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14. ABSTRACT

Bone marrow failure syndromes (BMF) are characterized by ineffective hematopoiesis due to impaired fitness of hematopoietic stem cells (HSC). BMFs can be acquired during bone marrow stress or innate are associated with driver genetic mutations. BMFs are at higher risks 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 factors, but remain to be understood in detail. The goals of this project are to determine the role of inflammation and stress signaling as non-genetic factors initiating and contributing BMF/MDS. We show that increased TGFβ signaling plus acute pIC challenge result in chronic pancytopenia, and increased bone marrow dysplasia 3-4 months after stress, phenotypes similar to human bone marrow failure syndromes. Mechanistically, this disease phenotype is uniquely associated with enhanced caspase-1 activity. Further, we show that the phenotype is MAVS-dependent. Together, these findings uncover chronic increased TGFβ signaling modifies an acute immune response to drive bone marrow failure without the need for pre-existing genetic insult. Hence, non-genetic factors in combination are sufficient to drive bone marrow failure.

15. SUBJECT TERMS
None listed.

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1-Introduction.

Bone marrow failure syndromes (BMFS) are life threatening diseases. They are classified into two major subgroups, inherited or acquired. BMFS are characterized by ineffective hematopoiesis leading to the absence of one or more hematopoietic lineages in the peripheral blood. [1-4] BMF or poor hematopoietic cell functions can develop under stress conditions such as after allogenic or autologous hematopoietic stem cell transplantation (HSCT) or after myeloablative chemotherapy. BMFS patients are at high risk of developing secondary clonal hematopoietic neoplasms, including myelodysplastic syndrome (MDS) and/or acute myeloid leukemia (AML). [5] [6] with some patients developing secondary MDS/AML within 6 years of autologous HSCT. [7] Although BMFS have established clinical and pathophysiological features, the exact causes of acquired BMFS, especially in the context of post-stress hematopoiesis, are ill-defined. This lack of knowledge has hindered our effort to develop effective therapeutic intervention of BMF. BMF/MDS are always associated with a defined numbers of somatic mutations. Yet, not everyone carrying similar somatic mutations will develop the diseases, underscoring the need for better understand which factors, genetic and/or non-genetic, predispose to BMF/MDS. The central goal of this proposal is to *identity non-genetic factors that drive BMF, with a specific focus on inflammatory and stress-induced cytokines*.

2- **KEYWORDS:**

Bone marrow failure

Myelodysplastic syndrome

Hematopoietic stem cell

Inflammation

Stress signaling

Innate immune signaling

DNA damage

3- ACCOMPLISHMENTS:

Major Goals:

Aim1 Define how cooperation between polyIC and TGFβ causes BMF/MDS

Aim2 Investigate the role of MAVS in inflammation-dependent BMF/MDS development in the context of increased TGFβ signaling

Accomplishments

Aim1:

Major Task 1: use genetic mouse models of gain of TGFβ1 protein (we have these mice) in HSCs, and determine effects of inflammatory stress on hematopoietic cell survival, HSC self-renewal and blood cell production

SubTask 1 examine Effect of polyIC and TGFβ on HSC, progenitors and mature blood cell production in vivo

We examined the effect of acute innate immune stress in the context of enhanced TGFβ signaling. To this end, mice were challenged again with 3 injections of pIC, one month following aTGFβ overexpression and then analyzed 2 and 90 days later. Mice re-challenged with pIC were termed $TgCre^{-}$ pIC^{+} and $TgCre^{+}$ pIC^{+}, respectively. Remarkably, $TgCre^{+}$ pIC^{+} mice developed significant PB pancytopenia, including neutropenia, lymphopenia and thrombocytopenia, beginning 3 months after pIC stress compared to $TgCre^{-}$ pIC^{+} mice. A persistent anemia was also noted in $TgCre^{+}$ pIC^{+} mice, that was more pronounced than without pIC challenge. $TgCre^{+}$ pIC^{+} mice had larger spleens with an expansion of the white pulp, whereas total BM cell count and density remained unchanged compared to control. (Figure 1B) Three experiments were performed; two experiments were done and analyzed prior the DOD and served as preliminary data, one experiment was done and analyzed under the DOD-y1.

We previously examined BM 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. Granulocyte/macrophage progenitor (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. Interestingly, 90 days after pIC stress, $TgCre^{+}$ mice exhibited higher MPP and SLAM HSC numbers compared to control mice, whereas MEP cells remained lower. Under the DOD we examined BM at 9M following pIC stress, $TgCre^{+}$ mice exhibited lower numbers of HSC in BM, indicative of HSC exhaustion following stress (Figure 2A). Further, $TgCre^{+}$ pIC^{+} mice displayed increased frequency of myeloblasts and promyelocytes but reduced frequency of mature neutrophils in BM , that was associated with significant degree of myeloid cell dysplasia, including hypersegmented neutrophils, and increased cell size and cytoplasm to nuclear ratio in myeloblasts and promyelocytes at 3M post pIC (Figure 1D, E, F) but less so at 9M post-pIC (Figure 2B). 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 that is characterized by an expanded HSPC 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.

SubTask2 Effect on HSC quiescence and survival

We examined the effect of acute innate immune stress in the context of enhanced TGFβ signaling on HSC cell cycle and survival. Mice were challenged again with 3 injections of pIC, one month following aTGFβ overexpression and then analyzed 2 (DOD) and 90 days (prior to DOD) later. Two days after pIC challenge, frequency of LSK-CD48 and LK cells in S phase was significantly lower in Tg-Cre+ mice than in Tg-Cre-, suggesting that enhanced TGFβ signaling prevents cell cycle progression in response to inflammatory challenge (Figure 3). However, at 90 days, LSK-SLAM cells from both genotype were fully quiescent, indicating that the effect of TGFβ signaling on HSC proliferation was transient. No difference in HSC survival, as examined using Annexin V staining, were noted (DOD-Figure 4).

Subtask3: effect on HSC self-renewal will be examined in subsequent transplant studies.

To do this, LSK-SLAM cells were isolated 3M following pIC stress and transplanted into sublethally irradiated recipient mice. Donor-derived cell chimerism in the peripheral blood was examined monthly for 4-6 months. This experiment was not conclusive because the transplanted cells derived from Tg-Cre+ mice did not show significant expression of the TGFβ transgene. This experiment needs to be repeated and will be in the next year of this award.

Major Task2 Assess effect on genotoxicity in HSC and progenitors: this task will be done in year 2 of this award, instead of year 1, due to COVID and changes in work organization. Instead major task 3 was started, and experiments related to Aim2 were also started.

Major Task3 test effect on initiation, selection and expansion of mutant hematopoietic clones whole exome sequencing

For this LSK cells were isolated 3M following pIC stress, DNA was prepared and will be sent for sequencing in the next month; results will be analyzed in year 2.

Aim2:
Major Task 3 test the functional importance of MAVS activity using MAVS-deficient mice in the context of inflammation and enhanced TGFβ signaling

Subtask 1 Effect on HSC, progenitors and mature blood cell production in vivo will be examined (30% completed)

To this end, Tg-Cre+ mice were crossed with MAVS-deficient mice [Mavs-/-] assess if MAVS deficiency prevents polyIC+TGFβ-driven BMF/MDS development. Mice were challenged with 3 injections of pIC, one month following aTGFβ overexpression and peripheral blood was analyzed between month 3 and 6 following pIC stress. Remarkably, $TgCre^{+}$ pIC^{+} mice crossed with Mavs-/- mice did not develop anemia or neutropenia. However, these mice developed thrombocytopenia. These data indicate that pIC+TGFb-induced anemia and neutropenia is MAVS-dependent, but thrombopenia is not MAVS-dependent. (Figure 5)

Major Task 4 Identify which signaling pathways mediates MAVS signaling focusing on PYCARD/inflammasome (20% complete)
Subtask 1: Examine NLPR3 inflammasome activity

We examined effect of aTGFβ overexpression on pIC-induced caspase-1 activity using the FAM-FLICA caspase-1 assay. Mice were challenged with 3 injections of pIC, one month following aTGFβ overexpression and then analyzed 2, 90 and 180 days later. Two days after pIC challenge, LSK-SLAM from both genotype exhibited significant increase in caspase 1 activity, indicating that pIC causes activation of the inflammasome to similar extend in Tg-Cre+ HSC and in Tg-Cre- HSC. Interestingly, at 90 and 180 days, caspase-1 activity was no longer detectable in Tg-Cre- HSC whereas it was sustained in $TgCre^{+}$ pIC^{+} SLAM HSC, compared to no pIC challenge control (Figure 6A, one experiment was analyzed un the DOD). At 180 days, caspase and pyroptosis were elevated in $TgCre^{+}$ pIC^{+} SLAM HSC (Figure 6B, 2 independent experiments under the DOD). These data suggest that aTGFβ1-overexpression modifies pIC-induced innate immune response to prevent termination of the pathways. The next step will be to determine whether this increased in caspase-1 activity is MAVS-dependent or not.

-Opportunities for training and professional development

A graduate student performed all experiments; he generated and analyzed the data; he wrote a manuscript that is now submitted. He graduated in May 2021.

- How were the results disseminated to communities of interest?
A manuscript has been submitted for publication

What do you plan to do during the next reporting period to accomplish the goals?
The goals are to examine the effect of TGFb on DNA damage and repair pathways, as proposed in aim1 of SOW, and complete Aim2.

