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Murine Neonatal CD4⁺ Cells Are Poised for Rapid Th2 Effector-Like Function

Shawn Rose, Mathias Lichtenheld, Monica R. Foote, and Becky Adkins

Murine neonates typically mount Th2-biased immune responses. This entails a cell-intrinsic component whose molecular basis is unknown. We found that neonatal CD4⁺ T cells are uniquely poised for rapid Th2 function. Within 24 h of activation, neonatal CD4⁺ cells made high levels of IL-4 and IL-13 mRNA and protein. The rapid high-level IL-4 production arose from a small subpopulation of cells, did not require cell cycle entry, and was unaffected by pharmacologic DNA demethylation. CpG methylation analyses in resting neonatal cells revealed pre-existing hypomethylation at a key Th2 cytokine regulatory region, termed conserved noncoding sequence 1 (CNS-1). Robust Th2 function and increased CNS-1 demethylation was a stable property that persisted in neonatal Th2 effectors. The transcription factor STAT6 was not required for CNS-1 demethylation and this state was already established in neonatal CD4 single-positive thymocytes. CNS-1 demethylation levels were much greater in IL-4-expressing CD4 single-positive thymocytes compared with unactivated cells. Together, these results indicate that neonatal CD4⁺ T cells possess distinct qualities that could predispose them toward rapid, effector-like Th2 function.


In murine neonates, Th cell responses to protein Ags are typically Th2 dominant (1). Strong Th2 responsiveness seems to be intrinsic to neonatal cells, as made evident by experiments that have separated neonatal CD4⁺ T cells from the neonatal environment. For instance, unlike adult cells, neonatal T cells produce high levels of the Th2 cytokine IL-4 within 48 h of in vitro activation with adult APCs (2). Moreover, adoptively transferred neonatal CD4⁺ cells demonstrate enhanced Th2 function in vivo, when compared with transferred adult cells (3). Thus, Th2 immune deviation in neonates is increasingly understood at the cellular level, yet the molecular mechanisms responsible for this phenomenon are largely unknown.

It is striking that naive neonatal T cells produce large amounts of IL-4 relatively early after activation, because adult cells generally require several days to weeks of development to express high levels of Th2 cytokines. This suggests that neonatal cells, unlike naive adult cells, are preprogrammed for rapid, robust Th2 function. The acquisition of the capacity for high-level Th2 cytokine expression has been extensively studied in adult CD4⁺ cells. The Th2 cytokine locus contains the Il5, Il13, and Il4 genes arranged in tandem on a roughly 150-kb region of mouse chromosome 11. In naive adult CD4⁺ cells, this locus exists in a transcriptionally silent state characterized by condensed chromatin and locus-wide hypermethylation of CpG residues. Activation under Th2-polarizing conditions leads to subsequent remodeling of the locus and high-level transcription of the Th2 cytokine genes (4). The transition of the Th2 cytokine locus to a fully open, highly transcribed state involves several processes including cellular proliferation (5, 6), the up-regulation of lineage-determining transcription factors (7), the appearance of DNase I hypersensitivity sites (8–11), histone modifications (11–15), and extensive DNA demethylation (10, 11, 14, 16, 17). Although the various processes controlling Th2 differentiation and function have been extensively studied in adult cells, it is not known how naive neonatal CD4⁺ cells achieve such high levels of Th2 function early after activation.

There is ample evidence that DNA methylation is an important regulator of Th2 cytokine expression in adult CD4⁺ cells. Several laboratories have demonstrated that the murine Th2 cytokine locus becomes demethylated as naive adult CD4⁺ cells differentiate into effector cells (6, 11, 14, 16, 17). Demethylation proceeds with different kinetics at the various parts of the locus (6, 11, 14, 16, 17). Some regulatory regions, such as rad50 hypersensitivity site 7 (RHS7) within the newly identified locus control region (LCR),⁴ become almost fully demethylated within several days of Th2 polarization (11). Other regulatory regions, such as conserved noncoding sequence 1 (CNS-1), are only partially demethylated within the same time frame (16). In contrast, nonregulatory regions within the Il4 gene require weeks of Th2 polarization before complete demethylation is achieved (16). The clear importance of DNA methylation as a nonredundant repressor of Th2 cytokine expression has recently been shown in adult mice deficient for the maintenance DNA methyltransferase Dnmt1. In these mice, Il4, Il13, and Il5 expression is markedly enhanced (18).

In this report, we focused on the prototypical Th2 cytokines IL-4 and IL-13 to understand the molecular regulation of Th2 cytokine expression in neonatal CD4⁺ T cells. We found that recently activated neonatal CD4⁺ cells expressed higher levels of Il4 and Il13 mRNA and protein compared with adult cells. Using the bisulfite genomic sequencing technique (19), we discovered that a coordinate regulatory region of Th2 cytokine expression, CNS-1 (20), was uniquely demethylated in resting neonatal CD4⁺ cells. The

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3 Abbreviations used in this paper: LCR, locus control region; CNS-1, conserved noncoding sequence 1; SP, single positive; CS-1, consensus sequence 1; RHS, rad50 hypersensitivity site; LIP, lymphopenia-induced proliferation.

