Persistent DNA damage and oncogenic stress-induced Trem1 promotes leukemia in mice

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Received: November 22, 2021.
Accepted: April 8, 2022.
Prepublished: April 21, 2022.

https://doi.org/10.3324/haematol.2021.280404

Abstract

The immune receptor TREM1 (Triggering receptor expressed on myeloid cells 1) is a master regulator of inflammatory response. Compelling evidence suggests important pathological roles for TREM1 in various types of solid tumors. However, the role of TREM1 in hematologic malignancies is not known. Our previous study demonstrated that TREM1 cooperates with diminished DNA damage response to induce expansion of pre-leukemic hematopoietic stem cells (HSC) in mice deficient for the Fanconi anemia gene *Fanca*. Here we investigated TREM1 in leukemogenesis using mouse models of the DNA repair-deficient *Fanca*$^{-/-}$ and the oncogenic *MLL-AF9* or *Kras*^{G12D}. We found that *Trem1* was highly expressed in pre-leukemic HSC and leukemia stem cells (LSC). By selective deletion of the *Trem1* gene in the hematopoietic compartment, we showed that ablation of Trem1 reduced leukemogenic activity of the pre-leukemic HSC and LSC in mice. Trem1 was required for the proliferation of the pre-leukemic HSC and LSC. Further analysis revealed that *Trem1* expression in pre-leukemic HSC and LSC was associated with persistent DNA damage, prolonged oncogenic stress, and a strong inflammatory signature. Targeting several top Trem1 inflammatory signatures inhibited the proliferation of pre-leukemic HSC and LSC. Collectively, our observations uncover previously unknown expression and function of TREM1 in malignant stem cells, and identify TREM1 as a driver of leukemogenesis.

Introduction

Triggering receptor expressed on myeloid cells 1 (TREM1, also known as CD354) is a member of the super immunoglobulin family initially found expressed on a select group of myeloid cells.[1,2] Compelling evidence suggests important pathological roles for TREM1 not only in acute infection-induced reactions but also in chronic inflammatory disorders including various types of cancers.[2] In fact, TREM1 is found overexpressed in a variety of cancers,[3] including colorectal cancer,[4] hepatocellular carcinoma,[5] lung cancer,[6] and prostate tumors.[7] Recent studies showed that TREM1 expression in patients with non-small cell lung cancer is associated with cancer recurrence and poor survival, suggesting that TREM1 may play an important role in cancer progression.[6] Furthermore, pharmacological inhibition of TREM1 attenuates tumor growth and prolongs survival in experimental pancreatic cancer[8] and lung cancer.[9] Analysis of transcriptome data of 33 cancers from The Cancer Genome Atlas using UALCAN (*http://ualcan.path.uab.edu/*)[10] showed that TREM1 is upregulated in at least 14 types of cancers, especially kidney renal clear cell carcinoma, cervical squamous cell carcinoma and glioblastoma, and that TREM1 expression is closely correlated with poor prognosis in kidney renal clear cell carcinoma and cervical squamous cell carcinoma. Overexpression of TREM1 is also found in hematologic malignancies, and it was shown that a high level of TREM1 expression was correlated with poor prognosis in five subsets of acute myeloid leukemia (AML) cells (BloodSpot). However, the underlying mechanisms of action of TREM1 in cancer development are poorly understood.

DNA damage response/repair (DDR) is a complex signal transduction network that is required for the preservation of the integrity of the genome and for ensuring its accurate transmission through generations. To counteract DNA damage, DDR machinery orchestrates DNA damage checkpoint activation and facilitates the removal of DNA lesions. Unrepaired damage results in cellular senescence or apoptosis while erroneously repaired DNA lesions can lead to mutations.[11] Dysregulation of the DDR and repair

systems can cause human disorders, which are associated with susceptibility to cancer, accelerated aging, and developmental abnormalities.[12] Given the enormous regenerative potential coupled with lifetime persistence of hematopoietic stem cells (HSC) in the body, tight control of HSC genome stability is demanded. In fact, the DDR has been considered as an evolutionary trade-off between blood regeneration and leukemia suppression.[13] Indeed, failure to accurately repair DNA damage in HSC is associated with bone marrow (BM) failure and leukemogenesis.[13] Using a mouse model deficient for the major Fanconi anemia (FA) gene *Fanca*, our recent studies demonstrated a temporal correlation of diminished DDR with elevated immune response in *Fanca*$^{-/-}$ pre-leukemic HSC and argued for the effectiveness of DDR as the cellular machinery preventing the transition of the initiating pre-leukemic HSC population into a leukemia stem cell (LSC) population with transformed properties.[14]

It is known that oncogene-driven proliferation must be associated with inhibition of apoptosis and senescence to allow malignant outgrowth.[15,16] In response to oncogenic activation, normal cells induce genetically encoded programs, mainly growth arrest, apoptosis and senescence, which prevent deregulated proliferation and thus protect multicellular organisms from cancer progression.[17,18] Mixed lineage leukemia (MLL) is an H2Kme3-depositing protein active during early development.[19] *MLL*-rearrangements are present in about 10% of acute leukemias. Among these, the translocation t(9;11)(p22;q23) is mainly associated with AML and fuses *AF9* to *MLL* (*MLL-AF9*).[20] Oncogenic Ras mutations (mutations of *NRAS* and *KRAS* genes) also occur in various leukemias,[21] including AML,[22] chronic myelomonocytic leukemia[23] and juvenile myelomonocytic leukemia.[24] However, the underlying mechanisms by which these oncogenes promote malignant transformation in leukemia remains controversial.

In this study, we investigated the role of TREM1 in leukemogenesis and show that persistent DNA damage and prolonged oncogenic stress induces the expression of *Trem1* in pre-leukemic HSC and LSC. This aberrant *Trem1* expression promotes leukemic development and is associated with enhanced proliferation and inflammatory response.

Methods

Mice and treatment

Trem1 conditional knockout mice (*Trem1*-floxed) were generated by the Transgenic core facility at Cincinnati Children's Hospital Medical Center using CRISPR-Cas9 technology. Mutant mice were produced on a C57BL/6J background. To generate *Trem1*$^{flox/+}$ mice, guide RNA (gRNA) targeting exon 2 of the mouse *Trem1* locus (5' gRNA [GTGGAGGTTGAAGGTCCTCA] and 3' gRNA [AGAGGTGGGAAGGGCCAAA]), along with Cas9 were injected into single-cell embryos to create the conditional knockout allele. To genotype the *Trem1*$^{flox/+}$ mouse line, forward primer, 5'- ATCTTTGGCAGGGACAAGATAGTC-3' and reverse primer, 5'- AGGGGAATCGACGCACAGGAAC-3' were used to detect the Exon2 wild-type allele (156 bp) or Exon2 floxed allele (173 bp). Gender- and age-matched littermates were selected and used for the following experiments.

Fanca$^{+/-}$ mice were provided by Dr. Madeleine Carreau (Laval University).[25] *MLL-AF9* transgenic mice[20] were obtained from Jackson Laboratory (Stock #: 009079) and interbred with *Trem1*$^{fl/fl}$*Vav1Cre* mice. LSL-Kras^{G12D} mice (Jackson Laboratory, Stock #: 008179)[26] were crossed with a tamoxifen-inducible deleter strain (CreER; Jackson Laboratory, Stock #: 008463)[27] to generate LSL-*Kras*^{G12D};*CreER* offspring. For Cre-mediated gene deletion, animals were injected intraperitoneally with 100 μL of tamoxifen (20 mg/mL; 80 mg/kg body weight; Sigma-Aldrich, St. Louis, MO, USa) once every 24 h for a total of 5 consecutive days.[28]

The DNA damage-induced *Fanca*$^{-/-}$ pre-leukemia model was established as previously described.[14] Briefly, 6- to 8-week-old mice were intraperitoneally injected with 0.3 mg/kg of mitomycin C (MMC; Sigma-Aldrich, St Louis, MO, USA) weekly for 6 weeks. All the animals, including BoyJ (C57BL/6: B6, CD45.1+) recipient mice, were maintained in the animal facility at the Hillman Cancer Center at the University of Pittsburgh. All experimental procedures conducted in this study were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh.

Bone marrow transplantation

One thousand freshly isolated BM LSK cells from *Fanca*$^{-/-}$*;Trem1*$^{fl/fl}$*Vav1Cre* mice and the *Fanca*$^{-/-}$*;Trem1*$^{fl/fl}$ control mice, 3,000 BM c-Kit^{+} cells from *MLL-AF9;Trem1*$^{fl/fl}$*Vav1Cre* mice and the *MLL-AF9;Trem1*$^{fl/fl}$ control mice, or 3,000 green fluorescent protein (GFP)-sorted virus-transduced BM c-Kit^{+} cells from 2-month-old *MLL-AF9* mice (CD45.2^{+}), along with 2X10^{5} protector cells from congenic BoyJ mice (CD45.1^{+}), were transplanted into lethally irradiated (11.75 Gy) BoyJ mice. Recipients were subjected to total body irradiation at the indicated time points. For serial bone marrow transplantation (BMT), 1-3x10^{6} whole BM cells from primary recipients were pooled and injected into sublethally irradiated (7.0 Gy) BoyJ recipients. Donor-derived chimera were detected by flow cytometry at 16 weeks after transplantatiion using antibodies against CD45.1 and CD45.2.

