The Role of Signal Regulatory Protein Alpha in Hemophagocytic and Cytokine Storm Syndromes

Koby Kidder

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The Role Of Signal Regulatory Protein Alpha
In Hemophagocytic And Cytokine Storm Syndromes

by

Koby Kidder

Under the Direction of Yuan Liu, M.D., Ph.D.

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of
Doctor of Philosophy
in the College of Arts and Sciences
Georgia State University
2021
ABSTRACT

The immune system not only eliminates pathogens and other untoward factors but effectively does so without incurring host-compromising collateral damage. Among the many leukocytes buttressing this balancing act, macrophages reside at the helm of innate immunity and orchestrate finely tuned immune responses by acquiring a phenotype bespoke to the underlying inflammatory insult, the optimal result of which is the swift removal of noxious stimuli and reversion to homeostasis thereafter. On rare occasion, macrophages become hyper-activated and potentiate inflammation that often proves life-threatening rather than life-saving, as is the case in hemophagocytic lymphohistiocytosis (HLH) and other cytokine storm syndromes (CSS). Improving our understanding of the mechanistic bases by which macrophage phenotypic plasticity and function are regulated in health and disease will better inform the diagnosis and management of these often-fatal disorders of inflammation. In our study, we explore the role of signal regulatory protein alpha (SIRPα) in pro-inflammatory macrophage activation under toll-like receptor (TLR) 9 agonist-driven secondary HLH (S-HLH) and cytokine storm. We show that depleting SIRPα (SIRPα−/−) in mice during TLR9-driven inflammation exacerbates and accelerates the onset of fulminant S-HLH, in which hemophagocytosis, hypercytokinemia, cytopenias, hyperferritinemia, and other HLH hallmarks were apparent. In contrast, mice expressing SIRPα, including those deficient of the SIRPα ligand CD47 (CD47−/−), do not phenocopy SIRPα deficiency and fail to fully develop S-HLH. Although interferon gamma (IFNγ) is largely considered a driver of HLH pathology, IFNγ neutralization did not prevent the precipitation of S-HLH in TLR9-inflamed SIRPα−/−
mice, whereas macrophage depletion attenuated S-HLH in SIRPα−/− mice. Mechanistic studies confirmed that SIRPα not only restrains macrophages from acquiring a hemophagocytic phenotype, but also tempers their pro-inflammatory cytokine and ferritin secretion by negatively regulating Erk1/2 and p38 activation downstream of TLR9 signaling. In addition to TLR9 agonists, TLR2, TLR3 or TLR4 agonists, as well as TNFα, IL-6, or IL-17A, but not IFNγ, similarly induced S-HLH in SIRPα−/− mice but not SIRPα+ mice. The collective findings of this study suggest that CD47-dependent and CD47-independent SIRPα inhibition play a negative role in HLH pathogenesis by precluding macrophages from becoming hemophagocytic and hyper-activated under inflammation.

INDEX WORDS: SIRPA, CD47, Macrophage, HLH, Cytokine Storm
The Role Of Signal Regulatory Protein Alpha
In Hemophagocytic And Cytokine Storm Syndromes

by

Koby Kidder

Committee Chair:  Yuan Liu

Committee:  Andrew Gewirtz

Didier Merlin

Electronic Version Approved:

Office of Graduate Services
College of Arts and Sciences
Georgia State University
December 2021
DEDICATION

This work is dedicated to my family and friends whose unwavering love and support have enabled me to accomplish all that I have. To my parents, words cannot truly convey my gratitude for your years of love, compassion, direction, and sacrifice that have molded me into the man I am today, and I hope I may continue to make you both proud. To my brother Noah, I know I haven’t always been the best big brother, but I am grateful for our “sibling rivalry” that has pushed us both in our pursuit to achieve great things in this life, the joys of which I look forward to sharing with you in the future. To my friends, I am so fortunate to have so many people in my life that are truly close and kind to me, and it is your support that has made this wild adventure all the more surmountable. I love you all dearly.
ACKNOWLEDGEMENTS

As though it were yesterday, I vividly recall the moment my P.I., Dr. Yuan Liu, responded (albeit reluctantly) to my email on the prospect of an undergraduate research internship, recharting the course of my life for the better. Yuan, words cannot express how deeply grateful I am for your being my advisor, my guardian, sometimes my adversary, but most importantly, my friend. These few years of working together have been some of the most taxing and challenging of times but also some of the most rewarding and joyful of times. Thank you for teaching me how to thoroughly scrutinize the scientific rigor of all data, how to persevere in the face of adversity, and how to recognize my strengths and weaknesses to better position myself for success. I know our chapter as advisor and student is coming to a bittersweet close, but I look forward to passing on all the wonderful things you have so kindly given me and using all that you have taught me in my future endeavors.

My sincerest thanks to the post-doctoral fellows in our lab, Dr. Zhen Bian and Dr. Lei Shi. Zhen and Stone, thank you both for your guidance and instruction in the design of experiments and execution of scientific techniques, as well as your instrumental support in troubleshooting and the analysis of otherwise esoteric data. Most crucially, you both taught me invaluable lessons on how to not get Boss upset – for that, I am in your debt.

I would finally like to graciously thank my committee members, Dr. Didier Merlin and Dr. Andrew Gewirtz, the sincere critique, advice, and support from whom has equally fostered my critical thinking and facilitated the completion of my dissertation.
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<th>Full Form</th>
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<tr>
<td>Ag</td>
<td>antigen</td>
</tr>
<tr>
<td>αIFNγ</td>
<td>anti-IFNγ neutralizing monoclonal antibody</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>APC</td>
<td>allophycocyanin</td>
</tr>
<tr>
<td>BMM</td>
<td>bone marrow-derived macrophage</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CD</td>
<td>cluster of differentiation</td>
</tr>
<tr>
<td>CFSE</td>
<td>carboxyfluorescein succinimidy1 ester</td>
</tr>
<tr>
<td>CL2MDP</td>
<td>clodrosome-containing liposome</td>
</tr>
<tr>
<td>COVID-19</td>
<td>coronavirus disease 2019</td>
</tr>
<tr>
<td>CpG ODN</td>
<td>cytosine-phosphorothioate-guanine oligodeoxynucleotides</td>
</tr>
<tr>
<td>CSS</td>
<td>cytokine storm syndrome</td>
</tr>
<tr>
<td>DAPI</td>
<td>4′,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cell</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein-Barr Virus</td>
</tr>
<tr>
<td>EL</td>
<td>empty liposome</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>granulocytic myeloid colony stimulating factor</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>hematoxylin and eosin staining</td>
</tr>
<tr>
<td>HLH</td>
<td>hemophagocytic lymphohistiocytosis</td>
</tr>
</tbody>
</table>
HPS – hemophagocytic syndrome
HRP – horseradish peroxidase
HSC – hematopoietic stem cell
HSD – honestly significant difference
IF – immunofluorescent
IFNγ – interferon gamma
IgG – immunoglobulin G
IHC – immunohistochemical staining
iHPC – inflammatory hemophagocyte
IL – interleukin
IL-18BP – interleukin 18 binding protein
ITIM – immunoreceptor tyrosine-based inhibitory motif
ITSM – immunoreceptor tyrosine-based switch motif
JAK – janus kinase
LCMV – lymphocytic choriomeningitis virus
LILRB – leukocyte immunoglobulin-like receptor B
LPS – lipopolysaccharide
MAPK – mitogen-activated protein kinase
MAS – macrophage activation syndrome
MC – monocyte
mCD47.ex – extracellular domain of murine CD47
MCMV – murine cytomegalovirus
MCP-1 – monocyte chemoattractant protein 1
MHC – major histocompatibility complex
N/D – not determined
NF-kB – nuclear factor kappa B
NS – not significant
OCT – optimal cutting temperature
P-HLH – primary hemophagocytic lymphohistiocytosis
PB – pacific blue
PBC – peripheral blood cell
PBS – phosphate-buffered saline
PD-1 – programmed cell death protein 1
PD-L1 – programmed cell death protein ligand 1
PE – phycoerythrin
PerCP – peridinin chlorophyll protein
PI3K – phosphoinositol 3’-kinase
PKO – perforin-knockout mice
PMN – polymorphonuclear cell
Poly I:C - polyinosinic-polycytidylic acid
PRF – perforin
RBC – red blood cell
RPM – red pulp macrophage
rpm – revolutions-per-minute
S- HLH – secondary hemophagocytic lymphohistiocytosis
sCD25 – soluble CD25
SDS-PAGE – sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM – standard error mean
SHP-1/2 – SH2 domain-containing tyrosine phosphatase 1/2
SIRPα – signal regulatory protein alpha
TAM – tumor-associated macrophage
TLR – toll-like receptor
TNFα – tumor necrosis factor alpha
WT – wild-type
XMEN – X-linked immunodeficiency, magnesium defect, EBV infection and neoplasia syndrome
XLP-1/2 – X-linked lymphoproliferative disorder type 1/2
1 INTRODUCTION

1.1 Fidelity of the Immune System

Multicellular organisms have evolved complex immune systems that execute rapid, effective, and self-resolving inflammatory responses against a wide range of threats (Boehm, Iwanami, & Hess, 2012; Cooper & Alder, 2006; Hoffmann, Kafatos, Janeway, & Ezekowitz, 1999; Medzhitov & Janeway, 2000). Likened to a double-edged sword, an efficient and potent immune response is essential to insulating the host from further tissue damage but in doing so may incite unwarranted inflammation that compromises host survival (Chovatiya & Medzhitov, 2014; Kotas & Medzhitov, 2015). To toe the fine line between mounting appropriate and disproportionate inflammatory responses, immune cell activation and suppression must be stringently controlled with layers of redundancy that collectively ensure the shift from homeostasis to inflammation, and vice versa, does not diverge from an “acceptable” range (Meizlish, Franklin, Zhou, & Medzhitov, 2021). Extreme deviation from this framework of regulated inflammation may beget a host of issues ranging from a state of immune paralysis that increases susceptibility to subsequent infection to a state of tissue-damaging pro-inflammation that jeopardizes organ function (Butterfield, Best, & Merrick, 2006; Sriskandan & Altmann, 2008). In light of the recent global pandemic borne of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), states of host-compromising hyper-inflammation have garnered more appreciation from the scientific community, and as a result, greater focus has been given to resolving therapeutic interventions that pacify exaggerated inflammatory responses such as cytokine storm (Hu, Huang, & Yin, 2021; Jose & Manuel, 2020; Sun et al., 2020).
1.2 Cytokine Storm

To effectively protect the host from pathogens and other noxious agents, innate and adaptive immune cells rely on well-coordinated communication that is in large part predicated upon cytokines (Arango Duque & Descoteaux, 2014). Cytokines are small proteins, the sizes of which range between 5 kDa to 25 kDa, with short half-lives that elicit autocrine, paracrine, or endocrine signaling by binding to receptors on the producing cell, other proximal cells, or distant cells, respectively (Dinarello, 2007; J.-M. Zhang & An, 2007). Depending on the presumed function, cellular source or target of action, cytokines may be classified as interleukins, chemokines, interferons, et cetera. Virtually all nucleated cells carry the capacity to produce cytokines, but immune cells such as lymphocytes and macrophages are often the primary producers of cytokines, especially in response to infections (Curtsinger & Mescher, 2010; Lacy & Stow, 2011). Indeed, cytokines are most instrumental in the effective coordination of effector cell networks and in delivering regulatory signals that fine-tune, propagate, and resolve the immune response (Paul & Seder, 1994; Striz, Brabcova, Kolesar, & Sekerkova, 2014). However, aberrant production of cytokines leads to excessive levels in the circulation that can have deleterious, widespread effects and cause irreparable damage to vital tissues and organs (Jaffer, Wade, & Gourlay, 2010).

