MECHANISMS UNDERLYING NEONATAL CD8+ T CELL DEVELOPMENT AND RESPONSES

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Neonatal infection is a major cause of morbidity and mortality worldwide. While adults generate robust immunity to most intracellular pathogens, neonates have an impaired ability to generate long-lasting immunity. As CD8+ T cells are essential for clearing intracellular pathogens, it is crucial to understand why these cells behave differently during infection in early life.

We previously showed that neonatal CD8+ T cells rapidly become terminally differentiated and fail to form long-term memory following infection. However, the underlying basis for these age-related differences is unclear. In this thesis, experiments were designed to investigate how neonatal and adult CD8+ T cells behave differently and what underlying mechanisms contribute to these differences. We demonstrated that different aged CD8+ T cell progenitors have different gene expression profiles. Different aged progenitors also give rise to CD8+ T cells with distinct characteristics in the periphery when developed in the same thymic environment. Ectopic expression of the developmentally regulated protein Lin28b in adult CD8+ T cells granted them neonate-like traits. Our data indicate that different aged CD8+ T cells behave differently because they have different origins and Lin28b may regulate CD8+ T cell behaviors in early life. Neonatal CD8+ T cells also acquire a much more active metabolic profile compared to adult CD8+ T cells, which may contribute to their rapid contraction and poor memory formation following infection.

Extrinsic differences between different aged animals were also studied by transferring adult CD8+ T cells into neonatal mice. Neonatal environment was able to modify adult CD8+ T cells to become phenotypically and functionally different. Neonatal-experienced adult CD8+ T cells proliferated more rapidly and formed insufficient memory. These findings suggest that in addition to cell-intrinsic factors, extrinsic factors may also contribute to age-related differences in CD8+ T cell behaviors.

In summary, these findings advance our knowledge of neonatal CD8+ T cell responses and cast light on potential therapeutic targets early in life, such as Lin28b, mTOR and components of metabolic pathways. A more thorough understanding of neonatal immune responses is pivotal for combating diseases in neonates and improving their wellbeing.

BIOGRAPHICAL SKETCH

Jie (Jocelyn) Wang was born in Anhui, China. At the age of 17, She moved with her parents to Toronto, Canada where she earned her B.S. degree in Pharmacology at the University of Toronto. She spent a lot of her free time volunteering and working in different labs. She dabbled in psychological, statistical and biological research throughout her undergraduate years.

In 2012, Jocelyn was accepted into Biological and Biomedical Sciences (BBS) PhD Program at Cornell University. Because she was impressed by the complexity and applicability of research in neonatal immunity, she decided to join the lab of Dr. Brian Rudd in the field of Immunology and Microbiology. During her years in graduate school, she thrived as a young investigator. Her thesis work focused on understanding the underlying basis of age-related differences in CD8+ T cell responses.

As someone with broad interests, Jocelyn also participated in several outreach activities during graduate school. She led workshops in local elementary schools and served as a student council member for the BBS program. She designed and taught mini-workshops in Expanding Your Horizons (EYH) conferences for two years. She also helped organize a regional communication conference (ComSciCon-Cornell) and was the co-founder and Vice President of the BBS Graduate Student Society (BBSGSS).

For my family and friends. Thanks for always being there for me and loving me unconditionally.

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I also owe a great gratitude to my parents who have always been on my side. I would not have made it this far in education without their endless love, encouragement

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LIST OF ABBREVATIONS

AGM	aorta-gonad-mesonephros
APC	antigen presenting cell
BCR	B cell receptor
BM	bone marrow
CFSE	carboxyfluorescein diacetate succinimidyl ester
CMV	cytomegalovirus
DC	dendritic cell
DN	CD8-CD4- double negative
DP	CD4+CD8+ double positive
dpi	days post infection
ECAR	extracellular acidification rate
ETC	electron transport chain
FAO	fatty acid oxidation
GSH	glutathione
GSSG	glutathione disulfide
HP	homeostatic proliferation
HSC	hematopietic stem cell
LN	lymp nodes
MP	memory phenotype
MPEC	memory precursor effector cells
mTORC1	mTOR complex 1
mTORC2	mTOR complex 2
NKT	natural killer T cells
OCR	oxygen consumption rate
OXPHOS	oxidative phosphorylation
PKC	protein kinase C
PPP	pentose phosphate pathway
ROS	reactive oxygen species
RTE	recent thymic emigrant
SLEC	short-lived effector cells
SP	single positive
SRC	spare respiratory capacity
T_{CM}	central memory T cells
TCR	T cell receptor
T_{EM}	effector memory T cells
TFAM	mitochondrial transcription factor A
TN	true naïve
T_{rm}	tissue-resident memory cells
WT	wild-type

CHAPTER ONE

Introduction

Cytotoxic CD8+ T Cells

Thanks to our immune system, most of us are healthy and able to perform daily activities in this non-sterile world. One of the important branches of adaptive immunity is consisted of cytotoxic CD8+ T cells, also known as killer T cells. As suggested by their name, they specialize in defending the hosts by killing infected and cancer cells.

Like all other immune cells, CD8+ T cells are derived from the hematopoietic stem cells (HSCs). HSCs largely reside in the bone marrow, where they give rise to common lymphoid progenitors. The common lymphoid progenitors then travel to the thymus to differentiate into CD8-CD4- double negative (DN) thymocytes, which will undergo 4 stages of differentiation to give rise to CD4+CD8+ double positive (DP) thymocytes[1]. In order to commit to either CD8 or CD4 single positive (SP) lineage, the DP thymocytes need to go through positive and negative selection to eliminate cells that bind too weakly or too vigorously to self-ligand, respectively. The SP cells can then leave the thymus to go to the periphery.

Even though there were early graft-rejection experiments in the 1920s implicated the identity and function of CD8+ T cells[2,3], it wasn't until later in 1964 that researchers showed small lymphocytes constantly recirculating from the thoracic duct[4]. This piece of evidence laid the groundwork for extensive subsequent studies characterizing the traits and function of small lymphocytes[5–7]. Around the same time, the concept of two lymphocytes subsets (B and T) that matured from different origins (bone marrow and thymus) was also proposed[8–10].

In the 1970s, cellular and humoral immunities were proven to be two distinct immune responses mediated largely by T and B cells, respectively. By 1975, researchers depleted CD8 expressing cells and found that the cellular immunity (cytotoxicity) was eliminated, which allowed them to distinguish between CD8+ and CD4+ T cells were distinguished based on their phenotypes and functions[11–13]. Soon after that, the protein and genetic compositions of T cell receptors (TCR) were identified by several groups[14–19], which facilitated the field to better understand how T cells recognize antigens and elicit subsequent signaling to perform killing.

As of today, with all of the pioneering work, we have a better understanding of how cytotoxic CD8+ T cells perform their jobs. However, there are still many knowledge gaps in CD8+ T cell biology. Therefore, extensive studies are in action to address the open questions in the field and advance our knowledge on CD8+ T cells.

CD8+ T Cell Responses

Upon infection, dendritic cells (DC) will present infectious agents to CD8+ T cells in lymph nodes (LN) to induce activation (priming)[20,21]. Once activated, CD8+ T cells expand to form effector cells, which are consist of functionally and phenotypically distinct cell populations. Short-lived effector cells (SLECs) and memory precursor effector cells (MPECs) are the two major groups of effector cells that have been well-characterized[22,23]. SLECs and MPECs can be distinguished based on their expression of different surface markers. SLECs express high levels of killer-cell lectin-like receptor G1 (KLRG1) and low levels of IL-7 receptor α (CD127), whereas MPECs express low level of KLRG1 but high level of CD127[22,23]. SLECs are more terminally differentiated and are eliminated once infections are cleared, however, MPECs are less terminally

differentiated and have the potential to differentiate into long-lived memory cells[23,24].

Activated CD8+ T cells migrate to sites of infection via lymph and blood by upregulating expression of chemokine receptors and adhesion molecules[25]. It is still controversial whether activated CD8+ T cells follow a Brownian motion-like (random) or a non-Brownian motion-like behavior to detect infected target cells. However, once CD8+ T cells bind to the peptide-MHC-I complex on target cells, they can induce apoptosis in infected cells by releasing perforin, granzymes and/or upregulating FasL (CD95L)[26]. CD8+ T cells can also perform their cytotoxic functions by producing IFNγ and TNFa to activate other effector cells (macrophages and neutrophils) to eliminate pathogens via their microbicidal activities[27]. Once CD8+ T cells clear the infections, effector CD8+ T cells will contract to form memory cells, which are responsible for rapid secondary responses to combat repeated infections. Thus, CD8+ T cells protect against the infections by differentiating into effector cells and convey long-term protection via memory formation.

CD8+ T Cell Memory

One of the main characteristics of the adaptive immune system is its ability to form “immunological memory”. Efficient memory cells are capable of maintaining their homeostasis with slow division and acquiring effector function rapidly in response to re-infection. Memory cells generated after experiencing antigens are commonly known as “true memory” cells. True memory CD8+ T cells can be further divided into 3 major types: central memory (T_{CM}), effector memory (T_{EM}) and a newly identified subset tissue-resident memory T cells (T_{rm}). T_{CM} cells and T_{EM} cells can be distinguished based on the expression of molecules such as the chemokine receptor CCR7 and the

lymphoid homing marker CD62L. T_{CM} cells express both markers whereas T_{EM} cells lack both markers because those molecules promote entry to lymphoid organs[28]. T_{CM} cells largely circulate within lymphoid organs (lymph nodes, spleen and blood), and T_{EM} cells mainly circulate among non-lymphoid organs such as liver, lung and intestines[28]. Whereas both T_{CM} and T_{EM} cells have the ability to circulate among organs, T_{rm} cells are confined to specific tissue without circulating (ex. mucosal layers). Because they are not detectable in blood, T_{rm} cells were identified and characterized later than T_{CM} and T_{EM} cells. T_{rm} cells normally resides at tissues prone to infections, thus they can respond to local infection immediately and produce progenies that are capable of migrating to other sites of infection[29].

Naïve CD8+ T cells can also acquire memory phenotype and characteristics without experiencing antigens. Those cells are known as “innate memory T cells” reflecting their responses to internal rather than external environments like the “true memory” cells. Currently, innate memory cells can be grouped into two subsets: lymphopenia-induced and IL-4 induced memory cells.

Lymphopenia-induced memory cells were identified when proliferation and phenotypic changes were observed in naïve T cells that were transferred into lymphopenic hosts[30]. Lymphopenia-induced memory cells share many characteristics with true memory cells including phenotypic markers to genetic profiles. However, there are still some markers that can be used to distinguish between the two. One of the well-accepted markers is CD49d (α4-integrin), which is highly expressed on true memory cells but low on lymphopenia-induced memory cells[30].

Certain cytokines have been shown to be important in forming and maintaining

lymphopenia-induced memory cells. IL-7, a cytokine known to be essential for T cell homeostasis, is also critical for the formation and survival of lymphopenia-induced memory T cells. IL-15 has also been shown to be important in maintaining lymphopenia-induced memory T cells[30]. CD4+ T cells are more complicated as they can still proliferate in lymphopenic environment yet they fail to acquire memory phenotypes and sufficient effector traits[31].

IL-4 induced memory cells are generated by exposing to exogenous IL4 produced by natural killer T (NKT) cells[30]. IL-4 induced memory cells are first generated in thymus when single positive (CD8+CD4- or CD8-CD4+) thymocytes are exposed to IL-4[30]. The cells can also be detected in the periphery at steady state in mice. Larger proportion of CD8+ IL-4 induced memory cells been observed than CD4+ IL4 induced memory cells[30]. CD8+ IL-4 induced memory cells express the transcription factor Eomes, which is different than lymphopenia- and antigen-induced memory cells (express Tbet)[30].

Hematopoietic Stem Cells During Development

As discussed briefly in part 1, like all other immune cells, CD8+ T cells are derived from HSCs, which largely reside in the bone marrow in adults. However, there is substantial evidence suggesting that distinctive waves of hematopoiesis occur during different developmental stages of life[32,33]. In mammals, the first wave of hematopoiesis is more primitive and produces limited blood cell lineages, such as macrophages[34]. This initial wave results from the yolk sac and is HSC-independent[32–34]. A more definitive wave arises later in development and is HSC-dependent[32–34]. The earliest HSCs were identified in aorta-gonad-mesonephros (AGM)[32–34]. Soon after that, hematopoiesis will

migrate to placenta and fetal liver. And then starts in spleens subsequently. Hematopoiesis will eventually shift to bone marrow shortly before birth in mammals[32–34].

Not all HSCs that arise throughout fetal life are the same, as evidence suggests that there is heterogeneity among HSCs[35]. With serial transplantation and cell sorting experiments, several groups have attempted to group HSC subtypes based on their proliferation patterns, self-renewal ability and differential contributions to blood cell lineages[35,36]. The factors segregating HSCs into their subtypes are complex and multifactorial. Among all of the potential factors, strong developmental-linked correlation has been identified, which can be at least partially explained by differences in microenvironments throughout development[32]. At least in mice, young and aged mice tend to have different subtypes of HSCs[37–39]. Young adult mice have a large amount of HSCs with similar preferences to give rise to lymphoid and myeloid cell lineages[37–39]. On the other hand, aged adults have more HSCs preferentially give rise to myeloid cell lineage[37–39].

On the other end of the spectrum, fetal/early life HSCs also appear to be quite different than adult HSCs. Unlike mostly quiescent adult bone marrow HSCs, HSCs isolated from fetal livers have been shown to be highly proliferative and are more capable of self-renewal[40–42]. Interestingly, there is evidence suggesting that fetal liver HSCs preferably support their proliferation with energy-generating oxidative metabolic pathways, such as oxidative phosphorylation (OXPHOS) and TCA cycle[43]. This could be potentially driven by larger mitochondria in fetal liver HSCs[43].

In addition to differences in proliferation, the progeny of fetal and adult HSCs are also different, and these changes happen around the time when hematopoiesis

relocates from fetal liver to bone marrow. Among fetal liver HSCs, the majority of them are myeloid-lymphoid balanced HSCs with comparable productions of myeloid and lymphoid cells[44]. The proportion of the HSCs preferentially give rise to myeloid lineage significantly increases after birth[44]. As mentioned above the lymphoid-deficient HSCs dramatically increases, resulting in the development of more myeloid cells.

Layered Immune System

As discussed above, HSCs that arise at different developmental stages have different characteristics. The next question is whether these HSCs give rise to same or different immune cells in the periphery. Multiple studies have shown that fetal-derived B cells demonstrate different traits than adult-derived B cells. B-1 and B-2 are the two major populations of B cells. B-1 B cells are known to participate in innate immune responses, whereas B-2 B cells are more involved in adaptive responses[45]. B-1 B cells respond rapidly in a B cell receptor (BCR)-independent manner, and these cells demonstrate a dampened responsiveness to BCR cross-linking[46–49].

There has been extensive debate regarding the origins of B-1 and B-2 B cells for a long time. The two most popular hypotheses are that a) differentiation of B-1 and B-2 B cells are dependent on specific pathogens and b) B-1 and B-2 B cells develop independently and originate from distinct progenitors (layered immune system)[50]. Over the years, extensive evidence has accumulated to support the idea of a layered immune system. Experiments transplanting fetal liver and adult bone marrow cells into irradiate recipients have shown that fetal progenitors can give rise to both B-1 and B-2 B cells, yet adult progenitor are unable to produce B-1 B cells efficiently[50]. These experiments suggest that there are different origins for these different subsets of B cells. Murine fetal

B-1 B cell progenitors were later identified with phenotype: lineage negative (Lin-) CD93+CD45R-/loCD19+[51]. Few years later, human B-1 B cell progenitors were also identified, further supporting layered immune system[52].

Similar phenomena have been observed with T cells, as evidence has shown that fetal HSCs give rise to certain type of T cells[53]. Both human and murine neonatal CD4+ T cells display a Th2 biased response upon activation, which could be attributed to the fact that they are derived from a different set of progenitors[54–56]. In mice, neonatal CD4+ T cells produce large amount of Th2 cytokines IL-4 and IL-13 upon activation[56,57]. Neonatal CD4+ T cells still possessed strong Th2 responses following stimulation by adult antigen presenting cells (APCs) or transplantation into adult recipients, suggesting that the observed differences are likely due to intrinsic differences between adult and neonatal CD4+ T cells[56,58,59].

Recently, we demonstrated that different aged progenitors give rise to distinct CD8+ T cells in the periphery[60]. We showed that fetal progenitors (CD4-CD8- thymocytes) give rise to CD8+ T cells with unique behaviors in the periphery[60]. CD8+ thymocytes isolated from different aged animals demonstrate different genetic profiles[60]. Neonatal CD8+ thymocytes demonstrate a more effector like genetic profile than their adult counterparts[60]. Fetal thymocytes give rise to mostly memory phenotype naïve CD8+ T cells in the peripheral and fail to form sufficient immunological memories post infection[60]. They are also skewed to form SLECs post-infection[60].

Different aged animals also have recent thymic emigrants (RTEs) that are functionally and phenotypically different[61,62]. RTEs, as their name suggests, are the newest T cells that enter the periphery from thymus. The cells demonstrate distinct

characteristics compared to more mature peripheral T cells. It takes about 3 weeks for RTEs to transit into more mature naïve T cells in the periphery[61]. During this process, RTEs will downregulate markers such as CD24 and PD-1, and upregulate markers including Qa2 and CD45RB[61]. RTEs are also functionally different than more mature T cells. RTEs are capable of responding to IL-7 without other stimuli but are less immunocompetent[61]. In neonates, it is not surprising that most peripheral CD4+ T cells are RTEs. However, neonatal CD4+ RTEs are phenotypically and functionally different than adult CD4+ RTEs, providing more evidence of layered immune responses[62]. Neonatal CD4+ RTEs express higher CD24, CD3, and CD28 and lower Qa2 than adult CD4+ RTEs[62]. Upon activation, neonatal CD4+ RTEs also produce higher levels of the cytokines IL-4, IL-2 and IFNγ than adult CD4+ RTEs[62]. Neonatal CD4+ RTEs also proliferate more rapidly than adults in response to IL-7[62]. Similar to CD4+ RTEs, neonates also have a large percentage of CD8+ RTEs in the periphery. Whereas there is no direct comparison between adult and neonatal CD8+ RTEs yet, similar with bulk neonatal CD8+ T cells, CD8+ RTEs preferentially differentiate into SLECs as compared with mature CD8+ T cells[61]. However, the differences observed between different aged RTEs are not solely due to intrinsic differences in their progenitors, as it is likely that the lymphopenic peripheral environment in neonates also contribute to RTEs behaviors[62].

Thus, there is a substantial amount of evidence in supporting the idea that immune system does not develop in a linear fashion. Alternatively, existing evidence suggests that the immune system consist of different layers that arise at different developmental stages. It is likely that both intrinsic and extrinsic factors contribute to the differences observed among all the layers.

CD8+ T cell Metabolism

Cellular metabolism plays an important role in supporting cellular functions, survival, proliferation and differentiation. In the past decade, extensive studies have investigated the contribution of metabolic programs in regulating CD8+ T cell responses. As such, six metabolic pathways have been extensively studied in respect to CD8+ T cell behavior, including glycolysis, oxidative phosphorylation (OXPHOS), fatty acid oxidation, fatty acid synthesis, pentose phosphate and amino acid metabolism[63–65] (Figure 1).

Glycolysis is a glucose metabolism pathway that converts extracellular glucoses to lactic acid. In non-proliferating, differentiated tissues, when there is sufficient oxygen availability, glucose metabolism is carried out via OXPHOS and the TCA cycle to produce more ATP (up to 36 ATPs)[66,67]. When oxygen levels are scarce, glucose is metabolized through glycolysis and results in lactic acid production in the cytosol[66,67]. However, in heavily proliferating tissues (ex. tumors), glucose is always metabolized through glycolysis. This phenomenon is known as the Warburg effect[66,67]. While this catabolic pathway is inefficient at producing energy (generates 2 ATPs), it generates many building blocks and cofactors to support the generation of new daughter cells. Hence, glycolysis is the preferential pathway used by activated T cells[68].

OXPHOS is used as the predominant ATP producing pathway by naïve cells when oxygen is abundant. This pathway occurs in mitochondria when electron transfer chain (ETC) couples oxygen consumption with ATP production, driven by NADH and $FADH_2$ produced by the TCA cycle[64,65]. The ETC is composed of 5 complexes including ATP synthase, also known as complex V, and other complexes (I, II, III and IV) that are

responsible for transporting electrons. Complexes I and II oxidize NADH and $FADH_2$ to transfer electrons to complexes III and IV via Coenzyme Q and cytochrome c. Throughout the transferring process, protons are constantly pumped through the mitochondrial inner member to create a gradient, which can be used to drive ATP production by complex V. Because of its extraordinary ATP production, OXPHOS is the major pathway utilized by quiescent T cells (naïve and memory) to support their homeostatic activities[68].

Fatty acids are the major components of membranes for organelles and cells, thus they are important building blocks utilized by proliferating or growing cells[64,65]. Fatty acid synthesis generates fatty acids using metabolites from cellular metabolic pathways (ex. glycolysis, TCA cycle and pentose phosphate pathway). Fatty acid synthesis is often utilized by activated T cells[64,65].

Fatty acid oxidation (FAO, beta oxidation) is the fatty acid metabolism pathway. FAO produces acetyl-CoA, which can feed into the TCA cycle and result in more ATP via OXPHOS[64,65]. FAO also produces its own NADH and $FADH_2$, which can be utilized by the ETC to produce ATP. Quiescent memory T cells prefer to use this efficient ATP producing pathway over glycolysis[64,65,68].

Activated T cells also utilize Pentose phosphate pathway (PPP) and amino acid metabolic pathways. PPP uses glucose-6-P produced by glycolysis to generate nucleotides and amino acid precursors[64,65]. This pathway also generates NADPH, which can be used in fatty acid synthesis and is important in maintaining redox homeostasis[64,65]. Amino acids are fundamental building units that can be used by several metabolic pathways in cells. Since amino acids are largely used to construct

proteins, they involve heavily in anabolic pathways[64,65]. Hence, just like PPP, amino acids metabolism is also very active in actively proliferating cells[64,65].

Metabolic programs involved in CD8+ T cell responses are crucial and complex. Depending on the specific metabolic program and demands of the cell, energy is created and distributed differently. Since the layered immune response model suggest that it will be important to investigate if different aged CD8+ T cells utilize different metabolic pathways.

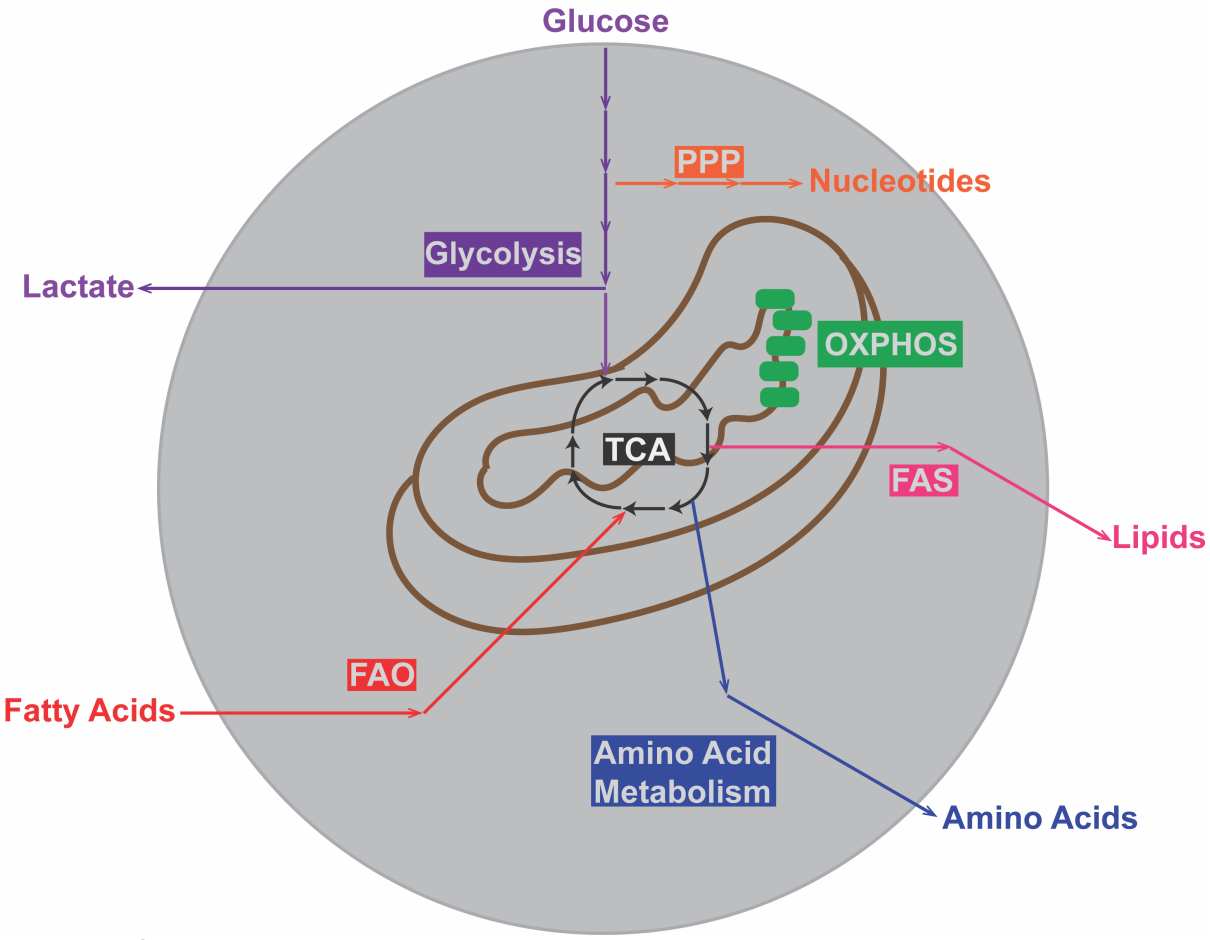

Figure 1.1. Six major metabolic pathways in T cells.

Neonatal CD8+ T cell Response

Many have noticed that young children are constantly sick and are more prone to infections. Globally, more than a third of the deaths in young children (less than 5 years

old) happen during the neonatal phase (first month of life)[69]. While morbidity and mortality are much reduced in young children due to expanding maternal vaccines, better pre-natal care and advance in medical care. Neonatal death still remains a significant concern[70,71]. Several leading causes of death in infants are of infectious origin[71,72]. Therefore, it is critical to gain a better understanding of differences between adult and neonatal immunity in order to deign more effective therapeutic regimens.

My thesis focuses on understanding and identifying the underlying mechanisms for the differences between adult and neonatal CD8+ T cells. Due to the complexity and ethical concerns of human studies, we, like many other researchers, choose to use murine models to address immunological questions. Murine models provide a genetically tractable system to investigate the basic biology of infant immunity.

Historically, neonates were thought to have immature adaptive immune systems with compromised immune responses, rendering them more susceptible to infections. Surprisingly, we found that neonatal CD8+ T cells are capable of forming effector cells post-infection[73]. In fact, neonatal CD8+ T cells respond vigorously to activation, neonatal CD8+ T cells proliferate more rapidly than adult CD8+ T cells and become quickly terminal differentiated[73,74,31]. Thus, neonatal CD8+ T cells have a significantly larger percent of SLECs and less MPECs than adult CD8+ T cells after infection[73]. Neonatal CD8+ T cells form poor immunological memory post-infection, which contribute to their poor ability to combat secondary infection. This could be due to their more terminally differentiated status[73]. Our data suggest that neonatal neonates are more susceptible to repeated infections not because they have immature immune systems, but due to their failure to form protective long-term memory (Figure 2).

