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

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**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 demonstrates that TREM1 cooperates with diminished DNA damage response to induce expansion of pre-leukemic hematopoietic stem cells (HSCs) in mice deficient for the Fanconi anemia gene *Fanca*. Here we investigate TREM1 in leukemogenesis using mouse models of the DNA repair-deficient *Fanca*<sup>-/-</sup> and the oncogenic *MLL-AF9* or *Kras*<sup>G12D</sup>. We found that *Trem1* was highly expressed in pre-leukemic HSCs and leukemia stem cells (LSCs). By selective deletion of the *Trem1* gene in the hematopoietic compartment, we showed that ablation of Trem1 reduced leukemogenic activity of the pre-leukemic HSCs and LSCs in mice. Trem1 was required for the proliferation of the pre-leukemic HSCs and LSCs. Further analysis revealed that *Trem1* expression in pre-leukemic HSCs and LSCs was associated with persistent DNA damage, prolonged oncogenic stress, and a strong inflammatory signature. Targeting several top Trem1 inflammatory signatures inhibits the proliferation of pre-leukemic HSCs and LSCs. 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.\textsuperscript{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.\textsuperscript{2} In fact, TREM1 is found overexpressed in variety of cancers,\textsuperscript{3} including colorectal cancer (CRC),\textsuperscript{4} hepatocellular carcinoma (HCC),\textsuperscript{5} Lung cancer,\textsuperscript{6} and prostate tumors.\textsuperscript{7} Recent studies show that TREM1 expression in patients with non-small cell lung cancer (NSCLC) is associated with cancer recurrence and poor survival, suggesting that TREM1 may play an important role in cancer progression.\textsuperscript{6} Furthermore, pharmacological inhibition of TREM1 attenuates tumor growth and prolongs survival in experimental pancreatic cancer\textsuperscript{8} and lung cancer.\textsuperscript{9} Analysis of 33 cancer transcriptome data from The Cancer Genome Atlas (TCGA) using UALCAN (http://ualcan.path.uab.edu/)\textsuperscript{10} show that TREM1 is upregulated in at least 14 types of cancers, especially kidney renal clear cell carcinoma (KIRC), cervical squamous cell carcinoma (CESC) and glioblastoma, and that TREM1 expression is closely correlated with poor prognosis in KIRC and CESC. Overexpression of TREM1 is also found in hematologic malignancies, and it has been shown that high level of TREM1 expression is correlated with poor prognosis in 5 subsets of AML cells (BloodSpot). However, the underlying mechanisms of TREM1 in cancer development is poorly understood.
DNA damage response/repair (DDR) is a complex signal transduction network that is required for preserving the integrity of 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. Dysregulation of DDR and repair systems can cause human disorders, which are associated with cancer susceptibility, accelerated aging, and developmental abnormalities. Given the enormous regenerative potential coupled with lifetime persistence of hematopoietic stem cells (HSCs) in the body, tight control of HSC genome stability is demanded. In fact, DDR has been considered as an evolutionary trade-off between blood regeneration and leukemia suppression. Indeed, failure to accurately repair DNA damage in HSCs is associated with bone marrow (BM) failure and leukemogenesis. Using a mouse model deficient for the major Fanconi anemia (FA) gene Fanca, our recent studies demonstrate a temporal correlation of diminished DDR with elevated immune response in Fanca−/− pre-leukemic HSCs and argue the effectiveness of DDR as the cellular machinery alone in preventing the transition of the initiating pre-leukemic HSC population into a leukemia stem cell (LSC) population with transformed properties.

It is known that oncogene-driven proliferation must be associated with inhibition of apoptosis and senescence to allow malignant outgrowth. 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. Mixed lineage leukemia
(MLL) is an H2Kme3-depositing protein active during early development.\textsuperscript{19} MLL rearranged (MLL\textsubscript{r}) leukemia are responsible for about 10\% of acute leukemia. Among them, the translocation t(9;11)(p22;q23) is mainly associated with acute myeloid leukemias (AML) and fuses AF9 to MLL (MLL-AF9).\textsuperscript{20} Oncogenic Ras mutations (mutations of \textit{NRAS} ad \textit{KRAS} genes) also occur in various leukemia,\textsuperscript{21} including AML,\textsuperscript{22} chronic myelomonocytic leukemia (CMML),\textsuperscript{23} and juvenile myelomonocytic leukemia (JMML) patients.\textsuperscript{24} However, the underlying mechanisms by which these oncogenes promote malignant transformation in leukemia remains controversial.

In this study, we investigate the role of TREM1 in leukemogenesis and show that persistent DNA damage and prolonged oncogenic stress induced the expression of \textit{Trem1} in pre-leukemic HSCs and LSCs. This aberrant \textit{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 Transgenic core facility at Cincinnati Children’s Hospital Medical Center (CCHMC) using the CRISPR-Cas9 technology. Mutant mice were produced on a C57BL/6J background. To generate Trem1\(^{flox/+}\) mice, gRNAs targeting exon 2 of 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 i.p. with 100 μl of tamoxifen (20 mg/ml; 80 mg/kg body weight; Sigma-Aldrich, St. Louis, MO) once every 24 hours for a total of 5 consecutive days.\(^{28}\)
The DNA damage-induced Fanca\(^{-}\) pre-leukemia model was established as previously described.\(^{14}\) Briefly, 6-8-week-old mice were intraperitoneal injected with 0.3 mg/kg of mitomycin C (MMC; Sigma-Aldrich, St Louis, MO) weekly for 6 weeks. All the animals including BoyJ (C57BL/6: B6, CD45.1\(^{+}\)) recipient mice were maintained in the animal facility at University of Pittsburgh Medical Center (UPMC) Hillman Cancer Center. All experimental procedures conducted in this study were approved by the Institutional Animal Care and Use Committee (IACUC) of University of Pittsburgh.

