Human basophils and eosinophils are the direct target leukocytes of the novel IL-1 family member IL-33

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Introduction

Cytokines of the interleukin (IL)–1 family are major proinflammatory and immunoregulatory mediators that act through receptors of the Toll-like/IL-1-receptor (TLR/IL-1R) superfamily. Due to homologous intracellular Toll/interleukin-1 receptor (TIR) domains, IL-1 family members and TLR-ligands activate very similar signaling pathways leading to NF-κB- and MAPK-activation. IL-1α and IL-1β, the prototypic family members, are generated in response to exogenous and endogenous danger signals and act as chief inflammatory mediators in many inflammatory conditions. Recent studies indicate that IL-1 may also participate in inflammatory pathologies and auto-immune diseases involving Th1-type T-helper cells. By contrast, IL-18 is best known for its role in Th1-type immune responses because it strongly amplifies IFN-γ production in natural killer (NK) cells and Th1 cells in synergy with IL-12. However, several lines of evidence mainly derived from mouse models indicate that IL-18 can also play a role in allergic diseases and defense against helminths. For example, in the absence of IFN-γ signaling, IL-18 increases immunoglobulin E (IgE) levels and promotes a Th2-type pathology. It has been suggested that this is due to the antigen-independent action of IL-18 on cells of the “innate allergic response,” basophils and mast cells. Bone marrow-derived c-Kit+/FceRI+ mouse “basophil-like” cells express IL-18 receptors (IL-18R), and IL-18 induces IL-4 and IL-13 expression in an antigen-independent manner as efficiently as IL-3. In the human system, IL-18 primes basophilic KU812 cells for enhanced leukotriene C4 (LTC4) production. Furthermore, a recent screen of novel CD antigens revealed that blood basophils express IL-18R and IL-18R-accessory protein (IL-18Rap), indicating that IL-18 may also act on human basophils. A soluble form of an IL-1R family member ST2 (sST2; also called T1) has been cloned many years ago. sST2 is formed by many cells and increased sST2 levels are found in inflammatory conditions, including allergic asthma. The expression of transmembrane ST2-receptor (ST2L), however, is largely restricted to cells of hematopoietic origin. ST2L is expressed by mouse bone marrow-derived mast cells (BMMCs) and is a stable marker of mouse, but not human, Th2-lymphocytes. Although the interpretations were sometimes conflicting, most studies indicate an important contribution of ST2 in Th2-type immune responses and allergic inflammation. The biology of ST2 is further complicated by a possible direct antiinflammatory action of sST2, and by the fact that the TIR domain of ST2L appears to be a negative regulator of IL-1 and TLR signaling. Research on ST2 strongly gained momentum by the recent discovery of its ligand, the novel IL-1 family member IL-33. Like IL-1α, the human IL-33 precursor (proIL-33) is a nuclear protein and was first identified as a gene (termed NF-HEV) differentially expressed by high endothelial venules of lymphatic tissues. Like IL-1β and IL-18, human and mouse proIL-33 can be cleaved by caspase-1, at least in vitro. Infusion of IL-33 in mice leads to increased IL-4, IL-5 and IL-13 expression, high IgE levels, eosinophilia, and mucosal pathologies in vivo. After binding to ST2 and recruitment of the IL-1Rap as accessory receptors, IL-33 activates the classical TIR/IRAK signaling pathway. It induces the production of proinflammatory cytokines.
and IL-13 in mouse BMMCs, and enhances the release of anti-CD3/CD28-induced IL-5 and IL-13 (but not IL-4) by in vitro polarized Th2 cell lines.21,23-27

Only little is known about the biology of IL-33 and ST2 in the human system. A role of IL-33 in human allergic diseases is suggested because of the association of functional SNPs in the promoter of the ST2 gene with atopic dermatitis.19 Recent studies in immature cord blood–derived mast cells (MCs) indicate that IL-33 may affect human tissue MCs. Similar to IL-1, IL-33 promotes the secretion of IL-5, IL-13, and certain chemokines in synergy with thymic stromal lymphopoietin (TSLP), and enhances the stem cell factor (SCF)–induced maturation of MC-progenitors.28-30 Furthermore, IL-33 appears to increase the frequency of cells with a polarized shape in freshly reactivated Th2 cell lines, suggesting that IL-33 may be involved in the trafficking of T cells to an inflammatory site.31 However, it is unknown whether IL-33 can activate certain primary human cell types isolated from human blood or tissues, and the direct target cells for IL-33 in the human system remain to be identified.

