Preterm neonates show marked leukopenia and lymphopenia that are associated with increased regulatory T-cell values and diminished IL-7

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INTRODUCTION: Current advances in neonatology have improved survival among preterm and low-birth-weight infants. However, the risk of neonatal death in preterm infants is much greater than in full-term neonates and is frequently associated with infections.

METHODS: Little is known about the immune status of preterm neonates; therefore, we analyzed the frequency and absolute counts of different immune populations in 211 cord blood samples taken from very-preterm to full-term neonates.

RESULTS: We found that absolute counts of all the immune subsets analyzed (i.e., monocytes, granulocytes, B cells, natural killer (NK) cells, CD4+, and CD8+ T cells) were markedly lower in preterm infants than in full-term infants. Surprisingly, we observed that regulatory T cells (Tregs) were the only cell subset that did not decrease in preterm infants, and their frequency was even higher than in full-term infants.

DISCUSSION: Tregs are crucial to maternal–fetal tolerance, but their suppressive role could be also implicated in the leukopenia observed in preterm infants. We did not observe differences in thymic function, but we found that plasma levels of interleukin (IL)-7 and the frequency of its receptor were significantly decreased in preterm infants. Our results could help to identify leukopenia and to implement immune therapies that significantly diminish mortality in preterm neonates.

Recent developments in neonatology and improvements in technology to manage preterm neonates have significantly increased the survival of very-preterm neonates (less than 28 wk of gestation) and very-low-birth-weight infants (less than 1,500 g) (1). However, the relative risk of neonatal death is much greater for preterm infants than for full-term infants, and prematurity has become the leading cause of perinatal morbidity and mortality (2,3). Along with decreasing gestational age (GA), low birth weight is also associated with increased perinatal morbidity and mortality (4). Among the causes responsible for this increased mortality, neonatal sepsis and bronchopulmonary dysplasia are the most frequent causes of death in premature newborns (3).

In fact, the most important factors that predispose to infection are prematurity and/or low birth weight.

Little is known about innate immunity in premature neonates and the capacity of their immune systems to fight against infections. Several authors have reported differences in phenotype and function of lymphocytes between healthy newborns, children, and adults (5). The immune response of preterm infants is assumed to be immature, although quantitative data on differences in immune capacity between preterm and full-term infants are scant.

The objective of this study was to investigate whether the high incidence of infections in preterm infants could be due to an immunodeficiency and, if so, to identify which immune cell populations are implicated in the absence of an adequate immune response. We performed a comprehensive analysis of the frequency and absolute counts of several cell populations in preterm and full-term infants to establish immune status according to GA and weight. We also studied the presence of regulatory T cells (Tregs) in preterm infants; this cell population may play a key role in preventing immune deregulation resulting in acute infections or chronic immune modulation. Tregs play a crucial role in maternal–fetal tolerance, and their levels are increased during pregnancy (6,7). However, little information is available about the frequency of these cells in neonates, particularly in preterm infants, or about their potential role in immune homeostasis during this period of life. Finally, establishing reference ranges for immune cell subpopulations in preterm infants could help the neonatologist to identify severe leukopenia and thus prevent infections. In addition, it could constitute a tool for implementing immunostimulatory treatments that could significantly decrease mortality in preterm infants.

RESULTS

We analyzed the percentage and absolute counts of different immune populations in fresh cord blood from 211 healthy neonates. Infants were stratified into two groups according to GA, and differences between these groups were studied. The

preterm group comprised 117 infants with a GA <37 wk; the full-term group comprised 94 infants with a GA ≥ 37 wk.

Clinical Parameters

The analysis of clinical parameters after delivery revealed that prematurity was associated with lower weight and length. Preterm neonates showed significantly lower weight ($P < 0.001$) and length ($P = 0.044$) than full-term neonates (**Table 1**), and weight and length values were directly correlated with GA ($P < 0.001$). Arterial pH values were higher in preterm infants (**Table 1**), although values were higher than 7.15 (which has been defined as the cutoff for acidosis

and increased mortality) in 97% of the studied samples (8). Therefore, no differences were established in the incidence of acidosis between preterm and full-term infants, and no correlation was established between prematurity and maternal age ($P = 0.390$) (**Table 1**).

