Cytokines and cytotoxicity: Dissecting susceptibility to lethal hyperinflammation

by

Emily Elizabeth Landy

BS Biochemistry, University of Missouri, 2016

Submitted to the Graduate Faculty of the

School of Medicine in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

University of Pittsburgh

2023

UNIVERSITY OF PITTSBURGH

SCHOOL OF MEDICINE

This dissertation was presented

by

Emily Elizabeth Landy

It was defended on

November 3, 2023

and approved by

Daniel Kaplan, MD, PhD
Professor, Department of Dermatology and Department of Immunology

Lawrence P. Kane, PhD
Professor and Vice Chair for Education, Department of Immunology

Mandy McGeachy, PhD
Associate Professor, Department of Microbiology and Immunology

Yi-Nan Gong, PhD
Assistant Professor, Department of Immunology

Dissertation Director: Scott W. Canna, MD
Attending Physician, Assistant Professor of Pediatrics, Children's Hospital of Philadelphia

Cytokines and cytotoxicity: Dissecting susceptibility to lethal hyperinflammation

Emily Elizabeth Landy, PhD

University of Pittsburgh, 2023

Hemophagocytic Lymphohistiocytosis (HLH) and Macrophage Activation Syndrome (MAS) are life-threatening hyperinflammatory syndromes. Familial HLH is caused by genetic impairment of granule-mediated cytotoxicity (e.g., perforin-deficiency). MAS is linked to excess activity of the inflammasome-activated and pro-inflammatory cytokine Interleukin 18 (IL-18). Both diseases share a similar set of clinical phenotypes such as hepatosplenomegaly, anemia, hemophagocytosis, and recurrent fevers. Patient samples from multiple organs show enhanced numbers of activated CD8 T cells. Together this suggests that these hyperinflammatory diseases (and maybe others) share a common pathogenic pathway. Additionally, burgeoning evidence suggests that heterozygous defects in cytotoxicity can predispose patients with other rheumatic diseases to a hyperinflammatory status. Understanding the underlying mechanisms of hyperinflammation is necessary for quality patient treatment to these deadly diseases. To study these mechanisms, we employ mouse models of hyperinflammation, in mice with either perforin deficiency ($Prf1^{-/-}$) or excess IL-18 (Il18tg) have little to no clinical features on their own/without provocation. However, when infected with LCMV these mice exhibit hyperinflammation nearly identical to that of HLH and MAS patients. Additionally, mice with dual susceptibility ($Prf1^{-/-}$ Il18tg; DS) succumb to spontaneous, lethal hyperinflammation. We hypothesized that these factors converged on pathogenic hyperactivated CD8 T cells with a decreased activation threshold, amplified cytokine response, and extended lifespan.

In Il18tg mice, IL-18 effects on CD8 T cells drove MAS following a viral (LCMV), but not innate (TLR9), trigger. In vitro, CD8 T cells required TCR stimulation to fully respond to IL-18, and $Prf1^{-/-}$ cells were resistant to restimulation-induced cell death (RICD). Concomitant with hyperinflammation, dual susceptibility mice developed massive post-thymic oligoclonal CD8 T cell expansion in their spleens, livers, and bone marrow detectable even in $Prf1^{+/-}$Il18tg mice. These cells proliferated, produced excessive IFNγ, but also expressed receptors and transcription factors associated with exhaustion. In addition, DS mice with a restricted TCR repertoire still developed spontaneous HLH and were strongly enriched for T cells not expressing the transgenic TCR.

Together, these data suggest that hyperinflammation is a result of a unique, unrelenting, and targetable state of CD8 T cell hyperactivation that is terminal, pathogenic, and has an activation state combining features of exhaustion and effector function.

Table of Contents

List of Tables

List of Figures

Preface

Earning a PhD has been one heck of a ride, but you would be hard pressed to find someone with a life sciences PhD to tell you any different. The fact remains that from the COVID-19 pandemic, being adopted into a new lab after my PI left the institution, and starting a family along the way, this has been a journey for the books. I have been extraordinarily lucky to have family, friends, coworkers, mentors, and thesis committee members for their support along the way.

None of the work discussed in this thesis would be possible without the guidance and support of my mentor, Dr. Scott Canna. While he has an MD rather than a PhD he likely has enough knowledge to support both titles. Scott has taught me how to think critically, exponentially expand my bandwidth, and become a confident and independent scientist. However, I would be remis if I said he was my only mentor. Dr. Larry Kane has not only been a key member in my thesis committee, but also graciously adopted me into his lab to finish my career. Dr. Kane has provided me with the space to hone my independence while still providing critical insights into my project. Additionally, Dr. Mandy McGeachy, has been there as a pillar of stability, counsel, and model of a thriving woman PI in science. Dr. Dan Kaplan, who does carry both MD and PhD titles, and Dr. Yi-Nan Gong have been there to help me get to the heart of the questions we wanted to answer and keeping me focused on a goal which is often harder than it sounds.

My mentors have been spectacular, but I also wouldn't have gotten this far without my friends and family. To my parents, thank you for providing me with constant support and encouragement from grade school till now. I quite literally would not be here without you. To my husband James, thank you for supporting me through early mornings that inevitably end up leading to long nights in the lab. To my son, Carter, which this dissertation is devoted to, you have taught

me more about dedication and time management than anyone else. To my friends and coworkers, I would be so lost if it weren't for the mountain biking dates, culinary adventures, and check ins to see how I am doing, the hardest part of moving on will be leaving you behind.

Science is amazing, rewarding, unforgiving, and relentless. The following is a list and explanations of the proverbs, adages, axioms, and pearls of wisdom, that have kept my sanity (mostly) intact throughout my time as a graduate student.

Teamwork makes the dream work: Making the most of your experiments often leads to harvest days expanding to levels unmanageable by a single person. When you have 25+ mice and need terminal bleeds, mouse weights, spleen weights, splenic touch preps, CBCs, plasma collection, liver perfusions and formalin preservation of five other organs *per mouse* you need help. I have experienced doing this in solitude and with the help of others. The latter has always proven more successful, but it is important to clarify that this goes *both* ways. You have to be willing to recieve help and provide help to others to make this work. Everything is just better when the entire lab is invested in each other because as a team your success is their success.

When you're here, your family: Science isn't forgiving. Experiments fail, then fail again, sometimes you are running flow till 3am, sometimes your mice don't want to breed, and sometimes your future husband decides to propose when you are out to dinner, but it was a surprise and you had to come back to lab to finish your experiment. When those times hit, a hug or a helping hand from a lab member who knows your project and what you need to get done makes everything better.

We stand on the shoulders of giants: Modern day science is built upon years of other people's findings. It can be easy to think what we do is less valuable than discovering how vaccines

work but embracing this quality is part of the ever- evolving nature of science. One day someone will think the work you did was equally important.

The cone of science: While having a fun and lively lab atmosphere can be amazing, sometimes you need to focus. You would think ‘zone’ would be the correct word here but for some reason in the Canna lab we used to say “In the cone, I’ll talk to you after!”. This later got translated into a literal birthday hat cone that you could wear when you needed no disruptions. I swear this was one of the best ideas we ever had.

Dad jokes: When your boss works in pediatrics, things can get a little punny. Here are some of the lab favorites (in no particular order) that keep lab life fully of laughter.

- **Have you been to the Canna Supermarket?** There's always a clean-up on (aisle) IL-18!
- **Flow Cytometrists never have opinions**, they only share the FACS!
- If you only get sick on weekdays you must have a **Weekend** (weakened) immune system.
- **The red rock joke:** Three guys are walking in the desert. Guy #1 finds a yellow rock, looks at it and says "Hey I can throw this rock in the air and I bet it'll come down in like 10 seconds." Other guys nod and agree sure. So he throws the yellow rock in the air and it comes down after 10 seconds. They keep walking for a bit then Guy #2 finds a blue rock and says 'I could throw this rock in the air and it'll come back down in 5 minutes. Everyone is like, "Wow you must have a killer arm to throw that far." Guy says yeah I played baseball. So he throws the rock in the air and sure enough, 5 minutes later it comes back down. They keep walking and Guy #3 finds a red rock. He says, "I can throw this rock in the air and it'll never come back down”. Everyone is like, "No way, gravity has to bring it down, that's impossible." Guy #3 takes the red rock throws it... and it never comes back down."- see **Supplemental File 6: *The long joke***

1.0 Introduction:

Portions of this chapter were compiled and published in a review article that is under review at *Nature Reviews Rheumatology* titled:

Landy E, Carol H, Ring A, and Canna SW. Biologic and clinical roles of IL-18 in inflammatory diseases. *Nature Reviews Rheumatology*. ***In Revision***.

1.1 The World of Hyperinflammation

The immune response consists of a potent set of defense mechanisms required for survival in a pathogen-filled world, but it is also tightly regulated to prevent unwanted damage. Hyperinflammation is a pathologic exaggeration of the systemic inflammatory response. Like other inflammatory response syndromes, there are many contributors to hyperinflammation: infection, trauma, surgery, ischema (lack of blood to a part of the body), autoimmune, autoinflammatory, atopic, and malignant/leukoproliferative- disorders. Clinically, hyperinflammation describes a temporal immunophysiologic syndrome characterized by recurrent fevers, high ferritin levels, low cell counts, disseminated intravascular coagulation, hepatitis, coagulopathy, hepatosplenomegaly, hemophagocytosis, central nervous system inflammation and too-often progression to organ failure and death[1]. Importantly, hyperinflammation is a description of this life-threatening immunophysiologic state, ***not*** an explanation of its origins. Used broadly, the term Hemophagocytic Lymphohistiocytosis (HLH) is somewhat synonymous with hyperinflammation and is defined by classification criteria developed by consensus in 2004[2]. HLH

can be considered primary- caused by high penetrance genetic defects- or secondary to any of the variety of contributors described above. In mechanistic studies, the term "HLH" is often used to describe a specific subset of primary HLH, familial HLH, caused by profound genetic impairment or granule-mediated cytotoxicity[3]. Hereafter, we will use the term "hyperinflammation" to describe the broad immunophysiologic state described above, and "HLH" to describe hyperinflammation due to profound cytotoxic impairment.

The term Macrophage Activation Syndrome (MAS) is used to denote HLH when it occurs secondary to a rheumatic disorder, most commonly Systemic JIA/Still's Disease and Systemic Lupus Erythematosus. Together HLH and MAS account for at least 11% of systemic inflammatory response syndrome patients[4]. Clinically, HLH and MAS have been difficult to distinguish from each other as they share a number of symptoms (***Figure 1***). Defining and targeting the underlying disease states and inflammatory triggers is crucial to prevent organ damage and death. This project is designed to determine the mechanisms by which two known susceptibility factors of hyperinflammation drive this disease. Currently the standard treatment for HLH patients is allogeneic hematopoietic stem cell transplantation (HSCT) which often carries a substantial risk of engraftment failure, opportunistic infections and at least 5-10% risk of overall mortality. By contrast, MAS patients have a far more favorable prognosis and are typically treated with anti-inflammatory and immunosuppressive medication. Finding the underlying mechanisms that are distinct or shared between HLH and MAS is imperative for the development of more rapid diagnoses and more efficient treatment options.

1.1.1 Susceptibility Factors of hyperinflammation

Much of what we know about hyperinflammation comes from genetic causes, initially those passed throughout family lineages, but more recently through identification of de novo mutations (variants not present in either parent). Genetic causes of HLH/MAS represent a minority of all cases (particularly in adults), but their discovery has led to diagnostic and treatment advances. Infection is the most common acute trigger of HLH/MAS. In children, infection is the most common etiology, with a specific triggering pathogen identified in over 50% of new HLH/MAS presentations[1]. This section outlines the factors that have been shown to cause susceptibility to hyperinflammation (genetic or otherwise).

1.1.1.1 Cytotoxic Impairment

The Inborn Errors of Immunity (IEI) include nearly 500 genetically defined disorders[5], in which HLH/MAS is rarely found as a complication. The canonical high-penetrance genetic causes of HLH are those that profoundly impair granule-mediated cytotoxicity. Importantly, HLH/MAS due to cytotoxicity defects tends to present in infancy and early childhood, whereas HLH/MAS in other IEI present in a broader age range including older children[6].

Mechanistically, cytotoxic defects have been found to play a profound role in the function of the lytic/ cytotoxic immunological synapse (IS) occurring between cytotoxic and target cells. At the IS, cytotoxic cells integrate signals from antigen presenting cells and activate inflammatory and proliferation programs. Under typical circumstances, the IS is ultimately terminated by their own stimulation through target cell apoptosis via release of cytotoxic granules. When cytotoxicity is absent, NK's and CD8 T cells have a prolonged immunological synapse and thereby experience continuous stimulation[7-9]. During this period, NK/CD8 T cells produce copious pro-inflammatory

cytokines and drive immunopathology in HLH. In short, these genetic disorders lead to chronic high levels of inflammation cause impaired clearance of pathogens and infected cells and the production of copious amounts of pro-inflammatory cytokines production.

Table 1: Genes associated with HLH/MAS susceptibility due to impaired granule-mediated cytotoxicity

Adapted from: Shakoory B., et al, 2022 [10]					
Gene	**Protein**	**Disease Acronym**	**Frequency of HLH/MAL**	**Clinical Associations**	**OMIM ID**
Prf1	Perforin	FHL2	High	early onset, isolated CNS	603553
Unc13d	Munc13-4	FHL3	High	Isolated CNS involvement	608898
Stx11	Syntaxin11	FHL4	High	Variable age at onset, possible risk for MDS/leukemia	603552
Stxbp2	Munc18-2	FHL5	High	IBD,SNHL, Hypogammaglobulinemia	613101
Rab27a	Rab27a	GS2	High	albinism, infection	607624
Lyst	LYST	CHS	Moderate	albinism, infection	214500
Ap3b1	AP3	HPS2	Moderate/Low	Albinism, infection, bleeding	608233
Rohg	RhoG	-	Unknown	-	-

1.1.1.2 Chronic Excess IL-18

IL-18 is stable and easy-to-measure in peripheral blood, and serum IL-18 levels are elevated in nearly every inflammatory disease. However, a defining characteristic of MAS patients is extremely high serum levels of IL-18[11] which is considered a susceptibility factor of the disease. Case reports suggest that MAS patients respond well to IL-18 blockade therapy[12], thus highlighting the significance of IL-18 in this disease. However, the mechanistic role of IL-18 in MAS is not fully elucidated. It is important to note that patients with both genetic (cytotoxicity mutations) and non -genetic subtypes of HLH have elevated levels of IL-18 that is unbound by the endogenous

inhibitor IL-18 binding protein[11]. However, these levels are completely dwarfed by the levels of free IL-18 found in MAS patients (***Figure 2***). IL-18 has also been implicated in diseases such as inflammatory bowel diseases[13-15], atherosclerosis[16,17], Crohn's Disease[18], atopic dermatitis[19], asthma[20], and sepsis[21]. Measurement of total IL-18 is quickly becoming essential, particularly in pediatric rheumatology, for the diagnosis of multiple rheumatic and/or autoinflammatory diseases. The profound, and in many cases chronic, elevation of total IL-18 (with attendant detectable free IL-18) has delineated a cluster of largely autoinflammatory diseases that may be aptly called IL-18opathies. The majority of these disorders involve increased susceptibility to MAS. Though the association of IL-18 with SJIA, AOSD, and MAS was made over a decade ago, the discovery of monogenic inflammasome-mediated Inborn Errors of Immunity (IEI) causing MAS and chronic IL-18 elevation has dramatically elevated its likely role in pathogenesis. High levels of IL-18 have been noted in patients both with active and inactive disease. In most all IL-18opathies, the degree of elevated IL-18 correlates to the degree of disease activity[22].

We now appreciate there are a number of IL-18opathies: Heterozygous mutations in the inflammasome nucleating protein NLRC4 have been associated with increased inflammasome activity and hyperinflammation. The inflammasome is an assembly of cytosolic proteins which can be activated to oligomerize in response to innate pattern associated- and/or damage associated-molecular pattern receptor signaling. Inflammasome activation leads to the proteolytic activation of IL-18 (and IL-1β)[23] and their passive release via gasdermin D (GSDMD) pores (**Figure 1**). Deficiency of the X-linked inhibitor of apoptosis protein (XIAP) causes a wide variety of inflammatory phenotypes, most notably an (often EBV-triggered) HLH-like syndrome known as Type 2 X-linked lymphoproliferative syndrome (XLP2)[22,24]. Cell division control protein 42 (CDC42) is a ubiquitous Rho-GTPase associated with a variety of critical immune and unrelated

functions. Patients with variants at the C-terminus of CDC42 can have fevers, rashes, and fatal HLH as part of a syndrome recently dubbed NOCARH (Neonatal-Onset Cytopenia, Autoinflammation, and Recurrent HLH) syndrome[25-27]. All tested patients appear to have dramatically elevated serum IL-18 levels even after clinical treatment[25,27]. IL-18 may pay a primary role in other IEI diseases, such as those resulting from mutations in *MEFV*, *WDR*, *PNP*, *IL18BP*, and *PSTPIP1* are less characterized and potentially have different (non-HLH like) immune manifestations.

1.1.1.3 Other genetic mutations that can manifest as hyperinflammation

Many other genetic defects exist that impart susceptibility to hyperinflammation (**Table 2**). While a few selected genetic modifications have been studied in detail in the context of hyperinflammation in concert with their clinical association, the vast majority have been recognized as case reports. Of note, SAP (*Sh2d1a)* deficient mouse models have shown a Th1 skewed lymphoproliferation when challenged with lymphocyte choriomeningitis virus (LCMV) or Toxoplasma gondi[28]. On the other hand, *Itk* knockout mice have been shown to have reduced effector T cell function but enhanced T regulatory (Treg) development[29-32]. This along with other studies on mice, mentioned have put T cells in the forefront of investigation of hyperinflammatory diseases.

Table 2: Other genes associated with HLH/MAS susecptability

Adapted from[33]	Gene	Protein	Disease Acronym	Frequency of HLH/MAS	Clinical Associations	Mouse Models Highlight	OMIM ID
Impaired EBV control	***Sh2d1a***	SAP	XLP1	High	lymphoma	Th1 Response[28]	308240
	Itk	ITK	LPFS1	Moderate	lymphoma	Treg[29-32]	613011

	Cd27	CD27	LPFS2	Moderate	lymphoma	Teffector[34]	615122
	Cd70	CD70	LPFS3	Moderate	lymphoma	CD8 T cell[35]	618261
	Magt1	MAGT1	XMEN	Moderate	lymphoma	B cells [36]	300853
	Ctps1	CTSP1	-	Moderate	lymphoma		615897
	Rasgrp1	RASGRP1	-	Moderate	lymphoma	CD4 activation[37]	618534
	Pik3cd	PI3Kδ	APDS	low		Increased AICD	615513
Impaired inflammasome activation	***Xiap/Birc4***	XIAP	XLP2	Moderate	IBD	T cells[38]	300635
	Nlrc4	NLRC4	AIFEC, MAS	Moderate	Early onset, IBD	IL-18[11,39]	616050
	Cdc42	CDC42	NOCARH	High	Early onset, rash		-
Other immune dysregulation	***Nckap1l***	HEM1	-	Unknown	Infection, autoimmunity	Innate immunity[40]	618982
	Rc3h1	ROQUIN	-	Unknown		T follicular[41,42]	618998
	Havcr2	TIM3	-	Moderate	lymphoma	Antigen presentation[43]	606652
Metabolism dysregulation	***Slc7a7***	SLC7A7	LPI	Moderate	Enteral protein intolerance		222700

1.1.1.4 Triggers of hyperinflammation

As mentioned, genetic mutation rarely sufficient for hyperinflammation. Nearly every infection has been associated with hyperinflammation to some degree, and infection is an

important trigger even in monogenic causes of hyperinflammation[1]. Several specific pathogens seem predisposed to hyperinflammatory responses. Understanding how hyperinflammation arises and persists relies on determining the pathways involved in both infection-mediated and genetic hyperinflammatory syndromes. Below are brief descriptions of the *known* associations with hyperinflammatory syndrome.

- ***Viral Infections*** are the leading cause of secondary hyperinflammation. They can lead to HLH-like disease in a number of ways including continuous pathogen receptor triggering (TCR, TLR, PRRs) which can lead to bone marrow exhaustion[44], infection of inflammatory cells[45] and interfere with cytotoxic function of CD8 and NK cells[46-48]. Below are how each specific infection has been shown to be associated with hyperinflammation.
    - ***EBV:*** Chronic or severe EBV infection of T cells can lead to excessive T cell proliferation, cytotoxic activity, and cytokine release[49]. Alternatively, EBV leads to decreased NK cell function allowing for decreased antigen clearance and increased immune cell activation.[50].
    - ***H1N1:*** NK cell cytotoxicity is directly targeted by the H1N1 influenza virus, which inhibits NK cytotoxicity by enhancing inhibitory signals preventing appropriate activation and NK directed killing[51].
    - ***HIV:*** HIV has been associated with HLH as both initial triggering infection or with a other underlying associated disorders[52,53]. HIV infection leads to CD4 T cell specific pyroptosis[54,55], in the absence of CD4 T cell helper functionality this leads to an acquired immunodeficient state. Acquired

cellular immunodeficiency can further lead to uncontrolled infection, and possibly defects in cytotoxicity[56].

- ***IRIS:*** Immune Reconstitution Inflammatory Syndrome (IRIS) associated hyperinflammation happens when the immune system is reinvigorated in patients receiving anti-retroviral treatment for HIV infection. In this case, HIV itself does not trigger hyperinflammation but as the immune system begins to come back to life it will recognize other pathogens that are present in the patient[57].

- ***CART:*** Nearly all responding patients receiving a Chimeric Antigen Receptor T cell (CART) therapy will undergo a cytokine release syndrome (CRS) associated with massive amounts of CART proliferation, target cell death, and release of inflammatory cytokines[58]. When CRS becomes so elevated that other manifestations of HLH arise such as hyperferritinemia, cytopenias, and hemophagocytosis it is considered carHLH[59]. However, the determination of the underlying factors determining the elevation of CRS to carHLH are still under investigation.
- ***Injury:*** Injury/trauma is a rare but known contributor to hyperinflammation[60-62]. Trauma can cause priming of the immune system to a hyperinflammatory state, with some studies suggest catecholamines [63] and/or inflammasome activation[64,65] priming the immune system for hyperinflammation. Injury to cells at barrier surfaces allow inflammatory cell infiltration into tissues that are typically protected. Some case reports suggest macrophage infiltration, proliferation, and cytokine release due to endothelial cell injury[66,67].

1.1.2 Hyperinflammation is a multifactorial disease

Patients with NLRC4 defects, despite chronically elevated IL-18, are not chronically hyperinflammed suggesting the need for a triggering event and as highlighted in the previous section, hyperinflammation is commonly a complication of other pre-existing diseases/conditions[1]. The cornerstone of my project is the finding that MAS patients with heterozygous defects in cytotoxicity exist and are enriched 2-3 times more than in the general population[68,69]. This suggests that there may be unexplored synergy between excess IL-18 and cytotoxic deficiencies warranting further investigation. The majority of this dissertation is focused on how perforin deficiency and excess IL-18 have individual impacts on CD8 T cells that work in concert to evolve into a hyperinflammatory state.

1.2 Immunological compartments involved in hyperinflammation

Hyperinflammation is, at its core, an overreaction of the immune system leading to excess pro-inflammatory cytokine production and organ damage. The immune system is made up of a myriad of cell types each having distinct roles yet working together in the removal of pathogens. In the case of hyperinflammation, distinct cell types also play specific roles in the pathogenesis of this disease. This section details the cells involved in hyperinflammation and the most up to date knowledge of their roles in this condition.

1.2.1 The myeloid compartment

The myeloid compartment of the immune system has a central role in hyperinflammation by directly causing some of the clinical damage/symptoms seen in patients as well as being the cell type that patho-genetic mutations affect. Below describes the specific myeloid cell types and their known involvment in hyperinflammation

1.2.1.1 Antigen Presenting Cells

Antigen presenting cells (APCs) including dendritic cells, macrophages, and B cells play critical functions in the immune system as translators of danger sensing into productive and directive antigen presentation. APCs can act as the 1st wave of proinflammatory cytokine release as they succumb to caspase-induced cell death (**Figure 1**) releasing both intracellular danger signals (DAMPs) and cleaved forms of pro-inflammatory cytokines like IL-1β and IL-18 into the surrounding microenvironment. During this initiation of the immune response, APCs rely heavily on danger signal sensing using receptors such as Toll- like receptors (TLR9[70], TLR4[71]) and cytosolic danger sensors (NLRC4[39,72], NLRP3[73]) to elicit downstream inflammasome activation. Hyperinflammatory disease can arise from mutations in these receptors lead to altered regulation and interactions with companion proteins[74].

APCs, when not undergoing caspase-induced cell death, also act to further propagate an inflammatory response by presenting antigen to CD4 and CD8 T cells. In this context the production of a certain cytokines (.i.e. IL-12, IL-18, IL-4) can enhance proliferation and skew the trajectories of interacting cells into differing effector types [75]. Hyperinflammation can occur alongside excessive antigen presentation by APCs during chronic infections such as HIV[76] and EBV[5,77] infection.

Macrophages are especially important in the pathophysiology of hyperinflammation as they are a major producer of pro-inflammatory cytokines as they undergo caspase-induced cell death. Additionally, they are the key cell type engaged in hemophagocytosis[78]. Hemophagocytosis is defined as the phagocytosis of red blood cells and white blood cells. Pathologists often observe dramatically increased numbers of macrophages engaging in hemophagocytosis are observed in HLH and MAS, although this is neither sensitive nor specific for hyperinflammation. While the mechanisms leading to hemophagocytosis are largely undefined, it is likely due to aberrant activation of macrophages by IFNγ[79] and/or IL-4[80].

1.2.1.2 The Baso/Neutro/Eosino -phils

Neutrophils are key for fighting off bacterial and fungal infections. They release granules with potent antimicrobial and cytotoxic components. They are also known to cause fibrosis through their release of TGFβ and PDGF. In the setting of hyperinflammation neutropenia is often described[1]. Both IFNγ and TLR activation can be broadly suppressive of hematopoiesis and specifically IFNγ can have potent effects on neutrophil production and activity[81]. Basophils are key for the recruitment of cells from the blood to the site of infection. They express high levels of the IL-18 receptor and in response can produce Th2 skewing cytokines. Eosinophils can similarly recruit other immune cells and produce histamines, reactive oxygen species, enzymes, and antiparasitic molecules like MBP[82] and EPO[83]. Eosinophils are also responsive to IL-18 and can become pathogenic under chronic stimulation[84]. Both IFNγ and TLR activation can be broadly suppressive of hematopoiesis, and IFNγ may have particularly potent effects on neutrophil production and activity[81].

1.2.2 CD4 and CD8 T cells

T cells are the workhorses of the adaptive immune system and are commonly the pathogenic cell type in autoimmune and autoinflammatory diseases[85]. Specifically in hyperinflammatory patients, CD8 T cells usually predominate CD4 T cells[3,86,87] . This section provides a broad overview of the known T cell differentiation states and their potential role in hyperinflammation.

1.2.2.1 Naïve

Naïve T cells are important for the initial pool of both CD4 and CD8 T cells that can be activated and have effector functions. Naïve T cells have little expression of IL-18 receptor components and cytotoxic genes[88]. While naïve T cells are a necessary cell type, in this disease they are an unlikely target for pathogenic effects during hyperinflammation.

1.2.2.2 Activation

Naïve CD8 T cells become activated when they are exposed to antigenic peptides presented by MHC I in an inflammatory environment. In order to become fully activated cells must have three signal inputs: 1) TCR:MHC I, 2) co-stimulation (CD28, CD40L, CD27), and 3) polarizing cytokine signals. Together these signals determine what type of effector T cell the naïve cell will become[89]. Of note, IL-18 (alongside IL-12) can help differentiate CD8 T cells into Th1 effector cells that are cytotoxic and can produce IFNγ and TNFα[90]. IL-18 and IL-4 have been shown to work together to promote a Th2 effector cell however this seems to be preferentially effective on CD4 T cells[91].

In hyperinflammatory patients, there is a preponderance of activated CD8 T cells in peripheral blood[86,87] and affected tissues[92-94]. Similarly in experimental hyperinflammatory mouse models, mice infected with typically-benign lymphocytic Choriomeningitis virus (LCMV), both excess IL-18 and perforin deficiency led to an HLH-like phenotype[95,96], and CD8 T cells are essential for disease in $Prf1^{-/-}$ mice[97].

1.2.2.3 Exhaustion

Under circumstances of persistent antigen exposure or chronic viral infection, development of memory T cells is impaired and under these conditions, progressively lose effector function and gain expression of multiple inhibitory receptors[98-101]. The term 'exhausted' is commonly used to define these cells although the consensus on the definition of T cell exhaustion is still highly contested[102]. Hyperinflammatory triggers include chronic viral infections such as EBV or HIV. CD8 T cells from patients with these infections often have expression of inhibitory receptors such as PD-1, Tim3, and Lag3[103]. Despite chronic antigen stimulus in viral hyperinflammation, hyperactivated/hyperinflammatory CD8 T cells maintain an activated phenotype including expression of *Il18r1*, which is typically lost on exhausted CD8 T cells[104]. Furthermore, hyperinflammatory CD8 T cells do not show repressed effector function[95] suggesting they are not 'functionally' exhausted[86,87].

1.2.2.4 Memory

Without persistent antigen exposure, some effector CD8 T cells enter into a more quiescent state termed memory. Regardless of their residency in the circulation or tissues, memory CD8 T cells are poised for rapid proliferation and effector function upon antigen recall. Memory CD8 T cells can be restimulated with either their cognate antigen or with potent cytokines such as IL-

18[105-107]. Memory CD8 T cells have the potential to become bystander-activated in a hyperinflammatory milieu, especially if onset is due to infection[108]. In theory, these bystander cells could even contribute to elevated IFNγ levels found in hyperinflammation[109]. Furthermore, in peptide immunization of HLH mouse models, pre-existing memory CD8 T cells have been shown to elicit more severe, and IFNγ- independent hyperinflammation[110]. However, studies have been unable to determine if hyperinflammatory CD8 T cells originate from aberrant memory cell activation or through *de novo* activation.

1.3 Excess IL-18 and perforin deficiency in hyperinflammation

While there are many genetic and non-genetic defects that can lead to hyperinflammation as explained in the previous section, this dissertation will focus on perforin deficiency and excess IL-18 by way of perforin deficient and Il18tg mice, respectively. This section details the relevant biology and known hyperinflammatory mechanisms involving these two susceptibility factors. Much of this chapter was compiled and published in a review article that was published in *Nature Reviews Rheumatology* titled:

Landy E, Carol H, Ring A, and Canna SW. Biologic and clinical roles of IL-18 in inflammatory diseases. *Nature Reviews Rheumatology. **In Revision***.

1.3.1 Perforin biology and history

While other mutations that impair cytotoxic function (**Table 1**) and lead to HLH//MAS are known, the most canonical genetic defect, is loss of function in the *Prf1* gene encoding the

cytotoxic molecule perforin. In 2004, this defect was converted into a mouse model of HLH/MAS that has shown high translational fidelity and is frequently used in this dissertation. Perforin is naturally expressed in cytotoxic CD8 T cells and natural killer cells (NK cells), indeed, the study of $Prf1^{-/-}$ mice revealed that $CD8^{+}$ T cells are the drivers of acute LCMV-induced HLH[95]. Under normal circumstances, wild type (WT) mice have efficient viral clearance occurring with minimal hyperinflammation. However, in mice with perforin deficient CD8 T cells the inability to kill LCMV infected macrophages leads to prolonged immunological synapse duration[7], increased proinflammatory cytokine production, including IFNγ, which can be detected systemically[7].

1.3.2 IL-18 in hyperinflammation

Levels of IL-18 are used to diagnostically distinguish hyperinflammation (specifically MAS) from the other rheumatic diseases[11]. Understanding how IL-18 functions in the context of hyperinflammation can not only lead to better treatment options for patients with hyperinflammatory disease but also can be repurposed as a tool to re-invigorate hypo-inflammatory situations such as in solid and liquid cancers[111,112]. As described below and depicted in **Figure 1**, IL-18 is a potent IL-1/TLR family signal that is tightly regulated at multiple levels. A careful understanding of its biology is necessary to interpret clinical findings related to its augmentation or inhibition and to avoid adverse effects.

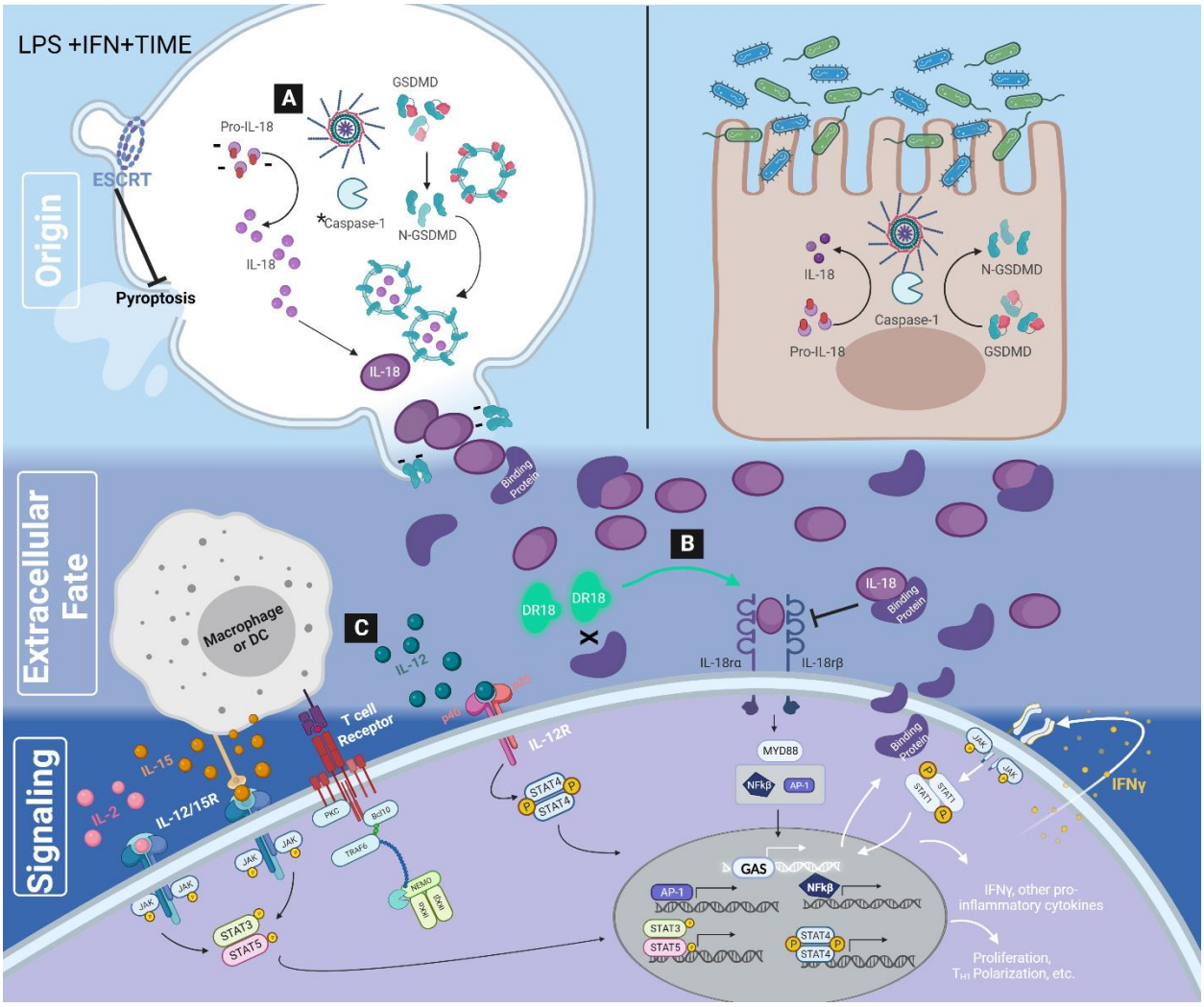


Figure 1: ***Origins, Extracellular Fate, and Signaling of IL-18:*** *Origin:* (A) Whether constitutively produced (epithelia and some tissue macrophages) or induced by complex stimuli (e.g. combination of LPS, IFN, and time or intestinal dysbiosis), pro-IL-18 is cleaved by caspase-1 into its active form where it is free of electrostatic hindrance to exit cells via inflammasome-dependent GSDMD pores. Prevention of pyroptosis due to GSDMD pores can be provided by the ESCRT pathway. *Extracellular Fate:* Once released, IL-18 is typically bound and neutralized by IL-18 binding protein (IL-18BP), which is itself made by most cell types in response to IFNγ. (B) Unbound or "decoy resistant" IL-18 (DR-18) signals through the IL-18 receptor heterodimer (IL-18R), typically exerting its effects in concert with other signals. *Signaling:* IL-18R signals through a conventional IL-1 family/MyD88-dependent pathway. It can drive both NF-κB and/or MAPK signals to promote proliferation, expression of pro-inflammatory cytokines, and/or other effector programs (like cytotoxicity). (C) The diverse effects of IL-18 depend on the type and state of the cell on which it is acting as well as the presence of other signals like TCR, IL-2, myeloid-cell derived IL-15, IL-12, and/or IFNγ (forming a positive feedback loop). *Other enzymes known to cleave pro-IL-18 are caspase-8, granzyme-B2, proteinase-33, and chymase.

1.3.2.1 Sites and signals of IL-18 production

Il18 mRNA (encoding pro-IL-18), is constitutively produced by multiple myeloid and epithelial populations. Among hematopoietic cells, the primary homeostatic sources of *Il18* mRNA in both humans and mice are lung alveolar and splenic red pulp macrophages[113-115]. It is unclear what specific role IL-18 may play in these organs, but the pattern suggests some noteworthy clinical correlations as discussed below. However, by far the greatest transcriptional and immunohistochemical abundance of pro-IL-18 is found in barrier epithelial cells of the skin, upper airway, and entirety of the gastrointestinal tract[11,116-121]. Studies in mice and humans have identified continuous production of pro-IL-18 by these epithelial cells, suggesting it may serve homeostatic barrier maintenance and/or alarmin functions[113,114]. A phenotype of genetic deficiency of human *IL18* has not been described, and very preliminary experience with IL-18 blockade has not identified obvious side effects at barrier sites.

IL18 transcription can be induced by inflammatory stimuli such as LPS, interferons (IFNα may be more potent than IFNγ), or signals from both[122,123,124]. In the murine gut, IL-22 derived from intestinal type 3 innate lymphoid cells (ILC3) acts on intestinal epithelia to drive (among other things) *IL18* transcription, which then acts back on ILC3 to form a barrier-maintaining loop[125]. In intestinal epithelia, even germ-free mice exhibit strong *Il18* transcription[11]. Pro-IL-18 production by epithelial cells is further induced by inflammatory stimuli at most barrier sites[126]. Colonization by both dysbiotic bacteria and commensal protists causes epithelial induction of *IL18*[119,127-129]. Transcriptional induction of *IL18* by myeloid cells is also unique relative to most other cytokines. Most inflammatory cytokines exhibit endotoxin tolerance: a burst of production with a single dose of LPS that dwarfs the production following any subsequent LPS stimulations. Unlike IL-1β, TNF, IL-6, etc., the levels of IL-18 after repeated LPS stimulation rose as high or

higher in human serum with a second stimulation than with initial exposure. In primary human monocytes, the induction of significant *IL18* may require a poorly-understood combination of inflammatory (e.g. TLR) signaling[130], IFN signaling, and time[131]. How this *in vitro* observation informs the highly variable expression of *Il18* by resident macrophages of different tissues is unclear. Thus, IL-18 is an inducible inflammatory cytokine, albeit in a pattern distinct from other myeloid-derived inflammatory cytokines.

1.3.2.2 IL-18 activation & release

The pro-form of IL-18 is not known to have biologic activity. Cleavage of pro-IL18 is best-described downstream of inflammasome-mediated activation of caspase-1, which is also responsible for the cleavage and activation of pro-IL-1β[132-134]. Though there is no biochemical evidence to suggest more efficient IL-18 processing by any specific inflammasome, those most well-described to activate IL-18 are NLRP3, pyrin, NLRC4, NLRP6, and NLRP9B[23,135-137]. Among these, multiple genetic connections have been made specifically between systemic IL-18 levels and NLRC4 activity[12,23,138]. Non-inflammasome proteases have also been shown to activate IL-18. Macrophages stimulated with Fas-ligand release active IL-18 in a process that is mediated by caspase-8[139]. Granzyme-B, which is found in cytotoxic granules can also cleave recombinant and keratinocyte-derived pro-IL-18 into an active form[140,141]. Other potential activators of IL-18 include proteinase-3 in human oral epithelial cells and chymase in stimulated mast cells[142,143]. Interestingly, proteinase -3 is also abundant in human azurophilic neutrophil granules[144], and has been implicated in proteolytic activation of IL-1β[145]. Furthermore, intact and enzymatically-active NLRP3-driven inflammasome particles may be released from macrophages and drive caspase-1 mediated cleavage of substrates extracellularly or upon phagocytosis by adjacent

macrophages[73,146]. This has been demonstrated for pro-IL-1β and it likely extends to pro-IL-18 as well.

Activated IL-1β and IL-18 lack a signal peptide and must find their way across the cell membrane to signal. This is achieved via preferential transit of cleaved IL-1β and IL-18 through plasma membrane pores composed of N-terminal fragments of the protein Gasdermin-D (**Figure 1**). Caspase 1 performs a dual function of proteolytically activating both pro-cytokines as well as their means of exit, gasdermin-D. caspase 4/5 (caspase-11 in mice) can also cleave Gasdermin-D but has less activity on proIL-1β and proIL-18. Gasdermin-D plasma membrane pores are necessary for the extracellular release of IL-1β and IL-18 as well as an inflammatory form of programmed cell death known as pyroptosis[147-149]. Electrochemical repulsion between the intracellular portions of Gasdermin-D pores and the pro-forms of IL-1β and IL-18 may explain how cells can selectively allow activated IL-1β and IL-18 to be released[150]. These findings place inflammasome activation upstream of cytokine activation, pore formation, cytokine release, and pyroptosis[151,152].

A great many inflammasome activators have been identified, including both pathogen products and endogenous triggers. Inflammasome activation/pyroptosis are themselves regulated by complex processes beyond the scope of this review[153]. Notably, pyroptosis downstream of Gasdermin-D pore formation requires the surface protein NINJ1[154] and is negatively regulated by the ESCRT complex. Thus, the presence of pores allowing IL-18 release does not always result in cell death[155]. Overall, the production, activation, and release of IL-18 provide a basis for its diverse roles in homeostasis, alarmin activity, and chronic inflammation.

1.3.2.3 Extracellular IL-18

Consistent with its potency *in vivo*, secreted IL-18 is subject to many mechanisms of negative regulation before it can exert its biologic effects. Reinforcing or circumventing this regulation is of critical importance to those wishing to therapeutically block or augment its effects.

1.3.2.4 Sequestration by IL-18 binding protein

Once the active form of IL-18 leaves its cell of origin it has a half-life of ~16h[156]. The extracellular activity of IL-18 is then opposed by an abundant, high-affinity "decoy-receptor" called IL-18 binding protein (IL-18BP[138]) whose sole function seems to be the inhibition of IL-18. The overwhelming majority of circulating IL-18 (>95% in healthy individuals[138,157]) is bound by the soluble antagonist IL-18BP. IL-18BP binds free IL-18 at an affinity comparable to most neutralizing antibodies (Kd ~1.1pM)[111], and all data suggest IL-18 in complex with IL-18BP remains biologically inactive[115,158]. The strength of this interaction makes hypotheses, such as the idea that IL-18BP provides a depot of cleaved IL-18, implausible. IL-18BP may also interact with another IL-1 family cytokine, IL-37, to both neutralize IL-18 and limit its signaling[159].

In contrast with IL-18, IL-18BP is produced by a wide array of hematopoietic and non-hematopoietic cells. Interferon-gamma (IFNγ) is a potent *IL18BP* inducer[160], forming an indirect IL-18/IFNγ/IL-18BP negative-feedback loop[161]. As such, IL-18BP induction has been exploited by various viruses (Pox, Chikungunya, Hepatitis C)[162-166] and neoplasms[138,167-169], resulting in immune evasion and active suppression.

1.3.2.5 IL-18 Signaling

Free, unbound, IL-18 can signal through a heterodimeric cell surface receptor composed of the IL-18 receptor α and β chains (encoded by *IL18R1* & *IL18RAP*, respectively). IL-18Rα can bind IL-18 with low affinity on its own[170]. Once bound to IL-18, the IL-18Rβ subunit is recruited[171] and the full, high-affinity receptor can signal[171-174]. The signaling cascade induced by IL-18 is nearly identical to the IL-1β signaling cascade. The TIR domains of the α and β subunits become auto-phosphorylated, allowing binding of the adapter proteins TRAM[175] and MYD88[176]. MYD88 phosphorylation leads to the activation of the MAPK and NF-κB pathways[177-179], leading to an increase in the production of other cytokines including IFNγ (**Figure 2**).

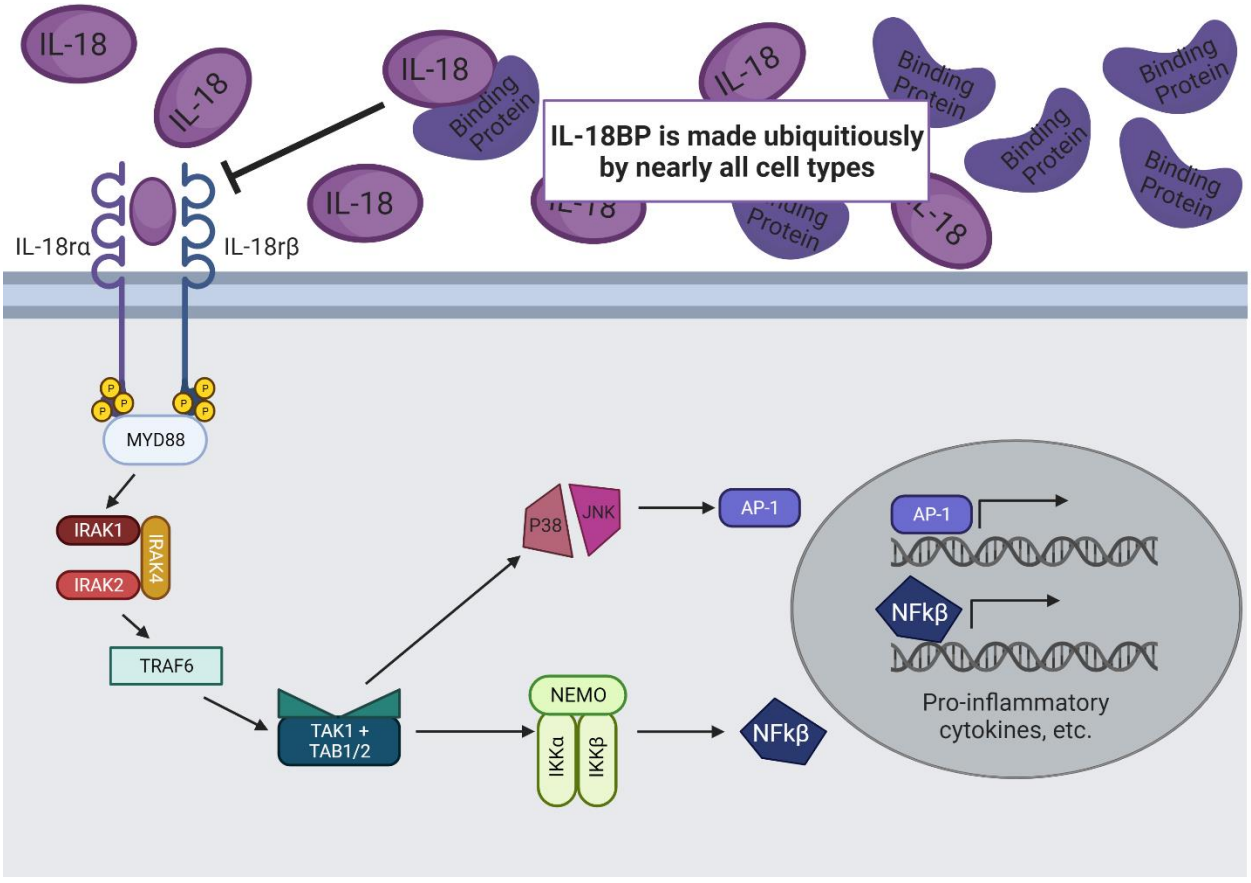


Figure 2: ***IL-18 Signaling Cascade:*** IL-18 signals through the IL-18rα and IL-18rβ heterodimeric receptor. When free IL-18 is bound to this cognate receptor the intracellular domains are phosphorylated and begin a cascade of

signaling events beginning with MYD88 activation and ending with both AP-1 and NFkβ translocation into the neuclus.

Il18r1 and *Il18RAP* expression seems restricted to immune cells, particularly lymphocytes like NK, NK-like ILC, and activated CD4 and CD8 T cells (**Table 3**). Expression on B cells[180], γδ-T cells, basophils[181,182] and to a much lower level in dendritic cells[181,182], synovial macrophages[183], mast cells[184], neutrophils[185], and skin (but not gut) resident ILC2 cells[186] has been reported but of unclear significance (. *IL18R1*-independent IL-18 signaling via a Na-Cl co-transporter has been reported in endothelia and adipocytes and may be important in atherosclerosis and obesity[187,188]. IL-37 also binds the alpha subunit of the IL-18R and can recruit the decoy receptor IL1-R8 (encoded by *SIGIRR*) to initiate anti-inflammatory signals[189,190].

Table 3: IL-18 recptor component expression

Cell Type:		Relative expression of:	
		IL-18r1 (α)	IL-18rap (β)
CD4 T cells	Thymic	+	-
	Naïve	++	-
	Effector	+	-
	$T_{regulatory}$	+	+
CD8 T cells	Thymic	+	-
	Naïve	++	+
	Terminal Effector	++++	++++
	$T_{memory\ precursor}$	+++++	+++
	$T_{central\ memory}$	+++++	+++
	$T_{effector\ memory}$	+++++++	++++
NKT cells		++++	+++
γδ T- cells		+++	++
NK Cells		++++	++++++
Innate Lymphoid Cells	ILC2	++++	++
	ILC3	++++	+++
(Sourced from ImmGen consortium)			

Thymocytes and naïve T cells have extremely low levels of IL-18 receptor genes, but antigen experience[191] and/or stimulation by inflammatory cytokines, such as IL-12, IL-15 and IL-2 promote the expression of *Il18r1* and *Il18RAP*. Recent TCR signaling has been shown to be a requirement for IL-18 to act on CD8 T cells[192]. This suggests that IL-18 acts on these cells in a context dependent manner. NK, NKT, and NK-like ILCs have constitutive expression of both receptor subunits suggesting an important role in sensing the alarmin function of IL-18.

1.3.2.6 Functions of IL-18

The described functions of IL-18, derived from a wealth of in vitro and murine pre-clinical data, include augmentation of an array of effector lymphocyte programs including T regulatory function. However, a consistent thread is that IL-18 plays a powerful role in the induction and maintenance of cellular states associated with IFNγ production.

1.3.2.7 IL-18 in immune and tissue homeostasis

Despite its continuous expression by a variety of macrophage and epithelial (and possibly other) cells, there is little evidence for a non-redundant homeostatic role for IL-18. Its contributions to barrier maintenance, including IL-22 induction and epithelial antigen presentation, are only beginning to be recognized[125,129,193]. No human disease has yet been attributed to deficiency of IL-18 or its receptors, and the genes for all three proteins show no evidence for evolutionary intolerance of haploinsufficiency[194]. The only spontaneous phenotype associated with global murine IL-18 deficiency is hyperphagia leading to obesity [195], but any endocrine or brain-specific effects of IL-18 are poorly understood.

