Contribution of Toll-Like Receptor 4 to Infection-Induced Hemophagocytosis

By

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Thesis directed by Professor Corrella Detweiler

Abstract

Hemophagocytes are a unique macrophage subset that accumulate during infection with many different microbes and are identified by their engulfment of erythrocytes and leukocytes. Hemophagocytes express anti-inflammatory markers and persist into chronic stages of infection; however, how hemophagocytes recognize blood cells for uptake and the signaling pathway(s) required to initiate hemophagocytosis during *Salmonella* infection are unknown.

I identified both resident and inflammatory monocytes as the main splenic hemophagocyte populations in *Salmonella enterica* serovar Typhimurium-infected mice. Furthermore, I established that structurally conserved microbial products stimulate macrophages to hemophagocytose through Toll-like receptor (TLR) recognition and subsequent NF-κB activation. Previous work in the laboratory showed that hemophagocytic macrophages harbor live *S.* Typhimurium during chronic infection and that both *S*. Typhimurium and *Yersinia pseudotuberculosis* stimulated hemophagocytosis *in vitro*. Since these pathogens survive within macrophages, I hypothesized that prolonged exposure to microbes is key to stimulating hemophagocytosis. To support this hypothesis, I showed that macrophages become hemophagocytic upon prolonged exposure to heat-killed *S*. Typhimurium, *Y. pseudotuberculosis*, *Bacillus subtilis*, or *Mycobacterium marinum*. Furthermore,

conserved microbial products were sufficient to stimulate macrophages to hemophagocytose. *S*. Typhimurium LPS (a TLR4 agonist) induced hemophagocytosis in resting and IFN-γ-pretreated macrophages, whereas lipoteichoic acid (a TLR2 agonist) and synthetic unmethylated deoxycytidine-deoxyguanosine dinucleotides, which mimic bacterial DNA (a TLR9 agonist), induced hemophagocytosis only in IFN-γ-pretreated macrophages. I identified a requirement for TLR4 in the induction of hemophagocytosis: chemical inhibition of TLR4 or genetic ablation of TLR4 prevented both *Salmonella*- and LPS-stimulated hemophagocytosis. Thus, LPS is the major constituent of *S*. Typhimurium that stimulates hemophagocytosis during infection. To demonstrate that signaling pathways downstream of TLR4 were required, I blocked activation of the transcription factor NF-κB and inhibited hemophagocytosis. In addition, I investigated whether phosphatidylserine (PS) recognition was required for erythrocyte uptake; treatment with antibodies to block PS-receptor interactions decreased the fraction of LPS- and *Salmonella*-induced hemophagocytes in IFN-γ-pretreated macrophages; there was no effect on resting macrophages. These findings show that prolonged TLR signaling and activation of NF-κB reprograms a subset of primary macrophages to hemophagocytose and that macrophage recognition of PS on erythrocytes is not the dominant mechanism driving hemophagocytosis.

Dedication

I would like to dedicate this work to my husband, Jeffrey Akkerman – you are my best friend and the love of my life and your support has made all of this possible

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Chapter 1 Introduction

I. Macrophage Function in Innate Immunity

Macrophages are white blood cells with a multitude of immune functions, including phagocytosing and destroying pathogens, initiating inflammatory responses, and regulating inflammatory responses (Varol et al., 2015). Macrophages can be generally categorized as either classically activated M1 macrophages or alternatively activated M2 macrophages, although in truth, macrophages fall along a spectrum between these two extremes(Mosser and Edwards, 2008). Classically activated macrophages produce pro-inflammatory cytokines, reactive oxygen species, and reactive nitrogen species after stimulation with Interferon gamma (IFN-γ) and Lipopolysaccharide (LPS). Alternatively activated macrophages are differentiated by exposure to IL-4, IL-10 or IL-13 and diverge from classically activated macrophages in that alternatively activated macrophages induce collagen production and help regulate inflammatory responses (Mosser and Edwards, 2008; Murray and Wynn, 2011).

Macrophages detect microbial infection via pattern recognition receptors (PRRs), which recognize structurally conserved microbial associated molecular patterns (MAMPs), such as cell wall components (e.g. lipopolysaccharide, LPS) and microbial DNA. One family of PRRs is the Toll-Like Receptors (TLRs) (Pandey et al., 2015). Toll receptor was first identified as an essential gene for Drosophila embryonic development (Nüsslein-Volhard and Wieschaus, 1980). Later, based on similarities in the signaling pathway for dorsoventral axis establishment and initiation of immune responses, it was determined that Toll is required to control the antifungal response in Drosophila

(Lemaitre et al., 1996; Wasserman, 1993). Shortly after this discovery, the human homologue of the toll receptor was identified (Medzhitov et al., 1997), and the following year, TLR4 was identified as the receptor that bound to, and responded to, LPS (Poltorak et al., 1998).

TLRs belong to the Interleukin-1 receptor (IL-1R) superfamily due to conserved structural similarity. Both TLRs and IL-1R are type I transmembrane proteins and both contain a cytoplasmic Toll interleukin (IL)-1 receptor (TIR) domain. The extracellular domain of TLRs contains Leucine Rich Repeat regions, whereas IL-1R's extracellular region is composed of three immunoglobulin domains (Botos et al., 2011).

TLRs can be divided into cell-surface TLRs and endosomal TLRs. The surface TLRs include TLR5, TLR4, TLR2, TLR1, TLR6, while the endosomal TLRs include TLR3, TLR7, TLR8, TLR9, and TLR13. Upon recognition of specific MAMPs, TLRs either homo-dimerize or hetero-dimerize, and various signaling pathways are activated; the specificity of the TLR response, i.e., which pathway is stimulated, depends on which TIR domain-containing adaptor protein(s) is recruited to the cytoplasmic TIR domain. The four TIR domain-containing adaptors are myeloid differentiation factor 88 (MyD88), MyD88 adaptor-like protein (MAL), TIR domain-containing adaptor-inducing IFN-β (TRIF), and TRIF-related adaptor molecule (TRAM) (Botos et al., 2011; Pandey et al., 2015).

Surface TLRs (TLR5, TLR4, TLR2, TLR1 and TLR6) bind and signal through the MyD88-dependent pathway. MyD88-dependent signaling leads to the activation of NFκB, p38 and JUN. Endosomal TLR3 signals through TRIF, whereas endosomal

TLR9 signals through MyD88. TLR4, which signals through MyD88 and MAL when on the surface, signals through TRIF and TRAM once it is endocytosed (Botos et al., 2011; Pandey et al., 2015). For an overview of the pathway, see Figure 1-1.

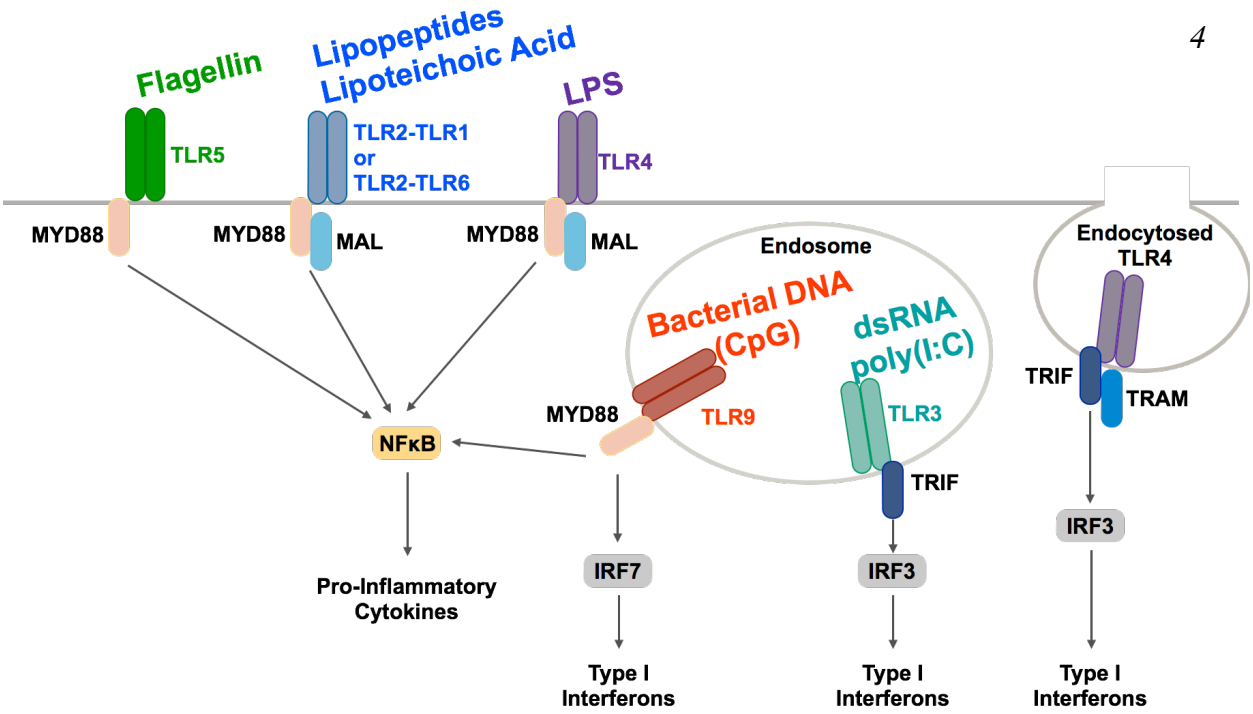


Figure 1-1. Toll-Like Receptors: Ligands and Overview of signaling pathways

TLR signaling leads to the induction of pro-inflammatory cytokines and/or Type I Interferon secretion. TLR stimulation leads to an MI, or classically activated, macrophage phenotype; these macrophages produce high levels of nitric oxide and reactive oxygen intermediates, secrete high levels of IL-1, IL-6, IL-12, IL-23 and TNF-α, have increased expression of the co-stimulatory molecule CD86, and have high antigen presentation, as evidenced by increased MHC-II expression (Mantovani et al., 2004; Martinez and Gordon, 2014; Wang et al., 2014). See table for an overview of the responses specific for each TLR.

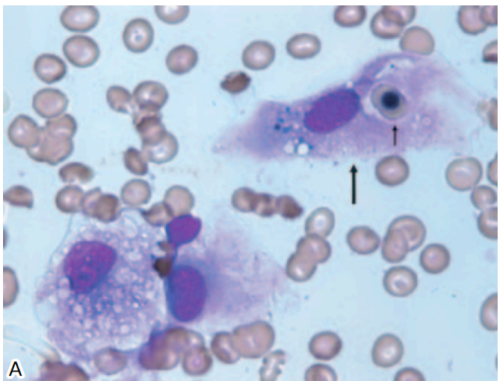
TLR Dimer	***PAMP***	***TIR Adaptor***	***Transcription Factors***	***Effector Response***
TLR1-2	Triacylated Lipopeptides	MyD88 & MAL	NFκB, AP1	IL-6, TNF-α, IL-8, MCP-1, RANTES
TLR2-6	Diacylated Lipopeptides	MyD88 & MAL	NFκB, AP1	IL-6, TNF-α, IL-8, MCP-1, RANTES
TLR3	dsRNA	TRIF	IRF3	IFN-β
TLR4	LPS	MyD88 & MAL; TRIF & TRAM	NFκB, AP1 IRF3	IL-6, TNF-α, IFN-β, IP-10
TLR5	Flagellin	MyD88	NFκB, AP1	TNF-α
TLR9	CpG DNA	MyD88	NFκB, IRF7	IFN-α

Table 1-1. TLR signaling leads to shared and unique effector responses.
Table adapted by author from (Pandey et al., 2015).

II. Hemophagocytes – An indicator of inflammation

Hemophagocytes (HMs) are macrophages that have engulfed hematopoietic cells, such as erythrocytes, white blood cells, and their precursors (Figure 1-2). Hemophagocytes are a diagnostic indicator of hypercytokine syndromes, such as Hemophagocytic Lymphohistiocytosis (HLH), Macrophage Activation Syndrome (MAS), and sepsis.

A



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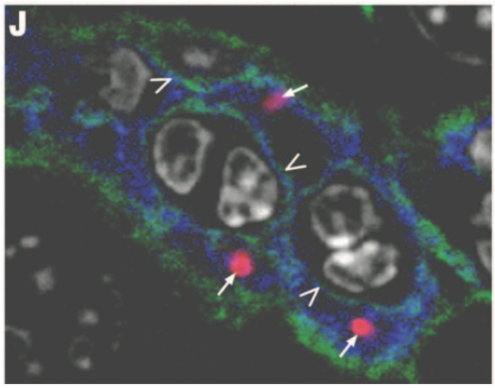


Figure 1-2 Hemophagocytes

A. A bone marrow smear from a patient with bacteremia. The hemophagocyte (large arrow) has engulfed an erythroblast (small arrow). Image from (Ito et al., 2006).
B. Confocal fluorescence microscopy of 50-μm-thick liver sections from a 1-wk-infected Slc11a1 (Nramp1) wild-type mouse. *S*. Typhimurium (O-antigen, arrows) are red, macrophages (F4–80 and MOMA-2) are blue, DNA (DAPI) is gray, and phalloidin (actin) is green. Image from (Nix et al., 2007).

HLH and related hyperinflammatory immune disorders, such as MAS, result in the hyper-activation and expansion of lymphocytes and macrophages. These hyper-activated cells secrete pro-inflammatory cytokines, resulting in a cytokine storm. HLH is classified as either primary HLH (also known as familial HLH) or acquired HLH (also known as secondary HLH, acquired HLH, or reactive HLH). Primary HLH is caused in infants due to genetic lesions in granule-mediated cytotoxicity. Secondary HLH is caused by infection, malignancies, or autoimmune disorders and usually occurs much later in life. A common feature of both syndromes is that infection is an underlying trigger: lymphocytes become hyperactivated due to lack of immune regulation and pathogen clearance (Table 1-2, Table 1-3). MAS is a serious, often fatal, complication of rheumatic diseases, and its etiology is unknown. The pathophysiology of MAS and HLH is similar: an overwhelming inflammatory response with heightened inflammatory cytokine levels, particularly of IFN-γ, enlargement of the liver and spleen, and an increase of hemophagocytes (Brisse et al., 2015a; Canna and Behrens, 2012). In 2004, the Histiocyte Society presented diagnostic guidelines for HLH, and in 2005, Ravelli et al., proposed guidelines for the diagnosis of MAS that are similar to the diagnostic criteria used to identify HLH (Table 1-4) (Henter et al., 2007; Ravelli et al., 2016; Weaver and Behrens, 2014).

DISEASE	GENETIC DEFECT	FUNCTION
Familial HLH (fHLH) without hypopigmentation		
Type 1 fHLH	Unknown	N/A
Type 2 fHLH	*PRF1*	Encodes the cytolytic protein perforin; major protein in cytotoxic granules produced by NKs and CTLs.
Type 3 fHLH	*UNC13D*	Encodes Munc13-4, a protein required in vesicle priming in cytotoxic cells. Munc13-4 is required for degranulation.
Type 4 fHLH	*STX11*	Encodes syntaxin 11, a member of the family of soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs). It regulates vesicle membrane fusion, a step required for lytic granule release from NKs and CTLs.
Type 5 fHLH	*STXBP2*	Encodes Munc18-2 or syntaxin-binding protein 2; also required for vesicle priming in cytotoxic cells (see Type 3 fHLH).
SH2D1A deficiency (XLP1)	*SH2D1A*	Encodes the signaling lymphocyte activation molecule associated protein (SAP). Binds an activating receptor in NK cells and costimulatory molecules in T cells.
XIAP deficiency (XLP2)	*XIAP/BIRC4*	Encodes the X-linked inhibitor of apoptosis (XIAP) protein.
Familial HLH (fHLH) with hypopigmentation		
Chediak-Higashi syndrome	*LYST*	Encodes the lysosomal trafficking regulator (LYST) protein; involved

		in lysosomal trafficking.
Griscelli syndrome, type 2	*RAB27A*	Encodes a small GTPase that regulates vesicular trafficking and membrane fusion, specifically regulating the docking of cytolytic granules to the plasma membrane.
Hermansky-Pudlak syndrome, type 2	*AP3B1*	Encodes the b3A subunit of the adaptor protein 3 complex; regulates protein trafficking from the Golgi apparatus to lysosomes.
Immunodeficiency		
CD27 deficiency	*CD27 (TNFRSF7)*	Encodes a receptor protein belonging to the Traf-linked tumor necrosis factor receptor family; T cell costimulatory molecule required for long-term maintenance of T cell immunity.

Table 1-2. Immune Dysregulation and Immunodeficiencies that cause familial HLH.
Table adapted by author from (Brisse et al., 2015a, 2016a; Côte et al., 2009).

TRIGGERING FACTORS AND PREDISPOSING DISEASES	EXAMPLES
Infections	
Viral	Epstein Barr-virus; Human Immunodeficiency virus (HIV); Cytomegalovirus
Bacterial	*Salmonella enterica* Typhimurium; *Mycobacterium tuberculosis*; *Rickettsia* spp.
Parasites	*Toxoplasma* spp.; *Leishmania* spp.; *Plasmodium* spp.
Fungi	*Histoplasma* spp.

Malignancy	Lymphoma, Leukemia, Other Neoplasms
Autoimmune/Autoinflammatory Disorders Macrophage Activation Syndrome (MAS)	Systemic lupus erythematosus Systemic onset-juvenile idiopathic arthritis

Table 1-3. Acquired HLH and Associated Triggers.
Table adapted by author from (Brisse et al., 2015b; Ramos-Casals et al., 2014).

Diagnostic Criteria*	Lab Results
Fever	
Splenomegaly	
Cytopenias (affecting at least 2 of 3 lineages in peripheral blood)	Hemoglobin < 90 g/L (infants < 4 weeks: hemoglobin < 100 g/L) Platelets < 100 x 10^9/L Neutrophils < 1.0 x 10^9/L
Hypertriglyceridemia and/or Hypofibrinogenemia	Fasting triglycerides $\geq$ 3.0 mmol/L Fibrinogen $\leq$ 1.5 g/L
Hemophagocytosis in bone marrow, spleen, or lymph nodes	If hemophagocytic activity is not conclusively determined at time of clinical presentation, further search for hemophagocytic activity is advised, including serial marrow aspirates over time.
Hyperferritinemia	Ferritin $\geq$ 500 μg/L
High levels of soluble IL-2r	sIL-2r $\geq$ 2,400 U/mL

Table 1-4. Histiocyte Society HLH-2004 Diagnostic Criteria
Patients must meet 5 of the 8 diagnostic criteria to be diagnosed with HLH; patients with a molecular diagnosis consistent with HLH do not need to fulfill the diagnostic criteria. Table adapted by author from (Henter et al., 2007).

Primary HLH is autosomal recessive and is caused by loss of function mutations in genes involved in the development, transportation, or activity of cytotoxic granules. These cytotoxic granules are the basis of the cytotoxic function of Natural Killer (NK) cells and CD8+ T-cells. For example, mice deficient for perforin 1 and infected with lymphocytic choriomeningitis virus develop primary HLH, including hemophagocyte accumulation in the spleen and bone marrow. NK and CD8+ T-cells kill target cells (virally infected or tumorigenic cells) by exocytosis of cytotoxic granules. If target cells are not killed, the immune response continues to expand, promoting inflammation and activating immune cells, such as macrophages and T-cells. This overwhelming and uncontrolled inflammatory response drives the disease pathogenesis (Brisse et al., 2015b).

Acquired HLH is defined as the clinical manifestations of primary HLH in the absence of a known genetic cause. Acquired HLH is due to activation of the immune system, which may be triggered by infection, malignancies, and/or autoimmune disorders. MAS also falls under this definition. There are established mouse models to study infection-associated HLH, autoinflammation-associated HLH, and autoimmunity-associated HLH. The Detweiler lab characterized the first reported animal model for infection-associated secondary HLH using immunocompetent (SV129S6) mice and the bacterium *S. enterica* serotype Typhimurium, a natural host-pathogen relationship (Brown et al., 2010). The SV129S6 mouse line encodes the intact cation transporter Nramp1/Slc11A1; mouse strains that lack this cation transporter (such as C57Bl/6 and BALB/c) succumb to infection with *S.* Typhimurium (and other intracellular parasites) within days (Vidal et al., 1995). Thus, mice infected with *S.* Typhimurium via an oral

route mimic natural typhoid-like infections; acute infections transition into chronic infections. *Salmonella* Typhimurium has been found to reside in HMs as early as one week post-infection to three weeks post-infection, which is diagnosed as chronic infection (Nix et al., 2007). Recently, another animal model for acquired HLH was characterized; immunodeficient, humanized mice infected with Epstein-Barr virus develop 4 of the 8 clinical signs of HLH, including the accumulation of hemophagocytes in the bone marrow, spleen and liver (Sato et al., 2011).

Acquired HLH can also be studied using wild-type animals chronically infected with virus, or stimulated with prolonged exposure to a TLR agonist. The first mouse model to demonstrate MAS/HLH like symptoms is wild-type animals with no known genetic defects that underwent repeated TLR9 stimulation with CpG. This mouse model also demonstrated the protective effect of IL-10; when mice were treated with an antibody to IL-10 receptor and CpG, they developed more severe disease and accumulated hemophagocytes (Behrens et al., 2011). In this model, IFN-γ is required and depletion of lymphocytes and NK cells reduced the severity of the disease, implying that innate immune cells are also responsible for this disease. This work demonstrated that TLR9 activation of innate immune cells is sufficient to initiate an HLH/MAS-like pathological state. Wild type BALB/c mice infected with β-herpesvirus murine CMV develop virus-associated secondary HLH (Brisse et al., 2016b). Injection of a high dose of unmethylated CpG DNA into wild type C57BL/6 mice induces peripheral blood monocyte-derived dendritic cells to hemophagocytose erythrocytes (Ohyagi et al., 2013). In this particular model of hemophagocytosis, CpG injection increased the fraction of phosphatidylserine-positive erythroid cells, and these apoptotic erythroid cells

were recognized and taken up by hemophagocytes via the “eat-me” signal receptors Tim1, Tim4, and $\alpha_v\beta_3$ integrin. In this same model, it was determined that chronic infections of mice with LCMV variant clone 13 induced hemophagocytosis. Prior to my research, the link between TLR agonists and induction of disease was only correlative. Using TLR4-deficient bone marrow-derived macrophages, I showed that TLR4 signaling is required for the induction of hemophagocytosis in our cell culture model.

Even though hemophagocytes are found in inflammatory settings, it is hypothesized that hemophagocytes play an immunoregulatory role. Murine hemophagocytes express anti-inflammatory markers, including scavenger receptor class B1 (also known as CD36), mannose receptor C type 1 (also known as CD206), and signaling lymphocyte activation molecule 1 (SLAMF1; CD150) (McCoy et al., 2012). Hemophagocytes isolated from mice repeatedly stimulated by a TLR9 agonist have a transcriptional profile similar to that of alternatively activated macrophages (Canna et al., 2014). LCMV infection of mice induces the formation of hemophagocytic dendritic cells, which are the main producers of the anti-inflammatory cytokine IL-10 *in vivo* (Ohyagi et al., 2013). Furthermore, this study identified a functional role for HMs during LCMV infection; blocking hemophagocyte formation during infection, or inhibiting the production of IL-10 by hemophagocytes, decreased survival rates of LCMV-infected mice, most likely due to excessive inflammatory responses (Ohyagi et al., 2013).

Despite research into HLH and other hyperinflammatory syndromes, an understanding of the mechanism(s) by which macrophages become hemophagocytic during *Salmonella* infection is not known. Furthermore, it is not clear if similar mechanisms drive the formation of hemophagocytes in the different disease states, and

it is unknown if hemophagocytes perform the same function(s) in the various hemophagocytic syndromes. For example, the onset for primary HLH is from infancy to early childhood and is usually fatal. The only curative treatment is a bone marrow transplant. The onset of secondary HLH usually occurs much later in life and is treated by ameliorating the inflammatory response (Das et al., 2016; Wang et al., 2015). Thus, macrophage activation may have completely different outcomes in primary and secondary HLH.

Delivery of IFN-γ to the intraperitoneal cavity of wild-type C57/BL6 mice results in hemophagocyte accumulation in the spleen, leading to the suggestion that IFN-γ stimulates hemophagocytosis (Zoller et al., 2011). In contrast, work done by Brisse et al. demonstrates that IFN-γ most likely plays a regulatory role in secondary HLH, as opposed to the pathogenic role it is attributed in primary HLH (Brisse et al., 2016b). In LCMV chronically infected mice, type I interferons are important for the induction of hemophagocytosis; in this model, interferon-α receptor 1 knockout mice have a reduction in hemophagocytes after LCMV infection (Ohyagi et al., 2013). Lastly, although TLR ligands have been used to induce HLH models in mice, or other macrophage activation syndromes (like MAS or sJIA), the direct role of TLR signaling has not been examined.

In discussing hemophagocytes, I will focus on the model pathogen *Salmonella enterica* serotype Typhimurium. *S*. Typhimurium is related to *S*. *Typhi*, the causative agent of Typhoid Fever. The connection between *S*. *Typhi* and hemophagocytes has been known since the early 19th century, when Frank Mallory, a renowned pathologist at Boston City Hospital, first described these cells after performing autopsies on typhoid

fever patients – “They are much dilated and filled with large phagocytic cells containing principally red blood globules, usually in very large numbers, but also lymphoid and plasma cells and polymorphonuclear leucocytes” (Mallory, 1898). As detailed above, immunocompetent wild-type mice infected with S. Typhimurium develop the clinicopathological features of acquired HLH, making this a valid model to study hemophagocytes (Brown et al., 2010).

III. *Salmonella* Pathogenesis

Salmonella enterica subspecies are bacterial pathogens responsible for Typhoid fever, gastroenteritis, and both acute and chronic infection of the spleen and liver. These pathogens remain a significant worldwide threat to human health, particularly to children, the elderly and the immunocompromised. In the most current census, there are 21 million cases of typhoid every year and 216,000 deaths per annum from typhoid fever (Crump et al., 2004). In addition, the number of multi-drug resistant strains of *Salmonella* continues to increase (Glynn et al., 1998; Holt et al., 2011; Kingsley et al., 2009). *Salmonella* infection is acquired orally through contaminated food and/or water. *Salmonella* survive the acidic stomach and subsequently breach the intestinal epithelium via a Type III Secretion System (T3SS), an “injection apparatus” that exports bacterial proteins into the host cell cytoplasm (Hansen-Wester and Hensel, 2001). These proteins modulate the host response, including inducing host cells, such as epithelial cells, to engulf the bacteria, which promotes intracellular survival of the bacteria. Once phagocytosed, *Salmonella* modifies the phagosome, making it a *Salmonella*-containing vacuole (SCV). In order to survive, *Salmonella* must regulate lysosome-phagosome fusion, through secreted effectors. Many of the secreted

Salmonella effectors are proteins that interact with and regulate host Rab GTPases. Rab proteins are important mediators of numerous fusion events, including endosomal vesicle formation and trafficking. *Salmonella* controls these fusion events through specific effectors (Stein et al., 2003). *Salmonella* preferentially survive and propagate in specialized intestinal epithelial cells (M cells and Peyer's patches), leading to the induction of a local immune response (Jones et al., 1994). In response to inflammation in the intestine, the host initiates a systemic immune response. Phagocytic white blood cells (monocytes, macrophages and dendritic cells) migrate into the small intestine. Once localized to the infected area, these phagocytic white blood cells engulf the bacterium and deliver it to the lymph nodes that drain the intestine, the spleen, and the liver, where *Salmonella* survives within macrophages and *Salmonella*-infected macrophages disseminate in these tissues (Buchmeier and Heffron, 1989; Monack et al., 2004; Tsolis et al., 2011).

IV. Synopsis of Results

In the current work, I explore the mechanism that induces macrophages to hemophagocytose. I report that prolonged exposure to bacterial molecular structures is key to stimulating hemophagocytosis. Exposure to either evolutionarily diverse heat-killed bacteria or single microbial-associated molecular patterns (MAMPs) stimulated macrophages to hemophagocytose. Heat-killed Gram-negative bacteria and LPS, a MAMP from Gram-negative bacteria, induced the greatest fraction of hemophagocytes. In these studies, I also discovered a difference between resting and IFN-γ-pre-treated macrophages. In resting macrophages, LPS and Gram-negative bacteria were more potent stimulators of hemophagocytosis than LTA or Gram-positive bacteria. This is

consistent with reports of TLR4 being more highly expressed by resting macrophages compared to TLR2 and TLR9 expression. I established a requirement for TLR signaling by demonstrating that LPS and S. Typhimurium induce hemophagocytosis via TLR4. Macrophages were treated with a chemical inhibitor of TLR4 signaling, TAK242, which prevents MyD88-dependent and MyD88-independent signaling. In both resting and IFN-γ-pretreated macrophages, treatment with TAK242 inhibited LPS- and bacterium-induced hemophagocytosis. To confirm a role for TLR4 in hemophagocyte formation, I compared TLR4-/- and TLR4+/+ macrophages; LPS treatment or infection with live *S*. Typhimurium stimulated hemophagocyte formation only in the presence of TLR4. Thus, TLR4 is required for hemophagocytosis in response to LPS or *S*. Typhimurium, and LPS appears to be the major constituent of *S*. Typhimurium that stimulates hemophagocytosis. Furthermore, TLR4 activation via exposure to *Salmonella* LPS induced hemophagocytosis of Jurkats (white blood cells). *Brucella abortus*, a Gram negative pathogen with LPS that lacks a marked pathogen-associated molecular pattern (Conde-Álvarez et al., 2012; Park et al., 2009), did not induce hemophagocyte formation. This supports the observation that robust activation of TLR4 is critical for the induction of hemophagocytosis in response to infection with Gram-negative pathogens. Upon examination of signaling pathways downstream of TLR4, I found that NF-κB activation is required; BMS-345541, a specific inhibitor of NF-κB, prevented *S*. Typhimurium and LPS-induced hemophagocytosis.

