Molecular Cancer Research

The PKR-Like Endoplasmic Reticulum Kinase Promotes the Dissemination of Myc-Induced Leukemic Cells

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Abstract

Hyperactive oncogenic Myc stimulates protein synthesis that induces the unfolded protein response, which requires the function of the eukaryotic translation initiation factor 2-alpha kinase 3, also known as protein kinase R (PKR)-like endoplasmic reticulum kinase (PERK). Activated PERK acts to limit mRNA translation, enable proper protein folding, and restore the homeostasis in the endoplasmic reticulum. Given that Myc activation contributes to many types of lymphoid and myeloid human leukemias, we used a mouse model to examine the importance of PERK in development and progression of Myc-induced leukemias. We found that genetic ablation of *Perk* does not suppress the generation of the leukemic cells in the bone marrow. However, the cell-autonomous *Perk* deficiency restricts the dissemination of leukemic cells into peripheral blood, lymph nodes, and vital peripheral organs. Whereas the loss of the IFNAR1 chain of type I IFN receptor stimulated leukemia, *Perk* ablation did not stabilize IFNAR1, suggesting that PERK stimulates the leukemic cells' dissemination in an IFNAR1-independent manner. We discuss the rationale for using PERK inhibitors against Myc-driven leukemias.

Implications: The role of PERK in dissemination of Myc-induced leukemic cells demonstrated in this study argues for the use of PERK inhibitors against leukemia progression.

Introduction

Eukaryotic translation initiation factor 2-alpha kinase 3, also known as protein kinase R (PKR)-like endoplasmic reticulum kinase (PERK) is a critical regulator of the unfolded protein response, which includes several signaling pathways that act to restore and maintain the homeostasis of the endoplasmic reticulum (ER; ref. 1). Protein and lipid kinase activities of PERK play a central role in determining the viability of cells undergoing the unfolded protein response (2). Whereas earlier studies clearly suggested that PERK functions as a proapoptotic regulator, more recently it has been demonstrated that, in the context of oncogene- and tumor microenvironment–induced stresses associated with tumor growth, activation of PERK might be required for cell survival and tumor progression (2, 3). These cell viability–supporting roles of PERK are supported by its ability to activate autophagy (3, 4) and induce the prosurvival miRNAs (5–7).

In addition, PERK plays a central role in suppressing the proapoptotic effects mediated by type I IFN (8–11). The latter effect is conferred by the ER stress–induced activation of PERK, which in turn, stimulates the p38 stress–activating kinase and, together with casein kinase 1α, phosphorylates the IFNAR1 chain of IFN receptor (8, 12–18). This phosphorylation enables the recruitment of the β-TrCP E3 ubiquitin ligase, which facilitates ubiquitination, endocytosis, and degradation of IFNAR1 leading to attenuation of IFN signaling and IFN-induced inhibition of cell proliferation and viability (19–21).

Accordingly, PERK plays a complex role function in tumorigenesis (2, 22, 23). PERK is induced during tumor development and progression in response to the stress stimuli originating from activation oncogenes (such as c-Myc) or/and deficit of glucose and oxygen in the tumor microenvironment (1). Whereas tempered PERK activities (due to hypomorphic mutants or haploinsufficiency) may promote initiation of melanomas (24), the complete ablation or inhibition of PERK is incompatible with solid tumor growth (4, 24–27). These findings prompted academic and industrial efforts to develop potent and selective inhibitors of PERK (28).

Hyperactive Myc oncogenes were shown to promote robust protein synthesis that can outmatch the ability of the ER to quickly fold these proteins or degrade unfolded protein leading to the induction of the unfolded protein response and activation of PERK (4, 29). Importantly, activated PERK contributes to tumor progression in lymphomas and mammary adenocarcinomas (4, 6, 25). Whereas these studies clearly indicate the importance of PERK in development and progression of solid tumors, little is known about the role of PERK in leukemias in general and in the context of Myc-driven leukemias in particular. Myc is overexpressed in human acute and chronic myeloid leukemias (30–32). In addition, Myc translocations, rearrangements, amplification, or overexpression are also found in acute lymphoblastic leukemias (33–35). Here we used a mouse model that

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involved expression of c-Myc in transplanted bone marrow cells combined with conditional and inducible cell-autonomous genetic ablation of *Perk* to determine the role of PERK in Myc-induced leukemia. We found that PERK plays an important role in supporting the dissemination of leukemic cells.

Materials and Methods

Plasmids and retroviruses

The plasmids MSCV-cMyc-IRES-GFP (MIGR1-cMyc)/vector control (MIGR1-Ctrl), and the packaging vectors (PCGP and PHIT) were kindly provided by Dr. Warren Pear (University of Pennsylvania, Philadelphia, PA). These plasmids were cotransfected into 293T cells (ATCC CRL-3216) by using the Calcium Phosphate Transfection Kit according to the manufacturer's instructions (Invitrogen). Retroviral supernatant was harvested 24 and 48 hours after transfection, filtered, and stored at −80°C. The titer of the infectious virus was determined by flow cytometry using NIH3T3 cells (ATCC CRL-1658) infected with serial dilution of virus in the presence of 8 μg/mL Polybrene (Sigma). Both the 293T and NIH3T3 cells were purchased from ATCC and maintained in DMEM (Gibco) supplemented with 10% FBS (HyClone) and 100 U/mL penicillin–streptomycin (Gibco). Cell lines were regularly tested for *Mycoplasma*.