For Trem1^{+} and Trem1^{-} cell transplantation, 100 sorted Trem1^{+}SLAM (Lin^{-}Sca1^{+}c-kit^{+}CD150^{+}CD48^{-} cells) and Trem1^{-} SLAM cells from *Fanca*$^{-/-}$ pre-leukemic mice, along with 2x10^{5} protector cells from congenic BoyJ mice (CD45.1^{+}),

were transplanted into lethally irradiated BoyJ recipients. Donor-derived chimera were detected by flow cytometry at 16 weeks after transplantation. For secondary transplants, 1x10^6 CD45.2$^+$ cells from the primary recipients at 4 months after BMT were transplanted into sublethally irradiated BoyJ recipients. Donor-derived chimerism and myeloid expansion were determined by flow cytometry. Cytological and morphological analyses were conducted using Wright-Giemsa staining. Survival of the recipients was plotted using the Kaplan-Meier curve method.

Statistical analysis

A paired or unpaired Student *t*-test was used for two-group comparisons, and one-way analysis of variance was used for comparisons of more than two groups. *P* values less than 0.05 were considered statistically significant. Results are presented as mean ± standard deviation. In the figures, * indicates $P<0.05$; ** indicates $P<0.01$ and *** indicates $P<0.001$.

Results

Trem1 is expressed in pre-leukemic hematopoietic stem cells and leukemic stem cells

We previously showed that the immune receptor TREM1 cooperates with a diminished DDR to induce pre-leukemic HSC expansion using a mouse model deficient for the *Fanca* gene.[14] To further understand the role of Trem1 in leukemogenesis, we employed two leukemic models (Figure 1A) to investigate the underlying mechanisms *in vivo*: (i) the DNA damage-induced *Fanca*$^{-/-}$ pre-leukemic model; and (ii) the *MLL-AF9* (*MA9*) transgenic model, in which the expression of the oncogenic MLL-AF9 fusion protein results in development of AML beginning around 5 months of age.[20] We first established pre-leukemic and leukemic *Fanca*$^{-/-}$ mice as previously described,[14] and measured the levels of *Trem1* expression in the pre-leukemic HSC and LSC by quantitative polymerase chain reaction (qPCR). We found that *Trem1* mRNA level was significantly elevated in the SLAM (LSKCD48$^-$CD150$^+$ cells; enriched for HSC; *Online Supplementary Figure S1A*) cells of the pre-leukemic *Fanca*$^{-/-}$ mice, as compared to those in wild-type (WT) mice (Figure 1B). This was accompanied by a 40-50% increase in Trem1$^+$SLAM cells in the pre-leukemic mice over the number of such cells in WT controls (Figure 1C). *Trem1* expression and the percentage of Trem1$^+$SLAM cells were even higher in the *Fanca*$^{-/-}$ leukemic mice than in the *Fanca*$^{-/-}$ pre-leukemic mice (Figure 1D, E). We also observed a progressive increase in *Trem1* expression in BM c-Kit$^+$ cells (enriched for *MA9* LSC)[29] of the *MA9* mice over the period of 5 months (Figure 1F). Leukemia was confirmed by accumulation of Mac1$^+$Gr1$^+$ cells in peripheral blood by flow cytometry (*Online Supplementary Figure S1B*) and Wright-Giemsa staining of peripheral blood (*Online Supplementary Figure S1C*) from the *Fanca*$^{-/-}$ and *MA9* leukemic mice. Thus, these data indicate that Trem1 is expressed in pre-leukemic HSC and LSC.

Deletion of *Trem1* does not alter steady-state hematopoiesis

To further investigate the role of Trem1 in leukemogenesis, we employed CRISPR-Cas9 technology to generate a conditional *Trem1* knockout mouse strain (*Trem1*$^{fl/fl}$) (*Online Supplementary Figure S2A*). By using a previously described *Vav1Cre* deleter strain,[30] we were able to selectively ablate Trem1 expression in the hematopoietic compartment at both mRNA and protein levels (*Online Supplementary Figure S2B-D*).

To determine whether ablation of Trem1 affected normal hematopoiesis, we first analyzed blood counts, BM cellularity, the frequency, and absolute numbers of total hematopoietic stem and progenitor cells (HSPC: LSK [Lin$^-$Sca1$^+$c-Kit$^+$] cells) and HSC (SLAM) in *Trem1*$^{fl/fl}$*Vav1Cre* mice. We found no significant differences between *Trem1*$^{fl/fl}$*Vav1Cre* mice and the *Trem1*$^{fl/fl}$ controls under homeostatic conditions, as evidenced by comparable complete blood counts (Figure 2A), total BM cell counts (Figure 2B) and flow cytometry-based phenotypic analysis of BM LSK and SLAM populations (Figure 2C). These data suggest that Trem1 is not required for the maintenance of normal hematopoiesis.

To determine HSPC proliferation, we sorted LSK cells from *Trem1*$^{fl/fl}$*Vav1Cre* and the *Trem1*$^{fl/fl}$ control mice and performed colony-forming-unit-cell (CFU-C) assays. We found that HSPC from *Trem1*$^{fl/fl}$*Vav1Cre* and *Trem1*$^{fl/fl}$ control mice produced comparable CFU-C colonies (Figure 2D). Competitive *in vivo* transplantation assays showed no significant differences in repopulating capacity between *Trem1*$^{fl/fl}$*Vav1Cre* HSC and the *Trem1*$^{fl/fl}$ control HSC (Figure 2E). Contributions to myeloid and lymphoid lineages were also similar between *Trem1f*$^{fl/fl}$*Vav1Cre* and *Trem1*$^{fl/fl}$ HSC (Figure 2F). However, we observed that the repopulating capacity of *Trem1*$^{fl/fl}$*Vav1Cre* HSC in secondary recipients was significantly higher than that of *Trem1*$^{fl/fl}$ HSC (Figure 2G). These data suggest that *Trem1* deletion does not influence hematopoiesis under homeostatic conditions.

Trem1 expression in pre-leukemic hematopoietic stem cells and leukemic stem cells promotes leukemogenesis

To determine the role of Trem1 in leukemia development *in vivo*, we established three leukemogenic models (Figure 3A): (i) *Fanca*$^{-/-}$ leukemia expressing the conditional *Trem1*$^{fl/fl}$; (ii) *MA9* leukemia expressing the conditional *Trem1*$^{fl/fl}$; and (iii) *MA9* leukemia expressing the Trem1 protein. We then analyzed LSC expansion and leukemia development in the transplanted recipients in the context of Trem1 expression. We found that deletion of *Trem1* signifi-

cantly reduced LSC-enriched donor SLAM cells in the recipient mice transplanted with LSK cells from *Fanca*$^{-/-}$*;Trem1*$^{fl/fl}$*Vav1Cre* mice compared to those from the *Fanca*$^{-/-}$*;Trem1*$^{fl/fl}$ controls (Figure 3B). Consistently, ablation of Trem1 markedly prolonged the survival of the recipients transplanted with *Fanca*$^{-/-}$*;Trem1*$^{fl/fl}$*Vav1Cre* cells compared to those transplanted with the *Fanca*$^{-/-}$*;Trem1*$^{fl/fl}$ control cells (Figure 3C). Similar results were obtained with the re-

