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Mast Cells, Basophils, and Eosinophils Acquire Constitutive IL-4 and IL-13 Transcripts during Lineage Differentiation That Are Sufficient for Rapid Cytokine Production

André Gessner,2*, Katja Mohrs,2† and Markus Mohrs3†

Mast cells, basophils, and eosinophils are myeloid cells that are distinguished by their capability to produce IL-4 and IL-13. However, it is not clear how this potential is related to the lineage differentiation of these subsets. In the present study we used bicistronic IL-4 reporter (4get) mice to directly visualize IL-4 expression by nonlymphoid cells in vitro and in vivo at the single-cell level. Our data show that frequent expression of both IL4 alleles is initiated and maintained during ontogeny by an IL-4Rα- or Stat6-independent mechanism. Despite the constitutive presence of cytokine transcripts in differentiated cells under steady state conditions, cytokine production is not detectable in the absence of stimulation. Moreover, mature mast cells, basophils, and eosinophils also constitutively express IL-13. Both preformed IL-4 and IL-13 mRNAs are sufficient for rapid cytokine production upon stimulation. Our data show that mast cells, basophils, and eosinophils are programmed for IL-4 and IL-13 expression early in ontogeny. These novel findings have important implications for the prevention and therapeutic intervention of allergic and asthmatic diseases.


However, it is not clear how this potential is related to the lineage differentiation of these nonlymphoid cells. In the present study we used bicistronic IL-4 reporter (4get) mice (11) to directly visualize the expression of IL-4 by mast cells, basophils, and eosinophils at the single-cell level. Additionally, we analyzed the expression of IL-13 and elucidated the mechanism underlying the rapid production of both cytokines.

Materials and Methods

Mice and infections

4get mice were generated as previously described (11) and backcrossed to BALB/c for 10 generations. 4get mice were bred to IL-4Ra−/− (14) mice on a BALB/c genetic background to obtain 4get×IL-4Ra−/− mice that were homozygous for both targeted alleles. The same strategy was used to generate 4get×Stat6−/− mice (12). Experimental animals were kept under specific pathogen-free conditions in filter-top cages at the animal facility of Trudeau Institute and were usually used at 8–12 wk of age. In some experiments peritoneal cells were sorted from retired 4get breeders at 6–9 mo of age. Infective third-stage larvae of Heligmosomoides polygyrus were prepared as previously described (17). Experimental animals were inoculated by oral gavage with 200 larvae. All experimental procedures involving mice were approved by the institutional animal care and use committee at Trudeau Institute.

Tissue sampling and preparation

Peritoneal exudate cells (PEC)4 were obtained by lavage with FACS buffer (1% BSA and 0.1% azide in PBS; complete RPMI (10% heat-inactivated FCS, 50 μM 2-ME, 2 mM L-glutamine, and 100 U/ml penicillin/streptomycin) was used when cells were purified for culture). Erythrocytes were removed from blood and bone marrow by ammonium chloride lysis. Single cells from ear skin and small intestine were obtained as previously described (18, 19). Mucosal mast cells were enriched in the pellet of a discontinuous 60/40% Percoll (Amersham Biosciences) gradient (20). Embryos and fetal blood from the umbilical vein were obtained from timed matings on day 15.5 postcoitum (21).

Cell culture

Bone marrow-derived cultures were initially cultured at 5 × 106/ml in complete RPMI 1640 supplemented with IL-3 (30 ng/ml; R&D Systems)
or IL-3 and stem cell factor (SCF; 50 ng/ml; R&D Systems) as indicated. Nonadherent cells were passaged semweekly at 1 × 10^6/ml into fresh medium supplemented with the respective cytokines.

To assess cytotoxic production, cells were cultured at 1 × 10^6/ml in the absence or the presence of ionomycin (500 ng/ml; Sigma-Aldrich), ionomycin and PMA (50 ng/ml, Sigma), or anti-IgE (1 ng/ml; BD Biosciences; clone UH297). The transcriptional inhibitor actinomycin D (0.1–5 μg/ml; Sigma) and the translational inhibitor cycloheximide (8 μg/ml; Sigma-Aldrich) were added 10 min before stimulation.

**Flow cytometry**

mAbs were purchased from Caltag Laboratories or BD Biosciences unless otherwise stated. All samples were first incubated with anti-CD16/32 (clone RM4-5), anti-CD4 (clone RM4-5), anti-CD8 (clone 53-2.1), anti-Thy1 (clone 53-2.1), anti-IL-4 (clone 11B11), anti-CD3 (clone SPE-7), and the Cy-ChipKIT chimera (BD Biosciences). The following mAbs against mouse Ags were used: anti-CD4 (clone RM4-5), anti-CD8 (clone 2B8), anti-IgE (clone UH297), anti-CCR3 (R&D Systems; clone 83101), anti-Thy1 (clone 53-2.1), anti-IL-4 (clone 11B11), and streptavidin-allophycocyanin.

Briefly, samples were incubated for 45 min with mouse anti-DNP IgE (10 μg/ml; Sigma-Aldrich; clone SPE-7), washed, and subsequently stained with biotinylated anti-mouse IgE. Finally streptavidin-allophycocyanin was added to detect the FcεRI/IgE complex. Omission of IgE sensitization had no effect on FcεRI staining on cells derived ex vivo from IL-4R−/− mice. IgE sensitization was included for all samples when experimental groups included IL-4R−/− mice. IgE sensitization was included for all samples when experimental groups included IL-4R−/− mice.

