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IL-5 Triggers a Cooperative Cytokine Network That Promotes Eosinophil Precursor Maturation

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Eosinophils originate in the bone marrow from an eosinophil lineage–committed, IL-5Rα–positive, hematopoietic progenitor (eosinophil progenitor). Indeed, IL-5 is recognized as a critical regulator of eosinophilia and has effects on eosinophil progenitors, eosinophil precursors, and mature eosinophils. However, substantial levels of eosinophils remain after IL-5 neutralization or genetic deletion, suggesting that there are alternative pathways for promoting eosinophilia. In this study, we investigated the contributory role of IL-5 accessory cytokines on the final stages of eosinophil differentiation. IL-5 stimulation of low-density bone marrow cells resulted in expression of a panel of cytokines and cytokine receptors, including several ligand–receptor pairs. Notably, IL-4 and IL-4Rα were expressed by eosinophil precursors and mature eosinophils. Signaling through IL-4Rα promoted eosinophil maturation when IL-5 was present, but IL-4 stimulation in the absence of IL-5 resulted in impaired eosinophil survival, suggesting that IL-4 cooperates with IL-5 to promote eosinophil differentiation. In contrast, CCL3, an eosinophil–produced chemokine that signals through CCR1, promotes terminal differentiation of CCR1-positive eosinophil precursors in the absence of IL-5, highlighting an autocrine loop capable of sustaining eosinophil differentiation. These findings suggest that brief exposure to IL-5 is sufficient to initiate a cytokine cooperative network that promotes eosinophil differentiation of low-density bone marrow cells independent of further IL-5 stimulation.

Eosinophils are derived from an eosinophil lineage–committed progenitor (EoP) that is derived from the granulocyte–macrophage progenitor in mice and the common myeloid progenitor in humans (1, 2). The EoP is identified via surface expression of CD34, IL-5Rα (also known as CD125), and low levels of c-kit (CD117). EoPs reside in small numbers primarily in the bone marrow (~0.05% of lineage-negative CD34+ cells), with even lower levels found in peripheral blood and in human umbilical cord blood (2). Importantly, EoP numbers in the bone marrow increase substantially with allergen exposure or helminth infection and in patients with eosinophil-associated disorders (2–4). Furthermore, EoPs are found within allergic nasal and pulmonary mucosa and in the sputum from asthmatic patients, suggesting that eosinophil differentiation in situ contributes to the accumulation of mature eosinophils in tissues (5–7).

PreEos mature into granule-containing eosinophil precursors (preEos) that maintain proliferative capacity and surface expression of IL-5Rα (Fig. 1). PreEos are an ill-defined heterogeneous population that matures into fully differentiated eosinophils that have a characteristic segmented nucleus (bilobed in humans and circular in mice) and a cytoplasm rich with eosin-staining granules. IL-5 is recognized as a critical regulator of eosinophilia and has effects on multiple eosinophil-lineage developmental stages (8). Although IL-3, IL-5, and GM-CSF have been shown to induce eosinophil-containing colony formation from bone marrow progenitors, IL-5 stimulation of bone marrow progenitors selectively produces eosinophils (9). Overexpression of IL-5 in mice results in profound eosinophilia, suggesting that IL-5 not only directs differentiation, but also has a proliferative effect on EoPs and/or preEos (10, 11). IL-5–deficient animals show a near-normal steady-state eosinophil production, suggesting that other unidentified mediators can promote eosinophil development in the absence of IL-5 (12). Notably, although treatment with anti-IL-5 lowers blood and tissue eosinophil levels in mice and man with established disease (13), there are still substantial residual eosinophils, especially in the bone marrow (14–18). These studies highlight the need to identify mediators responsible for persistent tissue eosinophilia after IL-5 is neutralized via targeted treatment.

We used an ex vivo culture system to identify eosinophil-promoting pathways that are initiated by IL-5 and could maintain an eosinophil-promoting environment after IL-5 is withdrawn. We report that IL-5 stimulation of low-density bone marrow (LDBM) cells for 4 d is sufficient to induce a cooperative cytokine network that can promote terminal eosinophil differentiation in the absence of further IL-5 stimulation. This cooperative network includes expression of a panel of cytokines and cytokine receptors, including ligand–receptor pairs that have the potential to influence eosinophil maturation. Notably, IL-4 and its receptor IL-4Rα are expressed during the full spectrum of eosinophil developmental stages. IL-4Rα signal transduction in LDBM cells promoted eosinophil differentiation in the presence of IL-5, whereas IL-4 stimulation of preEos in the absence of IL-5 impaired eosinophil
survival, highlighting a cooperative relationship between IL-4 and IL-5 signal transduction. In contrast, preEos express CCL3 and the CCL3 receptor CCR1, which can promote terminal differentiation of preEos in the absence of IL-5. As CCL3 expression is induced in eosinophil-associated disorders (19, 20), our data provide mechanistic insight into the limitations observed in solely targeting IL-5, as other mediators (e.g., CCL3) can promote eosinophil maturation after IL-5 removal.

