INTERLEUKIN-33 PROMOTES THE PROLIFERATION OF MOUSE MAST CELLS THROUGH ST2/MyD88 AND p38 MAPK-DEPENDENT AND Kit-INDEPENDENT PATHWAYS

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Interleukin-33 (IL-33), a member of the IL-1 cytokine family, is emerging as a new modulator of immune and inflammatory responses. Although IL-33 and its associated receptor ST2 are reportedly expressed in mast cells (MCs), the precise role of IL-33 in modulating MC function has not been determined. In the present studies, we explored IL-33 effects on MCs in vivo and in vitro. IL-33 increased the number of peritoneal and skin MCs in vivo. IL-33 also resulted in increased proliferation of MCs in vitro, as explored by WST assay. Cell cycle analysis further confirmed this result by showing increased G2 cell populations in MCs stimulated with IL-33. We found that IL-33-mediated MC proliferation requires ST2 and MyD88, is independent of Kit, and is mediated through a p38 MAPK-dependent pathway. IL-33 did not induce degranulation and was not cytotoxic for MCs. This novel mechanism for increasing MC proliferation and numbers further defines the role of IL-33 in MC-dependent diseases including allergies and may help to develop novel approaches for the treatment of these disorders.

Mast cells (MCs) are major effector cells in both innate and adaptive immunity (1). Most prominently, MCs are involved in the pathophysiology of allergic diseases, such as allergic rhinitis and asthma (2). MCs are tissue resident cells and may survive for years (3). Their location in the skin, gastrointestinal tract and lung places them strategically as a first line of defence against invading pathogens.

Marked increases in tissue MC numbers have been reported in murine models of several allergic conditions and asthma (4). For example, skin MCs are reportedly increased in murine oxazolone-induced dermatitis (5), and higher numbers of MCs in the bronchial epithelium and airway smooth muscle, associated with pulmonary inflammation, are found in mouse models of allergic airway inflammation (6). Increased MC numbers have also been reported in the bronchi of asthmatic patients after allergen exposure (7). MC hyperplasia seen in these conditions is thought to result, at least in part, from increased proliferation (8). However, the precise mechanisms leading to the increased MC numbers in these settings are still largely elusive and a number of different mechanisms may be involved.

IL-33 is a recently discovered cytokine of the IL-1 family, which is a ligand for T1/ST2/IL-1R4 (9). T1/ST2/IL-1R4 is preferentially expressed on Th2 cells and MCs (10). IL-33 can promote the production of IL-4 (11), IL-5 and IL-13 by Th2 cells and induce NF-κB phosphorylation and MAP kinase activation in MCs (9, 12). IL-33 is reported to have a strong association with MCs in different allergic diseases (13) and may play a critical role in the regulation of MC proliferation. Recently, IL-33 was described to

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be up regulated in allergic conditions and to activate MCs (14). Interestingly, increases in MC numbers have repeatedly been linked to MC activation by IgE. For example, in pulmonary inflammation induced by allergen challenge in mice, involvement of IgE was shown to be responsible for the subsequent increase in MCs in the lungs (6). A recent report suggests that MCs themselves can secrete IL-33 after IgE cross-linking (11) and act as target cells for IL-33. We, therefore, hypothesized that IL-33 contributes to the accumulation of MCs at sites of inflammation by inducing the proliferation of MCs, and we tested this hypothesis by using in vivo and in vitro mouse models.

MATERIALS AND METHODS

Assessment of IL-33 effects on MC numbers in vivo

Mouse rIL-33 (500 ng in 20 µL PBS, Enzo Life Sciences AG, Lausen, Switzerland) or PBS was injected intradermally (once daily) into the ear pinna of mice on three successive days. Mice were sacrificed on day 4, and the ears were excised, fixed in buffered formalin, and processed into paraffin-embedded sections. Specimens were stained with Giemsa stain to identify MCs. Cutaneous MC numbers were assessed by quantitative morphometry in 1 mm² areas. Images were captured and analyzed using the AxioVision 4.6 image processing software (Carl Zeiss).

To explore whether IL-33 treatment leads to increased numbers of peritoneal MCs, wild-type C57BL/6 mice were injected intraperitoneally daily with PBS or 500 ng rIL-33 for 3 days. The mice were sacrificed on day 4, and peritoneal lavage was collected. Numbers of peritoneal MCs were analyzed by flow cytometry by using MACS Quant (Miltenyi). The following antibodies were used in this study: FITC conjugated anti-mouse FcεRI (MAR-1) and anti-mouse CD117 (Kit) mAb 2B8 or FITC-conjugated rat IgG2b isotype control (both from BD Pharmingen, San Diego, USA), FITC-conjugated anti-mouse FcεRI-α mAb MAR-1, or FITC conjugated Armenian hamster IgG isotype control (eBioscience, Frankfurt, Germany). MCs (1x10⁶ cells/ml) were treated for 24 or 96 h with different concentrations of IL-33. In separate experiments to investigate the involvement of distinct signalling pathways, MCs were treated with the JNK inhibitor SP600125 (1 µM, Sigma), the ERK inhibitor PD 98059 (1 µM, Calbiochem, Germany) or the p38 MAPK inhibitor SB203580 (10 µM, Calbiochem) in the absence and presence of IL-33 for 24 h to 96 h.

