Received: 7 September 2018 | Revised: 8 January 2019 | Accepted: 24 January 2019

DOI: 10.1111/cas.13959

ORIGINAL ARTICLE

WILEY Cancer Science

GATA2 hypomorphism induces chronic myelomonocytic leukemia in mice

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Funding information
Japan Society for the Promotion of Science KAKENHI, Grant/Award Number: 26860204, 15H04759 and 15H02507; Japan Agency for Medical Research and Development, Grant/Award Number: JP18am0101095; Uehara Memorial Foundation

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 ($G2^{fGN/fGN}$) 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 $G2^{fGN/fGN}$ mice suffered from exacerbated leukocytosis concomitant with progressive anemia and thrombocytopenia and eventually developed massive granulomonocytosis accompanied by trilineage dysplasia. The reconstitution activity of $G2^{fGN/fGN}$ mouse stem cells was impaired. Furthermore, $G2^{fGN/fGN}$ progenitors showed myeloid lineage-biased proliferation and differentiation. Myeloid progenitor accumulation started at a younger age in $G2^{fGN/fGN}$ mice and appeared to worsen with age. $G2^{fGN/fGN}$ 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

1 | INTRODUCTION

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′.[1] 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.[2–11] In hematopoietic tissues, GATA2 is abundantly expressed in hematopoietic stem cells (HSCs) and contributes to long-term reconstitution activity.[12] GATA2-knockout HSCs fail to contribute to adult hematopoiesis in chimeric mice,[2] whereas forced GATA2 expression interferes with the repopulation function of HSCs due to reduced self-renewal capacity.[13-15] Furthermore, HSC reconstitution activity is impaired by haploinsufficiency of the *Gata2* gene.[16] Intriguingly, the low-dose expression of exogenous GATA2 increases the clonogenic potential of HSCs.[17] 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.[18,19] Indeed, a small increase in the G*ata2* gene guides progenitor cells to differentiate into $Gr1^+$ myeloid cells,[17] whereas forced high expression levels of the gene restrict granulocyte-monocyte progenitors (GMPs) to develop exclusively into eosinophils and mast cells.[19] In contrast, *Gata2* gene haploinsufficiency reduces the GMP cell population but not the CMP cell population in mice,[20] 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 ($G2^{fGN/fGN}$), in which GATA2 expression level is reduced to 20% of WT mice.[7,8] 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.[7,8] The penetrance of this urological abnormality is not complete; some mice show only unilateral or no obstruction. Therefore, $G2^{fGN/fGN}$ mice that have at least one functional kidney reach adulthood.

In this regard, whether (and how) reduced *Gata2* gene expression in $G2^{fGN/fGN}$ mice affects adult hematopoiesis remains to be determined. In this study, we found that $G2^{fGN/fGN}$ mice were prone to developing granulomonocytosis with trilineage dysplasia after 6 months, and these neoplasms closely resemble those observed in chronic myelomonocytic leukemia (CMMoL) in humans. We also analyzed HSC reconstitution activity in $G2^{fGN/fGN}$ 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 $G2^{fGN/fGN}$ mice, and the $G2^{fGN/fGN}$ mice were sensitive to lipopolysaccharide (LPS) stimulation and easily developed granulomonocytosis. Taken together, these findings indicate that GATA2 hypomorphism in $G2^{fGN/fGN}$ 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 $G2^{fGN/fGN}$ mice was described previously.[7] 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 FACSCalibur or FACSAria 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 ReverTra-Ace (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

(A)

		n	Age (d)	WBC ($\times 10^2/\mu L$)	Pt ($\times 10^4/\mu L$)	RBC ($\times 10^4/\mu L$)	Hct (%)	Hb (g/dL)	MCV (fl)	MCH (pg)	MCHC (%)
Young	WT	51	61 ± 17	76 ± 12	81.9 ± 12.3	1032 ± 67	49.6 ± 2.1	14.9 ±0.8	48.2 ± 2.7	14.5 ± 0.9	30.1 ± 1.7
	$G2^{fGN/fGN}$	21	71 ± 14	73 ± 31	62.6¶ ± 17.0	941*** ± 13	48.0 ± 3.8	15.7*** ±1.1	51.3† ± 3.4	16.8¶ ± 1.4	32.8¶ ± 1.2
Middle	WT	24	151 ± 37	78 ± 4	100.3 ± 17.9	1039 ± 66	49.0 ± 3.1	15.3 ±1.2	47.2 ± 2.7	14.7 ± 0.8	30.2 ± 2.3
	$G2^{fGN/fGN}$	37	167 ± 45	156 ± 175	67.4¶ ± 20.1	922¶ ±123	47.4 ± 4.1	15.6 ±1.2	51.8¶ ± 4.1	17.1¶ ± 1.8	33.0** ± 1.5
Elder	WT	20	399 ± 82	73 ± 29	99.0 ± 26.0	903 ± 79	44.4 ± 3.9	13.2 ±1.3	49.2 ± 2.5	14.7 ± 0.6	30.0 ± 1.9
	$G2^{fGN/fGN}$	25	332* ± 80	212* ± 245	55.4¶ ± 19.8	841* ± 96	44.5 ± 4.6	14.8† ±1.5	53.2† ± 4.2	17.7¶ ± 1.7	33.3¶ ± 1.6

