Immunomodulatory role of megakaryocytes in the hematopoietic niche of myeloproliferative neoplasms

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Abstract

Myeloproliferative neoplasms (MPN) are clonal stem cell disorders characterized by dysregulated megakaryopoiesis and expansion of neoplastic hematopoietic stem cells (HSC). Megakaryocytes (MK) not only regulate HSC function, but also shape immune responses within the marrow niche. Using an aging murine model of MPN with MK-restricted *JAK2*^{V617F} expression, we investigated the immunomodulatory roles of mutant MK. Compared to wild-type MK, aged mutant MK exhibit enhanced antigen uptake and MHC I presentation, secretion of pro-inflammatory cytokines (PF4, TGFβ, IL-1β), and induction of T-cell dysregulation in the marrow niche. In chimeric murine models with co-existing wild-type and *JAK2*^{V617F} mutant hematopoietic cells, enhanced MK immune activity correlates with mutant cell expansion and MPN development. Single-cell RNA sequencing revealed that aging amplifies *JAK2*^{V617F} MK–driven immune remodeling. Notably, aged mutant MK showed marked upregulation of long-interspersed element-1 (LINE-1) retrotransposon transcripts alongside elevated innate immune sensors cyclic GMP–AMP synthase (cGAS) and stimulator of interferon genes (STING), implicating retrotransposon activity in niche inflammation. In human MPN marrow, immunohistochemistry detected LINE-1–encoded protein ORF1p in MK from 12 of 13 MPN patients, but not in orthopedic controls (N=5). These findings identify MK as active immune regulators in MPN, with *JAK2*^{V617F} mutation and aging synergizing to reprogram MK into inflammatory, immune-modulatory niche cells. *LINE-1* activation emerges as a potential driver of chronic marrow inflammation, and a targetable mechanism in clonal hematopoiesis and MPN progression.

Introduction

Megakaryocytes (MK), traditionally recognized as the precursors of platelets, have emerged as key regulators of hematopoietic stem cell (HSC) function. Through the production of cytokines, chemokines, and extracellular matrix components, MK influence both steady-state and stress hematopoiesis.[1-5] Recent studies also suggest that MK contribute to pathogen surveillance and immune regulation.[6-11] While abnormal megakaryopoiesis is a common feature of many hematologic malignancies,[12-16] how diseased MK alter their immunomodulatory function and impact HSC behavior in normal and neoplastic hematopoiesis remains poorly understood.

Myeloproliferative neoplasms (MPN), including polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF), are clonal stem cell disorders characterized by hematopoietic stem/progenitor cell (HSPC) expansion and an increased risk of transformation to acute leukemia. The acquired kinase mutation *JAK2*^{V617F} plays a central role in these diseases.[17,18] MK hyperplasia is a hallmark feature of MPN, with advanced disease often associated with morphologically and functionally altered MK, raising the possibility that diseased MK contribute to disease progression.[12,14,15,19] Both MPN incidence and leukemia transformation risk increase significantly with aging,[17,18] suggesting a strong interplay between *JAK2*^{V617F}-driven hematopoiesis and age-related changes in the marrow microenvironment.

We previously established a MPN model in which *JAK2*^{V617F} mutant MK drive a myeloproliferative syndrome characterized by modest thrombocytosis, splenomegaly, increased marrow MK, and expansion of wild-type HSC compared to age-matched control mice.[20] Using competitive repopulation

assays, we found that while HSC from young mutant MK mice exhibited enhanced engraftment potential, 2-year-old *JAK2*^{V617F} mutant MK mice displayed features of age-related HSC dysfunction: reduced engraftment, myeloid-skewed hematopoiesis with expansion of CD41^{+} myeloid-biased HSC, and decreased quiescence.[21] These findings suggest that *JAK2*^{V617F} mutant MK progressively impair wild-type HSC function with age. In the current study, we investigate how mutant MK contribute to MPN development through immunomodulation. Given that chronic inflammation is a hallmark of MPN and is known to favor the expansion of *JAK2*^{V617F} mutant over co-existing wild-type cells,[22] we hypothesize that during aging *JAK2*^{V617F} mutant MK acquire immune modulatory functions, drive marrow inflammation, and promote neoplastic clone expansion.

Methods

Experimental mice

All mouse experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee at Stony Brook University. *JAK2*^{V617F} Flip-Flop (FF1) mice (which carry a Cre-inducible human *JAK2*^{V617F} gene driven by the human JAK2 promoter) were provided by Radek Skoda (University Hospital Basal, Switzerland). Pf4-Cre mice (which express Cre under the promoter of platelet factor 4; JAX stock #008535) were crossed with the FF1 mice to generate a transgenic mouse line with human *JAK2*^{V617F} expression in the MK lineage (Pf4^{+}FF1^{+}). Tie2-cre mice were obtained from Jackson Laboratory. All mice used were housed in a pathogen-free mouse facility at Stony Brook University. No randomization or blinding was used to allocate experimental groups.

Patient samples

Archived bone marrow biopsies from MPN patients and age-matched orthopedic surgery controls were obtained from Stony Brook University Hospital. Sample collection and analysis were conducted under an Institutional Review Board-approved protocol.

Additional information on the methods used can be found in the *Online Supplementary Methods*.

