

Functional Maturation of CD4⁺CD25⁺CTLA4⁺CD45RA⁺ T Regulatory Cells in Human Neonatal T Cell Responses to Environmental Antigens/Allergens¹

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A number of laboratories have reported cord blood T cell responses to ubiquitous environmental Ags, including allergens, by proliferation and cytokine secretion. Moreover, the magnitude of these responses has been linked with risk for subsequent expression of allergy. These findings have been widely interpreted as evidence for transplacental priming and the development of fetal T memory cells against Ags present in the maternal environment. However, we present findings below that suggest that neonatal T cell responses to allergens (and other Ags) differ markedly from those occurring in later life. Notably, in contrast to allergen-responsive adult CD4⁺ T cell cultures, responding neonatal T cell cultures display high levels of apoptosis. Comparable responses were observed against a range of microbial Ags and against a parasite Ag absent from the local environment, but not against autoantigen. A notable finding was the appearance in these cultures of CD4⁺CD25⁺CTLA4⁺ T cells that de novo develop MLR-suppressive activity. These cells moreover expressed CD45RA and CD38, hallmarks of recent thymic emigrants. CFSE-labeling studies indicate that the CD4⁺CD25⁺ cells observed at the end of the culture period were present in the day 0 starting populations, but they were not suppressive in MLR responses. Collectively, these findings suggest that a significant component of the reactivity of human neonatal CD4⁺ T cells toward nominal Ag (allergen) represents a default response by recent thymic emigrants, providing an initial burst of short-lived cellular immunity in the absence of conventional T cell memory, which is limited in intensity and duration via the parallel activation of regulatory T cells. *The Journal of Immunology*, 2004, 173: 3084–3092.

The ability of cord blood mononuclear cells (MNC)³ to proliferate and produce cytokines in response to a variety of environmental allergens has been described by many groups (1–5). This observation has generated much interest in the possibility that priming of the fetal immune system to allergen occurs before birth, and has focused attention on the impact that this might have on the development of allergic disease. Such responses have been postulated to offer a target for preventative strategies to limit the development of IgE-mediated allergic diseases, which have been increasing in prevalence over the last 30 years.

Despite this interest, the cord blood MNC response to allergen remains poorly characterized. The requirement for MHC class II presentation, the classical Ag presentation pathway for exogenous Ags, and thereby CD4⁺ T cells, is not yet formally established. Human umbilical cord blood MNC are used commonly as a source of human naive T cells, as greater than 90% of CD3⁺ T cells are

CD45RA⁺, in contrast to adults in which CD45RO⁺ T cells become increasingly dominant with age (6–8). CD45RA⁺CD3⁺ neonatal T cells also express CD38 (6), which although used as an activation marker, also characterizes resting thymocytes. CD38 expression, IL-7R α expression and responsiveness to IL-7, and the high frequency of TCR excision circles within the neonatal CD45RA⁺ T cell population collectively indicate that these cells are more akin to recent thymic emigrants (RTE) than the naive (CD45RA⁺) T cells found in the adult circulation (9).

The memory function of the low numbers of CD45RO⁺ T cells found in the fetal circulation remains undetermined. Although proliferative responses to grass pollen allergens could be partially abrogated by the depletion of CD45RO⁺ cells from cord blood MNC preparations, this only occurred in ~50% of donors, with the remainder still responsive to allergen upon removal of so-called memory T cells (1).

There is increasing evidence to suggest that allergens can indeed traverse the human placenta (10, 11), although whether fetal dendritic cells (DC) are exposed to allergen in a form that permits uptake, processing, and presentation to fetal T cells remains unknown. Moreover, recently published studies indicate, that at least for house dust mite (HDM) allergen, there is no association between the level of environmental exposure and the extent of HDM-induced proliferation by cord blood MNC (3). In addition, earlier studies from our laboratory involving T cell epitope mapping of cord blood responses to allergen indicated that allergen-responsive T cells in neonates lacked the fine specificity of adult T memory cells (12), further questioning the phenotype of the cord blood responders. Therefore, we undertook to identify the Ag-presenting pathway(s) used in cord blood MNC responses to allergens and to characterize these responses in detail.

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³ Abbreviations used in this paper: MNC, mononuclear cell; DC, dendritic cell; HDM, house dust mite; MBP, myelin basic protein; rGST, schistosomal rGST; RTE, recent thymic emigrant.

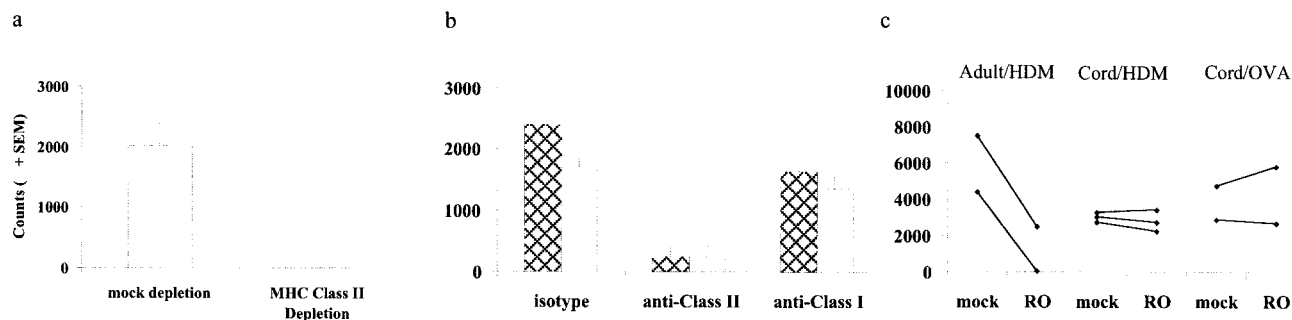


FIGURE 1. MHC class II⁺, but not CD45RO⁺ cells are required for allergen-induced proliferation by cord blood MNC proliferation. *a*, The depletion of MHC class II-positive cells abrogated the ability of cord MNC to respond to OVA ($n = 4$; $p < 0.02$) compared with mock-depleted MNC preparations, and *b*, blocking MHC class II, but not MHC class I with a specific Ab inhibited the proliferative response to both OVA (unshaded; $p < 0.002$) and HDM (shaded; $p < 0.03$) compared with proliferation in the presence of a matched isotype control (murine IgG2a) Ab. *c*, Diminished HDM-stimulated proliferation by adult MNC occurred after CD45RO⁺ depletion compared with mock-depleted preparations, whereas the removal of CD45RO⁺ cells did not affect the ability of cord blood MNC to proliferate in response to either HDM or OVA.

