GATA2 hypomorphism induces chronic myelomonocytic leukemia in mice

Nobuhiko Harada1,2 | Atsushi Hasegawa1 | Ikuo Hirano1 | Masayuki Yamamoto3,4 | Ritsuko Shimizu1,4

1Department of Molecular Hematology, Tohoku University Graduate School of Medicine, Sendai, Japan
2Department of Laboratory Animal Medicine, Tohoku University Graduate School of Medicine, Sendai, Japan
3Department of Medical Biochemistry, Tohoku University Graduate School of Medicine, Sendai, Japan
4Tohoku Medical Megabank Organization, Tohoku University, Sendai, Japan

Correspondence
Ritsuko Shimizu, Department of Molecular Hematology, Tohoku University Graduate School of Medicine, Sendai, Japan.
Email: rshimizu@med.tohoku.ac.jp

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The transcription factor GATA2 regulates normal hematopoiesis, particularly in stem cell maintenance and myeloid differentiation. Various heteroallelic GATA2 gene mutations are associated with a variety of hematological neoplasms, including myelodysplastic syndromes and leukemias. Here, we report that impaired GATA2 expression induces myelodysplastic and myeloproliferative neoplasm development in elderly animals, and this neoplasm resembles chronic myelomonocytic leukemia in humans. GATA2 hypomorphic mutant (G2fGN/fGN) mice that were generated by the germline insertion of a neocassette into the Gata2 gene locus avoided the early embryonic lethality observed in Gata2-null mice. However, adult G2fGN/fGN mice suffered from exacerbated leukocytosis concomitant with progressive anemia and thrombocytopenia and eventually developed massive granulomonocytosis accompanied by trilineage dysplasia. The reconstitution activity of G2fGN/fGN mouse stem cells was impaired. Furthermore, G2fGN/fGN progenitors showed myeloid lineage-biased proliferation and differentiation. Myeloid progenitor accumulation started at a younger age in G2fGN/fGN mice and appeared to worsen with age. G2fGN/fGN mice showed increased expression of transcripts encoding cytokine receptors, such as macrophage colony-stimulating factor receptor and interleukin-6 receptor, in granulocyte-monocyte progenitors. This increased expression could be correlated with the hypersensitive granulomonocytic proliferation reaction when the mice were exposed to lipopolysaccharide. Taken together, these observations indicate that GATA2 hypomorphism leads to a hyperreactive defense response to infections, and this reaction is attributed to a unique intrinsic cell defect in the regulation of myeloid expansion that increases the risk of hematological neoplasm transformation.

KEYWORDS
aging, GATA2 hypomorphism, myelodysplastic syndrome, myeloproliferative disorder, stem cell dysfunction
GATA2 is a member of the GATA family of transcription factors, which recognize a target site conforming to the consensus sequence 5′- (A/T)GATA(A/G)-3′. GATA2 is expressed in a variety of organs/cells, including hematopoietic tissues, endothelial cells, the nervous system, kidney and urinary tract, uterus, and pituitary gland; GATA2 regulates a variety of genes essential for organogenesis as well as organ functions. In hematopoietic tissues, GATA2 is abundantly expressed in hematopoietic stem cells (HSCs) and contributes to long-term reconstitution activity. GATA2-knockout HSCs fail to contribute to adult hematopoiesis in chimeric mice, whereas forced GATA2 expression interferes with the repopulation function of HSCs due to reduced self-renewal capacity. Furthermore, HSC reconstitution activity is impaired by haploinsufficiency of the Gata2 gene. Intriguingly, the low-dose expression of exogenous GATA2 increases the clonogenic potential of HSCs. Currently available lines of evidence support the notion that changes in GATA2 expression levels can determine HSC stemness.

Gata2 gene expression profiles in mice change dramatically during hematopoiesis. Gata2 gene expression levels are decreased when HSCs begin to differentiate. Notably, after the common myeloid progenitor (CMP) stage, Gata2 gene expression levels show distinct patterns depending on the differentiation direction. Thus, Gata2 gene expression levels play a role in determining specific myeloid lineage fates. Indeed, a small increase in the Gata2 gene guides progenitor cells to differentiate into Gr− myeloid cells, whereas forced high expression levels of the gene restrict granulocyte-monocyte progenitors (GMPs) to develop exclusively into eosinophils and mast cells. In contrast, Gata2 gene haploinsufficiency reduces the GMP cell population but not the CMP cell population in mice, suggesting that haploinsufficiency might confer myeloid abnormalities independent of the sequelae of impaired stem cell functions. GATA2 expression at the appropriate level at the appropriate time is required for maintaining hematopoietic homeostasis.