**BM Transplantation**

1,000 freshly isolated BM LSK cells from Fanca\(^{+}\);Trem1\(^{fl/fl}\)Vav1Cre mice and the Fanca\(^{-}\);Trem1\(^{fl/fl}\) control mice, 3000 BM c-Kit\(^{+}\) cells from MLL-AF9;Trem1\(^{fl/fl}\)Vav1Cre mice and the MLL-AF9;Trem1\(^{fl/fl}\) control mice, or 3000 GFP-sorted virus-transduced BM c-Kit\(^{+}\) cells from 2-month-old MLL-AF9 mice (CD45.2\(^{+}\)), along with 2\(\times\)10\(^{5}\) protector cells from congenic BoyJ mice (CD45.1\(^{+}\)), were transplanted into lethally irradiated (11.75 Gy) BoyJ mice. Recipients were subjected to TBI at the indicated time points. For serial BMT, 1-3 million WBMCs 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 posttransplant 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 2\(\times\)10\(^{5}\) 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 posttransplant. For secondary transplant, one million CD45.2\(^{+}\)
cells from the primary recipients at 4-month post BMT were transplanted into sublethally
irradiated BoyJ recipients. Donor-derived chimera and myeloid expansion was
determined by flow cytometry. Cytological and morphological analysis were examined
by Wright-Giemsa staining. Survival of the recipients was plotted by Kaplan-Meier curve
method.

**Statistical analysis**

Paired or unpaired student’s *t*-test was used for two-group comparison, and one-way
ANOVA for more than two-group comparison. Values of *p* less than 0.05 were
considered statistically significant. Results are presented as mean ± SD. * indicates
*p*<0.05; ** indicates *p*<0.01; *** indicates *p*<0.001.
Results

Trem1 is expressed in pre-leukemic HSCs and LSCs

We previously showed that the immune receptor TREM1 cooperates with diminished DNA damage response (DDR) to induce pre-leukemic HSC expansion using a mouse model deficient for the *Fanca* gene. To further understand the role of Trem1 in leukemogenesis, we employed two leukemic models (Fig. 1A) to investigate the underlying mechanisms *in vivo*: 1) the DNA damage-induced *Fanca*−/− pre-leukemic model; 2) the *MLL-AF9* (MA9) transgenic model, in which the expression of the oncogenic MLL-AF9 fusion protein results in development of acute myeloid leukemia (AML) beginning around 5 months of age. We first established pre-leukemic and leukemic *Fanca*−/− mice as previously described, and measured the levels of *Trem1* expression in the pre-leukemic HSCs and LSCs by qPCR. We found that *Trem1* mRNA level was significantly elevated in the SLAM (LSKCD48−CD150+ cells; enriched for HSCs; Fig. S1A) cells of the pre-leukemic *Fanca*−/− mice, as compared to those in wild-type (WT) mice (Fig. 1B). This was accompanied with 40-50% increase in Trem1+SLAM cells in the pre-leukemic mice over WT controls (Fig. 1C). *Trem1* expression and the percentage of Trem1+SLAM cells were even higher in the *Fanca*−/− leukemic mice than that in the *Fanca*−/− pre-leukemic mice (Fig. 1D, 1E). We also observed a progressive increase in *Trem1* expression in BM c-Kit+ cells (enriched for MA9 LSCs) of the MA9 mice over the period of 5 months (Fig. 1F). Leukemia was confirmed by accumulation of Mac1+Gr1+ cells in peripheral blood (PB) by flow cytometry (Fig. S1B) and Wright-Giemsa staining of PB (Fig. S1C) from the *Fanca*−/− and MA9 leukemic mice. Thus, these data indicate that Trem1 is expressed in pre-leukemic HSCs and LSCs.
Deletion of *Trem1* does not alter steady state hematopoiesis

To further investigate the role of Trem1 in leukemogenesis, we employed the CRISPR-Cas9 technology to generate a conditional *Trem1* knockout mouse strain (*Trem1*^{fl/fl}; Fig. 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 (Fig. S2B-2D).

To determine whether ablation of Trem1 affected normal hematopoiesis, we first analyzed blood counts, BM cellularity, the frequency, and absolute numbers of total HSPCs (LSK: Lin^−^Sca1^+^c-Kit^+^ cells; hematopoietic stem progenitor cells) and HSCs (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 (CBC; Fig. 2A), total BM cell counts (Fig. 2B) and flow cytometry-based phenotypic analysis of BM LSK and SLAM populations (Fig. 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 HSPCs from *Trem1*^{fl/fl}/*Vav1Cre* and *Trem1*^{fl/fl} control mice produced comparable CFU-C colonies (Fig. 2D). Competitive *in vivo* transplantation assays showed no significant differences in repopulating capacity between *Trem1*^{fl/fl}/*Vav1Cre* and the *Trem1*^{fl/fl} control HSCs (Fig. 2E). Contribution to myeloid and lymphoid lineages was also similar between *Trem1*^{fl/fl}/*Vav1Cre* and *Trem1*^{fl/fl} HSCs (Fig. 2F). However, we observed significantly higher repopulating capacity of *Trem1*^{fl/fl}/*Vav1Cre* HSCs in
secondary recipients than that of Trem1fl/fl HSCs (Fig. 2G). These data suggest that Trem1 deletion does not influence hematopoiesis under homeostatic conditions.

**Trem1 expression in pre-leukemic HSCs and LSCs promotes leukemogenesis**

To determine the role of Trem1 in leukemia development *in vivo*, we established three leukemogenic models (Fig. 3A): 1) Fanca−/− leukemia expressing the conditional Trem1fl/fl; 2) MA9 leukemia expressing the conditional Trem1fl/fl; and 3) 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 significantly reduced LSC-enriched donor SLAM cells in the recipient mice transplanted with LSK cells from Fanca−/−;Trem1fl/flVav1Cre mice compared to those from the Fanca−/−;Trem1fl/fl controls (Fig. 3B). Consistently, ablation of Trem1 markedly prolonged the survival of the recipients transplanted with Fanca−/−;Trem1fl/flVav1cre cells compared to those with the Fanca−/−;Trem1fl/fl control cells (Fig. 3C). Similar results were obtained with the recipients transplanted with donor cells from leukemic MA9 mice at age of 5 months old (when the mice develop AML), 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;Trem1fl/flVav1cre mice compared to the recipients transplanted with cells from age-matched MA9;Trem1fl/fl mice (Fig. 3D). Consequently, ablation of Trem1 greatly extended latency in the recipients of MA9;Trem1fl/flVav1cre cells compared to those of MA9;Trem1fl/fl cells (Fig. 3E).