In this thesis, I demonstrate that IFN-γ is not required in our model of *S*. Typhimurium-induced hemophagocytosis, although pre-treatment with IFN-γ does increase the overall fraction of hemophagocytes. Furthermore, IFN-γ was required to

induce a macrophage dose response to erythrocytes. Thus, IFN-γ may drive expression of a receptor, or increase sensitivity of a receptor for its ligand, leading to enhanced erythrocyte uptake.

In our cell model of hemophagocytosis, repeated exposure to TLR agonists decreased the formation of hemophagocytes, with the most dramatic reduction being found in the IFN-γ-treated population. These results strongly suggest that tolerant macrophages decrease expression of a key receptor or have decreased sensitivity to a key ligand involved in hemophagocytosis.

In addition, I adapted an advanced flow cytometry gating scheme to analyze hemophagocytosis *in vivo*. After *Salmonella* infection, I identified hemophagocytic inflammatory monocytes and resident macrophages in the spleens of infected mice.

These findings lead to a model whereby TLR signaling and subsequent NFκ-B activation induce macrophages to hemophagocytose.

Chapter 2 Bacterial Stimulation of Toll-Like Receptor 4 Drives Macrophages to Hemophagocytose

I. Introduction

Chronic infections cause significant damage to the host. Therefore, the host must balance tissue damage and pathogen load. In the early 1970s, plant biologists coined the term “tolerance,” the endurance of a severe disease without loss in yield or quality (Caldwell et al., 1958; Schafer, 1971). This concept has recently been applied to mammalian-pathogen interactions, whereby disease tolerance enables a host to survive infection by reducing or limiting disease severity without eliminating the pathogen (Ayres and Schneider, 2012).

Hemophagocytes are a specialized class of macrophage that have engulfed platelets, erythrocytes and leukocytes. Hemophagocytes express anti-inflammatory markers and accumulate in patients with severe systemic infections with pathogens such as *Salmonella enterica*, *Mycobacterium tuberculosis*, Epstein-Barr and mumps viruses, and species of the malarial parasite *Plasmodium* (Brisse et al., 2015b). Hemophagocyte accumulation is also associated with inflammatory diseases, including sepsis, hemophagocytic lymphohistiocytosis (HLH), macrophage activation syndrome, and systemic inflammatory response syndrome (Castillo and Carcillo, 2009). Although hemophagocytes from human typhoid victims were described over a century ago, these cells remain poorly understood (Mallory, 1898).

The mechanism(s) that trigger hemophagocyte formation are unknown. Hemophagocytes have been studied in several different mouse models of HLH, a human disease syndrome triggered by infection and characterized by fever,

splenomegaly, cytopenias, hemophagocytosis in bone marrow and spleen, hyperferritinemia, and hypofibrinogenemia (Brisse et al., 2015b; Henter et al., 2007). HLH is categorized as either primary HLH or secondary HLH. Primary HLH is the outcome of loss-of-function mutations that inhibit killing of target cells by lymphocytes, thus leading to an overwhelming production of interferon (IFN-γ), which activates T-cells and macrophages. Mice deficient for perforin 1 and infected with lymphocytic choriomeningitis virus develop HLH, including hemophagocyte accumulation in the spleen and bone marrow (Jordan et al., 2004). In contrast, infection, autoinflammatory or autoimmune diseases, or malignant diseases trigger secondary HLH. Hemophagocytes also accumulate in immunodeficient, humanized mice infected with Epstein-Barr virus (Sato et al., 2011). The common factor amongst these HLH models is an underlying severe inflammatory condition. Further studies have demonstrated that the driving cytokines differ between primary and secondary HLH. Delivery of IFN-γ to the intraperitoneal cavity of wild-type mice results in hemophagocyte accumulation in the spleen, leading to the suggestion that IFN-γ is sufficient to stimulate hemophagocytosis (Brisse et al., 2015b; Brown and Neher, 2012; Canna and Behrens, 2012; Cnops et al., 2015; Pachlopnik Schmid et al., 2009; Zoller et al., 2011). Yet, in secondary HLH, IFN-γ may play an immunoregulatory role, as IFN-γ-deficient mice developed a more severe HLH-like disease after cytomegalovirus infection (Brisse et al., 2016b).

TLR agonists have been used to induce an HLH-like state in mice, but whether TLRs are required for this condition was unknown. TLRs are pattern recognition receptors expressed by many kinds of cells. TLRs recognize structurally conserved

microbial ligands, such as bacterial cell wall components, bacterial DNA, and viral RNA. Macrophages become microbicidal when TLRs bind to these or other microbial associated molecular patterns (MAMPs). TLR activation also stimulates macrophages to secrete proinflammatory cytokines and danger-associated molecules, such as IFN-γ and HMGB1, respectively. Left unchecked, inflammation causes significant host damage. However, macrophages have the potential to tip the balance in favor of tolerance; these same cells produce anti-inflammatory cytokines and also products that heal wounds and repair tissue (Koh and DiPietro, 2011). TLR activation contributes to the transition from inflammatory to regulatory macrophage. For instance, TLR activation followed by CD39 activation results in macrophage anti-inflammatory cytokine production (Cohen et al., 2013), and TLR signaling leads to the production of miRNAs that negatively regulate TLRs (Liu et al., 2009).

To study hemophagocytes in a natural host-pathogen relationship, I used immunocompetent (SV129S6) mice and the bacterium *S. enterica* serotype Typhimurium (Brown et al., 2010). *S*. Typhimurium infection of mice via an oral route elicits monocyte infiltration into the small intestine. Monocytes engulf the bacterium and deliver it to the lymph nodes that drain the intestine, the spleen, and the liver, where *S*. Typhimurium survives within macrophages (Tsolis et al., 2011). Macrophages that harbor the bacteria include hemophagocytic macrophages, which accumulate in the liver and spleen as early as 1 week after inoculation (McCoy et al., 2012; Nix et al., 2007). During the acute stage of infection, these mice develop clinical signs consistent with human HLH, in addition to modeling human typhoid fever (Brown et al., 2010; Ruby et al., 2012; Tsolis et al., 2011). Approximately 3 to 5% of total splenic macrophages are

hemophagocytic at 4 weeks post inoculation, based on increased DNA content (flow cytometric analysis of DAPI (2-(4-amidinophenyl)-1H-indole-6-carboxamidine)-stained macrophages) (McCoy et al., 2012). Confocal microscopy identified *S*. Typhimurium within hemophagocytes in the liver from 1 to 8 weeks post infection (Nix et al., 2007). These cells may be a survival niche for *S*. Typhimurium by providing the bacteria with iron or other cell break-down products (Nagy et al., 2014). In addition, hemophagocytes appear to have an alternatively activated phenotype (Brisse et al., 2015b; Canna et al., 2014; McCoy et al., 2012), suggesting that they are less adept at killing pathogens than other kinds of macrophages (Benoit et al., 2008).

The Detweiler lab recently reported that in cell culture, treatment with IFN-γ is not sufficient to drive macrophages to hemophagocytose (Pilonieta et al., 2014). Here, I address the molecular events behind bacterium-driven hemophagocytosis. I found that prolonged exposure to heat-killed bacteria from diverse phyla, or to purified MAMPs was sufficient to promote hemophagocytosis. Inhibition of TLR4 activation prevented purified lipopolysaccharide (LPS) and infection with live *S*. Typhimurium from stimulating hemophagocytosis. These observations indicate that hemophagocytosis is an evolutionarily conserved response to microbial products and potentially outlines a role for hemophagocytes in disease tolerance.

II. Results

Prolonged macrophage exposure to heat-killed *S*. Typhimurium stimulates hemophagocytosis.

Infection of macrophages with *S*. Typhimurium, but not with *E. coli*, stimulates hemophagocytosis (Pilonieta et al., 2014). However, the *E. coli* strain tested was a

laboratory strain that macrophages kill within 2 hours (h) (Silva-Herzog and Detweiler, 2010). I hypothesized that prolonged exposure to bacteria is key to stimulating hemophagocytosis, and that *E. coli* does not provide enough stimulation within the 2 h to promote hemophagocytosis. Hemophagocytosis was monitored by a flow cytometry intracellular staining protocol; macrophages that engulfed erythrocytes stain positive for the erythrocyte marker TER119 (Fig 2-1) (Chen et al., 2009; Nagy et al., 2014). Infection of macrophages with live *S*. Typhimurium was compared to continuous exposure to heat-killed *S*. Typhimurium over 18 h, and both conditions stimulated significant hemophagocytosis (Fig 2-2). IFN-γ-pre-treatment alone does not appear to enhance erythrocyte uptake. Thus, prolonged exposure, i.e., $\geq$12 h, to either live or dead bacteria stimulates hemophagocytosis.

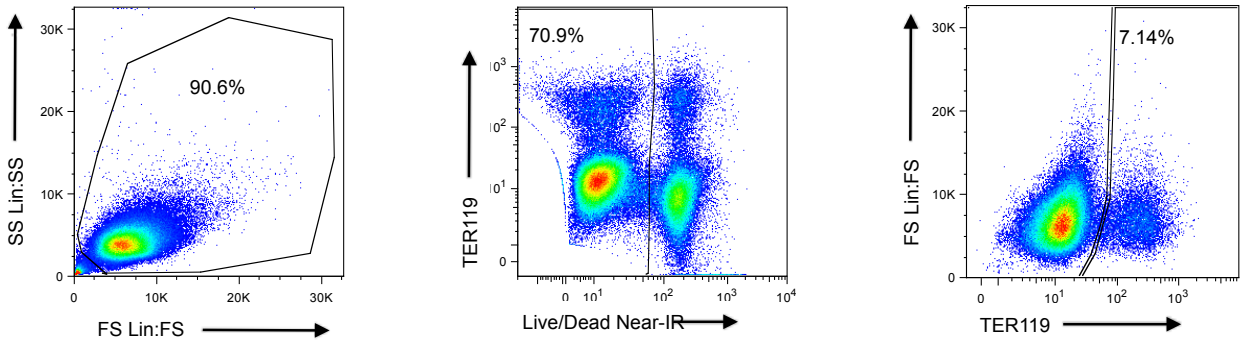


Figure 2-1. Flow cytometry gating scheme used to identify hemophagocytes *in vitro*

Using side scatter versus forward scatter, debris was removed from analysis. Live cells were then discriminated based on lack of Live/Dead Near-IR staining. Finally, TER119^{+} hemophagocytes were identified. A minimum of 10,000 live cells was collected for final analysis.

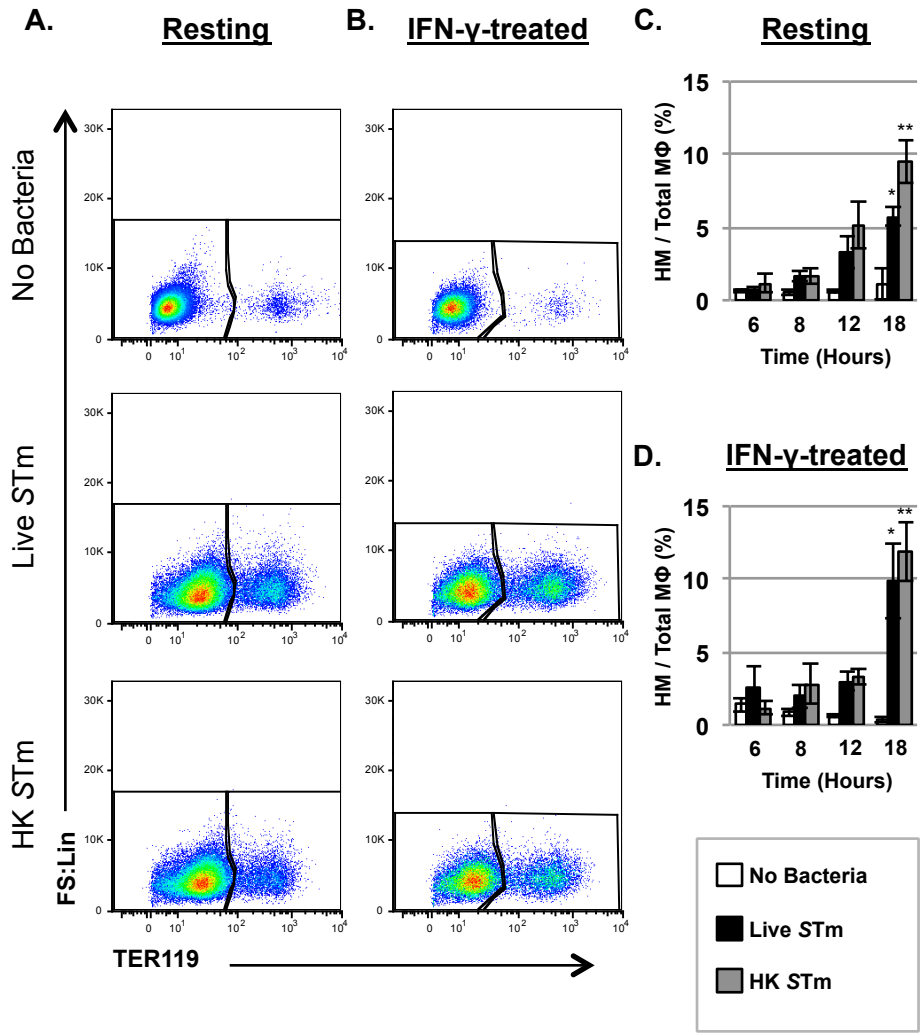


Figure 2-2. Infection with live *S*. Typhimurium (*S*Tm) or prolonged exposure to heat-killed (HK) *S*Tm stimulates macrophages to hemophagocytose
Resting **(A and C)** or IFN-γ-pretreated **(B and D)** macrophages were cocultured with erythrocytes at a 1:1 ratio in the absence of bacteria or with *S*Tm (MOI of 10), or HK *S*Tm (MOI of 10). At the times indicated, extracellular erythrocytes were lysed, and macrophages were processed for intracellular flow cytometry. **(A and B)** Representative gating of TER119- and TER119+ macrophages at 18 h post exposure to live or HK *S*Tm. **(C and D)** Mean percentage of TER119+ macrophages at the indicated time points (□, without bacteria; ■, with live STm; ■, with HK STm). Means ± the standard errors of the mean (SEM) of aggregated data were determined from three biological replicates. P values were determined by ANOVA with a Dunnett's posttest. **, $P \leq 0.0001$; * , $P \leq 0.05$ (compared to no bacteria at 18 h); ns, not significant. HM, hemophagocytes.

The fraction of macrophages that hemophagocytose saturates with increasing infectious dose.

I then exposed resting and IFN-γ-pretreated macrophages to a 10-fold dilution series of *S*. Typhimurium to establish whether hemophagocytosis is responsive to bacterial dose. At a multiplicity of infection (MOI) of 100, the macrophage monolayer was largely destroyed within 18 h, and therefore no other data were collected. In the remaining samples, hemophagocytosis and infection were monitored by flow cytometry. The percentage of resting or IFN-γ-pretreated macrophages that became hemophagocytic increased and then leveled off at an MOI of 1.0, at approximately 10 to 15% of the total macrophages. This observation suggests that only a fraction of macrophages has the capacity to hemophagocytose under these conditions (Fig 2-3). In contrast, the percentage of macrophages that became infected and the total CFU both rose with increasing MOI (Fig 2-3). At 18 h post infection, non-hemophagocytes were as likely as hemophagocytes to be infected at all bacterial dosages tested (Fig 2-3), a finding consistent with engulfment of erythrocytes after prolonged infection (Fig 2-2). Altogether, bacterium-driven hemophagocytosis appears to be dose responsive and saturable, suggesting the process requires recognition of a bacterial ligand(s) by macrophage receptors.

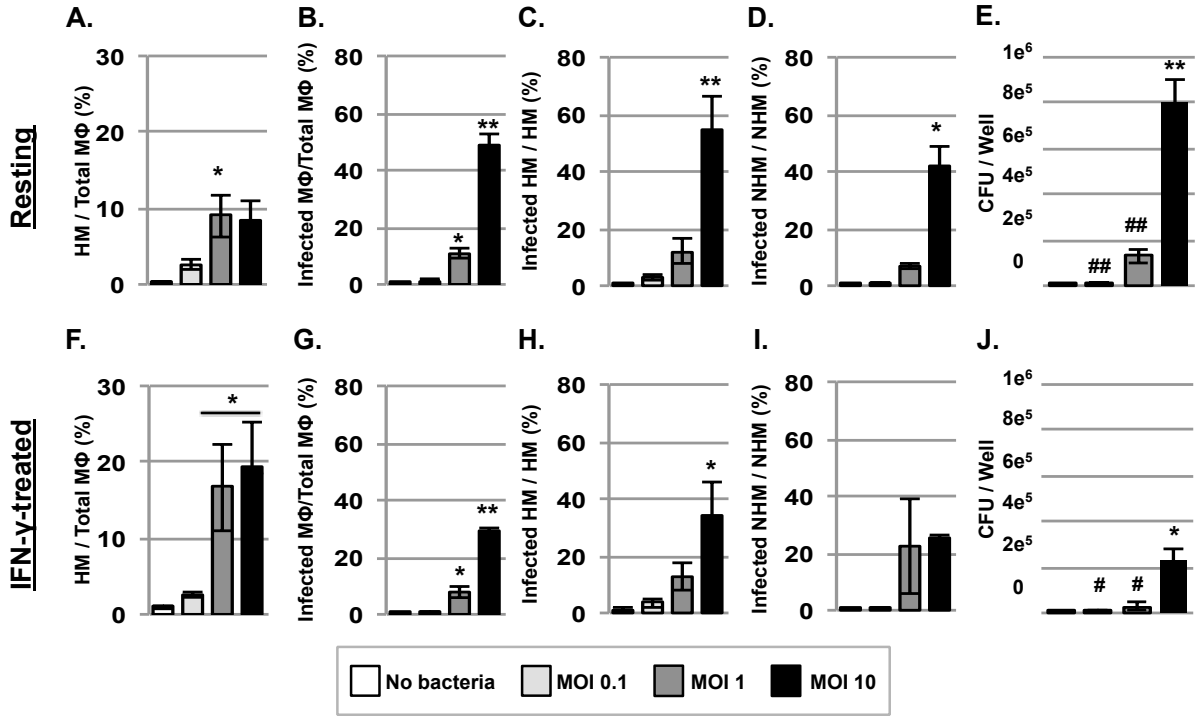


Figure 2-3. The fraction of macrophages that hemophagocytose saturates in response to increasing bacterial dose

Resting **(A to E)** or IFN-γ-treated **(F to J)** macrophages were cocultured with erythrocytes at a ratio of 1:10 without bacteria (□), or with live, GFP-expressing *S*Tm at MOIs of 0.1 (□), 1 (■), or 10 (■) for 18 h. Extracellular erythrocytes were then lysed and macrophages processed for flow cytometry. **(A and F)** Mean percentage of TER119+ macrophages (hemophagocytes). **(B and G)** Mean percentage of GFP-expressing *S*Tm-infected macrophages. **(C and H)** Mean percentage of GFP-expressing *S*Tm-infected hemophagocytes (out of total hemophagocytes). **(D and I)** Mean percentage of GFP-expressing *S*Tm-infected nonhemophagocytes (out of total nonhemophagocytes). **(E and J)** CFU/well. Means ± the SEM of aggregated data were determined from three biological replicates. P values were determined by ANOVA with a Dunnett's posttest. **, $P \leq 0.0001$; *, $P \leq 0.05$ (compared to no bacteria). ##, $P \leq 0.0001$; #, $P \leq 0.05$ (compared to an MOI of 10).

Evolutionarily diverse bacteria stimulate hemophagocytosis and reveal differences between resting and IFN-γ-treated macrophages.

To establish whether bacteria outside the *Enterobacteriaceae* family stimulate hemophagocytosis, I tested heat-killed *Bacillus subtilis* (a representative Gram-positive bacterium) and *Mycobacterium marinum*. In both resting and IFN-γ-pretreated macrophages, heat-killed Gram-negative bacterial species promoted the largest fraction of macrophages to hemophagocytose, followed by *M. marinum* (Fig 2-4). Heat-killed *B. subtilis* stimulated hemophagocytosis only in the IFN-γ-pretreated macrophages. The stimulation of hemophagocytosis by bacteria from distinct phyla mirrors observations in humans with severe infections caused by diverse pathogens (Fisman, 2000).

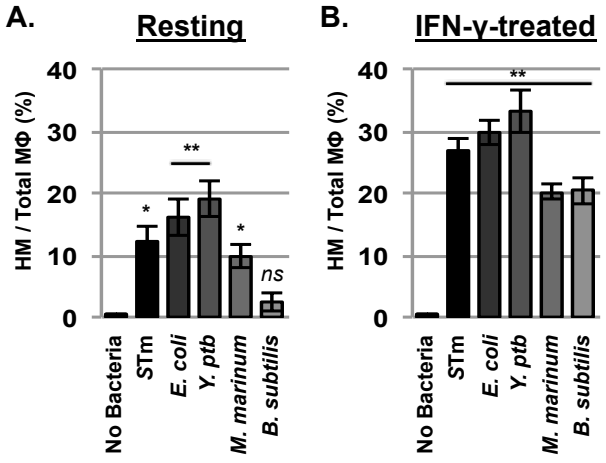


Figure 2-4. Prolonged macrophage exposure to HK bacteria from different phyla stimulates hemophagocytosis

Resting **(A)** or IFN-γ-pretreated **(B)** macrophages were cocultured with erythrocytes at a ratio of 1:10 without bacteria or with HK bacteria (MOI of 10). After 18 h, macrophages were processed for flow cytometry. Means ± the SEM of aggregated data were determined from three biological replicates. P values were determined by ANOVA with a Dunnett's posttest. **, $P \leq 0.0001$; *, $P \leq 0.05$; (compared to no bacteria). ns, not significant (compared to no bacteria).

TLR4, TLR2, or TLR9 agonists induce hemophagocytosis.

TLRs are key macrophage receptors for recognition of bacterial ligands. I hypothesized that whole heat-killed bacteria induced hemophagocytosis via TLR activation and that a single MAMP is sufficient. Macrophages were exposed to increasing doses of purified *S*. Typhimurium LPS, which activates TLR4 (Hoshino et al., 1999). LPS induced hemophagocytosis in a dose-dependent manner in resting and IFN-γ-pretreated macrophages (Fig 2-5). To determine whether the duration of macrophage exposure to LPS affects whether or not hemophagocytosis occurs, I incubated macrophages with LPS and erythrocytes for 2, 4, 8, or 18 h. At the end of each interval, macrophages were washed and then re-exposed to only erythrocytes for the remainder of the experiment, and hemophagocytes were quantified by flow cytometry at 18 h. The percentage of hemophagocytes increased with the length of exposure to LPS, particularly in IFN-γ-pretreated macrophages (Fig 2-5). Thus, 2 h of exposure to purified LPS induces hemophagocytosis in IFN-γ-pretreated macrophages, but longer exposure times are needed for resting macrophages.

I also examined the ability of purified LTA from *Staphylococcus aureus* (a TLR2 agonist), class B CpG oligodeoxynucleotides (a TLR9 agonist), poly(I·C) (a TLR3 agonist), and purified *S*. Typhimurium flagellin (a TLR5 agonist) to stimulate hemophagocytosis. Each agonist was tested at concentrations known to stimulate TLRs on macrophages (Arpaia et al., 2011; Hong et al., 2014; Józefowski et al., 2006; Sheikh et al., 2014; Yang et al., 2012). Flagellin did not stimulate hemophagocytosis, which is consistent with the lack of TLR5 activity in murine macrophages (Feuillet et al., 2006). Poly(I·C) also did not stimulate hemophagocytosis despite reported TLR3 expression by

mouse macrophages (Alexopoulou et al., 2001). However, prolonged exposure to LTA or CpG significantly increased the fraction of hemophagocytes, but only in IFN-γ-pretreated macrophages (Fig 2-5). These data are consistent with observed increases in expression of TLRs in IFN-γ-pretreated compared to resting macrophages (Arpaia et al., 2011; Spiller et al., 2008). Thus, macrophages may be more likely to hemophagocytose as acute infection develops and proinflammatory cytokines stimulate increased TLR expression.

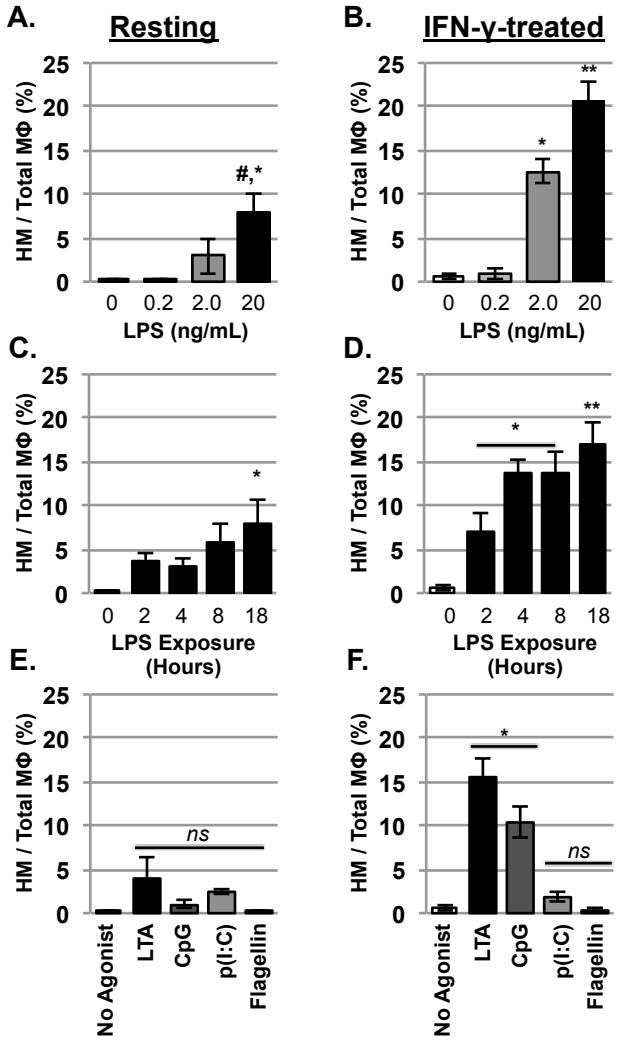


Figure 2-5. Select TLR agonists induce hemophagocytosis
Resting **(A, C, and E)** or IFN-γ-treated **(B, D, and F)** macrophages were cocultured with erythrocytes at a ratio of 1:10 with or without a TLR agonist as indicated. After 18 h, macrophages were processed for flow cytometry. **(A and B)** LPS titration. **(C and D)** Samples were incubated with 20 ng of LPS/ml for the period of time indicated. **(E and F)** 0.01 mg/ml LTA, 1 μM CpG, 10 μg/ml poly(I·C), or 0.05 μg/ml flagellin. Means ± the SEM of aggregated data were determined from three biological replicates. P values were determined by ANOVA with a Tukey's (A and C) or Dunnett's **(B, D, E, and F)** posttest. **, $P \leq 0.0001$; *, $P \leq 0.05$ (compared to 0 ng/ml LPS or 0 h of LPS exposure time). #, $P \leq 0.05$ (compared to 0.2 ng/ml LPS). For panels **A** and **E** and panels **B** and **F**, the 0-ng/ml and 0-h data are from the same experiment. ns, not significant (compared to no agonist).

***Salmonella* LPS induces macrophages to hemophagocytose Jurkats.**

Given that LPS is potent enough to induce hemophagocytosis of erythrocytes, I asked if TLR4 activation by LPS would induce uptake of white blood cells (Jurkats; T-cells). This is important, since hemophagocytes *in vivo* engulf a variety of blood cells, including T-cells (McCoy et al., 2012; Nix et al., 2007). To differentiate between engulfed Jurkats versus attached Jurkats, Jurkats were stained with the pH-sensitive dye pHrodo PE. This dye has minimal fluorescence at neutral pH and fluoresces in acidic environments, which allowed tracking of engulfed (versus attached) Jurkats. Similar to hemophagocytosis of erythrocytes, macrophage exposure to LPS significantly increased the fraction of hemophagocytes in both the resting and the IFN-γ-pretreated macrophages (Fig 2-6).

