

ORIGINAL ARTICLE

EXPERIMENTAL ALLERGY AND IMMUNOLOGY

***In vitro* differentiation of near-unlimited numbers of functional mouse basophils using conditional Hoxb8**

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Abstract

Background: Basophils constitute a rare leukocyte population known for their effector functions in inflammation and allergy, as well as more recently described immunoregulatory roles. Besides their low frequency, functional analysis of basophils is hindered by a short life span, inefficient *ex vivo* differentiation protocols, and lack of suitable cell models. A method to produce large quantities of basophils *in vitro* would facilitate basophil research and constitute a sought-after tool for diagnostic and drug testing purposes.

Methods: A method is described to massively expand bone marrow-derived basophils *in vitro*. Myeloid progenitors are conditionally immortalized using Hoxb8 in the presence of interleukin-3 (IL-3) and outgrowing cell lines selected for their potential to differentiate into basophils upon shutdown of Hoxb8 expression.

Results: IL-3-dependent, conditional Hoxb8-immortalized progenitor cell lines can be expanded and maintained in culture for prolonged periods. Upon shutdown of Hoxb8 expression, near-unlimited numbers of mature functional basophils can be differentiated *in vitro* within six days. The cells are end-differentiated and short-lived and express basophil-specific surface markers and proteases. Upon IgE- as well as C5a-mediated activation, differentiated basophils release granule enzymes and histamine and secrete Th2-type cytokines (IL-4, IL-13) and leukotriene C4. IL-3-deprivation induces apoptosis correlating with upregulation of the BH3-only proteins BCL-2-interacting mediator of cell death (BIM) and p53 upregulated modulator of apoptosis (PUMA) and downregulation of proviral integration site for Moloney murine leukemia virus 1 kinase (PIM-1).

Conclusion: A novel method is presented to generate quantitative amounts of mouse basophils *in vitro*, which moreover allows genetic manipulation of conditionally immortalized progenitors. This approach may represent a useful alternative method to isolating primary basophils.

Abbreviations

4-OHT, 4-hydroxytamoxifen; BIM, BCL-2-interacting mediator of cell death; C5a, complement component C5a; GM-CSF, granulocyte macrophage colony-stimulating factor; IgE, immunoglobulin E; IL-3/4/13, interleukin-3/4/13; LTC4, leukotriene C4; mMCP, mouse mast cell protease; PIM-1, proviral integration site for Moloney murine leukemia virus 1; PUMA, p53 upregulated modulator of apoptosis; qPCR, quantitative RT-PCR; SCF, stem cell factor; Th2, T helper lymphocyte type 2; XIAP, X-linked inhibitor of apoptosis.

Basophils develop from hematopoietic progenitors in the bone marrow and are released into the blood upon maturation. They constitute the least common granulocyte population, representing <1% of circulating leukocytes in the mouse (1). Research on basophils is hindered by their low frequency, short expected half-life of a few days, and their phenotypic similarities to mast cells (1). Both basophils and mast cells express the high-affinity receptor for immunoglobulin E (FcεRI) and contain granules rich in histamine and specific enzymes, which, together with *de novo* produced important cytokines and lipid mediators, are released upon

activation (2). However, in contrast to basophils, mast cells exit the bone marrow as progenitors and mature in peripheral tissue where they can persist for prolonged times (1). Phenotypically, mouse basophils can be distinguished from mast cells by their lack of surface c-kit (CD117) expression and differential expression of several mouse mast cell proteases (3).

Recently, new tools for functional *in vivo* analysis of basophils have been developed, including basophil-depleting antibodies (4–7) and basophil-deficient mouse models (8, 9). It has since become clear that basophils possess previously unrecognized functions in immune regulation and allergic responses. Basophils are now recognized as both initiators and effectors of chronic allergy, as important players in IgG (but not IgE)-mediated systemic anaphylaxis, and to possess a driving role in Th2 cell differentiation (reviewed in (1)). Basophils are an important source of Th2-type cytokines and, in fact, one of the first sources of interleukin-4 (IL-4), inducing and/or amplifying Th2 polarization in response to various stimuli, including signaling through FcεRI (1, 5, 10–12).

Interleukin 3 (IL-3) constitutes the best-characterized basophil growth factor and differentiation cytokine for human and mouse basophils and mouse mast cells (13, 14). Human and mouse basophils express high levels of IL-3 receptor, and IL-3 increases the life span of human basophils (15, 16). However, the mechanisms of IL-3-promoted basophil survival are poorly understood. Both phosphatidylinositol-3 kinase (PI₃K) and the serine/threonine kinase proviral integration site for Moloney murine leukemia virus 1 (PIM-1) have been proposed to act downstream of IL-3 in human basophils (15, 16). In the mouse, prosurvival IL-3 signaling has mainly been studied in other hematopoietic lineages (17–19).

Basophils are difficult to differentiate *ex vivo* in sufficient numbers and purity as mast cells predominate rapidly upon culture of bone marrow cells with IL-3 (20). Importantly, no suitable mouse or human model cell line is available that convincingly mimics basophil function. Recently, Wang et al. described a system to generate large amounts of mouse neutrophils and macrophages *ex vivo* using conditional Hoxb8 (21). The transcription factor Hoxb8 is able to promote the expansion of hematopoietic progenitors, and enforced expression of Hoxb8 blocks differentiation of stem cell factor (SCF)- or granulocyte macrophage colony-stimulating factor (GM-CSF)-dependent myeloid progenitors (22). Here, we present a modification of the method by Wang et al. to generate IL-3-dependent, conditional Hoxb8-immortalized myeloid progenitors, which can be differentiated 'on demand' and in near-unlimited amounts into functional basophils. This is to our knowledge the first description of quantitative production of basophils.

Methods

Mice and reagents

C57BL/6 wild-type mice were maintained under pathogen-free conditions. Animal experiments were approved by the

animal experimentation review board of the Canton of Bern (41/08 and BE31/11).

RPMI 1640/GlutaMAXTM medium was from Life Technologies (Zug, CH). Fetal calf serum (FCS, PAA Clone) was from PAA Laboratories GmbH, AT. 4-hydroxytamoxifen (4-OHT) was from Sigma-Aldrich (Buchs, CH). Q-VD-OPh was from SM Biochemicals (Anaheim CA, US). Recombinant mouse C5a was from HyCult Biotech (Uden, NL).

Generation of IL-3^{-cond}Hoxb8 progenitor lines and differentiation into basophils

The coding sequence of mouse Hoxb8 was cloned into the pF-5xUAS-SV40-puro-Gev16 lentiviral vector system (23) as described elsewhere (Salmanidis et al., *in revision*). Hematopoietic progenitor cells were enriched from bone marrow of C57BL/6 mice by magnetic cell separation using a lineage depletion cocktail (BD IMagTM, BD Biosciences Europe) following the manufacturer's instructions. 5×10^5 freshly isolated, or cryoconserved, lineage-negative cells were incubated for 24 h or 36–48 h, respectively, in complete RPMI medium (RPMI 1640/GlutaMAXTM, 10% FCS, 1% Penicillin/Streptomycin, 50 μM 2-mercaptoethanol) in the presence of ≥ 200 pg/ml IL-3 (added as WEHI-3B cell conditioned medium as a source for murine IL-3; concentration quantified by ELISA, BioLegend, San Diego, US). Cells were then spin infected with pF-5xUAS-Hoxb8(mm)-SV40-puro-GEV16 lentiviral particles in the presence of 8 μg/ml polybrene at 30°C for 90 min. Cells were cultivated in complete RPMI/IL-3 and Hoxb8 expression induced by addition of 100 nM 4-OHT. 3–5 days after infection, cells were selected with 1 μg/ml puromycin for a minimum of 3 weeks. Outgrowing IL-3-dependent cell lines were selected for absence of CD117 expression for further analysis. To differentiate progenitors into mature basophils, cells were washed twice in PBS and reseeded at 7.5×10^4 cells/ml in complete RPMI medium containing IL-3 (≥ 200 pg/ml) for 6 days.

