ARTICLE

STEM CELL BIOLOGY

The GPI-anchored protein CD109 protects hematopoietic progenitor cells from undergoing erythroid differentiation induced by TGF-β

Mikoto Tanabe[1,12], Kohei Hosokawa[1,12], Mai Anh Thi Nguyen[1,2,12], Noriharu Nakagawa[1], Kana Maruyama[1], Noriaki Tsuji[1], Ryota Urushihara[1], Luis Espinoza[3], Mahmoud I. Elbadry[4], Md Mohiuddin[5], Takamasa Katagiri[3], Masanori Ono[6], Hiroshi Fujiwara[6], Kazuhisa Chonabayashi[7,8], Yoshinori Yoshida[7], Hirohito Yamazaki[9], Atsushi Hirao[10,11] and Shinji Nakao[1]✉

Although a glycosylphosphatidylinositol-anchored protein (GPI-AP) CD109 serves as a TGF-β co-receptor and inhibits TGF-β signaling in keratinocytes, the role of CD109 on hematopoietic stem progenitor cells (HSPCs) remains unknown. We studied the effect of CD109 knockout (KO) or knockdown (KD) on TF-1, a myeloid leukemia cell line that expresses CD109, and primary human HSPCs. CD109-KO or KD TF-1 cells underwent erythroid differentiation in the presence of TGF-β. CD109 was more abundantly expressed in hematopoietic stem cells (HSCs) than in multipotent progenitors and HSPCs of human bone marrow (BM) and cord blood but was not detected in mouse HSCs. Erythroid differentiation was induced by TGF-β to a greater extent in CD109-KD cord blood or iPS cell-derived megakaryocyte–erythrocyte progenitor cells (MEPs) than in wild-type MEPs. When we analyzed the phenotype of peripheral blood MEPs of patients with paroxysmal nocturnal hemoglobinuria who had both GPI(+) and GPI(−) $CD34^+$ cells, the CD36 expression was more evident in $CD109^-$ MEPs than $CD109^+$ MEPs. In summary, CD109 suppresses TGF-β signaling in HSPCs, and the lack of CD109 may increase the sensitivity of *PIGA*-mutated HSPCs to TGF-β, thus leading to the preferential commitment of erythroid progenitor cells to mature red blood cells in immune-mediated BM failure.

Leukemia; https://doi.org/10.1038/s41375-021-01463-3

INTRODUCTION

An increase in the number of glycosylphosphatidylinositol-anchored protein (GPI-AP)-deficient (GPI[−]) leukocytes is thought to represent the immune pathophysiology of bone marrow (BM) failure, based on the high response rate to immunosuppressive therapy in patients harboring these surface marker-lacking cells [1–3]. However, what kind of immune mechanisms are involved in the selection of *PIGA*-mutated GPI(−) hematopoietic stem progenitor cells (HSPCs) remains unclear [4, 5]. HSPCs express several GPI-APs that potentially affect the sensitivity to hemato-inhibitory cytokines such as transforming growth factor-beta (TGF-β) and interferon-gamma (IFN-γ) [6, 7]. It is plausible to suppose that the lack of these GPI-APs in HSPCs may contribute to the preferential commitment of GPI(−) cells in immune-mediated BM failure where the inhibitory cytokines are abundant, although GPI-APs on HSPCs have not been highlighted in this context by previous studies.

TGF-β is the founding member of a large family of secreted polypeptide growth factors, consisting of over 30 members in humans, including activins, bone morphogenetic proteins, and others [8]. The TGF-β family constitutes a multifunctional set of cytokines that regulate a complex array of cellular processes. While TGF-β members regulate tissue homeostasis and regeneration in the adult organism, they play an important role in regulating HSPC behavior, particularly quiescence and self-renewal [9–12]. TGF-β ligands bind type I and type II receptors at the cell surface, and the type I receptor becomes phosphorylated by the type II receptor. This leads to phosphorylation of SMAD2 (pSMAD2) and SMAD3 (pSMAD3), which form a complex with SMAD4. Activated complexes accumulate in the nucleus

[1]Department of Hematology, Faculty of Medicine, Institute of Medical Pharmaceutical and Health Sciences, Kanazawa University, Kanazawa, Japan. [2]Pediatric Department, University of Medicine and Pharmacy at HCMC, Ho Chi Minh City, Vietnam. [3]Department of Clinical Laboratory Sciences, Kanazawa University Graduate School, Kanazawa, Japan. [4]Department of Internal Medicine, Division of Hematology, Faculty of Medicine, Sohag University, Sohag, Egypt. [5]Department of Respiratory Medicine, Kanazawa University, Kanazawa, Japan. [6]Department of Obstetrics and Gynecology, Graduate School of Medical Sciences, Kanazawa University, Kanazawa, Japan. [7]Center for iPS Cell Research and Application, Kyoto University, Kyoto, Japan. [8]Department of Hematology and Oncology, Graduate School of Medicine, Kyoto University, Kyoto, Japan. [9]Division of Transfusion Medicine, Kanazawa University Hospital, Kanazawa, Japan. [10]Division of Molecular Genetics, Cancer and Stem Cell Research Program, Cancer Research Institute, Kanazawa University, Kanazawa, Japan. [11]WPI Nano Life Science Institute, Kanazawa University, Kanazawa, Japan. [12]These authors contributed equally: Mikoto Tanabe, Kohei Hosokawa, Mai Anh Thi Nguyen. ✉email: snakao8205@staff.kanazawa-u.ac.jp

Received: 15 March 2021 Revised: 9 October 2021 Accepted: 21 October 2021
Published online: 06 November 2021

where they cooperate with DNA-binding co-factors to regulate target gene transcription. SMAD2 and SMAD3 also bind to TIF1-γ. TIF1-γ-SMAD2/3 promotes erythroid differentiation of HSPCs, whereas SMAD4- SMAD2/SMAD3 complexes inhibit proliferation [13].

CD109 is a GPI-AP expressed by keratinocytes and blood cells in humans, including activated T cells and platelets, and a subset of $CD34^+$ BM cells containing megakaryocyte progenitors [14, 15]. Recent studies have revealed that CD109 is expressed in many malignant tumors, including various squamous cell carcinomas and adenocarcinomas, and plays a role as a multifunctional co-receptor [16–19]. CD109 attenuates TGF-β induced SMAD2/3 phosphorylation in keratinocytes [14, 20, 21]. However, little is known about the role of CD109 in the regulation of hematopoiesis, despite the fact that TGF-β plays essential roles in the regulation of hematopoiesis.

Given the above, we hypothesized that CD109 on HSPCs might decrease the sensitivity to TGF-β, and the deficiency of CD109 may lead to augmented erythroid differentiation of *PIGA*-mutated HSPCs in response to TGF-β. To gain insight into the role of CD109 on HSPCs, we used TF-1 cells, a pluripotent erythroleukemia cell line that expresses CD109 and lacks glycophorin A (GPA) expression [22]. TF-1 cells were reported to undergo erythroid differentiation in response to hemin and delta-aminolevulinic acid (δ-Ala), suggesting that the cell line is ideal for assessing the erythroid differentiation associated with the loss of CD109 [22, 23].

In the present study, we established a *PIGA*-mutated TF-1 cell line, and a CD109-knockout (CD109 KO) TF-1 cell line using the CRISPR-Cas9 system and assessed their changes in differentiation. We also assessed the role of CD109 on human HSPCs and iPS cell-derived HSPCs in the differentiation induced by TGF-β using knockdown (KD) of the CD109 expression with siRNA, as well as the difference in the erythroid differentiation between $CD109^+$ and $CD109^-$ HSPCs of patients with paroxysmal nocturnal hemoglobinuria (PNH).