To compare the ability of Trem1+ vs Trem1− pre-leukemic HSCs to induce leukemia, we purified Trem1+SLAM and Trem1−SLAM cells from Fanca−/− pre-leukemic mice (Fig 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⁺ HSCs exhibited donor BM hypercellularity (Fig. S3B) and increased accumulation of donor-derived phenotypic HSCs in the BM (Fig. S3C). Furthermore, 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 post BMT (Fig. S3D). Leukemia was confirmed by accumulation of Mac1⁺Gr1⁺CD45.2⁺ cells in PB (Fig. S3E) and Wright-Giemsa staining of PB (Fig. S3F). These data indicate that Trem1⁺ pre-leukemic HSCs have higher leukemogenic potential than Trem1⁻ pre-leukemic HSCs.

To substantiate the observation that Trem1 promotes leukemia, we transduced c-kit⁺ cells from MA9 mice at age of 2 months old, at which time Trem1 expression is not detectable (see Fig. 1F above), with a lentiviral vector expressing eGFP or eGFP-Trem1 (Fig. 3A). We achieved >5-fold higher Trem1 expression in GFP-Trem1 cells than eGFP control cells (Fig. S4A, S4B). Remarkably, overexpression of Trem1 greatly increased LSC-enriched CD45.2⁺c-Kit⁺ cells in the recipients transplanted with eGFP-Trem1-transduced MA9 cells compared to the recipients transplanted with eGFP control cells (Fig. 3F). Consistently, overexpression of Trem1 markedly shortened latency in the recipients of GFP-Trem1-transduced MA9 cells compared to the recipients of eGFP control cells (Fig. 3G). Together, these data suggest that Trem1 expression in pre-leukemic HSCs and LSCs promotes leukemogenesis.

*Trem1 expression in pre-leukemic HSCs and LSCs is associated with increased proliferation but has no effect on apoptosis sensitivity*
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 of Fanca<sup>−/−</sup>;Trem1<sup>fl/fl</sup>Vav1Cre or MA9;Trem1<sup>fl/fl</sup>Vav1Cre mice, 4 weeks after transplantation. We found that deletion of Trem1 significantly reduced myeloid colony formation by CD45.2<sup>+</sup>LSK cells from the leukemic mice transplanted with the Fanca<sup>−/−</sup>;Trem1<sup>fl/fl</sup>Vav1Cre cells compared to those of Fanca<sup>−/−</sup>;Trem1<sup>fl/fl</sup> control cells, as determined by CFU assay (Fig. 4A). Furthermore, there was a lower percentage of proliferating (Ki67-positive) CD45.2<sup>+</sup>LSK cells from the leukemic mice transplanted with the Fanca<sup>−/−</sup>;Trem1<sup>fl/fl</sup>Vav1Cre cells compared to those of Fanca<sup>−/−</sup>;Trem1<sup>fl/fl</sup> control cells, as determined by nuclear Ki67 staining (Fig. 4B). We obtained similar results with the experiments with the MA9;Trem1<sup>fl/fl</sup>Vav1Cre and MA9;Trem1<sup>fl/fl</sup> leukemic cells, in which ablation of Trem1 significantly inhibited the proliferation of the LSC-enriched CD45.2<sup>+</sup>c-kit<sup>+</sup> cells (Figs. 4C and 4D). However, deletion of Trem1 did not significantly increase apoptosis in the leukemia from Fanca<sup>−/−</sup>;Trem1<sup>fl/fl</sup>Vav1Cre pre-leukemic cells or MA9;Trem1<sup>fl/fl</sup>Vav1Cre leukemic cells, as compared to those from Fanca<sup>−/−</sup>;Trem1<sup>fl/fl</sup> or MA9;Trem1<sup>fl/fl</sup> control cells, respectively (Fig. 4E, 4F). Furthermore, ablation of Trem1 did not render either Fanca<sup>−/−</sup> or MA9 leukemic cells more sensitive to growth factor-deprived culture conditions, as detected by MTS assay (Fig. 4G). Taken together, these results indicate that Trem1 promotes leukemic cell proliferation but has limited effect on apoptosis sensitivity.

**Trem1 expression in pre-leukemic HSCs and LSCs is associated with persistent DNA damage and prolonged oncogenic stress**
Since our previous studies demonstrate that Trem1 cooperates with diminished DNA damage response in pre-leukemic HSC expansion, we asked whether Trem1 expression in Fanca−/− pre-leukemic HSCs 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 2h and performed flow cytometry analysis for γ-H2AX, an established marker of double strand breaks (DSBs), at different times after treatment. We found that MMC induced robust expression of γ-H2AX at 4 hours post treatment in both Fanca−/− and WT cells; however, WT cells efficiently repaired DSBs, as evidenced by a progressive decline of γ-H2AX within 16 hours after MMC treatment (Fig. 5A). In contrast, the MMC-treated Fanca−/− cells retained high levels of γ-H2AX throughout the 16-hour period (Fig. 5A), indicative of persistent DNA damage. Correlated with this DNA damage kinetics, Trem1 expression were persistently elevated during the 8-16h period of observation in Fanca−/− cells, whereas Trem1 expression remained undetectable in WT cells (Fig. 5B).