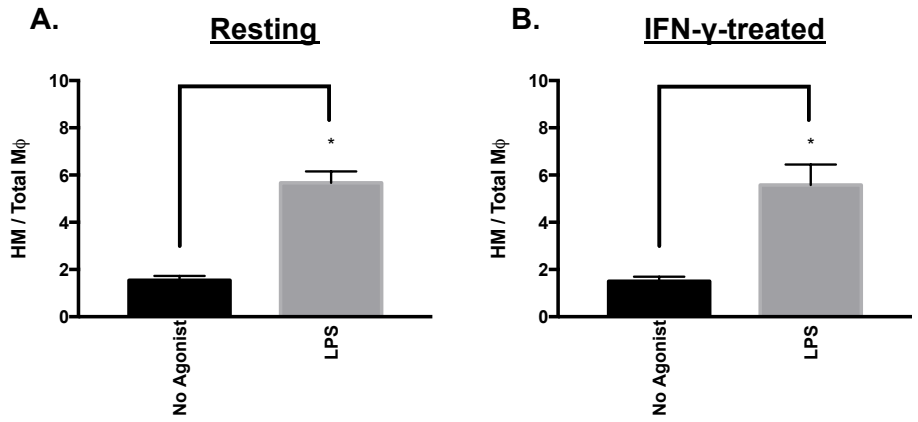


Figure 2-6. Exposure to LPS stimulates macrophages to hemophagocytose T-cells

Jurkats (T-cells) were stained with the pH-sensitive dye pHrodo PE. Resting **(A)** or IFN-γ-treated **(B)** macrophages were co-cultured with pHrodo PE Jurkats at a ratio of 1:1 with or without LPS (20 ng/mL) for 18 h. Macrophages were then processed for flow cytometry. **(A and B)** Mean percentage of pHrodo PE macrophages (hemophagocytes; HMs). Means $\pm$ the SEM of aggregated data from three biological replicates. P values were determined by a Student's t-test. *, $P \leq 0.05$.

TLR4 signaling through NF-κB induces hemophagocytosis

I used chemical and genetic approaches to establish whether LPS and/or *S*. Typhimurium induce macrophage hemophagocytosis via TLR4. Macrophages were treated with a chemical inhibitor of TLR4 signaling, TAK242, which binds the intracellular domain of TLR4 and blocks association with the TIRAP and TRAM adaptor proteins, effectively preventing MyD88-dependent and MyD88-independent signaling (Matsunaga et al., 2011). In both resting and IFN-γ-pretreated macrophages, treatment with TAK242 inhibited LPS-and bacterium-induced hemophagocytosis (Fig 2-7 and Fig 2-8). TAK242 did not affect the percentage of infected macrophages (Fig 2-8) but significantly increased bacterial survival in IFN-γ-pretreated macrophages (Fig 2-8). To confirm a role for TLR4 in hemophagocyte formation, I compared TLR4+/+ and TLR4-/- macrophages; LPS treatment or infection with live *S*. Typhimurium stimulated hemophagocyte formation only in the presence of TLR4. The percentages of *S*. Typhimurium-infected hemophagocytes were similar (Fig 2-9). Thus, TLR4 is required hemophagocytosis in response to LPS or *S*. Typhimurium, and LPS appears to be the major constituent of *S*. Typhimurium that stimulates hemophagocytosis.

TLR4 signaling activates the transcription factors NF-κB and IRF3/7. BMS-345541 is a specific inhibitor of the IKK kinase complex: IκBα sequesters NF-κB in the cytoplasm, until IKK phosphorylates IκBα, which targets it for degradation (Burke et al., 2003). Treatment of macrophages with BMS-345541 prevented LPS-induced hemophagocytosis (Fig 2-10), suggesting that activation of NF-κB is needed to reprogram macrophages to hemophagocytose.

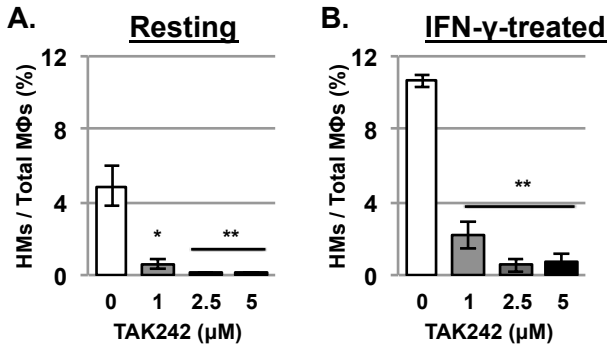


Figure 2-7. Treatment with the TLR4 inhibitor TAK242 blocks LPS-induced hemophagocytosis

Resting **(A)** or IFN-γ-pretreated **(B)** macrophages were treated for 1 h with vehicle (DMSO; □) or TAK242 at 1 μM (□), 2.5 μM (□), or 5 μM (■). After 1 h, erythrocytes were added at a ratio of 1:10, and LPS (20 ng/ml) was added to stimulate hemophagocytosis. After 18 h, macrophages were processed for flow cytometry. **(A and B)** Mean percentages of hemophagocytes. The means ± the SEM of aggregated data were determined from at least three biological replicates. P values were determined by ANOVA with a Tukey's (A) or Dunnett's (B) posttest. **, $P \leq 0.0001$; *, $P \leq 0.05$ (compared to vehicle).

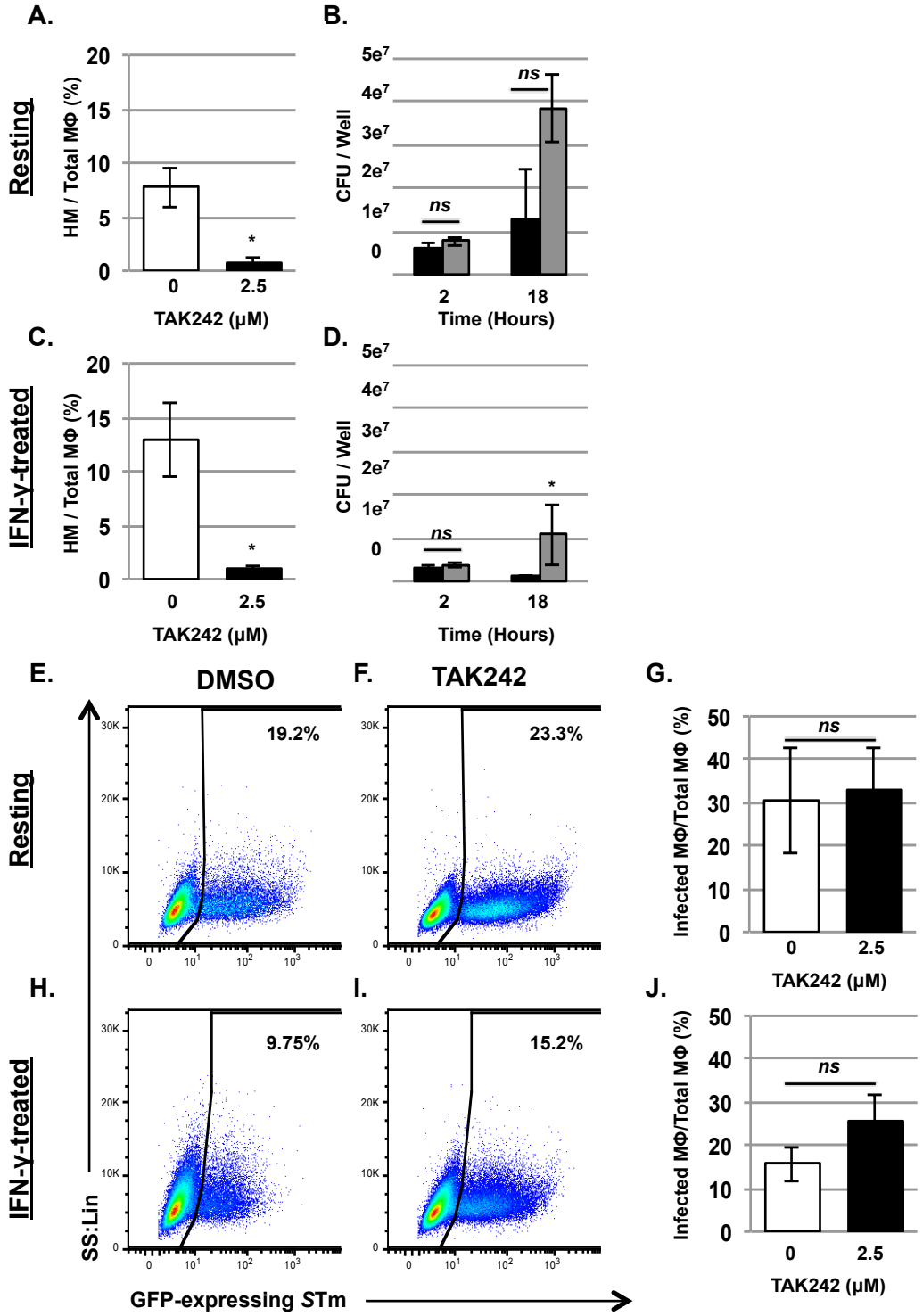


Figure 2-8. Treatment with the TLR4 inhibitor TAK242 blocks *S*Tm-induced hemophagocytosis

Resting **(A, B, E, F, and G)** or IFN-γ-pretreated **(C, D, H, I, and J)** macrophages were treated for 1 h with vehicle (DMSO) or 2.5 μM TAK242. Macrophages were infected with GFP-expressing *S*Tm (MOI of 10) and cocultured with erythrocytes at a ratio of 1:10. After 18 h, macrophages were processed for flow cytometry, and parallel samples were processed for CFU. **(A and C)** Mean percentages of hemophagocytes. **(B and D)**

CFU/well at 2 and 18 h post infection. **(E, F, H, and I)** Representative dot plots showing macrophages infected with *sifB*::GFP *S*Tm. **(G and J)** Mean percentages of infected macrophages from flow cytometry data. Means $\pm$ the SEM of aggregated data were determined from at least three biological replicates. *P* values were determined by ANOVA with a Tukey's test (B and D) or a Student *t* test **(A, C, G, and J)**. **, $P \leq 0.0001$; *, $P \leq 0.05$ (compared to vehicle).

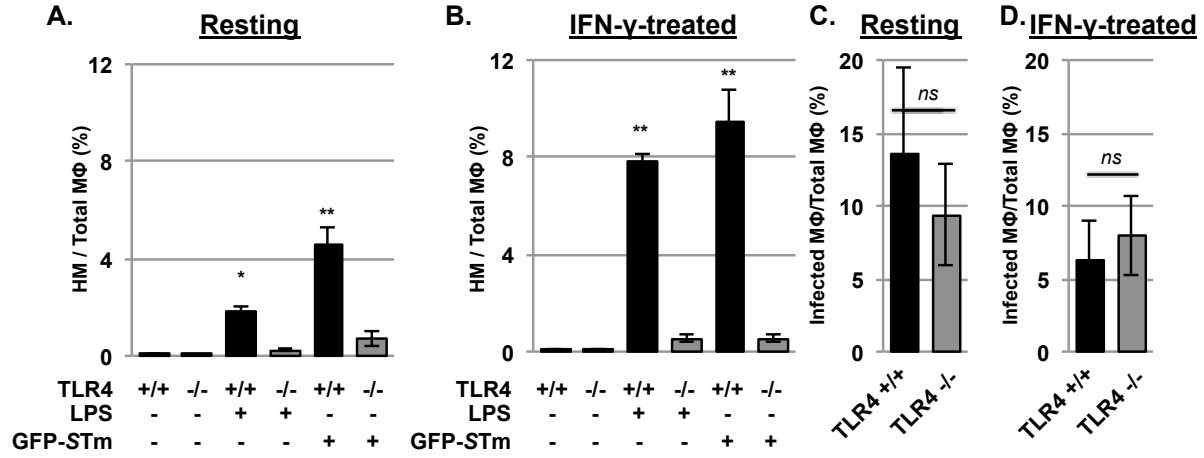


Figure 2-9. LPS or *S*Tm induction of hemophagocytosis is TLR4 dependent

C3H/HeOuJ (*Tlr4*$^{+/+}$) and C3H/HeJ (*Tlr4*$^{-/-}$) macrophages were left resting **(A and C)** or pretreated with IFN-γ **(B and D)** and were cocultured with erythrocytes at a ratio of 1:10 with or without LPS (20 ng/ml) or with or without live *S*Tm (MOI of 10). After 18 h, macrophages were processed for flow cytometry. **(A and B)** Mean percentages of TER119^{+} macrophages. **(C and D)** Mean percentages of GFP-expressing *S*Tm-infected macrophages. Means ± the SEM of aggregated data were determined from three biological replicates. *P* values were determined by ANOVA with a Dunnett's **(A and B)** or a Tukey's **(C and D)** posttest. *, $P \leq 0.05$; **, $P \leq 0.0001$ (compared to TLR4 [+/+], LPS [-], or live *S*Tm [-], as indicated). *ns*, not significant.

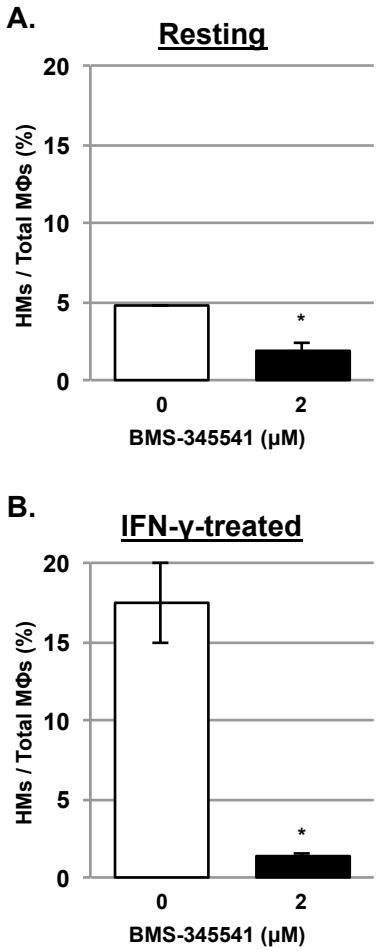


Figure 2-10. Treatment with the NF-κB inhibitor BMS-345541 blocks LPS-induced hemophagocytosis

Resting **(A)** or IFN-γ-pretreated **(B)** macrophages were treated for 1 h with vehicle (DMSO; □) or with 2 μM BMS-345541 (■). After 1 h, the macrophages were cocultured with erythrocytes at a ratio of 1:10, and LPS (20 ng/ml) was added. After 18 h, the macrophages were processed for flow cytometry. Means ± the SEM of aggregated data were determined from at least three biological replicates. *P* values were determined by ANOVA with a Dunnett's posttest. *, $P \leq 0.05$ (compared to vehicle).

III. Discussion

Here I report that TLR signaling and subsequent activation of NF-κB induces macrophages to hemophagocytose erythrocytes. I found that the key to inducing hemophagocytosis was either infection with a pathogen capable of surviving within macrophages, such as *S*. Typhimurium, or prolonged exposure to either heat-killed bacteria or purified MAMPs. The failure of infection with live, nonpathogenic *E. coli* to induce hemophagocytosis (Pilonieta et al., 2014) likely reflects that laboratory strains are killed and digested by macrophages within 2 h of their addition and that the *E. coli* that remained extracellular was washed away (Silva-Herzog and Detweiler, 2010). In contrast, prolonged extracellular exposure to HK *E. coli* may provide sufficient sustained TLR4 stimulation to induce hemophagocytosis. Prolonged exposure to purified MAMPs also promoted hemophagocytosis, increasing the fraction of hemophagocytes by 5- to 20-fold over that observed in the absence of bacteria. The concentrations of MAMPs needed to induce hemophagocytosis are consistent with those that stimulate TLR signaling (Arpaia et al., 2011; Hong et al., 2014; Hoshino et al., 1999; Józefowski et al., 2006). In resting macrophages, LPS and Gram-negative bacteria were more potent stimulators of hemophagocytosis than LTA or Gram-positive bacteria, which is consistent with reports of TLR4 expression by resting macrophages and TLR2 and TLR4 induction upon macrophage activation (Schroder et al., 2004; Spiller et al., 2008). LPS- and *S*. Typhimurium-induced hemophagocytosis requires TLR4, indicating a novel role for TLR signaling in the reprogramming of macrophages to hemophagocytose.

TLR signaling is transmitted through adaptor proteins, and those that signal through MyD88 may be needed to drive hemophagocytosis. TLR4, TLR2, and TLR9 all

stimulate NF-κB through MyD88, whereas TLR3 signaling is MyD88-independent (Jiang et al., 2004), and a TLR3 agonist did not promote hemophagocytosis. NF-κB activates some downstream targets within minutes and other targets within hours. For example, in cultured monocytes and macrophages, HMGB1 does not accumulate in the cytoplasm or in the serum for 4 to 18 h after stimulation with LPS (Gardella et al., 2002; Wang et al., 1999; Youn et al., 2011). Likewise, NF-κB-induced mRNA for IL-12p40 and TNF-α increases within 4 h, but the corresponding proteins require 4 to 16 h to accumulate (Bode et al., 2009; Cohen et al., 2013). NF-κB also regulates miRNAs, the effects of which are complex and may require hours (Zhou et al., 2009). In other words, the requirement for prolonged macrophage exposure to microbial stimuli may reflect the involvement of an NF-κB target that is not rapidly activated.

Hemophagocytosis driven by TLR activation in cell culture is consistent with reports demonstrating stimulation of hemophagocytosis *in vivo* following systemic delivery of TLR agonists in murine models (Brisse et al., 2015b). For example, a single intravenous dose of CpG (200 μg) stimulates hemophagocytosis, and intraperitoneal delivery of 5x10^8 CFU heat-killed *Brucella abortus* stimulates splenic macrophages to bind and engulf erythrocytes (Gardenghi et al., 2014; Ohyagi et al., 2013). In humans and mice, hemophagocytic diseases correlate with high serum proinflammatory cytokines and, in mice, repeated delivery of IFN-γ to the peritoneal cavity in the absence of an infectious agent is sufficient to promote hemophagocytosis in the spleen (Fisman, 2000; Zoller et al., 2011). It has been therefore suggested that IFN-γ drives hemophagocytosis (Brisse et al., 2015b; Brown and Neher, 2012; Canna and Behrens, 2012; Cnops et al., 2015). However, IFN-γ is not required for hemophagocytes to

accumulate in mice or in culture (Canna et al., 2013; Pilonieta et al., 2014). TLR and IFN-γ signaling activate different transcription factors, NF-κB and IRFs versus STAT1, respectively. In addition, IFN-γ increases expression of pathogen recognition receptors, including TLRs, and decreases the threshold concentration of MAMPs required to stimulate TLRs and induce cytokines and microbicidal activities (Costelloe et al., 1999; Schroder et al., 2006). We postulate that IFN-γ indirectly stimulates hemophagocytosis when delivered systemically to mice. IFN-γ activates neutrophils (Amulic et al., 2012; Ellis and Beaman, 2004), which cause bystander tissue damage and the release of danger-associated molecular patterns (DAMPs). DAMPs in turn activate TLRs (Schaefer, 2014) and may thereby drive macrophages to hemophagocytose, even in the absence of infection. Thus, TLR activation via DAMPs may explain why exogenous cytokines are sufficient to drive hemophagocytosis *in vivo*. Whether hemophagocytosis driven by systemic delivery of IFN-γ requires TLRs and NF-κB-dependent gene expression remains to be seen.

Animals from bony fish to humans accumulate hemophagocytes in response to severe infections caused by diverse microbes (Brisse et al., 2015b; MacWilliams et al., 2007). These observations suggest that the process of hemophagocytosis has been conserved and may be of benefit to the host. We speculate that hemophagocytes contribute to the suppression of inflammation that is needed to recover from acute infection and are a mechanism of disease tolerance. Once under way, an inflammatory response has the potential to overwhelm and kill the host regardless of whether an invading pathogen is containable. Therefore, the suppression of inflammation is hard-wired into the inflammatory response (Mosser and Edwards, 2008), and

hemophagocytosis may represent an additional mechanism of immunosuppression. Hemophagocytes express anti-inflammatory markers and produce the anti-inflammatory cytokine interleukin-10 (IL-10) (Brisse et al., 2015b; Ohyagi et al., 2013). In addition, hemophagocytes may also contribute to tolerance via the breakdown of heme from erythrocytes, since both carbon monoxide and biliverdin have anti-inflammatory properties (Mosser and Edwards, 2008; Otterbein et al., 2000). In our cell culture system, we consistently observe that only a fraction (5 to 30%) of macrophages hemophagocytose under the conditions tested, suggesting that not all macrophages have this capability. Altogether, the data suggest that a subset of macrophages is triggered by TLR signaling to become hemophagocytes, cells that may contribute to the suppression of inflammation essential for recovery from immune mediated damage.

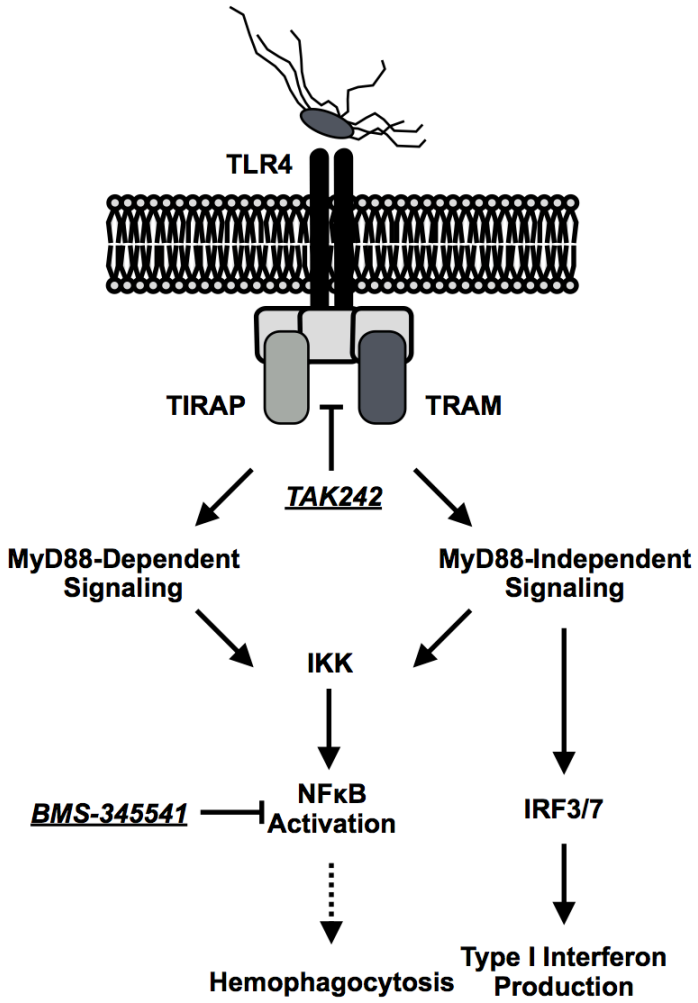


Figure 2-11. Predicted pathway of TLR4-induced hemophagocytosis, including the point-of-action of inhibitors used in the present study

IV. Materials and Methods

Bacterial strains and growth conditions

Salmonella enterica subsp. *enterica* serovar Typhimurium wild-type strain SL1344 transformed with pRFPTag (ESH829, Figures 1, 2 and 4) (Pilonieta et al., 2014), or transduced with GFP and a Kanamycin resistance cassette at *sifB* (*sifB*::GFP-Kan STm, CSD1021) (Barat et al., 2012). *S*. Typhimurium was grown overnight at 37°C in Luria-Bertani broth with aeration. Antibiotics were used at the following concentrations: streptomycin, 30 μg/mL, ampicillin, 300 μg/mL and kanamycin, 30 μg/mL. There were no significant differences in the fraction of hemophagocytes formed in the TER119 median fluorescence intensity (MFI) of hemophagocytes after infection with ESH829 or CSD1021 ($p > 0.05$).

For preparation of heat-killed bacteria, bacteria were grown overnight and then OD600 was recorded. The bacteria were washed twice in 1X PBS and then resuspended in 1 mL of PBS and boiled in a beaker of water for 30-45 minutes. After boiling, a small aliquot of the heat-killed culture was streaked out onto the appropriate media and incubated overnight to ensure complete killing had occurred.

Bone Marrow-Derived Macrophage (BMDM) generation

The University of Colorado Institutional Committee for Animal Care and Use approved research protocols. Bone marrow derived macrophages (BMDMs) were derived using 30% 3T3-MCSF cell-conditioned supernatant as previously described (Pilonieta et al., 2014). Briefly, marrow was flushed from the femurs and tibias of 2- to 4-month-old 129SvEvTac mice (Taconic Laboratories), C3H/HEJ ($TLR4^{-/-}$), or C3H/HeOuJ ($TLR4^{+/+}$) mice (Jackson Laboratories). Cells were resuspended in 1X Phosphate

Buffered Saline (PBS) and penicillin-streptomycin (50 IU/mL penicillin and 50 μg/mL streptomycin). Cells were overlaid onto an equal volume of Histopaque-1083 (Sigma-Aldrich) and centrifuged at 500 x g for 20 min. Monocytes at the interface were harvested, washed twice in 1X PBS and penicillin-streptomycin, resuspended in Dulbecco modified Eagle medium (DMEM) (Sigma-Aldrich) supplemented with fetal bovine serum (10%), L-glutamine (2 mM), sodium pyruvate (1 mM), beta-mercaptoethanol (50 μM), HEPES (10 mM), penicillin-streptomycin (50 IU/mL penicillin and 50 μg/mL streptomycin) and 30% macrophage-colony stimulating factor (M-CSF; to promote differentiation of adherent monocytes into macrophages) supernatant from 3T3 fibroblasts. The bone marrow progenitor cells were seeded at 3 mL and were incubated for 7 days at 37°C in 5% CO_2. The cells were fed at day three with 2 mL of the M-CSF supplemented media described above.

BMDM uptake of erythrocytes and Jurkats

Where indicated, BMDMs were pre-treated with 20 U/mL of IFN-γ for 18-24 hours prior to the addition of erythrocytes, bacteria, and/or purified bacterial molecules. After IFN-γ treatment (or after leaving the cells resting), the fold-replication of the BMDMs was determined: BMDMs were washed, scraped and used to make a 1:10 dilution in trypan blue. These cells were then counted and the fold replication determined. Murine erythrocytes were freshly isolated by cardiac puncture, harvested by centrifugation for 10 minutes, and added to BMDMs at a 1:1 or 10:1 erythrocyte:BMDM ratio. At the higher ratio, more hemophagocytes were typically observed. For Figure 2-6, Jurkats were used instead of erythrocytes. Jurkats are a T lymphocyte cell line (ATCC). Jurkats were maintained in suspension in complete RPMI media plus 10% FBS. Jurkats were

stained for one hour using the pH-sensitive dye pHrodo PE (Thermo Fisher Scientific); 1x10^9 Jurkats were stained with 1 uL of dye. After one hour, the cells were incubated with 15 mL of DMEM for 15 minutes, washed twice with DMEM and resuspended in a final volume of DMEM. For the studies on purified bacterial molecules, LPS (Sigma-Aldrich) was added at 20 ng/mL, 2 ng/mL, or 0.2 ng/mL; LTA (Sigma-Aldrich) at 0.01 mg/mL; CpG (InvivoGen) at 1 μM; poly I:C (gift from H. Yin) at 10 μg/mL or 25 μg/mL (data not shown); flagellin (Sigma-Aldrich) at 0.05 μg/mL. For addition to BMDMs, bacterial molecules or heat-killed bacteria were resuspended to their final concentration in complete DMEM supplemented with gentamicin (10 *μ*g/mL), along with the appropriate concentration of erythrocytes. For infection with live *S*. Typhimurium, macrophages were washed 30 minutes after infection and incubated for 1.5 hours at 37°C in fresh media supplemented with gentamicin (100 *μ*g/mL) to kill extracellular bacteria. Media was then exchanged for media supplemented with gentamicin (10 *μ*g/mL) to prevent extracellular bacterial growth and erythrocytes were resupplied at the same concentration after each media change (Pilonieta et al., 2014). To determine that media exchange, which involves washing the macrophages and adding back fresh media containing erythrocytes and purified bacterial molecules or heat-killed bacteria, did not significantly change the final fraction of hemophagocytes, I compared samples that had undergone media exchange with samples that had not undergone a media exchange. No significant differences were observed (Figure 2-15).

For the studies on NF-κB inhibition, BMDMs were pretreated with 2 μM of BMS-345541 (Sigma-Aldrich) or vehicle (DMSO) control for 60 minutes before addition of LPS (20 ng/mL). For the studies on TLR4 inhibition, BMDMs were pretreated with 1 μM, 2.5

μM or 5 μM of TAK242 (Invivogen) or vehicle (DMSO) control for 60-90 minutes before addition of LPS (20 ng/mL) or infection with live *S*. Typhimurium. The infection studies in the presence of TAK242 were carried out as described above, except that erythrocytes were added when 10 *μ*g/mL of gentamicin was added. To control for changes in erythrocytes during storage at 37C with 5% CO2 for up to 8 hours, a parallel set of BMDMs remained unexposed to LPS and were incubated with stored erythrocytes. By 18 hours, the fraction of hemophagocytes did not differ from BMDMs exposed to fresh erythrocytes.

