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IL-33 promotes eosinophilia *in vivo* and antagonizes IL-5-dependent eosinophil hematopoiesis *ex vivo*

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Abstract

IL-33 is an IL-1 family cytokine that elicits IL-5-dependent eosinophilia *in vivo*. We show here that IL-33 promotes minimal eosinophil hematopoiesis via direct interactions with mouse bone marrow progenitors *ex vivo* and that it antagonizes eosinophil hematopoiesis promoted by IL-5 on SCF and Flt3L primed bone marrow progenitor cells in culture. SCF and Flt3L primed progenitors respond to IL-33 by acquiring an adherent, macrophage-like phenotype, and by releasing macrophage-associated cytokines into the culture medium. IL-33-mediated antagonism of IL-5 was reproduced in part by the addition of GM-CSF and was inhibited by the actions of neutralizing anti-GM-CSF antibody. These findings suggest that the direct actions of IL-33 on bone marrow progenitors primed with SCF and Flt3L are antagonistic to the actions of IL-5 and are mediated in part by GM-CSF.

1. Introduction

Eosinophils represent only a small fraction of the total leukocytes in peripheral blood at homeostasis. Interleukin-5 (IL-5), a cytokine produced by Th2 lymphocytes in response to asthma provocation, in atopic diseases, and during helminth infections elicits eosinophilia (reviewed in [1]). Numerous studies have documented the pivotal role played by IL-5 in promoting differentiation of eosinophils from committed hematopoietic progenitors in the bone marrow, a crucial component of this response [2]. We have used IL-5 in combination with other cytokines to generate eosinophils from bone marrow progenitors; our current protocol uses IL-5 on stem cell factor (SCF) and Flt3 Ligand (Flt3L)-stimulated unselected bone marrow cells to produce virtually pure eosinophils by day 10 to 12 of culture. We have characterized these cells extensively both phenotypically and functionally [3].

The recent identification and characterization of interleukin-33 (IL-33) has added a significant new dimension and complexity to our understanding of mechanisms promoting systemic eosinophilia. IL-33 is a member of the IL-1 cytokine family and has been

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Authorship and Conflict of Interest Statements

Kimberly D. Dyer conceived and performed the experiments and prepared the manuscript. KDD declares that there are no competing financial interests.

Caroline M. Percopo provided technical assistance and provided editorial advice on final draft of paper. CMP declares that there are no competing financial interests.

Helene F. Rosenberg provided oversight on the design and execution of the project and preparation of manuscript. HFR declares that there are no competing financial interests.

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implicated as a molecule of interest in a number of diseases including asthma, atherosclerosis, arthritis and obesity [4]. IL-33 is expressed in epithelial cells, endothelial cells, fibroblasts and adipocytes [5–7]. IL-33 is an alarmin, and modulates Th2-type proinflammatory signals upon its release from necrotic cells and/or via atypical secretory pathways [5,8]. The IL-33 receptor ST2 (also known as IL1RL1) is found primarily on Th2 cells [9], but also on basophils [10], eosinophils [11] and mast cells [12]. Recently, natural helper cell [13], nuocyte [14], and type 2 innate cell [15] responses to IL-33 have also been documented. While the biology of this cytokine remains to be completely elucidated, IL-33 contributes to the synthesis and release of IL-5 from one or more of these numerous target cells, and thereby promotes eosinophilia [16]. In addition to these activities, several direct interactions between IL-33 and eosinophils have been examined and defined. IL-33 induces eosinophil degranulation and production of superoxide [11], elicits cytokine secretion in association with phosphorylation of intracellular signaling molecules ERK and I κ B [17], augments expression of CD11b and enhanced adhesion [18], and modulates responsiveness to Siglec 8 [19].

Given these observations, our intent was to explore the direct interactions of IL-33 with bone marrow hematopoietic progenitors in order to determine whether IL-33 might promote eosinophil hematopoiesis *ex vivo* in a well-characterized bone marrow culture system.

