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The STAT5–GATA2 Pathway Is Critical in Basophil and Mast Cell Differentiation and Maintenance

Yapeng Li,* Xiaopeng Qi,*† Bing Liu,*† and Hua Huang*‡

Transcription factor GATA binding protein 2 (GATA2) plays critical roles in hematopoietic stem cell survival and proliferation, granulocyte–monocyte progenitor differentiation, and basophil and mast cell differentiation. However, precise roles of GATA2 in basophil and mast cell differentiation and maintenance have not been delineated. We have identified GATA2 as an essential transcription factor in differentiation of newly identified common basophil and mast cell progenitors into basophils and mast cells. We observed Gata2 haploinsufficiency for mast cell differentiation, but not for basophil differentiation. We examined the precise role of GATA2 in maintaining the expression of a wide range of genes that are important for performing basophil or mast cell functions. The effects of GATA2 on gene expression were broadly based. We demonstrated that GATA2 was required for maintaining Fcer1a mRNA and FcεRIα protein expression on both basophils and mast cells, as well as for maintaining Kit mRNA and c-Kit protein expression on mast cells. GATA2 was required for histamine synthesis and was also critical for Il4 mRNA expression in basophils and Il13 mRNA expression in mast cells. We demonstrate a STAT5–GATA2 connection, showing that the STAT5 transcription factor directly bound to the promoter and an intronic region of the Gata2 gene. Overexpression of the Gata2 gene was sufficient to direct basophil and mast cell differentiation in the absence of the Stat5 gene. Our study reveals that the STAT5–GATA2 pathway is critical for basophil and mast cell differentiation and maintenance.

The processes of basophil and mast cell differentiation have received increased attention in recent years. Immature basophils differentiate and undergo maturation in the bone marrow. Mature basophils circulate in the bloodstream and enter inflamed tissues. In contrast, immature mast cells develop in the bone marrow before taking residence in tissues, where they undergo further maturation (2). The nature of precursors of these cells is a subject of intense debate. Galli and colleagues (8, 9) identified mast cell lineage–restricted progenitors (MCPs) in the bone marrow and proposed that MCPs are derived from multiple potential progenitors, but not from common myeloid progenitors or granulocyte–monocyte progenitors (GMPs). In contrast, Akashi and colleagues (10) determined that both basophils and mast cells are derived from common myeloid progenitors and GMPs. In addition, they described a subset of cells in the spleen, but not in the bone marrow, termed basophil and mast cell progenitors (BMCPs). These cells are suggested to create both basophils and mast cells (10). However, whether BMCPs are authentic bipotential BMCPS was challenged by a recent study (11) and our data (12), which indicate that BMCPS mainly create mast cells. Furthermore, data from proliferation-tracking experiments support the conclusion that most new basophils are generated in the bone marrow, rather than in the spleen (13).

We have identified a novel population of common BMCPS in the bone marrow (12). These progenitors were highly enriched in the capacity to differentiate into basophils and mast cells while retaining a limited capacity to differentiate into myeloid cells. Because it was determined that the common BMCPS were more mature than GMPs and because they possessed great potential to differentiate into basophils and mast cells but had not yet fully committed into bipotential basophil–mast cell potential progenitors, we have designated these progenitor cells “pre-basophil and mast cell progenitors” (pre-BMCPS). We showed that pre-BMCPS differentiated into basophils and mast cells at the clonal level in vitro and at the population level in vivo (12). We also demonstrated that STAT5 signaling was required for the differentiation

Abbreviations used in this article: BaP, basophil lineage–restricted progenitor; BMCSP, basophil and mast cell progenitor; BMPC, bone marrow–derived mast cell; ChIP, chromatin immunoprecipitation; CMP, common myeloid progenitor; DC, dendritic cell; GATA2, GATA binding protein 2; GMP, granulocyte–monocyte progenitor; HSC, hematopoietic stem cell; IL-7; 4HT, 4-hydroxytamoxifen; IDMEM, Iscove’s modification of DMEM; IL-3C, IL-3 and anti–IL-3 Ab complex; MCP, mast cell lineage–restricted progenitor; MFI, mean fluorescence intensity; MITF, microphthalmia–associated transcription factor; pre-BMP, pre-basophil and mast cell progenitor; qPCR, quantitative PCR; YFP, yellow fluorescent protein.

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of pre-BMPs into both basophils and mast cells, and was critical for inducing two downstream transcription factors, C/EBPα and microphthalmia-associated transcription factor (MITF). We identified C/EBPα as the critical transcription factor for specifying basophil cell fate and MITF as the crucial transcription factor for specifying mast cell fate. We demonstrated that C/EBPα and MITF silenced each other’s transcription in a directly antagonistic fashion (12).

GATA binding protein 2 (GATA2) is a member of the GATA family of zinc finger transcription factors. GATA2 plays critical roles in survival and proliferation of hematopoietic stem cells (HSCs) (14, 15). It has been implicated to play a role in GMP differentiation (16). GATA2 has been shown to be critical in both basophil and mast cell differentiation (17, 18). The order of GATA2 and C/EBPα expression has been suggested to be crucial in determining basophil cell fate. When GATA2 expression preceded C/EBPα expression at the GMP stage, GATA2 together with C/EBPα drove basophil differentiation. Conversely, when C/EBPα expression preceded GATA2 expression, C/EBPα together with GATA2 drove eosinophil differentiation (18). However, it remains unknown whether GATA2 plays a role in the differentiation of pre-BMPs into basophils and mast cells, and in the maintenance of basophil and mast cell identities.

