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IL-4 Utilizes an Alternative Receptor to Drive Apoptosis of Th1 Cells and Skews Neonatal Immunity toward Th2

Lequn Li,[1,4,5] Hyun-Hee Lee,[1,4] J. Jeremiah Bell,[1] Randal K. Gregg,[1] Jason S. Ellis,[1] Andre Gessner,[2] and Habib Zaghouani[1,3]

[1]Department of Molecular Microbiology and Immunology
University of Missouri School of Medicine
M616 Medical Sciences Building
Columbia, Missouri 65212

[2]Institut für Klinische Mikrobiologie
Immunologie und Hygiene
Wasserturmstrasse 3
91054 Erlangen
Germany

Summary

Primary neonatal Th1 cells develop alongside of Th2 upon priming of the newborn but undergo apoptosis upon recall with antigen. These Th1 cells were isolated, and their death was correlated with elevated IL-13Rα1 chain expression. Strikingly, neutralization of Th2s′ IL-4 reduced apoptosis, sustained recall responses, and the live Th1 cells displayed a decrease in IL-13Rα1 expression. Blockade of IL-13Rα1 or IL-4Rα also restores recall and secondary Th1 responses. Adult T cells primed within the neonatal environment did not upregulate IL-13Rα1 chain or undergo apoptosis and developed recall Th1 responses. These observations indicate that developmental expression of IL-13Rα1 along with IL-4Rα provides a receptor through which IL-4 induces death of Th1 cells and skews neonatal immunity toward Th2.

Introduction

Neonatal exposure to antigen usually leads to tolerance (Billingham et al., 1956; Gammon et al., 1986; Garza et al., 1997). Initially, T cell deletion/inactivation was considered the leading mechanism for such tolerance. Lately, however, it has been shown that immunity develops in the newborn (Ridge et al., 1996; Sarzotti et al., 1996), but in most cases the responses manifest an indubitable bias toward Th2 (Forsthuber et al., 1996; Chen and Field, 1995; Singh et al., 1996). Furthermore, when immunoglobulins (Igs) were used for peptide delivery to enhance presentation and provide adjuvanticity, biased Th2 immunity remained dominant (Min et al., 1998; Pack et al., 2001). Although this outcome could be beneficial for transplantation and suppression of Th1-mediated autoimmunity, it may place the neonate at risk for microbial infections, which require Th1 cells. In fact, a great deal of interest has been devoted lately toward defining regimens that could promote neonatal Th1 immunity (Siegrist, 2001). Thus, understanding how neonatal exposure to antigen skews Th1 and favors Th2 cells remains the key feature for progress toward the development of effective pediatric vaccines. The lack of an adaptable model for investigation of primary neonatal responses has been a major limitation in neonatal immunity. Transfer of TCR transgenic (Tg) T cells into animals allowed for proper homing to lymphoid organs and facilitated analysis of T cell responses in vivo (Kearney et al., 1994). Recently, we have used the ovalbumin (OVA) 323-339-specific TCR transgenic DO11.10 T cells and developed a T cell transfer system suitable for tracking neonatal T cells and analyzing their responses upon exposure to antigen (Li et al., 2001). Accordingly, transfer of neonatal (1 day old) DO11.10 T cells into 1 day old Balb/c mice sustains homing of the DO11.10 T cells to the hosts' lymphoid organs. Moreover, priming of the Balb/c hosts with Ig-OVA, an Ig chimera genetically engineered to express the OVA peptide, circumvents the use of adjuvant and promotes a secondary response skewed toward Th2 upon challenge with OVA peptide at an adult age (Li et al., 2001). Herein, the neonatal DO11.10 TCR Tg T cell transfer system was used to investigate the primary neonatal response to determine whether the Th2 bias was due to a lack of development of primary Th1 cells or to a defective recall of neonatally primed Th1 cells. The results indicate that primary Th1 cells do develop alongside of Th2 counterparts upon neonatal priming. However, recall with antigen drives the Th2 cells to respond but the Th1 lymphocytes undergo apoptosis. Moreover, when the Th2 cytokines are neutralized either during neonatal priming or recall with antigen, Th1 responses develop alongside of Th2. Interestingly, adult T cells primed within the neonatal environment give rise to primary Th1 and Th2 that are both able to respond to a recall with antigen. Finally, gene expression analysis indicated a developmental upregulation of IL-13Rα1 chain in neonatal Th1 that correlated with an inability of the cells to balance pro and antiapoptotic factors leading to death of the Th1 subset. However, blockade of IL-13Rα1 or IL-4Rα during either priming or rechallenge with antigen prevents apoptosis and restores Th1 responses.

Results

Exposure of Neonatal T Cells to Antigen Induces Cell Activation and Division

Figure 1 shows that neonatal DO11.10 T cells transferred into newborn Balb/c mice are activated and undergo cell division upon exposure to antigen. Indeed, ex vivo cell surface staining analysis indicated that only 9% of DO11.10 T cells stained positive for CD25 expression in animals that were not given the antigen, as opposed to those injected with OVA peptide or Ig-OVA where upregulation of CD25 was seen in 35% and 28% of their splenic DO11.10 T cells, respectively (Figure 1A). Moreover, both CD62L and CD45RB (Figures 1B and 1C) were downregulated, indicating that contact with

[3]Correspondence: zaghouanih@health.missouri.edu
[4]These authors contributed equally to this work.
[5]Present address: Dana Farber Cancer Institute, 44 Binney Street, Mayer Building 547, Boston, Massachusetts 02115.

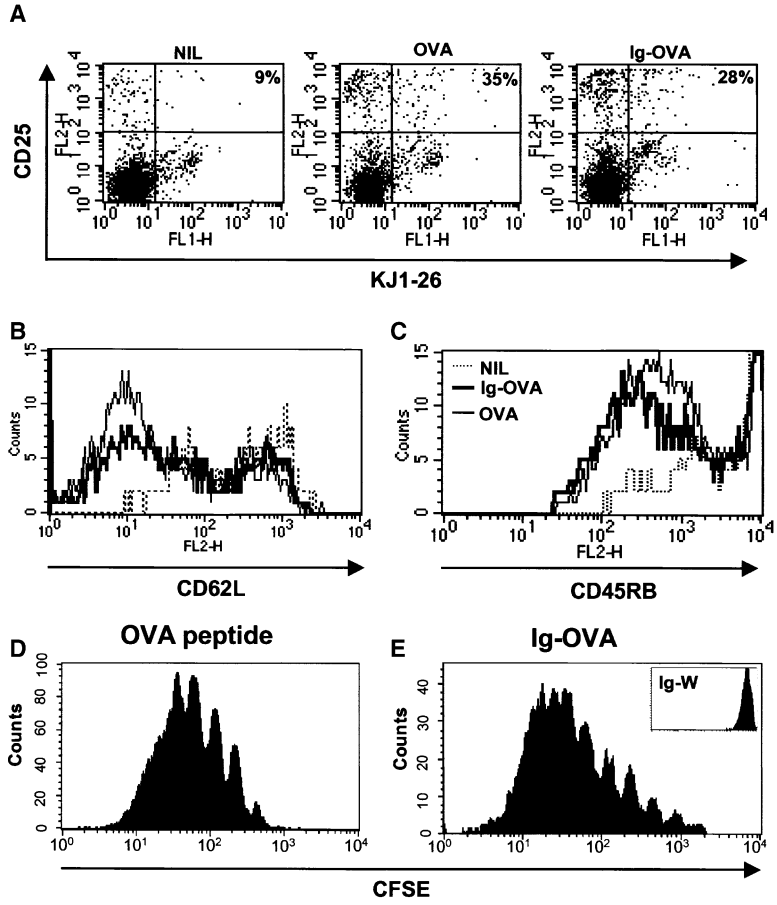

Figure 1. Neonatal Exposure to Antigen Induces T Cell Activation and Division

Neonatal DO11.10/scid T cells were transferred into 1 day old Balb/c mice and the hosts were given the antigen within a few hours. CD25 (A), CD62L (B), and CD45RB (C) expression as well as cell division (D and E) were then analyzed. In the top panel, staining of splenic cells with KJ1-26, anti-CD4, and anti-CD25 mAb was performed 2 days after injection of PBS (NIL), OVA peptide (100 μg/100 μl PBS), or Ig-OVA (100 μg/100 μl PBS). Dot plots were gated on CD4 and the percentage of CD25-positive among KJ1-26$^+$ cells is shown. In the median panel, the splenic DO11.10 T cells were harvested on day 7 after injection of antigen and stained with KJ1-26, anti-CD4, and anti-CD62L (B) or anti-CD45RB (C). In the bottom panel, CD4-purified, CFSE-labeled neonatal DO11.10/scid cells were transferred into Balb/c newborns and the hosts were given OVA peptide (D), Ig-OVA (E), or the parental Ig-W not containing any OVA peptide (insert in E). Four days later the splenic cells were purified on Miltenyi anti-CD4 beads, stained with anti-CD4 and KJ1-26 mAb, and analyzed by flow cytometry. Histogram plots are gated on the CD4$^+$-KJ1-26$^+$ population. The experiments presented in this and all other figures have been repeated at least twice.

the antigen has occurred and the cells abandoned their naive phenotype. Upon activation, naive T cells divide and enter the effector/memory stage. In contrast, unproductive antigen stimulation may promote death at the naive stage. To address this issue, neonatal DO11.10 T cells were labeled with the dye CFSE prior to transfer into the newborn Balb/c mice and cell division was analyzed by flow cytometry subsequent to exposure to antigen in vivo. Figures 1D and 1E show that both OVA peptide and Ig-OVA chimera induced a substantial reduction in cytoplasmic CFSE relative to the control Ig-W chimera, not containing OVA peptide, indicating that a number of cell divisions and most likely differentiation have occurred in vivo as a consequence of antigen recognition. Thus, neonatal exposure to antigen induces activation and division of neonatal T cells.

