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## Cord blood molecular biomarkers of eosinophilopoiesis: Kinetic analysis of GATA-1, MBP1 and IL-5R $\alpha$ mRNA expression

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### Abstract

Eosinophil/basophil (Eo/B) progenitor phenotype and function in cord blood (CB) are associated with atopic risk at birth and infant clinical outcomes. Molecular analyses of eosinophil–basophil differentiation events could identify clinically predictive biomarkers. To determine CB kinetic patterns of Eo/B lineage-associated gene expression (GATA-1, MBP1 and IL-5R $\alpha$ ) after IL-5 stimulation, CB non-adherent mononuclear cells were isolated from random fresh and frozen samples and incubated in the presence of recombinant human interleukin-5. Some underwent CD34<sup>+</sup> positive selection using magnetic cell separation. At various time-points, mRNA expression of GATA-1, MBP1 and IL-5R $\alpha$  (total transcripts) was determined utilizing multiplex quantitative polymerase chain reaction (Q-PCR). Relative expression levels of the IL-5R $\alpha$  soluble vs. transmembrane isoforms were also analyzed. Stimulation of the non-adherent mononuclear cells with IL-5 resulted in early up-regulation of GATA-1, peaking at 48 h, followed by decreasing expression and down-regulation by 96 h. The CD34<sup>+</sup> enriched population demonstrated an equivalent expression pattern ( $r = 0.963$ ,  $p = 0.0349$ ). MBP1 mRNA expression [non-adherent mononuclear cells (NAMNCs) and CD34<sup>+</sup> alike;  $r = 0.988$ ,  $p = 0.012$ ] was slowly up-regulated in response to IL-5, maximal at 96 h. Total IL-5R $\alpha$  expression appeared stable over the time-course, mediated by differential expression of the soluble and transmembrane isoforms (i.e., initial increase in the transmembrane contribution followed by a predominance of the soluble isoform by 48–72 h). Multiplex Q-PCR analysis of mRNA from CB demonstrates expression of critical eosinophil-basophil lineage-specific events that are consistent with current understanding of eosinophil differentiation and maturation. The non-adherent mononuclear cell population provides a surrogate signal for the CD34<sup>+</sup> progenitor population.

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## Keywords

cord blood; eosinophils; hematopoiesis; IL-5R; transcription factors; major basic protein-1

The prevalence of allergic disease has increased dramatically in the developed world in the last quarter century. Allergic airway conditions are associated with high morbidity and significant burden to the affected individuals, (1) and importantly, because of these recent epidemiologic changes, they have emerged as the most common chronic illness in childhood (3). The reasons for this are not yet fully elucidated, but it has become apparent that there is an inflammatory basis for these conditions, and thus, efforts to understand the development of these inflammatory responses, their basis in immunity and factors underlying their increased prevalence have intensified over the last decade and are critical to the goal of slowing the 'Allergy Epidemic'.

Identification of a biomarker in cord blood (CB) that is strongly predictive of later allergy could allow for early intervention with appropriate treatment and/or avoidance strategies that could potentially slow or halt the 'atopic march' (the recognized tendency toward atopic progression over time in an affected patient from atopic dermatitis → food allergy → asthma/rhinitis) (5).

Several birth cohort studies in which we have recently undertaken CB analyses have shown that the number and functional phenotype of CD34+CB stem cells (hematopoietic progenitors) are altered in infants at high atopic risk compared to low-risk infants (6–9), particularly in the eosinophil/basophil (Eo/B) lineage-committed progenitor cell population (10). It was additionally noted that differences in these CD34+ populations were associated with increased risk of fever and wheeze with respiratory illness in the first year of life (11). The collective results of these studies demonstrate that in infants at risk of atopy (by parental history and/or maternal allergy skin tests), these CB progenitors are reduced in number and show reduced expression of cell surface cytokine receptors such as IL-5R $\alpha$  or GM-CSFR $\alpha$  than those at low risk of atopy. These findings have been interpreted as a possible consequence of *in utero* exposure of progenitors to comparatively higher ambient levels of Th<sub>2</sub> cytokines and suggest that the resultant CB progenitor phenotype may be predictive of future atopic outcomes (7, 10).

## Rationale for current study

All studies to date have evaluated these progenitors via a labor-intensive, 14-day methylcellulose colony assay for functional outcomes, and by flow cytometry for phenotypic outcomes, but not by analyses of the molecular biomarkers involved in eosinophil lineage commitment and differentiation. Potential molecular targets of interest include GATA-1, a pivotal transcription factor required for eosinophil development (12), the IL-5R $\alpha$  subunit of the high affinity IL-5 receptor that binds IL-5 (13) and the eosinophil major basic protein-1 (MBP1), a major constituent of the large specific (secondary) granule in the terminally differentiated eosinophil (14). In a very preliminary evaluation of kinetic changes in the expression of GATA-1 over time, our laboratory was able to show that GATA-1 was up-regulated early in CB non-adherent mononuclear cells (NAMNCs) stimulated with

recombinant human interleukin-5 (rhIL-5) in a SYBR Green-based RT-PCR, peaking at 48 h, with subsequent diminishments in expression (9), but the other aforementioned target molecular biomarkers had not been investigated. Of relevance to the IL-5R $\alpha$  subunit, the full-length IL-5R $\alpha$  gene can produce two mRNA species that correspond to alternatively spliced soluble and transmembrane isoforms (sol-IL-5R $\alpha$ , Tm-IL-5R $\alpha$ ) (15–18). The Tm-IL-5R $\alpha$  is the functional isoform that allows for signal transduction and initiation of responses mediated via the IL-5R $\alpha$  and  $\beta$ c chain shared with IL-3 and GM-CSF receptors. The sol-IL-5R $\alpha$  subunit binds IL-5 and neutralizes the biologic activity of the cytokine *in vitro* (15–17).

Developing and applying an understanding of early molecular events in Eo/B lineage commitment with specific focus on mRNA expression in CB progenitors of GATA-1, IL-5R $\alpha$  and MBP1 would not only enhance our current understanding of hematopoietic (especially Eo/B progenitor) development in the neonate but could also potentially provide a very early marker of atopic risk in the newborn.