Preterm Infants Showed Marked Leukopenia

We compared the percentages of different immune subsets between the groups and observed that preterm infants had a higher percentage of total lymphocytes than full-term neonates. We found that there were no significant differences in the percentage of monocytes. In addition, the percentage of

Table 1. Clinical data and percentages of immune cells in cord blood samples from preterm and full-term infants

	n	Preterm	n	Term	P value
Infant characteristics					
Wk	117	33 (31–35)	94	39.6 (38–40.9)	0.000
Weight (g)	110	2,050 (1,539–2,540)	91	3,300 (3,070–3,570)	0.000
Length (cm)	16	47.7 (44.2–49)	19	49.5 (48–51)	0.044
Arterial pH	107	7.34 (7.29–7.38)	81	7.28 (7.22–7.33)	0.000
Maternal age	108	31 (26.3–35)	85	31 (28–35)	NS
% Total lymphocytes	21	45.93 (36.3–59.4)	17	31.09 (26.96–35.3)	0.000
% CD4⁺T[a]	103	47.8 (42.4–54.5)	83	44.38 (38.01–51.1)	0.011
% CD8⁺T[a]	103	17.96 (14.5–22.2)	83	16.15 (13.56–19.6)	0.048
% NK cells[a]	105	7.09 (3.8–11.8)	84	10.62 (4.98–18.4)	0.004
% B cells[a]	103	15.84 (11.9–20.8)	83	17.91 (14.1–23.07)	0.029
% T cells CD127⁺[a]	29	90.9 (89.8–92.2)	18	92.3 (90.9–93)	0.029
% Monocytes[b]	23	9.73 (7.2–11.1)	18	8.69 (7.48–10.11)	NS
% Basophils[b]	23	1.14 (0.89–1.44)	18	0.84 (0.61–1)	NS
% Granulocytes[b]	23	40.03 (26.1–51.5)	18	56.49 (53.03–59.9)	0.000
% Neutrophils[c]	23	84.12 (73.3–87.2)	18	89.42 (84.2–92.85)	0.002
% Eosinophils[c]	23	9.66 (5.19–13.53)	18	4.04 (2.85–6.67)	0.020
% Immature Grns.[c]	23	8.15 (3.72–9.75)	18	6.06 (3.22–8.48)	NS
% CD4⁺T cell[d]					
% Naive	80	92.3 (71.4–95.6)	77	90.08 (70.3–95.9)	NS
% Memory	78	2.77 (1.6–4.6)	77	3.03 (1.8–4.79)	NS
% Activated	15	0.99 (0.7–2.4)	16	1.48 (0.98–1.92)	NS
% Effector	80	0.10 (0.03–0.2)	77	0.05 (0.02–0.17)	NS
% RTEs	23	51.7 (46.5–60.6)	17	64.79 (49.11–69.7)	NS
% Tregs	29	8.97 (7.25–10.07)	19	6.91 (6.12–7.91)	0.001
% CD4⁺CD127⁺	29	89.2 (88–90.7)	18	90.9 (89.9–92.1)	0.013
% CD8⁺T cells[d]					
% Naive	78	88.5 (81.4–93)	77	89.43 (85.61–93.2)	NS
% Memory	78	1.85 (0.35–4.68)	77	2.32 (0.63–4.2)	NS
% Activated	15	0.31 (0.23–0.39)	16	0.26 (0.21–0.38)	NS
% Effector	78	1.06 (0.29–3.12)	77	0.89 (0.24–2.77)	NS
% RTEs	23	77.06 (70.8–84.8)	17	80.31 (76.01–84.2)	NS

Values are given as median (25th–75th percentiles). *P* value ≥ 0.05 by two-sided test was considered nonsignificant.

Grns., granulocytes; NK, natural killer; NS, nonsignificant; RTEs, recent thymic emigrants; Tregs, regulatory T cells.

[a]Percentages of cells in the total lymphocyte population. [b]Percentages of cells in the total of live cells. [c]Percentages of cells in the total granulocyte population. [d]Percentages of subpopulations in the CD4⁺ or CD8⁺ T cells.

total granulocytes was lower, probably due to the significantly lower percentage of neutrophils in preterm infants ($P = 0.002$) (**Table 1**). We also observed that the proportion of immature granulocytes was similar in both groups ($P = 0.132$). Regarding lymphocytes, the percentage of natural killer (NK) and B cells was lower in preterm infants; however, the percentage of both CD4^{+} and CD8^{+} T cells was significantly higher in preterm infants than in full-term infants ($P = 0.011$ and $P = 0.048$, respectively) (**Table 1**).

