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Murine Neonatal Lymphocytes Show Rapid Early Cell Cycle Entry and Cell Division¹

Becky Adkins,² Tonya Williamson, Patricia Guevara, and Yurong Bu

Neonatal animals are highly susceptible to infectious agents. At least part of this susceptibility is due to the virtual absence of immunological memory in newborns. One of the hallmarks of memory is the rapidity of the response. We show in this study that neonates may make up for their lack of memory, at least in part, by the rapid entry of large proportions of naive lymphocytes into the cell cycle. Following activation, greater percentages of both CD4⁺ and CD8⁺ neonatal, as compared with adult, lymph node cells showed early cell cycle entry; this was assessed by propidium iodide staining, CFSE labeling profiles, [³H]thymidine uptake, and up-regulation of early activation markers. This rapid cycle entry was observed following polyclonal activation with anti-CD3 or with PMA and ionomycin and in both C57BL/6 and BALB/c mice. Stimulation with specific peptide also elicited more rapid proliferative responses from neonatal vs adult TCR transgenic CD4⁺ cells. In addition, more rapid cycle entry was observed in vivo, in lymphopenic RAG2⁻/⁻ hosts. For both CD4⁺ and CD8⁺ cells, this phenomenon was observed out to 3 wk of life, although the differences between neonatal and adult cells became smaller with increasing time postbirth. These properties of peripheral neonatal T cells appeared to be inherited from their thymic precursors, because CD4⁺/-8⁻ single-positive cells in the neonatal thymus also showed more rapid cycle entry, compared with their counterparts in the adult thymus. Interestingly, rapid early cycling was also observed among activated neonatal B cells, compared with adult B cells. Thus, early cell cycle entry by large proportions of cells may allow the naive lymphocyte population to efficiently mobilize responses against the broad range of pathogens first encountered in neonatal life. The Journal of Immunology, 2003, 170: 4548–4556.

Immune responses during neonatal life in the mouse are typically weak in magnitude and poorly protective. This period of life is characterized by first exposures to a vast array of potentially pathogenic microorganisms and environmental Ags. Because strong immunological reactions can have deleterious effects, mature responses to many of these Ags simultaneously would probably be detrimental to neonates. Overly strong responses in newborns appear to be prevented by several mechanisms. First, the relatively low number of immune cells present in neonates almost certainly contributes to dampened responses when neonates receive the same doses of agents as do adults (1–3). Second, the cells participating in an immune response may be developmentally immature. One striking example of immature function is seen in the APC compartment. Numerous reports have demonstrated that neonatal APC are deficient in both number and function (4–7). Third, neonatal immune responses may be deviant. This phenomenon is well characterized for murine neonatal T cell responses. In particular, exposure to Ag during the neonatal period often results in Th2-dominant responses in later life (reviewed in Refs. 8–10). Because Th2 responses are anti-inflammatory in nature, this deviance undoubtedly contributes to the relatively poor capacity of neonates to mount protective cellular immune responses. Last, unlike in adult animals, T cells in the peripheral organs of neonates are almost exclusively naive. The absence of memory cells in the neonate would be expected to result in responses that are both slower to develop and lower in magnitude.

Although it may be important to limit immune responses in early life, it is almost certainly equally important to allow sufficient responsiveness to achieve protection from disease and death. Given the restrictions to responsiveness described in the paragraph above, how can this be achieved? In this study, we describe a phenomenon that may partially compensate for the limitations in immune cell function in neonates. Following CD3/TCR-dependent or -independent activation, greater proportions of neonatal T cells entered the cell cycle more rapidly than did adult T cells. This rapid cell cycle entry occurred both in vitro and in vivo, during homeostatic proliferation in lymphopenic hosts. Experiments in which exogenous cytokines or anti-cytokine mAb were added indicated that the differences between neonatal and adult early cycling were not dependent on differences in early cytokine production. In addition to T cells, greater proportions of neonatal, compared with adult, B cells also showed more rapid cycling following lineage-specific activation. Thus, populations of lymphocytes present in early life have the capacity to enter the cell cycle more quickly and hence may be able to mobilize a more rapid response from an otherwise completely naive population.