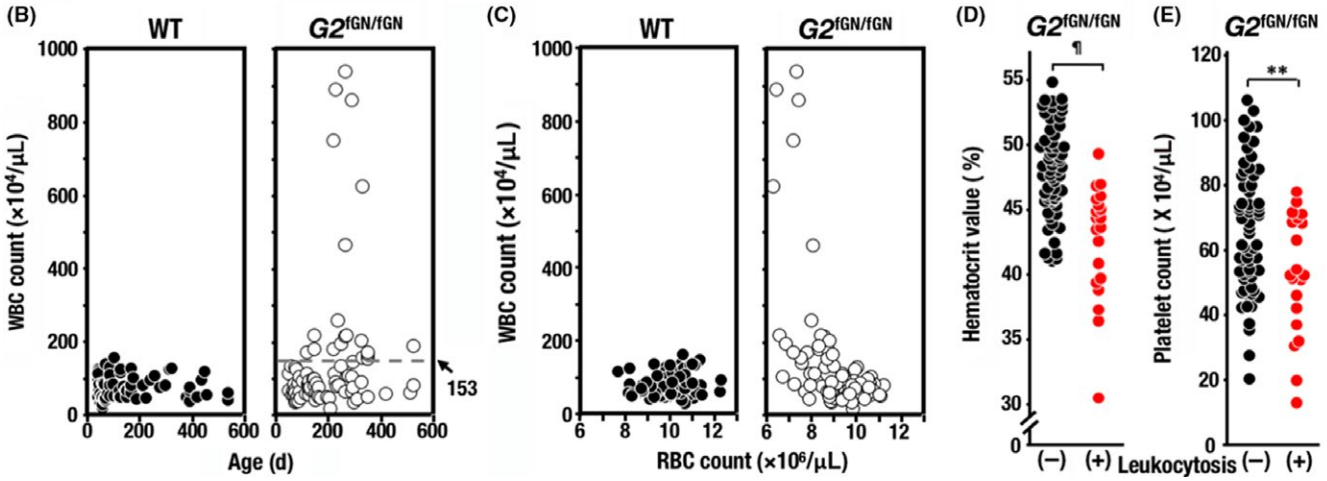

FIGURE 1 Leukocytosis occurs in aged $G2^{fGN/fGN}$ mice. A, Hematopoietic indices of young, middle-aged and elderly mice. B, Measurement of the correlation between white blood cell (WBC) count and age in WT (left) and $G2^{fGN/fGN}$ (right) mice. The dotted horizontal line (153×10^4 cells/μL) indicates the leukocytosis border, which was determined by double counting the mean WBC counts of a group of WT mice. C, Measurement of the correlation between WBC and red blood cell (RBC) counts in WT (left) and $G2^{fGN/fGN}$ (right) mice. D,E, Comparison of hematocrit values (D) and platelet (Pt) counts (E) in $G2^{fGN/fGN}$ mice suffering from leukocytosis (red circles) and $G2^{fGN/fGN}$ mice with normal WBC counts (black circles; $67\text{-}83 \times 10^4$ cells/μL; 99% confidence interval for the mean WBC count of the WT mice). $^*P < .05$, $^{**}P < .01$, $^{***}P < .005$, $^{\dagger}P < .001$, $^{\ddagger}P < .0005$, $^{\P}P < .00005$ by Student's *t* test. Hb, hemoglobin; Hct, hematocrit; MCH, erythrocyte mean corpuscular hemoglobin; MCHC, erythrocyte mean corpscular hemoglobin concentration; MCV, erythrocyte mean corpscular volume

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 | $G2^{fGN/fGN}$ 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 $G2^{fGN/fGN}$ mice in a C57BL/6 and DBA/2 mixed background. We utilized this cohort of $G2^{fGN/fGN}$ 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 $G2^{fGN/fGN}$ mice suffered from thrombocytopenia throughout their lives and from leukocytosis at an elderly age (Figure 1A). Notably, increased white blood cell (WBC) counts in $G2^{fGN/fGN}$ mice correlated with the age of the mice with a Spearman's rank correlation coefficient of 0.3024 ($P < .001$) (Figure 1B). Consequently, elderly $G2^{fGN/fGN}$ mice eventually suffered from overt leukocytosis, although some $G2^{fGN/fGN}$ mice maintained WBC counts within a permissible range (Figure 1B). We also found that the erythrocytes from $G2^{fGN/fGN}$ 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 $G2^{fGN/fGN}$ 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 $G2^{fGN/fGN}$ mice with a Spearman's rank correlation coefficient of −0.5383 ($P < .0001$), but not in WT mice (Figure 1C). Thus, the progression of leukocytosis coincided with anemia. In fact, the hematocrit values and the platelet counts were significantly lower in $G2^{fGN/fGN}$

