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IFN Consensus Sequence Binding Protein (Icsbp) Is Critical for Eosinophil Development

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IFN consensus sequence binding protein (Icsbp) (IFN response factor-8) is a hematopoietic transcription factor with dual functions in myelopoiesis and immunity. In this study, we report a novel role of Icsbp in regulating the development of eosinophils. Loss of Icsbp in mice leads to a reduction of eosinophils in different tissues. During parasite infection with the nematode *Nippostrongylus brasiliensis*, Icsbp-deficient mice fail to mount eosinophilia despite a vigorous IL-5 response. Numbers of phenotypically defined eosinophil progenitors are decreased and those progenitors have, on a per-cell basis, reduced eosinophil differentiation potential. The transcription factor *Gata1*, crucial for eosinophil development, is reduced expressed in committed eosinophil progenitors in wells as mature eosinophils. These findings identify Icsbp as a novel transcription factor critical for the development of the eosinophil lineage. *The Journal of Immunology, 2008, 181: 5045–5053.*

Eosinophils, synonymous to eosinophil granulocytes, account only for a minute fraction of leukocytes (around 1–3% in humans and mice) in the peripheral blood. Eosinophils are involved in the immunity against parasite infections and play a major role in the pathology of diverse allergic diseases (1–4). The most common cause of eosinophilia in humans worldwide is helminthic infection. Contrasting the knowledge about eosinophils in different diseases, little is known about the ontogeny of eosinophils, their precursors and maturation steps and the molecular control of eosinophil lineage commitment. The recent description of an eosinophil lineage-committed progenitor helps to further elucidate eosinophil development (5). At the molecular level, several studies have implicated *Gata1* (6, 7), and the C/EBP family, notably α (8), β (9), and κ (10, 11) as critical transcriptional regulators of transcripts encoding typical proteins of the eosinophil as well as the formation of mature eosinophils.

Icsbp, IFN consensus sequence binding protein, synonymous to Irf8 (IFN response factor-8) is an IFN-γ-induced transcription factor that regulates IFN responsive genes (12–14). In contrast to most other members of the Irf-family, Icsbp is preferentially expressed in the hematopoietic system. Proteins of the Irf-family bind to the IFN-stimulated response element and control genes harboring this element within their promoters (14). Furthermore, Icsbp and Irf4 interact with Pu.1, another important hematopoietic transcription factor for the development of eosinophils on a specific DNA (EICE) sequence (15, 16).

Targeted deletion of Icsbp in mice results in an increased susceptibility toward certain viruses (17), intracellular bacteria (18), and parasites (19). Moreover, loss of Icsbp in mice leads to a myeloproliferative syndrome characterized by accumulation and expansion of mature neutrophil granulocytes and myeloid progenitor cells at the expense of monocytes and macrophages. This defect has been localized to the committed granulocyte-monocyte progenitor (GMP) (20), but the molecular mechanisms underlying the aberrant myeloid development are not understood today.

We noted a reduction of eosinophils in the peripheral blood and bone marrow in Icsbp-deficient mice. In this study, we provide insight into the cellular and molecular basis for this defect in eosinophil numbers. To this end, we have examined the development of eosinophils in Icsbp-deficient mice under normal and parasite-infected conditions. We observed that Icsbp is a critical transcription factor for the regulation of the development of eosinophils under physiological but especially under pathological conditions. Challenged with the parasite *Nippostrongylus brasiliensis*, Icsbp-deficient mice fail to develop eosinophilia. This is due to an abnormal developmental potential of the eosinophil progenitor (EoP) in the absence of Icsbp.

Materials and Methods

Animals and parasites

Icsbp-deficient mice and wild-type littermates (background C57BL/6 and C57BL/6 × 129Sv) have been previously described (17). Mice were bred and maintained under specific pathogen-free conditions in the Forschungseinrichtung für Experimentelle Medizin der Charité, Universitätsmedizin Berlin, Campus Benjamin Franklin. Sex- and age-matched mice were 8 to 16 wk of age when used. Each experiment was repeated at least three times unless otherwise indicated. A mouse-adapted strain of *N. brasiliensis* was maintained and passed in Lewis rats at the Zentrum für Infektionsforschung, Universität Wuerzburg, Wuerzburg, Germany (Dr. Klaus Erb). Feces were collected from stock rats and served as a source of L3 after incubation of the fecal slurry. In brief 750 L3 larvae were injected s.c. to establish infection. All animal experiments were approved by the local authorities according to the German Federal Animal Protection Act.
Peritoneal lavage, immunostaining, and isolation of eosinophils