Materials and Methods

Mice

In-house–bred male and female wild-type (BALB/c), IL-4 knockout (KO) (21), IL-4–KOxKO (purchased from Taconic, Hudson NY), IL-5KO (12), and IL-5–enhanced GFP reporter (22) mice were used as the source of LDBM cells. All mice were housed under specific pathogen-free conditions and handled under approved protocols of the Institutional Animal Care and Use Committee of Cincinnati Children’s Hospital Medical Center (CCHMC).

LDBM cell cultures

LDBM cultures were performed as described previously (23), unless indicated otherwise, to differentiate preEos and mature eosinophils (Fig. 1). Cell-free culture supernatants were collected after 5, 8, and 11 d of IL-5 stimulation. For the IL-5 withdrawal (IL-5W) model (Fig. 2), LDBM cells were cultured in 24-well plates in media containing IL-5 (10 ng/ml) for 4 d and then washed with culture media to remove IL-5 and replaced and cultured in media without added IL-5. For the IL-5 removal (IL-5V) model (Fig. 2), LDBM cells were cultured in 24-well plates in media containing IL-5 (10 ng/ml) for 4 d and 6 d and then washed with culture media to remove IL-5 and replated and cultured in media without added IL-5 for the remainder of the culture period (6 d). 50–75% of the culture media was removed every 2 d and replaced with media without added IL-5 (3 times). For the IL-5 removal (IL-5V) model (Fig. 2), LDBM cells were cultured in 24-well plates in media containing IL-5 (10 ng/ml) for 4 d and 6 d and then washed with culture media to remove IL-5 and replated and cultured in media without added IL-5 for the remainder of the culture period (6 d). Flow cytometry analysis, along with cell-free supernatants for ELISA analysis. For some experiments, the neutralizing Ab anti–IL-5 (50 mg/ml, clone TRFK5; R&D Systems, Minneapolis, MN), anti–GM-CSF (50 mg/ml, clone MP122E9; R&D Systems), anti–CXCL10 (12 mg/ml), R&D Systems), and anti–CCL3 (0.2 mg/ml, R&D Systems) or anti–CCL4 (12 mg/ml, R&D Systems) or appropriate isotype IgG control Abs (rat IgG2a or goat IgG; R&D Systems) were added to media at the indicated time points.

Flow cytometry staining, data acquisition, and analysis

Cells were washed twice and resuspended in 100 μl flow buffer (1× PBS containing 1 mM EDTA) at a concentration of 1–100 × 106 cells/ml. Blocking was performed in flow buffer with 0.5–5.0 μg rat anti-mouse CD16/CD32 (BD Bioscience, San Diego, CA) for 5 min at 4˚C. Cells were then washed with culture media to remove IL-5 and replated and cultured in media without added IL-5 for the remainder of the culture period (6 d). Cell–cell interactions were determined using protocols of the Institutional Animal Care and Use Committee of Cincinnati Children’s Hospital Medical Center (CCHMC). Data were acquired using LSR II, FACS Canto, and LSR Fortessa flow cytometer (BD Biosciences, San Diego, CA) thermal cycling using the following program: 95˚C for 10 min followed by 40 cycles of 95˚C for 10 s and 60˚C for 30 s. Expression was calculated using the ΔΔCt method (Livak) and normalized to Gapdh.

Cytokine levels

Cytokine levels were measured in cell-free supernatants from cultured LDBM cells or cultured, isolated preEos via multiplex assay using the Mouse Cytokine/Chemokine Panel I (Millipore) by the Flow Cytometry Core Facility at CCHMC or by ELISA per the manufacturer’s instruction.

