation in both *in vivo* and *in vitro* models. Additionally, we showed that both bioactive forms of TNF were upregulated; however, tmTNF alone may be sufficient for BMDM priming which mediated signaling that improved macrophage clearance of *S. aureus*. In Chapter 3, we outlined the protocol we developed for extraction, differentiation, cryopreservation, and resuscitation of bone marrow derived macrophages from mice. In addition, we also provided data

on the viability and functionality of these cryopreserved primary bone marrow derived macrophages for use in *in vitro* assays. To assess how tmTNF could be priming macrophages prior to bacterial infection, in Chapter 4 we hypothesized that tmTNF reverse signaling could be involved in priming BMDMs following TLR stimulation. Collectively, our studies interrogated the signaling events following TLR2 or TLR4 PAMP recognition which primed BMDMs for improved clearance of a subsequent *S. aureus* infection. We identified that tmTNF may be important for BMDM priming following PAMP recognition which in turn, mediated subsequent BMDM response to bacterial infection. Furthermore, we presented preliminary data which suggested that tmTNF reverse signaling could be involved in BMDM priming following TLR stimulation; however, more studies are needed to confirm this hypothesis.

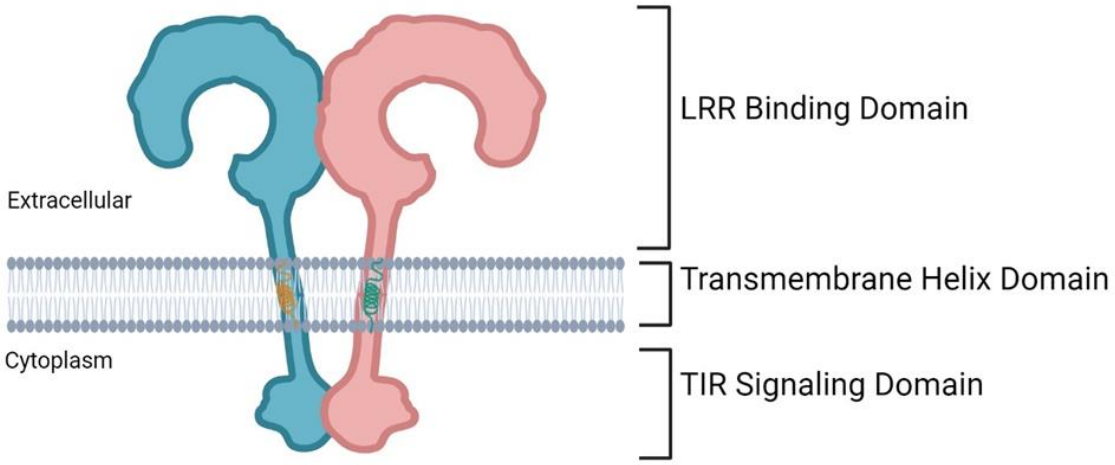

Figure 1. General TLR structure. The extracellular N-terminal ectodomain of TLRs consists of 19-25 highly conserved leucine rich repeats (LRRs) motifs. The transmembrane domain consists of a single helix and the intracellular C-terminal contains a Toll IL-1 Receptor (TIR) signaling domain.

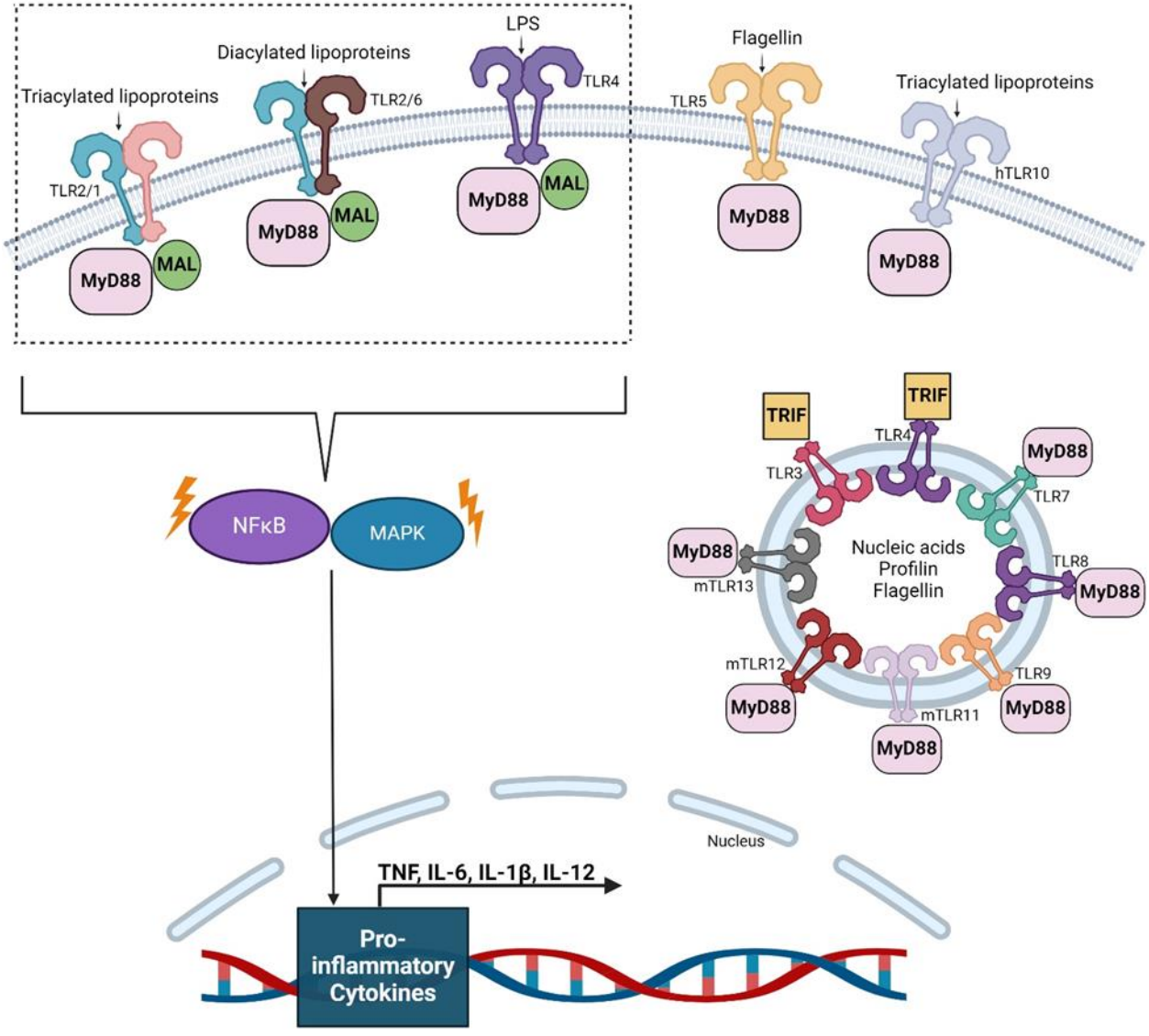

Figure 2. Summary of TLR receptors and ligands. Ligation of either TLR2/6, TLR2/1, or TLR4 recruits the signaling adaptor proteins MyD88 and MAL. These signaling adaptor proteins subsequently initiate MAPK and NFkB signaling pathways, leading to the production of pro-inflammatory cytokines.

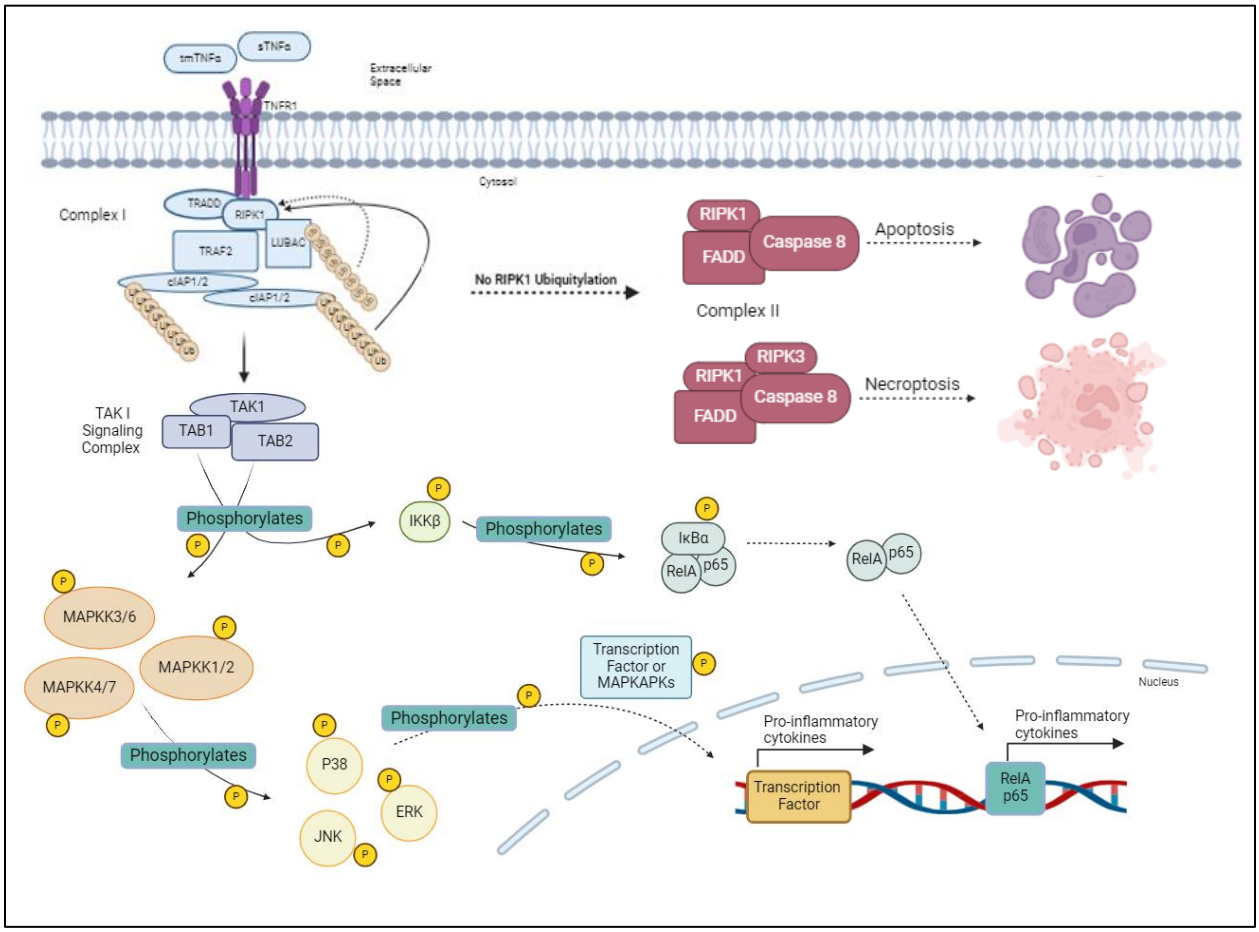

Figure 3. TNFR1 signaling cascade in response to sTNF or tmTNF ligation. Ligand binding to TNFR1 results in TRADD and RIPK1 recruitment to the death domain present on TNFR1 cytoplasmic tail. TRADD and RIPK1 recruit TRAF2 and cIAP1/2 which leads to cIAP1/2 ubiquitylation of RIPK1. Ubiquitylation of RIPK1 provides docking sites for LUBAC which further ubiquitylates RIPK1. This leads to the recruitment and assembly of the TAK1 signaling complex which initiates a series of phosphorylation cascades that ends with NFkB and MAPK activation and proinflammatory gene expression. Alternatively, at the start of the signaling cascade if RIPK1 is not ubiquitylated, it dissociates from TNFR1 and forms a complex with caspase 8 and FADD which leads to cellular death pathways.

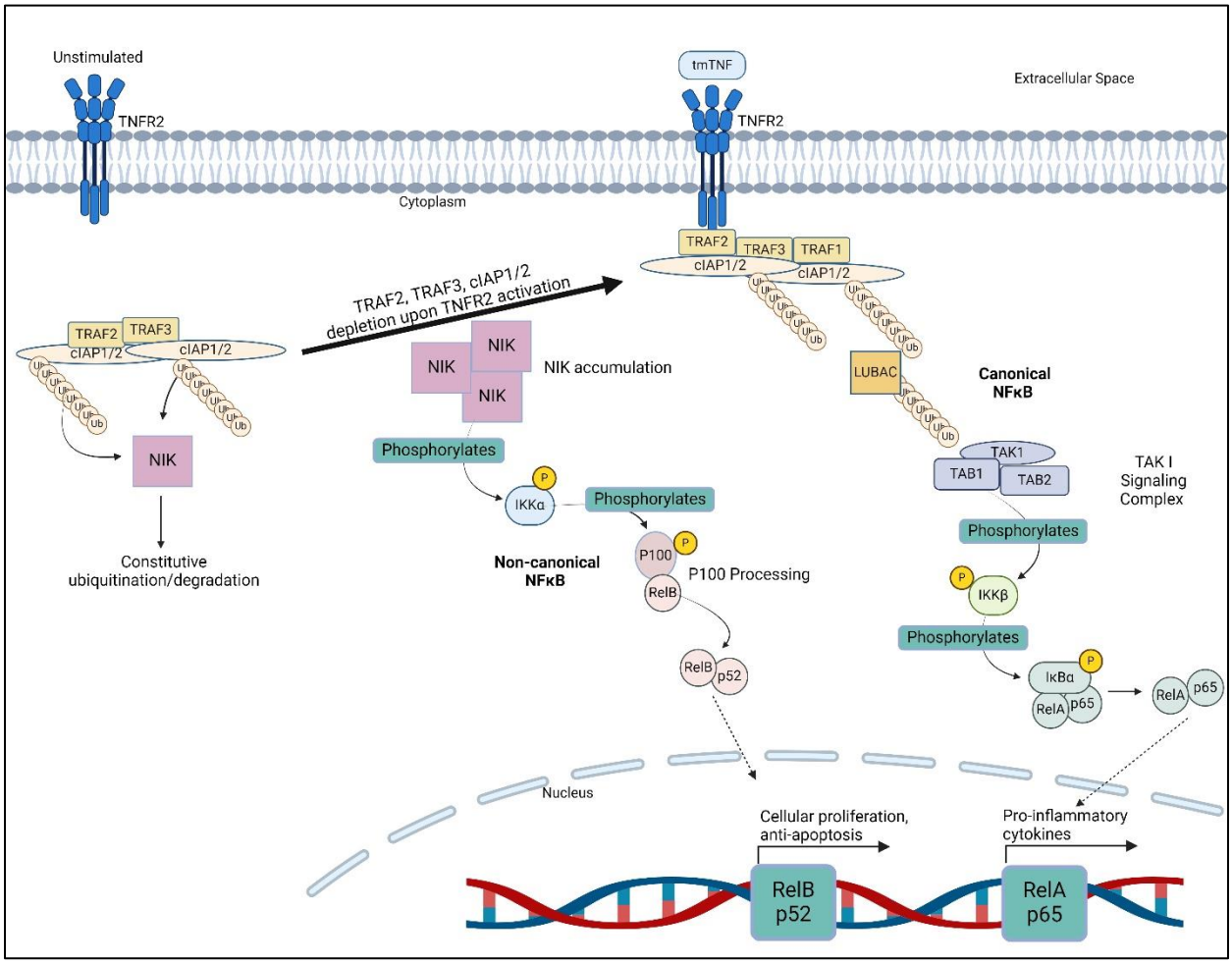

Figure 4. TNFR2 signaling cascade in response to tmTNF ligation. Ligand binding to TNFR2 results in direct recruitment of TRAF1, TRAF2, TRAF3, and cIAP1/2 to the cytoplasmic tail of TNFR2. Recruitment of TRAF2, TRAF3, and cIAP1/2 prevents TRAF2/3/cIAP1/2 mediated constitutive ubiquitination of NIK, allowing it to accumulate in the cytoplasm. NIK phosphorylates IKKα. Together, NIK and IKKα phosphorylate P100 and initiate P100 processing which results in release of the transcription factors p52 and RelB. RelB and p52 translocate to the nucleus to induce genes associated with cellular proliferation, anti-apoptosis, and secondary lymphoid development.

TLR2 OR TLR4 STIMULATION INDUCES TMTNF-DRIVEN PRIMING IN MACROPHAGES WHICH RESULTS IN IMPROVED CLEARANCE OF A SUBSEQUENT STAPHYLOCOCCUS AUREUS INFECTION

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Abstract

Introduction: Toll-like receptor (TLR) engagement on macrophages can improve responsiveness to infection. TNF is upregulated following TLR2 or TLR4 stimulation. We sought to determine whether and how the two bioactive forms of TNF may be contributing to macrophage priming which improved responsiveness to subsequent *Staphylococcus aureus* infection.

Methods: RNA sequencing and cytokine quantification assays identified differentially upregulated cytokines in response to TLR2 stimulation. Assays using immortalized and primary bone marrow derived macrophages (BMDMs) coupled with receptor blocking and cytokine supplementation were used to investigate whether/how prior TNF-primed macrophages improved *S. aureus* clearance.

Results: TLR2 or TLR4 stimulated TNF-/- BMDMs failed to efficiently clear a subsequent *S. aureus* infection compared to TLR stimulated WT BMDMs. Depletion of sTNF from TLR-stimulated WT BMDMs retained improved *S. aureus* clearance. Exogenous sTNF supplementation to TNF-/- BMDMs did not rescue improved *S. aureus* clearance. Cell density assays showed cell-to-cell contact was important for TLR-induced improvement of *S. aureus* clearance. Conversely, blocking TNFR2 reduced BMDM clearance of *S. aureus*, despite TLR2 stimulation.

Conclusions: Our results demonstrated that TNF produced in response to TLR stimulated BMDMs was required for improved clearance of a subsequent *S. aureus* infection. Between the two biologically active forms of TNF, we found that sTNF did not contribute to this priming, which suggested that tmTNF may be critical for BMDM priming which leads to improved *S. aureus* clearance.

Introduction

Macrophage recognition of microbes or their associated products, termed pathogen-associated molecular patterns (PAMPs) occur via ligation to host pattern recognition receptors (PRRs) (Nie et al., 2018; X. Zhang & Mosser, 2008). One class of PRRs that recognizes PAMPs are toll-like receptors (TLRs). TLRs encompass a group of evolutionarily conserved transmembrane receptors capable of recognizing PAMPs both extracellularly and intracellularly (Dasu et al., 2010; Hatton & Guerra, 2022). Two particularly important TLRs for extracellular PAMP recognition are TLR2 and TLR4, which are known to induce inflammation in response to a wide range of bacteria, including gram-negative and gram-positive microbes as well as mycobacteria (Dasu et al., 2010; Hatton & Guerra, 2022; Heldwein et al., 2003). PAMP ligation to TLR2 or TLR4 on immune cells is critical for initiating intracellular signaling cascades which promote host defense via inflammation (McCoy et al., 2021; Netea et al., 2002; Parker et al., 2004). During this inflammatory response, tumor necrosis factor alpha (TNF) is one of the first and most robustly produced cytokines, and it plays a major role in perpetuating the inflammatory host response (Raoust et al., 2009; Takeuchi et al., 1999).

TNF is a pleiotropic cytokine that exists in two bioactive forms. The 26kDa transmembrane TNF (tmTNF) is the precursor protein anchored to the cellular membrane. Due to this anchoring, tmTNF requires cell-to-cell contact to signal effectively. This limits tmTNF signaling outcomes to nearby cells (Kaymakcalan et al., 2009; Liang et al., 2022). The metalloprotease ADAM Metallopeptidase Domain 17 (ADAM17) cleaves tmTNF to produce the mature soluble form of TNF (sTNF), a 17kDa protein that is able to exert distal systemic effects (Kaymakcalan et al., 2009; Liang et al., 2022). Both forms of TNF are capable of binding either

of the two TNF receptors (TNFR1 or TNFR2). However, the inability of sTNF to efficiently signal through TNFR2 makes TNFR1 the primary receptor for sTNF (Grell et al., 1995, 1998). Broadly, ligation of TNFR1 by sTNF leads to classical pro-inflammatory responses via activation of canonical NFκB or MAPK signaling pathways (Liang et al., 2022; Lo et al., 2019; McFarlane et al., 2002; Wong et al., 2010). Conversely, TNFR2 is thought to be the primary receptor for tmTNF. Although tmTNF is also capable of signal transduction via TNFR1, tmTNF displays higher binding avidity for TNFR2 (Grell et al., 1995, 1998; Horiuchi et al., 2010; Liang et al., 2022). Ligation of TNFR2 leads to processes involved in cellular proliferation via activation of non-canonical NFκB or PI3K/AKT (Borghi et al., 2018; Naserian et al., 2020). Several studies suggest that differences in cellular expression between TNFR1 and TNFR2 could contribute to distinct signaling effects by sTNF and tmTNF (Al-Lamki et al., 2005; Wicovsky et al., 2009; Yang et al., 2019). Unlike TNFR1, which is ubiquitously expressed across cell types, TNFR2 expression is primarily found on immune cells (Hijdra et al., 2012; Naserian et al., 2020; Wajant & Siegmund, 2019; Yang et al., 2019). Furthermore, TNFR2 is a strictly inducible receptor, whereas TNFR1 is constitutively expressed (Hijdra et al., 2012; Naserian et al., 2020; Wajant & Siegmund, 2019; Yang et al., 2019). Together, the different affinities of TNF isoforms to TNFR1 and TNFR2 and the differential TNFR expression patterns allow for functional pleiotropy of TNF-induced inflammation.

Here, we hypothesize that tmTNF produced as a result of TLR stimulation may be critical for macrophage priming, which leads to increased clearance of a subsequent *Staphylococcus aureus* infection by primed macrophages. The primary goals of this study were to 1) characterize

both forms of TNF within our cell culture system and 2) determine whether/which bioactive form of TNF is required for macrophage priming that leads to improved clearance of *S. aureus.*

Although effects of signaling by either bioactive form of TNF induced during infection have been characterized, not much is known regarding whether and how sTNF and tmTNF induced prior to infection contribute to innate priming. A better understanding of the function of TNF following TLR stimulation (PAMP recognition) in macrophages could provide novel insights into the early key signaling events that could improve macrophage response to infection.

Materials and Methods

Mice

Six-to-eight-week-old female wild type (C57BL6) or TNF-/- mice were purchased from Jackson Laboratory. Mice were maintained at the Montana State University (MSU) Animal Resource Center. All care and procedures were in accordance with the recommendations of the National Institutes of Health, the U.S. Department of Agriculture, and the Guide for the Care and Use of Laboratory Animals (8^{th} ed) (*Guide for the Care and Use of Laboratory Animals*, 2011). Animal protocols were reviewed and approved by the MSU Institutional Animal Care and Use Committee (IACUC). For RNA sequencing experiments, WT mice were intratracheally inoculated with either 10µg of PAM2CSK4 or PAM3CSK4 (Invivogen) in 100µl volume, equal volume sterile PBS, or were left untreated (naïve). At 6 hours post-treatment (0 hours for naïve), mice were euthanized, lungs were extracted, cut into small pieces (<0.5cm) and were immediately stored in RNA-later (Thermofisher). RNA was extracted from the lungs by homogenizing the lung pieces with a TissueRuptor II (Qiagen) in 3mL of TRIzol Reagent (Invitrogen) with TissueRuptor disposable probes. Following homogenization, RNA was

extracted from half of the sample (other half flash frozen). Briefly, 0.3mL chloroform was added to the 1.5mL of TRIzol homogenate, vortexed, incubated 3 minutes, and the aqueous phase was collected into a new tube following a 15-minute centrifugation at 12000xg at 4C. Equal volume of 70% ethanol was added to the aqueous layer, mixed, and added to a RNeasy spin column. Following the Qiagen RNeasy protocol, the column was spun, flow through discarded, washed 2x with RPE Buffers. The column was dried with a brief centrifugation and the RNA was eluted. The quality of RNA was measured for each sample using a Bioanalyzer (Agilent) and only RNA samples with a RIN number >9.0 and OD260/280 >2.0 were sent off for sequencing. Processing of RNA for library generation and sequencing on an Illumina Platform PE150 instrument was done by NovoGene Corporation (Sacramento, CA, USA).