Materials and Methods

Mice

BALB/c mice, originally obtained from Charles River Laboratories (Wilmington, MA), OVA-specific TCR transgenic DO11.10 mice (The Jackson Laboratory, Bar Harbor, ME), and C57BL/6 mice (The Jackson Laboratory) were bred and housed under barrier conditions in the Division of Veterinary Resources at the University of Miami Medical School. Periodic screening showed the colony to be free of commonly occurring infectious agents. Females from timed matings were monitored closely from days 19–21 of gestation, and the date of delivery was recorded. Birth day was called day 0.
RAG2−/− mice were purchased from Taconic (Germantown, NY) and similarly bred and housed under barrier conditions.

Cell preparation

Lymph node preparations. Pools of tissues from ≥10 neonatal (day 7) or ≥2 adult (6–8 wk old) mice were used for the cell preparations. Mesenteric, inguinal, axillary, brachial, and cervical lymph nodes were collected to prepare total lymph node cell suspensions (11).

Enriched CD4+ thymocyte preparations. Thymus cell suspensions were incubated with anti-CD8 microbeads (Miltenyi Biotec, Auburn, CA), and CD8+ cells were depleted on negative-selection columns, following the manufacturer’s suggested protocol. The resultant population contained <1% CD8+ cells and ~50% CD4+ 8- and 50% CD4+ 8- cells.

CFSE labeling

CFSE was purchased from Molecular Probes (Eugene, OR), Total lymph node cells were labeled with CFSE according to the manufacturer’s instructions, with the concentrations of cells and CFSE in the labeling reaction adjusted to 5.0 × 10^6/ml and 0.5 μM, respectively.

Cell culture conditions

Total lymph node cells (5 × 10^5) were cultured in 200 μl of medium in 96-well culture dishes in the presence or absence of various activators. Culture medium consisted of RPMI 1640 (Life Technologies, Grand Island, NY) containing 1 mM sodium pyruvate (Life Technologies), 2 mM l-glutamine (Life Technologies), 5 × 10^{-2} mM 2-ME (Life Technologies), 1% penicillin-streptomycin (Life Technologies), and 10% heat-inactivated (56°C; 30 min) FCS (HyClone, Logan, UT). For cytokine assays, culture supernatants were harvested at 24 h, and γ-IFN and IL-4 content were assessed using mouse-specific cytokine ELISA kits (Pierce Endogen, Rockford, IL).

Cell activators

Peripheral T cell activation. 145-2C11 mAb, anti-murine CD3-ε (12), ascites fluid was used at a 1/2000 dilution, unless indicated otherwise. In some cases, cultures were supplemented with the indicated concentrations of IL-2 or IL-4 (R&D Systems, Minneapolis, MN) or with neutralizing anti-IL-4 mAb (11B11; BD PharMingen, San Diego, CA). PMA (Sigma-Aldrich, St. Louis, MO) was used at 12 nM in combination with ionomycin (Calbiochem, La Jolla, CA) at 0.15 μM for adult cells or 0.30 μM for neonatal cells (13). OVA peptide 323–339 (Calbiochem) was added to a final concentration of 5 μg/ml. After an additional 45 min at 37°C, samples were analyzed on a BD Biosciences (San Jose, CA) LSR equipped with a 488 nm laser line and an HeCd 325 nm UV laser.

Staining with Hoechst and pyronin Y

Cells (1 × 10^6) were stained with fluorescein-conjugated anti-CD4 mAb and then fixed and permeabilized in 200 μl of Cytofix/Cytoperp (BD PharMingen) for 45 min at 4°C. Cells were washed once with Perm/Wash (BD PharMingen) and resuspended in 1.0 ml 5 μg/ml Hoechst 33342 (Molecular Probes) in PBS containing 0.1% glucose. The cells were incubated at 37°C for 45 min, and pyronin Y (in PBS, 0.1% glucose) was added to a final concentration of 5 μg/ml. After an additional 45 min at 37°C, samples were analyzed on a BD Biosciences (San Jose, CA) LSR equipped with a 488 nm laser line and an HeCd 325 nm UV laser.

