

Children with egg allergy have evidence of reduced neonatal CD4⁺CD25⁺CD127^{lo/-} regulatory T cell function

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Background: The role of regulatory T (Treg) cells in allergic predisposition is not known.

Objective: This study compared the frequency and function of cord blood Treg cells from nonallergic children (n = 18) with those from children who have egg allergy (n = 15) in the first year of life.

Methods: CD4⁺ effector T cells and autologous antigen-presenting cells isolated from cord blood mononuclear cells were cocultured with or without CD4⁺CD25⁺CD127^{lo/-} Treg cells, and cytokine responses to staphylococcal endotoxin B were assessed after 48 hours.

Results: The addition of Treg cell populations to cord blood mononuclear cell cultures resulted in significant reduction in IL-10 ($P = .002$), IL-13 ($P = .012$), and IFN- γ ($P < .001$) production. Consistent with other reports, effector CD4⁺ T-cell responses (IFN- γ and IL-13) tended to be lower in the allergic group. These neonates showed less significant Treg cell-associated suppression of IFN- γ ($P = .015$) compared with that seen in the nonallergic group ($P = .001$). The allergic group was also less likely (44%) to show Treg cell-associated suppression of IFN- γ effector responses compared with that seen in the nonallergic group (78%, $P = .015$). The magnitude of suppression (change in IFN- γ level when CD4⁺CD25⁺CD127^{lo/-} Treg cells were added to responding effector T-cell cultures) was significantly lower in the allergic group ($P = .004$). There were no between-group differences in the circulating CD4⁺CD25⁺CD127^{lo/-} Treg cells (as a percentage of cord blood T cells) or in the FOXP3 expression of these cells.

Conclusion: This study confirms the presence and activity of Treg cells in cord blood and provides preliminary evidence of differences in neonates who progress to allergic disease in the first year of life. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

Key words: Regulatory T cells, cord blood, neonates, food allergy, allergic disease, egg allergy, cytokines, infants

Regulatory T (Treg) cells play a master role in controlling adaptive immune response and maintaining tolerance to innocuous self-proteins and environmental proteins. There is growing speculation that impairment in these pathways could be central in the pathogenesis of many immune diseases. This is the basis of the emerging hypothesis that modern environmental changes might be impairing the development of these pathways and increasing the susceptibility to both allergic disease and autoimmunity.¹ Although several studies suggest deficiencies in allergen-specific Treg cell numbers or function in allergic disease,²⁻⁴ others have shown the opposite.^{5,6} This might be due, at least in part, to difficulties in accurately identifying CD4⁺ Treg cells by using conventional cell markers. Although CD4⁺ T cells typically express CD25 (the IL-2 receptor α chain), so do activated effector T cells. Similarly, FOXP3, a characteristic marker of regulatory activity,⁷ might also be transiently expressed by effector T cells during activation.⁸ Furthermore, because FOXP3 is expressed intracellularly, it cannot be used readily to isolate cells for functional studies. More recently, CD127 (the IL-7 receptor α chain) expression has been used (in combination with other markers) to better discriminate Treg cells (that have significantly lower CD127 expression) from conventional T cells.⁹ However, thus far, few studies have used this technique to examine for potential differences in Treg cell function in association with allergic disease.

With escalating rates of allergic disease in infancy, it is evident that immune dysregulation is an early event. However, it is not clear whether the relative immaturities that have been associated with allergic disease¹⁰⁻¹⁶ extend to regulatory pathways. The only preliminary study to examine Treg cells in this context found that neonates at high atopic risk (familial allergic disease) generated reduced numbers of CD4⁺CD25⁺ cells after stimulation.¹⁷ However, this study did not examine regulatory function directly. For the first time, in the present study, we have used the more definitive strategy for identifying CD4⁺CD25⁺CD127^{lo/-} regulatory cells⁹ and identifying functional properties to examine for pre-symptomatic differences in the activity and frequency of these populations in cord blood from allergic and nonallergic children.

METHODS

Study population

Eighteen nonallergic children and 15 children with documented allergic disease (sensitization to egg and evidence of allergic symptoms) were selected from ongoing prospective birth cohorts. Only children of nonsmoking mothers were included. The allergic status of all mothers was assessed based on a clinical history and skin prick test (SPT) reactivity to a standard panel of allergens comprising house dust mite (HDM), perennial rye, molds, cat pelt,

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Abbreviations used

CBMC: Cord blood mononuclear cell
HDM: House dust mite
OVA: Ovalbumin
SEB: Staphylococcal endotoxin B
SPT: Skin prick test
Treg: Regulatory T

southern grass mix, cockroach extracts, dog pelt and feathers (Hollister-Stier Laboratories, Spokane, Wash).

Definitions of infant allergic outcomes

Infants' allergic status was based on clinical evaluations at 12 months of age, when children had a detailed history and examination by the same pediatric allergist (Professor Susan Prescott). *Nonallergic* children were defined as those without sensitization or any clinical disease at 1 year of age. The *allergic* children were selected if they had a positive SPT response to egg and evidence of clinical allergic disease (food reactivity, associated atopic dermatitis, or both). The diagnostic criteria conformed to the recently published clinical guidelines.¹⁸ *IgE-mediated food allergy* was defined as a history of immediate (within 60 minutes) symptoms after contact with food, ingestion of food, or both and a positive SPT response to the implicated food. A diagnosis of atopic dermatitis was made in infants with typical skin lesions¹⁹ responsive to topical steroids. Other symptoms were also assessed based on parental recording (on prospective diary cards). Parents who noted "noisy breathing" of any kind were asked to give detailed descriptions of this to the pediatrician (if not already confirmed by another physician). Only children with a convincing history of "wheeze" (or physician-documented wheeze) were classified in this category.

Allergen SPTs were performed with a standardized technique and common allergen extracts (milk, peanut, house dust mite, cat, grass, and mold; Hollister-Stier Laboratories) and whole egg, as well as histamine as a positive control and glycerin as a negative control. A wheal diameter of 3 mm or larger was considered positive.

Umbilical cord blood samples

Blood samples were collected by means of venipuncture of the umbilical cord from deliveries at term (>37 weeks) into heparinized RPMI (Life Technology, Paisley, United Kingdom) tissue culture medium. Cord blood mononuclear cells (CBMCs) were isolated by means of density gradient centrifugation with Lymphoprep (Nycomed Pharma, Oslo, Norway) and cryopreserved by using standard techniques for future batch analysis, as previously published.²⁰ It was not logistically feasible to perform the cell-separation experiments (below) on fresh samples, and we have previously shown that cryopreservation does not significantly alter cell function (see Introduction text and Figs E1 and E2 in the Online Repository at www.jacionline.org).