RNA Sequencing Analysis

Downstream analysis was performed by NovoGene Corporation and their protocol is as described below. A combination of programs including STAR (v2.6.1), HTseq (v0.6.1), Cufflink and NovaGene Corporation wrapped scripts were utilized. Alignments were parsed using STAR program and differential expressions were determined through DESeq2 (v2_1.6.3)/edgeR (v3.16.5). Gene fusion and difference of alternative splicing event were detected by Star-fusion and rMATS software. Reference genome and gene model annotation files were downloaded from genome website browser (NCBI/UCSC/Ensembl) directly (GRCm38 Mus Musculus genome). Indexes of the reference genome was built using STAR and paired-end clean reads were aligned to the reference genome using STAR (v2.5). STAR used the method of Maximal Mappable Prefix (MMP) which can generate a precise mapping result for junction reads. STAR will count number reads per gene while mapping. The counts coincide with those produced by htseq-count

with default parameters. Fragments per kilobase of exon per million mapped fragments (FPKM) of each gene was calculated based on the length of the gene and reads count mapped to this gene. FPKM considers the effect of sequencing depth and gene length for the reads count at the same time, and is currently the most commonly used method for estimating gene expression levels (Mortazavi et al., 2008). For DESeq2 with biological replicates, differential expression analysis between two conditions/groups (two biological replicates per condition) was performed using the DESeq2 R package (1.14.1). DESeq2 provide statistical routines for determining differential expression in digital gene expression data using a model based on the negative binomial distribution. The resulting P-values were adjusted using the Benjamini and Hochberg's approach for controlling the False Discovery Rate (FDR). Genes with an adjusted P-value <0.05 found by DESeq2 were assigned as differentially expressed.

For edgeR without biological replicates, prior to differential gene expression analysis, for each sequenced library, the read counts were adjusted by edgeR program package through one scaling normalized factor. Differential expression analysis of two conditions was performed using the edgeR R package (3.16.5). The P values were adjusted using the Benjamini & Hochberg method. Corrected P-value of 0.05 and absolute foldchange of 1 were set as the threshold for significantly differential expression.

RNA sequencing data received from NovoGene Corporation analysis was organized and plotted using Linux and R provided by the National Center of Genomics Research (NCGR; Santa Fe, NM, USA). Pathway analysis was performed using Cytoscape and ClueGo software (access provided by NM-INBRE and the National Center of Genomics Research).

Isolation and Culture of Primary BMDMs

Primary BMDMs were collected from WT C57BL6 or TNF-/- mice purchased from Jackson laboratories. Mice were euthanized according to IAUCUC protocol and both pairs of femurs, tibias, and fibulas were collected per mouse and bones were soaked in 70% ethanol for 5 minutes before bone marrow was flushed from the bones with 1X Roswell Park Memorial Institute 1640 (RPMI; Cytiva, Marlborough, MA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Atlas Biologicals, Fort Collins, CO, USA), 0.1M Hepes (Fisher Scientific, Waltham, MA, USA), 10000U/ml penicillin-streptomycin (Fisher Scientific, Waltham, MA, USA), 4mg/ml glutamine (Gibco, Grand Island, NY, USA), and $1.792x10^{-3}$ mM 2-Mercaptoethanol (Fisher Scientific, Waltham, MA, USA). Bone marrow was centrifuged at 408xg for 5 minutes at 4˚C and subsequently incubated with ACK lysis buffer (Fisher Scientific, Waltham, MA, USA) for 4 minutes at room temperature. Bone marrow cells were centrifuged, resuspended, and differentiated into macrophages in 1X RPMI1640 supplemented with 10% heat-inactivated FBS, 0.1M Hepes, 10000U/ml penicillin-streptomycin, 4mg/ml glutamine, $1.792x10^{-3}$ mM 2-Mercaptoethanol, and L929 conditioned media for 6 days at 37˚C with 5% CO_2.

Cell Culture

Immortalized bone marrow macrophages derived from WT, $TLR2^{-/-}$ and $TLR6^{-/-}$ mice on a C57BL6 background (NR-9456, NR-9457, and NR-19972) were obtained from BEI Resources, NIAID, NIH. Cells were maintained in Dulbecco's modified Eagles medium (DMEM; Cytiva, Marlborough, MA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Atlas Biologicals, Fort Collins, CO, USA), 2mM glutamine (Gibco, Grand Island, NY, USA)

1mM sodium pyruvate (Gibco, Grand Island, NY, USA), and 10ug/ml ciprofloxacin (Bioworld, Dublin, OH, USA) and cultured at 37°C with 5% CO_2 until 90% confluency was reached.

Preparation of *Staphylococcus aureus*

Staphylococcus aureus (USA300 LAC) was a kind gift from Dr. Jovanka Voyich, Department of Microbiology and Cell Biology at MSU. BBL Trypticase soy broth (TSB; BD, Franklin Lakes, NJ, USA) was inoculated with frozen *S. aureus* and grown overnight at 37°C with 250rpm shaking. The following morning, fresh TSB was inoculated with the overnight culture 1:100 and cultured for 2 hours 15 minutes at 37°C with 250rpm shaking to achieve a day culture of *S. aureus*. 1ml of day culture or uninfected TSB (blank) was transferred to a cuvette and OD_{600} was determined. Bacteria were then diluted to appropriate concentration based on growth curve.

Bacterial Clearance Assay

Murine immortalized macrophage cell line NR-9456, NR-9457, or NR-19972 (BEI Resources; Manassas, Virginia, USA) were grown and maintained as previously described until 90% confluency was achieved. Cells were lifted with a sterile-filtered solution of 3mM EDTA (MilliporeSigma; Burlington, MA, USA) dissolved in dPBS (Cytiva, Marlborough, MA, USA) for 10 minutes at 37°C with 5% CO_2. Cells were washed once in sterile dPBS, resuspended in complete media, and counted before being plated in 24-well plates at a cell density of $5x10^5$ cells/well and allowed to adhere for 12 hours at 37°C with 5% CO_2. Cells were then treated for 12 hours with 500ul complete media containing either equal volume PBS, 1ug/ml Pam2CSK4 (PAM2; InvivoGen, San Diego, CA, USA), 1µg/ml Pam3csk4 (PAM3; InvivoGen, San Diego, CA, USA), or 1µg/ml ultrapure lipopolysaccharide (LPS; InvivoGen, San Diego, CA, USA) with

or without 100µg/ml of p75TNFR:Fc, a dimeric fusion protein composed of two extracellular ligand binding domains of the 75kDa receptor for TNF linked to the FC fragment of human IgG1, a kind gift from Amgen Inc., 1µM TMI-005 (Cayman Chemical, Ann Arbor, MI, USA), or 1µg anti-mouse TNFR2 antibody (BioXcell, Lebanon, NH, USA). After treatment, cells were washed once with dPBS and incubated with *S. aureus* at an MOI of 2:1 for 5 hours at 37°C with 5% CO_2. Cell lysis was performed with 1% saponin (MilliporeSigma; Burlington, MA, USA) and CFUs were determined based on plating serial dilutions using the drop plate method onto tryptic soy agar (Sithisarn et al., 2021) (TSA; Fisher Scientific, Waltham, MA, USA). Cellular supernatants before treatment, after treatment, and after *S. aureus* were collected into sterile Eppendorf tubes and frozen at -80°C for cytokine analysis.

Western Blot

Cells were seeded into 6 well plates at $2x10^6$ cells/well and allowed to adhere for 12 hours at 37°C with 5% CO_2. Cells were treated for 12 hours with 1ml complete media containing either equal volume PBS, 0.5µg/ml PAM2, 0.5µg/ml PAM3, or 0.5µg/ml LPS. Subsequently, media was removed, cells were washed once with ice cold PBS and lysed with 500µl RIPA buffer (ThermoFisher Scientific; Waltham, MA, USA) supplemented with protease inhibitor cocktail (MilliporeSigma; Burlington, MA, USA) at 1:100 dilution and phosphatase inhibitor cocktail 2 (MilliporeSigma; Burlington, MA, USA) at 1:500 dilution. Cells were rocked at 4°C for 15 minutes before being transferred to Eppendorf tubes and centrifuged at top speed for 10 minutes at 4°C. Supernatant was collected and BCA (ThermoFisher Scientific; Waltham, MA, USA) was performed to assess total protein concentration. Total protein was separated on 10% SDS-PAGE gels at constant amperage (80mA) and transferred to nitrocellulose membranes

(MilliporeSigma; Burlington, MA, USA) for 1 hour at constant amperage (300mA). Membranes were blocked in 3% (w/v) bovine serum albumin (BSA; ThermoFisher Scientific; Waltham, MA, USA) for 1 hour with rocking at room temperature and subsequently incubated overnight with rocking at 4°C with the following primary antibodies (1:1000 dilution): anti-β-actin, anti-GAPDH (Cell Signaling Technologies, Danvers, MA; USA), or anti-TNF (Abcam, Cambridge, MA, USA). Membranes were then incubated 1:2000 in secondary anti-rabbit IgG HRP-linked antibody (Cell Signaling Technologies, Danvers, MA; USA) for 45 minutes with rocking at room temperature before detection with ECL substrate and imaging (BioRad, Hercules, CA, USA).

Quantitative Reverse Transcription PCR (qRT-PCR)

Cells were seeded into 24-well plates at $5x10^5$ cells/well and allowed to adhere for 24 hours at 37°C with 5% CO_2. Cells were then treated for 3 hours with 500µl complete media containing either equal volume PBS, 1µg/ml Pam2CSK4, 1µg/ml Pam3csk4, or 1µg/ml ultrapure lipopolysaccharide (PAM2; PAM3; LPS; all from InvivoGen, San Diego, CA, USA). Treatments were removed, cells were washed with sterile dPBS, and lysed with 400µl TRIzol Reagent (Invitrogen, Waltham, MA, USA). RNA was extracted using chloroform (ThermoFisher Scientific; Waltham, MA, USA), 70% ethanol (MSU chemistry store), Buffer RPE (Qiagen, USA), RNase free water (Invitrogen, Waltham, MA, USA), and RNA Mini spin columns (Epoch Life Science Inc, Missouri City, TX, USA) before quantification of RNA via nanodrop. All RNA samples were normalized to a concentration of 20ng/µl prior to cDNA library synthesis. The cDNA library was created using high-capacity cDNA Reverse Transcription kit (Applied Biosystems, Waltham, MA, USA) according to manufacturers' recommendation with a total input of 100ng RNA. qPCR was performed in triplicate using SsoAdvanced Universal SYBR

Green Supermix (BioRad, Hercules, CA, USA) according to manufacturers' recommendation with TNF, β-actin, LTα, and GAPDH primers (all from IDT, Coralville, IA, USA). All qRT-PCRs were run on QuantStudio 5 Real-Time PCR system (ThermoFisher Scientific; Waltham, MA, USA). A total of forty cycles were run for each qRT-PCR and a CT of ≤25 cycles was used as a cutoff for positive values. Relative fold change was calculated using delta delta CT method and data was normalized to β-actin.

TNF sequence primer 1: 5'AGACCCTCACACTCAGATCA-3'

TNF sequence primer 2: 5' TCTTTGAGATCCATGCCGTTG-3'

β-actin sequence primer 1: 5' GATTACTGCTCTGGCTCCTAG-3'

β-actin sequence primer 2: 5' GACTCATCGTACTCCTGCTTG-3'

GAPDH sequence primer 1: 5'-AATGGTGAAGGTCGGTGTG-3'

GAPDH sequence primer 2: 5'-GTGGAGTCATACTGGAACATGTAG-3'

LTα sequence primer1: 5'-CTCAGAAGCACTTGACCCAT-3'

LTα sequence primer 2: 5'-TCTCCAGAGCAGTGAGTTCT-3'

ELISA

Cellular supernatants were collected from bacterial clearance assays before treatment, after treatment, and after *S. aureus* infection and frozen back at -80°C for later cytokine analysis. Cellular supernatants collected post *S. aureus* infection were centrifuged at 835x*g* for 15 minutes before aliquoting and storage at -80°C. ELISAs were performed with ELISA MAX Deluxe Set Mouse TNF-α kit according to manufacturers' recommendations (BioLegend, San Diego, CA, USA).

Immunocytochemistry

Cells were seeded into 24-well plates containing sterile collagen coated coverslips (Neuvitro Corporation, Vancouver, WA, USA) at $5x10^5$ cells/well and allowed to adhere for 12 hours at 37°C with 5% CO_2. Cells were then treated for 12 hours with 500ul complete media containing either equal volume PBS, 1ug/ml Pam2CSK4 (PAM2; InvivoGen, San Diego, CA, USA), 1µg/ml Pam3csk4 (PAM3; InvivoGen, San Diego, CA, USA), or 1µg/ml ultrapure lipopolysaccharide (LPS; InvivoGen, San Diego, CA, USA) with or without the ADAM17 inhibitor, TMI-005 (Cayman Chemical, Ann Arbor, MI, USA) or TNF inhibitor p75TNFR:Fc (Amgen Inc., Thousand Oaks, CA, USA), for 12 hours at 37°C with 5% CO_2. Coverslips were washed twice with ice cold dPBS, fixed with 4% PFA (ThermoFisher Scientific; Waltham, MA, USA) for 15 minutes at room temperature, and blocked/permeabilized in 400ul 2% BSA (ThermoFisher Scientific; Waltham, MA, USA) with 0.1% Triton X-100 (MilliporeSigma; Burlington, MA, USA) for 45 minutes on ice. Coverslips were then blocked with 1x avidin/biotin blocking solution for 15 minutes at room temperature with washing steps in-between each blocking step (ThermoFisher Scientific; Waltham, MA, USA). Primary rat anti-mouse TNF antibody conjugated to biotin (a kind gift from Dr. Mark Jutila at MSU) was diluted 1:250 in 2% BSA and incubated with coverslips for 1 hour at room temperature protected from light. Coverslips were washed with dPBS and incubated with secondary avidin-HRP antibody (BD Pharmingen Inc, Franklin Lakes, New Jersey, USA) at 1:1000 dilution in 2% BSA for 1 hour at room temperature protected from light. Subsequently, coverslips were washed with dPBS and incubated with 3,3-diaminobenzidine chromogen (ImmPACTDAB; Vector Labs, Burlingame CA, USA) for 10 minutes at room temperature. Nuclear staining was performed with methyl

green counterstain (Vector Labs, Burlingame CA, USA) for 5 minutes at 60°C. Visualization was performed with standard compound light microscope.

Statistical Analysis

To account for the variance within and between individual experiments, bacterial clearance assay dilutions were plated onto two agar plates. On each plate every dilution was in duplicate and each duplicate included 5 drops. The values of CFU counts for all 5 drops were averaged to obtain one value per dilution and the same was done for the duplicate dilution on each plate. The two values were averaged to obtain a replicate. Shown is the average of two replicates per experiment. Results from at least two independent experiments are shown for bacterial clearance assays. Western blots were performed a minimum of three times independently. Data from ELISA assays included three technical replicates and were performed a minimum of two times independently. Western blot data were analyzed using AzureSpot Pro software and plotted with GraphPad Prism software. Quantitative RT-PCR was performed a minimum of two times independently, with each experiment containing three technical replicates. Relative fold change was calculated using delta delta CT method and data was normalized to β-actin. All other data were analyzed and plotted using either GraphPad Prism or R Studio software. Data were expressed as geometric mean with geometric standard deviation. Geometric means were calculated by taking the log means of experimental values and calculating antilog. Geometric standard deviations were calculated from the variance of log experimental values. Statistical significance was determined using either one-way analysis of variance (ANOVA), two-way ANOVA, or student's t-test and only statistically significant comparisons are shown in figures ($p \leq 0.05$).

Results

TNF Produced in Response to Either TLR2 or TLR4 Induced Priming of BMDMs Contributes to Improved Clearance of Subsequent *S. aureus* Infection

We found that prior stimulation of immortalized wild type (WT) bone marrow derived macrophages (BMDMs) with TLR2/6 agonist (Pam2CSK4; PAM2), TLR4 agonist (lipopolysaccharide; LPS), or TLR2/1 agonist (Pam3CSK4: PAM3) reduced bacterial burden of a subsequent *Staphylococcus aureus* infection by ~40% when compared to mock stimulated WT BMDMs (Figures 1A and S1A). Consistent with the findings of others (Hoogerwerf et al., 2010; Kulsantiwong et al., 2017; Kumar et al., 2010; Ware et al., 2019), BMDMs stimulated with these agonists required their cognate TLR2 or TLR4 receptors to productively clear bacterial infection (Figures 1B, S1B, S1C). Together, this indicated that signaling through either TLR2 or TLR4 was critical for improving subsequent BMDM response to *S. aureus* infection. Here, we sought to investigate the intracellular signaling cascade initiated by the detection of PAMPs (through TLR2 or TLR4) that provided BMDMs with improved capacity for clearance of a subsequent bacterial infection.

Stimulation of innate immune cells with PAMPs often results in rapid secretion of proinflammatory cytokines such as IL-1b, IL-6, and TNF (Heydarian et al., 2024). Thus, we analyzed transcript expression in whole lung homogenates of WT mice intratracheally inoculated with either PAM2 (TLR2/6) or PAM3 (TLR2/1) agonist for 6 hours. We found that TNF was among one of the most heavily upregulated genes in response to TLR2 stimulation (Figure 1C). As such, we focused our subsequent investigation on macrophages, which are the primary producers of TNF (Imaizumi et al., 2000; Nau et al., 2002) and one of the first innate immune

cells to respond to microbial invasion (Moon et al., 2011). Dependent on the isoform (tmTNF or sTNF), TNF protein signals through TNFR1 or TNFR2. Pathway analysis of RNA seq data within the Reactome database identified that the tumor necrosis factor receptor type II (TNFR2) and non-canonical NFkB pathway were upregulated among mice that received either PAM2 or PAM3 (Figure S2). TNFR2 has two naturally occurring ligands, TNF and lymphotoxin α (LTα) (Grell et al., 1995; Kucka et al., 2021) both of which were identified to be upregulated in the RNA sequencing dataset. However, LTα upregulation (≤1.1 log fold) was mild in comparison to the robust upregulation of TNF (≥3.8 log fold) and as such, we chose to focus our analysis on TNF.

Next, we quantified TNF gene expression in immortalized WT BMDMs stimulated with either TLR2 or TLR4 agonists. Indeed, we found that at 3 hours following WT BMDM stimulation, TNF gene expression was increased at least 5-fold relative to PBS (Figures 1D, S3A). Because LTα is known to bind TNFR2 and was mildly upregulated in our RNA seq dataset, we also quantified LTα gene expression within our system and found no notable increase in LTα gene expression in WT BMDMs stimulated with either TLR2 or TLR4 agonist (Figure S3B). This suggested that LTα did not play a major role in the TLR signaling-dependent priming of BMDMs.

We next sought to address whether the observed increase in TNF expression in WT BMDMs was indeed dependent on the TLR2 or TLR4 agonists binding their cognate receptors. We stimulated immortalized murine TLR2-/- or TLR4-/- with their respective ligands for 3 hours before quantifying TNF transcript via qRT-PCR. In TLR2-/- BMDMs stimulated with either PAM2 or PAM3, the absence of TLR2 receptor subunit resulted in deficiency of TNF gene

expression (Figures 1E, S3C). To the contrary, even in the absence of the TLR2 receptor subunit, BMDMs upregulated TNF gene expression in response to LPS stimulation (Figure 1E). In TLR4-/- BMDMs, LPS stimulation did not induce TNF gene expression. However, TNF gene was expressed in TLR4-/- BMDMs following either PAM2 or PAM3 stimulation (Figures 1F, S3D).

Next, we asked whether TNF mediated priming of BMDMs was necessary for improved clearance of subsequent bacterial infection. Unlike WT BMDMs (Figure 1A), primary TNF-/- BMDMs stimulated with either TLR2 or TLR4 agonists did not improve their clearance of a subsequent *S. aureus* infection when compared to PBS treated primary TNF-/- BMDMs (Figures 1G, S4). This suggested the TNF produced as a direct result of TLR2 or TLR4 stimulation played a central role in priming BMDMs prior to *S. aureus* infection. Based on these data, we sought to further elucidate the mechanism of TLR-induced TNF mediated priming which resulted in improved clearance of a subsequent bacterial infection.

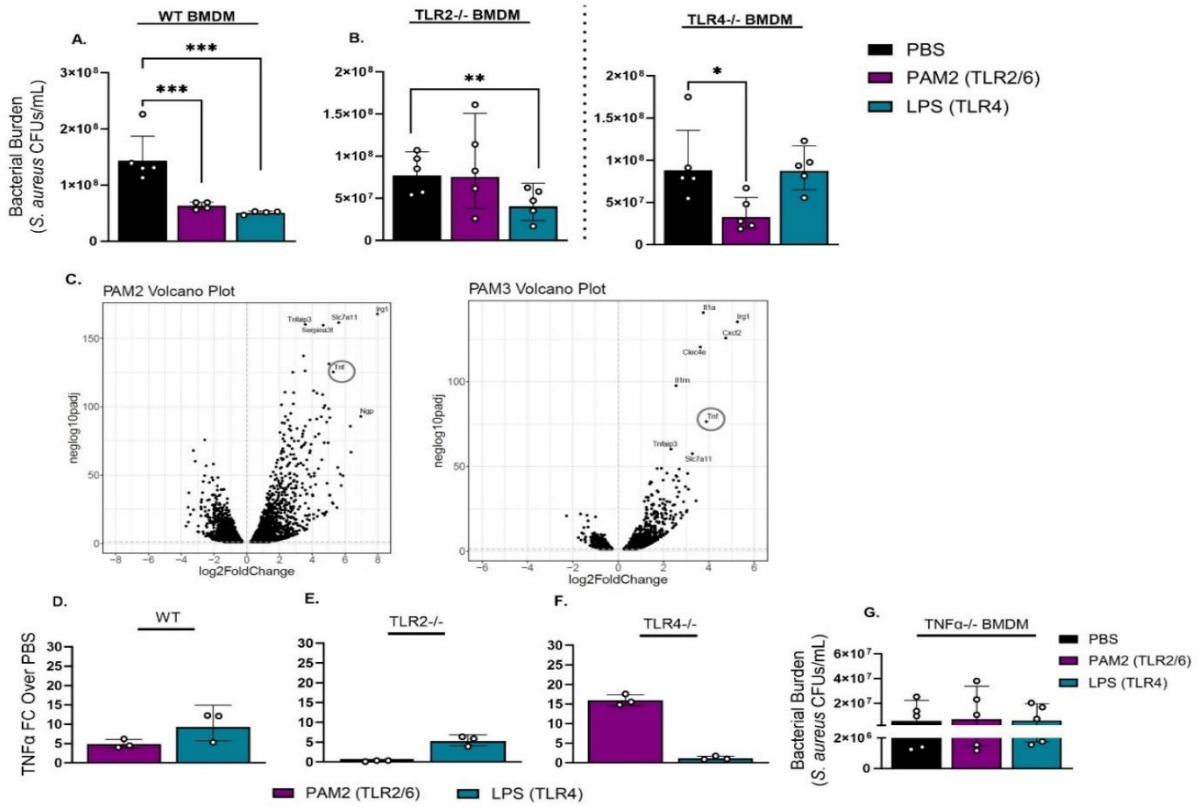


Figure 1. TLR-induced TNF is required for improved macrophage clearance of *S. aureus*. (A) WT BMDMs, (B) TLR2-/- and TLR4-/- BMDMs were treated with either 1µg/ml PAM2, LPS, or equal volume PBS for 12 hours followed by S. aureus infection for 5 hours (MOI 2:1). Bacterial burdens (CFUs) were determined after overnight incubation on TSA plates. Results shown in panels A and B were obtained from 5 independent experiments per cell line. Each data point represents the average of two technical replicates per experiment. A ROUT outlier test was performed and identified outliers were removed from the datasets prior to statistical analysis. Statistical analysis was performed with ordinary one-way ANOVA ***P≤0.0009 (WT BMDMs), *P≤0.0148 (TLR4-/- BDMDMs) or with one-sided paired t-test **P≤0.0084 (TLR2-/- BMDMs). (C) WT mice were intratracheally inoculated with 100µl of either 10µg PAM2, PAM3, or equal volume PBS. Lung homogenates collected 6 hours post-inoculation were processed and analyzed by RNA sequencing. Volcano plots were generated in R studio. (D-F) TNF transcript 3 hours following PAM2 or LPS stimulation of immortalized murine WT BMDMs (D), TLR2-/- BMDMs (E), and TLR4-/- BMDMs (F). Data was normalized to reference gene β-actin, and fold change was calculated using ΔΔCT method. Results were obtained from 3 independent experiments that had three technical replicates each. Each data point represents the average of three technical replicates. (G) Primary TNF-/- BMDMs were treated with either 1µg/ml PAM2, LPS, or equal volume PBS for 12 hours followed by S. aureus infection for 5 hours (MOI 2:1). Bacterial burdens (CFUs) were determined after overnight incubation on TSA plates. Data was obtained from BMDMs isolated from 5 mice. BMDMs from each mouse were ran as an independent assay which contained 2 technical replicates. Each data point represents the average of two technical replicates.