Surface staining for FcεRI was performed as previously described (22). Briefly, samples were incubated for 45 min with mouse anti-DNP IgE (10 μg/ml; Sigma-Aldrich; clone SPE-7), washed, and subsequently stained with biotinylated anti-mouse IgE. Finally streptavidin-allophycocyanin was added to detect the FcεRI/IgE complex. Omission of IgE sensitization had no effect on FcεRI staining on cells derived ex vivo from IL-4R−/− mice. IgE sensitization was included for all samples when experimental groups included IL-4R−/− mice, which have a defect in IgE class switch (13, 14). Dead cells were identified by addition of 4′,6-diamidino-2-phenylnilidine (DAPI; 0.1 μg/ml; Sigma-Aldrich) or propidium iodide (PI; 3 μg/ml; Sigma-Aldrich).

Samples were acquired on a FACSCalibur (BD Biosciences) or a CyAn (DakoCytomation) flow cytometer. Data were analyzed using FlowJo (TreeStar) software. Electronic compensation matrices for data acquired on the CyAn cytometer were determined using the FlowJo compensation platform. All samples were first incubated with anti-CD16/32 (clone RM4-5), anti-CD4 (clone RM4-5), anti-CD8 (clone 2B8), anti-IgE (clone UH297), anti-CCR3 (R&D Systems; clone 83101), anti-Thy1 (clone 53-2.1), anti-IL-4 (clone 11B11), and streptavidin-allophycocyanin.

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**Cell sorting**

Twelve-day bone marrow mast cell (BMMC) cultures were stained with c-Kit and sorted as indicated (Fig. 1E). PEC were stained with c-Kit to identify and sort mast cells (Fig. 5A). Basophils (GFP+/CCR3+/CD4+), eosinophils (GFP+/CCR3+/CD4+), and CD4+/GFP+ or CD4−/GFP− cells were sorted simultaneously from blood of 4get mice 2 wk after infection with H. polygyrus (Fig. 5D). Bone marrow from naive mice was sorted into GFP+ and GFP− populations, avoiding any forward/side scatter (FSC/SVSC) restriction. Samples were sorted on a FACSVantage (BD Biosciences) flow cytometer equipped with DiVa electronics. Purities varied between 85% (PEC mast cells) and 98%.

**Cytokine analysis**

Intracellular cytokine staining was performed using standard methods as previously described (23). Briefly, BMMCs were cultured for 4 h with or without ionomycin in the presence of brefeldin A, subsequently fixed with 4% formaldehyde (Sigma-Aldrich), permeabilized with 0.5% saponin (Sigma-Aldrich), and stained for IL-4. ELISPOT assays were performed as previously described (24). Cells were cultured in triplicate serial 3-fold dilutions on anti-IL-4 (clone 11B11)-coated, 96-well, MultiScreen plates (Millipore). Subsequently the plates were washed, incubated with the biotinylated paired anti-IL-4 (clone BVD6-24G2) Ab, then with streptavidin-conjugated alkaline phosphatase (Caltag), and finally developed with 5-bromo-4-chloro-3-indolyl-phosphate-NBT (Sigma-Aldrich).

Cytokines in culture supernatants were quantified in standard ELISA using paired Abs for IL-4 (clones 11B11 and BVD6-24G2), IL-13 (clone 38213 and polyclonal goat anti-mouse IL-13) and IL-2 (clones JES6-1A12 and JES6-5H4). Recombinant mouse IL-4, IL-13 (R&D Systems), and IL-2 (BD Biosciences) were used as standards. The detection limits were routinely 20 pg/ml for IL-4 and 40 pg/ml for IL-13 and IL-2.

Quantitative real-time RT-PCR was performed using an ABI PRISM 7700 Sequence BioDetector (Applied Biosystems) according to the manufacturer’s instructions (TaqMan; PerkinElmer). Total RNA was extracted using the RNAqueous-4PCR kit (Ambion) and reverse transcribed with the manufacturer’s instructions (TaqMan; PerkinElmer). Total RNA was extracted using the RNAqueous-4PCR kit (Ambion) and reverse transcribed with the Superscript II RNase H− kit (Invitrogen Life Technologies) using oligo(dT)18 priming. Specific primers and probes have been described previously (25, 26). GAPDH transcripts were quantified using the following primers and probe: forward, 5′-CTGTCGTCGGATACAAATGG-3′; reverse, 5′-AACACTCCATTTGCGCATTCA-3′; and GAPDH probe, FAM-5′-CGTTTGCCGTATGGGCGC-3′-TAMRA. Cycle threshold

