Inactivation of p53 provides a competitive advantage to del(5q) myelodysplastic syndrome hematopoietic stem cells during inflammation

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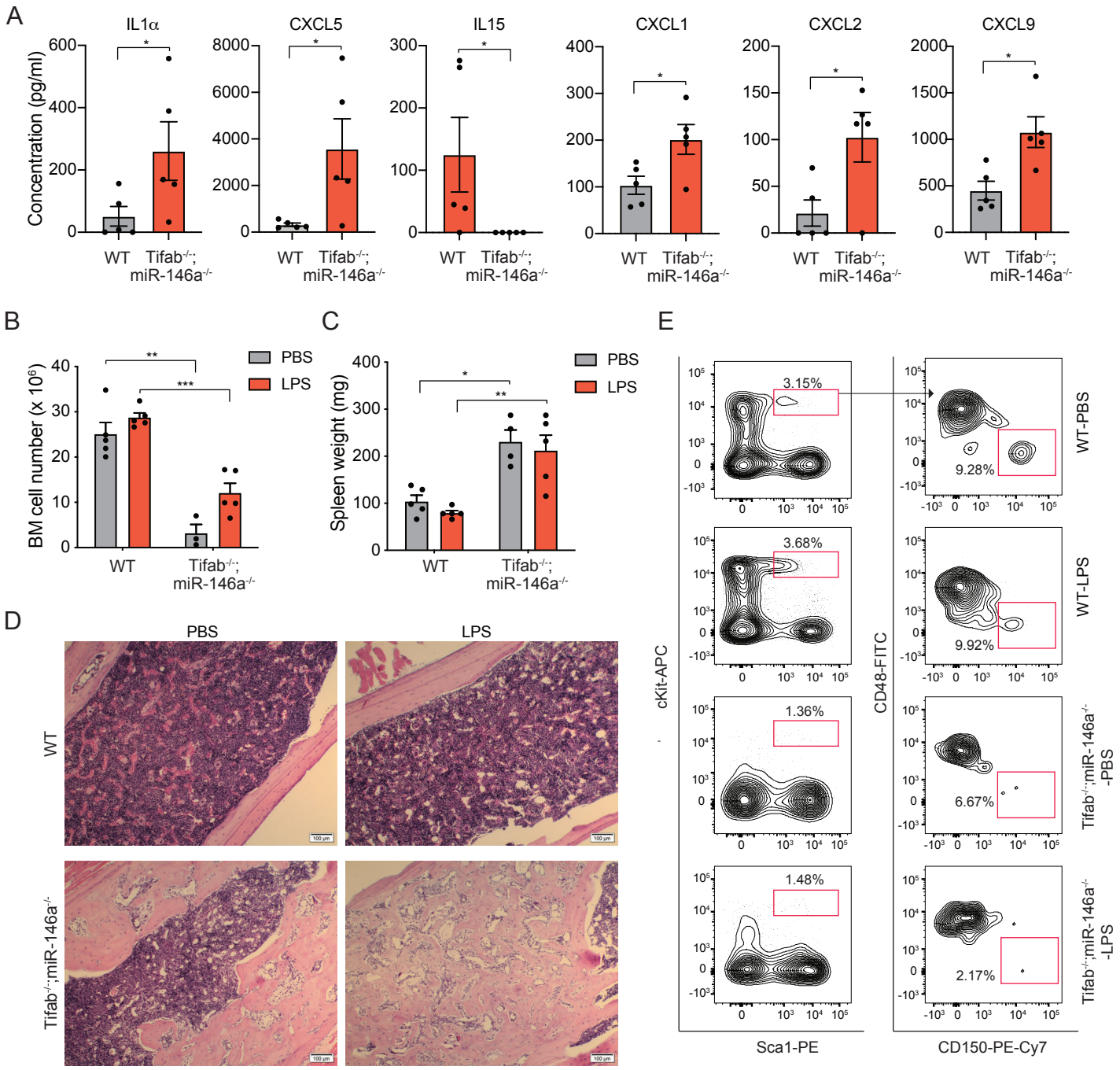
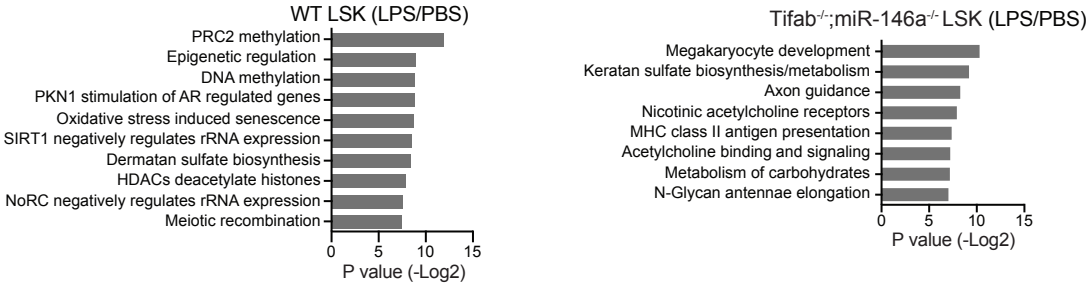
Supplemental Figure 1.