Microarray analysis

For RNA extraction, cultured LDBM cells were collected before IL-5 stimulation and after 4 and 10 d of IL-5 stimulation. No selection for any specific cell type was performed before RNA extraction. The Affymetrix exon Chip MoGene-1.0 was used to compare gene expression profiles between LDBM cells before IL-5 stimulation (0 d) and LDBM cells stimulated with IL-5 for 4 or 10 d. Microarray data were analyzed using the GeneSpring software (Agilent Technologies, Santa Clara, CA). Statistical significance was determined at p < 0.05 with one-way ANOVA and Benjamini–Hochberg multiple testing correction. Pathway enrichment analysis was performed with single-experiment analysis in GeneSpring. The data discussed in this publication have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus (24) and are accessible through GEO Series accession number GSE55386 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=etsaamelnixdcx&acc=GSE55386).

Statistics

Data were analyzed using either a two-tailed, unpaired Student t test or a one-way ANOVA with Bonferroni post hoc test as appropriate (GraphPad Prism). Differences were considered statistically significant when p < 0.05. Data are presented as mean ± SEM except for representative experiments, in which data are presented as mean ± SD.

Results

IL-5 withdrawal has little effect on final eosinophil maturation

To investigate the importance of IL-5 during the later stages (after lineage commitment) of eosinophil differentiation (Fig. 1), we used an ex vivo model of IL-5W in which the culture media initially contains high-dose IL-5 (10 ng/ml) to promote differentiation and expansion of EoPs and preEos for 4 d, after which no further IL-5 was added to the cultures (Fig. 2A). Eosinophil yield and maturation as measured by surface expression of Siglec-F and CCR3 were minimally affected by withdrawal of IL-5 for the last 6 d of differentiation (Fig. 2B, 2C). There was an increase in the proportion of 24-well plates at 1 × 106 cells/ml. Cells and cell-free supernatants were collected after 24 and 48 h. Total RNA was isolated with the Direct-zol RNA MiniPrep (Zymo Research, Irvine, CA) following the manufacturer’s instructions, and cell-free supernatants were collected for ELISA analysis.

Gene expression

Gene expression in isolated preEos was determined using primers described in Supplemental Table I. Reaction mixtures contained 10 ng cDNA template, 100 nM of each primer, and 1× FastStart Universal SYBR Green Master with Rox (Roche Diagnostics, Indianapolis, IN). Real-time quantitative PCR was performed with an ABI 7900HT (Applied Biosystems, Foster City, CA) thermal cycling using the following program: 95˚C for 10 min followed by 40 cycles of 95˚C for 10 s and 60˚C for 30 s. Expression was calculated using the ΔΔCt method (Livak) and normalized to Gapdh.

Eosinophil Differentiation

EoP PreEos Eos

IL-5Ra+ IL-5Ra+ IL-5Ra+

IL-5Rb+ IL-5Rb+ IL-5Rb+

SiglecF+ SiglecF+ SiglecF+

CCR3+ CCR3+ CCR3+

FIGURE 1. Eosinophil differentiation schematic in low-density bone marrow cultures. LDBM cells include the IL-5 receptor α (IL-5Ra)–expressing eosinophil-lineage committed progenitor (EoP), which differentiates into eosinophil precursors (preEos), which express IL-5Ra and Siglec-F on their surface. The preEos further differentiates into a mature eosinophil (Eos) identified by surface expression of IL-5Rb, Siglec-F, and CCR3.
of Siglec-F⁺CCR3⁻ cells in the cultures, which suggests that final eosinophil maturation and upregulation of CCR3 are delayed or stalled (Fig. 2C). Neutralizing the IL-5 that remained in the cultures resulted in declining yield of mature eosinophils in the cultures with time (Fig. 2D). Because the number of eosinophils declined with IL-5 neutralization, these data suggest that IL-5 is important for survival of the newly matured eosinophils (Fig. 2D).

To evaluate the contribution of IL-5 on preEos differentiation, we removed IL-5 from the cultures after 4 or 6 d (IL-5V; Fig. 2E) and determined the effect on mature eosinophil yield. Stimulating LDBM cells with IL-5 for 4 or 6 d followed by 6 or 4 d of culture with media alone (IL-5V), respectively, still yielded a population of mature eosinophils (Fig. 2F, 2G), albeit a significantly smaller one than in the presence of IL-5. The proportion of mature eosinophils in the cultures increased with longer IL-5 exposure (6 d versus 4 d, Fig. 2H), suggesting that IL-5 is critical for preEos expansion. Together, these data suggest that exposure of preEos to IL-5 resulted in a developmental program capable of driving the final stages of eosinophil differentiation after IL-5 is withdrawn, neutralized, or removed.