METHODS

Detailed Materials and methods are provided in Supplementary Data.

BM and umbilical cord blood (UCB) samples

Clinical samples from a total of seven PNH patients seen at Kanazawa University Hospital between April 2018 and January 2021 were used in this study. Supplementary Table 1 shows the PNH patients' characteristics. All PNH patients and healthy volunteers provided their informed consent on the peripheral blood (PB) and BM donation and normal pregnant women provided their informed consent for the UCB donation in accordance with Declaration of Helsinki.

This study protocol was approved by the medical ethics committee of Kanazawa University (nos. 2016-284, 2018-017).

Cell culture

TF-1 cells were provided by Prof. T Kitamura (Institute of Medial Sciences, The University of Tokyo, Japan) [22]. Leukemia cell lines (K562, MV4-11, KHYG-1, and Jurkat) were purchased from ATCC (Manassas, USA). TF-1 Cells were maintained in RPMI1640 medium (Wako, Japan) supplemented with 10% inactivated fetal bovine serum (FBS) (Biological Industries, Israel) and 5 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF) (R&D Systems, USA). To assess the erythroid differentiation, transient CD109-KD $CD34^+$ cells and scramble controls were cultured for 4 days on 12-well plates with serum-free medium (StemPro-34 SFM; Thermo Fisher Scientific, USA) supplemented with 20 ng/mL human recombinant stem cell factor (SCF), 20 ng/mL GM-CSF, 4 U/mL EPO, and 20 ng/mL thrombopoietin, according to a previous report [24].

Flow cytometry (FCM)

Hematopoietic stem cells (HSCs) and HSPC subpopulations, including common myeloid progenitor cells (CMPs), megakaryocyte–erythrocyte progenitor cells (MEPs), and granulocyte-macrophage progenitor cells (GMPs), in the BM and UCB were studied for the CD109 expression using FCM, as previously described [25]. HSCs and multipotent progenitors (MPPs) were defined as Lineage (Lin)$^-$$CD34^+CD38^-$ $CD90^+$ $CD45RA^-$ and $Lin^-CD34^+CD38^-$ $CD90^-$ $CD45RA^-$ cells, respectively, according to a previous report [26]. The expression of CD109 on HSCs and HSPCs was determined using monoclonal antibodies specific to anti-human lineages cocktail-APC (cat no. 348803; Biolegend, USA), anti-CD34-APC/Cy7 (cat no. 343514; Biolegend), anti-CD38-PE/Cy7 (cat no. 560677; Biolegend), anti-CD45RA-Pacific Blue (cat no. 562885; Biolegend), anti-CD90-FITC (cat no. 328108; Biolegend), anti-CD123-PerCP-Cy5.5 (cat no. 45-1239-42; Thermo Fisher Scientific), anti-CD49f-BV510 (cat no. 563271; BD Biosciences, USA), and anti-CD109-PE antibodies (cat no. 2-1099-42; Thermo Fisher Scientific). To determine the GPI(−) cell percentage in the BM HSPCs, we used FLAER (cat no. FL2-R; Cedarlane, Canada) in combination with other antibodies. To examine the GPA or CD36 expression in the HSPCs, we used anti-GPA-FITC (cat no. F0870; Wako) or anti-CD36-FITC (cat no. 561820; BD Biosciences) in combination with other antibodies. Dead cells were excluded using the Zombie Aqua™ Fixable Viability Kit (cat no. 423101; BioLegend). The expression of all surface proteins was analyzed using a FACSCanto II® instrument (BD Biosciences) with the Flowjo 10.0 software program (Tree Star, Inc., USA).

Statistical analyses

The *t* test was used for the comparison of continuous variables. All statistical analyses were performed using the EZR software package (Saitama Medical Center/Jichi Medical University, Japan) [27]. Graphs were generated using the GraphPad PRISM 7.0 software program (GraphPad Software Inc., USA). *P* values <0.05 were considered statistically significant (two sided).

RESULTS

TGF-β induces GPA expression in TF-1 cells in a dose-dependent manner

In accordance with previous reports [22], incubation in the presence of 0.5 mM δ-Ala for 72 h induced erythroid differentiation of TF-1 cells that was demonstrated by the GPA expression (Fig. 1A). Likewise, incubation in the presence of TGF-β with various concentrations (1–5 ng/mL) for 72 h induced the GPA expression of TF-1 cells in a dose-dependent manner (Fig. 1B). The minimum concentration of TGF-β capable of inducing erythroid differentiation of TF-1 cells was 1 ng/mL.

Effect of GPI-AP depletion on erythroid differentiation of TF-1 cells

We next examined whether or not GPI-APs on TF-1 cells are involved in their erythroid differentiation. The PIPL-C treatment of TF-1 cells for 1 h reduced the expression of CD55 and CD59, as expected (Fig. 1C, left). The transient depletion of GPI-APs after PIPL-C treatment resulted in the increased GPA expression on TF-1 cells from 1.27 to 42.3% (Fig. 1C, right).

To ascertain the effect of GPI-AP deficiency on the erythroid differentiation of TF-1 cells, we established GPI(−) TF-1 cells by culturing wild-type (WT) TF-1 cells in the presence of α-toxin for several months (Fig. 1D, upper). GPI(−) TF-1 cells expressed more GPA than WT TF-1 cells (Fig. 1D, lower), exhibited erythroid morphology (Fig. 1E, upper), and were positive for iron granules (Fig. 1E, lower). These results suggest that some GPI-APs inhibit TF-1 cells from undergoing erythroid differentiation during culture in FBS-containing medium.

Effect of CD109 knockout (KO) on erythroid differentiation of TF-1 cells

Given that FBS contains low amount of TGF-β [28], the depletion of CD109, a GPI-AP that works as a co-receptor for TGF-β, may have been responsible for the erythroid differentiation of GPI(−) TF-1 cells. FCM revealed the CD109 expression on WT TF-1 cells but not on other leukemia cell lines, such as K562, MV4-11, KHYG-1, and Jurkat cell lines (Fig. S1A). We then generated CD109-KO TF-1 cells using the CRISPR-Cas9 system (Fig. 1F, upper). Similar