## Materials and methods

This study was reviewed and approved by the Hamilton Health Sciences & McMaster Health Sciences Research Ethics Board. All CB donors gave written informed consent to participate.

### Materials

Materials were purchased as follows: Accuprep<sup>®</sup> was obtained from Accurate Chemicals Inc. (Westbury, NY, USA); McCoy's 5A medium, penicillin, streptomycin and FBS were obtained from Gibco BRL (Gaithersburg, MD, USA); heparin, dextran (molecular weight 260,000), from Sigma-Aldrich (Oakville, ON, Canada); and 2-mercaptoethanol from BDH Inc. (Toronto, ON, Canada).

### Preparation of cord blood cells

Immediately after delivery, umbilical CB was collected in a heparin-containing syringe. For this preliminary evaluation to verify the validity of our proposed multiplex quantitative polymerase chain reaction (Q-PCR), we only recruited anonymous, random CB donors with unknown atopic status. The CB was depleted of red cells by unit gravity sedimentation in 1% (vol/vol) dextran at 37°C for 30 min, followed by isolation of mononuclear cells on an Accuprep<sup>™</sup> density gradient (2000 rpm for 20 min at 20°C). After washing, cells were re-suspended in McCoy's 5A supplemented with 15% FBS, penicillin/streptomycin and 2-mercaptoethanol and depleted of adherent cells by incubation in plastic flasks for 2 h at 37°C. Cell viability was assessed by the trypan blue staining method. The NAMNCs were then incubated in the presence of rhIL-5 (1 ng/ml). Half of the samples utilized in these experiments were from frozen CB samples prepared as mentioned earlier but stored in liquid nitrogen after appropriate cryopreservation with freezing media. Frozen NAMNCs were flash-thawed at 37°C for 30 s then washed with ice-cold McCoy's 5A and similarly incubated with IL-5 after viability assessment and enumeration. Average recovery was 38% with 96% viability from the frozen samples. At 0, 24, 48, 72 and 96 h post-stimulation, cells

were lysed in Buffer RPE (Qiagen, Valencia, CA, USA) with added 2-mercaptoethanol and stored at  $-80^{\circ}\text{C}$  for future batch processing to ensure consistency of processing.

In addition to the NAMNC analyses, some CB samples underwent positive selection for CD34+ hematopoietic progenitors. This was achieved by taking the mononuclear layer and processing it through a CD34+ magnet-activated positive selection cocktail (EasySep; Stem Cell Technologies, Vancouver, BC, Canada). A cell count was performed at the end of the last purification step to determine the number of time-points that could be analyzed, aiming for a total of 4. The cells were re-suspended in an appropriate volume of media (complete RPMI), the zero-hour samples were immediately treated with lysis buffer (Buffer RLT with beta-mercaptoethanol), and the others were incubated with IL-5. At 24, 48 and 72 h, and for some experiments, 1-wk post-stimulation, cells were lysed and frozen as described earlier.

### RNA isolation and reverse transcription

RNA was extracted from each cell lysate sample using RNeasy<sup>®</sup> Mini-kit columns (Qiagen) according to manufacturer's instructions. DNA contamination was removed using a DNA Free kit<sup>®</sup> containing DNase-1 buffer and DNase-1 mix (Ambion, Austin, TX, USA) according to manufacturer's instructions. Total RNA in each sample was quantified using a UV spectrophotometer, and a fixed amount of RNA was reverse-transcribed for each sample (determined via lowest concentration of RNA per sample, as calculated via total RNA quantification). Reverse transcription (RT) was completed using a Stratascript cDNA synthesis kit (Stratagene, Cedar Creek, TX, USA), with 2.97 ml random hexamer primers and 0.03 ml oligo (dT) primer (both 100 ng/ml).

### Multiplex Q-PCR analysis

mRNA expression of GATA-1, IL-5R $\alpha$  (total) and MBP1 was determined utilizing comparative Q-PCR in a multiplex reaction (Stratagene MX4000; Stratagene). The housekeeping gene was GAPDH, which was selected using the TaqMan Human Endogenous Control Plate and had been established previously in our laboratory as a successful reference gene for GATA-1 in the NAMNC population (9). Primer/probe sets (including housekeeping gene) and master mix were obtained from Qiagen. Standard curves were run in duplicate using a commercially available total RNA (Stratagene) that was reverse-transcribed in tandem with the experimental samples, along with no RT and no template controls. Each gene was analyzed in separate individual reactions first, and then efficiencies and standard curves compared when run in concert in a multiplex reaction, with no evidence of inhibition or competition as a result of multiplexing. The sequences utilized for GATA-1, MBP1, IL-5R $\alpha$  (total) and GAPDH are detailed in Table 1. The IL-5R $\alpha$  soluble (Sol) and transmembrane (Tm) isoforms were then analyzed using a SYBR green-based assay and a BioRad iCycler (iQ5; BioRad, Hercules, CA, USA) with beta-2-microglobulin as the housekeeping gene as previously standardized and described for CD34+ cells differentiated to eosinophils (19). Standard curves were run in duplicate using RNA purified from the IL-5R $\alpha$ +AML14.3D10 eosinophil myelocyte cell line (19). The primer sequences for the Tm-, Sol-IL-5R $\alpha$  and beta-2-microglobulin are similarly detailed in Table 1. If the efficiency of the reaction (either for the housekeeping gene or any of the given genes of interest) was less than 85%, the data were rejected. Similarly, only reactions with  $r^2$  values  $> 0.97$  were

considered acceptable for analysis. Primer pairs used for both multiplex and individual real-time Q-PCR for the genes of interest (Table 1) were first analyzed by standard RT-PCRs for 40 cycles. We then used total RNA purified from the AML14.3D10 eosinophilic myelocyte cell line to confirm their amplification of single cDNA (mRNA) products of predicted size (Table 1) with standard 1.5% agarose DNA gel electrophoresis (Fig. S1). As well, melting curves were performed using real-time Q-PCR to confirm that these primer sets produced single peaks at high efficiencies with total cDNA generated from commercially available RNA (BioRad).

