

**DEFINING THE ROLES OF DEVELOPMENT, METABOLISM, AND THE ENVIRONMENT
IN THE CD8+ T CELL RESPONSE TO INFECTION**

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In contrast to adults, neonates experience high morbidity and mortality from recurrent intracellular infections. CD8+ T cells are responsible for killing intracellularly infected cells. Therefore, uncovering the mechanisms that regulate age-related differences in the CD8+ T cell compartment will allow us to develop approaches to enhance immunity for this vulnerable population.

Neonatal CD8+ T cells provide robust protection early in infection but become terminally differentiated and lose their ability to form memory whereas adult CD8+ T cells excel at memory formation. The mechanisms that instruct neonatal and adult CD8+ T cells to adopt divergent fates following infection are unknown. In this dissertation, I investigated the cellular and molecular mechanisms that promote the neonatal program of immunity. The conventional theory is that the neonatal immune system is immature because their CD8+ T cells are younger and have undergone less post-thymic maturation. However, I show neonatal CD8+ T cells are not immature, rather, CD8+ T cells made at different times of life arise distinct progenitors give rise to unique protective programs. Next, I asked whether differences in metabolic

programming contribute to the age-related program of protection. I discovered Lin28b promotes elevated levels of glycolysis in neonates and blocking neonatal cell entry into glycolysis led to sufficient memory formation and recall responses. Finally, fetal- and adult-derived CD8⁺ T cells co-exist in adulthood but we do not know if the developmental architecture can be modified or if the functions of fetal-derived cells can be altered. In the fourth chapter, I developed a novel approach to expose mice to a diverse microbial environment for the entirety of early development. I found high microbial exposure in early life allowed fetal-derived cells to integrate into the adult CD8⁺ T cell compartment at higher proportions and enhance their protective capabilities.

Ultimately, my doctoral work shows neonatal CD8⁺ T cells represent a distinct population that is uniquely suited to respond to the challenges present in early life. Findings from my dissertation will contribute to the conceptual framework that explains CD8⁺ T cell ontogeny, how individual variation in cell-mediated immune responses arises, and the programs that underlie immune cell instruction.

BIOGRAPHICAL SKETCH

Cybelle Tabilas was born in a small, rural barangay in the Cagayan Valley of the Philippines. At two years old, she moved to Woodland, California. Cybelle graduated from Willamette University and received a National Institutes of Health fellowship to study the signaling pathways that underlie thymus organogenesis under the guidance of Dr. Nancy Manley. This experience sparked her love for the thymus and T cells. Cybelle matriculated into Cornell University where she joined Dr. Brian Rudd's lab in 2017. Here, she investigated the life history of the CD8⁺ T cell compartment. Cybelle has been the recipient of three National Institutes of Health fellowships.

To remind herself science is more than laboratory work, Cybelle grounded herself by giving back to the communities she cares deeply about. She spent five years organizing the Expanding Your Horizons conference, four years designing a series of lessons entitled "Of Microbes and Men" to teach third-graders about the immune system and the tiny ecosystem of good and bad germs that surround them, and attended four national conferences to encourage individuals from marginalized communities to pursue graduate training at Cornell. Outside of science, she served as an advisor to the Cornell chapter of her collegiate sorority. In 2021, her efforts as chapter advisor were recognized by Alpha Chi Omega Headquarters and she was given the 'Outstanding Collegiate Advisor' award.

While Cybelle does not want to think about her life post-Rudd lab, Cybelle can only hope that it is as amazing and fulfilling as her time in the Rudd lab was.

For my mom and dad. Who left the land, culture, and people they loved with the hope of a life with fewer limitations.

To Shriners Hospital for Children for giving me and countless others excellent care.

In memory of the friends and family that I lost while in school. Especially those who passed from and because of COVID-19.

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My achievements were possible because my parents were the image of tenacity. And thanks to my sister, who can find calm in any situation.

During my Ph.D., I was met with countless tests and seemingly endless wandering, much like Odysseus on his way back to Ithaca. But I had great friends with me throughout my journey! I want to thank everyone who celebrated and commiserated with me. I'd like to particularly thank: Denise Poltavski for being my best friend. Ellie Larson for her beautiful friendship. Simon Früh for our chats about life, innate and adaptive immunity, and everything in-between. Monique Theriault for constantly reminding me to be proud of myself. Rachel Martini for somehow always sensing when I needed a pick me up. Brent Allman, it was life-changing having another first-gen BIPOC by my side. And finally, thank you to all the Rudd Rats, past and present, but especially Norah Smith for her wisdom on literally everything.

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Finally, I would like to give the biggest and most heart-filled thank you to Brian Rudd, the eternal optimist. Years after earning my Ph.D., my impact on immunology may change and I probably could no longer juggle multiple experiments in a single day

while maintaining my sanity but the intellectual skill set and the lessons will stay with me. When first-year students ask what to look for when choosing a lab, I tell them to prioritize mentorship because of my experience in Brian's lab. I am forever grateful for everything that I've learned from him, the thought experiments he made me do, and his constant reminders to rest. Brian showed empathy, patience, and compassion are the actions that foster immense growth. Without his mentorship, I would not have the confidence or achieved the same level of success that I have.

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LIST OF ABBREVIATIONS

ko	knock out
2-DG	2-deoxy-D-glucose
ACT	adoptive cell transfer
ATAC-seq	assay for transposase-accessible chromatin
ATP	adenosine triphosphate
CAR-T	chimeric antigen receptor T cell therapy
DC	dendritic cell
DE	differentially expressed
DOAHD	developmental origin of adult health and disease
dpi	days post infection
ECAR	extracellular acidification rate
GO	gene ontology
HP	homeostatic proliferation
HSC	hematopoietic stem cell
IFN	interferon
Lin28b tg	Lin28b transgenic
LM-gB	Listeria monocytogenes-glycoprotein B
LN	Lymph node
MHC-I	major histocompatibility complex-I
miR /miRNA	micro RNA
MPEC	memory precursor effector cell
n-addition	nucleotide addition
OCR	oxygen consumption rate
qPCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
RNA-seq	ribonucleic acid-sequencing
RTE	recent thymic emigrant
SLEC	short lived effector cell
SLO	secondary lymphoid organ
spMHC	self-peptide major histocompatibility complex
SRC	spare respiratory capacity
TCR	t cell receptor
TCR:pMHC	t cell receptor peptide presented on major histocompatibility complex
TdT	terminal deoxynucleotidyl transferase
TN	true naïve
ts	timestamp

VM	virtual memory
wt	wildtype

CHAPTER 1: Introduction

The Immune System

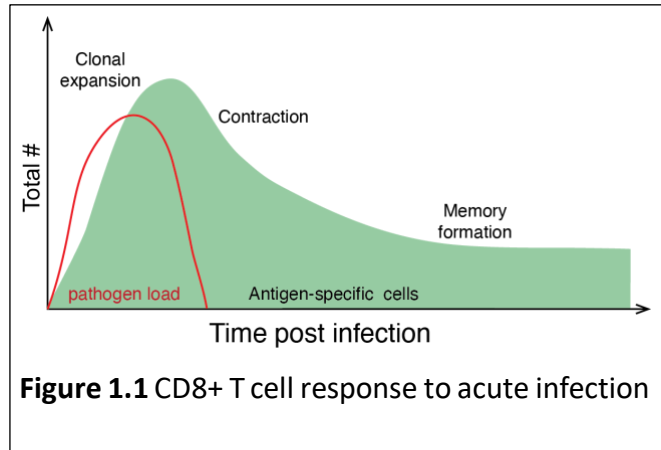
The mammalian immune system represents an extremely complex network of cells that maintain homeostasis for a host through elegant modes of communication. To maintain homeostasis, cells of the immune system must first be able to recognize and react to harmful foreign pathogens or environmental agents. Second, cells of the immune system must be able to eradicate the pathogen or bring the infection under control then provide protection against repeated assault. The high degree of coordination required to achieve this goal is a testament to the level of evolutionary sophistication present in the natural world.

CD8+ T Cells

CD8+ cytotoxic T lymphocytes, or CD8+ T cells, belong to the adaptive immune system and kill intracellularly infected or transformed cells. They mediate death through classical activation via T cell receptor (TCR) engagement with cognate antigen or through heterologous immunity termed 'bystander activation' ^{1,2}. A defining feature of CD8+ T cells is the expression of a unique T cell receptor (TCR) that is specific for a discrete antigen. Classical CD8+ T cell activation is triggered once a CD8+ T cell encounters and engages with their cognate antigen. CD8+ T cells are potent killers and as a result, their activation requires the integration of multiple signals to prevent inappropriate responses. Signal 1 is antigen presentation by a major histocompatibility class I (MHC-I) expressing cell, signal 2 is co-stimulation which involves the

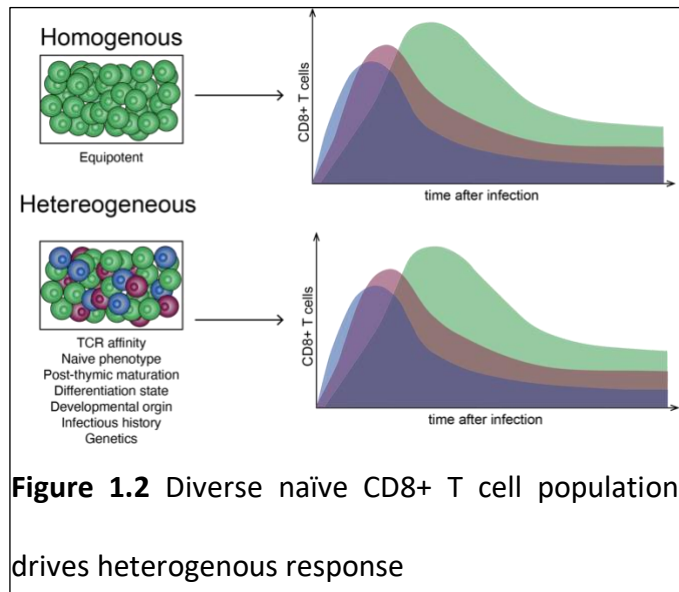
engagement of CD28 with B7-1/2, and signal 3 is a combination of inflammatory signals present at the site of activation ³⁻¹⁰. The absence of any of the three signals dampens the T cell response or renders a T cell anergic ^{11,12}.

A comprehensive review of CD8+ T cells is incomplete without showing the graph that depicts the three phases of the T cell response to acute infection: clonal expansion, T cell



contraction, and memory formation (fig 1). In consideration of Occam's razor, having the same antigen specificity should render CD8+ T cells equipotent. However, in clear violation of this theoretical principle, individual T cells with similar antigen specificities adopt divergent fates upon TCR:peptide-MHC (TCR:pMHC) engagement ¹³⁻¹⁸. Over the past three decades, there has been a renaissance in our understanding of CD8+ T cell potential with the discovery of several fate determinants beyond TCR specificity that promote the diversity of effector and memory subsets present in the responding population ¹⁹⁻²². As knowledge of CD8+ T cell biology has advanced, this popular graph now represents a reductive view of CD8+ T cell population dynamics because it overlooks the fate potentials that exist within various subsets of T naïve, effector, and memory cells ¹⁸.

An intense amount of research effort has been placed in identifying factors that alter the quantity and quality of effector and memory cells made in response to antigenic stimulation. The naïve T cell population is composed of a diverse subset of T



cells, though how these unique subsets contribute to the effector response has been largely ignored. There are a handful of theories that exist to explain variation in the responding population such as TCR affinity, asymmetric cell

division, and decreasing potential model ^{18,21,23}. Our lab focuses on understanding how the developmental origin of a CD8+ T cell alters its response to infection. Understanding the pathways by which a naïve T cell decides to contribute to infection can markedly improve approaches to promote ideal responses, such as enhanced killing capacity or generation of a robust memory population in vulnerable populations.

Post-Thymic Maturation

T cell development is a highly regulated process that occurs in the thymus. Thymic education is undoubtedly the most difficult trade school to graduate from, only 3-5% of the starting population survives and is exported into the periphery ²⁴. Once a T cell leaves the thymus, they are termed 'recent thymic emigrants' (RTEs) ^{25,26}. The

RTE period consists of the first three weeks post-thymic export ²⁷. During this period, RTEs undergo gradual changes in their phenotypic and functional properties then transition into the mature population. Despite extensive research, the biological importance of the post-thymic maturation period or the triggers that signal RTEs to transition into the mature naïve T (MNT) cell stage remains elusive.

Due to the absence of definitive markers, RTEs are broadly defined as being Qa2^{lo}CD24^{hi} ²⁵. 100% of cells with the Qa2^{lo}CD24^{hi} phenotype are RTEs but this scheme

fails to identify ~85% of RTEs ²⁷. In the past, a range of imperfect tools has been used to study RTEs (Table 1) ^{26,28,29}. The Fink lab has adopted the Rag2p-GFP mouse to identify RTEs. This mouse model has been instrumental in advancing our

Approach	Advantage	Disdvantage
FITC injection into thymus	Identify newly exported T cells	Mature T cells circulate through thymus FITC has short-half life
Thymic lobe transplantions	Easy identification of donor cells Control of environment	Non-sterile inflammation Need to irradiate host for adult thymus to graft
Rag2p-GFP	GFP is expressed for 3 weeks	GFP is diluted with each round of division
TRECs (hu-mans)	Non-invasive marker	One daughter cell will inherit TREC

Table 1.1 Approaches to identify 'Recent Thymic Emigrants'

understanding of RTE biology. In this mouse model, GFP expression is 'turned on' when Rag2 is activated during TCR rearrangement ³⁰. Thus, all T cells that have completed thymic development express GFP and GFP expression persist ~3 weeks post-thymic egress.

Studies using the Rag2p-GFP mouse found RTE and MNT cells represent distinct subsets with unique contributions to immunity ^{31–33}. RTEs express higher concentrations of TCR on their surface but lower levels of CD28 when compared to MNT cells ³⁴. This leads to the poor generation of co-stimulatory signals and renders

RTEs more susceptible to anergy ³⁵. As a result of poor co-stimulation, RTEs exhibit reduced proliferative capacities in response to *in vitro* α CD3/28 activation ³⁶. Upon *in vivo* antigenic stimulation, RTEs produce fewer effector molecules and undergo less clonal expansion showing RTEs have diminished ability to productively contribute to the antigen-specific response ²⁶. This hypo-reactivity is seen in both CD4+ and CD8+ T cells. Under T helper 1 polarizing conditions, CD4+ RTEs produce lower levels of interferon-gamma (IFN γ) ³⁶. Similarly, CD8+ RTEs produce lower levels of effector molecules and fail to transition into the memory population after infection ^{32,36}. The diminished production of effector molecules has led many to classify CD8+ RTEs as hyporesponsive. While RTEs often present as less productive versions of their MNT counterparts, a trend in the literature is that RTEs still provide sufficient protection during *in vivo* challenges.

100% of neonatal CD8+ T cells are RTEs. Because of this, the neonatal CD8+ T cells have undergone less post-thymic maturation. Consequently, the neonatal CD8+ T cell response has been classified as immature or deficient. Thus, the motivation to understand RTE biology has been associated with using RTEs as a surrogate for neonatal immunity ^{37,38}. However, the first direct comparison of neonatal and adult RTEs was performed on CD4+ T cells and demonstrated the neonatal and adult RTE populations are not phenotypically and functionally equivalent ³⁹. Neonatal RTEs proliferate more in response to homeostatic cytokines and produce higher levels of cytokines than adult RTEs in response to activation. While this study shows neonatal and adult cells behave differently following *in vitro* stimulation, we still do not know

whether functional differences observed between neonatal and adult RTEs arise from cell-intrinsic properties. Opiela *et al.* sorted RTEs from Rag-2pGFP neonatal or adult mice. This approach still leaves neonatal RTEs amenable to pressures associated with a lymphopenic environment which may lead to confounding factors in the interpretation of the data. Overall, Opiela *et al.* clearly showed RTE biology cannot be used as a surrogate for neonatal population dynamics. We still do not know whether these differences translate to altered protection against infection or what promotes age-related differences in cell-mediated immunity. In this dissertation, I asked whether neonatal RTEs provide a unique program of protection for a host and if so, does this altered program of protection arise as a result of developmental origin or arise from lymphopenic environment experienced in early life.

Homeostatic Proliferation and Virtual Memory Cells

The T cell compartment must recognize a multitude of foreign antigens, this is achieved by having a diverse TCR repertoire. The number of T cells is relatively constant within a healthy individual because the 'space' and resources T cells have access to are limited ⁴⁰. To maintain a diverse TCR repertoire, the naïve T cell compartment represents a balance between RTEs and existing MNT cells. Survival of naïve T cells depends on contact with self-peptides presented on MHC (spMHC) and IL-7 consumption ^{41–43}. The homeostatic expansion of existing T cell clones and the continued generation of new T cell clones (RTEs) creates ongoing competition for finite resources. Successful acquisition of these resources by T cells determines the diversity

of the T cell pool. While TCR engagement with self-peptide or consumption of homeostatic cytokines is necessary for T cell survival, interaction with these two factors also triggers homeostatic proliferation (HP) ⁴². For this dissertation, I will refer to the drivers of HP for naïve CD8+ T cells as the parameters that promote HP in memory T cells and CD4+ T cells are slightly different ⁴⁴. While HP is a slow and gradual process, HP has been the subject of intense investigation not only because of its importance in maintaining the naïve T cell pool but in recent years, the cell-intrinsic properties that promote HP have been found to influence a cell's resting phenotype and reactivity to a foreign antigen ^{45–50}.

Positive selection allows for cells with TCRs that exhibit low, yet adequate, reactivity towards self-peptides to progress through thymic development ⁵¹. The strength of TCR:peptide-MHC (TCR:pMHC) affinity is set during thymic development and remains stable for the lifetime of a T cell ⁵². There is a spectrum of TCR affinities present in the repertoire and TCR:pMHC affinity is positively correlated with CD5 expression ⁵³. To identify how affinity for spMHC promotes HP, researchers have utilized different strains of TCR transgenic (tg) mice. TCR tg cells that have higher affinity TCRs undergo HP at higher levels compared to lower affinity TCR tg cells ⁵⁴. In addition to TCR affinity promoting HP, high-affinity TCRs are recruited more efficiently to infection and have a gene expression profile that favors effector responses ^{20,55}.

Extensive homeostatic turnover leads to the adoption of a 'virtual memory' (VM) phenotype ^{56,57}. Unlike 'true naïve' (TN) cells, VMs display a CD44^{hi}CD122^{hi} phenotype and are associated with having a high affinity for self-peptides. VMs are

separated from antigen-experienced, 'true memory' CD8⁺ T cells by low expression of the surface marker CD49d and VMs represent ~5-20% of CD8⁺ T cells in circulation ⁵⁷. It was initially thought that VM cells arose from basal levels of antigen exposure from commensal pathogens but VM cells have been identified in all mice, including germ-free mice, suggesting they arise from pathogen-independent mechanisms ⁵⁸. CD122, a component of the IL-15 receptor, is used to identify VM cells ⁵⁶. IL-15 is important for the survival of true memory cells and was previously thought to be dispensable for the survival antigen-inexperienced cells ^{41,59,60}. However, the generation of VM cells is highly dependent on IL-15 as there are no VM cells present in IL-15^{-/-} mice ⁽⁴³⁾. This further exemplifies how the naïve phenotype of a T cell can impact the long-term fate of a cell. The adoption of a VM phenotype is highly consequential for the CD8⁺ T cell responses to infection ⁴⁶. RNA-seq on VM cells reveals transcriptional profiles similar to memory cells ¹⁹. Furthermore, VMs behave like memory cells as seen in their high capacity for proliferation, rapid activation, and increased cytotoxicity in antigen-specific and antigen-independent manners ^{20,55,57,61}. HP and cell survival are reduced if cytokines (particularly IL-7 alongside other γ chain cytokines) are not present, or if MHC-I expression is absent ^{42,62}. Dysregulation of lipid rafts disrupts T cell receptor complexes and cytokine receptors, showing the interaction of the two promotes HP ^{63,64}.

When naïve T cells experience a lymphopenic environment, they undergo more rapid proliferation known as lymphopenic-induced proliferation (LIP) ⁴⁶. LIP is driven by an abundance of IL-7 and increased access to spMHC. The peripheral compartment

of neonates is largely void of T cells, leading to LIP^{65,66}. Neonates have a large population of VM cells, this suggests the unique behavioral profile of neonatal cells may be explained by extensive proliferation rather than being pre-programmed during early development. In a clever experiment, Smith *et al.* created a dual reporter system in which they transplanted a tamoxifen-inducible RFP-expressing neonatal thymus under the kidney capsule of a tamoxifen-inducible GFP-expressing lymphoreplete adult recipient⁶⁷. They found neonatal T cells that are exported into a 'normal' environment still preferentially adopted a VM phenotype. Furthermore, Wang *et al.* adoptively co-transferred VM cells from neonatal and adult animals and found VMs from different aged mice continued to adopt distinct fates following infection. These findings support our model that state VM cells arise from a LIP-independent manner and provides evidence for developmentally regulated mechanisms that control for population dynamics of CD8+ T cells. It would be of interest to identify the programs that promote preferential recruitment of neonatal VM population early in infection and what cell-intrinsic programs underlie the altered program of CD8+ T cell immunity observed in neonatal and adult mice. I investigated this question in chapters 3 and 4 of my dissertation.

Retrospective of Neonatal Immunity

In 1960, Sir Medawar received the Nobel Prize in Medicine or Physiology for showing allogenic transplantation of tissues in early life does not lead to rejection even in the absence of conditioning while transplantation in adulthood led to rejection. This

served as the theoretical basis for organ transplantations and is now referred to as 'neonatal tolerance'. This underlying principle also led to early descriptions of the neonatal immune system as 'immature' because cells are rapidly tolerized in the presence of antigens ^{35,68}. This principle ignited interest in understanding cell-mediated immunity between differently aged individuals. The belief that the neonatal immune system is immature has been reinforced by epidemiological studies showing neonates are at an increased risk of morbidity and mortality from infectious diseases ⁶⁹⁻⁷². Furthermore, infants are also at an increased risk for repeated intracellular infections early in life, indicating a decreased capacity to form immunological memory. Indeed, many vaccinations given in the first 15 months of life require multiple doses to achieve the desired level of immune protection. Approaches to enhance cell-mediated immunity in this vulnerable population is the topic of intense investigation by many researchers including our lab. While the neonatal response to infection has been defined as deficient, there has been a shift in our understanding of neonatal T cell immunology. Paradigm-shifting studies performed in the past few years have shown neonatal CD8+ T cells provide robust protection against intracellular infection. The mechanisms underlying this response are currently under investigation.

Age-Related Alterations in T Cell Receptor Development

Terminal deoxynucleotidyl transferase (TdT) is a developmentally regulated enzyme that adds random nucleotide additions (n-additions) to the TCR ^{73,74}. Through this process, it is estimated that the TCR recombinational process can result in 10^{15}

unique TCRs ^{75,76}. Lymphocytes made early in life do not express TdT and as a result, have a limited repertoire compared to that in adulthood ^{77,78}. A limited repertoire may reduce an individual's probability to recognize potential pathogens. This could explain why neonatal T cell responses have historically been classified as deficient and why neonates are susceptible to infection. However, studies using TdT^{-/-} mice show CD8⁺ T cells provide sufficient protection against multiple intracellular infections and are promiscuous to multiple antigens when compared to the cells that have TdT expression ^{78,79}. Increased promiscuity of TCRs is driven by high-affinity TCRs. In fact, neonatal thymocytes bearing low-affinity TCRs are preferentially deleted and high-affinity TCRs are enriched in early life ⁸⁰. Perhaps the bias towards high-affinity TCRs in early life is two-fold: first, it increases the ability of cells to recognize a wide array of antigens. Second, high-affinity TCRs undergo HP at higher rates which allow neonatal T cells to quickly 'fill' the T cell compartment further enhancing the protective capabilities of the nascent immune compartment. Therefore, neonatal CD8⁺ T cells simply represent a distinct, not deficient, population that has evolved to meet the needs of the young host.

Age-Related Differences in CD8⁺ T Cell Function

Our lab has used multiple approaches to understand the programs that drive age-related differences in CD8⁺ T cells. A defining feature of neonatal CD8⁺ T cells is their ability to respond vigorously and rapidly to activation while failing to transition into the long-lived memory pool ^{44,81-86}. The quick kinetics of neonatal CD8⁺ T cells

biases cells toward a terminally differentiated short-lived effector (SLEC, KLRG1^{hi}CD127^{lo}) fate. *In vitro* stimulation models show quick responses to activation through extensive proliferation causes division-linked terminal differentiation, reducing the potential of neonatal cells to transition into the memory population ^{44,81}.

This finding led us to ask, how do neonatal T cells rapidly respond to activation? Naïve neonatal T cells express high levels of CD44 and Ly6C as well as transcription factors t-bet and Eomes, all of which are markers of differentiation ⁸⁵. In the past, expression of these markers has only been associated with activated or memory cells but the expression of these transcription factors by neonatal T cells may explain their rapid kinetics and further exemplify how they are separate from their adult counterparts. Preferential expression of transcription factors is not exclusive to the CD8⁺ T cell compartment, neonatal CD4⁺ T cells express high levels of Foxp3, increasing their likelihood of becoming regulatory T cells ⁸⁷. Perhaps the propensity of neonatal CD4⁺ T cells to become regulatory T cells serves as a ‘check and balance’ to ensure T cell responses present in early life are not overtly reactive to every antigen a neonatal CD8⁺ T cell may encounter. Overall, these findings show regulatory profiles and cell-intrinsic programs promote the adoption of unique behavioral programs observed in neonatal CD8⁺ T cells.

Age-Related Differences in CD8⁺ T Cell Programming

To further understand the mechanisms that underlie the unique behavioral program of neonatal CD8⁺ T cells, we investigated how the gene regulatory landscape

of different aged cells differed. RNA-sequencing of naïve and effector CD8⁺ T cells show that neonatal CD8⁺ T cells are biased towards an ‘activated’ gene expressional profile and that alterations in gene expression profile poise adult and neonatal cells to adopt unique functions ⁸¹. There are also several temporally and developmentally regulated micro-RNAs that modulate immunity ⁸⁸. Among the most differentially expressed miRNAs between neonatal and adult CD8⁺ T cells is microRNA-29 (miR-29) which is highly expressed in naïve adult CD8⁺ T cells ^{83,84}. Interestingly, possible targets of miR-29 are t-bet and Eomes ⁸⁹. This led us to hypothesize that miR-29 may regulate the age-related differences of CD8⁺ T cells. Overexpression of miR-29 in neonatal CD8⁺ T cells led to a T cell response that mimics adult kinetics and enhanced secondary responses ⁸⁹. This finding established molecular roles in developmentally dependent programming of CD8⁺ T cells. Another potential developmental regulator of immunity is Lin28. Lin28 is an RNA-binding protein and is expressed in contrasting amounts at different ends of the lifespan with neonatal cells having high expression of Lin28 ⁹⁰. Multiple lines of evidence implicate Lin28 as the ‘master regulator’ between neonatal and adult cellular functions. Ectopic expression of Lin28 in adult CD8⁺ T cell progenitors leads to the adult-derived T cells phenocopying the neonatal immune program ⁸⁵. Altogether, this shows neonatal CD8⁺ T cells’ behavioral program is linked to their molecular profile.

