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*J Immunol* 1998; 160:4217-4224; ;
http://www.jimmunol.org/content/160/9/4217

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Newborn Mice Develop Balanced Th1/Th2 Primary Effector Responses In Vivo But Are Biased to Th2 Secondary Responses

Becky Adkins and Rong-Qing Du

Newborn mice are impaired in their abilities to mount protective immune responses. For decades, it was generally held that the poor responses of newborns were largely due to the developmentally immature state of the T cells. In vitro studies showing that neonatal T cells were deficient in Th1 cytokine production, proliferation, and secondary responsiveness strongly supported this idea. Recently, several studies have challenged this view; animals exposed to Ag as neonates were shown to have mature Th1 responses in adulthood. However, it is not clear whether the mature immune responses were actually mounted by T cells generated after the neonatal stage. We have reexamined this issue by analyzing the capabilities of neonatal lymph node T cells to develop into Ag-specific effector cells during the actual neonatal period. Our results demonstrate that the capacity to develop a balanced Th1/Th2 primary effector response is fully mature within the first week of life. However, while neonatal and adult primary cytokine profiles were very similar, Th2 secondary responses predominated in animals first immunized as newborns. Moreover, we have observed other differences between adults and neonatal responses, including 1) the kinetics of cytokine production and responsiveness to adjuvant during the primary response, and 2) the contribution of spleen and lymph node to secondary responses. We propose that these differences reflect developmental regulation of effector cell function that has important consequences to neonatal immune function.

Received for publication August 14, 1997. Accepted for publication December 31, 1997.

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The experiments reported in this work were designed to reveal the true capacity of neonatal T cells in situ. We have examined the development of primary immune responses in the neonate during the first week of life. The production of IL-2, IL-4, and IFN-γ by lymph node T cells was analyzed 6 days after immunization of ≤24-h-old newborns with Ag in PBS. We found that the lymph node response of newborns is very similar to that of adults. Both Th1 and Th2 primary effector cell populations were generated in the neonate, and the levels of cytokine produced were comparable with those produced by adult cells. Thus, during the first week of life, lymph node T cells are fully competent to develop into Th1 effector cells in vivo. This capacity was already fully mature as early as 1 day postbirth since neonatally thymectomized mice also produced copious amounts of IFN-γ during a primary response. Although neonatal and adult primary effector responses were similar, they were not identical. Differences were observed in the kinetics of cytokine production and in responses to adjuvant. Moreover, even though a balanced Th1/Th2 primary response was observed, the secondary responses (cytokines, IgG isoforms) of the same animals were skewed toward Th2. Therefore, while neonates appear to be capable of mounting adult-like primary Th1 responses, Th1-like cells are apparently not well maintained in early life, and secondary responses become dominated by the preferential persistence of the Th2 population.

Materials and Methods

Mice

BALB/c mice, originally obtained from Charles River Laboratories (Wilmington, MA), were bred and housed under barrier conditions in Division of Veterinary Resources at University of Miami Medical School (Miami, FL). Periodic screening showed the colony to be free of commonly occurring infectious agents. Females from timed matings were monitored closely at about day 20 of gestation (the average day of birth in BALB/c mice), and the date of delivery was recorded. Birth day was called day 0. Neonatal animals were defined as ≤24 h old.

Immunization

Adult (6–8 wk old) and newborn mice were immunized with 5 μg/g keyhole limpet hemocyanin (KLH) (CalBiochem, San Diego, CA) alone or DNP-conjugated KLH (DNP-KLH) (CalBiochem), as indicated. The Ags were suspended in PBS for immunization, except for the experiments in Figure 7, in which the adjuvant CFA was also used. Each mouse was injected in three sites, i.p. and s.c. between the shoulder blades, and at the base of the tail. Adults received 100 μl and babies 10 μl per site.

Cell preparations

Pools of tissues from ≥2 adults or ≥10 newborn animals were used for the cell preparations.

Total lymph node or spleen cell suspensions

Total spleen cell suspensions were prepared and RBC were removed by incubation in hypotonic lysis buffer (0.15 M NH4 Cl, 0.001 M KHCO3, and 0.1 mM EDTA). Mesenteric, inguinal, axillary, brachial, and cervical lymph nodes were mixed and used for total lymph node cell suspensions.