Next, we asked whether prolonged oncogenic stress could also lead to aberrant expression of Trem1 in LSCs. We utilized two leukemia models, MLL-AF9 and KrasG12D, to induce prolonged oncogenic stress in vitro. For the MLL-AF9 model, we infected WT LSK cells with retroviral vector expressing eGFP-MLL-AF9 or eGFP alone, and subjected the transduced cells to culture in growth factors-supplemented medium for different periods of time. We observed persistent elevation of Trem1 expression during 4-12 days of culture in cells expressing MLL-AF9, whereas Trem1 expression remained undetectable in those expressing eGFP alone (Fig. 5C). For the KrasG12D model, we isolated LSK cells from KrasG12D CreER mice and cultured the cells in growth factors-
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 persistent increase in Trem1 expression during 3-9 days of culture, as compared to negligible Trem1 expression in the cells cultured in the absence of 4-OHT (Fig. 5D). These results indicate that prolonged oncogenic stress induces aberrant expression of Trem1 in LSC-enriched leukemic cells.

**Trem1 expression in pre-leukemic HSCs and LSCs 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 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 (Fig. 6A); and in 5-month-old MA9;Trem1\(^{fl/fl}\) mice compared with those of age-matched MA9;Trem1\(^{fl/fl}\)Vav1Cre mice (Fig. 6A).

Among the top 5 upregulated inflammatory genes, we noticed that Ccr1, Il1r1, Nlrp3 and Tlr2 were upregulated in both Fanca\(^{-/-}\) and MA9 cells (Figs. S5A, S5B). CCR1 and its ligand CCL3 has recently 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 are found in MDS patients.\(^{36}\) Recent
studies have shown that overall survival of AML patients with higher TLR2 expression was significantly shorter than that of patients with lower expression. To investigate the functional relevance of these TREM1-inflammation signatures to the expansion of Fanca−/− and MA9 leukemic cells, we performed blockade experiments using the CCR1 antagonist, BX471, the IL-1R antagonist, AF12198, the covalent inhibitor for NLRP3, or the neutralizing antibody against TLR2. LSK cells of pre-leukemic Fanca−/− mice and 5-month-old MA9 mice were cultured in growth factors-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 (Fig. 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 (Fig. 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 neutralization antibodies compared to the untreated control cells (Fig. 6D). These data suggest that targeting Trem1 inflammation signatures could suppress the expansion of Fanca−/− and MA9 LSCs.
Discussion

The immune system, consisted of immune cells, immune factors, and immune microenvironment, plays essential roles in tumorigenesis. The multifaceted effects of tumor-related immunity include destroying genome stability, generating genetic modification, promoting the proliferation of cancer cells, stimulating angiogenesis, and shaping tumor microenvironment. In this study, we show 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 demonstrate that Trem1 is highly expressed in pre-leukemic HSCs and LSCs. Moreover, we have 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 HSCs and LSCs. Our study thus sheds new insights into the role of TREM1 in leukemogenesis.

TREM-1 is found constitutively expressed on a selective group of myeloid cells including macrophages, monocytes and neutrophils in peripheral blood. It has also been identified on airway epithelial cells, hepatic endothelial cells and liver resident macrophages, NK cells, dendritic cells (DCs), B and T cells. TREM1 belongs to the immunoglobulin superfamily and is engaged in amplifying inflammatory cascades. It can be induced at high levels on neutrophils and monocytes and further amplifies Toll-like receptor (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. Although high-mobility group box 1
(HMGB1) and peptidoglycan recognition protein 1 (PGLYRP1) were identified as ligand of the pathways, the ligand for TREM1 remains to be characterized. A growing body of evidence suggest that TREM1 plays critical pathological role in chronic inflammatory disorders including cancer. In fact, novel TREM1 inhibitors are shown to attenuate tumor growth and prolong survival in experimental cancer models. By employing three leukemogenic mouse models: DNA damage-induced Fanca-/- pre-leukemic model; oncogenic MLL-AF9 transgenic model and KrasG12D CreER model, we show that Trem1 was highly expressed in pre-leukemic HSCs and LSCs, and dysregulated expression of Trem1 in these malignant stem cells enhanced their leukemogenic potential. Conversely, deletion of Trem1 in these pre-leukemic HSCs and LSCs compromised proliferation and delayed leukemia development in vivo. It is in this context that our study provides new insights to the current understanding of 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. The Fanconi anemia (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. Our previous studies showed that chronic DNA damage stress could transform Fanca-/- HSCs into pre-leukemic stem cells possessing leukemogenic activity in transplanted recipients. 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.\textsuperscript{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 HSCs and LSCs. 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 aberrant immune response.

Another notable finding of the present study is that the upregulated Trem1 expression in pre-leukemic HSCs and LSCs is associated with enhanced inflammation. Certain chronic inflammatory conditions have long been known to link to cancer.\textsuperscript{32, 33} Mounting evidence supports that chronic inflammation increases the risk of various human cancers.\textsuperscript{57-61} In these pathological conditions, unresolved inflammation provokes cell turnover coupled with the generation of reactive oxygen species (ROS) at the sites of inflammation, leading to chromosomal DNA mutations and malignant transformation.\textsuperscript{62, 63} Our current studies established a link between persistent DNA damage, prolonged oncogenic stress and enhanced inflammation. Although further mechanistic investigation remains needed, our finding that upregulated Trem1 expression enhances inflammation in pre-leukemic HSCs and LSCs, highlights the crucial role of inflammation in leukemia development.
References:


**Figure legend**

**Fig 1. Trem1 is expressed in pre-leukemic HSCs and LSCs.** A. Schematic presentation of experimental design. B. Aberrant Trem1 expression in Fanca<sup>−/−</sup> pre-leukemic HSCs. WT and Fanca<sup>−/−</sup> mice were intraperitoneal injected 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 qPCR analysis using primers listed in Table S1 (n= 8-10/group). C. Increased Trem1-expressing HSCs in Fanca<sup>−/−</sup> pre-leukemic mice. Left: representative flow cytometry analysis of percentages of BM Trem1<sup>+</sup>SLAM cells in the mice described in (B). Right: quantification (n= 8-10/group). D. Increased Trem1 expression in LSCs of Fanca<sup>−/−</sup> leukemic mice. FACS-isolated LSK cells from the mice described in (B) were subjected to two rounds of BMT to establish Fanca<sup>−/−</sup> 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/group). E. Quantification of flow cytometry analysis of percentages of BM Trem1<sup>+</sup> SLAM cells the secondary recipients (n= 12/group). F. Progressive increase in Trem1 expression in MLL-AF9 (MA9) LSCs. BM c-Kit<sup>+</sup> cells from 1- 5-month-old MA9 mice were subjected to qPCR for Trem1 expression. WT mice were used as control (n= 6/group).