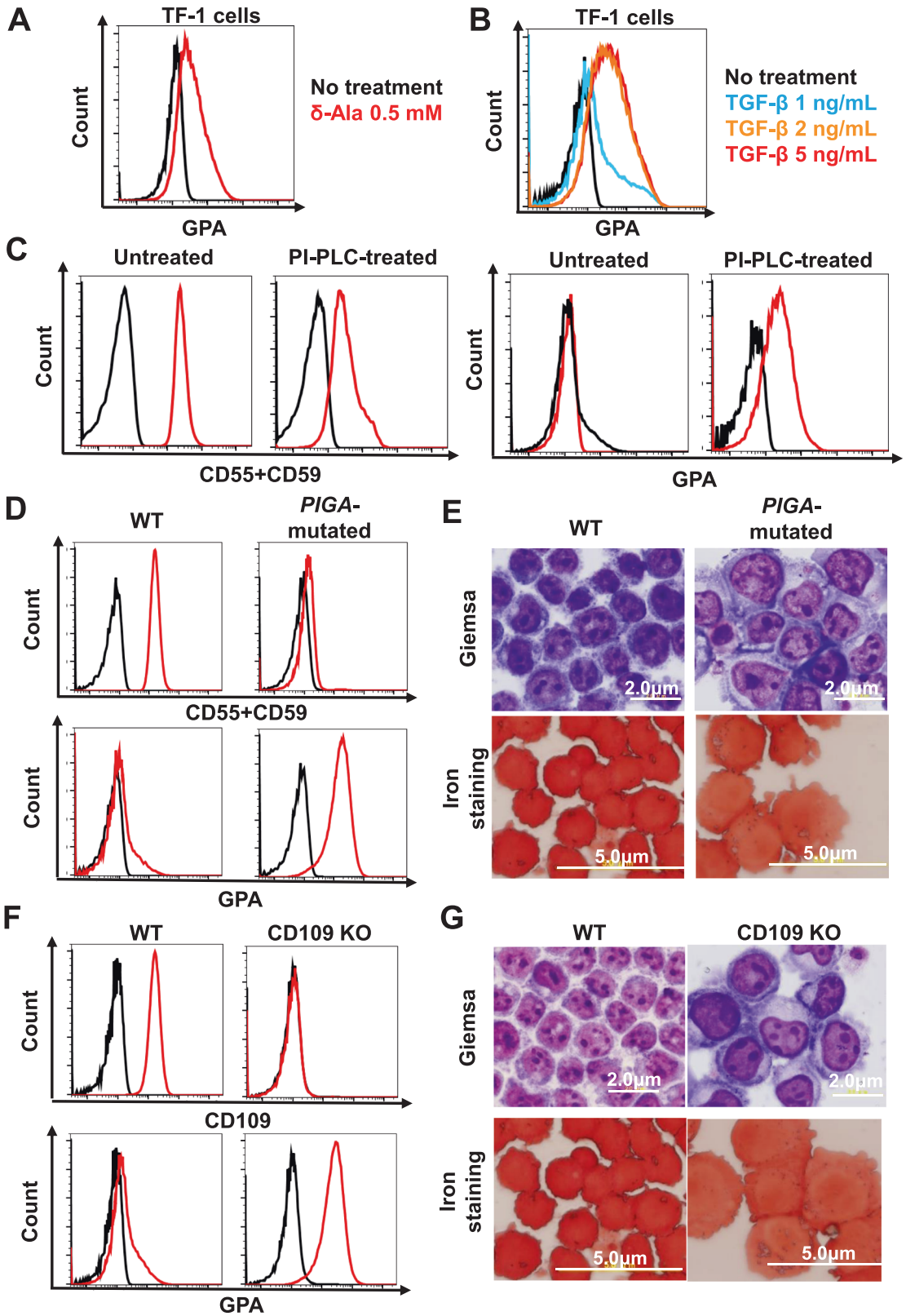
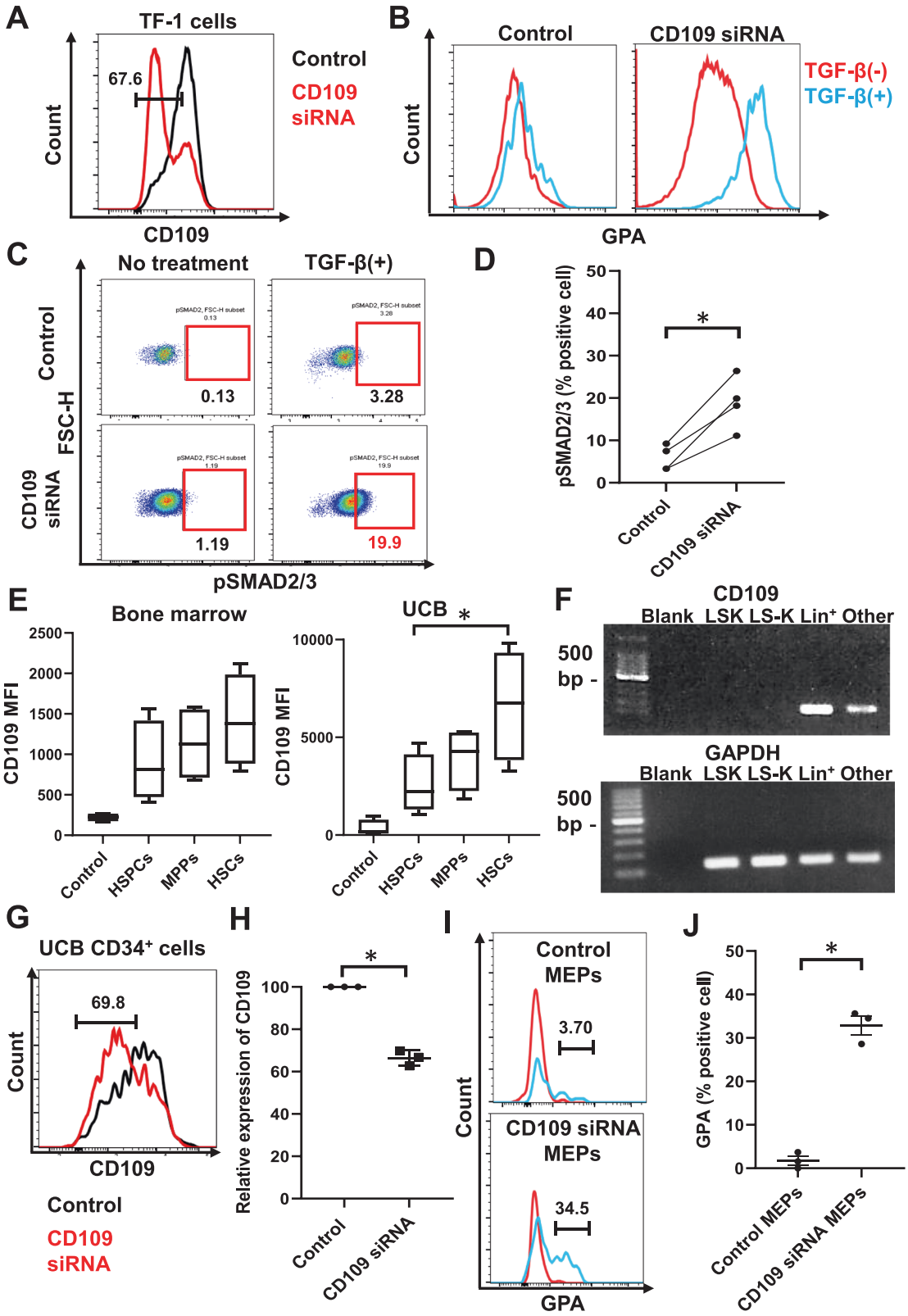


Fig. 1 Erythroid differentiation of TF-1 cells induced by TGF-β and GPI-AP depletion. A FCM histograms showing the GPA expression in TF-1 cells incubated in the presence or absence of δ-Ala (0.5 mM) for 72 h. **B** FCM histograms showing the GPA expression in TF-1 cells incubated in the presence (1, 2, and 5 ng/mL) or absence of TGF-β for 72 h. **C** The CD55 and CD59 expression by PI-PLC-treated or untreated TF-1 cells for 1 h (upper) and the GPA expression by PI-PLC-treated or untreated TF-1 cells (lower) that were cultured for 7 days in the FBS-containing culture medium are shown. **D** Histograms showing the GPI-AP expression (CD55 and CD59, upper) and GPA expression (lower) on wild-type (WT) and PNH-type TF-1 cells. **E** Giemsa (upper) and iron staining (lower) of WT and PNH-type TF-1 cells. **F** The CD109 (upper) and GPA (lower) expression by wild-type (WT) and CD109-KO TF-1 cells. **G** Giemsa (upper) and iron staining (lower) of WT TF-1 cells and CD109-KO TF-1 cells. δ-Ala delta-aminolevulinic acid, GPA glycophorin A. Black lines in (**C**), (**D**), and (**F**) indicate negative controls.



to PNH-type TF-1 cells, CD109-KO TF-1 cells expressed more GPA than WT TF-1 cells (Fig. 1F, lower), exhibited erythroid morphology (Fig. 1G, upper), and were positive for iron granules (Fig. 1G, lower), suggesting that the GPI-AP responsible for the inhibition of erythroid differentiation was CD109.