Results

Neonatal CD4+ and CD8+ cell populations divide more rapidly than adult cells in response to CD3/TCR-dependent or -independent stimulation

We previously observed that freshly isolated neonatal T cells, unlike adult T cells, produced large quantities of IL-4 relatively early (within 48 h) following activation with anti-CD3 mAb (13). For adult T cells, IL-4 production generally begins after ≥3 days and a number of rounds of proliferation (17–19). Therefore, we wondered whether the early appearance of IL-4 in neonatal cultures

FIGURE 1. Both neonatal CD4+ and CD8+ cell populations show more rapid division than their adult counterparts in response to anti-CD3 stimulation. Total lymph node cells from 7-day-old or adult BALB/c mice were labeled with CFSE and cultured in the absence (unactivated) or presence (activated) of anti-CD3 mAb. Twenty-four or 42 h later, the cells were harvested and stained with anti-CD4 or anti-CD8 mAb. Shown are the percentage of gated CD4+ cells and three individual experiments for CD8+ cells is shown.
was due to accelerated proliferation by neonatal T cells. To test this idea, total lymph node cells from 7-day-old neonatal or adult BALB/c mice were CFSE labeled and stimulated with soluble anti-CD3 mAb. Parallel cultures were prepared in the presence of medium only (unactivated). Twenty-four and 42 h after the initiation of culture, the cells were stained with anti-CD4 or anti-CD8 mAb and analyzed on the flow cytometer (Fig. 1). At 24 h of activation, there was no obvious division by either neonatal or adult cells. By 42 h of activation, both cell types showed clear division peaks. As has been recently reported for Ag-driven stimulation (20), adult CD4+ cells showed more limited proliferation than adult CD8+ cells. This relative phenomenon was also seen for neonatal CD4+ and CD8+ cells. However, among both the CD4+ and CD8+ populations, neonatal CD4+ cells had clearly undergone more extensive division than the corresponding adult cells. This more rapid division did not require neonatal APC, because similar results were obtained using purified neonatal or adult CD4+ cells and adult APC (data not shown).

We next sought to determine more about the conditions that elicit rapid division by neonatal T cells. We first found that CD4+ cells from the same strain of mice (BALB/c) also proliferated more quickly than adult CD4+ cells when stimulation bypassed the TCR, using PMA plus ionomycin (Fig. 2, left column). The phenomenon of more rapid division was not limited to the BALB/c strain of mice because CD4+ cells from C57BL/6 neonates also demonstrated more rapid proliferation in response to anti-CD3 stimulation (Fig. 2, second column from the left). Third, direct stimulation through the Ag receptor using OVA peptide elicited more rapid proliferation by neonatal DO11.10 TCR transgenic CD4+ cells (Fig. 2, second column from the right). Last, the phenomenon was not limited to in vitro activation, because CD4+ cells (data not shown) and CD8+ cells from BALB/c neonates also showed more rapid division in vivo, upon transfer to lymphopenic RAG2−/− hosts (Fig. 2, right column). Together, these results indicate that faster kinetics of proliferation is a universal phenomenon among neonatal T cell populations—it occurs both in vivo and in vitro, it occurs in several strains of mice, and it occurs whether stimulation is through the CD3/TCR complex or is TCR independent.

Faster kinetics of division are due to early cell cycle entry by greater proportions of neonatal T cells

In the CFSE experiments described in the previous section, division peaks were not detectable among either neonatal or adult cultures until ≥40 h. The cell population present at this time is the cumulative product of the ongoing processes of cell survival, cell proliferation, and cell death. For example, there may be relatively more death among undivided cells within the neonatal population. If so, the proportion of cells in the divided CFSE peaks would be increased proportionally, even if the amount of division was similar in the neonatal and adult cultures. Thus, to minimize the effects of longer term culturing, neonatal and adult cells were analyzed earlier, using PI staining. This method allowed us to monitor two things: 1) the percentage of cells in the different phases of the cell cycle and 2) the presence of apoptotic or sub-G0 cells. Neonatal or adult BALB/c lymph node cells were activated with soluble anti-CD3, and 21 h later, the cells were stained with anti-CD4 mAb, fixed and permeabilized, and stained with PI. Among freshly isolated cells, a small but consistently greater (~2-fold) percentage of neonatal CD4+ cells were in the S plus G2/M stages of the cell cycle, compared with adult cells (Fig. 3, left panel). At 21 h of activation, nearly 50% of neonatal CD4+ cells had entered the cell cycle, compared with ~20% of adult CD4+ cells (Fig. 3, right panel). This cell cycle entry was clearly dependent on activation, because similarly cultured but unactivated cells showed low levels of cycling (Fig. 3, middle panel). These experiments also demonstrated similar modest proportions (<10%) of apoptotic (sub-G0) cells among the activated neonatal or adult CD4+ populations at this early culture time, and although the proportions increased

