

Retinoic acid modulates IL-5 receptor expression and selectively inhibits eosinophil-basophil differentiation of hemopoietic progenitor cells

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Background: IL-5 plays a central role in eosinophil and basophil differentiation, exerting its effects through the IL-5 receptor (IL-5R α). Currently, little is known concerning regulation of IL-5R α expression in the context of commitment of hemopoietic progenitor cells to the eosinophil and basophil lineages.

Objective: Because all-trans retinoic acid (ATRA) is known to modulate some aspects of hemopoietic differentiation, we examined the effects of ATRA on eosinophil-basophil differentiation and IL-5R α expression.

Methods: Progenitor cells were obtained from bone marrow aspirates and cord blood samples. Enriched populations of CD34⁺ cells were isolated by means of positive immunomagnetic selection with MACS beads.

Results: In semisolid methylcellulose cultures of normal human bone marrow, ATRA (10⁻⁶ mol/L) selectively suppressed eosinophil-basophil colony-forming units but had no effect on granulocyte-macrophage colony-forming units. Similarly, ATRA (10⁻⁶ mol/L) inhibited eosinophil-basophil differentiation of cord blood CD34⁺ cells in liquid culture, whereas neutrophil differentiation proceeded without impediment.

Most importantly, these effects of ATRA (10⁻⁸ to 10⁻⁶ mol/L) on CD34⁺ cells were associated with a dose-dependent inhibition of IL-5R α but no change in GM-CSF receptor expression, as detected with flow cytometry.

Conclusions: These findings indicate that retinoids can differentially regulate expression of IL-5R α , but not GM-CSF receptor, and that these effects have functional consequences *in vitro* on eosinophil and basophil differentiation. (*J Allergy Clin Immunol* 2002;109:307-13.)

Key words: Retinoic acid, hemopoiesis, eosinophils, basophils, IL-5 receptor

Abbreviations used

ATRA: All-trans retinoic acid
CFU: Colony-forming unit
GM-CSFR α : GM-CSF receptor
IL-5R α : IL-5 receptor
MNC: Mononuclear cell

The production and differentiation of eosinophils and hemopoietic lineage-related basophils are principally regulated by a group of cytokines that use a common receptor-signaling system (ie, IL-3, GM-CSF, and IL-5).^{1,2} Of these, IL-5 is unique in its ability to specifically promote the terminal differentiation and maturation of eosinophil-basophil lineage-committed progenitors in liquid and semisolid cultures,^{3,4} as well as promoting the priming, activation, and enhanced survival of mature eosinophils and basophils. A central role for IL-5 in eosinophil biology is supported by the profound eosinophilia observed in IL-5 transgenic mice,⁵ the ability of recombinant IL-5 delivered into the airways to exacerbate bronchial eosinophilia,⁶ and the overexpression of IL-5 that is observed in many eosinophil-associated diseases, such as asthma.^{7,8} Moreover, in animal models of asthma, IL-5 deficiency⁹ and neutralizing anti-IL-5 mAbs^{10,11} inhibit pulmonary eosinophilia.

IL-5 mediates its effects through the IL-5 receptor (IL-5R) which consists of 2 subunits: a unique α chain (IL-5R α) that contains the specific ligand-recognition site that binds IL-5 and a common β subunit shared with the IL-3 and GM-CSF receptors.^{1,2} In human subjects IL-5R expression is essentially limited to the eosinophil and basophil lineages,¹ and it is this restricted expression of IL-5R that determines the lineage specificity of IL-5. In contrast, in transgenic mice that express IL-5R α on all hemopoietic progenitors, IL-5 can also be shown to function as a pluripotent growth factor.¹² Although IL-5R expression is often thought to be restricted to mature eosinophils and basophils and to late lineage-committed CD34-CD33⁺ cells,¹³ the recent demonstration that IL-5R α is also present on primitive CD34⁺ progenitor cells¹⁴ suggests that expression of IL-5R may be a critical and very early hemopoietic event that can determine commitment to the eosinophil-basophil lineage. Although IL-5 clearly plays a central role in eosinophil-basophil differ-

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entiation, there is limited and somewhat conflicting information currently available on molecular regulation of the IL-5R α that mediates responsiveness to IL-5.