Animals

All experiments with animals were carried out under the protocol 803995 approved by the Institutional Animal Care and Use Committee of The University of Pennsylvania (Philadelphia, PA). Mice were maintained in a specific pathogen-free facility in accordance with American Association for Laboratory Animal Science guidelines. C57BL/6 *Ifnar1*$^{-/-}$ mice were generously provided by Dr. Susan Weiss (University of Pennsylvania, Philadelphia, PA). *Ubc9-CreERT2* mice (gift from E. Brown, University of Pennsylvania) were crossed with *Perk*$^{fl/fl}$ mice (kindly provided by J.A. Diehl) to generate *Ubc9-CreERT2*$^{+/0}$; *Perk*$^{fl/fl}$ littermates. The littermates were all C57BL/6 and 129 mix background. Genotyping of mice using tail DNA was performed by PCR as described previously (11).

Bone marrow transplantation

Bone marrow cells were collected from 6- to 8-week-old wild-type (WT; *Ifnar1*$^{+/+}$), *Ifnar1*$^{-/-}$, *Perk*$^{fl/fl}$ *Ubc9-CreERT2*$^{-}$, or *Perk*$^{fl/fl}$ *Ubc9-CreERT2*$^{+}$ mice 4 days after intraperitoneal administration of 5-fluorouracil (5-FU, 150 mg/kg) and retrovirally transduced *ex vivo* in the presence of IL3 (6 ng/mL), IL6 (5 ng/mL), SCF (100 ng/mL), and polybrene (4 μg/mL) twice. Retroviral supernatants with equal titers were used to produce similar transduction efficiencies. The c-Myc/Ctrl transduced bone marrow cells were mixed with noninfected bone marrow cells isolated from WT mice in the ratio of 9:1, with a total of 1×10^6 cells retroorbitally injected into each lethally irradiated (915 rad) C57BL/6 recipient mouse. Chimeric mice were maintained on antibiotics for 2 weeks.

Tamoxifen treatment

To induce *Perk* deletion in the donor cells, 1 month after bone marrow transplantation, tamoxifen (Sigma) was dissolved in maize oil (Sigma) and given once daily via oral gavage for 5 consecutive days at a dose of 0.2 mg/g of body weight/day. Time after tamoxifen treatment was determined after the last treatment.

Analysis of mice

Mice were monitored for disease development three times per week by palpation and observation. Peripheral blood was obtained by either cutting tail or cardiac puncture after CO_2 euthanization. Blood smear Wright–Giemsa staining and blood cell counts were performed in the Department of Pathology, School of Veterinary Medicine in University of Pennsylvania (Philadelphia, PA). Mononuclear-cell suspensions of spleen, bone marrow, or lymph nodes were made by passing tissue through nylon mesh cell strainers. Tissues from the mice were stored in a 4% PFA-PBS. Formalin-fixed tissue slides were stained with hematoxylin and eosin and were imaged using an Olympus BX40 F4 Microscope (Olympus Optical). Pictures were taken with an Olympus DP70 digital camera using DPController software. The quantification of the area of lesions in the tissues was performed by Image J.

Flow cytometric analysis

Single-cell suspensions prepared from peripheral blood, bone marrow, spleen, and lymph node were resuspended in FACS buffer (PBS plus 1% BSA) and blocked with anti-mouse CD16/32 antibodies for 15 minutes prior to staining with specific antibodies. Antibody against cell surface IFNAR1 (anti-IFNAR1-PE) was purchased from BioLegend. Samples were mixed with DAPI (1 μg/mL) and acquired on LSRFortessa Flow Cytometer (BD Biosciences), and data were analyzed with FlowJo Software (Tree Star).

Statistical analysis

Data were expressed as mean ± SEM. Statistical analysis was performed using GraphPad Prism 6 Software (GraphPad Prism Software Inc.). Unpaired Student *t* test was used for the comparison between two groups. One-way ANOVA or two-way ANOVA were used for the multiple comparisons. Differences were considered significant at $P < 0.05$. All described results are representative of at least three independent experiments.

Results

In solid tumors, function of PERK was shown to be important for survival of cells harboring activated Myc (3–5, 36). Previous studies that utilized the retroviral-mediated expression of diverse Myc oncogenes in murine bone marrow cells followed by transplantation of these cells into lethally irradiated host described development of acute leukemia in these models (37–41). We have used this approach to determine the importance of PERK in Myc-induced leukemia.

To this end, bone marrow progenitors from 5-FU-treated *Perk*$^{fl/fl}$ *Ubc9-CreER*$^{-}$ or *Perk*$^{fl/fl}$ Ubc9-CreER^{+} donor mice were transduced with retroviruses for expressing c-Myc and GFP (or GFP alone), mixed with bone marrow cells from naïve WT mice (to ensure normal hematopoiesis) and transplanted into irradiated WT recipients. This approach was chosen to first ensure proper reconstitution of bone marrow in recipients and then enable us to follow the fate of GFP^{+} cells harboring or not Myc (Fig. 1A). Indeed, 30 days after transplantation, the recipient animals did not display any visible signs of illness and their peripheral blood analysis showed normal parameters including numbers of white and red blood cells and hematocrit (Fig. 1B). Interestingly, at this time point where total leukocytes numbers have not yet changed, a statistically significant increase in

