

Interleukin-18 enhances the production of interferon-gamma (IFN- γ) by allergen-specific and unspecific stimulated cord blood mononuclear cells

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Abstract

Background: IL-18 is a pleiotropic cytokine involved in the polarisation of T-cell response. This study was performed to determine whether or not IL-18 is detectable in phytohemagglutinin (PHA) or betalactoglobulin (BLG) stimulated supernatants of cord blood mononuclear cells (CBMC) and to study the *in vitro* effect of IL-18 on the interferon (IFN)- γ and IL-13 release of CBMC of healthy neonates.

Methods: CBMC of neonates were isolated by Ficoll density centrifugation. The cytokines IFN- γ , IL-13 and IL-18 in the cell culture supernatants were measured using the ELISA technique following stimulation with a unspecific (PHA 20 μ g/ml) and an allergen-specific stimulus (BLG 25 μ g/ml). In order to study the *in vitro* effect of IL-18, CBMC were stimulated either with medium alone or with IL-18, IL-18 + PHA and IL-18 + BLG.

Results: IL-18 levels in supernatants of CBMC were low and did not vary significantly between unstimulated and PHA or BLG stimulated cell cultures (median 21.4; 23.5 and 15.5 pg/ml, respectively). IFN- γ and IL-13 levels were significantly higher in response to PHA and BLG (PHA: IFN- γ , 6154; IL-13, 4357; BLG: IFN- γ , 801; IL-13, 249 pg/ml) compared to unstimulated cell cultures. The addition of IL-18 to PHA or BLG stimulated CBMC significantly enhanced the IFN- γ release (PHA: 6154; PHA + IL-18: 13474, $p = 0.0001$; BLG: 801; BLG + IL-18: 1077, $p = 0.008$). In comparison to incubation without IL-18, the release of IL-13 was invariable or even reduced, when CBMC were stimulated with PHA + IL-18 (4026, $p = 0.16$) or BLG + IL-18 (124, $p = 0.0001$) compared to stimulation of CBMC with PHA (4357 pg/ml) or BLG (249 pg/ml) alone.

Conclusions: IL-18 is detectable in supernatants of CBMC. We observed a significant effect of IL-18 + PHA as well as IL-18 + BLG on IFN- γ release *in vitro*. Based on our findings we conclude that IL-18 could act as a strong TH1-inducing factor on stimulated CBMC also *in vivo*.

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Keywords: Atopy; IL-18; IL-13; IFN- γ ; IgE-regulation; Cord blood; Allergen; Early sensitisation

Abbreviations: BLG, betalactoglobulin; CBMC, cord blood mononuclear cells; GBS, group B streptococci; IL, interleukin; PBMC, peripheral blood mononuclear cells; PHA, phytohemagglutinin; TH cells, T-helper cells

1. Introduction

CD4⁺ T cells can be subdivided into at least two different subsets characterised by TH1 and TH2 cytokine patterns [1]. The imbalance between TH1 and

TH2 cytokines is important for the outcome of several infectious and autoimmune diseases. TH1 cells producing IFN- γ and IL-12 are important for defence against intracellular pathogens and mediate inflammatory responses, whereas TH2 cells producing IL-4, IL-5 and IL-13 are mainly associated with eradication of extracellular parasites and are responsible for the regulation of immunoglobulin E (IgE) in allergic diseases [1–4].

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Cytokine production of cord blood cells in response to mitogens and allergens has been investigated by several groups. It is now generally accepted that supernatants of stimulated CBMC show a TH2 dominated cytokine pattern and only low TH1 cytokine production [5–7]. Comparing neonates with the later development of allergic diseases and non-atopic children, IFN- γ production is lower in individuals with an atopic family history and increases more slowly from birth to the age of 5 years [6,7]. Although the pathophysiology of the early TH2 response of neonates has been intensively investigated, the mechanism leading to their preferential differentiation remains elusive. Cytokines are considered the main determining factors in the initial differentiation of precursor T cells into these distinct subsets.

Interleukin (IL)-18, initially identified as IFN- γ -inducing factor [8], is a cytokine of approximately 18 kDa, which is made as an inactive 24 kDa precursor molecule without a signal peptide cleaved by caspase-1 to produce an active cytokine [9]. Different cell types have been reported to produce IL-18, including macrophages and dendritic cells [10], Kupffer cells [11], astrocytes and microglia [12], intestinal and airway epithelial cells [13], keratinocytes [14] and osteoblasts [15].