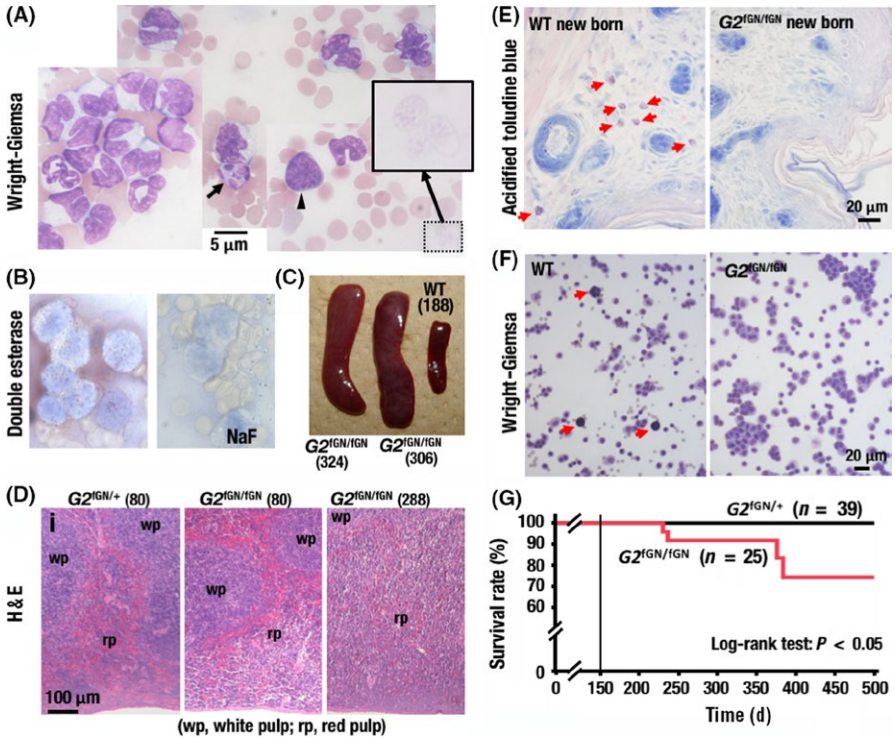

FIGURE 2 *G2*$^{fGN/fGN}$ mice have a chronic myelomonocytic leukemia-like phenotype. A,B, Examination of the peripheral blood smears of *G2*$^{fGN/fGN}$ mice with leukocytosis by Wright-Giemsa (A) and double-esterase staining (B). The arrow and arrowhead indicate nuclear fragmentation and blast-like cells, respectively, and the inset shows a partially enlarged image of hypogranular platelets. Naphthyl-butyrate activity is inhibited by sodium fluoride in the right panel of B. C,D, Macroscopic (C) and microscopic (D) views of the spleens of *G2*$^{fGN/fGN}$ mice. Age at analysis (days) shown in parentheses. E, Acidified toluidine blue staining of skin sections. Mast cells appear dark bluish-purple (red arrows). F, Wright-Giemsa staining of the peritoneal lavage fluid. Red arrows indicate mast cells with dark purple granules. G, A comparative survival evaluation of *G2*$^{fGN/+}$ (black) and *G2*$^{fGN/fGN}$ (red) mice that survived more than 150 d after birth

mice suffering from overt leukocytosis than in *G2*$^{fGN/fGN}$ mice with normal WBC counts (Figure 1D,E). Thus, these hematological data from *G2*$^{fGN/fGN}$ 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*$^{fGN/fGN}$ 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$^+$ or Mac1$^+$ myeloid cells in the PB samples of *G2*$^{fGN/fGN}$ mice (Figure S1). Importantly, we also observed morphological abnormalities in the PB cells from *G2*$^{fGN/fGN}$ 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*$^{fGN/fGN}$ mice without massive leukocytosis were practically normal (data not shown). These findings indicate that *G2*$^{fGN/fGN}$ mice eventually develop certain hematological disorders, resembling the features of CMMoL in humans.

Elderly *G2*$^{fGN/fGN}$ mice with massive leukocytosis showed marked splenomegaly (Figure 2C). Histological examinations revealed that the splenic architecture was substantially preserved in the young *G2*$^{fGN/fGN}$ mice without leukocytosis (Figure 2D), whereas the enlarged spleens of the elderly *G2*$^{fGN/fGN}$ 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.[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.[21,22] In our study, mast cells were rarely observed in *G2*$^{fGN/fGN}$ mouse skin (Figure 2E), and mast cells with characteristic morphology could not be found in peritoneal lavage fluid from *G2*$^{fGN/fGN}$ mice (Figure 2F). Thus, mast cell development appears to be impaired in *G2*$^{fGN/fGN}$ mice, at least in part, due to skewed differentiation toward the granulomonocytic lineage.

We also undertook a cohort study using heterozygous mutant (*G2*$^{fGN/+}$) mice as a control group. The results showed that *G2*$^{fGN/fGN}$ mice were prone to die earlier than *G2*$^{fGN/+}$ mice even if they avoided

