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Thymic and extrathymic contributions to T helper cell function in murine neonates

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A B S T R A C T

Murine neonatal CD4+ responses are often biased to Th2 function. There is increasing evidence that this phenomenon may be regulated both at the level of the thymus and the peripheral lymphoid compartment. In particular, residual fetal influence on the neonatal thymus may lead to an imprinting of developing T cells that is maintained in CD4+ cells when they emigrate to peripheral organs. Such imprinting may involve epigenetic modification of the Th2 cytokine gene locus and acquisition of the capacity to undergo rapid cell cycling. These properties, coupled with the homeostatic proliferation occurring in the peripheral tissues of neonates, shape a CD4+ population with the capacity for enhanced Th2 responsiveness.

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Neonatal T helper cell responses

Over the past decade, it has become clear that murine neonates are competent to develop mature CD4-mediated inflammatory responses, leading to adult-level Th1 and CTL function.[1] However, these mature responses are generally only achieved under very potent Th1-driving conditions. What is more often seen in murine neonates is a deviance to Th2 function.[2-4] Notably, mice initially immunized as neonates mount Th2-dominant memory responses when re-exposed to the same antigens as adults. Moreover, the primary antigen-specific responses that develop *in vivo* during neonatal life are Th2-skewed.[5] This phenomenon is apparently widespread since it has been observed in neonatal mice of both the BALB/c and C57BL/6 strains and with a variety of different antigens under conventional immunization conditions. Furthermore, a bias to Th2 function is also evident in vitro since freshly isolated neonatal, but not adult, T cells rapidly produce high levels of the Th2 cytokine IL-4 upon polyclonal stimulation in culture.[6] Interestingly, the in utero exposure of human fetuses to common environmental antigens also results in universally Th2-biased responses.[7-9] Thus, murine neonatal responses may be considered an excellent model for understanding human Th2 pathologies that begin in early life, such as asthma and allergy.

While the observation of a neonatal Th2 bias is well established, the mechanisms underlying the phenomenon are less clear. This is due, in part, to the complex nature of regulatory events during ontogeny. We propose that the robust Th2 responses of murine neonates are governed by processes occurring both within the thymus and post-thymically. Emerging evidence in support of this idea is summarized in the sections to follow.

Cell cycling and the acquisition of Th2 function in neonatal CD4+ T cells

For adult CD4+ T cells, the acquisition of high level Th2 function is linked to cell division. Reiner and colleagues[10] originally made the observation that IL-4 production occurs at increased frequencies in cells that have undergone at least three cell divisions. The rapid production of IL-4 by neonatal T cells in vitro prompted us to propose that neonatal T cells may make high levels of IL-4 early because they divide more rapidly than adult cells. Experiments to test this idea revealed that, as a population, CD4+ lymph node cells from neonates showed earlier cell cycle entry and cell division, compared with adult peripheral CD4+ cells.[11] This was observed for polyclonal or antigen-specific stimulation through the TCR and with TCR-independent activation. Interestingly, this characteristic appeared to be *inherited* from thymic precursors since CD4 single-positive cells in the neonatal thymus also showed more rapid cycle entry, compared with CD4 single-positive adult thymocytes. This capacity for rapid division, possibly conferred within the neonatal thymus, may contribute to the ability of neonatal CD4+ cells to rapidly acquire high-level Th2 function.

Epigenetic modifications and Th2 function in neonatal CD4+ cells

In both humans and mice, the Th2 cytokine locus contains the Il5, Il13, and Il4 genes. In naïve adult CD4+ T cells, this locus exists in a silent state characterized by condensed chromatin and locus-wide hypermethylation of CpG dinucleotide residues.[12-15] Activation under Th2-polarizing conditions leads to epigenetic remodeling of the locus including the appearance of DNase I hypersensitivity sites,[16-19] histone modifications,[20,21] and extensive DNA demethylation.[17,18,22,23] During T helper cell differentiation, these epigenetic modifications may be critical to achieve a permissive state at the Th2 locus, allowing Th2 cytokine gene expression.[24] In particular, there is compelling evidence that the transition from DNA hyper- to hypomethylation plays an important role in the development of Th2 effectors producing large amounts of Th2 cytokines. Many of the DNA demethylation events occur at important regulatory regions within the Th2 locus. One of these regions, termed conserved non-coding sequence 1 (CNS-1), acts as an enhancer and coordinate regulator of Th2 cytokine gene expression.[25] This region is highly methylated at CpG residues in naïve adult CD4+ cells. CNS-1 demethylation occurs over a period of many days to weeks of Th2 differentiation, coinciding with the development of the capacity for high level Th2 cytokine expression.[23]

