

Reduced expression of hemopoietic cytokine receptors on cord blood progenitor cells in neonates at risk for atopy

John W. Upham, MD, PhD,* Lisa M. Hayes, BSc, Joachim Lundahl, MD, Roma Sehmi, PhD, and Judah A. Denburg, MD *Hamilton, Ontario, Canada*

Background: Recent findings point to an association between allergic asthma in adults and increased responsiveness of myeloid progenitor cells to certain hemopoietic growth factors. However, it is not clear at what age these changes in progenitor cells first become manifest, although increasing evidence suggests that the allergic phenotype may begin to emerge in very early life.

Objective: We sought to compare expression of hemopoietic cytokine receptors on CD34⁺ progenitor cells in cord blood from normal infants ("low risk" for subsequent atopy) and infants with at least one atopic first degree relative ("at risk" for subsequent atopy).

Methods: Cord blood was obtained from 21 neonates. Nonadherent mononuclear cells were stained with mAbs directed against CD45, CD34, and the α -chains of the GM-CSF, IL-3, and IL-5 receptors and analyzed by flow cytometry.

Results: No differences in absolute CD34⁺ numbers were observed between the 2 groups. However, expression of GM-CSF receptor on CD34⁺ cells was reduced in the "at-risk" compared with the "low-risk" group ($P = .021$), although no significant differences were noted between the 2 groups with respect to IL-3 and IL-5 receptor expression.

Conclusion: The functional sequelae of reduced GM-CSF receptor expression on CD34⁺ cells remain to be determined. Nonetheless, these findings show an association between genetic risk for atopy and changes in the expression of hemopoietic cytokine receptors on cord blood progenitor cells and support the notion that the allergic phenotype may begin to evolve in the perinatal period. (*J Allergy Clin Immunol* 1999;104:370-5.)

Key words: Cytokine receptors, atopy, hemopoiesis, neonate, CD34

Human allergic disease is characterized by tissue infiltration with eosinophils, mast cells, and T lymphocytes, although the mechanisms that govern the supply and delivery of inflammatory cells to involved tissues remain incompletely understood. Previous findings from our laboratory have highlighted the links between allergic airway inflammation and increased numbers of inflammatory cell progenitors, both in the circulation and in the bone marrow. Compared with normal control subjects, circulating eosinophil-basophil colony-forming units are constitutively increased in atopic individuals,¹ and in asthma, numbers of these cells fluctuate in association with changes in clinical severity of the disease.² Furthermore, increased numbers of CD34⁺ cells are present in the blood and bone marrow of atopic individuals,³ and in allergic asthma, allergen inhalation is associated with specific upregulation of the α -chain of the IL-5 receptor (IL-5R α) on these CD34⁺ bone marrow cells.⁴ Although it is clear that myelopoiesis in allergic adults is skewed toward eosinophil differentiation, it is not clear at what age this first becomes manifest.

In recent years a number of investigators have proposed that the allergic phenotype may begin to emerge in the perinatal period. Allergen-specific T-cell proliferative responses can be detected both in cord blood^{5,6} and in very early infancy,⁷ implying intrauterine allergen exposure with resulting T-cell priming. Moreover, there is evidence for altered T-cell function in those infants "at risk" for the development of subsequent allergic disease. Reduced production of IFN- γ by cord blood mononuclear cells has been reported both in newborns with a family history of atopic disease⁸ and also in those infants in whom atopic eczema subsequently develops.⁹ Similarly, T cells from infants who are genetically at risk for atopy show reduced expression of the IL-2 receptor¹⁰ and a reduced proliferative capacity.^{11,12}

Whether these immunologic phenomena are associated with alterations in the phenotype of hemopoietic progenitor cells remains unclear. Even though various alterations of myelopoiesis and hemopoietic growth factor production have been identified in the neonatal peri-

From the Division of Clinical Immunology and Allergy, Department of Medicine, McMaster University, Hamilton.

*Dr Upham is currently affiliated with the University of Western Australia Department of Medicine, Queen Elizabeth II Medical Centre, Perth, Australia.

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Reprint requests: Judah A. Denburg, MD, Room 3V46, Health Sciences Centre, McMaster University, 1200 Main St West, Hamilton, Ontario L8N 3Z5, Canada.

