SUPPLEMENTAL INFORMATION

Aging drives *Tet2*$^{+/-}$ clonal hematopoiesis via IL-1 signaling

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CONTENTS:

Supplemental Methods

Isolation of bone marrow and peripheral blood cells

BM cells were harvested from femurs and tibias. Single cell suspensions were generated by flushing bones in FACS buffer (2%FBS, 2mM EDTA in PBS). Red blood cells were lysed with 5ml red blood cell lysis buffer (150 mM NH4Cl, 10 mM KHCO3, and 0.1 mM EDTA) for 2 minutes and cells were washed in 5 ml FACS buffer. Cell debris was removed with 70-μm filters (BD). For peripheral blood cell analysis 50-75 ul of blood was drawn from sublingual veins. Red blood cells were lysed with 1ml of red cell lysis buffer for 2 minutes and cells were washed once with 1 ml FACS buffer.

Flow Cytometry

All staining protocols were performed in FACS buffer. Cells were incubated with appropriate antibodies on ice for 30 or 45 minutes. For chimerism analysis PB samples cells were stained with anti-CD45.1 (A20), anti-CD45.2 (104), anti-CD19 (1D3), anti-CD3e (2C11), anti-Gr-1 (RB6-8C5), anti-CD11b (M1/70). For HSPC BM immunophenotyping, cells were stained for lineage and stem cells markers. In detail, BM cells were stained with biotinylated antibodies against the lineage antigens: B220 (RA3-6B2), CD3ε (145-2C11), Ter119 (Ter119), NK1.1 (PK136), CD11b (M1/70), CD4 (RM4-5), CD8α (53-6.7) and Gr-1 (RB6-8C5). Cells were subsequently stained with c-Kit (2B8), Sca-1 (D7), CD34 (RAM34), Flt3 (A2F10), IL-7Rα (A7R34), CD150 (TC15-12F12.2), CD48 (HM48-1), CD16/32 (2.4G2), QDot605-conjugated or CF594-conjugated streptavidin. All antibodies were purchased from Biolegend (San Diego, California).

BM lysate collection IL-1 quantification by ELISA

BM lysates were obtained by flushing a single femur bone with 300ul of 1x RayBio Lysis Buffer supplemented with proteinase inhibitor (cOmplete ULTRA Tablets, Mini, EDTA-free, EASYpack, Roche). ELISA kits for mouse IL-1α, IL-1β were purchased from R&D systems. ELISAs were performed according to manufacturer's manual.

Cell culture

All cultures were performed at 37°C in a 5% CO2 water jacket incubator (Thermo Scientific). For colony formation assays, 1000 BM Lin^- c-Kit^+ $Sca\text{-}1^+$ cells were cultured in methylcellulose (Stem Cell Technologies, M3231) supplemented with SCF (25 ng/ml), Flt3L (25 ng/ml), IL-11 (25 ng/ml), IL-3 (10 ng/ml), GM-CSF (10 ng/ml), Epo (4 U/ml) and Tpo (25 ng/ml) (Peprotech). IL-1α (Peprotech) was added at 25 ng/ml and colonies were scored visually after 7-10 days. For serial replating, cells from primary methylcellulose cultures in 3 cm dishes were disaggregated and washed three times in PBS, and 10000 total cells were re-plated in fresh methylcellulose without IL-1β. Colonies were scored visually after 7-10 days. For mature cells culture cells were seeded at a concentration of $16x10^5$ cells/ml in DMEM, 4.5g/l glucose, 10%(v/v) FBS, 100U/ml penicillin, 100ug/ml streptomycin. Cells were either stimulated with LPS (100ng/ml) or PBS, 24 hours later supernatant was collected and subjected for ELISA.

RT–PCR

Cells were subjected DNA to isolation (Qiagen, Blood & Tissue Kit) or RNA isolation (Qiagen, RNeasy Plus Micro Kit) and cDNA synthesis (Applied Biosystems, High Capacity cDNA Reverse Transcription Kit, #4368814). qPCR with TaqMan Assays-on-Demand probes on 7500 Fast Real Time PCR System (AppliedBiosystems). qPCR for Il1a, Il1b, Il1r1, Il1r2, Il1ra, Tnfa, Il6, Ifng, Tet2 and Gapdh were performed using TaqManAssays-on-Demand probes (Mm00439620_m1, Mm00434228_m1, Mm00434237_m1,Mm00439629_m1, Mm00446186_m1, Mm00443258_m1, Mm00446190_m1, Mm01168134_m1, Mm01312907_m1 Mm99999915_g1) on 7500 Fast Real Time PCR System (Applied Biosystems). Relative mRNA expression of each gene was calculated against expression of Gapdh. For *Tet2* gene quantification in genomic DNA, we preformed qPCR with SYBR green reagent (Applied Biosystems) on 7500 Fast Real Time PCR System (AppliedBiosystems). *Tet2* and *Gapdh* gene amplification was performed using primers directed to Tet2 exon 3[1]: Forward – agagcctcaagcaaccaaaa; Reverse – acatccctgagagctcttgc and to Gapdh: Forward – catcactgccacccagaagactg; Reverse – atgccagtgagcttcccgttcag.

