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The Generation of Th Memory in Neonates Versus Adults: Prolonged Primary Th2 Effector Function and Impaired Development of Th1 Memory Effector Function in Murine Neonates

Becky Adkins, Yurong Bu, and Patricia Guevara

Immunization during the neonatal period often results in Th2-biased secondary responses. To understand the regulation of this phenomenon, we have examined all phases of Th development, from the generation of primary effectors to the duration of the primary effector stage to the production of memory effector function. First, we had previously reported that although primary responses in the neonatal lymph nodes are mature, mixed Th1/Th2-like, primary responses in the spleens of the same animals are exclusively Th2-like. To determine whether Th2-dominant secondary responses are due to the Th2-polarized primary function in the spleen, neonates were splenectomized before immunization. Even in the absence of primary neonatal splenic responses, the secondary responses of neonates were Th2 dominant. Thus, the overwhelmingly Th2 primary responses in the neonatal spleen are not required to generate Th2-dominant memory in the lymph nodes. Second, we have compared the kinetics of the primary response phase in neonates and adults. In adults, Ag-specific Th2 function disappeared rapidly from both the lymph nodes and spleen. In contrast, primary Th2 function persisted out to 5 wk in both neonatal organs. Third, the generation of Th memory responses was examined in animals initially immunized as neonates and in adults. These experiments demonstrated that neonates are selectively impaired in the development of Th1 memory effector function. Together, these results indicate that neonates are biased to Th2 function at all phases of an immune response.

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There are two major types of Th cells, Th1 and Th2, defined by their distinctive functions and cytokine secretion patterns (reviewed in Refs. 1–3). Th1 cells are crucial for cell-mediated immune responses and are characterized by the production of the signature cytokine IFN-\(\gamma\). Th2 cells promote humoral immunity and secrete IL-4, IL-5, and IL-10. Both Th1 and Th2 function are required for comprehensive immunity. However, the inappropriate skewing of a response to either the Th1 or Th2 lineage can result in pathological conditions such as autoimmunity or the failure to clear infectious agents. Thus, achieving and maintaining the appropriate balance between Th1 and Th2 function are critical for protective and nonpathological immune responses. This is especially important for the neonatal period of life when tolerance to self-Ags as well as reactivity to a host of novel non-self-Ags must be established.

Neonatal animals are considered to be at high risk for infectious agents which have relatively minor effects in adults. In many cases, vigorous Th1-mediated responses appear to play a critical role in protecting the adult animals. Therefore, it had been widely thought that neonates were deficient in Th1 lineage development in vivo.

Despite this expectation, it has recently been shown that neonates are fully competent to develop Ag-specific Th1 responses in situ (reviewed in Ref. 4). These mature Th1 responses were elicited with the use of strong Th1-promoting agents, including DNA vaccines (5–10), strong Th1-promoting adjuvants (11), CpG-containing oligonucleotides (12), or IL-12 (13).

Although Th1 responses can be elicited from neonates, Th2-biased responses often emerge. In the early 1990s, Streilein and his colleague (14) first discovered a Th2 bias of neonatal allogeneic responses. Subsequently, Siegrist and his colleague (7) observed Th2 skewing in neonatal mice immunized with a variety of Ags precipitated with aluminum, the only adjuvant approved for pediatric use in humans (12). Bona and colleagues (9, 10) reported Th2 responses in neonates immunized with live or attenuated virus while comparably immunized adults developed Th1 responses. Using protein Ag (keyhole limpet hemocyanin (KLH)3) in PBS, we have also found that animals initially immunized as neonates show Th2-dominant memory responses (15). Finally, Zaghouani and colleagues (16–18) have described Th2-polarized responses in the lymph nodes of adult animals originally tolerated as neonates to peptide Ag. Thus, under many conditions, neonates appear to be biased to Th2 lineage function.