IL-18 was found to be elevated both after bacterial [16] and after viral infection [17]. A major target of IL-18 is cytokine production. Early studies of IL-18 highlighted the role of IL-18 as an IFN- γ -inducing factor and therefore as an inducer of TH1 response [8,18]. In combination with IL-12, IL-18 can induce IFN- γ production without TcR engagement in TH1 cells and non-polarized T cells, and induces IFN- γ release in B cells, NK cells, macrophages and dendritic cells.

More recently IL-18 was reported to induce the production of the TH2 cytokine IL-13 from T cells and NK cells in vitro [19]. Moreover, IL-18 directly stimulates basophils and mast cells to produce TH2 cytokines and histamine independently of IgE [20].

Therefore the question arises whether IL-18 is critically involved in the TH1/TH2 balance and the pathophysiology leading to specific IgE production at the beginning of the “allergic march” early in life. In the present study we analysed the release of IL-18, IL-13 and IFN- γ of CBMC of healthy neonates. Additionally the in vitro effects of IL-18 on IFN- γ and IL-13 production by CBMCs of neonates were studied.

2. Methods

2.1. Subjects and study design

We recruited $n = 57$ families who were randomly selected from women giving birth at a local hospital.

The infants were born between the 37th and 42nd week of gestation (median 40th week of gestation). Way of delivery, birth weight and medical as well as family history of atopy were obtained with a questionnaire and only families with uneventful pregnancy and delivery were included in our study. Human umbilical cord blood was drawn on heparin (15 IU heparin/ml blood) before expulsion of the placenta [21]. As a comparison group we recruited eight voluntary healthy adults (mean age 29 years).

The study protocol was approved by the ethics committee of the University of Freiburg and written consent was obtained from the parents.

2.2. Cell preparation

Cord blood mononuclear cells (CBMC) from umbilical cord blood and peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation (Pharmacia, Uppsala, Sweden) and washed three times with a saline buffer containing 3% heat-inactivated fetal calf serum (Biochrom, Berlin, Germany). The cells were frozen gradually to -70°C in a freezing container. Thereafter the tubes were stored in liquid nitrogen at -196°C . Before use, the cells were thawed quickly in a 37°C water bath, diluted and washed in Ultra culture medium (Bio Whittaker, Walkersville, USA) supplemented with 1% heat-inactivated human serum, $20\text{ }\mu\text{M}$ 2-mercaptoethanol (Merck, Darmstadt, Germany), 2 mM L-glutamine (Sigma, Taufkirchen, Germany) and 170 mg/l Gentamicin (Sigma).

2.3. Stimulation of CBMCs

For cytokine analysis CBMC were cultured in the medium alone or stimulated with PHA ($20\text{ }\mu\text{g/ml}$; Sigma). Based on methodological experiments using 10, 20 and $40\text{ }\mu\text{g/ml}$ PHA, respectively, this was the optimal dose of PHA for the maximal cytokine release.

As an allergenic stimulus we used BLG ($25\text{ }\mu\text{g/ml}$; Sigma) [21]. Cells were incubated in concentrations of 100,000 cells/well.

The supernatants of the unstimulated and the PHA stimulated cells were obtained after 48 h of culture at 37°C in humidified 5% CO_2 /95% O_2 . The supernatants of allergen stimulated cells were obtained after 5 days [21]. They were then analysed using the commercially available ELISA technique (for IL-18: human IL-18 ELISA-kit, MBL, Nagoya, Japan; for all other cytokines: OptEIA™, Pharmingen, San Diego, USA) in accordance with the manufacturer's instructions. All assays of the specifically stimulated CBMC were performed in duplicate. The detection level was 4 pg/ml (IL-18), 5 pg/ml (IFN- γ) and 3 pg/ml (IL-13), respectively.

2.4. In vitro release of IFN- γ and IL-13 in response to IL-18

In order to study the in vitro release of IFN- γ and IL-13 in response to IL-18 we used a sterile, endotoxin free, recombinant human IL-18 (Bender MedSystems, Vienna, Austria) and stimulated the isolated CBMC either with medium alone, with PHA (20 μ g/ml), with IL-18 alone (200 ng/ml), with IL-18 (200 ng/ml) + PHA (20 μ g/ml) and with IL-18 (200 ng/ml) + BLG (25 μ g/ml) for 5 days. Supernatants were harvested after 48 h (PHA) and 5 days (BLG), respectively, and IFN- γ and IL-13 concentrations were analysed using the ELISA technique.