Both Th1 and Th2 Cells Develop in the Primary Neonatal Response but Recall with Antigen Causes Substantial Apoptosis in the Th1 Population

Neonatal exposure to antigen usually gives rise to Th2 biased secondary responses. To date, it is still not clear whether a lack of priming or a defect in the recall of Th1 immunity is responsible for such a Th2 bias. To test this premise, ex vivo intracellular cytokine staining was adapted to the neonatal DO11.10 T cell transfer system, thus making analysis of T cell differentiation possible. It is shown that, upon transfer of neonatal DO11.10 T cells into the newborn recipients and exposure to Ig-OVA, some of the cells differentiated into Th1 lymphocytes and produced IFNγ. Others evolved along the Th2 pathway and secreted IL-4 (Figure 2A). Such Th1/Th2 priming is antigen specific, as intracellular cytokines were undetectable when Ig-W was used instead of Ig-OVA. Thus, both Th1 and Th2 primary responses develop in the neonate as was observed previously (Adkins and Du, 1998). However, when these cells were recalled in vitro with OVA peptide IL-4 and IL-10 were produced but IFNγ was lacking (Figure 2B). Analysis of the fate of primary Th1 and Th2 cells indicated that recall with antigen induces substantial apoptosis among the Th1 population because Annexin V staining was much more prominent on IFNγ- rather than IL-4-producing cells (Figure 2C). These results indicate a susceptibility of neonatal Th1 cells to antigen-driven apoptosis.

Neutralization of Th2 Cytokines during Priming or Recall with Antigen Diminishes Apoptosis and Restores Responses of the Th1 Counterparts

Upon recall with antigen, the neonatal Th2 cells respond and produce IL-4 and IL-10. Both of these cytokines have been shown to display negative effects on the development of Th1 cells (Reiner and Seder, 1999; Morel and Oriss, 1998). To test whether IL-4 and/or IL-10 influenced the recall of the differentiated primary Th1 cells, in vitro stimulation with OVA peptide was performed in the presence of anti-IL-4 and/or anti-IL-10. Surprisingly, a Th1 response developed and IFNγ production oc-

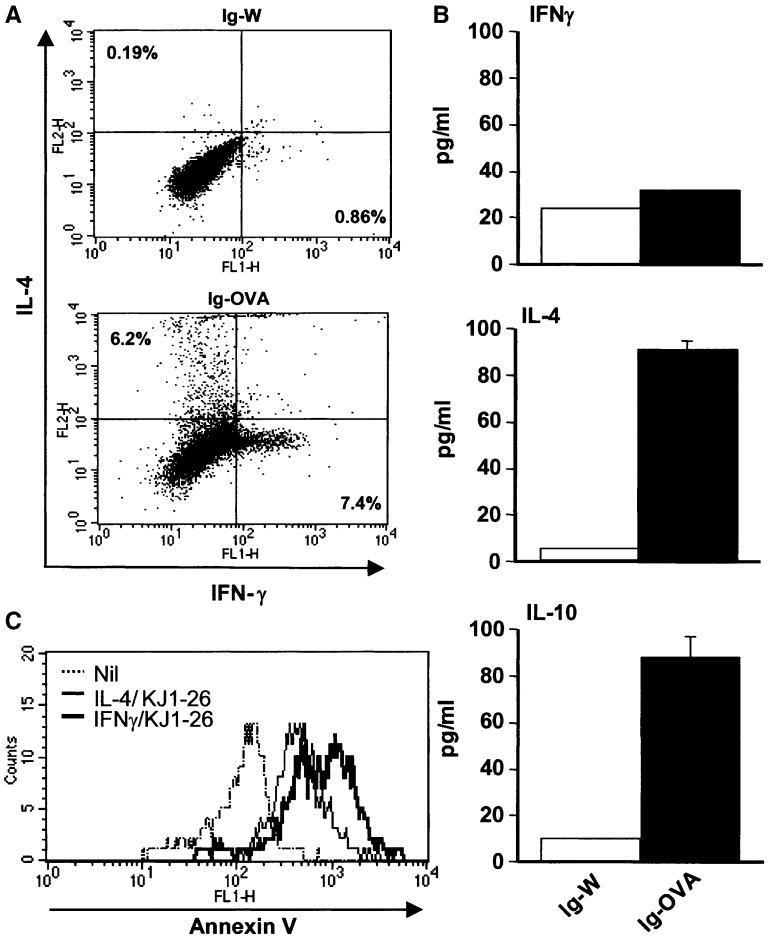

Figure 2. Neonatal Exposure to Antigen Primes Both Th1 and Th2 Lymphocytes but Th1 Cells Undergo Apoptosis upon Recall with Antigen

One day old mice recipient of neonatal DO11.10/scid T cells were given Ig-OVA and 14 days later the DO11.10 T cells were analyzed both ex vivo (A), and upon restimulation in vitro with OVA peptide (B) for production of Th1 and Th2 cytokines. Annexin V staining (C) was also used to determine apoptosis on both Th1 and Th2 cells. In (A), the splenic cells were subject to a rapid stimulation with OVA peptide (ex vivo conditions), the CD4 cells were isolated on anti-CD4-microbeads, labeled with KJ1-26 antibody, and tested for intracellular production for IL-4 and IFNγ. Dot plots show IL-4 and IFNγ production by KJ1-26 gated T cells. In (B), the splenic cells (1×10^6 cells/well) were stimulated with 10 μM OVA peptide for 24 hr and IFNγ, IL-4, and IL-10 were measured by ELISA. Each bar represents the mean ± SD of triplicate wells. In (C), the splenic cells (1×10^6 cells/ml) were stimulated with 10 μM OVA peptide for 4 hr and BFA was added and the culture was continued for an additional 8 hr. The cells were then stained with KJ1-26 and Annexin V and tested for intracellular IL-4 and IFNγ. The histograms show staining with Annexin V on IFNγ/KJ1-26 or IL-4/KJ1-26 double-positive cells. IFNγ/KJ1-26 positive cells that were not stained with Annexin V (Nil) were included for control purposes.

curred when IL-4 was neutralized (Figure 3A). Furthermore, although anti-IL-10 mAb alone did not restore IFNγ production, neutralization of both IL-4 and IL-10 simultaneously led to the production of even higher amounts of IFNγ (Figure 3A). It may be that IFNγ restoration is related to inhibition of apoptosis. To test this premise, the splenic cells from the primed Balb/c hosts were stimulated with OVA peptide in the presence of anti-IL-4 and/or anti-IL-10 and assessed for intracellular production of IFNγ and evidence of apoptosis. Figure 3B shows that neutralization of IL-4 and/or IL-10 during recall with OVA peptide reduced the number of Annexin V-positive cells among the IFNγ-producing Th1 population, indicating that Th2 cytokines are involved in apoptosis of Th1 cells. It should be noted that Th1 cells are identified in the culture by production of intracellular IFNγ, which requires a 12 hr stimulation before addition of Brefeldin A (BFA). This leads to a basal level of antigen-induced cell death that could not be prevented by cytokine neutralization and therefore complete inhibition may not be achieved.

Apoptosis of Th1 cells may reflect an immediate sensitivity to the Th2 cytokines or a susceptibility that was acquired during neonatal priming with antigen. To address this question, the newborn Balb/c hosts were given Ig-OVA accompanied with anti-IL-4 and/or anti-IL-10 and their recall cytokine responses were measured. Figure 3C shows that hosts given Ig-OVA alone developed only Th2 recall responses while those given anti-IL-4 and/or anti-IL-10 mAb along with Ig-OVA had Th1 recall responses and IFNγ was produced. This suggests that the sensitivity of Th1 cells to Th2 cytokines is acquired during priming with antigen at the neonatal stage.