The rapid production of high levels of IL-4 by neonatal CD4+ cells led us to postulate that, unlike in adults, the Th2 cytokine locus may pre-exist in a relatively permissive state in some neonatal T cells. To test this idea, the genomic bisulfite sequencing technique was used to analyze the extent of DNA methylation at several important regulatory regions within the Th2 cytokine locus. To date, CNS-1 is the only examined regulatory region to show differential methylation patterns between neonatal and adult lymph node CD4+ cells (manuscript submitted). At CNS-1, we found extensive demethylation of CpG residues in unactivated naïve neonatal lymph node CD4+ cells. This contrasts sharply with naïve adult CD4+ cells, in which the CNS-1 region is highly methylated.[26] Strikingly, the demethylated state observed in neonates preceded emigration to the periphery since the CNS-1 region of CD4 single positive neonatal thymocytes was also relatively demethylated (manuscript submitted). Thus, in addition to the capacity to cycle faster, neonatal thymocytes and their immediate descendants, naïve peripheral CD4+ T cells, appear to be epigenetically "poised" for robust Th2 differentiation and function.

Neonatal CD4+ cell function: the fetal connection

During murine ontogeny, the fetal thymus is first seeded by a wave of hematopoietic precursors between embryonic days 12-14.[27-29] These precursors proliferate and differentiate throughout the rest of fetal life. Near birth, ~19-21 days of gestation in inbred mice, there is a second major colonization by hematopoietic cells.[27,30] These two precursor entries are referred to as the fetal and adult waves, respectively. Following their entry, the adult precursors also proliferate and differentiate and are thought to eventually completely replace cells from the fetal wave. In postnatal life, the processes of expansion and maturation within the thymus are estimated to take approximately two weeks.[31] Therefore, the T cells present in the peripheral lymphoid organs in early life are mostly, if not entirely, derived from the fetal wave of thymopoiesis.

How the fetal versus the adult waves of thymopoiesis contribute to the development of the immune system is a fascinating area. The distinct nature of these two waves has been clearly demonstrated in genetically manipulated mice: some genes appear to be more critical during fetal than during adult thymopoiesis while the converse is true for other genes.[32-35] In addition, we have demonstrated that there are major phenotypic and functional differences between mature, peripheral CD4+ cells of fetal and adult origin.[36-38] Notably, fetal-derived CD4+ cells develop much greater antigen-specific Th2 function *in vivo* than do adult-derived cells, leading to Th2-skewed responses at low to intermediate antigen concentrations. In addition, like neonatal CD4+ cells, the CD4+ progeny of fetal precursors also cycle more rapidly than cells derived from adult precursors.[38] Thus, the unique immune responses of neonatal life may be due, at least in part, to the fetal origin of the majority of the cells.

Homeostatic proliferation and the neonatal Th2 bias

Due to the small numbers of immune cells in murine neonates, the peripheral lymphoid environment can be characterized as lymphopenic. Indeed, recent studies[39,40] have shown that both CD4+ and CD8+ adult T cells proliferate spontaneously (i.e., undergo homeostatic proliferation) and acquire Th1/Tc1 effector function when transferred to 1-day-old neonatal mice. This spontaneous cycling was associated with the upregulation of the surface molecule CD44, a marker of previous antigen exposure and proliferation. Le Campion *et al.*[41] demonstrated directly that a small fraction of endogenous neonatal T cells in mice are actively proliferating in vivo at any one time during the first week of life. This observation is supported by additional findings in humans[42] and mice[43] which showed that proportionally more freshly isolated neonatal T cells are cycling spontaneously, relative to adult T cells. Togeth-