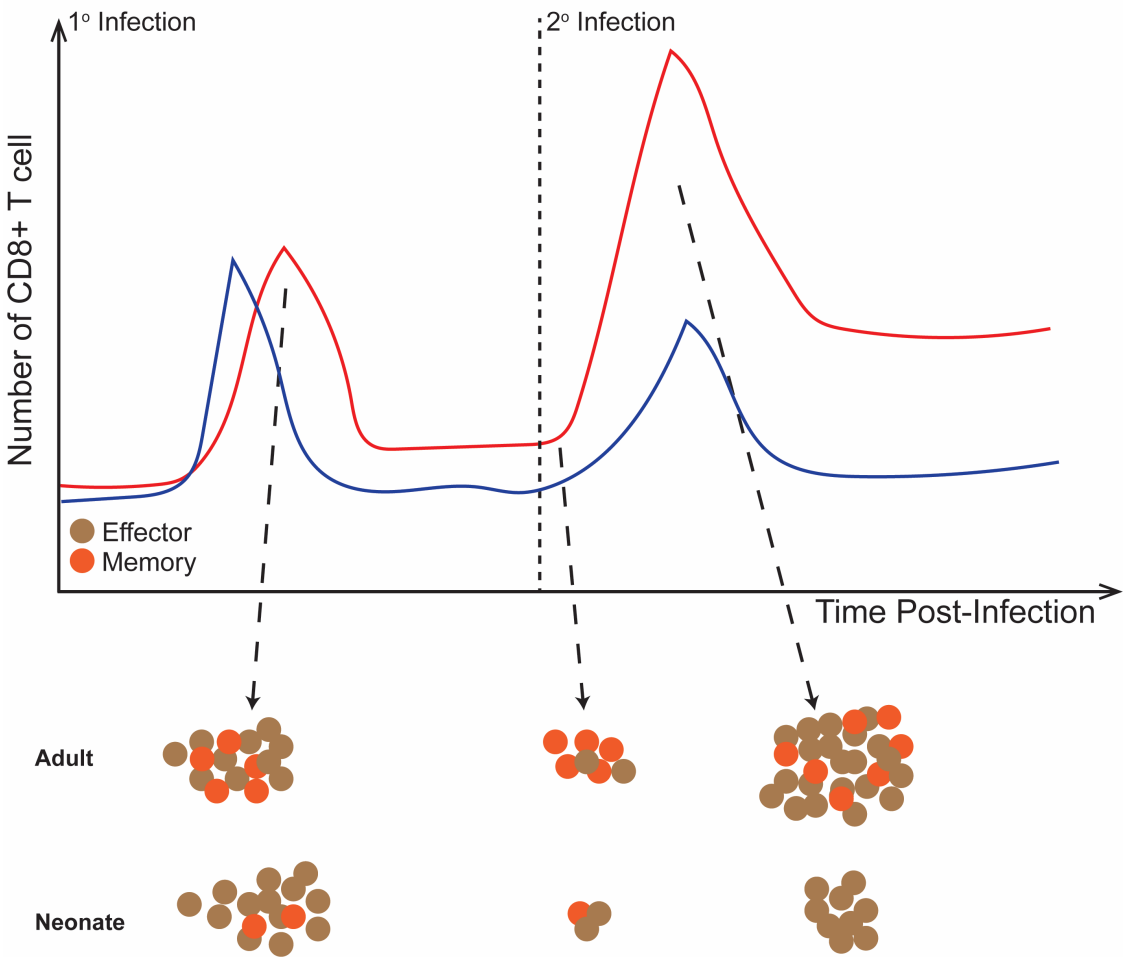

Figure 1.2. Schematics of adult and neonatal CD8+ T cell responses.

Naïve neonatal CD8+ T cells contain a larger percent of virtual memory cells than adults measured by expression of the memory phenotype markers, CD44 and CD122[60]. This is at least partially due to a more lymphopenic environment in early life[75]. Age-dependent differences in percentages of virtual memory cells are not likely driven by IL-4 induction because even after developing within the same thymic environment with similar IL-4 availability, neonatal thymocytes still gave rise to more memory phenotype CD8+ T cells in the periphery compared to adult thymocytes[60].

As mentioned above in the layered immune system section, we have further shown that neonatal CD8+ T cells adopt different fates than adult cells because they originate from different progenitors[60]. Our data also suggest that a developmental-

regulated RNA binding protein Lin28b plays an important role in shaping neonatal CD8+ T cell response[60].

Lin28 is a RNA binding protein that is well conserved across species, ranging from Drosophila to human[76,77]. Lin28a and Lin28b are the two isoforms of Lin28 proteins. They are considered equivalent since they share almost identical sequence[76]. Lin28 is highly expressed in early life and has been shown to promote fetal hematopoiesis[78–80]. The best-known target of Lin28, microRNA let-7 is highly expressed in adulthood[76,77]. Lin28 can inhibit let-7 biogenesis, which explains the reverse relationship of their level of expression during development[76,77]. Let-7 represses a wide array of targets, including oncogenes (Myc, Hmga2, etc.), insule-PI3K-mTOR pathway and cell cycle regulators (cyclins and cyclin-dependent kinase)[76,77]. Lin28 promotes the activities of let-7 downstream targets by inhibiting let-7 production. Another proposed target of Lin28 is mTOR pathway[77,81]. In addition to act on mTOR in a let-7 dependent manner, it has been shown that Lin28 can also directly promote mTOR activities by stabilizing some critical components (Igf2, etc.) transcriptionally[81].

Previous studies have shown that mTOR is important in regulate CD8+ T cell differentiation at least in adult animals, and mTOR acts mostly through mTOR complex 1 (mTORC1)[82,83]. mTOR is an ubiquitously expressed protein kinase that carries out its function by phosphorylating downstream targets. mTOR has been proved to regulate cellular metabolism and growth[83]. There are 2 complexes (mTORC1 and mTORC2) formed by mTOR with other proteins. mTORC1 has been shown to be the major regulatory complex of cell metabolism, and it is better studied and understood than mTORC2 in T cells. Its role in neonatal CD8+ T cells has been examined in this thesis,

yet more experiments are required to understand the mechanism better.

The underlying mechanisms for these age-related differences still remain unknown. Future studies are called for improve our understanding on CD8+ T cells response in early life.

Research Objectives

Understanding of CD8+ T cell biology has advanced drastically in the past several decades, but there are still many gaps in our knowledge of CD8+ T cell biology, particularly regarding the differences in CD8+ T cell behavior in early life. The overarching objective of this thesis is to investigate the developmental differences between adult and neonatal CD8+ T cell responses and the factors contributing to their different behaviors. We hypothesized that neonatal CD8+ T cells are derived from a distinct progenitor and utilize different metabolic program. In this thesis, experiments have been conducted to investigate the layered CD8+ T cell responses, the metabolic pathways used by different aged CD8+ T cells and the environmental impact on CD8+ T cell development. The findings provide a more comprehensive picture of neonatal CD8+ T cell response and how it is regulated. Our work also identify cellular pathways that are differentially utilized in neonatal CD8+ T cells, which can serve as potential drug targets in therapy development.

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CHAPTER TWO

Fetal and Adult Progenitors Give Rise to Unique Population of CD8+ T Cells[§]

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SUMMARY

During the ontogeny of the mammalian immune system, distinct lineages of cells arise from fetal and adult hematopoietic stem cells (HSCs) during specific stages of development. However, in some cases, the same immune cell type is produced by both HSC populations, resulting in the generation of phenotypically similar cells with distinct origins and divergent functional properties. In this report, we demonstrate that neonatal CD8+ T cells preferentially become short-lived effectors and adult CD8+ T cells selectively form long-lived memory cells after infection because they are derived from distinct hematopoietic stem cells. Notably, we find that naïve neonatal CD8+ T cells originate from a progenitor cell that is distinguished by expression of Lin28b. Remarkably, ectopic expression of Lin28b enables adult progenitors to give rise to CD8+ T cells that are phenotypically and functionally analogous to those found in neonates. These findings suggest that neonatal and adult CD8+ T cells belong to separate lineages of CD8+ T cells, and potentially explain why it is challenging to elicit memory CD8+ T cells in early life.

INTRODUCTION

Neonates often generate incomplete immunity against intracellular bacteria and viruses. Because CD8+ T cells play a critical role in protecting the host against these pathogens, it is important to understand how and why neonatal CD8+ T cells respond to infection differently than in adults. Recent studies suggest that neonatal CD8+ T cells fail to become memory cells because of an inherent propensity to rapidly proliferate and become terminally differentiated after antigenic stimulation.[1-3] However, the underlying basis for these age-related differences remains unknown.

Several models might explain why neonatal CD8+ T cell adopt fates different from those of adults during infection. First, the “proliferation model” posits that developmental changes in the CD8+ T-cell response relate to differences in homeostatic proliferation before infection. When naïve CD8+ T cells enter a lymphopenic environment, they divide rapidly in response to homeostatic cytokines and upregulate phenotypic markers (CD44, CD122) indicative of cell differentiation.[4,5] Thus, because newborn mice are nearly devoid of peripheral CD8+ T cells, it is possible that neonatal CD8+ T cells are less likely to develop into memory CD8+ T cells because the starting population is more differentiated than adults before infection.

Another possibility relates to the distinct hematopoietic stem cell (HSC) lineages that generate neonatal and adult CD8+ T cells (“origin model”). Although neonatal CD8+ T cells are derived from fetal liver HSCs that colonize the thymus during mid-gestation (approximately embryonic day [e] 13), adult CD8+ T cells are produced from bone marrow (BM) HSCs that seed the thymus just before birth (~e20). Importantly, fetal HSCs turn over more rapidly[6] and preferentially give rise to innate-like lymphocytes compared with adult HSCs.[7] Thus, it is also possible that neonatal CD8+ T cells fail to form memory cells because they are created from distinct progenitor cells.

To discriminate between the proliferation and origin models, we compared neonatal and adult CD8+ T cells that had undergone equivalent homeostatic proliferation in the periphery, or were at the same stage of development in the thymus. We also compared T-cell maturation by fetal and adult precursors in the adult thymus and examined whether fetal-derived CD8+ T cells respond differently to infection than their adult counterparts. Collectively, our data reject the proliferation model and support

the origin model, and imply that neonatal and adult CD8+ T cells adopt different fates after infection because they belong to separate lineages of naïve CD8+ T cells derived from distinct progenitors.

MATERIALS AND METHODS

Mice

B6-Ly5.2/Cr mice were purchased from Charles River Laboratories (Frederick, MD). TCR transgenic mice specific for the HSV-1 glycoprotein B498-505 peptide SSIEFARL8 (gBT-I mice) were provided by Janko Nikolich-Zugich (University of Arizona, Tucson, AZ) and crossed with Thy1.1 or C57BL/6 mice purchased from Jackson Laboratories (Bar Harbor, ME). Neonatal and adult gBT-I animals were used at 6 to 8 days old and at 2 to 4 months old, respectively. Mice with a tetracycline-inducible copy of human *LIN28B* on a C57BL/6 background (iLin28b mice) were obtained from George Daley (Harvard University, Cambridge, MA).[9] Male mice were used for all experiments, and 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.

Antibodies and flow cytofluorimetric analysis

Antibodies were purchased from eBioscience (San Diego, CA), Biolegend (San Diego, CA), Invitrogen (Carlsbad, CA), or BD Biosciences (Mountain View, CA). Sheep anti-human Lin28b was obtained from R&D Systems (Minneapolis, MN), and Alexa Fluor 488 rabbit-anti-sheep from Jackson Immunoresearch (West Grove, PA). Flow cytofluorimetric data were acquired using DiVa software from an LSRII equipped with 4 lasers (BD Biosciences). Analysis was performed with FlowJo (Tree Star, Ashland, OR).

Cell sorting

To purify subsets of $CD44^{hi}CD122^{hi}$ and $CD44^{lo}CD122^{lo}$ CD8+ T cells from neonatal and adult gBT-I mice, CD8+ T cells were enriched using anti-CD8a microbeads (Miltenyi Biotec) and were subsequently labeled with antibodies against CD4, CD8, CD44, and CD122 and sorted to >95% purity on a fluorescence-activated cell sorting (FACS) Aria III (BD Biosciences). To purify single-positive (SP; CD8+CD4–) thymocytes from neonatal and adult gBT-I mice, CD4+ thymocytes were depleted by negative immunomagnetic selection via biotinylated antibody followed by streptavidin-coated microbeads (Miltenyi Biotec). Thymocytes were labeled with antibodies against CD4, CD8, Va2, and Vb8, and FACS-sorted to >90% purity. To isolate donor CD8+ T cells from chimeric mice, CD4+, CD19+, MHC-II+, and Ter119+ cells were depleted by negative immunomagnetic selection. Cells were labeled with antibodies against CD8, CD4, CD45.1, CD45.2, Thy1.1, and Thy1.2, and FACS-sorted to >98% purity for adoptive cotransfers. Antibodies against Va2 and Vb8 were added for sorts intended for RNA isolation.

***In vitro* stimulation of CD8+ T cells**

SP CD8+ thymocytes were FACS-sorted from neonatal and adult gBT-I mice and labeled with carboxyfluorescein diacetate succinimidyl ester, as described,[1] and cells were stimulated with plate-bound anti-CD3 (5 mg/mL) and anti-CD28 (20 mg/mL).

Intrathymic injection

To compare the progeny of different aged progenitor cells, we adapted a protocol from Adkins B.[10] B6-Ly5.2 recipient mice were sublethally irradiated with 600 rads and injected intrathymically with 1×10^6 double-negative (DN, CD8–CD4–) thymocytes from

fetal gBT-I Thy1.2 mice (14 days' gestation) and adult gBT-I Thy1.1 mice. CD8–CD4– thymocytes were isolated from adult mice by depleting CD8+ and CD4+ cells using negative magnetic separation. Because 100% of thymocytes on embryonic day 14 are CD8–CD4– cells,[10] whole thymocyte preparations were used from fetal gBT-I mice. Splenic CD8+ T cells were recovered 4 weeks posttransfer and cotransferred into new B6-Ly5.2 (CD45.1+Thy1.2+) recipients.

Adoptive cotransfers

To compare primary and secondary responses to infection, FACS-sorted splenic neonatal and adult $CD44^{hi}CD122^{hi}$ or $CD44^{lo}CD122^{lo}$ CD8+ cells, or donor CD8+ cells from thymic transfers or BM chimeras were combined at a 1:1 ratio. Combined cells were suspended at 2×10^5 cells per mL of PBS and 100 ul of cells was injected i.v. into adult B6-Ly5.2 recipient mice. The next day, recipient mice were infected with WT LM-gB (5×10^3 CFU, i.v.) and later challenged with WT LM-gB (5×10^4 CFU, i.v.), as described.[1]

Bone marrow chimeras

Single and mixed BM chimeras were generated by obtaining BM from congenically marked gBT-I (Thy1.1+CD45.2+) and gBT-I iLin28b (Thy1.2+CD45.2+) mice. CD4+ and CD8+ T cells were depleted by magnetic separation, and 1×10^7 cells were injected (5×10^6 cells per donor for mixed chimeras) into lethally irradiated (990 rads) 7- to 8-week-old adult B6-Ly5.2 recipients. Forty-eight hours posttransplantation, mice were administered 1 mg/mL doxycycline with 10 mg/mL sucrose via drinking water to induce *LIN28B* expression for 8 weeks during reconstitution.

RNA sequencing

For mRNA sequencing, libraries were generated using 25 to 200 ng total RNA with the NEBNext Ultra RNA Library Prep Kit for Illumina (NEB) and sequenced on an Illumina HiSequation 2500, generating 100-nucleotide reads. The analysis was performed as previously described.[3] Reads were aligned with Tophat[11] (mm9), and CuffDiff was used to quantify the abundance and differential expression of each mRNA, using a false discovery rate of 5% for significance.[12] Small RNA libraries were generated using 50 to 100 ng of total RNA using the NEBNext Small RNA Library Kit (NEB), and sequenced on an Illumina HiSequation 2500, generating 50-nucleotide reads. The analysis was performed as previously described.[3] miRNAs were mapped and quantified using MirDeep2[13] (MirBase version 21) with Bowtie[14] (mm9). The data have been deposited in the GEO Small Read Archive[15] under accession number GSE80597.

Clustering was performed using the partitioning around the medoids method[16] in R. Gene ontology was performed with DAVID[17,18] to find enriched biological process terms with Benjamini-corrected P values $< .05$. Gene sets for enrichment statistics were downloaded from the Molecular Signatures Database (MSDB).[19] For each cluster, we found the number of genes present in both the cluster and the data set (b), the total number of genes present in that cluster (n), the number of genes in the MSDB data set (B), and the total number of genes that had been clustered (N). Enrichment was calculated as (b/n)/(B/N). One-sided Fisher exact tests were used to measure significance.

Statistical analysis

Statistical analysis was performed using Prism (GraphPad Software, Inc, La Jolla, CA). Error bars represent standard error of the mean. Significance was determined by Student t test or one-way analysis of variance followed by Tukey multiple comparisons test. Significance is denoted as: $*P < .05$, $**P < .01$, $***P < .001$, and $****P < .0001$.

RESULTS

Neonatal and adult CD8+ T cells exhibit distinct phenotypes

We first sought to test the proliferation model and determine whether differences in homeostatic proliferation contribute to cell-intrinsic changes in neonatal CD8+ T cells. To examine this possibility, we stained CD8+ T cells from uninfected neonatal and adult gBT-I mice for CD44 and CD122, which are used to define cells that have undergone significant amounts of homeostatic proliferation. Such $CD44^{hi}CD122^{hi}$ are referred to as *memory phenotype naïve cells*, because they display certain characteristics of memory cells without having encountered foreign antigen.[20,21] The neonatal CD8+ T cells were comprised of twofold more $CD44^{hi}CD122^{hi}$ memory phenotype (MP) cells, whereas the adult CD8+ T cells were made up of more $CD44^{lo}CD122^{lo}$ true naïve (TN) cells (Figure 1A-B). Importantly, we did not observe significant numbers of MP cells in the thymi of neonatal mice, and their appearance in the periphery coincided with the relative number of CD8+ T cells that are actively dividing, as measured by Ki-67 expression (supplemental Figure 1, available on the *Blood* Web site). Together, these data are consistent with earlier reports indicating that large number of CD8+ T cells undergo homeostatic expansion in early life.[22,23] We also compared the phenotype of neonatal

and adult MP CD8+ T cells in the periphery, and observed multiple age–related differences in the expression of natural killer cell receptors, activation markers, and T-box transcription factors (Figure 1C). Thus, neonatal CD8+ T cells that have undergone homeostatic expansion in the periphery express a distinct phenotypic signature, even when compared with matched adult counterparts (Figure 1C).

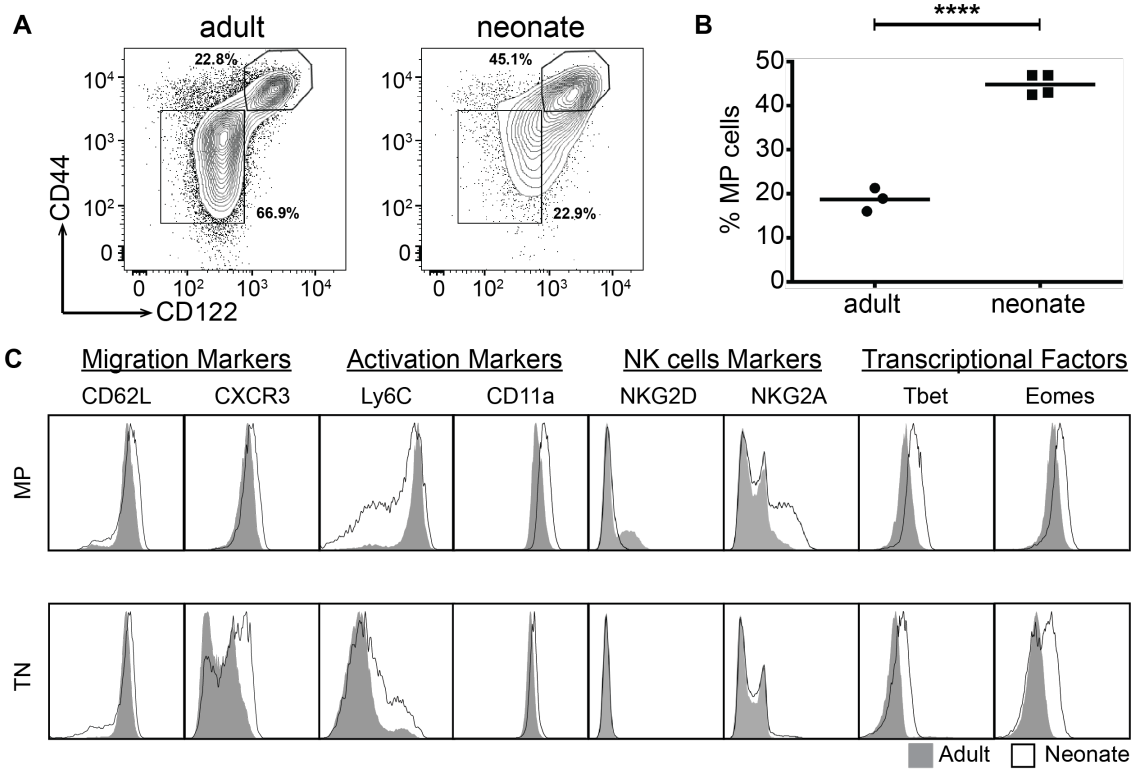

Figure 2.1. Memory phenotype CD8+ T cells are more abundant in early life and express different surface markers and transcription factors. (**A**) Surface expression of CD44 and CD122 by splenic CD8+ T cells from uninfected neonatal and adult gBT-I. (**B**) Statistical analysis of the percentages of MP cells from neonatal and adult gBT-I mice. (**C**) Comparison of various surface markers and transcription factors expressed in TN and MP cells from uninfected neonatal (line) or adult (shaded) gBT-I mice. Data are representative of at least 3 experiments. Significance was determined by student *t* test. ****$P < .0001$.

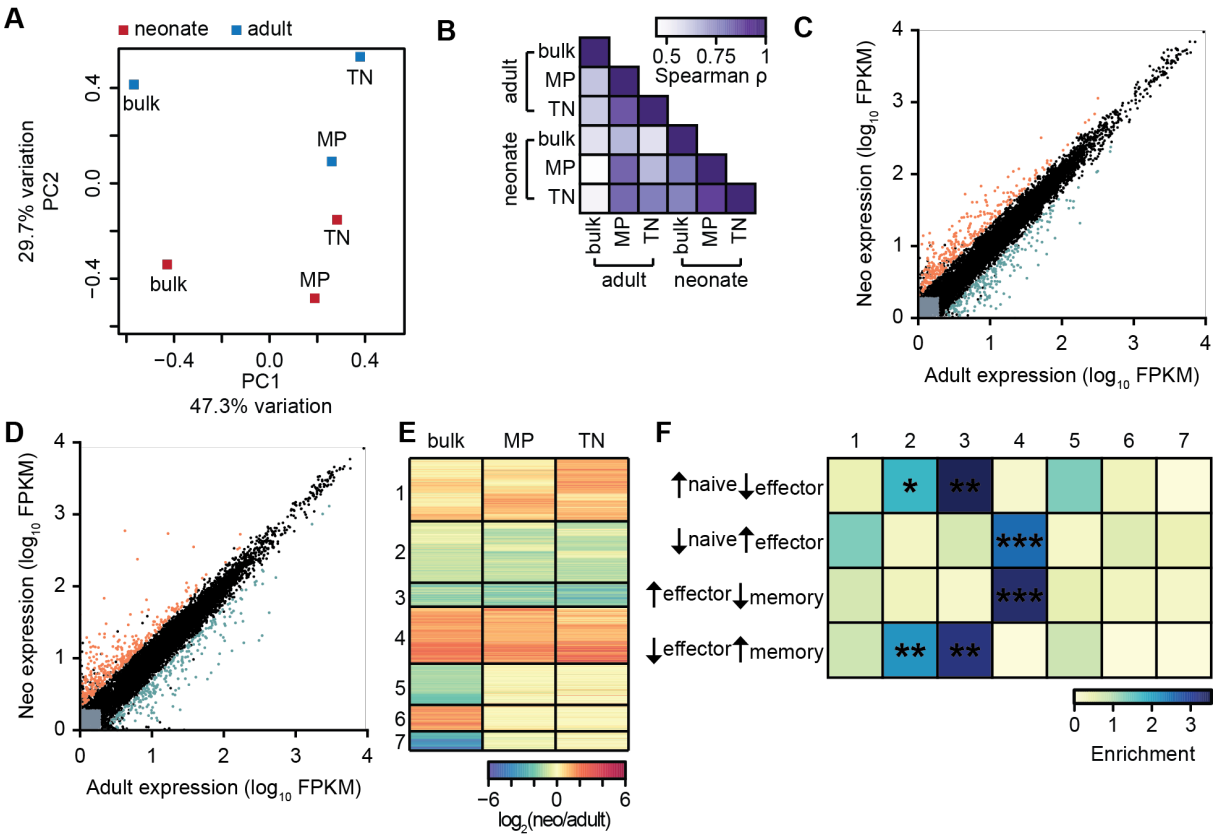

Figure 2.2. Both TN and MP CD8+ T cells from uninfected neonatal gBT-I mice express different genes from adults. (A) Principal component analysis on RNA-Seq data. Mean FPKM values from well-expressed genes were used from adult and neonatal bulk (total), TN, and MP cells. Bulk adult, MP adult, and MP neonatal samples consist of 3 pooled biological replicates; bulk neonatal, TN adult, and TN neonatal samples consist of 2 pooled biological replicates. The percentage of the overall variation accounted for by principal components 1 (x-axis) and 2 (y-axis) is indicated for each axis. Gene loadings for principal components 1 and 2 are shown in supplemental Figure 2. (B) Color-coded pairwise Spearman rank correlation coefficients comparing FPKM values for genes that are significantly differentially expressed between adults and neonates in at least 1 sample; $P<10^{15}$ for all comparisons. (C) Gene expression values for adults and neonates in MP cells. Gray indicates lowly expressed genes, black indicates nondifferentially expressed genes, orange indicates the 126 genes upregulated in neonatal cells, and blue indicates the 159 genes upregulated in adult cells. (D) Gene expression values for adults and neonates in TN cells, where 204 genes are upregulated in neonatal cells and 195 genes are upregulated in adult cells. (E) Clustering of genes in all samples. Fold-change differences for significantly differentially expressed genes were calculated between adults and neonates. Clustering was performed to identify genes with similar differences in expression in each sample; fold-change for each gene is plotted in each sample, and genes are shown in their clusters. (F) Genes in each cluster were compared with genes that define naïve cells before infection and effector or memory cells after infection. Enrichment was calculated as number of genes in each cluster compared with the number expected. Significance was determined by Fisher exact tests; $*P < .05$, $**P < .005$, $***P < .0005$. See supplemental Table 1 for gene expression values and clustering.

TN and MP CD8+ T cells from different aged mice express distinct gene expression profiles

We previously found that splenic neonatal CD8+ T cells upregulate genes needed for cell cycle progression and effector function,[3] similar to adult MP cells.[24] Thus, gene expression differences observed between bulk adult and neonatal CD8+ T cells could be explained by the different proportions of MP and TN cells. To investigate this possibility, we sequenced transcriptomes from TN, MP, and bulk splenic cells from neonatal and adult mouse CD8+ T cells (supplemental Table 1). Instead, we found that adult cells were distinct from neonatal cells for all populations, which we visualized using principal component analysis (Figure 2A-B; see supplemental Figure 2 for transcripts contributing to differences). Hundreds of genes were differentially expressed between adults and neonates in both the TN and MP populations (Figure 2C-D), several of which were consistent with protein abundances observed in Figure 1C. Clustering genes by their expression patterns showed that most genes had equivalent expression differences between adults and neonates (clusters 1-4), whereas the remainder had age-dependent expression changes in the bulk CD8+ population alone (clusters 5-7, Figure 2E; supplemental Table 1). Upon comparing the genes in each cluster (Figure 2E) with gene sets that characterize CD8+ T cells at different infection states,[19,25] we found that genes upregulated in neonates in both MP and TN cells (cluster 4) are significantly enriched in genes typifying effector cells (Figure 2F) and gene ontology terms related to cell division ($P < 10^{-8}$, supplemental Table 1). Similarly, genes downregulated in neonates in both MP and TN cells (clusters 2 and 3) are enriched in naïve and memory cell genes, consistent with previous data.[3] Most gene expression

differences between adults and neonates therefore cannot be attributed simply to the increase in MP cells observed in early life.

