Published in final edited form as:

Immunol Rev. 2023 May ; 315(1): 108–125. doi:10.1111/imr.13185.

Shaping immunity for life: layered development of CD8+ T cells

Cybelle Tabilas[1,2], **Norah L. Smith**[1,2], **Dr. Brian D. Rudd**[1,*]

[1]Department of Microbiology and Immunology, Cornell University, Ithaca, NY 14853, USA

[2]Co-first author

Summary

Historically, the immune system was believed to develop along a linear axis of maturity from fetal life to adulthood. Now, it is clear that distinct layers of immune cells are generated from unique waves of hematopoietic progenitors during different windows of development. This model, known as the layered immune model, has provided a useful framework for understanding why distinct lineages of B cells and γδ T cells arise in succession and display unique functions in adulthood. However, the layered immune model has not been applied to CD8+ T cells, which are still often viewed as a uniform population of cells belonging to the same lineage, with functional differences between cells arising from environmental factors encountered during infection. Recent studies have challenged this idea, demonstrating that not all CD8+ T cells are created equally, and that the functions of individual CD8+ T cells in adults are linked to when they were created in the host. In this review, we discuss the accumulating evidence suggesting there are distinct ontogenetic subpopulations of CD8+ T cells and propose that the layered immune model be extended to the CD8+ T cell compartment.

Keywords

CD8+ T cells; Immune development; Hematopoietic progenitor cells

1 Introduction

Almost 30 years ago, Leonard and Leonore Herzenberg proposed the layered immune system hypothesis, which posits that the immune system is stratified into layers of distinct immune cells that have developed sequentially from waves of different hematopoietic stem cells[1]. Since then, studies have described unique populations of B cells, γδ T cells, CD4+ T cells, and macrophages that arise during early stages of development and are derived from distinct progenitors[2–6]. These studies have provided an enormous amount information for some (but not all) immune cell types. For example, we now know the precise hematopoietic progenitors in mice that give rise to different subsets of macrophages (microglial, Langerhans cells, and Kupffer cells)[7,8]. We also know the exact days during

*Correspondence: Dr. Brian D. Rudd, Department of Microbiology and Immunology, C5 147 VMC, Cornell University, Ithaca, NY 14853., bdr54@cornell.edu.

Conflicts of interest

The authors have declared no conflicts of interest.

mouse gestation that different subsets of γδ T cells (Vγ3, Vγ4, Vγ2, Vγ5 cells) are produced[9]. In contrast, our understanding of the role of ontogeny in determining CD8+ T cell phenotype and function is incomplete. CD8+ T cells play an important role in protecting the host against intracellular pathogens[10]. Despite their importance, the field has yet to fully integrate developmental biology into the calculus of *how* CD8+ T cells protect the host. This is due, in part, to the fact that hematopoiesis, thymic selection, and functions of mature CD8+ T cells are often studied separately, preventing us from putting together a complete picture of CD8+ T cell ontogeny. In this review, we discuss how recent findings from these areas of research are interconnected, describing the functions of CD8+ T cells through the lens of immune development.

A major premise of the layered immune system model is that the functions of immune cells are linked to when they were produced. This model has led to our understanding of how the developmental origins of B cells, macrophages, and γδ T cells shape their phenotype and function. Reviews (and reviews[11–13]) of these important cell types often devote a significant amount of time discussing their developmental origins. For CD8+ T cells, the case is different. Despite a long list of landmark discoveries (Fig. 1), the developmental origin of CD8+ T cells is rarely mentioned in relation to their function[14,15]. One reason for this may be the focus on CD8+ T cell phenotype and function in the periphery, where understanding the differentiation from naïve to effector and memory CD8+ T cells (and subsets thereof) has presented a significant challenge by itself. Another reason may be that those studying hematopoiesis have primarily focused on other immune cell types (B cells, γδ T cells, macrophages) or simply use bulk CD3+ T cells as the readout for their assays. Hence, the canonical view has been that CD8+T cells in the periphery of the adult represent a single lineage of CD8+ T cells, which only acquire distinct functional programs after stimulation. However, recent work now shows that there are distinct lineages of neonatal- and adult-derived CD8+ T cells in the naïve pool, which are made during specific stages of development and play unique roles during infection[16–20].

The dominant question we pursue is whether there is sufficient evidence to extend the layered immune system model to CD8+ T cells. To address this question, it is important to first consider the key features of the layered immune system model. In general, 1) fetal lineages (e.g., fetal erythrocytes, B1a, certain γδ T subsets, microglial cells) are derived from fetal progenitors that arise from multiple anatomical sites during ontogeny, whereas adult lineages (e.g., B2 B cells, adult CD4+ T cells) are made from adult hematopoietic stem cells (HSCs) (Fig. 2)[21]. 2) Fetal progenitors give rise to more fast-acting innate-like lineages, whereas adult HSCs generate slower-acting adaptive lineages[4,6,18,22]. For example, fetal-derived lineages are more capable of exerting antigen-independent cytokine responses, due to their ability to acquire effector functions during development, which means they are less reliant on their immunoreceptors (e.g., T cell receptors (TCRs) or B cell receptors (BCRs)) for activation[23–27]. In contrast, adult-derived lineages exhibit a more diverse repertoire of immunoreceptors, exist in a more naïve state, and more efficiently develop into long-lived memory cells[16,28,29]. 3) Fetal-derived lineages persist into adulthood, creating an immune compartment comprised of developmental layers[4,30–33].

In this review, we will discuss how many of the hallmark characteristics of the layered immune system model can be found during the ontogeny of the CD8+ T cell compartment. We have elected to focus on the key experiments and approaches from our lab that have opened our eyes to the possibility that the CD8+ T cell compartment is layered. The developmental origins of CD8+ T cells may be a major and previously unappreciated determinant of individual cell function.

2. Age-related differences in CD8+ T cells

If the layered immune model pertains to CD8+ T cells, we should expect to find functionally distinct subpopulations of cells made at different stages of development. However, before addressing whether the behavior of CD8+ T cells depends on when they are produced, it is important to first describe the functions of CD8+ T cells. In general, there are two goals of the CD8+ T cell response to infection: 1) eliminate the pathogen; and 2) remember previous encounters with infectious agents. The division of labor is thought to arise following priming in the lymph node, where a small number of naïve cells become activated and either quickly differentiate into short-lived effectors (to protect the host) or more slowly develop into memory cells (to remember the pathogen). Based on their ability to recognize specific microbial peptides via their T cell receptors (TCRs) and form memory after the pathogen is cleared, the dogmatic view of CD8+T cells is that they are an adaptive immune cell; this conclusion is based on studies done mostly in adults. However, there is a subset of CD8+ T cells in the starting pool with more innate-like features, reminiscent of the B cell compartment, which has been found to have subsets of cells with both adaptive and innate functions[16,34–36]. This subset can be distinguished from other antigen-inexperienced CD8+ T cells because it expresses markers more commonly found on memory CD8+ T cells and can be activated by innate cytokines alone[37]. Since activation occurs in the absence of TCR stimulation, it is often referred to as bystander activation[38]. Although bystander activation of CD8+ T cells does not lead to clonal expansion, it does result in the production of effector molecules (e.g., IFNγ, gzmB), which can help to limit pathogen growth during early stages of infection[39,40]. Thus, CD8+ T cells can protect the host via an antigen-dependent (adaptive) response *and* an antigen-independent (innate) response. In this section, we will discuss recent data from studies done in neonatal (1 week of age) and adult (8–12 weeks of age) mice suggesting that CD8+ T cell functions are linked to when they are created in the host.

2.1 Not all CD8+ T cells are created equally

An intriguing feature of lymphocytes (B cells and CD4+ T cells) in neonatal mice is that they undergo more cell divisions than their adult counterparts after stimulation[41]. Around 10 years ago, our lab sought to determine whether this property also applied to CD8+ T cells, so we compared the ability of neonatal and adult CD8+ T cells to proliferate following in vitro stimulation[19]. For this experiment, we isolated CD8+ T cells from neonatal and adult mice, coated them with a proliferation dye (CFSE), and measured the number of divisions that each group of cells underwent at various times after stimulation via the TCR. Interestingly, CD8+ T cells from neonatal mice divided sooner and faster than their adult counterparts (Fig. 3A). The neonatal CD8+ T cells also underwent increased division-linked

differentiation (differentiated more per round of division) than adult CD8+ T cells[42]. These data indicated that there are cell-intrinsic differences between neonatal and adult CD8+ T cells, and 'rapid proliferation' is a functional trait that is shared among different immune cell types made in early life.

Another key feature of lymphocytes made early in life is that they have a limited capacity to 'remember' past infections. For example, B1 B cells and γδ T cells are the first to respond to infection, but they fail to form memory[43,44]. In contrast, B2 cells respond more slowly but are superior at becoming memory cells[45]. To see if neonatal and adult CD8+ T cells also exhibit different abilities to form memory, we adoptively co-transferred an equivalent number of CD8+ T cells from neonatal and adult donor mice into the same recipient and compared their number and phenotype at different times after infection (Fig. 3B)[19]. Similar to B1 B cells, the neonatal CD8+ T cells responded sooner than adult CD8+ T cells, but they rapidly became terminally differentiated and failed to transition into the long-lived memory pool. As a result, the recall response to a secondary challenge was largely dominated by the adult donor CD8+ T cells.

