RESEARCH LETTER

blood advances

TO THE EDITOR:

Elevated RIPK3 correlates with disease burden in myelofibrosis

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Recent work has strengthened the central role of inflammatory signaling in the pathogenesis and maintenance of myeloproliferative neoplasms (MPN).[1,2] In myelofibrosis (MF) particularly, symptom burden, fibrogenesis, and prognosis are tightly linked to the secretion of proinflammatory cytokines such as interleukin-8, vascular endothelial growth factor and basic fibroblast growth factor (b-FGF).[3-6] Above all, tumor necrosis factor (TNF) is a disease driver.[5,7] The inflammatory phenotype of MF and the common presence of cytopenia suggest a role in inflammatory cell death, such as regulated necrosis (necroptosis), which has not been studied further. So far, TNF signaling has been the best-characterized necroptotic pathway with receptor-interacting protein kinase 3 (RIPK3) serving as inducer and mixed lineage kinase domain like pseudokinase (MLKL) as executor of necroptotic cell death.[8-11] Furthermore, RIPK3 possesses strong, cell death-independent inflammatory capacity.[8,12,13] Despite recent advances in understanding the molecular mechanisms of necroptotic signaling, its relevance for the initiation and progression of hematologic malignancies is still uncertain.

Here, we aimed to elucidate the role of RIPK3 signaling in MF. Therefore, RIPK3 protein expression was quantified by flow cytometry in permeabilized peripheral blood mononuclear cells (PBMCs) from a cohort of 48 patients with MPN, compared with healthy individuals. The detailed experimental approach is explained in the supplemental Files. At the protein level, RIPK3 was significantly increased in the blood of patients with MF, when compared with other MPN subtypes and healthy controls (Figure 1A). By contrast, disease progression from MF to secondary acute myeloid leukemia (sAML) was accompanied by a significant decrease in RIPK3 expression in line with RIPK3 being a potent tumor suppressor in FLT3-ITD$^+$ AML.[14] When focusing on the MF subgroup, increased RIPK3 protein levels were detected across all MF samples including primary myelofibrosis, post-essential thrombocythemia (ET) MF, and post-polycythemia vera (PV) MF (supplemental Figure 2A). Moreover, the RIPK3 protein expression pattern was independent of the individual driver mutation (Figure 1A; supplemental Figure 2B). This is not unexpected because mutations in *JAK2* V617F, *CALR,* or *MPL* all result in the activation of STAT signaling promoting cytokine production.[15,16] Notably, therapy with ruxolitinib did not affect RIPK3 levels in patients with MF (supplemental Figure 2C). The study was approved by the local ethics committee of the Technical University Munich (362/19S) and conducted by the Declaration of Helsinki.

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Data are available on request from the corresponding author, Veronika Dill (veronika.dill@tum.de).

The full-text version of this article contains a data supplement.

