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IL-33 Activates Unprimed Murine Basophils Directly In Vitro and Induces Their In Vivo Expansion Indirectly by Promoting Hematopoietic Growth Factor Production

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IL-33, a new member of the IL-1 family, has been described as an important inducer of Th2 cytokines and mediator of inflammatory responses. In this study, we demonstrate that murine basophils sorted directly from the bone marrow, without prior exposure to IL-3 or FcεR cross-linking, respond to IL-33 alone by producing substantial amounts of histamine, IL-4, and IL-6. These cells express ST2 constitutively and generate a cytokine profile that differs from their IL-3-induced counterpart by a preferential production of IL-6. In vivo, IL-33 promotes basophil expansion in the bone marrow (BM) through an indirect mechanism of action depending on signaling through the βL chain shared by receptors for IL-3, GM-CSF, and IL-5. IL-33 can still signal through its specific βIl-3 chain in these mutant mice, which implies that it is not the unique growth-promoting mediator in this setup, but requires IL-5 and/or GM-CSF. Our results support a major role of the latter growth factor, which is readily generated by total BM cells as well as sorted basophils in response to IL-33 along with low amounts of IL-3. Furthermore, GM-CSF amplifies IL-3-induced differentiation of basophils from BM cells, whereas IL-5 that is also generated in vivo, affects neither their functions nor their growth in vitro or in vivo. In conclusion, our data provide the first evidence that IL-33 not only activates unprimed basophils directly, but also promotes their expansion in vivo through induction of GM-CSF and IL-3. The Journal of Immunology, 2009, 183: 3591–3597.

Interleukin 33 has recently been identified as the specific ligand of the orphan receptor ST2, expressed primarily on Th2 and mast cells (1–3). This novel member of the IL-1 family was initially discovered as a NF (NF-high endothelial venules) abundant expressed by endothelial cells in lymphoid tissues (4, 5). In accordance with the previously established Th2 effector functions of ST2 (6), IL-33 induced the expression of IL-4, IL-5, and IL-13 in vivo along with severe inflammatory lesions in the lung and the digestive tract with eosinophilic infiltrates, epithelial cell hyperplasia, and increased production of mucus (1). A role in allergic diseases is also supported by the increased risk of developing atopic dermatitis associated with a single nucleotide mutation resulting in enhanced ST2 expression in humans (7). It is further consistent with the constitutive expression of human IL-33 transcripts in airway epithelial cells and bronchial smooth muscles (1).

Because of their low incidence and their poor phenotypic and morphological characterization (8), the contribution of basophils to the immune response has long been neglected. This situation has changed recently since their role in orchestrating the immune response through their ability to generate rapidly a pro-Th2 cytokine profile in response to various stimuli has been established (9–13). We provided the first characterization of this basophil population in murine bone marrow (BM), where it constitutes an excellent source of pro-Th2 cytokines and histamine, in response to growth factors like IL-3 and GM-CSF (14). Upon recruitment to peripheral organs in pathological situations like nematode infection or allergic responses (9, 15, 16), these cells exert beneficial or deleterious effects, respectively, designating them as plausible targets for IL-33. Indeed, recent reports on human and murine basophils isolated from blood and derived from bone marrow cells, respectively, show that IL-33 can effectively enhance the typical biological activities of these populations (17–19). However, it is not entirely clear whether this enhancement requires priming by IL-3 or FcεR engagement, in particular in the murine experiments, currently performed in the presence of IL-3 to ensure survival.

To address this issue, we purified basophils from freshly isolated BM cells as CD49b+ c-kit+ rather than FcεR+ c-kit+ cells after depletion of NK cells to avoid activation through FcεR signaling, and assessed the effect of IL-33 in the absence of IL-3. We found that IL-33 could induce the typical basophil functions in the absence of IL-3 and generate a cytokine profile distinguished by a preferential expression of IL-6 rather than IL-4. By contrast, the basophil expansion in the BM that occurred upon in vivo treatment with IL-33 was not direct, but mediated through the induction of growth factors that we have investigated herein.