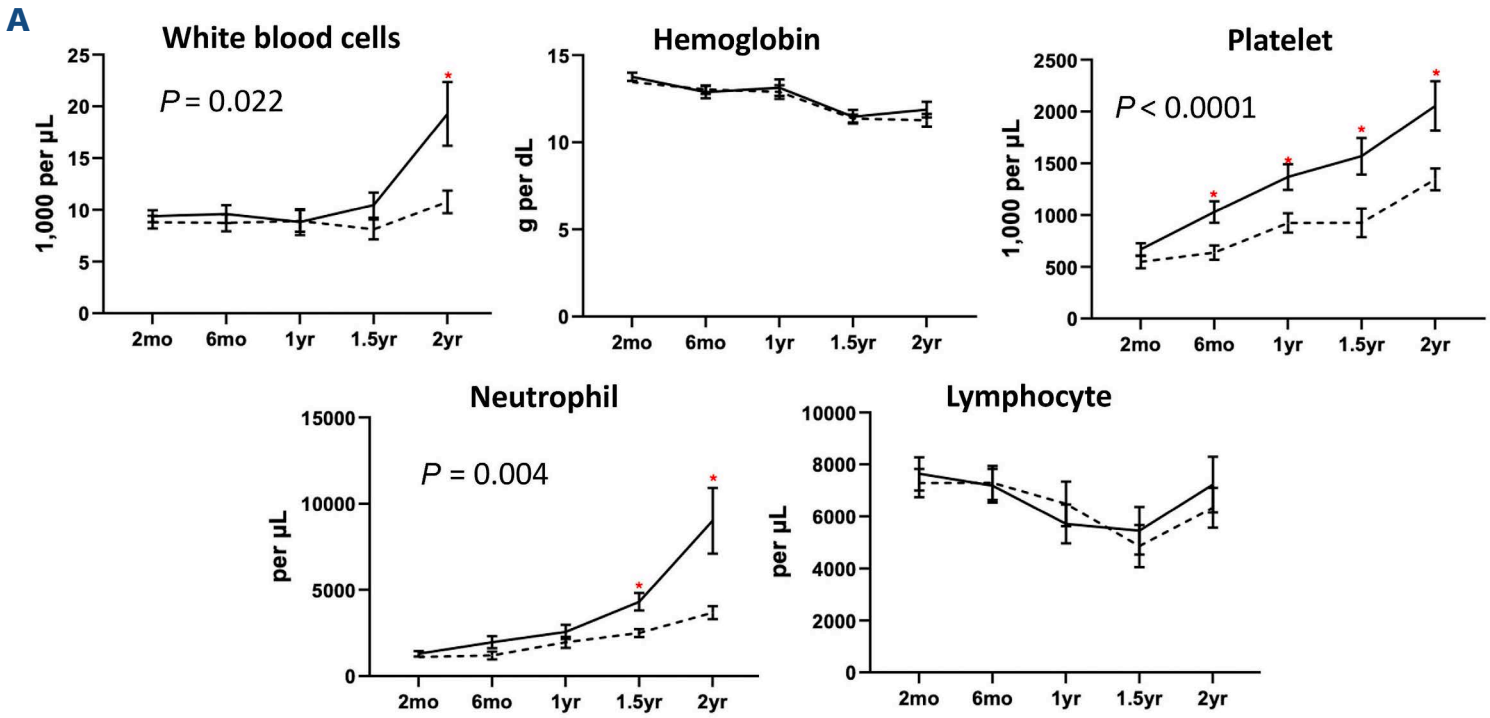
Results

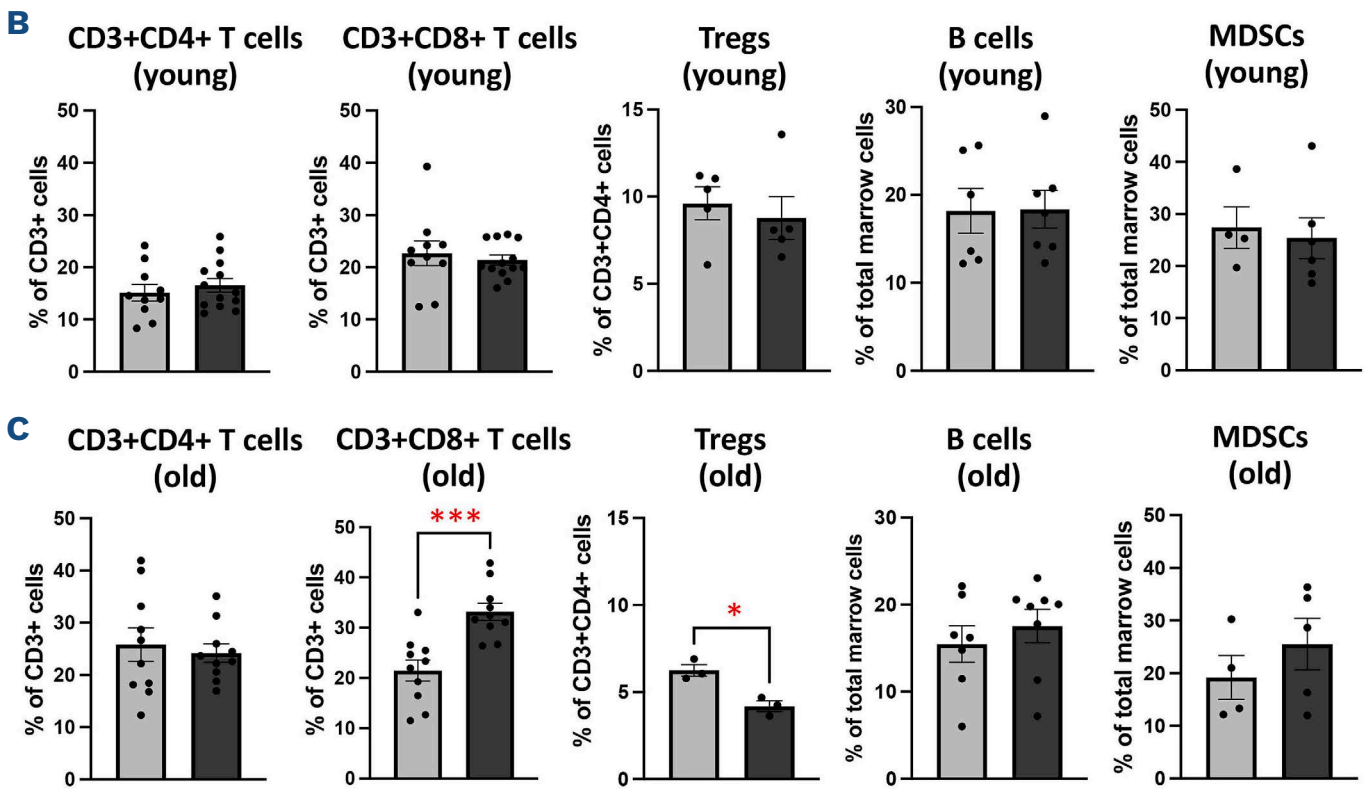
JAK2^{V617F} mutant megakaryocytes remodel the bone marrow immune microenvironment in Pf4^{+}FF1^{+} mice during aging

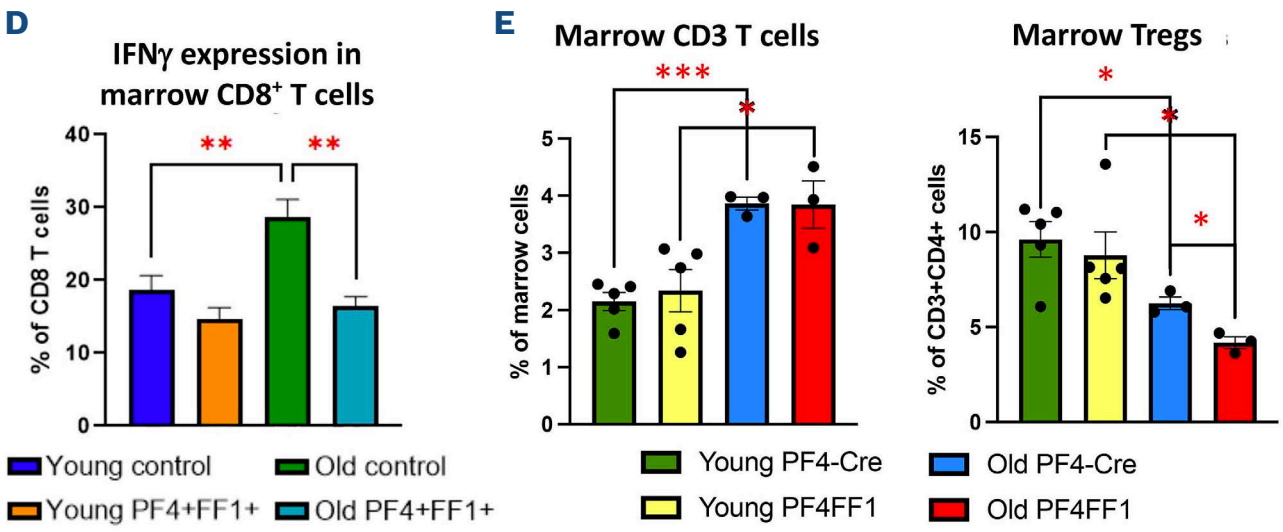
To investigate the immunomodulatory functions of *JAK2*^{V617F} mutant MK *in vivo*, we crossed mice with a Cre-inducible human *JAK2*^{V617F} transgene (FF1)[23] with the Pf4-cre mice, in which Cre recombinase is driven by the MK-specific platelet factor 4 promoter.[24] Although the Pf4-cre model has some limitations, including its expression in other cell lineages, it has remained a gold standard for creating efficient MK-specific transgene expression.[25] Using rigorous and sensitive assays (e.g., expression of fluorescent reporter genes, expression of the *JAK2*^{V617F} transgene, functional analysis of JAK/STAT downstream signaling), we and others verified the specific transgene expression and/or activation in MK, but not in HSC.[3,20,21,26,27] Previously, we had demonstrated that *JAK2*^{V617F} mutant MK drive a myeloproliferative syndrome characterized by thrombocytosis, splenomegaly, and expansion of HSPC.[20] We also showed that these mutant MK accelerate HSC aging, leading to myeloid-skewed hematopoiesis, expansion of CD41^{+} HSC, and reduced engraftment, self-renewal, and differentiation capacity compared to age-matched controls.[21] Thus, Pf4-cre^{+}FF1^{+} (Pf4^{+}FF1^{+}) mice provide a unique model to study the immunomodulatory functions of MK and their effects on the marrow microenvironment of MPN during aging.

Consistent with our previous reports,[20,21] Pf4^{+}FF1^{+} mice developed an essential thrombocythemia phenotype, exhibiting modest neutrophilia and sustained thrombocytosis over a 2-year follow-up (Figure 1A). In aged (2-year-old) mice, flow cytometry revealed a marked increase in CD3^{+}CD8^{+} T cells accompanied by a reduction in regulatory T (Treg) cells, changes not observed in young mice (Figure 1B, C). Despite the numeric increase, CD8^{+} T cells from aged Pf4^{+}FF1^{+} mice exhibited impaired cytotoxic function, as evidenced by reduced intracellular IFNγ levels (Figure 1D). Overall, both control and mutant mice exhibited an age-associated expansion of marrow CD3^{+} T cells and a decline in Treg cells, with these changes being more pronounced in Pf4^{+}FF1^{+} mice (Figure 1E). Collectively, these findings suggest that, in the context of aging, *JAK2*^{V617F} mutant MK can alter the bone marrow immune landscape. Previously, we demonstrated that the human JAK2 transgene is not expressed in HSC from either young (6 months)[20] or old (2 years)[21] Pf4^{+}FF1^{+} mice using sensitive reverse transcription-polymerase chain reaction (RT-PCR) assays. In the present study, we further confirmed that the human *JAK2* gene is not expressed in flow-sorted long-term HSC (Lin^{-}cKit^{+}Sca1^{+}CD150^{+}CD48^{-}), short-term HSC (Lin-cKit+Sca1+CD150-CD48-), MPP2 (Lin^{-}cKit^{+}Sca1^{+}CD150^{+}CD48^{+}), OR MPP3 (Lin^{-}cKit^{+}Sca1^{+}CD150^{-}CD48^{+}) populations,[28,29] nor in sorted T cells (CD45^{+}Gr1^{-}CD11b^{-}B220^{-}CD3^{+}), B cells (CD45^{+}Gr1^{-}CD11b^{-}B220^{+}CD3^{-}), macrophages (CD45^{+}Gr1^{+}CD11b^{+}F4/80^{+}, and myeloid cells (CD45^{+}Gr1^{+}CD11b^{+}F4/80^{-}) from 2-year-old Pf4^{+}FF1^{+} mice (Figure 1F). These findings confirm that the human *JAK2* transgene is not expressed outside the MK lineage.