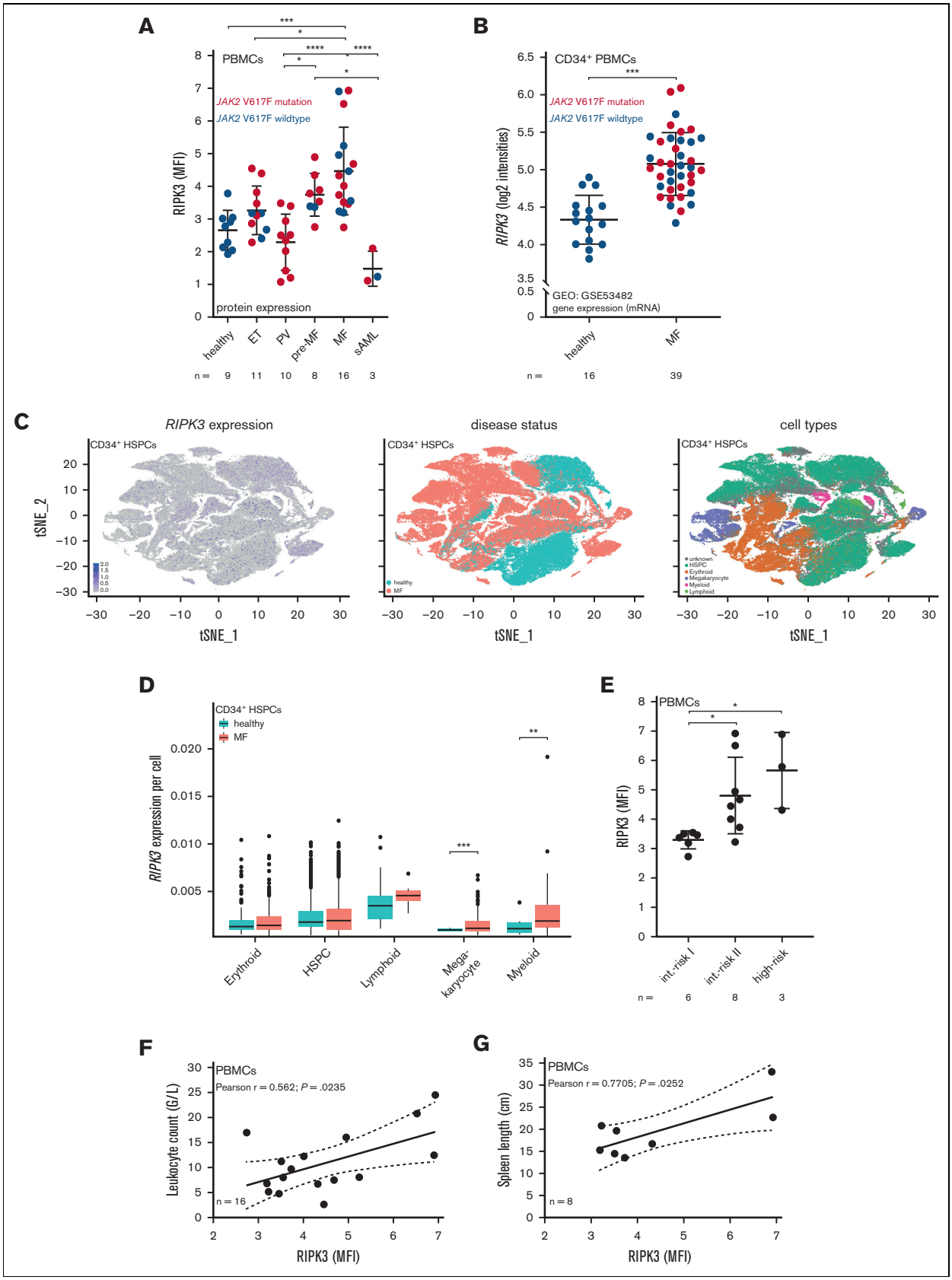

Figure 1.

Gene expression data of *RIPK3* were analyzed in a cohort sample of patients with MF and healthy control subjects from a public database (GEO: GSE53482). Messenger RNA analysis revealed a significant increase in *RIPK3* gene expression in CD34$^+$ PBMCs for patients with MF (Figure 1B), again without demonstrating an influence of the *JAK2* V617F mutational status. Of note, elevated RIPK3 levels were detected in MF bone marrow (BM) by flow cytometry and immunohistology (supplemental Figures 2D and 4). Immunohistochemical staining also showed elevated protein levels of the necroptotic effector MLKL in granulopoiesis of patients with MF compared to other MPN subtypes (supplemental Figure 5). Interestingly, MLKL was also highly expressed in healthy granulopoiesis, possibly consistent with the known dependence of neutrophil extracellular traps formation on MLKL.[17,18] Analyzing single-cell RNA sequencing data of human CD34$^+$ hematopoietic stem and progenitor cells from a public database (GEO: GSE144568),[19] we found an increased *RIPK3* expression in MF megakaryocytes and myeloid cells (Figure 1C), mediated by an increased expression within RIPK3 positive cells (Figure 1D; supplemental Figure 6). Elevated *RIPK3* in myeloid cells might be due to excessive TNF in MPN monocytes.[20]

We also aimed to detect clinical parameters correlating with RIPK3 expression in patients with MF (including primary myelofibrosis, post-ET MF, and post-PV MF). When stratified according to the common clinical Dynamic International Prognostic Scoring System, patients with advanced MF disease (classified as "intermediate-risk II" and "high-risk") featured significantly higher RIPK3 protein levels than patients with lower-risk MF disease (classified as "intermediate-risk I") (Figure 1E), suggesting that RIPK3 expression increases with disease progression. When correlated with clinical parameters, RIPK3 protein expression was associated with leukocytosis (Figure 1F) and spleen size (Figure 1G) in patients with MF. These data propose that RIPK3 might be involved in inflammation-driven proliferation and extramedullary hematopoiesis. Several studies have reported that leukocytosis in MPN itself is associated with increased levels of proinflammatory cytokines such as TNF and b-FGF.[21-23]. Splenomegaly has been attributed to specific cytokines,[21,22] as well as the expansion of the malignant clone from the BM to extramedullary organs, which is thought to be particularly mediated by TNF.[5] Unlike a recent study showing that gene expression of the necroptotic effector *MLKL* correlated with anemia in myelodysplastic syndromes,[24] no significant correlation of RIPK3 with hemoglobin levels (supplemental Figure 3A) or thrombocyte count (supplemental Figure 3B) was detected. Remarkably, there was no significant correlation observed between RIPK3 levels and age (supplemental Figure 3C) suggesting that chronic age-related inflammation, often referred to as "inflammaging,"[25] does not influence RIPK3 protein levels.

