Transforming growth factor-β signaling modifies the hematopoietic acute inflammatory response to drive bone marrow failure

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Extended Methods

Mouse Model

Transgenic Tg-b1glo$^{+/Flox}$ mice (Jackson Labs, Stock No:018393) were crossed with Mx1-Cre mice to generate MxCre^{+}; Tg-b1glo$^{+/Flox}$ (TgCre+) and MxCre^{-}; Tg-b1glo$^{+/Flox}$ mice (TgCre–). To express Cre recombinase, 6-8 week old mice were treated with 3 injections of 10 mg/kg/mouse polyinosinic:polycytidlic acid intraperitoneally (pIC, GE Healthcare), every other days. pIC-stressed mice were allowed to recover for at least 4 weeks prior to reinjection with the same pIC regimen. All animals were bred at a pathogen-free facility in house, and all studies conducted with protocols approved by the Animal Care Committee of Cincinnati Children's Hospital Medical Center.

Flow Cytometry

Peripheral blood was collected by retro-orbital puncture, then red cell lysed with lysis buffer (BD Biosciences-Pharmingen). Cells were then stained with CD45 PerCP-Cy5.5/APC Cy7, B220 APC/PE Cy7, Gr1 Alexa Fluor 700, Mac1/CD11b, CD3ε APC/PE, CD4 PE, CD8a APC/PE. All antibodies were obtained from BD Biosciences.

Whole bone marrow cells were isolated by flushing bones with Hanks' Buffered Saline Solution (Corning) with 10% fetal bovine serum (Atlanta). Cells were stained as above for mature lineages. Cell were also stained for LSK SLAM with biotin-conjugated anti-mouse lineage antibodies (Ter119, B220, Gr1, CD11b, CD3ε) followed by staining for streptavidin V500/eF450 (eBioscience (eF450)), c-Kit APC eF780/APC (eBioscience (APC eF780)), Sca1 PE Cy7, CD48 AF700/BV605 (Biolegend (AF700)) CD150 APC/PE (eBioscience (APC); Fisher Scientific (PE)). Bone marrow cells were further stained with CD16/32 PE (eBioscience) and CD34 eF450 (eBioscience) to immunostain for committed progenitor populations. For mitochondrial function, cells immunostained for LSK SLAM were then washed and incubated at 37°C and 5% CO_2 for 30 minutes with either MitoSOX Deep Red Reagent (1 μM, to measure mitochondrial superoxide, Invitrogen) or tetramethylrhodamine ester (0.1 μM, to measure mitochondrial membrane potential, Sigma Aldrich). To assess active caspase 1 content, cells were incubated with 200 μL of 1X FLICA 660 in 1X DPBS (Corning) for 1 hour, 37°C and 5% CO_2. Samples were then analyzed using BD LSR II, BD LSR Fortessa, or BD Canto III (BD Biosciences). All antibodies were obtained from BD Biosciences, unless otherwise noted.

ELISA Assays

Bone marrow fluid was isolated by flushing bones with 500 μL HBSS without serum, and pelleting the cell fraction at 5000 RPM for 10 minutes. The supernatant was then removed and aliquoted before freezing at -80 C. Aliquots were then assessed for active TGFβ1 using the Mouse TGF-beta 1 DuoSet ELISA Kit (R&D Systems) and DuoSet ELISA Ancillary Reagent Kit 1 (R&D Systems).

Bone marrow and spleen histology

Spleens were isolated from mice and kept in 10% formalin for 48 hours before placing in 70% ethanol for 5 days at room temperature. Femurs were removed with heads intact and kept in 10% formalin for 48 hours before placing in 5% EDTA for 4 days at room temperature, changing the solution every day to ensure de-calcification. Samples were then sent to the pathology core in Cincinnati Children's Hospital Medical Center for sectioning and staining. Whole bone marrow cells were also prepared by cytospin and stained using Kwik-Diff (Fisher Scientific) to asses frequency of dysplasia by light microscopy using a Leica microscope at 40X magnification.