### FIGURE 2
More rapid division by neonatal CD4+ or CD8+ cells is seen with TCR-independent stimulation, in different strains of mice, in response to specific peptide, and in vivo in lymphopenic hosts. **Left panel,** Total lymph node cells from day 7 or adult BALB/c mice were labeled with CFSE and stimulated with PMA and ionomycin for 42 h. The CFSE profiles of gated CD4+ cells are shown. **Second panel from left,** Total lymph node cells from day 7 or adult C57BL/6 mice were labeled with CFSE and stimulated with anti-CD3 mAb for 44 h. The CFSE profiles of gated CD4+ cells are presented. **Third panel from left,** Total lymph node cells from day 7 or adult TCR transgenic DO11.10 mice were CFSE labeled and stimulated with 0.1 μg/ml OVA peptide for 41 h. The CFSE staining of gated CD4+ cells is shown. **Right panel,** Total lymph node cells from day 7 or adult BALB/c mice were CFSE labeled, and 5 x 10^5 cells were injected i.v. into RAG2−/− hosts. Forty-eight hours later, spleen and lymph node cells were pooled and stained with anti-CD8 and anti-TCRβ mAb. The CFSE profiles of gated CD8+ TCRβ+ cells are shown. Each experiment was performed at least twice.

### FIGURE 3
Earlier division of neonatal CD4+ cells is preceded by more rapid cell cycle entry. Total lymph node cells from day 7 or adult BALB/c mice were cultured for 21 h in the presence (activated) or absence (unactivated) of anti-CD3. The cells were harvested and stained with anti-CD4 mAb and PI. Freshly isolated lymph node cells (fresh) from day 7 or adult animals were stained in parallel. The PI stainings of gated CD4+ cells are shown. The percentages of cells in the S plus G2/M stages of the cell cycle are indicated. One experiment typical of five separate analyses is shown.
later, neonatal and adult CD4+ cells also showed similar percentages of apoptotic cells at 48 h (data not shown) (16). Similar results were obtained when the CD8+ populations were compared, with 47–68% of activated neonatal CD8+ cells in cycle compared with 12–16% of adult CD8+ cells in S plus G2/M at 21 h of culture (data not shown). These experiments argue strongly that a greater proportion of activated neonatal, compared with adult, T cells enter the cell cycle early.

In the experiments described thus far, neonatal and adult T cells were activated with concentrations of reagents previously determined to be optimal for cytokine secretion or for later proliferation assessed by [3H]TdR incorporation (13). Nonetheless, it seemed possible that these conditions were not optimal for the early cycle entry of adult T cells, thus leading to the apparent discrepancies in kinetics between neonatal and adult cells. To investigate this issue, neonatal and adult total lymph node cells from DO11.10 TCR transgenic mice were stimulated with a 1000-fold range of specific peptide concentration. Twenty-four hours later, the cells were stained with anti-CD4 mAb, fixed and permeabilized, and stained with PI (Fig. 4). In the absence of stimulation (no peptide), both neonatal and adult CD4+ cells showed substantial proportions of cells in the sub-G1/apoptotic regions and minimal proportions of cells in S plus G2/M. This contrasts with BALB/c mice in which <10% of the cells are apoptotic in the absence of stimulation (data not shown) and may be a reflection of the relative heterogeneity of the TCR repertoire in normal and transgenic mice. With increasing peptide concentration, the percentages of apoptotic cells declined while the proportions in S plus G2/M increased, for both neonatal and adult cells. For all concentrations of peptide, a greater proportion of neonatal, compared with adult, T cells were in the S plus G2/M stages of the cell cycle. Neonatal CD4+ cells showed relatively large increases in the percentages of cells in S plus G2/M out to 50 μg/ml peptide. However, there was minimal further increase for adult cells between 5 and 50 μg/ml, indicated that optimal activation conditions for adult cells were obtained by a concentration of 5 μg/ml peptide. Thus, under conditions clearly optimal for the early proliferation of adult T cells, greater proportions of neonatal vs adult CD4+ cells showed early cell cycle entry.