When we analyzed the absolute values (cells/µl) of these immune cells, we observed that all the subsets studied (i.e., lymphocytes, monocytes, and granulocytes) were significantly decreased in preterm infants ($P < 0.001$) (**Table 2**, **Figure 1a**), and there was a significant positive correlation between GA and the absolute counts of these cells ($P < 0.001$ in all cases). We found a positive correlation between absolute counts of lymphocytes, monocytes, and granulocytes and weight ($P < 0.001$ in all cases). Regarding granulocytes, all basophil, neutrophil, and eosinophil counts were diminished in preterm infants (**Table 2**, **Figure 1a**).

Lymphocyte Subsets Were Decreased in Preterm Neonates
The lymphopenia observed in preterm infants affected all the lymphocyte subsets, and absolute counts of CD4^{+}, CD8^{+}, NK, and B cells were significantly lower than in full-term infants (**Table 2**, **Figure 1b**). Moreover, the values of these cells showed a marked positive correlation with GA ($P < 0.01$) and birth weight ($P < 0.05$), meaning that the more premature the infant, the lower the lymphocyte values. We determined the proportion of naive (CD27^{+}CD45RA^{+}), memory (CD45RO^{+}), activated (HLA-DR^{+}), and effector (CD27^{-}CD45RA^{+}) cells in both CD4^{+} and CD8^{+} T cells, but we did not find any differences between preterm and full-term infants (**Table 1**). Thus, the deficiency in T cells seems to be generalized to all T lymphocytes, and a decrease in or expansion of a specific subpopulation can be ruled out as the cause of T-cell lymphopenia.

A possible explanation for the decreased values of T lymphocytes could be that thymic production of T cells is lower in preterm infants; therefore, we analyzed the presence of T-cell receptor excision circles (TRECs) in peripheral blood mononuclear cells or purified T cells (9). We measured the ratio between the signal-joint TREC (sj-TREC) and β-TRECs, as previously described (10). No differences were observed in TREC content between preterm and full-term infants in either peripheral blood mononuclear cells or CD4^{+} T cells ($P = 0.502$, $P = 0.707$, respectively) (**Table 2**). There was no correlation between TREC values and T-cell counts ($P > 0.05$). We also measured the presence of protein tyrosine kinase 7, which has also been described as a marker of thymic production of CD4^{+} and CD8^{+} T cells (11). We did not detect the presence of protein tyrosine kinase 7 in CD8^{+} T cells, and we did not find significant differences between the two groups for CD4^{+} T cells ($P = 0.077$) (data not shown). Thus, there is no evidence that lower thymic production could explain the deficiency of T cells observed in preterm infants.

Table 2. Absolute counts of immune cells and thymic function in cord blood samples

	n	Preterm	n	Term	P value
Total lymphocytes[a]	103	3,372 (2,274–4,516)	85	4,378 (3,507–5,957)	0.000
CD4^{+} T	102	1,605 (1,140–2,248)	81	1,942 (1,396–2,458)	0.005
CD4^{+}RTEs	23	807 (538–961)	14	1,134 (821–1,937)	0.025
CD8^{+} T	102	584 (383–794)	81	739 (533–931)	0.002
CD8^{+}RTEs	23	382 (318–531)	14	621 (366.2–774.8)	0.008
Tregs	29	130 (100–166)	17	146 (90–179)	NS
NK cells	103	222 (131.7–421)	78	469 (206–861)	0.000
B cells	101	518 (348–804)	77	746 (554–1,056)	0.000
Monocytes[a]	23	601 (440–804)	17	1,241 (975–1,504)	0.000
Granulocytes[a]	23	2,252 (1,222–3,959)	17	7,930 (6,491–10,142)	0.000
Basophils	23	61 (48–106)	17	130 (88–159)	0.047
Neutrophils	23	1,780 (753–3,125)	17	6,781 (5,498–8,991)	0.000
Eosinophils	23	171 (122–426)	17	346 (223–559)	0.037
Immature Grns.	23	145 (71–303)	17	577 (240–772)	0.010
Thymic function[b]					
TRECs PBMCs	33	218 (128–1,210)	13	284 (41–680)	NS
TRECs CD4^{+}	17	160 (94–371)	19	154 (102–589)	NS
IL-7 (pg/ml)	85	0.50 (0.2–0.8)	68	0.95 (0.32–2.3)	0.001

Values are given as median (25th–75th percentiles). P value ≥ 0.05 by two-sided test was considered nonsignificant.