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transcription factor STAT6 was dispensable for CNS-1 demethylation and this state was already established in neonatal CD4 single-positive (SP) thymocytes. IL-4–expressing neonatal thymocytes exhibited substantially greater levels of CNS-1 demethylation compared with unactivated cells. Our data suggests that the relatively hypomethylated state of CNS-1 in neonatal CD4+ cells facilitates their rapid, high-level expression of the If4 and Il13 genes. Thus, the developmental regulation of epigenetic patterns at the Th2 cytokine locus may serve to maintain an anti-inflammatory state in early life.

Materials and Methods

Mice

Wild-type, STAT6-deficient, and DO11.10 TCR-transgenic mice on a BALB/c background were bred and housed in pathogen-free conditions in the Division of Veterinary Resources at the University of Miami Miller School of Medicine. All animal studies were approved by the University of Miami Animal Care and Use Committee. Neonatal animals were 7 days old and adult mice were 6–10 wk old. For the CD4 SP thymocyte experiments, neonates were 2 days old.

Cell culture

Cell suspensions were made from pools of mesenteric, inguinal, axillary, brachial, and cervical lymph nodes. CD4+ cells were positively selected from the cell suspensions on Miltenyi Biotec MACS MS+ columns using the manufacturer’s protocol. Cell purity was routinely >97%. For the DNA methylation studies, neonatal and adult CD4+ cells were stained with anti-CD4 CyChrome (BD Biosciences/BD Pharmingen) and anti-CD44 biotin (BD Pharmingen), followed by PE-coupled streptavidin (Jackson Immunoresearch Laboratories). CD4+CD44hi cells were purified on a Vantage SE cell sorter (BD Biosciences). For the neonatal thymocyte experiments, the cells were stained with anti-CD4 FITC (Caltag Laboratories) and anti-CD8 PE (BD Pharmingen), followed by sorting for CD4 SP thymocytes. A total of 2 × 106 CD4+ cells were activated in 96-well plates with 10 μg/ml precoated anti-CD3e (mAb 1452C11, purified from ascites) and 0.5 μg/ml soluble anti-BCR (mAb 375S1; BD Pharmingen). To measure Ag-specific responses, 2 × 105 CD4+ cells from DO11.10 mice were activated in the presence of 4 × 105 adult splenic APC (APC preparation previously described in Ref. 2) and various concentrations of chicken OVA peptide (amino acids 323–339). Culture medium contained RPMI 1640 (Invitrogen Life Technologies) supplemented with 1 mM sodium pyruvate (Invitrogen Life Technologies) and 10% heat-inactivated (56°C, 30 min) FCS (HyClone).

For the cell cycle arrest or 5-aza-deoxycytidine experiments, resting and activated cells were stained with anti-CD69 biotin (BD Pharmingen) or anti-CD25 biotin (Caltag Laboratories), respectively, followed by FITC-coupled streptavidin (BD Pharmingen). The cells were then processed for propidium iodide (Sigma-Aldrich) staining as previously described (23).

For the intracellular cytokine staining experiments, activated CD4+ cells were washed and cultured for 4 h in medium containing 10 nm PMA, 0.25 μM ionomycin, and 1 μM monensin (eBioscience). The cells were then washed and fixed for 20 min in 250 μl of Cytofix/Cytoperm (BD Pharmingen). Resting cells were fixed concurrently with 250 μl of Cytofix/Cytoperm. After two washes with 1× Perm/Wash buffer (BD Pharmingen), the cells were stained with anti-IL-4 PE (mAb 1B11; BD Pharmingen) and anti-IFN-γ FITC (mAb XMGI1.2; BD Pharmingen) for 30 min. Isotype control staining for IFN-γ was performed in parallel using a FITC-coupled rat IgG1 mAb A110-1 (BD Pharmingen). The specificity of IL-4 staining was determined by incubating the cells with an unlabeled anti-IL-4 mAb 1B11 purified from ascites for 30 min, followed by a PE-coupled anti-IL-4 mAb 1B11 for another 30 min. After staining, the cells were washed twice with 1× Perm/Wash buffer followed by a single wash with staining buffer.

RNA isolation and RT-PCR

Total RNA was isolated using RNeasy Mini kits (Qiagen). A total of 5 μg of total RNA was reverse-transcribed in reactions containing 125 U of Powerscript reverse transcriptase (BD Biosciences/BD Clontech), 1× First Strand buffer (BD Clontech), 10 μg recombinant RNase inhibitor (BD Clontech), 1 mM dNTPs, and 1 μM oligo dT16 primer (Applied Biosystems). The conditions for the reaction were as follows: 10 min at 70°C, 90 min at 42°C, and 15 min at 70°C. For the quantitative RT-PCR assays, cDNA from reactions containing 125–250 ng of input total RNA was amplified via PCR using LightCycler Fast DNA SYBR Green 1 kits (Roche Diagnostics) and a Roche LightCycler in accordance with the manufacturer’s instructions. Primer sequences for Il4, Il13, and Actb are listed below. The cycling parameters for both Il4 and Actb were as follows: 1 cycle at 95°C for 10 min; 36 cycles at 95°C for 10 s, 65°C for 8 s, 72°C for 45 s, and a single acquisition point for each cycle after 10 s at 85°C. For Il13, the cycling parameters were as follows: 1 cycle at 95°C for 10 min; 33 cycles at 95°C for 10 s, 72°C for 15 s, 72°C for 15 s, and a single acquisition point for each cycle after 10 s at 85°C. Standard curves for Il4, Il13, and Actb were generated using serial dilutions of plasmid DNA containing cloned PCR products for each gene. Using this method, the amplification efficiencies were determined to be equivalent for the Il4, Il13, and Actb primers. For each sample, the absolute levels of Il4, Il13, and Actb were extrapolated from the standard curves using Roche LightCycler data analysis software version 3.5. The Il4 or Il13 levels were normalized to those of Actb for individual samples. The specificity of the reactions was determined by melting curve analysis and by agarose gel electrophoresis. Control reactions lacking reverse transcriptase ensured that the products were derived from RNA templates. All reactions were performed in triplicate at the University of Miami/Sylvester Molecular Core Analysis Facility at the University of Miami Miller School of Medicine.

