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IL-33 Markedly Activates Murine Eosinophils by an NF-κB–Dependent Mechanism Differentially Dependent upon an IL-4–Driven Autoinflammatory Loop

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Eosinophils are major effector cells in type 2 inflammatory responses and become activated in response to IL-4 and IL-33, yet the molecular mechanisms and cooperative interaction between these cytokines remain unclear. Our objective was to investigate the molecular mechanism and cooperation of IL-4 and IL-33 in eosinophil activation. Eosinophils derived from bone marrow or isolated from Il5-transgenic mice were activated in the presence of IL-4 or IL-33 for 1 or 4 h, and the transcriptome was analyzed by RNA sequencing. The candidate genes were validated by quantitative PCR and ELISA. We demonstrated that murine-cultured eosinophils respond to IL-4 and IL-33 by phosphorylation of STAT-6 and NF-κB, respectively. RNA sequence analysis of murine-cultured eosinophils indicated that IL-33 induced 519 genes, whereas IL-4 induced only 28 genes, including 19 IL-33–regulated genes. Interestingly, IL-33 induced eosinophil activation via two distinct mechanisms, IL-4 independent and IL-4 secretion/autostimulation dependent. Anti–IL-4 or anti–IL-4Rα Ab-treated cultured and mature eosinophils, as well as II4- or Stat6-deficient cultured eosinophils, had attenuated protein secretion of a subset of IL-33–induced genes, including Retnla and Ccl17. Additionally, IL-33 induced the rapid release of preformed IL-4 protein from eosinophils by a NF-κB–dependent mechanism. However, the induction of most IL-33–regulated transcripts (e.g., Il6 and Il13) was IL-4 independent and blocked by NF-κB inhibition. In conclusion, we have identified a novel activation pathway in murine eosinophils that is induced by IL-33 and differentially dependent upon an IL-4 auto-amplification loop. The Journal of Immunology, 2013, 191: 4317–4325.

Interleukin-33 is a member of the IL-1 family, which includes IL-1 and IL-18, and binds to its heterodimeric receptor, IL-33R, consisting of the ST2 molecule and IL-1R accessory protein. Binding of IL-33 to its receptor typically triggers NF-κB phosphorylation and translocation to the nucleus to activate transcription of target genes. However, a recent study in fibroblasts overexpressing ST2 has demonstrated that IL-33 can signal through phospho-ERK, independently of NF-κB, indicating two distinct pathways of activation (1). Whether this pathway is operational in other cells remains unclear. IL-33 potently polarizes Th2 cells (2) and activates mast cells (3), basophils (4), and alternatively activated M2 macrophages (5). Recently, a pathogenic role of IL-33 in inflammatory airway disease has been reported [for review (6)]. Notably, asthmatic patients exhibit high levels of IL-33 in bronchoalveolar lavage fluid and in bronchial epithelial and airway smooth muscle cells (7, 8). Interestingly, intranasal administration of IL-33 in naive mice induces inflammatory airway responses (5) and exacerbates eosinophil-mediated airway inflammation (9), suggesting a key role of IL-33 in the development of allergic disease pathogenesis. Although eosinophils have been shown to respond to IL-33 (9), little is known about the molecular mechanism involved.

During immune responses, eosinophils are recruited from the circulation to inflammatory sites, rich in cytokines (e.g., IL-4, IL-13, and IL-33) and chemokines (e.g., CCL11); become activated; and modulate the response through diverse mechanisms. Upon activation, eosinophils undergo degranulation by releasing cationic proteins, which consequently induce cell damage and dysfunction (10). In vitro studies have shown that eosinophil granule constituents are toxic to a variety of tissues such as the intestine, skin, and trachea (10). In addition, activated eosinophils secrete an array of cytokines (e.g., IL-6, IL-10, and IL-13) and chemokines (e.g., CCL3, CCL5, and CCL17) capable of activating T cells (9, 10). Moreover, eosinophils have a profibrogenic role by producing TGF-αß, resistin-like molecule α (RELM-α), metalloproteinase 9, and fibroblast growth factor 2 (11–13). Notably, eosinophils have the capacity to auto-activate themselves as they express and respond to GM-CSF, which promotes their survival (14, 15). It has not yet been determined whether similar auto-activation loops exist for other eosinophil-derived cytokines, especially IL-4, as eosinophils are often a chief source of IL-4 (16–18).