2.5. Statistical analysis

The analysis was carried out using the Statistical Analysis System. In order to determine the statistical significance of differences between the subgroups of atopy and non-atopy prone neonates *p*-values were calculated and the Wilcoxon test was employed.

3. Results

Fifty-seven cord blood samples from full-term healthy neonates and eight blood samples from healthy voluntary adults (mean age 29 years) were collected for the different experiments. The time of delivery varied between the 37th and 42nd week of gestation (median 40th week of gestation). Preterm infants, neonates with neonatal sepsis and mothers with any complications during pregnancy were excluded from our study.

In methodological experiments we cultured CBMC with PHA for 24, 48 or 72 h and observed higher cytokine levels over time. However, the variability of the IL-13 and IFN- γ release in the supernatants of two parallel culture preparation was lower after 24 and 48 h compared to 72 h.

Additionally we tested the optimal concentration of IL-18 as an additional stimulus. Higher IFN- γ levels were observed culturing CBMC with PHA/BLG + 200 pg/ml IL-18 compared to PHA/BLG + 100 pg/ml IL-18. No significant differences were observed comparing 200 and 400 pg/ml IL-18.

Based on these methodological experiments we continued the assays, incubating CBMC for 48 h and 5 days for PHA and BLG, respectively, adding 200 ng/ml IL-18 (Fig. 1).

3.1. IL-18 release in response to PHA and BLG

In Fig. 2 the IL-18 concentrations in the supernatants of unstimulated CBMC and CBMC after stimulation with PHA and BLG are presented (*n* = 19). IL-18 was

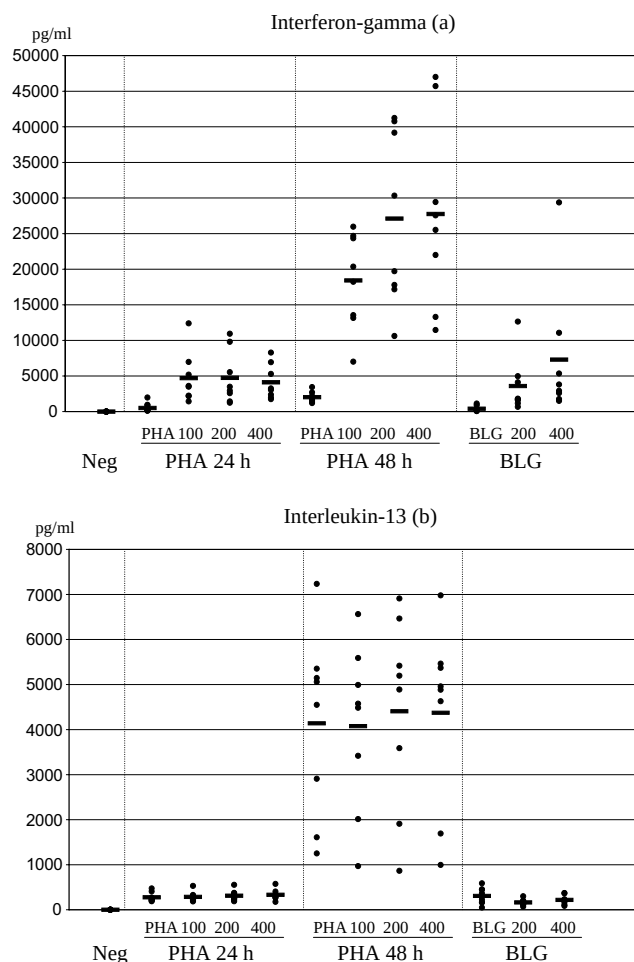


Fig. 1. IFN- γ (a) and IL-13 (b) concentrations in supernatants of CBMC in response to PHA after 24 and 48 h with different concentrations of IL-18 (100, 200 and 400 pg/ml) and in response to BLG after 5 days. The mean value is indicated as a bar.

detectable in all BLG stimulated cell cultures, whereas in three samples of unstimulated and PHA stimulated CBMC supernatants IL-18 was below the detection limit of 4 pg/ml. There was no difference in the IL-18 levels when comparing supernatants of unstimulated CBMC (median 21 pg/ml) and in response to PHA (23 pg/ml) or BLG (15.5 pg/ml). Similar levels of IL-18 in response to medium alone (median IL-18 release: 16 pg/ml) and PHA (29 pg/ml) were observed in supernatants of PBMC from healthy adults.