DNA isolation and bisulfite sequencing

Genomic DNA was isolated using QIAamp DNA Mini kits (Qiagen). The DNA was sheared by passage through a 30-gauge needle 20 times followed by denaturation in 0.3 M NaOH for 20 min at 30°C. Sodium bisulfite modification was performed with CpGenome kits (Chemicon International) using the manufacturer’s protocol. Bisulfite-modified DNA was amplified via touchdown PCR in reactions containing 0.625 U of iTaq DNA Polymerase (Bio-Rad), 1× PCR buffer (Bio-Rad), 1.5 mM MgCl2 (Bio-Rad), 0.2 mM dNTPs, and 0.25 μM primers specific for Il4 intron 1-exon 2, the CNS-1 region, the consensus sequence 1 (CS-1) region of the Il13 promoter, or Rhsf7 of the rad50 gene. The cycling parameters were as follows:

**Il4 intron 1-exon 2.** One cycle at 95°C for 5 min; five touchdown cycles decreasing the annealing temperature 2°C per cycle at 95°C for 30 s, 70–64°C for 30 s, and 72°C for 45 s; 35 cycles at 95°C for 30 s, 62°C for 30 s, and 72°C for 45 s; 1 cycle at 72°C for 7 min.

**CNS-1.** One cycle at 95°C for 5 min; five touchdown cycles decreasing the annealing temperature 1°C per cycle at 95°C for 30 s, 70–64°C for 30 s, and 72°C for 45 s; 40 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 45 s; 1 cycle at 72°C for 7 min.

**Il13 CS-1.** One cycle at 95°C for 5 min; five touchdown cycles decreasing the annealing temperature 1°C per cycle at 95°C for 30 s, 70–64°C for 30 s, and 72°C for 45 s; 35 cycles at 95°C for 30 s, 58°C for 30 s, and 72°C for 45 s; 1 cycle at 72°C for 7 min.

**rad50 Rhsf7.** One cycle at 95°C for 5 min; five touchdown cycles decreasing the annealing temperature 2°C per cycle at 95°C for 30 s, 62–54°C...
Bisulfite sequencing.

The nucleotide sequences for Il4 promoter CS-1 oligo 1: 5’-GGTAAGGGTAGTAGATTTTTTGGAG-3’, Il4 promoter CS-1 oligo 2: 5’-AAATTCGTAATTCCTTGAGTAGAAGAA-3’, rad50 RHS7 oligo 1: 5’-AAGTGTGATTGATGAGTAGAGACAGAAG-3’, rad50 RHS7 oligo 2: 5’-CTTACCTTCCCCAATAATAC-3’.

Sorting of Il4-expressing cells

CD4 SP thymocytes from day 3 neonatal DO11.10 mice were enriched by negative selection using Miltenyi CD8 beads followed by passage through Miltenyi MACS MS columns. A total of 4 x 10⁵ cells were activated with 10 µg/ml plate-bound anti-CD3ε mAb and 0.5 µg/ml soluble anti-CD28 mAb in the presence of 10 ng/ml rIL-2. After 2 days, the cells were harvested, washed, and suspended in medium solution containing 10 nM PMA and 0.25 µg/ml soluble anti-CD28 mAb. After 2 h, the thymocytes were stained for IL-4 expression using Miltenyi IL-4 Cell Enrichment and Detection kits in accordance with the manufacturer’s instructions. Following additional staining with anti-CD4 mAb, CD4 Il4⁺ cells were sorted on a BD Biosciences FACSARIA. The cells were then prepared for lysis and DNA
Results

Neonatal CD4+ cells produce high levels of IL-4 and IL-13 early after activation

We have previously observed that freshly activated naive total T cells from neonatal mice produce much greater levels of IL-4 than do adult cells when stimulated with soluble anti-CD3ε and adult APC (2). These findings suggest that enhanced Th2 function is an intrinsic property of neonatal T cells. To study this at the molecular level, we first needed to examine Th2 cytokine expression in purified neonatal CD4+ T cells. Thus, we activated DO11.10 TCR-transgenic neonatal or adult CD4+ cells with plate-bound anti-CD3ε and soluble anti-CD28 mAb. After 24 h, the levels of IL-4 or IL-13 protein in the culture supernatants were measured by ELISA. Compared with adult cells, neonatal CD4+ cells produced greater levels of both IL-4 (4.4- to 16.0-fold higher) and IL-13 (1.2- to 24.9-fold higher) at this early time point (Fig. 1A).