Adult T Cells Exposed to Antigen within the Neonatal Environment Develop Primary Th1 Cells that Do Respond to Recall with Antigen

To determine whether acquisition of sensitivity to Th2 cytokines represents a developmental feature associated with the neonatal stage or whether it could occur with adult T cells, the newborn Balb/c hosts were adoptively transferred with DO11.10 T cells from adult mice and primed with Ig-OVA in saline. The splenic cells were later stimulated in vitro with OVA peptide and their recall responses were analyzed. The findings indicate that adult T cells primed within the neonatal environment resist Th2 cytokines and generate Th1 cells that respond to recall with antigen. Indeed, Figure 4 shows that the spleen cells of the Balb/c hosts produce IFNγ, as well as IL-4 and IL-10 upon in vitro stimulation with OVA peptide (Figures 4A, 4B, and 4C). Such responses are antigen specific, as mice primed with the control Ig-W did not mount measurable cytokine responses. Neutralization of IL-4 and/or IL-10 during in vitro stimulation with OVA peptide did not significantly increase the Th1 cytokine responses indicating that IFNγ production by

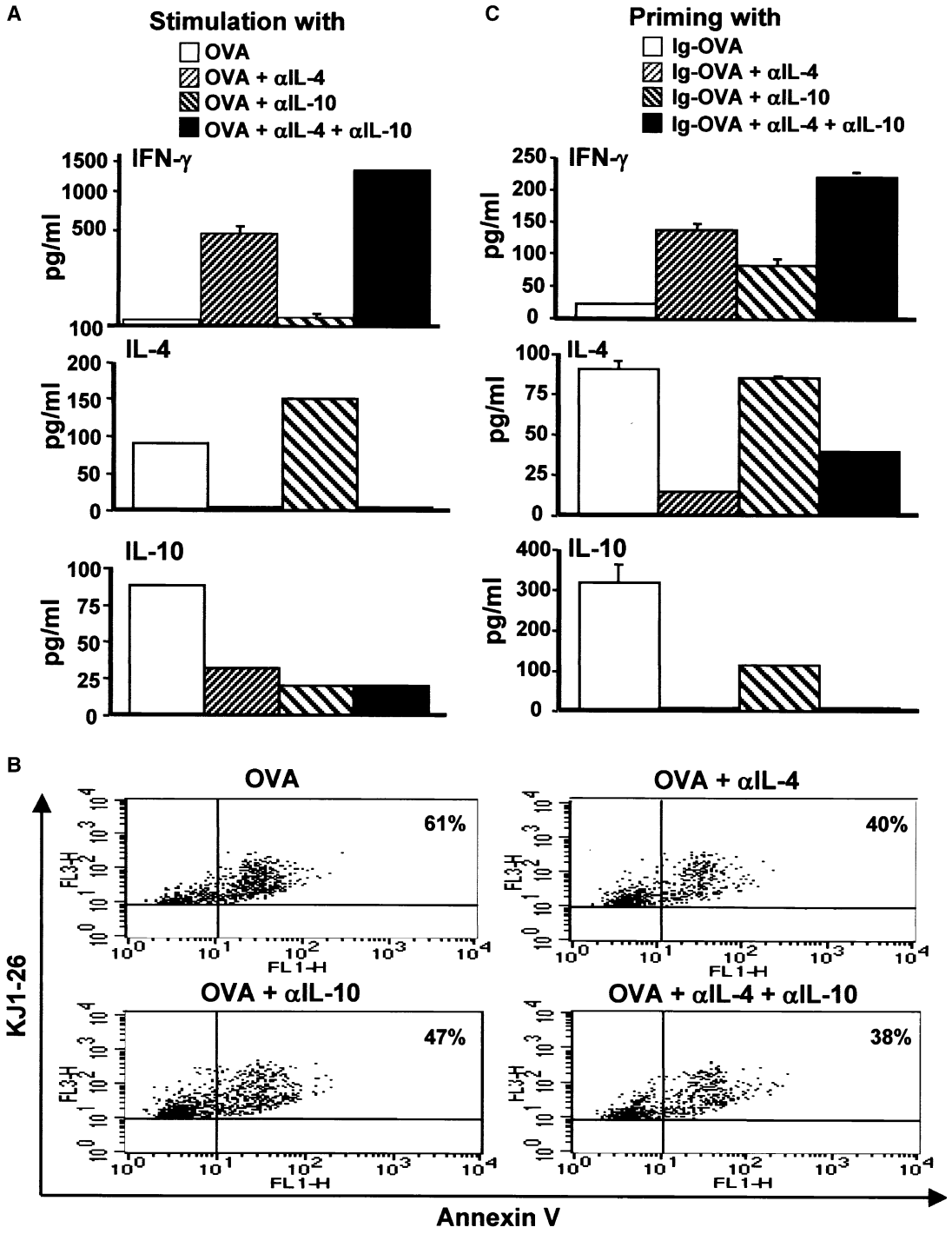

Figure 3. Neutralization of IL-4 and IL-10 during Neonatal Priming or Recall with Antigen Decreases Apoptosis and Restores Response in Neonatal Th1 Cells

One day old Balb/c mice recipient of neonatal DO11.10 T cells were given 100 μg Ig-OVA alone (A and B) or in combination with 100 μg anti-IL-4 and/or 100 μg anti-IL-10 (C). Fourteen days later, the splenic cells were stimulated in vitro with 10 μM OVA peptide (C) alone or (A and B) in the presence of 20 μg anti-IL-4 and/or 20 μg anti-IL-10 antibody. Cytokine production was measured by ELISA 24 hr later. Each bar represents the mean ± SD of triplicate wells. For cell surface staining with Annexin V (B), the splenic cells (1×10^6 cells/ml) were stimulated for 24 hr with 10 μM OVA peptide in the presence of 20 μg/ml of anti-IL-4 and/or 20 μg anti-IL-10 mAb with addition of BFA during the last 8 hr of incubation. The cells were then labeled with KJ1-26 and Annexin V and stained for production of intracellular IFNγ. Dot plots were gated on IFNγ-producing cells.

the Th1 cells was optimal and not subject to regulation by the cytokines of the Th2 counterparts (Figures 4D, 4E, and 4F). Analysis of early apoptosis of the responding adult splenic cells indicated that IFNγ-producing Th1 cells stained with Annexin V to the same extent as their IL-4-producing Th2 counterparts (Figure 4G).

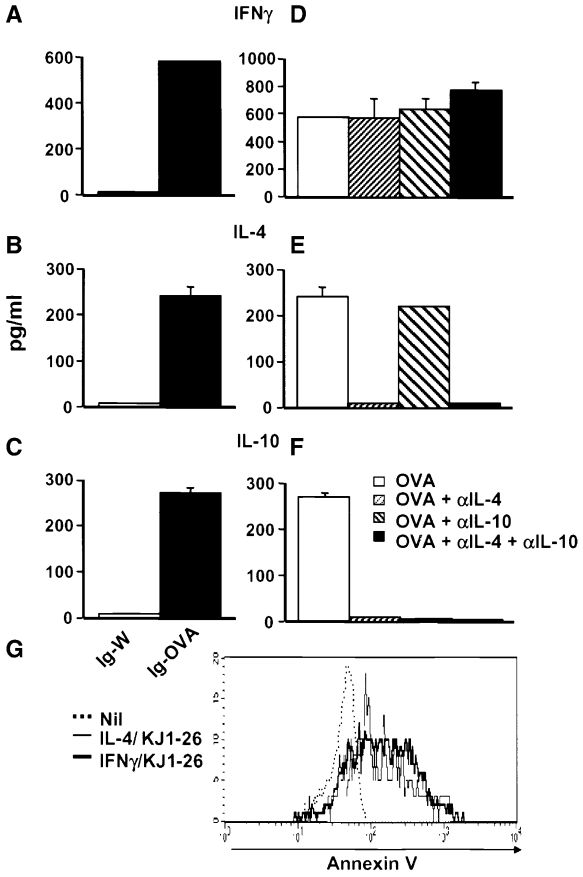

Figure 4. Priming of Adult T Cells within a Neonatal Environment Induces Th1 Cells that Can Be Recalled with Antigen without Undergoing Substantial Apoptosis

Newborn Balb/c mice were given 3×10^4 purified CD4-positive adult DO11.10 T cells, injected with 100 μg Ig-OVA or Ig-W in saline and 2 weeks later, the splenic cells (1×10^6 cells/well) were stimulated with 10 μM OVA peptide in the absence (A, B, and C) or presence (D, E, and F) of 20 μg/ml anti-IL-4 and/or anti IL-10 mAb and tested for the production of IFNγ (A and D), IL-4 (B and E), and IL-10 (C and F) by ELISA. Each bar represents the mean ± SD of triplicate wells. In (G) the splenic cells (1×10^6 cells/ml) were assessed for Th1 and Th2 apoptosis by Annexin V staining as described for neonatal T cells in Figure 2C.

Therefore, adult Th1 cells placed in a neonatal environment resisted the cytokines of the Th2 counterparts, did not undergo substantial apoptosis, and responded to a recall with antigen. These results suggest that acquisition of sensitivity to Th2 cytokines is under developmental control.