Grns., granulocytes; NK, natural killer; NS, nonsignificant; PBMC, peripheral blood mononuclear cell; RTEs, recent thymic emigrants; sj-TREC, signal-joint TREC; TREC, T-cell receptor gene rearrangement excision circle; Tregs, regulatory T cells.

[a]Absolute counts are shown as number of cells per µl of total cord blood. [b]TRECs values are shown as the ratio between sj-TRECs and β-TRECs.

Percentage of Treg Cells Was Increased in Preterm Infants

Treg cells are a subpopulation of CD4$^+$ T cells that play a crucial role in establishing and maintaining self-tolerance and immune homeostasis (12), and they can inhibit the proliferation of other immune cells. We analyzed the levels of this population identified as CD4$^+$CD25$^+$CD127low cells in neonates (**Figure 2a** (13)). Surprisingly, Tregs were the only subpopulation of immune cells that was not significantly decreased in preterm infants. In fact, absolute counts of Tregs were similar in preterm and full-term infants (median = 130 and 146, respectively; *P* = 0.860) (**Table 2, Figure 2b**), although the percentage of Tregs was significantly higher in preterm infants than in full-term infants (*P* = 0.001) (**Table 1, Figure 2c**). We also observed a significant inverse correlation between the percentage of Tregs and GA (*P* < 0.001), namely, the lower the GA, the higher the frequency of Treg cells (**Figure 2d**).

## Interleukin (IL)-7 Deficiency Could Contribute to Lymphopenia

We measured the IL-7 concentration, which plays a role in peripheral T-cell survival and expansion (14). Of note, the plasma concentration of IL-7 was markedly lower in preterm infants than in full-term infants (*P* = 0.001) (**Table 2, Figure 3a**). We also measured the IL-7 receptor (IL-7Rα or CD127) in both total T cells and CD4$^+$ T cells and found a significant decrease in the percentage of expression of CD127 in total T cells (*P* = 0.029) and in CD4$^+$ T cells (*P* = 0.013) (**Table 1**) in preterm infants. Moreover, we found a positive correlation between the frequency of CD127$^+$ cells and the absolute counts for both CD4$^+$ and CD8$^+$ T cells (*P* = 0.023 and *P* = 0.003, respectively) (**Figure 3b,c**). We did not observe differences in the mean fluorescence intensity of CD127 in total T cells (preterm = 3,576.8 ± 154, full-term = 3,755.3 ± 195; *P* = 0.204) or CD4$^+$ T cells (preterm = 6,499.2 ± 212, full-term = 7,117 ± 214; *P* = 0.092) between preterm and full-term infants. Thus, there were no differences in the quantity of IL-7 receptors per cell, but we did find significantly lower values of IL-7Rα–positive T cells in preterm infants. We also studied the percentage and absolute counts of recent thymic emigrants (RTEs), identified as CD45$^+$CD4$^+$CD45RA$^+$CD31$^+$ (15), which are the precursors of naive T cells and, therefore, responsible for the generation of the T-cell repertoire. We did not observe differences in the percentage of RTEs between preterm and full-term infants, although the results showed significantly lower absolute counts of both CD4$^+$ RTEs and CD8$^+$ RTEs in preterm infants (*P* = 0.025 and *P* = 0.008, respectively) (**Table 2**). Moreover, the absolute counts of RTEs for both CD4$^+$ and CD8$^+$ T cells were positively correlated with the frequency of IL-7Rα–positive T cells (*P* < 0.05) (**Figure 3d,e**).