To understand how neonates arrive at Th2-dominant memory responses, we have examined all phases of the immune response, from naive cells to memory effectors. First, we have tested the influence of primary Th1/Th2 function on subsequent memory responses. We had previously found that naive neonatal lymph nodes develop mature, mixed Th1/Th2 primary effectors (15, 19) but that the splenic primary effectors are exclusively Th2-like (19). The possibility that the exclusive Th2 primary responses in the spleen...
account for Th2-biased memory responses was addressed. Experiments using splenectomized neonates showed no significant differences in the Th2-skewed memory responses in the lymph nodes. Therefore, the Th2-biased primary responses in the neonatal spleen are not required to generate Th2-dominant memory in neonates. Second, we have compared the duration of the primary effector phase in neonates and adults. These experiments showed that although primary Th2 function in adult lymphoid organs disappears rapidly, high-level primary Th2 function is retained in both the spleen and lymph nodes of neonates for weeks. Third, we have analyzed the kinetics of the generation of memory effector cells in neonates and adults. These studies revealed that animals initially immunized as neonates are deficient in the capacity to generate Th1 memory effector function. Together, these data demonstrate that neonates are biased to Th2 function at all stages of the immune response.

Materials and Methods

Mice

BALB/c mice, originally obtained from Charles River Breeding Laboratories (Wilmington, MA), 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. Neonatal animals were defined as ≤1-day old.

Splenectomy

Neonatal mice ≤1-day old were anesthetized by burial up to the neck in wet ice for ~2–3 min. Adult mice were anesthetized by injection of a mixture of xylazine (Cetus, Rockville Center, NY) and ketaset (Fort Dodge Laboratories, Fort Dodge, IA) i.p. at, respectively, 0.2 and 1 mg/10 g body weight. Animals were placed on their stomachs and a small incision was made on the right back side, just below the rib cage. Spleens were pinched off with forceps and the wounds were closed with silk braided sutures (Ethicon, attached 5-0 needle) for neonates or with surgical staples for adults.

Immunization

Unless otherwise indicated in the text, ≤1-day-old neonatal or adult (6–8 wk old) mice were immunized with, respectively, 10 or 100 μg KLH (Calbiochem, San Diego, CA). Mice >1 day but <6 wk old were weighed and immunized with 5 μg/μl KLH. In most cases, a solution of KLH in PBS was used for immunization. For the experiment shown in Fig. 6, KLH precipitated with aluminum potassium sulfate (alum) was used. For alum immunization, a 1:1 (v/v) mixture of 1 mg/ml KLH and a 10% (w/v) solution of aluminum potassium sulfate dodecahydrate was prepared. A sufficient volume (~20% of the final volume) of 1.0 N NaOH was added to achieve a pH of 6.5. The mixture was allowed to stand for 30 min at room temperature and then was diluted with an excess of PBS and centrifuged for 5 min (1000 rpm). The pellet was resuspended with PBS to achieve a final ratio of 0.5 mg aluminum potassium sulfate/10 μg KLH.

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 neonates received 10 μl/site.

Preparation of total spleen and lymph node cells

Pools of tissues from ≥2 adults or ≥6 newborn animals were used for the cell preparations. Total spleen cell suspensions were prepared (15) and RBC were removed by incubation in hypotonic lysis buffer (0.15 M NaCl, 0.001 M KHCO₃, and 0.1 mM EDTA). Mesenteric, inguinal, axillary, brachial, and cervical lymph nodes were pooled and used for total lymph node cell suspensions (15).

Preparation of CD4⁺ cells

Enriched (95–98% CD4+) CD4⁺ cells were positively selected (MS⁺ columns) using the Miltenyi Biotec (Bergisch-Gladbach, Germany) MACS system, precisely per the manufacturer’s directions.

Preparation of adult splenic APC

Total spleen cells from naïve adult animals were treated with anti-Thy-1 (mAb 42–21) plus complement, followed by treatment with 50 μg/ml mitomycin C, as described earlier (20, 21).

Culture conditions for ELISAs

For cytokine production by total lymph node or spleen cells, 5 × 10⁶ cells were plated in 200 μl of culture medium and stimulated with 50 μg/ml KLH. 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 production by CD4⁺ T cells, 2 × 10⁶ CD4⁺ (prepared as described above) were coplated in 200 μl of culture medium with 4 × 10⁶ adult splenic APC (as above) and stimulated with 50 μg/ml KLH. Culture supernatants were harvested 72 h later and IFN-γ and IL-4 content were assessed using mouse-specific cytokine ELISA kits (Endogen, Woburn, MA), precisely according to the manufacturer’s directions.