**FIGURE 1.** GFP and c-kit expression during in vitro differentiation of 4get bone marrow. A, Flow cytometric ex vivo analysis of bone marrow (BM) from 4get (top panel) and wt (bottom panel) mice. Bone marrow from 4get and wt mice was cultured in the presence of IL-3 (B) or IL-3 plus SCF (C). Flow cytometric analysis was performed after 12 and 30 days. B, Flow cytometric ex vivo analysis of bone marrow from 4get (top panel) and 4get×IL-4R−/− (bottom panel) mice. C, Flow cytometric analysis of bone marrow from 4get (top panel) and 4get×IL-4R−/− (bottom panel) mice. D, Flow cytometric ex vivo analysis of bone marrow from 4get (top panel) and 4get×IL-4R−/− (bottom panel) mice. E, Flow cytometric analysis of bone marrow from 4get (top panel) and 4get×IL-4R−/− (bottom panel) mice. F, Flow cytometric analysis of bone marrow from 4get (top panel) and 4get×IL-4R−/− (bottom panel) mice. G, Flow cytometric analysis of bone marrow from 4get (top panel) and 4get×IL-4R−/− (bottom panel) mice. H, Flow cytometric analysis of bone marrow from 4get (top panel) and 4get×IL-4R−/− (bottom panel) mice. I, Flow cytometric analysis of bone marrow from 4get (top panel) and 4get×IL-4R−/− (bottom panel) mice. J, Flow cytometric analysis of bone marrow from 4get (top panel) and 4get×IL-4R−/− (bottom panel) mice. K, Flow cytometric analysis of bone marrow from 4get (top panel) and 4get×IL-4R−/− (bottom panel) mice. L, Flow cytometric analysis of bone marrow from 4get (top panel) and 4get×IL-4R−/− (bottom panel) mice. M, Flow cytometric analysis of bone marrow from 4get (top panel) and 4get×IL-4R−/− (bottom panel) mice. N, Flow cytometric analysis of bone marrow from 4get (top panel) and 4get×IL-4R−/− (bottom panel) mice. O, Flow cytometric analysis of bone marrow from 4get (top panel) and 4get×IL-4R−/− (bottom panel) mice. P, Flow cytometric analysis of bone marrow from 4get (top panel) and 4get×IL-4R−/− (bottom panel) mice.
values for GAPDH were routinely between 16 and 19 cycles, and normalization to β2-microglobulin gave similar results.

Histology
Histology was performed as previously described (24). Briefly, sorted cells were cytospun onto slides and stained with a modified Wright-Giemsa stain (Diff-Quick; Baxter Scientific).

Results
Mast cells and basophils derived from 4get bone marrow are constitutively GFP fluorescent
Populations of BMMC produce IL-4 upon stimulation (2, 27). To address how this potential is related to lineage differentiation at the single-cell level, we analyzed the GFP fluorescence of IL-3-treated and IL-3- plus SCF-treated bone marrow cultures from 4get and wild-type (wt) controls. A low frequency of GFP⁺ cells was detected in the bone marrow from naïve 4get mice (Fig. 1A). Unexpectedly, a substantial fraction of differentiating cells became spontaneously GFP⁺ even in the absence of stimulation (Fig. 1, B and C). Mast cells, identified by the expression of c-kit and FcεRI (data not shown), were GFP⁺ in short term (12-day) and long term (30-day) cultures with IL-3 or IL-3 plus SCF (Fig. 1, B and C). In addition, a c-kit⁻/FcεRI⁻/GFP⁺ population was transiently present after 12 days of culture in IL-3 only (Fig. 1B). This population was no longer detectable on day 30 and was entirely absent when SCF was added to the culture (Fig. 1C). FcεRI⁺/c-kit⁻ cells in short term IL-3 cultures have previously been identified as basophils (28). As we show in this study, basophils are in 4get cells additionally distinguished by their spontaneous GFP fluorescence.

Next we wanted to determine whether the expression of IL-4 in nonstimulated cells is dependent on IL-4Rα-mediated signals. Freshly isolated bone marrow from 4get and 4get×IL-4Rα⁻/⁻ mice revealed a comparable frequency of GFP⁺ cells (Fig. 1D). Upon short term culture in IL-3, both mast cells and basophils from 4get×IL-4Rα⁻/⁻ mice were uniformly GFP⁺ (Fig. 1E). Similar results were obtained in bone marrow-derived IL-3 cultures from 4get×Stat6⁻/⁻ mice (data not shown). Long term bone marrow cultures from all genotypes revealed unimpaired GFP fluorescence (data not shown).