2. Materials and Methods

2.1 Mice

Six to eight-week old wild-type BALB/c mice were purchased from Division of Cancer Technologies (DCT, Rockville, MD). Interleukin-5 deficient (IL-5^{-/-}) [20] and eosinophil-deficient Δ dblGATA mice [21], both on the BALB/c background, are maintained on site. This study was reviewed and all protocols were carried out in accordance with the NIAID Animal Care and Use Committee Guidelines (Animal Study Proposal LAD 7E).

2.2 Intraperitoneal injections, tissue collection and cell counts

IL-33 (R&D Systems) was administered via intraperitoneal injection at 0.1 μ g per mouse per day for 7 consecutive days, after which serum samples were collected from individual mice on day 8 and tissues harvested on day 9. Cell recruitment in response to IL-33 was determined by flushing the peritoneal cavity of each mouse with 10 – 12 mL of 1% BSA in PBS; the total cell number was determined by hemacytometer counts, and 50,000 cells in 100 μ L of 0.1% BSA in PBS were placed in a cytofunnel (Thermo Scientific) and centrifuged in a Shandon Cytospin 4 at 500 g for 5 minutes. The cytospin slides were stained with Diff Quik (Siemens, Newark, DE) as described [22]. Each slide was counted under a 64x oil immersion objective and eosinophils were reported as percent of total cells. Bone marrow cell suspensions were prepared from total cells from femurs and tibias flushed with RPMI 1640 (Invitrogen Life Technologies, Carlsbad, CA); red blood cells were subjected to hypotonic lysis, and the remaining cells were suspended in 0.1% BSA in PBS prior to determination of total cell number and preparation of cytospin slides. Single cell suspensions from spleen were obtained by cutting the tissue into small pieces in Hanks' Buffered saline solution supplemented with 1% fetal bovine serum (FBS; Lonza Bio Whittaker, Walkersville, MD) and 10 mM HEPES (Invitrogen). Fragments were passed through a 70 μ m strainer followed by a 40 μ m strainer. The red blood cells were subjected to hypotonic lysis, and the remaining cells were suspended in 0.1% BSA in PBS prior to determination of total cell number and preparation of cytospin slides.

2.3 Cytokines in serum and cell culture supernatants

Bone marrow eosinophil culture supernatants were collected and stored at -80°C prior to use. Macrophage colony stimulating factor (M-CSF) and granulocyte macrophage colony stimulating factor (GM-CSF) were evaluated by ELISA (R&D). Mouse cytokines evaluated by multibead assay (Bio-Rad) included: IL-1 α , IL-1 β , IL-2, -3, -4, -5, -6, -9, and -10, IL-12(p40), IL-12(p70), IL-13, IL-17A, eotaxin-1/CCL11, G-CSF, GM-CSF, IFN γ , KC, MCP-1, MIP-1 α , MIP-1 β , RANTES/CCL5 and TNF α .

2.4 *In vitro* culture of eosinophils

The method for eosinophil culture from unselected bone marrow progenitors was as previously described [3]. Briefly, the femur and tibia were flushed with RPMI 1640 (Invitrogen Life Technologies, Carlsbad, CA), the red blood cells were lysed, and the remaining cells were suspended RPMI 1640 (Invitrogen). The cells were seeded at $10^6/\text{mL}$ in 10 – 15 mL media containing RPMI 1640 with 20% FBS (Cambrex, East Rutherford, NJ), 100 IU/mL penicillin and 10 $\mu\text{g}/\text{mL}$ streptomycin (Cellgro Mediatech, Inc., Manassas, VA), 2 mM glutamine (Invitrogen), 25 mM HEPES, 1x non-essential amino acids, 1 mM sodium pyruvate (Invitrogen) and 50 μM β -mercaptoethanol (Sigma-Aldrich, St. Louis, MO), supplemented with 100 ng/mL stem-cell factor (SCF; PeproTech, Rocky Hill, NJ) and 100 ng/mL Flt3L (PeproTech); cells were maintained in this medium from day 0 to day 4. On day 4, the medium containing SCF and Flt3L was replaced with fresh medium containing 10 ng/mL recombinant mouse interleukin-5 (IL-5), 10 ng/mL recombinant mouse interleukin-33 (IL-33) or a combination of the two cytokines (R&D Systems, Minneapolis, MN). One-half of the media was replaced with fresh cytokine-containing media every other day. On day 8 the flask containing the cells was changed. In experiments in which IL-5 was supplemented with M-CSF or GM-CSF (R&D), these cytokines were used at 1 ng/mL. In experiments involving the neutralizing rat monoclonal anti-M-CSF or anti-GM-CSF antibodies and isotype controls (R&D), these reagents were added to a final concentration of 5 $\mu\text{g}/\text{mL}$ from day 4 of culture onward. The total cell number and percent eosinophils was determined in both suspension and adherent cultures as indicated.

