The Cancer Therapy-Related Clonal Hematopoiesis Driver Gene *Ppm1d* Promotes Inflammation and Non-Ischemic Heart Failure in Mice

Yoshimitsu Yura, Emiri Miura-Yura, Yasufumi Katanasaka, Kyung-Duk Min, Nicholas Chavkin, Ariel H. Polizio, Hayato Ogawa, Keita Horitani, Heather Doviak, Megan A. Evans, Miho Sano, Ying Wang, Katharina Boroviak, George Philippos, Ana Filipa Domingues, George Vassiliou, Soichi Sano, Kenneth Walsh

**RATIONALE:** Cancer therapy can be associated with short- and long-term cardiac dysfunction. Patients with cancer often exhibit therapy-related clonal hematopoiesis (t-CH), an aggressive form of clonal hematopoiesis that can result from somatic mutations in genes encoding regulators of the DNA-damage response (DDR) pathway. Gain-of-function mutations in exon 6 of the protein phosphatase Mg2+/Mn2+ dependent 1D (*PPM1D*) gene are the most frequently mutated DNA-damage response gene associated with t-CH. Whether t-CH can contribute to cardiac dysfunction is unknown.

**OBJECTIVE:** We evaluated the causal and mechanistic relationships between *Ppm1d*-mediated t-CH and nonischemic heart failure in an experimental system.

**METHODS AND RESULTS:** To test whether gain-of-function hematopoietic cell mutations in *Ppm1d* can increase susceptibility to cardiac stress, we evaluated cardiac dysfunction in a mouse model where clonal hematopoiesis-associated mutations in exon 6 of *Ppm1d* were produced by CRISPR-Cas9 technology. Mice transplanted with hematopoietic stem cells containing the mutated *Ppm1d* gene exhibited augmented cardiac remodeling following the continuous infusion of Ang II (angiotensin II). *Ppm1d*-mutant macrophages were impaired in DDR pathway activation and displayed greater DNA damage, higher reactive oxygen species generation, and an augmented proinflammatory profile with elevations in IL-1β and IL-18. The administration of an NLRP3 (NLR family pyrin domain containing 3) inflammasome inhibitor to mice reversed the cardiac phenotype induced by the *Ppm1d*-mutated hematopoietic stem cells under conditions of Ang II–induced stress.

**CONCLUSIONS:** A mouse model of *Ppm1d*-mediated t-CH was more susceptible to cardiac stress. Mechanistically, disruption of the DDR pathway led to elevations in inflammatory cytokine production, and the NLRP3 inflammasome was shown to be essential for this augmented cardiac stress response. These data indicate that t-CH involving activating mutations in *PPM1D* can contribute to the cardiac dysfunction observed in cancer survivors, and that anti-inflammatory therapy may have utility in treating this condition.

**GRAPHIC ABSTRACT:** An online graphic abstract is available for this article.

**Key Words:** cardiotoxicity • clonal hematopoiesis • CRISPR-Cas Systems • DNA damage • heart failure • inflammasome • macrophages

*Meet the First Author, see p 601*
Yura et al. Ppm1d Clonal Hematopoiesis Promotes Heart Failure

marrow niche. This condition is prevalent in elderly individuals who lack overt hematologic disorders, and it has been referred to as age-related clonal hematopoiesis and clonal hematopoiesis of indeterminate potential.3,4 Epidemiological studies show that mutations in genes encoding epigenetic regulators, including ten-eleven translocation-2 (TET2), DNA methyltransferase 3A (DNMT3A), additional sex combs like 1 (ASXL1), are prevalent in individuals with age-related clonal hematopoiesis. 5–8 These mutant HSPC give rise to circulating immune cells that harbor the mutant driver gene, 9 and, in some cases, it has been shown that the mutation alters the function of the progeny immune cells.1,2 Although age-associated clonal hematopoiesis in otherwise healthy individuals was initially reported decades ago, only recently has it been appreciated that this condition is associated with an increased risk of all-cause mortality.5,6,8,10 While clonal hematopoiesis increases the risk of hematologic cancer, this overall risk is small, and the increased mortality associated with this condition is largely due to an increased incidence of cardiovascular disease (CVD).6,11–14 Our laboratory has provided experimental evidence that clonal

Novelty and Significance

What Is Known?
• Patients who receive cancer therapy frequently develop short- and long-term cardiac dysfunction.
• Patients with cancer often exhibit therapy-related clonal hematopoiesis (t-CH), an aggressive form of clonal hematopoiesis.
• Whether t-CH contributes to development of cardiac dysfunction in cancer survivors is unknown.