We previously generated GATA2 hypomorphic mutant mice (Gata2<sup>fGN/fGN</sup>), in which GATA2 expression level is reduced to 20% of WT mice. Importantly, Gata2 gene expression in the mice circumvents the early embryonic lethality observed in Gata2-null mice. However, this hypomorphic expression of the gene does not fully support urological organogenesis. The penetrance of this urological abnormality is not complete; some mice show only unilateral or no obstruction. Therefore, Gata2<sup>fGN/fGN</sup> mice that have at least one functional kidney reach adulthood.

In this regard, whether (and how) reduced Gata2 gene expression in Gata2<sup>fGN/fGN</sup> mice affects adult hematopoiesis remains to be determined. In this study, we found that Gata2<sup>fGN/FGN</sup> mice were prone to developing granulomonocytosis with trilineage dysplasia after 6 months, and these neoplasms closely resemble those observed in chronic myelomonocytic leukemia (CMML) in humans. We also analyzed HSC reconstitution activity in Gata2<sup>fGN/FGN</sup> mice and found it to be severely impaired. Thus, a high proportion of myeloid-biased differentiation was present. Notably, the expression of myeloid lineage-associated cytokine receptor genes was increased in GMPs derived from Gata2<sup>fGN/FGN</sup> mice, and the Gata2<sup>fGN/FGN</sup> mice were sensitive to lipopolysaccharide (LPS) stimulation and easily developed granulomonocytosis. Taken together, these findings indicate that GATA2 hypomorphism in Gata2<sup>fGN/FGN</sup> mice severely compromises hematopoietic stem and progenitor cells, which leads to the development of hematopoietic neoplasms. These data imply the possible involvement or contribution of GATA2 hypomorphism to human leukemogenesis.

2 | MATERIALS AND METHODS

2.1 | Mice

The generation of Gata2<sup>fGN/FGN</sup> mice was described previously. All experimental procedures conformed to the Regulations for Animal Experiments and Related Activities at Tohoku University (Sendai, Japan).

2.2 | Cell preparation, flow cytometry and colony assays

Bone marrow (BM) and spleen mononuclear cells were isolated using Histopaque (Sigma-Aldrich, St Louis, MO, USA), and peripheral mononuclear cells were purified by lysing the erythrocytes in an ammonium chloride lysis solution. Peritoneal cells were aspirated using Tyrode's buffer. Flow cytometry was carried out with a FACS Calibur or FACS Aria II system (Becton-Dickinson, Franklin Lakes, NJ, USA) using fluorescein-conjugated or biotinylated Abs. For negative selection, the undesired cells were labeled with a cocktail of biotinylated Abs specific for CD8, CD4, B220, Gr1, Mac, Ter119, and interleukin-7R (IL-7R), followed by BioMag streptavidin (Qiagen, Venlo, Netherlands) or Texas Red-conjugated streptavidin. An anti-Sca1 biotinylated Ab was also used. Information regarding Abs is described in Table S1. For the side population assays, cells were analyzed by FACSVantage after staining with Hoechst 33342 solution (Molecular Probes, Eugene, OR, USA). Methocult M3434 (Stem Cell Technologies, Vancouver, BC, Canada) was used for the colony assays according to the manufacturer’s instructions.

2.3 | Real-time quantitative PCR and microarray analyses

RNA was isolated using ISOGEN-LS (Nippon Gene, Tokyo, Japan). Subsequently, first-strand cDNA was synthesized using ReverTraAce (Toyobo, Osaka, Japan). Real-time quantitative PCR was carried out with an ABI PRISM 7300 sequence detector system and StepOnePlus (Applied Biosystems, Foster City, CA, USA) using Thunderbird SYBR qPCR Mix (Toyobo). The data were normalized to the Hprt mRNA level. Information of primer sequences is described
in Table S2. For microarray analyses, RNA quality was verified with an Agilent-2100 Bioanalyzer; RNA was labeled with Cy3 using a Low Input Quick Amp Labeling Kit and hybridized to a Whole Mouse Genome Oligo Microarray (4 × 44K; Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer’s instructions. Microarray slide scanning was undertaken with an Agilent DNA microarray scanner. The expression data were normalized using GeneSpring software (Agilent Technologies).