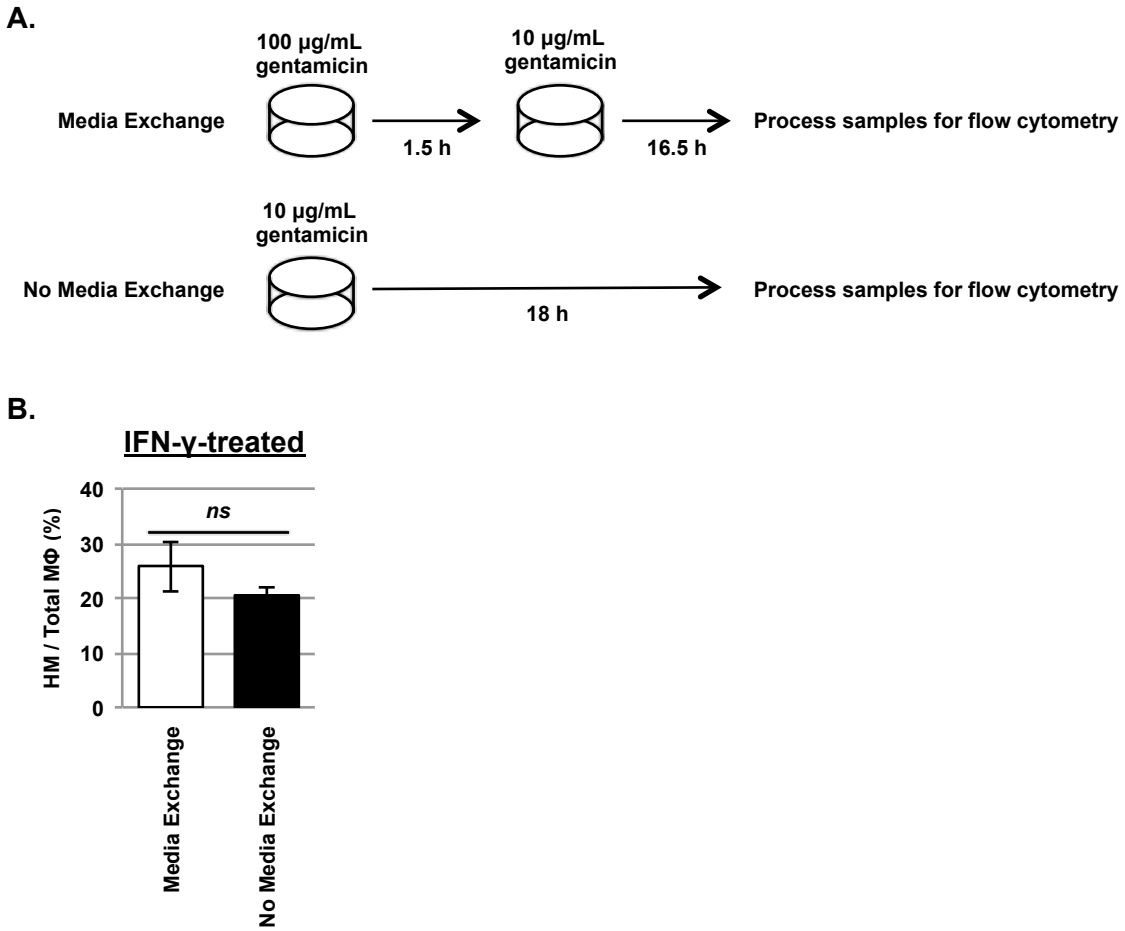


Figure 2-12. No significant differences found between samples that had media exchanged versus those that did not
(A) For addition to macrophages, HK *S*Tm were resuspended to their final concentration in complete DMEM supplemented with either 100 *µ*g/mL gentamicin (Media Exchange) or 10 *µ*g/mL gentamicin (No Media Exchange). The appropriate concentration of erythrocytes was added to the media. For samples undergoing media exchange, after 1.5 h, the macrophages were washed and media was then exchanged for media supplemented with gentamicin (10 *µ*g/mL) and HK *S*Tm at their final concentration, along with erythrocytes at their appropriate concentration. **(B)** After 18 h, the macrophages were processed for flow cytometry. Means ± the standard deviation of aggregated data were determined from two biological replicates. *P* values were determined by a Student's T-test. *ns*, no significance.

Flow Cytometry for *in vitro* hemophagocytosis assay

Resting or activated (20 U/mL IFNγ for 18-24 hours) BMDMs were washed with 1 mL of 1X PBS and external erythrocytes were lysed using 500 μL of 1X ammonium chloride-potassium bicarbonate (ACK) lysis buffer (Bossuyt et al., 1997) and harvested by gentle scraping. Cells from each condition were equally distributed into 96-well plates and resuspended in 1X PBS. Cells were stained with Live/Dead Near-IR stain (Life Technologies) at 1:3000, followed by incubation in FACS staining buffer (PBS plus 1% fetal bovine serum (FBS), 0.02% azide) containing anti-mouse CD16/32 (eBioscience, San Diego, CA) to block Fc receptors. Cells were then fixed in 1% paraformaldehyde-1% sucrose, permeabilized in staining buffer with 0.1% saponin for 10-minutes, and then incubated in permeabilization buffer containing 1:200 anti-mouse TER-119-APC (eBioscience). Compensation was performed using single color controls prepared from the resting or activated BMDMs. Fluorescently labeled cells were analyzed using a CyAn ADP flow cytometer (Beckman Coulter) and FlowJo software version 8.8.7 (Tree Star, Inc.). A minimum of 10,000 live cells was collected for analysis. All percentages reported are expressed as a percentage of live cells.

Median fluorescent intensity (MFI) was calculated for all experiments. MFI was normalized by subtracting background fluorescence (i.e., the MFI of the APC signal in the TER119- population was subtracted from the MFI of the APC signal in the TER119+ population). Statistics were not calculated for MFI. MFI values for all figures in Chapter 2 are shown in Appendix A.

Statistics

Data were analyzed using JMP Pro Version 11.2.0. (SAS Institute Inc.) and Prism 7. Two-way comparisons were analyzed with a Student's t-test (for parametric data). Multiple comparisons were analyzed by ANOVA with a Tukey's (parametric data) or Dunnett's (nonparametric data) post-hoc test.

Chapter 3 Hemophagocytosis Does Not Require Erythrocyte Surface Phosphatidylserine

I. Introduction

Hemophagocytic lymphohistiocytosis (HLH) is a life-threatening immunological disorder characterized by uncontrolled inflammation and organ damage in which hyper-activated lymphocytes and macrophages secrete pro-inflammatory cytokines, resulting in a cytokine storm. This clinical syndrome is associated with a variety of underlying genetic polymorphisms and there is a huge spectrum of sensitivity. On one end of the spectrum are genetic lesions that affect granule-mediated cytotoxicity and present early in infancy upon infection with any number of agents, versus the other extreme, in which infection, malignancies, and/or autoimmune disorders result in disease much later in life. A common feature is that infection can trigger HLH, and lymphocytes become hyperactivated due to lack of immune regulation or pathogen clearance. A clinical marker, and the hallmark feature, used in the diagnosis of HLH and other hyperinflammatory syndromes is the presence of hemophagocytes, a subset of macrophages that have engulfed morphologically intact erythrocytes, platelets, and/or white blood cells(Brisse et al., 2015a; Canna and Behrens, 2012).

Hemophagocytosis is observed in many animal models of HLH. These models include: a natural host-pathogen relationship using immunocompetent (Sv129S6) mice and the bacterium *Salmonella enterica* serotype Typhimurium (Brown et al., 2010), wild-type C57BL/6J mice injected with a single high dose of a Toll-like receptor 9 (TLR9) agonist or infected with lymphocytic choriomeningitis virus (LCMV) (Ohyagi et al., 2013), and wild-type C57BL/6J mice infected with *Trypanosoma brucei* (Guegan et al., 2013; Kitani et al., 2004; Namangala et al., 2001). Hemophagocytosis is also seen in animal

models that develop some of the clinical signs of HLH: wild-type rabbits infected with Herpesvirus papio (HVP) (Hsieh et al., 2007), wild-type C57BL/6J mice repeatedly injected with a TLR9 agonist and treated with anti-IL-10 receptor antibodies (Behrens et al., 2011), IFN-$\gamma^{-/-}$ mice injected with complete Freund's adjuvant (Avau et al., 2014), and immunodeficient, humanized mice infected with Epstein-Barr virus, a human pathogen (Sato et al., 2011). Animal models of anemia in the context of acute or chronic inflammation are also characterized by hemophagocytes: C57BL/6J mice injected with either *Toxoplasma gondii* or subcutaneously infused with IFN-γ (Zoller et al., 2011), BALB/c mice injected with LPS (Ballin et al., 2013), or C57BL/6J mice injected with heat-killed *Brucella abortus* (a select BSL-3 agent) (Gardenghi et al., 2014; Kim et al., 2014; Sasu et al., 2010).

Although hemophagocytosis is observed in the majority of these models, few of them have characterized the mechanisms leading to erythrocyte uptake. For example, HVP infection of rabbits stimulates hemophagocytosis via triggering antibody coating of erythrocytes *in vivo*, leading to Fc receptor-mediated uptake by macrophages (Hsieh et al., 2007). Hemophagocytosis induced by trypanosome infection may have multiple contributing factors: an *ex vivo* assay demonstrated that trans-sialidases produced by the parasite desialylate glycophorins on erythrocytes, leading to erythrocyte uptake by macrophages (Guegan et al., 2013), whereas an *in vivo* model demonstrated that high levels of IFN-γ induced by trypanosome infection resulted in hemophagocytosis (Zoller et al., 2011). Less well understood is the mechanism by which bacterial infection or TLR agonists induce erythrocyte uptake. Hemophagocytosis after infection with LCMV or after activating TLR9 receptors with a bacterial DNA mimic is hypothesized to be due to

phosphatidylserine (PS) externalization on blood cells; this is supported by the fact that a mixture of blocking antibodies to key PS receptors reduces the fraction of hemophagocytes *in vivo* (Ohyagi et al., 2013). The mechanism(s) driving other models of TLR- and infection-induced hemophagocytosis is currently unknown.

The Detweiler lab recently developed a cell culture model to study *S*. Typhimurium-induced hemophagocytosis (McDonald et al., 2015; Nix et al., 2007; Pilonieta et al., 2014). Using this model, we discovered that TLR4 signaling and NFκB activation are required for *S*. Typhimurium-induced hemophagocytosis by macrophages (McDonald et al., 2015), but the specific receptor(s)/ligand(s) involved are unknown. In the present work, I have utilized our cell culture model to prove that PS recognition is not required for *S*. Typhimurium-induced hemophagocytosis. Furthermore, I show that IFN-γ-treatment is crucial to increase expression of a receptor involved in this process and that endotoxin tolerance can regulate hemophagocytosis, most likely by decreased expression of a key receptor. Finally, I have extended our studies to demonstrate that both inflammatory monocytes and resident macrophages in the spleen hemophagocytose erythrocytes in a mouse model of *S*. Typhimurium infection.

II. Results

In vivo, hemophagocytes are derived from both resident macrophages and inflammatory monocytes

Infection of Sv129 mice with S. Typhimurium results in the accumulation of hemophagocytes that express MOMA-2, which broadly detects monocytes and macrophages (Brown et al., 2010; McCoy et al., 2012; Nix et al., 2007), but the source of these macrophages is unknown. Tissue mononuclear phagocytes from which

hemophagocytes may derive include resident macrophages, which arise from circulating monocytes that have entered healthy tissues, circulating monocytes, which enter tissues to become inflammatory monocyte-macrophages during inflammation (Yang et al., 2014), or plasmacytoid dendritic cells (pDCs), tissue monocyte-derived phagocytes that produce large amounts of type I interferons in response to viral and bacterial stimuli (Asselin-Paturel et al., 2001; Björck, 2001; Nakano et al., 2001; O'Keeffe et al., 2002). In the liver and spleen, for example, resident macrophages phagocytose senescent or damaged erythrocytes for recycling (Knutson and Wessling-Resnick, 2003).

To establish the identity of splenic hemophagocytic macrophages in S. Typhimurium infected mice, Sv129S6 mice were orogastrically inoculated with approximately 1 x 10^9 of a virulent strain (SL1344) with a chromosomally-integrated rpsM::gfp reporter construct (Vazquez-Torres et al., 1999). We focused on resident and inflammatory monocytes because previous work indicated that MOMA-2+ hemophagocytes in the livers of mice harbor S. Typhimurium (Nix et al., 2007); pDCs are MOMA-2 negative and thus we did not include them in our analysis (Döring et al., 2012). We initially tried to identify infected splenic macrophages and hemophagocytes using the GFP reporter expressed by S. Typhimurium, but were unable to separate bacterial GFP signal from macrophage autofluorescence by flow cytometry. Instead, we monitored splenic bacterial loads by plating tissue extracts and counting colony-forming units (CFU). We also identified hemophagocytes in dissociated spleen cells using a flow cytometry gating scheme which included a live/dead stain, a lineage dump channel, and two monocyte markers (Figure 3-1). This gating scheme distinguished resident macrophages and inflammatory monocytes (Rose et al., 2012). We then detected cells

containing engulfed erythrocytes using intracellular staining for the erythroid specific marker TER119 (Chen et al., 2009; McDonald et al., 2015; Pilonieta et al., 2014).

Hemophagocytes included both resident macrophages and inflammatory monocytes. When splenic bacterial loads were highest, at one week post-infection (Figure 3-2 A), the fraction of resident hemophagocytic macrophages was significantly greater in the infected spleens versus control spleens (Figure 3-3 E). There was no difference in the fraction of hemophagocytic inflammatory monocytes between control and infected mice at either time point (Figure 3-3 F). Splenomegaly also increased (Figure 3-2 C-D), supporting infiltration and extramedullary hematopoiesis (Jackson et al., 2010). The fraction of monocytes and macrophages that were hemophagocytes did not increase with increasing time post-infection (Figure 3-3 E-F), in contrast with previous observations (McCoy et al., 2012). However, this may reflect that, in the current experiments, bacterial load in the spleen diminished by two weeks post-infection (Figure 3-2 B), suggesting containment of the microbe and a return to homeostasis. Infected mice had fewer resident macrophages than control mice (Figure 3-3 B), most likely due to these cells dying in response to infection or tissue damage. However, the key observation is that both resident macrophages and inflammatory monocytes hemophagocytose.

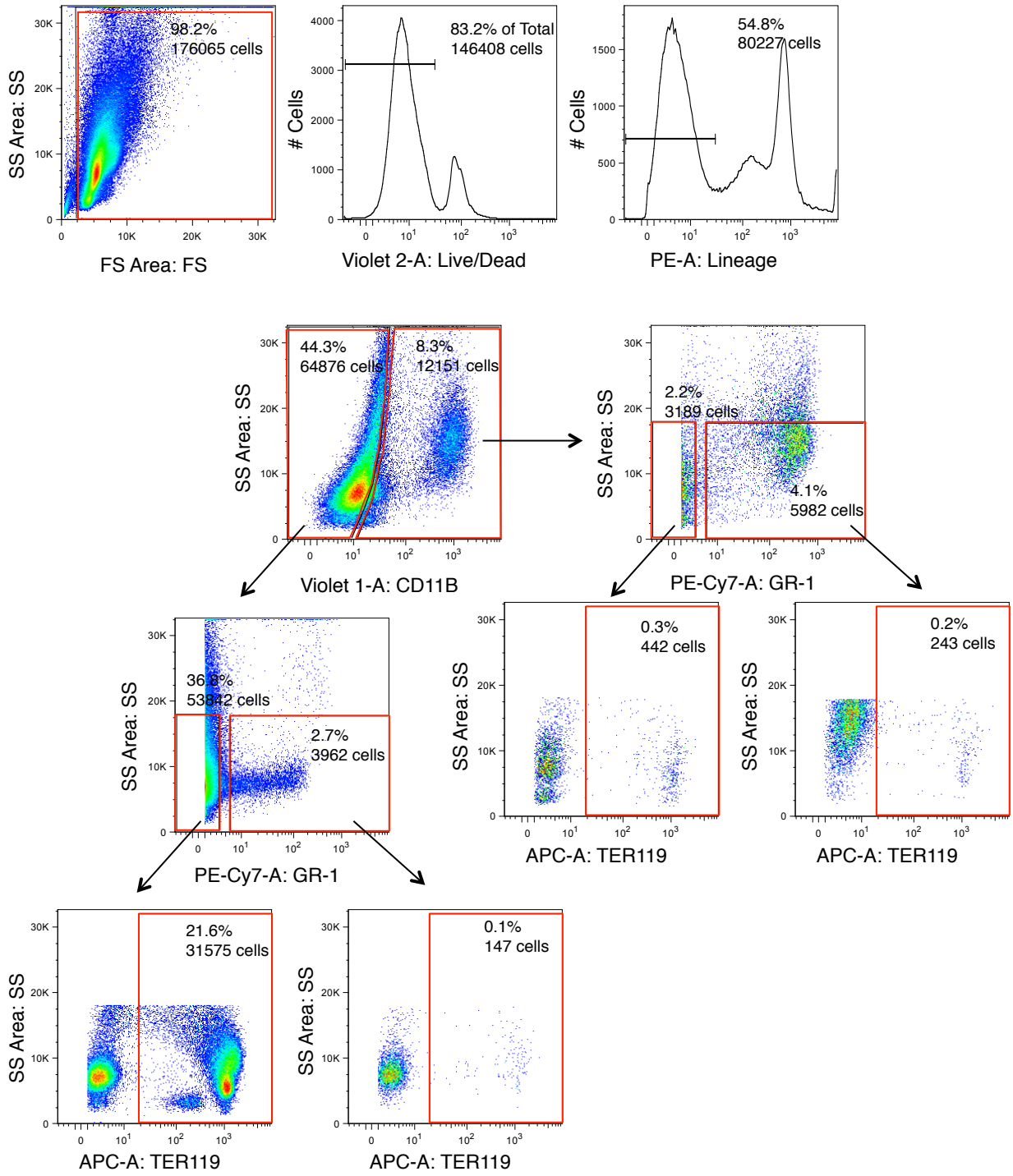


Figure 3-1. Flow cytometry gating scheme for identifying hemophagocytic macrophages and monocytes in the mouse spleen using TER119 antigen
Mice were orogastrically inoculated with 1 x 10^9^9 SL1344 strain with a chromosomally-integrated *rpsM::gfp* reporter construct. At one and two weeks post-infection, mice were sacrificed and the spleens were harvested and processed into a single-cell suspension. Red blood cell lysis was performed using 1 mL/spleen of ACK Lysis buffer. Single cell suspensions were stained according to the methods. Using side scatter (SS) versus forward scatter (FS), debris was excluded from total splenocytes. B220+ (B-cells), Ly-

6G+ (neutrophils), NK1.1+ (NK and NKT cells) and CD11c+ (monocytes including Dendritic cells) cells were gated out by exclusion of PE+ (lineage) cells. CD11b+ and CD11b- cells were then gated out from the PE- population. The CD11b+ cells were sorted into Gr-1- (resident macrophages) and Gr-1+ (inflammatory monocytes) populations. These two subpopulations were then further subdivided into TER119+ populations. The CD11b- population was also examined, as the majority of TER119+ cells are found in this population. The $CD11b^{-}Gr\text{-}1^{-}$ population was found to contain the majority of TER119+ cells. These cells are most likely erythrocyte precursors. Percent of live cells are shown, unless otherwise indicated.

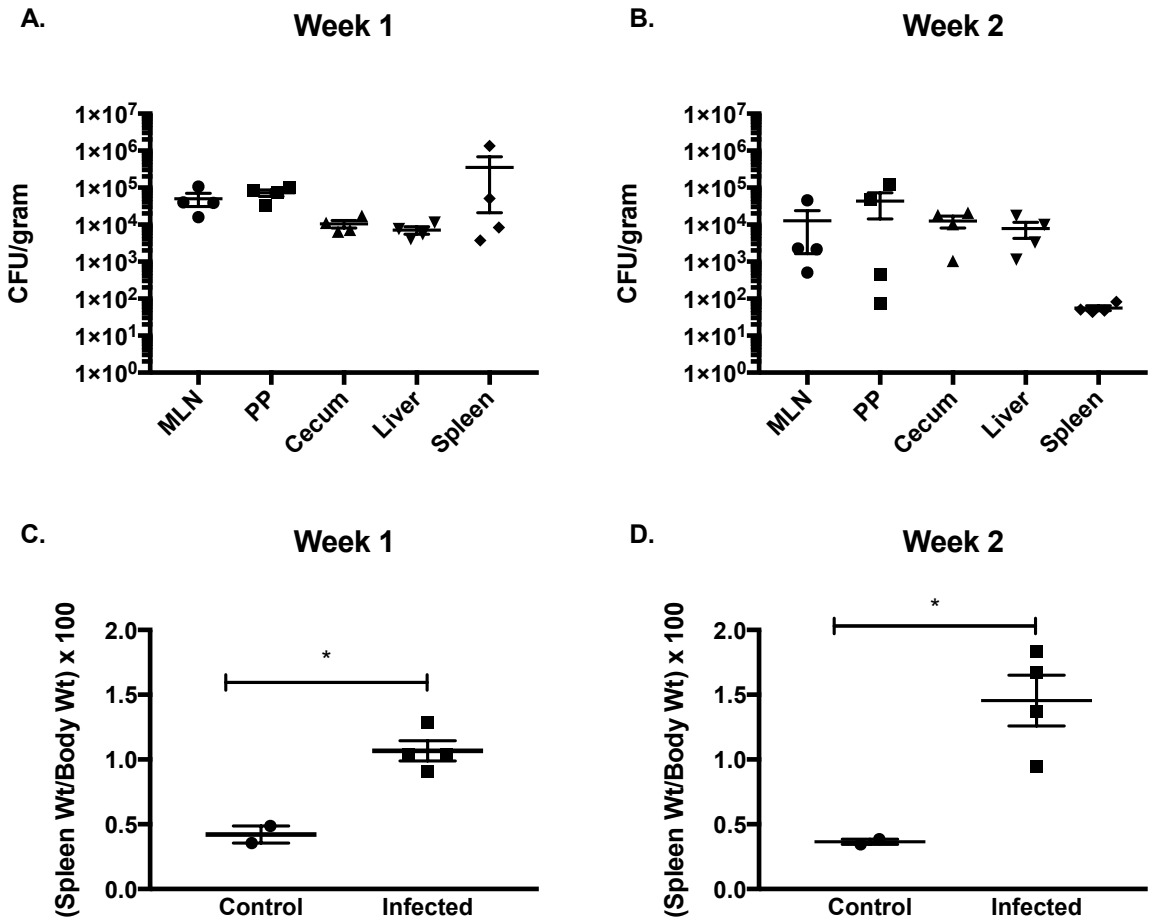


Figure 3-2. Infected mice develop splenomegaly
Mice were orogastrically inoculated with 1 x 10^9^{9} SL1344 strain with a chromosomally-integrated *rpsM::gfp* reporter construct. At one (A) and two (B) weeks post infection, mice were sacrificed and the mesenteric lymph nodes (MLN), Peyer's patches (PP), cecum, liver and spleen were homogenized and plated to determine colony forming units (CFU) per gram of organ. Splenomegaly was quantified at one (C) and two (D) weeks post infection. The ratio of (spleen to body weight) x 100 was measured. Mean $\pm$ SEM are shown. Each symbol represents one mouse: n = 2 control mice, n = 4 infected mice. *P* values were determined by an unpaired t-test. *, $P \leq 0.05$ (compared to vehicle).

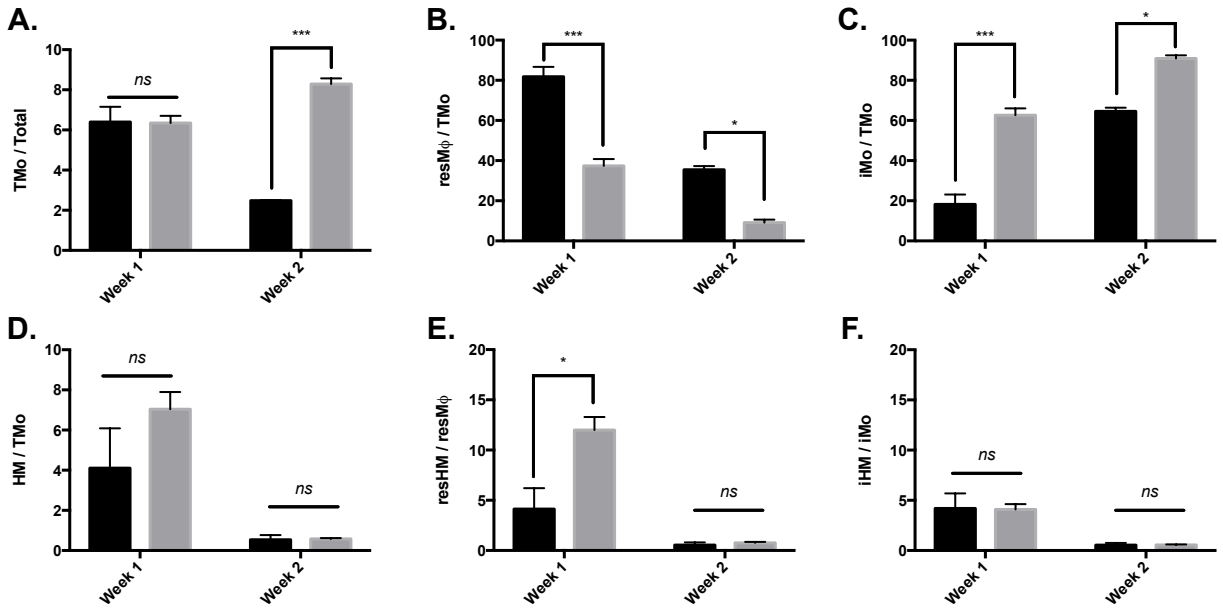


Figure 3-3. Resident macrophages and inflammatory monocytes hemophagocytose

Mice were orogastrically inoculated with 1 x 10^9 SL1344 strain with a chromosomally-integrated *rpsM::gfp* reporter construct. At one and two weeks post-infection, mice were sacrificed and the spleens were harvested and processed into a single cell suspension. Red blood cell lysis was performed using 1 mL/spleen of ACK Lysis buffer. Single cell suspensions were stained according to the methods (A) Percentage of live monocytes. (B) Percentage of resident macrophages out of total monocytes. (C) Percentage of inflammatory monocytes out of total monocytes. (D) Percentage of hemophagocytic monocytes out of total monocytes. (E) Percentage of hemophagocytic resident macrophages out of resident macrophages. (F) Percentage of hemophagocytic inflammatory monocytes out of inflammatory monocytes.. Mean + SEM are shown. n = 2 control mice, n = 4 infected mice. P values were determined by a 2 way ANOVA and Tukey's post hoc test. *, $P \leq 0.05$; ***, $P \leq 0.0001$ (compared to control mice). TMo is Total Monocytes; resMϕ is resident macrophages; iMo is inflammatory monocytes; HM is hemophagocyte.

In cell culture, antibodies to phosphatidylserine receptors inhibit hemophagocytosis in IFN-γ-treated macrophages

Hemophagocyte accumulation in animals and humans correlates with pro-inflammatory conditions, including increased serum levels of TNF-α (Chatterjee, 1999; Lang et al., 2004, 2005). TNF-α may increase erythrocyte externalization of phosphatidylserine (PS) by increasing plasma levels of sphingomyelinase, which is secreted from macrophages and other leukocytes (Chatterjee, 1999; Lang et al., 2004). Sphingomyelinase breaks down the phospholipid sphingomyelin to generate ceramide, which acts as a second messenger leading to surface exposure of PS by erythrocytes (Lang et al., 2004, 2005). Thus, it has been hypothesized that PS recognition by macrophages mediates hemophagocytosis (Ohyagi et al., 2013; Zoller et al., 2011). For these reasons, we addressed whether blocking antibodies to PS receptors alters TLR-agonist stimulated hemophagocytosis.

Macrophages express multiple PS receptors: TIM-4, CD51/CD61 ($\alpha_V\beta_3$, vitronectin receptor), $\alpha_V\beta_5$ integrin complex (fibronectin receptor), and the TAM family of tyrosine kinase receptors (Tyro3, Axl, and MerTK) (Armstrong and Ravichandran, 2011; Korns et al., 2011). We decided to focus on TIM-4 and CD51/CD61 since the other receptors have been reported to be lowly expressed ($\alpha_V\beta_5$ integrin complex), or not expressed by M-CSF derived BMDMs (Axl and Tyro3), or have decreased expression upon LPS treatment (MerTK) (Dransfield et al., 2015; Fujimori et al., 2015; Huang et al., 1995; Kaner et al., 1999; Zagórska et al., 2014). TIM-4 is a cell-surface glycoprotein that directly binds PS (Kobayashi et al., 2007), whereas CD51/CD61 binds a bridging molecule (milk factor globule epidermal growth factor 8 (MFG-E8)), which in turn binds

PS (Fadok et al., 1992a). First, to demonstrate that our primary macrophages recognize and engulf PS+ erythrocytes, we caused erythrocytes to externalize PS by oxidizing the erythrocytes with treatment of 0.2 mM $CuSO_4$ and 5 mM L-ascorbate (Sambrano and Steinberg, 1995; Tanaka et al., 2001). To determine what fraction of the erythrocytes had externalized PS, we stained the erythrocytes with a fluorescently labeled annexin V, a PS binding protein. We found that approximately 80% of the erythrocytes bound to annexin V (Figure 3-4 A, C). As expected, macrophages ingested approximately 30% of the oxidized erythrocytes within two hours (Sambrano and Steinberg, 1995; Tanaka et al., 2001), compared to approximately 1% of control erythrocytes (Figure 3-4 D). Next, we confirmed that the anti-PS receptors antibody mixture effectively bound the corresponding receptors: incubation of macrophages with the anti-PS receptors antibody mixture decreased supernatant nitrite levels (Figure G-H). When PS receptors are engaged, either by binding PS on an apoptotic cell, or by an antibody binding the PS receptors, nitric oxide synthase (iNOS) is inhibited. When iNOS is inhibited, nitrite levels decrease (Benoit et al., 2008; Cavicchi et al., 2000).