In summary, the percentages of the different immune populations studied vary between preterm and full-term infants. However, all these immune subsets were quantitatively decreased in preterm neonates except for Treg cells. In the case of T lymphocytes, depletion seems to be independent of thymic function and could be related to a decrease in the concentration of IL-7 and the frequency of the IL-7 receptor in T cells.

DISCUSSION

The point during gestation when the human embryo begins to develop a competent immune system has been placed at around 20–24 wk. It had been widely assumed that neonatal T cells differ qualitatively from adult T cells. However, some authors propose that the problem with neonatal T cells is strictly quantitative: neonates contain far fewer T cells than adults (16). Several authors have described reference values for immune cell subpopulations in children from birth (full-term
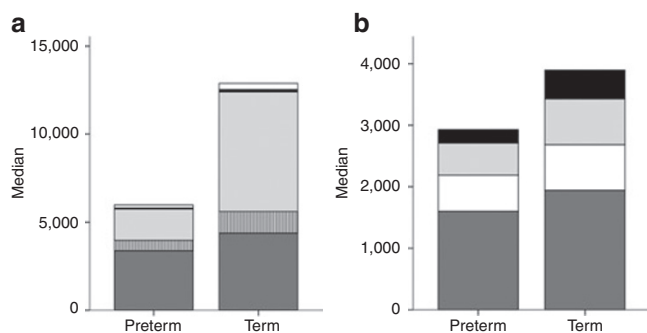
Figure 1. Leukopenia and lymphopenia in preterm infants. (**a**) Median of absolute counts for different immune populations of leukocytes: white, eosinophils; black, basophils; light gray, neutrophils; striped, monocytes; dark gray, lymphocytes. (**b**) Median of absolute counts for different subtypes of lymphocytes: black, NK; light gray, B cells; white, CD8$^+$ T cells; dark gray, CD4$^+$ T cells. NK, natural killer.
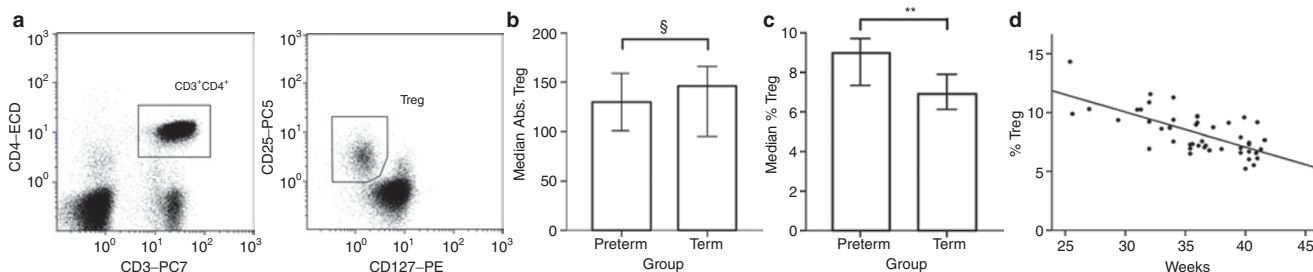
Figure 2. Increased Treg percentages in preterm infants. (**a**) Gating strategy for Treg cells in a representative subject. Treg cells were defined as CD4$^+$CD25$^+$CD127low. (**b**) Median values of absolute counts (Abs.) of Tregs; (**c**) percentage of Tregs in CD4$^+$ T cells; and (**d**) correlation between percentage of Tregs and GA (*R* = 0.498; *P* < 0.001). Error bars represent 95% confidence intervals. §, Nonsignificant differences; **, *P* value = 0.001. GA, gestational age; Treg, regulatory T cell.
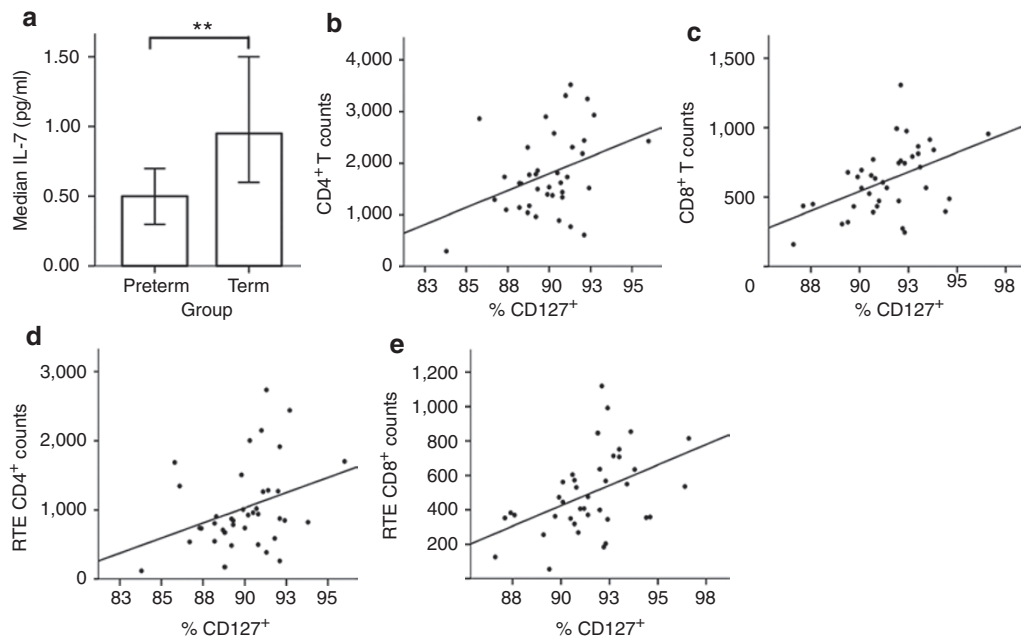
Figure 3. Concentration of IL-7 and frequency of IL-7 receptor. (**a**) Plasma levels of IL-7 expressed as pg/ml. Error bars represent 95% confidence intervals. Correlation between percentage of CD127⁺ (IL-7 receptor) cells and absolute counts of (**b**) CD4⁺ T cells ($R = 0.156$; $P = 0.023$), (**c**) CD8⁺ T cells ($R = 0.331$; $P = 0.003$), (**d**) RTECD4⁺ cells ($R = 0.114$; $P = 0.036$), and (**e**) RTECD8⁺ cells ($R = 0.208$; $P = 0.004$). **P value = 0.001. IL, interleukin; RTE, recent thymic emigrant.