TLR2 or TLR4-Induced Priming Leads to Production of Both Bioactive Forms of TNF Protein in BMDMs

TNF exists in two bioactive forms, soluble/secreted (sTNF) and transmembrane (tmTNF), both of which bind distinct receptors to elicit differential signaling outcomes (Alexopoulou et al., 2006; Ardestani et al., 2013; Liang et al., 2022; Lis et al., 2014). Cellular supernatants collected from WT BMDMs after 12-hour stimulation with either TLR2 or TLR4 agonist exhibited a significant increase in sTNF protein production compared to PBS treated BMDMs (Figure 2A). Immunocytochemisty (ICC) and western blot analyses following 12-hour stimulation with either TLR2 or TLR4 agonists also demonstrated an increase in tmTNF protein production compared to mock treated BMDMs (Figures 2B, 2C). To address the possibility that this early sTNF and tmTNF could improve subsequent bacterial clearance by simply enhancing TNF production, we quantified TNF in supernatants from TLR2 or TLR4 stimulated WT BMDMs compared to PBS stimulated WT BMDMs after a 5-hour infection period with *S. aureus*. PBS, PAM2, PAM3, and LPS stimulated BMDMs all exhibited comparable concentrations of sTNF after *S. aureus* infection (~7000pg/ml) (Figure 2D, S5). This suggested the presence of TNF following TLR2 or TLR4 stimulation was not influencing the subsequent production of TNF during active *S. aureus* infection.

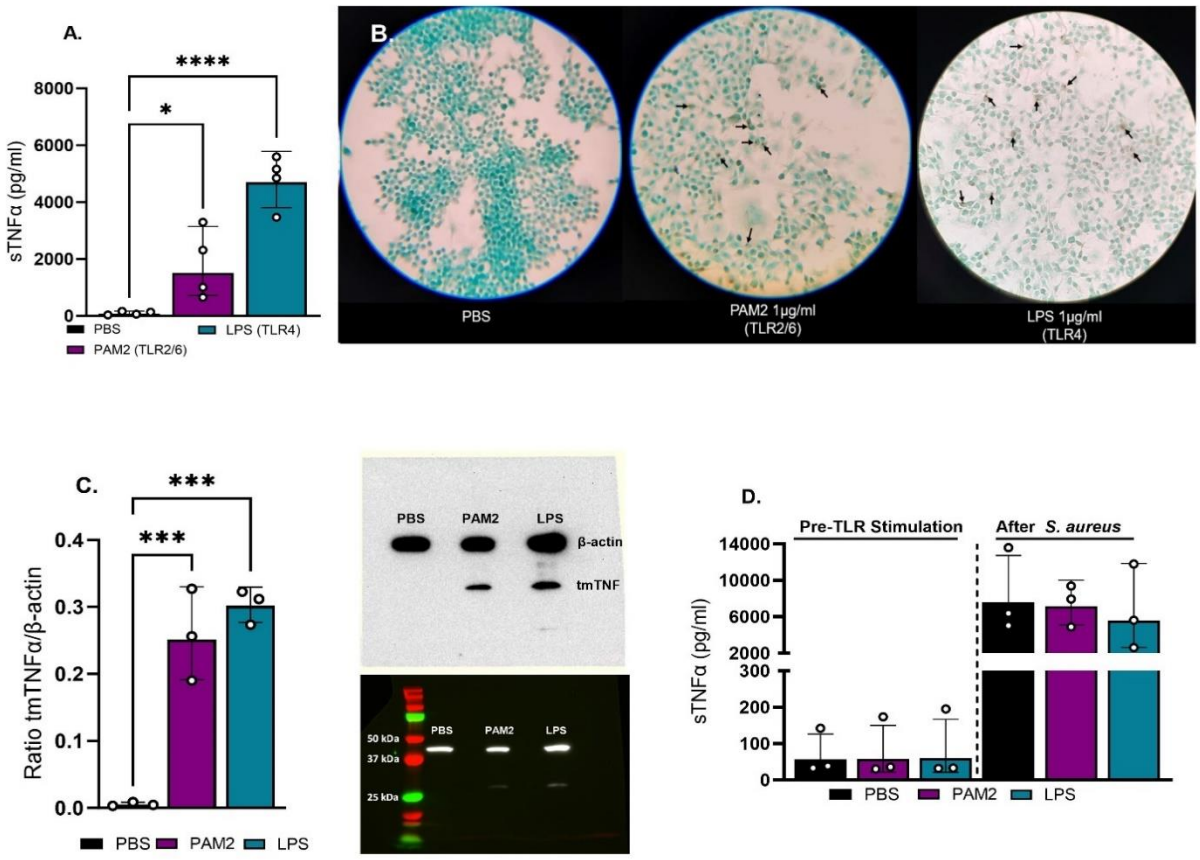


Figure 2. TLR stimulation of BMDMs upregulates both bioactive forms of TNF. (A) Soluble TNF was quantified from immortalized WT BMDM cellular supernatants after stimulation for 12 hours with either 1µg/ml PAM2, LPS, or equal volume PBS. Results were obtained from 4 independent experiments. ELISAs had a minimum of 2 technical replicates for each sample. Each data point represents the averaged technical replicate in each experiment. Statistical analysis was performed with one-way ANOVA *P≤0.0485 **** P≤0.0001. (B) Transmembrane TNF protein visualized by immunocytochemistry (presence of brown indicates tmTNFα; black arrows). ICC pictures were obtained from different experiments. (C) The tmTNF quantified by western blot and presented as the ratio of tmTNF over reference protein β-actin using AzureSpot Pro software. Western blot ratios were calculated from 3 independent experiments; however, western blot band data shown (right) is from one representative experiment. Statistical analysis was performed with one-way ANOVA ***P≤0.0008. (D) The sTNF protein was quantified from immortalized WT BMDM supernatant before TLR stimulation (Pre-TLR Stimulation; left) and after infection with S. aureus for 5hrs (After S. aureus; right). Results were obtained from three independent experiments which included 3 technical replicates each. Each data point represents the average of 3 technical replicates. Data in "Pre-TLR Stimulation" condition was obtained from separate experiments than data in "After S. aureus" condition.

Soluble TNF is Not Sufficient to Prime BMDMs For Improved Clearance of a Subsequent *S. aureus* Infection

In Figure 1G, we demonstrated that PAM2 or LPS stimulated BMDMs deficient in TNF were not able to improve their ability to clear a subsequent *S. aureus* infection. Because either TLR2 or TLR4 stimulation of WT BMDMs resulted in abundant production of both sTNF and tmTNF, we sought to investigate if both bioactive forms of TNF equally participated in BMDM priming that granted BMDMs improved clearance of a subsequent *S. aureus* infection.

We first tested whether sTNF is sufficient to mediate the priming event. PAM2 or PBS stimulated TNF-/- BMDMs were supplemented with 2000pg/ml of exogenous murine recombinant sTNF or equal concentration of irrelevant protein control (OVA). Our exogenous sTNF dosage choice was guided by results in Figure 2A, which demonstrated that WT BMDMs stimulated with TLR2 or TLR4 agonist induced a minimum of 2000pg/ml sTNF. The validation of TNF-/- BMDMs prior to exogenous sTNF supplementation confirmed deficiency of sTNF production both after 12-hour stimulation with TLR2 or TLR4 stimulation and after *S. aureus* infection (Figure S6).

Consistent with results in Figure 1G, we found that TLR2 stimulation in the absence of TNF was insufficient for BMDM priming (Figure 3A). More importantly, we found that addition of exogenous sTNF to TLR2 stimulated TNF-/- BMDMs was also not sufficient to prime BMDMs prior to bacterial infection. This was evidenced by no change in *S. aureus* clearance between treatment groups that lacked exogenous sTNF and treatment groups that were supplemented with exogenous sTNF (Figure 3A).

Next, we employed a reductionist approach to complement experiments which supplemented sTNF. The tmTNF is the bioactive precursor to mature sTNF and as such, the

extracellular domain of tmTNF and sTNF have nearly homologous sequences (Deora et al., 2017; Miao et al., 2020). This posed an obstacle for selective TNF inhibition. To address this issue, we employed two approaches which target TNF protein regulation at different cellular levels. To ensure that our approaches yielded depletion to baseline concentrations, we first quantified sTNF concentrations in PBS treated BMDMs to establish a baseline. We determined that in the absence of TLR stimulation, sTNF concentrations in these cells was on average ~100pg/ml (Figure S7).

In our first approach, we leveraged the critical function of tumor necrosis factor-α-converting enzyme (TACE; ADAM17) for regulating TNF. ADAM17 is the enzyme responsible for cleavage of the precursor form of tmTNF to its secreted sTNF form (Deora et al., 2017; Miao et al., 2020). Therefore, we postulated the use of ADAM17 small molecule inhibitor, TMI-005 (Apratastat) would prevent cleavage of tmTNF, thereby reducing sTNF protein levels. Indeed, inhibition of ADAM17 allowed us to enrich cell surface associated tmTNF protein while simultaneously reducing concentrations of sTNF protein by ~75% to ~500pg/ml (Figure 3B).

Despite this reduction of sTNF protein, WT BMDMs stimulated with either TLR2 or TLR4 agonist retained their improved ability to clear subsequent *S. aureus* infection (Figure 3C). While a ~75% decrease in sTNF was a substantial reduction, 500pg/ml was still representative of a 3-fold increase of sTNF compared to PBS treated BMDMs. Thus, in our second approach we employed a murine biologic, p75TNFR:Fc, which acts as a soluble decoy receptor for sTNF. Similar to TMI-005 treatment, cellular supernatants collected from WT BMDMs treated with increasing concentrations of murine p75TNFR:Fc in the presence of TLR2 agonist showed a dose dependent reduction of sTNF. The optimal dosage of p75TNFR:Fc was determined to be

100μg/ml, which reduced sTNF by >90% from ~2000pg/ml to ~125pg/ml (Figure S8).

Treatment of WT BMDMs with 100μg/ml p75TNFR:Fc for 12 hours concurrent with TLR2 stimulation effectively reduced sTNF protein concentrations to near baseline concentrations, achieving a >90% reduction of sTNF to ~171pg/ml (Figure 3D). Importantly, reduction of sTNF to near baseline levels (prior to bacterial infection) did not abrogate improved clearance of *S. aureus* following TLR2 stimulation (Figure 3E). Cumulatively, our results to this point indicate that the presence of sTNF following either TLR2 or TLR4 stimulation was not required for BMDM priming which led to improved clearance of a subsequent *S. aureus* infection. This suggested that tmTNF signaling in response to TLR2 or TLR4 stimulation may be the critical signal that primed BMDMs.

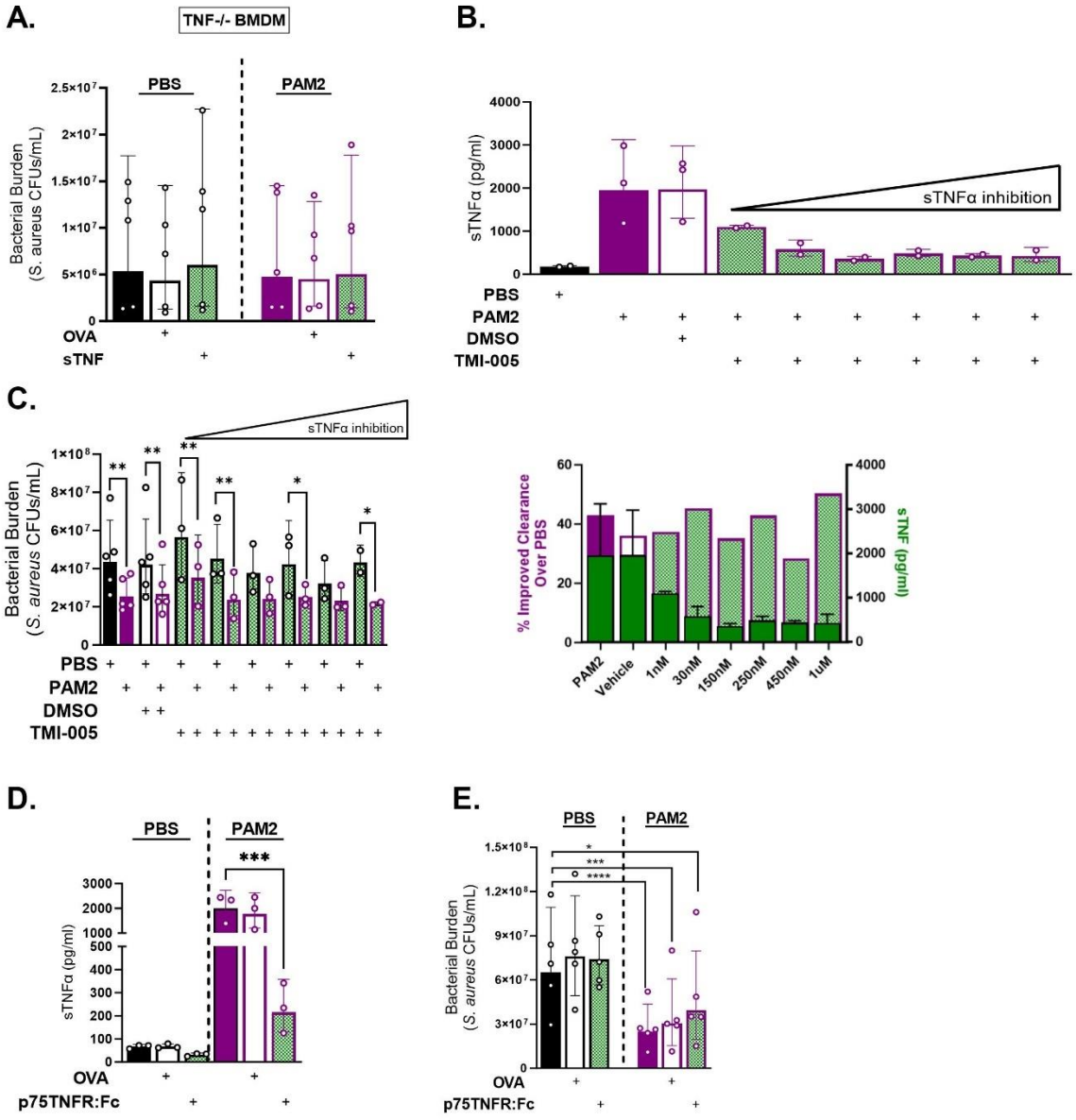


Figure 3. sTNF is not required for priming of BMDMs. (A) Bone marrow cells were extracted from TNF-/- and differentiated for 6 days in L929 conditioned culture media and subsequently seeded into 24-well plates at 1x106 cells/well and treated with either 1μg/ml PAM2 or equal volume of PBS for 12 hours. BMDMs were then treated with either 2000pg/ml murine TNF (sTNF), or ovalbumin for 1 hour before S. aureus infection for 5 hours at MOI 2:1. Data was obtained from BMDMs isolated from 5 mice. BMDMs from each mouse were ran as an independent assay which contained 2 technical replicates. Each data point represents the average of two technical replicates. (B) Soluble TNF protein was quantified from cellular supernatants collected from WT BMDMs treated with either equal volume PBS only, 1μg/ml PAM2 only, DMSO vehicle control, or PAM2 plus increasing concentrations of TMI-005 (green bars in order from left to right; 1nM, 30nM, 150nM, 250nM, 450nM, or 1μM). Results were obtained from

two independent experiments (for various TMI-005 concentrations) and from three independent experiments for PAM2 and PAM2+DMSO control; all conditions in each independent experiment had three technical replicates. (C) WT BMDMs were pre-treated for 1hr with TMI-005 at increasing concentrations and then subsequently treated with either 1μg/ml PAM2 or equal volume PBS with or without increasing concentrations of TMI-005 (concentrations in order from left to right; 1nM, 30nM, 150nM, 250nM, 450nM, or 1μM) for 12 hours followed by S. aureus infection for 5 hours (MOI 2:1). Bacterial burdens (CFUs) were determined after overnight incubation on TSA plates. Results were obtained from five independent experiments and pooled. Two experiments included only lower concentrations of TMI-005 (1nM-150nM). One experiment included only TMI-005 concentrations 250nM and 450nM, one experiment included concentrations 250nM, 450nM, 1μM, one experiment included 1nM-1μM. PBS, PBS+Vehicle control, PAM2, and PAM2+Vehicle control were included in all five experiments and each experiment had two technical replicates. Statistical analysis was performed with two-way ANOVA *P≤0.0204, **P≤0.0074 (left panel). Right panel: Overlay of bacterial clearance data (Fig. 3C-left panel) with sTNF protein quantification (Fig. 3B). Bacterial clearance assay displayed as percent improved S. aureus clearance over PBS and corresponding sTNF concentrations for each treatment group are overlayed on top of bacterial clearance data. (D) Soluble TNF protein quantified from WT BMDM cellular supernatants treated with either 1μg/ml PAM2 or equal volume PBS, 1μg/ml PAM2 or equal volume PBS in the presence of 100μg/ml protein control (ovalbumin), or 1μg/ml PAM2 or equal volume PBS in the presence of 100μg/ml p75TNFR:Fc. Results were obtained from three independent experiments which had 3 technical replicates each. Statistcal analysis was performed with ordinary one-way ANOVA ***P≤0.0005. (E) WT BMDMs treated with either 1μg/ml PAM2 or equal volume PBS with or without 100μg/ml p75TNFR:Fc or OVA protein control for 12 hours followed by S. aureus infection for 5 hours (MOI 2:1). Bacterial burdens (CFUs) were determined after overnight incubation on TSA plates. Results were obtained from 5 independent experiments. Each experiment included 2 technical replicates and each data point represents the average of 2 technical replicates. Statistical analysis was performed with two-way ANOVA *P≤0.0165, ***P≤0.0005, ***P≤0.0001.

TNF Signaling Through TNFR2 Contributes to Improved Clearance of a Subsequent *S. aureus* Infection by TLR2-Primed BMDMs

Despite TNF expression being necessary for TLR-induced BMDM priming, sTNF protein was neither sufficient nor required for improved *S. aureus* clearance by primed BMDMs. Thus, we next sought to examine the contribution of tmTNF to TLR2-induced priming of BMDMs. Interestingly, regardless of the mode for sTNF inhibition, both TMI-005 and

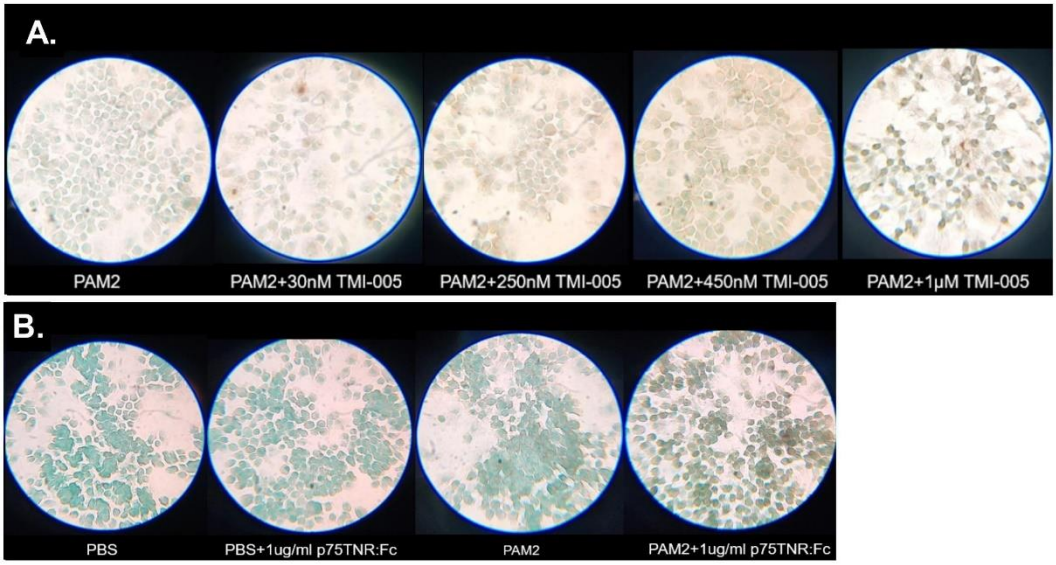
p75TNFR:Fc reduced sTNF while enriching tmTNF protein on WT BMDMs in a dose dependent manner (Figures 4A, 4B).

To investigate whether tmTNF contributed to BMDM priming following TLR stimulation, we leveraged the physical proximity constraint inherent to tmTNF signaling. Because tmTNF is anchored to the cell membrane (Alexopoulou et al., 2006; Deora et al., 2017), it requires cell-to-cell contact for ligand/receptor binding and signaling to occur. We capitalized on this cell-to-cell contact requirement to assess tmTNF involvement in BMDM priming prior to infection with *S. aureus.* Specifically, we performed bacterial clearance assays in which WT BMDMs were plated at various cell densities prior to TLR2 stimulation. The MOI was scaled for each cell density group, such that MOI was 2:1 for all conditions. At low cell density with limited cell-to-cell contact, bacterial burden remained the same across treatment groups, indicating that BMDMs were deficient in clearing *S. aureus* (Figure 4C). As cell density and cell-to-cell contact increased, bacterial burden decreased in TLR2 stimulated WT BMDMs. At our final concentration of 2 million cells/well ($4x10^6$ cells/ml) no TLR2-induced priming advantage in bacterial clearance was observed, which correlated with an overabundance of cells (Figure 4C). This suggested that cell-to-cell contact may be important for TLR-induced BMDM priming that leads to improved *S. aureus* clearance.

To more directly address a possible role of tmTNF in BMDM priming following TLR stimulation, we targeted TNFR2 by antibody-based blocking. TNFR2 is the primary receptor for tmTNF, whereas sTNF cannot signal through TNFR2 (Grell et al., 1995, 1998). WT BMDMs stimulated with TLR2 agonist in the presence of TNFR2 blocking antibody prior to infection lost their ability to efficiently clear subsequent *S. aureus* infection. In fact, the bacterial burden of

TLR2 stimulated BMDMs in the presence of TNFR2 blocking antibody resembled that of PBS treated BMDMs (Figure 4D). This suggested that signaling through tmTNF may be important for priming BMDMs following TLR2 stimulation. Interestingly, WT BMDMs stimulated with PBS in the presence of TNFR2 blocking antibody showed a subsequent improved ability for *S. aureus* clearance.