2.4 | Statistical analyses

JMP software (JMP-13.1.0; SAS Institute, Cary, NC, USA) was used for the statistical analyses.

3 | RESULTS

3.1 | G2^GN/IGN mice are prone to developing leukocytosis at an old age

To investigate whether GATA2 deficiency confers hematological disease risk, we propagated a large number of G2^GN/IGN mice in a C57BL/6 and DBA/2 mixed background. We utilized this cohort of G2^GN/IGN mice to first examine hematopoietic indices in young (42–99 days old), middle-aged (100–249 days old), and elderly (250 days old or more) mice.

We found that G2^GN/IGN mice suffered from thrombocytopenia throughout their lives and from leukocytosis at an elderly age (Figure 1A). Notably, increased white blood cell (WBC) counts in G2^GN/IGN mice correlated with the age of the mice with a Spearman’s rank correlation coefficient of 0.3024 (P < .001) (Figure 1B).

Consequently, elderly G2^GN/IGN mice eventually suffered from overt leukocytosis, although some G2^GN/IGN mice maintained WBC counts within a permissible range (Figure 1B). We also found that the erythrocytes from G2^GN/IGN mice showed macrocytic and hyperchromic features, accompanied by increased hemoglobin levels and decreased red blood cell (RBC) counts, but their hematocrit index values were within normal levels (Figure 1A).

The number of erythrocytes in WT mice decreased substantially as their age increased, and G2^GN/IGN and WT mice showed similar trends (Figure 1A). We examined the paired correlation between RBC and WBC counts and found a statistically significant negative correlation between RBC and WBC counts in G2^GN/IGN mice with a Spearman’s rank correlation coefficient of –0.5383 (P < .00005) by Student’s t test. Hb, hemoglobin; Hct, hematocrit; MCH, erythrocyte mean corpuscular hemoglobin; MCHC, erythrocyte mean corpuscular hemoglobin concentration; MCV, erythrocyte mean corpuscular volume

**FIGURE 1** Leukocytosis occurs in aged G2^GN/IGN mice. A, Hematopoietic indices of young, middle-aged and elderly mice. The dotted horizontal line (153 × 10^4 cells/μL) indicates the leukocytosis border, which was determined by double counting the mean WBC counts of a group of WT mice. B, Measurement of the correlation between white blood cell (WBC) count and age in WT (left) and G2^GN/IGN (right) mice. The dotted horizontal line (153 × 10^4 cells/μL) indicates the leukocytosis border, which was determined by double counting the mean WBC count of a group of WT mice.

C, Measurement of the correlation between WBC and red blood cell (RBC) counts in WT (left) and G2^GN/IGN (right) mice. D,E, Comparison of hematocrit values (D) and platelet (Pt) counts (E) in G2^GN/IGN mice suffering from leukocytosis (red circles) and G2^GN/IGN mice with normal WBC counts (black circles; 67-83 × 10^6 cells/μL; 99% confidence interval for the mean WBC count of the WT mice). *P < .05, **P < .01, ***P < .001, ****P < .005, ****P < .0005, ****P < .00005 by Student’s t test. Hb, hemoglobin; Hct, hematocrit; MCH, erythrocyte mean corpuscular hemoglobin; MCHC, erythrocyte mean corpuscular hemoglobin concentration; MCV, erythrocyte mean corpuscular volume

mice suffering from overt leukocytosis than in G2\textsuperscript{IGN/IGN} mice with normal WBC counts (Figure 1D,E). Thus, these hematological data from G2\textsuperscript{IGN/IGN} mice revealed the presence of leukocytosis, which appears with the exacerbation of thrombocytopenia and anemia in the elderly stage.

