Modeling the dynamics of neonatal CD8+ T-cell responses

Arnold Reynaldi1, Norah L Smith2, Timothy E Schlub3, Vanessa Venturi1, Brian D Rudd2 and Miles P Davenport1

Neonates are particularly susceptible to a number of infections, and the neonatal CD8+ T-cell response demonstrates differences in both the phenotype and magnitude of responses to infection compared with adults. However, the underlying basis for these differences is unclear. We have used a mathematical modeling approach to analyze the dynamics of neonatal and adult CD8+ T-cell responses following in vitro stimulation and in vivo infection, which allows us to dissect key cell-intrinsic differences in expansion, differentiation and memory formation. We found that neonatal cells started dividing 8 h earlier and proliferated at a faster rate (0.077 vs 0.105 per day) than adult cells in vitro. In addition, neonatal cells also differentiated more rapidly, as measured by the loss in CD62L and Ly6C expression. We extended our mathematical modeling to analysis of neonatal and adult CD8+ T cells responding in vivo and demonstrated that neonatal cells divide more slowly than adult cells after day 4 post infection. However, neonatal cells differentiate more rapidly, upregulating more KLRG1 per division than adult cells (20% vs 5%). The dynamics of memory formation were also found to be different, with neonatal effector cells showing increased death (1.0 vs 2.45 per day). Comparison of the division of human cord blood and adult naive cells stimulated in vitro showed more division in cord blood-derived cells, consistent with the observations in mice. This work highlights differences of the cell-intrinsic division and differentiation program in neonatal CD8+ T cells.

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CD8+ T cells have an important role in the control and clearance of viral infection. During acute infection, virus-specific CD8+ T cells undergo activation, followed by massive expansion and differentiation.1 Following viral control and clearance, most activated T cells will die by apoptosis leaving only a small proportion of virus-specific memory cells to provide enhanced protection from subsequent infection. Neonatal individuals show an increased susceptibility to infection compared with adults, which is thought to arise from differences in both innate and acquired immune responses to infection.2,3 In the case of CD8+ T-cell responses to infection, there are a variety of environmental and cell-intrinsic factors that may affect the neonatal response. Previous work by Kollman et al. showed that the neonatal immune environment differs substantially from the adult.4 Neonatal mononuclear cells secrete less interferon-alpha, interferon-gamma and interleukin (IL)-12 following stimulation with toll-like receptor agonist.5,6 In contrast, neonatal cells produced more IL-10, IL-6 and IL-23. These data suggest that neonates may be more susceptible to intracellular pathogens due to a reduced capacity to initiate strong Th1 and CD8+ T-cell responses. Other groups have also reported developmental differences in the number and composition of the dendritic cell population, which may further limit the induction of robust cellular immunity.7,8

Cell-intrinsic differences between adult and neonatal CD8+ T cells include the limited diversity of the neonatal T-cell receptor (TCR) repertoire compared with adults. The generation of TCR diversity is accomplished by the somatic recombination of the V–D–J gene segments9 and the addition of random nucleotides (N-addition) mediated by the TdT enzyme.10 The TdT enzyme is absent before birth in mice, and thus neonatal T cells show a lower diversity in their TCR repertoire responding to infection.11–15 This limited diversity persists as neonatal cells’ transition into the memory pool, limiting their ability to undergo robust recall responses.16 In addition to the TCR, neonatal T cells may also respond differently to identical stimuli, having different rates of proliferation and/or differentiation in response to the same stimulus.

Given the large number of cell-intrinsic and environmental differences between neonates and adults, we used a reductionist approach to understand the relative influence of these factors in the development of CD8+ T-cell responses. Recently, we focused on cell-intrinsic differences in neonatal responses by assuring identical TCR (using TCR-transgenic mice) and identical host environment (using in vitro assays and co-transfer of congenically marked neonatal and adult donor CD8+ T cells into the same recipient animal).17 Consistent with previous studies,18 our data showed faster early growth of neonatal CD8+ T cells both in vivo and in vitro compared...
with the adult. Our previous in vitro studies indicated that neonatal cells proliferate more during the first 72 h of stimulation. Furthermore, neonatal cells were present in higher numbers at early stages of in vivo infection, and showed a more differentiated phenotype at this time. Despite this faster early growth, we also showed neonatal cells have a smaller peak in primary responses, and also made a poor memory recall response to secondary infection. These kinetic observations raise a number of questions about the differences in the cell-intrinsic differentiation and proliferation program between neonatal and adult CD8+ T cells. The simplest explanation would be that neonatal cells divided sooner than adult cells, and also differentiated faster than adult cells. However, as division has been shown to be associated with differentiation in many circumstances, it may be that neonatal cells differentiate at the same rate per division, but just divided more rapidly than adult cells. Similarly, the poor memory formation in neonatal infection may occur because all neonatal cells survive poorly, or just because the neonatal response was dominated by terminally differentiated effectors at the peak, and the underlying death rate of memory-precursor cells may be the same in adult and neonate.

Understanding the fundamental mechanisms that alter neonatal T-cell behavior is important for developing more rational approaches to enhance immunity in early life. However, traditional methods of measuring T-cell proliferation and memory differentiation only provide a snapshot of the response, and it is often difficult to differentiate the underlying mechanisms of behavior. Mathematical modeling allows the dissection of these factors by analyzing cell number and cell division profiles over time. In the present study, we have combined new experimental data with mathematical modeling to understand the key differences in behavior between adult and neonatal CD8+ T cells.