4- **IMPACT:**
-What was the impact on the development of the principal discipline(s) of the project?

The goals are to investigate how inflammation and TGFβ signaling together cause BMF/MDS. This is critical in order to identify key regulatory elements and inflammatory conditions that promote the initiation and progression of BMF/MDS. These studies are important because it could explain why patients with HSCT are more at risks of developing clonal hematopoietic neoplasm after viral infection; and why not everyone carrying similar somatic mutations will develop the disease. [24] BMFS are HSC diseases that are almost universally fatal.

Although it can be ameliorated by stem cell transplantation or immunosuppressive drug therapy for some BMFS, it is not sufficient and HLA-donor match are not always available. Hence, the risk of relapse and/or clonal evaluation to either MDS or AML is high in BMFS patients (10-20% of acquired AA and 40% in some inherited BMFS, most patient with HSCT and poor graft function will develop MDS/AML). This emphasizes the importance of understanding the factors that drive the onset and progression of BMF. Our hypothesis will provide evidence for a novel cooperation of factors, in BMF initiation and progression. It will have far-reaching implications for our fundamental understanding of basic mechanism of BMFS and provide rationale for the development of novel therapeutic interventions.

- **What was the impact on other disciplines?**
Nothing to report

-What was the impact on technology transfer?

Nothing to report

-What was the impact on society beyond science and technology?

Nothing to report

5- **CHANGES/PROBLEMS:**
As noted previously, the transplant experiment was not conclusive because the transplanted cells derived from Tg-Cre+ mice did not show significant expression of the TGFβ transgene. This experiment needs to be repeated and will be in the next year of this award. Also, major task2 of Aim1 will be done in year 2 instead of year1. Major task 3 and 4 of Aim2 were started in year 1 instead of year 2. Results from these experiments is described under accomplishment.

Because of shortage of mice, collaborative project under this award has not started, but will start next month. Subcontract was issued only recently.

6- **PRODUCTS**

A manuscript has been submitted for publication

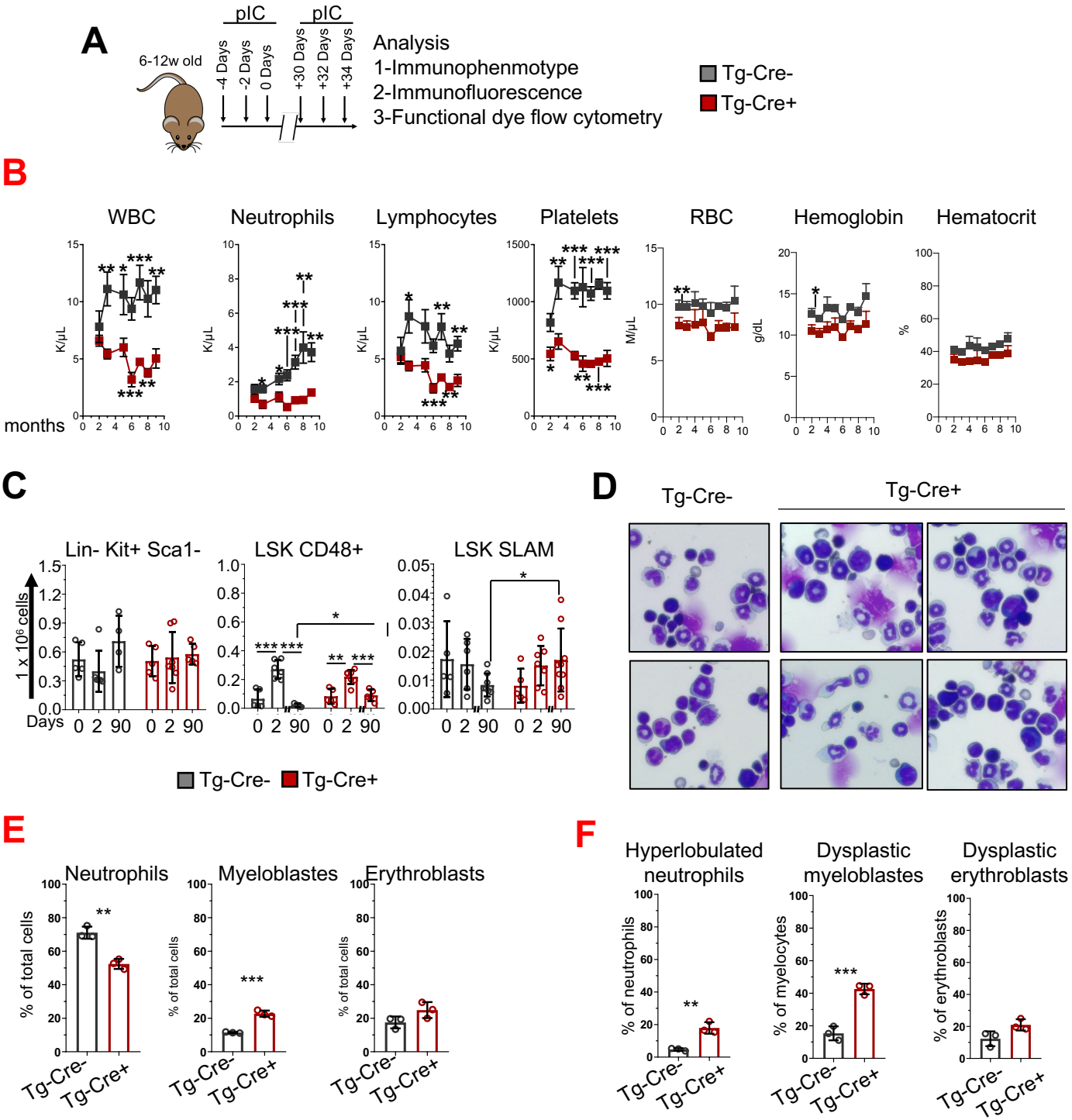
7- **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**

Name:	*Jose Javier*
Project Role:	*Graduate Student*
Contribution to Project:	performed all experiments; he generated and analyzed the data; he wrote a manuscript

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

What other organizations were involved as partners?
Nothing to report
SPECIAL REPORTING REQUIREMENTS
Nothing to report for this period

9-Appendice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pIC
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Lymphocytes
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Hematocrit
K/µL
M/µL
g/dL
%
months
C
Lin- Kit+ Sca1-
LSK CD48+
LSK SLAM
1 x 10⁶ cells
Days
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Tg-Cre+
D
Tg-Cre-
Tg-Cre+
E
Neutrophils
Myeloblastes
Erythroblasts
% of total cells
Tg-Cre-
Tg-Cre+
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Tg-Cre-
Tg-Cre+

Figure 1

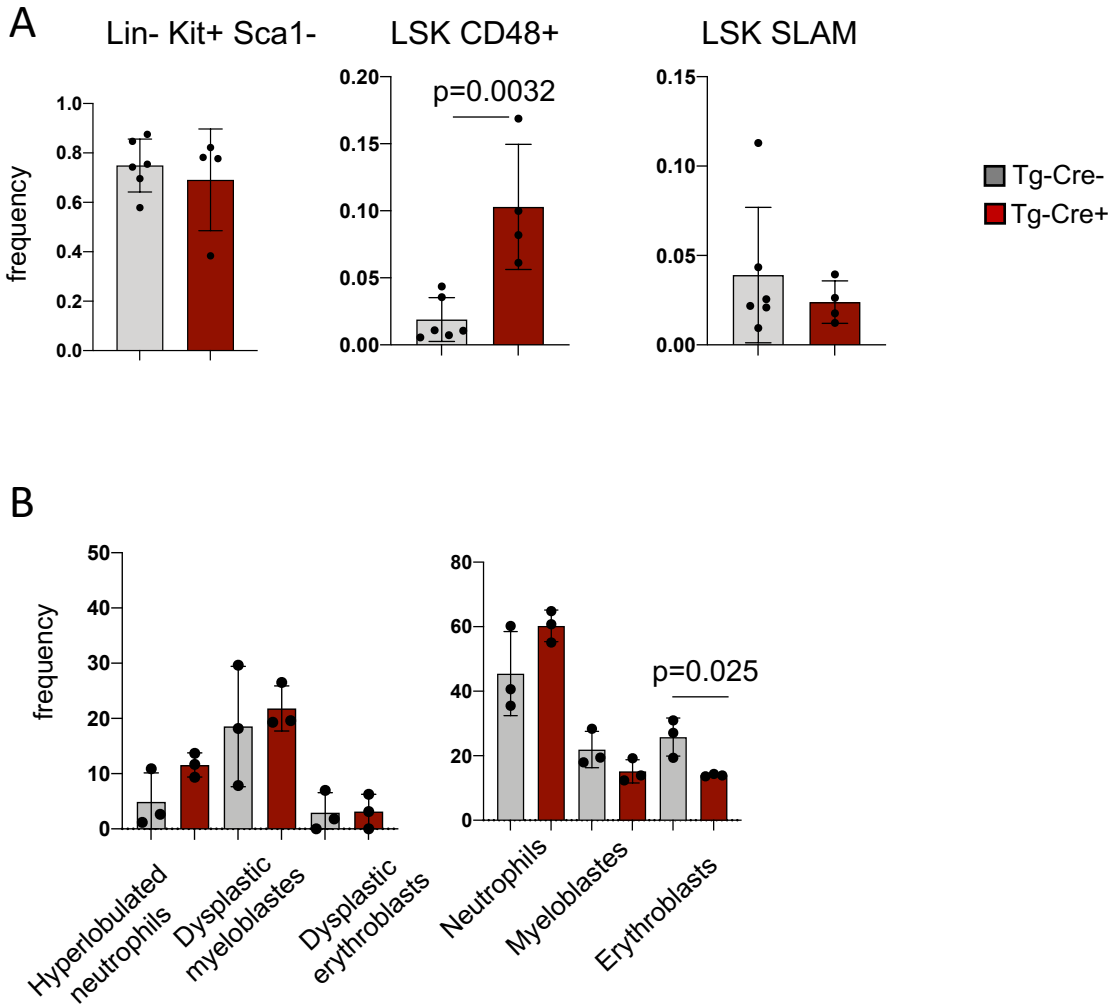
A
Lin- Kit+ Sca1-
LSK CD48+
LSK SLAM
frequency
p=0.0032
Tg-Cre-
Tg-Cre+
B
frequency
p=0.025
Hyperlobulated neutrophils
Dysplastic myeloblastes
Dysplastic erythroblasts
Neutrophils
Myeloblastes
Erythroblasts