3.2. IFN- γ and IL-13 release in response to IL-18 alone

We observed a significant increase of IFN- γ release in cell cultures stimulated with IL-18 alone, compared to cell cultures without an additional stimulus in supernatants harvested after 120 h but not after an incubation period of 48 h (Table 1; *p* < 0.0001, Wilcoxon test).

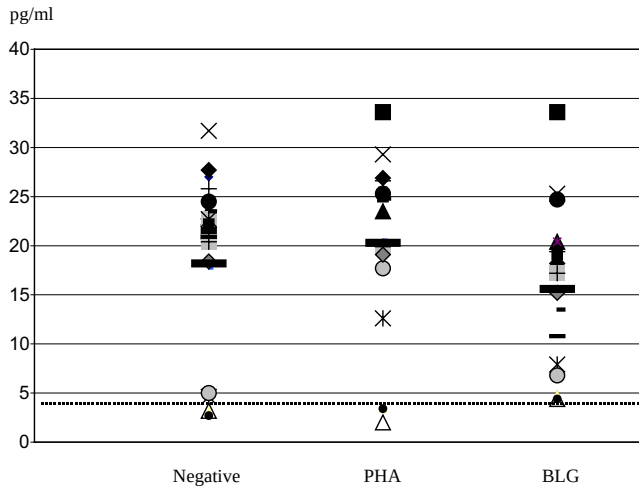


Fig. 2. IL-18 concentrations (pg/ml) in the supernatants of unstimulated CBMC samples (Negative) as well as after stimulation with PHA and BLG are presented ($n = 19$). The bar indicates the mean value and the dotted line the detection limit of 4 pg/ml.

Under the same culture conditions we determined the IL-13 concentration in supernatants of unstimulated and IL-18 stimulated CBMC (Table 1). Similar to IFN- γ , the release of IL-13 was significantly higher in response to IL-18 compared to the negative control after an incubation period of 120 h ($p < 0.0001$). In adults we observed only a significant higher IFN- γ release in response to IL-18 after 120 h (unstimulated: 99; IL-18: 233 pg/ml) but no changes in IL-13 release (unstimulated: 7.4; IL-18: 6.2 pg/ml) compared to the negative control.

3.3. IFN- γ and IL-13 release in response to PHA and BLG in addition to IL-18

When stimulated with PHA or BLG the IFN- γ as well as IL-13 release in the supernatants of CBMC and PBMC increased significantly compared to the negative control ($p < 0.0001$).

When stimulated with the combination of PHA + IL-18, the median value of IFN- γ production increased significantly compared to the cell cultures stimulated

Table 1
IFN- γ and IL-13 concentrations in supernatants of unstimulated CBMC (negative control) and in response to IL-18

	Negative (48 h)	IL-18 (48 h)	IL-18 (120 h)
IFN- γ (pg/ml)	4.3 2.5–146.1	6.7 3–342.1	102.1* 4.3–1652
IL-13 (pg/ml)	1.8 1–43	6.4 1–24.8	44* 2.5–142.1

Supernatants were harvested after 48 and 120 h, respectively. The median, 5th and 95th percentile are presented (pg/ml) and the asterisk (*) indicates statistical significance (compared to unstimulated control).

with PHA alone ($p = 0.0001$; Fig. 3) and with IL-18 alone (Table 1; $p < 0.0001$).

When stimulating CBMC with PHA + IL-18 we observed no increase in the IL-13 levels compared to PHA alone (Fig. 4; $p = 0.16$).

Similar to neonates, we observed a statistical significant increase in the IFN- γ release of adults, when PBMC were stimulated with PHA + IL-18 in contrast to PHA alone (PHA: 6069; PHA + IL-18: 10648 pg/ml; $p = 0.03$). Again, no significant differences in the IL-13 release in response to PHA and PHA + IL-18 can be detected in adult blood (PHA: 8462; PHA + IL-18: 9953 pg/ml).

We determined the IFN- γ and IL-13 concentration in CBMC in response to BLG. Again the IFN- γ production was significantly higher in response to BLG + IL-18 compared to BLG alone ($p = 0.008$; Fig. 3;) or to IL-18 alone ($p < 0.0001$; Table 1).