RESULTS

Earlier onset and faster division of neonatal CD8+ T cells in vitro

Previous studies have shown that neonatal CD8+ T cells stimulated in vitro undergo a higher number of divisions than adult CD8+ T cells. However, this may have occurred because of earlier division, higher divisions rates or lower death rates of neonatal cells. Our goal in this study was to quantify the cell-intrinsic differences in behavior between adult and neonate CD8+ T cells undergoing the same stimulus. To answer this question, naïve adult and neonatal transgenic gBT-I CD8+ T cells were labeled with carboxyfluorescein succinimidyl ester (CFSE) and stimulated with peptide in the presence of IL-2. First, we compared the expression of CD3, Vβ8, IL2-Rα, CTLA-4 and CD62L between neonate and adult cells. Naïve neonatal cells express slightly lower levels of CD3 and Vβ8 but have similar expression of CD25 (IL2-Rα), CTLA-4 and CD62L (Supplementary Figure 1). As previously observed, we found that neonatal CD8+ T cells divided more than adult cells (Figures 1a and b). To dissect whether these gross differences in proliferation could be best explained by differences in
time-to-first-division, division rate or death rate of neonatal cells (Methods), cells were collected at multiple time points (4, 16, 28, 40, 52 and 64 h). Our analysis of the in vitro CD8\(^+\) T-cell proliferation kinetics (Equations (1) and (2)) revealed that neonatal CD8\(^+\) T cells divide faster than adult cells, with the duration of each division ~13 h for adult and 9 h for neonate (Figure 1c, \(P<0.001\), Table 1). Neonatal CD8\(^+\) T cells also enter their first division earlier (about 30 h for neonate and 38 h for adult). We also estimated death rates of adult and neonatal cells (Figure 1d) and found that neonatal CD8\(^+\) T cells were more susceptible to death before their first division in culture (\(P=0.001\), Table 1). However, neonatal cells underwent less death in each subsequent division compared with adult cells (Figure 1e, \(P<0.001\), Table 1). Thus, despite higher death rate before their first division, neonatal CD8\(^+\) T cells enter their first division earlier than adult cells and proliferate faster than adult cells over the first 64 h in vitro. To verify that these differences between neonatal and adult cells are not dependent on the choice of model, we also applied the alternative cyton method,\(^{28}\) which is a more complex version of the original model that we use here. Information about the cyton model and application to these data are provided in Supplementary Information, with the results presented in Supplementary Figure 2 and Supplementary Table 1.

Faster rate of differentiation of neonatal CD8\(^+\) T cells in vitro

Our previous studies\(^{17}\) demonstrated a more differentiated phenotype of neonatal cells in vivo. However, given that neonatal CD8\(^+\) T cells also proliferate more (as described above), it is not clear whether this difference in differentiation is simply because neonatal cells have undergone more rounds of division (with the same rate of differentiation per division), or if they also differentiate more per division than adult cells. The frequent sampling of cell division in vitro allowed us to model the differentiation patterns of adult and neonatal CD8\(^+\) T cells. That is, we analyzed the proportion of cells of a given phenotype in each division (identiﬁed by CFSE dilution) and applied a formula for the rate of differentiation per division (Methods). These results first conﬁrmed that the data are consistent with a model of both adult and neonatal cells differentiating with a constant rate of differentiation per division. However, when we compared this rate of differentiation in neonatal vs adult cells, we observed a more rapid loss of CD62L\(^{+}\) in neonatal cells (0.8% per division vs 4% per division, \(P=0.008\) (using non-linear regression to evaluate the effect of group (adult and neonate)) in neonatal cells up to 64 h in vitro (Figure 2a). The same was true for the expression of Ly6C\(^{+}\), where we observed that neonatal cells differentiated at a rate of 13% per division, whereas adult cells differentiated at a rate of 9% per division (\(P=0.01\), Figure 2b). Thus, in addition to faster division, it appears neonatal cells are also programmed for faster differentiation per division.

Adult and neonatal CD8\(^+\) T-cell responses are correlated after in vivo co-transfer

To understand more about the differences between adult and neonatal CD8\(^+\) T-cell responses during primary infection, we also compared the dynamics of adult and neonatal transgenic gBT-I CD8\(^+\) T cells in vivo using an adoptive co-transfer experiment. We transferred equal numbers of adult (Thy1.1\(^+\) and CD45.2\(^+\)) and neonatal (Thy1.2\(^+\) and CD45.2\(^+\)) transgenic donor CD8\(^+\) T cells into adult Ly5.2\(^+\) recipients (Thy1.1\(^+\) and CD45.1\(^+\)) (Figure 3a). Using this method, cells are exposed to the same host and antigenic environment, allowing us to focus on the cell-intrinsic factors affecting the responses. In order to analyze the detailed dynamics of T-cell growth and differentiation in vivo, we required regular (daily) sampling of CD8\(^+\) T-cell numbers. Two cohorts of mice were analyzed in parallel, to allow regular sampling without the risk of excessive bleeding (Figure 3a).

Our experimental design, whereby adult and neonatal donor CD8\(^+\) T cells were transferred into the same host and paired samples for the response to antigen of adult and neonatal CD8\(^+\) T cells were obtained at each time point, enabled us to first investigate the impact of host environment on the response of these cells. Both adult and neonatal CD8\(^+\) T cells showed a wide variation in the rate of growth and differentiation between individual recipient mice. However, when we compared these features within an individual animal, we found that the adult and neonatal donor cells behaved similarly. That is, although the overall average growth rate was different between adult- and neonatal-derived cells, if one donor population grew more slowly in a given recipient, the other donor population also tended to grow slowly. Features including early magnitude of response (day 4), peak level of response (day 6) and memory level of donor cells (day 33) were highly correlated between neonatal and adult cells within the same recipient (Supplementary Figure 3A). The level of differentiation, as assessed by surface marker expression at the peak response, was also highly correlated (Supplementary Figure 3B). Overall, this highlights the importance of co-transfer of adult and neonatal cells in our study, and the important role of host environment in determining CD8\(^+\) T-cell kinetics. To account for host environment as a factor in our investigations of the dynamics and differentiation of adult and neonatal CD8\(^+\) T cells, we modeled the cell number and surface marker expression data accounting for the pairing of the sampled of neonatal and adult CD8\(^+\) T-cell populations obtained from the same recipient animals.