Hypercytokinemia, more commonly referred to as cytokine storm, is a pathological state of inflammation characterized by extremely high levels (the threshold for which is defined in clinical context-specific manner) of pro-inflammatory cytokines, such as IL-1β, IL-6, IL-
10, IL-12, IL-17, IL-18, GM-CSF, IFNγ, and TNFα, within the circulation (D'Elia, Harrison, Oyston, Lukaszewski, & Clark, 2013). To achieve elevated concentrations of cytokines in the circulation that warrant labeling of “cytokine storm”, leukocytes including T cells, NK cells, monocytes, and macrophages must first become hyper-activated following a disproportionate response to that which is perceived as “danger” (Heil & Land, 2014). Immune hyper-activation may take place in response to many different scenarios: (i) when sensing an exorbitant level of pathogen-associated or damage-associated molecular patterns as in sepsis or burns (Chousterman, Swirski, & Weber, 2017; Y. Y. Tang, Lou, & He, 2021), (ii) in the absence of pathogen as in genetic disorders involving aberrant inflammasome activation or systemic juvenile idiopathic arthritis (Shimizu, 2019), (iii) insufficient amplification of immune effector responses in the face of persistent antigenic stimulation as in immunotherapy-elicited cytokine release syndrome (Shimabukuro-Vornhagen et al., 2018), (iv) under uncontrolled infections coupled with protracted activation of effector immune cells as in hemophagocytic lymphohistiocytosis (Halyabar et al., 2019), or (v) when inflammation is unsuccessfully resolved and homeostasis is not re-established as in macrophage activation syndrome (McGonagle, Ramanan, & Bridgewood, 2021). Regardless of the etiology, these disease states all involve an imbalance of immune activation and suppression brought on by a loss of essential negative feedback circuits that otherwise preclude immune hyper-activation and multi-organ failure putatively driven by cytokine storm (Fajgenbaum & June, 2020; Tisoncik et al., 2012; Yang et al., 2021).
Though cytokine storm has long been diagnosed in patients with severe infections, malignancy, or autoimmunity (Clark, 2007; Fajgenbaum & June, 2020; Yu et al., 2011), clinical case reports and academic literature recognizing cytokine storm and dissecting the underlying mechanisms by which it arises have been few and far between until the advent of COVID-19 (Figure 1.1). Numerous studies characterizing the cytokine profiles of COVID-19 patients found considerable evidence supporting a strong correlation between the presence of cytokine storm and severe COVID-19 typified by lung injury, multi-organ failure, and unfavorable prognosis (Hojyo et al., 2020; Melo et al., 2021; Sinha, Matthay, & Calfee, 2020). Investigations into the pathogenesis of COVID-19-associated cytokine storm point to a key role for macrophages and an impaired type I interferon response that undermines effective clearance of SARS-CoV-2, the result of which is persistent antigenic stimulation, hyper-activation of immune cells, and pathological levels of IL-1β, IL-6, IFNγ, and TNFα in the circulation (Blanco-Melo et al., 2020; Galani et al., 2021; Hadjadj et al., 2020; Jafarzadeh, Chauhan, Saha, Jafarzadeh, & Nemati, 2020; Meidaninikjah et al., 2021; Ramasamy & Subbian, 2021; J. Zhang et al., 2021). It remains to be determined whether these states of heightened and sustained cytokine production are merely an unwanted trade-off of our robust immune systems that has yet to be extirpated by evolutionary pressures or are rather a necessary means to effectively control extensive pathogen burden.
Figure 1.1 Literature search for cytokine storm on PubMed
The data were generated from two queries on PubMed that searched for publications on “cytokine storm” that excluded (CS) or included (CS-COVID19) the term “COVID-19”.

After finding evidence that suggests disease severity of COVID-19 associates with cytokine storm, clinicians explored the use of therapeutic interventions that directly or indirectly disrupt cytokine signaling (Cron, Caricchio, & Chatham, 2021). Neutralizing antibodies against IL-1R (anakinra), IL-6 (siltuximab), IL-6R (tocilizumab), TNFα (infliximab), or IFNγ (emapalumab) and pharmacological pan-inhibitors against JAK-STAT (ruxolitinib and tofacitinib) have been explored as treatments for patients with severe COVID-19 (Gritti et al., 2021; Kyriazopoulou et al., 2021; F. La Rosée et al., 2020; Magro, 2020; Rosas et al., 2021; Stallmach et al., 2020). However, the efficacies of these modalities have been moderate and inconsistent, suggesting the complex interplay between these cytokines and cellular targets must be considered. Beyond their use in
COVID-19-associated cytokine storm, these cytokine-directed treatments have found considerable success in other highly lethal hyper-inflammatory disorders including immunotherapy-associated cytokine release syndrome, macrophage activation syndrome and hemophagocytic lymphohistiocytosis.

1.3 Hemophagocytic Lymphohistiocytosis

First documented and described as histiocytic medullary reticulosis in 1939 (Bodley Scott & Robb-Smith, 1939), hemophagocytic lymphohistiocytosis (HLH) is a life-threatening syndrome driven by unabated activation of cytotoxic CD8 T cells, natural killer (NK) cells and macrophages that, if not promptly treated, culminates in cytokine storm-driven multi-organ failure and death (Scott W. Canna & Behrens, 2012). For individuals to develop HLH, essential negative feedback loops that temper activated leukocytes are inherently absent or omitted, resulting in hyper-activated macrophages that phagocytose healthy blood cells (i.e., hemophagocytosis), pathological production of pro-inflammatory cytokines, and widespread tissue damage (Weaver & Behrens, 2014). Per the identification of an underlying mutation, classification of HLH is bifurcated into either familial/primary (P-HLH) or acquired/secondary (S-HLH), with the former arising in individuals with genetically defective CD8 T cells or NK cells and the latter arising secondary to infections, autoimmunity (termed macrophage activation syndrome), malignancy or immunotherapies in the absence of the P-HLH-associated mutations. Though individuals diagnosed with HLH span all ages and ethnicities, P-HLH is more common in children whereas S-HLH is more frequently observed in adults, the treatment
of whom represents 40% of all HLH cases. Despite having different pathoetiologies, P-HLH and S-HLH patients similarly present with a constellation of symptoms such as anemia, leukopenia, thrombocytopenia, splenomegaly, hepatomegaly, fever, hyperferritinemia, hypertriglyceridemia, hypofibrinogenemia, and hemophagocytosis (Castillo & Carcillo, 2009; Hutchinson, Tattersall, & Manson, 2019). Manifestation of these clinical and pathological features putatively occurs due to excessive production and release of various cytokines by macrophages, CD8 T cells and NK cells in the face of persistent antigenic stimulation, the most common source of which is viral infection (Akira Morimoto, Yozo Nakazawa, & Eiichi Ishii, 2016). Aside from controlling the underlying inflammatory trigger – for example, by administering antibiotics for bacterial infection – the mainstay treatment for HLH is a combination of myeloablative and lymphoablative chemotherapies and other immunosuppressive treatments such as high-dose corticosteroids (Jordan, Allen, Weitzman, Filipovich, & McClain, 2011). Despite best practices, mortality for HLH patients remains near 40% since management of this disease is complicated by our relatively poor understanding of pathogenesis and the lack of clarity surrounding recognition, diagnosis, and treatment of this deleterious and complex disorder (Ramos-Casals, Brito-Zerón, López-Guillermo, Khamashta, & Bosch, 2014).

1.3.1 Primary Hemophagocytic Lymphohistiocytosis

First recognized as a hereditary disorder in 1952 (Farquhar & Claireaux, 1952), primary HLH (P-HLH) is the form of HLH associated with inherited genetic defects that impair lymphocyte cytotoxicity against viral infections or alter inflammasome activity (Cetica,
Pende, Griffiths, & Aricò, 2010), which is why P-HLH is most commonly diagnosed in children. Accordingly, the most common precipitant for individuals with P-HLH are viral infections, the clearance of which is impaired by mutated genes encoding for proteins necessary in cytolysis of virally infected cells or in the activation and regulation of T cells, NK cells or macrophages (G. E. Janka, 2012). There are currently more than fifteen genes recognized to predispose individuals for HLH when loss-of-function or gain-of-function mutations are introduced. Sequencing the genomes of patients with P-HLH has often found homozygous or compound heterozygous mutations in genes encoding for proteins involved in the exocytosis or formulation of cytolytic granules, but P-HLH may also arise in individuals with other mutation-driven immunodeficiencies (Côte et al., 2009; Feldmann et al., 2003; Rohr et al., 2010). Generally, the many forms of P-HLH fall into one of five classifications: familial, pigment abnormality, lymphoproliferative, Epstein-Barr virus (EBV) susceptible, and other (Table 1.1).
Table 1.1 Forms of primary hemophagocytic lymphohistiocytosis and their genetic defects

<table>
<thead>
<tr>
<th>NAME</th>
<th>CLASSIFICATION</th>
<th>GENE</th>
<th>FUNCTION</th>
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<tr>
<td>HLH Type 1</td>
<td>Familial</td>
<td>ND; 9q21</td>
<td>ND</td>
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<td>HLH Type 2</td>
<td>Familial</td>
<td>PRF1</td>
<td>Pore formation</td>
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<td>RAB27A</td>
<td>Vesicle docking</td>
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<td>Pigment Abnormality</td>
<td>LYST</td>
<td>Vesicle trafficking</td>
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<tr>
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<td>XLP-1</td>
<td>Lymphoproliferative</td>
<td>SH2D1A</td>
<td>NK, NKT and T cell signaling</td>
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<td>XLP-2</td>
<td>Lymphoproliferative</td>
<td>XIAP</td>
<td>NF-kB signaling</td>
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<td>EBV Susceptible</td>
<td>MAGT1</td>
<td>TCR-dependent T cell activation</td>
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<td>EBV Susceptible</td>
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<td>T cell signaling</td>
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<td>Costimulatory molecule</td>
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<td>EBV Susceptible</td>
<td>CD70</td>
<td>Costimulatory molecule</td>
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<td>Cryopyrin-Associated Periodic Syndrome</td>
<td>Other</td>
<td>NLRC4</td>
<td>Inflammasome activation</td>
</tr>
<tr>
<td>Takenouchi-Kosaki Syndrome</td>
<td>Other</td>
<td>CDC42</td>
<td>Inflammasome activation</td>
</tr>
</tbody>
</table>

There are currently five forms of familial HLH (HLH Type 1-5) recognized based on the underlying mutation. While the gene specifically affected remains to be identified, patients with familial HLH type 1 carry an aberration in the 9q21 locus on chromosome 9 (Ohadi et al., 1999). Familial HLH type 2 arises from mutations in the gene encoding for perforin, \( PRF1 \), which is the most common P-HLH-associated mutation and prevents CD8 T cells and NK cells from lysing cognately engaged target cells infected with the underlying virus (Risma, 2005) Familial HLH type 3, type 4 and type 5 manifest when there are mutations
in the genes encoding for Munc-13-4 (UNC13D), syntaxin 11 (STX11), and syntaxin-binding protein 2 (STXBP2), which respectively function to mature, transport, and release cytolytic granules (Feldmann et al., 2003; U. zur Stadt, 2005; Udo zur Stadt et al., 2009). Similarly, the pigmentary disorders found to precipitate HLH involve mutations in genes associated with the trafficking and release of granules, as well as other functions in platelets, melanocytes, and neutrophils (Pachlopnik Schmid et al., 2009). The canonical X-linked lymphoproliferative diseases not only impair lymphocyte cytotoxicity but also confer resistance to activation-induced cell death (Morra et al., 2001). XIAP deficiency, in particular, also instigates inflammasome dysfunction that concurrently exacerbates disease (Yabal et al., 2014), which is paralleled in individuals carrying NLRC4 or CDC42 mutations that lead to heightened activation of inflammasomes and IL-1 family cytokine production (Scott W. Canna et al., 2014; Lam et al., 2019). Though EBV is the most prevalent virus found in P-HLH patients, especially in individuals with inborn errors in genes that lead to EBV susceptibility (Marsh, 2018b), other viruses including cytomegalovirus (CMV), varicella-zoster virus, human immunodeficiency virus, adenovirus, hepatitis B virus, hepatitis C virus, influenza A, and most recently SARS-CoV-2 have been observed as infectious triggers (Blaney, Thotakura, & Sisco, 2021; Censoplano et al., 2018; Mostaza-Fernández, Guerra Laso, Carriedo Ule, & Ruiz de Morales, 2014; Prilutskiy et al., 2020). In contrast to P-HLH, S-HLH is loosely and broadly defined and occurs on the background of many other diseases and symptomatic triggers.
1.3.2 Secondary Hemophagocytic Lymphohistiocytosis

Though not generally considered to involve genetic defects in cytotoxic machinery or other anti-viral attributes and functions of CD8 T cells and NK cells, S-HLH, also known as reactive or acquired HLH, may associate with certain genetic predispositions (Cetica et al., 2016), which is most well-documented in S-HLH arising in patients with autoimmunity, i.e., macrophage activation syndrome (Schulert & Cron, 2020). In contrast to P-HLH, S-HLH is predominantly diagnosed in adults, may or may not be associated with disease-prediecting mutations, and involves an unknown or identifiable trigger on the background of infection, malignancy, autoimmunity, immune-modulating therapies in an iatrogenic setting, or unknown, i.e., an idiopathic setting (G. Janka, Imashuku, Elinder, Schneider, & Henter, 1998; Ramachandran, Zaidi, Aggarwal, & Gera, 2017).

EBV remains the prevailing infectious trigger most often identified in patients with S-HLH (Marsh, 2018a). However, EBV reactivation in immunocompromised adults is observed in S-HLH whereas primary EBV infection in children is commonly observed in P-HLH (Al-Samkari & Berliner, 2018). Paralleling P-HLH, S-HLH can arise from infection with other viruses, particularly herpesviruses, that elicit hyper-activation of immune cells, but unlike P-HLH, infection-associated S-HLH may involve other pathogens including bacteria (mycobacterium tuberculosis), fungi (histoplasma), and parasites (leishmania, plasmodium, and toxoplasma) (Ramos-Casals et al., 2014). Aside from extensive infections, S-HLH may manifest in patients with malignancies originating from hematopoietic lineage cells such as B cells, T cells, and NK cells (H. Wang, Xiong, Tang,
Zhou, & Li, 2017). Malignancy-associated S-HLH accounts for half of all adult HLH cases and may occur before, during, or after cancer treatment (Tamamyan et al., 2016). Although cancer treatment often results in immunosuppression and increased susceptibility to nosocomial infections that trigger S-HLH in adults (Griffin, Shenoi, & Hughes, 2020), some therapeutic modalities such as immune checkpoint inhibitors and chimeric antigen receptor (CAR) T cells may elicit iatrogenic S-HLH or cytokine release syndrome (CRS) by introducing antibodies that disrupt intrinsic negative feedback loops in T cells or infusing exogenously-modified genetically engineered T cells with enhanced effector function and hypo-responsiveness to inhibitory signals (Ceschi, Noseda, Palin, & Verhamme, 2020; Frey & Porter, 2019). One recent clinical trial (NCT04227275) was halted after patients administered CAR T cells, which targeted prostate-specific membrane antigen (PSMA) and were armored with dominant-negative TGF-β receptors, had incurred severe CRS that involved systemic hyper-activation of macrophages (Kloss et al., 2018; Narayan et al., 2019).