**IL-5 stimulation induces expression of a panel of cytokines by LDBM cells**

To identify cytokines that might contribute to the final stages of eosinophil differentiation, we used two unbiased approaches to identify active cytokine signaling pathways in LDBM cells after IL-5 stimulation. Initially, we kinetically measured cytokine levels...
in the culture supernatants. Cytokines previously associated with eosinophils, including IL-4, IL-9, and GM-CSF, were detected after 5, 8, and 11 d of IL-5 stimulation of LDBM cells (Fig. 3A). Notably, in the supernatants, we also detected TNF-α accumulation that declined as the eosinophils matured, and levels of vascular endothelial growth factor increased as the eosinophils matured (Fig. 3B). In addition, we detected IL-6 (12 ± 2 pg/ml), IL-10 (7 ± 0.7 pg/ml), and IL-12p40 (16 ± 2 pg/ml) in the supernatants after 5 d of IL-5 stimulation, but these cytokines were undetectable in the supernatants at later time points. These data suggest that IL-5 stimulates LDBM cells to express a panel of cytokines that changes as mature eosinophils become more predominant in the cultures.

To screen for mediators and receptors that are induced by IL-5 and might contribute to eosinophil differentiation, we performed gene expression profiling on LDBM cells cultured with IL-5 for 4 or 10 d. Expression of 681 or 1055 genes was significantly (p < 0.05) induced ≥2-fold after 4 or 10 d of IL-5 stimulation, respectively, when compared with expression levels prior to IL-5 stimulation (Fig. 3C, Supplemental Table II). As expected, the genes with the greatest induction in LDBM cells after 4 d of IL-5 stimulation encoded constituents of eosinophil granules, including eosinophil ribonucleases and eosinophil peroxidase. Two genes, Ccr3 and Alox15, that encode for proteins important for mature eosinophil effector functions were the most induced genes in LDBM cells stimulated with IL-5 for 10 d. We also identified distinct subsets of genes that could be delineated on the basis of peak expression level after 4 or 10 d of IL-5 stimulation, when preEos and mature eosinophils predominate in the cultures (Fig. 3C). Pathway analysis revealed significant enrichment for cytokine signaling pathways, including IL-5, chemokines, IL-3, and IL-4 (Table I). We further explored changes in gene expression specific to cytokine-mediated signaling (Fig. 3D, Supplemental Table III) and chemokine receptors (Fig. 3E). This analysis revealed panels of genes with

Table I. Pathway analysis of differentially expressed genes

<table>
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<tr>
<th>Pathway</th>
<th>p Value</th>
<th>Genes in Pathway</th>
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</thead>
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<tr>
<td>Mm_IL-5_Signaling_Pathway_WP151_69175</td>
<td>9.36E-08</td>
<td>25</td>
</tr>
<tr>
<td>Mm_Chemokine_signaling_pathway_WP2292_53116</td>
<td>1.84E-06</td>
<td>44</td>
</tr>
<tr>
<td>Mm_IL-3_Signaling_Pathway_WP373_69196</td>
<td>6.42E-06</td>
<td>28</td>
</tr>
<tr>
<td>Mm_IL-4_signaling_Pathway_WP93_41293</td>
<td>1.28E-03</td>
<td>16</td>
</tr>
</tbody>
</table>
IL-5–mediated differential expression during eosinophil development from LDBM cells, suggesting that eosinophilopoiesis could be differentially influenced by mediators, depending on the developmental stage.

