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

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Abstract

Bone marrow failure syndromes are characterized by ineffective hematopoiesis due to impaired fitness of hematopoietic stem cells. They can be acquired during bone marrow stress or innate and are associated with driver genetic mutations. Patients with a bone marrow failure syndrome are at higher risk of developing secondary neoplasms, including myelodysplastic syndromes and leukemia. Despite the identification of genetic driver mutations, the hematopoietic presentation of the disease is quite heterogeneous, raising the possibility that non-genetic factors contribute to the pathogenesis of the disease. The role of inflammation has emerged as an important contributing factor, but remains to be understood in detail. In this study, we examined the effect of increased transforming growth factor-β (TGFβ) signaling, in combination or not with an acute innate immune challenge using polyinosinc:polycytidilic acid (pIC), on the hematopoietic system without genetic mutations. We show that acute rounds of pIC alone drive a benign age-related myeloid cell expansion and increased TGFβ signaling alone causes a modest anemia in old mice. In sharp contrast, increased TGFβ signaling plus acute pIC challenge result in chronic pancytopenia, expanded hematopoietic stem and progenitor cell pools, and increased bone marrow dysplasia 3-4 months after stress, which are phenotypes similar to human bone marrow failure syndromes. Mechanistically, this disease phenotype is uniquely associated with increased mitochondrial content, increased reactive oxygen species and enhanced caspase-1 activity. Our results suggest that chronic increased TGFβ signaling modifies the memory of an acute immune response to drive bone marrow failure without the need for a pre-existing genetic insult. Hence, non-genetic factors in combination are sufficient to drive bone marrow failure.

Introduction

Bone marrow failure (BMF) syndromes are rare hematologic diseases characterized by impaired fitness of hematopoietic stem cells (HSC) and ineffective hematopoiesis, resulting in the absence of one or more hematopoietic lineages in the peripheral blood.[1] BMF syndromes can be inherited[2] or induced by inflammatory stress, allogenic or autologous HSC transplantation, myeloablative chemotherapy, or abnormal activation of the auto-immune T-cell system.[3,4]

Most BMF syndromes and myelodysplastic syndromes (MDS) are associated with genetic mutations, in particular in epigenetic regulators. Nevertheless, BMF syndromes are quite heterogeneous disorders raising interest in understanding what factors contribute to the disease development, in addition to driver mutations. Substantial clinical data show that hyperactivity of inflammatory cytokines, including tumor necrosis factor-α, interleukin-6, and transforming growth factor–β (TGFβ), as well as innate immune signaling pathways contribute to the pathogenesis of BMF.[5,6] In particular, the TGFβ signaling pathway is known to be hyperactive in Fanconi anemia,[7] MDS,[8-11] Shwachman-Diamond syndrome,[12] and myelofibrosis.[13] TGFβ1 is a myelosuppressive cytokine: it is secreted as an inactive protein complex bound to latency-associated

peptide (LAP), and the latent TGF-beta1 binding protein-1 (LTBP1). Dissociation from the complex is necessary for biological activity. Active TGFβ1 proteins then signal through two serine/threonine kinases, type I and type II receptors and trigger several signaling pathways.[14] The functions of TGFβ1 are complex and highly context-dependent.[15] In the hematopoietic system, TGFβ1 inhibits or promotes cell growth, varying from cell to cell and in a dose-dependent manner. TGFβ controls HSC quiescence,[16,17] homing, and survival.[18] During aging, TGFβ1 can promote the expansion of a myeloid-biased HSC population.[16] TGFβ can also suppress erythropoiesis and myelopoiesis. Work in our laboratory previously showed that TGFβ contributes to a functional decline in HSC in murine transplant models by promoting HSC differentiation at the expense of self-renewal.[19] We found that TGFβ signaling, including canonical p-Smad2 and non-canonical pp38^{MAPK}, remains high in HSC after bone marrow transplantation in mice due to increased expression of the active form of TGFβ in HSC. Interestingly, pharmacological inhibition of TGFβ signaling during bone marrow reconstitution following transplantation improved HSC functions, suggesting that this increased TGFβ signaling causes HSC functional decline after bone marrow transplantation.[19] In mouse models of BMF or Fanconi anemia, pharmacological inhibition of TGFβ signaling also restores effective hematopoiesis *in vivo*.[7] Moreover, inhibitors of TGFβ signaling have shown promising results in improving hematopoiesis in MDS patients.[20] However, only 30% of patients respond to the treatment, indicating that other factors contribute to BMF.[8,10,20] Dysregulation of several innate immune pathways are also known factors contributing to BMF or MDS. Toll-like receptors or their signaling effectors are often overexpressed in MDS samples compared to healthy controls, enhancing a type I interferon response through NF-κB, MAPK, and IRF3.[5] Other innate immune pathways, including the inflammasome and the necrosome, are also elevated in patients with BMF or MDS and contribute to ineffective hematopoiesis.[21,22]

Although inflammatory pathways are strongly implicated in human BMF syndromes and MDS, their exact contribution to the pathogenesis of the diseases remains to be understood. It is still unclear whether deregulated TGFβ signaling and/or inflammation are secondary events that contribute to the pathogenesis of the disease or can initiate the disease, and if so in which context.[23] In this study, we used a transgenic conditional mouse model overexpressing constitutively active TGFβ1 (aTGFβ1)[19] to further investigate the role of TGFβ1 in BMF/MDS. We show that a physiological increase in aTGFβ1 production in the bone marrow only produces mild neutropenia and anemia in mice during aging. However, the combination of increased TGFβ signaling plus polyinosinic:polycytidilic acid (pIC)-driven acute inflammatory stress drives chronic BMF with phenotypes similar to those of the human disorders associated with ineffective hematopoiesis, including BMF syndromes and MDS. Mechanistically, TGFβ prevents the termination of an acute pIC response causing permanent alteration in mitochondrial functions and increased caspase-1 activity. Our findings therefore suggest that BMF syndromes can be initiated solely by multiple inflammatory hits in the context of increased TGFβ signaling, and that disease outcome is dependent on the inflammatory context. Increased TGFβ signaling plus pIC thus represents a novel non-genetic-driven mouse model of human BMF-like diseases.

Methods

Mouse model

Transgenic Tg-b1glo$^{+/Flox}$ mice (Jackson Labs, Stock 018393) were crossed with Mx1-Cre mice to generate MxCre^{+}; Tg-b1glo$^{+/Flox}$ (TgCre^{+}) and MxCre^{-}; Tg-b1glo$^{+/Flox}$ mice (TgCre^{-}). Cre recombinase expression was induced with three injections of 10 mg/kg/mouse pIC (GE Healthcare), every other day. 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 an in-house, pathogen-free facility, and all studies were conducted with protocols approved by the Animal Care Committee of Cincinnati Children's Hospital Medical Center.