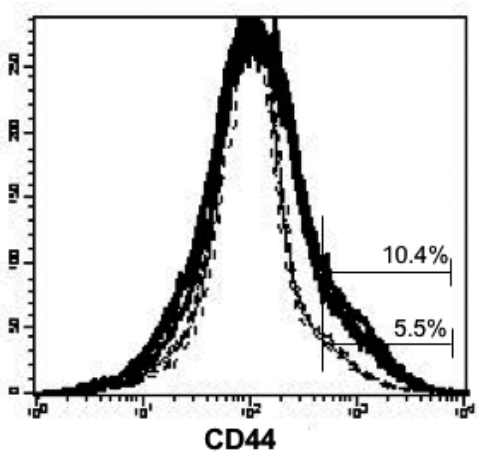

Figure 1. A greater proportion of neonatal CD4$^+$ cells are CD44^{hi}, compared with adult CD4$^+$ cells. Freshly isolated lymph node cells from six individual 7-day-old neonatal or 8-week-old adult BALB/c mice were stained with anti-CD4 and anti-CD44 antibodies. The neonatal mice were each taken from different litters. CD44 staining on gated CD4$^+$ cells is depicted. Tracings from individual neonates are overlaid with the bold lines; tracings from individual adults are overlaid with the dashed lines.

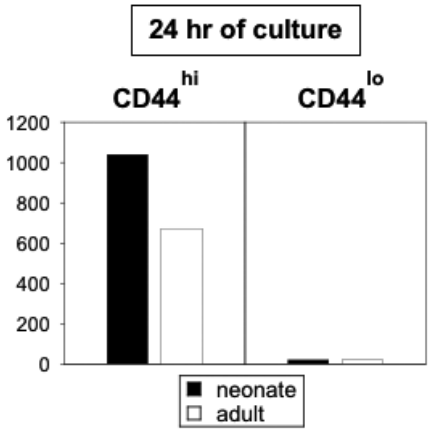

Figure 2. Very rapid IL-4 production by neonatal CD4$^+$ cells is confined to the population that has undergone homeostatic proliferation. CD44^{hi} and CD44^{lo} CD4$^+$ cells from day 7 neonatal and adult BALB/c lymph nodes were purified by cell sorting. The cells were then activated with plate bound anti-CD3 (0.5 µg/well) and anti-CD28 (0.5 µg/mL) for 24 hr. Supernatants were harvested and the IL-4 content was measured by specific ELISA.

er, these observations lead to the hypothesis that the homeostatic proliferation of endogenous neonatal T cells may be accompanied by the development of effector function.

To better understand how homeostatic proliferation might influence T helper cell function, we examined 7-day-old neonatal lymph nodes to determine the proportions of CD4$^+$ cells which had undergone proliferation during the first week of life. Freshly explanted neonatal and adult lymph node CD4$^+$ cells were stained for the expression of CD44 (Figure 1). The proportions of CD4$^+$ cells that were CD44^{hi} in either neonates or adults were remarkably similar among individual animals, such that the expression curves were nearly identical from one animal to the next within each age group. This was observed among neonatal animals even when individual pups from different litters were assessed, suggesting that the process of homeostatic proliferation in the neonate is actually a stringently regulated process. However, there were clear differences in expression of CD44 between neonates and adults – approximately twice as many CD4$^+$ cells in the neonate were CD44^{hi}, relative to adult cells.

To determine how previous proliferation in the neonate might influence cytokine expression, neonatal and adult CD4$^+$ cells were separated by cell sorting into CD44^{hi} and CD44^{lo} populations and activated with anti-CD3 antibody *in vitro*. Since we have previously seen high levels of IL-4 production by total neonatal CD4+ cells as early as 24 hr after activation (manuscript submitted), we harvested supernatants and assessed the IL-4 content at both 24 and 48 hr of activation. Interestingly, only the CD44^{hi} subset of both the neonatal and adult CD4$^+$ populations produced IL-4 within 24 hr of activation (Figure 2). However, within the naïve CD44^{lo} populations, a clear difference between neonatal and adult cells became apparent by 48 hr of activation (Figure 3). At this time point, neonatal CD44^{lo} cells produced copious amounts of IL-4 while IL-4 production by adult CD44^{lo} cells was undetectable.

Although both neonatal and adult CD44^{hi} cells made IL-4 rapidly, the development of this capacity may occur via different mechanisms in neonatal and adult life. In adults, a very small percentage of cells are proliferating spontaneously *in situ*.[41] Thus, in the adult, high levels of CD44 expression are probably primarily due to the presence of memory cells generated at earlier times in the life of the animal, in response to exogenous antigen. This memory-like CD44^{hi} adult population is capable of very rapid IL-4 production. In contrast, the high levels of CD44 expression on 7-day-old neonatal CD4$^+$ cells are probably acquired as the cells undergo homeostatic proliferation to endogenous antigens occurring throughout the first week of life. During this proliferation, the neonatal cells apparently develop into cells with Th2 effector-like function. Since IL-4 production at 24 hr was confined to the CD44^{hi} subset, all of the very rapid *in vitro* Th2 cytokine production by neonatal cells may result from previ-