**PreEos express a specific panel of cytokines**

Because the LDBM cultures used for our gene expression profiling represent a heterogeneous population of cells that include preEos until late in the cultures, when mature eosinophils predominate, we evaluated the specific expression of cytokines and cytokine receptors by preEos (Siglec-FCCR3+) sorted from IL-5–stimulated LDBM cultures. The mRNA for the receptors for IL-3, IL-5, GM-CSF, the common β-chain, and IL-4 was detected in preEos sorted from cultures after 4 d of IL-5 stimulation (Fig. 4A), suggesting that preEos may be responsive to these cytokines. Levels of mRNA for these cytokine receptors, with the exception of the β-chain, were similar to that of Siglec-F, which is also expressed on the surface of preEos. We investigated IL-5–stimulated cytokine expression by the sorted preEos and detected the cytokines IL-4, IL-6, and IL-13 in the supernatants (Fig. 4B). Notably, no IL-9 or GM-CSF was detected in the supernatants from the purified preEos cultured with IL-5, suggesting that the IL-9 and GM-CSF detected in the supernatants from the LDBM cultures is produced and secreted by other bone marrow–derived cells in those cultures (Fig. 3A). No significant difference in levels of mRNA for IL-5Rα, CCL3, IL-4, or the granule proteins major basic protein and eosinophil peroxidase were observed in preEos sorted from LDBM cells stimulated with IL-5 at 10, 1, or 0.1 ng/ml (Fig. 4C; data not shown), suggesting that IL-5 stimulates an expression profile in preEos that is independent of the concentration of IL-5. We next evaluated the kinetic expression of IL-4 mRNA by preEos during maturation using an IL-4 fluorescent reporter (4get) mouse line (22). The absolute number of cells that express both IL-4 and Siglec-F increased after 7 d of IL-5 stimulation (Fig. 4D), and few cells that expressed Siglec-F did not express IL-4 (Fig. 4D). These data reveal that mRNA for IL-4 is expressed by both preEos and mature eosinophils and that IL-5–stimulated preEos produce and secrete multiple chemokines and cytokines, including IL-4 and IL-13.

**IL-4Rα signaling cooperates with IL-5 to promote eosinophil differentiation**

As preEos express the mRNA for IL-4Rα (Fig. 4A) and secrete the IL-4Rα ligands IL-4 and IL-13 (Fig. 4B), we investigated the contribution of IL-4Rα signal transduction in promoting preEos differentiation. Deficiency in IL-4Rα, but not IL-4, resulted in decreased eosinophil yield under IL-5W conditions (Fig. 5A), suggesting that signaling through IL-4Rα (via IL-4 or IL-13) can contribute to preEos maturation. In contrast, although preEos also express mRNA for GM-CSF receptor (Fig. 4A), neutralizing GM-CSF after IL-5W did not result in a significant change in eosinophil yield, suggesting that GM-CSF does not promote preEos differentiation (Fig. 5B). We next investigated the potential for IL-4, GM-CSF, IL-9, and IL-33 to promote preEos maturation when IL-5 was completely removed from the cultures. The addition of IL-4, GM-CSF, IL-9, and IL-33 to cultures stimulated with IL-5 for 10, 1, or 0.1 ng/ml (Fig. 4C; data not shown), suggesting that IL-5 stimulates an expression profile in preEos that is independent of the concentration of IL-5. We next evaluated the kinetic expression of IL-4 mRNA by preEos during maturation using an IL-4 fluorescent reporter (4get) mouse line (22). The absolute number of cells that express both IL-4 and Siglec-F increased after 7 d of IL-5 stimulation (Fig. 4D), and few cells that expressed Siglec-F did not express IL-4 (Fig. 4D). These data reveal that mRNA for IL-4 is expressed by both preEos and mature eosinophils and that IL-5–stimulated preEos produce and secrete multiple chemokines and cytokines, including IL-4 and IL-13.

**FIGURE 4.** IL-5 stimulation of eosinophil precursors induces cytokine expression. Normalized expression (mean ± SD) of cytokine receptors (A) in sorted eosinophil precursors (preEos) after 4 d of IL-5 stimulation is shown from a representative of three independent experiments with three wells per time point per experiment. (B) Cytokine level (mean ± SD) in the supernatants from sorted preEos after stimulation with IL-5 for 24 or 48 h is shown from a representative of three independent experiments with three wells per time point per experiment. (C) Normalized gene expression (mean ± SEM; n = 3 independent experiments) in preEos sorted from LDBM cells after 4 d of IL-5 at 10, 1, or 0.1 ng/ml is shown. (D) Absolute number (mean ± SD) of Siglec-F+ (solid line) and Siglec-F+GFP+ (dashed line) cells per well from LDBM cells cultured in IL-5 for the indicated number of days is shown from a representative of three independent experiments with three wells per time point in each condition. βc, β-chain; R, receptor.
IL-9, GM-CSF, or IL-33 to the culture media for 2 d in the absence of IL-5 did not result in enhanced preEos maturation (as measured by upregulation of CCR3 on the surface of the Siglec-F+ preEos) compared with media alone (Fig. 5C), but the frequency of preEos (Siglec-F+CCR3+) in the cultures increased with the addition of GM-CSF (Fig. 5D). Notably, the increase in preEos with GM-CSF stimulation was greater than with IL-5 (Fig. 5E), suggesting that GM-CSF promotes preEos expansion. The addition of IL-4 resulted in decreased preEos maturation compared with media alone (Fig. 5C) with an increase in the frequency of preEos (Fig. 5D, 5E), suggesting that IL-4 signaling in the absence of IL-5 results in decreased survival of the newly matured eosinophils. Indeed, the percentage of nonviable cells after 48 h of IL-4 stimulation in the absence of IL-5 was significantly greater compared with media alone (Fig. 5F). In addition, deficiency in IL-4Rα resulted in a significant reduction in the baseline frequency of mature eosinophils in the bone marrow compared with bone marrow from wild-type animals (Fig. 5G). IL-4 or IL-5 deficiency had no significant effect on baseline eosinophil production in vivo (Fig. 5G). Together, these data suggest that IL-4Rα