**Fig 2. Deletion of Trem1 does not alter steady state hematopoiesis.** A. Complete blood count (CBC) of 8-week-old Trem1<sup>fl/fl</sup>Vav1Cre mice and the Trem1<sup>fl/fl</sup> controls (n= 7-8). B. BM cell counts (n= 7-8). C. BM LSK and SLAM cell counts (n= 7-8), and D. CFC counts are shown for the Trem1<sup>fl/fl</sup>Vav1Cre mice and the Trem1<sup>fl/fl</sup> controls (n= 6). E. Deletion of Trem1 does not affect hematopoietic repopulation in primary transplant
recipient. 100 SLAM cells from \( Trem1^{fl/fl} \) \( Vav1\text{Cre} \) mice and the \( Trem1^{fl/fl} \) controls were transplanted, along with \( 2 \times 10^5 \) competitor cells from congenic mice, into lethally irradiated BoyJ recipients. Donor derived chimera were determined by flow cytometry at different time points post BMT (n= 10). F. Deletion of \( Trem1 \) does not affect multi-lineage reconstitution in primary transplant recipient. Percentage of donor-derived Myeloid, T, B cells were measured by flow cytometry 16 weeks post BMT (n= 10). G. Deletion of \( Trem1 \) increases long-term hematopoietic repopulation. 1-3 million of WBMCs from the primary recipients were pooled and injected into sublethally irradiated BoyJ recipients. Donor derived chimera were determined by flow cytometry at different time points post BMT (n= 12).

**Fig 3.** \( Trem1 \) expression in pre-leukemic HSCs and LSCs promotes leukemogenesis. A. Schematic presentation of experimental design. B. Deletion of \( Trem1 \) suppresses \( Fanca^{-/-} \) LSC expansion in transplant recipient mice. LSK cells from \( Fanca^{-/-};Trem1^{fl/fl} \) \( Vav1\text{Cre} \) mice and the \( Fanca^{-/-};Trem1^{fl/fl} \) controls subjected to 6-week MMC treatments, were transplanted , along with \( 2 \times 10^5 \) competitor cells from congenic mice, into lethally irradiated BoyJ recipients. Donor-derived CD45.2⁺SLAM cells were analyzed by flow cytometry 4 weeks post BMT (n= 10). C. Deletion of \( Trem1 \) extends the latency of \( Fanca^{-/-} \) leukemia. Survival of the recipient mice groups described in (B) was monitored and plotted by Kaplan-Meier method (n = 9-10 per group). D. Ablation of Trem1 suppresses \( MLL-AF9 \) LSC expansion in transplant recipients. BM c-Kit⁺ cells from \( MLL-AF9;Trem1^{fl/fl} \) \( Vav1\text{Cre} \) mice and the \( MLL-AF9;Trem1^{fl/fl} \) controls were transplanted , along with \( 2 \times 10^5 \) competitor cells from congenic mice, into lethally irradiated BoyJ recipients. Donor-derived CD45.2⁺c-Kit⁺ cells were analyzed by flow
cytometry 4 weeks post BMT (n= 10). 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 per group). F. Forced expression of Trem1 increases MLL-AF9 LSC expansion in transplant recipients. BM 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 2×10^5 competitor cells from congenic mice, into lethally irradiated BoyJ recipients. Donor-derived GFP+ c-Kit+ cells were analyzed by flow cytometry 4 weeks post BMT (n= 10). 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 Kaplan-Meier method (n = 10-13). B & C: Fanca−/− leukemia model; D & E: MA9 model; F & G: MA9+Trem1 model.

**Fig 4.** Trem1 expression in pre-leukemic HSCs and LSCs is associated with increased proliferation. A. Deletion of Trem1 reduces myeloid colony formation by Fanca−/− LSC-enriched donor LSK cells. LSK cells from Fanca−/−;Trem1fl/flVav1Cre mice and the Fanca−/−;Trem1fl/fl controls subjected to 6-week MMC treatments, were transplanted, along with 2×10^5 competitor cells from congenic mice, into lethally irradiated BoyJ recipients. Donor-derived CD45.2+LSK cells were subjected to CFU assays 4 weeks post BMT (n= 6). 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). C. Ablation of Trem1 reduces myeloid colony formation by MLL-AF9 LSC-enriched donor c-Kit+ cells. BM c-Kit+ cells from MLL-
AF9;Trem1\textsuperscript{fl/fl}Vav1Cre mice and the MLL-AF9;Trem1\textsuperscript{fl/fl} controls were transplanted, along with 2× 10\textsuperscript{5} competitor cells from congeneric mice, into lethally irradiated BoyJ recipients. Donor-derived CD45.2\textsuperscript{+}c-Kit\textsuperscript{+} cells were subjected to CFU assays 4 weeks post BMT (n= 6). D. Ablation of Trem1 reduces Ki67\textsuperscript{+} proliferating MLL-AF9 LSC-enriched donor c-Kit\textsuperscript{+} cells. The CD45.2\textsuperscript{+}c-Kit\textsuperscript{+} cells from the recipient mice in (C) were gated for Ki67-positive nuclear stain (n= 8-9). E. Deletion of Trem1 does not increase apoptosis in Fanca\textsuperscript{-/-} LSC-enriched donor LSK cells. The CD45.2\textsuperscript{+}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). F. Deletion of Trem1 does not increase apoptosis in MLL-AF9 LSC-enriched donor c-Kit\textsuperscript{+} cells. The CD45.2\textsuperscript{+}c-Kit\textsuperscript{+} cells from the recipient mice in (C) were gated for Annexin V-positive staining (n= 7-8). G. Ablation of Trem1 does not render Fanca\textsuperscript{-/-} LSC-enriched donor LSK cells or MLL-AF9 LSC-enriched donor c-Kit\textsuperscript{+} cells more sensitive to growth factor-deprived conditions. The CD45.2\textsuperscript{+}LSK cells from the recipient mice in (A) or the CD45.2\textsuperscript{+}c-Kit\textsuperscript{+} cells from the recipient mice in (C) were cultured in serum-free StemCell medium supplemented with SCF, Flt3 ligand and TPO for 72h followed by factor withdrawal. Cell viability was determined by absorbance using CellTiter 96 Aqueous One Solution Cell Proliferation (MTS) assay after factor withdrawal for 24h (n = 6-8).