Flow cytometry

Peripheral blood samples were stained with CD45 PerCP-Cy5.5/APC Cy7, B220 APC/PE Cy7, Gr1 Alexa Fluor 700, Mac1/CD11b, CD3ε APC/PE, CD4 PE, and CD8a APC/PE.

Whole bone marrow cells were stained as above for mature lineages. Cells were also stained for Lineage^{-} Sca1^{+} Kit^{+} CD48^{-} CD150^{+} (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 incubated at 37°C in 5% CO_2 for 30 min with either MitoSOX Deep Red Reagent (1 μM, Invitrogen) or tetramethylrhodamine ester (0.1 μM, Sigma Aldrich). Caspase 1 activity was determined using the FLICA assay (Corning), according to the manufacturer's recommendations. Samples were then analyzed using a BD LSR II, BD LSR Fortessa, or BD Canto III (BD Biosciences). All antibodies were obtained from BD Biosciences, unless otherwise noted.

Enzyme-linked immunosorbent assays

Active TGFβ1 was assessed in bone marrow fluid 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

Tissues were fixed with 10% formalin, and stained with hematoxylin and eosin. Whole bone marrow cells were also prepared by cytospin and stained using Kwik-Diff (Fisher Scientific).

RNA sequencing

cDNA from 500 SLAM HSC was 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). Sequencing was done by the Cincinnati Children's Hospital Medical Center core and Alt-analyze was used for the analyses,[24] as previously described.[25,26] Differentially expressed genes were then analyzed using the ENRICHR database.[27]

Immunofluorescence assays

SLAM HSC were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton-X 100, 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 were taken using the Nyquist limit setting (0.1 μm XY pixel size) at 100X magnification. Images were analyzed using the surface building Matlab extension in Imaris software for at least 30 cells in each group.

Statistical analyses

Experiments were done in two or three replicates unless specified otherwise. Statistical analyses were performed using an unpaired Student *t*-test, unless specified otherwise.

Results

Increased TGFβ1 causes mild neutropenia with age

To further investigate the role of increased TGFβ signaling in hematopoiesis, we crossed a transgenic mouse model containing a transgene of aTGFβ1 with an Mx1-Cre mouse. The transgene contains two point mutations (C223S and C225S) to prevent the inhibitory latent associated peptide (LAP) from binding upon expression, permitting the produced ligand to initiate downstream signaling immediately after expression (Figure 1A).[28] Offspring containing both Mx1-Cre and aTGFβ1 constructs ($TgCre^+$) were then used for further experiments, with mice containing the transgene but without Cre ($TgCre^-$) as controls – thus preventing confounding issues associated with the leakiness of Mx1-Cre (*Online Supplementary Figure S1A*).[29] Mice were injected with pIC 10 mg/kg/mouse three times, once every 48 h, to cause aTGFβ1 overexpression (Figure 1B). We previously confirmed that aTGFβ1 levels are higher in bone marrow LSK cells ($Lin^-Sca1^+c\text{-}Kit^+$) from $TgCre^+$ mice.[19] Because TGFβ ligands are secreted in the extracellular matrix of tissues, we here examined levels of aTGFβ1 that were released in the bone marrow microenvironment. Bone marrow fluid of $TgCre^+$ mice contained higher levels of aTGFβ1 compared to control mice, up to 6 months following aTGFβ overexpression (Figure 1C). In this model, aTGFβ1 levels were in the range of 50 to 100 pg/mL. Levels of the activated form of the canonical TGFβ signaling transcription factor Smad2 in phenotypically identified SLAM HSC ($Lin^-Sca1^+c\text{-}Kit^+CD48^-CD150^+$) 3-4 weeks after inducing overexpression were higher in $TgCre^+$ SLAM HSC (Figure 1D). No signs of fibrosis were observed in the bone marrow of these mice up to 6 months after inducing overexpression (*Online Supplementary Figure S1B*). Hence, in this model, the increased TGFβ1 levels remain physiological, not higher than in some clinical MDS samples and much lower than in other aTGFβ1-overexpressing mouse models associated with myelofibrosis.[9,30-32]

To understand the impact of increased TGFβ signaling on the hematopoietic system, mice were analyzed for up to 12 months after overexpression had been induced (Figure 1B). Differential blood count analysis indicated that mice overexpressing aTGFβ1 developed modest neutropenia and anemia by 6 to 12 months of age compared to control animals (Figure 1E). Examination of the peripheral blood by flow cytometry analysis confirmed a mild reduction in myeloid cell frequency but increased T-cell frequency (*Online Supplementary Figures S1C* and *S2A*). We next examined the consequence of aTGFβ1 overexpression in the bone marrow and spleen 3 months after TGFβ overexpression. Bone marrow cellularity was not significantly different between the groups (*Online Supplementary Figure S1D*). However, $TgCre^+$ mice had splenomegaly (*Online Supplementary Figure S1E*). Hematoxylin and eosin staining of sections of the femur and spleen taken from $TgCre^+$ and $TgCre^-$ mice showed no gross abnormalities (*Online Supplementary Figure S1F*).

Further analysis indicated that the total count of each hematopoietic stem or progenitor cell population in bone marrow was similar between $TgCre^+$ and $TgCre^-$ mice during aging (*Online Supplementary Figures S1G, H* and *S2*). At 6 months, a modest dysplasia in bone marrow neutrophils and myeloid progenitors of $TgCre^+$ mice was observed (Figure 1F-H, *Online Supplementary Figure S3*). A small percentage of neutrophils was hyper-nucleated; some promyelocyte/myelocytes had increased size with a higher cytoplasm to nucleus ratio (Figure 1F-H, *Online Supplementary Figure S3*). This effect did not persist and was no longer observed at 12 months, perhaps due to