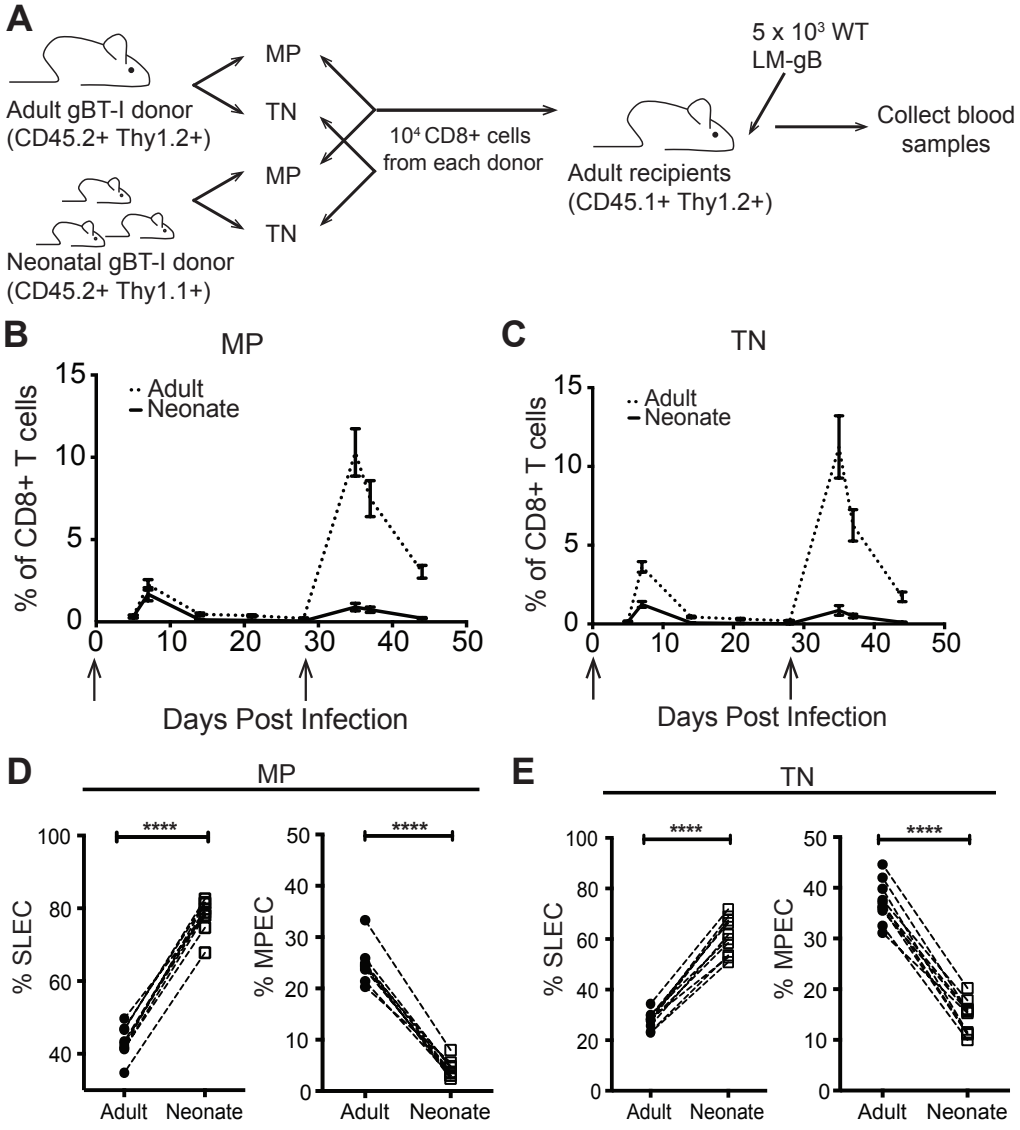

Figure 2.3. Both TN and MP CD8+ T cells from neonatal gBT-I mice preferentially become short-lived effectors after infection. (A) Schematic of experimental design: TN and MP cells were sorted from congenically marked gBT-I adult (CD45.2, Thy1.2) and neonatal (CD45.2, Thy1.1) mice and adoptively cotransferred (1:1 ratio) into adult recipient mice (CD45.1, Thy1.2). These recipients were infected with 5×10^3 colony-forming unit (CFU) LM-gB and serially bled to examine primary CD8+ T-cell responses. Recipients were also challenged at 28 dpi with 5×10^4 WT LM-gB to assess the memory recall response. Arrows indicate days of infections. Relative numbers and ratios of TN (B) and MP (C) donor cells from different aged mice at various times after infection. Percentages of MP (D) and TN (E) donor cells from different aged mice that display a short-lived effector cell (SLEC) or memory precursor effector cell (MPEC) phenotype at the peak of the primary response (7 dpi). Data are representative of 2 experiments (n=9-12 mice/group). Significance was assessed by Student t test (****$P < .0001$). DPI, days postinfection.

TN and MP CD8+ T cells from different aged mice respond differently to infection

Next, we wanted to address whether neonatal CD8+ T cells preferentially become terminally differentiated during an immune response because a larger proportion of the naïve pool has undergone homeostatic proliferation before infection. We cotransferred equivalent phenotypic subsets (MP or TN cells) from neonatal and adult gBT-I mice into the same adult recipient mice (Figure 3A) and tracked their response to infection. Regardless of their initial phenotype, neonatal donor cells preferentially differentiated into short-lived effectors (SLECs; $KLRG1^{hi}CD127^{lo}$) during the effector stage (Figure 3D-E) and failed to transition into the long-lived memory pool. As a consequence, the memory recall response was almost entirely dominated by adult donor–derived cells (Figure 3B-C). Collectively, these data argue against the hypothesis that age-related differences in homeostatic proliferation underlie the altered behavior of different-aged CD8+ T cells after infection.

Single positive CD8+CD4- thymocytes in neonatal mice exhibit unique genetic properties compared with adults

We next sought to test the origin model and determine whether cell- intrinsic differences between neonatal and adult CD8+ T cells relate to their distinct HSC origin. We compared gene expression profiles in single-positive (SP) CD8+ T cells from neonatal and adult thymi to compare transcriptomes at the same stage of thymic development, and to control for age-related differences in maturation and proliferation (supplemental Table 1; validated protein levels of selected genes in supplemental Figure 3). Overall, we found greater variation between splenic and thymic CD8+ T cells, regardless of age (Figure 4A-B). Widespread gene expression differences do exist

between adults and neonates in both types of cells (Figure 4C-D and supplemental Table 1), however, indicating that neonatal CD8+ T cells exhibit a divergent pattern of gene expression even at the time they are initially created. To gain insight into these differences, we clustered genes based on their coexpression patterns (Figure 4E-F; supplemental Table 1). Genes with differential expression between adults and neonates in the spleen generally have similar differences in the thymus (clusters 1 and 5), yet additional genes show differences in the thymus only. Interestingly, genes that are upregulated only in the neonatal thymus (cluster 3) are enriched in effector-cell genes, suggesting that neonatal CD8+ T cells are more effectorlike. Genes that are downregulated in splenic neonates with little difference in thymic cells (cluster 4) have memory like expression, whereas genes downregulated in neonates in both the thymus and spleen (cluster 5) are enriched for genes found in naïve cells. Overall, differences in gene expression between neonatal and adult CD8+ T cells, beginning at the cells' generation and continuing throughout the course of development, suggest neonatal CD8+ T cells represent a distinct and more effector like population of lymphocytes that associates with their progenitor cell origin.

To uncover functional differences between thymic CD8+ T cells from neonatal and adult mice, we compared the ability of SP CD8+ T cells from neonatal and adult thymi to proliferate after in vitro stimulation. We found that neonatal SP CD8+ T cells had divided significantly more than their adult counterparts (supplemental Figure 5). Thus, the enhanced ability of peripheral neonatal CD8+ T cells to proliferate after stimulation appears to originate in their immediate precursors in the thymus.

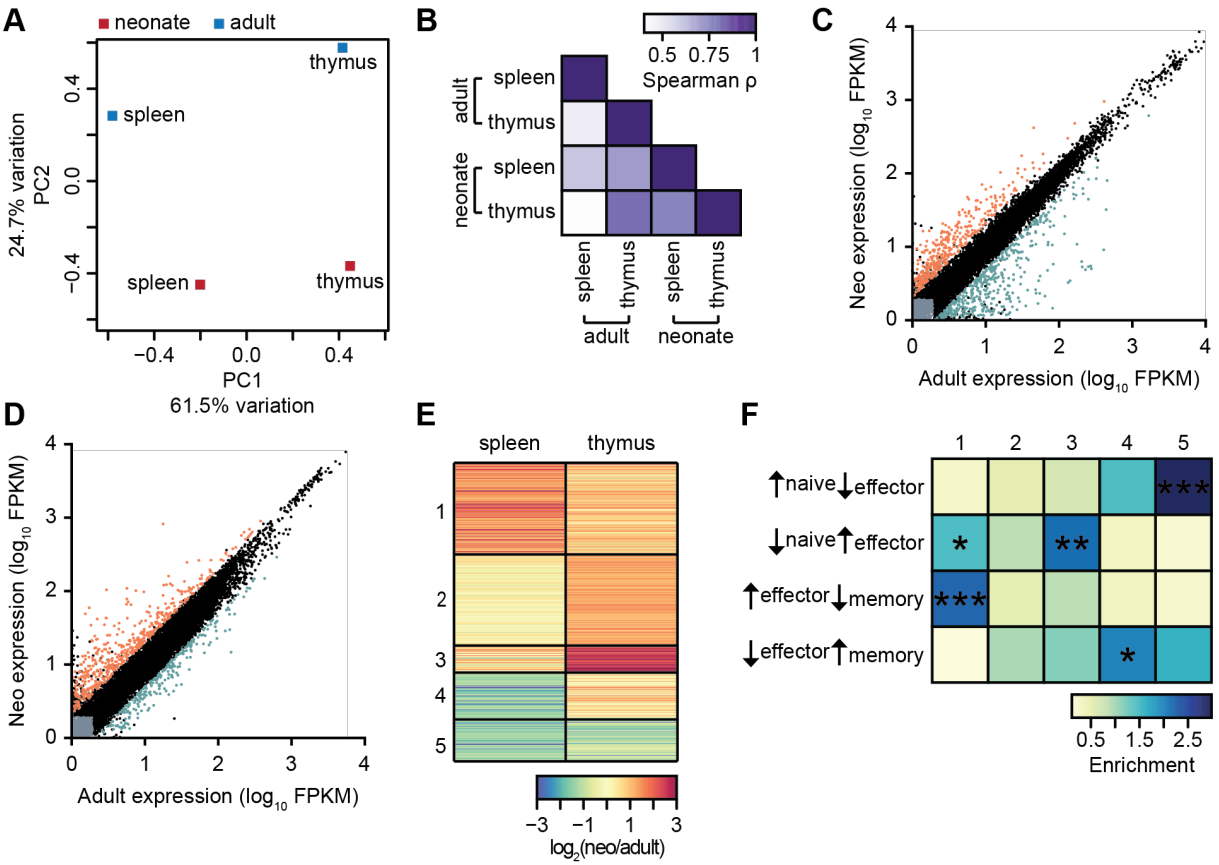

Figure 2.4. Neonatal single-positive CD8+ T cells from the thymus express different genes and proliferate faster than adults. (A-F) RNAseq was performed on neonate and adult gBT-I CD8+ thymocytes and splenocytes. (A) Principal component analysis. Mean FPKM values from well-expressed genes were used from adult and neonatal naïve splenic and thymic CD8+ T cells. The naïve splenic adult sample consists of 3 pooled biological replicates; the remaining samples consist of 2 pooled biological replicates. The percentage of the overall variation accounted for by principal components 1 (x-axis) and 2 (y-axis) is indicated for each axis. Gene loadings are shown for principal components 1 and 2 in supplemental Figure 4. (B) Color-coded pairwise Spearman rank correlation coefficients comparing FPKM values for genes that are significantly differentially expressed between adults and neonates in at least one sample; $P<10^{15}$ for all comparisons. (C) Gene expression values for adults and neonates in splenic cells. Gray indicates lowly expressed genes, black indicates nondifferentially expressed genes, orange indicates the 118 genes upregulated in neonatal cells, and blue indicates the 153 genes upregulated in adult cells. (D) Gene expression values for adults and neonates in thymic cells, where 264 genes are upregulated in neonatal cells and 199 genes are upregulated in adult cells. Clustering of genes in naïve splenic and thymic CD8+ T-cell transcriptomes. Fold-change differences for significantly differentially expressed genes were calculated between adults and neonates. (E) Clustering was performed to identify genes with similar differences in expression in each sample; fold-change for each gene is plotted in each sample, and genes are shown in their clusters. (F) Genes in each cluster were compared with genes that define naïve, effector, or memory cells. Enrichment was calculated as number of genes in each cluster compared with the number expected. See supplemental Table 1 for gene expression values and clustering.

Fetal progenitors give rise to mature CD8+ T cells that adopt distinctive fates after infection

To confirm that thymic precursors in neonate and adult mice give rise to distinct lineages of CD8+ T cells, we intrathymically injected equivalent numbers of DN thymocytes from fetal and adult gBT-I mice into adult recipient mice, allowing us to control for age-related differences in the thymic stromal environment (Figure 5A). Four weeks after injection, we collected the peripheral cells and compared their phenotype. Strikingly, nearly 100% of fetal-derived CD8+ T cells were MP cells, whereas adult-derived CD8+ T cells were comprised of many more TN cells (Figure 5B-C). To determine whether the progeny of fetal and adult precursors behaved similarly to neonatal and adult CD8+ T cells after microbial challenge, we cotransferred fetal- and adult-derived CD8+ T cells into adult recipient mice and compared their ability to respond to infection. We found that fetal-derived cells exhibited a terminally differentiated phenotype at the peak of the primary response (7dpi), and failed to survive during later stages of the infection (Figure 5D-E). As a result, the memory recall response was largely comprised of adult-derived CD8+ T cells (Figure 5D). Collectively, these data support the origin model, and suggest that neonatal and adult CD8+ T cells adopt different fates after infection because they are derived from different progenitor cells.

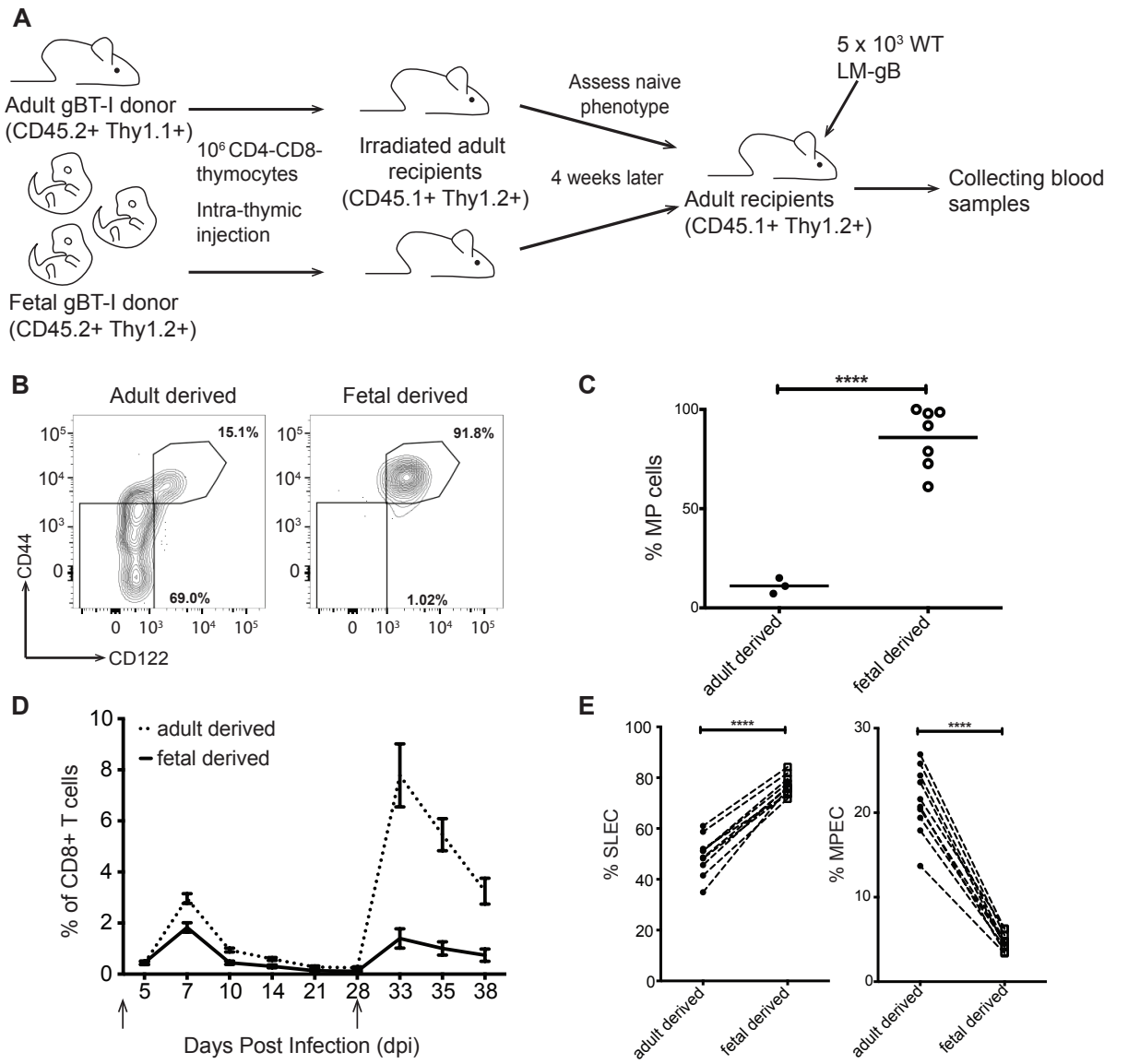

Figure 2.5. Fetal progenitors give rise to mature CD8+ T cells that adopt different fates after infection. (A) Schematic of experimental design: CD8-CD4- progenitors from congenically marked gBT-I adult (CD45.2, Thy1.1) and neonatal (CD45.2, Thy1.2) mice were transferred by intrathymic injection into sublethally irradiated congenic recipient mice (CD45.1, Thy1.2) separately. Four weeks later, progenitor-derived splenic CD8+ T cells were sorted and adoptively co-transferred (1:1 ratio) into new congenic recipient mice (CD45.1, Thy1.2). These recipients were infected with 5×10^3 LM-gB and serially bled to examine CD8+ T-cell responses. (B-C) Naïve phenotypes of progenitor-derived CD8+ T cells before adoptive cotransfer. (B) Representative plots displaying CD44 and CD122 expression by gBT-I progenitor-derived CD8+ T cells 4 weeks post–intrathymic transfer in blood. (C) Statistical analysis of the percentages of MP cells from gBT-I adult and fetal progenitor-derived cells. (D) Relative numbers of gBT-I adult (dashed) and fetal (solid) progenitor-derived donor CD8+ T cells postinfection and re-challenge (5×10^4 WT LM-gB); arrows indicate days of infections. (E) Percentages of gBT-I adult and fetal progenitor-derived donor CD8+ T cells that are SLECs and MPECs. Data are representative of 2 experiments (n=4-11 mice/group) (****$P<.0001$).

Lin28b reprograms adult CD8+ T cells to behave more like neonatal cells

Previous reports suggest that many key biological differences between fetal and adult HSCs can be attributed to changes in the expression of Lin28b, a conserved and developmentally regulated RNA-binding protein that represses the let-7 microRNA,[26,27] among other roles. Importantly, Lin28b is selectively expressed in fetal progenitors (supplemental Figure 6), and overexpression of Lin28b in adult BM cells promotes the development of fetal-like B cells (B-1a B cells, marginal zone B cells) and innate-like T cells (NKT cells, γδT cells).[28] If Lin28b underlies key developmental-related differences in CD8+ T cells, we reasoned that ectopic expression of Lin28 in adult progenitor cells would result in the development of "neonatal-like" cells. To test this, we generated BM chimeras by reconstituting lethally irradiated mice with gBT-I WT or iLin28b hematopoietic cells (Figure 6A). After reconstitution, robust expression of Lin28b was confirmed in CD8+ T cells (supplemental Figure 7A-B), and its expression was sufficient to downregulate all let-7 family members except let-7c, which is not subject to control by Lin28[29] (Figure 6B; supplemental Table 1). Phenotypic analysis of CD8+ T cells from chimeric mice revealed that forced expression of Lin28b in adult progenitor cells promotes the generation of MP cells that develop after thymic egress, reminiscent of neonatal cells (Figure 6C-D; supplemental Figure 7C-D). Notably, in mixed BM chimeras, wild-type (WT) CD8+ T cells produced in the same thymus as iLin28b CD8+ T cells did not give rise to more MP CD8+ T cells (supplemental Figure 8), suggesting that Lin28b drives the development of MP CD8+ T cells in a cell-intrinsic manner.

To determine whether Lin28b expression influences the fate of CD8+ T cells after infection, we adoptively cotransferred equal numbers of WT and iLin28b CD8+ T cells

from BM chimeras into adult recipients, and infected the recipients (Figure 6A). The data revealed that WT cells predominate as the immune response progressed. The few iLin28b CD8+ cells that remained at day 28 responded poorly to secondary challenge, and the recall response was entirely comprised of WT cells (Figure 6E). We also examined changes in the expression of KLRG1 and CD127 at the peak of the primary response, which revealed that most iLin28b cells expressed a SLEC phenotype, whereas WT cells exhibited a balance of SLEC and MPEC phenotypes (Figure 6F). Together, these data demonstrate that ectopic expression of Lin28b in adult progenitor cells enables them to give rise to CD8+ T cells that are phenotypically and functionally analogous to those found in neonates, indicating that increased expression of Lin28b in neonatal progenitor cells underlies their preferential terminal differentiation into SLECs during infection.

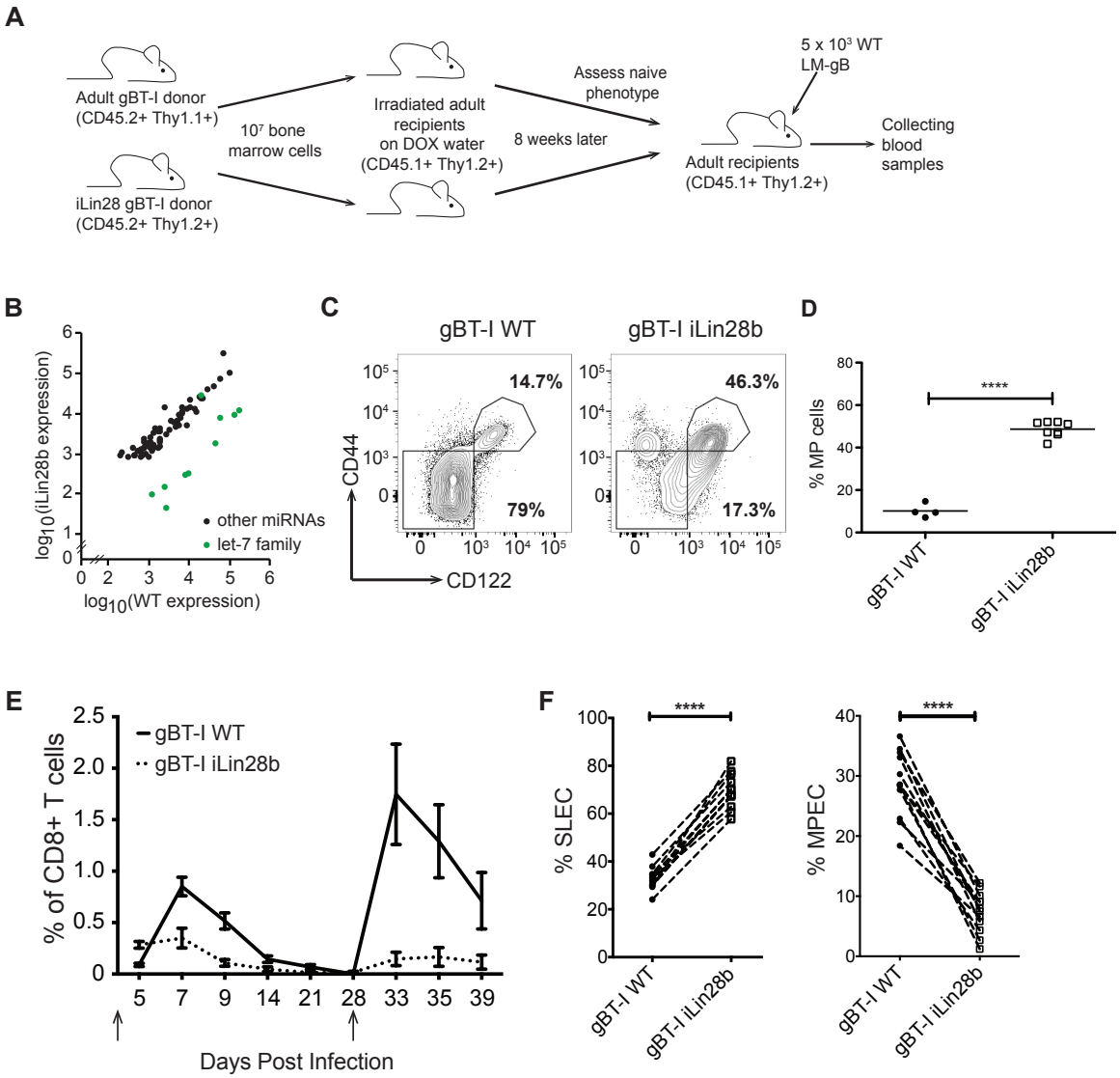

Figure 2.6. Lin28b reprograms adult CD8+ T cells to behave more like neonatal cells. (A) Schematic of experimental design: congenic gBT-I adult (Thy1.1) and iLin28b gBT-I adult (Thy1.2) BM cells were transferred into lethally irradiated congenic WT recipient mice (CD45.1) separately. Lin28b expression was induced by DOX in drinking water. Eight weeks later, splenic donor CD8+ T cells were sorted and adoptively cotransferred into congenic WT recipient mice (CD45.1). These recipients were infected with 5×10^3 WT LM-gB and serially bled to examine CD8+ T-cell responses. (B-D) Naïve phenotypes of gBT-I WT or gBT-I iLin28b CD8+ T cells before adoptive cotransfer. (B) Expression of miRNAs in adult WT gBT-I and adult iLin28b splenic donor CD8+ T cells at 8 weeks after reconstitution. (C) Representative plots showing CD44 and CD122 expression by gBT-I BM-derived splenic CD8+ T cells 8 weeks after reconstitution. (D) Statistical analysis of the percentages of MP cells from WT gBT-I adult and iLin28b adult CD8+ T cells. (E) Relative numbers of iLin28b gBT-I adult (dashed) and WT gBT-I adult (solid) BM-derived donor CD8+ T cells postinfection and re-challenge (5×10^4 WT LM-gB); arrows indicate days of infections. (F) Percentages of gBT-I adult and fetal progenitor-derived donor CD8+ T cells that are SLECs and MPECs. Data are representative of 2 experiments (****$P<.0001$).

Discussion

Previous work has demonstrated that the ontogeny of the immune system does not progress in a linear manner from fetal life to adulthood.[30-34] Instead, the prevailing model argues that distinct “steps” or “layers” develop sequentially from progenitor cells that are metabolically and epigenetically distinct.[35-39] As a consequence, unique populations of immune cells (B1a-B cells and dendritic epidermal T cells) are generated during different windows of development.[40-42] In this report, we provide compelling support for the existence of a distinct layer of CD8+ T cells that is produced in early life and demonstrate that neonatal and adult CD8+ T cells respond differently to infection because they are derived from distinct progenitor cells.

The “layered immune hypothesis” may also be relevant to the ontogeny of MP CD8+ T cells. Previous work has suggested that MP cells are driven by lymphopenia-induced proliferation during early stages of development.[22,43] However, our work demonstrates that fetal- derived CD8+ T cells preferentially acquire a memory phenotype, even when placed in the same environment as adult-derived cells (Figure 5). These findings suggest that MP cell development cannot be attributed solely to age-related changes in the environment and indicates that cell-intrinsic differences between neonatal and adult CD8+ T cells should also be considered. It will be important to determine whether sensitivity to homeostatic cytokines and/or proximity toward IL-7- and IL-15- dependent niches in lymphoid tissue is linked to their developmental origin. Indeed, perhaps MP cells in young adult mice are derived largely from the fetal layers of HSCs.

Intrathymic transfer experiments allowed us to control for differences in the

amount of post-thymic maturation. As recent thymic emigrants (RTEs) make up a larger percentage of naïve cells in neonatal mice (100%) compared with adult mice (~10%-20%)[44] and skew toward the short-lived effector lineage after infection,[45] there has been some speculation that neonatal CD8+ T cells produce fewer memory cells because they have yet to undergo a sufficient amount of post-thymic maturation. Although we were unable to directly compare neonatal and adult RTEs because of dramatic differences in the rates of homeostatic proliferation, it is worth pointing out that we have compared the progeny of fetal and adult progenitor cells after giving these cells the same length of post-thymic maturation (~4 weeks), and we still observed cell-intrinsic differences (Figure 5). Thus, although the age of the cell clearly influences its ability to respond to stimulation, the age of the animal in which the cell is created may ultimately be a greater determining factor for how a cell will respond after infection.