![Figure 5](http://www.jimmunol.org/)

**FIGURE 5.** Effects of IL-4Rα signaling on eosinophil differentiation in the absence of IL-5. (A) Eosinophil yield (mean ± SEM, n = 3 independent experiment with 3–4 wells per condition in each experiment) from wild-type (WT), IL-4–deficient (IL-4KO), and IL-4Rα–deficient (IL-4RKO) LDBM cells cultured with IL-5 continuously (solid bars) or IL-5 withdrawal (IL-5W, hatched bars) is shown. *p < 0.05. (B) Eosinophil yield (mean ± SEM, n = 3 independent experiment with 4–6 wells per condition in each experiment) from LDBM cells cultured with IL-5 continuously or IL-5W with anti–GM-CSF Ab or isotype control Ab (IgG). Absolute number [(C), mean ± SD] of mature eosinophils (CCR3+Siglec-F+), dot blots (D) showing surface CCR3 and Siglec-F expression, fold change [(E), mean ± SEM] in percentage of mature eosinophils (Siglec-F+CCR3+) and preEos (Siglec-F−CCR3−), and percentage of nonviable cells (F) from LDBM cells cultured for 4 d in IL-5 and then washed and replated in media alone (NONE) or with the indicated cytokines for an additional 2 days is shown. (C–F) are from a representative of three independent experiments with three wells for each condition in each experiment. (F) is the mean ± SEM of the three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 when compared with fold change when IL-5 is added back to culture media. (G) Frequency (mean ± SEM, n = 4 independent experiments with 3–4 mice per group per experiment) of mature eosinophils (Eos) in the whole bone marrow (WBM) of WT, IL-4KO, IL-4RKO, and IL-5KO mice is shown. *p < 0.05.
FIGURE 6. CCL3 promotes eosinophil differentiation. (A and B) Chemokine levels (mean ± SD) in the supernatants from LDBM cells stimulated with IL-5 for 5, 8, or 11 d is shown from a representative of two independent experiments with three wells per time point per experiment. *p < 0.05, ***p < 0.001, ****p < 0.0001 when compared with levels after 5 d of IL-5 stimulation. (C) Cytokine level (mean ± SD) in the supernatants from eosinophil precursors (preEos) sorted from LDBM cells cultured in IL-5 for 4 d and subsequently stimulated with IL-5 for 24 and 48 h is shown from a representative of two independent experiments with three wells per time point per experiment. ****p < 0.0001 when compared with level present after 24 h. (D) Percentage of preEos (Siglec-F+) and mature eosinophils (Siglec-F+CCR3+) with surface expression of indicated chemokine receptors in LDBM cells stimulated for 4 d with IL-5 is shown from a representative of three independent experiments. (E) Eosinophil yield (mean ± SD) from LDBM cells cultured with IL-5 continuously or IL-5W with anti-CXCL10, anti-CCL3, anti-CCL4, or isotype control Ab (Goat IgG) or no Ab is shown from a representative of three independent experiments with three wells per condition in each experiment. Absolute number [(F), mean ± SD] of mature eosinophils (Siglec-F+ CCR3+) and dot blots (G) showing surface expression of Siglec-F and CCR3 from LDBM cells cultured for 4 d in IL-5 and then (Figure legend continues)
CCL3 promotes eosinophil differentiation