Earlier studies from our laboratory (13) found that activated murine neonatal T cells are highly impaired, compared with adult T cells, in [3H]TdR incorporation. How can we reconcile these earlier findings to the data presented in this study showing faster early cell cycle kinetics among neonatal T cells? The previous results were obtained by adding [3H]TdR 48 h after activation and allowing incorporation to occur over the next 18–20 h. In this study, we have reported events occurring within the initial 48 h of activation. Therefore, the simplest reconciliation of the two results may be the differences in times of analysis. To test this idea, neonatal and adult lymph node cells were activated with different concentrations of soluble anti-CD3 mAb, and [3H]thymidine was added, and the cultures were incubated for an additional 20 h. One experiment typical of two independent setups is shown.
Rapid cell cycle entry also occurs among neonatal CD4⁺ 8⁻ thymocytes and begins to disappear among peripheral T cells by 3 wk of life

Newborn mice contain greatly reduced (50- to 100-fold) numbers of peripheral lymphocytes. As a result, neonates can be considered lymphopenic relative to adults. T cells isolated from the secondary lymphoid organs of neonates may be primed by peripheral homeostatic signals to undergo rapid cycle entry. Alternatively, the rapid cycling of peripheral T cells in the neonate may be due to their derivation from the neonatal vs the adult thymus. To distinguish between these possibilities, we compared cycling among neonatal vs adult thymocytes. To exclude the major population of immature thymocytes (CD4⁺ 8⁺ double-positive cells) from the analyses, thymocytes from day 1, day 3, and adult animals were enriched for CD4⁺ 8⁻ cells (see Materials and Methods). The enriched populations were activated for 24 h with plastic-immobilized anti-CD3 mAb with or without anti-CD28 mAb; control unactivated cultures were plated in medium only in non-coated wells. The cells were then stained with anti-CD4 mAb, fixed, permeabilized, and stained with PI (Table I). The proportions of apoptotic CD4⁺ 8⁻ cells was higher, especially among unactivated cells, than generally observed for peripheral CD4⁺ 8⁻ cells (see, for example, Fig. 3). However, in common with peripheral T cells, neonatal CD4⁺ 8⁻ thymocytes showed somewhat more (~3-fold) spontaneous proliferation, compared with adult thymocytes, in the absence of activation. Moreover, when activated with anti-CD3 or with anti-CD3 plus anti-CD28, greater proportions of neonatal, compared with adult, CD4⁺ 8⁻ thymocytes entered the cycle (percentage of S plus G₂/M). Therefore, the rapid cell cycle entry of peripheral T cells in the neonate appears to be inherited from their immediate precursors in the thymus.

In these experiments, we defined neonatal mice as 7 days old. An immediate question that arises is how long postbirth this phenomenon continues to occur. To address this question, BALB/c mice 1, 2, and 3 wk postbirth were compared with adult mice. Total lymph node cells were activated with anti-CD3 mAb. Twenty-four hours later, the cells were stained with anti-CD4 or anti-CD8 mAb, fixed and permeabilized, and stained with PI (Table II). Results were expressed as the percentage of CD4⁺ or CD8⁺ cells staining with anti-CD69 or anti-CD25 mAb, compared with adult cells. The percentages of gated CD4⁺ or CD8⁺ cells in BALB/c mice and for CD4⁺ cells in DO11.10 mice with comparable results.

Table I. More rapid cell cycle entry also occurs among neonatal CD4⁺ 8⁻ thymocytes

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<th></th>
<th>Anti-CD3</th>
<th>Anti-CD28</th>
<th>% Apoptotic</th>
<th>% G₀/G₁</th>
<th>% S</th>
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*Enriched CD4⁺ 8⁻ thymocytes from day 1, day 3, or adult BALB/c mice were cultured in medium alone (−/−) or activated with plate-bound anti-CD3 with or without soluble anti-CD28. Twenty-four hours later, cells were stained with anti-CD4 mAb and PI.