Mice were injected with 3% thioglycollate in PBS 72 h before harvesting of peritoneal cells. The peritoneum was flushed with cold PBS and cytospins were prepared, stained with May-Gruenwald-Giemsa, and differentially counted. Lavage cells were harvested, plastic adherent cells were depleted, and remaining cells were stained with FITC-labeled F4/80 (Serotec), PE-labeled CCR3 (R&D Systems), and allopurinol-labeled CD11b (clone M1/70, BD Biosciences) conjugated Abs. Cells were sorted on a MoFlow Cytometer (Cytomation) and eosinophils were isolated. Sample cytopsins of sorted cells were prepared and stained with May-Gruenwald-Giems. For induction of apoptosis, purified eosinophils were plated in IMDM supplemented with 10% FCS (Biochrom), 1% penicillin/streptomycin, and decreasing concentrations of mIL-5 (R&D Systems), as indicated. After overnight incubation, apoptotic cells were detected by flow cytometry, using FITC coupled Annexin V (BD Pharmingen), according to the manufacturer’s protocol.

Histopathology

Mice were anesthetized with isofluorane inhalation and bled by cardiac puncture at 13 days after infection. Their small intestines were fixed in a solution containing 10% formalin, 70% ethanol, and 5% acetic acid. Sections of small intestines were stained with H&E. Numbers of goblet cells in the small intestine were determined microscopically in two randomly chosen areas of two villi in at least three mice per experimental group.

Blood eosinophil numbers

Blood smears were prepared and analyzed for numbers of eosinophils following May-Gruenwald-Giems staining (Merck). One hundred white cells were counted per mouse and the percentage of eosinophils was determined.

Lymphocyte culture and cytokine responses

Spleens were removed from mice, and single-cell suspensions were prepared. Cells were resuspended in RPMI 1640 (Invitrogen) supplemented with 10% FCS (Biochrom), 2 mM l-glutamine, 100 U of penicillin, and 100 μg of streptomycin (Biochrom) per ml and left untreated or stimulated at 37°C and 5% CO2, with predetermined optimal concentration of either ConA (5.0 μg/ml) (Sigma-Aldrich) or Toxoplasma-lyse-Ag (5.0 μg/ml). Cell-free supernatants were harvested after 48 h and stored at -70°C. Cytokine analysis was conducted by sandwich ELISA using paired mAbs to detect IL-4 (clones 11B11 and BVD6 -24G2) and IL-5 (clones TRFK5 and TRFK4). Cytokines were quantified by reference to commercially available recombinant murine standards (all BD Pharmingen).

CFU-eosinophil and CFU-granulocyte-monocyte/macrophage assay

Mononuclear cells were isolated from the total bone marrow cell suspension by density gradient centrifugation in Biscoll solution (density 0.909, Biochrom). After 25 min centrifugation on 2500 rpm, 20°C, the layer of mononuclear cells was collected, washed once in PBS, and plated at indicated densities (10⁴/ml or 10⁵/ml) in the methylcellulose-based medium, containing 1% methylcellulose (Methocult H3100, Stem Cell Technologies), 30% FCS (Biochrom), 2 mM glutamine, 0.1 mM 2-ME, and 2% penicillin/streptomycin (Life Technologies). Differentiation of cells was induced by 50 ng/ml mIL-5 (R&D Systems) or 5 ng/ml mGM-CSF (PeproTech). Cultures were performed in duplicates, incubated at 37°C with 5% CO2, and colonies were scored 8–10 days after plating.