Materials and Methods

Umbilical cord blood samples

Blood samples were collected by venipuncture of the umbilical cord from singleton deliveries at term (>37 wk of gestation by last menstrual period). MNC were prepared by density gradient centrifugation (Lymphoprep; Nycomed, Oslo, Norway) and cryopreserved using standard techniques. Preliminary experiments established that the responses of cryopreserved MNC were not distinguishable from those of MNC that had not been cryopreserved.

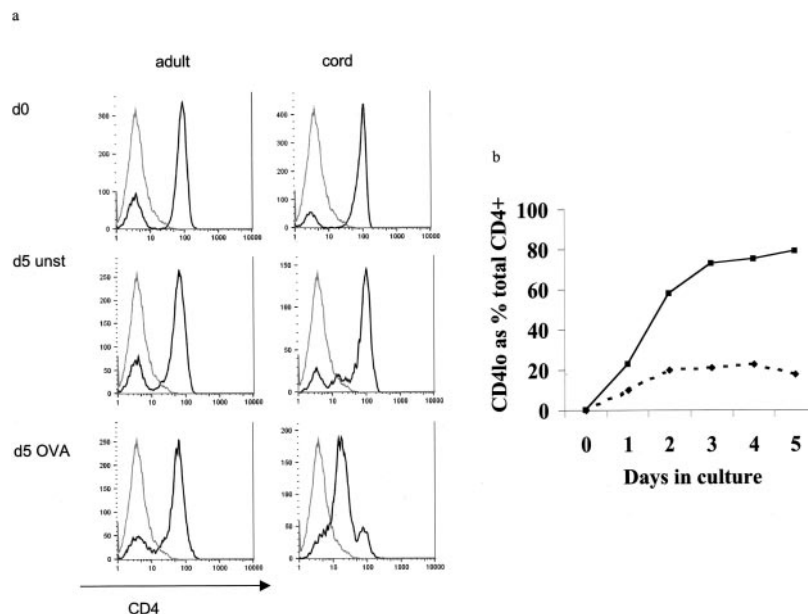
Ag response assays

MNC were cultured in round-bottom plates ($2 \times 10^5/200 \mu\text{l}/\text{well}$; Nunc, Roskilde, Denmark) or culture tubes ($1 \times 10^6/\text{ml}/\text{tube}$; Falcon). Initial experiments in this series compared the use of the commercial serum-free medium AIM-V (Invitrogen Life Technologies, Rockville, MD) supplemented with 2-ME (5×10^{-4} M; Invitrogen Life Technologies), with RPMI 1640/2-ME supplemented with 10% FCS; the only difference detected was increased background proliferation in RPMI 1640/FCS, and accordingly, experiments were continued in AIM-V/2-ME. Cells were cultured alone or with optimal stimulatory concentrations of HDM (10 $\mu\text{g}/\text{ml}$; CSL, Victoria, Australia), OVA (100 $\mu\text{g}/\text{ml}$; Sigma-Aldrich, St. Louis, MO), β -lactoglobulin (100 $\mu\text{g}/\text{ml}$; CSL), the major cat allergen Fel d 1 (30 $\mu\text{g}/\text{ml}$; ALK, Copenhagen, Denmark), purified protein derivative (10 $\mu\text{g}/\text{ml}$; CSL), diphtheria toxin (0.5 limit flocculation units per milliliter), tetanus toxin (0.5 limit flocculation units per milliliter), schistosomal rGST (rGST; 30 $\mu\text{g}/\text{ml}$; kind gift of W. Thomas, Institute for Child Health

Research, Perth, Australia), and myelin basic protein (MBP; 30 $\mu\text{g}/\text{ml}$; kind gift of M. Pender, University of Queensland, Brisbane, Australia). The immunogenicity of the MBP preparation was confirmed in cultures of adult MNC (30% of subjects tested were positive, consistent with published data from other laboratories). For lymphoproliferation assays, DNA synthesis was measured by incorporation of [³H]thymidine incorporation after pulse labeling for 16–18 h before harvest on day 5 (setup day being day 0) and expressed as cpm per culture. Background proliferation (in the absence of Ag; range, 124–1053 cpm; median, 370 cpm; $n = 19$) was subtracted from proliferation in the presence of Ag, and this figure was used for all analyses. All other experiments were terminated at various times, as noted in Results. For MHC-blocking experiments, anti-MHC class II (clone L243), anti-MHC class I (clone W6/32), or isotype control (murine IgG2a; BD Pharmingen, San Diego, CA) at 5 $\mu\text{g}/\text{ml}$ was added at the beginning of culture. Recombinant human cytokines were added at the initiation of culture at preoptimized concentrations based on reported biologically active concentrations: IL-2 (20 and 200 IU/ml), IL-4 (10 ng/ml), IL-7 (10 ng/ml), IL-10 (10 ng/ml), IL-12p70 (2 ng/ml), IL-13 (10 ng/ml), IL-15 (10 ng/ml), IFN- γ (2 ng/ml), and TGF- β (3 ng/ml).

In some experiments, cord blood MNC were cocultured with autologous monocyte-derived DC, generated from cord blood monocytes, as described previously (13). Monocytes were isolated by negative selection using anti-CD2, anti-CD7, anti-CD16, anti-CD56, anti-CD19, and glycophorin A Abs, followed by anti-mouse Ig magnetic beads (Dynal Biotech, Oslo, Norway). These monocytes (90% pure) were then cultured with GM-CSF (50 ng/ml) and IL-4 (20 ng/ml) for 7 days. The resulting cord blood-derived DC were functionally similar to adult monocyte-derived DC, as determined

FIGURE 2. The response of umbilical cord blood MNC to allergens/Ag is characterized by the emergence of a CD4^{low} T cell population. *a*, Cells were gated on CD3 expression vs side scatter and CD4 expression (open histogram) compared with isotype control (shaded histogram). After 5 days of in vitro culture of neonatal, but not adult MNC with OVA, a population of CD4^{low}CD3⁺ T cells emerges. A representative example is shown. *b*, During in vitro culture with (solid line) or without (dashed line) OVA, a CD4^{low} population (as a percentage of total CD4⁺ T cells) increases over the 5 days in culture.



by capacity to synthesize IL-12p70 (13), and by their ability to stimulate a vigorous allogeneic MLR. Cord blood MNC (2×10^5) were cocultured with 2×10^4 DC plus Ag for 5 days, as described above; stained with Abs against annexin V, CD3, and CD4; and analyzed by flow cytometry.

