Multiplexed Analysis of Primary Cord Blood Adherent-Mononuclear Cell-Supernatants Related to Maternal Atopy

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Abstract

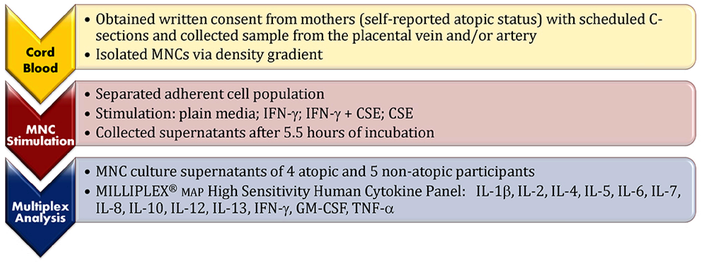
RATIONALE: We have previously reported decreased levels of Interleukin(IL)-10 in primary cord-blood derived adherent-mononuclear cell-cultures (AMNCs) from atopic versus non-atopic mothers following Control Standard Endotoxin (CSE) stimulation. In the current study we examined a multiplexed panel of 13 cytokines in these cultures. This may potentially identify a set of atopic-risk biomarkers that can be employed at birth.

METHODS: Cord blood samples were obtained from mothers undergoing elective C-sections and atopic or non-atopic status was self-reported. AMNCs were isolated and cultured with or without Interferon(IFN)-gamma and/or CSE for 5.5 hours, following which supernatants were collected and frozen at -80 °C. MILLIPLEX® analysis was performed utilizing the 'High Sensitivity Human Cytokine Magnetic Bead' kit (EMD Millipore) to determine the following cytokine concentrations: IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IFN-γ, GM-CSF and TNF-α.

RESULTS: Stimulation with IFN-γ and CSE, as well as CSE alone, significantly upregulated IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-13, GM-CSF and TNF-α concentrations (P<0.05, compared to media control). Also, IFN-γ was significantly elevated (P<0.05) in atopic supernatants when stimulated with IFN-γ and IFN-γ together with CSE in comparison to the non-atopic controls. Furthermore, we observed a trend towards upregulated levels of IL-2, IL-4, IL-5, IL-7 and IL-13 as well as lower IL-10 levels in the atopic samples when compared to non-atopics.

CONCLUSIONS: Using a small set of atopic and non-atopic samples, preliminary differences were noted in a specific set of cytokines between atopic and atopic mothers following CSE treatment.

Methods



Informed consent was obtained from mothers undergoing elective Caesarian-sections at Kingston General Hospital for the procurement of cord blood samples. Atopic or non-atopic status was self-reported. Cord blood was obtained from the placental vein and/or artery using an 18 G needle on a heparinized syringe. From these samples, mononuclear cells (MNCs) were isolated via density gradient (Lymphoprep ™, via Cerdarlane®) centrifugation and temporarily cryopreserved.
Upon thawing, adherent-mononuclear cells (AMNCs) were separated by culturing $5x10^6$ MNCs per condition for 2 hours (37 °C and 5 % CO_2) in RPMI (Life Technologies ™) complete media containing 10 % FBS (via VWR), 1 % Penicillin/Streptomycin (Life Technologies ™) and 2.5 % HEPES (Fisher Scientific) using BD Primaria ™ culture plates (via VWR).
The AMNCs were then incubated in one of three conditions: plain RPMI complete media, IFN-γ (at 1 μg/ml, Sigma Aldrich) or IFN-γ (at 1 μg/ml) together with CSE (at 10 ng/ml, via MJS BioLynx Inc.). The cells were incubated under these conditions at 37 °C and 5 % CO_2 for 5.5 hours. Afterwards, supernatants were collected and aliquots were frozen at -80 °C.
The MILLIPLEX® MAP High Sensitivity Human Cytokine Magnetic Bead Kit (EMD Millipore) was utilized to determine 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 levels.

The samples used in this project are as follows:

Atopic status of mother	samples per condition [n]			
	Plain media	IFN-γ	IFN-γ + CSE	CSE
Atopic	4	3	3	4
Non-Atopic	5	3	3	5

Table 1: Summary of cord blood samples (self-reported atopic status) used per condition.