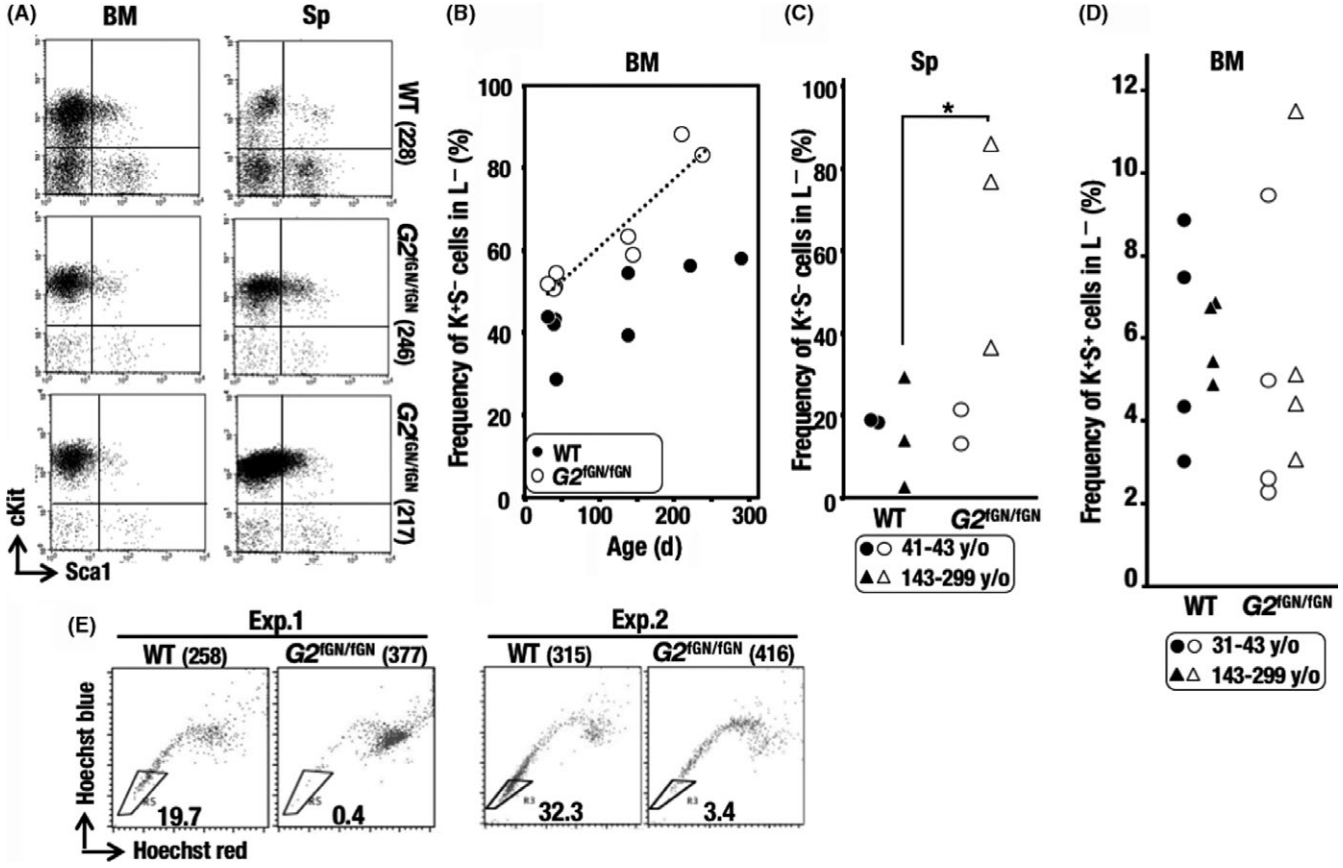

FIGURE 3 Hematopoietic progenitors are increased in $G2^{fGN/fGN}$ mice. A, Representative flow cytometry plots for cKit and Sca1 in lineage-negative cells from the bone marrow (BM) and spleens (Sp) of mice. Age at analysis (days) shown in parentheses. B,C, Correlations of the proportion of cKit-positive and Sca1-negative (K^+S^-) cells in lineage-negative (L^-) BM (B) and splenic (C) hematopoietic cells with the age of the mice. The proportions of K^+S^- cells in L^- cells were significantly correlated with the age of the mice for the BM samples ($P < .005$ by Spearman's rank correlation coefficient test [dotted line]) and spleens ($P < .05$ by Mann-Whitney U test). D, Proportion of K^+S^+ cells in L^- BM. E, Reduced number of cells with a Hoechst 33342 low-fluorescent profile in $K^+S^+L^-$ cells from $G2^{fGN/fGN}$ BM. Age at analysis (days) shown in parentheses

congenital anomalies of the kidney and urinary tract (Figure 2G). These results further support the notion that $G2^{fGN/fGN}$ mice eventually suffer from hematological disorders.

3.3 | Hematopoietic progenitors are increased in $G2^{fGN/fGN}$ mice with age

Because GATA2 is a transcription factor involved in the maintenance of hematopoietic stem and progenitor cells,[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 $G2^{fGN/fGN}$ 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.[23,24] Although the proportion of K^+S^+ cells in the lineage-negative fraction ($K^+S^+L^-$) was maintained in $G2^{fGN/fGN}$ mice (Figure 3D), the cells were still heterogeneous and mixed with lineage-primed multipotent progenitors and short-term and long-term HSCs.[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.[26] We found that SP cells were decreased considerably in the $K^+S^+L^-$ cell population from the BM of $G2^{fGN/fGN}$ mice (Figure 3E).