1.3.2.8 Interferon-γ, cytotoxicity, and type I immunity

Type I immunity is directed against intracellular pathogens and defined by the combined effector programs of granule-mediated cytotoxicity and IFNγ. When T cells and NK cells receive signals from IL-18 and another (usually Jak/STAT) cytokine, which induce IFNγ production far beyond any individual signals[161,196]. Cytokine cocktails containing IL-18 produce the strongest CD8 T cell responses[197], though synergy with IL-12 is typically strongest[90,198]. Although it is not immediately clear why IL-18 exerts such minimal effects in isolation, it is likely that cells with high expression (activated T cells and NK-like ILCs) typically require integration of multiple

positive signals for robust effector activity. Alternatively, IL-18 could act to prime cells for further reactions toward other cytokines like IL-12[199]. Furthermore, pretreatment of NK cells with this combination of cytokines has led to memory-like NK cell populations in a tumor model[200]. However, even without IL-12 support, IL-18 can increase perforin and FasL-dependent cytotoxic activities in NK and CD8+ T cells[201,202].

IL-18 has been shown to promote nearly all *in vivo* models of type 1 immune responses to pathogens. Most studies include mice lacking IL-18 or its receptor, but more recent work in mice with excess free IL-18 demonstrate IFNγ-mediated immunopathology during LCMV infection, repeated TLR9 stimulation, and/or toxoplasma gondii infection[11,96,158,203,204]. In the intestinal tract, IL-18 may act as a type 1 immune-skewing alarmin. Murine models of intestinal microbial infection suggest a requirement for IL-18 in maintaining mucosal integrity and maintenance[128], though lack of IL-18 regulation may cause increased mucosal inflammation and colitis in many murine models[119,205-208]. IL-18 appears to protect against enteric pathogens by activating local/infiltrating T and NK cells to produce IFNγ and/or IL-22, promoting cellular defense against intracellular pathogens, promoting barrier functions (e.g. anti-microbial peptides and mucous), and promoting antigen presentation, potentially by intestinal epithelia[125,129]. In sum, these studies suggest that IL-18 enhances existing type 1 immune responses through stimulation of innate or adaptive IL-18 responsive cells.

1.3.2.9 Participation in Th2 and Th17 responses

IL-18 can also amplify other types of inflammatory responses. In combination with IL-3, IL-18 can stimulate the production of IL-4 and IL-13 by basophils, and IL-13 by mast cells[184] further enhancing Th2 responses to parasites and bacterial infections. In conjunction with IL-2, IL-18 induces production of IL-3, IL-9 and IL-13 by NK cells, CD4+ NKT cells and splenic

CD4+T cells[196,209,210]. IL-18 is particularly important in the skin[186], where lack of IL-18 signaling in ILC2s impairs their ability to infiltrate skin both homeostatically and during inflammation. Confirming the impact of IL-18 in the skin, overexpression of IL-18 in keratinocytes leads to an IgE/IgG1 independent dermatitis[19]. IL-18rα bearing ILC2s can also be found in murine lung and bone marrow, but not GI tract, suggesting tissue-specific roles for IL-18 in Th2 responses[186].

IL-18 can be seen as a tissue damage alarmin and is sufficient to induce a switch of CD8+ Tc17 cells into a type-2 cytokine (IL-5 and IL-13) secreting cell[211]. Interestingly this Tc17 response switch comes at the expense of CD4+ Th17 cells. However, in the context of IL-23, IL-18 is able to intensify inflammatory responses by promoting IL-17 secretion from γδ– T cells[212]. In EAE, where a mixed type 1 and 17 inflammation drives CNS autoimmunity, the role of IL-18 remians unclear, with some systems showing inhibition and others showing induction of inflammatory responses[212-215].

1.3.2.10 Effects on Tregs

The ability of IL-18 to promote expansion of effector T cells likely comes at the cost of other cell types like regulatory T cells (Tregs). While there are minimal studies on the effects of IL-18 directly acting on Tregs, in the context of obesity, IL-18 treated Tregs had increased proteasomal degradation of *FoxP3* and fail to control primary graft dysfunction in the lung[216]. Similarly, PBMC's from patients with rheumatoid arthritis, blocking IL-18 signaling with IL-18BP allowed for more Treg differentiation *in vitro*[217]. Conversely, IL-18 signaling led to expression of key Treg effector molecules and was found to be critical for Treg-mediated control of intestinal inflammation[218]. Together these studies suggest that the impact of IL-18 on Tregs is highly context dependent and is capable of inhibiting or enhancing Treg function.

1.3.2.11 Effects on neutrophils

Neutrophils are abundant in circulation, critical for host defense, capable of causing tremendous immunopathology, and can express both IL-18ra and IL-18rap. IL-18 is thus capable of promoting neutrophil activation and effector function[116,185,219]. Neutralization of IL-18 prior to LPS challenge in mice reduces levels of the neutrophil myeloperoxidase (MPO) enzyme within affected tissues[220]. The specific effects of IL-18 on neutrophils *in vivo* are poorly understood.

1.3.2.12 Effects of IL-18 on T cell exhaustion

This are conflicting data regarding the role of IL-18 CD8 T cell exhaustion in chronic infections and tumors. Some reports showed IL-18 receptor (IL-18R) upregulation by tumor infiltrating lymphocytes[111] while others have shown downregulation in terminally exhausted T cells in chronic infection[104]. In tumor settings, IL-18R signaling has been shown to induce the expression of inhibitory receptors (PD-1, TIM-3, LAG-3) and transcription factors (Eomes, TOX) that are associated with exhaustion[221]. Moreover, IL-18R signaling activates the IL-2/STAT5/mTOR pathway, which regulates metabolic processes and gene expression in exhausted CD8 T cells. Indeed, IL-18R-deficient CD8 T cells show enhanced antitumor activity and cytokine production in mouse models of pancreatic cancer[221]. This contradicts studies showing decoy-resistant IL-18's potent and T cell dependent ability to shrink tumors in mouse cancer models[111]. These findings require more careful study, but may suggest that rather than recovering function from exhausted cells, IL-18 promotes effector T cells to a degree that prevents/delays exhaustion.

1.3.2.13 Effects on DCs and Antigen Presentation

IL-18 can both be produced by and act on dendritic cells. IL-18 can have a wide variety of effects on dendritic cells based on the poised state of the individual cell. IL-18 signaling on

immature DCs (but not monocytes) can lead to their maturity[222] while directed free IL-18 secretion by immature DCs can initiate NK cell activation[223]. Furthermore, free IL-18 released at the DC:T cell interface leads to enhanced T cell activation[106].

1.3.3 Mouse models of excess IL-18

In order to study IL-18opathies in the context of hyperinflammation several mouse models fo excess IL-18 have been developed. Some are inspired by known genetic variants such as the Nlrc4 T337S mutant mouse developed by our lab[11]. These mice have elevated IL-18 that is derived from the intestinal epithelia but minimal pathology due to excess IL-18BP prevents elevated free IL-18[11]and /or because its intestinal origin is associated with less ability to provoke immunopathology. To gain a broader interpretation of how IL-18 can lead to hyperinflammation we have used models with notable free IL-18. In particular, the mice with excess IL-18 signaling used in this dissertation include the following:

- ***Il18bp$^{-/-}$ mice:*** Global knockout of the endogenous IL-18 antagonist. As such, any active IL-18 that is released is unobstructed by IL-18BP. These mice have no spontaneous phenotype, but have been previously reported to manifest a severe MAS phenotype upon repeated TLR9 stimulation[158].
- ***Il18tg mice:*** Mice in which a transgene encoding the mature form of IL-18 was fused with the signal peptide of the mouse Ig kappa-chain and placed under the control of the human Eu enhancer and mouse IgVH promoter, which allows expression of IL-18 in B and mature T cells[203]. These mice ae phenotypically normal, but have chronically elevated IL-18 and modestly elevated serum IFNγ.

1.4 Murine models of hyperinflammation

Within this dissertation, we use multiple models of inducible hyperinflammation to understand the roles that proposed susceptibility factors play in disease. We define murine hyperinflammation similarly to the human clinical phenotype: Anemia (hemoglobin > 12g/dL, platelets > $5x10^5$/uL), splenomegaly (<2% body weight), cytokine storm (IFNγ > 1000pg/mL), liver damage (aspartate aminotransferase [AST] >50U/L), and hemophagocytosis. Many stimuli are capable of including these parameters in mice, so a second condition of study is the inclusion of biomarkers or well-established genetic risk factors for human HLH/MAS into the murine system. Below is a brief explanation of the models used:

1.4.1 Lymphocytic choriomeningitis virus infection

Lymphocytic choriomeningitis virus (LCMV) is a classic murine virus used for decades to study the response to viral infection of the adaptive immune system. LCMV is a valuable tool for immunological research, as it can induce different types of immune responses depending on the strain, dose and route of infection. There are two main commonly used strains of LCMV that differ by their chronicity. The Armstrong variant models acute viral infections, while the Clone-13 variant models chronic viral infection. Infection by either strain induces a strong virus specific CD8 T cell expansion which peaks at day 8[224,225]. In ***LCMV-Armstrong***, contraction occurs after the virus has been cleared (typically around day 9) leaving memory CD8T cells. In ***LCMV-Clone 13***, due to persistent antigen exposure, CD8 T cells enter a state of functional exhaustion in which they are unable to clear the virus[100]. CD8 T cell exhaustion can be seen in mice by day 15 after

initial infection[98] and can persist for upwards of 22 weeks in immune-competent C57/BL/6 mice[226].

1.4.2 TLR9 Stimulus by CpG-ODNs

In order to study the innate immune response to IL-18 in the absence of a direct TCR stimulated response, we use a previously published ***TLR9 agonist*** model[70] in which mice are repeatedly injected with unmethylated CpG-B DNA fragments resembling bacterial DNA. Mice develop non-lethal MAS-like inflammation in a manner dependent on IFNγ but independent of B and T cells. Such mice develop a prominent population of activated hepatic CD8 t cells that may be an important source of immunoregulatory IL-10[227]. In short, CpG fragments are recognized by the TLR9 pattern recognition receptor and initiate the production of type 1 interferons and an innate immune response. TLR9 stimulus can also activate the adaptive immune system in a Th-1/B cell mediated manner[228]. In the presence of systemically high IL-18 this has been shown to induce a MAS-like phenotype.

2.0 CD8 T cells are important in susceptible models of hyperinflammation

Portions of this chapter have been published in one of the following articles:

- Paul Tsoukas, **Emily Rapp**, Lauren Van Der Kraak, Eric S. Weiss, Vinh Dang, Corinne Schneider, Edwin Klein, Jennifer Picarsic, Rosalba Salcedo, C. Andrew Stewart, Scott W. Canna; Interleukin-18 and cytotoxic impairment are independent and synergistic causes of murine virus-induced hyperinflammation. Blood 2020; 136 (19): 2162–2174. doi: https://doi.org/10.1182/blood.2019003846
- **Emily Landy**, Jemy Varghese, Vinh Dang, Andrea Workman, Lawrence Kane, Scott Canna; Complementary HLH Susceptibility Factors Converge on CD8 T-cell Hyperactivation. Blood Adv 2023; bloodadvances.2023010502. doi: https://doi.org/10.1182/bloodadvances.2023010502

2.1 Summary

Patients with hyperinflammatory disease have a preponderance of activated CD8 T cells in peripheral blood[86,87] and affected tissues (i.e. spleen, liver, bone marrow)[92-94]. We wondered if this activation phenotype existed in a mouse model of hyperinflammation. In Section 2.3.1 we explore the impact of IL-18 in acute and chronic viral infection on candidate cell types and subsequent inflammatory disease. We show that both Il18tg and Prf1$^{-/-}$ mice develop a hyperinflammatory phenotype upon infection with acute LCMV (Armstrong). We also show that CD8 T cells from these mice have an activated phenotype, with effector levels of IFNγ production, a state hereafter

termed “hyperactivated”. We then demonstrate that IL-18 responsive CD8 T cells are the main pathogenic cell type driving hyperinflammation, by using antibody-mediated depletion and cell-specific deletion of IL-18r1. We demonstrate that during chronic LCMV infection (Armstrong), $Prf1^{-/-}$ mice succumb to disease due to viremia, whereas excess IL-18 culminated in lethal hyperinflammation. In **Section 2.3.2**, we show that the TLR9-MAS model (using repeated injections of CpG-1826) in perforin deficient mice had little-to-no impact on inflammation and were largely spared from the hyperinflammation seen in Il18tg mice. We further attempted to understand if CD8 T cells played a role in this context; however, the loss of IL-18 signaling on CD8 T cells did not ameliorate disease. This suggests that further investigation is necessary to determine the potential contributions of other cell types to pathology in TLR triggered hyperinflammatory responses.

Together these data suggest that not only are CD8 T cells necessary for (at least) viral triggered hyperinflammation, but that they are in fact the pathogenic cell type in this setting. These “hyperactivated” pathogenic CD8 T cells respond to IL-18, produce excess IFNγ, and exhibit expression and transcriptional profiles with features of both terminal effector activation and exhaustion which we term “hyperactivation”.

2.2 Methods

2.2.1 Murine models and stimuli

All animals were kept in specific pathogen-free conditions under an animal protocol approved by the University of Pittsburgh. Animals originated from Jackson Laboratories with the

following exceptions: Il18tg mice were a kind gift of Tomoaki Hoshino (Kurume University). Il18bp$^{-/-}$ mice were obtained from the Knockout Mouse Project[158]. Splenocytes from P14 mice were a kind gift from Larry Kane (University of Pittsburgh). CD4^{Cre}; Il18r1$^{fl/fl}$ mice were provided by Dr. Giorgio Trinchieri (NIH, Bethesda, MA). Specificity of deletion was assured by quantitative polymerase chain reaction (qPCR; data not shown) and on an ongoing basis by flow cytometry.

CpG1826 was administered repeatedly as in Behrens et al.[70]. Mice were injected intraperitoneally with 2x10^5 plaque-forming units of LCMV, Armstrong strain[95].

2.2.2 Assessment of systemic hyperinflammation

Complete blood counts were obtained using a HemaVet950 (Drew Scientific). Aspartate transaminase (AST) activity was assessed by colorimetric assay per the manufacturer's instructions (Sigma Aldrich). Mouse serum cytokines were measured by Cytometric Bead Array (BD)[11]. We used an offset of +1/4 the lowest concentration of each analyte's standard curve to each experimental value to minimize floor effects of data range. To measure viral load, splenic tissue was placed in Trizol immediately after euthanasia and cDNA was reverse transcribed (Bio-Rad iScript cDNA Synthesis Kit). qPCR was performed using LCMV-specific primers[229] and normalized to eukaryotic 18s (Taqman, Applied Biosystems).

2.2.3 Antibodies

To deplete CD8- or NK1.1-expressing cells, the following antibodies were injected intraperitoneally on days −3, 0, 3, and 6 of infection: YTS169 (0.5 mg, CD8), PK136 (0.2 mg,

NK1.1). Depletion was confirmed by flow cytometry of spleens and/or livers. IFNγ was neutralized with XMG1.2 (0.5 mg) on days −1, 3 and 5, relative to LCMV per Buatois et al[230].

2.2.4 Flow cytometry, tetramers, assays, and stimulations

Murine tissues were prepared in single cell suspensions in FACS buffer as in[11] and flow cytometry was performed on a BD LSRII or LSR Fortessa and analyzed using FlowJo v9.9.4 (Treestar). T-distributed Stochastic Neighbor Embedding (tSNE) analysis was carried out using the EXCYT2 program on Matlab R2020a[231]. LCMV-Armstrong specific tetramers (GP33 and NP396) were obtained from the National Institutes of Health tetramer core facility. Peptide (10ug/mL) and phorbol myristate acetate (50ng/mL)/ionomycin (1ug/mL) stimulations were performed in single cell suspensions of $4e^6$ cells/well were stimulated in the presence of 2 μg/ml brefeldin A (Sigma), and 2 μM monensin (eBioscience) for 5 hours at 37°C.

2.2.5 RNASeq analysis

On day 8 after LCMV-Arm infection, splenic GP33 tetramer+ CD8 T cells were isolated by fluorescence-activated cell sorting directly into lysis/stabilization media. Messenger RNA purification and fragmentation, complementary DNA synthesis, and target amplification were performed with the Smart-Seq v4 ultra-low-input RNA sequencing preparation kit. Pooled complementary DNA libraries were sequenced on the Illumina NextSeq500, mapped to Mus_musculus_ensembl_v80 reference sequence, and quantified using CLC Genomics Workbench software (V8.1.6, Qiagen). Differentially expressed genes were those defined as having a maximum group mean reads per kilobase million > 5, |fold change| > 2, and false

discovery rate of $P < .05$. Fold changes were not calculated directly from reads per kilobase million values but from the generalized linear model, which corrects for differences in library size between the samples and the effects of confounding factors. Gene Set Enrichment Analysis was performed on untrimmed transcripts per million data comparing $Prf1^{-/-}$ vs. Il18tg cells using GSEAv4.0.3 with the following parameters: Gene sets database = MSigDB C7.all.V7.1 (Immunologic Signatures); permutations = 1000; permutation type = gene_set. A principal component analysis plot with scaling by covariance was generated on Partek Genomics Suite v7.18.0723 using unfiltered transcripts per million values.

2.3 Results

2.3.1 CD8 T cells are the pathogenic cell type in viral-triggered hyperinflammation

Previously, it was shown that in mice infected with typically benign acute lymphocytic choriomeningitis virus (LCMV-Armstrong), either chronic excess IL-18 or perforin deficiency led to an HLH-like phenotype[95], and that CD8 T cells are essential for disease in $Prf1^{-/-}$ mice[97]. Using perforin deficient mice as a hyperinflammatory comparator, we explore the impact of IL-18 in both acute and chronic infection on candidate cell types and how they lead to disease.

2.3.1.1 Either perforin deficiency or excess IL-18 leads to CD8 T cell expansion and hyperinflammation during acute LCMV infection

As discussed in **Section 1.3.2.8**, IL-18 can promote cytotoxic function[232,233]. Therefore, we hypothesized that (unlike perforin deficiency) excess IL-18 would result in normal or enhanced

clearance of LCMV-Armstrong (Arm) infection, and minimal immunopathology. However, we found that Il18tg mice developed hyperinflammation in a similar timeline to Prf1$^{-/-}$ mice, but with less mortality. Specifically, Il18tg mice developed significant cachexia, splenomegaly, pancytopenia, transaminitis, and hemophagocytosis (**Figure 3**). Additionally, while Prf1$^{-/-}$ mice succumbed to viremia, Il18tg mice cleared LCMV infection at the same rate as WT mice (**Figure 3E**). As expected, serum IL-18 levels were highest in Il18tg mice, although Single Susceptibility (Prf1$^{-/-}$ or Il18tg) mice also developed elevated levels of IFNγ and tumor necrosis factor (TNF; **Figure 4**). Together this suggests that both perforin deficiency and excess IL-18 are susceptibility factors to LCMV-triggered hyperinflammation, and that this clinical phenotype can be seen regardless of active infection.

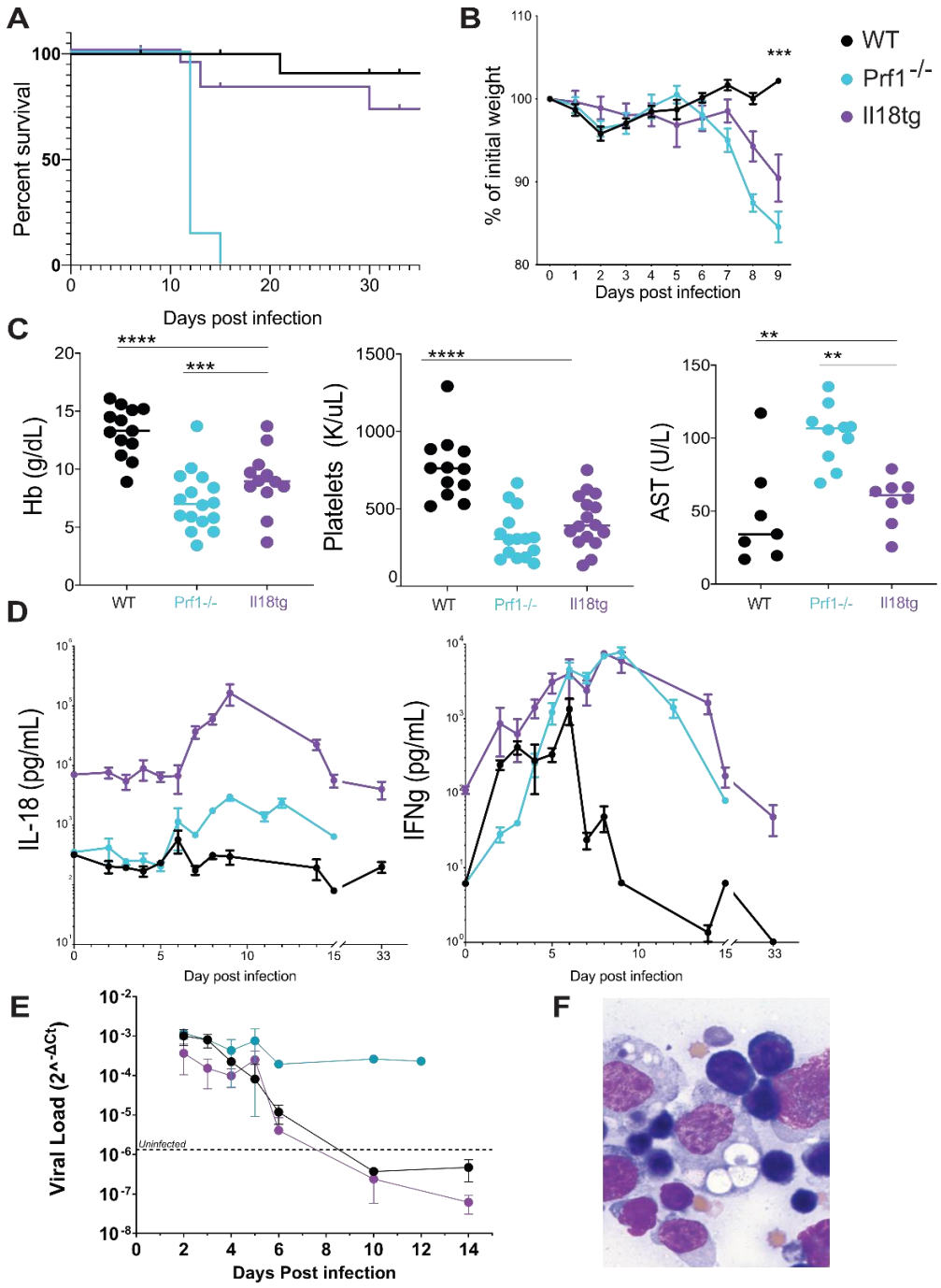


Figure 3: ***Acute LCMV Armstrong infection leads to hyperinflammation in Prf1$^{-/-}$ and Il18tg mice:*** The indicated genotypes were infected with 200,000 pfu of LCMV Armstrong and assessed for (A) survival and (B) weight (statistical significance for WT vs. Il18tg comparison), (C) hemoglobin, platelet count, and serum aspartate transaminase (AST), (D) serum IFNγ & IL-18. (E)Viral clearance (up to day 14 as were below threshold thereafter) and (F) representative splenic touch preparation from Il18tg mouse on day 8 (Wright-Giemsa). Data are composites of at least three independent experiments with a minimum of three mice per genotype, apart from AST, which is a composite of two experiments. Daily cytokine measurements in (D) represent a minimum of 4 mice per genotype. *Adjusted $p<0.05$, **$p<0.01$, ***$p<0.001$, ***$p<0.0001$ by one-way ANOVA with Tukey post-test on day 8 values. Significance is only shown for comparisons where adjusted $p<0.05$. Error bars represent standard error of the mean (SEM).

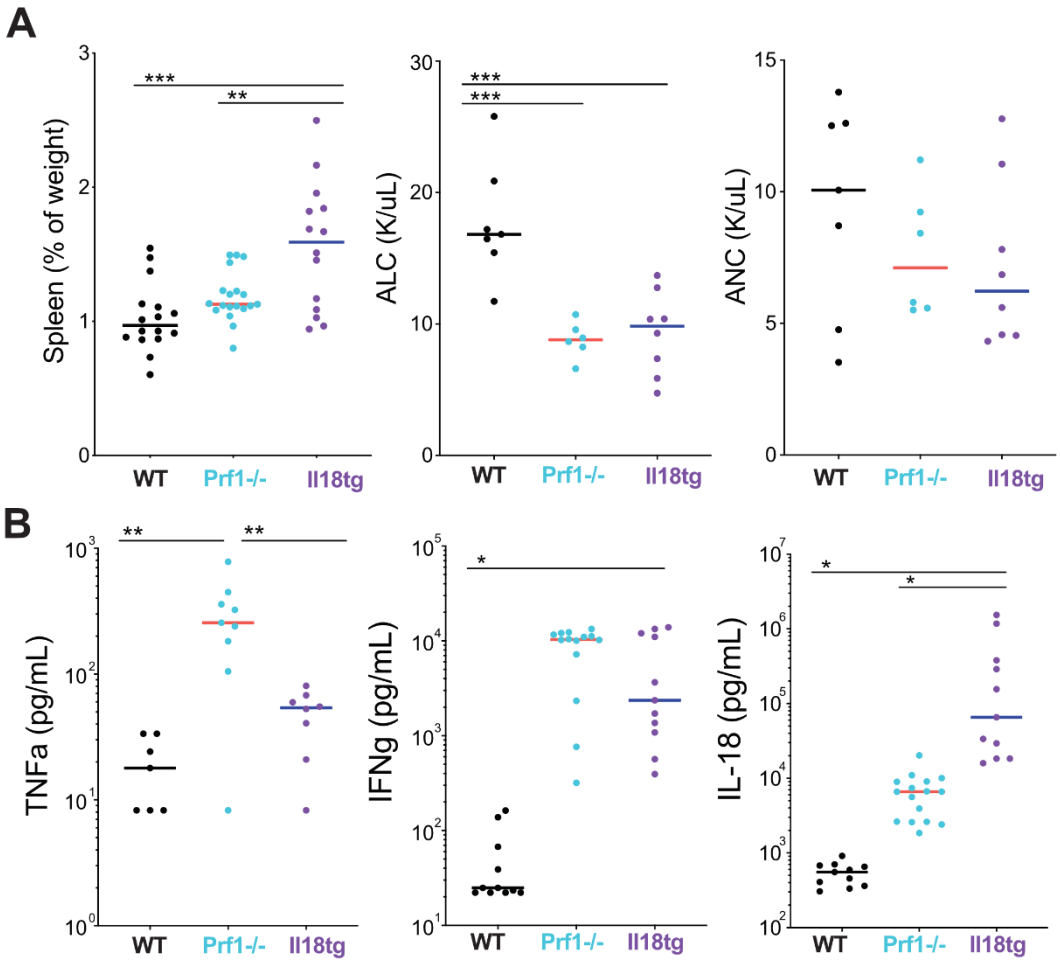


Figure 4: ***LCMV induces hyperinflammation in Il18tg mice:*** The indicated genotypes were infected with 200,000 pfu of LCMV Armstrong for 8 days and assessed for (A) spleen weight, serum lymphocyte counts, neuorophil counts, as well as (B) serum TNF, IFNγ and IL-18. Data are composites of at least two independent experiments with a minimum of three mice per genotype. *Adjusted p<0.05, **p<0.01, ***p<0.001, ***p<0.0001 by one-way ANOVA with Tukey post-test. Significance is only shown for comparisons where adjusted p<0.05.

2.3.1.3 CD8 T cells are hyperactivated in Il18tg and Prf1$^{-/-}$ mice during LCMV infection

We next wanted to identify the pathogenic cell type in murine hyperinflammation. We predicted that CD8 T cells would be a likely target due to: a) their upregulation of activation receptors including Il18r1; b) the striking preponderance of activated CD8 T cells in human hyperinflammation[92-94]; and c) the requirement of CD8 T cells for viral clearance during LCMV infection[234-236]. As expected, CD8 T cells were the dominant immune cell type in the spleens of

LCMV infected mice. Absolute numbers of total and activated CD8 T cells were similar between genotypes. However Single Susceptibility mice had CD8 T cells that had increased cytokine production (**Figure 5**) when compared to WT mice under the same conditions. These "hyperactivated" CD8 T cells showed increased levels of IFNγ as well as programmed cell death protein 1 (PD-1). Il18tg CD8 T cells were predominately PD-$1^{+}CD39^{-}$, suggesting activation rather than exhaustion[237]. $Prf1^{-/-}$ CD8 T cells, while still producing excess IFNγ and therefore termed hyperactivated, expressed both PD-1 and CD39 suggesting these CD8 T cells were prone to exhaustion[238].

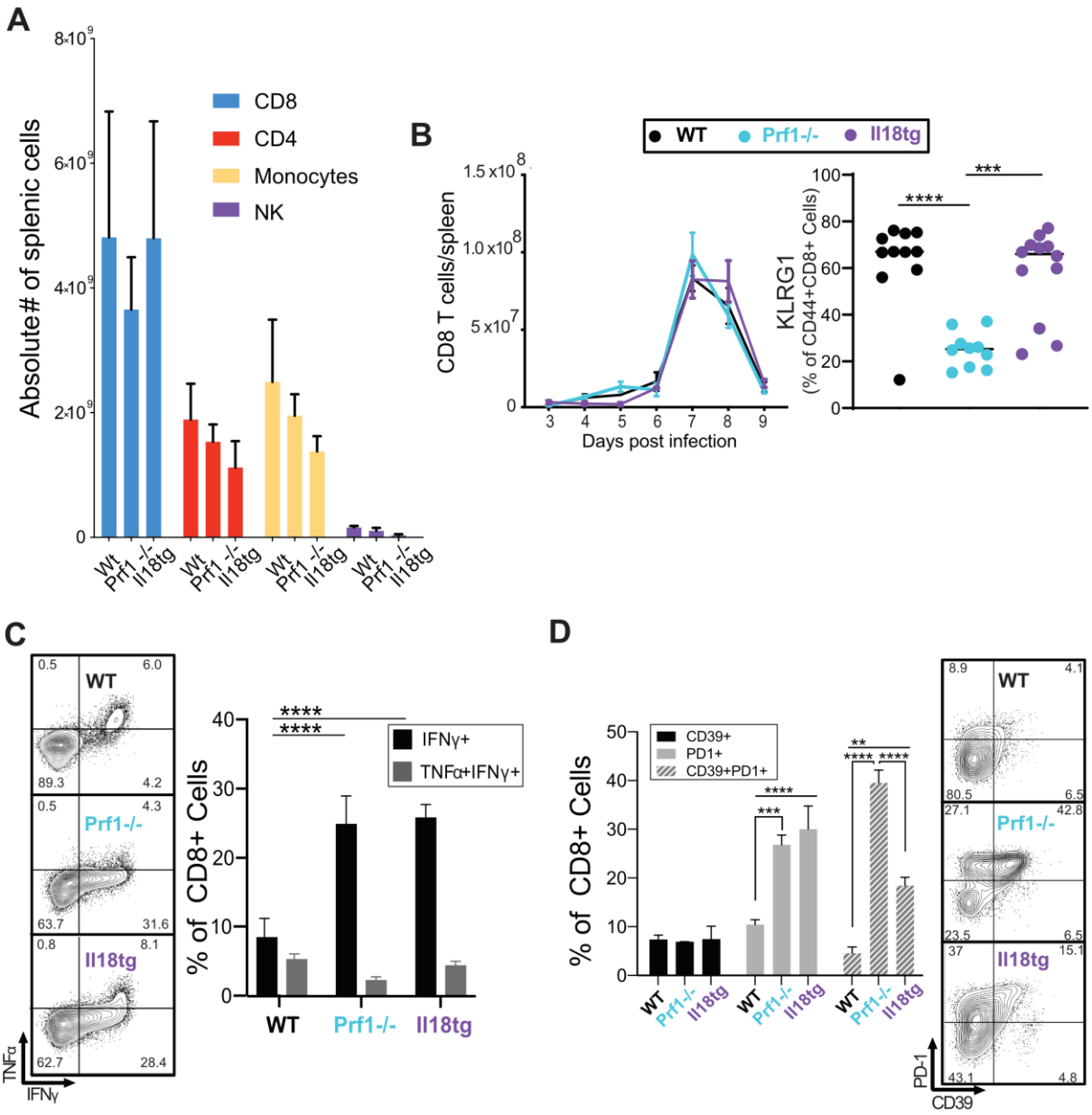


Figure 5: ***CD8 T cell expansion and hyperactivation in Single Susceptability 8 days post LCMVa infection****:* The indicated genotypes were infected with 200,000 pfu of LCMV Armstrong for 8 days and assessed for (A) absolute number of splenic immune cells at day 8 of infection, (B) absolute number of splenic CD8+ T lymphocytes, the percentage of splenic CD8+ T lymphocytes expressing KLRG1+, (C) cytokine expression after stimulation with GP33 peptide, and (D) the percentage of effector CD8 T lymphocytes expressing inhibitory markers. *Adjusted P < .05, **P < .01, ***P < .001, ***P < .0001 by 1-way ANOVA with Tukey post-test. Significance is only shown for comparisons where adjusted P < .05. Error bars represent SEM. Results are representative of at least 2 independent experiments with a minimum of 3 mice per genotype.

In an effort to further understand hyperactivated CD8 T cells at the transcriptional level, we employed bulk RNA sequencing of CD8 T cells in WT and Single Susceptibility mice 8 days post LCMV Armstrong infection. We observed that based on differential gene expression (DEG), WT and Il18tg CD8 T cells were relatively similar. Additionally, we saw similar upregulation of *Ifng* in both Prf1$^{-/-}$ and Il18tg CD8 T cells. Il18tg CD8 T cells showed increased transcription of effector- like molecules such as *Il1rl1* (encoding ST2), *Csf2* (encoding GM-CSF), and *Il12rb2* (encoding IL-12 receptor). Whereas we observed increased transcriptional expression of inhibitory receptors and transcription factors indicative of exhaustion in Prf1$^{-/-}$ CD8 T cells such as *Lag3*, *Havcr2* (encoding Tim-3), and *Tox* (**Figure 6**).

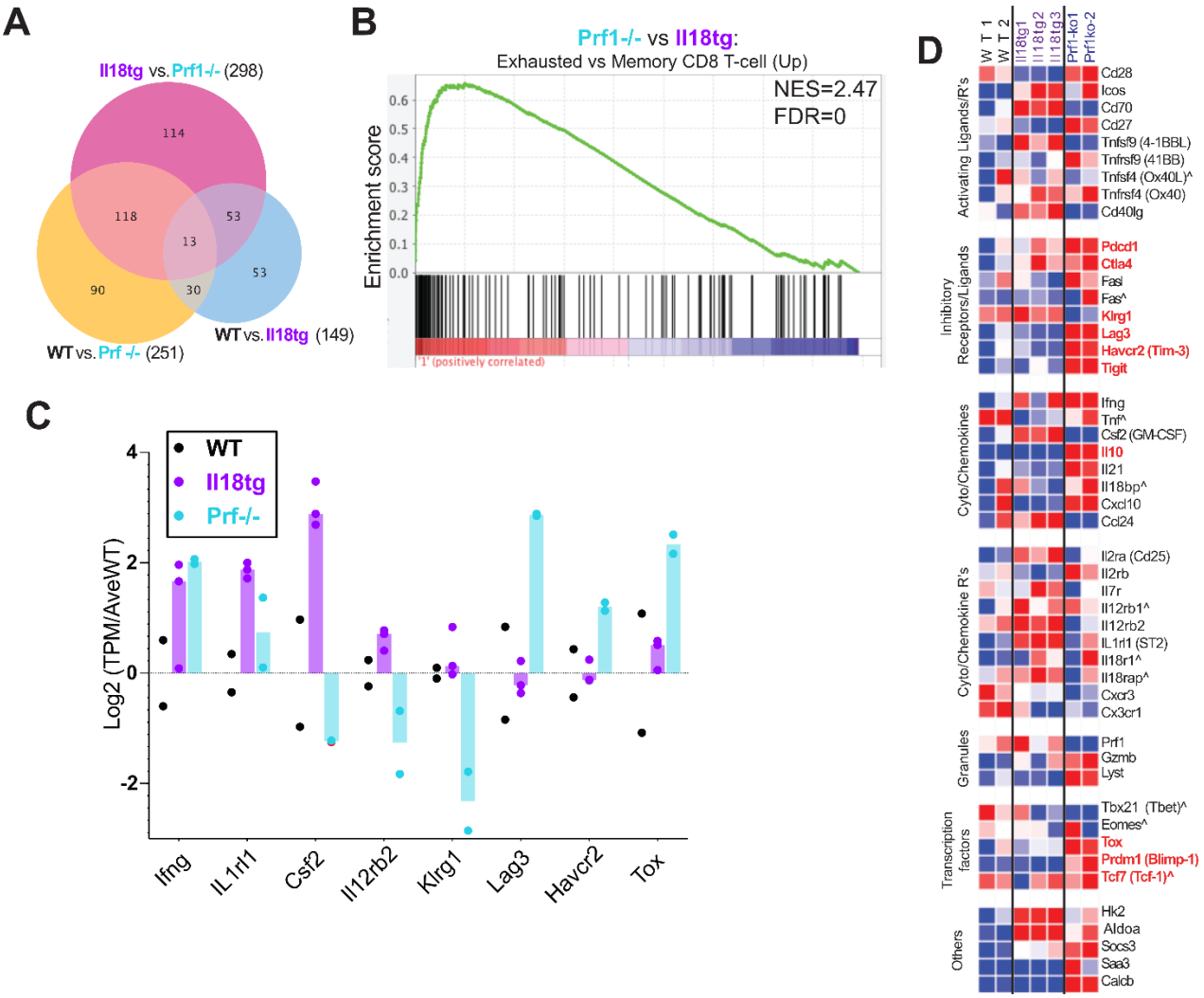


Figure 6: ***Il18tg CD8 T cells are transcriptionally distinct from Prf1$^{-/-}$ during LCMVa infection****:* RNASeq analysis of GP33-antigen specific CD8 T cells including (A) Venn diagram of differentially expressed genes, (B) Gene Set Enrichment Analysis (GSEA) of the Prf1$^{-/-}$ vs Il18tg comparison showing the most highly enriched MSigDb C7 (immunologic signature) CD8 T cell upregulation gene set (from GSE9650), (C) relative expression of selected genes (normalized to the average expression of WT), and (D) Heatmap of selected genes in WT, Prf1$^{-/-}$, and Il18tg CD8 T cells. See Supplemental Data for complete differential expression and GSEA data. FDR, false discovery rate; NES, normalized enrichment score.

Interestingly, upregulation of *Tox*, a purported master regulator for exhaustion[239], was also seen in Il18tg mice yet to a less extent when compared to Prf1$^{-/-}$ mice. We next wanted to see if IL-18 would eventually lead to exhaustion and if normal memory cells would form. We followed Il18tg and WT mice +30dpi with LCMV (Armstrong). We found that Il18tg CD8 T cells after 30dpi had similar levels of LCMV-specific memory cells but showed increased PD-1 expression (**Figure 7**). This suggests that IL-18 does not impede memory formation but may leave memory cells in a more activated state.

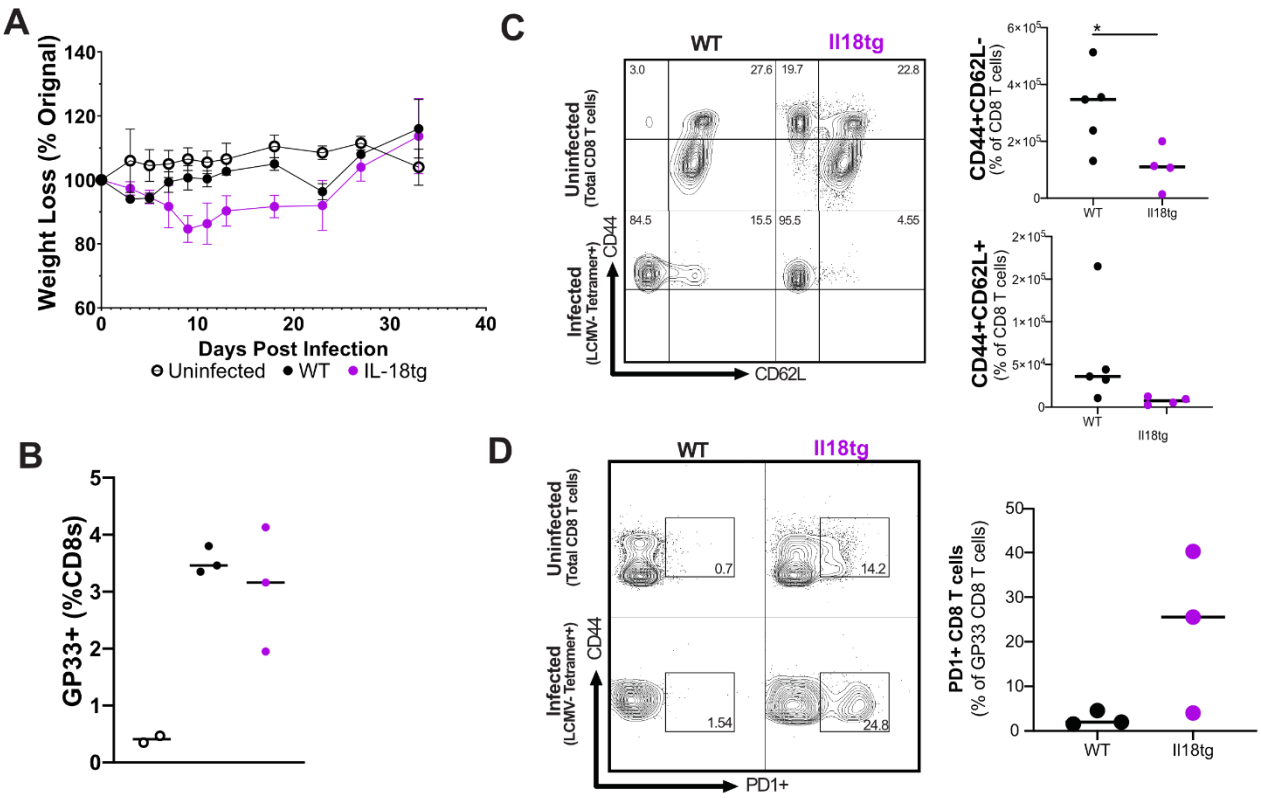


Figure 7: ***CD8 T cell responses at memory timepoints following virus-induced hyperinflammation:*** Mice of the indicated genotypes were infected with LCMV Armstrong and assessed 33 days later for (A) weight loss, (B) LCMV-specific CD8 T cell levels, (C) splenic central and effector memory CD8 T cells and (D) PD-1 positive viral specific CD8 T cells. Data are representative of at least two independent experiments with a minimum of three mice per genotype. *Adjusted $p<0.05$ by unpaired t-test. Significance is only shown for comparisons where adjusted $p<0.05$.

2.3.1.4 CD8 T cells drive disease in LCMV induced hyperinflammation

CD8 T cell outgrowth and activation is a typical and healthy response to LCMV infection. In the case of mice susceptable to hyperinflammation, hyperactivated CD8 T cells appeared to bethe pathogenic cell type. To test if CD8 T cells were directly responding to excess IL-18 and causing immunopathology we employed antibody mediated depletions and neutralizations in mice during LCMV infection. Treatments for patients with hyperinflmamatory disease include IFNγ neutralization (emapalumab[240,241]) and JAK inhibitors (ruxolitinib[242-244] NCT05137496). Since one of the main phenotypes of hyperactivated CD8 T cells was excess IFNγ producdion, we hypothesized that IFNγ was main contributor to the immunopathology seen in both Prf1$^{-/-}$ and Il18tg mice. To this end we treated susceptible mice with IFNγ neutralizing antibody (XMG1.2) during LCMV-Armstrong infection and assesed for clinical hyperinflammation 8dpi. In both Prf1$^{-/-}$ and Il18tg mice we saw reduced splenomegaly, anemia and liver damage in treated mice versus isotype controls (**Figure 8A-C**). We also determined that global pro-inflammatory cytokine/chemokine levels in treated mice we decreased as well (**Figure 8D**). Together this suggested that IFNγ was a major contributor to the mouse hyperinflammatory phenotype.

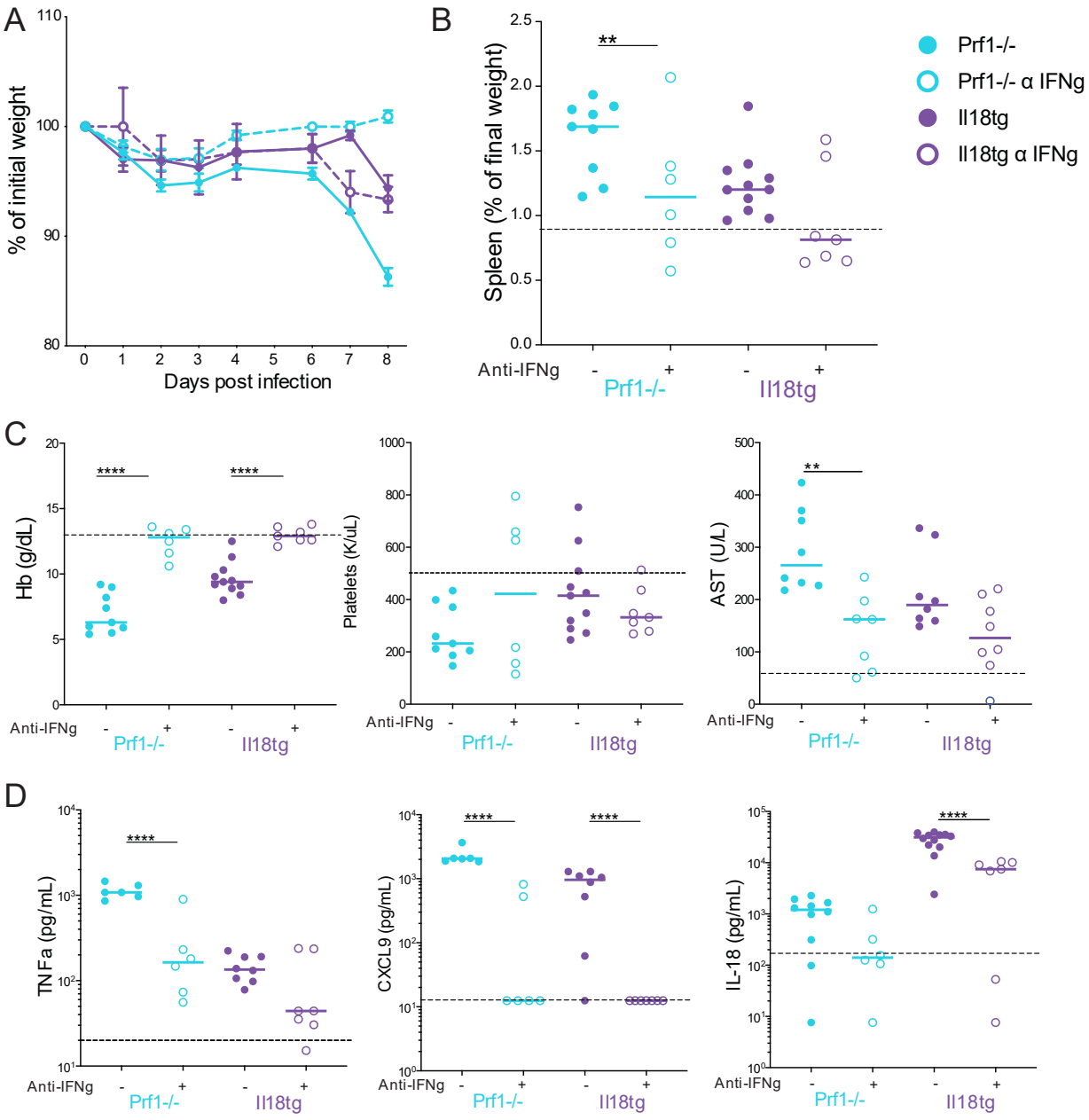


Figure 8: ***Neutralization of IFNγ partially ameliorates LCMV- induced hyperinflammation in Single susceptability mice:*** Mice of the indicated genotypes were infected with LCMV Armstrong and treated with IFNγ-neutralizing (clone XMG1.2) or control antibody. 8 days post infection, mice were assessed for (A) weight loss, (B) splenomegaly, (C) hemoglobin (Hb), Platelets, and liver damage (AST). Mice were also tested for serum (D) TNF, CXCL9, and IL-18. Horizontal dashed lines indicate the median value of uninfected WT control mice for reference. Results are composites of at least two independent experiments with a minimum of three mice per group.*Adjusted $p<0.05$, **$p<0.01$, ***$p<0.001$, ***$p<0.0001$ by one-way ANOVA with Tukey post-test on Day 8 values. Significance is only shown for comparisons where adjusted $p<0.05$. Error bars represent standard error of the mean (SEM).

Next, to determine if CD8 T cells were the pathogenic cell drivign disease in LCMV Armstrong-induced hyperinflammation we attempted to remove them via antibody mediated depletion (YTS169). We saw that depletion of CD8 T cells during LCMV infection in susceptable mice greatly reduced the hyperinflammatory phenotype similar to IFNγ neutralization. Specifcally CD8 T cell depletion in either $Prf1^{-/-}$ or Il18tg mice led to the mitigation of severe weight loss, anemia, splenomegaly, and serum IFNγ levels (**Figure 9**). Interestingly, treated $Prf1^{-/-}$ mice had less liver damage and significantly lower serum TNFa levels than untreated controls. by contrast, treated Il18tg mice showed increased AST levels and nearly identical levels of serum TNF. While levels of TNF and AST were mediated by IFNγ treatement in these mice, the lack of control by CD8 T cell depletion suggests there may be another source of IFNγ in the IL-18tg mice.