Figure 2

Cell cycle analysis 48h post-pIC challenge

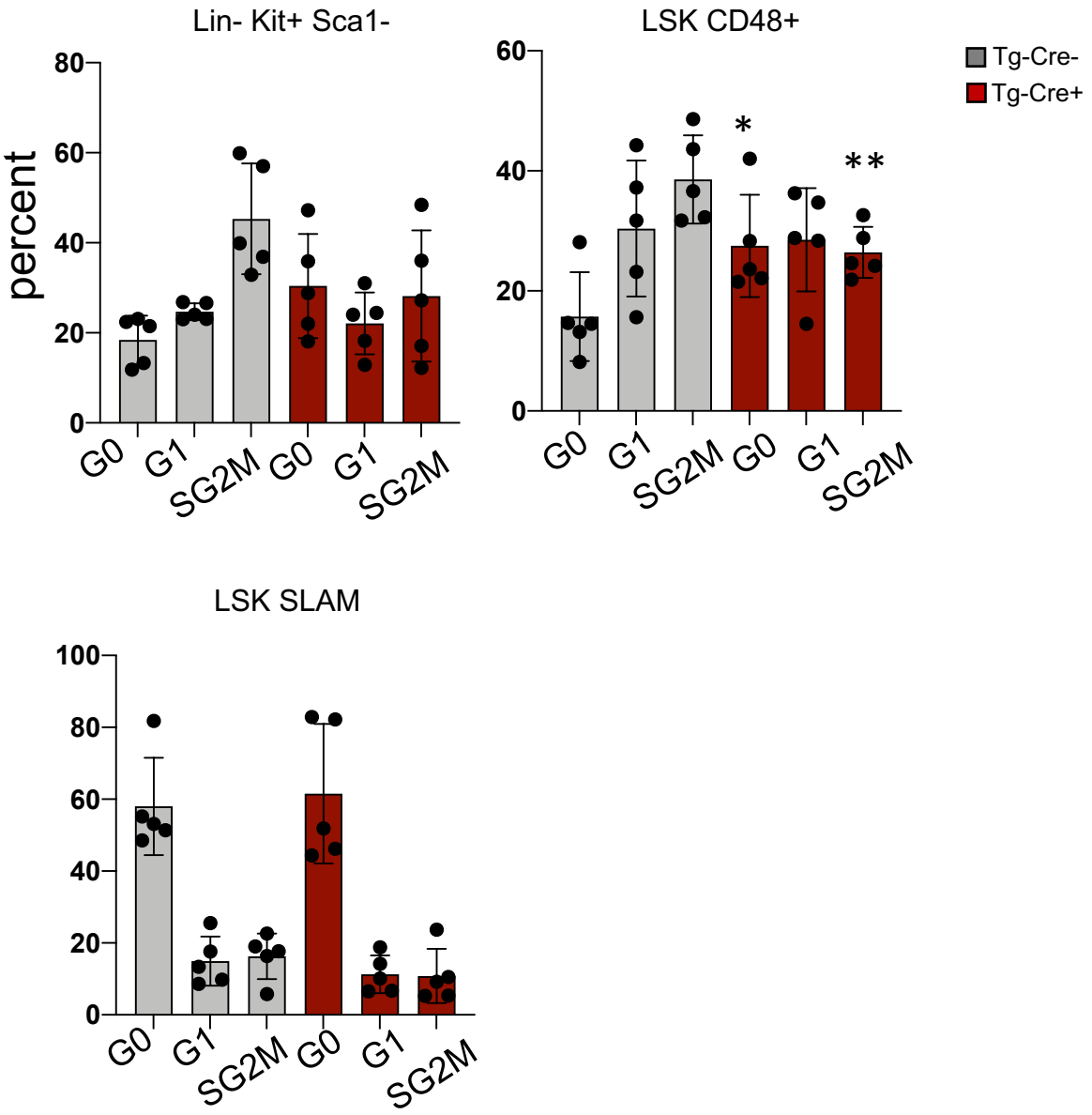

Figure 3

Apoptosis (annexin V)

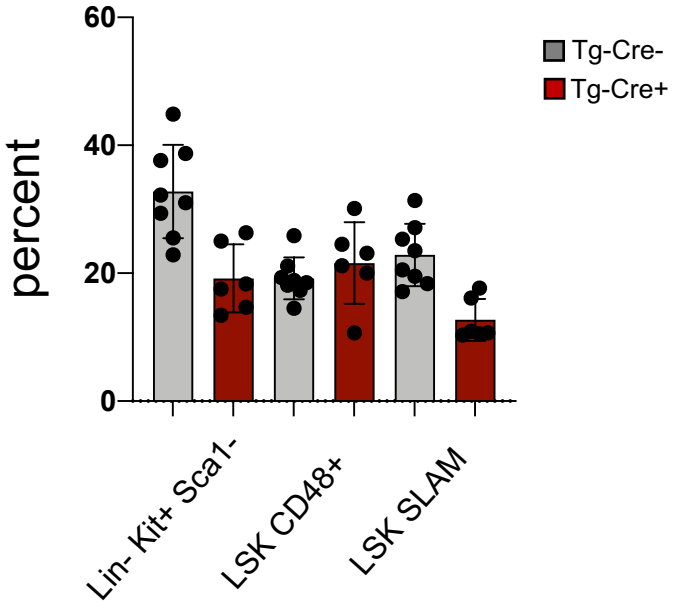

Figure 4

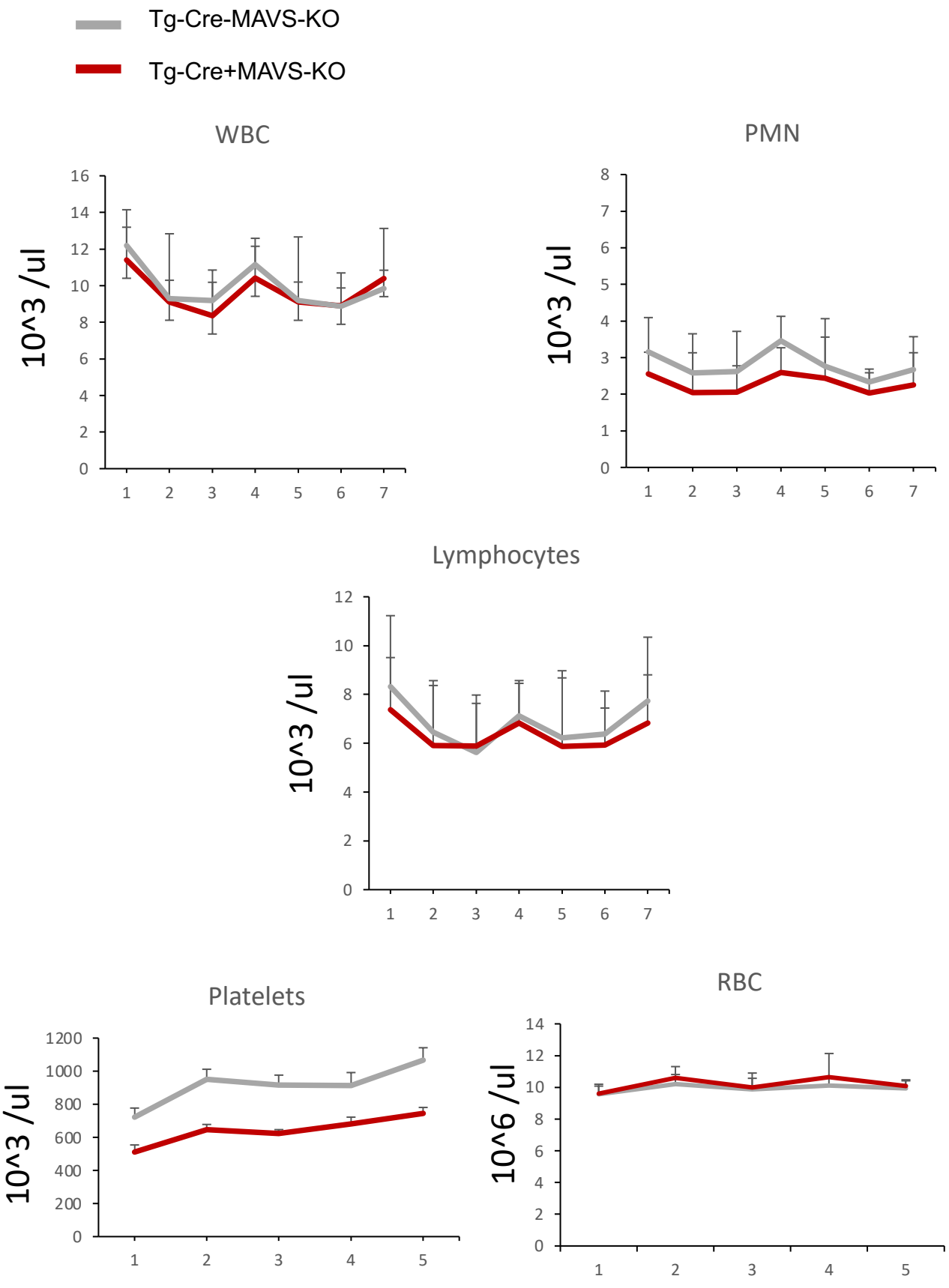
Tg-Cre-MAVS-KO
Tg-Cre+MAVS-KO
WBC
10^3 /ul
PMN
10^3 /ul
Lymphocytes
10^3 /ul
Platelets
10^3 /ul
RBC
10^6 /ul