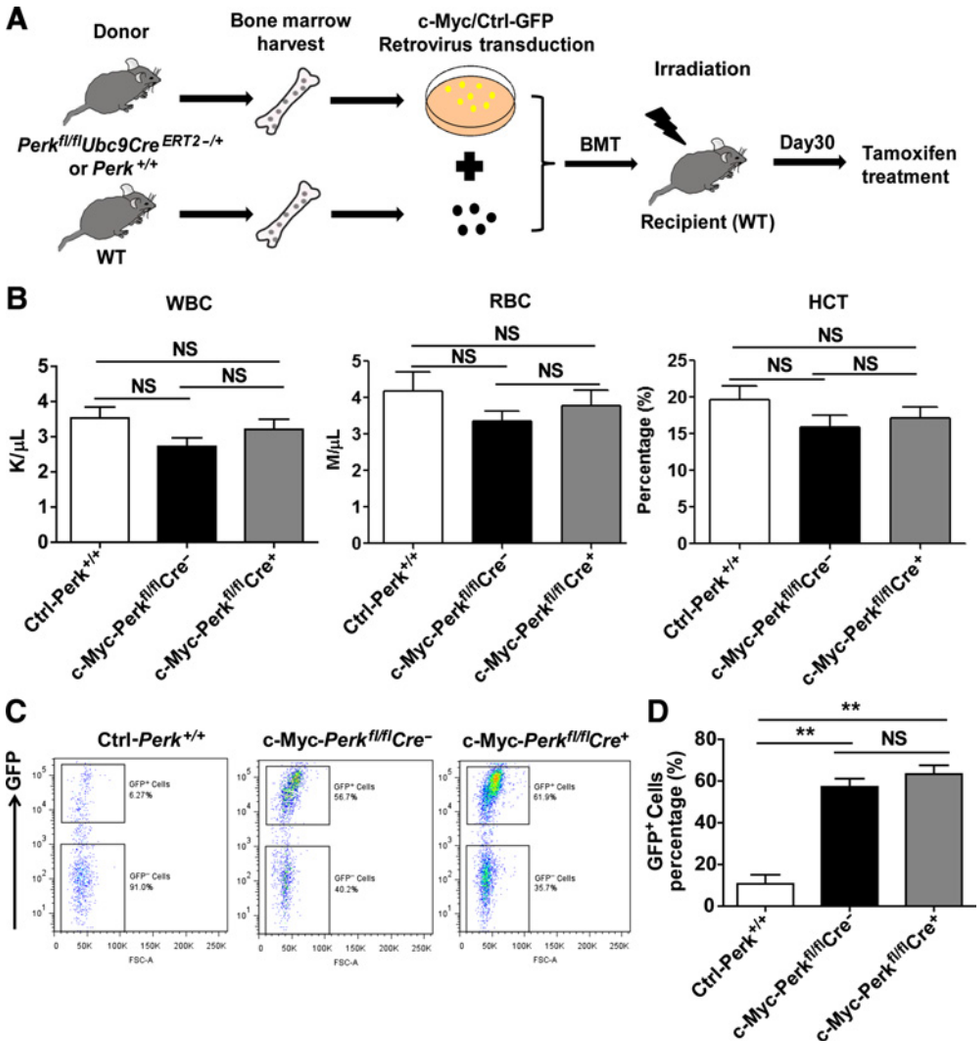

Figure 1.

A, Schematic illustration for the experiment design. Bone marrow cells were harvested from 5-FU injected donors (*Perk*$^{fl/fl}$*Ubc9-CreERT2*$^{+}$ and *Perk*$^{fl/fl}$*Ubc9-CreERT2*$^{-}$) and transduced with c-Myc-GFP or control (GFP alone) retrovirus. The c-Myc/Ctrl transduced bone marrow cells were mixed with noninfected bone marrow cells isolated from WT mice in the ratio of 9:1, with a total of 1×10^6 cells retroorbitally injected into each lethally irradiated recipient mouse. Thirty days after bone marrow transplantation (BMT), mice were orally gavaged with tamoxifen for 5 days to ablate *Perk* gene. WT mice that were transplanted with control retrovirus transduced bone marrow cells isolated from WT (*Perk*$^{+/+}$) mice set as a control. **B,** Blood cell counts in the peripheral blood of mice at day 30 after bone marrow transplantation, but before tamoxifen treatment. WBC, white blood cells; RBC, red blood cells; HCT, hematocrit; NS, no significance. Data shown as means ± SEM ($n = 3$–4). **C,** Representative FACS analysis of percentage of GFP^{+} cells in the peripheral blood of mice at day 30 after bone marrow transplantation, but before tamoxifen treatment. **D,** Quantification of the percentage of GFP^{+} cells as described in **C**. Data shown as means ± SEM ($n = 3$–4; **, $P < 0.01$; NS, no significance).

percent of GFP^{+} cells was already observed in mice that receive cells transduced by c-Myc (Fig. 1C and D).

To determine the importance of PERK in Myc-induced proliferation of hematopoietic cells, we have further examined the numbers of GFP^{+} cells in different compartments upon *Perk* genetic ablation, which was induced by treatment of recipient mice with tamoxifen (Fig. 1A). Analysis of GFP^{+} bone marrow cells in recipient mice at 40–60 days after transplantation revealed that *Perk* was successfully deleted upon tamoxifen treatment (Fig. 2A). *Perk* ablation did not affect either the levels of c-Myc expression (Fig. 2A) or the percentage of GFP^{+} cells in bone marrow (Fig. 2B). The latter result is consistent with a recent report that *Perk* is dispensable for the maintenance of normal hematopoietic stem and progenitor cells (42). Intriguingly, we observed a modest (but not significant) trend toward a decrease in GFP^{+} cells in the spleen (Fig. 2C). Importantly, deletion of *Perk* significantly decreased the numbers of GFP^{+} cells in the lymph nodes and peripheral blood (Fig. 2D and E) suggesting that Perk might contribute to dissemination of GFP^{+} c-Myc–expressing cells.