### Statistical considerations

All data were normalized to pre-treatment controls with an equal quantity of viable cells; no medium-only controls were analyzed. Normalized relative expression ratios (RERs) between stimulated and un-stimulated cells were calculated using the delta-delta Ct method ( $2^{-\Delta\Delta C_t}$ ), with the zero-hour time-point as reference. Statistical analyses were completed using SIGMASTAT v2.0 for Windows (Systat Software, Inc. Chicago, IL, USA). The mean RERs were compared using the Mann–Whitney *U* test and/or ANOVA on ranks for non-parametric data, as appropriate. Correlation analyses between mRNA expression and Eo/B colony forming units (CFUs) were completed with Pearson's and/or Spearman's correlation coefficients, as the normality of the data dictated.

## Results

### Cord blood NAMNC population results

We were able to successfully determine the mRNA expression of each of the genes of interest from CB NAMNCs utilizing a multiplex reaction (Fig. 1). Stimulation of CB NAMNC with rhIL-5 resulted in the up-regulation of GATA-1 mRNA expression that peaked at 48 h (mean fold-increase of 10.3), followed by decreasing expression by 72 h, and clear down-regulation by 96 h (mean fold-decrease of -8.3). These kinetic changes were statistically significantly different over time;  $p = 0.04$ . MBP1 gene expression was up-regulated in a slowly progressive pattern, with maximal up-regulation at 96 h (mean fold-increase of 2.3). The MBP1 mRNA kinetic changes were also statistically different;  $p < 0.001$ . When the pattern of GATA-1 up-regulation and subsequent down-regulation was examined alongside changes in MBP1 mRNA expression, GATA-1's role as a co-promoter of MBP1 expression was apparent (Fig. 2), as GATA-1 expression preceded MBP1 expression.

There was an *inverse* correlation between early MBP1 expression and total number of Eo/B CFUs in the CB samples. This relationship existed at both the 24-h and 48-h time-points ( $r = -0.602$ ,  $p = 0.046$ ; and  $r = -0.660$ ,  $p = 0.0275$ , respectively).

Although the expression of total IL-5R $\alpha$  transcripts appeared comparatively stable over all time-points studied (Fig. 1), with no significant differences in the means ( $p = 0.488$ ), there was differential (reciprocal) expression of the TM- and sol-IL-5R $\alpha$  isoforms over time (Fig. 3). Specifically, we observed an initial up-regulation followed by progressive decrease in the

proportion of the transmembrane (functional) isoform compared to the soluble (antagonistic) isoform.

### Cord blood CD34+ progenitor cell studies

Subjecting the CB samples to CD34+ cell purification resulted in a significant decrease in the number of cells available for culture (average number per time-point =  $9.3 \times 10^4$ ). As a result, the total RNA available for analysis was reduced almost 100-fold for these studies. Despite this, we indeed found that stimulation of a purified fraction of CD34+ cells resulted in a pattern of GATA-1, IL-5R $\alpha$  and MBP1 expression that mirrored that seen in the non-adherent fraction (Fig. 4). These expression patterns in CD34+ cells were highly correlated to the patterns seen with the NAMNC population ( $r = 0.963$ ,  $p = 0.035$  for GATA-1;  $r = 0.988$ ,  $p = 0.012$  for MBP1). The expression of the IL-5R $\alpha$  subunit was again comparatively unchanged over time when total IL-5R $\alpha$  transcripts (Sol + Tm) were assessed.

As seen in the CB NAMNC samples, the mRNA for the two isoforms of the IL-5R $\alpha$  was differentially expressed in the cultured CD34+ cells over time, such that we observed an initial increase in the percentage of the expression of the transmembrane isoform, which was quickly replaced by an up-regulation in the percentage of the soluble IL-5R $\alpha$  splice variant (Fig. 5).

## Discussion

In this study, we were able to successfully measure kinetic patterns of expression of three key mRNA species: GATA-1, IL-5R $\alpha$  and MBP1, as biomarkers of eosinophil lineage commitment and maturation, in a multiplex Q-PCR in both CB non-adherent cells and a CD34+ purified progenitor fraction.

GATA-1 is not only necessary and sufficient for eosinophilopoiesis (20), it has been clearly established as a pivotal transcription factor for eosinophil lineage specification, as revealed by the loss of the eosinophil lineage in mice harboring a targeted transgenic deletion of the high-affinity GATA-binding site in the HS-2 region of the murine GATA-1 promoter (21) and in the GATA-1 knockout mouse (12). GATA-1 is critical in the early stage of eosinophil differentiation (12), and furthermore, others have shown that GATA-1 can transactivate the expression of the eosinophil-specific MBP1 P2 promoter (22, 23).

In this study, we saw an appropriate up-regulation of GATA-1 mRNA expression upon stimulation of the non-adherent mononuclear cell population with IL-5, suggesting activation of cellular events leading to eosinophil development. Our results suggest that IL-5 is responsible for driving the maturation of the eosinophil lineage, with early peaking of GATA-1 followed by later up-regulation of MBP1, a secondary granule protein product produced almost exclusively by eosinophils. Importantly, while undergoing maturation, eosinophil progenitors will express detectable mRNA levels of MBP1 (24), but mature eosinophils do not express any mRNA for MBP1 or other granule cationic proteins (25). Additionally, mature blood eosinophils no longer express GATA-1 protein, so its expression is also down-regulated as eosinophils complete their terminal differentiation into mature



cells. Our results support the observations of others that GATA-1 is a potent transcriptional activator of MBP1 gene expression (22, 23), as the up-regulation of MBP1 followed the initial expression of GATA-1, and was closely associated with peak GATA-1 expression (Fig. 2).