Flow cytometry

Nonspecific binding of Abs to Fc receptors was controlled by preincubating MNC ($5 \times 10^5/50 \mu\text{l}$ /FACS tube; BD Biosciences, San Jose, CA) with human Ig (50 $\mu\text{g}/\text{ml}$; CSL). Optimized amounts of fluorochrome-conjugated Abs were then added: anti-CD4 allophycocyanin or FITC (clone RPA-T4), anti-CD3 PerCP (clone SK3), anti-CD25 PE or allophycocyanin (clone M-A251), anti-CD38 FITC (clone T16), anti-CD45RA FITC (clone L48), anti-CD45RO (clone UCHL1), anti-CD69 PE (clone FN50), anti-HLA-DR (clone L243), and anti-CTLA4 allophycocyanin (clone BNI3) (Abs were from BD Biosciences or Beckman Coulter, Fullerton, CA). Appropriate isotype controls were always included. After staining, cells were fixed in 1% paraformaldehyde and analyzed within 24 h on a FACSCalibur (BD Pharmingen) using CellQuest software. Intracellular flow cytometry for CTLA4 was performed with 10^6 cells/test using Cell Fix/Perm (BD Pharmingen), according to the manufacturer's instructions. Stained cells were resuspended in PBS/0.2% BSA/0.05% sodium azide and analyzed immediately.

Preparation of depleted/enriched populations

MHC class II/CD45RO depletion. MHC class II or CD45RO-positive cells were depleted from total MNC with anti-MHC class II or anti-CD45RO Dynabeads (Dyna Beads), according to the manufacturer's instructions. Depletions were monitored by flow cytometry, and routinely >98% of the target population was removed.

Cell sorting. After staining cells at days 0 and 5 with anti-CD4 FITC and anti-CD25 PE, as described above, cells were sorted using FACSVantage (BD Biosciences). $\text{CD4}^+\text{CD25}^-$ (day 0 only) of >95% were obtained routinely, and $\text{CD4}^+\text{CD25}^+$ ($\text{CD4}^{\text{high}}\text{CD25}^+$ at day 5) preparations were typically enriched to ~85%.

Removal/Enrichment of CD25^+ T cells. Nucleated RBC were removed from cord blood MNC preparations with anti-glycophorin microbeads, and then CD25^+ cells were positively selected using CD25 microbeads, according to the manufacturer's instructions (Miltenyi Biotec, Surrey, U.K.). Monitoring by flow cytometry revealed that >99% of CD25^+ cells were depleted from the total MNC pool and that 85–90% of the positively selected population were $\text{CD4}^+\text{CD25}^+$. The CD25^+ fraction of cells was then stained with 500 μl of 5 μM CFSE (Molecular Probes, Eugene, OR) for 10 min at 37°C. After washing the cells twice with AIMV/10% FCS, they were returned to the CD25^- fraction, and MNC were cultured at 1×10^5 cells/ml with and without OVA as before.

Apoptosis assays

Annexin V/Propidium iodide. MNC at various times of culture were stained for flow cytometry, as described, using Annexin V^{FITC} (BD Pharmingen), CD3 PerCP, and CD4 allophycocyanin, or propidium iodide (BD Pharmingen) and CD4 allophycocyanin. Cells were suspended in PBS/0.2% BSA/0.05% sodium azide and analyzed immediately on a FACSCalibur using CellQuest software.

DNA laddering. CD8^+ , CD14^+ , and CD19^+ cells were removed with specific Dynabeads (Dyna Beads), according to the manufacturer's instructions. RNase A-treated genomic DNA was prepared from the remaining cells, and 1 μg was run on a 1.5% agarose gel containing ethidium bromide. DNA was then visualized under UV light and compared with a base pair ladder.

Regulatory T cell assays

$\text{CD4}^+\text{CD25}^+$ and $\text{CD4}^+\text{CD25}^-$ T cells were prepared from freshly thawed cord blood MNC by FACS sorting, as described above, and compared with $\text{CD4}^+\text{CD25}^+$ T cells sorted from MNC that had been cultured with OVA for 5 days. The use of cryopreserved material enabled day 0 and day 5 cells to be prepared on the same day. To assess the proliferative response to PHA (1 $\mu\text{g}/\text{ml}$), 25,000/well of the relevant T cell subset were cocultured with autologous irradiated accessory cells (CD3-depleted MNC preparations; 50,000/well) for 3 days. For suppression experiments, day 0 $\text{CD4}^+\text{CD25}^-$ T cells were used as the responders (25,000/well) in an MLR with allogeneic irradiated CD3-depleted MNC as the stimulators (50,000/well). Day 0 or day 5 $\text{CD4}^+\text{CD25}^+$ (12,500/well) T cells, as appropriate, were added at the initiation of culture. To control for the increased number of potential responder cells per well, an additional control was included, to which extra day 0 $\text{CD4}^+\text{CD25}^-$ cells were added (12,500 or 25,000/well). DNA synthesis was measured by incorporation of [^3H]thymidine after

pulse labeling for 16–18 h before harvest on day 3 (PHA response) or day 5 (MLR).

Statistical analysis

Results were compared using Student's *t* test (Microsoft Excel 2000).

Results

Requirement for MHC class II-positive cells for *in vitro* proliferation

The removal of MHC class II-positive cells completely abrogated the proliferative responses of cord blood MNC to OVA (Fig. 1a; $n = 4$; $p < 0.02$) and HDM allergen (data not shown). Similarly, blocking MHC class II ($n = 6$), but not MHC class I ($n = 3$) also inhibited the proliferative response to both OVA ($p < 0.002$) and HDM ($n = 5$; $p < 0.03$) (Fig. 1b). This confirms the requirement for MHC class II and thereby CD4^+ T cells in the allergen-stimulated proliferation response by cord blood MNC.

