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Analysis of the Human Neonatal Thymus: Evidence for a Transient Thymic Involution

Alberto Varas,2* Eva Jiménez,* Rosa Sacedón,* Margarita Rodríguez-Mahou,‡ Enrique Maroto,§ Agustín G. Zapata,*, and Angeles Vicente†

The neonatal period is marked by the impairment of the major components of both innate and adaptive immunity. We report a severe depletion of cortical CD4+CD8+ double-positive thymocytes in the human neonatal thymus. This drastic reduction in immature double-positive cells, largely provoked by an increased rate of cell death, could be observed as early as 1 day after birth, delaying the recovery of the normal proportion of this thymocyte subset until the end of the first month of postnatal life. Serum cortisol levels were not increased in newborn donors, indicating that the neonatal thymic involution is a physiological rather than a stress-associated pathological event occurring in the perinatal period. Newborn thymuses also showed increased proportions of both primitive CD34+CD1+ precursor cells and mature TCRαβCD69+CD1+CD45RO+/RA and CD45RO+RA+ cells, which presumably correspond to recirculating T lymphocytes into the thymus. A notable reinforcement of the subcapsular epithelial cell layer as well as an increase in the intralobular extracellular matrix network accompanied modifications in the thymocyte population. Additionally neonatal thymic dendritic cells were found to be more effective than dendritic cells isolated from children’s thymuses at stimulating proliferative responses in allogeneic T cells. All these findings can account for several alterations affecting the peripheral pool of T lymphocytes in the perinatal period. The Journal of Immunology, 2000, 164:6260–6267.

T
he neonate, whether premature or of normal gestational age, is a unique host from an immunological perspective.

A certain reduction of the functional capabilities of many components of the immune system observed in neonates compared with adults has been explained on the basis of both the immaturity and the naive state of the neonatal immune system (1–3). Immunological differences between neonates and adults concern both innate and acquired immunity. Decreased bone marrow neutrophil storage pool reserve, reduced adherence/chemotaxis, and diminished microbicidal activity of neonatal neutrophils (4, 5); reduced serum complement levels and activity (6); impaired function of NK and lymphokine-activated killer cells (7, 8); and deficient APC function exhibited by spleen B cells and blood dendritic cells (DC) (9, 10) have been reported in neonates. In addition, newborns show a limited ability to produce Igκ (11), and make effective responses to a limited variety of Ags, principally failing to mount an Ab response to bacterial capsular polysaccharides (12).

Even for protein Ags, which do elicit an Ab response, neonates show a restricted use of the Ig V, D, and J gene repertoire (13), which limits the diversity of the response, as well as a reduced ability to switch from IgM to IgG or IgA responses (14), restricting the range of Ab-mediated functions available to the neonate. Neonatal Ab responses show little evidence of somatic mutation of Ig genes and a poor maturation of Ab affinity (15), factors that imply a limited ability to develop immunological memory.

Among peripheral T lymphocytes, most neonatal T cells exhibit an antigenically naive cell surface phenotype (i.e., CD45RA−) and a correspondingly naive functional program (16). The cytokine profile of neonatal T cells is unique, and it has been reported that T cells from newborns produce less IFN-γ, IL-2, IL-4, IL-10, and TNF-α than adult T cells in response to various stimuli (17–19). The CD40 ligand activation marker, which is involved in T-B cell interactions, is reduced on neonatal T cell activation (20), and the expression of a number of cytokine receptors, including the α, β, and γ chains of the IL-2 receptor, is also diminished compared with adult cells (21, 22). Normal responses to alloantigens and improvement of both proliferative responses and cytokine production after enhancement of costimulatory signals suggest that neonatal T cells have altered thresholds of responsiveness (1).

Despite the large number of results showing the impairment of newborn immunity, very little information is available about the neonatal condition of the thymus gland, a key organ for the maturation of an efficient immune system. In this regard, a decrease in the proportion of CD4+CD8+ TCRαβ high thymocytes has been reported in the neonatal rat and mouse thymus, concomitant with the start of differentiation of a new wave of T cell precursors colonizing the organ (23–25). In the present report, we analyze the different cellular components of the neonatal human thymus, demonstrating that a transient severe depletion of immature CD4+CD8− thymocytes occurs after birth, along with remarkable phenotypic and functional modifications of the thymic microenvironment components.
Materials and Methods

Tissue and cell preparation

Human thymus samples were obtained from neonates (age range, 1–20 days; n = 9) and children (age range, 1 month to 10 years; n = 11) undergoing corrective cardiovascular surgery at the Hospital Gregorio Marañón (Madrid, Spain). All donors were born at term after either spontaneous onset labor or cesarean section in the absence of labor; and at the time of sampling, donors were free of treatment that may influence immune functions, such as irradiation, steroids, glucocorticoids, or other immunosuppressive treatments.