3.4 | Hematopoietic reconstitution is impaired in $G2^{fGN/fGN}$ mice

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

We transplanted 1×10^5 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.[25]

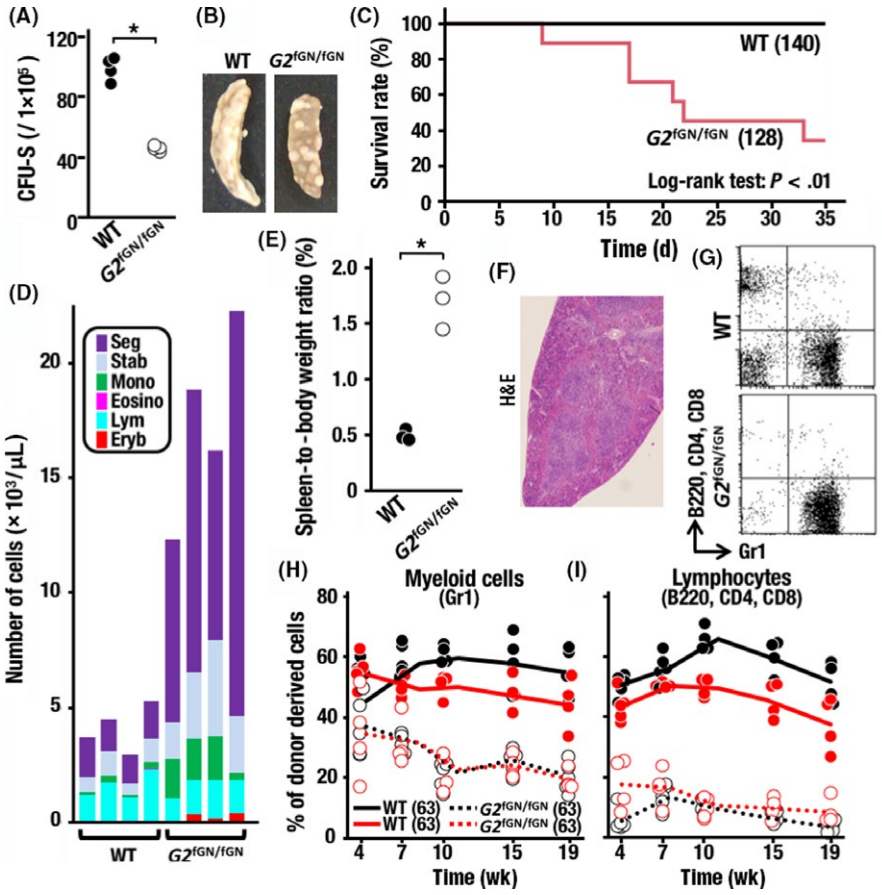

FIGURE 4 Reconstitution activity is impaired in hematopoietic progenitors and stem cells from $G2^{fGN/fGN}$ mice. A,B, Results of the CFU-S8 assays of 4 recipient mice transplanted with WT and $G2^{fGN/fGN}$ bone marrow (BM) cells. Spleens of recipient mice were collected 8 d after transplantation and fixed in Tellesniczky's solution, then macroscopically visible colonies were counted. Representative macroscopic images of the recipient spleens transplanted with WT (left) and $G2^{fGN/fGN}$ (right) BM cells are shown in B. *P < .05, Mann-Whitney U test. C, Comparative survival analysis of recipient mice transplanted with WT (black; 140 d old) or $G2^{fGN/fGN}$ (red; 128 d old) BM cells. D, Hematopoietic indices of recipient mice 21 d after transplantation. Four recipient mice of each group in (C) were used. E, Spleen-to-body weight ratios of the recipient mice 35 d after transplantation. *P < .05, Mann-Whitney U test. F, Representative histological features of the spleen sections from recipient mice transplanted with $G2^{fGN/fGN}$ mouse BM cells. G, Representative flow cytometry plots of lymphoid and myeloid BM antigens from recipient mice 35 d after transplantation. H,I, Percent reconstitution of myeloid and lymphoid cells tracked in peripheral blood over a period of 19 wk. Age of donor mice (days) are shown in parentheses. Data from 2 WT and 2 $G2^{fGN/fGN}$ mice are indicated with black/red solid and dotted lines, respectively

Although $K^+S^-L^-$ cells accumulated in $G2^{fGN/fGN}$ mice (Figure 3A-C), CFU-S8 formation was significantly lower in $G2^{fGN/fGN}$ mice than in WT mice (Figure 4A-B), suggesting that progenitor functions might be impaired in the $K^+S^-L^-$ cells of $G2^{fGN/fGN}$ mice.

To examine reconstitution ability, we transplanted 1×10^6 BM cells obtained from 140-day-old WT and 128-day-old $G2^{fGN/fGN}$ 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 $G2^{fGN/fGN}$ 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 $82.4 \pm 14.5 \times 10^4/\mu L$, respectively [n = 7]), while significant anemia and thrombocytopenia were observed in mice transplanted with $G2^{fGN/fGN}$ BM cells (574 ± 175 and 33.5 ± 12.2 [n = 4]). Significant leukocytosis was observed in mice transplanted with $G2^{fGN/fGN}$ BM cells, in which granulocytes were predominant (Figure 4D). Furthermore, spleens of the recipient mice transplanted with $G2^{fGN/fGN}$ BM cells were enlarged and the splenic architecture was destroyed 5 weeks after the transplantation (Figure 4E,F). The BM of mice transplanted with $G2^{fGN/fGN}$ BM cells contained primarily $Gr1^+$ myeloid cells (Figure 4G). These results suggest that progenitors skew toward myeloid lineage differentiation in $G2^{fGN/fGN}$ 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 $G2^{fGN/fGN}$ mice were significantly reduced (Figure 4H,I),

indicating that long-term reconstitution capacity is impaired in *G2*$^{fGN/fGN}$ mice. Notably, although the population of *G2*$^{fGN/fGN}$-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*$^{fGN/fGN}$ 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*$^{fGN/fGN}$ 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*$^{fGN/fGN}$ mice from an early age

We found that *G2*$^{fGN/fGN}$ 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*$^{fGN/fGN}$ 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*$^{fGN/+}$ parent in the F1 generation of ICR and C57BL/6 mice (Figure S2A). We used 10-week-old *G2*$^{fGN/fGN}$ mice generated by brother-sister inbreeding of *G2*$^{fGN/+}$ 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*$^{fGN/fGN}$ 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*$^{fGN/fGN}$ 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*$^{fGN/fGN}$ mice, but there were no significant differences between the body weights of WT and *G2*$^{fGN/fGN}$ mice (Figure 5B-D).