In this study, we demonstrated that GATA2 was essential for the differentiation of pre-BMPs into basophils and mast cells, and for the maintenance of basophil and mast cell identities. GATA2 haploinsufficiency was observed for mast cell differentiation, but not for basophil differentiation. We further demonstrated that the STAT5 transcription factor directly regulated Gata2 gene expression and that overexpression of the STAT5 transcription factor directly regulated the expression of basophil and mast cell identities.

**Materials and Methods**

**Mice**

C57BL/6J (B6) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Stat5ab+/−, RosaΔYFP/Wt; Cre mice and Stat5ab+/−, RosaΔYFP/Wt; Cre mice were generated as described previously (12). Gata2−/−RosaΔYFP/Wt TgCreEr2loxmis mice were generated by crossing Gata2−/− mice (Gata2tm1Sac [19], the mutant mouse regional resource center at University of California-Davis, Davis, CA) to the mice with a yellow fluorescent protein (YFP) Cre activity reporter gene knocked in the Rosa locus (B6.129X1-It (Rosa26)Sor10M1CreERT2/J [20]; short name: RosaΔYFP/Wt; Jackson Laboratory) and to the transgenic mice with an inducible Cre enzyme (TyCreEr2loxmis [21]; Jackson Laboratory). Gata2−/−RosaΔYFP/Wt TgCreEr2loxmis mice were generated by crossing Gata2−/− RosaΔYFP/Wt TgCreEr2loxmis mice with Gata2−/−RosaΔYFP/Wt TgCreEr2loxmis mice, which were generated by crossing RosaΔYFP/Wt to TgCreEr2loxmis mice. All animal experiments were approved by the National Jewish Health Institutional Animal Care and Use Committee.

**FACS analysis and sorting**

For FACS analysis of basophils, mast cells, T cells, B cells, dendritic cells (DCs), neutrophils, and macrophages, cells obtained from various tissues or cell cultures were stained with the following Abs: basophils and mast cells were stained with aliphosphocyanin-CY7-conjugated anti-c-Kit (2B8) and PE-CY7-conjugated anti-FcRc (MAR-1) Abs; T and B cells were stained with PE-CY7-conjugated anti-CD45 (13-0521) and PE-CY7-conjugated anti-CD19 (1D3) Abs; DCs were stained with aliphosphocyanin-conjugated anti-MHC class II (M5/114.15.2) and PE-conjugated anti-CD11c (N418) Abs; neutrophils and macrophages were stained with PE-conjugated anti-Gr-1 (RB6-8C5) and aliphosphocyanin-conjugated anti-CD11b (M1/70) Abs. Dead cells were stained with propidium iodide and excluded in all FACS plots. Stained cells were acquired by using CyAn (DakoCytomation, Glostrup, Denmark) and analyzed using the FlowJo software (Tree Star, Ashland, OR). The absolute number of positive cells was calculated by multiplying the total number of cells with the percentages of positive cells. Mean fluorescence intensities (MFIs) were calculated by using the FlowJo software. The percentage of reduction was calculated by multiplying the total number of cells with the percent-age of positive cells. Mean fluorescence intensities (MFIs) were calculated by using the following formula: percent of reduction = [MFI (control) − MFI (inducible knockout)]/MFI (control) × 100%. Regular GMPs (FcrRc−/− GMPs) and pre-BMPs (FcrRc+ GMPs) were stained and FACS-sorted according to the published protocol (12). In brief, regular GMPs were FACS-sorted as Lin IL-7Ra− Sca-1− c-kit− CD34−FcyRI/III−/FcγRllc− cells. Pre-BMPs were FACS-sorted as Lin IL-7Ra− Sca-1− c-kit−CD34+/FcyRI/III+/FcγRllc+ cells. The stained cells were FACS-sorted using a MoFlo machine (DakoCytomation, Glostrup, Denmark). All Abs used for FACS analysis and sorting were purchased from BD Pharmingen (San Diego, CA) or eBioscience (San Diego, CA).

**In vitro gene deletion**

The floxed genes in the cultured cells were deleted by the 4-hydroxymexafmine (4HT; Calbiochem, Billerica, MA) treatment using a concentration of 25 nM. For the myelocellule culture, 4HT was included in the culture for 9 d without washing (the 25-nM concentration was determined to generate maximum floxed gene deletion with the least amount of toxicity). For the liquid cultures, 4HT was washed after 3 d of treatment. The washed cells were cultured under the original conditions without 4HT until they were subjected to analysis. The deletion of the floxed Gata2 gene in the FACS-sorted YFP+ basophils or YFP+ mast cells was determined to be near 100%. The deletion of the floxed Stat5ab genes in the FACS-sorted YFP+ basophils or YFP+ bone marrow–derived mast cells (BMMCs) was also highly effective (12).

**In vitro differentiation of progenitors**

To analyze the differentiation of pre-BMPs into basophils and mast cells, we FACS-sorted pre-BMPs from Gata2−/−RosaΔYFP/Wt TgCreEr2loxmis mice and Gata2−/−RosaΔYFP/Wt TgCreEr2loxmis mice. The sorted pre-BMPs were seeded in a 35-mm Nunclish (Thermo Fisher Scientific, Rochester, NY) at a density of 1000 cells/dish in 1 ml of 1% methylcellulose containing complete Iscove’s modification of DMEM (IMDM) supplemented with 50 μM 2-ME (Sigma-Aldrich, St. Louis, MO), 20 ng/ml IL-3, and 25 nM 4HT for 9 d (8, 22). Cells were then collected and analyzed by FACS.