Careful examination revealed the signs of Myc-induced leukemia in recipient mice observed on days 40–60 after bone marrow transplantation as differences between mice that received bone marrow transduced with c-Myc and control mice harboring WT bone marrow transduced with GFP only. The Myc-induced

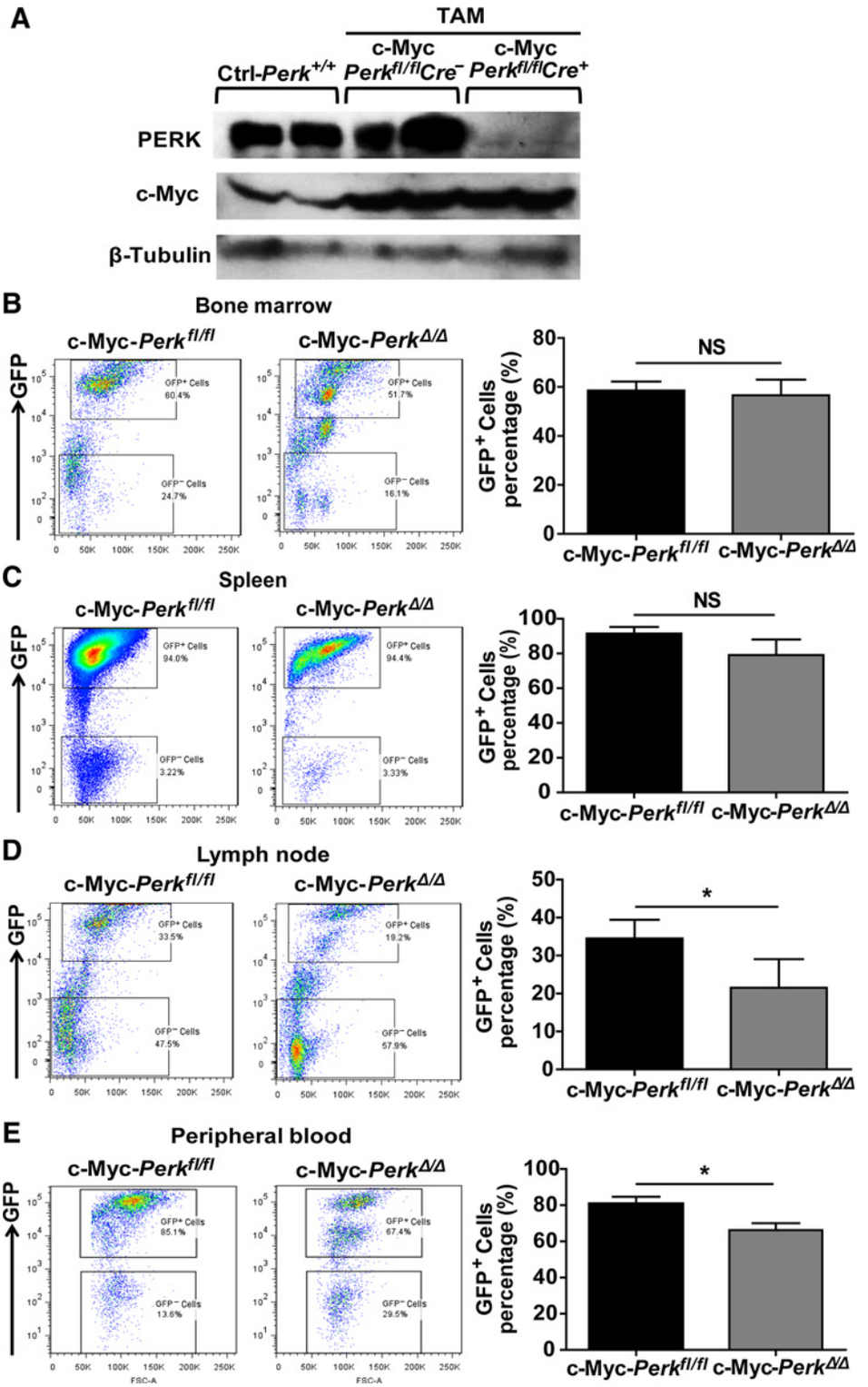

Figure 2.

A, Representative Western blot analysis of PERK and c-Myc expression in the bone marrow cells isolated from the recipient mice [reconstituted with indicated genotype bone marrow cells transduced with c-Myc or control (ctrl) retroviruses] after tamoxifen (TAM) treatment. Independent experiments were done three times with similar results. **B,** Representative FACS analysis (left) and the quantification (right) of GFP^+ cells in the bone marrow from the mice reconstituted with the indicated genotype bone marrow cells transduced with c-Myc retrovirus after tamoxifen treatment. Data shown as means ± SEM ($n = 3$). NS, no significance. **C,** Representative FACS analysis (left) and the quantification (right) of GFP^+ cells in the spleen from the mice reconstituted with the indicated genotype bone marrow cells transduced with c-Myc retrovirus after tamoxifen treatment. Data shown as means ± SEM ($n = 3$). NS, no significance. **D,** Representative FACS analysis (left) and the quantification (right) of GFP^+ cells in the lymph node from the mice reconstituted with the indicated genotype bone marrow cells transduced with c-Myc retrovirus after tamoxifen treatment. Data shown as means ± SEM ($n = 3$; *, $P < 0.05$). **E,** Representative FACS analysis (left) and the quantification (right) of GFP^+ cells in the peripheral blood from the mice reconstituted with the indicated genotype bone marrow cells transduced with c-Myc retrovirus after tamoxifen treatment. Data shown as means ± SEM ($n = 3$; *, $P < 0.05$).