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Abbreviations used

NAMNC: Nonadherent mononuclear cell
PE: Phycoerythrin

od,^{13,14} there has been no systematic attempt to relate these to subsequent risk of allergic disease. We hypothesized that atopic risk would be associated with alterations in hemopoietic lineage commitment, in particular that of the eosinophil pathway. Accordingly, this study focused on cord blood CD34⁺ cells and compared their expression of the relevant hemopoietic growth factor receptors in 2 groups of infants: those with low genetic risk for atopy and those at increased genetic risk for atopy.

METHODS

Study design

Pregnant women scheduled to have their babies delivered by elective Cesarean section were contacted by telephone late in the third trimester and invited to participate in the study. One to 2 weeks before delivery, women attended the laboratory to complete a questionnaire and physician interview that detailed personal and family history of allergic disease and to verify the absence of obstetric or medical complications of the pregnancy. Allergic sensitization of the mothers was assessed by skin prick testing with a panel of 19 allergen extracts plus negative and positive (histamine) controls. For practical reasons, it was not possible for fathers to complete their own questionnaires or to have skin testing. Neonates were thus classified as at risk for the later development of atopic disease when either the mother's skin test response was positive (2 or more allergens causing a wheal response at least 3 mm larger than that produced by the negative control) or when one or more first-degree relatives (including the mother) had a history of allergic disease that had previously been confirmed by a physician. Low-risk neonates were those in whom the maternal skin test response was negative and in whom there was no history of confirmed allergic disease in first-degree relatives. The study was approved by the Ethics Committee of McMaster University, and each mother gave her written informed consent.

Materials

Materials were purchased as follows. Lymphoprep was obtained from NycoMed Pharma AS (Oslo, Norway); McCoy's 5A medium, penicillin, streptomycin, and FCS were obtained from Gibco BRL (Gaithersburg, Md); heparin, dextran (molecular weight, 260,000), sodium azide, and paraformaldehyde were obtained from Sigma Aldrich Canada (Oakville, Ontario); and 2-mercaptoethanol was obtained from BDH Inc (Toronto, Ontario).

Antibodies

FITC-conjugated CD45 (anti-HLE-1, IgG1), phycoerythrin (PE)-conjugated CD34 (anti-HPCA-2, IgG1), and PE-conjugated isotype control antibody (mouse IgG1) were purchased from Becton Dickinson, Canada (Mississauga, Ontario); and streptavidin-conjugated CyChrome and HLA-DR CyChrome (anti-G46-6, IgG2a) were purchased from Pharmingen Canada (Mississauga, Ontario). Nonneutralizing mAbs directed against the α -chains of the GM-CSF receptor (GM-CSFR α ; clone 6E10) and IL-3 receptor (IL-3R α ; clone 7G2) were kind gifts from Dr A. Lopez (Institute of Medical and Veterinary Science, Adelaide, Australia); a similar antibody directed against the α -chain of the IL-5 receptor (IL-5R α ;

clone α 16) was graciously provided by Roche Laboratories (Ghent Belgium). These cytokine receptor antibodies and their respective isotype-matched controls were biotinylated in our laboratory as previously described.⁴

Preparation of cord blood cells

Immediately after delivery, umbilical cord blood was collected in a heparin-containing syringe. Blood smears were stained with Diff-Quik (VWR Canlab, Mississauga, Canada), and eosinophil counts were determined by microscopic examination of 200 leukocytes. Cord blood was depleted of red cells by unit gravity sedimentation in 1% (vol/vol) dextran at 37°C for 30 minutes, followed by isolation of mononuclear cells on a Lymphoprep density gradient (400g for 20 minutes at 20°C). After washing, cells were resuspended in McCoy's 5A supplemented with 15% FCS, antibiotics, and 2-mercaptoethanol and depleted of adherent cells by incubation in plastic flasks for a minimum of 2 hours at 37°C. To enumerate CD34⁺ progenitor cells, an aliquot of these nonadherent mononuclear cells (NAMNCs) was diverted for immunofluorescent staining (see below). To examine cytokine receptor expression on progenitor cells, it was necessary to first enrich for CD34⁺ cells by using immunomagnetic depletion, given that this population typically is composed of 1% or less cord blood NAMNCs. Cells were thus labeled with biotinylated anti-CD3 and anti-CD16 antibodies followed by streptavidin-coated MACS microbeads (Miltenyi, Auburn, Calif), and the negative fraction was collected after passage over a MACS-depletion column. Cell viability was assessed by trypan blue.

