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Abstract

Objective/Purpose: Using colony assays and flow cytometry, we have recently shown that eosinophil/basophil (Eo/B) progenitor phenotype and function are associated with atopic risk at birth and infant clinical outcomes. The current study aimed to utilize real-time polymerase chain reaction (Q-PCR) to ascertain the kinetic patterns of expression of CB Eo/B-lineage specific genes, GATA-1, MBP and IL-5R α in order to develop molecular markers of Eo/B differentiation.

Methods: CB non-adherent mononuclear cells (NAMNCs) were isolated from random fresh and frozen samples, and incubated in the presence of IL-5 (1 ng/mL). At 24, 48 and 72h post-stimulation, RNA was isolated, reverse transcribed, and expression of IL-5R α , GATA-1, and MBP were determined utilizing comparative Q-PCR in a multiplex reaction. The relative expression ratios between stimulated and un-stimulated cells were calculated using the delta-delta Ct method.

Findings: Stimulation with IL-5 resulted in an up-regulation of GATA-1 expression, which peaked between 24 and 48hrs. In contrast, MBP was up-regulated in a slowly progressive pattern, with maximal up-regulation at 72h, while there was a stable, minor down-regulation of IL-5R α . In keeping with these molecular kinetics, Eo/B colony-forming cells, grown in 14 day methylcellulose cultures, were found to be present in relation to timing of GATA-1 expression.

Relevance: Multiplex Q-PCR analysis of mRNA from CB mononuclear cells stimulated with IL-5 demonstrates sequential expression of critical lineage-specific events, and can be used as a surrogate, molecular marker of CB Eo/B differentiation in basic research and clinical studies.

Introduction

• We have previously identified that the number and phenotype of cord blood (CB) eosinophil/basophil (Eo/B) progenitor cell relate to atopic risk and infant clinical outcomes

• All previous evaluations utilized either 14 day methylcellulose colony assays or flow cytometry analysis

• We have previously demonstrated the kinetics of GATA-1 expression in CB mononuclear cells with IL-5 stimulation, and shown these to be in concert with subsequent Eo/B colony forming units (CFU's) in methylcellulose culture

• We have not examined the expression of other molecular markers of Eo/B lineage commitment such as the IL-5R or Major Basic Protein (MBP)

Objective

• To ascertain the kinetic patterns of expression of CB Eo/B-lineage specific genes: GATA-1, MBP and IL-5R α via a multiplex Q-PCR reaction, in order to develop molecular markers of Eo/B differentiation

Methods

• Cord blood (CB) non-adherent mononuclear cells (NAMNCs) were isolated from random fresh and frozen samples, and incubated in the presence of rhIL-5 (1 ng/mL) (10 million cells included for each condition)

• At 24, 48 and 72h post-stimulation:
 • RNA extracted using RNeasy® Mini-Kit columns (Qiagen) according to manufacturer's instructions
 • DNA contamination removed using DNA Free kit® containing DNase-1 buffer and DNase-1 mix (Ambion) according to manufacturer's instructions
 • Total RNA in each sample was quantified using UV spectrophotometer
 • 2 mg RNA reverse-transcribed for each sample (volume calculated via Total RNA quantification)
 • Reverse-transcription completed with 2.97 mL random hexamer primers and 0.03 mL oligo (dT) primer (both 100ng/mL)

• Expression of IL-5R α , GATA-1, and MBP were determined utilizing comparative Q-PCR in a multiplex reaction (Stratagene MX4000); Housekeeping gene was GAPDH

• Normalized relative expression ratios between stimulated and un-stimulated cells were calculated using the delta-delta Ct method ($2^{-\Delta\Delta Ct}$)

Results

• Stimulation of CB NAMNC's with rhIL-5 resulted in the following patterns of expression:

• GATA-1: Upregulation that peaked between 24 and 48hrs followed by down-regulation at 72hrs

• IL-5R α : Stable expression throughout all time-points when evaluating total expression; this was later sub-analyzed to show relative contributions of the transmembrane vs. the soluble isoforms
 • Transmembrane – initially comprises the majority of IL-5R α expression, and is later down-regulated
 • Soluble – slow and steady up-regulation, peaking at 72hrs

• MBP: Up-regulation in a slowly progressive pattern, with maximal up-regulation at 72hrs

• Eo/B colony-forming cells, grown in 14 day methylcellulose cultures, confirmed this lineage commitment, and number of colonies were positively correlated with MBP expression at 72h and negatively correlated with IL-5R and GATA-1 expression at 72h, suggesting early turn off of GATA-1 when Eo/B lineage is further advanced.