**Fig 5. Trem1 expression in pre-leukemic HSCs and LSCs is associated with persistent DNA damage and prolonged oncogenic stress.** A. Persistent DNA damage in Fanca\textsuperscript{-/-} pre-leukemic HSCs. LSK cells from WT and pre-leukemic Fanca\textsuperscript{-/-} mice were treated with MMC (dose) in culture for 2h and performed flow cytometry analysis for \(\gamma\)-H2AX at different time points post-treatment. Representative flow plots
(Left) and MFI kinetics (Right) are shown. 0 h, untreated control (n= 6). B. *Trem1* expression is specifically induced by persistent DNA damage in *Fanca<sup>−/−</sup>* pre-leukemic HSCs. RNAs were then extracted from the cells described in (A) followed by qPCR analysis for *Trem1* expression using primers listed in Table S1. Samples were normalized to the level of *GAPDH* mRNA (n= 6 per group). ** MMC vs untreated control (0 h). C. Prolonged oncogenic stress induces *Trem1* expression in *MLL-AF9* 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 factors-supplemented medium. RNAs were then extracted from the sorted GFP<sup>+</sup> LSK cells at different timepoints 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<sup>G12D</sup>* LSC-enriched cells. LSK cells from *Kras<sup>G12D</sup>CreER* and *Kras<sup>G12D</sup>* control mice were cultured in growth factors-supplemented medium in the presence of 4-OHT. RNAs were then extracted from the sorted LSK cells at different timepoints followed by qPCR analysis for *Trem1* expression. Samples were normalized to the level of *GAPDH* mRNA (n= 6).

**Fig 6. Trem1 expression in pre-leukemic HSCs and LSCs is associated with enhanced inflammation.** A. Inflammatory gene expression (by NanoString analysis) in LSK cells of pre-leukemic *Fanca<sup>−/−</sup>;Trem1<sup>fl/fl</sup>* (*Trem1-WT*) mice compared with those of *Fanca<sup>−/−</sup>;Trem1<sup>fl/fl</sup>Vav1Cre* (*Trem1-KO*) mice (n= 3 assays; Left) and LSK cells of *MA9;Trem1<sup>fl/fl</sup>* (*Trem1-WT*) mice compared with those of *MA9;Trem1<sup>fl/fl</sup>Vav1Cre* (*Trem1-KO*) mice (n=3 assays; Right). Heatmap of inflammatory genes (log2 fold change; FC) differentially expressed among *Fanca<sup>−/−</sup>;Trem1<sup>fl/fl</sup> and *Fanca<sup>−/−</sup>;Trem1<sup>fl/fl</sup>Vav1Cre* cells or
MA9;Trem1\textsuperscript{fl/fl} and MA9;Trem1\textsuperscript{fl/fl}Vav1\textit{Cre} cells. B. Blockade of CCR1, IL1R1, NLPR3, or TLR2 reduces colony formation of Fanca\textsuperscript{−/−} and MA9 leukemic cells. LSK cells from Fanca\textsuperscript{−/−} 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 followed by CFU-C assay (n= 6 assays). C. Blockade of CCR1, IL1R1, NLPR3, or TLR2 suppresses the expansion of Fanca\textsuperscript{−/−} and MA9 LSCs. The cultured cells described in (B) were transplanted the cultured cells, along with 2X10\textsuperscript{6} protector cells from congenic mice, into lethally irradiated recipients. Donor-derived CD45.2\textsuperscript{+}LSK cells from the recipient mice were analyzed 4 months post-BMT by flow cytometry (n= 10). D. Blockade of CCR1, IL1R1, NLPR3, or TLR2 inhibits the proliferation of Fanca\textsuperscript{−/−} and MA9 LSCs. The recipient mice described in (C) were analyzed for Ki67\textsuperscript{+} CD45.2\textsuperscript{+}LSK cells 4 months post-BMT by flow cytometry (n= 10).
Fig 2

A  
Hemoglobin (g/dl)  
- Trem1^{fl/fl}  
- Trem1^{fl/fl} Vav1Cre  

B  
% Neutrophils  
- Trem1^{fl/fl}  
- Trem1^{fl/fl} Vav1Cre  

% Lymphocytes  
- Trem1^{fl/fl}  
- Trem1^{fl/fl} Vav1Cre  

WBMCs (X10^7)  
- Trem1^{fl/fl}  
- Trem1^{fl/fl} Vav1Cre  

C  
LSKs (X10^3)  
- Trem1^{fl/fl}  
- Trem1^{fl/fl} Vav1Cre  

SLAM (X10^3)  
- Trem1^{fl/fl}  
- Trem1^{fl/fl} Vav1Cre  

D  
CFCs/200 LSKs  
- Trem1^{fl/fl}  
- Trem1^{fl/fl} Vav1Cre  

plating  
1  
2  

E  

F  
% cells  
- Mac1^+  
- Gr1^+  
- B220^+  
- CD3e^+  

G  

*  
**  

*  
**
Supplementary materials

Wright-Giemsa staining

For cytological and morphological analysis, cytospin preparations or blood smear were stained with Wright-Giemsa staining solution (Fisher Scientific, Hampton, NH) and visualized by microscopy.