Cell preparation and isolation techniques

Except where specified, a solution of RPMI 1640 medium (without phenol red; Gibco, Life Technology) supplemented with 2% heat-inactivated FCS (Australian Biosearch, Perth, Australia) and 10 μ g/mL Deoxyribonuclease 1 (DN-25; Sigma Chemical Company, St Louis, Mo) was used for all washes and cell resuspensions. All washes were performed at 500g for 10 minutes at 4°C. CBMCs (40×10^6) were thawed, counted, and assessed for viability by means of trypan blue exclusion. An aliquot of 2×10^6 CBMCs from each individual were depleted of CD3 cells by labeling with magnetic CD3 Dynalbeads (Dynal Biotech, Invitrogen, Victoria, Australia), according to the manufacturer's instructions. The remaining cells were stained simultaneously with anti-human CD4 fluorescein isothiocyanate (Becton Dickinson Immunocytometry Systems, San Jose, Calif), phycoerythrin-cyanin 5 mouse anti-human CD25 (BD PharMingen, San Diego, Calif), and phycoerythrin-conjugated mouse anti-human CD127 (BD PharMingen) for 25 minutes on

ice in the dark. After this, the cells were washed and filtered through a sterile piece of nylon gauze (Australian Filter Specialists, Maddington, Australia) to remove cell clumps.

Cells were sorted with a FACSaria (BD Biosciences, San Jose, Calif) by first gating on their forward-scatter/side-scatter profiles, and then CD4⁺ cells were excluded (performed by Dr Michelle R. Tourigny, PhD, Research Assistant and Instrument Specialist). Cells were then sorted into 2 populations based on whether they expressed CD25 and their levels of CD127 expression. Specifically, cells were sorted into 2 populations: CD4⁺CD25⁺CD127^{low} (Treg cells) and CD4⁺CD25⁺CD127⁺ (CD4⁺ effector T cells). Cells were collected into RPMI 1640 medium (Gibco, Life Technology) supplemented with 10% heat-inactivated FCS and 10 μ g/mL DNase. Purities of each of these populations were routinely between 91% and 99.9%.

CD3 depletions were routinely monitored by using standard flow cytometry. Briefly, cells were labeled with fluorescein isothiocyanate mouse anti-human CD3 (BD PharMingen) for 25 minutes on ice in the dark before being washed and fixed in 1% paraformaldehyde. Purity was assessed with a FACSCalibur (BD Biosciences) and FlowJo software (TreeStar, Ashland, Ore), and greater than 98% of the target population was routinely removed.

Cell culture

After sorting, cells were counted and cultured as outlined below in AIM-V serum-free medium (Gibco, Life Technologies) supplemented with 4×10^{-5} mol/L 2-mercaptoethanol (Sigma) in 96-well round-bottom culture plates (NUNC, Roskilde, Denmark). Briefly, 5×10^4 CD3-depleted CBMCs were cocultured with 2.5×10^4 autologous CD4⁺CD25⁺ cells (effector cells) and then either with or without 2.5×10^4 autologous Treg cells (defined as CD4⁺CD25⁺CD127^{low}). Autologous CD4⁺CD25⁺ effector cells (2.5×10^4) were irradiated to impair function and added to the wells that did not contain Treg cells to control for the increased number of cells per well in the cultures that contained Treg cells. In separate experiments irradiation was shown to markedly inhibit the production of cytokines by neonatal effector cells (generally by more than 100-fold) and not to alter the suppressive activity of Treg cells (see Introduction text and Figs E3-E5 in the Online Repository at www.jacionline.org). Cultures were performed in a final volume of 200 μ L at 37°C with 5% CO₂ either alone (control) or stimulated for 48 hours with ovalbumin (OVA; 100 μ g/mL; Sigma, Castle Hill, Australia) or staphylococcal endotoxin B (SEB; 200 ng/mL; Sigma-Aldrich, St Louis, Mo).

Cytokine detection

At the end of the culture period, cell supernatants were collected and stored at -20°C before cytokine analysis, and cell pellets were resuspended in 100 μ L of RNA-later (Ambion, Austin, Tex) and stored at -20°C for RNA extraction and subsequent RT-PCR. Cytokine protein levels above background were determined by using an in-house time-resolved fluorometry detection system (DELTA; Wallace, Turku, Finland), as previously published.²¹ The limit of detection was 10 pg/mL for each cytokine. Cytokine data are expressed as Δ values between stimulated and control cultures and as detected (>10 μ g/mL) or not detected.

mRNA analysis (FOXP3 and IL-2)

Total RNA was extracted from cell pellets that had been stored in RNeasy by using the RNeasy 96 Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions, and included a DNase treatment step to remove confounding genomic DNA. Briefly, 3.5 μ L of Turbo DNase buffer (Ambion) and 0.7 μ L of Turbo DNase (Ambion) were added per 35 μ L of RNA, and samples were placed on a heat block at 37°C for 20 to 30 minutes. Then 1.4 μ L of 0.125 M EDTA (Affymetrix, Santa Clara, Calif) was added to each RNA sample, and samples were heated at 75°C for 10 minutes.

Reverse transcription was performed with the Omniscript Kit (Qiagen) with Oligo-dT (Promega, Madison, Wis) and RNasin Ribonuclease Inhibitor (Promega), according to the manufacturer's protocol. Reverse-transcribed RNA samples were diluted 1:5 and stored at -20°C. A liquid-handling robot with vacuum manifold (Multiprobe II Robotic 4 Tip Liquid Handling System, PerkinElmer, Fremont, Calif) was used to set up the real-time PCR reactions

TABLE I. Clinical characteristics of the population at 12 months of age

Clinical characteristics	Nonallergic group (n = 18)	Allergic group (n = 15)	P value
Positive maternal SPT response, (n)	0% (0)	100% (15)	.001
Infant sex, (n)			
Male	39% (7)	53% (8)	.87
Female	61% (11)	47% (7)	
Positive infant SPT response to egg at 1 y, (n)	0% (0)	100% (15)	.001
Positive infant SPT response to other allergens at 1 year, (n)	0% (0)	66.7% (10)	.001
Peanut	—	53.3% (8)	
Cow's milk	—	13.3% (2)	
Other foods	—	6.7% (1)	
Dust mite	—	20.0% (3)	
Cat	—	13.3% (2)	
Pollen	—	6.7% (1)	
Allergic disease Diagnosed at 1 y, (n)	0% (0)	100% (15)	.001
Acute clinical reactivity (<1 h) to egg at 1 y, (n)	0% (0)	100% (15)	.001
Urticaria	—	80.0% (12)	
Angioedema	—	40.0% (6)	
Respiratory distress	—	13.3% (2)	
Gastrointestinal	—	6.7% (1)	
Atopic dermatitis at 1 of age, (n)	0% (0)	80% (12)	.001
Any allergic Symptoms at 1 y, (n)	0% (0)	100% (15)	.001
Recurrent wheeze at 1 y, (n)	0% (0)	33.3% (5)	.001
SPT ⁺	—	26.6% (4)	
SPT ⁻	—	6.7% (1)	

(courtesy of Telethon Institute for Child Health Research, Western Australia). FOXP3 and IL-2 expression was quantitated by means of real-time PCR with QuantiTect SYBR Green Master Mix (Qiagen) and the ABI PRISM 7900HT (Applied Biosystems, Uppsala, Sweden). The FOXP3 primer sequence used was produced commercially by Proligo (Sigma-Aldrich; forward primer: GAA ACA GCA CATT CCC AGA GT; reverse primer: ATG GCC CAG CGG ATG AG). Melting-curve analysis was used to assess the specificity of the assay. Copy numbers were determined by using 10-fold serial dilutions of either PCR product or plasmid standards and normalized to the reference gene ubiquitin-conjugating enzyme E2D2.²² Data are expressed as expression level above background (level in stimulated cells minus unstimulated cells).