Culture conditions for enzyme-linked immunospot (ELISPOT) assays

To activate cells for the ELISPOT assays, lymph node or spleen cells from immunized mice were cultured at 5 × 10⁵ cells/200 μl of culture medium containing 50 μg/ml KLH. At 36–48 h later, the cells were cultured and processed for ELISPOT, as previously described in detail (19). Briefly, Nunc Maxisorp plates (Nunc, Naperville, IL) were coated by overnight incubation at room temperature with 100 μl of a 5-μg/ml solution of anti-mouse IL-4 (PharMingen, San Diego, CA) or anti-mouse IFN-γ mAb (PharMingen). The plates were washed and the wells were then blocked with 100 μl of culture medium for 1 h at room temperature. Different dilutions of the harvested cells (above) were added to the wells and the plates were incubated for 20 h at 37°C in an atmosphere of 5% CO₂. The plates were then washed and 100 μl of biotinylated anti-IL-4 or anti-IFN-γ mAb (PharMingen) was added to each well. Following a 90-min incubation at room temperature and additional washes, 100 μl of 0.2 μg/ml streptavidin–alkaline phosphatase (Jackson ImmunoResearch, West Grove, PA) was added to each well. The wells were incubated for 60 min at room temperature, washed, and 100 μl of a 1:4 mixture (v/v) of 3% melted low EEO type 1 agarose (Sigma, St. Louis, MO) and 2.3 mM 5-bromo-4-chloro-3-indolyl phosphate (Sigma) in AMP buffer was added to each well. AMP buffer was prepared by mixing 75 mg MgCl₂, hexahydrate, 50 μl Triton X-405, 500 mg Na₃citrate, and 47.9 ml 2-amino-2-methyl-1-propanol (Sigma) in 350 ml of H₂O. The mixture was brought to pH 10.25 with HCl and the final volume was then adjusted to 500 ml with H₂O. The developed spots were counted with the aid of a dissecting scope.

Results

Th2-dominant primary responses in the neonatal spleen are not required for the development of Th2-skewed memory responses

We had previously found that the nature of the primary Th1/Th2 response in neonates is highly dependent on the site of initial Ag exposure. In the lymph nodes, neonates develop primary, mixed Th1/Th2 effector function that is indistinguishable from that found in adults (15, 19). In striking contrast, there is the exclusive development of Th2 responses in the neonatal spleen (19). Since neonatal memory responses become Th2 skewed under similar immunization conditions, the question that arises is, are the Th2-dominant memory responses of neonates due to the preferential development of primary splenic Th2 function? To address this question, we wished to separate the lymph node from the splenic responses in situ. In particular, we wanted to eliminate the primary neonatal splenic responses to test whether memory responses in the lymph nodes remained Th2 biased or became more Th1 dominant, as in adult animals (15). For this purpose, ≤1-day-old or adult BALB/c mice were splenectomized. Two to 4 h later, the neonatal and adult animals were immunized both s.c. and i.p. with, respectively, 10 and 100 μg of KLH in PBS. Nonsplenectomized age-matched control animals were similarly immunized in parallel. Two weeks later, the animals were reimmunized with KLH in...
PBS; adults were reimmunized with 100 μg and the 2-wk-old animals were weighed and immunized with 5 μg/g KLH. Following an additional 2 wk, the animals were sacrificed and CD4+ lymph node cells were cultured with adult splenic APC and 50 μg/ml KLH. Seventy-two hours later, supernatants were harvested and tested for IFN-γ and IL-4 content using cytokine-specific ELISA kits. As shown in Fig. 1, there was little difference in the cytokine secretion patterns in the splenectomized vs the control animals for either neonatal or adult animals; animals initially immunized as neonates still produced less IFN-γ and more IL-4 than did adults. This Th2 bias observed in vitro appeared to reflect the in vivo availability of cytokines since secondary serum antihapten Ab responses were skewed to the Th2-associated IgG1 isotype in splenectomized mice (data not shown), as previously seen in nonsplenectomized neonates (15). Therefore, even in the absence of the neonatal spleen, Th2-skewed memory responses ensue. From these experiments, we conclude that the exclusive Th2 primary responses in the neonatal spleen are not required for the development of Th2-dominant memory in neonates.