To investigate the effects of the depletion of CD45RO^+ cells, HDM was used as the Ag, as this enabled mite-sensitized adult donors to be used as a positive control. Although the removal of CD45RO^+ cells dramatically reduced the HDM-induced proliferation of two adult donors, there was no effect on the proliferative response by cord blood MNC to HDM ($n = 3$) or OVA ($n = 2$) (Fig. 1c).

Emergence of an apoptotic CD4^{low} T cell population after Ag exposure

Having identified a requirement for CD4^+ T cells in the Ag-specific response by cord blood MNC, the expression of activation markers (CD25, CD69, HLA-DR) by CD4^+ T cells during the response to allergen was examined. After 5 days of culture, a population expressing lower levels of CD4 (hereafter termed CD4^{low}) appeared in cord blood, but not in adult peripheral blood MNC treated in the same way (Fig. 2a). Time course analysis revealed that the CD4^{low} population emerged over 5 days of culture (Fig. 2b), and this population accounted for up to 92.9% of the total CD4^+ population after Ag stimulation. For cord, but not adult MNC, the emergence of this CD4^{low} population of T cells was a common phenomenon occurring in the unstimulated control and increased in response to all of the Ags tested (Table I).

Further examination revealed that apoptosis was a prominent feature in the cord blood MNC preparations, and was most marked in cultures stimulated with nominal Ag, as exemplified by the results illustrated for OVA-treated cord MNC populations (Fig. 3). The staining of the CD4^{low} , but not the CD4^{high} population with propidium iodide (Fig. 3a) indicated cell death was occurring; an apoptotic mechanism was confirmed by staining for annexin V (Fig. 3b) and by DNA laddering (Fig. 3c). The emergence of this

Table I. Emergence of a CD4^{low} T cell population in cord blood MNC occurs in response to a variety of allergens/Ags

Ag	n	CD4^{low} as % of Total CD4^+ (mean $\pm$ SEM)	p Value ^a
Unstimulated	12	38.4 $\pm$ 6.2	
OVA	12	71.9 $\pm$ 4.5	< 0.0002
β -lactoglobulin	5	86.9 $\pm$ 2.4	< 0.005
HDM	10	78.7 $\pm$ 4.8	< 0.0002
Fel d 1 (cat)	4	78.9 $\pm$ 3.3	< 0.005
Purified protein derivative	6	72.8 $\pm$ 7.2	< 0.01
Diphtheria toxoid	7	70.9 $\pm$ 9.3	< 0.05
Tetanus toxoid	7	58.5 $\pm$ 10.7	NS

^a Compared with unstimulated control.

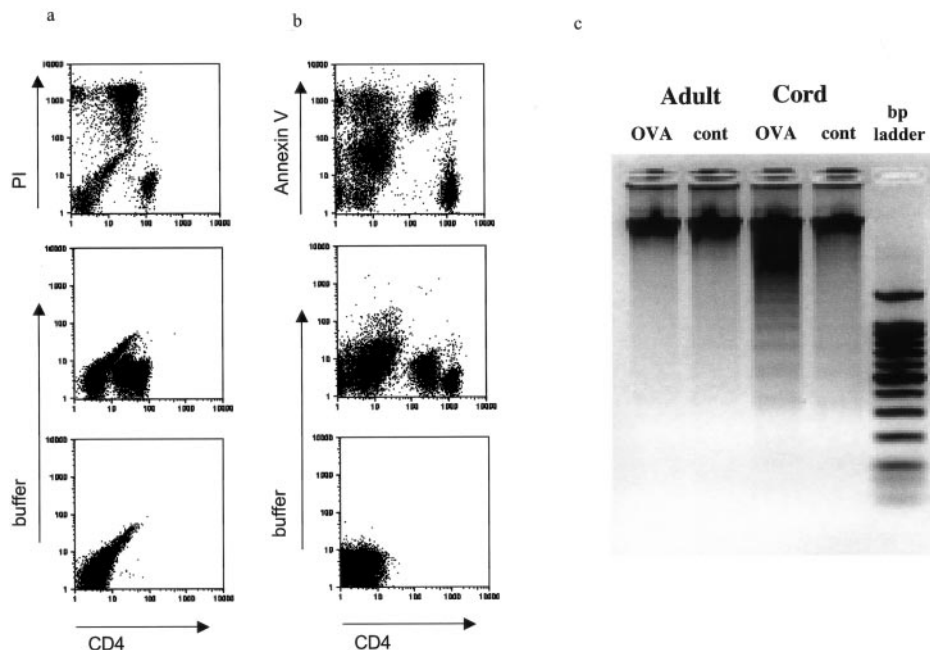


FIGURE 3. The CD4^{low} T cells that emerge during the neonatal response to allergen/Ag are apoptotic. Flow cytometric analysis revealed that the CD4^{low} population stained with propidium iodide (PI) (a) and annexin V (b) after 5 days of in vitro culture with OVA. A representative experiment of three, including the isotype control (for CD4) and buffer control (for propidium iodide/annexin V), is shown. c, DNA laddering of total CD4⁺ T cells prepared by negative selection with magnetic beads from cord or adult blood MNC after 5 days of in vitro culture with and without OVA. A representative experiment of three is shown.

CD4^{low} apoptotic T cell population could be abrogated in our experiments by treatment with the common γ -chain cytokines IL-4 (10 ng/ml) and IL-7 (10 ng/ml). However, IL-15 (10 ng/ml) did not exert an antiapoptotic effect, and only high doses (200 IU/ml), but not low doses (20 IU/ml) of IL-2 had this effect (Fig. 4).

We next considered the response of cord MNC to a self Ag (MBP), reactivity to which is likely to be limited by intrathymic mechanisms that edit the peripheral T cell repertoire (14), and also responses to a nominal Ag to which the Western mother/fetus is unlikely to have been exposed (rsGST from *Schistosoma japonicum*). Both proliferation (9 of 14; data not shown) and apoptosis significantly greater than spontaneous levels (4 of 4) were observed in cultures containing the neoantigen rsGST, suggesting that pre-exposure was not required to prime for this response. In marked contrast, apoptosis was not observed, and only 1 of 12 proliferated in response to the self Ag MBP, consistent with the prior elimination of the bulk of specifically responsive cells in the thymus (data not shown).