Immunofluorescent staining

Triple color immunofluorescent staining was performed as previously described,⁴ although with minor modifications. Briefly, CD34-enriched cells (0.5×10^6 per tube) were stained with saturating amounts of biotin-conjugated anti-GM-CSFR α , anti-IL-3R α , anti-IL-5R α , or IgG1 isotype control antibodies in ice-cold PBS containing 0.1% sodium azide, 2.5% normal mouse serum, and 2.5% normal human serum for 30 minutes on ice. After washing with PBS/0.1% azide, cells were then stained with optimal concentrations of streptavidin CyChrome, and the direct conjugates CD45-FITC, CD34-PE, and isotype controls were kept on ice for a further 30 minutes. The cells were then washed in PBS/0.1% azide, fixed in 400 μ L of PBS plus 1% paraformaldehyde, and refrigerated until ready for analysis. NAMNCs (0.5×10^6 per tube) were stained in a similar manner, although only with the direct conjugates CD45-FITC and CD34-PE and relevant isotype controls.

Flow cytometry

Cells were acquired and analyzed without knowledge of clinical details by using a FACScan flow cytometer (Becton Dickinson), as previously described.⁴ For each sample, a minimum of 40,000 events were acquired. Compensation settings were established by using Calbrite beads (BDIS) and confirmed with NAMNCs stained with CD45-FITC, CD34-PE, or HLA-DR CyChrome alone.

Analysis was performed by using PCLysis software (BDIS). To eliminate cell populations that nonspecifically take up anti-CD34 from the analysis, we used a multiparameter sequential gating strategy that has previously been shown to accurately enumerate CD34⁺ progenitor cell numbers (Fig 1). Originally developed by Sutherland et al,^{15,16} this technique has now been established in our laboratory.^{3,4} True CD34⁺ blast cells (region R4) are thus identified as cells with a high expression of CD34, low to intermediate expression of CD45, and scatter properties characteristic of lymphoblastoid cells. Within the CD34⁺ population, specific staining of CyChrome-linked cytokine receptor antibodies was determined by subtracting the staining attributable to the isotype control.