FcεRI crosslinking and degranulation assay

1×10^6 cells were sensitized in RPMI-1640/GlutaMAXTM medium containing 0.2% BSA (Sigma) with mouse monoclonal anti-trinitrophenyl (TNP) IgE (IgE1-b4, ATCC), added as 15% hybridoma supernatant for 90 min at 37°C. Cells were washed and activated with 100 ng/ml TNP-BSA (Biosearch Tech Inc., Novato, US) for 30 min. 140 μl of cellular supernatant was added to 60 μl of 8 mM p-nitrophenol N-acetyl-β-D-glucosaminide (Sigma) in 0.08 M citrate solution, pH 4.5, and incubated for two hours at 37°C. The reaction was stopped by addition of 0.2 M glycine, pH 10.7, and results analyzed on a spectrometer plate reader (SpectraMax M2^e, Molecular Devices, λ = 405 nm).

qPCR analysis

Total RNA was extracted from 5×10^6 cells (SV total RNA isolation system, Promega AG, Dübendorf, CH), and 1 μg

RNA was reverse-transcribed using oligo(dT) primer and M-MLV reverse transcriptase following the manufacturer's instructions (Promega). Quantitative RT-PCR (qPCR) analysis was performed using HOT FIREPol® EvaGreen® qPCR Mix Plus (Solis Biodyne, Tartu, EE) on a multicolor Real-Time PCR detection system (iQ5, BIO-RAD). Primer sequences specific for the various mast cell proteases were: *Prss34* (mMCP-11, amplicon 149 bp) Fw 5'-GGACATTGCTCTGCTGAAAC, Rev 5'-GCAGTGGCATGTAGTTCTCG; *Mcpt8* (mMCP-8, amplicon 134 bp) Fw 5'-TCATTCTGTCTAGTGAAGCC, Rev 5'-GCTCTTTGGAAGGGCAATAG; *Mcpt4* (mMCP-4, amplicon 128 bp) Fw 5'-TGAGGGAGGTGAACTGAGA, Rev 5'-TCCAGAGTCTCCCTGTATGTC; *Cmal* (mMCP-5, amplicon 74 bp) Fw 5'-AGCACAAAGCTGGAGAGAT, Rev 5'-TCCAGATAGGCCATGTAGGG. *Hprt1* (24) (HPRT, amplicon 124 bp) was used as reference gene: Fw 5'-TGGATACAGGCCAGAC TTTGTT, Rev 5'-CAGATTCAACTTGCCTCATC.

Statistical analysis

Statistical analysis was performed using a Student's t-test. $P < 0.05$ was considered statistically significant.

Online Supporting Information

See the online Supporting Information for additional description of methods.

Results

Generation of mouse basophils *in vitro* using conditional Hoxb8

Freshly isolated lineage-negative progenitors from bone marrow of C57BL/6 mice were incubated for 24 h in IL-3-containing medium prior to transduction with lentiviral

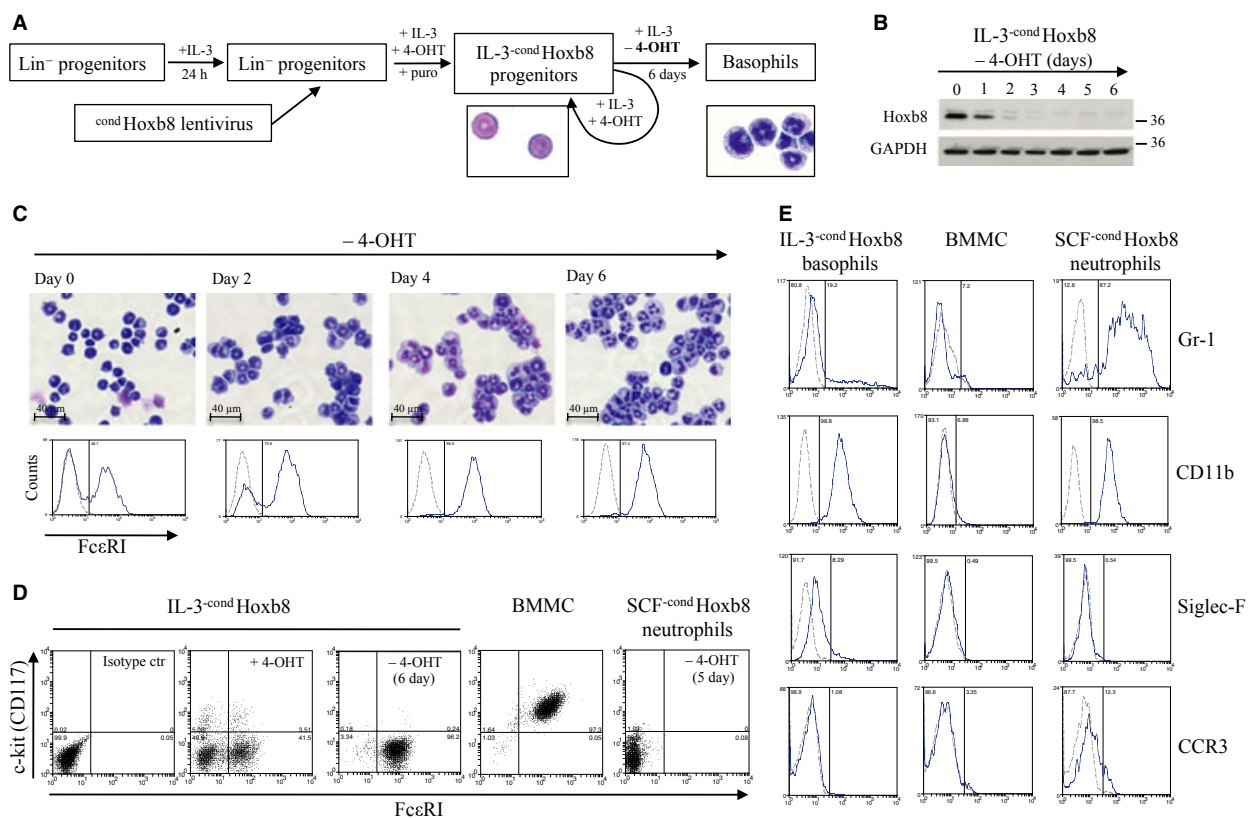


Figure 1 Generation of IL-3^{cond}Hoxb8 basophils. (A) Schematic overview. (B) Western blot analysis of exogenous Hoxb8 protein levels upon removal of 4-OHT. Re-probing with anti-GAPDH antibody served as loading control. Representative immunoblots of at least three independent experiments are shown. (C) H&E staining and FcεRI surface expression analysis of differentiating (-4-OHT) IL-3^{cond}Hoxb8 cells. Representative examples of at least three independent experiments are shown. Isotype controls

are shown as dotted lines. (D) Representative dot plots ($n \geq 3$) of surface c-kit and FcεRI expression of IL-3^{cond}Hoxb8 progenitors, differentiated basophils, differentiated SCF^{cond}Hoxb8 neutrophils, and bone marrow-derived mast cells (BMMC). (E) Flow cytometric analysis for the indicated surface markers of cells as in (D). Representative histograms of at least three independent experiments are shown; isotype controls are shown as dotted lines.

particles carrying an inducible expression system for Hoxb8 (Fig. 1A). Hoxb8 overexpression was induced by addition of 100 nM 4-OHT and successfully transduced cells selected with puromycin. Outgrowing cell lines, termed IL-3^{-cond}Hoxb8, were cycling and maintaining their progenitor status over prolonged periods (up to several months) in culture. IL-3^{-cond}Hoxb8 progenitors displayed a bipolar surface staining for FcεRI, and lines that stained negative for c-kit (CD117) were selected for further analysis (Fig. 1C,D).