Neonatal Th1 Cells Display Differential Expression of Apoptosis-Related Genes Relative to the Th2 Counterparts and Restore Their IFNγ Response upon Inhibition of Caspases

The results presented above do not discriminate whether IL-4 of the Th2 counterparts is simply driving death of Th1 cells or is also interfering with IFNγ expression. To test this premise, we sought to prevent apoptosis by blockade of caspases and assess for IFNγ production. The results in Figure 5A show that upon stimulation with OVA peptide, caspases are activated in the Th1 cells and 87% of the detectable lymphocytes bound Z-VAD, a synthetic substrate that binds to activated caspases. However, when IL-4 of the Th2 cells was neutralized with anti-IL-4 antibody, many more cells remained alive and a significant number (39%) of them showed reduced Z-VAD binding activity. Thus, caspases are activated during OVA stimulation and such activation is inhibitable by anti-IL-4. Subsequently, unlabeled Z-VAD was used to block caspase activity and the cells were tested for IFNγ production and Annexin V binding. Figure 5B shows that addition of Z-VAD during stimulation with OVA peptide rescues a significant number of cells from apoptosis. Indeed while 81% of IFNγ-producing cells bound Annexin V during OVA-stimulation, only 59% displayed Annexin V binding activity when Z-VAD was added to the primary cell culture. Moreover, the rescued Th1 cells secreted IFNγ and such cytokine production was proportional to the amount of Z-VAD added (Figure 5C). These results provide direct evidence that apoptosis is responsible for the lack of IFNγ production and the bias of neonatal recall responses toward Th2.

To gain insight into the susceptibility of neonatal Th1 cells to the cytokines of their Th2 counterparts, we elected to separate the two subsets on the basis of cytokine secretion (Ouyang et al., 2000) and analyze their pattern of gene expression. RNA extracted from purified Th1 and Th2 cells was used to generate labeled cDNA probes and expression of genes involved in apoptosis was assessed by microarray technology. Th1 cells derived from host recipients of adult DO11.10 T cells at the neonatal stage were also isolated and included in this analysis to assess for developmental control of the susceptibility of neonatal Th1 cells to Th2 cytokine-induced apoptosis. Figures 5D and 5E show that upon recall with OVA peptide expression of LT-β (Pokholok et al., 1995), TNFR1 (Ashkenazi and Dixit, 1998), and TNFRSF11A (Theill et al., 2002) were significantly increased in both neonatal Th1 and Th2 cells. However, the MCL-1 antiapoptotic factor (Kozopas et al., 1993; Lomo et al., 1996) was upregulated only in Th2 cells. Th1 cells derived from the neonatal hosts recipient of adult DO11.10 cells showed upregulation of LT-β only (Figure 5E). Interestingly, when recall with antigen of neonatal Th1 cells was carried out in the presence of anti-IL-4 and anti-IL-10, the gene expression pattern was similar to the adult DO11.10-derived Th1 cells and only LT-β was upregulated. These results support the observation obtained with Annexin V staining and suggest that the inability to upregulate MCL-1 may be responsible for IL-4-induced Th1 apoptosis.

Upregulation of IL-13Rα1 in Neonatal Th1 Cells Is under Control by IL-4 of the Th2 Counterparts

Neutralization of IL-4 during priming with Ig-OVA or recall with OVA peptide restored significant responses of Th1 cells while anti-IL-10 had a much lower effect unless combined with anti-IL-4 antibody (Figure 3). These observations along with the fact that only IL-4 is seen in most antigen systems of biased neonatal immunity led us to believe that IL-4 had a primary role on the induction of apoptosis in neonatal Th1 cells. On the other hand, it is known that IL-4 receptor, although expressed on both Th1 and Th2 cells, signals only in the Th2 subset (Huang and Paul, 1998). Therefore, it is intriguing how

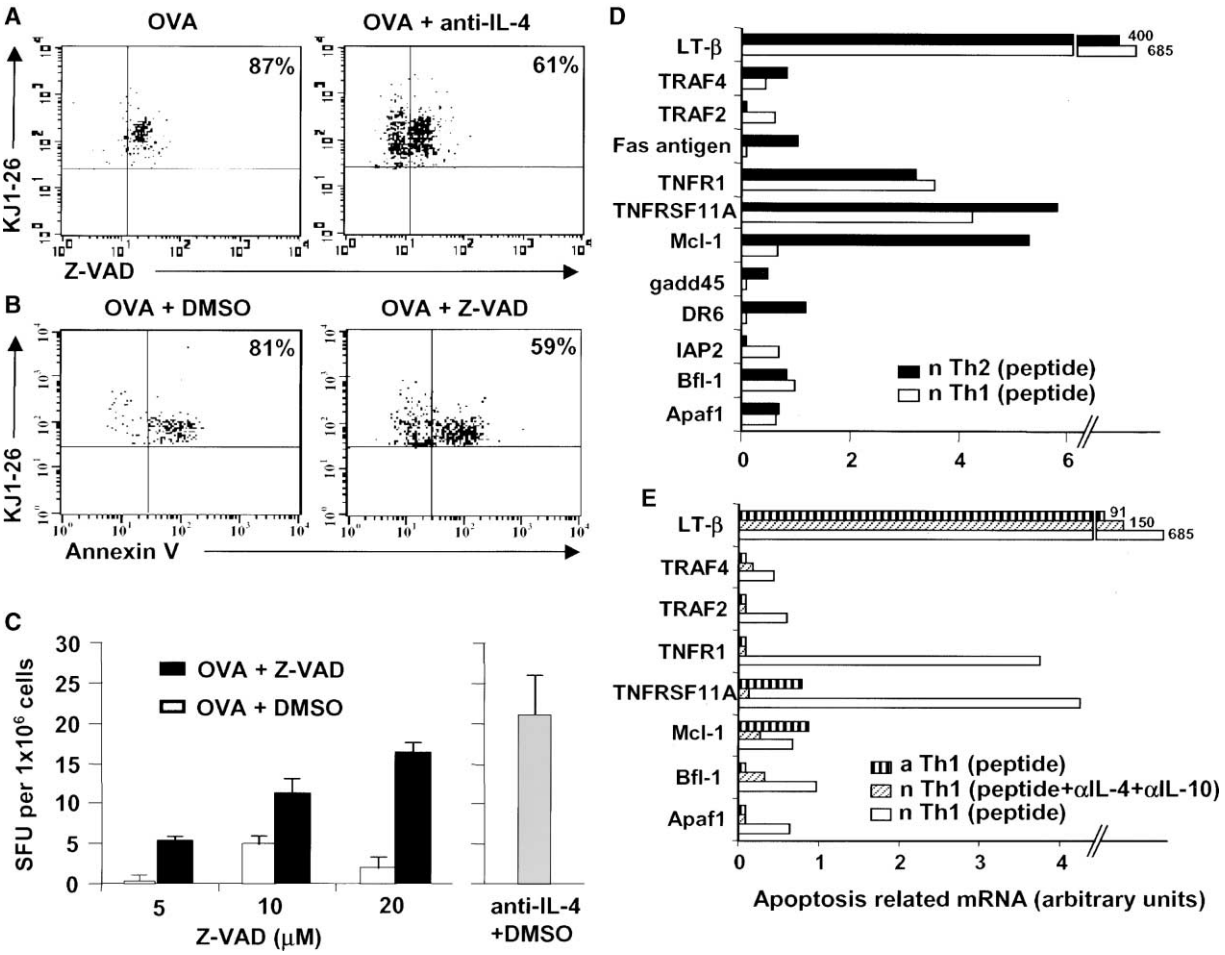

Figure 5. Restoration of Recall Responses by Caspase Inhibitor and Pattern of Apoptosis-Related Gene Expression in Neonatal Th1 Cells

One day old Balb/c mice recipient of neonatal DO11.10 T cells were given 100 μg Ig-OVA and 14 days later the splenic cells were stimulated for 24 hr with OVA peptide (10 μM) in the (A) absence or (B and C) presence of the pan-caspase inhibitor, Z-VAD. The cells were then assessed for apoptosis by staining with (A) FITC-Z-VAD, or (B) Annexin V and for (C) IFNγ production by ELISPOT. In (A) the stimulation was carried out with (right panel) or without (left panel) 20 μg/ml anti-IL-4 antibody and in (B) with (right panel) or without (left panel) 20 μM Z-VAD. The cells were then stained with Annexin V, anti-IFNγ antibody, and KJ1-26. The plots were gated on IFNγ$^+$/KJ1-26$^+$ cells. Each bar in (C) represents the mean ± SD of triplicate wells. (D) shows a GEArray comparison of the expression of apoptosis-related genes in neonatal Th1 versus neonatal Th2 and (E) illustrates similar comparison in neonatal versus adult Th1 cells. Gene expression in neonatal Th1 cells isolated subsequent to peptide stimulation in the presence of anti-IL-4 and anti-IL-10 is included in (E) for control purposes. In (D and E) the newborn Balb/c mice were given 3×10^5 CD4-purified neonatal or adult DO11.10 T cells and 24 hr later injected with 100 μg Ig-OVA in saline. Two weeks later the splenic cells (1×10^7 cells/ml) were stimulated with 10 μM OVA peptide for 10 hr and Th1 and Th2 cells were separated using Miltenyi's IFNγ and IL-4 secretion kits. Gene array was performed as described in Experimental Procedures. The mRNA levels were estimated by normalization with β-actin intensity after deduction of background. Each bar represents an arbitrary unit corresponding to the ratio of a test sample spot intensity divided by the β actin spot intensity.