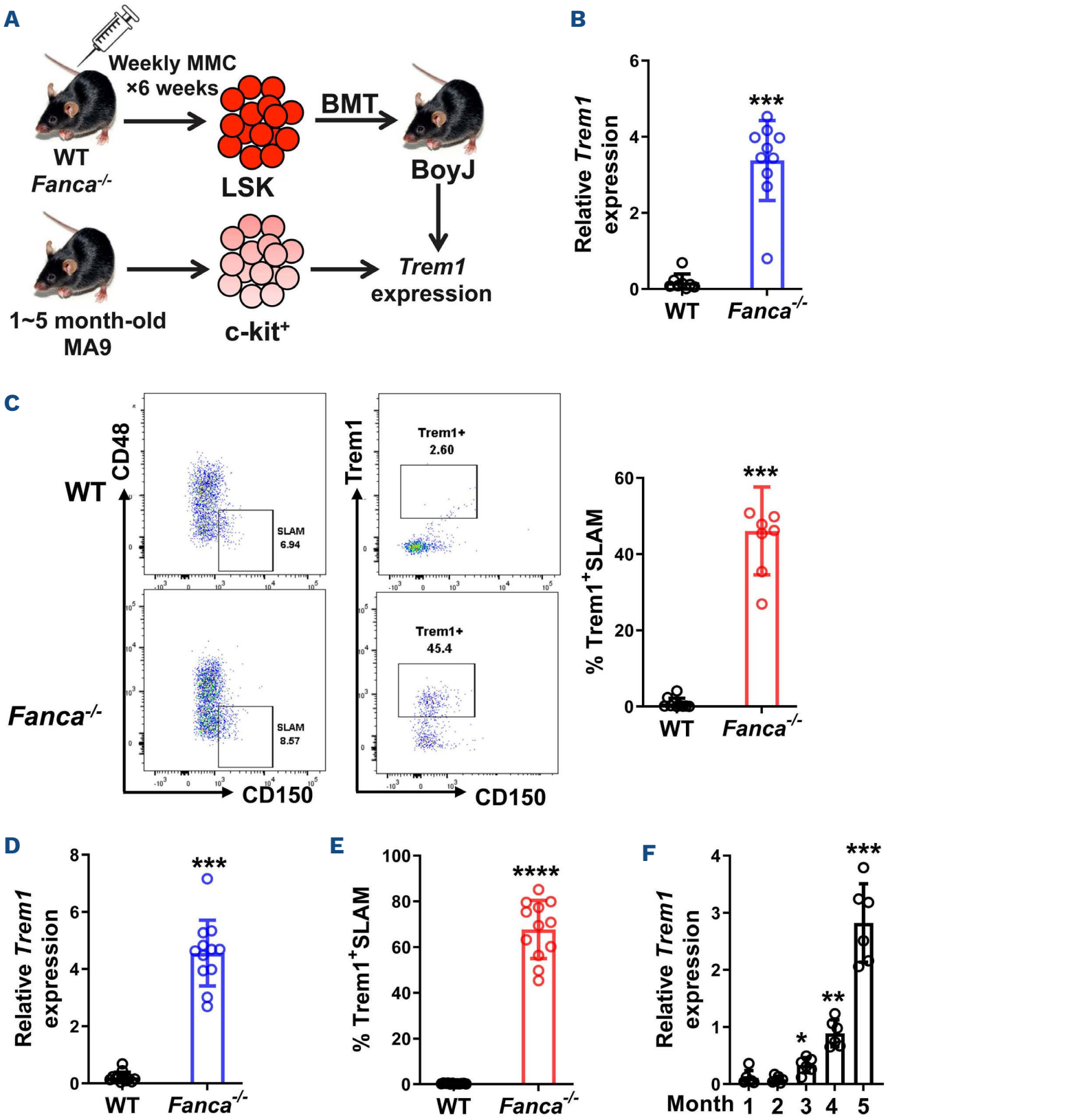

Figure 1. *Trem1* is expressed in pre-leukemic hematopoietic stem cells and leukemic stem cells. (A) Schematic presentation of the experimental design. (B) Aberrant *Trem1* expression in *Fanca*$^{-/-}$ pre-leukemic hematopoietic stem cells (HSC). Wild-type (WT) and *Fanca*$^{-/-}$ mice were injected intraperitoneally with 0.3 mg/kg of mitomycin C (MMC) weekly for 6 weeks. RNA was extracted from SLAM cells isolated from the indicated mice followed by quantitative polymerase chain reaction (qPCR) analysis using the primers listed in *Online Supplementary Table S1* (n=8-10 mice/group). (C) Increased Trem1-expressing HSC in *Fanca*$^{-/-}$ pre-leukemic mice. Left: representative flow cytometry analysis of percentages of bone marrow Trem1^{+} SLAM cells in the mice described in (B). Right: quantification (n=8-10 mice/group). (D) Increased *Trem1* expression in leukemia stem cells (LSC) from *Fanca*$^{-/-}$ leukemic mice. FACS-isolated LSK cells from the mice described in (B) were subjected to two rounds of bone marrow transplantation (BMT) to establish *Fanca*$^{-/-}$ leukemia in secondary transplanted recipient mice. Donor-derived SLAM cells were isolated from the secondary recipients and subjected to qPCR analysis for *Trem1* expression (n=12 mice/group). (E) Quantification of flow cytometry analysis of percentages of bone marrow Trem1^{+} SLAM cells in the secondary recipients (n=12 mice/group). (F) Progressive increase in *Trem1* expression in *MLL-AF9* (*MA9*) LSC. Bone marrow c-Kit^{+} cells from 1- to 5-month-old *MA9* mice were subjected to qPCR for *Trem1* expression. WT mice were used as controls (n=6 mice/group).

cipients transplanted with donor cells from leukemic *MA9* mice at the age of 5 months old (when the mice develop AML),[29] in which deletion of *Trem1* significantly reduced LSC-enriched CD45.2$^+$c-Kit$^+$ cells in the recipients transplanted with cells from 5-month-old *MA9;Trem1$^{fl/fl}$Vav1cre* mice compared to those in the recipients transplanted with cells from age-matched *MA9;Trem1$^{fl/fl}$* mice (Figure 3D). Consequently, ablation of Trem1 greatly extended latency in the recipients of *MA9;Trem1$^{fl/fl}$Vav1cre* cells compared to those of *MA9;Trem1$^{fl/fl}$* cells (Figure 3E).

To compare the ability of Trem1$^+$ and Trem1$^-$ pre-leukemic HSC to induce leukemia, we purified Trem1$^+$SLAM and Trem1$^-$-SLAM cells from *Fanca$^{-/-}$* pre-leukemic mice (*Online Supplementary Figure S3A*), and performed serial BMT. We found that although all primary recipients survived for more than 12 months without signs of leukemia, the recipients transplanted with Trem1$^+$ HSC exhibited donor BM hypercellularity (*Online Supplementary Figure S3B*) and increased accumulation of donor-derived phenotypic HSC in the BM (*Online Supplementary Figure S3C*). Furthermore,