In allergic airway inflammation, the overexpression of IL-4 and IL-13 has been demonstrated to have a role in the development of pulmonary eosinophilia (19, 20). IL-4 mediates its effects through either the type I IL-4R (composed of IL-4Rα and the common γ-chain) or the type II IL-4R (composed of IL-4Rα and IL-13Rα1), which can also mediate IL-13 signaling (21–23). Engagement of both receptors induces the phosphorylation of STAT-6, which subsequently dimerizes and translocates to the nucleus to induce transcription of specific genes (22, 23). Despite the similar intracellular cascades of IL-4 and IL-13, IL-4 can mediate specific signals independently of IL-13 (24). In asthma, IL-13Rα1 has...
been highlighted as having a critical role in the pathogenesis of allergic lung responses, regulating different subsets of genes according to the stimulus (IL-4, IL-13, or allergen) (25).

In this study, we demonstrated that murine eosinophils directly respond to IL-4 and IL-33, but not IL-13, by the phosphorylation of STAT-6 and NF-κB, respectively. Using transcriptome analysis, we identified the genes associated with the IL-33 and IL-4 pathways. We found that IL-33 upregulated 519 genes, whereas IL-4 only induced 28 genes, including 19 genes that were also IL-33 induced. Interestingly, IL-33 induced eosinophil activation via two distinct mechanisms depending upon IL-4 secretion and autostimulation. Anti-IL-4 or anti–IL-4Rx Ab-treated cultured and mature eosinophils, as well as H4- or Stat6-deficient cultured eosinophils, had attenuated protein secretion of a subset of IL-33–induced genes, including Retina and Ccl17. However, the induction of most IL-33–regulated transcripts (e.g., Il6 and Il13) was independent of IL-4 and blocked by an inhibitor of NF-κB. Our study provides evidence for a novel eosinophil activation pathway that is triggered by IL-33 and involves an IL-4/STAT-6 auto-amplification loop or an IL-4–independent/NF-κB–dependent pathway.

Materials and Methods

Mice

BALB/c wild-type, Il13ra1−/− (IL-13Rα1), Il4−/− (IL-4), Il5 (IL-5) CD2-transgenic, or Stat6−/− (STAT-6) mice were analyzed at 4–8 wk of age. All mice were housed under specific pathogen-free conditions and treated according to institutional guidelines.

Bone marrow–derived eosinophil culture

Bone marrow–derived eosinophil culture was modified from Dyer et al. (26). Briefly, total bone marrow cells were isolated, and erythrocytes were lysed by RBC lysis buffer (Sigma-Aldrich). After a density gradient of Histopaque 1083 (Sigma-Aldrich), the low-density bone marrow cells were collected and plated at 1 × 106 cells/ml in IMDM (Life Technologies) supplemented with 10% PBS (HyClone), penicillin-streptomycin (Life Technologies), 200 mM L-glutamine (Life Technologies), and 55 μM 2-ME (Sigma-Aldrich). During the first 4 d, the medium also contained stem cell factor (PeproTech) and Fms-like tyrosine kinase 3 ligand (PeproTech) at 100 ng/ml each. From day 4 to day 14, the cells were cultured in medium containing 10 ng/ml IL-5 (PeproTech). The medium was changed every 2 d until day 14.

For eosinophil activation, cells were collected, pooled, and plated for at least 1 h in a tissue culture dish, to remove any contaminating cells, such as stromal cells or macrophages. Then the nonadherent cells were collected, washed, counted, and incubated with different treatment, according to the experiments. Murine Rl-4 and Rl-13 were purchased from PeproTech, and IL-33 was purchased from R&D Systems. The NF-κB inhibitor BAY 11-7082 was purchased from Santa Cruz Biotechnology and was administered at 5 μM for all of the experiments in which it was used.

Eosinophil purification from Il5-transgenic mice

Briefly, spleen from Il5-transgenic mice was collected and crushed onto a 40-μm cell strainer (BD Falcon). After RBC lysis, the T and B cells were depleted by using microbeads anti-CD90.2 and anti-CD19 (MACS; Miltenyi Biotec). The cell suspension was incubated for 1 h in a tissue culture dish to deplete the adherent cells (macrophages). Then the cells were collected, plated at a density of 2.5 × 106 cells/ml, and activated for 24 h in complete IMDM (see details in bone marrow–derived eosinophil culture section) supplemented with 10 ng/ml IL-5.