2.5 Flow cytometric analysis

Bone marrow cells were suspended in 0.1% BSA (Sigma, St. Louis, MO) in PBS and probed with fluorescent-tagged antibody or isotype control. Each antibody was used in the presence of 0.5 μg blocking antibody, anti-CD16/CD32 (clone 2.4G2, BD Pharmingen) and Live-Dead stain (Invitrogen) and incubated for 1 hour at 4°C in the dark. After washing in 0.1% BSA in PBS, 100,000 events were collected on a LSRII flow cytometer (BD Biosciences) and the data was analyzed in FlowJo 7.5 (Tree Star, Ashland, OR). Compensation was performed in FlowJo post-collection. All analyses were performed on cells within the live gate and the data are reported as percentage of live cells. Antibodies recognizing Siglec F (BD Pharmingen #552126) and recommended isotype control (rat IgG2aK #553930) were used.

2.6 Statistical analysis

All analyses were performed in GraphPad Prism 5 (GraphPad Software, La Jolla, CA). Each dataset was examined by ANOVA followed by either Bonferroni's Multiple Comparison post-test or as indicated in the figure legends.

3. Results

3.1. Systemic interleukin-33 (IL-33) elicits eosinophilia *in vivo*

Previous studies focused on IL-33 and its receptor, ST2/IL1RL1 documented a role for this cytokine-signaling pathway in promoting synthesis and release of Th2 cytokines, resulting in blood and tissue eosinophilia [16,23]. Systemic administration of IL-33 (0.1 µg IL-33 per BALB/c mouse per day for 7 days) resulted in increased serum levels of the Th2 cytokines IL-4, IL-5 and IL-13 (Figure 1A). Total cells in the bone marrow increased from 2.3 to 4.0 × 10⁷ cells/mouse (Figure 1B) in association with a dramatic increase in percent eosinophils (5% to nearly 80%), identified with Diff-Quik stain (Figure 1C) and by flow cytometry with anti-Siglec F antibody (Figure 1D). Profound eosinophil accumulation was also noted in the peritoneal cavity and in the spleen (data not shown). IL-33 was unable to circumvent eosinophil deficiency secondary to the Δ dblGATA deletion (Figure 1C and 1D), although previous studies indicate that there are conditions under which this is possible [24]. IL-33 was also unable to induce eosinophilia in IL-5^{-/-} mice (Figure 1C and 1D) confirming the IL-5 dependence of this response.