leukemia manifested itself as leukocytosis, splenomegaly, and lymphadenopathy (Fig. 3A–E), as well as leukemic lesions found in the liver, lungs, and kidneys (Fig. 3F and G). Importantly, ablation of *Perk* resulted in significant decrease in the number of peripheral blood leukocytes (Fig. 3A and B), size of the lymph nodes (Fig. 3C and E), and the number of leukemic lesions (Fig. 3F and G). Taken together with a trend for attenuated splenomegaly (Fig. 3C and D), these results further suggest that

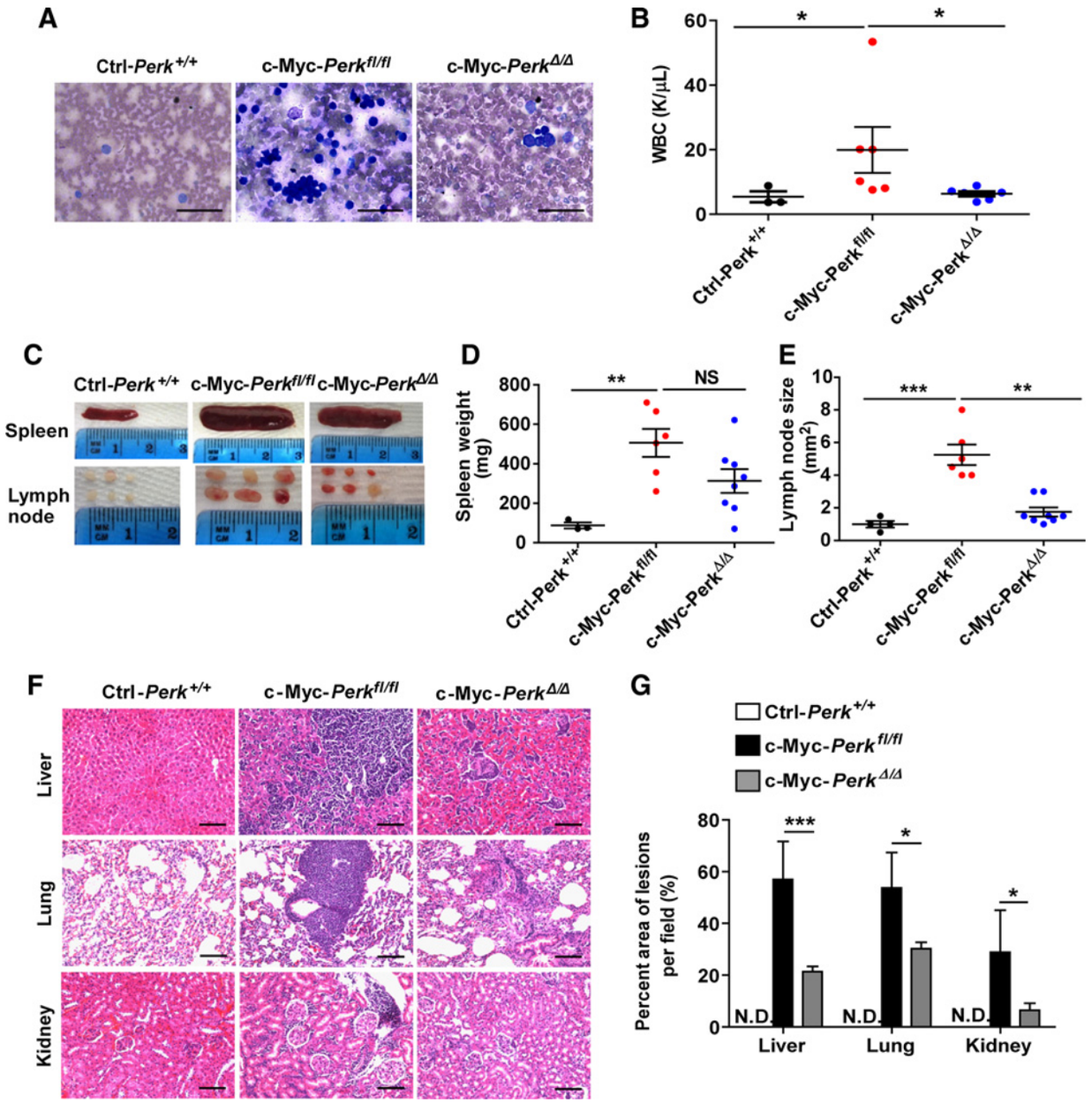

Figure 3.

A, Wright–Giemsa-stained peripheral blood smear from the mice reconstituted with the indicated genotype bone marrow cells transduced with c-Myc retrovirus. Scale bar, 50 μm. **B,** White blood cell (WBC) counts in the peripheral blood of the mice reconstituted with the indicated genotype bone marrow cells transduced with c-Myc retrovirus. Data shown as means ± SEM ($n = 3$–6; *, $P < 0.05$). **C,** Representative photographs of spleens and lymph nodes derived from the mice reconstituted with the indicated genotype bone marrow cells transduced with c-Myc retrovirus. **D,** The quantification of spleen weight of the indicated mice as shown in **C**. Data shown as means ± SEM ($n = 3$–8; **, $P < 0.01$). NS, no significance. **E,** The quantification of lymph node size of the indicated mice as shown in **C**. Data shown as means ± SEM ($n = 4$–8; **, $P < 0.01$; ***, $P < 0.001$). **F,** Histology analysis of liver, lung, and kidney of the indicated tumor-burdened mice with one representative field shown in each group. Scale bar, 100 μm. **G,** Quantification of leukemic lesions in the tissues as shown in **F**. Data shown as means ± SEM ($n = 3$; *, $P < 0.05$; ***, $P < 0.001$; N.D., not detected).