In contrast to IFN- γ , the production of IL-13 significantly decreased after stimulation with BLG + IL-18 compared to stimulation with BLG alone (Fig. 4; $p = 0.0001$).

4. Discussion

This study was performed to elucidate the role of IL-18 in human cord blood samples of term neonates. Here we were able to demonstrate that IL-18 is detectable in supernatants of human cord blood mononuclear cells. To investigate the functional properties of IL-18 we analysed the IFN- γ and IL-13 release of CBMC. Our data consistently demonstrate that CBMC stimulated with PHA + IL-18 or BLG + IL-18 release significantly enhanced amounts of the TH1 cytokine IFN- γ compared to CBMC stimulated with PHA or BLG alone. With regard to the TH2 cytokine IL-13 no differences were seen after stimulation with PHA or PHA + IL-18. Contrariwise, in response to BLG + IL-18 we observed a significant decrease in the IL-13 concentration. We

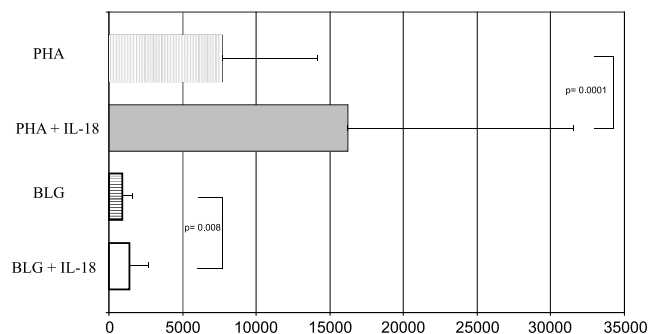


Fig. 3. IFN- γ release [pg/ml] in response to PHA and PHA + IL-18, BLG and BLG + IL-18. The mean value and the standard deviation are indicated. The p -value is reported, when the Wilcoxon test shows a significant result (< 0.05).

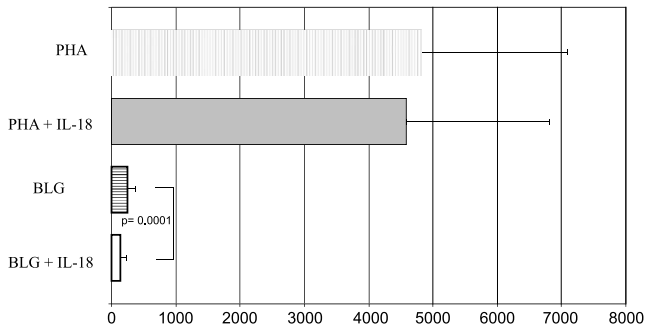


Fig. 4. IL-13 release [pg/ml] in response to PHA and PHA + IL-18, BLG and BLG + IL-18. The mean value and the standard deviation are indicated. The p -value is reported, when the Wilcoxon test shows a significant result (<0.05).

speculate that the overwhelming IFN- γ production in BLG + IL-18 stimulated cell cultures is causally related to the significantly decreased IL-13 levels compared to cell cultures stimulated with BLG alone. As the decrease in IL-13 levels was only observed in response to BLG after incubation over 5 days, the lack of effect on IL-13 when stimulated with PHA + IL-18 might be caused only by the short duration of culture period.

IL-18 is a pleiotropic cytokine involved in the polarisation of T-cell response. There are multiple sources of IL-18. Recently Takahata and coworkers analysed human milk and reported IL-18 levels in colostrum as well as in mature milk of up to 1300 pg/ml, which were found to be elevated after preterm delivery and pregnancy complications of the mother [23].

Here we observed low levels of IL-18 in healthy neonates without any significant difference between unstimulated and PHA or BLG stimulated cell cultures. Similar to our findings, IL-18 levels in cord blood of healthy term infants were reported to be low, whereas increased levels of IL-18 in the amniotic and fetal compartments were observed in cases of microbial invasion of the amniotic cavity [24]. Furthermore, IL-18 is a cytokine which is typically released after cell damage, for example after periventricular leukomalacia, a neonatal white matter damage of the brain of preterm infants [25], after severe lung injury [26] and after acute graft-versus-host disease [27].