Dynamics of adult and neonatal CD8\(^+\) T-cell responses in vivo

Previous work has shown that neonatal CD8\(^+\) T cells showed lower peak responses and poorer memory formation than adult cells.\(^{17}\) Our observation of the primary response to antigen of adoptively co-transferred neonatal and adult donor CD8\(^+\) T cells (described in the previous section) revealed the same pattern (Figure 3b). Consistent with the observations in vitro, this work showed that at 3 days post infection, there were higher numbers of neonatal cells, and they were more differentiated than adult cells. However, after day 4 neonatal cells proliferated more slowly, and also formed poor memory responses. In order to understand the differences in the cell-intrinsic differentiation program between adult and neonatal CD8\(^+\) T cells in vivo, we repeated these experiments using a fine time-sampling, and applied a mathematical model (Methods section, Equations (3) and (4)) and non-linear mixed effect fitting (Figure 3c) of T-cell growth to fit the data. The model assumes that CD8\(^+\) T cells replicate

| Table 1 | In vitro data analysis |
|---------|-----------------------|
| Name    | Units | Adult | Neonate | P-value |
| Division rate | Per day | 0.077 (0.07, 0.08) | 0.105 (0.10, 0.11) | <0.001 |
| Death before division | Per day | 0.029 (0.02, 0.03) | 0.037 (0.034, 0.04) | 0.001 |
| Death rate per division | Per day | 0.422 (0.39, 0.55) | 0.278 (0.23, 0.33) | <0.001 |

Abbreviation: CI, confidence interval. Estimated parameters from the analysis of the in vitro data obtained for n = 4 replicate samples of adult and neonate gBT-I CD8\(^{+}\) T cells. A linear regression analysis was performed on various data (Figures 1c-e). P-value is the significance of the treatment effect between adult and neonate determined using the Wald test from nlme package in R. 95% CI was obtained using R function intervals in library nlme.
exponentially during the expansion phase until the peak response (day 6). We also accounted for the pairing between the expanded neonatal and adult donor CD8+ T-cell populations sampled from the same recipient. Adult CD8+ T cells were found to have a higher net-growth rate during the expansion phase compared with neonatal cells (1.86 and 1.48 per day ($P<0.001$, Table 2)). This equates to a doubling time for adult cells of ≈8.9 h, and for neonatal cells of ≈11.2 h. Thus, whereas our in vitro analysis demonstrates more rapid proliferation by neonatal cells in the first 3 days post stimulation, from day 4 to 6 in vivo this has slowed down. It is difficult to directly analyze antigen-specific CD8+ T cells in vivo before day 4, because of the low cell numbers involved. One approach to overcoming low cell numbers is simply to transfer more cells. Using higher cell numbers we have previously shown that neonatal cells outnumber adult cells at day 3 post infection,12 consistent with our in vitro observations. In addition, using high cell transfer numbers we find that neonatal cells have diluted CFSE more at day 3 (data not shown). Although using higher cell transfer numbers may not be directly comparable,21 we believe that the cell numbers and CFSE dilution (at high cell transfer numbers) and the more differentiated phenotype of neonatal cells observed at day 4 (Figure 4b) are consistent with the rapid early proliferation and differentiation of neonatal CD8+ T cells in vivo.

Differentiation of CD8+ T cells during early infection

The investigation in the previous section of the dynamics of the adoptively co-transferred neonatal and adult donor CD8+ T cells during the primary response shows lower peak numbers of neonatal CD8+ T cells compared with adult (Figure 3b). However, our phenotypic analysis of these neonatal- and adult-derived CD8+ T-cell populations during the primary response showed that neonatal CD8+ T cells consist of a more effector-like, terminally differentiated cell population (Figure 4a). We found that at the peak neonatal CD8+ T cells consist of a higher proportion of KLRG1hi cells at the peak ($P=0.001$), and a higher proportion of CD62Llow cells at the peak ($P=0.001$), and a higher proportion of CD127low cells ($P=0.001$; Figure 4b). This phenotypic difference arose during the expansion of donor CD8+ T cells during the response to primary infection, as the phenotype of donor cells was similar before transfer.13 Previous work by ourselves and others has suggested that the rate of differentiation is in some cases well-correlated with cell division both in vitro22,23 and in vivo.24,25 Therefore, just as in the in vitro situation above, these phenotypic differences may have either arisen as a result of more divisions by neonatal cells or a higher differentiation rate per division, or both. We used a model of CD8+ T-cell differentiation (Methods section, Equations (5)–(8)) to analyze the rate of differentiation of adult and neonatal T cells during the expansion phase in order to determine (i) whether differentiation was correlated with cell division, and if so (ii) whether the more rapid differentiation in neonatal cells occurred simply because of more rapid division, or because of a higher rate of differentiation per division. Although different combinations of marker expression form an observed cellular phenotype we analyzed the immune markers independently of each other. Similar to the modeling for the investigation of the CD8+ T-cell dynamics, our cell differentiation model also accounted for the pairing between the neonatal- and adult-derived CD8+ T-cell populations sampled from the same host. Our assumption is that CD62Lhi cells have a probability of giving rise to CD62Llow cells in each division. This behavior is also assumed in CD27 cells (CD27hi cells will give rise to CD27low cells). However, for KLRG1, the assumption is that in each division, KLRG1low cells will give rise to KLRG1hi cells. To investigate this, we first estimated the number of divisions that cells in individual animals had undergone from day 4 to the peak of the response. Then, we compared this with the change in the proportion of cells of different phenotypes over the same period (Figure 4c). In keeping with our previous work, we observed the proportion of cells remaining CD62Lhi was nicely predicted by a model of division-linked differentiation.24 Neonatal cells started with a lower proportion of CD62Lhi cells than adult cells (16.9% vs 42.7%, $P<0.001$; Table 3). The rate of loss of CD62L per division was also slightly higher in neonatal cells compared with adult cells, although this was not significant (19.1% per division vs 15.2% per division, $P=0.35$). The opposite effect was seen in KLRG1 expression. That is, the starting proportion of KLRG1low cells was similar (adult 91.7%, neonate 86.7%, $P=0.08$). However, the upregulation of KLRG1 per division was much greater for neonatal cells (21.2% per division for neonates vs 5.1% for adults, $P<0.001$). Finally, for CD27 expression we observed a significant difference in both the starting proportion of CD27hi cells, as well as the rate of differentiation per cell division (Table 3). We explored another scenario assuming that the observed difference in growth rate (during the expansion phase) was due to increased death in neonatal cells. Even when we took into account this increased death, we could see still see the difference in differentiation rate (Supplementary Table 3)

Overall, this suggests a faster rate of differentiation per division for neonatal cells in vivo between day 4 and 6 of infection.