In order to drive IL-3^{-cond}Hoxb8 progenitors into differentiation, 4-OHT was removed and cells reseeded at low density in the presence of IL-3. This resulted in the rapid loss of Hoxb8 protein within 2 days (Fig. 1B) and appearance of mature granulocytes within 6 days (Fig. 1C). The overall cellular expansion during differentiation was six- to eightfold, whereas cells stopped to cycle at around day 4 (not shown). The percentage of FcεRI⁺c-kit⁻ cells increased gradually during differentiation and was routinely above 95% at day 4 (Figs. 1C and S1). The surface marker staining profile on differentiated cells was FcεRI⁺c-kit⁻CD123⁺CD88⁺Gr-1⁻CD11b⁺Siglec-F^{low}CCR3⁻, matching known mouse basophil markers (25) and clearly distinguishing them from bone marrow-derived mast cells (BMMC), neutrophils, or eosinophils (Figs. 1E, 4A, 5A and not shown). Terminally differentiated cells underwent spontaneous apoptosis (Fig. 2A). Of note, spontaneous apoptosis of mature IL-3^{-cond}Hoxb8 basophils was significantly slower than of mature SCF^{-cond}Hoxb8 neutrophils, correlating with higher levels of anti-apoptotic BCL-2 in 5-day differentiated basophils compared with 5-day differentiated neutrophils (Fig. 2A–C).

To further analyze the FcεRI bipolar staining of IL-3^{-cond}Hoxb8 progenitors, we sorted FcεRI⁺ and FcεRI⁻

populations by flow cytometry. Both FcεRI⁺c-kit⁻ and FcεRI⁻c-kit⁻ populations rapidly re-adapted a bipolar FcεRI surface staining and were able to differentiate into basophils to comparable extents, with no evidence for enhanced cell death in either differentiating population (Fig. S2A and not shown). Addition of monoclonal IgE to IL-3^{-cond}Hoxb8 progenitors did not shift the percentage of FcεRI-positivity (Fig. S2B).

IL-3^{-cond}Hoxb8 basophils selectively express basophil-specific mouse mast cell proteases

Ugajin et al. showed that primary mouse basophils specifically express high levels of the mouse mast cell proteases *Mcpt8* (mMCP-8) and *Prss34* (mMCP-11), but no (or very little of the) mast cell-specific *Mcpt4* (mMCP-4) and *Cmal* (mMCP-5), respectively (3). As shown in Figure 3A, qPCR analysis confirmed that differentiated IL-3^{-cond}Hoxb8 basophils express high levels of *Prss34* and *Mcpt8*, but non-detectable (or very low) levels of *Mcpt4* and *Cmal*. On the other hand, BMMC expressed *Mcpt4* and *Cmal*, but no *Prss34* or *Mcpt8* (Fig. 3B). Analysis of mMCP expression during differentiation revealed that *Prss34* and *Mcpt8* are already expressed at the progenitor stage and further increase during basophil differentiation, whereas *Mcpt4* and *Cmal* remain low (Fig. 3C).

In summary, phenotypic analyses as well as qPCR analysis of basophil and mast cell-specific mMCPs strongly indicate that in the presence of IL-3 conditional Hoxb8-immortalized progenitors can be terminally differentiated in large amounts into basophils.

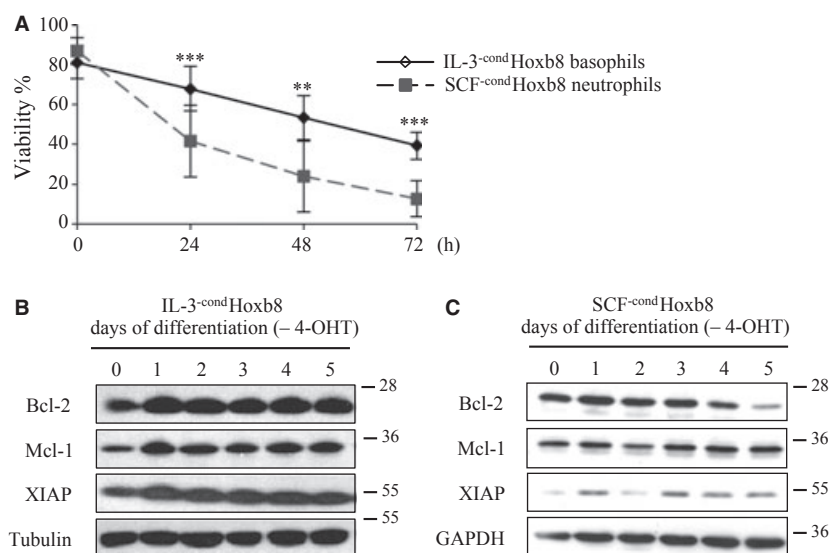


Figure 2 End-differentiated IL-3^{-cond}Hoxb8 basophils undergo spontaneous apoptosis in culture. (A) Analysis of spontaneous cell death (in the presence of cytokines) in 6-day differentiated IL-3^{-cond}Hoxb8 basophils and SCF^{-cond}Hoxb8 neutrophils, respectively. Values represent means $\pm$ SD of at least

three independent experiments. ** $P < 0.01$; *** $P < 0.005$. Western blot analysis (representative immunoblots of ≥ 2 independent experiments) for the indicated proteins in differentiating IL-3^{-cond}Hoxb8 basophils (B) and SCF^{-cond}Hoxb8 neutrophils (C).

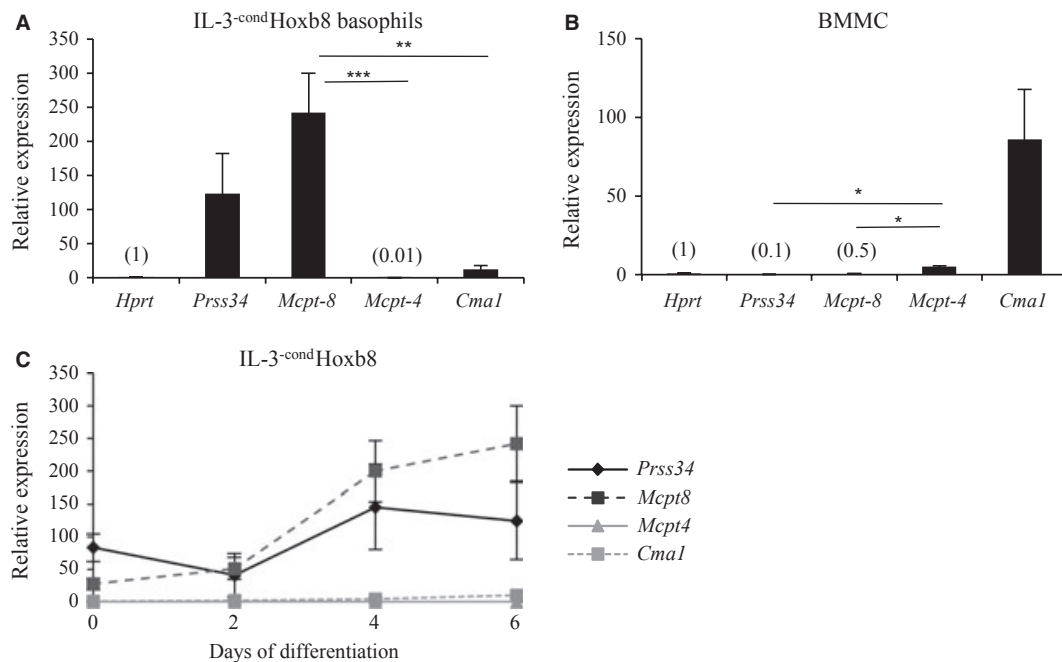


Figure 3 IL-3^{-cond}Hoxb8 basophils express high levels of basophil-specific mouse mast cell proteases. Quantitative RT-PCR (qPCR) analysis of basophil-specific (*Prss34*, *Mcpt8*) and mast cell-specific (*Mcpt4*, *Cma1*) mouse mast cell proteases in mature (A) and differenti-

ating (C) IL-3^{-cond}Hoxb8 basophils and bone marrow-derived mast cells (BMMC) (B). Data represent means $\pm$ SEM of 4 (A, C) and three (B) independent experiments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$. *Hprt* was used as reference gene.