Thymuses were dissected free of surrounding connective tissue and then gently disrupted with a Potter homogenizer until completely disaggregated. Thymocyte suspensions were washed and resuspended in RPMI 1640 supplemented with 2 mM glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin (all products from Life Technologies, Eragny, France), and 5% FCS (Biosy, Compiègne, France), hereafter referred to as complete medium.

Immunofluorescence and flow cytometry

mAbs of the following specificities were used in our study: CD4 (RPA-T4-FTTC or -PE), CD8 (RPA-T8-PE), CD1 (HI149-biotin or -FITC), CD45RO (UCHL1-FTTC), CD45RA (HI100-PE), CD11c (B-ly6-PE), CD69 (FN50-FTTC), CD54 (81-FTTC), CD80 (BB1-FTTC), CD86 (2331-biotin), CD40 (5C3-FTTC), and HLA-ABC (G46-2.6-FTTC) were obtained from PharcMingen; CD3 (SK7-FITC), and HLA-DR (L243-FTTC or -PE or -PerCP) from Becton Dickinson (San Jose, CA); CD54 (15.2-FTTC) from Southern Biotechnology (Birmingham, AL); and CD49d (HP2/1-FTTC) from Immunotech (Marseille, France).

Two- and three-color immunofluorescence stainings were performed by incubating the cells in PBS containing 1% FCS and 0.1% NaN₃ in the presence of saturating amounts of FITC-, PE-, PerCP-, and biotin-labeled Abs. Isotype-matched irrelevant Abs were used as negative controls to define background fluorescence.

Stained cells were analyzed in a FACScan flow cytometer (Becton Dickinson) and were excluded from the analysis by forward light scatter (FSC), side light scatter (SSC), and propidium iodide gating, and analysis was performed on at least 10,000 events. The data were analyzed using PC-lysis research software (Becton Dickinson).

Cell cycle analysis

To determine the proportion of proliferating cells, 2–3 × 10⁵ cells were stained with anti-CD4-FTTC and anti-CD8-PE for 30 min at 4°C. Cells were washed twice with PBS and fixed in 30% ethanol for a minimum of 30 min, but usually overnight at 4°C. The cells were then washed, resuspended in a solution of 25 μg/ml 7-amino actinomycin D (Sigma España) in PBS with 0.025% Nonidet P-40 (Sigma), and incubated in the dark at 4°C for 2 h. Analysis was conducted in a FACScan, using Cell Fit and PC-lysis software (Becton Dickinson).

Apoptosis assay

After cells were stained with anti-CD3-PE and washed twice with PBS containing 1% FCS, cells were stained with annexin V-FTTC (Boehringer Mannheim, Mannheim, Germany) according to the instructions of the manufacturer. Cells were analyzed on a FACScan and gated according to FSC, SSC, and their ability to exclude propidium iodide. Apoptotic cells were considered as those annexin V-positive and propidium iodide negative.

Serum cortisol levels

Blood samples were collected before surgery in nonheparinized tubes and, after four h at room temperature, centrifuged at 2,200 rpm for 15 min at 4°C. Serum was stored at −70°C until assayed. A fluorescence polarization immunoassay kit (TDX/TDX Cortisol, Abbott Laboratories, Abbott Park, IL) was used for the determination of serum cortisol levels according to the commercial supplier’s instructions.

Immunohistological staining

Thymic crossections 7 μm thick were air-dried for 2 h at room temperature and fixed in acetone for 10 min. Sections were incubated for 1 h at room temperature in the presence of the following unlabeled Abs: anti-cytokeratin (NCL-Pan-CK) from Novocastra (Newcastle, U.K.); anti-laminin (LAM-89), anti-fibronectin (IST-4), and anti-type-I collagen (COL-1) from Sigma; anti-HLA-DR (L243) from Becton Dickinson; anti-CD1a (HI149) and anti-CD11c (B-ly6) from Pharmingen; and anti-cortical epithelial cells (TE-3) and anti-medullary/subcapsular epithelial cells (TE-4) kindly provided by Dr. B. F. Haynes (Duke University Medical Center, Durham, NC). Control slides were incubated with antithyroglobulin Abs or normal mouse IgG (Dako, Glostrup, Denmark).