A question that arises is, why would it be beneficial for the host to produce CD8+ T cells poor at forming memory? One possibility comes from an evolutionary standpoint. It is more important to produce CD8+ T cells capable of rapid proliferation and differentiation early in life because if the host does not survive infection, it is unimportant what happens later. If we accept this reasoning, CD8+ T cells in neonatal mice mount a vigorous early response to keep the host alive, but it comes at the expense of forming memory. However, another possibility is that CD8+ T cells are less efficient at forming memory because they are wired more for innate defense in ways that go beyond rapid proliferation. Indeed, a unique function of lymphocytes in neonatal mice is that they are highly responsive to inflammation and can rapidly produce a wide variety of cytokines. For example, in neonatal mice, different subsets of γδ T cells secrete either IFNγ and IL-4 or IL-17 upon activation[24,25]. Neonatal B1 B cells also display significant heterogeneity, with some subsets producing IL-10 while others make GM-CSF and IL-3[26,27].

To determine whether the same heterogeneity applies to CD8+ T cells, we recently compared the ability of CD8+ T cells from different-aged mice to respond to innate cytokines (IL-12 and IL-18) alone, in the absence of TCR stimulation (Fig. 3A)[20]. Strikingly, we found that neonatal CD8+ T cells were much more responsive to inflammation than adult CD8+ T cells and underwent a distinct program of innate immune activation. In addition to producing larger amounts of canonical effector molecules (IFNγ, gzmB), the neonatal CD8+ T cells secreted a broad spectrum of cytokines (e.g., IL-13, IL-10, GMCSF, IL-17, IL-22) not typically associated with CD8+ T cells. To understand how innate functions are stratified across the population, we also performed high dimensional flow cytometry and single cell RNAseq and discovered that neonatal CD8+ T cells are not comprised of a single subset of polyfunctional cells ('generalist'), but rather multiple subsets of cells ('specialist') that produce distinct combinations of cytokines and effector molecules upon stimulation.

We wondered whether the unique program of innate immune activation by neonatal CD8+ T cells corresponded to better or worse infection outcomes. To test this, we adoptively transferred CD8+ T cells from neonatal and adult CD8+ TCR transgenic mice and compared their ability to mediate bystander immune protection against irrelevant strains of bacterial, helminth, and viral infections[20]. Interestingly, the recipients containing neonatal donor CD8+ T cells had dramatically reduced pathogen burdens compared to their adult counterparts. Thus, while neonatal CD8+ T cells are less efficient at forming memory, they exhibit an enhanced ability to protect the host against a wide range of pathogens in the absence of T-cell receptor (TCR) signaling. Our data demonstrate that CD8+ T cells in neonatal mice possess more rapid innate-like functions, whereas those in adult mice exhibit slower adaptive features.

2.2 Factors that alter the biology of CD8+ T cells made in early life

In this subsection, we will describe a series of experiments that allowed our lab to dissect out the key developmental factors contributing to the altered functions of CD8+ T cells in neonatal mice. To gain insight into why neonatal and adult CD8+ T cells behave differently, an important first step is to compare their surface phenotype. In general, most CD8+ T cells in the spleen and lymph node exhibit a naïve phenotype (CD62L+CD44−)[46]. However, there is a small subset of antigen-inexperienced cells in adult mice that express markers (CD44, CD122) that are more commonly found on memory cells[37,46]. These memory phenotype cells not only resemble antigen-experienced memory CD8+ T cells but also display similar functional traits, including rapid proliferation and production of effector molecules after stimulation[47]. Interestingly, when we compared the phenotype of CD8+ T cells from different-aged mice, we discovered that CD8+ T cells from neonatal mice are comprised of a much larger fraction of memory phenotype cells[18]. In contrast, most of the CD8+ T cells in adult mice exhibited a naïve phenotype[18].

Memory phenotype CD8+ T cells can be further categorized as innate memory CD8+ T cells (if they upregulate memory markers during thymic development) or as virtual memory CD8+ T cells (if they upregulate memory markers in the periphery)[36]. This latter subset in neonatal mice, the virtual memory (VM) CD8+ T cells, is what we focus on in this review, since they acquire their memory phenotype after thymic egress[18]. Although it seems counterintuitive to generate more responsive CD8+ T cells in early life, virtual memory CD8+ T cells in mice do not have an enhanced capacity to induce autoimmune diseases (e.g., type I diabetes) compared to naïve CD8+ T cells, which may relate to their reduced ability to upregulate CD25 and CD49d in response to suboptimal antigens[48]. Thus, the acquisition of a virtual memory phenotype in early life may enable neonatal CD8+ T cells to provide enhanced immune defense (akin to antigen-experienced memory cells) during critical stages of development, while still being as self-tolerant as naïve CD8+ T cells.

In some ways, the increased percentage of VM cells we found in the neonatal mice was not surprising, as earlier studies have suggested that CD8+ T cells undergo more homeostatic proliferation in neonatal mice because they are lymphopenic[49,50]. Due to this, the dogmatic view was that neonatal CD8+ T cells are exposed to greater amounts of homeostatic cytokines (IL-7 and IL-15) than adult CD8+ T cells and undergo more

homeostatic proliferation in an attempt to ‘fill the space’. To test whether it is true that CD8+ T cells proliferate more in early life to fill an empty periphery, we transplanted a newborn thymus under the kidney capsule of an adult mouse and examined the phenotype of the donor CD8+ T cells four weeks later (Fig. 4A)[17]. Interestingly, we found that newborn CD8+ T cells proliferate no matter what; that is, they preferentially acquire a VM phenotype regardless of whether they mature in a neonatal (lymphopenic) or adult (lymphoreplete) host. These data suggest that neonatal CD8+ T cells are comprised of more VM cells not because of age-related differences in the environment, but rather because the neonatal cells are inherently more proliferative than their adult counterparts.

Since VM cells are the first to respond and become effectors after infection, we considered it likely that CD8+ T cells from neonatal mice adopt different fates than those from adults because they are comprised of a larger fraction of VM cells. To examine this possibility, we sorted out naïve (CD44-CD122−) and VM (CD44+CD122+) CD8+ T cells from neonatal and adult donor mice and directly compared their ability to respond to infection in the same host (Fig. 4B)[18]. Surprisingly, even when comparing phenotype-matched subsets of cells from different-aged donor mice, the neonatal donor CD8+ T cells still preferentially gave rise to short-lived effectors and the adults more efficiently became memory precursors. Moreover, the naïve and VM subsets from different-aged mice exhibited distinct gene expression profiles; the genes that were upregulated in neonatal CD8+ T cells represented those more commonly found in effector CD8+ T cells[18,51]. Together, these data indicate that the effector bias by neonatal CD8+ T cells is not due to age-related changes in the starting phenotype of cells prior to infection.

After ruling out age-related changes in homeostatic proliferation and starting phenotype, we next asked whether CD8+ T cells from neonatal and adult mice adopt different fates during infection because they are derived from distinct hematopoietic progenitor cells (fetal liver progenitors vs. adult bone hematopoietic stem cells). Briefly, during immune ontogeny, the thymus, which is a specialized primary lymphoid organ, is colonized by distinct waves of hematopoietic progenitors that give rise to unique lineages of immune cells[6,52–55]. To test our hypothesis, we first examined the gene expression profiles of CD8+ T cells at the same stage of thymic development in neonatal and adult mice, which control for age-related differences in post-thymic maturation. Interestingly, the single-positive (SP) CD8+ T cells in the thymus from neonatal mice expressed more effector genes than their adult counterparts, suggesting that neonatal CD8+ T cells don’t become different in the periphery, but rather are made more effector-like during development[18].

We found more definitive evidence that fetal and adult HSCs generate different subsets of CD8+ T cells when we performed intrathymic injection experiments. In these experiments, we injected double negative thymocytes from a fetal (embryonic day 14) or adult thymus (8 weeks) into the thymus of adult recipient mice (8 weeks) and the progeny were examined 4 weeks later (Fig. 4C). Remarkably, the fetal progenitors gave rise to CD8+ T cells that were almost exclusively virtual memory CD8+ T cells. In contrast, the majority of adult progenitors generated CD8+ T cells with a naïve phenotype and an enhanced capacity to form memory after infection. We also transferred the fetal- and adult-derived CD8+ T cells into congenic recipients and tracked their response to infection. Whereas the fetal-derived

CD8+ T cells predominantly gave rise to short-lived effectors, the adult-derived CD8+ T cells preferentially became memory CD8+ T cells. Collectively, these studies provide strong support for the notion that CD8+ T cells in neonatal and adult mice behave differently because they come from distinct developmental origins.

3. CD8+ T cells and developmental layering

In the previous section, we described the phenotype and function of CD8+ T cells present in neonatal versus adult mice. Based on our findings, and because the primary function of CD8+ T cells is to protect the host against pathogens, an important question was whether neonatal CD8+ T cells contribute to the CD8+ T cell response to infection in adults. Previous work has shown that approximately 1% of the total thymic cell content is exported from the thymus per day (~10^6 recent thymic emigrants per day in an adult)[56]. Once CD8+ T cells leave the thymus, they survive for ~3 months in the periphery of mice[57], though some naïve T cells can persist for up to 1 year[58]. As thymic output peaks early in life and naïve T cells are long-lived, it seemed reasonable to assume that a large fraction of the naïve CD8+ T cell compartment in young adults is made up of cells produced in fetal and neonatal life. In the section below, we describe how we developed tools to track murine CD8+ T cells made at different stages of development, so we could examine their phenotype and behavior in adulthood.