Full *in-vitro* differentiation of eosinophils from CB CD34+ cells takes around 15–17 days, so the fact that the increase in MBP1 mRNA expression by 72 h was only two-fold is not particularly surprising. The pilot data extending to 1 wk of incubation showed a mean up-regulation of ~four-fold, suggesting that a plateau may not yet be reached. Presumably then, the silencing of MBP1 mRNA does not occur until the end stages of terminal differentiation, as it is known that once fully matured, eosinophils no longer express mRNA for MBP1 (25). The parameters of this evaluation do not allow us to comment on when precisely this plateau in kinetic expression occurs.

IL-5 is unquestionably an important factor in the functional maturation of eosinophils in terms of priming and activation, and their tissue survival (27, 28). Cellular responses to IL-5 are mediated through the high affinity IL-5R, a heterodimer consisting of a ligand-binding 60 kDa IL-5-specific  $\kappa$  subunit and a 130 kDa signal-transducing  $\beta$  subunit (also referred to as the common  $\beta$  chain, or  $\beta_c$ ), which is identical to the signal-transducing subunit of the IL-3R and GM-CSFR (29).

The IL-5R $\alpha$  subunit is mainly restricted to mature eosinophils and basophils (30), but this IL-5-binding subunit of the receptor has also been shown to be present on late-lineage-committed CD34–CD33+ cells and has also recently been demonstrated to be on the surface of some CD34+ cells, suggesting its presence to be a marker of an early hematopoietic event that can indicate commitment to the eosinophil-basophil lineages (31). Human eosinophil progenitors (EoP) were recently re-defined as IL-5R $\alpha$ + CD34+CD38+IL-3R $\alpha$ +CD45RA– constituents of the common myeloid progenitor (CMP) pool (32). The full-length IL-5R $\alpha$  gene can produce two mRNA species that correspond to alternatively spliced soluble and transmembrane isoforms (sol-IL-5R $\alpha$ , Tm-IL-5R $\alpha$ ) (15–18). The Tm-IL-5R $\alpha$  is the functional isoform that allows for signal transduction and initiation of responses mediated via the IL-5R $\alpha$  and  $\beta_c$  chain. The sol-IL-5R $\alpha$  subunit binds IL-5 and neutralizes the biologic activity of the cytokine *in vitro* (15–17), suggesting that the sol-IL-5R $\alpha$  subunit may play a role in the immunoregulation of eosinophilia *in vivo*. It has been proposed that eosinophils are able to control their responsiveness to IL-5 by regulated expression of the IL-5R $\alpha$  isoforms (17). In the current evaluation, the mRNA expression of total IL-5R $\alpha$  transcripts in CB NAMNCs and CD34+ populations remained apparently stable after stimulation with rhIL-5, but subsequent analysis demonstrated reciprocal contributory roles of the soluble and transmembrane variants of the IL-5R $\alpha$  subunit, with progressive down-regulation of the Tm-IL-5R $\alpha$  isoform concomitant with progressive up-regulation of the soluble isoform. This is in keeping with the work from others who have shown a similar switch from the predominantly soluble isoform to Tm-IL-5R $\alpha$  in early IL-5-driven eosinophil development from human umbilical cord CD34+ cells (34), but predominant expression of the sol-IL-5R $\alpha$  isoform in mature eosinophils (35). We would expect these findings to be specific to IL-5 stimulation (as opposed to IL-3 or GM-CSF stimulation), as signaling through the common

beta-chain shared by the IL-3R, IL-5R and GM-CSFR is still dependent upon specific activation of the relevant alpha subunits (29).

The Eo/B CFU correlations in this study identified an inverse relationship between early MBP1 up-regulation and the number of Eo/B CFUs in the CB samples. This has biologic plausibility, as earlier peaking of MBP1 expression would indicate a population of a more further-differentiated eosinophil progenitor population in the sample, as opposed to one with a higher number of undifferentiated CD34+ cells (indicated by a larger number of Eo/B CFUs in the methylcellulose colony assay), which would need to follow the stimulation → GATA-1 → MBP cycle before showing an increase in MBP mRNA expression.

The results obtained in the experiments utilizing a purified CD34+ fraction mirrored those found in the less purified NAMNC population, indicating that these studies can be pursued using the NAMNC cell population as a surrogate for the responses of purified CD34+ progenitors. This results in a less complicated, less laborintensive assay that could more easily be adopted by other laboratories.

We are currently applying these techniques to CB samples from infants with increased risk of the development of atopy to determine whether differences in either the kinetic patterns or the maximal expression of these biomarkers can be applied as predictors of atopy at birth.

### Relevance of this study

Based on our newly enhanced knowledge of, and assays for, these hematopoietic biomarkers, it may be possible to elucidate molecular mechanisms and patterns that are associated with atopic risk and potentially influence the subsequent development of atopy and/or asthma.

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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### Abbreviations:

<b>C/W</b>	compared with
<b>CB</b>	cord blood
<b>CD34</b>	cluster of differentiation marker 34
<b>CFU</b>	colony forming unit
<b>Eo/B</b>	eosinophil/basophil

