Multiplexed Cytokine Analysis of Cord Blood Non-Adherent Mononuclear Cells from Infants with Attributable Risk Following IL-5 Stimulation

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Background and Objective

Atopy is described as a genetic predisposition to develop IgE in response to otherwise harmless antigens[1]. The mechanisms that influence atopic disease development in infants are not fully understood, however it has been shown that the atopic risk in infants primarily relates to the atopic status of the mother[2-4]. It is thought that immunological interactions of the mother affect the fetal cytokine environment and thus the overall immunological programming of the fetus[5]. Therefore, it has been hypothesized that the allergic phenotype begins to emerge during the prenatal period[2,3,5]. Studying umbilical cord blood provides a non-invasive platform to examine these early life events. The identification of biomarkers of atopy in umbilical cord blood would promote early detection and successful implementation of prevention strategies.

We ultimately aim to determine a cytokine profile that identifies atopic risk as early as delivery.

Methods

Consent was given by expecting mothers and the umbilical cord blood (UCB) was collected after delivery. The mother's atopic status was self-reported as atopic (A) and non-atopic (NA). The atopic status was confirmed 3 months post-delivery by skin prick testing (SPT). The UCB samples were collected within 20 minutes of the birth in a 60 ml heparinated syringe and processed within 12 hours from the birth. All samples were depleted of erythrocytes via unit gravity sedimentation in 1% Dextran, followed by Accuprep® density gradient to isolate mononuclear cells (MNCs). The MNCs were incubated in McCoy's 3+ media for 2 hours at 37°C to isolate the non-adherent mononuclear cell population (NAMNCs). The NAMNCs were then reseeded at 4.5 to 5 million cells per plate. A 0 hour unstimulated control was collected and the remaining cells were stimulated with recombinant human Interleukin-5 (rhIL-5) (1ng/mL) to promote eosinophilopoeisis. NAMNCs were collected at 24, 48 and 72 hours post-stimulation.
The cell supernatants were collected and frozen. One supernatant aliquot was examined via Luminex xMAP system. The MILLIPLEX High Sensitivity Human Cytokine Magnetic Bead Kit (Millipore) was used to measure IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IFN-γ, GM-CSF, TNF-α. Statistical analysis included Mann-Whitney test as well as repeated measures ANOVA. Statistics were performed using GraphPad Prism.

Fig 1. Summary of umbilical cord blood procurement following collection immediately after birth.

Atopic status	Number of samples
Atopic (A)	10
Non-atopic (NA)	6

Table 1. Summary of cord blood samples used for cytokines analysis. All atopic status were confirmed by skin prick testing (SPT)

Results

Cytokine	Detection Status
IL-1β	Detected
IL-2	Detected
IL-4	Detected
IL-5	Not analyzed
IL-6	Detected
IL-7	Detected
IL-8	Detected
IL-10	Detected
IL-12	Detected
IL-13	Not detected
IFN-γ	Detected
GM-CSF	Detected
TNF-α	Detected

Table 2. Summary of cytokines measured using Luminex technology.