3.1 Timestamping: Function follows origin

The key question was whether neonatal CD8+ T cells persist into adulthood and retain their ability to rapidly respond to infection. An alternative possibility was that the neonatal CD8+ T cells would ‘mature’ and behave more like adult CD8+ T cells in adulthood. To differentiate between these possibilities, we devised a strategy using mice with CD4-driven tamoxifen-inducible Cre (CD4cre-ERT2) to induce expression of fluorescent reporter protein (TdTomato), allowing us to permanently label, or ‘timestamp,’ a wave of CD8+ T cells made in the thymus at the time of tamoxifen exposure (Fig. 5A)[17,59]. This strategy works because CD8+ T cells only express CD4 for a brief period of time during thymic development (at the double positive stage) before becoming a single positive CD8+ T cell. We marked one group of mice at birth to label neonatal-derived CD8+ T cells and a second group at 28 days to label adult-derived CD8+ T cells. What we found was that both groups still existed in adulthood 8 weeks later (Fig. 5A). The majority of neonatal-derived CD8+ T cells exhibited a VM phenotype and were enriched in the liver. The adult-derived CD8+ T cells, on the other hand, expressed genes more traditionally associated with naïve cells and were preferentially found in the lymph nodes. These data suggested that the phenotype and distribution of CD8+ T cells in adult mice was linked to their developmental origins.

To determine whether neonatal- and adult-derived CD8+ T cells retained their cell-intrinsic properties in adulthood, we repeated the *in vitro* stimulation and adoptive transfer experiments described above[17]. The neonatal-derived CD8+ T cells were the first to respond and become effectors after infection, while the adult-derived CD8+ T cells responded with slower kinetics but were more efficient at transitioning into the long-lived memory pool. To better understand why neonatal- and adult-derived cells adopt different fates after infection

in adults, we performed genome-wide mapping of chromatin accessibility and found that neonatal-derived CD8+ T cells exhibit increased accessibility to transcription factors that drive effector cell differentiation *(Tbx21, Id2, Eomes*). Adult-derived CD8+ T cells, on the other hand, have increased accessibility for genes that promote memory formation (*Foxo1, Foxo3*). Thus, even before priming, the neonatal- and adult-derived CD8+ T cells are biased towards becoming different types of effectors (short-lived effectors vs. memory precursors). The neonatal-derived CD8+ T cells also retained their enhanced ability to produce effector molecules after innate cytokine stimulation. These findings led to the new idea that there are distinct lineages of CD8+ T cells generated during different windows of development, which co-exist in adulthood and have predictable fates during infection.

The timestamp experiments were important because they demonstrated that the behavior of CD8+ T cells was determined, at least in part, by when the cells were created in the host. However, an important and unanswered question was whether the neonatal layer of CD8+ T cells was essential for the host to control infection. To address this question, we designed experiments to examine how susceptibility to infection changes in mice that only contain the neonatal or adult layer of CD8+ T cells[60] (Fig. 5B). To deplete the neonatal layer, we administered an anti-CD8 depleting antibody to mice at 2 weeks of age and let the mice age to 8 weeks. To deplete the adult layer, we performed thymectomies in another cohort of mice at 2 weeks of age, so that at 8 weeks of age the mice would only contain the neonatal layer. We then adoptively transferred CD8+ T cells from each group (neonate-depleted, adult-depleted) into separate T cell-deficient recipient mice and compared their ability to clear *Listeria monocytogenes*. Remarkably, the recipient mice lacking the neonatal layer of CD8+ T cells had ~10 times more bacteria in the spleen compared to those lacking the adult layer. This experiment indicated that neonatal-derived CD8+ T cells may be more useful for protecting adults against primary infection, whereas adult-derived CD8+ T cells may play a larger role in providing immune protection against secondary infections. In this way, the division of labor in the CD8+ T cell compartment is mediated by distinct subsets of innate and adaptive cells, similar to B cells (B1a, B1b, B2), which have unique functional capabilities[16,34].

3.2 Factors that alter the developmental layering of CD8+ T cells

The above studies point toward a new model to explain why individual CD8+ T cells follow unique patterns of differentiation after infection. In the past, researchers have placed their focus on understanding how various cues during activation (e.g., cytokines, APCs, signal strength) lead to phenotypic diversity in the effector and memory pool[61–63]. However, our more recent studies suggest the diversity of effector and memory cells that arise after microbial challenge is linked to the composition of naïve CD8+ T cells prior to infection. Rather than exclusively focus on events that occur after infection, we felt it was important to also identify the early factors that alter the developmental layering of the CD8+ T cell compartment, since the number of neonatal- and adult-derived CD8+ T cells present in the starting pool has a significant impact on the outcomes of infection[60].

A major factor that would influence the proportion of neonatal- and adult-derived CD8+ T cells in the starting pool is, of course, the age of the host. While fate-mapping can be useful

to mark waves of CD8+ T cells present during the window that tamoxifen is present, it is challenging to use this approach to mark the entire layer of neonatal- or adult-derived CD8+ T cells, since prolonged exposure to tamoxifen is toxic for young mice. Thus, it was not feasible to use fate-mapping alone to estimate the number of neonatal- and adult-derived CD8+ T cells that were present at different ages. To get around this issue, we formed a collaboration with Miles Davenport's group at the University of New South Wales, and together we combined fate mapping and mathematical modeling to obtain the first ever glimpse of the age structure of the CD8+ T cell compartment over the lifetime of the host[59]. The results demonstrated that the survival of CD8+ T cells is a function of their age of production (cells early in life have a fast initial decay rate) and the age of the cell (older cells have slower decay rates). While it is generally the case that neonatal-derived CD8+ T cells initially decay at faster rates than adult-derived cells, we found a subset of neonatal-derived CD8+ T cells that survives for the life of the host, which is reminiscent of the B1 B cells that also have the capacity to self-renew and persist in adulthood. Importantly, this study revealed how the developmental architecture of the CD8+ T cell compartment dynamically changes with age. The constant restructuring of the CD8+ T cell compartment has implications for predicting the outcomes of infection in different-aged animals[17,64,65].

While the age structure study was useful for establishing a baseline measurement of the number of neonatal- and adult-derived CD8+ T cells across the lifespan, all the work was performed in mice raised in specific pathogen-free (SPF) conditions, which is an abnormally clean environment[66]. Thus, we wondered how the CD8+ T cell compartment would change if the mice were raised in a more microbially diverse setting. There is justification for this question. Previous epidemiological studies have suggested that children exposed to microbially diverse environments (i.e., farms, daycares, etc.) are better protected against the development of T cell-mediated diseases, such as asthma, inflammatory bowel disease and diabetes[67–70]. Lim et al. found that the development of immune cells can be shaped by infections that occur *in utero*[71]. However, how early microbial exposure shapes immunity is poorly understood. To gain mechanistic insight into how immunity is set in early life, we recently exposed SPF laboratory mice to a 'dirty' pet-shop environment and found that their offspring had enhanced immunity against intracellular infection in adulthood[60]. One possible explanation is environmental; that is, enhanced immune protection in dirty mice is because the environment enhanced the function of all cells in a uniform manner. Alternatively, early microbial exposure could alter immune responsiveness by changing the developmental structure, or layering, of the CD8+ T cell compartment. To delineate between the 'uniform' and 'layered' model, we used timestamp mice and demonstrated that the enhanced immunity we saw was due to an expansion of the 'fast-acting' neonatal-derived CD8+ T cells, which have increased protective capabilities. We found nearly 5 times more neonatal-derived CD8+ T cells in dirty mice versus clean mice, and ablating the neonatal layer in dirty mice made them as susceptible to infection as clean mice. Thus, the developmental layering of the CD8+ T cell compartment can be remodeled by early microbial exposure and alter the set-point for immune susceptibility in adulthood.

3.3 Parallels in humans

The evidence is accumulating for developmental layering of the CD8+ T cell compartment, based on our studies in mice and mathematical modeling. But is developmental layering of CD8+ T cells conserved in humans? There is data suggesting the behavior of human CD8+ T cells is linked to their derivation from distinct hematopoietic progenitors. For example, bone marrow transplant studies have indicated that CD8+ T cells from cord blood share more features with newborn cells, whereas those from adult bone marrow more closely match adult CD8+ T cells[72]. In addition, numerous reports have indicated that human CD8+ T cells in early life possess more innate-like functions, as evidenced by their innate-like receptor expression (TLRs, complement receptors, NK cell receptors), response to inflammation and danger signals, and ability to deploy nonspecific defense mechanisms typically associated with innate cells[17,73–77]. There is also evidence that large numbers of memory phenotype (CD45RA-CD45RO+) CD8+ T cells are present prior to birth[78]. However, since human research precludes the possibility of genetically encoded fate-mapping, researchers have turned their attention to performing single cell RNA sequencing (scRNAseq) to map the contribution of neonatal and early life ‘layers’ of CD8+ T cells to the total pool over the course of early development.

The strategy of these studies is to determine whether changes in the composition of CD8+ T cells at different stages of life can be explained by the ‘layered model’ or by what is known as the ‘gradual change’ model. As we know, the layered model posits that the proportion of fetal- and adult-derived CD8+ T cells changes with age, but the individual cells within each layer retain their distinctive phenotypic and functional properties. The gradual change model proposes that the change in ‘average phenotype’ of CD8+ T cells at different stages of life comes from a maturation of individual cells along a linear axis, where individual CD8+ T cells made early in life progressively lose their fetal phenotype and acquire a more adult phenotype with age.