Figure 5

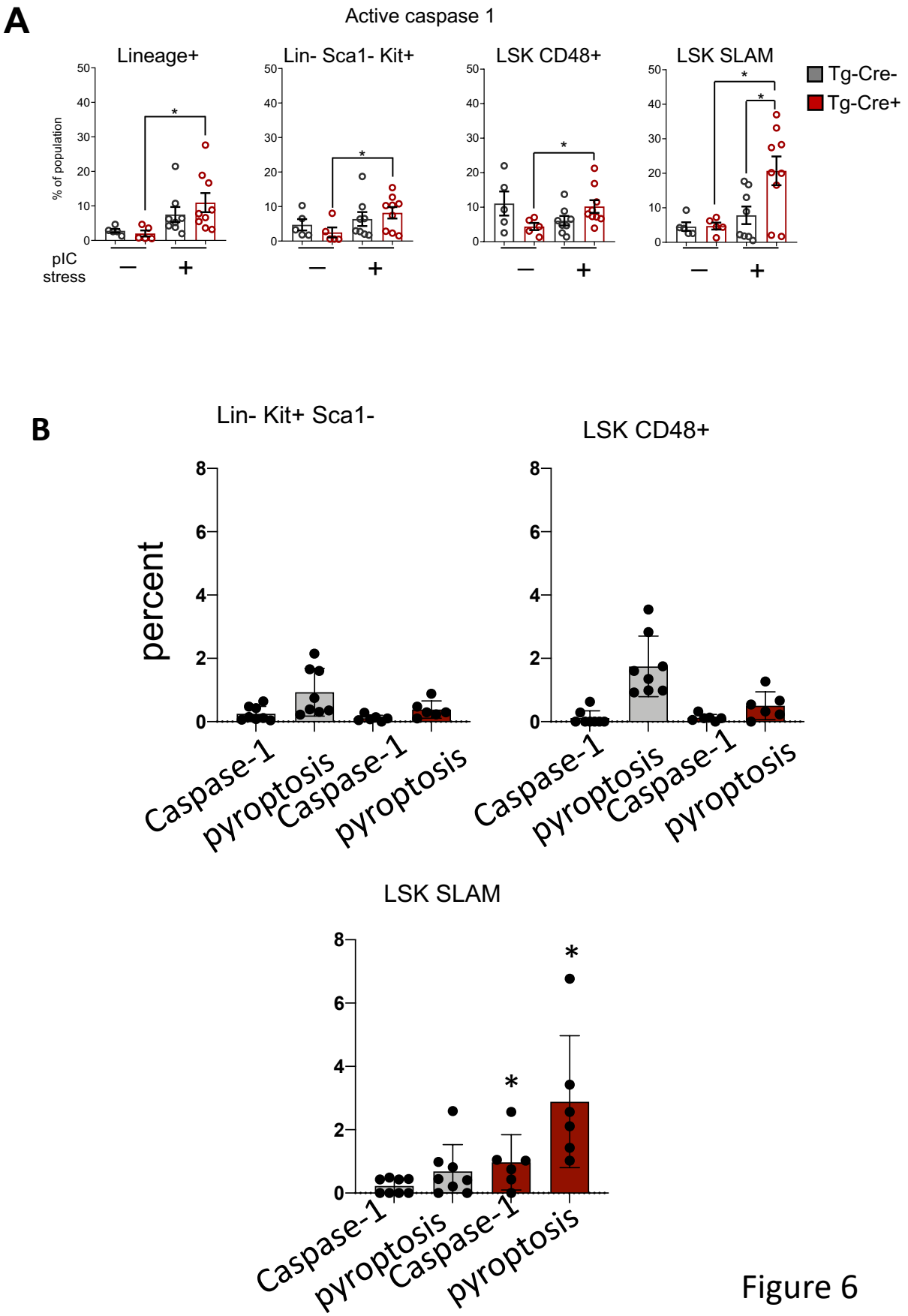
A
Active caspase 1
Lineage+
Lin- Sca1- Kit+
LSK CD48+
LSK SLAM
% of population
pIC stress
Tg-Cre-
Tg-Cre+
B
Lin- Kit+ Sca1-
LSK CD48+
percent
Caspase-1
pyroptosis
LSK SLAM

Figure 6

Figure legends

Figure 1. Mice overexpressing active TGFβ1 develop ineffective hematopoiesis with cell dysplasia after acute pIC stress.(A) Schema of experiment. Mice were challenged with 3 injections of 10 mg/kg/mouse pIC every other day, performed at least 4 weeks after inducing aTGFβ1 overexpression. **(B)** Differential peripheral blood counts. N=6 mice/group **(C)** Bone marrow cell counts of LSK, LSK CD48+ and LSK SLAM. N=5 mice/group (No stress), N=7 mice (2 days), N=8 $TgCre^-$ mice, 9 $TgCre^+$ mice (90 days). **(D)** Wright-Giemsa staining on bone marrow cells 3-4 months after pIC stress. Normal arrows denote hyper-lobulated neutrophils; block arrows denote dysplastic myeloblastes; arrow heads denote dysplastic erythroblastes. N=3 mice/group **(E)** Frequency of indicated cell types in BM, N=3 mice/group. **(F)** Frequency of dysplastic cells within each population, N=3 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 independent Student's T-test. ***P<0.001, **P<0.01, *P<0.05.

Figure 2. Mice overexpressing active TGFβ1 develop ineffective hematopoiesis with cell dysplasia after acute pIC stress.(A) Bone marrow cell counts of LSK, LSK CD48+ and LSK SLAM 9M post-pIC. N=7 mice (2 days), N=6 $TgCre^-$ mice, 4 $TgCre^+$ mice **(B)** Frequency of indicated cell types in BM, N=3 mice/group. Frequency of dysplastic cells within each population, N=3 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 independent Student's T-test. ***P<0.001, **P<0.01, *P<0.05.

Figure 3. Mice overexpressing active TGFβ1 show slower cell cycle after acute pIC stress. Bone marrow cell LSK, LSK CD48+ and LSK SLAM were stained with Ki67 and Hoescht to analyze cell cycle 2 days after pIC challenge. Frequency of indicated cell types in G0, G1, SG2M of the cell cycle, N=5 mice/group. Experiments was performed twice, with data shown as the mean ± standard error of the mean (SEM). Statistical significance was assessed using independent Student's T-test. **P<0.01, *P<0.05.

Figure 4. Mice overexpressing active TGFβ1 do not have increased apoptosis 9M post-pIC. Bone marrow cell LSK, LSK CD48+ and LSK SLAM were stained with AnnexinV to analyze apoptosis 180 days after pIC challenge. Frequency of Annexin V positive of indicated cell types N=5 mice/group. Experiments was performed twice, with data shown as the mean ± standard error of the mean (SEM).

Figure 5. Mice overexpressing active TGFβ1 crossed with MAVS-KO mice do not develop ineffective hematopoiesis after acute pIC stress. Differential peripheral blood counts. N=9 mice/group, 3 independent experiments

Figure 6: Active TGFβ1-overexpressing SLAM HSCs display sustained caspase-1 activity long-term following acute pIC stress.

Active caspase 1 was measured using the FAM-FLICA 660 kit. . **A.** N= 5 mice/group (no stress), N=8 $TgCre^-$, 9 $TgCre^+$ mice (3 months after pIC stress). **B** Active caspase 1 and annexin V were measured 180 days post-pIC. N=5 mice/group. Data are from at least two independent experiments and statistics were performed using independent Student's T-test. ***P<0.001, **P<0.01, *P<0,05.

