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# The interferon-alpha and interleukin-10 responses in neonates differ from adults, and their production remains partial throughout the first 18 months of life

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## Summary

**Previous studies have suggested that the susceptibility of newborns to infections is linked to the immaturity of their immune system, but very few data are available on the early stages of maturation of the immune response. Therefore, we decided to investigate the evolution of the interferon (IFN)-α and interleukin (IL)-10 responses in neonatal mononuclear cells. To this end, mononuclear cells isolated from cord blood and peripheral blood of 2-, 6- and 18-month-old children and adults were stimulated with unmethylated cytosine-phosphate-guanosine oligodeoxynucleotide (CpG-ODN) 2216 (IFN-α response) or lipopolysaccharide (LPS) (IL-10 response) for 24 h. The production of IFN-α and IL-10 was then measured in culture supernatants using enzyme-linked immunosorbent assay (ELISA) or a 6-plex cytokine array, respectively. Compared to adults, we found a significant impairment in both the IFN-α and IL-10 responses of neonatal mononuclear cells. Interestingly, both responses had increased significantly after 2 months, but remained lower than the adult responses throughout the first 18 months of life. This study shows that although the immune response of neonates tends to mature fairly quickly, it remains different when compared to the adult immune response throughout the first 18 months of life. This could have important consequences on children's ability to mount an appropriate immune response to various challenges and to establish tolerance and immune homeostasis.**

**Keywords:** cord blood, LPS, newborns, oligodeoxynucleotide, peripheral blood mononuclear cell

## Introduction

Newborns have been shown to have a higher susceptibility to infection and a lower response to vaccination than adults [1,2]. This is thought to be due to the immaturity of both their innate and adaptive immune system [3,4]. This immaturity could be related to impaired T cell responses [5] and to deficient functions of antigen-presenting cells (APC). Indeed, impaired responses of either myeloid or plasmacytoid dendritic cells (DC), the major inducers of effector T cell responses or tolerance [6], to various Toll-like receptor (TLR) ligands have been described extensively in human newborns [7–14]. However, most studies are performed with whole blood, rather than isolated mononuclear cells, potentially highlighting the effects of factors contained in the blood itself, and are restricted to one or a few time-points in early childhood. In this study, we wanted to investigate the differences between mononuclear cells of neonates and adults and follow the evolution of the immune response over the first 18 months of life.

Interferon (IFN)-α is a cytokine belonging to the type 1 IFN family that is fundamental for the anti-viral responses [15], but may also mediate immunoregulatory functions [16]. Indeed, type 1 IFNs promote a T helper type 1 (Th1)-like immune response with the production of IFN-γ and interleukin (IL)-10 in humans [17–20]. Virtually all nucleated cells are able to produce IFN-α, but among peripheral blood mononuclear cells (PBMC), plasmacytoid DC (pDC) are the major source of IFN-α because these cells are able to produce large amounts of IFN-α within the first 24 h in response to viral DNA and RNA or bacterial DNA [16,18,21–24]. TLRs are involved mainly in the control of innate immunity in response to pathogens. Among these, TLR-3, -7, -8 and -9 mediate immune responses to viral proteins or nucleic acids with the production of type 1 IFN (reviewed in [25]).

Synthetic TLR-9 ligands, such as the unmethylated cytosine-phosphate-guanosine oligodeoxynucleotide (CpG-ODN) 2216, have been shown to trigger *in vitro* the production of high level of IFN-α by pDC [7,8]. Such TLR-9 ligands are therefore proposed as adjuvants for vaccines against intracellular pathogens, cancer or atopic disorders (reviewed in [26] and [27]).

Interleukin (IL)-10 is a type II cytokine produced by a wide array of immune and non-immune cells (reviewed in [28]), including Th2 cells, regulatory T cells and DCs. Its potent regulatory and anti-inflammatory properties make it a key component of the immune system. Indeed, IL-10 production prevents tissue damage by limiting the inflammatory response [28]. In addition, IL-10 can inhibit both Th1 and Th2 responses and plays an essential role in immune homeostasis [28,29]. Finally, IL-10 contributes to the development of tolerance and is therefore believed to play a role in various disorders, including allergy [29].

In order to shed light on the maturation of the immune response of neonates, we followed the production of IFN-α and IL-10 by mononuclear cells over the first 18 months of life and compared it to adult responses. These results could have an application in vaccination strategy in early life.