Enriched CD4+ and CD8+ cells

Total lymph node cell suspensions were passed over enrichment columns for mouse CD4+ or CD8+ cells (R&D Systems, Minneapolis, MN), precisely according to the manufacturer’s instructions. Enriched CD4+ suspensions were contaminated with ≤1% CD8+ cells; enriched CD8+ suspensions were contaminated with ≤1% CD4+ cells.

Adult splenic APC

Total spleen cells from adult animals were treated with anti-Thy-1 (42–21) plus complement, followed by treatment with 50 μg/ml mitomycin C, as described earlier (5, 14).

Thymectomies

Adult mice were anesthetized by i.p. injection of a mixture containing 0.2 mg and 1 mg per 10 g of body weight of, respectively, xylazine (Rugby Laboratories, Rockville Center, NY) and ketamine HCl (Fort Dodge Laboratories, Fort Dodge, IA). Neonatal mice were anesthetized by cooling in a reservoir of wet ice for approximately 1 min. A midline incision was made in the skin of the upper thoracic region to expose the sternum, and a second, longitudinal incision of the sternum was made to expose the tops of both thymic lobes. Both thymic lobes were then removed with a Pasteur pipette under a gentle vacuum. The chest was compressed to eliminate air in the thoracic cavity. The wounds in the adults were closed using surgical staples (Clay Adams, Parsippany, NJ), and the wounds in the newborns were sutured with Ethicon 6-0 sterile surgical braided silk (Roboz, Rockville, MD).

Cultures for cytokine production

A quantity amounting to 5 × 106 total lymph node cells was plated in 200 μl of culture medium (RPMI containing 10% FCS) and stimulated with the indicated concentrations of KLH. For cytokine production by subsets of T cells, 2 × 106 CD4+ or CD8+ cells (prepared as described above) were coplated in 200 μl culture medium with 4 × 103 adult splenic APC (as above) and stimulated with the indicated concentrations of KLH. Culture supernatants were harvested at the times indicated in the text.

Cytokine and serum ELISAs

Sandwich ELISA kits specific for mouse IL-2, IL-4, and IFN-γ were purchased from Endogen (Cambridge, MA) and performed precisely per the manufacturer’s instructions. Statistical analyses of the results were performed using the paired Student’s t test. p values ≤0.005 were considered significant.

Sera from individual animals were analyzed individually in ELISA assays specific for mouse IgG1 or IgG2a. Nunc Maxisorb plates were coated with 0.4 μg/well DNP-albumin (Calbiochem) overnight at r.t. The wells were then blocked with PBS containing 1% BSA for 1 h at r.t. The plates were washed with 50 mM Tris and 0.2% Tween-20, pH 7 (wash buffer), and 200 μl of the indicated serum dilutions was added to each well. After a second overnight incubation at r.t., the plates were washed with wash buffer, and 200 μl of a 1:2000 dilution of anti-mouse IgG1 horseradish peroxidase (Cappell, Durham, NC) or anti-mouse IgG2a horseradish peroxidase (Cappell) was added to each well and incubated for 2 h at r.t. The plates were washed again, and 100 μl of TMB substrate (Dako, Carpinte- ria, CA) were added to each well. Thirty minutes later, the reaction was stopped by the addition of 100 μl of 0.18 M sulfuric acid and the plates were read on an ELISA reader (OD450–OD630).

Results

One-day-old newborns develop balanced, adult-like Th1/Th2 primary effector responses

Several recent reports (11–13) have suggested that the newborn immune system is capable of mounting mature protective immune responses in vivo. However, in those experiments, the actual responses were not tested until weeks later, after the animal had grown into adulthood. Because of this experimental delay, the possibility that the mature responses may actually have been made by cells produced later in life could not be excluded. For these experiments, we wished to examine the responses of T cells present during the actual neonatal period. Although the neonatal spleen contains virtually undetectable proportions of T cells, the lymph nodes in neonates contain proportions of T cells similar to those found in adults (5). Therefore, we chose to compare the Ag-specific recall responses of lymph node T cells in adults and in neonates during the first week of life.