For immunohistochemistry stainings, endogenous peroxidase activity was inhibited with 1% H₂O₂ in methanol for 20 min, and sections were then incubated for 45 min with a 1/40 solution of peroxidase-conjugated rabbit anti-mouse IgG in PBS (Dako). The peroxidase reaction was developed with 0.05% 3,3′-diaminobenzidine (Sigma) in PBS with 0.1% H₂O₂ for 10 min. Sections were counterstained with methylene blue, dehydrated, and mounted in DePeX.

For immunofluorescence stainings, binding of primary mAbs was detected by incubation with a 1/100 solution of FITC-conjugated Fab’₂ fragment of rabbit anti-mouse IgG (Jackson Immunoresearch Laboratories, West Grove, PA). Slides were mounted in Vectashield (Vector Laboratories, Burlingame, CA) and then examined using a Zeiss Axiosplan-2 microscope.

Histological study

Thymic fragments were fixed by immersion in 4% cold glutaraldehyde, buffered to pH 7.3 with Millonig’s fluid, postfixed in 1% osmium tetroxide in the same buffer, and dehydrated in acetone for embedding in Araldite (Fluka, Buchs, Switzerland). Semithin sections (1–2 μm) were obtained with a Reichert OM-U3 ultramicrotome (Reichert-Jung, Wien, Austria) and stained with alkaline toluidine blue for the histological studies.

MLR assay

Thymic DC were isolated according to a modified procedure described by Beaulieu et al. (26). Briefly, unFractionated human thymic cells were first depleted of CD2⁺ thymocytes by incubation with neuraminidase-treated sheep RBC followed by Ficoll separation. Recovered cells were then depleted of T, B, myeloid, and NK cells by treatment with anti-CD3 (UCHT1), anti-CD7 (M-T701), anti-CD19 (B43), anti-CD14 (MSL9), and anti-CD56 (B159) (all from Pharmingen) bound to sheep anti-mouse IgG-coated magnetic beads (Dynal, Oslo, Norway). Subsequently, thymic DC were used at different numbers (10 cells to 5 × 10⁵ cells) as stimulators for resting allogeneic T cells (2 × 10⁵) isolated from peripheral blood. The cultures were performed in 96-well flat-bottom culture plates, using 0.2 ml complete medium. After 5 days at 37°C in a 5% CO₂-air incubator, the cultures were pulsed for 12 h with 10 µM 5-bromo-2′-deoxyuridine (BrdU). A specific kit from Boehringer Mannheim (Brdu Labeling and Detection Kit III) was used to measure BrdU incorporation into newly synthesized DNA. Briefly, the labeling medium was removed, and cells were washed (2 h at 1000 × g). The cultures were then incubated for 30 min at 37°C, treated with nuclease (30 min at 37°C), and then incubated with peroxidase-conjugated Fab’ fragments of mouse anti-BrdU (30 min at 37°C). The peroxidase reaction was developed with ABTS substrate, and the sample absorbance was measured using an ELISA reader at 405 nm with a reference wavelength at 492 nm.

Results

A drastic reduction in the numbers of CD4⁺CD8⁺ thymocytes occurs in the human neonatal thymus