3.2 | GATA2 hypomorphism increases the number of monocytes in elderly mice

We then undertook morphological examinations of the peripheral blood (PB) samples of G2\textsuperscript{IGN/IGN} mice with overt leukocytosis and found numerous mature monocytes (Figure 2A) that were positive for α-naphthyl-butyrate esterase but negative for chloroacetate esterase staining (Figure 2B). Plenty of neutrophils were also identified on the film (Figure 2A). Flow cytometry analyses confirmed the expansion of Gr1\textsuperscript+ or Mac1\textsuperscript+ myeloid cells in the PB samples of G2\textsuperscript{IGN/IGN} mice (Figure S1). Importantly, we also observed morphological abnormalities in the PB cells from G2\textsuperscript{IGN/IGN} mice with leukocytosis; these abnormalities included anisocytotic erythrocytes, hypogranular platelets, nuclear fragmentation, and blast-like cells, which are typical in myelodysplastic syndrome (Figure 2A). In contrast, PB samples from G2\textsuperscript{IGN/IGN} mice without massive leukocytosis were practically normal (data not shown). These findings indicate that G2\textsuperscript{IGN/IGN} mice eventually develop certain hematological disorders, resembling the features of CMMoL in humans.

Elderly G2\textsuperscript{IGN/IGN} mice with massive leukocytosis showed marked splenomegaly (Figure 2C). Histological examinations revealed that the splenic architecture was substantially preserved in the young G2\textsuperscript{IGN/IGN} mice without leukocytosis (Figure 2D), whereas the enlarged spleens of the elderly G2\textsuperscript{IGN/IGN} mice contained more red pulp (Figure 2D), indicating increased extramedullary hematopoietic activity.

GATA2 is upregulated to induce basophil, eosinophil, and mast cell differentiation at the divergence point of neutrophils and monocytes.\textsuperscript{19} It has been reported that GATA2 plays important roles in mast cell differentiation, and mast cell progenitors retain the potential to differentiate into neutrophils and macrophages.\textsuperscript{21,22} In our study, mast cells were rarely observed in G2\textsuperscript{IGN/IGN} mouse skin (Figure 2E), and mast cells with characteristic morphology could not be found in peritoneal lavage fluid from G2\textsuperscript{IGN/IGN} mice (Figure 2F). Thus, mast cell development appears to be impaired in G2\textsuperscript{IGN/IGN} mice, at least in part, due to skewed differentiation toward the granulomonocytic lineage.

We also undertook a cohort study using heterozygous mutant (G2\textsuperscript{IGN/+}) mice as a control group. The results showed that G2\textsuperscript{IGN/+} mice were prone to die earlier than G2\textsuperscript{IGN/IGN} mice even if they avoided
congenital anomalies of the kidney and urinary tract (Figure 2G). These results further support the notion that G2\textsuperscript{fGN/fGN} mice eventually suffer from hematological disorders.

### 3.3 Hematopoietic progenitors are increased in G2\textsuperscript{fGN/IGN} mice with age

Because GATA2 is a transcription factor involved in the maintenance of hematopoietic stem and progenitor cells,\textsuperscript{2,12} we examined the expression profiles of cKit and Sca1 in lineage-negative hematopoietic cells. We found that lineage-negative, cKit-positive, and Sca1-negative (K’S’L’) myeloid progenitors accumulated abundantly in the BM and spleens of G2\textsuperscript{fGN/IGN} mice (Figure 3A). K’S’L’ myeloid progenitor accumulation was observed to some extent in mice of all ages; however, this effect appeared to be stronger in elderly mice (Figure 3B,C).

Impaired stem cell function is associated with age and is usually accompanied by skewed myeloid differentiation and impaired HSC self-renewal.\textsuperscript{23,24} Although the proportion of K’S’ cells in the lineage-negative fraction (K’S’L’) was maintained in G2\textsuperscript{fGN/IGN} mice (Figure 3D), the cells were still heterogeneous and mixed with lineage-primed multipotent progenitors and short-term and long-term HSCs.\textsuperscript{25} Therefore, we used the Hoechst-effluxing side population (SP) assay because long-term repopulating HSCs are known to be highly concentrated in the SP fraction.\textsuperscript{26} We found that SP cells were decreased considerably in the K’S’L’ cell population from the BM of G2\textsuperscript{fGN/IGN} mice (Figure 3E).