RNA sequencing

The RNA extraction was performed following the protocol of ArcturusTM PicoPureTM RNA isolation kit (Applied Biosystems). 10’000-30’000 cells were sorted directly in the 100ul of extraction buffer of the kit. Quality and quantity of RNA was measured using Agilent 4200 TapeStation System. The libraries were prepared following the Smart-seq2 protocol[2]. Following reverse transcription, the quality of the cDNAs was evaluated using an Agilent 2100 Bioanalyzer. 0,5 ng of cDNA from each sample were tagmented and amplified using Illumina Nextera XT kit. The resulting libraries were pooled, double-sided size selected (0.5x followed by 0.8x ratio using Beckman Ampure XP beads) and quantified using an Agilent 4200 TapeStation System. The pool of libraries was sequenced in an Illumina NovaSeq6000 sequencer (single-end 100 bp) with a depth of around 20 Mio reads per sample.

Bioinformatics analysis

Raw reads were first cleaned by removing adapter sequences, trimming low quality ends, and filtering reads with low quality (phred quality <20) using Fastp (Version 0.20)[3]. Sequence pseudo alignment of the resulting high-quality reads to the Mouse reference genome (build GRCm39) and quantification of gene level expression (gene model definitions based on GENCODE release M26) was carried out using Kallisto (Version 0.43.1)[4]. Differential expression was computed using the generalized linear model implemented in the Bioconductor package DESeq2 (R version: 4.2.0, DESeq2 version: 1.36.0)[5]. Genes showing altered expression with adjusted (Benjamini and Hochberg method) p-value < 0.05 were considered differentially expressed. Gene ontology analysis and transcription factor co-occurrence analysis was preformed using Enrichr tool[6-8].

Data and Code Availability

The accession number for the RNA sequencing data described in this publication is PRJEB56666.