S-HLH occurring in patients with autoimmune diseases such as adult-onset Still disease, Kawasaki disease, systemic juvenile idiopathic arthritis, and systemic lupus erythematosus is referred to as macrophage activation syndrome (MAS) due to the more pronounced involvement of over-activated macrophages (McGonagle et al., 2021). The propensity to develop MAS in chronic rheumatological diseases involves a complex interplay between inborn genetic errors, protracted or chronic inflammation, and immunosuppression (Crayne, Albeituni, Nichols, & Cron, 2019). Indeed, a considerable
frequency of patients with MAS present with heterozygous mutations that are commonly affiliated with familial HLH (Bae, Jung, Kim, & Suh, 2015; E. M. Behrens, Beukelman, Paessler, & Cron, 2007; Minoia et al., 2014), and in combination with repeated stimulation of pathogen receptors such as toll-like receptors (TLR) (Edward M. Behrens et al., 2011; Strippoli et al., 2012), the compounded impact of activation signals drives macrophages into an uncontrolled state of pro-inflammatory polarization – the result of which is excessive production of cytokines that reinforce this heightened state of inflammation and promote incipient HLH-associated pathophysiology.

1.3.3 Pathophysiology and management of HLH

Regardless of the form of HLH, symptomatic onset is associated with some sort of trigger whether that be pathogenic, autoimmune, malignant, iatrogenic, or idiopathic (Soy, Atagündüz, Atagündüz, & Sucak, 2021). Given the largely confined nature and defined role of the genetic mutations associated with P-HLH and that the symptomatic trigger is most often a viral infection, the pathophysiology of P-HLH is more clearly understood than that of S-HLH. Under physiological conditions, both innate and adaptive immune cells recognize virally infected cells and release cytolytic granules comprising perforin, which forms pores in the virally infected cell’s membrane, and a cocktail of granzymes, which elicit programmed cell death upon entering the target cell’s cytosol (Kägi et al., 1994). Individuals born with mutations in genes that encode for perforin, granzyme, exocytosis-associated molecules, or proteins involved in CD8 T cell or NK cell activation or survival have a compromised immune response against viral infection. This dysregulated immune
response by and large results in uncontrolled activation of lymphocytes and macrophages that do not receive proper negative feedback signals to resolve their pro-inflammatory responses and consequently manifest HLH pathology (A. Morimoto, Y. Nakazawa, & E. Ishii, 2016). Extensive studies in animal models of familial HLH and using patient samples suggest that the sustained activation of CD8 T cells and NK cells results in excessive IFNγ production (Jordan, Hildeman, Kappler, & Marrack, 2004; Schmid et al., 2009; Tesi et al., 2015), which then potently activates macrophages (C. Wu et al., 2014). Thereafter, activated macrophages over-produce other pro-inflammatory cytokines including IL-1β, IL-6, IL-12, IL-18, and TNFα (Schulert & Grom, 2014b), which in turn amplify pro-inflammatory activation of CD8 T cells, NK cells, macrophages, and other cellular targets. Without essential resolution of immune activation, this feed-forward loop perpetuates uncontrollably and inevitably culminates in multi-organ failure and death in the HLH patient.

Unlike P-HLH, the pathogenesis of S-HLH is not as well understood as that of P-HLH. One proposed mechanism is the aberrant stimulation of TLR leads to excessive activation of and cytokine production by innate immune cells, particularly macrophages (Edward M. Behrens et al., 2011). In addition to notable TLR-mediated inflammation, inflammasome-dependent mechanisms leading to overproduction of IL-18 by myeloid and epithelial cells have been implicated in the onset of secondary HLH, especially in MAS patients with gain-of-function mutations in NLRC4 inflammasomes (Scott W. Canna et al., 2014). Similar to IL-12, excess IL-18 augments lymphocyte production of IFNγ (Girard-
Guyonvarc'h et al., 2018), which putatively drives extensive macrophage activation and HLH immunopathology. Though it is clear that HLH pathophysiology involves some sort of genetic or non-genetic predisposition and some sort of trigger that culminate in HLH diagnostic hallmarks, it remains to be determined how hyper-activation of macrophages leads them to become hemophagocytic in HLH and it is equally unclear whether macrophages are pathogenic drivers or bystanders in HLH (Figure 1.2).

**Figure 1.2 Overview of primary and secondary hemophagocytic lymphohistiocytosis pathophysiology and outstanding questions surrounding the emergence of HLH hallmarks**
The difficulty in managing HLH is extremely complicated by the ambiguity surrounding what HLH is and how HLH may be recognized, diagnosed, and properly treated (P. La Rosée et al., 2019). The majority of HLH-related deaths occur in the first 8 weeks of symptomatic onset, and if not promptly treated, HLH carries a greater than 90% mortality rate. However, over the past couple decades, improvement in recognizing and managing HLH has decreased the mortality rate to nearly 40% (Al-Samkari & Berliner, 2018). The current standard-of-care comprises high-dose corticosteroids co-administered with the chemotherapeutic agent etoposide and other immunosuppressive medications such as cyclosporin A and methotrexate, with the primary goal being the dampening of T cell activation and proliferation that are key attributes in hemophagocytic and cytokine storm syndromes (Scott W. Canna & Marsh, 2020).

However, regardless of the etiology, primary and secondary HLH similarly precipitate a cytokine storm variably involving IL-1β, IL-6, IL-10, IL-12, IL-18, IFNγ, TNFα and GM-CSF (Fajgenbaum & June, 2020), the sum of which putatively leads to life-threatening immunopathology, organ failure and death. This more nuanced understanding of cytokine-driven HLH pathology has begun to shift away from non-specific treatment strategies toward precision-based medicine comprising antibody-mediated cytokine neutralization (e.g., anti-IFNγ, anti-TNFα, anti-IL-6) or pan-cytokine signaling inhibitors (e.g., JAK-1/2 inhibitors) to treat HLH and other forms of CSS (Ahmed et al., 2019; Lounder, Bin, de Min, & Jordan, 2019). While it is clear that these cytokines and their signaling pathways are integral to HLH pathogenesis, there remains a lack of
understanding insofar as how and why HLH patients and preclinical models variably respond to these treatment strategies.

1.4 Macrophage Activation and Regulation

The central role of macrophages, both tissue-resident and inflammatory monocyte-derived, in inflammation has long been recognized (Murray, 2017). Macrophages often dominate the immune landscape within any tissue niche and are frequently responsible for the initial immune response to innocuous or injurious stimuli (Gasteiger et al., 2017). Non-stochastic acquisition of an activation phenotype commensurate to the type, concentration and location of the underlying inflammation-inciting stimuli enables macrophages to effectively drive an inflammatory response that rapidly removes the stimulus and resolves inflammation thereafter (Orecchioni, Ghosheh, Pramod, & Ley, 2019). In contrast, dysregulated macrophage-driven inflammation may give rise to life-threatening pathological conditions that compromise host survival. Indeed, hyper-activated macrophages have been reported to contribute to other cardinal features commonly presenting in fulminant S-HLH including hyperferritinemia, hypertriglyceridemia and hypercytokinemia, the latter being the putative driver of multi-organ failure and death (Crayne et al., 2019). Moreover, previous reports have shown that dysregulation of pro-inflammatory macrophage activation can contribute to HLH/CSS pathogenesis in various models of sterile and non-sterile inflammation (S. Mahajan et al., 2019; A. Wang et al., 2019). While cytokines such as IFNγ have been implicated in over-activating macrophages, thereby driving them to phagocytose healthy blood cells (i.e.,
hemophagocytosis) (Zoller et al., 2011), hemophagocytic macrophages have been shown to arise in IFNγ-deficient mice under TLR9-induced inflammation (Burn et al., 2019; S. W. Canna et al., 2013). Concordant with this observation, we previously showed that macrophages deficient in signal regulatory protein alpha (SIRPα) may become both hemophagocytic and hyperactivated under TLR9 signaling in an IFNγ-independent manner (Kidder, Bian, Shi, & Liu, 2020).

1.4.1 SIRPα regulation of macrophage activation

SIRPα is a transmembrane protein that comprises an extracellular region with three immunoglobulin superfamily domains and an intracellular region with two immunoreceptor tyrosine-based inhibitory motifs (ITIMs; I/V/L-xYxx-I/V/L) and two immunoreceptor tyrosine-based switch motifs (ITSMs; T/S-xYxx-I/V) (Figure 1.3) (A. Neil Barclay & Berg, 2014). When SIRPα becomes ligated by CD47 and/or macrophages are stimulated by cytokines or TLR agonists (Shi, Bian, Kidder, Liang, & Liu, 2021; Shi, Kidder, et al., 2021), SIRPα ITIMs/ITSMs become phosphorylated by distinct tyrosine kinases (e.g., Src family kinases, Bruton’s tyrosine kinase, etc.) in a stimulation-specific manner to subsequently recruit the promiscuous tyrosine phosphatases, SHP-1 (PTPN6) and/or SHP-2 (PTPN11) (M. Garg, Wahid, & Khan, 2020). Thereafter, SHP-1 and/or SHP-2 mediate an array of functions often by dephosphorylating tyrosine residues, the most common result of which is inhibition of various immune cell activities including macrophage activation and phagocytosis (Kant et al., 2002).
Figure 1.3 Overview of SIRPα structure including tyrosine-based signaling domains and downstream effector phosphatases
This image was generated using BioRender.com.

Though SIRPα canonically functions to inhibit macrophage phagocytosis of host cells, recent studies have provided evidence that SIRPα protein expression and signaling profoundly regulate macrophage activation in response to pro-inflammatory or anti-inflammatory stimuli (Figure 1.4). Stimulation of macrophages with IL-4 or IL-13 has long been known to elicit alternative activation of macrophages, which exhibit an immunosuppressive phenotype typified by expression of anti-inflammatory markers including CD206, arginase-1, IL-10, and TGF-β (Siamon Gordon, 2003). Shi et al. demonstrated that macrophages deficient of SIRPα largely fail to undergo alternative activation in response to either IL-4 or IL-13 (Shi, Kidder, et al., 2021). SIRPα expression was found to be essential in IL-4/13-driven anti-inflammatory activation of macrophages by spatially restricting SHP-2 from IL-4R and IL-13R, the signaling through which would
otherwise be inhibiting by SHP-2-mediated dephosphorylation. Furthermore, IL-4 or IL-13 stimulation was found to elevate SIRPα expression on macrophages in a dose-dependent and time-dependent manner, suggesting that SIRPα is essential to amplifying IL-4/13-driven anti-inflammatory polarization of macrophages. These observations were most tangible in an animal model of ulcerative colitis where SIRPα deficiency was found to protract inflammation and impair wound healing by limiting the emergence of immunosuppressive activation of macrophages in the colon. On the other hand, infusion of alternatively activated macrophages with elevated SIRPα expression into SIRPα-deficient mice accelerates the resolution of colitis and enhances wound healing.

Figure 1.4 Overview of SIRPα regulation in macrophages stimulated by anti-inflammatory or pro-inflammatory stimuli
This image was generated using BioRender.com.
Aside from the role of SIRPα in promoting anti-inflammatory macrophage activation, other studies have demonstrated SIRPα negatively regulates pro-inflammatory macrophage activation. Kidder et al. found that SIRPα potently inhibits CpG-driven TLR9 signaling in macrophages by reducing the phosphorylation of the mitogen-activated protein kinases, p38 and Erk1/2 (Kidder et al., 2020). Furthermore, the study suggests that SIRPα is necessary for preventing excessively TLR9-stimulated macrophages from becoming hemophagocytic and producing extreme levels of pro-inflammatory cytokines. Dovetailing with this study, the recent work by Shi et al. demonstrated that SIRPα inhibits PI3K-Akt2 signaling and pro-inflammatory macrophage polarization by activating SHP-1 in macrophages (Shi, Bian, et al., 2021). SIRPα was found to be a chief negative regulator in macrophages responding to TLR agonists against TLR3, TLR4, and TLR9 or to pro-inflammatory cytokines such as IFNγ. SIRPα negatively regulates these signaling pathways by recruiting and activating the inhibitory phosphatase SHP-1, which then moderately represses JAK-STAT, MAPK, and NF-κB signaling and potently suppresses PI3K-Akt2 signaling. SIRPα deficiency in macrophages responding to these pro-inflammatory stimuli results in augmented production of pro-inflammatory cytokines and expression of immunogenic antigen presentation machinery, which cumulatively exacerbates disease in mice with type I diabetes or peritonitis. Collectively, these studies support that SIRPα potently regulates macrophage activation by inhibiting pro-inflammatory signaling and promoting anti-inflammatory signaling, whereas the absence of SIRPα regulation predisposes macrophages to acquire a hyper-inflammatory phenotype that worsens or drives immunopathology.
1.4.2 SIRPα regulation of macrophage phagocytosis

Phagocytosis is a cellular function predominantly conducted by professional phagocytes such as macrophages and involves the engulfment of large particles, cells, debris or otherwise (Flannagan, Jaumouillé, & Grinstein, 2012). Although phagocytosis is not the sole tool at their disposal, macrophages utilize phagocytosis as a first-line defense against pathogens (S. Gordon, 2016). Aside from being vital in host defense against infection, phagocytosis is equally indispensable in maintaining tissue homeostasis, with billions of cells undergoing programmed death daily. Phagocytosis of apoptotic bodies (i.e., efferocytosis) ensures proximal immune cells remain quiescent and do not incite unnecessary inflammation that otherwise manifests when dying cells are not aptly removed and instead undergo secondary necrosis (Doran, Yurdagul, & Tabas, 2020). The fidelity of this process, and phagocytosis in general, relies upon a suite of receptor-ligand pairs that are bifurcated into ‘eat-me’ and ‘don’t-eat-me’ signals, which respectively promote or inhibit macrophage phagocytosis of foreign targets and self-cells (Freeman & Grinstein, 2014). In general, self-cells are not phagocytosed unless certain prerequisites are met: i) self-cells must harbor ‘eat-me’ markers, such as phosphatidylserine (PS), calreticulin (CRT), or opsonins (antibodies or complement components), to a greater extent than they express ‘don’t-eat-me’ molecules, such as CD31, sialoglycoproteins, or CD47 (Brown et al., 2002); ii) macrophages express appropriate activating receptors, such as TIM1/4, LRP1, FcγRI-III, or CR1/3/4 (Uribe-Querol & Rosales, 2020), that recognize ‘eat-me’ signals on self-cells and outcompete ‘don’t-eat-me’ signals downstream of their inhibitory receptors, such as CD31, Siglec-10, or SIRPα, which
Comprise immuno-tyrosine inhibitory motifs (ITIMs) that recruit and activate tyrosine phosphatases (SHP-1/2) to dampen phagocytosis (Barkal et al., 2019; Crocker, Paulson, & Varki, 2007). Physiological conditions, response to stressors (radiation, chemotherapy), infection and autoimmunity are all capable of fulfilling these conditions and leading macrophages to phagocytose altered self-cells (Bass, Tuscano, & Tuscano, 2014; A. D. Garg, Romano, Rufo, & Agostinis, 2016; Nagata, 2018), which may either be dying (apoptotic), dead (necrotic), “stressed but viable”, infected or opsonized.