a cytokine that does not support signaling in Th1 cells can drive apoptosis of these cells. In the face of this dilemma, we sought to carry out gene array analysis for expression of cytokine receptors to determine whether neonatal Th1 cells display any discrepancy that could be correlated with the effects of IL-4. Accordingly, Th1 cells were isolated on the basis of IFNγ secretion and were assessed for cytokine receptor expression in comparison with their Th2 counterparts and adult Th1 cells primed within the neonatal environment. The results presented in Figure 6 indicate that among the 23 cytokine receptor genes tested only IL-13Rα1 chain is upregulated in neonatal Th1 but not neonatal Th2 or adult Th1 cells (Figure 6). Indeed, the spot intensity in Th1 cells increased by 11-fold relative to GAPDH (Figure 6A), while Th2 cells had no such increase (Figure 6B). Adult Th1 cells that mounted recall responses also had no upregulation of IL-13Rα1 chain (spot intensity was 0.98 relative to GAPDH). Some levels of IL-8, IL-10, and IL-18 receptor chains were observed but these were not significant. These results indicate that the sensitivity of neonatal Th1 cells to apoptosis mediated by the cytokines of their Th2 counterparts may be related to a developmental upregulation of IL-13Rα1 chain. This data bodes well with the lack of IL-13Rα1 expression on adult T cells (Graber et al., 1998).

IL-13R includes two chains, α1 and α2, which can be expressed on a variety of cell types and mediate diverse

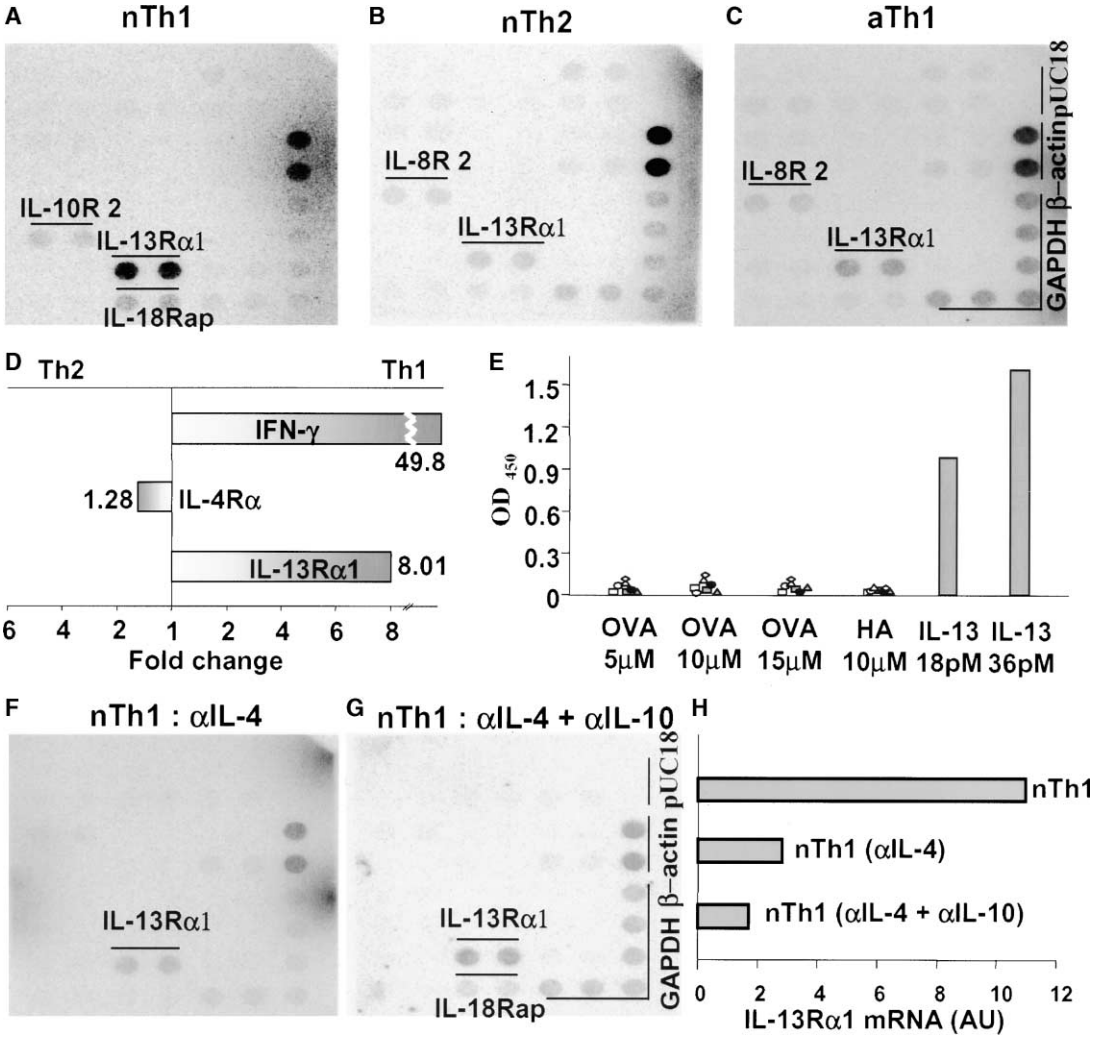

Figure 6. Control of IL-13Rα1 Chain Expression on Neonatal Th1 by IL-4 of the Th2 Counterparts

Newborn Balb/c mice were given 5×10^5 purified CD4-positive neonatal or adult DO11.10 T cells, injected i.p. with 100 μg Ig-OVA in saline, and 2 weeks later the splenic cells (1×10^7 cells/ml) were stimulated for 10 hr with 10 μM OVA peptide (A, B, C, and D) alone, or in the presence of (F) 20 μg /ml anti-IL-4 antibody or (G) 20 μg/ml anti-IL-4 and 20 μg/ml anti-IL-10 antibody. Subsequently, Th1 and Th2 cells were separated and RNA was extracted and used for analysis of cytokine receptor gene expression by array technology (A, B, C, F, and G), and real-time PCR (D). RNA (4.5 μg) from (A) neonatal Th1 (nTh1), (B) neonatal Th2 (nTh2), (C) adult Th1 (aTh1), (F) neonatal Th1 isolated from cells stimulated in the presence of anti-IL-4 (nTh1, αIL-4) and (G) neonatal Th1 isolated from culture stimulated in the presence of both anti-IL-4 and anti-IL-10. Each gene is analyzed in duplicate spots as underlined. The control GAPDH is provided in six spots. The spot intensity of IL-13Rα1 in (A), (F), and (G) are illustrated in (H) as arbitrary units that were estimated as described in Experimental Procedures. In (D), the real-time PCR used 100 ng RNA, and the QuantiTect SYBR Green RT-PCR kit from Qiagen. In (E) splenic cells (1×10^6 cells/well) from seven newborn Balb/c mice were stimulated with OVA peptide and IL-13 production was measured using a Quantikine M kit from R&D Systems as described in Experimental Procedures. The OD_{450} represents the value of the test sample minus the background of splenic cells incubated without peptide.

IL-13-associated functions (Obiri et al., 1997). Interestingly, IL-13Rα1 (Hilton et al., 1996), which binds IL-13, can associate with IL-4Rα and provide a receptor through which both IL-4 and IL-13 can signal (Murata et al., 1998; McKenzie et al., 1999). The next step then was to determine whether neonatal Th1 cells express IL-4Rα. Using real- time PCR, the neonatal Th1 cells were found to have as much IL-4Rα chain mRNA as their Th2 counterparts. Indeed, the fold change in Th2 cells was only 0.28 higher than in Th1 (Figure 6D). In fact, the threshold cycle (C_T) was 7.02 for Th1 and 6.66 for Th2, indicating that both subsets had significant but similar IL-4Rα chain mRNA level. The IL-4Rα chain mRNA found in Th1 cells is not the result of contamination with Th2 cells during the isolation process because the Th2 preparation showed no mRNA for IFNγ while Th1 cells had highly significant levels of such mRNA (Figure 6D). Furthermore, Th1 cells had a significant amount of IL-13Rα1 chain mRNA while Th2 cells displayed a minimal level of such mRNA confirming the results obtained with the gene array analysis.