PERK functions to support the dissemination of Myc-induced leukemic malignant cells.

Previous reports demonstrated that activated PERK acts to downregulate the IFNAR1 chain of type I IFN receptor and to suppress type I IFN pathway (8–11). As ablation of *Perk* stabilized IFNAR1 in melanoma cells, normal fibroblasts, and pancreatic cells (8, 11), and stabilization of IFNAR1 in the host was shown to limit dissemination of transplanted syngeneic acute leukemia cells (43) we next sought to determine whether the loss of IFNAR1 contributes to the phenotypes of Myc-induced leukemia. To this

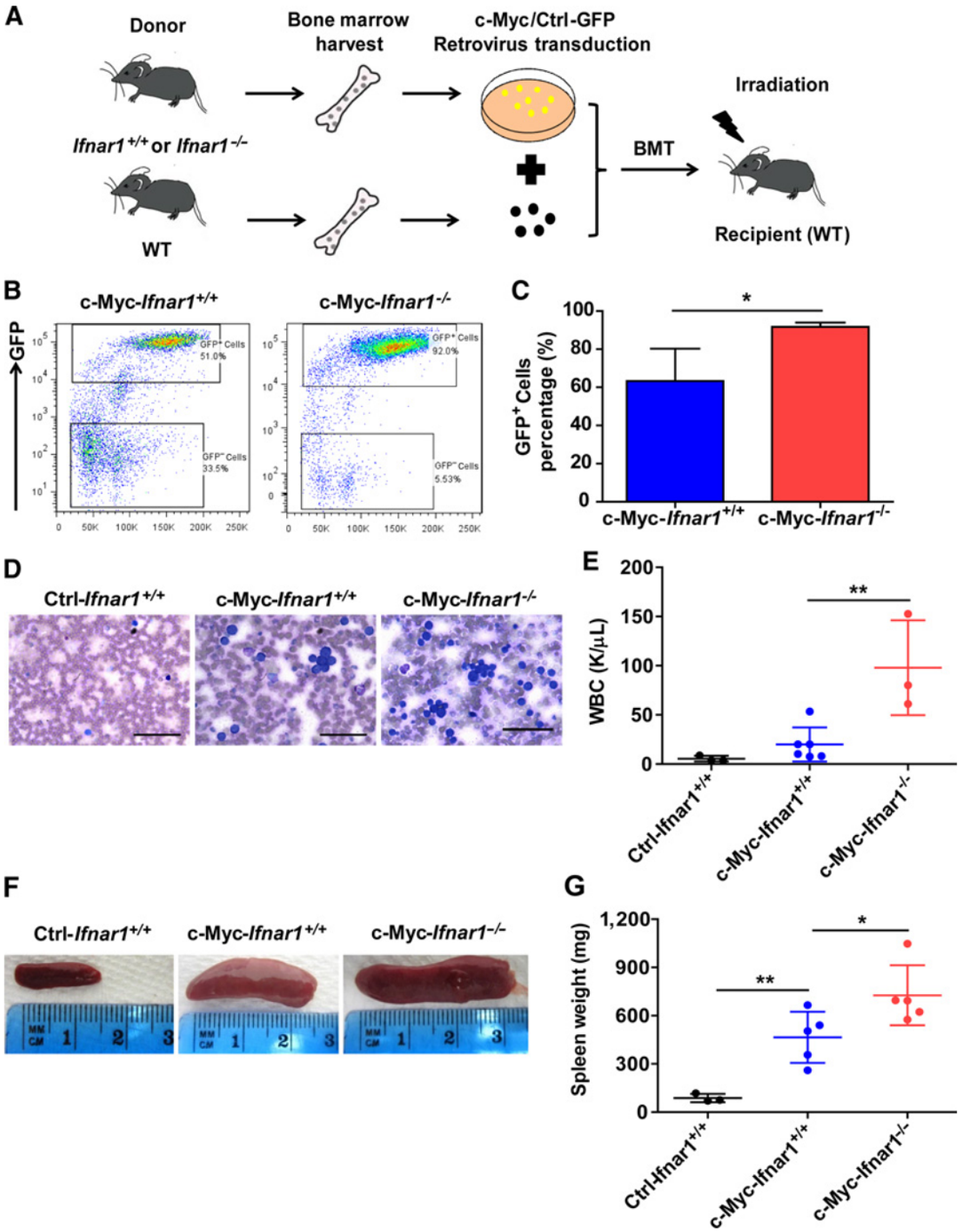

Figure 4.