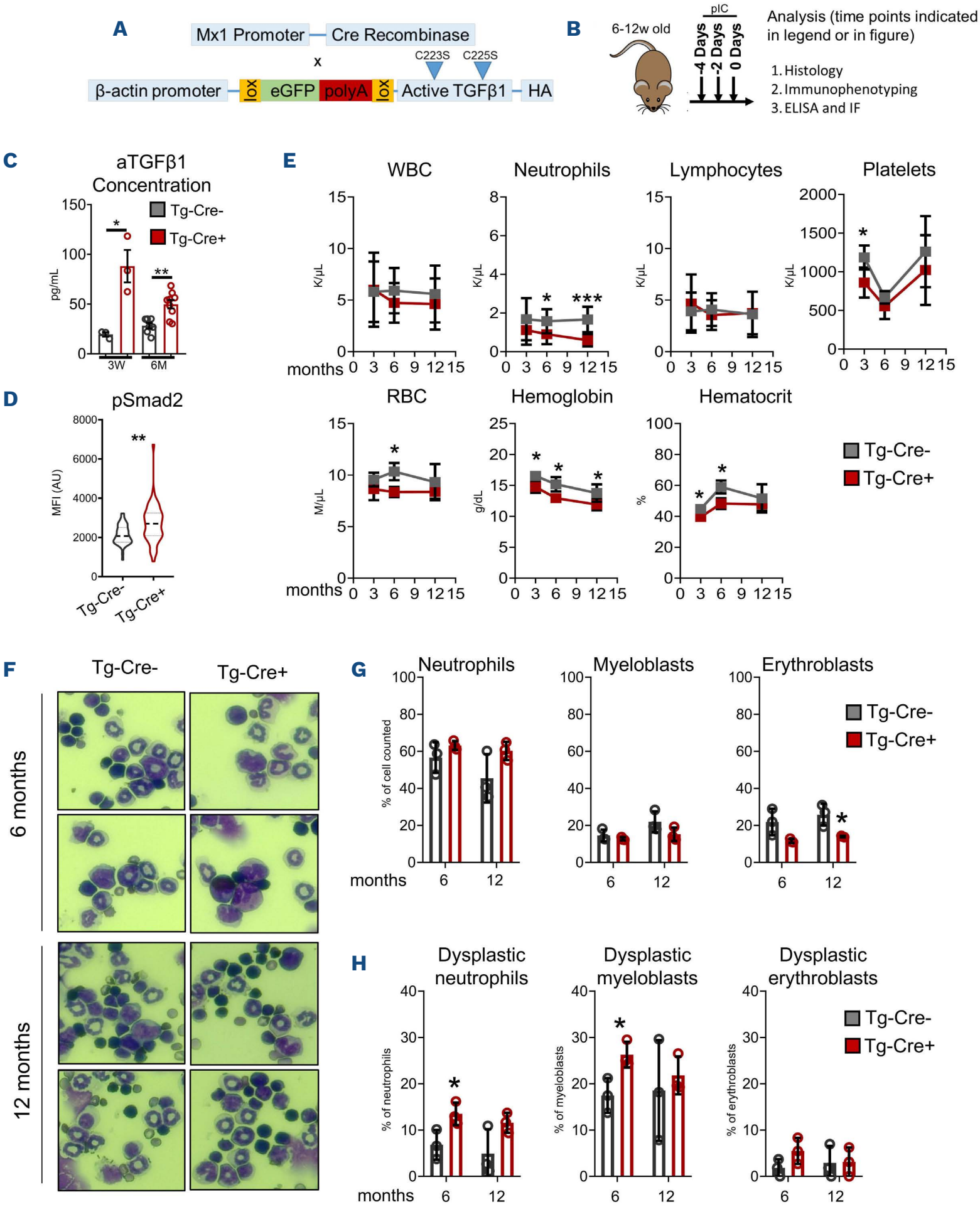


Figure 1. Mice overexpressing active TGFβ1 do not develop overt hematopoietic phenotypes during steady-state hematopoiesis. (A) Schema of the mouse model of active TGFβ1 (aTGFβ1) overexpression. (B) Schema of the experiments. (C) Levels of aTGFβ1 protein in bone marrow fluid from mice 3 weeks (3 mice/group) and 6 months (7 mice/group) after aTGFβ1 overexpression determined using an enzyme-linked immunosorbent assay. (D) Analysis of the TGFβ signaling effector phospho-Smad2 using immunofluorescence in LSK SLAM cells 4 weeks after aTGFβ1 overexpression, 50 cells/mouse, three mice/group. (E) Differential peripheral blood counts at indicated time points following aTGFβ1 overexpression. Five mice/group (3 months), seven TgCre^{-} mice, eight TgCre^{+} mice (6 months), seven mice/group (12 months). (F) Wright-Giemsa staining of bone marrow cytospins 6 and 12 months after aTGFβ1 overexpression, three mice/group. (G) Frequency of indicated cell types in bone marrow, three mice/group. (H) Frequency of dysplastic cells within each population, three mice/group. All experiments were conducted at least twice, with data shown as the mean ± standard error of the mean (SEM). Statistical significance was assessed using an independent Student *t*-test. ***P<0.001, **P<0.01, *P<0.05. ELISA: enzyme-linked immunosorbent assay; IF: immnofluorescence; WBC: white blood cells; RBC: red blood cells.

confounding aging effects. Hence, aTGFβ1 overexpression causes mild hematopoietic defects.

Mice overexpressing active TGFβ1 show significant long-term bone marrow failure after acute polyinosinic:polycytidilic stress

Several studies have previously demonstrated the role of innate immune signaling in the development of BMF and MDS.[5,6] The effects of multiple inflammatory stressors are however poorly defined. We examined the effect of acute innate immune stress in the context of enhanced TGFβ signaling. To this end, mice were challenged again with three injections of pIC, 1 month following aTGFβ overexpression (Figure 2A) and then analyzed 2 and 90 days later (Figure 2A). Mice re-challenged with pIC were termed TgCre$^-$ pIC$^+$ and TgCre$^+$ pIC$^+$, respectively. Remarkably, compared to TgCre$^-$ pIC$^+$ mice, TgCre$^+$ pIC$^+$ mice developed significant peripheral blood pancytopenia, including neutropenia, lymphocytopenia and thrombocytopenia, beginning 3 months after pIC stress (Figure 2B). Persistent anemia was also noted in TgCre$^+$ pIC$^+$ mice and this was more pronounced than without pIC challenge (Figure 2B). TgCre$^+$ pIC$^+$ mice had larger spleens with an expansion of the white pulp (*Online Supplementary Figure S4A, B*), whereas total bone marrow cell count and density remained unchanged compared to those of control mice (*Online Supplementary Figure S4C, D*).