While we have identified several important cell-intrinsic mechanisms that explain age-related differences in immunity. It is also important to discern how the peripheral environment contributes to age-related programs. For example, the

neonatal T cell compartment is largely lymphopenic (the consequences of which are explained more in-depth in the 'homeostatic proliferation and virtual memory' subsection of this chapter) ²⁸. An explanation for the extreme differences observed in neonates and adults is that the neonatal periphery is 'empty' and T cells undergo extensive proliferation to fill the periphery. This may explain why neonatal T cells have a more differentiated program prior to infection and, are, therefore, more reactive to infection. To test this conventional hypothesis, Wang *et al.* injected fetal or adult progenitors into recipients and found fetal-derived cells continued to adopt a SLEC phenotype and failed to transition into the memory population while adult-derived CD8+ T cells continued to preferentially give rise to MPECs ⁸⁵. This provides further support for a unique program of behavior that arises largely independent of environmental influences.

The advancement and refinement of immunological approaches have allowed researchers to take reductionist approaches to study neonatal immunity. Assembling evidence generated by multiple labs has led to a revision in our understanding of neonatal T cell biology. Rather than the previous paradigm that defines neonatal T cells as immature forms of adult T cells, we now recognize neonatal and adult CD8+ T cells as distinct populations that are separated by naïve behavioral programs which impact their responses to infection and their long-term fates.

CD8+ T Cell Metabolism

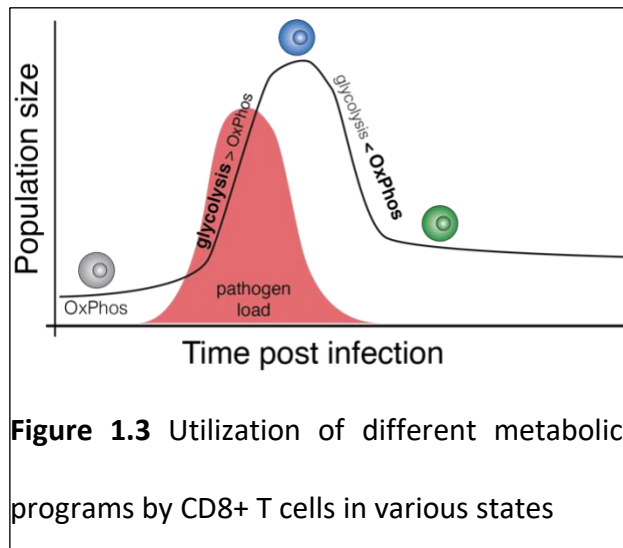
Differentiation of naïve CD8+ T cells into various effector or memory subsets is tightly regulated through soluble factors, receptor engagement, and alterations in

transcriptional programs. This highlights the plasticity contained within the CD8+ T cell population. Activation of CD8+ T cells not only causes alterations in the behavioral profile of the antigen-specific T cell population but also results in metabolic reprogramming⁹¹. Each T cell activation state is broadly associated with a bias towards a particular metabolic profile because various activation states have different requirements for energy. The dynamic processes in which CD8+ T cells must alter their energy creation and consumption have made them an excellent model to understand functional outcomes of metabolic programming.

There are many cellular metabolic pathways but the two most widely studied in respect to CD8+ T cell biology are oxidative phosphorylation (OxPhos) and glycolysis. OxPhos is a mitochondrial pathway characterized by the transfer of electrons from NADH or FADH₂ to the electron transport chain (ETC). This can generate up to 36 adenosine triphosphate (ATP) molecules⁹². Glycolysis occurs in the cytosol and converts extracellular glucose into lactic acid. On average, this generates 2 ATP molecules⁹³. CD8+ T cells preferentially utilize glycolysis following activation. When measuring the efficiency of OxPhos or glycolysis with the sole parameter of ATP generation, glycolysis seemingly falls short. An obvious question then is: why would an activated CD8+ T cell preferentially adopt an inefficient mode of energy creation? In addition to ensuring T cells can meet the energy demands necessary for their activation state, activated T cells need to rapidly proliferate during the expansion phase. Intermediate metabolites generated through glycolysis can be used by other pathways to support the biosynthesis of amino acids and nucleotides⁹⁴. Preferential

utilization of glycolysis by effector T cells ensures cells have the bio-intermediates necessary to synthesize daughter cells. This highlights metabolic reprogramming as a critical component for ensuring proper immune responses and can be used as a metric to understand what guides T cells to adopt various short-term or long-term fates and if a pathogen can be controlled.

Our understanding of the metabolic drivers in T cell immunology has led to significant advances in the past decade. The strong links between metabolism and cell behavior have caused researchers to find ways to alter metabolism to leverage desired



immunological outcomes. Elevated glycolysis favors differentiation of effector CD4+ T cell subsets, formation of SLECs, and prevents anti-tumor activity in CD8+ T cells^{95–97}. It would be interesting to understand how different subsets of

naïve cells experience metabolic reprogramming in different immune settings such as differentiation status or age of the cell. For example, do cells that undergo more extensive HP have an inherent bias towards glycolysis? Is the differentiated profile of naïve neonatal CD8+ T cells and expansion of VM cells supported by an active program of metabolism which allows neonatal cells to rapidly adopt an effector fate upon antigen stimulation? Understanding how metabolism is modulated in different aged

cells could provide important insight into understanding how to promote desired immunological outcomes.

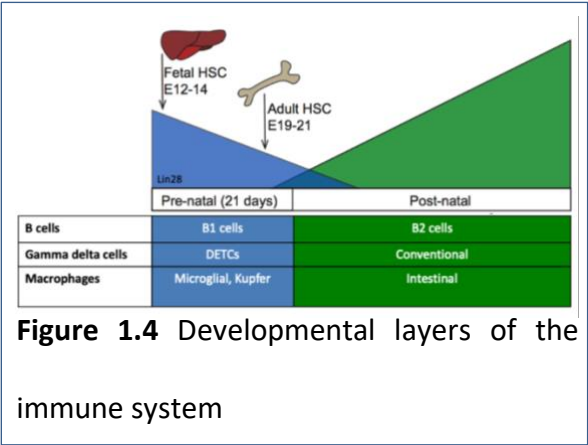
Developmental Layering of the Immune System

While all cells of the body can contribute to immunity, immune cells, in the classic definition, arise from a population of long-lived, self-renewing hematopoietic stem cells (HSCs) ⁹⁸. In the late 1980s, paradigm-shifting theories posited that different layers of HSCs arise sequentially and temporally. The Herzenberg group named this ‘the layered immune system’ ⁹⁹. The layered immune system encompasses the idea that maturation of the immune system occurs in waves and these waves facilitate the development of the immune system as a whole. Unique layers of the immune system are identified from their HSC origin, or the site in which the HSC colonized such as the primordial yolk sac, fetal liver, and bone marrow in mice. While the first HSCs arise in early development (yolk sac- and fetal-liver derived) do not retain their self-renewing capacity, different ‘layers’ co-exist in adulthood. From an evolutionary perspective, the functional layers of the immune system likely arose to protect against age-associated exposures throughout life but in the present day, the functional layers potentially exist to provide division of labor.

An extensive review of all layers of the immune system is not within the scope of this dissertation but I will briefly touch on the layers present in the innate and adaptive immune system that persist into adulthood. HSCs are first observed in the primordial yolk sac. Yolk sac HSCs give rise to monocytes that can differentiate into

tissue-resident macrophages, most notably to the central nervous system microglia ¹⁰⁰.

HSCs then travel from the yolk sac and aorta to colonize the liver at embryonic day 10 ^{101,102}. Several studies have provided robust evidence that suggests B1a B cells arise from fetal-derived HSCs ^{103,104}. B1 B cells rapidly secrete



IgM through T cell-independent mechanisms providing broad and quick protection. In contrast, B2 B cells, which are thought to arise from bone marrow HSCs are likely to have a more specific antigen recognition and develop long-lived memory. Along these lines, other lymphocytes that arise in early life are defined as more innate-like due to their responses to inflammatory signals over B cell receptor or TCR engagement, expression of innate-like receptors, and broad protective mechanisms ^{104–107}. Evidence from our lab suggests that fetal-derived T cells also exhibit more innate-like properties than T cells that arise from bone marrow resident HSCs (Watson *et al.* Manuscript in prep.).

Studies from the Adkins group have hinted towards layered immunity in the T cell compartment and the McCune group found distinct lineages of HSCs give rise to T cells with unique behavioral properties ^{39,108,109}. In 2017, Wang *et al.* found CD8+ T cells that arise from distinct progenitors provide unique forms of protection ⁸⁵. In 2018, seminal studies formally extended the layered immune hypothesis to the CD8+ T cell compartment by showing fetal-derived CD8+ T cells exist in the adult compartment

and retain their functional differences throughout their lifetime ^{67,110}. However, the mechanisms that drive the switch in the fetal/neonatal to the adult program are unknown.

Lin28 is a developmentally regulated RNA-binding protein and it is expressed at contrasting levels in fetal- and adult-derived HSCs ¹⁰⁷. As mentioned in the 'age-related differences in T cell immunity' subsection, Lin28 has been implicated as the master regulator of fetal lymphopoiesis by instructing the 'innate-like' program of fetal-derived lymphocytes ⁸⁵. While it is unknown exactly how Lin28 promotes an innate-like program, one possibility is through directing metabolism. It is hypothesized that Lin28 promotes the stability of transcriptional components that are critical in activating the mechanistic target of rapamycin (mTOR) pathway, a key regulator in metabolic processes ^{111,112}. mTOR, specifically mTORC1, controls glycolysis ¹¹³. As reviewed previously, metabolic programs drive the activation profile of CD8+ T cells towards effector programs. From these associations, I hypothesized that Lin28 promotes neonatal programs of immunity via glycolysis induction, and so, I examined the role of Lin28 in regulating metabolic activity in this dissertation.

Influences on T Cell Immunity

The 'hygiene hypothesis' attempts to associate the decreased incidence of infectious diseases present in Western countries with the increased prevalence of inflammation-based diseases such as atopy and autoimmune disorders ^{114–116}. These

epidemiological observations are supported by mouse models that control for pathogen exposure. For example, mice exposed to germ-free conditions have increased levels of mediators of allergies, such as IgE and ILC2s^{117,118}.

To define the influence that the environment has on immunity, we first need to understand the extent to which environmental or genetic factors drive variations in immunity.

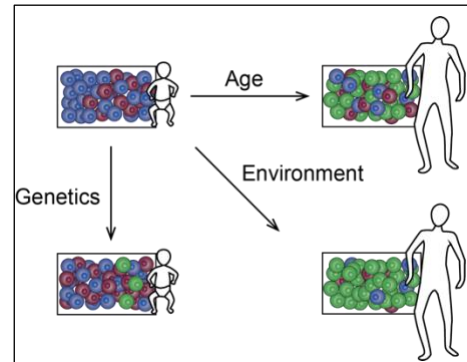


Figure 1.5 Parameters governing heterogeneity in the naive CD8+ T cell compartment

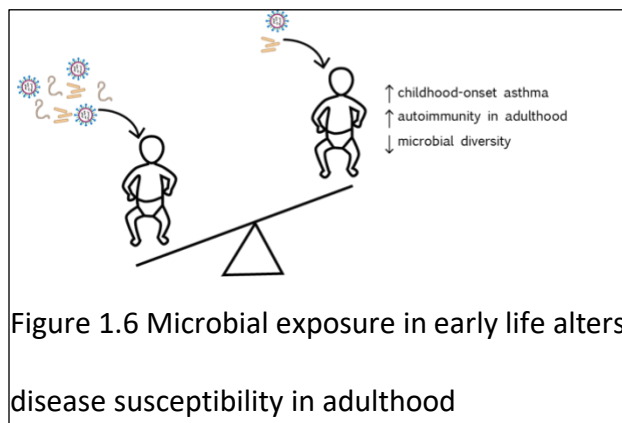
Studies found specific components of the immune response, such as cytokine production, arise largely from heritable factors¹¹⁸. However, studies on multi-generational families show 87% of immune variance does not arise from genetic inheritance¹¹⁹. Twin studies continue to show overwhelming deviations in immune function, further underscoring the stark variation in immunity within genetically alike individuals¹²⁰. This shows the complexity of disentangling environmental versus genetic factors in predicting and understanding immune variation at the population level while highlighting the importance that non-heritable traits have in promoting immunity.

Consistent with the hygiene hypothesis, children raised in close proximity to diverse microbial environments, such as farm children, children with pets, or those from large families, experience atopy at lower rates compared to children that were largely protected from diverse microbial exposure in adulthood^{121–125}. In addition to

decreased susceptibility to atopic diseases, populations exposed to large microbial communities are largely protected from developing autoimmune disorders later in life ^{126,127}. Brodin *et al.* found 58% of immune variations present in twins are non-heritable and can be associated through infectious histories, such as infection with cytomegalovirus ¹²⁰. This provides additional evidence that environmental exposures and infectious history can have profound impacts on immunity.

The nascent immune system rapidly transitions from a tolerant program *in utero* to a protective program after birth. During this time, a neonate first experiences a diverse microbial environment. This rapid transition highlights the high degree of plasticity present within the neonatal immune system ¹²⁸. Early life experiences can lay a profound foundation for determining immune responses later in life. The developmental origins of health and disease (DOHAD) indicates early life experiences during critical periods of development have profound impacts on long-term health even when the stressor or exposure has long-passed ^{121,129,130}. While not typically associated with microbial colonization, the DOHAD hints towards a defined window of exposure that can either favorably or unfavorably imprint on the immune system.

Evidence in support of a critical window is exemplified by the mode of birth ^{131,132}. Individuals born through cesarean section (c-sections) are more prone to developing asthma and allergies



compared to vaginally delivered individuals, though these findings are controversial^{132–136}. Researchers found exposing germ-free mice to microbiota before weaning led to protection against allergy, autoimmunity, and alterations in protective chemokine levels¹³⁷. Protection against allergic responses was ablated in germ-free mice when microbiota was introduced after weaning. The ‘weaning reaction’ is a period where infants encounter diverse antigens from the introduction of new foods¹³⁷. This creates a defined window of opportunity in which microbial exposure or tolerance must be established, otherwise, susceptibility to infection or autoimmunity is increased later in life. It would be of interest to understand if such a window exists and if a window exists, how to target that window to promote favorable immune outcomes.

The laboratory mouse serves as the workhorse for immunological discoveries. Our deep understanding of the mammalian immune system was made possible because of their use. The use of laboratory mice has allowed researchers to selectively manipulate the microbiota. Thus, mouse models that contain a spectrum of microbial colonizations exist, this includes germ-free, gnotobiotic, specific-pathogen-free, and ‘dirty’ mice. The latter represents the most recent addition to our understanding of microbial influences in health and disease. Using a combination of these mouse models has allowed biomedical researchers to identify contributions by discrete pathogens or learn how host-pathogen interactions contribute to immunity. Many iterations of the ‘dirty’ mouse exist and each has found diverse microbial exposure alters immunity^{138–143}. Importantly, dirty mouse models are more representative of what is seen in human immunology^{142,143}. However, dirty mouse models expose mice to a diverse microbial

environment in adulthood. The layered immune model shows fetal liver-derived immune cells co-exist with bone marrow-derived immune cells in adulthood and that each layer contributes uniquely to immunity. Therefore, a salient question to ask is what are we missing by only exposing adult-derived cells to a diverse microbial environment? If we expose mice to a diverse microbial environment for the entirety of early life, can we uncover the mechanisms by which early life exposures continue to impact health and immunity long after the perinatal period has passed?

Research Objectives

In the last several decades, there have been great strides in our general understanding of CD8⁺ T cell biology. Perhaps the most commonly asked question within the field is “what drives T cell fate?” Many labs have placed focus on understanding how external and cellular cues present in activated cells drive the quality and quantity of effector responses and memory formation. However, this lens neglects the heterogeneity that exists within the naïve compartment. When I started my thesis work, cellular age and the developmental origin of the cell were still largely ignored as factors driving immune variation though it predicts the short- and long-term fates of a CD8⁺ T cell.

Previous findings from our lab show that CD8⁺ T cells from fetal- or adult-derived T cell exhibit unique responses to activation and that fetal-derived cells persist into adulthood. We still do not know what underlies the behavioral program that promotes different fates of cells or when these differences arise. In this dissertation, I

investigated the mechanistic drivers of neonatal immunity. In chapter 2, I challenged the predominant theory present in the CD8⁺ T cell field that suggests neonatal CD8⁺ T cells are 'immature' because they have undergone less post-thymic maturation. I show RTE biology is not synonymous with neonatal immunity, rather, lineage-based decisions underlie the neonatal response to infection. In chapter 3, I extended our understanding of the mechanisms underlying lineage-based decisions by showing neonatal T cells are biased towards a short-lived effector fate because they undergo more aerobic glycolysis. Blockade of glycolysis during activation allowed neonatal cells to progress into the memory pool and contribute to secondary infection. In chapter 4, I showed microbial exposure during early life can alter the proportion and function of fetal-derived CD8⁺ T cells. I provided compelling evidence that the developmental architecture of the CD8⁺ T cell alters the course of infection and microbial exposure enhances or dampens CD8⁺ T cell protection in a layer-dependent manner. Work from my doctoral research identified multiple mechanisms that underlie variation in T cell behavior in an age-dependent manner.

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**CHAPTER 2: LIMITED POST-THYMIC MATURATION DOES NOT UNDERLIE NEONATAL
CD8+ T CELL IMMUNITY**

ABSTRACT

The recent thymic emigrant population represents the youngest T cells in the periphery and are functionally distinct from their more widely studied mature naïve counterparts. While most of our knowledge of recent thymic emigrants comes from studies performed on adult mice, recent thymic emigrant biology has been associated with neonatal immunology. We have previously demonstrated CD8⁺ T cells from different developmental origins have disparate fates during infection suggesting findings from adult RTEs may not translate to neonatal population dynamics. In this report, we show RTEs are not a homogenous population. Rather, RTEs from neonatal and adult animals display unique phenotypic markers in the naïve state which informs their fate in response to infection. This suggests a singular approach based on surface markers cannot be used to identify different aged RTEs. Importantly, when we controlled for the peripheral environment RTEs are exported into and mature in, we found neonatal RTEs had an enhanced ability to proliferate *in vitro* and produced more effector molecules in response to an *in vivo* challenge. Collectively, these data demonstrate the functional properties of RTEs are dependent on their time of creation and highlight the importance of understanding developmental heterogeneity within subsets of T cell populations.

INTRODUCTION

The conventional view that the naïve CD8⁺ T cell compartment is homogenous is being disrupted. Several labs have reported multiple layers of heterogeneity within the resting naïve population and show the initial phenotype of a cell serves as a predictive measure of a cell's fate during activation. For example, adoption of the 'virtual memory' phenotype allows naïve cells to behave with 'memory-like' properties, expression of higher affinity T cell receptors promotes rapid responses to infection, upregulation of chemokine receptors allows naïve cells to efficiently home and respond to infection, and development origin guides the long-term fate of a cell ¹⁻⁵. One level of resting heterogeneity is the post-thymic maturation status of a cell ⁶⁻⁸.

Recent thymic emigrants (RTEs) are the youngest cells in the peripheral T cell compartment and are considered distinct from their more well-studied mature naïve T (MNT) cell counterparts. RTEs and MNT cells make unique contributions to the immune response during infection ¹⁰⁻¹². As their name suggests, RTEs have recently completed thymic development, left the thymus, and have occupied the periphery for less than three weeks ¹². While RTEs represent approximately 20% of the naïve T cell compartment in healthy adults, several populations possess T cell compartments that are over-represented by RTEs including neonates, cancer patients who have undergone radiation treatment, and transplantation recipients ¹¹. Lymphopenic individuals are at an increased risk for infections and have poor responses to vaccinations ¹³⁻¹⁷. Thus, it is important to understand how RTEs provide immune protection for at-risk populations.

Since RTEs comprise most, if not all, of the neonatal T cell compartment. Findings from RTE studies have been used as a surrogate for understanding neonatal immunity. Moreover, T cells in neonates are viewed as immature because they have undergone less extensive post-thymic maturation because of their RTE status. Indeed, RTEs in adult mice preferentially adopt a terminally differentiated phenotype, similar to neonatal T cells ^{10,18}. Though, few studies exist that characterize the differences between neonatal and adult RTEs, making it difficult to infer T cell dynamics in neonates to adult RTE behavior. RTEs are defined as less responsive versions of their MNT counterparts, further supporting the false attribution that T cell responses in neonates are defective ^{7,8,19}. However, the Adkins group found neonatal CD4⁺ RTEs produce higher levels of Th1/Th2 cytokines in response to *in vitro* TCR stimulation ²⁰. This study shows neonatal and adult CD4⁺ RTEs represent phenotypically and functionally distinct populations. To our knowledge, there is no published work that identifies if differences between different aged CD8⁺ RTEs exist. Findings that characterize neonatal RTEs as more responsive to stimulation are reminiscent of studies from our lab showing neonatal CD8⁺ T cells produce more cytolytic molecules in response to *in vitro* TCR activation ^{18,20}. Therefore, it is reasonable to hypothesize neonatal and adult CD8⁺ RTEs also represent distinct populations and contribute differently during infection.

Recent discoveries from our lab have shown neonatal and adult T cells are derived from unique progenitors ^{4,5,21}. This is an important distinction because the progenitor serves as a predictor of a cell's phenotype during the steady-state and

guides its behavior in response to activation. While these studies are of particular interest because it shows age-related differences in immunity, these studies were designed to understand the functional properties present MNT cells from disparate developmental origins. Therefore, we still lack a comprehensive framework describing the population dynamics of neonatal CD8⁺ RTEs and if developmental origin influences RTE behavior.

To investigate whether neonatal and adult RTEs are equivalent populations, we used a fate-mapping mouse model to track cells made at discrete developmental windows. By using this mouse model, we can examine the phenotypic and behavioral properties of different aged RTEs following thymic egress. In this report, we offer the first piece of evidence showing neonatal and adult CD8⁺ RTEs display distinct phenotypes and behaviors before and after stimulation. Moreover, variations between neonatal and adult RTEs do not arise from the lymphopenic environment that neonatal RTEs experience. Rather, we found their unique properties are pre-programmed during thymic development and continue to arise irrespective of the peripheral environment. Our results provide insight into the factors that underlie age-related differences and demonstrate the CD8⁺ RTE population is not equivalent in different aged animals. This further highlights the need to understand how lineage-based decisions drive CD8⁺ T cell responses to infection.

RESULTS

This study aims to determine whether RTEs from neonatal and adult mice have similar phenotypic and functional properties or if they represent unique populations. To identify and track RTEs in neonatal and adult mice, we used a T cell fate-mapping strategy we refer to as ‘timestamping’. In this model, mice express a transgene for a TCR δ -promoter that is driven by a tamoxifen-inducible-Cre cassette (TCR δ -CreERT2) and lox-stop-lox fluorescent protein reporter ²². This allows us to permanently mark a wave of cells at a discrete time during T cell thymic development ²³. We administered tamoxifen to mice at 1d of age to mark cells from the fetal-liver progenitors (fetal-derived or neonatal cells) and 4w of age to mark cells from adult bone marrow progenitors (adult-derived or adult cells) (Fig 1a) ^{24,25}. After two weeks, we isolated and stained CD8+ T cells from fetal-derived or adult-derived mice for RTE markers, Qa2 and CD24 ²⁶. RTEs express lower levels of Qa2 and elevated levels of CD24 compared to MNT cells. We found fetal-derived expressed higher levels of CD24 and lower levels of Qa2 compared to their adult-derived counterparts (Fig 1b, c). This shows fetal-derived RTEs exhibit enhanced phenotypic properties associated with RTEs compared to their adult-derived counterparts. We next asked whether fetal-derived and adult-derived RTEs displayed different phenotypic markers indicative of disparate long-term fates. Eomes and CD44 expression have been attributed to the memory CD8+ T cell population ^{27,28}. However, recent advances in CD8+ T cell biology have found a subset of naïve CD8+ T cells express these markers and high expression of these markers is indicative of extensive homeostatic proliferation and/or differentiation ^{28–30}. We found

naïve fetal RTEs express higher levels of eomes and CD44 (Fig 1d, c). While fetal and adult RTEs have occupied the periphery for equal amounts of time, neonatal RTEs appear more differentiated.

One possible reason fetal-derived RTEs express markers indicative of differentiation is that they are exported into a lymphopenic environment ^{31,32}. To ensure adequate protection for a host, RTEs must rapidly proliferate to fill the periphery. Extensive proliferation may alter the program of neonatal RTEs. Another possible explanation is differences observed are pre-programmed during development and arise independently of peripheral experiences ^{31,33}. To distinguish between these two possibilities, we transplanted a thymic lobe from a 1d old (RFP timestamp reporter) under the kidney capsule of a 6w old mouse (GFP timestamp reporter) then administered tamoxifen to mice (Fig 2a) and treated the recipient mice with tamoxifen to uniformly mark thymocytes from the donor and endogenous thymii ³⁴. By doing so, fetal- and adult-derived RTEs experienced the same lymphoreplete post-thymic environment. Two weeks post-transplantation and tamoxifen treatment, we analyzed fetal and adult RTEs. We found fetal-derived RTEs continue to express similar patterns of RTE cell surface marker expression that were observed in an unmanipulated animal (fig 1). Notably, fetal-derived RTEs from continued to express higher levels of CD24 and increased Ki67 activity (Fig. 2b,c). This indicates the altered phenotype of neonatal RTEs is not a result of lymphopenic induced proliferation (LIP). Rather, fetal-derived are hard-wired to undergo extensive proliferation³⁵. Furthermore, fetal-derived RTEs continued to express markers associated with a memory phenotype that was first

observed in our non-transplantation experiments further suggesting the differentiation profile arises from cell-intrinsic factors (fig 1, fig 2f-g). Collectively, these results show the unique fetal-derived RTE program does not arise from the maturation in a lymphopenic environment but arises due to cell-intrinsic factors that are pre-programmed during thymic development.

RTEs have been characterized as less functional than their MNT counterparts^{10,36–38}. The enhanced accumulation of the virtual memory population in the fetal-derived RTE population prompted us to hypothesize that fetal-derived cells would be more reactive to TCR activation when compared to the adult RTE population (Fig 2f, g). To determine whether reduced functionality is a ubiquitous property for all RTEs, we performed an *in vitro* activation assay by stimulating purified CD8⁺ T cells from the dual-reporter thymic transplant mice with anti-CD3/CD28 (Fig 3a). 48h post-TCR stimulation, we found fetal-derived RTEs underwent similar rates of proliferation when compared to the non-stamped population (Fig 3b). Consistent with past reports, adult-derived RTEs underwent less extensive proliferation and had a lower division index compared to both the fetal-derived and non-stamped populations (Fig 3c)⁷. We also assessed the ability of fetal-derived and adult-derived RTEs to produce the cytolytic molecule, Granzyme B. Again, we observed similar levels of Granzyme B production between fetal-derived and non-stamped populations while adult-derived RTEs had lower levels of Granzyme B production (Fig 3d, e). Collectively, this suggests defining RTEs as hypofunctional is only appropriate for adult-derived RTEs because fetal-

derived RTEs possess similar rates of functionality when compared to the non-stamped population.