## Materials and methods

### Cells isolation and culture

*Human subjects.* The present study was approved by the University and Hospital ethical committee and written informed consent was obtained from each individual or parents before blood sampling. Umbilical cord blood samples were collected from healthy full-term neonates. Children were included in a prospective cohort with regular clinical follow-up.

*Cord blood mononuclear cells (CBMC)/PBMC isolation.* CBMC/PBMC from healthy children/adults were isolated by density gradient centrifugation using Ficoll-Paque (GE Healthcare, Diegem, Belgium) on heparinized blood. CBMC/PBMC were collected, washed three times with Hanks' balanced salt solution (HBSS) (GibcoBRL, Merelbeke, Belgium) and resuspended in RPMI-1640 (GibcoBRL) supplemented with 1% of penicillin/streptomycin (GibcoBRL), 2 mM L-glutamine (GibcoBRL), 1% non-essential amino acids (GibcoBRL) and 10% fetal calf serum (FCS) (AEScientific, Marcq, Belgium). Cell viability estimated by blue trypan exclusion was >95%.

*CBMC/PBMC stimulation.* To study the IFN-α response, $5 \times 10^5$ CBMC/PBMC were cultured for 24 h in a volume of 500 µl in a 48-well plate, in the presence of 20 µg/ml of either unmethylated CpG-ODN (CpG 2216) or non-stimulatory CpG-ODN (CpG-CTRL). The CpG-ODN were purchased from Tib Molbiol (Berlin, Germany): CpG 2216, 5′-ggGGGACGATCGTCgggggG-3′; CpG-CTRL, 5′-TgCTgCTTTTgTgCTTTTgTgCTT-3′. The CpG-ODN concentration used in this assay was selected according to previous dose–response studies performed in a whole-blood model (15; 28). To study the IL-10 response, $5 \times 10^5$ CBMC/PBMC were cultured for 24 h, in a volume of 500 µl in a 48-well plate, in the presence or absence of lipopolysaccharide (LPS) (1 µg/ml). After 24 h of stimulation, the supernatants were collected and stored at −20°C until use.

### Cytokine quantification

The IFN-α produced in response to CpG stimulation were measured in the culture supernatants by enzyme-linked immunosorbent assay (ELISA) from PBL Biomedical Laboratories (detection limit: 6·8 pg/ml) (Invitrogen, Merelbeke, Belgium) according to the manufacturer's instructions and the data were analysed with a Multiskan EX absorbance reader (Thermo, Aalst, Belgium). IL-10 produced in response to LPS stimulation was measured in the culture supernatants using the a 6-plex bead array (detection limit: 0·45 pg/ml) (Luminex Technology, Biorad Laboratories, Eke, Belgium) according to the manufacturer's instructions and the data were analysed using the Bio-plex manager software. The results, obtained by subtracting the control values from the stimulated values, are expressed in pg/ml.

### Statistical analysis

Data were compared using the non-parametric Mann-Whitney unpaired *t*-test or the non-parametric Wilcoxon's paired *t*-test. *P*-values $\leq 0{\cdot}05$ were considered significant; *P*-values $\leq 0{\cdot}01$ were considered very significant; and *P*-values $\leq 0{\cdot}001$ were considered highly significant; n.s.: not significant.

## Results

### Deficiency in the IFN-α response of mononuclear blood cells persists throughout the first 18 months of life

In order to determine the evolution of the neonatal immune response to viral infections, we isolated mononuclear cells from cord blood as well as peripheral blood from 2-, 6- and 18-month-old children and compared their ability to produce IFN-α in response to CpG 2216 to that of adult controls. The clinical characteristics of the cohort are shown in Tables 1 and 2. We found that the response of neonatal mononuclear cells to CpG 2216 was impaired compared to that of adult PBMC (Fig. 1). Indeed, IFN-α production was significantly lower in CBMC than in adult PBMC (6·35 pg/ml ± 1·37 *versus* 118·93 pg/ml ± 30·89; $P \leq 0{\cdot}001$), and increased at 2 months (44·44 pg/ml ± 7·77) and 6 months (43·27 pg/ml ± 12·32) while remaining statistically lower

than adult levels ($P \leq 0{\cdot}05$ and $P \leq 0{\cdot}01$, respectively). IFN-α levels at 18 months were still less than half the adult levels (58·33 pg/ml ± 23·77), but the difference did not reach statistical significance ($P = 0{\cdot}1068$), due probably to the smaller number of patients in this group. No correlation was found between IFN-α levels and clinical data regarding the delivery, feeding, early signs of allergy or vaccinations of the children at the time of blood collection (data not shown). In order to determine whether IFN-α was the only CpG-induced response to be affected, we also measured the production of IL-10 or IL-12p70 in the culture supernatants and found no age-related modulation of the response, although most of our values were at the limit of detection of assay both in infants and adult controls (data not shown).