Extraction of mRNA and quantitative RT-PCR analysis

Total RNA was isolated with the RNeasy mini kit (Qiagen), according to the manufacturer’s protocol. cDNA was synthesized from 1 or 0.5 μg RNA using the iScript synthesis kit (Bio-Rad). Quantitative RT-PCR (qRT-PCR) was performed using a 7900HT Fast Real-Time PCR system (Applied Biosystems) with FastStart Universal SYBR Green Master mix (Roche). Primers will be provided upon request.

RNA sequencing analysis

FASTQ files from Illumina Pipeline were aligned by TopHat (version 1.4.1) (27), with -T and -G parameters and iGenome annotation from Illumina (08/30/11) for the mm9 genome. The -T parameter is used to align reads to the mouse transcriptome, and the -G parameter is used to provide transcriptome annotation. Produced bam files were fed to cuffdiff (version 1.3.0) (27) annotated with the same GTF file. Only genes for which at least one isoform was significantly changed and had fragment per kb per million mapped reads >2 in at least one condition were analyzed. Experimental conditions were compared with appropriate controls, and a total set of 1593 genes was generated. To generate heat maps, genes were clustered using Gene Cluster 3.0 (28) with the following parameters: clustering average linkage and correlation (centered) similarity. For visualizing of clustered data, Java Tree View (29) software was applied: http://jtreeview.sourceforge.net/. The database is available following this link: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=mflrwcwiweseda&acc=GSE43660

Flow cytometry

Eosinophil markers were assessed by FACS staining for CCR3 and Siglec-F (BD Biosciences) at day 14 of culture. For the detection of phospho-p38 or phospho–STAT-6, the cells were activated with IL-33 (100 ng/ml) for 5 min and IL-4 or IL-13 (100 ng/ml) for 30 min, washed with PBS, then processed for fixation (1% formaldehyde) and permeabilization (90% methanol), and stained with 1 μg/ml anti-mouse phospho-p38 (Cell Signaling Technology) or phospho–STAT-6 Ab (BD Biosciences) per 1 × 106 cells. For the intracellular staining, bone marrow–derived cultured eosinophils, activated overnight by IL-4 or IL-33, were incubated for 4 h with 5 μg/ml bretelidin A (Sigma-Aldrich) to block the protein secretion. Cells were washed with PBS and then processed for the fixation, permeabilization, and staining with anti-mouse Siglec-F PE (BD Biosciences), anti-mouse RELM-α (PeproTech), anti–ST2 (R&D Systems), and anti–IL-4Rx (BD Biosciences) Abs and secondary Ab: goat anti-rabbit Alexa Fluor 488 (Life Technologies, Invitrogen). All Abs were used at 1 μg/ml per 106 cells. Flow cytometry was performed on FACScMalibur, and data were analyzed with FlowJo software (Tree Star).

For cell culture, samples were incubated with 10 μg/ml anti-mouse IL-4 (eBioscience) or anti-mouse IL-4Rx (BD Biosciences) Ab for 4 or 24 h, depending on the experiment. Control IgG1 and IgG2a Abs were purchased from BD Biosciences.

Western blot

Proteins extracted with radioimmunoprecipitation assay buffer were loaded in equal amounts on 4–12% polyacrylamide gradient gels (Invitrogen) and subjected to Western blot with anti-phospho NF-κB (BD Biosciences) or phospho–STAT-6 Ab (BD Biosciences) per 1 × 106 cells. Flow cytometry data were analyzed with FlowJo software (Tree Star).

ELISA

Murine IL-4 and IL-6 levels were analyzed by ELISA using a kit from BioLegend. Murine IL-13 and CCL17 were measured by ELISA using the DuoSet ELISA Development Systems from R&D Systems. For the detection of murine RELM-α, purified anti–RELM-α and biotinylated anti–RELM-α (PeproTech) Abs were used according to a protocol provided by the manufacturer. The lower detection limits for IL-4 and IL-13 were 1.95 and 62.5 pg/ml, respectively, and for IL-6, CCL17, and RELM-α were 15.6 pg/ml.

Statistical analysis

Statistics were done by using the Student t test or one-way ANOVA with Bonferroni’s correction. The p values <0.05 were considered as significant.