Activated IL-3^{-cond}Hoxb8 basophils degranulate, release histamine, and secrete Th2 cytokines and leukotriene C4

To test whether Fc ϵ RI expressed on IL-3^{-cond}Hoxb8 basophils is functional, we provoked IgE-dependent degranulation and quantified the release of N-acetyl- β -D-hexosaminidase and histamine from intracellular granules. We also tested the effect of complement component C5a, as IL-3^{-cond}Hoxb8 basophils expressed C5a receptor, CD88 (Fig. 4A). Both N-acetyl- β -D-hexosaminidase and histamine were specifically released after Fc ϵ RI crosslinking, as well as after stimulation with C5a (100 nM), in IL-3^{-cond}Hoxb8 basophils (Fig. 4B,C). Whereas intracellular histamine levels were low in progenitors and increased during differentiation (Fig. 4D), N-acetyl- β -D-hexosaminidase was already present in progenitors, even though release of the enzyme upon activation was only detectable at later stages of differentiation (Fig. 4B and not shown).

Basophils are recognized as an important source of Th2 type cytokines. As reported for human basophils, differentiated IL-3^{-cond}Hoxb8 basophils secreted IL-4 and IL-13 in response to Fc ϵ RI crosslinking or C5a (Fig. 4E–G). IL-3^{-cond}Hoxb8 basophils also generated the lipid mediator leukotriene C4 (LTC4) upon Fc ϵ RI but not C5aR stimulation (Fig. 4H). Taken together, we demonstrate that IL-3^{-cond}Hoxb8 basophils are functional and respond to IgE-dependent activation in an identical manner as reported for primary mouse and human basophils. They also respond to the IgE-independent trigger C5a in a fashion very similar to primary human basophils (26).

Mechanisms of IL-3 withdrawal-induced apoptosis

Both IL-3^{-cond}Hoxb8 progenitors and basophils expressed high levels of the IL-3 receptor CD123 on their surface and rapidly underwent apoptosis upon IL-3-withdrawal (Fig. 5A, B). Procasase-9 was rapidly processed and effector caspases-3 and caspases-7 activated upon IL-3 removal, whereas X-linked inhibitor of apoptosis (XIAP), an anti-apoptotic protein known to directly bind and inhibit active caspase-3, caspase-7, and caspase-9 (27), was downregulated (Fig. 5D). Interestingly, the processed and partially active p19 large subunit of caspase-3 was already present in lysates from healthy cells and rapidly further processed into the highly active p17 subunit upon IL-3 removal (Fig. 5D). In contrast to cytokine withdrawal in BMMC, cytokine-deprived IL-3^{-cond}Hoxb8 progenitors and basophils could only be protected from apoptosis by addition of high (100 μ M) concentrations of the pan-caspase inhibitor Q-VD-OPh (Fig. 5B,C).

Interleukin-3 withdrawal resulted in a rapid increase in the pro-apoptotic BH3-only protein BCL-2-interacting mediator of cell death (BIM) in differentiated basophils, whereas BIM protein levels were low in Hoxb8-expressing progenitors (Fig. 6A). *Bim* upregulation was confirmed by qPCR analysis, which further revealed that the mRNA encoding for another BH3-only protein, *Puma*, was similarly induced by IL-3-deprivation (Fig. 6B).

Prosurvival IL-3 signaling in human basophils was reported to involve the activity of PI3K. In accordance, we

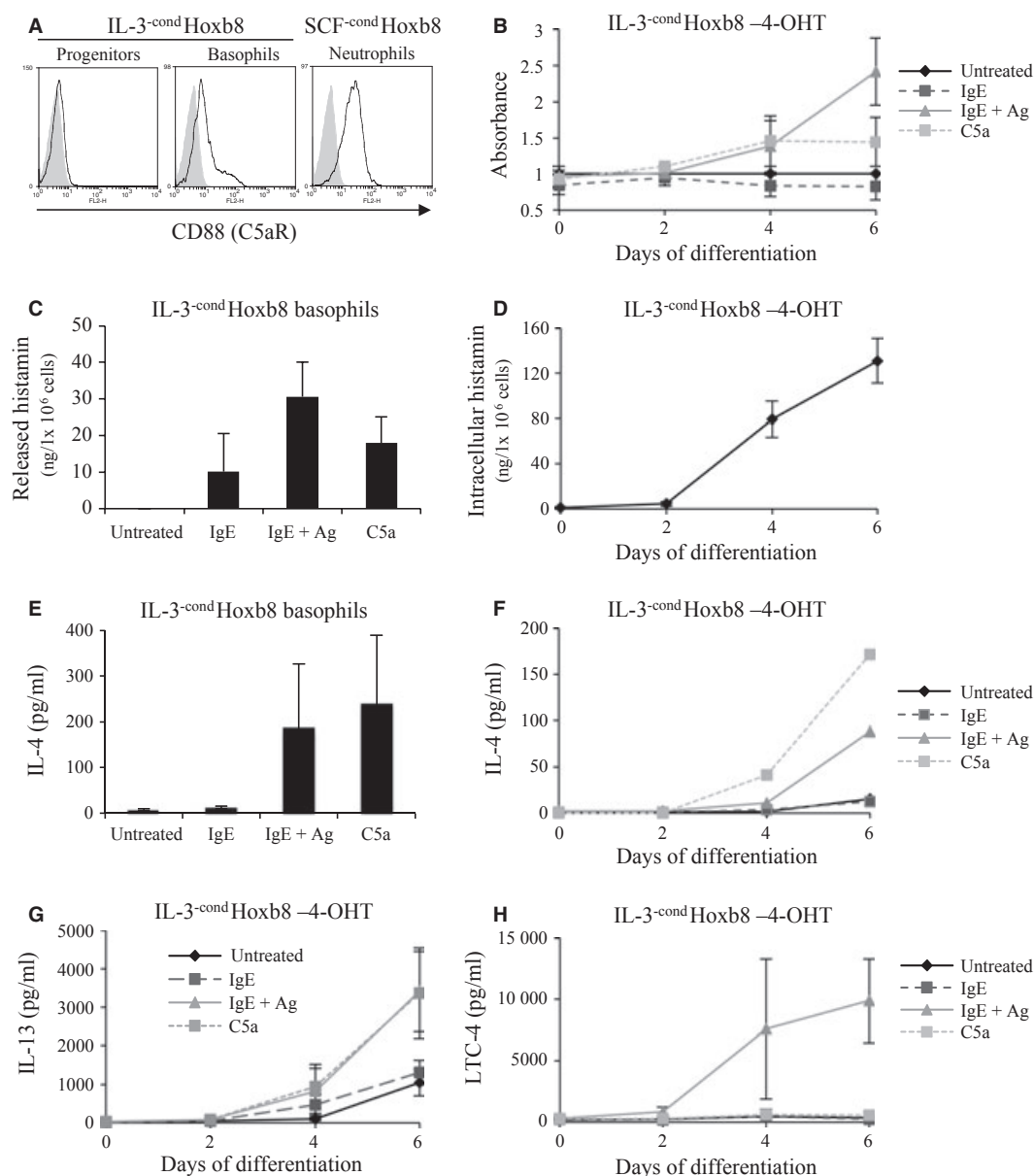


Figure 4 IL-3^{-cond}Hoxb8 basophils release granule contents, Th2-type cytokines and LTC₄ upon activation. (A) CD88 (C5aR) is expressed on the surface of differentiated IL-3^{-cond}Hoxb8 basophils but not of progenitors. SCF^{-cond}Hoxb8 neutrophils served as positive control. Isotype controls are shown in gray. (B) N-acetyl-β-D-hexosaminidase release from IL-3^{-cond}Hoxb8 basophils at indicated stages of differentiation upon FcεRI crosslinking or activation with C5a for 30 min. (C) Histamine release from differentiated basophils activated as in (B) for 30 min. (D) Intracellular histamine levels of