**BMMC culture**

BMMCs were prepared by culturing bone marrow cells from Gata2−/−RosaΔYFP/Wt TgCreEr2loxmis mice, Gata2−/−RosaΔYFP/Wt TgCreEr2loxmis mice, Stat5ab+/−RosaΔCreErt2loxmis mice, or Stat5ab+/−RosaΔCreErt2loxmis mice in complete IMDM supplemented with 50 μM 2-ME and 20 ng/ml IL-3 for 4 wk (23). Greater than 99% of BMMCs were mast cells (FcrRc− c-Kit−) as determined by FACS analysis.

**Basophil culture**

To obtain committed basophils, we mixed together IL-3 (10 μg) and anti–IL-3 Ab (5 μg; MP2-8F8; BD Pharmingen) at room temperature for 1 min according to published methods (24). IL-3 and anti–IL-3 Ab complex (IL-3C) was i.p. injected into Gata2−/−RosaΔYFP/Wt TgCreEr2loxmis mice, Gata2−/−RosaΔYFP/Wt TgCreEr2loxmis mice, Stat5ab+/−RosaΔCreErt2loxmis mice, or Stat5ab+/−RosaΔCreErt2loxmis mice 3 d before bone marrow harvest. Bone marrow cells from the treated mice were cultured in complete IMDM containing 50 μM 2-ME and 20 ng/ml IL-3 for an additional 3 d. CD34 expression on the cultured basophils was no longer detectable and these cells expressed lineage markers Gr-1 and CD11b, indicating that they exhibited a mature phenotype.

**In vivo treatment of mice**

Gata2−/−RosaΔYFP/Wt TgCreEr2loxmis mice, Gata2−/−RosaΔYFP/Wt TgCreEr2loxmis mice, or Gata2−/−RosaΔYFP/Wt TgCreEr2loxmis mice were injected i.p. with 100 μl tamoxifen (Sigma-Aldrich) mixed with sunflower seed oil (2 ng/100 μl; Spectrum Chemical, Gardena, CA) once daily for 5 consecutive days. Two weeks after the last injection, cells and tissues from the treated mice were collected and subjected to FACS and histological analysis.

**Retroviral infection**

Full-length Stat5a and Gata2 cDNAs were cloned into the retroviral expression vector MSCV2.2-Ires-Thyla through BglII and Not sites. The retroviral particles were prepared as described previously (25). For retroviral infection, bone marrow cells from the control (Stat5ab+/− RosaΔCreErt2loxmis, Stat5ab+/−) mice and inducible Stat5 knockout (Stat5ab+/− RosaΔCreErt2loxmis, Stat5ab+/−) mice were stimulated with stem cell factor (50 ng/ml). IL-6 (50 ng/ml) and IL-3 (20 ng/ml) overnight. The stimulated cells (2 × 106) were centrifuged at 1800 rpm for 90 min at room temperature with 1 ml viral supernatant containing recombinant retrovirus containing the Stat5a,
Gata2, or Thy1 (CTRL) genes in the presence of polybrene (8 μg/ml; Millipore, Billerica, MA). Infected cells were cultured in complete IMDM with 20 ng/ml IL-3 and treated with 25 nM 4HT for 3 d to delete the Stat5ab gene. Ten days after the initial treatment of 4HT, cells were collected and analyzed by FACS. Infected cells were identified by the expression of Thy1.1 using allophtoocyanin-labeled anti-Thy1.1 Ab (clone HIS51; eBioscience).

Quantitative PCR and ELISA

Total RNA from FACS-sorted GMPs and pre-BMPs, basophils, or BMCCs was isolated with an RNeasy Mini Kit (Qiagen, Valencia, CA) according to manufacturer’s instructions. For cytokine gene mRNA analysis, the cells were stimulated with PMA (50 ng/ml; Sigma-Aldrich) and ionomycin (1 μM; Sigma-Aldrich) for 6 h or IgE cross-linking (1 μg/ml IgE [clone D8406; Sigma-Aldrich] and 1 μg/ml anti-IgE Ab [clone R35-72; BD Pharmingen]) overnight; then total RNA was prepared from the treated cells. cDNA was synthesized by reverse transcription. Quantitative PCR (qPCR) was performed in an ABI PRISM 7700 Sequence Detection System. Primer sequences are listed in Supplemental Table I. Relative mRNA amounts were calculated as follows: Relative mRNA amount = 2^([Ct(input)] - [Ct(input)]) × 100%

Measurement of histamine content and release in basophils and mast cells

For histamine content, basophils or BMCCs were frozen and thawed three times. Histamine in the cell lysates was measured by using a histamine enzyme immunoassay kit (Beckman Coulter, Fullerton, CA) following the manufacturer’s instructions. For histamine release, basophils or BMCCs were stimulated with PMA (50 ng/ml) and ionomycin (1 μM) for 6 h or IgE cross-linking (1 μg/ml IgE and 1 μg/ml anti-IgE Ab). Histamine in the supernatants was measured by ELISA (BD Pharmingen).

Histology

Ear sections from the tamoxifen-treated mice were fixed in 4% paraformaldehyde and stained with toluidine blue. Histological images were captured on a Nikon E800 microscope (Nikon, Melville, NY).

Chromatin immunoprecipitation assay

Anti-STAT5 Ab was purchased from Cell Signaling Technology (Beverly, MA). Chromatin immunoprecipitation (ChIP) experiments were performed using an EpiTect Chip One-Day Kit (Qiagen, Valencia, CA) as described previously (12). The quantity of DNA precipitated by anti-STAT5 Ab was calculated as fold of enrichment using the following formula: Fold enrichment = 2^[ΔCt(input) - ΔCt(IP)], where ΔCt(IP) = Ct(IP) - Ct(input) - Log2(input dilution factor), ΔCt(Input) = Ct(Input) - [Ct(input) - Log2(input dilution factor)] (12).

