Differing activation status and immune effector molecule expression profiles of neonatal and maternal lymphocytes in an African population

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Summary

Higher susceptibility of newborns to infections has been attributed to the hypo-responsiveness of their cellular immune system. Here we compared the activation status and expression of cytokines and cytotoxic molecules of cord versus maternal peripheral blood mononuclear cells in an African population. Human leucocyte antigen-DR was expressed on a lower percentage of cord compared to maternal γδ and $CD3^+$ T cells. Similarly, a lower proportion of cord versus maternal γδ and $CD3^+$ T cells displayed perforin, granzyme B and cytokine activity either *ex vivo* or following non-specific stimulation *in vitro*. In contrast, comparable proportions of cord and maternal $CD94^+$ $CD3^-$ natural killer (NK) cells showed perforin and granzyme B expression *ex vivo*. We conclude that cord blood γδ and $CD3^+$ T cells are functionally hypo-responsive as reflected by reduced numbers of such cells expressing either an activation marker, T helper 1 (Th1) and Th2 cytokines or cytotoxic effector molecules. The similarity in numbers of cord and maternal $CD94^+$ $CD3^-$ cells expressing cytotoxic effector molecules suggests that neonatal Africans' NK cells may be functionally mature.

Keywords: neonate; innate immunity; cytokines; cordblood; perforin

Introduction

Infectious diseases are a major cause of child mortality especially in developing countries and often cause morbidity and mortality already in the neonatal period.[1]

The cellular immune system of newborns is generally considered to be immature and hypo-responsive in comparison to that of adults. This is reflected in clinical observations showing that neonates are more highly susceptible to infection than adults and exhibit more severe or prolonged symptoms when infected.[2] However there is still controversy about the factors underlying this hypo-responsiveness. The dogmatic view that the neonatal immune system has intrinsically less immunological capacity *per se* than that of adults has recently been challenged by reports suggesting that neonatal cells are naive but nevertheless immunologically competent, that the observed differences are of a quantitative nature and that neonatal cells have the capacity to mount adult-like responses under appropriate conditions of stimulation.[3–5]

Because immune cells mediate many of their functions by secretion of cytokines, differences in cytokine production capacity help to characterize functional differences in neonatal and adult immune responses. Several studies have compared cytokine production by neonatal and adult cells in non-African populations.[6–8] Most studies directly compare cord blood to adult peripheral blood cells collected in different settings without taking into account the specific situation of pregnancy and delivery during which the levels of several immunomodulatory substances, including cortisol, prolactin, prostaglandin E2, oestrogens, are known to be altered in maternal and cord blood.[9–12] There are, in addition, substantial differences with the respect to global immune status in African versus European populations in general that are considered to be the result both of genetic differences as well as of

Abbreviations: CBMC, cord blood mononuclear cells; NK cells, natural killer cells; IPP, isopentenylpyrophosphate; PMA, phorbol 12-myristate 13-acetate.

environmental influences.[13–15] The latter include infectious diseases such as malaria.

The study described here therefore aimed to characterize the activation status and cytokine production capacity of Gabonese cord blood mononuclear cells (CBMC) in comparison to corresponding mothers' peripheral blood mononuclear cells (PBMC) collected shortly after delivery, in an attempt to further elucidate factors contributing to the perceived immunological immaturity of the neonate. We focused our analyses in particular on cells comprising components of the innate immune system. These include γδ T cells that represent 1–5% of the lymphocytes in the peripheral blood of adults, but a lower percentage in cord blood, and are cells that are involved in immune responses to several micro-organisms, including *Mycobacterium tuberculosis*, *Listeria monocytogenes and Brucella abortus*.[16–20] Natural killer (NK) cells are also important in this context, comprising approximately 15% of all circulating lymphocytes, and capable of lysing target cells as well as secreting cytokines, via which they activate antigen-presenting cells and direct the T helper (Th) cell type in the Th1 direction.[21] NK cells are usually defined by expression of CD56 and by a lack of CD3 on their surface. The $CD56^{bright}$ subset of NK cells is characterized by a high expression of NK receptor CD94.[22]