IL-4 and IL-13 can both use a type II receptor comprising IL-13Rα1 and IL-4Rα chains to perform similar functions (for review see Nelms et al., 1999). Therefore, the

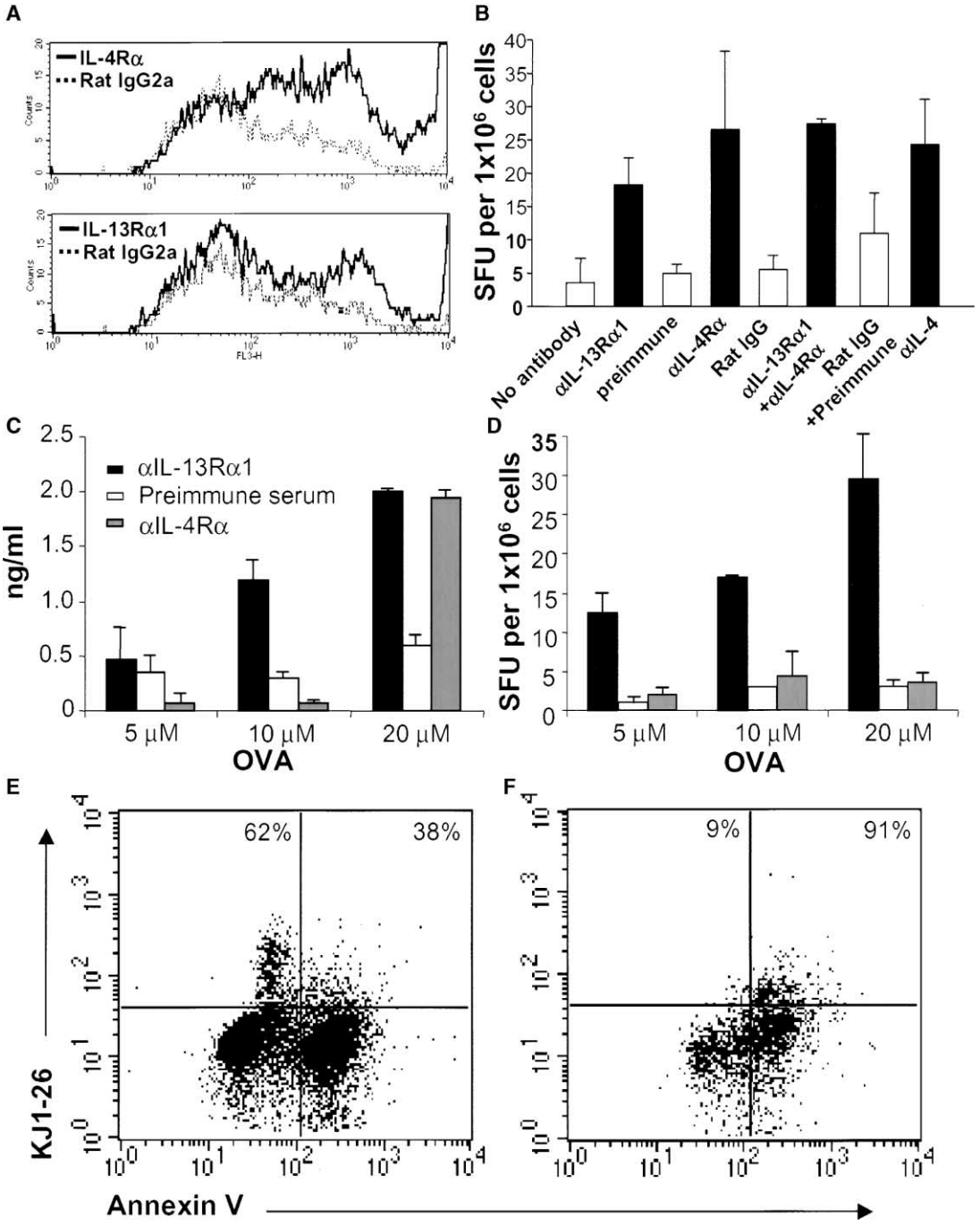

Figure 7. In Vivo Blockade of IL-13Rα1 or IL-4Rα Restores Th1 Responses in Neonatally Primed Animals

Newborn Balb/c mice were given 30 × 10^3 neonatal DO11.10 T cells, and injected i.p. with 100 μg Ig-OVA in saline. (A) Expression of IL-13Rα1 and IL-4Rα chains on the surface of KJ1-26$^+$/IFNγ$^+$ splenic cells was assessed on day 15 post priming. The cells were stimulated with 10 μM OVA peptide for 14 hr with the addition of 10 μg/ml BFA during the last 4 hr, stained with anti-IL-4Rα antibody (M1: Amgen), anti-IL-13Rα1 antibody, C41, or rat IgG2a isotype control and traced with KJ1-26 and intracellular IFNγ. (B) In vitro splenic IFNγ recall responses to OVA peptide were measured by ELISPOT in the presence of 2 μl anti-IL-13Rα1 or control preimmune rabbit serum; 20 μg/ml anti-IL-4Rα antibody, muIL-4R-M1, or 20 μg/ml control rat IgG. (C) Splenic IFNγ secondary responses were measured by ELISA. One month after priming, the mice were challenged with 125 μg OVA peptide in CFA and given 100 μl anti-IL-13Rα1, 100 μg anti-IL-4Rα antibody, or 100 μl preimmune serum 4 hr before and 24 and 48 hr after challenge. The splenic cells were stimulated with 10 μM OVA peptide for 24 hr 10 days after the challenge. (D) Recall splenic IFNγ responses from mice that received 50 μl anti-IL-13Rα1, 10 μg anti-IL-4Rα antibody, or 50 μl control preimmune rabbit serum 4 and 48 hr after neonatal priming with Ig-OVA were measured by ELISPOT on day 15 following priming. (E and F) Apoptosis level in secondary Th1 lymphocytes was measured by Annexin V staining of KJ1-26$^+$/IFNγ$^+$ splenic cells from mice that were challenged with OVA peptide in CFA accompanied by (E) anti-IL-13Rα1 or (F) preimmune serum 1 month after priming with Ig-OVA. The stimulation with 10 μM OVA peptide lasted 24 hr with addition of BFA during the last 8 hr.

primary neonatal Th2 cells may be producing IL-13 alongside of IL-4 and utilize such a cytokine to affect their Th1 counterparts. Alternatively, they may not secrete IL-13, but instead use IL-4 through the type II receptor to drive apoptosis of the Th1 cells. To address this issue, the splenic cells of individual mice recipient of neonatal DO11.10 cells and primed with Ig-OVA were stimulated with OVA peptide and tested for production of IL-13. The results in Figure 6E indicated that IL-13 was below the limit of detectability and the OD_{450} values were similar to those obtained with the negative control HA peptide. Together the findings suggest that IL-4 utilizes a type II IL-13Rα1/IL-4Rα receptor to drive death of Th1 cells. If this hypothesis is correct, then neutralization of IL-4, which prevents apoptosis, may induce downregulation of the expression of IL-13Rα1 on the live neonatal Th1 cells. The lower panels of Figure 6 show that this is indeed the case and IL-13Rα1 chain mRNA expression was reduced from 11 to 2 to 3 units upon neutralization of IL-4 or both IL-4 and IL-10 (Figures 6F, 6G, and 6H). These results indicate that IL-13Rα1 expression on Th1 cells is under control of IL-4 of the Th2 counterparts.

Blockade of IL-4Rα and/or IL-13Rα1 Restores Neonatal Th1 Recall and Secondary Responses

If IL-4 of the Th2 cells is utilizing a heteroreceptor to drive apoptosis of Th1 cells then blockade of IL-4Rα and/or IL-13Rα1 should restore the recall as well as secondary neonatal Th1 responses. Before addressing this issue, we investigated whether IL-13Rα1 and IL-4Rα proteins are expressed on the surface of Th1 cells. Figure 7A shows that neonatal T cells producing intracellular IFNγ and positive for KJ1-26 bind IL-4Rα-specific muIL-4R-M1 (top panel) antibody much higher than the rat IgG2a isotype control. Also, the cells were significantly stained with the IL-13Rα1-reactive nonneutralizing C41 antibody (lower panel). These results indicate that primary Th1 cells express IL-4Rα and IL-13Rα1 on the surface. These receptors play a role in the downregulation of primary neonatal Th1 cells by IL-4 because blockade with anti-IL-4Rα and/or anti-IL-13Rα1 antibodies restores IFNγ recall responses of the Th1 cells (Figure 7B). Indeed, splenic cells from mice recipient of DO11-10 T cells and given Ig-OVA on the day of birth were able to produce IFNγ upon in vitro stimulation with OVA peptide in the presence of rabbit anti-IL-13Rα1 anti-serum, and/or anti-IL-4Rα antibody to the same extent as neutralization of IL-4 cytokine. Rabbit preimmune serum and/or rat IgG controls had no significant effect on the response and IFNγ was at levels similar to stimulation with OVA peptide without any blockade. These results indicate that both IL-4Rα and IL-13Rα1 are involved in IL-4-mediated targeting of primary Th1 cells during recall with OVA peptide in vitro. Subsequently, we investigated whether blockade of IL-4Rα and/or IL-13Rα1 in vivo during a later challenge with OVA peptide restores Th1 secondary responses. The findings indicate that mice recipient of DO11.10 T cells and Ig-OVA/saline on the day of birth and 1 month later administered with anti-IL-13Rα1 antiserum or anti-IL-4Rα antibody during immunization with OVA peptide in CFA develop IFNγ responses (Figure 7C). Preimmune serum used as control did not allow for secondary IFNγ responses. It is worth noting that development of IFNγ responses upon in vivo blockade of IL-4Rα required high amounts of OVA peptide for stimulation in vitro. As Th1 cells manifest high expression of IL-4Rα (Figure 7A), it may be that fewer cells were rescued by anti-IL-4Rα antibody, thus leading to the requirement for high peptide concentration to read out a low frequency of cells. Overall, these results support the in vitro data and indicate that both IL-4Rα and IL-13Rα1 are involved in IL-4-mediated targeting of Th1 cells in vivo during challenge with OVA peptide.