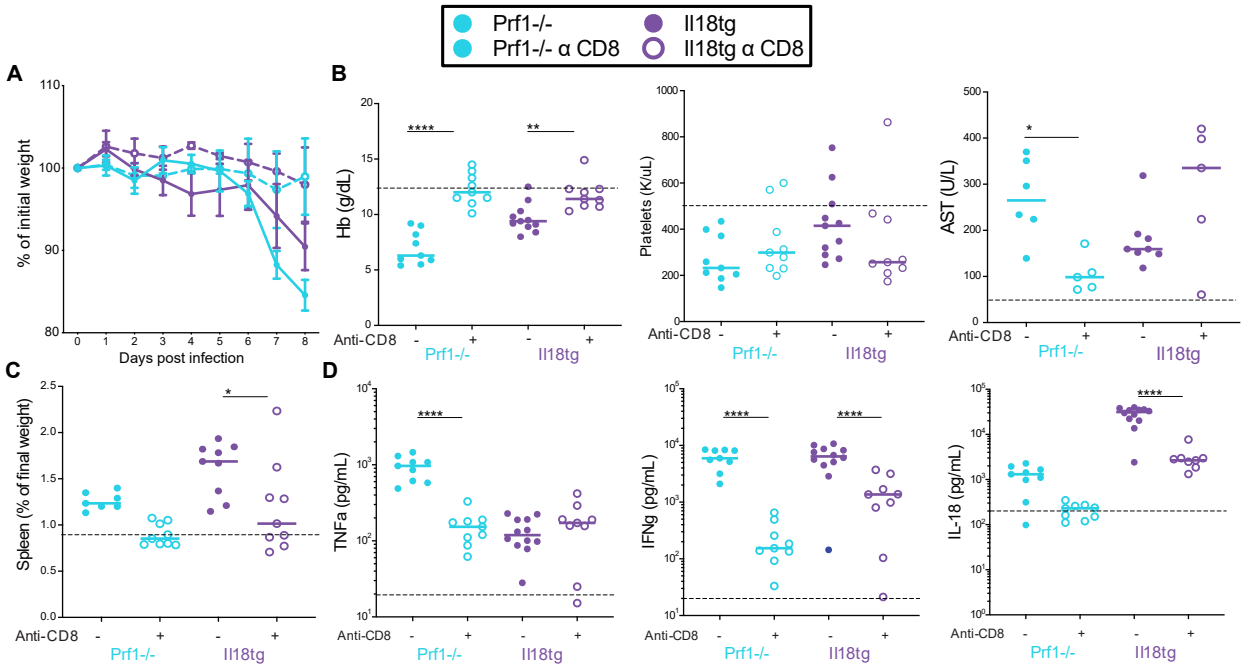


Figure 9: ***CD8 Depletion in single susceptability mice ameliorates LCMV-induced hyperinflammation:*** (A) Mice of the indicated genotypes were treated with CD8-depleting (clone YTS169) or control antibody, infected with LCMV Arm, and assessed at day 8 for weight loss, spleen weight, hemoglobin (Hb), serum aspartate transaminase (AST), serum TNF , IFNγ and IL-18. Horizontal dashed lines indicate the median value of uninfected WT control mice. Results are composites of at least two independent experiments with a minimum of three mice per group.*Adjusted $p<0.05$, **$p<0.01$, ***$p<0.001$, ***$p<0.0001$ by one-way ANOVA with Tukey post-test on day 8 values. Significance is only shown for comparisons where adjusted $p<0.05$. Error bars represent standard error of the mean (SEM).

Another candidate pathogenic cell type in hyperinflammation are NK cells. These cells, while not antigen specific, are a major producer of IFNγ and have consitiutive expression of IL-18 receptor components. NK cells contribute to tht early containment of LCMV infection but are quite dispensible for viral clearance[245]. In order to determine if NK cells were playing a pathogenic role in LCMV-caused hyperinflammation in susceptible mice, we used antibody-mediated depletion (PK136) of NK1.1 expressing cells during infection. There was little effect on hyperinflammation in susceptible mice depeted of NK cells when compared to Isotype treated controls (**Figure 10**). This along with the other studies mentioned in this section suggest that

hyperactivated CD8 T cells are the pathogenic cell type in this model of hyperinflammation due to their excess production if pro-inflammatory IFNγ.

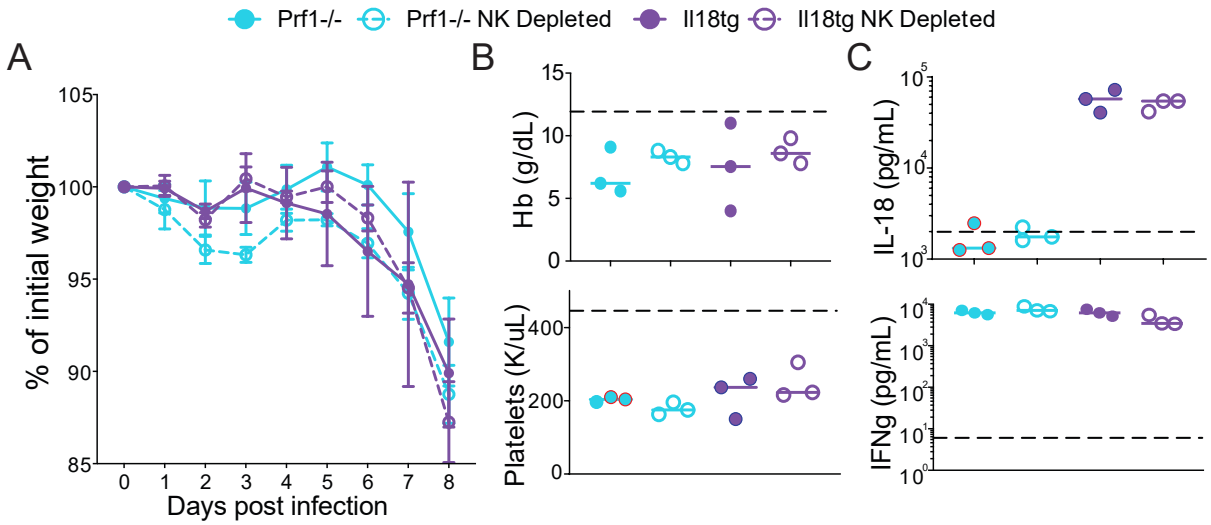


Figure 10: ***NK Cell depletion does NOT ameliorate LCMV-induced hyperinflammation in single susceptability mice:*** (A) Mice of the indicated genotypes were infected with LCMV Armstrong, treated with NK1.1 depleting (PK136) or control antibody, and assessed 8 days post infection. Infected mice were assessed for (A)weight loss (B) anemia (Hemoglobin and Platelets) and (C) serum IFNγ and IL-18. Horizontal dashed lines indicate the median values of uninfected WT control mice for reference. No statistically significant differences between NK cell depleted and non-depleted mice were detected using one-way ANOVA with Tukey post-test.

2.3.1.5 IL-18r1 expression on CD8 T cells is required for their hyperactivation during LCMV-induced hyperinflammation

Given the dramatic expansion of (especially CD8) T cells with LCMV infection, and their upregulation of IL-18R1 (**Figure 6D**), we hypothesized that T cells were responding to IL-18 to drive immunopathology in this model. To test this, we developed Il18tg mice lacking the IL-18 receptor specifically on T lymphocytes (CD4Cre^{+}; Il18tg; Il18r1$^{flox/flox}$ Il18tg, Il18r$^{\Delta T}$). We have consistently observed less IL-18R expression on NK cells from Il18tg mice. Interestingly, NK cell downregulation of the IL-18R1 was less profound in Il18tg IL18r$^{\Delta T}$ mice than Il18tg mice, suggesting T cell responses to IL-18 result in diminished expression of IL-18R1 on NK cells under homeostatic conditions. Upon LCMV infection, Il18tg, Il18r$^{\Delta T}$ mice responded like WT mice,

whereas CD4Cre negative controls developed immunopathology similar to Il18tg mice (**Figure 11**). Specifically, infected Il18tg Il18r$^{\Delta T}$ mice, despite retaining elevated serum IL-18, did not lose weight, develop splenomegaly or anemia, or exhibit elevated serum IFNγ. These data demonstrate that LCMV-induced hyperinflammation in Il18tg mice is dependent on IL-18 sensing by T cells.

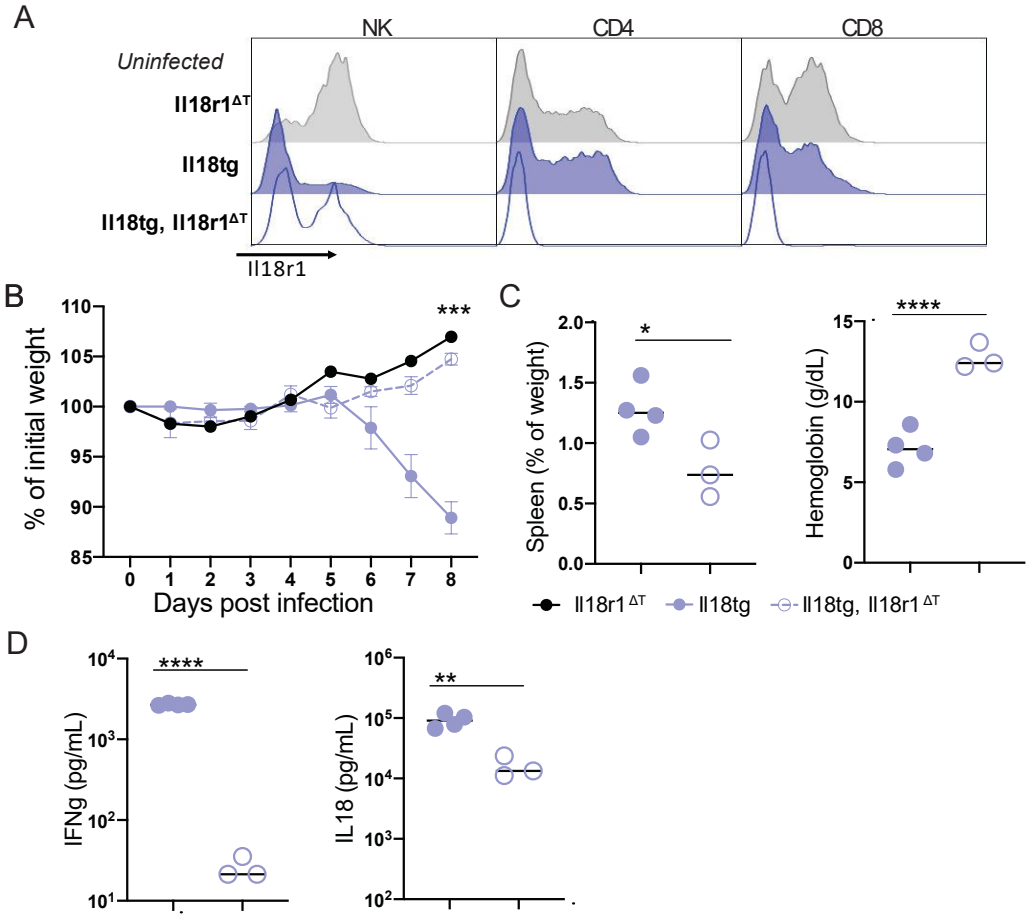


Figure 11: ***Loss of Il18r1 on T cells ameliorates LCMV-induced hyperinflammation in Il18tg mice****:* A) Il18r1 expression by flow cytometry of indicated genotypes under homeostatic conditions. Mice of the indicated genotypes were infected with LCMV-Arm and assessed for (B)weight loss, (C) splenomegaly, anemia, and (D) serum IFNγ and IL-18. Dashed horizontal lines represent the median values of infected CD4Cre Il18r1$^{fl/fl}$ mice. Weight statistical significance compares Il18tg CD4Cre Il18r1$^{fl/fl}$; and Il18tg CD4Cre Il18r1$^{fl/fl}$ mice. Data are representative of two independent experiments with a minimum of three mice per genotype. *Adjusted p<0.05, **p<0.01, ***p<0.001, ***p<0.0001 by unpaired t-test on day 8 values. Significance is only shown for comparisons where adjusted p<0.05. Error bars represent standard error of the mean (SEM).

Since the development of LCMV-induced MAS in Il18tg mice required *Il18r1* expression by T cells and CD8 T cells seemed to be the pathogenic cell type we hypothesized that the effects of excess IL-18 were acting specifically on CD8 T cells. To test this, we temporally and selectively deleted *Il18r1* in CD8 T cells using E8i$^{ERT2\text{-}Cre\text{-}GFP}$ Il18r1$^{flox/flox}$ mice (Il18r1$^{\Delta 8}$)[246]. Upon LCMV-Armstrong infection, Cre+ mice were protected from mortality, severe weight loss, and anemia (**Figure 12A-B**). By itself, tamoxifen treatment substantially (but incompletely) reduced the population of IL-18R1+ CD8 T cells in the blood, but not CD4 T cells (~70% in control mice to ~20% in Cre+ mice, (**Figure 13A-B**).

Nearly all CD8 T cells expressed IL-18r1 in infected control (Cre-) mice. However, there was more "leak-through" IL-18R1 expression on CD8 T cells from Il18tg Il18r1$^{\Delta 8}$ mice than those without excess IL-18. Likewise, CD8 T cells from Il18tg Il18r1$^{\Delta 8}$ mice produced IFNγ comparably to Il18r1-sufficient Il18tg controls, but showed less activation as measured by PD-1 expression (**Figure 12C-E**). These findings suggest cell-intrinsic activation, survival, and/or proliferative advantages conferred by IL-18 on CD8 T cells during infection. In addition to more "leak-through" IL-18R1 on CD8 T cells, Il18tg Il18r1$^{\Delta 8}$ mice also showed more CD4 T cell activation (**Figure 12C,E**), suggesting that IL-18 responsive CD8 T cells compete for cytokines/growth factors and/or suppress CD4 T cell responses. This apparent compensation by CD4 T cells may help explain the persistence of serum IFNγ elevation in Il18tg Il18r1$^{\Delta 8}$ mice (**Figure 13D**).

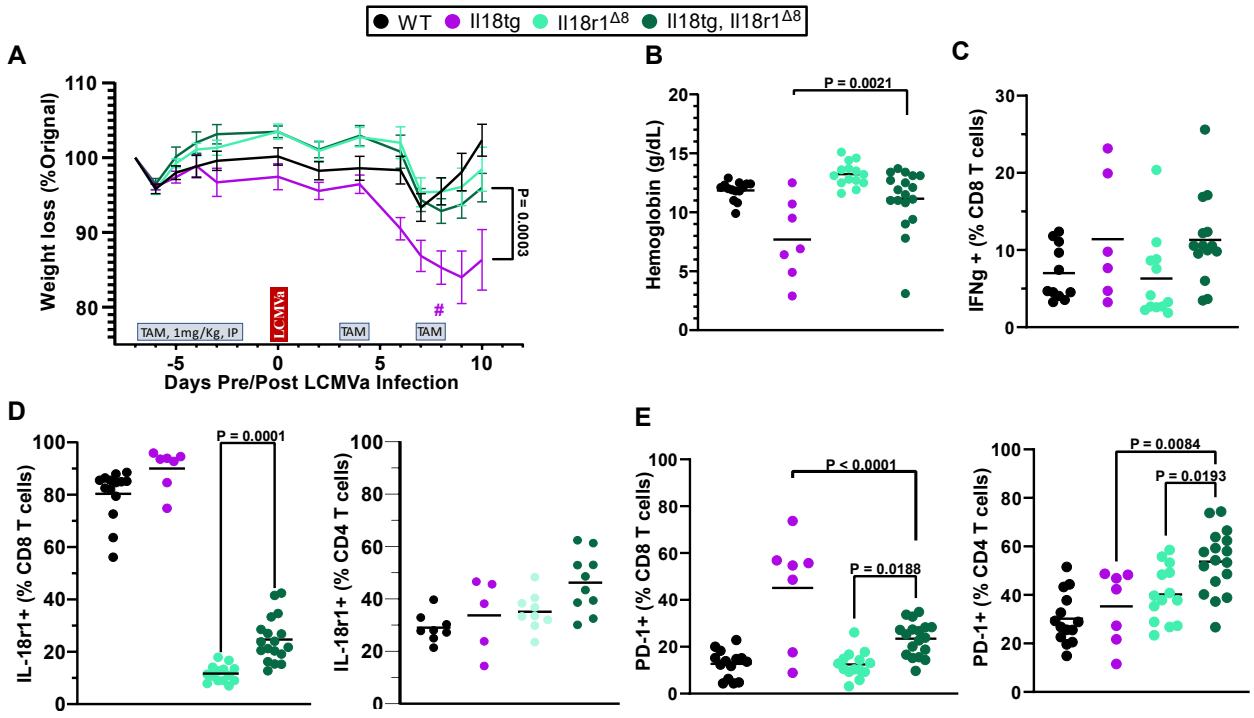


Figure 12: ***Loss of Il18r1 on CD8 T cells reduces LCMV-induced hyperinflammation without CD8 T cell expansion:*** (A) Weight loss throughout tamoxifen treatment and LCMV-Armstrong (2e5 PFU/mouse) infection. Il18tg and Il18tg;Il18r1$^{\Delta 8}$ mice were compared by mixed effects modeling because only 63% of Il18tg mice survived until experimental endpoint. (#, mice succumbed 7-8 days post infection, dpi). No other mice succumbed to infection. (B) Anemia in surviving mice 10 dpi. (C) Percentage of splenic CD8 T cells producing IFNγ by intracellular flow cytometry after 6h GP33 stimulation 10dpi. (D) Percentage of splenic CD8 and CD4 T cells expressing IL-18R1. (E) Percentage of splenic CD8 and CD4 T cells expressing PD-1 in mice directly *ex vivo* 10dpi. A-E show combined data from 3+ experiments. P-values in B and E are calculated using one-way ANOVA with Tukey's post-test of comparisons between Il18tg Il18r1$^{\Delta 8}$ and all other groups. Significance for comparisons between Il18tg and l18tg Il18r1$^{\Delta 8}$ groups are shown if adjusted p-value <0.05.

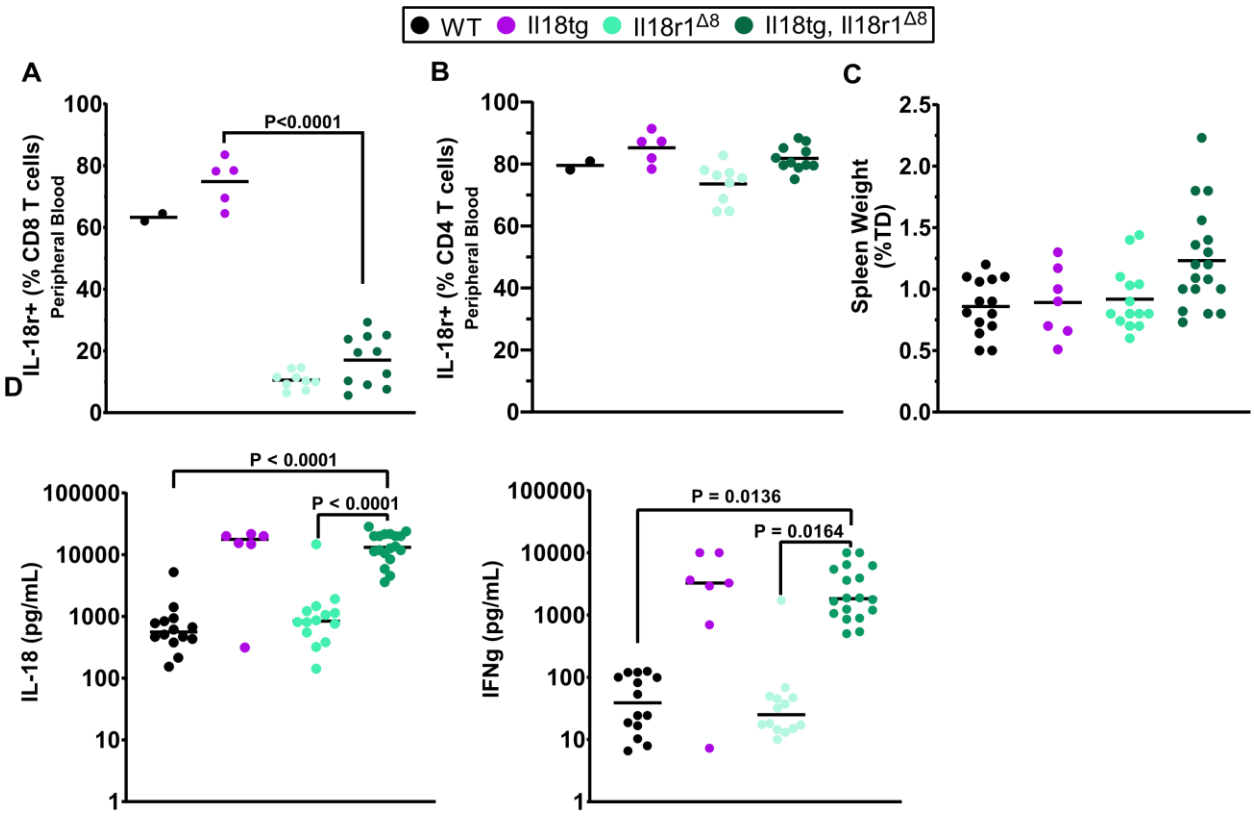


Figure 13: ***Temporal loss of Il18r1 is specific to CD8 T cells but has no effect on splenomegaly and serum IFNγ:*** (A) Percentage of IL-18r1 expressing peripheral blood (A) CD8 and (B) CD4 T cells after 5 days of tamoxifen (prior to infection). (C) Splenomegaly and (D) plasma IL-18 and IFNγ levels 10dpi. All graphs are combined data from 3+ experiments. P values calculated by one-way ANOVA with Tukey multiple comparisons test evaluating all comparisons against Il18tg Il18r1$^{\Delta 8}$.

2.3.2 Minimal effects of IL-18 on CD8 T cells in the absence of T cell receptor stimulation

In both humans and mice with cytotoxic defects such as the lack of *Perforin* cause viral persistence and allows for T cell and macrophage activation[245]. However, the role of cytotoxicity in noninfectious hyperinflammation has not been well characterized. Thus, we investigated the effects of complete cytotoxic impairment on TLR9-driven MAS, an innate, infection-free model. We hypothesized that lack of perforin in these mice could lead to dysregulated CD8 T cell expansion and related immunopathology. Interestingly however, Prf1$^{-/-}$ mice showed comparable

TLR9-MAS severity to wild type (WT), suggesting cytotoxic impairment has a limited role in TLR9-driven hyperinflammation (**Figure 14**).

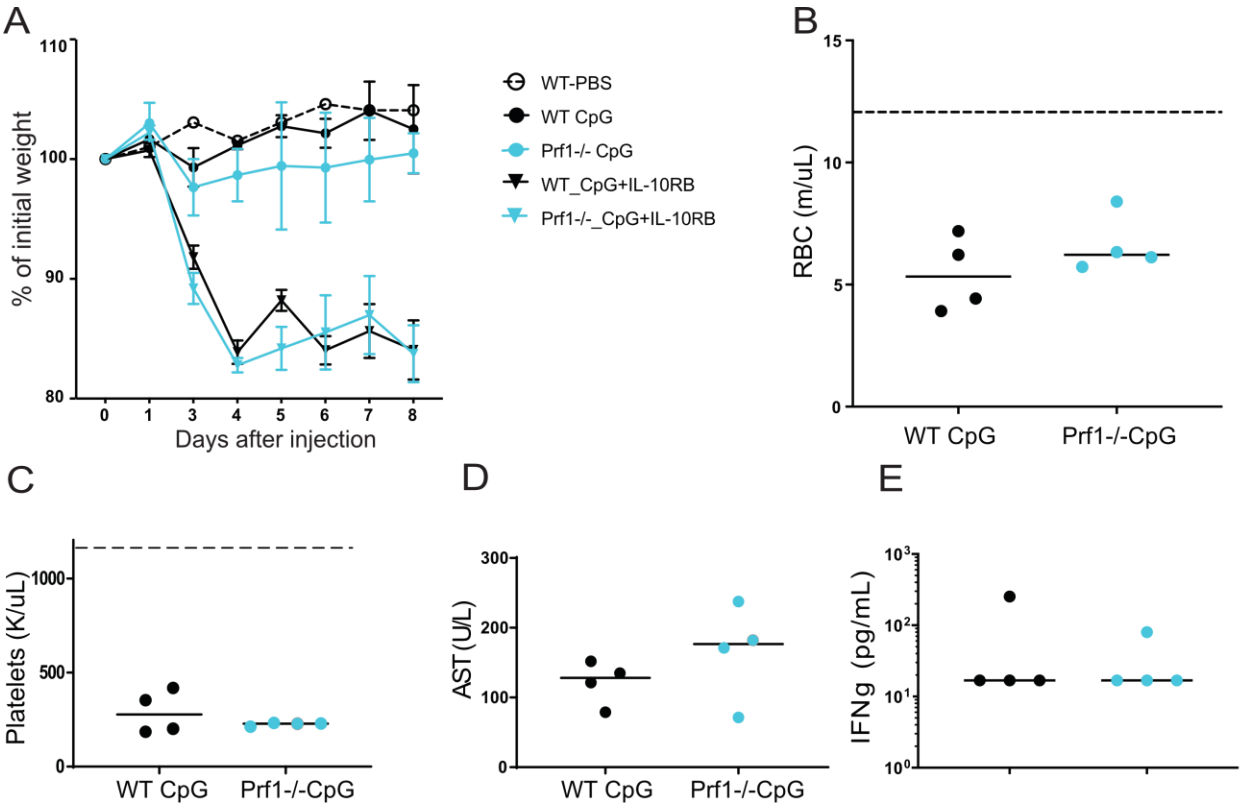


Figure 14: ***TLR9-hyperinflammation is not effected by perforin deficiency****:* WT or Prf1$^{-/-}$ mice were treated with repeated CpG with or without IL-10 receptor blockade and assessed for systemic immunopathology: (A) weight loss, (B) anemia, (C) thrombocytopenia, (D) aspartate transaminase (AST), and serum IFNγ. Dashed lines indicate median values of PBS-treated WT control mice. Data are represen- tative of two independent experiments composed of a minimum of 3 mice per genotype. No statistically significant differences between WT and Prf1$^{-/-}$ mice treated with CpG were detected using unpaired t-test.

The work described in **Section 2.3.1** demonstrated that the development of LCMV-induced MAS in Il18tg mice required *Il18r1* expression. IL-18 is known to amplify disease in the TLR9-driven model of MAS[158], which typically does not require T cells[11,70]. Nevertheless, cytokines like IL-12 can provoke effector responses from activated and memory T cells in the absence of TCR-stimulation[247,248]. Thus, we predicted that mice specifically lacking *Il18r1* in all T cells might be protected from increased inflammation in the setting of systemic TLR9 agonism, which profoundly induces systemic IL-12 production[249]. However, Il18tg mice lacking *Il18r1* on all T cells (Il18tg;Il18r1$^{\Delta T}$) mice were not protected from increased weight loss, splenomegaly, or thrombocytopenia (**Figure 15A-C**). Interestingly, T cell responses to IL-18 made a significant

contribution to increased systemic IFNγ levels, suggesting IL-18 mediated more severe MAS in this model via early effects of IFNγ and/or factors other than IFNγ (**Figure 15D**).

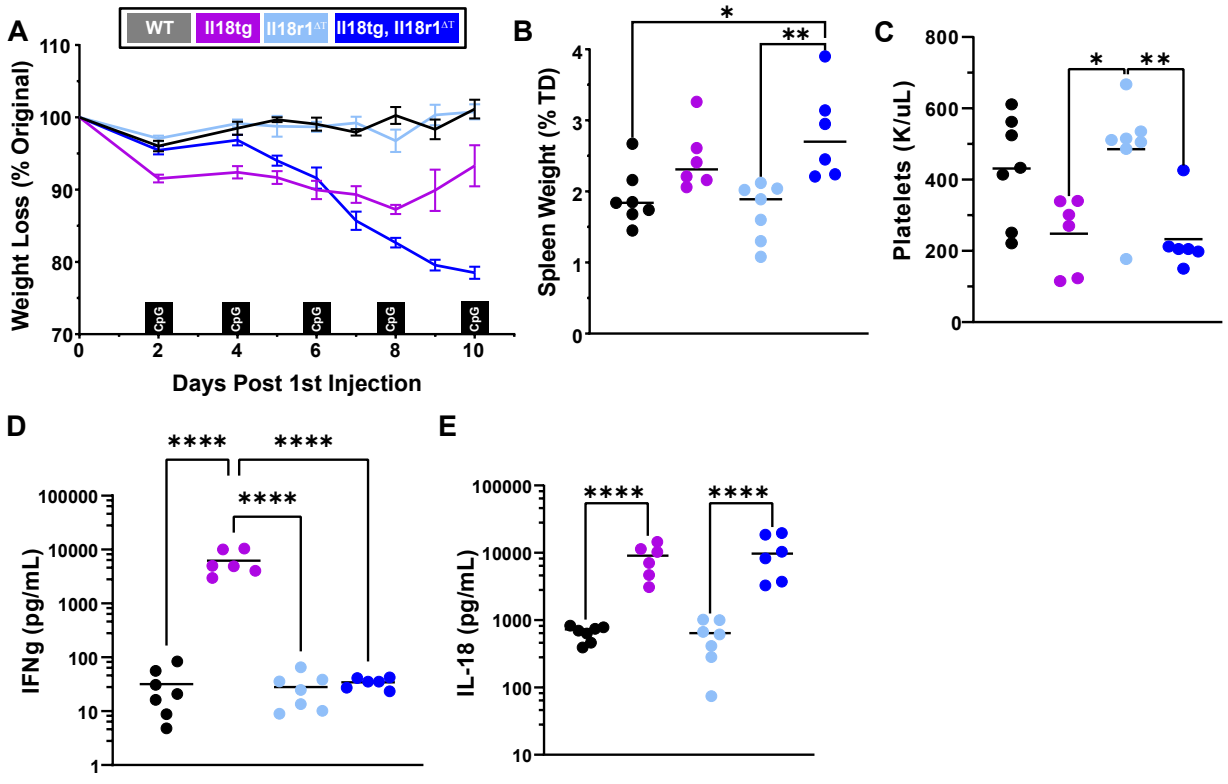


Figure 15: ***Loss of Il18r1 in CD8 T cells does not amelorate TLR9- hyperinflammation****:* (A) Weight loss over the course of experiment (2.5ug/g CpG-ODN every 2-3 days IP). (B) Splenomegaly, (C) Anemia, (D) plasma IFNγ and (E) IL-18 at end of experiment. Data from 2+ experiments, each data point represents a single mouse. P values calculated by one-way ANOVA with Tukey multiple comparisons test evaluating all comparisons between genotypes. **** P<0.0001, ** P<0.01, * P<0.1.

2.4 Discussion

Together the data in the is section provide evidence that in mice harboring single susceptibility factors (perforin deficiency or excess chronic IL-18) CD8 T cells are necessary, respond to IL-18, and become hyperactivated during LCMV induced hyperinflammation. First, we show that both Prf1$^{-/-}$ and Il18tg mice have a hyperinflammatory response to LCMV infection

which is characterized by cachexia, splenomegaly, cytopenia, transaminitis, cytokine storm and hemophagocytosis (**Figure 3**). However, Il18tg mice have viral clearance levels nearly identical to WT mice, suggesting that the hyperinflammation was not due to viremia. During infection, both $Prf1^{-/-}$ and Il18tg show highly elevated levels of the proinflammatory cytokine IFNγ. Neutralization of IFNγ in Il18tg mice ameliorates most LCMV-dependent hyperinflammation and human hyperinflammatory patients have successfully been treated with IFNγ neutralization (emapalumab[240,241]). This suggests that while the susceptibility factors in these mice differ, the mechanism leading to hyperinflammation converges on the generation of systemic excess IFNγ.

We further show that in both mouse models during hyperinflammation, CD8 T cells expand and have a "hyperactivated" phenotype (T_{HYP}) which harbors characteristics of both activation (IL-18r1, CD44, PD-1) and exhaustion (*Tox, CTLA4*). T_{HYP} CD8 T cells also have increased IFNγ production over WT $T_{EFFECTOR}$ CD8 T cells, suggesting they are the pathogenic cell type contributing to the disease in both $Prf1^{-/-}$ and Il18tg mice. Hyperinflammatory susceptibility factors are poised to have effects on CD8 T cells. Cytotoxic defects inherently affect CD8 T cells as they are a major cytotoxic cell type utilizing directed release of granules containing perforin and granzymes. IL-18 exerts its effects in a more dynamic manner on CD8 T cells due to their unique upregulation of Il18r1 upon activation. We also confirmed that in the context of excess IL-18, CD8 T cells require Il18r1 expression to become hyperactivated. It is important to note that cytotoxic defects and IL-18 could also affect NK cells, however only CD8 T cell depletion and not NK cell depletion was able to ameliorate hyperinflammation during LCMV infection in these mice. This suggests that cytotoxic defects, at least perforin deficiency, and excess IL-18 converge on IFNγ over-producing CD8 T cells to drive LCMV-hyperinflammation.

We and other groups have shown that repeated TLR9 stimulation in the context of excess IL-18 lead to the clinical manifestations of hyperinflammation[70,158], we were curious whether cytotoxic defects and CD8 T cells would also play a role. CpG stimulus is a potent inducer of IL-12[250] and studies suggest that IL-12 and IL-18 work together to promote Th1 responses[90,200,210,251]. Our studies indicate that perforin deficiency does not have a large impact on TLR9 driven hyperinflammation as these mice reacted nearly identical to WT mice. We assessed if IL-18 signaling on T cells was leading to hyperinflammation in Il18tg mice and surprisingly loss of Il18r1 on T cells did not ameliorate disease. In this case, IL-12 stimulated B cells upregulate Il18r1[251] and respond to IL-18 by producing IFNγ, leading to hyperinflammatory phenotypes. This highlights how the contextual environment in which excess IL-18 is present leads to differential pathogenic cell types in hyperinflammation.

3.0 Perforin Deficiency and excess IL-18 synergistically promote spontaneous hyperinflammation

Host susceptibility to hyperinflammation is multifactorial in that bi-allelic or/hemizygous defects require triggering events (e.g. infection with EBV, HIV, etc.) in order for hyperinflammation to occur. Furthermore, heterozygous/monoallelic defects in cytotoxicity genes are common in the general population but enriched up to four-fold among patients with existing hyperinflammatory syndromes[68,252,253]. We wondered whether/how excess, IL-18 and heterozygous defects in cytotoxicity might work in concert. In this section we explore the role of combinations of susceptibility factors in an attempt to understand the mechanisms driving hyperinflammation.

Portions of this chapter have been published in one of the following articles:

- Paul Tsoukas, **Emily Rapp**, Lauren Van Der Kraak, Eric S. Weiss, Vinh Dang, Corinne Schneider, Edwin Klein, Jennifer Picarsic, Rosalba Salcedo, C. Andrew Stewart, Scott W. Canna; Interleukin-18 and cytotoxic impairment are independent and synergistic causes of murine virus-induced hyperinflammation. Blood 2020; 136 (19): 2162–2174. doi: https://doi.org/10.1182/blood.2019003846
- **Emily Landy**, Jemy Varghese, Vinh Dang, Andrea Workman, Lawrence Kane, Scott Canna; Complementary HLH Susceptibility Factors Converge on CD8 T-cell Hyperactivation. Blood Adv 2023; bloodadvances.2023010502. doi: https://doi.org/10.1182/bloodadvances.2023010502

3.1 Summary

During acute LCMV infection Il18tg mice have similar kinetics of viral clearance when compared to WT mice. However, mice lacking functional cytotoxicity ($Prf1^{-/-}$), succumb to immunopathology, responses to viremia due to the inability to kill virally infected cells. (**Figure 3**). Monoallelic defects in cytotoxicity genes are common but enriched up to four-fold among patients with SJIA-MAS[68,252]. Using a model of chronic viral infection (LCMV Clone 13) we show that excess chronic, free, IL-18 synergizes with the inability to clear antigen during chronic infection causing Il18tg mice to succumb to hyperinflammation rather than establishing T cell exhaustion. In a more direct correlation to MAS patients due to monoallelic defects in cytotoxicity, we also show that mice harboring both perforin deficiency and transgenic expression of IL-18, so-called dual susceptibility (DS) mice, develop spontaneous, lethal, IFNγ-dependent hyperinflammation. Paralleling hyperinflammation, DS mice displayed massive post-thymic oligoclonal CD8 T cell hyperactivation in their spleens, livers, and bone marrow as early as three weeks of age. These cells had increased proliferation and IFNγ production contrasted with increased expression of receptors and transcription factors associated with exhaustion. Broad-spectrum antibiotics and anti-retroviral agents failed to ameliorate disease. In addition, restricting T cell antigen specificity by breeding to TCR transgenic mice also resulted in spontaneous HLH and hyperactivation., highlighting the preferential expansion of precisely those few T cells that did not express the transgenic TCR. Together these data strongly support the hypothesis that cytotoxic defects and excess IL-18 contribute to, but are not individually sufficient for, hyperinflammation. Our data reinforce the primacy of CD8 T cells and IFNγ in hyperinflammation, identify an effector/exhaustion state arising in spontaneous CD8 T cell activation; and reveal synergy between

CD8 TCR stimulation, signal amplification (e.g., IL-18 and LCMV), and impaired immunoregulation.

3.2 Methods

3.2.1 Mouse lines:

Mice were housed in specific pathogen-free conditions under a protocol approved by the University of Pittsburgh Institutional Animal Care and Use Committee (IACUC). All animals originated from Jackson Laboratories except: Il18tg mice were a gift from Dr. Tomoaki Hoshino (Kurume University)[203], mice bearing the *Il18r1*$^{f/f}$ allele were a gift from G. Trinchieri (NCI)[96], and mice bearing the E8iCre$^{ERT2/GFP}$ allele were a gift from D. Vignali (University of Pittsburgh)[246].

3.2.2 Tissue Harvesting:

Spleens, lymph nodes, bone marrows, and thymuses were processed in RPMI supplemented with 10% FBS (R10) and filtered through 100 μM strainers. Livers were perfused with 10 mL of cold PBS then processed in R10 supplemented with 0.5 units/ml Collagenase D and 20 μg/mL DNase, processed using GentleMACS C Tubes, and purified using a 40% Percoll gradient. Tissues with visible red blood cell contamination underwent ACK lysis. Single cell suspensions were prepared in PBS + 2% Fetal Bovine Serum for flow cytometry or flow sorting.

3.2.3 Flow Cytometry and Analysis:

Peptide and phorbol myristate acetate/ionomycin stimulations were performed as in *Burn et al*.[254] Flow cytometric data were collected on a 5 laser Cytek Aurora Spectral Cytometer and analyzed using FlowJo v10.9.0 (Treestar). T-distributed Stochastic Neighbor Embedding (tSNE) analysis was carried out using the EXCYT2 program[231] on Matlab R2022a. GP^{33-41} tetramer (GP33) was obtained from the National Institutes of Health tetramer core facility.

3.2.4 In vivo neutralization/depletion:

Adult mice were given 500 μg/mouse of either anti-CD4 (GK1.5), anti-CD8 (YTS169.4), or control (LTF-2) antibody i.p. on day one, and 250 μg/mouse every third day, for four consecutive treatments.

3.2.5 In vivo tamoxifen treatment and LCMV infections:

Mice were injected i.p. with 1mg tamoxifen for five consecutive days and rested for two days prior to infection i.p. with $2x10^5$ pfu of LCMV Armstrong. Mice were given 1 mg of tamoxifen on days four and seven after infection and assessed on day nine. For chronic infection, $2x10^6$ pfu of LCMV Clone 13 was injected i.v. retro-orbitally in mice.

3.2.6 Bulk CD8 T cell RNAseq and TCRseq:

mRNA purification and fragmentation, complementary DNA synthesis and target amplification were performed with Smart-Seq v4 ultra-Low input RNA Kit with Nextera XT. Pooled complementary DNA libraries were sequenced on the Illumina NextSeq500, mapped to Mus_musculus_ensembl_v80 reference sequence, gene track Mus_musculus_ensembl_v86, and quantified using CLC Main Workbench software (V22, Qiagen). Reads were converted to TPM and a 0.5 TPM offset added to all transcripts. Batch correction between in-house data and publicly available data was completed using ComBat-seq[255] which uses a negative binomial regression to model and adjust for batch effects. Heatmaps were generated using Morpheus analysis software[256]https://software.broadinstitute.org/morpheus. PCA plots were generated from ComBat adjusted TPM tables using the R package pcaExplorer[257]. Differential gene expression between ComBat adjusted datasets was determined using the BioJupies online analysis suite[258].

T cell receptor (TCR) sequencing was performed on bulk splenic CD8 T cells using the TakaraBio SMARTer Mouse TCR α/β Profiling Kit. Libraries were sequenced, mapped, and quantified as above. TCRseq analysis and visualizations were performed using the R packages Immunarch (V0.6.8)[259], Mixcr[260], VDJtools,[261] and packcircles (Bubble Plots), utilizing the University of Pittsburgh Center for Research Computing.

3.2.7 Single Cell RNAseq and TCRseq:

Our single cell data encompasses data from a total of 10 mice of varying genotypes in duplicate. WT, Il18tg, $Prf1^{-/-}$, DS^{HET} and DS^{KO} splenocytes were first enriched for CD8 T cells using magnetic separation (BioLegend Cat: 480008) then individual mice were stained as in

Section 3.2.3. Cells from individual mice were antibody barcoded with hashtags (TotalSeq C0301-C0310) before FACS sorting of live cells. All cells were pooled into a single cell suspension of 12.5% WT, 12.5% Prf1$^{-/-}$, 25% Il18tg, 25% DS^{HET} and 25% DS^{KO}. The pooled suspension of +90% viable (AOPI) cells was provided to the University of Pittsburgh Single Cell Core and was loaded into a 10x Genomics Chromium system using Chromium Next GEM Single Cell 5′ v1.1 to generate two GEM wells of which 5′ GEX libraries, TCR libraries and Cell Hashtag libraries were prepared. Libraries were sequenced by the UPMC Genome Center on a NovaSeq 6000 with the S4 200 Cycle kit. Cellranger-7.0.1 program was used to perform demultiplexing, alignment, filtering, Barcode counting, and UMI counting of raw BCL sample files using The University of Pittsburgh's Center for Research Computing. The resulting GEX processed files were then analyzed using Cellenics (www.biomage.net) software for differential gene expression and cell type identification. TCR analysis was performed using Loupe Browser 6.5.0 from 10X Genomics.

3.2.8 Data Accessibility

The Bulk RNAseq and TCRseq data discussed in this section have been published and have been deposited in NCBI's Gene Expression Omnibus[262]. They are accessible through GEO Series accession number GSE226126 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE226126). scRNAseq and scTCRseq data can be acquired by contacting Scott Canna at cannas@chop.edu.

3.3 Results

3.3.1 Chronic viral infection is lethal in Il18tg mice but not in Il18bp$^{-/-}$ mice

Although, defects in cytotoxicity lead to excessive antigen exposure, not all hyperinflammatory patients have genetic defects in cytotoxicity. Some patients with difficulty controlling infections like Herpes viruses (EBV[263], CMV[264], HHV8[265]) and HIV [266]have been associated with secondary HLH/MAS manifestations. Under these circumstances, chronic antigen exposure meets a threshold, obviating the need for defects in cytotoxicity, in order to trigger hyperinflammation provided there are other susceptibility factors present. We wondered if excess IL-18 and chronic antigen exposure synergized leading to severe hyperinflammation. We subjected WT, Il18tg, and IL-18 binding protein deficient (IL-18BP$^{-/-}$) mice to LCMV clone 13 infection and assessed for hyperinflammation. We also wondered how much IL-18 was necessary to elicit a hyperinflammatory response in this situation. Il18bp$^{-/-}$ mice do not inherently produce excess IL-18 but rather due to their complete lack of IL-18 binding protein, all IL-18 produced can freely bind *Il18r1*. Perhaps unsurprisingly based on hyperinflammation in Il18tg mice during LCMV Armstrong, upon LCMV Clone 13 infection these mice succumbed to hyperinflammation. While these data are preliminary, this suggested to us that chronic antigen stimulation in combination with excess IL-18 was additive, if not synergistic, to hyperinflammation. Interestingly, IL-18BP$^{-/-}$ mice did *not* succumb to hyperinflammation or viremia. These mice responded similarly to WT mice in terms of weight loss, viral clearance, and systemic inflammation by 29 dpi (**Figure 16A-D**). In IL-18BP $^{-/-}$ CD8 T cells there was more functional exhaustion marked by an increased proportion of PD-1^{+}Tim-3^{+} cells and fewer IFNγ-producing CD8 T cells than in WT mice (**Figure 16E-F**). We speculate there is likely little sustainable IL-18

driven hyperactivation in IL-18BP $^{-/-}$ CD8 T cells due to minimal IL-18 production and the relatively short half-life (~16hr[156]) of IL-18 in the absence of IL-18BP. Thus this data highlights that while chronic antigen presentation and IL-18 could cooperate to create hyperinflammation, a heightened level of IL-18 is required for the combination of these susceptibility factors to result in hyperinflammation.

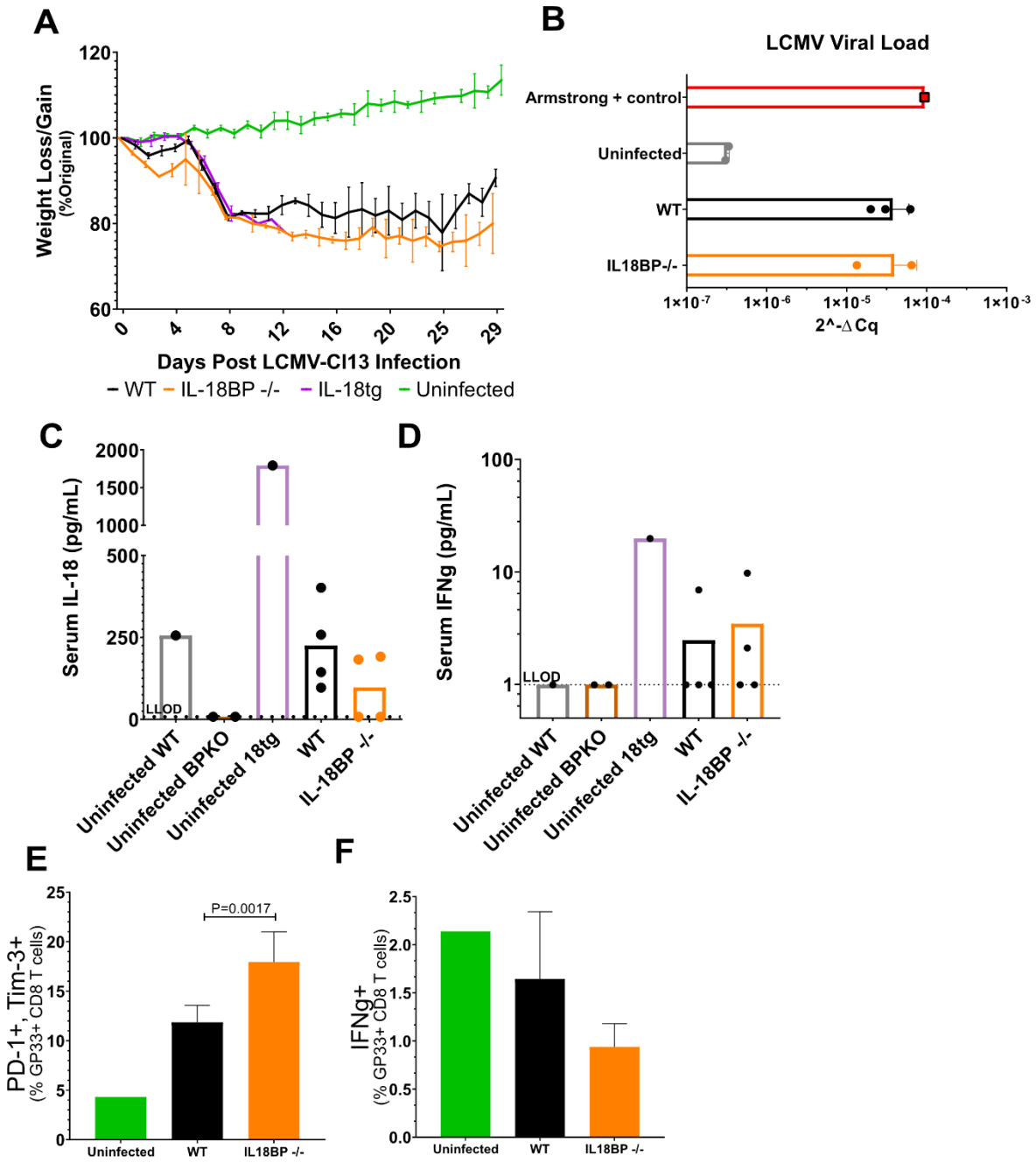


Figure 16: ***Chronic viral infection leads to lethal hyperinflammation in Il18tg but not IL-18BP$^{-/-}$ mice:*** Mice were infected with 2x10^6 pfu LCMV Clone 13 i.v. and monitored for (A) weight loss over the course of infection. 29 days post infection mice were harvested and assesed for (B) splenic viral load, serum levels of (C) IL-18 and (D) IFNγ. LCMV-specific (GP33+) CD8 T cells were assesed for (E) inhibitory receptor expression and (F) after 3hr stimulation with PMA/ionomycin we assesed for IFNγ production. Statistical significance was determined through one-way ANOVA with Tukey post-test.

3.3.2 Dual susceptibility mice develop spontaneous, lethal hyperinflammation

Monoallelic defects in cytotoxicity genes are common in the general population, but are enriched up to four-fold in patients who harbor chronically elevated IL-18 and possibly in other hyperinflammatory scenarios[68,252,253,267]. Based on the data discussed in **Section 3.3.1,** we hypothesized that under conditions of chronically elevated IL-18, cytotoxic impairment would demonstrate at least additive effects on LCMV-induced immunopathology. To test this, we generated $Prf1^{+/-}$;Il18tg and $Prf1^{-/-}$;Il18tg (dual susceptibility; DS) mice with the intention of assessing their responses to LCMV. We were unable to ethically infect DS mice because they spontaneously developed lethal immunopathology characterized by cachexia, splenomegaly, hepatitis, and cytokinemia indicative of hyperinflammation. However, unlike in LCMV-hyperinflammation, we provided no exogenous trigger initiating hyperinflammation. DS succumb to lethal hyperinflammation as early as seven weeks of age (**Figure 17A**). Compared with $Prf1^{-/-}$ and Il18tg controls, 4-week-old $Prf1^{-/-}$;Il18tg (DS^{KO}) mice were found to have severe splenic and hepatic extramedullary hematopoiesis, obscuring all normal splenic architecture (data not shown). Liver histology revealed marked hepatic sinusoidal distension with modest hepatitis (**Figure 17B**). Histologic analyses of brain, lung, skin, bone marrow, heart, and small and large intestine showed no remarkable differences (data not shown). Notably, even $Prf1^{+/-}$;Il18tg (DS^{HET}) mice developed an intermediate hyperinflammatory phenotype across all parameters.