Subjects and methods

Study population

A total of 120 pregnant women and their 126 newborns were recruited shortly after delivery in the maternity unit of the Albert Schweitzer Hospital, Lambaréné, Gabon, a town of ~20 000 inhabitants situated in a rural tropical rainforest area, close to the equator, where malaria is hyperendemic and perennially transmitted. Mothers were of mixed ethnicity but predominantly from the locally most prevalent groups, i.e. Fang, Kele, Myene. Samples of maternal peripheral venous and of cord blood were taken into heparinized tubes after written informed consent to participate in the study had been obtained. For technical reasons, including low blood volumes and/or low recovery of viable cells as well as lower limits set for inclusion of flow cytometry-derived data (see below), variable numbers of samples (as indicated in the figures) were tested and analysed. Because *in vitro* rather than *ex vivo* assays were expected to provide the most relevant information, the former were favoured over the latter. Restricted availability of cells consequently resulted in a maximum of 10 maternal and 31 cord blood samples being used for *ex vivo* assays, and, for non-specific stimulation of cells *in vitro*, of 48 maternal and 74 cord blood samples, and 20 cord blood as well as 10 maternal blood samples for isopentenylpyrophosphate (IPP) stimulation. The study was approved by the ethics committee of the International Foundation of the Albert Schweitzer Hospital in Lambaréné.

Mitogens and antigens

The combination of phorbol 12-myristate 13-acetate (PMA), and ionomycin (both from Sigma, Deisenhofen, Germany) was used for mitogenic stimulation of mononuclear cells (see below). For specific stimulation of the γδ T-cell subset, IPP (Sigma), was used.

PBMC/CBMC cultures

All blood samples were processed within 4 hr of collection. Maternal PBMC or CBMC were isolated from heparinized blood using routine density centrifugation methods with Ficoll (Amersham Biosciences, Uppsala, Sweden). For stimulation cells were cultured at a concentration of $1{\cdot}25 \times 10^6$/ml in medium (Ultra Culture, BioWhittaker, Walkersville, MD) supplemented with L-glutamine (2 mM), 2-mercaptoethanol (3·5 μl/l, Sigma) and gentamicin (170 μg/ml). A mixture of PMA (10 ng/ml) and ionomycin (1·25 μM) was used to stimulate cells for 4 hr at 37° in 5% CO_2 in the presence of brefeldin A (10 μg/ml, Sigma). IPP (10 μg/ml) was used as a specific stimulus of γδ T cells for 18 hr and in this case brefeldin A (10 μg/ml) was added for the last 12 hr of incubation.

At the end of culture periods, cells were harvested on ice, washed twice in phosphate-buffered saline (PBS) and fixed with a 2% solution of formaldehyde (Merck, Darmstadt, Germany) in PBS for 20 min, again washed twice in PBS and then stored in Hank's balanced salt solution (HBSS, containing 0·1% sodium azide, both Sigma) supplemented with 0·3% bovine serum albumin (Serva, Heidelberg, Germany) at 4° in the dark until used for staining. These procedures follow those originally described by Winkler and colleagues.[23]

Flow cytometric analysis

For immunostaining, cells were washed once in PBS followed by surface staining in PBS at room temperature for 25 min. Cells were then permeabilized with PBS/0·1% saponin (Sigma). Intracellular staining was carried out in PBS/0·1% saponin for 30 min at room temperature, using appropriate combinations of the antibodies described below, followed by washing in PBS/0·1% saponin and PBS. Four-colour staining was performed and 2×10^5 cells were acquired on a FACSCalibur flow cytometer (Becton Dickinson, Heidelberg, Germany). Gates were set according to the profiles obtained with antibody isotype controls. Data derived from samples from which insufficient cells (i.e. <100 γδ T cells, <2000 $CD3^+$ T cells) could be acquired were discarded. Data were analysed using CellQuest 3.3 software (Becton Dickinson).

Antibodies and reagents

The following monoclonal antibodies (mAb) were used: fluoroscein isothiocyanate (FITC)–anti-interferon-γ (IFN-γ) mAb, FITC–anti-perforin mAb, FITC–anti-human leucocyte antigen (HLA)-DR mAb, phycoerythrin (PE)–anti-CD69 mAb, PE–anti-interleukin (IL)-13 mAb, PE–anti-IL-10 mAb, PE–anti-IL-5 mAb, PerCP–anti-CD3 mAb, allophycocyanin (APC)–anti-T-cell receptor (TCR) γδ mAb, APC–anti-CD94 mAb (all Becton Dickinson/Pharmingen), PE–anti-granzyme B mAb (Caltag, Hamburg, Germany). Isotype controls were included as appropriate.