To confirm the results and ensure that the differences seen were not due to different patient groups, we focused upon the IFN-α production in 24 children for whom blood samples were available at 0 and 2 months. In those samples, the IFN-α production in response to CpG 2216 was decreased in three of 24 individuals or only maintained in six of 24 individuals at the age of 2 months compared to birth. However, using the non-parametric Wilcoxon's paired *t*-test, we confirmed a very significant increase of the IFN-α production after 2 months ($P \leq 0{\cdot}01$) (Fig. 2).

Together, these data demonstrated an impairment of the neonatal IFN-α response to viral products, which persisted from birth up to 18 months of age, despite a significant increase already evident in the first 2 months of life.

**Table 1.** Clinical data of the cohort.

| | *n* (%) | Months |
|---|---|---|
| Sex | | |
| Male | 39 (45%) | |
| Female | 47 (55%) | |
| Delivery | | |
| Midwifery | 72 (84%) | |
| Caesarean | 14 (16%) | |
| Feeding | | |
| Unknown | 6 (7%) | |
| Breast milk | 61 (71%) | |
| Mean duration ± s.d. | | 6·2 ± 3·9 |
| Median duration (range) | | 6 (1–18) |
| Powder milk | 19 (22%) | |
| Cumulative allergic status at 18 months | | |
| Unknown | 16 (19%) | |
| No allergy | 49 (58%) | |
| Allergy | 20 (23%) | |

s.d., standard deviation.

**Table 2.** Delay between blood test and vaccination.

| | *n* (%) | Days |
|---|---|---|
| 2 months ($n = 43/43$) | | |
| Not yet vaccinated | 24 (51%) | |
| Already vaccinated | 23 (49%) | |
| Mean ± s.d. | | 12 ± 9 |
| Median (range) | | 9 (2–39) |
| 6 months ($n = 35/39$) | | |
| Mean ± s.d. | | 53 ± 19 |
| Median (range) | | 56 (14–74) |
| 18 months ($n = 12/14$) | | |
| Mean ± s.d. | | 81 ± 51 |
| Median (range) | | 81 (14–186) |

s.d., standard deviation.

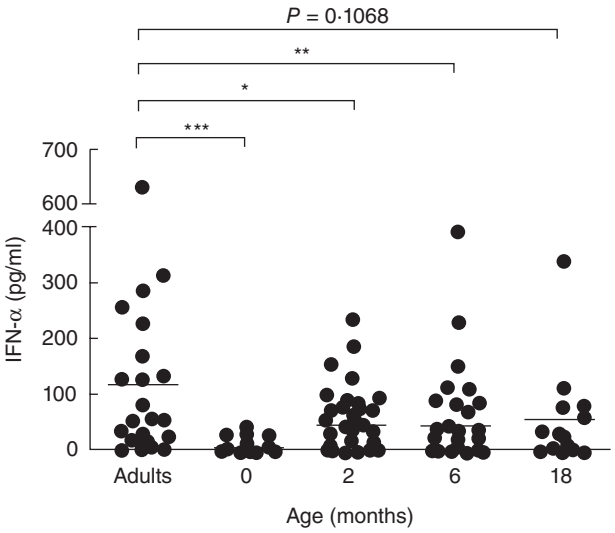


**Fig. 1.** Interferon (IFN)-α production in peripheral blood mononuclear cells from adults and neonates. Cord blood mononuclear cells ($n = 47$) or peripheral blood mononuclear cells from adults ($n = 23$), 2-month ($n = 47$), 6-month ($n = 39$) or 18-month ($n = 14$)-old children were cultured in a 48-well plate ($10^6$ cells/ml) in the presence of 20 μg/ml of either unmethylated cytosine-phosphate-guanosine-rich oligodeoxynucleotides (CpG-ODN) (CpG 2216) or non-stimulatory CpG-ODN (CpG-CTRL). After 24 h of stimulation, IFN-α production was evaluated in the culture supernatants using enzyme-linked immunosorbent assay. The results, obtained by subtracting the control values from the stimulated values, are expressed in pg/ml. **P*-values ≤ 0·05 were considered significant; ***P*-values ≤ 0·01 were considered very significant; ****P*-values ≤ 0·001 were considered highly significant.