neonates) until adolescence (17), although few have analyzed the same immune parameters in preterm infants (18,19). The novelty of our work is that we analyzed not only the percentage but also the absolute counts of almost all the relevant immune populations, including Tregs. The absolute values are not affected by the relative frequencies of other subsets and constitute a more reliable indicator of physiological immune status. We found discrepancies between the percentages we obtained for some subsets and the percentages reported in other studies (18). These differences are probably due to the lower number of neonates enrolled in some of these studies or to different staining strategies.

Our results for absolute counts of immune subsets enable us to conclude that preterm infants have marked leukopenia and lymphophenia and that this could play a crucial role in the increased frequency of infections in these neonates. Unfortunately, we do not have data about preeclampsia or pregnancy-induced hypertension in our cohort, which have been also associated to immune alterations (reviewed in ref. 20), and this limitation must be taken in consideration to explain the observed leukopenia. We observed that granulocytes, and notably neutrophils, were significantly decreased in the preterm group. Because the frequency of immature granulocytes was comparable in the two groups, the decrease did not seem to be due to a defect in the production of these cells. The median neutrophil count, considered one of the main defenses against infection, was 3.8-fold lower in preterm infants than in full-term infants. Therefore, these results reflect the presence of neutropenia in preterm infants. Neutropenia has been correlated with increased incidence of neonatal sepsis (21), and a recent study established a direct association between neutropenia and early sepsis and death in

preterm or very-low-birth-weight infants (22). Therefore, the neutropenia observed in the preterm group could be one of the factors responsible for the higher incidence of sepsis in preterm infants.