Prolongation of primary Th2 effector function in neonatal lymph nodes and spleen

The splenectomy experiments indicated that the generation of dominant Th2 memory in neonates was likely regulated by an event(s) downstream of primary effector development. We next decided to compare the primary effector phase in neonates and adults. In one from an elegant series of in vivo studies, Swain and colleagues (22) earlier described the kinetics of Th1/Th2 primary effector development in adult lymph nodes. Following a single immunization with KLH, both Th1 and Th2 primary effectors developed rapidly, peaking in cytokine secretion activity 5–7 days after immunization. The production of both IFN-γ and IL-4 declined rapidly thereafter and was evident only at low levels by 2 wk after immunization. To examine similarly the primary effector phase in our system, neonates and adults were immunized with KLH in PBS and cytokine production by lymph node cells restimulated with KLH in culture was analyzed 1 and 2 wk after immunization (Fig. 2). Between 1 and 2 wk after immunization, IFN-γ secretion declined modestly in both neonatal and adult lymph node cell cultures. Thus, neonatal and adult Th1 effector function showed similar changes with time following immunization. A different picture emerged when IL-4 production was examined. As was previously observed by Swain and colleagues (22), IL-4 production by adult lymph node cells was virtually undetectable by 2 wk after immunization. In contrast, neonatal lymph node cells continued to produce high levels of IL-4, as late as 2 wk following a single immunization. Thus, unlike adults, neonates appear to retain substantial Th2 primary effector function in the lymph nodes for at least 2 wk after immunization.

To determine how long neonatal Th2 primary effector function lasted as well as to examine the kinetics of the effector phase in the neonatal spleen, similar analyses were performed out to 5 wk after immunization using both lymph node and spleen (Fig. 3). Moderate changes in IFN-γ production were observed among 1, 2, and 5 wk after immunization in neonatal and adult lymph nodes and in adult spleen. Neonatal spleens showed a large increase in IFN-γ production between 1 and 5 wk, likely due to the greatly increased numbers of T cells accumulating over the intervening period of development (Ref. 23; B. Adkins, unpublished observation). Nonetheless, the corresponding lymphoid organs in neonates and adults showed similar levels of IFN-γ production at the end of the 5-wk period. As previously seen (Fig. 2), IL-4 secretion was undetectable as early as 2 wk after immunization in adult lymph nodes, and a similar drastic reduction was seen in the adult spleen. In neonatal lymph nodes, IL-4 production decreased 5- to 10-fold by 5 wk after immunization but was nonetheless readily detectable. As observed for IFN-γ production, IL-4 secretion in the neonatal spleen increased markedly by 5 wk after immunization but was nonetheless readily detectable. These experiments had all been performed using total spleen or lymph node cells, APC function was provided by the
The experiment was performed twice.

The prolonged Th2 effector function seen among KLH was undetectable; spontaneous IFN-γ production was assessed by ELISA. Spontaneous IL-4 production in the absence of antigen (Ag) was measured, as described for Fig. 1. The IFN-γ production of neonatal and adult mice was even more dramatic among CD4+ cells than among total neonatal lymph node and spleen cells. Total neonatal lymph node and spleen cells produced 10- to 600-fold (lymph node) more IL-4 than did adult CD4+ cells. Therefore, the retention of the capacity for high-level primary Th2 function in neonates is not dependent on the presence of neonatal APC in culture.

**Prolonged Th2 primary effector function in neonates is not related to Ag dose, specific types of carrier, or the continual presence of the thymus**

The dissimilarities in the duration of primary Th2 activity in neonates and adults might result from differential Ag clearance. For example, if neonates clear Ag less efficiently than do adults, the persistence of Ag may result in the continual recruitment of new Th2 primary effectors. Similarly, if adults efficiently eliminate Ag, new Th2 cells could not continue to be enlisted. We have tested this idea using three approaches. First, we have immunized neonates with 10-fold less KLH to reduce the amount of Ag that is present late during the primary response phase. We have also immunized adults with 10-fold more KLH to provide the potential opportunity for late-phase recruitment of new Th2 cells. Immunizing neonates with 10-fold less or adults with 10-fold more KLH resulted in minor differences in IFN-γ production 1 or 2 wk later, in either the lymph node or spleen (Fig. 5). A similar major decline in IL-4 production was observed in both the lymph nodes and spleens of adults immunized with either the standard amount, 100 μg, of KLH or with 10-fold more. Immunization of neonates with 10-fold less KLH did not reduce the levels of IL-4 secreted at 2 wk and, indeed, resulted in a major increase from 1 to 2 wk, especially in the lymph nodes. Thus, it seems unlikely that large differences in effective Ag dose in vivo account for the retention of prolonged Th2 function in neonates and the corresponding loss of this function in adults.