What are the molecular mechanisms that underlie the developmental switch from fetal to adult CD8+ T-cell production? Our work points toward a role for Lin28b in this process, which is highly expressed in fetal animals and then downregulated later in development. Previous reports have suggested that many posttranscriptional and metabolic differences between fetal and adult HSCs can be attributed to the expression of Lin28b.[26,46,47] One way in which Lin28b regulates fetal HSCs is by blocking the biogenesis of let-7 miRNAs, which control the developmental timing in a wide range of species.[28,48-51] More recently, let-7 has gained attention for its ability to behave as a tumor suppressor in human cancer cells by repressing cell proliferation,[52-54] potentially by targeting cell cycle factors and genes in the mTOR pathway.[9] In this report, we demonstrate that ectopic Lin28b expression in adult progenitor cells gives rise to CD8+

T cells that undergo more rapid proliferation and effector cell differentiation (Figure 6). To our knowledge, this is the first study demonstrating how Lin28 expression alters the CD8+ T-cell response to infection. However, additional studies are required to determine the degree to which Lin28b is controlling CD8+ T-cell responses by reducing expression of let-7, enhancing the translation of metabolic enzymes55 or other factors.[56,57] It will be essential to examine more closely the changes in gene expression at the individual cell level to uncover the molecular circuitry regulated by Lin28b and let-7 in CD8+ T cells. We are currently using both gain- and loss-of-function gene targeting approaches to identify these networks. Clearly, more work studying the role of let-7/Lin28 in CD8+ T-cell development is warranted.

It is intriguing to speculate whether Lin28b was co-opted during the evolution of mammalian immune system to serve as rheostat of effector and memory cell differentiation during stages of life. A common feature of lymphocytes derived from Lin28b[1] fetal HSCs is that they tend to respond more rapidly to infection than their adult-derived counterparts.[1,58] Responding with a more vigorous primary CD8+ T cell response may help protect the host at critical stages of immune development, but likely comes at the cost of not being able to form long- lived memory cells. However, because the neonatal CD8+ T-cell repertoire is extremely limited, it is likely that neonatal cells are of lesser value in the memory pool, because more “fit” CD8+ T cells will arise later in adulthood. Although this model remains to be tested, our data provide insight into the developmental-related factors that influence effector and memory CD8+ T-cell differentiation during periods of life.

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SUPPLEMENTARY FIGURES:

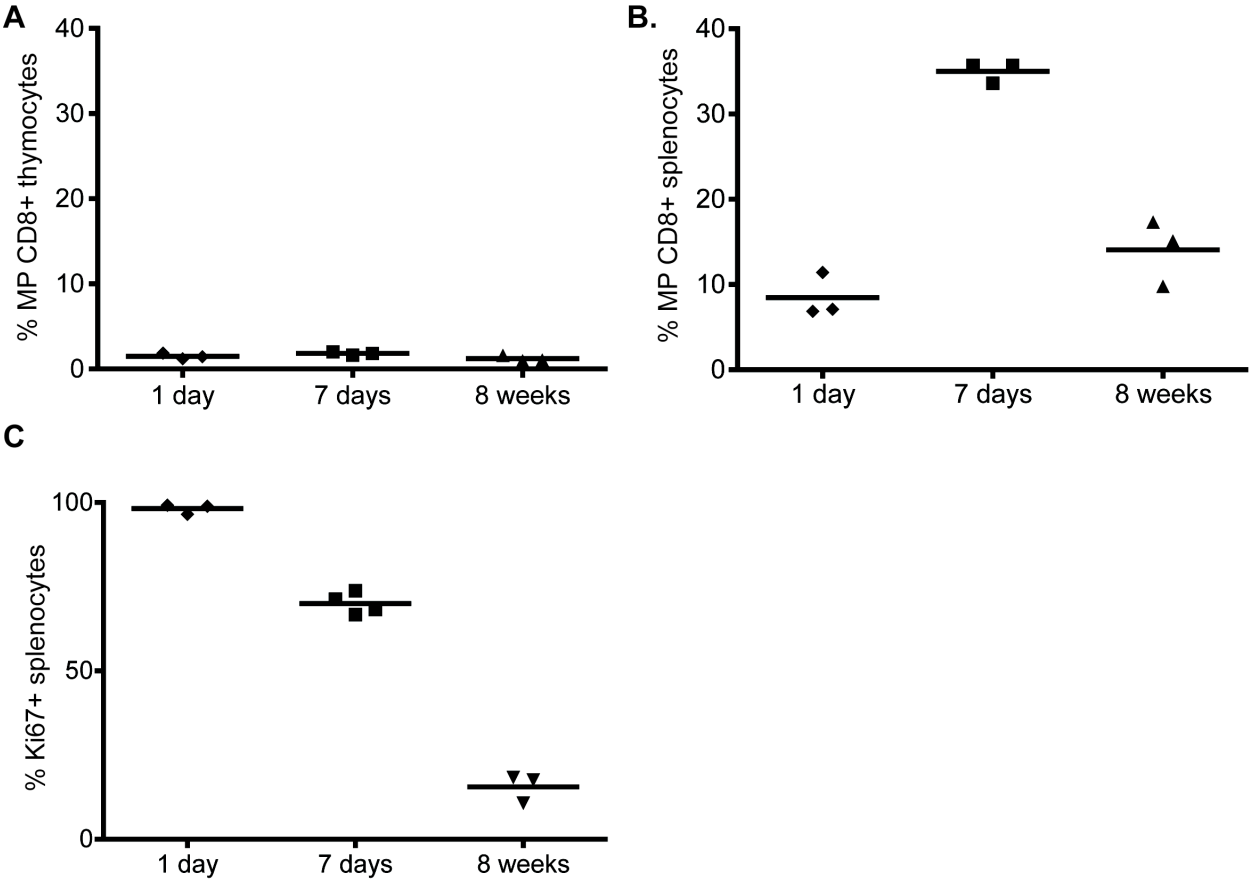

Figure 2.S1. Naïve CD8+ T cells acquire a memory phenotype in the periphery during early life. Percentages of thymic (A) and splenic (B) CD8+ T cells from uninfected gBT-I mice that exhibit a memory phenotype (MP, $CD44^{hi}$ $CD122^{hi}$) at various ages. (C) Relative number of splenic CD8+ T cells that are actively proliferating (Ki-67 positive) in different aged gBT-I mice. Data are representative of 2 experiments.

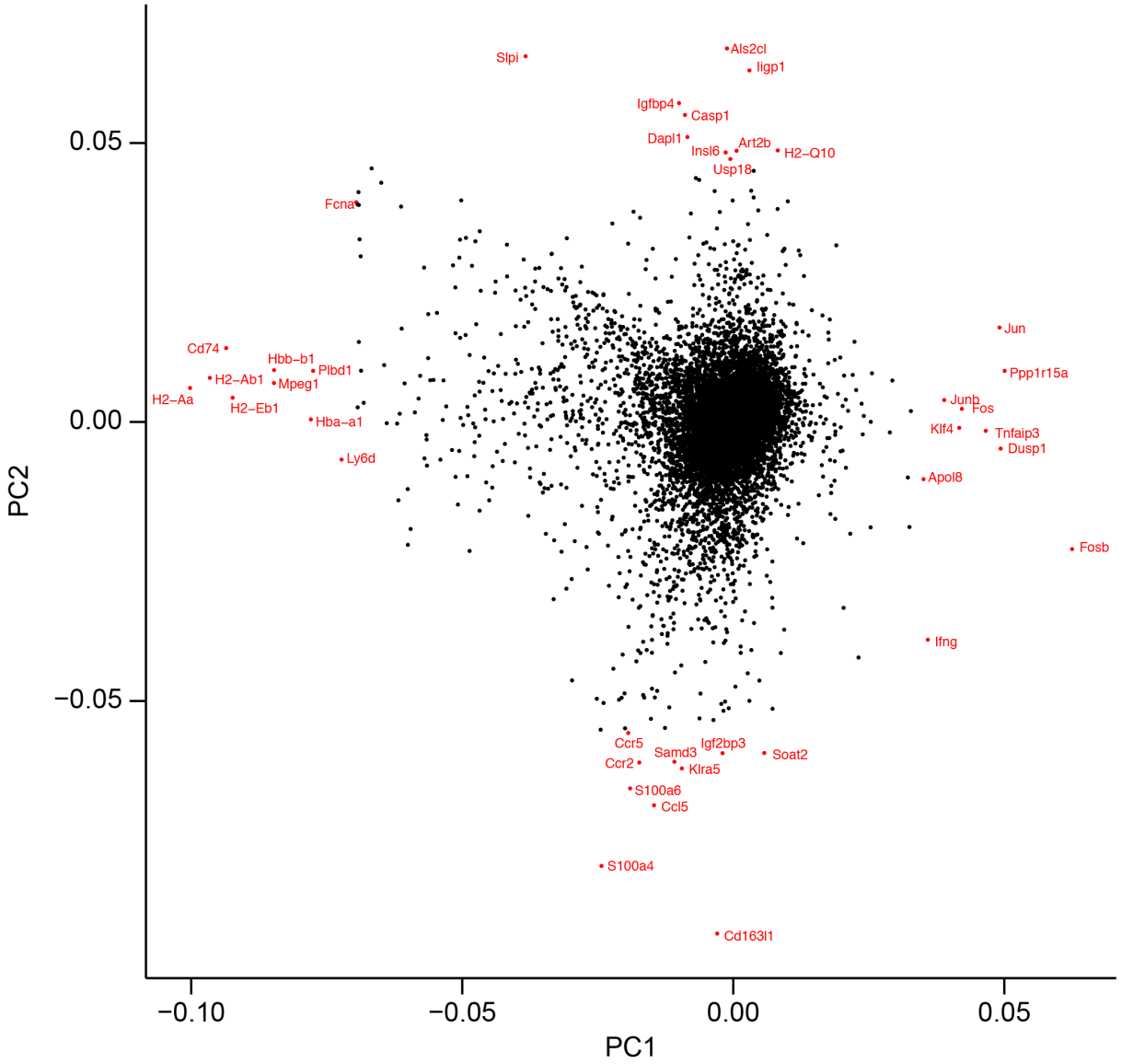

Figure 2.S2. Gene loadings for principal component analysis. The values for all genes corresponding to the samples used in Figure 2A are shown. Values on the x-axis correspond to the placement of samples on the x-axis; the same is true for the y-axis. In red are the genes with the highest or lowest values for PC1 and PC2, demonstrating they had the largest effect on the PCA.

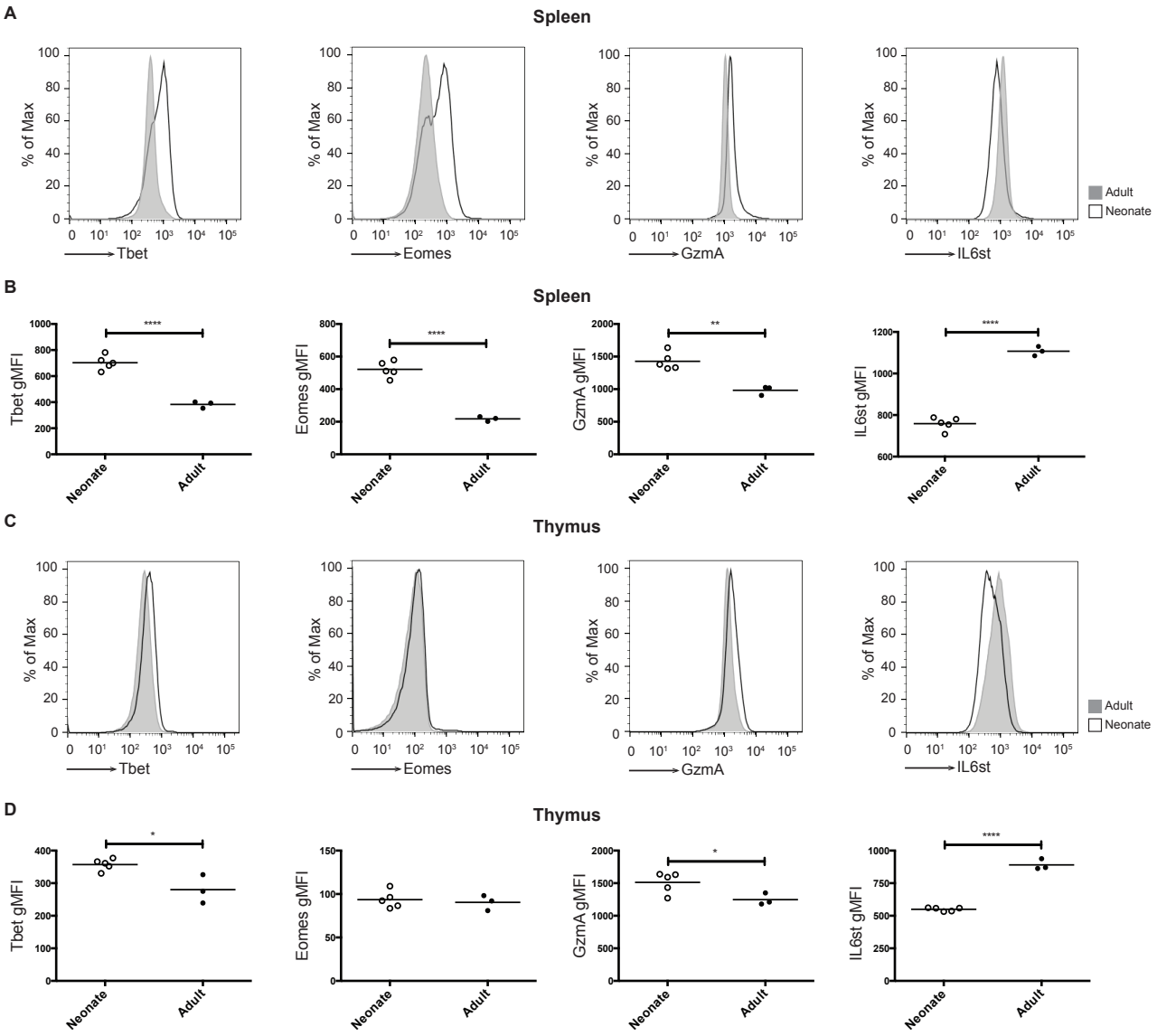

Figure 2.S3. Differential gene expression between neonatal and adult CD8+ splenocytes and thymocytes measured by flow cytometry. Flow cytometry analysis of CD8-enriched splenocytes (A-B) and bulk thymocytes (C-D) from neonatal (line) and adult (shaded) animals. Representative histograms shown in A, C, and geometric mean fluorescent intensities depicted in B, D; n=3-5 mice/group. Significance was assessed by student *t*-test; **P* < .05, ***P* < .01, ****P* < .001, *****P* < .0001.

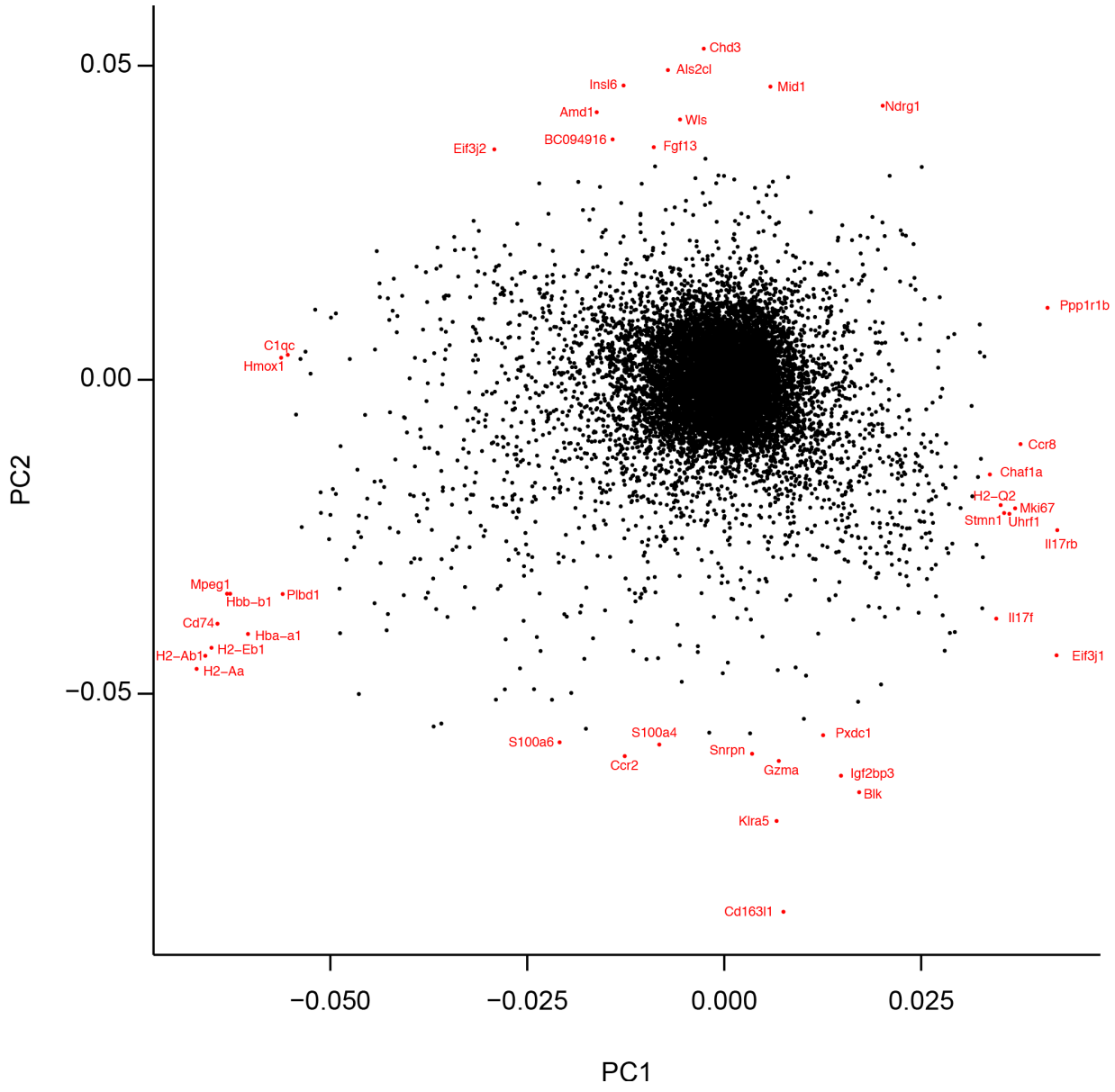

Figure 2.S4. Gene loadings for principal component analysis. The values for all genes corresponding to the samples used in Figure 4A are shown. Values on the x-axis correspond to the placement of samples on the x-axis; the same is true for the y-axis. In red are the genes with the highest or lowest values for PC1 and PC2, demonstrating they had the largest effect on the PCA.

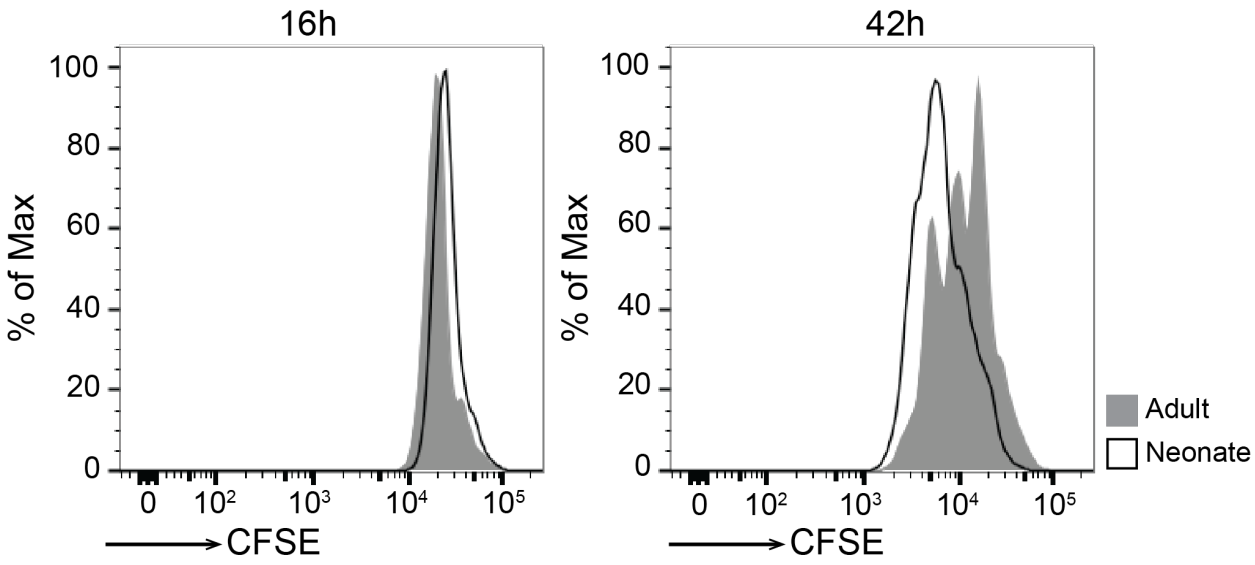

Figure 2.S5. Neonatal CD8+ thymocytes proliferate more than their adult counterparts following *in vitro* stimulation. Single positive CD8+ thymocytes from gBT-I adult (shaded) and neonatal (line) mice were labeled with CFSE and stimulated with plate-bound anti-CD3/anti-CD28. Data is representative of 2 experiments.

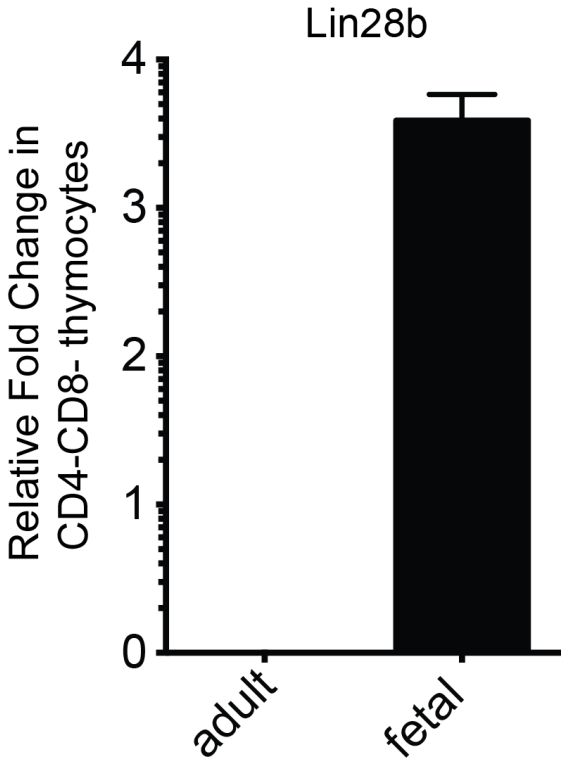

Figure 2.S6. Double-negative thymocytes preferentially express Lin28b in fetal life. RNA was isolated from double negative (DN) thymocytes obtained from fetal and adult gBT-I mice and the relative amount of Lin28b was examined by qPCR.

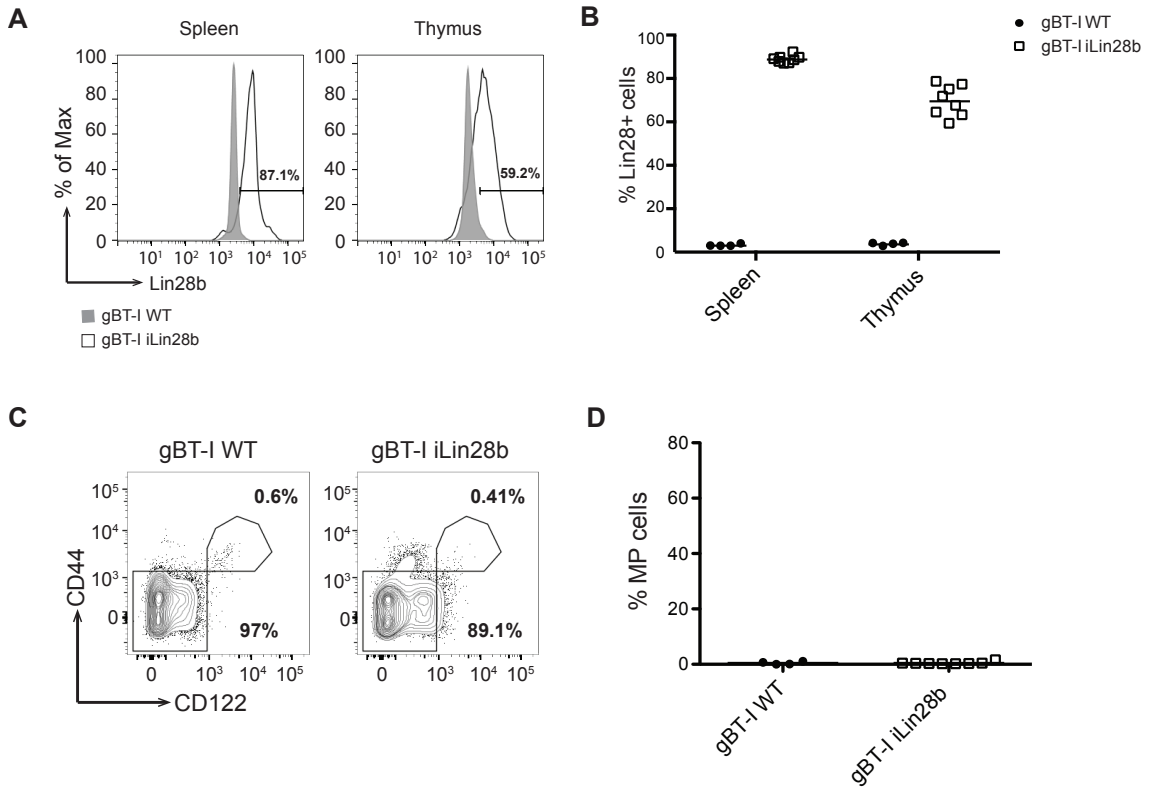

Figure 2.S7. Upregulation of Lin28b does not result in more memory phenotype cells in the thymus. (A) Representative flow cytometry data showing levels of human Lin28b in WT (shaded histogram) and iLin28b (open histogram) CD8+ T cells from the spleen and thymus of chimeric mice, and (B) percentages of Lin28b+ cells. Also depicted are representative plots (C) showing CD44 and CD122 expression in thymic CD8+ T cells, and (D) percentages of MP cells. Data is representative of 2 experiments.

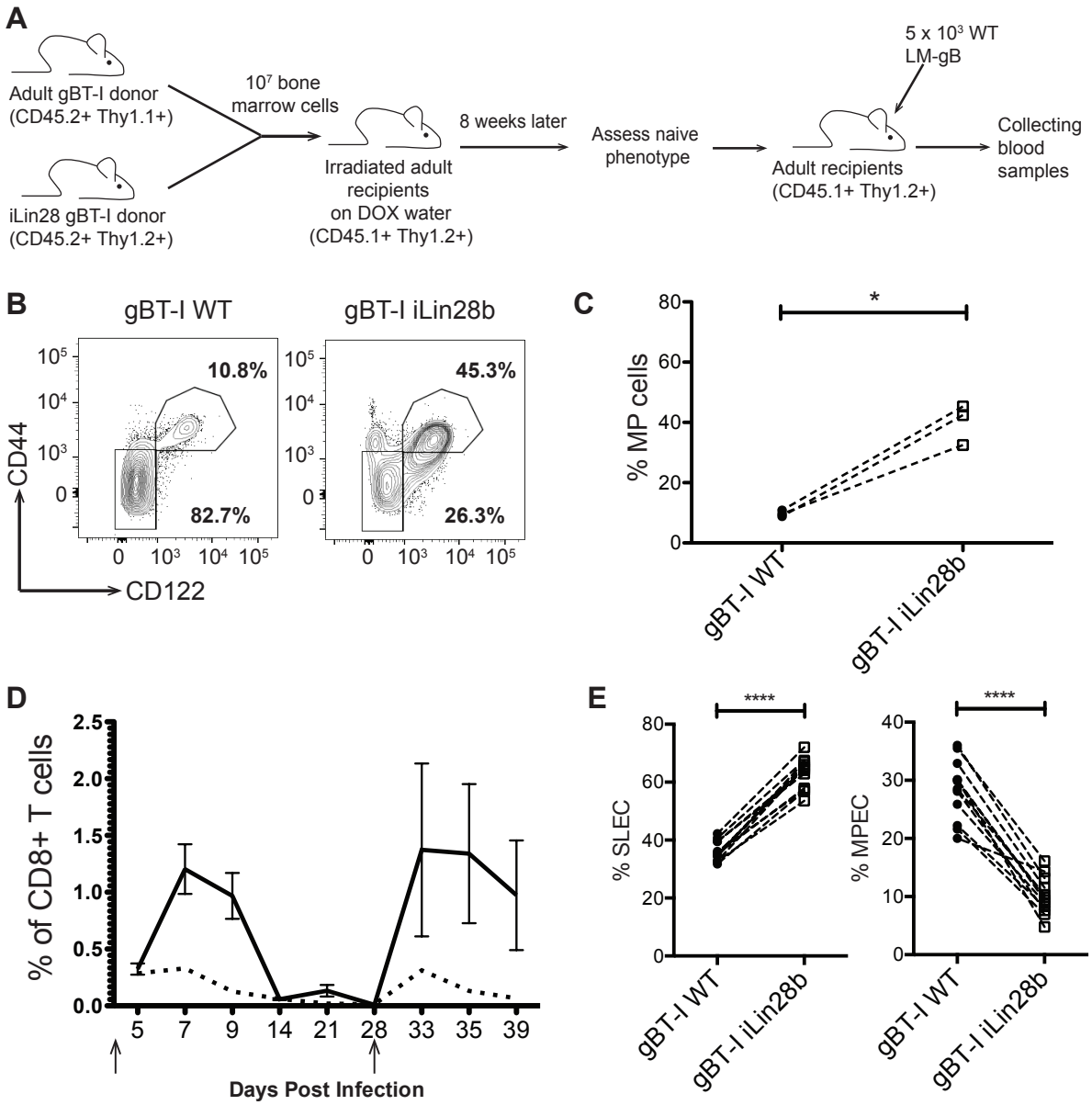

Figure 2.S8. Lin28b reprograms adult CD8+ T cells in a cell-intrinsic manner. (A) Schematic of experimental design: congenic gBT-I adult (Thy1.1) and iLin28b gBT-I adult (Thy1.2) bone marrow cells were co-transferred into the same lethally irradiated congenic WT recipient mice (CD45.1). Lin28b expression was induced by DOX in drinking water. 8 weeks later, bone marrow-derived splenic CD8+ T cells were sorted and adoptively co-transferred into congenic WT recipient mice (CD45.1). These recipients were infected with 5×10^3 WT LM-gB and serially bled to examine CD8+ T cell responses. (B-C) Naïve phenotypes of gBT-I WT and gBT-I iLin28b CD8+ T cells prior to adoptive co-transfer. (B) Representative plots showing CD44 and CD122 expression by gBT-I bone marrow-derived splenic CD8+ T cells, and (C) percentages of MP cells. (D) Relative numbers of iLin28b gBT-I adult (dashed) and WT gBT-I adult (solid) bone marrow-derived donor CD8+ T cells post infection and re-challenge (5×10^4 WT LM-gB); arrows indicate days of infections. (E) Percentages of WT gBT-I adult and iLin28b gBT-I adult CD8+ T cells that are SLECs and MPECs at 7dpi; **P* < .05, *****P* < .0001.