To investigate how *JAK2*^{V617F} mutant MK alter the marrow immune microenvironment, we performed single-cell RNA-seq (scRNAseq) on unfractionated marrow cells from young (4-month-old) and aged (1-year-old) Pf4^{+}FF1^{+} and control mice (N=1 per group). Following data integration, doublet removal and quality control (see Methods), 47,730







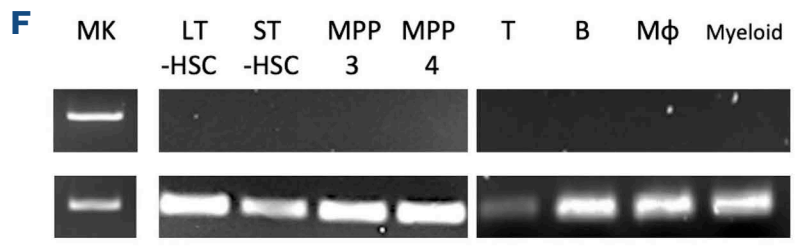


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Figure 1. Inflammatory and immune activation signatures in *JAK2*^{V617F} mutant megakaryocytes linked to skewed marrow T-cell populations during aging of Pf4^{+}FF1^{+} mice. (A) Peripheral blood cell counts of Pf4^{+}FF1^{+} (black line) and Pf4-cre control mice (dotted line) (N=10-23 mice in each group). (B and C) Frequency of bone marrow T cells (CD3^{+}CD4^{+} helper T cells, CD3^{+}CD8^{+} cytotoxic T cells, and CD3^{+}CD4^{+}CD25^{+}FoxP3^{+} regulatory T [Treg] cells), B cells (CD3^{-}B220^{+}), myeloid-derived suppressor cells or myeloid-derived suppressor cells (MDSC) (both CD11b^{+}Ly6C^{high}Ly6G^{-} M-MDSC and CD11b^{+}Ly6C^{low}Ly6G^{+} PMN-MDSC) in young (C) and old (D) Pf4-Cre control (gray) and Pf4^{+}FF1^{+} (black) mice (young: N=5-11 mice in each group; old: N=3-10 mice in each group). (D) Flow cytometry quantification of intracellular IFN-γ protein levels in bone marrow CD8^{+} T cells (N=4-5 mice per group). (E) Overall comparison of marrow CD3^{+} T cells and Treg cells in control and Pf4^{+}FF1^{+} mice across aging (N=3-6 per group). (F) As determined by RT-PCR, human JAK2 gene was expressed in megakaryocytes (MK), but not in long-term hematopoietic stem cell (HSC), short-term HSC, MPP2, MPP3, T, B, macrophages, or myeloid cells isolated by flow cytometry from the bone marrow of 2 aged Pf4^{+}FF1^{+} mice. *P<0.05; **P<0.01; ***P<0.001.

cells were analyzed from 4 mice (9,800-15,678 cells per mouse). Unsupervised clustering of all the data identified 29 clusters of marrow cells, visualized by uniform manifold approximation and projection (UMAP). Cell identity was manually assigned to clusters based on the differential expression of known marker genes, identifying distinct immune and hematopoietic populations including neutrophil, monocyte-macrophage, T-natural killer (NK) cell, B cell, dendritic cell, granulocyte-monocyte progenitor, MK-HSPC, eosinophil-basophil-mast cell, and erythrocyte cell populations (Figure 2A). We performed an integrated cluster analysis of the mixed MK/HSPC population. HSPC were identified by enriched expression of CD34, Flt3, and CD27, while MK were characterized by high expression of Pf4, Gp9, Mpl, and vWF (Figure 2B). We did not detect any distinct “immune MK” populations with high Ccl3, Ccl4, Cd53, Lsp1, or Cxcr4 expression,[11,30-32] likely because our scRNAseq was performed on unfractionated marrow cells rather than MK-enriched samples.

Consistent with previous findings of decreased HSC proliferation in young Pf4^{+}FF1^{+} mice but increased activity in aged Pf4^{+}FF1^{+} mice,[21,33] gene sets associated with proliferation (e.g., MYC targets, G2/M checkpoint, E2F targets) were significantly up-regulated in HSC from aged Pf4^{+}FF1^{+} mice compared to age-matched controls, contrasting with their expression patterns in young mice (Figure 2C). Gene set enrichment analysis (GSEA) of MK revealed that immune response pathways (e.g., interferon gamma response, interferon alpha response, and TNFα signaling via NFκb) were significantly up-regulated in aged Pf4^{+}FF1^{+} mice, but not in young mice, reinforcing the idea that aging amplified mutant MK-driven immune modulation (Figure 2D). Beyond HSPC and MK, cell proliferation (e.g., MYC targets, G2/M checkpoint, E2F targets), inflammation and immune activation (e.g., PI3K AKT MTOR signaling, reactive oxygen species pathway, IL6 JAK STAT3 signaling, oxidative phosphorylation, TNFα signaling via NFκb, Interferon alpha response), and metabolic pathways (e.g., cholesterol homeostasis, fatty acid metabolism, glycolysis, MTORCS signaling) were highly up-regulated in neutrophils and T cells in aged Pf4^{+}FF1^{+} mice compared to age-matched controls, reflecting a pro-inflammatory marrow immune microenvironment (Figure 2E). In contrast, while cell proliferation pathways are significantly up-regulated in monocytes and macrophages from aged Pf4^{+}FF1^{+} mice, IL6/JAK/STAT3, TNFα/NFkB, interferon, and inflammatory response genes are notably down-regulated, indicating a shift toward a less immune-active phenotype of these cells with aging (*Online Supplementary Figure S1*). Taken together, these findings suggest that *JAK2*^{V617F} mutant MK drive chronic inflammation and immune activation in the marrow, accompanied by skewed T-cell populations (Figure 1B, C), impaired T-cell function (Figure 1D), and an immune-suppressive monocyte phenotype (Figure 2F).

The modulatory functions of *JAK2*^{V617F} mutant megakaryocytes in both innate and adaptive immunity

We assessed the ability of *JAK2*^{V617F} mutant MK to process exogenous protein antigens.[8,10] Marrow MK from aged wild-type and Pf4^{+}FF1^{+} mice were cultured with 200 ug/mL DQ-Ovalbumin (DQ-Ova) and cell fluorescence was measured by flow cytometry and fluorescence microscope (Figure 3A, *Supplementary Figure S2A*) 2 hours (hr) later as a measure of Ova processing. (DQ-Ova fluorescence is only activated upon cellular uptake and proteolytic cleavage.) Antigen presentation on MHC I was evaluated using an anti-MHC class I-Ova antibody. *JAK2*^{V617F} mutant MK from aged Pf4^{+}FF1^{+} mice showed enhanced Ova antigen uptake, processing, and presentation on MHC I compared to wild-type MK from aged control mice.

To validate murine findings in human cells, we assessed the antigen-processing ability of *JAK2*^{V617F} mutant human MK. For this study, we obtained a *JAK2*^{V617F} homozygous mutant-induced pluripotent stem (iPS) cell line derived from an MPN patient (PVB1.4) and a *JAK2* wild-type iPS cell line derived from a healthy donor (BC1).[34] Their genotypes and *JAK2* gene expression were confirmed by a nested allele-specific PCR assay[35] (*Supplementary Figure S3A-C*). These iPS cells can be differentiated into CD34^{+}CD45^{+} hematopoietic progenitors using the spin-embryoid body method, harvested after 2-3 weeks, and further differentiated into CD41^{+}CD42^{+} mature MK[36-38] (*Supplementary Figures S2A* and *S3D, E*). Wild-type (BC1-derived) or *JAK2*V617F mutant (PVB1.4-derived) MK were cultured with 200 ug/mL Ovalbumin-Alexa Fluor 647 for 2 hr at 37°C. Consistent with our findings in murine MK (Figure 3A), *JAK2*^{V617F} mutant human MK also show increased Ova uptake and MHC class I presentation compared to wild-type MK (Figure 3B, *Online Supplementary Figure S2A*), validating the mouse

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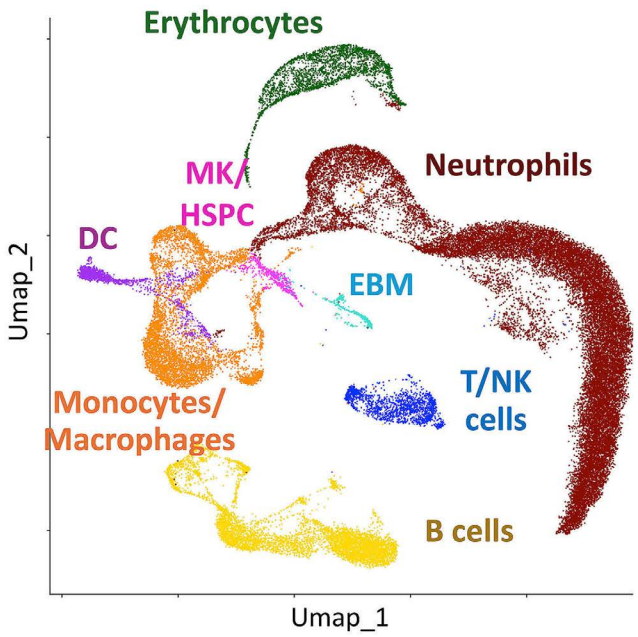

Neutrophils:
S100a8, S100a9, Ly6G
Monocytes-Macrophages:
Ccr2, Csf1r
B cells:
Cd79a
T cells-NK cells:
Cd3d, Ncr1
Dendritic cells (DCs):
Siglech
MK-HSC:
Itga2b, Cd34
Eosinophil-Basophil-Mast cells:
Cd200r3, Fcer1a, Mcpt8, Prss34

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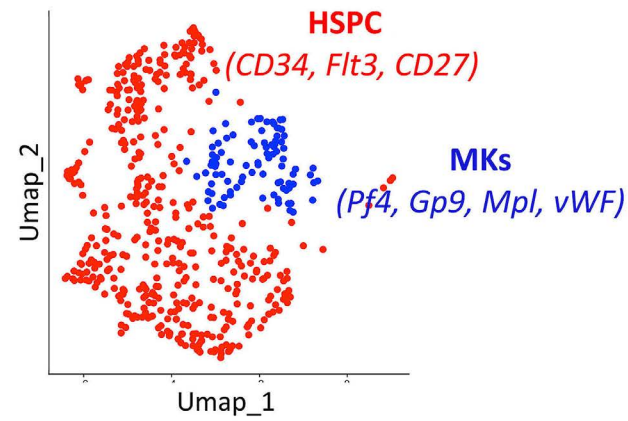