We then treated LPS- or *S.* Typhimurium-stimulated macrophages with a mixture of antibodies that block the interaction of PS with the TIM-4 and the CD51/CD61 complexes (Miyanishi et al., 2007; Ohyagi et al., 2013; Xu et al., 2015) and monitored the fraction of cells that hemophagocytose. As previously noted, IFN-γ treatment increases the fraction of hemophagocytes (McDonald et al., 2015; Pilonieta et al., 2014), possibly because activation increases expression of a key macrophage receptor(s) for hemophagocytosis. Treatment with the anti-PS receptors antibody mixture decreased hemophagocytosis by IFN-γ-treated, LPS-stimulated macrophages

(33% reduction in hemophagocytosis), but had no significant effect on hemophagocytosis by resting macrophages stimulated with LPS or infected with *S*Tm, or on *S*Tm-infected IFN-γ treated macrophages (Figure 3-4 E-F). This indicates that the majority of hemophagocytosis is not PS-dependent. It is possible that activation of macrophages by LPS or *S*Tm causes production of DAMPs or other molecules that stimulate PS exposure on erythrocytes during the hemophagocytosis assay. However, only 2-4% of erythrocytes have surface-exposed PS after washing the cells with PBS (Figure 3-4 C) or 18 hours of coculture with resting or IFN-γ-activated macrophages (Figure Appendix B-1). These data are also consistent with reports that erythrocyte clearance in the spleen is independent of phosphatidylserine recognition.

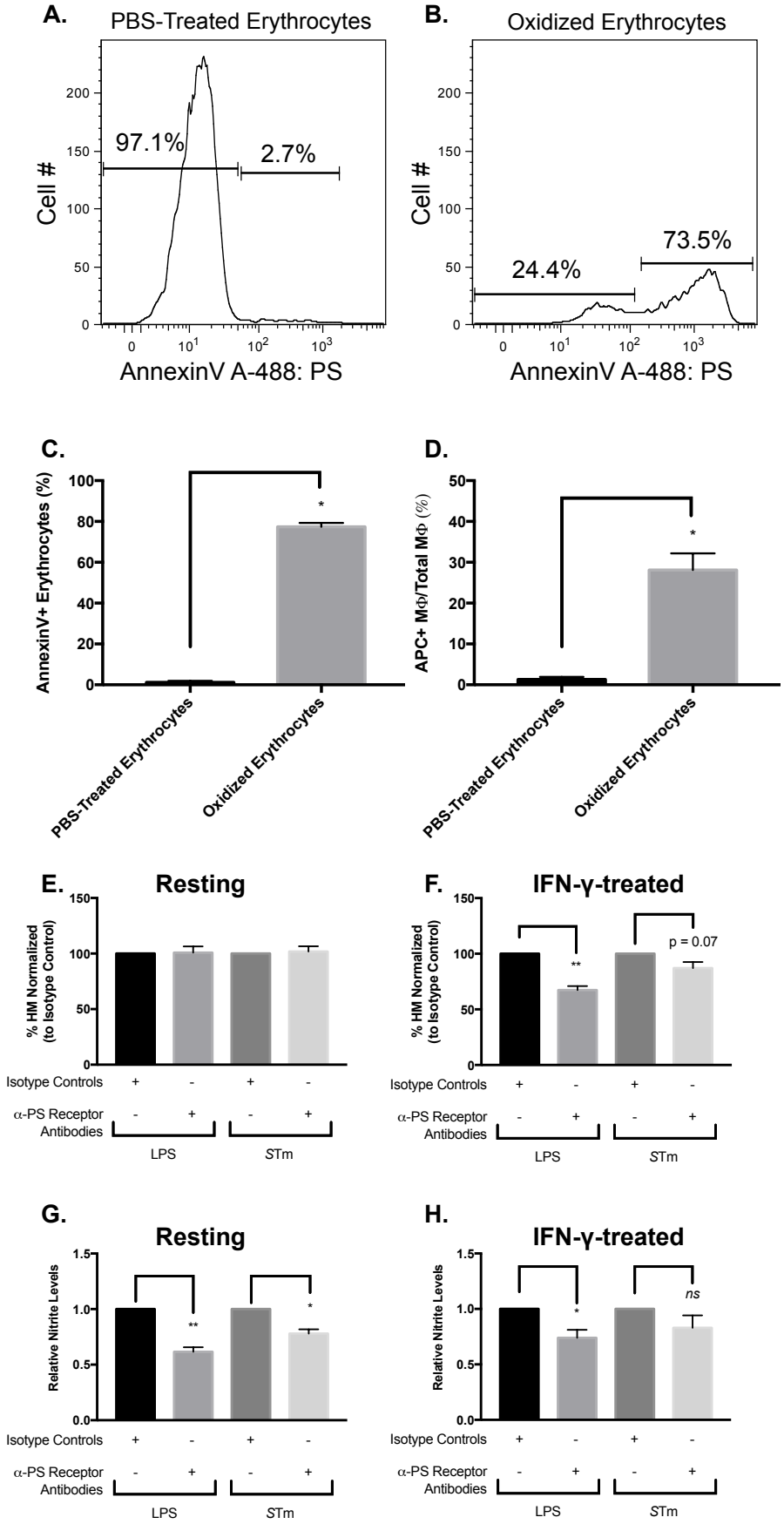

A.
PBS-Treated Erythrocytes
Cell #
97.1%
2.7%
AnnexinV A-488: PS
B.
Oxidized Erythrocytes
Cell #
24.4%
73.5%
AnnexinV A-488: PS
C.
AnnexinV+ Erythrocytes (%)
PBS-Treated Erythrocytes
Oxidized Erythrocytes
D.
APC+ MΦ/Total MΦ (%)
PBS-Treated Erythrocytes
Oxidized Erythrocytes
E.
Resting
% HM Normalized (to Isotype Control)
Isotype Controls
α-PS Receptor Antibodies
LPS
STm
F.
IFN-γ-treated
% HM Normalized (to Isotype Control)
p = 0.07
Isotype Controls
α-PS Receptor Antibodies
LPS
STm
G.
Resting
Relative Nitrite Levels
Isotype Controls
α-PS Receptor Antibodies
LPS
STm
H.
IFN-γ-treated
Relative Nitrite Levels
ns
Isotype Controls
α-PS Receptor Antibodies
LPS
STm

Figure 3-4. Treatment with phosphatidylserine (PS) receptor blocking antibodies reduces the fraction of IFN- treated macrophages that hemophagocytose
Erythrocytes were stained with Cell Tracker Deep Red (detected in the APC channel). (A-D) Stained erythrocytes underwent oxidation treatment or were PBS-treated as a control. (A-C) After treatment, erythrocytes were stained with Annexin V tagged with Alexa 488. (A-B) Representative histograms of Annexin V staining. (C) Mean percentage of Annexin V+ erythrocytes after oxidation treatment or PBS treatment (control) of erythrocytes isolated by cardiac perfusion. (D) Macrophages were pre-treated with IFN-γ and then co-incubated with oxidized or PBS-treated erythrocytes at a ratio of 10:1 (Erythrocyte:BMDM). After 2 hours, macrophages were processed for flow cytometry. Mean percentage of APC+ macrophages. (E-H) Macrophages were left resting (E,G) or pre-treated with IFN-γ (F,H) and were exposed to either LPS (20 ng/mL) or infected with wild-type *S*Tm containing the fluorescence dilution plasmid pDiGi. Cell Tracker Deep Red-stained erythrocytes were added at a ratio of 10:1 (Erythrocyte: BMDM). 8 hours later, anti-phosphatidylserine receptor antibodies (αCD51, αCD61, αTIM-4, or isotype controls; 5 *μ*g/mL each) were spiked into each well. At 18 hours post-infection, the macrophages were processed for flow cytometry. (G,H) The Griess assay was performed on the cell culture supernatants (from E,F) to detect nitrite levels by reading absorbance at 540 nm. Mean +/- SEM of aggregated data from three biological replicates. p-values were determined by one-way ANOVA (A-B) or unpaired student's t-test (C-D, G-H). * $p < 0.05$, ** $p < 0.001$, ns, not significant.

IL-10 and TGF-β1 production are not affected by treatment with PS receptor antibodies

Apoptotic cell and senescent erythrocyte uptake by macrophages is PS-dependent (Fadok et al., 1992b, 1998; Hoffmann et al., 2001) and induces IL-10 and TGF-β1 production (Byrne and Reen, 2002; Chung et al., 2007; Voll et al., 1997). We therefore determined whether these cytokines are reduced by PS receptor antibody treatment. Despite the significant decrease in hemophagocytosis by IFN-γ- and LPS-treated macrophages upon exposure to anti-PS receptor antibodies (Figure 3-4 F), there was no difference in IL-10 production in any of the conditions tested (Figure 3-5 A-B). Furthermore, TGF-β1 was not detected. This is consistent with hemophagocytosis being a PS-independent process. Macrophages produce high levels of TGF-β1 within 18 hours of apoptotic cell uptake and production of TGF-β1 requires PS recognition (Chung et al., 2007; Huynh et al., 2002). The IL-10 production in this assay is most likely due to TLR4 activation and not uptake of senescent erythrocytes; macrophages exposed to LPS and either cultured in the presence or absence of erythrocytes produce similar levels of IL-10 (data not shown) and it is known that *S.* Typhimurium infection (and LPS exposure) induces IL-10 production by macrophages (Barsig et al., 1995; Uchiya et al., 2004). These data do not support the hypothesis that hemophagocytosis requires PS recognition and instead suggest a PS-independent process is the major factor.

One caveat of the experiment above is that we do not separate hemophagocytes from nonhemophagocytes prior to measuring cytokines. Therefore, we repeated the experiment using FACS-sorted hemophagocytes and nonhemophagocytes, and

examined cytokine expression in the purified populations by RT-PCR. Consistent with the protein level results, hemophagocytes and nonhemophagocytes did not differ in expression of these cytokines (Figure 3-6). These data suggest a PS-independent process is critical for hemophagocytosis.

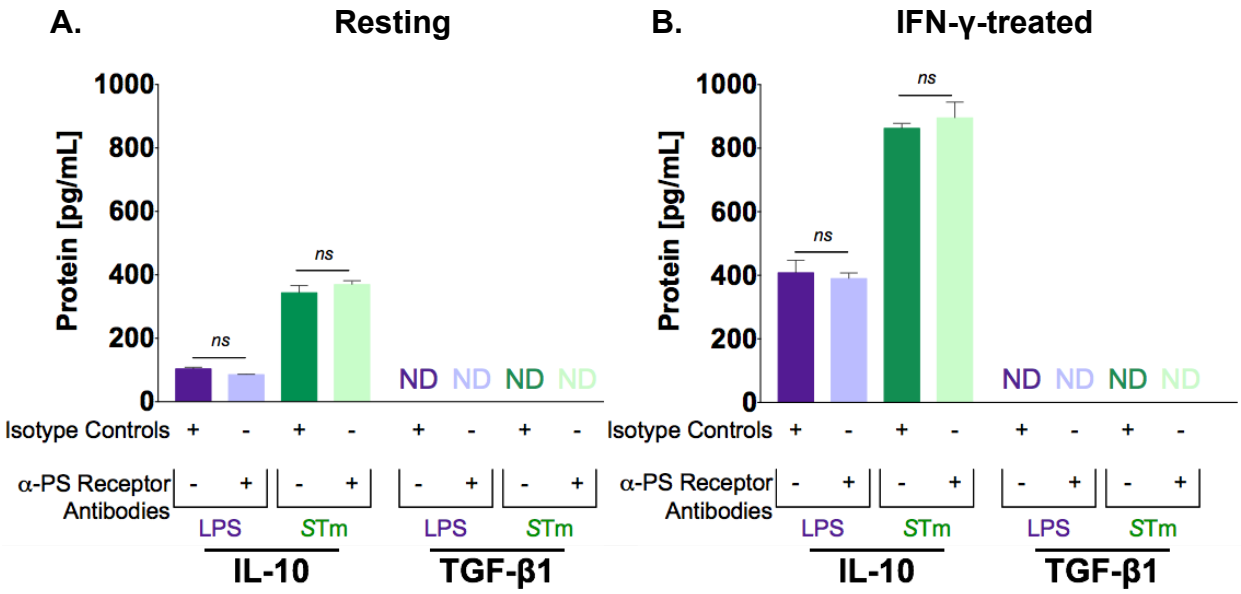


Figure 3-5. Treatment with PS receptor blocking antibodies does not affect IL-10 protein production

Macrophages were treated as in Figure 2. At 18 hours post-infection, ELISA was performed on supernatants from each condition. Mean +/- SEM of aggregated data from two biological replicates. p-values were determined by ANOVA. ns, not significant; ND, not detectable.

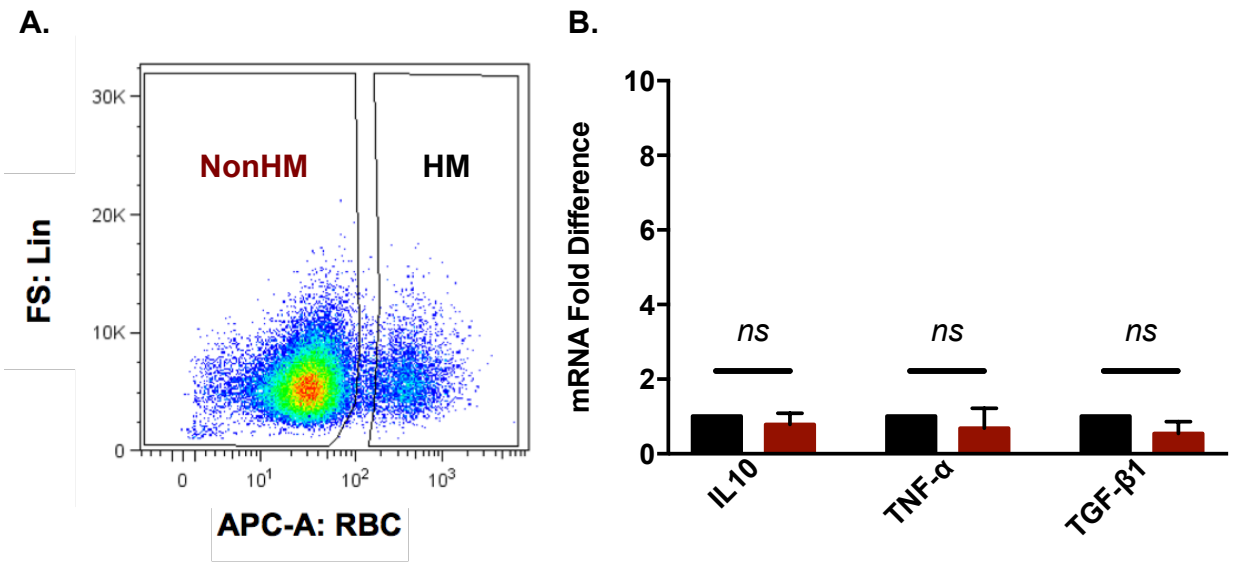
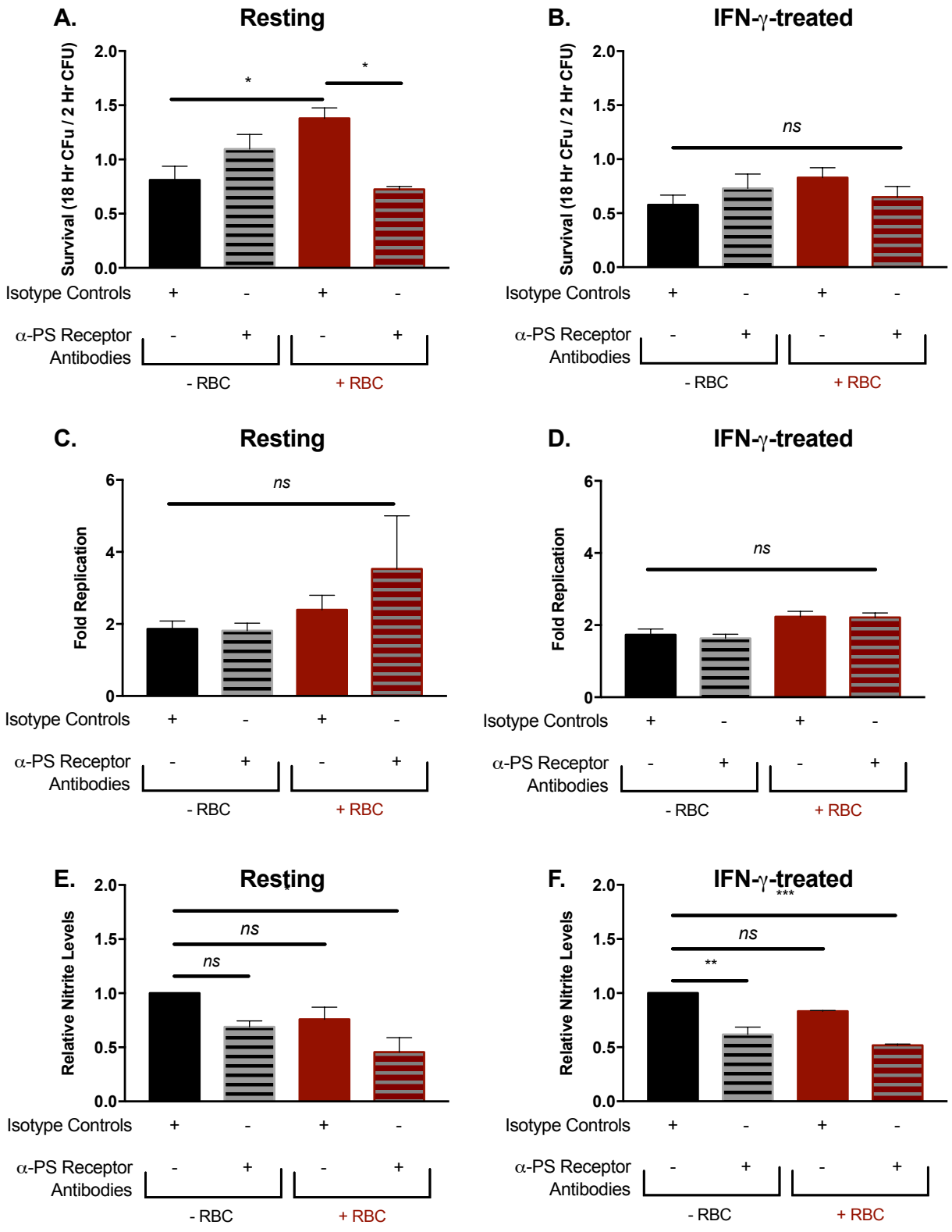


Figure 3-6. Hemophagocytes and nonhemophagocytes do not differ in expression of IL-10, TGF-β1 or TNF-α

Cell Tracker Deep Red-labeled erythrocytes were cocultured with IFN-γ-treated macrophages at a ratio of 10:1, and LPS (20 ng/mL) was added to stimulate hemophagocytosis. After 18 h, external erythrocytes were lysed, and macrophages were stained with a live/dead marker while on ice. (A) Samples were sorted into two populations: Nonhemophagocytes (NonHMs; black bars) and hemophagocytes (HMs; red bars). (B) Total RNA was immediately extracted. The levels of IL-10, TNF-α and TGF-β1 mRNA were assayed using real-time reverse transcription-PCR (SYBR Green). The expression results were normalized to those of the internal controls, Hprt and GAPDH mRNA. The fold difference in target gene expression in HMs was then calculated relative to the mean of the normalized expression level for NonHMs ($2^{-\Delta\Delta CT}$ method). Mean +/- SD of aggregated data from three biological replicates, each performed in duplicate. ns, not significant. All samples were kept on ice throughout the procedure, except during the sorting process, which took one to two hours total. Samples were sorted into ice-cold FACS buffer.

Treatment with PS receptor antibodies decreases *S*Tm survival in macrophages

The anti-PS receptor antibody mixture we used triggers PS receptors to signal, based on the decrease in nitrite levels observed after treatment (Figure 3-4 G-H). Since *Salmonella* can reside within hemophagocytes (Nix et al., 2007), we established whether treating macrophages with antibodies to PS receptors affected *S*. Typhimurium replication and/or survival. To test this notion, we infected macrophages with wild-type *S*. Typhimurium containing the fluorescence dilution plasmid pDiGi (Helaine et al., 2010), which drives arabinose-inducible expression of the DsRed fluorescent protein. We then treated macrophages with the mixture of anti-PS receptor antibodies. At two and 18 hours post-infection, macrophages were lysed; released *S*. Typhimurium were analyzed for DsRed fluorescence using flow cytometry to assess replication, and a portion of the macrophage lysate was plated for CFU. As previously observed, bacterial survival was increased in co-cultures of resting macrophages and red blood cells (Figure 3-7 A) (Nix et al., 2007), but this survival was lost upon treatment with the anti-PS antibody mixture (Figure 3-7 A). Neither coculture with red blood cells nor anti-PS antibody treatment affected bacterial replication (Figure 3-7 C-D). Therefore, the increased survival observed is most likely due to reduced killing of *S*. Typhimurium. Despite the decrease in nitrite levels upon anti-PS receptor antibody signaling (Figure 3-7 E-F), these data suggest that signaling through PS receptors decreases bacterial survival in resting macrophages cocultured with blood cells. The anti-TIM-4 antibody used in the anti-PS antibody mixture has been reported to increase LPS-induced NFκB signaling and macrophage activation (Xu et al., 2010, 2015).


A.
Resting
Survival (18 Hr CFu / 2 Hr CFU)
2.0
1.5
1.0
0.5
0.0
*
*
Isotype Controls + - + -
α-PS Receptor Antibodies - + - +
- RBC
+ RBC
B.
IFN-γ-treated
Survival (18 Hr CFu / 2 Hr CFU)
ns
Isotype Controls + - + -
α-PS Receptor Antibodies - + - +
- RBC
+ RBC
C.
Resting
Fold Replication
6
4
2
0
ns
Isotype Controls + - + -
α-PS Receptor Antibodies - + - +
- RBC
+ RBC
D.
IFN-γ-treated
Fold Replication
ns
Isotype Controls + - + -
α-PS Receptor Antibodies - + - +
- RBC
+ RBC
E.
Resting
Relative Nitrite Levels
*
ns
ns
Isotype Controls + - + -
α-PS Receptor Antibodies - + - +
- RBC
+ RBC
F.
IFN-γ-treated
Relative Nitrite Levels
***
ns
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Isotype Controls + - + -
α-PS Receptor Antibodies - + - +
- RBC
+ RBC

Figure 3-7. Treatment with PS receptor blocking antibodies decreases *S.* Typhimurium survival in resting macrophages co-cultures with erythrocytes
Erythrocytes were cocultured with resting macrophages (A,C,E) or IFN-γ-pre-treated macrophages (B,D,F) at a ratio of 10:1 after infection with wild-type STm containing the fluorescence dilution plasmid pDiGi. 8 hours later, anti-phosphatidylserine receptor antibodies (αCD51, αCD61, αTIM-4, or isotype controls; 5 μg/mL each) were spiked into each well. At 18 hours post-infection, the Griess assay was performed on the cell culture supernatants to detect nitrite levels by reading absorbance at 540 nm (A, B), and a portion of the macrophage lysate was plated for CFU (C, D), and bacteria were isolated and analyzed for DsRed fluorescence using flow cytometry (E, F). Mean +/- SEM of aggregated data from three biological replicates (Griess assay has two biological replicates), each performed in duplicate. For (A, B) a minimum of 20,000 bacteria were collected for each condition. p-values were determined by one-way ANOVA. *$p < 0.05$,**$p < 0.001$, ns, not significant.

Repeated exposure to TLR agonists decreases HM formation

Macrophage pre-treatment with both IFN-γ and LPS versus IFN-γ alone reduced the fraction of hemophagocytes formed (Pilonieta et al., 2014). These data suggest that endotoxin tolerance may interfere with hemophagocytosis. Endotoxin tolerance is defined as reduced macrophage responsiveness to LPS upon repeated stimulation (West and Heagy, 2002). We challenged macrophages with two doses of LPS, or a dose of LPS followed by lipoteichoic acid (LTA), and then monitored hemophagocytosis (Figure 3-8 A). Under these circumstances, resting macrophages reduced hemophagocytes by 25 and 52%, after the second challenge with LPS or LTA, respectively (Figure 3-8 B). The depressive effect of endotoxin tolerance was even greater in the IFN-γ treated macrophages (Figure 3-8 C). Tolerized macrophages have an alternatively activated (M2) phenotype, as shown by global transcriptional profiling and bioinformatic analyses (Mages et al., 2007; Nomura et al., 2000; Pena et al., 2011; Porta et al., 2009). They also have an increased phagocytic capability, due to increased expression of scavenger receptors and opsonin-dependent receptors, so it is surprising that the fraction of macrophages that hemophagocytosed declined, and suggests that hemophagocytosis is governed by a receptor-ligand interaction (Jing et al., 2013). Moreover, the data indicate that there is a window of time after TLR stimulation during which macrophages are able to hemophagocytose, and this may be reflected by whether the transcriptional activating form or the transcriptional inhibitor form of NFκB predominates. In canonical TLR signaling, the transcriptional activating form, the p65/p50 heterodimer, of NFκB is activated. During endotoxin tolerance, the inhibitory p50/p50 homodimer form of NFκB dominates, thus repressing transcription of NFκB

target genes (Kastenbauer and Ziegler-Heitbrock, 1999; Ziegler-Heitbrock, 2001). These results support a hypothesis whereby tolerance decreases either a key signaling component, pathway or receptor involved in the process of hemophagocytosis.

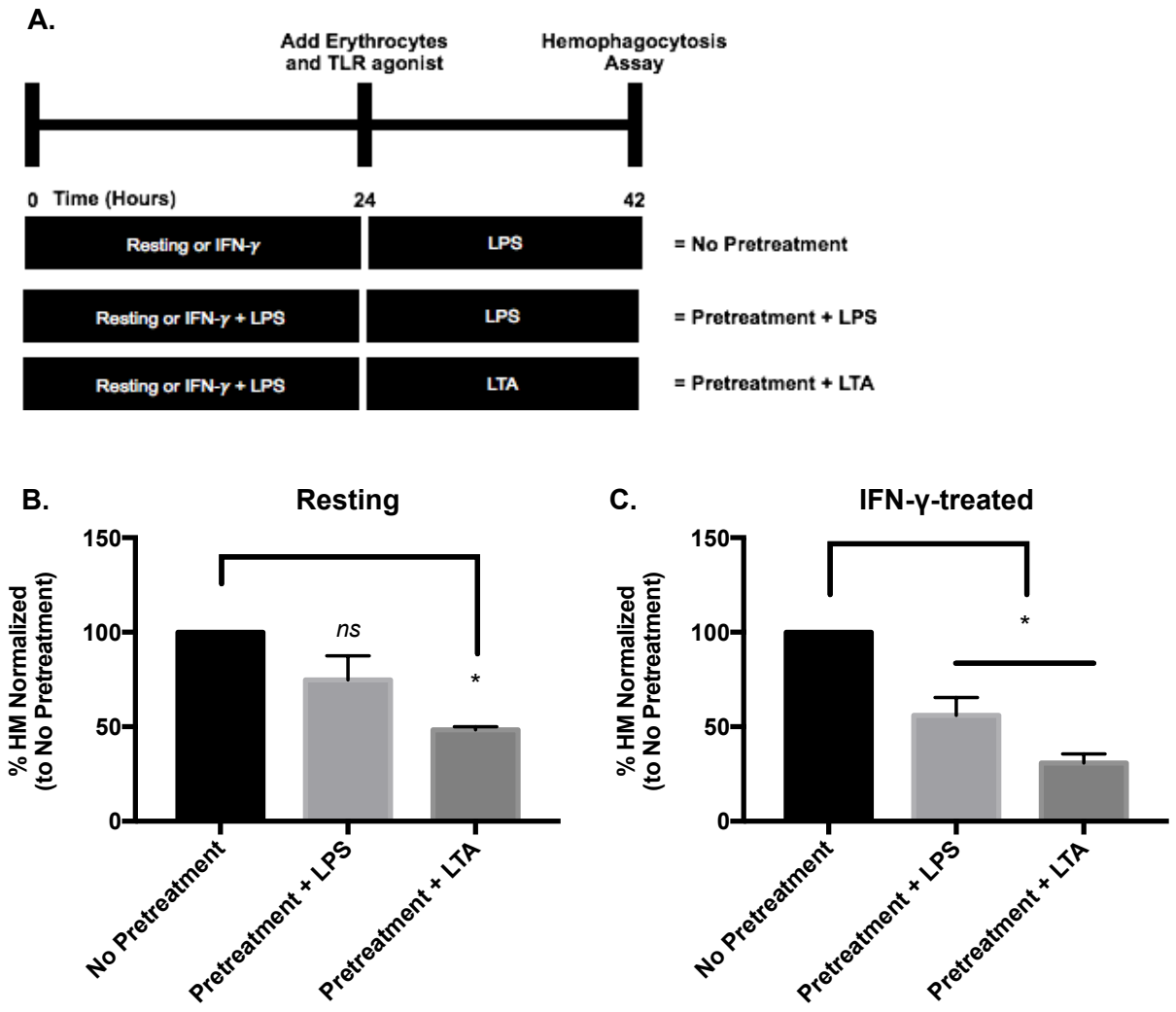

Figure 3-8. Multiple exposures to a TLR agonist decrease HM formation
(A) Macrophages were either challenged with one dose of LPS (after being left resting or treated with IFN-γ; this is the "No Pretreatment" condition), or with two doses of LPS, or a dose of LPS followed by challenge with lipoteichoic acid (LTA). See figure for timeline. At 42 hours (18 hours post single or second TLR agonist exposure), extracellular erythrocytes were lysed and the macrophages were processed for flow cytometry. (B, C) Mean percentage of TER119+ macrophages (hemophagocytes). Mean +/- SEM of aggregated data from two biological replicates. p-values were determined by ANOVA. *$p < 0.05$, ns, not significant.