References

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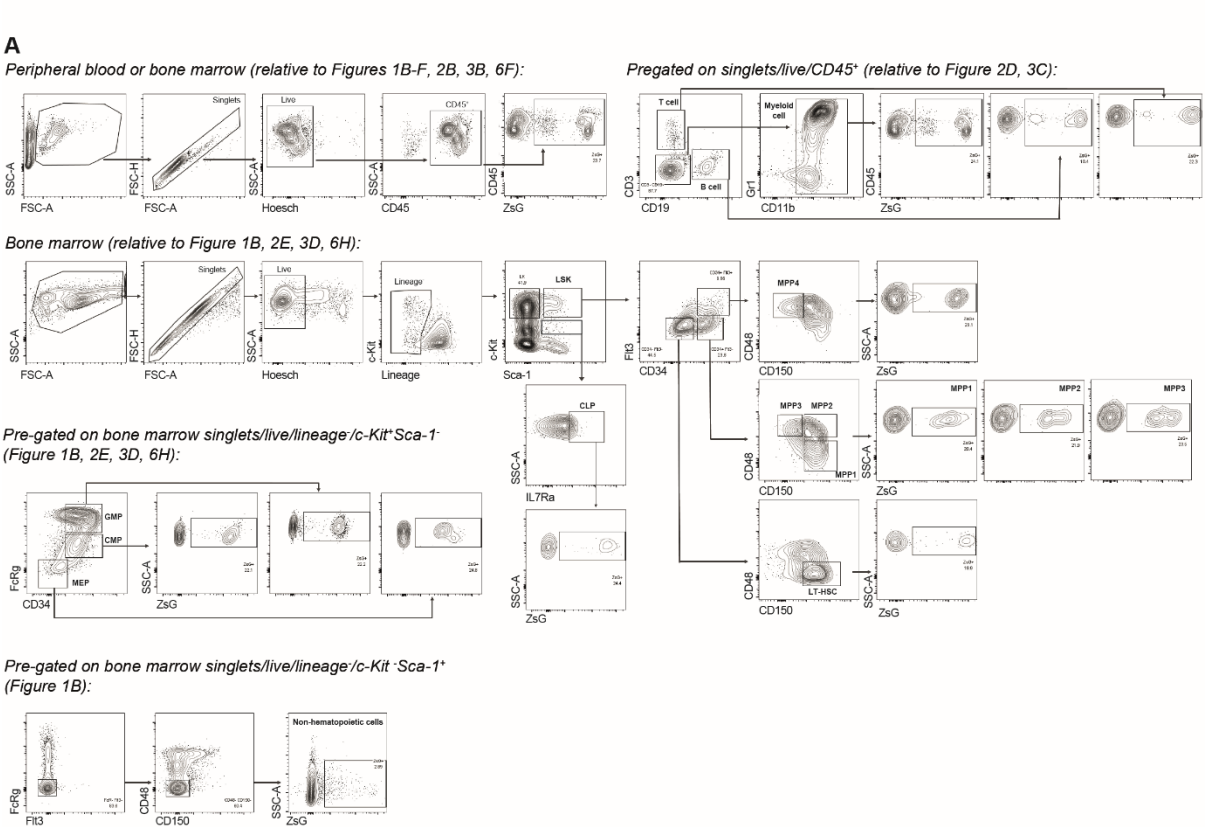
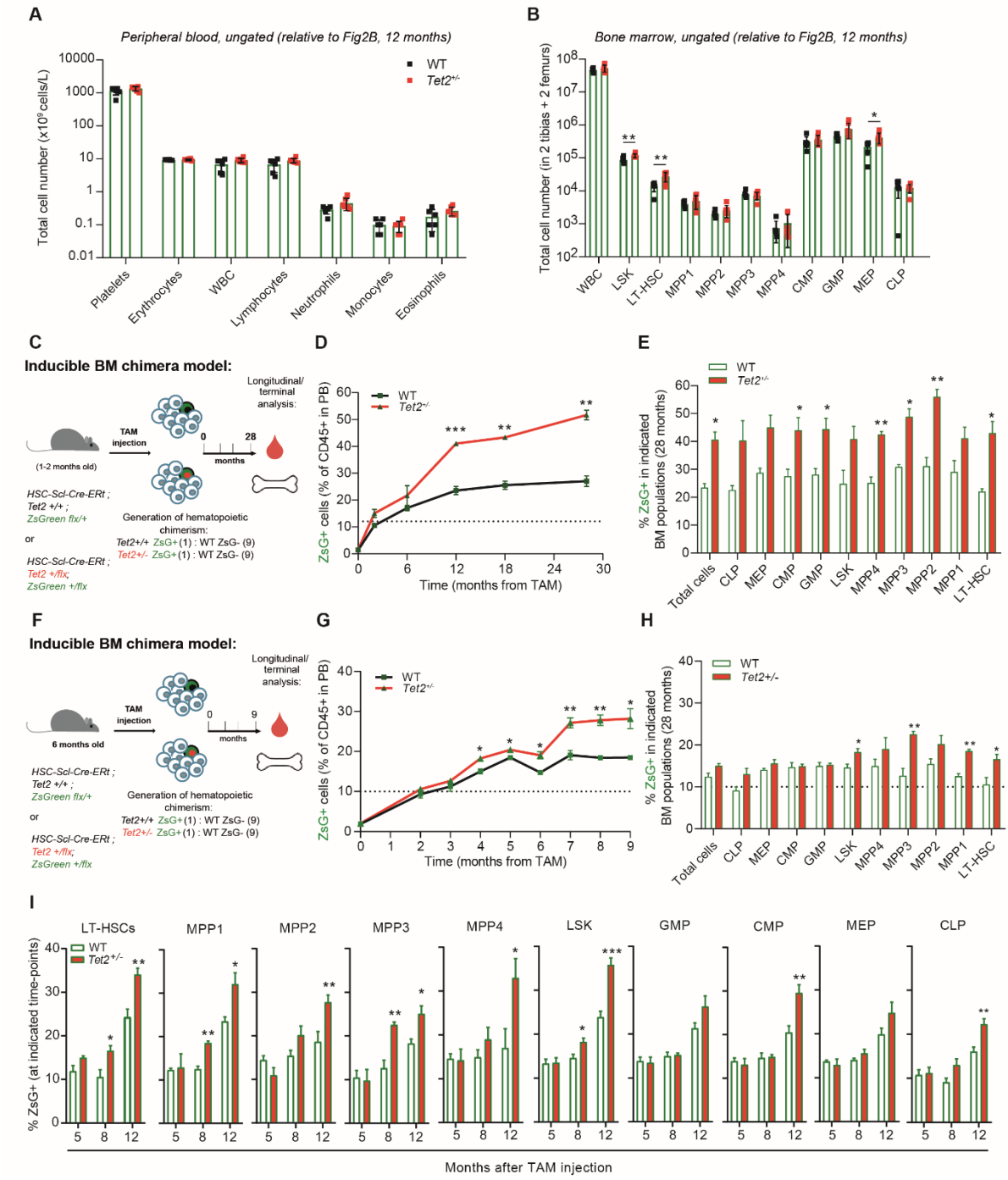
Supplemental Figure 1