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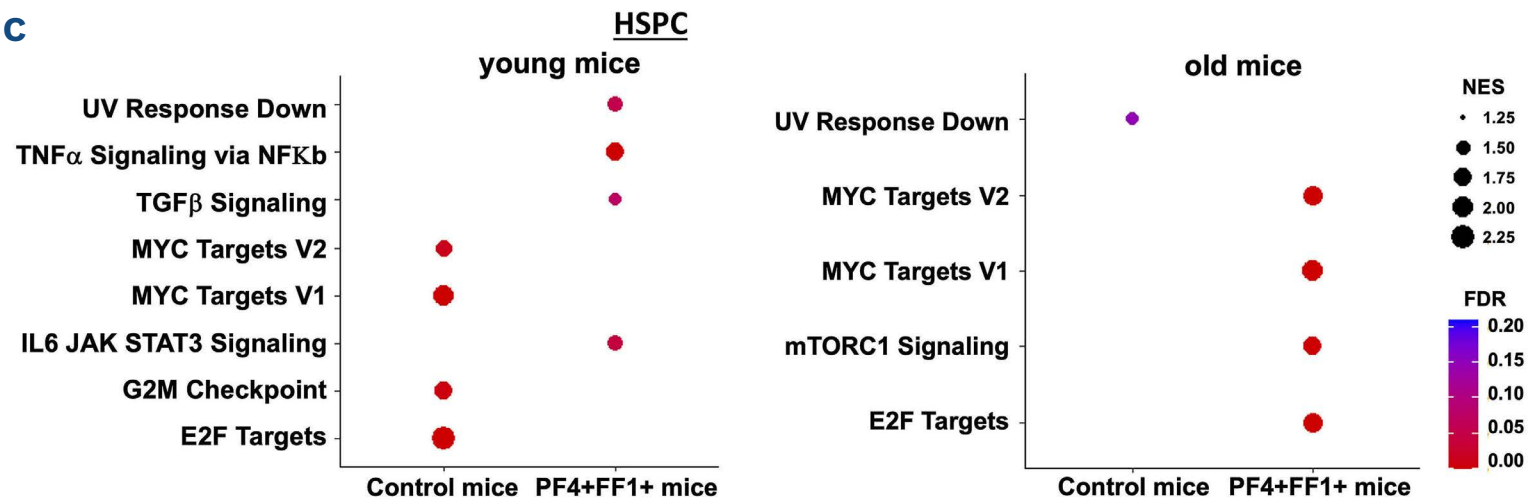

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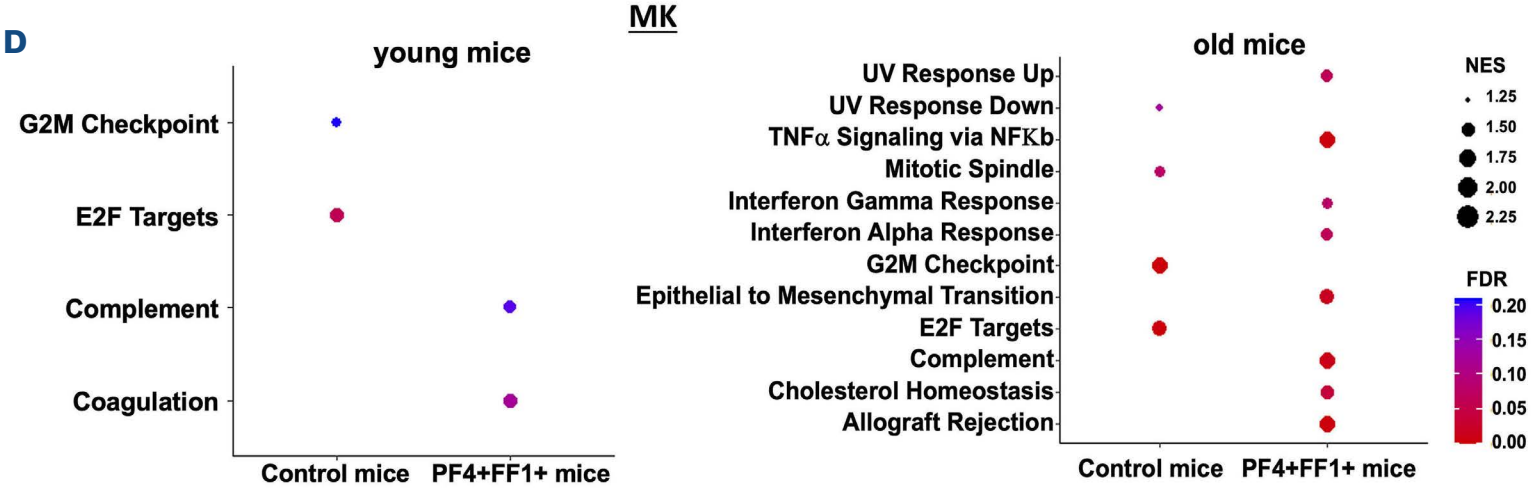

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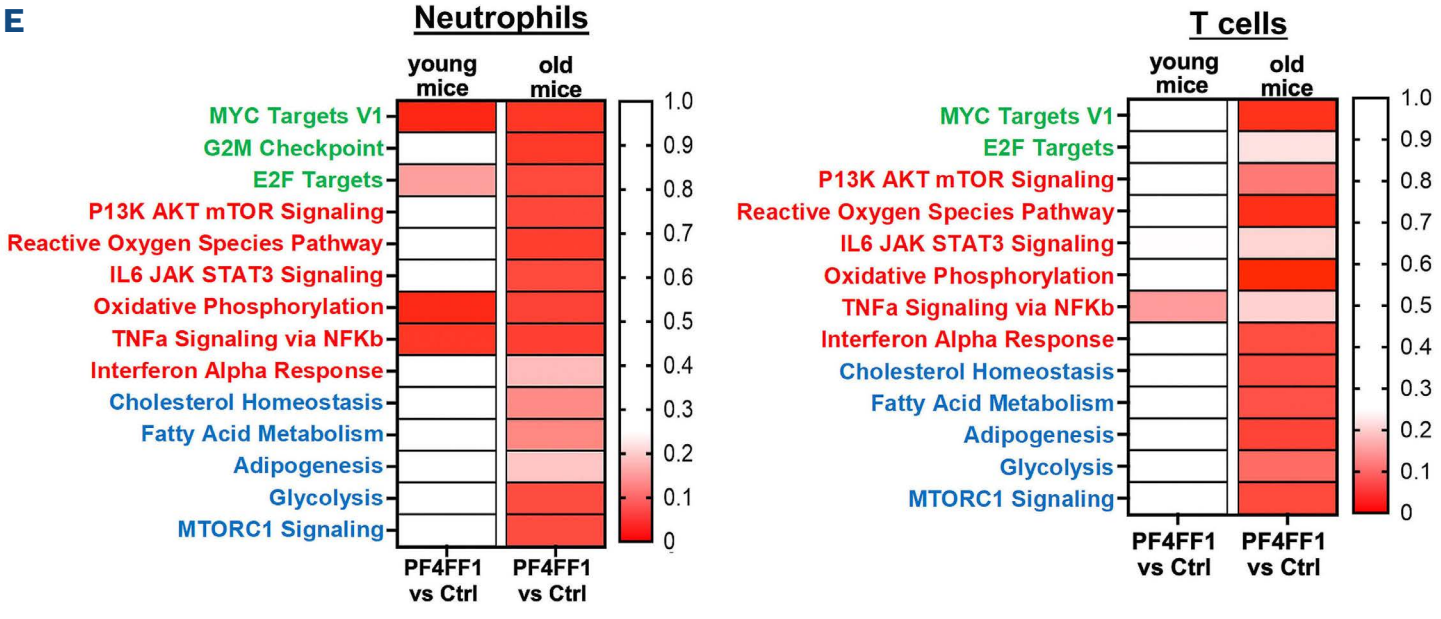

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Figure 2. scRNAseq analysis of marrow cells from young and aged Pf4^{+}FF1^{+} mice. (A) Uniform manifold approximation and projection (UMAP) plot of unfractionated marrow cells, with key markers used to infer cell identity listed. (B) UMAP visualization of the megakaryocyte (MK) / hematopoietic stem cell (HSC) population, with key markers used for cell identity listed. (C and D) Gene set enrichment analysis of hematopoietic stem/progenitor cells (HSPC) (C) and MK (D) from young (left) and old (right) mice. (E) Gene sets associated with cell proliferation (green), inflammation and immune activation (red), and metabolism (blue) in neutrophils and T cells from both young and old Pf4^{+}FF1^{+} mice, each compared with age-matched controls.

observations.