Materials and Methods

Mice and cytokines

Male and female 6- to 8-wk-old C57BL/6J, βL−/−, and βL/βIl-3−/− mice on a C57BL/6J genetic background were purchased from The Jackson

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Laboratory and maintained under pathogen-free conditions in our animal facility. MyD88<sup>−/−</sup> and TLR4<sup>−/−</sup> mice were bred in our facility on a C57BL/6J genetic background (at least 10 backcrosses). Murine Rl3-3, GM-CSF, IL-5, and IL-33 were purchased from R&D Systems. For some in vivo experiments, human Rl3-3 (112-270) was produced in Escherichia coli BL21pLysS (Novagen) and purified on Ni-NTA-agarose (Qiagen) according to the manufacturer’s instructions. Animal experiments were performed according to the recommendations of the French Institutional Committee.

In vivo treatment, cell cultures, and flow cytometry
Mice received daily i.p. injections of 4 μg of human or 1 μg of murine recombinant (rm) IL-33, GM-CSF, IL-5, IL-3, or saline for 4 or 7 days. BM and spleen cells were recovered as reported before (15) and adjusted to 10<sup>6</sup> and 10<sup>7</sup>/ml, respectively, in culture medium (MEM) supplemented with 10% horse serum (all from Life Technologies). Histamine and cytokines were measured in supernatants after a 24-h incubation with or without 10 ng/ml IL-3. For ex vivo basophil enrichment, BM cells were depleted for NK1.1<sup>+</sup> cells followed by positive selection of CD49b<sup>+</sup> cells using the RoboSep automaton (Stem Cell Technologies). This population was further sorted electronically as CD49b<sup>+</sup>/FcεRI<sup>+</sup> or CD49b<sup>+</sup>/FceRα<sup>+</sup>/FcγRI<sup>+</sup> cells using a FACSVantage cell sorter (BD Biosciences). Basophil-enriched populations were also generated from total BM cells cultured for 8 days with IL-3, as described before (20), followed by positive magnetic selection of CD49b<sup>+</sup> basophils. The same protocol was used to evaluate the effect of GM-CSF and/or IL-5 on basophil differentiation with or without IL-3. Sorted cells were >95% pure upon reanalysis. For cytokine assays, they were incubated for 24 h at a concentration of 10<sup>5</sup> cells/ml in the presence of 10 ng/ml IL-3, IL-33, or both in round-bottom 96-well plates (Falcon). The following appropriately labeled Abs were used: anti-mouse CD11b (28B), CD11b (M1/70), CD49b (Hdc2), FceRαa (MAR-1), and FceRαβ (PK136), and isotype controls (all from BD Pharmingen). FITC-conjugated anti-ST2 mAb was from BD Biosciences. Cells were acquired on a FACScanto flow cytometer (BD Biosciences) and analyzed with FlowJo software (Tree Star). IL-3, IL-4, IL-5, IL-6, and GM-CSF production was measured by ELISA (R&D Systems) and histamine was quantified by an automated continuous flow spectrophotometric technique (21).

Statistical analysis
Data are expressed as means ± SEM. Statistical difference between experimental groups was evaluated by Student’s unpaired t test. Values of p < 0.05 were considered statistically significant.