IL-4 and IL-13 production is dependent on stimulation despite constitutive presence of IL-4 transcripts
The spontaneous GFP fluorescence, and therefore IL-4 mRNA expression, of BMMC and basophils was puzzling given the absence of stimulatory signals. Thus, we wanted to determine whether IL-4 production occurs in the absence of stimulation. Long term cultures of 4get and wt BMMC (Fig. 1B) were seeded for 24 h in the absence or the presence of ionomycin, and the supernatants were analyzed for IL-4 and IL-13. Neither cytokine could be detected in nonstimulated cultures (Fig. 2A) despite robust GFP fluorescence of 4get cells (Fig. 1, B and C). As expected, stimulation resulted in substantial cytokine production (>500-fold increase for IL-4; >1000-fold increase for IL-13). We used ELISPOT assay to corroborate that IL-4 production was dependent on stimulation at the single-cell level (Fig. 2B). To correlate GFP fluorescence with IL-4 production at the single-cell level, we performed intracellular cytokine staining on short term bone marrow cultures that contained both basophils and mast cells (Fig. 2C). As expected, the GFP fluorescence was substantially reduced due to fixation and permeabilization (29). A low level of IL-4 staining above the isotype control value was detected in resting cells. However, the staining for IL-4 was largely enhanced by stimulation, and the GFP brightness correlated directly with the fluorescence intensity of IL-4 staining. Because basophils express higher levels of GFP than mast cells (Fig. 1B), and GFPhigh cells stained more brightly for intracellular IL-4 (Fig. 2C), we speculated that basophils produce larger amounts of IL-4. To test this, mast cells (c-kit⁻/GFP⁺), basophils (c-kit⁻/GFP⁻), and the remaining undefined population were sorted from short term IL-3 cultures and seeded for 24 h in the absence or the presence of ionomycin (Fig. 1, B and D). Because our own and published studies have demonstrated that mast cells and basophils do not require IL-4Rα-mediated signals for IL-4 expression (Fig. 1D) (16), IL-4Rα⁻/⁻ bone marrow was cultured to increase the sensitivity of IL-4 and IL-13 detection by eliminating cytokine consumption. IL-4 and IL-13 could not be detected in nonstimulated culture supernatants of mast cells or c-kit⁻/GFP⁻ cells (Fig. 2D). Unstimulated basophils in some (Fig. 2D), but not all, experiments (data not shown) produced detectable amounts of IL-4, whereas IL-13 was undetectable in all experiments. The production of both IL-4 and IL-13 increased distinctly upon stimulation in all GFP⁺ populations. As hypothesized, purified basophils produced substantially larger amounts of IL-4 than mast cells, whereas IL-13 production was comparable.

Constitutive IL-4 expression of mast cells, basophils, and eosinophils in vivo
Bone marrow-derived cultures are widely used to generate and study homogeneous BMMC populations (2, 28, 30). However,
BMMCs are considered immature (31, 32) and do not reflect the in vivo diversification of mast cells into at least two prominent subsets, connective tissue mast cells (CTMC) and mucosal mast cells (30). To analyze the GFP fluorescence of CTMCs ex vivo, we obtained cells from skin, peritoneal cavity, and joint (data not shown) of 4get and wt mice and stained for c-kit (Fig. 3A), FcεRI, CCR3, and CD4 (data not shown) to identify mast cells (c-kit+/FcεRI+/CCR3+/CD4+/SSC<sub>high</sub>). FACS analysis revealed that dermal and serosal mast cells in naive mice were uniformly GFP<sup>+</sup>. The histology of GFP<sup>+</sup> c-kit<sup>+</sup>/CCR3<sup>+</sup> cells sorted from the peritoneal cavity exhibited cytoplasmic granules characteristic of mast cells (Fig. 3A) (21). Mucosal mast cells are rare in naive mice, but the number of mast cells is substantially induced upon infection (1). However, the small population of mucosal mast cells (c-kit<sup>+</sup>/FcεRI<sup>+</sup>/CCR3<sup>+</sup>/CD4<sup>+</sup>/SSC<sub>high</sub>) in the intestine of naive mice was also GFP<sup>+</sup> and expressed FcεRI (Fig. 3B).

Basophils are present in the bone marrow and peripheral blood of naive mice, whereas mature mast cells are extremely rare in the bone marrow and are virtually absent in the blood (33). Staining for FcεRI (Fig. 3C), CCR3, c-kit, and CD4 (data not shown) was used to identify basophils (FcεRI<sup>+</sup>/CCR3<sup>+</sup>/c-kit<sup>+</sup>/CD4<sup>+</sup>/SSC<sub>low</sub>) in these tissues. Basophils from both sites were uniformly GFP<sup>+</sup> in naive mice (Fig. 3C). The histology of FcεRI<sup>+</sup> cells sorted from the blood revealed a basophil phenotype that is clearly distinct from mast cells and eosinophils (see below).

The flow cytometry of peripheral blood and bone marrow revealed an additional GFP<sup>+</sup> population that did not express FcεRI (Fig. 3C). These cells were identified as eosinophils with an FcεRI<sup>−</sup>/CCR3<sup>−</sup>/c-kit<sup>−</sup>/CD4<sup>−</sup>/SSC<sub>high</sub> phenotype (Figs. 3D and 4 and data not shown) (24, 34). The histology of eosinophils (CCR3<sup>−</sup>/GFP<sup>+</sup>) sorted from the peripheral blood confirmed this interpretation (24, 34).

Although we have demonstrated that mast cells and basophils do not require IL-4Rα or Stat6 for IL-4 expression in vitro (Fig. 1E and data not shown), we wanted to corroborate our finding in vivo. Moreover, it is not known whether IL-4Rα or Stat6 are required for IL-4 expression by eosinophils. IL-4Rα-deficient 4get mice revealed unimpaired GFP fluorescence of mast cells (Fig. 3E), basophils, and eosinophils (Fig. 3F). Similar results were obtained with Stat6-deficient 4get mice (data not shown). Of note, IL-4Rα<sup>−/−</sup> mice are defective in IgE isotype switching and have no detectable serum IgE (13, 14). Because the expression of FcεRI is enhanced by IgE binding (22), the surface expression of FcεRI was markedly reduced (Fig. 3F).