There is increasing evidence for a central role of mouse c-Kit+/FceRI+ “basophil-like” cells and human basophil granulocytes in the initiation and amplification of Th2-type immune responses.32-35 Despite important differences between mouse and human basophils, both are able to rapidly produce the key Th2 cytokines IL-4 and IL-13 in response to antigen-dependent and -independent activation. Human basophils also release other important mediators of allergic inflammation, such as histamine and LTC4. Although several cytokines, such as nerve growth-factor (NGF), IL-5, and granulocyte macrophage colony-stimulating factor (GM-CSF), can transiently prime basophils for enhanced mediator release,36-38 several studies from our and other groups showed that IL-3 is a unique and potent regulator of the phenotype and function of mature basophils.39-48 Among a large number of ligands studied, including the priming cytokines and IL-1, IL-3 is the only known cytokine that directly induces secretion of low levels of IL-4 and IL-13, and most efficiently promotes cytokine expression in synergy with C5a or FceRI-activation.39,43,44

Here, we investigated receptor expression, the signaling pathways, and biologic effects of IL-33 and IL-18 in blood basophils and also examined which cell types in peripheral blood are the direct targets of IL-33.

Cell stimuli and measurements of mediators and cytokines

Ligands were obtained as follows: rhIL-3, rhGM-CSF (Novartis, Basel, Switzerland); rhIL-5, anti-FcεRI mAb 29C6 (Roche, Basel, Switzerland); rhIL-1β (PeproTech, London, United Kingdom); rhTNF-α (Bender Med-Systems, Vienna, Austria); rhIL-33 (Alexis, Lausen, Switzerland). IL-18 was from 2 commercial sources (PeproTech and MBL, Woburn, MA), and both were active on human T cells. Human C5a was purified from complement activated plasma.36 Cytokines in cell-free supernatants were quantified as follows: IL-4, IL-13, IL-5, and IFN-γ using Eli-Pair kits (Diaclone, Stamford, CT), IL-8 using the MiniKit from Endogen (Rockford, IL) and sST2 using the Kit purchased from MBL. In some experiments, cytokines were also measured with a multiplex assay (Bioplex; Bio-Rad, Hercules, CA). LTC4 was measured using the CAST-ELISA (Buchmann Laboratories, Allschwil, Switzerland). Histamine was measured by fluorometry as described.36 Anti-human-ST2 mAbs 2A5, FB9, HB12 were from MBL and affinity-purified polyclonal antibodies (AF523) from R&D Systems (Minneapolis, MN).

Protein sample preparation and Western blot analysis

Sample preparation and Western blotting was performed as described39,47 using primary antibodies at a 1:1000 dilution. More details and the list of antibodies used are provided in Document S1 (available on the Blood website; see the Supplemental Materials link at the top of the online article). Blots were developed using peroxidase-labeled secondary antibodies and enhanced chemiluminescence detection system (ECL-Kit; Pierce Chemical, Rockford, IL). Immunoreactive bands were analyzed using the Image Analyzer LAS-3000 and AIDA software (FujiFilm, Tokyo, Japan). To allow an accurate comparison of the signals under the different experimental conditions, the blots of each figure were obtained from the same representative experiment. All experiments were repeated at least 3 times with an identical pattern of signals.