Statistical analysis

All of the error bars in this report represent the SD. For ELISA and qPCR analyses, mean ± SD was derived from triplicate measurements. Pooled data are indicated in the figure legends. The difference between two samples was analyzed with Student’s t test.

Results

GATA2 is essential for the differentiation of pre-BMPs into basophils and mast cells

To analyze the role of GATA2 in the differentiation of pre-BMPs into basophils and mast cells, we first examined Gata2 mRNA expression in the FACS-sorted pre-BMPs and found that the levels of Gata2 mRNA in the pre-BMPs were 60-fold higher than those in regular GMPs (FceR1α− GMPs), albeit that expression was still lower compared with that detected in basophils or mast cells (Fig. 1A). We then tested whether GATA2 was necessary for the differentiation of pre-BMPs into basophils and mast cells. We took advantage of the inducible gene deletion system, in which mice with a floxed Gata2 gene were crossed to mice with an inducible Cre enzyme and to mice with a YFP Cre activity reporter (Gata2fl/fl; Rosa26R26R; TgCreEr22; Rosa26R26R; TgCreEr22) mice. We refer to these mice as the inducible Gata2 knockout mice and to Gata2+/+;Rosa26R26R; TgCreEr22;Rosa26R26R; TgCreEr22 mice as the control mice hereafter. We deleted the Gata2 gene in the FACS-sorted pre-BMPs of the inducible Gata2 knockout mice with 4HT. As a control, we treated the FACS-sorted pre-BMPs of the control mice with 4HT. YFP+ pre-BMPs of the inducible Gata2 knockout mice (Gata2−/−) failed to differentiate into basophils and mast cells but differentiated into neutrophils and macrophages normally, whereas YFP+ pre-BMPs of the control mice (Gata2+/−) differentiated into basophils and mast cells (throughout this report, YFP+ cells represent YFP+ propidium iodide−live cells at the time of analysis; Fig. 1B). These results indicate that GATA2 plays an essential role in the differentiation of pre-BMPs into basophils and mast cells.

GATA2 haploinsufficiency is observed for mast cell differentiation, but not for basophil differentiation

GATA2 haploinsufficiency (one copy of a gene is not sufficient to carry out the gene’s biological functions) has been reported in both mice and humans. In mice, Gata2 haploinsufficiency leads to reduced production and expansion of HSCs in the aorta-gonad-mesonephros region (14). In humans, Gata2 haploinsufficiency results in immune deficiency (26). Although FceR1α− GMPs failed to differentiate into basophils, these progenitors differentiated into mast cells (data not shown), suggesting that there might exist multiple mast cell progenitors (27). In this study, we wished to examine whether there is a Gata2 haploinsufficiency for basophil and mast cell development using experimental systems that assess basophil and mast cell developmental potential of all possible BMCPs. In vitro, we used semi-solid 1% methylcellulose culture conditions, in which only progenitors can grow colonies, to assess collectively the capacity of mixed bone marrow BMCPS, including basophil lineage–restricted progenitors (BaPs), MCPs, uncharacterized mast cell progenitors, and pre-BMPs, to differentiate into basophils or mast cells. Whole bone marrow cells of homozygous or heterozygous inducible Gata2 knockout mice, or control mice were cultured in 1% methylcellulose–containing medium with 4HT. We found that Gata2−/− BMCPS differentiated into basophils, but not mast cells, whereas Gata2+/− myeloid progenitors differentiated into neutrophils and macrophages comparable with Gata2+/+myeloid progenitors (Fig. 2A). To verify this finding in vivo, we treated heterozygous inducible Gata2 knockout mice with tamoxifen (one copy of the Gata2 gene was deleted in all cells). Direct ex vivo analysis of cells prepared from the treated mice revealed that Gata2 haploinsufficiency was indeed detected in the differentiation of mast cells, but not in basophils. We did not observe a Gata2 haploinsufficiency in the differentiation of other cell lineages with the exception of DCs (Fig. 2B and 2C). Histological analysis showed that the number of mast cells in the ears of heterozygous inducible Gata2 knockout mice treated with tamoxifen was reduced greatly to a level similar to that of tamoxifen-treated homozygous mice (Fig. 2D). Together, these data demonstrate a Gata2 haploinsufficiency in mast cell differentiation.

GATA2 is critical for maintaining FceR1α expression on basophils and FceR1α and c-Kit expression on mast cells

Transcription factors that regulate developmental process leading to cell lineage commitment might not always be needed for maintaining the committed molecular signatures once progenitor cells have differentiated into specific cell types. To determine whether GATA2 is required for the maintenance of basophil and
mast cell identities, we deleted the Gata2 gene after progenitors were committed to basophils or mast cells. For the basophil maintenance study, we prepared basophil-enriched bone marrow cells from the inducible Gata2 knockout or control mice injected with IL-3C, which rapidly expanded BaPs and basophils in vivo (28, 29). The IL-3C–expanded BaPs and basophils were cultured in the presence of IL-3 for an additional 3 d to ensure that basophils were committed. We then deleted the Gata2 gene in the committed basophils with the 4HT treatment. We first examined mRNA and protein expression of FcεRIα. We found that in the absence of the Gata2 gene, FcεRIα protein expression on YFP+ Gata2+/− basophils began to decrease at day 5 after the initial 4HT treatment, reaching the lowest levels at day 7 after the initial 4HT treatment (Fig. 3A and upper panel of 3B). Directly ex vivo Fcer1a mRNA expression in the FACS-sorted YFP+ Gata2+/− basophils decreased even more dramatically when compared with FcεRIα protein expression on YFP+ Gata2+/− basophils at day 5 after the initial 4HT treatment (Fig. 3A and lower panel of Fig. 3B). The number of YFP+ Gata2+/− basophils in the culture was significantly reduced compared with that of YFP+ Gata2+/+ basophils, indicating a pivotal role of GATA2 in basophil survival (Fig. 3C).