Statistical analysis

Statistical analysis was performed with SPSS software (version 11 for Mac OS X, SPSS, Inc, Chicago, Ill). Groups were compared by using the Mann-Whitney *U* test for (nonparametric) unpaired responses. The Wilcoxon matched-pairs signed-rank test was used to examine paired data (ie, differences between cultures with or without Treg cells present). Between-group differences in dichotomous data (ie, whether Treg cells suppressed effector responses) were determined by using the χ^2 test. Correlations (ie, between FOXP3 expression and Treg cell function) were assessed by using the Spearman or Kendall tau (τ) b (where a proportion of the variables of interest shared zero values) to avoid the problems associated with ties within the data. A *P* value of less than .05 was considered statistically significant for all analyses.

RESULTS

Population characteristics

Two discrete groups were selected for comparison, and the characteristics of these are shown in Table I. The allergic group (*n* = 15) was selected to have sensitization to egg with clinical

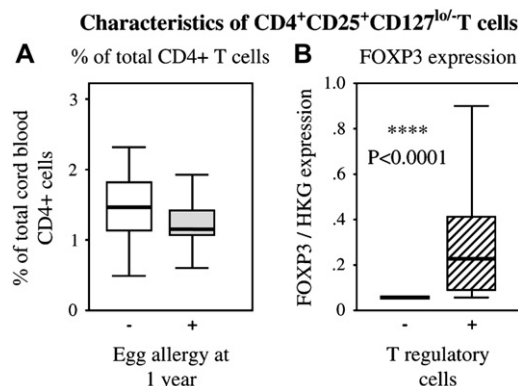


FIG 1. Comparison of CD4⁺CD25⁺CD127^{lo/-} cells as a proportion of total CD4⁺ T cells and FOXP3 mRNA expression in these regulatory populations. **A**, The percentage of CD4⁺CD25⁺CD127^{lo/-} Treg cells as a proportion of total CD4⁺ cells in cord blood is shown for the allergic (shaded) and nonallergic (unshaded) groups. **B**, FOXP3 mRNA expression was assessed in unstimulated cell culture expression relative to the UBE2D2 housekeeping gene (*HKG*). Levels were compared in CBMCs cultured either with (+) or without (-) CD4⁺CD25⁺CD127^{lo/-} Treg cells.

symptoms. Of these, 12 of (80%) 15 had atopic dermatitis, and 10 (66.6%) of 15 also had sensitization to other allergens. In contrast, the nonallergic group (*n* = 18) was selected to have no evidence of sensitization, symptoms, or allergic disease at 1 year of age.

Comparison of the frequency of CD4⁺CD25⁺CD127^{lo/-} Treg cells in cord blood of the allergic and nonallergic groups

There were no differences in the frequency of Treg cell populations in the neonates with subsequent allergic disease, although there was a trend toward lower proportions in the allergic group (*P* = .38; Fig 1, A). Similarly, there were no differences in CD4⁺CD25⁻ effector T-cell populations in the allergic and nonallergic groups (data not shown). There were no significant relationships between the frequency of Treg cells in cord blood and the function of these cells in subsequent cultures (after isolation). The expression of the regulatory marker FOXP3 (as mRNA) was markedly higher (*P* < .0001) in the cultures containing Treg cells (Fig 1, B) compared with that seen in effector cell populations (without Treg cells). Effector cells also expressed negligible FOXP3 mRNA after polyclonal activation (with SEB; data not shown). This also confirms that the selected CD4⁺CD25⁺CD127^{lo/-} population represents Treg cells.

Effect of CD4⁺CD25⁺CD127^{lo/-} Treg cells in culture

Neonatal CD4⁺CD25⁺CD127^{lo/-} Treg cells were isolated from mononuclear cell populations to assess their inhibitory capacity (as described in the Methods section). PBMC populations were sorted to obtain pure (>97%) populations of CD4⁺CD25⁻ effector T cells, and these were cultured with CD3-depleted autologous antigen-presenting (accessory) cells, either in the presence or absence of a purified population of sorted (CD4⁺CD25⁺CD127^{lo/-}) Treg cells. Initial experiments confirmed that the presence of CD4⁺CD25⁺CD127^{lo/-} Treg cells in culture was associated with marked inhibition of both T_H1 (IFN- γ) and T_H2 (IL-13) cytokine responses (*P* < .001) after polyclonal stimulation (data not shown). We then proceeded to examine this in the study population (Fig 2), confirming that these

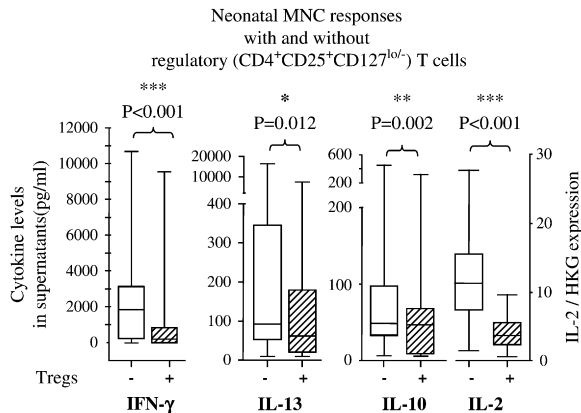


FIG 2. The effects of adding $CD4^+CD25^+CD127^{lo/-}$ regulatory cells to mononuclear cell cultures. CBMC populations were sorted to obtain populations of $CD4^+CD25^-$ effector T cells (>97% pure), which were cultured with autologous antigen-presenting cells either in the presence (+) or absence (-) of a purified population of $CD4^+CD25^+CD127^{lo/-}$ Treg cells. These were stimulated for 48 hours with SEB, and the cytokine response (IFN- γ , IL-13, and IL-10) was measured in the supernatants. Significance levels are denoted as follows: * $P < .05$, ** $P < .01$, and *** $P < .001$. HKG, Housekeeping gene.

neonates also showed a significant reduction in the production of IL-10 ($P = .002$), IL-13 ($P = .012$), and IFN- γ ($P < .001$) in the presence of Treg cells.

Comparison of Treg cell function in neonates with and without subsequent allergic disease

Next we compared this functional measure of neonatal regulatory activity according to subsequent allergic status. Consistent with other reports,¹⁰⁻¹⁶ there was a trend toward reduced IFN- γ responses from $CD4^+CD25^-$ effector T cells in the allergic group (Fig 3, A). Both groups showed significant inhibition of SEB-induced IFN- γ responses when $CD4^+CD25^+CD127^{lo/-}$ Treg cells were added (Fig 3, A); however, significantly fewer of the neonates who later had egg allergy (44%) showed Treg cell-associated suppression of IFN- γ effector responses compared with neonates without subsequent allergy (78%; $P = .015$; data not shown). In this analysis suppression was defined as any reduction in cytokine production in neonates with detectable responses.