Peritoneal lavage, immunostaining, and isolation of eosinophils

Mice were injected with 3% thioglycollate in PBS 72 h before harvesting of peritoneal cells. The peritoneum was flushed with cold PBS and cytospins were prepared, stained with May-Gruenwald-Giems, and differentially counted. Lavage cells were harvested, plastic adherent cells were depleted, and remaining cells were stained with FITC-labeled F4/80 (Serotec), PE-labeled CCR3 (R&D Systems), and allopurinol-labeled CD11b (clone M1/70, BD Biosciences) conjugated Abs. Cells were sorted on a MoFlow Cytometer (Cytomation) and eosinophils were isolated. Sample cytopsins of sorted cells were prepared and stained with May-Gruenwald-Giems. For induction of apoptosis, purified eosinophils were plated in IMDM supplemented with 10% FCS (Biochrom), 1% penicillin/streptomycin, and decreasing concentrations of mIL-5 (R&D Systems), as indicated. After overnight incubation, apoptotic cells were detected by flow cytometry, using FITC coupled Annexin V (BD Pharmingen), according to the manufacturer’s protocol.

Flow cytometry staining, isolation, and culture of EoP and isolation of erythroid progenitors (EP)

EoP were sorted according to Iwasaki and coworkers (5). In brief, bone marrow cells were stained with anti-IL-5Rα-chain, anti-CD34, and anti-c-KIT, as well as a lineage mixture supplemented with anti-Sca-1. EoP were purified as Lin- Sca-1-CD34+ IL-5Rα– c-Kit–cells. Following biotinylated lineage markers were used: B220 (clone RA3–6B2), CD3 (clone 17A2), Gr-1 (clone RB6–8C5), Mac1 (clone M1/70), CD4 (clone H129–19), CD8 (clone 53–6,7), CD19 (clone 1D3), TER119 (all BD Biosciences), and CD127 (IL-7Rα, Clone A7R34; Cymbus Biotechnology). Further Abs were: biotin-labeled anti-Sca-1 (clone D7), allopurinol-labeled anti-CD117 (clone 2B8), FITC-labeled anti-CD34 (clone RAM34; all BD Biosciences), and Alexa 647 (Molecular Probes) self-labeled anti-IL-5Rα (clone H7; Wako). Second step were streptavidine-PE-C7 from BD Biosciences. Progenitors were sorted using high-speed cell sorter (Aria; BD Biosciences). Sorted EoP were cultured for 10 days in a methylcellulose medium (MethoCult M3231; Stem Cell Technologies) in the presence of IL-3 (1% cell line supernatant; Ref. 21), IL-5 (50 ng/ml; R&D Systems), rm stem cell factor (50 ng/ml; R&D Systems), rm stem cell factor (50 ng/ml; R&D Systems), and rm GM-CSF (1 ng/ml; R&D Systems). Each colony was picked, cytopsin, and analyzed after May-Gruenwald-Giems staining.

RT-PCR

Total RNA was isolated from purified EoPs and eosinophils using the RNeasy kit including DNase digestion (Qiagen). Total RNA from purified mature eosinophils was isolated using TriFast-reagent according to the manufacturer’s protocol (Peqlab). RNA was prepared according to the manufacturer’s recommendation. First strand cDNAs were synthesized from 1 μg RNA using PowerScript reverse transcriptase kit (Clontech Laboratories). PCR amplification was performed in 25 μl volume, containing 1 × PCR buffer, 1.5 mM MgCl₂, 0.2 mM dNTP mix, 0.3 mM each of primer.
primer, and 0.1U/μl TaqDNA Polymerase (Bioline). All PCR were done in duplicate for each cDNA sample. Products were ran on the 1% agarose gel and visualized with EtBr staining to confirm the product identity. Primer sequences and product specificities for each target gene are available upon request.

**Real-time PCR**

To analyze the expression of transcripts of typical eosinophil genes in mature eosinophils, "nested" PCR method was used. The first PCR amplification was performed in 25 μl volume, containing 1× PCR buffer, 1.5

**FIGURE 2.** Icsbp-deficient mice show increased intestinal goblet cell numbers and increased IL-4 and IL-5 production in response to Nippostrongylus brasiliensis infection, but no increase in eosinophil numbers. **A**, Blood smears were prepared and analyzed morphologically for numbers of eosinophils after May-Gruenwald-Giemsa staining. **B**, Numbers of goblet cells in the small intestine were determined microscopically in two randomly chosen areas of two villi. Data shown are mean values ± SD from three mice/group. One representative of three independent experiments is shown. **C**, Supernatants from spleen cells were analyzed for IL-4 and IL-5 by ELISA. Data shown are mean values ± SD from three mice/group. One representative of three independent experiments is shown. Statistically significant differences between Icsbp+/+(○) and −/− (●) mice samples are indicated above bars.