Unlike altered self-cells primed for removal by macrophages, healthy self-cells very rarely become targeted for phagocytosis. Nevertheless, under systemic hyperinflammatory conditions such as HLH, iatrogenic cytokine release syndrome (I-CRS), sepsis and in some cases of COVID-19, tissue-resident macrophages have been observed to phagocytose healthy hematopoietic lineage cells (erythrocytes, thrombocytes, and leukocytes), referred to as hemophagocytosis, even in the absence of opsonization (auto-antibodies or complement factors) or markers of stress (calreticulin) (Maude, Barrett, Teachey, & Grupp, 2014; Schae, Schae, Schoedon, Imhof, & Kurrer, 2006; L. V. Tang & Hu, 2021). Thus, there remains the need to identify the receptor-ligand pair(s) primarily responsible for precluding and permitting hemophagocytosis in HLH and other diseases.

Although the physiological relevance remains unclear, macrophages harbor the capability to phagocytose healthy self-cells, the prevention of which is putatively enforced by the CD47-SIRPα signaling axis. Initially characterized for its association with integrins, CD47...
is a transmembrane protein involved in numerous cellular and molecular functions (Chung, Wang, Lindberg, & Frazier, 1999; P. Jiang, C. F. Lagenaur, & V. Narayanan, 1999; Oronsky, Carter, Reid, Brinkhaus, & Knox, 2020; Reinhold, Lindberg, Kersh, Allen, & Brown, 1997), albeit most well-known for its negative regulatory role in phagocytosis of self-cells as the cognate ligand for SIRPα. Oldenburg et al. first discovered the importance of CD47 and SIRPα in ascertaining self from non-self when they adoptively transferred erythrocytes from CD47-deficient mice into wild-type recipients, which led to rapid clearance of the transferred CD47⁻/⁻ erythrocytes by red pulp macrophages in the absence of SIRPα-mediated inhibition (P. A. Oldenborg et al., 2000).

Despite being well known for more than 20 years (P.-A. Oldenborg, Gresham, & Lindberg, 2001; Yamao et al., 2002), the mechanism by which the CD47-SIRPα axis attenuates phagocytosis of healthy self-cells remains without consensus. CD47-SIRPα-SHP-1 has been shown to reduce phosphotyrosine on non-muscle myosin IIA and thereby inhibit the contractile force necessary to engulf antibody-opsonized targets (Sosale et al., 2015; Tsai & Discher 2008). On the other hand, CD47 has been shown to sterically reposition SIRPα within the immunologic synapse and thereby inhibit inside-out CD11b/CD18 integrin activation, which then attenuates cell spreading and antibody- or complement-dependent phagocytosis of self-cells such as cancer cells (Morrissey, Kern, & Vale, 2020). Although both studies provide valuable insight into how CD47-SIRPα negatively regulates phagocytosis of opsonized self-cells, this inhibitory axis also suppresses phagocytosis of non-opsonized healthy self-cells (P. A. Oldenborg et al., 2000).
Numerous studies have fortified the role of CD47-SIRPα in preventing macrophages from phagocytosing healthy self-cells, thus it would stand to reason that an absence of this inhibitory axis would lead to extensive tissue damage in vivo. However, a systemic deficiency of either CD47 or SIRPα in mice does not elicit widespread autoimmunity manifest by phagocytic elimination of healthy tissue (P. A. Oldenborg et al., 2000). An explanation for this paradoxical observation was provided when Bian et al. reported that the act of phagocytosing healthy self-cells in the concurrent absence of opsonization and SIRPα inhibition does not occur in vitro or in vivo unless certain activation signaling is provided (Bian et al., 2016). Macrophages unrestrained by SIRPα inhibition phagocytose (non-opsonized) healthy self-cells only when macrophages are also stimulated by various factors such as toll-like receptor (TLR) agonists (e.g., LPS, poly I:C, zymosan, CpG, etc.) or pro-inflammatory cytokines such as IL-1, IL-6, IL-17 or TNFα (Figure 1.5). Interestingly, the same study found that stimulating SIRPα-deficient macrophages with IFNγ did not license them to phagocytose healthy self-cells – a finding that challenges a previous study reporting that IFNγ-stimulated macrophages phagocytose healthy red blood cells in the presence of CD47-SIRPα inhibition (Zoller et al., 2011).
Bian et al. further show that TLR agonists or the aforementioned pro-inflammatory cytokines drive protein kinase C (PKC)-Syk-mediated inside-out activation of an uncharacterized phagocytic receptor that binds to an unknown ligand ubiquitously expressed on healthy self-cells, the phagocytosis of which depends on Mac-1 (CD11b/CD18) and may also be repressed by IL-10 signaling (Bian et al., 2016; Chen et al., 2017). When these prerequisites are contemporaneously met, SIRPα-positive macrophages may phagocytose CD47-negative self-cells, and SIRPα-negative macrophages may phagocytose CD47-positive self-cells, which is most evident in inflamed SIRPα-deficient or CD47-deficient mice whose red pulp macrophages quickly phagocytose many circulating red blood cells, healthy and aged, and induce anemia and splenomegaly. The framework in which SIRPα functions to prevent macrophages from become hemophagocytic was later explored in a study using an animal model of S-HLH (Kidder et al., 2020). This study was the first to suggest an unappreciated role for SIRPα in the emergence of hemophagocytic macrophages in S-HLH and other CSS, but additional investigations are necessary to determine whether disruption or abrogation of SIRPα regulation inherently occurs in HLH or CSS pathogenesis.
2 INFLAMMATION UNRESTRAINED BY SIRPA INDUCES SECONDARY HEMOPHAGOCYTIC LYMPHOHISTIOCYTOSIS INDEPENDENT OF INTERFERON-GAMMA

2.1 Introduction

Cytokine storm syndromes (CSS) such as secondary hemophagocytic lymphohistiocytosis (S-HLH) are life-threatening disorders associated with bacterial and viral infections and have recently been considered a cause for mortality in persons infected with SARS-CoV-2 (Al-Samkari & Berliner, 2018; Cao, 2020; Gritta E. Janka, 2007; Mehta et al., 2020). Also known as macrophage activation syndrome (Bracaglia, Prencipe, & De Benedetti, 2017; Nikiforow & Berliner, 2020), S-HLH is associated with hyper-activated macrophages that adopt a hemophagocytic phenotype and thus aberrantly engulf other healthy hematopoietic-lineage cells such as red blood cells (RBC), leukocytes and platelets, often leading to consumptive cytopenias (i.e., anemia, leukopenia and thrombocytopenia) (Bian et al., 2016; A. Wang et al., 2019; Zoller et al., 2011). The underlying mechanism driving macrophages to become hemophagocytic under inflammatory conditions, however, has yet to be clearly determined (Crayne et al., 2019).

Though an ensemble of proteins regulate macrophage phagocytosis, the inhibitory receptor that is well-known to prevent phagocytosis of healthy host cells is SIRPα (A. N. Barclay & Van den Berg, 2014; S. Gordon, 2016). The SIRPα-CD47 axis serves as the quintessential ‘don’t-eat-me’ signal to prevent unwanted phagocytosis of healthy host
cells (Ide et al., 2007; Lemke, 2019; Londino, Gulick, Isenberg, & Mallampalli, 2015; Takizawa & Manz, 2007). As an inhibitory receptor highly expressed on myeloid leukocytes including macrophages, monocytes (MC), dendritic cells (DC) and neutrophils (PMN), SIRPα is critical to prohibiting phagocytosis of CD47-expressing host cells such as RBC, leukocytes and platelets (Y. Liu et al., 2002; Yi et al., 2015). Despite the importance of this safety mechanism, the absence of SIRPα or CD47 alone does not confer an autoimmune phenotype, as mice devoid of SIRPα (SIRPα−/−) or CD47 (CD47−/−) are healthy like their wild-type (WT) littermates (Bian et al., 2016; P. A. Oldenborg et al., 2000). Providing insight into this paradoxical phenomenon, our prior study revealed that macrophages will not phagocytose healthy host cells unless two criteria are simultaneously met: 1) SIRPα-CD47 inhibition must be removed and 2) pro-inflammatory activation signaling must be present, presumably to activate the uncharacterized macrophage phagocytic receptor recognizing healthy host cells (Bian et al., 2016). While this bipartite ‘fail-safe’ mechanism has yet to be fully understood, we have shown that various TLR agonists or certain pro-inflammatory cytokines such as IL-1β, IL-6, TNFα or IL-17A, but not IFNγ, are capable of driving the required activation signaling (Bennett et al., 2019; Bian et al., 2016).

Aside from phagocytosing healthy blood cells, overactivated macrophages have been reported to contribute to other cardinal features commonly presenting in fulminant S-HLH including hyperferritinemia, hypertriglyceridemia and hypercytokinemia, the latter being the putative driver of multi-organ failure and death (Bian et al., 2016; Bracaglia et al.,
2017; Cohen et al., 2010; George, 2014; Grom & Mellins, 2010; Lerkvaleekul & Vilaiyuk, 2018; Ravelli, Grom, Behrens, & Cron, 2012). Indeed, previous reports have shown that dysregulation of pro-inflammatory macrophage activation can contribute to HLH/CSS pathogenesis in various models of sterile and non-sterile inflammation (S. Mahajan et al., 2019; Sahil Mahajan, Mellins, & Facci, 2020; A. Wang et al., 2019). Along these lines, SIRPα not only canonically inhibits macrophage phagocytosis but has become increasingly appreciated to also negatively regulate macrophage-driven pro-inflammation, with inflammatory diseases such as colitis, peritonitis and diabetic nephropathy being exacerbated under SIRPα deficiency (Bian et al., 2016; Li et al., 2019; Shi, Bian, & Liu, 2017; Zen et al., 2013). Thus, we hypothesized that SIRPα likely prevents the overactivation of macrophages to the point of becoming hemophagocytic under pro-inflammation and potentially serves as a critical barrier in the macrophage-intrinsic contribution to S-HLH/CSS pathogenesis.

2.2 Materials and Methods

2.2.1 Mouse Models

Mice were eight to eleven weeks of age and sex-matched for all studies. Wild-type (C57BL/6J) and CD47−/− (B6.129-CD47tm1Fpl/J) mice were purchased from The Jackson Laboratory. SIRPα−/− mice were established as previously described (Bian et al., 2016). All mice were housed in a specific-pathogen-free facility. All animal experiments and procedures for handling were approved by the Institutional Animal Care and Use Committee of Georgia State University.
In cytosine guanine dinucleotide (CpG) experiments, WT, SIRPα⁻/⁻ or CD47⁻/⁻ mice were intraperitoneally (IP) injected with 50 μg of CpG-B (ODN1826; InvivoGen) suspended in 50 μL phosphate-buffered saline (PBS) every other day for a total of three injections and were euthanized forty-eight hours (day 6) thereafter for analyses. To neutralize IFNγ, mice were intravenously (IV) injected with anti-IFNγ antibody (500 μg/mouse; XMG1.2, Bio X cell) or isotype control (BioLegend) two hours prior to receiving CpG injections. For macrophage depletion experiments, mice were IP injected with 100 μL of empty or clodronate-containing liposomes (5 mg/mL, Encapsula Nano Sciences) once a day for two consecutive days, with the initial injection given forty-eight hours prior (day -2) to the first CpG injection. In other experiments, WT or SIRPα⁻/⁻ mice were injected with zymosan A (500 μg/mouse, IP; Sigma), poly I:C (10 mg/kg, IP; InvivoGen), lipopolysaccharide (LPS) (0.25 mg/kg, IP; Sigma, E. coli O111:B4), recombinant TNFα (10 μg/kg, IV; PeproTech), recombinant IL-6 (10 μg/kg, IV; PeproTech), recombinant IL-17A (10 μg/kg, IV; PeproTech) or recombinant IFNγ (10 μg/kg, IV; PeproTech) suspended in PBS every other day for a total of three injections and were euthanized forty-eight hours thereafter for analyses (Bian et al., 2016).