**FIGURE 3.** Ex vivo GFP expression by nonlymphoid cells. A. Flow cytometric analysis of mast cells derived from the ear dermis (skin) or peritoneal cavity (PEC) of 4get and wt mice for GFP and c-kit expression. GFP<sup>+</sup> c-kit<sup>+</sup> cells were sorted from PEC as indicated by the bold upper right quadrant, cytospun, and stained with modified Wright-Giemsa. B. Intestinal cells were isolated from 4get mice and analyzed for GFP, c-kit (top panel), and FcεRI (bottom panel) expression. C. The bone marrow (left panels) and blood (right panels) from naive 4get and wt mice were analyzed for GFP and FcεRI expression. GFP<sup>+</sup>/FcεRI<sup>+</sup> cells were sorted from the blood as indicated by the bold upper right quadrant, cytospun, and stained with modified Wright-Giemsa. D. The blood samples from C were analyzed for GFP and CCR3 expression. GFP<sup>+</sup>/CCR3<sup>+</sup> cells were sorted as indicated by the bold upper right quadrant, cytospun, and stained with modified Wright-Giemsa. E. Peritoneal exudate cells from 4get (top panel) and 4get×IL-4Rα<sup>−/−</sup>/CCR3<sup>−/−</sup> (bottom panel) mice were analyzed for GFP and c-kit expression. F. Blood samples of 4get (top panel) and 4get×IL-4Rα<sup>−/−</sup>/CCR3<sup>−/−</sup> (bottom panel) mice were analyzed for GFP, FcεRI (left panels), and CCR3 (right panels) expression. All depicted dot plots were gated on live (PI<sup>−</sup>) cells, omitting additional FSC/SSC gate restriction. Numbers indicate the percentage of cells in the respective quadrant. FACS plots are representative of two or more experiments.

**FIGURE 4.** GFP fluorescence of heterozygous and homozygous 4get mice. Mast cells (c-kit<sup>+</sup>/FcεRI<sup>+</sup>) derived from the peritoneal cavity (A) or basophils (CCR3<sup>+</sup>/FcεRI<sup>+</sup>) or eosinophils (CCR3<sup>−</sup>/FcεRI<sup>−</sup>) from the peripheral blood (B) of homozygous (bold lines) or heterozygous (dotted lines) 4get mice and wt controls were analyzed for GFP fluorescence. All depicted histograms were gated on live (PI<sup>−</sup>) cells, omitting any additional FSC/SSC gate restriction. The gates were set to contain <1% wt cells. FACS plots are representative of two experiments.
Constitutive IL-4 expression of mast cells, basophils, and eosinophils is biallelic

The 4get mice were generated by targeting the bicistronic reporter into the 3’-untranslated region of the endogenous Il4 locus. Thus, the expression of the marked allele can be positively identified on a heterozygous background. To visualize the activation of individual alleles, we analyzed peritoneal mast cells (c-kit+/FceRI+) and basophils (CCR3−/FceRI+) and eosinophils (CCR3−/FceRI+) in the blood of homozygous or heterozygous 4get mice and wt controls for GFP fluorescence. All populations were even in heterozygous 4get mice virtually exclusively GFP+. This requires the frequent expression of both IL-4 alleles, because otherwise a substantial population would be GFP−. The reduced fluorescence of heterozygous 4get cells is consistent with our previous work (11).

Ex vivo IL-4 and IL-13 production by mast cells, basophils, and eosinophils does not occur in the absence of stimulation despite abundant cytokine transcripts

The constitutive GFP fluorescence of mast cells, basophils, and eosinophils indicated the presence of IL-4 transcripts even in non-stimulated cells. However, as shown in Fig. 2, cytokine secretion does not occur in the absence of stimulation. To verify this observation in vivo, mast cells (c-kit+/GFP+; Fig. 5A) were sorted from the peritoneal cavity of 4get mice and analyzed by RT-PCR for the presence IL-4 and IL-13 transcripts (Fig. 5B). Th2 cells (11, 24)