Results

Analytes involved in Th2 responses

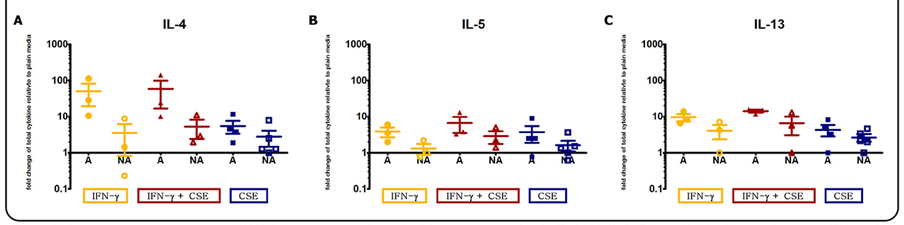


Figure 1: Adherent mononuclear cells (AMNCs) responded with a significant upregulation in IL-4 (A), IL-5 (B) and IL-13 (C) secretion upon IFN-γ + CSE as well as CSE stimulation (P<0.05, compared to media control). Atopic AMNC cultures exhibited slightly elevated levels of these cytokines when compared to non-atopic AMNCs. Shown are the fold changes of total cytokine relative to media control. Error bars represent the standard error of the mean. Cultures of atopic and non-atopic AMNCs were plated at $5x10^6$ cells per condition. Following 5.5 hours incubation with either plain media, 1 μg/ml IFN-γ, or 1 μg/ml IFN-γ and 10 ng/ml CSE, supernatants were collected and analyzed.

Analytes involved in Th1 responses

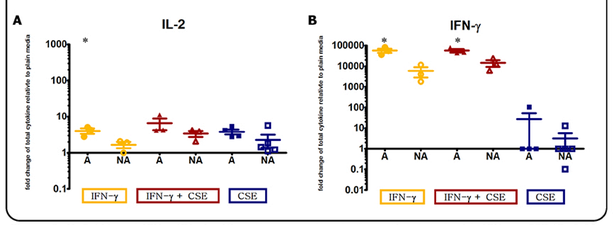


Figure 2: IL-2 (A) and IFN-γ (B) significantly upregulated when stimulated with IFN-γ + CSE (P<0.05, compared to media control). Atopic AMNC cultures exhibited slightly elevated levels of IL-2 and significantly higher levels of IFN-γ. IL-12 concentrations (data not shown) were below detection limit. Shown are the fold changes of total cytokine relative to media control. Error bars represent the standard error of the mean.

Analyte involved in chemotaxis

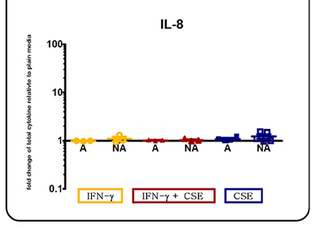


Figure 3: IL-8 concentrations remained unchanged upon stimulation when compared to media control. Shown are the fold changes of total cytokine relative to media control. Error bars represent the standard error of the mean.

Anti-inflammatory and pro-inflammatory analytes

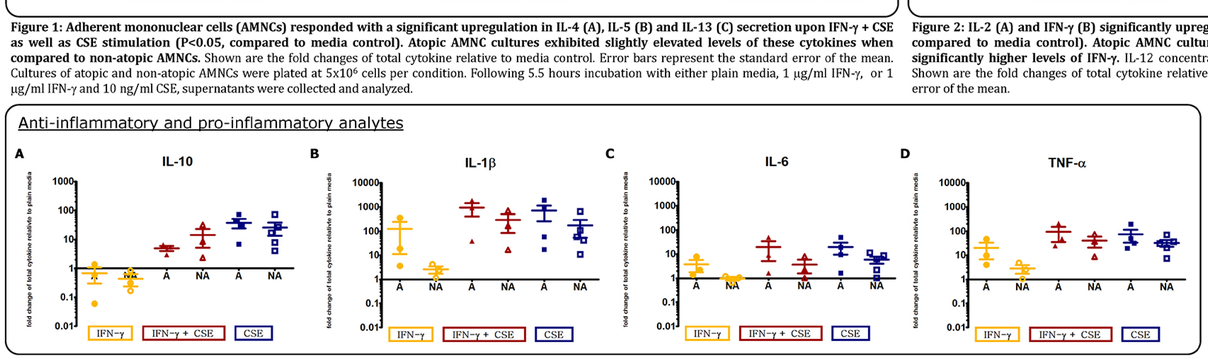


Figure 4: The anti-inflammatory cytokine IL-10 (A), as well as the pro-inflammatory cytokines IL-1β (B), IL-6 (C) and TNF-α (D) were significantly upregulated in AMNC cultures after IFN-γ + CSE stimulation and with CSE alone (P<0.05, compared to media control). Shown are the fold changes of total cytokine relative to media control. Error bars represent the standard error of the mean. Cultures of atopic and non-atopic AMNCs were plated at $5x10^6$ cells per condition. Following 5.5 hours incubation with either plain media, 1 μg/ml IFN-γ, or 1 μg/ml IFN-γ and 10 ng/ml CSE, supernatants were collected and analyzed.