The sensitivity of CD109-KD TF-1 cells to TGF-β

To determine the role of CD109 on TF-1 cells, we attempted to examine the sensitivity of CD109-KO TF-1 cells to TGF-β. However, the attempt was unsuccessful because the established CD109-KO TF-1 cells had undergone differentiation into erythroid cells

Fig. 2 Effect of transient CD109 KD by siRNA on the erythroid differentiation of TF-1 cells and UCB HSPCs. A CD109 expression in CD109 siRNA-treated (red line) and mock-transfected (black line) TF-1 cells cultured in serum-containing medium (RPMI1640 medium + 10% FBS) for 72 h. A representative histogram is shown. **B** The GPA expression by mock-transfected (left) and CD109 siRNA-transfected (right) TF-1 cells that were cultured in the presence (5 ng/mL) or absence of TGF-β. Representative histograms are shown. GPA glycophorin A. **C** Representative dot plots showing the pSMAD2/3 expression in mock-transfected (upper) and CD109 siRNA-treated (lower) TF-1 cells that were cultured in the presence (20 ng/mL) or absence of TGF-β. **D** The percentages of pSMAD2/3-positive cells in mock-transfected and CD109 siRNA-transfected TF-1 cells. Data were obtained from four independent experiments. $^*P < 0.05$. **E** The box plots showing the CD109 expression in each $CD34^+$ cell subset. Data were obtained from four independent experiments (means ± SEM). $^*P < 0.05$. **F** The CD109 gene expression by different hematopoietic cells from C57BL/6 mice. Amplified products of CD109 and GAPDH cDNA from sorted $Lin^-Sca\text{-}1^+c\text{-}kit^+$ (LSK), $Lin^-Sca^-1^-c\text{-}kit^+$ (LSK), all lineage (+) cells, and $Lin^-c\text{-}kit^-$ (others) BM cells. **G** The CD109 expression by CD109 siRNA-treated (red line) and mock-transfected (black line) UCB $CD34^+$ cells. Transfected $CD34^+$ UCB cells were cultured in serum-free medium for 72 h. Representative histograms are shown. **H** The expression of CD109 in siRNA-transfected $CD34^+$ cells relative to that of CD109 in mock-transfected $CD34^+$ cells. Data were obtained from three independent experiments (means ± SEM). $^*P < 0.05$. **I** The GPA expression in mock-transfected (left) and CD109 siRNA-treated (right) MEPs cultured in the presence (12 ng/mL) or absence of TGF-β for 4 days. Representative histograms are shown. **J** Percentages of GPA-positive cells in mock-transfected and CD109 siRNA-transfected MEPs. Data were obtained from three independent experiments (means ± SEM). $^*P < 0.05$. GPA glycophorin A.

(Fig. 1F, G). We then designed siRNA that transiently downregulated the CD109 expression by TF-1 cells. Treatment of TF-1 cells with CD109 siRNA for 72 h significantly reduced the CD109 expression to 67.6% of that in the mock-transfected cells ($P < 0.05$, Figs. 2A, and S2A). When the transfected TF-1 cells were cultured for 4 days in RPMI1640 medium with 10% FBS with or without TGF-β (5 ng/mL), significantly higher levels of GPA were observed in TGF-β-treated CD109-KD cells than in mock-transfected TF-1 cells (Fig. 2B). The results were similar when TF-1 cells were cultured in serum-free medium (data not shown).

We next analyzed the expression of pSMAD2/3, which is a key downstream molecule associated with erythroid differentiation, in TF-1 cells after treatment with TGF-β (20 ng/mL) for 20 min. The expression of pSMAD2/3 induced by TGF-β in CD109-KD TF-1 cells was greater than that observed in mock-transfected TF-1 cells ($P < 0.05$, Fig. 2C, D). These results suggested that the transient downregulation of CD109 increases the sensitivity of TF-1 cells to TGF-β in TF-1 cells, thus inducing erythroid differentiation.

The CD109 expression in human HSCs and HSPCs from BM and UCB

Next, $CD34^+$ cell subsets were extensively analyzed to determine the difference in the CD109 expression according to their maturational stage in BM from healthy individuals and UCB. The representative gating strategy for HSCs, MPPs, and HSPCs from a UCB sample is shown in Fig. S3A. In both the BM of healthy individuals and UCB, CD109 was more abundantly expressed in $Lin^-CD34^+CD38^-CD90^+CD45RA^-$ HSCs than in $Lin^-CD34^+CD38^-CD90^-CD45RA^-$ MPPs and $Lin^-CD34^+CD38^+$ HSPCs (Figs. 2E, and S3B). Furthermore, the CD109 expression was only detected in activated T cells among mature leukocytes from healthy controls (data not shown).

We attempted to examine the CD109 expression of murine LSK cells and LSK cells using FCM, but antibodies specific to murine CD109 were unavailable. Therefore, LSK, LSK, Lin^+, and other cell populations were sorted using FACS, and the CD109 mRNA expression was evaluated by quantitative polymerase chain reaction. CD109 mRNA was detected in mature blood cells (Lin^+) and $Lin^-c\text{-}kit^-$ cells but not in LSK or LSK cells of C57BL/6 mice (Fig. 2F).

Effect of CD109 KD on the sensitivity to TGF-β in UCB HSPCs

We used UCB for further analyses because of the better availability and higher CD109 expression in UCB HSPCs than in BM HSPCs (Fig. 2E). The treatment of UCB $CD34^+$ cells with CD109 siRNA significantly reduced the CD109 expression in $Lin^-CD34^+CD38^+$ cells to 66.9–69.8% of that in mock-transfected cells ($P < 0.05$, Fig. 2G, H). CD109 or mock siRNA-transfected $CD34^+$ cells were cultured in serum-free medium containing SCF, TPO, and EPO with or without TGF-β (12 ng/mL) for 4 days. The TGF-β treatment induced the GPA expression in CD109 siRNA-transfected MEPs ($Lin^-CD34^+CD38^+CD123^-CD45RA^-$) to a greater degree than in mock-transfected MEPs ($P < 0.05$, Fig. 2I, J), suggesting that CD109 may also inhibit human $CD34^+$ cells from undergoing erythroid differentiation in response to TGF-β.

Effect of CD109 KD on the sensitivity of iPSC-derived HSPCs to TGF-β

To confirm the increased sensitivity of CD109-KD $CD34^+$ cells to TGF-β, we used the HSPCs induced from iPS cells that we had previously established [29]. $CD34^+$ cells were isolated from cultured iPSCs using microbeads and treated in the same way as UCB $CD34^+$ cells. The siRNA treatment reduced the CD109 expression on iPSC-HSPCs by up to 88.6% (67.8–88.6%) (Figs. 3A, S4A). Similar to UCB $CD34^+$ cells, the CD109-KD by itself slightly increased the GPA expression of iPSC-HSPCs, and further increased GPA levels were observed following TGF-β treatment in CD109-KD iPSC-HSPCs, especially in MEPs (Fig. 3B).

The sufficient number of iPSC-derived $CD34^+$ cells available enabled us to assess the change in the level of pSMAD2/3 induced by TGF-β. After 24 h of siRNA treatment, transfected iPSC-derived $CD34^+$ cells were incubated in the presence of TGF-β (12 ng/mL) for 30 min. A significantly greater number of pSMAD2/3(+) cells was revealed among CD109-KD $CD34^+$ cells than among mock-transfected $CD34^+$ cells by phospho-FCM ($P < 0.05$, Figs. 3C, S5A). The more abundant pSMAD2/3 proteins in CD109-KD $CD34^+$ cells than in mock-transfected cells was confirmed by Western blotting (Fig. 3D). The results clearly showed the increased expression of pSMAD2, but not pSMAD3, in response to TGF-β. This is consistent with the findings of previous studies, as the phosphorylation of SMAD2 was robust and more sensitive in relaying TGF-β than that of SMAD3 [30, 31]. These results confirmed the increased sensitivity of CD109-deficient HSPCs to TGF-β.