In neonates, RTEs are the sole drivers of cell-mediated immunity. It is, therefore, pertinent that we define the contributions of fetal-derived RTEs in protection against intracellular infection. Thus far, we have shown fetal-derived RTEs express markers indicative of a more differentiated phenotype and display enhanced proliferative capacities during *in vitro* activation. This led us to hypothesize fetal-derived RTEs will mount a more robust *in vivo* effector response. To address this hypothesis, we again performed our dual reporter thymic transplantation experiments (Fig 4a). Two weeks post-transplantation, we directly infected mice with a recombinant strain of *Listeria monocytogenes* (LM) that expresses the gB peptide so that we could assess antigen-specific CD8⁺ T cell responses of fetal and adult RTE populations. When compared to the non-stamped antigen-specific population, we found a three-fold reduction in the proportion of antigen-specific fetal- and adult-derived RTEs present at 5 days post-infection (dpi) (fig 4b). While we did not discover differences in the clonal expansion of the antigen-specific fetal or adult RTE populations, we observed divergence in the phenotype of the responding cells. The majority of the antigen-specific fetal-derived RTEs acquired a terminally-differentiated short-lived effector cell phenotype (SLEC, CD127^{lo}KLRG1^{hi}) whereas the SLEC population represented half of the responding adult population and non-stamped population (Fig 4c). Interestingly, adult RTEs and the non-stamped population formed similar proportions of SLECs. This is in direct disagreement with past studies that show RTEs from adults are skewed

towards a SLEC phenotype ¹⁰. Moreover, a higher percentage of adult RTEs expressed the memory precursor phenotype (MPEC, CD127^{hi}KLRG1^{lo}) suggesting they had an enhanced propensity to transition into the long-lived CD8⁺ T cell population (Fig 4d). In addition to a bias towards becoming SLECs, fetal-derived RTEs produced higher levels of effector molecules when compared to the adult RTE population and bulk, unstamped population (fig e-g). Thus, while fetal- and adult-derived RTEs are recruited to the response at equal proportions, fetal-derived CD8⁺ T cells contribute more productively to the primary response by producing higher levels of effector molecules. Overall, these data indicate published work describing the population dynamics of RTEs is not representative of RTEs from all developmental lineages. Rather, we show the importance that lineage-based decisions have in promoting the RTE response to infection.

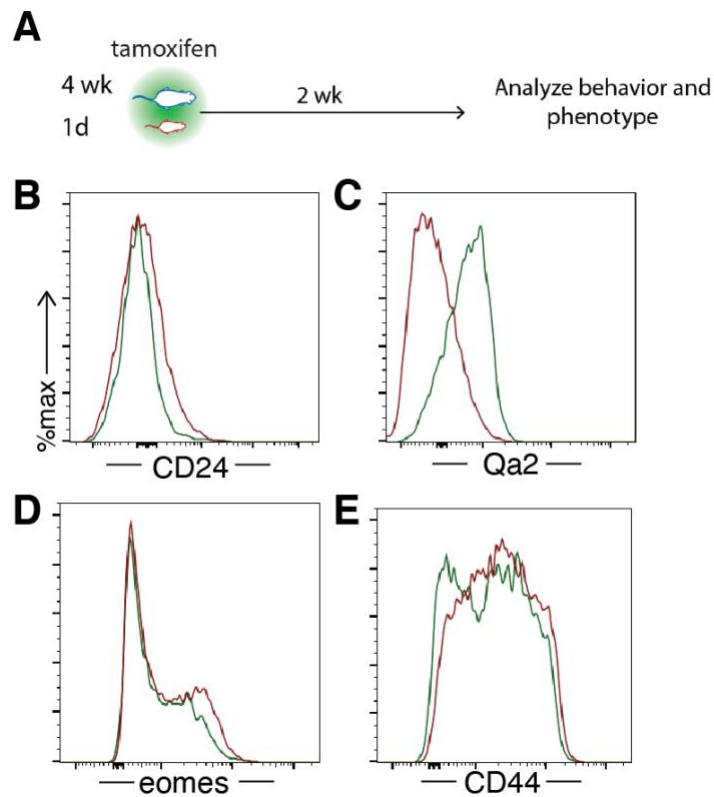


Figure 2.1 Neonatal RTEs exhibit a unique phenotypic profile. (A) Experimental strategy to ‘timestamp’ and identify fetal- and adult-derived RTEs. **(B)** Representative expression of CD24 **(C)** Qa2 **(D)** Eomes, and **(E)** CD44 in fetal- and adult-derived RTEs. Histograms are representative of staining profiles from two independent experiments using 6-8 independent mice

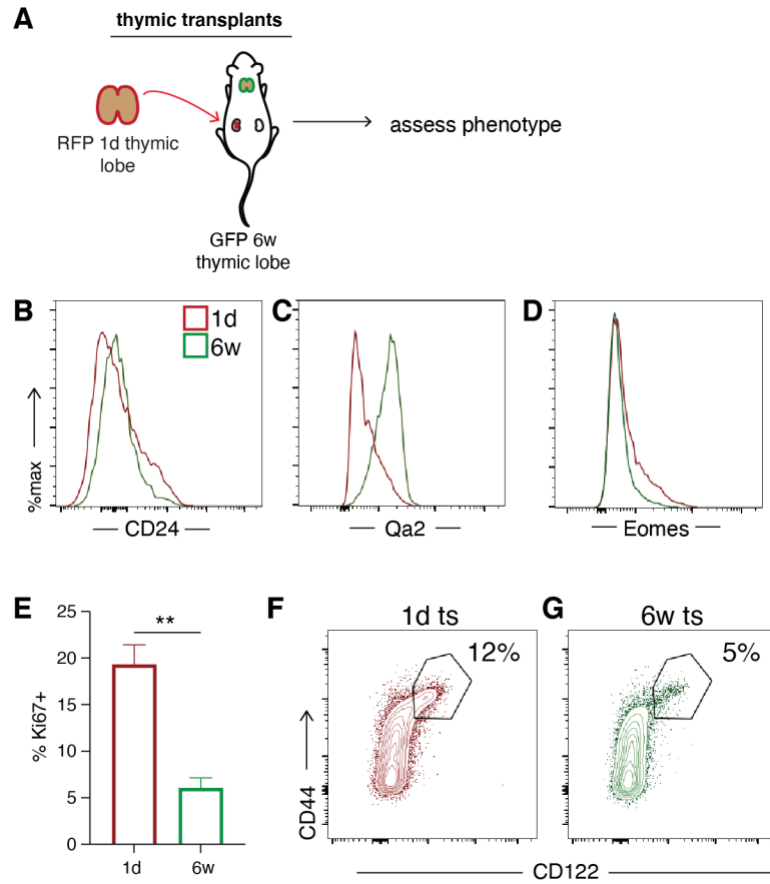


Figure 2.2 Different aged RTEs acquire unique phenotype independent of peripheral environment. **(A)** Experimental strategy for dual-reporter thymic transplantation experiments. **(B)** Representative expression of CD24, **(C)** Qa2, **(D)** and Eomes. **(E)** Percentage of cells that express Ki67, **(F)** representative plot showing proportion of virtual memory cells present in fetal- **(G)** and adult-derived RTEs. Data representative of two independent experiments with 6-8 biological replicates per group. ** $p < 0.005$ by unpaired Student t test.

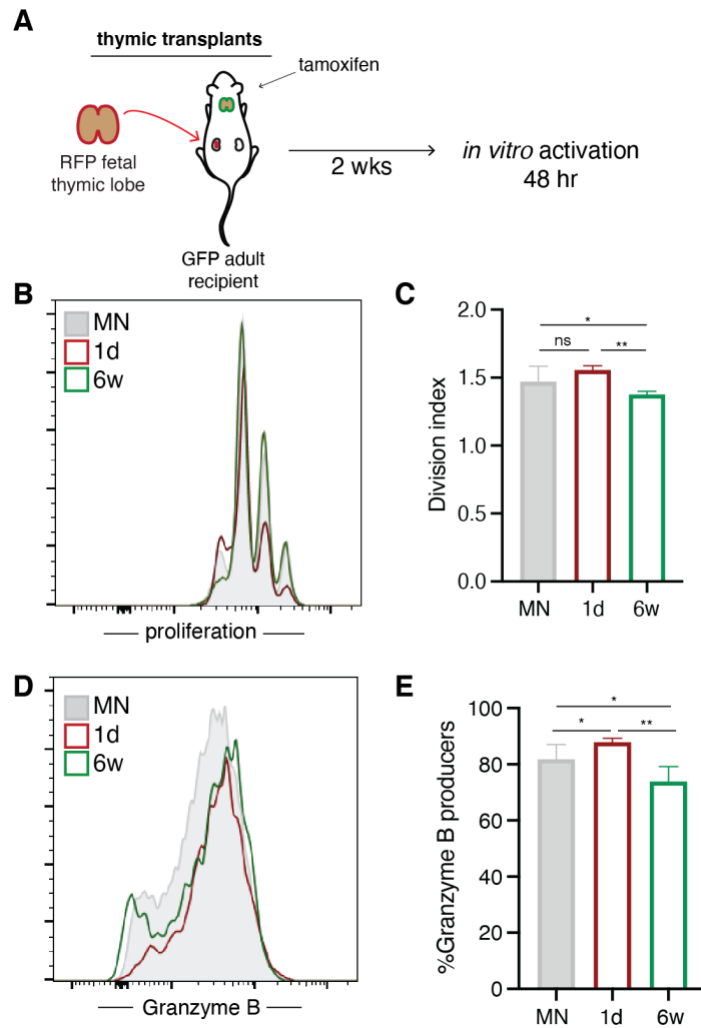


Figure 2.3 Adult-derived RTEs display reduced proliferative capacities. (A) Experimental strategy for dual-reporter thymic transplantation experiments. **(B)** Representative histogram showing proliferation peaks at 48 h post-CD3/28 *in vitro* stimulation. **(C)** Division index after 48h *in vitro* stimulation. **(D)** Representative histogram depicting granzyme B production. **(E)** Proportion of cells that are producing granzyme B. Histograms are representative of staining profiles from two independent experiments using 6-8 independent mice. ** $p < 0.005$ by unpaired Student t test.

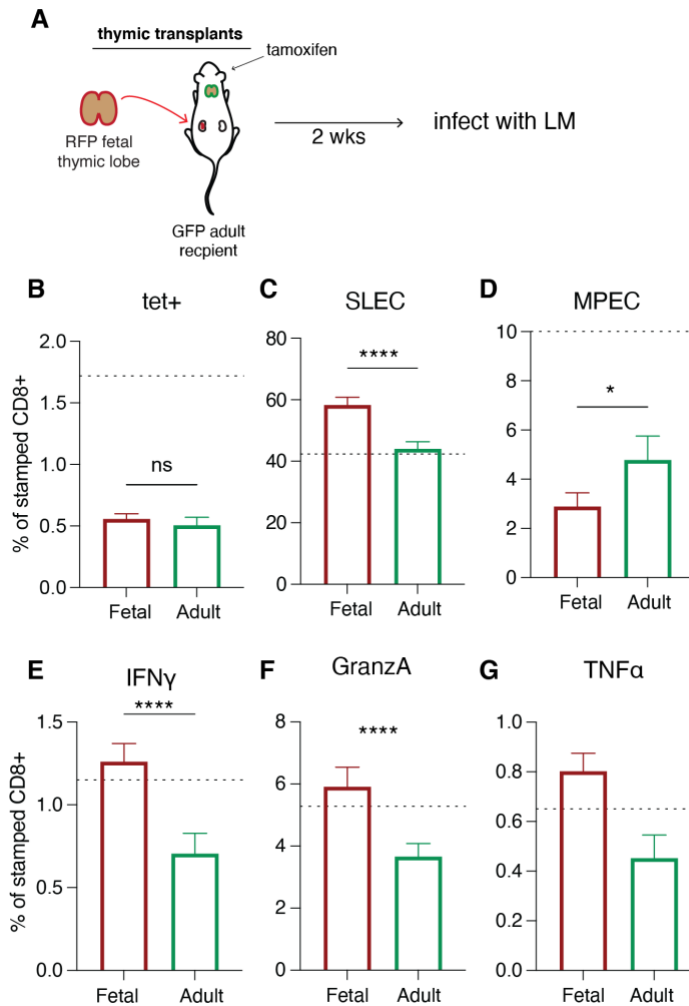


Figure 2.4 Fetal-derived RTEs are not defective in their functional capacity. (A) Experimental strategy for dual-reporter thymic transplantation experiments. **(B)** Percentage of tetramer+ cells. **(C)** Proportion of antigen-specific cells that are SLECS and **(d)** MPECs at 5 dpi. **(E)** Percentage of stamped cells that are producing IFN γ **(F)** Granzyme A, and **(G)** TNF α . Data representative of two independent experiments with 6-8 biological replicates per group. ns not significant, * $p < 0.05$, *** $p < 0.0005$, **** $p < 0.00005$ by unpaired Student t test.

DISCUSSION

In the past few decades, an immense amount of effort has been put forth to understand the factors that give rise to the functional heterogeneity of T cells. Separate models exist to describe how the naïve T cell population gives rise to a heterogeneous response, such as the TCR signal strength and decreasing potential model^{39–41}. More recently, evidence implicating developmental origin as a driver of T cell fate has risen in popularity^{18–20,42–45}. To this end, we wanted to determine if lineage-based decisions influence RTE behavior.

We designed a set of experiments to determine whether neonatal and adult RTEs were equivalent populations that contribute similarly to the immune response against intracellular infections. Using a fate-mapping approach, we were able to systematically track CD8⁺ T cells made at different times of life and analyze their behavior during the RTE period. Through this approach, we demonstrated the developmental origin of an RTE plays a deterministic role in their resting phenotype and response to *in vitro* and *in vivo* stimulation. The prevailing notion is that RTE population dynamics can be used as a surrogate for neonatal cell-mediated immunity^{9,10}. Rather than representing a homogenous subset that is hyporesponsive to intracellular infections, we show this label is only accurate for adult RTEs^{9–11,37}. Our work asserts that neonatal RTEs are equally as functional as MNT cells and parallels previous work showing neonatal CD4⁺ RTEs produce elevated levels of cytokines compared to their adult RTE counterparts²⁰. Overall, we provide further evidence that the neonatal T cell compartment is not defective, rather, lymphocytes that arise from

the early waves of HSCs contain a program that protects against the unique challenges encountered early in life.

The prevailing hypothesis is that the neonatal T cell compartment is immature because their T cells have undergone less post-thymic maturation. Another hypothesis is LIP may give rise to the memory-like phenotype of fetal-derived RTEs³². Extensive LIP could explain why fetal-derived RTEs upregulate markers indicative of differentiation in the steady-state²⁷. However, it is unclear whether fetal-derived RTEs are inherently more proliferative or if it is a result of the lymphopenic environment they are exported into. CD24 is used to identify RTEs and its expression promotes the accumulation and proliferation of T cells in lymphopenic environments¹⁸. Through our thymic transplantation experiments, we found fetal-derived RTEs expressed higher levels of CD24 and Ki67. Even when fetal RTEs were exported into a lymphoreplete environment, neonatal RTEs are more proliferative suggesting alterations in programming are cell-intrinsic. Enhanced CD44 and eomes expression may have also arisen from LIP but our transplantation experiments showed fetal-derived RTEs continued to adopt a differentiated phenotype when placed in an 'adult' and lymphoreplete environment. Together, this shows environmental cues that are unique to the neonatal environment do not contribute to extensive proliferation or differentiation status observed in fetal-derived RTEs. Rather, fetal-derived RTEs continue to adopt a more differentiated phenotype when exported into lymphoreplete environments indicating cell-intrinsic factors are involved in governing the behavior of different aged RTEs.

Our findings show fetal-derived RTEs are highly functional during both *in vitro* and *in vivo* activation. This raises the question: what is the teleological benefit of generating more reactive RTEs early in life? Neonates are vulnerable to opportunistic intracellular infections because the lymphocyte compartment is still being established. Therefore, having RTEs that can induce strong cytotoxic responses is beneficial to clear and survive an infection. Indeed, we found the fetal-derived RTE population produced effector molecules in levels equivalent to those produced by the unstamped population. Additionally, the majority of the antigen-specific fetal-derived RTEs were SLECs. SLECs rapidly expand in response to infection and once the pathogen is cleared, die via apoptosis. While the formation of a robust effector response is beneficial to quickly resolve infection, this comes at a cost of decreased memory potential at the population level. Nevertheless, expending energy to create immunological memory in anticipation of subsequent infections is not beneficial if a neonate can neither clear nor survive the initial infection.

Lymphopenic adults have issues controlling infection, which can be fatal ^{46,47}. Additionally, bone marrow transplantation donors are often adults resulting in the reconstitution of cells skewed towards memory formation rather than strong primary responses. Therefore, it is also important to understand the contributions adult-derived RTEs have to immunity. In this study, we found adult RTEs provide a less potent response compared to the fetal-derived RTE population despite being recruited at similar proportions and have decreased functionality compared to the non-stamped population. The decreased capacity to produce cytotoxic cytokines may underlie the

inability of lymphopenic adults or those that receive bone marrow transplantations to control primary intracellular infection. Smith *et al.* discovered fetal- and adult-derived MNT cells co-exist in adulthood, which results in the formation of cells that provide diverse types of protection against intracellular infection^{5,43}. It would be valuable to determine if protection against intracellular infection in lymphopenic adults can be enhanced if we manipulate a subset of adult-derived RTEs to adopt the neonatal CD8+ T cell program of immunity^{22,48}.

In conclusion, our study demonstrates population dynamics of RTEs are not uniform across different aged individuals. Findings from this report will provide valuable insight into medical interventions for different aged individuals whose T cell compartments are overrepresented by RTEs. For example, we show neonatal RTEs are defective in forming memory. Thus, early vaccination programs must be designed to promote the formation of immunological memory in neonates by directly perturbing the expansion of the SLEC subsets. Moreover, vulnerable lymphopenic adult populations have little to no memory T cells and are highly susceptible to primary infections. Therefore, it is of utmost importance to find ways to enhance the cytolytic capacity of adult RTEs to efficiently kill infected cells. Overall, these findings will inform future strategies to enhance cell-mediated therapies and vaccine development for different aged individuals experiencing lymphopenia.

MATERIALS AND METHODS

Mice

ZsGreen, TdTomato, and TCR δ Cre-ERT2 mice were obtained from Jackson Laboratories. All experiments utilized timed matings to ensure mice were of similar ages. Mice were maintained under specific pathogen-free conditions at the College of Veterinary Medicine. The facilities are accredited by the American Association of Accreditation of Laboratory Animal Care. All protocols regarding animal use were reviewed and approved by the Institutional Animal Care and Use Committee at Cornell University.

Timestamping method

Timestamp mice were generated by crossing a TCR δ Cre-ERT2 mouse with a ZsGreen or TdTomato reporter mouse. To active fluorescent reporter expression in fetal-derived T cells, 2.5 mgs of tamoxifen was administered to dams by oral gavage three times in 12-hour intervals over a 24-hour period and 0-1d old pups received tamoxifen through lactation. To active fluorescent reporter expression in adult-derived T cells, 2.5 mgs was administered to mice by oral gavage in 24-hour intervals for two days.

Thymic transplants

Thymic transplants were performed as previously described ⁴⁹. Briefly, thymus lobes were isolated from 0-1d old timestamp mice. The thymus lobes were separated into individual lobes and were placed under the kidney capsule of a 6-week-old mouse. To mark thymocytes from the donor thymus lobe, 5 mg of tamoxifen was administered

from 0-3d post-transplantation. Recipients were serially bled post-surgery through a cheek bleed and spleens were isolated to assess phenotype and behavior through flow cytometry.

Infection

Mice were infected with 5×10^3 CFU of wild-type (WT) *Listeria monocytogenes* expressing the gB-8p peptide (Lm-gB) as previously described ¹⁸.

Flow cytometry

For flow cytometry, cells were stained with antibodies purchased from eBioscience, BioLegend, Invitrogen, or BD Biosciences and concentrations were used as recommended by the manufacturer. Flow cytofluorimetric data were acquired using FACSDiva software from a BD FACSsymphony equipped with five lasers (BD Biosciences). Analysis was performed with FlowJo (Tree Star, Ashland, OR).

***In vitro* stimulation**

CD8⁺ T cells were isolated from the spleen using positive magnetic selection with CD8 microbeads (Miltenyi). Following magnetic bead purification, cells were labeled with Cell Trace Violet. Cells were stimulated with plate-bound anti-CD3 (2C11) then cultured with complete RPMI supplemented with 100 U/ml human IL2 and 4 μ g CD28/ml (37.51). Cells were harvested at the timepoints indicated in the figure legends and stained for surface markers then analyzed for flow cytometry.

Statistical analysis

Error bars are represented by SD. Statistical significance was determined by a student's t-test or by a 2-way ANOVA followed by an appropriate post-test as indicated in the figure legends. Significance is denoted by the following: ns not significant * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$

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**CHAPTER 3: ELEVATED GLYCOLYTIC METABOLISM LIMITS THE FORMATION OF
MEMORY CD8⁺ T CELLS IN EARLY LIFE**

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ABSTRACT

Neonates often develop poor immunity against intracellular pathogens. Because CD8⁺ T cells are essential for eliminating infectious agents, it is crucial to understand why they behave differently in early life. Previous studies in mice have demonstrated that neonatal CD8⁺ T cells fail to form memory because of an intrinsic propensity to differentiate into short-lived effectors. However, the underlying mechanisms remain undefined. We now show that neonatal CD8⁺ T cells exhibit higher glycolytic activity than adult CD8⁺ T cells post-infection, which may be due to age-related differences in Lin28b expression. Importantly, when glycolysis is pharmacologically inhibited, the impaired formation of neonatal memory CD8⁺ T cells can be restored. Collectively, these data suggest that neonatal CD8⁺ T cells are inherently biased toward undergoing glycolytic metabolism post-infection, which compromises their ability to develop into memory CD8⁺ T cells in early life.

INTRODUCTION

Infections remain a major cause of neonatal morbidity and mortality. Repeated infections with the same intracellular pathogen (respiratory syncytial virus, rhinovirus) are common in early life (1), indicating a reduced capacity to develop neonatal memory CD8⁺ T cells. However, the basic mechanisms that prevent neonates from generating robust memory CD8⁺ T cell responses are poorly understood, and the lack of this knowledge has limited our ability to develop more rational strategies to enhance immunity in early life. During infection, naive Ag-specific CD8⁺ T cells undergo massive clonal expansion and differentiate into effector CD8⁺ T cells capable of eliminating infected cells (2–4). Once the infection has been controlled, the majority (~90–95%) of CD8⁺ T cells undergo apoptosis. However, a small percentage (~5–10%) of cells survive and differentiate into long-lived memory cells, protecting the host against reinfection. Recent studies have demonstrated that neonatal CD8⁺ T cells are intrinsically defective at forming memory CD8⁺ T cells (5–7). Surprisingly, this impairment is not due to a lack of responsiveness or proliferation but rather to an inherent propensity of neonatal CD8⁺ T cells to rapidly become terminally differentiated, thereby losing their potential to transition into the long-lived memory pool (7).

The propensity for CD8⁺ T cells to become terminally differentiated in early life relates to the unique properties of the hematopoietic stem cells (HSCs) from which neonatal CD8⁺ T cells are derived (8). Whereas neonatal CD8⁺ T cells are produced from highly proliferative fetal HSCs that originate in the liver (9, 10), adult CD8⁺ T cells

are generated from more quiescent adult HSCs in the bone marrow (9, 11). Notably, fetal HSCs use distinct metabolic pathways compared with adult HSCs, which are largely regulated by Lin28b (12–14). Lin28b is a classic oncofetal gene that creates a metabolic program conducive for rapid cell growth in fetal life as well as in aggressive cancers (15). Whether this metabolic program is retained in neonatal CD8⁺ T cells and alters their fate during infection remains an open question.

In adults, CD8⁺ T cells undergo extensive changes in their metabolic properties throughout the course of infection. Following activation, naive CD8⁺ T cells switch their glucose metabolism from oxidative phosphorylation to aerobic glycolysis, similar to cancer cells (16–18). This phenomenon (known as the Warburg effect) is required to mobilize sufficient amounts of proteins, nucleic acids, lipids, and carbohydrates to undergo massive clonal expansion (19) and acquire effector functions (20). Once the infection has been cleared, effector CD8⁺ T cells must decrease anabolic activity to become more quiescent memory cells. This is accomplished by switching from glycolysis back to fatty acid oxidation (21–23). In this study, we investigated whether age-related differences in metabolic programming underlie the impaired development of neonatal memory CD8⁺ T cells.

RESULTS

Neonatal CD8⁺ T cells exhibit higher glycolytic metabolism after in vitro stimulation

Previous studies have demonstrated that neonatal T cells from mice (6–8, 28, 29) and humans (7, 30, 31) are inherently more reactive than their adult counterparts

after *in vitro* stimulation. To understand the metabolic changes that occur during this process, we activated CD8⁺ T cells from neonatal and adult TCR transgenic mice (gBT-I mice) and used the Seahorse Extracellular Flux Analyzer to compare their bioenergetics profiles. After 2 d of stimulation via the TCR and CD28, we found that the basal OCR, an indicator of oxidative phosphorylation, was higher in adult cells (Fig. 1A, 1B). In contrast, neonatal CD8⁺ T cells exhibited a higher basal ECAR (Fig. 1C), which is used as a readout for aerobic glycolysis. Overall, neonatal CD8⁺ T cells possessed a lower OCR/ECAR ratio (Fig. 1D), suggesting that CD8⁺ T cells in early life preferentially use glycolytic metabolism poststimulation.

To extend our extracellular flux data, we performed quantitative PCR (qPCR) and compared the expression levels of genes in the glycolysis pathway in neonatal and adult CD8⁺ T cells at 8 h poststimulation. We found that most of the key genes involved in glycolysis were highly upregulated in neonatal CD8⁺ T cells after activation (Fig. 1F). We also used liquid chromatography/high-resolution mass spectrometry to determine how glycolytic metabolites are altered in different-aged CD8⁺ T cells at 18 h poststimulation. In line with our qPCR data, neonatal CD8⁺ T cells produced higher amounts of metabolites involved in the glycolysis pathway (e.g., pyruvate, lactate) than adults (Supplemental Fig. 1). Collectively, these findings demonstrate that neonatal CD8⁺ T cells undergo augmented glycolysis following TCR activation.