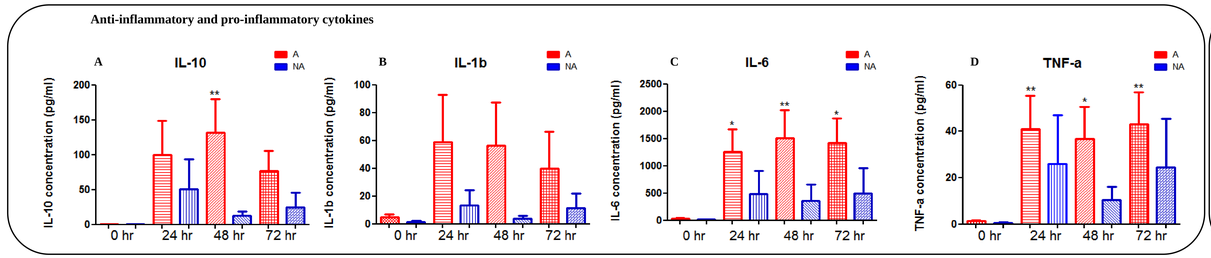

Fig 2. NAMNCs from atopic samples show a significant upregulation in expression of IL-10 (A) , IL-6 (C) and TNF-α (D) over time (P< 0.05, compared to 0 hr unstimulated control by repeated measures ANOVA). Non-adherent mononuclear cells (NAMNCs) stimulated with IL-5 display an upregulation of the anti-inflammatory cytokine IL-10 (A), as well as the pro-inflammatory cytokines IL-1β (B), IL-6 (C), and TNF-α (D). Shown are the observed concentrations of cytokines (pg/ml) at 0, 24, 48 and 72 hr. Error bars represent the standard error of the mean. The atopic and non-atopic NAMNCs were plated at 4.5 to 5 million cells per time point (0, 24, 48 and 72 hr) following IL-5 stimulation at 37 C°(5% CO_2).(*= P ≤ 0.05, **= P ≤ 0.01, ***= P ≤ 0.001).

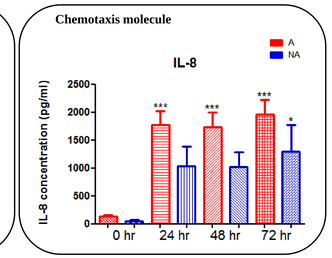

Fig 3. IL-8 concentration in NAMNCs from atopic samples show a significant upregulation at all time points, whereas non-atopic samples show significant upregulation at 72 hr (P< 0.05). Error bars represent the standard error of the mean.

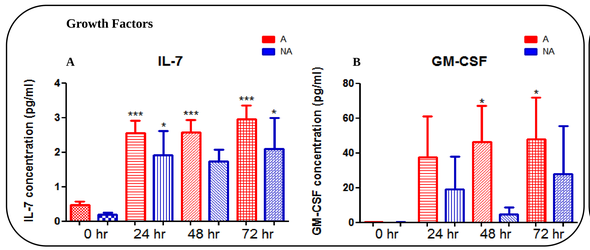

Fig 4. Growth factors, IL-7 (A) and GM-CSF (B) show an upregulation in concentration over time following IL-5 stimulation of NAMNCS. Atopic samples show a significant upregulation in the expression of IL-7 at all time points, whereas GM-CSF expression is significantly upregulated at 48 and 72 hr post-IL-5 stimulation (P< 0.05, compared to 0 hr unstimulated control). Shown are the observed concentrations of cytokines (pg/ml) over time. Error bars represent the standard error of the mean. The atopic and non-atopic NAMNCs were plated at 4.5 to 5 million cells per time point following IL-5 stimulation at 37 C°(5% CO_2)

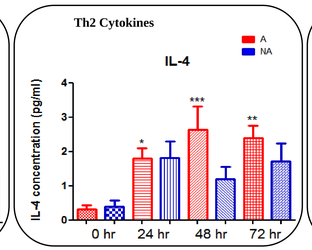

Fig 5. IL-4 concentration is upregulated following IL-5 stimulation in NAMNCs over time. Atopic samples are significantly upregulated at 24, 48 and 72 hr post-IL-5 stimulation (P< 0.05, compared to 0 hr unstimulated control). Error bars represent the standard error of the mean.