To assess the inflammatory phenotype of patients with MF, central proinflammatory cytokines were quantified via cytometric bead assay (CBA) in the serum of patients with MF and healthy controls (supplemental Figure 7). Here, levels of interleukin-8 (Figure 2A) and vascular endothelial growth factor (Figure 2B) were significantly elevated in MF. Interestingly, RIPK3 protein levels correlated positively with the b-FGF levels of the respective MF patient sample (Figure 2C). These data confirm that RIPK3 is particularly elevated in patients with MF with an inflammatory phenotype and highlight the close connection between inflammation, proliferation, and RIPK3 signaling in this disease.

To functionally characterize the role of RIPK3, PBMCs from patients with MF, patients with sAML after a history of MPN, and healthy controls were treated with the RIPK3-inhibitor GSK'843 (1 μM) for 72 hours. Dimethyl sulfoxide (dimethyl sulfoxide [DMSO]; 1:1000) was used as soluble control. Cytokine analysis of the supernatant revealed that RIPK3 inhibition significantly reduced TNF levels of 6 patients with MF compared to DMSO control in vitro (Figure 2D). Additionally, the viability of PBMCs after RIPK3 inhibition was analyzed by flow cytometry. Here, RIPK3 inhibition significantly increased the viability of PBMCs from patients with MF compared to DMSO control in vitro (Figure 2E). Of note, pharmacological inhibition of RIPK3 did not affect the viability of PBMCs from healthy controls or patients with sAML. Furthermore, the increase in cell viability was significantly higher in patients with MF than in healthy individuals, and patients with sAML, suggesting a disease-specific effect of RIPK3.

To evaluate the longer-term effects of RIPK3 inhibition on the stem/progenitor cell level, BM mononuclear cells from patients with

Figure 1. RIPK3 is significantly increased in MF and correlates with disease burden. (A) Intracellular RIPK3 protein expression of PBMCs from 9 healthy control subjects, 11 patients with ET, 10 patients with PV, 8 patients with pre-MF, 16 patients with MF, and 3 patients with sAML after a history of MPN were quantified via flow cytometry. RIPK3 protein expression was calculated as the ratio of stained antibody MFI divided by isotype control MFI. Shown are the mean and error bars denoting standard deviation. The presence of a *JAK2* V16F mutation is indicated by the coloration of the individual patient samples. One-way ANOVA $P < .0001$; P values from post hoc analysis with Tukey test as indicated in the figure. (B) *RIPK3* gene expression (mRNA) was analyzed in primary human CD34$^+$ PBMCs from 39 patients with PMF (including 18 *JAK2* V16F mutated and 21 *JAK2* V617F wild-type patients) and 16 healthy control subjects using the Human Genome U219 Array (GEO: GSE53482). Shown are the mean and error bars denoting standard deviation. P values from Student t test as indicated in the figure. (C-D) Analysis of single-cell RNA sequencing data of primary human CD34$^+$ HSPCs from a public database (GEO: GSE144568). (C) Dimensionality reduction by t-SNE summarizing all MF and control cells analyzed. Each dot represents a single cell. Left panel: t-SNE colored by *RIPK3* expression (low [gray] or high [blue]). Middle panel: t-SNE colored by disease status (healthy donors [blue] or MF [red]). Right panel: t-SNE colored by hematopoietic cell types according to the expression of lineage signature genes as indicated in the figure. (D) Boxplots indicating *RIPK3* expression within positive cells in MF and control cells, normalized by the number of positive cells. (E-G) RIPK3 protein expression in PBMCs was analyzed and calculated as described in Figure 1A. (E) RIPK3 protein expression in PBMCs from 17 patients with MF (including PMF, post-PV MF, and post-ET MF) including 6 patients with DIPSS-based classification as intermediate-risk I, 8 intermediate-risk II patients, and 3 high-risk patients. Shown are the mean and error bars denoting standard deviation. One-way ANOVA $P = .0133$; P values from post hoc Tukey test as indicated in the figure. (F) Linear regression analysis of RIPK3 protein levels and leucocyte count (G/L) in patients with MF (including PMF, post-ET MF, and post-PV MF) ($n = 16$). Pearson $r = 0.562$, $P = .0235$, $y = 2.552x - 0.5553$. (G) Linear regression analysis of RIPK3 protein levels with spleen length (cm) in patients with MF (including PMF, post-ET M, F, and, post-PV MF; $n = 8$). Pearson $r = 0.7705$, $P = .0252$, $y = 3.095x + 5.833$. All panels: level of significance .05: $^*P < .05$, $^{**}P < .005$, $^{***}P < .0005$ and $^{****}P < .0001$. ANOVA, analysis of variance; DIPSS, Dynamic International Prognostic Scoring System; HSPC, hematopoietic stem and progenitor cell; MFI, mean fluorescence intensity; mRNA, messenger RNA; PMF, primary myelofibrosis; sAML, secondary acute myeloid leukemia; t-SNE, t-distributed stochastic neighbor embedding.