TGFβ signaling modifies hematopoietic acute inflammatory response to drive bone marrow failure

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Conflict of interest

The authors declare no conflict of interest

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Author contributions

Conceptualization: J.J., A.H., M-D.F.; Methodology: J.J., A.H., J.B., J.X..; Investigation: J.J., A.H., J.B., J.X.,; Formal analysis: M-D.F.; Writing-original draft: J.J., M-D.F.; Writing – review & editing: M-D.F.; Funding acquisition: J.J., M-D.F.; Supervision: M-D.F. Founding source: The work was supported by NIH (DK102890 [MDF]; DOD (BM 190093 [MDF]); a Pilot Innovative Project (CCHMC [MDF]); a Pelotonia fellowship [J.J.].

Abstract

Bone marrow failure syndromes (BMF) are characterized by ineffective hematopoiesis due to impaired fitness of hematopoietic stem cells (HSC). BMFs can be acquired during bone marrow stress or innate are associated with driver genetic mutations. BMFs are at higher risks 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 factors, but remain to be understood in detail. In this study, we examined the effect of increased 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, increased TGFβ signaling alone causes a modest anemia on old mice. In sharp contrast, increased TGFβ signaling plus acute pIC challenge result in chronic pancytopenia, expanded hematopoietic stem and progenitor pools, and increased bone marrow dysplasia 3-4 months after stress, 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 pre-existing genetic insult. Hence, non-genetic factors in combination are sufficient to drive bone marrow failure.

Introduction

Bone marrow failure syndromes (BMFS) 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]. BMFS can be heritable [2] or induced by inflammatory stress, allogenic or autologous hematopoietic stem cell transplantation (HSCT), myeloablative chemotherapy, or abnormal activation of the auto-immune T cell system [3, 4].

Most of BMFS and myelodysplastic syndromes (MDS) are associated with genetic mutations, in particular in epigenetic regulators. Yet, BMFS are quite heterogenous disorders raising the interest in understanding which additional factors contribute to the disease development, in addition to driver mutations. Substantial clinical data show that hyperactivity of inflammatory cytokines, including TNFα, IL-6, and transforming growth factor–β (TGFβ), as well as innate immune signaling pathways contribute to the pathogenesis of bone marrow failures [5, 6]. In particular, the TGFβ signaling pathway is known to be hyperactive in Fanconi anemia [7], myelodysplastic syndromes, [8-11], Shwachman-Diamond syndrome [12], myelofibrosis [13]. TGFβ1 is a myelosuppressive cytokine. TGF-β1 is secreted as inactive complex protein bound to latency-associated peptide (LAP), and the latent TGF-beta1 binding protein-1 (LTBP1). Dissociation from the complex is necessary for their biological activity. Active TGF-β1 proteins then signals through two serine/threonine kinases, type I and type II receptors and trigger several signaling pathways[14]. TGFβ1 functions 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 myeloid-biased HSC population [16]. TGFβ can also suppress erythropoiesis and myelopoiesis. Our lab has previously shown that TGFβ contributes to HSC functional decline 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 HSCs after bone marrow transplantation in mice due to increased expression of the active form of TGFβ in HSCs. Interestingly, pharmacologic inhibition of TGFβ signaling during bone marrow reconstitution following transplantation improved HSC functions, suggesting that this increased TGFβ signaling causes HSC functional decline after BMT [19]. In mouse models of bone marrow failure or Fanconi Anemia, pharmacologic 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% patients respond to the treatment, indicating that other factors contribute to BMF. [8, 10, 20]. Dysregulation of several innate immune pathways are also known contributing factors of BMF or MDS. Toll-like receptors (TLRs) 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 BMF/MDS patients and contribute to ineffective hematopoiesis [21, 22].

Although inflammatory pathways are strongly implicated in human BMFS and MDS, their exact contribution to the pathogenesis of the diseases still remain to be understood. It is still unclear whether deregulated TGFβ signaling 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 drive chronic bone marrow failure with phenotypes similar to human disorders associated with ineffective hematopoiesis, including BMF 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 BMFS can be initiated solely by multiple inflammatory hits in the context of increased TGFβ signaling, and that disease outcome is inflammatory context-dependent. 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 3 injections of 10 mg/kg/mouse polyinosinic:polycytidlic acid (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 were 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.

Whole bone marrow cells were stained as above for mature lineages. Cells 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 incubated at 37°C and 5% CO_2 for 30 minutes 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 manufacture recommendations. 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

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 hematoxylin and eosin stained. Whole bone marrow cells were also prepared by cytospin and stained using Kwik-Diff (Fisher Scientific).

RNA sequencing

cDNA from 500 SLAM HSCs were 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 then done by the CCHMC core. Analyses were done using Alt-analyze [24], as previously described [25, 26]. Differentially expressed genes were then analyzed using the ENRICHR database [27].

Immunofluorescence assays

SLAM HSCs 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 Nyquist limit setting (0.1 μm XY pixel size) at 100X magnification. Image analyses were done using the surface building Matlab extension in Imaris software from at least 30 cells in each group.

Statistical analyses

Experiments were done in 2-3 replicates unless specified. Statistics were performed using unpaired Student T-test, unless specified.

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 active TGFβ1 (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 (Supplementary Figure S1A) [29]. Mice were injected with 10 mg/kg/mouse polyinosinic:polycytidilic acid (pIC) three times, once every 48 hours, to cause aTGFβ1 overexpression (Figure 1B).We previously confirmed that aTGFβ1 levels are higher in BM LSK cells 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 (phenotypically identified as Lineage- Sca1+ Kit+ CD48- CD150+, hereafter termed SLAM HSCs) 3-4 weeks after inducing overexpression were higher in $TgCre^{+}$ SLAM HSCs (Figure 1D). No signs of fibrosis were observed in the bone marrow of these mice up to 6 months after inducing overexpression (Supplementary Figure S1B). Hence, in this model, increased TGFβ1 remain physiological, not higher than some clinical MDS samples and much lower than 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 was 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 (Figure 1E). Examination of the peripheral blood by flow cytometry analysis confirmed mild reduction in myeloid cell frequency but increased T-cell frequency (Supplementary Figure S1C and Figure S2A). We next examined the consequence of aTGFβ1 overexpression in the bone marrow (BM) and spleen 3 months after TGFβ overexpression. BM cellularity was not significantly different between the groups (Supplementary Figure S1D). However, TgCre^{+} mice had splenomegaly (Supplementary Figure S1E). H&E staining section of the femur and spleen taken from TgCre^{+} and TgCre^{-} mice showed no gross abnormalities (Supplementary Figure S1F).

Further analysis indicated that total count of each hematopoietic stem or progenitor cell (HSPC) population in BM was similar between TgCre^{+} and TgCre^{-} mice during aging (Supplementary Figure S1G,H and S2). At 6 months, a modest dysplasia in BM neutrophils and myeloid progenitors of TgCre^{+} mice was observed (Figure 1F-H, Figure S3). Low percent of neutrophils were hyper-nucleated; some promyelocyte/myelocytes had increased size with higher cytoplasm to nucleus ratio (Figure 1F-H, Figure S3). This effect did not persist and was no longer observed at 12 months, perhaps due to confounding aging effects. Hence, aTGFβ1 overexpression causes mild hematopoietic defects.

Mice overexpressing active TGFβ1 show significant bone marrow failure long-term 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 3 injections of pIC, one 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, TgCre^{+} pIC^{+} mice developed significant PB pancytopenia, including neutropenia, lymphopenia and thrombocytopenia, beginning 3 months after pIC stress compared to TgCre^{-} pIC^{+} mice (Figure 2B). A persistent anemia was also noted in TgCre^{+} pIC^{+} mice, that was more pronounced than without pIC challenge (Figure 2B). TgCre^{+} pIC^{+} mice had larger spleens with an expansion of the white pulp (Supplementary Figure S4A,B), whereas total BM cell count and density remained unchanged compared to control (Supplementary Figure S4C,D).

We then examined BM 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 progenitor (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 (Supplementary Figure S4E). Interestingly, 90 days after pIC stress, TgCre^{+} mice exhibited higher MPP and SLAM HSC numbers compared to control mice, whereas MEP cells remained lower (Figure 2C and supplementary FigureS3E). Further, TgCre^{+} pIC^{+} mice displayed increased frequency of myeloblasts and promyelocytes but reduced frequency of mature neutrophils in BM (Figure 2D,E), that was associated with 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, 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 that is characterized by an expanded HSPC 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.

dsRNA and enhanced TGFβ signaling cause permanent changes in gene expression profile of HSC.

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 4 groups of SLAM HSCS: (1) from 3 M-old Tg-Cre^{-} mice, (2) from 3 M-old TgCre^{+} mice, (3) from Tg-Cre^{-} mice 3 months after pIC re-challenge and (4) from TgCre^{+} mice 3 months after pIC re-challenge. For the pIC re-challenge groups, we chose to collect the SLAM HSC when pancytopenia begins to manifest, i.e 3 months after pIC re-challenge (Figure 3A). Data were analyzed using unsupervised principle component analysis (PCA) and supervised hierarchical clustering in AltAnalyze^{R}. [25, 26] PCA separated the 4 groups of cells into 4 distinct clusters, indicating that each group possess a unique transcriptional signature (Figure 3B). Hierarchical clustering indicated that pIC re-challenge profoundly altered the transcriptional landscape in SLAM HSCs from both Tg-Cre^{-} 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, 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 genes, and were downregulated by pIC challenge, but more so in TgCre^{+} SLAM HSC. Finally, specific differences in gene expression between Tg-Cre^{-} or TgCre^{+} SLAM HSC after pIC challenge were noted, and are underscored by white boxes (Figure 3C). Thus, a transient pIC challenge causes long-lasting transcriptional changes in HSCs.