Growth factors

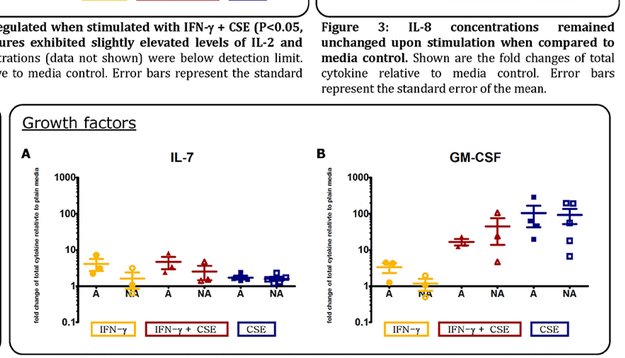


Figure 5: Both IL-7 (A) and GM-CSF (B) were significantly upregulated when stimulated with IFN-γ + CSE or CSE alone (P<0.05, compared to media control. Shown are the fold changes of total cytokine relative to media control. Error bars represent the standard error of the mean.

Summary and Discussion

In this preliminary study we investigated supernatants of primary adherent-mononuclear cell (AMNC) populations derived from four atopic and five non-atopic cord blood specimen. Stimulation of these cells with IFN-γ (at 1 μg/ml) and CSE (at 10 ng/ml), as well as CSE alone (at 10 ng/ml), resulted in a significant upregulation of IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-13, GM-CSF and TNF-α concentrations (P<0.05, compared to media control). Interestingly, IL-2 and IFN-γ levels were significantly elevated (P<0.05) in the supernatants from mothers with self-report of atopic disease when stimulated with IFN-γ and IFN-γ together with CSE (IFN-γ analyte only) in comparison to the non-atopic controls. Furthermore, we observed a trend towards heightened levels of IL-4, IL-5, IL-7 and IL-13 as well as lower IL-10 levels in the self-reported atopic samples when compared to non-atopics. No differences could be noted between atopic and non-atopic culture supernatants in the unstimulated media control, mainly due to cytokine concentrations below the limit of detection.
The elevated levels of IL-2 and IFN-γ - analytes usually involved in Th1 responses - were unexpected and need to be further investigated. Atopy is commonly characterized by a deficiency of Th1 related cytokines and increased concentrations of cytokines involved in Th2 responses.
Elevated concentrations of those cytokines involved in Th2 responses - IL-4, IL-5 and IL-13 - observed in the atopic AMNC cultures are consistent with the current literature. It is a well accepted model that Th2 cells, by releasing IL-4, IL-5 and IL-13, stimulate IgE production, eosinophil recruitment and their differentiation. Multiple research groups previously demonstrated that allergens, IgG and IgA antibodies can be directly transferred by the placenta. As such, the fetus' immune response to allergens could already begin to develop in the womb. It is because of the maternal-infant interface and hence an unique link between the maternal and fetal immune systems, that cord blood derived AMNCs were chosen for this study.
Using a small set of atopic and non-atopic samples, preliminary differences were noted in a specific set of cytokines between atopic and non-atopic mothers following treatment with IFN-γ and IFN-γ together with CSE. The intra-group variation of both, the atopic sample group as well as the non-atopic sample group, was high and is believed to be the main reason as to why no significant differences could be identified. A power calculation of this data set revealed that a group number of n=7 or more might be needed to reach statistical significance for the analysis of IL-5 and IL-13. Also, the intra-group differences for IL-10 will require a group number of n=50 or more to reach statistical significance.
Furthermore, the accuracy required to reliably identify the atopic status in this cohort remains an ongoing challenge. Verification of the self-reported atopic or non-atopic status by a more consistent technique, like skin-prick testing, could improve the intra-group variation observed, especially in the self-reported non-atopic group. Another limitation of the study is our relatively small number of samples, which could influence the results.
It is also possible that changes between atopic and non-atopic samples might not reach significance due to the mixed population of cells (AMNC) used in the assay. Further isolation of specific cell populations such as monocytes or dendritic cells need to be considered.

Future Directions

Using this small set of atopic and non-atopic samples, preliminary differences were noted in a specific set of cytokines between atopic and atopic mothers following CSE treatment. We are currently working on increasing the cord blood sample size to ten or more for each atopic and non-atopic status to confirm these results.
Furthermore, the accuracy required to reliably identify the atopic status in this cohort remains an ongoing challenge. Verification of the self-reported atopic or non-atopic status by a more consistent technique, like skin-prick testing, could improve the intra-group variation observed, especially in the self-reported non-atopic group, in this study.

References

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Acknowledgements

This study was supported by the Allergy, Genes and the Environment Networks of Centres of Excellence, the Canadian Foundation for Innovation, the PSI Foundation and the Department of Medicine at Queen's University.