RNA sequencing

SLAM HSCs were obtained by isolating whole bone marrow cells as above, followed by immunostaining with CD117 magnetic separation beads (Milteryi Biotec) before running samples through an AutoMACS Pro magnetic cell separator (Miltenyi Biotec). The cKit+ enriched fraction was stained as above for LSK SLAM, then sorted using a BD FACS Aria II. cDNA from 500 SLAM HSCs from each sample were then made using the Smart-seq v4 Ultra Low Input RNA Kit (Takara/Clontech). A barcoded DNA library was then made using the Nextera XT DNA Library Preparation Kit (Illumina). Lastly the quality of the DNA library was assessed using the Agilent High Sensitivity DNA kit (Agilent Technologies) and an Agilent 2100 Bioanalyzer (Agilent Technologies). Sequencing was then done by the CCHMC core. The open-source software Alt-analyze [24] was then used to perform supervised hierarchical clustering and principle component analysis of the samples, as previously described [25, 26]. Differentially expressed genes were then analyzed using the ENRICHR database [27].

Immunofluorescence assays

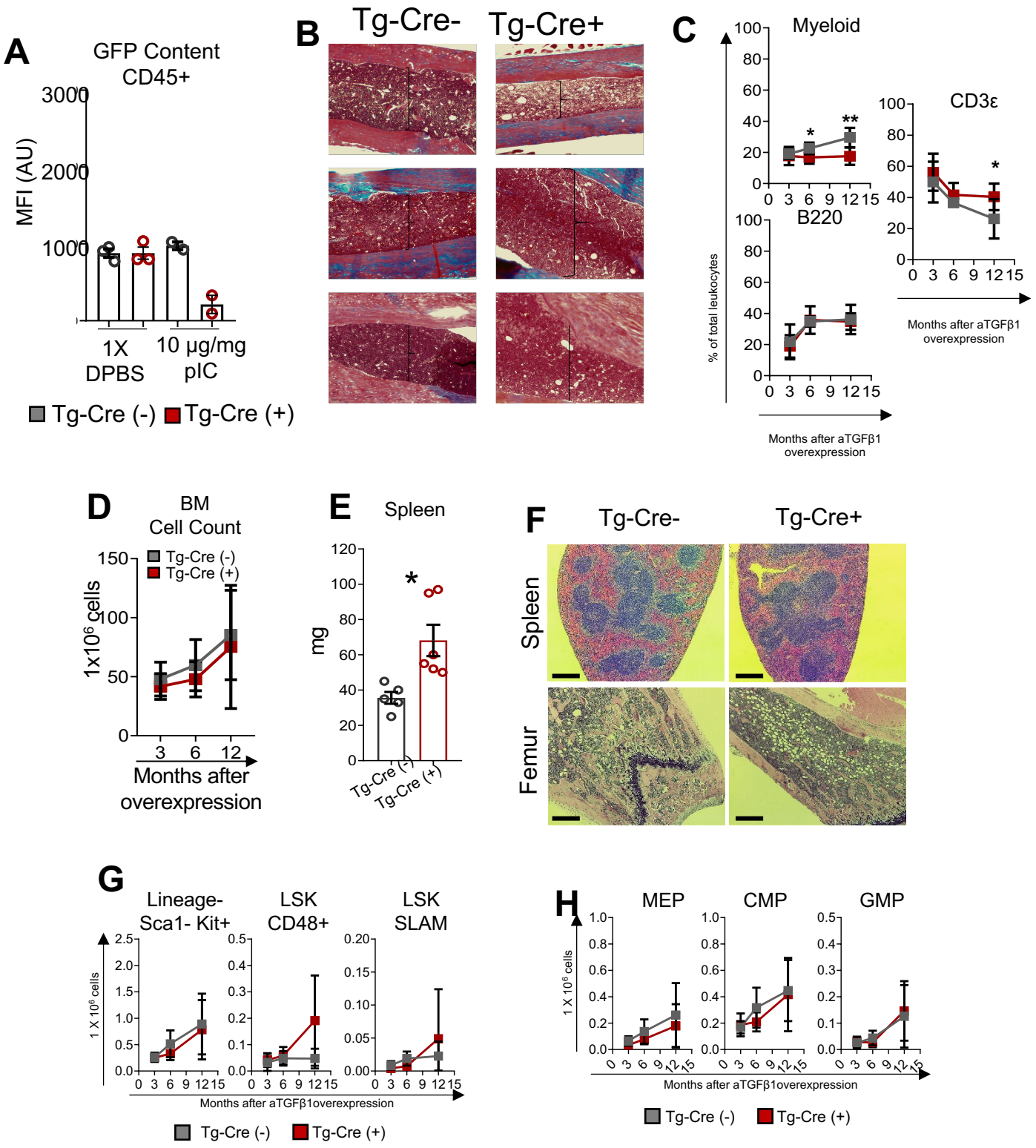
SLAM HSCs were sorted and incubated on retronectin (Takara)-coated 8-well chamber slides at 37°C and 5% CO_2 for 1 hour, before fixing with 4% paraformaldehyde at room temperature for 15 minutes. Cells were then washed and permeabilized with 0.1% Triton-X 100 for 3 minutes before blocking with 2% bovine serum albumin in PBS. Cells were then immunostained for mitochondria using rabbit anti-Tomm20 conjugated to Alexa Fluor 555 (Abcam). The cells were then mounted with Slowfade Glass with DAPI (Invitrogen). Images of cells were then taken using Nyquist limit setting (0.1 µm XY pixel size) at 100X magnification using 1.2 AU pinhole. Z stacks were acquired at 0.125 µm step-size, and 100-150 Z sections were taken.