### 3.4 Hematopoietic reconstitution is impaired in G2\textsuperscript{fGN/IGN} mice

To further evaluate HSC functions in G2\textsuperscript{fGN/IGN} mice, we carried out hematopoietic reconstitution assays with the BM cells. C57BL/6 and DBA/2 mixed background G2\textsuperscript{fGN/+} mice were back-crossed more than 6 generations onto the C57BL/6 genetic background to generate C57BL/6 G2\textsuperscript{fGN/IGN} mice.

We transplanted 1 × 10\textsuperscript{5} BM cells obtained from 73-day-old mice into 9.3-Gy lethally irradiated C57BL/6 recipient mice and evaluated CFU-S8 generation in the recipient mice. It has been reported that K’S’L’ cells contribute most significantly to CFU-S8 formation.\textsuperscript{25}
Although K'S'L' cells accumulated in G2\textsuperscript{IGN/IGN} mice (Figure 3A-C), CFU-S8 formation was significantly lower in G2\textsuperscript{IGN/IGN} mice than in WT mice (Figure 4A-B), suggesting that progenitor functions might be impaired in the K'S'L' cells of G2\textsuperscript{IGN/IGN} mice.

To examine reconstitution ability, we transplanted 1 × 10\textsuperscript{6} BM cells obtained from 140-day-old WT and 128-day-old G2\textsuperscript{IGN/IGN} mouse BM into eight and nine recipient mice per donor mouse, respectively, and monitored for survival and clinical signs of cachexia daily. Notably, 5 of 9 recipient mice that were transplanted with G2\textsuperscript{IGN/IGN} BM cells did not survive for more than 3 weeks after transplantation, whereas all mice transplanted with WT BM cells survived the entire observation period (Figure 4C).

We examined the hematopoietic indices of mice that survived 3 weeks after transplantation and found that RBC and platelet values were restored in mice transplanted with WT BM cells (962 ± 39 and 82.4 ± 14.5 × 10\textsuperscript{5}/μL, respectively [n = 7]), while significant anemia and thrombocytopenia were observed in mice transplanted with G2\textsuperscript{IGN/IGN} BM cells (574 ± 175 and 33.5 ± 12.2 [n = 4]). Significant leukocytosis was observed in mice transplanted with G2\textsuperscript{IGN/IGN} BM cells, in which granulocytes were predominant (Figure 4D). Furthermore, spleens of the recipient mice transplanted with G2\textsuperscript{IGN/IGN} BM cells were enlarged and the splenic architecture was destroyed 5 weeks after the transplantation (Figure 4E,F). The BM of mice transplanted with G2\textsuperscript{IGN/IGN} BM cells contained primarily Gr1\textsuperscript{+} myeloid cells (Figure 4G). These results suggest that progenitors skew toward myeloid lineage differentiation in G2\textsuperscript{IGN/IGN} mice.

We next transplanted 1:1 BM mixtures of 63-day-old donor mice and WT mice into 4 recipient mice per donor mouse. The donor-derived cells can be distinguished from the competitive cells by CD45 isotype markers. We measured the proportion of donor-derived cells in the PB of recipient mice and found that both myeloid and lymphoid cells recovered from G2\textsuperscript{IGN/IGN} mice were significantly reduced (Figure 4H,I).
indicating that long-term reconstitution capacity is impaired in G2\textsuperscript{fGN/fGN} mice. Notably, although the population of G2\textsuperscript{fGN/IGN} derived myeloid cells recovered at 4 weeks after transplantation, the number of these cells gradually decreased (Figure 4H). Reconstitution toward the lymphoid lineage was disturbed more severely in G2\textsuperscript{fGN/fGN} cells than in myeloid lineage cells (Figure 4H,I). We surmise that these findings are due, at least in part, to the myeloid-biased differentiation feature of progenitor/stem cells from G2\textsuperscript{fGN/fGN} mice. Taken together, these transplantation analyses indicate that hypomorphic GATA2 mutations impair the reconstitution function of HSCs and induce myeloid-biased proliferation and HSC differentiation.