Results

Gene expression profiles in murine eosinophils activated by IL-4 or IL-33

We first generated murine bone marrow–derived eosinophils, primarily as per Dyer et al. (26). After 14 d of culture, we confirmed that the cells were mature eosinophils, characterized by multilobed nuclei (Fig. 1A) and the expression of Siglec-F and CCR3 (Fig. 1B) and the expression of eosinophil granule proteins by staining for eosinophil peroxidase activity (data not shown). To minimize the possibility that noneosinophils could be contaminating cells and responsible for the signaling induction by IL-4 or IL-33, adherent cells were removed prior to cellular activation, and eosinophils represented ≥99% of the cells. IL-33 signaling triggered the phosphorylation of p65, a subunit of NF-κB, within 5 min; total NF-κB (p65) did not change over time, demonstrating...
that eosinophils respond to IL-33 (Fig. 1C). We also confirmed that IL-33 induced the phosphorylation of p38 within 5 min of stimulation (Fig. 1D). Similarly, IL-4 induced STAT-6 phosphorylation in eosinophils within 30 min, whereas IL-13 had no effect on STAT-6 phosphorylation (Fig. 1E).

We generated genome-wide transcriptome data based on RNA sequence analysis from eosinophils activated with IL-4 or IL-33. We analyzed two time points, 1 and 4 h, to determine early and late gene induction. We found that multiple genes were differentially expressed after 1 and 4 h of IL-4 or IL-33 exposure (Fig. 2A). Each treatment induced a specific pattern of gene expression, with certain genes being regulated (≥2-fold change) by IL-33, but not by IL-4 and vice versa (Fig. 2B). IL-33 exhibited a more profound effect compared with IL-4, inducing 519 genes. IL-4 induced 28 genes, with only 9 being specifically induced by IL-4. Notably, 19 genes were induced by both IL-33 and IL-4. Functional analysis revealed that the IL-33–induced genes were involved in cytokine production, immune response, and NF-κB signaling, whereas IL-4–induced genes were involved in cellular homeostasis and Jak/STAT signaling (data not shown) (http://toppgene.cchmc.org). Validating genes by qRT-PCR, we confirmed that Cxcl2, Cxcl10, Clec4e, Adora2b, Il6, and Il13 were specifically upregulated by IL-33, but...
IL-33 activates murine eosinophils

Because IL-33 induced Il6 and Il13 expression, we evaluated whether these two cytokines were released in response to 24 h of exposure to different concentrations of IL-33. Eosinophil secretion of IL-6 and IL-13 increased in response to IL-33 in a dose-dependent manner (Fig. 3A). This response was specific to IL-33, as IL-4 had no effect. Similarly, CCL17 release increased in a dose-dependent manner in response to different doses of IL-33, but not IL-4 (Fig. 3B).

Eosinophils also secreted IL-4 in response to IL-33 in a dose-dependent manner (Fig. 3C). Notably, CCL17 and IL-4 release increased following IL-33 exposure, but IL-33 did not regulate Ccl17 or Il4 expression as determined by RNA sequencing. Indeed, Il4 mRNA was not induced by IL-33 at 2, 6, or 24 h of exposure, suggesting that the protein is preformed in the cells and released when the eosinophils were activated (Fig. 3D). By ELISA, we detected IL-4 in a total protein lysate of unstimulated eosinophils compared with the negative control, Il4-deficient eosinophils (data not shown). In contrast, Ccl17 was increased after 6 h of IL-33 treatment and decreased at 24 h (Fig. 3D). For comparison, Il6 was induced at 2 and 6 h and diminished at 24 h. Finally, we observed that 24 h of exposure to different concentrations of IL-4 or IL-33 induced significant, dose-dependent production of RELM-α by eosinophils (Fig. 3E). Additionally, we confirmed that IL-4– and IL-33–treated eosinophils did indeed express RELM-α by flow cytometry analysis (Fig. 3F).