Formation of immune memory

Having used the donor-derived adult and neonatal CD8+ T-cell populations responding to primary infection in the adoptive
co-transfer experiments to compare the expansion kinetics of adult and neonatal CD8\(^+\) T cells from infection to day 6, we also compared the dynamics of memory formation in these animals. One approach to identify putative 'memory precursor' cells is to analyze cellular surface markers. An alternative is to look at the kinetics of the response, and estimate the proportion of cells present at the peak of the response that exhibit a long-lived memory phenotype (by their later persistence). From the peak of CD8\(^+\) T cells at day 6, we assumed that there exist two populations, namely short-lived effector CD8\(^+\) T cells and long-lived memory-precursor CD8\(^+\) T cells. Each population may subsequently die, with different death rates. Thus, we aimed to estimate the initial proportions of short-lived and long-lived cells, as well as the death rates for each population, and compare these between adult and neonatal cells. We used a non-linear mixed effects model of the in vivo CD8\(^+\) T-cell dynamics (Equations (3) and (4)) to fit our experimental data on total numbers of donor adult/neonate cells over time (Figure 3c). We used the paired adult and neonatal cells from the same host to evaluate the effect of group (adult and neonate as treatment factor).

Figure 3 Kinetics of adult and neonatal CD8\(^+\) T-cell responses in vivo. (a) Co-transfer experiment: 10,000 adult gBI-T CD8\(^+\) T cells and 10,000 neonatal gBI-T CD8\(^+\) T cells were co-transferred into an adult recipient. Recipients were infected with VACV-gB 1 day after transfer. Two groups of mice (n=11 for each) were bled on indicated days post infection to observe the kinetics CD8\(^+\) T cells of both donor populations. (b) Kinetics of adult and neonatal CD8\(^+\) T cells, with error bars represent s.e.m. from 11 samples. (c) Non-linear mixed effect model fit for the data. We have a fixed peak response at day 6. The solid black line represents the global fit (pooled from group 1 and group 2), and the dashed colored lines represent the individual fits (varying for each mouse). Although the response varies considerably between individual animals, the adult and neonatal responses are highly correlated within an individual animal (Supplementary Figure 3). Our analysis involved pair-wise consideration of adult and neonatal responses in each animal. Significance was determined by using Wald test from the package *nlme* in *R*. For parameters, see Table 2. A full color version of this figure is available online at the *Immunology and Cell Biology* website.
Using this approach we found that the effector death rate was higher in neonates (2.45 per day) than adults (1.003 per day) although this did not achieve significance \( (P=0.05, \text{Table 2}) \). This equates to a half-life of effector cells of 6.8 for neonates vs 16.6 h for adults. In contrast to this rapid death of effectors, we observed a more stable pool of memory CD8+ T cells in neonates compared with adults (decay of 0.11 vs 0.17 per day \( (P=0.007, \text{Table 2}) \)). This equates to an early half-life of memory cells of 6.3 days in neonates and 4.1 days in adults. Finally, the model also estimated that the fraction of putative memory precursors present at the peak of the response was very similar between adult and neonatal cells (31% vs 26% \( (P=0.33) \)).

Thus, the major difference between neonatal and adult cells appears to be the more rapid death of short-lived effector cells following the peak response, consistent with a more terminally differentiated state of these cells in the neonate. We also tested the data to another model with a contraction of effector cells and the forming of memory cells,\(^{26}\) however this did not provide a better fit (for a more detailed description, see Supplementary Information, with the results presented in Supplementary Figure 4 and Supplementary Table 2).

**Table 2 Dynamics of adult and neonatal CD8+ T-cell responses in vivo**

| Name               | Units | Adult Value | 95% CI     | Neonate Value | 95% CI     | P-value |
|--------------------|-------|-------------|------------|---------------|------------|---------|
| Growth rate        | Per day | 2.11        | 1.94, 2.28 | 1.2           | 0.81, 1.6  | <0.001  |
| Effector death rate| Per day | 1.003       | 0.6, 1.41  | 2.45          | 0.6, 4.3   | 0.05    |
| Memory death rate  | Per day | 0.17        | 0.14, 0.2  | 0.11          | 0.04, 0.15 | 0.007   |
| Fraction of memory |        | 0.31        | 0.22, 0.4  | 0.26          | 0.04, 0.46 | 0.33    |

Abbreviation: CI, confidence interval.

The data consisted of paired adult and neonatal CD8+ T-cell samples from blood obtained from the same host at multiple time points for two groups of 11 mice each. We fitted a linear regression on log-transformed data (from day 4 to day 6) to find the growth rate. Then, for effector death rate, memory death rate and fraction of memory we fitted the data from day 6 to day 51 on the model described in Methods section. The values here are the global parameter estimation of a mixed effect model; \( P \)-value is the significance of the difference between adult and neonate determined using the Wald test from \texttt{nlime} package in R. 95% CI was obtained using \texttt{R} function \texttt{intervals} in library \texttt{nlme}.