To differentiate between these models in humans, Bunis et al. applied a single-cell developmental stage score to sort populations of naïve CD8+ T cells ($CD8^+CD45RA^+CD27^+CCR7^+CD95^-$) at different stages of human development (fetal, birth, adult)[79]. What they found is that the naïve CD8+ T cells at birth clustered as a distinct population between the fetal and adult clusters. They also found that CD34+ hematopoietic stem and progenitor cells (HSPCs) at birth were positioned at an intermediate range, between the fetal and adult HSPCs. One interpretation here is that CD8+ T cell ontogeny does, in fact, progress along a spectrum of maturity. However, since lineage tracing is not possible, it is unclear whether the changes in gene expression were occurring in the same population of CD8+ T cells or coming from a distinct wave of CD8+ T cells produced before birth[80]. Indeed, some fetal progenitors are developmentally restricted and contribute to hematopoiesis in early life but then disappear after birth[81]. An alternative explanation was that there are at least 3 distinct layers of CD8+ T cells that exist along a continuum of development. This conclusion recalls previous work in quail/chick chimeras suggesting there are three distinct waves of thymocytes that colonize the thymus, and studies in mice suggesting there are 3 lineages of B cells (B1a, B1b, B2) and γδ T cells (Vγ3, Vγ4, Vγ5/Vγ1) that arise in succession[82]. Nonetheless, the current studies support the idea that

different types of CD8+ T cells are produced at different stages of life from an evolving hematopoietic progenitor cell population.

Our own studies in humans have made use of scRNAseq to investigate changes in the composition of human CD8+ T cells at different stages of life[20]. However, instead of sorting for CD8+ T cells with a naïve phenotype, we stimulated bulk CD8+ T cells from different-aged individuals with innate cytokines (IL-12 and IL-18), since inflammation responsiveness is a unique feature of neonatal-derived CD8+ T cells. We identified multiple, distinct innate-like fetal clusters at birth, some of which disappeared with progressing age. Interestingly, the most innate-like cluster of CD8+ T cells in adults possessed a transcriptome similar to subsets of CD8+ T cells found at birth, suggesting that this cluster may represent the population of neonatal CD8+ T cells that persists into adulthood. These data lend support for the layered immune system model, implicating that a progressive shift from innate to adaptive functions is mediated by developmental-related changes in the abundance of phenotypically and functionally distinct subpopulations of CD8+ T cells. It is possible that the subset of putative fetal CD8+ T cells that persists into adulthood was not observed in previous studies because analysis is typically restricted to naïve CD8+ T cells, so any fetal-derived CD8+ T cells that have lost their expression of naïve markers would be excluded. This is an important point because many of the fetal-derived CD8+ T cells that are ‘timestamped’ in fate-mapping mice no longer exhibit a naïve phenotype in adulthood[17]. Thus, a broader and more comprehensive analysis of the CD8+ T cell compartment may be needed to capture the dynamic nature of CD8+ T cell ontogeny across different stages of development.

4. Programming of neonatal and adult CD8+ T cells

It is now clear that we must take into consideration the developmental origins of CD8+ T cells to understand how CD8+ T cells are programmed differently at various stages of life. In mice, discrete hematopoietic waves give rise to different types of immune cells at different stages of development[21]. The first, or primitive, wave of hematopoiesis begins at embryonic (E) day 7.5 in the yolk sac, with the emergence of primitive erythrocytes, macrophages, and megakaryocytes[83–85]. The second, or pro-definitive, wave develops at ~E8.25, with the formation of erythromyeloid progenitor (EMP), which gives rise to both fetal and adult erythrocytes and tissue resident macrophages (e.g., microglia, Langerhans cells, and Kupffer cells)[86–88]. The third, or definitive, wave begins at E10.5, with the emergence of HSCs from the aorta-gonad-mesonephros (AGM)[89]. The hematopoietic stem cells (HSCs) and earlier hematopoietic stem cell progenitor cells (HSPCs) migrate to the liver. Fetal liver HSPCs colonize the thymus around mid-gestation (~E12.5) and give rise to the first wave of CD8+ T cells[90]. Later in development (~E18), the HSCs home to the bone marrow, where they generate all lineages of immune cells, including adult CD8+ T cells[91]. Importantly, neonatal CD8+ T cells are likely derived from the same waves of fetal hematopoietic progenitors that give rise to unique subsets of B cells and γδ T cells. Yet, we know significantly less about how functional variation of CD8+ T cells is linked to their derivation from distinct hematopoietic progenitors. In this section, we discuss the key regulators in fetal and adult hematopoietic progenitors that promote distinct functions in CD8+ T cells produced at different stages of life.

4.1. Lin28: The key to being rapid and innate

What are the transcriptional regulators in fetal progenitors that promote rapid and innate functions in CD8+ T cells made in early life? A seminal study by Yuan et al. found that ectopic expression of Lin28b in adult progenitors is sufficient to permit the development of innate-like lineages of lymphocytes (B1a B cells, marginal zone B cells, Vγ1.1+Vδ6.3 T cells, NKT cells) that are typically only produced during fetal and neonatal stages of development[92]. These findings defined a hematopoietic role for Lin28b, a developmentally regulated RNA binding protein that fine-tunes gene expression in early life[93]. More recent studies from our lab and others indicate that Lin28b converts CD8+ T cells into a more innate-like subset with a virtual memory phenotype[18,20,94,95]. Importantly, virtual memory cells from Lin28b Tg mice undergo a program of bystander activation that is nearly identical to that found in neonatal CD8+ T cells and provides bystander immune protection against unrelated pathogens, in the absence of TCR stimulation[20]. Thus, Lin28b may serve as a gatekeeper, opening the door to a hidden diversity of innate phenotypes in neonatal CD8+ T cells that are not present in the adult population.

Lin28b has also been implicated in altering the fates of fetal- and adult-derived CD8+ T cells after infection. We demonstrated that Lin28b induction in adult progenitors results in the generation of CD8+ T cells that respond rapidly to primary infection but quickly become terminally differentiated[18]. As a result, adult CD8+ T cells overexpressing Lin28b fail to transition into the long-lived memory pool and mount poor recall responses to secondary infections, which phenocopies the neonatal-derived CD8+ T cell response to infection. Although the underlying mechanisms require further investigation, recent data suggest that Lin28b promotes rapid effector CD8+ T cell differentiation by driving a more glycolytic metabolic program[96], which is consistent with studies in other cell types demonstrating Lin28b is a key regulator of glucose metabolism[97]. Induction of Lin28b in adult CD8+ T cells results in significantly higher rates of glycolytic metabolism, and pharmacological inhibition of glycolysis in neonatal CD8+ T cells restores their ability to develop into memory cells[96]. Thus, Lin28b may facilitate rapid proliferation by altering metabolic reprogramming so that neonatal-derived CD8+ T cells have a greater ability to use glucose and respond quickly to infection, albeit at the expense of forming memory.

4.2 Let-7: ‘Letting’ adult CD8+ T cells be adaptive

The expression of Lin28b rapidly decreases in the first week of life and is nearly undetectable at 4 weeks of age[92], which mirrors the drop in the proliferative capacity of CD8+ T cells[41]. In adult HSCs, this loss of Lin28b allows for expression of let-7 microRNA family members, suggesting that they may regulate the adaptive functions of CD8+ T cells. The let-7 miRNA family has been identified as a controller of developmental timing in a wide range of species (nematodes, mice, humans)[92,98–101]. More recently, let-7 has gained attention for its ability to behave as a tumor suppressor in human cancer cells and repress cell proliferation[102–104]. In mice, there are 9 slightly different let-7 family members, though all express the identical core sequence required for specifying mRNA targets and are believed to perform nearly identical functions[105]. Importantly, the let-7 family is deeply conserved, and many let-7 targets are shared in mice and humans[106,107]. Of note, let-7 is predicted to target a variety of genes in the mTOR pathway (IGF1R, PI3K, ATK2, TSC1)

for degradation[108,109]. Thus, the rise in expression of let-7 in adult-derived CD8+ T cells may help to create a transcriptional landscape that is conducive for slow homeostatic cell proliferation and maintenance of naïve CD8+ T cells.

Let-7 has been shown to directly regulate key functions of CD8+ T cells. For example, Wells et al. demonstrated that let-7 was essential for maintaining the naïve state in CD8+ T cells[110]. When let-7 was blocked, the CD8+ T cells in the spleen and lymph node underwent increased rates of proliferation and acquired a VM phenotype. The authors proposed that let-7 may reduce proliferation by directly inhibiting expression of cell cycle genes. Notably, the transcription factor Eomes is expressed in thymic precursors of virtual memory cells[111] and has been shown to be a target of let-7[110]. Thus, it is possible that adult progenitors give rise to fewer VM cells because they express lower amounts of let-7, resulting in the downregulation of Eomes. Let-7 has also been shown to limit proliferation and differentiation of CD8+ T cells after TCR stimulation[110], potentially explaining why adult-derived CD8+ T cells are more efficient than neonatal-derived CD8+ T cells at forming immunological memory. Collectively, these studies suggest that innate and adaptive properties of CD8+ T cells are derived from developmental changes in let-7/Lin28b expression in the hematopoietic stem cell compartment.

5. Thymic education of neonatal and adult CD8+ T cells

In the previous sections, we discussed how the ontogeny of CD8+ T cells is similar to B cells and macrophages, with distinct layers of CD8+ T cells arising from unique HSPCs at different stages of life. However, a unique feature of CD8+ T cell development is that the hematopoietic progenitors must first travel to the thymus to generate mature CD8+ T cells. While the process of thymic development is an area of intense research, the ways in which CD8+ T cells are educated in the neonatal thymus has received less attention. In this section, we discuss how developmental events that occur in the neonatal thymus may set the stage for the behavioral differences observed in T cells made in early life.