Erythroid differentiation tendency unique to CD109(−) MEPs in vivo

The lack of CD109 may affect erythroid differentiation of HSPCs in patients with PNH, where the low concentration of TGF-β is involved in the regulation of inductive hematopoiesis under immunological stress. When analyzing $CD34^+$ cell subsets in the BM of three PNH patients (Fig. S6A), the percentages of BM $CD34^+CD38^+$ cells and $CD34^+CD38^-$ cells of total Lin^- cells in PNH patients were shown to be significantly lower than that of healthy controls, although their absolute reticulocyte count (ARC) was significantly higher than that in healthy controls ($P < 0.05$, Fig. 4A). The CD109 expression was also detected in FLAER(+) $CD34^+$ subpopulations of the three PNH patients, with no expression of CD109 noted in FLAER(−) subpopulations, as expected (Fig. 4B). In accordance with the results of BM from healthy individuals, CD109 was more abundantly expressed in

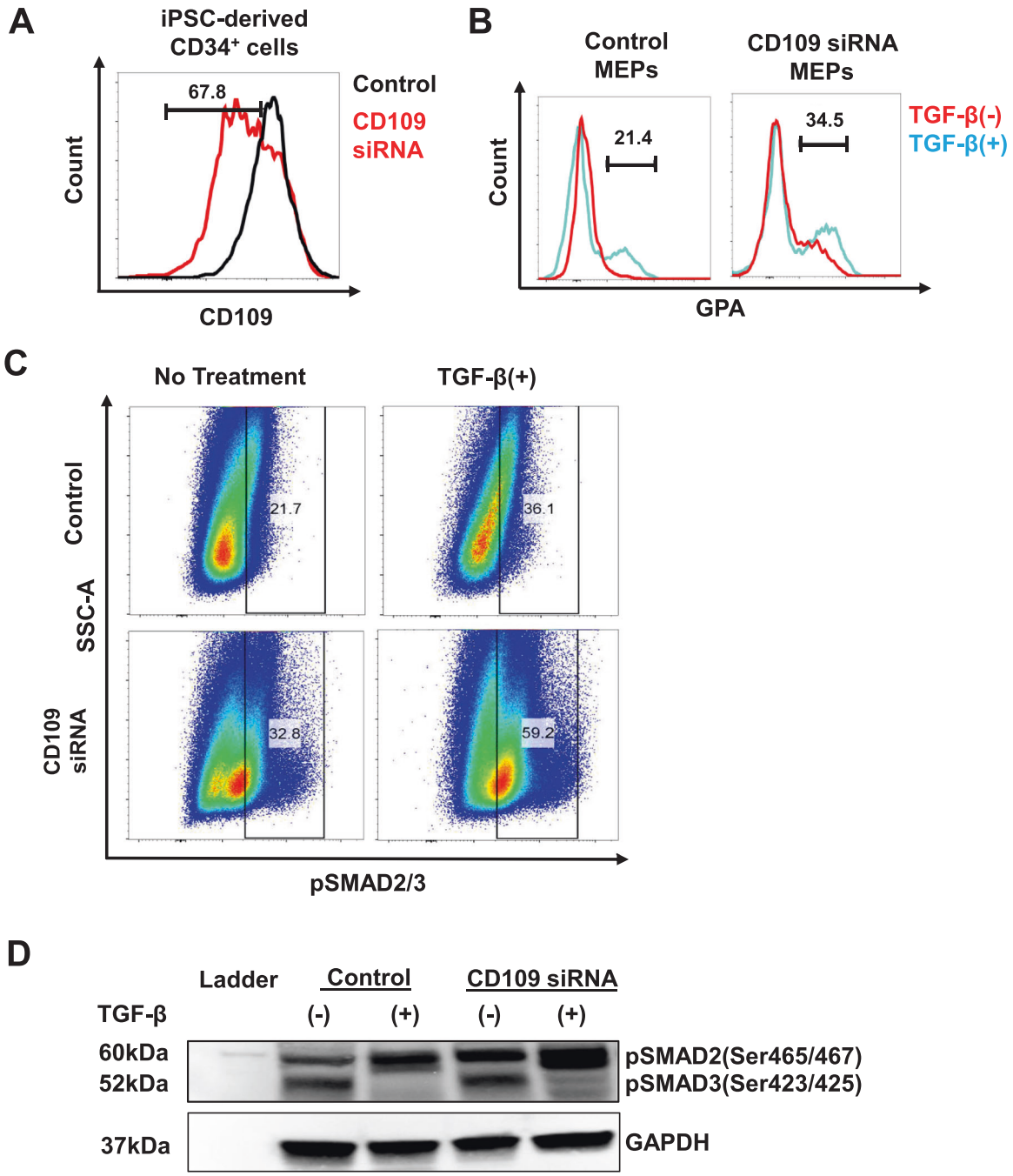


Fig. 3 Effect of transient CD109 KD on the erythroid differentiation of iPSC-derived HSPCs. A The CD109 expression by CD109 siRNA-transfected (red line) and mock-transfected (black line) iPSC-derived HSPCs. Representative histograms are shown. **B** The GPA expression by mock-transfected (left) and CD109 siRNA-transfected (right) MEPs cultured in the presence (12 ng/mL) or absence of TGF-β for 4 days. Representative histograms are shown. **C** Representative dot plots showing the pSMAD2/3 expression in mock-transfected (upper) and CD109 siRNA-transfected (lower) iPSC-HSPCs cultured in the presence (12 ng/mL) or absence of TGF-β for 30 min. **D** The same cultured cell populations as (**C**) were subjected to immunoblotting using antibodies specific to pSMAD2, pSMAD3, and GAPDH. The results are representative of three independent experiments. GPA glycophorin A.

$Lin^-CD34^+CD38^-CD90^+CD45RA^-$ HSCs than in $Lin^-CD34^+CD38^+$ HSPCs (CMPs, MEPs, and GMPs) (Fig. 4B, lower). Because of the difficulty in obtaining BM samples from patients with PNH, we focused on PB HSPCs for further analyses. To determine whether or not the lack of CD109 affects the erythroid differentiation of MEPs in vivo, we compared the expression of CD36, which is a marker of erythroid differentiation and highly expressed on MEPs [24], between $CD109^+$ and $CD109^-$ MEPs in two patients with PNH (Fig. 4C). As shown in representative zebra plots, the individual percentages of $CD36^+$ cells were higher in $CD109^-$ MEPs than in $CD109^+$ MEPs (UPN 3 and UPN 5, Fig. 4C, right). Furthermore, when the CD36 expression by MEPs was compared between PNH patients and healthy individuals, the percentages of $CD36^+$ cells were significantly higher in PNH patients than in healthy controls (mean 27.6% [11.8–40.1%] vs. 10.1% [1.67–24.4%], $P < 0.05$, Fig. 4D), suggesting that the CD109 deficiency may favor erythroid differentiation of *PIGA*-mutated HSPCs over *PIGA*-normal HSPCs in PNH patients.