*Percentage among gated CD4⁺ cells.
Compared with adult cells, elevated percentages of both CD4+ and CD8+ cells from animals up to 3 wk old showed early cell cycle entry. Nonetheless, the proportions of cells entering the cycle early declined between 1 and 3 wk of life, especially for CD4+ cells. These results demonstrate that rapid responses to activation stimuli are gradually lost over the first few weeks of life.

Differences in early cell cycle entry are not due to differences in early cytokine levels or to differences in the cell cycle status of unactivated neonatal and adult T cells

When neonatal lymph node cells are activated with anti-CD3, i.e., the mode of activation used for most of the cycling experiments, we found that they produced high levels of both Th1 and Th2 cytokines as early as 24 h of activation (Fig. 7). This contrasts with adult total lymph node cell cultures that contained markedly less of the Th1 cytokine γ-IFN and no detectable IL-4 (Th2 cytokine) at this early time point. Thus, one possible explanation for the faster cycling kinetics of neonatal T cells is that the high levels of cytokine present early act to drive cell cycle entry. This possibility was tested in two ways. First, adult cells were activated in the presence of exogenous IL-2. The prediction here is that early cycle entry by neonatal T cells may be promoted by their high IL-4 production, something completely lacking in the early adult cultures. In this case, elimination of the IL-4 signal should slow down cycle entry to resemble adult T cell cycling. Again, however, the presence of anti-IL-4 in the cultures had no effect on the proportions of cycling neonatal CD4+ cells (Table III).

Another possibility is that the rapid cycle entry of neonatal T cells may be because they are actually ahead of adult T cells before activation. That is, adult T cells taken from unprimed animals are largely in the quiescent G0 phase of the cell cycle. If freshly isolated neonatal T cells were already in the G0 phase of the cell cycle, their progression into the S phase would appear to be more rapid. To test this idea, we took advantage of a staining method that allows the discrimination between the G0 and G1 stages (21). During cell cycle progression, there is a constant increase of cellular RNA, largely because of the increased production of rRNA (22, 23). Simultaneous staining of viable cells with the RNA-specific fluorochrome pyronin Y and the DNA-specific fluorochrome Hoechst can be used to determine cell cycle stages and, particularly, differentially mark cells in G0 vs G1 (21). Total lymph node cells from day 7 or adult BALB/c mice were isolated and stained with anti-CD4 mAb followed by fixation and permeabilization and were activated in the presence of neutralizing anti-IL-4 mAb. The rationale here is that early cycle entry by neonatal T cells may be promoted by their high IL-4 production, something completely lacking in the early adult cultures. In this case, elimination of the IL-4 signal should slow down cycle entry to resemble adult T cell cycling. Again, however, the presence of anti-IL-4 in the cultures had no effect on the proportions of cycling neonatal CD4+ cells (Table III).

![FIGURE 7](https://www.jimmunol.org/)

**FIGURE 7.** Neonatal T cells produce high levels of both Th1 and Th2 cytokines earlier than do adult T cells. Total lymph node cells from day 7 or adult DO11.10 mice were activated with soluble anti-CD3 mAb. Twenty-four hours later, supernatants were harvested, and the IFN-γ and IL-4 content was determined by specific ELISA. One experiment typical of three is shown.

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<th>CD4+ (S + G2/M)</th>
<th>CD8+ (S + G2/M)</th>
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</tr>
<tr>
<td>Adult</td>
<td>19.4</td>
<td>46.6</td>
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</table>

* Total lymph node cells from BALB/c mice of the indicated ages were activated with anti-CD3. Twenty-four hours later, the cells were stained with PI and anti-CD4 or anti-CD8.

![TABLE II](https://www.jimmunol.org/)

**TABLE II.** Rapid cell cycle entry by peripheral neonatal T cells begins to disappear by 3 wk postbirth

<table>
<thead>
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<th>Age (wk)</th>
<th>CD4+</th>
<th>CD8+</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>52.7</td>
<td>66.2</td>
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* Total lymph node cells from BALB/c mice of the indicated ages were activated with anti-CD3. Twenty-four hours later, the cells were stained with PI and anti-CD4 or anti-CD8.