**FIGURE 3.** **A** and **B**, Icsbp-deficient bone marrow cells show increased colony formation in response to GM-CSF and decreased colony formation in response to IL-5. Bone marrow cells were cultured at 10^4 cells (GM-CSF) or 10^5 cells (IL-5) per 1 ml methylcellulose medium supplemented with 5 ng/ml GM-CSF (**A**) or 50 ng/ml IL-5 (**B**), as described in experimental procedures. Cultures were performed in duplicates, incubated at 37°C with 5% CO2, and colonies were counted 8–10 days after plating. **C**, Icsbp-deficient eosinophils show reduced response to IL-5 dependent apoptosis rescue. Eosinophils were isolated from the peritoneal cavity of Icsbp+/+(○) and −/− (●) mice by high speed FACS, plated in medium supplemented with increasing IL-5 concentrations (as indicated) and cultured over night. The percentage of annexin V positive cells was determined by flow cytometry.
mM MgCl₂, 0.2 mM dNTP mix, 0.3 mM of each primer, and 0.1U/μl TaqDNA Polymerase (Bioline). Number of preamplification cycles was optimized for each gene analyzed to get crossing points in the second amplification step, which was performed as real-time PCR on the LightCycler System (Roche Diagnostics) using LightCycler FastStart DNA MasterPLUS SYBR Green1 kit and primers located within the sequence amplified in the first step (“nested” primers). Ccr3 and Gapdh (reference transcript) were analyzed directly from 1 μl of cDNA, without preamplification step. For quantification, the normalized target/reference gene amounts in Icsbp−/− samples were calculated as relative amounts of normalized target/reference gene amounts in Icsbp+/− samples (without the correction for PCR efficiency). The formula used is: Relative Gene Amount = 2^((Cp(target, wt) – Cp(reference, wt)) - (Cp(target, ho) – Cp(reference, ho))). All PCR were done in duplicate for each cDNA sample. Products from the second amplification step were ran on the 1% agarose gel and visualized with EtBr staining to confirm the product identity.

Statistics
Levels of significance were determined using student’s t test. Results are presented as mean ± SD.

Results
Numbers of eosinophils are reduced in Icsbp-deficient mice
Enumeration of cytopsins prepared from bone marrow of normal or Icsbp-deficient mice revealed an ~8-fold reduction in the percentage of eosinophils (Fig. 1A). Absolute numbers of nucleated cells in the bone marrow were about 2-fold increased in Icsbp-deficient mice demonstrating an absolute decrease of eosinophils in the bone marrow cavity. Normal peritoneal lavage fluid contained only low numbers of eosinophils. Following i.p. injection of thioglycollate, percentages of eosinophils significantly increased in normal mice (Fig. 1B, and Louahed and coworkers, Ref. 22). Reflecting the well-known neutrophil granulocytosis of this mutant, percentages of peritoneal neutrophils were relatively high in Icsbp-deficient mice (Fig. 1B, and Holtschke and coworkers, Ref. 17). Absolute numbers of nucleated cells in the lavage fluid did not differ significantly. The inflammatory exudate in wild-type mice in response to thioglycollate was characterized in particular through a significant percentage of eosinophils. Although Icsbp-deficient mice responded also with an increase in eosinophils, percentages of eosinophils infiltrating the peritoneal cavity were significantly lower. Notably, in Icsbp-deficient mice, numbers of eosinophils were reduced both under normal (in the bone marrow) and inflammatory conditions (in the peritoneal cavity).