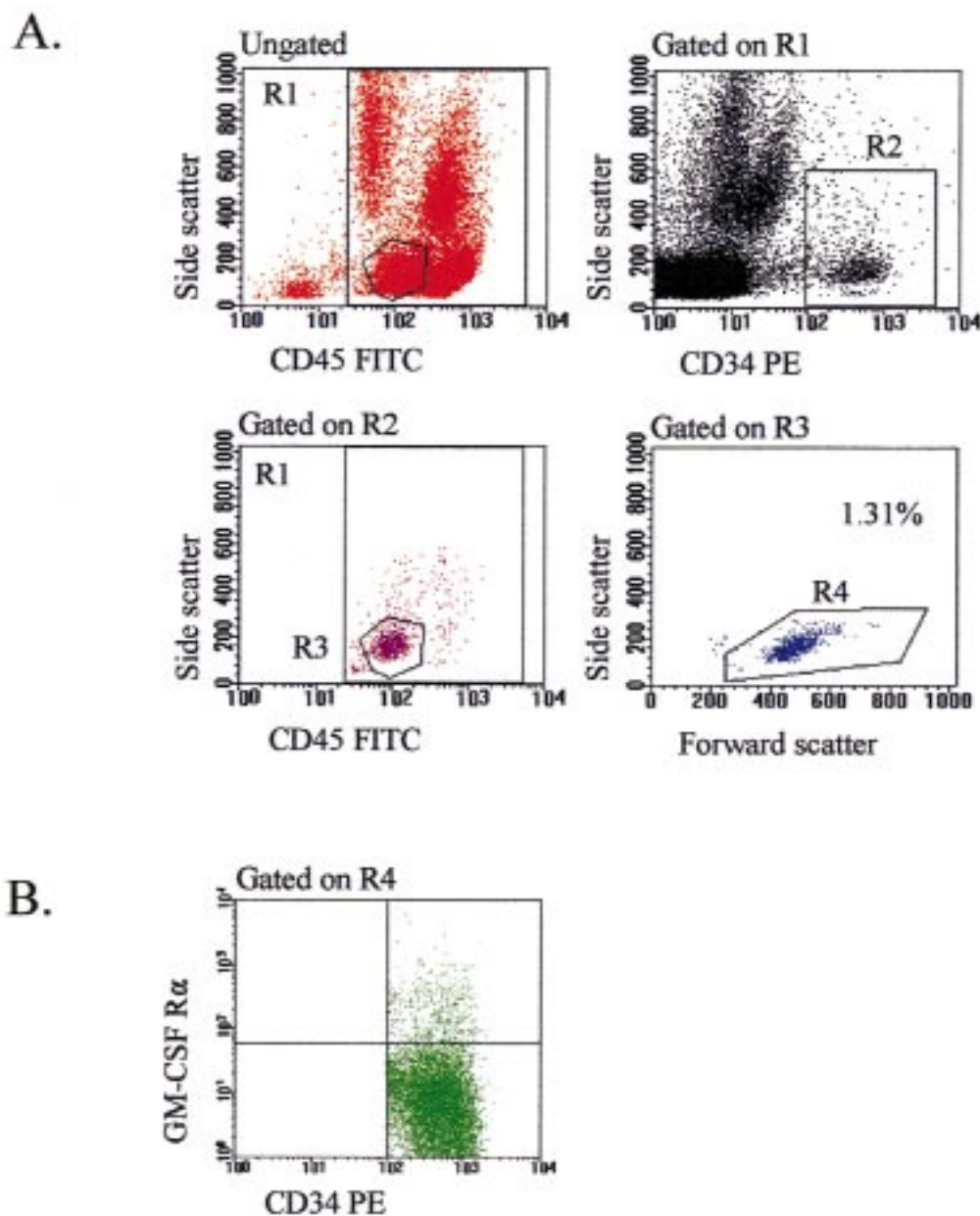


FIG 1. A, Enumeration of CD34⁺ progenitor cells in cord blood. NAMNCs were stained with CD45-FITC and CD34-PE and analyzed by flow cytometry. Numbers of true CD34⁺ blasts were determined by expressing cell numbers in region R4 as a percentage of cell numbers in region R1. Nonspecific staining with isotype controls was less than 1% in all experiments. A representative experiment is shown. **B,** Cytokine receptor expression on CD34⁺ cells (gated on region 4). A representative experiment of GM-CSF α -chain staining is shown. Quadrant markers were set such that only 1% or less of cells stained with isotype control.

Statistical analysis

Except where indicated, data are expressed as means $\pm$ SEM. Statistical analysis was performed by a 2-tailed *t* test for unpaired data, and comparison groups were considered significantly different if *P* values were less than .05.

RESULTS

Umbilical cord blood samples were obtained from 21 full-term neonates. All of the pregnancies were uncomplicated,

and none of the mothers had received systemic corticosteroids at any time during the pregnancy. Thirteen of the mothers had positive skin test responses. Six neonates had no atopic first-degree relatives and were classified as being at low risk. In 11 of the neonates, atopy was present in one first-degree relative, whereas 4 neonates had 2 or more atopic relatives. For the sake of convenience, these 15 were combined and are hereafter referred to as the at-risk group.

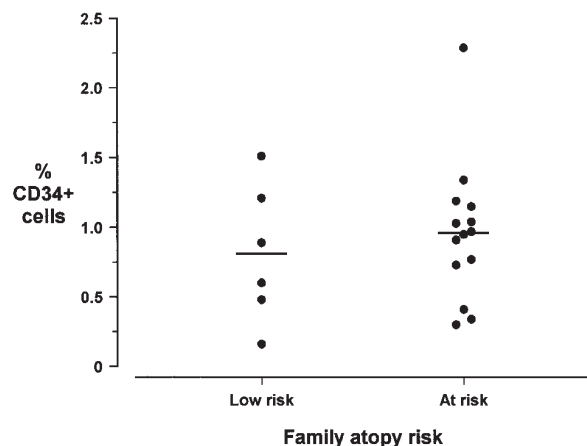


FIG 2. CD34⁺ cell numbers in cord blood NAMNCs. Data from low-risk and at-risk cord blood samples are shown. Horizontal bars refer to mean values ($P = .54$, low risk versus at risk).

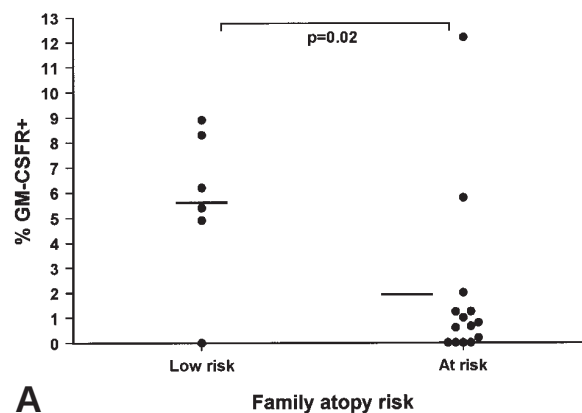
CD34⁺ progenitor cells were enumerated as outlined in the Methods section and expressed as a percentage of cord blood CD45⁺ NAMNCs (Fig 1). No significant differences were noted in absolute CD34 counts when comparing the low-risk and at-risk groups ($0.81\% \pm 0.20\%$ vs $0.96\% \pm 0.13\%$, respectively; $P = .54$; Fig 2). Likewise, no differences were observed between the 2 groups with respect to either cord blood eosinophil counts or HLA-DR expression on CD34⁺ cells (data not shown).