**Weak neonatal CD8+ T-cell expansion in secondary challenge**

Using the same cohort of animals involved in our investigations of the dynamics and differentiation during primary infection of adoptively co-transferred adult and neonatal CD8+ T cells, we also compared the recall response between adult and neonatal CD8+ T cells 51 days after primary challenge. We measured the growth rate from the initial day of re-challenge up to the peak of the response (day 5 post re-challenge). We found the growth rate during the expansion phase was significantly slower in neonatal compared with adult CD8+ T cells (doubling time of 8 h for adult and 11 h for neonate, \( P=0.004, \text{Figure 5a} \)). This is consistent with our previous observation that

![Figure 4 Phenotypic analysis of adult and neonatal CD8+ T cells in vivo. (a) Expression of various markers from day 4 to the peak response in vivo. (b) Phenotype of adult and neonatal donor at the peak of the primary challenge (day 6). Overall, we observed more differentiated phenotype in neonatal cells. Significance was determined by paired Wilcoxon test. (c) In vivo one-way division-linked differentiation from day 4 post infection up to the peak response (day 6). Number of divisions was estimated from net change in cell numbers. On each division, we assumed a probability that KLRG1low cells will become KLRG1hi cells, CD62Lhi cells will become CD62Llow cells and CD27hi cells will become CD27low cells. Experimental data for individual animals are shown as dots, and best-fit model of division-linked differentiation is shown as lines. Significance was determined by using Wald test from the package \texttt{nlime} in R.](image-url)
neonatal CD8+ T cells contributed less to secondary immune responses.\textsuperscript{17,27} However, although on average there is a statistical significance between adult and neonatal growth rates, this difference is small, and would only result in a median growth rate of 1.56 (adult) and 1.46 per day (neonate). Moreover, the very low starting levels of memory CD8+ T cells in most mice (Supplementary Figure 5) also increases the possibility of errors in calculating these growth rates. We also looked at the differentiation pattern from the initial day of re-challenge up to the peak of the secondary response. We modeled division-linked differentiation using the same method as primary infection, and observed that the expression of various markers was correlated with cellular division (Figure 5b). Neonatal cells started with a higher proportion of CD62L\textsuperscript{hi} cells than adult cells (68% for neonate vs 42% for adult, \textit{P}=0.003). However, in contrast to primary infection, the rate of loss of CD62L per division was higher in adult cells compared with neonatal cells (28% per division in adult vs 18% per division in neonate, \textit{P}=0.02). In the case of KLRG1\textsuperscript{hi}, the starting proportion of KLRG1\textsuperscript{hi} cells was not significantly different (adult 47% and neonate 53%, \textit{P}=0.34). However, the upregulation of KLRG1 per division was slightly higher in adult, although not significantly different (7% per division for adult vs 5% for neonate, \textit{P}=0.17). Finally, for the expression of CD27\textsuperscript{hi}, we found no significant difference in the starting proportion of CD27\textsuperscript{hi} cells (92% for adult and 83% for neonate, \textit{P}=0.52), and there was no difference in rate of differentiation on each cell division (12.2% for adult and 12.8% for neonate, \textit{P}=0.82). Overall, based on this analysis of division and differentiation, we found no evidence of faster differentiation in neonatal cells during secondary challenge (based on the differentiation pattern of KLRG1 and CD27), and if anything the results suggest that the rate of effector acquisition may be lower in neonatal CD8+ T cells compared with adult (based on CD62L marker).

Human cord blood CD8+ T cells divide earlier than adult cells \textit{in vitro}

To determine whether our observations with a murine model can be extended to humans, we purified naive CD8+ T cells from human

### Table 3 Phenotype and differentiation of adult and neonate CD8+ T cells \textit{in vivo}

| Phenotype | Type | Initial proportion (%) | Gain or loss rate (% per division) |
|-----------|------|------------------------|------------------------------------|
| KLRG1\textsuperscript{hi} | Adult | 91.7 | 86.4, 96.99 | 0.08 | 5.1 | −6.9, −3.25 | <0.001 |
| CD62L\textsuperscript{hi} | Adult | 42.7 | 38.97, 46.42 | <0.001 | −15.2 | −17.7, −12.6 | 0.35 |
| CD27\textsuperscript{hi} | Adult | 95.1 | 92.36, 97.84 | 0.045 | −2.27 | −3.3, −1.23 | <0.001 |

Abbreviation: CI, confidence interval.

The data consisted of the phenotypes of paired adult and neonatal CD8+ T-cell samples from blood obtained from the same host at multiple time points for two groups of 11 mice each. Estimation of initial proportion and gain/loss rate per division. We assumed that on each division, cells will lose or gain certain amounts of cell surface markers (Methods). The values here are the global parameter estimation of the mixed effect model. The 95% CI was obtained using R function intervals in library nlme. \textit{P}-value is the significance of the treatment effect between adult and neonate determined using the Wald test from the package \textit{nlme} in R.

**Figure 5** Division and differentiation in secondary challenge. (a) We measured the growth rate from the initial day of re-challenge (day 51 post-primary infection) up to the peak of the response (day 5 post re-challenge). Neonatal T cells showed reduced growth compared with adult T cells during secondary challenge (paired Wilcoxon test). (b) Differentiation pattern from the initial day of re-challenge up to the peak of the secondary response. Neonatal cells also showed the same (for KLRG1 and CD27) or slower differentiation (CD62L) per division than adult cells. Overall, we found no evidence of faster differentiation in neonatal cells during secondary challenge. Significance was determined by using Wald test from the package \textit{nlme} in R.
cord blood (neonates) and peripheral blood (adults). Cells were labeled with CFSE and stimulated with microbeads coated with anti-CD3, anti-CD28 and anti-CD2 in the presence of IL-2. We collected the cells at 16, 40, 64, 88 and 111 h, and analyzed the CFSE levels to assess cell division. We found more peak of division in human cord blood CD8+ T cells compared with adult cells (Figure 6a). We then asked whether some features of CD8+ T-cell proliferation observed in mice could also be found in human cells. Our analysis (based on the precursor cohort method, as the experiment to obtain human data was not setup to facilitate the more complicated cyton method) revealed that the division rate for human cord blood CD8+ T cells was similar to adult cells, with the duration of each division ~20 h (Figure 6b; P=0.79). However, consistent with the results observed in mice, human cord blood CD8+ T cells entered their first division earlier (about 48 h for cord blood cells and 58 h for adult cells, P<0.001). This suggests that cell-intrinsic differences in neonatal CD8+ T-cell responses are also seen in humans.