## IL-10 production remains impaired significantly during the first 18 months of life

In order to determine the evolution of other critical immune responses in neonates, we studied IL-10 production by mononuclear cells in response to LPS stimulation (Fig. 3). We found that neonatal mononuclear cells produced significantly lower levels of IL-10 following LPS stimulation than adult PBMC (124·93 pg/ml ± 16·55 *versus* 804·41 pg/ml ± 141·57, $P \leq 0{\cdot}001$). In addition, we noted a progressive increase in IL-10 production with time (178·79 pg/ml ± 28·47 at 2 months, 284·97 pg/ml ± 27·24 at 6 months

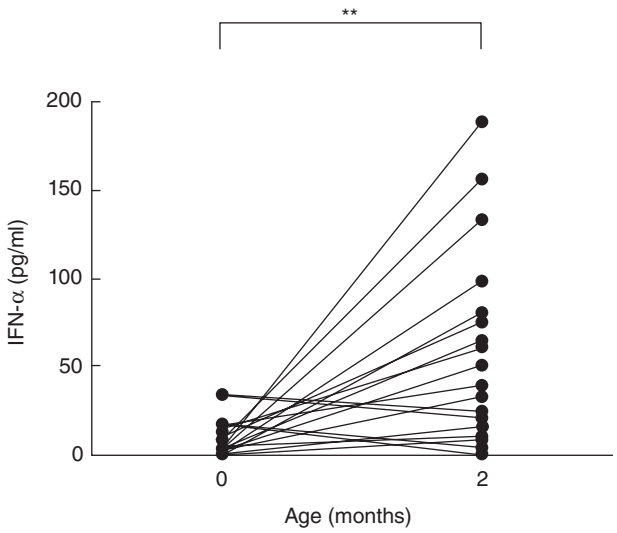


**Fig. 2.** Production of interferon (IFN)-α in children for whom blood was available at 0 and 2 months of age. Cord blood mononuclear cells ($n = 24$) and related 2-month-old ($n = 24$) peripheral blood mononuclear cells were cultured in a 48-well plate ($10^6$ cells/ml) in the presence of 20 μg/ml of either unmethylated cytosine-phosphate-guanosine-rich oligodeoxynucleotides (CpG-ODN) (CpG 2216) or non-stimulatory CpG-ODN (CpG-CTRL). After 24 h of stimulation, IFN-α production was evaluated in the culture supernatants using enzyme-linked immunosorbent assay. The results, obtained by subtracting the control values from the stimulated values, are expressed in pg/ml. $^{**}P$-values ≤ 0·01 were considered very significant.

and 212·07 pg/ml ± 127·1 at 18 months), although IL-10 levels remained significantly lower than in adults throughout the first 18 months of life ($P \leq 0{\cdot}001$). Together, these data demonstrated a deficiency in the IL-10 response of neonates, which was not corrected completely during the first 18 months of life.

## Discussion

In this study, we have demonstrated that the immune response of neonates is impaired compared to adults throughout the first 18 months of life. Indeed, both the IFN-α response elicited by the TLR-9 ligand, CpG 2216, and the IL-10 response elicited by LPS were reduced significantly in CBMCs when compared to adult PBMCs. Interestingly, both responses had already increased significantly by 2 months of age and continued to increase over the 18-month period, but remained lower than adult levels.