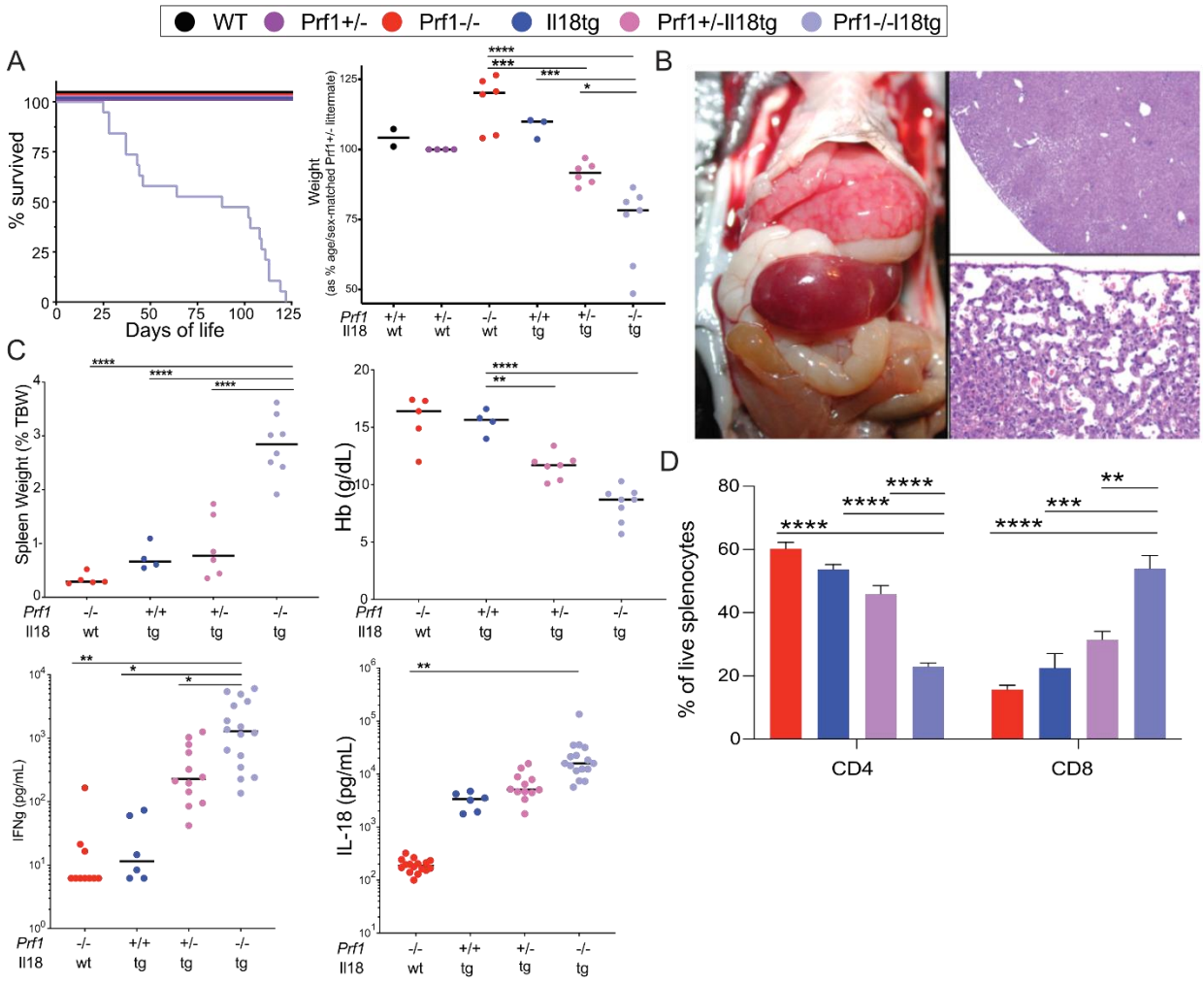


Figure 17: ***Perforin Deficiency and Chronic excess IL-18 together lead to lethal spontaneous hyperinflammation with excess CD8 T cells:*** Mice of the indicated genotypes were bred and assessed for (A) survival and body weight. $Prf1^{-/-}$, $Prf1^{+/-}$, DS^{HET}, and DS^{KO} littermates were compared with littermate sex-matched controls. WT and Il18tg mice were littermates, and their weights were compared with age- and sex-matched $Prf^{+/-}$ controls. (B) Gross morphology and liver histology in a 5-week-old DS^{KO} mouse. (C) Spleen weight, Hb, serum IFNγ, and IL-18. (D) percentage of splenic T cells in the indicated genotypes. *Adjusted P < .05, **P < .01, ***P < .001, ***P < .0001 by 1-way ANOVA with Tukey post-test. Significance is only shown for comparisons where adjusted P < .05. Error bars represent SEM. Results are a composite of at least 2 independent experiments.

3.3.3 Prominent CD8 T cell expansion in DS mice establishes a "hyperactivated" immunophenotype

We performed a flow cytometric survey of multiple organs in DS mice, focusing on lymphocytes as potential sources of pathogenic cytokines like IFNγ[96]. In patients with

hyperinflammation, there is an increased proportion of activated CD8 T cells[92-94]. Similarly, we found that DS^{KO} mice had expanded CD8 T cells in reticuloendothelial organs (bone marrow, liver, and spleen) **(Figure 19A-B**). Those CD8 T cells showed dramatically increased PD-1 and IL-18R1 expression, indicative of activation. CD8 T cell numbers and activation states in mesenteric and inguinal lymph nodes and thymus were comparable between DS and non-DS (particularly Il18tg control) mice (**Figure 18A-B**).

Flow cytometry analysis using unsupervised t-stochastic neighbor embedding (tSNE) clustering of splenic T cells identified two clusters of CD8 T cells specific to DS mice. Both clusters showed high expression of IL-18R1 and PD-1 and low expression of CD62L. Combined with co-expression of Tim-3, Lag-3, and PD-1 in the CD8 T cells in these clusters (**Figure 18C-D**), these data suggest a unique CD8 T cell state exhibiting both effector and exhaustion phenotypes[99,100,268-270] that is preferentially expanded in DS^{KO} mice. Of these, one cluster was clearly activated, but distinguished by low CD44 expression and was more prominent in DS^{KO} than DS^{HET} mice, making up nearly 60% of DS^{KO} splenic CD8 T cells (**Figure 19C**). This CD44/CD62L double negative CD8 T cell population may be comparable to terminally differentiated CD8 T cells induced *in vitro* with repeated TCR stimulation and intact PI3K[271]. The combination of expression of multiple inhibitory receptors and retained effector function (possibly including CD44 downregulation) suggestive of a state of CD8 T cell hyperactivation (CD8 T_{HYP}).

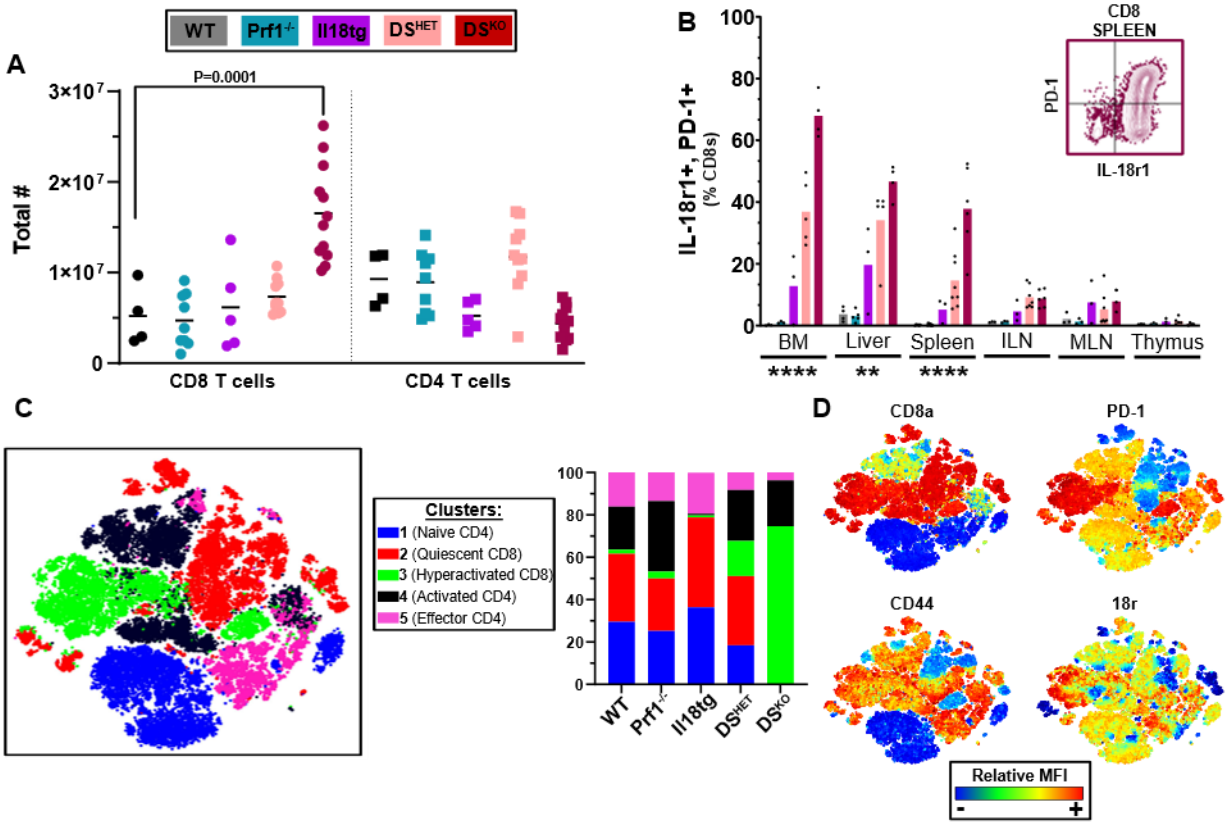


Figure 18: ***Dual Susceptability mice have excess CD8 T cells with a hyperactiavated immunophenotype:*** (A) Total number of splenic CD8 and CD4 T cells. (B) Percentage of CD8 T cells homeostatically expressing activation markers IL-18r1 and PD-1 in various organs. Graph shows combined data from 3+ experiments, each data point represents a mouse. P value based on one-way ANOVA with Tukey post test of comparisons between Il18tg to DSKO. ****P<0.0001, **<0.001, *P<0.05. (C) Unbiased K means clustering t-distributed stochastic neighbor embedding (tSNE) projection of splenic T cells (7649 cells/mouse) from 3 mice of each genotype with representation of each cluster within each genotype. (D) heatmap of relative geometric mean fluorescent intensity

(MFI) of selected markers.

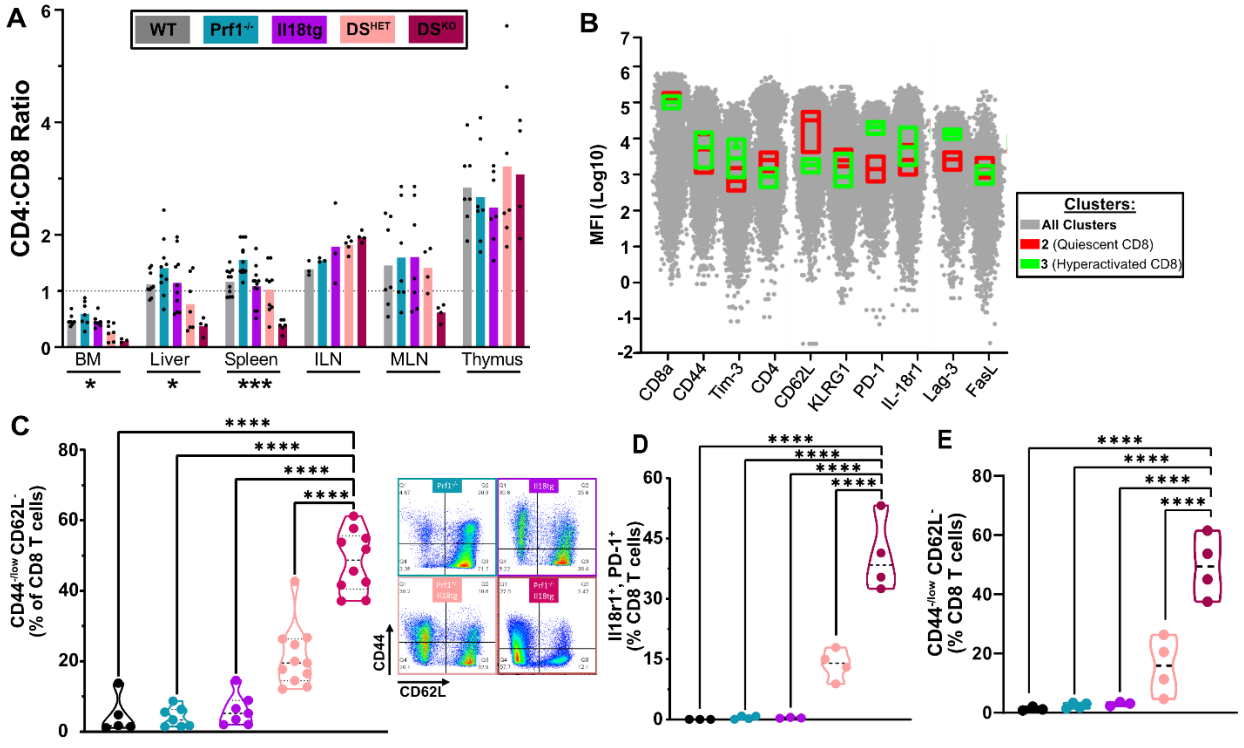


Figure 19: ***Dual susceptibility leads to CD8 T cell specific expansion and activation in reticuloendothelial organs:*** (A) CD4:CD8 ratio in various organs under homeostatic conditions. Comparison of Il18tg vs DSKO is depicted below organ. (B) Box and whisker plots (median and quartiles shown) representing MFI of various markers based on tSNE clusters depicted in Figure 3c. (C) Percentage of CD44$^{-/Low}$, CD62L^{-} CD8 T cells and representative flow plots in each genotype. (D) Percentage of *Il18r1*$^{+}$, PD-1^{+} and (E) CD44$^{-/LOW}$, CD62L^{-} splenic CD8 T cells from mice ≤ 3wks old. Data of at least three experiments, each data point represents a single mouse. P values calculated by one-way ANOVA with Tukey multiple comparisons test evaluating all comparisons against DSKO within the same organ. **** P<0.0001, *** P<0.001, ** P<0.01, * P<0.1

3.3.4 Dual susceptibility CD4 T cells are minimally affected in reticuloendothelial organs

Though far less numerous, CD4 T cells in DS mice showed slightly greater PD-1 and IL-18R1 expression (**Figure 20**). As previously demonstrated[96], CD8 (but not CD4) T cells from DS mice produced more IFNγ after *ex vivo* PMA/ionomycin stimulation (**Figure 20B, C**). Because Foxp3^{+} regulatory CD4 T cells (Treg) are reduced in the Prf1$^{-/-}$-LCMV model[272], we hypothesized

that Treg deficiency might contribute to immunopathology in DS mice but observed only a small decrease that was largely restricted to the bone marrow (**Figure 20D**).

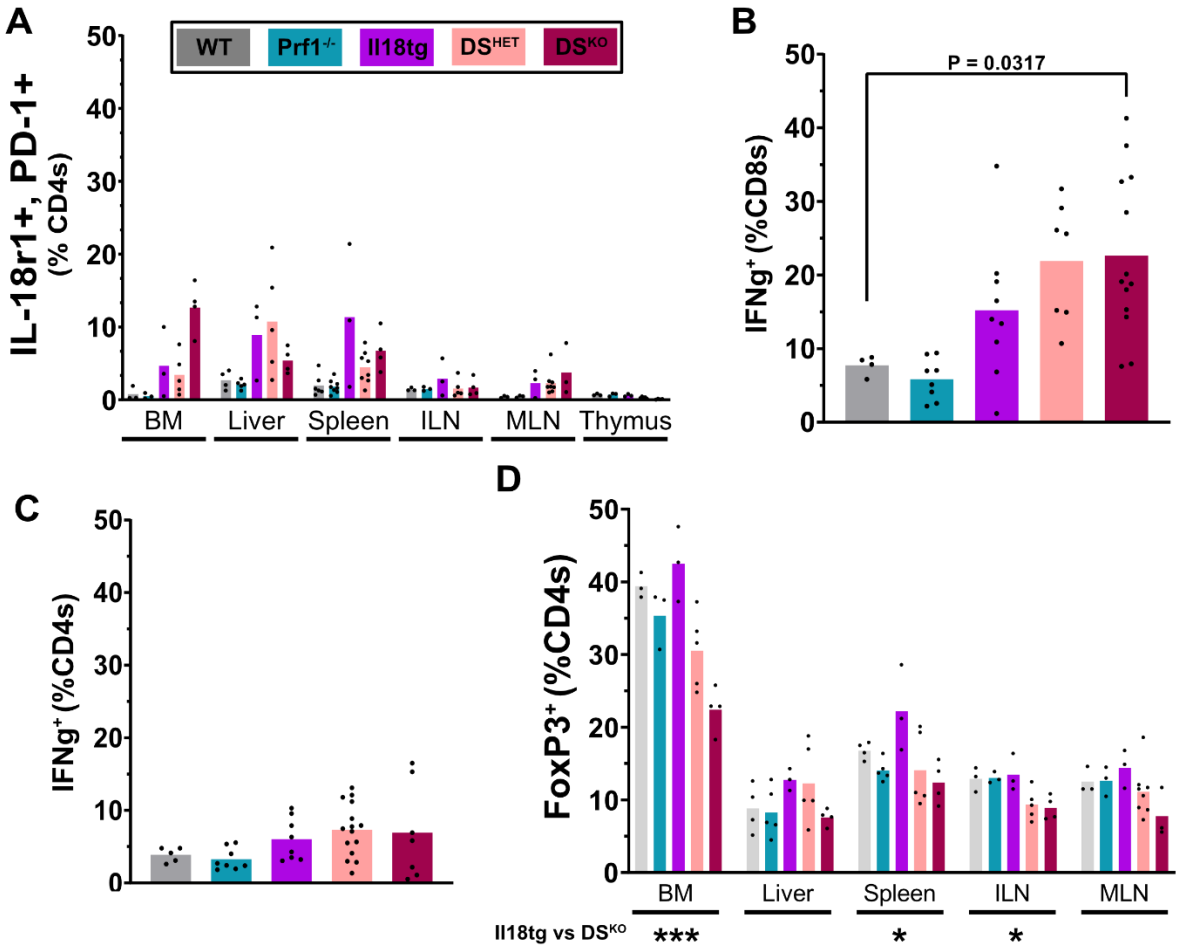


Figure 20: ***Dual Susceptability CD4 T cells are minimally effected:*** (A) Percent of activated (IL-18r1+, PD-1+) CD4 T cells in various organs under homeostatic conditions. (B) Percent of IFNγ producing CD8 and (C) CD4 splenic T cells after 4hr PMA and Ionomycin stimulation. (D) Percentage of regulatory (FoxP3$^+$) CD4 T cells and (E) NK cells (NK1.1+, TCRβ-) within various organs under homeostatic conditions. Data ofat least two experiments: each data point represents a single mouse. P values calculated by one-way ANOVA with Tukey multiple comparisons test evaluating all comparisons against DS^{KO} within the same organ. *** P<0.001, **P<0.01, *P<0.1

3.3.5 CD8 T cells and IFNγ are pathogenic in dual susceptibility mice

Despite the profound CD8 T cell expansion/activation, minimal CD4 T cell activation, and near absence of NK1.1+ innate lymphoid cells, antibody-mediated depletion of either T cell subset

was not sufficient to rescue hyperinflammation in DS mice. CD8 depletion resulted no effect in hyperinflammatory phenotype yet substantial increase in the activation state and IFNγ production of CD4 T cells (**Figure 21**).

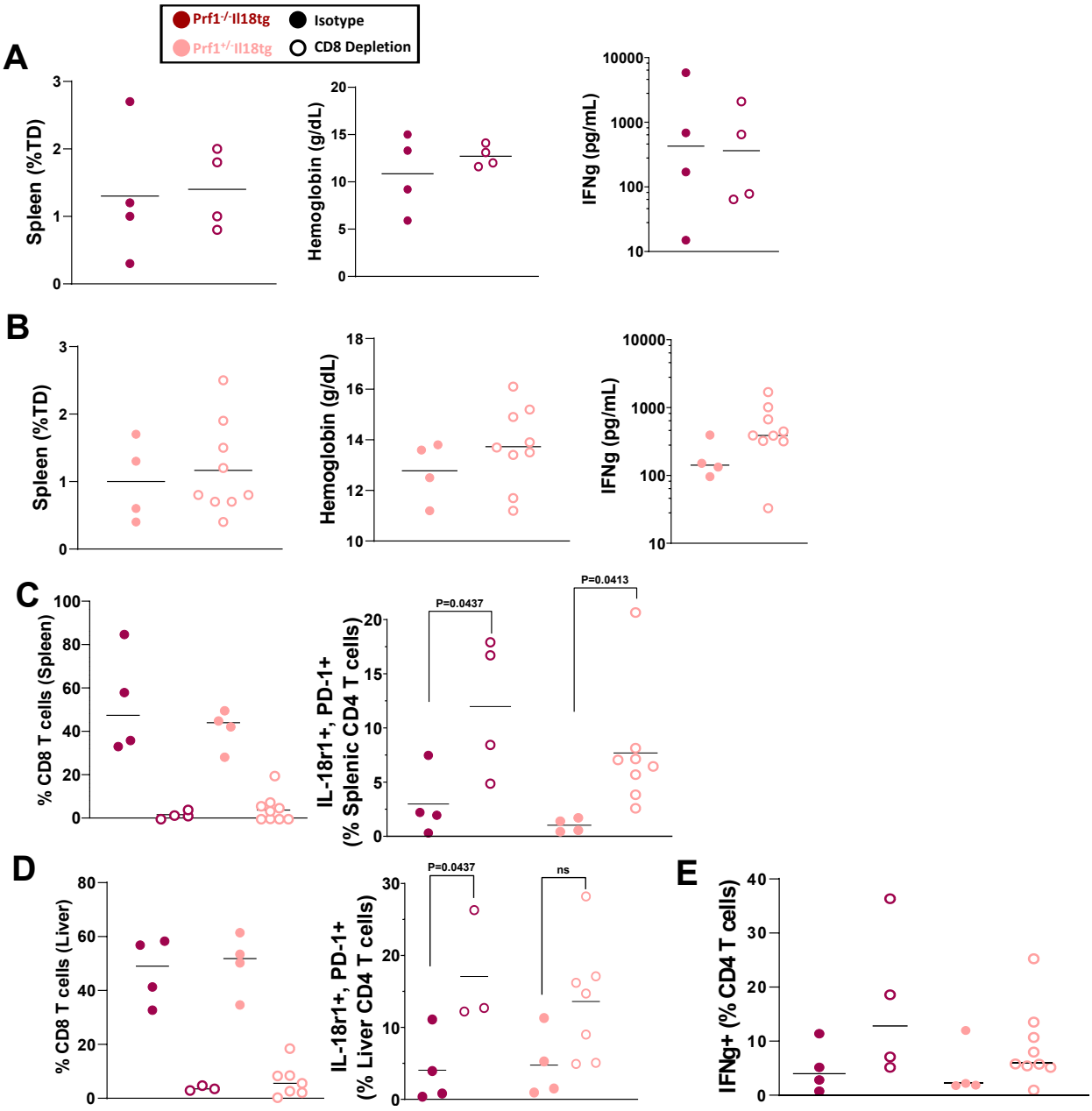


Figure 21: ***CD8 depletion does not ameliorate disease in Dual Susceptability mice:*** Splenomegaly, anemia, and serum IFNγ in (A) DS^{KO} and (B) DS^{HET} mice after CD8 T cell depletion. Percent CD8 T cells and percentage of activated (IL-18r1+, PD-1+) in (C) Spleen and (D) Liver (E) IFNγ producing CD4 T cells after CD8 Depletion. P values calculated for each genotype separately using unpaired t test between treatment groups.

Given the surprising lack of effect of CD8 T cell depletion, and increase in CD4 activation, we wondered whether CD4 T cells were responsible for driving hyperinflammation Depletion of CD4 T cells affected neither hyperinflammatory features nor the extent of CD8 T cell activation (**Figure 22**). These data together suggest temporal depletion of CD8 T cells is insufficient to blunt the chronic effects of IFNγ overproduction by lymphocytes while also highlighting the compensatory capacity of CD4 T cell activation in DS mice.

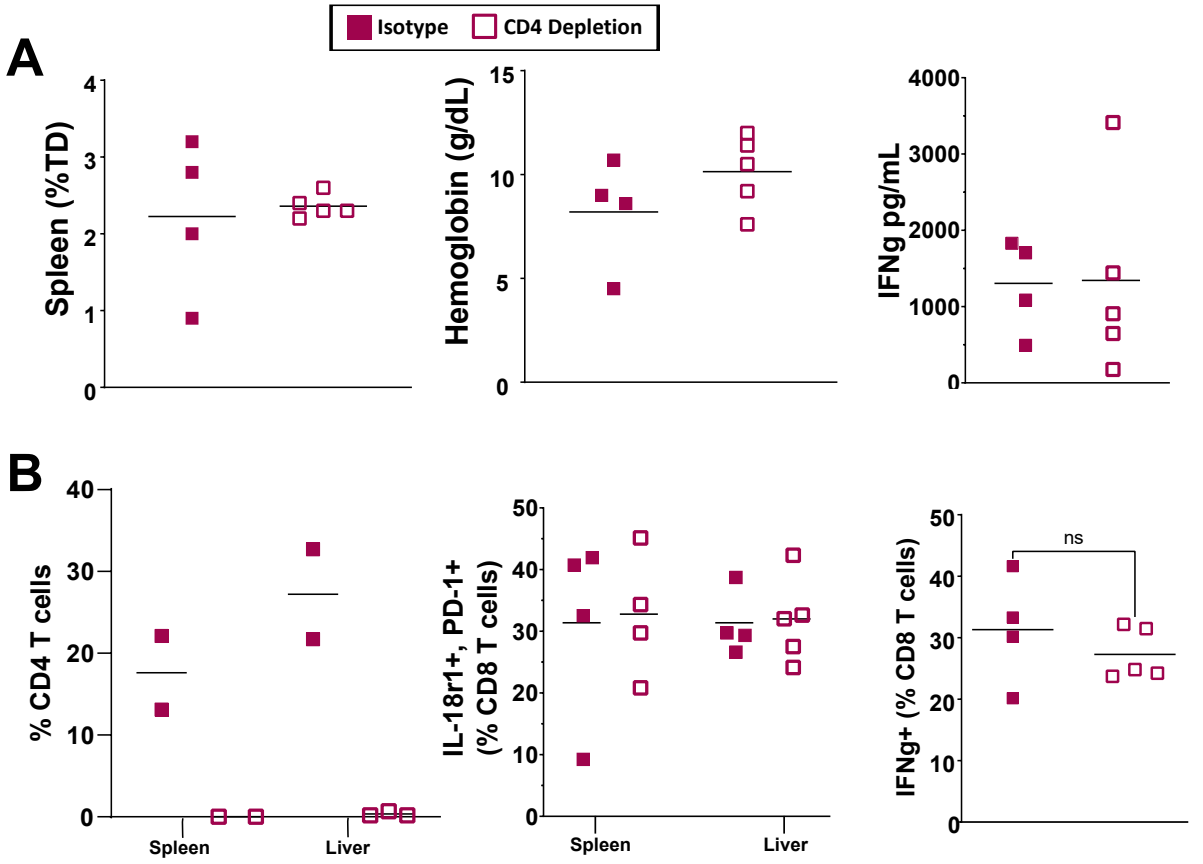


Figure 22: ***CD4 Depletion does not ameliorate disease in Dual Susceptability mice :*** (A) DSKO splenomegaly, anemia, serum IFNγ levels, (B) percent of CD4 T cells (spleen and liver), and percentage of activated (IL-18r1+, PD-1+) CD8 T cells, and IFNγ producing CD8 T cells after CD4 depletion. P values calculated for each genotype separately using unpaired t test between treatment groups.

It is well known that IL-18 can augment cells other than T cells, including NK cells and sometimes B- and myeloid cells under varying conditions. Like in SJIA patients[273], NK cells are

less abundant in mice with excess IL-18, including DS mice (**Figure 23A**). Excess IL-18 has been reported to induce NK cell defects and death however the mechanism is not fully understood[274-277]. In the context of hyperinflammation, NK cell dysfunction and cell death may allow for the manifestation of other underlying diseases that can lead to hyperinflammation such as EBV or HIV. Additionally, Il18tg and DS mice show a near absence of CD19+ B cells (**Figure 23B, C**). While IL-18 has been suggested to induce autoantibody production by B cells, the lack of B cells and lack of autoimmunity in Il18tg or DS mice suggest that hyperinflammation is *not* an autoimmune disease and highlights the role that the timing and chronicity of IL-18 exposure to B cells can have varying effects on their function and longevity. Together this data suggests that the major pathogenic cell type in DS hyperinflammation is CD8 T cells, but that CD4 T cells have the capacity to become hyperactivated in the absence of CD8 T cells.

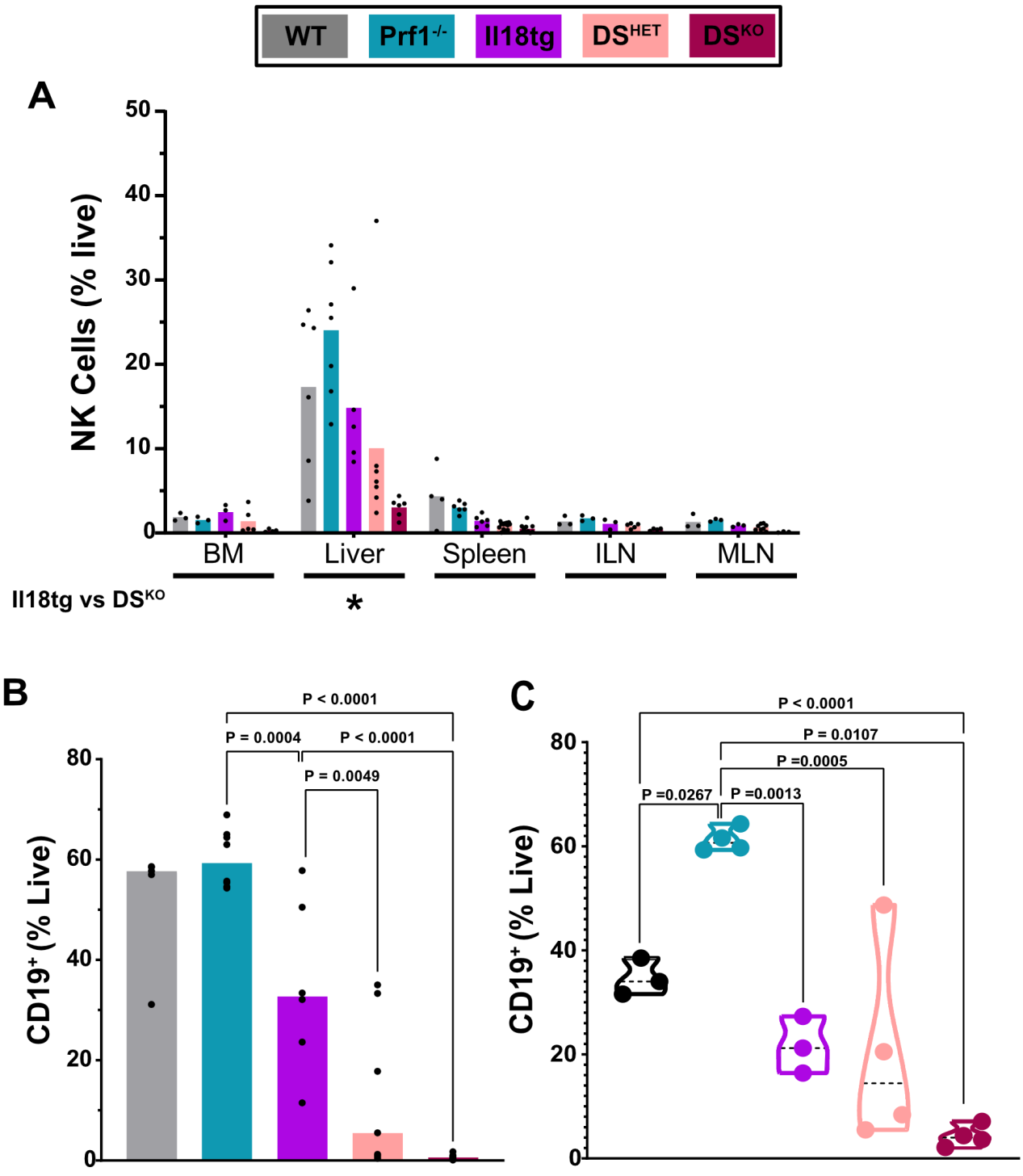


Figure 23: ***Dual Susceptability mice have reduced levels of NK and B cells*****:** (A) NK cells and (B) splenic B cells (CD19+) in *adult* DS mice under homeostatic conditions. (C) Splenic B cells (CD19+) in DS *pups* ≤ 3 weeks old. (A) P values calculated by one-way ANOVA with Tukey multiple comparisons test evaluating all comparisons against DS^{KO} or (B-C) within the same organ or between all genotype pairs . *** P<0.001, **P<0.01, *P<0.1

3.3.6 DS CD8 T cells have a distinct hyperactivated transcriptional signature

We determined that CD8 T cells were the major pathogenic cell type in hyperinflammation and based on the typical immune profiling through flow cytometric methods, it was obvious that these T_{HYP} CD8 T cells were not of a canonical nature requiring a more in-depth evaluation. Therefore, we next performed bulk RNAseq of both $CD44^{+}$ and the unique $CD44^{lo/-}$/$CD62L^{-}$ CD8 T cell populations. We found these populations were transcriptionally similar within genotypes (although Cd44 was downregulated), but that DS^{KO} CD8 T cells were transcriptionally distinct from control genotypes (**Figure 24**). $CD44^{+}$ CD8 T cells from $Prf1^{-/-}$ mice had a central-memory phenotype (CD8 T_{cm}) typical of an unstimulated mouse, with high expression of progenitor/memory markers *Tcf7* and *Il7r* and high expression of *Sell* (encoding CD62L, **Figure 25-B**). Cytokine receptors *Il12rb1, Il15r, Il2rb,* and *Il2rg* were increased in DS CD8 T cells, consistent with an effector phenotype (**Supplemental File 2**). CD8 T cells from DS mice showed evidence of terminal differentiation, with high expression of effector transcription factors like *Eomes*, *Tgfb1, Ifng*, *Gzmk*, and *Il10* and low expression of *Il7r* and *Tcf7* (TCF-1)[278-280]. Despite the absence of foreign antigen, the most differentially upregulated transcripts in DS CD8 T cells were exhaustion-associated inhibitory receptors like *Pdcd1* (PD-1), *Havcr2* (Tim-3), *Ctla4, Entpd1* (CD39), and *Tigit* (**Figure 25-B**). These cells also expressed *Tox*, a key transcription factor driving the exhaustion program[239,281,282].

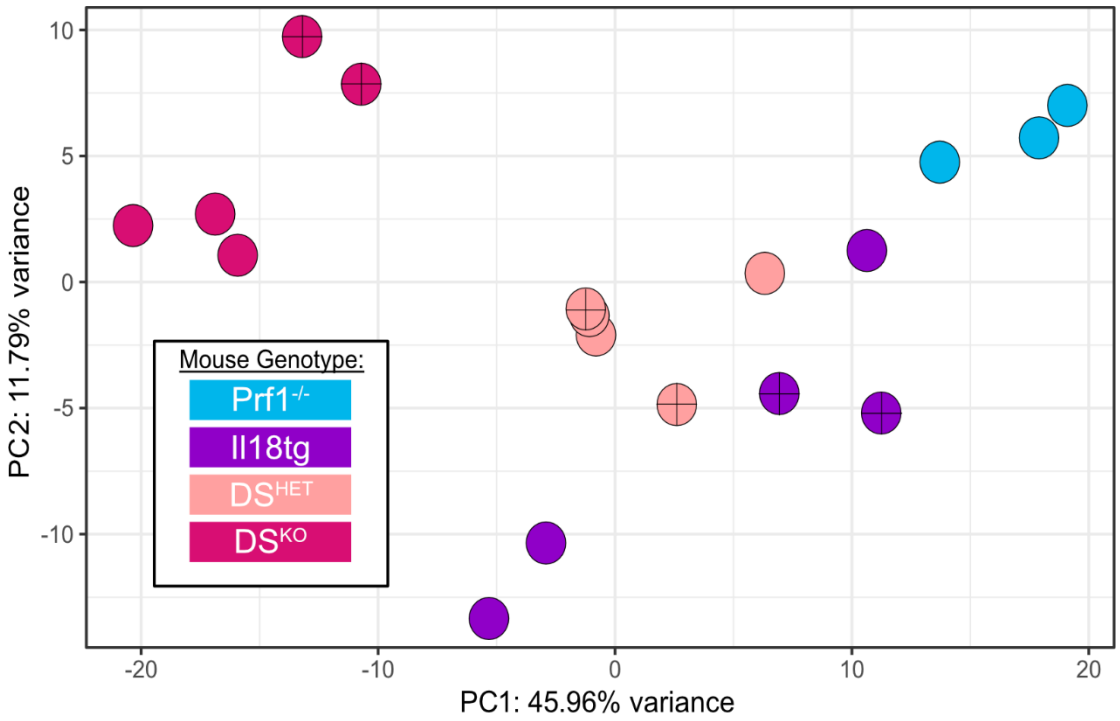


Figure 24: ***DSKO CD8 T cells are transcriptionally unique:*** Principal Component Analysis (PCA) from bulk CD8 T cell RNA-seq from at least 3 adult mice from each genotype under homeostatic conditions. Crosses within data point indicate $CD44^{Lo}$ $CD62L^{-}$ CD8 T cells, otherwise $CD44^{+}$ CD8 T cells.

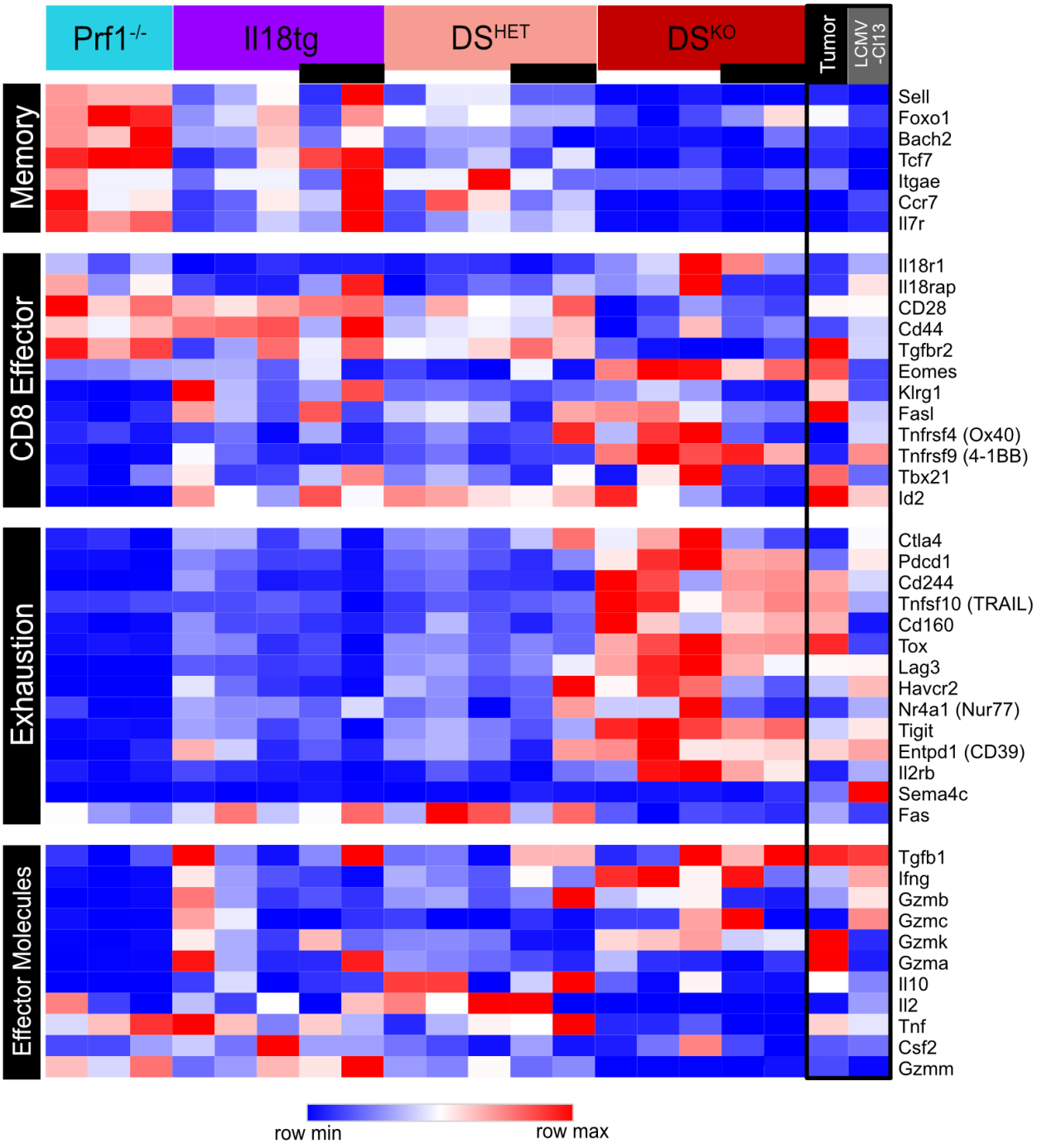


Figure 25: ***DSKO CD8 T cells have a similar yet distinct transcriptional profile when compared to terminally exhausted CD8 T cells:*** Heatmap comparison of selected genes of homeostatic CD8 T cells and terminally-exhausted CD8 T cells from tumor and chronic infection (LCMV-Cl13)65. Black bars under genotype indicate $CD44^{Lo}CD62L^{-}$ CD8 T cells, otherwise $CD44^{+}$ CD8 T cells. Rows are Z-score normalized**.**

Given the transcriptional overlap with CD8 T cell exhaustion, we analyzed the datasets discussed above alongside published transcriptomes of terminally exhausted (T_{EX}, PD-1^{+}Tim-3^{+}) and precursor exhausted (T_{PEX}, PD-1^{Int}Tim-3^{-}) CD8 T cells[283]. CD8 T cells from DS mice clustered separately from both populations, but the trajectory of change from CD8 T_{PEX} to T_{EX} paralleled that of CD8 T cells from Prf$1^{-/-}$ (mostly effector/central memory) to DS^{KO} (**Figure 26**). CD8 T cells Differentially expressed genes between DS^{KO} and T_{EX} included adhesion/chemotaxis (e.g. *Nek7, Tgfbr2, Ccr5, PECAM1)* and T cell hyperactivation (e.g. *Il18r1, Sh2d1a, Ikzf2*) genes **(Figure 27**, **Supplemental File 2**) suggesting that hyperactivated CD8 T cells may be able to circulate throughout the body. Thus, transcriptomes of CD8 T cells from DS mice (both $CD44^{+}$ and $CD44^{lo/-}$/$CD62L^{-}$ populations) include features of both terminal effector differentiation and exhaustion.

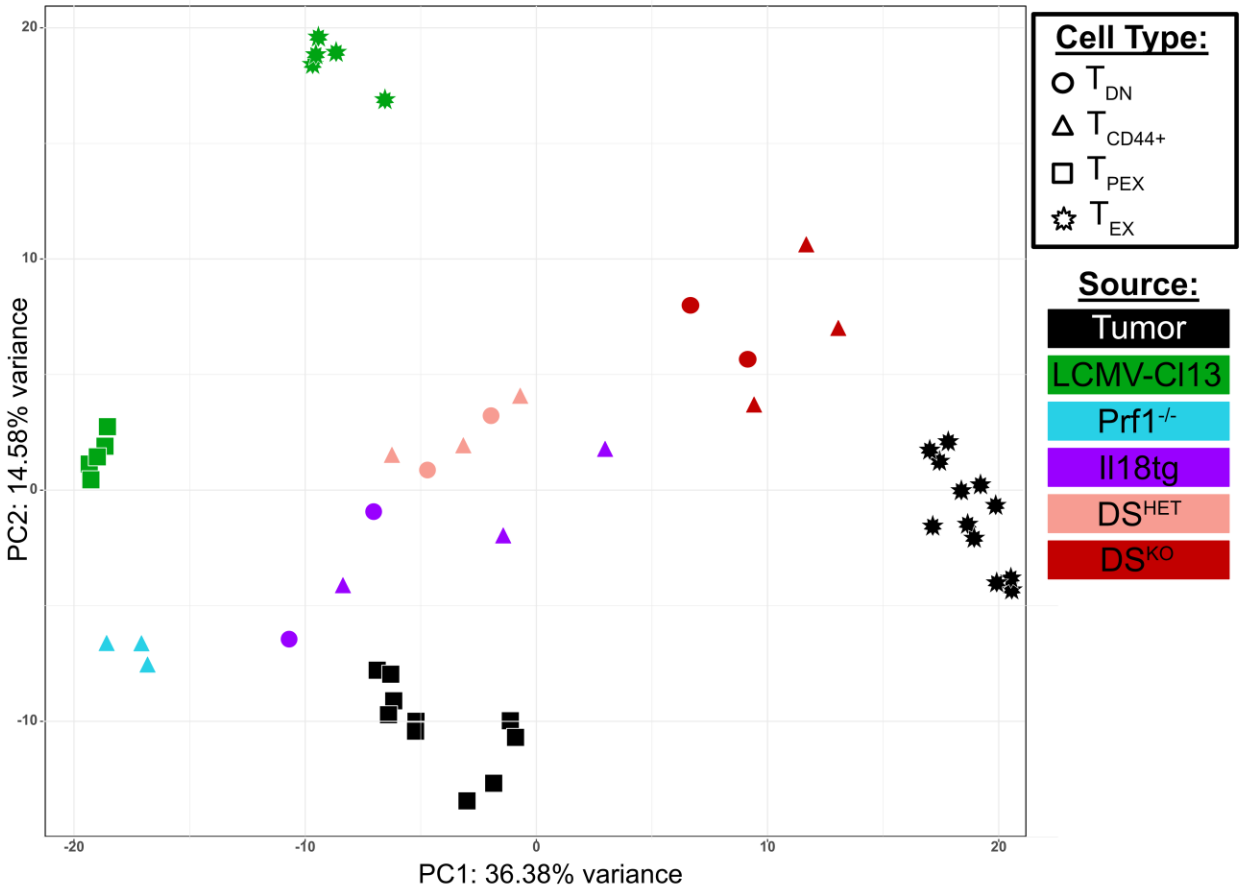


Figure 26: ***Comparison of DS hyperactivated CD8 T cells to terminally exhausted CD8 T cells.*** PCA analysis of exhaustion precoursor (T_{PEX}), terminaly exhausted (T_{EX}), activated (T_{CD44+}), and hyperactivaed (T_{DN}) CD8 T cells.

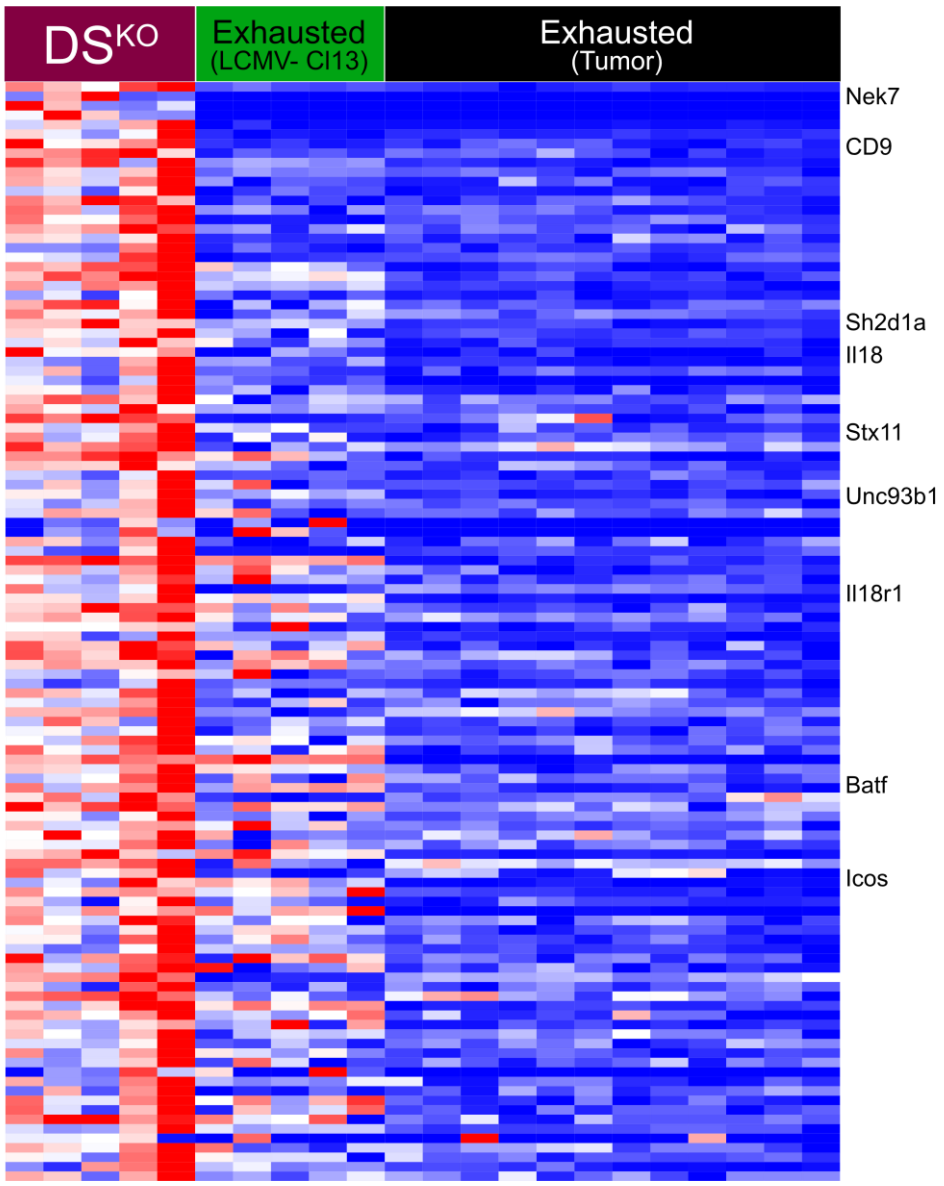


Figure 27: ***DS CD8 T cells have a distinct transcriptional profile when compared to exhausted CD8 T cells.*** Heatmap of the top 100 differentially expressed genes (FDR ≤ 0.1) in a comparison of DSKO and Terminally exhausted cells from both tumor and LCMV-Clone13.

3.3.7 Oligoclonality of hyperactivated CD8 T cells are hyperexpanded

Given that their CD8 T cells show increased proliferation, expression of exhaustion markers, and TCR-dependent IL-18 responses (*in vitro*), we sought to understand the nature of the TCR stimuli driving CD8 T cell expansion in DS mice. We analyzed the TCR sequences of splenic CD8 T cells from DS and control mice. We found that a very high proportion of the sequenced clonotypes belonged to the most prominent clones (up to 50%) in DS^{KO} CD8 T cells, as opposed to controls. This oligoclonality correlated with the degree of genetic susceptibility to hyperinflammation, as it was present to a lesser extent in DS^{HET} and Il18tg mice (**Figure 28A,B**). TCR clones of known antigen-specific TCRs present in available self peptide and pathogenic databases[284,285] were equally rare in DS and control mice (**Supplemental File 3**). Likewise, there were no shared clonotypes between the hyperexpanded clones of individual DS mice (**Figure 28**), suggesting that clonal expansion was not do to a common shared antigen. No significant differences in TCR variable region gene usage were found between DS mice and controls (**Figure 29A,B, Supplemental File 4**). Together, these analyses suggest that CD8 T cell expansion in DS mice is oligoclonal but not TCR-restricted.