Icsbp is required for eosinophilia during parasite infection
Eosinophilia is a functionally important hallmark of helminthic infection. To study whether Icsbp-deficient mice can mount parasite-induced eosinophilia, mice were infected with the gastrointestinal nematode N. brasiliensis. This infection develops in distinct stages and immunity depends on an intact Th2 response. Although percentages of eosinophils in uninfected Icsbp-deficient blood were decreased (wt: mean 1.3%; Icsbp-deficient 0.7%) the absolute numbers were increased, albeit not significantly, owing to the well-known high total leukocyte counts in these mice. After infection with N. brasiliensis, numbers of eosinophils in peripheral
blood increased significantly in wild-type mice, whereas numbers of eosinophils did not differ significantly in Icsbp-deficient mice before and after infection (Fig. 2A). In contrast, both wild-type and Icsbp-deficient mice displayed increased percentages of goblet cells, a nonhematopoietic cell type that expands during *N. brasiliensis* infection in the small intestine (Fig. 2B). At the same time point (13 days after infection), both wild-type and Icsbp-deficient mice infected with *N. brasiliensis* mounted a strong Th2-response in spleens, as shown by high levels of Th2-cytokines, IL-4, and IL-5 (Fig. 2C). Induction of high amounts of IL-5, a crucial eosinophilic growth factor (23–25), implied that lack of IL-5 was not the cause of the reduced eosinophil numbers in Icsbp-deficient mice. In fact, following infection, concentrations of IL-4 and IL-5 were significantly higher in Icsbp-deficient mice than in wild-type controls. This alteration might be due to the constitutive lack of Th1 cytokines in these animals (19, 26, 27). Collectively, Icsbp-deficient mice were capable of mounting normal or even enhanced Th2 responses in this helminth infection. This strong Th2 cytokine response contrasted with the absent eosinophilia.

**Icsbp-deficient mice have reduced overall eosinophil developmental potential, and reduced numbers of eosinophil progenitors**

Reduced numbers of eosinophils in Icsbp-deficient mice could be due to defects in the generation of eosinophils from stem/progenitor cells, or due to defects in the maintenance of this lineage. To address the former possibility, we first determined the overall eosinophil generation potential by measuring frequencies of IL-5-responsive eosinophil colonies in wild-type and Icsbp-deficient mice. For comparison, frequencies of GM-CSF-responsive colonies were determined (Fig. 3A). In line with the predominant neutrophil granulopoiesis in Icsbp-deficient bone marrow (17, 28), frequencies of GM-CSF colonies were increased. Conversely, frequencies of IL-5 colonies were strongly reduced in Icsbp-deficient mice (Fig. 3B). Because IL-5 and GM-CSF receptors share the common \( \beta \)-chain (\( \beta_5 \)) (29), signaling through \( \beta_5 \) is not compromised in Icsbp-deficient progenitor cells. Levels of IL-5 receptor \( \alpha \)-chain were measured when the eosinophil progenitor was analyzed more directly (see below). To exclude apoptosis of mature eosinophils as a potential cause for the observed lack of eosinophils, we measured apoptosis of eosinophils isolated from the peritoneal cavity. Eosinophils were isolated by flow cytometry after surface staining with F4/80 (30), CD11b, and CCR3 (see Fig. 7A). Starvation-provoked apoptosis could be rescued by adding increasing concentrations of IL-5 to the culture in wild type (up to 90%) as well as Icsbp-deficient (up to 70%) eosinophils (Fig. 3C).

The observed difference in response to IL-5 does not explain the lack of eosinophilia in parasite-infected mice. Furthermore, those data demonstrate again that signaling through \( \beta_5 \) is not severely compromised in Icsbp-deficient eosinophils.

Next, we determined numbers and developmental potential of the recently reported, prospective EoP in bone marrow (5). We defined EoP as lineage-marker (B220, CD3, Gr-1, Mac1, CD4, CD8, CD19, and TER119) negative (Lin\(^{-}\)), Sca-1\(^{-}\), IL-7 receptor (IL-7R)\(^{-}\), CD34\(^{-}\) and c-Kit\(^{int}\) (Fig. 4). In addition, and in line with the role of IL-5 in eosinophil-development, EoP are IL-5R\(^{+}\) (5). IL-5R\(^{+}\) cells with either higher or lower c-kit staining (represented by dots above or below the gate shown in Fig. 4, respectively) did not show clonogenic growth of eosinophil colonies.
FIGURE 7. Reduced expression of typical eosinophil transcripts in mature eosinophils derived from Icsbp-deficient mice. A, Isolation of eosinophils from the peritoneal cavity. Icsbp-deficient and wild-type mice were injected with 2 ml of 3% thioglycollate i.p. Seventy-two hours after injection, the peritoneum was flushed, adherent cells were depleted, and the remaining cells were sorted using FITC-labeled F4/80, PE-labeled CCR3 and allophycoceyanin-labeled CD11b. B, Measurement of transcripts from eosinophils isolated from the peritoneal cavity. Total RNA was isolated, converted to cDNA and the expression of Gata2, Il5, Il5Ra, Ccr3, Prg2, and Epx were analyzed by a nested real-time PCR approach. C, Expression of Ccr3 on eosinophils. Expression of surface Ccr3 is shown in histograms of eosinophils pregated for Cd11b and F4/80 as shown in the oval region in the dot blots.
(data not shown). We observed a depletion of cells with this phenotype in Icsbp-deficient bone marrow. Related to the numbers of CD34<sup>+</sup> cells, percentages of EoP were reduced ~3-fold comparing wild-type and Icsbp-deficient mice (Fig. 4). Mean fluorescent intensities of IL-5Rα staining of cells of this phenotype were not significantly different between wild-type (224 ± 30 arbitrary units) and Icsbp-deficient mice (182 ± 12; n = 5 each genotype). This marked reduction of phenotypically defined EoP could, at least in part, account for the defect in peripheral eosinophils observed in Icsbp-deficient mice.