Results
IL-33 targets basophils directly to induce their typical biological activities
Knowing that basophils are most frequent in the BM (14), where they represent on average 1.24 ± 0.07% (mean ± SEM from four experiments), we first examined how total cells from this organ responded to IL-33 in terms of histamine and cytokine production compared with IL-3, their main growth and differentiation factor. As shown in Fig. 1A, a 24-h exposure to IL-33 at optimal concentrations promoted a substantial increase of histamine and IL-6 production. Compared with IL-3, it induced more IL-6, but less histamine and even less IL-4 that did not attain statistically significant levels. The preferential effect on IL-6 production occurred at all IL-33 concentrations tested as shown in the dose-response curves in comparison to different concentrations of IL-3 (supplemental Fig. 1). In support of a direct, growth factor-independent activation, IL-33 enhanced IL-6 production by BM cells that cannot respond to IL-3, IL-5, and GM-CSF because the genes encoding their common β<sub>3</sub> signaling chain as well as the IL-3-specific β<sub>3</sub> chain have been disrupted (Fig. 1B). Note that the proportion of medullary basophils was lower in these mutant mice than in their wild-type counterpart (0.6 vs 1.2%), which explains why IL-6 production was comparatively reduced and IL-4 not consistently detected (data not shown). As expected for a member of the IL-1 family, IL-33 failed to activate basophils from MyD88-deficient mice (data not shown).

To prove that histamine and cytokines were specifically produced by IL-33-stimulated basophils, we sorted this subset directly from the BM using the CD49b marker for positive magnetic selection after depletion of NK1.1<sup>+</sup> cells (>95% pure). To avoid activation by staining with the anti-FcεRI mAb, basophils were then sorted electronically as CD49b<sup>+</sup>/CD11b<sup>+</sup> cells (Fig. 2A), which overlapped completely with their CD49b<sup>+</sup>/FceRα<sup>+</sup> counterpart (Fig. 2B) and did not display c-kit, as assessed upon reanalysis. All sorted cells expressed the IL-33R ST2 constitutively at low levels and responded to IL-33 alone by concomitant production of histamine, IL-4, and, most prominently, IL-6 (Fig. 2A). The effect of IL-3 or IL-33 on this purified population was ~100-fold higher than on total BM cells, in accordance with the specific involvement of basophils. Sorting based on CD11b expression resulted in low or undetectable spontaneous activity, contrasting with purified CD49b<sup>+</sup>/FceRα<sup>+</sup> cells that produced 2–10 times more histamine and cytokines in culture medium alone, revealing their activation upon staining with anti-FceR Ab (Fig. 2B). Histamine levels were particularly increased, possibly because of partial degranulation. These FceRα-stained cells responded better to IL-3 than their CD49b<sup>+</sup>/CD11b<sup>+</sup> counterpart, while preactivation reduced the effect of IL-33 on histamine and IL-4 but not on IL-6 production (Fig. 2B). Note that ex vivo-sorted basophils generated less histamine and IL-4 in response to IL-33 than in response to IL-3 alone, while only IL-6 production added up to some extent. This decreased responsiveness was not explained by a down-regulation of IL-3R expression, which was not significantly modified in response to IL-33 (supplemental Fig. 2).

To assess the effect of previous exposure to IL-3 on the histamine and cytokine profile induced by IL-33, we compared basophils sorted ex vivo with their BM-derived basophil (BMDB)
counterpart purified from the cell population generated during 8 days of culture in the presence of IL-3. Basophils identified as CD49b+/c-kit+/cells represented on average 30%, (Fig. 3A). They were >95% pure after positive magnetic selection of the CD49b+ population and analysis of ST2 expression (the gray line corresponds to isotype control). Histamine, IL-4, and IL-6 production was measured in supernatants after a 24-h exposure to 10 ng/ml IL-3, IL-33, or a combination of both at a concentration of 10^5 cells/ml. Data are means ± SD from two separate experiments. B, Data represent a typical experiment of two performed with sorted CD49b+/FcεRIα+c-kit+basophils.

In vivo treatment with IL-33 increases the incidence of basophils

We took advantage of the experimental protocol in which the Th2 effector functions of IL-33 had been established in vivo (1) to examine the impact of this treatment on the incidence and activity of basophils in terms of IL-3-induced histamine and cytokine production. As shown in Fig. 4A, BM cells from mice having received
repeat injections of IL-33 for 7 days markedly increased their histamine and cytokine production in response to IL-3 compared with saline controls. A similar enhancement occurred in TLR4-deficient mice which ruled out the involvement of contaminating LPS (supplemental Fig. 3).