We then examined bone marrow parameters in response to pIC stress. The acute response to pIC was comparable between the groups. Each group showed an increase in multipotent progenitors (MPP, Lineage$^-$ Sca1$^+$ Kit$^+$ CD48$^+$) whereas SLAM numbers were unchanged 2 days after pIC challenge (Figure 2C). Granulocyte/macrophage progenitors (GMP, Lineage$^+$ Sca1$^-$ Kit$^+$ CD34$^+$ CD16/32$^+$) increased; common myeloid progenitors (CMP, Lineage$^+$ Sca1$^-$ Kit$^+$ CD34$^+$ CD16/32$^-$) and megakaryocyte/ erythrocyte progenitors (MEP, Lineage$^+$ Sca1$^-$Kit$^+$ CD34$^-$CD16/32$^-$) decreased (*Online Supplementary Figure S4E*). Interestingly, 90 days after pIC stress, TgCre$^+$ mice had higher numbers of MPP and SLAM HSC compared to control mice, whereas MEP numbers remained lower (Figure 2C, *Online Supplementary Figure S3E*). Furthermore, TgCre$^+$ pIC$^+$ mice had an increased frequency of myeloblasts and promyelocytes but reduced frequency of mature neutrophils in the bone marrow (Figure 2D,E), which was associated with a significant degree of myeloid cell dysplasia, including hypersegmented neutrophils, and increased cell size and cytoplasm to nuclear ratio in myeloblasts and promyelocytes (Figure 2D,F, *Online Supplementary Figure S4F*). This myeloid dysplasia is reminiscent of bone marrow cytology present in mouse models of MDS.[33]

Taken together, our data suggest that TgCre$^+$ pIC$^+$ mice develop ineffective hematopoiesis which is characterized by an expanded hematopoietic stem or progenitor cell pool, pancytopenia and myeloid cell dysplasia. Thus, multiple inflammatory hits – increased TGFβ signaling plus acute pIC challenge – together cause a disease that recapitulates features of human BMF/MDS-like diseases, suggesting that non-genetic factors can initiate the onset of long-lasting BMF/MDS disorders.

Double-stranded RNA and enhanced TGFβ signaling cause permanent changes in the gene expression profile of hematopoietic stem cells

To understand how an acute pIC challenge in the context of enhanced TGFβ signaling causes long-lasting ineffective hematopoiesis, we analyzed the global transcriptome profile of four groups of SLAM HSC: (i) from 3-month-old TgCre$^-$ mice, (ii) from 3-month-old TgCre$^+$ mice, (iii) from TgCre$^-$ mice 3 months after pIC re-challenge and (iv) from TgCre$^+$ mice 3 months after pIC re-challenge. For the pIC re-challenge groups, we chose to collect the SLAM HSC when pancytopenia began to manifest, i.e., 3 months after pIC re-challenge (Figure 3A). Data were analyzed using unsupervised principle component analysis and supervised hierarchical clustering in AltAnalyze®.[25,26] Principal component analysis separated the four groups of cells into four distinct clusters, indicating that each group possesses a unique transcriptional signature (Figure 3B). Hierarchical clustering indicated that pIC re-challenge profoundly altered the transcriptional landscape in SLAM HSC from both TgCre$^-$ and TgCre$^+$ mice in comparison to controls (Figure 3C), even 3 months following the transient pIC challenge. A large number of these differentially expressed genes were downregulated by pIC challenge and belonged to chromosome organization, mitochondrion, and cell cycle (Figure 3C, D - cluster 1). Cluster 2 represents genes that were upregulated by pIC; these genes mostly relate to mitochondrion and the respiratory chain complex (Figure 3C, E). Genes highlighted in cluster 3 relate to signal transduction, and were downregulated by pIC challenge, but more so in TgCre$^+$ SLAM HSC. Finally, specific differences in gene expression between TgCre$^-$ or TgCre$^+$ SLAM HSC after pIC challenge were noted, and are underscored by white boxes (Figure 3C). Thus, a transient pIC challenge caused long-lasting transcriptional changes in HSC.

A more detailed examination of which genes are differentially expressed in TgCre$^+$ SLAM HSC specifically after pIC challenge revealed that they belong to two main categories, interferon response genes and nuclear-encoded genes related to mitochondrial regulation. The MarkerFinder algorithm in AltAnalze identified that myeloid and innate immune genes, including TLR2/4/6 co-receptor *cd14*, *cxcl10*, *anxa3*, *olfm4*, and *s100a8/s100a9* were upregulated in TgCre$^-$ pIC$^+$ SLAM HSC but not in TgCre$^+$ pIC$^+$ SLAM HSC (Figure 3D, F, and *Online Supplementary Figure S5A*), thus correlating with the increase in peripheral blood myeloid cells in these

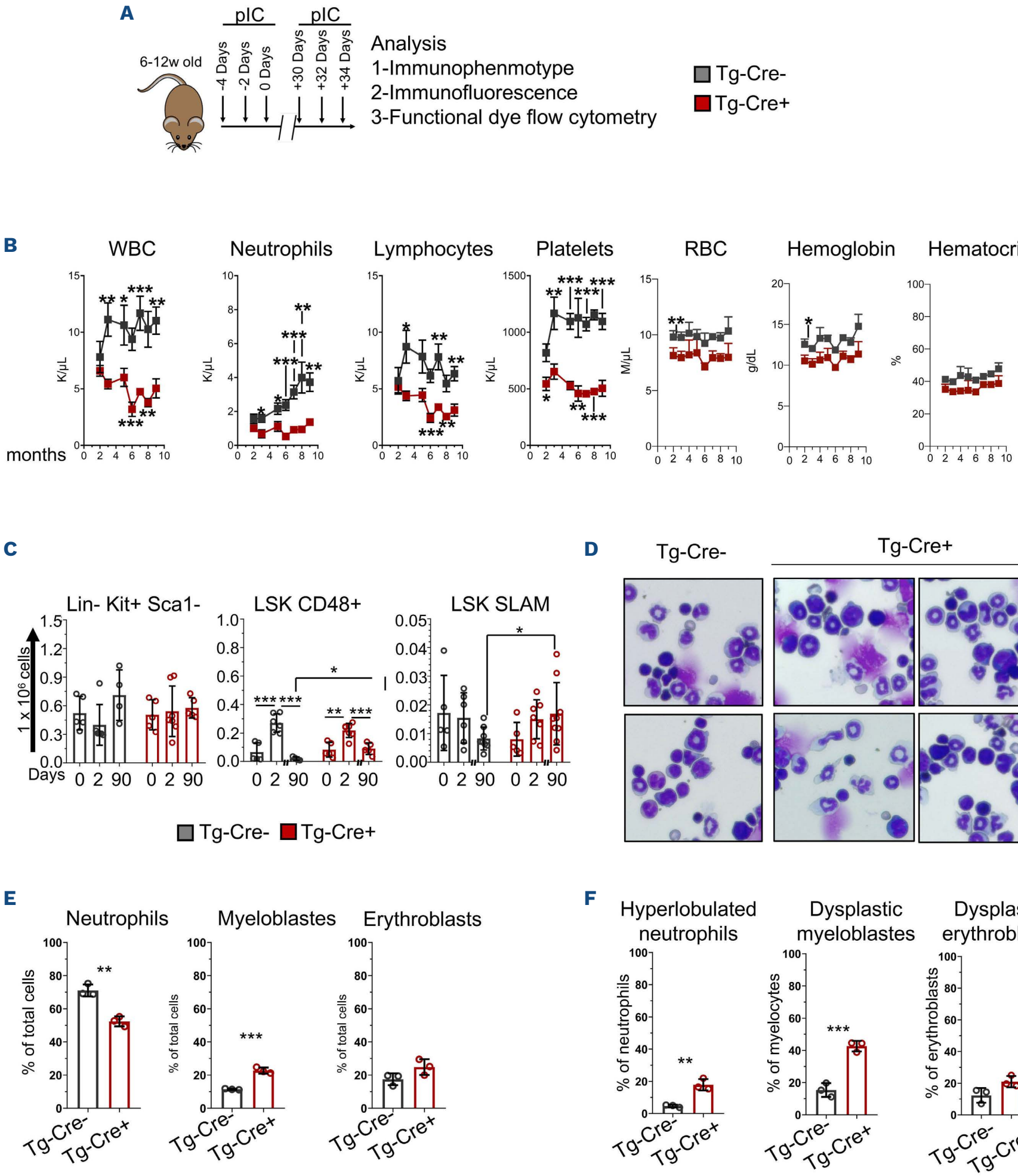
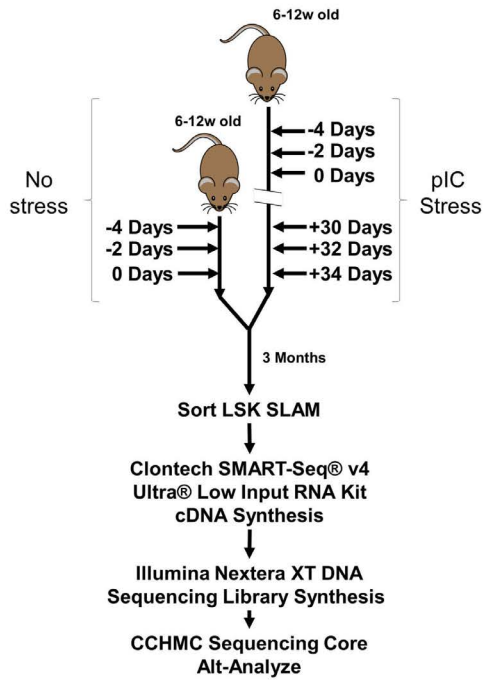