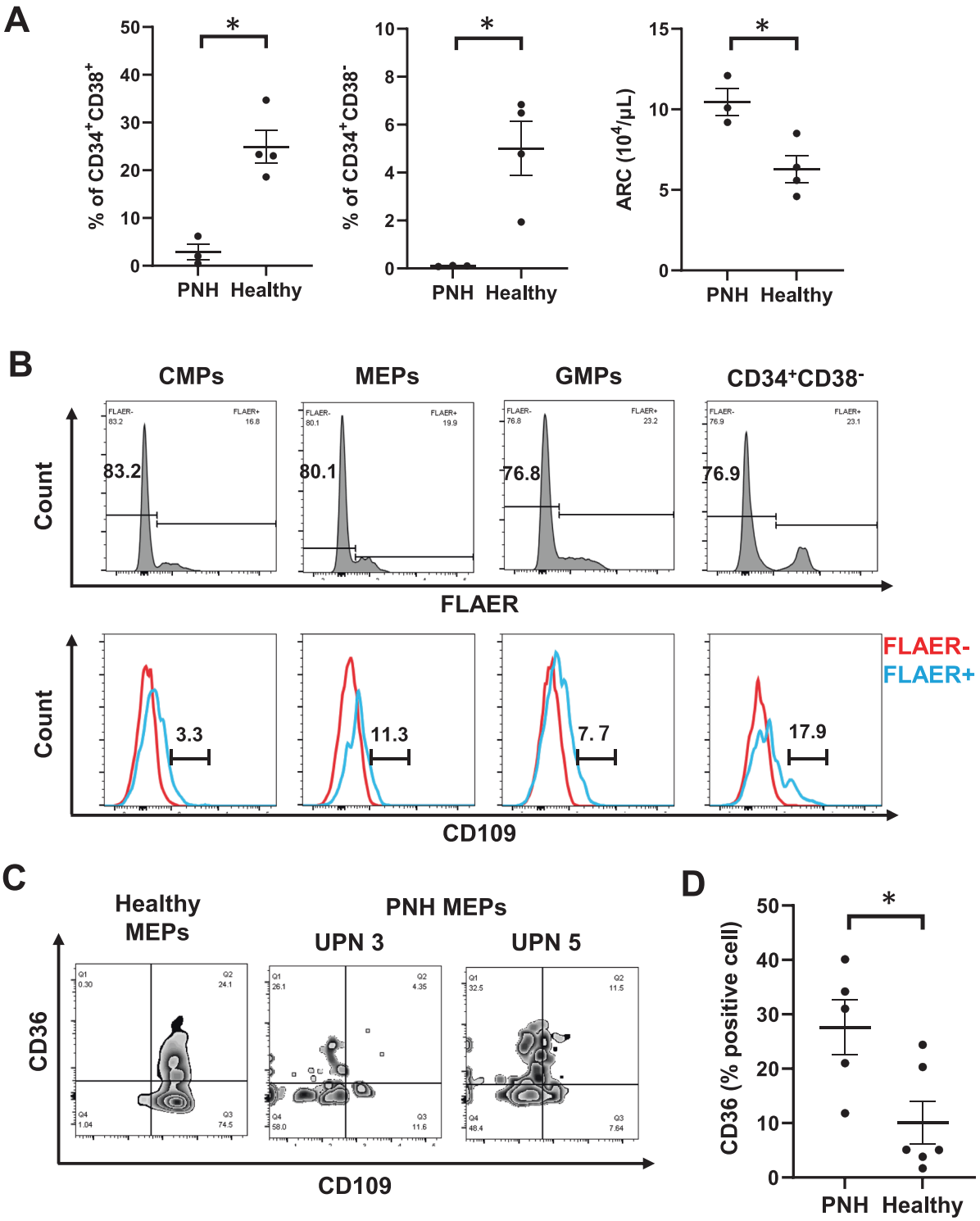


Fig. 4 Erythroid differentiation of CD109$^+$ and CD109$^-$ HSPCs from patients with PNH. A The percentages of CD34$^+$CD38$^+$ and CD34$^+$CD38$^-$ cells of BM, and absolute reticulocyte count (ARC) in the peripheral blood from three PNH patients and four healthy volunteers. *$P < 0.05$. **B** Percentages of GPI(−) cells (upper) and CD109$^+$ (lower) cells in different CD34$^+$ cell subsets of a patient with PNH. The CD109 expression by GPI(−) (in red) and GPI(+) cell (in blue) populations was differentially analyzed. **C** The expression of CD36 by CD109$^+$ and CD109$^-$ MEPs from healthy controls (left) and PNH patients (UPN 3 and UPN 5, right). Representative zebra plots are shown. **D** The percentages of CD36$^+$ cells in CD109$^-$ MEPs of PNH patients ($n = 5$) and in CD109$^+$ MEPs of healthy individuals ($n = 6$). *$P < 0.05$.

DISCUSSION

In this study, we investigated the role of CD109, a GPI-linked co-receptor of TGF-β, in the differentiation of HSPCs triggered by TGF-β and its relevance to pathophysiology of PNH. Both TF-1 and human primary HSPCs underwent erythroid differentiation following complete or partial depletion of CD109 from the cell surface during the culture in the presence of TGF-β. The increased sensitivity to TGF-β in CD109-deficient HSPCs was confirmed by a

greater increase in the level of pSMAD2 in CD109-KD iPSC-HSPCs than in WT iPSC-HSPCs. The tendency toward erythroid differentiation of $CD109^-$ HSPCs was supported by the in vivo finding that the proportion of $CD36^+$ cells in $CD109^-$ MEPs was higher than that in $CD109^+$ MEPs. Taken together, these findings suggest that CD109 inhibits differentiation of HSPCs from undergoing erythroid differentiation induced by TGF-β. Inhibitory roles of CD109 in the signaling of TGF-β have been demonstrated in keratinocytes and melanoma [14, 32]. On the cell surface, CD109 negatively regulates the TGF-β1 signaling pathway via the formation of a receptor complex with TGF-βR1 and TGF-βR2 in human keratinocytes [14]. Bizet et al. demonstrated that CD109 associates with caveolin-1 and promotes TGF-β receptor endocytosis [33]. In addition, CD109 promotes localization of the TGF-β receptors into the caveolar compartment in the presence of ligand and facilitates TGF-β receptor degradation. CD109 may thus dampen TGF-β responses in order to avoid a deleterious effect of excess TGF-β that often leads to many human diseases, such as tissue fibrosis [34] and cancer metastasis [35]. In inductive human hematopoiesis, CD109 may also blunt the response of HSPCs to TGF-β to inhibit their erythroid differentiation. The present study is the first to clarify a similar role of CD109 in the HSPC differentiation triggered by TGF-β.

Our study revealed that among different subsets of human $CD34^+$ cells, CD109 was the most abundantly expressed on HSCs, and its expression decreased as HSCs differentiated, a finding suggestive of the important role of CD109 in the regulation of primitive HSCs. TGF-β inhibits cell-cycling of HSCs, causing them to remain in a dormant state [36]. Although we were unable to assess the effect of CD109 depletion on the quiescence of HSCs induced by TGF-β due to the paucity of human HSCs, CD109 might also inhibit TGF-β signaling in HSCs. If this hypothesis is correct, CD109-deficient HSCs are more likely to remain in the dormant state than WT HSCs during inductive hematopoiesis, which conflicts with the preferential commitment of *PIGA*-mutated HSCs that occurs in patients with PNH. TGF-β is reported to exert bimodal effects on target cells depending on its concentration [37]. In immune-mediated BM failure, TGF-β may actually induce cell cycle in HSCs, and the lack of CD109 may lead to the enhanced commitment of HSCs.