The flow cytometric analysis of CD4 and CD8 Ag expression by thymocytes from newborns and children showed that the proportion of double-positive (DP) CD4⁺CD8⁺ thymocytes was dramatically reduced in neonatal thymuses. On the contrary, the percentages of single-positive (SP) CD4⁺CD8⁺ thymocytes and, mainly, double-negative (DN) CD4⁻CD8⁻ cells were notably higher than those found in children (Fig. 1A). Furthermore, although the total proportion of TCRαβ⁺ thymocytes hardly changed, the subset of TCRαβ⁺ high thymocytes dropped from 35–45% to 5–20% whereas mature TCRαβ⁺ low thymocytes accumulated in newborns (Fig. 1B). Within each CD4⁺CD8⁻ defined cell population, the relative distribution of TCRαβ expression remained unaltered, except for a 2- to 3-fold increase in the proportion of TCRαβ⁺ high cells detected within the CD4⁺CD8⁻ subset (Fig. 1B). No important variations in CD4⁺CD8⁺ cell ratio were observed (newborns, 2.2; children, 2.7).
A further immunohistochemical analysis, by using CD1 as a cell marker for cortical thymocytes (27), confirmed the depletion of CD4<sup>+</sup>CD8<sup>+</sup> thymocytes from neonatal thymuses. As shown in Fig. 2, thymic glands from children showed a well-developed cortex, filled by CD1<sup>+</sup> cells. In contrast, neonatal thymuses exhibited a reduction in the size of thymic cortex, where only a few scattered CD1<sup>+</sup> cells appeared. This neonatal depletion of cortical DP thymocytes was evident in all samples from donors with ages ranging from 1 to 20 – 30 days. From the second month of postnatal life, all donors exhibited normal proportions of thymocyte subsets (Fig. 1C).

Differences in the proportions of thymocyte subpopulations between newborns and children may be caused by variations in the proliferative rate of the different cell subsets. To test this possibility, a cell cycle analysis was conducted using 7-amino actinomycin D in combination with anti-CD4 and anti-CD8 mAbs. Comparison of the percentage of cycling cells in all CD4:CD8 populations did not show significant differences between newborns and children (Table I). Therefore, variations in proliferation cannot account for the differences found in the proportion of the thymocyte subsets.

We next analyzed whether the reduction in the numbers of DP thymocytes was due to an increased incidence of cells undergoing apoptosis in the neonatal thymus. Apoptosis was measured by staining with FITC-labeled annexin V after gating out dead cells using FSC, SSC, and propidium iodide exclusion. The proportion of thymocytes undergoing apoptosis was three-four times higher in newborns than in children (Fig. 3). When cells were gated according to their CD3 expression, the highest proportion of cells undergoing apoptosis was found in the CD3<sup>lo</sup> and, mainly, CD3<sup>low</sup> cell subsets, where almost one-half of the population stained with annexin V (Fig. 3). The incidence of cells undergoing apoptosis in the mature CD3<sup>hi</sup> cell subset was low in both newborns and children (Fig. 3). These data show that it is predominantly the CD4<sup>+</sup>CD8<sup>+</sup>CD3<sup>low</sup> cell population that is undergoing apoptosis, therefore largely accounting for the decreased proportion of DP thymocytes found in the neonatal thymus.

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**Serum cortisol levels in newborns and children**

Thymocyte depletion observed in newborns could be reflecting an acute stress as a consequence of the complex congenital cardiac
defects exhibited by these neonatal donors. We then determined the levels of cortisol in the serum of newborns and children, just before cardiac surgery. Our results demonstrate that there is no correlation between the levels of circulating cortisol and the percentage of DP thymocytes (Fig. 4). In addition, serum cortisol levels from all donors used in this study were in the same range of values than those obtained from healthy newborns (range, 2–11 μg/dl) and children (3–21 μg/dl) (28). We can therefore conclude that the transient thymic involution is a physiological rather than a stress-associated pathological event occurring in the neonates.

**Characterization of neonatal mature SP thymocytes**

The nature of mature TCRαβ<sup>hi</sup> thymocytes which increased in neonatal thymuses was investigated by analyzing the expression of different cell markers, including CD69, CD1, CD45RO, and CD45RA, which define phenotypically distinct stages during terminal differentiation of human thymocytes (29, 30). Three parameter analysis of the neonatal TCRαβ<sup>hi</sup> thymocyte subset showed an accumulation of CD69<sup>+</sup>CD1<sup>−</sup>CD45RO<sup>−</sup>CD45RA<sup>dull</sup>/CD45RO<sup>+</sup>CD45RA<sup>dull</sup> cells (Fig. 5), the phenotype of these cells being identical with that found in T cells from cord blood and neonatal peripheral blood (Fig. 5).