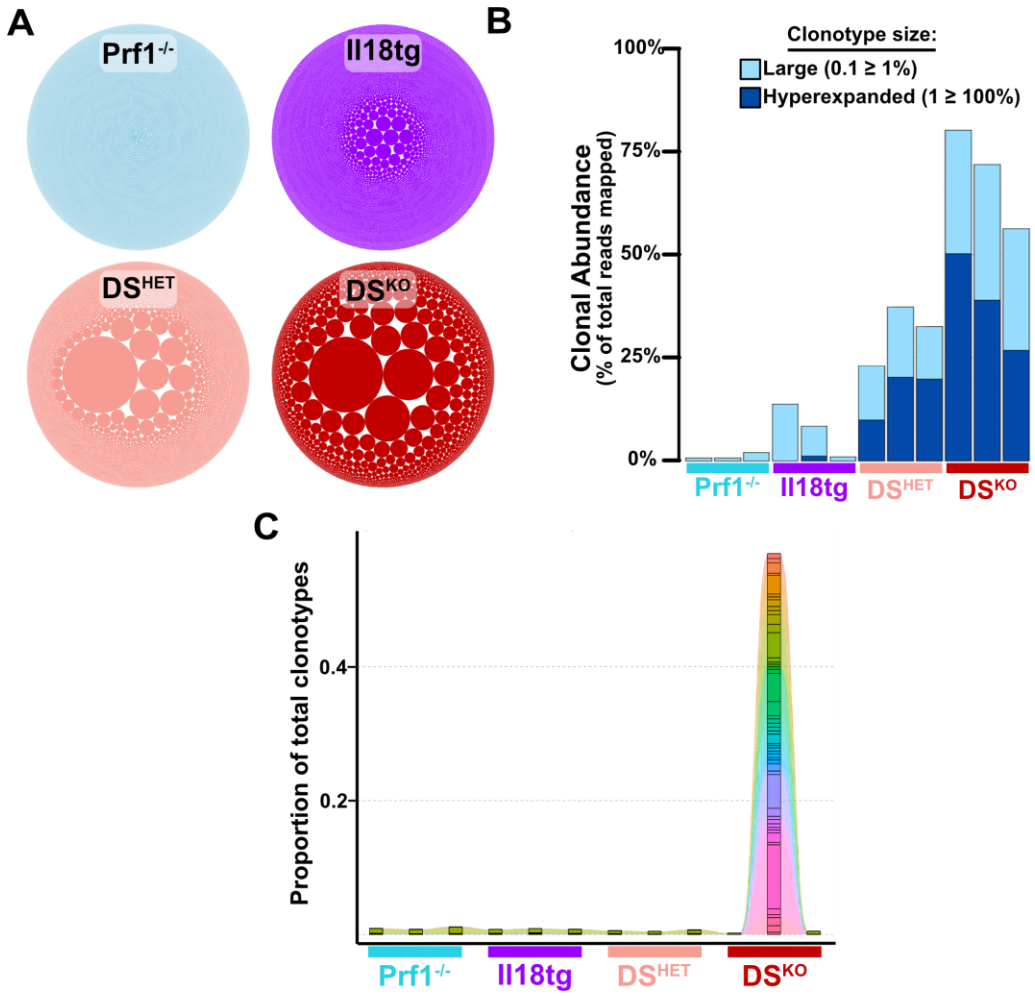


Figure 28: ***IL-18 and Perforin deficiency drive oligoclonal CD8 T cell hyperexpansion:*** (A) Bubble plot representing the proportion of mapped TCRs attributable to individual clonotypes within Prf1$^{-/-}$, Il18tg, DSHET, and DSKO mice. (B) Sums of the proportion of mapped TCRs attributable to clonotypes that individually occupy a large (0.1 to 1%) or hyperexpanded (1 to 100%) proportion of all mapped TCRs. (C) Representative plot showing that the top 50 hyperexpanded clonotypes in a single DSKO mouse account for ~50% of all mapped TCRs in that mouse, but the same clonotypes account for a negligible proportion of all mapped TCRs in all other sequenced mice.

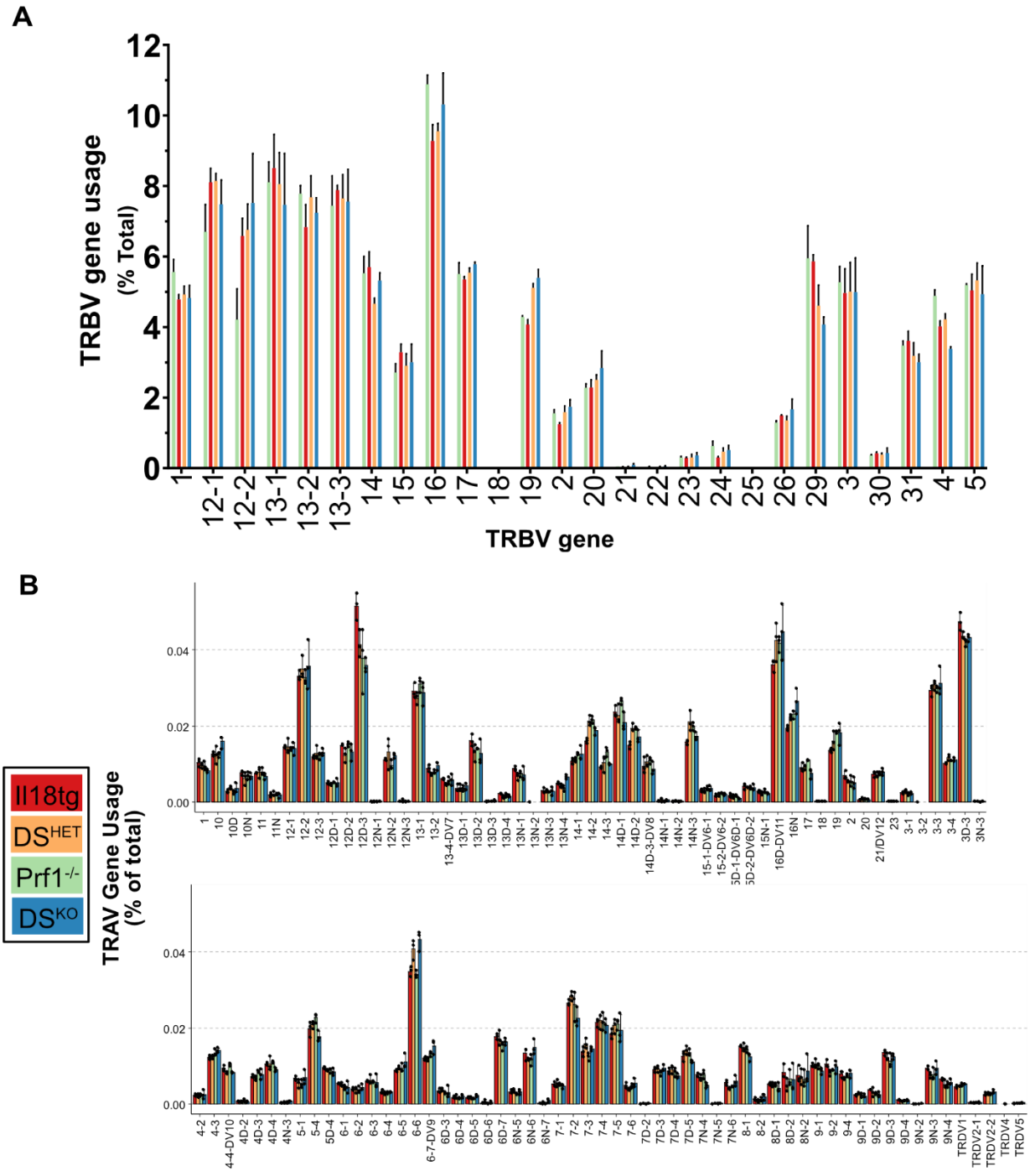


Figure 29: ***CD8 T cell TCR variable gene usage in dual susceptibility mice:*** Percentage of (A) TRBV and (B) TRAV gene usage in Prf1$^{-/-}$ (green), Il18tg (red), DSHET (orange), and DSKO (blue) CD8 T cells.

3.3.8 Single cell RNAseq of DS CD8 T cells

Based on the lack of shared TCR clones and variable gene usage in the CD8 T cells from DS mice, we hypothesized that hyperactivation was arising in one of two differentiation states in the CD8 T cell population. Since IL-18 has been linked to increased CD8 T cell proliferation, hyperactivation could be eliciting its effects on a progenitor population in which all daughter cells would have a hyperactivated, clonal phenotype. Alternatively, the act of becoming hyperactivated occurs when expanded daughter cells become activated and substantially upregulate *Il18r1*. To answer this, we attempted to utilize joint single cell RNAseq and TCRseq on activated CD8 T cells from DS and control mice. This would allow for transcriptome and TCR sequence analysis on the same cell to determine if clonal expansion occurred prior to or after transcriptional hyperactivation was noted. We provided roughly 60,000 highly viable CD8 T cells for input into the 10X controller which encompassed five genotypes (WT, $Prf1^{-/-}$, Il18tg, DS^{HET}, and DS^{KO}) in duplicate. We read the samples, at a depth of $2x10^5$ reads per cell for transcriptome, 5000 reads per cell for TCR sequencing, and 5000 reads per cell for feature barcodes (CellHashing). We anticipated obtaining a minimum of $2x10^4$ cells available for analysis however we only recovered data from roughly 10% of that (2048 cells) with only 50% providing TCR sequencing data making this dataset severely underpowered. The reason for such profound cell loss is still unclear however we were able to perform preliminary analyses on the data received to compare with our bulk RNAseq and TCRseq datasets.

After calculating UMAP reduction and cluster detection with the Louvain clustering method the dataset produced eight transcriptionally unique clusters to which each mouse genotype had varying degrees of contribution to (**Figure 30A-B**). The majority of WT and $Prf1^{-/-}$ CD8 T cells inhabited clusters 1 and 5. While clusters 2, 3, 4, and 6 were highly enriched for Il18tg and

DS CD8 T cells. Cluster 7 was enriched for CD8 T cells from Il18tg mice, while Cluster 8 was almost exclusively comprised of DS^{KO} CD8 T cells. Initial analysis based on unbiased marker gene selection, clusters 3 and 4 were composed of CD8 T cells that had similar transcriptional profiles to that seen in our DS CD8 T cell bulk RNAseq dataset (**Figure 30C**). This included elevated expression of *Lag3*, *Tigit*, *Pdcd1*, and *Gzmk*.

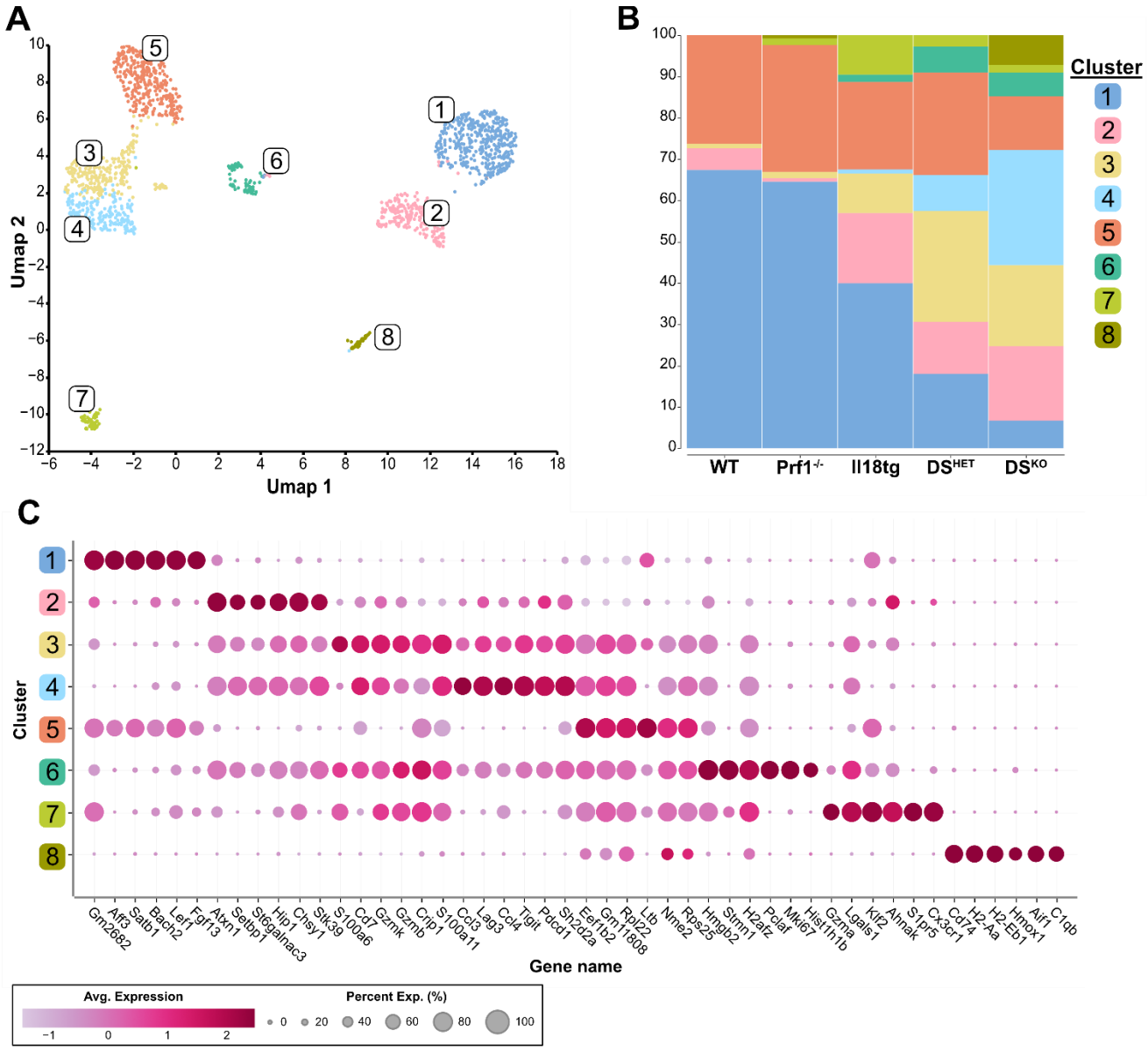
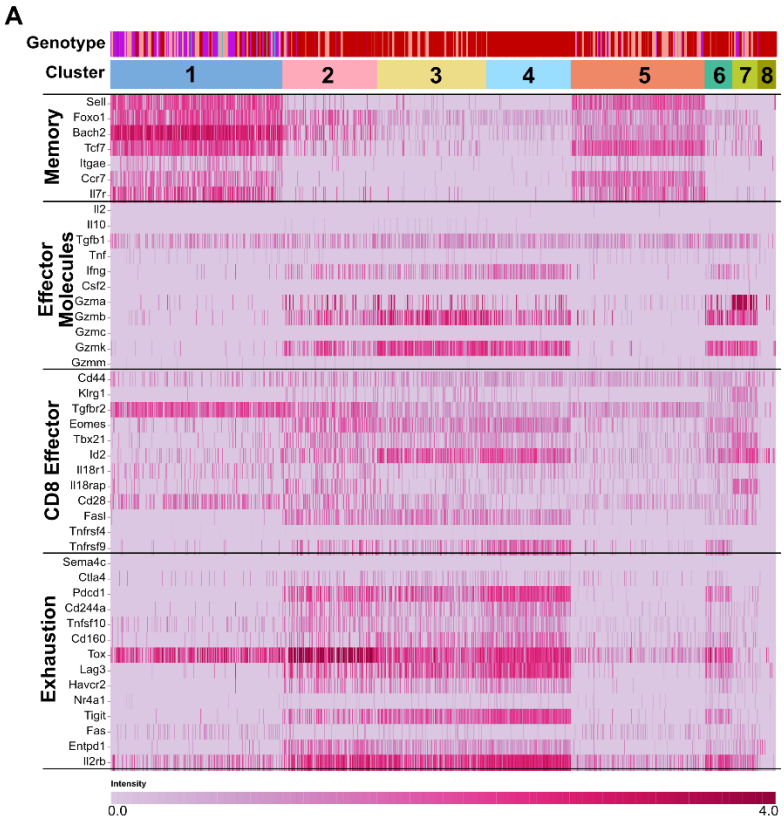
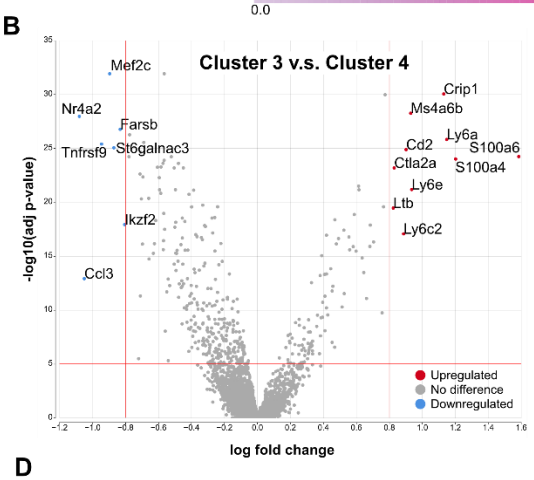


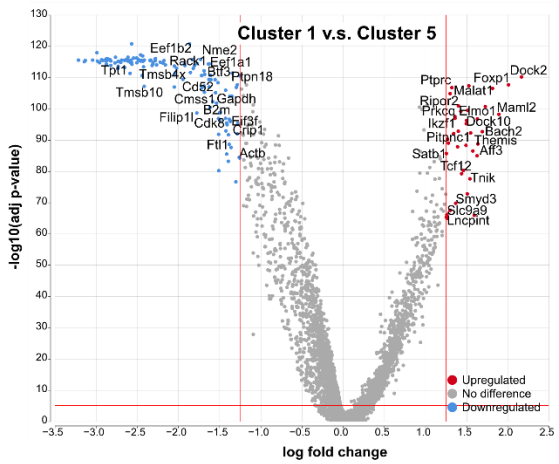
Figure 30: ***Single Cell RNAseq in DS CD8 T cells:*** Single cell RNAseq analysis of 2048 splenic CD8 T cells from indicated genotypes were analyzed and high dimensional data clustering was performed using (A) Louvain clustering projected on a UMAP. (B) Cluster distribution within each genotpes and (C) top 6 marker genes defining each cluster are shown.

A more directed analysis revealed clusters 3 and 4 both resembled T_{HYP} CD8 T cells however cluster 4 had higher expression of *Ccl3* and *Tnfrsf9* (encoding the potent costimulatory receptor 4-1BB) (**Figure 31A-B**). CCL3 has been shown to cause recruitment and differentiation of other CD8 T cells[286-288]. 4-1BB on the other hand can be used to distinguish a highly exhausted CD8 T cell with proliferative potential[289]. Cluster 2 also resembled T_{HYP} CD8 T cells however when compared to cluster 4, had increased expression of *Tox* combined with decreased expression of *Id2* and granzymes (**Figure 31A,C)**. High expression of *Tox* and less effector function is indicative of terminally exhausted cells[239]. Clusters 1 and 5 had a relatively similar transcriptional profile yet the majority of the cells in cluster 5 were DS CD8 T cells. Additionally, differential gene expression between cluster 1 and cluster 5 revealed that cluster 1 had elevated transcripts of *Tox* alongside *Bach2*, and *Ikzf1*(Ikaros) (**Figure 31D**). Bach2 expression is thought to limit the expression on other TCR driven genes and dampen TCR-driven effector programs[290]. Similarly *Ikzf1* has been shown to dampen CD8 T cell immune function[291,292] suggesting cluster 1 may represent a more undifferentiated, naïve population, although further testing is needed. Within the smaller clusters, cluster 6 had high levels of *Mki67,* suggesting active proliferation. Recent evidence of *Mki67+* proliferative exhausted CD8 T cells that are not part of the canonical progenitor subset have been found [293], potentially paralleling the cells within this cluster. Cluster 7 had increased expression of *Gzma, Gzmb* and *Gzmk* yet lack expression of canonical effector CD8 T cells like *Cd44.* Cluster 8 lacked *Cd3e* expression and had elevated transcripts of MHC II, *CD74,* and *Hmox1*, suggesting this very small population could derive from contamination of CD8+ antigen presenting cells (cDCs), despite multiple rounds of purification. Both cluster 7 and 8 were very small and their incompletely defined transcriptomic signature likely places them outside of the realm of hyperinflammatory CD8 T cells.

Initially, we had intended to trace the clonality of the CD8 T cells back to the individual differentiation states within our dataset, however the sequencing for the TCR repertoire was not ideal and severely underpowered. We were able to successfully identify 1411 cells with corresponding TCRα and TCRβ sequences. Within these cells, we found that DS CD8 T cells were more clonal than the controls as determined by the ratio of clonotypes per cell within the genotype (**Figure 31E**). Together the gene expression and clonality found in our single cell data, while underpowered, was consistent with our bulk RNAseq findings and indicative of a hyperactivated phenotype in DS CD8 T cells.







E

Genotype	Clonotypes	Barcodes (cells)	RATIO
WT	60	60	1.0000
Prf1-/-	87	87	1.0000
Il18tg	266	313	0.8498
DSHET	281	400	0.7025
DSKO	357	551	0.6479
TOTAL	1051	1411	

Figure 31: ***Directed scRNAseq and scTCRseq parallels bulk RNAseq****:* (A) Heatmap of selected genes in each cluster , Volcano plots of differential gene expression between (B) cluster 3 and cluster 4, (C) cluster 2 and cluster4, as well as (D) cluster 1 and cluster5. (E) Comparison of the number of clonotypes (TCRa+TCRb pair) to the number of cells (barcodes) within the dataset. DEG lists can be found in **Supplemental File 5**.

3.4 Discussion

HLH is a recognizable clinical syndrome of systemic inflammation whose proper management requires prompt identification of its life-threatening immunopathology and simultaneous consideration of its diverse contributors[1]. Although widely described in diverse contexts, the factors that contribute to HLH are incompletely understood. Monoallelic defects in cytotoxicity genes are common but enriched up to four-fold in SJIA-MAS patients[68,252] which suggests that excess IL-18 and defects (even partial) in cytotoxicity are cumulative (at minimum) in hyperinflammation. How these contributors converge on this phenotype remains unclear and a source of clinical confusion. Patients with chronic viral infections like Herpes viruses (EBV[263], CMV[264], HHV8[265]) and HIV [266]have been associated with secondary HLH/MAS manifestations. Through chronic antigen exposure, this could circumvent the need for defects in cytotoxicity to trigger hyperinflammation, provided there are other susceptibility factors also present. Our studies suggest that the chronicity of these other factors is a requirement. Chronic LCMV under conditions of normal production, but unopposed signaling of IL-18, as in Il18bp$^{-/-}$ mice, does not lead to hyperinflammation above what is seen in WT mice (**Figure 16**). However, under conditions of chronic LCMV infection, chronically elevated levels of free IL-18 (Il18tg) result in lethal hyperinflammation.

Our data suggests that IFNγ plays a prominent role in hyperinflammation. Neutralization of IFNγ helped decrease anemia and weight loss, but did not affect CD8 T cell activation[96]. Further

exploring T cell directed therapies for HLH in DS^{KO} mice, we found that HLH and CD8 T cell activation were remarkably un- attenuated. At least four factors may contribute to this resistance: ***1)*** Most likely, non-T cells may be critical contributors to increased IFNγ production and related immunopathologies, although we observed a dramatic reduction of $NK1.1^{+}$ cells and B cells in DS mice (**Figure 23**). ***2)*** Morbidity in these mice arises spontaneously but, we suspect, follows many weeks' accumulation of immunopathology. Antibody-based depletion of neither CD4 nor CD8 T cells was sufficient to ameliorate immunopathology, but longer-term depletion is not possible due to the development of neutralizing antibodies[294]. ***3)*** Antibody-based depletion may not be complete[295] (**Figure 21 and Figure 22**). ***4)*** We found that CD4 T cells "compensated" by increasing their activation status and producing more IFNγ when CD8 T cells were depleted (**Figure 21E**).

Hyperactivated CD8 T cells were found specifically in reticuloendothelial organs: sites of both homeostatic and HLH-associated hemophagocytosis[296]. These may be sites where CD8 T cells encounter antigen and exert local effects like producing IFNγ[79], an established driver of hemophagocytosis. Similarly the local sensing other signals such as IL-15 and type 1 interferons by CD8 T cells causing activation[297]. Interestingly, the protein and transcriptional signatures of CD8 T cells in DS mice consisted of both terminal effector and exhaustion features. Although typically considered immunosuppressive, exhausted CD8 T cells are generally not thought to be intrinsically anti-inflammatory. Terminally exhausted cells require continuous antigen stimulation and can still produce IFNg until the latest stages of exhaustion[298]. Features of exhaustion in CD8 T cells from DS mice may indicate progression of naïve cells through an IL-18-amplified effector state and through to terminal exhaustion[299,300], a path that DS CD8 T cells are unable to complete. The inflammatory effects of IL-18 on partially and terminally exhausted T cells are being actively

explored in cancer immunotherapy studies[111,112,301]. Terminally exhausted CD8 T cells do not express *Il18r1*[104], yet this receptor is highly expressed (at transcript and protein levels) on hyperactivated CD8 T cells in DS mice (**Figure 25, Figure 31A**)further demonstrating this is a unique population. As a population, these "hyperactivated" CD8 T cells could represent a pathogenic transitional T effector-like phenotype[302].

CD8 T cells from DS mice showed extremely high levels of clonal hyperexpansion however the precise nature of TCR stimulation in the DS mice remains an enigma. Classic autoimmunity seems unlikely, as DS mice did not appear to develop autoimmune immunopathology, had disturbed splenic germinal centers[96], and had few $CD19^+$ B cells. Our data cannot entirely rule out a role for CD8 T "virtual memory"[303]. Virtual memory CD8 t cells have an antigen-naïve, harbor a memory phenotype, and were shown to produce IFNγ in response to IL-18/IL-12/IL-15 stimulation[304,305]. Additionally, our data cannot account for responses to self-antigens, commensal or endogenous retroviral antigens, or even selection for above-normal peptide independent TCR stimulation are possible. Rather than continuous/cumulative expansion of the same clones, DS mice may experience rolling waves of immunization, hyperactivation, and ultimately exhaustion – a hypothesis that would fit our data in 2-3 week old pups (**Figure 19C, D**) as well as the absence of overlapping hyperexpanded clonotypes between DS mice in bulk and single cell TCR sequencing data (**Figure 28C, Figure 31E**).

Overall, our data reinforce the primacy of CD8 T cells and IFNg; identify an effector/exhaustion state arising in spontaneous CD8 T cell activation; and reveal synergy between CD8 TCR stimulation, signal amplification (e.g., IL-18 and LCMV), and impaired immunoregulation. Blocking convergent CD8 T cell effector functions (e.g. with steroids and cytokine blockade, e.g. IFNγ) may be feasible tempering measures, but our data reinforce the

ultimate need to identify and minimize chronic drivers of T cell hyperactivation by targeting type 1 inflammatory stimuli (e.g. IL-18), restoring T cell regulation (e.g. cytotoxicity), and clearing relevant antigens. Circumstantial observations that oligoclonal CD8 T cells dominate peripheral blood[86,306], liver[92,93], and nodular parenchymal lesions[94] of diverse HLH patients reinforces the translational validity of these murine results. Thus, we believe our findings support a novel framework for the classification of HLH/MAS based on multiple mechanistic contributors. Rethinking HLH in this way may lead to novel diagnostic strategies, and more-precise personalized, multi-pronged treatment strategies to improve outcomes in this often fatal syndrome.

4.0 Enigmatic, non-pathogen TCR stimulation drives CD8 T-cell hyper activation in DS mice.

While the previous sections have described the outcome of hyperinflammation due to excess IL-18 and impaired cytotoxicity, the exact mechanism(s) behind their convergence on CD8 T cells has not yet been explored. This section attempts to define the mechanisms at play during CD8 T cell hyperactivation and hyperinflammation.

Portions of this chapter have been published in the following article:

- **Emily Landy**, Jemy Varghese, Vinh Dang, Andrea Workman, Lawrence Kane, Scott Canna; Complementary HLH Susceptibility Factors Converge on CD8 T-cell Hyperactivation. Blood Adv 2023; bloodadvances.2023010502. doi: https://doi.org/10.1182/bloodadvances.2023010502

4.1 Summary

In **Section 3.3.7**, we showed that hyperactivated CD8 T cells were clonally hyperexpanded suggesting that antigen recognition by T cells is involved in hyperactivation. *Il18r1* expression is highly upregulated in CD8 T cells only after they have been activated, further suggesting that CD8 T cell activation is a requirement for IL-18 augmented hyperactivation. While naïve CD8 T cells do not express IL-18R components, we hypothesized that activation was a requirement for IL-18 mediated hyperactivation. We were interested if either cognate antigen exposure or cytokine activation[307,308] was responsible for hyperactivation and excess IFNγ production. In the studies

described in this section we show that neither, IL-18 alone, nor IL-12 in combination with IL-18 (in the absence of CD3/CD28) significantly induced IFNγ production. However, CD8 T cells required active TCR stimulus with IL-18 to become hyperactivated *in vitro.*

To understand why hyperactivated CD8 T cell expansion was being left unchecked, we determined that *in vitro,* IL-18 increased reactivation induced cell death (RICD). In contrast to IL-18, perforin deficiency (even haploinsufficiency) lead to decreased RICD. Thus, when perforin deficient CD8 T cells were exposed to IL-18 in the context of CD3/CD28, the effects of IL-18 on RICD were abrogated. This suggested that lack of perforin in CD8 T cells prevented the natural response to excess IL-18. RICD, leading activated DS CD8 T cells to persist and drive immunopathlolgy. We also tested if IL-18 or perforin deficiency led to increased proliferation *in vivo*. We found that Il18tg and DS mice, unlike $Prf1^{-/-}$ mice, had significantly increased EdU incorporation in their CD8 T cell compartment.

Further substantiating a requirement for active TCR engagement in CD8 T cell hyperactivation, DS mice on a P14 TCR transgenic background without cognate antigen exposure (i.e. LCMV infection) had significant expansion of non P14 CD8 T cells which had escaped the allelic exclusion typically seen in P14 control mice. DS-P14 mice succumbed to lethal hyperinflammation nearly identical to that of their non-P14 controls. Increased IFNγ production has been shown to be an outcome of IL-18 stimulation [133]. Perforin deficiency[7] in CD8 T cells has also been shown to produce excess INFγ, however this is thought to be due to increased immune synapse durations. We hypothesized that this would also be the case with CD8 T cells receiving excess IL-18 stimulation. Live imaging of CD8:BMDC immune synapse formation and dissociation showed that IL-18 decreased IS duration to less than that of non-IL-18 stimulated interactions. However, co-culture supernatants revealed that even in this shortened IS duration,

IFNγ production was elevated. IL-18 drastically reduced the elongated IS duration in $Prf1^{+/-}$ mice, but IL-18 only partially reduced IS duration in $Prf1^{-/-}$ CD8 T cell co-cultures. IFNγ production was elevated in co-cultures with IL-18 as compared to the same co-cultures without. This suggests that perforin deficiency and IL-18 converge on hyperactivated CD8 T cell IFNγ production.

Given the clonality and requirement of TCR engagement in CD8 T cell activation we attempted to determine the source of antigen. We found that neither broad spectrum antibiotics, nor anti-retroviral treatment ameliorated DS hyperinflammation. This suggested that neither commensal bacteria nor (ART susceptible) ERVs were driving spontaneous hyperactivation and lethal hyperinflammation in DS mice.

4.2 Methods

4.2.1 Mouse lines:

Mice were housed in specific pathogen-free conditions under a protocol approved by the University of Pittsburgh Institutional Animal Care and Use Committee (IACUC). All animals originated from Jackson Laboratories except Il18tg mice were a gift from Dr. Tomoaki Hoshino (Kurume University)[203].

4.2.2 In Vitro stimulation assays:

Whole splenocytes from P14 or $Prf1^{-/-}$ P14 mice (bearing a transgenic T cell receptor recognizing the LCMV GP^{33-42} peptide) were processed as described and then cultured for 24 hours

with cognate GP^{33-42} peptide (1 μg/mL) in complete R10 media (RPMI supplemented with 10% FBS, 1X Penicillin-streptomycin, 2 mM L-Glutamine, 1 mM sodium pyruvate, 10mM non-essential amino acids, 50 μM 2-mercaptoethanol, and 12 mM HEPES) supplemented with 50 U/mL IL-2. Peptide was removed and cells were resuspended in complete media with 50 U/mL IL-2 (+/- 50 ng/mL IL-18) and allowed to rest for three days. Every other day thereafter, live cells were cultured in fresh complete media with 25 U/mL IL-2 (+/- 50 ng/mL IL-18). At 9-11 days after activation cells were reactivated via anti-CD3 (3 μg/mL coated plate) and anti-CD28 (5 μg/mL) in complete media (25 U/mL IL-2, +/- 50 ng/mL IL-18) and assessed for activation markers and cytokine production via flow cytometry.

4.2.3 In vitro synapse time and IFNγ production assays:

P14 transgenic CD8 T cells were purified by fluorescent-activated cell sorting (FACS) and cultured in IL-2 for 72 hrs, target cells were derived from bone marrow derived macrophages (BMDCs) generated from C57BL/6 mouse bone marrow via culture in GM-CSF^{309} for 72hrs. CD8 T cells were then co-cultured with BMDCs at a ratio of 3:1 for immunological synapse (IS) duration and IFNγ production as described below.

Immunological synapse (IS) initiation was measured by two parameters: 1) CD8 T cell and target cell proximity and 2) calcium flux (using Fluo-4 dye (ThermoFisher Cat: F10489) added to CD8 T cells prior to imaging). Termination of the IS was determined at three distinct stages including, a) uptake of PI target cell, indicating loss of membrane integrity, b) change in cellular structure measured by real-time phase contrast imaging, and c) detachment of cells. Image analysis was performed using LAS AF (Leica), Fiji, and MetaMorph Imaging Series 7 software packages.

Cytokine production was measured in co-culture supernatants at indicated timepoints using Cytometric Bead Array (BD)[11]. We used an offset of +1/4 the lowest concentration of each analyte's standard curve to each experimental value to minimize floor effects of data range.

4.2.4 Antibiotic treatment:

Adult mice were provided with broad-spectrum antibiotics *ad libitum* in drinking water for five weeks. Antibiotic water supplemented with saccharin contained metronidazole (0.5 mg/mL), vancomycin (0.5 mg/mL), neomycin (1 mg/mL), and ampicillin (1 mg/mL). Control mice received only saccharin supplemented water.

4.2.5 Antiretroviral and Etoposide Treatment:

For antiretroviral treatment (ART) adult mice were given 200uL of combined tenofovir (12.5 mg/mL) and emtricitabine (7.5 mg/mL) daily for three weeks via oral gavage as in Lima-Junior et al[310]. Mice were harvested one day after last ART treatment. Etoposide was administered to adult mice at 30 mg/kg i.p. twice weekly for four doses and mice were evaluated one week after last treatment as in Koh et al[311].

4.3 Results

4.3.1 Minimal effects of IL-18 on CD8 T cells in the absence of T cell receptor stimulation

IL-18 is known to amplify disease in the TLR9-driven model of MAS, which typically does not require T cells[11,70]. Nevertheless, cytokines like IL-12 can provoke effector responses from activated and memory T cells in the absence of TCR-stimulation[247,248]. To further assess the effects of IL-18 with and without TCR stimulation, we cultured CD8 T cells with IL-18 during *in vitro* TCR restimulation. Consistent with a role for IL-18 as an amplifier of IFNγ production, we found that maximal IFNγ production required the interaction of CD3/CD28 and IL-18 (**Figure 32A**). Interestingly, however, previously activated CD8 T cells in culture with IL-18 in the absence of CD3/CD28 stimulation had minimal IFNγ production. In CD8 T cells restimulated *in vitro* (up to two weeks after initial TCR stimulation), we found that neither IL-12 alone nor IL-18 plus IL-12 substantially impacted IFNγ production by CD8 T cells in the absence of TCR re-stimulation (**Figure 32B**). Together, these data suggest that the effects of chronic IL-18 on T cells may require active or very recent TCR signaling.

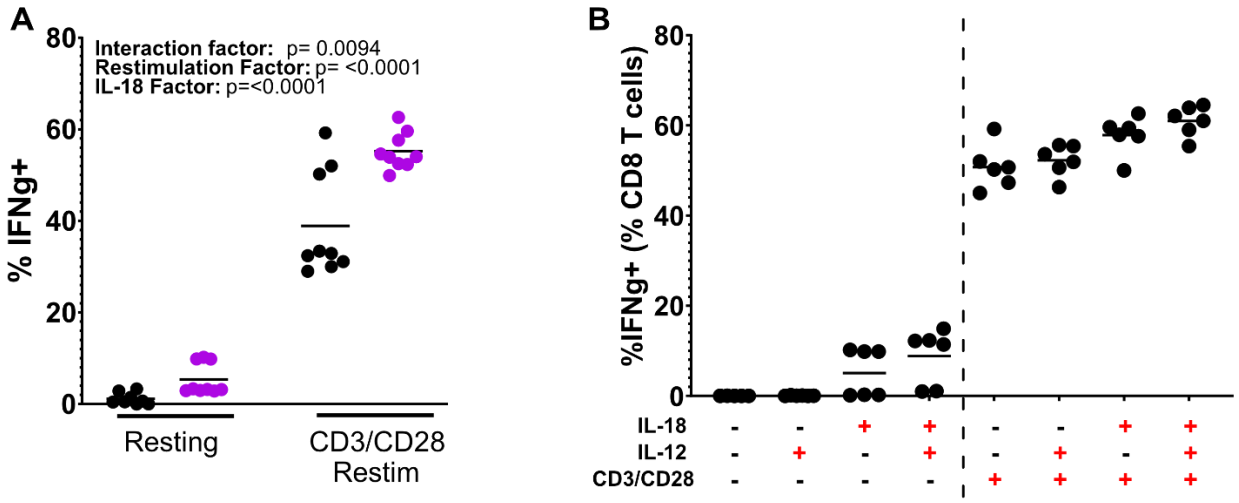


Figure 32: ***CD8 T cells require TCR stimulus in the presence of IL-18 for hyperactivation.*** (A) Percentage of CD8 T cells producing IFNγ following reactivation with or without TCR restimulation for 24 hours in the presence (purple) or absence of 50ng/mL recombinant murine IL-18. Each point represents an average of three wells in an experiment, 9 experiments shown. Analysis by two-way ANOVA. (B) Percentage of CD8 T cells producing IFNγ following reactivation with or without TCR restimulation for 24 hours in the presence or absence of 50ng/mL recombinant murine IL-18 and/or 10ng/mL recombinant murine IL-12. Graph shows representative data from two independent experiments, each dot is a technical replicate. P-value is based on one way ANOVA with Tukey post-test comparing cytokine addition within resting and CD3/CD28 groups separately.

4.3.2 Effects of HLH susceptibility factors on CD8 T cell restimulation induced cell death (RICD) and proliferation promote hyperinflammation

Impaired reactivation-induced cell death (RICD) has been implicated in the pathogenesis of X-linked lymphoproliferative syndrome type 1 (XLP1), a type of primary HLH due to SAP (*Shd2a*) deficiency that is usually triggered by EBV infection[312]. Perforin may also help mediate activation-induced apoptotic cell death, specifically at early timepoints prior to Fas-mediated killing[313,314]. To determine whether perforin deficiency and IL-18 conferred CD8 T cell resistance to cell death, we tested the effects of these two factors (individually and in concert) in an *in vitro* RICD system. We found that the addition of IL-18 significantly *increased* RICD (**Figure 33A**). Consistent with prior results[313,315], perforin deficiency caused a slight decrease in RICD.

Surprisingly, this persisted even in the presence of IL-18. This reinforces the notion that perforin prevents hyperinflammation both by killing target (e.g., dendritic) cells[97] and other cytotoxic cells ("fratricide")[245]. IL-18 has also been linked to increased CD8 T cell proliferation[107,191], and we observed greater EdU incorporation in DS than in WT or Prf1$^{-/-}$ CD8 T cells (**Figure 33B**). Importantly, Il18tg CD8 T cell EdU incorporation was comparable to that in DS mice, but such mice have far less profound CD8 T cell activation (**Figure 18**). Thus, inflammatory/proliferative stimuli like excess IL-18 may increase demand for immunoregulatory RICD, a demand that goes unmet when cytotoxicity is (even partially) impaired.

Etoposide is a cornerstone of the regimen used to dampen hyperinflammation and prepare familial HLH patients for hematopoietic stem cell transplant (HSCT)[316]. It also selectively ablates dividing, pathogenic CD8 T cells and ameliorates hyperinflammation in Prf1$^{-/-}$ mice infected with LCMV[317]. Due to increased proliferation of CD8 T cells in DS mice, we hypothesized that a similar etoposide treatment regimen might ameliorate HLH. We observed no appreciable improvement in splenomegaly, anemia, or levels of hyperactivated CD8 T cells in DS mice after two weeks' treatment (**Figure 34**).

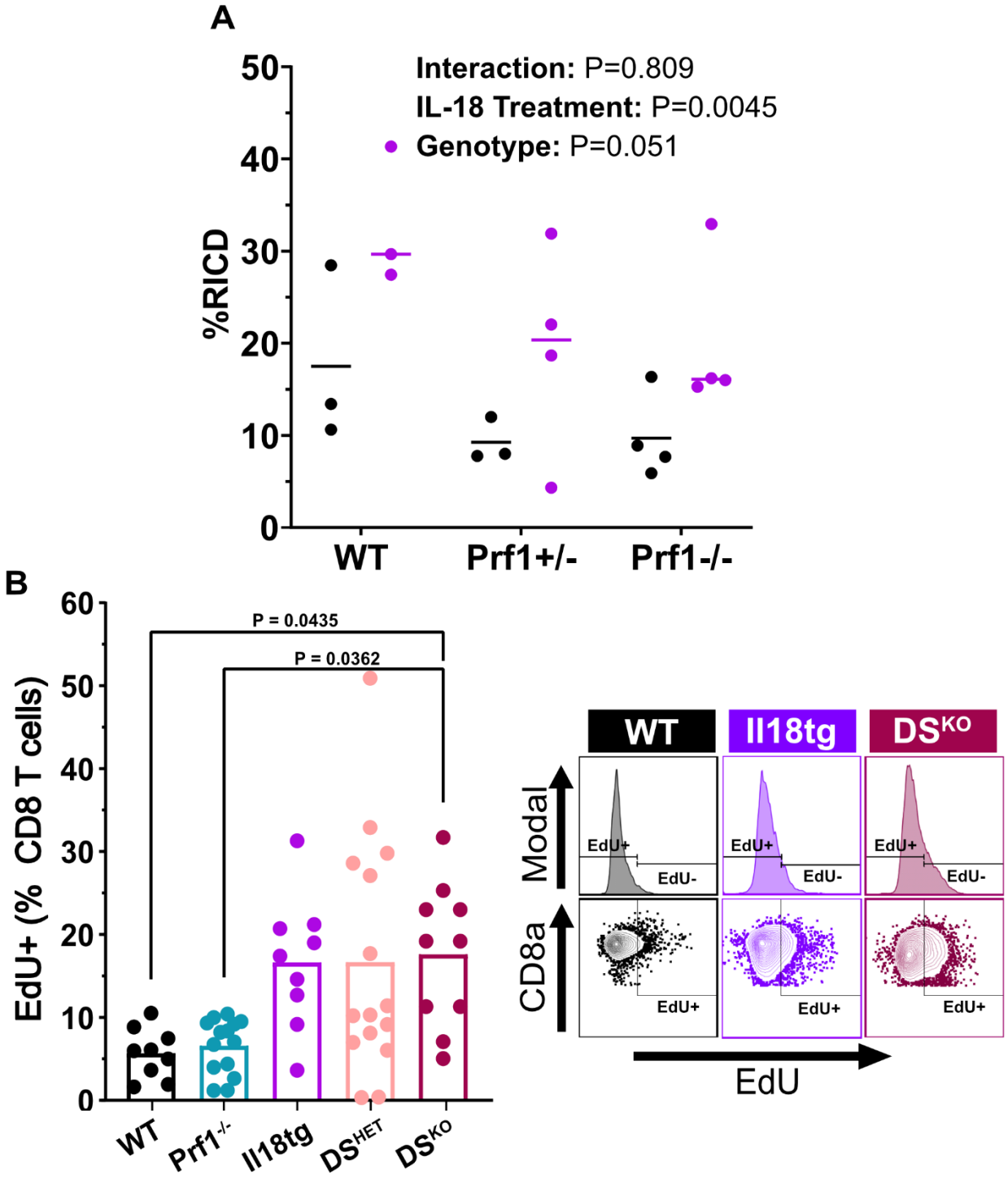


Figure 33: ***IL-18 and Perforin deficiency alter CD8 T cell RICD and proliferation.*** (A) Reactivation induced cell death (RICD) in CD8 T cells in the presence or absence of 50 ng/μL IL-18 following 24h restimulation with CD3/CD28. Each data point represents the average of technical triplicates within one experiment. P values are determined using two-way ANOVA. (B) Percent and representative graphs of in vivo EdU incorporation in CD8 T cells 19h after systemic administration. Data from 3+ separate experiments, each data point represents a single mouse. P values calculated by one-way ANOVA with Tukey multiple comparisons test evaluating all comparisons with DSKO- group. Only adjusted $p<0.05$ shown.

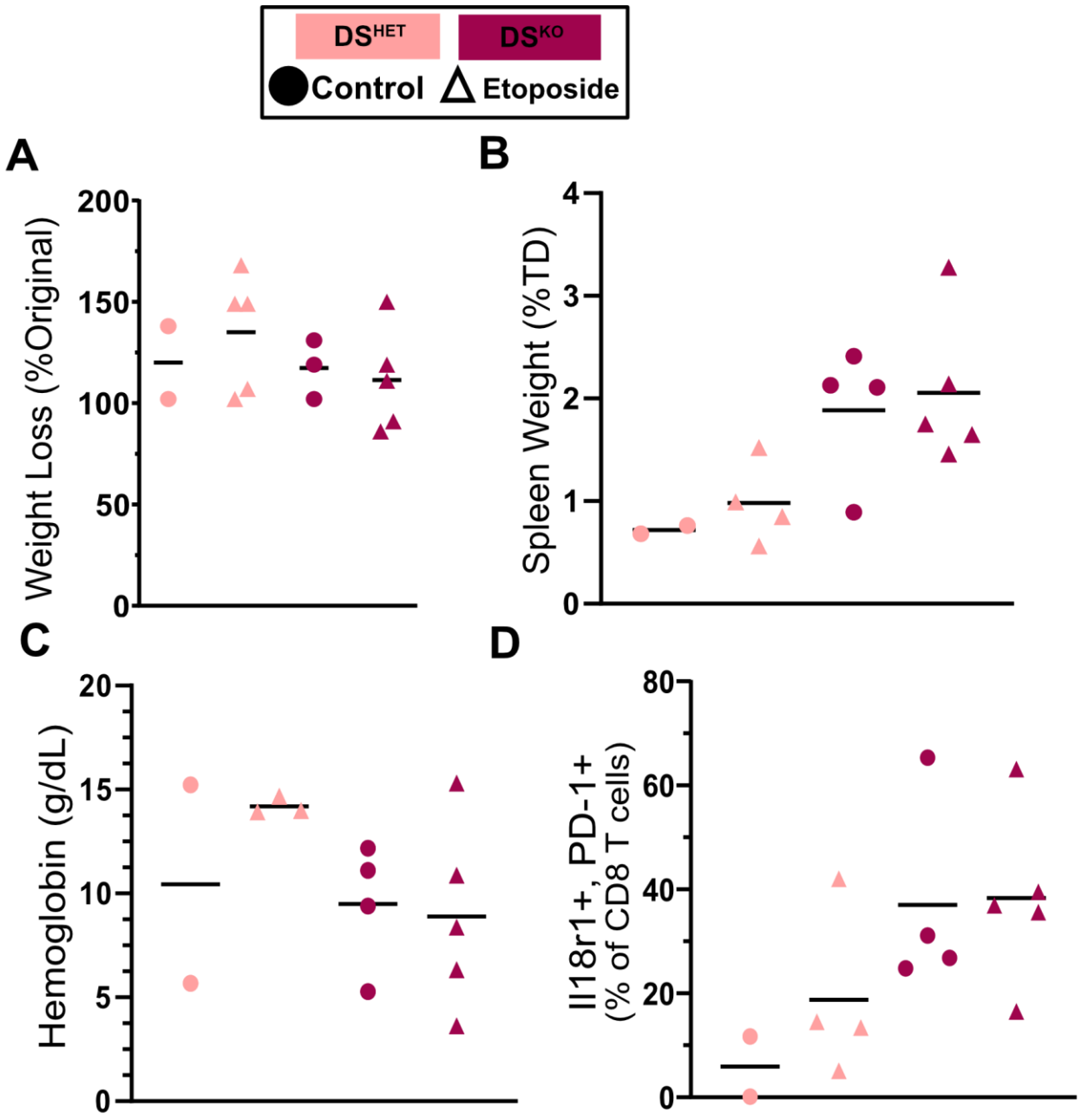


Figure 34: ***T cell ablation does not ameliorate disease in dual susceptibility mice****:* DS^{HET} and DS^{KO} mice were given 30mg/kg of etoposide twice weekly for two weeks. (A) Weight loss, (B) splenomegaly, (C) anemia, and (D) CD8 T cell hyperactivation (IL-18r1+, PD-1+) were assessed one week after final treatment.

4.3.3 Chronic, systemic, elevated IL-18 is required for sustained CD8 T cell hyperactivation

In the context of chronic elevated levels of IL-18, perforin deficiency leads to spontaneous lethal hyperinflammation. However we wondered if the chronicity of elevated IL-18 played a role in this hyperinflammatory phenotype. We crossed perforin deficient mice with IL-18BP$^{-/-}$ mice, producing mice that completely lacked perforin and had unopposed, although in normal abundance, IL-18. These mice did not succumb to lethal hyperinflammation like DS^{KO} mice, yet they developed some moderate signs of spontaneous hyperinflammation with age. Prf1$^{-/-}$Il-18BP$^{-/-}$ mice had slightly decreased levels of platelets and increased splenomegaly (**Figure 35A, B**). While CD8 levels were similar to Prf1$^{-/-}$ and IL-18BP$^{-/-}$ controls, more IL-18r1+ and CD44$^{-/LOW}$, CD62L^{-} CD8 T cells (also seen in DS mice) were present in Prf1$^{-/-}$Il-18BP$^{-/-}$ (**Figure 35C-E**). This together suggests that IL-18 signaling (even at low levels) can synergize with perforin deficiency to cause, albeit low-grade, hyperinflammation.

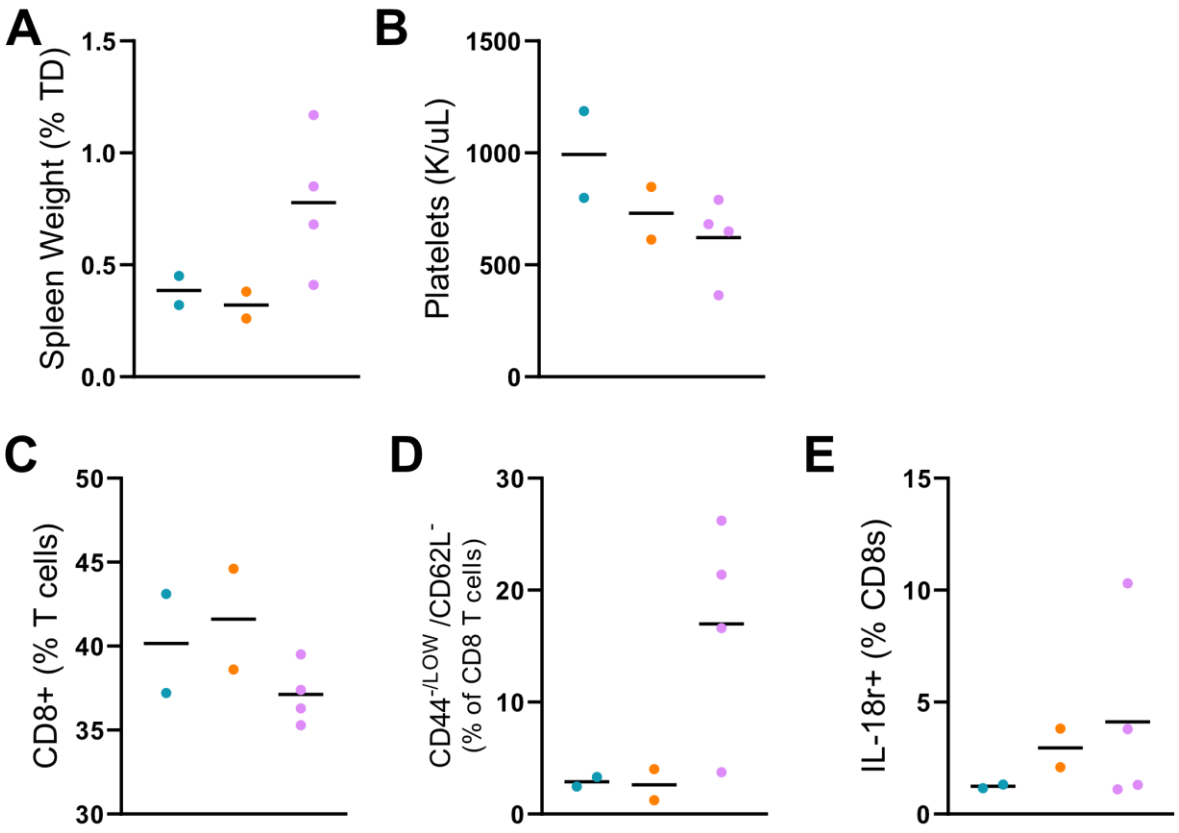


Figure 35: ***Prf1$^{-/-}$IL-18BP$^{-/-}$ mice have mild spontaneous hyperinflammation:*** Mice were aged out to over 9 months under homeostatic conditions and then assesed for (A)splenomegaly, (B)anemia, and (C) expansion of CD8 T cells. CD8 T cells were then further assesed for a hyperinflammatory phenotype using previously noted (D) CD44$^{-/Low}$ CD62L^{-} and (E) IL-18r1status. Statistical significance was assesed using one-way ANOVA and Tukey mutliple comparisons.