In addition to cytokine expression (Fig. 3A), we also investigated IL-5–mediated expression of chemokines by cultured LDBM cells. Accumulation of a subset of chemokines (CCL2, CCL5, and CXCL10) in the culture supernatants changed significantly during IL-5–induced eosinophil differentiation (Fig. 6A), whereas protein levels of another subset of chemokines (CCL3, CCL4, CXCL2, and CXCL9) remained relatively constant throughout the culture period (Fig. 6B). To determine which chemokines are specifically expressed by preEos within the LDBM cultures, we sorted preEos and replated the purified cells to measure cytokine levels in the supernatants after 24 and 48 h of IL-5 stimulation. CCL2, CCL3, and CXCL10 protein were detected in the supernatants from purified preEos (Fig. 6C). We next evaluated expression of chemokine receptors by preEos to identify potential chemokine receptor-ligand pairs that might influence preEos maturation. Surface expression for CCR1 (receptor for CCL3), CCR5 (receptor for CCL3 and CCL4), and CXCR3 (receptor for CXCL10 and CXCL9) on preEos (Siglec-F+/CCR3−) and mature eosinophils (Siglec-F+/CCR3+) was measured by flow cytometry on cultured LDBM cells. We detected CCR1 on the surface of a subset (approximately one third) of the preEos after 4 d of IL-5 stimulation, whereas CCR5 and CXCR3 were detected on much smaller subsets of preEos (Fig. 6D). No significant expression of CCR1, CCR5, or CXCR3 was detected on the surface of mature eosinophils (Fig. 6D). Together, these data suggest that surface expression of chemokine receptors changes during IL-5–mediated eosinophil development and that preEos express ligands for chemokine receptors on their surface, which can result in developmental stage-specific responses to chemokines.

Although preEos express the receptor and ligand pairs, individually neutralizing the chemokines CCL3, CCL4, and CXCL10 resulted in no significant change in eosinophil yield following IL-5W (Fig. 6E). We next investigated the potential for CCL3, CCL4, and CXCL10 to promote preEos maturation after the removal of IL-5. Although there was no enhanced preEos maturation above media alone with the addition of CCL4 or CXCL10, adding CCL3 to the culture media after IL-5 removal modestly promoted preEos maturation greater than media alone did (Fig. 6F, 6G), suggesting that CCL3 can promote eosinophil differentiation in the absence of IL-5.

Discussion

The aim of IL-5–targeted therapy has been to reduce blood and tissue eosinophilia safely to prevent eosinophil-mediated tissue damage. In a range of eosinophil-associated disorders, anti–IL-5 treatment results in a partial reduction in tissue eosinophilia (14–18) and a greater inhibitory effect on the later eosinophil developmental stages in the bone marrow (25). Similar to observations in IL-5–deficient mice (12), IL-5–targeted treatments do not reduce homeostatic levels of tissue eosinophils (26). Together, these data highlight the presence of clinically relevant IL-5–dependent pathways that promote eosinophil development and tissue accumulation. As disease-associated eosinophilia primarily results from IL-5–mediated enhancement of eosinophil production, we aimed to identify cytokines that could promote persistent eosinophil differentiation after IL-5 is withdrawn. We demonstrate that IL-5 stimulation of LDBM cells results in expression of stage-specific subsets of cytokines and chemokines that can influence eosinophilopoiesis and survival of developing and newly formed eosinophils. We also demonstrate differences in chemokine receptor surface expression between preEos and eosinophils, suggesting that stage-specific chemokine responses could influence eosinophil development and mobilization of preEos versus eosinophils from the bone marrow and into inflamed tissues. Our study highlights an IL-5–initiated cooperative cytokine pathway that contributes to persistent eosinophil differentiation and survival even after IL-5 is completely withdrawn.

In our culture system, in which progenitor-enriched LDBM cells are stimulated with high-dose IL-5 for 4 d, we demonstrate that preEos are still capable of expanding and differentiating for at least an additional 48 h after IL-5 is removed from the culture media without the addition of any additional cytokines. This finding suggests that IL-5 stimulation initiates a developmental program that can continue in its absence. Our data further highlight that IL-5 is critical to promote the survival of the mature eosinophils that result from the differentiating preEos. In this study, we pursued the identification of cytokines and cytokine receptors that are components of the IL-5–initiated developmental program using unbiased approaches to measure cytokines produced and genes expressed by the LDBM cells. We report the IL-5–mediated gene expression profile from LDBM cells (GEO Series GSE55386). The kinetic analysis reveals an expression profile that changed as the preEos mature into eosinophils. After 4 d of IL-5 stimulation, the genes with the greatest induction of expression are granule proteins, including several eosinophil-associated ribonucleases and eosinophil peroxidase (Supplemental Table II), which is consistent with a prior study investigating IL-5–dependent changes in gene expression in an infection model (27). We focused our expression analysis on cytokines and cytokine receptors produced by the LDBM cells and investigated their ability to influence eosinophilopoiesis. We show that the level of mRNA that encode for multiple cytokine receptors, including CCR1, CCR3, and IL-5Rα, was increased in LDBM cells stimulated with IL-5 for 4 d and further increased after 10 d of IL-5 stimulation. Importantly, expression of the CCR1 ligand CCL3 was also induced by IL-5. Interestingly, we show that expression of mRNA that encodes for CXCR3 and its ligand CXCL10 peaked after 4 d of IL-5, but then declined with further IL-5–mediated differentiation. The expression analysis suggests that IL-5 stimulation of progenitor-enriched LDBM cells results in a developmental stage-specific transcriptome that ultimately leads to differential cytokine responsiveness between preEos and mature eosinophils.