We originally planned to study the effect of CD109-KD on HSCs using murine models. Unexpectedly, however, CD109 was not expressed by LSK cells of C57BL/6 mice although its expression was detected in mature blood cells. There were no marked differences in the complete blood counts and proportion of LSK cells between WT mice and CD109-KO mice (data not shown). These findings suggest that CD109 has little involvement in murine hematopoiesis. Similar to CD109, some proteins, such as Robo1, are known to be expressed by human HSCs but not by murine HSCs [38–40]. Given that the lack of CD109 on human HSPCs appears to be responsible for the preferential erythroid commitment of GPI(−) HSPCs in patients with PNH, the absence of CD109 on murine HSCs may account for the lack of PNH in mice.

By evaluating PNH patients whose HSPCs comprised GPI(+) and GPI(−) cells, we confirmed that CD109 was expressed on $FLAER^+$ $CD34^+$ cell subsets. Similar to $CD34^+$ cells of healthy individuals, CD109 was more abundantly expressed on $FLAER^+$ HSCs than on $FLAER^+$ HSPCs of PNH patients. The absence of CD109 on $FLAER^-$ HSPCs in these patients may make the *PIGA*-mutated HSPCs more sensitive to TGF-β, leading to their preferential erythroid differentiation over WT HSPCs. Indeed, the proportion of $CD36^+$ cells in $CD109^-$ MEPs was greater than that in $CD109^+$ MEPs among PNH patients (Fig. 4C). The higher sensitivity to TGF-β in GPI(−) HSPCs than in GPI(+) HSPCs may partly explain the predominance of GPI(−) erythrocytes in immune-mediated BM failure, including PNH. Although escape from inflammatory cytokine due to the lack of GPI-APs on HSPCs has been speculated to be a mechanism underlying the survival advantages of *PIGA*-mutated HSPCs over wild-type HSPCs, no studies have implicated the lack of specific GPI-APs in the preferential commitment of GPI (−) HSPCs. This study is the first to demonstrate that the lack of a GPI-AP CD109 augments the response to the inflammatory cytokine TGF-β, thereby causing enhanced erythroid differentiation of HSPCs. Some other GPI-APs, such as semaphorin7A (CD108) and glypican-3, may also be involved in the preferential commitment of HSPCs in patients with PNH (Fig. S7A) [41, 42]. These GPI-APs might work as receptors of cytokines, such as TGF-β or IFN-γ, which eventually affect the differentiation of HSCs. Although we were unable to assess the effects of TGF-β inhibitors on the commitment of WT and GPI(−) HSPCs due to the limited clinical material available, TGF-β inhibition may help not only restore hematopoiesis by WT HSPCs but also suppress the preferential commitment of GPI(−) HSPCs in patients with immune-mediated BM failure and prevent them from developing florid PNH.

In conclusion, our study clarified the role of CD109 in the HSPC differentiation induced by TGF-β. The lack of CD109 may be one of the mechanisms underlying the preferential commitment of *PIGA*-mutated HSPCs in immune-mediated BM failure, wherein HSPCs in the BM are exposed to excessive inflammatory cytokines, including TGF-β. We previously showed that iPSC-derived HSPCs can reconstitute human hematopoiesis in immunodeficient mice [29]. Comparing the engraftment ability between WT and CD109-KD iPSC-HSPCs may help further clarify the role of CD109 on HSPCs in inductive hematopoiesis.

REFERENCES

1. Tutelman PR, Aubert G, Milner RA, Dalal BI, Schultz KR, Deyell RJ. Paroxysmal nocturnal haemoglobinuria phenotype cells and leucocyte subset telomere length in childhood acquired aplastic anaemia. Br J Haematol. 2014;164:717–21.
2. Kulagin A, Lisukov I, Ivanova M, Golubovskaya I, Kruchkova I, Bondarenko S, et al. Prognostic value of paroxysmal nocturnal haemoglobinuria clone presence in aplastic anaemia patients treated with combined immunosuppression: results of two-centre prospective study. Br J Haematol. 2014;164:546–54.
3. Sugimori C, Chuhjo T, Feng X, Yamazaki H, Takami A, Teramura M, et al. Minor population of CD55-CD59- blood cells predicts response to immunosuppressive therapy and prognosis in patients with aplastic anemia. Blood. 2006;107:1308–14.
4. Gargiulo L, Papaioannou M, Sica M, Talini G, Chaidos A, Richichi B, et al. Glycosylphosphatidylinositol-specific, CD1d-restricted T cells in paroxysmal nocturnal hemoglobinuria. Blood. 2013;121:2753–61.
5. Hosokawa K, Katagiri T, Sugimori N, Ishiyama K, Sasaki Y, Seiki Y, et al. Favorable outcome of patients who have 13q deletion: a suggestion for revision of the WHO 'MDS-U' designation. Haematologica. 2012;97:1845–9.
6. Zhang H, Kozono DE, O'Connor KW, Vidal-Cardenas S, Rousseau A, Hamilton A, et al. TGF-beta inhibition rescues hematopoietic stem cell defects and bone marrow failure in fanconi anemia. Cell Stem Cell. 2016;18:668–81.
7. de Bruin AM, Voermans C, Nolte MA. Impact of interferon-gamma on hematopoiesis. Blood. 2014;124:2479–86.
8. Massague J. TGFbeta signalling in context. Nat Rev Mol Cell Biol. 2012;13:616–30.
9. Garbe A, Spyridonidis A, Mobest D, Schmoor C, Mertelsmann R, Henschler R. Transforming growth factor-beta 1 delays formation of granulocyte-macrophage colony-forming cells, but spares more primitive progenitors during ex vivo expansion of CD34+ haemopoietic progenitor cells. Br J Haematol. 1997;99:951–8.
10. Batard P, Monier MN, Fortunel N, Ducos K, Sansilvestri-Morel P, Phan T, et al. TGF-(beta)1 maintains hematopoietic immaturity by a reversible negative control of cell cycle and induces CD34 antigen up-modulation. J Cell Sci. 2000;113:383–90.
11. Sitnicka E, Ruscetti FW, Priestley GV, Wolf NS, Bartelmez SH. Transforming growth factor beta 1 directly and reversibly inhibits the initial cell divisions of long-term repopulating hematopoietic stem cells. Blood. 1996;88:82–8.
12. Fortunel NO, Hatzfeld A, Hatzfeld JA. Transforming growth factor-beta: pleiotropic role in the regulation of hematopoiesis. Blood. 2000;96:2022–36.
13. He W, Dorn DC, Erdjument-Bromage H, Tempst P, Moore MA, Massague J. Hematopoiesis controlled by distinct TIF1gamma and Smad4 branches of the TGFbeta pathway. Cell. 2006;125:929–41.
14. Finnson KW, Tam BY, Liu K, Marcoux A, Lepage P, Roy S, et al. Identification of CD109 as part of the TGF-beta receptor system in human keratinocytes. FASEB J. 2006;20:1525–7.