3.2. IL-33 antagonizes IL-5-mediated eosinophil differentiation

Our protocol for generating phenotypically and functionally mature eosinophils *ex vivo* depends directly on the actions of IL-5 [3]. As shown in Figure 2, IL-33 initially promotes proliferation of the SCF and Flt3L-stimulated progenitors, but ultimately redirects IL-5-mediated eosinophil differentiation. Cells cultured in IL-33 alone or in IL-33 together with IL-5 from day 4 forward proliferate dramatically up to day 8, after which the population of cells in suspension undergoes rapid decline (Figure 2A). IL-33 alone does not support the differentiation of progenitors into eosinophils, and actually antagonizes the effects of IL-5 (Figure 2B). While the cells cultured with IL-5 alone remain predominantly in suspension and acquire eosinophilic characteristics (Figure 2C), there are relatively few eosinophils among the cells in suspension that include IL-33. Most of cells in cultures that include IL-33 become adherent, and acquire a macrophage-like phenotype (Figure 2D and 2E). The percent eosinophils in suspension and among the adherent cell populations in each culture condition (measured as Siglec F⁺ cells) are as indicated in Figure 2F; very few (< 5%) are Siglec F⁺, and most are F4/80⁺ (data not shown) as would be anticipated for bone marrow-derived macrophages. Even at peak cell population (day 8, Fig. 2A), cultures that include IL-33 alone generate comparatively few total eosinophils (Table 1, Supplemental Figure 1). Cultures that include both IL5 and IL-33 generate 3-fold fewer total eosinophils than cultures with IL-5 alone, documenting clearly both the antagonistic effect and the dominance of IL-33 in this culture system.

3.3. Cytokines Detected in Bone Marrow Culture

Numerous cytokines were detected in SCF and Flt3L-primed bone marrow progenitor cultures differentiated with IL-33 either alone or IL-33 with IL-5 (Figure 3). Interestingly, many of these cytokines are among those characterized as products of monocytes and macrophages [25–28]. However, none of these are unique lineage markers, and several are also eosinophil secretory mediators [3,29]. IL-10 and RANTES were the only mediators on the panel that were detected under all conditions utilized (Supplemental Figure 2).

3.4 GM-CSF contributes to the IL-33-mediated antagonism of IL-5

In order to explore the mechanisms via which IL-33 antagonizes IL-5 and induces macrophage development, we first examined the expression of macrophage colony stimulating factor (M-CSF) and granulocyte macrophage colony stimulating factor (GM-CSF) in culture media collected from bone marrow cells differentiated in the presence of

IL-5 and IL-33. We found that both M-CSF (Figure 4A) and GM-CSF (Figure 4B) could be detected in supernatants from cells in culture with IL-33. Expression of GM-CSF was low (< 10 pg/ml) and transient while the expression of M-CSF was more robust and sustained. We found that supplementation of SCF and Flt3L-primed progenitors grown in IL-5 with 1 ng/ml GM-CSF resulted in a drop in the percentage of eosinophils (from 97% to 24% when evaluated on day 12) although not to the level detected when cells were cultured in IL-5 and IL-33 (Figure 4C). In contrast, addition of M-CSF (1 ng/mL) to SCF and Flt3L-primed progenitors grown in IL-5 (with or without GM-CSF) had no effect on percentage eosinophils achieved. These results suggest that M-CSF was not contributing to the IL-33-mediated inhibition of eosinophil differentiation. Likewise, antibody-mediated neutralization of GM-CSF in cultures differentiated with both IL-5 and IL-33 resulted in a partial recovery of eosinophil hematopoiesis when compared to cultures provided with an equivalent amount of isotype control (Figure 4D). Neutralizing antibody directed against M-CSF had no impact on eosinophil hematopoiesis in these experiments.

4. Discussion

Among our findings, we confirmed that IL-33 administered systemically elicited profound IL-5-dependent eosinophilia [16]. Recent studies indicate that IL-33 elicits IL-5 production from sources other than traditional Th2 lymphocytes. Among these, Neill and colleagues [14] have described nuocytes, distinct non-T non-B innate effector cells. Likewise, Ikutani and colleagues [30] have defined a distinct population of innate non-T IL-5 producing cells that are crucial for promoting eosinophil recruitment to the lung tissue specifically in response to IL-33.