Image Analysis

Mean fluorescence intensity of Tomm20 fluorescent signals was measured by using the surface building Matlab extension in Imaris software from at least 30 cells in each group.

Statistical analyses

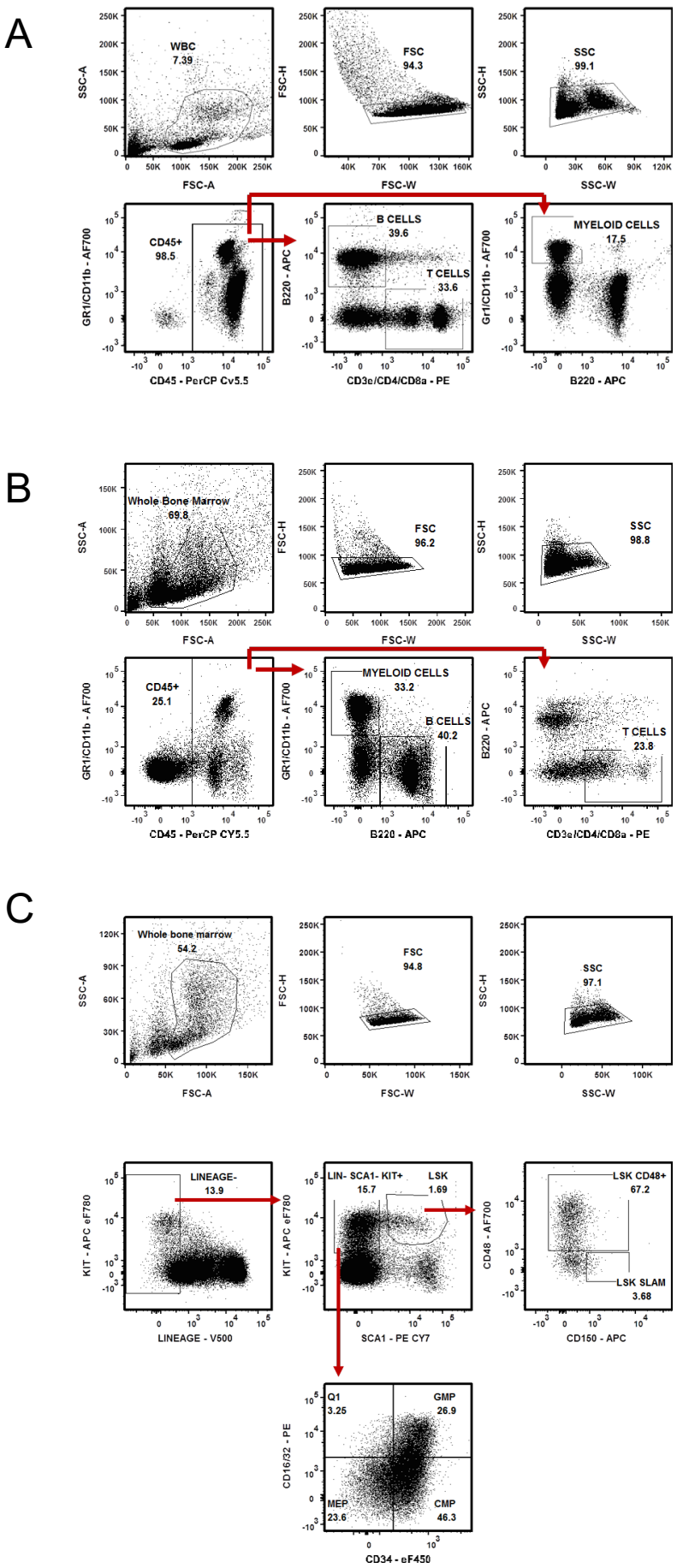
Results are presented as mean ± standard deviation or mean ± standard error of the mean. Experiments were done in 2-3 replicates unless specified. Statistics were performed using unpaired Student T-test, unless specified.