15. Murray LJ, Bruno E, Uchida N, Hoffman R, Nayar R, Yeo EL, et al. CD109 is expressed on a subpopulation of CD34+ cells enriched in hematopoietic stem and progenitor cells. Exp Hematol. 1999;27:1282–94.
16. Zhang JM, Murakumo Y, Hagiwara S, Jiang P, Mii S, Kalyoncu E, et al. CD109 attenuates TGF-beta1 signaling and enhances EGF signaling in SK-MG-1 human glioblastoma cells. Biochem Biophys Res Commun. 2015;459:252–8.
17. Mii S, Murakumo Y, Asai N, Jijiwa M, Hagiwara S, Kato T, et al. Epidermal hyperplasia and appendage abnormalities in mice lacking CD109. Am J Pathol. 2012;181:1180–9.
18. Chuang CH, Greenside PG, Rogers ZN, Brady JJ, Yang D, Ma RK, et al. Molecular definition of a metastatic lung cancer state reveals a targetable CD109-Janus kinase-Stat axis. Nat Med. 2017;23:291–300.
19. Litvinov IV, Bizet AA, Binamer Y, Jones DA, Sasseville D, Philip A. CD109 release from the cell surface in human keratinocytes regulates TGF-beta receptor expression, TGF-beta signalling and STAT3 activation: relevance to psoriasis. Exp Dermatol. 2011;20:627–32.
20. Hagiwara S, Murakumo Y, Mii S, Shigetomi T, Yamamoto N, Furue H, et al. Processing of CD109 by furin and its role in the regulation of TGF-beta signaling. Oncogene. 2010;29:2181–91.
21. Xue Q, Yu C, Wang Y, Liu L, Zhang K, Fang C, et al. miR-9 and miR-124 synergistically affect regulation of dendritic branching via the AKT/GSK3beta pathway by targeting Rap2a. Sci Rep. 2016;6:26781.
22. Kitamura T, Tange T, Terasawa T, Chiba S, Kuwaki T, Miyagawa K, et al. Establishment and characterization of a unique human cell line that proliferates dependently on GM-CSF, IL-3, or erythropoietin. J Cell Physiol. 1989;140:323–34.
23. Klampfer L, Zhang J, Nimer SD. GM-CSF rescues TF-1 cells from growth factor withdrawal-induced, but not differentiation-induced apoptosis: the role of BCL-2 and MCL-1. Cytokine. 1999;11:849–55.
24. Mori Y, Chen JY, Pluvinage JV, Seita J, Weissman IL. Prospective isolation of human erythroid lineage-committed progenitors. Proc Natl Acad Sci USA. 2015;112:9638–43.
25. Manz MG, Miyamoto T, Akashi K, Weissman IL. Prospective isolation of human clonogenic common myeloid progenitors. Proc Natl Acad Sci USA. 2002;99:11872–7.
26. Majeti R, Park CY, Weissman IL. Identification of a hierarchy of multipotent hematopoietic progenitors in human cord blood. Cell Stem Cell. 2007;1:635–45.
27. Kanda Y. Investigation of the freely available easy-to-use software 'EZR' for medical statistics. Bone Marrow Transplant. 2013;48:452–8.
28. Oida T, Weiner HL. Depletion of TGF-beta from fetal bovine serum. J Immunol Methods. 2010;362:195–8.
29. Espinoza JL, Elbadry MI, Chonabayashi K, Yoshida Y, Katagiri T, Harada K, et al. Hematopoiesis by iPSC-derived hematopoietic stem cells of aplastic anemia that escape cytotoxic T-cell attack. Blood Adv. 2018;2:390–400.
30. Liu L, Liu X, Ren X, Tian Y, Chen Z, Xu X, et al. Smad2 and Smad3 have differential sensitivity in relaying TGFbeta signaling and inversely regulate early lineage specification. Sci Rep. 2016;6:21602.
31. Frick CL, Yarka C, Nunns H, Goentoro L. Sensing relative signal in the Tgf-beta/Smad pathway. Proc Natl Acad Sci USA. 2017;114:E2975–82.
32. Ohshima Y, Yajima I, Kumasaka MY, Yanagishita T, Watanabe D, Takahashi M, et al. CD109 expression levels in malignant melanoma. J Dermatol Sci. 2010;57:140–2.
33. Bizet AA, Liu K, Tran-Khanh N, Saksena A, Vorstenbosch J, Finnson KW, et al. The TGF-beta co-receptor, CD109, promotes internalization and degradation of TGF-beta receptors. Biochim Biophys Acta. 2011;1813:742–53.
34. Vorstenbosch J, Al-Ajmi H, Winocour S, Trzeciak A, Lessard L, Philip A. CD109 overexpression ameliorates skin fibrosis in a mouse model of bleomycin-induced scleroderma. Arthritis Rheum. 2013;65:1378–83.
35. Zhou S, da Silva SD, Siegel PM, Philip A. CD109 acts as a gatekeeper of the epithelial trait by suppressing epithelial to mesenchymal transition in squamous cell carcinoma cells in vitro. Sci Rep. 2019;9:16317.
36. Yamazaki S, Iwama A, Takayanagi S, Eto K, Ema H, Nakauchi H. TGF-beta as a candidate bone marrow niche signal to induce hematopoietic stem cell hibernation. Blood. 2009;113:1250–6.
37. Langer JC, Henckaerts E, Orenstein J, Snoeck HW. Quantitative trait analysis reveals transforming growth factor-beta2 as a positive regulator of early hematopoietic progenitor and stem cell function. J Exp Med. 2004;199:5–14.
38. Hosokawa K, Mizumaki H, Elbadry MI, Saito C, Espinoza JL, Thi Thanh Dao A, et al. Clonal hematopoiesis by SLIT1-mutated hematopoietic stem cells due to a breakdown of the autocrine loop involving Slit1 in acquired aplastic anemia. Leukemia. 2019;33:2732–66.
39. Geutskens SB, Andrews WD, van Stalborch AM, Brussen K, Holtrop-de Haan SE, Parnavelas JG, et al. Control of human hematopoietic stem/progenitor cell migration by the extracellular matrix protein Slit3. Lab Investig. 2012;92:1129–39.
40. Shibata F, Goto-Koshino Y, Morikawa Y, Komori T, Ito M, Fukuchi Y, et al. Roundabout 4 is expressed on hematopoietic stem cells and potentially involved in the niche-mediated regulation of the side population phenotype. Stem Cells. 2009;27:183–90.
41. Khurana S, Margamuljana L, Joseph C, Schouteden S, Buckley SM, Verfaillie CM. Glypican-3-mediated inhibition of CD26 by TFPI: a novel mechanism in hematopoietic stem cell homing and maintenance. Blood. 2013;121:2587–95.
42. Mine T, Harada K, Matsumoto T, Yamana H, Shirouzu K, Itoh K, et al. CDw108 expression during T-cell development. Tissue Antigens. 2000;55:429–36.

ACKNOWLEDGEMENTS

This work was supported by MEXT KAKENHI (Grant-in-Aid for Scientific Research (B), Grant Number: 24390243) to SN, MEXT KAKENHI (Grant-in-Aid for Young Scientists (B), Grant Number: 26860363) to TK, MEXT KAKENHI (Grant-in-Aid for Young Scientists, Grant Number: 17K16184 and 19K17823) to KH, MEXT KAKENHI (Grant-in-Aid for Scientific Research (C), Grant Number: 18K08318) to HY. We are grateful to the following doctors providing us with technical advice: Prof. Toshio Kitamura (The institute of Medial Sciences, The University of Tokyo, Japan) and Shinji Mii (Nagoya University). We thank the patients and donors and their physicians for contributing to this study and the Advanced Preventive Medical Sciences Research Center, Kanazawa University for the use of facilities. MT is a Ph.D. candidate at Kanazawa University, and this work is submitted in partial fulfillment of the requirements for the Ph.D.

AUTHOR CONTRIBUTIONS

MT, KH, NN, NT, RU, HY, and SN collected clinical data and blood and BM samples. MT, KH, MATN, NN, LE, MIE, and MM, performed most of the in vitro experiments. KM, TK, and AH, performed in vivo experiments. MO and HF collected cord blood samples. KC and YY generated induced pluripotent stem cells. MT, KH, and SN designed the research and wrote the paper. All authors critically reviewed the paper and checked the final version. MT, KH, and MATN contributed equally to this work.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41375-021-01463-3.

Correspondence and requests for materials should be addressed to Shinji Nakao.

Reprints and permission information is available at http://www.nature.com/reprints

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.