Neonatal CD8⁺ T cells possess an inherent propensity to undergo glycolytic metabolism following infection

We next assessed the metabolic changes that occur in neonatal and adult CD8⁺ T cells post infection *in vivo*. Our strategy was to cotransfer the same number of neonatal (Thy1.2) and adult (Thy1.1) gBT-I TCR transgenic cells into congenic recipients (Ly5.2 mice), which were subsequently infected with recombinant LM-gB (Fig. 2A). By comparing equal numbers of monoclonal neonatal and adult CD8⁺ T cells in the same environment, we were able to focus on cell-intrinsic differences in metabolic reprogramming. Consistent with our earlier work (6), neonatal CD8⁺ T cells preferentially gave rise to SLECs (KLRG1⁺, CD127⁺), whereas adult CD8⁺ T cells formed more memory precursor cells (MPECs; KLRG12, CD127⁺) post-infection (Fig. 2B). To compare their metabolic profiles, we used FACS to isolate neonatal and adult donor cells at the peak of infection (7 d post-infection [dpi]) and performed extracellular flux analysis under basal conditions and after drug-induced stress. Neonatal donor CD8⁺ T cells exhibited a lower basal and maximum OCR and a higher basal and maximum ECAR compared with their adult counterparts (Fig. 2C, 2D). Moreover, neonatal CD8⁺ T cells had significantly lower levels of spare respiratory capacity (SRC) (Fig. 2E), which has previously been shown to be critical for the development of memory CD8⁺ T cells (32). These results suggest that neonatal CD8⁺ T cells fail to transition into the long-lived memory pool because of an inability to undergo oxidative metabolism in response to increased stress.

To better understand why neonatal CD8⁺ T cells use different metabolic programs than adults post-infection, we next performed a series of experiments to control for phenotypic differences that are present before and post-infection. First, we

considered that age-related changes in metabolic reprogramming might be due to differences in the proportion of naive and VM cells in the starting pool. Indeed, we previously found that neonatal CD8⁺ T cells comprise nearly twice as many VM cells (Ag-inexperienced cells with a memory phenotype) as adults prior to infection (8). To control for these initial phenotypic differences, we repeated our co-transfer experiments with sorted populations of CD44^{lo}CD122^{lo} TN and CD44^{hi}CD122^{hi} VM donor cells from neonatal and adult gBT-I mice and directly compared their metabolic profiles at the peak of infection (Supplemental Fig. 2A, 2B). Regardless of the initial phenotype, the neonatal donor cells (both TN and VM) use an elevated glycolytic program compared with their adult counterparts (Supplemental Fig. 2C–G). These data suggest that age-related changes in metabolic reprogramming cannot be simply explained by the different phenotypes of cells that are present prior to infection.

Second, we tested the possibility that metabolic changes observed in neonatal and adult effector CD8⁺ T cells are due to phenotypic differences that are present at the peak of the response. For example, the lower levels of OCR and SRC observed in neonatal cells could be due to a higher proportion of SLECs in the bulk population rather than a difference in metabolic programming in the responding cells. To examine this possibility, we repeated our co-transfer experiment. However, this time we sorted out an equivalent phenotypic subset (SLECs) from neonatal and adult donor cells at the peak of the response (7 dpi) and directly compared their metabolic profiles (Supplemental Fig. 2H). The adult SLECs still showed higher rates of basal and maximum OCR and exhibited a larger SRC compared with neonatal SLECs

(Supplemental Fig. 2I–K). In contrast, the neonatal SLECs displayed higher basal and maximum ECAR (Supplemental Fig. 2L, 2M), which is consistent with the overall metabolic programs observed in the bulk neonatal and adult populations. Taken together, these data suggest that age-related differences in metabolic reprogramming of CD8⁺ T cells are due to inherent changes at the individual cell level rather than shifts in the representation of naive or effector subsets in the mixed population.

Lin28b drives a more glycolytic metabolic program in CD8⁺ T cells

Another key question is, how are neonatal CD8⁺ T cells programmed to use glycolysis postinfection? We previously showed that neonatal and adult CD8⁺ T cells adopt different fates during infection because they are derived from distinct progenitor populations, which are specified, at least in part, by their differential expression of Lin28b (8). Interestingly, Lin28b is preferentially expressed in fetal HSCs and was recently shown to be a major positive regulator of glucose metabolism (14). Thus, we hypothesized that metabolic changes between neonatal and adult CD8⁺ T cells could be attributed to developmentally-related differences in Lin28b expression. To test this, we crossed gBT-I mice with Lin28b Tg mice to generate a source of adult donor CD8⁺ T cells that expressed Lin28b. We then cotransferred an equal number of WT adult (Thy1.1) and Lin28b Tg adult (Thy1.2) donor CD8⁺ T cells into the same recipient mice (Ly5.2) and asked whether forced expression of Lin28b in adult CD8⁺ T cells drives a more “neonatal-like” metabolic program postinfection with LM-gB (Fig. 3A). Consistent with our previous work, we found that Lin28b Tg adult donor cells preferentially became SLECs during infection, similar to neonatal CD8⁺ T cells (Fig. 3B).

At 7 dpi, we sorted out WT and Lin28b Tg donor CD8⁺ T cells and compared their metabolic profiles via extracellular flux analysis. Induction of Lin28b in adult CD8⁺ T cells resulted in lower rates of cellular respiration and a decrease in SRC (Fig. 3C–E), levels similar to those observed in neonatal donor CD8⁺ T cells (Fig. 2). The Lin28b Tg cells also displayed higher rates of glycolytic metabolism (Fig. 3F, 3G), suggesting that Lin28b promotes rapid differentiation of CD8⁺ T cells in early life by enhancing glucose metabolism.

Inhibiting glycolytic metabolism restores the formation of neonatal memory CD8⁺ T cells

Our data thus far indicate that increased glycolysis in neonatal CD8⁺ T cells is associated with more rapid effector cell differentiation. However, whether changes in glucose metabolism are ultimately responsible for altering the fate of neonatal CD8⁺ T cells during infection is unclear. Recent studies have demonstrated that CD8⁺ T cells with high glycolytic metabolism are short-lived and quickly die postinfection, whereas those with low glycolytic metabolism persist and transition into the long-lived memory pool (33). Thus, we hypothesized that neonatal CD8⁺ T cells are impaired at forming memory because they undergo a more pronounced glycolytic flux after antigenic stimulation. To test this hypothesis, we designed an experiment to limit glucose metabolism in neonatal CD8⁺ T cells before priming and examined changes in their ability to respond to infection. Our strategy involved stimulating neonatal and adult CD8⁺ T cells in vitro in the presence or absence of a competitive inhibitor for glucose (2-DG) and then cotransferring adult donor CD8⁺ T cells with either neonatal (2-DG–

treated) or neonatal (control) donor cells into an LM-gB–infected matched recipient (Fig. 4A). At various times postinfection, we examined the phenotype of each donor population and found that inhibiting glycolysis in neonatal cells resulted in significantly more MPECs and fewer SLECs (Fig. 4B, 4C). In fact, the neonatal cells treated with 2-DG exhibited a phenotype comparable to the adult donor CD8⁺ T cells, indicating that a sizeable portion of 2-DG–treated cells were able to transition into the memory stage (Fig. 4B, 4C). Strikingly, 2-DG treatment also enabled neonatal CD8⁺ T cells to survive during contraction and mount a more proliferative recall response following reinfection with LM-gB, similar to adult donor CD8⁺ T cells (Fig. 4D). Thus, limiting entry into glycolysis is sufficient to limit terminal differentiation and promote the development of neonatal memory CD8⁺ T cells.

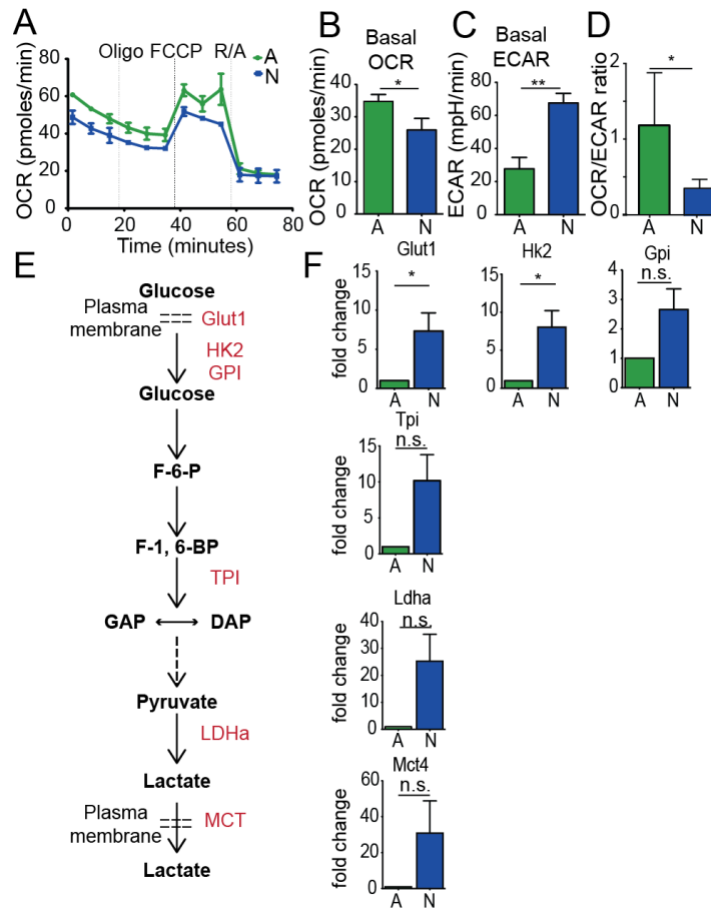


Figure 3.1 Neonatal CD8⁺ T cells exhibit higher glycolytic metabolism after in vitro stimulation. (A) OCR measurements of adult and neonatal CD8⁺ T cells at 48 h after anti-CD3 and anti-CD28 activation. (B) Basal OCR, (C) basal ECAR, and (D) basal OCR/ECAR ratios in adult and neo- natal CD8⁺ T cells at 48 h after anti-CD3 and anti-CD28 activation. (E) Pathway of critical proteins in glycolysis. (F) Fold change in mRNA ex- pression of proteins 8 h poststimulation using qPCR. Data representative of two independent experiments with three to five biological replicates per group. *p , 0.05, **p , 0.005 by unpaired Student t test.

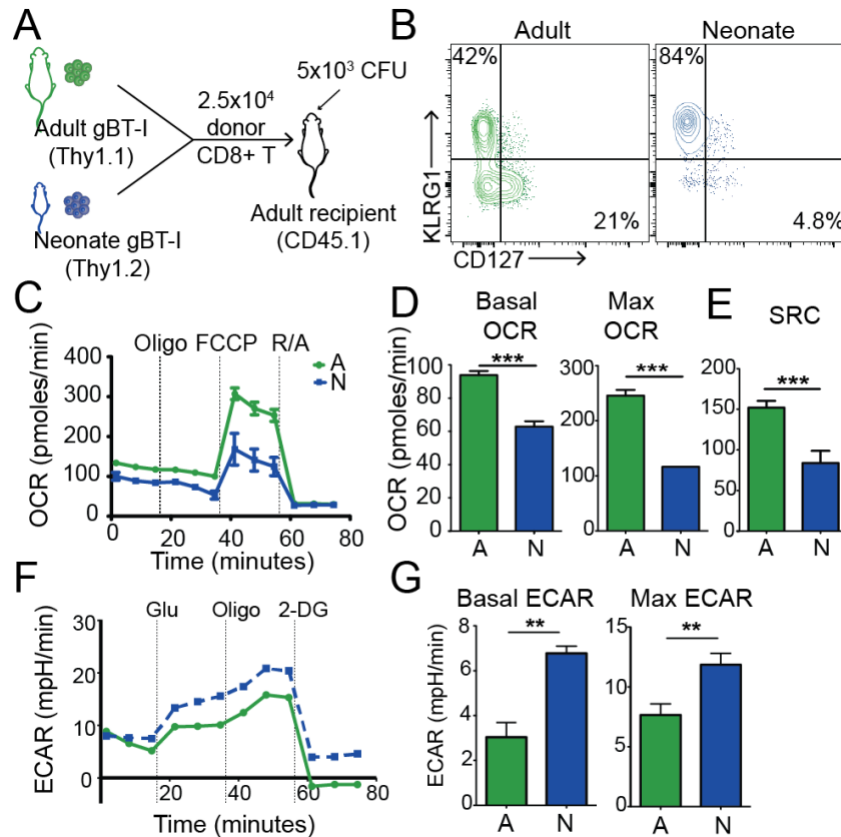


Figure 3.2 Neonatal CD8⁺ T cells possess an inherent propensity to undergo glycolytic metabolism following infection. (A) The experimental design to examine the metabolic programs of neonatal and adult CD8⁺ T cells during infection. (B) Representative contour plots of KLRG1 and CD127 expression at 7dpi. (C) OCR measurements, (D) basal and maximum OCR, and (E) SRC values in adult and neonatal CD8⁺ T cells during a mitochondrial stress test at 7 dpi. (F) ECAR measurements and (G) basal and maximum ECAR values of adult and neonatal CD8⁺ T cells during a glycolysis stress test at 7 dpi. Data representative of two independent experiments with three biological replicates per group. **p , 0.005, ***p , 0.0005 by unpaired Student t test.

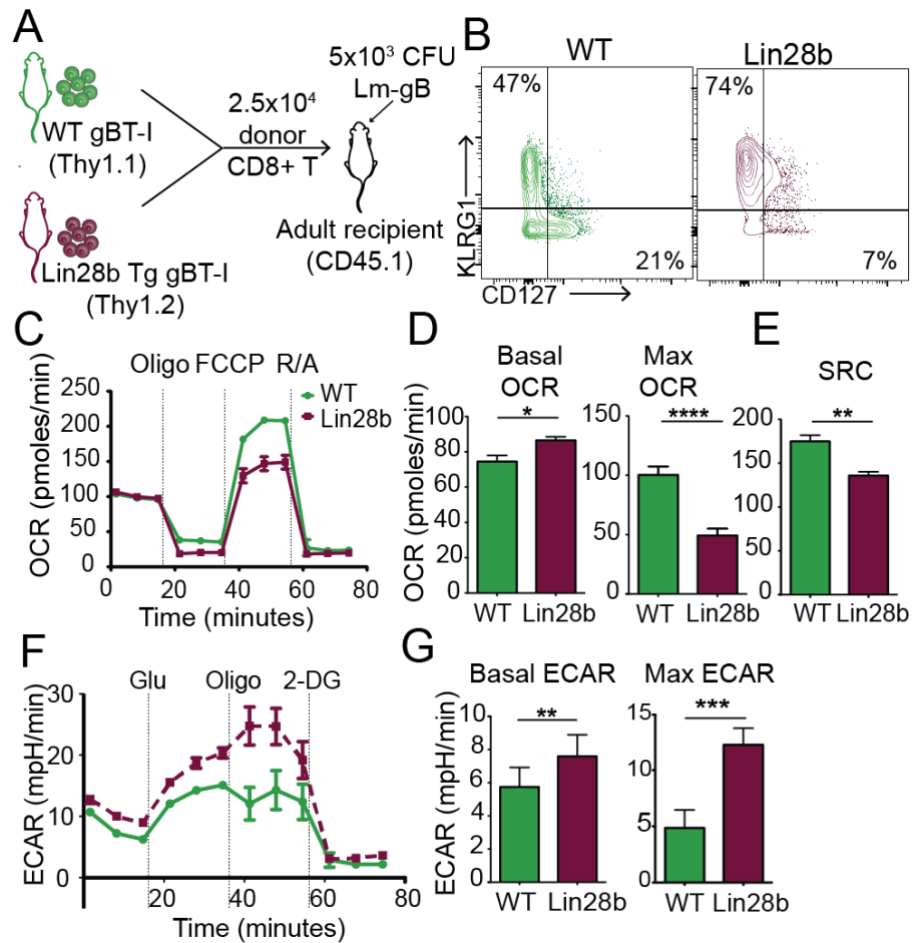


Figure 3.3 Lin28b drives a more glycolytic metabolic program in CD8⁺ T cells (A) The experimental design to examine the metabolic programs of WT adult and Lin28b Tg adult CD8⁺ T cells during infection. (B) Representative contour plot of KLRG1 and CD127 expression at 7 dpi. (C) OCR measurements, (D) basal and maximum OCR, and (E) SRC values in adult and neonatal CD8⁺ T cells at 7 dpi from a mitochondrial stress test. (F) ECAR measurements and (G) basal and maximum ECAR values in WT and Lin28b Tg CD8⁺ T cells from a glycolysis stress test at 7 dpi. Data representative of two independent experiments with three biological replicates per group. *p , 0.05, **p , 0.005, ***p , 0.0005, ****p , 0.000 by unpaired Student t test.

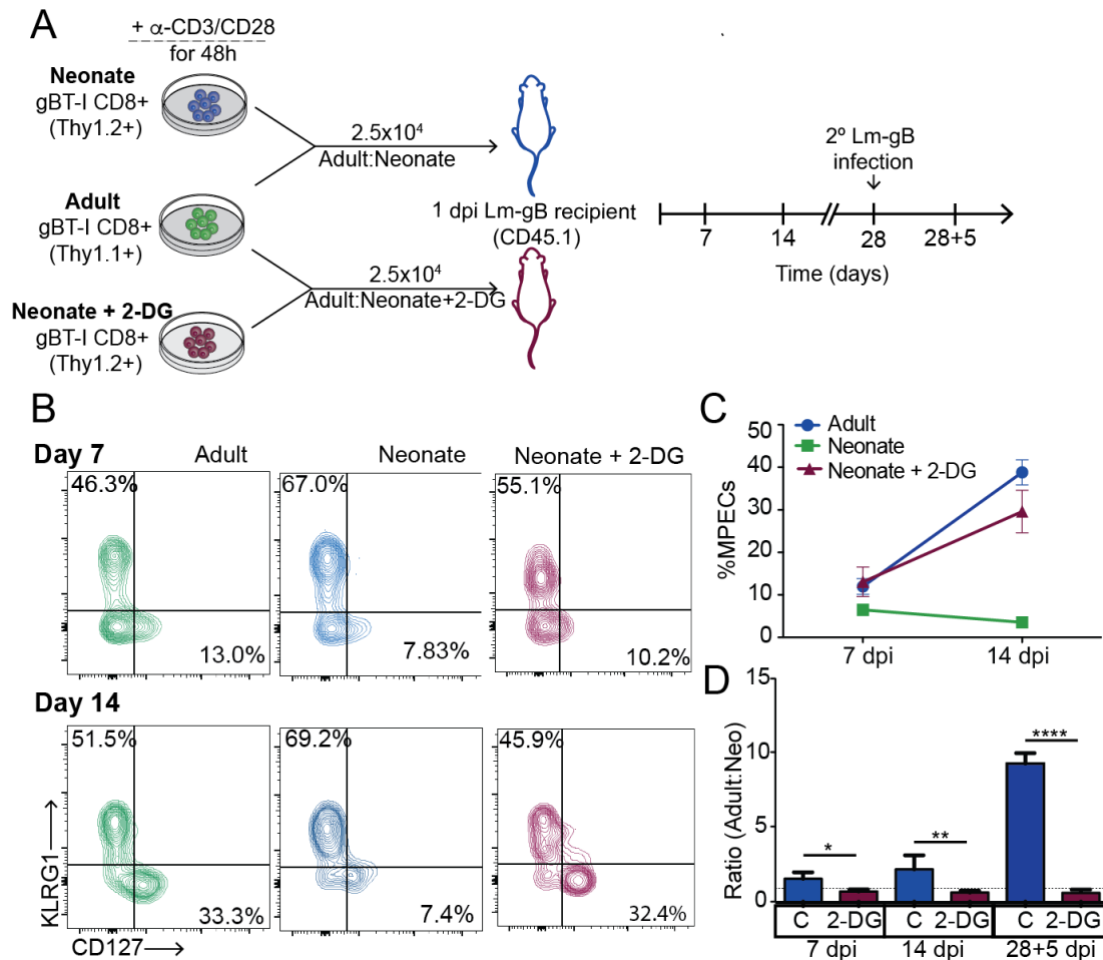


Figure 3.4 Inhibiting glycolytic metabolism restores the formation of neonatal memory CD8+ T cells. (A) Schematic of the experimental design: adult and neonatal CD8+ T cells were stimulated with anti-CD3 and anti-CD28 in the presence of a vehicle control or 0.3 mM 2-DG for 48 h. Then, 2.5×10^4 activated adult (Thy1.1) and neonatal (Thy1.2) cells were cotransferred i.v. into congenic WT recipients (CD45.1) that were infected with 5×10^3 CFU of LM-gB the previous day. Recipients were rechallenged with 5×10^4 CFU of LM-gB at 28 dpi. Phenotypes of donor cells were assessed through bleeds at indicated time points. (B) Representative contour plots of KLRG1 and CD127 expression at 7 and 14 dpi. (C) Percentage of adult, neonatal, and

2-DG neonatal cells that are MPECs at 7 and 14 dpi. (D) Ratio of adult and neonatal donor populations with respect to time. Data representative of three independent experiments with four to six biological replicates per group. *p , 0.05, **p , 0.005, ****p , 0.00005 by unpaired Student t-test

DISCUSSION

In summary, these findings demonstrate that neonatal and adult CD8⁺ T cells are intrinsically different and respond to Ag stimulation with dissimilar metabolic programs, leading them to adopt different fates. Higher glycolytic flux biases neonatal CD8⁺ T cells to become short-lived effectors at the expense of forming memory cells. Perhaps counterintuitively, reducing glycolytic metabolism during priming can enhance the survival of neonatal memory CD8⁺ T cells. These findings suggest that many of the intrinsic differences between neonatal and adult CD8⁺ T cells can be attributed to age-related changes in metabolic reprogramming and demonstrate that neonatal memory T cell responses can be therapeutically enhanced.

Although the underlying basis for why neonatal and adult CD8⁺ T cells use distinct metabolic programs requires further investigation, our findings suggest that neonatal CD8⁺ T cells exhibit a different metabolic program than adults because they are derived from fetal-liver HSCs. Thus, it would be interesting to examine the metabolic programs in other fetal-derived lymphocytes to see if they also have a higher glycolytic flux than their adult counterparts. It is also interesting to speculate that Lin28b serves as a metabolic rheostat, balancing the need to generate different amounts of effector and memory cells at various stages of life. In the future, it will be important to investigate how the different metabolic states in neonatal and adult CD8⁺ T cells influence their ability to give rise to different subsets of memory cells (e.g., tissue resident memory, central memory, and effector memory). Such studies could

provide us with novel strategies to fine-tune the development of memory CD8⁺ T cells during critical stages of development.

SUPPLEMENTAL MATERIAL

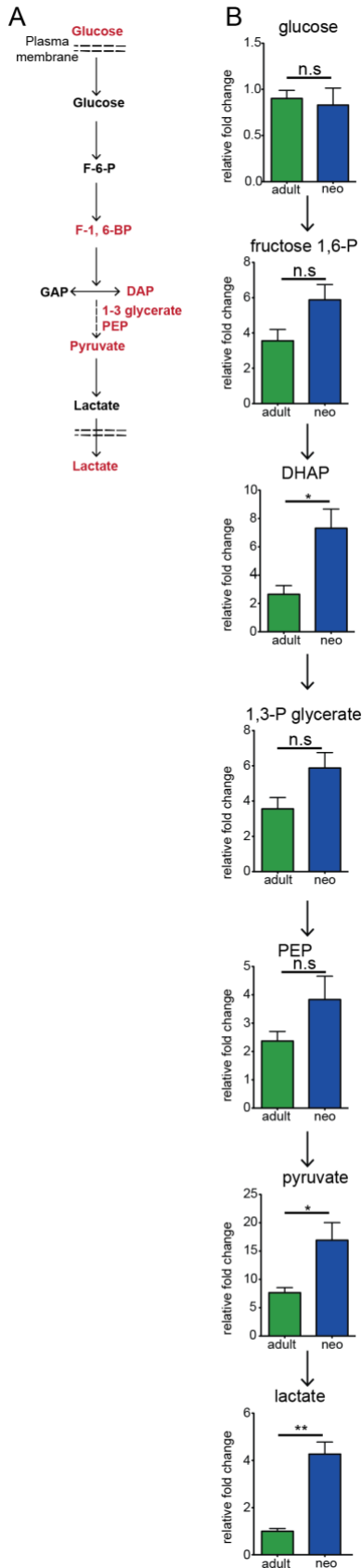


Fig S3.1 Measurements of metabolites in OXPHOS and glycolytic pathways post-activation in vitro.

(a) Pathway of critical metabolites in glycolysis. (b) Metabolomics was performed on adult and neonatal gBT-I CD8+ T cells 18-hour post-activation in vitro. Data has been normalized to measurements collected prior to stimulation. Significance was determined by student t test. Data representative of two independent experiments with 3 biological replicates/group. *P < .05 and **P < .005.

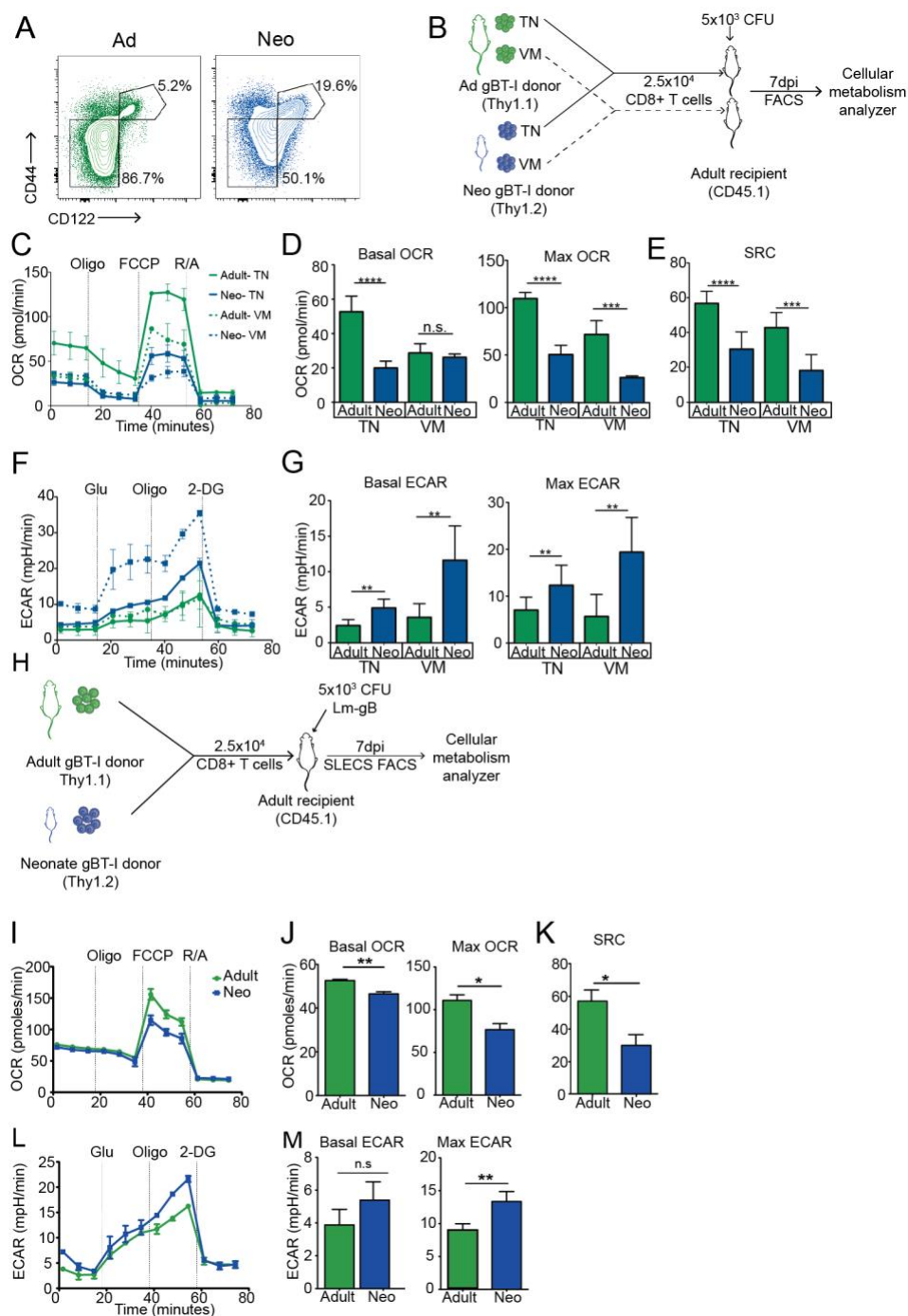


Figure S3.2 Metabolic programs are governed by cell-intrinsic factors. (a)

Representative contour plots of the TN and VM populations in uninfected adult and neonatal mice. (b) The experimental design to examine if the starting phenotype plays a deterministic role in metabolic programs (c) OCR measurements (d) Basal and

max OCR values, (e) SRC values in mice at 7 dpi from a mitochondrial stress test (f) ECAR measurements, (g) basal and max ECAR values from a glycolysis stress test at 7 dpi. Data are representative of two independent experiments. (h) Schematic of the experimental design to examine if the effector phenotype influences metabolic differences between neonatal and adult T cells (i) OCR measurements, (j) Basal and max OCR, (k) SRC values in adult and neonatal SLECs during a Mitochondrial Stress Test at 7 dpi (l) ECAR measurements, (m) Basal and max ECAR values of adult and neonatal CD8⁺ T cells during a Glycolysis Stress Test at 7 dpi. Data representative of 2-4 independent experiments with 3 biological replicates/group. * p **** P<0.005, *** P<0.0005, **** P<0.00005 BY AN A TWO-WAY ANOVA FOLLOWED BY A TUKEY POST-HOC TEST (C-G) AND AN UNPAIRED STUDENT T-TEST (I-M)

MATERIALS AND METHODS

Mice

B6-Ly5.2/Cr mice were purchased from Charles River Laboratories. TCR transgenic mice specific for the HSV-1 glycoprotein gB498–505 peptide SSIEFARL [gBT-I mice (24)] were provided by J. Nikolich-Zugich (University of Arizona, Tucson, AZ) and crossed with Thy1.1 or C57BL/6 mice purchased from The Jackson Laboratory. Lin28b transgenic (Lin28b Tg) mice (25) driven under the CD2 promoter were provided by L. Pobezinsky (University of Massachusetts) and crossed with gBT-I mice. Neonatal and adult gBT-I animals of both sexes were used at 5–7-d and 2–4-mo-old, respectively. Mice were housed under specific pathogen-free conditions at Cornell University

College of Veterinary Medicine, accredited by the Assessment and Accreditation of Laboratory Animal Care.