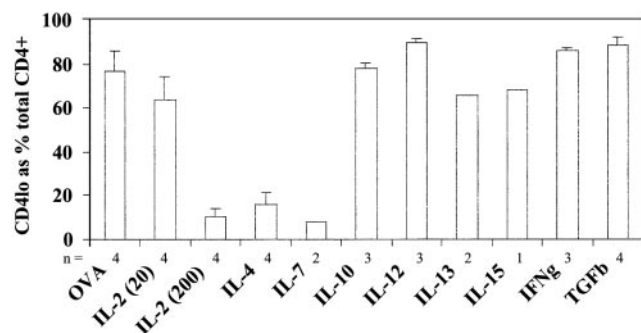


FIGURE 4. IL-4 and IL-7 prevent apoptosis by cord blood CD4⁺ T cells. Cord MNC were treated with OVA $\pm$ various cytokines, as described in *Materials and Methods*. CD4^{low} cells as percentage of total CD4⁺ T cells after 5 days of in vitro culture were evaluated using flow cytometry.

Survival of a CD4⁺CD25⁺ regulatory T cell population

Further examination of expression of the T cell activation markers CD25, CD69, and HLA-DR revealed that over the course of 5 days of culture with Ag, there was negligible change in the expression of CD69 or HLA-DR (data not shown) by either the CD4^{low} or CD4^{high} populations of neonatal CD4⁺ T cells. In contrast, CD25 was preferentially expressed on the nonapoptotic, CD4^{high} population (Fig. 5; only days 0 and 5 shown) such that although CD4⁺CD25⁺ T cells comprised $\sim$ 3–10% (median, 7.36; range, 2.99–9.98; $n = 13$) of CD4⁺ T cells at the initiation of culture, after 5 days of culture with allergen/Ag, 20–60% (median, 41.71; range, 19.42–59.52; $n = 13$) of the surviving nonapoptotic CD4^{high} T cell population coexpressed CD25. The CD4^{high}CD25⁺ population was shown to be a surviving population rather than a population of newly generated cells. If CD25⁺ cells were removed before culture, there were negligible CD4^{high}CD25⁺ cells present in the CD25-depleted culture after 5 days of stimulation with Ag, and the emergence of CD4^{low} cells in OVA-stimulated cultures was not affected ($n = 4$; data not shown). This indicates that CD25 expression is not induced on CD25-negative cells during the culture period, and that the CD25⁺ subset does not have a direct role in triggering the apoptosis process itself. Consistent with the suggestion that CD25⁺CD4⁺ cells are a surviving population, if CD25⁺ cells were removed before culture initiation, labeled with CFSE, and returned to the MNC preparation, virtually all of the surviving CD4^{high}CD25⁺ cells 5 days later were CFSE^{high}, indicating that they were present in the starting CD4⁺CD25⁺ population (Fig. 6).

In the present experiments, further phenotypic analysis of the cord blood CD4⁺CD25⁺ population at time 0 revealed that intracellular CTLA4 expression was comparable to the adult-equivalent population (Fig. 7). Flow cytometric analysis for CD45RA (data not shown), CD45RO, and CD38 revealed that neonatal CD4⁺CD25⁺ T cells were predominantly CD45RO⁺CD45RA⁺

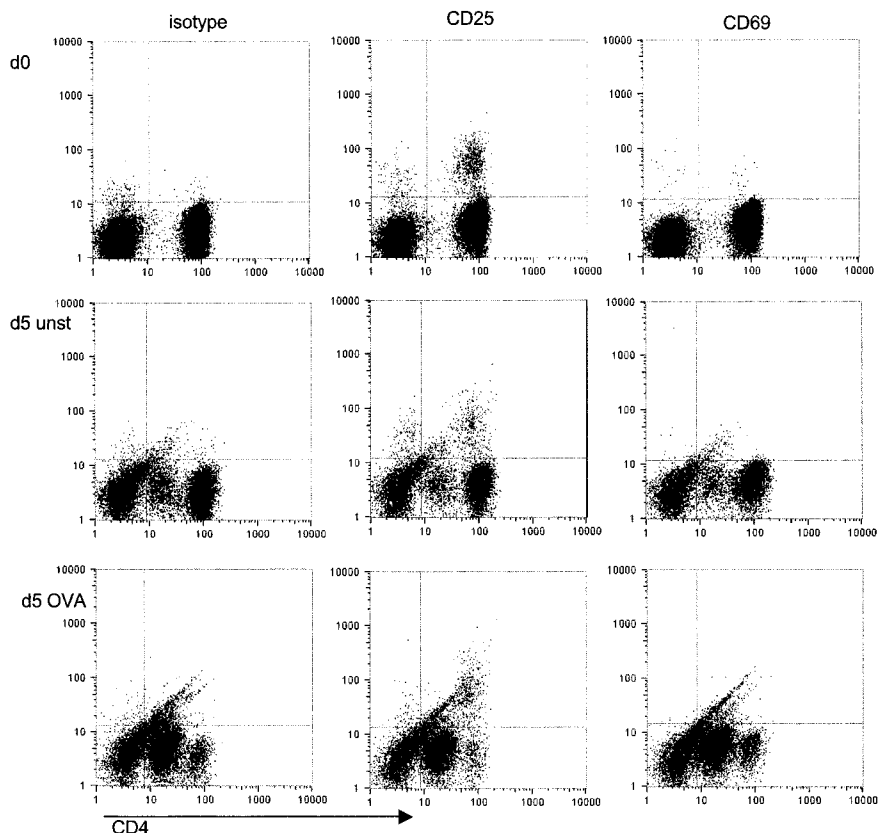


FIGURE 5. The expression of T cell activation markers by Ag stimulated by cord blood $CD4^+$ T cells. After 5 days of in vitro culture with OVA, the expression of CD25, CD69, and HLA-DR (and an appropriate isotype control) by $CD4^+$ T cells was examined by flow cytometry. Dot plots generated after gating on lymphocytes (by forward vs side scatter) are shown.

$CD38^+$ in contrast to adult $CD4^+CD25^+$ T cells that were predominantly $CD45RO^+CD45RA^-CD38^-$.

The $CD4^{high}CD25^+$ T cell population surviving in 5-day cord blood MNC cultures expressed surface phenotypic markers identical with the pattern shown for day 0 cord cells in Fig. 8, including CTLA4 (data not shown). $CD4^+CD25^+$ and $CD4^+CD25^-$ neonatal T cells collected by FACS sorting before culture (time 0) proliferated in response to PHA (Fig. 8a) and showed no evidence of suppressor activity in an MLR (Fig. 9b). However, $CD4^+CD25^+$ neonatal T cells collected after 5 days of culture with Ag (OVA) were both anergic (poor response to PHA stimulation; Fig.