Newborns or adult animals were immunized both s.c. (between the shoulder blades and at the base of the tail) and i.p. with 5 μg/g KLH in PBS. Six to seven days later, lymph node cells were prepared and restimulated in vitro with increasing amounts of KLH. Supernatants were collected at the times indicated in the figure legends and assessed for IL-2, IL-4, or IFN-γ content by specific ELISA. To guard against variation from experiment to experiment in the absolute amounts of cytokine produced, adults and neonates

3 Abbreviations used in this paper: KLH, keyhole limpet hemocyanin; r.t., room temperature.
were compared directly within each experiment. The production of all three cytokines by neonatal lymph node effector cells was very similar to their production by adult lymph node T cells (Fig. 1). This pattern of cytokine production was not limited to the Ag KLH, but was also observed for recall responses to OVA (not shown). These experiments indicate that the lymph node T cells present in the first few days of life are fully mature in their capacity to develop into Th1 and Th2 primary effector cells in vivo. Moreover, they demonstrate that neonates and adults generate equivalent Th1 and Th2 primary effector populations in vivo. Finally, these results indicate that the APC population present in neonatal life is sufficiently mature to present Ag efficiently and promote the development of Th effector cells in situ.

To determine whether the in vitro cytokine profiles were an accurate reflection of the cytokines available in vivo, neonates and adults were immunized with KLH coupled to the hapten DNP. One month later, serum anti-DNP Ig levels were assessed (Fig. 2). The two isotypes examined were IgG1 and IgG2a, whose production is promoted by, respectively, IL-4 and IFN-γ. All animals immunized as adults or neonates produced anti-DNP IgG1 Abs (Fig. 2, top), and the relative levels of IgG1 produced by the neonates and adults were comparable. The anti-DNP IgG2a responses were different; not all animals responded, but the same proportion (roughly one-half) of adults and neonates made anti-DNP IgG2a Abs. In addition, among the responders, animals immunized as neonates produced the same levels (relative to the control unimmunized littermates) of anti-DNP IgG2a Abs as did adults.

As described above, several different experimental approaches have led to the idea that newborns are skewed toward Th2 responses. The corollary to this idea is that newborns are deficient in Th1 responses. Our experiments, in which newborns produce adult levels of IFN-γ (above), argue against that idea. However, it could still be maintained that the IFN-γ was actually being made by more developmentally mature T cells that were produced after day 1, but before day 7, of life (i.e., the time of analysis). To investigate this possibility, ≤24-h-old newborns or adult mice were thymectomized or sham thymectomized and immunized with KLH. Six days later, lymph node cell suspensions were restimulated with KLH, and supernatants were collected for cytokine measurements (Fig. 3). There was no significant difference in the amount of IL-4 produced by T cells from sham vs thymectomized adult animals. However, IL-4 production by T cells from thymectomized newborn mice was not detectable. In contrast, IFN-γ production was affected similarly in newborns and adults with thymectomy, resulting in a modest (≈fourfold) increase. The IFN-γ production by cells from the thymectomized neonates was remarkable since there was such a severe reduction in T cell proportion in these animals. While total lymph node cell suspensions from thymectomized and sham-treated adults contained similar proportion of T cells, the percentage of T cells was reduced up to 10-fold in suspensions from thymectomized compared with sham neonates ( sham newborns, 53 ± 0.5% CD4+; 16.4 ± 0.7% CD8+; thymectomized newborns, 5.5 ± 0.5% CD4+; 1.3 ± 0.1% CD8+). Thus, the capacity to develop IFN-γ-secreting Th1 effector cells is...
fully mature on the first day of life in mice, whereas the ability to develop Th2 responses appears to require output by the thymus postbirth.

Although cytokines produced by the total lymph node population appeared adult-like, it was possible that subsets of T cells showed developmental immaturity. To address this possibility, similar experiments were performed using populations enriched for CD4+ or CD8+ lymph node T cells (Fig. 4). In two separate experiments, the major producer of IFN-γ among neonatal T cells was the CD8+ population, whereas IL-4 was produced primarily by CD4+ cells. This pattern was also observed for adults (not shown). Therefore, mice ≤1 wk old contain CD4+ and CD8+ populations capable of developing into adult-like cytokine-secreting effector cells.