It has been known for some time that all-trans retinoic acid (ATRA) and various synthetic analogues have potent modulating effects on hemopoiesis, influencing the differentiation of both neoplastic and normal hemopoietic cells. In particular, ATRA induces granulocytic differentiation of leukemic cell lines in vitro^{15,16} and promyelocytic blasts in vivo.¹⁷ Moreover, retinoids can also alter lineage commitment: ATRA suppresses erythroid maturation of normal pluripotent progenitor cells, forcing differentiation toward the neutrophil lineage,¹⁸ and it has recently been shown that ATRA inhibits eosinophil-basophil maturation of the HL-60 cell line.¹⁹ More direct evidence that physiologic concentrations of retinoic acid influence differentiation of myeloid progenitors comes from the work of Tsai et al,²⁰ who transfected a pluripotent murine cell line with a dominant negative retinoic acid α receptor, such that these cells were no longer able to respond to retinoic acid. Consequently, the cell line differentiated almost exclusively into basophils, whereas cells with a normal retinoic acid receptor differentiated into a population dominated by neutrophils. These findings imply that under normal conditions, ambient concentrations of retinoids induce neutrophil differentiation of myeloid progenitors, whereas in the absence of a physiologic retinoic acid response, basophil (and accompanying eosinophil) differentiation predominates.

However, because much of the preceding work had focused on transformed cell lines, it was important to establish whether ATRA influences eosinophil-basophil differentiation of normal, nontransformed progenitor cells and surface expression of IL-5R α on differentiating CD34⁺ cells.

METHODS

Materials

Materials were purchased as follows: Lymphoprep was obtained from NycoMed Pharma AS; McCoy's 5A medium, penicillin, streptomycin, and FCS were obtained from Gibco BRL; heparin, dextran (molecular weight, 260,000 kD), sodium azide, ATRA, and paraformaldehyde were obtained from Sigma Aldrich Canada; 2-mercaptoethanol was obtained from BDH Inc; recombinant IL-3, IL-5, and GM-CSF were obtained from Pharmingen Canada; and methylcellulose (Methocel A4M premium) was obtained from Dow Chemicals. The human T-lymphocyte leukemia cell line (Mo) was kindly supplied by Dr D. Golde. Conditioned medium from this cell line was prepared as previously described²¹ and used as a source of GM-CSF in some experiments.

Antibodies

Phycoerythrin-conjugated anti-CD34 (anti-HPCA-2, IgG1) and phycoerythrin-conjugated isotype control (mouse IgG1) were purchased from Becton Dickinson, Canada, and streptavidin-conjugated CyChrome was purchased from Pharmingen. Nonneutralizing mAbs directed against IL-5R α (clone a16) and the GM-CSF receptor (GM-CSFR α ; clone 6E10) were graciously provided by Roche Laboratories and Dr A. Lopez (Institute of Medical and Veterinary Science, Adelaide, Australia), respectively. These cytokine receptor antibodies

and their respective isotype-matched controls were biotinylated in our laboratory, as previously described.¹⁴

Cell processing and culture

Samples of normal bone marrow were obtained preoperatively from the sternum of nonatopic patients undergoing cardiac surgery, as described previously.²² Exclusion criteria included patients receiving H1 and H2 antagonists and corticosteroids. Our previous experience with bone marrow samples obtained in this way has shown no effect of preoperative medications on either the phenotype or function of bone marrow progenitor populations. Low-density mononuclear cells (MNCs) were isolated by means of density gradient centrifugation on Lymphoprep and depleted of adherent cells by means of incubation in plastic flasks for 2 hours at 37°C. Nonadherent bone marrow MNCs were washed and cultured at 0.25×10^6 cells per 10 mm of tissue-culture dish (Falcon) in Iscove's modified Dulbecco's medium supplemented with 20% FCS, 0.9% methylcellulose, 1% penicillin-streptomycin, 0.5 mmol/L 2-mercaptoethanol, and ATRA to enumerate bone marrow colony-forming units (CFUs). Conditioned medium from the Mo cell line used as a source of GM-CSF was added to the cultures at a final concentration of 10%. Duplicate cell cultures were set up for each condition, and CFUs were enumerated on day 14 by means of inverted light microscopy. Colonies of greater than 40 cells were classified as either eosinophil-basophil CFUs or as granulocyte-macrophage CFUs, as previously described.³