Supplemental Figure 1 – Gating strategies.

A. Gating strategies used in indicated figure panels.

Supplemental Figure 2

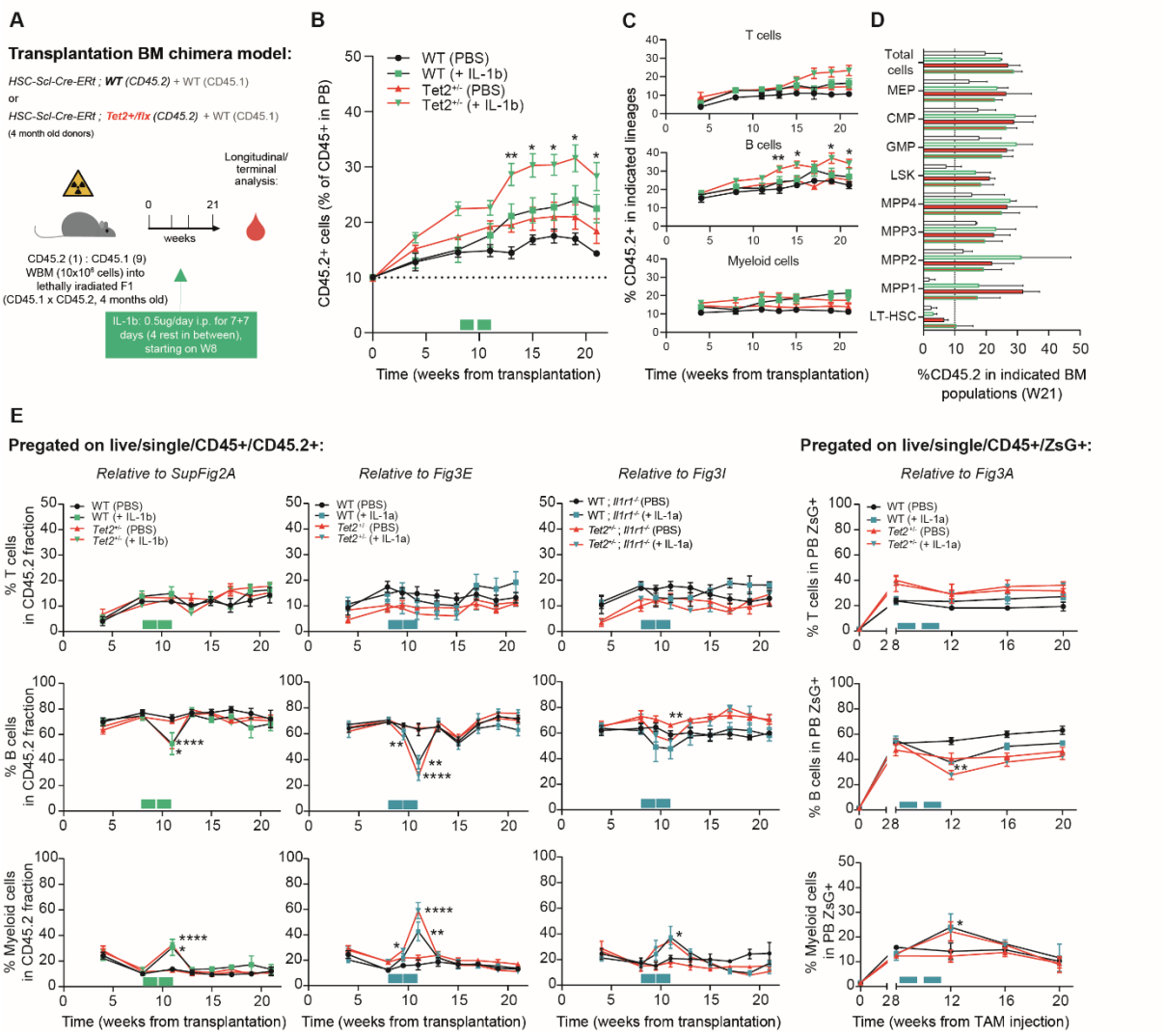


Supplemental Figure 2 - Hematopoietic *Tet2*$^{+/-}$ clonal expansion associates with advanced age.