In some experimental systems, the dose of Ag appears to play an important role in whether Th1 or Th2 responses are elicited (12, 15–17). The dose of immunogen may be especially important for comparisons between adult and newborn responses. Although newborns weigh about 10-fold less than adults, they contain ≥100-fold fewer T cells (5, 11). Therefore, for comparative responses with immunized adults, it is not immediately obvious how to adjust the dose of immunogen for neonates. To address this issue, we immunized newborns with a 50-fold range and adults with a 25-fold range of KLH amounts. Six days later, IFN-γ and IL-4 production by lymph node T cells in response to restimulation in vitro were assessed (Fig. 5). Over this broad range in immunogen dose, there did not appear to be any single dose that preferentially stimulated Th1 or Th2 cytokines. In general, if a dose resulted in increased (or decreased) IFN-γ, there would also be increased (or decreased) IL-4 production. This pattern was observed for both neonates and adults. Therefore, at least when immunogens are delivered in PBS, neonates and adults produce both Th1 and Th2 effector responses over a wide range of immunogen dose.

The primary responses of neonates and adults are not identical: differences in kinetics of cytokine production and response to adjuvant

Although neonates and adults showed many similarities in their development of Th1 and Th2 primary effector populations, they were not identical. The first difference was revealed by experiments to chart the kinetics of cytokine production by neonatal vs adult T cells using specific column isolation. CD4+ and CD8+ T cells among neonatal and adult responses varied, and differences in kinetics of cytokine production and response to adjuvant were seen for IL-4 and IFN-γ, except at later time points. By 48 h of activation, neonates produced high levels of both cytokines. In contrast, adults produced no detectable IL-4 and IFN-γ until

![Graph showing cytokine production](http://www.jimmunol.org/)

**FIGURE 3.** Newborns thymectomized at birth develop mature primary Th1 effector responses. Newborns ≤24 h old and adult animals were thymectomized or sham thymectomized and immunized, as for Figure 1. Six days later, lymph node cells were stimulated in vitro with 25 μg/ml KLH, and supernatants were harvested at 72 h for ELISA determination of cytokine content. Average values ± SD from triplicate ELISA wells are plotted. Background cytokine production in the absence of KLH was undetectable for IL-4. The IFN-γ values are presented with the background cytokine values subtracted out. One experiment typical of three independent attempts is depicted.

![Graph showing cytokine production](http://www.jimmunol.org/)

**FIGURE 4.** Both CD4+ and CD8+ T cells in newborns mature to cytokine-producing effector cells. Newborn mice ≤24 h old were immunized with KLH, as described in the legend for Figure 1. Six days later, lymph node cell suspensions were prepared and separately enriched for CD4+ or CD8+ T cells using specific column isolation. CD4+ cells were enriched to 91% of the population with <1% contamination with CD8+ cells; purified CD8+ populations consisted of 72% CD8+ cells with <1% contamination with CD4+ cells. A quantity amounting to 2 × 10^5 CD4+ or CD8+ cells was cultured in 200 μl of medium with 4 × 10^6 anti-Thy-1 plus complement-treated adult splenic APC and 25 μg/ml KLH. Supernatants were harvested at 72 h of culture and assayed by ELISA; average values ± SDs from three replicate ELISA wells are shown. Background cytokine production in the absence of KLH, as follows: experiment 1, undetectable IFN-γ, <37 pg/ml IL-4; experiment 2, <6.5 × 10^3 pg/ml IFN-γ, undetectable IL-4.
Thus, cytokine production by cells from neonatal animals proceeded with more rapid kinetics than that by adult cells; neonatal T cells produced high levels of Th1 and Th2 cytokines up to 24 h earlier than adult T cells following restimulation in vitro.

The second difference between neonatal and adult responses was found in experiments comparing the effects of delivering Ag in CFA vs PBS. Newborns ≤24 h old and adult animals were immunized with 5 μg/g KLH in either CFA or PBS. Six days later, lymph node cells were restimulated with 12.5 or 25 μg/ml KLH, and supernatants were harvested at 24 h (day 1 IL-2), 48 h (adult IL-2), or 72 h (IL-4 and IFN-γ). Maximal cytokine levels were graphed and were produced at 12.5 μg/ml (IL-2) and 25 μg/ml (IFN-γ and IL-4) KLH. Average values ± SDs from triplicate ELISA wells are shown. Background cytokine produced in the absence of KLH was undetectable for IL-4, <3 × 10^3 pg/ml for IFN-γ, and was subtracted out for IL-2. One experiment typical of three independent ones is shown.