**Intrathymic T cell precursors**

The earliest T cell precursors in the thymus have been shown to be cortical thymocytes contained within the CD3<sup>−</sup>CD4<sup>−</sup>CD8<sup>−</sup> triple negative (TN) population that express the human pluripotent stem cell marker CD34 (31). Using three-color flow cytometric analysis, we found that TN CD34<sup>+</sup> cells represented 1.1% (range, 0.8–1.5%) of total thymocytes from children, whereas their proportion was increased 3–4 times in neonatal thymuses (mean, 3.9%; range, 2.9–6.1%). In addition, a higher proportion of TN CD34<sup>+</sup> cells did not express CD1 in newborns (70–75%) compared with children (40–50%), indicating that the most immature intrathymic precursors accumulate in the newborn thymus. This increased levels of primitive precursor cells could be related with the regeneration of the thymus gland which occurs by the end of the first month of postnatal life.

**Analysis of neonatal thymic microenvironment components**

An immunohistochemical analysis of thymic samples was conducted to assess whether the modifications found in neonatal thymocytes correlate with changes in the thymic stroma, including thymic epithelial cells (TEC) and extracellular matrix (ECM) components.

When comparing thymuses from children and newborns, no significant differences could be observed in the keratin-positive epithelial reticulum appearing in both thymic cortex and medulla (Fig. 6, a and b), by using an anti-pan cytokeratin Ab defining all epithelial cell subsets. Similarly, the pattern of distribution of TE-3<sup>−</sup> and TE-4<sup>+</sup> cells, which corresponded to cortical and medullary TEC, respectively, was not modified in neonatal thymuses (data not shown). It is important, however, to emphasize the notable reinforcement of the subcapsular epithelial cell layer occurring in the thymus of newborns, as evidenced by staining with anticytokeratin and TE-4 Abs (Fig. 6, c–f). By contrast, important changes occurred in the pattern of immunostaining of class II MHC molecules on TEC. Whereas in children’s thymuses a regular network of class II-positive epithelial cell processes occupied the whole thymic cortex (Fig. 6g), in neonatal thymuses the cortical areas exhibited a confluent pattern of staining (Fig. 6h), similar to that observed in the thymic medulla from children (Fig. 6g).

Type I collagen expression in children’s thymuses was restricted to the interstitial spaces of both capsule and septa and to the blood vessels, mainly those from the corticomedullary junction (Fig. 7a).

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**Table I. Cell cycle analysis of thymocyte subsets defined according to CD4 and CD8 expression**

<table>
<thead>
<tr>
<th>% of Cells in S + G2 + M</th>
<th>Total cells</th>
<th>CD4&lt;sup&gt;+&lt;/sup&gt;CD8&lt;sup&gt;−&lt;/sup&gt;</th>
<th>CD4&lt;sup&gt;+&lt;/sup&gt;CD8&lt;sup&gt;+&lt;/sup&gt;</th>
<th>CD4&lt;sup&gt;+&lt;/sup&gt;CD8&lt;sup&gt;+&lt;/sup&gt;</th>
<th>CD4&lt;sup&gt;+&lt;/sup&gt;CD8&lt;sup&gt;+&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Children</td>
<td>9.3 ± 1.2</td>
<td>9.7 ± 1.5</td>
<td>12.4 ± 2.6</td>
<td>2.0 ± 0.8</td>
<td>3.9 ± 0.4</td>
</tr>
<tr>
<td>Newborns</td>
<td>8.0 ± 2.6</td>
<td>13.8 ± 3.5</td>
<td>13.8 ± 3.3</td>
<td>1.9 ± 0.6</td>
<td>2.1 ± 0.7</td>
</tr>
</tbody>
</table>

* Data are expressed as percent of cells in $S + G_2 + M$ phases. Results are the mean ± SEM of three to four different individuals from each group.

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**FIGURE 3.** Apoptosis in thymocytes from children and newborns. Annexin V staining was measured by flow cytometry on cells gated by staining with Abs against CD3 as CD3<sup>+</sup>, CD3<sup>low</sup>, and CD3<sup>hi</sup>. Annexin V binding to total thymocytes is also shown. Dead cells were excluded by FSC, SSC profile and by their inability to exclude propidium iodide. Results are representative of 9–11 experiments.