A. Number of indicated cell populations per liter of peripheral blood (PB) in SCL-Cre+/- ; Ai6+/-; WT and SCL-Cre+/-; Ai6$^{+/-}$; *Tet2*$^{+/-}$ mice (n=6-7). Relative to figure 2B at 12 months. **B.** Number of indicated cell populations in total bone marrow (BM, two femurs and 2 tibias) of SCL-Cre+/-; Ai6+/-; WT and SCL-Cre+/-; Ai6+/-; *Tet2*$^{+/-}$ mice (n=6-7). Relative to figure 2B at 12 months. **C.** Experimental design. TAM – Tamoxifen. **D.** Longitudinal quantification of the percentage of PB CD45+ WT ZsG+ (n=3, black line)

and CD45+ *Tet2*$^{+/-}$ ZsG+ (n=3, red line) cells over 28 months post TAM induction. **E.** Percentage of WT ZsG+ (n=3) and *Tet2*$^{+/-}$ ZsG+ (n=3) on indicated BM populations. **F.** Experimental design. **G.** Longitudinal quantification of the percentage of PB CD45+ WT ZsG+ (n=3, black line) and CD45+ *Tet2*$^{+/-}$ ZsG+ (n=3, red line) cells over 9 months post TAM induction. **H.** Percentage of WT ZsG+ (n=3) and *Tet2*$^{+/-}$ ZsG+ (n=3) on indicated BM populations. **I.** Percentage of WT ZsG+ and *Tet2*$^{+/-}$ ZsG+ on indicated BM populations at 5 months (n=4-6), 8 months (n=3) or 12 months (n=6-7) post TAM induction. Data from 5 months and 12 months are derived from Figure 2E and Figure 3D. * P<0.05; ** P<0.01; *** P<0.001; **** P<0.0001 by unpaired t-test. Error bars represent standard error of the mean (SEM).