A weakly immunostimulatory TLR4 agonist does not induce hemophagocytosis

In cultured macrophages, TLR4 activation via *S*. Typhimurium is required to stimulate hemophagocytosis (McDonald et al., 2015). The alpha-proteobacterium *Brucella abortus* has an LPS that is not well recognized by TLR4 due to long acyl chains on its lipid A LPS core (Barquero-Calvo et al., 2007; Conde-Álvarez et al., 2012; Park et al., 2009). Nevertheless, delivery of a single intraperitoneal injection of 5 x 10^8 heat-killed *B. abortus* (HK *BA*) to C57BL/6 mice induces hemophagocytosis after two days (Gardenghi et al., 2014; Kim et al., 2014; Sasu et al., 2010). Prior to hemophagocytosis in this model, there is transient inflammation, as IL-6 serum levels peak at 6 hours but decline to baseline within 24 hours (Sasu et al., 2010). Whether HK *BA* may directly or indirectly induce macrophages to hemophagocytose in this model is not clear.

To establish whether HK *BA* may stimulate hemophagocytosis directly, we exposed cell culture macrophages with or without TLR4 to equivalent numbers of HK *BA* or live *S*. Typhimurium. Previous work demonstrated that treatment with 1 x 10^7 live or dead *S*. Typhimurium*, Yersinia pseudotuberculosis* or *Escherichia coli,* all bacteria with LPS that is potent with regard to TLR4 activation, is sufficient to stimulate macrophages to hemophagocytose (McDonald et al., 2015; Pilonieta et al., 2014). We also treated macrophages with purified *S*. Typhimurium LPS (20 ng/mL) as a control, as this dosage, but not 100-fold less, induces macrophages to hemophagocytose (McDonald et al., 2015). Macrophages treated with HK *BA* did not engulf erythrocytes, in contrast to macrophages treated with purified *Salmonella* LPS or macrophages infected with live *S*. Typhimurium (Figure 3-9 A-B). It has been shown that 5,000 times more *Brucella* LPS than *S*. Typhimurium LPS is needed to induce macrophages to secrete comparable

levels of TNF-α, a TLR4 regulated process (Weiss et al., 2005). These data suggest that *Brucella* LPS does not stimulate sufficient TLR4 activity to drive macrophages to hemophagocytose. We suggest that, in mice, intraperitoneal delivery of HK *BA* allows for the accumulation of hemophagocytes two days later (Gardenghi et al., 2014) because other TLR agonists such as lipoproteins, the major constituent of HK *BA* sensed by TLRs (Giambartolomei et al., 2004), stimulate inflammatory responses in the host. This idea is supported by the observation that other TLR agonists also stimulate hemophagocytosis in cultured macrophages in a dose responsive manner (McDonald et al., 2015). Alternatively, or in addition, *in vivo* macrophages may be primed to hemophagocytose by multiple stimuli, including IFN-γ, an observation made in cell culture (McDonald et al., 2015). Along these lines, intraperitoneal injection of C57BL/6 mice with HK *BA* increases serum IFN-γ levels (Xavier et al., 2013). Finally, it is also possible that TLR stimulation *in vivo* may drive differentiation of monocytes into different subtypes of macrophage, only some of which can hemophagocytose. These macrophages may express the correct suite of receptors required for hemophagocytosis. In other words, primary bone marrow-derived macrophages may not fully respond appropriately to HK *BA* stimulation.

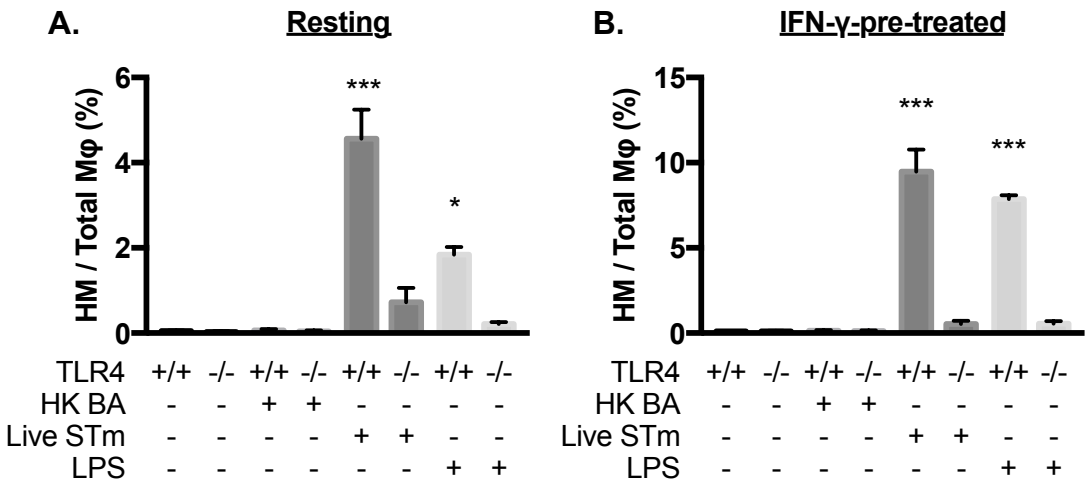

Figure 3-9. Heat-killed *Brucella abortus* does not induce hemophagocyte formation

C3H/HeOuJ (*Tlr4*$^{+/+}$) and C3H/HeJ (*Tlr4*$^{-/-}$) macrophages were left resting (A) or pre-treated with IFN-γ (B) and were cocultured with erythrocytes at a ratio of 1:10 with or without heat-killed *B. abortus* (HK BA; 1 x 10^7 cells/mL), or with or without live S. Typhimurium (Live *S*Tm; 1 x 10^7 cells/mL), or with or without purified *Salmonella* Typhimurium LPS (20ng/mL). After 18 hours, macrophages were processed for flow cytometry. (A-B) Mean percentage of TER119+ macrophages. Mean +/- SEM of aggregated data from three biological replicates. *p*-values were determined by ANOVA with a Dunnett's post test. $^{*}p \leq 0.05$, $^{**}p \leq 0.0001$, compared to TLR4 +/+, - HK BA, - Live *S*Tm, - LPS. TLR4 +/+ and TLR4-/- macrophages exposed to Salmonella LPS or infected with live S. Typhimurium was previously published; macrophage exposure to HK BA was not previously published.

IFN-γ-treatment increases macrophage engulfment of erythrocytes

Previous work demonstrated that pre-activating macrophages with IFN-γ and co-culturing with erythrocytes in the absence of a bacterial component was not sufficient to induce hemophagocytosis (McDonald et al., 2015; Pilonieta et al., 2014). Both resting and IFN-γ treated macrophages require prolonged exposure to bacteria, or a bacterial component, to become hemophagocytes (McDonald et al., 2015). Yet *in vivo*, hemophagocytes accumulate in pro-inflammatory conditions, including increased serum levels of IFN-γ (Zoller et al., 2011). This led us to ask if whether IFN-γ increases a macrophage's capacity to hemophagocytose after bacterial stimulation. We therefore asked whether hemophagocytosis is responsive to erythrocyte dose and whether macrophage activation is key to this response. Erythrocytes were added to macrophages exposed to live or heat-killed bacteria at ratios of 1:1 and 10:1 (Erythrocyte:BMDM). At the higher ratio, IFN-γ-pre-treated macrophages were more likely to engulf erythrocytes upon exposure to live or heat-killed *S*. Typhimurium, but the fraction of hemophagocytes in the resting macrophage population remained unchanged (Figure 3-10 A-B). Taken together, our results indicate that pre-treatment with IFN-γ and exposure to bacterial components are required for maximum frequency of macrophage hemophagocytes, suggesting that IFN-γ treatment increases macrophage expression of a key receptor(s) for erythrocytes.

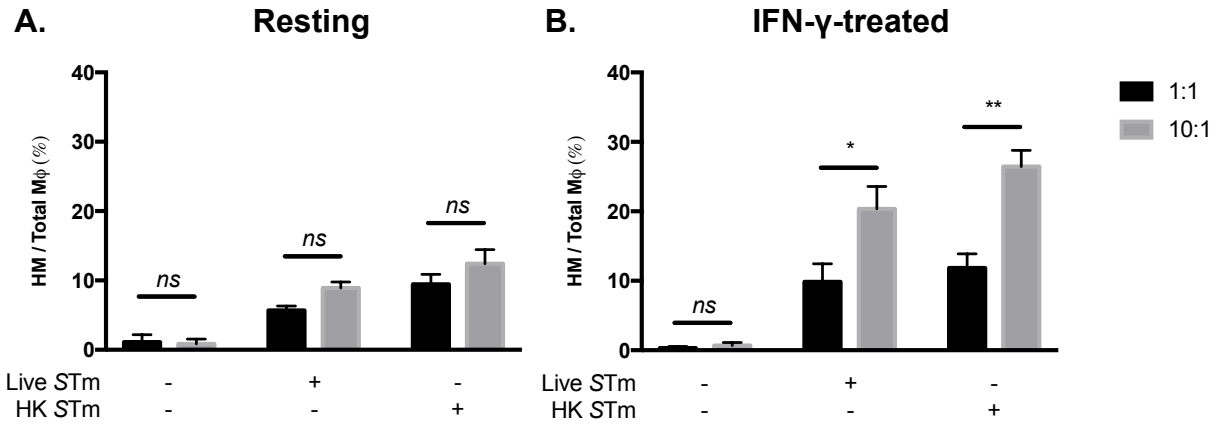

Figure 3-10. The fraction of hemophagocytic macrophages increases with increasing erythrocyte dose only in the presence of a bacterial component and with IFN-γ-pre-treatment

Erythrocytes were cocultured with resting (A) or IFN-g-pretreated (B) macrophages at a ratio of 1:1 or 10:1 without bacteria, or with live STm, or with heat-killed (HK) STm, at an MOI of 10 for 18 h. Extracellular erythrocytes were then lysed and macrophages processed for flow cytometry. (A and B) Mean percentage of TER119+ macrophages (hemophagocytes). Means + the SEM of aggregated data were determined from at least three biological replicates. P values were determined by a two-way ANOVA. *, $P < 0.05$, ***$P < 0.005$, ns, not significant.

III. Discussion

Hemophagocytes are an indicator of hyperinflammatory conditions and are a clinical diagnostic for HLH in humans. Hemophagocytes are identified by their engulfment of non-apoptotic erythrocytes, white blood cells, and platelets (Brisse et al., 2015b; Canna and Behrens, 2012; Henter et al., 2007). Sv129 mice infected with *S*. Typhimurium are a model of human Typhoid fever, and develop the clinico-pathological features of HLH, including the accumulation of hemophagocytes in the liver, bone marrow and spleen (Brown et al., 2010; McCoy et al., 2012; Nix et al., 2007). The type of macrophage(s) that becomes hemophagocytic *in vivo* and the underlying mechanism(s) of blood cell uptake during *S*. Typhimurium-induced hemophagocytosis remain poorly understood.

A flow cytometry gating scheme was adapted to delineate splenic myeloid subsets and found that both resident macrophages and inflammatory monocytes are hemophagocytic. Between one and two weeks post-infection, splenic bacterial loads declined dramatically and the fraction of monocytes that were inflammatory monocytes did not increase greater than two-fold. The fraction of hemophagocytes in the splenic resident macrophage population was significantly greater in infected mice than in control mice. No significant differences were found between control and infected mice when the fraction of hemophagocytic inflammatory monocytes was compared.

These data indicate that both resident macrophages and inflammatory monocytes appear to be similarly capable of hemophagocytosis. These findings are novel, as hemophagocytes remain largely uncharacterized. Our lab previously characterized these cells as CD68+, a monocyte/macrophage-specific lineage marker,

and not as dendritic cells (McCoy et al., 2012). Whether these hemophagocytic macrophages derived from inflammatory monocytes was unknown. Other mouse models that identified *in vivo* hemophagocytes either used the pan-macrophage marker F4/80 (Zoller et al., 2011) or used histological stains, such as Hematoxylin and Eosin or Wright-Giemsa, to distinguish macrophages that had engulfed erythrocytes (Avau et al., 2014; Behrens et al., 2011; Hsieh et al., 2007). In a TLR9-agonist induced hemophagocytosis model, circulating inflammatory CD11c+ monocytes were the main population of hemophagocytes (Ohyagi et al., 2013). This supports our model whereby infection induces the recruitment of inflammatory monocytes into the spleen, and these inflammatory monocytes are competent to hemophagocytose. In another mouse model, both inflammatory monocytes and pDCs increased their rate of erythrocyte engulfment in response to intraperitoneal delivery of poly(I:C), a TLR3 agonist (Richards et al., 2016). However, resident splenic macrophages remained the major monocyte-derived cells responsible for erythrocyte uptake in both control and treated animals (Richards et al., 2016), which agrees with our current findings.

We attempted to identify *S*. Typhimurium-infected monocytes and macrophages in the spleens of mice using *Salmonella* that express GFP from a chromosomal ribosomal promoter, but infected monocyte-macrophages could not be distinguished from background autofluorescence by flow cytometry. It is possible that the bacteria shut down GFP expression *in vivo,* or that they lost the GFP marker. *S*. Typhimurium was previously identified in anti-inflammatory/M2-like macrophages *in vivo*, but the flow cytometry gating scheme used in that study employed an isotype control antibody to identify a *S*. Typhimurium-positive gate, whereas our gating scheme attempted to rely

on bacterial expression of GFP (Eisele et al., 2013). Efforts to identify *S*. Typhimurium infected cells using antibodies to *Salmonella* LPS or antibodies to GFP protein were unsuccessful.

The data presented in the remainder of the paper support a model whereby hemophagocytosis is driven by a receptor-ligand interaction, although the identity of both the receptor and ligand remain unknown. PS-mediated recognition was hypothesized to drive hemophagocytosis, as the pro-inflammatory cytokine TNF-α, a major cytokine produced during HLH and other hyperinflammatory syndromes (Brisse et al., 2015b; Chang et al., 2006), may induce externalization of PS on erythrocytes (Chatterjee, 1999; Lang et al., 2004, 2005). Also, previous work demonstrated that TLR activation leads to increases in damaged erythrocytes that are recognized by phagocytes for uptake: Intravenous injection of 200 μg of CpG DNA into C57BL/6 mice increases the fraction of annexin V+ (PS+) erythrocytes in the peripheral blood and increases the fraction of peripheral blood monocytes that hemophagocytose. Furthermore, blocking antibodies to the PS receptors TIM-1, TIM-4 and CD51/CD61 significantly decreased hemophagocytes in this model (Ohyagi et al., 2013). These receptors do not seem to play a role in *S*. Typhimurium-induced hemophagocytosis, as treatment with a mixture of anti-TIM-4 and anti-CD51/CD61 receptor antibodies only minimally decreased the fraction of hemophagocytes in our cell culture model. It is possible that other PS receptors could be responsible for the remaining hemophagocytes after treatment. However, this seems unlikely due to the complete lack of an effect on hemophagocyte formation when PS-receptor antibodies were used to treat resting macrophages. In addition, macrophages secrete the anti-inflammatory

mediators IL-10 and TGF-β1 after PS-mediated cell uptake (Byrne and Reen, 2002; Chung et al., 2007; Huynh et al., 2002; Voll et al., 1997). However, in our model system, ELISA failed to demonstrate a difference in IL-10 levels in supernatants from heterogeneous cultures of hemophagocytes and nonhemophagocytes treated with or without anti-PS receptor antibodies. In addition, TGF-β1 was not detected at the protein level in any conditions tested. In addition, cytokine expression levels of IL-10 and TGF-β1, as monitored by RT-PCR, did not differ between hemophagocytes versus nonhemophagocytes. These data are also consistent with reports that erythrocyte clearance by macrophages in the spleen is independent of phosphatidylserine recognition (Lee et al., 2011; Terpstra and van Berkel, 2000).

Hemophagocytes have been hypothesized to be a survival niche for *S*. Typhimurium *in vivo*. Despite the anti-PS antibody mixture having no effect on the fraction of hemophagocytes in the resting macrophage population, *S*. Typhimurium did not survive as well after anti-PS antibody mixture treatment. The anti-TIM-4 antibody used in the anti-PS antibody mixture has been reported to increase LPS-induced NFκB signaling and macrophage activation (Xu et al., 2010, 2015). Therefore, the decreased survival of *S*. Typhimurium after treatment with the anti-PS antibody mixture may be due to increased macrophage activation.

Although IFN-γ is not required to induce hemophagocytosis in our model, we had previously observed that IFN-γ treatment increases the fraction of hemophagocytes upon TLR stimulation, or upon infection, as compared to resting macrophages (McDonald et al., 2015; Pilonieta et al., 2014). Work presented here demonstrates IFN-γ is required to increase the fraction of hemophagocytes in response to an increased

concentration of erythrocytes upon exposure to live or heat-killed *S.* Typhimurium. The fraction of hemophagocytes in the resting macrophage population remained unchanged in the presence of an increased concentration of erythrocytes. Thus, IFN-γ may drive expression of a receptor, leading to erythrocyte uptake. Preliminary data obtained by Dr. Heidi Nick show that CD163 is more highly expressed on hemophagocytes than nonhemophagocytes (data not shown). CD163 is a scavenger receptor that binds haptoglobin-hemoglobin complexes that form after erythrocyte lysis. CD163 expressing macrophages have been identified in humans with MAS and many other inflammatory conditions (Graversen and Moestrup, 2015; Grom and Mellins, 2010). A role for the CD163 receptor in phagocytosis of intact blood cells has not been described, although CD163 expression is negatively regulated by IFN-γ treatment (Buechler et al., 2000).

In contrast to IFN-γ treatment, macrophages tolerized by repeated exposure to LPS had a decreased fraction of hemophagocytes. Tolerized macrophages have increased phagocytic capability due to increased expression of opsonin-dependent receptors and scavenger receptors (Biswas and Lopez-Collazo, 2009; del Fresno et al., 2009; Jing et al., 2013); thus, these particular classes of receptors may not be involved in hemophagocytosis, as a decreased fraction of tolerized macrophages were hemophagocytes. Microarray analyses have reported differences in gene expression between tolerized and non-tolerized macrophages: Tolerized macrophages have decreased expression of certain receptors on the plasma membrane, such as DARC, the Duffy antigen/chemokine receptor, and CD44, an adhesion receptor, and tolerized macrophages undergo strong down regulation of the TLR4 to NFκB signaling pathway (Lehner et al., 2001; Nomura et al., 2000; Pena et al., 2011; Sly et al., 2004). This

supports our model that NFkB signaling is required for the induction of hemophagocytosis (McDonald et al., 2015). Furthermore, these observations support a model whereby a key receptor(s) is down regulated, most likely a NFκB-regulated receptor, leading to a decreased fraction of hemophagocytes.

Previous work demonstrated that TLR signaling is required for *S*. Typhimurium-induced hemophagocytosis. HK *BA* did not induce hemophagocytosis in our cell culture model, most likely due to the long acyl chains on lipid A hindering recognition by TLR4 (Barquero-Calvo et al., 2007; Conde-Álvarez et al., 2012; Park et al., 2009). While others have shown that hemophagocytes can accumulate upon a single injection of C57BL/6 mice with $5x10^8$ heat-killed *B. abortus* (Gardenghi et al., 2014), their model used a dose of heat-killed bacteria optimized such that 80%-90% of the mice developed inflammation or anemia (Sasu et al., 2010). Therefore, the use of 25-fold less HK *BA* used in the current study most likely resulted in a lack of TLR stimulation, and thus, macrophages were not induced to hemophagocytose.

In conclusion, we have shown that *S*. Typhimurium- and LPS-induced hemophagocytosis does not require erythrocyte surface PS. A mixture of PS-receptor antibodies only minimally reduced the fraction of IFN-γ-treated macrophages that hemophagocytosed and had no effect on the resting macrophage population. The lack of an effect in the resting macrophage population strongly suggests that PS-recognition is not the major mechanism driving hemophagocytosis. Evidence for a specific receptor/ligand interaction driving hemophagocytosis is supported by the decrease in hemophagocytes upon endotoxin tolerance, which suggests expression of a key surface receptor is down regulated. Furthermore, IFN-γ-treatment increases hemophagocytes

in response to an increased concentration of erythrocytes, demonstrating that IFN-γ likely drives increased expression of a receptor involved in hemophagocytosis.

IV. Materials and Methods

Bacterial strains and growth conditions

Salmonella enterica subsp. enterica serovar Typhimurium wild-type strain SL1344 transduced with GFP and a Kanamycin resistance cassette at *rpsM* (SM022) (Vazquez-Torres et al., 1999). For fluorescence dilution experiments, we found that use of pDiGc, which encodes GFP under the control of *rpsM*, significantly hindered *Salmonella* infection, presumably due to high GFP expression from multiple plasmid copies. Thus, strains were transformed with pDiGi and we employed P22 phage transduction to mark strains with GFP and kanamycin resistance at the *rpsM* locus to enable identification of bacteria by flow cytometry. Strains were induced overnight in media containing 170 mM MES pH 5.0, 5 mM KCl, 7.5 mM $(NH_4)_2SO_4$, 0.5 mM K_2SO_4, 1 mM KH_2PO_4, 10 mM $MgCl_2$, 0.3% glycerol, 0.1% casamino acids, 10 mM arabinose with appropriate antibiotics prior to infection (Helaine et al., 2010). *S*. Typhimurium was grown overnight at 37°C in Luria-Bertani broth with aeration. Antibiotics were used at the following concentrations: streptomycin, 30 μg/mL, ampicillin, 300 μg/mL and kanamycin, 30 μg/mL.

Animals

Sv1296S/SvEvTac (Sv129) mice were bred in-house and maintained in a pathogen free barrier facility. All experiments involving mice were approved by the IACUC at University of Colorado, Boulder. Mice were fasted for 4 h prior to orogastric inoculation with a total of 1x10^9 CFU in 100 uL PBS; a kanamycin-resistant strain of

Salmonella expressing GFP driven by the ribosomal promoter *rpsM* (SM022) (Vazquez-Torres et al., 1999) were used for the *in vivo* hemophagocytosis studies. Control mice were orogastrically inoculated with 100 uL PBS. The infectious dose was verified by plating for CFU on selective LB agar. Mice were monitored daily for signs of poor grooming. At one week and two weeks post-inoculation, infected mice and control mice were euthanized by CO2 asphyxiation followed by cervical dislocation. Spleen, liver, mesenteric lymph nodes, Peyer's patches, and cecum were collected, homogenized in 1 mL PBS, and then serially diluted for plating to enumerate CFU.

Bone Marrow-Derived Macrophage (BMDM) generation

The University of Colorado Institutional Committee for Animal Care and Use approved research protocols. Bone marrow derived macrophages (BMDMs) were derived using 30%-35% 3T3-MCSF cell-conditioned supernatant as previously described (McDonald et al., 2015; Pilonieta et al., 2014). Briefly, marrow was flushed from the femurs and tibias of 2- to 4-month-old 129SvEvTac mice (Taconic Laboratories), C3H/HEJ ($TLR4^{-/-}$), or C3H/HeOuJ ($TLR4^{+/+}$) mice (Jackson Laboratories). Cells were resuspended in 1X Phosphate Buffered Saline (PBS) and penicillin-streptomycin (50 IU/mL penicillin and 50 μg/mL streptomycin). Cells were overlaid onto an equal volume of Histopaque-1083 (Sigma-Aldrich) and centrifuged at 500 x g for 20 min. Monocytes at the interface were harvested, washed twice in 1X PBS and penicillin-streptomycin, resuspended in Dulbecco modified Eagle medium (DMEM) (Sigma-Aldrich) supplemented with fetal bovine serum (10%), L-glutamine (2 mM), sodium pyruvate (1 mM), beta-mercaptoethanol (50 μM), HEPES (10 mM), penicillin-streptomycin (50 IU/mL penicillin and 50 μg/mL streptomycin) and 30%

macrophage-colony stimulating factor (M-CSF; to promote differentiation of adherent monocytes into macrophages) supernatant from 3T3 fibroblasts. The bone marrow progenitor cells were seeded at 1e5 cells/mL (3 mL for 6-well plates and 1 mL for 24-well plates) and were incubated for 7 days at 37°C in 5% CO_2. The cells were fed at day three with 2 mL of the M-CSF supplemented media described above.

BMDM uptake of erythrocytes and Jurkats

Where indicated, BMDMs were pre-treated with 20 U/mL of IFN-γ for 18-24 hours prior to the addition of erythrocytes, bacteria, and/or purified bacterial molecules. After IFN-γ treatment (or after leaving the cells resting; resting cells were treated with same volume as PBS as their IFN-γ treated counterparts), the fold-replication of the BMDMs was determined: BMDMs were washed, scraped and used to make a 1:10 dilution in trypan blue. These cells were then counted and the fold replication determined. Murine erythrocytes were freshly isolated by cardiac puncture, harvested by centrifugation for 10 minutes. For Figures 3-8, 3-9, and 3-10, erythrocytes were added to the BMDMs at a 1:1 or 10:1 erythrocyte:BMDM ratio. For Figures 3-4, 3-5, 3-6 and 3-7, 1x10^8 erythrocytes/mL were stained with 0.5 μM Cell Tracker Deep Red (Thermo Fisher Scientific) for 30 minutes, washed twice in PBS, resuspended in a final volume of DMEM, and then added to the BMDMs at the appropriate concentration. This dye is detected in the APC channel and removes the need to permeabilize fixed macrophages and staining with a primary antibody to TER119 (an erythrocyte marker). For the studies using purified bacterial molecules, LPS (Sigma-Aldrich) was added at 20 ng/mL and LTA (Sigma-Aldrich) at 0.01 mg/mL. For addition to BMDMs, bacterial molecules or heat-killed bacteria were resuspended to their final concentration in complete DMEM

supplemented with gentamicin (10 μg/mL), along with the appropriate concentration of erythrocytes. Heat-killed *B. abortus* was obtained from R.M. Roop II (East Carolina University). For infection with live *S*. Typhimurium, macrophages were washed 30 minutes after infection and incubated for 1.5 hours at 37°C in fresh media supplemented with gentamicin (100 μg/mL) to kill extracellular bacteria. Media was then exchanged for media supplemented with gentamicin (10 μg/mL) to prevent extracellular bacterial growth. Erythrocytes were added at the appropriate concentration after the final media change. For treatment with the anti-PS receptor antibodies, a mixture of αCD51 clone RMV-7 (Biolegend), αCD61 clone C29.G2 (Biolegend), αTIM-4 clone RMT4-54 (Biolegend), or isotype controls, 5 μg/mL each, was used. Eight hours after infection with live *S*. Typhimurium or exposure to LPS, the mixture of anti-phosphatidylserine receptor antibodies (αCD51, αCD61, αTIM-4, or isotype controls; 5 μg/mL each) was spiked into each well. Ten hours after addition of the mixture, the cells were processed for flow cytometry.

Fluorescence Dilution Protocol

For fluorescence dilution experiments, bacteria from overnight cultures grown in fluorescence-dilution inducing media overnight with 10 mM arabinose and appropriate antibiotics (Helaine et al., 2010) were washed in 1x PBS and then diluted to 3x10^7 cfu/ml in complete cell culture media and used to infect BMDMs. We found that this protocol reproducibly yielded robust (70-80%) infection of BMDMs as measured by flow cytometry. After 45 minutes, media was exchanged for media containing 100 μg/ml gentamicin to kill extracellular bacteria. At 2 hours post infection, media is exchanged for media containing 10 μg/ml gentamicin. Fix 100 uL of the inoculum by first washing

the inoculum in 1X PBS and then resuspending in 1.6% paraformaldehyde for at least 2 minutes; store protected from light at 4oC. Erythrocytes were added at the appropriate concentration after the final media change. At each time point, wash the wells three times with 1 mL of 1X PBS. Add 100 μL of 0.1% Triton X-100 and incubate at room temperature for 10-15 minutes. After macrophages are lysed, add 500 μL of 1X PBS to each well. Transfer 300 μL into a U-bottom 96-well plate and centrifuge at 2547xg (3500rpm) for 20min at 10oC; use remaining 300 uL to plate for colony forming units (CFU). After spin, resuspend the pelleted bacteria in 100 μL of 1.6% paraformaldehyde and incubate for 10 minutes at room temperature (or 2-24 hours at 4oC). After bacterial cells are fixed, centrifuge at 2547xg (3500rpm) for 20min at 10oC and resuspend in 400 μL of 1X PBS. Samples were run on a CyAn ADP flow cytometer (Beckman Coulter) with appropriate compensation controls. Compensation controls include uninfected lysed macrophages (to distinguish macrophage debris from GFP+ bacteria), WT SL1344 in FD-inducing media with arabinose at 10 mM, pDiGc SL1344 (Alr1212; FITC control) in FD-inducing media without arabinose, and pDiGi (Alr1211) in FD-inducing media with arabinose at 10 mM (PE control). Data were analyzed with FlowJo. Samples were gated for GFP-positive bacteria. Bacterial fold replication was calculated as R0/R, where R is the DsRed geometric mean of the GFP+ population at 18 hours and R0 is the DsRed geometric mean of the GFP+ population at two hours.