**DISCUSSION**

Although it is well understood that there are limitations to neonatal immunity, there has been to date no rigorous quantitative investigation of the underlying mechanisms. In this study, we used statistical analysis and mathematical modeling to uncover the key differences in the dynamics of neonatal and adult CD8+ T cells following activation. Such an approach is necessary to calculate the rates of proliferation, death and formation of memory as opposed to just simply measuring the proportion of cells that are proliferating or dying at individual time points. In doing so we have been able to obtain a greater understanding of why neonatal CD8+ T cells more rapidly become terminally differentiated and fail to develop into memory cells after infection.

For example, we previously showed that neonatal CD8+ T cells undergo more cell divisions than adult cells following *in vitro* stimulation, but the underlying mechanism that allows neonatal cells to divide more than adults was unclear. Here we performed new experiments in order to analyze cell number and cell division profiles over time and report that neonates divide more than adults because they enter division earlier and have a faster cell cycle time. Consistent with our observations in mice, neonatal CD8+ T cells from humans were also found to divide sooner than their adult counterparts. In addition, we also used mathematical modeling to understand the suboptimal generation of neonatal memory CD8+ T cell after infection *in vivo*. Earlier work indicated that fewer neonatal effector cells transition to the long-lived memory pool but the underlying basis for this imbalance in effector and memory cell differentiation remained undefined. By repeating these experiments with many more time points, we were able to understand which stages of the response are altered and identify the major factors contributing to age-related differences (for example, increased death and slower proliferation). A number of novel observations emerged from this analysis. We made an assumption that the precursor cells seed recipient mice in a comparable manner based on our previous observations that both neonatal and adult donor cells express similar levels of CD62L, a major lymph node homing molecule and the ‘take’ is the same in the spleen after the adoptive transfer.17

Perhaps one of the most remarkable findings from our study was that neonatal CD8+ T cells differentiate more per division than adults. For example, we found that neonatal CD8+ T cells were more likely to upregulate KLRG1 per round of division (20% in neonates vs 5% in adult cells) following infection. As a result, neonatal CD8+ T cells consist mainly of terminally differentiated KLRG1+ cells at the peak of the response. One feature of KLRG1+ effector cells is a decreased proliferative potential,28 which may explain why rapid proliferation in neonatal CD8+ T cells was not maintained at later time points. Although it is unclear why neonatal cells undergo increased rates of differentiation, one possibility may relate to differences in asymmetric cell division.29–31 Previous data have shown that proximal daughter cells receive more stimulation and are fated to become effector cells, whereas distal daughter cells receive less stimulation and are biased to the memory cell lineage. Thus, it is possible that neonates...
preferentially differentiate into short-lived effectors because of reduced amounts of asymmetric cell division, which may also coincide with increased rates of proliferation.

Another interesting finding from our study was that neonatal CD8+ T cells exhibit less death per division than adult cells early after stimulation in vitro but exhibit a higher death rate than adult effector cells after infection in vivo. A key difference between the in vitro and in vivo experiments is the presence of pro-inflammatory cytokines (for example, type I interferons and IL-12) in host mice after infection. Pro-inflammatory cytokines can promote or inhibit CD8+ T-cell survival, which depends in large part on the amount and timing of exposure relative to TCR signaling.32,33 As the threshold to survival, which depends in large part on the amount and timing of TCR signaling.

CD8+ T cells exhibit less death per division than adult effector cells early after infection, but exhibit a higher death rate than adult effector cells early after stimulation in vitro. A key difference between the in vitro and in vivo experiments is the presence of pro-inflammatory cytokines (for example, type I interferons and IL-12) in host mice after infection. Pro-inflammatory cytokines can promote or inhibit CD8+ T-cell survival, which depends in large part on the amount and timing of exposure relative to TCR signaling.32,33 As the threshold to survival, which depends in large part on the amount and timing of TCR signaling.

As the threshold to pro-inflammatory signaling is known to increase in T cells with progressing age,34 it would be interesting to investigate whether neonatal CD8+ T cells are hypersensitive to type I interferons or IL-12. CD8+ T cells are simply 'wired' differently than primary cells and follow a different pathway toward terminally highly differentiated effector cells. An alternative explanation is that the subsets of naive neonatal cells that have an enhanced capacity to proliferate and differentiate are lost during the primary response to infection. As a result, neonatal and adult memory CD8+ T cells exhibit a more uniform pattern of differentiation during memory recall. Clearly, more studies are warranted to better understand the ontogeny of the CD8+ T-cell response and its implications for immune control of infection.

The technical difficulties of comparing neonatal and adult responses in vitro provide a powerful analytical approach, but also a number of limitations. First, adoptive transfer always provides some risks of manipulation of cells. Second, the need to track the responses in individual animals longitudinally meant that we were restricted to analyzing cells in blood. As we have shown previously, the ratio of adult and neonatal cells can vary slightly in different tissues.17 However, tissue sampling would mean different animals at each time point, which would have severely limited comparisons. Another limitation is our assumption about cell divisions in vitro, as we used a simple method to estimate the number of relative divisions based on the net change in cell numbers. The differences during expansion may be a product of differences in division rates or differences in death rates, and these cannot be differentiated from cell numbers alone. However, we have analyzed the possibility that differences in observed growth rate occurred due to increases in death rate during proliferation (Supplementary Information).

Overall, our current study highlights the importance of modeling in vitro and in vivo cell dynamics to obtain greater insights into the key differences between neonatal and adult memory CD8+ T cells. The demonstration that neonatal and adult CD8+ T cells respond differently during various stages of the response reveals new opportunities of therapeutic intervention. Further understanding the intrinsic and extrinsic factors that shape the development of neonatal memory CD8+ T cells will help guide the development of more effective vaccines that can be safely administered in early life.

METHODS

Mice

B6-Ly5.2/Cr mice were purchased from the National Cancer Institute colony (Fredrick, MD, USA). gBT-1 TCR transgenic mice (mice transgenic for TCRαβ specific for the HSV-1 glycoprotein B498-505 peptide SIIEFLR) were provided by Dr Janko Nikolich-Zugich (University of Arizona, Tucson, AZ, USA) and crossed with B6- Thy1.1(Cy) mice, which were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). For experiments, male mice were used (6- to 7-day-old pups were considered neonates and adults were 2–4 months of age) and all mice were maintained under pathogen-free conditions at Cornell University College of Veterinary Medicine, accredited by the American Association of Accreditation of Laboratory Animal Care. The experiments in this study were performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocols were approved by the Institutional Animal Care and Use Committee at Cornell University.