To determine whether MK interact with T cells in an antigen-specific manner, we used OT-I and OT-II transgenic mice, which recognize MHC I-restricted Ova257-264 and MHC II-restricted Ova323-339 peptides, respectively.[39,40] Wild-type mice were treated with a single dose of 25 ug Ova257-264, Ova323-339, or PBS via intraperitoneal injection; 1 hr later, marrow MK were isolated and co-cultured with naïve OT-I CD8^{+} or OT-II CD4^{+} T cells. IFNγ expression and cell proliferation (by carboxyfluorescein succinimidyl ester, or CFSE label dilution) were assessed. Ova257-264-loaded marrow MK significantly enhanced CD8^{+} T-cell proliferation and transiently increased IFNγ production, peaking at day 2. In contrast, Ova323-339-loaded marrow MK had no effect on CD4^{+} T cells (Figure 3C). To confirm that Ova257-264-stimulated MK directly activate CD8^{+} T cells, wild-type marrow MK were pulsed with Ova257-264 *in vitro* (2.5 ug/mL, 2 hr at 37°C) before co-culture with OT-I splenic CD8^{+} T cells. A mouse IFNγ enzyme-linked immunospot (ELISpot) assay revealed a significant increase in IFNγ-secreting CD8^{+} T cells, consistent with *in vivo* findings (Figure 3D). We observed no significant differences in T-cell proliferation or activation between wild-type and *JAK2*^{V617F} mutant MK (*data not shown*), suggesting that both MK types can engage T cells in an antigen-specific manner, although the potency of Ova257-264 may mask their functional differences.

An important function of innate immunity is the production of cytokines to modulate immune responses. To assess their cytokine profiles, we performed targeted cytokine arrays (Abcam®, Cat. 133995) on wild-type and *JAK2*^{V617F} MK isolated from young and old mice. Marrow MK from 2-4 mice per group were pooled to reduce the effects of individual sample variability. In aged Pf4^{+}FF1^{+} mice, mutant MK exhibited elevated expression of multiple inflammatory cytokines compared to wild-type MK from age-matched controls (*Online Supplementary Figure S2B*). Next, we analyzed marrow plasma (collected by flushing each murine femur with 0.5 mL PBS followed by centrifugation at 2,000 g for 15 minutes) using ELISA. This revealed significantly increased levels of platelet factor 4 (PF4), transforming growth factor beta (TGFβ), and interleukin-1 beta (IL1β) in aged Pf4^{+}FF1^{+} mice compared to controls, whereas no difference was observed in young mice (Figure 3E). MK are the primary source of PF4 in the marrow,[4] and its plasma levels are known to be elevated in many MPN patients.[41,42] PF4 has been implicated in immune cell recruitment,[43] monocyte differentiation,[44,45] and CD8^{+} T-cell inhibition.[44,46] Therefore, PF4 upregulation may contribute to immune dysfunction driven by *JAK2*^{V617F} mutant MK.

Overall, *JAK2*^{V617F} mutant MK up-regulate inflammatory and immune-regulatory genes, produce pro-inflammatory cytokines, present antigens, and modulate T-cell functions, all of which may contribute to the inflammatory marrow niche that characterizes MPN and promotes expansion of mutant over wild-type cells.[22]

Increased inflammatory and immunomodulatory gene expression in megakaryocytes correlates with higher mutant cell burden in a murine model with co-existing wild-type and *JAK2*^{V617F} mutant cells

To test whether this heightened immune modulatory MK signature is linked to MPN disease progression, we generated chimeric murine models harboring both wild-type and *JAK2*^{V617F} mutant hematopoietic cells at varying ratios to model MPN mutant cell expansion. We used the Tie2-cre^{+}FF1^{+} murine model we had previously established in which human *JAK2*^{V617F} is expressed in all hematopoietic cells.[47-49] In brief, lethally irradiated wild-type mice (CD45.1) were transplanted with both wild-type marrow cells (isolated from CD45.1 wild-type mice) and *JAK2*^{V617F} mutant marrow cells (isolated from CD45.2 Tie2-cre^{+}FF1^{+} mice) mixed at ratios of 100:0, 90:10, and 10:90. These chimeric mice exhibited a “dose-dependent” MPN phenotype, with those receiving a higher proportion (90%) of mutant donor cells developing leukocytosis, thrombocytosis, and an increased marrow Lin^{-}cKit^{+}Sca1^{+} (LSK) and Lin^{-}cKit^{+}Sca1^{+}CD150^{+}CD48^{-} (HSC) 16 weeks post transplantation, while those receiving a lower proportion (10%) of mutant donor cells remained asymptomatic throughout the 4-month follow-up period (Figure 4A).

We conducted scRNAseq analysis on unfractionated marrow cells from 3 chimeric mice (A, B, and C), characterized by varying mutant cell burdens and distinct hematologic phenotypes at different time points post transplantation (Figure 4B). After quality filtering, a total of 15,403 cells from 3 mice were included in the analysis (Figure 4C). MK were identified by expression of established marker genes, including Itga2b, Pf4, Gp9, vWF, and MPL. Comparative analysis of gene expression profiles revealed significant enrichment of cell proliferation, inflammation, and immune activation gene sets in MK from the high mutant burden MPN mouse (Mouse C) compared to MK from a low mutant burden, non-MPN mouse (Mouse A). Similarly, MK from Mouse C showed the same enrichment when com-

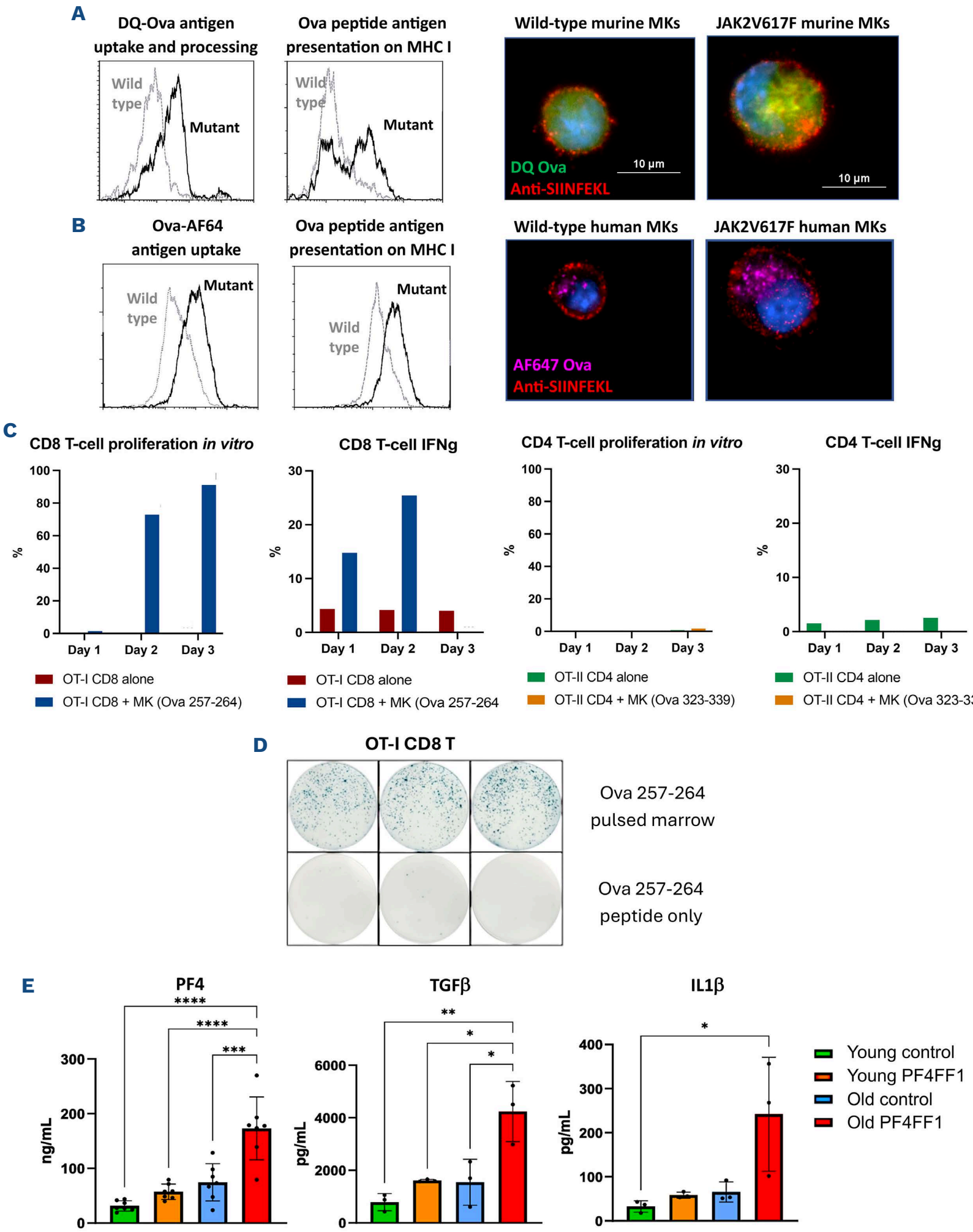

Figure 3. The immunomodulatory functions of *JAK2*^{V617F} mutant megakaryocytes. (A) Representative flow cytometry analysis (left) and fluorescence microscope images (right) showing wild-type and *JAK2*^{V617F} mutant megakaryocytes (MK) processing DQ-Ova (green fluorescence) and presenting Ova peptide antigen on MHC I molecules (red fluorescence). Data are from one of 3 independent experiments that gave similar results. (B) Representative flow cytometry analysis (left) and fluorescence microscope images (right) of wild-type and *JAK2*^{V617F} mutant iPS-derived human MK uptake Ova (purple fluorescence) and present Ova peptide antigen on MHC I molecules (red fluorescence). Magnification: 1000x. (C) OT-I CD8$^+$ T cell and OT-II CD4$^+$ T-cell proliferation and IFNγ expression following co-culture with marrow MK from Ova-treated mice. Data are from one of 2 independent experiments that gave similar results. (D) IFNγ ELISpot assays showing OT-I CD8$^+$ T-cell activation after co-culture with Ova257-284 pulsed MK. Data are from one of 3 independent experiments that gave similar results. (E) Protein levels of PF4, TGFβ, and IL1β in marrow plasma from young and old Pf4$^+$FF1$^+$ mice and aged-matched controls (N=3-7 mice per group). *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.