The difference between neonatal and adult responses was found in experiments comparing the effects of delivering Ag in CFA vs PBS. Newborns ≤24 h old and adult animals were immunized with KLH either in PBS or CFA. Six days later, lymph node cells were restimulated with different concentrations of KLH, and supernatants were harvested for ELISA analyses (Fig. 7). Unlike that observed with immunization using PBS as the vehicle, IL-4 production by either adults or neonates immunized with KLH in CFA was undetectable. Adults immunized using CFA showed a modest increase (not statistically significant) in IL-2 secretion. In contrast, adult IFN-γ production was enhanced 10-fold (p < 0.004) when immunization was performed with CFA. The effects of CFA on IL-2 and IFN-γ production by neonates were strikingly different. IL-2 production was reduced severely (p < 0.004), while IFN-γ was not enhanced (p > 0.01). Only single time points are shown, but supernatants from 24 to 72 h were examined to ensure that differences between CFA and PBS were not simply due to differences in the kinetics of cytokine production. Therefore, unlike in adults, CFA does not act to enhance the development of primary Th1 effector cells during early neonatal life.
Although neonates mount balanced Th1/Th2 primary responses, mice immunized at birth are skewed toward Th2 secondary responses

It has been reported recently (9, 10) that mice immunized with Ag in adjuvant at birth become primed to develop highly skewed Th2 secondary responses. Since we had seen a balanced Th1/Th2 primary response using PBS as the vehicle, we wished to know whether our mice were nonetheless biased toward Th2 secondary responses. Newborns ≤24 h old or adult animals were immunized with KLH, as previously described. Four weeks later, the mice were reimmunized with 5 μg/g KLH in PBS. Eight days later, the animals were bled and the anti-DNP IgG1 and IgG2a serum responses were measured in ELISA assays. Each line represents a different mouse; each point represents the average ± SD from triplicate ELISA wells. ○ = unimmunized control animals; ● = immunized animals. All mice immunized as neonates (n = 8) or adults (n = 4) produced anti-DNP IgG1 Abs. Two representative immunized animals of each age group are shown. All immunized adults produced high levels of anti-DNP IgG2a Abs, and two representative individual animals are shown. Four of the eight mice immunized as neonates produced intermediate levels of anti-DNP IgG2a Abs, and the other four produced low levels. One immunized neonate from each of these two response patterns is shown.

Discussion

We have analyzed the in vivo generation of Th1 and Th2 effector cells in the lymph nodes during the neonatal period of life. Newborns were immunized within the first 24 h of birth, and the in vitro recall responses of their lymph node cells were analyzed 6 days later. We found that newborns were as effective as adults in developing primary Ag-specific T cell populations secreting IL-2, IL-4, and IFN-γ. Thus, the T cell population is sufficiently mature within the first week of life to develop balanced Th1/Th2 primary effector populations similar to those produced by adults. Because the development of Th cells requires APC, this result also suggests that the APC population in newborns is functionally mature. Although the primary cytokine profiles of neonatal and adult animals were very similar, other aspects of the immune responses showed major differences. Upon in vitro restimulation, primary effector cells from neonates produced IL-2, IL-4, and IFN-γ up to 24 h earlier than did primary adult effectors. While adult IFN-γ was enhanced ≥10-fold by CFA, adjuvant had little to no effect on the generation of primary IFN-γ-secreting cells in the neonate. Finally,
despite balanced Th1/Th2 primary responses, the secondary responses of mice immunized as neonates were skewed toward the Th2 pathway. These results demonstrate that neonates are developmentally mature in their capacity to mount primary, adult-like Th1/Th2 responses in vivo. However, neonates are developmentally immature in their kinetics of cytokine production, responsiveness to adjuvant, and the maintenance and/or generation of Th1 secondary responses.

One of the major differences seen between newborn and adult primary effectors was in the kinetics of cytokine production. The production of maximal amounts of all three cytokines, IL-2, IL-4, and IFN-γ, by neonatal T cells occurred ≥24 h earlier than their production by adult T cells. The mechanism(s) underlying more rapid response kinetics is unknown. However, the rapid cytokine production by neonatal effector cells may be just one reflection of a developmental state in which the T cells are poised to respond quickly. In support of this idea, we have also found that polyclonally activated, naive newborn (4-day-old) T cells enter the cell cycle with more rapid kinetics than do adult T cells (not shown). In addition to starting earlier, the responses of neonatal T cells also end earlier. For example, while IL-2 is present in cultures of neonatal T cells at 24 h, it is no longer detectable at 48 h, presumably because it has been utilized by the cells. Adult cultures, on the other hand, contain copious amounts of IL-2 at 48 h of activation. Similarly, polyclonally activated, naive neonatal T cells appear to enter the cell cycle earlier and subsequently revert to cultures containing largely G0-G1 cells 1 full day earlier than adult T cells (unpublished observation). A possible consequence of these rapid kinetics is that the overall immune response may be temporally limited in neonates. It is tempting to speculate that developmental regulation of the kinetics of immune responsiveness contributes to the relatively immunodeficient state of newborns.