The second approach we have taken is to prolong the presence of Ag in adults by introducing the Ag in adjuvant. Neonates or adults were immunized with the same Ag, KLH, precipitated with aluminum potassium sulfate (alum). One and 2 wk later, spleen and lymph node cells were restimulated with KLH in vitro and the cytokines produced were analyzed (Fig. 6). As we had seen for immunization with KLH in PBS, neonates and adults showed similar patterns of IFN-γ production. In the lymph nodes, IFN-γ decreased modestly between 1 and 2 wk, whereas in the spleen, IFN-γ increased modestly. Contrasting patterns were observed between neonates and adults for IL-4 production. Between 1 and 2 wk of immunization in adults, IL-4 production decreased ≥5-fold. On the other hand, IL-4 production increased in both the lymph nodes and spleens of neonates. Therefore, the use of adjuvant does not obviously prevent the decline in primary Th2 function in adults nor does it interfere with the prolonged retention of this activity in neonates.

The rates of migration of newly generated cells from the thymus to peripheral organs appear to be similar in neonates and adults (24). However, the relative proportions of recent thymic emigrants, compared with resident cells, are much higher in neonates than in adults. Thus, in the third approach, we tested the possibility that, in neonates, prolonged Th2 primary function resulted from the continued recruitment of new thymic emigrants into the response. Two groups of 1-day-old neonates were immunized with KLH. One week later, animals in one group were thymectomized while animals in the other group were kept as unmanipulated controls. One additional wk later (i.e., 2 wk after the initial immunization), CD4+ lymph node and spleen cells were restimulated with KLH in the presence of adult splenic APC and supernatants were harvested for ELISA (Fig. 7). IFN-γ production was increased 2- to 4-fold in

**FIGURE 4.** Prolonged primary neonatal Th2 effector function is independent of in vitro neonatal APC function. Neonatal and adult mice were immunized as described for Fig. 1. Two weeks later, portions of the total lymph node (LN) or spleen (SP) cell suspensions were cultured (5 × 10^3 well) with or without 50 μg of KLH. CD4+ cells were prepared from the remaining cells and cultured with adult splenic APC with or without KLH as described for Fig. 1. The IFN-γ and IL-4 present in 72-h supernatants were assayed by ELISA. Spontaneous IL-4 production in the absence of KLH was undetectable; spontaneous IFN-γ levels were ≤5.0 × 10^3 pg/ml. The experiment was performed twice.
the lymph nodes and spleens of thymectomized, as compared with control, neonates. Similarly, IL-4 production increased ~4 fold in the lymph nodes of thymectomized vs control neonates while IL-4 production was similar in the spleens of the two groups. Therefore, removal of the thymus 1 wk after primary immunization did not result in a reduction, and actually resulted in an increase in the lymph nodes, in prolonged Th2 primary function.

Major differences in cytokine secretion but relatively minor differences in the frequencies of late-stage primary Th2 effectors in neonates and adults

The large differences in IL-4 secretion seen in bulk cultures of adult and neonatal cells at late times following primary immunization could result in one of at least two possible ways. First, neonatal tissues may contain many more IL-4-secreting cells compared with adult tissues. Second, neonatal and adult Th2 cells may be present at similar frequencies but neonatal cells may be capable of greater IL-4 production per cell. To distinguish between these possibilities, the frequencies of IFN-γ- and IL-4-producing cells were measured by ELISPOT analyses 1 and 5 wk after immunization (Fig. 8). In the lymph nodes, neonates developed 2- to 3-fold more of both IFN-γ- and IL-4-secreting cells than did adults 1 wk after immunization. As we have previously reported (19), the neonatal spleen contained very few IFN-γ-secreting cells but mature levels of IL-4-producing cells 1 wk after immunization. With the exception of the large increase in the neonatal spleen, the frequencies of IFN-γ-producing cells showed modest changes from 1 to 5 wk after immunization in both neonates and adults. The frequencies of IL-4-producing cells declined in the lymph nodes of both neonates and adults between 1 and 5 wk of immunization. Surprisingly, the fold decline was not very different between the