CHAPTER THREE

Adult and neonatal CD8+ T cells utilize different metabolism programs

SUMMARY

CD8+ T cells from neonatal and adult mice respond differently to infection. Upon activation, neonatal CD8+ T cells rapidly become more terminal differentiated and undergo more contraction than adult CD8+ T cells following pathogen clearance. However, the underlying mechanisms contributing to these differences are unclear. Historically, metabolic programs have been known to regulate a diverse array of cellular functions. In recent years, metabolism has become an intense area of investigation by immunologists and many studies have revealed the importance of different metabolic programs in T cell activation and differentiation. In this study, we show that neonatal CD8+ T cells acquire a more metabolically active status after stimulation, demonstrated by less OXPHOS and more glycolytic activities. We also found that the impaired development of neonatal memory CD8+ T cells could be rescued by limiting mTOR, which may be elevated in neonatal CD8+ T cell because of age-related differences in Lin28b expression. Collectively, our data suggest that neonatal CD8+ T cells preferentially become short-lived effector cells after infection because they exhibit an inherently more metabolically active program after antigenic stimulation.

INTRODUCTION

Neonates are more susceptible to repeated infections from the same intracellular pathogen because they exhibit insufficient immunological memory formation. CD8+ T cells are critical in eliminating intracellular pathogens from the hosts. Hence, it is important to gain insight into why neonatal CD8+ T cells respond differently to infections

than adults. Recent studies suggest that neonatal CD8+ T cells fail to form sufficient memory because they proliferate rapidly and quickly become terminal differentiated post-activation[1–3]. Neonatal and adult CD8+ T cells display these age-related differences because they are derived from distinct progenitor cells[1]. However, the underlying mechanisms that regulate these age-related different behaviors in CD8+ T cells are unknown.

The role of metabolic pathways in T cells was largely overlooked until recently. In the past few years, a tremendous amount of studies have demonstrated the critical regulatory role of metabolic programs in T cell response. Currently, the common notion in the field is that CD8+ T cells utilize different metabolic programs upon activation[4,5]. Naïve CD8+ T cells prefer using high ATP producing oxidative phosphorylation (OXPHOS) as their major energy source. Upon activation, OXPHOS is still utilized[6]. However, in order to meet the high demand of extensive proliferation, CD8+ T cells prefer to use catabolic glycolysis to provide sufficient building units to construct progeny cells[4,5,7]. This is a phenomena known as “Warburg Effect” that has been shown to be particularly important in rapidly proliferating cells[8]. In order to survive and differentiate into memory cells, CD8+ T cells need to acquire a more quiescent metabolic prolife by utilizing pathways like OXPHOS and fatty acid oxidation (FAO) to support their survival[4,7,9–11]. Since neonatal CD8+ T cells proliferate rapidly and fail to form sufficient memory post-activation, we hypothesize that neonatal CD8+ T cells preferably use a excessively active metabolic program upon activation, which results in a failure to acquire a more quiescent metabolic prolife needed to make memory cells.

To address this question, we measured the metabolic activities in different aged

CD8+ T cells post-infections. We found that neonatal CD8+ T cells demonstrated lower OXPHOS and higher glycolytic activities post-infection, which is consistent with our hypothesis. mTOR pathway is heavily involved with cellular metabolism and has been shown to regulate CD8+ T cell differentiation[12–14]. Hence, we limited mTOR activities to assess its role in regulating metabolic programs in neonatal CD8+ T cells and found that limiting mTOR allowed neonatal cells to behave more analogously to adult cells. Moreover, our data suggest that Lin28b may regulate CD8+ T behaviors through mTOR, implicating mTOR as a key regulator driving age-related differences in the CD8+ T cell response to infection.

MATERIAL AND METHODS

Mice

B6-Ly5.2/Cr mice were purchased from Charles River Laboratories (Frederick, MD). TCR transgenic mice specific for the HSV-1 glycoprotein B498-505 peptide SSIEFARL8 (gBT-I mice) were provided by Janko Nikolich-Zugich (University of Arizona, Tucson, AZ) and crossed with Thy1.1 or C57BL/6 mice purchased from Jackson Laboratories (Bar Harbor, ME). Neonatal and adult gBT-I animals were used at 5 to 7 days old and at 2 to 4 months old, respectively. Mice with a tetracycline-inducible copy of human *LIN28B* on a C57BL/6 background (iLin28b mice) were obtained from George Daley (Harvard University, Cambridge, MA)[15]. 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.

Antibodies and flow cytofluorimetric analysis

Antibodies were purchased from eBioscience (San Diego, CA), Biolegend (San

Diego, CA), Invitrogen (Carlsbad, CA), or BD Biosciences (Mountain View, CA). Flow cytofluorimetric data were acquired using DiVa software from an LSRII equipped with 4 lasers (BD Biosciences). Analysis was performed with FlowJo (Tree Star, Ashland, OR).

BM chimeras

Single BM chimeras were generated by obtaining BM from congenically marked gBT-I (Thy1.1+CD45.2+) and gBT-I iLin28b (Thy1.2+CD45.2+) mice. CD4+ and CD8+ BM cells were depleted by magnetic separation, and 1×10^7 cells were injected into lethally irradiated (990 rads) 7- to 8-week-old adult B6-Ly5.2 recipients. Forty-eight hours posttransplantation, mice were administered 0.1 mg/ml doxycycline with 10 mg/ml sucrose via drinking water to induce *LIN28B* expression for 6-8 weeks during reconstitution.

Adoptive transfer

Microbeads (Miltenyi Biotec) enriched gBT-I splenic neonatal and adult CD8+ cells, or FACS-sorted CD8+ cells from BM chimeras were combined at a 1:1 ratio. Combined cells were suspended at 5×10^5 cells per ml of PBS and 100 ul of cells was injected i.v. into adult B6-Ly5.2 recipient mice. The next day, recipient mice were infected with WT LM-gB (5×10^3 CFU, i.v.), as described.[2] For adoptive transfers in the presence of rapamycin, rapamycin (LC Laboratories or Chemie Tek) was given at 100 ng/g i.p. daily from 0-7 dpi.

Cell sorting

To purify CD8+ T cells from adoptive cotransfer recipient mice to perform cellular metabolism analysis (Agilent Seahorse), CD8+ T cells were enriched using microbeads (Miltenyi Biotec) and were subsequently labeled with antibodies against CD8, CD45.1,

CD45.2 and Thy1.1 and sorted to >95% purity on a fluorescence-activated cell sorting (FACS) Aria III (BD Biosciences). To isolate CD8+ T cells from chimeric mice, CD4+, CD19+, MHC-II+, and Ter119+ cells were depleted by negative immunomagnetic selection[1]. Cells were labeled with antibodies against CD8, CD45.1, CD45.2 and Thy1.1 and FACS-sorted to >95% purity for adoptive cotransfers. Two additional antibodies against CD127 and KLRG1 were used when sorting on SLEC populations from the donor cell groups.

Metabolism assays

3×10^5 cells plated in buffer-free media with 2 mM glutamine $\pm$ 25 mM glucose and $\pm$ 1 mM sodium pyruvate were used to measure oxygen consumption rates (OCR, with glucose and sodium pyruvate) or extra-cellular acidification rates (ECAR, no glucose and sodium pyruvate) using XFp Extracellular Flux Analyzer (Agilent Seahorse). OCR was measured following the addition of 1 uM oligomycin, 1 uM FCCP and 0.5 uM rotenone/antimycin A. ECAR was measured following the addition of 10 mM glucose, 1 uM oligomycin and 50 uM 2-Deoxy-D-glucose (2DG).

***In vitro* stimulation and proliferation**

Cells were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE), as described[2], and cells were stimulated with plate-bound anti-CD3 (5 mg/ml) and anti-CD28 (20 mg/ml).

Statistical analysis

Statistical analysis was performed using Prism (GraphPad Software, Inc, La Jolla, CA). Error bars represent standard error of the mean. Significance was determined by Student t test. Significance is denoted as: $*P < .05$, $**P < .01$, $***P <$

.001, and ****$P < .0001$.

RESULTS

Naïve adult and neonatal utilize different metabolic programs

First, we wanted to examine the metabolic programs utilized by naïve CD8+ T cells collected at different ages. Naïve CD8+ T cells were sorted from both 7-day-old neonate and 2-3 month old adult gBT-I mice to measure their oxygen consumption rate (OCR) and extracellular acidification rate (ECAR), which are indicators of OXPHOS and glycolytic activities, respectively. Surprisingly, naïve neonatal CD8+ T cells demonstrated higher basal and maximal OCR than naïve adult CD8+ T cells (Figure 1A and 1B), indicating higher OXPHOS activity in naïve CD8+ T cells. Naïve neonatal CD8+ T cells also have slightly higher spare respiratory capacity (SRC, difference between maximal and basal OCR) (Figure 1B). SRC is an indicator of how much extra capacity mitochondria have to provide energy upon increased demand. It has been shown to be positively correlated with memory T cell formation[10]. Naïve neonatal CD8+ T cells also exhibited lower basal and maximal ECAR than adult CD8+ T cells (Figure 1C and 1D), suggesting lower glycolytic activity in naïve neonatal CD8+ T cells. These differences could be attributed to the higher percentage of CD122+ (IL15 receptor) cells in naïve neonatal CD8+ T cells[1]. IL15 has been shown to promote mitochondrial biogenesis, which can result in higher OXPHOS level[10]. Collectively, our data suggest that neonatal CD8+ T cells display higher OXPHOS and lower glycolytic activities prior to infection.

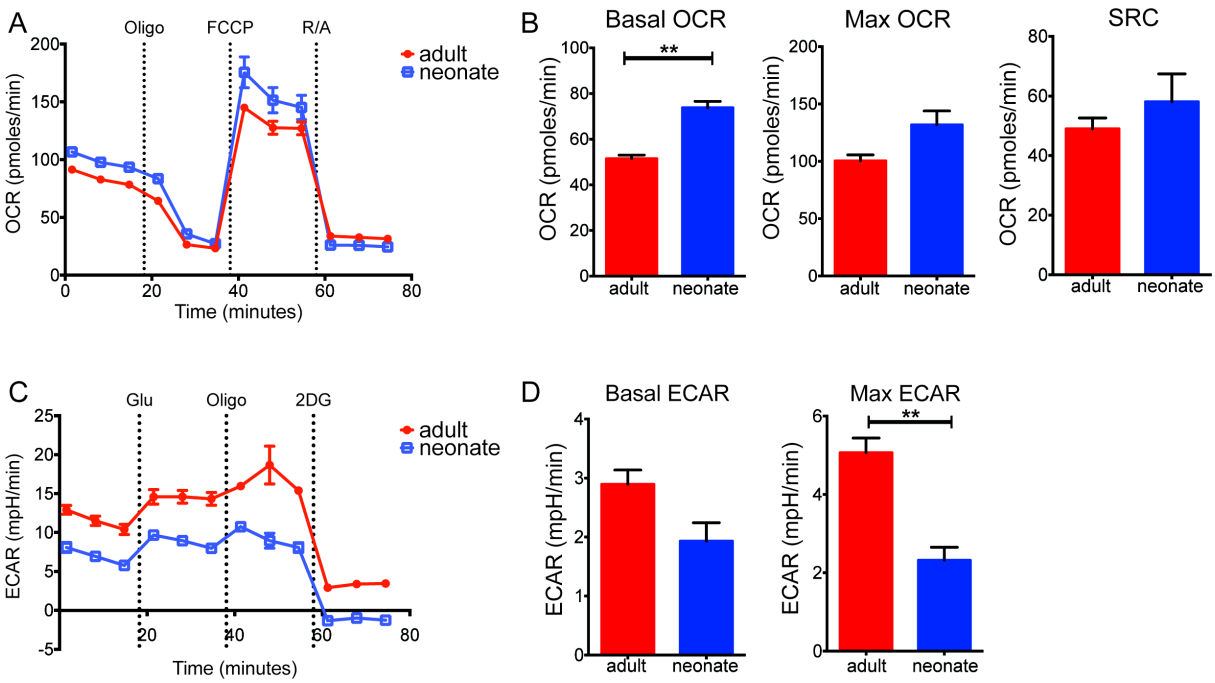

Figure 3.1. Naïve neonatal CD8+ T cells demonstrate higher OXPHOS and lower glycolytic activities compare to adult. (**A**) OCR measurements of sorted naïve adult and neonatal CD8+ T cells. (**B**) Statistical analysis of basal, max OCR and SRC of naïve adult and neonatal CD8+ T cells. (**C**) ECAR measurements of sorted naïve adult and neonatal CD8+ T cells. (**D**) Statistical analysis of basal and max ECAR of naïve adult and neonatal CD8+ T cells. Data are representative of 2 experiments. Significance was determined by student *t* test. ***P* < .01.

Neonatal CD8+ T cells demonstrate higher metabolic activities post-activation *in vitro*

Neonatal CD8+ T cells can undergo vigorous proliferation and quickly become terminal differentiated post-activation, which we hypothesize require support from more active catabolic pathways based on previous studies[4,5]. In an effort to uncover the specific metabolic program utilized by each age group, we first performed quantitive PCR on glycolytic proteins in adult and neonatal gBT-I CD8+ T cells collected at 8-hour post *in vitro* stimulation (Figure 2). Glut1 encodes glucose transporter, which is very critical in managing glucose uptake. Mutation in Glut1 can result in systemic disorders[16]. HK2 is one of the glycolytic enzymes we examined, and it encodes hexokinase 2, which

mediates the first step in glucose metabolism and has been shown to be up-regulated in cancer cells with Warburg Effect[17]. GPI, TPI and LDHa are other glycolytic enzymes we examined (Figure 2). MCT encodes another transporter transferring lactate outside of the cell membrane[18]. We found that the mRNA of all the major components and enzymes involved in glycolysis were highly up-regulation post-activation in neonatal CD8+ T cells, which indicate that neonatal CD8+ T cells have the potential to launch a more metabolic active program upon stimulation.

We then investigated how metabolites from major pathways are regulated post-activation. We performed liquid chromatography/tandem mass spectrometry based metabolomics on adult and neonatal gBT-I CD8+ T cells to profile their metabolites post *in vitro* stimulation at 18-hour (Figure 3). We found that activated neonatal CD8+ T cells demonstrated higher level of most of the intermediate metabolites in glycolysis (Figure 3). In contrast, the metabolites involved in OXPHOS (TCA cycle) were not significantly different in neonatal and adult CD8+ T cells, implying that OXPHOS activity either preferentially increased in adults cells or decreased in neonatal cells following activation (Figure 1 and 3).

Collectively, our data suggest that neonatal CD8+ T cells could quickly acquire a more metabolic active profile compare to their adult counterparts upon activation.

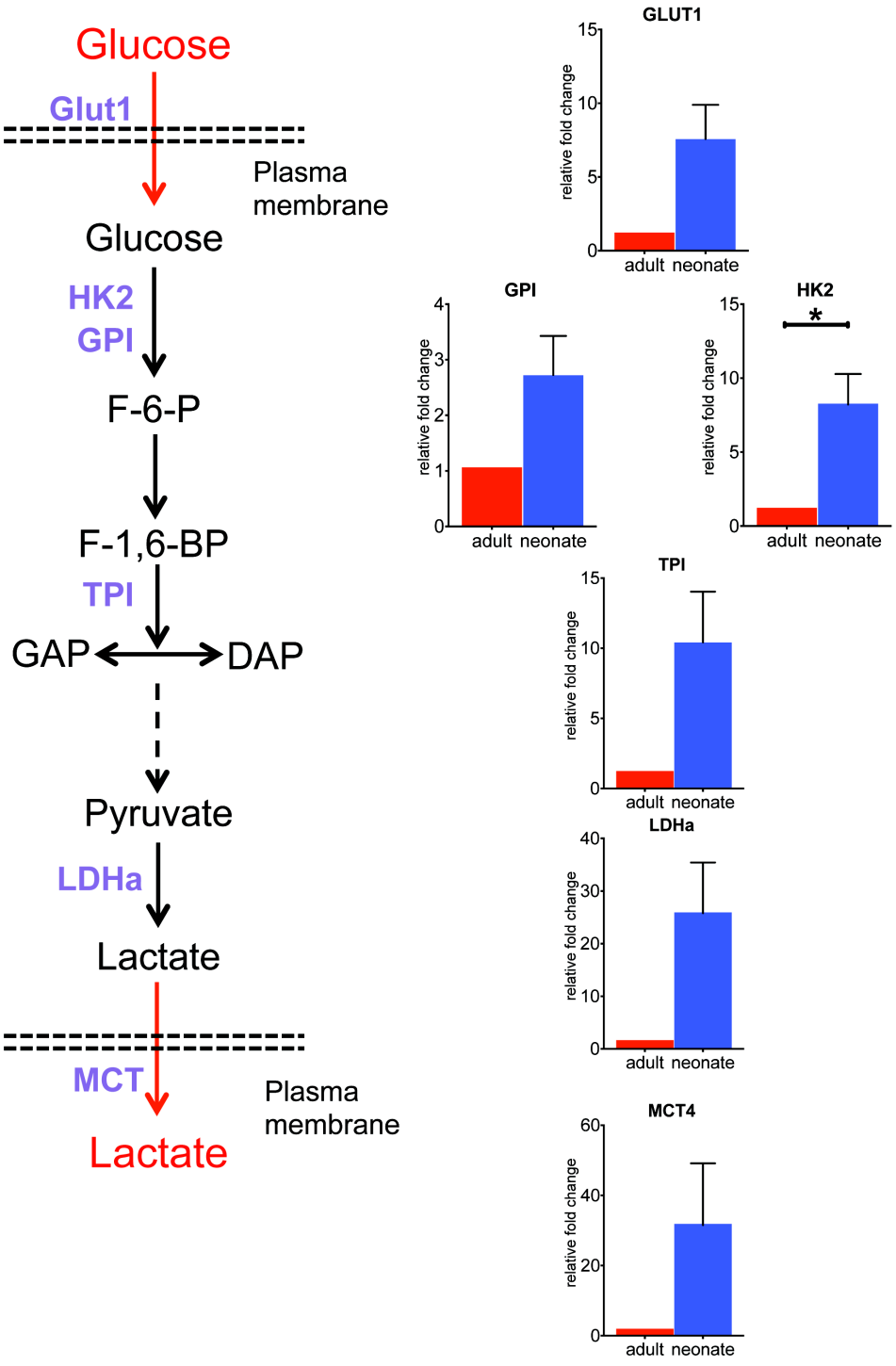

Figure 3.2. mRNA of glycolytic proteins are up-regulated in neonatal CD8+ T cells post-activation *in vitro*. mRNA level of several important proteins were measured using quantative PCR at 8-hour post plate bound αCD3/CD28 stimulation. Significance was determined by student *t* test. **P* < .05.

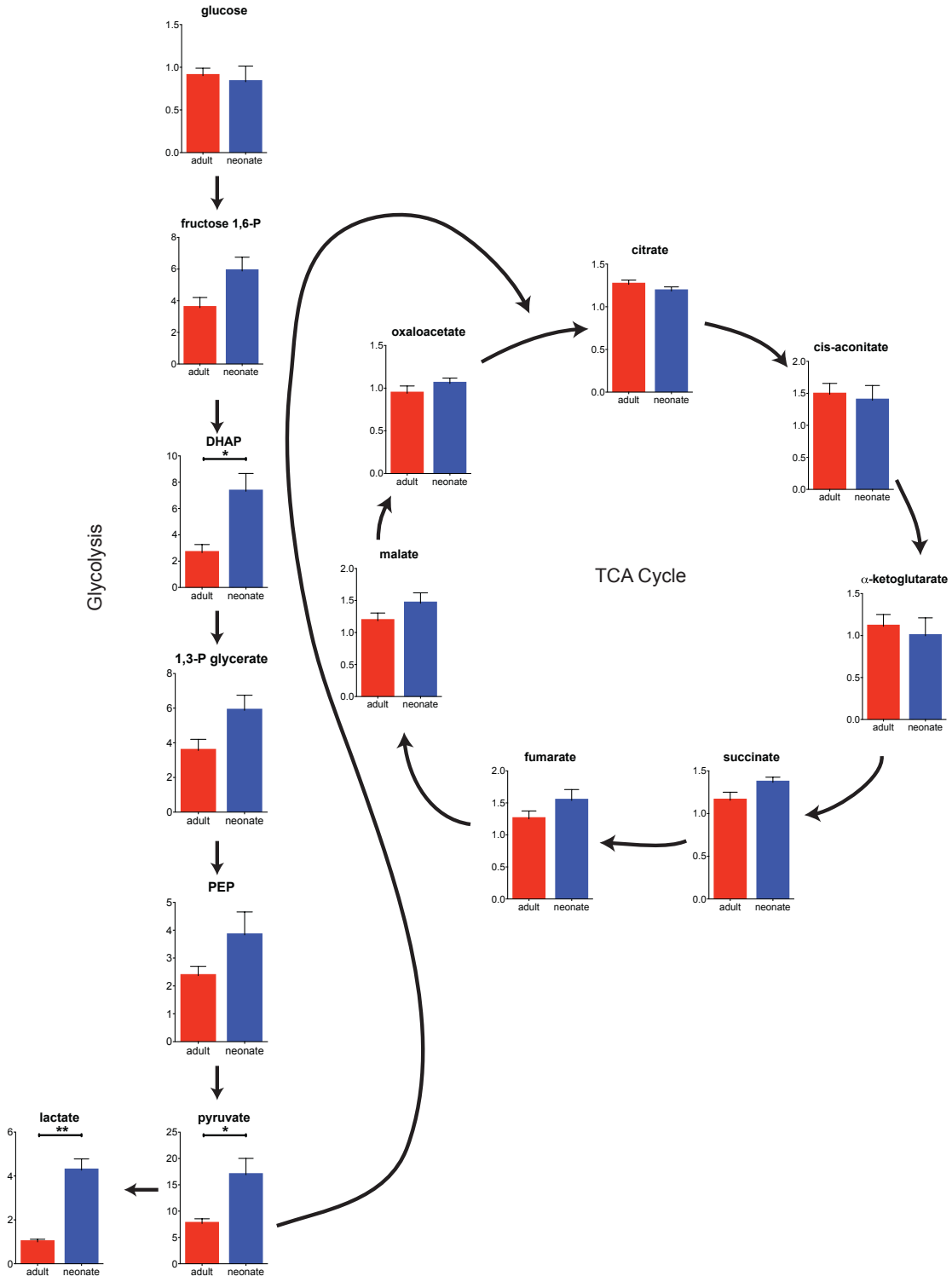

Figure 3.3. Metabolomics measurements of metabolites in OXPHOS and glycolytic pathways post-activation *in vitro*. Metabolomics was performed on adult and neonatal gBT-I CD8+ T cells 18-hour post-activation *in vitro*. Data has been normalized to measurements collected at naïve stage. Significance was determined by student *t* test. *$P < .05$ and **$P < .01$.

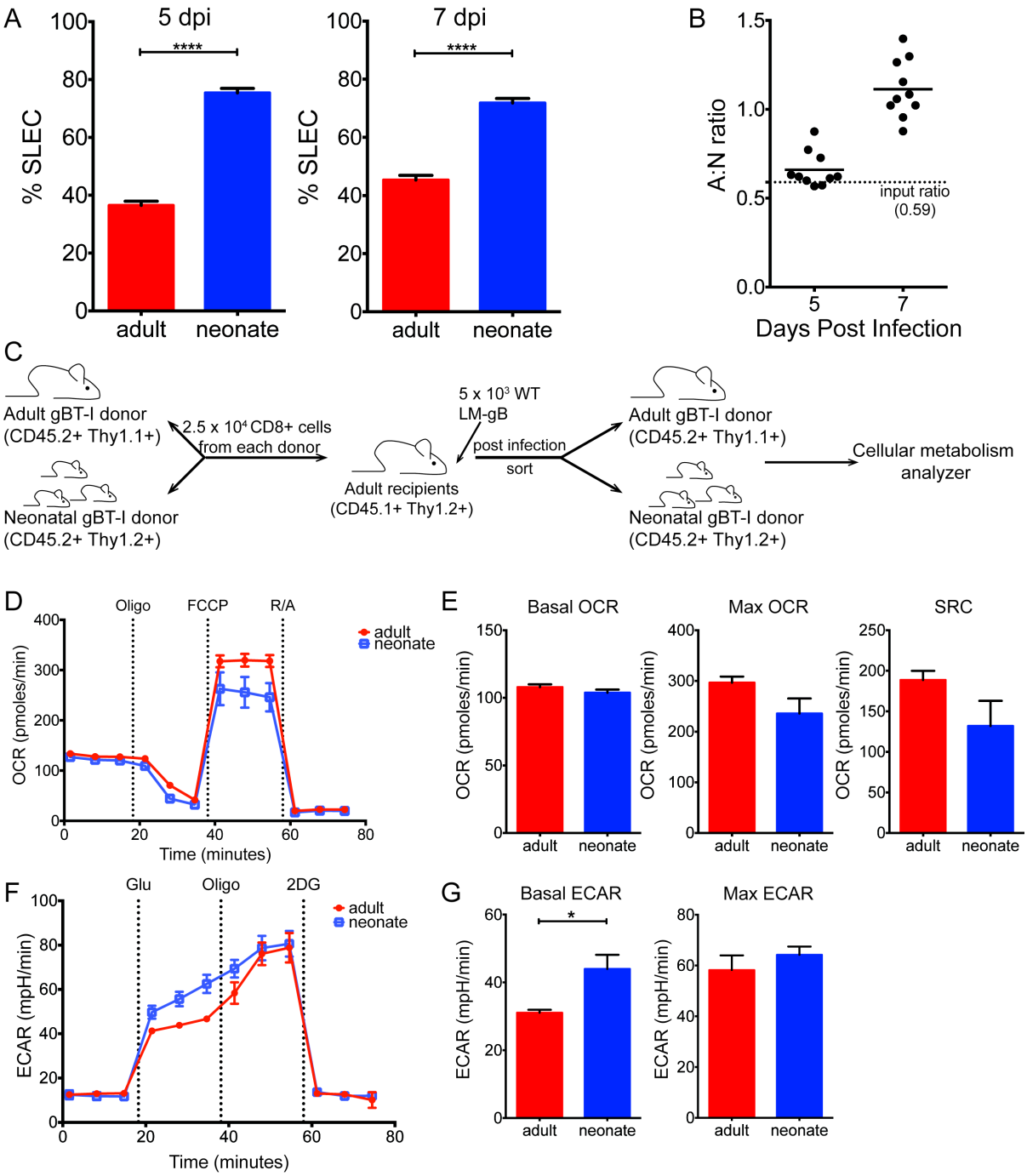

Figure 3.4. Infected neonatal CD8+ T cells have higher glycolytic and lower OXPHOS activities prior to the peak of response. (**A**) SLEC populations of adult and neonatal CD8+ T cells on 5 and 7 dpi. (**B**) Ratios of adult to neonatal CD8+ T cells on 5 and 7 dpi. (**C**) Schematic of experimental design: adoptively co-transferred adult and neonatal CD8+ T cells were sorted for metabolic analysis. (**D**) OCR measurements of 5 dpi adult and neonatal CD8+ T cells. (**E**) Statistical analysis of basal, max OCR and SRC of adult and neonatal CD8+ T cells on 5 dpi. (**F**) ECAR measurements of 5 dpi adult and neonatal CD8+ T cells. (**G**) Statistical analysis of basal and max ECAR of 5 dpi adult and neonatal CD8+ T cells. Data are representative of 2 experiments. Significance was determined by student *t* test. **P* < .05.