RT-PCR

RNA isolation, reverse transcription, and quantitative reverse transcriptase–polymerase chain reaction (RT-PCR) were performed as described.39 Validated TaqMan primers and probes for ST2L (long, IL1RL1 isoform 1; Hs00249389_m1), sST2 (soluble, IL1RL1 isoform 2/3; Hs01073297_m1), ST2 all (all isoforms, IL1RL1 isoform 1/2/3; Hs00545033_m1) and β2-microglobulin (B2M; Hs00187842_m1) were from Applied Biosystems. The relative expression of ST2 in comparison to the reference of the house-keeping gene β2M was calculated by the formula POWER (2,-dCT) and given as RQ (relative quantity).

Flow cytometry

Cell surface staining was performed in PBS (phosphate-buffered saline)2/2% FCS. Receptor expression was analyzed using anti–ST2-FITC (MBL), anti–IL-1R-PE (R&D Systems) and isotype matched control Abs (BD Bioscience). For phosphoprotein analysis, freshly isolated PBMCs were stained before activation with anti–CRTH2-Alexa Fluor 647 (BD Bioscience). Flow cytometry was performed with a FACSCalibur (BD Bioscience). Representative flow cytometry experiments are shown in the figures. All experiments were repeated 5 to 10 times with identical results.

Immunofluorescence microscopy

Basophils plated on poly-L-lysine–coated glass slides were fixed and stained as described previously39 using the following primary antibodies: anti–granulyme B mAb (2C5) sc-8022 (Santa Cruz Biotechnology, Santa Cruz, CA); final concentration of 2 μg/mL; anti–ST2, a mixture of 3 mAb (2A5, FB6, H121; MBL), final concentration of 1.5 μg/mL each.
Results

IL-3 induces expression of the soluble and transmembrane form of ST2

To investigate whether IL-33 and IL-18 may regulate functional responses of human blood basophils, we first analyzed surface expression of ST2 and IL-18 receptors on basophils cultured with or without IL-3. Consistent with published data, basophils constitutively express high levels of IL-18 receptors (IL18R1 and IL18RAP; Figure 1A, and data not shown). By contrast, ST2 was detectable only on the surface of IL-3–stimulated cells. Time course analysis of total and surface ST2 expression induced by IL-3 revealed that freshly isolated cells weakly express sST2 but no detectable membrane ST2L (Figure 1B). IL-3 promoted a rapid induction of sST2 already after 2 hours of stimulation, whereas ST2L became detectable after approximately 8 hours (Figure 1B). Measurement of mRNA levels of ST2 isoforms using real-time RT-PCR indicated that sST2 and ST2L are transcriptionally regulated by IL-3 (Figure 1C).

IL-3 has been shown to induce a prominent change in the content of basophil granules. The observation that a secreted form of ST2 protein remains cell-associated in IL-3–stimulated basophils prompted us to examine whether it localizes to the granule compartment. Figure 2 demonstrates that de novo formed sST2 is sorted to basophil granules and can be mobilized by degranulation in response to IgE-dependent or -independent stimuli. This granular localization of sST2 in basophils was an unexpected finding, because, in contrast to Granzyme B (GzB), which is a known granule protein, sST2 (like ST2L) contains a signal sequence for transport through the cellular membrane.

IL-33, but not IL-18, is an efficient and direct activator of blood basophils

Ligand binding to receptors of the IL-1/TLR family, including IL-33 stimulation of mouse ST2-expressing cells, leads to the recruitment of MyD88, IRAK1, IRAK4, and TRAF6 with formation of a signaling complex that results in downstream activation of...
NF-κB and MAP-kinases. We therefore analyzed whether IL-33 is able to stimulate these signaling pathways in freshly isolated human basophils. Figure 3A demonstrates that IL-33 rapidly activated ERK1/2, JNK, p38, and NF-κB signaling cascades, as evidenced by phosphorylation of MAP kinases and phosphorylation and degradation of the NF-κB inhibitor IκB-α. In contrast to mouse cells, in which IL-33 has been reported to induce prominent ERK activation, it strongly activates p38 MAPK in human basophils. The ability of IL-33 to stimulate freshly isolated basophils expressing no detectable ST2L suggested a ST2-dependent mechanism of activation. To investigate the involvement of ST2L in the bioactivity of IL-33, we first tested in pilot experiments the capacity of commercially available anti-human-ST2 monoclonal and polyclonal antibodies to block p38 activation in the human mast cell-line HMC-1 that we found to constitutively express ST2L. The most efficient, albeit incomplete, inhibition of IL-33–induced p38 activation in HMC-1 cells was obtained by combining 3 mAbs (data not shown). Figure 3B shows that pre-incubation of basophils with this mixture of anti-ST2 antibodies (a mixture of 3 mAbs, clones 2A5, FB9, and HB12; 3 μg/mL each) or anti-CD28 (control mAb; at 10 μg/mL) for 15 minutes at 37°C. Cells were stimulated with 2 ng/mL IL-33 for 15 minutes as indicated. Erk and p38 activation and the degradation of IκB-α were assessed by Western blotting.