Nomura and coworkers analysed the effect of IL-18 on IFN- γ release in different T-cell subsets of neonates and adults [22]. After stimulation with or without IL-18 the IFN- γ levels from cord blood T cells were lower than those from adult T cells. However, CD45RA(+) T cells from cord blood and from adult blood produced similar amounts of IFN- γ after stimulation with IL-18 + IL-12. Here we chose a different approach. We aimed to analyse the effect of mitogen and allergen stimuli when cocultured with or without IL-18. Our data indicate that IL-18 preferentially induces an IFN- γ release even in the absence of IL-12 in adults as well as

neonates, whereas no significant IL-13 release was observed.

One possible explanation for the lack of IL-13 increase in response to IL-18 might be the fact that CBMC of neonates are already TH2 biased [5–7,21]. Recently we were able to demonstrate that IL-13 levels of BLG stimulated CBMC in neonates were much higher than in their mothers [21]. Under such a high baseline production, coculturing CBMC with IL-18 might not result in an additive effect on the release of IL-13 in cell cultures already TH2 biased. This might also explain the differences between our results and recently published data. Looking at patients with bronchial asthma or atopic dermatitis, significantly higher IL-18 concentrations were observed in comparison to non-allergic controls, suggesting that IL-18 is involved in the pathogenesis of atopic disorders [30,31]. Moreover a strong correlation between IL-18, eosinophils and allergic inflammation in the human lung could be demonstrated [32]. Experimental animal models indicate that IL-18 upregulate the TH2 cytokines IL-4 and IL-13 and IgE production. In addition we were very recently able to identify polymorphisms in the IL-18 promotor region which are associated with high IgE levels, specific sensitisation to common allergens and seasonal allergic rhinitis [29]. Taken together, the data indicate that IL-18 plays a direct role in the pathogenesis of TH2-mediated allergic disorders.

In contrast to data reported from animal models and adults, we found that IL-18 in neonates acts as a strong TH1-inducer when CBMC were cultured with PHA or BLG. In preliminary experiments we additionally analysed the effect of IL-4 and IL-4 + IL-18 on cytokine release of PHA and BLG stimulated CBMC ($n = 10$). However, even there no effects of IL-4 or IL-4 + IL-18 were seen on IL-13 or IFN- γ release in stimulated CBMC (data not shown).

As IL-18 upregulates the IFN- γ release in CBMC we hypothesise that IL-18 is a driving force in the redirection of the TH2 response of a neonate towards a TH1 response later in life. Therefore IL-18 might be protective in respect of the development of allergy during a susceptible period. We admit that this is a speculative view on the early development of allergic sensitisation. However, there is some evidence from epidemiological studies supporting our hypothesis. In the hygiene hypothesis the increase of allergic disorders in Western countries is attributed to reduced microbial exposure in early life [34]. Based on this hypothesis at least two events after birth are believed to be associated with the outcome of subsequent atopic sensitisation. One is the acquisition of an appropriate commensal gut flora after delivery, which has the potential to skew the immune system towards TH1 cytokine production. Isolauri recently demonstrated a prevention of early atopic disease in children at high risk supplemented with

Lactobacillus GG [33]. Interestingly in vitro experiments have shown that IL-18 production in human peripheral blood mononuclear cells is upregulated in response to Lactobacilli strains. The second hallmark of early events influencing the immune system is repeated infections, which are believed to be protective for the development of allergy. Again, enhanced levels of IL-18 release are described after viral [17,28] as well as after bacterial infections [16].

Very recently, La Pine and coworkers studied the IFN- γ response to group B streptococci (GBS) in addition to IL-18 and IL-12 [35]. In accordance to our data they were able to show that CBMC production of IFN- γ is enhanced by adding recombinant IL-18 (100 ng/ml). Interestingly, the addition of anti-IL-18 Mab completely blocked the IL-18 enhanced response to GBS, suggesting that IL-18 is crucially involved in neonatal IFN- γ production.

In conclusion we were able to show that IL-18 is detectable in supernatants of CBMC cultures. By analysing the in vitro effects of IL-18 on the release of TH1 and TH2 cytokines, we observed a significant in vitro effect of IL-18 on IFN- γ release in the presence of PHA or BLG. In contrast, IL-18 reduced the release of IL-13 compared to stimulation with BLG alone. Thus our findings point out that IL-18 is a strong TH1-inducing factor on CBMC stimulated in vitro.

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