![FIGURE 8](https://www.jimmunol.org/)

**FIGURE 8.** Similar percentages of cells in G0 among freshly isolated neonatal or adult CD4+ cells. Total lymph node cells from day 7 or adult BALB/c mice were isolated and stained with anti-CD4 mAb followed by Hoechst and pyronin Y staining. Control cells stained in parallel consisted of 24-h anti-CD3 activated adult lymph node cells. The Hoechst and pyronin staining profiles of gated CD4+ cells are shown. One example of three separate experiments is shown.
staining with pyronin Y and Hoechst (Fig. 8). To allow definition of cells in various stages of the cycle, lymph node cells activated 48 h earlier with anti-CD3 mAb were stained in parallel (Fig. 8, left panel). Using the activated cells as a standard, a region defining cells in G₁ was drawn. A small percentage of freshly isolated adult CD44+ cells appeared within this G₁ region. Neonatal CD44+ cells showed a small but consistent increase (~1.5-fold) over adult CD44+ cells in the percentage of cells in G₁. These findings are consistent with the PI results presented in Figure 3, indicating that ~1.5–2 times as many CD44+ cells in neonates, compared with adults, appear to be spontaneously cycling. Nonetheless, the percentage in cycle is low, and it seems unlikely that spontaneous cycling contributes substantially to the large proportion of neonatal CD44+ cells in the S plus G₂/M stages of the cell cycle after only 24 h of activation (see Fig. 3).

Like neonatal T cells, greater proportions of neonatal than adult B cells show early cell cycle entry

The rapid responses of neonatal T cells to activation signals may be a quality unique to T lineage cells. Alternatively, rapid cycle entry may be a feature common to all lymphoid cells of this developmental age. To distinguish between these possibilities, lymph node cells from neonatal or adult BALB/c mice were stimulated with LPS or with anti-CD40 mAb plus rIL-4 for 24 h. The cells were then stained with anti-B220 mAb, to detect B cells, fixed and permeabilized, and stained with PI. Unlike the T cell populations, freshly isolated and unactivated cultured B cells from both neonates and adults showed a considerable percentage (10–20%) of cells in S plus G₂/M (Fig. 9, first and second panels from the left). Following 24 of activation with either agent, there was essentially no increase in cycling among adult B cells (Fig. 9, third and fourth panels from the left). In contrast, the percentage of cycling cells in the neonatal B cell population increased at least 2-fold in response to either LPS or anti-CD40 plus rIL-4 stimulation. Similar results were obtained when cells were stimulated with anti-μ mAb (data not shown). Thus, both the B and T cell populations present in early life respond to lineage-specific activation with rapid cell cycle entry.

Discussion

We have shown in this study that greater proportions of neonatal lymphocytes, including CD4+ and CD8+ T cells and B cells, respond to lineage-specific activation by entering the cell cycle earlier than their adult counterparts. This is likely to be a physiologically significant event, because 1) it occurs in vitro in response to direct TCRαβ activation, 2) it occurs in vivo in response to the peptides and/or signals stimulating homeostatic proliferation (reviewed in Refs. 24 and 25), and 3) it is seen in several strains of mice. At least in part, early cell cycle entry may compensate for the lack of immunological memory in neonates, allowing the rapid mobilization of immune effector mechanisms.

The major finding that led to these experiments was that neonatal T cells, unlike adult T cells, make high levels of IL-4 within 48 h of initial activation (13). For adult T cells, Reiner and colleagues (17) made the original observation that IL-4 production occurs at increased frequencies in cells that have undergone at least three cell divisions. We reasoned that neonatal T cells may make high levels of IL-4 early because they divide more rapidly than adult cells. While proportionally more neonatal than adult T cells entered the cell cycle early, no cell division was detected before ~36 h of activation (data not shown; see Figs. 1 and 2). In these experiments, we found that large quantities of IL-4 were secreted by neonatal T cells within 24 h of activation (Fig. 7). Thus, neonatal T cells produce high levels of IL-4 before undergoing a single round of cell division. In this regard, neonatal T cells resemble the relatively small proportion of adult cells that can acquire IL-4-secreting potential in the absence of proliferation (26, 27). However, it is important to point out that ≥37 h of culture time was required before IL-4 production by undivided adult cells was apparent. Thus, whether the cells proliferate or not, some extended time period may be required to remodel the IL-4 locus (17, 28–31) to allow efficient gene expression. Because neonatal T cells produce IL-4 so rapidly, in a division-independent fashion, it is tempting to speculate that the IL-4 locus is relatively more accessible, e.g., less methylated, in neonates compared with adults. Experiments to test this intriguing possibility are currently underway.