To determine expression of the α -chains of the receptors for GM-CSF, IL-3, and IL-5, CD34⁺ progenitor cells were enriched approximately 8-fold by depleting NAMNCs of CD3⁺ and CD16⁺ cell populations. Fig 3 outlines the proportion of CD34⁺ cells that expressed these cytokine receptors. As shown in Fig 3, A, GM-CSFR α was detectable on a significantly lower proportion of CD34⁺ cells from at-risk neonates ($1.92\% \pm 0.89\%$) than on CD34⁺ cells from low-risk neonates ($5.62\% \pm 1.30\%$; $P = .021$).

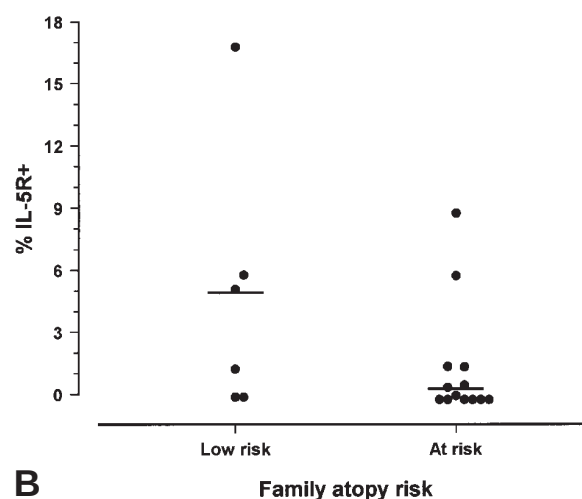
A similar trend for reduced expression of IL-5R α on CD34⁺ cells from the at-risk group was also seen, but this difference did not reach statistical significance ($P = .12$; Fig 3, B). A subsequent power analysis based on our data suggested that any future study would require approximately 38 subjects to have a 90% power to demonstrate a significant difference in IL-5R α expression between the 2 groups of neonates. In contrast, there was no difference in IL-3R α expression between the 2 groups (Fig 3, C). When results for individual subjects were analyzed, there was a trend for many of the at-risk infants to have very little expression of all 3 cytokine receptors on their CD34⁺ cells, whereas there was a much greater heterogeneity of receptor expression in the low-risk group.

DISCUSSION

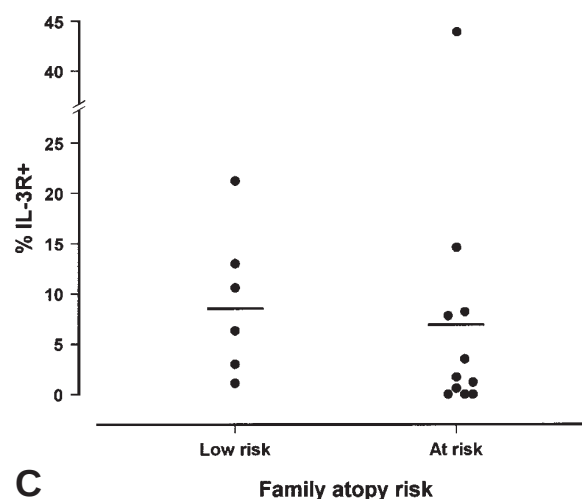
This study examined cytokine receptor expression on cord blood progenitor cells, comparing infants from nonatopic families with those who were genetically at



A



B



C

FIG 3. Surface expression of cytokine receptors on CD34⁺ cells. Cytokine receptor expression was determined relative to the isotype control. Data from cord blood samples of low-risk and at-risk newborns are shown. Horizontal bars refer to mean values. **A**, Expression of GM-CSFR α -chain ($P = .02$, low risk vs at risk). **B**, Expression of IL-5R α -chain ($P = .12$, low risk vs at risk). **C**, Expression of IL-3R α -chain ($P = .72$, low risk vs at risk).

increased risk for subsequent atopy. Two lines of evidence led us to hypothesize that the phenotype of progenitor cells might be altered in infants born into an atopic family. First, recent findings have pointed to upregulation of an IL-5-responsive subset of bone marrow progenitor cells in adult subjects with asthma.⁴ Second, increasing evidence suggests that the allergic phenotype may begin to emerge in the perinatal period,^{5,6,8-12} leading us to predict that expression of receptors for hemopoietic cytokines on progenitor cells might be altered in at-risk, compared with low-risk, infants.