Oxidized RBC protocol

Murine erythrocytes were freshly isolated by cardiac puncture, harvested by centrifugation for 10 minutes. Erythrocytes were resuspended at 1x10^8 cells/mL and stained with 0.5 μM Cell Tracker Deep Red (as described above). After staining and

washing the cells, the erythrocytes were resuspended at 8x10^6 cells/mL in PBS. These cells were then spun down and resuspended in 1 mL of oxidation buffer (0.2 mM $CuSO_4$; 5 mM L-ascorbate in PBS; oxidation buffer must be prepared immediately before use. L-ascorbic acid is extremely light sensitive when in solution.). Incubate erythrocytes at 37oC in the dark for 90 minutes. Erythrocytes were washed twice with 0.2% EDTA in PBS and washed a final time in 1x PBS before being resuspended in 1 mL PBS. To stain erythrocytes with Annexin-A488, aliquot 4e6 erythrocytes/mL to tubes. Pellet the erythrocytes and remove the supernatant. Resuspend in 100 uL of 1X Annexin Binding Buffer (ABB). Add 2 uL of Annexin-A488 and stain for fifteen minutes at room temperature. Keep cells protected from the light. After fifteen minutes, add 400 uL of 1X PBS and spin down. Wash the cells with 2 mL of ABB. Spin down the erythrocytes and resuspend in 500 uL ABB and transfer to a FACS tube and run flow cytometry immediately (Sambrano et al., 1997; Tanaka et al., 2001).

ELISAs

Primary macrophages were isolated and differentiated as described above. Supernatant was collected at the end of the 18 hour time point for each experiment. The supernatant was spun down at 500 x g at 4°C for 10 minutes to pellet debris and cells. The supernatant was removed and stored at -20°C. Quantikine ELISAs (R&D Systems) were performed for mouse IL-10 and mouse/rat/porcine/canine TGF-β1. All ELISAs were performed according to the manufacturer's instructions. For the TGF-β1 ELISA, a complete DMEM control was run to determine the baseline concentration of TGF-β1 in the media, which is present in the 10% FBS used to supplement the cell culture medium (as per the manufacturer’s instructions).

Nitrite Measurement

Primary macrophages were isolated and differentiated as described above. Supernatant was collected at the end of the 18 hour time point for each experiment. The supernatant was spun down at 500 x g at 4°C for 10 minutes to pellet debris and cells. 50 μL of the supernatant was removed and added to a 96 well flat bottom plate and allowed to reach room temperature. 50 μL of the Griess reagent (Sigma) was added. After 15 minutes, the absorbance at 540 nm was read.

RT PCR

Primary macrophages were isolated and differentiated as described above. After differentiation, the macrophages were pre-treated with 20 U/mL of IFN-γ for 18-24 hours prior to the addition of Cell-Tracker Deep Red stained erythrocytes (10:1 erythrocyte:BMDM ratio) and LPS (20 ng/mL). After 18 hours, the media was removed and the cells were washed twice with 5 mL of 1X PBS. Next, external erythrocytes were lysed using 500 mL of 1X ammonium chloride-potassium bicarbonate (ACK) lysis buffer (Bossuyt et al., 1997) and harvested by gentle scraping. Cells were washed twice in 5 mL of 1X PBS and stained with Live/Dead Near-IR stain (Life Technologies) at 1:3000 for 20 minutes at 4°C. After staining, the cells were washed twice and then pipetted into a Falcon 5 mL round bottom polystyrene test tube with cell strainer snap cap (has a 35μm nylon mesh). Cells were two-way sorted by the MoFlo XDP Cell sorter after compensation; live nonhemophagocytes and live hemophagocytes were collected. After collection, the cells were spun down at 500xg for 10 minutes at 4°C. RNA was isolated using the RNeasy Mini kit (Qiagen), including on-column DNase treatment, according to the manufacturer's instruction. First-strand cDNA was synthesized from

250 ng of total RNA using the iScript cDNA synthesis kit [Bio-Rad; 170-8891; a blend of oligo(dT) and random hexamers] according to the manufacturer's protocol. The cDNA was subsequently diluted 10-fold with nuclease-free water. RT-PCR assays were carried out using *Hprt* and *Gapdh* for normalization; validation experiments determined that the Ct values for each reference gene were not different from each other in the nonHM vs HM populations (Figure 3-11). The qRT-PCR mixtures (25 µl) contained 10 µL of 10-fold diluted cDNA, 1 µL of each the forward and reverse primers (equivalent to a final concentration of 0.2 µM of each primer; both primers at 5 µM working concentration), 10 µL of the 2x SYBR Green Master Mix (Thermo Fisher Scientific) and 3 µL of nuclease-free water. Reactions were run on a Mastercycler RealPlex 2 (Eppendorf) under the following cycling conditions: 10 min at 95°C, and then 40 cycles at 95°C for 15 s and 60°C for 60 s. No-RNA and no-reverse transcriptase controls were included for each gene assayed. Amplification results were baseline corrected using the epRealplex software (Eppendorf), and the quantification cycle (*Cq*) threshold for each primer pair was recorded. The data were then exported to Microsoft Excel for further analysis. The relative gene expression was determined according to the Livak and Schmittgen method (Livak and Schmittgen, 2001) as follows: (i) *IL-10*, *TNF-alpha*, and *TGF-β1* expression for each sample were normalized to the geomean of the Ct of *Hprt* and *Gapdh* (ΔC_T); the mean of the target genes of the nonhemophagocytes was set as the calibrator for calculation of the $\Delta\Delta C_T$ values for each sample; (iii) the fold difference in target gene expression for each sample was then calculated using the $2^{-\Delta\Delta CT}$ equation. Reaction efficiencies for each primer-probe set were assessed by performing real-time PCR on serial 10-fold dilutions of cDNA, plotting the threshold cycle (C_T)

values for each dilution against the log of the input concentration, and determining the efficiency using the following equation: efficiency = $-1 + 10^{(-1/\text{slope})}$. Statistical analysis of the mean fold changes between nonhemophagocytes and hemophagocytes was carried out using one-way analysis of variance (ANOVA).

Gene	Forward and Reverse Primer (5' → 3')	Product (bp)	E (%)	References
GAPDH	AGG AGA GTG TTT CCT CGT CCC ACG GCC AAA TCC GTT CAC AC	63	98	Primer3
HprT	CAC AGG ACT AGA ACA CCT GC GCT GGT GAA AAG GAC CTC T	1,087	105.3	(Keller et al., 1993)
IL-10	CAG CCG GGA AGA CAA TAA CTG GCA GCT CTA GGA GCA TGT	65	90.72	(Masocha et al., 2008)
TGF-β1	TCG ACA TGG AGC TGG TGA AA GAG CCT TAG TTT GGA CAG GAT CTG	71	93.56	(O'Connell et al., 2013)
TNFα	CGT CAG CCG ATT TGC TAT CT CGG ACT CCG CAA AGT CTA AG	206	89.5	(Yan et al., 2012)
SPIC	TCC GCA ACC CAA GAC TCT TCA A GGG TTC TCT GTG GGT GAC ATT CCA T	1,965	105.8	(Haldar et al., 2014)

Table 3-1. Primers for Reference genes (*GAPDH* and *HprT*) and Target genes

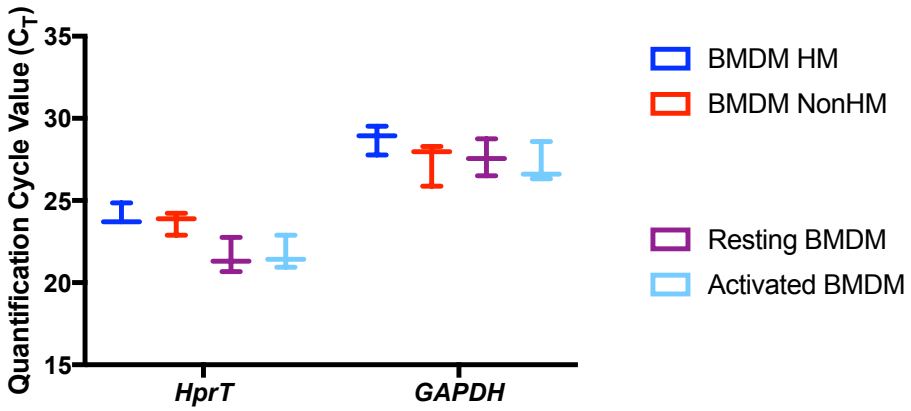

Figure 3-11. C_T Values for reference genes across different conditions

Flow Cytometry for *in vitro* hemophagocytosis assay

Resting or activated (20 U/mL IFNγ for 18-24 hours) BMDMs were washed with 1 mL of 1X PBS and external erythrocytes were lysed using 500 mL of 1X ammonium chloride-potassium bicarbonate (ACK) lysis buffer (Bossuyt et al., 1997) and harvested by gentle scraping. Cells from each condition were equally distributed into 96-well plates and resuspended in 1X PBS. Cells were stained with Live/Dead Near-IR stain (Life Technologies) at 1:3000, followed by incubation in FACS staining buffer (PBS plus 1% fetal bovine serum (FBS), 0.02% azide) containing anti-mouse CD16/32 (eBioscience, San Diego, CA) to block Fc receptors. Cells were then fixed in 1% paraformaldehyde-1% sucrose. For experiments with unstained erythrocytes, the macrophages were then permeabilized in staining buffer with 0.1% saponin for 10-minutes, and then incubated in permeabilization buffer containing 1:200 anti-mouse TER-119-APC (eBioscience). Compensation was performed using single color controls prepared from the resting or activated BMDMs. Fluorescently labeled cells were analyzed using a CyAn ADP flow cytometer (Beckman Coulter) and FlowJo software version 8.8.7 (Tree Star, Inc.). A minimum of 30,000 live cells was collected for analysis. All percentages reported are expressed as a percentage of live cells.

Splenic Cell Preparation (to identify *in vivo* hemophagocytes)

7-week old 129SvEvTac mice (Taconic Laboratories; bred in house) mice were orogastrically inoculated with $1x10^{9}$ SL1344 strain with a chromosomally-integrated rpsM::gfp reporter construct. At one and two weeks post infection, mice were sacrificed and the spleens were placed in a polypropylene tube containing 1 mL Pen/Strep 1X PBS. The spleen was processed in the tissue culture hood. A cell strainer (70 µM) was

placed into a petri dish with 10 mL of DMEM. Using the plunger end of a syringe, the spleen was mashed through the cell strainer into the petri dish. The cell strainer was rinsed with an additional 5 mL DMEM. The suspended cells were transferred to a 15 mL conical. The cells were spun at 500xg for 3 minutes. The supernatant was discarded and the pellet was re-suspended in 1 mL 1x ACK Lysis Buffer and incubate at RT for 5-10 minutes. 9 mL of DMEM was added and the cells were spun as before. The pellet was re-suspended in 1 mL PBS, and any dead cell mass was discarded. 10 µL of the cell suspension was diluted in trypan blue and was counted using a hemocytometer. For flow cytometry immunophenotyping experiments, 2×10^{6} cells to 6×10^{6} cells per tube were stained as described below.

Flow Cytometry for *in vivo* hemophagocytes

Cell viability was assessed by incubating cells in the LIVE/DEAD Fixable Aqua Dead Cell Stain (Thermo Scientific) at a 1:3000 dilution in 1X PBS for 30 minutes in the dark at 4°C, followed by a single wash in 1x PBS. For all experiments, cells were incubated in anti-mouse CD16/32 (eBioscience, San Diego, CA) for 10 minutes at RT. Surface staining was performed in the dark for 30 minutes at 4°C in FACS staining buffer. Cells were then washed twice with FACS followed by fixation in a 1% paraformaldehyde (Fisher Scientific) and 1% sucrose solution in 1X PBS. A comprehensive list of surface markers for these experiments includes: CD45R (B220) clone RA3-6B2 PE (1:250, BD Pharminogen), Ly-6G clone IA8 PE (1:500, BD Pharminogen), NK-1.1 clone PK136 PE (1:160, BD Pharminogen), CD11c clone HL3 PE (1:125, BD Biosciences), CD11b clone M1/70 eFluor 450 (1:160, eBioscience), GR-1 clone RB6-8C5 PE Cy-7 (1:250, eBioscience), and TER119 APC (1:200,

eBioscience). For flow cytometry experiments, a CyAN ADP cytometer (BD Immunocytometry Systems) equipped with 405 nm, 488 nm, and 640 nm excitation lasers was used. This cytometer was located at the University of Colorado Boulder Flow Cytometry Core Facility. Data analyses were performed using FlowJo software (Tree Star, Ashland, OR, USA). Fluorescence minus one (FMO) controls were used for gating analyses to distinguish positively from negatively staining cell populations. Compensation was performed using single color controls prepared from either BD Comp Beads (BD Biosciences) or splenic cells. HMs are rare events. The average frequency of HMs in the infected splenic population is 0.16% for Week 1 and 0.04% for Week 2. The coefficient of variation allows one to compare variation between populations; when studying rare events, it describes the number of live events you need to collect to obtain a number of rare events that can be used for statistical analysis. For a coefficient of variation of 10% for 0.1% HMs, 100,000 live cells must be collected. For a coefficient of variation of 10% for 0.01% HMs, 1,000,000 live cells must be collected. The average number of live cells collected for Week 1 was 141,365 cells; the average number of live cells collected for Week 2 was 944,340 cells. Compensation matrices were calculated and applied using FlowJo software (Tree Star).

After exclusion of debris and doublets, a live/dead stain was used to exclude nonviable cells. From this population of viable cells, cells positive for a subset of lineage markers (Neutrophils, NK and NK-T cells, Dendritic cells, and B-cells) were excluded using a dump channel. The lineage negative population, which contains macrophages, monocytes, and eosinophils, was further classified as CD11b+ or CD11b-. Inflammatory monocytes and resident macrophages are CD11b+ GR-1high and

CD11b+ GR-1low, respectively. The two gates subdividing GR-1high from GR-1low do not overlap, therefore there are some monocytes that are not counted as resident or inflammatory. The two monocyte subpopulations were then further analyzed for positive staining of TER119 (an erythrocyte specific protein; associates with the membrane of erythrocytes at all stages of erythrocyte development) (Chen et al., 2009). To calculate total hemophagocytes out of total live monocytes, I used a TER119+ gate on the CD11b+ population. Since some hemophagocytes will be found in between the GR-1high and GR-1low gate, the fraction of resident hemophagocytes out of total hemophagocytes and the fraction of inflammatory monocyte hemophagocytes out of total hemophagocytes will not add up to 100%.

Statistics

Data were analyzed using JMP Pro Version 11.2.0. (SAS Institute Inc.) and Prism 7. Two-way comparisons were analyzed with a Student's t-test (for parametric data). Multiple comparisons were analyzed by ANOVA with a Tukey's (parametric data) or Dunnett's (nonparametric data) post-hoc test.

Chapter 4 Closing Remarks and Future Directions

Introduction

Hemophagocytes are an indicator of severe inflammatory conditions and are key diagnostic criteria for the clinical syndrome HLH. Primary HLH is identified in infants due to genetic lesions in granule-mediated cytotoxicity. Secondary HLH is caused by infection, malignancies, or autoimmune disorders and usually occurs much later in life. A common feature of both syndromes is that infection is the trigger: Lymphocytes become hyperactivated due to lack of immune regulation and pathogen clearance. A clinical marker, and the hallmark feature, used in the diagnosis of HLH and other hyperinflammatory syndromes is the presence of hemophagocytes, a subset of macrophages that have engulfed erythrocytes, platelets, and/or white blood cells(Brisse et al., 2015b; Canna and Behrens, 2012; Henter et al., 2007). The underlying mechanism of blood cell uptake during this inflammatory condition remains poorly understood.

The relationship between microbe detection by TLRs and the induction of hemophagocytosis was undefined at the time I began my studies. TLR agonists had been utilized to induce secondary/infection-associated HLH in various animal models, but the requirement for TLR signaling in stimulating macrophages to hemophagocytose had not been discovered. Furthermore, the ligand-receptor interactions required for infection- and TLR-induced hemophagocytosis are yet to be identified. Using co-cultures of primary bone marrow derived macrophages and erythrocytes I demonstrated that structurally conserved microbial products stimulate macrophages to hemophagocytose through Toll-like receptor (TLR) recognition and subsequent NF-κB signaling (McDonald

et al., 2015). In addition, I provide evidence that hemophagocytosis in this context does not require erythrocyte externalization of phosphatidylserine (PS).

TLR4 signaling is required for STm-induced hemophagocytosis

We developed a cell culture model to study infection-induced hemophagocytosis (McDonald et al., 2015; Nix et al., 2007; Pilonieta et al., 2014). Both tissue resident and circulating phagocytic cells have the capacity to hemophagocytose (Brisse et al., 2015a; Brown et al., 2010; Ohyagi et al., 2013). Erythrophagocytosis is the engulfment of senescent or damaged erythrocytes by phagocytic cells, such as macrophages and dendritic cells. Although both processes involve engulfment of blood cells, the contexts in which these processes occur are very different. Erythrophagocytosis is a normal turnover process that occurs in healthy tissues, whereas excessive hemophagocytosis is associated with inflammation and disease. Erythrophagocytosis is modeled in cell culture by incubating macrophages with aged erythrocytes or chemically oxidized erythrocytes, to mimic senescence. Damaged erythrocytes are engulfed by macrophages at much higher rates than healthy non-senescent erythrocytes, and this process occurs very quickly, within minutes to a few hours. Externalization of PS and decreased expression of CD47 on erythrocytes are thought to be the primary drivers of rapid erythrophagocytosis (Burger et al., 2012; Gottlieb et al., 2012; Oldenborg et al., 2000). Primary macrophages do not phagocytose healthy erythrocytes to a significant extent; only 2.8% of human peripheral blood monocytes phagocytose or have adherent erythrocytes when co-cultured with non-senescent erythrocytes for three hours (Biondi et al., 2002) and less than 5% of bone-marrow derived macrophages from C57Bl/6J mice phagocytose erythrocytes from healthy control mice after 90 minutes of co-culture

(Gottlieb et al., 2012). In our hemophagocytosis cell culture model, less than 3% of bone-marrow derived macrophages from SV129 mice hemophagocytose erythrocytes between six to eighteen hours of co-culture (McDonald et al., 2015). Furthermore, less than 2% of erythrocytes co-cultured with primary macrophages for eighteen hours externalize phosphatidylserine, as evidenced by AnnexinV binding (Figure 3-4 G; Appendix B-1). It is only upon prolonged TLR stimulation, via TLR agonists or infection with *S*. Typhimurium, that an increased fraction of bone-marrow derived macrophages engulf erythrocytes (McDonald et al., 2015; Pilonieta et al., 2014).

I show here that TLR4 is required for the induction of hemophagocytosis during *S*. Typhimurium infection of primary macrophages. In this work, I demonstrated that prolonged exposure to TLR agonists and NF-κB activation were required to induce hemophagocytosis. Specifically, Pilonieta et al., and I demonstrated that infection with *S*. Typhimurium or *Yersinia pseudotuberculosis*, but not *Escherichia coli*, stimulated hemophagocytosis. Further work suggested that this was due to prolonged survival within macrophages (*E. coli* does not survive in macrophages). To support the hypothesis that prolonged TLR signaling is key, I found that exposure to heat-killed bacteria, or exposure to purified TLR agonists, for greater than twelve hours stimulated hemophagocytosis. These results are consistent with animal models of secondary HLH requiring extended immune activation. C57BL/6 mice intraperitoneally injected with high concentrations of heat-killed *B. abortus* develop anemia and accumulate hemophagocytes and BALB/c mice infected with Murine Cytomegalovirus develop secondary HLH because the mice are unable to clear the virus (Brisse et al., 2016b; Gardenghi et al., 2014). Wild-type C57BL/6 mice injected with a TLR9 agonist develop

an HLH/MAS-like syndrome, and IFN-$\gamma^{-/-}$ mice injected with Freund's complete adjuvant containing heat-killed mycobacteria develop features of systemic juvenile idiopathic arthritis and HLH (Avau et al., 2014; Behrens et al., 2011; Ohyagi et al., 2013). Hemophagocytosis is observed in all the above models.

Using chemical and genetic approaches, I established a requirement for TLR signaling in *S*. Typhimurium-induced hemophagocytosis. TAK242, a chemical inhibitor of TLR4 signaling, blocks association of the TLR4 signaling domain with TIRAP and TRAM adaptor proteins and prevents both MyD88-dependent and -independent signaling. In both resting and IFN-γ-pretreated macrophages, TAK242 treatment inhibited LPS and bacterium-induced hemophagocytosis. To confirm a role for TLR4 in hemophagocyte formation, I compared TLR4-/- and TLR4+/+ macrophages; LPS treatment or infection with live *S*. Typhimurium stimulated hemophagocyte formation only in the presence of a functional TLR4 receptor. Thus, TLR4 is required for hemophagocytosis in response to LPS or *S*. Typhimurium, and LPS appears to be the major component of *S*. Typhimurium that stimulates hemophagocytosis.

A key feature of our model is that IFN-γ is not required for infection-induced hemophagocytosis. This is in contrast to primary HLH animal models, where IFN-γ is required for the development of HLH. In this work, I showed that primary macrophages become hemophagocytic in the absence or presence of interferon-γ upon infection with Gram-negative bacterial pathogens or prolonged exposure to heat-killed species of *S*. Typhimurium*, Y. pseudotuberculosis, E. coli,* or *Mycobacterium marinum*, all of which are known to activate TLR4. This is consistent with other animal models of secondary HLH. Brisse et al. showed that IFN-γ plays a protective role in MCMV induced HLH and

Avau et al. 2014 established that hemophagocytes and HLH can develop in the absence of IFN-γ. Hemophagocytes also develop in C57BL/6 mice exposed to high levels of IL-4, and IFN-γ neutralization does not inhibit hemophagocyte formation in this model (Milner et al., 2010).

Hemophagocytosis is likely driven by a receptor-ligand interaction

Although IFN- γ is not required to induce hemophagocytosis in our model, IFN-γ treatment did increase the fraction of hemophagocytes upon TLR stimulation or upon infection as compared to resting macrophages. Also, IFN- γ was required to increase the fraction of hemophagocytes in response to an increased concentration of erythrocytes upon exposure to live or heat-killed *S*. Typhimurium; the fraction of hemophagocytes in the resting macrophage population remained unchanged in the presence of an increased concentration of erythrocytes. Thus, IFN-γ may drive expression of a receptor, leading to erythrocyte uptake.

Our cell culture model also demonstrates that other TLR agonists stimulate hemophagocytosis. TLR2 and TLR9 agonists only induced hemophagocytosis in IFN-γ-primed macrophages. TLR2 surface levels are decreased on resting macrophages, but IFN-γ treatment increases surface expression and sensitivity of TLR2. Similarly, IFN-γ treatment increases TLR9 signaling by increasing macrophage surface expression of pattern recognition receptors that bind microbial DNA (receptors include Mannose Receptor 1, and MARCO) and by recruiting TLR9 to endolysosomes, where lysosomal proteases activate TLR9 (Gordon, 2002; Józefowski et al., 2006; Moseman et al., 2013; Mouchess et al., 2011). Once these pattern recognition receptors have bound microbial DNA, they are taken up by endosomes, which fuse with TLR9 containing

endolysosomes. The microbial DNA dissociates from the PRR and binds to TLR9 to induce signaling. Increased surface levels of TLR2 and DNA-binding receptors (such as MARCO) may explain why TLR2 agonists and a TLR9 agonist only stimulated hemophagocytosis in IFN-γ-treated macrophages.

TLR4 activation via exposure to *S*. Typhimurium LPS induced hemophagocytosis of Jurkats (white blood cells). This is consistent with reports of *S*. Typhimurium infection resulting in F4/80+ splenic macrophages co-localizing with and engulfing CD4+ and CD8+ T-cells (Rosche et al., 2015). In contrast, the alpha-proteo bacterium *Brucella abortus* has an LPS that is not well recognized by TLR4 due to long acyl chains on its lipid A LPS core (Barquero-Calvo et al., 2007; Conde-Álvarez et al., 2012; Park et al., 2009). Heat-killed *B. abortus* did not induce hemophagocyte formation at the dosage (1x10^7 bacteria/mL) tested. While others have shown that hemophagocytes can accumulate upon a single injection of C57BL/6 mice with 5x10^8 heat-killed *B. abortus*, their model used a dose of heat-killed bacteria optimized such that 80%-90% of the mice developed inflammation or anemia (Gardenghi et al., 2014; Kim et al., 2014; Sasu et al., 2010). Furthermore, it has been shown that 5,000 times more *Brucella* LPS than *S*. Typhimurium LPS is needed to induce macrophages to secrete comparable levels of TNF-α, a TLR4 regulated process (Weiss et al., 2005). These data suggest that *Brucella* LPS does not stimulate sufficient TLR4 activity to drive macrophages to hemophagocytose. I used 25-fold less *B. abortus* in my *in vitro* experiments and thus most likely did not stimulate enough of a response to induce macrophages to hemophagocytose. This supports the model that robust activation of TLR4 is critical for the induction of hemophagocytosis in response to infection with Gram-negative

pathogens (McDonald et al., 2015). Therefore, the dosage of heat-killed *Brucella* used, the lack of robust activation of TLR4 by *Brucella* LPS, combined with the fact that the major component of heat-killed *Brucella* sensed by TLRs are lipoproteins, a TLR2 agonist, make a compelling case as to why *B. abortus* did not stimulate hemophagocytosis in our cell culture model (Giambartolomei et al., 2004; Huang et al., 2003).

Previous work in the lab showed that primary macrophages activated with both LPS and IFN-γ and then infected with *S*. Typhimurium have a smaller fraction of hemophagocytes than macrophages activated with IFN-γ alone and then infected with *S*. Typhimurium. Macrophages repeatedly exposed to TLR agonists become "tolerant"; these macrophages have a dampened response to LPS and other TLR agonists (Lehner et al., 2001; Sly et al., 2004). In our cell culture model of hemophagocytosis, repeated exposure to TLR agonists decreased the formation of hemophagocytes, with the most dramatic reduction being found in the IFN-γ population exposed to LPS and then to LTA. These results strongly suggest that tolerant macrophages decreased expression of a key receptor or have decreased sensitivity to a key ligand involved in hemophagocytosis. Microarray analyses have reported many differences in gene expression between tolerant and non-tolerant macrophages. Strong differences in gene expression include wound healing proteins, which are strongly up-regulated in tolerized macrophages, and the TLR4 to NFκB signaling pathway, which is strongly down-regulated in tolerized macrophages (Nomura et al., 2000; Pena et al., 2011; Sly et al., 2004). This supports our model of NFκB signaling being required for the induction of hemophagocytosis. Furthermore, there is decreased expression of certain receptors on

the plasma membrane, such as CD44, an adhesion receptor and DARC, the Duffy antigen/chemokine receptor. These observations support a model whereby a key receptor(s) is decreased in expression, leading to a decreased fraction of hemophagocytes.

As stated above, the ligand-receptor interactions required for infection- and TLR-induced hemophagocytosis have yet to be identified. When hemophagocytes accumulate in animals and humans they correlate with pro-inflammatory conditions including increased serum levels of TNF-α (Brisse et al., 2015b; Chang et al., 2006). There are reports that link TNF-α to increased erythrocyte externalization of phosphatidylserine (PS) (Chatterjee, 1999; Lang et al., 2004, 2005). Thus receptors that bind phosphatidylserine were considered potential candidates for hemophagocytosis. Macrophages bind phosphatidylserine via multiple receptors. Two key receptor complexes are TIM-4 and the CD51/CD61 vitronectin complex. TIM-4 is a cell surface glycoprotein expressed on human and mouse macrophages. TIM-4 specifically binds phosphatidylserine (Kobayashi et al., 2007; Miyanishi et al., 2007). Other family members in the TIM family include TIM-1, TIM-2 and TIM-3; TIM-1 and TIM-3 are expressed on T cell subsets, whereas TIM-2 is expressed on B-cells, bile duct epithelial cells and renal tubule cells (Chen et al., 2005). TIM-1 and TIM-4 have a critical domain that contains the ligand-binding cavity for PS; this cavity is missing from TIM-2 (Cao et al., 2007; Santiago et al., 2007) and the entire domain is absent in TIM-2 (Kobayashi et al., 2007). The CD51/CD61 complex is also known as the vitronectin complex. This complex binds the bridging molecule milk factor globule epidermal growth factor 8 (MFG-E8); MFG-E8 binds phosphatidylserine via its discoidin domain (Fadok et al.,

1992b). Treatment with phosphatidylserine receptor antibodies reduced the fraction of IFN-γ-treated macrophages that hemophagocytose, but had no effect on the resting macrophage population. The lack of an effect in the resting macrophage population strongly suggests that PS-recognition is not the only mechanism driving hemophagocytosis during infection; it could be that IFN-γ driven inflammation damages a certain fraction of erythrocytes, leading to their clearance by engagement of PS receptors, but that the remaining fraction of undamaged erythrocytes is taken up by a different mechanism. Support for this hypothesis comes from *in vivo* studies on erythrophagocytosis by splenic macrophages: erythrocyte clearance in the spleen is independent of PS recognition; PS liposomes did not inhibit uptake of oxidized erythrocytes by splenic macrophages (Lee et al., 2011; Terpstra and van Berkel, 2000).