8a) and suppressive in MLR responses (Fig. 8b). The functional properties of $CD4^+CD25^-$ cells after 5 days of culture were not examined, as insufficient numbers of $CD4^{high}CD25^-$ cells could be obtained and $CD4^{low}CD25^-$ cells are apoptotic, leading to very poor recovery after FACS sorting.

APC function is known to be impaired in the neonatal period (15, 16), so it was important to determine the extent to which the high levels of apoptosis observed in neonatal T cells might be attributed to APC immaturity. For these experiments, we took advantage of our previous observation that cord blood monocytes cultured with GM-CSF and IL-4 develop into functionally mature

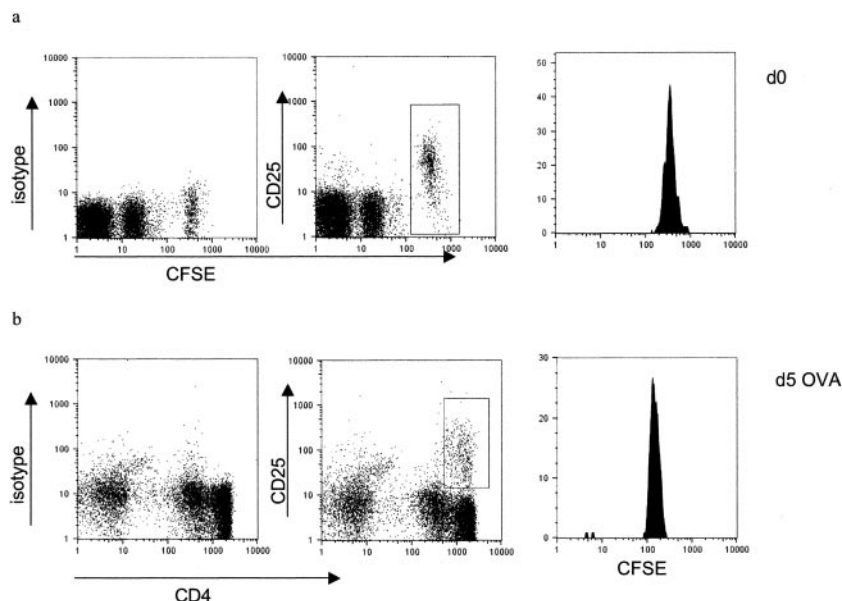
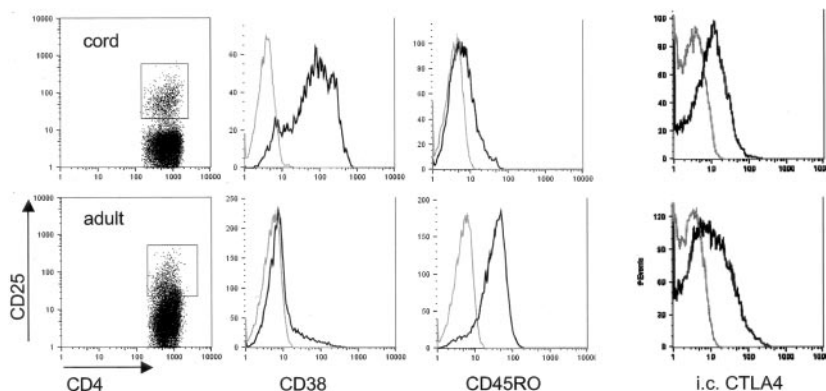


FIGURE 6. $CD4^{high}CD25^+$ T cells survive in vitro culture with Ag. $CD25^+$ MNC were positively selected from total MNC for staining with CFSE, returned to the $CD25^-$ fraction, and then cultured with and without OVA. After 5 days, the expression of CFSE by the $CD25^+$ subset at days 0 (a) and 5 (b) after stimulation with OVA was examined using flow cytometry.

FIGURE 7. The phenotype of cord vs adult blood $CD4^+CD25^+$ T cells. After setting a gate on $CD4^+$ T cells ($CD4$ expression vs side scatter), a further gate (R2) was set on $CD4^+CD25^+$ T cells. The expression of $CD38$, $CD45RO$, and intracellular CTLA4 by cells within this gate was then compared with an appropriate isotype control. A representative of at least three experiments is shown.



DC with adult-equivalent function (13). As shown in Fig. 9, supplementation of Ag-stimulated cord blood MNC with small numbers of autologous monocyte-derived functionally mature DC resulted in reduced apoptosis and increased $CD4^+$ T cell survival.

Discussion

This is the first study to characterize the *in vitro* response of cord blood MNC to common environmental allergens. Although a role is demonstrated for MHC class II-positive APC in allergen-induced activation of cord MNC, there appears to be no requirement for classical memory T cells ($CD45RO^+$) in this response. Although $CD45RO$ expression is not restricted to memory T cells and is also found on some non-T cell populations that might also be removed by the magnetic depletion protocol used in this study, the dramatic difference observed between the effects of $CD45RO$ depletion on adult and cord donors supports the postulate that cord blood MNC proliferative reactivity to allergen does not reside within the conventional memory T cell population. Moreover, the demonstration of rsGST-reactive T cells in cord MNC in the absence of apparent prepriming *in vivo*, taken together with recent findings pointing to a similar situation relative to HDM allergen

(3), suggest that these responses are unlikely to involve conventional memory T cells.