What New Information Does This Article Contribute?
• Somatic mutations in protein phosphatase Mg2+/Mn2+ dependent 1D (Ppm1d), that frequently occur in cancer survivors with t-CH, contribute to the development of nonischemic heart failure in a murine model.
• Activating mutations in exon 6 of Ppm1d lead to disruption of the DNA-damage response pathway and contribute to a proinflammatory state in macrophages.
• Inhibition of the NLRP3 (NLR family pyrin domain containing 3) inflammasome was effective in ameliorating the cardiac dysfunction observed in the Ppm1d-mutant model of t-CH.

While both cardiac dysfunction and t-CH are commonly observed in individuals treated for cancer, it was unknown whether a causal relationship exists between these conditions. By focusing on the DNA-damage response gene Ppm1d, this study indicates that t-CH can contribute to nonischemic heart failure. Macrophages harboring mutations in exon 6 of Ppm1d were impaired in DDR pathway activation, displayed an augmented proinflammatory profile, and promoted cardiac dysfunction in a model of nonischemic heart failure. Inflammasome inhibition reversed the cardiac phenotype observed in the mice transplanted with Ppm1d-mutant hematopoietic cells. These data suggest that t-CH could be predictive of heart failure in cancer survivors and that anti-inflammatory therapies may have utility in treating this condition.

Nonstandard Abbreviations and Acronyms

AIM2 absent in melanoma 2
Ang II angiotensin II
ATM ataxia telangiectasia mutated
BMT bone marrow transplantation
CCR2 C-C motif chemokine receptor 2
cGAS cyclic GMP-AMP synthase
CHK1 checkpoint kinase 1
CVD cardiovascular disease
DDR DNA-damage response
dsDNA double strand DNA
HDAC histone deacetylase
HSPC hematopoietic stem and progenitor cell(s)
IFN-γ interferon γ
NBS1 nijmegen breakage syndrome 1
NF-κB nuclear factor kappa-light-chain-enhancer of activated B cells
NLRP3 NLR family pyrin domain containing 3
PPM1D protein phosphatase Mg2+/Mn2+ dependent 1D
ROS reactive oxygen species
sgRNA single guide RNA
TagRFP tag red fluorescent protein
t-CH therapy-related clonal hematopoiesis
TEMPOL 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl

TLR4 toll-like receptor 4
WIP1 wild-type p53-induced phosphatase 1
WT wild type
γH2AX γH2A histone family member X

Circulation Research. 2021;129:684–698. DOI: 10.1161/CIRCRESAHA.121.319314 September 3, 2021  685
hematopoiesis attributed to mutations in TET2, DNMT3A, and Janus kinase 2 genes (ie, JAK2)
can causally contribute to CVD in various animal models by conferring proinflammatory
phenotypes to the progeny leukocytes that are derived from the HSPC clones.

In contrast to age-related clonal hematopoiesis, therapy-related clonal hematopoiesis (t-CH) refers to the aberrant clonal expansions that are often detected in the blood of cancer survivors. This form of clonal hematopoiesis occurs as a result of the selective pressure that the genotoxic stresses of radiation and chemotherapy exert on the HSPC. Mutations associated with t-CH typically occur in genes that encode regulators of DNA-damage response (DDR) pathway, such as PPM1D (protein phosphatase, Mg2+/Mn2+ dependent 1D), TP53 (tumor protein 53), and checkpoint kinase 2 (CHEK2).

Of these, PPM1D, also referred to as WIP1 (wild-type [WT] p53-induced phosphatase 1), is the most frequently mutated DDR gene associated with t-CH. The PPM1D mutations that result in t-CH are localized in exon 6. These mutations result in the stabilization and activation of the PPM1D protein, and they are often referred to as gain-of-function. Although somatic PPM1D mutations in blood cells have been detected in patients with therapy-related myelodysplastic syndrome, PPM1D is often overlooked as a clonal hematopoiesis driver gene because it is typically not detected in patients with acute myeloid leukemia (AML).