2.2.2 Hematology

Disease progression was monitored daily by changes in hemoglobin, which was assessed by serial sampling of blood (30-40 μL) via the saphenous vein. Hemoglobin concentration was determined by adding 25 μL of whole blood to diluted Drabkin reagent (Sigma) and then measuring the spectral absorbance of hemiglobincyanide at 540nm.
Percent change in hemoglobin was determined by: \((100 - \frac{\text{measured hemoglobin}}{\text{initial hemoglobin}}) \times 100\). To analyze peripheral blood at the treatment endpoint, mice were euthanized, and blood was extracted by cardiac puncture into heparinized tubes. 100 μl of whole blood was then used to isolate platelets (Aurbach, Spindler, Haining, Bender, & Pleines, 2019), which were then counted on a Cellometer Auto2000 (Nexcelom). The remaining whole blood was then centrifuged \((1000 \times g)\) to isolate the plasma for later quantification of cytokines and other biomarkers. The buffy coat and RBC pellet were resuspended in lysis buffer \((150 \text{ mM ammonium chloride, 10 mM potassium bicarbonate, and 0.1 mM EDTA})\) for RBC lysis and the remaining leukocytes were then counted on a Cellometer Auto2000.

2.2.3 Histology and Microscopy

Freshly isolated spleens and livers were embedded in OCT compound, flash frozen in liquid nitrogen and then stored at -80°C. Tissues were cryostat sectioned at 6-10 μm and then immediately fixed in 4% paraformaldehyde (PFA). Hematoxylin and eosin (H&E) stains were performed for histological analysis of hemophagocytosis in bone marrow smears, spleens and livers. For adoptive transfer experiments, mice were first IV injected with \(~10^9\) CFSE-stained RBC and after 1h, mice were IP injected with CpG (50 μg) or PBS (Bian et al., 2016). After twelve hours, mice were euthanized and their spleens removed. In some experiments, spleens were digested and splenocytes were isolated for flow cytometry to quantify the percentage of hemophagocytic \((\text{CFSE}^+)\) RPM. For immunofluorescent (IF) staining, sections were blocked with 5% BSA and incubated with
PE-conjugated rat-anti-mouse F4/80 (BM8; BioLegend). DAPI was used to stain nuclei. For immunohistochemistry (IHC) analyses, spleen tissue sections were treated with 0.3% H$_2$O$_2$, blocked with 10% BSA and then stained with biotin-conjugated rat anti-mouse F4/80 (BM8; BioLegend) at 4°C for eighteen hours. After washing, slides were incubated with HRP-conjugated streptavidin (Thermo Fisher) and exposed to DAB (Thermo Fisher). Thereafter, sections were H&E counterstained. All images were acquired with a BZ-X700 All-In-One Fluorescent Microscope (Keyence).

### 2.2.4 Flow Cytometry Analyses of Tissue-Resident Leukocytes

To isolate splenocytes, spleens were minced, digested in a cocktail of collagenase (0.17 mg/mL, Sigma) and DNase1 (40 μg/mL, Sigma) in RPMI-1640 at 37°C, passed through a 70-μm cell strainer and then contaminating RBC were removed with lysis buffer and several washes. All staining was done in the presence of rat anti-mouse CD16/CD32 antibody (Fc block, BioLegend) and in the dark at 4°C. To assess phagocytosis of RBC, splenocytes were additionally blocked with purified anti-mouse Ter-119 (BioLegend) prior to cell surface staining with fluorescently labeled antibodies (Akilesh et al., 2019; Ohyagi et al., 2013; Zoller et al., 2011). Cells were then stained with antibody cocktails comprising: CD45.1-Pacific Blue (A20), CD11b-APC (M1/70), CD11c-FITC (N418), F4/80-PE (BM8), Ly6C-BV650 (HK1.4) and Ly6G-PerCP (1A8) (all from BioLegend) for myeloid cells. To determine lymphocyte populations, cells were stained for the following markers: CD45.1-Pacific Blue (A20), B220-BV605 (RA3-6B2), CD4-APC (RM4-4), CD8-PE and NK1.1-FITC (PK136) (all antibodies from BioLegend). Cells were then washed
and incubated with 7-AAD to determine live cells. To assess phagocytosis of RBC, cells were again washed and then prepared for intracellular staining with fixation and permeabilization buffer (BD Biosciences), washed in perm/wash buffer (BD Biosciences) and then stained with anti-Ter-119-PE/Cy7 (Ter-119, BioLegend). Data were acquired on a BD LSRFortessa (BD Biosciences) and analyzed using FlowJo 10.6.01 (Tree Star).

2.2.5 Cytokine and Biomarker Quantification

Twelve hours following a single injection of PBS, CpG or LPS, plasma levels of IL-1β, IL-6, IL-10, IL-12, TNFα, GM-CSF, or MCP-1 were determined by standard enzyme-linked immunosorbent assay (ELISA) using capture, detection and HRP-conjugated antibodies (all from BioLegend). Similarly, free IL-18 in plasma was assayed using the Mouse IL-18 ELISA Kit (MBL). Plasma levels of triglyceride, ferritin and soluble CD25 (IL-2Rα) were determined using the Triglyceride Assay Kit (Abcam), Mouse Ferritin ELISA Kit (Abcam) and Mouse CD25/IL-2R alpha DuoSet ELISA (R&D Systems), respectively. To assay BMM cytokine or ferritin production, BMM were treated with PBS or CpG (1 μg/mL) ± mCD47.ex for twelve hours. Thereafter, cell-free BMM-conditioned medium was assessed by ELISA.

2.2.6 Macrophage Phagocytosis Assay

Bone marrow-derived macrophages (BMM) were generated as previously described (Bian et al., 2016; Ha et al., 2013). To assess phagocytic activity, BMM were treated with PBS, CpG (1 μg/mL), Poly I:C (100 ng/mL), LPS (20 ng/mL), TNFα (20 ng/mL) or IFNγ
(20 ng/mL) for twelve hours, and then were washed and incubated with peripheral blood cells (PBC) for thirty minutes at 37°C. BMM-PBC co-cultures were then washed, extracellular RBC were lysed and then cells were visualized by microscopy. For IF staining, PBC were labeled with CFSE prior to co-culture with BMM. BMM were then labeled with PE-conjugated anti-F4/80 and fixed with 4% PFA. After fixation, nuclei were stained with DAPI and cells were visualized by fluorescent microscopy.

2.2.7 Soluble Murine CD47 Extracellular Domain Fusion Protein

The AP-tag2 plasmid containing the extracellular domain of murine CD47 (mCD47.ex) and alkaline phosphatase (AP) was a generous gift of V. Narayanan (University of Pittsburgh School of Medicine) (P. H. Jiang, C. F. Lagenaur, & V. Narayanan, 1999). After transfecting COS cells, recombinant mCD47.ex fusion proteins were produced, affinity purified and stored in PBS as previously described (P. H. Jiang et al., 1999; Y. Liu et al., 2002; Y. Liu et al., 2004; Y. Liu et al., 2007; Lv et al., 2015). Prior to use, the binding capacity of mCD47.ex was confirmed by binding to murine SIRPα extracellular domain fusion protein (mSIRPα.ex-Fc) as well as adhesion to macrophages in a CD47-SIRPα-dependent manner.

2.2.8 SDS-PAGE and Immunoblot Analyses

BMM were either non-treated or treated with CpG (1 μg/mL) in the presence or absence of mCD47.ex for thirty minutes at 37°C. Thereafter, BMM were washed and then incubated with ice-cold lysis buffer containing 25mM Tris-HCl, pH 7.4, 150mM NaCl, 1%
Triton X-100, protease inhibitors (Protease Inhibitor Cocktail, Sigma-Aldrich), phosphatase inhibitors (Phosphatase Inhibitor Cocktail 1 and 2, Sigma), 3mM PMSF and 2mM pervanadate. After centrifugation at 12,000rpm for five minutes, cell lysates were collected and then heated at 95°C for three minutes in SDS-PAGE sample buffer. After electrophoresis in acrylamide gels, proteins were transferred onto nitrocellulose, followed by blocking with 3% BSA and probed for: SIRPα (clone P84, BioLegend); phospho-Erk1/2 (D13.14.4E); phospho-p38 (D3F9); and beta-actin (13E5) (all from Cell Signaling Technology). Densitometric analyses were performed using Image J (NIH).

2.2.9 Statistical Analyses

All graphs and statistical analyses were generated and performed using Prism 6.0 (GraphPad Software). Statistical significance was calculated using Student’s t test (k = 2) or analysis of variance (ANOVA; one- or two-way) (k > 2). For post-hoc analyses, Dunn’s or Tukey’s HSD test was used to determine statistical significance among multiple comparisons, with an experiment-wise error rate of 0.05. When P < 0.05, samples were considered statistically significant. At least three independent experiments were performed for each set of data, which were represented as individual values, mean ± SEM or both.
2.3 Results

2.3.1 SIRPα deficiency exacerbates and accelerates the onset of TLR9-induced secondary hemophagocytic lymphohistiocytosis in mice

As a TLR9 agonist, cytosine guanine dinucleotide (CpG) is both a relevant TLR stimulus in S-HLH and a viable trigger for activating SIRPα<sup>−/−</sup> phagocytes to engulf healthy host cells (Edward M. Behrens et al., 2011; Bian et al., 2016). To examine the role of SIRPα in TLR9-induced S-HLH, we repeatedly injected WT or SIRPα<sup>−/−</sup> mice with CpG. The development of S-HLH was monitored by measuring changes in hemoglobin. As shown (Figure 2.1A, left panel), whereas WT mice did not exhibit anemia, i.e., ≥ 20% reduction in hemoglobin, until 24h after the third injection of CpG, SIRPα<sup>−/−</sup> mice experienced a ~25% drop in hemoglobin after merely one dose of CpG. With every additional dose of CpG, SIRPα<sup>−/−</sup> mice became increasingly more anemic and decreased their hemoglobin in excess of 60% within 48 hours after three injections (day 6), which led us to terminate the study (Figure 2.1A). By day 6, CpG-treated WT mice were only slightly anemic, whereas SIRPα<sup>−/−</sup> mice had severe pancytopenia (Figure 2.1, A-B). Assaying other pathological features of S-HLH, we found that plasma ferritin, triglycerides and soluble CD25/IL-2Rα (sCD25) were highly elevated in CpG-treated SIRPα<sup>−/−</sup> mice, whereas WT mice only had a slight increase in sCD25 (Figure 2.1, C-E) (Al-Samkari & Berliner, 2018). Screening resected organs found that WT mice had normal livers and only moderate splenomegaly (Figure 2.2, A-B). However, CpG-treated SIRPα<sup>−/−</sup> mice had severe hepatosplenomegaly, accounting for ≥ 10% of their body weight. Histological examination
of CpG-treated spleens revealed the splenic white pulp architecture remained uniform in WT mice but was greatly disrupted in SIRPα−/− mice (Figure 2.3A).

A) Disease severity was monitored by measuring daily changes in hemoglobin (left). A-B) Following euthanization on day 6: anemia (A; right), leukopenia (B; left) and thrombocytopenia (B; right) were quantified by peripheral blood analyses; C-E) additional blood markers of plasma ferritin (C), triglycerides (D) and soluble CD25 (E) were determined by ELISA. The data are pooled from twelve independent experiments and all data were measured in triplicate. Each symbol represents an individual mouse, with the mean ± SEM also shown. Two-way ANOVA and Tukey’s HSD post-hoc analyses were used determine statistical significance among multiple comparisons: not significant (ns) = P > 0.05; *P < 0.05; **P < 0.01; ****P < 0.0001.

**Figure 2.1 SIRPα deficiency exacerbates and accelerates the onset of TLR9-induced secondary HLH-associated pancytopenia and other inflammatory peripheral biomarkers**

A-I) Wild-type (WT) or SIRPα−/− mice were challenged with three intraperitoneal (IP) injections of CpG-B (ODN 1864; 50 µg/mouse; n = 9/group) or PBS (100 µl/mouse; n = 5/group) on days 0, 2 and 4 and were euthanized on day 6 for analyses. A) Disease severity was monitored by measuring daily changes in hemoglobin (left). A-B) Following euthanization on day 6: anemia (A; right), leukopenia (B; left) and thrombocytopenia (B; right) were quantified by peripheral blood analyses; C-E) additional blood markers of plasma ferritin (C), triglycerides (D) and soluble CD25 (E) were determined by ELISA. The data are pooled from twelve independent experiments and all data were measured in triplicate. Each symbol represents an individual mouse, with the mean ± SEM also shown. Two-way ANOVA and Tukey’s HSD post-hoc analyses were used determine statistical significance among multiple comparisons: not significant (ns) = P > 0.05; *P < 0.05; **P < 0.01; ****P < 0.0001.
Figure 2.2 SIRPα deficiency exacerbates organomegaly in TLR9-induced S-HLH
Livers (A) and spleens (B) were weighed and normalized to their respective total body weight. Representative image of splenomegaly (B; right). C) Myeloid population frequencies (left) and counts (right) among CD45+ splenocytes were determined by flow cytometry: CD11b-F4/80+ red pulp macrophages (RPM), CD11b+Ly6G+Ly6C+ monocytes (MC), CD11b+Ly6Ghi neutrophil (PMN) and CD11c+ dendritic cells (DC). The data are pooled from twelve independent experiments and all data were measured in triplicate. Each symbol represents an individual mouse, with the mean ± SEM also shown. Two-way ANOVA and Tukey’s HSD post-hoc analyses were used to determine statistical significance among multiple comparisons: not significant (ns) = P > 0.05; * P < 0.05; *** P < 0.001; **** P < 0.0001.
Figure 2.3 SIRPα deficiency exacerbates inflammation-driven extramedullary leukocytosis in the spleens of TLR9-induced S-HLH mice
A) Hematoxylin and eosin staining of spleen tissue sections from CpG-treated WT or SIRPα−/− mice; magnification 20x. B-D) Following CpG or PBS injections, splenocytes were isolated from WT and SIRPα−/− mice. B) The number of CD45+ leukocytes among splenocytes were determined by flow cytometry. C) The frequency (left) and count (right) of lymphocyte populations. D) Summary of splenic leukocyte frequencies among the total CD45+ splenocytes within WT versus SIRPα−/− spleens. The data are pooled from twelve independent experiments and all data were measured in triplicate. Each symbol represents an individual mouse, with the mean ± SEM also shown.