Collectively, our results provide evidence that tmTNF, but not sTNF, induced in response to TLR stimulation contributed to BMDM priming that enabled these cells to improve their ability to clear a subsequent *S. aureus* infection.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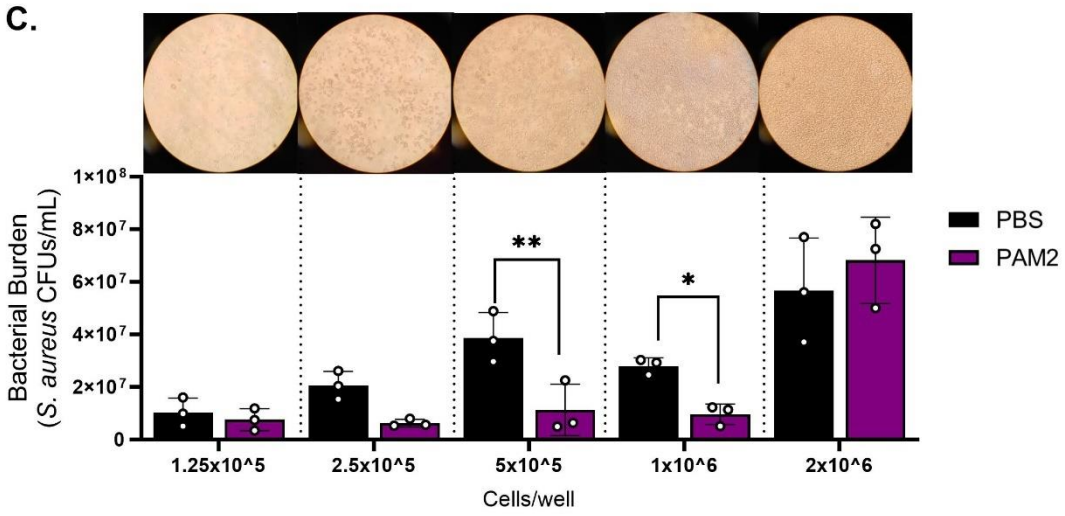
C.
Bacterial Burden
(S. aureus CFUs/mL)
1×10^8
8×10^7
6×10^7
4×10^7
2×10^7
0
**
*
1.25x10^5
2.5x10^5
5x10^5
1x10^6
2x10^6
Cells/well
PBS
PAM2

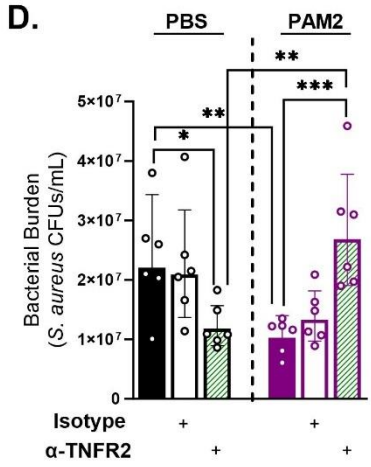
D.
PBS
PAM2
**
***
**
*
Bacterial Burden
(S. aureus CFUs/mL)
5×10^7
4×10^7
3×10^7
2×10^7
1×10^7
0
Isotype
α-TNFR2
+
+
+
+

Figure 4. Addition of exogenous sTNF to TNF-/- BMDMs is not sufficient to prime BMDMs. (A, B) Transmembrane TNF protein visualized by immunocytochemistry (presence of brown indicates tmTNF) in WT BMDMs treated with either (A) TMI-005 (B) or p75TNFR:Fc. (C) Immortalized WT BMDMs seeded into 24-well plates at either (left to right) $1.25x10^5$, $2.5x10^5$, $5x10^5$, $1x10^6$, and $2x10^6$ cells/well and stimulated for 12 hours with either 1μg/ml PAM2 or equal volume PBS followed by infection with S. aureus for 5 hours at MOI 2:1. Microscopy images highlighting cell density were taken after S. aureus infection at 20x. Results were obtained from three independent experiments that had two technical replicates each. The average of the two technical replicates is shown for each experiment. Statistical analysis was performed using 2-way ANOVA * $P<0.0319$, ** $P<0.0027$. (D) WT BMDMs were treated with Fc receptor block (1:6400) prior to 1 hour pre-treatment with IgG isotype control or TNFR2 blocking antibody (1μg/ml). Subsequently, BMDMs were stimulated with PAM2 (1μg/ml) or equal volume PBS in the presence of isotype control or TNFR2 blocking antibody for 12 hours followed by S. aureus infection for 5 hours at MOI 2:1. Results were obtained from 6 independent experiments that contained two technical replicates each. Each data point represents the average of two technical replicates per experiment. Statistcal analysis was performed using 2-way ANOVA *$P\leq0.0305$, **$P\leq0.0060$, ***$P\leq0.0008$.

Discussion

Microbial structures or products are collectively known as pathogen associated molecular patterns (PAMPs) and upon invasion bind to host membrane pattern recognition receptors known as toll-like receptors (TLRs). TLR binding of PAMPs triggers a myriad of intracellular signaling events which orchestrate a complex network of cellular activation (Nie et al., 2018; Peroval et al., 2013). Although TLRs are among one of the most well studied family of pattern recognition receptors (Mokhtari et al., 2021), there is still much of the TLR signaling cascade that needs to be unraveled. A deeper understanding of the TLR signaling cascade will provide insight into the *individual signaling components* that comprise cellular activation post PAMP ligation by TLRs.

This study demonstrated that treatment of macrophages with TLR2 or TLR4 agonists prior to infection improved their capacity to clear *S. aureus* infection due to TNF mediated priming. We found that stimulation of WT BMDMs with TLR2 or TLR4 agonists significantly upregulated both bioactive protein forms of TNF (tmTNF; sTNF). Interestingly, however, we

found that sTNF was not required for BMDM priming following TLR stimulation. Depletion of sTNF in WT BMDMs prior to *S. aureus* infection did not compromise improved bacterial clearance. Supplementation of exogenous sTNF to TNF-/- BMDMs in the presence of TLR stimulation was not sufficient to restore improved clearance capacity. While we did not explicitly determine that tmTNF is required for macrophage priming, we did confirm that both cell-to-cell contact and TNFR2 signaling, which are critical for tmTNF but not sTNF signaling, are required to improve bacterial clearance capacity of primed BMDMs.

TLR2 stimulation in the absence of TNF (TNF-/- BMDMs) was insufficient to establish signaling which resulted in BMDM priming. This was reflected by the loss of improved *S. aureus* clearance by TLR2 stimulated TNF-/- BMDMs. TLR2 stimulation followed by exogenous sTNF addition prior to bacterial infection also was not sufficient to rescue the signaling that occurs in TLR stimulated WT BMDMs prior to *S. aureus* infection. Altogether, these data support our hypothesis that tmTNF signaling resulting from TLR stimulation prior to *S. aureus* infection could prime BMDMs by enhancing their capacity for bacterial clearance.

To assess whether tmTNF was required for BMDM priming, we leveraged the physical constraint inherent to membrane-bound proteins such as tmTNF. These experiments revealed an association between cell-to-cell contact and BMDM *S. aureus* clearance ability. Because MOI was scaled and consistent between treatment groups, the possibility of *S. aureus* overgrowth and overwhelming BMDMs was limited. Additionally, we did not observe any significant cell death between different cell density treatment groups. Cell death was assessed via visual microscopic analysis and defined as cells which lost adherence ability and remained in media as floaters. This

suggested that lack of improved *S. aureus* clearance by TLR2 stimulated BMDMs at lower cell densities could be due to limited cell-to-cell contact, which is required for tmTNF signaling.

To more directly examine tmTNF involvement in BMDM priming, we blocked TNFR2 to prevent signaling following TLR2 stimulation. In TLR2 stimulated BMDMs, blocking tmTNF/TNFR2 signaling led to deficiencies in *S. aureus* clearance compared to TLR2 stimulated BMDMs capable of signaling through TNFR2. This provided evidence that tmTNF signaling following TLR stimulation could be a key signaling event involved in priming BMDMs prior to infection which leads to improved *S. aureus* clearance.

While blocking TNFR2 in TLR2 stimulated BMDMs reduced responsiveness of these cells to a subsequent infection, we also found that blocking TNFR2 in PBS treated BMDMs had an opposite effect. Not much is known about TNFR2 in regards to macrophage priming, nonetheless this result was unexpected. There are several important differences between PBS- and TLR2 stimulated macrophages that could help explain this phenomenon. BMDMs stimulated with PBS produce very little to no TNF, therefore for this treatment condition, TNF was primarily produced in response to *S. aureus* infection. Blocking TNFR2 in PBS stimulated BMDMs likely directed sTNF produced during *S. aureus* infection to bind and signal through TNFR1. Because signaling through TNFR1 is known to initiate and perpetuate inflammatory host responses, it is possible that funneling TNF through TNFR1 *in the presence of an active infection* bolstered macrophage inflammatory responses and aided in the improved clearance of *S. aureus.* On the other hand, TLR2 stimulated BMDMs produced both sTNF and tmTNF prior to infection. Thus, blocking TNFR2 in TLR2 stimulated BMDMs likely directed TNF to bind and signal through TNFR1 prior to *S. aureus* infection. Accordingly, tmTNF was blocked from

signaling through its cognate receptor, TNFR2, which could be important for priming BMDMs to respond to *S. aureus* infection.

Studies highlighting the importance of TLR activation and subsequent sTNF production in host defense against infection have prompted interest in therapeutic manipulation of TLRs or sTNF, especially in the context of antibiotic resistance (Fremond et al., 2005; Fromm et al., 2012; Goulopoulou et al., 2016; Isogawa et al., 2005; W.-J. Lee et al., 2018; Porte et al., 2015). However, immunotherapies which directly target TLRs or sTNF can result in numerous global off-target effects which are disadvantageous for the host. Several studies suggest that prolonged activation of TLRs can lead to prolonged inflammation which can contribute to a hyperinflammatory host environment which damages native host cellular structures and is associated with auto-immune disease (Brentano et al., 2005; Lucas & Maes, 2013; C. G. McCarthy et al., 2014; Stewart et al., 2010; Viglianti et al., 2003). On the other hand, studies suggest that sustained suppression of sTNF signaling is associated with increased host susceptibility to infection (Ali et al., 2013; Crawford & Curtis, 2008; Furst, 2010; W.-J. Lee et al., 2018).

In the present study, we found that a critical signaling event for activation of macrophage microbial defense via either TLR2 or TLR4 ligation may rely on signaling by the protein tmTNF, which is the bioactive precursor form of sTNF. Interestingly, we also found that the mature secreted protein form of TNF, sTNF, was not necessary for this priming event. This finding was unexpected because the majority of the existing literature which characterizes the importance of TNF in resolving infection has historically focused on the role of sTNF, while the role of tmTNF remained poorly defined (Flynn et al., 1995; Giacomini et al., 2001; Van den Bosch et al., 2000).

Nonetheless, more recent reports suggest that tmTNF, independent of sTNF, may be sufficient for protection against mycobacterium and listeria infection (Fremond et al., 2005; Torres et al., 2005). These studies found that the presence of tmTNF alone was sufficient to either partially or fully rescue mice from succumbing to mycobacterial or listeria infection as compared to mice globally deficient in TNF, which exhibited 100% mortality (Fremond et al., 2005; Torres et al., 2005). The findings from these reports attribute the protective function of tmTNF to tmTNF's retained ability to functionally induce signaling (Fremond et al., 2005; Torres et al., 2005). Thus, these studies highlight the overlooked importance and contribution that tmTNF could have on infection resolution. Although the function of tmTNF is beginning to be elucidated, there are still many gaps in our understanding of tmTNF signaling and how it may influence resolution of bacterial infections, specifically in the context of macrophages. While our study did not evaluate whether/how tmTNF is directly involved in resolution of bacterial infection, we provided evidence of tmTNF contribution to macrophage priming. To our knowledge this is the first report of tmTNF conferring an early advantage which improved subsequent *S. aureus* clearance.

In conclusion, we have shown that TLR2 or TLR4 activation of BMDMs results in signaling which led to increased BMDM ability to clear *S. aureus.* We sought to understand what specific signaling events were critical to promoting BMDM priming following early TLR stimulation (PAMP detection) that led to this increased *S. aureus* clearance efficiency. We found that sTNF signaling was not required to prime BMDMs, however, tmTNF signaling contributed to priming of BMDMs, allowing them to efficiently respond to a subsequent *S. aureus* infection. These findings will provide insight into the TLR-based downstream signaling mechanisms which drive host pathogen resolution mechanisms.

Acknowledgements

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Statement of Ethics

This work was approved by Montana State University's Institutional Biosafety Committee (IBC). Protocol number 2022-127-IBC. Animal protocols were reviewed and approved by Montana State University's Institutional Animal Care and Use Committee (IACUC). Protocol number 2022-49-IA.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Data Curation: AML, AAH

Project Administration/Oversight: AML, ARA

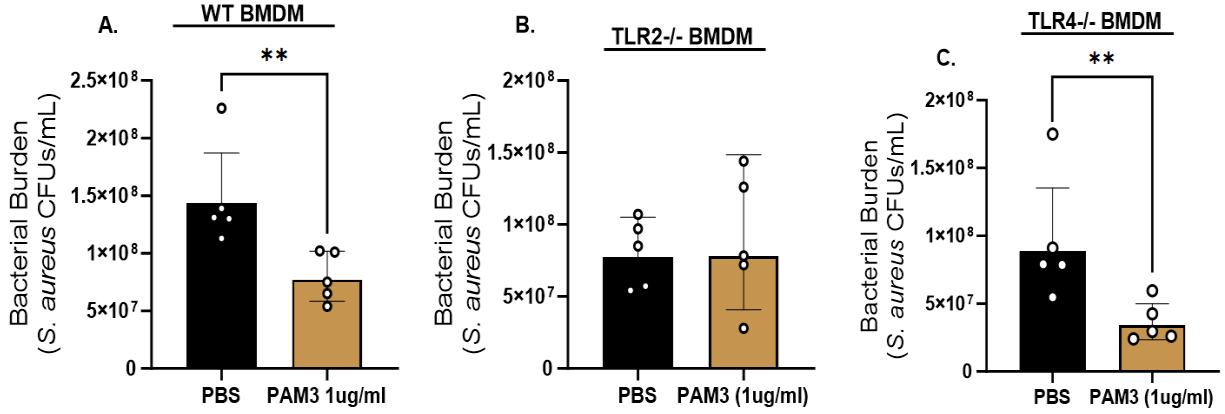
Writing Original Draft: AML, KMS

Writing Revisions and Editing: AML, AAH, MNH, KMS, EB, ARA

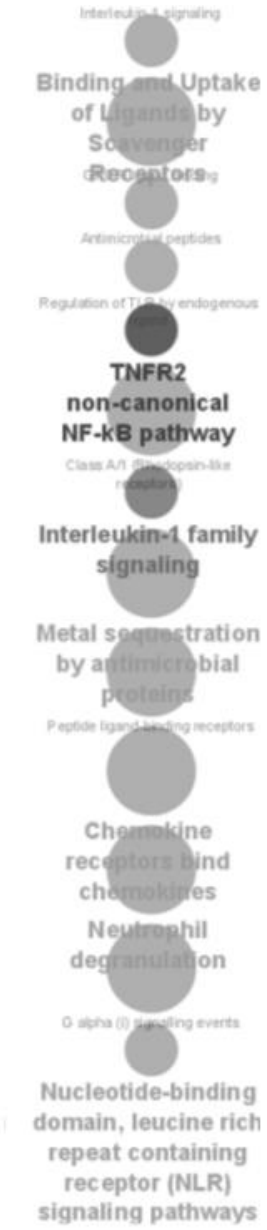
Data Availability Statement

All data generated or analyzed during this study are included in this article and its supplementary material files. Further enquiries can be directed to the corresponding author.

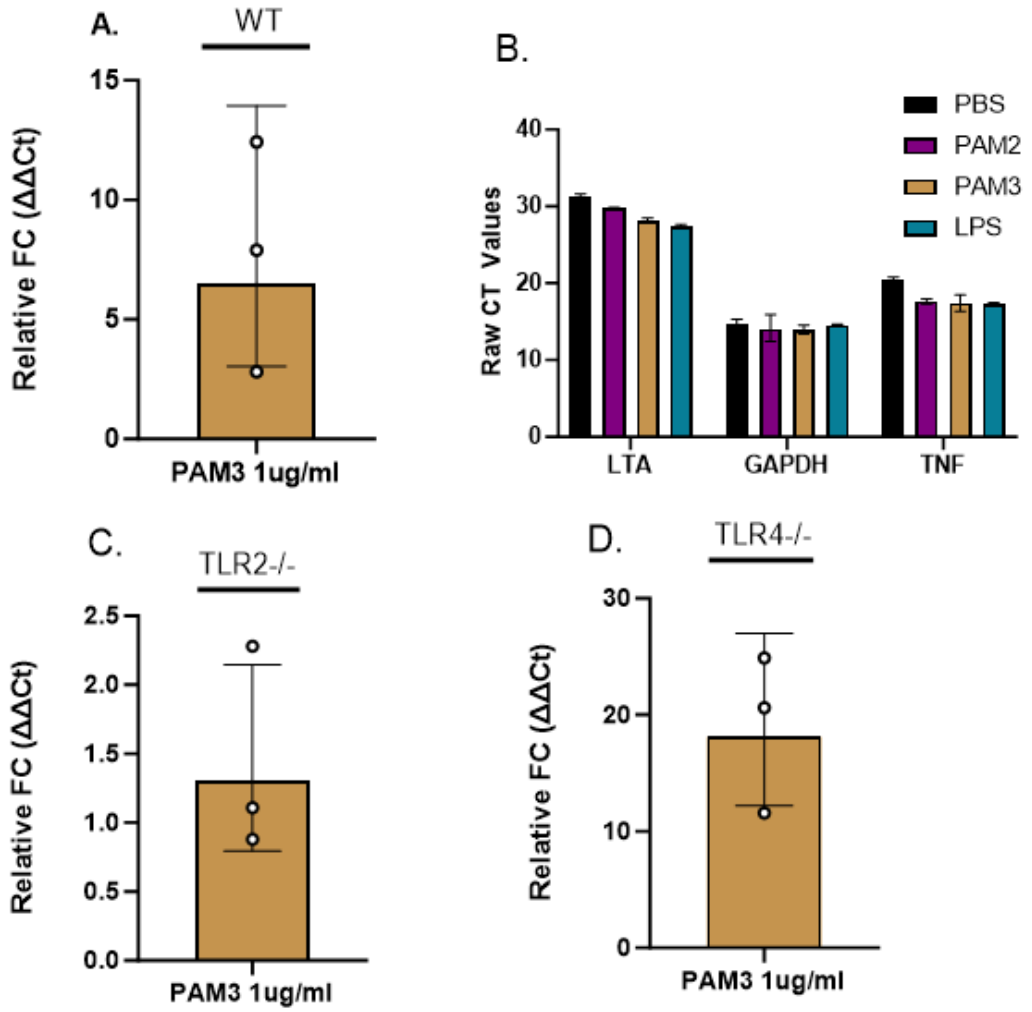
Supplemental Figures



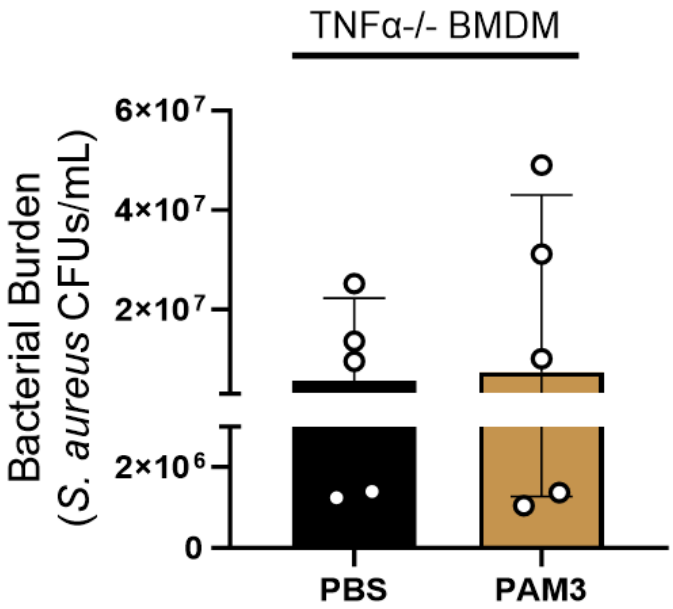
Supplementary Figure 1. *S. aureus* bacterial burden following PAM3 stimulation. (A) WT BMDMs, (B) TLR2-/- BMDMs or (C) TLR4-/- BMDMs were treated with 1µg/ml PAM3 or equal volume PBS for 12 hours followed by *S. aureus* infection for 5 hours (MOI 2:1). Bacterial burdens (CFUs) were determined after overnight incubation on TSA plates. Results shown were obtained from 5 independent experiments per cell line. Each data point represents the average of two technical replicates per experiment. A ROUT outlier test was performed and identified outliers were removed from the datasets prior to statistical analysis. Statistical analysis was performed with a paired one-sided student's t-test **P≤0.0098 (WT BMDMs), **P≤0.0076 (TLR4-/- BMDMs).



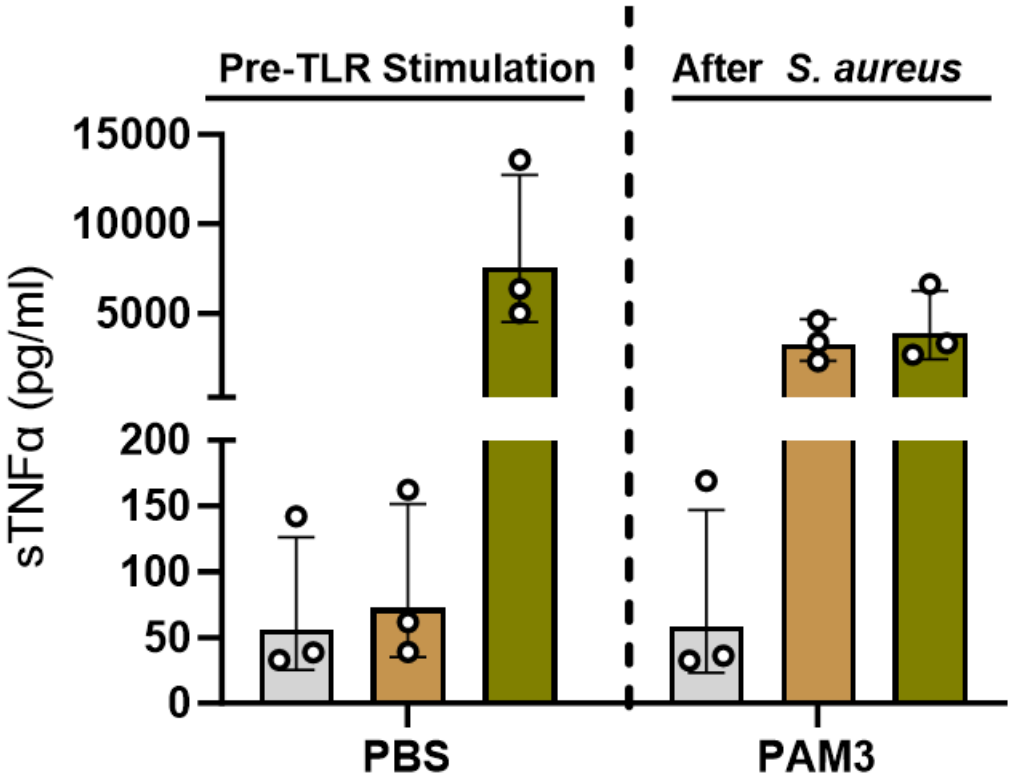
Supplementary Figure 2. Reactome Pathway analysis. Common differentially upregulated genes identified from RNA sequencing analysis between PAM2 and PAM3 inoculated mice were run through the pathway analysis software Cytoscape. Conditions for analysis included use of Reactome pathway database, medium network specificity (levels 3-8), and enrichment with right-sided hypergeometric test.