untreated IL-3^{-cond}Hoxb8 basophils at indicated stages of differentiation. (E) Released IL-4 from 5-day differentiated IL-3^{-cond}Hoxb8 basophils upon FcεRI crosslinking or stimulation with C5a for 24 h. IL-4 (F, representative example) and IL-13 (G) release from differentiating IL-3^{-cond}Hoxb8 basophils stimulated as in (E) for 24 h. (H) Released LTC₄ from cells stimulated as in (E) for 30 min. Values represent means ± SEM of ≥ 4 (B, E) and three (C, D, G, H) independent experiments.

observed increased cell death when IL-3^{-cond}Hoxb8 progenitors and basophils, in the presence of IL-3, were treated with the PI₃K inhibitors LY29004 (20 μM) or high concentrations (1 μM) of Wortmannin (Fig. 6C and not shown). However, Wortmannin at 100 nM, a concentration highly specific for PI₃K (28), did not induce apoptosis (Fig. 6C). LY294002, at

the concentration used, is known to also inhibit proviral integration site for Moloney murine leukemia virus 1 (PIM-1), a Ser/Thr kinase downstream of IL-3 in human basophils (29). PIM-1 kinase activity is not subjected to strong posttranslational regulation and thus correlates with PIM-1 protein levels (30). We found that PIM-1 protein levels rapidly

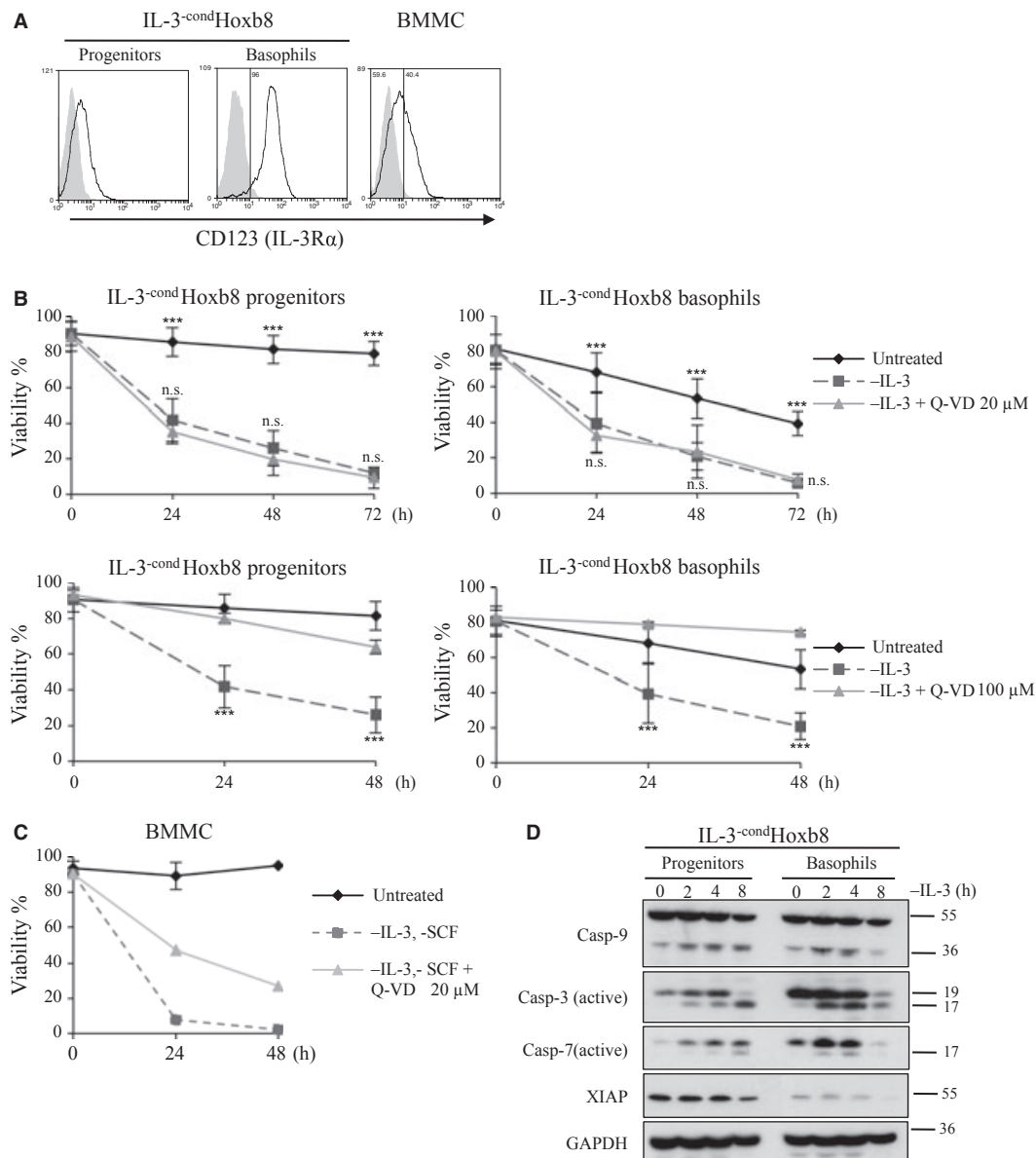


Figure 5 IL-3^{-cond}Hoxb8 cells depend on IL-3 for their survival. (A) Cell surface expression of CD123 (IL-3Rα) on IL-3^{-cond}Hoxb8 progenitors and basophils. Bone marrow-derived mast cells (BMMC) are shown as positive control. Representative histograms of three independent experiments are shown; isotype controls are shown in gray. Survival curves of IL-3^{-cond}Hoxb8 progenitors and 6-day differentiated basophils (B) and BMMC (C)

decreased in IL-3^{-cond}Hoxb8 progenitors and basophils upon IL-3-removal (Fig. 6A).

Taken together, we show that the survival of both IL-3^{-cond}Hoxb8 progenitors and basophils critically depends on IL-3. Upon IL-3-deprivation, rapid loss of the prosurvival kinase PIM-1 and transcriptional upregulation of the pro-apoptotic BH3-only genes *Bim* and *Puma* are observed, correlating with induction of caspase-dependent apoptosis.

upon cytokine deprivation with or without addition of the pan-caspase inhibitor Q-VD-OPh. Values represent means ± SD of at least three independent experiments. ****P* < 0.005. (D) Western blot analysis of the indicated proteins in IL-3-deprived progenitors and 6-day differentiated IL-3^{-cond}Hoxb8 basophils. Presented immunoblots are representative of two independent experiments.