**FIGURE 4.** Comparison of the percentage of CD4<sup>+</sup>CD8<sup>−</sup> thymocytes from all thymic samples according to the serum levels of cortisol. No correlation between the proportion of CD4<sup>+</sup>CD8<sup>+</sup> thymocytes and the serum cortisol levels in newborns and children was observed.
Fibronectin and laminin were detected in the basement membranes, as well as forming a fine network within the medulla of the thymic lobes (Fig. 7, c and e). On the contrary, a remarkable ECM pattern was consistently found in neonatal thymuses. A thickening of the basement membrane bordering the capsule and septa was observed after staining with anti-laminin and anti-fibronectin Abs (Fig. 7d). The cortical areas showed an increased expression of any ECM component (Fig. 7, b and d), partially associated with numerous enlarged blood vessels (Fig. 7, g and h). In addition, there was an increase in the number of fibronectin-positive thin fibers localized in the thymic medulla (Fig. 7f).

Comparison of the phenotypic and functional characteristics of thymic DC from children and newborns

Thymic DC constitute a relative sparse cell population which is thought to be involved in intrathymic negative selection of autoreactive thymocytes. Then, we first analyzed the pattern of expression of several surface markers on thymic DC from children and newborns. Virtually all thymic DC isolated from children and newborns were positive for CD11c, class II and I MHC molecules, CD86, ICAM-1, and VLA-4, although they lacked CD80 and CD1 expression (Fig. 8A). The level of expression of these molecules was, however, modified when both DC populations were compared, being that the levels of CD86 and MHC Class II molecules increased and those of the adhesion molecules ICAM-1 and VLA-4 decreased in neonatal DC (Fig. 8A). Likewise, both the proportion of positive cells and the level of expression of CD40 were increased in the neonatal DC population (Fig. 8A).

To know whether the phenotypic modifications exhibited by neonatal DC correlated with a differential allostimulatory capacity, thymic DC isolated from children and newborns were tested for their accessory function in allogeneic MLRs. As shown in Fig. 8B, neonatal thymic DC were more efficient than thymic DC from children. We demonstrate that a severe cortical CD4⁺ CD8⁺ thymocyte depletion occurs in newborn thymuses, largely as a consequence of the augmented rate of apoptosis exhibited by the DP cell subpopulation during the neonatal period. Additionally, the current results indicate that this transient thymic involution is a physiological event and is not reflecting a severe stress as a consequence of the complex heart defects exhibited by the neonatal donors. In agreement, Anand et al. (32) measured several hormonal and metabolic stress responses in neonates undergoing cardiac surgery, and showed that newborns are not stressed before surgical operation. Similar results have been also described by Milne et al. (33) in older infants undergoing cardiac surgery.
To our knowledge, a similar thymocyte depletion in the neonatal period has not been previously reported in humans, although Bertho et al. (34) have described a reduced thymic cellular density during the first weeks after birth. Decreased proportions of CD4⁺CD8⁺TCRβ⁺low thymocytes have been also pointed out in the thymus of neonatal mice and rats (24, 25). The underlying mechanisms to this process have not been formally proved, but two distinct mechanisms are known to be involved in the intrathymic cell death of DP thymocytes. One seems to be dependent of endogenous glucocorticoids (35), and the other operates via TCR and involves thymic DCs (36). The elevation of endogenous glucocorticoid levels has been demonstrated to result in a massive apoptosis of thymocytes, largely immature DP cells, and loss of thymic cellularity (37, 38). In this regard, it has been reported that fetal levels of corticosteroids rise very markedly by 3- to 12-fold throughout late gestation (from weeks 24 to 26 until the onset of labor), and sharply decline under adult levels immediately after birth (39 – 41). This physiological increase in fetal corticosteroid levels taking place by the end of gestation has a critical and unique function in the fetus in inducing a wide range of enzyme systems before birth that have little or no function during fetal life, but on which survival after birth is dependent. These maturation events have been reviewed by Liggins (42) and include stimulation of enzymes responsible for glycogen accumulation and gluconeogenesis in the liver; maturation of β cells of the pancreas; induction of cytodifferentiation of type II alveolar cells and synthesis and release of pulmonary surfactant; induction of epithelial maturation and alkaline phosphatase activity of the small intestine; and increase in the production of triiodothyronine and catecholamines.

Then, the prolonged exposure of fetal thymus to the increased levels of corticosteroids could explain the cellular depletion affecting the neonatal thymus. The finding that reduced numbers of DP cells can be detected in thymus samples from 1-day-old donors strongly suggests that the depletion of DP thymocytes begins to occur some time before birth. The normal proportions of thymocyte subsets are, however, recovered by the end of the first month of postnatal life, which indicates that the perinatal thymus recovers slowly after glucocorticoid exposure. In agreement with this point,
Bakker et al. (43) described that the prenatal exposure to glucocorticoids induce long-lasting effects on the neonatal rat thymus, which exhibited decreased T cell numbers.