5.1 T cell receptor-mediated instruction of cell fate

While we propose that CD8+ T cells made in early life have an increased propensity to become a VM cell with innate-like properties because they are derived from an alternative progenitor, there is also evidence to suggest that the development of VM cells is a T cell receptor (TCR)-mediated process that begins in the thymus, with acquisition of the phenotype completed in the periphery. Using retrogenic mice, Drobek et al. first demonstrated that certain TCRs can specify a VM or naïve fate[48]. Later, Miller et al. extended these findings by showing that VMs across individual mice have similar TCRs and are distinct from their true naïve (TN) counterparts[111]. By expressing recurrent VM TCRs in retrogenic mice, they showed that clonotypes with TCRs associated with VM cells preferentially give rise to cells with a virtual memory phenotype. In addition to bearing the surface markers associated with virtual memory status, the VM cells from retrogenic mice exhibited behavior associated with VM CD8+ T cells, such as enhanced reactivity towards self-antigens and higher levels of Eomes during the later stages of thymic development. Eomes identifies thymic precursors that later become VM cells, suggesting an analogous

developmental trajectory to Foxp3 in the formation of T regulatory cells (Tregs)[111]. For example, the development of VM and Tregs both involve a three-step process starting with an initial TCR-mediated event in the thymus, expression of a master regulator (Eomes or FoxP3), and consolidation in the periphery to acquire the VM or Treg phenotype and function (Fig. 6).

The work by Drobek and Miller convincingly demonstrates that VM fate is set in the thymus, potentially explaining why CD8+ T cells from a newborn thymus give rise to a similar proportion of VM cells, regardless of whether they are matured in a neonatal or adult peripheral environment[17]. However, the link between VMs and TCRs has only been made in adult mice, and we have established that CD8+ T cells in early life have a greater propensity to become VMs. Thus, another factor that needs to be taken into consideration is developmentally regulated differences in the TCR repertoire and how these differences may promote the innate-like program of developing thymocytes. Importantly, HSPCs derived from the fetal liver do not express Terminal deoxynucleotidyl Transferase (TdT)[112]. As a result, fetal- and neonatal-derived CD8+ T cells express shorter and more germline-encoded TCRs, which are more peptide promiscuous, or cross-reactive[113]. Enhanced promiscuity in germline-encoded clonotypes may allow for more efficient interactions with MHC peptides during positive selection and promote enhanced homeostatic proliferation in the periphery, leading to an expansion of the VM population[114]. Thus, it is possible that limiting TdT expression in early life helps to rapidly fill the peripheral pool with VM cells with innate-like functions to provide broad immune protection until more diverse clonotypes are generated.

5.2 Increased self-reactivity in early life

Another common feature of innate-like lymphocytes (B1a B cells, γδ T cells) is the expression of more self-reactive immunoreceptors, which may enable their basal existence in a more activated state. In the case of CD8+ T cells, wide-ranging TCR affinities for self-peptide MHC complexes are permissible for developing T cells to progress past positive selection. However, both CD8+ and CD4+ T cells in early life are skewed towards high self-reactivity. This conclusion is based on studies in mice and humans showing that neonatal thymocytes express higher levels of the surface marker CD5[40,115–117]. CD5 expression levels are set during positive selection and serve as an accurate indicator of reactivity towards self-peptides. Importantly, several distinct behaviors of neonatal CD8+ T cells can be attributed to high levels of CD5, including enhanced homeostatic proliferation, enrichment for the VM phenotype, rapid recruitment during infection, and high expression of transcription factors that promote effector differentiation (e.g., Eomes)[18,19,42,48,96,118]. Expression of more self-reactive TCRs may divert CD8+ T cells toward a rapid innate-like lineage of cells in early life.

Why are highly reactive CD5hi cells enriched in early life? To better understand age-related differences in CD5 expression, Dong et al. compared CD5 levels in CD8+ T cells during the same stage of development in the thymus or at the same cellular age in the periphery. In both cases, CD5 levels were elevated in neonatal thymocytes compared to their adult counterparts, suggesting that age-related differences in homeostatic proliferation and post-

thymic maturation do not explain the higher levels of CD5 expression in CD8+ T cells in early life. There was also no difference observed in CD5 expression in single positive CD8+ T cells from adult wild-type and TdT KO mice, indicating that germline-encoded clonotypes did not explain the increased CD5 expression on neonatal CD8+ T cells either. Instead, the data indicated that the thymic selection threshold was shifted in early life and thymocytes expressing a low-affinity TCR were not efficiently selected into the neonatal pool. Thus, the TCR repertoire of fetal-derived CD8+ T cells is enriched for self-reactive TCR simply because those with low affinity TCR fail to develop in early life. Although the molecular mechanisms are unknown, studies thus far suggest that strong signals mediated by TCRs during thymic development correspond to the more rapid innate-like functions of neonatal-derived CD8+ T cells.

We are just beginning to understand how the diversity of functions in CD8+ T cells is linked to events that occur during thymic development. It is clear that distinct hematopoietic progenitor populations arrive in the thymus and give rise to CD8+ T cells that express unique TCRs, which provide additional environmental sensing capabilities. At the same time, the thymic environment may offer other instructions that are integrated and ultimately establish a more innate or adaptive program[119]. However, there is much more to be learned. In some ways, the CD8+ T cell field is just beginning to ask the same questions that the γδ T cell and B cell fields have been addressing for many years.

6. Conclusion

The studies discussed in this review make a compelling case for extending the layered immune system model to CD8+ T cells. Similar to other fetal-derived lineages, neonatal CD8+ T cells possess a more effector-like program, preferentially localize to peripheral organs (e.g., the liver), use more restricted TCRs, and offer rapid innate-like immune protection. Likewise, adult-derived CD8+ T cells resemble the adult-derived lineages in the layered immune model, as they are the subset that exhibit a naïve phenotype, accumulate in the lymph node, express a diverse TCR repertoire, and preferentially form memory after infection. The neonatal-derived CD8+ T cells persist into adulthood and exhibit unique roles during infection, providing the host with developmental layers of cells with distinctive phenotypes and functions.

The experiments that allowed researchers to conclude that fetal CD4+ T cells represent a distinct lineage compared to adult CD4+ T cells have largely been recapitulated in CD8+ T cells in mice. For example, both fetal-derived CD4+ and CD8+ T cells exhibit a distinct gene expression profile prior to thymic egress, are derived from alternative hematopoietic progenitors, and require the master regulator of fetal lymphopoiesis (Lin28b) for their development[6,17,18,120]. However, whereas CD4+ T cells in early life are biased toward becoming Tregs, fetal CD8+ T cells have an increased propensity to form VM cells. The same machinery that regulates the switch from tolerogenic to immunogenic in the CD4+ T cell compartment may also be involved in regulating the switch from innate to adaptive in CD8+ T cells.

The work discussed in this review points toward a new model explaining why CD8+ T cells follow a unique pattern of differentiation after infection. While earlier studies have suggested that the fates of CD8+ T cells were linked to TCR usage[62], asymmetric cell division[61], or environmental cues in the host environment following microbial challenge[63], it is now evident that CD8+ T cell fate decisions are not solely determined in the periphery, but also shaped by when they are produced in an individual. Importantly, what we call the 'developmental origins' model of effector cell differentiation, similar to the layered immune system model but for CD8+ T cells, need not be viewed independently from previous models. For example, both neonatal- and adult-derived CD8+ T cells likely undergo asymmetric cell division after stimulation. However, the neonatal-derived CD8+ T cells simply divide more rapidly and undergo a different intrinsic program of differentiation when compared to adult-derived CD8+ T cells. Similarly, it is also possible that CD8+ T cells with early developmental origins encounter different cues in the environment after infection. For example, CD8+ T cells produced early in life express higher levels of CXCR3 and may cluster in regions of the lymph node and spleen that receive greater amounts of stimulation during infection[121]. Lastly, TCR usage may contribute to the altered fates of CD8+ T cells after infection, as well as to their time of production. Thus, the 'developmental origins' model of effector cell differentiation can be reconciled with other prevailing models in the field.

It is now apparent that the phenotype of CD8+ T cells is linked to their developmental origins. However, our knowledge of how different hematopoietic precursors give rise to functionally distinct CD8+ T cells is far behind what has been described for B cells, macrophages, and γδ T cells. Specifically, the source of progenitors for fetal-derived CD8+ T cells is unclear, due to the shifting landscape of discrete hematopoietic progenitors that arise from different anatomical sites during ontogeny. Accumulating evidence suggests that CD8+ T cells in early life may also be derived from non-HSC progenitors[122]. Indeed, there is a wave of hematopoietic cells that initiates at ~E9 and is characterized by a sudden rise in multipotent progenitors, including some with a lymphoid bias, known as the lymphoid-primed multipotent progenitors (LMPPs)[123–127]. The LMPPs are responsible for the production of some innate lymphocytes, including NK cells, T cells, and B1a B cells. More recently, lineage tracing studies have shown that other T cell subsets, such as the epidermal γδ T cells (DETCs), are not made from HSCs, but instead originate from the yolk sac[128]. Although still somewhat controversial[55], there is also evidence that alpha beta T cells develop independently from definitive HSCs. For example, Yoshimoto demonstrated that progenitors from the yolk sac (~E9.5) are capable of giving rise to both γδ (Vγ3+, Vγ4+, Vγ5+) and αβ T cells in neonatal recipients[127]. These studies are consistent with related experiments performed in zebrafish showing that the first wave of T cells occurs independently and prior to the development of LT-HSCs[129,130]. However, the CD8+ T cells that are produced via the HSC-independent pathways remain poorly characterized. In the future, it will be important to use mouse lineage tracing models and in vivo reconstitution assays to determine whether the more innate-like lineages of CD8+ T cells are produced from a non-HSC progenitor in early life.