Discussion

The characterization of mouse basophils by functional, biochemical, and genetic methods is close to impossible due to their low frequency and the excessive numbers of animals needed for even simplest experiments. Here, we describe a novel cellular model for the study of mouse basophils. The method is based on the report by Wang et al. describing the *ex vivo* production of neutrophils and macrophages using

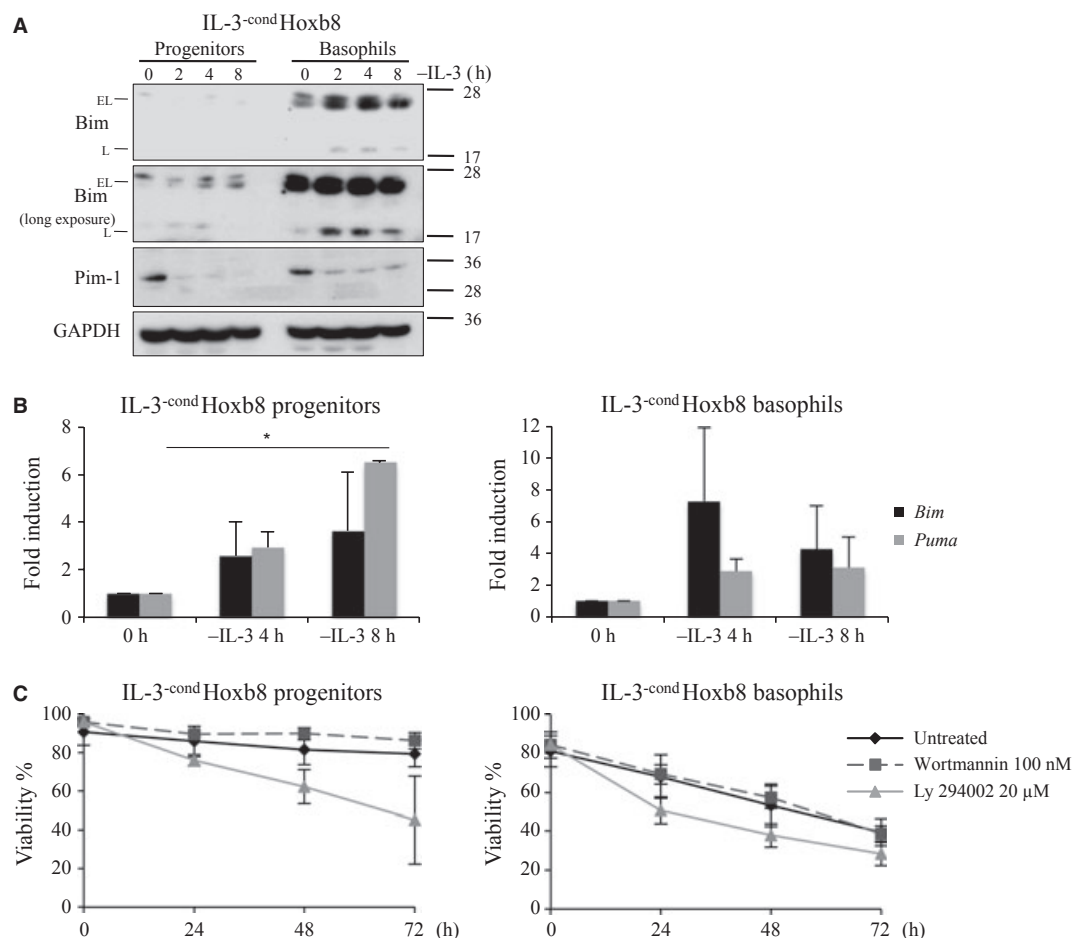


Figure 6 Initiation of IL-3 withdrawal-induced apoptosis. (A) Western blot analysis of the indicated proteins in IL-3-deprived IL-3^{-cond}Hoxb8 progenitors and 6-day differentiated basophils. Presented immunoblots are representative of two independent experiments. (B) Quantitative RT-PCR (qPCR) analysis of *Bim* and *Puma*

levels upon IL-3-deprivation. Values represent means $\pm$ SEM of three independent experiments. (C) Survival curves of IL-3^{-cond}Hoxb8 progenitors and basophils treated with Wortmannin or Ly294002 in the presence of IL-3. Values represent means $\pm$ SD of three independent experiments.

conditional Hoxb8 (21). The major difference to Wang et al. is that Hoxb8-mediated immortalization was achieved in the presence of IL-3 instead of SCF, thereby favoring commitment to the basophil lineage. Also, instead of a Hoxb8-estrogen receptor fusion protein, we conditionally expressed wild-type Hoxb8 using a lentiviral system (23) that lacks any exogenous Hoxb8 expression in the absence of 4-OHT.

IL-3^{-cond}Hoxb8-immortalized cell lines maintain their growth potential and progenitor status for prolonged times. Cell lines can be potentially generated from any genetically modified mouse strain, given that it contains multipotent myeloid progenitors in the bone marrow or fetal liver. The latter is of particular interest for strains presenting with severe phenotypes. Another advantage over primary cells is the possibility to genetically manipulate IL-3^{-cond}Hoxb8 progenitors by standard protocols (e.g., transgenesis, RNA interference). Near-unlimited amounts of mature basophils can be generated 'on demand' and in high purity upon shutdown of exogenous Hoxb8 expression, enabling or facilitating experiments requiring larger cell numbers. Besides known

markers (25), we show that differentiated basophils express C5aR (CD88) and Siglec-F, the closest functional paralog to human Siglec-8, which is expressed on human basophils (31).

Like primary human and mouse basophils, IL-3^{-cond}Hoxb8 basophils release preformed granule enzymes and histamine, and newly generated LTC₄, IL-4, and IL-13 upon IgE-mediated activation. As the cells are constantly kept in an IL-3-rich environment, we found increased IL-13 levels in the supernatants of unstimulated basophils (Fig. 4G); this is in accordance with findings in human primary basophils (26). Interestingly, even though a high percentage of immature basophils express surface FcεRI and contain good amounts of granule enzymes, efficient IgE-mediated release was only apparent in mature cells. A similar unresponsiveness was found for the production of LTC₄ and Th2 cytokines, indicating a possible lack of FcεRI signal coupling in immature basophils, requiring further investigation. We also tested the effect of the complement-derived chemotaxin C5a known to activate human basophils. Indeed, we found that mature IL-3^{-cond}Hoxb8 basophils express C5a

receptor and strongly respond to C5a, comparable to IgE-mediated activation. C5a not only induced degranulation but also promoted the production of IL-4 and IL-13 indicating that such an innate, antigen-independent pathway for the production of these crucial Th2 cytokines may also operate in mice. It will be interesting to determine whether complement activation plays a role in the induction and/or amplification of certain Th2-mediated inflammatory conditions. Interestingly, C5a did not induce an LTC4 response despite the presence of IL-3 for unknown reasons requiring further investigation.

It is controversially discussed how basophils develop from hematopoietic progenitors. In humans, bipotent eosinophil/basophil and megakaryocyte/basophil progenitors have been proposed (32). In the mouse, however, a strong lineage relationship exists between basophils and mast cells where IL-3 is a major differentiation cytokine for both lineages. This is underlined by the outgrowth of both basophils (minor and transient population) and mast cells (dominating after 3 weeks in culture) upon incubation of bone marrow with IL-3 and the description of a common basophil/mast cell progenitor in the mouse (20, 33, 34). The IL-3^{-cond}Hoxb8 basophil model may represent a useful tool to study individual stages of basophil development as well as the developmental relationship between mouse basophils and mast cells.

As reported for human basophils, we observed spontaneous apoptosis of end-differentiated basophils to be significantly slower than of neutrophils (Fig. 2A), a finding supported by the very high levels of BCL-2 in mature basophils compared with hardly detectable BCL-2 in mature neutrophils (Fig. 2B,C and (16)). IL-3^{-cond}Hoxb8 progenitors and basophils both critically depended on IL-3 for their survival as IL-3 deprivation led to rapid apoptosis. Somewhat surprisingly, caspase-3 was already partially activated in healthy cells. Interestingly, the basophil-specific mast cell protease mMCP-8 is highly homologous with granzyme B (35), and it will be interesting to test the potential of mMCP-8 to directly activate procaspases. We found that IL-3 deprivation leads to a rapid upregulation of the pro-apoptotic BH3-only proteins BIM and PUMA, which is in accordance with reports in other IL-3-dependent systems (17–19). Hoxb8 seems to negatively regulate Bim expression, as indicated by the lack of BIM in IL-3^{-cond}Hoxb8 progenitors (Fig. 6A and Salmanidis et al., *in revision*). Our data further support recent reports on an essential role for PIM-1 kinase in prosurvival IL-3 signaling in human basophils (16) and *Pim-1* downregulation in a murine system (36).