We next tested whether the differences in IL-4 and IL-13 protein production between activated neonatal and adult CD4+ cells were also evident at the mRNA level. Quantitative RT-PCR analysis revealed more abundant Il4 (4.2- to 18.5-fold higher) and Il13 (3.7- to 5.1-fold higher) transcripts in neonatal cells, compared with adult cells, after 24 h of activation (Fig. 1B). The differences in Il4 or Il13 mRNA between activated neonatal and adult cells were within the same range as the differences in IL-4 and IL-13 protein measured in culture supernatants by ELISA (Fig. 1A). Moreover, activation of neonatal cells resulted in a de novo increase in Il4 and Il13 mRNA (Fig. 1C), suggesting that Th2 cytokine expression is transcriptionally regulated in neonatal CD4+ T cells, as is in adult cells (27).

To determine whether Ag-specific Th2 responses were also enhanced in neonatal cells, neonatal and adult CD4+ cells from DO11.10 TCR-transgenic mice were activated with chicken OVA peptide in the presence of adult splenic APC. Two days later, OVA-specific cytokine responses were measured by ELISA. Neonatal CD4+ cells clearly made more IL-4 (range 2.7- to 16.3-fold higher) than did adult cells at all concentrations of OVA peptide tested (Fig. 1D). In contrast, neonatal and adult cells made similar levels of IL-2. These findings indicate that recently activated, naive neonatal CD4+ cells mount greater Ag-specific IL-4 responses, but similar IL-2 responses, compared with adult cells.

Greater frequencies of neonatal than adult CD4+ cells produce IL-4 early after activation

The differences in IL-4 production between recently activated neonatal and adult CD4+ cells could potentially be explained by differences in the frequencies of responding cells. To investigate this possibility, intracellular cytokine staining was performed on neonatal or adult DO11.10 CD4+ cells after 24 h of activation (Fig. 2A). Although the frequencies of neonatal and adult IFN-γ-producing cells were similar, only neonatal cells stained positively for IL-4. Despite their early high-level Th2 function (Fig. 1A), the frequencies of IL-4-producing neonatal CD4+ cells were surprisingly low, ranging from 0.5 to 1.1% at this time point (Fig. 2A and data not shown). IL-4 and IFN-γ were not detectable in freshly isolated neonatal CD4+ cells, suggesting that neonatal cells do not contain preformed stores of intracellular IL-4 protein, as has previously been described for mast cells (28).

Because IL-4 was undetectable in activated adult cells by intracellular staining, we used a more sensitive assay, the ELISPOT, to compare differences in the frequencies of IL-4-secreting neonatal and adult CD4+ T cells (Fig. 2B). After 24 h of activation, the frequencies of IL-4-secretors were 4.6- to 12.2-fold higher among neonatal CD4+ cells compared with adult cells. This range is similar to the relative differences in total IL-4 protein or Il4 mRNA at 24 h (Fig. 1). The frequencies of IL-4-producing neonatal CD4+ cells ranged from roughly 0.5 to 2% at this time point (Fig. 2B), corroborating our intracellular cytokine staining results (Fig. 2A). Therefore, the robust early IL-4 responses of murine neonates arise from a small population of IL-4-secreting CD4+ cells that are nonetheless present in greater numbers in comparison with adult mice.

Early IL-4 production by neonatal CD4+ cells does not require cell cycle entry

It has previously been shown that IL-4 expression increases with successive cell divisions as naive adult CD4+ cells develop into Th2 effectors (5, 6). This is thought to coincide with the opening of the Th2 cytokine locus and the stable imprinting of epigenetic modifications that are propagated to daughter cells with each division. Once these modifications are established, adult Th2 effectors rapidly express IL-4 in a cell cycle-independent manner (6).
Thus far, our findings have indicated that at least a subset of neonatal CD4+ cells exist in a state that is permissive for high-level IL-4 production early after activation. Therefore, it is possible that neonatal cells resemble adult Th2 effectors by making IL-4 without requiring cell cycle entry. CD4+ cells from day 7 neonatal DO11.10 mice were activated polyclonally for 24 h. Some cultures contained the cell cycle inhibitors aphidicolin (G1-S arrest), hydroxyurea (early S arrest), or paclitaxel (G2-M arrest). A. The IL-4 content in culture supernatants was determined by ELISA. The results are graphed as the mean ± the SD of the triplicate values from a representative experiment. B. After ethanol fixation, the cells were stained with propidium iodide to measure the percentages of apoptotic cells (left marker) and actively cycling cells (S + G2-M, right marker). C. Before propidium iodide staining, the cells were stained for surface expression of the early activation marker CD69 (solid line). Unactivated cells were stained as controls (dotted line). The experiment in C was electronically gated on nonapoptotic cells. All results are representative of at least three independent experiments.