Figure 3. IL-33, but not IL-18, activates MAP-kinase and NF-κB signaling pathways in blood basophils.

(A) IL-33 rapidly activates signaling pathways of the TLR/IL-1R family in freshly isolated blood basophils. Cells were stimulated with IL-33 or IL-18 (50 ng/mL each) for the indicated time periods. Western blot analysis of cell extracts shows the rapid activation of the MAP-kinases Erk, JNK, and p38, and the phosphorylation and degradation of IκB-α in response to IL-33, but not to IL-18. The lack of responsiveness of human basophils to IL-18 was confirmed with different preparations of IL-18 from 2 different suppliers. (B) Anti-ST2 antibodies block IL-33–induced basophil activation. Basophils were left untreated, or pre-incubated with anti-ST2 antibodies (a mixture of 3 mAbs, clones 2A5, FB9, and HB12; 3 μg/mL each) or anti-CD28 (control mAb; at 10 μg/mL) for 15 minutes at 37°C. Cells were stimulated with 2 ng/mL IL-33 for 15 minutes as indicated. Erk and p38 activation and the degradation of IκB-α were assessed by Western blotting.

IL-33 promotes Th2 cytokine secretion and enhances IgER-induced mediator release as efficiently as IL-3

It is now well established that IL-3 is a very effective and unique regulator of cytokine expression in human basophils, and no other ligand with a similar efficacy has been identified so far.

IL-3 and IL-33 activate distinct signaling pathways in blood basophils

Despite the similar bioactivities and efficacies in promoting cytokine secretion and enhancing IgER-induced mediator release, the signaling pathways activated by IL-3 and IL-33 in freshly isolated basophils differ markedly (Figure 5A). While IL-3 (but not IL-33) activates the Jak/Stat-pathway leading to the phosphorylation of Stat3/Stat5, IL-33 (but not IL-3) strongly activates NF-κB as evidenced by the phosphorylation and degradation of IκB-α. Furthermore, IL-3 and IL-33 exhibit a preference in activating the MAP-kinases Erk and p38, respectively ( Figures 5A, S3, S4). The marked difference between IL-3 and IL-33 in promoting the activating phosphorylation of ERK and p38, respectively, was not
only evident at different time points after activation with maximally effective concentrations of the cytokines (Figure 5A), but was also observed at lower doses of ligands (Figure S4). We also examined whether IL-3–induced up-regulation of ST2 altered the signaling pathways or signaling strength in response to IL-33. However, while culture of basophils with IL-3 diminished Stat3/5 phosphorylation in response to restimulation with IL-3, it hardly influenced IL-33 signaling (Figure S5).