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

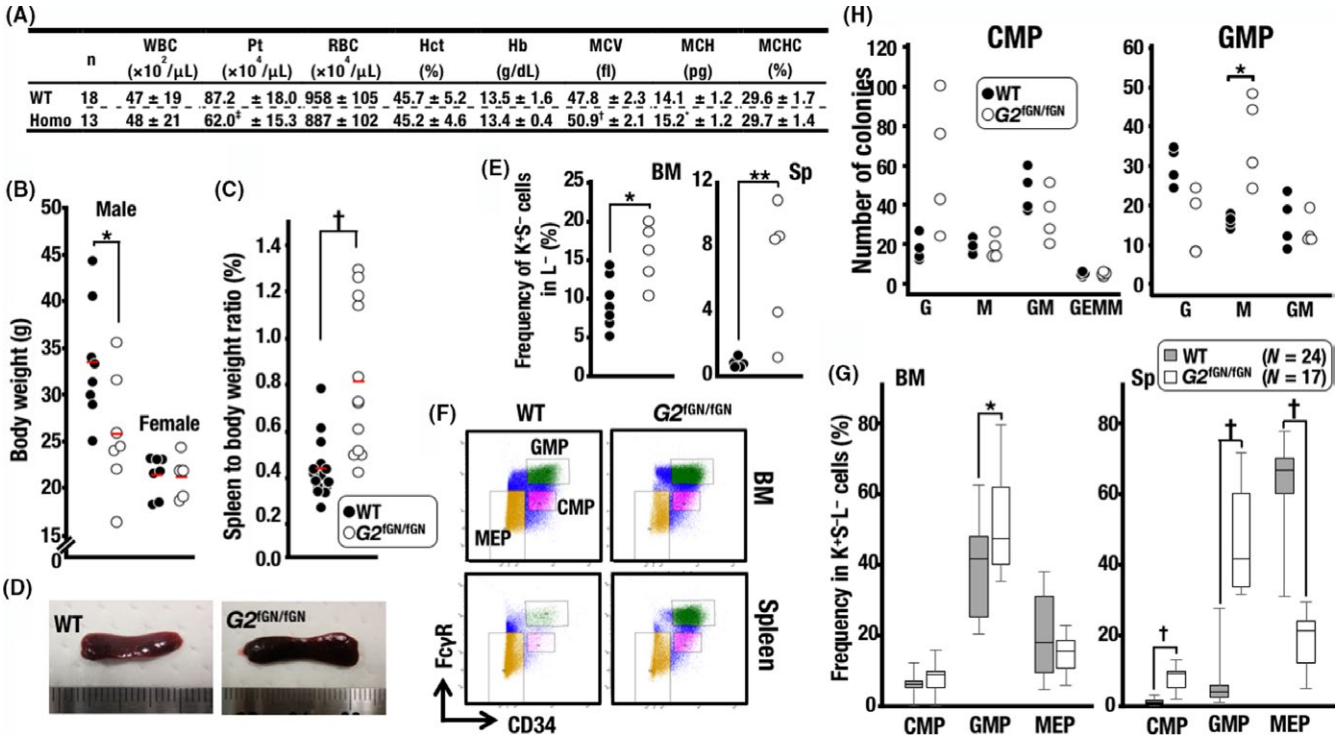

FIGURE 5 Myeloid-biased differentiation and proliferation occur in young *G2*$^{fGN/fGN}$ mice. A, Hematopoietic indices of young *G2*$^{fGN/fGN}$ mice. B-D, *G2*$^{fGN/fGN}$ mice have enlarged spleens. Comparison of body weights (B) and spleen/body weight ratios (C) of *G2*$^{fGN/fGN}$ mice with WT control mice. Horizontal red lines indicate average values. Representative macroscopic appearance of enlarged spleens from young male *G2*$^{fGN/fGN}$ mice are shown (D). E, Proportion of cKit-positive and Sca1-negative (K^+S^-) cells in lineage-negative bone marrow (BM) and splenic (Sp) hematopoietic cells from young *G2*$^{fGN/fGN}$ mice. F, Representative flow cytometry plots for FcγR and CD34 in K^+S^- lineage-negative (L^-) cells from BM samples (upper) and spleens (lower) of WT (left) and *G2*$^{fGN/fGN}$ (right panels) mice. Note the marked increase in the granulocyte-monocyte progenitor (GMP) population in *G2*$^{fGN/fGN}$ 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. $^*P < .05$, $^{**}P < .01$, $^{\dagger}P < .001$, $^{\ddagger}P < .0005$ 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 corpscular hemoglobin concentration; MCV, erythrocyte mean corpscular volume