Supplementary Figure S1

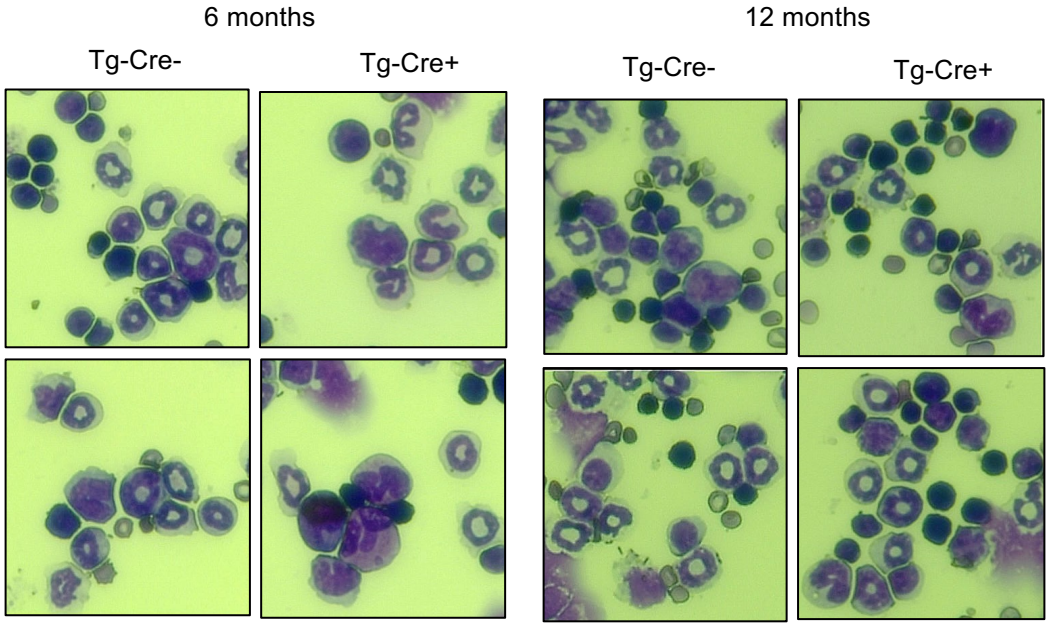
Supplementary figure S1:

(A) TgCre^{+} and TgCre^{-} mice were treated with PBS or pIC. Efficiency of excision of eGFP cassette was evaluated by flow cytometry. N=3 mice/group. **(B)** aTGFβ1-overexpressing mice do not develop fibrosis compared to controls during steady state. Femurs were taken from control and aTGFβ1-overexpressing mice 6 months and Masson's trichrome staining was performed. Brackets denote the bone marrow space. **(C)** Flow cytometry analysis of mature leukocyte lineages in the peripheral blood. N=5 mice total/group (3 months), N=4 TgCre^{-} mice, 6 TgCre^{+} mice (6 months), N=7 TgCre^{-} mice, 8 TgCre^{+} mice (12 months)**. (D)** Bone marrow cell counts at indicated time. N=5 mice total/group (3 months), N=7 TgCre^{-} mice, 9 TgCre^{+} mice (6 months), N=7 TgCre^{-} mice, 8 TgCre^{+} mice (12 months) **(E)** Spleen weight at 3M. N=5 TgCre^{-} mice, 6 TgCre^{+} mice **(F)** Histology using hematoxylin/eosin staining on spleens and femurs 3 months after aTGFβ1 overexpression. N=3 mice/group **(G-H)** Bone marrow cell counts committed progenitors (Lineage- Sca1- Kit+, LK), multipotent progenitors (LSK CD48+) and SLAM HSCs in G, and of megakaryocyte/erythrocyte progenitors (MEP), common myeloid progenitors (CMP), granulocyte/monocyte progenitors (GMP) in H, at indicated time points after aTGFβ1 overexpression. N=5 mice total/group (3 months), N=7 TgCre^{-} mice, 8 TgCre^{+} mice (6 months), 7 mice total/group (12 months).