Pharmacologic demethylation does not affect IL-4 production by neonatal CD4+ cells

DNA methylation is an epigenetic silencing mechanism whose manner of inheritance is inexorably linked to the cell cycle. After associating with replicating loci during S phase, the maintenance DNA methyltransferase Dnmt1 faithfully copies DNA methylation patterns from the parental to the newly synthesized DNA strand (29). It is intriguing that neonatal CD4+ cells still made moderately high levels of IL-4 despite inhibition of DNA synthesis with the DNA polymerase inhibitor aphidicolin (Fig. 3A). This raised the possibility that neonatal cells could already be hypomethylated at the Th2 cytokine locus, and thus would be unaffected by the repressive effects of maintenance DNA methylation. To test this hypothesis, neonatal or adult CD4+ cells from day 7 neonatal or adult DO11.10 mice were activated polyclonally in the presence or absence of the demethylating agent 5-aza-deoxycytidine. After 72 h, the (A) IL-4 or (B) IFN-γ content in culture supernatants was determined by ELISA. The results are graphed as the mean ± the SD of the triplicate values from a representative experiment. The fold difference in (A) IL-4 or (B) IFN-γ secretion between treated and untreated neonatal or adult cells is indicated. C. After 48 h, 5-aza-deoxycytidine (5-aza-dc) treated cells (filled histogram) or untreated cells (open histogram) were stained for surface expression of the activation marker CD25. Unactivated cells were also stained as controls (dotted line). The experiment in C was electronically gated on nonapoptotic cells. All results are representative of four independent experiments.
by neonatal cells was unchanged in treated cells compared with untreated controls (Fig. 4A). The differences between neonatal and adult cells were specific for IL-4 because production of the Th1 cytokine IFN-γ was enhanced by 5-aza-deoxycytidine treatment to a similar degree in neonatal and adult cells (Fig. 4B).

Differences in cell activation, apoptosis, or cycling could trivially account for the distinct IL-4 responses of neonatal and adult cells to treatment with this agent. Therefore, we investigated the cellular activation status and the percentages of apoptotic cells and cells in cycle after 48 or 72 h of culture with 5-aza-deoxycytidine. For both neonatal and adult cells, expression of the activation marker CD25 was comparable between treated and untreated cells (Fig. 4C and data not shown). Higher percentages of both neonatal and adult cells underwent apoptosis when treated with 5-aza-deoxycytidine relative to their respective untreated controls (Fig. 4D). However, the percentages of apoptotic cells were similar between treated neonatal and adult cells at both 48 and 72 h (Fig. 4D and data not shown). The percentages of cells in the S, G2, and M phases of the cell cycle were also comparable between neonatal and adult cells whether or not they were treated with 5-aza-deoxycytidine (Fig. 4D and data not shown). Altogether, these findings indicate that the demethylating effects of 5-aza-deoxycytidine did
not affect IL-4 production by neonatal CD4+ cells, suggesting that neonatal cells are less methylated at the Th2 cytokine locus than are adult cells.

The CNS-1 region pre-exists in a demethylated state in resting neonatal CD4+ T cells

Pharmacologic demethylation of neonatal and adult CD4+ T cells (Fig. 4A) suggested that neonatal cells may be relatively hypomethylated at the Th2 cytokine locus (Fig. 5A). To test this directly, we used the bisulfite genomic sequencing method (19) to compare DNA methylation patterns between neonatal and adult 5-day DO11.10 Th2 effectors (Fig. 5, B and C). Fourteen CpG sites were analyzed within a highly conserved, coordinate regulatory region of the Th2 cytokine expression, termed CNS-1 (20). As expected (14, 16), CpG demethylation was detected in adult Th2 effectors (Fig. 5B). However, a comparison of DNA methylation levels at each individual CpG site revealed that demethylation was 1.5- to 5.6-fold greater in neonatal Th2 cells relative to adult Th2 cells (Fig. 5C). Strikingly, all 14 CpG sites were completely demethylated in 58% of the clones from neonatal Th2 effectors compared with only 7% of the clones from adult Th2 effectors (Table I). In contrast to the Th2 effectors, adult Th1 cells exhibited little demethylation at each of the CpG sites within the CNS-1 region and none of the clones from Th1 effectors was completely demethylated (Fig. 5B). The levels of demethylation strongly reflected the capacity to express IL-4, because neonatal Th2 cells had greater numbers of IL-4-secreting cells than did adult Th2 cells and very few adult Th1 cells made IL-4 (Fig. 5D).

Greater demethylation at CNS-1 was observed in neonatal CD4+ cells, relative to adult cells, after 5 days of Th2 polarization in vitro (Fig. 5, B and C, and Table I). This raised the intriguing possibility that resting neonatal cells could pre-exist in a demethylated state at this region. To investigate this idea, CpG methylation patterns at CNS-1 were analyzed in resting naive adult (sorted CD4+CD44low) and neonatal CD4+ cells from DO11.10 mice (Fig. 6, A and B). Although demethylated CpG dinucleotides were infrequent in resting adult CD4+ cells (range = 0–12% demethylated), resting neonatal cells were demethylated to varying degrees (range = 11–47% demethylated) at each of the CpG sites analyzed (Fig. 6A). Eight percent of the clones from resting neonatal CD4+ cells were demethylated at all 14 CpG sites whereas none of the clones from resting adult cells was completely demethylated (Table I). The differences between neonates and adults were not due to the selective analysis of naive cells in adults because similar levels of CNS-1 demethylation were observed when CD4+CD44low naive phenotype neonatal cells were purified from the total cell population (Fig. 6C). Thus, subsequent experiments were performed on the total resting CD4+ cell population in neonates. Taken together, these findings demonstrate that a subset of resting neonatal CD4+ cells have pre-existing demethylation at the CNS-1, which could allow for high-level IL-4 and IL-13 production early after activation.