Examining in more details which genes are differentially expressed in TgCre^{+} SLAM HSC specifically after pIC challenge uncovered that they belong to 2 main categories, interferon response genes and nuclear-encoded genes related to mitochondrial regulation. MarkerFinder algorithm in AltAnalze identified that myeloid and innate immune genes, including TLR2/4/6 co-receptor *cd14*, *cxcl10*, *Anxa3*, *Olfm4*, *s100a8/s100a9* were up-regulated in TgCre^{-} pIC^{+} SLAM HSCs but not in TgCre^{+} pIC^{+} SLAM HSCs (Figure 3D,F and Supplementary Figure S5A), thus correlating with the increase in PB myeloid cell in these mice (Figure 2). We specifically interrogated differential gene expression of IFN alpha and beta signaling pathway. They were more downregulated in TgCre^{+} SLAM HSC (Supplementary Figure S5B). We also interrogated genes related to mitochondrial regulation. Genes important for the regulation of mitochondrial translation such as *mrpl46* were up-regulated only in TgCre^{+} SLAM HSCs after pIC stress, (Figure 3D,F and supplementary Figure S5B). On the other hand, genes encoding regulators of mitophagy were downregulated in TgCre^{+} SLAM HSCs after pIC stress (supplementary Figure S5B).

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

Active TGFβ1-overexpressing SLAM HSCs 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 active form of IRF3, and confirmed that IRF3 was not chronically activated in HSC from TgCre^{+} pIC^{+} mice (Supplementary Figure S5D). We then focused on mitochondria, as suggested by the transcriptional profile of TgCre^{+} pIC^{+} SLAM HSCs. 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 MDS patient samples exhibited 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 predictive of secondary MDS development 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 3-dimensional reconstruction analyses. Mitochondrial content was similar in SLAM HSCs from both $TgCre^{-}$ and $TgCre^{+}$ mice before pIC stress (Figure 4A,B). However, 3 months after pIC stress, $TgCre^{+}$ pIC^{+} SLAM HSCs had higher mitochondrial content compared to control. This is consistent with gene expression signature of elevated regulators of mitochondria biogenesis and reduced regulators of mitophagy. Mitochondrial membrane potential (MMP), analyzed using tetramethylrhodamine ester, showed several differences between the groups. Shortly after pIC re-challenge (2 days and 7 days), MMP increased in SLAM HSC, MPP and CP populations from both $TgCre^{-}$ pIC^{+} and $TgCre^{+}$ pIC^{+} mice (Figure 4C). At longer term (3 months), MMP returned to base line 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. in the committed progenitor pool (CP, Lineage- Sca1- Kit+), MPP and SLAM HSCs, 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

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 seen by increased caspase-1 in $TgCre^{+}$ pIC^{+} SLAM HSCs up to 3 months after pIC challenge, compared to control (Figure 4E).

These data suggest that aTGFβ1-overexpressing SLAM HSCs maintain more active mitochondria, have increased ROS levels and caspase-1 activity compared to control 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 chronic bone marrow failure associated with myelodysplasia. Mechanistically, acute pIC imposes permanent transcriptional changes in HSC, which, in the context of increased TGFβ signaling, are associated with long-lasting increased 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 also may 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 on using combinatorial therapies for treating human bone marrow failure syndromes.

Inflammation has long been associated with acquired bone marrow failure syndromes. [5, 6] There is also strong correlation between inflammation (regardless of cause, duration and frequency) and the development of MDS. Independent 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 myelodysplastic syndromes and has been implicated in aplastic anemia, Fanconi Anemia (FA) and Shwachman-Diamond Syndrome [7, 9, 11, 12, 41]. Previous studies exploring the relationship between TGFβ signaling and bone marrow failure have used 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 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 suggest that a modest increase in TGFβ signaling alone is not sufficient to drive severe bone marrow failure 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 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 BMFS and MDS. Crosstalk between pIC and TGFβ signaling in BMF development was previously described in the context of Fanconi anemia. FA is caused by mutations in DNA repair proteins via homologous recombination. The group of Dr Milsom dissected the response of HSC to pIC. They show that in response to pIC, HSC exiting from quiescence sustain DNA damage that can be resolved by the FA-mediated DNA repair response (DDR). As such, WT mice recover from pIC stress. In contrast, *Fanca-deficient* mice demonstrated reduced numbers of HSC, unresolved DNA damage and developed severe BMF

[44]. Interestingly, Zhang *et al.* showed that enhanced TGFβ signaling, known to be up-regulated in Fanconi anemia patients, contributes to pIC-induced BMF of in *Fanca-deficient* mice by modifying DDR to pIC-induced DNA damage. When TGFβ signaling is high, HSC use the 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 in *Fanca-deficient* mice [7]. Together, these findings support the idea that TGFβ is a 'modifier' of HSC functions that predisposes to BMF/MDS development. It also raises the interesting possibility that a TGFβ-modified pIC-induced could contribute to our phenotype. Something that 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 if this resembles the recently described trained immunity phenomenon, [45-47] whether other innate immune insults similarly synergizes with TGFβ1 in disease development and what are the exact mechanisms behind this synergy.

TGFβ signaling is mostly known to signal 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 hematological phenotypes of varying degrees, such as Pearson syndrome, which presents with pancytopenia, and Barth syndrome, which presents with neutropenia. A study of MDS and AML patients revealed transcriptional dysregulation of their mitochondria [50]. Several studies have also implicated mitochondrial dysfunction and increased intracellular ROS in driving 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 functions contribute to ineffective hematopoiesis. Our study further suggests that there may be direct links between mitochondrial dysfunction and altered TGFβ signaling. It will remain 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 the abnormal activation of caspase-1 activity, which can contribute to bone marrow failure [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 HSCs post pIC stress in our mouse model may therefore not only have direct genotoxic effects after pIC stress, but may also synergize with and amplify pIC-mediated caspase 1 activation to drive bone marrow failure. 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 targeting 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 (SREBPs), 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, our study describes a mouse model of bone marrow failure that results from acute inflammatory challenge in the context of increased TGFβ signaling. This model recapitulates phenotypes of human bone marrow failure syndromes that are linked to TGFβ signaling. This mouse model will help not only furthering our understanding of the pathogenesis of BMFS/MDS associated with TGFβ signaling but also provides an *in vivo* model to test effects of combinatorial therapy to cure these disorders.

Data availability

All data are available; scRNA-seq accession codes, GEO accession number. Figure 1-4 have associated raw data. Raw Imaging are available upon requests

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Figure legends

Figure 1: Active TGFβ1 overexpressing mice do not develop overt hematopoietic phenotypes during steady state hematopoiesis.

(A) Schema of the mouse model of aTGFβ1 overexpression. **(B)** Schema of experiments. **(C)** Levels of aTGFβ1 protein in bone marrow fluid from mice 3 weeks (N= 3 mice/group) and 6 months (N=7 mice/group) after aTGFβ1 overexpression using ELISA. **(D)** Analysis of the TGFβ signaling effector phospho-Smad2 using immunofluorescence in LSK SLAM 4 weeks after aTGFβ1 overexpression. N=50 cells/mouse, 3 mice/group **(E)** Differential peripheral blood counts at indicated time points following aTGFβ1 overexpression. N=5 mice/group (3 months), N=7 $TgCre^-$ mice, 8 $TgCre^+$ mice (6 months), N=7 mice/group (12 months). **(F)** Wright-Giemsa staining of bone marrow cytospins 6 and 12 months after aTGFβ1 overexpression. N=3 mice/group **(G)** Frequency of indicated cell types in BM, N=3 mice/group. **(H)** Frequency of dysplastic cells within each population, N=3 mice/group. All experiments were conducted at least twice, with data shown as the mean $\pm$ standard error of the mean (SEM). Statistical significance was assessed using independent Student's T-test. ***P<0.001, **P<0.01, *P<0.05.

Figure 2: Mice overexpressing active TGFβ1 develop ineffective hematopoiesis with cell dysplasia after acute pIC stress.(A) Schema of experiment. Mice were challenged with 3 injections of 10 mg/kg/mouse pIC every other day, performed at least 4 weeks after inducing aTGFβ1 overexpression. **(B)** Differential peripheral blood counts. N=6 mice/group **(C)** Bone marrow cell counts of LSK, LSK CD48+ and LSK SLAM. N=5 mice/group (No stress), N=7 mice (2 days), N=8 $TgCre^-$ mice, 9 $TgCre^+$ mice (90 days). **(D)** Wright-Giemsa staining on bone marrow cells 3-4 months after pIC stress. Normal arrows denote hyper-lobulated neutrophils; block arrows denote dysplastic myeloblastes; arrow heads denote dysplastic erythroblastes. N=3 mice/group **(E)** Frequency of indicated cell types in BM, N=3 mice/group. **(F)** Frequency of dysplastic cells within each population, N=3 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 independent Student's T-test. ***P<0.001, **P<0.01, *P<0.05.

Figure 3: Active TGFβ1-overexpressing SLAM HSCs display unique transcriptional signature long-term following acute pIC stress.