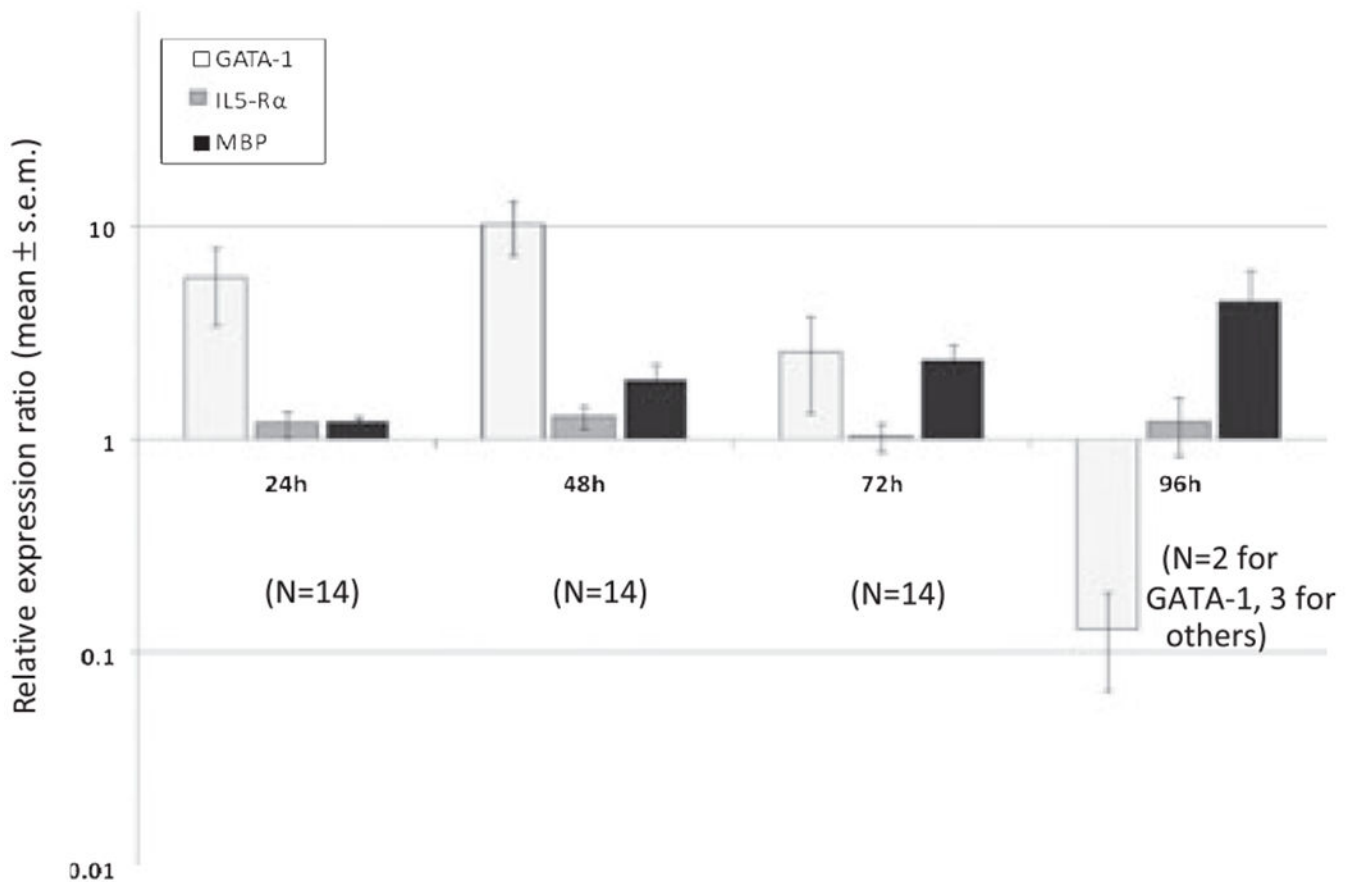


<b>GM-CSFR<math>\alpha</math></b>	granulocyte-macrophage colony stimulating factor receptor $\alpha$ -subunit
<b>IL-5R<math>\alpha</math></b>	interleukin-5 receptor $\alpha$ -subunit
<b>MBP1</b>	major basic protein-1
<b>MNCs</b>	mononuclear cells
<b>NAMNCs</b>	non-adherent mononuclear cells
<b>PCR</b>	polymerase chain reaction
<b>Q-PCR</b>	quantitative polymerase chain reaction
<b>RER</b>	relative expression ratio
<b>rhIL-5</b>	recombinant human interleukin-5