Mast cell proliferation

BMMCs (1x10⁶ cells/ml) were cultured in RPMI1640 medium containing 10% FCS and rIL-33 (10-100 ng/ml) at 37°C for 24, 48, 72, or 96 h. For monitoring cell proliferation, a colorimetric assay (WST-1, Roche Diagnostics, Mannheim, Germany) was used. 20,000 cells in 200 µl culture medium per well were seeded in 96-well plates and treated for 24 to 96 h with 10, 50, or 100 ng/ml rIL-33, before 10 µl WST-1 reagent was added, followed by incubation of the cells at 37°C for 1 to 3 h until colour changes were seen (17). The optical density of samples was determined at 450 nm in an ELISA plate reader (Dynatech Laboratories). For background correction, the values of untreated controls were subtracted.

Quantification of apoptosis and proliferation by cell cycle analysis

BMMCs (1x10⁶ cells/ml) were cultured in RPMI1640 medium containing 10% FCS and rIL-33 (10-100 ng/ml) at 37°C for 24, 48, 72, or 96 h. For quantification of apoptosis and proliferation, cell cycle analyses were carried out using propidium iodide staining (17). BMMCs were harvested after treatment with IL-33 at different time points (24 to 96 h), stained with PBS buffer containing Triton-X 100, sodium citrate and propidium iodide (200 mg/ml, Sigma), centrifuged, with PBS
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and analyzed by flow cytometry in a FACS (BD FACS Calibur).

Mast cell activation and cytokine measurement

For detection of the granular enzyme β-hexosaminidase, an enzymatic colorimetric assay was used as described previously (18). Briefly, 60 μl of supernatant was transferred to a 96-well plate and mixed with an equal volume of substrate solution (7.5 mM p-nitrophenyl-N-acetyl-β-D-glucosaminide dissolved in 80 mM citric acid, pH 4.5). The mixture was incubated on a rocker platform for 2 h at 37°C. After incubation, 120 μl of glycine (0.2 M, pH 10.7) was added to each well, and the absorbance at 405 and 490 nm was measured. Levels of IL-6 in the supernatants were measured by ELISA according to the manufacturer’s instructions (eBioscience).

Mast cell viability and cytotoxicity

BMMCs were treated with different concentrations of IL-33 for 24 to 96 h. Cells were re-suspended in 200 μl medium without FCS and penicillin/streptomycin, and 5 μM calcine-AM was added. The cells were then incubated for 1 h (5% CO₂, 37°C), washed and resuspended in PBS, and measured immediately by flow cytometry. Cytotoxicity was determined by measuring LDH activity following the assay manufacturer’s instructions (Roche Diagnostics, Basel, Switzerland).

Statistical analyses

All results are expressed as means ± standard errors (SEM) and all statistical analyses were performed using Student’s t-test. *P* < 0.05 was considered statistically significant.

RESULTS

IL-33 treatment increases mast cell numbers in the skin and peritoneum of mice

When we assessed the effects of IL-33 on MC numbers in vivo, we found that intradermal injections of IL-33 markedly increased the numbers of skin MCs at the site of treatment as compared to vehicle injected sites (87±6 vs 163±7 / mm², *P*<0.01, Fig. 1 A and B). Also, intraperitoneal injections of IL-33 increased peritoneal MC numbers assessed by cytometry (Kimura stain, data not shown) and by FACS staining for Kit and FcεR1, albeit not statistically significantly (Fig. 1C). Together, these data suggest that IL-33 treatment can increase murine MC numbers in vivo. It is possible that IL-33 may result in the recruitment of MC progenitors and the migration of mast cells to the peritoneum and their proliferation and that increases in mast cell numbers are due to a combination of these effects. When peritoneal MCs were tested for proliferation in response to IL-33, we found that IL-33 induces significant proliferation at concentrations ranging from 10 to 100 ng/ml (Fig. 1D).

**IL-33 induces proliferation, but not degranulation, of mast cells in vitro**

To further explore the effect of IL-33 on MC proliferation, we assessed bone marrow-derived MCs and found that they also showed significant dose dependent and sustained proliferation in response to IL-33 (Fig. 2A). IL-33 did not induce MC degranulation as assessed by β-hexosaminidase release assays (Fig. 2B). Furthermore, IL-33 did not exhibit cytotoxic effects, as determined by lactate dehydrogenase (LDH) release assays (Fig. 2C) and Calcein AM staining (to assess cell viability) followed by subsequent flow cytometric analysis at 24 h to 96 h of treatment (Fig. 2D).