In a second model of eosinophil-basophil differentiation, we examined the effects of ATRA on differentiation of purified cord blood CD34⁺ cells in liquid culture. Cord blood from normal full-term deliveries was collected into heparin-containing syringes. Samples were depleted of erythrocytes by means of unit gravity sedimentation in 1% (vol/vol) dextran at 37°C for 30 minutes, followed by isolation of nonadherent MNCs, as described above. CD34⁺ cells were enriched by means of positive immunomagnetic selection with MACS beads and resuspended in McCoy's 5A medium supplemented with 15% FCS, antibiotics, and 2-mercaptoethanol. These highly enriched CD34⁺ cells (average purity, approximately 70%) were cultured at a concentration of 1×10^5 per milliliter in 24-well plates (Nunc) together with 1.0 ng/mL IL-3, 1.0 ng/mL IL-5, and 10 ng/mL GM-CSF. Fresh medium and cytokines were added on day 3, and on day 7, the expression of IL-5R α and GM-CSFR α was examined by means of flow cytometry and RT-PCR. For comparison, some experiments also examined expression of IL-5R α on fresh cord blood CD34⁺ cells by using methods described elsewhere.¹⁴ ATRA was removed from cultures in some experiments after 3 days by washing the cells and resuspending them in fresh medium and cytokines to determine whether ATRA needs to be present throughout the period of cell culture.

In other experiments these liquid cultures of CD34⁺ cells were maintained for a further 3 weeks with IL-3, IL-5, and GM-CSF to allow granulocyte differentiation to proceed further. Cultures were fed twice weekly with cytokines, and at day 28, cell morphology was examined on cytospin preparations stained with DiffQuik. Differential cell counts were calculated by counting at least 200 cells per slide.

Immunofluorescent staining and flow cytometry

Immunostaining was performed by using methods detailed elsewhere, albeit with minor modifications.¹⁴ Briefly, cells (0.5×10^6 /tube) were stained with saturating amounts of biotin-conjugated anti-GM-CSFR 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 stained with streptavidin

CyChrome for a further 30 minutes on ice. 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. Samples were analyzed with a FACScan flow cytometer (BDIS), and at least 30,000 events were acquired. Offline analysis was performed with PC-Lysis software (BDIS). Analysis gates were set to exclude dead cells, and specific staining for cytokine receptors was determined relative to staining with the isotype control antibody. Data are expressed both as percentages of positively staining cells and as the relative fluorescence intensity (expressed as a percentage of fluorescence intensity of the isotype control).

Statistics

Data are presented as means $\pm$ SE. Except where indicated, the statistical significance of differences between groups was determined by using the Student *t* test. *P* values of less than .05 were regarded as significant.

RESULTS

ATRA selectively inhibits eosinophil-basophil CFUs

In initial experiments sternal bone marrow samples were obtained preoperatively from patients undergoing cardiac surgery, and CFUs were enumerated in 14-day methylcellulose-based assays, with Mo-conditioned medium as a source of GM-CSF. Data are expressed as CFUs per 2.5×10^5 nonadherent bone marrow cells. As shown in Fig 1, ATRA (10^{-6} mol/L) reduced eosinophil-basophil CFUs from 21 ± 5.9 to 9.25 ± 2.7 colonies (*P* < .05), associated with no significant change in the proportion of granulocyte-macrophage CFUs and no significant change in total numbers of colonies (eosinophil-basophil CFUs plus granulocyte-macrophage CFUs). In the absence of conditioned medium, no colonies were observed.

ATRA selectively inhibits eosinophil-basophil differentiation of enriched CD34⁺ cells in liquid culture

Previous studies have shown that retinoids influence hemopoiesis both by means of a direct effect on purified progenitor cells²³ and indirectly by enhancing cytokine production by bone marrow accessory cells.²⁴ Hence it was important to examine the effect of ATRA on a highly enriched progenitor population by using an established in vitro model of eosinophil-basophil differentiation.^{25,26} Twenty-eight-day liquid culture of cord blood CD34⁺ cells in the presence of IL-3, IL-5, and GM-CSF yielded a population of cells dominated by eosinophilic or basophilic progeny, with relatively few neutrophils (Table I). Conversely, in the presence of ATRA, eosinophil and basophil differentiation was inhibited, and the population of cells obtained after 28 days was predominantly neutrophilic, although the total yield of leukocytes obtained was not altered.