Neonatal CD8+ T cells acquire a less quiescent program upon infection

Next, we wanted to assess the metabolic changes in different aged CD8+ T cells upon infection directly. We first assessed different aged CD8+ T cell responses upon listeria monocytogenes-gB (LM-gB) infection. Consistent with previously studies[2], neonatal CD8+ T cells quickly became more terminal differentiated and contract more rapidly than adult CD8+ T cells post-infection. Neonatal CD8+ T cells have higher percentages of more terminally differentiated short-lived effector precursors (SLECs, KLRG1+CD127-) on 5 and 7 days post infection (dpi) than their adult counterparts (Figure 4A). Adult to neonatal CD8+ T cells ratios increased post-infection, indicating that neonatal CD8+ T cells peak sooner than adults within the same period post-infection (Figure 4B). Adoptively co-transferred adult and neonatal CD8+ T cells were sorted back out on 5 and 7 dpi to measure their metabolic activities (Figure 4C). Early after infection, neonatal CD8+ T cells started to have lower OXPHOS activities indicated by lower basal and maximal OCR measurements compared to adults on 5 dpi (Figure 4D and 4E). Neonatal CD8+ T cells also showed lower SRC than adults (Figure 4E). Lower neonatal SRC implied that they have lower memory formation potential, which is consistent with previous findings[2]. Glycolytic activity in neonatal CD8+ T cells also started to increase on 5 dpi, indicated by higher basal ECAR (Figure 4F and 4G).

On 7 dpi, the adult peak of response post-infection, the differences in metabolic programs between different aged CD8+ T cells became more drastic (Figure 5). Neonatal CD8+ T cells showed significantly higher basal and maximal OCR than adult CD8+ T cells, suggesting higher OXPHOS activity in neonatal CD8+ T cells (Figure 5A and 5B). Neonatal CD8+ T cells exhibited much lower SRC than their adult counterparts

(Figure 5B), which implies that as infection progresses neonatal CD8+ T cells quickly lose their potential to form memory cells. Basal and maximal ECAR also increased significantly in neonatal CD8+ T cells, indicating neonatal T cells utilize a lot more glycolysis at the peak of infection (Figure 5C and 5D). Overall, our data showed that upon infection, neonatal CD8+ T cells preferably using glycolysis more than OXPHOS and quickly lose memory forming potential. This is consistent with previously shown rapid proliferation and quick terminal differentiation in activated neonatal CD8+ T cells.

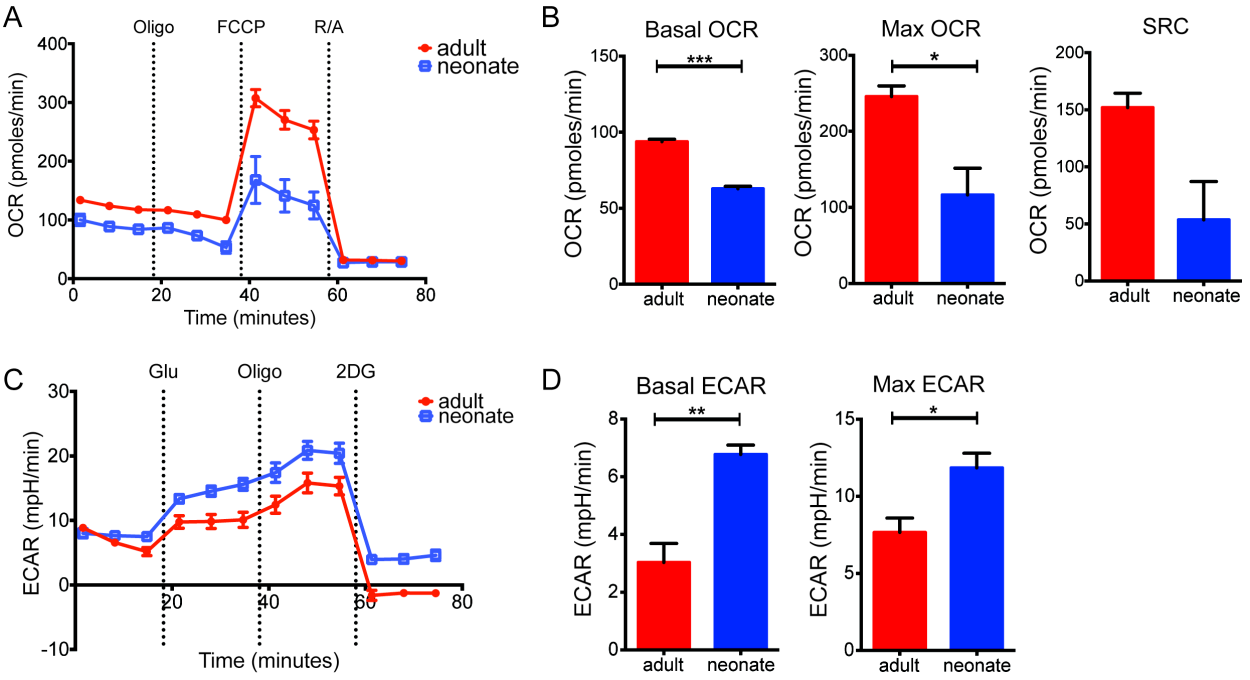

Figure 3.5. Infected neonatal CD8+ T cells have higher glycolytic and lower OXPHOS activities at the peak of response. (**A**) OCR measurements of 7 dpi adult and neonatal CD8+ T cells. (**B**) Statistical analysis of basal, max OCR and SRC of adult and neonatal CD8+ T cells on 7 dpi. (**C**) ECAR measurements of 7 dpi adult and neonatal CD8+ T cells. (**D**) Statistical analysis of basal and max ECAR of 7 dpi adult and neonatal CD8+ T cells. Data are representative of 2 experiments. Significance was determined by student *t* test. *P < .05, **P < .01, and ***P < .001.

Neonatal and adult effector precursors utilize different metabolic programs

Neonatal CD8+ T cells exhibit a higher percentage of terminally differentiated SLECs than adult CD8+ T cells. In order to test whether the more active metabolic

program (lower OXPHOS and higher glycolytic) in neonatal donor CD8+ T cells can be explained by the higher amount of SLECs, KLRG1+CD127- CD8+ T cells were sorted from recipient mice on 7 dpi and cell numbers were normalized to compare their metabolic activities (Figure 6A, B). Interestingly, neonatal CD8+ T cells still showed higher OXPHOS activity demonstrated by higher basal and maximal OCR (Figure 6C and 6D). Neonatal SLECs displayed lower SRC compared with adult SLECs suggesting lower memory forming potential (Figure 6D). The basal ECAR also increased in neonatal SLEC cells indicating higher glycolytic activity (Figure 6E and 6F). These findings are consistent with the observed metabolic differences in bulk adult and neonatal CD8+ T cells (Figure 4 and 5). Thus, the more metabolic active program observed in neonatal donor CD8+ T cells upon infection does not appear to be due to the higher percentage of more terminal differentiated SLECs at the peak of the response, but instead because they are intrinsically different than adult CD8+ T cells.

Lin28b regulates CD8+ T cell development through mTOR

Previously, we have shown that Lin28b plays an important role in regulating the neonatal CD8+ T cell response[1]. Lin28b is highly expressed in early life and plays an important role in fetal hematopoiesis[19,20]. When Lin28b is overexpressed in adult CD8+ T cells, they can acquire a neonatal CD8+ T cell phenotype and become more terminal differentiated post-infection[1]. Lin28b can also promote proliferation in adult gBT-I CD8+ T cells upon *in vitro* stimulation compare to wild type (WT) adult gBT-I CD8+ T cells (Figure 7A).

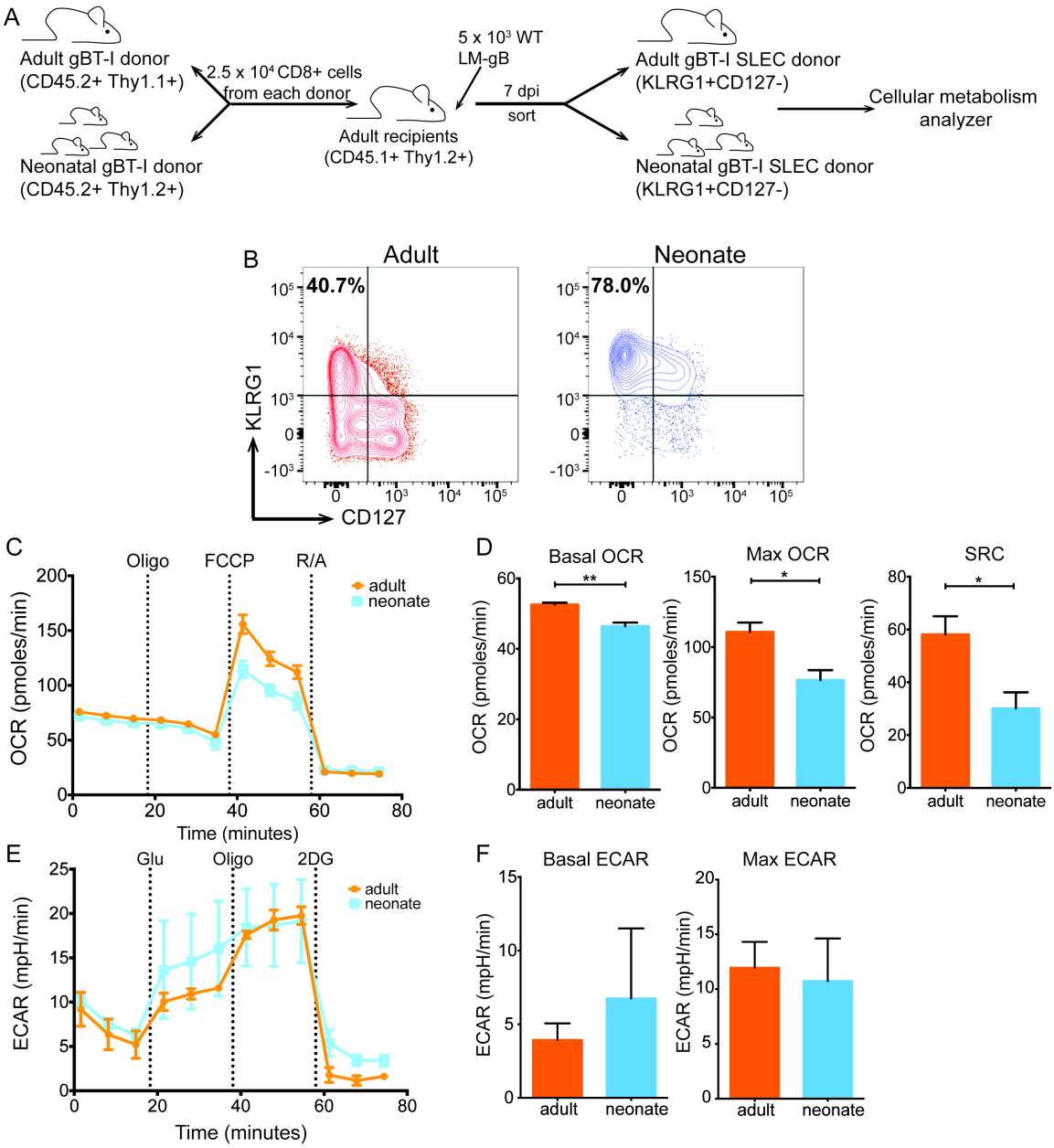

Figure 3.6. Neonatal SLEC CD8+ T cells have higher glycolytic and lower OXPHOS activities at the peak of response. (**A**) Schematic of experimental design: SLEC populations from adoptively co-transferred adult and neonatal CD8+ T cells were sorted for metabolic analysis. (**B**) Gating strategy of adult and neonatal SLECs (KLRG1+CD127-). (**C**) OCR measurements of 7 dpi adult and neonatal SLEC CD8+ T cells. (**B**) Statistical analysis of basal, max OCR and SRC of SLEC adult and neonatal CD8+ T cells. (**C**) ECAR measurements of 7 dpi adult and neonatal SLEC CD8+ T cells. (**D**) Statistical analysis of basal, max ECAR and glycolysis reserve of SLEC adult and neonatal CD8+ T cells. Significance was determined by student *t* test, **P* < .05 and ***P* < .01.

However, the mechanism of how Lin28b influences the fate of CD8+ T cells after infection still remains unclear. Mammalian target of rapamycin (mTOR) is one of the

proposed targets of Lin28, and recent data has indicated that mTOR is a major regulator in CD8+ T cell metabolism and function[13,21–23]. Lin28 has been proposed to promote mTOR activities which lead to increased glycolysis[15]. Here, we wanted to assess whether the impact of Lin28b in adult CD8+ T cells is mediated by mTOR activity. To do that, we utilized the same doxycycline-inducible Lin28b mouse model as described before[1]. We induced Lin28b expression in adult gBT-I CD8+ T cells (iLin28b) and compared their behaviors with WT adult CD8+ T cells. WT and iLin28b adult CD8+ T cells were adoptively co-transferred into the same recipients. Rapamycin or PBS was given daily for 7 days post-infection (Figure 7B). Consistent with previous study, adult CD8+ T cells with ectopic expression of Lin28b responded to infection in a more neonatal-like manner with more rapid contraction and insufficient memory formation (Figure 7C, left panel). Interestingly, in the presence of rapamycin, iLin28b adult CD8+ T cells formed similar level secondary response post-challenge as their WT adult counterparts, which suggest that limiting mTOR activities in CD8+ T cells can rescue the impaired development of Lin28b-induced CD8+ T cells (Figure 7C, right panel). This data is consistent with previous reports indicating that mTOR is a downstream target of Lin28[15,24,25]. We also examined how rapamycin influences the kinetics and phenotype of adult CD8+ T cells induced with Lin28b. In line with previous observations, we found that Lin28b promotes terminal differentiation of effector CD8+ T cells and limited their ability to transition into the long-lived memory pool. However, in the presence of rapamycin, Lin28b-induced cells appear more phenotypically similar to wild-type cells (Figure 7D). Also, limiting mTOR rescued the ability of Lin28b-induced CD8+ T cells to form memory CD8+ T cells (Figure 7E). These data suggested that rapamycin

promotes development of Lin28b-induced memory CD8+ T cells by limiting terminal differentiation and enhancing survival during contraction.

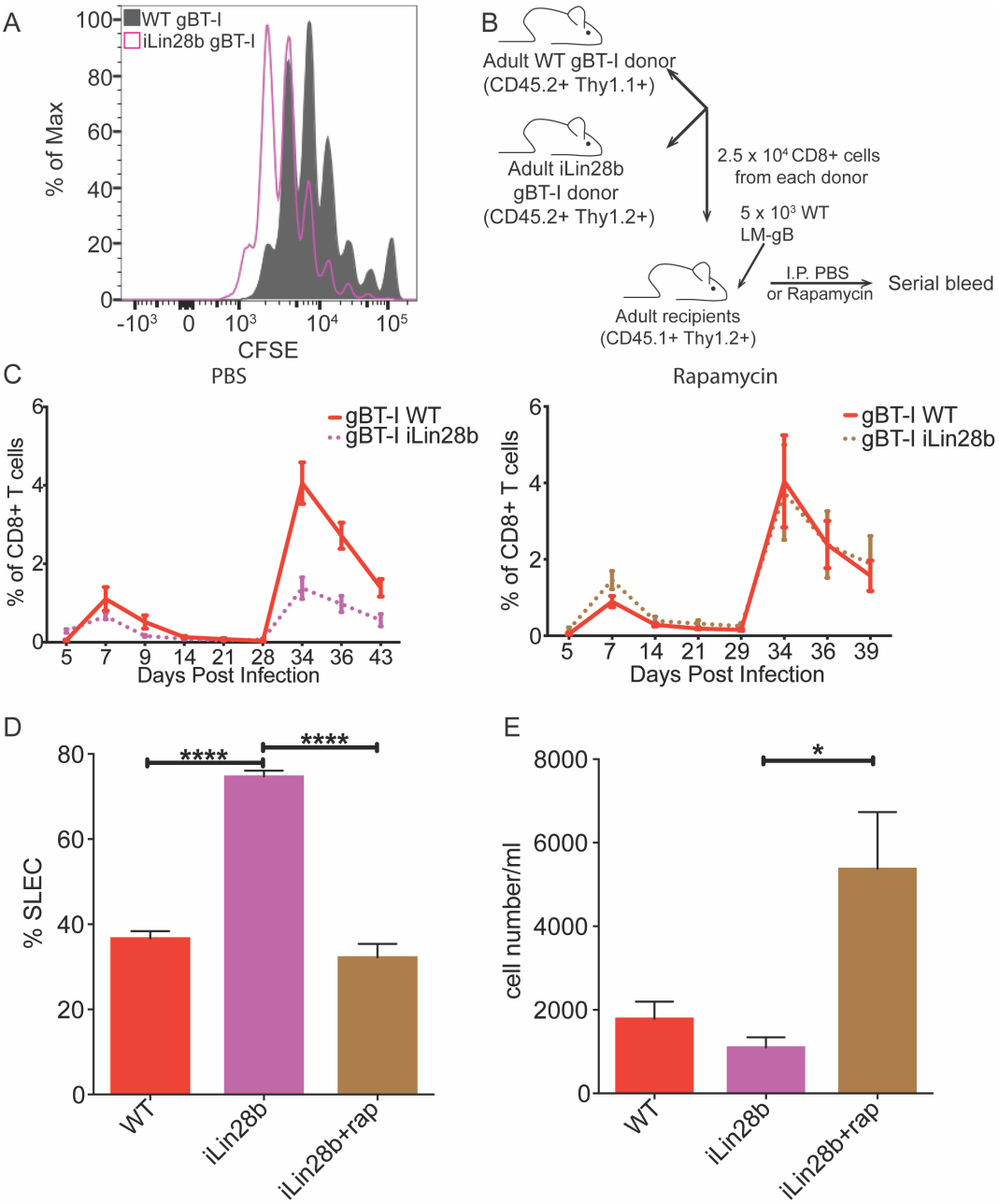

Figure 3.7. Lin28b regulates CD8+ T cell development through mTOR. (**A**) Overexpressing Lin28b promote proliferation in adult CD8+ T cells *in vitro* at 68 hour post-stimulation. (**B**) Experimental design: WT and iLin28b gBT-I CD8+ T cells were sorted from bone marrow chimera and adoptively co-transferred into recipients for serial bleed. PBS or rapamycin were given I.P. post-LmgB infection for 7 days. (**C**) SLEC populations of WT and iLin28b adult CD8+ T cells with and without rapamycin on 7 dpi. (**D**) Cell number of of WT and iLin28b adult CD8+ T cells with and without rapamycin on 21 dpi. Data are representative of 2 experiments. Significance was determined by student *t* test. **P* < .05 and *****P* < .0001.

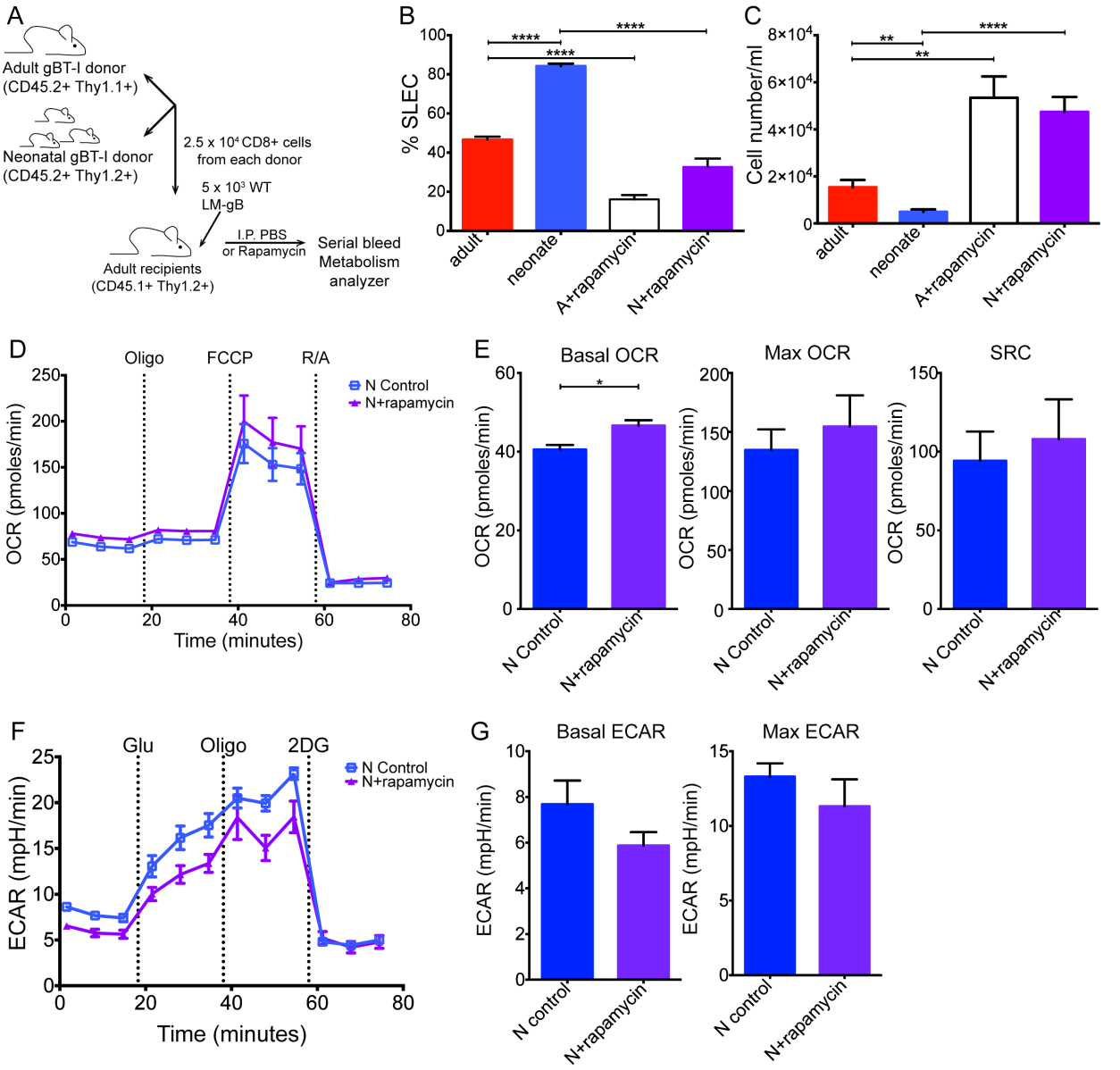

Figure 3.8. Limiting mTOR activities improve neonatal CD8+ T cells survival and alter their metabolism. (**A**) Schematic of experimental design: adoptively co-transferred adult and neonatal CD8+ T cells in the presence or absence of rapamycin were bled or sorted for metabolic analysis. (**B**) SLEC populations of different groups with and without rapamycin. (**C**) CD8+ T cell number of different groups with and without rapamycin on 25 dpi. (**D**) OCR measurements of 7 dpi neonatal control and neonatal+rapamycin CD8+ T cells. (**E**) Statistical analysis of basal, max OCR and SRC of (D). (**F**) ECAR measurements of 7 dpi neonatal control and neonatal+rapamycin CD8+ T cells. (**G**) Statistical analysis of basal, max ECAR and glycolysis reserve of (F). Data are representative of 2 experiments. Significance was determined by student *t* test. **P* < .05, ***P* < .01, and *****P* < .0001.

Limiting mTOR activities improve neonatal CD8+ T cells survival and alter their metabolism

Up to this point, we have showed that different aged CD8+ T cells utilize different metabolic programs post-infection, and Lin28b potentially regulate CD8+ T cells responses through mTOR. However, the role of mTOR and Lin28b in CD8+ T cells were restricted to adult CD8+ T cells. Thus, a key remaining question is whether we can make neonatal CD8+ T cells behave more similarly to adult CD8+ T cells by manipulating mTOR or their metabolic activities.

To investigate the link between mTOR and metabolism in neonatal CD8+ T cells, we first used rapamycin to limit mTOR activity in different aged CD8+ T cells post-infection in adoptively co-transferred recipients (Figure 8A). In the presence of rapamycin, both adult and neonatal CD8+ T cells became less terminal differentiated with smaller amount of SLECs than the control groups (Figure 8B). Next, we sought to examine whether limiting mTOR activity improved neonatal cell survival post-infection. We enumerated the cell numbers of all groups at during the resting memory stage on 25 dpi. We found that limiting mTOR activity significantly increased the numbers of neonatal memory CD8+ T cells on 25 dpi, which suggest that control neonatal CD8+ T cells fail to form memory because of an excessive amount of mTOR activity (Figure 8C).

To understand how mTOR alters neonatal CD8+ T cell metabolism, we sorted neonatal CD8+ T cells treated with PBS and rapamycin from infected recipient animals on 7 dpi and measured their glycolytic and OXPHOS activities. In the presence of rapamycin, neonatal CD8+ T cells displayed higher OXPHOS with higher basal and maximal OCR measurements (Figure 8D and 8E). Limiting mTOR activity also improved

memory forming potential in neonatal CD8+ T cells indicated by higher SRC, which indirectly validate that rapamycin treated neonatal CD8+ T cells gained more memory forming potential (Figure 8E). In addition, the catabolic glycolytic pathway also decreased in the presence of rapamycin (Figure 8F and 8G). Neonatal CD8+ T cells responding in the presence of rapamycin showed lower basal and maximal ECAR compared to the control group (Figure 8G). These data indicate that when mTOR activity is limited, neonatal CD8+ T cells engage in less glycolysis and more OXPHOS, which likely explains why neonatal effector CD8+ T cells become less terminally differentiated and are more capable of transitioning into the memory pool.

In all of our studies up to this point, rapamycin was administered after infection. Thus, we wondered whether we could enhance neonatal memory CD8+ T cell formation by limiting mTOR in neonatal CD8+ T cells prior to antigenic stimulation. To test this, we gave daily injections of either PBS or rapamycin to 7-day-old gBT-I donor mice for 3 days and then harvested their CD8+ T cells to examine their responses to infection following adoptive transfer into congenic recipients. (Figure 9A). Interestingly, CD8+ T cells from neonatal gBT-I mice exposed to rapamycin exhibited a less memory like phenotype prior to infection, when compared to their counterparts exposed to PBS. (Figure 9B). As mTOR has been shown to be positively correlated with CD44 and CD122 expression in NK and T cells[26–28], it is possible that the reduced expression of these markers indicate that rapamycin was able to access CD8+ T cells in neonates and modify them. To test whether pre-treatment with rapamycin alters the fate of neonatal CD8+ T cells after infection, we next purified and adoptively co-transferred both PBS and rapamycin treated donor CD8+ T cells into recipients and examined their

response to infection *in vivo*. Rapamycin treated neonatal CD8+ T cells surprisingly did not exhibit a markedly different phenotype after infection. Indeed, both the PBS and rapamycin-treated groups displayed a similar amount of SLECs at the peak of the responses, suggesting that limiting mTOR activities in neonatal CD8+ T cells prior to stimulation is insufficient in altering their response to infection (Figure 9C). To validate this, cell numbers of WT and rapamycin treated neonatal CD8+ T cells were also evaluated on 7 and 14 dpi. There were no significant changes in cell numbers between the the PBS and rapamycin treated groups on 7 and 14 dpi, which further confirm that treating neonatal CD8+ T cells prior to infection does not change their ability to respond to intracellular pathogens (Figure 9D). These data suggest mTOR must be present during the activation and expansion phase of the response to alter the neonatal CD8+ T cells response to infection.