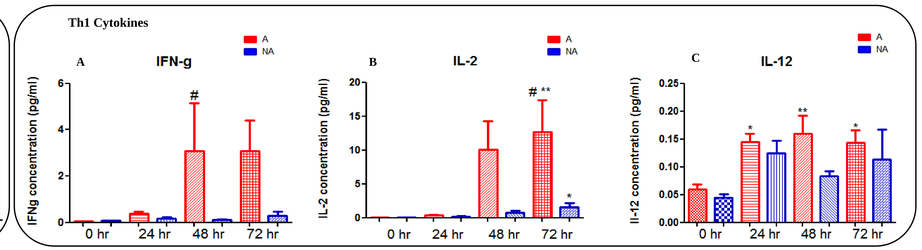

Fig 6. Th1 related cytokines IFN-γ (A), IL-2 (B) and IL-12 (C) expression is upregulated over time following IL-5 stimulation. NAMNCs from atopic samples show a significant upregulation of IL-2 concentration at 72 hr (P< 0.05, compared to 0 hr unstimulated control). Non-atopic NAMNCs show a significant upregulation in the concentration of IL-2 at 72 hr post-IL-5 stimulation (P< 0.05). IL-12 concentration is significantly upregulated over time (P< 0.05). There is a significant difference in IFN-γ and IL-2 concentrations between atopic and non-atopic samples at 48 and 72 hrs, respectively (P< 0.05, by Mann-Whitney test). Shown are the observed concentrations of cytokines (pg/ml) over 0, 24, 48 and 72 hr. Error bars represent the standard error of the mean. (*= P ≤ 0.05, **= P ≤ 0.01, ***= P ≤ 0.001, #= P<0.05 compared to non-atopic sample group).

Summary and Discussion

In the present study, umbilical cord blood was collected at birth from mothers confirmed to be either atopic or non-atopic by skin prick testing. Cord blood non-adherent mononuclear cells (NAMNCs) from 10 atopic and 6 non-atopic participants were stimulated with human recombinant IL-5 (1 ng/ml) for 24, 48 and 72 hours and the cell supernatants were then analyzed. Overall, all cytokine concentrations detected were upregulated over time following IL-5 stimulation. Atopic samples stimulated with IL-5 displayed a significant upregulation in the concentration of TNF-α, IL-12, IL-7, and IL-4 at all time points (P<0.05 compared to 0 hour unstimulated control by repeated measures ANOVA). In addition, both IL-10 and IL-2 concentrations were significantly increased at 48 and 72 hours, respectively. Similarly, growth factor GM-CSF concentration was elevated at 48 and 72 hours compared to the 0 hour unstimulated control (P< 0.05). In NAMNC supernatants from non-atopic participants IL-7 concentration was significantly upregulated at 24 and 72 hours (P<0.05 compared to 0 hr unstimulated control). Additionally, both concentrations of IL-2 and IL-8 were elevated at 72 hours post-IL-5 stimulation (P< 0.05).

Overall, we observed a trend towards increased levels of all cytokines in the atopic samples compared to non-atopics. In particular, the upregulation of both IFN-γ and IL-2 concentrations were significantly different between atopic and non-atopic samples (P<0.05, by Mann-Whitney test). IFN-γ concentration was upregulated at 48 hours in the atopic samples only. Likewise, IL-2 concentration was elevated at 72 hours post-IL-5 stimulation compared to the non-atopic samples (P< 0.05). Atopic individuals are characterized by an imbalanced Th2 weighted immune response. In fact, research has shown an inverse relationship between IFN-γ production and the development of atopic disorders[6]. However, these results are quite heterogeneous, which is potentially due to varying cell culture protocols and stimulations. Less consistently, it has also been found that heightened IFN-γ production might be related to atopy[7].

In this study, atopic cultures displayed a trend of hyper-responsiveness to IL-5 across all cytokines when compared to non-atopic samples. This might suggest that atopic NAMNCs are inherently primed to mount larger immune responses. However, given the complex interplay of Th1/Th2 disproportion involved in atopy, specific cytokines differences could be found between atopic and non-atopic individuals. Therefore, it is important to continue to conduct specifically targeted cytokine profile studies in order to obtain more insight into the immunological development of atopic disorders. We ultimately aim to find a cytokine profile that could be used as a potential predictor of atopy in infants with attributable risk.

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