Upon challenge with acute LCMV (Armstrong), WT and IL-18BP$^{-/-}$ mice behaved as previously described[96], however Prf1$^{-/-}$ and Prf1$^{-/-}$Il-18BP$^{-/-}$ mice were similar in terms of cachexia, splenomegaly, and anemia. Prf1$^{-/-}$Il-18BP$^{-/-}$ mice had elevated levels of CD8 T cells and IFNγ+ CD8 T cells over that of any other genotype of mouse suggesting combinatorial effects within the CD8 T cell compartment. There was a higher proportion of GP33 tetramer positive CD8 T cells in Prf1$^{-/-}$ mice than in Prf1$^{-/-}$IL-18BP$^{-/-}$ mice. This could indicate increased turnover of clonally expanded CD8 T cells can be driven by IL-18 signaling on CD8 T cells akin to our observations *in vitro* showing increased RICD in Prf1$^{-/-}$ CD8 T cells when co-cultured with IL-18 (**Section 4.3.2**).

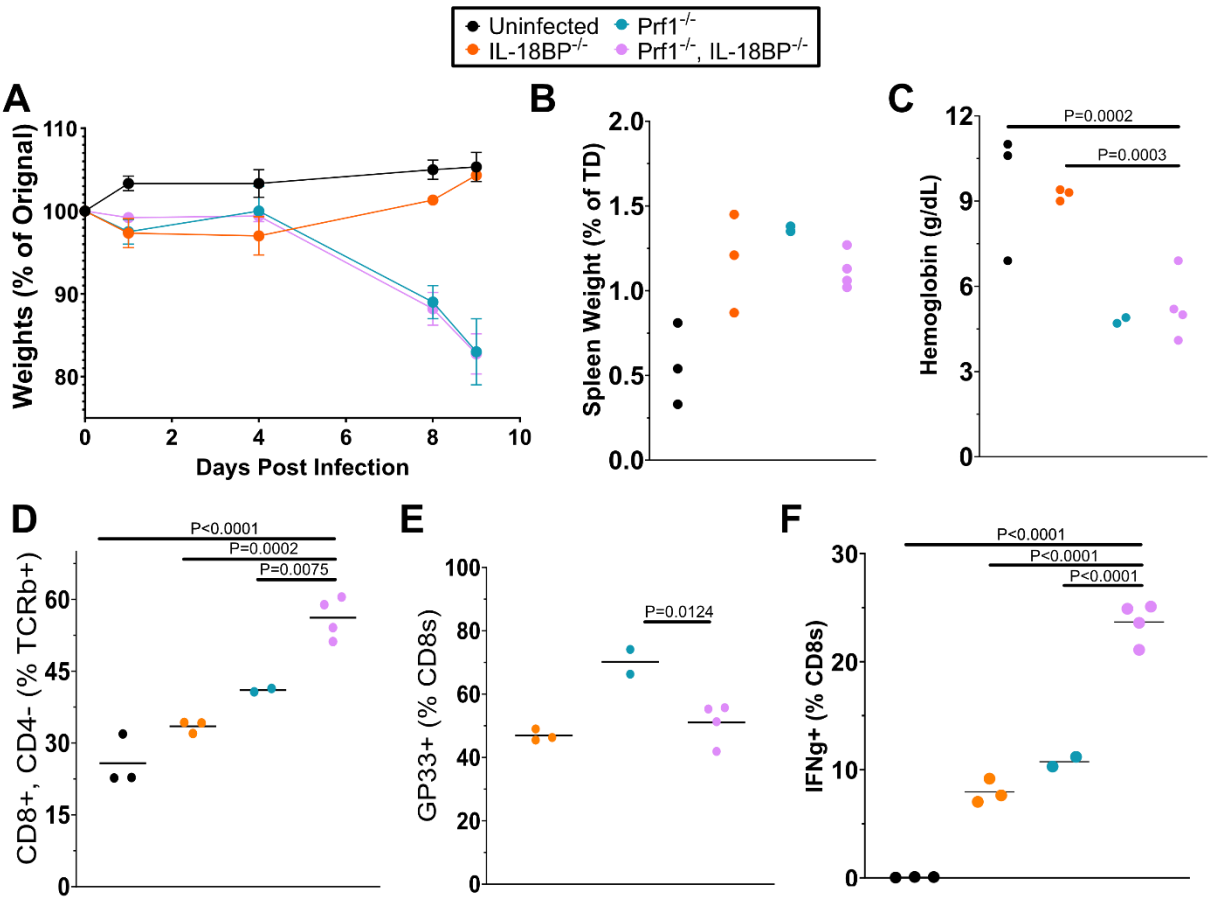


Figure 36: ***Perforin deficience combined with un-apposed IL-18 signaling leads to lethal LCMV hyperinflammation****:* (A) Weight loss during LCMV Armstrong infection in in Prf1$^{-/-}$, IL-18bp$^{-/-}$, and Prf1$^{-/-}$IL-18bp$^{-/-}$ mice with uninfected controls as comparison. 9dpi mice were assesed for (B) splenomegaly, (C) hemolgobin as well as CD8 T cell (D) expansion, (E) LCMV specificity, and (F) post 5hr GP33 peptide stimulation cytokine production.Statistical significance was determined using One-way ANOVA with Tukey multiple comparisons. Only showing significance when compared to Prf1$^{-/-}$IL-18bp$^{-/-}$.

4.3.4 TCR engagement is required for CD8 T cell hyperactivation in vivo

Based on our *in vitro* data suggesting TCR signaling is required for IL-18-mediated elevated IFNγ production by CD8 T cells (**Figure 32**), we wondered whether we could prevent hyperinflammation and CD8 T cell hyperactivation in DS mice by genetically limiting T cell encounters with cognate antigen. To test this, we bred DS mice to express the "P14" transgene, generating DS-P14 mice. The P14 transgene encodes a preformed TCR that is preferentially

expressed (by allelic exclusion) on CD8 T cells. The transgenic TCR recognizes an immunodominant peptide of the LCMV glycoprotein, GP^{33-41}. Despite this attempt to "fix" their TCRs, DS^{KO}-P14 mice developed spontaneous immunopathology nearly identical to control DS^{KO} mice, including splenomegaly, thrombocytopenia, and systemic IFNg (**Figure 37A-C**). Like control DS^{KO} mice, DS^{KO}-P14 mice developed (almost exclusively) CD8 T cell hyperactivation. However, the majority of CD8 T cells in DS-P14 mice were $GP33^{Tet}$-negative (**Figure 37D, E**). This enrichment for $GP33^{Tet}$-negative CD8 T cells was highly concentrated in the most activated (IL-18R1+) cells. These "breakthrough" CD8 T cells produced IFNg at a significantly higher rate than GP33 tetramer-positive cells in DS-P14 mice (**Figure 38A**).

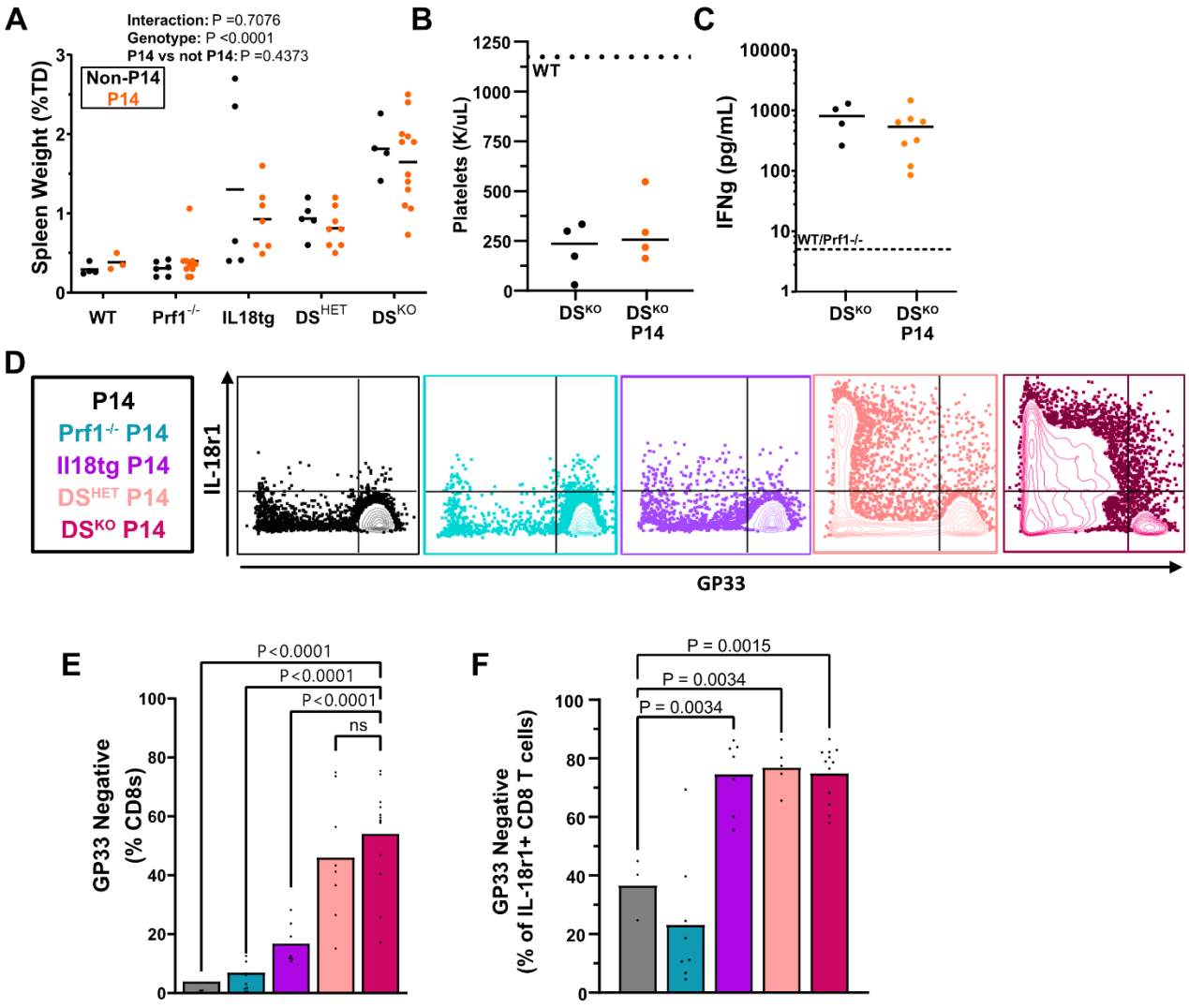


Figure 37: ***Dual Susceptibility mice bearing the P14 transgene succumb to hyperinflammation but develop "breakthrough" GP33TET-negative CD8 T cell activation:*** (A-C) Splenomegaly, thrombocytopenia, and systemic IFNγ in DSKO, DSKO-P14, and control mice. (D) Representative flow cytometry plots of splenic CD8 T cells. (E) Proportion of splenic CD8 T cells that bind $GP33^{Tet}$. (F) Proportion of the activated (IL-18R1+) CD8 T cell pool occupied by $GP33^{Tet}$-negative cells. Graphs show combined data from 3+ experiments, each point represents an individual mouse. P-values determined by two way ANOVA (A) or one way ANOVA with Tukey multiple comparisons test of pairwise comparisons against WT-P14 group.

The P14 TCR includes the TCRVβ8.1 chain[318]; and as expected, $GP33^{Tet}$-negative CD8 T-cells expressed TCRVβ chains other than TCRVβ8.1. Since we surmised that "breakthrough" CD8 T cells were responding to a peptide other than GP^{33-41}, we predicted that the few $GP33^{TET}$-positive T cells in DS^{KO}-P14 mice would also express another TCR. As predicted, the $GP33^{TET}$-positive cells from DS-P14 mice showed increased co-expression of TCRVβ8.1 and other TCRVβ chains (**Figure 38B, C**). Thus, though all T cells in DS-P14 mice are deficient in perforin and exposed to the same high levels of IL-18, those bearing the GP33-specific TCR (typically the vast majority) are specifically excluded from becoming hyperactivated. Simultaneously, CD8 T cells bearing "other" TCRs, and thereby capable of encountering cognate peptides, come to dominate the pool of hyperactivated T cells. These data underscore the necessity of antigen stimulation for IL-18 and perforin-deficiency to exert their synergistic effects on CD8 T cell hyperactivation.

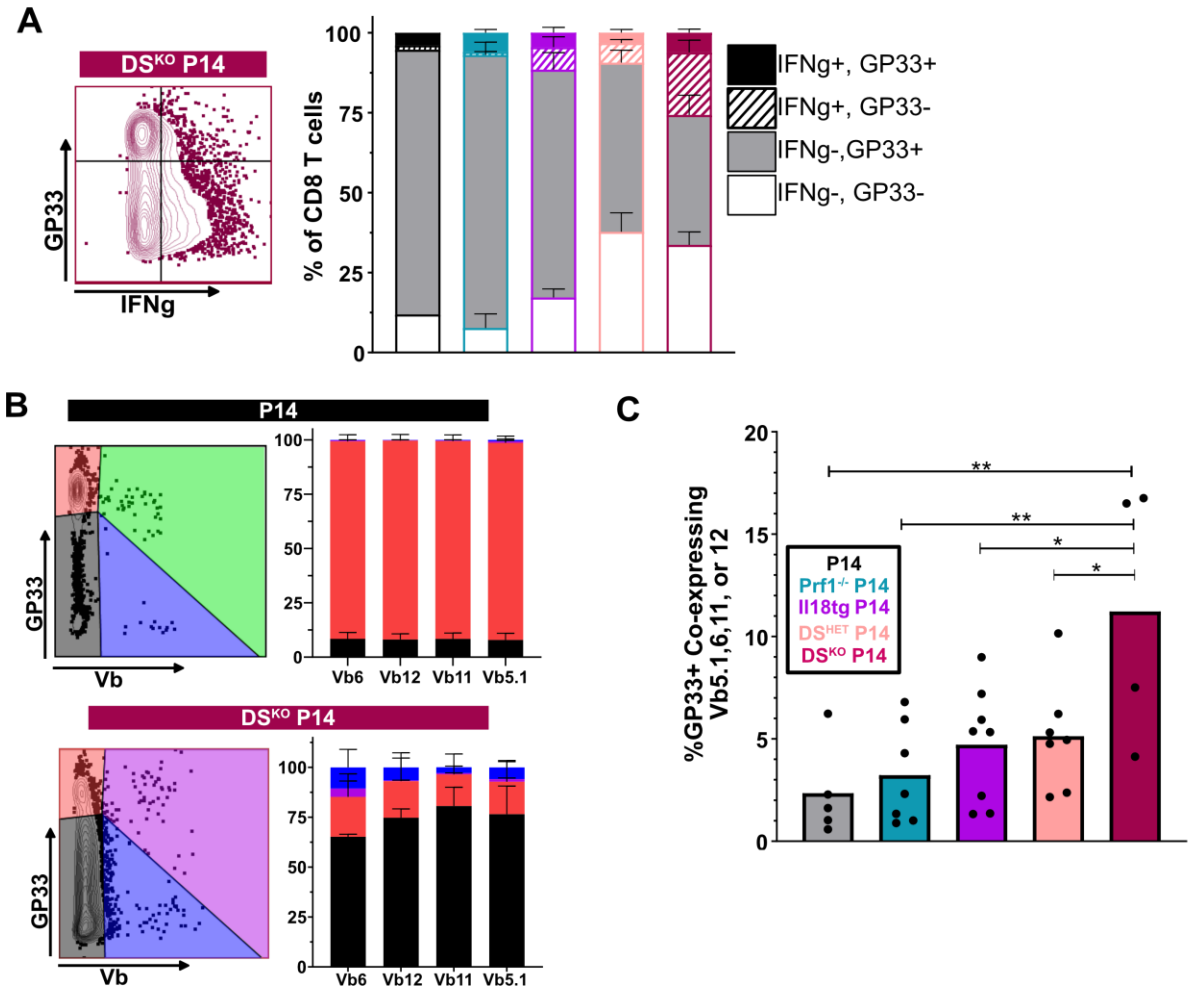


Figure 38: ***Dual Susceptibility P14 mice have increased co-expression of other TCRs****:* (A) Percentage of GP33+ and GP33- IFNg producing CD8 T cells after PMA/Ionomycin stimulation. The P14 insert includes a gene encoding the TCRVβ8.1 chain. (B) Representative flow plots and bar graphs of individual expression of TCRVb 6,12, 11, and 5.1/5.2 in P14 and DSKO P14 mice. (C) Proportion of GP33+ CD8 T cells co-expressing TCRVb 6,12, 11, or 5.1/5.2 in DS P14 and control mice.P values calculated by one-way ANOVA with Tukey multiple comparisons test. **<0.005, *P<0.05.

4.3.5 Prf1$^{-/-}$ CD8 T cells in high IL-18 environments have altered responses to TCR engagement

From the data in **Section 4.3.1,** TCR stimulus seems to be required for IL-18 driven excess IFNγ production in CD8 T cells. Studies performed in Prf1$^{-/-}$ CD8 T cells suggest that they have longer engagement times with antigen presenting cells[7], resulting in increased production of IFNγ.

We were interested if IL-18 was augmenting the immune synapse in a similar manner. In our experiments, we confirmed that perforin deficiency, even haploinsufficiency, led to longer synapse times, however, IL-18 addition to perforin sufficient CD8 T cells decreased immune synapse times. In combination, excess IL-18 on $Prf1^{+/-}$ CD8 T cells almost completely reverted synapse duration back down to that of a WT synapse (**Figure 39A**). However, in $Prf1^{-/-}$ CD8 T cells, excess IL-18 was only able to partially reduce the elongated synapse duration.

We also saw that either excess IL-18 in perforin-sufficient cells, or perforin deficiency itself resulted in an increase in IFNγ secretion. Together, we saw that while IL-18 decreased the immune synapse duration in Perforin insufficient mice, the production of IFNγ was more than that of perforin insufficiency alone (**Figure 39B**). This suggests that these dual susceptibility factors not only converge on CD8 T cells, but that they synergize to create hyperactivated IFNγ 'super-producers'.

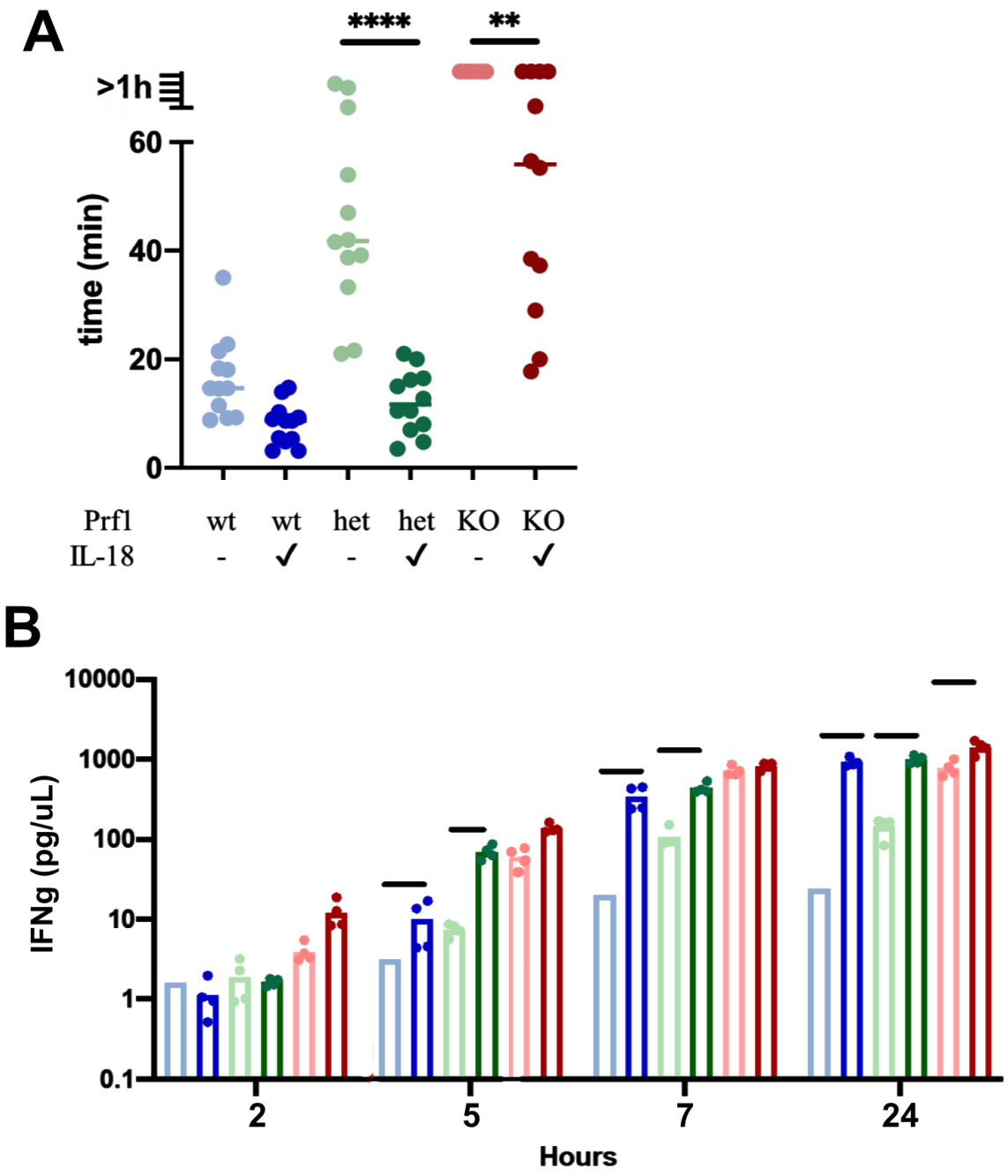


Figure 39: ***In vitro dual susceptability leads to shorter synapse time but increased IFNγ:*** (A) Synapse duration as calculated by the difference between initial calcium flux and target cell death (PI) and (B) IFNγ levels in supernatant after designated times in $Prf1^{+/+}$, $Prf1^{+/-}$, and $Prf1^{-/-}$ CD8 T cells in co-culture with cognate antigen presenting cells with or without excess IL-18. This work was performed and given express persmission for use in this thesis by **Anastasia Frank-Kamenetskii, PhD**.

4.3.6 Non-commensal antigens may drive CD8 T cell hyperactivation in DS mice

Given that CD8 T cells in DS mice showed increased proliferation, expression of exhaustion markers, and TCR-dependent IL-18 responses (*in vitro*), we sought to understand the nature of the TCR stimuli driving CD8 T cell activation. We found a very similar proportion of CD8 T cells were hyperactivated in 2-3 week old DS pups as in adult DS mice (**Figure 19D, E**), suggesting that the antigen(s) and immunologic circumstances needed to promote hyperactivation were present continuously. Bulk and single cell TCR sequencing substantiated this and further suggested that CD8 T cell expansion in DS mice is oligoclonal but not TCR-restricted.

Although DS mice do not develop mucosal immunopathology[96], one potential source of antigen driving T cell activation and expansion in DS mice could be commensal microbes. We therefore hypothesized that broad-spectrum antibiotics would decrease bacterial antigen load and diminish CD8 T cell activation and immunopathology. Aggressive decolonization with continuous, *ad libitum* ingestion of ampicillin, vancomycin, metronidazole, and neomycin did not appreciably alter CD8 T cell activation or clinical features of hyperinflammation in DS mice (**Figure 40**).

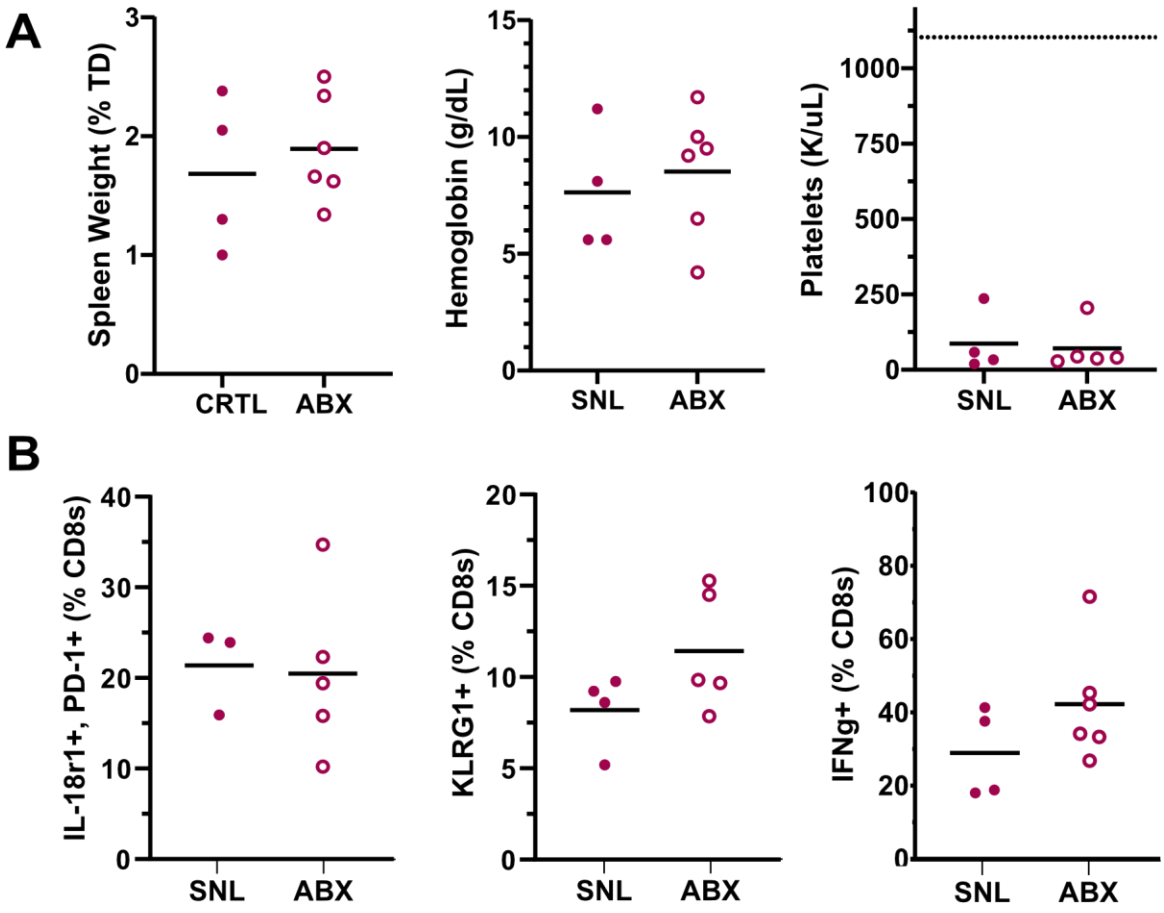


Figure 40: ***Dual Susceptability hyperinflammation is not ameliorated by broad-spectrum antibiotic treatment.*** Splenomegaly, anemia, and thrombocytopenia in DSKO mice after 30+ days of antibiotic treatment with metronidazole, neomycin, ampicillin, and vancomycin. Dotted line represents average platelet count for WT mice. (E) CD8 T cell activation and ex vivo stimulated IFNγ production in DSKO mice after treatment. All graphs are combined from 2 experiments, each point represents an individual mouse.

We then hypothesized that endogenous retroviruses (ERV) might be driving T cell activation in DS mice as they do in multiple sclerosis[319] and some primary human immunodeficiency virus (HIV) infections[320]. Treatment of adult DS mice with the anti-retroviral (ART) drugs emtricitabine and tenofovir daily for three weeks[310] which is sufficient to ablate susceptible retro viruses but did not significantly alter CD8 T cell hyperactivation or clinical features of hyperinflammation (**Figure 41**). Thus, neither commensal bacteria nor (ART-susceptible) ERVs appear necessary for driving spontaneous activation of CD8 T cells in DS mice.

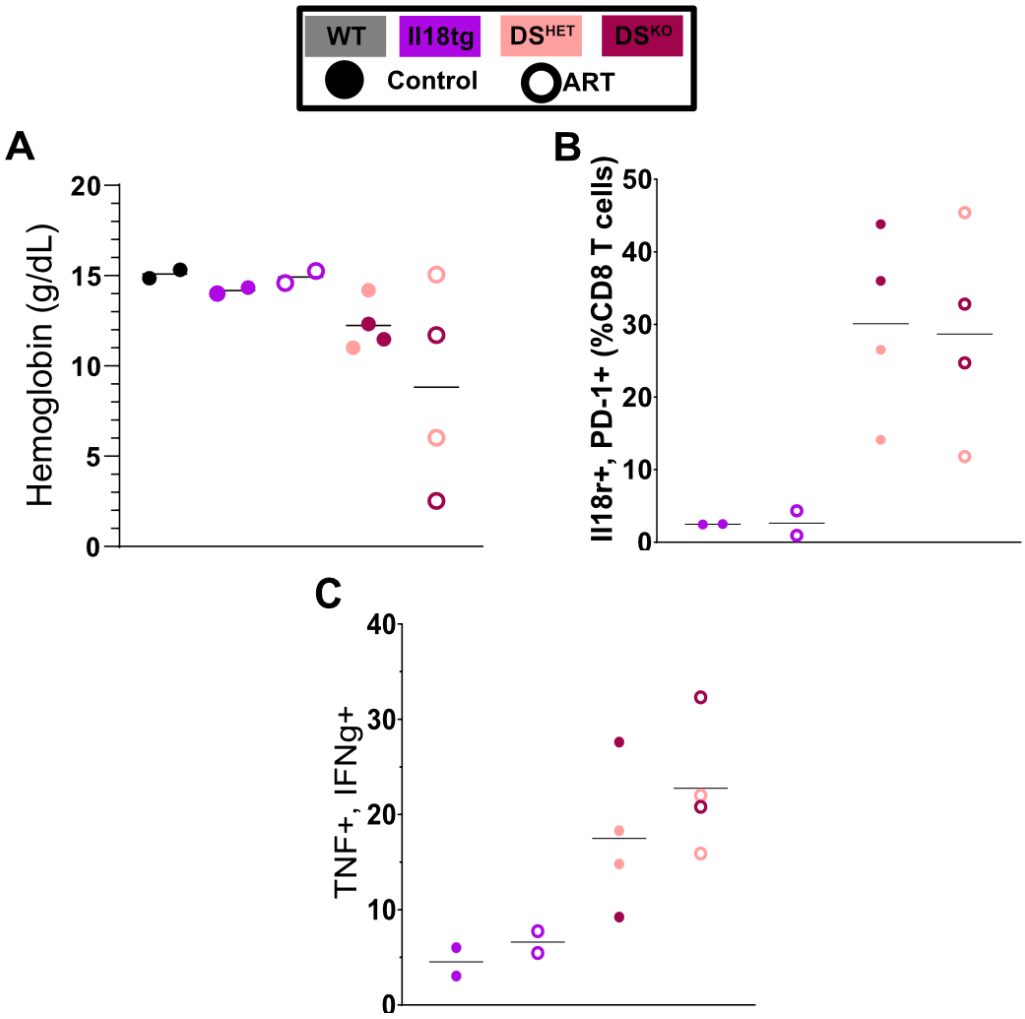


Figure 41: ***Antiretroviral treatment does not ameliorate disease in dual susceptibility mice :*** Il18tg and DS mice were provided daily (o.g.) with a combination of tenofovir (2.5 mg) and emtricitabine (1.5 mg) for three weeks and then assessed for hyperinflammation. (A) Hemoglobin levels, (B) percent of activated (IL-18r1+, PD-1+) splenic CD8 T cells, and (C) Percent of IFNγ and TNF producing CD8 splenic T cells after 4hr PMA and ionomycin stimulation.

4.4 Discussion

Mechanistically, DS mice may reveal the nature of immune defects likely to promote CD8 T cell hyperactivation and HLH. IL-18 appears to be a potent and possibly CD8-tropic amplifier of cytokine production, proliferation, and cytotoxicity. Compounding the effects of IL-18 induced IFNγ production with longer TCR engagement times as seen in $Prf1^{-/-}$ co-cultures with IL-18,

results in severely high levels of IFNγ production. In addition to inducing IL-18 binding protein via IFNg, our data suggest AICD/RICD are also important mechanisms regulating IL-18 effects. Thus, IL-18 (like LCMV, EBV, or other HLH stimuli) may stress cytotoxic function and thus increase sensitivity to common, partial defects like Prf1 haploinsufficiency[321]. Without sufficient cytotoxic capacity, these stimuli, can drive proliferation, persistence, cytokine production, and potentially inflammatory cell death (e.g., necroptosis) or exhaustion.

Spontaneous HLH in DS mice gave rise to an oligoclonal expansion of hyperactivated CD8 T cells, but different DS mice did not share CD8 TCR clonotypes. The introduction of the P14 TCR transgene created an in vivo competition between the abundant GP33-specific CD8 T cells and the rare CD8 T cells that escaped allelic exclusion. CD8 T cells not expressing the P14 TCR were at a clear advantage for expansion driven by IL-18 and longevity driven by perforin-deficiency failing to meet the increased demand for regulation. Notably, $Prf1^{-/-}$ CD19 chimeric antigen receptor T cells (CD19 CAR-T) may expand even after elimination of target antigen when WT CD19 CAR-T have contracted[322], suggesting an important cell-intrinsic or fratricidal role for *Prf1* in CD8 T cells.

T cell receptor stimulation appears to be critical to both susceptibility factors. In vitro, IL-18 became relatively inactive on T cells after just a few days without TCR stimulation. Hyperactivated CD8 T cells have shown remarkable persistence despite (attempted) IL-18 receptor deletion, broad-spectrum antibiotics, antiretrovirals, etoposide, and attempts at TCR fixation. Etoposide (chosen for its CD8-specificity in the $Prf1^{-/-}$ LCMV infection model[317]) was ineffective in treating spontaneous HLH in DS mice, but longer use would likely compound HLH phenotypes due to bone marrow suppression[323].

5.0 Extended Discussions and Summary

5.1 Fas deficiency in the context of excess chronic IL-18 may lead to CD8 T cell hyperactivation

Considering the significant finding of synergy between perforin and IL-18 in DS mice, we were curious to see if we could extend our findings to encompass other cell death factors. While our data suggests that perforin plays a role in activation-induced cell death, Fas/FasL signaling has canonically been considered as the major mechanism of terminating an immune response via T cell contraction after pathogen clearance. There are two main modes of CTL termination by Fas/FasL, autologous killing aka 'suicide' by FasL triggering Fas on the same cell or 'fratricide' in which FasL on T cells trigger Fas on neighboring T cells[324-326].

Mutations in *Fas* or *Fasl* lead to autoimmune lymphoproliferative syndrome (ALPS) which is characterized by organomegaly, antibody-mediated cytopenias (differing mechanism of cytopenias than HLH), and risk lymphoma[327]. In mice, the lpr strains harbor a mutation causing pre-mature termination in the transcription in the *Fas* gene[328]. These mice spontaneously develop systemic lupus erythematosus (SLE) and provide a mouse model for this disease in humans. Studies in B6.MRL-Fas^{lpr}/J mice (JAX #000482) infected with acute LCMV show normal levels of viral clearance, CD8 T cell expansion and contraction with no reports of a hyperinflammatory clinical phenotype[329].

We crossed B6.MRL-Fas^{lpr}/J mice with Il18tg mice to assess if lack of functional Fas would synergize with elevated IL-18 similar to the effects of perforin deficiency in settings of chronic excess IL-18. Preliminary assessments in adult Il18tg mice that were homozygous for the

Fas^{lpr} mutation (Fas^{KO}Il18tg) showed no signs of lethal hyperinflammation up to 10 weeks of age. Splenomegaly was mild in Fas^{KO}Il18tg mice compared to DS^{KO} mice, although Fas^{KO}Il18tg mice did show increased levels of splenic CD8 T cells and an increased level of $CD44^{-/Low}CD62L^{-}$ CD8 T cells, similar to DS^{KO} mice. Interestingly, the levels of IL-18 responsive (IL-18r1+) CD8 T cells are only elevated to the same degree as Il18tg and DS^{HET} mice. This suggests, consistent with previous findings, that perforin deficiency offers a unique circumstance for IL-18 augmented CD8 T cell hyperactivation. However, more studies need to be completed on this mouse model to assess IFNγ production and responses to infection.

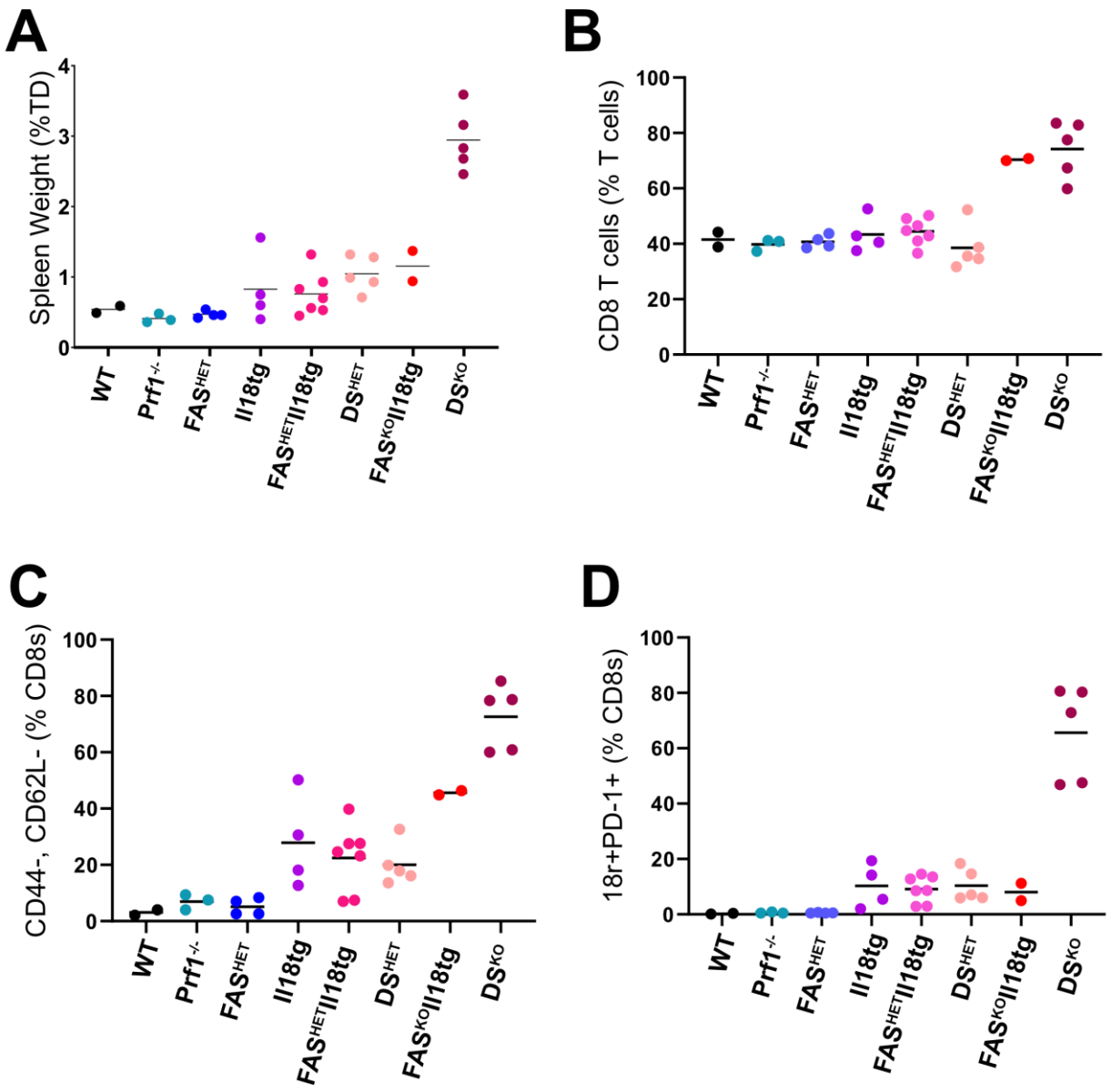


Figure 42: ***CD8 T cell have robust expansion in Fas deficienct Il18tg mice:*** Adult (8-10 weeks old) mice of indicated genotypes were assesed for (A) splenomegaly and (B) splenic CD8 T cell expansion. Indication of CD8 T cell hyperactivation was also assesed by measure of (C) $CD44^{-/LOW}$ $CD62L^{-}$ and (D) IL-18r1 expression. Statistical significance was determine usign one way ANOVA with Tukey multiple comparisons. Significance is only shown for comparisons to FAS^{KO}Il18tg.

5.2 Recent implementations of IL-18 and Perforin in human health

5.2.1 CART therapy

One of the greatest recent achievements for bench to bedside translational sciences has been the use of chimeric antigen receptor T (CAR-T) cells for use as cancer therapeutics. So far, there have been six variations of CAR T cell therapy approved[330]. Our data, as well as that of many others have shown that IL-18 can induce a potent Th1 phenotype in CD8 T cells which could prove beneficial in the face of immunosuppression imposed by cancers. In the mid-2000's, phase I trials tested recombinant IL-18 in a variety of advanced cancers. These trials demonstrated safety, but showed no significant evidence of efficacy so these development programs were halted[331,332]. Now, several studies have revived interest in IL-18 as a potential cancer therapeutic, and findings from these studies could help to explain earlier failures.

The addition of *IL18* to CART lentiviral[191] or retroviral[333] constructs resulted in amplified $CD4^+$ and $CD8^+$ CART cell responses in vitro and in animal models. This construct reportedly included the complete *IL18* cDNA, encoding full-length proIL-18, yet transduced cells secreted bioactive IL-18. Thus, how bioactive IL-18 was processed and released from CART cells remains unanswered questions. Early results in eight patients with refractory lymphoma who were treated with IL18-secreting CD19 CART cells showed promising results and, somewhat surprisingly, no apparent increase in the frequency or severity of CRS[334].

Perforin deficiency in CAR-T cells has, for obvious efficacy reasons, not be used in humans. They have still been useful in understanding therapeutic outcomes. Interestingly, perforin is dispensable during the initial phase of target cell clearance[335] due to redundancies in clearance like Fas/FasL and augmentation of the tumor microenvironment[336]. Perforin-deficient CAR-T cells

have been used in mouse models of cancer to recapitulate late-onset inflammatory toxicities often seen in patients after CAR T therapy. Interestingly in these studies, Perforin-deficient CAR-T cells have been shown to re-expand after the targets of these cells are cleared, leading to mild hyperinflammation[337].

5.2.2 Decoy Resistant IL-18

Our studies of IL-18 provide clear evidence that IL-18 is a potent stimulator of IFNγ-driven inflammation in the setting of TCR stimulus. Naturally, attempts to harness the pro-inflammatory power of IL-18 for immunotherapeutic applications against immunosuppressive targets like cancer have been made. Early failures of recombinant IL-18 treatment were due, at least in part, to the abundance of IL-18BP in patients with cancer, particularly in the tumor microenvironment. One group has. demonstrated robust IL-18BP production by a variety of tumor types, and engineered a 'decoy-resistant' version of IL-18 (DR-18) that agonized the IL-18 receptor but was resistant to neutralization by IL-18BP[111]. Murine DR-18 showed dramatic efficacy in several liquid and solid tumors and specifically augmented graft-versus-tumor (rather than graft-versus-host) effects in a transplant model of multiple myeloma treatment[111,338]. Engineered T cells with mRNA encoding decoy resistant IL-18 alongside IL-12 have been shown to mediate significantly enhanced tumor clearance in B16 melanoma mouse models[339]. Trials of DR-18 in human malignancies are underway[340].

5.3 Overall Summary

Hyperinflammatory diseases account for nearly 11% of a large and heterogeneous group of diseases collectively called systemic inflammatory response syndromes (SIRS)[341] and are defined as an overactivation of the inflammatory response to triggers such as infection or injury resulting in unwanted destruction of healthy cells and tissues. HLH and MAS are similar in their hyperinflammatory presentation, but MAS patients have been noted to have extremely elevated serum IL-18. Standard treatment for these patients is bone marrow transplantation which can only happen *after* inflammation has decreased to a manageable level which itself requires pretreatment that delays potentially curative bone marrow transplant. Thus, in this dissertation I aimed to define the underlying mechanisms that are shared or distinct between HLH and MAS.

Section 2.0, explored how either perforin deficiency or excess chronic IL-18 is sufficient for the development of hyperinflammation. In response to infection, both $Prf1^{-/-}$ and Il18tg mice show clinical manifestations of hyperinflammation including anemia, splenomegaly, cytokine storm, liver damage, and hemophagocytosis. Both of these susceptibility factors independently led to CD8 T cell specific hyperactivation causing excess IFNγ production with a requirement of antigenic stimulus.

Section 3.0, detailed the synergistic effects of perforin deficiency and excess IL-18 on CD8 T cell hyperactivation in dual susceptibility (DS) mice. These mice recapitulate emerging case reports showing a 4-5-fold increase of hemizygous cytotoxic gene defects within cohorts of patients with underlying rheumatic hyperinflammation such as sJIA/Still's Disease. DS^{KO} mice showed spontaneous, lethal hyperinflammation, with DS^{HET} mice even having intermediate yet pronounced hyperinflammation. While CD4 and other immune cells were relatively unaffected,

pathogenic CD8 T cells in DS mice were hyperexpanded, hyperactivated and had hyperclonal TCR repertoires.

Section 2.0 aimed to understand the mechanisms driving pathological CD8 T cell hyperactivation and how each susceptibility factor contributed to this phenotype. *In vitro* and *in vivo* studies showed that CD8 T cells required active TCR stimulation to allow IL-18 driven hyperactivation and enhanced IFNγ production. In the case of DS mice, the source of this might be non-commensal self-antigens. IL-18 also decreased immune synapse duration, but this was mildly abrogated in $Prf1^{-/-}$ CD8 T cells under the same conditions *in vitro* while further increasing IFNγ production. *In vivo* chronic, excess IL-18 production in Il18tg and DS mice led to CD8 T cell specific proliferation. Additionally, *in vitro,* IL-18 signaling increased reactivation-induced cell death, likely a natural immunosuppressive checkpoint, although perforin deficiency circumvented this allowing for extended cell survival.

Our data suggest that the hyperinflammatory susceptibility factors of perforin deficiency and chronic excess IL-18 independently, and synergistically converge on clonally expanded, hyperactivated CD8 T cells. Evidence suggests, but is not yet conclusive, that these cells are responding to self-antigen *in vivo*. Hyperactivated CD8 T cells contribute to the pathogenesis of hyperinflammation by means of decreased activation induced cell death allowing for a longer duration of IFNγ production.

One question still left unanswered surrounds the nature and timing of the antigenic stimulus required for initial activation of T_{HYP}. While our antibiotic experiments in DS mice suggest commensal antigens are not the trigger, the hyperinflammation seen in these mice are likely accumulated over time. Antibiotic treatment at an earlier age or rederiving these mice into a germ-free environment may be able to provide a clearer answer to whether commensal antigens play a

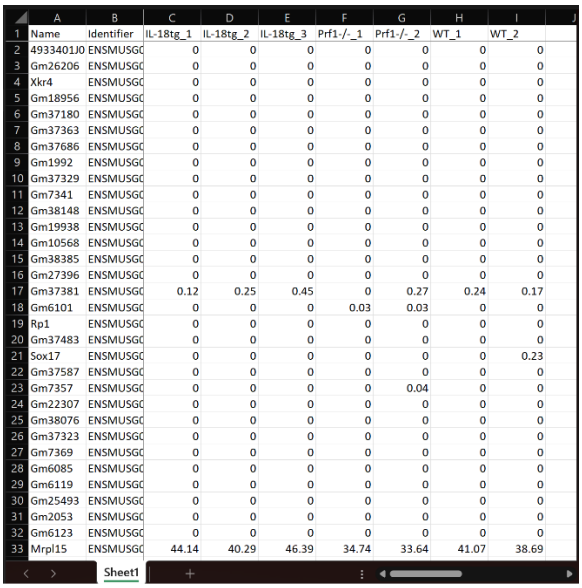
role in CD8 T cell hyperactivation. Understanding the timing of antigenic stimulus in CD8 T_{HYP} could provide valuable information for the treatment courses given to hyperinflammatory patients. Studies involving repeated, non-identical, immunizations in DS mice would allow for analyzing T_{HYP} responses to controlled antigen exposures. Similary, longitudinal TCR sequencing within the same mouse could provide valuable information of the terminal velocity, bystander activation, and overall evolution of T_{HYP} CD8 T cells throughout the mouse lifespan.

While we believe that T_{HYP} CD8 T cells are a driving pathogenic cell type in hyperinflammation, simple depletion of CD8 T cells was not enough to ameliorate disease in our DS mouse model. It remains unknown what other cell types might be involved in the hyperinflammation seen in DS mice. Since DS mice have abnormally low levels of NK and CD19+ B cells they are likely not a major player in hyperinflammation. Myeloid cells, on the other hand, may play a role in antigen presentation and skewing towards a Th1 immune response. Antigenic stimulus through TCR:MHC I interactions is a requirement for T_{HYP} and is likely highly impacted by APCs. Treatment of DS mice with compounds blocking antigen presentation, like cyclosporin A^{342}, may provide insights into the role of APCs in this context. Myeloid cells are particularly responsive to IFNγ, elevated levels of IFNγ as seen in DS mice, likely promote a more pro-inflammatory phenotype in the myeloid population further propagating a hyperinflammatory phenotype.

Together the data in this dissertation have provided key insights into the mechanistic underpinnings of hyperinflammation. Specifically, we have shown that hyperinflammation driven by pathogenic CD8 T cells requires antigenic stimulus, however this antigenic source may be self or “virtual” in nature. We also have shown a requirement for the loss (even partial) of cell death signals that are typically control immune responses. Furthermore, we have defined a new CD8 T

cell hyperactivated phenotype. This T_{HYP} has a mixed transcriptional profile of both effector and terminal exhaustion components. T_{HYP} CD8 T cells likely represent the cell type involved in the damaging pathway from naïve to exhausted CD8 T cells under the circumstances of chronic TCR stimulus and excess CD8-tropic inflammatory signals like IL-18.