Figure 2. Mice overexpressing active TGFβ1 develop ineffective hematopoiesis with cell dysplasia after acute pIC stress. (A) Schema of the experiment. Mice were challenged with three injections of pIC 10 mg/kg/mouse every other day, performed at least 4 weeks after the induction of active TGFβ1 (aTGFβ1) overexpression. (B) Differential peripheral blood counts. Six mice/group. (C) Bone marrow cell counts of LSK, LSK CD48$^+$ and LSK SLAM. Five mice/group (No stress), seven mice (2 days), eight TgCre$^-$ mice, and nine TgCre$^+$ mice (90 days). (D) Wright-Giemsa staining of bone marrow cells 3-4 months after pIC stress. Normal arrows denote hyper-lobulated neutrophils; block arrows denote dysplastic myeloblasts; arrow heads denote dysplastic erythroblasts. Three mice/group. (E) Frequency of indicated cell types in bone marrow, three mice/group. (F) Frequency of dysplastic cells within each population. Three mice/group. Experiments were performed at least twice, with data shown as the mean ± standard error of the mean (SEM). Statistical significance was assessed using an independent Student *t*-test. ***P<0.001, **P<0.01, *P<0.05. WBC: white blood cells; RBC: red blood cells.

A



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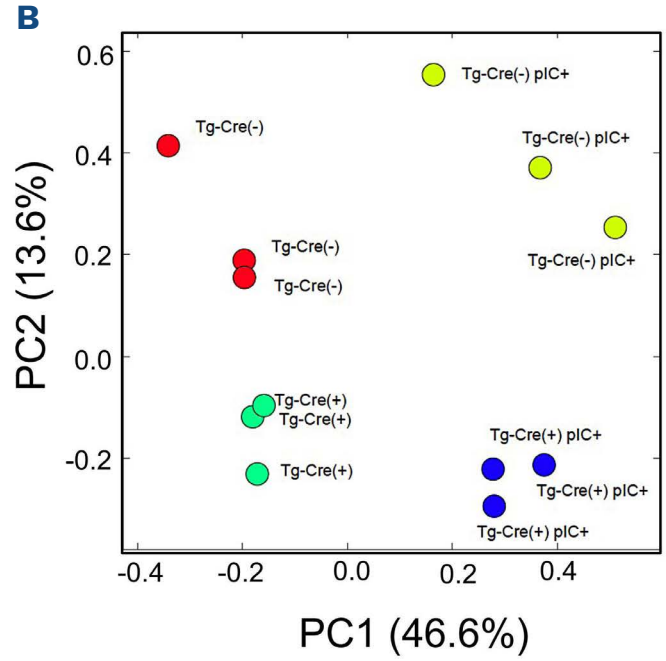

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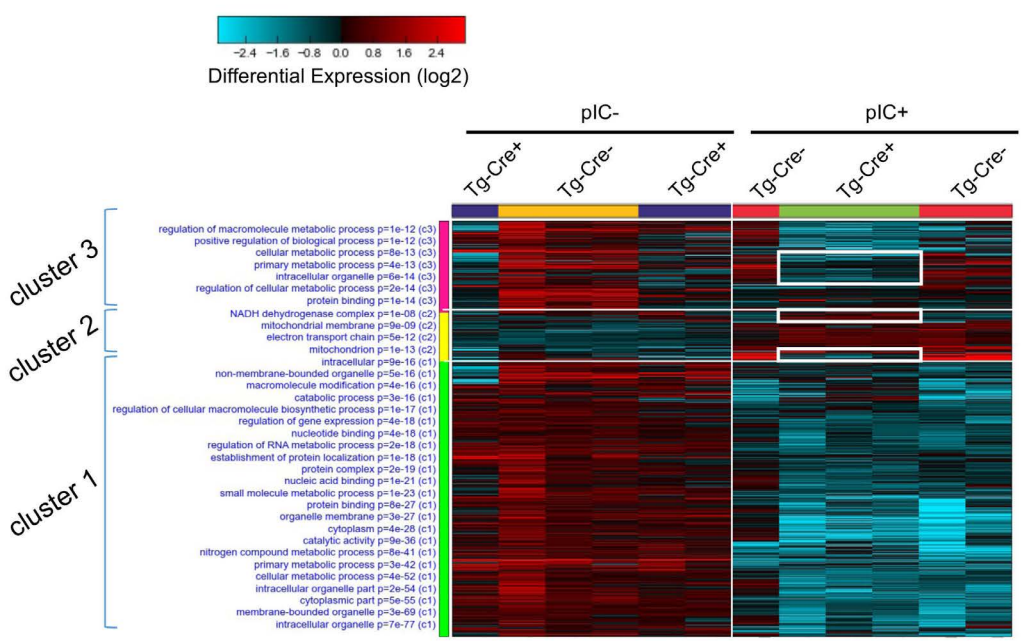