IL-33 induces RELM-α and CCL17 expression through an IL-4 autocrine mechanism

Because eosinophils secreted RELM-α in response to IL-4 or IL-33 and released IL-4 after IL-33 exposure, we hypothesized that an IL-4 autocrine loop is involved in IL-33–induced RELM-α. We first generated Il4−/− and wild-type bone marrow–derived eosinophils. We monitored the cultures and did not observe any differences in terms of proliferation, differentiation, and morphology between the IL-4–deficient and wild-type eosinophils. In addition, IL-4–deficient eosinophils expressed CCR3, Siglec-F, IL-4Rα, and ST2 at similar levels compared with wild-type eosinophils (data not shown). We then activated Il4−/− and wild-type bone marrow–derived eosinophils (obtained at day 14 of culture) for 24 h with IL-4 or IL-33 and compared RELM-α as well as IL-6, IL-13, and CCL17 expression at the mRNA and protein levels. Notably, RELM-α was not induced in Il4−/− eosinophils stimulated with IL-33 compared with wild-type eosinophils; however, Il4−/− eosinophils responded to IL-4 by releasing RELM-α (Fig. 4A). Il4−/− eosinophils stimulated with IL-33 secreted IL-6 and IL-13 at similar levels to IL-33–stimulated wild-type eosinophils (Fig. 4B). Interestingly, the release of CCL17 was lower in IL-33–stimulated Il4−/− eosinophils in comparison with IL-33–stimulated wild-type eosinophils, suggesting a role for an IL-4 autocrine loop in CCL17 expression (Fig. 4C). However, in Fig. 3B, the treatment by IL-4 did not induce CCL17 expression. Thus, we tested whether the addition of IL-4 with IL-33 could induce the expression of CCL17. Il4-deficient eosinophils treated with IL-33 and IL-4 produced CCL17 at similar levels compared with wild-type eosinophils (data not shown). However, the cotreatment did not increase the expression of CCL17 in wild-type eosinophils, suggesting that the IL-4 has an additional, but not synergistic effect. By qRT-PCR analysis, we confirmed that, compared with IL-33–treated wild-type eosinophils, IL-33–treated Il4−/− eosinophils displayed decreased Retnla and a trend for decreased Ccl17 mRNA expression, but unchanged Il6 and Il13 mRNA expression (Fig. 4D). Collectively, these data suggest the existence of an IL-4 autocrine loop that is responsible for IL-33–induced RELM-α and, to a lesser extent, CCL17. In contrast, IL-6 and IL-13 are independent of the IL-4 autocrine loop and may be directly induced by IL-33.

IL-33 induces eosinophil activation through IL-4–dependent and –independent pathways

To further investigate the role of IL-4 in mediating activation of eosinophils by IL-33, we examined the effect of IL-4–specific...
neutralizing Ab. Eosinophils were incubated for 24 h with anti–IL-4 Ab in the presence of IL-33 (10 ng/ml) in the absence or presence of IgG1 (isotype control) or anti–IL-4 Ab (10 μg/ml). Gene expression of Cxcl2, Cxcl10, Clec4e, Il6, Il13, Adora2b, Ccl17, Tfec, and Retnla was analyzed by qRT-PCR (F). The levels of IL-4 (E), RELM-α and CCL17 (G), and IL-6 and IL-13 (H) in the supernatants were measured by ELISA. Bars represent the mean of two wells, and the error bars represent the SEM values. Data are representative of three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001. \( \bar{p} = 0.055, \#p = 0.083 \). ND, Not detected; ns, not significant.

Next, we aimed to determine whether IL-4R type I and/or II are required for eosinophil activation. We first treated eosinophils with IL-33 and IgG2a, an isotype control. Neutralizing IL-4R α inhibited the IL-33–induced expression and production of RELM-α by eosinophils (Fig. 6A, 6B). Although the IL-33–induced expression of Ccl17 mRNA was not significantly decreased by anti–IL-4R α treatment (Fig. 6A), the production of CCL17 protein after 24 h of IL-33 and anti–IL-4R α treatment was dramatically decreased in comparison with IL-33 and IgG2a treatment (Fig. 6B). Treatment with anti–IL-4R α Ab did not change the production of IL-4 induced by IL-33 (Fig. 6B). In addition, IL-33–induced IL-6 and IL-13 expression and production were not affected by the anti–IL-4R Ab treatment (Fig. 6A, 6B). Similarly, the IL-33–induced expression of Cxcl2, Cxcl10, Clec4e, and Adora2b was not affected by the treatment with the anti–IL-4R Ab, indicating that these genes are directly regulated by IL-33. IL-33–induced Tfec expression tended to be decreased in the presence of IL-33 and anti–IL-4R Ab compared with IL-33 and IgG2a (Fig. 6A), indicating that this gene may be dependent on the IL-4 pathway. In summary, we found that IL-33 requires IL-4Rα to induce RELM-α and CCL17 secretion.