A second major difference between the primary responses of neonates and adults was seen when Ag was introduced in CFA. In adult animals, CFA markedly enhanced Th1 responses. IFN-γ production was enhanced ≥10-fold over that produced when Ag was delivered in PBS. In contrast, IFN-γ was largely unaffected in neonates immunized with CFA. This contrasts with reports (9, 13) by others that different adjuvants could contribute to the relatively immunodeficient state of newborns. It is possible that the adult-like Th1 responses observed in the latter systems actually resulted from cells produced after the immediate neonatal period. Nonetheless, these results illustrate that the choice of adjuvant (or lack thereof) is critical in determining the type of Th response generated in the neonate. Moreover, our results clearly demonstrate that one cannot predict a newborn response to a particular adjuvant (e.g., CFA) from the type of response elicited in adults. Due to the strong relevance to vaccine development, it will be important to more clearly define the effects of different adjuvants on both the primary and secondary responses of neonates.

Since neonates develop fully mature primary Th1 effector populations, the question that arises is why are they relatively deficient in producing Th1 responses to secondary immunization? There are at least two possible explanations for this phenomenon. First, Th1 cells capable of responding to secondary stimulation may be present, but unable to respond, i.e., they may be anergized. This possibility seems unlikely because endogenous IL-2, a cytokine that usually reverses nonresponsiveness, is present at similar levels in cultures prepared from twice immunized adults and neonates (not shown). The second possibility is that the Th1 cells generated initially may show poor survival, and hence, their numbers would be limiting in a secondary response. There are in vitro data that make this possibility appealing. Several years ago, we showed (14) that approximately one-half of the lymph node T cells from 4-day-old animals undergo apoptosis in response to a single round of stimulation with anti-CD3 Ab. It could be proposed that apoptosis also occurs in vivo following the initial effector phase, and the major population affected is the Th1 cell type. This is an exciting possibility because it suggests a mechanism that could account for tolerance induction during the neonatal period.

In addition to showing skewed Th2 secondary responses, neonates differed from adults in the organs contributing to secondary responses. In newborns, both lymph node and spleen cells made IL-4 and IFN-γ. In contrast, production of both cytokines in the adult was largely confined to the spleen. This was not due to a generalized inactivity of the adult lymph node cells since the same cells produced large amounts of IL-2 in response to the Ag and high levels of IFN-γ in response to anti-CD3 stimulation (not shown). It is not clear how this arises, but it may be that recirculation of primed/memory cells is different in early life and adulthood. For example, primed/memory cells may not recirculate efficiently from the lymph nodes to the spleen in newborns and juvenile mice. In that case, relatively more activity would be retained in the lymph nodes. This could have important implications for vaccine responsiveness, i.e., an immunization route that reaches both the lymph nodes and spleen may be more important in early life.

The experiments performed with euthymic mice suggest that the capacity to develop adult-like Th1 and Th2 primary effector responses is mature within the first week of life. To determine whether this was a true property of the cells already resident on day 1 of life, we similarly examined thymectomized newborns. Total lymph node cells from thymectomized mice produced IFN-γ at levels similar to those produced by cells from sham-thymectomized newborns. In contrast, IL-4 production was greatly reduced in thymectomized newborns. In the thymectomized mice, the proportion of T cells in total lymph node suspensions was reduced approximately 10-fold. The poor IL-4 production may have resulted from the limited numbers of T cells present. However, it is clear that there were sufficient numbers of cells present for the production of copious amounts of IFN-γ. Thus, an alternative explanation for poor IL-4 production is that cells capable of developing into Th2 effectors may not be produced efficiently in the thymus until after the first day of life. We are currently conducting experiments to test this hypothesis.

References