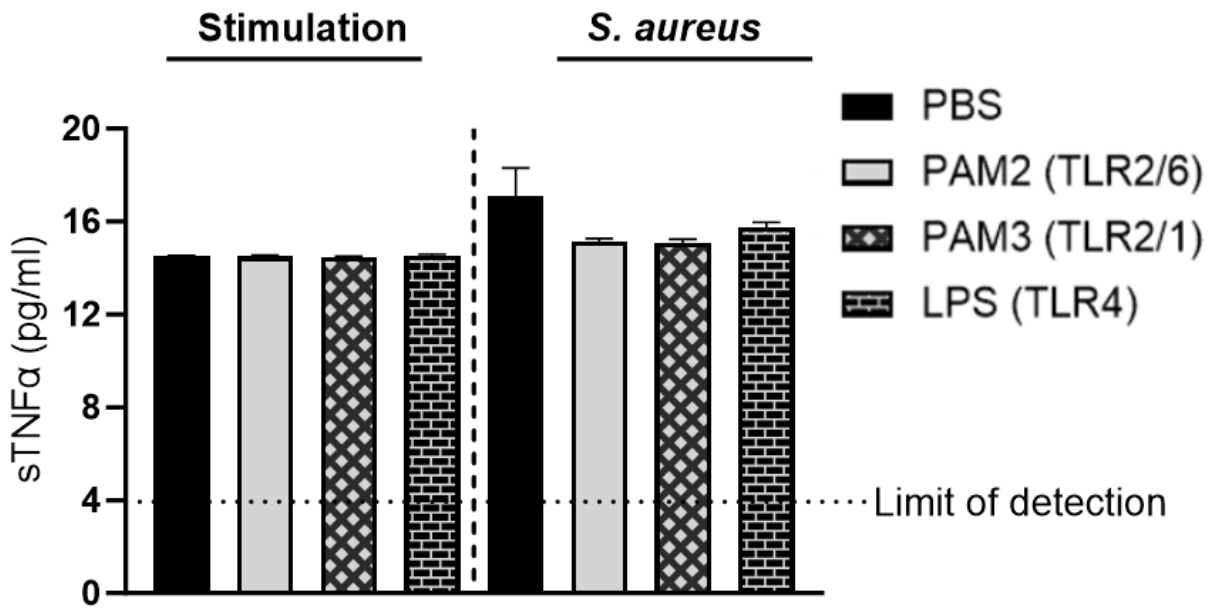
Supplementary Figure 3. TNF transcript induced in response to PAM3 stimulation. TNF transcript 3 hours post TLR stimulation. BMDMs were washed with PBS and lysed with Trizol for RNA extraction. cDNA library was prepared and qRT-PCR was performed with 40 amplification cycles using either β-actin or GAPDH as a housekeeping control. PAM3 was used to stimulated (A) WT BMDMs (C) TLR2-/- BMDMs, and (D) TLR4-/- BMDMs. Data was normalized to reference gene β-actin, and fold change was calculated using ΔΔCT method. Results were obtained from 3 independent experiments that had three technical replicates each. (B) WT BMDMs were stimulated with either 1μg/ml PAM2, PAM3, LPS or equal volume PBS for 3 hours. Raw CT values are shown. Results were obtained from three independent experiments that had three technical replicates for each sample. Data shown are the average of the three technical replicates from each experiment.



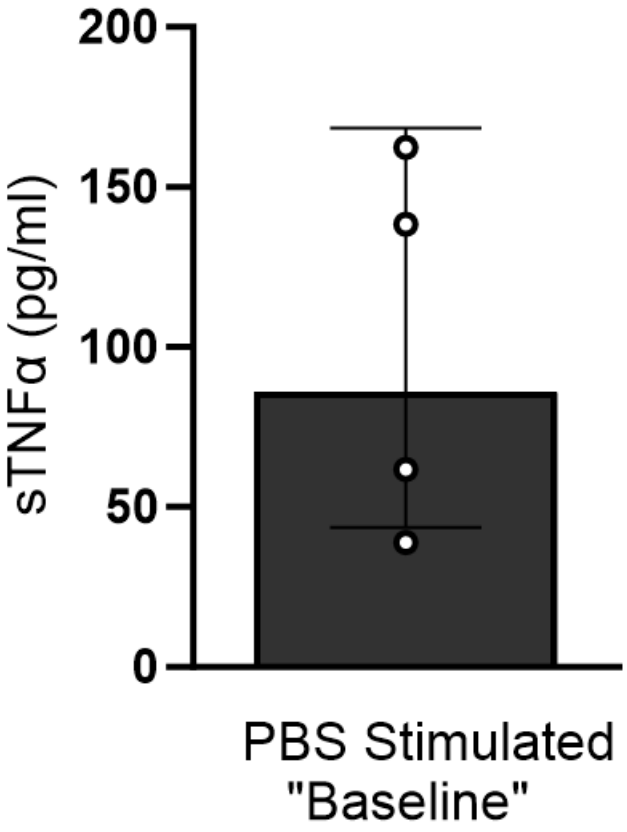
Supplementary Figure 4. PAM3 stimulation in TNF-/- BMDMs does not rescue phenotype. Primary TNF-/- BMDMs were treated with either 1µg/ml PAM3 or equal volume PBS for 12 hours followed by *S. aureus* infection for 5 hours (MOI 2:1). Bacterial burdens (CFUs) were determined after overnight incubation on TSA plates. Data was obtained from BMDMs isolated from 5 mice. BMDMs from each mouse were ran as an independent assay which contained 2 technical replicates. Each data point represents the average of two technical replicates.



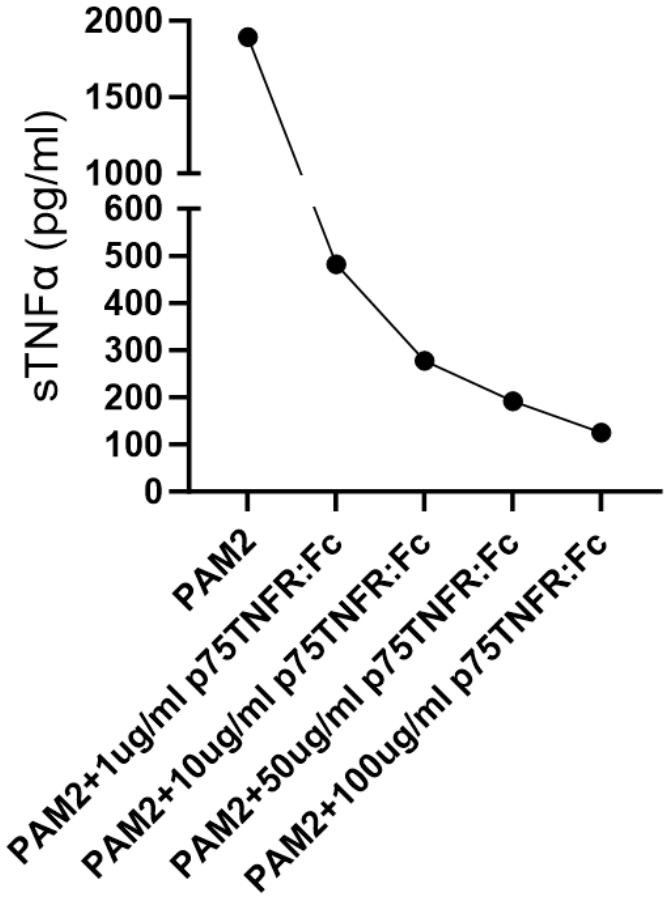
Supplementary Figure 5. The presence of early sTNF following TLR stimulation does not increase sTNF after *S. aureus* infection. Soluble TNF was quantified from immortalized WT BMDMs cellular supernatants before stimulation (pre-TLR Stimulation) and after *S. aureus* infection for 5 hours at MOI 2:1 (After *S. aureus*). Results were obtained from 3 independent experiments which had 3 technical replicates each. Each data point represents the average of 3 technical replicates.



Supplementary Figure 6. Primary TNF-/- BMDMs are deficient in sTNF protein. Cellular supernatants from primary TNF-/- BMDMs were collected at two timepoints. Stimulation samples (left) were collected after BMDM stimulation with either 1µg/ml PAM2, PAM3, LPS, or equal volume PBS for 12 hours. *S. aureus* samples (right) were collected after BMDM stimulation with either 1µg/ml PAM2, PAM3, LPS, or equal volume PBS for 12 hours followed by infection with *S. aureus* for 5 hours (MOI 2:1). Supernatants from both of these timepoints were run on murine TNF ELISA to validate TNF deficiency in these mice. Assay limit of detection is 4pg/ml.



Supplementary Figure 7. Baseline concentration of sTNF in PBS stimulated WT BMDMs. Cellular supernatants from WT BMDMs were collected following 12-hour stimulation with equal volume PBS. Protein concentration of sTNF was measured via ELISA. Results were obtained from 3 independent experiments which had 3 technical replicates each. Each data point represents the average of 3 technical replicates.



Supplementary Figure 8. Murine p75TNFR:Fc reduces sTNF by >90%. WT BMDMs were stimulated with 1μg/ml, 10μg/ml, 50μg/ml, or 100μg/ml p75TNFR:Fc in the presence of 1μg/ml PAM2 for 12 hours. Cellular supernatants were collected for sTNF quantification via ELISA to determine optimal p75TNFR:Fc concentration for sTNF reduction. Treatment of WT BMDMs with 100μg/ml p75TNFR:Fc reduced sTNF protein concentrations to near baseline (PBS-treated) concentrations, reducing sTNF concentrations to ~171pg/ml.

A COMPREHENSIVE PROTOCOL FOR THE COLLECTION, DIFFERENTATION, CRYOPRESERVATION, AND RESUSCITATION OF PRIMARY MURINE BONE MARROW DERIVED MACROPHAGES (BMDMs)

Contribution of Authors and Co-Authors

Manuscript in Chapter 3

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Abstract

The field of immunology has undoubtedly benefited from the *in vitro* use of cell lines for immunological studies; however, due to the "immortal" nature of many cell lines, they are not always the best model. Thus, direct collection and culture of primary cells from model organisms is a solution that many researchers utilize. To the best of our knowledge, there is not a singular protocol which encompasses the entire process of bone marrow cell collection through cryopreservation and resuscitation of cells from a murine model. Herein, we present a comprehensive protocol for the collection, differentiation, cryopreservation, and resuscitation of primary murine bone marrow derived macrophages for use in cell culture.

Background

Macrophages are essential immune cells which have diverse functions. Under normal physiological conditions, macrophages are responsible for maintaining homeostasis via the clearance of cellular debris (Hirayama et al., 2017; Lendeckel et al., 2022). Under pathological conditions, however, macrophages become effector cells that are critical in the early defense against invading pathogens. During infection, macrophages play an important role in the secretion of pro-inflammatory cytokines as well as phagocytosis and clearance of both pathogens and infected/dead cells (Hirayama et al., 2017; Lendeckel et al., 2022). As a result, the *in vitro* study of macrophages using immortalized cell lines has played a pivotal role in aiding and furthering our understanding of how macrophages function (Cutolo et al., 2005; Khadaroo et al., 2003; Pearlman et al., 1988).

Although studies utilizing immortalized cell lines have been invaluable in helping to elucidate cellular functions, cell lines have limitations and, thus, are not always the best model. Because cell lines are transformed, their native responses and functions have strong potential to be altered (Castell et al., 2006; Parson et al., 2005). Additionally, extended culture and multiple passages of cell lines can result in genotypic and phenotypic drift (Castell et al., 2006; Parson et al., 2005). To omit these limitations, many researchers may instead choose to utilize primary cells for culture. Primary cells are extracted directly from tissues or organs of interest for *in vitro* culture (Philippeos et al., 2012). Herein, we present a comprehensive protocol for the collection/isolation, differentiation, cryopreservation, and resuscitation of primary murine bone marrow derived macrophages (BMDMs). Importantly, we found that primary BMDMs were both viable and functional following cryopreservation and resuscitation protocol. However, we observed that cryopreserved/resuscitated primary BMDMs exhibited a reduced ability to clear a subsequent *S. aureus* infection compared to "freshly" isolated/differentiated primary BMDMs which did not undergo cryopreservation. However, despite this reduction in bacterial clearance, cryopreserved primary BMDMs retained an overall ability to clear *S. aureus*.

Methods

Materials

-Sterile surgical tools (tweezers, scissors)

-70% ethanol

-1X sterile PBS

-Dimethyl sulfoxide (DMSO)

-Fetal Bovine Serum (FBS)

-1X Roswell Park Memorial Institute 1640 (RPMI)

-ACK lysis buffer (150mM NH_4Cl, 10mM $KHCO_3$, .01mM EDTA dissolved in deionized water and pH=7.3, sterile filtered)

-Complete media (1X RPMI, 20% FBS, 30% L929 supernatant, 0.1M Hepes, 10000U/ml penicillin-streptomycin, $1.792x10^{-3}$ mM 2-Mercaptoethanol, and 4mg/ml glutamine)

Mice

6–8-week-old female TNF-/- mice were purchased from Jackson Laboratory. 8–9-week-old female Elp+/LoxP and Wild Type (WT) C57BL6 mice were bred and housed at Montana State University's Animal Resource Center. All mice were bred on a C57BL6 genetic background. Mice were maintained at the Montana State University (MSU) Animal Resource Center. All care and procedures were in accordance with the recommendations of the National Institutes of Health, the U.S. Department of Agriculture, and the Guide for the Care and Use of Laboratory Animals (8^{th} ed) (*Guide for the Care and Use of Laboratory Animals*, 2011). Animal protocols were reviewed and approved by the MSU Institutional Animal Care and Use Committee (IACUC).

Isolation of Murine Bone Marrow Cells

NOTE: For anatomical pictures of murine bones described in this protocol, please reference Supplementary Figures.

1. Mice were euthanized according to IACUC protocol. After euthanasia, mice were sprayed down with 70% ethanol, and a small superficial incision was made midline at the abdomen, taking care to remove only the fur and fascia.

2. Taking hold of the fur to the top and bottom of the incision, the incision was widened and made to extend around the entire abdominal circumference of the mouse by pulling the fur-we refer to this as “depants-ing” because the separation of the skin/fur gives the appearance of pants.

3. From the midline, or “the top of the pants,” the fur was pulled downwards towards the tail. Scissors may be needed to make small incisions of the fur at the tail and feet in order to fully remove “pants.”

4. Once “pants” have been removed, scissors were used to cut away the muscle near the articulation point between the head of the femur and the pelvis. The articulation point can be felt/estimated by manipulating the hind leg while palpitating the pelvis area. It will feel like a bony protrusion that moves as the hind leg is manipulated. Take care not to accidently cut into the bone during this step.

5. Once the muscle surrounding this articulation point has been removed, the head of the femur articulating in the pelvic socket (acetabulum) should become visible and will appear as a small white ball.

6. There are three ligaments that attach the head of the femur to the acetabulum. All three ligaments need to be cut to detach the femur from the mouse. The ligaments will appear as small, taught white strands that go from the head of the femur into the acetabulum.

7. Repeat steps 5-6 for the other hind leg.

8. Remove foot by bending/breaking it in the opposite direction at the talus/calcaneus until it becomes loose.

9. Place kneecap on your thumb while gently grasping the femur between your index finger and thumb. With your other hand, pull broken foot downwards, in the direction that it was broken,

while pushing up on the kneecap. Some ligaments may need to be cut if there is a lot of resistance (these will appear as white strands). This step will remove the foot and make proceeding muscle removal easier.

10. Gently grasp the femur between your thumb and index finger. With your other hand, break the kneecap by bending it in the opposite direction. Place your thumb at the distal end of the fibula/tibia, where the foot was broken, and push the fibula/tibia out towards the direction of the broken kneecap. This step should cleanly free the fibula/tibia and remove the majority of the muscle surrounding these bones. The fibula is a very thin bone, it is likely that it will break during this step if it has not already. This is okay. Breakage of the fibula will not contaminate/affect collection of bone marrow from the tibia.

11. Using sterile scissors, carefully remove the muscle surrounding the femur.

12. Any remaining muscle can be removed using a paper towel that has been soaked in 70% ethanol and air dried prior to use.

13. Repeat steps 8-12 for the other hind leg.

14. Pour 70% ethanol into a petri dish (enough to submerge bones) and soak collected bones for 5 minutes. This step is critical for sterilization of the bones.

15. All proceeding steps following 70% ethanol soak of bones MUST be performed with new sterile tools that have also been UV-sterilized to mitigate the risk of contamination.

16. Pour ~30ml of 1X Roswell Park Memorial Institute 1640 (RPMI) into a sterile petri dish.

17. Aspirate 10ml of RPMI into a 10ml syringe, attach a 26.5-gauge needle. Using a NEW pair of sterile scissors and tweezers to manipulate the bones, rinse the bones off with 1X RPMI.

18. Cut small portions of both ends of the bones off. Insert needle attached to syringe and flush out bone marrow into a sterile 50ml conical. Repeat as needed until all bone marrow has been removed and the bones take on a translucent appearance. The needle may not go into the bone easily during the first insertion. Gently rotating/small circular motions can aid needle insertion into the bone.

19. Repeat this process for the remaining bones.

20. Place a sterile 100µm filter strainer into a new sterile 50ml conical tube.

21. After all of the bone marrow has been collected into a 50ml conical, use a sterile 10ml serological to pipette up and down and break up any clumps. Pipette bone marrow solution onto 100µm filter strainer.

22. Discard strainer, replace cap onto 50ml conical containing bone marrow cells and centrifuge at 408 *x g* for 5 minutes at 4˚C.

23. In a tissue culture cabinet, remove supernatant using a sterile 10ml serological. Resuspend cells in 1ml ACK lysis buffer for 5 minutes at room temperature.

24. Add 10ml 1X RPMI and centrifuge cells at 408 *x g* for 5 minutes at 4˚C.

25. In a tissue culture cabinet, remove supernatant and resuspend cells in 12ml pre-warmed (37˚C) complete media.

26. Add 17ml complete media to 6 sterile petri dishes (100mmx15mm) and add 2ml of re-suspended cells to each dish (4 bones=6petri dishes with 2ml of cell suspension each). If counting the cells, add ~5 to 8million cells per petri dish.

27. For differentiation to macrophages, culture cells at 37˚C with 5% CO_2 for 6 days in complete media containing M-CSF. Change media on day 3. The source of M-CSF can be sourced from L929 cell supernatant (Boltz-Nitulescu et al., 1987a).

28. On day 6, cells have completed differentiation and are now ready for use in assays. To lift cells for assay plating, wash cells with 5ml ice-cold sterile 1X PBS two times and incubate on ice for 5minutes with 5ml sterile 1X PBS.

29. Using a pipette, gently wash cells off petri dish and collect in a 50ml conical.

30. Centrifuge cells at 408 *x g* for 5 minutes at 4˚C.

31. Count cells and re-suspend to desired cell density. We have found that a typical 6-9-week-old female mouse bred on a C57BL6 genetic background yields a total of ~30 million cells following 6-day differentiation period (assuming 2 femurs and 2 tibias were collected)

32. Once seeded into culture plates, cells will adhere within a few hours.

Cryopreservation of Murine BMDMs

1. To cryopreserve extra cells, label sterile cryopreservation vials and keep on ice. DMSO reactions with water are *extremely exothermic*, so it is critical to pre-chill tubes and ensure that all reagents are ice cold.

2. Re-suspend cells in 90% FBS and 10% DMSO at $6x10^6$ cells/ml.

3. Aliquot 1ml cell suspension into each cryopreservation vial and place vials either in a Mr. Frosty (ThermoFisher) or sandwiched between Styrofoam and placed in -80˚C for 24 hours.

4. Transfer vials to liquid nitrogen for long term storage.

Resuscitation of Murine BMDMs

1. Using a water bath set to 37˚C, warm up complete media.

2. Place cryopreservation vial in water bath until suspension has completely thawed.

3. Transfer 1ml of thawed cell suspension into 15ml conical tube and add 7ml pre-warmed (37˚C) complete media to conical tube containing cell suspension.

4. Centrifuge at room temperature for 7 minutes at 408 *x g.*

5. Pipette 17ml of pre-warmed complete media into two petri dishes (100mmx15mm).

6. Re-suspend in 1ml pre-warmed complete media and add 500µl of cell suspension to each dish.

7. Culture at 37˚C with 5% CO_2 for 6 days in complete media containing M-CSF. Change media on day 3. The source of M-CSF can be sourced from L929 cell supernatant (Boltz-Nitulescu et al., 1987a).

8. To lift cells for assay plating, wash cells with 5ml ice-cold sterile 1X PBS two times and incubate on ice for 5minutes with 5ml sterile 1X PBS.

9. Using a pipette, gently wash cells off petri dish and collect in a 50ml conical.

10. Centrifuge cells at 408 *x g* for 5 minutes at 4˚C.

11. Count cells and re-suspend to desired cell density. We have found that a typical yield for cryopreserved/resuscitated primary murine BMDMs (frozen back at ~6million cells) yields a total of ~18 million cells following 6 day in resuscitation/culture.

Results

As described in the above protocol, primary bone marrow cells were cultured in differentiating media for 6 days and either used immediately (“fresh”) or cryopreserved. Cryopreserved bone marrow derived macrophages (BMDMs) were resuscitated and cultured in differentiating media again for 6 days prior to use (“frozen”). The cryopreservation recovery rate

of “frozen” BMDMs on day 6 of culture was ~30%. This was determined by dividing the number of live cells ($1.35x10^6$cells/ml) by the total number of cells frozen back ($5x10^6$cells/ml).

Undifferentiated bone marrow cells (Day 0), Day 6 “fresh”, or Day 6 “frozen” BMDMs were placed into a funnel and centrifuged onto microscope slides (Cytospin) prior to DiffQuick staining. Figure 1 demonstrates the diverse cellularity of bone marrow cells immediately following isolation (Day 0) and the similarly uniform macrophage phenotype of Day 6 “fresh”, and Day 6 “frozen” BMDMs. Figure 2 demonstrates the cellular morphology of “fresh” and “frozen” BMDMs on days 0, 3, and 6 in culture.