FIGURE 5. Ex vivo analysis of cytokine transcripts and protein of cells isolated from 4get mice. A, GFP+/c-kit+ mast cells were purified by cell sorting from the peritoneal cavity of 4get mice. Pre- and postsort analyses are depicted. B, Naive/Th1 cells, Th2 cells, and mast cells (MC) were sorted ex vivo from the peritoneal cavity of 4get mice and analyzed by real-time RT-PCR for the relative abundance of IL-4 (■) and IL-13 (□) transcripts normalized to GAPDH. C, The indicated populations were cultured in the absence (−) or the presence (+) of ionomycin only (Iono) or PMA plus ionomycin (P+I) as indicated. Supernatants were collected after 24 h and analyzed for IL-4 by ELISA. D, 4get mice were infected with the helminth H. polygyrus, and peripheral blood was collected 2 wk later. Within the GFP+ population (left panel), three distinct subsets were identified by their CCR3 and CD4 surface phenotypes and were isolated by cell sorting as indicated by the bold quadrants (middle panel). The remaining GFP+/CCR3+/CD4− cells were homogeneously FceRI+ and c-kit− (right panel). E, Naive/Th1 cells, Th2 cells, basophils (Baso), and eosinophils (Eos) were sorted ex vivo and analyzed by real-time RT-PCR for the relative abundance of IL-4 (■) and IL-13 (□) transcripts normalized to GAPDH. F, The indicated ex vivo sorted populations were cultured in the absence (−) or the presence (+) of ionomycin only (Iono), PMA plus ionomycin (P+I), or anti-IgE (aIgE) as indicated. Supernatants were collected after 24 h and analyzed for IL-4 by ELISA. All depicted plots were gated on live (PI− or DAPI−) cells, omitting any additional FSC/SSC gate restriction. Numbers indicate the percentage of cells in the respective quadrant. The detection limit for the real-time RT-PCR and the IL-4 ELISA are indicated as dotted lines. Naive/Th1 cells (GFP−/c-kit+/CD4−), Th2 cells (GFP+/c-kit−/CD4+ in PEC and GFP+/CCR3+/CD4− in blood), mast cells (MC; GFP+/c-kit+/CD4−), basophils (Baso; GFP+/CCR3−/CD4−), and eosinophils (Eos; GFP+/CCR3+/CD4−) are shown. ND, not determined. Data are representative of two experiments.
and naive and/or Th1 cells were sorted concurrently as references for transcript levels. IL-4 and IL-13 transcripts were abundant in mast cells and Th2 cells, whereas GFP⁺ cells contained no detectable (>1000-fold less) IL-4 transcripts, and the abundance of IL-13 was at least 100-fold lower. Consistent with the in vitro results (Fig. 2), IL-4 protein was undetectable in nonstimulated cultures despite robust GFP fluorescence of the sorted cells. Upon stimulation, both mast cells and Th2 cells produced large amounts of IL-4, whereas cytokine production by GFP⁻ T cells was not detectable (Fig. 5C).

To analyze basophils and eosinophils for IL-4 and IL-13 transcript levels and cytokine production, we infected 4get mice with *H. polygyrus* and sorted the respective populations from the blood 2 wk later (Fig. 5D). FcεRI staining was omitted to avoid activation by receptor cross-linkage (Fig. 5F), and basophils were unambiguously identified as GFP⁺/CCR3⁺/CD11b⁻ cells (see Fig. 5D). Th2 cells (11, 24) and naive and/or Th1 cells were sorted concurrently as references for transcript levels. Basophils and Th2 cells contained abundant IL-4 and IL-13 transcripts, whereas GFP⁺/CD11b⁻ cells had no detectable (>1000-fold less) IL-4 transcripts and substantially lower (>100-fold) IL-13 levels (Fig. 5E). Eosinophils revealed similar IL-4 transcript abundance as Th2 cells, whereas IL-13 mRNA levels were lower. The production of IL-4 was generally restricted to GFP⁺ cells and dependent on stimulation (Fig. 5F). Activation of basophils by cross-linkage of the FcεRI with anti-IgE resulted in similar IL-4 production. Unstimulated eosinophils produced small amounts of IL-4 in some, but not all, experiments.

**Committed mast cell precursors do not express IL-4**

The earliest committed precursors of the mast cell lineage have been identified in fetal blood of day 15.5 embryos (21) and have a c-kit⁺Thy1⁻ phenotype. To determine whether committed precursors already express IL-4, we analyzed the fetal liver, which contains substantial amounts of fetal blood, and fetal blood (Fig. 6A and data not shown) from 4get and wt embryos. Cells with a c-kit⁺Thy1⁻ phenotype were in fetal blood and liver GFP⁻, demonstrating the absence of IL-4 transcripts in committed precursors. Subsequent in vitro culture of fetal livers with IL-3 and SCF differentiated GFP⁻/c-kit⁺ mast cells from GFP⁺ precursors (Fig. 6B).

Mast cell progenitors arise in adult animals in the bone marrow, where mature mast cells are extremely rare (data not shown) (30, 33). To test whether precursors in adult mice are also GFP⁺, we sorted GFP⁺ and GFP⁻ cells from the bone marrow of naive 4get mice (Fig. 6C) and cultured the purified populations in vitro. IL-3 cultures of GFP⁺ cells gave rise to both mast cells and basophils with similar kinetics and frequency as unseparated bone marrow (compare Figs. 6D and 1B), demonstrating the presence of precursors for both lineages in the GFP⁻ fraction. The development of basophils was also suppressed by the addition of SCF (compare Figs. 6E and 1C). In contrast, the lineage differentiation of GFP⁺ cells was clearly delayed despite the addition of irradiated bone marrow feeder cells (35) (Fig. 6D). The development of mast cells was strikingly impaired even in long term culture. Addition of SCF suppressed the development of basophils, but did not restore mast cell development (Fig. 6E).

**Preformed IL-4 and IL-13 transcripts in mast cells and basophils are sufficient for cytokine production**

Next we wanted to determine whether preformed IL-4 and IL-13 transcripts contribute to rapid cytokine production by mast cells. In addition to various type 2-associated cytokines, mast cells produce IL-2 (36). Long term IL-4R⁻/⁻ mast cells differentiated GFP⁺/CCR3⁻/CD11b⁻ cells were suppressed the development of basophils, but did not restore mast cell development. None of the cytokines was detected at any time in nonstimulated cultures.