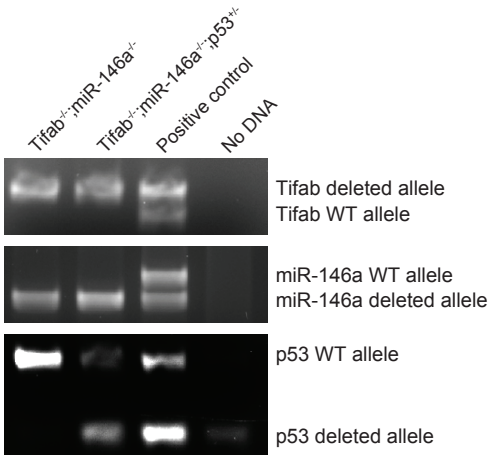
Supplemental Figure 1. Characterization of moribund del(5q)-like MDS model. (A) PB plasma cytokine analysis. **(B)** Total BM cells numbers in moribund mice transplanted with Tifab$^{-/-}$;miR-146a$^{-/-}$ BM cells (n = 4-5 per group). WT recipient mice are age-matched controls. **(C)** Spleen weights of moribund mice. WT recipient mice are age-matched controls. **(D)** H&E staining of BM from moribund mice transplanted with Tifab$^{-/-}$;miR-146a$^{-/-}$ BM cells (n = 4-5 per group). WT recipient mice are age-matched controls. **(E)** Representative flow cytometric analysis and gating strategy of moribund mice transplanted with Tifab$^{-/-}$;miR-146a$^{-/-}$ BM cells. WT recipient mice are age-matched controls.

Supplemental Figure 2.



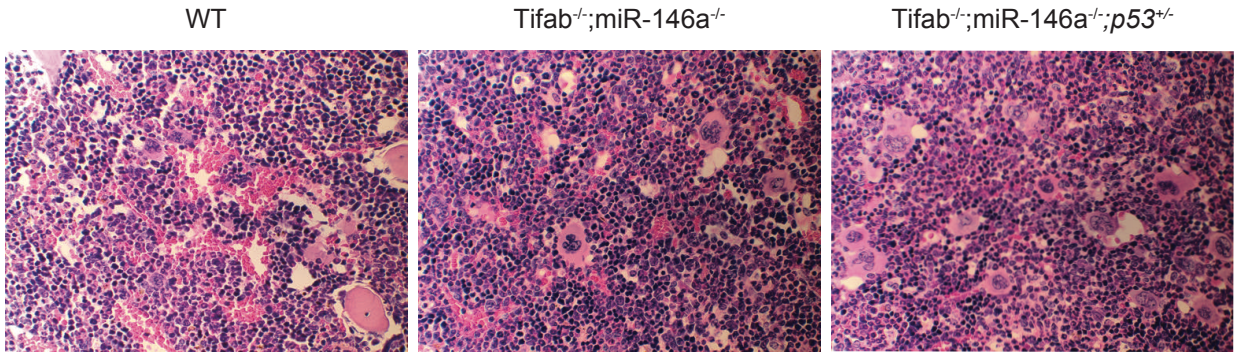
Supplemental Figure 2. Effects of low-grade inflammation on del(5q)-like MDS HSPCs. Pathway analysis of LPS-stimulated WT LSK cells (relative to WT LSK cells treated with PBS) and LPS-stimulated Tifab$^{-/-}$;miR-146a$^{-/-}$ LSK cells (relative to Tifab$^{-/-}$;miR-146a$^{-/-}$ LSK cells treated with PBS).