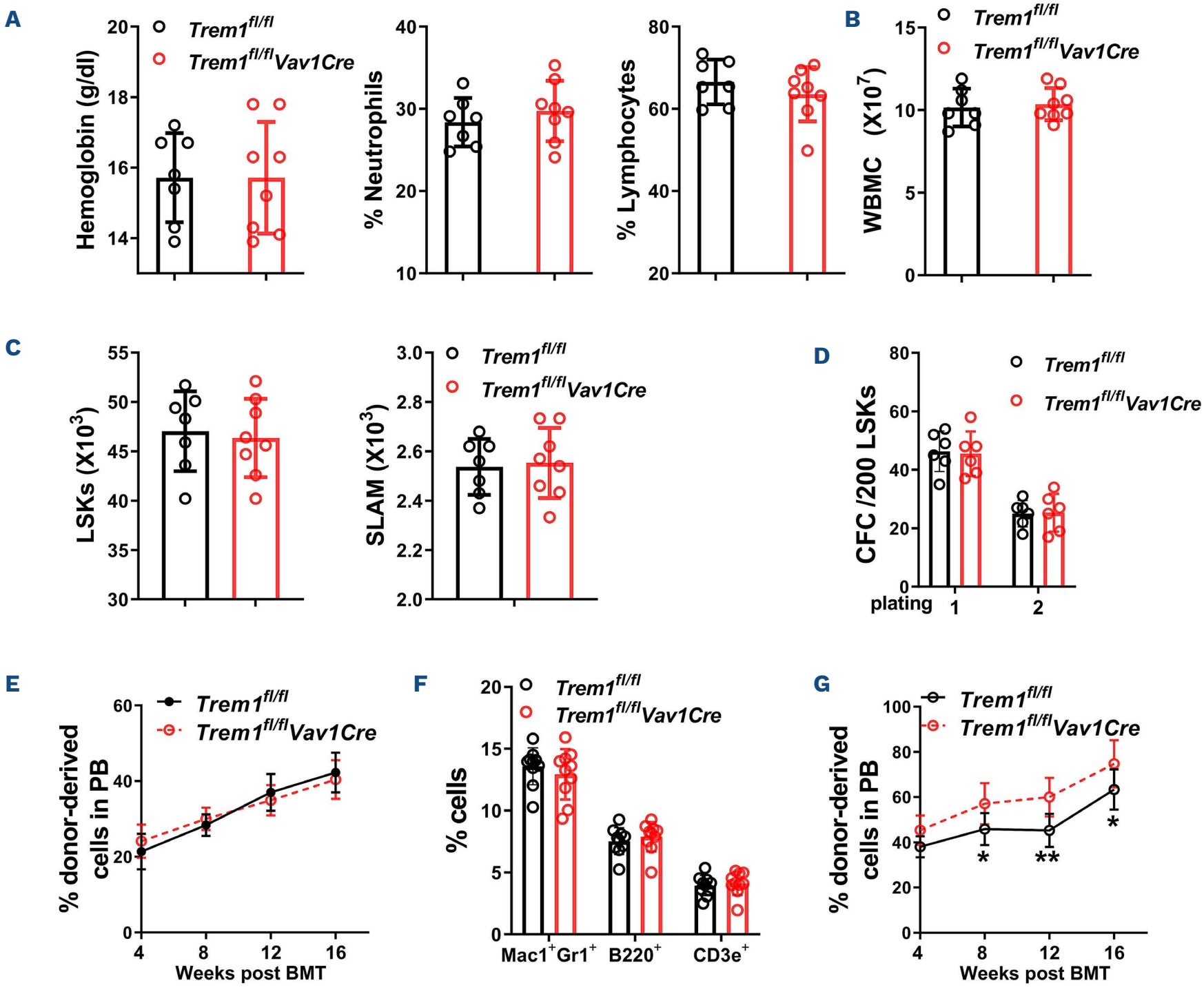

Figure 2. Deletion of *Trem1* does not alter steady-state hematopoiesis. (A) Complete blood count of 8-week-old *Trem1$^{fl/fl}$Vav1Cre* mice and the *Trem1$^{fl/fl}$* controls (n=7-8 mice). (B) Whole bone marrow cell (WBMC) counts (n=7-8 mice). (C) Bone marrow LSK and SLAM cell counts (n=7-8), and (D) colony-forming cell (CFC) counts are shown for the *Trem1$^{fl/fl}$Vav1Cre* mice and the *Trem1$^{fl/fl}$* controls (n=6 mice). (E) Deletion of *Trem1* does not affect hematopoietic repopulation in primary transplant recipients. One hundred SLAM cells from *Trem1$^{fl/fl}$Vav1Cre* mice and *Trem1$^{fl/fl}$* controls were transplanted, along with 2x10^5 competitor cells from congenic mice, into lethally irradiated BoyJ recipients. Donor-derived chimerism was determined by flow cytometry of peripheral blood (PB) at different time points after bone marrow transplantation (BMT) (n=10 mice). (F) Deletion of *Trem1* does not affect multi-lineage reconstitution in primary transplant recipients. Percentages of donor-derived myeloid, T, and B cells were measured by flow cytometry 16 weeks after BMT (n=10 mice). (G) Deletion of *Trem1* increases long-term hematopoietic repopulation. One to three million WBMC from the primary recipients were pooled and injected into sublethally irradiated BoyJ recipients. Donor-derived chimerism was determined by flow cytometry at different time points after BMT (n=12 mice).

the secondary recipients of Trem1$^+$ cohorts gave rise to lethal leukemias within 100 days, while the majority of recipients transplanted with Trem1$^-$ cells survived over 100 days after BMT (*Online Supplementary Figure S3D*). Leukemia was confirmed by accumulation of Mac1$^+$Gr1$^+$CD45.2$^+$ cells in peripheral blood (*Online Supplementary Figure S3E*) and Wright-Giemsa staining of peripheral blood (*Online Supplementary Figure S3F*). These data indicate that Trem1$^+$ pre-leukemic HSC have greater leukemogenic potential than that of Trem1$^-$ pre-leukemic HSC.

To substantiate the observation that Trem1 promotes leukemia, we transduced c-kit+ cells from *MA9* mice at the age of 2 months, at which time *Trem1* expression is not detectable (see Figure 1F above), with a lentiviral vector expressing eGFP or *eGFP-Trem1* (Figure 3A). We achieved >5-fold higher *Trem1* expression in GFP-*Trem1* cells than in eGFP control cells (*Online Supplementary Figure S4A, B*). Remarkably, overexpression of *Trem1* greatly increased LSC-enriched CD45.2$^+$c-Kit$^+$ cells in the recipients transplanted with eGFP-*Trem1*-transduced MA9 cells