We also found decreased absolute counts for all NK, B, CD4⁺, and CD8⁺ T cells in preterm infants. These subsets are essential in the innate and adaptive immune responses and play a key role in the coordination and regulation of other immune cells. Deficient NK cytotoxicity has been described in both neonatal sepsis and recurrent infections in full-term infants (23). As mentioned earlier, monocytes are also diminished in preterm infants, and these cells play a key role as antigen-presenting cells in the activation and function of T cells, contributing to deficiency in the functionality of T cells. Regarding relative proportions, we observed that the percentage of total lymphocytes and frequency of CD4⁺ and CD8⁺ T cells was increased in preterm infants. Several studies have also reported higher percentages of lymphocytes in neonates (24) and preterm infants (18,19) than in children or adults. Those percentages could be interpreted as meaning preterm infants possess a similar immune capacity to that of full-term infants. However, we found that absolute counts of lymphocyte subsets were markedly diminished in preterm infants. Consequently, it seems likely that immune capacity would be severely impaired in these infants.

In the case of T lymphocytes, the decreased counts could be due to lower thymic production of T cells in preterm infants. However, we did not find differences in TREC content, and the proportions of both CD4⁺ RTEs and CD8⁺ RTEs were comparable in preterm and full-term infants. In fact, the thymus has been shown to be functional from very early phases of pregnancy (25), and therefore there is no evidence that

lower thymic production can explain the deficiency of T cells observed in preterm infants.

In preterm infants, we found a deficiency of IL-7 that has also been observed in low-birth-weight neonates (26). IL-7 is essential for the survival of T cells, especially RTEs. In fact, RTEs are greatly enriched in neonates and in cord blood (as compared with adult blood (27)), and they show increased proliferation to IL-7 (as compared with long-term resident peripheral T cells (14)). Moreover, the survival of immature umbilical cord lymphocytes is limited, and their viability is much more dependent on IL-7 than on resident naive or memory T cells (28). We also found reduced expression of IL-7Rα in preterm infants and a direct correlation between the frequency of this receptor and absolute counts of RTEs and T lymphocytes. IL-7Rα plays a critical role in the differentiation of T cells, and a partial deficiency in this receptor is sufficient to abrogate T-cell development and cause severe combined immunodeficiency (29). In summary, decreased counts of T lymphocytes, notably RTEs, in preterm infants could be a consequence of the lower concentrations of IL-7 and IL-7 receptor. The reduction in the RTE population seriously compromises the generation of an adequate repertoire of T cells capable of mounting an immune response against potential pathogens and could be related to reduced responses to immunization described in preterm infants (30). Under conditions of lymphopenia, IL-7 supports the homeostatic proliferation of peripheral T lymphocytes by promoting expansion of T cells with a diverse T-cell receptor repertoire (31,32). We do not know if this mechanism is also capable of compensating the lymphopenia observed in neonates, but it can be compromised in preterm infants because of decreased levels of IL-7 and IL-7 receptor.

Another factor that could be implicated in the lymphopenia observed in preterm infants is the presence of increased values of Treg cells. Tregs are a specialized subpopulation of CD4+ T cells that exert a regulatory effect on immune cells by suppressing the proliferation of naive T cells, the effector function of differentiated CD4 and CD8 T cells, and the function of NK cells, B cells, macrophages, osteoclasts, and dendritic cells (33). Recent studies support the concept that normal pregnancy is associated with an elevation in the number of Treg cells, and this may be important in maintaining maternal–fetal tolerance (34). Mold *et al.* (7) have shown that a substantial number of maternal cells cross the placenta to reside in fetal lymph nodes, inducing the development of CD4+CD25high FoxP3+ Tregs that suppress fetal antimaternal immunity. This fact could explain the presence of high values of Tregs in neonates and the negative correlation between Treg frequency and GA. In fact, we observed that absolute counts and percentage of Tregs in both preterm and full-term infants are higher than the values reported in the literature for children (17) and adults (35). The presence of increased values of Tregs in preterm infants would play a protective role in maternal–fetal tolerance but could have a secondary effect by which Tregs inhibit the survival and proliferation of lymphocytes and other immune cells. Moreover, unlike other T cells, Tregs do not require IL-7, because of the at least partially overlapping actions of IL-7 and

thymic stromal lymphopoietin for the development of Treg cells (36). It seems likely that the decreased concentration of IL-7 in preterm infants does not affect the survival or the frequency of Tregs, unlike other T lymphocytes.