The salient findings to emerge were compatible with our hypothesis. However, the specific alterations we found were somewhat surprising. Although cord blood CD34 counts were similar in the 2 groups of infants, expression of GM-CSFR α was significantly reduced on cord blood progenitors from at-risk newborns compared with low-risk newborns. A similar trend was observed for downregulation of IL-5R α , but this did not achieve statistical significance. To our knowledge, no previous attempts have been made to relate risk of atopy at birth to early events in hemopoietic differentiation, although others have suggested that atopy may be associated with cord blood eosinophilia¹⁷ or cord blood thrombocytopenia.¹⁸

The finding of downregulation of GM-CSFR α at first might appear counterintuitive given that CD34⁺ numbers and IL-5R α expression are higher in adults with established allergic disease. One possible explanation is that the cord blood does not accurately reflect the overall hemopoietic changes because tissue distribution of progenitors may be altered in the at-risk newborns. Thus early lineage-committed CD34 cells, of the type seen in asthmatic adults,⁴ might be preferentially sequestered in the bone marrow or other sites, rather than in the cord blood. However, the similar cord blood absolute CD34 counts in the 2 groups make this explanation somewhat less likely. If CD34⁺ cells were indeed sequestered outside the circulation, one might expect reduced numbers of cord blood CD34⁺ cells in the at-risk group.

Alternatively, this reduction in GM-CSFR α expression by progenitor cells may indicate an alteration in hemopoietic maturation in the at-risk group of neonates or may have occurred as a consequence of exposure to cytokines. With respect to this latter mechanism, others have documented that exposure of progenitors to particular hemopoietic growth factors can downregulate expression of both homologous and heterologous receptors,^{19,20} and further studies are required to establish the relevance of this mechanism in the current setting.

At present we can only speculate on the functional consequences of reduced GM-CSFR α expression on progenitor cells. These could include alterations in the evolution of mature eosinophils, neutrophils, and antigen-presenting cells (monocytes and dendritic cells). Future experiments will need to examine the relationship between atopy risk and (1) *in vitro* responsiveness of progenitor cells to GM-CSF and other cytokines, (2) the presence in cord blood of factors that are able to suppress

particular hemopoietic lineages, and (3) the postnatal kinetics of progenitor cell maturation. Whether the changes in GM-CSFR α expression we have observed are intrinsic to progenitors themselves or are caused by the elaboration of maternally derived factors that modulate receptor expression is unclear at present. If maternally derived factors are indeed responsible for these changes, one might expect to see differences between neonates born to atopic mothers as compared with atopic fathers, an issue that we plan to address in a forthcoming, much larger study.

It is acknowledged that certain limitations must be placed on the interpretation of our findings. First, in the absence of long-term follow-up, we cannot confidently predict which of the neonates we studied will eventually become atopic. Current methods of assigning atopic risk on the basis of family history or cord blood IgE lack sufficient predictive power to reliably identify all infants in whom allergic disease will develop,²¹ and therefore we plan to address these issues in a prospective study. Second, our study was limited to babies born by elective Cesarean section, although we have no reason to expect that the findings would have been any different had we also studied cord blood samples from spontaneous vaginal deliveries. Third, it is clear that the nature of the study led to the preferential recruitment of atopic women who are more likely to be included in such a study. Fourth, although we cannot fully exclude contamination of cord blood samples by maternal blood, this seems unlikely given the magnitude of the CD34⁺ cells counts, which are considerably in excess of those found in normal adults. Finally, although it is conceivable that flow cytometry may not be sufficiently sensitive to detect low-level receptor expression on progenitor cells, thereby underestimating the percentage of receptor-positive cells, it is unlikely that this would cause a systematic bias in our results. Moreover, the percentage of GM-CSF, IL-3, and IL-5 receptor-bearing CD34⁺ cells that we found in the current study is similar to that reported previously by Kurata et al.¹⁹ Despite the aforementioned limitations, we were able to identify clear differences between cord blood progenitor cells from the 2 subject groups.

In conclusion, we have shown reduced expression of GM-CSFR α on progenitor cells in cord blood from neonates at risk for the subsequent development of allergic disease. These findings may shed light on the mechanisms that facilitate the eventual emergence of the mature atopic phenotype.

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