Supplemental Figure 3.



Supplemental Figure 3. Generation of p53-deficient Tifab$^{-/-}$;miR-146a$^{-/-}$ mice. Genotyping analysis of p53-deficient Tifab$^{-/-}$;miR-146a$^{-/-}$ mice.

Supplemental Figure 4.



Supplemental Figure 4. BM analysis of p53-deficient Tifab$^{-/-}$;miR-146a$^{-/-}$ mice. Hematoxylin and eosin staining of BM from WT, Tifab$^{-/-}$;miR-146a$^{-/-}$, and Tifab$^{-/-}$;miR-146a$^{-/-}$;p53$^{+/-}$ mice.

Supplemental Methods

Mice

Tifab$^{-/-}$ C57Bl/6 mice were described previously[1]. Briefly, a target vector was designed to replace exon 3, which contains all coding regio*n* of *Tifab*, with a β-galactosidase-loxP-neomycin resistant gene cassette-loxP fragment. Diphtheria toxin A gene cassette was inserted at the downstream end of the short arm. The targeting construct was electroporated into a 129-derived embryonic stem (ES) cell line. G418-resistant ES clones were screened for homologous recombination by Southern blot analysis. ES clones containing the correctly targeted *Tifab* locus were injected into C57BL/6 blastocystes. Male chimeric mice were mated with C57BL/6 J, and agouti pups carrying the correct target locus were used as F1 mice. F1 mice were crossed with transgenic mice expressing CRE under control of CAG promoter[2]. Pups lacking neomycin-resistant gene cassette were further backcrossed into the C57BL/6 J background. *miR-146a*$^{-/-}$ C57BL/6 mice were obtained from Dr. David Baltimore as previously described[3]. Generation of *Tifab*$^{-/-}$;*miR-146a*$^{-/-}$ mice was previously described[1,4]. *Tifab*$^{-/-}$;*miR-146a*$^{-/-}$ mice were crossed with *Trp53*$^{-/-}$ mice (Jackson Laboratories, 002101). All mouse experiments were performed in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care-accredited animal facility of Cincinnati Children's Hospital.

Statistical analysis

Differences among multiple groups were assessed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison posttest for all possible combinations. Comparison of two group was performed using the Mann-Whitney test or the Student's *t* test (unpaired, two tailed) when sample size allowed. Unless otherwise specified, results are depicted as the mean ± standard deviation or standard error of the mean. A normal distribution of data was assessed for data sets >30. D'Agostino and Pearson and Shapiro-Wilk tests were performed to assess data distributions. For Kaplan-Meier analysis, Mantel-Cox test was used. All graphs and analysis were generated using GraphPad Prism software or using the package ggplot2 from R[5].

Colony forming assay

Either chronic LD-LPS (1 μg/g) or PBS to WT and *Tifab*$^{-/-}$;*miR-146a*$^{-/-}$ mice was administered via intraperitoneal injection twice a week for 30 days, and BM cells were harvested afterwards. Twenty thousand BM cells per replicate were plated in methylcellulose (3434; Stemcell Technologies). Colonies propagated in culture were scored at day 14.

Hematological analysis

Blood counts were measured with a hemacytometer (HEMAVET).

Flow cytometry

For immunophenotypic analysis of lineage positive cells, PB samples were processed with 1 x RBC lysis buffer, and then incubated with CD11b-PE-cy7 (25-0112-81, eBiosciences), Gr1-eFluor450 (48-5931-82, eBiosciences), CD3-PE (12-0031-83, eBiosciences), and B220-APC (17-0452-82, eBiosciences). To distinguish donor from recipient hematopoietic cells, PB were stained with CD45.1-Brilliant Violet 510 (110741, BioLegend), and CD45.2-APC-eFluor780 (47-0454-82, eBiosciences) or CD45.2- eFluor450 (48-