CpG methylation patterns at other control regions of the Th2 cytokine locus are similar in resting neonatal and adult CD4+ cells

CNS-1 was relatively demethylated in resting neonatal CD4+ cells (Fig. 6 and Table I). However, widespread hypomethylation of the Th2 cytokine locus was not a feature of neonatal cells, because six CpG sites within Il4 intron I and exon 2 were only 0–18% demethylated, even in 5-day neonatal Th2 effectors (Fig. 7A and Table I). We also tested whether neonatal cells were demethylated at another coordinate control region of Il4 and Il13 expression, rad50 RHS7 of the Th2 cytokine LCR, and 13 CpG sites within CS-1 of the distal Il13 promoter. The percentages of DNA clones that were completely demethylated at every CpG site for each of the regions analyzed are indicated. The presented data is pooled from two independent cell preparations for each cell type.

The transcription factor STAT6 is not required for CNS-1 demethylation and early IL-4 expression in neonatal CD4+ cells

STAT6 is a key positive regulator of Th2 effector cell development in adult CD4+ T cells (31–33). STAT6 has been shown to be required for demethylation of a Th2 cytokine regulatory region (9). However, its role in CNS-1 demethylation and, in particular, its

| Table 1. The percentages of clones that were completely demethylated at every CpG site for each genomic region analyzed within the Th2 cytokine locusa |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Cell Type       | CNS-1 (%)       | Il4 Intron 1-Exon 2 (%) | rad50 RHS7 LCR (%) | Il13 CS-1 (%)   |
| Neo resting CD4+| 8               | 0               | 0               | 90              |
| Adult resting CD4+| 0             | 0               | 0               | 95              |
| Neo Th2         | 58              | 0               | Not done        | Not done        |
| Adult Th2       | 7               | 0               | Not done        | Not done        |

a CD4+ lymph node cells were enriched from day 7 neonatal or adult DO11.10 mice. Genomic DNA was isolated from naive resting cells or from 5-day Th2 effectors. CpG methylation was assessed via the bisulfite genomic sequencing method. Four regions of the Th2 cytokine locus were analyzed: 14 CpG sites within the CNS-1 region, 6 CpG sites within intron I-exon 2 of the Il4 gene, 9 CpG sites within or adjacent to rad50 RHS7 of the Th2 cytokine LCR, and 13 CpG sites within CS-1 of the distal Il13 promoter. The percentages of DNA clones that were completely demethylated at every CpG site for each of the regions analyzed are indicated. The presented data is pooled from two independent cell preparations for each cell type.
function in neonatal CD4^+ cells is currently unknown. Thus far, our DNA methylation analyses have been performed entirely on DO11.10 TCR-transgenic CD4^+ cells. Therefore, before investigating the STAT6 dependence of CNS-1 demethylation, it was first necessary to determine whether this state also occurs in resting nontransgenic neonatal cells. For this reason, CNS-1 DNA methylation patterns were compared in resting CD4^+ lymph node cells from day 7 neonatal DO11.10, wild-type BALB/c, and STAT6-deficient mice on a BALB/c background. CNS-1 demethylation clearly occurred in wild-type BALB/c neonatal cells, often at levels comparable to those in DO11.10 TCR-transgenic neonatal cells (Fig. 8A). For the majority of the CpG sites within CNS-1, STAT6-deficient neonatal CD4^+ cells had similar levels of DNA demethylation relative to their wild-type BALB/c counterparts (Fig. 8A). These findings indicate that CNS-1 demethylation occurs in nontransgenic neonatal CD4^+ T cells and that STAT6 is not required to establish this state. To determine whether recently activated STAT6-deficient neonatal CD4^+ cells are competent to make IL-4, the frequencies of IL-4-producing cells were measured by ELISPOT after 24 h of polyclonal stimulation. As in adult cells, IL-4-secreting neonatal cells were only slightly
diminished among STAT6-deficient cells compared with wild-type BALB/c cells (Fig. 8B). This confirmed that, like CNS-1 demethylation, IL-4 production by recently activated neonatal CD4+ cells does not require STAT6.

**CNS-1 demethylation is already present in unactivated neonatal CD4 SP thymocytes and this epigenetic state is greatly expanded in IL-4-producing cells**

Around the time of birth, the first wave of mature CD4 SP thymocytes exits the thymus and colonizes the peripheral lymphoid compartments (34). Because resting neonatal peripheral CD4+ lymphocytes were already demethylated at CNS-1 (Figs. 6 and 8A), we next tested whether demethylation was established during T cell development in the thymus. CNS-1 methylation levels were assessed in resting CD4 SP thymocytes sorted from day 2 DO11.10 neonates. For most of the CNS-1 CpG sites, the levels of CNS-1 demethylation in neonatal cells, did not require cell cycle entry, but were already demethylated at CNS-1 (Figs. 6 and 8A). These findings demonstrate that CNS-1 demethylation is initially patterned within the thymus during early life.