Neutralization of IL-4 during priming with Ig-OVA on the day of birth restored Th1 responses (Figure 3). Blockade of IL-13Rα1 in vivo during priming with Ig-OVA also restored IFNγ recall responses while preimmune serum used as control did not (Figure 7D). However, blockade of IL-4Rα during priming with Ig-OVA did not restore IFNγ responses. This is possibly due to the fact that naive T cells may not polarize toward Th2 as IL-4Rα is blocked and transient IL-4 needed for development of Th1 cells may be lacking (Biedermann et al., 2001).

Finally, blockade of IL-13Rα1 in vivo during challenge with OVA peptide reduced apoptosis among Th1 cells as only 38% of cells were stained with Annexin V (Figure 7E). Mice given preimmune serum instead of anti-IL-13Rα1 antiserum had 91% of their KJ1-26-positive IFNγ-producing cells stain positive with Annexin V (Figure 7F). These results indicate that blockade of IL-13Rα1 on primary Th1 cells during challenge with OVA peptide allows the cells to remain alive and develop secondary responses.

Discussion

Upon priming with antigen, neonatal T cells were activated, abandoned their naive phenotype, and proliferated in vivo (Figure 1). Some of the cells differentiated into Th2 cells but others, surprisingly, developed as Th1 cells (Figure 2). Intriguingly, however, upon reencounter with the antigen, the Th2 responded and produced both IL-4 and IL-10 but the Th1 cells were unable to sustain IFNγ production and died by apoptosis (Figure 2). Blockade of the Th2 cytokines during recall with antigen in vitro reduced Th1 apoptosis and sustained IFNγ production (Figure 3). Similarly, when anti-Th2 cytokine antibodies were administered to the host mice during neonatal priming with antigen, recall Th1 responses developed (Figure 3). Furthermore, when adult T cells were placed within the neonatal environment and primed with antigen, Th1 recall responses also arose (Figure 4). Together, these results indicated that the Th2 cytokines are not affecting the development of Th1 from naive cells (Stetson et al., 2002), but are most likely sensitizing differentiated Th1 cells for apoptosis. In addition, such sensitivity seems to reflect a developmental trait unique to neonatal Th1 cells as adult T cells primed within the neonatal environment generated Th1 cells resistant to the Th2 cytokines.

IL-4 of the Th2 cells seems to interfere with Th1 recall responses by triggering apoptosis of primary Th1 cells rather than inhibition of differentiation into IFNγ-producing cells. Indeed, when recall of the cells was performed

in the presence of a caspase inhibitor, apoptosis was significantly reduced and IFNγ production was restored (Figures 5A, 5B, and 5C). Furthermore, gene array analysis indicated that primary Th1 cells display unbalanced apoptotic machinery relative to their Th2 counterparts as well as adult T cells primed within the neonatal environment (Figures 5D and 5E). Seemingly, an inability to upregulate the antiapoptotic factor MCL-1 may be responsible for IL-4-induced Th1 apoptosis.

IL-4 usually directs differentiation of naive T cells toward Th2 but has no defined downregulatory functions on differentiated Th1 cells (Reiner and Seder, 1999; Morel and Oriss, 1998; Nelms et al., 1999). IL-10 has been shown to display antiproliferative (anergic) functions on Th1 cells mostly through downregulation of accessory molecules on APCs or through inhibition of IL-12 production by the APCs (Moore et al., 2001). The results presented here show that the unresponsiveness and apoptosis of differentiated neonatal Th1 cells are primarily due to negative effects by IL-4, with IL-10 playing only a secondary role (Figures 2 and 4). Gene array analysis of cytokine receptor expression on neonatal Th1 cells indicated that expression of the IL-13Rα1 chain is upregulated in neonatal Th1 but not in neonatal Th2, or even adult Th1 cells. Also, such upregulation is decreased when IL-4 is neutralized (Figure 6). It is well established that the conventional IL-4R (IL-4Rα/common γ) mediates IL-4 signaling in Th2 but not in Th1 cells (Huang and Paul, 1998). However, IL-13Rα1 can associate with IL-4Rα and provide a receptor through which both IL-4 and IL-13 can signal (Hilton et al., 1996; Murata et al., 1998; McKenzie et al., 1999). Given that the neonatal Th2 did not produce IL-13 and neutralization of IL-4 leads to a reduction in Th1 apoptosis and a decrease in IL-13Rα1 expression (Figure 6), it may be that IL-4 utilizes an IL-4Rα/IL-13Rα1 heterodimer complex to signal in neonatal Th1 cells as has been shown in other cells (Murata et al., 1998; McKenzie et al., 1999). IL-4Rα is constitutively expressed on adult Th1 and Th2 cells (Huang and Paul, 1998) and real-time PCR analysis indicated that neonatal Th1 and Th2 cells also express IL-4Rα mRNA (Figure 6). Thus, knowing that there has been no defined direct effect on differentiated Th1 cells by IL-10 (Moore et al., 2001) and neutralization of IL-4 diminishes both upregulation of IL-13Rα1 and death among Th1 cells, it is possible that IL-4 operates through an alternative heteroreceptor comprising IL-4Rα and IL-13Rα1 chains to induce apoptosis of neonatal Th1 cells. This is supported by findings indicating that IL-4Rα and IL-13Rα1 proteins are expressed on the surface of the neonatal Th1 cells and blockade of either receptor during in vitro stimulation supported recall of the Th1 cells (Figures 7A and 7B). In addition, when the blockade of either IL-4Rα or IL-13Rα1 was performed in vivo during rechallenge with antigen in CFA at the adult stage, secondary Th1 responses developed (Figure 7C). Again, the restoration of the secondary IFNγ response correlated with reduction in Th1 apoptosis (Figures 7E and 7F). Another piece of evidence that the expression of IL-13Rα1 is under developmental control originates from the observation that administration of anti-IL-13Rα1 during priming at the neonatal stage restored recall responses of the Th1 cells. Blockade of IL-4Rα, however, did not produce similar effects, possibly due to the lack of polarization to Th2, which are required to produce some IL-4 needed for induction of IL-12 production by dendritic cells and differentiation of Th1 cells (Biedermann et al., 2001).

Overall, it is shown that priming with antigen at the neonatal stage induces both Th1 and Th2 primary responses. However, IL-4 of the Th2 cells induces a developmental upregulation of IL-13Rα1 on the neonatal Th1 counterparts and this receptor chain along with IL-4Rα provides an alternative receptor that marks the Th1 cells for death if reencounter with antigen occurs in an IL-4-containing environment. It is possible that neonatally primed Th1 cells succumb to IL-4 due to signaling through an IL-4Rα/IL-13Rα1 heteroreceptor that unbalances expression of pro- and antiapoptotic factors.

Experimental Procedures

Mice

Balb/c mice (H-2^d) were purchased from Harlan Sprague Dawley (Indianapolis, IN) and used as hosts for adoptive transfer of T cells. DO11.10/scid transgenic mice expressing a TCR specific for OVA peptide were used as donors of T cells.

Antigens

OVA peptide (SQAVHAAHAEINEAGR) encompasses aa residues 323-339 of OVA and is immunogenic in Balb/c (H-2^d) mice. Influenza virus hemagglutinin (HA) aa residues 110-120 peptide (SFERFEIF PKI), which is also immunogenic in Balb/c mice, was used as negative control. Ig-W, a Balb/c IgG2b Ig molecule generated by transfection of the 91A3 anti-arsonate antibody heavy and light chains into the non-Ig secreting myeloma B cell line SP2/0 was described (Min et al., 1998). Ig-OVA, expressing OVA peptide within the heavy chain variable region of Ig-W, was also described (Li et al., 2001).