For the mast cell maintenance study, we prepared 4-wk BMMCs from the Gata2 inducible knockout or control mice and used them as committed mast cells (although BMMCs are often considered as immature mast cells, they are committed because they cannot differentiate into other cell lineages even under culture conditions that are appropriate for other cell lineage differentiation [data not shown]). We found that in the absence of the Gata2 gene, FcεRIα and c-Kit protein expression on YFP+ Gata2+/− mast cells (FcεRIα+ and c-Kit+) decreased beginning at day 3 (Fig. 3D and upper panel of 3E). At day 7 after the initial 4HT treatment, one portion of mast cells completely lost c-Kit expression, whereas a smaller percentage of mast cells completely lost FcεRIα expression. By day 11, in addition to greater percentages of mast cells that lost either c-Kit or FcεRIα expression, significant percentages of mast cells lost both c-Kit and FcεRIα expression (Fig. 3D). Although Gata2+/− mast cells that have lost FcεRIα, c-Kit, or both expressions no longer met the phenotypical definition of mast cells, they could still maintain mast cell molecular signatures. In contrast, Gata2+/− mast cells that expressed c-Kit and FcεRIα expression could still lose mast cell molecular signatures. Thus, we analyzed YFP+ FcεRIα+/− and/or c-Kit+/− cells collectively and refer to these cells as “Gata2+/− mast cells” hereafter. MFIs of c-Kit and FcεRIα expression on YFP+ Gata2+/− mast cells reached the lowest level at day 11 after the initial 4HT treatment (Fig. 3E, upper panel). Fcer1a and Kit mRNA expression in the FACS-sorted YFP+ Gata2+/− mast cells at day 11 after the initial 4HT treatment were reduced significantly (Fig. 3E, lower panel). In regard to GATA2 dosage requirement for maintaining FcεRIα and c-Kit expression, committed mast cells, unlike differentiating mast cells, did not exhibit a Gata2 haploinsufficiency (Supplemental Fig. 1).

Interestingly, in contrast with its role in basophil survival, GATA2 did not appear to affect mast cell survival. Although mast cells lost much of FcεRIα and c-Kit protein expression in the absence of the Gata2 gene, the number of YFP+ Gata2+/− mast cells at day 11 after the initial 4HT treatment was comparable with that of YFP+ Gata2+/+ mast cells (Fig. 3F), suggesting that GATA2 is not a survival factor for mast cells.

GATA2 is crucial for maintaining the expression of genes that are important in performing basophil or mast cell functions and in histamine synthesis

We analyzed further whether deletion of the Gata2 gene affects the expression of genes known to carry out basophil or mast cell functions, which could be expressed in both basophils and mast cells, genes that we previously identified as those that are highly expressed in basophils but not in mast cells, or genes that are highly expressed in mast cells but not in basophils (12). Genes that are highly expressed in basophils but not in mast cells include genes encoding protease (Mcp1, chemokine (Ccl3), and receptors (Ilgam, Itgae2, and Il3ra). Genes that are highly expressed in mast cells but not in basophils include those that encode cytokines (Il4, Il6, Il13, and Tslp) and a gene encoding for an enzyme required for histamine synthesis. As a control, we included Cd63 and Alox5, which are commonly expressed in both basophils and mast cells. For cytokine gene expression analysis, we analyzed Il4 gene expression in basophils and Il13 gene expression in mast cells because the cytokines encoded by these two genes perform major basophil and mast cell functions (30, 31). We activated basophils and mast cells with IgE cross-linking to determine the effect of reduced FcεRIα expression on cytokine gene expression (to assess the indirect effects of Gata2 gene deletion on cytokine expression) and with PKC stimulator PMA and Ca2+...
influx stimulator ionomycin (activate basophils and mast cells bypassing FcεRI) to assess more directly the effects of GATA2 on cytokine gene expression. Fig. 4A (upper panel) shows that Il4 mRNA and IL-4 protein expression were greatly diminished in the FACS-sorted YFP+ Gata2−/− basophils stimulated either with PMA and ionomycin or with IgE cross-linking. Similarly, Il13 mRNA and IL-13 protein expression in the FACS-sorted YFP+ Gata2−/− mast cells were nearly abolished (Fig. 4A, lower panel). Cytokine gene expression in WT or Gata2−/− basophils and mast cells was low without stimulation. These results demonstrate that GATA2 plays a critical role in maintaining the expression of the Il4 gene in basophils and the Il13 gene in mast cells.