ATRA selectively inhibits surface expression of IL-5R α but not GM-CSFR α

We next focused on the effects of ATRA on IL-5R α expression during progenitor differentiation, given that

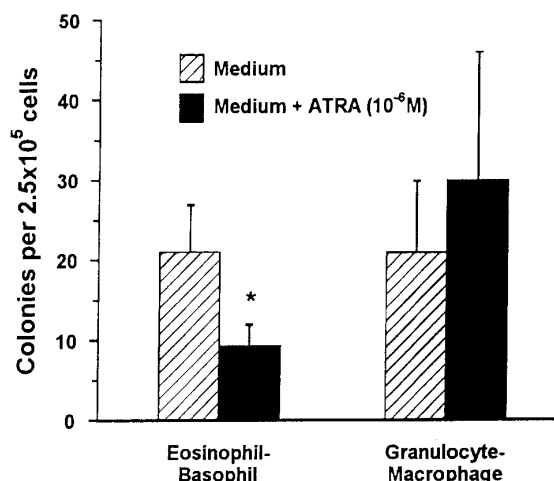


FIG 1. ATRA inhibits eosinophil-basophil, but not granulocyte-macrophage, CFUs. Normal bone marrow was obtained from nonatopic subjects undergoing cardiac surgery. Low-density, nonadherent bone marrow cells were cultured in conventional semisolid colony-forming assays and supplemented with conditioned medium in the presence or absence of ATRA 10^{-6} mol/L. CFUs were identified and enumerated on day 14 by means of inverted light microscopy and classified as either eosinophil-basophil CFUs or as granulocyte-macrophage CFUs by using standard morphologic criteria.³ Data are expressed as mean of 6 experiments. **P* < .05.

this may be a key early event in eosinophil-basophil lineage commitment. Cord blood CD34⁺ cells were again studied in liquid culture in the presence of IL-3, IL-5, and GM-CSF. In preliminary experiments only $1.8\% \pm 0.64\%$ of freshly isolated CD34⁺CD45⁺ cells expressed detectable IL-5R α , whereas by day 7, low-level surface expression of IL-5R α could be detected by means of flow cytometry in almost 10% of cells cultured in the absence of ATRA. In contrast, ATRA reduced the proportion of cells expressing IL-5R α and the intensity of receptor expression in a dose-dependent manner (Figs 2 and 3) associated with a significant inhibition of subsequent eosinophil-basophil differentiation in 28-day liquid cultures, as discussed earlier (Table I). These effects were observed regardless of whether ATRA was present throughout the 7-day period of culture or was washed out after 3 days (data not shown). In contrast, ATRA had minimal effects on GM-CSFR α expression. Although a minor change in the shape of the histogram was seen at the highest concentration of ATRA (Fig 2, *F*), this was not associated with significant changes in the proportion of cells expressing GM-CSFR α relative to the isotype control (Fig 4) and did not result in any change in the ability of progenitor cells to undergo neutrophilic differentiation in 28-day liquid cultures, as discussed earlier (Table I). It did not appear that these effects were due to the nonspecific toxic properties of ATRA, given that cell viability was not altered (as assessed by trypan blue staining) and that GM-CSFR α expression was not affected (Figs 2 and 4).