3.5 | Myeloid progenitors accumulate in G2\textsuperscript{fGN/fGN} mice from an early age

We found that G2\textsuperscript{fGN/IGN} embryos from the C57BL/6 congenic strain were prone to die in utero due to severe anemia (Figure S2A,B). This outcome hampered further analysis of adult hematopoiesis. Therefore, we decided to change the background of the mice. In our search for a suitable mouse background, we found that the G2\textsuperscript{fGN/fGN} genotype did not provoke significant embryonic lethality in ICR mice (Figure S2A,C). The incidence of embryonic lethality was reduced when we used one G2\textsuperscript{fGN} parent in the F1 generation of ICR and C57BL/6 mice (Figure S2A). We used 10-week-old G2\textsuperscript{fGN/fGN} mice generated by brother-sister inbreeding of G2\textsuperscript{fGN/+} mice on the C57BL/6 and ICR mixed background for further experiments and WT littermates in the breeding colony as controls. The expression of GATA2 protein was decreased to approximately 40% of the WT level (Figure S3). We confirmed that adult G2\textsuperscript{fGN/IGN} mice with this background escaped from the incidence of congenital anomalies of the kidney and urinary tract (Figure S4).

The C57BL/6 and ICR mixed background G2\textsuperscript{fGN/IGN} mice had thrombocytopenia as well as macrocytic and hyperchromic erythrocytes (Figure 5A), similar to the C57BL/6 and DBA/2 mixed background mice (Figure 1A), suggesting that the hematological phenotypes are not related to the genetic background. Furthermore, we found that the spleen was enlarged in young G2\textsuperscript{fGN/IGN} mice, but there were no significant differences between the body weights of WT and G2\textsuperscript{fGN/IGN} mice (Figure 5B-D).

We found that K\textsuperscript{S−L−} myeloid progenitors accumulated in G2\textsuperscript{fGN/IGN} mice, particularly in their spleens (Figure 5E), but the proportion of K\textsuperscript{S+L−} cells did not change (data not shown). Intriguingly, we found that the proportion of GMPs was increased in the BM and spleens

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**Figure 5** Myeloid-biased differentiation and proliferation occur in young G2\textsuperscript{fGN/IGN} mice. A, Hematopoietic indices of young G2\textsuperscript{fGN/IGN} mice. B-D, G2\textsuperscript{fGN/IGN} mice have enlarged spleens. Comparison of body weights (B) and spleen/body weight ratios (C) of G2\textsuperscript{fGN/IGN} mice with WT control mice. Horizontal red lines indicate average values. Representative macroscopic appearance of enlarged spleens from young male G2\textsuperscript{fGN/IGN} mice are shown (D). E, Proportion of cKit-positive and Sca1-negative (K\textsuperscript{S+}) cells in lineage-negative bone marrow (BM) and splenic (Sp) hematopoietic cells from young G2\textsuperscript{fGN/IGN} mice. F, Representative flow cytometry plots for Fc\textgamma R and CD34 in K\textsuperscript{S+} lineage-negative (L\textsuperscript{−}) cells from BM samples (upper) and spleens (lower) of WT (left) and G2\textsuperscript{fGN/IGN} (right panels) mice. Note the marked increase in the granulocyte-monocyte progenitor (GMP) population in G2\textsuperscript{fGN/IGN} mice. G, Box-and-whisker plots of the common myeloid progenitor (CMP), GMP, and megakaryocyte-erythrocyte progenitor (MEP) populations in the BM samples (left) and spleens (right). H, Numbers of colonies formed 14 d after the start of culture using 250 BM CMPs (left) and GMPs (right). Colonies consisting of more than 30 cells were counted. *P < .05, **P < .01, †P < .001, ‡P < .0005 by Student’s t test (A) or Mann-Whitney U test (B, C, E, G, H). Hb, hemoglobin; Hct, hematocrit; MCH, erythrocyte mean corpuscular hemoglobin; MCHC, erythrocyte mean corpuscular hemoglobin concentration; MCV, erythrocyte mean corpuscular volume.
from G2\textsuperscript{fGN/IGN} mice (Figure 5F-G). In addition, the proportion of CMPs was also increased in the spleens of G2\textsuperscript{fGN/IGN} mice, while that of megakaryocyte-erythrocyte progenitors (MEPs) was decreased in the spleens (Figure 5G). Notably, although MEPs are the majority of K+S\textsuperscript{-} myeloid progenitors in the spleens of WT mice, approximately half of the K+S\textsuperscript{-} cells in the G2\textsuperscript{fGN/IGN} mouse spleens were GMPs. We also found that monocyte colony-forming efficiency was significantly increased in the GMP fraction from WT mice, (Figure 5H). Thus, in G2\textsuperscript{fGN/IGN} mice, myeloid lineage cells with differentiation capacity toward the granulomonocytic cell lineage were increased at a young age.