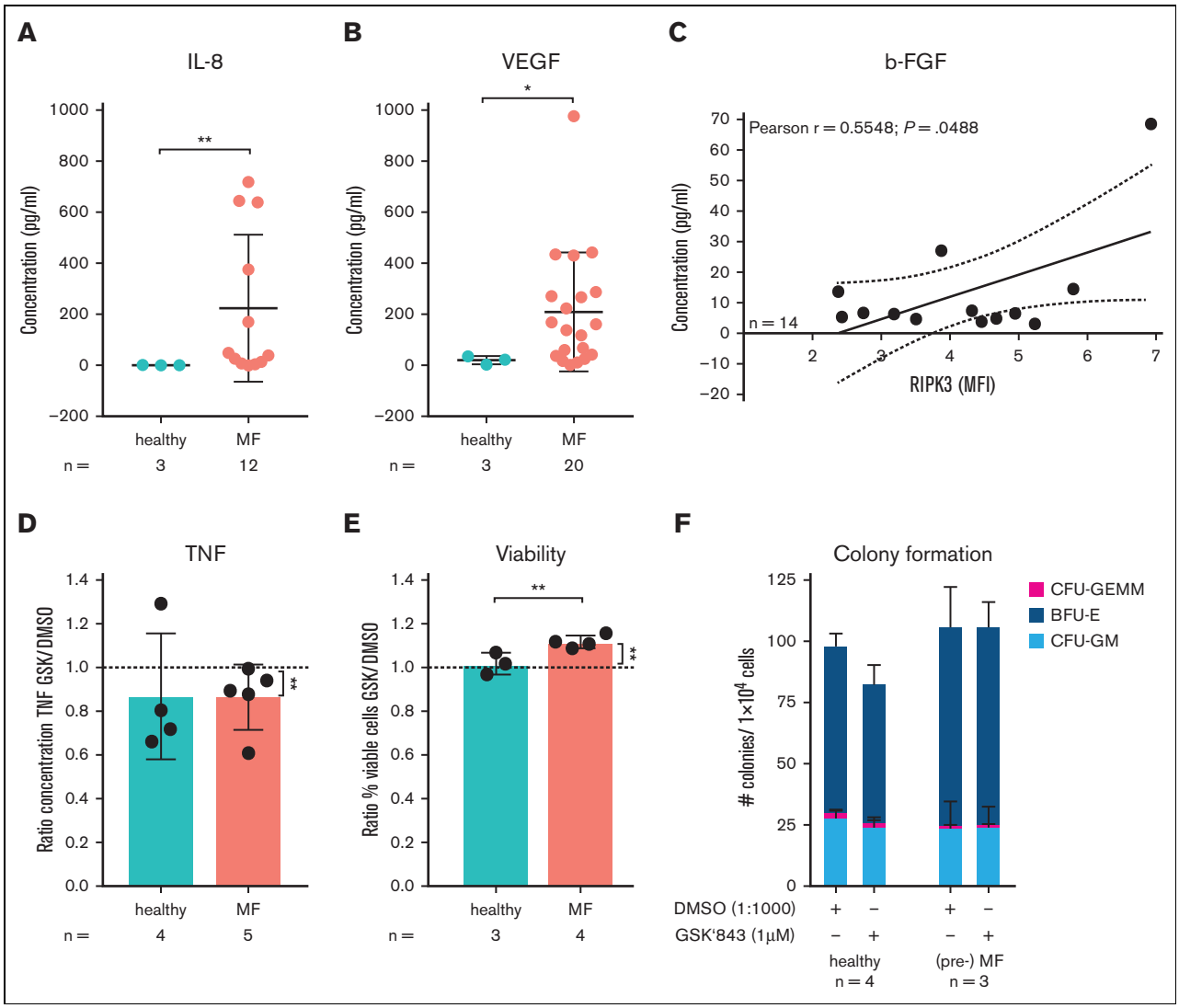

Figure 2. RIPK3 inhibition reduces TNF levels in MF supernatant and increases the viability of MF PBMCs. (A) Cytokine levels of IL-8 were determined by CBA in the PB serum of 3 healthy control subjects and 1 patient with MF. Data are shown as dot plots reporting mean and standard deviation. *P* values from Mann-Whitney test as indicated in the figure. (B) Cytokine levels of VEGF were determined by CBA in the PB serum of 3 healthy control subjects and 20 patients with MF. Data are shown as dot plots reporting mean and standard deviation. *P* values from Mann-Whitney test as indicated in the figure. (C) RIPK3 protein levels were quantified in PBMCs from 14 patients with MF as described in Figure 1A and correlated with corresponding b-FGF levels (pg/mL) analyzed by CBA. The strength of the association was calculated by Pearson correlation coefficient. The functional relationship was characterized by linear regression analysis. Pearson r = 0.5548, *P* = .0488, y = 7.301X -17.37. (D) Cytokine levels of TNF were determined by CBA in the supernatant of liquid culture of PBMCs from 6 patients with MF, and 4 healthy control subjects after treatment with the RIPK3 inhibitor GSK'843 (1 μM) and vehicle control DMSO (1:1000) for 72 hours. Data are shown as dot plots reporting mean and standard deviation. Student *t* test as shown in the figure. (E) Cell viability of PBMCs from 3 healthy control subjects and 4 patients with MF was analyzed by flow cytometry after staining with 7AAD and Annexin V. Shown is the ratio between the percentage of viable PBMCs after treatment with soluble control (DMSO 1:1000), and the percentage of viable PBMCs after treatment with GSK'843 (1 μM) for 72 hours. Shown are the mean and error bars denoting standard deviation. For comparison between entities, one-way ANOVA was performed (*P* = .0037) with a post-hoc Tukey test as shown in the figure. For comparison between treatment (GSK 1 μM) and soluble control (DMSO 1:1000), Student *t* test was used as shown in the figure. (F) BMMCs were treated for 72 hours with GSK'843 (1 μM) and vehicle control (DMSO 1:1000), before being plated in cytokine-enriched methylcellulose. The number of colonies was counted