**IL-33 shifts cell cycling to proliferation, not apoptosis**

To delineate the mechanisms of the pro-proliferative effects of IL-33 on MCs, we next examined the effects of IL-33 on cell cycling by propidium iodide (PI) nuclear staining and subsequent flow cytometry (Fig. 3A). IL-33 prominently enhanced the G2 population in BMMCs without affecting the sG1 population within the first 24 h of treatment and for up to 96 h (Fig. 3 B-C). G2 populations were increased 2.8, 3.5, and 4-fold after treatment with 10, 50, and 100 ng/ml, respectively, at 24 hr. Similar effects were observed after 48, 72, and 96 h, indicating that IL-33 induces sustained DNA replication (Fig. 3B). There was no significant increase of sub-G1 cell populations, i.e. no enhancement of apoptosis in BMMCs in response to IL-33 treatment as compared with vehicle-treated cells (Fig. 3C).

**IL-33-mediated mast cell proliferation depends on ST2 and MyD88**

Since IL-33 is reported to signal through ST2 and MyD88, we investigated the involvement of this pathway in IL-33-mediated MC proliferation.
**Fig. 1.** IL-33 treatment induces mast cell proliferation in the skin as well as in the peritoneum. A, B) increased numbers of skin mast cells (arrows in A) in ears of mice treated with IL-33 (left, 500 ng daily for 3 days, intradermal injections) or vehicle (PBS). Sections shown in (A) are stained with Giemsa (magnification 200X, scale bar 50 μm). C) Peritoneal MCs as assessed by FACS (Kit⁺ and FcεRI⁺) on day 4 after treatment with IL-33 (500 ng, i.p. daily for three days, n=3 for each group) or vehicle. The data shown are from three independent experiments with 3 mice per group and experiment. D) Peritoneal MC proliferation was explored by use of the WST-1 assay, and values are expressed as fold increase vs vehicle control. Cells were harvested at different time points e.g. 24, 48, 72 or 96 h after stimulation. **p < 0.01, ***p < 0.001 indicates significant differences from the respective controls. Results are represented as the means ± SEM of 3 independent experiments.
Kit is not needed for IL-33-induced mast cell proliferation

To explore whether IL-33-induced MC proliferation involves Kit, the receptor for stem cell factor, we used BMMCs from Kit-deficient Kit−/−/W−/− mice which express ST2 but no functional Kit (19). Kit-deficient BMMCs showed IL-33-induced increases in proliferation that were similar to those of control MCs, indicating that Kit is not critically involved in IL-33-induced MC proliferation (Fig. 5A). To confirm this, we used the specific Kit inhibitor imatinib, which completely abolished MC proliferation induced by SCF but had no effect on IL-33-mediated MC proliferation (Fig. 5B). Imatinib had no effect on IL-33-induced IL-6 production (Fig. 5C). On the other hand, the combination of IL-33
This study shows that IL-33 can induce murine MC proliferation in vivo as well as in in vitro via an ST2/MyD88/p38 MAPK-dependent and Kit-independent pathway. In contrast, IL-33 did not induce MC degranulation or affect MC viability.

Treatment with IL-33 increases MC numbers in the skin as well as the peritoneum, which is in support of earlier reports. For example, Hueber et al. (20) observed increased MC numbers in the ears of mice after intradermal injection of IL-33 on alternate days up to day 16. In contrast, Enoksson
migration of MC precursors or MCs to site of IL-33 treatment and increased differentiation of MC precursors at such sites. Our WST-1 results indicate, to our knowledge for the first time, that IL-33 can significantly enhance MC proliferation in vitro, which is confirmed by the results of cell cycle analyses. IL-33 increases proliferating G2 populations in vehicle treated cells were 0.53, 0.52, 0.39 and 0.38 at 24 h, 48 h, 72 h and 96 h, respectively. The mean values for sG1 populations in vehicle treated cells were 0.45, 0.69, 1.5, and 0.90 at 24 h, 48 h, 72 h and 96 h, respectively. D) BMMCs from MyD88-deficient mice do not proliferate after treatment with IL-33. Fold increases of G2 (E) and sG1 populations (F) of IL-33 treated MCs from MyD88−/− mice. The mean values for G2 populations in vehicle treated cells were 0.29, 0.20, 0.19, and 0.23 at 24 h, 48 h, 72 h, and 96 h, respectively. The mean values for sG1 populations in vehicle treated cells were 1.44, 4.50, 3.8, and 2.5 at 24 h, 48 h, 72 h and 96 h, respectively. Pooled results are presented as the means ± SEM of 3 independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001.