A, Schematic illustration for the experiment design. Bone marrow cells were harvested from 5-FU injected donors (*Ifnar1*$^{+/+}$ and *Ifnar1*$^{-/-}$) and transduced with c-Myc-GFP retrovirus. The c-Myc/Ctrl transduced bone marrow cells were mixed with noninfected bone marrow cells isolated from WT mice in the ratio of 9:1, with a total of 1×10^6 cells retroorbitally injected into each lethally irradiated recipient mouse. WT mice that were transplanted with control retrovirus-transduced bone marrow cells isolated from WT mice set as a control. BMT, bone marrow transplantation. **B,** Representative FACS analysis of percentage of GFP$^+$ cells in the peripheral blood of the mice reconstituted with c-Myc–transduced WT (c-Myc-*Ifnar1*$^{+/+}$) or IFNAR1 knockout (c-Myc-*Ifnar1*$^{-/-}$) bone marrow cells around 30 days after bone marrow transplantation. **C,** Quantification of the percentage of GFP$^+$ cells in the peripheral blood as described in **B**. Data shown as means ± SEM ($n = 3$; *, $P < 0.05$). **D,** Wright–Giemsa-stained peripheral blood smear from the indicated mice around 30 days after bone marrow transplantation. Scale bar, 50 μm. **E,** White blood cell (WBC) counts in the peripheral blood of the indicated mice. Data were shown as means ± SEM ($n = 3$–6; **, $P < 0.01$). **F,** Representative photographs of spleens derived from the indicated mice. **G,** Quantification of the spleen weight of the indicated mice as shown in **F**. Data shown as means ± SEM ($n = 3$–5; *, $P < 0.05$; **, $P < 0.01$).

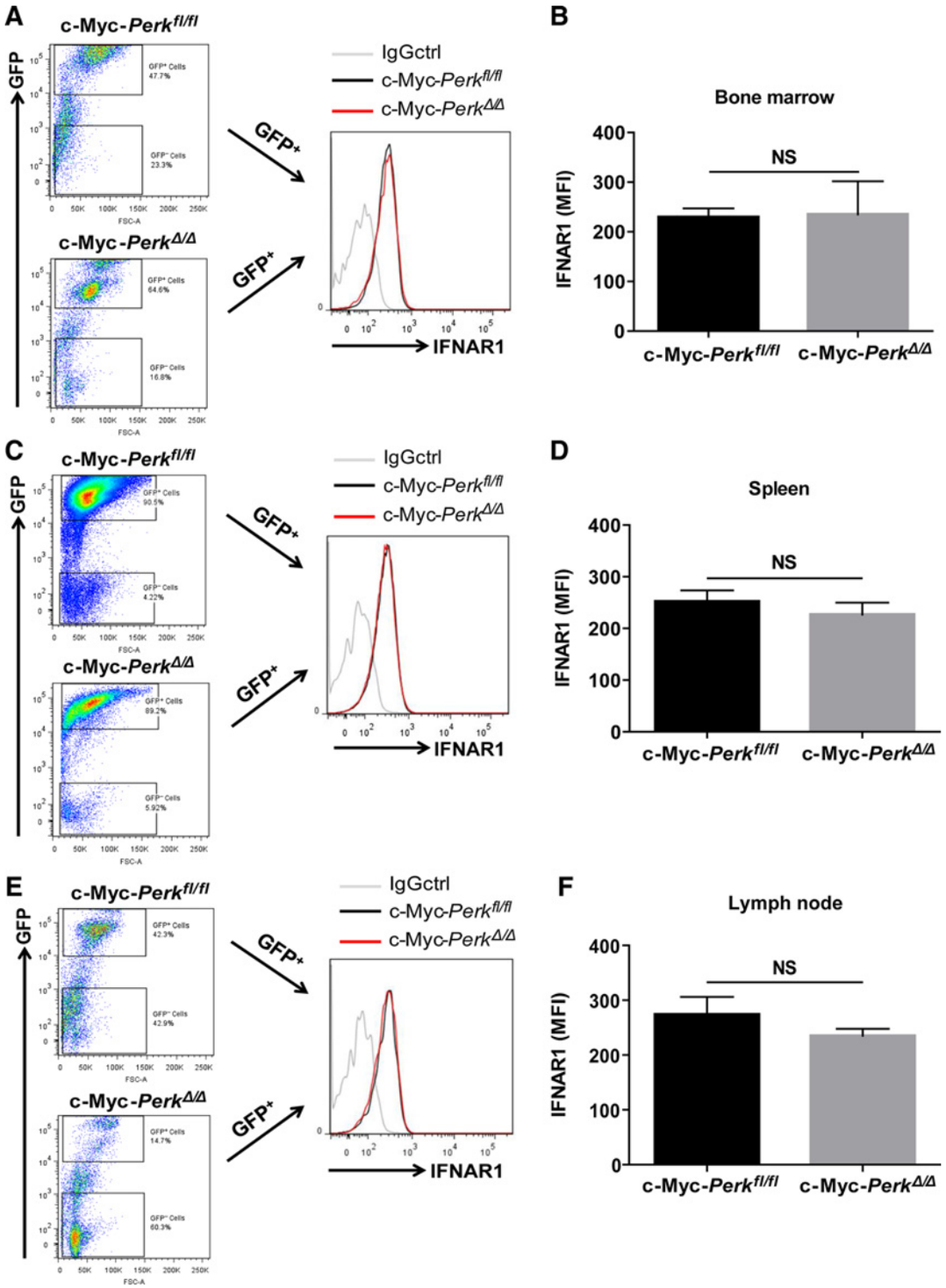

Figure 5.