FIGURE 2. GATA2 haploid insufficiency is observed for mast cell differentiation, but not for basophil differentiation. (A) Bone marrow cells (not FACS-sorted) of Gata2+/+, Gata2+/f, or Gata2−/− mice were cultured in methylcellulose containing medium in the presence of IL-3 and 25 nM 4HT for 9 d. YFP+ cells are shown in the FACS plots. (B) FACS analysis of cells from the tamoxifen-treated mice. YFP+ cells are shown. T cells, B cells, and DCs were prepared from spleen; basophils (Ba), neutrophils (Neu), and macrophages (MΦ) from bone marrow cells, and mast cells (MC) from peritoneal cavity of the treated mice. Data represent two independent experiments with similar results. (C) Total numbers of YFP+ cells (mean ± SD, n = 6). (D) Toluidine blue staining of ear sections (original magnification ×40; inset, ×100). Mast cells are indicated by arrows. Right panel shows the average number of mast cells in 10 different fields (original magnification ×40) randomly selected from the sections of ears (mean ± SD, n = 3). The percentages indicate the percentages of reduction in mast cell numbers in the ear sections.
In addition to de novo cytokine synthesis, basophils and mast cells synthesize histamine and store the synthesized histamine in preformed granules. Activated basophils and mast cells then secrete the preformed granules through a cellular process known as degranulation. Histamine is synthesized from the decarboxylation of the amino acid histidine, a reaction catalyzed by the enzyme called histidine decarboxylase (32). Mice deficient in the histidine decarboxylase gene (\(Hdc\)) fail to synthesize histamine. IgE-mediated anaphylactic reactions are absent in \(Hdc\)-deficient mice (33, 34). To determine whether GATA2 affects the histamine synthesis or histamine release in basophils and mast cells, we measured histamine content in the FACS-sorted YFP\(^+\) Gata2\(^{+/+}\) basophils and Gata2\(^{+/+}\) mast cells and found that the histamine contents were greatly reduced in those cells (Fig. 4B, left two panels). Similar levels of reduction in histamine release by the FACS-sorted YFP\(^+\) Gata2\(^{+/+}\) basophils and Gata2\(^{+/+}\) mast cells...
FIGURE 4. GATA2 is crucial for maintaining the expression of genes that are important in carrying out basophil or mast cell functions and for histamine synthesis. (A) qPCR and ELISA analyses of Il4 mRNA and IL-4 protein in basophils or Il13 mRNA and IL-13 protein in mast cells not treated or treated with 4HT. YFP+ Gata2+/+ or Gata2−/− basophils at day 5 after the initial 4HT treatment and YFP+ Gata2+/+ mast cells or Gata2−/− mast cells at day 11 after the initial 4HT treatment were FACS-sorted and stimulated with PMA and ionomycin for 6 h or stimulated with IgE cross-linking overnight. (B) ELISA measurement of histamine content and qPCR analysis of Hdc mRNA expression in the FACS-sorted YFP+ Gata2−/− basophils and Gata2−/− mast cells. (C) qPCR analysis of mRNA expression of basophil genes in the FACS-sorted YFP+ Gata2−/− basophils at day 5 after the initial 4HT treatment. Different scales were used to present data generated in the same experiment. (D) qPCR analysis of mRNA expression of mast cell genes in the FACS-sorted YFP+ Gata2−/− mast cells or Gata2−/− mast cells at day 11 after the initial 4HT treatment. The percentages of reduction are indicated in all panels. Data represent mean ± SD (triplicates) and two independent experiments with similar results.
in response to IgE cross-linking were also observed (data not shown). Consistent with a critical role of GATA2 in histamine synthesis, we showed that Hdc mRNA expression in FACS-sorted YFP+ Gata2−/− basophils and Gata2−/− mast cells was greatly reduced (Fig. 4B, right two panels). These results demonstrate that GATA2 is required for histamine synthesis in basophils and mast cells.

For expression analysis of the remaining genes, the FACS-sorted basophils and Gata2−/− mast cells were not stimulated because expression of those genes generally does not require stimulation. We showed that the majority of basophil- or mast cell–specific genes (except Ly6g and Tslp) depended on GATA2 for their expression, whereas the two commonly expressed genes did not (Fig. 4C and 4D). We did not detect re-expression of the Cebpa and Mitf gene in Gata2−/− mast cells and the Mtf gene in Gata2−/− basophils. We also did not detect re-expression of cell-surface markers unique to T cells (CD3), B cells (CD19), DCs (MHC class II and CD11c), and eosinophils (CCR3 and Siglec-F) or genes that are expressed in macrophages (Mmp12, Mpg-1, and Msr1), or neutrophils (Ela2, Prtn3, and Lactoferrin) in Gata2−/− mast cells, indicating that GATA2 is not a cell fate-altering factor (Supplemental Fig. 2). Taken together, our data revealed that GATA2 is a crucial transcription factor in the molecular programs that regulate gene expression necessary for maintaining basophil and mast cell identities, and for carrying out basophil and mast cell functions. Our data also demonstrate that GATA2 is imperative in basophil survival but not in mast cell survival.

STAT5 is required for maintaining FcεRIα expression on basophils and FcεRIα and c-Kit expression on mast cells

Our and other’s previous studies (12, 28, 35) demonstrated that STAT5 is critical in basophil and mast cell development. However, it remains to be determined whether STAT5 is needed for the maintenance of basophil and mast cell identities. We showed that in the absence of the Stat5a/b gene, FcεRIα protein expression on YFP+ Stat5a/b−/− basophils began to decrease at day 3 after the initial 4HT treatment, reaching the lowest levels at day 7 after the initial 4HT treatment (Fig. 5A and 5B). The number of YFP+ basophils was reduced by 3%, 33%, and 56% at days 3, 5, and 7, respectively, after the initial 4HT treatment (Fig. 5B). Similarly, we observed a decrease in FcεRIα expression on YFP+ Stat5a/b−/− mast cells (Fig. 5C). The number of YFP+ mast cells was reduced by 12%, 14%, and 24% at days 3, 7, and 11, respectively, after the initial 4HT treatment (Fig. 5F). These results demonstrate that STAT5 is required for maintaining FcεRIα expression on basophils and FcεRIα and c-Kit expression on mast cells.