We are also beginning to appreciate how the functions of CD8+ T cells produced at different stages of life are programmed during thymic development. As previously discussed,

numerous studies have found that CD8+ T cells made in early life express a more self-reactive TCR, which may alter where they fall along the innate to adaptive spectrum. An important question is whether the bias towards more self-reactive TCRs comes at the risk of predisposing individuals to autoimmunity later in life. Although we mentioned the VM phenotype may not promote autoimmunity in early life, the production of more self-reactive TCRs is a different issue. For example, cells bearing autoreactive Vβ chains are readily detected in the thymus and secondary lymphoid organs of neonatal mice[131]. These so-called 'forbidden TCRs' arise only in early life and are deleted in adults; the moniker is given because of their high reactivity toward self-peptides, which should result in preferential deletion. While the forbidden TCRs in adult mice are likely unresponsive most of the time, they do have the potential to induce autoimmune disease[132]. There have also been studies showing that susceptibility to autoimmunity peaks during specific windows of development[133]. Thus, future studies are needed to more closely examine how age-related changes in tolerance contribute to autoimmunity in adulthood.

In conclusion, we are just starting to understand how the diversity of functions in CD8+ T cells is linked to changes in immune development. Going forward, it will be important to incorporate lessons and tools from hematology in order to move the field of developmental immunology forward. It will also be critical to consider how host genetics and environmental factors (maternal diet, microbiome, infections) alter immune ontogeny, as they have important implications for understanding the biology of CD8+ T cells in health and disease. There is still much to be learned, including how the ontogenetic layers of CD8+ T cells work both together and with other immune cells to protect the host against infection. In the future, taking advantage of higher resolution genomic techniques will provide a clearer picture of how the CD8+ T cell compartment is 'put together' in mice and humans, which has important implications for understanding how CD8+ T cells protect the host against infection across the lifespan.

Acknowledgments

The authors would like to thank past and present members of the Rudd laboratory for their hard work and invaluable contributions toward our working model. We also thank Miles Davenport (University of New South Wales), Ellen Rothenberg (Caltech), Jeff Mold (Karolinska Institutet), and Momoko Yoshimoto (University of Texas) for their helpful insights into the manuscript. The authors are grateful to the National Institutes of Health (NIH) for support through grants R01HD107798, R01AI05265, R01AI110613, F31AI157236, and U01AI131348.

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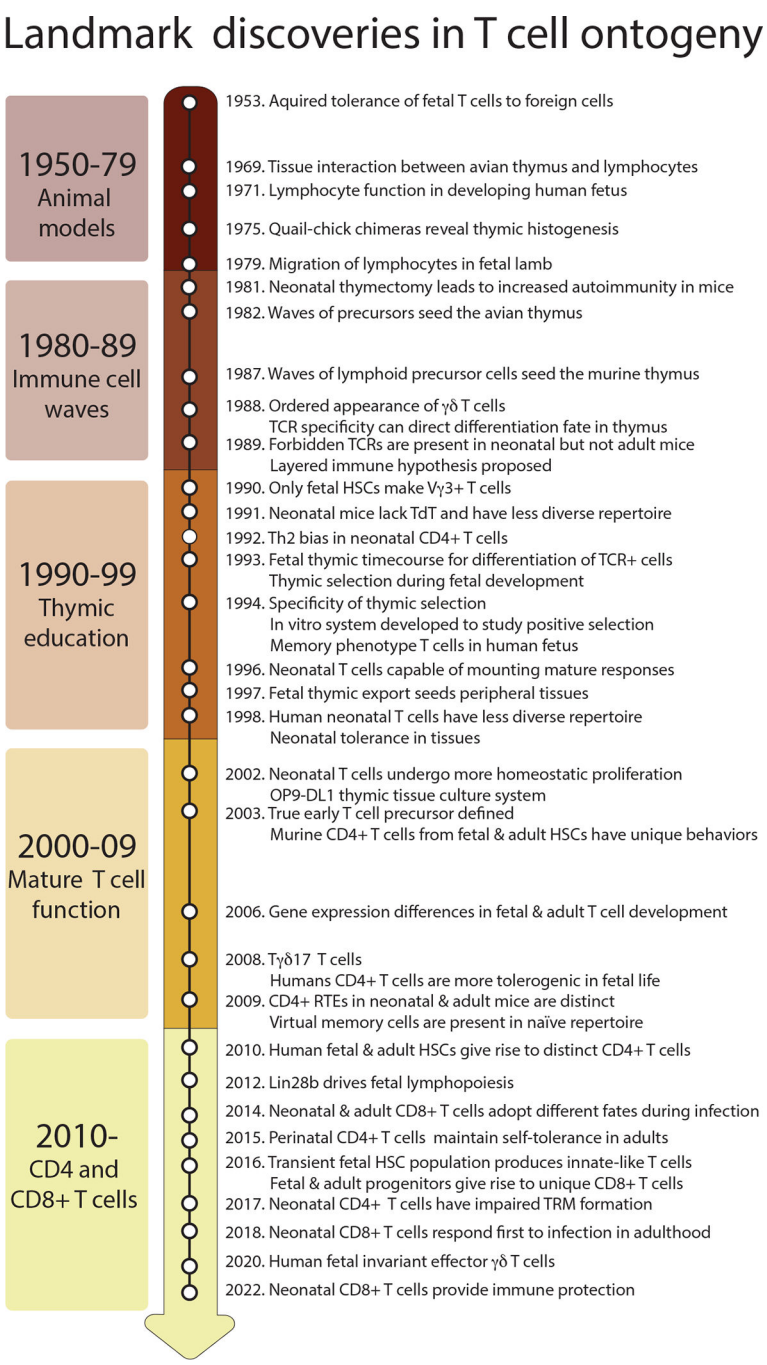

Figure 1. Timeline of key discoveries supporting developmental layering of CD8+ T cells.

In the 1950s to late 1970s, different animal models were used to study the origins of T cells[134–138]. In the 1980s, numerous groups reported the existence of developmental waves of T cells, which possessed unique functions[1,5,52,139–142]. In the 1990s, immunological approaches were developed to understand the key drivers of thymocyte development and T cell function in early life[4,78,143–153]. In the 2000s, more attention was placed on linking the functions of adult T cells in the periphery with their developmental origins[37,90,120,154–159]. In the 2010s to the present day, novel tools were used to identify the persistence of

fetal-derived CD8+ and CD4+ T cells and the molecular drivers that promote their unique behavior[6,17–20,33,60,81,92,160,161].

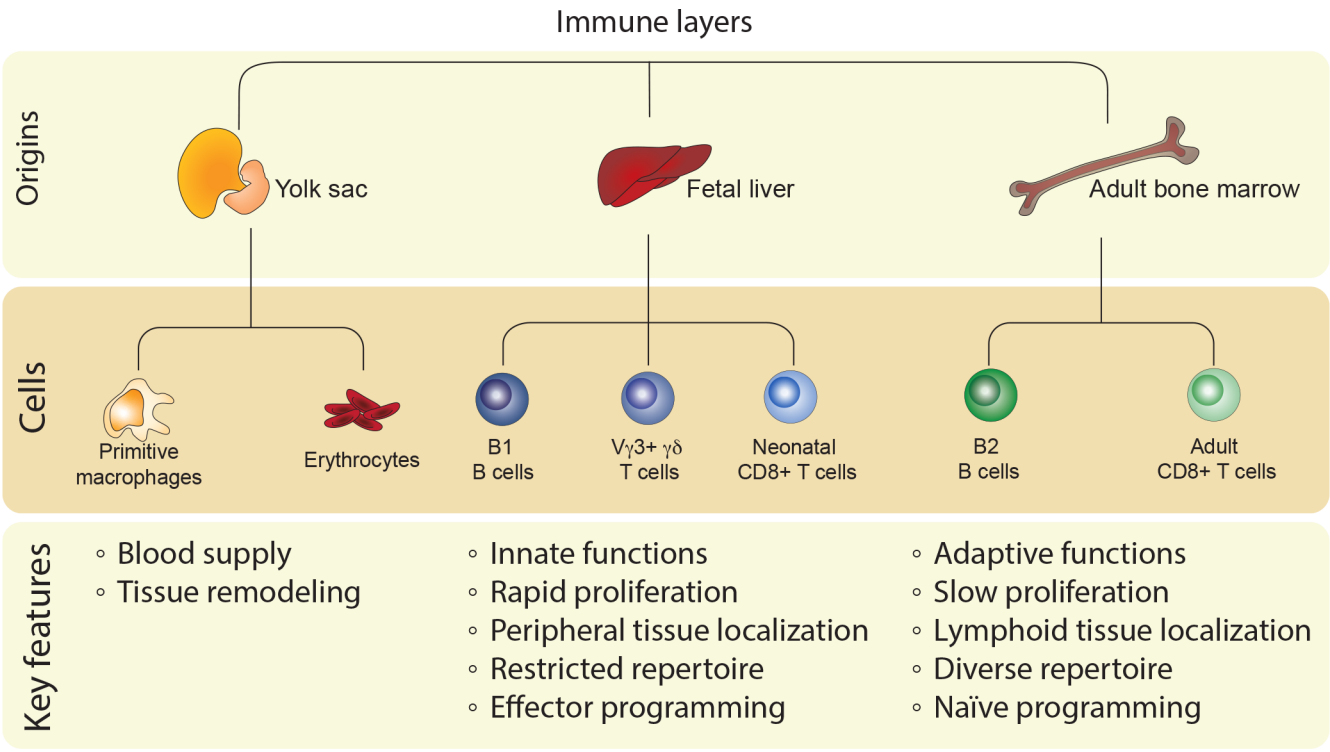

Figure 2. Layered hierarchy of immune cell ontogeny.