after 14 days. Experiments were performed in duplicates. Shown is the number of colonies from 3 patients with pre-MF (including pre-MF and PMF) and 4 healthy control subjects. The colony subtypes are indicated in the legend. Two-way ANOVA was performed. All panels: level of significance: .05; **P* < .05, ***P* < .005. ANOVA, analysis of variance; BMMCs, BM mononuclear cells; CBA, cytometric bead assay; IL-8, interleukin-8; PMF, primary myelofibrosis; VEGF, vascular endothelial growth factor.

pre-MF and healthy controls were treated with GSK'843 (1 μM) for 72 hours before being transferred in growth-factor supplemented methylcellulose. DMSO (1:1000) was used as soluble control. Assessing colony-forming capacity after 14 days, no statistically significant difference was revealed between healthy, and MF BM (Figure 2F). Despite mediating a short-term survival benefit of PBMCs, RIPK3 appears not to promote relevant levels of cell death at the MF stem/progenitor cell compartment.

In summary, our work shows that RIPK3 is elevated in MF and associated with disease burden. Peripheral RIPK3 levels might predominantly result from myeloid cells. We postulate that RIPK3 promotes MF disease progression by maintaining inflammatory signaling. The degree to which RIPK3 might also contribute to the reduction of healthy hematopoiesis via MLKL-dependent cell death remains less elusive. In the future, additional studies including in vivo models will be required to confirm our findings and to further evaluate the role and prognostic significance of RIPK3 signaling in this disease.

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Contribution: V.D. and C.V.W. performed the experiments, analyzed the data, and wrote the manuscript; T.O.O., L.B., and R.T.H. performed the experiments and analyzed the data; V.D. and U.H. performed the gene expression analysis; J.S.-H. and E.C.K. performed immunohistochemistry and analyzed the data; F.J.F.-H. and S.V. analyzed the single-cell sequencing data; K.S., C.S., J.S.-H., P.H., B.S., S.J., and M.S. provided primary samples and clinical data; U.H., F.B., S.J., and C.B. gave conceptual advice; and P.J.J. conceived and supervised the project.

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