No suitable mouse or human basophil cell lines exist to date, and, with the exception of the slow growing LAD2 mast cell line, there are no human cell lines expressing the high-affinity IgE receptor. Comparable, IgE receptor positive

cell lines of human origin would therefore be of great interest, also with respect to potential applications in diagnostics and for drug testing purposes.

In summary, we describe a novel and potentially useful cellular model to produce large amounts of mature mouse basophils *in vitro*. As with any model system, *in vitro* differentiated basophils cannot be seen as a general substitute for primary cell analysis or *in vivo* studies. However, given the rare frequency of basophils *in vivo*, we are convinced that the here described model constitute a powerful tool to dissect cellular and molecular aspects of basophil biology and may prove a valid alternative method to isolating primary basophils in many respects.

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Author contributions

UG and TK designed and performed research and wrote the manuscript. TR, NE, and DB performed research. CAD provided critical inputs, designed, and performed research. MS, GB, PGE, and HUS provided critical tools and intellectual inputs.

Conflict of interest

The authors declare no conflict of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Differentiation IL-3^{-cond}Hoxb8 progenitors into mature basophils within six days upon removal of 4-OHT.

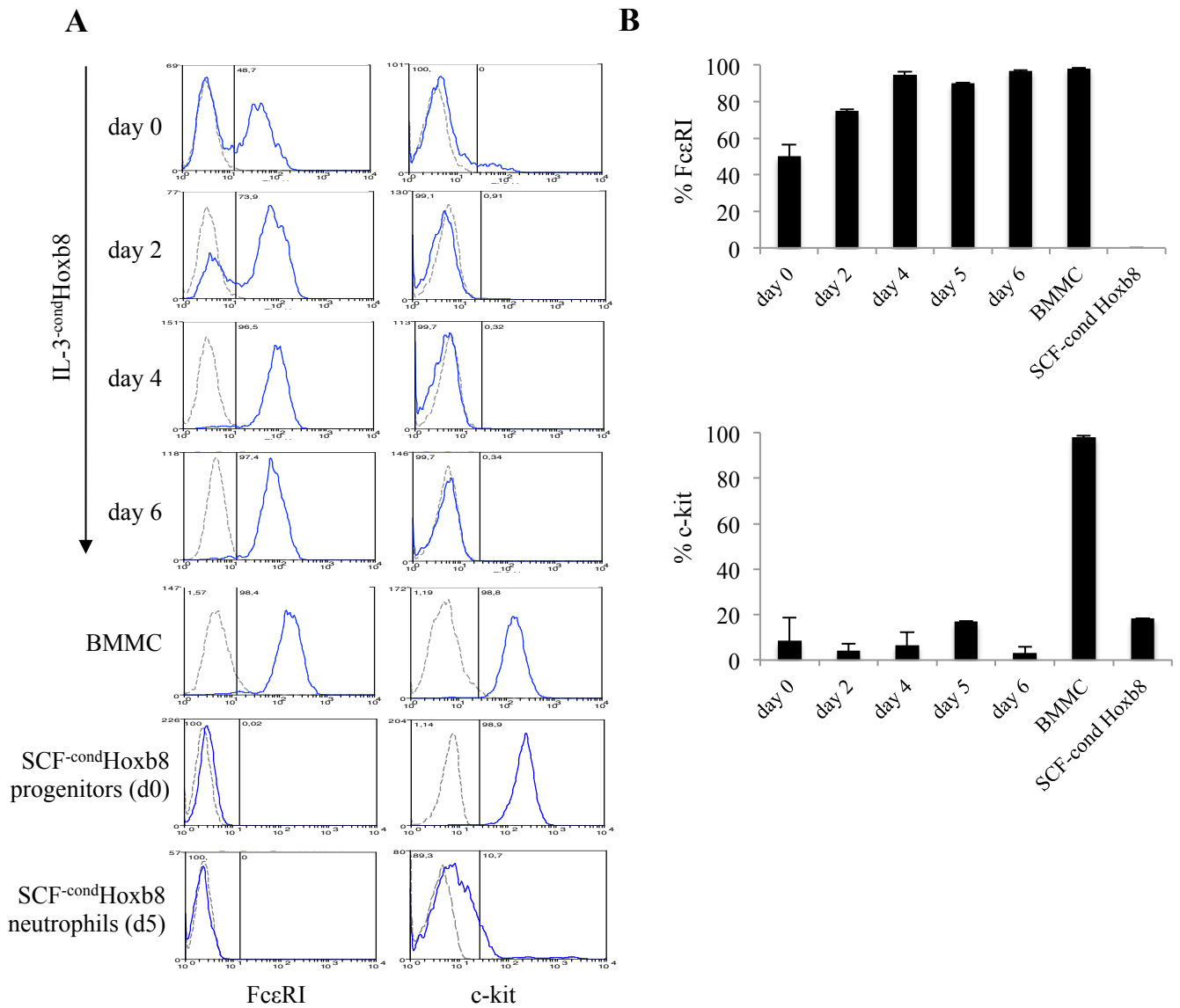
Figure S2. Basophil-committed IL-3^{-cond}Hoxb8 progenitors display bipolarity in FcεRI surface expression.

Data S1. Materials and methods.

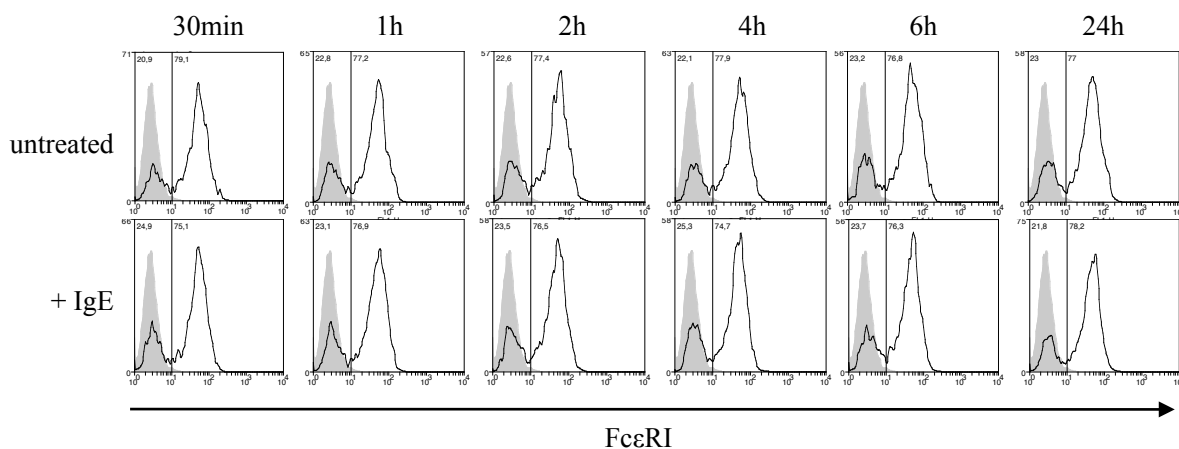
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Supplementary Figure S1 Differentiation IL-3-^{cond}Hoxb8 progenitors into mature basophils within six days upon removal of 4-OHT. (A) Flow cytometric analysis of FcεRI and c-kit (CD117) surface staining of differentiating IL-3-^{cond}Hoxb8 basophils, mature bone marrow-derived mast cells (BMMC) and 0 and 6 day-differentiated SCF-^{cond}Hoxb8 neutrophils. Representative histograms of at least 3 independent experiments are shown; isotype controls are presented as dotted lines. (B) Quantitative analysis of FcεRI and c-kit (CD117) surface staining on differentiating IL-3-^{cond}Hoxb8 basophils.