Because cord blood NK cells may lack CD56 expression,[24] and also for technical reasons, the combination of $CD3^-$ $CD94^+$ was used to gate NK cells.

Statistical analyses

Statistical analyses were performed using the Statview software programme version 5.0.1 (SAS Institute, Cary, NC). Because the numbers of paired mother–cord samples varied and was frequently small, e.g. only six pairs for *ex vivo* analyses, only unpaired comparisons of continuous variables were made using the non-parametric Mann–Whitney *U*-test where a value of $P < 0{\cdot}05$ was considered significant.

Results

In this study CBMC and maternal PBMC were compared with respect to expression of activation markers, perforin and granzyme B expression *ex vivo*, as well as to cytokine production and perforin/granzyme B expression after stimulation with PMA/ionomycin or IPP.

CD69 and the major histocompatibility complex class II molecule HLA-DR are considered early and late T-cell activation markers, respectively, the latter playing a role in antigen presentation. *Ex vivo* analyses showed a lower percentage of $HLA\text{-}DR^+$ cord γδ and $CD3^+$ T cells in CBMC compared to PBMC (Fig. 1a, b). This pattern was identical following stimulation with PMA/ionomycin (Fig. 1c, d). Expression of CD69 was not detected on γδ and $CD3^+$ T cells *ex vivo* with the exception of a small number of CBMC and PBMC samples in which low percentages of $CD69^+$ cells were detected (data not shown).

Perforin and granzyme B are important effector molecules of cytotoxic T and NK cells that mediate target cell lysis and induction of apoptosis.[25] Expression of these molecules was examined both *ex vivo* and after non-specific stimulation. Maternal γδ and $CD3^+$ T cells expressed both perforin and granzyme B *ex vivo* but expression by cord γδ or $CD3^+$ T cells was minimal (Table 1). This pattern was unaffected by stimulation with PMA/ionomycin, although a certain amount of degranulation was suggested

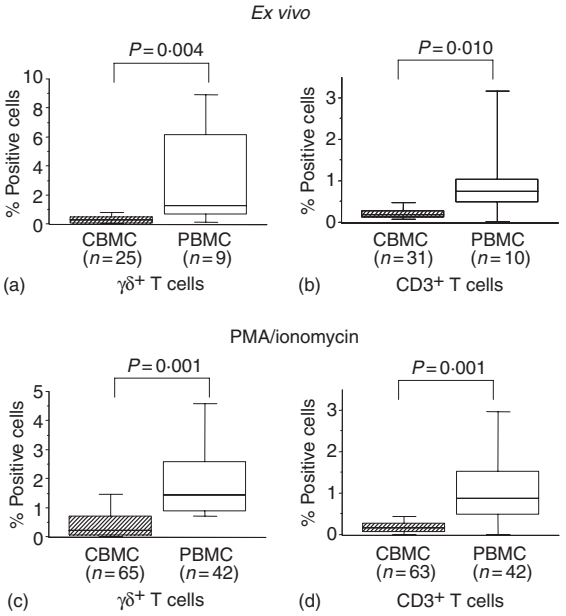

Figure 1. Expression of HLA-DR on CBMC and maternal PBMC *ex vivo* (a, b) or stimulated with PMA/ionomycin for 4 h in the presence of brefeldin A (c, d), fixed and stained for CD3, TCR-γδ, HLA-DR. A gate was set on $CD3^+TCR\gamma\delta^+$ cells (a, c) or on $CD3^+$ cells (b, d). Box-whisker plots show medians of percentages of cells expressing either HLA-DR with 25th and 75th percentiles in boxes and 10th/90th percentiles as whiskers. *P*-values refer to significance of differences between percentages considered as continuous variables and compared between groups using the non-parametric Mann–Whitney *U*-test.

by the slightly (but not statistically significantly) lower proportions of maternal γδ T cells with detectable granzyme B after stimulation (Table 1). In *ex vivo* samples of CBMC and PBMC the proportions of NK cells ($CD94^+$ $CD3^-$ cells) expressing perforin and granzyme B did not show statistically significant differences, but after PMA/ionomycin stimulation a higher proportion of maternal $CD94^+$ $CD3^-$ cells expressed granzyme B compared to cord cells (Table 1).