**EoP in Icsbp-deficient mice have a deviated differentiation potential**

The EoP population has been reported to be endowed with clonogenic, eosinophilic lineage committed differentiation potential (5). The fact that the relative frequency of EoP increases in response to helminth infection suggests that EoP represent an important stage in eosinophil production in vivo. To determine whether the defect in eosinophil development in Icsbp-deficient mice was evident already at the EoP stage, EoP were purified from wild-type and Icsbp-deficient mice by fluorescent activated cell sorting. Cells were placed in methylcellulose cultures in the presence of IL-3, IL-5, SCF, and GM-CSF, and the colony frequency and colony lineage were determined after 10 days. The majority (>75%), but not all, EoP from wild-type mice generated pure eosinophil colonies, or mixed colonies that included eosinophils plus neutrophils, or eosinophils plus monocytes, or eosinophils plus mast cells. In Icsbp-deficient mice, the EoP phenotype had a markedly altered developmental potential. Although frequencies of colony forming cells on per cell basis were similar in wild-type (one colony per eight cells plated) and Icsbp-deficient mice (one per seven), only ~25% of Icsbp-deficient EoP had single or mixed eosinophil potential. The vast majority of colonies arising from Icsbp-deficient EoP were monocytes (Fig. 5). Enumeration of the cells with EoP phenotype and determination of their differentiation potential has therefore uncovered two defects in Icsbp-deficient mice: reduction of EoP in bone marrow, and a loss of eosinophil differentiation potential within this compartment. The deviation of EoP toward monocyte potential is remarkable in view of the fact that, bone marrow cells in general (17, 28), and GMP in particular (G. Terszowski; unpublished observation) showed a strong bias for neutrophils and against monocytes in Icsbp-deficient mice.

**Gata1 expression in EoPs and mature eosinophils in Icsbp-deficient mice is reduced**

Colony assays described above indicated a cell-intrinsic defect at the level of the EoP in Icsbp-deficient mice. EoP were originally defined via expression of Gata1, a transcription factor that is essential for the development of erythroid, megakaryocytic, and notably also eosinophil lineages (6, 31–33). Because of the crucial role of Gata1 in eosinophil development, we compared the expression of this gene in EoP from wild-type and Icsbp-deficient mice. RNA from cell sort-purified EoP was transcribed into cDNA. These templates were carefully titrated for PCR-amplification of Actin to normalize the amount of cDNA that was used to examine expression of Gata1. Gata1-expression was reduced ~2-fold in Icsbp-deficient EoP compared with wild-type EoP (Fig. 6A). To further validate those findings, we also investigated Gata1 expression in mature eosinophils. As shown in Fig. 6B, expression of Gata1 was also reduced in mature eosinophils from Icsbp-deficient mice. Gata1 is also expressed in committed EP. To study whether the observed reduction of Gata1-expression was restricted to the eosinophil lineage, we also analyzed Gata1 expression in EP from Icsbp-deficient and wild-type mice. Numbers of EP are strongly reduced in Icsbp-deficient bone marrow but these EP have normal erythrocyte generation potential on a per cell basis (20). In line with the normal potential of EP from Icsbp-deficient mice, Gata1-expression was comparable in wild-type and Icsbp-deficient EP (Fig. 6C). These data show that Gata1 expression is selectively lost in cells of the eosinophil lineage in Icsbp-deficient mice. Several other transcription factors regulate the expression of typical eosinophil transcripts and may also contribute to the physiological development of EoP. We therefore measured the mRNA expression of Sfi1 (encoding Pu.1), Cebpα, Cebpβ, Zfpml (encoding Fog), and Gata2 in EoP in primary as well as serially diluted cDNA samples. Although Sfi1 and Cebpα were expressed rather equally in EoP derived from wild-type and Icsbp-deficient mice (data not shown), expression of Zfpml and Gata2 was equally low and could not be detected in serially diluted samples. Expression of Cebpα was lower in EoP from wild-type mice and could be detected in diluted samples only from Icsbp-deficient mice (Fig. 6D). In B cell development, expression of Irf4 and Irf8 can substitute for each other (34). A similar mechanism may be operative in eosinophil development. We therefore investigated expression of Irf4 in EoP but were unable to detect expression of Irf4 in EP derived from wild-type or Icsbp-deficient mice (data not shown).