Indeed, splenic leukocyte expansion was commensurate to the severity of splenomegaly, with CpG-treated SIRPα−/− mice having 2-3-fold more CD45+ splenocytes than WT mice (Figure 2.3B), as determined by flow cytometry (Figure 2.4). Healthy SIRPα−/− mice had slightly lower frequencies of DC and CD4 T cells (Figure 2.2C and Figure 2.3, C-D), corroborating previous findings (Bian et al., 2016; Y. Saito et al., 2010; Yasuyuki Saito et al., 2017). After CpG treatment, SIRPα−/− mice had lower splenic frequencies of B cells and CD4 T cells than WT mice; however, adjusting for cell count mitigated these differences, as SIRPα−/− mice had 20% more B cells and similar a quantity of CD4 cells as WT mice (Figure 2.2C, right panel). Whereas neither the frequency nor quantity of splenic
myeloid leukocytes increased in CpG-treated WT mice, PMN, MC and RPM had increased 2-3-fold in SIRPα−/− mice (Figure 2.2C and Figure 2.3D). Thus, myeloid cell and CD8 T cell expansion appeared to account for the reduced B cell frequency in CpG-treated SIRPα−/− spleens (Figure 2.3, C-D).

Figure 2.4 Flow cytometry gating strategy for lymphocyte, myeloid and hemophagocyte populations within the spleen
Representative gating strategy of live, singlet leukocytes (CD45+): F4/80+CD11b+ RPM; CD11b+Ly6G-Ly6C+ MC; CD11b+Ly6Ghi PMN; CD11c+ DC; B cells (B220+NK1.1−); CD4 T cells (CD4+CD8−); CD8 T cells (CD8+CD4−); & NK cells (NK1.1+). Hemophagocytes were determined by first extracellular saturation with non-conjugated anti-mouse Ter-119 antibody followed by intracellular staining with fluorescently labeled anti-Ter-119-PE/Cy7 and were gated within CD45+F4/80+CD11b+ RPM, CD45+CD11b+Ly6G-Ly6C+ MC, CD45+CD11b+Ly6Ghi PMN and CD45+CD11c+ DC.
Being a CSS, S-HLH is typified by elevated pro-inflammatory cytokines, with overactivated macrophages being a major source (Edward M. Behrens & Koretzky, 2017; Scott W. Canna & Behrens, 2012; Scott W. Canna et al., 2014; Schulert & Grom, 2014a). As an absence of SIRPα worsens disease conditions (Bian et al., 2016; Kong et al., 2007; Li et al., 2019; Londino et al., 2015; Shi et al., 2017; Zen et al., 2013), we assessed whether SIRPα deficiency would exacerbate CpG-induced hypercytokinemia.

Figure 2.5 SIRPα deficiency exacerbates hypercytokinemia in TLR9-induced S-HLH
Concentrations of plasma cytokines (IL-1β, IL-6, IL-12, TNFα, MCP-1 and GM-CSF) from WT or SIRPα−/− mice 12 hours after receiving an injection of PBS or CpG. The data are pooled from twelve independent experiments and all data were measured in triplicate. Each symbol represents an individual mouse, with the mean ± SEM also shown. Two-way ANOVA and Tukey’s HSD post-hoc analyses were used determine statistical significance among multiple comparisons: not significant (ns) = P > 0.05; *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.
Healthy WT and SIRPα<sup>−/−</sup> mice mostly exhibited similar cytokine levels; however, under TLR9 stimulation, SIRPα<sup>−/−</sup> mice had 2-4-fold higher levels of IL-1β, IL-6, IL-12, IL-18 and TNFα than WT mice (Figure 2.5). The exacerbated phenotype in CpG-treated SIRPα<sup>−/−</sup> mice was not due to a stunted anti-inflammatory response, as both mice produced comparable amounts of IL-10 after CpG treatment. Next, we assayed cytokines associated with granulopoiesis and myeloid leukocyte chemotaxis to determine what led to the expanded myeloid population in CpG-treated SIRPα<sup>−/−</sup> spleens (Bian et al., 2018; Kantari, Pederzoli-Ribeil, & Witko-Sarsat, 2008; Khanna-Gupta & Berliner, 2018). As with IL-10, GM-CSF was low in SIRPα<sup>−/−</sup> mice prior to TLR9 signaling, but after CpG, circulating GM-CSF significantly increased. The chemoattractant MCP-1 (CCL2) was also produced almost 3-fold greater in SIRPα<sup>−/−</sup> mice than in WT mice responding to TLR9 agonism. Collectively, these data suggest that an absence of SIRPα enhances both the kinetics and severity of S-HLH onset induced by TLR9-driven inflammation (Girard-Guyonvarc'h et al., 2018; A. Wang et al., 2019).

2.3.2 Absence of SIRPα during TLR9 stimulation induces hemophagocytic leukocytes

Our previous study showed that acute inflammation in SIRPα<sup>−/−</sup> mice led to erythrophagocytosis, consumptive anemia and splenomegaly (Bian et al., 2016), thus we next assessed the extent of hemophagocytosis within the spleen following TLR9 stimulation. To quantify splenic hemophagocytes, splenic leukocytes (CD45<sup>+</sup>) were intracellularly stained for the RBC-associated antigen Ter-119 (Akilesh et al., 2019; J. Liu
et al., 2013; Ohyagi et al., 2013; Zoller et al., 2011). Regardless of genotype, about 10-20% of RPM were Ter-119+ under healthy conditions (Figure 2.6, A-B), corroborating previous reports (Akilesh et al., 2019; Zoller et al., 2011). However, whereas the frequency of Ter-119+ RPM did not significantly increase in WT mice following CpG treatment, over 50% of RPM were Ter-119+ in the absence of SIRPα. Since CpG-treated SIRPα−/− spleens were highly infiltrated with other myeloid leukocytes that innately express SIRPα, we also assessed the frequency of Ter119+ cells among MC, PMN and DC (Ohyagi et al., 2013). Without inflammatory stimulation, no differences in Ter-119+ MC, PMN and DC frequency were detected. After CpG treatment, however, all SIRPα−/− myeloid cells exhibited robust hemophagocytosis (Figure 2.6, A-B), whereas the quantity of Ter-119+ cells did not significantly increase in WT mice. On average, CpG-treated SIRPα−/− mice spleens comprised 18-fold more Ter-119+ leukocytes than CpG-treated WT mice (Figure 2.6B).
**Figure 2.6** Absence of SIRPa during TLR9 stimulation induces hemophagocytic leukocytes

A) Frequencies of hemophagocytic CD45+ splenocytes (F4/80+CD11b+ RPM, CD11b+Ly6G-Ly6C+ MC, CD11b+Ly6G+ PMN and CD11c+ DC) were determined by intracellular staining for RBC (Ter-119+). B) Frequencies (left), individual counts (middle) and total counts (right) of hemophagocytes from PBS- or CpG-treated mice (n = 5/group). The data are pooled from eight independent experiments and each symbol represents an individual mouse, with the mean ± SEM also shown. Two-way ANOVA and Tukey’s HSD post-hoc analyses were used to determine statistical significance among multiple comparisons: ns = P > 0.05; ****P < 0.0001.

Indeed, hematoxylin and eosin staining of spleen and liver tissue sections, as well as bone marrow smears, were insufficient to detect hemophagocytosis in CpG-treated WT mice (not shown), whereas hemophagocytosis was apparent in tissues isolated from SIRPa−/− mice (Figure 2.7A). To determine whether this difference in hemophagocytosis may occur after just one CpG injection, we adoptively transferred fluorescence-labeled (CFSE) RBC with CpG or PBS concurrently injected (Bian et al., 2016). In mice receiving CpG, 7% of RPM were CFSE+ in WT mice whereas 40% were CFSE+ in SIRPa−/− mice (Figure 2.7B). Immunofluorescent staining of spleen sections from CpG-treated mice
supported our flow cytometry data (Figure 2.7C, inset), with the frequency of CFSE⁺F4/80⁺ macrophages (yellow) being much more apparent in spleens of CpG-treated SIRPα⁻/⁻ mice.

Figure 2.7 Activated SIRPα-deficient macrophages phagocytose healthy red blood cells
A) Representative images of hemophagocytes within the bone marrow, spleen and liver of CpG-treated SIRPα⁻/⁻ mice as determined by hematoxylin and eosin staining (scale bar: 10 µm) (n = 3/group). B) Histogram frequency of CD45⁺CD11b⁻F4/80⁺ RPM with intracellular RBC (CFSE⁺). C) Representative image of immunofluorescent stained spleen tissue sections after RBC (10⁹) transfer in CpG-treated WT and SIRPα⁻/⁻ mice; (scale bar: 100 µm). The data are representative of eight independent experiments.

2.3.3 CD47 deficiency does not phenocopy SIRPα deficiency under TLR9 agonism

Serving as a ligand for SIRPα, CD47 is a critical self-recognition molecule expressed on healthy host cells, which are normally not phagocytosed (P. A. Oldenborg et al., 2000). One study previously reported that serum from HLH patients can down-regulate CD47 on hematopoietic stem cells (HSC), which led macrophages to phagocytose HLH serum-
treated HSC in vitro. However, the question remains: do CpG-treated CD47<sup>−/−</sup> mice similarly develop fulminant S-HLH as in SIRPα<sup>−/−</sup> mice? Following CpG treatment, hemoglobin sharply dropped in CD47<sup>−/−</sup> mice, which largely mimicked the kinetics and severity in CpG-treated SIRPα<sup>−/−</sup> mice (Figure 2.8A). However, unlike CpG-treated SIRPα<sup>−/−</sup> mice, CD47<sup>−/−</sup> mice did not develop leukopenia or thrombocytopenia (Figure 2.8B). CpG-treated CD47<sup>−/−</sup> mice had slightly elevated ferritin, albeit not comparably as severe in SIRPα<sup>−/−</sup> mice (Figure 2.8C). Furthermore, both plasma triglycerides and sCD25 were not highly elevated in CpG-treated CD47<sup>−/−</sup> mice (Figure 2.8, D-E).

**Figure 2.8** CD47 deficiency does not phenocopy SIRPα deficiency under TLR9 agonism

SIRPα<sup>−/−</sup> or CD47<sup>−/−</sup> mice were injected with CpG or PBS (n = 5/group) on days 0, 2 and 4 and euthanized on day 6 for analyses. A) Daily changes in hemoglobin (left). Following euthanization on day 6: anemia (A; right), leukopenia (B; left) and thrombocytopenia (B; right) were quantified and additional blood markers of plasma ferritin (C), triglycerides (D) and soluble CD25 (E) were determined. The data are pooled from nine independent experiments and all data were measured in triplicate. Each symbol represents an individual mouse, with the mean ± SEM also shown. Two-way ANOVA and Tukey’s HSD post-hoc analyses were used to determine statistical significance among multiple comparisons: ns = P > 0.05; *P < 0.05; **P < 0.001; ***P < 0.0001.
As anticipated, CD47⁻/⁻ mice developed splenomegaly after TLR9 stimulation (Figure 2.9A), the result of which was associated with a largely similar expanded presence of CD45⁺ leukocytes (Figure 2.9B) (Bian et al., 2016). However, the only leukocyte population expanded to a greater extent in CD47⁻/⁻ spleens than in SIRPα⁻/⁻ spleens was B cells – but only after adjusting for cell count (Figure 2.9C). Indeed, an absence of CD47 only led to moderate expansion of RPM and a slight increase in splenic infiltration of PMN (Figure 2.9, D-E). Despite exhibiting a noticeable increase in Ter-119⁺ RPM, CpG-treated CD47⁻/⁻ spleens comprised exceptionally less hemophagocytes than SIRPα⁻/⁻ spleens, which had nearly 5-fold more Ter-119⁺ cells than CD47⁻/⁻ mice (Figure 2.9, F-G). Assessing plasma cytokines found that CD47⁻/⁻ mice were appreciably less inflamed than SIRPα⁻/⁻ mice, which likely associated with the relatively less prominent extramedullary myelopoiesis in the spleens of CD47⁻/⁻ mice (Figure 2.9H). Collectively, these data suggest that SIRPα negatively regulates inflammation in a CD47-independent manner and that while CD47 deficiency is sufficient to develop hemophagocytosis, anemia and splenomegaly, it does not fully mimic SIRPα deficiency with respect to fully developing S-HLH.
Figure 2.9 CD47 deficiency fails to develop fulminant S-HLH under TLR9 agonism
Spleens were isolated to determine splenomegaly (A), the count of CD45+ leukocytes (B), the frequency (C; left) and count (C; right) of lymphocytes, and the frequency of myeloid leukocytes (D). splenic hemophagocytes. I) Plasma cytokines (MCP-1, GM-CSF, IL-6, and TNFα) assayed 12h following a single CpG or PBS injection. The data are pooled from nine independent experiments and all data were measured in triplicate. Each symbol represents an individual mouse, with the mean ± SEM also shown. Two-way ANOVA and Tukey’s HSD post-hoc analyses were used to determine statistical significance among multiple comparisons: ns = *P > 0.05; *P < 0.05; ***P < 0.001; ****P < 0.0001.