Flow cytometry analysis

Femurs and tibias were flushed to dissociate the bone marrow (BM) fraction. Cells were resuspended in 5mL PBS/0.5% BSA and filtered through a 70-μm filter (BD Biosciences, Cat #: 3523350, San Jose, CA). The mononuclear cells were isolated by Ficoll-Paque (GE Healthcare, Cat #: 95040-394, Pittsburgh, PA) gradient centrifugation. For LSK (Lineage-Sca-1+c-Kit+) staining, cells were labeled by the biotin conjugated anti-lineage antibody cocktail (BioLegend, Cat #: 133307, San Diego, CA) followed by staining with a secondary PerCP-Cy5.5-anti Streptavidin antibody (BioLegend, Cat #: 405214, San Diego, CA), PE-Cy7-anti-Sca1 antibody (BD Biosciences, Cat #: 558162, San Jose, CA), and APC-Cy7-anti-c-Kit antibody (BD Biosciences, Cat #: 553356, San Jose, CA). To access long-term HSC subpopulation, cells were stained with LSK antibodies in addition to CD45.2-eFluore 450 (eBioscience, Cat #: 48-0454-82, Waltham, MA). Flow cytometry was performed on LSRFortessa (BD Biosciences, San Jose, CA) and analysis was done with FCS Express 6 software (De Novo Software, Los Angeles, CA) and FlowJo software (FlowJo, Ashland, OR). For Trem1 staining, cells were labeled with anti-Trem1 antibody (Miltenyi Biotech, Auburn, CA).
For donor derived chimera analysis, peripheral blood (PB) from the recipient mice were subjected to staining using PE-anti-CD45.1, APC-anti-CD45.2 (Both from BD Biosciences, Cat #: 553776 and 558702, San Jose, CA) antibodies followed by Flow cytometry analysis.

For cell cycle analysis, surface marker-stained cells were fixed and permeabilized using Cytofix/Cytoperm buffer (BD PharMingen, Cat #: 554722, San Jose, CA) followed by intensive wash using Perm/Wash Buffer (BD PharMingen, Cat #: 554723, San Jose, CA). Cells were then labeled with anti-mouse Ki67 antibody (BD PharMingen, San Jose, CA) and DAPI (Sigma-Aldrich, St Louis, MO) at room temperature for 30 minutes followed by Flow cytometry analysis on CD45.2+ SLAM (Lin-Sca1+c-kit+CD150+CD48-) gated population.

For intracellular staining, surface marker-stained cells were fixed and permeabilized using Cytofix/Cytoperm buffer (BD PharMingen, Cat #: 554722, San Jose, CA) followed by intensive wash using Perm/Wash Buffer (BD PharMingen, Cat #: 554723, San Jose, CA). Cells were then incubated with antibodies against γ-H2AX (biotin-conjugated, Millipore, Cat #: 16-193, Billerica, MA) at 4°C for 30 minutes. After washing, cells were then incubated with secondary antibody and analyzed by Flow Cytometry analysis.

For the cell sorting, lineage negative cells were enriched using lineage depletion reagents (Miltenyi Biotec, Cat #: 130-090-858, Auburn, CA) according to the manufacturer’s instruction. The LSK (Lin-Sca-1+c-Kit+) or c-Kit+ cell fractions were acquired by using the FACSaria II sorter (BD Biosciences, San Jose, CA).
CFC assays, HSC culture
CFC assays (colony-forming unit-granulocyte monocyte (CFU-GM), burst-forming unit-erythroid (BFU-E), and colony-forming unit-mix (CFU-GEMM) were performed as previously described.\(^1\) For \textit{in vitro} cultures, BM CD34\(^+\) KSL, KSL, and lineage-negative cells were cultured in TSF medium (IMDM, 10 % FBS, 1% penicillin-streptomycin, 20 ng/ml thrombopoietin (TPO), 125 ng/ml SCF, 50 ng/ml, Flt3 ligand). SCF, Flt3 ligand and TPO were purchased from R&D systems.

For inflammation neutralization, pre-leukemic LSK cells or c-Kit\(^+\) cells were cultured in the presence or absence of CCR1 antagonist BX471 (10\(^{-7}\) M; Sigma-Aldrich, St. Louis, MO);\(^2\) IL1R antagonist AF12198 (1 mM; R&D Systems, Minneapolis, MN),\(^3,4\) or anti-mouse TLR2 antibody (25 mg/ml; R&D Systems, Minneapolis, MN)\(^5\) for 5 days followed by CFU or BMT assays. Oridonin (Sigma-Aldrich, St. Louis, MO) was used to block NLRP3 (0.5 \(\mu\)M).\(^6\)

qPCR analysis
Total RNA was extracted using RNeasy Mini Kit (QIAGEN) following the manufacturer’s procedure. Reverse transcription was carried out at 42\(^\circ\)C for 60 minutes and stopped at 95\(^\circ\)C for 5 minutes using random hexamers and Superscript II RT (Invitrogen, Thermo Fisher Scientific, Cat #: 18064014). Primers was used for quantitative PCR analysis using primers listed in Supplemental Table 1. Samples were normalized to the level of \textit{GAPDH} mRNA.

MTS (3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H-tetrazolium) assay
Proliferation of pre-leukemic cells from the indicated mice was measured using the Cell-Titer 96 AQueous One Solution Cell Proliferation Assay (MTS; Promega) according to the manufacturer’s instructions. In this assay, the MTS tetrazolium compound (Owen’s reagent) was bioreduced by metabolically active cells into a colored formazan product. The quantity of formazan product was measured in a 96-well plate by absorbance at 490 nm.