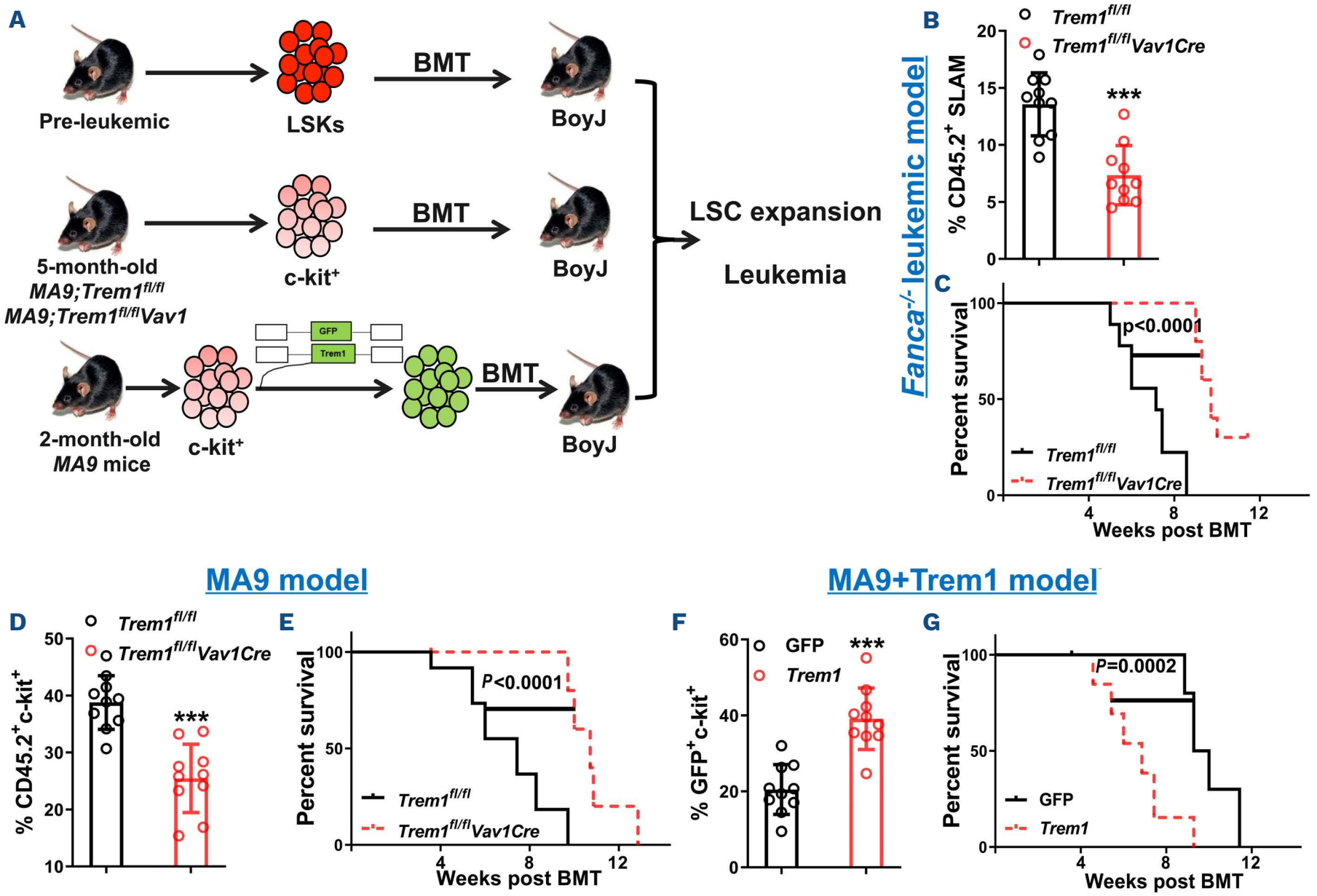

Figure 3. *Trem1* expression in pre-leukemic hematopoietic stem cells and leukemic stem cells promotes leukemogenesis. (A) Schematic presentation of the experimental design. (B) Deletion of *Trem1* suppresses *Fanca*$^{-/-}$ leukemic stem cell (LSC) expansion in transplant recipient mice. LSK cells from *Fanca*$^{-/-}$*;Trem1*$^{fl/fl}$*Vav1Cre* mice and the *Fanca*$^{-/-}$*;Trem1f*$^{l/fl}$ controls subjected to 6 weeks of treatment with mitomycin C (MMC) were transplanted, along with 2x10^5 competitor cells from congenic mice, into lethally irradiated BoyJ recipients. Donor-derived CD45.2$^+$ SLAM cells were analyzed by flow cytometry 4 weeks after bone marrow transplantation (BMT) (n=10 mice). (C) Deletion of *Trem1* extends the latency of *Fanca*$^{-/-}$ leukemia. Survival of the recipient mice groups described in (B) was monitored and plotted by the Kaplan-Meier method (n=9-10 mice/group). (D) Ablation of Trem1 suppresses *MLL-AF9* LSC expansion in transplant recipients. Bone marrow c-Kit$^+$ cells from *MLL-AF9;Trem1*$^{fl/fl}$*Vav1Cre* mice and the *MLL-AF9;Trem1*$^{fl/fl}$ controls were transplanted, along with 2x10^5 competitor cells from congenic mice, into lethally irradiated BoyJ recipients. Donor-derived CD45.2$^+$c-Kit$^+$ cells were analyzed by flow cytometry 4 weeks after BMT (n=10 mice). (E) Ablation of Trem1 extends the latency of *MLL-AF9* leukemia. Survival of the recipient mice groups described in (D) was monitored and plotted by Kaplan-Meier method (n=10-11 mice/group). (F) Forced expression of Trem1 increases *MLL-AF9* LSC expansion in transplant recipients. Bone marrow c-Kit$^+$ cells from 2-month-old *MLL-AF9* mice were transduced with lentiviral vector expressing eGFP-Trem1 or eGFP alone. The transduced GFP$^+$ cells were transplanted, along with 2x10^5 competitor cells from congenic mice, into lethally irradiated BoyJ recipients. Donor-derived GFP$^+$ c-Kit$^+$ cells were analyzed by flow cytometry 4 weeks after BMT (n=10 mice). (G) Forced expression of Trem1 shortens the latency of *MLL-AF9* leukemia. Survival of the recipient mice groups described in (F) was monitored and plotted by the Kaplan-Meier method (n=10-13 mice). (B & C): the *Fanca*$^{-/-}$ leukemia model; (D & E) the MA9 model; and (F & G) the MA9$^+$ Trem1 model.

compared to the recipients transplanted with eGFP control cells (Figure 3F). Consistently, overexpression of *Trem1* markedly shortened latency in the recipients of GFP-*Trem1*-transduced MA9 cells compared to the latency in recipients of eGFP control cells (Figure 3G). Together, these data suggest that *Trem1* expression in pre-leukemic HSC and LSC promotes leukemogenesis.

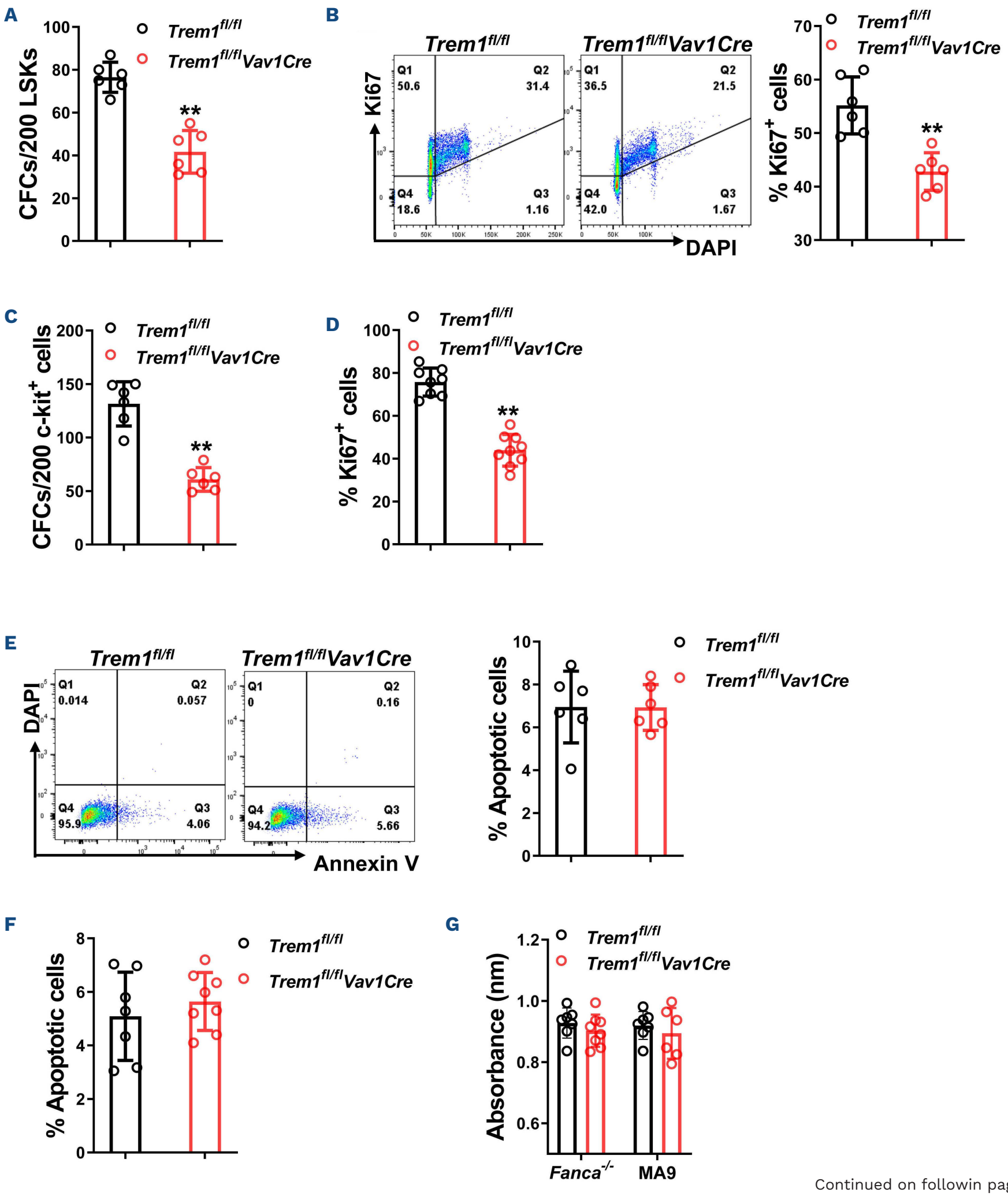

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Figure 4. Trem1 expression in pre-leukemic hematopoietic stem cells and leukemic stem cells is associated with increased proliferation. (A) Deletion of *Trem1* reduces myeloid colony formation by *Fanca*$^{-/-}$ leukemia stem cell (LSC)-enriched donor LSK cells. LSK cells from *Fanca*$^{-/-}$*;Trem1*$^{fl/fl}$*Vav1Cre* mice and the *Fanca*$^{-/-}$*;Trem1*$^{fl/fl}$ controls subjected to 6 weeks of treatment with mitomycin C (MMC) were transplanted, along with 2x10^5 competitor cells from congenic mice, into lethally irradiated BoyJ recipients. Donor-derived CD45.2$^+$LSK cells were subjected to colony-forming unit (CFU) assays 4 weeks after bone marrow transplantation (BMT) (n=6 mice). (B) Deletion of *Trem1* reduces Ki67$^+$ proliferating *Fanca*$^{-/-}$ LSC-enriched donor LSK cells. The CD45.2$^+$LSK cells from the recipient mice in (A) were gated for Ki67-positive nuclear stain. Representative flow cytometry (left) and quantification (right) are shown (n=6 mice). (C) Ablation of Trem1 reduces myeloid colony formation by *MLL-AF9* LSC-enriched donor c-Kit$^+$ cells. Bone marrow c-Kit$^+$ cells from *MLL-AF9;Trem1*$^{fl/fl}$*Vav1Cre* mice and the *MLL-AF9;Trem1f*$^{l/fl}$ controls were transplanted, along with 2x10^5 competitor cells from congenic mice, into lethally irradiated BoyJ recipients. Donor-derived CD45.2$^+$c-Kit$^+$ cells were subjected to CFU assays 4 weeks after BMT (n=6 mice). (D) Ablation of Trem1 reduces Ki67-positive proliferating *MLL-AF9* LSC-enriched donor c-Kit$^+$ cells. The CD45.2$^+$c-Kit$^+$ cells from the recipient mice in (C) were gated for Ki67-positive nuclear stain (n=8-9 mice). (E) Deletion of *Trem1* does not increase apoptosis in *Fanca*$^{-/-}$ LSC-enriched donor LSK cells. The CD45.2+LSK cells from the recipient mice in (A) were gated for annexin V-positive staining. Representative flow cytometry (left) and quantification (right) are shown (n=6 mice). (F) Deletion of *Trem1* does not increase apoptosis in *MLL-AF9* LSC-enriched donor c-Kit$^+$ cells. The CD45.2$^+$c-Kit$^+$ cells from the recipient mice in (C) were gated for annexin V-positive staining (n=7-8 mice). (G) Ablation of Trem1 does not render *Fanca*$^{-/-}$ LSC-enriched donor LSK cells or *MLL-AF9* LSC-enriched donor c-Kit$^+$ cells more sensitive to growth factor-deprived conditions. The CD45.2$^+$LSK cells from the recipient mice in (A) or the CD45.2$^+$c-Kit$^+$ cells from the recipient mice in (C) were cultured in serum-free StemCell medium supplemented with stem cell factor, Flt3 ligand and thrombopoietin for 72 h followed by factor withdrawal. Cell viability was determined by absorbance using a CellTiter 96 Aqueous One Solution Cell Proliferation (MTS) assay after factor withdrawal for 24 h (n=6-8 assays).