In vitro T cell stimulation assays

Splenic CD8⁺ T cells were isolated from gBT-I mice by positive immunomagnetic selection (Miltenyi Biotec), stimulated with plate-bound anti-CD3 (5 mg/ml) and anti-CD28 (20 mg/ml), and cultured in complete media supplemented with 10 U/ml IL-2 (BioLegend) for 48 h. For glycolytic inhibition experiments, cells were cultured with media supplemented with 0.3 mM 2-deoxy-D-glucose (2-DG; Sigma-Aldrich).

Dual adoptive transfer experiments

gBT-I splenic neonatal and adult CD8⁺ T cells were enriched by positive immunomagnetic selection (Miltenyi Biotec). Combined cells were suspended at 53105 cells/ml of PBS and 100 µl was injected i.v. into adult B6-CD45.1 recipient mice. The next day, recipient mice were infected (53103 CFU, i.v.) with wild-type (WT) *Listeria monocytogenes* expressing the gB-8p peptide (LM-gB), as previously described (6).

Abs and flow cytofluorimetric analysis

Abs were purchased from eBioscience, BioLegend, Invitrogen, or BD Biosciences, and concentrations were used as recommended by the manufacturer. Flow cytofluorimetric data were acquired using FACSDiva software from an LSRII equipped with four lasers (BD Biosciences). Analysis was performed with FlowJo (Tree Star, Ashland, OR).

Cell sorting

To perform metabolic flux analysis, donor CD8⁺ T cells were recovered from recipient mice by positive immunomagnetic selection (Miltenyi Biotec) and subsequently labeled with Abs against CD8 (53-6.7), CD4 (Gk1.5), CD45.1 (A20), CD45.2 (104), and Thy1.1 (OX-7). For experiments that isolated true naive (TN) and virtual memory (VM) populations, Abs against CD122 (Tm-b1) and CD44 (IM7) were used. For experiments that isolated the short-lived effector cell (SLEC) population, Abs against KLRG1 (2F1) and CD127 (A7R34) were used. The cells were then sorted to .95% purity on a FACS Aria III (BD Biosciences).

Metabolic bioassays

To measure oxygen consumption rates (OCR) and extracellular acidification rates (ECAR), 2.5–3 × 10⁵ sorted CD8⁺ T cells were plated in buffer-free media containing 2 mM glutamine and 1 mM sodium pyruvate. Twenty-five millimolars glucose was additionally supplemented to measure OCR. Using a Seahorse XFp Extracellular Flux Analyzer (Agilent, Santa Clara, CA), OCR was measured following the addition of 1 mM oligomycin, 1 mM FCCP, and 0.5 mM rotenone/antimycin A, and ECAR was measured following the addition of 10 mM glucose, 1 mM oligomycin, and 50 mM 2-DG.

Quantitative PCR

RNA was isolated using TRIzol (Life Technologies), and cDNA was generated with the iScript Reverse Transcription Supermix kit (Bio-Rad). Real-time RT-PCR was performed on an Applied Biosystems 7900HT with primers that have been previously described (26). Gene expression was calculated relative to Actb.

Metabolomics

Metabolite extraction from activated neonatal and adult CD8⁺ T cells was performed exactly as described in a previous study (27). An liquid chromatography-mass spectrometry (Q Exactive mass spectrometer; Thermo Fisher Scientific) was used for metabolite profiling, and the relative abundance of each metabolite was calculated using the commercially available software Sieve 2.2 (Thermo Fisher Scientific) according to previously described procedures (27).

Statistical analysis

Statistical analysis was performed using Prism (GraphPad Software, La Jolla, CA). Error bars represent SEM and are representative of biological replicates within an experiment. Significance was determined by Student t test or a two-way ANOVA followed by a Tukey post hoc test as indicated in the figure legends. The p values ,0.05 were considered significant.

ACKNOWLEDGMENTS

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**CHAPTER 4: EARLY MICROBIAL EXPOSURE SHAPES ADULT IMMUNITY BY ALTERING
CD8+ T CELL DEVELOPMENT**

Abstract

Microbial exposure during development can elicit long-lasting effects on the health of an individual ¹⁻⁵. However, how microbial exposure in early life leads to permanent changes in the immune system is unknown. Here, we show that the microbial environment alters the setpoint for immune susceptibility by altering the developmental architecture of the CD8⁺ T cell compartment. In particular, early microbial exposure results in the preferential expansion of highly responsive fetal-derived CD8⁺ T cells that persist into adulthood and provide the host with enhanced immune protection against intracellular pathogens. Interestingly, microbial education of fetal-derived CD8⁺ T cells occurs during thymic development rather than in the periphery and involves the acquisition of a more effector-like epigenetic program. Collectively, our results provide a new conceptual framework for understanding how microbial colonization in early life leads to lifelong, and potentially irreversible, changes in the immune system.

Results and Discussion

Microbial exposure in early life can permanently program an individual's immune system and alter lifelong disease risk. For example, children exposed to farm environments are less likely to develop asthma, whereas antibiotic use in early life is associated with an increased risk of developing inflammatory bowel disease and diabetes^{2,3,6,7}. This phenomenon, often referred to as 'developmental programming', is founded on the idea that various organ systems adapt a phenotype that is best suited for the environment during early development⁸. However, most of these studies are based on epidemiological associations, and the underlying mechanisms remain undefined. While previous reports have highlighted aspects of immune development that can be altered by the early microbial environment (e.g., thymic output, lymph node growth, and epigenetic modifications)⁹⁻¹³, one key variable left unexamined is the developmental layering of the immune system¹⁴⁻¹⁶.

The development of the immune system was long thought to progress in a linear manner from fetal life to adulthood. Now, numerous studies have established that the immune system is stratified into layers of distinct immune cells, which develop sequentially from unique waves of hematopoietic stem cells (HSCs)¹⁷⁻²¹. In general, fetal HSCs produce a layer of fast-acting lymphocytes (e.g., neonatal CD8+ T cells, B1 B cells, gamma/delta T cells) that persist into adulthood and represent the early effectors, while adult HSCs produce a layer of slower-acting lymphocytes (e.g., adult CD8+ T cells, B2 B cells) that preferentially contribute to the memory

population ²²⁻²⁴. These fetal and adult developmental layers of the CD8+ T cell pool exhibit unique roles during infection ^{18,25}. Interestingly, the developmental switch between fetal and adult lymphopoiesis occurs at the same time the host is transitioning from the protected environment of the uterus to the antigen-rich environment of the outside world ²⁶⁻²⁸. An important and unanswered question is whether the early microbial environment alters lifelong disease risk by modulating the layering of immune cells during ontogeny.

To examine the long-term impact of early microbial exposure on immune susceptibility, we developed a mouse model in which pups were raised in either a 'dirty' (pet shop-exposed) or 'clean' (specific pathogen-free) environment (Fig. 1A). The pups raised in a dirty environment were born to mothers that were uniformly exposed to material from pet-shop mice prior to mating (Table S1, Fig. S1). The pups raised in a clean environment correspond to mice raised in a standard lab facility. First, we challenged the offspring with *Listeria monocytogenes* (LM), a pathogen that requires CD8+ T cells for clearance. Despite having a similar number of CD8+ T cells before infection (Fig. S2), the mice raised in a dirty environment exhibited an enhanced ability to eliminate LM (Fig. 1B) and mounted a more robust antigen-specific CD8+ T cell response (Fig. 1C-E). We considered the possibility that the dirty environment primed CD8+ T cells to respond more quickly. However, CD8+ T cell adoptive transfer experiments showed that even when placed in the same environment, dirty cells were more protective than their clean counterparts (Fig. 1F, G). Collectively, these data demonstrate that microbial exposure induces changes to

the CD8+ T cell compartment that enhance its ability to protect the host against infection.

We next sought to understand how a more microbially diverse environment in early life leads to a more protective CD8+ T cell compartment in adulthood. On the one hand, the microbial environment may enhance the ‘average phenotype’ or function of all CD8+ T cells in the starting pool in a similar manner (the ‘uniform’ model). Alternatively, microbes could alter immune responsiveness by changing the proportion of cells that are derived from different developmental periods (the ‘layered’ model). To distinguish between the ‘uniform’ and ‘layered’ models of microbial education, we first determined whether the developmental layering of CD8+ T cells was altered in dirty mice. To do this, we used fate-mapping ‘timestamp’ mice (see methods) to permanently mark or ‘timestamp’ a wave of cells at discrete times of thymic development. We timestamped ‘dirty’ and ‘clean’ pups at birth, to mark fetal-derived cells ²⁹, and at 28 days, to mark adult-derived cells (Fig. 2A) ²⁷. At 8 wks of age, we found a higher proportion of fetal-derived CD8+ T cells and a lower proportion of adult-derived CD8+ T cells present in dirty mice (Fig. 2B, C). This pattern persisted across multiple stages of development (Fig. S3). Importantly, cells with different developmental origins had an antigen-inexperienced (CD49d^{lo}) phenotype, indicating that the increased proportion of fetal-derived CD8+ T cells in dirty mice was not due to an expansion of antigen-specific cells, but rather to changes in the developmental layering of the naïve CD8+ T cell compartment. We also found that delaying microbial exposure to adulthood did not lead to an increase

of CD8⁺ T cells in the fetal layer (Fig. S4), indicating that there is a critical window of opportunity in early life when the developmental layers of the CD8⁺ T cell compartment can be altered.

The timestamping experiments suggest dirty mice mount a more protective CD8⁺ T cell response because they are comprised of a larger proportion of fast-acting, fetal-derived cells prior to infection. However, we wanted to know whether fetal-derived CD8⁺ T cells provide enhanced protection against intracellular pathogens in adulthood, and whether the observed changes in the proportions of fetal- and adult-derived CD8⁺ T cells in dirty mice were functionally significant. To address these questions, we designed experiments to see if we could normalize susceptibility in clean and dirty mice by manipulating the developmental layers in the CD8⁺ T cell compartment, specifically by removing the fetal layer. To ablate the fetal layer, we administered a CD8 α depleting antibody to clean or dirty mice at 2 wks of age. Once the mice reached 8 wks of age, we transferred an equal number of purified CD8⁺ T cells from depleted donor mice into T cell-deficient recipients and infected the recipients with LM (Fig. 2D). Importantly, recipient mice that received cells from dirty mice lacking the fetal layer had a pathogen load comparable to clean mice with a normal CD8⁺ T cell compartment (Fig. 2E). To test whether immune susceptibility was changed in mice lacking the adult layer of CD8⁺ T cells, we performed thymectomies on clean and dirty mice at 2.5-3 wks of age to prevent the adult layer from forming (Fig. 2F). When the mice were 8 wks old, we again transferred an equal number of donor CD8⁺ T cells into the T cell-deficient recipients

and infected the mice with LM. Notably, the recipient mice that received cells from clean mice with only the fetal layer had a similar pathogen load compared to recipient mice that received cells from dirty mice (Fig. 2G). Together, these results establish the fetal layer as both necessary and sufficient for enhanced immunity in dirty mice.

One of the most intriguing findings was that recipient mice receiving fetal-derived cells from dirty mice had the lowest pathogen load (Fig. 2G), suggesting that fetal cells from dirty mice may be even more protective than their counterparts from clean mice. To examine the impact of microbial exposure on the behavior of fetal- and adult-derived CD8⁺ T cells, we compared the function and phenotype of the developmental layers in clean and dirty mice after direct infection with LM (Fig. 3A). Interestingly, fetal-derived cells from dirty mice mounted an enhanced response and produced more of the effector protein granzyme B compared to their clean counterparts (Fig. 3B, C). In contrast, adult-derived cells from dirty mice contributed less to the response while producing lower levels of granzyme B (Fig. 3D, E). We also observed similar layer- dependent alterations in clean and dirty mice infected with Vaccinia virus, suggesting that the more robust response by fetal-derived cells is not pathogen dependent (Fig. S5). These data show that the microbial environment preferentially enhances the functions of fetal-derived CD8⁺ T cells.

We considered the possibility that the enhanced responsiveness of fetal-derived cells in dirty mice was due to cell-intrinsic differences. To test this, we compared the ability of cells from clean and dirty mice to proliferate after *in vitro*

stimulation via the T cell receptor (TCR). Fetal-derived cells from dirty mice turned out to be more proliferative than their clean counterparts (Fig. 3F, G). We also considered whether this enhanced ability of dirty fetal-derived cells to proliferate could relate to differences in their starting phenotype. Indeed, we found that fetal-derived CD8⁺ T cells from dirty mice have the highest proportion of virtual memory (VM) cells, a subset of antigen-inexperienced cells that are functionally similar to memory cells, which could explain their more proliferative response after stimulation (Fig. S6A, B) ^{30,31}. However, even after controlling for their initial phenotype status, we found that dirty fetal-derived CD8⁺ T cells still underwent division at higher rates in the steady-state and in response to TCR stimulation (Fig. S6 C-E). This indicates that the microbial environment programs the fetal layer of CD8⁺ T cells to be highly responsive to antigenic stimulation at the individual cell level.

An important question is, when does the microbial environment ‘instruct’ fetal-derived CD8⁺ T cells to behave differently? The simplest explanation is that fetal-derived CD8⁺ T cells are programmed differently in clean and dirty mice by environmental factors in the periphery. However, recent studies have suggested that ‘training’ of immune cells can occur during the early stages of their development ³²⁻³⁴, raising the possibility that the microbial education of CD8⁺ T cells takes place in progenitors during thymic development. To differentiate between these possibilities, we took newborn timestamp thymii from clean or dirty donor mice and transplanted them under the kidney capsule of clean or dirty adult

recipients (Fig. 3H). We administered tamoxifen immediately after thymic transplantation to mark a wave of fetal-derived thymic precursors and tracked their behavior after maturing in either a clean or dirty peripheral environment. Interestingly, we observed a preferential accumulation of fetal-derived CD8⁺ T cells from dirty mice that was largely independent of the microbial status of the peripheral environment (Fig S7). In addition, the fetal-derived CD8⁺ T cells from dirty mice exhibited an enhanced ability to proliferate after stimulation, regardless of whether they were matured in a clean or dirty environment (Fig. 3I, J). Collectively, these data demonstrate that microbial programming of fetal-derived CD8⁺ T cells occurs in the progenitors and is retained in the mature cells after they have exited the thymus.

Lastly, we sought to understand the regulatory basis of how the microbial environment enhances the ability of peripheral fetal-derived CD8⁺ T cells to respond to infection. We previously reported that fetal-derived CD8⁺ T cells respond more rapidly to infection because they have increased chromatin accessibility at regulatory sites for effector genes before stimulation ²⁵. However, the enhanced immune functions in dirty mice suggest that fetal-derived CD8⁺ T cells may undergo additional programming when developed in a dirty environment. To address this possibility, we compared the transcriptomes (RNA-seq) and chromatin landscapes (ATAC-seq) of antigen-inexperienced fetal-derived CD8⁺ T cells from clean and dirty mice at 8 wks of age (Fig. 4A). Consistent with their unique behavior during infection, fetal-derived cells from dirty mice exhibited distinct transcriptomic and epigenomic

profiles (Fig 4B, C). To understand the nature of these differences, we compared the profiles of upregulated genes in clean and dirty cells to establish gene sets that reflect different CD8⁺ T cell states. Whereas the dirty fetal-derived cells exhibited increased expression and accessibility for genes corresponding to effector or memory cells, the clean cells more closely resembled naïve CD8⁺ T cells (Fig. 4D-F). Since fetal-derived cells have higher proportions of VM cells, we also compared the gene expression profiles and chromatin landscapes of phenotype-matched cells from clean and dirty mice and found that the distinct loss of the naïve state in dirty mice persisted in phenotypically similar populations (Fig. S8). Gene ontology (GO) analysis of the poised genes, which are not differentially expressed but have altered accessibility in the corresponding promoter and enhancer regions, revealed that the epigenome of dirty fetal-derived cells was more associated with immune defense (innate immunity, inflammatory response, TCR activation) and cytokine response (type I IFN, cytokine response), while their clean counterparts overlapped with more general developmental processes (Fig. 4G, Fig. S9). Overall, these data suggest that mice raised in a dirty environment mount stronger responses to infection because their T cells are epigenetically 'primed' for stimulation.

Our work provides a new conceptual framework for understanding how the microbial environment alters immune susceptibility in adulthood. By tracking T cells produced at different stages of life, we uncovered a link between microbial exposure, developmental layering, and immune susceptibility. In response to increased microbial exposure, the developing immune system adapts by expanding

the 'fast-acting' fetal layer of CD8⁺ T cells, allowing the host to mount a more targeted response to early infection in adulthood. When microbial exposure is reduced, the slower-acting adult layer of cells preferentially accumulates, leaving the host more susceptible to intracellular pathogens. These findings offer insight into how variation in microbial exposure in early life can program lifelong intra-individual variation in the immune system and may allow us to predict infection outcomes and disease risk based upon the ratio of fetal- to adult-derived CD8⁺ T cells present in adults.

Our findings also provide a novel perspective on immune training, which currently focuses on understanding how innate immune cells, such as natural killer cells and macrophages, retain memory of past infections³⁵. The trained immune phenotype in innate cells is associated with epigenetic reprogramming, allowing them to respond more strongly to subsequent infections. We found that fetal-derived CD8⁺ T cells in dirty mice similarly exhibit enhanced functions related to increased accessibility at effector genes, indicating that immune training is not limited to innate immune cells and that CD8⁺ T cells are also programmed by the microbial environment. In the future, it will be important to examine how other environmental factors, such as stress, diet, and toxins alter the developmental layering and programming of CD8⁺ T cells. Knowledge gained from these studies will broaden our fundamental understanding of immune ontogeny and cell-mediated immunity.

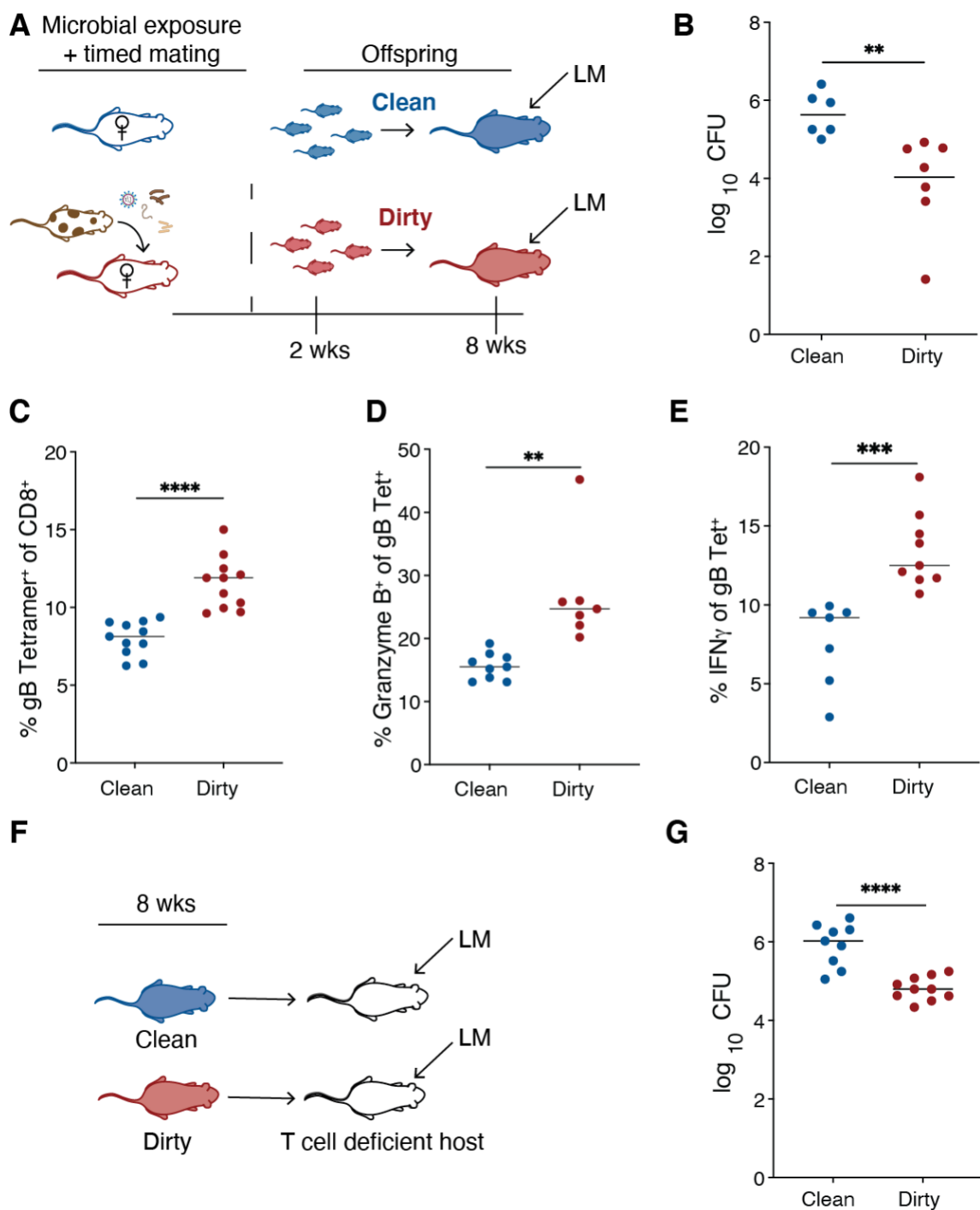


Fig 1. Early microbial exposure enhances immunity in adulthood. (A) Approach to generate 'dirty' mice: Female laboratory mice were exposed to fecal and cage contents of pet-shop mice for four weeks. Their offspring were maintained in a 'dirty'

environment. **(B)** Bacterial load at 3 days post-infection in 8-week old mice that were directly infected with 1×10^4 CFU of wildtype *Listeria monocytogenes-gB* (LM). **(C)** Percent of CD8+ T cells that are positive for Kb:gB₄₉₈₋₅₀₅ tetramer **(D)** Granzyme B and **(E)** interferon-gamma following infection **(F)** Approach to transfer purified CD8s into T cell deficient hosts **(G)** Bacterial load at 3 dpi from TCR α KO recipients that received 5×10^6 purified CD8s from 'clean' or 'dirty' 8wk old donor mice and subsequently infected with LM-gB. Data for B are pooled from 2 independent experiments (n=3-4 mice/group) and are \pm SEM. Data from C-E are pooled from 2 independent experiments (n=4-5 mice/group) and are \pm SEM. Data for G are pooled from 2 independent experiments (n=4-5 mice/group) and are mean \pm SEM. Statistical significance was determined by a student's t-test (** P<0.005. **** P<0.00005)

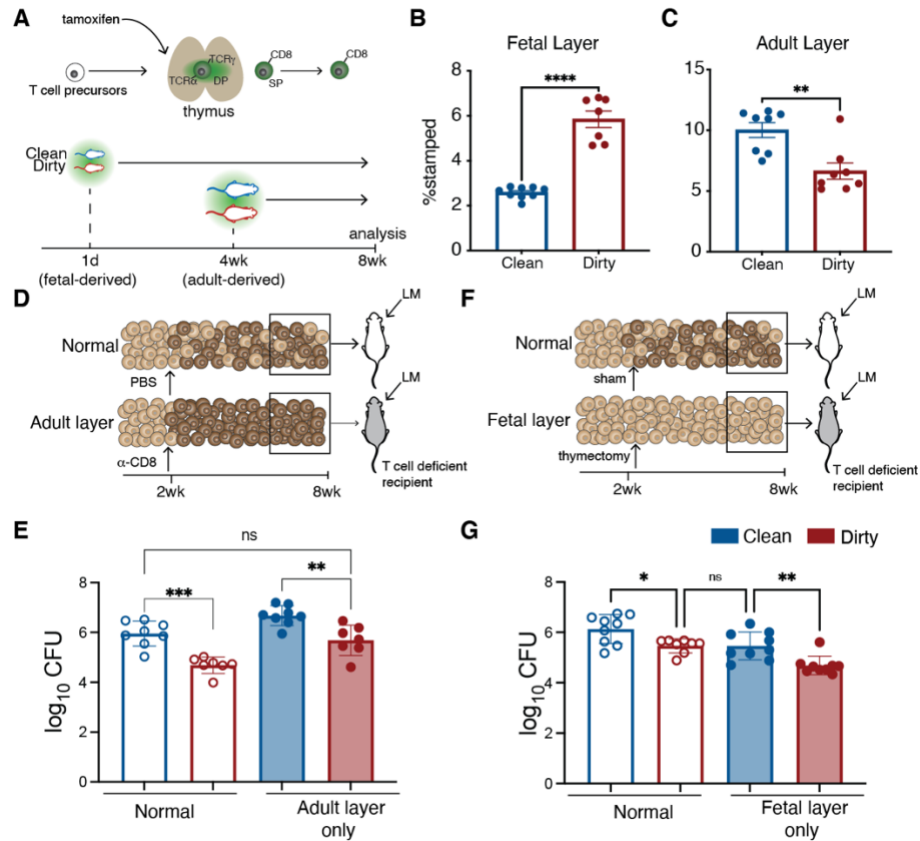


Fig 2. Expansion of the fetal layer drives enhanced protection in dirty mice. (A) Approach to timestamp the fetal and adult layer in clean and dirty mice. **(B)** The proportion of fetal- and **(C)** adult-derived CD8⁺ T cells in 8 wk old clean and dirty mice. **(D)** Schematic showing the approach to deplete the fetal layer. **(E)** Pathogen burden in clean and dirty mice lacking the fetal layer of CD8⁺ T cells. **(F)** Schematic showing the approach to prevent the adult layer from forming. **(G)** Pathogen burden in clean and dirty mice lacking the adult layer of CD8⁺ T cells. Data for B, C are \pm SEM and are pooled from 2 independent experiments (n=3-4/group). Data for E, G are mean \pm SEM and are pooled from 2 independent experiments (n=3-4/group). Statistical significance for B, C was determined by a student's t-test. Significance for E, F was determined by a one-

way ANOVA with a Tukey multiple comparison test (ns not significant, * $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$, **** $P < 0.00005$)