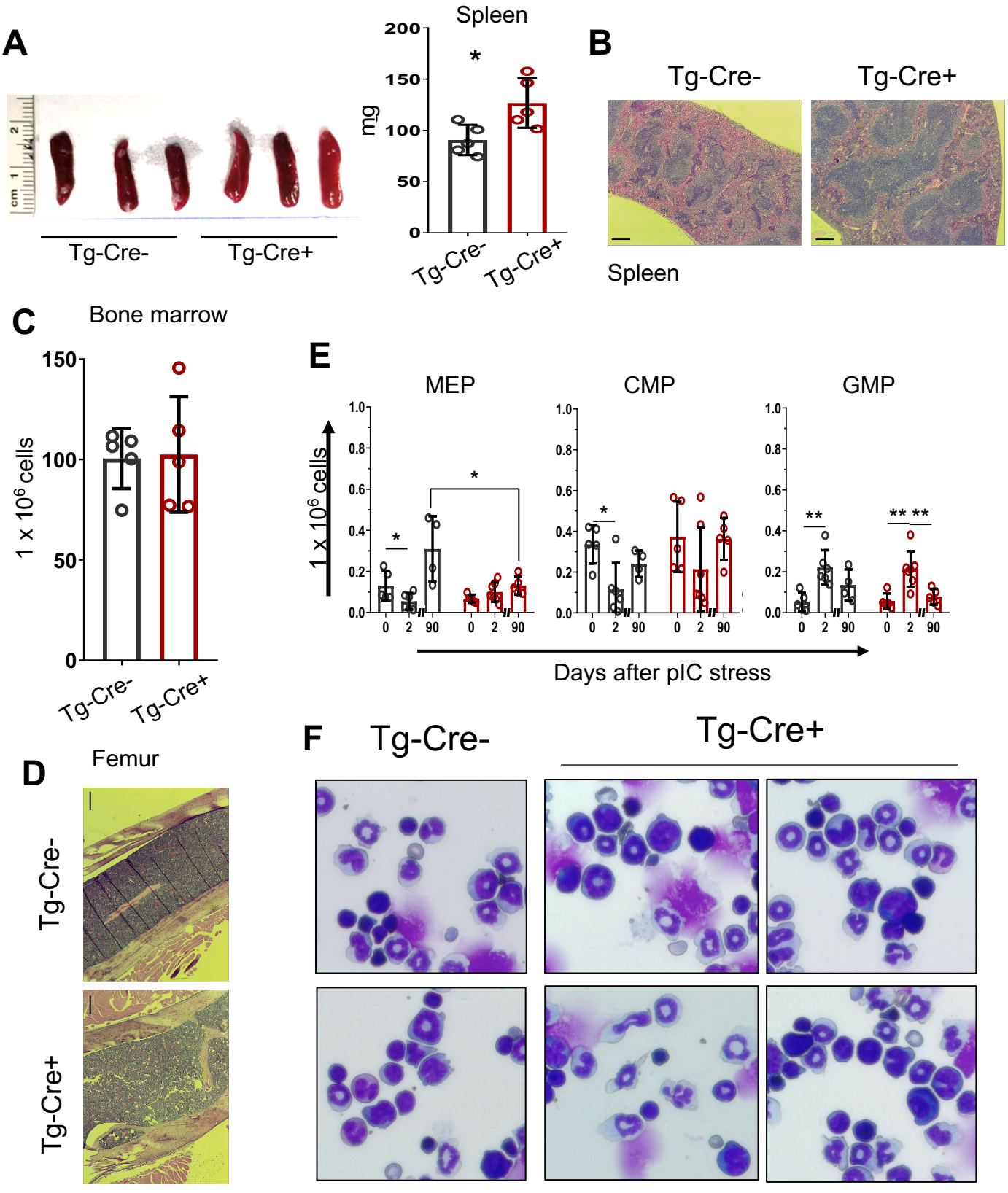
Supplementary Figure S2

Supplementary figure S2: Representative flow cytometry bone marrow and peripheral blood immunophenotyping analysis during steady state hematopoiesis. (A) Peripheral blood mature lineage **(B)** bone marrow mature lineage **(C)** bone marrow stem and progenitor cells



Supplementary figure S3.

Wright-Giemsa staining of bone marrow cytospins 6 and 12 months after aTGFβ1 overexpression, full size images shown in figure 1

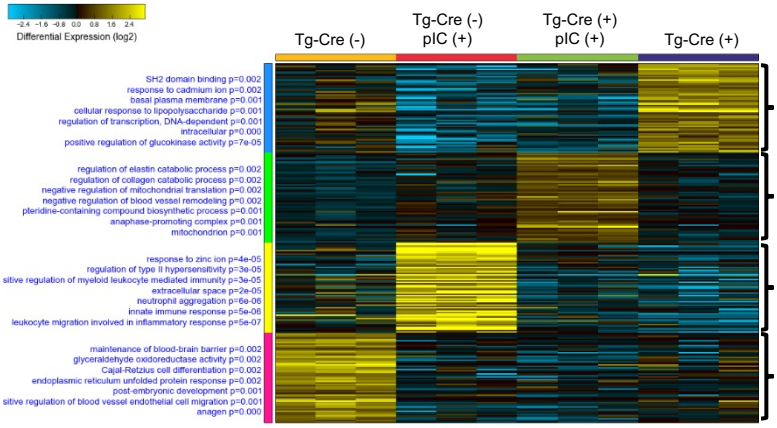


Supplementary Figure S4

Supplementary figure S4.

(A) Images and weight (N=5 mice/group) of spleens 3 months after pIC stress. **(B)** Histology and hematoxylin/eosin staining of spleens **(C)** Total bone marrow cell counts 3 months after pIC stress. (N=5 mice/group). **(D)** Histology and hematoxylin/eosin staining of femurs 3 months after pIC stress. N=3 mice/group **(E)** Cell counts of MEP, CMP, GMP in BM. N=5 mice total/group (No stress), N=7 mice (2 days), N=8 $TgCre^-$ mice, 9 $TgCre^+$ mice (90 days). **(F)** Wright-Giemsa staining of bone marrow cytospins 6 and 12 months after aTGFβ1 overexpression, full size images shown in figure 2