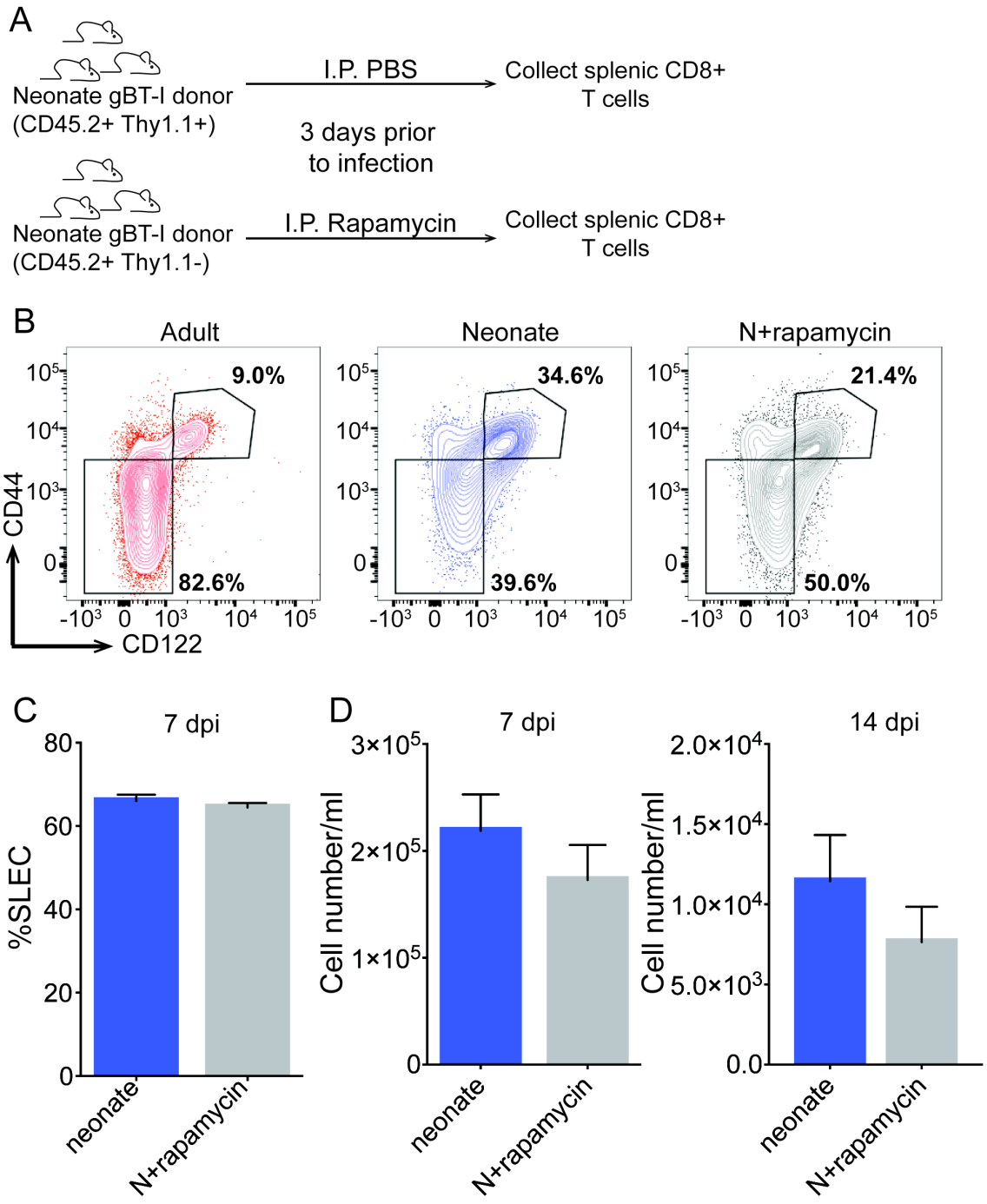

Figure 3.9. Rapamycin requires infection to improve neonatal CD8+ T cells survival. (**A**) Experimental design: daily rapamycin (100 ng/g) or PBS was given to 7-day-old neonates I.P. for 3 days prior to infection, splenic neonatal CD8+ T cells from each group was collected. (**B**) Phenotype (CD44 and CD122) of WT adult, WT neonatal and neonatal with rapamycin CD8+ T cells prior to infection. (**C**) SLEC population of WT neonatal and neonatal with rapamycin CD8+ T cells post-LmgB-infection on 7 dpi. (**D**) Cell number of WT neonatal and neonatal with rapamycin CD8+ T cells post-LmgB-infection on 7 dpi and 14 dpi.

DISCUSSION

Extensive studies have shown that immune system does not develop from early life into adulthood in a linear fashion[29–32]. Instead, the immune system is formed from distinct developmental layers throughout life[29–32]. Metabolic programs have been shown to play critical roles in regulating T cell activation and differentiation in adulthood[4,5,14]. This report demonstrated many age-related differences in the CD8+ T cell response to infection is due to the utilization of different metabolic programs by CD8+ T cells at different stages of life.

We previously found that neonatal mice have a significantly higher percentage of memory phenotype (CD44+CD122+) cells than their adult counterparts[1]. However, this phenotypic differences on the population levels does not explain why neonatal cells preferentially become short-lived effectors and are impaired at forming memory cells[1]. However, the larger percentage of memory phenotype cells in early life could contribute to the higher mitochondrial but lower glycolytic activities in naïve neonatal CD8+ T cells we found in this report. Since IL15 promote mitochondrial biogenesis, having larger composition of CD122+ (IL15 receptors) cells could grant naïve neonatal CD8+ T cells higher capacity to carry out mitochondrial activity[10]. It has been shown that memory phenotype cells are capable of proliferate faster and expand better than naïve CD8+ T cells upon activation[33], which fits what we observed in neonatal CD8+ T cells. Future studies examining responses to infection in CD8+ T cells with different amount of OXPHOS activities (ex. with mitochondrial inhibitors) are needed to verify this hypothesis.

Glucose metabolism plays an important role in supporting both CD8+ and CD4+

T cell proliferation and effector function[7,8,34,35]. CD8+ T cells heavily depend on extracellular glucose level for proliferation upon activation. Their proliferation decrease significantly when glucose level is low[36,37]. Even though we did not compare glucose uptake efficiency between adult and neonatal CD8+ T cells, we found that neonatal CD8+ T cells upregulate glucose receptor Glut1 and quickly improve the usage of glycolysis upon activation. This could explain why neonatal CD8+ T cells are able to proliferate rapidly post-infection. Neonatal CD8+ T cells also have lower SRC, which suggest lower mitochondrial fatty acid oxidation (FAO) activity[10]. Since FAO is critical in CD8+ T cell memory formation, this indicates that neonatal CD8+ T cells have lower memory forming potential[10]. There is also evidence suggesting that increased SRC and the ability to utilize OXPHOS are not essential in memory formation, rather the amount of ATP produced by the metabolic pathways used[34]. Further studies comparing different aged CD8+ T cell responses when ATP productions are normalized are called for to disentangle this question. Since more active metabolic profile (low OXPHOS and high glycolytic activities) are correlated with terminal differentiation status[38,39], we wanted to assess the metabolic programs used by different aged CD8+ SLECs (Figure 6). Consistent with the layered immune system model, mostly fetal-layer derived neonatal CD8+ SLECs still demonstrated lower OXPHOS and higher glycolysis than adult SLECs.

There are other important metabolic pathways supporting and regulating CD8+ T cell activation and differentiation we did not assess in this report. In addition to OXPHOS and glycolysis, some other well-studied pathways include fatty acid oxidation, fatty acid synthesis, pentose phosphate and amino acid metabolism[4,5]. All these pathways are critical in creating building blocks and meeting the cellular upon

activation[4,5]. Future experiments measuring more comprehensive metabolic profiles in different aged CD8+ T cells are called for to learn more about metabolic programs contributing to their different behaviors.

Lin28 is a developmental-regulated protein that is highly expressed in early life[20]. Lin28a and Lin28b are the 2 paralogs of Lin28. They share high sequence identity and have been mostly considered equivalent[20]. There is accumulative evidence showing that Lin28b regulates immune responses[1,19,40,41]. Lin28 can promote mTOR activity either through suppressing its well-studied microRNA target let7 or directly stabilizing the translation of several critical proteins involved in mTOR pathway[15,24]. mTOR has also been studied extensively and has been shown to play an important role in T cell differentiation[12,13]. mTOR activity has been shown to increase glucose uptake and promote glucose metabolism in mice[15,24,25]. Limiting mTOR activities with its inhibitor rapamycin has been shown to abolish the increased glucose uptake induced by overexpressing Lin28[15]. Limiting mTOR can also promote adult CD8+ T cell memory formation[22]. We sought to investigate whether Lin28b can regulate neonatal CD8+ T cells responses through mTOR in this report. We demonstrated that iLin28b adult CD8+ T cells behaved more similarly to neonatal CD8+ T cells, and limiting mTOR activities in iLin28b adult CD8+ T cells restrained their inherent propensity to differentiate into short-lived effectors and rescued their memory formation. This data proved that Lin28b regulates CD8+ T cell responses at least partially through mTOR. Some further experiments directly measuring the mTOR activities and the metabolic programs in iLin28b adult CD8+ T cells are required to validate the relationship between Lin28b-mTOR axis and cell metabolism.

We also attempted to assess the role of mTOR activities in neonatal CD8+ T cells and how it regulates cell metabolism. We found that limiting mTOR activities in neonatal CD8+ T cells reduced their contraction and terminal differentiation level post-infection. Limiting mTOR also reduced OXPHOS and improved glycolysis in neonatal CD8+ T cells compared with their WT counterparts. We have previously demonstrated that neonatal CD8+ T cells have high level of Lin28b expression[1]. This data imply that upon activation, neonatal Lin28b promotes mTOR activities, which then result in a more metabolic active profile. Nearly all of the previous studies performed with adult T cells used rapamycin to limit mTOR activities after infection[11,22,23]. In this report, we sought to examine whether the time of administration would change its impact on neonatal CD8+ T cells behaviors, and found that rapamycin requires active infection to have its impact on neonatal CD8+ T cells (Figure 9).

In conclusion, our study demonstrates that many of the cell-intrinsic differences between neonatal and adult CD8+ T cells during infection are driven by age-related changes in the metabolism. We propose a model whereby higher levels of Lin28b in neonatal CD8+ T cells drives an excessive amount of mTOR and glycolysis, which enables them to respond rapidly to infection, albeit at the expense of forming memory. Further studies are required to validate this model. However, to our knowledge, this study is the first report to examine metabolic programs in neonatal CD8+ T cells after infection and raises the possibility that the formation of neonatal memory CD8+ T cells can be therapeutically enhanced by manipulating key metabolic pathways.

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CHAPTER FOUR

The neonatal environment alters the ability of CD8+ T cells to respond to infection

SUMMARY

CD8+ T cells from different aged mice have been shown to behave differently in response to infection. Neonatal CD8+ T cells proliferate rapidly and quickly become more terminal differentiated. They also fail to form sufficient memory and respond poorly to secondary infections. Many of these age-related changes in the CD8+ T cell response are due to cell-intrinsic differences. Different progenitors at different developmental stages give rise to CD8+ T cells with distinct traits. However, the impact of extrinsic factors on different aged CD8+ T cells remains unclear. Neonates possess a more lymphopenic peripheral environment than adults, which is believed to impact T cell homeostasis. In this report, we sought to address how age-related differences in the environment influence the CD8+ T cell response to infection. We found that after "parking" adult donor CD8+ T cells in neonatal recipient mice for a week, they start to acquire the characteristics of a neonatal CD8+ T cell. For example, they became phenotypically similar to neonatal CD8+ T cells, proliferated more rapidly after stimulation and lost ther ability to differentiate into memory cells. We also obtained some data to suggest that ROS production from neonates might serve as a potential signaling molecule at mediating these differences. Collectively, these findings suggest that, in addition to intrinsic developmental differences, there are age-related differences in the environment, which also impact the CD8+ T cell response to infection.

INTRODUCTION

Previous studies have shown that adult and neonatal CD8+ T cells display

different characteristics and can respond differently to infection[1–3]. Neonatal CD8+ T cells are more proliferative and quickly become more terminal differentiated post-infection. They also contract rapidly and fail to form sufficient memory cells to carry out efficient secondary response[1–3]. These discrepancies can be attributed to the intrinsic differences between different aged CD8+ T cells, which are largely due to their distinct progenitors throughout development[3]. However, whether extrinsic factors also contribute to age-related differences in CD8+ T cell responses remains an open question.

Following thymic selection, neonatal and adult CD8+ T cells enter very different peripheral environments. In particular, neonatal mice are lymphopenic, which results in an increased exposure to homeostatic cytokines on a per cell basis and consequently elicits a greater amount of homeostatic proliferation (HP)[4]. Homeostatic proliferation does not require contact with foreign antigen. Instead, naïve cells are believed to undergo spontaneous proliferation following stimulation by self peptide:MHC complexes and cytokines, such as IL-7 and IL-15[4–6]. While previous studies have demonstrated that the neonatal environmental supports lymphopenia-induced proliferation, it is currently not known whether these changes that occur prior to infection alter their ability to respond to intracellular pathogens[5–7]. Here, we addressed this question by 'parking' adult donor CD8+ T cells in neonatal recipients for one week and comparing their phenotype and function after infection.

Interestingly, we found that neonatal-experienced adult CD8+ T cells acquire a phenotype mimicking neonatal CD8+ T cells, as exhibited by higher expression of markers (CD44, CD122) typically associated with memory cells. Both neonatal cells and

adult cells exposed to the neonatal environment contained significantly higher levels of reactive oxygen species (ROS), which could explain why these cells are able to mount a more rapid proliferative response after in vitro stimulation. Lastly, we found that adult CD8+ T cells parked in the neonatal environment exhibit an imbalance in effector and memory cell differentiation that is characteristic of neonatal cells.

MATERIAL AND METHODS

Mice

B6-Ly5.2/Cr mice were purchased from Charles River Laboratories (Frederick, MD). TCR transgenic mice specific for the HSV-1 glycoprotein B498-505 peptide SSIEFARL8 (gBT-I mice) were provided by Janko Nikolich-Zugich (University of Arizona, Tucson, AZ) and crossed with Thy1.1 or C57BL/6 mice purchased from Jackson Laboratories (Bar Harbor, ME). Neonatal and adult gBT-I animals were used at 6 to 8 days old and at 2 to 4 months 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.

Antibodies and flow cytofluorimetric analysis

Antibodies were purchased from eBioscience (San Diego, CA), Biolegend (San Diego, CA), Invitrogen (Carlsbad, CA), or BD Biosciences (Mountain View, CA). Flow cytofluorimetric data were acquired using DiVa software from an LSRII equipped with 4 lasers (BD Biosciences). Analysis was performed with FlowJo (Tree Star, Ashland, OR).

Adoptive transfer

Microbeads (Miltenyi Biotec) enriched gBT-I splenic adult and neonatal experienced adult CD8+ cells were combined at a 1:1 ratio. Combined cells were

suspended at 2×10^5 cells per ml of PBS and 100 ul of cells was injected i.v. into adult B6-Ly5.2 recipient mice. The next day, recipient mice were infected with WT LM-gB (5×10^3 CFU, i.v.) and later challenged with WT LM-gB (5×10^4 CFU, i.v.), as described.[2]

***In vitro* stimulation and proliferation**

CD8+ T cells were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE), as described[2], and cells were stimulated with plate-bound anti-CD3 (5 mg/ml) and anti-CD28 (20 mg/ml).

Mitotracker and ROS staining

Splenic CD8+ T cells were stained with 50 nM mitotracker green (ThermoFisher, M7514) or 2 uM H2DCFDA (ThermoFisher, D399) for 30 minutes at 37^{o}C and ran on flow cytometer non-fixed.

Statistical analysis

Statistical analysis was performed using Prism (GraphPad Software, Inc, La Jolla, CA). Error bars represent standard error of the mean. Significance was determined by Student t test. Significance is denoted as: $*P < .05$, $**P < .01$, $***P < .001$, and $****P < .0001$.

RESULTS

Neonatal-experienced adult CD8+ T cells become more phenotypically and functionally similar to neonatal cells

In order to access how the neonatal environment affects CD8+ T cell development and differentiation, we transferred $1X10^6$ adult gBT-I CD8+ T cells into 7-day-old gBT-I neonates and “parked” them for a week (Figure 1A). We first assessed their expression of the memory-phenotype markers CD44 and CD122 by flow

cytometry. Neonatal-experienced adult CD8+ T cells acquired a higher percentage of memory phenotype cells (Figure 1B). There are also other markers known to be upregulated with HP, such as Ly6C, chemokine receptor CXCR3 and integrin CD11a. Neonatal-experienced adult CD8+ T cells showed significantly higher expression of Ly6C, CXCR3 and CD11a than wild type (WT) adult CD8+ T cells (Figure 1C). These data suggest that adult CD8+ T cells undergo a significant amount of homeostatic proliferation in neonatal recipient mice.

One of the hallmark features of neonatal CD8+ T cells is that they proliferate rapidly in response to activation. Thus, we next sought to test whether neonatal environment enables adult CD8+ T cells to respond more quickly to TCR stimulation. Adult and neonatal-experienced gBT-I adult CD8+ T cells were coated with proliferation dye CFSE and stimulated with plate-bound anti-CD3 and anti-CD28 antibodies. We found that neonatal-experienced adult gBT-I CD8+ T cells did indeed proliferate more than control adult CD8+ T cells after stimulation (Figure 1D), as evidenced by their increased dilution of CFSE dye. Overall, our data suggest that being in a neonatal environment can change both the phenotype and function of CD8+ T cells.

Different aged animals have different level of reactive oxygen species (ROS)

Next we wondered how the neonatal environment enhances the ability of adult CD8+ T cell to respond to *in vitro* stimulation. Previously, we found that different aged CD8+ T cells utilize different metabolic programs (discussed in Chapter 3). This led us to consider the possibility that homeostatic proliferation increases certain signaling molecules or metabolic products, which might contribute to the differences we observed in neonatal-experienced adult CD8+ T cells. In our liquid chromatography/tandem mass

spectrometry based metabolomics analysis from CD8+ T cells harvested from different animals, one of the intriguing differences we observed was their distinct oxidative stress level. Both naïve and activated neonatal CD8+ T cells displayed significantly higher GSSG:GSH ratios than adult CD8+ T cells (Figure 2A). Antioxidant enzyme glutathione (GSH) can scavenge ROS and get oxidized to form glutathione disulfide (GSSG). The GSSG to GSH ratio is therefore a useful indicator of the oxidative stress level in cells. The higher the value, the more oxidative stressed the cells are.

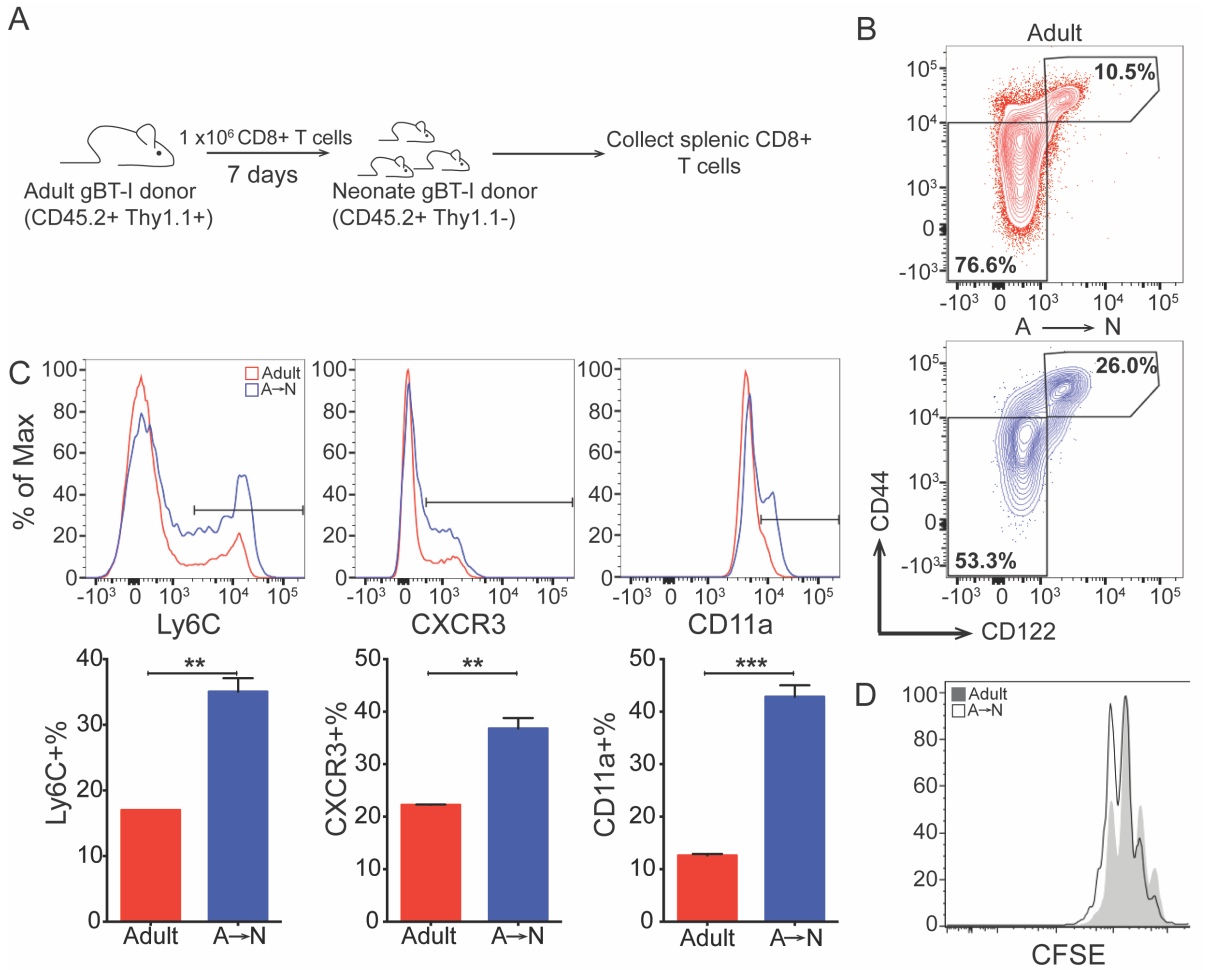

Figure 4.1. Adult CD8+ T cells in neonatal environment acquire neonatal phenotype and become more proliferative. (**A**) Experimental design: adult CD8+ T cells were transferred into neonates. 7 days later, neonatal-experienced adult CD8+ T cells were harvested for testing. (**B**) Memory phenotype (CD44 and CD122) of adult and neonatal-experienced adult (A→N) CD8+ T cells. (**C**) Homeostatic proliferation markers on adult and neonatal-experienced adult CD8+ T cells. (**D**) Neonatal-experienced CD8+ T cell proliferated faster than control adult CD8+ T cells 2 days post-activation. $^{**}P < .01$ *and* $^{***}P < .001$.

Reactive oxygen species (ROS) have historically been viewed as a metabolic byproduct that can cause cellular damage and induce apoptosis[8,9]. However, recent studies suggest that ROS can also enhance cellular signaling transduction[8,10]. Thus, we wondered whether elevated levels of ROS might explain why neonatal CD8+ T cells respond more quickly to stimulation but also exhibit increased death during contraction. To test this possibility, we wanted to assess the ROS producing capacity of CD8+ T cells from different aged animals. Mitochondria are known as the major powerhouse and the primary site of ROS production in cells[11]. We therefore reasoned that the amount of mitochondria could be used as an indicator of ROS producing potential. To examine the mitochondrial content in different aged CD8+ T cells, we first measured the expression of mitochondrial transcription factor A (TFAM), since it has previously been shown to regulate mitochondrial biogenesis[12]. Neonatal CD8+ T cells displayed significantly higher levels of TFAM mRNA, which indicate that they have a bigger potential to contain higher mitochondrial content (Figure 2B).

In order to directly measure mitochondrial mass, we stained cells with mitotracker green for their total mitochondrial content and confirmed that neonatal CD8+ T cells have higher mitochondrial content than their adult counterparts (Figure 2C). Since the neonatal environment is capable of affecting the phenotype of adult CD8+ T cells, we wanted to also test whether neonatal-experienced adult CD8+ T cells have higher mitochondrial mass that can result in higher ROS production. In fact, neonatal-experienced adult gBT-I cells also showed higher mitochondrial mass compared with WT adult CD8+ T cells (Figure 2D).

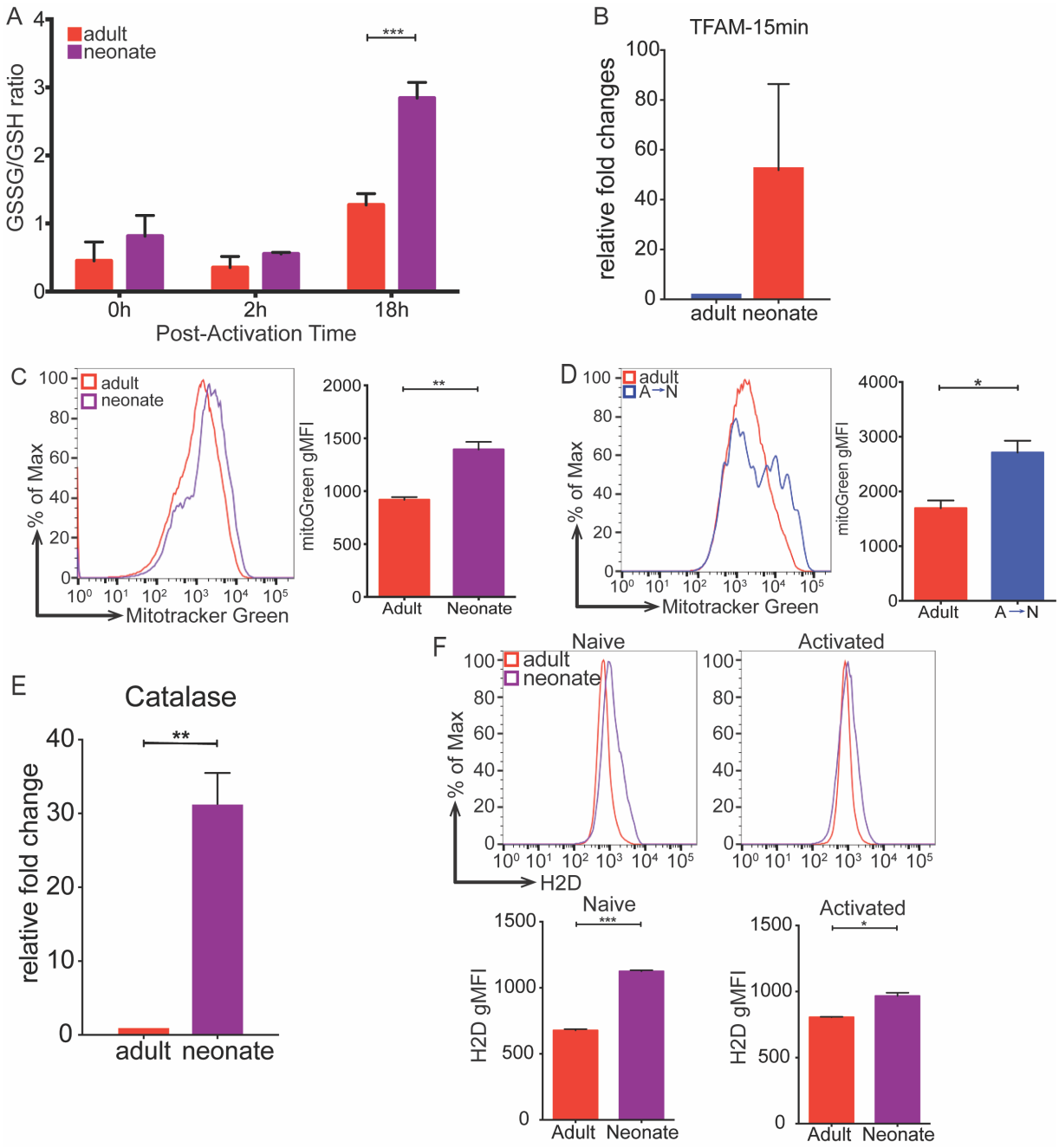

Figure 4.2. Adult and neonatal CD8+ T cells processes different amount of mitochondrial and ROS. (**A**) Neonatal CD8+ T cells showed higher oxidized glutathione (GSSG) to glutathione (GSH) ratios than adult CD8+ T cells. (**B**) Neonatal CD8+ T cells showed higher level of TFAM at 15 min post-activation. (**C**) Neonatal CD8+ T cells showed bigger mitochondrial mass. (**D**) Neonatal-experienced adult CD8+ T cells showed higher mitochondrial mass. (**E**) Neonatal CD8+ T cells showed higher antioxidant enzyme catalase. (**F**) Both naïve and activated (15 min) neonatal CD8+ T cells showed higher hydrogen peroxide level. $^{*}P < .05$. $^{**}P < .01$, $^{***}P < .001$ and $^{****}P < .0001$.