FIGURE 5. STAT5 is required for maintaining FcεRIα expression on basophils and FcεRIα and c-Kit expression on mast cells. (A) Bone marrow cells prepared from the IL-3C–injected Stat5a/b−/− Rosa26CreERT2/Yfp (Stat5a/b−/) or Stat5a/b−/− Rosa26CreERT2/Yfp (Stat5a/b−/) mice were cultured in the presence of IL-3 for 3 d. The resulting cells were treated with 25 nM 4HT for an additional 3 d. Three, 5, or 7 d after the initial 4HT treatment, the cells were analyzed by FACS. (B) MFIs of FcεRIα expression on YFP+ Stat5a/b−/− Stat5a/b−/− basophils (mean ± SD, n = 6). (C) Total numbers of YFP+ Stat5a/b−/− Stat5a/b−/− basophils (mean ± SD, n = 6). (D) FACS analysis of BMMCs treated with 4HT. (E) MFIs of FcεRIα and c-Kit expression on YFP+ Stat5a/b−/− Stat5a/b−/− mast cells (mean ± SD, n = 6). (F) Total numbers of YFP+ Stat5a/b−/− Stat5a/b−/− mast cells (mean ± SD, n = 6). YFP+ cells are shown (A and D). Percentages of reduction are indicated (B and E).
Stat5a/b−/− basophils in the culture after the 4HT treatment was significantly reduced compared with that of YFP+ Stat5a/b−/− basophils, indicating a pivotal role of STAT5 in basophil survival (Fig. 5C). Similarly, we found that in the absence of the Stat5a/b gene, FceRα and c-Kit expression on YFP+ Stat5a/b−/− mast cells decreased beginning at day 3, reaching the lowest levels at day 11 after the initial 4HT treatment (Fig. 5D and 5E). The absence of the Stat5a/b gene had a less severe effect on FceRα and c-Kit expression than the absence of the Gata2 gene because we did not observe a complete loss of FceRα and c-Kit expression on YFP+ Stat5a/b−/− mast cells. The number of YFP+ Stat5a/b−/− mast cells decreased at day 7 after the initial 4HT treatment, reaching the lowest numbers at day 11 after the initial 4HT treatment (Fig. 5F), suggesting that STAT5 is a survival factor for mast cells. Thus, our data demonstrate that STAT5 signaling is essential for maintaining FceRα expression on basophils and FceRα and c-Kit expression on mast cells, and for basophil and mast cell survival.

STAT5 directly regulates the Gata2 gene, and overexpression of the Gata2 gene is sufficient to direct basophil and mast cell differentiation in the absence of the Stat5 gene

Stat5 deficiency and Gata2 deficiency resulted in the same developmental phenotype: a failure of pre-BMPs to differentiate into both basophils and mast cells. These results suggested that STAT5 and GATA2 might operate in the same pathway to regulate basophil and mast cell development. To define the relationship of STAT5 and GATA2 in basophil and mast cell differentiation, we searched the STAT5-binding sites (TTCCNNGAA, N means any nucleotide) in the ±30 kb from the transcription start site of the Gata2 gene and found three STAT5-binding sites in the Gata2 promoter (S5b1, S5b2, and S5b3) and two STAT5-binding sites in the +2 kb intronic region of the Gata2 gene (S5b4 and S5b5; Fig. 6A, upper panel). Using ChIP assay, we found that STAT5 was recruited into the promoter and the +2 kb intronic region of the Gata2 gene (Fig. 6A, lower panel). Overexpression of the Gata2 gene in the Stat5a/b−/− bone marrow BMPCs rescued basophil and mast cell differentiation to the levels that were comparable with those driven by Stat5a gene overexpression (Fig. 6B). We also noticed that the Stat5a virus-infected or Gata2 virus-infected Stat5a/b−/− or Stat5a/b−/+ bone marrow progenitors consistently differentiated into higher percentages of basophils and mast cells than the control bone marrow progenitors with vector virus transduction, presumably because of higher than WT levels of STAT5A and GATA2 protein expression in the infected progenitors. Compared with STAT5A, GATA2, when overexpressed, appeared to possess a higher capacity to drive mast cell differentiation (Fig. 6B). These results demonstrate that the Gata2 gene is a direct STAT5 target gene in the signaling pathway that drives the differentiation of bone marrow BMPCs into basophils and mast cells.

Discussion

In this study, we examined the role of GATA2 in the differentiation of pre-BMPs into basophils and mast cells. GATA2 has been shown to be important for mast cell differentiation (17). Enhanced expression of GATA2 together with PU.1 drives the differentiation of mast cells from myeloid progenitors (36). GATA2 has also been implicated to play a role in basophil development. Akashi and colleagues (18) showed that the order of GATA2 and C/EBPs expression is a deciding factor in driving GMPs into basophils. However, the previous approaches of deleting the Gata2 gene in the germ cells or overexpressing the Gata2 gene in GMPs failed to determine the precise role of GATA2 in basophil and mast cell development. Because GATA2 exerts its functions at the multiple developmental stages of hematopoiesis, it is pivotal to analyze the precise role of GATA2 in the defined progenitors of basophils and mast cells. We used an approach combining prospective FACS sorting with an inducible gene deletion system to analyze the precise role of GATA2 in the differentiation of pre-BMPs into basophils and mast cells. Our analyses demonstrate that GATA2 is required for the differentiation of pre-BMPs into basophils and mast cells.