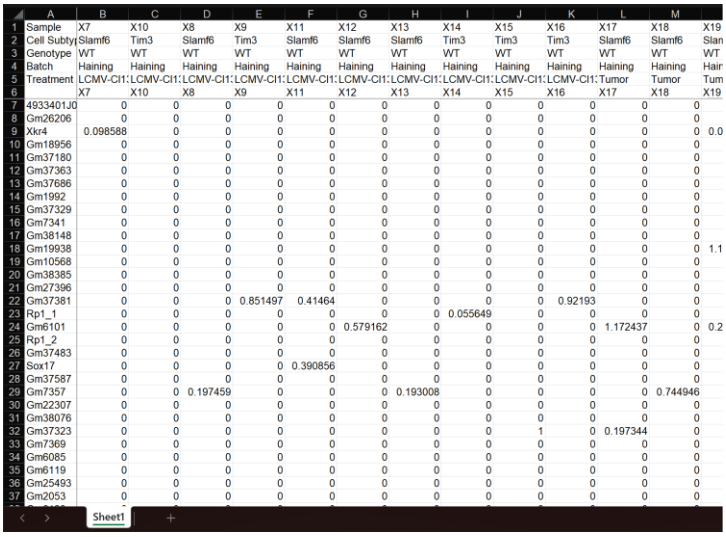
Appendix A Supplemental Data and Files



	A	B	C	D	E	F	G	H	I
1	Name	Identifier	IL-18tg_1	IL-18tg_2	IL-18tg_3	Prf1-/-_1	Prf1-/-_2	WT_1	WT_2
2	4933401J0	ENSMUSG0	0	0	0	0	0	0	0
3	Gm26206	ENSMUSG0	0	0	0	0	0	0	0
4	Xkr4	ENSMUSG0	0	0	0	0	0	0	0
5	Gm18956	ENSMUSG0	0	0	0	0	0	0	0
6	Gm37180	ENSMUSG0	0	0	0	0	0	0	0
7	Gm37363	ENSMUSG0	0	0	0	0	0	0	0
8	Gm37686	ENSMUSG0	0	0	0	0	0	0	0
9	Gm1992	ENSMUSG0	0	0	0	0	0	0	0
10	Gm37329	ENSMUSG0	0	0	0	0	0	0	0
11	Gm7341	ENSMUSG0	0	0	0	0	0	0	0
12	Gm38148	ENSMUSG0	0	0	0	0	0	0	0
13	Gm19938	ENSMUSG0	0	0	0	0	0	0	0
14	Gm10568	ENSMUSG0	0	0	0	0	0	0	0
15	Gm38385	ENSMUSG0	0	0	0	0	0	0	0
16	Gm27396	ENSMUSG0	0	0	0	0	0	0	0
17	Gm37381	ENSMUSG0	0.12	0.25	0.45	0	0.27	0.24	0.17
18	Gm6101	ENSMUSG0	0	0	0	0.03	0.03	0	0
19	Rp1	ENSMUSG0	0	0	0	0	0	0	0
20	Gm37483	ENSMUSG0	0	0	0	0	0	0	0
21	Sox17	ENSMUSG0	0	0	0	0	0	0	0.23
22	Gm37587	ENSMUSG0	0	0	0	0	0	0	0
23	Gm7357	ENSMUSG0	0	0	0	0	0.04	0	0
24	Gm22307	ENSMUSG0	0	0	0	0	0	0	0
25	Gm38076	ENSMUSG0	0	0	0	0	0	0	0
26	Gm37323	ENSMUSG0	0	0	0	0	0	0	0
27	Gm7369	ENSMUSG0	0	0	0	0	0	0	0
28	Gm6085	ENSMUSG0	0	0	0	0	0	0	0
29	Gm6119	ENSMUSG0	0	0	0	0	0	0	0
30	Gm25493	ENSMUSG0	0	0	0	0	0	0	0
31	Gm2053	ENSMUSG0	0	0	0	0	0	0	0
32	Gm6123	ENSMUSG0	0	0	0	0	0	0	0
33	Mrpl15	ENSMUSG0	44.14	40.29	46.39	34.74	33.64	41.07	38.69

Sheet1

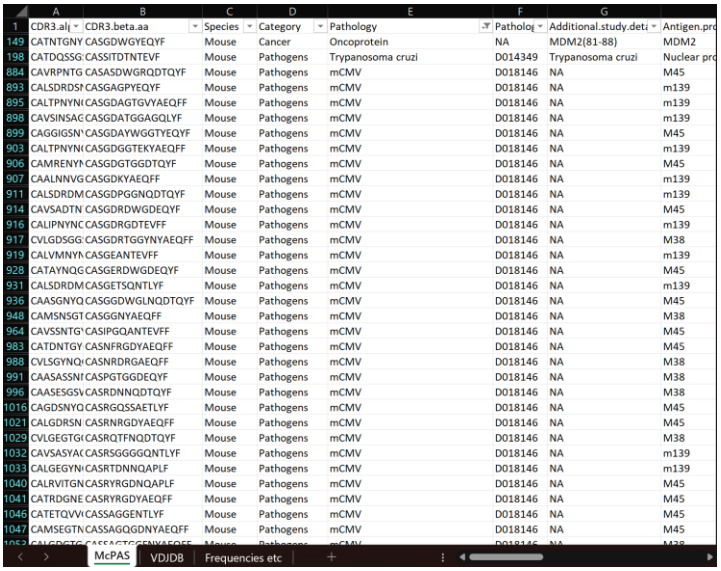
Supplemental File 1: ***Bulk RNAseq Expression Matrix of CD8 T cells in LCMV infected mice:*** Expression of Il18tg, Prf1$^{-/-}$, and Wild type splenic CD8 T cells 8dpi with acute LCMV (armstrong). The file can be found for download at https://d-scholarship.pitt.edu/45415/2/CD8_gene%20expression%20browser.xlsx



	A	B	C	D	E	F	G	H	I	J	K	L	M	
1	Sample	X7	X10	X8	X9	X11	X12	X13	X14	X15	X16	X17	X18	X19
2	Cell Subty	Slamf6	Tim3	Slamf6	Tim3	Slamf6	Slamf6	Slamf6	Tim3	Tim3	Tim3	Slamf6	Slamf6	Slan
3	Genotype	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
4	Batch	Haining	Haining	Haining	Haining	Haining	Haining	Haining	Haining	Haining	Haining	Haining	Haining	Hair
5	Treatment	LCMV-Cl1	LCMV-Cl1	LCMV-Cl1	LCMV-Cl1	LCMV-Cl1	LCMV-Cl1	LCMV-Cl1	LCMV-Cl1	LCMV-Cl1	LCMV-Cl1	Tumor	Tumor	Tum
6		X7	X10	X8	X9	X11	X12	X13	X14	X15	X16	X17	X18	X19
7	4933401J0	0	0	0	0	0	0	0	0	0	0	0	0	
8	Gm26206	0	0	0	0	0	0	0	0	0	0	0	0	
9	Xkr4	0.098588	0	0	0	0	0	0	0	0	0	0	0	0.0
10	Gm18956	0	0	0	0	0	0	0	0	0	0	0	0	
11	Gm37180	0	0	0	0	0	0	0	0	0	0	0	0	
12	Gm37363	0	0	0	0	0	0	0	0	0	0	0	0	
13	Gm37686	0	0	0	0	0	0	0	0	0	0	0	0	
14	Gm1992	0	0	0	0	0	0	0	0	0	0	0	0	
15	Gm37329	0	0	0	0	0	0	0	0	0	0	0	0	
16	Gm7341	0	0	0	0	0	0	0	0	0	0	0	0	
17	Gm38148	0	0	0	0	0	0	0	0	0	0	0	0	
18	Gm19938	0	0	0	0	0	0	0	0	0	0	0	0	1.1
19	Gm10568	0	0	0	0	0	0	0	0	0	0	0	0	
20	Gm38385	0	0	0	0	0	0	0	0	0	0	0	0	
21	Gm27396	0	0	0	0	0	0	0	0	0	0	0	0	
22	Gm37381	0	0	0	0.851497	0.41464	0	0	0	0	0.92193	0	0	
23	Rp1_1	0	0	0	0	0	0	0	0.055649	0	0	0	0	
24	Gm6101	0	0	0	0	0	0.579162	0	0	0	0	1.172437	0	0.2
25	Rp1_2	0	0	0	0	0	0	0	0	0	0	0	0	
26	Gm37483	0	0	0	0	0	0	0	0	0	0	0	0	
27	Sox17	0	0	0	0	0.390856	0	0	0	0	0	0	0	
28	Gm37587	0	0	0	0	0	0	0	0	0	0	0	0	
29	Gm7357	0	0	0.197459	0	0	0	0.193008	0	0	0	0	0.744946	
30	Gm22307	0	0	0	0	0	0	0	0	0	0	0	0	
31	Gm38076	0	0	0	0	0	0	0	0	0	0	0	0	
32	Gm37323	0	0	0	0	0	0	0	0	1	0	0.197344	0	
33	Gm7369	0	0	0	0	0	0	0	0	0	0	0	0	
34	Gm6085	0	0	0	0	0	0	0	0	0	0	0	0	
35	Gm6119	0	0	0	0	0	0	0	0	0	0	0	0	
36	Gm25493	0	0	0	0	0	0	0	0	0	0	0	0	
37	Gm2053	0	0	0	0	0	0	0	0	0	0	0	0	

Sheet1

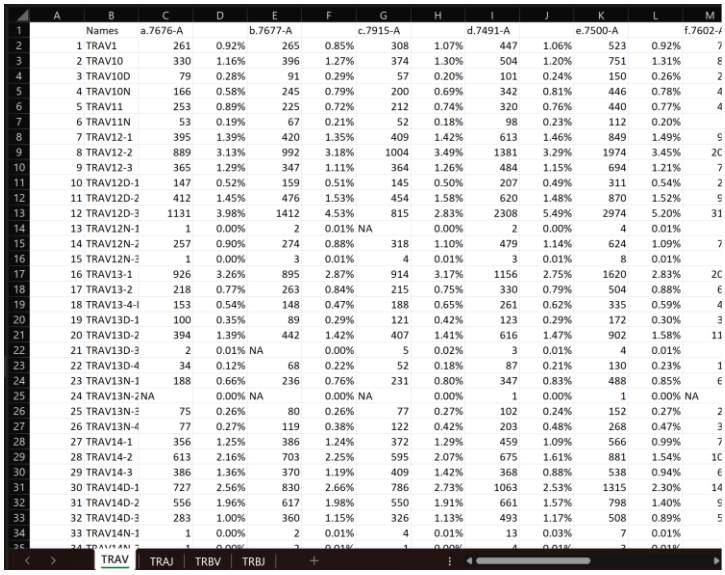
Supplemental File 2: ***Combat adjusted Dual Susceptability, Teminally Exhausted, and Exhaustion Progenitor CD8 T cell Bulk RNAseq Expression Matrix :*** This file can be found for download at https://d-scholarship.pitt.edu/45415/1/HAINING_HOMEOSTATIC_COMBATADJ-metadata_Annotated.xlsx



	A	B	C	D	E	F	G	
1	CDR3.alp	CDR3.beta.aa	Species	Category	Pathology	Patholog	Additional.study.deta	Antigen.pro
149	CATNTGNY	CASGDWGYEQYF	Mouse	Cancer	Oncoprotein	NA	MDM2(81-88)	MDM2
198	CATDQSSG	CASSITDTNTEVF	Mouse	Pathogens	Trypanosoma cruzi	D014349	Trypanosoma cruzi	Nuclear pro
884	CAVRPNTG	CASASDWGRQDTQYF	Mouse	Pathogens	mCMV	D018146	NA	M45
893	CALSDRDS	CASGAGPYEQYF	Mouse	Pathogens	mCMV	D018146	NA	m139
895	CALTPNYN	CASGDAGTGVYAEQFF	Mouse	Pathogens	mCMV	D018146	NA	m139
898	CAVSINSA	CASGDATGGAGQLYF	Mouse	Pathogens	mCMV	D018146	NA	m139
899	CAGGIGSN	CASGDAYWGGTYEQYF	Mouse	Pathogens	mCMV	D018146	NA	M45
903	CALTPNYN	CASGDGGTEKYAEQFF	Mouse	Pathogens	mCMV	D018146	NA	m139
906	CAMRENYN	CASGDGTGGDTQYF	Mouse	Pathogens	mCMV	D018146	NA	M45
907	CAALNNVG	CASGDKYAEQFF	Mouse	Pathogens	mCMV	D018146	NA	m139
911	CALSDRDM	CASGDPGGNQDTQYF	Mouse	Pathogens	mCMV	D018146	NA	m139
914	CAVSADTN	CASGDRDWGDEQYF	Mouse	Pathogens	mCMV	D018146	NA	M45
916	CALIPNYN	CASGDRGDTEVFF	Mouse	Pathogens	mCMV	D018146	NA	m139
917	CVLGDSGG	CASGDRTGGYNYAEQFF	Mouse	Pathogens	mCMV	D018146	NA	M38
919	CALVMNYN	CASGEANTEVFF	Mouse	Pathogens	mCMV	D018146	NA	m139
928	CATAYNQG	CASGERDWGDEQYF	Mouse	Pathogens	mCMV	D018146	NA	M45
931	CALSDRDM	CASGETSQNTLYF	Mouse	Pathogens	mCMV	D018146	NA	m139
936	CAASGNYQ	CASGGDWGLNQDTQYF	Mouse	Pathogens	mCMV	D018146	NA	M45
948	CAMSNSGT	CASGGNYAEQFF	Mouse	Pathogens	mCMV	D018146	NA	M38
964	CAVSSNTG	CASIPGQANTEVFF	Mouse	Pathogens	mCMV	D018146	NA	M45
983	CATDNTGY	CASNFRGDYAEQFF	Mouse	Pathogens	mCMV	D018146	NA	M45
988	CVLSGYNQ	CASNRDRGAEQFF	Mouse	Pathogens	mCMV	D018146	NA	M38
991	CAASASSN	CASPGTGGDEQYF	Mouse	Pathogens	mCMV	D018146	NA	M38
996	CAASESGS	CASRDNNQDTQYF	Mouse	Pathogens	mCMV	D018146	NA	M38
1016	CAGDSNYQ	CASRGQSSAETLYF	Mouse	Pathogens	mCMV	D018146	NA	M45
1021	CALGDRSN	CASRNRGDYAEQFF	Mouse	Pathogens	mCMV	D018146	NA	M45
1029	CVLGEGTG	CASRQTFNQDTQYF	Mouse	Pathogens	mCMV	D018146	NA	M38
1032	CAVSASYA	CASRSGGGGQNTLYF	Mouse	Pathogens	mCMV	D018146	NA	m139
1033	CALGEGYN	CASRTDNNQAPLF	Mouse	Pathogens	mCMV	D018146	NA	m139
1040	CALRVITG	CASRYRGDNQAPLF	Mouse	Pathogens	mCMV	D018146	NA	M45
1041	CATRDGNE	CASRYRGDYAEQFF	Mouse	Pathogens	mCMV	D018146	NA	M45
1046	CATETQVV	CASSAGGENTLYF	Mouse	Pathogens	mCMV	D018146	NA	M45
1047	CAMSEGTN	CASSAGQGDNYAEQFF	Mouse	Pathogens	mCMV	D018146	NA	M45

McPAS | VDJDB | Frequencies etc

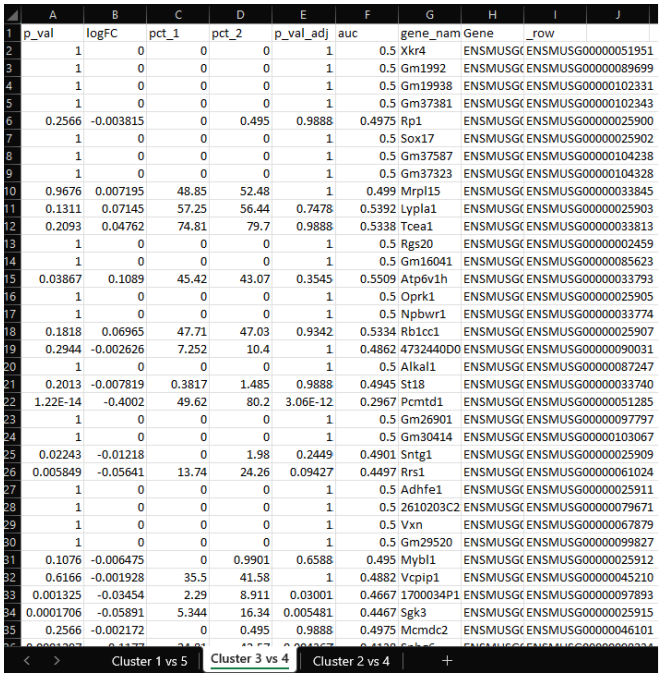
Supplemental File 3: ***Analysis of known TCR epitope frequencies in dual susceptanility CD8 T cells****:*CDR3 sequences from Prf1$^{-/-}$, Il18tg, DS^{HET}, and DS^{KO} CD8 T cells were compared to kown epitope CD3 sequences found in McPAS and VDJDB databases. This file can be downloaded at https://d-scholarship.pitt.edu/45415/3/KnownEpitopes.xlsx



	A	B	C	D	E	F	G	H	I	J	K	L	M
1		Names	a.7676-A		b.7677-A		c.7915-A		d.7491-A		e.7500-A		f.7602-A
2	1	TRAV1	261	0.92%	265	0.85%	308	1.07%	447	1.06%	523	0.92%	7
3	2	TRAV10	330	1.16%	396	1.27%	374	1.30%	504	1.20%	751	1.31%	8
4	3	TRAV10D	79	0.28%	91	0.29%	57	0.20%	101	0.24%	150	0.26%	2
5	4	TRAV10N	166	0.58%	245	0.79%	200	0.69%	342	0.81%	446	0.78%	4
6	5	TRAV11	253	0.89%	225	0.72%	212	0.74%	320	0.76%	440	0.77%	4
7	6	TRAV11N	53	0.19%	67	0.21%	52	0.18%	98	0.23%	112	0.20%	
8	7	TRAV12-1	395	1.39%	420	1.35%	409	1.42%	613	1.46%	849	1.49%	9
9	8	TRAV12-2	889	3.13%	992	3.18%	1004	3.49%	1381	3.29%	1974	3.45%	20
10	9	TRAV12-3	365	1.29%	347	1.11%	364	1.26%	484	1.15%	694	1.21%	7
11	10	TRAV12D-1	147	0.52%	159	0.51%	145	0.50%	207	0.49%	311	0.54%	2
12	11	TRAV12D-2	412	1.45%	476	1.53%	454	1.58%	620	1.48%	870	1.52%	9
13	12	TRAV12D-3	1131	3.98%	1412	4.53%	815	2.83%	2308	5.49%	2974	5.20%	31
14	13	TRAV12N-1	1	0.00%	2	0.01%	NA	0.00%	2	0.00%	4	0.01%	
15	14	TRAV12N-2	257	0.90%	274	0.88%	318	1.10%	479	1.14%	624	1.09%	7
16	15	TRAV12N-3	1	0.00%	3	0.01%	4	0.01%	3	0.01%	8	0.01%	
17	16	TRAV13-1	926	3.26%	895	2.87%	914	3.17%	1156	2.75%	1620	2.83%	20
18	17	TRAV13-2	218	0.77%	263	0.84%	215	0.75%	330	0.79%	504	0.88%	6
19	18	TRAV13-4-I	153	0.54%	148	0.47%	188	0.65%	261	0.62%	335	0.59%	4
20	19	TRAV13D-1	100	0.35%	89	0.29%	121	0.42%	123	0.29%	172	0.30%	3
21	20	TRAV13D-2	394	1.39%	442	1.42%	407	1.41%	616	1.47%	902	1.58%	11
22	21	TRAV13D-3	2	0.01%	NA	0.00%	5	0.02%	3	0.01%	4	0.01%	
23	22	TRAV13D-4	34	0.12%	68	0.22%	52	0.18%	87	0.21%	130	0.23%	1
24	23	TRAV13N-1	188	0.66%	236	0.76%	231	0.80%	347	0.83%	488	0.85%	6
25	24	TRAV13N-2	NA	0.00%	NA	0.00%	NA	0.00%	1	0.00%	1	0.00%	NA
26	25	TRAV13N-3	75	0.26%	80	0.26%	77	0.27%	102	0.24%	152	0.27%	2
27	26	TRAV13N-4	77	0.27%	119	0.38%	122	0.42%	203	0.48%	268	0.47%	3
28	27	TRAV14-1	356	1.25%	386	1.24%	372	1.29%	459	1.09%	566	0.99%	7
29	28	TRAV14-2	613	2.16%	703	2.25%	595	2.07%	675	1.61%	881	1.54%	10
30	29	TRAV14-3	386	1.36%	370	1.19%	409	1.42%	368	0.88%	538	0.94%	6
31	30	TRAV14D-1	727	2.56%	830	2.66%	786	2.73%	1063	2.53%	1315	2.30%	14
32	31	TRAV14D-2	556	1.96%	617	1.98%	550	1.91%	661	1.57%	798	1.40%	9
33	32	TRAV14D-3	283	1.00%	360	1.15%	326	1.13%	493	1.17%	508	0.89%	5
34	33	TRAV14N-1	1	0.00%	2	0.01%	4	0.01%	13	0.03%	7	0.01%	

TRAV | TRAJ | TRBV | TRBJ

Supplemental File 4: ***Variable and joining region gene usage in Dual Susceptability CD8 T cells:***This data was used to produce **Figure 29** and can be found at https://d-scholarship.pitt.edu/45415/4/GeneUsageALL.xlsx



	A	B	C	D	E	F	G	H	I	J
1	p_val	logFC	pct_1	pct_2	p_val_adj	auc	gene_nam	Gene	_row	
2	1	0	0	0	1	0.5	Xkr4	ENSMUSG(	ENSMUSG00000051951	
3	1	0	0	0	1	0.5	Gm1992	ENSMUSG(	ENSMUSG00000089699	
4	1	0	0	0	1	0.5	Gm19938	ENSMUSG(	ENSMUSG00000102331	
5	1	0	0	0	1	0.5	Gm37381	ENSMUSG(	ENSMUSG00000102343	
6	0.2566	-0.003815	0	0.495	0.9888	0.4975	Rp1	ENSMUSG(	ENSMUSG00000025900	
7	1	0	0	0	1	0.5	Sox17	ENSMUSG(	ENSMUSG00000025902	
8	1	0	0	0	1	0.5	Gm37587	ENSMUSG(	ENSMUSG00000104238	
9	1	0	0	0	1	0.5	Gm37323	ENSMUSG(	ENSMUSG00000104328	
10	0.9676	0.007195	48.85	52.48	1	0.499	Mrpl15	ENSMUSG(	ENSMUSG00000033845	
11	0.1311	0.07145	57.25	56.44	0.7478	0.5392	Lypla1	ENSMUSG(	ENSMUSG00000025903	
12	0.2093	0.04762	74.81	79.7	0.9888	0.5338	Tcea1	ENSMUSG(	ENSMUSG00000033813	
13	1	0	0	0	1	0.5	Rgs20	ENSMUSG(	ENSMUSG00000002459	
14	1	0	0	0	1	0.5	Gm16041	ENSMUSG(	ENSMUSG00000085623	
15	0.03867	0.1089	45.42	43.07	0.3545	0.5509	Atp6v1h	ENSMUSG(	ENSMUSG00000033793	
16	1	0	0	0	1	0.5	Oprk1	ENSMUSG(	ENSMUSG00000025905	
17	1	0	0	0	1	0.5	Npbwr1	ENSMUSG(	ENSMUSG00000033774	
18	0.1818	0.06965	47.71	47.03	0.9342	0.5334	Rb1cc1	ENSMUSG(	ENSMUSG00000025907	
19	0.2944	-0.002626	7.252	10.4	1	0.4862	4732440D0	ENSMUSG(	ENSMUSG00000090031	
20	1	0	0	0	1	0.5	Alkal1	ENSMUSG(	ENSMUSG00000087247	
21	0.2013	-0.007819	0.3817	1.485	0.9888	0.4945	St18	ENSMUSG(	ENSMUSG00000033740	
22	1.22E-14	-0.4002	49.62	80.2	3.06E-12	0.2967	Pcmtd1	ENSMUSG(	ENSMUSG00000051285	
23	1	0	0	0	1	0.5	Gm26901	ENSMUSG(	ENSMUSG00000097797	
24	1	0	0	0	1	0.5	Gm30414	ENSMUSG(	ENSMUSG00000103067	
25	0.02243	-0.01218	0	1.98	0.2449	0.4901	Sntg1	ENSMUSG(	ENSMUSG00000025909	
26	0.005849	-0.05641	13.74	24.26	0.09427	0.4497	Rrs1	ENSMUSG(	ENSMUSG00000061024	
27	1	0	0	0	1	0.5	Adhfe1	ENSMUSG(	ENSMUSG00000025911	
28	1	0	0	0	1	0.5	2610203C2	ENSMUSG(	ENSMUSG00000079671	
29	1	0	0	0	1	0.5	Vxn	ENSMUSG(	ENSMUSG00000067879	
30	1	0	0	0	1	0.5	Gm29520	ENSMUSG(	ENSMUSG00000099827	
31	0.1076	-0.006475	0	0.9901	0.6588	0.495	Mybl1	ENSMUSG(	ENSMUSG00000025912	
32	0.6166	-0.001928	35.5	41.58	1	0.4882	Vcpip1	ENSMUSG(	ENSMUSG00000045210	
33	0.001325	-0.03454	2.29	8.911	0.03001	0.4667	1700034P1	ENSMUSG(	ENSMUSG00000097893	
34	0.0001706	-0.05891	5.344	16.34	0.005481	0.4467	Sgk3	ENSMUSG(	ENSMUSG00000025915	
35	0.2566	-0.002172	0	0.495	0.9888	0.4975	Mcmdc2	ENSMUSG(	ENSMUSG00000046101	

Cluster 1 vs 5 | Cluster 3 vs 4 | Cluster 2 vs 4 | +

Supplemental File 5: ***Differential Expression between scRNAseq clusters:*** This data can be found at https://d-scholarship.pitt.edu/45415/5/DEG-Clusters.xlsx

So have you heard of the story between the Bird and the plane? So it's like the early 1950s and and plane were just getting popular amongst the rich. So this guy hops on the plane and sees there's a bird in a cage in the seat next to his. Probably just a rich person who bought the seat for the bird. So he sits down lights a cigar as the plane takes off (you could still smoke at the time on planes) and the bird peaks its head out of the cage, bites at the cigar, and then chews it up and spits it out. Guy is like "Oh weird ok." No problem, he has more cigars so he pulls another one out, lights it, and then starts smoking. Sure enough bird peaks its head out and chews it up and spits it out. So now the guy is a bit upset and says "Ok if this bird does it again, I'm going to throw it out of the plane." So he lights his cigar and of course bird chews it up and spits it out. So the guy takes the bird throws it out of the plane but the bird comes flying back in. It's choking on something. It's the red rock.

Supplemental File 6: ***The long joke:*** Congratulations you made it to the end of my work! This is the extension of the joke from the preface… this is intended to be told at a later time to cause confusion. Get it? The red rock never came back down because it got eaten by the bird. Haha. – courtsey of Vinh Dang.

Bibliography

1.Shakoory B, Geerlinks A, Wilejto M, et al. The 2022 EULAR/ACR points to consider at the early stages of diagnosis and management of suspected haemophagocytic lymphohistiocytosis/macrophage activation syndrome (HLH/MAS). *Ann Rheum Dis*. 2023.

2.Henter JI, Horne A, Arico M, et al. HLH-2004: Diagnostic and therapeutic guidelines for hemophagocytic lymphohistiocytosis. *Pediatr Blood Cancer*. 2007;48(2):124-131.

3.Canna SW, Cron RQ. Highways to hell: Mechanism-based management of cytokine storm syndromes. *J Allergy Clin Immunol*. 2020;146(5):949-959.

4.Carcillo JA, Halstead ES, Hall MW, et al. Three Hypothetical Inflammation Pathobiology Phenotypes and Pediatric Sepsis-Induced Multiple Organ Failure Outcome. *Pediatr Crit Care Med*. 2017;18(6):513-523.

5.Tangye SG, Al-Herz W, Bousfiha A, et al. Human Inborn Errors of Immunity: 2022 Update on the Classification from the International Union of Immunological Societies Expert Committee. *J Clin Immunol*. 2022;42(7):1473-1507.

6.Chinn IK, Eckstein OS, Peckham-Gregory EC, et al. Genetic and mechanistic diversity in pediatric hemophagocytic lymphohistiocytosis. *Blood*. 2018;132(1):89-100.

7.Jenkins MR, Rudd-Schmidt JA, Lopez JA, et al. Failed CTL/NK cell killing and cytokine hypersecretion are directly linked through prolonged synapse time. *J Exp Med*. 2015;212(3):307-317.

8.Anft M, Netter P, Urlaub D, Prager I, Schaffner S, Watzl C. NK cell detachment from target cells is regulated by successful cytotoxicity and influences cytokine production. *Cell Mol Immunol*. 2020;17(4):347-355.

9.Netter P, Anft M, Watzl C. Termination of the activating NK cell immunological synapse is an active and regulated process. *The Journal of Immunology*. 2017;199(7):2528-2535.

10.Shakoory B, Geerlinks A, Wilejto M, et al. POS0339 POINTS TO CONSIDER AT THE EARLIEST STAGES OF THE DIAGNOSIS AND MANAGEMENT OF HEMOPHAGOCYTIC LYMPHOHISTIOCYTOSIS/MACROPHAGE ACTIVATION SYNDROME (HLH/MAS). *Annals of the Rheumatic Diseases*. 2022;81(Suppl 1):422-422.

11.Weiss ES, Girard-Guyonvarc'h C, Holzinger D, et al. Interleukin-18 diagnostically distinguishes and pathogenically promotes human and murine macrophage activation syndrome. *Blood*. 2018;131(13):1442.

12.Canna SW, Girard C, Malle L, et al. Life-threatening NLRC4-associated hyperinflammation successfully treated with IL-18 inhibition. *Journal of Allergy and Clinical Immunology*. 2017;139(5):1698-1701.

13.Naftali T, Novick D, Gabay G, Rubinstein M, Novis B. Interleukin-18 and its binding protein in patients with inflammatory bowel disease during remission and exacerbation. *Isr Med Assoc J*. 2007;9(7):504-508.

14.Monteleone G, Trapasso F, Parrello T, et al. Bioactive IL-18 expression is up-regulated in Crohn's disease. *The Journal of Immunology*. 1999;163(1):143-147.

15.Leach ST, Messina I, Lemberg DA, Novick D, Rubenstein M, Day AS. Local and systemic interleukin-18 and interleukin-18-binding protein in children with inflammatory bowel disease. *Inflammatory bowel diseases*. 2007;14(1):68-74.

16.Duewell P, Kono H, Rayner KJ, et al. NLRP3 inflammasomes are required for atherogenesis and activated by cholesterol crystals. *Nature*. 2010;464(7293):1357-1361.
17.Gerdes N, Sukhova GK, Libby P, Reynolds RS, Young JL, Schonbeck U. Expression of interleukin (IL)-18 and functional IL-18 receptor on human vascular endothelial cells, smooth muscle cells, and macrophages: implications for atherogenesis. *J Exp Med*. 2002;195(2):245-257.
18.Kanai T, Uraushihara K, Totsuka T, et al. Macrophage-derived IL-18 targeting for the treatment of Crohn's disease. *Curr Drug Targets Inflamm Allergy*. 2003;2(2):131-136.
19.Konishi H, Tsutsui H, Murakami T, et al. IL-18 contributes to the spontaneous development of atopic dermatitis-like inflammatory skin lesion independently of IgE/stat6 under specific pathogen-free conditions. *Proc Natl Acad Sci U S A*. 2002;99(17):11340-11345.
20.Imaoka H, Gauvreau GM, Watson RM, et al. Interleukin-18 and interleukin-18 receptor-alpha expression in allergic asthma. *Eur Respir J*. 2011;38(4):981-983.
21.Emmanuilidis K, Weighardt H, Matevossian E, et al. Differential regulation of systemic IL-18 and IL-12 release during postoperative sepsis: high serum IL-18 as an early predictive indicator of lethal outcome. *Shock*. 2002;18(4):301-305.
22.Mudde ACA, Booth C, Marsh RA. Evolution of Our Understanding of XIAP Deficiency. *Front Pediatr*. 2021;9:660520.
23.Canna SW, de Jesus AA, Gouni S, et al. An activating NLRC4 inflammasome mutation causes autoinflammation with recurrent macrophage activation syndrome. *Nat Genet*. 2014;46(10):1140-1146.
24.Chiang SCC, Owsley E, Panchal N, et al. Quercetin ameliorates XIAP deficiency-associated hyperinflammation. *Blood*. 2022;140(7):706-715.
25.Coppola S, Insalaco A, Zara E, et al. Mutations at the C-terminus of CDC42 cause distinct hematopoietic and autoinflammatory disorders. *J Allergy Clin Immunol*. 2022;150(1):223-228.
26.Gernez Y, de Jesus AA, Alsaleem H, et al. Severe autoinflammation in 4 patients with C-terminal variants in cell division control protein 42 homolog (CDC42) successfully treated with IL-1β inhibition. *J Allergy Clin Immunol*. 2019;144(4):1122-1125.e1126.
27.Lam MT, Coppola S, Krumbach OHF, et al. A novel disorder involving dyshematopoiesis, inflammation, and HLH due to aberrant CDC42 function. *J Exp Med*. 2019;216(12):2778-2799.
28.Czar MJ, Kersh EN, Mijares LA, et al. Altered lymphocyte responses and cytokine production in mice deficient in the X-linked lymphoproliferative disease gene SH2D1A/DSHP/SAP. *Proc Natl Acad Sci U S A*. 2001;98(13):7449-7454.
29.Gomez-Rodriguez J, Wohlfert EA, Handon R, et al. Itk-mediated integration of T cell receptor and cytokine signaling regulates the balance between Th17 and regulatory T cells. *Journal of Experimental Medicine*. 2014;211(3):529-543.
30.Kannan A, Lee Y, Qi Q, et al. Allele-sensitive mutant, Itkas, reveals that Itk kinase activity is required for Th1, Th2, Th17, and iNKT-cell cytokine production. *European Journal of Immunology*. 2015;45(8):2276-2285.
31.Huang W, Jeong A-R, Kannan AK, Huang L, August A. IL-2–inducible T cell kinase tunes T regulatory cell development and is required for suppressive function. *The Journal of Immunology*. 2014;193(5):2267-2272.
32.Deakin A, Duddy G, Wilson S, et al. Characterisation of a K390R ITK kinase dead transgenic mouse–implications for ITK as a therapeutic target. *PLoS One*. 2014;9(9):e107490.
33.Canna SW, Marsh RA. Pediatric hemophagocytic lymphohistiocytosis. *Blood*. 2020;135(16):1332-1343.

34.Hendriks J, Xiao Y, Borst J. CD27 promotes survival of activated T cells and complements CD28 in generation and establishment of the effector T cell pool. *J Exp Med*. 2003;198(9):1369-1380.
35.Munitic I, Kuka M, Allam A, Scoville JP, Ashwell JD. CD70 deficiency impairs effector CD8 T cell generation and viral clearance but is dispensable for the recall response to lymphocytic choriomeningitis virus. *J Immunol*. 2013;190(3):1169-1179.
36.Gotru SK, Gil-Pulido J, Beyersdorf N, et al. Cutting Edge: Imbalanced Cation Homeostasis in MAGT1-Deficient B Cells Dysregulates B Cell Development and Signaling in Mice. *J Immunol*. 2018;200(8):2529-2534.
37.Priatel JJ, Chen X, Zenewicz LA, et al. Chronic immunodeficiency in mice lacking RasGRP1 results in CD4 T cell immune activation and exhaustion. *J Immunol*. 2007;179(4):2143-2152.
38.Jost PJ, Yabal M, Adler H, et al. A Mouse Model for XLP-2 Disease Uncovers a Critical Function for IL-1beta and TNF in Driving Hyper-Inflammation. *Blood*. 2014;124(21):1403-1403.
39.Kitamura A, Sasaki Y, Abe T, Kano H, Yasutomo K. An inherited mutation in NLRC4 causes autoinflammation in human and mice. *J Exp Med*. 2014;211(12):2385-2396.
40.Cook S, Lenardo MJ, Freeman AF. HEM1 Actin Immunodysregulatory Disorder: Genotypes, Phenotypes, and Future Directions. *Journal of Clinical Immunology*. 2022;42(8):1583-1592.
41.Vinuesa CG, Cook MC, Angelucci C, et al. A RING-type ubiquitin ligase family member required to repress follicular helper T cells and autoimmunity. *Nature*. 2005;435(7041):452-458.
42.Linterman MA, Rigby RJ, Wong RK, et al. Follicular helper T cells are required for systemic autoimmunity. *Journal of Experimental Medicine*. 2009;206(3):561-576.
43.Dixon KO, Tabaka M, Schramm MA, et al. TIM-3 restrains anti-tumour immunity by regulating inflammasome activation. *Nature*. 2021;595(7865):101-106.
44.Münz C, Lünemann JD, Getts MT, Miller SD. Antiviral immune responses: triggers of or triggered by autoimmunity? *Nature Reviews Immunology*. 2009;9(4):246-258.
45.Brisse E, Wouters CH, Andrei G, Matthys P. How Viruses Contribute to the Pathogenesis of Hemophagocytic Lymphohistiocytosis. *Frontiers in Immunology*. 2017;8.
46.Orange JS, Fassett MS, Koopman LA, Boyson JE, Strominger JL. Viral evasion of natural killer cells. *Nature immunology*. 2002;3(11):1006-1012.
47.Brinkmann MM, Dağ F, Hengel H, Messerle M, Kalinke U, Čičin-Šain L. Cytomegalovirus immune evasion of myeloid lineage cells. *Medical microbiology and immunology*. 2015;204:367-382.
48.Alcami A, Koszinowski UH. Viral mechanisms of immune evasion. *Trends in Microbiology*. 2000;8(9):410-418.
49.Hue SS-S, Oon ML, Wang S, Tan S-Y, Ng S-B. Epstein–Barr virus-associated T- and NK-cell lymphoproliferative diseases: an update and diagnostic approach. *Pathology*. 2020;52(1):111-127.
50.Shamriz O, Kumar D, Shim J, et al. T Cell-Epstein-Barr Virus–Associated Hemophagocytic Lymphohistiocytosis (HLH) Occurs in Non-Asians and Is Associated with a T Cell Activation State that Is Comparable to Primary HLH. *Journal of Clinical Immunology*. 2021;41(7):1582-1596.
51.Achdout H, Manaster I, Mandelboim O. Influenza virus infection augments NK cell inhibition through reorganization of major histocompatibility complex class I proteins. *J Virol*. 2008;82(16):8030-8037.

52.Tabaja H, Kanj A, El Zein S, Comba IY, Chehab O, Mahmood M. A Review of Hemophagocytic Lymphohistiocytosis in Patients With HIV. *Open Forum Infect Dis*. 2022;9(4):ofac071.
53.Doyle T, Bhagani S, Cwynarski K. Haemophagocytic syndrome and HIV. *Current Opinion in Infectious Diseases*. 2009;22(1):1-6.
54.Doitsh G, Galloway NL, Geng X, et al. Cell death by pyroptosis drives CD4 T-cell depletion in HIV-1 infection. *Nature*. 2014;505(7484):509-514.
55.Chun T-W, Fauci AS. HIV reservoirs: pathogenesis and obstacles to viral eradication and cure. *Aids*. 2012;26(10):1261-1268.
56.Lowdell MW, O'Toole CM. Defects in cell-mediated immunity in HIV-1 infection: implications for immunotherapy and vaccine development. *Serodiagnosis and Immunotherapy in Infectious Disease*. 1988;2(4):251-261.
57.Shelburne SA, Visnegarwala F, Darcourt J, et al. Incidence and risk factors for immune reconstitution inflammatory syndrome during highly active antiretroviral therapy. *Aids*. 2005;19(4):399-406.
58.Teachey DT, Lacey SF, Shaw PA, et al. Identification of Predictive Biomarkers for Cytokine Release Syndrome after Chimeric Antigen Receptor T-cell Therapy for Acute Lymphoblastic Leukemia. *Cancer Discovery*. 2016;6(6):664-679.
59.Lichtenstein DA, Schischlik F, Shao L, et al. Characterization of HLH-like manifestations as a CRS variant in patients receiving CD22 CAR T cells. *Blood*. 2021;138(24):2469-2484.
60.Lenz A, Franklin GA, Cheadle WG. Systemic inflammation after trauma. *Injury*. 2007;38(12):1336-1345.
61.Menger MD, Vollmar B. Surgical trauma: hyperinflammation versus immunosuppression? *Langenbeck's Archives of Surgery*. 2004;389(6):475-484.
62.Yao YM, Redl H, Bahrami S, Schlag G. The inflammatory basis of trauma/shock-associated multiple organ failure. *Inflammation Research*. 1998;47(5):201-210.
63.Rough J, Engdahl R, Opperman K, Yerrum S, Monroy MA, Daly JM. β2 Adrenoreceptor blockade attenuates the hyperinflammatory response induced by traumatic injury. *Surgery*. 2009;145(2):235-242.
64.Osuka A, Ogura H, Ueyama M, Shimazu T, Lederer JA. Immune response to traumatic injury: harmony and discordance of immune system homeostasis. *Acute Med Surg*. 2014;1(2):63-69.
65.Walsh SA, Hoyt BW, Rowe CJ, Dey D, Davis TA. Alarming Cargo: The Role of Exosomes in Trauma-Induced Inflammation. Biomolecules. Vol. 11; 2021.
66.Dokouhaki P, Van der Merwe D-e, Vats K, Said SM, D'Agati VD, Nasr SH. Histiocytic Glomerulopathy Associated With Hemophagocytic Lymphohistiocytosis. *Kidney Medicine*. 2022;4(2).
67.Schönfelder J, Seibold T, Morawe M, et al. Endothelial Protein kinase D1 is a major regulator of post-traumatic hyperinflammation. *Frontiers in Immunology*. 2023;14.
68.Zhang M, Behrens EM, Atkinson TP, Shakoory B, Grom AA, Cron RQ. Genetic defects in cytolysis in macrophage activation syndrome. *Curr Rheumatol Rep*. 2014;16(9):439.
69.Bracaglia C, Sieni E, Da Ros M, et al. Mutations of familial hemophagocytic lymphohistiocytosis (FHL) related genes and abnormalities of cytotoxicity function tests in patients with macrophage activation syndrome (MAS) occurring in systemic juvenile idiopathic arthritis (sJIA). *Pediatric Rheumatology Online Journal*. 2014;12(Suppl 1):P53-P53.
70.Behrens EM, Canna SW, Slade K, et al. Repeated TLR9 stimulation results in macrophage activation syndrome-like disease in mice. *J Clin Invest*. 2011;121(6):2264-2277.

71.Strippoli R, Carvello F, Scianaro R, et al. Amplification of the response to Toll-like receptor ligands by prolonged exposure to interleukin-6 in mice: Implication for the pathogenesis of macrophage activation syndrome. *Arthritis & Rheumatism*. 2012;64(5):1680-1688.
72.Canna SW, Costa-Reis P, Bernal WE, et al. Brief report: alternative activation of laser-captured murine hemophagocytes. *Arthritis Rheumatol*. 2014;66(6):1666-1671.
73.Baroja-Mazo A, Martín-Sánchez F, Gomez AI, et al. The NLRP3 inflammasome is released as a particulate danger signal that amplifies the inflammatory response. *Nat Immunol*. 2014;15(8):738-748.
74.Fernandes FP, Leal VNC, Souza de Lima D, Reis EC, Pontillo A. Inflammasome genetics and complex diseases: a comprehensive review. *Eur J Hum Genet*. 2020;28(10):1307-1321.
75.Gutcher I, Becher B. APC-derived cytokines and T cell polarization in autoimmune inflammation. *J Clin Invest*. 2007;117(5):1119-1127.
76.Fardet L, Lambotte O, Meynard J-L, et al. Reactive haemophagocytic syndrome in 58 HIV-1-infected patients: clinical features, underlying diseases and prognosis. *Aids*. 2010;24(9):1299-1306.
77.Imashuku S, Morimoto A, Ishii E. Virus-triggered secondary hemophagocytic lymphohistiocytosis. *Acta Paediatrica*. 2021;110(10):2729-2736.
78.Al-Samkari H, Berliner N. Hemophagocytic lymphohistiocytosis. *Annual review of pathology: mechanisms of disease*. 2018;13:27-49.
79.Zoller EE, Lykens JE, Terrell CE, et al. Hemophagocytosis causes a consumptive anemia of inflammation. *J Exp Med*. 2011;208(6):1203-1214.
80.Milner JD, Orekov T, Ward JM, et al. Sustained IL-4 exposure leads to a novel pathway for hemophagocytosis, inflammation, and tissue macrophage accumulation. *Blood, The Journal of the American Society of Hematology*. 2010;116(14):2476-2483.
81.Baratono SR, Chu N, Richman LP, Behrens EM. Toll-like receptor 9 and interferon-γ receptor signaling suppress the B-cell fate of uncommitted progenitors in mice. *Eur J Immunol*. 2015;45(5):1313-1325.
82.Denzler KL, Farmer SC, Crosby JR, et al. Eosinophil major basic protein-1 does not contribute to allergen-induced airway pathologies in mouse models of asthma. *The Journal of Immunology*. 2000;165(10):5509-5517.
83.Denzler KL, Borchers MT, Crosby JR, et al. Extensive eosinophil degranulation and peroxidase-mediated oxidation of airway proteins do not occur in a mouse ovalbumin-challenge model of pulmonary inflammation. *The Journal of Immunology*. 2001;167(3):1672-1682.
84.Venkateshaiah SU, Mishra A, Manohar M, et al. A critical role for IL-18 in transformation and maturation of naive eosinophils to pathogenic eosinophils. *J Allergy Clin Immunol*. 2018;142(1):301-305.
85.Sun L, Su Y, Jiao A, Wang X, Zhang B. T cells in health and disease. *Signal Transduction and Targeted Therapy*. 2023;8(1):235.
86.De Matteis A, Colucci M, Rossi MN, et al. Expansion of CD4dimCD8+T cells characterizes macrophage activation syndrome and other secondary HLH. *Blood*. 2022.
87.Chaturvedi V, Marsh RA, Zoref-Lorenz A, et al. T-cell activation profiles distinguish hemophagocytic lymphohistiocytosis and early sepsis. *Blood*. 2021;137(17):2337-2346.
88.Giles JR, Ngiow SF, Manne S, et al. Shared and distinct biological circuits in effector, memory and exhausted CD8+ T cells revealed by temporal single-cell transcriptomics and epigenetics. *Nature Immunology*. 2022;23(11):1600-1613.

89.St. Paul M, Ohashi PS. The Roles of CD8⁺ T Cell Subsets in Antitumor Immunity. *Trends in Cell Biology*. 2020;30(9):695-704.
90.Tominaga K, Yoshimoto T, Torigoe K, et al. IL-12 synergizes with IL-18 or IL-1beta for IFN-gamma production from human T cells. *Int Immunol*. 2000;12(2):151-160.
91.Xu D, Trajkovic V, Hunter D, et al. IL-18 induces the differentiation of Th1 or Th2 cells depending upon cytokine milieu and genetic background. *Eur J Immunol*. 2000;30(11):3147-3156.
92.Chapin CA, Burn T, Meijome T, et al. Indeterminate pediatric acute liver failure is uniquely characterized by a CD103(+) CD8(+) T-cell infiltrate. *Hepatology*. 2018;68(3):1087-1100.
93.Billiau AD, Roskams T, Van Damme-Lombaerts R, Matthys P, Wouters C. Macrophage activation syndrome: characteristic findings on liver biopsy illustrating the key role of activated, IFN-gamma-producing lymphocytes and IL-6- and TNF-alpha-producing macrophages. *Blood*. 2005;105(4):1648-1651.
94.Segal JE, Daley JD, Barnum JL, et al. Systemic and Nodular Hyperinflammation in a Patient with Refractory Familial Hemophagocytic Lymphohistiocytosis 2. *J Clin Immunol*. 2021;41(5):987-991.
95.Jordan MB, Hildeman D, Kappler J, Marrack P. An animal model of hemophagocytic lymphohistiocytosis (HLH): CD8+ T cells and interferon gamma are essential for the disorder. *Blood*. 2004;104(3):735-743.
96.Tsoukas P, Rapp E, Van Der Kraak L, et al. Interleukin-18 and cytotoxic impairment are independent and synergistic causes of murine virus-induced hyperinflammation. *Blood*. 2020.
97.Terrell CE, Jordan MB. Perforin deficiency impairs a critical immunoregulatory loop involving murine CD8(+) T cells and dendritic cells. *Blood*. 2013;121(26):5184-5191.
98.Angelosanto J, Blackburn S, Crawford A, Wherry EJ. Progressive Loss of Memory T Cell Potential and Commitment to Exhaustion during Chronic Viral Infection. *Journal of virology*. 2012;86:8161-8170.
99.Blackburn SD, Shin H, Haining WN, et al. Coregulation of CD8+ T cell exhaustion by multiple inhibitory receptors during chronic viral infection. *Nature immunology*. 2009;10(1):29-37.
100.Wherry EJ, Kurachi M. Molecular and cellular insights into T cell exhaustion. *Nat Rev Immunol*. 2015;15(8):486-499.
101.Attanasio J, Wherry EJ. Costimulatory and Coinhibitory Receptor Pathways in Infectious Disease. *Immunity*. 2016;44(5):1052-1068.
102.Jenkins E, Whitehead T, Fellermeyer M, Davis SJ, Sharma S. The current state and future of T-cell exhaustion research. *Oxford Open Immunology*. 2023;4(1).
103.Kelkar MG, Bargir UA, Malik-Yadav R, et al. CD8 + T Cells Exhibit an Exhausted Phenotype in Hemophagocytic Lymphohistiocytosis. *J Clin Immunol*. 2021;41(8):1794-1803.
104.Ingram JT, Yi JS, Zajac AJ. Exhausted CD8 T cells downregulate the IL-18 receptor and become unresponsive to inflammatory cytokines and bacterial co-infections. *PLoS Pathog*. 2011;7(9):e1002273.
105.Raué H-P, Brien JD, Hammarlund E, Slifka MK. Activation of virus-specific CD8+ T cells by lipopolysaccharide-induced IL-12 and IL-18. *The Journal of Immunology*. 2004;173(11):6873-6881.
106.Iwai Y, Hemmi H, Mizenina O, Kuroda S, Suda K, Steinman RM. An IFN-γ-IL-18 Signaling Loop Accelerates Memory CD8+ T Cell Proliferation. *PLOS ONE*. 2008;3(6):e2404.
107.Iwai Y, Hemmi H, Mizenina O, Kuroda S, Suda K, Steinman RM. An IFN-gamma-IL-18 signaling loop accelerates memory CD8+ T cell proliferation. *PLoS One*. 2008;3(6):e2404.