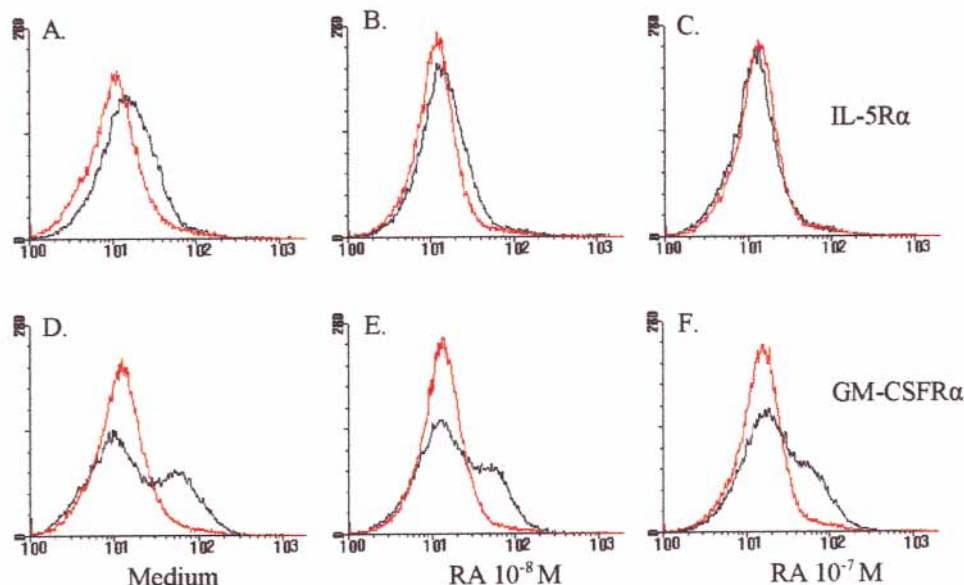


FIG 2. ATRA inhibits surface expression of IL-5R α but not GM-CSFR α . Cord blood CD34 $^{+}$ cells were isolated by means of positive immunomagnetic selection with MACS beads and studied after 7 days of liquid culture in the presence of 1.0 ng/mL IL-3, 1.0 ng/mL IL-5, and 10 ng/mL GM-CSF. Cells were stained with saturating amounts of either biotin-conjugated anti-IL-5R α , anti-GM-CSFR α (black line), or IgG1 isotype control (red line) antibodies, followed by streptavidin CyChrome. FACS analysis gates were set to exclude dead cells, and at least 30,000 events were acquired for each sample. Histograms of IL-5R α expression (A, B, and C) and GM-CSFR α expression (D, E, and F) from a representative experiment are shown. RA, All-trans retinoic acid.

TABLE I. Effects of ATRA (10^{-6} mol/L) on the progeny of cord blood CD34 $^{+}$ cells

ATRA	Eosinophil-basophil (%)		Neutrophil (%)	
	Myelocyte or metamyelocyte	Band or mature	Myelocyte or metamyelocyte	Band or mature
Nil	53.3 $\pm$ 16.6	36.8 $\pm$ 14.5	2.3 $\pm$ 1.0	7.8 $\pm$ 2.6
10^{-6} mol/L	4.5 $\pm$ 0.9	6.8 $\pm$ 2.5	12.5 $\pm$ 2.9	76.3 $\pm$ 5.0

Highly enriched cord blood CD34 $^{+}$ cells were cultured for 28 days with IL-5 and IL-3 with or without 10^{-6} mol/L ATRA. Cytospin preparations were stained with DiffQuik, counts were made of 200 cells per slide, and results were expressed as percentages. Data are from 4 experiments. ATRA had no effect on total cell numbers or viability.

DISCUSSION

Our findings demonstrate that ATRA has the ability to selectively inhibit eosinophil-basophil differentiation and surface expression of IL-5R α by hemopoietic progenitor cells in vitro. First, ATRA suppressed bone marrow eosinophil-basophil CFUs but had no effect on granulocyte-macrophage CFUs (Fig 1). Second, ATRA suppressed eosinophil-basophil differentiation of CD34 $^{+}$ cord blood cells in long-term liquid culture, whereas neutrophil differentiation proceeded without impediment (Table I). Finally, and most importantly, our results show that ATRA selectively inhibits the emergence of cells expressing IL-5R α in the early phases of progenitor cell differentiation (Figs 2-4). These effects of ATRA on IL-5R α expression are accompanied by functional changes in eosinophil-basophil differentiation.

Previous evidence from our group indicates that ATRA inhibits eosinophil-basophil differentiation of the pluripotent HL-60 leukemic cell line.¹⁹ Our findings

herein demonstrate that ATRA also inhibits eosinophil-basophil differentiation of nontransformed progenitor cells from normal bone marrow and cord blood. These effects were seen both in mixed cultures (bone marrow nonadherent cells) and in highly enriched CD34 $^{+}$ cells, which suggests, but does not prove, that ATRA is exerting its effects directly on progenitor cells, rather than acting indirectly through accessory cells. In the HL-60 system ATRA inhibits eosinophil-basophil differentiation in the complete absence of accessory cells and is most effective when added to cultures at early time points. Once commitment to eosinophil-basophil differentiation has occurred, further maturation along this pathway is insensitive to ATRA.¹⁹ Similarly, the ability of ATRA to induce a shift in the differentiation program of human fetal hemopoietic progenitor cells is most pronounced at early time points.²³ Our findings in the current study are also consistent with the notion that ATRA is having its effect at an early time point in lineage commitment, which coincides with the emergence of cells expressing