We next investigated whether the capacity for IL-4 expression corresponds with a demethylated state at CNS-1. To test this hypothesis, CD4 SP thymocytes from day 3 neonatal DO11.10 mice were activated for 2 days. The frequencies of IL-4-secreting cells were enumerated by ELISPOT as described in Fig. 2. The depicted experiment is representative of four independent experiments.

**Discussion**

Unlike naive adult cells, murine neonatal CD4+ T cells demonstrate effector-like Th2 function early after polyclonal stimulation in vitro. Although enhanced Th2 function in neonates is well-documented, the molecular processes that contribute to this phenomenon have not previously been studied. In this report, we found that the early high-level IL-4 production arose from a small population of neonatal CD4+ cells, did not require cell cycle entry, and was relatively unaffected by treatment with a global demethylating agent. An analysis of CpG methylation patterns at the Th2 cytokine locus revealed that resting neonatal CD4+ cells, unlike resting adult cells, were demethylated at a highly conserved control region, termed CNS-1 (20). This differential pattern of methylation between resting naive neonatal and adult CD4+ cells was unique to CNS-1, because methylation profiles were similar at other investigated regions of the Th2 cytokine locus. Enhanced Th2 function and greater CNS-1 demethylation was a stable property of neonatal cells, because the frequencies of IL-4 secretors and the levels of CNS-1 demethylation were both higher in 5-day neonatal Th2 effectors compared with adult Th2 cells. The transcription factor STAT6 was not required for establishing CNS-1 demethylation and this epigenetic state was already present in neonatal CD4 SP thymocytes. Lastly, CNS-1 demethylation was...
strongly overrepresented in IL-4-expressing neonatal CD4+ T cells, compared with unactivated cells. Taken together, these data indicate that neonatal CD4+ cells have several distinct properties that predispose them toward rapid, high-level Th2 function.

The poised state of neonatal CD4+ T cells contrasts sharply with that of naive adult cells, which have been shown to undergo extensive proliferation and epigenetic remodeling of the Th2 cytokine locus over a period of several days to weeks before high-level Th2 cytokine expression is achieved (4). Neonatal CD4+ cells possess at least three distinctive properties that could predispose them toward greater early Th2 effector development and function. First, we have found that a key enhancer region of Th2 cytokine expression, CNS-1 (20), was completely demethylated in a subset of resting neonatal CD4+ cells, but not in adult cells. Because the degree of demethylation at CNS-1 correlates strongly with the frequencies of Th2 cells that make IL-4 (14), this epigenetic state could promote early Th2 cytokine production by neonatal cells. Second, unlike adult cells (5, 6), neonatal CD4+ cells produced high levels of IL-4 protein early after activation. Because IL-4 drives Th2 differentiation (35), this could rapidly initiate the development of neonatal Th2 cells. Third, our laboratory has previously shown that activated neonatal CD4+ cells have more rapid cell division kinetics compared with adult cells (23). Thus, neonatal CD4+ cells could rapidly produce high levels of IL-4, which may be followed by more rapid proliferation of the IL-4-secreting neonatal cells, compared with adult cells. Together, these unique properties may give neonatal cells a “head start” over adult cells during early Th2 cell development.

Because CNS-1 was uniquely demethylated in resting neonatal CD4+ cells, it is tempting to speculate that this regulatory region alone is responsible for the differences in IL-4 and IL-13 production between recently activated neonatal and adult CD4+ cells. In transgenic adult mice, CNS-1 has been shown to be a coordinate regulator of human IL4 and IL13 gene expression (20) and a targeted deletion of the murine CNS-1 region disrupts Th2 differentiation in vitro and in vivo (36). It has been suggested that epigenetic modifications at this region are permissive for IL4 and IL13 gene transcription (37). We have demonstrated that two other regulatory regions of both IL4 and IL13 expression, rad50 RSH7 within the Th2 cytokine LCR (11) and CS-1 of the distal IL13 promoter (30), exhibited similar DNA methylation patterns in neonatal and adult cells. Thus, recently activated neonatal cells could initially use a demethylated CNS-1 region to enhance their early Th2 cytokine expression. Other control regions of the locus may also become involved as neonatal cells differentiate into Th2 effectors, similar to what occurs in adult cells. This would explain why there are initially large differences in the frequencies of IL-4-secreting neonatal and adult CD4+ cells, but smaller differences at the Th2 effector stage.