**FIGURE 4.** IL-33 requires the IL-4 autocrine loop to induce RELM-α and CCL17. Wild-type (WT) or Il4−/− eosinophils (4 × 10^6/ml) treated for 24 h with IL-4 (10 ng/ml) (A) or IL-33 (10 ng/ml) (A–D). RELM-α, CCL17, IL-6, and IL-13 levels in the supernatants were determined by ELISA (A–C). qRT-PCR from WT or Il4−/− eosinophils (4 × 10^6/ml) incubated for 4 h in the presence of IL-33 (10 ng/ml); graph represents the fold change of gene expression in IL-33–treated eosinophils compared with untreated (D). Eosinophils (4 × 10^6/ml) were activated for 4 h (F) or 24 h (E, G, H) by IL-33 (10 ng/ml) in the absence or presence of IgG1 (isotype control) or anti–IL-4 Ab (10 μg/ml). Gene expression of Cxcl2, Cxcl10, Clec4e, Il6, Il13, Adora2b, Ccl17, Tfec, and Retnla was analyzed by qRT-PCR (F).
We then tested whether IL-4R type II was required for eosinophil activation. Because anti-mouse IL-13Rα1 neutralizing Ab was not commercially available, we generated eosinophils from Il13ra1-deficient mice. Wild-type and Il13ra1-deficient eosinophils were treated for 24 h with IL-33. IL-33 induced RELM-α, CCL17, IL-4, and IL-13 secretion in Il13ra1−/− eosinophils at similar levels compared with wild-type eosinophils (Fig. 6C). Overall, these results demonstrated that IL-4Rα, but not IL-13Rα1, is required for the IL-33–mediated release of RELM-α and CCL17.

Role of STAT-6 in eosinophil activation

To further elucidate whether IL-4 signaling is involved in the expression of genes induced by IL-33, we generated Stat6-deficient bone marrow–derived eosinophils and examined their response to IL-33 or IL-4. We found that RELM-α production was not induced by either IL-4 or IL-33 in Stat6−/− eosinophils compared with wild-type eosinophils (Fig. 7A), which is consistent with our previous observations that RELM-α expression is IL-4/IL-4Rα dependent. In addition, IL-33 did not induce CCL17 production in Stat6−/− eosinophils (Fig. 7B). IL-4, IL-6, and IL-13 were secreted by wild-type and Stat6−/− eosinophils after IL-33 exposure (Fig. 7C). Although Stat6−/− eosinophils responded to IL-33 and expressed ST2 (data not shown), they secreted significantly less IL-4, IL-6, and IL-13 compared with wild-type eosinophils, suggesting a dysfunction in these cells, probably due to the loss of STAT-6.

NF-κB is involved in the expression of genes induced by IL-33

To determine whether the induction of genes by IL-33 occurs via NF-κB signaling, eosinophils were pretreated for 1 h with a NF-κB pathway inhibitor (Fig. 5).

FIGURE 5. Mature eosinophils respond to IL-33 and require IL-4 to induce RELM-α and CCL17. Eosinophils from Il5-transgenic mice were activated 24 h with IL-33 in the presence of anti–IL-4 Ab or isotype control (IgG1). IL-4, RELM-α, CCL17, IL-6, and IL-13 released in the supernatants were measured by ELISA. Bars represent the mean of two wells, and the error bars represent the SEM values. Data are representative of three independent experiments. *p < 0.05, **p < 0.001. ns, Not significant.

FIGURE 6. IL-4Rα, but not IL-13Rα1, is involved in IL-33–induced eosinophil activation. Eosinophils (4 × 10^6/ml) activated for 4 h (A) or 24 h (B) by IL-33 (10 ng/ml) in the absence or presence of IgG2a (isotype control) or anti–IL-4Rα Ab at 10 μg/ml. Gene expressions of Cxcl2, Cxcl10, Clec4e, Il6, Il13, Adora2b, Ccl17, Tfec, and Retnla were analyzed by qRT-PCR (A). The levels of RELM-α, CCL17, IL-4, IL-6, and IL-13 (B) in the supernatants were measured by ELISA. Wild-type (WT) or Il13ra1−/− eosinophils (4 × 10^6/ml) were treated for 24 h in the presence of IL-33 (10 ng/ml), and the levels of RELM-α, CCL17, IL-4, and IL-6 in the supernatants were measured by ELISA (C). Bars represent the mean of two wells, and the error bars represent the SEM values. Data are representative of three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. ND, Not detected; ns, not significant.
We previously showed that IL-33 is regulated directly or indirectly by NF-κB. We further demonstrated that IL-33 is a powerful activator of eosinophils, whereas IL-4 and IL-13 induce modest and no activation of eosinophils, respectively. We demonstrate that IL-33 mediates its effects by two pathways, distinguished by their dependence on IL-4. IL-33 directly stimulates eosinophils to release preformed IL-4, which then auto-activates eosinophils via IL-4Rα and STAT-6; additionally, IL-33 directly induces transcription of a myriad of gene products via NF-κB. Interestingly, functional analysis revealed that IL-33 mediates the expression of cytokine and chemokine gene products, such as IL-6, IL-13, CXC12, and CXC10, whereas IL-4 induces genes enriched in the STAT-6 signaling pathway, such as RELM-α and TFEC, which are known to be STAT-6 dependent (34, 35). Fig. 9 summarizes our findings, which support the ability of IL-33 to profoundly induce eosinophil activation, as measured by transcriptional induction and release of pleiotropic immunomodulatory mediators, and the induction of an IL-4–driven inflammatory loop that is likely to contribute to a variety of Th2 and/or innate immune responses. Whereas mature eosinophils are classically known as primarily granule protein-secreting cells, there are emerging data that these cells remain transcriptionally active and secrete a number of gene products, including IL-4 and RELM-α mRNA and protein (18, 36–38).