FIGURE 6. Immunization with alum-precipitated Ag also results in persistent Th2 primary function in neonates. Neonates and adults were immunized with alum-precipitated KLH, as described in Materials and Methods. One and 2 wk later, cytokine production by total lymph node (LN) or spleen (SP) cells was measured by ELISA. Background IL-4 production in the absence of KLH was undetectable in all cases; background IFN-γ levels were also undetectable in the absence of KLH. The experiment was performed twice.

FIGURE 7. Thymectomy at 1 wk does not diminish the prolonged primary Th2 function in neonates. Neonates were immunized with KLH in PBS. One week later, one-half of the animals were thymectomized, as described in Materials and Methods. The other one-half of the animals were kept as unmanipulated controls. An additional week later, CD4+ cells were prepared from spleens (SP) and lymph node (LN) and restimulated with adult splenic APC and KLH. IFN-γ and IL-4 content in 72-h supernatants was assessed by ELISA. Background levels of either cytokine, produced in the absence of KLH, were undetectable. One experiment representative of two independent experiments is shown.
two, a decrease of 2- to 3-fold for neonates and 3- to 4-fold for adults between 1 and 5 wk. In the spleen, neonates showed a modest increase in the frequency of IL-4-secreting cells while the frequency declined in adult spleens. However, the decline was modest, with close to 1000 IL-4-secreting cells/10^6 total cells still remaining in adult spleens 5 wk following immunization. Since total IL-4 production in bulk cultures is low to undetectable by this time point in both adult lymph node and spleens, primary adult Th2 cells may lose robust function while neonatal Th2 cells efficiently retain it.

Poor development of Th1 memory effector function in animals initially immunized as neonates

The prolongation of primary Th2 effector function in neonates could have a significant impact on the generation of memory effectors. Bradley et al. (25) have shown that IL-4 can drive the preferential development of adult Th2 memory effector cells. Accordingly, the increased IL-4 produced by long-lived Th2 primary effector function in neonates may serve to skew the development of memory effectors to the Th2 lineage. To compare memory effector development in neonatal and adult animals, the mice were immunized with KLH in PBS and then reimmunized 5 wk later. Th1/Th2 cytokine production was then assessed at different intervals following reimmunization (Fig. 9). The development of IL-4-secreting memory effector function in adults was very similar to that previously described by Bradley et al. (26). In both lymph nodes and spleen, IL-4 production appeared rapidly, peaking 3 days after reimmunization. Neonates showed similar rapid increases in IL-4 production although maximal production was observed as early as day 2 after reimmunization. There was also a rapid increase in IFN-γ production in adult lymph node and spleen. In striking contrast, there was no increase in IFN-γ production at any time point analyzed in either the neonatal lymph nodes or spleen. Similar results were obtained using CD4+ cell populations (data not shown). Thus, in addition to the prolongation of primary

![FIGURE 8. Relatively small differences between adult and neonatal tissues in the frequencies of IL-4-secreting cells at late times following a single immunization. Neonates and adults were immunized with KLH in PBS, as described for Fig. 1. At 1 and 5 wk after immunization, lymph node (LN) and spleen (SP) cells were prepared, restimulated with KLH, and processed by specific ELISPOT, as described in Materials and Methods. Each bar represents the average ± SD obtained from four replicate cultures. Secretors for each cytokine are normalized to the number per 10^6 total cells. The frequencies of spontaneous cytokine-secreting cells from unimmunized, age-matched control mice did not exceed 7% of the values obtained from immunized mice in all cases. One experiment representative of three individual experiments is shown.](image)

![FIGURE 9. Poor generation of Th1 memory effector function in animals initially immunized as neonates. Neonates and adults were immunized with KLH in PBS. Five weeks later, lymph node (LN) and spleen (SP) cells from a subset of the animals were restimulated in culture. The remaining animals were reimmunized and spleen and lymph nodes were prepared at the indicated times following reimmunization. Cytokine content in 72-h culture supernatants was analyzed by ELISA. Spontaneous cytokine production in the absence of KLH was as follows: neonatal or adult lymph node IL-4, ≤20 pg/ml; neonatal or adult spleen IL-4, ≤60 pg/ml; neonatal or adult lymph node IFN-γ, not detectable; neonatal spleen IFN-γ, ≤8 x 10^3 pg/ml; adult spleen IFN-γ, ≤15 x 10^3 pg/ml. The experiment was performed twice.](image)