The suppressive activity of Treg cells (ie, change in response) was calculated for neonates who had clear IFN- γ $CD4^+CD25^-$ effector responses (>200 pg/mL). Nonresponders (or weak responders) were excluded because a resulting small change would reflect the low initial response rather than impaired suppression in those individuals. Of the good responders, neonates who had subsequent egg allergy ($n = 13$) had significantly weaker inhibition (ie, change in response) when $CD4^+CD25^+CD127^{lo/-}$ Treg cells were added ($P = .004$) compared with that seen in the nonallergic group ($n = 12$; Fig 4, A). The same relationship was seen for neonates with responses of greater than 500 pg/mL ($n = 12$ nonallergic and 11 allergic, $P = .014$), greater than 1000 pg/mL ($n = 11$ nonallergic and 9 allergic, $P = .020$), and greater than 1500 pg/mL ($n = 10$ nonallergic and 9 allergic, $P = .006$).

For IL-13, only the nonallergic group showed significant $CD4^+CD25^+CD127^{lo/-}$ Treg cell-associated suppression of IL-13 ($P = .041$) responses (Fig 3, B). As with IFN- γ , the neonatal IL-13 responses were generally lower in the allergic group, and this is also consistent with observations in other populations.^{16,20} The lack of change in cytokine production when Treg cells were

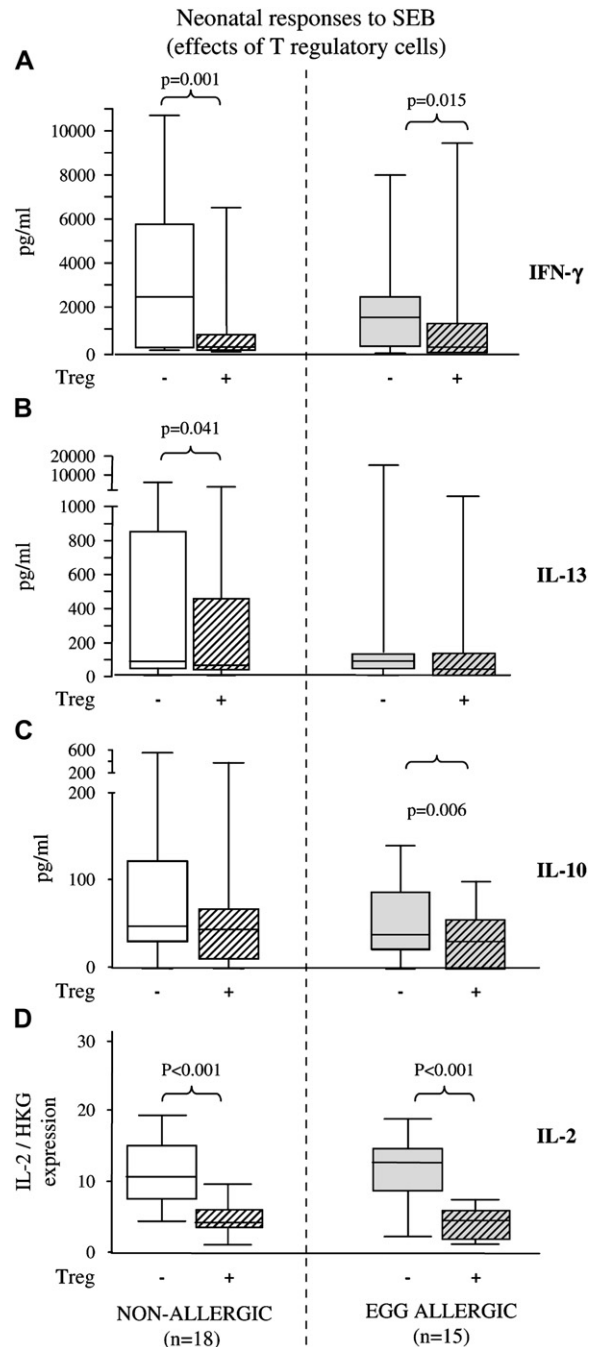


FIG 3. Comparison of the suppressive effects of neonatal $CD4^+CD25^+CD127^{lo/-}$ Treg cells in the allergic and nonallergic groups. The effects of adding $CD4^+CD25^+CD127^{lo/-}$ Treg cells to effector T-cell cultures ($CD4^+CD25^-$ effector T cells and autologous antigen-presenting cells) were compared in neonates who subsequently had allergic disease ($n = 15$) and those who remained nonallergic ($n = 18$). This is shown for IFN- γ (A), IL-13 (B), and IL-10 (C) protein. For IL-2 mRNA, the Treg cell-negative cultures (unshaded bars) were compared with the Treg cell-positive cultures (shaded bars). HKG, Housekeeping gene.

added might have been due to lower effector responses; however, when we analyzed neonates with clear IL-13 responses (>50 pg/mL), the allergic group did not show any reduction in response ($n = 10$, $P = .14$) after addition of Treg cells, whereas the nonallergic group did ($n = 12$, $P = .041$).

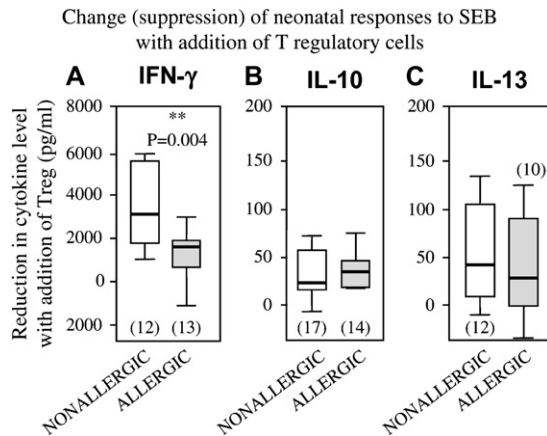


FIG 4. Comparison of the Treg cell-associated suppression in the allergic and nonallergic groups. The Treg suppressive activity (ie, change in response when $CD4^+CD25^+CD127^{lo/-}$ Treg cells were added) was compared in neonates who had subsequent egg allergy (shaded) and the nonallergic group (unshaded). The data shown are the difference between IFN- γ (A), IL-10 (B), and IL-13 (C) cytokine levels in Treg cell-negative and Treg cell-positive cultures. This analysis only included data from neonates who had clear $CD4^+CD25^-$ effector responses (>200 pg/mL for IFN- γ and greater than 20 pg/mL for IL-10 and IL-13).

IL-10 responses were similar for the neonates who had subsequent allergic disease and those who did not. IL-10 effector responses correlated with IFN- γ effector responses ($\tau = 0.31$, $P = .017$), but there were no other relationships between cytokines produced. Both groups showed suppression of IL-10 production when Treg cells were added, but this was only statistically significant in the group with egg allergy (Fig 3, B). There were no differences in the change (suppression) in IL-10 responses (Fig 4, B) or IL-2 responses (data not shown) after the addition of Treg cells in the allergic and nonallergic groups.

Although OVA-specific responses were assessed in the same manner, effector cytokine responses were too infrequent and of too low a level in this neonatal population to assess the effects of adding Treg cells (data not shown).