A, Representative FACS analysis of surface IFNAR1 level in the GFP$^+$ bone marrow cells from the mice reconstituted with the indicated genotype bone marrow cells transduced with c-Myc retrovirus. **B,** Quantification of IFNAR1 level as described in **A**. Data shown as means ± SEM ($n = 3$). NS, no significance. **C,** Representative FACS analysis of surface IFNAR1 level in the GFP$^+$ splenocytes from the mice reconstituted with the indicated genotype bone marrow cells transduced with c-Myc retrovirus. **D,** Quantification of IFNAR1 level as described in **C**. Data shown as means ± SEM ($n = 3$). NS, no significance. **E,** Representative FACS analysis of surface IFNAR1 level in the GFP$^+$ lymph node cells from the mice reconstituted with the indicated genotype bone marrow cells transduced with c-Myc retrovirus. **F,** Quantification of IFNAR1 level as described in **E**. Data shown as means ± SEM ($n = 3$). NS, no significance.

end, we used donor bone marrow cells from *Ifnar1*$^{+/+}$ (WT) or *Ifnar1*$^{-/-}$ mice for their transduction with c-Myc/GFP and transplantation into irradiated WT hosts (Fig. 4A).

Mice that received c-Myc/GFP-expressing cells lacking IFNAR1 displayed a greater number of GFP^{+} leukemic cells in their blood (Fig. 4B and C) indicating that the loss of type I IFN signaling may promote the leukemogenic effect of c-Myc. Indeed, animals that received IFNAR1-deficient bone marrow cells displayed a greater leukocytosis (Fig. 4D and E) and splenomegaly (Fig. 4F and G) further supporting the antitumorigenic role of IFNAR1 in pathogenesis of Myc-driven acute leukemia.

However, analysis of IFNAR1 on the surface of *Perk*-null or *Perk*-competent leukemic cells isolated from bone marrow, spleen, or lymph nodes showed that ablation of *Perk* did not affect IFNAR1 levels (Fig. 5A–F). These results are consistent with previous reports that PERK status does not affect IFNAR1 levels on normal hematopoietic stem and progenitor cells (42) suggesting that there are redundant pathways in hematopoietic cells that affect IFNAR1 status and type I IFN pathway. These data also indirectly suggest that antileukemogenic effects of *Perk* ablation might be mediated by pathways that are not dependent on IFNAR1.

Discussion

Previous reports that demonstrate the importance of PERK in development and progression of solid tumors driven by Myc oncogene (reviewed in refs. 2, 3) prompted us to examine the roles of PERK in Myc-induced leukemia. Here we show that ablation of *Perk* in Myc-expressing leukemic cells does not affect their numbers (Figs. 1 and 2). These data suggest that PERK is dispensable for the maintenance of proliferation or/and viability of hematopoietic cells harboring c-Myc. Intriguingly, it has been previously reported that ablation of *Perk* does not affect the fate and proliferating potential of normal hematopoietic stem cells and progenitors (42) indicating that activities of additional regulators may render PERK function redundant for these biological processes.

However, we observed that genetic ablation of PERK impedes the ability of Myc-expressing cells to disseminate to peripheral blood and form lesions in peripheral organs including lymph nodes, spleen, lung, liver, and kidney (Fig. 3). These results indicate that PERK functions to support the dissemination of the leukemic cells and, accordingly, might play an important role in progression of Myc-driven leukemias. Thus, it can be argued that pharmacologic inhibitors of PERK proposed to be used against solid tumors (11, 24) might not be very efficient in helping to eradicate the Myc-induced leukemias but could be useful in slowing down the progression of the disease.

The mechanisms by which PERK expression in the cells harboring activated Myc supports the dissemination of these cells remain to be elucidated. One of potential mechanisms we considered is the alteration of IFN signaling, which is known to suppress the metastatic processes and contribute to expression of numerous chemokines and their receptors that could guide leukemic cells' dissemination (44, 45). Indeed, previous reports suggested that PERK signals toward accelerated ubiquitination and degradation of the IFNAR1 chain of IFN receptor (9). Furthermore, our current data show that ablation of IFNAR1 indeed promoted the development and progression of the Myc-driven leukemia (Fig. 4). However, no perturbation of IFNAR1 levels was observed upon *Perk* ablation (Fig. 5). It is plausible that, in the context of Myc activation, stimulating effects of PERK on ubiquitination of IFNAR1 are counteracted by IFNAR1 deubiquitination, which is facilitated by the BRISC deubiquitination complex recruited to IFNAR1 via serine hydroxymethyltransferase-2 (46). Importantly, serine hydroxymethyltransferase-2 is known as a canonical Myc-induced protein (47). Thus, induction of serine hydroxymethyltransferase-2 and ensuing deubiquitination of IFNAR1 may offset stimulation of IFNAR1 ubiquitination driven by PERK.

Furthermore, although our results may be interpreted in a way that PERK is redundant in the bone marrow cells for the regulation of IFNAR1 stability and signaling, we cannot rule out that PERK status still affects the IFN pathway through acting on other mediators of IFN signaling (such as IFNAR2, Janus kinases, or STAT proteins). Future studies will test this possibility, as well as reveal the mechanisms by which PERK contributes to dissemination of leukemic cells and evaluate the usefulness of PERK inhibitors for the treatment of Myc-associated leukemias.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: J. Gui, J.A. Diehl, S.Y. Fuchs
Development of methodology: J. Gui
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J. Gui, K.V. Katlinski
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J. Gui, K.V. Katlinski, C. Koumenis, S.Y. Fuchs
Writing, review, and/or revision of the manuscript: J. Gui, C. Koumenis, S.Y. Fuchs
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S.Y. Fuchs
Study supervision: C. Koumenis, S.Y. Fuchs

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