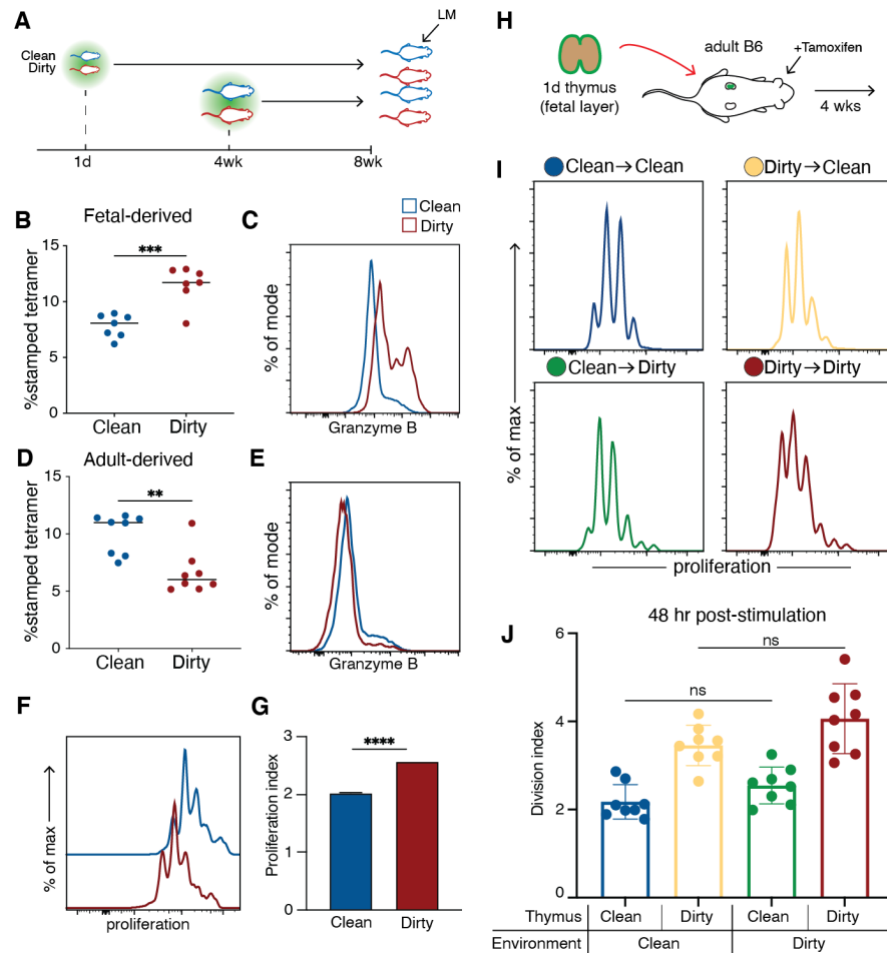


Fig 3. Fetal-derived cells in the dirty environment are more responsive to stimulation.

(A) Approach to infect clean or dirty timestamp mice with *Listeria*. **(B)** The proportion of antigen-specific fetal-derived cells at 5 days post-LM infection. **(C)** Representative histogram showing Granzyme B production after infection in fetal-derived cells. **(D)** The proportion of antigen-specific adult-derived CD8⁺ T cells at 5 days post-LM infection. **(E)** Representative histogram showing Granzyme B production in adult-derived cells. **(F)** Representative histograms of proliferation peaks after α CD3/28 activation. **(G)** Division index of clean or dirty fetal-derived cells **(H)** Experimental approach for kidney capsule thymic transplants surgeries. **(I)** Representative

proliferation peaks 48h after *in vitro* activation with aCD3/CD28. **(J)** Division index of fetal-derived cells after aCD3/28 stimulation. Data for B, D are \pm SEM and are pooled from 2 independent experiments (n=3-4 mice/group). Statistical significance for B, D was determined by a student's t-test. Statistical significance for J was determined by a one-way ANOVA with a Tukey multiple comparison test (ns not significant, * $P<0.05$, ** $P<0.005$, *** $P<0.0005$).

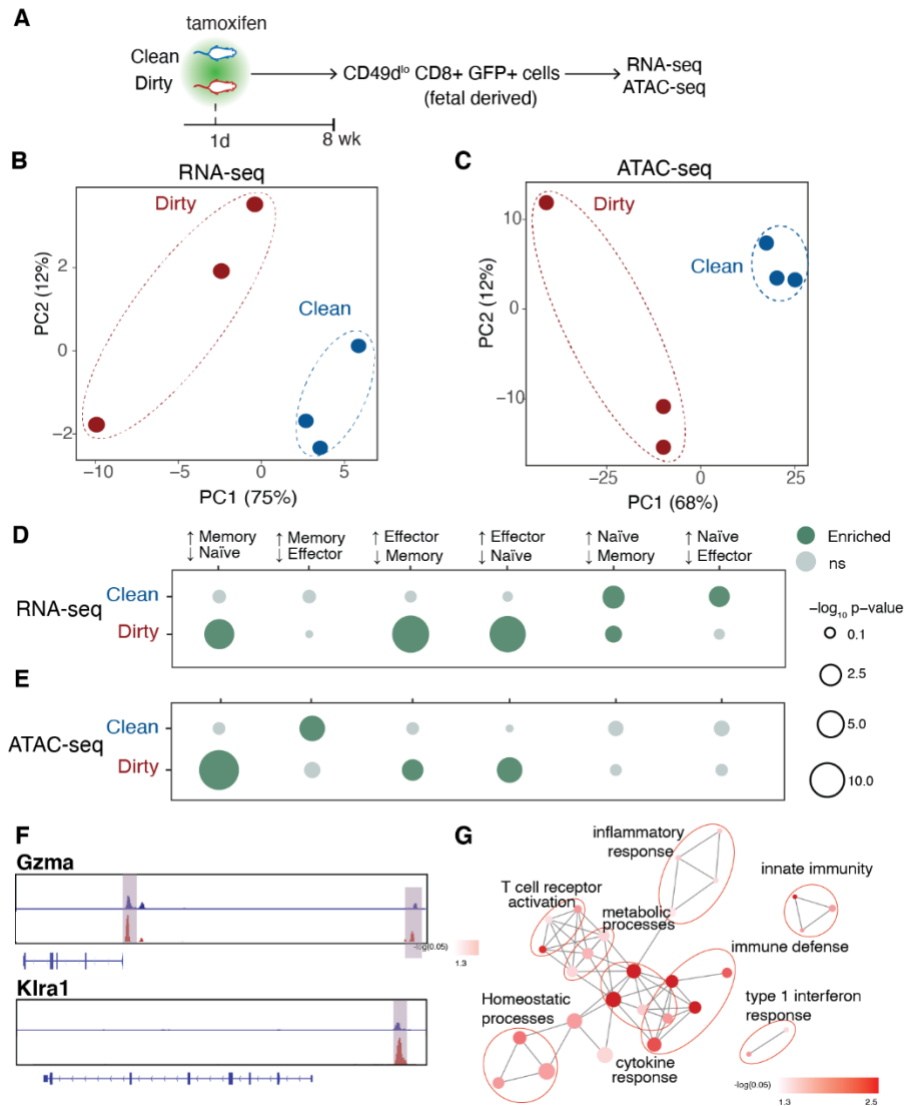
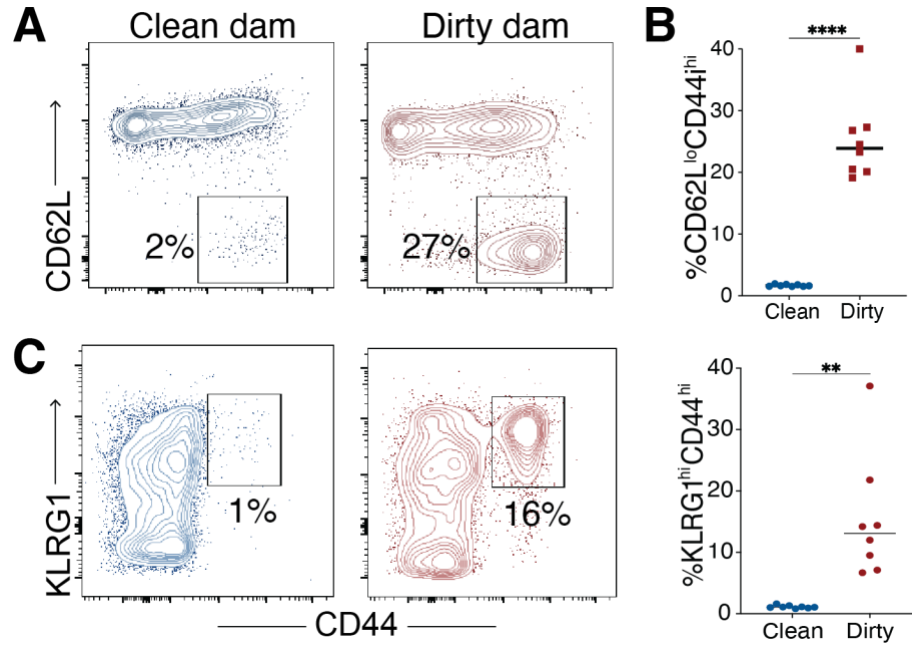


Fig 4. Dirty fetal-derived cells lose their naïve programming. (A) Schematic of sorting strategy. **(B)** Principle component analysis of genome-wide sequencing from RNA- and **(C)** ATAC-sequencing data sets. **(D)** Enrichment analysis for various CD8⁺ T cell states in genes with increased expression and/or **(E)** increased accessibility in clean and dirty cells. **(F)** Genome browser views of a dirty-upregulated gene and a dirty-poised gene. **(G)** Enrichment analysis for GO “biological process” terms in dirty-poised genes. Pathways are connected if they share 30% or more genes. Node sizes denote gene set

sizes. Statistical significance of differential expression and differential accessibility was determined by Wald test (D) and quasi-likelihood F test (E) with Benjamini-Hochberg correction ($p < 0.05$). Corrected p-values for enrichment or depletion denoted by dot size and color..Enrichment values in (D) and (F) were determined by hypergeometric test followed by Benjamini-Hochberg correction. ($p < 0.05$).

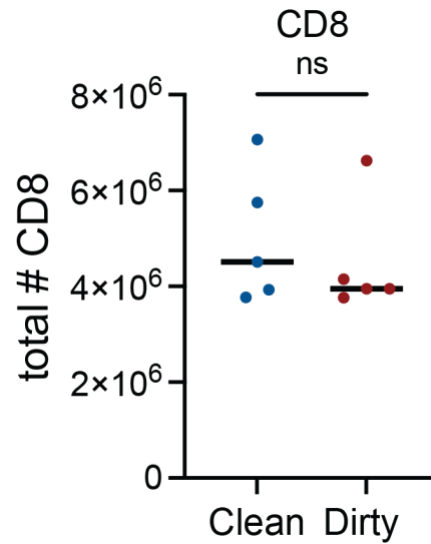
Supplemental Material



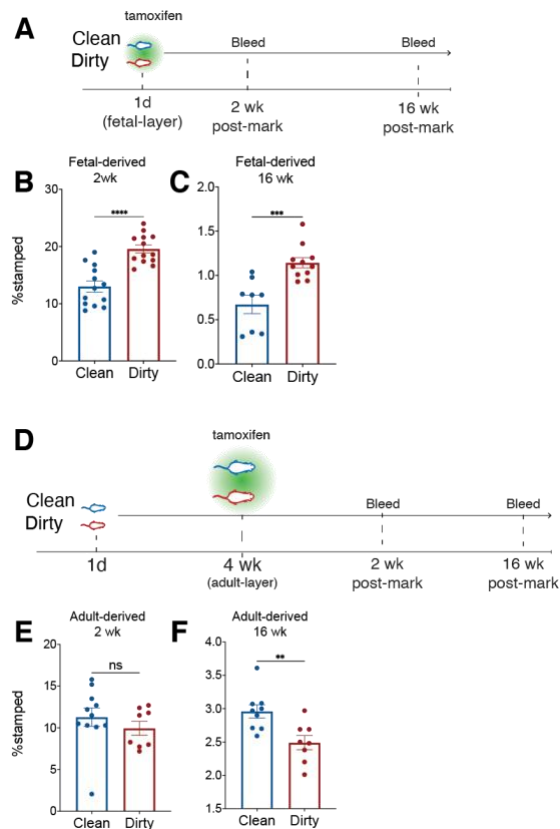
Supplemental Fig 1. Evidence that the CD8⁺ T cell compartment in dams is altered by exposure to pet-shop material. (A) Representative flow plots showing the CD44^{hi}CD62L^{lo} population in clean and dirty dams prior to mating. **(B)** Proportion of CD44^{hi}CD62L^{lo} CD8⁺ T cells. **(C)** Representative flow plots showing the CD44^{hi}KLRG1^{hi} population in clean and dirty dams prior to mating **(D)** Proportion of CD44^{hi}KLRG1^{hi} CD8⁺ T cells forward scatter **(D)** KLRG1 and **(E)** CD62L. Data is pooled from 2 independent experiments (n=4/group). Data for B-D are ±SEM. Statistical significance was determined by student's t-test (**** P<0.00005)

		Clean	Dirty	Pet shop
Bacteria	C. Jejuni	-	-	+
	Campylobacter	-	+	+
	Cryptosporidium	-	+	+
	H. bilis	-	-	+
	H. ganmani	-	+	+
	H. mastomyrinus	-	+	+
	H. typhlonius	-	+	+
	Helicobacter genus	-	+	+
	M. pulmonis	-	+	+
	R. heyliei	-	+	+
	R. pneumotropicus	-	+	+
	S. aureus	-	+	+
	Ectromelia	-	-	-
	Campylobacter Genus	-	+	+
	C. bovis	-	-	-
	C. kutscheri	-	-	-
	C. rodentium	-	-	-
	C. piliforme	-	-	-
	K. oxytoca	-	-	-
	K. pneumoniae	-	-	-
	Helicobacter	-	+	+
	M. pulmonis	-	-	+
	R. heyliei	-	+	+
	R. pneumotropicus	-	+	+
	Ps. aeruginosa	-	-	-
	Salmonella Genus	-	-	-
	S. aureus	+	+	-
	S. moniliformis	-	-	-
	S. pneumoniae	-	-	-
	Cryptosporidium	-	+	+
	Demodex	-	-	-
	Entamoeba	-	+	+
	P. mirabilis	+	+	+
Virus	MAV 1	-	+	+
	MAV 2	-	+	+
	MHV	-	+	+
	Mouse Norovirus	-	+	+
	Mouse Parvovirus	-	+	+
	Theiler's murine encephalomyelitis virus	-	+	+
Pinworms	LCMV	-	-	-
	Aspiculuris tetraptera	-	+	+
	Syphacia obvelata	-	-	+
Parasites	Entamoeba	-	-	-
	Giardia	-	-	-

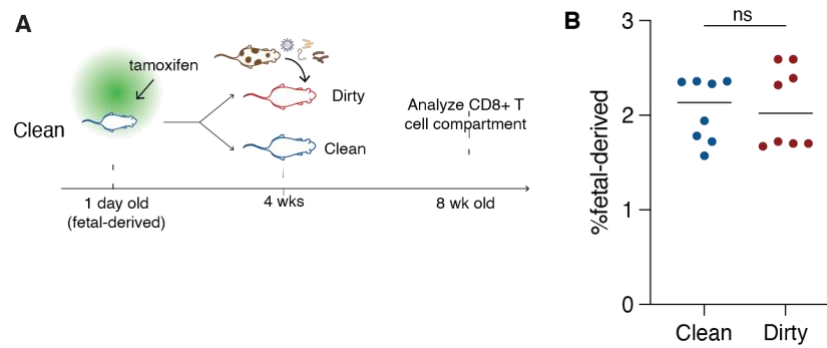
Supplemental Table 1. Microbial contents of clean and dirty dams, and pet shop mice. Bacterial, viral, pinworm, and parasitic load of clean and dirty and pet-shop mice.



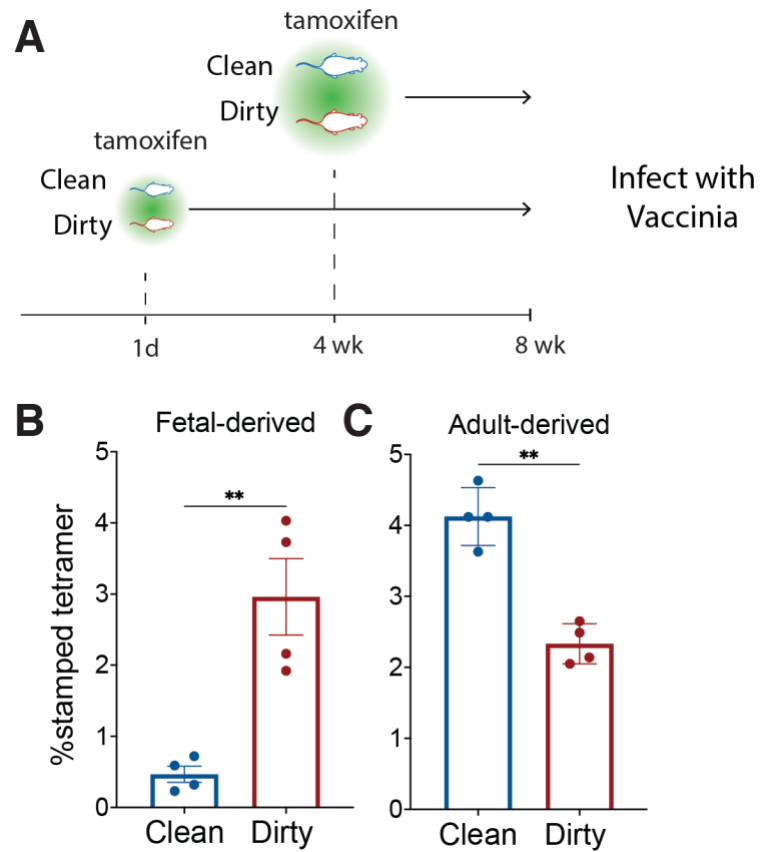
Supplemental Fig 2. Total number of CD8+ T cells in clean and dirty mice. Shown are the total numbers of CD8+ T cells in the spleens of mice at 8 wks of age. Data is representative of 2 independent experiments (n=4-5 mice/group). Statistical significance was determined by student's t-test (ns not significant).



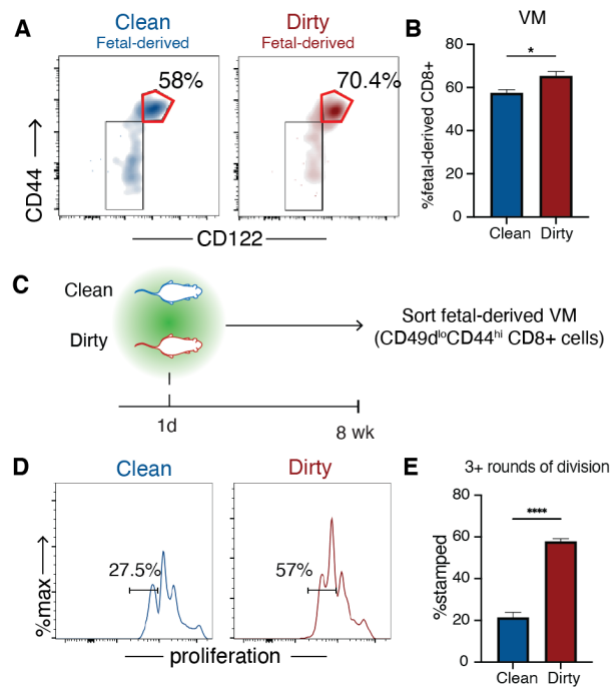
Supplemental Fig 3. Early microbial exposure permanently alters the developmental layering of the CD8+ T cell compartment. (A) Approach to mark and track fetal-derived cells. Proportion of stamped fetal-derived cells at **(B)** 2 wk and **(C)** 16 wk post-tamoxifen administration. **(D)** Approach to mark and track adult-derived cells. **(E)** Proportion of stamped fetal-derived cells at 2 wk and **(F)** 16 wk post-tamoxifen administration. Data are pooled from two independent experiments (n=4-5 mice/group). Statistical significance was determined by a student's *t*-test (ns not significant, ** $p < 0.005$, *** $p < 0.0005$, **** $p < 0.00005$)



Supplemental Fig 4. Expansion of fetal-layer is dependent on the timing of microbial exposure. (A) Experimental schematic showing timestamped mice exposed to a dirty environment during adulthood. **(B)** Proportion of fetal-derived CD8+ T cells present in adulthood. Data is pooled from two independent experiments (n=4 mice/group). Statistical significance was determined by a student's *t*-test (ns, not significant).



Sup Fig 5. Alterations in antigen-specific responses of different layers are not pathogen dependent. (A) Approach to infect timestamp mice with Vaccinia virus. **(B)** The proportion of antigen-specific fetal- and **(C)** adult-derived cells at 5 dpi. Data for B, C are \pm SEM. Statistical significance was determined by a student's *t*-test (** $p < 0.005$).



Supplemental Fig 6 Dirty fetal-derived cells are inherently more reactive (A)

Representative flow plots of VM population in fetal-derived clean or dirty CD8+ T cells

(B) Proportion of clean or dirty fetal-derived cells with a memory phenotype **(C)**

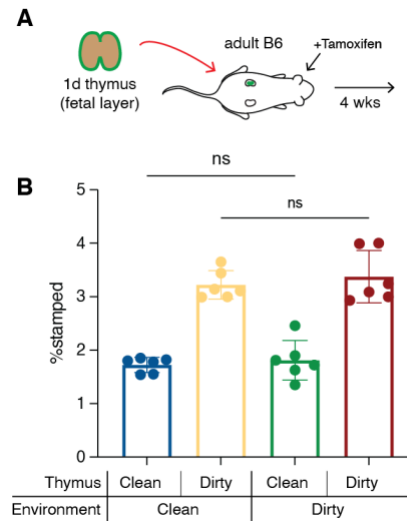
Experimental approach to sort out antigen-inexperienced VM population **(D)**

Representative graphs showing the proportion of cells in the last two rounds of division

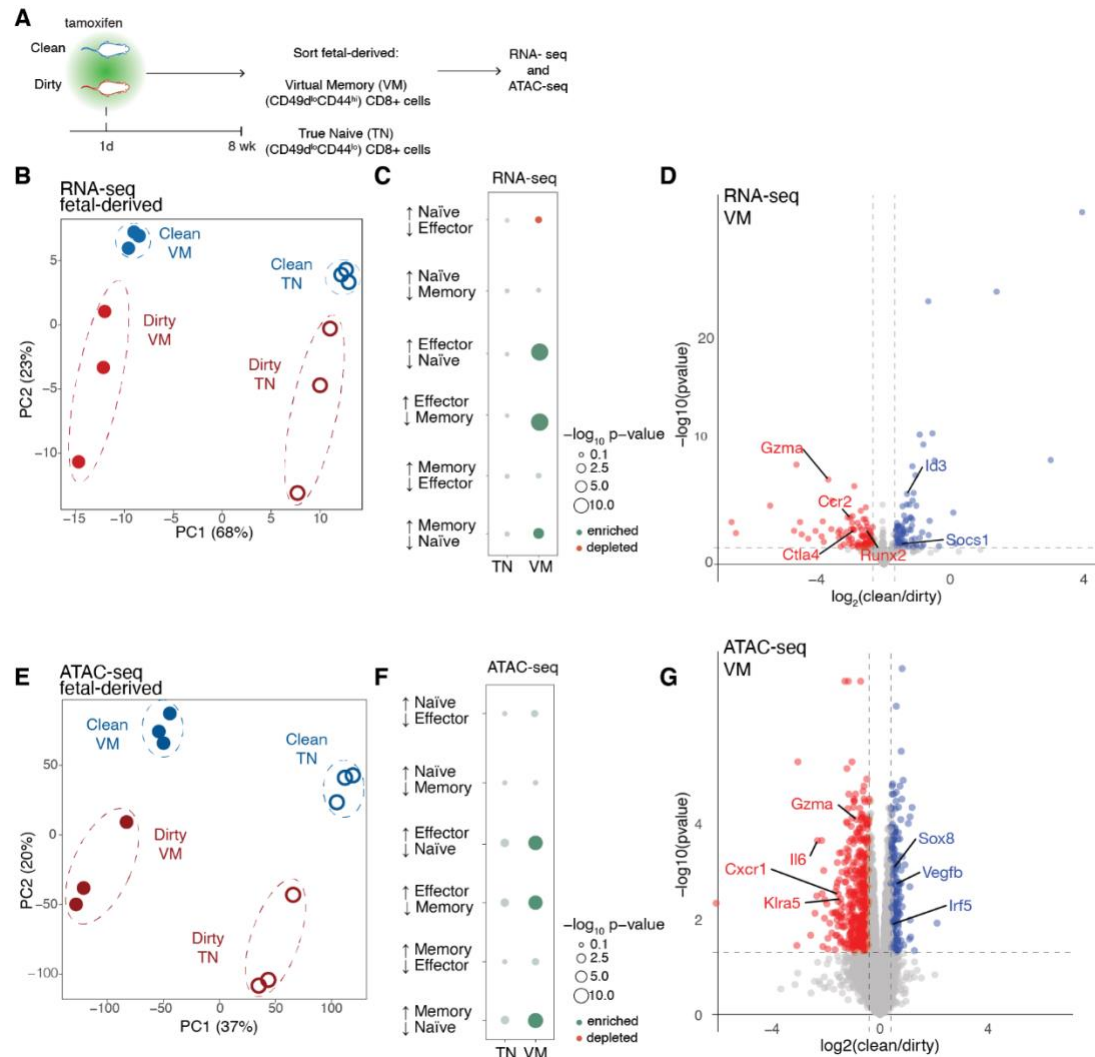
(E) Proportion of cells that underwent 3+ rounds of division. Data for B, E are mean \pm