Trem1 expression in pre-leukemic hematopoietic stem cells and leukemic stem cells is associated with increased proliferation but has no effect on sensitivity to apoptosis

To explore the underlying mechanism by which Trem1 promotes leukemogenesis, we next examined the rates of proliferation and apoptosis in the LSC-enriched donor cells from the leukemic mice transplanted with cells from *Fanca*$^{-/-}$*;Trem1*$^{fl/fl}$*Vav1Cre* or *MA9;Trem1*$^{fl/fl}$*Vav1Cre* mice, 4 weeks after transplantation. We found that deletion of Trem1 significantly reduced myeloid colony formation by CD45.2$^+$LSK cells from the leukemic mice transplanted with the *Fanca*$^{-/-}$*;Trem1*$^{fl/fl}$*Vav1Cre* cells compared to those of *Fanca*$^{-/-}$*;Trem1*$^{fl/fl}$ control cells, as determined by CFU assay (Figure 4A). Furthermore, there was a lower percentage of proliferating (Ki67-positive) CD45.2$^+$LSK cells from the leukemic mice transplanted with the *Fanca*$^{-/-}$*;Trem1*$^{fl/fl}$*Vav1Cre* cells compared to those of *Fanca*$^{-/-}$*;Trem1*$^{fl/fl}$ control cells, as determined by nuclear Ki67 staining (Figure 4B). We obtained similar results in the experiments with the *MA9;Trem1*$^{fl/fl}$*Vav1Cre* and *MA9;Trem1*$^{fl/fl}$ leukemic cells, in which ablation of Trem1 significantly inhibited the proliferation of the LSC-enriched CD45.2$^+$c-kit$^+$ cells (Figure 4C, D). However, deletion of Trem1 did not significantly increase apoptosis in the leukemia from *Fanca*$^{-/-}$*;Trem1*$^{fl/fl}$*Vav1Cre* pre-leukemic cells or *MA9;Trem1*$^{fl/fl}$*Vav1Cre* leukemic cells, as compared to those from *Fanca*$^{-/-}$*;Trem1*$^{fl/fl}$ or *MA9;Trem1*$^{fl/fl}$ control cells, respectively (Figure 4E, F). Furthermore, ablation of Trem1 did not render either *Fanca*$^{-/-}$ or *MA9* leukemic cells more sensitive to growth factor-deprived culture conditions, as detected by MTS assay (Figure 4G). Taken together, these results indicate that Trem1 promotes leukemic cell proliferation but has limited effect on apoptosis sensitivity.

Trem1 expression in pre-leukemic hematopoietic stem cells and leukemic stem cells is associated with persistent DNA damage and prolonged oncogenic stress

Since our previous studies demonstrated that Trem1 cooperates with diminished DDR in pre-leukemic HSC expansion,[14] we asked whether *Trem1* expression in *Fanca*$^{-/-}$ pre-leukemic HSC was associated with persistent DNA damage. To this end, we treated the SLAM cells from WT and pre-leukemic *Fanca*$^{-/-}$ mice with MMC in culture for 2 h and performed flow cytometry analysis for γ-H2AX, an established marker of double-strand breaks,[31] at different times after treatment. We found that MMC induced robust expression of γ-H2AX at 4 h after treatment in both *Fanca*$^{-/-}$ and WT cells; however, WT cells efficiently repaired double-strand breaks, as evidenced by a progressive decline of γ-H2AX within 16 h after MMC treatment (Figure 5A). In contrast, the MMC-treated *Fanca*$^{-/-}$ cells retained high levels of γ-H2AX throughout the 16-h period (Figure 5A), indicative of persistent DNA damage. Correlated with this DNA damage kinetics, *Trem1* expression was persistently elevated during the 8- to 16-h period of observation in *Fanca*$^{-/-}$ cells, whereas *Trem1* expression remained undetectable in WT cells (Figure 5B).

Next, we investigated whether prolonged oncogenic stress could also lead to aberrant expression of *Trem1* in LSC. We utilized two leukemia models, *MLL-AF9* and *Kras*^{G12D}, to induce prolonged oncogenic stress *in vitro*. For the *MLL-AF9* model, we infected WT LSK cells with a retroviral vector expressing eGFP-*MLL-AF9* or eGFP alone, and subjected the transduced cells to culture in growth factor-supplemented medium for different periods of time. We observed persistent elevation of *Trem1* expression during 4 to 12 days of culture in cells expressing *MLL-AF9*, whereas *Trem1* expression remained undetectable in those expressing eGFP alone (Figure 5C). For the *Kras*^{G12D} model,