Following stimulation with PMA/ionomycin the proportions of CBMC γδ and $CD3^+$ T cells expressing either IFN-γ or IL-13 were lower than in PBMC, indicating a stronger capacity of adult versus neonatal cells for production of both Th1- and Th2-type cytokines after polyclonal activation (Fig. 2a–d). A smaller proportion of CBMC $CD3^+$ T cells expressed IL-10 compared with PBMC $CD3^+$ T cells after stimulation (Fig. 2e), and expression of IL-5 was detected in only low numbers of CBMC $CD3^+$ T cells (Fig. 2f).

After stimulation with IPP significantly fewer CBMC γδ T cells expressed perforin and granzyme B compared to those in PBMC and expression of IFN-γ was not observed in cord γδ T cells (Fig. 3a–c). Similarly low proportions of γδ T cells expressed IL-13 after IPP stimulation,

Table 1. Perforin and granzyme B expression in CBMC and PBMC

	Ex vivo			PMA + Ionomycin stimulation		
	Cord blood (n = 24–31)	Maternal peripheral blood (n = 5–9)	P-value[2]	Cord blood (n = 60–74)	Maternal peripheral blood (n = 35–48)	P-value
Perforin						
γδ T cells	0·1 (0·5)[1]	14·2 (33·1)	0·005	0·2 (0·5)	5·8 (12·7)	<0·001
$CD3^+$ T cells	0·1 (0·2)	0·6 (6·3)	0·01	0·1 (0·2)	0·4 (0·8)	<0·001
$CD94^+$ $CD3^-$ cells	30·1 (53·1)	24·2 (34·6)	0·45	19·7 (26·5)	18·7 (19·8)	0·62
Granzyme B						
γδ T cells	0·4 (1·2)	60·6 (43·9)	<0·001	0·4 (1·0)	32·2 (16·6)	<0·001
$CD3^+$ T cells	0·2 (0·4)	11·4 (16·6)	<0·001	0·3 (0·5)	7·6 (7·7)	<0·001
$CD94^+$ $CD3^-$ cells	20·7 (32·5)	55·8 (59·4)	0·25	34·6 (38·7)	74·4 (17·0)	<0·001

[1]Values are medians (interquartile ranges) of percentage cells detected with perforin/granzyme B activity.
[2]Mann–Whitney U-test (cord blood versus maternal peripheral blood mononuclear cell).