2.3.4 Macrophage depletion but not IFNγ neutralization ameliorates TLR9-induced S-HLH in SIRPα-/- mice

Hypercytokinemia is largely considered to precede multi-organ failure and death in CSS/HLH. Accordingly, current efforts to treat HLH/CSS primarily revolve around neutralization of cytokines considered putative drivers of the immunopathology. IFNγ is one such cytokine that is considered critical to manifesting immunopathological features of HLH (Edward M. Behrens et al., 2011; George, 2014; Girard-Guyonvarc'h et al., 2018), and indeed, previous studies have shown that neutralization IFNγ may reverse S-HLH symptoms in the TLR9-induced S-HLH mouse model, even when exacerbated by co-administering antagonistic IL-10 receptor antibodies (Albeituni et al., 2019; Edward M. Behrens et al., 2011; S. W. Canna et al., 2013; Girard-Guyonvarc'h et al., 2018). Thus, we assessed whether IFNγ neutralization could similarly prove to be therapeutic in CpG-treated SIRPα-/- mice by co-administering anti-IFNγ antibody (αIFNγ; XMG1.2) or isotype control (IgG ctl.) (Figure 2.10). To our surprise, IFNγ neutralization was largely ineffective in preventing S-HLH onset in SIRPα-/- mice, suggesting S-HLH pathogenesis under SIRPα deficiency is not dependent upon IFNγ.
Our prior study implicated macrophages as drivers of anemia in inflamed SIRPα<sup>-/-</sup> mice (Bian et al., 2016), thus we assessed the therapeutic efficacy of macrophage depletion by administering empty (EL) or clodrosome-containing liposomes (CL2MDP) prior to (day -2 and -1) CpG treatment (Biewenga et al., 1995; Bu, Gao, Qu, & Liu, 2013; Ciavarra et al., 2005; Kirby, Beattie, Maroof, van Rooijen, & Kaye, 2009; S. Mahajan et al., 2019; Sahil Mahajan et al., 2020). In contrast to IFNγ neutralization, macrophage depletion by CL2MDP precluded S-HLH in CpG-treated SIRPα<sup>-/-</sup> mice. CL2MDP prevented the rapid onset of severe pancytopenia, with CpG-treated SIRPα<sup>-/-</sup> mice only exhibiting a 10% drop in Hemoglobin by day 6 (Figure 2.10, A-B). Furthermore, macrophage depletion drastically reduced plasma ferritin and triglyceride levels and slightly lowered sCD25 levels as well (Figure 2.10, C-E). Assessing resected spleens, CL2MDP effectively prevented splenomegaly and also led to a significant reduction in myeloid splenocyte frequency and count (Figure 2.10, F-H). While CL2MDP did not significantly affect the frequency of Ter-119<sup>+</sup> splenocytes, adjusting for cell count revealed a marked reduction in the quantity of splenic hemophagocytes (Figure 2.10I). Notably, CL2MDP largely abated hypercytokinemia, albeit GM-CSF remained moderately elevated (Figure 2.10J). These data suggest that phagocytes, chiefly macrophages, are primarily responsible for TLR9-driven S-HLH pathology in SIRPα<sup>-/-</sup> mice.
Figure 2.10 Macrophage depletion but not IFNγ neutralization precludes TLR9-induced S-HLH in SIRPα−/− mice

SIRPα−/− mice were treated with PBS (n = 5) or CpG co-administered with IgG isotype control (n = 5) or αIFNγ (XMG1.2; n = 5) on days 0, 2 and 4 and euthanized on day 6 for analyses. To deplete macrophages empty (EL; n = 5) or clodronate-containing liposomes (CL2MDP; n = 5) were administered prior (day -2 and -1) to CpG treatment. A) Daily changes in hemoglobin (left). Following euthanization on day 6: A-E) anemia (A; right), leukopenia (B; left) and thrombocytopenia (B; right) were quantified and additional blood markers of plasma ferritin (C),
triglycerides (D) and soluble CD25 (E) were determined. F-H) Spleens were isolated to determine splenomegaly (F), the frequency of myeloid leukocytes (G) and the frequency (H; left) and the count (H; middle and left) of hemophagocytes. I) Plasma cytokines (IL-1β, IL-6, IL-12, TNFα and GM-CSF) assayed 12 hours following a single injection of PBS, CpG + IgG isotype control, CpG + αIFNγ or CpG alone after EL or CL2MDP treatment. The data represent thirteen independent experiments and all data were measured in triplicate. Each symbol represents an individual mouse, with the mean ± SEM also shown. One-way ANOVA and Dunn’s post-hoc analyses were used determine statistical significance among multiple comparisons: not significant (ns) = P > 0.05; *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.

2.3.5 SIRPα negatively regulates TLR9-driven Erk1/2 and p38 activation to inhibit macrophage function

Given CL2DMP treatment abrogated S-HLH onset in CpG-treated SIRPα−/− mice, we sought to determine whether SIRPα directly affects TLR9 signaling in macrophages. As anticipated, SIRPα inhibited CpG-treated WT and CD47−/− bone marrow-derived macrophages (BMM) from phagocytosing WT (CD47+) peripheral blood cells (PBC) (Figure 2.11A). Indeed, fluorescent-labeled CpG-activated WT or CD47−/− BMM (red) failed to phagocytose CFSE-positive PBC (green), whereas similarly treated SIRPα−/− BMM were highly phagocytic after CpG treatment and engulfed many PBC, including RBC and nucleated cells (arrows). Aside from being more hemophagocytic, SIRPα−/− BMM were adept at secreting pro-inflammatory cytokines (Figure 2.11B). To determine the effect of SIRPα on BMM cytokine production, WT, CD47−/− and SIRPα−/− BMM were treated with CpG in the presence or absence of CD47 ligation via murine CD47 extracellular domain fusion protein (mCD47.ex) (P. H. Jiang et al., 1999; Y. Liu et al., 2002; Y. Liu et al., 2004; Y. Liu et al., 2007; Lv et al., 2015). In the absence of mCD47.ex, CpG-treated WT and CD47−/− BMM produced on average 2-fold less IL-1β, IL-6, IL-12 and TNFα than SIRPα−/− BMM (Figure 2.11B). Conversely, SIRPα−/− BMM produced markedly
less IL-10 than WT or CD47^{-/-} BMM in response to TLR9 signaling. Given that previous studies have shown macrophages are a major source of extracellular ferritin (Cohen et al., 2010; Rosário, Zandman-Goddard, Meyron-Holtz, D’Cruz, & Shoenfeld, 2013; Truman-Rosentsvit et al., 2018), BMM-conditioned culture medium was also assayed for extracellular ferritin (Figure 2.11C). Indeed, SIRPα^{-/-} BMM secreted significantly more ferritin after CpG treatment than WT or CD47^{-/-} BMM. Furthermore, the disparity between WT or CD47^{-/-} BMM and SIRPα^{-/-} BMM insofar as their capacities to secrete pro-inflammatory cytokines and ferritin was magnified in the presence CD47 ligation (+mCD47.ex) (Figure 2.11, B-C). Along these lines, CpG-treated WT and CD47^{-/-} BMM produced even more anti-inflammatory cytokine IL-10 in the presence of mCD47.ex, whereas SIRPα^{-/-} BMM production of IL-10 remained limited under TLR9 signaling. Given that CD47-SIRPα signaling appeared to impact BMM cytokine production in response to TLR9 stimulation, we examined potential differences in macrophage TLR9 signaling in the presence or absence of CD47-SIRPα signaling (Figure 2.11D). The extent to which Erk1/2 (p44/42) and p38 (p38) were phosphorylated was mostly similar among BMM after CpG treatment alone. However, in the presence of CD47 ligation (+mCD47.ex) and thus strong SIRPα signaling, CpG-treated WT and CD47^{-/-} BMM exhibited a level of phosphorylation Erk1/2 and p38 that paralleled that of non-treated BMM, whereas SIRPα^{-/-} BMM were unaffected and maintained MAPK activation. Together, these data suggest that SIRPα tempers TLR9 signaling in macrophages by inhibiting MAPK.
Figure 2.11 SIRPα negatively regulates TLR9-driven Erk1/2 and p38 activation to inhibit macrophage hemophagocytosis, pro-inflammatory cytokine production and ferritin secretion

A) Bone marrow-derived macrophages (BMM) were generated from WT, CD47⁻/⁻ or SIRPα⁻/⁻ mice, treated with CpG (1 µg/mL) and then co-cultured with peripheral blood cells (PBC) isolated from healthy WT mice. Representative microscopy images of immunofluorescent stained BMM (PE-F4/80; red) and PBC (CFSE; green), with nuclei stained by DAPI (blue). The data are pooled from three independent experiments and each phagocytosis assay was performed in triplicate. B-C)
WT, CD47−/− and SIRPα−/− BMM were treated with CpG (1 µg/mL) in the presence or absence of CD47 ligation (± mCD47.ex) for 12h and cell-free medium was then assayed by ELISA to quantify IL-1β, IL-6, IL-10, IL-12, TNFα (B) and ferritin (C) secretion. The data represent four independent experiments and each experimental group was performed in triplicate. The data are presented as mean ± SEM. Two-way ANOVA and Tukey’s HSD post-hoc analyses were used to determine statistical significance among multiple comparisons: not significant (ns) = P > 0.05; *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. D) Representative immunoblot analyses of SIRPα, p38 phosphorylation (p-p38), Erk1/2 phosphorylation (p-p44/42) and beta-actin protein abundance in WT, CD47−/− and SIRPα−/− BMM that were non-treated, treated with mCD47.ex alone or treated with CpG (1 µg/mL) in the presence or absence of CD47 ligation (± mCD47.ex) for 30 minutes. All immunoblots are representative of five independent experiments. Densitometric analysis was used to determine the relative change in phosphorylated p44/42 and p38 by normalizing against beta-actin.

2.3.6 Activation of SIRPα−/− macrophages by various inflammatory factors confers S-HLH-like disease

Given other TLR agonists and certain cytokines fulfill the requisite activation signaling for phagocytosis of healthy host cells (Bian et al., 2016), we treated mice with zymosan A (TLR2), poly I:C (TLR3), LPS (TLR4), TNFα, IL-6 or IL-17A. One study has shown that IFNγ alone induces a partial HLH phenotype in mice (Zoller et al., 2011), thus we also assessed IFNγ-treated mice. Following zymosan, LPS or IFNγ treatment, WT mice developed mild anemia (Figure 2.12A). LPS treatment also induced splenomegaly and elevated ferritin (Figure 2.12, D-E). Otherwise, all treatments failed to develop S-HLH symptoms in WT mice.
Figure 2.12 Treatment of WT mice with various pro-inflammatory factors does not induce S-HLH
A) WT mice (n = 4 mice/group) were injected with PBS, zymosan A (500 μg/mouse, IP), poly I:C (10 mg/kg, IP), LPS (0.25 mg/kg, IP), recombinant TNFα (10 μg/kg, IV), recombinant IL-6 (10 μg/kg, IV), recombinant IL-17A (10 μg/kg, IV) or recombinant IFNγ (10 μg/kg, IV) every other day for a total of three injections and were euthanized 48 after the last injection for analyses. Peripheral blood markers for HLH were assessed: anemia (A), leukopenia (B), thrombocytopenia (C), hyperferritinemia (E) and hypertriglyceridemia (F). D) Spleen weight to determine splenomegaly. The data represent eleven independent experiments and are presented as mean ± SEM.

In contrast, treating SIRPα−/− mice with any inflammatory stimuli, except IFNγ, led to an S-HLH phenotype as that induced by CpG (Figure 2.13). All hemophagocytosis-activating factors conferred a moderate to severe drop in hemoglobin (Figure 2.13A). While all treatments induced severe thrombocytopenia, poly I:C, TNFα and IL-6 only induced mild/moderate leukopenia (Figure 2.13, B-C). All treatments, except IFNγ, led to severe splenomegaly, hyperferritinemia and hypertriglyceridemia (Figure 2.13, D-F).
Figure 2.13 Activation of SIRPα− macrophages by various pro-inflammatory factors confers S-HLH-like disease in mice

SIRPα− mice (n = 5 mice per treatment) were injected with PBS, zymosan A (500 μg/mouse, IP), poly I:C (10 mg/kg, IP), LPS (0.25 mg/kg, IP), recombinant TNFα (10 μg/kg, IV), recombinant IL-6 (10 μg/kg, IV), recombinant IL-17A (10 μg/kg, IV) or recombinant IFNγ (10 μg/kg, IV) on days 0, 2 and 4 were then euthanized on day 6 for analyses. Peripheral blood markers for HLH were assessed: anemia (A), leukopenia (B), thrombocytopenia (C), hyperferritinemia (E) and hypertriglyceridemia (F). D) Spleens were also excised and weighed to determine splenomegaly. The data are pooled from sixteen independent experiments. Each symbol represents an individual mouse. One-way ANOVA and Dunn’s post-hoc analyses were used to determined statistical significance among multiple comparisons: NS = P > 0.05; *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.