Knowing that basophils are the only BM population responding to IL-3 by a concomitant increase of histamine, IL-4, and IL-6 production, we examined whether the increased biological activity coincided with basophil expansion in the BM. As shown in Fig. 4B, the incidence of this subset identified as CD49b<sup>FcγRIα</sup><sup>-/H11001</sup><sup>+</sup>/H9255<sup>-/H9251</sup><sup>+</sup> c-kit⁻ cells was significantly higher in mice having received IL-33, as was the total basophil count, notwithstanding the decrease of total cells per femur (11.33 ± 0.64 x 10⁶ vs 18.92 ± 1.2 x 10⁶ in controls; n = 12; p < 0.001) due to the development of splenomegaly. Basophil activities were also enhanced in spleens of mice having received IL-33, although less markedly, in accordance with a smaller rise in basophil frequencies that could not be assessed reliably by FACS analysis (supplemental Fig. 4).

Increased responsiveness as well as basophil expansion in vivo failed to occur in mice lacking the β<sub>2</sub> receptor chain required for IL-3, IL-5, and GM-CSF signal transduction (Fig. 5, A and B), which ruled out a direct effect of IL-33. In these mice, IL-3 remains fully functional since it can still signal through its specific β<sub>2</sub>-chain to induce normal histamine and cytokine production in

**FIGURE 4.** In vivo treatment with IL-33 increases the biological activity and incidence of basophils in the BM. A, Total BM cells were recovered 15 h after the last of seven daily injections of 1 μg of mrIL-33 per mouse and incubated for 24 h in culture medium or IL-3 (10 ng/ml) at a concentration of 2.5 x 10⁶ cells/ml. Supernatants were then collected and assayed for histamine, IL-4, and IL-6. Histograms represent means ± SEM from 7 to 13 mice. *p < 0.05 and **p < 0.001. B, The proportion of basophils in the BM of mice having received saline or IL-33 was determined by double staining with anti-FcεRIα and anti-CD49b Abs as illustrated in the right panel and the number of basophils per femur was calculated from total BM cell counts. Data are means ± SEM from 7 to 13 mice. **p < 0.001.

**FIGURE 5.** Basophil counts and activities are not increased after in vivo treatment with IL-33 in mice lacking the β<sub>2</sub> receptor chain. A, Total BM cells were recovered 15 h after the last treatment from WT and mutant mice after four daily injections of 1 μg of mrIL-33 per mouse as compared with saline-injected controls. They were incubated for 24 h in culture medium or IL-3 (10 ng/ml) at a concentration of 2.5 x 10⁶ cells/ml. Histamine, IL-4, and IL-6 were then determined in supernatants. Data represent means ± SD from two individual mice. B, The proportion of basophils in the BM of mice having received saline or IL-33 was established by double staining with anti-FcεRIα and anti-CD49b Abs and the number of basophils per femur was calculated from total BM cell counts. Data are means ± SD from two individual mice. C, Generation of basophil-enriched populations from BM cells recovered from wild-type (WT) and mutant mice after 8 days of culture with IL-3 (10 ng/ml). A typical experiment is shown.
total BM cells as well as basophil differentiation (Fig. 5C). Consequently, IL-33-induced endogenous IL-3 cannot support in vivo expansion of basophils per se without the contribution of IL-5 and/or GM-CSF, which are also responsible for the splenomegaly that does no longer occur in \( \beta_{5} \)-deficient mice (data not shown).

GM-CSF is the most likely mediator of basophil expansion in vivo, as it mimicked to some extent the effect of IL-33 in terms of responsiveness to IL-3 and basophil expansion after four daily injections (Fig. 6, A and B), while IL-5 had no such effect (data not shown). Furthermore, total BM cells could effectively respond to IL-33 by producing GM-CSF (114 pg/10^6 BM cells) that was mainly generated by basophils, as assessed by an \( \sim \)100-fold increase after sorting (Fig. 6C). Note that this purified population also secreted IL-3 at significant levels when stimulated with IL-33, conversely to IL-5. The latter was easily detected in the serum of mice after in vivo treatment with IL-33, conversely to GM-CSF or IL-3 (2301 pg/ml after three repeat injections of IL-33).