0454-82, eBiosciences). For HSC analysis, BM cells were washed and incubated for 30 minutes with biotin conjugated lineage markers (CD11b, Gr1, Ter119, CD3, B220, mouse hematopoietic lineage biotin panel, [88-7774-75 eBiosciences]), followed by staining with streptavidin eFluor780 (47-4317-82, eBiosciences), Sca-1-PE (12-5981-82, eBiosciences), c-Kit-APC (17-1171-81, eBiosciences), CD48-FITC (11-0481-85, Affymetrix), CD150-PE-cy7 (115914, BioLegend). SLAM-HSC were identified based on expression of $Lin^-Sca^-1^+c\text{-}Kit^+CD150^+CD48^-$; multipotent progenitor cells (MPP) were identified based on expression of $Lin^-cKit^+Sca1^+CD48^+CD150^-$; short-term hematopoietic stem cells (ST-HSC) were identified based on expression of $Lin^-cKit^+Sca1^+CD48^-CD150^-$**;** long-term hematopoietic stem cells (LT-HSC) were identified based on expression of $Lin^-cKit^+Sca1^+CD48^-CD150^+$).

Cell cycle and apoptosis analysis

BrdU (Sigma-Aldrich) was administered continuously to mice via drinking water (0.5 mg/ml). After 1 week, BrdU incorporation was analyzed using a BrdU Flow Kit (559619, BD Biosciences) according to the manufacture's recommendation. Annexin V viability staining was carried out according to manufacturer's instructions (C0556419, BD Biosciences).

Immunoblotting

Cell extract was prepared by lysing cells in sodium dodecyl sulfate (SDS) sample buffer followed by incubation with benzonase (70746, Millipore) on ice for 10 minutes. Nuclear and cytoplasmic fractionation was performed with Nuclear Extract Kit (40010, Active Motif) according to the manufacture's protocol. Samples were boiled at 95 °C for 5 minutes and loaded to SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membranes (162-0112, Bio-Rad). Immunoblot analysis was performed with the following antibodies: p53 (2524S, Cell Signaling), Vinculin (13901, Cell Signaling).

RNA sequencing

Total RNA was extracted from the cells using RNeasy Plus Micro Kit (Qiagen). The initial amplification step for all samples was done with the NuGEN Ovation RNA-Seq System v2. The assay was used to amplify RNA samples to create double stranded cDNA. The concentrations were measured using the Qubit dsDNA BR assay. Libraries were then created for all samples using the Illumina protocol (Nextera XT DNA Sample Preparation Kit). The concentrations were measured using the Qubit dsDNA HS assay. The size of the libraries for each sample was measured using the Agilent HS DNA chip. The concentration of the pool was optimized to acquire at least 15-20 million reads per sample. The analysis of RNA sequencing was performed with iGeak[6]. Gene set enrichment analysis (GSEA) was performed as previously described[7].

Cytokine analysis

Peripheral blood was obtained from each mouse in K_3 EDTA-coated tubes on ice, then samples were centrifuged for 10 minutes at 2,000 g within 30 minutes at 4ºC. After centrifugation, supernatant was immediately transferred to ice cold eppendorf tubes and frozen at -70ºC until further use. Samples were thawed on ice, vortexed thoroughly prior to being diluted 1:1 in assay buffer using the mouse cytokines/chemokines magnetic bead panel kit to quantify 32-plex mouse panel (Cat no. MCYTOMAG-70K; Millipore Sigma) following the manufacturer's instructions.

Hematoxylin and eosin staining of BM

Mouse femurs and tibia were dissected and fixed with 10% formalin at room temperature, sectioned and stained with hematoxylin and eosin by the CCHMC pathology core. Imaging performed on the Motic Type 102M Microscope and image capture, and processing was accomplished using Olympus LC Micro Imaging (Olympus) Software and Adobe Photoshop (Adobe).

Supplemental References

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Supplemental Tables (see excel files)

Supplemental Table 1.

Differentially expressed genes in Tifab$^{-/-}$;miR-146a$^{-/-}$ LSK cells treated with LPS relative to PBS.

Supplemental Table 2.

Differentially expressed genes in WT LSK cells treated with LPS relative to PBS.