Comparison of FOXP3 expression in neonates with and without subsequent allergic disease

FOXP3 mRNA expression was detected in unstimulated Treg cell-positive cultures, and this was significantly higher than in unstimulated Treg cell-negative cultures ($P = .002$, data not shown). After SEB stimulation, Treg cell-positive FOXP3 mRNA expression increased markedly in both the allergic ($P = .003$) and nonallergic ($P = .005$) groups (Fig 5). There were no differences in Treg cell-positive FOXP3 expression in the allergic and nonallergic groups for either the unstimulated cultures ($P = .70$) or for cultures stimulated with OVA ($P = .55$) or SEB ($P = .98$). There were also no significant correlations between the level (or change) in FOXP3 expression and the level of cytokine suppression when Treg cells were added. Although stimulation with SEB increased FOXP3 levels in the $CD4^+CD25^-$ (Treg cell-negative) effector T-cell population ($P = .002$), levels were more than 100 times lower than in the Treg cell-positive cell populations ($P < .0001$ for both groups, data not shown).

DISCUSSION

Treg cells play a key role in the inhibition of inappropriate immune responses; however, it is still not clear whether these cell

Foxp3 mRNA expression in $CD4^+CD25^+CD127^{lo/-}$ Treg populations

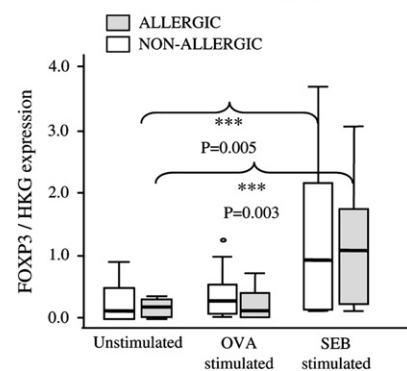


FIG 5. Comparison of FOXP3 expression in the allergic and nonallergic groups. Neonatal FOXP3 mRNA expression in Treg cell-positive cultures is shown for the allergic (shaded) and nonallergic (unshaded) groups. FOXP3 mRNA levels in Treg cell-negative cultures are not shown (but are at very low levels, as in Fig 1). The mRNA level is shown as FOXP3 gene expression relative to expression of the UBE2D2 housekeeping gene (HKG).

populations have a primary role in the pathogenesis of allergic disease. The aim of this study was to assess regulatory function of neonates who have a subsequent allergic phenotype. The experimental model was designed to specifically assess the suppressive function of neonatal Treg cells and not to reproduce the complexity of *in vivo* interactions. Although the limitations of this approach are acknowledged, we made a number of novel observations.

First, we observed that the lower neonatal T_H1 IFN- γ responses that have been well documented in association with the allergic phenotype¹⁰⁻¹⁶ were independent of any potential differences in Treg cell function. The lower IFN- γ responses were seen in the allergic group when $CD4^+CD25^+CD127^{lo/-}$ Treg cells were excluded from the culture. This lends further support to the notion that allergy might be associated with reduced ability of neonatal effector T cells to respond to polyclonal activation. In this system it was not possible to determine whether variations in antigen-presenting cell function contributed to this effect.

Second, this study demonstrated that neonatal $CD4^+CD25^+CD127^{lo/-}$ T cells demonstrate clear regulatory activity. These neonatal Treg cells were able to overcome the strong activation signals of SEB. This challenges the findings of Wing et al,²³ who observed that cord blood $CD25^+$ Treg cells failed to suppress SEB responses. The reason for this discrepancy is not clear. Although Wing et al relied only on $CD25^+$ to distinguish putative Treg cell populations, these were also characterized by significantly higher FOXP3 expression, as in the present study.

Third, we have also provided the first preliminary evidence that allergic disease might be associated with altered neonatal $CD4^+CD25^+CD127^{lo/-}$ Treg cell function. Specifically, the level of (IFN- γ) inhibition by $CD4^+CD25^+CD127^{lo/-}$ Treg cells was less in neonates who subsequently had allergic disease. It is possible that the smaller suppressive effect of adding Treg cells in the allergic group was due to lower initial effector response (also observed by many others¹⁰⁻¹⁶); however, the difference remained significant when only the strong responders were compared (>500 pg/mL). This effect was cytokine specific, with no associated effects on other cytokine responses.

The implication of these observations is not clear. Although relative deficiencies of T_H1 function in early life have been used

as an explanation for persistent T_H2 allergic responses,¹⁴ the ability to inhibit T_H1 function is important for fetal survival in pregnancy.²⁴ Although many hormonally driven pathways are involved in suppressing T_H1 maternofetal damage, the fetal contribution to this process is not clear. It has been largely presumed that the fetus is a passive contributor to this process with weak T_H1 function caused by immaturity. There is now evidence that T_H1 function might be actively regulated by the fetus through epigenetic mechanisms that alter methylation patterns and gene expression of the IFN- γ promoter in effector ($CD4^+$) T cells.²⁵ Here we demonstrate that cord blood also contains Treg cell populations that can actively suppress potent T_H1 -inducing superantigens. Although the apparently lower suppressive activity in the allergic group needs to be confirmed in further studies, this could represent further evidence of the wide-ranging immaturities in immune function that have been emerging in neonates at high risk of allergic disease.^{10-16,26,27} Our findings could be consistent with observations by Haddeland et al¹⁷ that neonates at "high risk" of allergy have reduced generation of putative Treg cell populations after LPS stimulation. Although that study used different methods of isolating Treg cells (relying on CD4 and CD25 only) and did not assess suppressive activity directly, the investigators did examine other markers of the regulatory phenotype (eg, cytotoxic T lymphocyte-associated antigen 4) and noted that these populations varied inversely with the capacity for lymphoproliferation.¹⁷ Reduced ability to regulate effector T-cell responses could be a logical contributing factor in the failure of normal tolerance mechanisms and needs to be investigated further. In this study the allergic group all had allergic mothers, and it is not possible to determine whether the observed differences are an effect of maternal allergy *per se* or due to the inherent infant phenotype, which might also not be specific for egg allergy.

Although there are preliminary reports of reduced $CD4^+CD25^+$ Treg cell function in adults with allergic disease,^{2,3} there is still no evidence that these cells play a primary role in disease pathogenesis, and the role during early disease development is not clear. Although our current findings provide preliminary evidence of reduced Treg cell function in neonates who have subsequent allergic disease, longitudinal studies are needed to assess this further.

Finally, we observed that although high FOXP3 expression was a defining feature of the $CD4^+CD25^+CD127^{lo/-}$ Treg population, as noted by others,⁹ it did not correlate well with variations in function within this Treg cell population. Specifically, FOXP3 levels and the level of suppressive activity (inhibition of effector cytokine responses) appeared to vary independently in these neonates. Although there are reports that FOXP3 can be nonspecifically upregulated in adult effector $CD4^+$ T cells during activation,⁸ this was negligible in the present study of neonates, with FOXP3 expressed predominantly by the $CD4^+CD25^+CD127^{lo/-}$ Treg cell population. This higher FOXP3 expression was evident in unstimulated Treg cell–positive cultures (compared with the Treg cell–negative cultures) and increased markedly with SEB stimulation. However, the level (or the change in level) of FOXP3 expression did not correlate with the capacity of the $CD4^+CD25^+CD127^{lo/-}$ Treg cell population to inhibit IL-13, IFN- γ , or IL-10 responses. Thus although the allergic group showed reduced Treg cell–associated suppression of IFN- γ responses, this was not associated with reduced FOXP3 expression (at either baseline or on stimulation). Collectively, these findings suggest that although FOXP3 is a useful marker of neonatal regulatory populations, it does not predict function of these cells, at least in this study population.