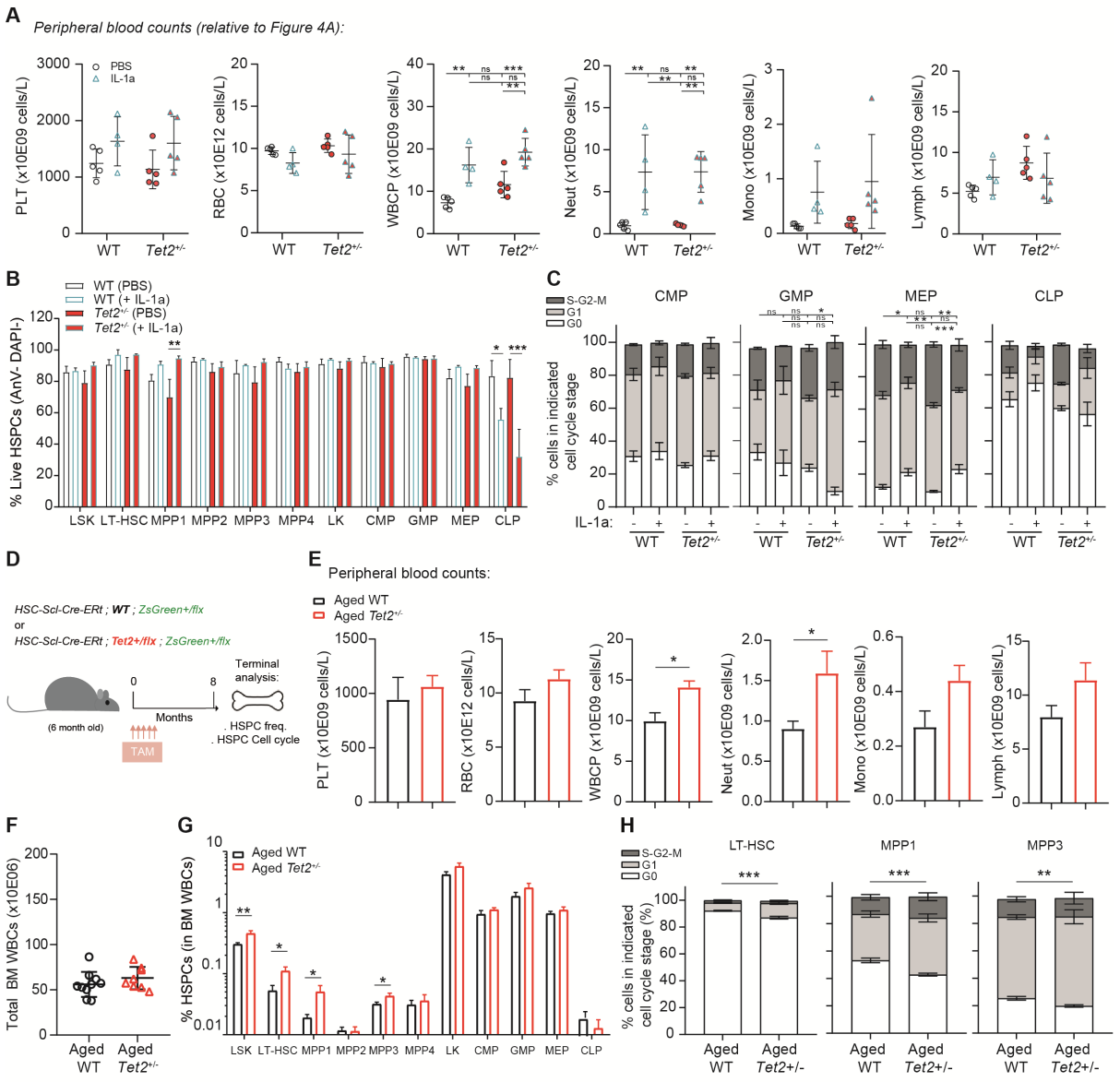
Supplemental Figure 3



Supplemental Figure 3 - IL-1β drives *Tet2*$^{+/-}$ clonal expansion via increased multilineage differentiation to a lesser extent than IL-1α.

A. Experimental design. WBM – Whole bone marrow. **B.** Longitudinal quantification of the percentage of CD45.2^{+} WT and CD45.2^{+} *Tet2*$^{+/-}$ in peripheral blood (PB) of mice exposed to PBS or IL-1β (n=4-5). Green boxes on X-axis indicate IL-1β exposure period. **C.** Percentage of WT or *Tet2*$^{+/-}$ CD45.2^{+} cells in PB T cells, B cells, myeloid cells of mice exposed to PBS or IL-1β (n=4-5). **D.** Percentage of WT or *Tet2*$^{+/-}$ CD45.2^{+} cells in indicated BM populations of mice exposed to PBS or IL-1β (n=4-5). **E.** Longitudinal quantification of the percentage of T cells, B cells and myeloid cells in CD45.2^{+} or ZsG+ (last column) cell fraction. Green boxes on X-axis indicate IL-1β exposure period. Main figures panels related to these graphs are indicated. * P<0.05; ** P<0.01; *** P<0.001; **** P<0.0001 by unpaired t-test (within the same genotype). Error bars represent standard error of the mean (SEM).