108.Beadling C, Slifka MK. Differential regulation of virus-specific T-cell effector functions following activation by peptide or innate cytokines. *Blood*. 2005;105(3):1179-1186.
109.Lertmemongkolchai G, Cai G, Hunter CA, Bancroft GJ. Bystander activation of CD8+ T cells contributes to the rapid production of IFN-γ in response to bacterial pathogens. *The Journal of Immunology*. 2001;166(2):1097-1105.
110.Taylor MD, Burn TN, Wherry EJ, Behrens EM. CD8 T Cell Memory Increases Immunopathology in the Perforin-Deficient Model of Hemophagocytic Lymphohistiocytosis Secondary to TNF-alpha. *Immunohorizons*. 2018;2(2):67-73.
111.Zhou T, Damsky W, Weizman OE, et al. IL-18BP is a secreted immune checkpoint and barrier to IL-18 immunotherapy. *Nature*. 2020;583(7817):609-614.
112.Hu B, Ren J, Luo Y, et al. Augmentation of Antitumor Immunity by Human and Mouse CAR T Cells Secreting IL-18. *Cell Reports*. 2017;20:3025-3033.
113.Jordan JA, Guo RF, Yun EC, et al. Role of IL-18 in acute lung inflammation. *J Immunol*. 2001;167(12):7060-7068.
114.Kitasato Y, Hoshino T, Okamoto M, et al. Enhanced expression of interleukin-18 and its receptor in idiopathic pulmonary fibrosis. *Am J Respir Cell Mol Biol*. 2004;31(6):619-625.
115.Harms RZ, Creer AJ, Lorenzo-Arteaga KM, Ostlund KR, Sarvetnick NE. Interleukin (IL)-18 Binding Protein Deficiency Disrupts Natural Killer Cell Maturation and Diminishes Circulating IL-18. *Front Immunol*. 2017;8:1020.
116.Uhlén M, Fagerberg L, Hallström BM, et al. Proteomics. Tissue-based map of the human proteome. *Science*. 2015;347(6220):1260419.
117.Pechkovsky DV, Goldmann T, Vollmer E, Müller-Quernheim J, Zissel G. Interleukin-18 expression by alveolar epithelial cells type II in tuberculosis and sarcoidosis. *FEMS Immunol Med Microbiol*. 2006;46(1):30-38.
118.Okazawa A, Kanai T, Nakamaru K, et al. Human intestinal epithelial cell-derived interleukin (IL)-18, along with IL-2, IL-7 and IL-15, is a potent synergistic factor for the proliferation of intraepithelial lymphocytes. *Clin Exp Immunol*. 2004;136(2):269-276.
119.Nowarski R, Jackson R, Gagliani N, et al. Epithelial IL-18 Equilibrium Controls Barrier Function in Colitis. *Cell*. 2015;163(6):1444-1456.
120.Wittmann M, Macdonald A, Renne J. IL-18 and skin inflammation. *Autoimmun Rev*. 2009;9(1):45-48.
121.Companjen AR, van der Velden VH, Vooys A, Debets R, Benner R, Prens EP. Human keratinocytes are major producers of IL-18: predominant expression of the unprocessed form. *Eur Cytokine Netw*. 2000;11(3):383-390.
122.Heng TS, Painter MW. The Immunological Genome Project: networks of gene expression in immune cells. *Nat Immunol*. 2008;9(10):1091-1094.
123.Mostafavi S, Yoshida H, Moodley D, et al. Parsing the Interferon Transcriptional Network and Its Disease Associations. *Cell*. 2016;164(3):564-578.
124.Chen G, Deutsch G, Schulert G, et al. Serum proteome analysis of systemic JIA and related pulmonary alveolar proteinosis identifies distinct inflammatory programs and biomarkers. *medRxiv*. 2021:2021.2001.2020.21250141.
125.Muñoz M, Eidenschenk C, Ota N, et al. Interleukin-22 induces interleukin-18 expression from epithelial cells during intestinal infection. *Immunity*. 2015;42(2):321-331.
126.Dinarello CA. Interleukin-18. *Methods*. 1999;19(1):121-132.

127.Rauch KS, Hils M, Menner AJ, et al. Regulatory T cells characterized by low Id3 expression are highly suppressive and accumulate during chronic infection. *Oncotarget*. 2017;8(61):102835-102851.
128.Chudnovskiy A, Mortha A, Kana V, et al. Host-Protozoan Interactions Protect from Mucosal Infections through Activation of the Inflammasome. *Cell*. 2016;167(2):444-456.e414.
129.Van Der Kraak LA, Schneider C, Dang V, et al. Genetic and commensal induction of IL-18 drive intestinal epithelial MHCII via IFNγ. *Mucosal Immunol*. 2021.
130.Zhu Q, Kanneganti TD. Cutting Edge: Distinct Regulatory Mechanisms Control Proinflammatory Cytokines IL-18 and IL-1β. *J Immunol*. 2017;198(11):4210-4215.
131.Verweyen E, Holzinger D, Weinhage T, et al. Synergistic Signaling of TLR and IFNα/β Facilitates Escape of IL-18 Expression from Endotoxin Tolerance. *Am J Respir Crit Care Med*. 2020;201(5):526-539.
132.Gu Y, Kuida K, Tsutsui H, et al. Activation of interferon-gamma inducing factor mediated by interleukin-1beta converting enzyme. *Science*. 1997;275(5297):206-209.
133.Fantuzzi G, Puren AJ, Harding MW, Livingston DJ, Dinarello CA. Interleukin-18 regulation of interferon gamma production and cell proliferation as shown in interleukin-1beta-converting enzyme (caspase-1)-deficient mice. *Blood*. 1998;91(6):2118-2125.
134.Ghayur T, Banerjee S, Hugunin M, et al. Caspase-1 processes IFN-gamma-inducing factor and regulates LPS-induced IFN-gamma production. *Nature*. 1997;386(6625):619-623.
135.Martinon F, Pétrilli V, Mayor A, Tardivel A, Tschopp J. Gout-associated uric acid crystals activate the NALP3 inflammasome. *Nature*. 2006;440(7081):237-241.
136.Levy M, Thaiss CA, Zeevi D, et al. Microbiota-Modulated Metabolites Shape the Intestinal Microenvironment by Regulating NLRP6 Inflammasome Signaling. *Cell*. 2015;163(6):1428-1443.
137.Zhu S, Ding S, Wang P, et al. Nlrp9b inflammasome restricts rotavirus infection in intestinal epithelial cells. *Nature*. 2017;546(7660):667-670.
138.Novick D, Kim SH, Fantuzzi G, Reznikov LL, Dinarello CA, Rubinstein M. Interleukin-18 binding protein: a novel modulator of the Th1 cytokine response. *Immunity*. 1999;10(1):127-136.
139.Bossaller L, Chiang PI, Schmidt-Lauber C, et al. Cutting edge: FAS (CD95) mediates noncanonical IL-1β and IL-18 maturation via caspase-8 in an RIP3-independent manner. *J Immunol*. 2012;189(12):5508-5512.
140.Omoto Y, Yamanaka K, Tokime K, et al. Granzyme B is a novel interleukin-18 converting enzyme. *J Dermatol Sci*. 2010;59(2):129-135.
141.Akeda T, Yamanaka K, Tsuda K, Omoto Y, Gabazza EC, Mizutani H. CD8+ T cell granzyme B activates keratinocyte endogenous IL-18. *Arch Dermatol Res*. 2014;306(2):125-130.
142.Sugawara S, Uehara A, Nochi T, et al. Neutrophil proteinase 3-mediated induction of bioactive IL-18 secretion by human oral epithelial cells. *J Immunol*. 2001;167(11):6568-6575.
143.Omoto Y, Tokime K, Yamanaka K, et al. Human mast cell chymase cleaves pro-IL-18 and generates a novel and biologically active IL-18 fragment. *J Immunol*. 2006;177(12):8315-8319.
144.Witko-Sarsat V, Cramer EM, Hieblot C, et al. Presence of proteinase 3 in secretory vesicles: evidence of a novel, highly mobilizable intracellular pool distinct from azurophil granules. *Blood*. 1999;94(7):2487-2496.
145.Chen KW, Groß CJ, Sotomayor FV, et al. The neutrophil NLRC4 inflammasome selectively promotes IL-1β maturation without pyroptosis during acute Salmonella challenge. *Cell Rep*. 2014;8(2):570-582.

146.Franklin BS, Bossaller L, De Nardo D, et al. The adaptor ASC has extracellular and 'prionoid' activities that propagate inflammation. *Nat Immunol*. 2014;15(8):727-737.
147.Tapia VS, Daniels MJD, Palazón-Riquelme P, et al. The three cytokines IL-1β, IL-18, and IL-1α share related but distinct secretory routes. *J Biol Chem*. 2019;294(21):8325-8335.
148.He WT, Wan H, Hu L, et al. Gasdermin D is an executor of pyroptosis and required for interleukin-1β secretion. *Cell Res*. 2015;25(12):1285-1298.
149.Heilig R, Dick MS, Sborgi L, Meunier E, Hiller S, Broz P. The Gasdermin-D pore acts as a conduit for IL-1β secretion in mice. *Eur J Immunol*. 2018;48(4):584-592.
150.Xia S, Zhang Z, Magupalli VG, et al. Gasdermin D pore structure reveals preferential release of mature interleukin-1. *Nature*. 2021;593(7860):607-611.
151.Tsuchiya K, Hosojima S, Hara H, et al. Gasdermin D mediates the maturation and release of IL-1α downstream of inflammasomes. *Cell Rep*. 2021;34(12):108887.
152.Kayagaki N, Stowe IB, Lee BL, et al. Caspase-11 cleaves gasdermin D for non-canonical inflammasome signalling. *Nature*. 2015;526(7575):666-671.
153.Chauhan D, Vande Walle L, Lamkanfi M. Therapeutic modulation of inflammasome pathways. *Immunological Reviews*. 2020;297(1):123-138.
154.Kayagaki N, Kornfeld OS, Lee BL, et al. NINJ1 mediates plasma membrane rupture during lytic cell death. *Nature*. 2021;591(7848):131-136.
155.Rühl S, Shkarina K, Demarco B, Heilig R, Santos JC, Broz P. ESCRT-dependent membrane repair negatively regulates pyroptosis downstream of GSDMD activation. *Science*. 2018;362(6417):956-960.
156.Hosohara K, Ueda H, Kashiwamura S, et al. Interleukin-18 induces acute biphasic reduction in the levels of circulating leukocytes in mice. *Clin Diagn Lab Immunol*. 2002;9(4):777-783.
157.Ha CT, Li X, Fu D, Xiao M. Circulating IL-18 Binding Protein (IL-18BP) and IL-18 as Dual Biomarkers of Total-Body Irradiation in Mice. *Radiat Res*. 2016;185(4):375-383.
158.Girard-Guyonvarc'h C, Palomo J, Martin P, et al. Unopposed IL-18 signaling leads to severe TLR9-induced macrophage activation syndrome in mice. *Blood*. 2018;131(13):1430.
159.Bufler P, Azam T, Gamboni-Robertson F, et al. A complex of the IL-1 homologue IL-1F7b and IL-18-binding protein reduces IL-18 activity. *Proceedings of the National Academy of Sciences*. 2002;99(21):13723-13728.
160.Harel M, Girard-Guyonvarc'h C, Rodriguez E, Palmer G, Gabay C. Production of IL-18 Binding Protein by Radiosensitive and Radioresistant Cells in CpG-Induced Macrophage Activation Syndrome. *The Journal of Immunology*. 2020:ji2000168.
161.Prencipe G, Bracaglia C, De Benedetti F. Interleukin-18 in pediatric rheumatic diseases. *Curr Opin Rheumatol*. 2019;31(5):421-427.
162.Chirathaworn C, Rianthavorn P, Wuttirattanakowit N, Poovorawan Y. Serum IL-18 and IL-18BP levels in patients with Chikungunya virus infection. *Viral immunology*. 2010;23(1):113-117.
163.Chirathaworn C, Poovorawan Y, Lertmaharit S, Wuttirattanakowit N. Cytokine levels in patients with chikungunya virus infection. *Asian Pacific journal of tropical medicine*. 2013;6(8):631-634.
164.Michels M, de Mast Q, Netea MG, et al. Normal free interleukin-18 (IL-18) plasma levels in dengue virus infection and the need to measure both total IL-18 and IL-18 binding protein levels. *Clinical and Vaccine Immunology*. 2015;22(6):650-655.
165.Kaser A, Novick D, Rubinstein M, et al. Interferon-α induces interleukin-18 binding protein in chronic hepatitis C patients. *Clinical & Experimental Immunology*. 2002;129(2):332-338.

166.Mele D, Mantovani S, Oliviero B, et al. Monocytes inhibit hepatitis C virus-induced TRAIL expression on CD56bright NK Cells. *Journal of hepatology*. 2017;67(6):1148-1156.
167.Nazarian SH, Rahman MM, Werden SJ, et al. Yaba monkey tumor virus encodes a functional inhibitor of interleukin-18. *Journal of virology*. 2008;82(1):522-528.
168.Smith VP, Bryant NA, Alcamí A. Ectromelia, vaccinia and cowpox viruses encode secreted interleukin-18-binding proteins. *Microbiology*. 2000;81(5):1223-1230.
169.Xiang Y, Moss B. IL-18 binding and inhibition of interferon γ induction by human poxvirus-encoded proteins. *Proceedings of the National Academy of Sciences*. 1999;96(20):11537-11542.
170.Hoshino K, Tsutsui H, Kawai T, et al. Cutting Edge: Generation of IL-18 Receptor-Deficient Mice: Evidence for IL-1 Receptor-Related Protein as an Essential IL-18 Binding Receptor. *The Journal of Immunology*. 1999;162(9):5041.
171.Tsutsumi N, Kimura T, Arita K, et al. The structural basis for receptor recognition of human interleukin-18. *Nature Communications*. 2014;5:5340.
172.Yasuda K, Nakanishi K, Tsutsui H. Interleukin-18 in Health and Disease. *Int J Mol Sci*. 2019;20(3).
173.Kim SH, Reznikov LL, Stuyt RJ, et al. Functional reconstitution and regulation of IL-18 activity by the IL-18R beta chain. *J Immunol*. 2001;166(1):148-154.
174.Wu C, Sakorafas P, Miller R, et al. IL-18 Receptor β-Induced Changes in the Presentation of IL-18 Binding Sites Affect Ligand Binding and Signal Transduction. *The Journal of Immunology*. 2003;170(11):5571.
175.Ohnishi H, Tochio H, Kato Z, et al. TRAM Is Involved in IL-18 Signaling and Functions as a Sorting Adaptor for MyD88. *PLOS ONE*. 2012;7(6):e38423.
176.Kaplanski G. Interleukin-18: Biological properties and role in disease pathogenesis. *Immunological Reviews*. 2018;281(1):138-153.
177.Cao Z, Henzel WJ, Gao X. IRAK: a kinase associated with the interleukin-1 receptor. *Science*. 1996;271(5252):1128-1131.
178.Adachi O, Kawai T, Takeda K, et al. Targeted disruption of the MyD88 gene results in loss of IL-1- and IL-18-mediated function. *Immunity*. 1998;9(1):143-150.
179.Rex DAB, Agarwal N, Prasad TSK, Kandasamy RK, Subbannayya Y, Pinto SM. A comprehensive pathway map of IL-18-mediated signalling. *J Cell Commun Signal*. 2020;14(2):257-266.
180.Airoldi I, Gri G, Marshall JD, et al. Expression and Function of IL-12 and IL-18 Receptors on Human Tonsillar B Cells. *The Journal of Immunology*. 2000;165(12):6880.
181.Gutzmer R, Langer K, Mommert S, Wittmann M, Kapp A, Werfel T. Human Dendritic Cells Express the IL-18R and Are Chemoattracted to IL-18. *The Journal of Immunology*. 2003;171(12):6363.
182.Tucci M, Quatraro C, Dammacco F, Silvestris F. Increased IL-18 production by dendritic cells in active inflammatory myopathies. *Ann N Y Acad Sci*. 2007;1107:184-192.
183.Gracie JA, Forsey RJ, Chan WL, et al. A proinflammatory role for IL-18 in rheumatoid arthritis. *The Journal of Clinical Investigation*. 1999;104(10):1393-1401.
184.Yoshimoto T, Tsutsui H, Tominaga K, et al. IL-18, although antiallergic when administered with IL-12, stimulates IL-4 and histamine release by basophils. *Proceedings of the National Academy of Sciences*. 1999;96(24):13962-13966.
185.Leung BP, Culshaw S, Gracie JA, et al. A Role for IL-18 in Neutrophil Activation. *The Journal of Immunology*. 2001;167(5):2879.

186.Ricardo-Gonzalez RR, Van Dyken SJ, Schneider C, et al. Tissue signals imprint ILC2 identity with anticipatory function. *Nature immunology*. 2018;19(10):1093-1099.
187.Zhang X, Luo S, Wang M, et al. Differential IL18 signaling via IL18 receptor and Na-Cl co-transporter discriminating thermogenesis and glucose metabolism regulation. *Nat Commun*. 2022;13(1):7582.
188.Wang J, Sun C, Gerdes N, et al. Interleukin 18 function in atherosclerosis is mediated by the interleukin 18 receptor and the Na-Cl co-transporter. *Nat Med*. 2015;21(7):820-826.
189.Molgora M, Barajon I, Mantovani A, Garlanda C. Regulatory Role of IL-1R8 in Immunity and Disease. *Front Immunol*. 2016;7:149.
190.Cavalli G, Justice JN, Boyle KE, et al. Interleukin 37 reverses the metabolic cost of inflammation, increases oxidative respiration, and improves exercise tolerance. *Proceedings of the National Academy of Sciences of the United States of America*. 2017;114(9):2313-2318.
191.Hu B, Ren J, Luo Y, et al. CAR T Cells Secreting IL18 Augment Antitumor Immunity and Increase T Cell Proliferation and Costimulation. *bioRxiv*. 2017:111260.
192.Landy E CS, Tsoukas P, Dang V, Varghese J. . Cytokine Storm Susceptibility Factors IL-18 and Cytotoxic Impairment Converge on Pathologic CD8 T-cell Hyperactivation [abstract]. *Arthritis Rheum*. 2022;74.
193.Sharma D, Malik A, Guy CS, Karki R, Vogel P, Kanneganti TD. Pyrin Inflammasome Regulates Tight Junction Integrity to Restrict Colitis and Tumorigenesis. *Gastroenterology*. 2018;154(4):948-964.e948.
194.Karczewski KJ, Francioli LC, Tiao G, et al. The mutational constraint spectrum quantified from variation in 141,456 humans. *Nature*. 2020;581(7809):434-443.
195.Netea MG, Joosten LA, Lewis E, et al. Deficiency of interleukin-18 in mice leads to hyperphagia, obesity and insulin resistance. *Nat Med*. 2006;12(6):650-656.
196.Nakanishi K, Yoshimoto T, Tsutsui H, Okamura H. Interleukin-18 is a unique cytokine that stimulates both Th1 and Th2 responses depending on its cytokine milieu. *Cytokine Growth Factor Rev*. 2001;12(1):53-72.
197.Freeman BE, Hammarlund E, Raué HP, Slifka MK. Regulation of innate CD8+ T-cell activation mediated by cytokines. *Proc Natl Acad Sci U S A*. 2012;109(25):9971-9976.
198.Cirella A, Bolaños E, Di Trani CA, et al. Intratumoral Gene Transfer of mRNAs Encoding IL12 in Combination with Decoy-Resistant IL18 Improves Local and Systemic Antitumor Immunity. *Cancer Immunology Research*. 2023;11(2):184-198.
199.Chaix J, Tessmer MS, Hoebe K, et al. Cutting Edge: Priming of NK Cells by IL-18. *The Journal of Immunology*. 2008;181(3):1627.
200.Ni J, Miller M, Stojanovic A, Garbi N, Cerwenka A. Sustained effector function of IL-12/15/18–preactivated NK cells against established tumors. *Journal of Experimental Medicine*. 2012;209(13):2351-2365.
201.Hashimoto W, Osaki T, Okamura H, et al. Differential antitumor effects of administration of recombinant IL-18 or recombinant IL-12 are mediated primarily by Fas-Fas ligand-and perforin-induced tumor apoptosis, respectively. *The Journal of Immunology*. 1999;163(2):583-589.
202.Osaki T, Péron JM, Cai Q, et al. IFN-gamma-inducing factor/IL-18 administration mediates IFN-gamma- and IL-12-independent antitumor effects. *J Immunol*. 1998;160(4):1742-1749.
203.Hoshino T, Kawase Y, Okamoto M, et al. Cutting Edge: IL-18-Transgenic Mice: In Vivo Evidence of a Broad Role for IL-18 in Modulating Immune Function. *The Journal of Immunology*. 2001;166(12):7014.

204.Clark JT, Weizman O-E, Aldridge DL, et al. IL-18BP mediates the balance between protective and pathological immune responses to Toxoplasma gondii. *Cell Reports*. 2023;42(3).
205.Kanai T, Watanabe M, Okazawa A, et al. Macrophage-derived IL-18-mediated intestinal inflammation in the murine model of Crohn's disease. *Gastroenterology*. 2001;121(4):875-888.
206.Siegmund B, Fantuzzi G, Rieder F, et al. Neutralization of interleukin-18 reduces severity in murine colitis and intestinal IFN-gamma and TNF-alpha production. *Am J Physiol Regul Integr Comp Physiol*. 2001;281(4):R1264-1273.
207.Ten Hove T, Corbaz A, Amitai H, et al. Blockade of endogenous IL-18 ameliorates TNBS-induced colitis by decreasing local TNF-alpha production in mice. *Gastroenterology*. 2001;121(6):1372-1379.
208.Elinav E, Strowig T, Kau AL, et al. NLRP6 inflammasome regulates colonic microbial ecology and risk for colitis. *Cell*. 2011;145(5):745-757.
209.Son YI, Dallal RM, Mailliard RB, Egawa S, Jonak ZL, Lotze MT. Interleukin-18 (IL-18) synergizes with IL-2 to enhance cytotoxicity, interferon-gamma production, and expansion of natural killer cells. *Cancer Res*. 2001;61(3):884-888.
210.Rodriguez-Galán MC, Bream JH, Farr A, Young HA. Synergistic effect of IL-2, IL-12, and IL-18 on thymocyte apoptosis and Th1/Th2 cytokine expression. *J Immunol*. 2005;174(5):2796-2804.
211.Harrison OJ, Linehan JL, Shih H-Y, et al. Commensal-specific T cell plasticity promotes rapid tissue adaptation to injury. *Science (New York, NY)*. 2019;363(6422):eaat6280.
212.Lalor SJ, Dungan LS, Sutton CE, Basdeo SA, Fletcher JM, Mills KHG. Caspase-1–Processed Cytokines IL-1β and IL-18 Promote IL-17 Production by γδ and CD4 T Cells That Mediate Autoimmunity. *The Journal of Immunology*. 2011;186(10):5738.
213.Millward JM, Løbner M, Wheeler RD, Owens T. Inflammation in the Central Nervous System and Th17 Responses Are Inhibited by IFN-γ–Induced IL-18 Binding Protein. *The Journal of Immunology*. 2010;185(4):2458.
214.Shi F-D, Takeda K, Akira S, Sarvetnick N, Ljunggren H-G. IL-18 Directs Autoreactive T Cells and Promotes Autodestruction in the Central Nervous System Via Induction of IFN-γ by NK Cells. *The Journal of Immunology*. 2000;165(6):3099.
215.Andoh T, Kishi H, Motoki K, Nakanishi K, Kuraishi Y, Muraguchi A. Protective effect of IL-18 on kainate- and IL-1 beta-induced cerebellar ataxia in mice. *J Immunol*. 2008;180(4):2322-2328.
216.Akimova T, Zhang T, Christensen LM, et al. Obesity-related IL-18 Impairs T-Regulatory Cell Function and Promotes Lung Ischemia-Reperfusion Injury. *Am J Respir Crit Care Med*. 2021;204(9):1060-1074.
217.Min HK, Kim S, Lee JY, Kim KW, Lee SH, Kim HR. IL-18 binding protein suppresses IL-17-induced osteoclastogenesis and rectifies type 17 helper T cell / regulatory T cell imbalance in rheumatoid arthritis. *J Transl Med*. 2021;19(1):392.
218.Harrison OJ, Srinivasan N, Pott J, et al. Epithelial-derived IL-18 regulates Th17 cell differentiation and Foxp3^{+} Treg cell function in the intestine. *Mucosal Immunol*. 2015;8(6):1226-1236.
219.Ma J, Lam IKY, Lau C-S, Chan VSF. Elevated Interleukin-18 Receptor Accessory Protein Mediates Enhancement in Reactive Oxygen Species Production in Neutrophils of Systemic Lupus Erythematosus Patients. *Cells*. 2021;10(5):964.

220.Netea MG, Fantuzzi G, Kullberg BJ, et al. Neutralization of IL-18 reduces neutrophil tissue accumulation and protects mice against lethal Escherichia coli and Salmonella typhimurium endotoxemia. *The Journal of Immunology*. 2000;164(5):2644-2649.
221.Lutz V, Hellmund V, Picard F, et al. IL18 Receptor Signaling Regulates Tumor-Reactive CD8+ T-cell Exhaustion via Activation of the IL2/STAT5/mTOR Pathway in a Pancreatic Cancer Model. *Cancer immunology research*. 2023.
222.Li J, Mbow ML, Sun L, et al. Induction of dendritic cell maturation by IL-18. *Cell Immunol*. 2004;227(2):103-108.
223.Semino C, Angelini G, Poggi A, Rubartelli A. NK/iDC interaction results in IL-18 secretion by DCs at the synaptic cleft followed by NK cell activation and release of the DC maturation factor HMGB1. *Blood*. 2005;106(2):609-616.
224.Wherry EJ, Blattman JN, Murali-Krishna K, van der Most R, Ahmed R. Viral persistence alters CD8 T-cell immunodominance and tissue distribution and results in distinct stages of functional impairment. *J Virol*. 2003;77(8):4911-4927.
225.De Boer RJ, Homann D, Perelson AS. Different dynamics of CD4+ and CD8+ T cell responses during and after acute lymphocytic choriomeningitis virus infection. *J Immunol*. 2003;171(8):3928-3935.
226.Stelekati E, Chen Z, Manne S, et al. Long-Term Persistence of Exhausted CD8 T Cells in Chronic Infection Is Regulated by MicroRNA-155. *Cell Reports*. 2018;23(7):2142-2156.
227.Rood JE, Canna SW, Weaver LK, Tobias JW, Behrens EM. IL-10 distinguishes a unique population of activated, effector-like CD8(+) T cells in murine acute liver inflammation. *J Leukoc Biol*. 2017;101(4):1037-1044.
228.Choi SS, Chung E, Jung YJ. Newly identified CpG ODNs, M5-30 and M6-395, stimulate mouse immune cells to secrete TNF-alpha and enhance Th1-mediated immunity. *J Microbiol*. 2010;48(4):512-517.
229.McCausland MM, Crotty S. Quantitative PCR technique for detecting lymphocytic choriomeningitis virus in vivo. *Journal of virological methods*. 2008;147(1):167-176.
230.Buatois V, Chatel L, Cons L, et al. Use of a mouse model to identify a blood biomarker for IFNγ activity in pediatric secondary hemophagocytic lymphohistiocytosis. *Translational Research*. 2017;180:37-52. e32.
231.Sidhom JW, Theodros D, Murter B, et al. ExCYT: A Graphical User Interface for Streamlining Analysis of High-Dimensional Cytometry Data. *J Vis Exp*. 2019(143).
232.Put K, Vandenhaute J, Avau A, et al. Inflammatory gene expression profile and defective interferon-γ and granzyme K in natural killer cells from systemic juvenile idiopathic arthritis patients. *Arthritis & Rheumatology*. 2017;69(1):213-224.
233.Dinarello C, Novick D, Kim S, Kaplanski G. Interleukin-18 and IL-18 Binding Protein. *Frontiers in Immunology*. 2013;4(289).
234.Masopust D, Murali-Krishna K, Ahmed R. Quantitating the magnitude of the lymphocytic choriomeningitis virus-specific CD8 T-cell response: it is even bigger than we thought. *Journal of virology*. 2007;81(4):2002-2011.
235.Van Der Most RG, Murali-Krishna K, Lanier JG, et al. Changing immunodominance patterns in antiviral CD8 T-cell responses after loss of epitope presentation or chronic antigenic stimulation. *Virology*. 2003;315(1):93-102.
236.Butz EA, Bevan MJ. Massive expansion of antigen-specific CD8+ T cells during an acute virus infection. *Immunity*. 1998;8(2):167-175.

237.Terawaki S, Chikuma S, Shibayama S, et al. IFN-α directly promotes programmed cell death-1 transcription and limits the duration of T cell-mediated immunity. *The Journal of Immunology*. 2011;186(5):2772-2779.
238.Gupta PK, Godec J, Wolski D, et al. CD39 Expression Identifies Terminally Exhausted CD8+ T Cells. *PLoS Pathog*. 2015;11(10):e1005177.
239.Khan O, Giles JR, McDonald S, et al. TOX transcriptionally and epigenetically programs CD8(+) T cell exhaustion. *Nature*. 2019;571(7764):211-218.
240.De Benedetti F, Grom AA, Brogan PA, et al. Efficacy and safety of emapalumab in macrophage activation syndrome. *Ann Rheum Dis*. 2023.
241.Locatelli F, Jordan MB, Allen C, et al. Emapalumab in Children with Primary Hemophagocytic Lymphohistiocytosis. *N Engl J Med*. 2020;382(19):1811-1822.
242.Keenan C, Nichols KE, Albeituni S. Use of the JAK Inhibitor Ruxolitinib in the Treatment of Hemophagocytic Lymphohistiocytosis. *Front Immunol*. 2021;12:614704.
243.Wang J, Wang Y, Wu L, et al. Ruxolitinib for refractory/relapsed hemophagocytic lymphohistiocytosis. *Haematologica*. 2020;105(5):e210-e212.
244.Chi Y, Liu R, Zhou Z-x, Shi X-d, Ding Y-c, Li J-g. Ruxolitinib treatment permits lower cumulative glucocorticoid dosing in children with secondary hemophagocytic lymphohistiocytosis. *Pediatric Rheumatology*. 2021;19(1):49.
245.Sepulveda FE, Maschalidi S, Vosshenrich CA, et al. A novel immunoregulatory role for NK-cell cytotoxicity in protection from HLH-like immunopathology in mice. *Blood*. 2015;125(9):1427-1434.
246.Andrews LP, Vignali KM, Szymczak-Workman AL, et al. A Cre-driven allele-conditioning line to interrogate CD4⁺ conventional T cells. *Immunity*. 2021;54(10):2209-2217.e2206.
247.Freeman BE, Hammarlund E, Raué H-P, Slifka MK. Regulation of innate CD8+ T-cell activation mediated by cytokines. *Proceedings of the National Academy of Sciences*. 2012;109(25):9971-9976.
248.Berg RE, Forman J. The role of CD8 T cells in innate immunity and in antigen non-specific protection. *Current opinion in immunology*. 2006;18(3):338-343.
249.Hou X, Hao X, Zheng M, et al. CD205-TLR9-IL-12 axis contributes to CpG-induced oversensitive liver injury in HBsAg transgenic mice by promoting the interaction of NKT cells with Kupffer cells. *Cell Mol Immunol*. 2017;14(8):675-684.
250.Krieg AM, Love-Homan L, Yi AK, Harty JT. CpG DNA induces sustained IL-12 expression in vivo and resistance to Listeria monocytogenes challenge. *J Immunol*. 1998;161(5):2428-2434.
251.Yoshimoto T, Takeda K, Tanaka T, et al. IL-12 up-regulates IL-18 receptor expression on T cells, Th1 cells, and B cells: synergism with IL-18 for IFN-gamma production. *J Immunol*. 1998;161(7):3400-3407.
252.Bracaglia C, Sieni E, Da Ros M, et al. Mutations of familial hemophagocytic lymphohistiocytosis (FHL) related genes and abnormalities of cytotoxicity function tests in patients with macrophage activation syndrome (MAS) occurring in systemic juvenile idiopathic arthritis (sJIA). *Pediatric Rheumatology*. 2014;12(S1):P53-P53.
253.Rocco JM, Laidlaw E, Galindo F, et al. Mycobacterial Immune Reconstitution Inflammatory Syndrome in HIV is Associated With Protein-Altering Variants in Hemophagocytic Lymphohistiocytosis-Related Genes. *The Journal of Infectious Diseases*. 2023.

254.Burn TN, Weaver L, Rood JE, et al. Genetic Deficiency of Interferon-γ Reveals Interferon-γ-Independent Manifestations of Murine Hemophagocytic Lymphohistiocytosis. *Arthritis Rheumatol*. 2020;72(2):335-347.
255.Zhang Y, Parmigiani G, Johnson WE. ComBat-seq: batch effect adjustment for RNA-seq count data. *NAR Genomics and Bioinformatics*. 2020;2(3).
256.Morpheus. https://software.broadinstitute.org/morpheus.
257.Marini F, Binder H. pcaExplorer: an R/Bioconductor package for interacting with RNA-seq principal components. *BMC Bioinformatics*. 2019;20(1):331.
258.Torre D, Lachmann A, Ma'ayan A. BioJupies: Automated Generation of Interactive Notebooks for RNA-Seq Data Analysis in the Cloud. *Cell Systems*. 2018;7(5):556-561.e553.
259.immunarch: An R Package for Painless Bioinformatics Analysis of T-Cell and B-Cell Immune Repertoires. 2019.
260.Bolotin DA, Poslavsky S, Mitrophanov I, et al. MiXCR: software for comprehensive adaptive immunity profiling. *Nat Methods*. 2015;12(5):380-381.
261.Shugay M, Bagaev DV, Turchaninova MA, et al. VDJtools: Unifying Post-analysis of T Cell Receptor Repertoires. *PLoS Comput Biol*. 2015;11(11):e1004503.
262.Edgar R, Domrachev M, Lash AE. Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. *Nucleic Acids Res*. 2002;30(1):207-210.
263.Chen CJ, Huang YC, Jaing TH, et al. Hemophagocytic syndrome: a review of 18 pediatric cases. *J Microbiol Immunol Infect*. 2004;37(3):157-163.
264.Oloomi Z, Moayeri H. Cytomegalovirus infection-associated hemophagocytic syndrome. *Arch Iran Med*. 2006;9(3):284-287.
265.Eloseily EM, Cron RQ. Macrophage Activation Syndrome. *The Microbiome in Rheumatic Diseases and Infection*. 2018:151-182.
266.Bhatia S, Bauer F, Bilgrami SA. Candidiasis-Associated Hemophagocytic Lymphohistiocytosis in a Patient Infected with Human Immunodeficiency Virus. *Clinical Infectious Diseases*. 2003;37(11):e161-e166.
267.Schulert GS, Zhang M, Fall N, et al. Whole-Exome Sequencing Reveals Mutations in Genes Linked to Hemophagocytic Lymphohistiocytosis and Macrophage Activation Syndrome in Fatal Cases of H1N1 Influenza. *J Infect Dis*. 2016;213(7):1180-1188.
268.Fourcade J, Sun Z, Benallaoua M, et al. Upregulation of Tim-3 and PD-1 expression is associated with tumor antigen–specific CD8+ T cell dysfunction in melanoma patients. *Journal of Experimental Medicine*. 2010;207(10):2175-2186.
269.Jin H-T, Anderson AC, Tan WG, et al. Cooperation of Tim-3 and PD-1 in CD8 T-cell exhaustion during chronic viral infection. *Proceedings of the National Academy of Sciences*. 2010;107(33):14733-14738.
270.Joshi NS, Kaech SM. Effector CD8 T Cell Development: A Balancing Act between Memory Cell Potential and Terminal Differentiation. *The Journal of Immunology*. 2008;180(3):1309.
271.Abu Eid R, Ahmad S, Lin Y, et al. Enhanced Therapeutic Efficacy and Memory of Tumor-Specific CD8 T Cells by Ex Vivo PI3K-δ Inhibition. *Cancer Research*. 2017;77(15):4135-4145.
272.Humblet-Baron S, Franckaert D, Dooley J, et al. IL-2 consumption by highly activated CD8 T cells induces regulatory T-cell dysfunction in patients with hemophagocytic lymphohistiocytosis. *J Allergy Clin Immunol*. 2016;138(1):200-209.e208.
273.Grom AA, Villanueva J, Lee S, Goldmuntz EA, Passo MH, Filipovich A. Natural killer cell dysfunction in patients with systemic-onset juvenile rheumatoid arthritis and macrophage activation syndrome. *J Pediatr*. 2003;142(3):292-296.

274.de Jager W, Vastert SJ, Beekman JM, et al. Defective phosphorylation of interleukin-18 receptor β causes impaired natural killer cell function in systemic-onset juvenile idiopathic arthritis. *Arthritis & Rheumatism*. 2009;60(9):2782-2793.
275.Takakura M, Shimizu M, Yakoyama T, Mizuta M, Yachie A. Transient natural killer cell dysfunction associated with interleukin-18 overproduction in systemic juvenile idiopathic arthritis. *Pediatrics International*. 2018;60(10):984-985.
276.Ohya T, Nishimura K, Murase A, et al. Impaired Interleukin-18 Signaling in Natural Killer Cells From Patients With Systemic Juvenile Idiopathic Arthritis. *ACR Open Rheumatology*. 2022;4(6):503-510.
277.Shimizu M, Kizawa T, Kato R, Suzuki T, Yachie A. Macrophage activation syndrome in neonates born to mothers with adult-onset Still's disease: perinatal effect of maternal IL-18. *Clinical Immunology*. 2019;207:36-39.
278.Raghu D, Xue H-H, Mielke LA. Control of lymphocyte fate, infection, and tumor immunity by TCF-1. *Trends in immunology*. 2019;40(12):1149-1162.
279.Schluns KS, Kieper WC, Jameson SC, Lefrançois L. Interleukin-7 mediates the homeostasis of naive and memory CD8 T cells in vivo. *Nature immunology*. 2000;1(5):426-432.
280.Kaech SM, Tan JT, Wherry EJ, Konieczny BT, Surh CD, Ahmed R. Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to long-lived memory cells. *Nature immunology*. 2003;4(12):1191-1198.
281.Page N, Klimek B, De Roo M, et al. Expression of the DNA-Binding Factor TOX Promotes the Encephalitogenic Potential of Microbe-Induced Autoreactive CD8(+) T Cells. *Immunity*. 2018;48(5):937-950.e938.
282.Kim K, Park S, Park SY, et al. Single-cell transcriptome analysis reveals TOX as a promoting factor for T cell exhaustion and a predictor for anti-PD-1 responses in human cancer. *Genome Medicine*. 2020;12(1):22.
283.Miller BC, Sen DR, Al Abosy R, et al. Subsets of exhausted CD8+ T cells differentially mediate tumor control and respond to checkpoint blockade. *Nature Immunology*. 2019;20(3):326-336.
284.Tickotsky N, Sagiv T, Prilusky J, Shifrut E, Friedman N. McPAS-TCR: a manually curated catalogue of pathology-associated T cell receptor sequences. *Bioinformatics*. 2017;33(18):2924-2929.
285.Bagaev DV, Vroomans RMA, Samir J, et al. VDJdb in 2019: database extension, new analysis infrastructure and a T-cell receptor motif compendium. *Nucleic Acids Research*. 2019;48(D1):D1057-D1062.
286.Honey K. CCL3 and CCL4 actively recruit CD8+ T cells. *Nature Reviews Immunology*. 2006;6(6):427-427.
287.Trifilo MJ, Bergmann CC, Kuziel WA, Lane TE. CC chemokine ligand 3 (CCL3) regulates CD8(+)-T-cell effector function and migration following viral infection. *J Virol*. 2003;77(7):4004-4014.
288.Galeano Niño JL, Pageon SV, Tay SS, et al. Cytotoxic T cells swarm by homotypic chemokine signalling. *eLife*. 2020;9:e56554.
289.Kim H-D, Park S, Jeong S, et al. 4-1BB Delineates Distinct Activation Status of Exhausted Tumor-Infiltrating CD8+ T Cells in Hepatocellular Carcinoma. *Hepatology*. 2020;71(3):955-971.
290.Roychoudhuri R, Clever D, Li P, et al. BACH2 regulates CD8(+) T cell differentiation by controlling access of AP-1 factors to enhancers. *Nat Immunol*. 2016;17(7):851-860.

291.Clambey ET, Collins B, Young MH, et al. The Ikaros Transcription Factor Regulates Responsiveness to IL-12 and Expression of IL-2 Receptor Alpha in Mature, Activated CD8 T Cells. *PLOS ONE*. 2013;8(2):e57435.
292.O'Brien S, Thomas RM, Wertheim GB, Zhang F, Shen H, Wells AD. Ikaros Imposes a Barrier to CD8+ T Cell Differentiation by Restricting Autocrine IL-2 Production. *The Journal of Immunology*. 2014;192(11):5118-5129.
293.Cheng D, Qiu K, Rao Y, et al. Proliferative exhausted CD8+ T cells exacerbate long-lasting anti-tumor effects in human papillomavirus-positive head and neck squamous cell carcinoma. *eLife*. 2023;12:e82705.
294.Abrams JS, Pearce MK. Development of rat anti-mouse interleukin 3 monoclonal antibodies which neutralize bioactivity in vitro. *J Immunol*. 1988;140(1):131-137.
295.Greenlaw RE, Gardner NJ, Farrar CA, et al. An Antibody Combination That Targets Activated T Cells Extends Graft Survival in Sensitized Recipients. *American Journal of Transplantation*. 2008;8(11):2272-2282.
296.Créput C, Galicier L, Buyse S, Azoulay E. Understanding organ dysfunction in hemophagocytic lymphohistiocytosis. *Intensive Care Med*. 2008;34(7):1177-1187.
297.Kim J, Chang DY, Lee HW, et al. Innate-like Cytotoxic Function of Bystander-Activated CD8(+) T Cells Is Associated with Liver Injury in Acute Hepatitis A. *Immunity*. 2018;48(1):161-173.e165.
298.Mackerness KJ, Cox MA, Lilly LM, Weaver CT, Harrington LE, Zajac AJ. Pronounced virus-dependent activation drives exhaustion but sustains IFN-γ transcript levels. *J Immunol*. 2010;185(6):3643-3651.
299.Oldstone MBA, Ware BC, Horton LE, et al. Lymphocytic choriomeningitis virus Clone 13 infection causes either persistence or acute death dependent on IFN-1, cytotoxic T lymphocytes (CTLs), and host genetics. *Proc Natl Acad Sci U S A*. 2018;115(33):E7814-e7823.
300.Cornberg M, Kenney L, Chen A, et al. Clonal Exhaustion as a Mechanism to Protect Against Severe Immunopathology and Death from an Overwhelming CD8 T Cell Response. *Frontiers in Immunology*. 2013;4.
301.Svoboda J, Gerson JN, Landsburg DJ, et al. Interleukin-18 Secreting Autologous Anti-CD19 CAR T-Cells (huCART19-IL18) in Patients with Non-Hodgkin Lymphomas Relapsed or Refractory to Prior CAR T-Cell Therapy. *Blood*. 2022;140(Supplement 1):4612-4614.
302.Chen Z, Ji Z, Ngiow SF, et al. TCF-1-Centered Transcriptional Network Drives an Effector versus Exhausted CD8 T Cell-Fate Decision. *Immunity*. 2019;51(5):840-855.e845.
303.Lee JY, Hamilton SE, Akue AD, Hogquist KA, Jameson SC. Virtual memory CD8 T cells display unique functional properties. *Proc Natl Acad Sci U S A*. 2013;110(33):13498-13503.
304.Akue AD, Lee JY, Jameson SC. Derivation and maintenance of virtual memory CD8 T cells. *J Immunol*. 2012;188(6):2516-2523.
305.White JT, Cross EW, Burchill MA, et al. Virtual memory T cells develop and mediate bystander protective immunity in an IL-15-dependent manner. *Nat Commun*. 2016;7:11291.
306.Chaturvedi V, Marsh RA, Lorenz AZ, et al. T cell activation profiles distinguish hemophagocytic lymphohistiocytosis and early sepsis. *Blood*. 2020.
307.Tough DF, Borrow P, Sprent J. Induction of bystander T cell proliferation by viruses and type I interferon in vivo. *Science*. 1996;272(5270):1947-1950.
308.Shim CH, Cho S, Shin YM, Choi JM. Emerging role of bystander T cell activation in autoimmune diseases. *BMB Rep*. 2022;55(2):57-64.

309.Inaba K, Inaba M, Romani N, et al. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *The Journal of experimental medicine*. 1992;176(6):1693-1702.
310.Lima-Junior DS, Krishnamurthy SR, Bouladoux N, et al. Endogenous retroviruses promote homeostatic and inflammatory responses to the microbiota. *Cell*. 2021;184(14):3794-3811.e3719.
311.Koh AJ, Sinder BP, Entezami P, Nilsson L, McCauley LK. The skeletal impact of the chemotherapeutic agent etoposide. *Osteoporos Int*. 2017;28(8):2321-2333.
312.Snow AL, Marsh RA, Krummey SM, et al. Restimulation-induced apoptosis of T cells is impaired in patients with X-linked lymphoproliferative disease caused by SAP deficiency. *J Clin Invest*. 2009;119(10):2976-2989.
313.Spaner D, Raju K, Radvanyi L, Lin Y, Miller RG. A role for perforin in activation-induced cell death. *J Immunol*. 1998;160(6):2655-2664.
314.Chen L, Woo M, Hakem R, Miller RG. Perforin-dependent activation-induced cell death acts through caspase 3 but not through caspases 8 or 9. *European Journal of Immunology*. 2003;33(3):769-778.
315.Spaner D, Raju K, Rabinovich B, Miller RG. A role for perforin in activation-induced T cell death in vivo: increased expansion of allogeneic perforin-deficient T cells in SCID mice. *J Immunol*. 1999;162(2):1192-1199.
316.Zondag TCE, Lika A, van Laar JAM. The role of etoposide in the treatment of adult patients with hemophagocytic lymphohistiocytosis. *Experimental Hematology & Oncology*. 2023;12(1):2.
317.Johnson TS, Terrell CE, Millen SH, Katz JD, Hildeman DA, Jordan MB. Etoposide selectively ablates activated T cells to control the immunoregulatory disorder hemophagocytic lymphohistiocytosis. *J Immunol*. 2014;192(1):84-91.
318.Tissot AC, Pecorari F, Plückthun A. Characterizing the functionality of recombinant T-cell receptors in vitro: a pMHC tetramer based approach. *J Immunol Methods*. 2000;236(1-2):147-165.
319.Garcia-Montojo M, Rodriguez-Martin E, Ramos-Mozo P, et al. Syncytin-1/HERV-W envelope is an early activation marker of leukocytes and is upregulated in multiple sclerosis patients. *European Journal of Immunology*. 2020;50(5):685-694.
320.Fitzgerald BP, Wojciechowski AL, Bajwa RPS. Efficacy of Prompt Initiation of Antiretroviral Therapy in the Treatment of Hemophagocytic Lymphohistiocytosis Triggered by Uncontrolled Human Immunodeficiency Virus. *Case Reports in Critical Care*. 2017;2017:8630609.
321.Jessen B, Kogl T, Sepulveda FE, de Saint Basile G, Aichele P, Ehl S. Graded defects in cytotoxicity determine severity of hemophagocytic lymphohistiocytosis in humans and mice. *Front Immunol*. 2013;4:448.
322.Ishii K, Pouzolles M, Chien CD, et al. Perforin-deficient CAR T cells recapitulate late-onset inflammatory toxicities observed in patients. *J Clin Invest*. 2020;130(10):5425-5443.
323.Gibson LF, Fortney J, Landreth KS, Piktel D, Ericson SG, Lynch JP. Disruption of bone marrow stromal cell function by etoposide. *Biol Blood Marrow Transplant*. 1997;3(3):122-132.
324.Lynch DH, Ramsdell F, Alderson MR. Fas and FasL in the homeostatic regulation of immune responses. *Immunology today*. 1995;16(12):569-574.
325.O'Connell J. Fas ligand and the fate of antitumour cytotoxic T lymphocytes. *Immunology*. 2002;105(3):263-266.
326.Rauf A, Khatri M, Murgia MV, Saif YM. Fas/FasL and perforin-granzyme pathways mediated T cell cytotoxic responses in infectious bursal disease virus infected chickens. *Results in immunology*. 2012;2:112-119.

327.Straus SE, Sneller M, Lenardo MJ, Puck JM, Strober W. An inherited disorder of lymphocyte apoptosis: the autoimmune lymphoproliferative syndrome. *Annals of internal medicine*. 1999;130(7):591-601.
328.Theofilopoulos AN, Dixon FJ. Murine models of systemic lupus erythematosus. *Advances in immunology*. 1985;37:269-390.
329.Lohman BL, Razvi ES, Welsh RM. T-lymphocyte downregulation after acute viral infection is not dependent on CD95 (Fas) receptor-ligand interactions. *Journal of Virology*. 1996;70(11):8199-8203.
330.Mitra A, Barua A, Huang L, Ganguly S, Feng Q, He B. From bench to bedside: the history and progress of CAR T cell therapy. *Frontiers in Immunology*. 2023;14.
331.Robertson MJ, Mier JW, Logan T, et al. Clinical and biological effects of recombinant human interleukin-18 administered by intravenous infusion to patients with advanced cancer. *Clin Cancer Res*. 2006;12(14 Pt 1):4265-4273.
332.Robertson MJ, Kirkwood JM, Logan TF, et al. A dose-escalation study of recombinant human interleukin-18 using two different schedules of administration in patients with cancer. *Clin Cancer Res*. 2008;14(11):3462-3469.
333.Jaspers JE, Khan JF, Godfrey WD, et al. IL-18–secreting CAR T cells targeting DLL3 are highly effective in small cell lung cancer models. *The Journal of Clinical Investigation*. 2023;133(9).
334.Svoboda J, Gerson JN, Landsburg DJ, et al. Interleukin-18 Secreting Autologous Anti-CD19 CAR T-Cells (huCART19-IL18) in Patients with Non-Hodgkin Lymphomas Relapsed or Refractory to Prior CAR T-Cell Therapy [abstract]. *64th ASH Annual Meeting and Exposition*. 2022.
335.Ishii K, Chien CD, Erwin-Cohen R, Young HA, Fry TJ. Perforin Is Dispensable for Anti-CD19 CAR-T Cell Cytotoxicity but Contributes to CAR-T Cell Contraction and Regulates Host Inflammatory Response. *Blood*. 2017;130(Supplement 1):1918-1918.
336.Benmebarek M-R, Karches CH, Cadilha BL, Lesch S, Endres S, Kobold S. Killing Mechanisms of Chimeric Antigen Receptor (CAR) T Cells. International Journal of Molecular Sciences. Vol. 20; 2019.
337.Ishii K, Pouzolles M, Chien CD, et al. Perforin-deficient CAR T cells recapitulate late-onset inflammatory toxicities observed in patients. *The Journal of Clinical Investigation*. 2020;130(10):5425-5443.
338.Minnie SA, Waltner OG, Ensbey KS, et al. Depletion of exhausted alloreactive T cells enables targeting of stem-like memory T cells to generate tumor-specific immunity. *Sci Immunol*. 2022;7(76):eabo3420.
339.Olivera I, Bolaños E, Gonzalez-Gomariz J, et al. mRNAs encoding IL-12 and a decoy-resistant variant of IL-18 synergize to engineer T cells for efficacious intratumoral adoptive immunotherapy. *Cell Reports Medicine*.
340.Simcha Il I. Phase 1a and Phase 2 Study for PK, PD, Safety and Preliminary Efficacy of ST-067; 2024.
341.Carcillo JA, Halstead Es Fau - Hall MW, Hall Mw Fau - Nguyen TC, et al. Three Hypothetical Inflammation Pathobiology Phenotypes and Pediatric Sepsis-Induced Multiple Organ Failure Outcome. (1529-7535 (Print)).
342.Lee YR, Yang IH, Lee YH, et al. Cyclosporin A and tacrolimus, but not rapamycin, inhibit MHC-restricted antigen presentation pathways in dendritic cells. *Blood*. 2005;105(10):3951-3955.