Schematic showing the shared features of immune cells derived from similar developmental origins. The first wave of immune cells arises from yolk sac progenitors and primarily serve to maintain blood homeostasis for the young host. The next wave of immune cells are generated from fetal progenitors that accumulate in the liver and give rise to lymphocytes with more innate-like features. The last broad wave of immune cells is made from adult progenitors in the bone marrow, which produce lymphocytes with more adaptive properties.

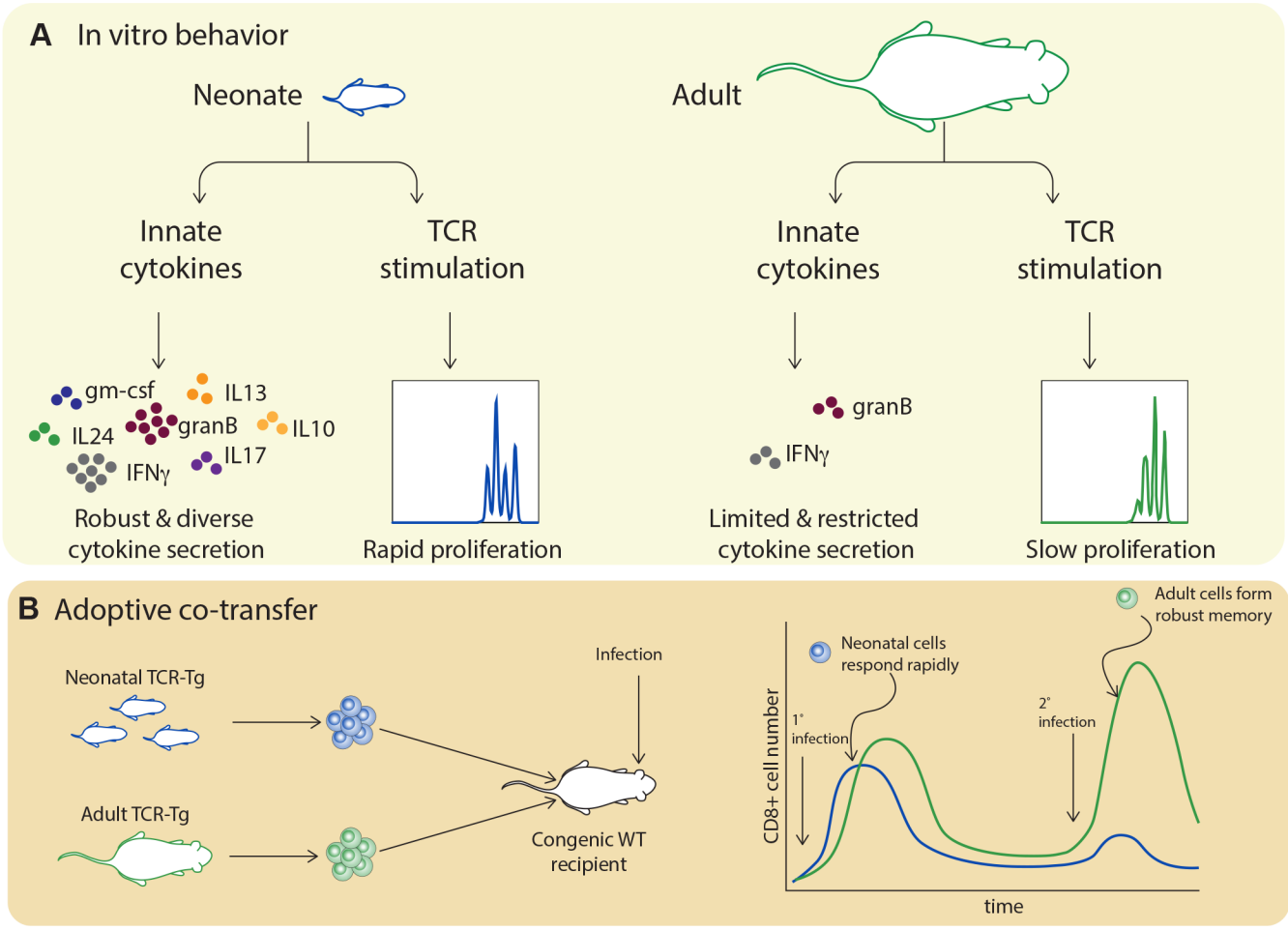

Figure 3. The distinct functional properties of neonatal and adult CD8+ T cells.

(A) A comparison of how CD8+ T cells from neonatal and adult mice respond to in vitro stimulation with innate cytokines (IL-12 + IL-18) or following TCR activation (anti-CD3/CD28 antibodies). After stimulation with innate cytokines, neonatal CD8+ T cells produce more effector molecules, including non-canonical cytokines that are not made from adult CD8+ T cells. In response to TCR stimulation, neonatal CD8+ T cells undergo more rounds of division than adult CD8+ T cells. **(B)** Adoptive co-transfer experiments using donor CD8+ T cells from different-aged TCR-transgenic mice show altered fates of neonatal and adult CD8+ T cells during infection is due to cell-intrinsic differences.

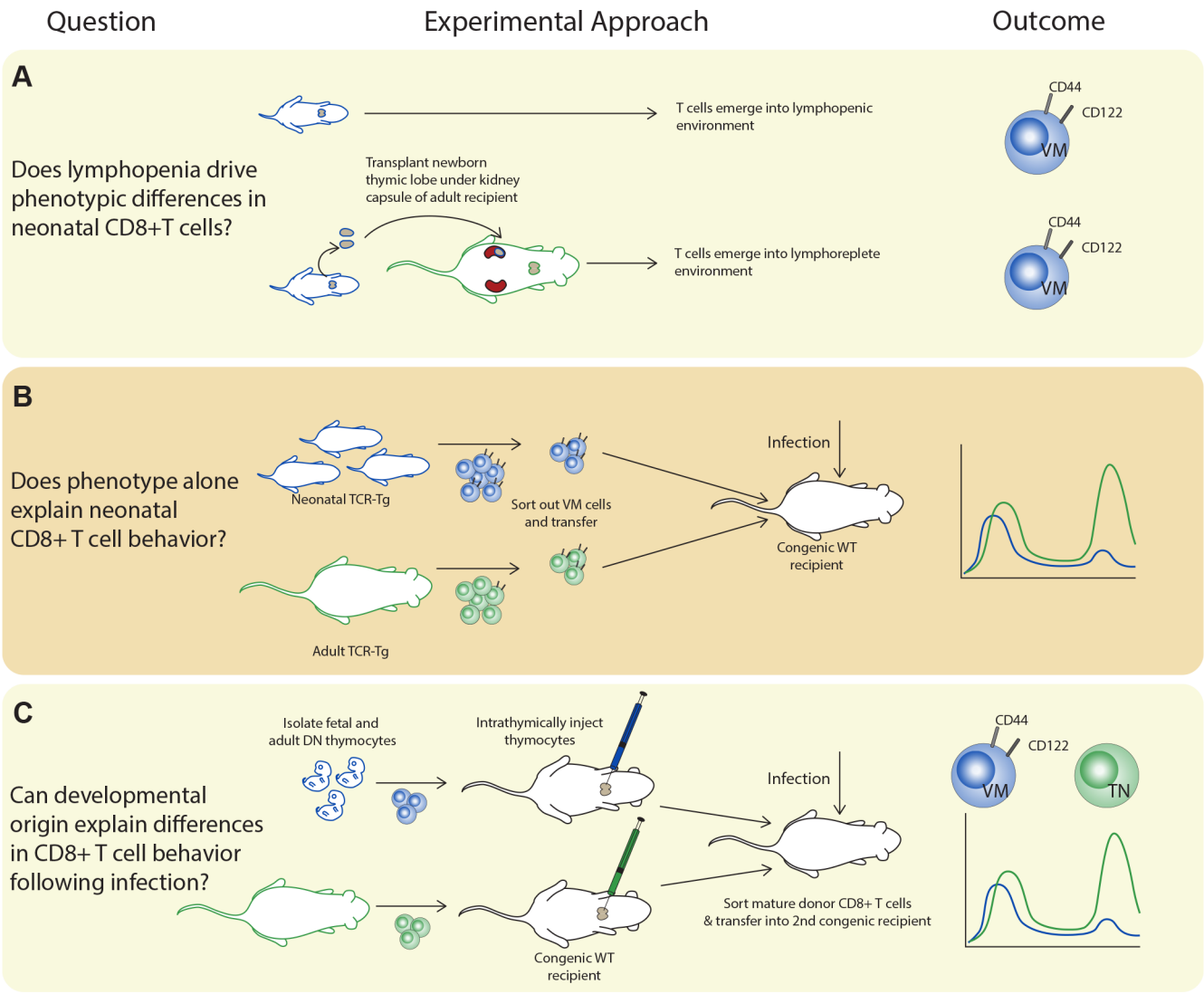

Figure 4. Experimental approaches used to understand why CD8+ T cells behave differently in early life.

(A) To determine whether the lymphopenic environment of neonates promotes the adoption of the virtual memory phenotype, a thymic lobe from a 1-day old animal was implanted under the kidney capsule of an adult. Independent of the peripheral environment, neonatal CD8+ T cells continued to preferentially adopt a virtual memory phenotype. **(B)** To determine whether the altered phenotype explains differences in the behavior of neonatal and adult CD8+ T cells, purified VM CD8+ T cells from neonatal and adult animals were adoptively co-transferred into a congenic recipient and then infected. When controlling for initial phenotype, neonatal CD8+ T cells still responded to infection with quicker kinetics and failed to transition into the memory pool. **(C)** To determine if an alternative source of progenitors explain neonatal CD8+ T cell behavior, DN thymocytes from fetal or adult animals were purified and intra-thymically injected into an adult recipient. CD8+ T cells developed from fetal-DN precursors continued to adopt a VM phenotype and respond with rapid kinetics following infection.