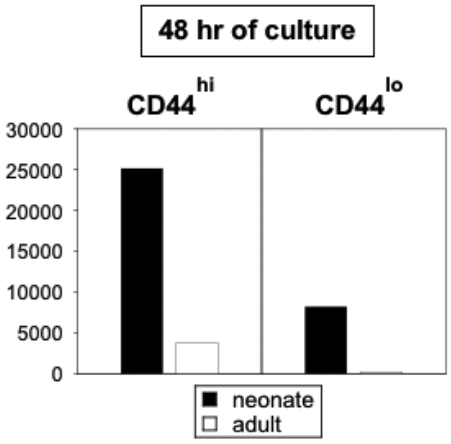

Figure 3. CD44^{lo} naïve neonatal CD4^{+} cells acquire Th2 effector function more rapidly than naive adult CD4^{+} cells. CD44^{hi} and CD44^{lo} CD4^{+} cells from day 7 neonatal and adult BALB/c lymph nodes were purified and activated as described for Figure 2, except that supernatants were collected and analyzed after 48 hr of culture.

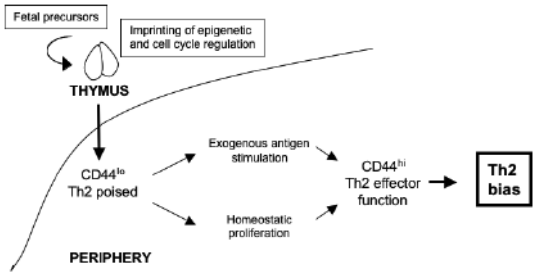

Figure 4. Model of the processes contributing to the enhanced Th2 function of murine neonates. Fetal precursor activity and perhaps the fetal/neonatal thymic environment contribute to the production of mature CD4^{+} thymocytes that cycle rapidly and contain epigenetic modifications that poise them for the rapid development of Th2 function. Upon exiting to the peripheral tissues, a portion of these cells will undergo homeostatic proliferation. This process appears to lead to the development of Th2 effector-like cells, competent to secrete Th2 cytokines very rapidly upon activation. Despite their immunologically naïve state, the remaining CD4^{+} cells are capable of rapidly acquiring robust Th2 function. Together, these two cell populations may make important contributions to the strong Th2 responses of murine neonates.

ously activated cells that have undergone homeostatic proliferation and developed into Th2 effectors. However, homeostatic proliferation is not absolutely required for robust Th2 function, since naïve neonatal CD4^{+} cells developed Th2 function more rapidly than naïve adult cells. Therefore, the Th2-biased state of neonates *in vivo* may be contributed to both by cells which have undergone homeostatic proliferation and by naïve cells.

Summary and model of the processes contributing to robust Th2 function in murine neonates

Both thymic and extrathymic events may contribute to the typically Th2-dominant responses of murine neonates (Figure 4). Unlike in adult life, the early neonatal thymus contains cells derived from fetal precursors. These cells have been shown to generate mature progeny with great potential for Th2 development.[38] Within the fetal and/or neonatal thymus, maturing T cells may acquire two characteristics that would support the efficient development of Th2 function. The first is the capacity to undergo more rapid cell cycle progression, compared with adult cells. The second is that, unlike in the adult thymus, the Th2 cytokine locus in neonatal CD4^{+} thymocytes becomes epigenetically poised for rapid Th2 effector-like function. These properties, which may be imprinted within the thymus, may subsequently contribute to the development of T helper function among mature T cells.

Mature thymocytes then leave the thymus and encounter the lymphopenic environment of the neonatal peripheral lymphoid tissues. There, a small portion of the cells undergo homeostatic proliferation and develop into effector cells. There is recent evidence[44] that the neonatal peripheral environment may play a role in selectively promoting the development of Th2 effectors. We propose that cell intrinsic properties, imprinted in the fetal and/or neonatal thymus, also act to efficiently elicit Th2 development during homeostatic proliferation. These same cell intrinsic properties may contribute to the rapid acquisition of Th2 effector development when naïve peripheral CD4+ cells initially encounter exogenous antigen. Thus, Th2 dominance in the neonate may be the product of multiple regulatory events within both the primary and secondary lymphoid tissues.

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