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

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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 ($Lin^-Sca1^+c\text{-}kit^+CD150^+CD48^-$) 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 ($Lin^-Sca\text{-}1^+c\text{-}Kit^+$) or $c\text{-}Kit^+$ 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 *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 $c\text{-}Kit^+$ 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 μM).[6]

qPCR analysis

Total RNA was extracted using RNeasy Mini Kit (QIAGEN) following the manufacturer's procedure. Reverse transcription was carried out at 42°C for 60 minutes and stopped at 95°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 *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 moue 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*$^{-/-}$*;Trem1*$^{fl/fl}$, *Fanca*$^{-/-}$*;Trem1*$^{fl/fl}$*Vav1Cre*, *MA9;Trem1*$^{fl/fl}$, *MA9;Trem1*$^{fl/fl}$*Vav1Cre*. Comparison was made: *Fanca*$^{-/-}$*;Trem1*$^{fl/fl}$ vs *Fanca*$^{-/-}$*;Trem1*$^{fl/fl}$*Vav1Cre*, *MA9;Trem1*$^{fl/fl}$ vs *MA9;Trem1*$^{fl/fl}$*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

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Supplementary table

Table S 1. Primers used for qPCR analysis

Gene	Forward	Reverse
Trem1	TGTGCGTGTTCTTTGTCTCA	TCTGGCTGTTGGCATACTTC
Ccr1	GCCAAAAGACTGCTGTAAGAGCC	GCTTTGAAGCCTCCTATGCTGC
Il1r1	CTGTTGGTGAGGAATGTGGCTG	GGCTCAGGATAACAGGTCTGTC
Nlrp3	TCACAACTCGCCCAAGGAGGAA	AAGAGACCACGGCAGAAGCTAG
Tlr2	CCAAAGAGCTCGTAGCATCC	AGGGGCTTCACTTCTCTGCT
Gapdh	GTCTCCTCTGACTTCAACAGCG	ACCACCCTGTTGCTGTAGCCAA

Supplementary figures

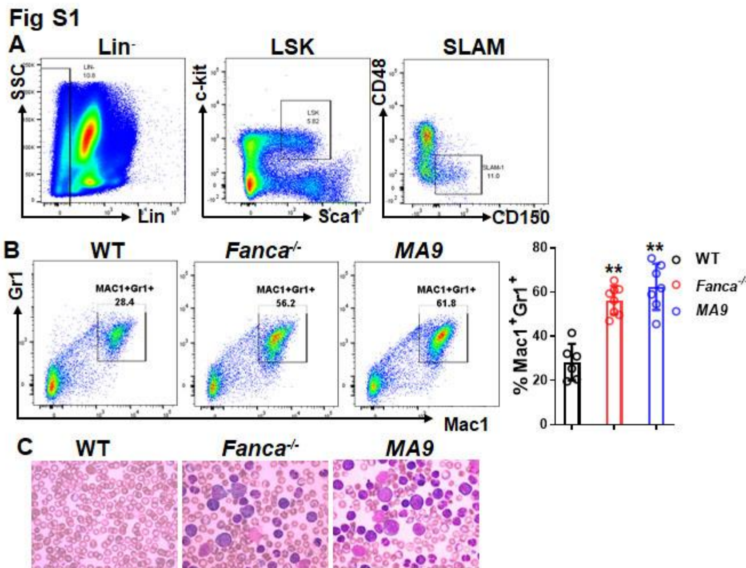

Fig S1. A. Gating strategy for flow cytometry analysis. Flow plots show gating strategies for Lin^-, LSK ($Lin^-Sca1^+c\text{-}kit^+$), SLAM ($LSKCD150^+CD48^-$) cells. B. Accumulation of $Mac1^+Gr1^+$ cells in peripheral blood (PB) of *Fanca*$^{-/-}$ and *MLL-AF9* leukemic mice.

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.

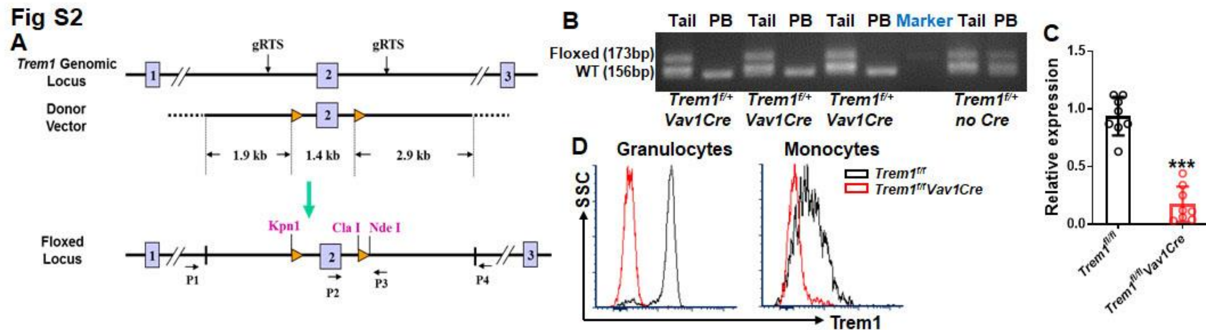

Fig S2. Generation of conditional *Trem1* knockout mice. A. Schematic diagram of *Trem1*$^{flox/flox}$ mice knock-out strategy. *Trem1* locus following homologous recombination and the final *Trem1* locus following excision of the *loxP*-flanked gene were illustrated. B. Genotyping of *Trem1*$^{flox/flox}$*Vav1Cre*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