D

Cluster 1	*P*-value
chromosome organization	1.121E-21
mitotic cell cycle	1.088E-20
organelle fission	1.445E-19
mitochondrion	3.826E-28
nuclear protein-containing complex	1.150E-21
organelle subcompartment	6.217E-20
transferase complex	1.999E-19
catalytic complex	2.381E-19
ribonucleotide binding	6.613E-13
RNA binding	1.176E-11
catalytic activity, acting on RNA	2.707E-11

E

Cluster 2	*P*-value
mitochondrial inner membrane	1.073E-16
mitochondrial protein-containing complex	1.916E-16
mitochondrial envelope	6.163E-14
organelle envelope	8.231E-13
mitochondrion	1.746E-12
respirasome	2.632E-12
respiratory chain complex	2.176E-10
mitochondrial respirasome	3.040E-10
oxidoreductase complex	1.899E-9
NADH dehydrogenase complex	3.830E-9

F

Cluster 3	*P*-value
negative regulation of signal transduction	9.063E-10
negative regulation of cell communication	1.594E-9
negative regulation of phosphate metabolic process	7.687E-9
protein phosphorylation	4.878E-8
response to cytokine	1.751E-7
positive regulation of signal transduction	2.259E-7
actin cytoskeleton organization	2.541E-7
positive regulation of protein catabolic process	2.924E-7
negative regulation of protein modification process	1.375E-6
myeloid leukocyte differentiation	1.421E-6
cytoplasmic pattern recognition receptor signaling pathway in response to virus	1.614E-6

Figure 3. Active TGFβ1-overexpressing SLAM hematopoietic stem cells display a unique transcriptional signature long-term following acute pIC stress. (A) Schematic of the workflow of the transcriptomic analysis from SLAM hematopoietic stem cells (HSC) before and 3 months after pIC stress. (B) Principle component analysis visualization. (C) Hierarchical clustering of differentially regulated genes using pairwise comparative analysis. Columns represent cell populations. Rows represent genes. Three mice/group. (D-F) Top gene ontology categories of differentially expressed genes identified in cluster 1 (D), cluster 2 (E) and cluster 3 (F).

mice (Figure 2). We specifically interrogated differential gene expression of the interferon-α and -β signaling pathway. These genes were more downregulated in TgCre$^+$ SLAM HSC (*Online Supplementary Figure S5B*). We also interrogated genes related to mitochondrial regulation. Genes important for the regulation of mitochondrial translation such as *mrpl46* were upregulated only in TgCre$^+$ SLAM HSC after pIC stress, (Figure 3D, F and *Online Supplementary Figure S5C*). On the other hand, genes encoding regulators of mitophagy were downregulated in TgCre$^+$ SLAM HSC after pIC stress (*Online Supplementary Figure S5C*).

These findings strongly suggest that pIC causes significant and permanent transcriptional changes in SLAM HSC, some of which are modified only by aTGFβ1 overexpression.

Active TGFβ1-overexpressing SLAM hematopoietic stem cells show aberrant mitochondrial polarization and increased caspase 1 activity long-term following pIC stress

To functionally validate the gene expression findings, we first examined nuclear localization of IRF3, representing the active form of IRF3, and confirmed that IRF3 was not chronically activated in HSC from TgCre$^+$ pIC$^+$ mice (*Online Supplementary Figure S5D*). We then focused on mitochondria, as suggested by the transcriptional profile of TgCre$^+$ pIC$^+$ SLAM HSC. Mitochondria have emerged as a central platform for the activation of intracellular innate immune responses, including the inflammasome, which can be activated in response to pIC.[34,35] These immune responses are known to depend on and subsequently to alter mitochondrial metabolism. Interestingly, several groups have shown that samples from MDS patients exhibit abnormal mitochondrial functions, including increased cellular reactive oxygen species (ROS) and hyperpolarized mitochondria.[36-38] Alteration in nuclear-encoding mitochondrial gene expression was also found to be predictive of the development of secondary MDS after chemotherapy or bone marrow transplantation.

We first examined mitochondrial content by immunostaining for the mitochondrial outer membrane protein Tomm20 and performing high resolution z-stacked imaging and three-dimensional reconstruction analyses. Mitochondrial content was similar in SLAM HSC from both TgCre$^-$ and TgCre$^+$ mice before pIC stress (Figure 4A, B). However, 3 months after pIC stress, TgCre$^+$ pIC$^+$ SLAM HSC had higher mitochondrial content compared to control cells. This is consistent with a gene expression signature of elevated regulators of mitochondrial biogenesis and reduced regulators of mitophagy. Mitochondrial membrane potential, analyzed using tetramethylrhodamine ester, showed several differences between the groups. Shortly after pIC re-challenge (2 days and 7 days), mitochondrial membrane potential increased in SLAM HSC, MPP and the committed progenitor pool (CP, Lineage$^-$ Sca1$^-$ Kit$^+$) populations from both TgCre$^-$ pIC$^+$ and TgCre$^+$ pIC$^+$ mice (Figure 4C). In the longer term (3 months), mitochondrial membrane potential returned to baseline in TgCre$^-$ pIC$^+$ SLAM HSC, MPP and CP populations. Interestingly, mitochondrial membrane potential remained high in TgCre$^+$ pIC$^+$ SLAM HSC but was reduced in TgCre$^+$ pIC$^+$ CP. Finally, we examined total cellular levels of ROS using CellROX staining. All hematopoietic cell populations, i.e, CP, MPP and SLAM HSC, from TgCre$^+$ pIC$^+$ mice displayed increased ROS levels compared to those from TgCre$^-$ pIC$^+$ mice (Figure 4D). Increased ROS in TgCre$^-$ pIC$^+$ HSC SLAM did not necessarily come from mitochondria since mitochondrial-driven superoxide levels, as assessed using MitoSOX Red dye, were not different between the groups (*data not shown*).

Caspase-1 activation can be induced by mitochondrial activation or intracellular ROS.[39,40] In other cell types, pIC can trigger the activation of caspase 1. We thus examined the effect of aTGFβ overexpression on pIC-induced caspase-1 activity using the FAM-FLICA caspase-1 assay. TgCre$^+$ pIC$^+$ SLAM HSC had sustained caspase-1 activity compared to TgCre$^-$ pIC$^+$ SLAM HSC, as indicated by increased caspase-1 in TgCre$^+$ pIC$^+$ SLAM HSC up to 3 months after pIC challenge, compared to controls (Figure 4E).