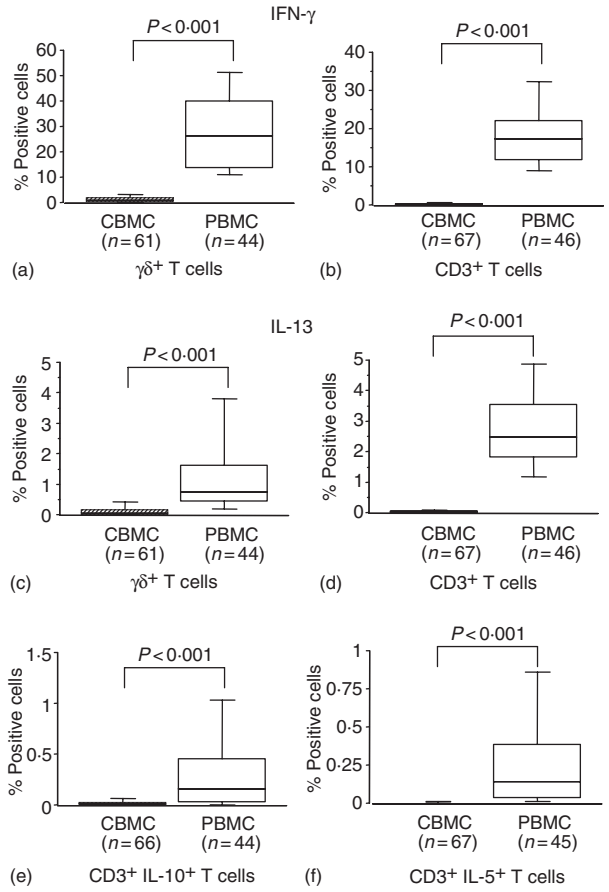

Figure 2. The percentages of IFN-γ- (a, b), IL13- (c, d), IL-10- (e) and IL-5-containing (f) CBMC and maternal PBMC detected by flow cytometry after stimulation with PMA/ionomycin for 4 hr in the presence of brefeldin A, followed by fixation, surface and intracellular staining and gating on $CD3^+$ $TCR\gamma\delta^+$ cells (a, c) or on $CD3^+$ cells (b, d, e, f). Box-whisker plots show medians of percentages with 25th and 75th percentiles in boxes and 10th/90th percentiles as whiskers. P-values refer to significance of differences between percentages considered as continuous variables and compared between groups using the non-parametric Mann–Whitney U-test.

with no difference between CBMC and PBMC (data not shown).

Discussion

Neonates are more susceptible to infection than adults and suffer from more severe or prolonged symptoms when infected. This has been attributed to an immature cellular immune system in newborns that is relatively hypo-responsive compared to that in adults.[2] The immaturity of the adaptive immune response in newborns is explained by lack of antigen contact and the high number of naïve neonatal T cells.[5,7]

Here, we aimed to compare the activation status and cytokine production capacities of neonatal in comparison with maternal mononuclear cells. Flow cytometry was used to examine different cell populations: $CD3^+$ T cells as part of the adaptive immune system, NK cells and γδ T cells as part of the innate immune system. The latter might be of special importance in neonates as they constitute the first line of defence against pathogens. Differences between Europeans and Africans with respect to immune status have been reported by others[13,15] that might result from different quantity and quality of *in utero* or postnatal antigen exposure. Here, we chose to focus only on an African population.

The expression of the early activation marker CD69 was equally low in both PBMC and CBMC, whereas the late activation marker HLA-DR was more frequently expressed on maternal γδ and $CD3^+$ T cells, possibly reflecting more frequent recent activation in mothers than in newborns. This finding is consistent with a study showing higher HLA-DR expression on Ethiopian adult $CD4^+$ and $CD8^+$ T cells than on those of neonates.[26] We extend this observation to the γδ T-cell population and to the context of pregnancy and delivery. In contrast to our observation the study by Hodge and colleagues did not find significant differences in HLA-DR expression on

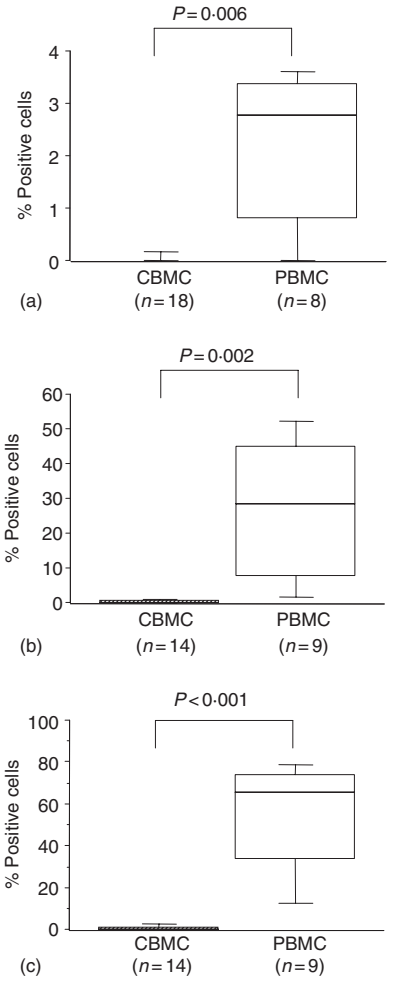

Figure 3. The percentages of IFN-γ- (a), Perforin- (b) and granzyme B-containing (c) CBMC and maternal PBMC detected by flow cytometry after stimulation with IPP for 18 hr in the presence of brefeldin A (12 hr), followed by fixation, surface and intracellular staining and gating on $CD3^+$ $TCR\gamma\delta^+$ cells. Box-whisker plots show medians of percentages with 25th and 75th percentiles in boxes and 10th/90th percentiles as whiskers. *P*-values refer to significance of differences between percentages considered as continuous variables and compared between groups using the non-parametric Mann–Whitney *U*-test.