**Molecular cloning and materials**

To generate lentiviral expression vectors for *Trem1*, the *Trem1* cDNA (purchased from Origene, Rockville, MD; Cat #: MR2219079) was cloned into the pLVX-IRES-GFP vector (Cat #: 128652; Addgene, Watertown, MA) as previously described.7

Lentivirus was produced in 293 T cells after transfection of 20 μg plasmid, 15 μg pCMVΔ8.91 helper plasmid and 6 μg pMD.G using standard calcium phosphate transfection procedures. Fresh medium change was performed 12 hours after transfection. Supernatants from the cell culture were collected 48 hours after transfection, filtered through 0.45 μm-pore-size filters, and concentrated at 25,000 rpm for 2.5 hours at 4°C to harvest viral particles. Virus pellet was resuspended in sterile PBS and stored at -80°C.

**Lentiviral Transduction**

Lentiviral transduction was performed as previously described.8 Briefly, sorted BM c-Kit⁺ cells from 2-month-old *MLL-AF9* mice were pre-stimulated for 5-10 hr in a 24-well dish in serum-free medium. The lentiviral media were added to the cells, spinoculated for 90 min
at 270G in the presence of 8 μg/ml polybrene (Cat #: TR-1003; Sigma-Aldrich, St Louis, MO). This process was repeated 24 hours later with a fresh batch of lentiviral media.

**NanoString analysis**

RNA was extracted and purified from the indicated cells using the miRNeasy Mini Kit according to the manufacturer’s instructions (QGN-217004, Qiagen, Germantown, MD). Total RNA was diluted to 20 ng/μl and probed using an nCounter© Mouse Inflammation Panel (NanoString Technologies, Seattle, WA) profiling 254 genes involved in mouse inflammation pathways. Gene expression was analyzed using NanoString nCounter platform (NanoString Technologies, Seattle, WA, USA) according to manufacturer’s instructions.

Pairwise differential expression and pathway analyses were performed using NanoStringDiff (v3.6.0)$^9$ with raw cell counts including negative and positive controls and housekeeping genes from NanoString nCounter. The analysis was comprised on 4 groups: Fanca<sup>−/−</sup>;Trem1<sup>fl/fl</sup>, Fanca<sup>−/−</sup>;Trem1<sup>fl/fl</sup>Vav1Cre, MA9;Trem1<sup>fl/fl</sup>, MA9;Trem1<sup>fl/fl</sup>Vav1Cre. Comparison was made: Fanca<sup>−/−</sup>;Trem1<sup>fl/fl</sup> vs Fanca<sup>−/−</sup>;Trem1<sup>fl/fl</sup>Vav1Cre, MA9;Trem1<sup>fl/fl</sup> vs MA9;Trem1<sup>fl/fl</sup>Vav1Cre. Differentially expressed genes (DEGs) were defined as those having a $p$ value < 0.05 compared to the controls. Student’s $t$-test was used for comparing continuous variables and Fisher’s exact test for comparing categorical variables. $p$ values were adjusted for multiple testing using the Benjamini-Hochberg method. $p$ values below 0.05 were considered significant.
Supplementary references


Supplementary table

Table S 1. Primers used for qPCR analysis

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Supplementary figures

**Fig S1.** A. Gating strategy for flow cytometry analysis. Flow plots show gating strategies for Lin⁻, LSK (Lin⁻Sca1⁺c-kit⁺), SLAM (LSKCD150⁺CD48⁻) cells. B. Accumulation of Mac1⁺Gr1⁺ cells in peripheral blood (PB) of Fanca⁻/⁻ and MLL-AF9 leukemic mice.
Representative flow cytometry (left) and quantification (right) are shown (n= 6-8). C. Representative Wright-Giemsa staining of PB from Fanca−/− and MLL-AF9 leukemic mice.

**Fig S2.** Generation of conditional Trem1 knockout mice. A. Schematic diagram of Trem1floxflox mice knock-out strategy. Trem1 locus following homologous recombination and the final Trem1 locus following excision of the loxP-flanked gene were illustrated. B. Genotyping of Trem1floxflox Vav1Cre mice by PCR. C. qPCR of Trem1 expression in BM cells from Trem1fl/fl Vav1Cre or Trem1fl/fl mice (n= 8). D. Flow cytometry analysis of Trem1 (CD354) protein in BM granulocytes and monocytes.

**Fig S3.** Trem1+ pre-leukemic HSCs induce leukemia in secondary recipients with a shorter latency. (A) Sorting strategy for separating the Trem1+SLAM and Trem1−SLAM
subsets. (B, C) BM cellularity (B) and phenotypic HSCs (C) in the BM of primary recipients were determined by flow cytometry 16 weeks post BMT. (D) Trem1+ pre-leukemic HSCs induce leukemia in secondary recipients. Survival of the recipients was plotted by Kaplan-Meier curve method. (E) Donor myeloid marker expression in PB of moribund mice at the endpoint of survival experiments. (F) Representative Wright-Giemsa staining of PB of moribund mice. *p<0.05, **p<0.01.

**Fig S4.** A. Flow cytometry analysis of BM c-Kit+ cells from MA9 mice transduced with lentiviral vector expressing GFP or GFP-Trem1. Flow plots of pre-sorting and post-sorting are shown. B qPCR of Trem1 expression in sorted transduced cells (n= 8 assays).

**Fig S5.** A. Top 5 differentially expressed genes in LSK cells of pre-leukemic Fanca−/−;Trem1−/− mice compared to those from LSK cells from the control Fanca−/−.
;Trem1\textsuperscript{fl/fl}Vav1Cre mice (Left), or 5-month-old MA9;Trem1\textsuperscript{fl/fl} mice compared to those of age-matched MA9;Trem1\textsuperscript{fl/fl}Vav1Cre mice (Right) by NanoString nCounter Mouse Inflammation assay. Log2FC and p value of the genes are shown. B. qPCR analysis of Ccr1, Il1r1, Nlrp3, and Tlr2 expression in LSK cells of Fanca\textsuperscript{−/−};Trem1\textsuperscript{fl/fl}Vav1Cre mice compared with those of Fanca\textsuperscript{−/−};Trem1\textsuperscript{fl/fl} mice and LSK cells of MA9;Trem1\textsuperscript{fl/fl}Vav1Cre mice compared with those of MA9;Trem1\textsuperscript{fl/fl} mice.