Immunohistochemical staining against F4/80 in spleen sections and in vitro phagocytosis assays with poly I:C, LPS or TNFα, but not IFNγ, demonstrated the exceptional capacity of activated SIRPα− phagocytes to uptake RBC (Figure 2.14A). Quantification of plasma cytokines in LPS-treated SIRPα− mice again demonstrated markedly worse hypercytokininemia under SIRPα deficiency (Figure 2.14B).
Figure 2.14 Activation of SIRPα− macrophages by various inflammatory factors confers hemophagocytosis in the spleen and hypercytokinemia in mice with HLH-like disease

A) Representative images of SIRPα− BMM treated with poly I:C (100 ng/mL), LPS (20 ng/mL), TNFα (20 ng/mL) or IFNγ (20 ng/mL) for 12 hours and then were incubated with RBC isolated from healthy mice (A; upper). Representative images of immunohistochemical staining against F4/80 among spleens isolated from poly I:C-, LPS-, TNFα- or IFNγ-treated SIRPα− mice (A; lower). B) 12h after one injection of LPS, the concentration of IL-1β, IL-6, IL-10, TNFα and GM-CSF were quantified. The data are pooled from sixteen independent experiments. Each symbol represents an individual mouse. Student’s t test was used to determined statistical significance among multiple comparisons: NS = $P > 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$.

H-scores were calculated and summarized in Figure 2.15. Collectively, these data indicate the importance of SIRPα in not only suppressing a hemophagocytic phenotype but also preventing the development of S-HLH/CSS under various inflammatory conditions.
Figure 2.15 Summary of HLH phenotype in mice with varied treatments

Six or five hallmarks were assessed in CpG-treated or other inflammatory stimuli-treated mice, respectively, in accordance with the HLH-2004 diagnostic criteria. An H-score $\geq 169$ accurately classifies 90% of individuals and corresponds to a sensitivity of 93% and specificity of 86% for HLH diagnosis. NS, not significant = $P > 0.05$; + = $P < 0.05$; ++ = $P < 0.01$; +++ = $P < 0.001$; ++++ $P < 0.0001$; ND, not determined.

2.4 Discussion

The appearance of aberrant histiocytes phagocytosing healthy host cells in S-HLH is largely attributed to the hypercytokinemic milieu (Hayden, Park, Giustini, Lee, & Chen, 2016; Schulert & Grom, 2015). However, the mechanism by which inflammatory signaling overactivates macrophages and other myeloid leukocytes to the point of manifesting a hemophagocytic phenotype remains unclear. This study identifies SIRP$\alpha$ as a critical deterrent against the induction of hemophagocytic myeloid leukocytes under inflammation and also the development of S-HLH/CSS. We found that activation of SIRP$\alpha^{-/-}$ leukocytes by TLR agonists (TLR2, TLR3, TLR4 or TLR9) or pro-inflammatory cytokines (TNF$\alpha$, IL-6 or IL-17A, but not IFN$\gamma$) leads to severe pancytopenia and other hallmarks of S-HLH (Al-Samkari & Berliner, 2018; Nikiforow & Berliner, 2020). While HLH-
associated cytokines down-regulate CD47 on HSC, allowing macrophages to phagocytose HSC in vitro (Kuriyama et al., 2012), we found that TLR9-driven inflammation in CD47−/− mice, unlike SIRPα−/− mice, does not induce S-HLH, but rather only leads to hemophagocytosis, anemia and splenomegaly (Figure 2.8, Figure 2.9 and Figure 2.15). Conceivably, the differences between SIRPα and CD47 in cellular expression and function underly the inability of CD47−/− mice to fully develop severe S-HLH under TLR9 agonism as in SIRPα−/− mice (A. N. Barclay & Brown, 2006; P.-A. Oldenborg, 2013). S-HLH symptoms associated with macrophage activation, such as hypercytokinemia, hypertriglyceridemia and hyperferritinemia, were less severe in CpG-treated CD47−/− mice than in SIRPα−/− mice (Cohen et al., 2010; George, 2014; Grom & Mellins, 2010). This disparity likely manifests due to the differing roles of SIRPα and CD47 in regulating macrophage activation, as we and others have shown that SIRPα tempers the pro-inflammatory macrophage phenotype (Figure 2.11) (Bian et al., 2016; Shi et al., 2017). Collectively, these observations suggest that there are CD47-independent SIRPα regulatory mechanisms that remain to be clarified.

Whereas SIRPα canonically suppresses phagocytosis of healthy host cells, several studies suggest that SIRPα also negatively regulates pro-inflammation, as SIRPα deficiency exacerbates disease conditions (Bian et al., 2016; Li et al., 2019; Shi et al., 2017; Zen et al., 2013). Similarly, SIRPα deficiency worsened and accelerated the onset of TLR9-driven S-HLH (Figure 2.1, Figure 2.2, Figure 2.3, Figure 2.5, Figure 2.6 and Figure 2.7). Comparable severity of the S-HLH phenotype has been shown to also
manifest in CpG-treated mice with intact SIRPα signaling if they are deficient in IL-18BP, which opposes IL-18 signaling (Girard-Guyonvarc'h et al., 2018). However, unlike other S-HLH models, TLR9-challenged SIRPα−/− mice rapidly exhibited severe anemia, hemophagocytosis and hypercytokinemia following the initial CpG injection, which likely points to the involvement of innate immunity in S-HLH pathology therein. Wang et al showed that a specific sequence of pathogen sensing, i.e., viral (TLR3) to bacterial (TLR4) challenge, leads to metabolic dysregulation in macrophages and subsequently induces a highly lethal hyperinflammatory state resembling HLH secondary to endotoxic shock (A. Wang et al., 2019). Similarly, Mahajan et al has shown that dysregulation of lipid signaling in macrophages can also confer macrophage-intrinsic S-HLH/CSS pathology (S. Mahajan et al., 2019; Sahil Mahajan et al., 2020). In parallel, our data suggests that macrophages, and likely other myeloid leukocytes, also become dysregulated and hyperinflammatory in the absence of SIRPα-dependent inhibition (Figure 2.10 and Figure 2.11), leading to severe and rapid onset of macrophage-intrinsic S-HLH/CSS pathology. Indeed, activating SIRPα−/− macrophages with TLR agonists or pro-inflammatory cytokines led to exuberant hemophagocytosis and macrophage-intrinsic fulminant S-HLH (Figure 2.13, Figure 2.14 and Figure 2.15), whereas depleting macrophages prevented S-HLH onset in TLR9-inflamed SIRPα−/− mice (Figure 2.10). Mechanistic studies suggest that this phenotype was partially due to SIRPα inhibiting TLR9-driven macrophage activation by negatively regulating MAPK pathways (Figure 2.11), thus removing SIRPα appears to ‘prime’ macrophages and facilitates their acquisition of a hyperinflammatory hemophagocytic state. Other studies have similarly
shown that SIRPα regulates M1 and M2 macrophage polarization by modulating PI3K-Akt, MAPK and NF-kB pathways (Kong et al., 2007; Shi et al., 2017). However, given that CL2MDP has been shown to be non-specific (Ciavarra et al., 2005; Danenberg Haim et al., 2002; Lee, Rosen, M.D, Van Rooijen, & Noble-Haeusslein, 2011; Zeisberger et al., 2006), further investigation is required to ascertain a definitive role for macrophages over other phagocytes in HLH/CSS pathogenesis in SIRPα−/− mice.

The pro-inflammatory cytokine IFNγ is considered a key molecule driving inflammation in HLH and is intimately associated with hemophagocytosis and anemia of inflammation (Burn et al., 2019; S. W. Canna et al., 2013; Das et al., 2016; Lounder et al., 2019; Zoller et al., 2011). For example, two HLH patients had not developed hemophagocytosis owing to an IFNγ-receptor deficiency (Tesi et al., 2015). Indeed, IFNγ administration alone is sufficient to induce hemophagocytic RPM in WT mice, leading to consumptive pancytopenia (Zoller et al., 2011). To that end, researchers have endeavored to treat HLH by neutralizing IFNγ, which has demonstrated exceptional efficacy in various settings (Edward M. Behrens et al., 2011; Girard-Guyonvarc'h et al., 2018; Lounder et al., 2019). In contrast, IFNγ neutralization was non-therapeutic in SIRPα−/− mice (Figure 2.10) and IFNγ treatment failed to induce any aspects of S-HLH in SIRPα−/− mice (Figure 2.13, Figure 2.14 and Figure 2.15), corroborating our previous study showing that, while many pro-inflammatory cytokines and TLR agonists may drive SIRPα−/− macrophages to become hemophagocytic, IFNγ has no such effect (Bian et al., 2016). Thus, future studies
are necessary to determine the role of IFNγ in driving macrophages to become hemophagocytic if not for providing the putative activation signaling (Bian et al., 2016).

Supporting IFNγ-independent mechanisms in HLH onset, Albeituni et al compared the therapeutic efficacies of αIFNγ and ruxolitinib – a JAK1/2 inhibitor – in murine models of HLH and found that ruxolitinib was superior, as it reduced neutrophil expansion and tissue infiltration (Ahmed et al., 2019; Albeituni et al., 2019; Maschalidi, Sepulveda, Garrigue, Fischer, & de Saint Basile, 2016). In parallel, an absence of SIRPα under TLR9-driven inflammation greatly increased granulopoiesis and PMN tissue infiltration (Figure 2.2), which may partially underly the exceptionally severe S-HLH phenotype in CpG-treated SIRPα−/− mice and the inefficacy of αIFNγ (Figure 2.10). Interestingly, CL2MDP depletion reduced circulating GM-CSF and also the frequency and number of splenic PMN in CpG-treated SIRPα−/− mice. Furthermore, we show the mechanism by which macrophages and other myeloid leukocytes may become hemophagocytic and drive HLH-like disease under SIRPα deficiency is partially redundant, as an array of TLR agonists and pro-inflammatory cytokines are capable of providing the activation signaling (Figure 2.13, Figure 2.14 and Figure 2.15). This heterogenous capacity to drive myeloid leukocytes toward a hemophagocytic phenotype likely underscores why hemophagocytosis is neither specific nor sensitive to HLH or CSS in general and may also lend an explanation as to why CSS/HLH patients differentially respond to current cytokine neutralization therapies (Al-Samkari & Berliner, 2018; Albeituni et al., 2019; Chinn et al., 2018; Schwartz et al., 2017; D. Wu & Yang, 2020; Xu et al., 2020; C. Zhang, Wu, Li, Zhao, & Wang, 2020).
Although our studies reveal a link between SIRPα and the nascence of both hemophagocytes and HLH-like disease, a major question remains unanswered: do events preceding the onset of HLH/CSS comprise a phase during which SIRPα becomes down-regulated or lost? Another puzzle is whether SIRPα can be down-regulated under physiological or pathological conditions? Although the first question is currently unanswered and certainly demands further investigation, some studies have shown particular TLR-driven inflammation or disease conditions such as diabetic nephropathy lead to or are associated with a loss of SIRPα expression (Bennett et al., 2019; Bian et al., 2016; Kong et al., 2007; Li et al., 2019). Conceivably, these conditions not only provide the pre-disposing condition, i.e., an absence of SIRPα, but also the necessary inflammatory activation, i.e., TLR or pro-inflammatory cytokine signaling, to drive leukocytes toward a hemophagocytic phenotype and potentially confer HLH/CSS-like disease. Akilesh et al recently showed that chronic TLR7 and TLR9 signaling reprograms myelopoiesis toward differentiating specialized monocytes, referred to as inflammatory hemophagocytes (iHPCs) (Akilesh et al., 2019). iHPCs arose in aged mice with constitutively active TLR7 (TLR7.1) or, to a lesser degree, in mice injected with R848 (TLR7 agonist) or CpG every day for 13 or 5 days, respectively. However, these iHPCs appear to represent a separate subset of hemophagocytes following long-term myelopoietic reprogramming (Grigoriou et al., 2020; Yvan-Charvet & Ng, 2019), and given the stark differences in the kinetics and severity of hemophagocytosis and cytopenias, these iHPCs likely differ from hemophagocytes that arise in the absence of SIRPα.
Nevertheless, our findings, as summarized in Figure 2.16, collectively demonstrate that SIRPα plays an indispensable role in preventing myeloid leukocytes from becoming hemophagocytic under inflammation may and also provide an explanation for why heterogenous inciting inflammatory factors can inevitably culminate in HLH/CSS pathology.

![Figure 2.16](image)

**Figure 2.16 Inflammation unrestrained by SIRPα precipitates macrophage-driven severe HLH in mice in an IFNγ-independent manner**
A graphical abstract demonstrating the impact SIRPα expression has on macrophages responses to pro-inflammatory cytokine and TLR signaling in mice. In the absence of SIRPα inhibition, macrophages become hemophagocytic, hyper-inflammatory and foster a dysregulated inflammatory response resembling HLH or other CSS. This image was generated using BioRender.com.
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