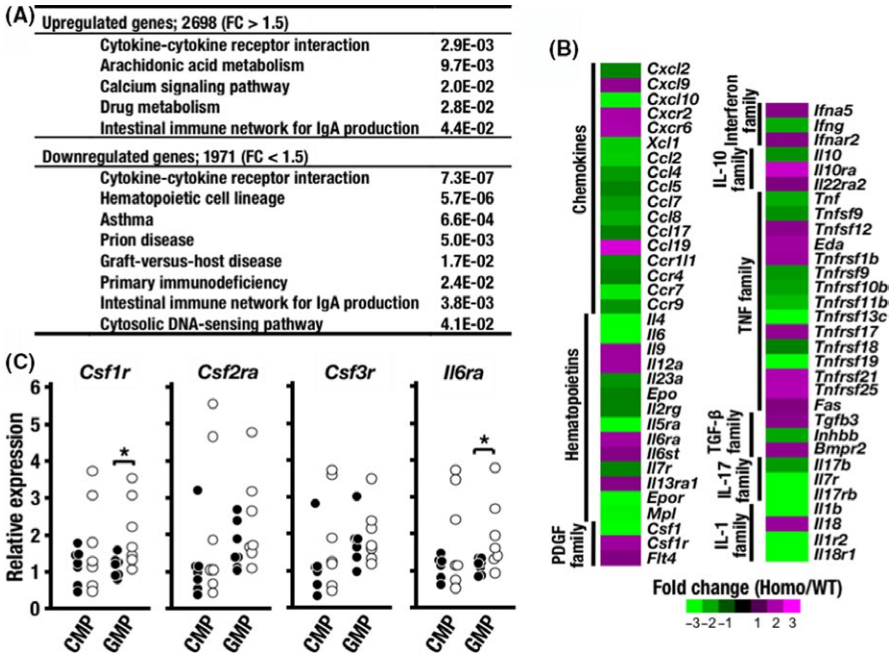

FIGURE 6 Gene expression analysis of $G2^{fGN/fGN}$ granulocyte-monocyte progenitor (GMP) cells. A, Differentially expressed genes in bone marrow GMPs from $G2^{fGN/fGN}$ mice according to Kyoto Encyclopedia of Genes and Genomes pathway analysis. B, Expression profiles of genes encoding cytokines or 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

from $G2^{fGN/fGN}$ mice (Figure 5F-G). In addition, the proportion of CMPs was also increased in the spleens of $G2^{fGN/fGN}$ mice, while that of megakaryocyte-erythrocyte progenitors (MEPs) was decreased in the spleens (Figure 5G). Notably, although MEPs are the majority of $K^+S^-L^-$ myeloid progenitors in the spleens of WT mice, approximately half of the $K^+S^-L^-$ cells in the $G2^{fGN/fGN}$ mouse spleens were GMPs. We also found that monocyte colony-forming efficiency was significantly increased in the GMP fraction from $G2^{fGN/fGN}$ mice (Figure 5H). Thus, in $G2^{fGN/fGN}$ 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 $G2^{fGN/fGN}$ myeloid progenitors

We next undertook a microarray gene expression analysis using BM GMPs isolated from 2 independent WT and $G2^{fGN/fGN}$ mice. We identified 2698 upregulated genes and 1973 downregulated genes in $G2^{fGN/fGN}$ 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 $G2^{fGN/fGN}$ 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.[27-29] We used real-time PCR to find that *Csf1r* and *Il6ra* gene expression was significantly increased in $G2^{fGN/fGN}$ 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 $G2^{fGN/fGN}$ GMPs (Figure S5). 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 | $G2^{fGN/fGN}$ mice overreact to LPS and produce granulomonocytes

In our long-term observations, we found that the leukocytosis and myeloid hyperplasia in $G2^{fGN/fGN}$ 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 $G2^{fGN/fGN}$ 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 $G2^{fGN/fGN}$ 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 $G2^{fGN/fGN}$ mice, whereas the number of B lymphocytes was markedly decreased (Figure 7A). Although the total WBC counts in young $G2^{fGN/fGN}$ mice appeared to be in the normal range, the monocyte and granulocyte numbers were both increased in $G2^{fGN/fGN}$ mice, even in the vehicle control group (Figure 7A). These findings indicated that