However, PPM1D-mediated clonal hematopoiesis has recently been reported to be prevalent in patients with chronic ischemic heart failure and associated with poor outcome. Furthermore, t-CH is predictive of inferior survival and elevated CVD events in patients receiving autologous stem cell transplantation for lymphoma, with PPM1D being the most frequently mutated gene in this cohort.

Cancer survivors display increased medium- to long-term risk for nonischemic heart failure. Notably, the mortality due to CVD in this population is typically greater than that of cancer itself after a 10 year follow-up. To date, the relationship between t-CH and CVD has not been evaluated in an experimental study. Given the mechanistic links between age-related clonal hematopoiesis and CVD, we wondered whether the DDR genes commonly mutated in t-CH could play a role in accelerating the nonischemic heart failure that is prevalent in cancer survivors. Thus, the current study was performed to test whether a causal relationship exists between t-CH and nonischemic heart failure by focusing on activating mutations in exon 6 of the Ppm1d gene as a test case.

**METHODS**

Data Availability
The detailed methods are available in the Data Supplement. Please see also the Major Resources Table in the Data Supplement. The supporting data are available from the corresponding author upon request.

**RESULTS**

Generation of a Ppm1d Model of Clonal Hematopoiesis
To establish the Ppm1d clonal hematopoiesis model, a lentivirus-mediated, CRISPR-Cas9 approach was employed to induce mutations in Ppm1d in bone marrow cells before transplantation into recipient mice. The PPM1D mutations commonly associated with clonal hematopoiesis are restricted to exon 6 that will promote protein stability and increase in phosphatase

**Statistical Analyses**

All statistical analyses were performed with GraphPad Prism 8 (GraphPad Software Inc, San Diego, CA). The Shapiro-Wilk normality test was used to evaluate data distribution. For normally distributed data with one experimental variable, statistical analyses were performed by parametric analysis: unpaired (2-tailed) Student t test for 2 groups and 1-way ANOVA with the Tukey multiple-comparison test for >2 groups. The data with 2 independent variables were evaluated by 2-way ANOVA with post hoc Sidak multiple comparison tests. For these tests, data are presented as mean±SEM. For non-normally distributed data with one experimental variable, statistical analyses were performed by nonparametric analysis: Mann-Whitney U test (2-tailed) for 2 groups and Kruskal-Wallis tests with post hoc Dunn multiple comparison tests for >2 groups. For these tests, data are represented as median (minimum, maximum).

**Mice**

C57BL/6J WT mice (No:000664), B6(C)-Ccr2tm1.1Cln/J (No: 027619), and B6(C)-Gt(Rosa)26Sorem1.1(CAG-cas9/-EGFP)Rsky/J (No: 028555) were obtained from Jackson Laboratories. Male mice were used for all in vivo experiments, and study protocols were approved by the institutional ACAC at the University of Virginia.

**Lentivirus Production**

pLKO5.sgRNA.EFS.tRFP (No. 57823), pLKO5.sgRNA.EFS, GFP (No. 57822), LentiCRISPRv2GFP (No. 82416), psPAX2 (No. 12260), and pMD2.G (No. 12259), were purchased from Addgene. Single gRNA targeting mouse Ppm1d (gtccacagt-gagatacgta or tgtcctaatcgaagtagcg), nontargeting sgRNA (acgggagctaacgctgca), or a noncoding sgRNA that targets an intron in the murine Actb gene (aggtgttcctgcaacac) were subcloned into the BsmB1 restriction enzyme site of the appropriate vector: pLKO5.sgRNA.EFS.tRFP was used for most of the in vivo experiments; pLKO5.sgRNA.EFS.GFP was used for competitive bone marrow transplantation experiments; and LentCRISPRv2GFP was used for cell culture experiments. For Ppm1d expression, the mouse cDNA corresponding to 1 to 1326 base pairs, under the spleen focus forming virus (SFFV) promoter, was subcloned into the lentivirus vector.
activity.20,23,24 Thus, bone marrow cells from transgenic mice expressing Cas9 and EGFP from the Rosa26 locus were transplanted with lentivirus particles expressing single guide RNA (sgRNA)/TagRFP (Tag red fluorescent protein) and delivered to lethally irradiated C57/BL6 wild-type (WT) mice. One month after bone marrow transplantation (BMT), peripheral blood was collected for flow cytometric analysis. B, Stable expression of the sgRNA/TagRFP vector at 4 wk post-BMT in monocyte, neutrophil, and lymphoid cells. C, Quantitative analysis of TagRFP-positive expression in cell populations derived from hematopoietic stem and progenitor cell (HSPC) transduced with lentiviral vectors encoding control (Ctrl) or Ppm1d-targeting sgRNA (n=8 per genotype). Statistical significance was evaluated by Mann Whitney U tests. D, Tracking of Indels by Decomposition analysis of the TagRFP-positive peripheral blood cells revealing insertions and deletions. E, The absolute number of white blood cells (WBC), red blood cells (RBC), hemoglobin (HGB), and platelets (PLT) in both experimental groups (n=8 per genotype). Statistical significance was evaluated by 2-tailed unpaired Student t test. F, Flow cytometric analysis of the peripheral blood at 4 wk after bone marrow reconstitution with HSPC transduced with control or Ppm1d-targeted lentiviral vectors. The absolute number of the major immune cell populations are shown (n=8 per genotype). Statistical significance was evaluated by multiple Student t test.