We found that IL-33 induces IL-4 release by eosinophils. However, IL-4 mRNA did not increase after IL-33 treatment. Interestingly, we found that IL-4 was prestored in murine eosinophils under homeostatic conditions (at day 14 of culture) (data not shown). Similarly, human eosinophils have been demonstrated to contain prestored IL-4 in vesicles and to rapidly release IL-4 upon stimulation (39, 40). Previous studies have reported that IL-33 can induce IL-4 production by human basophils (4, 41) and murine splenic eosinophils when these cells are cotreated with IL-33 and IL-5 or GM-CSF (30). Moreover, a recent study showed that, in a dextran sulfate sodium–induced colitis model, IL-33 exacerbates the disease via IL-4 (42). In addition, IL-33 has been demonstrated to enhance B cell activation through IL-4 secreted by mast cells and eosinophils (43). Notably, IL-4–producing eosinophils have been demonstrated to have a role in the initiation of type 2 immune responses. In a helminth infection model, eosinophils were identified as one of the major innate IL-4–producing cells in the lung of infected mice (16, 17), whereas IL-33 is known to initiate the immune response (44). Interestingly, in adipose tissue, eosinophil-derived IL-4 has been demonstrated to have a role in glucose metabolism through the maintenance of adipose M2 macrophages, indicating an anti-inflammatory role of IL-4–secreting eosinophils (18). Moreover, two recent studies have demonstrated that, after muscle or liver injury, eosinophil-derived IL-4 is required for tissue regeneration, through the regulation of the resident cells (45, 46). In addition, IL-33 protects mice from adipose tissue inflammation by increasing Th2 cells and macrophages (47). On the basis of this collective dataset as well as our data that IL-33 potently induces eosinophil production of a plethora of potent cytokines, we spec-

**FIGURE 8.** Effect of NF-κB inhibitor in the regulation in IL-33–induced gene expression. Eosinophils (4 × 10^6/ml) were pretreated for 1 h with NF-κB inhibitor (BAY11-7082 [BAY]) at 5 μM and then cultured for 4 h in the presence of IL-33 (A, B) or IL-4 (A) at 10 ng/ml. Gene expression of Cxcl2, Cxcl10, Clec4e, Il6, Il13, Adora2b, Ccl17, Tfec, and Retnla was analyzed by qRT-PCR (A), and IL-4 in the supernatant was measured by ELISA (B). Bars represent the mean of two wells, and the error bars represent the SEM values. Data are representative of three independent experiments. ***p < 0.01, ****p < 0.0001, ND, Not detected.
and IL-33 increased CCL17 expression in
induce the expression of CCL17 (Fig. 3B), treatment with IL-4
pression. Interestingly, although single treatment by IL-4 did not
using anti–IL-4 or anti–IL-4R
we generated eosinophils from
the specific role of IL-4 in IL-33–induced eosinophil responses,
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discovered that IL-33 requires IL-4 signaling to induce RELM-
as a potent coactivator of eosinophils in allergic reactions.