et al. (21) did not observe increased numbers of peritoneal MCs 6 h after treatment with IL-33, which is owed most likely, to the short duration of IL-33 treatment. Our findings suggest that IL-33 could play an important role in the increase of MC numbers and may be involved in the pathogenesis of diseases that are characterized by up regulation of IL-33 such as psoriasis (20) and atopic dermatitis (22). These findings suggest that the increases in MC numbers seen after intradermal and intraperitoneal injections of IL-33 are due to the induction of proliferation by direct effects of IL-33 on local MC populations. However, other effects of IL-33 on MCs, directly or indirectly, could contribute to the accumulation of MCs after IL-33 injections, including increased

Fig. 4. IL-33 promotes proliferation of MCs through a ST2- and MyD88-dependent pathway. A) Bone marrow mast cells from ST2-knockout mice treated with IL-33 do not show proliferation as determined by the WST-1 assay. Fold increases of G2 populations (B) and sG1 populations (C) after treatment with IL-33 or vehicle. The mean values for G2 populations in vehicle treated cells were 0.53, 0.52, 0.39 and 0.38 at 24 h, 48 h, 72 h and 96 h, respectively. The mean values for sG1 populations in vehicle treated cells were 0.45, 0.69, 1.5, and 0.90 at 24 h, 48 h, 72 h and 96 h, respectively. D) BMMCs from MyD88-deficient mice do not proliferate after treatment with IL-33. Fold increases of G2 (E) and sG1 populations (F) of IL-33 treated MCs from MyD88−/− mice. The mean values for G2 populations in vehicle treated cells were 0.29, 0.20, 0.19, and 0.23 at 24 h, 48 h, 72 h, and 96 h, respectively. The mean values for sG1 populations in vehicle treated cells were 1.44, 4.50, 3.8, and 2.5 at 24 h, 48 h, 72 h and 96 h, respectively. Pooled results are presented as the means ± SEM of 3 independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001.
IL-33 mediates its known effects through a heterodimeric receptor composed of IL-33R (T1/ST2) and IL1RAP (24). Our results indicate that this receptor is also involved in IL-33-induced MC proliferation. A BMMC from Kit<sup>W-sh</sup>/Kit<sup>W-sh</sup> mice show normal proliferation in response to stimulation with increasing concentrations (10-100 ng/ml) of IL-33 as determined by the WST-1 assay. The Kit inhibitor Imatinib does not affect IL-33 induced MC proliferation (B) or cytokine production (C). D) SCF and IL-33 act synergistically to enhance MC proliferation. BMMCs were incubated for 24 h to 96 h with the indicated concentrations of SCF (2.5 ng/ml), in the absence or presence of IL-33 (100 ng/ml). MC proliferation was determined by use of the WST-1 assay. Results are presented as the means ± SEMs for 3 independent experiments and values are expressed as fold change vs control. *p < 0.05, **p < 0.01, ***p < 0.001.

presence of SCF during BMMC generation, the absence of FCS in the medium during stimulation or the use of different detection methods (CFSE vs WST-1/PI).
differences in the experimental set-up and conditions (such as concentration of IL-33). We also found that IL-33-induced MC proliferation was unaltered in the absence of Kit. Genetically Kit-deficient MCs from Kit<sup>W-sh</sup> / Kit<sup>W-sh</sup> mice as well as MCs treated with the Kit-inhibitor imatinib showed normal proliferation in response to IL-33 treatment. On the other hand, SCF and IL-33 appear to induce MC proliferation in synergy as the stimulation of MCs with a combination of both induced more proliferation than either one alone in normal BMMCs.

Finally, we explored the involvement and importance of distinct IL-33 related signalling molecules, i.e. JNK, ERK1/2, and p38 MAPK, in the induction of MCs proliferation by IL-33. Only the inhibition of p38 MAPK, but not of JNK or ERK1/2, significantly inhibited MC proliferation in response to IL-33. Previous reports had indicated that all of these three signalling molecules are involved in the induction of IL-6 (11, 25, 26) and IL-13 production in BMMCs by IL-33 (27). Taken together, our results suggest that IL-33 may contribute to the accumulation of MCs in diseases characterized by...
high local levels of this cytokine, such as asthma (28, 29) and allergic rhinitis (30).

One must keep in mind, however, that the effects of IL-33 on human MC proliferation have not yet been investigated. IL-33 has been shown to prolong the survival of human cord blood MCs (31), and just like in murine MCs (26, 32), IL-33 does not induce the release of histamine and PGD$_2$ in human MCs (31, 33). Thus it appears likely that IL-33 also makes human MCs proliferate, which could be important for the pathogenesis of chronic inflammatory diseases.

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