It is generally believed that most fetal, and possibly neonatal, cells proliferate faster than adult cells. However, direct comparisons of cell cycle entry by fetal/neonatal and adult cells are generally lacking. What is clear is that fetal/neonatal cells appear to cycle spontaneously at a higher rate than their adult counterparts. For example, a higher percentage of freshly isolated fetal CD34+ hematopoietic stem cells appear to be in cycle compared with the adult CD34+ population (32, 33). This spontaneous proliferation is not limited to hematopoietic cells. In first-passage cultures, a greater proportion of newborn than adult lung fibroblasts spontaneously enter the S phase of the cell cycle at any time point (34). Smooth muscle cells from neonatal rats, unlike those from adult rats, appear to be competent to proliferate in the absence of exogenous mitogens (35). Among T cell populations, cord blood cells, but not naive adult cells, proliferate in response to IL-7, in the absence of stimulation through the TCR (36, 37). Indeed, in our system, although spontaneous proliferation is not high, there is a reproducibly greater (~2-fold) percentage of freshly isolated neonatal lymph node T cells that appear to be spontaneously in cycle (see Fig. 3, left panel). In addition, a recent report (38) describes significantly greater spontaneous proliferation of neonatal splenic T cells in situ. However, the observations reported in this study are unique in that rapid early entry into the cell cycle is observed following lineage-specific activation of fully differentiated cells.
The demonstration that greater proportions of neonatal B cells as well as T cells enter the cycle faster than their adult counterparts indicates that this phenomenon is common to all lymphocytes of this developmental stage. It will be interesting to determine whether other neonatal blood cells, like macrophages and dendritic cells, also share this property.

What are the characteristics of the neonatal lymphocyte population that lead to rapid early cell cycle progression? For T cells, one possibility we entertained was that a greater proportion of neonatal T cells were already in G1 and, thus, entered the S phase of the cycle faster. However, low proportions of both freshly isolated neonatal and adult T cells were found to be in G1 (see Fig. 8). Because neonatal T cells made high levels of cytokine more rapidly than adult T cells, we then postulated that these high early levels of cytokine drove proportionally more neonatal T cells into cycle early. This also did not appear to be the case because 1) adding saturating amounts of exogenous cytokine did not result in faster cell cycle progression by adult T cells and 2) neutralizing anti-IL-4 mAb did not slow down neonatal cell cycle progression (see Table III). Yet another possibility may be found in the relative compositions of neonatal and adult populations. For example, Turka and colleagues (39) have shown that up to 40% of adult T cells fail to progress through the cell cycle, despite showing evidence of an activated state. Due to developmental immaturity, neonatal lymphocytes may lack this population, resulting in a greater proportion of cells responding to activation with early proliferation. Thus, it will be important to measure the absolute number of precursors among the neonatal and adult populations that respond to activation with proliferation (39).

The activation of T cells results in signaling cascades that ultimately lead to cellular proliferation and the acquisition of effector function. Lineage-specific molecular signals are activated, often by phosphorylation, and these signals, in turn, interface with cell cycle machinery common to all cells. The more rapid cycling of neonatal T cells might result from the dysregulation of one or more of these signals. For example, the initial entry of all cells into cycle is controlled by a combination of the up-regulation of positive signals and the down-regulation of negative signals. p27kip is expressed in resting cells of all types and must be degraded to allow cell cycle entry (reviewed in Refs. 40 and 41). It is conceivable that resting neonatal T cells undergo low p27kip or that high p27kip is expressed at normal levels but degraded more rapidly upon activation. T lineage-specific signals may also be affected in neonates. For example, phospholipase C (PLC)-y1 is a central signaling molecule in T cells. Phosphorylation of PLC-y1 on multiple tyrosine residues is required for its activation (reviewed in Ref. 42). It is conceivable that these phosphorylation events occur more rapidly or efficiently in neonatal T cells or even that PLC-y1 may be partially tyrosine phosphorylated in resting neonatal T cells. Clearly, there are numerous candidate signals in the regulation of the relative cell cycle entry of neonatal and adult T cells. We are currently beginning experiments to distinguish among the many possibilities.

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References