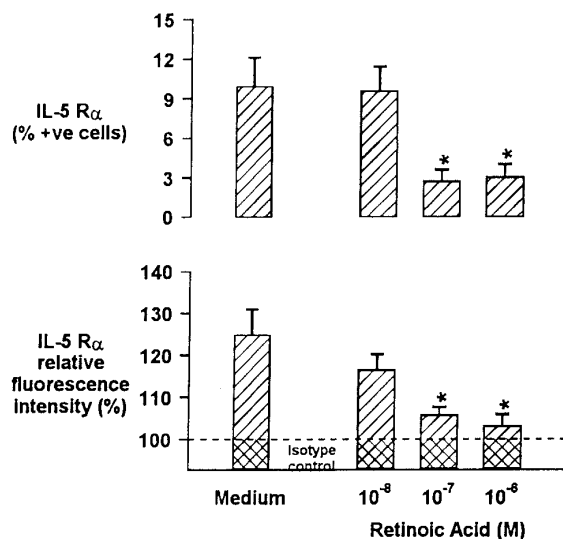


FIG 3. ATRA inhibits surface expression of IL-5R α in a dose-dependent manner. Group data are shown ($n = 5$). ATRA significantly reduced both the proportion of cells expressing IL-5R α (upper panel) and the intensity of receptor expression (lower panel). Fluorescence intensity of cytokine receptor staining was determined relative to fluorescence of the isotype control, indicated in the lower panel by the dashed line, and arbitrarily set at 100. * $P < .05$.

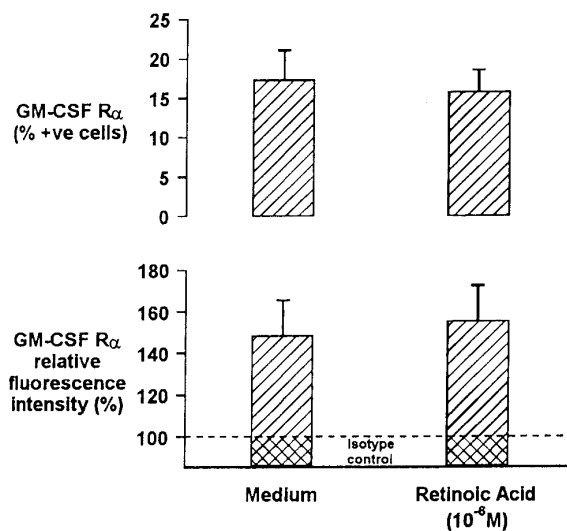


FIG 4. ATRA has no effect on surface expression of GM-CSFR α . Group data are shown, as in Fig 3. Data are shown only for 10⁻⁶ mol/L ATRA; 10⁻⁷ and 10⁻⁸ mol/L ATRA likewise had no effect on GM-CSFR α expression. The upper panel displays the proportion of cells expressing GM-CSFR α , whereas the intensity of receptor expression is shown in the lower panel. Fluorescence intensity of cytokine receptor staining was determined relative to fluorescence of the isotype control, indicated in the lower panel by the dashed line, and arbitrarily set at 100.

IL-5R α . ATRA reduced the number of cells expressing IL-5R α at day 7 in a dose-dependent manner (Fig 2). Importantly, these effects of ATRA on IL-5R α appeared to have functional significance, given that they were associated with selective inhibition of eosinophil-basophil differentiation in 28-day liquid cultures. Moreover, the observation that ATRA did not alter total numbers of progeny (ie, the sum of eosinophils, basophils, and neutrophils) in both semisolid and liquid culture systems makes it unlikely that these findings can be attributed to cytotoxicity. Rather, ATRA selectively inhibited both eosinophil-basophil differentiation and IL-5R α expression; concurrent neutrophil differentiation and GM-CSFR α expression were not affected by ATRA. Accordingly, we hypothesize that these phenomena reflect a shift of the committed myeloid progenitor cell differentiation program from eosinophil-basophil to neutrophil differentiation. It is noteworthy that ATRA has also recently been shown to inhibit differentiation of human mast cell progenitors,²⁷ providing additional support for the notion that retinoids have the capacity to modulate hemopoietic differentiation in a manner that is highly relevant to the pathogenesis of allergic disease.