![Th2 function in neonates, there appears to be the selective impairment in the development of Th1 memory effector function. Based on our analyses of the kinetics of the primary response phase in neonates, we postulated that the failure to generate Th1 memory effector function was due to a failure to develop memory effector cells. In particular, we reasoned that the IL-4 production by “long-lived” primary effectors, in combination with the IL-4 produced early in a memory response, was driving the overwhelming development of Th2 memory effectors and, at the same time, inhibiting Th1 memory effector development in neonates. To test this idea, memory effectors in neonates and adults were enumerated using the ELISPOT technique (Fig. 10). Two groups each of neonates and adults were immunized with KLH. Four weeks later, one group of each was reimmunized with KLH; the other group of each was not reimmunized. Three days later, lymph node and spleen cells from all four groups were restimulated with KLH and subsequently processed for ELISPOT, as described in Materials and Methods. For both adults and neonates, the frequencies of cells secreting IL-4 increased ≥10-fold following reimmunization. The frequency of IFN-γ-secreting cells also increased ~2-fold in adult lymph nodes and spleens. As we had expected, there was no increase in the frequency of IFN-γ-secreting neonatal spleen cells following reimmunization. However, the frequency of IFN-γ secretors did increase in neonatal lymph nodes and, surprisingly, the increase was at least as great as that seen in adult lymph nodes. Thus, in the lymph nodes, neonates developed Th1 memory effectors in frequencies similar to those that developed in adult lymph nodes. Since total IFN-γ secretion as assessed by ELISA did not increase (Fig. 9), these results imply that neonatal lymph node Th1 memory effectors may produce less IFN-γ per cell than do adult Th1 memory effectors.

Discussion

In many experimental settings, neonates appear to be biased to Th2 lineage function (reviewed in Ref. 27). To try to understand how this occurs, we have examined all phases of Ag-specific Th responses developing in vivo in neonates. First, we had previously found that naive T cells in neonatal lymph nodes develop mature,
Ag-specific Th1/Th2 primary effector responses, whereas those in the spleens of the same animals are exclusively Th2 (19). Here, we have addressed the possibility that the Th2-dominant primary responses in the neonatal spleen are responsible for Th2-skewed memory responses. Experiments using splenectomized neonates and adults indicate that the primary Th2 skewing in the neonatal spleen is not required to achieve Th2-dominant memory responses in the neonatal lymph nodes. Thus, although the exclusive Th2 responses in the neonatal spleen are almost certainly important during the neonatal period of life, they may have little influence on responses in later life. Second, we have described the prolonged appearance of primary Th2 effector function in both the lymph nodes and spleens of neonates. Although adult Th2 activity declines dramatically as early as 2 wk after a single immunization, neonates retain the capacity for high-level Th2 function as late as 5 wk after immunization. Finally, we have demonstrated that animals initially immunized as neonates are impaired in the capacity to generate Th1 memory effector function following reimmunization. Altogether, these results demonstrate that neonates are prone to Th2 lineage function at multiple stages of an immune response (Fig. 11).