SEM and are pooled from 2 independent experiments (n=4-6 mice/group). Statistical

significance was determined by a student's t-test (* P<0.05, ** P<0.005)

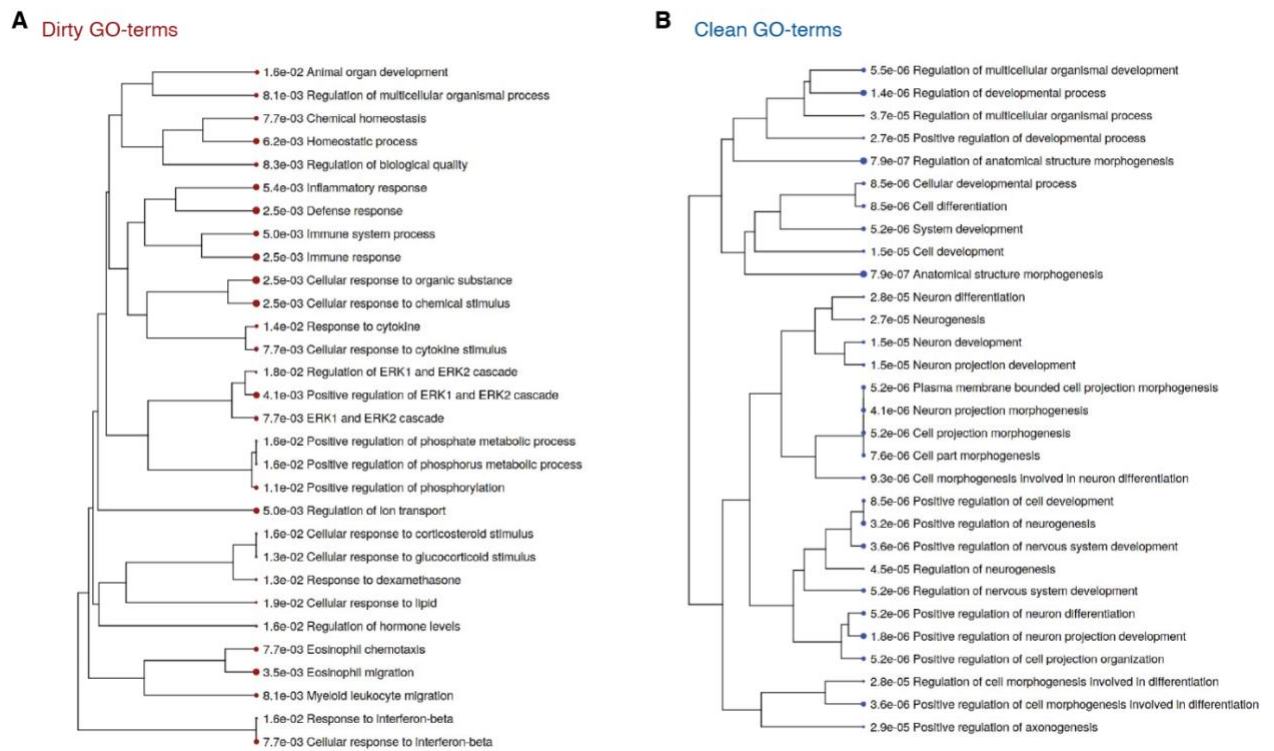


Supplemental Fig. 7. Fetal-derived CD8s from dirty thymus preferentially expand in the periphery. (A) Experimental approach to transplant a fetal thymus under the kidney capsule of an adult recipient. **(B)** Proportion of fetal-derived CD8⁺ T cells 4 weeks post-transplantation. Data is representative of two independent experiments (n=6 mice/group. are mean \pm SEM and are pooled from 2 independent experiments (n=4-6 mice/group). Statistical significance was determined by a one-way ANOVA with a Tukey multiple comparison test (ns not significant)



Supplemental Fig 8. Cell-intrinsic factors drive enhanced reactivity in fetal-layer. (A)

Schematic of sorting strategy for clean or dirty fetal-derived VM or TN cells **(B)** Principle component analysis, **(C)** Enrichment analysis and **(D)** Volcano plots showing differentially expressed genes using RNA-seq in clean or dirty fetal-derived with a VM phenotype **(E)** Principle component analysis, **(F)** Enrichment analysis and **(G)** Volcano plots showing differentially accessible genes using ATAC-seq from clean or dirty fetal-derived CD8⁺ T cells with a VM phenotype.



Supplemental fig 9. Dirty fetal-derived cells are poised towards effector-like

behavior (A) Dendrograms showing enriched GO-terms for poised genes in dirty and **(B)** clean fetal-derived cells.

Methods

Mice and microbial exposure

C57BL/6 mice were purchased from Charles River Laboratories. ZsGreen and TCR δ Cre-ERT2 mice were obtained from Jackson Laboratories. Pet shop mice were purchased from pet shops. All experiments utilized timed matings to ensure mice were of similar ages. Unless otherwise stated, mice were used at 8 weeks of age. Generation of 'dirty' mice occurred at an off-campus mouse facility. Microbial material from pet shop mice was transferred to female laboratory mice through an oral gavage from fecal material and bedding transfer for four weeks. All mice that survived the four-week microbial exposure period were used for timed matings. Clean mice were maintained under specific pathogen-free conditions at the College of Veterinary Medicine and mice exposed to pet-shop material were maintained in a separate facility. The facilities are accredited by the American Association of Accreditation of Laboratory Animal Care. All protocols regarding animal use were reviewed and approved by the Institutional Animal Care and Use Committee at Cornell University.

Timestamping method

Timestamp mice were generated by crossing a TCR δ Cre-ERT2 mouse with a ZsGreen reporter mouse. To activate fluorescent reporter expression in fetal-derived T cells, 2.5 mg of tamoxifen was administered to dams by oral gavage three times in 12-hour intervals over a 24-hour period and 0-1d old pups received tamoxifen through lactation^{12,13}. To activate fluorescent reporter expression in adult-derived T cells, 2.5 mg was administered to mice by oral gavage in 24-hour intervals for two days.

Thymic transplants

Thymic transplants were performed as previously described ¹⁶. Briefly, thymus lobes were isolated from 0-1d old timestamp mice. The thymus lobes were separated into individual lobes and were placed under the kidney capsule of a 6-week-old mouse. To mark thymocytes from the donor thymus lobe, 5 mg of tamoxifen was administered through oral gavage from 0-3d post-transplantation.

Manipulating developmental layers

To prevent the formation of the adult layer, 2.5-3 wk old mice were anesthetized using 1.2 mg of avertin and thymus lobes were removed through aspiration. A sham thymectomy was a complete surgery without thymic removal. At 8 wks of age, mice were visually inspected for the presence of thymic lobes and mice that had complete removal of both thymic lobes were included in experiments.

To 'delete' the fetal layer, 2 wk old mice received 0.1 mg of a CD8a mAb (BioX Cell) intraperitoneally every 24 hrs for two days.

Adoptive transfer and pathogen burden

CD8+ T cells from the spleens were purified using positive selection with CD8a microbeads (Miltenyi). 5×10^6 cells were injected (i.v.) into a TCR α KO recipient. The next day, mice were infected with 1×10^4 CFU of wild-type (WT) *Listeria monocytogenes* expressing the gB-8p peptide (Lm-gB) as previously described ¹⁷. At 3 days post-infection, lysed tissue homogenate from the spleen were serially plated and colony growth was enumerated.

***In vitro* stimulation**

CD8⁺ T cells were isolated from the spleen using positive magnetic selection with CD8 microbeads (Miltenyi). If fluorescence activated cell sorting was required, cells were stained with antibodies against CD4 (Gk1.5), CD44 (IM7), CD122 (TM-B1), CD49d (R1-2), and CD8a (53-6.7). VM cells were sorted for CD4⁻CD8⁺CD49d^{lo}CD44^{hi}. All samples were sorted to >95% purity as determined by a post-purity sort check. Following the cell sort or magnetic bead purification, cells were labeled with Cell Trace Violet. Cells were stimulated with plate-bound anti-CD3 (2C11) then cultured with complete RPMI supplemented with 100 U/ml human IL2 and 4 ugs CD28/ml (37.51). Cells were harvested at the time points indicated in the figure legends and stained for surface markers then analyzed for flow cytometry.

Flow cytometry

For flow cytometry, cells were stained with antibodies against CD4 (Gk1.5), CD44 (IM7), CD122 (TM-B1), CD49d (R1-2), CD62L (ML-14), CD8a (53-6.7), TCR g/d (GL3). All antibodies were purchased from eBioscience, BioLegend, Invitrogen, or BD Biosciences. Concentrations were used as recommended by the manufacturer. Flow cytometric data were acquired using FACSDiva software from a BD FACSsymphony equipped with five lasers (BD Biosciences). Analysis was performed with FlowJo (Tree Star, Ashland, OR).

RNA preparation and sequencing

Splenic CD8⁺ T cells were isolated and purified using positive selection with CD8a microbeads (Miltenyi). Purified CD8⁺ T cells were stained using antibodies against CD4 (Gk1.5), CD44 (IM7), CD122 (TM-B1), CD49d (R1-2), and CD8a (53-6.7). 'Bulk stamped'

CD8⁺ populations were sorted for CD4⁻CD8⁺CD49d^{lo}GFP⁺, ‘stamped VMs’ populations were sorted for CD4⁻CD8⁺CD49d^{lo}GFP⁺CD44^{hi}, and ‘stamped TN’ populations were sorted for CD4⁻CD8⁺CD49d^{lo}GFP⁺CD44^{lo}. Samples were sorted using a BD FACS Aria Fusion II equipped with four lasers. Samples were sorted to >95% cell purity as determined by a post-sort check. 10,000 FACS sorted cells were lysed in 1 mL Trizol according to the manufacturer’s instructions (Thermo Fisher) with the addition of a second chloroform extraction and Glycobule carrier (Thermo Fisher) before precipitation (1 hr at 4°C) and a second wash of the pelleted RNA in 70% ethanol. RNA integrity was confirmed with a Femto Pulse Fragment Analyzer (Agilent). RNAseq libraries were prepared by the Transcriptional Regulation and Expression (TReX) Facility using the NEBNext Ultra II RNA Library Prep Kit (New England Biolabs). All RNA-seq libraries were sequenced with 2x150 paired-end reads on a HiSeqX (Illumina) at Novogene (Sacramento, CA).

ATAC-seq library preparation and sequencing

Splenic CD8⁺ T cells were isolated and purified using positive selection with CD8a microbeads (Miltenyi). Purified CD8⁺ T cells were stained using antibodies against CD4 (Gk1.5), CD44 (IM7), CD122 (TM-B1), CD49d (R1-2), and CD8a (53-6.7). ‘Bulk stamped’ CD8⁺ populations were sorted for CD4⁻CD8⁺CD49d^{lo}GFP⁺, ‘stamped CD44^{hi}’ populations were sorted for CD4⁻CD8⁺CD49d^{lo}GFP⁺CD44^{hi}, and ‘stamped CD44^{lo}’ populations were sorted for CD4⁻CD8⁺CD49d^{lo}GFP⁺CD44^{lo}. Samples were sorted using a BD FACS Aria Fusion II equipped with four lasers. Samples were sorted using a BD FACS Aria Fusion II equipped with four lasers. Samples were sorted to >95% cell purity

as determined by a post-sort check. FACS sorted cells were permeabilized as previously described¹¹. The permeabilized cells were counted, snap-frozen in liquid nitrogen, and stored at -80°C. ATAC-seq libraries were prepared by the TReX Facility from 25,000 permeabilized using half-reaction volumes for the transposition reaction as previously described^{18,19}. Briefly, frozen permeabilized cells were thawed, washed twice in 1 mL cold ATAC-RSB buffer containing 0.1% Tween20, resuspended in 25 µL Omni-ATAC Transposition Mix (10 mM Tris pH 8, 5 mM MgCl₂, 10% DMF, 0.1% Tween20, 0.01% digitonin, 1.25 µL TDE1 enzyme (Illumina), and 8.25 µL PBS in final 25 µL volume per sample), incubated for 30 minutes at 37°C with shaking (900 rpm), with immediate addition of Zymo binding buffer. After Zymo column cleanup, libraries were amplified using Nextera PCR oligos (Nextera Index Kit, Illumina) and 2x HiFi Master Mix (New England Biolabs) with an initial incubation at 72°C for 5 minutes to extend the segmented ends and 11 PCR amplification cycles. The libraries were cleaned up twice (in series) with a 2:1 ratio of SPRIselect beads (Beckman Coulter). ATACseq libraries were sequenced with 2x150 paired-end reads on a HiSeqX (Illumina) at Novogene (Sacramento, CA).

RNA-seq analysis

Raw reads were evaluated, trimmed, and filtered using trim galore (v0.6.5; -j 8 — quality 20 -gzip —length 50 —paired —fastqc) and aligned to the mouse reference genome (mm10) using STAR (v2.7.0e) with default parameters^{24, 25}. Quality control metrics were calculated using multiqc (v1.8)²⁶. Mapped reads were assigned to genes using featureCounts under Rsubread (v2.4.3; minMQS=30,

countMultiMappingReads=FALSE, isPairedEnd=TRUE)²⁷. DESeq2 (v1.30.1) was then used to perform differential expression analysis and statistical testing of expressed genes (more than 5 overlapping reads in at least 2 samples)²⁸. For gene set enrichment analysis, genes were first ranked by $\log_2(\text{clean/dirty})$. Gene sets comparing naïve, effector, and memory CD8⁺ T cells²⁹ were downloaded from the molecular signature database (MSigDB), then fgsea (v1.16.0)³⁰ was used with default parameters except $\text{eps}=0$ to calculate normalized enrichment scores and p-values, followed by Benjamini-Hochberg correction.

ATAC-seq analysis

Raw reads were evaluated, trimmed, and filtered using trim galore (v0.6.5; -j 8 — quality 20 -gzip —length 50 —paired —fastqc) and aligned to the mouse reference genome (mm10) using bwa mem (-M -R)³¹, then processed using samtools view (v1.9; -b -h -F 0x0100). The reads were further sorted and indexed using samtools sort and samtools index³². Mitochondrial reads were filtered out using a custom shell script, and then picard MarkDuplicates was used to deduplicate the reads. Peaks were called using MACS3 (v3.0.0a6; callpeak -f BAMPE -g mm -B -q 0.01)³³, then filtered through Irreproducibility Discovery Rate (IDR) analysis, as previously described by the ENCODE Consortium³⁴. A unified peak list was generated by merging reproducible peaks from each cell type using bedtools (v2.29.2)³⁵, resulting in a total of 30,830 peaks. Peaks that matched blacklist of artifactual regions in mm10 were filtered out. The peaks were associated with their nearest genes based on the shortest distance between the peak and the gene's promoter, as annotated by GENCODE (release M27, mapped back from

mm39 to mm10) and defined as 1kb upstream and 500 bp downstream of the annotation transcription start site. featureCounts under Rsubread (v2.4.3) was used to calculate raw read counts per peak, which were then aggregated per gene for differential analysis. Raw reads were normalized to counts per million (CPM), then the EdgeR (v3.32.1) framework was used to determine differentially accessible genes across all genes accessible ($\text{cpm} \geq 0.5$) in at least one sample³⁶. Gene set enrichment analysis was performed as described above.

Poised Gene Analysis

Poised genes were defined as genes that have at least one associated peak with significantly ($q\text{-value} < 0.05$) higher accessibility in one sample compared to another per ATAC-seq signal, but do not have significant upregulation in transcripts per RNA-seq. Gene ontology on these genes were performed on ShinyGO (v0.66) with all annotated genes as background³⁷. After Benjamini-Hochberg correction, the significant terms ($q\text{-value} < 0.05$) were visualized using Cytoscape.

Track Visualization and Normalization

Replicates were combined using samtools merge (v1.9), indexed, then normalized to CPM using bamCoverage from deep tools (v3.5) to generate 125igwig files used for visualization. Tracks were visualized with igv (v2.9.1).

Infectious agent screening

‘Clean’, ‘dirty’ mice (before mating), and pet shop mice were screened for microbial load. Blood, feces, oral, and body swabs were collected per the instructions of Charles River Laboratories.

Statistical analysis

Error bars are represented by SEM or SD. Unless otherwise stated in the figure legends, statistical significance was determined by a student's t-test or by a 1- or 2-way ANOVA followed by an appropriate post-test as indicated in the figure legends. Significance is denoted by the following: * $p < 0.05$, ** $p < 0.05$, *** $p < 0.0005$, **** $P < 0.00005$

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CHAPTER 5: CONCLUSIONS

Summarized Findings

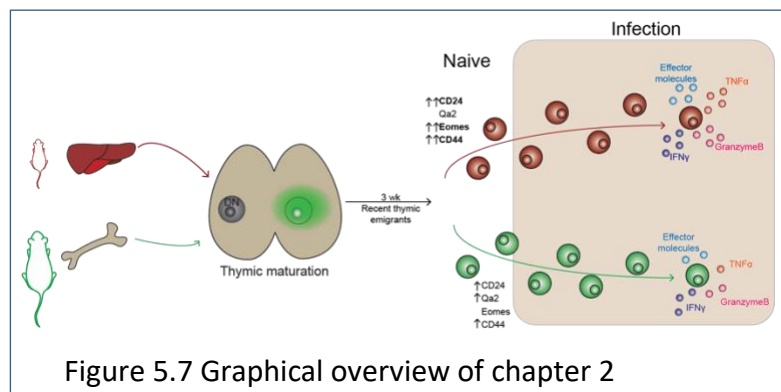
Neonates experience high mortality from recurrent intracellular infections ¹⁻⁴. The prevailing hypothesis is the immune system of neonates is immature or represents a deviant form of immunity when compared to their adult counterparts. However, accumulating evidence asserts neonatal, or fetal-derived, T cells are a distinct population of cells with unique programs and contributions to immunity. Fetal-derived CD8+ T cells respond rapidly to infection but become short-lived effector cells limiting their ability to transition into the memory pool ^{5,6}. Furthermore, fetal-derived cells persist into adulthood and retain their cell-intrinsic properties ^{7,8}. When I started my doctoral research, we did not know the mechanisms that promoted the neonatal program of immunity or if the developmental architecture of the adult CD8+ T cell compartment could be altered. To fill these gaps, I used a combination of reductionist *in vivo* experiments and fate-mapping approaches. By doing so, I made three important discoveries that assert neonatal CD8+ T cells are derived from a unique population of progenitors that contain different protective capacities which exist even before activation.

Chapter 2 summary

Insights into RTE biology have been associated with the population dynamics of the neonatal T cell compartment ^{9,10}. Because of this assumption, the common hypothesis in the CD8+ T cell field has been immunity in neonates is immature because

their CD8+ T cells are younger and have undergone less post-thymic maturation. Our lab has shown the developmental origin of a CD8+ T cell regulates its naïve phenotype and response to infection ⁸. Our past studies were purposefully designed to understand the behaviors of mature naïve T cells and their behaviors after activation. In the second chapter, I asked if the population dynamics of neonatal and adult RTEs were similar despite arising from different progenitor populations. I used the ‘timestamp’ fate-mapping mouse model to control for the time in which different aged cells occupy the periphery. I marked and tracked a discrete population of CD8+ T cells from the fetal and adult layers. I discovered neonatal RTEs do not express the canonical RTE markers at similar levels when compared to adult RTEs. This shows, at a superficial level, different aged RTEs cannot be classified as equivalent populations.

The neonatal CD8+ T cell compartment is largely void of T cells. When CD8+ T cells enter a lymphopenic



environment, they undergo rapid proliferation and upregulate markers associated with extensive differentiation. Thus, alterations in the phenotype observed in neonatal RTEs may be a consequence of the peripheral environment. When I controlled for the extra-thymic environment RTEs were exported into, I found neonatal RTEs continued to express the unique phenotype profile observed in an unmanipulated neonate.

Furthermore, I found antigen specific neonatal RTEs were equally, if not more, functional in response to activation when compared to the bulk T cell population. This observation is inconsistent with past studies that define RTEs as ‘hypo-responsive’ to activation when compared to the mature, naïve T cell population ^{11–14}. Those data were collected using RTEs from adult animals, my data challenges the notion that insights gained from adult CD8+ RTE biology can be translated to our understanding of neonatal immunity. Furthermore, my findings clearly show population dynamics of neonatal CD8+ T cells is not a byproduct of looking at cells newly released into the periphery. Rather, my findings assert fetal-liver HSCs give rise to a unique population of CD8+ T cells with distinct behavioral profiles. Findings from this chapter will aid in making informed decisions for therapeutics for neonates and create approaches to enhance cell-mediated responses in lymphopenic adults.

Chapter 3 summary

Interest in cellular metabolism has risen within the immunology community because findings from basic research can readily translate to pharmaceuticals or precision medicine. In the third chapter, I asked if metabolic programming promotes age-related differences in the CD8+ T cell response. Through a series of adoptive co-transfer experiments and Seahorse assays, I found that upon stimulation, neonatal CD8+ T cells rapidly acquire an active metabolic program by preferentially utilizing glycolysis over oxidative phosphorylation. The neonatal metabolic program and response to infection are driven by elevated expression of the RNA binding protein, Lin28b. While Lin28b’s role in regulating developmental processes and immunity is not

novel, my work was the first to directly associate Lin28b with eliciting enhanced catabolic programs in activated T cells^{15–19}.

By understanding the different molecular and metabolic pathways used by neonates, we can identify potential drug targets while developing immunotherapies

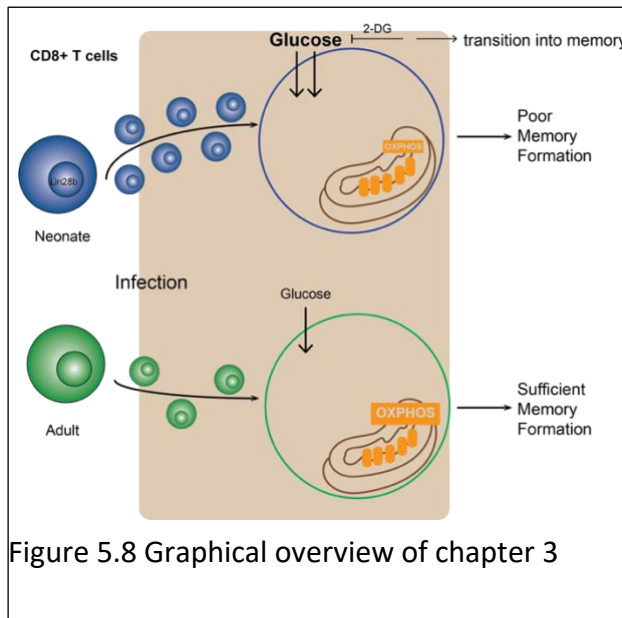


Figure 5.8 Graphical overview of chapter 3

or vaccinations. Indeed, blocking entry into glycolysis through 2-DG treatment increased the proportion of neonatal cells that survived the primary infection and transitioned into the memory stage. Importantly, 2-DG treated neonatal cells responded to secondary infection in

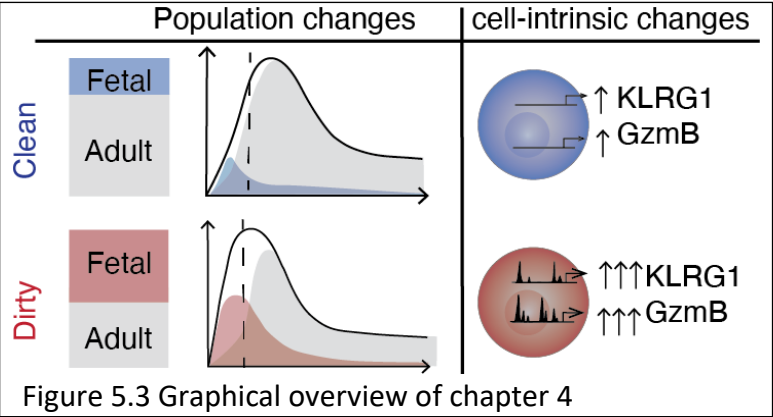
equal proportions when compared to the adult response during the secondary challenge. In the third chapter, I showed the failure of neonatal CD8+ T cells to form memory is driven by an overwhelming bias of catabolic metabolic programs utilized during infection and neonatal memory formation can be pharmacologically manipulated. Findings from this chapter will inform the development of future vaccination strategies.

Chapter 4

An interesting finding from chapter 3 is that neonatal CD8+ T cells retain their propensity to rapidly respond to infection independent of the peripheral environment as seen in our adoptive co-transfer experiments. The thymic transplantation

experiments in chapter 2 show neonatal CD8+ T cells retain their program of enhanced reactivity when exported into an adult periphery. This suggests alterations in T cell programming must occur prior to thymic egress. This led me to ask if the developmental architecture of the CD8+ T cell compartment can be altered or if there was a way to modify the functions of fetal-derived CD8+ T cells.

To address this question, I created a novel approach to expose laboratory mice to a diverse microbial environment for the entirety of early development. Epidemiological studies show early microbial experiences have a lasting and profound impact on an individual’s health. Yet, most iterations of the ‘dirty’ mouse model ignore early life experiences ^{20–23}. This chapter built off work from the lab showing fetal and adult CD8+ T cells co-exist in adulthood and fetal-derived CD8+ T cells retain their ability to rapidly respond to infection. I found antigen-inexperienced fetal-derived cells integrated into the adult CD8+ T cell compartment at higher proportions when developed in a diverse microbial environment. I found exposure to a microbially diverse environment for the entirety of early development enhanced the protective capabilities of fetal-derived cells through epigenetic modifications. This suggests there



is a critical window that exists prior to thymic development in which CD8+ T cells can be ‘reprogrammed’.

A question that our lab often gets asked is ‘what is the contribution of the fetal-derived layer during infection?’ In addition to showing microbial exposure during early development permanently reshapes the developmental architecture of the T cell compartment, work in chapter 4 defined the contributions of each layer during acute infection by using reductionist approaches. By preventing the adult layer from forming in both clean and dirty mice, I found the fetal layer provides superior protection against intracellular infection when compared to the adult layer. Together, this further supports our hypothesis that fetal-derived CD8⁺ T cells act as the early effector cells by providing robust protection against infection.

The mechanisms that alter immunity in adults long after childhood exposures were previously unknown. Findings from this chapter provide a conceptual framework to explain why early life experiences profoundly impact immunity long after the stressor or exposure has passed by showing the microbial environment alters the developmental architecture of the T cell compartment. Ultimately, this chapter provides insight into how an individual’s susceptibility to disease can be predicted based on their fetal-to-adult CD8⁺ T cell ratio.

Future Directions

Work from my doctoral research identified mechanisms that promote the neonatal program of immunity and confirmed distinct progenitor populations give rise to neonatal and adult CD8⁺ T cells with unique protective capacities. Using knowledge gained from my doctoral research, it would be intriguing to (i) understand if the

localization of neonatal CD8+ cells contributed to the quick effector profile observed in response to infection, (ii) identify a ‘critical window’ in which CD8+ T cells can alter their programming, and (iii) understand the translational implications that variations in the fetal-to-adult CD8+ T cell ratio may have on the efficacy of therapies.