### 3.6 Csf1r and Il6ra mRNA levels are increased in G2\textsuperscript{fGN/IGN} myeloid progenitors

We next undertook a microarray gene expression analysis using BM GMPs isolated from 2 independent WT and G2\textsuperscript{fGN/IGN} mice. We identified 2698 upregulated genes and 1973 downregulated genes in G2\textsuperscript{fGN/IGN} mice by comparing the average values of the 2 mice (fold-change cutoff: 1.5-fold) (Tables S3 and S4) and “cytokine–cytokine receptor interaction pathway” was most significantly enriched in both groups (Figure 6A). A differential expression analysis revealed that the variation of genes categorized in this pathway was broad in G2\textsuperscript{fGN/IGN} mice (Figure 6B).

We selected the macrophage colony-stimulating factor receptor (Csf1r) and interleukin-6 receptor α-chain precursor (Il6ra) genes for further examination based on their fold-change in the microarray analysis and their biologic relevance. Both are important for granulocyte and monocyte differentiation.\textsuperscript{27-29} We used real-time PCR to find that Csf1r and Il6ra gene expression was significantly increased in G2\textsuperscript{fGN/IGN} GMPs, whereas granulocyte-macrophage-colony-stimulating factor receptor α-subunit (Csf2ra) and granulocyte colony-stimulating factor receptor (Csf3r2) mRNA levels did not change much (Figure 6C). Flow cytometric analyses showed that cells highly expressing macrophage colony-stimulating factor (M-CSF) and IL-6 receptors were increased in the G2\textsuperscript{fGN/IGN} GMPs (Figure 6S). We surmise that high expression levels of the M-CSF and IL-6 receptors in myeloid progenitors might promote progenitor differentiation toward granulomonocytic lineages.

### 3.7 G2\textsuperscript{fGN/IGN} mice overreact to LPS and produce granulomonocytes

In our long-term observations, we found that the leukocytosis and myeloid hyperplasia in G2\textsuperscript{fGN/IGN} mice worsened with age. It is plausible that certain environmental factors might contribute to this phenotype. Because the M-CSF and IL-6 receptors were overexpressed in G2\textsuperscript{fGN/IGN} GMPs, myeloid progenitors might be highly sensitive to inflammatory stresses. Because LPS is known to trigger a distinctive pattern of pro-inflammatory cytokine release, we next examined the effects of LPS on 10-week-old G2\textsuperscript{fGN/IGN} mice.

Peripheral blood samples from the mice were analyzed 4 days after i.p. LPS administration at a dose of 5 mg/kg body weight or the same volume of vehicle. There were no significant changes in the total WBC count or any type of WBC count in WT mice treated with LPS; these data indicated that 5 mg/kg body weight LPS did not modify the hematopoietic process considerably in WT mice (Figure 7A). In sharp contrast, the numbers of monocytes and granulocytes were significantly increased after LPS treatment in G2\textsuperscript{fGN/IGN} mice, whereas the number of B lymphocytes was markedly decreased (Figure 7A). Although the total WBC counts in young G2\textsuperscript{fGN/IGN} mice appeared to be in the normal range, the monocyte and granulocyte numbers were both increased in G2\textsuperscript{fGN/IGN} mice, even in the vehicle control group (Figure 7A). These findings indicated that

\textit{FIGURE 6} Gene expression analysis of G2\textsuperscript{fGN/IGN} granulocyte-monocyte progenitor (GMP) cells. A. Differentially expressed genes in bone marrow GMPs from G2\textsuperscript{fGN/IGN} mice according to Kyoto Encyclopedia of Genes and Genomes pathway analysis. B. Expression profiles of genes encoding cytokines or cytokine receptors. C. Quantitative real-time PCR analyses of the cytokine receptor genes Csf1r, Csf2ra, Csf3r, and Il6ra in common myeloid progenitor (CMP) and GMP cells. The average value in WT CMPs was set to 1. *P < .05, Mann-Whitney U test. FC, fold change; IL, interleukin; PDGF, platelet-derived growth factor; TGF-β, transforming growth factor-β; TNF, tumor necrosis factor.
certain differentiation regulation toward these lineages occurs in the steady-state condition. Thus, quantitative GATA2 deficits render G2fGN/fGN mice hyperreactive to inflammatory stimuli and provoke granulomonocytic cell proliferation in response to the stimuli (Figure 7B).