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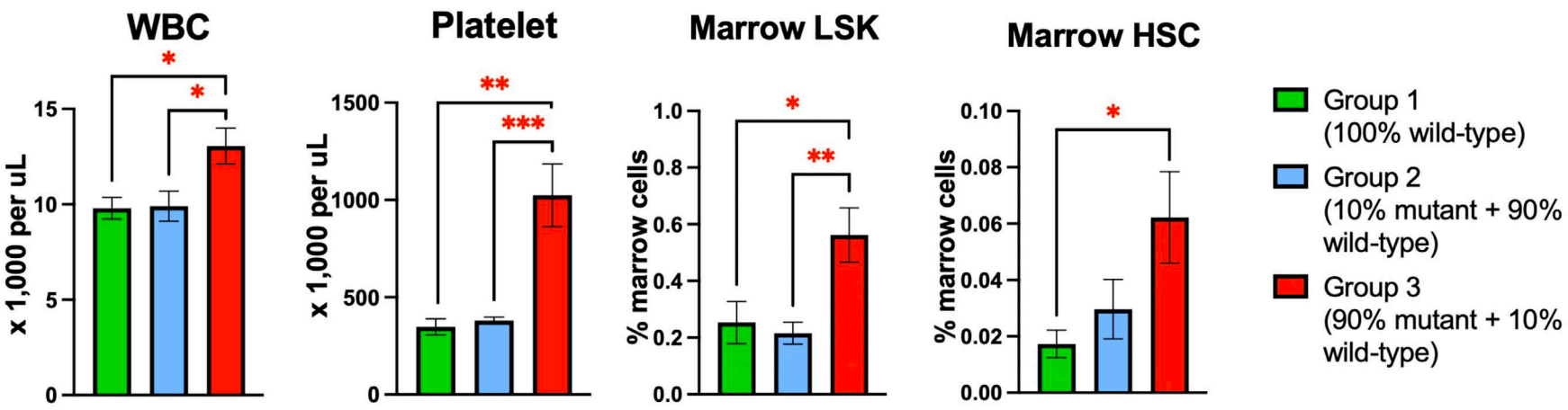

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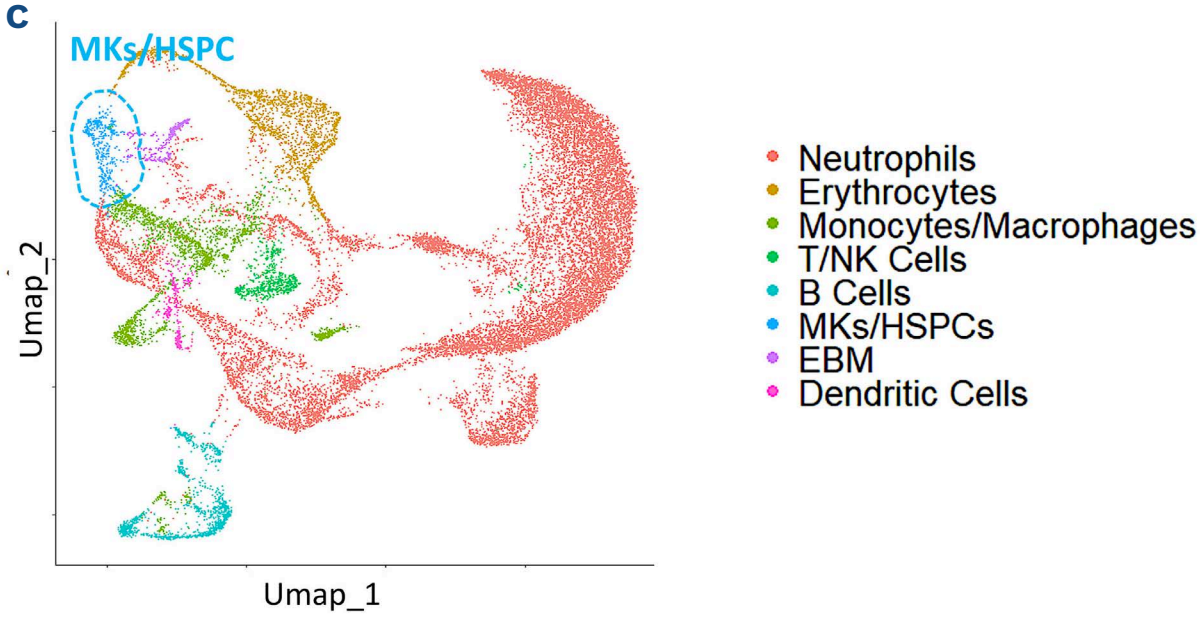
	Mutant: Wild-type donor ratio	Weeks after transplant	Blood count	Blood mutant donor chimerism	Marrow mutant donor chimerism	Marrow MKs	Marrow LSKs
Mouse A	10:90	16 wks	Normal	2%	14%	0.78%	0.156%
Mouse B	90:10	12 wks	Normal	63%	95%	0.56%	0.126%
Mouse C	90:10	16 wks	leukocytosis, thrombocytosis	95%	95%	3.73%	0.451%

C



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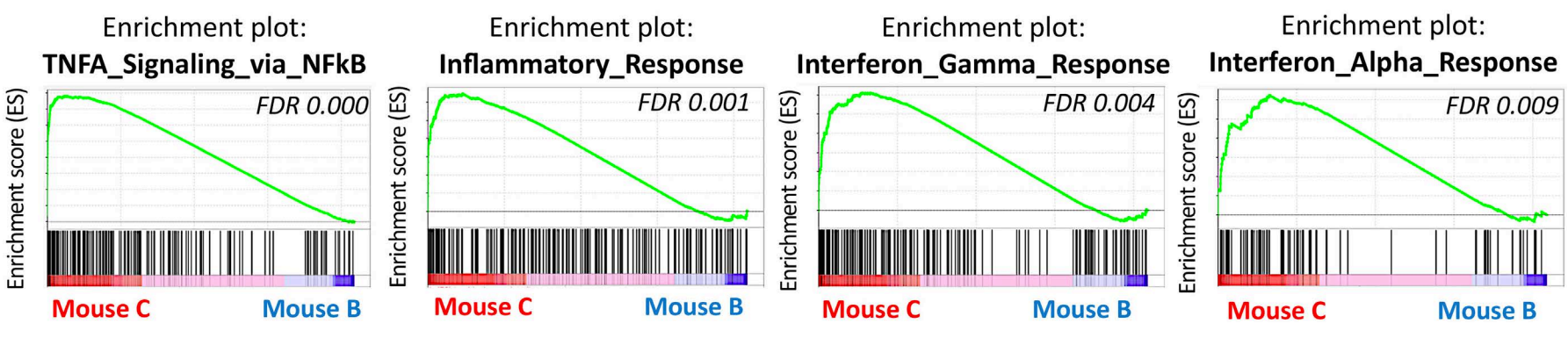

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Figure 4. Augmented inflammatory and immunomodulatory gene expression in megakaryocytes is associated with mutant cell expansion in myeloproliferative neoplasm. (A) Peripheral blood white blood cell (WBC) and platelet counts, marrow LSK and hematopoietic stem cell (HSC) numbers of the chimeric mice with varying mutant cell burdens (N=6-8 mice in each group). (B) Mice selected for scRNAseq analysis. (C) Uniform manifold approximation and projection (UMAP) plot showing cell clusters from unfractionated marrow cells. (D) Top gene sets significantly enriched in Mouse C (90% mutant) MK compared to mouse A (10% mutant) MK. (E) Top gene sets significantly enriched in Mouse C (90% mutant, positive myeloproliferative neoplasm [MPN] phenotype) MK compared to Mouse B (90% mutant, no MPN phenotype) MK. LSK: $Lin^{-}cKit^{+}Sca1^{+}$. *P<0.05; **P<0.01; ***P<0.001.

pared to MK from a comparable mutant burden, non-MPN mouse (Mouse B) (Figure 4D, E). These findings suggest that heightened immunomodulatory signatures in MK are associated with mutant cell burden and may contribute to MPN development (Mouse C *vs*. Mouse A) and that the immunomodulatory functions of mutant MK are not solely attributable to the presence of the *JAK2*^{V617F} mutation alone (Mouse C *vs*. Mouse B). In contrast, angiogenesis and oxidative phosphorylation pathways were significantly enriched in MK from Mouse B compared to MK from Mouse A, indicating a metabolically active and proangiogenic state in MK from mice with high mutant burden but no MPN blood phenotype (*Online Supplementary Figure S4*).

Elevated LINE-1 transcription in bone marrow megakaryocytes in myeloproliferative neoplasm murine models.

Half of the human genome consists of transposable elements, with LINE-1 being the only protein-coding transposon that remains active in humans.[50] While LINE-1 reverse transcriptase activity has been detected in human and mouse platelets,[51] little is known about its expression in MK. Since LINE-1 activation is linked to DNA replication and cell cycling,[52] and MK undergo endomitosis (a unique form of cell cycling during which MK undergo multiple rounds of DNA synthesis without cell division[53]), it is likely that LINE-1 activity increases in MK with aging.