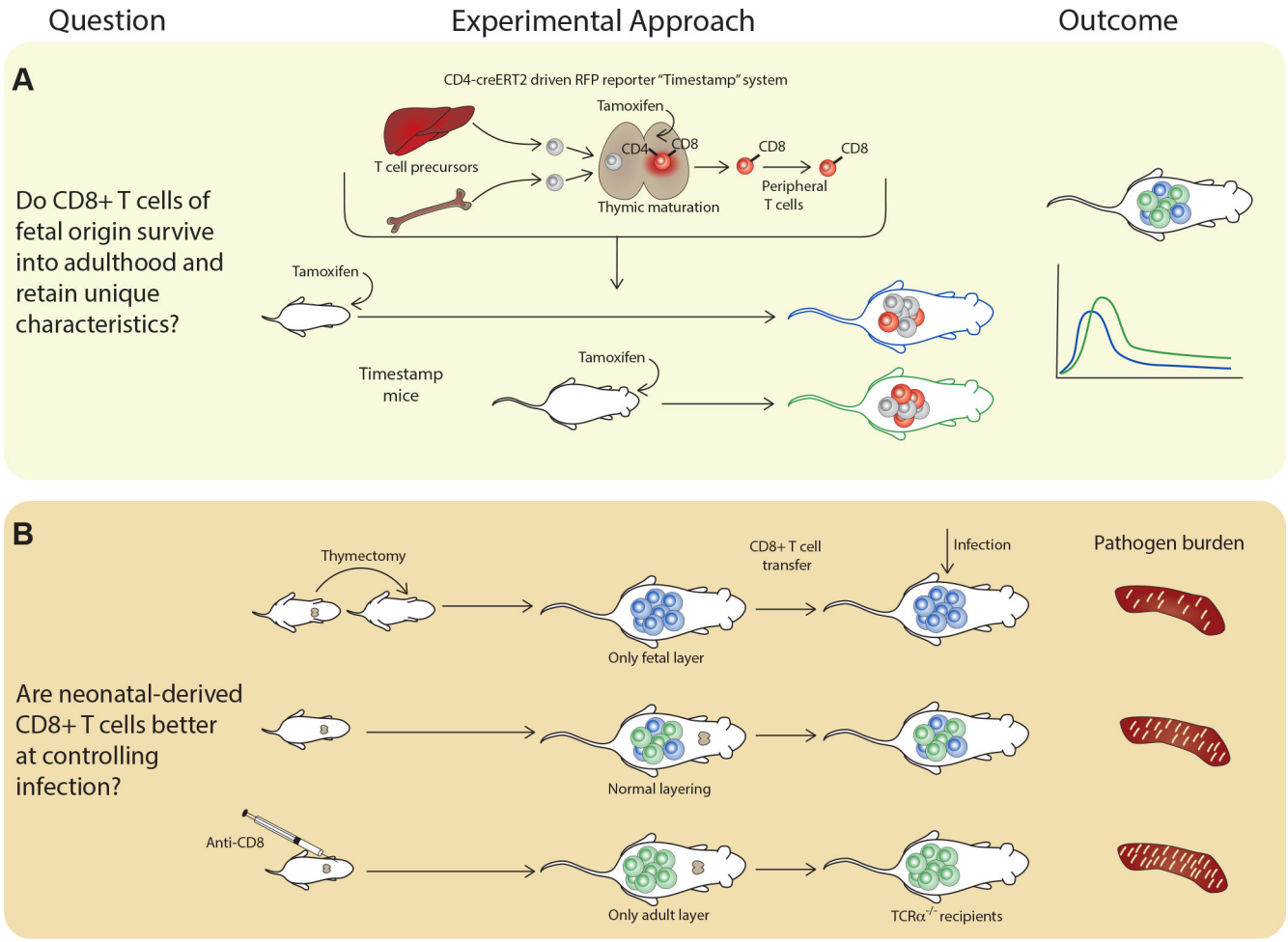

Figure 5. Defining the contributions of the neonatal-derived CD8+ T cells during infection in adulthood.

(A) Our approach to permanently mark or 'timestamp' CD8+ T cells from neonatal or adult origins and track their persistence in the adult CD8+ T cell compartment. The timestamp approach shows the adult CD8+ T cell compartment is composed of cells with developmentally heterogeneous origins, which retain their cell-intrinsic properties. **(B)** To identify whether neonatal-derived CD8+ T cells are necessary for controlling early infection, a series of adoptive transfer experiments were performed using CD8+ T cells from mice thymectomized at 2 wks of age (to prevent the adult layer from forming) or mice administered anti-CD8 antibody at 2 wks of age (to ablate the neonatal layer). Following adoptive transfers, mice were infected and the pathogen load was enumerated. Mice with only the neonatal layer had a lower pathogen load compared to mice with a normal CD8+ T cell compartment, and mice with only the adult layer had the highest pathogen burden. This shows neonatal CD8+ T cells are important for immune defense during primary infections.

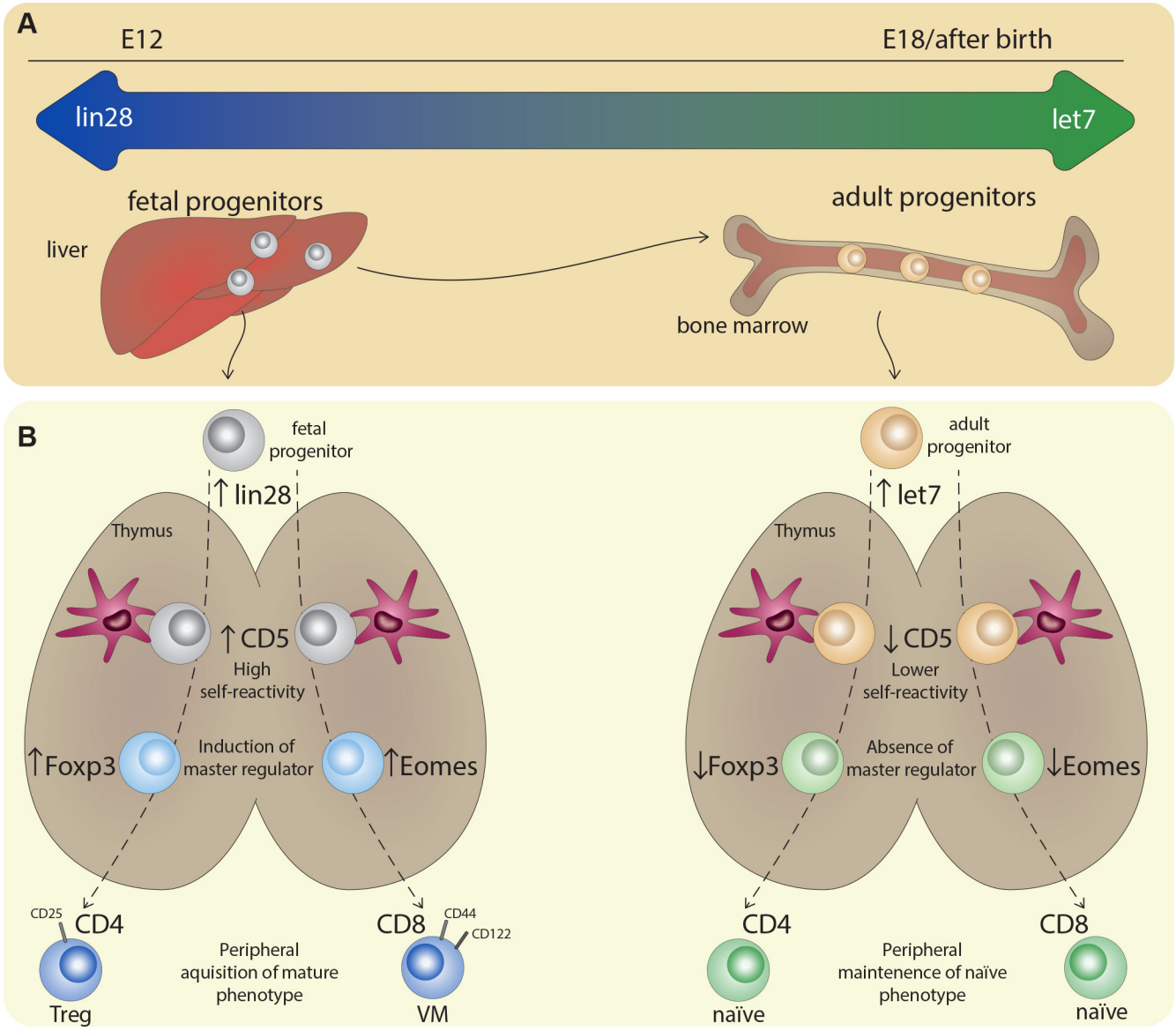

Figure 6. Working model highlighting the developmental parallels of regulatory CD4+ T cells and virtual memory CD8+ T cells.

(A) A unique aspect of fetal progenitors is their high expression of Lin28b, which tunes the fetal program towards an ‘innate-like’ program, whereas let-7 is upregulated in adult progenitors. **(B)** The induction of regulatory CD4+ T cells is dependent on the expression of a TCR with a high affinity for self-peptides and is induced by the master transcriptional regulator, Foxp3. Similarly, the virtual memory fate of CD8+ T cells is dependent on the expression of TCRs with a high affinity for self-peptides and is induced by the master regulator, Eomes. CD4+ Tregs and CD8+ VMs T cells are enriched in early life, and both require similar multi-step processes for the commitment of either fate, which begins in the thymus and continues following export into the periphery.