In vitro proliferation

For in vitro proliferation experiment, gBT-1 CD8+ T cells from adult and neonatal mice were isolated by positive magnetic selection. Purified cells were labeled with CFSE dye,86 resuspended in RPMI 1640 (Lonza, Basel, Switzerland) supplemented with 10% heat-inactivated serum (PAA Laboratories, Pasching, Austria) and stimulated with peptide (10−8 M) in the presence of IL-2 (100 U ml−1). Cells were collected at 4, 16, 20, 40, 52 and 64 h post stimulation. To determine total cell number, 1 × 10^6 unlabeled calibrate beads (BD Biosciences, Mountain View, CA, USA) were added to samples before staining procedures. Cells were stained with monoclonal antibodies to anti-CD8α (53-6.7, catalog # 48-0081-82, eBiosciences, San Diego, CA, USA), anti-CD-4 (GK1.5, catalog # 56-0041-82, eBiosciences), anti-CD62L (MEL-14, catalog # 562404, BD Biosciences, San Jose, CA, USA) and anti-Ly6c (H1K4.1, catalog # 47-5932-82, eBiosciences). To determine cell viability, cells were stained with fixable viability dye-e780 (65-0856-14, eBiosciences) according to the manufacturer's instruction. The number and phenotype of cells in each division was estimated using Flowjo's proliferation analysis (Treestar, Ashland, OR, USA). We used four replicates of adult and four neonatal cells in our in vitro experiment (based on our pilot study).

Human cord and adult peripheral blood in vitro proliferation

De-identified whole adult (18–55 years of age) and cord blood (39–41 weeks gestation) samples from healthy donors were obtained from New York Blood Center and National Disease Research Interchange, respectively. Cornell University’s Committee on the Use of Human Subjects does not consider the use of this material to be Human Subjects Research because all the samples are de-identified, and the research does not involve intervention or interaction with the donors. Mononuclear cells were isolated using Ficoll-paque Plus (GE Healthcare, Marlborough, MA, USA) and CD8+ T cells were enriched using CD8+ microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), both according to the manufacturer's instructions. Following magnetic enrichment of CD8+ cells, samples were labeled with anti-CD8 (fluor450, RPA-T8, catalog # 48-0088-42, eBiosciences), anti-CD4 (A700, RPA-T4, catalog # 56-0049-42, eBiosciences), anti-CD3 (eFluor610, HI100, catalog # 61-0458-42, eBiosciences) and CD45RO (PE-Cy7, UCHL1, catalog # 25-0457-42, eBiosciences). Naive CD8+ cells (CD45RA+ and CD45RO−) were fluorescence-activated cell sorting (FACS) sorted to <95% purity with an Aria (BD Biosciences). Sorted cells were labeled with CFSE and then plated in RPMI 1640 (Lonza) supplemented with 10% heat-inactivated serum (PAA Laboratories, Pasching, Austria), 20 Uml−1 IL-2 and prepared beads from human T-cell activation/expansion kit (Miltenyi), prepared according to the manufacturer's instructions. We used three replicates of adult and three replicates of cord in our in vitro experiment (based on our pilot study).

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In vivo CD8⁺ T-cell proliferation kinetics

We used the precursor cohort plot approach based on the method described by Gutt and Hodgkin, which assumes that the average division number varies linearly with time. Briefly, we calculated the fraction of cells in each division at a given time \( f(t) \) from the normalized cell count at a certain harvest time using this formula

\[
f(i, t) = \frac{n(i, t)}{\sum_{i=1}^{\infty} n(i, t)}
\]

Here \( n(i, t) \) is the non-normalized cell count in division \( i \) at harvest time \( t \). Then we found the average division number at a given harvest time

\[
d(t) = \sum_{i=0}^{\infty} f(i, t)
\]

Plotting these values against various harvest times will generate a linear relationship, in which the slope is the division rate. We can also find the time to first division, \( t_{\text{first}} = \frac{\log 2}{c} \). Here \( m \) and \( c \) are the gradient and intercept of the best-fit linear line, respectively.

We can also estimate two kinds of death rate: the initial death rate, which is the death of cells before they enter the first division (assumed to be approximately exponential); and subsequent death rate per division, which is the loss of cells through their division cycle. In order to estimate the initial death rate and death rate per division, we fitted an exponential function to the total numbers of live cells against time before the peak would be to allow a period of rapid effector death (due to apoptosis) as short-lived effectors. We then estimated both as the death rate of effector cells (\( \delta_1 \)) and memory cells (\( \delta_2 \)). For the decay phase, we have

\[
A = (1 - f) A_T e^{-\delta_1 (t - t_{\text{peak}})} + f A_T e^{-\delta_2 (t - t_{\text{peak}})}
\]

where \( A_T \) is the total number of CD8⁺ T cells at the peak.

In vivo co-transfer of adult and neonatal CD8⁺ T cells

These experiment were conducted as described previously. Briefly, we co-transferred \( 10^6 \) congenically marked donor CD8⁺ T cells from adult and neonatal gBT-I mice into adult Ly5.2 recipient mice that were then infected with VACV-gB (2 × 10⁵ plaque-forming units, intraperitoneal) the following day. To monitor donor responses, recipient mice were bled at regular intervals after infection. Both neonatal and adult cells were analyzed together within the same host animal to account for differences in the size of the overall response between animals. In order to study the fine kinetics of the response, we prepared two groups of mice (no blinding, \( n = 11 \) for each group, 22 mice total, based on our pilot study), and bled the groups alternatively on indicated days post infection (Figure 3a). To determine CD8⁺ T-cell numbers in the blood, \( 1 \times 10^6 \) unlabeled calibrate beads (BD Biosciences) were added to a known volume of blood before staining. Cells were stained with monoclonal antibodies anti-CD8α (53-6.7), anti-CD4 (GK1.5, catalog # 56-0041-82, eBiosciences), anti-CD45.1 (A20, catalog # 25-0543082, eBiosciences), anti-CD45.2 (104, catalog # 47-0454082, eBiosciences), anti-CD90.1/Thy1.1 (OX-1, catalog # 202526, Biolegend, San Diego, CA, USA), anti-KLRG1 (2F1, catalog # 46-5893-82, eBiosciences), anti-CD127 (A7R34, catalog # 12-1271-85, eBiosciences), anti-CD27 (LG7E9, catalog # 11-0271-85, eBiosciences) and anti-CD62L (MEL-14, catalog # 562404, BD Biosciences). For studies of secondary responses (\( n = 9 \), some mice died due to complication), mice were challenged with \( 5 \times 10^8 \) colony-forming units wild-type Listeria monocytogenes expressing gB peptide 51 days after initial exposure to VACV-gB.