Another well-studied antioxidant enzyme is catalase. The level of catalase is also a good indicator of ROS activity in cells. To see if catalase expression was different in neonatal and adult CD8+ T cells, we measured its expression by qPCR and found that neonatal CD8+ T cells express significantly higher levels than adults (Figure 2E). Lastly, we also stained the cells with ROS dye H2DCFDA to directly assess the hydrogen peroxide in either naïve or activated (15 mins) CD8+ T cells from different aged animals (Figure 2F). Both naïve and activated neonatal CD8+ T cells contained higher levels of hydrogen peroxide than their adult counterparts (Figure 2F).

Collectively, our data indicates that neonatal CD8+ T cells contain more ROS than adults, which could be a result of higher mitochondrial content in CD8+ T cells. The increase in mitochondrial mass may be a function of cells undergoing more homeostatic proliferation, as adult CD8+ T cells 'parked' in neonatal recipient mice also contain larger amounts of mitochondria. These findings raise the possibility that cells with more mitochondrial mass (neonates and neonatal-exposed adults) respond to stimulation with more rapid ROS production, which enhances signaling and activation.

Neonatal-experienced adult CD8+ T cells respond differently to infection

Given that we have shown that the neonatal environment can change how adult CD8+ T cells respond to stimulation *in vitro*, we next wanted to examine how the neonatal environment would alter the behavior of adult CD8+ T cells after infection *in vivo*. To test this, adult and neonatal-experienced adult donor CD8+ T cells were purified and co-transferred into congenic adult recipients, and then infected the next day with Listeria Monocytogenes-gB (Lm-gB) (Figure 3A).

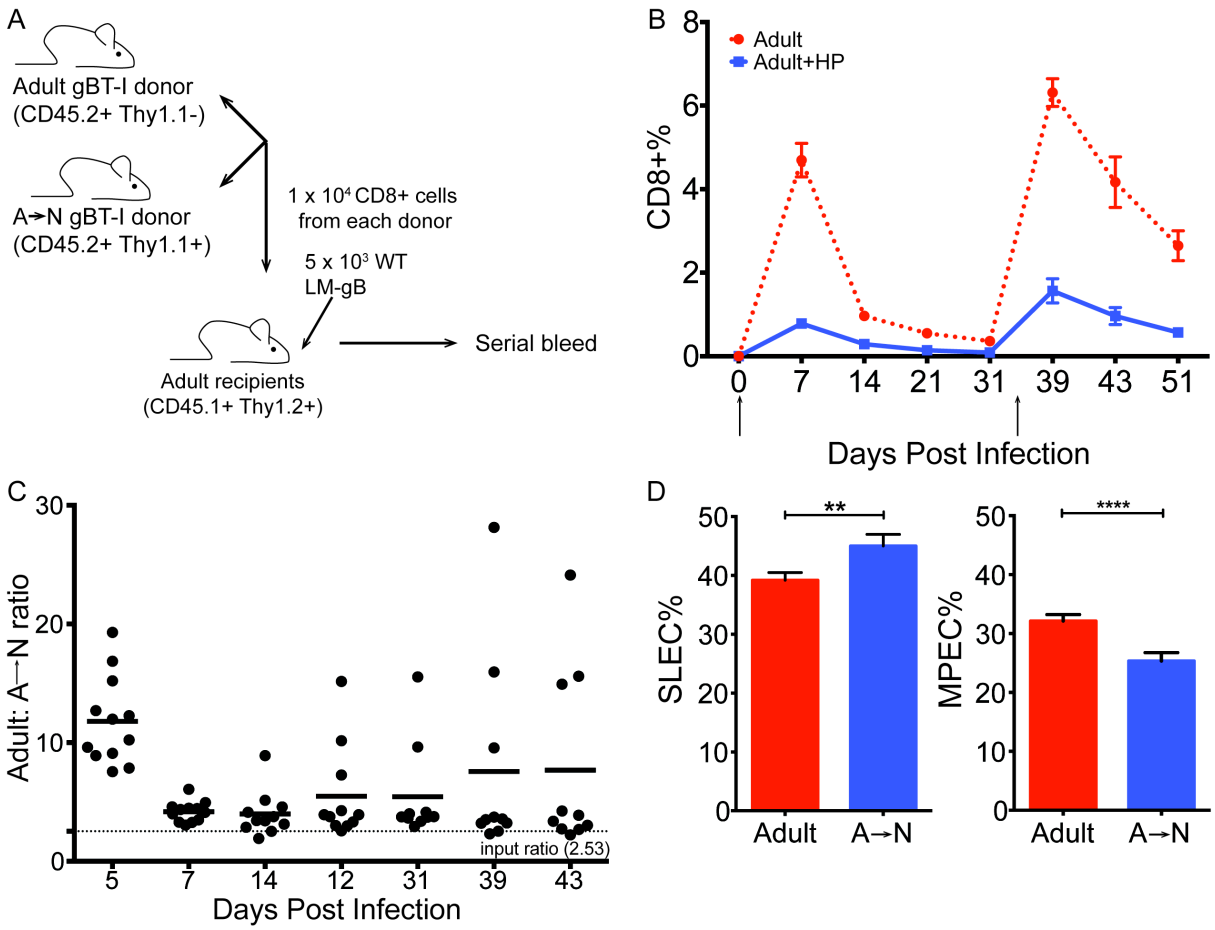

Figure 4.3. Adult CD8+ T cells in neonatal environment respond similar to neonatal CD8+ T cells post-infection *in vivo.* (**A**) Experimental design: adult and neonatal-experienced adult were adoptively co-transferred into recipients, then infected them with Lm-gB and serial bled. (**B**) Kinetics of adult and neonatal-experienced adult CD8+ T cells, neonatal-experienced adult CD8+ T cells failed to form sufficient memory. (**C**) Ratio of adult vs. neonatal-experienced adult CD8+ T cells throughout infection. (**D**) Percentages of SLEC and MPEC in adult and neonatal-experienced adult CD8+ T cells. $^{**}P < .01$ *and* $^{****}P < .0001$.

Interestingly, the neonatal-experienced adult gBT-I CD8+ T cells did not expand as well as WT adult gBT-I CD8+ T cells and failed to form sufficient memory response upon challenge (Figure 3B). This is similar what we previously observed in neonatal CD8+ T cells. The ratios of WT adult to neonatal-experienced adult gBT-I CD8+ T cells (A:A→N) throughout infection were bigger than the input ratio and peaked on 5 dpi, which suggest that neonatal-experienced adult CD8+ T cells contracted more than WT adult CD8+ T cells (Figure 3C). We also examined the phenotype of both groups of

donor cells at the peak of the response and found that the neonatal-experienced adult CD8+ T cells preferentially became short-lived effector cells (SLECs, CD127-KLRG1+) compared to WT adult CD8s (Figure 3D). Based on these results, we conclude that the neonatal environment is capable of changing how CD8+ T cells behave in response to infection and further exacerbates the imbalance in effector and memory cell differentiation in early life.

DISCUSSION

There are several possible extrinsic differences between the neonatal and adult peripheral environment. Neonates have peripheries with fewer immune cells and are therefore considered to be lymphopenic[4]. When mature neonatal CD8+ T cells leave the thymus and enter a periphery containing a low a number of T cells they undergo extensive HP and upregulate phenotypic markers associated with cell division[13]. In contrast, newly made CD8+ T cell entering an adult environment undergo significantly less homeostatic proliferation, because many more T cells occupy the peripheral environment. Lymphopenia-induced CD8+ T cells have undergone more rounds of proliferation and are known as “virtual memory” cells due to their memory phenotype[14]. Although they are still naïve cells in the sense that they have yet to encounter their cognate peptide, they respond differently than true naïve CD8+ T cells[14,15]. We found that neonatal-experienced adult CD8+ T cells upregulate HP related markers, indicating they have undergone more extensive HP.

Another possible difference in the environment of neonatal and adult animals could relate to the amount cytokines that are present in the periphery. Presently, there have not yet been any reports performed to directly compare the amounts of specific

homeostatic cytokines between neonatal and adult animals. However, some studies have reported different cytokine profiles in different aged animals and humans[16,17]. For example, infants have higher level of IL-2 and IL-4[17]. And aging mice tend to have gradually decreased level of IL-2 and IFNγ[16]. It is also well accepted that there are different amounts of DCs in neonatal and animals, which can produce different level of cytokines (e.g. IL-15) in extracellular environment[18–20]. In addition, the overall levels of metabolites (lactate, nucleotides, etc.) and cellular products (ROS, fatty acid, etc.) can also be different in neonates, which could participate in signaling pathway mediating CD8+ T cell behavior.

Mitochondria serve a critical role in powering cellular functions and regulate cellular processes to meet the current demand. Several major metabolic pathways involved in T cell responses rely on proper mitochondrial function[21–24]. Mitochondria play an important role in mediating cellular metabolic programs to support CD8+ T cell activation and differentiation[21–23]. Mitochondria are also the major production site of the common cellular product ROS. In theory, bigger mitochondrial implies different amount of metabolites accumulated extracellularly. We attempted to address this by measuring mitochondrial content in different CD8+ T cells harvested from neonatal and adult animals. Our data indicate that neonatal CD8+ T cells indeed have more mitochondrial content than adult CD8+ T cells. Neonatal environment can also promote mitochondrial biogenesis in adult CD8+ T cells resulting in larger mitochondrial mass. One recent study suggest that mitochondria itself can also affect how CD8+ T cells respond *in vitro* stimulation[25]. Mitochondria tend to modify and become fused in memory CD8+ T cells. Fused mitochondria have also been shown to promote fatty acid oxidation to support

memory formation and improve their anti-tumor activities[25]. In the future it would be interesting to more formally link mitochondrial mass and structure in different aged CD8+ T cells with their ability to respond to microbial challenge.

Our metabolomics data suggest that neonatal T cells are under a greater amount of oxidative stress, suggesting that there is higher ROS produced by neonatal cells. ROS are produced by the partial reduction of oxygen, which includes superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($HO^\cdot$). When the level of oxygen exceeds the cell's ability to carry out an antioxidant response, the condition is known as oxidative stress[8]. ROS is generally known as a detrimental cellular byproduct to induce cell death. However, several studies have shown that ROS plays essential role in activating T cells and fine-tuning their responses[21,26–28]. Since ROS can transduce signals and induce apoptosis in T cells, we suspect that ROS could affect CD8+ T cell behaviors. We attempted to address this question starting examining ROS production in different aged CD8+ T cells. We found that neonatal CD8+ T cells can produce higher amount of ROS than adult CD8+ T cells. The conclusion that neonatal CD8+ T cells are under higher level of oxidative stress was supported by data indicating that they also exhibit higher GSSG:GSH ratios and amounts of the antioxidant enzyme catalase. Collectively, our data suggest that neonatal environment might alter adult CD8+ T cell response by promoting mitochondrial biogenesis, which result in more ROS production to facilitate signaling.

An important question is how does ROS alter the function of CD8+ T cells? ROS has been shown to regulate cellular proliferation and survival by activating NFkB and NFAT1 in T cells, resulting in increased proliferation and differentiation. Additionally,

ROS can also activate other molecules, such as transcriptional factor AP1 and protein kinase C (PKC). AP1 and PKC are both important in T cell activation and AP1 is also involved in inducing apoptosis[9,29–33]. One recent study has proved that ROS triggers GSH activities, which can drive metabolic pathways supporting effector T cell functions[10]. This might explain why neonatal-experienced CD8+ T cells proliferate rapidly and became more terminal differentiated post-infection. However, further studies directly investigating the specific role of ROS on regulating CD8+ T cells with different level of extracellular ROS (inhibitor or enhancer) are warranted to fully understand how ROS influences the CD8+ T cell response.

Collectively, our findings indicate that neonatal environment is capable of altering adult CD8+ T cell responses, which further exacerbate the age-related changes due to cell intrinsic differences.

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CHAPTER FIVE

SUMMARY AND FUTURE DIRECTIONS

General Background

Neonates often generate incomplete immunity against intracellular pathogens. As CD8+ T cells are essential for clearing intracellular pathogens, it is crucial to understand why they behave differently than adults during infection. The long-standing belief is that neonates have impaired immunes systems, which make them more prone to repeated infections. Surprisingly, we discovered that neonatal CD8+ T cells are more susceptible to infection because they quickly become terminal differentiated and fail for form sufficient immunological memory[1]. Other researchers also found that neonatal CD4+ T cells develop a hypersensitive Th2 skewed response[2]. These findings suggest that neonates are more susceptible to infections not because they have immature immune systems, rather because their immune cells respond differently to infections early in life.

The underlying mechanisms contributing to the discrepancies between adult and neonatal CD8+ T cells are still unclear. Both intrinsic and extrinsic factors could impact different aged CD8+ T cells behaviors. In this thesis, I focused on understanding what factors are contributing to the different responses carried out by neonatal CD8 T cells.

Summarized Findings

I started by investigating intrinsic differences. In chapter 2, using T cell receptor transgenic mouse model, I showed that progenitors from different aged animals give rise to unique CD8+ T cells in the periphery. My findings are consistent with the current "layered immune system" model, which theorizes that cells of the immune system seed the periphery in distinct waves of cells throughout development[3–5]. Instead of

progressing in a linear fashion, the immune system is composed of multiple distinct waves of cells throughout development. This model challenges the common belief that neonatal immune cells are just underdeveloped immune cells. The finding that they in fact come from distinct lineages will be critical in development age-targeted immunotherapies. Effective treatments may need to involve new classes of drugs, and not simply adjusting the dosage of the adult medications for neonates.

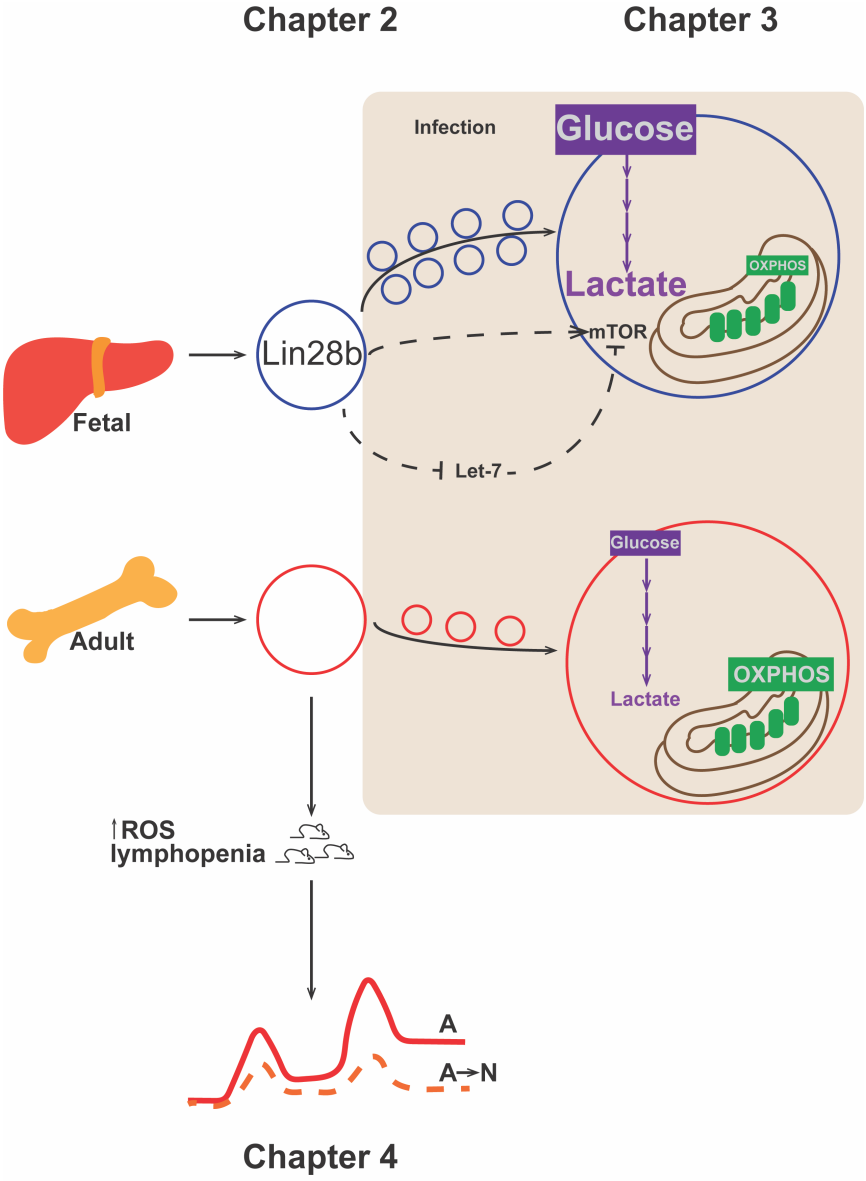

Figure 5.1. Overview of chapters.

Additionally, I provided evidence that a developmental-regulated RNA binding protein Lin28b could potentially regulate neonatal CD8+ T cell development. Lin28b is highly conserved across species ranging from Drosophila to human[6]. While human studies are relevant, they are generally limited to examining peripheral blood, in contrast to the in-depth analysis of our murine infection model allows. The role of Lin28b in regulating homeostasis is not a new concept. Other researchers have shown that Lin28b supports fetal-like lymphopoiesis in adult HSCs, resulting in increased productions of fetal B cells, natural killer T cells, regulatory T cells and γδT cells[7–9]. However, to our knowledge, our publication is the first report showing how Lin28b mediates neonatal-like responses to infection in CD8+ T cells.

In Chapter 3, I extended my cell intrinsic studies of different aged CD8+ T cells to better understand the underlying mechanisms that contribute to age-related different responses. Recently, T cell metabolism has drawn a lot of attention, due to its promising role in immunotherapy[10]. Cellular metabolism is essential in supporting cellular function and ensuring proper differentiation of cells. Dysregulation in cellular metabolism is known to lead to detrimental outcomes (e.g. cancer). Researchers have found key metabolic regulators that are deterministic of cell fate[11,12]. Since CD8+ T cells of developmental origin adopt different fates, I asked whether the metabolic profiles in different aged CD8+ T cells are also different. I found that neonatal CD8+ T cells acquire a more metabolic active profile (lower OXPHOS and higher glycolytic activities) compared to adult CD8+ T cells post-infection. This is consistent with studies demonstrating that effector T cells upregulate catabolic pathways to support their rapid proliferation, and a more quiescent metabolic program is required to sustain naïve T

cells and form memory T cells[11–14]. I also demonstrated that terminally differentiated CD8+ T cells from adult and neonates displayed different metabolic programs, suggesting that the discrepancies I observed are largely due to intrinsic properties, lending further support to the idea that there are developmentally distinct layers of CD8+ T cells.

I also found that by limiting mTOR activities with rapamycin, I reshaped the metabolic programs and improved the survival of neonatal CD8+ T cells. In the presence of rapamycin, neonatal CD8+ T cells acquired a more quiescent metabolic program (less glycolytic and more OXPHOS) compared with WT neonatal CD8+ T cells, which suggest that mTOR is important in regulating cellular metabolism neonatal CD8+ T cells. Rapamycin also rescued memory formation in iLin28b adult CD8+ T cells, indicating that Lin28b could elicit its effect via mTOR pathway.

My findings from Chapter 2 and 3 have successfully established the significance of intrinsic differences between adult and neonatal CD8+ T cells. How, it is likely that extrinsic factors also contribute to developmentally related differences in CD8+ T cell responses. In Chapter 4, I showed that neonatal environment is capable of modifying the phenotype and response of adult CD8+ T cells. It is not surprising that lymphopenic neonatal environment promote proliferation in adult CD8+ T cells since there is evidence showing lymphopenia can induce homeostatic proliferation[15]. However, it is intriguing to learn that the neonatal environment can change adult CD8+ T cell behaviors in response to infection *in vivo*. Neonatal environment also promoted mitochondrial biogenesis in adult CD8+ T cells, which could potential results in higher ROS production to mediate signaling. My findings suggest that extrinsic factors can

exacerbate the aged-related differences induced by cell-intrinsic properties.

Future Directions and Discussion

This thesis focused on post-thymic distinctions in different aged CD8+ T cells. However, thymic environment plays an important role in shaping CD8+ T cell responses. My intra-thymic transfer experiment (chapter 2) suggests that different aged progenitors give rise to unique cell populations within the same thymic environment. However, future experiments to directly examining the differences in thymic environment (cytokine level, progenitor sensitivity towards cytokines, etc) will be important for a more complete understanding of the effects of thymic environment on CD8+ T cell behaviors.

Although the layered immune system model is well characterized, how distinct developmental layers throughout life contributing to responses to infection are still perplexing. Fate-mapping experiments are needed to learn how these layers persist in life and how they could participate in combating infections. Current fate-mapping study in the lab suggests that CD8+ T cells made early in life proliferate more rapidly and are more sensitive to pro-inflammatory cytokine IL-12/IL-18 than cells made in older animals. CD8+ T cell made early in life also preferentially differentiate into SLECs during infection in adulthood. These data suggest that CD8+ T cell responses after infection are influenced by when the responding cells were made.

It will also be interesting to further investigate how Lin28b is involved in neonatal CD8+ T cell development. Moreover, it will be important to determine if it is necessary or if there maybe are other compensating pathways. Experiments investigating neonatal cells behavior without Lin28b at different development stages (fetal vs. neonatal) would help answering these questions. Since Lin28b is a RNA binding protein, sequencing

experiments comparing RNA profiles in neonatal CD8+ T cells with different level of Lin28b may assist in the identification of its potential targets.

I showed that rapamycin treatment dampens the metabolic programs in neonatal CD8+ T cells. In the future, it will be important to design experiments directly measuring mTOR activities in different aged CD8+ T cells. My data sheds light on how metabolic programs differ between adult and neonatal CD8+ T cells, and provide evidence that metabolic pathways could serve as potential drug targets in neonates. The metabolic profiles in different aged CD8+ T cells will be required to design optimal therapies in neonates.

Cellular metabolism is very complex and intertwined; it is unlikely that only one or two pathways direct cellular functions. In this thesis, I focused on 2 major pathways: OXPHOS and glycolysis. However, there are evidences showing that other metabolic pathways (ex. fatty acid oxidation, amino acid metabolism, pentose phosphate, etc.) are also involved in T cell activation and differentiation[11,13]. Therefore, future studies focusing on other metabolic pathways are required to reveal a comprehensive picture of neonatal metabolic profile. It will also be important to study when metabolic programs diverge in different aged CD8+ T cells. For example, they could be different at progenitor level or gain the differences in the peripheral post thymic maturation. To address these possibilities, a fate-mapping strategy would be useful. Investigating metabolic programs at different time (progenitors, recent thymic emigrants and mature T cells) and tracing experiments would be essential to answer this question.

ROS has long been classified as detrimental byproducts produced by cells, but recently, more functional roles have been assigned to ROS[16,17]. My data showed that

neonatal and neonatal-experienced adult CD8+ T cells both contain higher mitochondrial content, which might result in more ROS production. ROS production needs to be measured in neonatal-experienced adult CD8+ T cells. Manipulating ROS level in different aged CD8+ T cells is also critical in defining the functional role of ROS in shaping T cell responses. Amount of mitochondrial can also be regulated to investigate its relationship with ROS production.

My findings clearly showed that neonatal CD8+ T cells do not generate sufficient memory cells due to the intrinsic differences between neonatal and adult CD8+ T cells. It would also be interesting to investigate whether neonatal CD8+ T cell generate similar or different type of memory cells compared with adult CD8+ T cells. By definition, central memory T cells express CCR7 and CD62L, whereas effector memory T cells lack both markers but have higher Tbet level[18]. Since neonatal CD8+ T cells express lower CD62L and higher Tbet following infection compared with adult CD8+ T cells, it would be more likely that when neonates generate memory cells, they would preferentially generate effector memory T cells. Further experiments assessing the traits of memory cells generated by adult and neonatal CD8+ T cells are needed to address this question further.

There is evidence in the field suggesting that neonatal CD4+ respond differently (Th2 biased) compared with adult CD4+ T cells[2]. However, there is no directly comparison of CD4+ T cell progenies derived from different aged progenitors. My intra-thymic progenitor transfer experiment can be used to investigate whether different aged progenitors could give rise to distinct CD4+ T cells in the periphery. In this experiment set-up, in order to examine intrinsic differences between fetal and adult progenitors,

different aged progenitors are allowed to develop in the same thymic environment. Assessing the responses of different progenitor-derived CD4+ T cells to infection will allow us to conclude whether ontogeny of CD4+ T cells supports the layered immune system hypothesis.

There is also a large body of studies investigating metabolism in adult CD4+ T cells[13]. Th1, Th2 and Th17 effector cells preferentially use glycolysis, and Treg and memory T cells utilize more quiescent metabolic program, such as FAO[13]. Nevertheless, there is very limited knowledge on metabolism in neonatal CD4+ T cells. My findings suggest that different aged CD8+ T cells utilize different metabolic programs. It would be interesting to examine whether different metabolic programs underlie the differences between different aged CD4+ T cells by directly measuring their usage of different metabolic pathways following activation.

Infections can be quite complex, and cells respond differently depending on the specific type of infection. I only assessed neonatal CD8+ T cells response to acute systemic infections (Listeria-gB and vaccinia-gB) where they respond vigorously at the expense of memory formation. However, I have not investigated tissue specific infections (e.g. respiratory infection) or chronic infections (e.g. CMV).

Young children endure more severe symptoms from respiratory infections, in part due to their reduced tissue resident memory CD8+ T cells[19]. Neonatal mice are still able to produce effector CD8+ T cells in response to influenza infection[19]. However, the amount of neonatal lung resident memory CD8+ T cells is drastically reduced compared with adults[19]. Similar to our observations about neonatal effector T cells in response to acute systemic infection, effector cells generated after influenza infection in neonates

are also more terminal differentiated[19]. In addition to that, CDC (Centers for Disease Control and Prevention) do not recommend flu vaccines for children under 6 months old, which renders limited ways to protect infants from influenza infection. My findings suggest that development-regulated RNA binding protein Lin28 and metabolic programs are critical in regulating CD8+ T cell differentiation. Tempering Lin28 targeted pathways and/or metabolic programs can potentially promote better memory formation in CD8+ T cells to help protecting infants against early influenza infection. Manipulating Lin28 targeted pathways and T cell metabolism can also serve as adjuvants to maximize the protective effects from flu vaccines in young children (6 months – 5 years old).

Cytomegalovirus (CMV) is a chronic infection known to cause serious problems in fetuses and immune-compromised adults.[20,21] One hallmark about CMV infection is that there is a large amount of CMV specific memory cells in adulthood from infection acquired in early life, known as memory inflation.[20,21] Studies have found that fetuses are able to produce effector CD8+ T cells early in life and neonatal CD8+ T cell repertoire diversified after CMV infection[20–22]. However, the significance and persistence of the contribution from these effector T cells produced early in life have not been studied in details. My findings demonstrated that fetal-driven CD8+ T cells become highly differentiated. One would suspect that their contributions to the persisted CMV specific memory cell pool later in life might be limited. Fate-mapping experiments during CMV infections would help us to understand the role of fetal-driven CD8+ T cells in adulthood.

Chronic infections are also known to cause T cell exhaustion with impaired T cell functions[23]. Recent therapies targeting exhaustion marker PD-1 have shown promising

clinical effects in tumor treatments, which suggest that phenotypically exhausted T cells might play a functional role in diseases[23]. It would also be interesting to know how neonatal CD8+ T cells would behave in response to chronic infection. My findings suggest that neonatal CD8+ T cells contract a lot faster than their adult counterparts and are more responsive to similar level of stimuli. One can predict that there might not be an observable population of exhausted neonatal CD8+ T cells following chronic infection due to their rapid contraction rate, which could result in limited effectiveness of current PD-1 therapies in neonates. Examining neonatal CD8+ T cell responses in chronic infection and cancer models directly will help addressing this question.

Conclusions

Overall, this thesis demonstrated that fetal and adult progenitors give rise to CD8+ T cells with unique behaviors at different developmental stages, and distinctive metabolic programs utilized by different aged CD8+ T cells could underlie their responses to infection. In addition to the drastic intrinsic differences, extrinsic factors in the periphery of different aged animals might also provide a minor but significant contribution to their different behaviors. My findings advance the understanding of different aged CD8+ T cells, and cast light on identifying potential therapeutic targets in neonates.

From an evolutionary perspective, species are constantly undergoing natural selection to become better adapted in order to survive and reproduce. Insufficient memory formation in neonatal CD8+ T cells may not be a “defective” response. Neonatal immunity could have been shaped by natural selection to devote limited resources towards a vigorous primary response to combat the infections during this

more susceptible period. The trade-off is that they will not form long-lived memory cells. Later in life, selective pressure may have driven adult CD8+ T cell response towards the production of long-lived memory cells to improve their longevity.

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