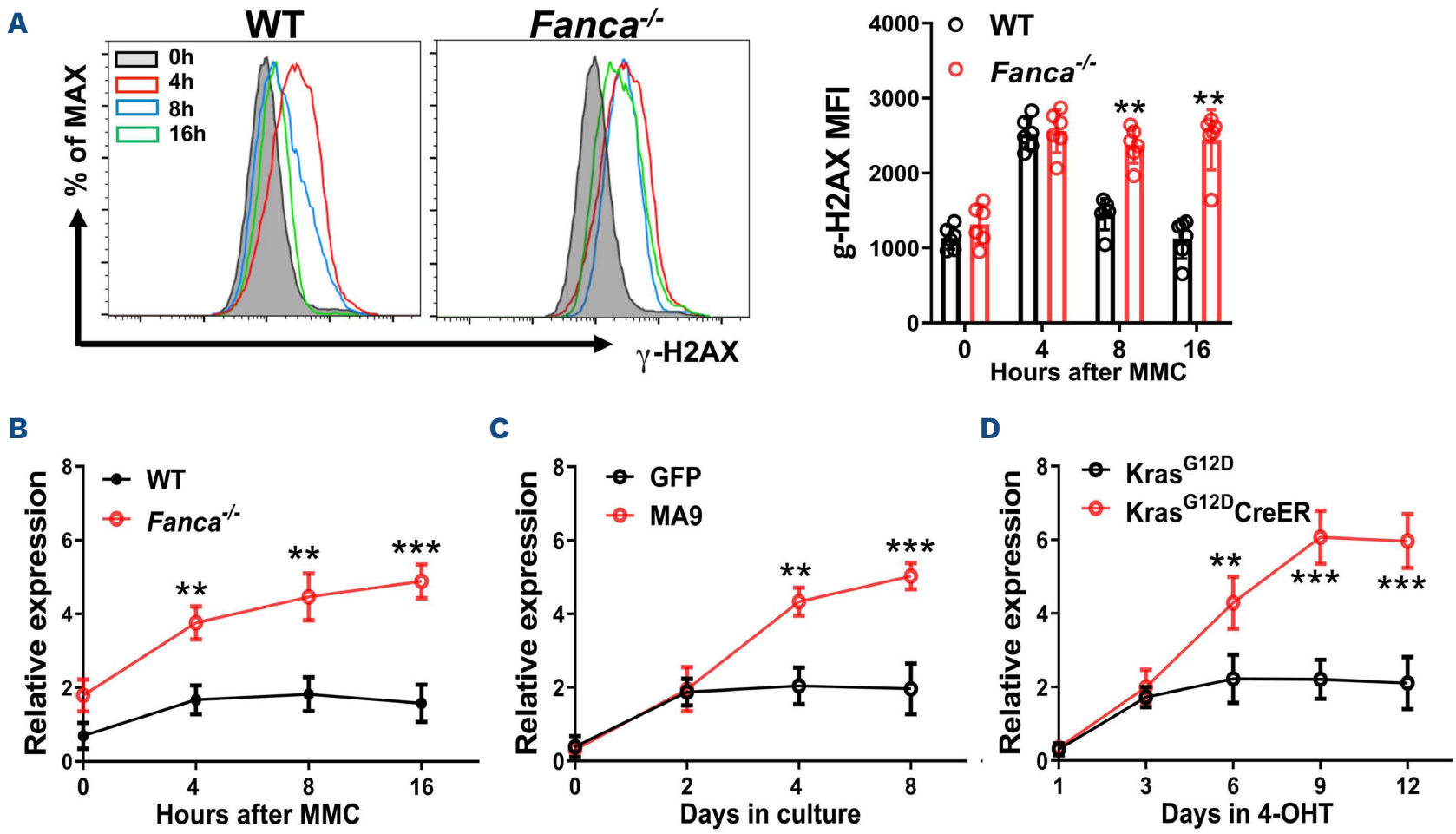

Figure 5. *Trem1* expression in pre-leukemic hematopoietic stem cells and leukemic stem cells is associated with persistent DNA damage and prolonged oncogenic stress. (A) Persistent DNA damage in *Fanca*$^{-/-}$ pre-leukemic hematopoietic stem cells (HSC). LSK cells from wild-type (WT) and pre-leukemic *Fanca*$^{-/-}$ mice were treated with mitomycin C (MMC) in culture for 2 h and analyzed by flow cytometry for γ-H2AX at different time points after treatment. Representative flow plots (left) and mean fluorescence intensity (MFI) kinetics (right) are shown. 0 h: untreated control (n=6 experiments). (B) *Trem1* expression is specifically induced by persistent DNA damage in *Fanca*$^{-/-}$ pre-leukemic HSC. RNA was then extracted from the cells described in (A) and subjected to quantitative polymerase chain reaction (qPCR) analysis for *Trem1* expression using the primers listed in *Online Supplementary Table S1*. Samples were normalized to the level of *GAPDH* mRNA (n=6 assays/group). **MMC *vs*. untreated control (0 h). (C) Prolonged oncogenic stress induces *Trem1* expression in *MLL-AF9* leukemic stem cell (LSC)-enriched cells. WT LSK cells were transduced with retroviral vector expressing eGFP-*MLL-AF9* or eGFP alone, and the transduced cells were subjected to culture in growth factor-supplemented medium. RNA was then extracted from the sorted GFP^{+} LSK cells at different time points followed by qPCR analysis for *Trem1* expression. Samples were normalized to the level of *GAPDH* mRNA (n=6 assays). (D) Prolonged oncogenic stress induces *Trem1* expression in *Kras*^{G12D} LSC-enriched cells. LSK cells from *Kras*^{G12D}*CreER* and *Kras*^{G12D} control mice were cultured in growth factors-supplemented medium in the presence of 4-OHT. RNA was then extracted from the sorted LSK cells at different time points followed by qPCR analysis for *Trem1* expression. Samples were normalized to the level of *GAPDH* mRNA (n=6 assays).

we isolated LSK cells from *Kras*^{G12D}*CreER* mice and cultured the cells in growth factor-supplemented medium in the presence of 4-hydroxytamoxifen (4-OHT; to induce Cre-mediated expression of *Kras*^{G12D}) for different periods of time. We found that prolonged treatment of the *Kras*^{G12D}*CreER* cells with 4-OHT induced a persistent increase in *Trem1* expression during 3 to 9 days of culture, as compared to negligible *Trem1* expression in the cells cultured in the absence of 4-OHT (Figure 5D). These results indicate that prolonged oncogenic stress induces aberrant expression of *Trem1* in LSC-enriched leukemic cells.

Trem1 expression in pre-leukemic hematopoietic stem cells and leukemic stem cells is associated with enhanced inflammation

Inflammation is a key feature of leukemia.[32,33] TREM1 is known to trigger and amplify inflammatory responses.[1,2] To explore the relationship between Trem1, inflammation and leukemia progression, we measured 248 inflammation-related genes using an nCounter Mouse Inflammation multiplex panel. We observed 20 significantly dysregulated genes in LSK cells of pre-leukemic *Fanca*$^{-/-}$*;Trem1*$^{fl/fl}$ mice compared to those from LSK cells from *Fanca*$^{-/-}$*;Trem1*$^{fl/fl}$*Vav1Cre* mice (Figure 6A); and in 5-month-old *MA9;Trem1*$^{fl/fl}$ mice compared with those of age-matched *MA9;Trem1*$^{fl/fl}$*Vav1Cre* mice (Figure 6A).

Among the top five upregulated inflammatory genes, we noted that *Ccr1*, *Il1r1*, *Nlrp3* and *Tlr2* were upregulated in both *Fanca*$^{-/-}$ and *MA9* cells (*Online Supplementary Figure S5A, B*). CCR1 and its ligand CCL3 have recently been shown to promote leukemogenesis.[34] IL1R1 and NLRP3 are key components of the NLPR3 inflammasome, which plays important roles in hematologic malignancies.[35] Increased expression of Toll-like receptor 2 (TLR2) and its functional binding partners, TLR1 and TLR6, is found in patients with