IL-4 and IL-13 transcripts were abundant in resting BMMC (Fig. 7B) and comparable to levels in ex vivo isolated mast cells.
was extremely rapid, because the presence of 1C). In fact, the remaining 15–20% IL-4 and IL-13 production in 7 critically more inhibited than the production of IL-4 or IL-13 (Fig. 5). The production of IL-2 at all tested concentrations was significantly level (39) and therefore serves as a sensitive internal control. Production of IL-2 is controlled almost entirely at the transcriptional level (40). Culture supernatants were harvested and analyzed for cytokines by ELISA. Values represent the cytokine concentrations as a percentage of maximum production in the absence of ActD. Data represent the mean and SD. The detection limits for the ELISAs and real-time RTPCRs are indicated. Asterisks indicate $p$ values determined by Student’s $t$ test: *, $p < 0.05$; **, $p < 0.01$. Data are representative of two or more experiments.

To analyze the kinetics and the contribution of preformed cytokine transcripts to the production of IL-4 and IL-13 by basophils, we sorted GFP+/-c-kit+ cells from short term IL-3 cultures of 4get×IL-4Rα−/− bone marrow. The secretion of both cytokines was extremely rapid, because ~75% of IL-4 and essentially all IL-13 (~95 and 105% in two independent experiments, respectively) were secreted within the first 4 h (Fig. 8A). In contrast, IL-2 accumulated with substantially slower kinetics, and 85% was produced after 4 h, demonstrating that the cells remained viable and capable of cytokine production. The production of IL-4 and IL-13 was very low in resting cultures, and IL-2 was undetectable.

IL-4 and IL-13 transcripts were abundant in resting, in vitro-derived basophils (Fig. 8B) and comparable to levels in ex vivo isolated basophils (Fig. 5E), whereas IL-2 transcripts were at the limit of detection within 40 cycles of amplification. As expected, stimulation increased IL-4 and IL-13 transcript levels robustly; however, the induction of IL-2 was substantially greater. This suggested that transcriptional activity is clearly required for IL-2 production, but might be less important for IL-4 and IL-13 production. To test the functional contribution of preformed IL-4 and IL-13 transcripts, we analyzed cytokine secretion upon stimulation in the presence of the transcriptional inhibitor actinomycin D. The production of IL-2 is controlled almost entirely at the transcriptional level (39) and therefore serves as a sensitive internal control. The production of IL-2 at all tested concentrations was significantly more inhibited than the production of IL-4 or IL-13 (Fig. 7C). In fact, the remaining 15–20% IL-4 and IL-13 production in the presence of 1 μg/ml actinomycin D was comparable to that in a similar study demonstrating the transcription-independent production of RANTES (40), and the production of IL-2 was inhibited ~98% at this concentration. The production of IL-4, IL-13, and IL-2 was undetectable when cycloheximide (40) was added, demonstrating that cytokine production requires de novo protein synthesis (data not shown).

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**Discussion**

Mast cells, basophils, and eosinophils are distinct bone marrow-derived cells of the myeloid lineage that are collectively regarded as key effectors of type 2 immunity and immunopathology (1, 2, 4). Despite their strikingly different development, homing properties, diversity of effector functions, and the exquisite phenotypic

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**FIGURE 7.** Kinetics of cytokine production by BMMC. BMMCs from 4get×IL-4Rα−/− mice were cultured in the presence (filled line) or the absence (open line) of ionomycin. **A,** Culture supernatants were harvested and analyzed for the indicated cytokines by ELISA. **B,** Cells were harvested from the same cultures as in A and analyzed by real-time RT-PCR for the relative abundance of the indicated cytokine transcripts normalized to GAPDH. **C,** BMMCs were stimulated for 4 h with ionomycin in the absence or the presence of the indicated concentrations of actinomycin D (ActD). Culture supernatants were harvested and analyzed for cytokines by ELISA. Values represent the cytokine concentrations as a percentage of maximum production in the absence of ActD. Data represent the mean and SD. The detection limits for the ELISAs and real-time RTPCRs are indicated. Asterisks indicate $p$ values determined by Student’s $t$ test: *, $p < 0.05$; ***, $p < 0.01$. Data are representative of two or more experiments.
heterogeneity of mast cells, they share the potential for IL-4 and IL-13 production upon stimulation (2, 30, 33, 41, 42). The release of these cytokines by FcεRI-bearing basophils and mast cells can be induced independent of T cell help (43, 44), the presence of Ag-specific IgE (45–47), or FcεRI cross-linking (48, 49). Thus, mast cells and basophils might be a potential source of innate IL-4, which promotes the development of an adaptive Th2 response (10). Basophils have indeed been shown to be major sources of IL-4 in vivo, potentially releasing IL-4 in larger quantities and more frequently than CD4+ T cells (46, 50). Mast cells, basophils, and eosinophils, moreover, are preferentially located in peripheral inflammatory sites and are therefore exquisitely positioned to mediate effector functions and orchestrate type 2 immunity (8, 24, 41, 46). In the present study we clearly demonstrate that mast cells, basophils, and eosinophils initiate and maintain the constitutive presence of functional IL-4 and IL-13 transcripts at the single-cell level under steady state conditions as an integral part of their lineage differentiation. This process is independent of IL-4Rα- or Stat6-mediated signals in these lineages. Despite the presence of abundant transcript levels, the production of IL-4 and IL-13 was not detectable in the absence of stimulation. Consistent with published studies (37, 38), stimulation resulted in rapid cytokine production, accompanied by increased transcript levels. However, as we demonstrate, the constitutively present IL-4 and IL-13 transcripts were sufficient for the rapid production of both cytokines under conditions where the de novo synthesis of mRNA was inhibited. These data suggest that preformed IL-4 and IL-13 transcripts contribute to the rapid production of both cytokines.