We investigated the role of IL-4 in promoting preEos expansion and differentiation in the presence and absence of IL-5, and we demonstrate that IL-4 and IL-4Rα are expressed during eosinophil development from preEos to mature eosinophils. In addition, preEos express and secrete IL-13, which also signals through IL-4Rα, highlighting the potential for this signaling pathway to influence eosinophilopoiesis. Deficiency of IL-4Rα, but not IL-4, resulted in decreased eosinophil yield from LDBM cells after IL-5 was withdrawn and in decreased numbers of baseline mature eosinophils in the bone marrow, suggesting that IL-13–mediated signaling through IL-4Rα can promote preEos differentiation and expansion. Our studies also propose a cooperative signaling
pathway between IL-4Rα and IL-5Rα, as the addition of IL-4 to the culture media when IL-5 is absent results in decreased eosinophil survival.

CCR1 expression on eosinophils varies between and within human donors (28, 29), is expressed by murine eosinophils as well (30, 31) and has been shown to be important for recruitment of mature eosinophils into tissues (32, 33). In addition, the CCR1/ CCL3 axis has been shown to enhance proliferation of lineage-committed myeloid progenitors (34). In this study, we demonstrate expression of CCR1 on the surface of preEos, an eosinophil lineage-committed precursor, and further demonstrate that CCL3 activation of preEos modestly enhanced the expansion and differentiation of preEos in the absence of IL-5. Our data focus on the single eosinophil lineage and are consistent with a prior study demonstrating that CCL3 stimulation of LDBM increases the number of GM-CSF–induced colonies containing granulocytes and macrophages (34). As CCL3 (19) and preEos (5) have been noted to be in the airways of patients with atopic disorders, it is possible that activation of CCR1-expressing preEos by CCL3 in the airways induces in situ eosinophil differentiation in the inflamed tissue and contributes to the persistent tissue eosinophilia seen in asthmatics treated with anti–IL-5 (15). Thus, although the heightened preEos differentiation and expansion mediated by CCL3 is modest, we propose that it is likely clinically relevant in inflamed tissues where small numbers of eosinophils have been associated with persistent disease and clinical symptoms even after IL-5–targeted therapy (15, 35).

After the removal of IL-5 from the culture media, the percentage of LDBM-derived cells that expressed neither CCR3 nor Siglec-F increased in the cultures. We propose that the increased frequency of Siglec-F–CCR3– cells results from the combination of cessation of preEos expansion and persistence of preEos maturation via differentiation-promoting pathways, such as CCL3/CCR1 as described above. However, we cannot rule out the possibility that the removal of IL-5 results in the downregulation of Siglec-F, thereby increasing the double-negative population. Our studies also revealed a significant increase in the percentage of preEos after the addition of GM-CSF to cultures in which IL-5 had been removed, with this increase being greater than the increase in preEos observed when IL-5 was added back to the media. As the absolute number of mature eosinophils that resulted in the presence of GM-CSF was similar to media alone, GM-CSF likely strongly promotes preEos proliferation but not differentiation.

In this article, we describe a novel, eosinophil-promoting cooperative cytokine network that is initiated by IL-5 stimulation of bone marrow progenitors and that propagates eosinophilia by an autocrine mechanism in the absence of further IL-5 exposure. Importantly, expression of the mediators and receptors identified, specifically IL-4/IL-13/IL-4Rα and CCL3/CCR1, has been noted in eosinophil-associated disorders. Our data provide mechanistic insight into the potential limitations observed in solely targeting IL-5 in eosinophil-associated disorders.

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