Evidence for PS recognition being key during viral-induced hemophagocytosis comes from Ohyagi et al. They demonstrated that after injection of 200 μg of CpG DNA into mice, AnnexinV+ erythrocytes in the peripheral blood increased to 30% by 4 hours, but then dropped back below 5% of total peripheral blood cells. This demonstrates that TLR stimulation damages erythrocytes. When Ohyagi et al. used blocking antibodies to TIM-1, TIM-4 and CD51/CD61, the proportion of hemophagocytes 18 hours after CpG injection decreased by approximately 75%, as compared to mice injected with isotype control antibodies. Similarly, I used antibodies to TIM-4 and CD51/CD61 to test PS recognition in my model; TIM-1 was excluded from my analysis because macrophages do not express it. It is possible that other PS receptors, such as MerTK and Brain-specific angiogenesis inhibitor 1 (Nishi et al., 2014; Park et al., 2007) could be responsible for the remaining hemophagocytes after treatment, but this seems unlikely

due to the complete lack of an effect when PS-receptor antibodies were used to treat resting macrophages.

Apoptotic cell uptake is PS-dependent (Fadok et al., 1992b; Hoffmann et al., 2001) and induces IL-10 and TGF-B1 production (Byrne and Reen, 2002; Chung et al., 2007; Voll et al., 1997). Recognition and binding of PS is required for the production of TGF-β1: if apoptotic cells are opsonized and engulfed in a Fcγ receptor-dependent manner, production of TGF-β1 is abolished (Huynh et al., 2002). Production of IL-10 and TGF-β1 after macrophage engulfment of PS+ cells also leads to an inhibition in production of inflammatory cytokines, which leads to arrest and resolution of inflammation (Fadok et al., 1998). I was also specifically interested in IL-10 as not only is it produced upon apoptotic cell uptake, but it is also produced by macrophages upon heme degradation . Senescent erythrocytes are removed by macrophages; the engulfed erythrocytes are broken down into heme and other by-products. Weigel et al. demonstrated that biliverdin, a heme breakdown product, induces the expression of biliverdin reductase (BVR). BVR catalyzes the breakdown of biliverdin into bilirubin, and this activity induces PI3K and AKT signaling, leading to the production of IL-10 (Wegiel et al., 2009, 2011). Furthermore, Ohyagi et al. conclusively demonstrated through the use of reporter mice that hemophagocytic monocytes are the primary producers of IL-10. In my model, both LPS exposure and *S*. Typhimurium infection triggered IL-10 production, consistent with previous findings (Barsig et al., 1995; Uchiya et al., 2004). The addition of PS receptor antibodies did not reduce IL-10 production. Unfortunately, I was unable to distinguish between the effects of heme from erythrocyte breakdown on IL-10 production versus the effects of *S*. Typhimurium infection on IL-10 production. The

IL-10 production in this assay is most likely due to TLR4 activation and not uptake of senescent erythrocytes; macrophages exposed to LPS and either cultured in the presence or absence of erythrocytes produce similar levels of IL-10 (data not shown). Thus, it seems likely that LPS stimulation of TLR4 drives production of IL-10 and heme breakdown does not play a major role. A better approach to analyze the contribution of heme breakdown on IL-10 production would be through the use of TLR4 or BVR knockout macrophages.

Despite the decrease in hemophagocytes in the IFN-γ population after treatment with PS receptor antibodies, I was unable to affect *S*. Typhimurium replication or net survival. This was not too surprising, as I previously showed that hemophagocytes were not more likely to be infected than non-hemophagocytes. Other work in the lab has shown that *S*. Typhimurium replication in macrophages slightly increases upon co-culture with erythrocytes, but that this increase is not dependent on *S*. Typhimurium being in a hemophagocyte. An interesting observation I made while studying *S*. Typhimurium replication during PS receptor antibody treatment was that the supernatants from macrophages co-cultured with erythrocytes had a decreased amount of nitrite (Griess assay). Others have demonstrated that erythrocytes can act as scavengers of nitric oxide (NO): erythrocytes can take up and inactivate NO via hemoglobin (Azarov et al., 2005; Cortese-Krott and Kelm, 2014).

A novel flow cytometry gating scheme identifies hemophagocytes in resident macrophage and inflammatory monocyte subsets

Previously, hemophagocytes were defined as multinucleate cells (i.e., cells containing 3 or more nuclei based on DAPI staining using flow cytometry, designated 6N+ cells) (McCoy et al., 2012). The logic behind this definition was that

hemophagocytes had engulfed nucleated cells, and that the vast majority of these nucleated cells were erythroblasts (an immature erythrocyte that still contains a nucleus). Erythroblasts accumulate during *S*. Typhimurium infection due to regenerative microcytic anemia (Brown et al., 2010; Jackson et al., 2010). This gating scheme did not contain a live/dead stain; instead, debris and dead cells were gated out based on low forward scatter (size) and low side scatter (complexity). The exclusion of a live/dead stain led to the inclusion of dead cells in the 6N+ gate. These dead cells are false positives, as dead cells stain positive for DAPI, which is the marker used to delineate 6N+ cells. McCoy et al. identified $CD68^{+}CD11c^{low/-}GRI^{int}$ macrophages as the main 6N+ cells that accumulate in the spleens of *S*. Typhimurium infected mice.

Previous work in the lab indicated that splenic hemophagocytes were MOMA-2 positive (McCoy et al., 2012; Nix et al., 2007). MOMA-2 is a monocyte marker, and so to delineate whether splenic hemophagocytes were resident macrophages or inflammatory monocytes, I adapted a five color gating scheme to distinguish between these cell types and included the use of the erythrocyte marker TER119, to identify which cells engulfed erythrocytes (Chen et al., 2009; Rose et al., 2012). My improved analysis demonstrated that both resident macrophages and inflammatory monocytes hemophagocytosed. Although the fraction of hemophagocytic monocytes did not differ between control and infected animals, the fraction of inflammatory versus resident hemophagocytes was in proportion to the abundance of their respective monocyte population. Infected mice had significantly more inflammatory monocytes than control mice at both time points, and this corresponded to the significant increase in inflammatory monocytes that had hemophagocytosed at both time points, as compared

to those populations in control mice. Conversely, infection reduced the fraction of resident macrophages and this same reduction was seen in the resident macrophage hemophagocyte population. These data suggest that both resident and inflammatory monocytes are similarly capable of hemophagocytosis.

Future Directions

In conclusion, our data show that TLR4 signaling induces hemophagocytosis in a NF-κB-dependent manner. Also, macrophage recognition of PS on blood cells is not the dominant mechanism of *S*. Typhimurium-induced hemophagocytosis. Thus, questions remain regarding what receptor(s) and ligand(s) drive uptake of blood cells during infection-induced hemophagocytosis. Since our data demonstrate that there is another mechanism at play for the recognition and phagocytosis of blood cells, using an unbiased approach to determine the receptor(s) involved in infection-induced hemophagocytosis is warranted. Such an approach could be taken with a genome-wide CRISPR-Cas9 screen. This type of screen has already been done in primary immune cells (Parnas et al., 2015). To identify genes involved in hemophagocytosis, I propose performing independent biological replicates of a genome-wide pooled CRISPR-Cas9 screen using immortalized BMDMs. Hemophagocytes and nonhemophagocytes can be sorted by FACS and sequenced to identify single guide RNAs differentially enriched in each population. By comparing the enriched genes and pathways between populations, we can identify regulators of hemophagocytosis. Preliminary experiments with immortalized BMDMs demonstrate that they are capable of hemophagocytosing erythrocytes after LPS exposure (data not shown).

A more targeted approach would be to determine expression levels of potential key receptors on hemophagocytes versus nonhemophagocytes. Candidate receptors that I propose looking at include CD11c, a monocyte/macrophage specific marker which binds the erythrocyte-specific marker ICAM-4 (Ihanus et al., 2007), and SIRPα, which binds CD47 on erythrocytes (Lutz and Bogdanova, 2013). CD47 can act as both a "don't eat me" signal and an "eat-me" signal. The CD47-SIRPa interaction on healthy erythrocytes negatively regulated phagocytosis. Aged erythrocytes express a variant of CD47 that binds thrombospondin-1, and these erythrocytes are rapidly engulfed (Burger et al., 2012; Sosale and Discher, 2012). Therefore, a change in either expression (or modification) of the receptor or ligand during macrophage-erythrocyte co-culture could affect the fraction of hemophagocytes. Preliminary data from Dr. Heidi Nick shows that CD163 is more highly expressed on hemophagocytes than nonhemophagocytes; CD163 is a scavenger receptor that binds the haptoglobin-hemoglobin complexes that forms after erythrocyte lysis. CD163 expressing macrophages have been identified in humans with MAS (Grom and Mellins, 2010) and in many other inflammatory conditions (Graversen and Moestrup, 2015). A role for the CD163 receptor in phagocytosis of intact blood cells has not been described.

Another important question remains: what is the biological function of hemophagocytes during hyperinflammatory conditions? In infection-associated HLH, they are hypothesized to play an anti-inflammatory role by producing IL-10 (Ohyagi et al., 2013), and hemophagocytes have been shown to express markers associated with alternatively activated macrophages (Canna et al., 2014; McCoy et al., 2012). It will be crucial to repeat the *in vivo* identification of splenic hemophagocytes in S. Typhimurium

infected Sv129 mice, but earlier time points should be analyzed. At earlier time points, inflammation will be greater and identifying a larger population of hemophagocytes would be made easier. These hemophagocytes could be sorted and single cell analyses, such as RNA-seq, performed. It will be interesting to see if inflammation changes which subsets of cells hemophagocytose, or markers associated with those cell types. Recently, Richards et al. showed that poly(I:C) induced inflammation influenced which phagocytic population in the spleen engulfed erythrocytes: resident macrophages were the main population that engulfed erythrocytes, but after poly(I:C) stimulation, the rate of erythrocyte consumption by tissue macrophages decreased whereas the rate of erythrocyte uptake by inflammatory monocytes and plasmacytoid dendritic cells increased. Lastly, whether the anti-inflammatory role of hemophagocytes is linked to their blood-eating function is a question that remains unanswered.

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Appendix A – Median Fluorescent Intensity Plots

Statistics were not calculated for MFI for multiple reasons. First, MFI is dependent on PMT voltages used; therefore, if voltages were increased or decreased between experiments performed on different days, the MFI values will increase or decrease, respectively. APC voltage was not kept consistent throughout experiments, as it had to be changed to align the negative population over the first decade of fluorescence for each single color control. In addition, MFI is sensitive to laser fluctuations, which occur over time due to instrument use. The effect on MFI due to laser fluctuations and changes in APC voltage could be corrected if the flow cytometer had been standardized for each experiment by running fluorescent beads to confirm a consistent MFI for the APC channel was obtained on each occasion. A second reason statistics were not calculated for MFI values is that MFI cannot be used to quantify expression, unless the antibody used is titrated and its expression calibrated via a standard curve, thus ensuring a saturating concentration of antibody is used for each experiment. This was not feasible for the experiments described above, as the TER119 antibody is used as a marker to denote which macrophages have phagocytosed red blood cells. It is unknown at which point in phagosomal degradation of erythrocytes the TER119 signal is lost. For these two reasons, huge fluctuations in TER119 MFI signal was observed. The third, and most important, reason statistics were not calculated for MFI values is that for some of the rare populations (the fraction of TER119+ macrophages in the erythrocyte only condition, or the fraction of TER119+ macrophages after treatment with TAK242 or BMS-345541) too few live cells were collected for these rare events to be considered true and accurate and robust enough to allow statistical analysis. The current accepted standard is a co-efficient of variation of 5% or better. This means that a minimum of 40,000 to 400,000 live cells must be collected for a frequency of rare events of 1% to 0.1% to be accepted (Dako Cytomation, Guide to flow cytometry). As stated above, a minimum of 10,000 live cells were collected for analysis.

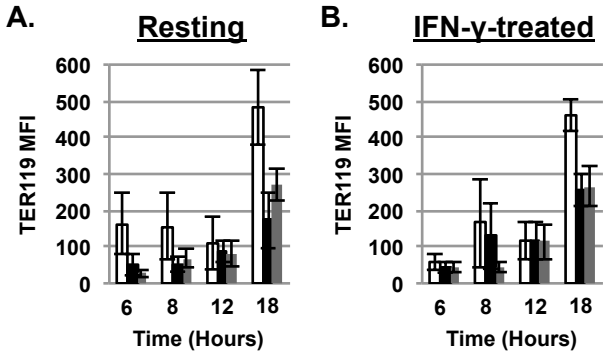

Figure A-1
Resting (A) or IFN-γ-pretreated (B) macrophages were cocultured with erythrocytes at a 1:1 ratio in the absence of bacteria or with STm (MOI of 10), or HK STm (MOI of 10). At the times indicated, extracellular erythrocytes were lysed, and macrophages were processed for intracellular flow cytometry. (A and B) Median Fluorescent Intensity (MFI) of TER119 signal in TER119+ macrophages at the indicated time points (□, without bacteria; ■, with live STm; ■, with HK STm). Means ± the standard errors of the mean (SEM) of aggregated data were determined from three biological replicates. MFI was calculated as described in the methods in Chapter 2.

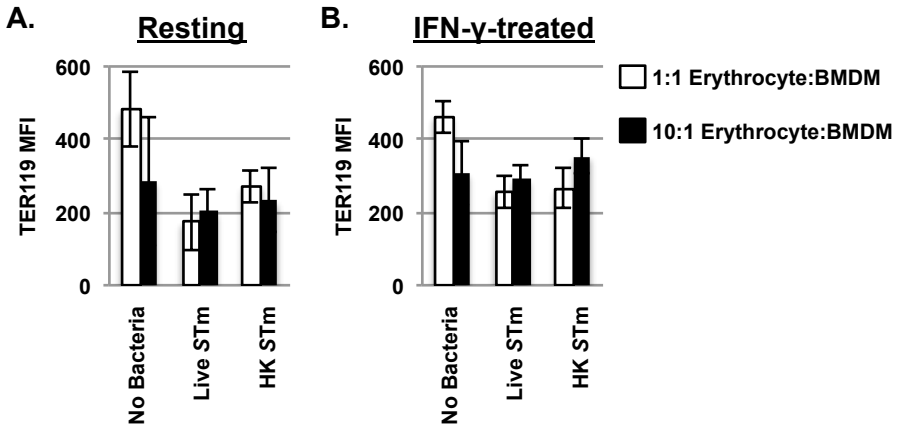

Figure A-2
Resting (A) or IFN-γ-pretreated (B) macrophages were cocultured with erythrocytes at a ratio of 1:1 or 1:10 without bacteria, or with live, RFP-expressing STm, or with heat-killed bacteria, at MOI of 10 for 18 h. Extracellular erythrocytes were then lysed and macrophages processed for flow cytometry. (A and B) Median Fluorescent Intensity (MFI) of TER119 signal in TER119+ macrophages. Means ± the SEM of aggregated data were determined from three biological replicates. MFI was calculated as described in the methods in Chapter 2.

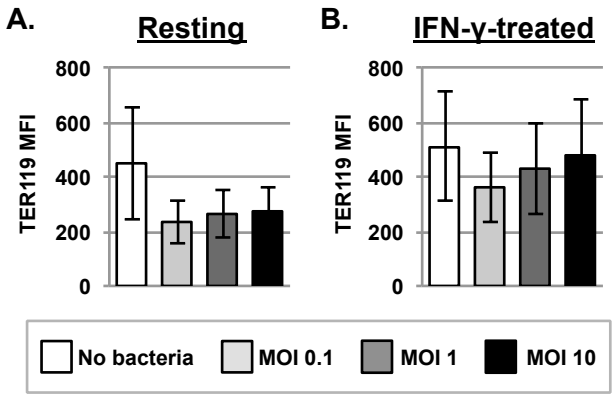

Figure A-3
Resting (A) or IFN-γ-pretreated (B) macrophages were cocultured with erythrocytes at a ratio of 1:10 without bacteria (□), or with live, GFP-expressing STm at MOIs of 0.1 (■), 1 (■), or 10 (■) for 18 h. Extracellular erythrocytes were then lysed and macrophages processed for flow cytometry. (A and B) Median Fluorescent Intensity (MFI) of TER119 signal in TER119+ macrophages. Means ± the SEM of aggregated data were determined from three biological replicates. MFI was calculated as described in the methods in Chapter 2.

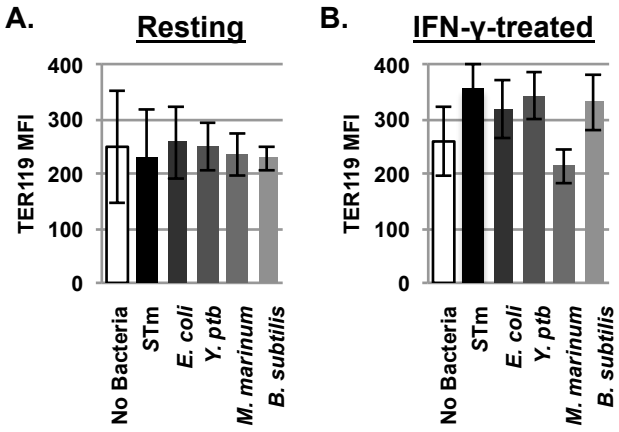

Figure A-4
Resting (A) or IFN-γ-pretreated (B) macrophages were cocultured with erythrocytes at a ratio of 1:10 without bacteria or with HK bacteria (MOI of 10). After 18 h, macrophages were processed for flow cytometry. (A and B) Median Fluorescent Intensity (MFI) of TER119 signal in TER119+ macrophages. Means ± the SEM of aggregated data were determined from three biological replicates. MFI was calculated as described in the methods in Chapter 2.

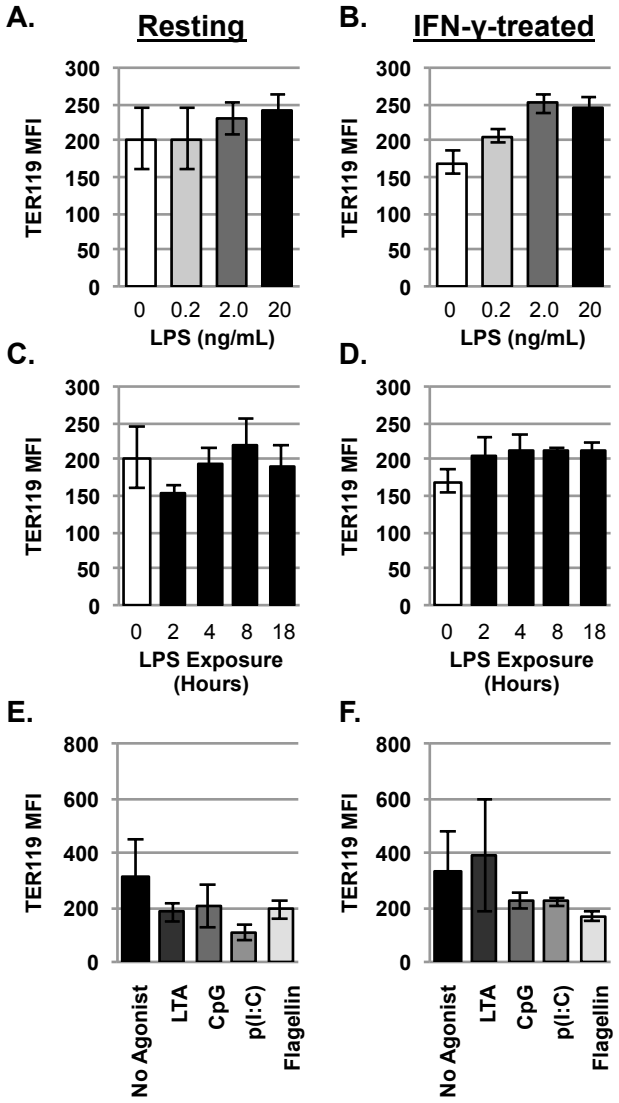

Figure A-5
Resting (A, C, and E) or IFN-γ-pretreated (B, D, and F) macrophages were cocultured with erythrocytes at a ratio of 1:10 with or without a TLR agonist as indicated. After 18 h, macrophages were processed for flow cytometry. (A and B) LPS titration. (C and D) Samples were incubated with 20 ng of LPS/ml for the period of time indicated. (E and F) 0.01 mg/ml LTA, 1 μM CpG, 10 μg/ml poly(I·C), or 0.05 μg/ml flagellin. (A - F) Median Fluorescent Intensity (MFI) of TER119 signal in TER119+ macrophages. Means ± the SEM of aggregated data were determined from three biological replicates. MFI was calculated as described in the methods in Chapter 2.

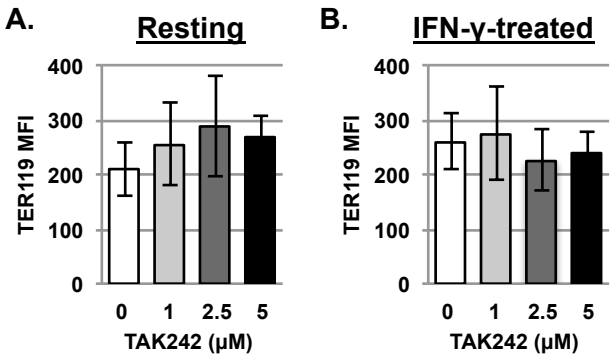

Figure A-6
Resting (A) or IFN-γ-pretreated (B) macrophages were treated for 1 h with vehicle (DMSO; □) or TAK242 at 1 μM (□), 2.5 μM (■), or 5 μM (■). After 1 h, erythrocytes were added at a ratio of 1:10, and LPS (20 ng/ml) was added to stimulate hemophagocytosis. After 18 h, macrophages were processed for flow cytometry. (A and B) Median Fluorescent Intensity (MFI) of TER119 signal in TER119+ macrophages. The means ± the SEM of aggregated data were determined from at least three biological replicates. MFI was calculated as described in the methods in Chapter 2.

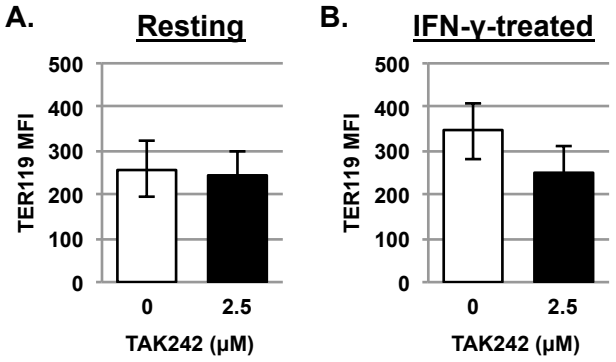

Figure A-7
Resting (A) or IFN-γ-pretreated (B) macrophages were treated for 1 h with vehicle (DMSO; □) or 2.5 μM TAK242 (■). Macrophages were infected with GFP-expressing *S*Tm (MOI of 10) and cocultured with erythrocytes at a ratio of 1:10. After 18 h, macrophages were processed for flow cytometry. (A and B) Median Fluorescent Intensity (MFI) of TER119 signal in TER119+ macrophages. The means ± the SEM of aggregated data were determined from at least three biological replicates. MFI was calculated as described in the methods in Chapter 2.

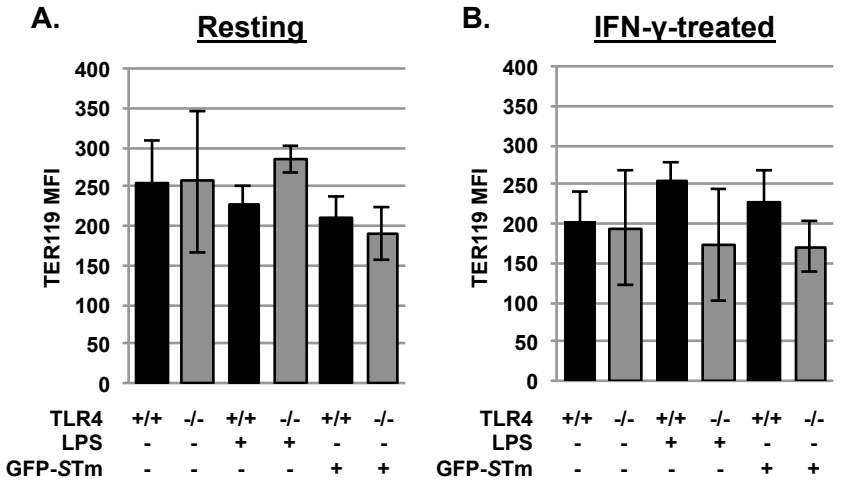

Figure A-8
C3H/HeOuJ (*Tlr4*+/+) and C3H/HeJ (*Tlr4*-/-) macrophages were left resting (A) or pretreated with IFN-γ (B) and were cocultured with erythrocytes at a ratio of 1:10 with or without LPS (20 ng/ml) or with or without live *S*Tm (MOI of 10). After 18 h, macrophages were processed for flow cytometry. (A and B) Median Fluorescent Intensity (MFI) of TER119 signal in TER119+ macrophages. The means ± the SEM of aggregated data were determined from at least three biological replicates. MFI was calculated as described in the methods in Chapter 2.

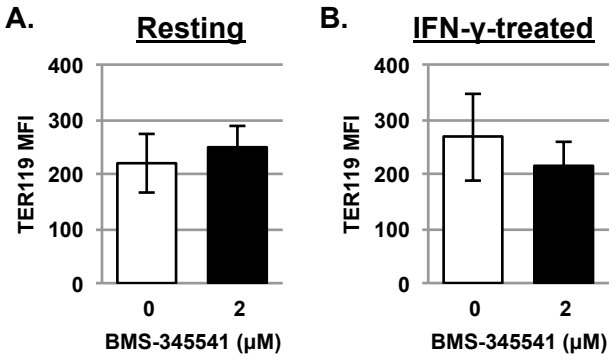

Figure A-9
Resting (A) or IFN-γ-pretreated (B) macrophages were treated for 1 h with vehicle (DMSO; white bars) or with 2 μM BMS-345541 (black bars). After 1 h, the macrophages were cocultured with erythrocytes at a ratio of 1:10, and LPS (20 ng/ml) was added. After 18 h, the macrophages were processed for flow cytometry. (A and B) Median Fluorescent Intensity (MFI) of TER119 signal in TER119+ macrophages. The means ± the SEM of aggregated data were determined from at least three biological replicates. MFI was calculated as described in the methods in Chapter 2.

Appendix B – TLR4 signaling does not increase the fraction of erythrocytes that externalize phosphatidylserine (PS)

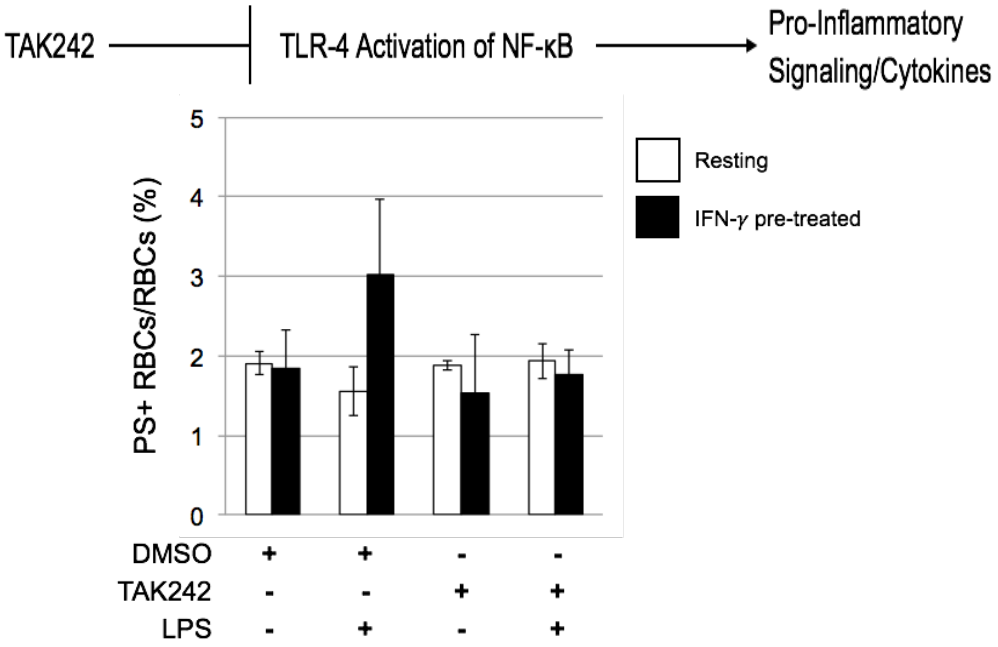

Figure B-1 TLR4 signaling does not increase the fraction of erythrocytes that externalize phosphatidylserine (PS)
Erythrocytes at a ratio of 10:1 were co-cultured with resting (□) or IFN-γ-pretreated (■) macrophages in the presence or absence of LPS (20 ng/mL). Prior to the addition of erythrocytes, subsets of the macrophages were pre-incubated for 1 hour with TAK242 or with DMSO (control). After 18 h, the erythrocytes were removed and stained with AnnexinV (which binds to phosphatidylserine). The mean percentage of PS+ erythrocytes was calculated. The graph shows the means ± the standard deviation of two technical duplicates from one biological replicate.