The use of 4get mice allows the faithful and direct visualization of IL-4 expression at the single-cell level without additional stimulation (11, 24). The detected mRNAs are mature, full-length IL-4 transcripts and cannot be attributed to truncated mRNAs containing only exons 3 and 4 (51) or transcripts initiated in intron 2 (52), because the primers and the probe for fluorogenic RT-PCR bind in exons 1 and 2, and we used oligo(dT)14 priming for RT (25). Our findings are also supported by published data showing low levels of IL-4 mRNA by Northern blot in resting transformed and normal mast cell lines in vitro, whereas IL-4 bioactivity is undetectable (27, 53). The levels of IL-4 transcripts in nonstimulated mast cells, basophils, and eosinophils are surprisingly high and, in fact, are comparable to those in ex vivo isolated GFP+ Th2 cells (Fig. 5, B and E). A recent study (8), also using 4get mice, is entirely consistent with our findings regarding the expression of IL-4. In contrast to this study, we performed extensive studies on the mast cell lineage, analyzed for IL-13 transcripts and protein, and demonstrated that preformed IL-4 and IL-13 transcripts are sufficient for rapid cytokine production.

We do not have similarly designed IL-13 reporter mice to directly visualize IL-13 expression at the single-cell level. However, our data strongly suggest that IL-13 transcripts are also induced and maintained during lineage differentiation of mast cells, basophils, and eosinophils. Both preformed IL-4 and IL-13 transcripts are sufficient for cytokine production by mast cells and basophils upon stimulation. Constitutive cytokine transcripts correlate moreover with an accessible gene locus poised for an instantaneous increase in transcriptional activity (54). The remarkably rapid IL-4 and IL-13 production might...
be attributed to both preformed transcripts and an accessible cytokine locus. Constitutive IL-4 transcripts, moreover, are potentially required to replenish intracellular stores of preformed IL-4 protein in basophils (Figs. 2C and 8C) (55, 56).

The developmental signals resulting in the constitutive presence of IL-4 and IL-13 transcripts in mast cells, basophils, and eosinophils are not mediated by IL-4Rα (Fig. 1D and Fig. 3, E and F) or Stat6 (data not shown). It has previously been demonstrated that Stat6<sup>−/−</sup> BMMC can produce IL-4 upon stimulation (16); however, our present study contributes numerous novel insights. We show that in addition to Stat6, which is only one signaling molecule associated with the IL-4R complex (57), the entire IL-4R α-chain is dispensable for both IL-4 and, previously not demonstrated, IL-13 production. It is conceivable that exogenous factors initiate IL-4 and IL-13 expression during the ontogeny of eosinophils and basophils at the stage of a common eosinophil/basophil progenitor (Eo/B-CFU) (58) in the bone marrow. Consistent with this interpretation, a substantial fraction of GFP<sup>+</sup> cells in the bone marrow cannot be identified as mature basophil, eosinophils, mast cells, NK T, or T cells (Fig. 1, A and D, and Fig. 3C). These cells might contain hemopoietic precursors, presumably of eosinophil/basophil lineage, already committed to IL-4 expression (59). In contrast, mast cells progenitors exit the bone marrow and mature in the periphery (2, 30). As clearly shown in Fig. 6A, committed fetal mast cell progenitors (21) do not yet express IL-4 transcripts. Mast cell progenitors in adult mice also do not express IL-4, because sorted GFP<sup>−</sup>, but not GFP<sup>+</sup>, bone marrow cells retain an unpaired potential to develop into BMMC (Fig. 6, C–E). The expression of IL-4 in the diverse mast cell subsets is apparently regulated by distinct factors, because common GFP<sup>−</sup> progenitors spontaneously mature in connective tissues into GFP<sup>+</sup> CTMC, whereas mucosal mast cells require T cell-derived factors (60).

Regardless of the elusive mechanism, both IL4 alleles are rendered accessible during lineage differentiation of mast cells, basophils, and eosinophils and are frequently expressed. Recent studies limited to eosinophils activated in the presence of IL-4 support our conclusion (42). In contrast to nonlymphoid cells, some (61–63), but not all (11), studies have reported the frequent expression of only one IL4 allele in CD4<sup>+</sup> T cells.

In addition to our present study of nonlymphoid cells, we have recently demonstrated the presence of constitutive IL-4 transcripts in NK T cells (54, 64). It is intriguing that cells of various lineages because sorted GFP<sup>−</sup>, but not GFP<sup>+</sup>, bone marrow cells retain an unpaired potential to develop into BMMC (Fig. 6, C–E). The expression of IL-4 in the diverse mast cell subsets is apparently regulated by distinct factors, because common GFP<sup>−</sup> progenitors spontaneously mature in connective tissues into GFP<sup>+</sup> CTMC, whereas mucosal mast cells require T cell-derived factors (60).

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