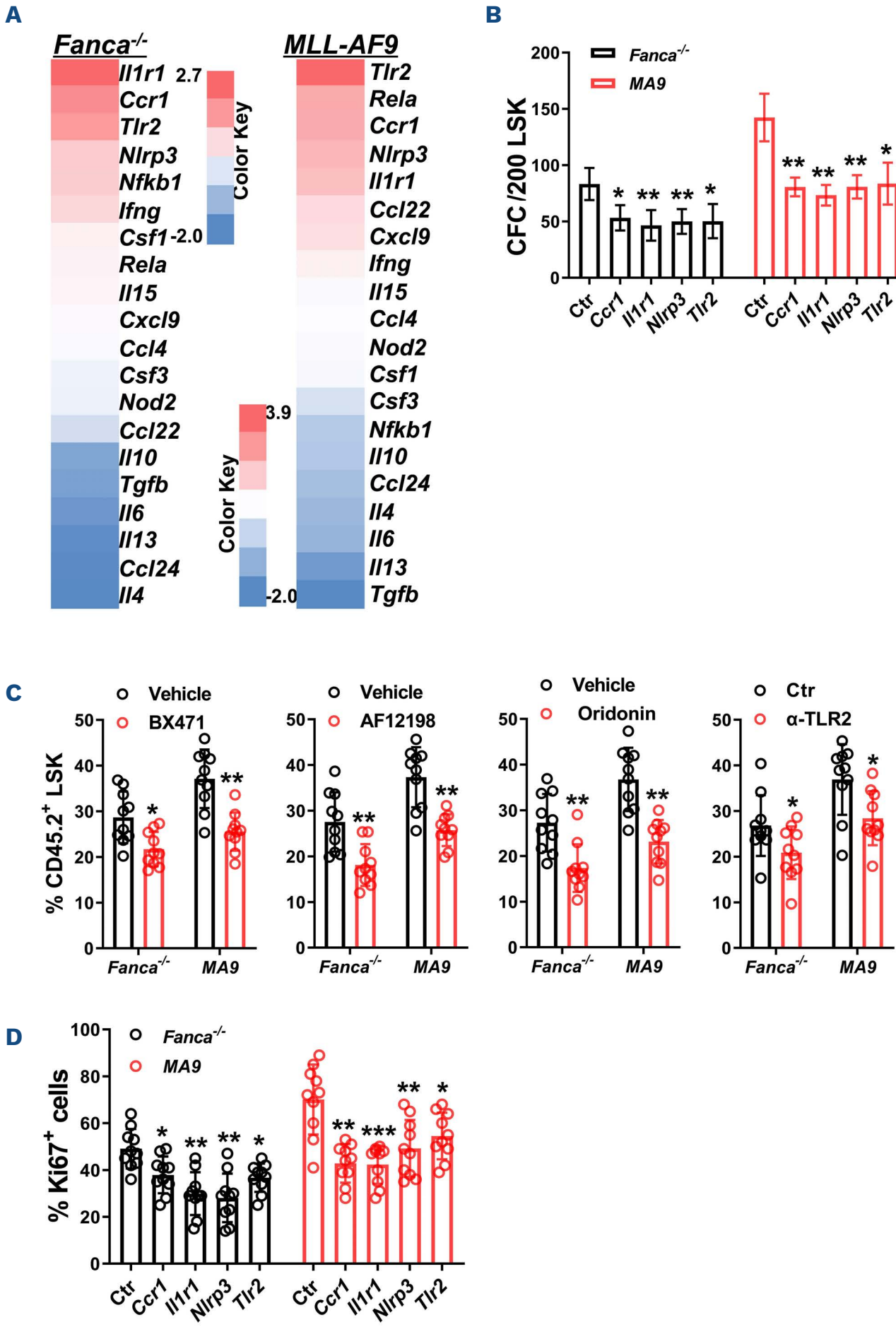

Figure 6. *Trem1* expression in pre-leukemic hematopoietic stem cells and leukemic stem cells is associated with enhanced inflammation. (A) Inflammatory gene expression (by NanoString analysis) in LSK cells of pre-leukemic *Fanca*$^{-/-}$;*Trem1*$^{fl/fl}$ (*Trem1-WT*) mice compared with those of *Fanca*$^{-/-}$;*Trem1f*$^{l/fl}$*Vav1Cre* (*Trem1-KO*) mice (n=3 assays; left) and LSK cells of *MA9;Trem1*$^{fl/fl}$ (*Trem1-WT*) mice compared with those of *MA9;Trem1*$^{fl/fl}$*Vav1Cre* (*Trem1-KO*) mice (n=3 assays; right). Heatmap of inflammatory genes (log_2 fold change; FC) differentially expressed among *Fanca*$^{-/-}$;*Trem1*$^{fl/fl}$ and *Fanca*$^{-/-}$;*Trem1*$^{fl/fl}$*Vav1Cre* cells or *MA9;Trem1*$^{fl/fl}$ and *MA9;Trem1*$^{fl/fl}$*Vav1Cre* cells. (B) Blockade of CCR1, IL1R1, NLPR3, or TLR2 reduces colony formation of *Fanca*$^{-/-}$ and *MA9* leukemic cells. LSK cells from *Fanca*$^{-/-}$ pre-leukemic mice or 5-month-old *MA9* mice were cultured in the presence of BX471, AF12198, oridonin, or anti-TLR2 antibodies for 5 days after which colony-froming unit-cell (CFU-C) assays were performed (n=6 assays). (C) Blockade of CCR1, IL1R1, NLPR3, or TLR2 suppresses the expansion of *Fanca*$^{-/-}$ and *MA9* leukemic stem cells (LSC). The cultured cells described in (B) were transplanted, along with 2x10^5 protector cells from congenic mice, into lethally irradiated recipients. Donor-derived CD45.2+LSK cells from the recipient mice were analyzed 4 months after bone marrow transplantation (BMT) by flow cytometry (n=10 mice). (D) Blockade of CCR1, IL1R1, NLPR3, or TLR2 inhibited the proliferation of *Fanca*$^{-/-}$ and *MA9* LSC. The recipient mice described in (C) were analyzed for Ki67$^+$ CD45.2$^+$LSK cells 4 months after BMT by flow cytometry (n=10 mice).

myelodysplastic syndromes.[36] Recent studies showed that overall survival of AML patients with higher TLR2 expression was significantly shorter than that of patients with lower expression.[37] To investigate the functional relevance of these TREM1-inflammation signatures in the expansion of *Fanca*$^{-/-}$ and *MA9* leukemic cells, we performed blockade experiments using the CCR1 antagonist, BX471,[38] the IL-1R antagonist, AF12198,[39] the covalent inhibitor for NLRP3,[40] or the neutralizing antibody against TLR2.[41] LSK cells of pre-leukemic *Fanca*$^{-/-}$ mice and 5-month-old *MA9* mice were cultured in growth factor-supplemented medium in the presence of neutralizing antibodies for 5 days, followed by CFU and BMT assays. We found that blockade of CCR1, the NLPR3 inflammasome, or TLR2 significantly reduced myeloid colony formation of both *Fanca*$^{-/-}$ and *MA9* leukemic cells (Figure 6B), indicating that these Trem1 inflammation signatures promote the proliferation of *Fanca*$^{-/-}$ and *MA9* leukemic cells.

To substantiate these findings, we transplanted the cultured cells, along with 2x10^5 protector cells from congenic mice, into lethally irradiated recipients. We observed significant reduction of LSC-enriched donor cells in the recipient mice transplanted with *Fanca*$^{-/-}$ and *MA9* leukemic cells cultured in the presence of neutralizing antibodies, compared to those cultured in the absence of neutralizing antibodies (Figure 6C). Furthermore, there were significantly fewer proliferating (Ki67-positive) donor-derived LSC-enriched CD45.2$^+$LSK cells in the recipient mice transplanted with *Fanca*$^{-/-}$ and *MA9* leukemic cells treated with neutralizing antibodies compared to the untreated control cells (Figure 6D). These data suggest that targeting Trem1 inflammation signatures could suppress the expansion of *Fanca*$^{-/-}$ and *MA9* LSC.

Discussion

The immune system, consisting of immune cells, immune factors, and the immune microenvironment, plays essential roles in tumorigenesis.[42] The multifaceted effects of tumor-related immunity include destroying genome stability, generating genetic modification, promoting the proliferation of cancer cells, stimulating angiogenesis, and shaping the tumor microenvironment.[43] In this study, we showed that one such immune factor, Trem1, is induced by persistent DNA damage and oncogenic stress and promotes leukemogenesis. Using our established *Fanca*$^{-/-}$ pre-leukemic model and the *MLL-AF9* AML model, we demonstrated that Trem1 is highly expressed in pre-leukemic HSC and LSC. Moreover, we generated an innovative conditional Trem1 knockout mouse model to show that selective ablation of Trem1 in the hematopoietic compartment significantly extended leukemic latency in mice, and that Trem1 was required for the proliferation of the pre-leukemic HSC and LSC. Our study thus provides new insights into the role of TREM1 in leukemogenesis.

TREM1 is constitutively expressed on a select group of myeloid cells including macrophages, monocytes and neutrophils in peripheral blood.[44,45] It has also been identified on airway epithelial cells, hepatic endothelial cells and liver résident macrophages, natural killer cells, dendritic cells, as well as B and T cells.[5,45-47] TREM1 belongs to the immunoglobulin superfamily and is engaged in amplifying inflammatory cascades.[48,49] It can be induced at high levels on neutrophils and monocytes and further amplifies TLR-initiated responses against microbial challenges, potentiating the secretion of pro-inflammatory cytokines with the help of DAP12 adaptor protein in response to bacterial and fungal infections.[50-52] Although high-mobility group box 1 (HMGB1) and peptidoglycan recognition protein 1 (PGLYRP1) were shown to associate with TREM1, the ligand for TREM1 remains to be characterized.[53,54] A growing body of evidence suggests that TREM1 plays critical pathological roles in chronic inflammatory disorders including cancer.[2] In fact, novel TREM1 inhibitors are being shown to attenuate tumor growth and prolong survival in experimental cancer models.[8] By employing three leukemogenic mouse models (a DNA damage-induced *Fanca*$^{-/-}$ pre-leukemic model, an oncogenic *MLL-AF9* transgenic model, and a *Kras*^{G12D}*CreER* model) we showed that Trem1 was highly expressed in pre-leukemic HSC and LSC, and dysregulated expression of *Trem1* in these malignant stem cells, enhancing their leukemogenic potential. Conversely, deletion of Trem1 in these pre-leukemic HSC and LSC compromised proliferation and delayed leukemia development *in vivo*. It is in this context that our study provides new insights into the current understanding of a pathological role for TREM1 in cancer.

One intriguing finding of our current study is the observation that Trem1 expression is induced both by persistent DDR and oncogenic stress. Genomic instability, found in most cancers, is considered one of the hallmark characteristics of cancer cells. It is regarded as a major driver of tumorigenicity.[55] The FA DNA repair pathway consists of a protein core complex that recognizes damage caused by inter-strand crosslinks, and a multi-subunit ubiquitin ligase that monoubiquitinates downstream DNA repair factors.[56] Our previous studies showed that chronic DNA damage stress could transform *Fanca*$^{-/-}$ HSC into pre-leukemic stem cells possessing leukemogenic activity in transplanted recipients.[14] Oncogene-induced replication stress and its role in cancer development have been studied comprehensively. Oncogene activation is an endogenous source of replication stress, disrupting replication regulation and inducing DNA damage.[55] Our current study shows that both could induce the expression of *Trem1*, which is required for maintaining the leukemogenic activity of the pre-leukemic HSC and LSC. Although further mechanistic investigation remains needed, it is in this context that our study unveils

for the first time an immune receptor linking leukemogenesis to multiple detrimental cellular stresses; that is, persistent DNA damage, prolonged oncogenic stress and an aberrant immune response.

Another notable finding of the present study is that the upregulated Trem1 expression in pre-leukemic HSC and LSC is associated with enhanced inflammation. Certain chronic inflammatory conditions have long been known to be linked to cancer.[32,33] Mounting evidence supports that chronic inflammation increases the risk of various human cancers.[57-61] In these pathological conditions, unresolved inflammation provokes cell turnover coupled with the generation of reactive oxygen species at the sites of inflammation, leading to chromosomal DNA mutations and malignant transformation.[62, 63] Our current studies established a link between persistent DNA damage, prolonged oncogenic stress and enhanced inflammation. While awaiting further mechanistic insights, our finding that upregulated Trem1 expression enhances inflammation in pre-leukemic HSC and LSC, highlights the crucial role of inflammation in leukemia development.

Disclosures

No conflicts of interest to disclose.

Contributions

XL performed the research and analyzed the data; SC, AW, LW, and NA performed some of the research and assisted with the data analysis. WD designed the research, analyzed the data, and wrote the paper.

Acknowledgments

We thank Dr. Madeleine Carreau (Laval University) for the Fanca$^{+/-}$ mice, and the Transgenic core facility at Cincinnati Children's Hospital Medical Center for generating the Trem1$^{fl/fl}$ mice. We thank Fabliha Chowdhury for technical support for some supplementary figures.

Funding

This work was supported by an NIH/National Heart, Lung, and Blood Institute grant (R01HL151390 to WD). This project used the Hillman Animal Facility that is supported in part by award P30CA047904. WD is a 2021-2022 Hillman Senior Fellow for Innovative Cancer Research at the University of Pittsburgh.

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