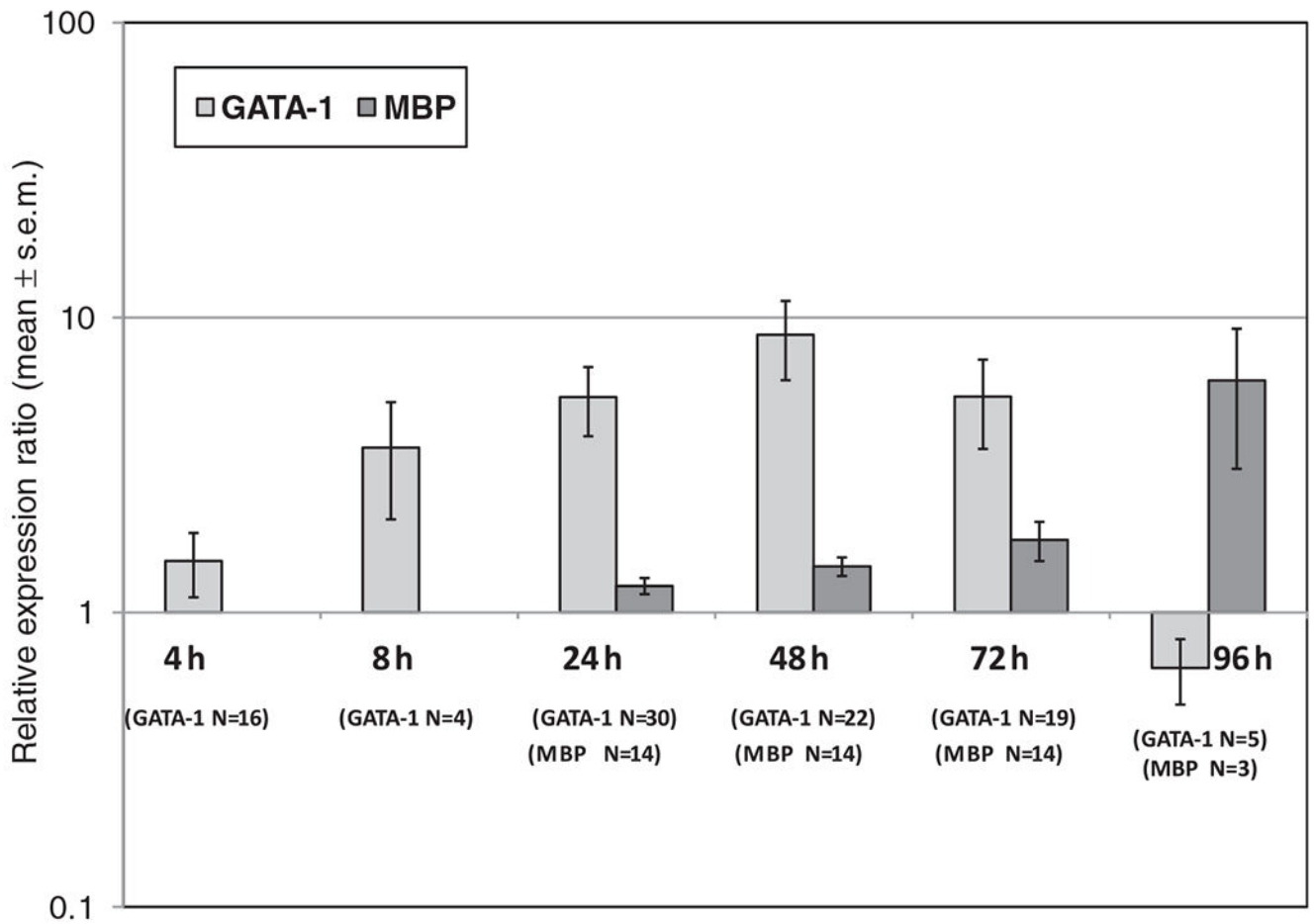
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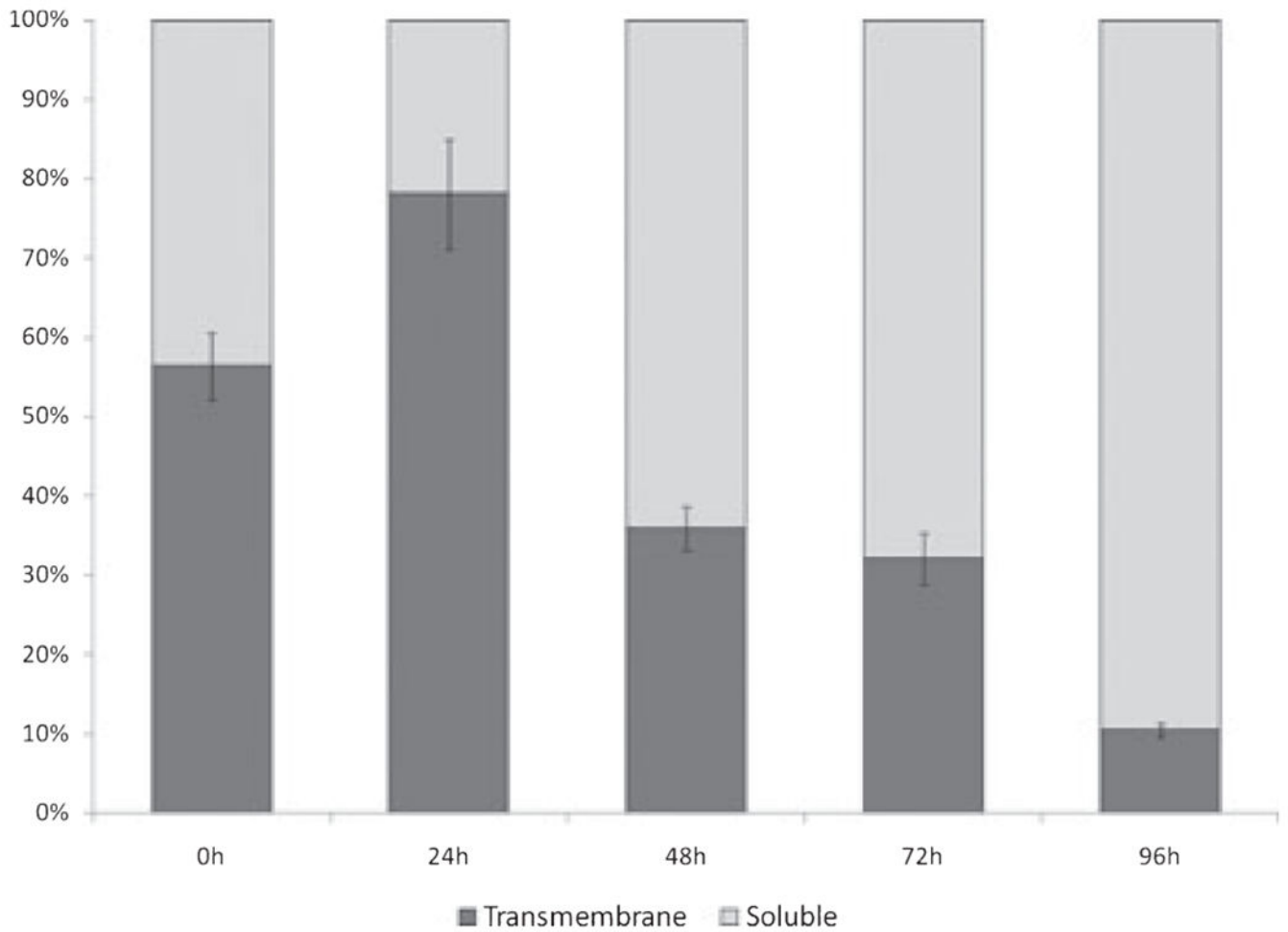
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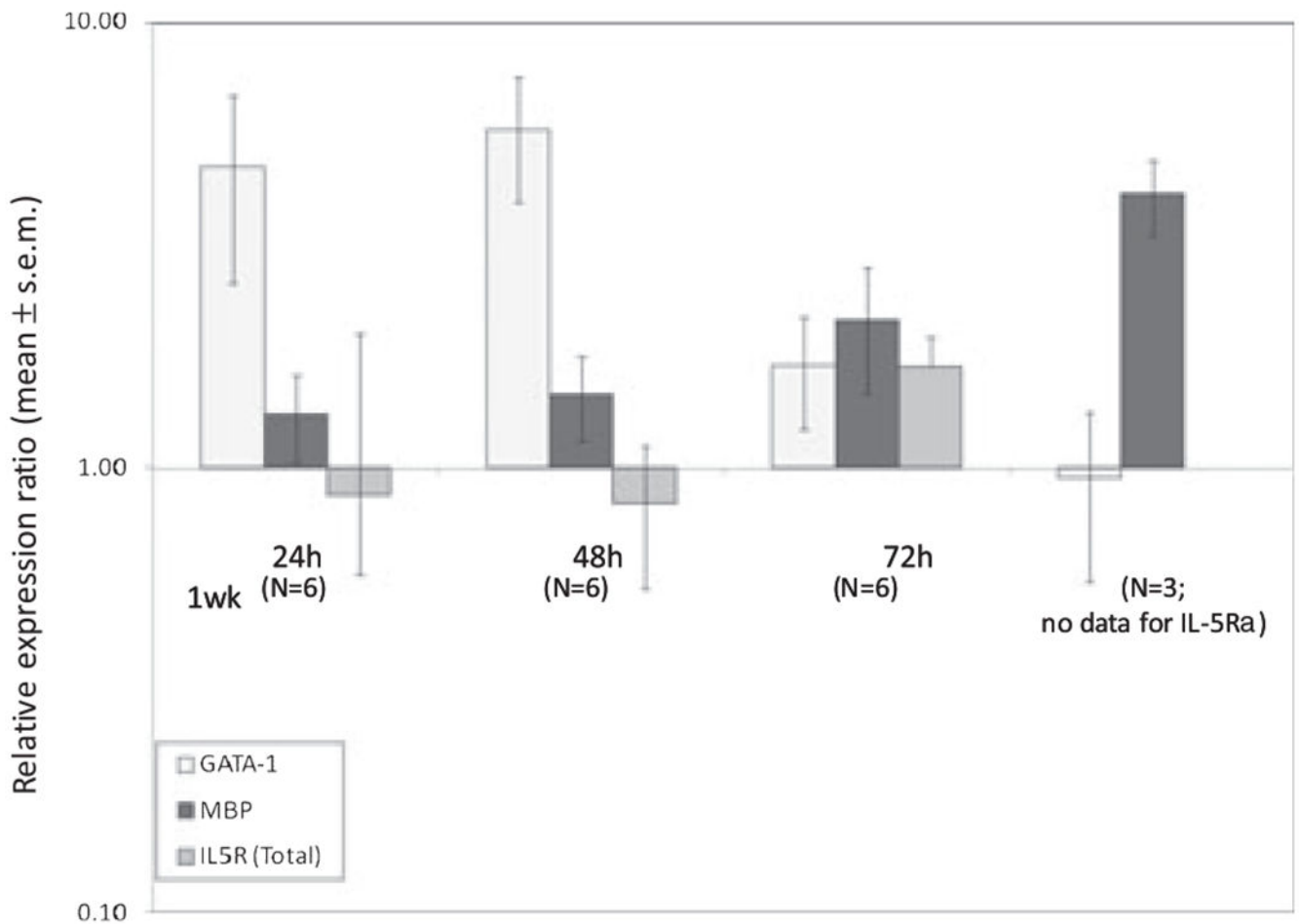
**Fig. 1.** Kinetic mRNA expression of GATA-1, MBP1, and IL-5Rα (total) from cord blood (CB) non-adherent mononuclear cells (NAMNCs) stimulated with IL-5. NAMNCs from random fresh and frozen CB samples were incubated in the presence of rhIL-5, and removed at various time points for cell lysis, RNA extraction, reverse-transcription and quantitative polymerase chain reaction (Q-PCR) analysis using a multiplex reaction. Displayed are relative expression ratios (normalized to housekeeping gene, GAPDH), calculated using the standard  $2^{-(C_t)}$  formula (GATA-1:  $p < 0.001$ ; MBP1:  $p < 0.001$ ; IL-5Rα means not different:  $p = 0.488$ ; ANOVA on ranks).