Results obtained in mice and in humans suggested that neonatal T cell-dependent responses are different in early life [5], although mature responses to vaccines or infectious pathogens were also demonstrated during the postnatal period (reviewed in [30]). Indeed, cord blood T cells and mononuclear cells respond normally when stimulated by allogeneic adult DC, suggesting that immature APC functions could be a reason for the defective immune response observed in neonates. In fact, neonatal monocyte-derived DC were shown to produce lower concentrations of the Th1-promoting cytokine, IL-12p70, in response to *Bordetella pertusis* toxin or CD40 ligation [9,10]. Moreover, various studies report that neonatal myeloid or plasmacytoid DC response to TLR-3 and TLR-4 [10–12,14] or to TLR-9 ligands [7,8] were impaired. The neonatal defect could not be attributed to lower expression of the corresponding TLR or to fewer cell counts in cord blood-derived cells [8,10,31–33]. So far, these observations are still controversial, as Gold *et al.* [34] found substantial IFN-α levels in neonates compared to adult levels in response to CpG-ODN, although IFN-α levels were significantly lower but not abolished completely in neonatal whole blood or purified pDC. This could be explained partially by the fact that the B cell response after TLR-9 triggering could also influence the pDC and Th1/Th2 T cell responses [35]. In this study, using freshly isolated CBMC or PBMC from healthy newborns or adults, we confirmed that IFN-α production in response to TLR-9 ligand was diminished significantly in neonates, and we demonstrated that this response remained lower than the adult response up to 18 months of age. These results are in line with the report by Nguyen *et al.* [36], showing that the type I IFN-dependent chemokines interferon gamma-induced protein (IP)-10 and monokine

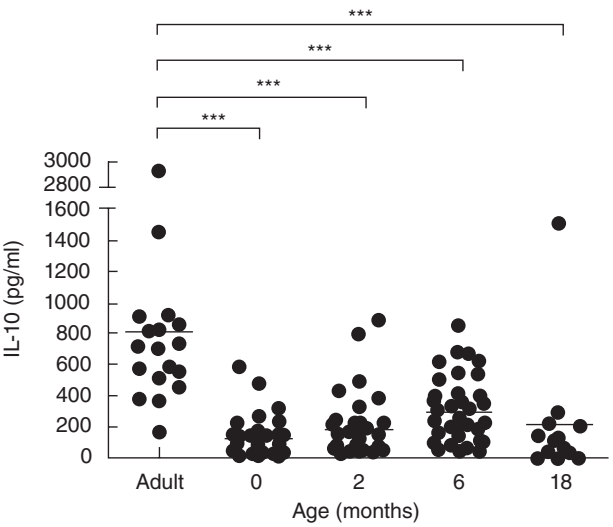


**Fig. 3.** Interleukin (IL)-10 production in peripheral blood mononuclear cells from adults and neonates. Cord blood mononuclear cells ($n = 46$) or peripheral blood mononuclear cells from adults ($n = 18$), 2-month ($n = 42$), 6-month ($n = 50$) or 18-month ($n = 10$)-old children were cultured in a 48-well plate ($10^6$ cells/ml) in the presence or absence of 1 μg/ml of lipopolysaccharide. After 24 h of stimulation, IL-10 production was evaluated in the culture supernatants using a 6-plex bead array. The results, obtained by subtracting the control values from the stimulated values, are expressed in pg/ml. $^{***}P$-values ≤ 0·001 were considered as highly significant.

induced by interferon-γ (MIG) levels were lower than the adult levels even at 1 year of age. It is interesting to note that clinical parameters such as delivery, feeding, allergy or vaccination status at the time of blood collection had no influence on the data.

Several studies seem to demonstrate higher IL-10 responses in cord blood compared to adult samples [12,36,37], but it has to be noted that these studies stimulated whole blood samples from cord blood or before 1 month of age and could also reflect the impact of factors present in the blood itself. Indeed, Belderbos *et al.* have demonstrated that adult PBMC produced higher levels of IL-10 in response to LPS when stimulated in the presence of neonatal plasma than when stimulated in the presence of adult plasma [37]. In this study, we used isolated mononuclear cells to investigate the immune status of the cell population itself. We found that, in contrast to the results obtained with whole blood stimulation, isolated neonatal cord blood mononuclear cells showed a deficient production of IL-10 in response to LPS after 2 months which persisted up to 18 months of age. In addition to the variation in the test samples themselves, the discrepancy between our results and previous reports could be explained by differences in kinetics and in the concentrations of LPS used as well as the stimulation protocol, as we used LPS alone, but some other studies have used LPS in the presence of IFN-γ [37]. Taken together, our results confirm the immaturity of the neonatal immune system by demonstrating a deficiency in both IFN-α and IL-10 responses, which persisted throughout the first 18 months of life, despite a significant amelioration with time. This deficient immune response could be relevant to the understanding of the susceptibility of newborns to infections, and to the use of CpG-ODN as vaccine adjuvant in order to boost immunity of newborns. In addition, this study brings new insight into the ability of the neonate to establish immune tolerance and homeostasis.

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## Disclosure

Authors have no disclosures to declare.

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