Full-length LINE-1 elements in mice span ~7kb, containing a 5' UTR, two open reading frames ORF1 and ORF2, and a 3' UTR with a poly(A) tail. RT-quantitative PCR (qPCR) using multiple primer pairs targeting active LINE-1 families[54] revealed a significant increase in 5'UTR, ORF1, ORF2, and 3'UTR transcripts in mutant MK from aged mice, but not young mice (Figure 5A). Such a trend was not observed in other marrow cell populations including $CD3^{+}$ T cells, $B220^{+}$ B cells, $CD11b^{+}$ myeloid cells, or $Lin^{-}cKit^{+}Sca1^{+}$ HSPC (Figure 5B). Treating marrow MK from aged control and $Pf4^{+}FF1^{+}$ mice with lamivudine (25 uM for 24 hr), a reverse transcriptase inhibitor used to suppress HIV replication, significantly reduced LINE-1 transcripts in mutant MK (Figure 5C). Although this is likely an indirect effect of reverse transcription inhibition, it suggests that retrotransposition contributes to the elevated LINE-1 RNA levels observed in *JAK2*^{V617F} mutant MK. Given LINE 1's role in aging and innate immunity, its upregulation in aged mutant MK may drive innate immune activation and inflammatory remodeling of the marrow.[54-57] Consistent with this, the innate immune sensors cGAS and stimulator of interferon genes (STING), which induce interferon and interferon-regulated genes,[50] are up-regulated in *JAK2*^{V617F} mutant MK from aged $Pf4^{+}FF1^{+}$ mice (Figure 5D).

LINE-1 ORF1p protein expression in marrow megakaryocytes of myeloproliferative neoplasm patients

Finally, we assessed LINE-1 expression in marrow biopsies from MPN patients (N=13, average age 71 yr) and age-matched controls from orthopedic surgery patients (4 hip marrow samples and 1 rib marrow samples; average age 71 yr) using immunohistochemistry (IHC) for CD41 and ORF1p. ORF1p staining was validated in adult testis tissue, known to have low ORF1p expression,[58] and MK were identified based on morphology and CD41 positivity (Figure 6A). No ORF1p protein was detected in any of the 5 control marrow samples. In contrast, ORF1p expression was observed in marrow MK in 12 out of 13 MPN patients, with varying levels of positivity (Figure 6B). Within individual MPN patients, both ORF1p-positive and ORF1p-negative MK co-existed, with 40-100% MK expressing ORF1p in affected patients (Figure 6C). Notably, while ORF1p expression was largely restricted to MK, diffuse ORF1p expression in non-MK marrow cells was observed in 2 patients with advanced disease: one transitioning from PV to myelodysplastic syndrome (MDS) (Patient #SL025) and another with accelerated-phase post-PV myelofibrosis (MF) (Patient #SL046) (Figure 6D). These findings suggest that LINE-1 activation is a hallmark of MPN marrow MK, with its expression increasing in advanced disease stages. The presence of ORF1p in non-MK marrow cells in progressive MPN cases further raises the possibility that LINE-1 activation extends beyond MK during disease evolution, potentially contributing to neoplastic clonal expansion.

Discussion

Megakaryocytes, long recognized for their role in platelet production, are increasingly understood to be key regulators of the hematopoietic and immune microenvironment. By producing inflammatory cytokines and immune mediators, MK actively participate in pathogen surveillance and immune responses in tissue microenvironment.[6-11,59] However, how diseased MK alter these functions to influence HSC behavior in neoplastic hematopoiesis remains unclear. Our

findings suggest that *JAK2*^{V617F} mutant MK may act as potent immunomodulators, contributing to the reprogramming of the marrow immune microenvironment and potentially promoting disease progression in MPN. Compared to wild-type MK, mutant MK exhibit heightened inflammation and innate immune activation, including increased antigen presentation, elevated pro-inflammatory cytokines (PF4, TGFβ, IL1β), skewed T-cell populations, and impaired T-cell functions in the *JAK2*^{V617F}-bearing MK niche *in vivo*. These changes are further amplified by aging, a major risk factor for MPN progression. To determine the impact of MK-driven immune dysregulation on MPN evolution, we developed a chimeric murine model with co-existing wild-type and *JAK2*^{V617F} mutant HSC, mimicking the competitive dynamics observed in MPN patients. Our results reveal that enhanced MK immunomodulatory function is linked to *JAK2*^{V617F} mutant

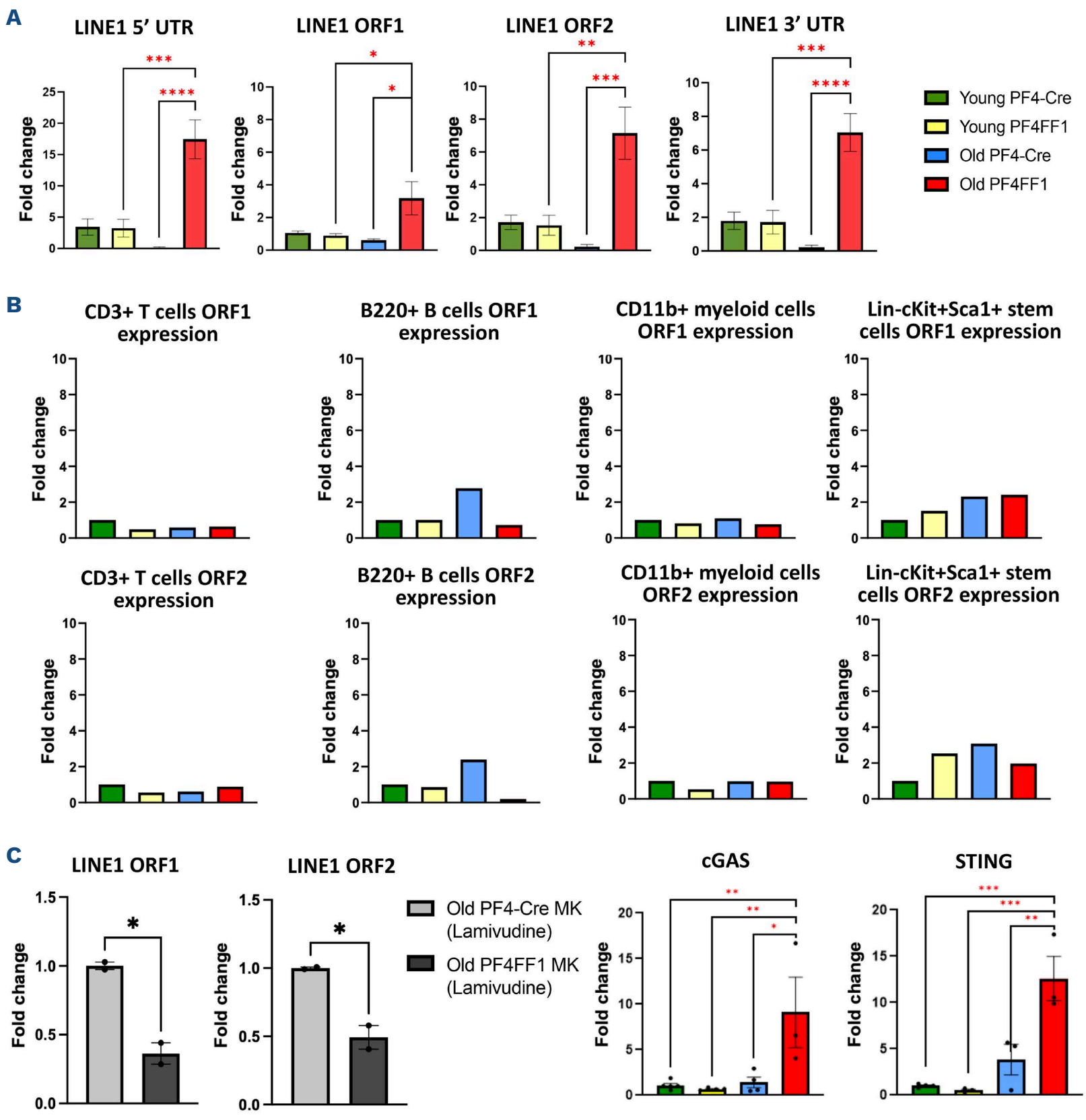
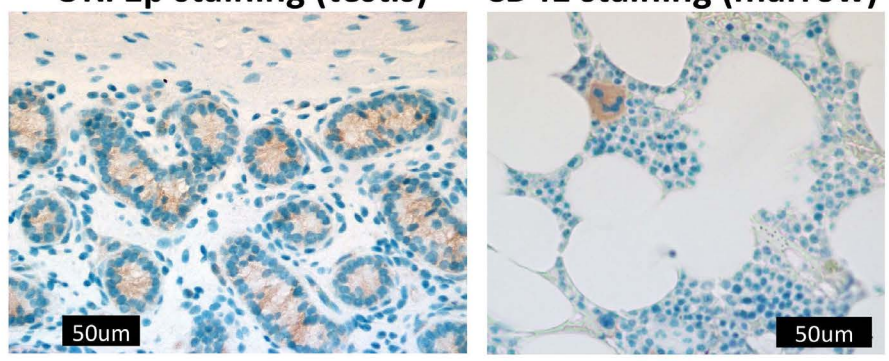


Figure 5. Elevated LINE-1 expression in *JAK2*^{V617F} mutant megakaryocytes during aging. (A) LINE-1 RNA levels measured using primers specific to the 5' UTR, ORF1, ORF2, and 3' UTR. The transcript levels are shown as fold changes relative to their expression levels in megakaryocytes (MK) from young control mice (N=3-4 mice in each group). (B) LINE-1 ORF1 (top) and ORF2 (bottom) expression in bone marrow T cells, B cells, myeloid cells, and Lin$^-$cKit$^+$Sca1$^+$ stem/progenitor cells isolated from young and aged mice, isolated by flow sorting. Each group represents pooled samples from 2 mice. (C) ORF1 and ORF2 RNA levels in control and *JAK2*^{V617F} mutant marrow MK after lamivudine treatment. (D) Cyclic GMP–AMP synthase (cGAS) and stimulator of interferon genes (STING) gene expression shown as fold change relative to their expression levels in MK from young control mice (N=3-4 mice in each group). **P*<0.05; ***P*<0.01; ****P*<0.001; *****P*<0.0001.