These data suggest that aTGFβ1-overexpressing SLAM HSC maintain more active mitochondria, and have increased ROS levels and caspase-1 activity compared to control cells in the long term following acute pIC stress.

Discussion

In this study, we found that while a physiological and chronic increase in TGFβ signaling alone has little impact on the hematopoietic system, an additional but acute insult with pIC leads to long-lasting ineffective hematopoiesis that closely resembles the chronic BMF associated with myelodysplasia. Mechanistically, acute pIC imposes permanent transcriptional changes in HSC, which, in the context of increased TGFβ signaling, are associated with a long-lasting increase in mitochondrial content, hyperpolarized mitochondria, increased intracellular ROS and caspase-1 activity. These results imply that inflammatory stresses are sufficient to cause long-lasting BMF/MDS-like disorders without the need for driver mutations. These findings may also provide insights into the causes of BMF/MDS-like disease heterogeneity in which disease outcome may vary with specific combinations of inflammatory insults and dosage of insult. Finally, these findings also have long-term implications for the use of combinatorial therapies for treating human BMF syndromes.

Inflammation has long been associated with acquired BMF syndromes.[5,6] There is also a strong correlation between inflammation (regardless of cause, duration and frequency) and the development of MDS. Independent

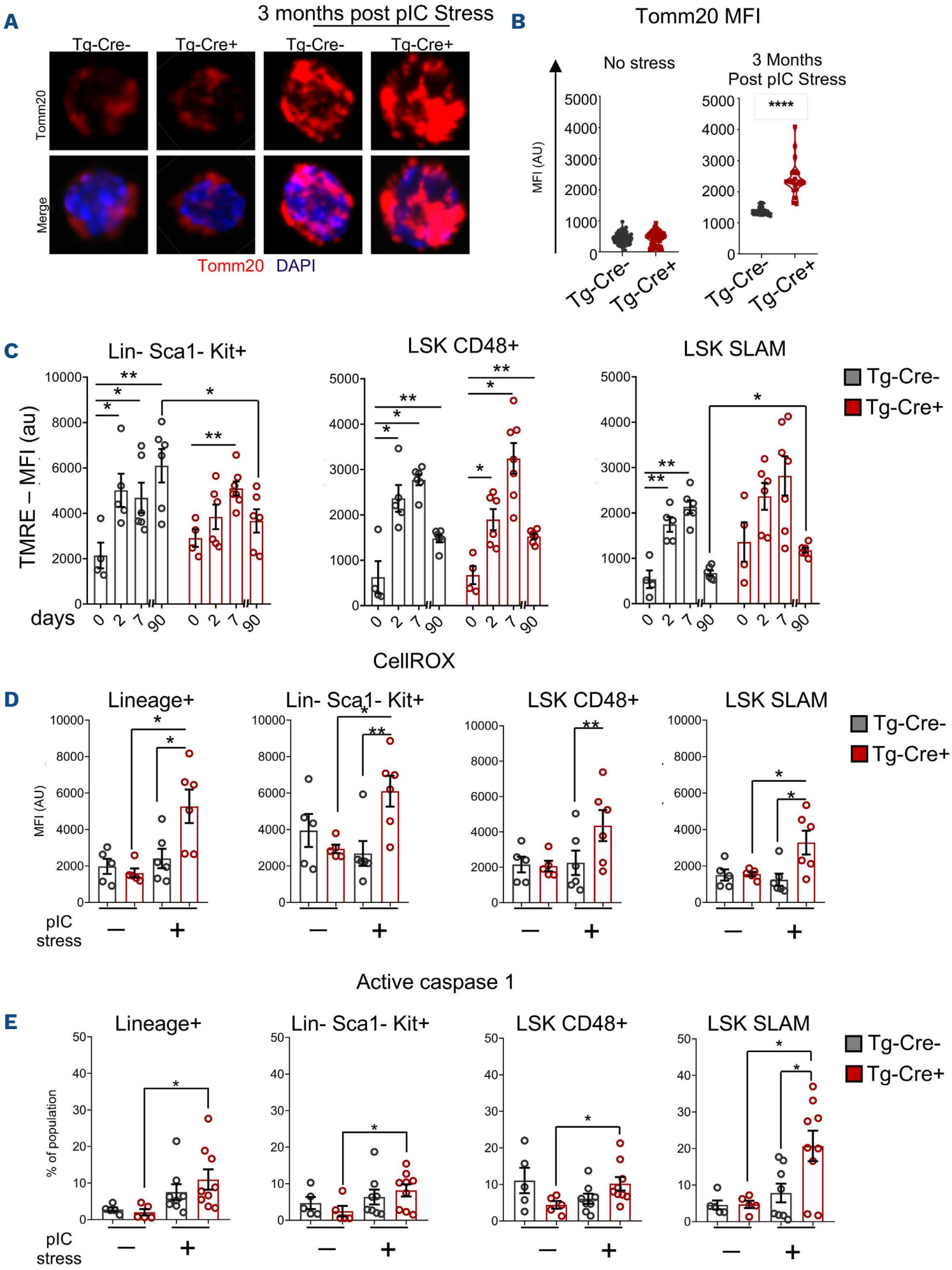

Figure 4. Active TGFβ1-overexpressing SLAM hematopoietic stem cells display sustained mitochondrial activity and caspase-1 activity long-term following acute pIC stress. (A,B) Mitochondrial content assessed in SLAM hematopoietic stem cells (HSC) using Tomm20 immunostaining. (A) Representative immunofluorescence images (Tomm20 in red, DAPI in blue). (B) Quantification of Tomm20 mean fluorescent intensity (MFI). Fifty cells from each mouse, six mice/group. (C) Mitochondrial membrane potential was assessed using tetra-methyl rhodamine ester dye (TMRE) staining at the indicated time after pIC stress. Four mice/group (0 days after pIC stress/no stress), Five TgCre^{-}, six TgCre^{+} (2 days post stress), six TgCre^{-}and seven TgCre^{+} (7 days after stress). Six mice/group (90 days after pIC stress). (D) Intracellular reactive oxygen species were measured using the CellROX Deep Red Reagent. Five mice/group (no stress), six mice/group (3 months after pIC stress). (E) Active caspase 1 was measured using the FAM-FLICA 660 kit. Five mice/group (no stress), eight TgCre^{-}, nine TgCre^{+} mice (3 months after pIC stress). Data are from at least two independent experiments and statistics were performed using an independent Student *t*-test. ***P<0.001, **P<0.01, *P<0,05. MFI: mean fluorescent intensity; AU: arbitrary units.