T cells or NK cells of Australian donors' PBMC and CBMC either *ex vivo* or after stimulation.[2] We speculate that this difference reflects the relatively higher exposure of sub-Saharan African populations to endemic parasitic infections for example, that, in the population studied here, include malaria and a variety of helminths.

Perforin and granzyme B are two effector molecules of cytotoxic cells. We found that higher proportions of maternal γδ T and $CD3^+$ T cells expressed these molecules, implying a higher capacity to lyse target cells than cord cells. γδ T cells of newborns are the main cellular effectors combating extracellular pathogens, but the cytolytic and proliferative activity of cord blood γδ T-cell clones is decreased compared with that of adult γδ T cell clones[27] a finding consistent with our own. Berthou and colleagues found no constitutive expression of perforin in cord blood γδ or $CD4^+$ T cells as opposed to adult blood cells, whereas cord blood $CD8^+$ T cells expressed perforin.[25] We therefore conclude that the perforin expressing $CD3^+$ T cells we detected here belong to the $CD8^+$ T cell subset.

No significant differences between CBMC and PBMC with respect to the proportion of NK cells containing perforin or granzyme B were observed, indicating that these innate immune cells may have similar cytotoxic potential in newborns and adults. In line with this a separate study reported no significant difference in neonatal versus maternal NK cell cytotoxicity.[28] Other studies have reported neonatal NK cell cytotoxicity to be low[6,25,29] but capable of being enhanced in the presence of exogenous IL-2[24] and to reach levels comparable to adults upon addition of IL-12.[6,25]

We found that, following stimulation, lower proportions of CBMC expressed cytokines of both the Th1 and Th2 types compared with maternal PBMC, a finding that we assume to be an indication of their decreased functional capacity. This observation is broadly in accordance with other studies of neonatal and adult cells in non-African populations[2,6–8] although it should be noted that comparability is hampered somewhat by the varying methods of stimulation used in these different studies. Our study, in addition, took into account the immunological changes associated specifically with pregnancy and delivery. Krampera and colleagues compared PMA/ionomycin-stimulated CBMC and PBMC of Italian donors and reported no differences in T cell Th2-type (IL-5, IL-10, IL-13) cytokine activity.[30] This is clearly inconsistent with the results we report here. As discussed already above, we speculate that the higher capacity of African adult cells' for such cytokine production that we observed simply reflects the prevalence of, for example, intestinal and other helminth infections in this population. These data are consistent with those reported by Wilfing and colleagues.[15] One recent study reported significantly elevated expression of IL-13 specifically by naïve cord blood $CD8^+$ T cells of Dutch donors.[31] Interestingly, IL-13 promotes Th2 cell differentiation in mice[32] and it is thought that the neonatal immune system is biased towards Th2-type responses unless exposure to infectious agents redirects it to Th1.[31,33] It should be noted that such exposure – *in utero* or postnatally – is more frequent in developing countries. Both here and in a separate study in the same site[34] we have observed lower proportions of CBMC T cells containing IL-13 following either specific or non-specific stimulation compared with maternal PBMC, but we have not measured IL-13 protein secretion during extended stimulation and culture of cells *in vitro*. One recent study of Kenyan mothers and their offspring,

however, did not detect differences in IL-13 production *in vitro* following parasite antigen-specific stimulation.[35] A bias in favour of IL-10 production by CBMC following either specific or nonspecific stimulation has also been reported[35,36] and may reflect fetal exposure in African populations in which *in utero* sensitization to certain parasite antigens is common.[35,37,38]

Clearly, however, cytokine production capacity alone cannot explain the differences observed in immune responses between adult and neonatal mononuclear blood cells. The expression of the corresponding cytokine receptors must also influence the biological effect exerted by cytokines. In this context, it has indeed been shown that a range of cytokine receptors are expressed at a lower level on CBMC.[39]

In conclusion we show that African cord blood γδ and $CD3^+$ T cells exhibit reduced expression of an activation marker, of Th-1- and Th-2-type cytokines as well as cytotoxic effector molecules compared to corresponding maternal cells, reflecting their functional hyporesponsiveness, whereas cord $CD94^+$ $CD3^-$ cells contain levels of cytotoxic effector molecules comparable to maternal cells.

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