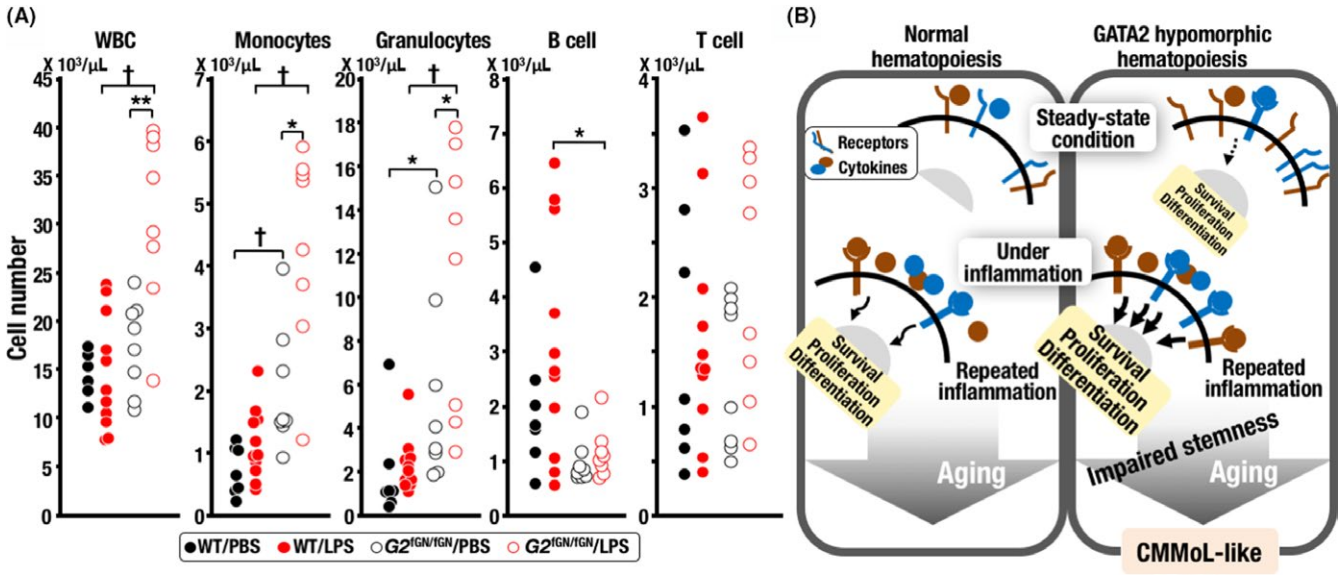

FIGURE 7 Overreaction of the *G2*$^{fGN/fGN}$ mouse hematopoietic system to lipopolysaccharide (LPS) challenge. A, Changes in the counts of total white blood cells (WBC) and monocytes (F4/80$^+$), granulocytes (Gr1$^+$F4/80$^-$), and B (B220$^+$) and T (CD4$^+$ or CD8$^+$) cells after LPS exposure. The number of cells was calculated based on the proportion of the cells defined by surface immune antigen expression. *P < .05, **P < .01, $^{\dagger}P$ < .001, Mann-Whitney U test. B, Model for the development of chronic myelomonocytic leukemia (CMMoL)-like disease caused by the hypomorphic expression of GATA2. *G2*$^{fGN/fGN}$ myeloid progenitors carrying an increased number of cytokine receptors are sensitive to cytokines, even in steady-state conditions. After repeated inflammatory signals throughout their lifetime, hematopoietic stem cells with impaired stemness occasionally transform into cells with a CMMoL-like appearance

certain differentiation regulation toward these lineages occurs in the steady-state condition. Thus, quantitative GATA2 deficits render *G2*$^{fGN/fGN}$ 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).[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.[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.[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.[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 *G2*$^{fGN/fGN}$ 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-hyperchromic erythrocytes. Notably, however, the numbers of granulocytes and monocytes increase with age, and a portion of *G2*$^{fGN/fGN}$ mice develop severe granulomonocytosis with trilineage morphological abnormalities. These features resemble CMMoL in humans. We surmise that *G2*$^{fGN/fGN}$ 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 *G2*$^{fGN/fGN}$ mice, whereas monocyte deficiency is one of the characteristic features of the incipient stage of GATA2 haploinsufficiency-related human diseases.[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 *G2*$^{fGN/fGN}$ mice. The reduction in GATA2 expression to 20% of the WT level might skew the myeloid progenitors

toward the granulocyte-monocyte lineage.[17,19] Indeed, although it has been reported that GMPs do not accumulate considerably in *Gata2*-haploinsufficient mice,[20] we found that GMPs accumulated in $G2^{fGN/fGN}$ mice in this study, further supporting the notion that low GATA2 expression levels in $G2^{fGN/fGN}$ mice elicit CMP differentiation bias toward GMPs.

We propose that myeloid progenitors in $G2^{fGN/fGN}$ 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 $G2^{fGN/fGN}$ mice, indicating that $G2^{fGN/fGN}$ mice are sensitive to environmental stimuli that usually do not cause leukocytosis in WT mice. We surmise that the increased *Csf1r* and *Il6r* gene expression in $G2^{fGN/fGN}$ GMPs is involved, at least in part, in the hyperreactive phenotype induced by LPS treatment. We also found that $G2^{fGN/fGN}$ HSC reconstitution is impaired concomitantly. Taken together, these results support our proposal that impaired GATA2 function in $G2^{fGN/fGN}$ mice increases the risk of developing hematological neoplasms in HSC and progenitors.

ACKNOWLEDGMENT

We thank Aya Goto and Eriko Naganuma for technical assistance. We also thank the Biomedical Research Core of Tohoku University Graduate School of Medicine for technical support. This work was supported in part by the Japan Society for the Promotion of Science KAKENHI (grant nos. 26860204 to N.H., 15H04759 to R.S., and 15H02507 to M.Y.), the Platform Project for Supporting Drug Discovery and Life Science Research (Basis for Supporting Innovative Drug Discovery and Life Science Research) from the Japan Agency for Medical Research Development (grant no. JP18am0101095 to M.Y. and R.S.), and the Uehara Memorial Foundation (to R.S.).

CONFLICT OF INTEREST

The authors declare no competing financial interests.

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SUPPORTING INFORMATION

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

How to cite this article: Harada N, Hasegawa A, Hirano I, Yamamoto M, Shimizu R. GATA2 hypomorphism induces chronic myelomonocytic leukemia in mice. *Cancer Sci*. 2019;110:1183–1193. https://doi.org/10.1111/cas.13959