In summary, this study has confirmed the presence and activity of Treg cells in cord blood and provided preliminary evidence of differences in neonates who progress to allergic disease in the first year of life. This now needs to be investigated further to determine the implications for allergy pathogenesis.

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Key messages:

- This study provides preliminary evidence of presymptomatic perinatal differences in Treg cell function in neonates who progress to allergic disease.
- The findings warrant further studies to determine the role of early Treg cells in the development of allergic disease.

REFERENCES

1. Bach JF. The effect of infections on susceptibility to autoimmune and allergic diseases. *N Engl J Med* 2002;347:911-20.
2. Akdis M, Verhagen J, Taylor A, Karamloo F, Karagiannidis C, Cramer R, et al. Immune responses in healthy and allergic individuals are characterized by a fine balance between allergen-specific T regulatory 1 and T helper 2 cells. *J Exp Med* 2004;199:1567-75.
3. Ling EM, Smith T, Nguyen XD, Pridgeon C, Dallman M, Arbery J, et al. Relation of $CD4^+CD25^+$ regulatory T-cell suppression of allergen-driven T-cell activation to atopic status and expression of allergic disease. *Lancet* 2004;363:608-15.
4. Bellinghausen I, König B, Böttcher I, Knop J, Saloga J. Regulatory activity of human $CD4^+CD25^+$ T cells depends on allergen concentration, type of allergen and atopy status of the donor. *Immunology* 2005;116:103-11.
5. Ou LS, Goleva E, Hall C, Leung DY. T regulatory cells in atopic dermatitis and subversion of their activity by superantigens. *J Allergy Clin Immunol* 2004;113:756-63.
6. Taylor AL, Hale J, Hales BJ, Dunstan JA, Thomas WR, Prescott SL. FOXP3 mRNA expression at 6 months of age is higher in infants who develop atopic dermatitis, but is not affected by giving probiotics from birth. *Pediatr Allergy Immunol* 2007;18:10-9.
7. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of $CD4^+CD25^+$ regulatory T cells. *Nat Immunol* 2003;4:330-6.
8. Pillai V, Ortega SB, Wang CK, Karandikar NJ. Transient regulatory T-cells: a state attained by all activated human T-cells. *Clin Immunol* 2007;123:18-29.
9. Seddiki N, Santner-Nanan B, Martinson J, Zaunders J, Sasson S, Landay A, et al. Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. *J Exp Med* 2006;203:1693-700.
10. Tang MLK, Kemp AS, Thorburn J, Hill D. Reduced interferon gamma secretion in neonates and subsequent atopy. *Lancet* 1994;344:983-5.
11. Warner JA, Miles EA, Jones AC, Quint DJ, Colwell BM, Warner JO. Is deficiency of interferon gamma production by allergen triggered cord blood cells a predictor of atopic eczema? *Clin Exp Allergy* 1994;24:423-30.
12. Martinez F, Stern D, Wright A, Holberg C, Taussig L, Halonen M. Association of interleukin-2 and interferon- γ production by blood mononuclear cells in infancy with parental allergy skin tests and with subsequent development of atopy. *J Allergy Clin Immunol* 1995;96:652-60.
13. Kondo N, Kobayashi Y, Shinoda S, Takenaka R, Teramoto T, Kaneko H, et al. Reduced interferon gamma production by antigen-stimulated cord blood mononuclear cells is a risk factor of allergic disorders—6-year follow-up study. *Clin Exp Allergy* 1998;28:1340-4.
14. Prescott S, Macaubas C, Smallacombe T, Holt B, Sly P, Loh R, et al. Development of allergen-specific T-cell memory in atopic and normal children. *Lancet* 1999;353:196-200.
15. Prescott SL, Macaubas C, Smallacombe T, Holt BJ, Sly PD, Loh R, et al. Reciprocal age-related patterns of allergen-specific T-cell immunity in normal vs. atopic infants. *Clin Exp Allergy* 1998;28(suppl 5):39-44; discussion 50-1.
16. Neaville WA, Tisler C, Bhattacharya A, Ankam K, Gilbertson-White S, Hamilton R, et al. Developmental cytokine response profiles and the clinical and

- immunologic expression of atopy during the first year of life. *J Allergy Clin Immunol* 2003;112:740-6.
17. Haddeland U, Karstensen AB, Farkas L, Bo KO, Pirhonen J, Karlsson M, et al. Putative regulatory T cells are impaired in cord blood from neonates with hereditary allergy risk. *Pediatr Allergy Immunol* 2005;16:104-12.
 18. Johansson SG, Bieber T, Dahl R, Friedmann PS, Lanier BQ, Lockey RF, et al. Revised nomenclature for allergy for global use: report of the Nomenclature Review Committee of the World Allergy Organization, October 2003. *J Allergy Clin Immunol* 2004;113:832-6.
 19. Hanifin J, Rajka G. Diagnostic features of atopic dermatitis. *Acta Derm Venereol (Stockh)* 1980;92(suppl 144):44-7.
 20. Prescott S, Macaubas C, Holt B, Smallacombe T, Loh R, Sly P, et al. Transplacental priming of the human immune system to environmental allergens: universal skewing of initial T-cell responses towards Th-2 cytokine profile. *J Immunol* 1998;160:4730-7.
 21. Rowe J, Heaton T, Kusel M, Suriyaarachchi D, Serralha M, Holt BJ, et al. High IFN-gamma production by CD8+ T cells and early sensitization among infants at high risk of atopy. *J Allergy Clin Immunol* 2004;113:710-6.
 22. Hamalainen HK, Tubman JC, Vikman S, Kyrola T, Ylikoski E, Warrington JA, et al. Identification and validation of endogenous reference genes for expression profiling of T helper cell differentiation by quantitative real-time RT-PCR. *Anal Biochem* 2001;299:63-70.
 23. Wing K, Larsson P, Sandström K, Lundin SB, Suri-Payer E, Rudin A. CD4+ CD25+ FOXP3+ regulatory T cells from human thymus and cord blood suppress antigen-specific T cell responses. *Immunology* 2005;115: 516-25.
 24. Wegmann TG, Lin H, Guilbert L, Mosmann TR. Bidirectional cytokine interactions in the maternal-fetal relationship: is successful pregnancy a Th2 phenomenon? *Immunol Today* 1993;14:353-6.
 25. White GP, Watt PM, Holt BJ, Holt PG. Differential patterns of methylation of the IFN-gamma promoter at CpG and non-CpG sites underlie differences in IFN-gamma gene expression between human neonatal and adult CD45RO- T cells. *J Immunol* 2002;168:2820-7.
 26. Macaubas C, de Klerk NH, Holt BJ, Wee C, Kendall G, Firth M, et al. Association between antenatal cytokine production and the development of atopy and asthma at age 6 years. *Lancet* 2003;362:1192-7.
 27. Upham JW, Hayes LM, Lundahl J, Sehmi R, Denburg JA. Reduced expression of hemopoietic cytokine receptors on cord blood progenitor cells in neonates at risk for atopy. *J Allergy Clin Immunol* 1999;104:370-5.