IL-5, GM-CSF, or IL-33 did not support differentiation of basophils from BM cells in vitro, whether separately or in combination. However, unlike IL-5, both GM-CSF and IL-33 increased the overall yield of basophils when added to cultures set up in IL-3 (Fig. 7A). The fact that this treatment did not affect their incidence indicates that other myeloid populations have likewise been expanded. Basophils are short-lived cells that cease to proliferate and die once they are fully differentiated. Increased survival during a 5-day incubation of sorted cells occurred not only in the presence of IL-3, their main survival factor, but also in cultures supplemented with GM-CSF plus IL-33 or, more moderately, with each factor alone, as shown in Fig. 7B. Yet, even though this prolonged life span might contribute to the increase of basophil counts in mice having received IL-33, it is certainly not its main cause. It can therefore be concluded that IL-33-induced basophil expansion in vivo is most likely due to the combined growth-promoting activity

**FIGURE 6.** GM-CSF mimics the effect of IL-33 on basophil functions and expansion. A, Total BM cells were recovered 15 h after the last treatment from wild-type and mutant mice having received four daily injections of 1 \( \mu \)g of mrIL-33 or mrGM-CSF compared with saline-injected controls. They were incubated in culture medium with or without IL-3 (10 ng/ml) for 24 h when supernatants were collected for histamine, IL-4, and IL-6 assays. Data are means ± SEM from five mice. \( * \), \( p < 0.05 \) and \( ** \), \( p < 0.001 \). B, The proportion of basophils in the BM of mice having received saline, IL-33, or GM-CSF was established by double staining with anti-FcεRIα and anti-CD49b Abs and the number of basophils per femur was calculated from total BM cell counts. Data are means ± SEM from five mice. \( * \), \( p < 0.05 \) and \( ** \), \( p < 0.001 \). C, Basophils were sorted electronically as CD49b^+ CD11b^+ cells after magnetic depletion of NK1.1^+ cells and positive selection of CD49b^+ cells and incubated at a concentration of 10^5 cells/ml for 24 h in culture medium with or without IL-33. GM-CSF and IL-3 were not detected in supernatants from unstimulated controls.

**FIGURE 7.** GM-CSF and IL-33 increase basophil yield and survival. A, Basophils generated from 4 \( \times \) 10^6 total BM cells in response to the growth factors were quantified after 8 days of culture as FcεRIα^- c-kit^- cells. The percentage of basophils among total cells was not modified by the presence of GM-CSF or IL-33, relative to IL-3 alone. A typical experiment is shown. B, The survival of basophils sorted ex vivo from BM cells was assessed by trypan blue exclusion after 5 days of incubation with different growth factors compared with cells incubated in culture medium alone that were all dead at this time point. Date represent a typical experiment quantified.
of GM-CSF in combination with low levels of IL-3. Both cytokines can be produced in an autocrine fashion by basophils themselves, while IL-5 that is also generated in response to IL-33 in vivo, derives from a different cell population.

Discussion

The recently reported pro-Th2 effector functions of IL-33 prompted us to investigate whether murine basophils, which are widely acknowledged for their role in the initiation of Th2 polarization, are targeted by this cytokine. We found that all freshly isolated mediatory basophils that have been activated neither by FceR engagement nor by IL-3 expressed the receptor ST2 at low levels and produced substantial amounts of histamine, IL-4, and IL-6 in response to IL-33. Even though the cytokines generated were the same as those induced by IL-3, their profile differed considerably, as IL-33 privileged the production of IL-6, while IL-3 induced IL-4 and histamine more efficiently at all concentrations tested. This differential effect is not surprising knowing that the signaling pathways triggered by either cytokine in human basophils have been shown to be distinct (22). It suggests that through basophil activation IL-33 could affect preferentially the inflammatory Th17 axis of the immune response (23), relative to the pro-Th2 effect mediated by IL-3 (24).