As IL-33 elicits eosinophilia, albeit indirectly via IL-5, and serves to activate peripheral blood eosinophils [11], our initial intent was to examine eosinophil hematopoiesis in direct response to IL-33. Stolarski and colleagues [31] examined this question, and found that c-kit (CD117, also known as the SCF receptor) - positive mouse bone marrow progenitors generated 10% eosinophils by day 5 in culture in response to either IL-5 or IL-33 alone. In contrast, we found that unselected bone marrow progenitors initially primed with SCF and Flt3L responded somewhat differently, with IL-33 antagonizing eosinophil-hematopoiesis promoted by IL-5. We include Flt3L and SCF in our culture system, as they are factors that sustain murine progenitors and promote hematopoiesis. SCF-mediated signaling through c-Kit/CD117 is absolutely crucial for normal hematopoiesis [32] and Flt3L, while dispensable for maintaining progenitors pre-transplantation [33], synergizes with SCF and promotes the growth of murine stem cells *ex vivo* [34]. As a result, we typically achieve 100 % eosinophils from SCF and Flt3L primed unselected progenitors from BALB/c mice cultured with IL-5 in conjunction with a 3 -10 fold increase in total cell number. In contrast, Ishihara and colleagues [35] found that IL-5 alone without SCF and Flt3L generated eosinophils from rat bone marrow progenitors *ex vivo*, but the cultures sustained an overall four-fold drop in cell number.

We were initially surprised to find that the addition of IL-33 to primed progenitors generated predominantly a macrophage-like phenotype, and even more so, that IL-33 would antagonize the IL-5 dependent eosinophil hematopoiesis. Several groups have reported IL-33-dependent differentiation of hematopoietic progenitors into other myeloid lineages [12,36] including dendritic cells (DCs [37]). Interestingly, IL-33-mediated generation of CD11c⁺ DCs was completely blocked by anti-GM-CSF, and was associated with no detectable levels of IL-12p70, in contrast to the findings presented here (Figure 3). In other studies, IL-33 has been implicated prominently as a factor promoting M1/M2 polarization and augmented inflammatory capacity [38–42]. At current writing, nuocytes are the only cells known to require IL-33 for development from progenitors *in vitro* [43].

5. Conclusions

In summary, we note a profound distinction between the outcome of IL-33 administration *in vivo* and the actions of IL-33 directed at SCF and Flt3L-primed bone marrow progenitors in culture. *In vivo*, IL-33 elicits the production of IL-5 leading to systemic eosinophilia. In contrast, IL-33 does not serve as a strong promoter of eosinophil growth and differentiation in an IL-5-dependent bone marrow culture system, but instead antagonizes the growth and differentiation of progenitors promoted, at least in part, via the actions of GM-CSF.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Research Highlights

- IL-33 mediated eosinophil hematopoiesis is IL-5 dependent *in vivo*
- IL-33 antagonizes the effects of IL-5 *in vitro* and inhibits IL-5 dependent eosinophil differentiation
- The autocrine release of GM-CSF in IL-33 treated cultures mediates, in part, the IL-33 inhibition of IL-5 dependent eosinophil differentiation

Figure 1. Administration of IL-33 alters Th2 serum cytokine levels in mice and promotes bone marrow eosinophilia

Mice received IL-33 (0.1 μ g/day for 7 days) via intraperitoneal injection (closed symbols) or were left untreated (open symbols); serum was collected from individual BALB/c mice on day 8; bone marrow was collected on day 9 from wild-type BALB/c, eosinophil-deficient Δ dbIGATA, and interleukin-5 gene-deleted mice. **(A)** Serum levels of IL-4, IL-5, IL-13; n = 5 – 14 mice per group. Groups were compared with the Mann-Whitney test, **p < 0.01. **(B)** Total number of bone marrow cells recovered from each mouse; **(C)** Percent eosinophils were determined by counting of Diff-Quik stained cells recovered from the bone marrow; **(D)** Cell surface expression of Siglec F was evaluated by flow cytometry. Each symbol represents data derived from a single mouse. Groups were compared with ANOVA followed by Bonferroni's multiple comparison post-test, *** p < 0.001.

Figure 2. IL-33 antagonizes IL-5-mediated eosinophil hematopoiesis *ex vivo*.