INTRODUCTION

Comparison of cytokine responses from cultures performed on cryopreserved versus freshly cultured cells

The following preliminary studies are provided to support the use of cryopreservation in cell culture and to demonstrate that it does not adversely affect biologic functions of mononuclear cells. These techniques have been in use by many groups for many years. This work was originally performed for the PhD studies of the senior author.^{E1}

Because of logistic difficulties in collecting large numbers of cord blood samples and processing them for lymphoproliferative and cytokine studies during the same time period, cryopreservation techniques were used. The cryopreservation techniques developed in the host laboratory were working consistently, with excellent viable cell yields and no significant changes in lymphoproliferative patterns. Earlier experiments in the host laboratory had failed to detect significant freeze-thawing effects on allergen-specific IL-4 or IFN- γ mRNA responses, but data were not available for cytokine protein measurements. Accordingly, the cytokine responses of freshly stimulated CBMCs were compared with responses by the same CBMCs after cryopreservation and thawing.

Methods. CBMCs from a series ($n = 10$) of cord blood samples were isolated in the usual way^{E2} and then either cryopreserved in dimethyl sulfoxide or placed fresh in bulk cultures (1×10^6 cells in 500 μ L of AIM-V) for 24 hours, with either HDM (10 μ g/mL), OVA (100 μ g/mL), PHA (1 μ g/mL), or no additives (negative control). The culture supernatants were then collected and frozen. After several days at -80°C , the frozen cells from the same individual were slowly and gently thawed. These cells were then cultured for 24 hours under identical conditions as the freshly processed cells above. These supernatants were also frozen overnight in the same manner as the freshly cultured cells. Standard ELISAs^{E2} were performed for all cultures from each individual on the same plate. The responses of fresh and thawed CBMC cultures were compared.

Findings. The results shown in Fig E1 illustrate that CBMCs show similar patterns of cytokine response whether processed fresh or after cryopreservation and thawing. Each individual showed the same pattern of allergen response, and although the magnitude of responses showed some variability, there were no statistically significant differences. In some cases the responses to PHA were, if anything, higher in cryobanked cells, but this was not consistent.

Interpretations. The major studies performed for this thesis were of the same order of magnitude as the preliminary experiments. There is expected variability, inherent to cell culture, but this appeared random in this subpopulation. Freezing clearly did not appear to inhibit cytokine responses, and although it is theoretically possible that the freezing process might in some way make cells more responsive to stimulation, this was not a consistent finding.

Cytokine responses do not follow a normal Gaussian distribution and show biologic variability within an individual. The purpose of these assays was to show trends suggesting biologic significance. Large differences between stimulated and unstimulated cultures indicate a positive response, and this is seen regardless of whether cells are frozen before culture. This study describes comparative trends in cytokine response between atopic and nonatopic individuals. There is no reason to suspect that cryopreservation has altered these findings.

All cord blood samples were processed after cryopreservation under similar conditions, so that the differences that have been subsequently detected between nonatopic and atopic individuals were unlikely to be due to this process.

These findings and the consistent observations that fresh and cryobanked cells show similar patterns of lymphoproliferation (as previously demonstrated and shown in Fig E2) support the use of cryopreservation, which has become a logistic necessity during studies such as this.

The justification for using irradiated effector cells in cultures (and data to support that this did not confound the results)

The aim of adding irradiated cells to the cultures was to ensure that the total cells in culture remained standard in all cultures. The studies below demonstrate that the addition of these cells was unlikely to significantly influence the findings.

First, we demonstrated that irradiated neonatal effector cells produce trivial levels of cytokines relative to nonirradiated effector cells (as shown above for 12 replicates). Thus although irradiated cells are capable of producing cytokines, in our system the levels were 100 times lower than those in nonirradiated cells and unlikely to significantly alter the cytokine levels in coculture experiments (Fig E3).

Second, we assessed the effects of coculturing, differing proportions of irradiated and nonirradiated $\text{CD4}^+\text{CD25}^-$ effector cells with antigen-presenting cells. This also demonstrated that most of the cytokine production in these coculture studies is derived from the nonirradiated cells and that irradiated cells are unlikely to have a significant influence of cytokine levels detected in the supernatants (as shown in Fig E4).

Finally, we assessed the potential role of the irradiated cells in the Treg cultures (as shown in Fig E5).

- If no irradiated cells were used, the addition of Treg cells was associated with a reduction in cytokine responses (ie, 2.5×10^4 effector cells + 2.5×10^4 Treg cell).
- If irradiated cells were included at 50%, similar Treg cell suppression was observed (ie, 2.5×10^4 effector cells/ 2.5×10^4 irradiated effector cells + 2.5×10^4 Treg cells).
- If only irradiated effector cells (and no nonirradiated effector cells) were used, there were no detectable responses.

Thus using irradiated cells did not increase the cytokine levels in cultures. Based on this, it is unlikely that the use of irradiated cells (to maintain the total cell numbers in all cultures) significantly contributed to the change in cytokine levels when Treg cells were added.

Because of the limited cell numbers available after involved cell-sorting experiments, it was not possible to repeat all these conditions on all the test subjects in the study.

REFERENCES

- E1. Prescott SL. Studies on the early postnatal development of T-cell immunity to environmental allergens" [thesis]. Perth, University of Western Australia; 1998. p. 149-50.
- E2. Prescott S, Macaubas C, Holt B, Smalacombe T, Loh R, Sly P, et al. Transplacental priming of the human immune system to environmental allergens: universal skewing of initial T-cell responses towards Th-2 cytokine profile. *J Immunol* 1998;160:4730-7.

BIBLIOGRAPHY

1. Disis ML, de la Rosa C, Goodell V, Kuan LY, Chang JC, Kuus-Reichel K, et al. Maximizing the retention of antigen specific lymphocyte function after cryopreservation. *J Immunol Methods* 2006;308:13-8.

2. Maecker HT, Moon J, Bhatia S, Ghanekar SA, Maino VC, Payne JK, et al. Impact of cryopreservation on tetramer, cytokine flow cytometry, and ELISPOT. *BMC Immunol* 2005;6:17.
3. García-Piñeres AJ, Hildesheim A, Williams M, Trivett M, Strobl S, Pinto LA. DNase treatment following thawing of cryopreserved PBMC is a procedure suitable for lymphocyte functional studies. *J Immunol Methods* 2006;313:209-13.
4. Reimann KA, Chernoff M, Wilkening CL, Nickerson CE, Landay AL. Preservation of lymphocyte immunophenotype and proliferative responses in cryopreserved peripheral blood mononuclear cells from human immunodeficiency virus type 1-infected donors: implications for multicenter clinical trials. The ACTG Immunology Advanced Technology Laboratories. *Clin Diagn Lab Immunol* 2000;7:352-9.