studies have demonstrated that innate immune signaling is responsible for phenotypes of some MDS subtypes, including del5q MDS. TGFβ signaling is a key driver of MDS and has been implicated in aplastic anemia, Fanconi anemia and Shwachman-Diamond syndrome.[7,9,11,12,41] Previous studies exploring the relationship between TGFβ signaling and BMF have used a similar aTGFβ1-overexpressing construct but under the control of the albumin promoter.[20,30] In the albumin-aTGFβ1 mouse model, aTGFβ1 overexpression produces concentrations much higher than in the mouse model used in our study, and higher than those found in MDS patients.[30] In this model, mice developed severe anemia, megakaryocyte dysplasia and marrow reticulin fibrosis within 3 weeks postpartum. Although informative, the acute presentation of the hematopoietic defects of this model prevented long-term assessment of the effects of a chronic increase in aTGFβ1 on disease development. TGFβ functions in a dose-dependent manner. In the hematopoietic system, low aTGFβ1 concentrations (pg/mL) stimulate HSC proliferation, whereas higher concentrations (ng/mL) are inhibitory.[42,43] Our model suggests that a modest increase in TGFβ signaling alone is not sufficient to drive severe BMF during steady-state hematopoiesis. Interestingly, an added acute innate immune signal allows a persistent HSC response leading to BMF. In the WT context, acute pIC challenge seems to cause an accelerated aging phenotype, at least related to myeloid expansion. In the context of enhanced TGFβ signaling, acute pIC challenge causes a BMF/MDS-like syndrome. These findings mean that disease outcome depends on a specific combination of inflammatory stressors, supporting the emerging hypothesis of the multiple inflammatory hit hypothesis to explain heterogeneity in BMF syndromes and MDS. Crosstalk between pIC and TGFβ signaling in the development of BMF was previously described in the context of Fanconi anemia, which is caused by mutations in DNA repair proteins via homologous recombination. Milsom's group dissected the response of HSC to pIC. They showed that in response to pIC, HSC exiting from quiescence sustain DNA damage that can be resolved by the Fanconi anemia-mediated DNA repair response. As such, WT mice recover from pIC stress. In contrast, *Fanca*-deficient mice had reduced numbers of HSC, unresolved DNA damage and developed severe BMF.[44] Interestingly, Zhang *et al.* showed that enhanced TGFβ signaling, known to be upregulated in patients with Fanconi anemia, contributes to pIC-induced BMF in *Fanca*-deficient mice by modifying the DNA repair response to pIC-induced DNA damage. When TGFβ signaling is high, HSC use error-prone non-homologous end-joining instead of homologous recombination, thus favoring DNA mutations. In this model, inhibition of TGFβ signaling rescued hematopoiesis in pIC-treated *Fanca*-deficient mice.[7] Collectively, these findings support the idea that TGFβ is a 'modifier' of HSC functions, which predisposes to the development of BMF/MDS. It also raises the interesting possibility that TGFβ-modified pIC-induced DNS damage could contribute to our phenotype. This will be interesting to examine further. The fact that an acute pIC challenge causes long-lasting effects in HSC is also notable. It means that pIC can induce long-lasting transcriptional memory in HSC. It will be interesting to examine whether this resembles the recently described trained immunity phenomenon,[45-47] whether other innate immune insults similarly synergize with TGFβ1 in disease development and what are the exact mechanisms behind this synergy.

TGFβ signaling is mostly known to act though canonical Smad signaling and non-canonical p38 MAPK signaling. Our data suggest that increased TGFβ signaling alters mitochondrial function and caspase-1 activity after pIC stress. The association between altered mitochondria and BMF/MDS is not unprecedented. Studies have demonstrated that Fanconi anemia patients have altered mitochondria[48] and respiratory chain defects.[49] Mitochondrial diseases themselves have hematologic phenotypes of varying degrees, such as Pearson syndrome, which presents with pancytopenia, and Barth syndrome, which presents with neutropenia. A study of patients with MDS or acute myeloid leukemia revealed transcriptional dysregulation of their mitochondria.[50] Several studies have also implicated mitochondrial dysfunction and increased intracellular ROS in driving the refractory anemia associated with MDS.[36-38] We recently reported that aberrant mitochondrial function is one source of abnormal HSC function.[26] Thus, our study supports the current knowledge that impaired or altered mitochondrial function contributes to ineffective hematopoiesis. Our study further suggests that there may be direct links between mitochondrial dysfunction and altered TGFβ signaling. It remains to be seen how TGFβ causes mitochondrial defects and how those defects contribute to ineffective hematopoiesis. One possibility is enhancing mitochondrial biogenesis, perhaps via Myc, which has been involved in BMF.[51] Another possibility would be abnormal activation of caspase-1, which can contribute to BMF.[21,52] Intracellular ROS, perhaps as a result of abnormal mitochondria, may be responsible for sustained caspase-1 activation.[39,40] The elevation of intracellular ROS in aTGFβ1-overexpressing SLAM HSC after pIC stress in our mouse model may therefore not only have direct genotoxic effects, but may also synergize with and amplify pIC-mediated caspase-1 activation to drive BMF. The functional outcome of enhanced caspase-1 activation in HSC remains unclear. In our model, it is unlikely that the outcome is only cell death since the SLAM HSC pool is in fact expanded in TgCre^{+} pIC^{+} mice. While caspase-1 is known to cause cell death by pyroptosis, other studies have shown that caspase-1 also controls glycolysis during *Salmonella typhimurium* infection by tar-

geting and cleaving key glycolytic enzymes such as aldolase, triose-phosphate isomerase and α-enolase.[53] Activated caspase-1 can also induce the activation of sterol regulatory element binding proteins, responsible for regulating lipid membrane biogenesis, to favor cell survival instead of causing cell death.[54] A careful examination of the role of caspase-1 activity in HSC is therefore needed.

In conclusion, we have described a mouse model of BMF that results from an acute inflammatory challenge in the context of increased TGFβ signaling. This model recapitulates phenotypes of human BMF syndromes that are linked to TGFβ signaling. This mouse model will help not only to further our understanding of the pathogenesis of BMF syndromes and MDS associated with TGFβ signaling but also provides an *in vivo* model to test effects of combinatorial therapy to cure these disorders.

Disclosures

No conflicts of interest to disclose.

Contributions

JJ, AH and M-DF conceived the study; JJ, AH, JB, and JX were responsible for the methodology; JJ, AH, JB, and JX conducted the investigations; M-DF was responsible for the formal analysis; JJ and M-DF wrote the original draft, reviewed and edited it; JJ and M-DF acquired funding for the study; M-DF provided supervision.

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Data availability

All data are available, including scRNA-sequencing accession codes and a Gene Expression Omnibus accession number. Figures 1-4 have associated raw data. Raw images are also available upon request

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