In neonatal lymph nodes and spleens, there is a prolongation in the primary Ag-specific production of both IFN-γ and IL-4. In our system, adult animals also show somewhat prolonged IFN-γ production. What sets neonates apart from adults is the extended period of time (≥5 wk) over which high-level IL-4 production remains evident. Why this occurs is currently unclear. In the adult, it is known that shortly after the peak of a primary response, at 5–7 days, the vast majority of Ag-specific cells either leave the lymph nodes or die in situ (reviewed in Refs. 28 and 29). This may not occur in neonates, i.e., neonatal Th2 cells may either fail to exit the nodes or fail to undergo apoptosis. If this were the case, the frequencies of IL-4-secreting cells in neonates would be expected to be much higher than in adults late following primary immunization. However, as late as 5 wk after a single immunization, the frequencies of IL-4-secreting cells in adults were only ~3-fold lower than the frequencies in the corresponding neonatal tissues. This sharply contrasts with total IL-4 production which, at this late time after primary immunization, is ~100 times lower in adult than in neonatal tissues. One important question that arises is, is the extended Th2 function in neonates due to the retention of robust primary function or to the continual recruitment of new effectors by residual Ag? We have tried to address this possibility by decreasing the amount of Ag used to immunize neonates. The rationale is that this approach may decrease the amount of Ag present at later times to below the threshold level for the activation of naive cells; the result would be greatly reduced Th2 function. However, immunizing neonates with 10-fold less Ag did not alter the late-phase retention of Th2 primary function. Moreover, thymectomy at 1 wk of life to eliminate any ongoing recruitment of new thymic emigrants into the response did not diminish the prolonged primary Th2 activity in neonates. This finding argues that the extended primary Th2 function in neonates is largely not due to the persistent activation of new naive T cells. Together, these data suggest that prolonged primary Th2 function in neonates is achieved, at least in part, by the retention of the capacity for high-level IL-4 production by individual Th2 cells.

Animals initially immunized as neonates mounted robust Th2 memory effector responses. However, as assessed by total IFN-γ levels measured by ELISA, there was essentially no development of Th1 memory effector function. In the spleen, this appears to be mainly due to the failure to develop Th1 memory effectors since there was no increase in the frequency of IFN-γ-secreting cells following reimmunization. In contrast, IFN-γ-secreting memory effectors did develop in neonatal lymph nodes and their frequencies were similar to those developing in adult lymph nodes. However, since the levels of IFN-γ produced in bulk cultures by neonatal lymph node cells were ≥5-fold less than those produced by adult lymph node cells, neonatal lymph node Th1 memory cells may secrete less IFN-γ per cell as compared with adult Th1 memory cells. Thus, animals initially immunized as neonates show regionally distinct patterns of attenuation of Th1 memory responses: in the spleen, Th1 memory effectors fail to develop; in the lymph nodes, these cells develop but show limited function. Regionally discrete
responses have been previously reported for neonates. First, we demonstrated that neonatal primary effector responses are mature, mixed Th1/Th2-like in the lymph nodes but exclusively Th2-like in the spleens (19). Second, Zaghouani and colleagues (16–18) have reported different regional responses in adult animals originallytoleranced as neonates to peptide Ag. In their case, Ag-specific responses in the lymph nodes were Th2-polarized while Ag-specific splenic T cells were anergized. Together, these results indicate that the spleen and lymph nodes of neonates are functionally distinct sites and the influence of the different environments can persist well into adult life.

One interesting observation is that the exclusive primary Th2 function in the neonatal spleen does not appear to be required to develop Th2-biased memory responses in the lymph nodes. Experiments using splenectomized neonates showed no major changes in Th1/Th2 cytokines produced in a memory response in neonatal lymph nodes. These results indicate that intrinsic properties of the lymph node T cells and/or the neonatal lymph node environment are sufficient to generate dominant Th2 memory responses. One straightforward way to test this idea is to separate the T cells from the environment by transfers of neonatal T cells to adoptive adult hosts and vice versa. Determining the relative contributions of T cell intrinsic properties and environmental influences is a major goal of the laboratory. Although primary splenic responses may not have a major effect on later memory responses, the clear Th2 bias in neonates is likely to be important during the neonatal period. Based on the regional responses in neonates, we have developed a working hypothesis to explain the differential responses: during early neonatal life, two opposing but necessary processes are occurring at frequencies that are almost certainly higher than in adult life. First, the vast majority of T cells are newly generated and are encountering many peripheral Ags not present in the thymus. Tolerance to these self-Ags must first be established during the neonatal period. Second, the neonate has no immunological experience and must, therefore, establish productive responses against a wealth of newly encountered non-self-Ags. We propose that, in the neonate, the spleen is the primary site of tolerance induction to self-Ags in the periphery whereas the lymph nodes are the site of immune responsiveness to foreign Ags. There are a number of specific predictions from this hypothesis, such as neonatal splenectomy should result in an increased incidence of early onset autoimmune disease. Using this and related approaches, we are currently trying to determine the relative roles of splenic vs lymph node responses in neonates.

References