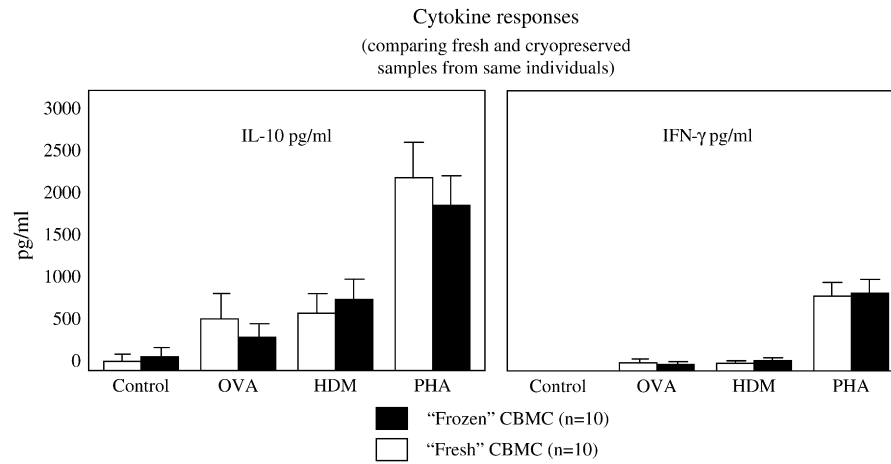


FIG E1. Comparison of cytokine responses of mononuclear cells cultured with OVA, HDM, and PHA within 4 hours of collection (*Fresh*) compared with those after cryopreservation (*Frozen*).

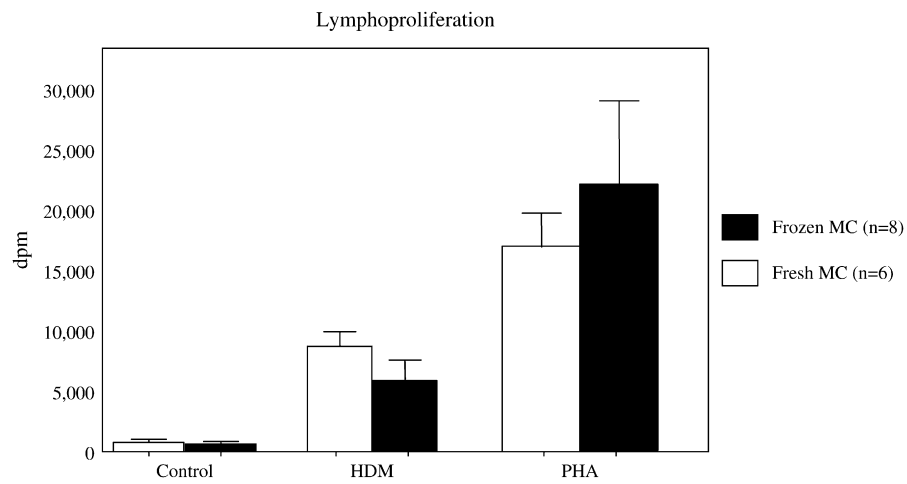


FIG E2. Comparison of lymphoproliferative responses from mononuclear cells cultured within 4 hours of collection (*Fresh*) compared with those after cryopreservation (*Frozen*).

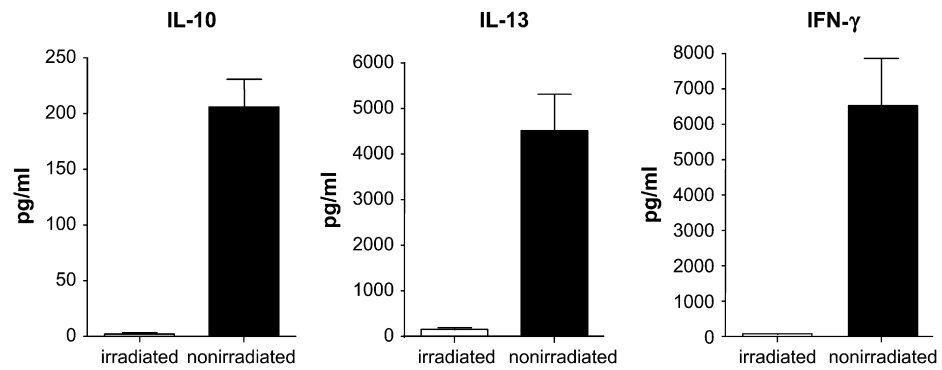


FIG E3. Effects of irradiation on neonatal cytokine responses (data from 12 replicates).

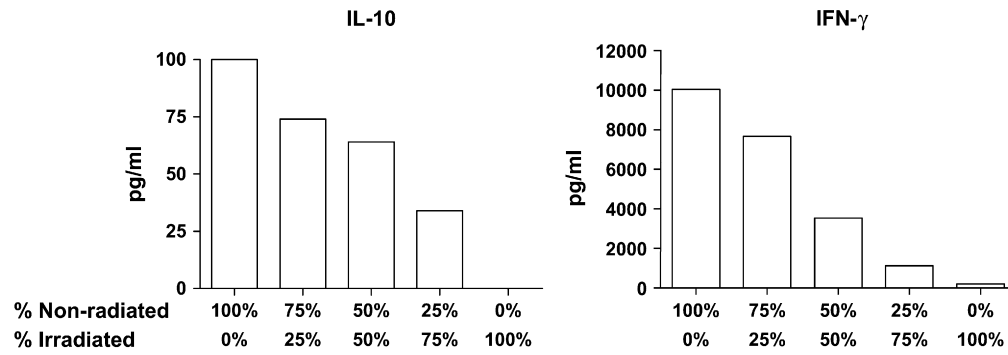


FIG E4. Effects of changing proportion of nonirradiated and irradiated CD4⁺CD25⁻ cells in culture (cocultured with autologous antigen-presenting cells and stimulated with SEB for 48 hours).

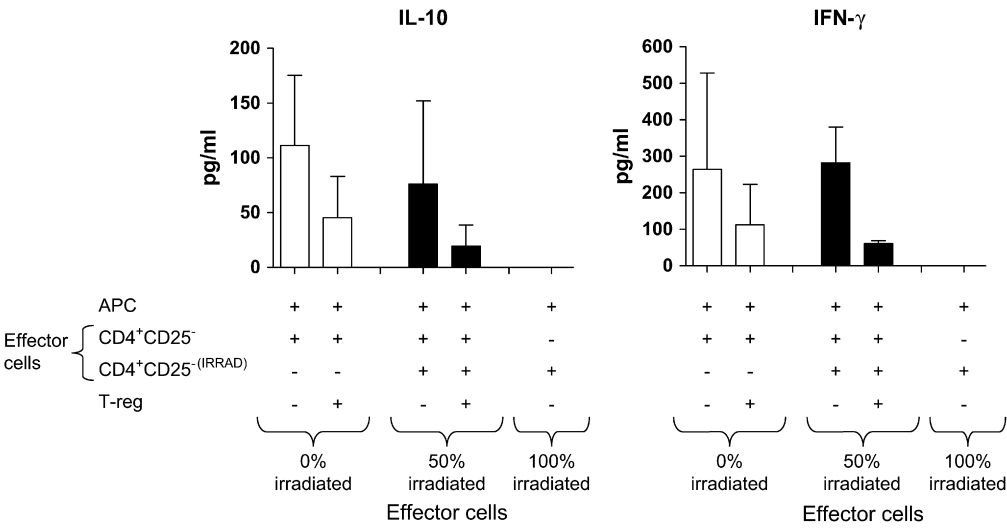


FIG E5. The effects of irradiation and Treg cells on effector T-cell responses. This demonstrates that irradiated effector T cells added to cultures do not contribute significantly to cytokine production, and Treg cells have similar suppressive effects if irradiated effector T cells were added to nonirradiated effector T-cell cultures to maintain cell numbers. *APC*, Antigen-presenting cells.