### IL-33 does not efficiently prime basophils for C5a-induced LTC4 generation

IL-3, IL-5, GM-CSF, and NGF all rapidly prime basophils for C5a-induced LTC4 synthesis.36-38 Figure 5B shows that, compared with IL-3, priming of cells with IL-33 leads to the generation of little LTC4 in response to C5a. This might be due to insufficient activation of cytosolic phospholipase A2 (cPLA2), whose activating...
phosphorylation is indeed weaker in response to IL-33 as compared with IL-3 (Figure 5A). This difference in cPLA2 phosphorylation is possibly related to the type of MAP-kinases that are preferentially activated by the 2 cytokines.49,50 Consistent with this interpretation, we find a complete block of C5a-induced LTC4 synthesis in IL-3–primed cells by the specific MEK1/2 inhibitor U0126 acting upstream of ERK (data not shown). Particularly large quantities of leukotrienes are formed in response to diverse agonists when basophils are cultured with IL-3 for 18 hours or longer, an effect that is unique for this cytokine, and has been termed late priming.39,42 Figure 5C demonstrates that cells cultured with IL-33 do not generate LTC4 in response to C5a. Furthermore, there was no enhancement of LTC4 formation in response to IgE crosslinking after prolonged culture with IL-33. The observation that IL-33 is not capable of promoting late priming, therefore, reveals another important difference between the bioactivity of IL-3 and IL-33. Finally, neither short nor prolonged preincubation with IL-33 enhanced C5a-induced histamine release, in contrast to the known priming effects of IL-3 (Figure S6).

**Basophils are the only cell type within mononuclear cells that directly respond to IL-33**

Basophil granulocytes are present in the fraction of mononuclear cells and display similar light scatter characteristic as lymphocytes. To search for other direct human leukocyte targets of IL-33, freshly isolated PBMCs were stimulated with IL-33 and analyzed by flow cytometry for p38 phosphorylation. Figure 6A demonstrates that, in contrast to basophils, monocytes or different lymphocyte populations, including CRTH2+ Th2-polarized T-helper cells, do not respond to IL-33 stimulation. By contrast, monocytes and lymphocyte subsets are activated to variable degrees by TNF-α used as a positive control, while basophils do not respond to this pro-inflammatory cytokine.

**IL-33 enhances CD3/CD28-mediated cytokine expression in primary in vivo polarized Th2 cells**

Ligation of CD3/CD28 in Th2 (but not Th1) human T-cell lines was reported to up-regulate ST2 mRNA, predominantly the soluble isoform.13 Recent studies showed an enhanced cellular migration of T-cell receptor (TCR)–activated in vitro polarized human Th2 cell lines in response to IL-33, indicating that IL-33 can also regulate functions of human T-helper cells.31 To get a first insight into the biologic effects of IL-33 on human blood T-cell subpopulations, we sorted CRTH2+CCR5+CD4+ Th2 and CCR5+/CRTH2++CD4+ Th1 cells. Figure 6B shows that the cytokine secretion pattern of the purified T-helper cell populations is in line with published data.51,52 Addition of IL-33 to CD3/CD28-activated CRTH2+ Th2 cells enhanced the expression of IL-4, IL-5, and IL-13 (Figure 6C). The effect of IL-33 was statistically significant for all 3 cytokines and most prominent for IL-5 (Figure S7). However, the enhancement of Th2 cytokine expression was rather weak compared with the more than 10-fold increased secretion of IFN-γ caused by IL-1 or IL-18 in CCR5+ Th1 cells. In contrast to the IL-1 family members IL-1 and IL-18, IL-33 did not enhance in a statistically significant manner the secretion of IFN-γ in CCR5+ Th1 cells (Figures 6C, S7). These data demonstrate that the engagement of CD3/CD28 renders primary human Th2 cells responsive to stimulation with IL-33. However, they also suggest that compared with the well established and potent costimulatory effect of IL-1 or IL-18 on Th1 cells, the regulation of Th2 cells may not be the most biologically relevant activity of IL-33 in the human system.

**Apart from basophils, IL-33 also activates eosinophils, but not neutrophils**

Next, we investigated whether IL-33 may be capable of activating granulocyte types other than basophils. For comparison, we also examined the activation of classical IL-1R–signaling pathways in response to other IL-1 family members and TNF-α (Figure 7A).
The characterization of the bioactivity of IL-33 on human basophils forms the main body of investigations presented here. Our study was initiated by the observation that IL-3, a major regulator of the phenotype and functions of basophils, strongly induces the IL-1R family member ST2. Both soluble as well as transmembrane isoforms of ST2 are induced at the mRNA and protein levels. In basophils, sST2 remains cell-associated, is stored in a granule compartment, and can be released by triggering degranulation. Thus, sST2 represents another example for the alteration in the basophil granule content that is induced by IL-3, as reported previously for GzB.39