It has been reported that several myeloid cell populations involved in inflammatory diseases and Th2-type immune responses, such as mast cells (25, 26), eosinophils (22, 27, 28), and basophils (17–19, 25), are targeted by IL-33 to enhance their proinflammatory activities. However, it is not entirely clear whether these cells, namely, murine basophils, respond to this cytokine without priming by growth factors or FceR cross-linking or whether the function of IL-33 consists primarily in providing a costimulatory signal to amplify both pro-Th2 and pro-Th1 immune responses (18). We found that in basophils sorted ex vivo, histamine and IL-4 production in response to IL-3 plus IL-33 was hampered rather than amplified, relative to IL-3 alone and only partially additive in BM-derived basophils, suggesting that these cytokines trigger opposing signaling pathways. Previous studies (17) were performed exclusively with BMDB cocultured with IL-3, which might differ from freshly isolated cells as to their state of activation and maturation. It is also noteworthy that the amounts of IL-4 generated in response to IL-33 were quite low relative to those induced by IL-3, when BMDB were magnetically sorted as CD49b+ cells, but increased markedly upon FceR staining, which induced a significant IL-4 production on its own. This observation is at variance with previous data showing that binding of the high-affinity mAb MAR-1 to FceRIα does not promote IL-4 production by basophils in vitro (13). Thus, even though basophils are directly targeted by IL-33, their responsiveness appears to be quite variable, depending on the initial stimuli encountered in the microenvironment and the stage of maturation. This result is consistent with the proposal that IL-33 behaves like an "alarmin" or endogenous danger signal initiating the immune response (29). It is supported by the finding that other cells contributing to the innate immune response, like NK and invariant NKT cells, are targeted by IL-33 as a costimulatory factor to promote both Th1 and Th2 responses (18, 30).

In vivo treatment with IL-33 increased the incidence as well as the number of basophils per femur, notwithstanding the reduction of total cell counts consecutive to the development of splenomegaly. This effect was not directly mediated by IL-33 since it failed to occur in mice deficient for the β3 chain shared by IL-3, IL-5, and GM-CSF receptors. These mutants conserve the IL-3-specific β-chain that is fully functional as a signal transducer of histamine and cytokine induction as well as basophil differentiation. IL-33-induced IL-3 can therefore not account for the in vivo expansion of this population per se, but depends on the contribution of IL-5 and/or GM-CSF. However, neither cytokine alone or in combination supported basophil differentiation from BM cells in vitro in the absence of IL-3, although IL-33 and GM-CSF could both amplify its growth-promoting activity and prolong the survival of purified basophils. IL-5 that was likewise generated and easily detected in serum after treatment with IL-33 had no such effect, making GM-CSF the most likely mediator for their in vivo expansion, together with low amounts of endogenous IL-3. This assumption is supported not only by previous evidence for a role of GM-CSF in basophil priming (31), but also by its effective production by BM cells stimulated with IL-33. Moreover, it can act in an autocrine fashion since it is secreted by purified basophils, which produce also IL-3 but no IL-5 in response to IL-33. This observation does not exclude other cells involved in innate immune responses as potential sources of GM-CSF and IL-3, such as invariant NKT cells that respond to IL-33 and can produce both growth factors upon stimulation with their cognate ligand e-galactosidase ceramide (32).

In conclusion, our study provides the first evidence that IL-33 targets murine basophils directly to induce their typical biological activities with a preferential effect on IL-6 rather than IL-4 production. On the other hand, it promotes basophil expansion in vivo indirectly by inducing GM-CSF and IL-3 production by basophils themselves and possibly by other innate immune cells. These findings strengthen the notion that this rare neglected population is important in early events leading from innate to adaptive immune responses through their capacity to respond directly and rapidly to a variety of stimuli (33) by generating a cytokine profile that will determine the final outcome of the immune response.

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Disclosures

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References