Neonatal thymic DC could also contribute to the decrease in the thymic DP cell numbers. Supporting this notion, we demonstrate that neonatal thymic DC exhibit an enhanced allostimulatory activity compared with DC isolated from children thymuses. This was in complete agreement with the up-regulated expression of CD86, CD40, and MHC class II molecules shown in the neonatal thymic DC population. The functional importance of MHC Class II Ags and CD86 and CD40 costimulatory molecules in the allo- genetic MLR stimulated by human DC has been repeatedly reported (44–46). In contrast, cord blood DC have been shown to be poor stimulators of the allogeneic MLR compared with adult DC (10). The differential function of these two types of DC populations, as well as their putative distinct origin (36), could explain the different results obtained.

An important issue which this study raises is the impact that the transient neonatal thymic involution could have on the peripheral T cell population. Interestingly, several authors have described a 20–70% reduction in the proportion of T cells in cord blood and neonatal peripheral blood when compared with adult blood (our unpublished observations and Refs. 47–49). The drastic reduction in the numbers of immature CD4+CD8- thymocytes and the subsequent depletion of their progeny, which includes the mature SP thymocytes population that will exit the thymus to the periphery, could account for the diminished numbers of peripheral T cell occurring in the perinatal period.

Another finding of this study is that the alterations in thymocyte subpopulations are accompanied by modifications of the thymic stromal cell components. Some of these alterations, such as the change in the pattern of expression of Class II MHC molecules, could just be reflecting the loss of cortical thymocytes. However, other modifications of the nonlymphoid thymic components described here would represent an unique situation of the perinatal period rather than a direct influence of the cortical thymocyte depletion. Our data demonstrate a remarkable reinforcement of the subcapsular epithelial cell layer, as well as the existence of numerous enlarged blood vessels throughout the neonatal thymic parenchyma, which suggests the occurrence of important changes in thymic permeability. Similarly, enlarged perivascular spaces appear throughout rat thymic parenchyma during the perinatal period (25). Also Martín et al. (50) described, after increasing sex steroid levels, an increase of thymic cortical and corticomedullary vascular permeability, along with an almost total disappearance of the transcapsular route, caused by the reinforcement of the subcapsular epithelial cell layer. High levels of circulating sex steroids occur in the transcapsular route, caused by the reinforcement of the subcapsular epithelial cell layer. High levels of circulating sex steroids occur in the perinatal period (51) and, therefore, could explain the changes in neonatal thymic permeability, which could favor the entry of cells from the periphery. In fact, the analysis of TCRαβhigh thymocyte subset from newborn thymuses showed a preferential accumulation of CD69+CD1-CD45RA+ cells. These cells exhibit the same phenotype as T cells from cord blood as well as peripheral blood from neonates and children, and would correspond to recirculating T lymphocytes that enter the thymus from the periphery. Supporting this, it has been pointed out that increased numbers of peripheral T cells with a resting/naïve phenotype readily enter the mouse thymic gland during the neonatal period (52).

Additionally, the emigration of thymocytes from the thymus could be also altered by the increased thymic vascular permeability. We and other authors (27, 47, 49) have shown the presence of immature T cell subpopulations in neonatal blood, including CD4+CD8- and CD1+ cells. A phenotypic characterization of human peripheral DP cells has been conducted by Res et al. (27), who conclude that these cells correspond to a mature stage of CD1+ DP thymocytes that have been submitted to positive selection. With regard to CD1+ cells, they must include SP thymocytes that have not conclude the maturation process by which functionally mature SP T cells are generated (29, 30).

Although most functionally immature thymocyte subsets are stationary, their migration activity has been shown to be stimulated by high concentrations of ECM proteins, such as fibronectin (53). Our results show an increased expression of different ECM components in the neonatal thymus, and mainly of fibronectin in the thymic medulla. Therefore, the increase in the intrathymic ECM network could explain the presence of immature T cell subsets in peripheral blood during the perinatal period.

In conclusion, our results provide evidence that a profound involution transiently affects the human neonatal thymus; therefore, this is another event to incorporate in the ever-increasing list of immunological alterations occurring in the perinatal period.

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