Although IL-5 clearly plays a central role in eosinophil-basophil differentiation, there is limited and somewhat conflicting information currently available on molecular regulation of the IL-5R α that mediates responsiveness to IL-5. In both normal human eosinophils²⁸ and HL-60 eosinophilic cells,^{29,30} cytokines such as IL-5, IL-3, and GM-CSF selectively inhibit IL-5R α chain gene transcription and surface receptor expression in vitro,

whereas in erythroleukemic TF-1 cells³¹ and eosinophils from hypereosinophilic patients,³² these same stimuli elicit either no change or may even upregulate IL-5R α . Importantly, a recent report indicates that IL-5 (but not IL-3 or GM-CSF) is able to regulate differential splicing of membrane and soluble isoforms of IL-5R α during in vitro differentiation of normal human progenitor cells.³³ Using a similar culture system to our own (ie, IL-5-driven eosinophil development from cord blood CD34⁺ cells), Tavernier et al³³ showed that IL-5 itself was able to induce a switch from predominantly soluble to membrane IL-5R α .

In allergic asthma it has recently been shown that allergen inhalation in vivo upregulates IL-5R α expression on bone marrow CD34⁺ cells,¹⁴ although the mechanism by which this phenomenon is mediated remains unclear, and it is likely that there are other factors (aside from IL-5, IL-3, and GM-CSF) that enhance IL-5R α expression in the context of human allergic disease.

In the current study we show for the first time that ATRA can inhibit IL-5R α expression and that this effect has functional consequences in established experimental models of hemopoiesis. Although the magnitude of the downregulation in surface expression of IL-5R is relatively modest, it nonetheless appears to be biologically relevant, given that it is associated with significant changes in eosinophil-basophil differentiation in vitro. Moreover, a similarly modest degree of upregulation of IL-5R α on bone marrow CD34⁺ cells in vivo has been associated with the development of significant pulmonary eosinophilia in patients with allergic asthma.¹⁴

In contrast to our findings herein, a previous study was unable to demonstrate modulation of IL-5R α expression by either retinoids or corticosteroids.³¹ However, in that study IL-5R α mRNA expression was investigated in a leukemic cell line only, and that cell line (as opposed to the HL-60 line) may have lost some of its capacity to be regulated by physiologic and pharmacologic agents.

The current study does not allow us to determine the molecular mechanisms by which ATRA inhibits eosinophil-basophil differentiation and IL-5R α expression. Future experiments will therefore need to examine the effects of ATRA on IL-5R α gene transcription. The α chain of the IL-5R is known to exist either as membrane-bound or soluble isoforms, depending on alternative splicing of IL-5R α mRNA,^{34,35} and the relative proportions of these 2 isoforms may have important functional consequences. The soluble isoform of IL-5R α is secreted into the extracellular fluid, where it competes with membrane IL-5R α for IL-5 binding, thereby inhibiting the effects of IL-5.³⁶ Indeed, there are data to indicate a link between the severity of asthma and the relative proportions of membrane versus soluble IL-5R α mRNA.³⁷ This is a potential site at which ATRA might also be exerting its effects. In addition, a number of promoter regions have been described upstream of the gene encoding IL5R α ,^{38,39} and it has recently been shown that one of the RFX family of proteins is a transcription factor that contributes to the activity of this promoter.⁴⁰ We are not aware of evidence for retinoid responsive elements within the IL-5R α promoter, although this issue clearly warrants further examination in the light of the findings presented herein.

As a result of our current study, we hypothesize that retinoids may play a role in the physiologic regulation of IL-5R α . Given that allergic diseases such as asthma are associated with increased expression of IL-5R α on bone marrow progenitor cells¹⁴ and given the involvement of eosinophil differentiation pathways in these conditions,⁴¹ it will be important for future studies to examine the possible role of ATRA-responsive genes in the pathogenesis of these diseases. Moreover, there is clearly a need to examine the effects of retinoids in animal models of allergic disease to establish the in vivo relevance of our findings.

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