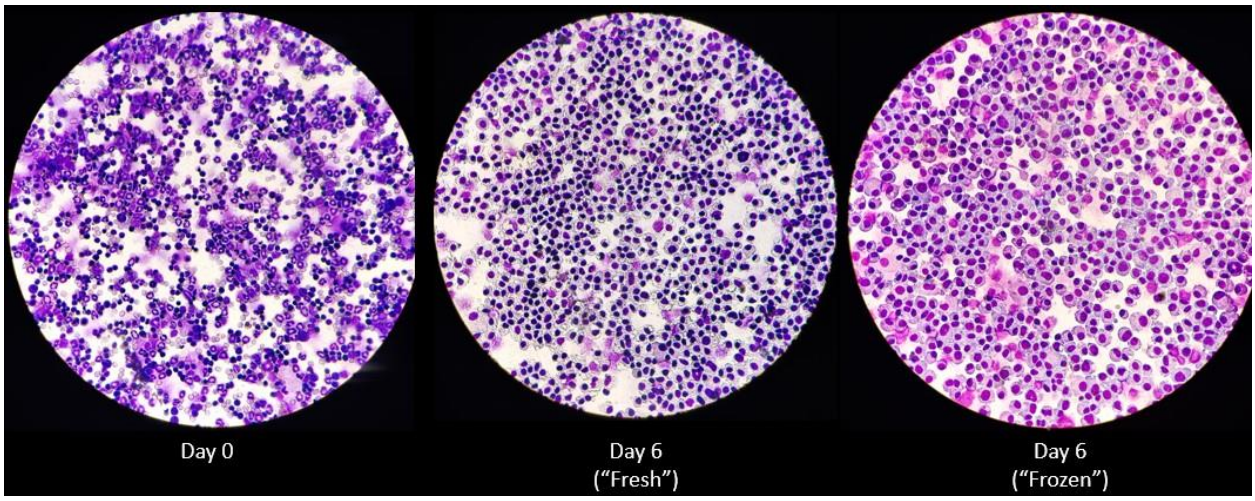

Figure 1. Differential staining of primary cells obtained from bone marrow. Primary cells collected from bone marrow and subjected to centrifugation using cytospin and stained with Diff Quik immediately (Day 0), after 6 days in macrophage differentiating media containing L929 (Day 6 “Fresh”), or following cryopreservation in liquid nitrogen, resuscitation, and culture for 6 days in macrophage differentiating media (Day 6 “Frozen”). Differential staining was performed on BMDMs isolated from three individual mice. Images at 40X magnification depict representative staining from one mouse.

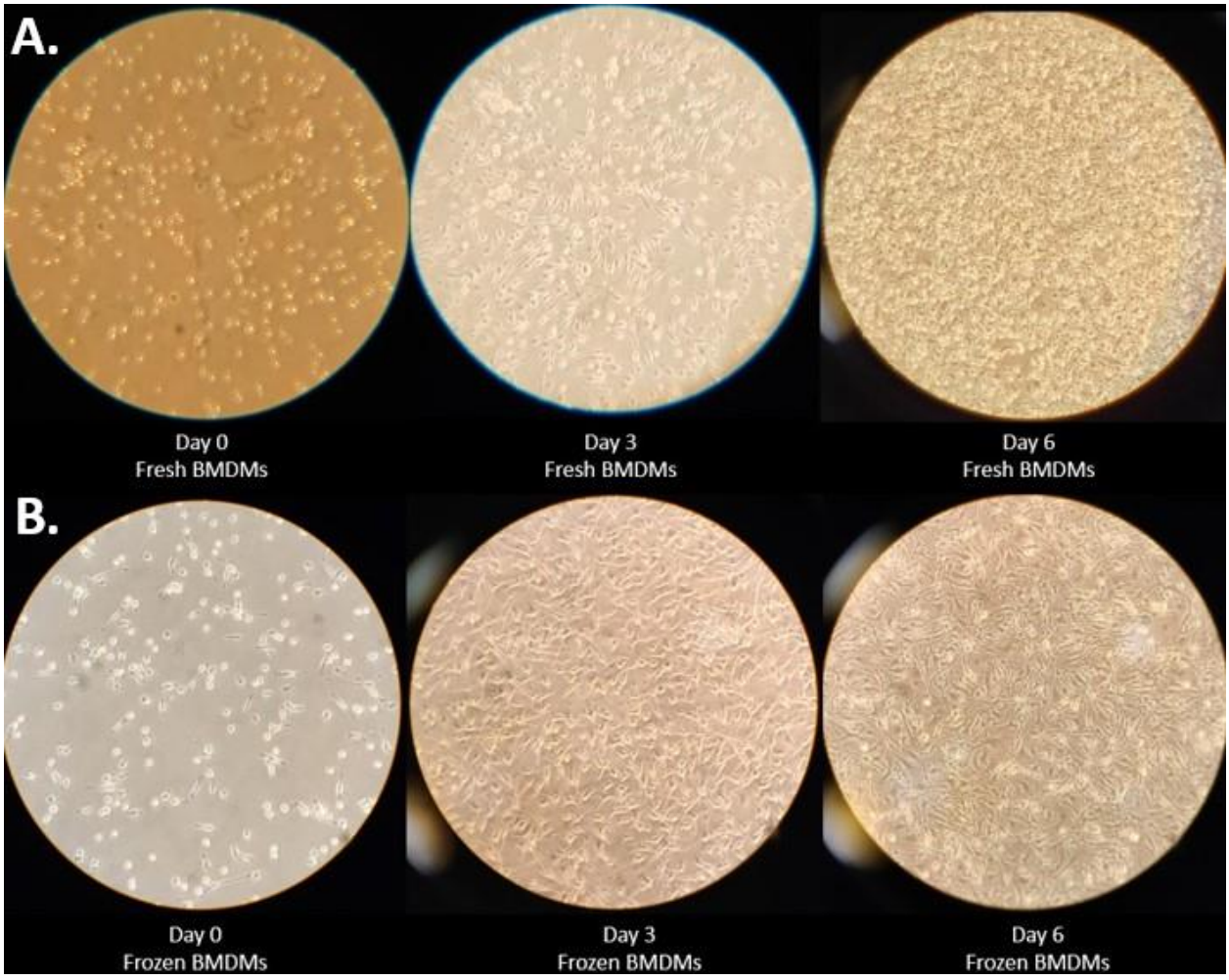

Figure 2. Similar morphology of "fresh" and "frozen" BMDMs. Freshly isolated bone marrow cells **(A)** or resuscitated cryopreserved BMDMs **(B)** were differentiated in media containing L929 for a total of 6 days. Microscopy images were taken at 40x magnification on day 0 (left), day 3 (middle), and day 6 (right) during culture. Images of BMDMs were taken from three individual mice. Data in this figure are representative of cells from one mouse.

In line with the existing body of literature which describes macrophage differentiation from bone marrow cells, we used M-CSF sourced from L929 supernatant in our protocol (Boltz-Nitulescu et al., 1987b; Haag & Murthy, 2021; Mendoza et al., 2022; Trouplin et al., 2013; Weischenfeldt & Porse, 2008). Following Day 6 of culture in L929 differentiating media, both "fresh" and "frozen" primary BMDMs exhibited macrophage-like morphology (Figure 1). However, to more directly characterize bone marrow cell differentiation to macrophages, we

performed immunocytochemical staining of BMDMs for expression of classical macrophage differentiation markers CD11b and CD206 (Bisgaard et al., 2016; Zajd et al., 2020; X. Zhang et al., 2008a). Being a classical T lymphocyte differentiation marker, CD4 is not expressed by murine macrophages and thus, served as a negative control (Moore et al., 1992). Figure 3 demonstrates BMDM positive staining (brown) for expression of the classical macrophage differentiation markers CD11b and CD206 and lack of staining for CD4.

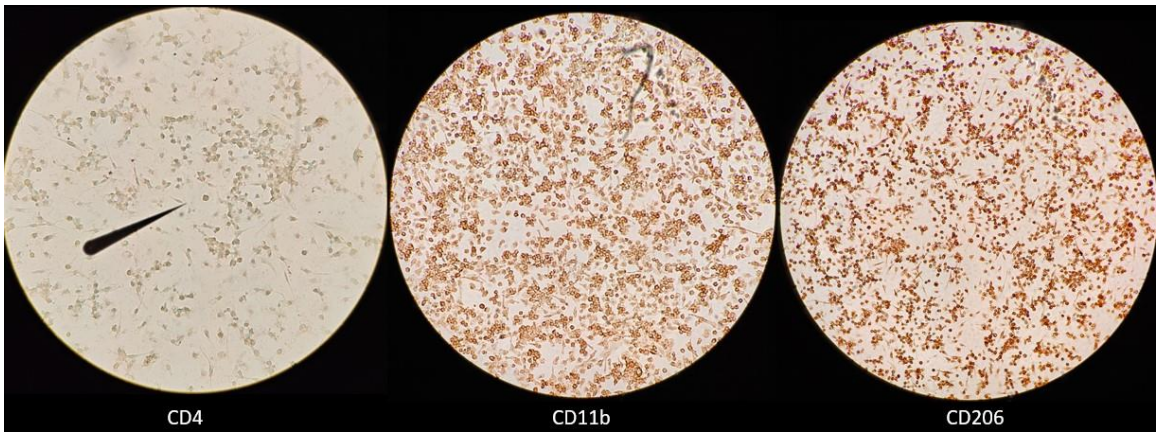

Figure 3. BMDMs express CD11b and CD206. Day 6 "Frozen" BMDMs were resuscitated and cultured in L929 containing media for 6 days. On day 6, BMDMs were lifted and seeded into 24-well plates containing collagen covers and subsequently incubated with biotinylated rat anti-mouse monoclonal IgG2 antibodies raised against CD4 (left; clone GK1.5), CD11b (middle; clone M1/70), or CD206 (right; clone MR5D3). All BMDMs received the same secondary avidin-HRP antibody and 3,3-diaminobenzidine chromogen. The presence of brown staining indicates expression of cell surface marker. Visualization was performed using standard inverted compound light microscope. Images depict staining from one representative experiment; however, two independent experiments were performed.

Figure 4 demonstrates that the viability of "frozen" and "fresh" BMDMs was comparable on day 6 of culture. In fact, the ratio of live/dead for "frozen" cells (6.4) was improved compared to the ratio of live/dead for "fresh" cells (2.2). Day 6 of culture for "fresh" BMDMs represents the completed differentiation period when BMDMs can be either cryopreserved or used in subsequent assays. As described in the above protocol, "fresh" and "frozen" BMDMs were lifted

from the plate using ice cold PBS, centrifuged, and re-suspended in media. Cell density and viability was determined after trypan blue staining.

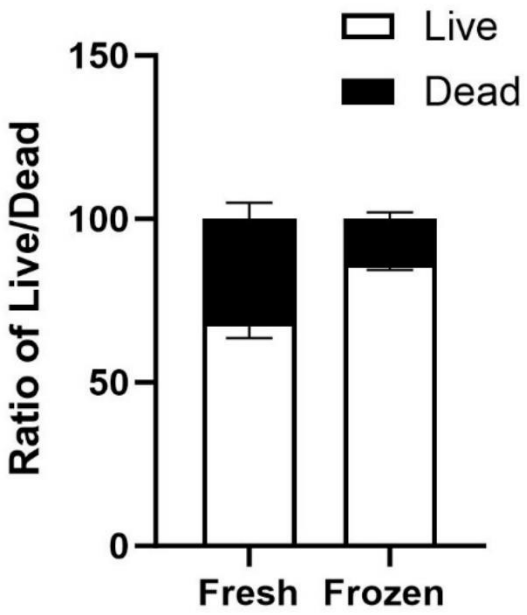

Figure 4. Similar viability of "fresh" and "frozen" BMDMs. Trypan blue staining assessing the viability of "fresh" BMDMs on day 6 of culture in L929 containing media (left) compared to viability of "frozen" BMDMs following resuscitation for 6 days in L929 containing media (right). Results show mean and SD of two independent experiments. Each experiment contained two technical replicates.

Having determined that primary BMDMs that had undergone cryopreservation followed by resuscitation retain a high degree of viability, we next sought to determine the functionality of "frozen" BMDMs. We compared the bacterial clearance ability of "fresh" BMDMs and "frozen" BMDMs. Figure 4 demonstrates that "frozen" BMDMs had a reduced ability to clear *S. aureus* infection compared to "fresh" BMDMs. For "fresh" BMDMs, treatments that contained only *S. aureus* (no BMDMs), had a median bacterial burden (CFUs/ml) that was approximately 52.90 times greater than the median bacterial burden for treatments that contained "fresh" BMDMs in

addition to *S. aureus*. For "frozen" BMDMs, treatments that contained only *S. aureus* (no BMDMs), had a median bacterial burden that was approximately 7.19 times greater than the median bacterial burden for treatments that contained "frozen" BMDMs in addition to *S. aureus*. These data demonstrated that in comparison to "fresh" BMDMs, "frozen" BMDMs display a significant decrease in *S. aureus* clearance efficiency. However, as evidenced by the decrease of *S. aureus* bacterial burden in treatments containing frozen/resuscitated BMDMs compared to *S. aureus* conditions alone, our results demonstrated that "frozen" BMDMs retained an overall ability for *S. aureus* bacterial clearance, albeit to a limited capacity.

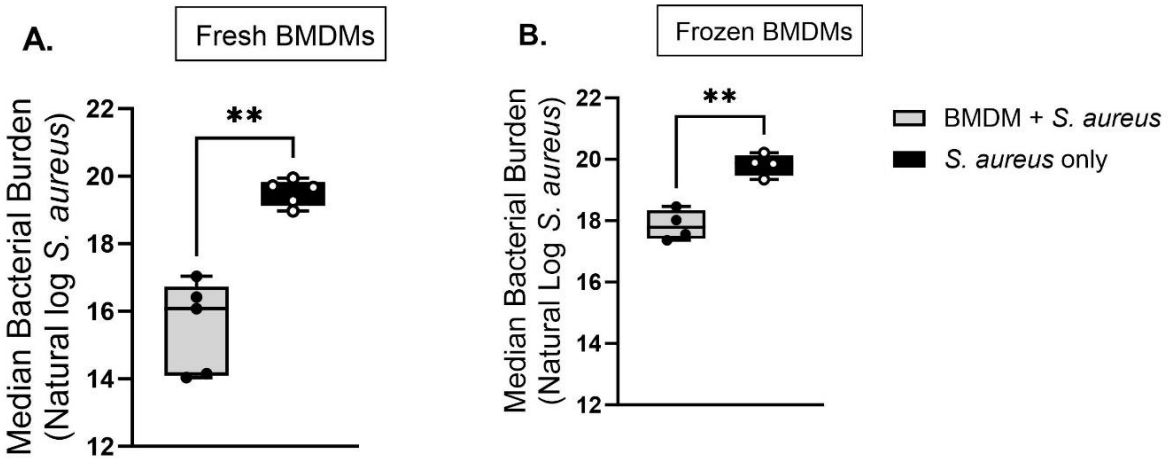

Figure 5. Cryopreservation reduces *S. aureus* clearance capacity of primary BMDMs. (A) After 6 days in macrophage differentiating media, primary TNF-/- cells were treated with saline and infected with *S. aureus* for 5 hours at MOI 2:1. "Fresh" BMDM CFU data shown here were used in another manuscript (Luu *et al,* submitted). Shown is data from five independent experiments that contained two technical replicates each. (B) Primary TNF-/- BMDMs were cryopreserved in liquid nitrogen, thawed, and resuscitated in macrophage differentiating media for an additional 6 days. Cells were treated for either 12hrs or 1hr with WT BMDMs fixed in 1% PFA before infection with *S. aureus* for 5 hours at MOI 2:1. Data was obtained from four independent experiments that contained two technical replicates each. (A, B) The average of two technical replicates per experiment is shown. Grey bars represent *S. aureus* bacterial burden (with BMDMs) compared to black bars which represent *S. aureus* uninhibited growth (without BMDMs) during a 5-hour period. Statistical analysis was performed using a one-sided paired *t*-test *p*-value=0.0014 (A) *p*-value=0.0061 (B) on log transformed data.

Conclusions

In vitro cell culture is a vital facet of immunological research, and the use of immortalized cell lines in research dates back as early as the 1900s (Mirabelli et al., 2019). Although there are many benefits to the use of cell lines in research (cost, cultivability, and published body of literature) limitations of cell lines include altered phenotype with increasing passage, differential native responses and functions, and genotypic drift (Castell et al., 2006; Parson et al., 2005). As a result, many researchers began to also utilize primary cells in their research. Here, we described an easy to follow and comprehensive protocol for the collection, isolation, cryopreservation, and resuscitation of primary murine bone marrow derived macrophages (BMDMs). We showed that the cryopreservation of these BMDMs did not result in marked alterations to their cellularity or morphology compared to "fresh" BMDMs. Additionally, cryopreservation of primary BMDMs did not impact their viability following resuscitation and culture protocol. We did find, however, that resuscitated "frozen" BMDMs exhibited reduced capacity for clearance of *S. aureus* compared to "fresh" BMDMs. DMSO in cryopreservation media is known to affect the epigenetic landscape of mammalian cells (Verheijen et al., 2019; Wei et al., 2020). Additionally, residual DMSO in "frozen" BMDMs could have provided a survival advantage to *S. aureus*. Supporting this, one study showed that DMSO inhibited killing of *S. aureus* by polymorphonuclear leukocytes (PMNLs) (Repine et al., 1981). Several studies also demonstrated protective effects of DMSO on bacterial survival (H. Han et al., 2023; Mi et al., 2016). Taken together, these factors could have contributed to the observed reduction of *S. aureus* clearance in "frozen" vs "fresh" BMDMs. Finally, while "fresh" and "frozen" BMDMs in our study were exposed to the same culture conditions, after 6 days in culture, "frozen" BMDMs

but not “fresh” BMDMs were additionally co-cultured with fixed BMDMs for 1-12 hours. While fixed BMDMs are not viable, this additional variable could have affected *S. aureus* clearance by “frozen” BMDMs. Additional experimentation is needed to further investigate our findings. Taken together, however, we demonstrated that “frozen” BMDMs were both viable and functional following our protocol.

Although there is a myriad of different protocols for the isolation of primary cells either from blood or bone marrow(Barcelo et al., 2018; Haag & Murthy, 2021; Weischenfeldt & Porse, 2008; X. Zhang et al., 2008b), we have found that these protocols can be difficult to follow for individuals newly entering immunology who have limited to no experience in mouse or wet lab work. Additionally, to the best of our knowledge, an up-to-date *comprehensive* protocol that encompasses the entire process of cell isolation, differentiation, cryopreservation, and resuscitation, especially as it pertains to primary murine bone marrow derived macrophages has not been published. Furthermore, we are not aware of a protocol that includes all of these components and also addresses the viability and functionality of cryopreserved BMDMs for subsequent assay use. Here, we present a comprehensive and easy to follow protocol for the collection, isolation, cryopreservation, and resuscitation of primary bone marrow derived macrophages as well as data showing the viability and functionality of primary BMDMs that have undergone liquid nitrogen cryopreservation.

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Disclosure Statement

The authors report there are no competing interests to declare.

Data Availability Statement

All data generated or analyzed during this study are included in this article and its supplementary material files. Further enquiries can be directed to the corresponding author.

Authorship

Conceptualization: AML, ARA

Methodology: AML, KMS

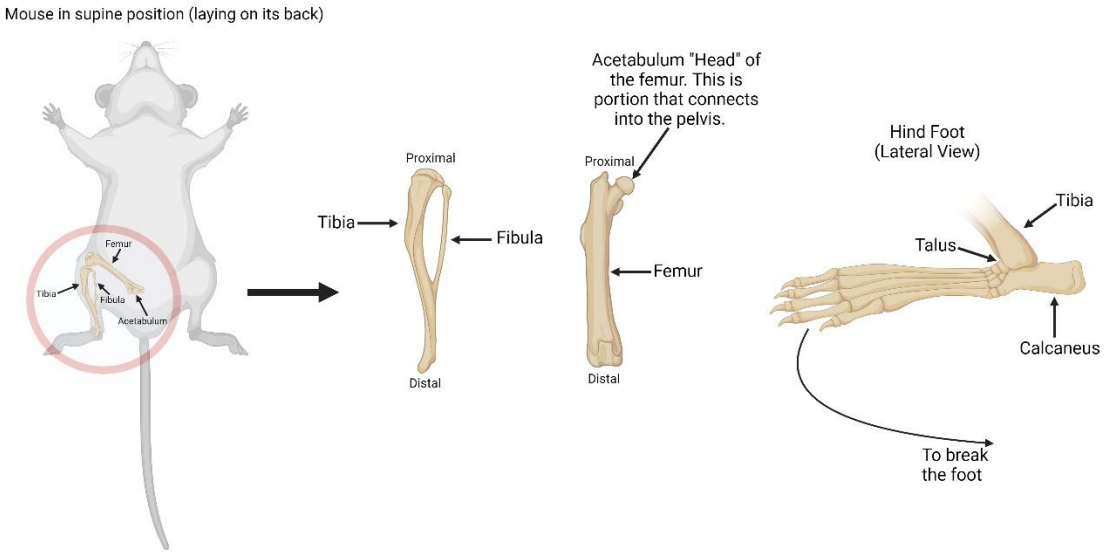
Data Curation: AML

Project Administration/Oversight: AML, ARA

Writing Original Draft: AML

Writing Revisions and Editing: AML, KMS, ARA

Supplemental Figure



Supplementary Figure 1: Murine hind leg bones. In the supine position, the murine hind legs are shown. The head of the femur will be located more medially in the legs are unmanipulated. Pulling the leg down will cause the head of the femur to move up and laterally. Proximal generally refers to “up” and distal to “down”. The mouse hind legs should be broken by pushing the top of the foot down, towards the calcaneus/distal portion of the tibia.

TRANSMEMBRANE TNF REVERSE SIGNALING-A POTENTIAL MECHANISM

Introduction

Among the many ligands present in the body, very few have been characterized to induce bi-directional signaling, which is defined as membrane bound ligands functioning as both a ligand as well as a receptor. The large majority of ligands capable of bi-directional signaling are associated with nervous system processes; however, ligands from the tumor necrosis factor superfamily (TNFSF) are also capable of bi-directional signaling (W.-H. Lee et al., 2019; Ross et al., 2016; L. Yu & Rao, 2009). TNFSF membrane bound ligands that have been described to induce bi-directional signaling include but are not limited to TNFSF14, TNFSF18, TNFSF13B, TNFSF13, and tmTNF (W.-H. Lee et al., 2019; Ross et al., 2016). Signaling through these ligands are involved in processes related to immune functioning (W.-H. Lee et al., 2019; Ross et al., 2016). Of the TNFSF membrane bound ligands described to induce bi-directional signaling, only tmTNF has been implicated in protective signaling during acute bacterial infection (Flynn et al., 1995; Fremond et al., 2005; Torres et al., 2005). Although tmTNF has been implicated in playing a protective role during acute bacterial infection, the mechanism and signaling through which it operates has not been elucidated. The existing body of literature which characterizes tmTNF forward intracellular signaling suggests that canonical and non-canonical NFkB are the primary signaling pathways (Fu et al., 2021; Thommesen & Laegreid, 2005). In contrast, much less literature characterizing the tmTNF reverse intracellular signaling cascade exists. To the best of our knowledge, the only identified component of the tmTNF reverse intracellular signaling cascade is the discovery of a casein kinase I (CKI) binding motif that has been shown to be

conserved across species, suggesting that CKI could be involved in the tmTNF reverse signaling cascade (Watts, 1999). Although the tmTNF reverse intracellular signaling cascade has not been elucidated, LPS tolerance (hypo-responsiveness to inflammatory stimuli) has been identified as one of the major outcomes of tmTNF reverse signaling (Diallo et al., 2021; Eissner et al., 2000; Krasselt et al., 2022; Pallai et al., 2016). Although controversial, IL-10 has also been shown to be upregulated in response to tmTNF reverse signaling (Diallo et al., 2021; Krasselt et al., 2022). Altogether, these studies highlight that very little is known about tmTNF reverse signaling and its outcomes. Therefore, we sought to investigate whether tmTNF reverse signaling was occurring within our system and whether reverse signaling could be a potential mechanism that contributed to tmTNF macrophage priming which led to TLR-induced improved *S. aureus* clearance described in Chapter 2.

In the following data, we found that IL-10 gene expression was increased in WT BMDMs stimulated with either TLR2 or TLR4 agonists, suggesting that tmTNF reverse signaling could be occurring. Based on the current body of literature that suggests LPS tolerance and increased anti-inflammatory IL-10 cytokine were among some of the major outcomes of tmTNF reverse signaling (Diallo et al., 2021; Krasselt et al., 2022), we sought to characterize whether tmTNF reverse signaling was occurring. Furthermore, we also sought to address whether tmTNF reverse signaling could be a potential mechanism for tmTNF-mediated priming which contributed to improved *S. aureus* clearance in TLR stimulated macrophages.