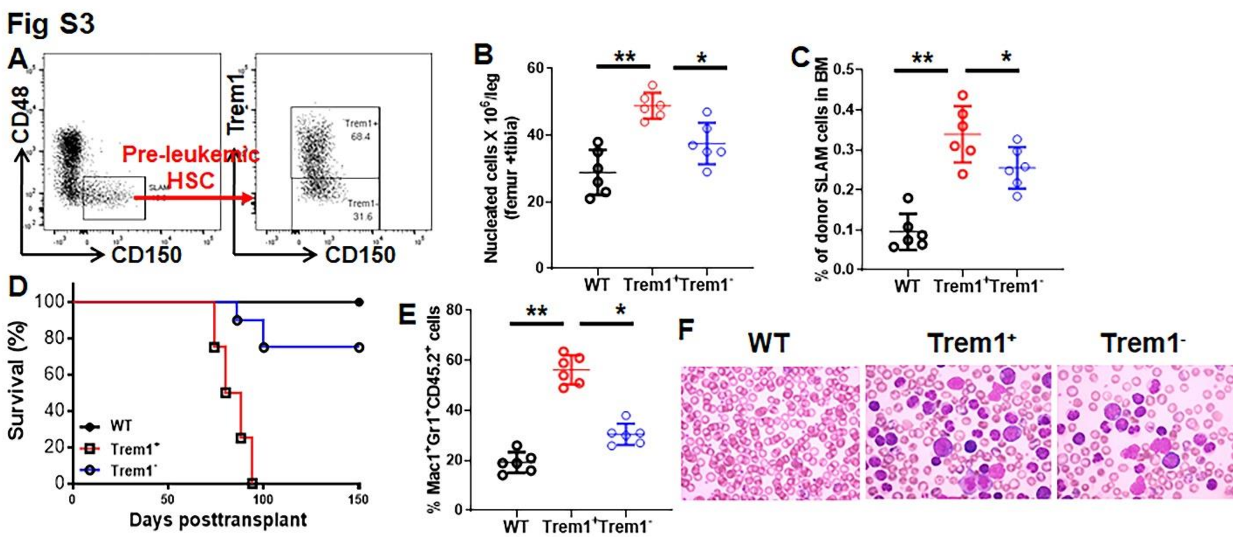

Fig S3. Trem1^{+} pre-leukemic HSCs induce leukemia in secondary recipients with a shorter latency. (A) Sorting strategy for separating the Trem1^{+}SLAM and Trem1^{-}SLAM

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.

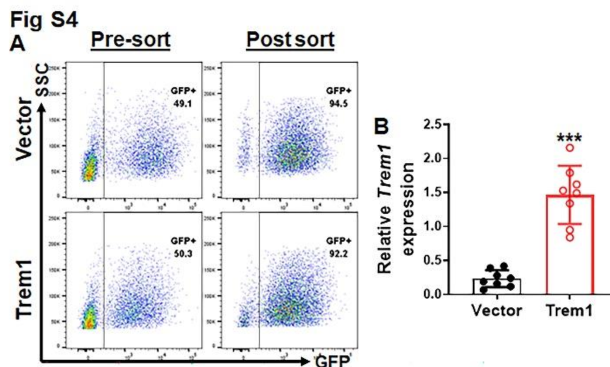

Fig S4. A. Flow cytometry analysis of BM c-Kit^{+} cells from *MA9* mice transduced with lentiviral vector expressing GFP or GFP-*Trem1*. Flow plots of pre-sorting and post-sorting are shown. B qPCR of *Trem1* expression in sorted transduced cells (n= 8 assays).

Fig S5

A

FA			MA9		
Gene	FC	p Value	Gene	FC	p Value
Il1r1	2.66	0.0077	***Tlr2***	3.94	0.0342
Ccr1	2.41	0.0217	***Rela***	2.46	0.0184
Tlr2	2.32	0.0051	***Ccr1***	2.33	0.0211
Nlrp3	1.93	0.0219	***Nlrp3***	2.17	0.0004
Nfkb1	1.92	0.0047	***Il1r1***	1.75	0.0358

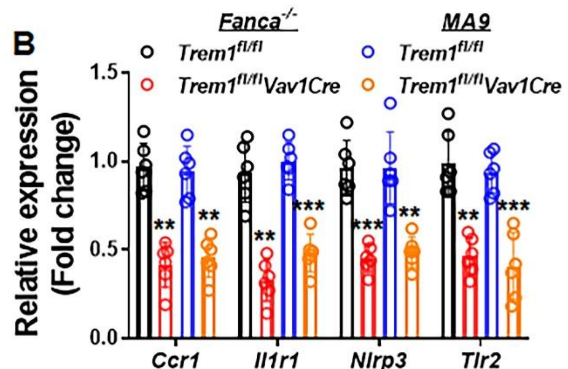

Fig S5. A. Top 5 differentially expressed genes in LSK cells of pre-leukemic *Fanca*$^{-/-}$ *;Trem1*$^{fl/fl}$ mice compared to those from LSK cells from the control *Fanca*$^{-/-}$

;Trem1$^{fl/fl}$*Vav1Cre* mice (Left), or 5-month-old *MA9;Trem1*$^{fl/fl}$ mice compared to those of age-matched *MA9;Trem1*$^{fl/fl}$*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*$^{-/-}$;*Trem1*$^{fl/fl}$*Vav1Cre* mice compared with those of *Fanca*$^{-/-}$;*Trem1*$^{fl/fl}$ mice and LSK cells of *MA9;Trem1*$^{fl/fl}$*Vav1Cre* mice compared with those of *MA9;Trem1*$^{fl/fl}$ mice.