Adoptive T Cell Transfer

Splenic cells from 1 day old DO11.10/scid mice containing the equivalent of 3×10^4 DO11.10 T cells were transferred into 1 day old Balb/c mice by intravenous (i.v.) injection through the facial vein using a 30-gauge needle. For transfer of adult T cells into newborn mice, DO11.10 T cells were purified from the spleen of adult DO11.10/scid mice with anti-CD4 coupled magnetic beads (Miltenyi Biotec, Auburn, CA) prior to injection into the host. This step was necessary to eliminate adult APCs that might interfere with antigen presentation within the neonatal environment. For isolation of Th1 and Th2 cells, 3×10^5 purified neonatal or adult CD4$^+$-T cells were transferred into each mouse. This increase in T cell transfer was required for the isolation of sufficient numbers of T cells for RNA extraction and gene array analysis.

Antireceptor Antibodies

The anti-IL-4Rα monoclonal antibody (M1) was provided by Amgen (Seattle, WA). The nonneutralizing anti-IL-13Rα1 monoclonal antibody C41 was previously described (Poudrier et al., 2000). Rabbit antiserum against the extracellular domain of the murine IL-13Rα1 chain was developed as previously described (Schnare et al., 1998).

Cell Surface and Intracellular Staining

For cell surface staining the spleen cells from Balb/c hosts were incubated (20 min at 4°C) with 5 μg/ml 2.4G2 mAb to block FcγRs on the cell surface prior to staining. The cells were then stained with the anti-TCR OVA clonotypic mAb, KJ1-26 (mouse IgG2a), and the culture was supplemented with FITC-anti-mouse IgG2a (clone R19-15), PerCP-anti-CD4 and PE-anti-CD25 (clone 3C7), PE-anti-CD62L (clone Mel-14), or PE-anti-CD45RB (clone 16A). For ex vivo intracellular cytokine staining, the cells were stimulated for a 4 hr short period of time to enhance cytokine accumulation and facilitate intracellular detection as has been previously defined (Moser et al., 2001). Cytokine secretion was blocked by addition of 10 μg/ml BFA (Epicentre, Madison, WI), and the cells were then fixed with 2% formaldehyde, permeabilized with 2% saponin (Sigma Chemical Co.)

in FACS buffer for 10 min at room temperature, and incubated with PE-anti-mouse IL-4, and FITC-anti-mouse IFN-γ. Isotype-matched controls were included in all experiments. All the data were collected using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA) and analyzed with CellQuest software (Becton Dickinson).

Analysis of Cell Division In Vivo

Purified $CD4^+$-neonatal DO11.10 T cells were labeled with the intracellular fluorescent dye, 5(and 6)-carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes, Eugene, OR), as described (Lyons and Parish, 1994) and transferred into newborn Balb/c mice (3×10^5 cells/mouse). The hosts were then injected i.p. with 100 μg OVA peptide, Ig-OVA, or Ig-W in saline. Four days later, the $CD4^+$-T cells were isolated from the spleen of Balb/c hosts and stained with KJ1-26 and anti-CD4 mAbs.

Cytokine ELISA and ELISPOT

Measurement of Cytokines by ELISA

Spleen cells containing both T cells and APCs were incubated with antigen in 96-well round-bottom plates and 24 hr later IL-4, IL-10, and IFNγ production was measured by ELISA using anti-cytokine antibodies according to BD PharMingen's instructions. IL-13 production was measured using the Quantikine M kit from R&D Systems (Minneapolis, MN) according to the manufacturer's instructions.

Measurement of IFNγ by ELISPOT

Detection of IFNγ by ELISPOT was carried out as described (Min et al., 1998). In brief, HA-multiscreen plates (Millipore, Bedford, MA) were coated with capture antibody and free sites were saturated with DMEM culture media containing 10% fetal calf serum. Subsequently, 1×10^6 splenic cells were added and the culture was stimulated with OVA peptide with or without blocking antibody. Biotinylated anti-IFNγ antibody (1 μg/ml) was added and bound antibody was revealed with avidin-peroxidase. Spots were counted under a dissecting microscope.

Detection of Early Apoptosis by Staining with Annexin V

Spleen cells (1×10^6 cells/ml) were stimulated with antigen, incubated for 30 min with KJ1-26 mAb, and the culture was supplemented with biotin-anti-mouse IgG2a. The cells were then incubated with PerCP-Avidin and 30 min later resuspended in Annexin V staining buffer. Subsequently, the cells were stained with FITC-labeled Annexin V (PharMingen) for 15 min at room temperature, washed, and fixed with 2% formaldehyde. To assess for early apoptosis of Th1 and Th2 lymphocytes, the cells were permeabilized and intracellular cytokine production was analyzed as described above.

Detection of Caspase Activation and Inhibition of Apoptosis by Z-VAD

Spleen cells (1×10^6 cells/well) were stimulated with 10 μM OVA peptide for 24 hr with addition of BFA (10 μg/ml) during the last 6 hr of incubation. Subsequently, the cells were stained with 5 μM FITC-conjugated Z-VAD (CaspACE FITC-VAD-fmk from Promega Corp., Madison, WI) for 30 min to detect activated caspases. For identification of Th1 cells, the culture was also costained with KJ1-26 and anti-IFNγ antibody. For blockade of apoptosis, OVA stimulation was done in the presence of graded amounts of unlabeled Z-VAD-fmk (Calbiochem, La Jolla, CA) for 24 hr with addition of BFA during the last 6 hr of incubation. The cells were then stained with Annexin V, KJ1-26, and anti-IFNγ antibodies. Since Z-VAD is solubilized in DMSO as recommended by the manufacturer, the controls are diluted in DMSO to account for cell death that could be mediated by side effects associated with DMSO.

Separation of Th1 and Th2 Cells

The separation of neonatal Th1 and Th2 cells requires an increased frequency of primary T lymphocytes. Thus, the transfer was performed with CD4-purified neonatal DO11.10/scid T lymphocytes instead of total splenic cells and the number of transferred cells was increased from 30 to 300-500 $\times 10^3$ cells per newborn Balb/c host. Accordingly each Balb/c newborn was given either neonatal (1 day old) or adult (8 week old) purified DO11.10/scid cells and injected i.p. with a saline solution containing 100 μg Ig-OVA. Two weeks later, the splenic T cells were stimulated for 10 hr with 10 μM OVA peptide and separated into Th1 and Th2 on the basis of cytokine secretion (IFNγ for Th1 and IL-4 for Th2) as described (Ouyang et al., 2000) using separation kits from Miltenyi Biotech. Usually, 10^8 splenic cells are used to obtain 0.5 to 1×10^6 Th1 or Th2 cells.

Gene Array Analysis

mRNA expression in isolated neonatal and adult T cells was analyzed using both the GEArray Q series kit including 96 key genes involved in apoptosis, and interleukin receptor GEArray kit comprising 23 genes of interleukin receptor subunits (SuperArray Inc., Bethesda, MD). Total RNA was extracted from 0.5–1×10^6 purified neonatal Th1, adult Th1 or neonatal Th2 cells using TRIzol reagent (Life Technologies, Rockville, Maryland). The RNA (3 to 4.5 μg) was then used in a reverse transcription reaction along with α-^{32}P-dCTP (ICN Biomedical, Inc., Aurora, OH) to generate labeled cDNA probes. Subsequently, for the Q series kit, the labeled cDNA probes were hybridized to the membrane, in a 0.75 ml hybridization buffer for 14 hr at 60°C. For the interleukin receptor membrane, the hybridization was carried out in a 5.1 ml buffer for 14 hr at 68°C. The intensity of radioactive spots was analyzed on a Bio-Rad molecular imager FX (Hercules, California) using Quantity One software. Results are expressed as arbitrary units estimated as follows: (mean spot intensity of sample – mean spot intensity of background) / (mean spot intensity of β-actin – mean spot intensity of background). The background represents the mean radioactive intensity obtained from pUC18 DNA spots included in the membrane.

Real-Time PCR

Reverse transcription and DNA amplification were performed according to a one-step protocol using 100 ng of total RNA and a QuantiTect SYBR Green RT-PCR kit from Qiagen (Valencia, CA) according to the manufacturers' instructions. Expression of IL-4Rα chain and IL-13Rα1 chain mRNA was assessed in both Th1 and Th2 neonatal T cells. Expression of IFNγ mRNA was included to assess for the purity of the Th1 and Th2 populations. β-actin was included to serve as a normalizer. The oligonucleotides used as specific primers were: sense IFNγ, TCAAGTGGCATAGATGTGGAAGAA; IL-4Rα, CTTGCAGACAATCCTGCCTA; IL-13Rα1, GCACAGAGTATAGG TAAGGAGCAA; β−actin, AGAGGGAAATCGTGCGTGAC; and antisense, IFN-γ, TGGCTCTGCAGGATTTTCATG; IL-4Rα, GGCTCCTCT TCTTCCAGATG; IL-13Rα1, ACAAAGACTGGAATGGTGAGTAAC; β−actin, 5′-CAATAGTGATGACCTGGCCGT. Real-time PCR was performed on a Cepheid Smart Cycler (Sunnyvale, CA) and the results were analyzed by the Comparative C_T method described in the Smart Cycler Software.

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