By dissecting the mechanism of IL-4 action on eosinophils, we
discovered that IL-33 requires IL-4 signaling to induce RELM-α and CCL17. First, we found that IL-4 induces RELM-α production
by eosinophils through the type I IL-4R. To further examine
the specific role of IL-4 in IL-33–induced eosinophil responses, we
generated eosinophils from Il4-deficient mice, which demon-
strated that eosinophils require IL-4 to induce RELM-α and
CCL17, but not IL-6 and IL-13. In complementary experiments
using anti–IL-4 or anti–IL-4Ra treatment, we validated the IL-4/-
IL-4Ra dependency of IL-33–induced RELM-α and CCL17 ex-
pression. Interestingly, although single treatment by IL-4 did not
induce the expression of CCL17 (Fig. 3B), treatment with IL-4
and IL-33 increased CCL17 expression in IL-4–deficient eosino-
phils, suggesting that STAT-6 and NF-κB signaling are both re-
quired for CCL17 production (data not shown). However, in wild-
type eosinophils, CCL17 expression was not increased by the
cotreatment with IL-4 and IL-33 compared with IL-33 alone.
These data indicate that CCL17 reached a maximal expression
after IL-33 treatment or cotreatment with IL-4 and suggest that
the signal cannot be amplified due to a saturation of the IL-4R by the
IL-4. By using in vivo eosinophils from Il5-transgenic mice, we
confirmed the in vitro findings showing that IL-33 elicits two
activation pathways. Although in vivo eosinophils respond to IL-
33 and IL-4, we found that the levels of cytokines released are
lower than bone marrow–derived eosinophils. In Il5-transgenic
mice, the overexpression of IL-5 may preactivate directly or in-
directly the eosinophils. It is known that activated eosinophils are
more sensitive to apoptosis. In addition, the eosinophil preparation
process could also weaken the cells and alter their viability, and
thus their potential to respond to a stimulus.

Moreover, we showed that the IL-33–induced expression of Tfec
was dependent on the IL-4 autocrine loop, whereas the IL-33–
mediated induction of Cxcl2, Cxcl10, Clec4e, and Adora2b tran-
scripts was not affected by neutralizing the IL-4/IL-4Ra axis.

Although IL-4 displays a lower effect than IL-33 on eosinophil
activation, these findings showed that IL-33 induces and amplifies
eosinophil activation partially via IL-4. Overall, we suggest that
IL-4 contributes to the IL-33–induced eosinophil activation by the
release of CCL17, which enhances the recruitment of Tcell+ cells,
and by producing RELM-α, which promotes fibrogenic responses
and eosinophil chemotraction (51–53).

In agreement with a recent study (9), we showed that Il6 and Il13
were rapidly expressed in eosinophils and released within 24 h of
treatment with IL-33. Moreover, our study showed that IL-6 and IL-
13 transcripts and proteins were not induced by IL-4 treatment alone.
Using a NF-κB inhibitor, we demonstrated that IL-33–induced Il6
and Il13 expression were dependent on NF-κB signaling, suggest-
ing that NF-κB binds to Il6 and Il13 promoters and consequently
induces their transcription. It is important to note that the IL-33–
induced expression of some genes (e.g., Il6 or Il13) was not entirely
abolished by NF-κB inhibition, indicating that NF-κB is not likely
the only signaling molecule involved in the activity of IL-33; other
mediators such as ERK, p38, or JNK could be involved as well.

In conclusion, we have demonstrated the ability of IL-33 to
directly and profoundly induce eosinophil activation by a NF-κB–
dependent mechanism. Additionally, we have determined that
IL-33 induces NF-κB–dependent eosinophil release of IL-4. Fur-
thermore, we have identified an IL-33–induced IL-4–driven eos-
phin autoinflammatory loop that most likely contributes to a variety of Th2 and/or innate immune responses, involving both
immature developing eosinophils and mature eosinophils. To date,
several papers have demonstrated the effect of IL-33 on human eosi-
nophils (4, 54, 55), and the transcriptome or gene expression profile
has not been shown. In this study, we demonstrated that IL-6 is
induced by IL-33 in murine eosinophils, similarly to reported
induction in human eosinophils (55). We also found that Ccl2 and
Ilcam1 are expressed in murine eosinophils after IL-33 treatment,
which is consistent with protein expression in IL-33–activated human eosinophils (55).

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References
cytokine that signals via the IL-1 receptor-related protein ST2 and induces T
edge: the ST2 ligand IL-33 potently activates and drives maturation of human
leukocytes of the novel IL-1 family member IL-33. Blood 113: 1526–1534.
pression of IL-33 in severe asthma: evidence of expression by airway smooth