Results

IL-10 Transcript Is Increased Following TLR2 or TLR4 Stimulation in Murine BMDMs

To address if tmTNF reverse signaling was occurring, we measured induction of IL-10 transcript in response to TLR2 or TLR4 stimulation in WT immortalized murine BMDMs. Interestingly, we detected IL-10 transcript as early as 15 minutes following TLR2 or TLR4 stimulation (Figure 1). Even more intriguing, IL-10 transcript appeared to match, if not exceed at some timepoints, TNF transcript. These results were unexpected because macrophage recognition of pathogen associated molecular patterns (PAMPs) via either TLR2 or TLR4 generates robust pro-inflammatory responses which are critical for mounting an effective immune response (L. Yu, Wang, et al., 2010). Therefore, the detection of transcript from a primarily anti-inflammatory cytokine almost immediately following PAMP recognition seemed counterintuitive to generation of a strong inflammatory defense response. Although early transcript upregulation of IL-10 in response to PAMP recognition was surprising, it is important to highlight the inverse relationship that exists at the protein level between IL-10 and TNF. Due to TNF's potent induction and propagation of highly inflammatory environments (Josephs et al., 2018), it is unsurprising that TNF signaling eventually elicits a robust anti-inflammatory IL-10 cytokine response. IL-10 signaling inhibits the primary acute phase proteins that drive inflammation such as TNF, IL-6, and IL-1β (Armstrong et al., 1996; Huynh et al., 2016). Functionally, IL-10 mediated reduction of inflammation prevents pathological inflammation that would otherwise cause severe damage to native host structures (Armstrong et al., 1996; Huynh et al., 2016). This inverse relationship between IL-10 and TNF protein has been characterized to occur well after an inflammatory host response has been established (Huynh et al., 2016);

however, we observed IL-10 transcript almost immediately following TLR2 or TLR4 stimulation (PAMP recognition). This led us to hypothesize that tmTNF reverse signaling was occurring within our system.

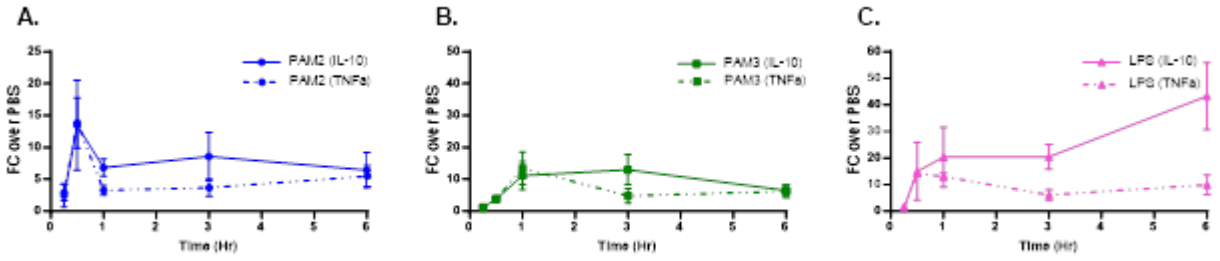


Figure 1: IL-10 cytokine transcript is upregulated in response to TLR2 or TLR4 stimulation. Immortalized WT murine BMDMs were stimulated with either 1μg/ml PAM2 (A), PAM3 (B), LPS (C) or equal volume PBS for 15 minutes, 30 minutes, 1 hour, 3 hour, or 6 hours before Trizol extraction, cDNA library synthesis, and qRT-PCR. Solid lines represent relative IL-10 transcript and dashed lines represent relative TNFα transcript compared to PBS treatment group. Transcripts were normalized to β-actin and fold change was calculated using ΔΔCT method. Results shown are from at least 2 independent experiments.

IL-10 is Dysregulated in The Absence of TNF

To investigate whether tmTNF reverse signaling was responsible for early upregulation of IL-10 transcript in response to TLR2 or TLR4 stimulation of WT murine BMDMs, we compared IL-10 transcript levels in immortalized WT murine BMDMs and primary TNF-/- BMDMs stimulated with either TLR2 or TLR4 agonists for 30 minutes, 1 hour, and 3 hours. We reasoned that if tmTNF reverse signaling was responsible for the observed IL-10 transcript in WT BMDMs immediately following TLR2 or TLR4 stimulation, then the absence of TNF protein in TNF-/- BMDMs should correlate with a reduction in IL-10 transcript. Unexpectedly, we observed that the absence of TNF resulted in dysregulation of IL-10 transcript following TLR2 or TLR4 stimulation. TNF-/- BMDMs stimulated with either PAM2, PAM3, or LPS

exhibited increased IL-10 transcript levels compared to WT BMDMs (Figure 2). Although these results were contrary to our hypothesis, research that describes global cytokine dysregulation, including IL-10, in the absence of TNF illuminated a potential explanation for our observed results (Tuazon Kels et al., 2020). Tuazon Kels et al., found that the absence of TNF resulted in overactivation of the transcription factor STAT3, which led to increased production of several cytokines, such as IL-6 and IL-10 in a murine model of infection. Based on the research by Tuazon Kels et al., it is possible that the absence of TNF in our primary TNF-/- BMDMs led to dysregulation of cytokine production, leading to increased IL-10 transcript compared to WT BMDMs.

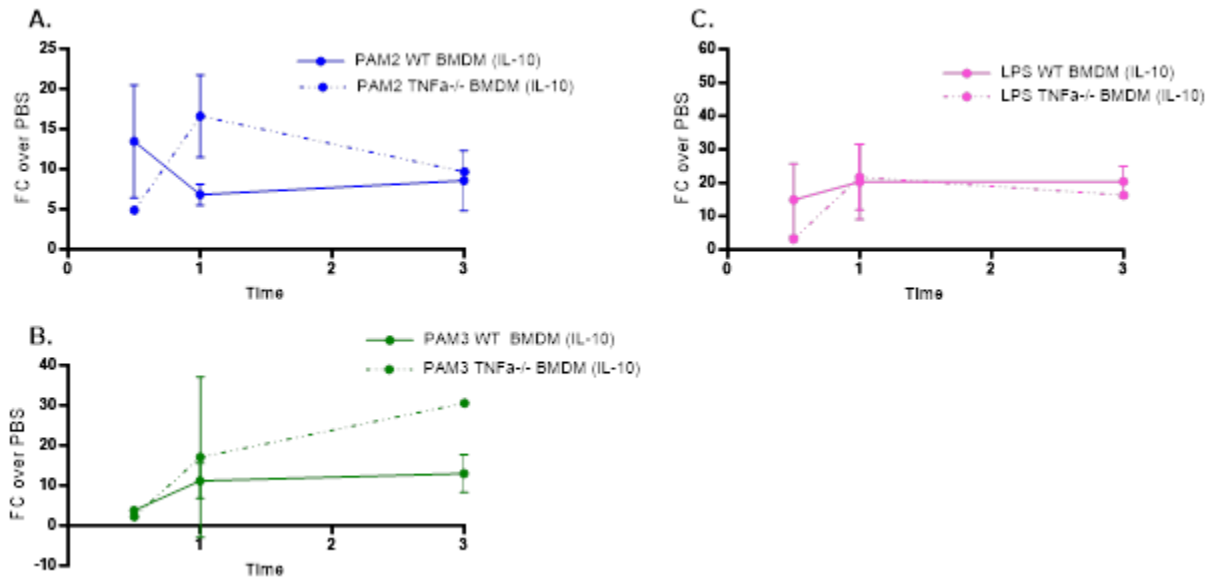


Figure 2: IL-10 is dysregulated in TNF-/- BMDMs. Relative IL-10 transcript was assessed in immortalized WT murine BMDMs (solid lines) or primary TNF-/- BMDMs (dashed lines) following 1µg/ml PAM2 (A), PAM3 (B), or LPS (C) stimulation for 30 minutes, 1 hour, and 3 hours relative to PBS treatment group. Transcripts were normalized to β-actin and fold change was calculated using ΔΔCT method. Results from 30 minute and 3-hour timepoint are from one experiment. Results from 1-hour timepoint are from 3 independent experiments.

IL-10 Protein Quantification Following TLR2 Stimulation

Based on our qRT-PCR data which showed robust expression of IL-10 transcript, we sought to assess protein production of IL-10. To the best of our knowledge, LPS tolerance is the best characterized outcome associated with tmTNF reverse signaling (Diallo et al., 2021; Eissner et al., 2000; Krasselt et al., 2022; Pallai et al., 2016). Thus, we reasoned that if LPS tolerance was occurring, it would result in higher IL-10 protein concentrations and could implicate a role for tmTNF reverse signaling. LPS tolerance was assessed by measuring the cytokine concentrations of TNF and IL-10 at two timepoints: 1) following 12-hour stimulation of TLR2 or TLR4 and 2) after 5-hour incubation with UV killed *S. aureus* at MOI 2:1. To isolate the differences between IL-10 and TNF cytokine concentrations which could be occurring due to tmTNF reverse signaling, a number of different treatment groups were included.

LPS tolerance was classically induced as previously described by others (Seeley & Ghosh, 2017; Virca et al., 1989) and used as a positive control for TNF cytokine measurements. Briefly, LPS tolerance results in decreased inflammatory cytokines and increased anti-inflammatory cytokines. To isolate the specific cytokine responses that may arise from tmTNF reverse signaling, the biologic p75TNFR:Fc and antibody based blocking of TNFR2 were used. As demonstrated in Chapter 2, treatment of murine WT BMDMs with the biologic p75TNFR:Fc resulted in suppression of sTNF and enrichment of tmTNF protein concentrations. Additionally, in Chapter 2 we also demonstrated that suppression of sTNF did not abrogate macrophage priming following TLR stimulation which led to enhanced clearance of subsequent *S. aureus* infection. Thus, we reasoned that enriching tmTNF using p75TNFR:Fc would increase the amount of reverse signaling, resulting in elevated IL-10 concentrations. Additionally, we

reasoned that WT BMDMs treated with TNFR2 blocking antibody would dampen tmTNF reverse signaling, resulting in higher TNF concentrations and lower IL-10 concentrations.

Interestingly, at the timepoints we assessed (12-hours following TLR2 stimulation and 12-hour TLR2 stimulation followed by 5-hour incubation with UV killed *S. aureus*) we found very little production of IL-10 in comparison to TNF production (Figure 3A, 3B). Consistent with our results in Chapter 2, TLR2 stimulation induced about ~2000pg/ml sTNF following a 12-hour stimulation period, and addition of p75TNFR:Fc led to an overall decrease of sTNF concentrations. Antibody blocking of TNFR2 in TLR2 stimulated WT BMDMs led to increased TNF production ~6000pg/ml, which was consistent with our hypothesis that tmTNF reverse signaling was occurring (Figure 3A). Unexpectedly, the concentration of IL-10 following 12-hour TLR2 stimulation was relatively low. Based on the IL-10 transcript data presented in Figure 1, we expected to see higher production of IL-10 cytokine. We found that IL-10 concentrations following 12-hour TLR2 stimulation was relatively low at ~60-40pg/ml (Figure 3A). Contrary to our hypothesis, the addition of p75TNFR:Fc did not lead to increased IL-10 production. Rather, p75TNFR:Fc addition had the opposite effect and resulted in lower IL-10 production. Interestingly, we saw a similar decrease of IL-10 in our LPS tolerant positive control, which is contrary to literature characterizing an increase of IL-10 during LPS tolerance (Nürnberger et al., 2021; Ziegler-Heitbrock, 1995). Although IL-10 production was unexpectedly decreased in our control, we also observed reduced TNF production, which was consistent with literature describing LPS tolerance. These differences could be due to the method used for LPS tolerance induction. Although we utilized an established protocol for LPS induction, there exist many different methods used for LPS induction (Mages et al., 2008; Natarajan et al., 2010; Nürnberger

et al., 2021; Seeley & Ghosh, 2017; Virca et al., 1989; Ziegler-Heitbrock, 1995). It is possible that different methods of LPS induction result in different cytokine expression profiles, which could offer an explanation for our IL-10 LPS tolerance results; however, more studies are needed to address this. Similar to the increase in sTNF production seen with TNFR2 blocking, a decrease in IL-10 production following TNFR2 blocking was consistent with our hypothesis, in which blocking TNFR2 would dampen tmTNF reverse signaling, leading to increased sTNF but decreased IL-10 production.

Even more interesting was the decrease in sTNF production following 5-hour incubation with UV killed *S. aureus* (Figure 3B). This decrease in sTNF production following incubation with UV killed *S. aureus* is in contrast to the increase in sTNF production following infection with live/active *S. aureus* that we described in Chapter 2. Overall, production of IL-10 following 5-hour incubation with UV killed *S. aureus* remained relatively consistent to IL-10 production following TLR2 stimulation, except for conditions that received either TNFR2 blocking antibody or p75TNFR:Fc (Figure 3B). In conditions where TLR2 stimulated WT BMDMs were treated with either TNFR2 blocking antibody or p75TNFR:Fc followed by incubation with UV killed *S. aureus* production of IL-10 was below the limit of detection (Figure 3B).

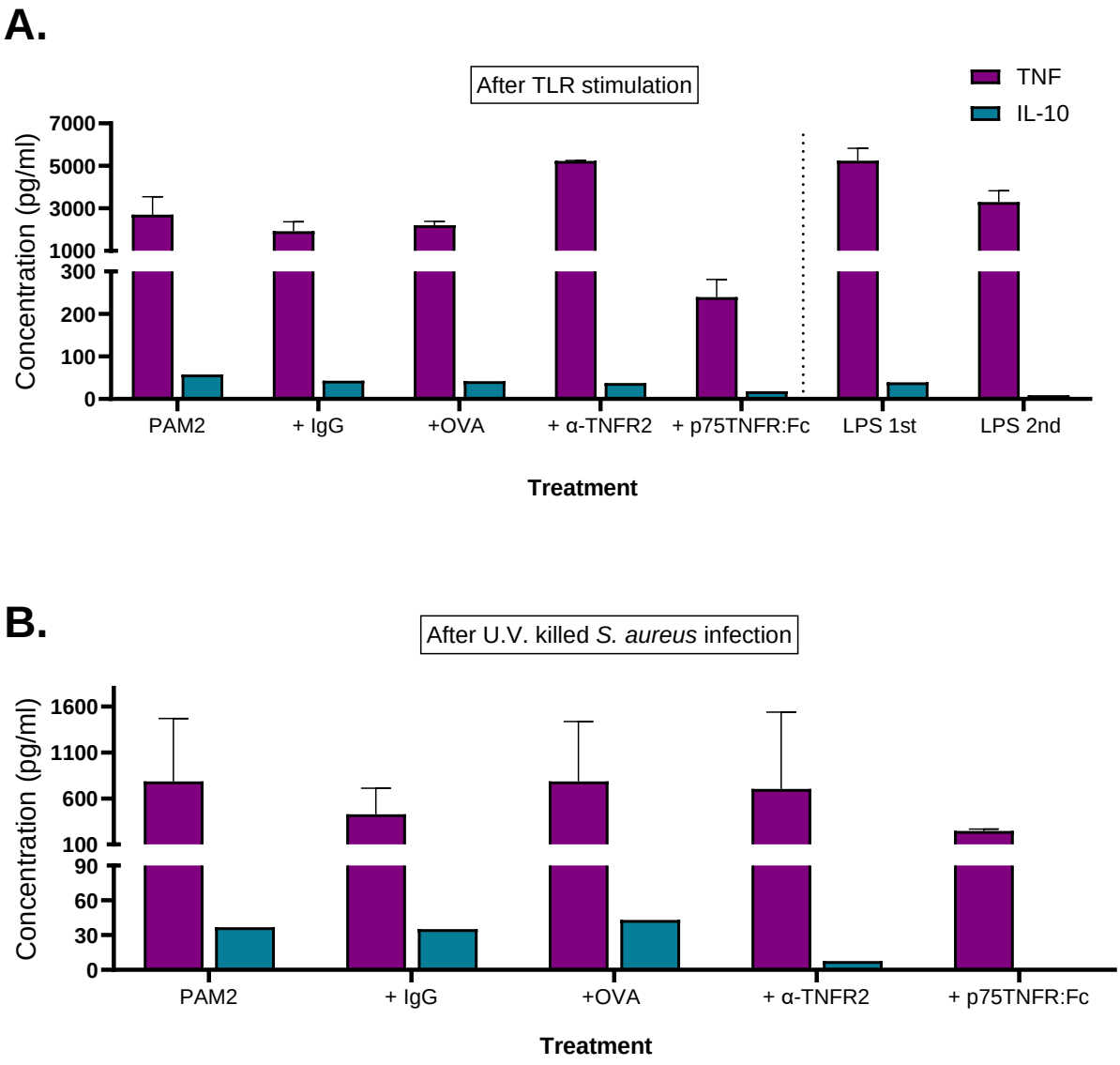


Figure 3. Comparison of IL-10 and TNF production. **(A)** Immortalized WT BMDMs were stimulated with either 1µg/ml PAM2 in the presence of 1µg/ml IgG (isotype control), 100µg/ml Ovalbumin (irrelevant protein control), 1µg/ml anti-TNFR2 blocking antibody, or 100µg/ml p75TNFR:Fc. Cells stimulated with 100ng/ml LPS for 24 hours (LPS 1 Stim) followed by a secondary stimulation with 100ng/ml LPS for 2 hours (LPS 2 Stim) served as a positive control for LPS tolerance. IL-10 and TNF production was analyzed from cellular supernatants following 12-hour stimulation period with corresponding treatment group. **(B)** IL-10 and TNF protein production was measured from WT BMDMs following 12-stimulation described above in (A) and subsequently infected with U.V. killed *S. aureus* at MOI 2:1 for 5 hours.

Preliminary Fixed Co-Culture System Attempts

To more directly investigate whether tmTNF reverse signaling was occurring following TLR stimulation of BMDMs, we attempted a co-culture system using fixed immortalized WT BMDMs and primary TNF BMDMs. Currently, two experimental methods have been described for effectively preventing tmTNF cleavage by ADAM17. These methods require either transfection of cells with an uncleavable form of tmTNF or a mutant form of tmTNF that lacks an intracellular domain, or the use of an inhibitory TNF antibody that competes with ADAM17. (Decoster et al., 1995; C. Li et al., 2019; H. Zhang et al., 2008). Due to time and resource constraints, we opted to use the system established by Li et al., who used activated fixed cells as a source of tmTNF (C. Li et al., 2019). We reasoned that if we could successfully isolate a reliable source of tmTNF in the absence of sTNF, we could begin to assess the role of tmTNF forward and reverse signaling as well as implicate tmTNF in TLR2 or TLR4-dependent enhanced macrophage clearance of a subsequent *S. aureus* infection as discussed in Chapter 2.

To enrich for tmTNF expression, WT BMDMs were stimulated with TLR2 or TLR4 agonist in the presence of TMI-005, an ADAM17 inhibitor. Following this, BMDMs were then washed and fixed. Cryopreserved primary TNF-/- BMDMs were collected and cultured, as described in Chapter 3, and were incubated for 1 hour with fixed BMDMs expressing tmTNF (Figure 4). TNF-/- BMDMs were utilized for this assay to prevent autocrine production of TNF which would confound our results. The results from these experiments were inconclusive. Because the WT BMDMs bearing tmTNF were fixed, they did not stay synchronized with the TNF-/- BMDMs at the bottom of culture plates. This introduced variable and inconsistent stimulation times between tmTNF bearing WT BMDMs and receptors on TNF-/- BMDMs. This inconsistency was reflected in our data measuring bacterial burden, most notably with TNF-/-

BMDMs that were incubated with WT BMDMs stimulated with LPS and then fixed (Purple Bar-Figure 4).

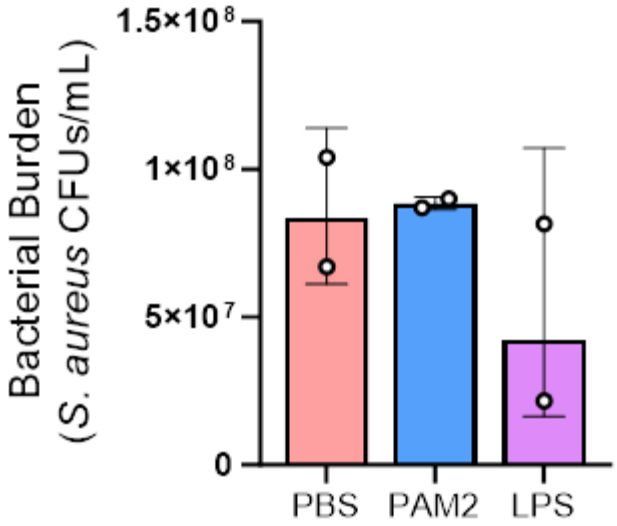


Figure 4: Bacterial burden of co-cultured cryopreserved primary BMDMs. Primary TNF-/- cells were cryopreserved in liquid nitrogen after 6 days in macrophage differentiating media, thawed, and resuscitated in macrophage differentiating media for 6 days. WT BMDMs were treated with either 1µg/ml PAM2, LPS, or equal volume PBS all in the presence of 1µM TMI-005. Subsequently, WT BMDMs were fixed in 1% PFA. TNFα-/- BMDMs were incubated with fixed WT BMDMs enriched from tmTNFα for 1 hour before subsequent infection with *S. aureus* for 5 hours at MOI 2:1. Bacterial burden between treatment groups was assessed via agar plating/CFU counts the following day. Data shown were obtained from two independent experiments that contained two technical replicates each.

Discussion

The preliminary data presented here demonstrate that IL-10 expression is upregulated, comparable to TNF expression following TLR2 or TLR4 stimulation. In line with literature characterizing tmTNF reverse signaling, LPS tolerance and IL-10 production have been associated with tmTNF reverse signaling (Diallo et al., 2021; Eissner et al., 2000; Krasselt et al., 2022; Pallai et al., 2016). Thus, our preliminary IL-10 transcript data led us to hypothesize that

tmTNF reverse signaling may be occurring within our system. To begin to test this hypothesis, we analyzed production of IL-10 and sTNF cytokine following 12-hour TLR2 stimulation and 12-hour TLR2 stimulation + 5-hour incubation with UV killed *S. aureus*. Additionally, we attempted to test this hypothesis using a fixed co-culture system in which tmTNF was isolated on stimulated and fixed cells.

We found that IL-10 production following 12-hour TLR2 stimulation was relatively low. Based on our previous TNF transcript data and the corresponding sTNF ELISA data, we expected IL-10 production to reflect concentrations similar to our TNF data (~2000pg/ml) since IL-10 and TNF transcript data were comparable. Although IL-10 production was lower than expected, one possible explanation could be the experimental timepoint we interrogated. IL-10 has been reported to exhibit bi-phasic production. IL-10 concentrations have been reported to peak early in response to stimuli, drop, and then peak again late in the response (Barsig et al., 1995; Huynh et al., 2016). In the absence of a proper time course for our data, we can only speculate on where our tested timepoints (12-hour following TLR2 stimulation and 12-hour TLR2 stimulation + 5-hour incubation with UV killed *S. aureus*) fall within the spectrum of early vs late responses and IL-10 production. Based on our previous IL-10 transcript data, however, we observed relatively high IL-10 expression after 1- and 3-hour stimulation with TLR agonists, which could suggest that IL-10 protein production occurs earlier than the 12-hour time point we assessed. Thus, it is possible that the experimental timepoint we interrogated was outside the "early" IL-10 peak concentration; however future studies which establish a time course for IL-10 production following TLR stimulation are needed to validate this.