(A) Workflow schematic of the transcriptomic analysis from SLAM HSCs 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. N=3 mice/group **(D-F)** Top gene ontology category of differentially expressed genes identified in cluster 1 (D), cluster 2 (E) and cluster 3 (F).

Figure 4: Active TGFβ1-overexpressing SLAM HSCs display sustained mitochondrial activity and caspase-1 activity long-term following acute pIC stress.

(A-B) Mitochondrial content assessed in SLAM HSCS using Tomm20 immunostaining. (A) Representative immunofluorescence images (Tomm20 in red, DAPI in blue). (B) Quantification of Tomm20 MFI. N=50 cells from each mouse, 6 mice/group **(C)** Mitochondrial membrane potential was assessed using tetra-methyl rhodamine ester dye (TMRE) staining at the indicated time after pIC stress. N=4 mice/group (0 days post pIC stress/no stress), N=5 $TgCre^-$, 6 $TgCre^+$ (2 days post stress), N=6 $TgCre^-$, 7 $TgCre^+$ (7 days post stress), N=6 mice/group (90 days post pIC stress) **(D)** Intracellular reactive oxygen species was measured using the CellROX Deep Red Reagent. N= 5 mice/group (no stress), N=6 mice/group, (3 months after pIC stress). **(E)** Active caspase 1 was measured using the FAM-FLICA 660 kit. . N= 5 mice/group (no stress), N=8 $TgCre^-$, 9 $TgCre^+$ mice (3 months after pIC stress). Data are from at least two independent experiments and statistics were performed using independent Student's T-test. ***P<0.001, **P<0.01, *P<0,05.

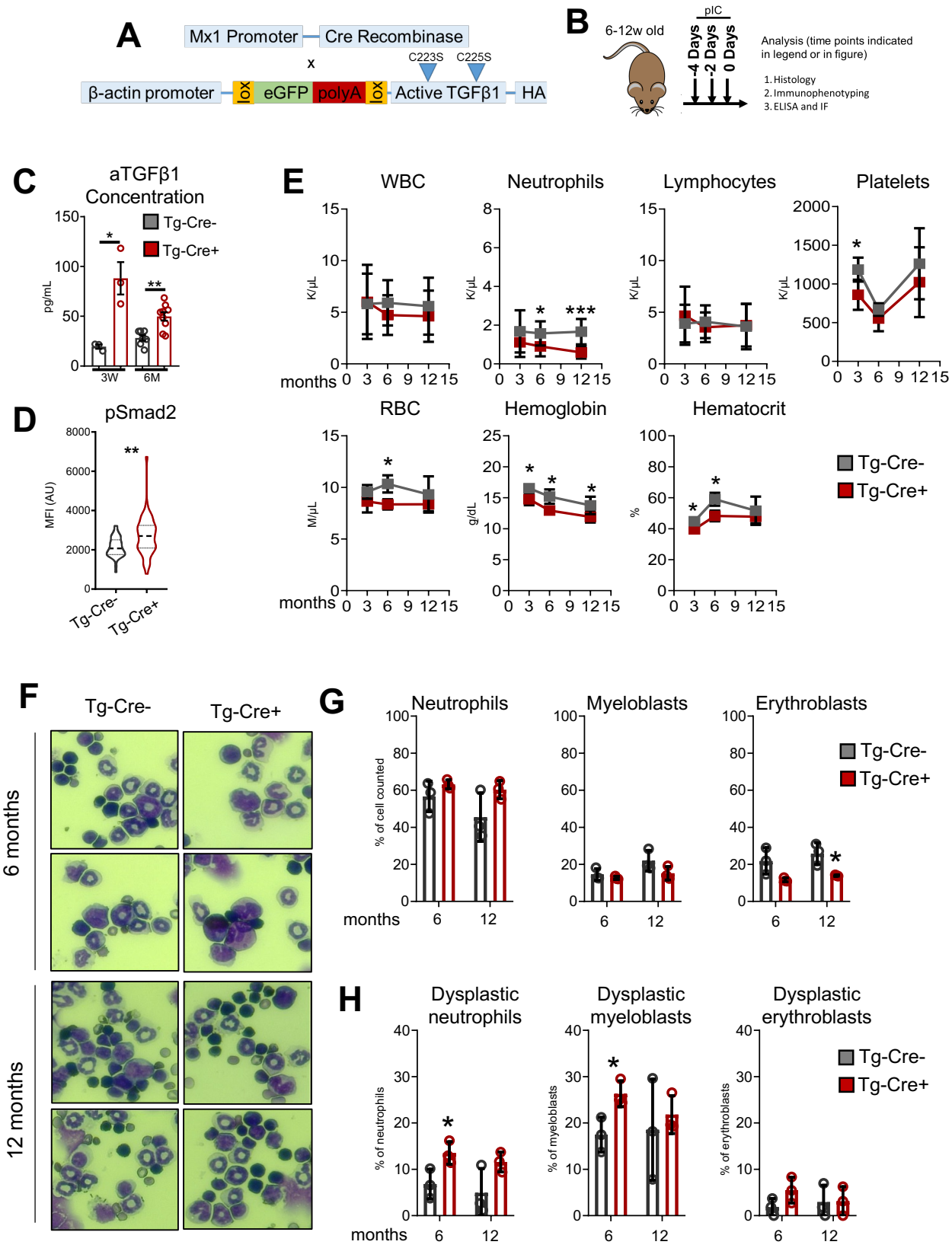

A
Mx1 Promoter
Cre Recombinase
x
C223S
C225S
β-actin promoter
lox
eGFP
polyA
lox
Active TGFβ1
HA
B
6-12w old
pIC
-4 Days
-2 Days
0 Days
Analysis (time points indicated in legend or in figure)
1. Histology
2. Immunophenotyping
3. ELISA and IF
C
aTGFβ1 Concentration
pg/mL
Tg-Cre-
Tg-Cre+
3W
6M
D
pSmad2
MFI (AU)
Tg-Cre-
Tg-Cre+
E
WBC
Neutrophils
Lymphocytes
Platelets
RBC
Hemoglobin
Hematocrit
K/µL
M/µL
g/dL
%
months
Tg-Cre-
Tg-Cre+
F
Tg-Cre-
Tg-Cre+
6 months
12 months
G
Neutrophils
Myeloblasts
Erythroblasts
% of cell counted
months
Tg-Cre-
Tg-Cre+
H
Dysplastic neutrophils
Dysplastic myeloblasts
Dysplastic erythroblasts
% of neutrophils
% of myeloblasts
% of erythroblasts
months
Tg-Cre-
Tg-Cre+

Figure 1

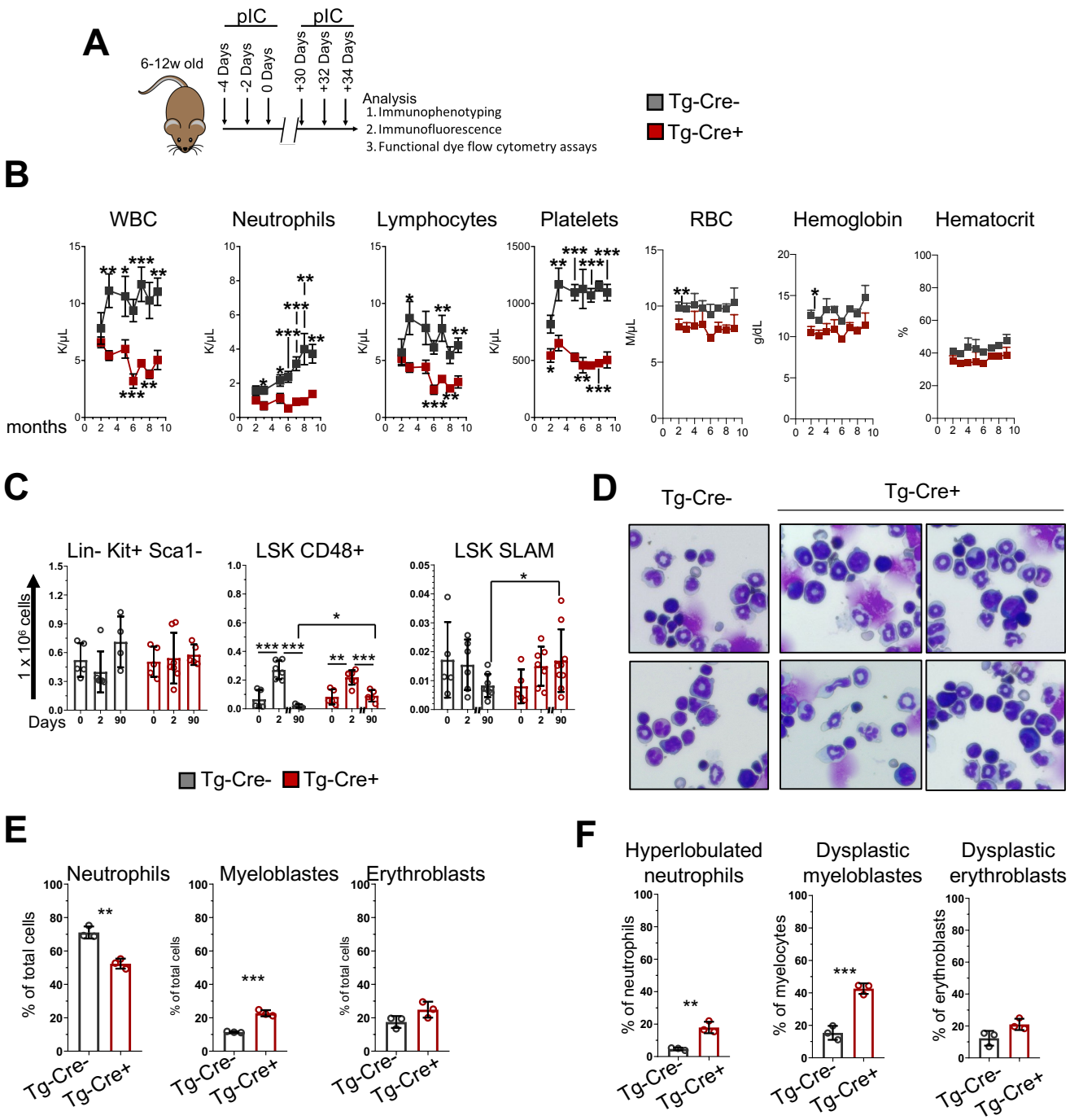
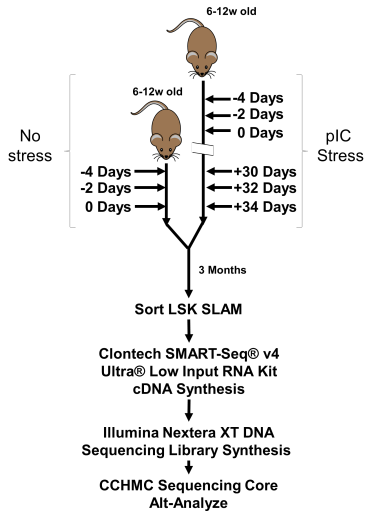
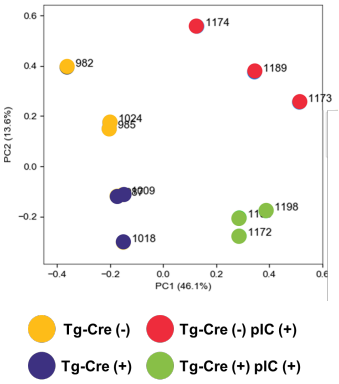