We document a Gata2 haploinsufficiency for mast cell differentiation, but not for basophil differentiation in vitro and in vivo. Gata2 haploinsufficiency has been reported for production and expansion of HSCs in the aorta- gonad-mesonephros region during embryonic development (14). In adult Gata2−/− mice, hematopoiesis appears to be normal. Only when a more robust test known as serial or competitive transplantation assay is used are defects in HSC self-renewal (14) and GMP functions in Gata2−/− mice (16) revealed. Our in vitro results that Gata2−/− BMPCs failed to differentiate into mast cells prompted us to further examine whether there is a Gata2 haploinsufficiency for mast cells in vivo. We demonstrate a selective Gata2 haploinsufficiency in mast cell differentiation. However, it is still unknown why a full dose of

FIGURE 6. STAT5 directly regulates the Gata2 gene, and overexpression of the Gata2 gene is sufficient to direct basophil and mast cell differentiation in the absence of the Stat5 gene. (A) STAT5-binding sites (S5b1–5, upper panel) and ChIP analysis of STAT5 binding to the Gata2 promoter and downstream region (lower panel). BMPCs without stimulation were used for the ChIP analysis. (B) Bone marrow cells of Stat5a/b−/− and Stat5a/b−/+ mice were infected with retrovirus containing Stat5a, Gata2, or Thy1a (CTRL) gene. Twenty-four hours postinfection, the infected cells were treated with 25 nM 4HT for 3 d. Ten days after the initial 4HT treatment, the cells were analyzed by FACS. YFP+ Thy1.1+ cell populations are shown. Data represent two independent experiments with similar results.
GATA2 is required for mast cell differentiation but not for basophil development or mast cell maintenance. It is likely that GATA2 inefficiently transcribes genes whose protein products are required for the mast cell progenitors to pass mast cell developmental checkpoint(s). Once mast cell progenitors pass the checkpoints, one copy of the Gata2 gene is sufficient to maintain the expression of those important developmental genes. Alternatively, GATA2 downstream genes that maintain the mast cell identities differ from those that direct mast cell differentiation. Thus, the mast cell molecular program that directs mast cell differentiation and the mast cell molecular program that maintains mast cell identities require a different dose of the Gata2 gene. Identification of GATA2 target genes that are important in mast cell differentiation and maintenance is needed to test these possibilities.

If a gene is required for basophil or mast cell differentiation, then deletion of the gene will result in loss of basophils or mast cells, making it difficult to study gene functions in these cells. For example, STAT5 (35), GATA1 (37), GATA2 (17), and MITF (38, 39) have each been demonstrated to be critical for mast cell differentiation, whereas STAT5 (28), Runx1 (11), GATA2, and C/EBPα (12, 18) have been shown as crucial transcription factors for basophil differentiation. Deletion of these transcription factors all resulted in loss or abnormal development of basophils and mast cells. Our approach has overcome this problem. We developed an experimental system, in which the Gata2 gene was deleted after basophils and mast cells become committed, to examine the precise roles of GATA2 in maintaining the expression of a wide range of genes that are important in carrying out basophil or mast cell functions, including genes encoding FcεRIα, c-Kit, and cytokines, genes that are expressed highly in basophils or mast cells, and genes that are commonly expressed in basophils and mast cells. We found that the effects of GATA2 on those gene expressions were broadly based. We demonstrate that GATA2 was required for maintaining FcεRIα mRNA and FcεRIα protein expression on basophils, as well as for maintaining FcεRIα mRNA, FcεRIα protein expression, Kit mRNA, and c-Kit protein expression on mast cells. We showed that GATA2 was also required for histamine synthesis by upregulating Hdc gene expression. Our data suggest that GATA2 regulates Il4 mRNA expression in basophils and Il13 mRNA expression in mast cells through both indirect and direct mechanisms. In the indirect mechanism, GATA2 regulates Il4 and Il13 gene transcription by regulating FcεRIα mRNA expression or critical signaling proteins. However, the reduced FcεRIα protein expression caused by the lack of the Gata2 gene could not fully account for the reduction in Il4 and Il13 mRNA expression in Gata2−/− basophils and Gata2−/− mast cells when those cells were activated by PMA and ionomycin, which bypass the FcεRI receptor stimulation to induce Il4 and Il13 gene transcription.

We propose that GATA2 might regulate Il4 and Il13 gene transcription by either binding to the Il4 and Il13 regulatory regions or by inducing transcription factors that bind to Il4 and Il13 regulatory regions.

Unlike the Cebpa or Mitf genes, deletion of which resulted in re-expression of Mitf mRNA in Cebpa−/− basophils and re-expression of Cebpa mRNA in Mitf mutant mast cells, and thus resulted in a basophil–mast cell fate conversion (12), deletion of the Gata2 gene did not lead to re-expression of Mitf mRNA in Gata2−/− basophils nor re-expression of Cebpa mRNA in Gata2−/− mast cells (Supplemental Fig. 2A), and thus did not lead to a basophil–mast cell fate conversion. We also did not detect re-expression of genes that are important for T cell, B cell, DC, cosinophil, neutrophil, or macrophage molecular programming. Moreover, deletion of the Gata2 gene in mast cells does not appear to affect mast cell survival. We found a significant number of live Gata2−/− cells that had lost FcεRIα and c-Kit expression at the end of culture. The identities of those Gata2−/− cells remain unknown, and further extensive analysis of gene expression profiles will be required.

Our experimental data also make a STAT5–GATA2 connection. Previous work has established that STAT5 plays critical roles in basophil and mast cell development (28, 35). Our work advances the understanding of STAT5 downstream transcription factor that exerts the STAT5-mediated biological functions. We demonstrate that STAT5 binds to the promoter and an intrinsic regulatory region of the Gata2 gene, and overexpression of the Gata2 gene is sufficient to direct basophil and mast cell differentiation in the absence of the Stat5 gene. The finding that STAT5 was required for mast cell survival is consistent with a published work that STAT5 is required for mast cell survival by maintaining the expression of prosurvival molecules, such as Bcl-2 and Bcl-x(L) (35). Together, our analyses of the roles of GATA2 using an improved approach reveal a novel STAT5–GATA2 pathway in the differentiation and maintenance of basophils and mast cells.

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Disclosures
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References