Based on our observed data with p75TNFR:Fc in Chapter 2, we reasoned that addition of p75TNFR:Fc would lead to an increase in IL-10 production by way of tmTNF enrichment and thus a possible increase in tmTNF reverse signaling. Unexpectedly, however, addition of p75TNFR:Fc in the presence of TLR2 stimulation for 12-hours led to a decrease in IL-10 production. This decrease in IL-10 protein was mirrored by the decrease in sTNF following p75TNFR:Fc treatment, which raises the possibility that sTNF could be contributing to IL-10 production. We believe that a time course assessing both sTNF and IL-10 protein production in response to TLR2 stimulation is needed to address the temporal aspect of cytokine regulation and expression. It is possible that sTNF contributed to IL-10 production at the tested 12-hour time point, however, whether this is applicable to other time points could be addressed with further experimentation.

To more directly test tmTNF reverse signaling involvement in our system, we attempted a fixed co-culture model that isolated an endogenous source of tmTNF. Although our results were inconclusive for these experiments, we believe that the variability observed in our data, especially in LPS stimulated/fixed WT BMDMs co-cultured with TNF-/- BMDMs is worth further pursing. One experiment showed no change in TNF-/- BMDMs (co-cultured with LPS stimulated/fixed WT BMDMs) bacterial clearance efficiency of *S. aureus*; however, a second biologic replicate experiment showed that TNF-/- BMDMs co-cultured with LPS stimulated/fixed WT BMDMs exhibited improved clearance of a subsequent *S. aureus* infection. While this variability could represent typical experimental variability between experiments, it could also reveal a potential finding that was masked by unforeseen experimental complications. For future studies, we suggest the use of cell culture membrane inserts to prevent fixed cells

bearing tmTNF from de-synchronizing with live cells and floating to the top of culture media. This may yield more reliable and consistent results.

Materials & Methods

qRT-PCR

Primary BMDMs were isolated, differentiated, cultured, cryopreserved, and resuscitated as outlined in Chapter 3. Immortalized bone marrow macrophages derived from WT mice on a C57BL/6 background (NR-9456) were obtained from BEI Resources, NIAID, NIH. Cells were maintained in Dulbecco's modified Eagles medium (DMEM; Cytiva, Marlborough, MA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Atlas Biologicals, Fort Collins, CO, USA), 2mM glutamine (Gibco, Grand Island, NY, USA) 1mM sodium pyruvate (Gibco, Grand Island, NY, USA), and 10ug/ml ciprofloxacin (Bioworld, Dublin, OH, USA) and cultured at 37°C with 5% CO2 until 90% confluency was reached. Following confluency, BMDMs were seeded onto 24-well plates (Corning, Corning, NY, USA) at a cell density of $1x10^6$ cells/ml. BMDMs were then stimulated with either 1µg/ml Pam2CSK4 (PAM2; Invivogen, San Diego, CA, USA), 1µg/ml Pam3CSK4 (PAM3; Invivogen, San Diego, CA, USA), 1µg/ml ultrapure lipopolysaccharide (LPS: Invivogen, San Diego, CA, USA), or equal volume PBS (Cytiva, Marlborough, MA, USA) for 3 hours. Subsequently, BMDMs were washed with PBS and lysed with 400µl TRIzol Reagent (Invitrogen, Waltham, MA, USA). RNA was extracted using chloroform (ThermoFisher Scientific; Waltham, MA, USA), 70% ethanol (MSU chemistry store), Buffer RPE (Qiagen, USA), RNase free water (Invitrogen, Waltham, MA, USA), and RNA Mini spin columns (Epoch Life Science Inc, Missouri City, TX, USA) before quantification of RNA via nanodrop. All RNA samples were normalized to a concentration of

20ng/µl prior to cDNA library synthesis. The cDNA library was created using high-capacity cDNA Reverse Transcription kit (Applied Biosystems, Waltham, MA, USA) according to manufacturers' recommendation with a total input of 100ng RNA. qPCR was performed in triplicate using 2X Universal SYBR Green Fast qPCR Mix (ABclonal, Woburn, MA, USA) according to manufacturers' recommendation with TNFα, IL-10, and β-actin primers (all from IDT, Coralville, IA, USA). All qRT-PCRs were run on QuantStudio 5 Real-Time PCR system (ThermoFisher Scientific; Waltham, MA, USA). A total of forty cycles were run for each qRT-PCR. Data was normalized to β-actin and relative fold change was calculated using delta delta CT method.

TNFα sequence primer 1: 5'AGACCCTCACACTCAGATCA-3'

TNFα sequence primer 2: 5' TCTTTGAGATCCATGCCGTTG-3'

IL-10 sequence primer 1: 5'GTCATCGATTTCTCCCCTGTG-3'

IL-10 sequence primer 2: 5'ATGGCCTTGTAGACACCTTG-3'

β-actin sequence primer 1: 5' GATTACTGCTCTGGCTCCTAG-3'

β-actin sequence primer 2: 5' GACTCATCGTACTCCTGCTTG-3'

Enzyme-Linked Immunosorbent Assay (ELISA)

IL-10 and TNFα protein concentrations were analyzed from cellular supernatants collected at two timepoints: 1.) following 12-hour stimulation and 2.) following 5-hour infection with U.V. killed *S. aureus*. Cellular supernatants were collected and frozen back at -80˚C. ELISAs were performed with ELISA MAX Deluxe Set Mouse TNF-α kit and ELISA MAX Deluxe Set Mouse IL-10 kit according to manufacturers' recommendations (BioLegend, San Diego, CA, USA).

Bacterial Clearance Assay

WT murine BMDMs (NR-9456; BEI Resources; Manassas, Virginia, USA) or primary TNFα-/- BMDMs (collected from TNFα-/- obtained from Jackson Laboratory, Bar Harbor, ME, USA) were seeded into 24-well plates (Corning, Corning, NY, USA) at a cell density of $1x10^6$ cells/ml. WT BMDMs were stimulated for 12 hours with either equal volume PBS, 1ug/ml Pam2CSK4 (PAM2; InvivoGen, San Diego, CA, USA), 1μg/ml Pam3csk4 (PAM3; InvivoGen, San Diego, CA, USA), or 1μg/ml ultrapure lipopolysaccharide (LPS; InvivoGen, San Diego, CA, USA) all in the presence with 1μM TMI-005 (Cayman Chemical, Ann Arbor, MI, USA) at 37°C with 5% CO_2. Subsequently, treatment was removed and WT BMDMs were washed 2x with sterile PBS. WT BMDMs were fixed with 1% sterile filtered paraformaldehyde (PFA; Fisher Scientific, Waltham, MA, USA) for 20 minutes at room temperature. Next, WT BMDMs were washed with sterile PBS 2x and resuspended in primary cell culture media and seeded into cell culture wells containing TNFα-/- BMDMs at 1:1 ratio. Cells were synchronized via centrifugation and incubated at 37°C with 5% CO_2 for 1 hour. Supernatant was removed and cells were washed 2x with sterile PBS. *Staphylococcus aureus* USA300 LAC (a kind gift from Dr. Jovanka Voyich, Department of Microbiology and Cell Biology at MSU) was U.V. killed for 15 minutes, diluted to the appropriate concentration based on prior growth curve equation, and used to infect TNFα-/- BMDMs at MOI 2:1 for 5 hours at 37°C with 5% CO_2. Following infection, cells were centrifuged and supernatant was collected/stored at -80˚C.

SUMMARY, CONCLUSIONS, AND FUTURE STUDIES

Summary and Conclusions

One of the major cornerstones of host immune defense against invading pathogens is the innate immune system. Within the innate immune system, macrophages and Toll-Like Receptors form a formidable barrier that pathogens must overcome to establish a productive infection. Understanding the interplay between host innate immune defense and pathogen response is critical for the development of antimicrobial therapeutics, especially in the context of antibiotic resistance. Because macrophages are one of the first immune cells to recognize and respond to pathogen insult (through TLRs and other PRRs) it is vital to fully characterize the intracellular signaling events propagated by pathogen recognition.

A deeper, holistic understanding of these early macrophage intracellular signaling events which mediate cellular activation and resolution of bacterial infection could reveal novel druggable targets aimed at either modulating host inflammatory responses (rheumatoid arthritis, systemic lupus erythematosus, etc.,) or manipulation of intrinsic host mechanisms for pathogen resolution (antimicrobial therapeutics). To that end, the research described in this dissertation sought to identify the key TLR2 or TLR4 induced signaling events involved in priming macrophages which led to their improved clearance efficiency of a subsequent *S. aureus* infection. Based on RNA sequencing data of murine whole lung stimulated with TLR2 agonists combined with subsequent pathway analysis, we hypothesized that the cytokine tmTNF contributed to macrophage priming following PAMP recognition (TLR stimulation), which led to increased clearance efficiency of a subsequent *S. aureus* infection.

In Chapter 1 we reviewed macrophage activation via TLR PAMP recognition and the resulting intracellular signaling pathways which are induced to initiate and promote an inflammatory environment. Additionally, we reviewed the function and signaling of TNF, one of the most potent inflammatory cytokines that mediates inflammation. The concept of reverse signaling was introduced and the current body of literature characterizing tmTNF reverse signaling was also discussed.

In Chapter 2 we investigated the TLR2 or TLR4-induced priming signals that contributed to improved macrophage clearance of a subsequent bacterial infection. In these studies, we observed that stimulation of TLR2 or TLR4 in macrophages resulted in improved clearance of a subsequent *S. aureus* infection compared to mock stimulated. RNA sequencing analysis on murine whole lung identified that TNF was among one of the most differentially upregulated genes in response to TLR2 or TLR4 stimulation at 6-hours. Subsequent pathway analysis revealed that signaling through TNFR2 is one of the primary pathways initiated following TLR2 PAMP recognition in murine whole lung. Thus, we developed and tested the hypothesis that the transmembrane bioactive form of TNF (tmTNF) may be contributing to macrophage priming following TLR2 or TLR4 stimulation which led to improved clearance of a subsequent *S. aureus* infection. We observed abundant concentrations of both bioactive forms of TNF (sTNF, tmTNF) in response to TLR2 or TLR4 stimulation. Ablation of both sTNF and tmTNF resulted in a loss of improved macrophage clearance of a subsequent *S. aureus* infection, despite prior TLR2 or TLR4 stimulation. This suggested that TNF was necessary for improved macrophage clearance of *S. aureus* following TLR2 or TLR4 stimulation. Depletion of only sTNF in a wild-type macrophage system did not abrogate improved macrophage clearance of *S. aureus* following

TLR2 or TLR4 stimulation. Conversely, addition of sTNF to TLR2 stimulated TNF-/- macrophages was not sufficient to prime macrophages prior to infection, which led to no differences in *S. aureus* clearance, even in the presence of TLR stimulation. These data suggested that the sTNF produced as a result of TLR stimulation may not be a critical signal involved in macrophage priming which led to increased clearance of a subsequent *S. aureus* infection. Leveraging the juxtacrine-dependent nature of tmTNF signaling (Kaymakcalan et al., 2009; Liang et al., 2022) by varying cell-to-cell contact in the presence of TLR2 stimulation informed us that cell-to-cell contact was important for TLR-induced priming of macrophages prior to bacterial infection. Additionally, we found that blocking TNFR2, tmTNF's primary receptor, abrogated TLR-induced improved clearance of *S. aureus* infection.

In Chapter 3 we described the protocol we developed for the extraction, differentiation, cryopreservation, and resuscitation of primary murine bone marrow derived macrophages (BMDMs). We provided data that compared the functionality and viability of freshly extracted/differentiated primary BMDMs ("fresh" BMDMs) and "frozen" primary BMDMs, which had undergone cryopreservation and resuscitation following extraction and differentiation. To the best of our knowledge, an all-encompassing protocol for the extraction, differentiation, cryopreservation, resuscitation, and functional assessment of primary murine BMDMs does not exist. Our developed protocol fills this gap and offers an alternative method for obtaining primary cell cultures, in comparison to approaches that euthanize mice and extract bone marrow on an as needed experimental basis. This is oftentimes time consuming and can add to increased mouse usage, which could otherwise be avoided by freezing back extra differentiated cells for

later use. Furthermore, we provided data which suggested that primary BMDMs that have undergone cryopreservation retained a high degree of viability and functionality.

In Chapter 4 we discussed preliminary work for which our aim was to begin to elucidate whether tmTNF reverse signaling was occurring within our cell culture system. Guided by the current body of literature on tmTNF reverse signaling, we assessed LPS tolerance and IL-10 production. We found increased expression of IL-10 in response to TLR2 or TLR4 stimulation, which was comparable to TNF expression. IL-10 protein analysis at 12 hours following TLR2 stimulation revealed low production of IL-10 in comparison to TNF production. Consistent with our hypothesis that tmTNF reverse signaling could be occurring within our system, TNFR2 blocking resulted in increased TNF but decreased IL-10 production following TLR2 stimulation. Interestingly, however, we found that enrichment of tmTNF (and depletion of sTNF) using the biologic p75TNFR:Fc led to decreased concentrations of IL-10, which was in opposition to our hypothesis. However, IL-10 has been reported to exhibit bi-phasic production following stimulus (Barsig et al., 1995; Huynh et al., 2016); therefore, it is possible the 12-hour time point we assessed was not the optimal time point for IL-10 production.

Furthermore, we attempted a fixed co-culture system that isolated endogenous tmTNF. Using this set-up, we intended to more directly address if tmTNF reverse signaling was contributing to TLR priming which resulted in improved clearance of a subsequent *S. aureus* infection. Although the results from these experiments were inconclusive due to unforeseen experimental complications, we believe that minor adjustments would resolve these issues and could produce an assay that could reliably test tmTNF involvement.

Taken as a whole, the data presented in this dissertation provided evidence that tmTNF produced as a result of TLR PAMP recognition could be involved in macrophage priming which improved clearance efficiency of a subsequent *S. aureus* infection. Additionally, preliminary work suggested that tmTNF reverse signaling could be involved, however more experimentation is needed to test this hypothesis.

Future Studies

The work presented here highlights an important and often overlooked feature of TNF signaling, which is that TNF exists in two functionally active protein forms. This is especially important in the context of macrophages which are primary producers of TNF during pathogenic insult (Imaizumi et al., 2000; Nau et al., 2002). Upon PAMP recognition, macrophages produce TNF which plays a major role in coordinating inflammatory host defense responses against infection (Imaizumi et al., 2000; Nau et al., 2002). Indeed, it has been shown that many pathogens produce homologues of TNF receptors (TNFR1 and TNFR2) which function to disrupt and reduce host inflammatory signaling (Rahman & McFadden, 2006; Tuazon Kels et al., 2020) . By targeting TNF signaling, these pathogens create deficiencies in host inflammatory defense mechanisms which allows the pathogen to proliferate and establish productive infection. A more in-depth understanding of how the two forms of TNF co-exist, their signaling mechanisms, outcomes, and ability for cross-talk and co-regulation is vital for expanding our current understanding of host-pathogen interactions. A better understanding of TNF signaling, especially in the context of both bioactive forms, could uncover novel signaling events that are critical for host defense. This could ultimately lead to the development of therapeutics which seek to bolster and enhance these signaling events to improve host outcome to infection.

In addition to the therapeutic potential of harnessing existing immune defense functions, a more holistic understanding of TNF signaling could have potential translational implications for autoimmune therapeutics. Currently, there are only 5 FDA approved TNF inhibitors on market to treat autoimmune diseases such as rheumatoid arthritis, ankylosing spondylitis, Crohn's disease, plaque psoriasis, and ulcerative colitis (Kalliolias & Ivashkiv, 2016; J. Li et al., 2021; Valerie Gerriets et al., 2023). Each of these inhibitors exhibit different degrees of efficacy and side effects (J. Li et al., 2021), which suggests that the regulation of TNF is highly dependent on its cellular environment which dictates the course of TNF signaling. A more in-depth analysis of TNF signaling in the context of infection could uncover unexplored TNF regulation mechanisms that pertain to the differences seen with current anti-TNF therapeutics and autoimmune disease.

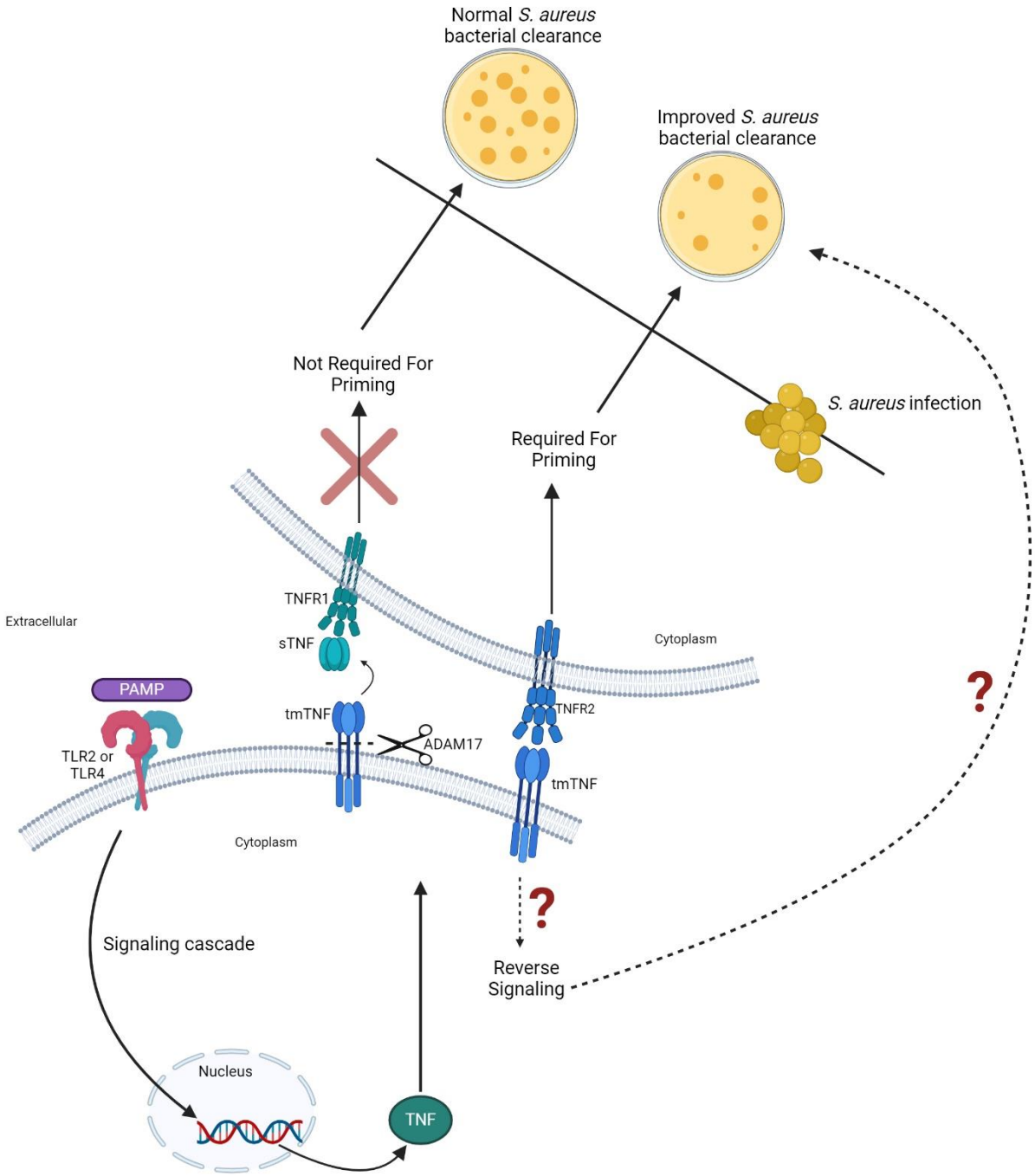
In Chapter 2, we did not directly assess the role of tmTNF signaling (forward or reverse) in macrophage priming following TLR stimulation; however, we provided evidence that sTNF is not necessary for TLR-induced priming which led to improved macrophage clearance of a subsequent *S. aureus* infection. Future studies could take advantage of transfection models which introduce uncleavable forms of tmTNF or reverse signaling incompetent forms of tmTNF into cells (Decoster et al., 1995; H. Zhang et al., 2008). These experiments would definitively and directly implicate a role for tmTNF in macrophage priming and address whether tmTNF is indeed responsible for mediating priming signals which directly lead to improved *S. aureus* clearance. Following the characterization presented in Chapter 2, we sought to lay the groundwork for experiments which could address a potential mechanism through which tmTNF priming occurred. In comparison to tmTNF forward signaling, little is known about the

intracellular signaling events which orchestrate tmTNF reverse signaling. Therefore, in Chapter 4 we sought to investigate tmTNF reverse signaling by assessing LPS tolerance. Although our results were preliminary and somewhat inconclusive, we believe a detailed time course of IL-10 compared to sTNF and tmTNF could improve the work performed in Chapter 4. In addition to this, we also believe a perfected fixed co-culture system could begin to address the intracellular signaling events of tmTNF reverse signaling, if it is indeed occurring within our system.

One of the major roadblocks with our current experimental set-up is that macrophages must be stimulated with TLR agonists in order to induce expression of both sTNF and tmTNF. Because of this cellular activation, overall kinase activity is heavily upregulated (Mogensen, 2009). This makes isolation and identification of intracellular tmTNF reverse signaling kinase activity indistinguishable from TLR-induced kinase activity. As discussed in Chapter 1, a conserved casein kinase 1 (CK1) binding motif has been identified in the cytoplasmic tails of a large majority of TNFSF proteins capable of reverse signaling (Ross et al., 2016; Watts, 1999). This suggested that CK1 could be involved in tmTNF reverse signaling. We believe a perfected fixed co-culture system in combination with existing uncleavable tmTNF mutants could be used to isolate the contribution of tmTNF forward and reverse signaling. A perfected and reliable fixed co-culture system could isolate and address kinase activity induced as a result of tmTNF forward signaling. Transfected cells bearing uncleavable forms of tmTNF could be used to isolate and address kinase activity associated with tmTNF reverse signaling, using CK1 as an initial starting point.

Research discussed in Chapters 2 and 4, raised several key questions that ventured beyond macrophages and sought to elucidate how the work presented here could be extended to

other cell types and interactions: Does tmTNF priming/signaling of macrophages change *in vivo*? What is the role of tmTNF on recruited immune cells during infection? This research provides a framework that could be utilized to address these questions. Although much knowledge has been acquired about the innate immune system following the initial discovery of TLRs (Weiss & O'Neill, 2022), there is still much that remains shrouded in mystery. We believe that a greater understanding of the early TLR-induced signaling events (PAMP recognition) which drive downstream infection resolution outcomes could reveal unknown targets that could eventually be leveraged to harness the innate immune system. As such, we sought to elucidate the key intracellular signaling events following TLR stimulation of macrophages which led to improved *S. aureus* clearance. We found that signaling through tmTNF, rather than sTNF, may be a critical signaling event involved in macrophage priming which led to improved *S. aureus* clearance. Furthermore, we provided preliminary data which suggested that tmTNF reverse signaling could be a potential mechanism through which tmTNF priming occurs. To the best of our knowledge, involvement of tmTNF signaling in macrophage priming has not been described. Thus, the work presented in this dissertation contributes to and expands upon the existing body of literature which implicates a protective role for tmTNF during mycobacterial or listeria bacterial infections by characterizing a priming role for tmTNF in macrophages which leads to increased clearance of a subsequent *S. aureus* infection (Fremond et al., 2005; Torres et al., 2005) (Summary Figure 1). Our work uncovered a portion of the intricate immune signaling that occurred following pathogen detection in one immune cell type. It is my hope that the research presented here inspires or offers insight to others who may build upon, improve, or revise the presented science to garner a more holistic understanding of TLR-based innate immune defense.



Summary Figure 1. Summary of dissertation research findings. PAMP ligation to its cognate TLR signaling subunit in macrophages induces TNF protein production which is expressed in both bioactive forms as tmTNF and sTNF. Binding and signaling of tmTNF through TNFR2 contributes to macrophage priming which leads to increased *S. aureus* clearance.

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