A
6-12w old
pIC
pIC
-4 Days
-2 Days
0 Days
+30 Days
+32 Days
+34 Days
Analysis
1. Immunophenotyping
2. Immunofluorescence
3. Functional dye flow cytometry assays
Tg-Cre-
Tg-Cre+
B
WBC
Neutrophils
Lymphocytes
Platelets
RBC
Hemoglobin
Hematocrit
K/µL
M/µL
g/dL
%
months
C
Lin- Kit+ Sca1-
LSK CD48+
LSK SLAM
1 x 10⁶ cells
Days
Tg-Cre-
Tg-Cre+
D
Tg-Cre-
Tg-Cre+
E
Neutrophils
Myeloblastes
Erythroblasts
% of total cells
Tg-Cre-
Tg-Cre+
F
Hyperlobulated neutrophils
Dysplastic myeloblastes
Dysplastic erythroblasts
% of neutrophils
% of myelocytes
% of erythroblasts
Tg-Cre-
Tg-Cre+

Figure 2

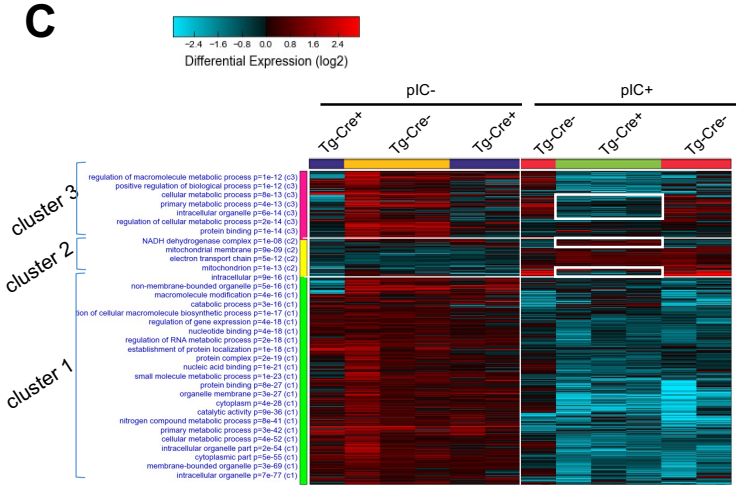
A



B



C



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

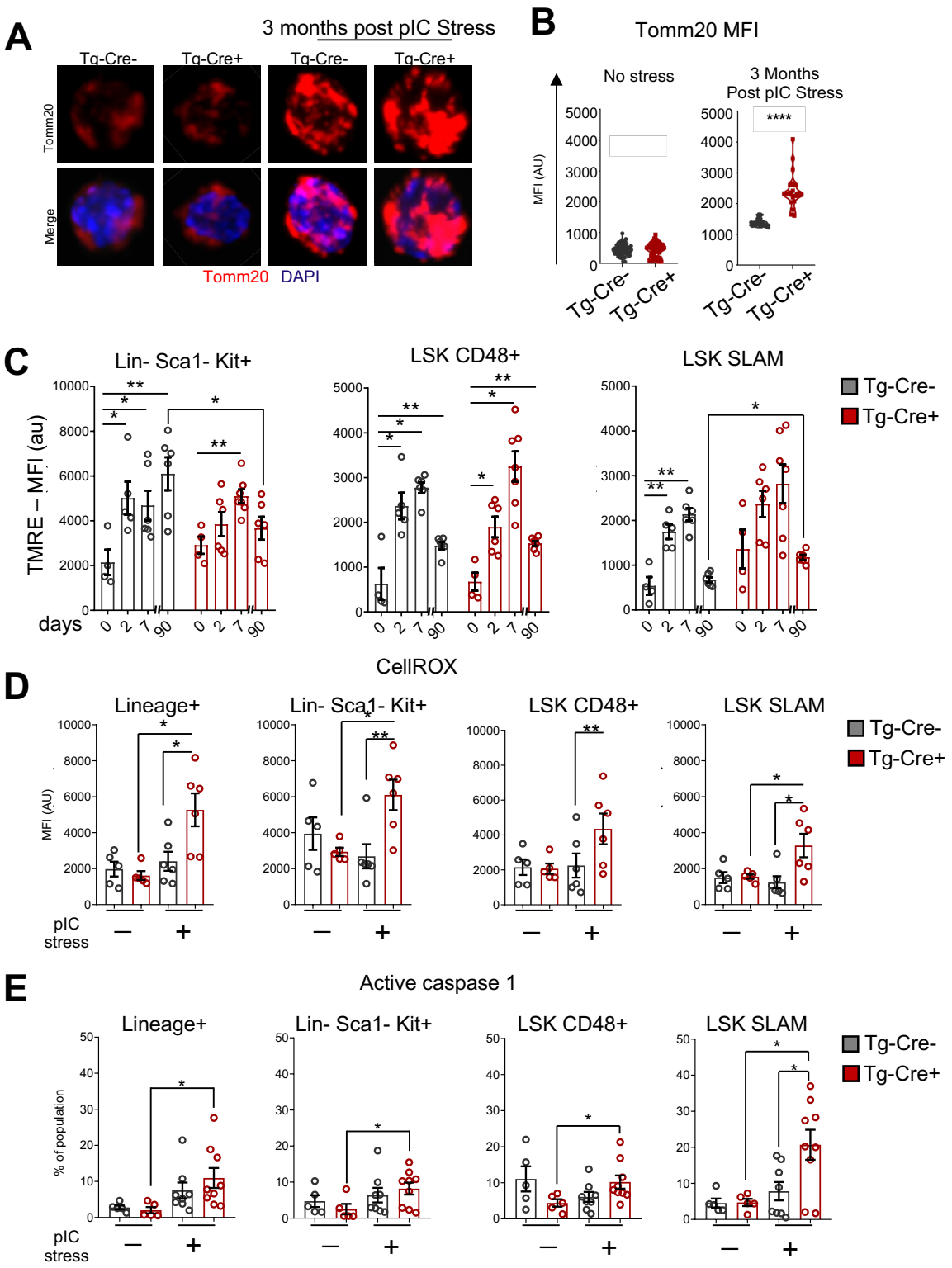

A
3 months post pIC Stress
Tg-Cre-
Tg-Cre+
Tg-Cre-
Tg-Cre+
Tomm20
Merge
Tomm20 DAPI
B
Tomm20 MFI
No stress
3 Months Post pIC Stress
MFI (AU)
****
Tg-Cre-
Tg-Cre+
C
Lin- Sca1- Kit+
LSK CD48+
LSK SLAM
TMRE – MFI (au)
days
Tg-Cre-
Tg-Cre+
D
CellROX
Lineage+
Lin- Sca1- Kit+
LSK CD48+
LSK SLAM
MFI (AU)
pIC stress
Tg-Cre-
Tg-Cre+
E
Active caspase 1
Lineage+
Lin- Sca1- Kit+
LSK CD48+
LSK SLAM
% of population
pIC stress
Tg-Cre-
Tg-Cre+

Figure 4