How could differences in DNA methylation levels between resting neonatal and adult CD4+ cells give rise to differences in IL4 and IL13 expression between recently activated neonatal and adult cells? CNS-1 contains putative binding sites for a number of transcriptional activators, including CREB, NFAT, ATF, AP-1, and c-Myb, and these binding sites each contain CpG dinucleotides (38). CpG methylation interferes with the binding of each of these transcription factors to their target sequences (39–42). Therefore, methylated CpG sites within CNS-1 could disrupt the binding of transcriptional activators to this region. Because CNS-1 has been shown to strongly interact with both the IL4 and IL13 promoters (43), it is possible that a demethylated CNS-1 region enhances IL4 and IL13 expression by bringing transcriptional activators in close proximity to the Th2 cytokine promoters. CNS-1 is relatively demethylated in resting neonatal CD4+ cells, a state that could facilitate the binding of transcriptional activators, resulting in high levels of IL4 and IL13 expression early after activation. In contrast, naive resting adult CD4+ cells exhibit high levels of CNS-1 methylation. Transcription factor binding could therefore be inhibited in adult cells, resulting in lower levels of IL4 and IL13 expression following activation.

It is worth noting that while CNS-1 demethylation may be indicative of an open, permissive gene structure, it is not sufficient, per se, for high-level Th2 cytokine expression by neonatal CD4+ cells. As has previously been described for adult Th2 clones (14), there was a discrepancy between the frequencies of neonatal cells that were completely demethylated at CNS-1 and the frequencies of cells that actually produced IL-4. This was true for both recently activated cells and for Th2 effectors. Thus, while CNS-1 demethylation may establish a competent state at the IL4/IL13 locus (37), it is not sufficient, by itself, for high-level Th2 cytokine production by neonatal CD4+ cells. Other factors, such as the local availability of transcriptional activators, are also likely to play a role.

In light of our present observations, it will be interesting to determine what factors are responsible for establishing the unique epigenetic profile of neonatal CD4+ cells. Early IL-4 production and CNS-1 demethylation in neonatal cells did not require STAT6, STAT6-independent IL-4 expression and modifications at the Th2 cytokine locus have often been attributed to its downstream mediator GATA-3 (44). GATA-3 could be important for patterning CNS-1 demethylation in wild-type neonatal cells, as it has been shown to bind within this region (38).

Reports on adult and neonatal thymocytes support the idea that GATA-3 could establish CNS-1 demethylation within the thymus during early life. The de novo DNA methyltransferase Dnmt3b has been shown to bind within the CNS-1 region in resting adult CD4 SP thymocytes (26). Following activation of adult CD4 SP cells, Dnmt3b is preferentially excluded from CNS-1, an event that is associated with a marked decline in CpG methylation levels at this region (26). It has been suggested that GATA-3 binding may obstruct the recruitment of DNA methyltransferases, leading to demethylation of CNS-1 (26). Because neonatal CD4 SP thymocytes are already substantially demethylated in the resting state, it is possible that GATA-3 blocks Dnmt3b binding during this stage of thymocyte maturation. It has previously been shown that neonatal CD4 SP thymocytes express higher levels of GATA-3 than do adult cells (45). If resting neonatal CD4 SP cells had higher levels of GATA-3 bound to CNS-1, this could hinder Dnmt3b binding and result in greater DNA demethylation compared with resting adult cells. Because GATA-3 is expressed at all stages of thymocyte maturation (46), a second possibility is that neonatal thymocytes become demethylated in response to activation signals received before the CD4 SP stage. Thus, exclusion of Dnmt3b from the CNS-1 region, and ensuing CNS-1 hypomethylation, could potentially be established at or before the CD4 SP stage of thymocyte development.

High-level IL-4 expression (47) and CNS-1 demethylation are characteristics of neonatal CD4 SP thymocytes that are retained in peripheral CD4+ lymphocytes. It is possible that processes occurring in the periphery, such as lymphopenia-induced proliferation (LIP), act to sustain these properties of neonatal cells. At birth, neonates are in a state of relative lymphopenia compared with adult animals. During the first week of life, the peripheral lymphoid organs are seeded by mature CD4 SP thymic emigrants. A proportion of these cells undergo LIP (48), a process that is associated with the acquisition of effector function when adult CD4+ T cells are adoptively transferred into neonatal hosts (49). It is not known whether endogenously proliferating neonatal T cells are
also capable of effector function. However, naive phenotype neonatal T cells also showed substantial demethylation at CNS-1, suggesting that mechanisms other than LIP contribute to the epigenetically poised state. Nonetheless, LIP may help to solidify the Th2 state in neonates. Experiments to address these and other related hypotheses are ongoing in our laboratory.

It is very clear that epigenetic modifications are critical for regulating gene expression during development. However, well-characterized examples, such as globin gene switching (50) and selective TCR Vγ usage (51), apply to genes that are nonmutually expressed in fetal and adult life. The developmental regulation of Il4 represents a unique strategy whereby epigenetic modifications modulate the frequency of expression of a gene that is expressed throughout life. The epigenetic regulation of cytokine expression in early life may be a common theme in mice and humans. In human newborns, repressive gene structures are thought to be responsible for the reduced expression (compared with adults) of IFN-γ in cord blood CD4+ T cells (52) and IL-12 in cord blood dendritic cells (53). Thus, it appears that certain cytokine response patterns of neonatal immune cells may already be preprogrammed at birth and that the epigenetic state of the cytokine genes promotes anti-inflammatory responses. Significant contributions from several laboratories now suggest that the Th2-biased immune responses of murine neonates arise through a combination of cell intrinsic (Refs. 3, 54, and the present manuscript) and environmental (55) factors that are unique to early life.

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Disclosures

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References