A



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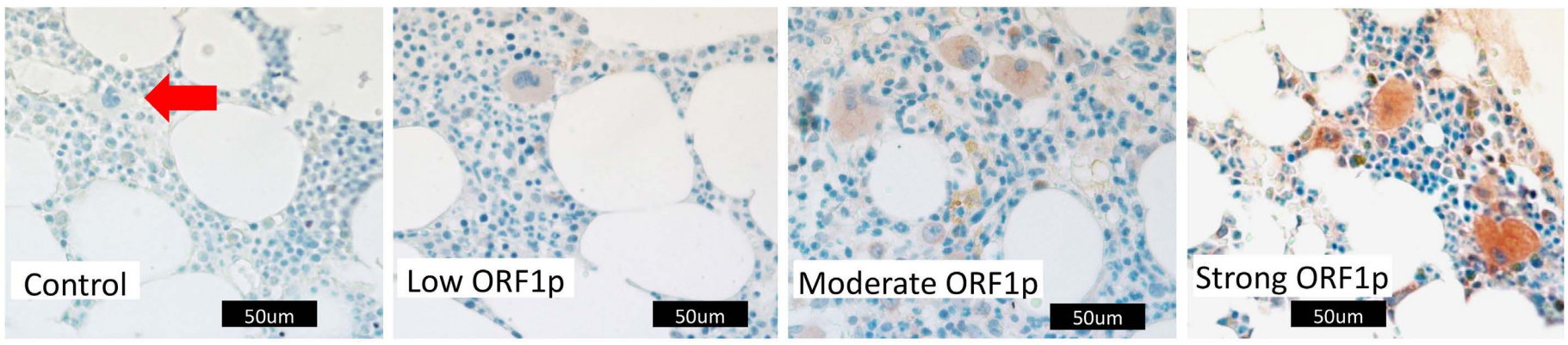

C

Patient N	Gender	Age	MPN diagnosis	Mutation status	ORF1p-positive MK %
SL001	Female	78	MPN, nos	JAK2V617F	69%
SL002	Male	80	MPN, nos	ND	90%
SL003	Male	82	ET	ND	94%
SL004	Male	47	PMF	ND	95%
SL007	Female	78	PMF	JAK2V617F	69%
SL012	Female	80	ET	CALR	50%
SL016	Male	65	ET	CALR	41%
SL019	Male	58	ET	CALR	98%
SL025	Male	63	PV, evolving into MDS	JAK2V617F	100%
SL030	Male	72	PV	JAK2V617F	80%
SL035	Male	69	ET	JAK2V617F	73%
SL039	Female	76	PV	JAK2V617F	0%
SL046	Female	76	Post-PV MF (accelerated phase)	JAK2V617F	100%

D

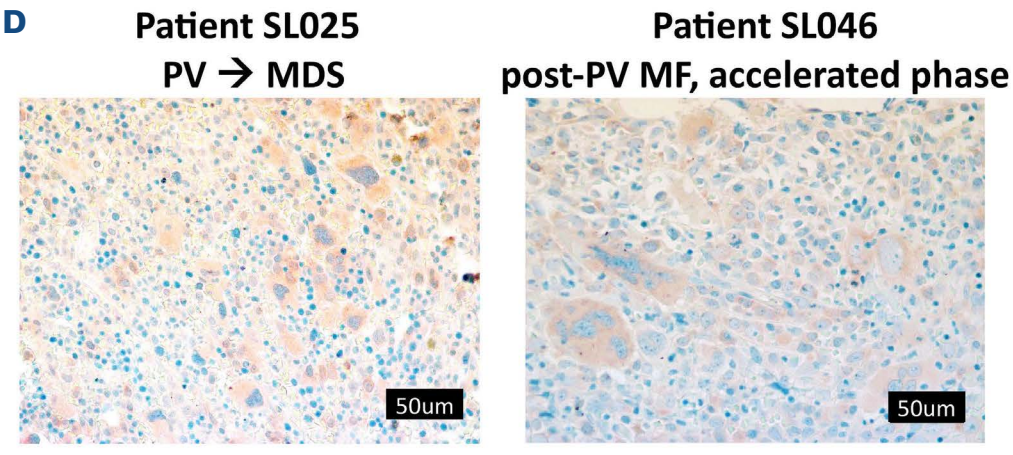

Figure 6. LINE-1 ORF1p protein expression in marrow megakaryocytes from myeloproliferative neoplasms patients. (A) Representative images of ORF1p-positive staining in a histologically normal adult testis (left) and CD41 staining in normal bone marrow (right). (B) ORF1p protein expression in marrow biopsies from a representative control patient and 3 myeloproliferative neoplasm (MPN) patients with low, moderate, and strong ORF1p expression. (C) Summary of gender, age, MPN diagnosis, and ORF1p-positive megakaryocytes (MK) (%) in the 13 MPN patients analyzed. (D) Diffuse ORF1p expression in the marrow of 2 MPN patients with disease progression. ORF1p-positive MK% = number of ORF1p-positive MK/total MK [ORF1p-positive + ORF1p-negative x 100%. An average of 70 MK were evaluated per sample. All images were captured at 400x magnification.

cell expansion, underscoring the role of MK as key drivers of clonal hematopoiesis. These findings were further validated in human cells using an MPN patient-derived iPS cell line model, which allowed us to confirm increased antigen processing and presentation in *JAK2*^{V617F} mutant MK compared to wild-type MK in a genetically controlled system. Approximately half of the human genome is composed of transposable elements, with LINE-1 being the only protein-coding transposon still active in humans.[50] As LINE-1 replicates itself using RNA intermediates via retrotransposition, LINE-1-derived endogenous nucleic acids can act as danger signals to trigger the innate immune response via pattern recognition receptors, leading to immune cell activation and the production of inflammatory cytokines.[60] LINE-1 retrotransposition activity peaks during cell cycling and DNA replication.[52] MK, which undergo endomitosis and accumulate large amounts of DNA and RNA, may therefore be particularly vulnerable to LINE-1 reactivation. However, the role of LINE-1 in MK has been largely unexplored. Here, we show that LINE-1 transcripts and the innate immune sensors cGAS and STING are significantly up-regulated in aged *JAK2*^{V617F} mutant MK in a murine model of MPN (Figure 5). This finding is corroborated by detection of LINE-1–encoded ORF1p protein in marrow MK from 13 of 14 MPN patients, but not in age-matched controls undergoing orthopedic surgery (Figure 6). Given that LINE-1 activation is a known driver of innate immune activation and is implicated in aging-related inflammation,[54-57] our findings suggest that elevated LINE-1 expression in diseased MK may contribute to the inflammatory marrow microenvironment characteristic of MPN.

While chronic inflammation is a well-established driver of HSC stress and aging-associated hematologic disorders, the specific niche cells responsible for inflammatory cytokine production remain poorly defined. Our findings suggest that MK play an important role in regulating niche inflammation in MPN during aging, and they can influence both innate and adaptive immunity to shape a marrow microenvironment that favors neoplastic HSC expansion over wild-type hematopoiesis. The translational significance of these findings extends beyond MPN. Abnormal megakaryopoiesis is a common feature of many hematologic malignancies,[12-16] and aging-associated clonal hematopoiesis is increasingly recognized as a risk factor for cardiovascular disease, immune dysregulation, and cancer.[61] Our study highlights a broader role for MK-driven inflammation in shaping both the marrow and systemic immune landscape, with potential implications for developing novel therapeutic strategies targeting inflammatory and immune pathways activated in mutant MK. Several important questions remain. For instance, how does *JAK2*^{V617F} promote LINE-1 activation in MK? Does MK-driven inflammation cause or accelerate MPN disease progression, or is it merely a consequence of clonal expansion? Answering these questions will be critical for establishing MK as key regulators of marrow inflammation and immune modulation, and for developing targeted therapies that disrupt the vicious cycle of mutant MK-driven inflammation, immune dysfunction, and clonal expansion.

Disclosures

No conflicts of interest to disclose.

Contributions

XY performed transgenic murine model experiments and data analysis, and conducted patient sample studies; SL performed transgenic murine model experiments and data analysis; KM contributed to transgenic murine model studies and scRNAseq data analysis; TA provided patient samples; LZ provided scientific and technical support for immunology studies; HZ designed and supervised the experiments, analyzed data, interpreted results, and wrote the manuscript. All authors read and approved the final version of the manuscript for publication.

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Data-sharing statement

RNA sequencing data will be deposited in the GEO data repository. Other data that support the findings of this study are available from the corresponding author upon reasonable request.

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