Supplementary Figure S2 Basophil-committed IL-3^{cond}Hoxb8 progenitors display bipolarity in FcεRI surface expression. (A) Flow cytometric analysis of FcεRI surface expression levels on FACS sorted FcεRI⁻ and FcεRI⁺ subpopulations over time. Both subpopulations differentiate into mature basophils upon removal of 4-OHT. (B) IL-3^{cond}Hoxb8 progenitors were treated for the indicated times with monoclonal IgE anti-TNP and surface expression levels of FcεRI were determined by flow cytometry. Isotype controls are shown in gray.

ONLINE SUPPLEMENTARY MATERIAL

SUPPLEMENTARY MATERIALS AND METHODS

Cloning and production of inducible Hoxb8 lentiviral particles

Inducible Hoxb8 Lentiviral particles were produced in HEK 293T cells using X-tremeGENE HP transfection reagent (Roche Diagnostics, Rotkreuz, CH). A total of 5 µg DNA was transfected per 10-cm tissue culture dish, using a ratio pMD2.VSV-G (envelope proteins) : pCMVδR8.2 (packaging elements) : pF-5xUAS-Hoxb8(mm)-Sv40puroGev16 of 2 : 5 : 3. Virus-containing supernatant was collected up to 48 hours after transfection, passed through a 0.2 µm filter and used fresh for infection or stored in aliquots at -80°C.

Generation of SCF-dependent conditional Hoxb8-immortalized neutrophil/macrophage progenitor lines

The protocol largely follows the method described by Wang et al. (1), with the modification of using a different inducible system for Hoxb8 expression (2). In short, C57BL/6 wildtype mice were sacrificed and bone marrow harvested from the femurs. Hematopoietic progenitor cells were enriched by magnetic cell separation using a mouse lineage depletion cocktail (BD IMagTM, BD Biosciences) following the manufacturer's instructions. $2-5 \times 10^5$ lineage-negative cells were incubated for 24 h in complete RPMI Medium (RPMI 1640/GlutaMAXTM, 10% FCS, 1% Penicillin/Streptomycin, 50 µM 2-mercaptoethanol) in the presence of stem cell factor, SCF (added as 10% of CHO/SCF(mm) cell conditioned medium as a source for murine SCF). Cells were then transduced with pF-5xUAS-Hoxb8(mm)-SV40puro-GEV16 lentiviral particles and Hoxb8 expression induced by addition of 100 nM 4-OHT. Successfully transduced cells were positively selected in the presence of 1.0 µg/ml puromycin for a minimum of 3 weeks and cells maintained in SCF

containing medium (added as 5% of CHO/mSCF cell conditioned medium). The resulting immortalized cell lines were termed SCF-^{cond}Hoxb8. For differentiation into neutrophils, 4-OHT was removed by 2 wash steps and cells seeded at 2×10^4 /ml in complete RPMI medium containing SCF. Mature neutrophils were obtained after 5-6 days.

Generation of bone marrow-derived mast cells

Bone marrow-derived mast cells (BMMC) were generated as described by Marsden et al. (3) In short, bone marrow was harvested from femurs of C57BL/6 wildtype mice and cultured at a density of 1×10^6 cells/ml in complete RPMI medium (see above) supplemented with 10% of WEHI3B cell conditioned medium as a source of murine IL-3 (corresponding to 200-500 pg/ml as determined by ELISA, BioLegend) and replated weekly for 3 weeks to exclude adherent cells. In the fourth week of culture, SCF was added (added as 10% of CHO/mSCF conditioned supernatant). Cells were subsequently maintained in SCF plus IL-3 and experiments were performed within 4 weeks.

FACS surface staining and determination of apoptosis

1×10^5 cells were stained with the following antibodies diluted in buffered salt solution containing 2% FCS, 20% anti-CD16/CD32 hybridoma conditioned medium (clone 2.4G2), 1% normal rat serum and 10 mM NaN₃: anti-FcεRI (MAR-1, BioLegend), c-kit (CD117) (2B8, BioLegend), Gr-1 (RB6-8C5, BioLegend), CD11b (M1/70, BioLegend), CD88 (20/70, BioLegend), CD123 (5B11, BioLegend), Siglec-F (E50-2440, BD Bioscience), CCR3 (TG14, BioLegend). Cells were analyzed by flow cytometry on a FACS CaliburTM (BD Biosciences). For morphologic analyses, cytopins were stained using Diff-Quik (Baxter) and photos were acquired with a light microscope connected to a digital camera (Axiovert 35 and AxioCam, Carl Zeiss,

Jena, Germany). Cellular viability was assessed by flow cytometry using GFP-Annexin V and propidium iodide (PI) double staining for early and late apoptotic cells. Purification of recombinant His₆-GFP-Annexin V is described in (4)

Cytokine, LTC₄ and histamine measurements

IL-3^{-cond}Hoxb8 cells at various stages of differentiation, or BMMC, were activated by IgE-crosslinking (see above) or by addition of recombinant mouse C5a (100 nM) and supernatants harvested after indicated times. Viability of cells was assessed by flow cytometry using GFP-Annexin V/PI exclusion. IL-4 and IL-13 cytokines were measured by ELISA (BioLegend) and analyzed on a spectrometer plate reader (SpectraMax M2^e, Molecular Devices, λ =405 nm). Histamine levels were quantified by an automatic fluorimetric method, as described in (5). LTC₄ was quantified by ELISA (Bühlmann laboratories AG, Schönenbuch, CH). Cell viabilities were determined for all conditions and were in all cases comparable between treated and untreated cells (not shown).

Gel electrophoresis and immunoblotting

1-5x10⁶ cells were washed with PBS and lysed in modified RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% Triton-X100, 0.5% Na-deoxycholate, 0.1% SDS, 2mM EDTA supplemented with 0.5 mg/ml pepstatin A and complete protease inhibitor cocktail (Roche Diagnostics, Rotkreuz, CH). Protein concentration was determined by BCA protein assay (Thermo Scientific) and denatured in reducing Lämmli buffer prior to separation by SDS/PAGE gel electrophoresis. Proteins were blotted on PVDF transfer membranes (Merck Millipore) and subsequently probed with the following primary antibodies: mouse anti-Hoxb8 (F48, Abnova), mouse anti-GAPDH (6C5, Merck Millipore), rat anti-Bim (3C5, Enzo Life Sciences, Lausen, CH), rabbit anti-caspase-3 (#9662 + #9661) and rabbit anti-caspase-7 active subunit

(#9491, Cell Signaling Technology), mouse anti-PIM-1 (12H8, Santa Cruz Biotechnology), mouse anti-XIAP (2F1, MBL). Secondary antibodies coupled to horseradish peroxidase were from Jackson ImmunoResearch Laboratories and signals developed by enhanced chemiluminescence (ECL western blotting substrate, Thermo Scientific and Luminata Forte, Merck Millipore) and detected on photosensitive film (ECL Hyperfilm, GE Healthcare).

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LEGENDS TO SUPPLEMENTARY FIGURES

Supplementary Figure S1 Differentiation IL-3^{-cond}Hoxb8 progenitors into mature basophils within six days upon removal of 4-OHT. (A) Flow cytometric analysis of FcεRI and c-kit (CD117) surface staining of differentiating IL-3^{-cond}Hoxb8 basophils, mature bone marrow-derived mast cells (BMMC) and 0 and 6 day-differentiated SCF^{-cond}Hoxb8 neutrophils. Representative histograms of at least 3 independent experiments are shown; isotype controls are presented as dotted lines. (B) Quantitative analysis of FcεRI and c-kit (CD117) surface staining on differentiating IL-3^{-cond}Hoxb8 basophils.

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