Contrasting the adult response, the response by cord MNC to various allergens/Ag is characterized by apoptosis and accompanied variably by proliferation. Ag-dependent apoptosis exceeded the characteristically high spontaneous apoptosis that is a commonly described feature of *in vitro* cultured cord blood MNC (17–19). Naive murine neonatal T cells have also been shown to proliferate strongly (in the absence of up-regulation of activation markers) in response to nominal Ag, and this has been suggested to be mediated largely by low affinity interactions with self peptide/MHC, although a contribution from exogenous peptide could not be excluded (20). Apoptosis was not examined in that study, but other studies have demonstrated that naive murine neonatal T cells also undergo apoptosis in response to primary TCR-mediated stimulation (21). Similarly, after activation with anti-CD3, naive human cord blood T cells show extensive apoptosis compared with adult naive T cells, a response postulated to arise subsequent to IL-2 consumption (17). Caspase-mediated cell death has been suggested as a mechanism of homeostatic control of Ag-independent expansion by cord blood RTE (22), although whether the increased

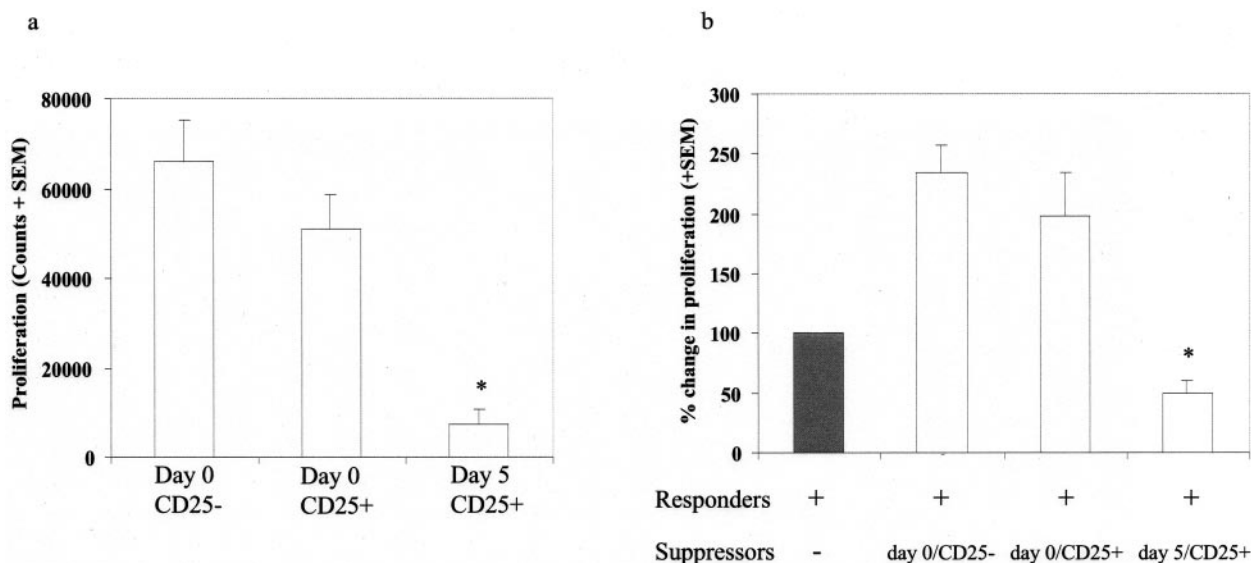


FIGURE 8. Functional maturation of $CD4^+CD25^+$ regulatory T cells after stimulation with Ag. $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells at day 0 and $CD4^+CD25^+$ T cells after 5 days of *in vitro* culture with OVA were prepared by cell sorting. *a*, Sorted cell populations (25,000/well) were cultured for 72 h with autologous $CD3$ -depleted MNC (50,000/well) with PHA, and the proliferative response (cpm $\pm$ SEM) was assessed ($n = 3$). *b*, Sorted cells (12,500/well) were cultured for 5 days with allogeneic irradiated $CD3$ -depleted MNC (50,000/well) as the stimulators and day 0 $CD4^+CD25^-$ T cells (25,000/well) as the responders. To control for the increased number of potential responder cells per well, the effect of increasing the number of responders (day 0 $CD4^+CD25^-$ cells were added; 12,500 or 25,000/well) was considered. Proliferation was assessed, and percentage of change in comparison with responders/stimulators only was determined (■, 100%; $n = 3$). *, $p < 0.05$.

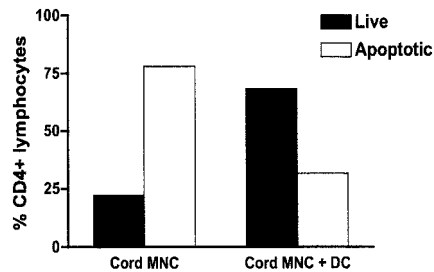


FIGURE 9. Mature DC enhance the survival of Ag-stimulated neonatal lymphocytes. Cord blood MNC were cocultured with autologous monocyte-derived DC and either OVA or HDM. Five days following Ag stimulation, live cells ($CD4^{\text{high}}$ annexin V $^{\text{negative}}$) and apoptotic cells ($CD4^{\text{low}}$ annexin V $^{\text{positive}}$) were enumerated by flow cytometry, after first gating on lymphocytes and excluding DC. Results are shown as the percentage of $CD4^+$ lymphocytes. A representative of three experiments is shown, in which MNC were stimulated with OVA. Absolute cell numbers were as follows: MNC cultured alone (live cells = 1,330; apoptotic cells = 4,591); MNC + DC (live cells = 25,080; apoptotic cells = 11,625). Similar results were also obtained with HDM stimulation.

apoptosis in the presence of Ag, as described in this work, uses the same pathways remains to be determined.

One plausible possibility, previously suggested in relation to comparable class I MHC-dependent $CD8^+$ responses in neonatal mice (23), is that promiscuous low affinity TCR/MHC-peptide interactions on the surface of functionally immature T cells might provide a mechanism for the expression of limited cellular immune responses against foreign Ags during the early postnatal period, preceding the development of conventional T cell memory mediated by high affinity T cells. It has been suggested that in neonatal mice this process may be mediated by a subset of T cells with CDR3 that are shorter than adult T cells (23). The TCR of these cells has been postulated to interact with the α -chain of the MHC molecule rather than with the peptide/peptide-binding groove, and thus explains promiscuity in neonatal T cell responses. An equivalent subset of such T cells in the human might account for the universality of the response we observed in this study, and there are reports of reduced CDR3 length in cord blood T cells that are consistent with this possibility (24). Indirect support for this suggestion is provided by results from earlier studies by our group on class II MHC-dependent $CD4^+$ T cell responses to environmental Ags such as OVA in human neonates (12). Notably, T cell epitope mapping of responses to OVA by cord blood MNC indicated reactivity to multiple regions of the molecule, as opposed to an average of ≤ 1 site when peripheral blood MNC from 5 year olds were tested.