Results - continued

Figure 1: Relative expression of GATA-1, IL-5R and MBP mRNA in CB NAMNC's incubated with rhIL-5

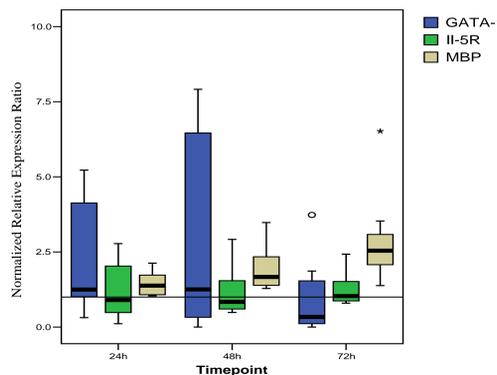


Figure 3: Cord blood Eo/B CFU positively correlate with MBP mRNA expression at 72h of stimulation with IL-5

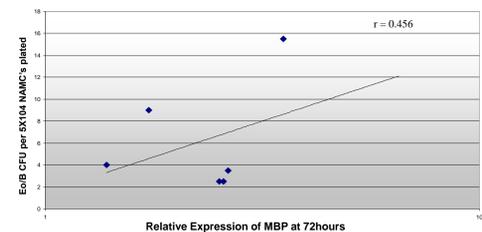


Figure 2: Kinetics of GATA-1 and MBP mRNA expression in CB NAMNC's incubated with IL-5; combination of current and previous investigations.

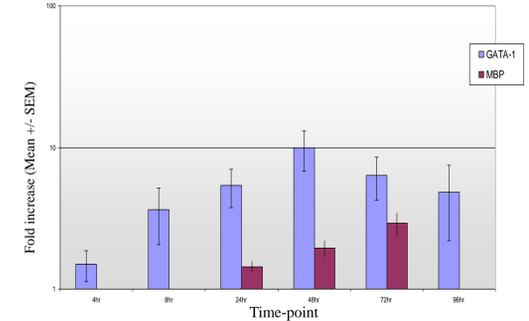


Table 1: Correlation coefficients between molecular marker expression at 72hrs of incubation with IL-5 and Eo/B CFU count after 2 wk incubation with IL-5 in methylcellulose culture.

Time-point	GATA-1	MBP	IL5-R α
24h	-0.356	-0.002	-0.358
48h	-0.383	0.355	-0.397
72h	-0.386	0.469	-0.450

Figure 4a: Relative expression of IL-5R in CB NAMNC's after stimulation with IL-5

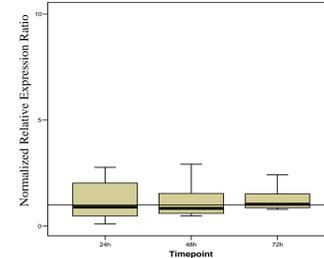
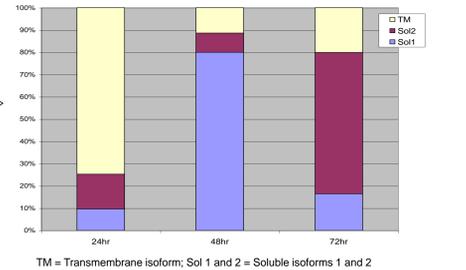


Figure 4b: Percentage composition of IL-5R expression in CB NAMNC's stimulated with IL-5



Discussion

The patterns of expression of each of the genes evaluated were consistent with our expectations, since GATA-1 is known to be an early, essential transcription factor in eosinophil lineage commitment. In addition, our findings in the current experiment were in concordance with previous evaluations in this laboratory of the kinetics of GATA-1 expression over time in CN MNC's.

MBP is produced much later in eosinophilopoiesis, and is a marker of mature Eo commitment. That the up-regulation seen of MBP mRNA was slow and steady with a 4 fold increase by 72 hrs was in keeping with previous evaluations demonstrating MBP production (i.e. product) peaking after 12 days of culture. In addition, MBP expression was positively correlated with later Eo/B CFU development in 2 wk culture.

It has been previously shown that IL-5 has the ability to downregulate its own receptor; we have shown that while total IL-5R α expression patterns remain stable, there is differential expression of the transmembrane and soluble isoforms: progressive up-regulation of the soluble isoform is accompanied by downregulation of the transmembrane.

Conclusions

• mRNA of GATA-1, IL-5R α , and MBP can be evaluated in a Multiplex Q-PCR reaction.

• Analysis of mRNA from cord blood mononuclear cells stimulated with rhIL-5 demonstrates sequential expression of critical Eo/B lineage-specific events

• Q-PCR analysis of the expression of these genes can be used as a surrogate, molecular marker of CB Eo/B differentiation in basic research and clinical studies

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