Modeling in vivo CD8⁺ T-cell dynamics

In order to compare proliferation kinetics between adult and neonatal cells, we used a piecewise fitting of growth and decay rates similar to that described by de Boer and colleagues. Briefly, in this model, it is assumed that CD8⁺ T cells proliferate exponentially at a rate \( \rho \) after initial antigen stimulation, until the peak response. Let \( A \) be the total number of CD8⁺ T cells, thus we have

\[
A = A_0 e^{\rho t}
\]

...where \( A_0 \) is the initial number of CD8⁺ T cells at day 4. We assumed equal numbers of precursor cells for adult and neonate, as they received approximately the same number of transgenic cells during adoptive transfer. We assumed that the precursor frequency of each type of transgenic CD8⁺ T cells was 0.003% of the total T-cell population at the time of infection (that is, total CD8⁺ T-cell number is around \( 3 \times 10^7 \), we add \( 10^6 \) of each type cell, so if we have a 10% take, we have \( 0.3 \) in \( 10^6 \) cells).

To compare the decay phase of CD8⁺ T cells after the peak, we modeled a fraction \( f \) of cells at the peak (day 6) as memory-precursor CD8⁺ T cells, and the remainder (1 - \( f \)) as short-lived effectors. We then estimated both as the death rate of effector cells (\( \delta_1 \)) and memory cells (\( \delta_2 \)). For the decay phase, we have

\[
A = (1 - f) A_T e^{-\delta_1 (t - t_{\text{peak}})} + f A_T e^{-\delta_2 (t - t_{\text{peak}})}
\]

where \( A_T \) is the total number of CD8⁺ T cells at the peak.

Modeling CD8⁺ T-cell differentiation

In order to understand the differentiation pathways of adult and neonatal CD8⁺ T cells we modeled the process of differentiation being linked to T-cell division. The assumption of this simple ‘division-linked differentiation’ is that on each division, cells will lose or gain certain amounts of specific cell surface markers. We cannot really measure both death and proliferation simultaneously, and what we observe is only the net effect from these two. However, we have discussed an alternative scenario where neonatal cells are allowed to proliferate as fast as adult cells (Supplementary Information). For CD62L and CD27, we assume that at each division there are certain proportions of high expressor cells (CD62Lhi or CD27hi) that will be converted to low expressors (CD62Llo or CD27lo), which will be converted to low expressors (CD62Llo or CD27lo). The model can be written as follows:

\[
H(n) = H_0 (1 - c)^n
\]

where \( H(n) \) is the proportion of CD62Lhi or CD27hi at division \( n \), \( H_0 \) is the initial proportion of CD62Lhi or CD27hi cells, \( n \) is the division number and \( c \) is the loss of CD62Lhi or CD27hi proportion on each division. For KLRG1 expression, we assume that on each division some proportion of KLRG1low cells will differentiate to become KLRG1hi cells. Thus, the proportion of cells KLRG1hi after \( n \) divisions (\( H(n) \)) is

\[
H(n) = 1 - L_0 (1 - c)^n
\]

where \( L_0 \) is the initial proportion of KLRG1low cells, \( n \) is the division number and \( c \) is the loss of KLRG1low proportion on each division.

To estimate the number of divisions cells have undergone, we compare the net difference of the cell numbers between the initial day (\( C_0 \)) and at day \( t \) (\( C(t) \)). Thus, we have the following relationships:

\[
n(t) = \frac{\log C(t)}{\log 2}
\]

\[
C(t) = 2^{n(t)} C_0
\]

in which \( n(t) \) is the division number.

Fitting procedures

To fit the models to our data, we used a non-linear mixed effect model with binary covariate to capture the difference between adult and neonatal CD8⁺ T cells. Significance was determined based on the value of this covariate (whether it was significantly different from zero), which can be calculated using the Wald test from the standard errors calculated in package nlme. All other parameters were treated as mixed effects (fixed effects plus random effects). Denoting \( \varphi \) as the parameter in the model described above, thus we have

\[
\varphi = \mu + \beta + r_i
\]
in which $\mu$ is the mean parameter for all individuals, $\beta$ is the fixed covariance (for adult and neonate) and $\gamma$ is the random effect associated with the $i$-th subject. We fitted a global model (pooled from group 1 and group 2 of our in vivo mice), and an individual model based on each unique individual.

The models were fitted with the non-linear mixed effect model $R$ (v3.0.2) function nlme in library nlme (v3.1-113). For the decay model, the fit was weighted using varPower to account for increases in error variance with fitted values. We assumed a diagonal variance-covariance matrix for the random effect using the pdDiag option in R. To find the 95% confidence interval, we used R function intervals in library nlme. The final model was chosen using backwards elimination starting from the full model (random effect for each parameter), and gradually removing each random effect. The best model was determined by the Akaike Information Criteria.

**Statistical analysis**

Throughout this paper, we use Spearman correlation coefficients to test for statistically significant correlations in the sample. Correlation analysis was performed using GraphPad Prism (version 6; San Diego, CA, USA). We also used the McNemar test for matched pair rank test (two-sided) to test for a difference between paired samples of adult and neonatal CD8+ T cells in the same donor mouse, and to determine the effectiveness of this pairing.

**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

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