4 | DISCUSSION

Chronic myelomonocytic leukemia is classified as a myelodysplastic/myeloproliferative neoplasm (MDS/MPN).\textsuperscript{30} The molecular mechanisms underlying the onset of CMMoL remain to be fully understood. We show here that GATA2 hypomorphism is one of the pathogenic factors of CMMoL development. We found that quantitative GATA2 deficiency provokes human CMMoL-like disease in mice, which involves defects in hematopoietic stem/progenitor cells. The hypomorphic expression of GATA2 reduces self-renewal activity in HSCs and skewed progenitor cell differentiation toward the granulomonocytic lineage. As summarized in Figure 7B, our data further suggest that with repeated inflammatory signals, HSCs with GATA2 hypomorphism and impaired stemness are prone to transformation into CMMoL-like cells.

The pathological natures of the diseases caused by heteroallelic loss-of-function mutations in the GATA2 gene have been an area of strong interest.\textsuperscript{31-34} Importantly, the types of mutations in the human GATA2 gene vary and include nonsense mutations, frameshift mutations, missense mutations in the DNA binding region, and regulatory mutations that lead to reduced expression.\textsuperscript{35,36} Many individuals with these GATA2 mutations have histories of recurrent infections, and these infections are relevant to the characteristic clinical features of B cell, natural killer cell, and monocyte deficiency. The affected individuals harbor an increased risk of developing a variety of hematopoietic malignancies with aging, including MDS, AML, MPN, and CMMoL.\textsuperscript{36} It is conceivable that complex mechanisms, including environmental factors, underlie the pathogenesis of the hematopoietic malignancies that occur with GATA2 gene mutations.

We found here that G2fGN/fGN mice, which have approximately 20% of the GATA2 expression of wild-type mice, show marginal hematological abnormalities in hematopoietic indices in young ages, with the exception of macrocytic-hypochromic erythrocytes. Notably, however, the numbers of granulocytes and monocytes increase with age, and a portion of G2fGN/fGN mice develop severe granulomonocytosis with trilineage morphological abnormalities. These features resemble CMMoL in humans. We surmise that G2fGN/fGN mice are predisposed to MPN/MDS and that the underlying pathological mechanisms are similar to those of human hematological diseases caused by heteroallelic GATA2 gene mutations.

Importantly, the numbers of peripheral granulocytes and monocytes are increased in asymptomatic G2fGN/fGN mice, whereas monocyte deficiency is one of the characteristic features of the incipient stage of GATA2 haploinsufficiency-related human diseases.\textsuperscript{31,32,37,38} We surmise that the GATA2 gene expression from one normal allele could cause the difference in myeloid development compared to the artificially modified GATA2 gene expression in G2fGN/fGN mice. The reduction in GATA2 expression to 20% of the WT level might skew the myeloid progenitors...
toward the granulocyte-monocyte lineage. Indeed, although it has been reported that GMPs do not accumulate considerably in Gata2-haploinsufficient mice, we found that GMPs accumulated in G2G2 mice in this study, further supporting the notion that low GATA2 expression levels in G2G2 mice elicit CMP differentiation bias toward GMPs.

We propose that myeloid progenitors in G2G2 mice are hypersensitive to cytokines that stimulate granulomonocyte differentiation. In fact, we found that injecting low-dose LPS, which does not cause leukocytosis in WT mice, worsens granulomonocytosis in G2G2 mice, indicating that G2G2 mice are sensitive to environmental stimuli that usually do not cause leukocytosis in WT mice. We surmise that the increased Csfr1 and Il6r gene expression in G2G2 GMPs is involved, at least in part, in the hyperreactive phenotype induced by LPS treatment. We also found that G2G2 HSC reconstitution is impaired concomitantly. Taken together, these results support our proposal that impaired GATA2 function in G2G2 mice increases the risk of developing hematological neoplasms in HSC and progenitors.

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CONFLICT OF INTEREST

The authors declare no competing financial interests.

ORCID

Ritsuko Shimizu https://orcid.org/0000-0001-6672-7606

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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