It is clear from our findings that current interpretations of the relationship of in vitro T cell reactivity in human infants to the generation of T cell memory associated with allergic disease expression in later life require revision, given that the fate of the majority of the putative allergen-responsive cells in these cultures is apoptotic death. However, given that 1) substantial cytokine production precedes apoptosis (2, 4, 5, 25–28), 2) the responses appear to be generated against virtually any antigenic specificity that has not been deleted previously from the T cell repertoire, and 3) the RTE responsible for these effects constitute $>90\%$ of the peripheral blood T cell compartment in infants (22), it is likely that these responses are functional, and may play a significant role in regulation of early immune responses to a broad range of Ags.

A burgeoning body of literature highlights the intrinsic functional differences of fetal/neonatal vs adult naive T cells, and cord blood T cells can be considered to represent a transitional popu-

lation between thymocytes and adult T cells (17). Likewise, the functions of cells arising from murine fetal thymic precursors are qualitatively and quantitatively different from those derived from the adult thymus (29). In particular, the more rapid and earlier entry to cell cycle after both polyclonal and Ag-specific stimulation by murine fetal/neonatal relative to adult naive T cells appears to be properties retained from their respective thymic precursors (29), and may represent a further adaptation of T cell reactivity in the neonates that compensates for the relative inefficiency of T cell memory generation mechanisms during this life phase. Similarly, the available data highlight phenotypic and functional differences in $CD4^+CD25^+$ regulatory T cells from cord and adult blood.

Originally described in mouse models, $CD4^+CD25^+$ T cells with functional and other phenotypic properties comparable to their mouse counterparts are now described in humans (30–37). $CD4^+CD25^+$ regulatory T cells are characterized by expression of the negative regulatory molecule CTLA4, and more recently the transcription repressor FOXP3 (38), in association with the functional properties of anergy and suppression. Although expression of intracellular CTLA4 on neonatal $CD4^+CD25^+$ T cells was comparable to the adult-equivalent population, neonatal $CD4^+CD25^+$ T cells (including those surviving in day 5 cultures) were predominantly $CD45RO^-CD45RA^+CD38^+$ in contrast to adult $CD4^+CD25^+$ T cells, which were $CD45RO^+CD45RA^-CD38^-$. The lack of CD45RO expression has been described by all groups who have examined the phenotype of these cells in cord blood (34, 37, 39) and suggests that cord blood $CD4^+CD25^+$ regulatory T cells are a naive ($CD45RO^-CD45RA^+$) population, contrasting with the memory ($CD45RO^+CD45RA^-$) population found in peripheral blood from adults. Similarly, far fewer cord than adult blood $CD4^+CD25^+$ T cells express glutathione transferase (40).

A direct comparison with adult peripheral blood $CD4^+CD25^+$ T cells was not undertaken. However, earlier published studies indicate that $CD4^+CD25^+$ T cells freshly isolated from adult blood have both suppressive and anergic properties (30–33, 35), but this was not seen with the cord MNC response in the present experiments, although this activity has been reported in cord MNC elsewhere (34, 40). A more recent study also described functional differences between cord and adult blood $CD4^+CD25^+$ T cells: although the ability to suppress mitogen-activated $CD4^+CD25^-$ T cells was comparable, the cord blood population, unlike their adult counterparts, did not suppress Ag-specific responses (40). The limited number of cells available for our experiments did not allow for extensive dose-ranging studies on putative suppressor cells, and only a ratio of 1:2 (suppressor:responder) could be considered. It is possible that covert suppressive activity might be unmasked in our resting cord $CD4^+CD25^+$ cells if the ratio of these was increased; nevertheless, our observations clearly indicate that this function is markedly up-regulated in the $CD4^+CD25^+$ T cells that survive within the Ag-stimulated cultures. The preferential survival of cord blood $CD4^+CD25^+$ T cells contrasts with findings in the adult in which this population seems to be particularly susceptible to apoptosis (36); however, murine double-negative regulatory T cells are naturally resistant to apoptosis induced by TCR cross-linking (41). Such differences highlight the need to undertake further detailed functional and phenotypic analysis of regulatory T cell populations at different ages in humans, particularly during the first few years of life when long-term immunological memory to a range of environmental Ags is programmed.

Current perceptions of how regulatory T cells develop are based primarily on animal models, which suggest that these cells are generated in the thymus by TCR agonists expressed on thymic cortical epithelium (42). However, our data suggest that additional

events in the periphery might be necessary to generate fully functional regulatory T cells and, as suggested (40), these cells emerge in a naive state from the thymus to become fully activated in the periphery. Regulation of this activation process is likely to involve a series of mechanisms, which include a central role for APC. Indeed, limited proliferation accompanied by apoptosis is a characteristic feature of T cell responses induced by immature/resting DC in the absence of inflammatory/danger signals (43), and DC immaturity has been suggested as an important factor favoring development of regulatory T cell activity in the neonatal period (44, 45). Cord blood DC are functionally immature (13, 46–48), and more detailed studies on their contribution to this process appear warranted. Our preliminary investigations revealed that supplementation of Ag-stimulated cord blood MNC cultures with small numbers of autologous DC derived by cytokine-driven differentiation of monocyte precursors resulted in reduced apoptosis and increased CD4⁺ T cell survival, suggesting that DC immaturity might be a contributing factor in the phenomena reported in this study. We plan follow-up studies to determine the relative importance of T cell vs APC function in the regulation of neonatal T cell survival and the emergence of T regulatory cell populations in this system.

In contrast to the apoptotic responses seen under standard conditions of *in vitro* culture, it is feasible that the outcome of these initial CD4⁺ T cell responses *in vivo* varies with the tissue microenvironment in which they occur. As noted in Fig. 4, apoptotic death in these responses is markedly reduced in the presence of IL-4 and IL-7, and accordingly the fate and functional phenotype of the Ag-responsive CD4⁺ T cells and the CD4⁺CD25⁺ T cells in cultures supplemented with these cytokines merit more detailed investigations. Additionally, as detailed above, the maturational status of DC populations at sites of Ag challenge may be a crucial factor in determining the outcome of these early immune responses. Studies from our laboratory and elsewhere (reviewed in Ref. 49) indicate that the kinetics of DC maturation varies markedly between tissues during infancy, and can be markedly accelerated at mucosal tissue sites via environmental stimuli, particularly within the upper respiratory tract mucosa, which is the principal site of contact with aeroallergens. The possibility that variations in this maturation process may modulate the development of regulatory T cells during these early immune responses to environmental allergens also merits further investigation.

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