Supplemental Figure 4

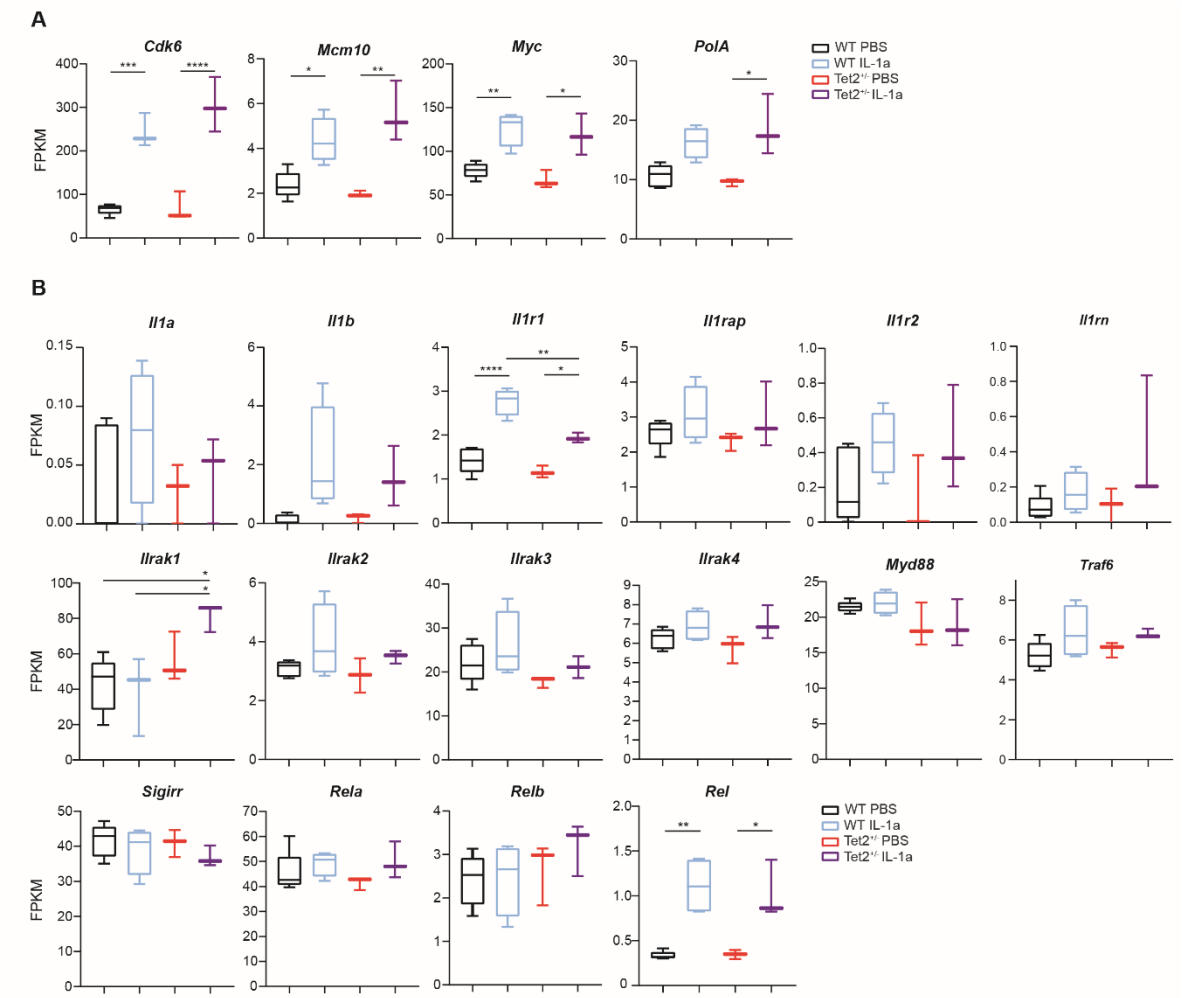


Supplemental Figure 4 - Aged *Tet2*$^{+/-}$ HSPCs maintain higher proliferative capacity than WT.

A. Number of indicated cell populations per liter of peripheral blood (PB) in WT and *Tet2*$^{+/-}$ mice exposed to PBS or IL-1α (n=4-5). **B.** Percent of viable cells (AnnexinV^{-} DAPI^{-}) in indicated BM population WT and *Tet2*$^{+/-}$ mice exposed to PBS or IL-1α (n=4-5). Relative to experiment depicted in 4A. **C.** Percent of indicated bone marrow (BM) populations in G0, G1 or S-G2-M phases of cell cycle from WT and *Tet2*$^{+/-}$ mice exposed to PBS or IL-1α (n=4-5). Statistical analysis performed for G0 stage. Relative to experiment depicted in 4A. **D.** Experimental design. HSPC – Hematopoietic stem and progenitor cells. **E.** Number of indicated cell populations per liter of blood in aged (14 months old) WT and *Tet2*$^{+/-}$ mice (n=8-10). **F.** Number of total white blood cells (WBC) in BM (2 femurs and 2 tibias) of aged WT and *Tet2*$^{+/-}$ mice (n=8-10). **G.**

Percentage of indicated BM populations in aged WT and *Tet2*$^{+/-}$ mice (n=8-10). **H.** Percent of indicated bone marrow (BM) populations in G0, G1 or S-G2-M phases of cell cycle from aged WT and *Tet2*$^{+/-}$ mice (n=8-10). Statistical analysis performed for G0 stage. * P<0.05; ** P<0.01; *** P<0.001; **** P<0.0001 by one-way ANOVA with Holm-Sidak's correction (graphs A-C) or unpaired t-test (graphs E-H). Error bars represent standard error of the mean (SEM).