**Fig. 2.** Kinetic mRNA expression of GATA-1 vs. MBP1 from cord blood (CB) non-adherent mononuclear cells (NAMNCs) stimulated with IL-5: presence of GATA-1 precedes MBP gene transcription ( $p < 0.001$ , ANOVA on ranks). NAMNCs from random fresh and frozen CB samples were incubated in the presence of rhIL-5, and removed at various time points for cell lysis, RNA extraction, reverse-transcription and quantitative polymerase chain reaction (Q-PCR) analysis using a multiplex reaction. Displayed are relative expression ratios (normalized to housekeeping gene, GAPDH), calculated using the standard  $2^{(-Ct)}$  formula.

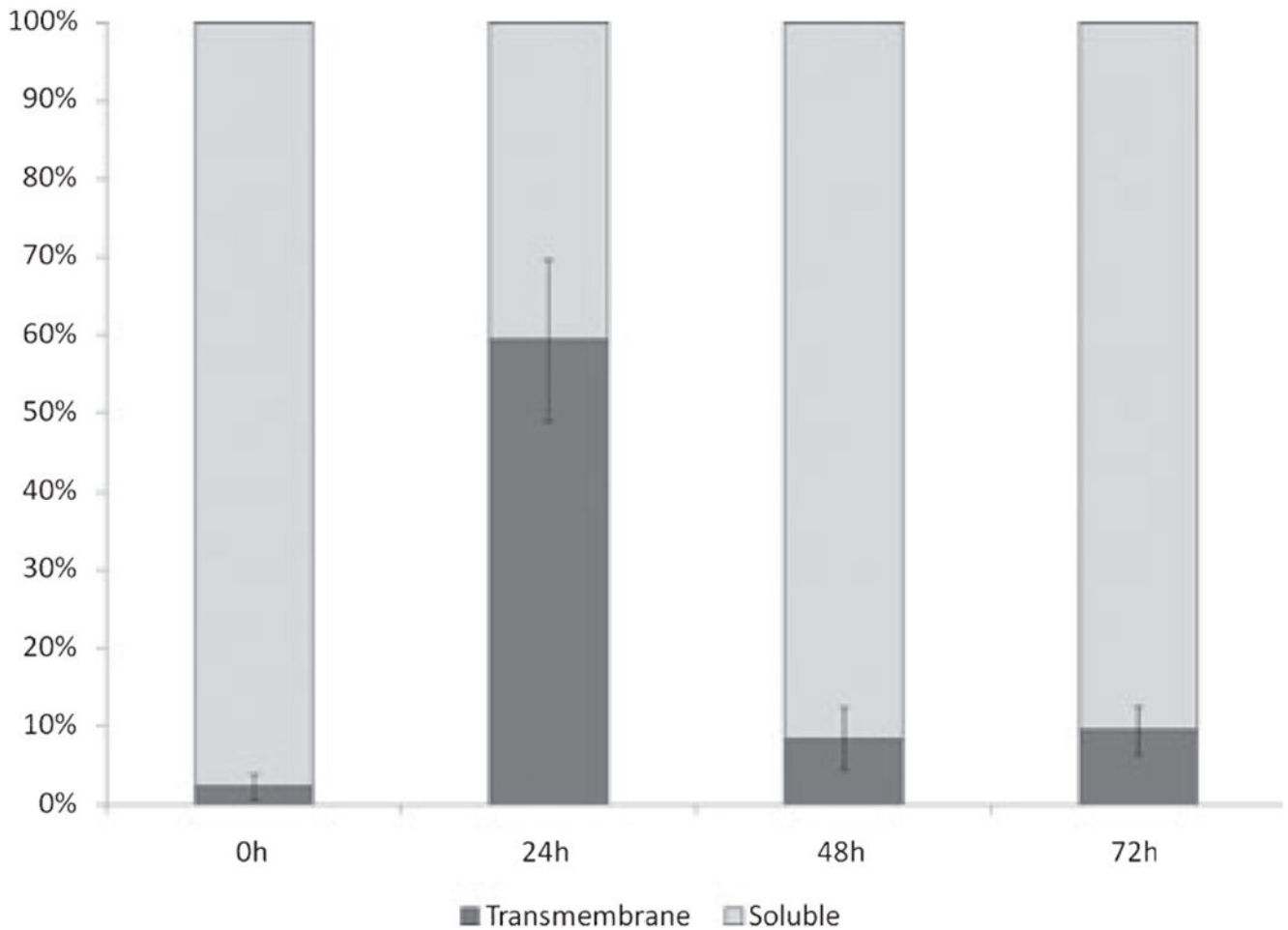


**Fig. 3.** Kinetic changes in the mRNA expression of the Tm- & Sol-IL-5R $\alpha$  isoforms in cord blood (CB) non-adherent mononuclear cells (NAMNCs) incubated with IL-5 (shown as percent of total IL-5R $\alpha$ ). NAMNCs from random fresh and frozen CB samples were incubated in the presence of rhIL-5, and removed at various time points for cell lysis, RNA extraction, reverse-transcription and determination of Tm-IL-5R $\alpha$  and Sol-IL-5R $\alpha$  mRNA expression patterns using SYBR green based quantitative polymerase chain reaction (Q-PCR) analysis.



**Fig. 4.** Kinetic mRNA expression patterns of GATA-1, MBP1 and IL-5R $\alpha$  (Total) from cord blood (CB) CD34<sup>+</sup> purified cell populations in response to stimulation with IL-5. Mononuclear cells from random fresh and frozen CB samples underwent magnet-activated cell separation using a CD34<sup>+</sup> selection kit. CB CD34<sup>+</sup> cells were incubated in the presence of rhIL-5, and removed at various time points for cell lysis, RNA extraction, reverse-transcription and determination of GATA-1, MBP1 and IL-5R $\alpha$  (Total) mRNA expression using a multiplex quantitative polymerase chain reaction (Q-PCR) reaction. Displayed are relative expression ratios (normalized to housekeeping gene, GAPDH), calculated using the standard  $2^{-(Ct)}$  formula (GATA-1:  $p = 0.013$ ; MBP1:  $p = 0.033$ ; IL5Ra (total) means not different:  $p = 0.68$ ; ANOVA on ranks).





**Fig. 5.** Kinetic changes in the mRNA expression of the Tm- & Sol-IL-5R $\alpha$  isoforms in cord blood (CB) CD34 $^{+}$  cells incubated with IL-5 (shown as percent of total IL-5R $\alpha$ ). Non-adherent mononuclear cells (NAMNCs) from random fresh and frozen CB samples underwent magnet-activated cell separation using a CD34 $^{+}$  selection kit. CB CD34 $^{+}$  cells were incubated in the presence of rhIL-5, and removed at various time points for cell lysis, RNA extraction, reverse-transcription and determination of Tm-IL-5R $\alpha$  and Sol-IL-5R $\alpha$  mRNA expression patterns using SYBR green based quantitative polymerase chain reaction (Q-PCR) analysis.

**Table 1.**

Primer/probe sequences for housekeeping genes and genes of interest

Gene	Sequence	Amplicon (bp)
Multiplex reactions		
GAPDH	Probe: AAGGCTGAGAACGGG Forward primer: GACCTCAACTACATGGTTT Reverse primer: CTGGAAGATGGTGATGGGATTT	238
GATA-1	Probe: ACTCTCCCTGCCTC Forward primer: AGCCTATTCCTCTCCAA Reverse primer: TCCGCAGTTCACACACTC	78
MBP1	Probe: GAGGAAGAGGAGGAGTG Forward primer: AGCAGGAGATGGAGGAGA Reverse primer: CTGGCACTGAGATAGACTCAAC	113
IL-5Ra	Probe: TTGGTTGTGG *GAGTT Forward primer: TAATGGCTATCTGGACGAG Reverse primer: AAGCAAAT *AT *GGA *GGAGGA	110
SYBR green-based reactions (no probe required)		
IL-5Ra soluble	Forward primer: GCAGCAGTGAGCTCCATGTG Reverse primer: TTCAGATACGGTGTGGGGCAG	124
IL-5Ra transmembrane	Forward primer: GCAGCAGTGAGCTCCATGTG Reverse primer: AGGGCTTGTGTCAICAITTCCC	87
$\beta$ -2-microglobulin	Forward primer: CCTGAATTGCTATGTGTCTGGG Reverse primer: TGATGCTGCTTACAGTCTCGA	245

\* Denotes superbases.