Gata1 regulates the expression of several essential proteins of the eosinophil, including major basic protein, or proteoglycan2 (Prg2), as well as eosinophil peroxidase (Eps). To investigate whether expression of transcripts coding for surface molecules or for eosinophil granule proteins were differentially regulated, we measured mRNA for Gata2, Il5ra, Ccr3, Prg2, and Eps in mature eosinophils isolated from the peritoneal cavity (Fig. 7A). As shown in Fig. 7B, expression of Gata2, Il5ra, Prg2, and Eps was reduced while expression of Ccr3 was not. Unaltered expression of surface Ccr3 was confirmed by flow cytometry (Fig. 7C). Therefore, consistent with the infiltration of eosinophils into the peritoneal cavity during sterile inflammation in Icsbp-deficient mice Ccr3 chemokine receptor expression was unaltered, whereas expression of transcripts of eosinophil granule proteins and, in contrast to EoP, Il5Ra expression was reduced in mature eosinophils. Reduced Il5Ra expression might explain reduced rescue from apoptosis we observed in Icsbp-deficient mice.

**Discussion**

Icsbp is known for its role in the regulation of macrophage (17, 28, 35) and B cell development (36). In this study, we have demonstrated an important and previously unappreciated role of Icsbp for eosinophilopoiesis. First, Icsbp-deficient mice had lower numbers of eosinophils under normal steady-state conditions. However, loss of Icsbp did not cause a complete block in eosinophil development, and the remaining mature eosinophils could migrate into the peritoneal cavity in response to a sterile inflammation. Furthermore, apoptosis rescue was only mildly impaired, indicating that the function of remaining mature eosinophils is largely preserved in Icsbp-deficient mice. Second, Icsbp-deficient mice could not mount significant eosinophilia in response to helminth infection. This is remarkable given that the Th1/Th2 “balance” in Icsbp-deficient mice is shifted toward a Th2-pattern due to reduced expression of the Th1 cytokines INF-γ and IL-12 (19, 26, 27). The Th2-bias should have favored the development of eosinophils. This did not occur despite higher expression of IL-5 in parasite-infected Icsbp-deficient mice. We conclude that the availability of IL-5 is not a limiting factor in Icsbp-deficient mice.

The role of eosinophils for helminth parasite clearance is controversial (37) and Icsbp-deficient mice have a complex disorder affecting the humoral as well as the cellular branch of the immune system.
systems with abnormalities in generation and function of neutrophils, monocytes/macrophages, dendritic cells, and B cells (36, 38–40). Thus, we did not attempt to investigate the function of eosinophils in clearance of parasites because it would be impossible to contribute the outcome of the parasite infection in Icsbp-deficient mice to the lack of eosinophils.

EoP from both wild-type and Icsbp-deficient mice expressed the IL-5 receptor, and at least some Icsbp-deficient EoP formed eosinophil colonies in response to IL-5 suggesting that the IL-5/IL-5 receptor system was functional in Icsbp-deficient mice. However, the low number of eosinophil colonies from EoP in Icsbp-deficient mice implies that extrinsic signals via IL-5/IL-5 receptor are insufficient for commitment and maturation of eosinophil progenitors toward terminally differentiated eosinophils. This is in keeping with the finding that enforced expression of IL-5Rα at the GMP stage did not lead to increased frequencies of EoP (5).