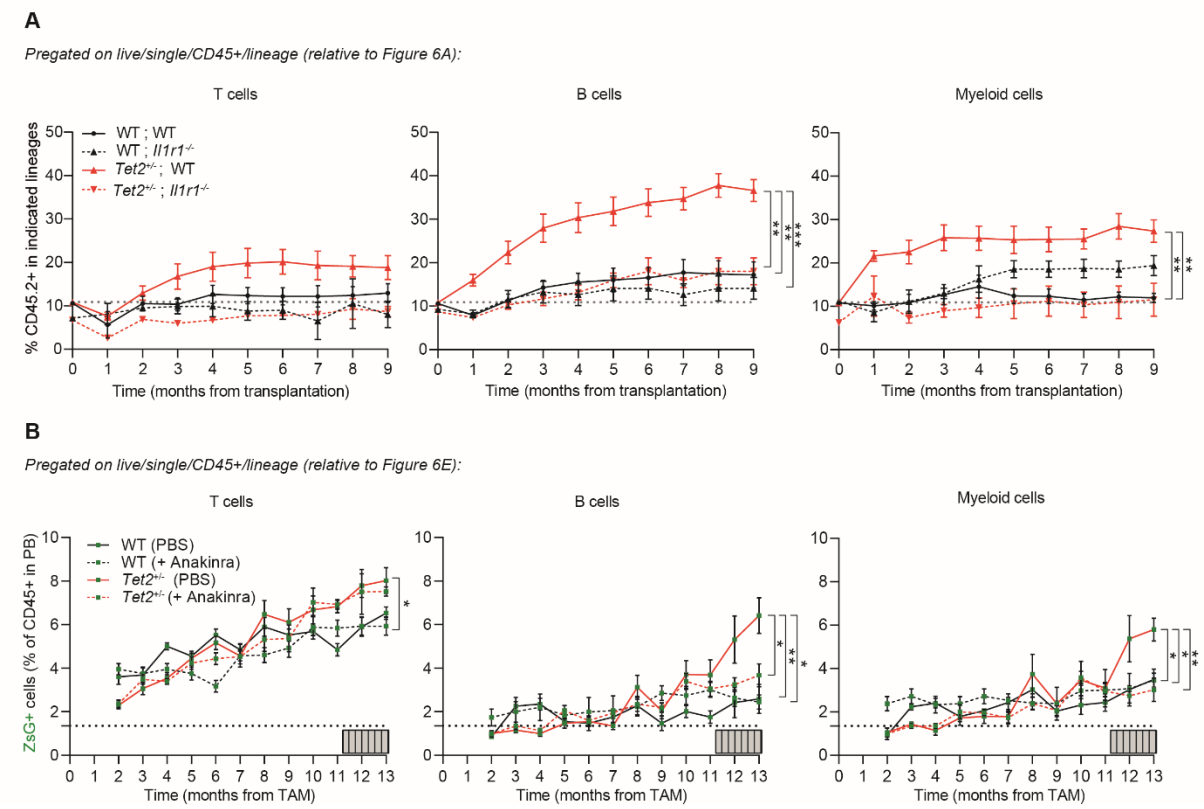
Supplemental Figure 5



Supplemental Figure 5 – IL-1 pathway members expression in WT and *Tet2*$^{+/-}$ HSCs exposed to PBS or IL-1α.

A. Boxplot depicting indicated gene expression values in fragments per kilobase million (FPKMs) in WT PBS (n=5), WT IL1-1a (n=4), *Tet2*$^{+/-}$ PBS (n=3) and *Tet2*$^{+/-}$ IL-1α (n=3) HSCs. **B.** Boxplot depicting indicated gene expression values in fragments per kilobase million (FPKMs) in indicated groups. * P<0.05; ** P<0.01; *** P<0.001; **** P<0.0001 by a One-way ANOVA with Tukey's correction.

Supplemental Figure 6



Supplemental Figure 6 – Lack of IL-1R1 signaling (either Il1r1-/- or antagonist treatment) attenuates *Tet2*$^{+/-}$ multilineage differentiation upon aging.

A. Longitudinal quantification of the percentage of CD45.2^{+} cells in peripheral blood (PB) T, B and myeloid cells from WT; WT, WT; *Il1r1*$^{-/-}$, *Tet2*$^{+/-}$; WT and *Tet2*$^{+/-}$; *Il1r1*$^{-/-}$ mice (n=3-4). **B.** Longitudinal quantification of the percentage of ZsG+ cells in T, B and myeloid PB cells of mice exposed to PBS or Anakinra treatment (hIL1ra) (n=3-5). * P<0.05; ** P<0.01; *** P<0.001; **** P<0.0001 by a Two-way ANOVA with Holm-Sidak's correction (for last time point). Error bars represent standard error of the mean (SEM).