Localization of neonatal cells

Our data showing neonatal CD8+ T cells rapidly respond to infection comes from flow cytometric analysis using homogenized tissue. From flow cytometric analysis, we cannot connect whether the rapid activation profiles of neonatal CD8+ T cells is coincident with their proximity to antigen, survival molecules, or co-stimulatory signals. T cells home to areas by sensing their environment through chemokine receptors and cytokine milieu ²⁴. It is becoming increasingly apparent that location is important in determining T cell activation ^{25,26}. Recent studies have suggested T cells at the periphery of the lymph node (LN) are likely to be fast-acting effector cells ²⁷. It is feasible to hypothesize fetal-derived cells display rapid responses to infection because they home to this location of the LN. Others have shown that CD8+ T memory cells use organ-specific cues to promote tissue-resident programs by shifting their metabolic programming ²⁸. A combination of published and unpublished data from our lab show that fetal-derived CD8+ T cells accumulate in higher proportions in peripheral organs such as the bone marrow and liver when compared to adult-derived CD8+ T cells ⁸. Perhaps the differential metabolic programs observed in chapter 2 arises from cues received from the microenvironments neonatal cells experience. Neonatal CD8+ T cells display a more differentiated phenotype compared to their adult counterparts.

This phenotype includes the upregulation of transcription factors associated with activation, such as t-bet. Interestingly, ectopic overexpression of t-bet led to cells preferentially migrating to the red pulp of the spleen, a site of high antigen density ²⁹. This suggests subsets of T cells localize in tissues based on their differentiation state. Thus, in support of these findings, it is reasonable to hypothesize because naïve neonatal CD8⁺ T cells display a differentiated phenotype, they likely occupy a unique niche within secondary lymphoid organs (SLOs) or peripheral organs. Understanding if differently aged cells occupy distinct compartments in the naïve state would contribute to our understanding of how CD8⁺ T cell population dynamics and response times to infection are determined.

Further evidence that indicates neonatal CD8⁺ T cells likely occupy special locations in tissues comes from their high CD122 expression. Interestingly, CD122 is a component of the receptor for IL-15 (IL-15R α), a homeostatic cytokine ³⁰. Enhanced expression of the IL-15R α suggests neonatal cells are likely to encounter IL-15 at higher rates compared to their adult counterparts. In SLOs, IL-15 is produced by specialized immune cells such as dendritic cells (DCs) ³¹. Unlike other soluble cytokines that are secreted, IL-15 is unstable and needs to be bound to the IL-15R α ³². For a cell to consume IL-15, it needs to be trans-presented through cell-to-cell contact ^{33–35}. Therefore, an obvious criterion for enhanced consumption of IL-15 by neonatal T cells is the location of the T cell in relation to the cell producing IL-15.

Another question that arises is if neonatal CD8⁺ T cells preferentially occupy a region of the LN that has a higher concentration of IL-15 producing DCs, how does this

alter their activation? In addition to producing IL-15, DCs are sentinels of the immune system and serve as a bridge between the innate and adaptive immune systems. DCs provide strong co-stimulation signals (signal 2) and efficiently present a large amount of antigen (signal 1) ³⁶. If neonatal CD8+ T cells preferentially localize in areas enriched for IL-15 producing DCs, this would also put them in a favorable position to encounter antigen. If this statement is true, then close proximity to high levels of antigen exposure and increased co-stimulation provided by DCs could explain why neonatal CD8+ T cells are the 'first responders' and produce high levels of effector molecules. Uncovering unique localization or motility patterns of neonatal CD8+ T cells could provide insight into why neonatal and adult CD8+ T cells display different activation dynamics in response to infections.

Expansion of the fetal layer

Researchers have utilized diverse animal husbandry approaches to control for microbial exposures of mice. This includes germ-free, gnotobiotic, specific pathogen-free, and various types of 'wild' or 'dirty' mice. The biomedical value of wild and dirty mice is their enhanced ability to phenocopy human immunology, increasing the translational value of murine models ^{20,21,23,37,38}. In chapter 4, I asked how non-regulated exposure during early-life altered immunity in adulthood. A critical next step is to identify a list of pathogens that elicit increases in the fetal-to-adult CD8+ T cell ratio in mice. Other groups have established roles for pathogens influencing seemingly unrelated immune responses, but favorable immunity is dependent on the infection and cell type specific ²². Researchers have defined a group of pathogens that promote

anti-tumor activity in CD8+ T cells ³⁹. Others have found prenatal infection promoted tissue-specific immunity in an IL-6-dependent manner ⁴⁰. Recent reports show type I interferons enhance naïve T cell survival and enhance peptide interactions ⁴¹. Interestingly, the CD8+ T cells that are responsive to type I interferons display similar phenotypic properties as fetal-derived CD8+ T cells. As neonatal cells preferentially respond to inflammatory cytokines (Watson *et al.* Manuscript in prep), exposing embryos or young mice to cytokines, such as type I interferons, may promote the expansion of fetal-derived cells and permanently enhance their protective properties at later times of life.

In chapter 4, I discovered microbial exposure for the entirety of early development but not adulthood led to an expansion of the antigen-inexperienced fetal-derived T cell compartment. Both epidemiological and experimental data suggest a 'critical window of exposure' exists ⁴²⁻⁴⁹. During this period, it is thought that microbial exposure has its most intense impact on the immune system. Therefore, it is prudent to ask that if this critical window exists, when is it open? If the enhanced protective program arises from microbial exposure during HSC colonization of the liver, then our timeline of microbial exposure is appropriate ⁵⁰. If microbial exposure is necessary during the switch from a tolerant program to a protective program present at birth, we can perform c-sections and cross-fostering experiments to expose mice developed in a dirty environment (*in utero*) to a clean environment (pre-wean period) to see if the enhanced fetal-to-adult program arises. Altering microbial exposure by

targeting developmental stages will allow us to identify the presence of critical windows of exposure.

Consequences of developmental layering

In this dissertation, I focused on the contribution of CD8+ T cells against acute intracellular infections but activated CD8+ T cells can also recognize and kill transformed cells and promote or alter the formation of autoimmune disorders. Understanding the neonatal response to prolonged antigen exposure is an area of active research in our lab and the CD8+ T cell response during chronic infection phenocopies their behavior against cancer⁵¹⁻⁵³. Defining the contributions of different aged cells against cancer may explain variation in responses against immunotherapies.

Immunotherapies have been heralded as a breakthrough cancer therapy but unfortunately, it has had limited successes on patients. CD8+ T cell mediated immunotherapies have two major pitfalls, the first is the induction of cytokine storms and the second loss of functionality within the tumor, perhaps due to exhaustion and/or terminal differentiation⁵⁴⁻⁵⁶. We discovered neonatal CD8+ T cells produce high levels of cytokines and quickly become terminally differentiated. Chapter 4 showed life experiences of an individual alters the fetal-to-adult ratio present in adulthood which impacts the protective capacity of the CD8+ T cell compartment. It would be of particular interest to understand if the fetal-to-adult ratio of individuals undergoing treatment contributes to variation of success for T cell therapies. My three research chapters show neonatal CD8+ T cells produce high levels of inflammatory cytokines and quickly become terminally differentiated compared to their adult

counterparts. It is interesting to speculate that patients who do not experience cytokine storms and successfully go into remission T cell immunotherapies have a larger proportion of adult-derived CD8+ T cells. If this hypothesis is true, could we give a glycolysis inhibitor and alter the T cell landscape to act more like adult-derived cells⁵⁷? Indeed, inhibiting glycolysis in CD8+ T cells *ex vivo* resulted in enhanced anti-tumor immunity which lends support for my hypothesis¹¹.

Work from chapter 4 found an expansion of fetal-derived CD8+ T cells in the adult compartment. Past studies show neonatal/fetal-derived CD8+ T cells are more reactive to spMHC^{58–60}. This suggests an expansion of the fetal-derived CD8+ T cells should result in an increased prevalence of autoimmune disorders. Paradoxically, epidemiological evidence that serves as the foundational framework for the hygiene hypothesis indicates a decreased incidence rate of autoimmunity in children who grew up in proximity to microbially-rich environments. Therefore, it is interesting to speculate that the conceptual framework described in chapter 4 can be extended to the CD4+ T cell compartment. An important question to address is if the decreased prevalence of autoimmunity present in children raised in microbially diverse environments is a result of alterations in the developmental architecture of the CD4+ T cell compartment. Work from the Mathis group found CD4+ T cells made early in life represent a distinct population of Aire-dependent T regulatory (Tregs) cells that persist into adulthood⁶¹. Interestingly, depletion of Tregs made early in life increased development of autoimmunity. To understand if exposure to a dirty environment leads to an expansion of fetal-derived Tregs and if fetal-derived Tregs developed in a dirty

environment have enhanced suppressive capabilities, we can isolate clean or dirty fetal-derived CD4⁺ T cells that express high levels of CD25 and perform *in vitro* suppression assays. By doing so, we can determine if decreased development of autoimmune disorders in individuals raised in microbially rich environments is coincident with an expansion of fetal-derived Tregs and if their fetal-derived Tregs provide superior mechanisms to promote self-tolerance even in the presence of more fetal-derived CD8⁺ T cells with increased affinity for spMHC.

Concluding Remarks

Neonates experience increased morbidity and mortality from intracellular infections compared to other groups, which have been associated with aberrant T cell behavior. This dissertation addressed many important questions in CD8⁺ T cell biology. I showed the importance of acknowledging heterogeneity within the naïve T cell compartment particularly from a developmental perspective. I found that cell-intrinsic properties drive age-related differences in immunity. Furthermore, these differences can be exacerbated by the microbial environment that a cell is exposed to during early development. The general view of the CD8⁺ T cell compartment is that a homogenous population gives rise to a heterogeneous response to infection. This heterogeneous response is filled with countless subsets of effector cells that either die after the infection is cleared or transition into memory subsets that persist in various forms and locations. My work shows heterogeneity exists in the naïve compartment and is a product of the developmental origin of a cell. By targeting metabolic processes, small

molecules (such as Lin28), developmental origin, and understanding environmental experiences, we can fine-tune the generation of therapeutics to enhance memory formation in neonates or enhance effector responses in the elderly, lymphopenic adults, and those undergoing cancer immunotherapy. Ultimately, my dissertation has created conceptual frameworks to explain why differences in T cell responses exist at the population level. In doing so, it has opened the door to many other questions such as those posed in this final chapter. That is the beauty of science.

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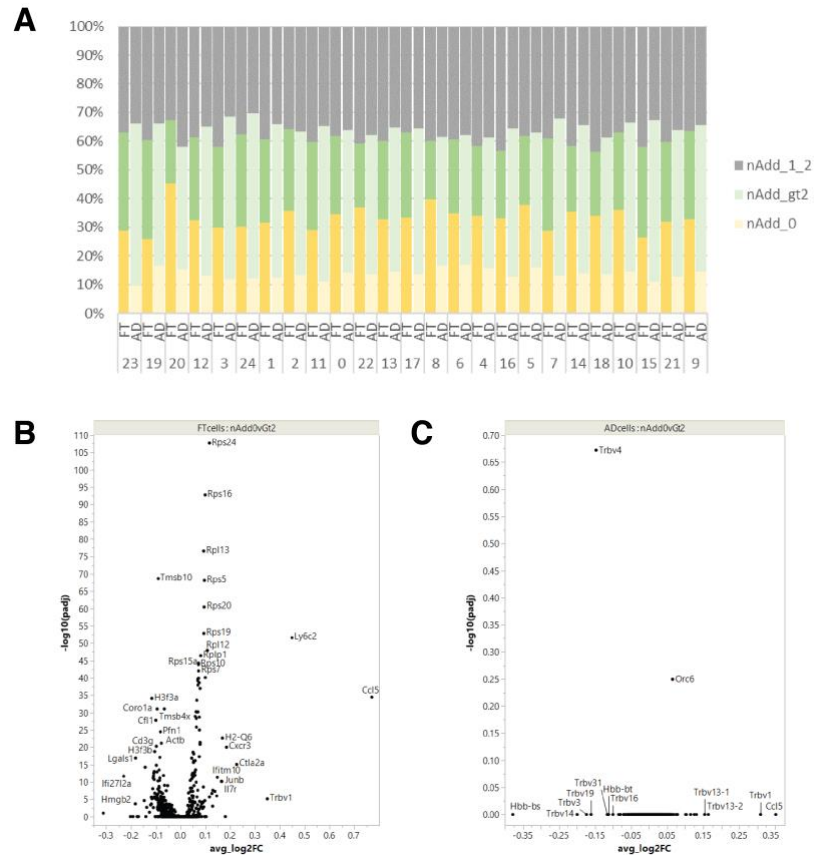
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APPENDIX

Appendix I: Germline-Encoded Fetal-Derived CD8+ T Cells Exhibit a Distinct Transcriptional Profile



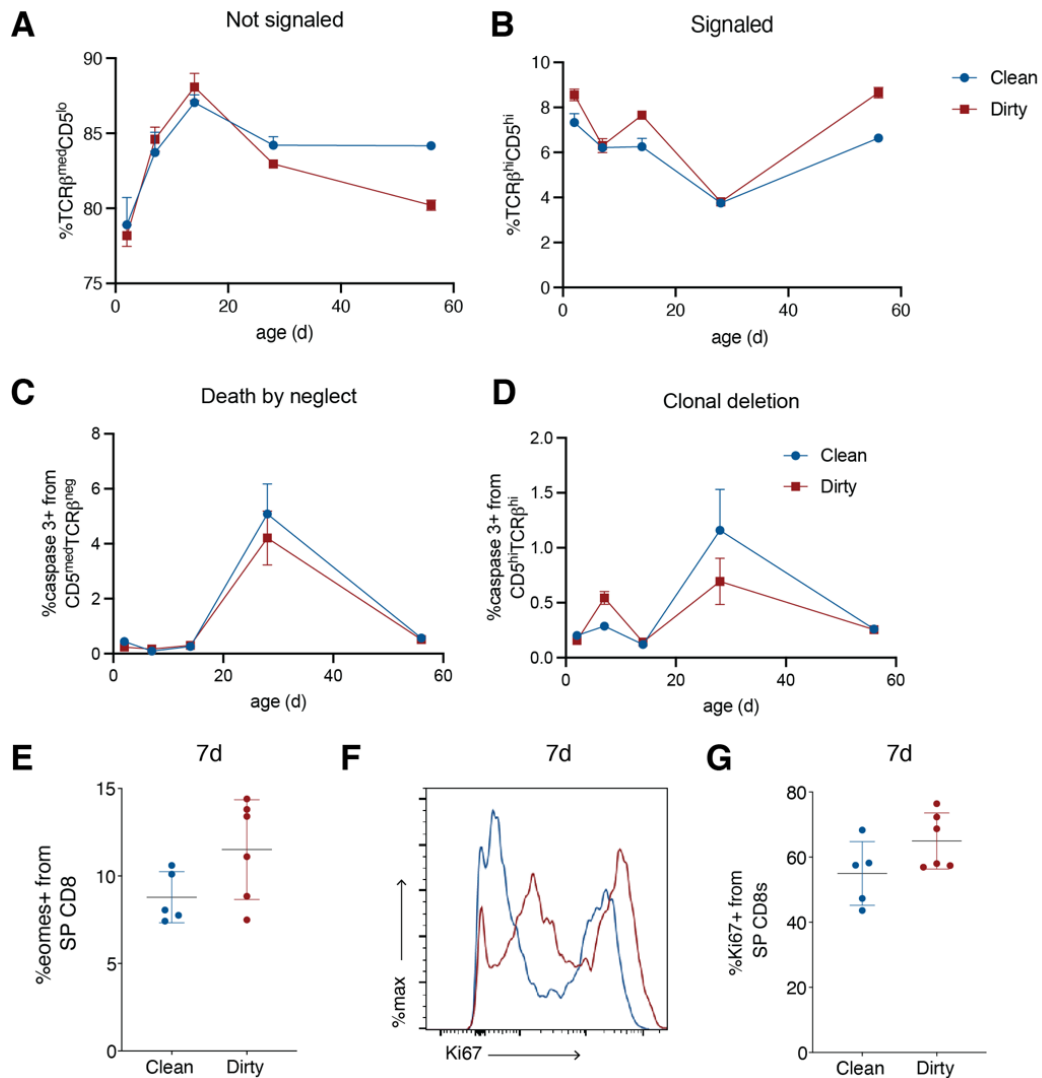
Appendix I: Germline-encoded fetal-derived CD8+ T cells exhibit a distinct transcriptional profile. (A) The proportion of fetal- or adult-derived RTEs in each cluster that have 0, 1-2, or >2 n-additions. **(B)** Volcano plot showing differentially expressed genes ($p < 0.05$) in fetal-derived and **(C)** adult-derived RTEs.

Summary

The T cell receptor (TCR) is the fundamental unit of a T cell and it dictates the specificity of a cell. Lymphocytes made in early life lack TdT expression, this leads to a less diverse TCR repertoire in early life which may result in limited cell-mediated immunity against a wide array of pathogens ^{1,2}. However, using TdT^{-/-} mice, Gavin and Bevan showed germline-encoded CD8⁺ T cells are promiscuous to multiple antigens and have increased affinity towards self-peptides ³. While these findings are often associated with neonatal CD8⁺ T cell biology, a caveat to Gavin and Bevan's work is their use of adult TdT^{-/-} mice. To my knowledge, there is no published work using neonatal TdT^{-/-} mice. Therefore, we still do not know how having a predominantly germline-encoded TCR repertoire alters the function and population dynamics of CD8⁺ T cells made early in life. To understand if TdT expression alters the behavioral program of CD8⁺ T cells in an age-dependent manner, we sorted out fetal- and adult-derived CD8⁺ T cells and used immunoprofiling technology from 10x Genomics to distinguish whether the distinct transcriptional program observed in fetal-derived CD8⁺ T cells can be attributed to a germline-encoded TCR repertoire. To address this question, we first asked whether fetal- and adult-derived CD8⁺ T cells had different compositions of germline vs non-germline encoded TCRs. In this appendix, I will focus on the RTE period (2 wk post-marking) in clean mice. Independent of clusters, fetal-derived CD8⁺ T cells always had an increased proportion of germline-encoded TCRs when compared to their adult-derived counterparts (Appendix I A). Next, we wanted to understand if germline-encoded TCRs expressed a unique transcriptional profile compared to cells

that had TCRs with more than 2 n-additions. Interestingly, only fetal-derived RTEs had differentially expressed (DE) genes in present in germline vs non-germline encoded TCRs ($p < 0.05$) while no DE genes were detected in adult-derived RTEs that were germline or non-germline encoded. This shows the number of n-additions is unimportant in adult-derived CD8⁺ T cells while it alters the transcriptional profile of fetal-derived cells (Appendix I B, C). In consideration of the striking differences described in chapter 4, it would be interesting to understand if alterations in the molecular programming and protective program observed between clean and dirty fetal-derived CD8⁺ T cells is a result of the TCR repertoire. Additionally, it will be important to understand how the TCR promotes CD8⁺ T cells to differentiate into various subsets following infection.

Appendix II: The Microbial Environment Alters Thymic Development



Appendix II The microbial environment alters the process of thymic development.

(A) Relative proportion of thymocytes that have not or (B) have received a signal through their TCR at different times of life. (C) Relative proportion of thymocytes that are undergoing death through neglect or (D) clonal deletion at different times of life. (E) Proportion of SP CD8+ T cells that express eomes at postnatal day 7. (F) Representative histogram showing Ki67+ expression in SP CD8+ thymocytes from

postnatal day 7 mice. **(G)** Proportion of SP CD8s+ thymocytes that express Ki67+ at postnatal day 7.

Summary

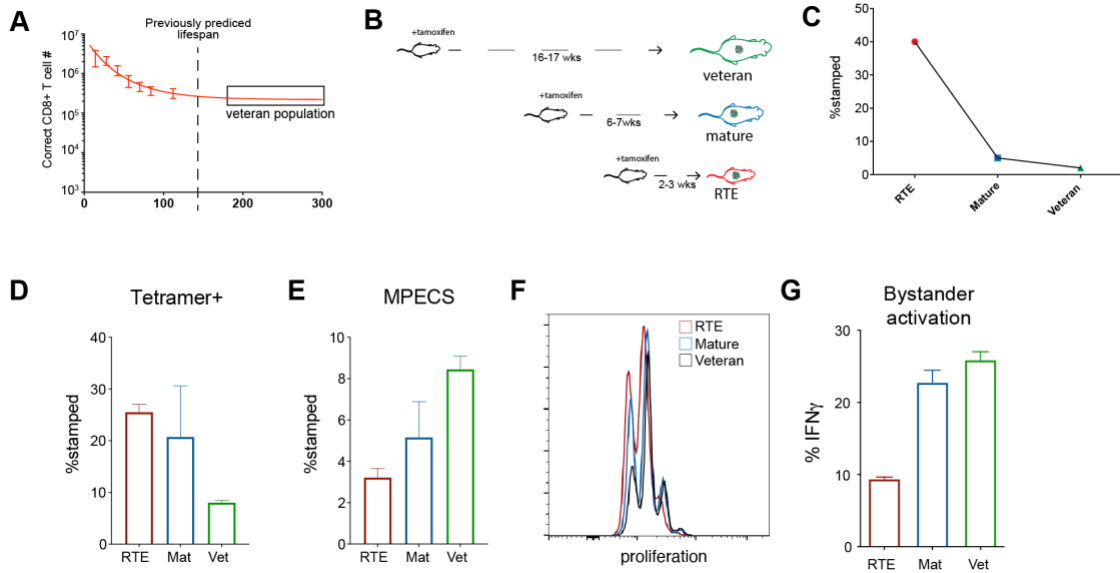
In chapter 4, I found fetal-derived cells from mice raised in a dirty environment persist in larger proportions in adulthood and acquire a virtual memory phenotype. Figures 4.3 and S4.7 show enhanced reactivity and expansion of fetal-derived cells is programmed during early thymic development. This led us to ask if thymic education is altered in clean and dirty mice. To address this question, I used Nur77-eGFP mice to measure thymocyte survival in a polyclonal population. Nur77 is 'turned on' when the TCR engages with a peptide and its expression level correlates to the strength of TCR signal. Using these mice, I found the proportion of thymocytes that receive signal through the TCR β chain is not significantly altered in clean or dirty mice (Appendix II A, B). However, thymocytes from dirty mice experienced death at lower rates at 3 wks of life, this coincides with the 'weaning reaction' (Appendix II C, D) ⁴. This provides further evidence that the dirty environment alters the program of development at critical stages of development.

Next, I wanted to address why mice raised in a dirty environment have a larger proportion of fetal-derived cells with a virtual memory phenotype (Fig. S4.6). To determine if an expansion of the VM subset in dirty mice is a result of altered programming during thymic development, I measured Eomes expression in single positive (SP) CD8⁺ thymocytes. Eomes has been implicated in directing SP CD8s towards a VM fate ⁵. Indeed, I found SP CD8s from 7 day old dirty mice expressed higher levels of Eomes (Appendix II E). This indicates the increased proportion of VM

cells present in dirty fetal-derived cells is triggered during thymic development. I also found SP CD8s from dirty mice underwent higher levels of proliferation (Appendix II F, G). Together, this shows the program of enhanced differentiation and expansion of the VM population in dirty fetal-derived cells likely arises during thymic development.

From observations made in Appendix II, it would be interesting to determine if the program of thymic selection determines the fate cells adopt following thymic export. Indeed, Poberzinsky *et al.* found autoreactive thymocytes are diverted towards the intraepithelial lymphocyte fate ⁶. Moreover, Miller *et al.* found Eomes directs SP CD8s to adopt the virtual memory fate by binding to particular ligands that preferentially promote a VM phenotype. In consideration of these past findings and my findings from chapter 4 and this appendix, it is reasonable to speculate that events that occur during thymic selection play a deterministic role in the fate of a naïve CD8+ T cell. The use of clean and dirty mice as a model can be an extremely powerful tool in understanding thymic selection. Knowledge gained from experiments that focus on thymic events in clean and dirty mice will provide insight into how early developmental events set the stage for immune protection and how heterogeneity arises within the naïve population.

Appendix III: Post-thymic Maturation Alterations TCR-mediated Responses



Appendix III Post-thymic maturation alters the response to TCR-mediated activation.

(A) Model showing the total number of CD8+ T cells present in a mouse. (B) Experimental strategy to control for time post-thymic maturation in 7d ts cells. (C) Proportion of 7d ts cells present at three times post-thymic export. (D) Proportion of antigen-specific cells following *Listeria* infection. (E) Proportion of MPECs at 7 dpi. (F) Representative proliferation peaks at 72 hrs post-*in vitro* TCR stimulation. (G) Production of IFN- γ 16 hr after *in vitro* bystander activation using IL-12/18.

Summary

In the past, the survival of T cells was measured by labeling cells with deuterated water or glucose and modeling the data to predict the average lifespan of cells within a population. Using these techniques, the average lifespan of murine CD8+ T cells was predicted to be 11 weeks ⁷. However, a limitation of this approach is that survival is inferred because it is assumed that all cells are homogenous. The timestamp mouse model allows us to track and enumerate cells made at precise times of life. By using this approach, we found a population of cells that survives past the estimated 7-week period and persists for the lifetime of a host (Appendix III A-C). We named this novel population the 'veteran' population. To understand how post-thymic maturation alters the protective program of CD8+ T cells made at the same time in development, we infected *Listeria-gB-ΔActA* and characterized the antigen-specific responses in cells at different times post-thymic maturation. We found veteran CD8+ T cells were recruited to the infection in lower proportions and were biased towards adopting an MPEC phenotype (Appendix III D, F). Numerous groups have reported CD8+ T cells that receive weaker signaling through their TCR are more likely to become memory precursor cells ⁸. This led us to hypothesize that veteran cells are less dependent on their TCR for activation. To determine if veterans were less sensitive to TCR stimulation, we performed an *in vitro* TCR activation assay using α CD3/28. We found veteran cells underwent less proliferation when compared to their younger counterparts (Appendix III F). Thus, if veteran cells are less dependent on their TCRs for activation, I hypothesized they will be more reactive to inflammation-based

activation. To test this hypothesis, I performed an *in vitro* bystander assay using IL-12/18. Indeed, veteran cells produced higher levels of IFN- γ compared to their younger counterparts when activated in a bystander manner. Overall, results from this appendix suggest extensive post-thymic maturation results in T cells being less dependent on their TCRs for activation.

What is the teleological benefit of having T cells that are less dependent on TCR-dependent activation later in life? As an individual ages, the thymus involutes and less T cells are exported into the periphery. Switching from a mainly TCR-dependent mode of activation towards heterologous immunity increases the probability that a host will react to and clear novel infections. This theory underscores the extent in which CD8⁺ T cells dynamically alter their protective programs to meet the needs of the host. In consideration of appendix I, it is also interesting to speculate that the TCR plays a deterministic role in promoting survival from the RTE phase to the mature or long-lived 'veteran' stage. To address this question, we would need to perform immune profiling paired with single-cell ATAC seq.

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