The route from hematopoietic stem cells via eosinophil progenitors to mature eosinophils is not fully understood, but the recent identification of EoP provides a basis to follow this pathway under pathological conditions, and in hematopoietic mutants. Direct comparison of EoP in normal and in Icsbp-deficient mice indicated a defect in eosinophil development at the progenitor stage. Absolute EoP numbers were reduced in Icsbp-deficient mice, indicating that Icsbp has a role at an early stage of eosinophil development. i.e., at the level of the generation or maintenance of EoP. The residual EoP in Icsbp-deficient mice had a normal cell surface phenotype. Like their wild-type counterparts, Icsbp-deficient EoP were purified based on their IL-5 receptor expression. The overall hematopoietic clonogenicity of Icsbp-deficient EoP was unchanged compared with wild-type EoP. In agreement with the reported potential of EoP in normal mice, almost 80% of wild-type EoP-derived colonies contained eosinophils; the remaining cells were mostly monocytes.

However, the developmental potential of EoP in Icsbp-deficient mice was markedly altered at the expense of eosinophils (down to ~25%), and in favor of monocytes, which represented >70% of colony progeny from EoP in Icsbp-deficient mice. Iwasaki and coworkers (5) identified EoP as a descendant of the GMP that expressed a Gata1-reporter, were IL-5Rα+ and expanded in response to helminth infection. Our direct analysis of purified EoP representation and differentiation potential in Icsbp-deficient mice demonstrates that Icsbp is an important factor regulating the generation of EoP itself, as well as the developmental decision between monocytes and eosinophils at this GMP descendant. Aberrant development of EoP explains the lack of eosinophilia in N. brasilienensis infected Icsbp-deficient mice. In contrast to the pathological state, eosinophilopoiesis was less affected in vivo under physiological conditions. This is in agreement with previous reports showing almost normal residual eosinophil numbers in mice deficient for IL-5 (25) or IL-5Rα (41). This indicates that steady state and emergency eosinophilopoiesis may be alternatively regulated.

Loss of Gata1 prevents eosinophil development (6). In various experimental systems, enforced expression of Gata1 promotes eosinophil development at the expense of myelomonocytic cells (42, 43). A model proposed by McNagny and Graf (44) incorporated these findings, as well as further data on other transcription factors, to suggest that 1) low or no expression of Gata1 favors granulocyte and monocyte development, 2) moderate expression of Gata1 favors the eosinophil fate, and 3) high expression of Gata1 favors the development of erythrocytes and megakaryocytes. Hence, expression of Gata1 does not act as an “on” or “off” switch in developmental decisions but, intriguingly, its fine tuned expression can regulate hematopoietic decisions. We noted an approximate 2-fold reduction in Gata1-expression in Icsbp-deficient EoP, and the potential of these cells switched from the normal eosinophil fate to an abnormal monocyte fate. It is known from other transcription factors that slight changes in expression might have dramatic impact on the developmental potential of hematopoietic progenitor cells (45). It should be further noted that levels of Gata1-expression may have a specific impact in different progenitor subsets, e.g., GMP, EoP, or megakaryocyte-erythrocyte progenitors. Indirectly, via loss of Icsbp, we have now identified a crucial role of the Gata1-expression strength at the level of the EoP. Why is Gata1-expression selectively reduced in Icsbp-deficient EoP but not in EP? Gata1-expression appears to be differentially regulated in erythroid or megakaryocytes vs eosinophil lineages (43, 46). Presently, we cannot determine how Icsbp regulates Gata1-expression in the eosinophil lineage. The development of eosinophils is to our knowledge not affected by IFNs and we were unable to detect any canonical Icsbp-binding sites in the Gata1 promoter.

Parallel to reduced Gata1-expression, expression of Cebpe was increased in EoP derived from Icsbp-deficient mice. Ectopic expression of Cebpe induces the expression of several transcripts typical for the macrophage lineage (47). Over expression of Cebpe in combination with reduced expression of Gata1 may explain the observed aberrant maturation of EoP in Icsbp-deficient mice (Fig. 5).

In conclusion, we have shown that Icsbp has crucial, and non-redundant roles for the development of eosinophils at the level of the generation of EoP and by impairing the developmental potential of EoP. This defect was associated with aberrant Gata1 and Cebpe expression, suggesting that the expression of those transcription factors must be tightly regulated at the committed eosinophil progenitor stage to faithfully maintain eosinophil lineage commitment.

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Disclosures

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