A



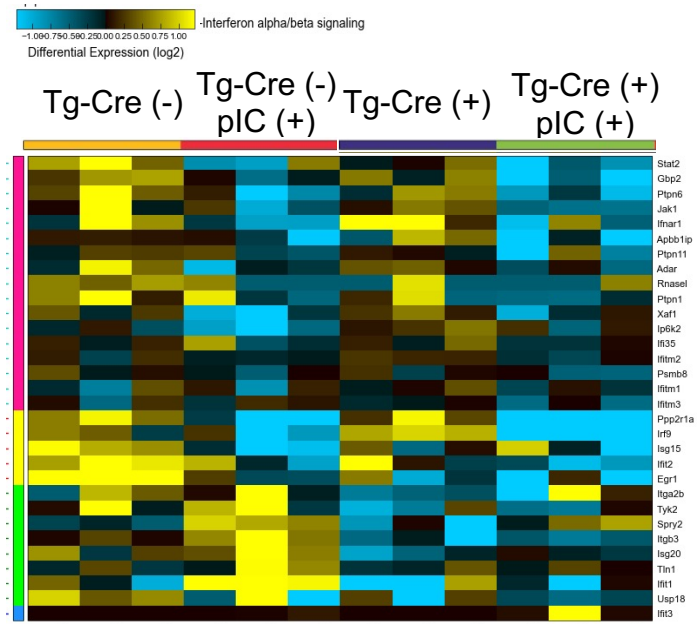
Tespa1 *Lat2*
Tnfsf9 *Hsf2*
Ccl3 *Pfkb1*
Cd34

Rpa3 *Aurkaip1* *Exosc7* *Pdcl3*
Mrpl17 *Cox14* *Ssbp1* *Bbip1*
Ndufs7 *Etfb* *Chtf18*
Gtg2h5 *Mrpl46* *Cst3*

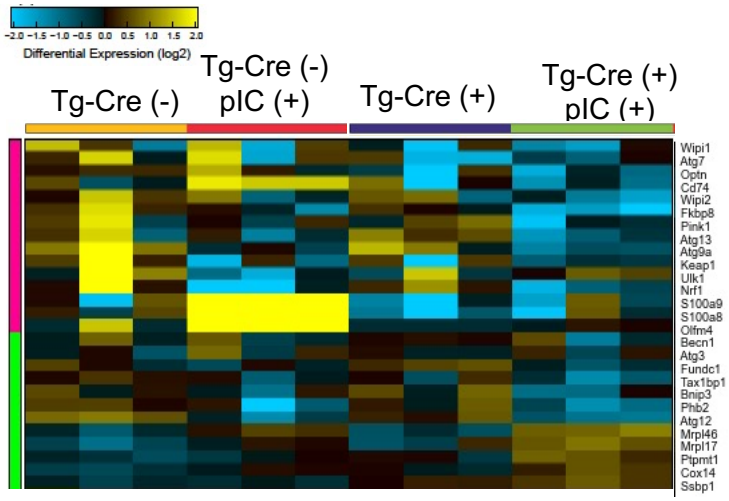
Cd74 *Orm1*
S100a8 *Apaf1*
S100a9 *Olfm4*
Fcert1g *Il1r2*

Angpt1
Ptgs2
Cxcl11
Csf1

B



C



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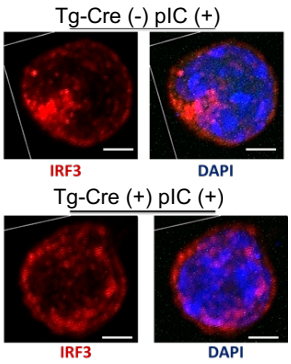

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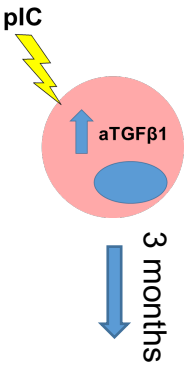

Increased:
1.Intracellular superoxide
2.Mitochondrial membrane potential
3.Active caspase 1

Bone marrow failure

Supplementary figure S5.

(A) Heatmap of differentially expressed genes identified by MarkerFinder analysis using AltAnalyze software. Columns represent cell population. Rows represent genes. **(B)** Heatmap of differentially expressed of IFN alpha and beta signaling pathway

(C) Heatmap of differentially expressed related to mitochondria. **(D)** SLAM HSCs from control and aTGFβ1-overexpressing mice were isolated 3 months after pIC stress and immunostained for the interferon α downstream effector IRF3, and counterstained with DAPI. N=50 cells from each mouse, using 6 mice/group. **(E)** Schematic representation of the main findings.