Our results enable us to conclude that preterm infants experience severe impairment of the different immune populations in comparison with full-term infants. This leukopenia affects all granulocyte and lymphocyte subtypes, which are essential in the correct development of immune responses. The increased frequency of Tregs and the diminished values of both plasma IL-7 and IL-7Rα expression probably play a key role in the leukopenia observed. Moreover, this leukopenia is not limited to the moment of birth, and several follow-up studies in preterm infants demonstrated that lymphopenia is prolonged until 7 mo after birth (30), which could be correlated with the high incidence of early- and late-onset sepsis in premature neonates (3).

Historically, the detection of immunodeficiency in neonates has been guided by the presence or absence of certain types of infection. Identifying infants with a more profound lymphocyte deficiency before these sequelae develop may enable clinicians to take preventive measures and thus reduce mortality. The reference values reported in this study could help neonatologists in this task. We must find effective ways to intensify or accelerate the development of the immune system in preterm infants, and therapies such as stem cell transplantation, transfusions, or treatments with proven efficacy in neutropenia or severe combined immunodeficiency disease (37) could dramatically reduce the mortality associated with infections in this group.

METHODS

Study Population and Sample Preparation

Cord blood samples were obtained from 211 healthy neonates directly after delivery in the Hospital General Universitario Gregorio Marañón of Madrid (Spain) and were processed in the Spanish HIVHGM Biobank (38). GA ranged between 25 and 42 wk. Cord blood samples were stratified into two groups including 117 preterm infants (<37 wk of GA) and 94 full-term infants (≥37 wk of GA). Inclusion criteria comprised healthy neonates and mothers without genetic alterations or major birth defects. Diabetes, chromosomal abnormalities, and infections were also exclusion factors for the study. The study was approved by the clinical ethical committee of the Hospital General Universitario Gregorio Marañón of Madrid (Spain), and written informed consent was obtained from all legal guardians in agreement with the Declaration of Helsinki. Cord blood was taken by venipuncture from the umbilicalcord attached to the placenta immediately following separation from the infant and collected in sterile EDTA tubes. All blood analyses were performed within 12 h after birth.

Analysis of Immune Populations

Percentage and complete blood counts were determined in total blood samples by flow cytometry using a Beckman Coulter FC-500 Cytometer (Beckman Coulter, Marseille, France). Mouse antihuman antibodies were used to identify immune populations as described in the literature (39,40):anti-hCD45-FITC, anti-hCD4-ECD, anti-hCD45RA-ECD, anti-hCD127-PE, anti-hCD25-PECy5, anti-hCD3-PECy7, anti-hCD27-PECy5, anti-hCD8-PECy7, anti-hCD31-FITC, anti-hCD19-ECD, anti-hCD16-PECy5, anti-hCD56-PE, and corresponding isotype controls (all from Beckman Coulter). Absolute number of immune subtypes was determined using Flow-Count Fluorospheres and Flow-Count method (Beckman Coulter).

Analysis of Cytokines

Plasma IL-7 concentration was quantified in plasma from cord blood samples using commercial enzyme-linked immunosorbent assay Quantikine HS Human IL-7 (R&D Systems, Abingdon, UK) following the instructions of the kit. The minimum detectable dose of IL-7 is typically less than 0.1 pg/ml.

Analysis of Thymic Function

Thymic function was studied by measuring with real-time PCR the frequency of TRECs (9,41,42) in total peripheral blood mononuclear cells or CD4 and CD8 T cells purified by immunomagnetic isolation (MiltenyiBiotec, Bergisch Gladbach, Germany). PCRs were performed in a LightCycler system using the LightCycler FastStart DNA MasterPLUS kit (Roche Molecular Biochemicals, Mannheim, Germany). We determined the signal joint/β- TREC ratio as previously described (10), which has shown to be a more accurate method to measure thymic function. Moreover, the presence of protein tyrosine kinase 7 has been also described as a marker of thymic production of CD4$^+$ and CD8$^+$ T cells (11). We analyzed by flow cytometry the frequency of protein tyrosine kinase 7 in T cells using anti-hPTK7-PE (from Miltenyi Biotec).

Statistical Analysis

All data are given as median (25th–75th percentiles). Statistical analysis was performed using SPSS software. Student's *t*-test was used for variables with normal distribution and nonparametric Mann–Whitney test for variables without normal distribution. Correlation between variables was established by Pearson's correlation test. *P* value < 0.05 by two-sided test was considered significant.

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