Bone marrow cells were cultured for 4 days in stem cell factor (SCF) and Flt3 ligand (Flt3L), then transferred into media containing IL-5, IL-33 or the combination of IL-5 + IL-33. **(A)** Total number of cells in suspension at various time points, day 4 and onward, $n = 3 - 6$ cultures from individual mice per time point. **(B)** Percentage eosinophils at each time point; groups were compared by ANOVA followed by Bonferroni's multiple comparison post-test, *** $p < 0.001$, representative experiment from a total of 4. **(C - E)** Cells fixed and stained with Diff-Quik; shown are cells in suspension (Su) from cultures transferred to IL-5; those in suspension from cultures transferred to IL-33; and cells adherent to the culture flask from cultures transferred to IL-33, all at day 8. Original magnification was 63x. **(F)**

Expression of Siglec F at day 8 on suspension cells (Su) and adherent cells (Ad) as indicated. Groups compared with ANOVA followed by Bonferroni's multiple comparison post-test, **** $p < 0.0001$.

Figure 3. Detection of cytokines in bone marrow progenitor cultures

Culture supernatants were collected at day 8 and evaluated for the expression of proinflammatory cytokines. Cultures with IL-5 only open bar; with IL-33 only, grey bars; with IL-5 + IL-33, black bars. Cytokines detected include: (A) IL-1 α , (B) IL-1 β , (C) IL-6, (D) IL-12p40, (E) IL-12p70, (F) G-CSF, (G) MCP-1, (H) MIP-1 α , (I) MIP-1 β (J) TNF- α (K) eotaxin-1 and (L) IL-13. Groups were compared by ANOVA followed by Bonferroni's multiple comparison post-test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; Data pooled from two experiments, $n = 7$ mice per data point.

Figure 4. GM-CSF but not M-CSF partially mediates the antagonistic effects of IL-33 on IL-5 dependent eosinophil differentiation

Cells were cultured as indicated and cell supernatants were collected and assayed by ELISA for the presence of: (A) M-CSF or (B) GM-CSF. (C) IL-5 (10 ng/ml) was supplemented with IL-33, M-CSF or GM-CSF or the combination of M-CSF and GM-CSF; percent eosinophils was determined on day 12 of culture. (D) Antibodies (5 μ g/ml) directed against either M-CSF or GM-CSF or isotype controls (IgG2a for anti-GM-CSF antibody and IgG2b for anti-M-CSF antibody) were added to IL-5 + IL-33 supplemented cultures and the percent eosinophils was determined on day 11 of culture. Groups were compared with unpaired t-test, ns = not significant; *** $p < 0.001$, $n = 4 - 7$ mice per group.

Table 1
IL-33 antagonizes eosinophil hematopoiesis promoted by IL-5

The total and percent eosinophils among cells in suspension and adherent to the culture flask at day 8 of culture were determined by Diff-Quik stain.

	Cultures Differentiated with:		
	IL-5	IL-33	IL-5 + IL-33
# cells in suspension ($\times 10^6$)	2.83 $\pm$ 0.3	7.6 $\pm$ 0.2	8.9 $\pm$ 0.7
% eosinophils	41.7 $\pm$ 1.8	0.95 $\pm$ 0.39	3.92 $\pm$ 1.02
Total eosinophils in suspension ($\times 10^6$)	1.16 $\pm$ 4.6^{**}	0.07 $\pm$ 0.29	0.34 $\pm$ 1.4
# adherent cells ($\times 10^6$)	0.24 $\pm$ 0.05	1.34 $\pm$ 0.16	1.25 $\pm$ 0.25
% eosinophils	4.0 $\pm$ 1.6	0.5 $\pm$ 0.29	1.08 $\pm$ 0.47
Total adherent eosinophils ($\times 10^6$)	0.01 $\pm$.01	0.01 $\pm$.01	0.01 $\pm$.01
Total eosinophils in culture ($\times 10^6$)	1.17 $\pm$.17^{**}	0.08 $\pm$ 0.02	0.35 $\pm$.09
Fold reduction in total eosinophils	-	14.6	3.3

^{**}
p < 0.01 by 1-way ANOVA with Bonferroni's multiple comparison post-test.