Most surprising was the finding that IL-33 potently activates freshly isolated basophils in a ST2-dependent manner, despite undetectable ST2L protein expression levels. A weak expression of transmembrane ST2L mRNA is, however, detectable by RT-PCR in freshly isolated blood basophils. Moreover, activation of basophils with IL-33 is inhibited by anti-ST2 Abs, demonstrating that IL-33 interacts with ST2 receptors. Thus, IL-33 is able to transmit strong activation signals by engaging only very few receptors. In contrast to mouse basophils, which release IL-4 and IL-13 upon stimulation with IL-18,6-8 human basophils do not respond to IL-18 even though they constitutively express abundant IL-18 receptors. These observations indicate that genomic or proteomic receptor expression profiles of leukocyte subsets do not always provide a reliable guide for predicting the responsiveness of cells to the corresponding ligands. We have no explanation for these intriguing findings other than to suggest that some unknown ligand-specific receptor or signaling component(s) may also dictate the responsiveness of cells to IL-18 or IL-33, respectively.

Although IL-3 and IL-33 activate different signaling pathways resulting in certain differences in bioactivity (eg, priming for C5a-induced LTC4 production), they share the ability to promote the secretion of IL-4, IL-13, and IL-8, and to enhance IgE-influenced mediator release and cytokine production. Therefore, IL-33 emerges as a second major regulator of these important basophil functions. IL-3 and IL-33 also induce cytokine expression in a synergistic manner, providing an efficient IgE-independent mechanism for the secretion of Th2 cytokines and chemokines in human basophils. Our finding that IL-33 can promote cytokine expression in human basophils is in part in agreement with more preliminary experiments with human basophils published very recently.53 However, in marked contrast to the prominent induction of IL-5 reported in this study,53 we never observed any secretion of IL-5 (detection limit of 5 pg IL-5 per million basophils) under any experimental condition (our unpublished observations). It should also be noted that in contrast to the well-established capacity of basophils to produce IL-4 and IL-13, the secretion of IL-5 by human basophils has never been reported and thus requires further confirmation.

The synergistic induction of immunoregulatory and pro-inflammatory cytokines by IL-33, IL-3 and IgE activation shown here provides a mechanism by which allergen-dependent and tissue-derived factors may cooperate to activate basophils at sites of inflammation. Although the regulation of expression and release of biologically active IL-33 in vitro and in vivo is still largely unknown,22,54 the expression of mRNA of proIL-33 seems to be restricted to resident tissue cells, in contrast to proIL-1 that is also strongly expressed in hematopoietic cells.22,55 Thus, IL-33 derived from inflamed tissue, IL-3 derived from activated mast cells or Th2 lymphocytes, and exposure to allergen, may all contribute to variable degrees to the activation state of basophils in chronic inflammation.

**Discussion**

Here we show that blood basophils and eosinophils, 2 related granulocyte types involved in allergic inflammatory diseases and in Th2-type immune responses to helminth infections, are the prime direct target leukocytes of the novel IL-1 family member IL-33.
The lack of p38 activation in PBMCs other than basophils indicates that monocytes and different lymphocyte populations, including in vivo polarized CRTH2+ Th2 cells, do not directly respond to IL-33. After CD3/CD28-ligation of CRTH2+ cells, however, we find a modestly increased secretion of TH2 cytokines, demonstrating that this cytokine is, apart from IL-3, a second major regulator of basophil functions. The data indicate that basophils and eosinophils represent the only direct leukocyte target of IL-33, suggesting a prominent role for these cells in the innate immune system in the biology of IL-33.

Acknowledgments
This work was supported by the Swiss National Science Foundation and the Stiftung 3R.

Authorship

Contribution: T.P.-P. and S.A.D. performed and analyzed most experiments and participated in writing; S.K. performed and analyzed experiments on basophils and eosinophils; N.S. performed and analyzed research on PBMCs and T cells; and C.A.D. designed and supervised the studies and wrote the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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References


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