Figure 1. Ppm1d (protein phosphatase Mg2+/Mn2+ dependent 1D) mutagenesis in hematopoietic stem and progenitor cells (HSPC) and baseline mouse characteristics.

A, Schematic of experimental design. Bone marrow, lineage-negative cells from Cas9 expressing mice were transduced with lentivirus particles expressing single guide RNA (sgRNA)/TagRFP (Tag red fluorescent protein) and delivered to lethally irradiated C57/BL6 wild-type (WT) mice. One month after bone marrow transplantation (BMT), peripheral blood was collected for flow cytometric analysis. B, Stable expression of the sgRNA/TagRFP vector at 4 wk post-BMT in monocyte, neutrophil, and lymphoid cells. C, Quantitative analysis of TagRFP-positive expression in cell populations derived from hematopoietic stem and progenitor cell (HSPC) transduced with lentiviral vectors encoding control (Ctrl) or Ppm1d-targeting sgRNA (n=8 per genotype). Statistical significance was evaluated by Mann Whitney U tests. D, Tracking of Indels by Decomposition analysis of the TagRFP-positive peripheral blood cells revealing insertions and deletions. E, The absolute number of white blood cells (WBC), red blood cells (RBC), hemoglobin (HGB), and platelets (PLT) in both experimental groups (n=8 per genotype). Statistical significance was evaluated by 2-tailed unpaired Student t test. F, Flow cytometric analysis of the peripheral blood at 4 wk after bone marrow reconstitution with HSPC transduced with control or Ppm1d-targeted lentiviral vectors. The absolute number of the major immune cell populations are shown (n=8 per genotype). Statistical significance was evaluated by multiple Student t test.

analyses of the blood from mice revealed that the absolute number of peripheral blood cells or hemoglobin levels did not differ between mice transplanted with the mutant cells compared with those transplanted with the control cells (Figure 1E), consistent with the clinical paradigm of clonal hematopoiesis. Furthermore, flow cytometric analysis of the major immune cell subset ratios was not different between the test and control groups (Figure 1F), indicating that exon 6 Ppm1d mutations in HSPC does not affect blood cell differentiation. Echocardiographic analysis at 1-month post-BMT did not reveal any overt abnormalities in cardiac function, and cardiac parameters were
not different between animals receiving control versus 
Ppm1d-mutant cells (Table I in the Data Supplement). An 
analysis of immune cells within the heart by flow cytomet-
ric analysis revealed no detectable differences in immune 
cell composition or the degree of chimerism between the 
control and Ppm1d-mutant groups (Figure III in the Data 
Supplement). Overall, these data indicate that baseline 
hematologic or cardiac abnormalities are not observed in 
a mouse model in which a substantial fraction of engrafted 
HSPC contains mutations in exon 6 of Ppm1d.

Mice With Hematopoietic Ppm1d Mutations 
Exhibit Augmented Cardiac Remodeling 
Following Infusion of Ang II

Next, we examined whether Ppm1d mutations in HSPC 
cause mice to be more susceptible to cardiac stress. 
For the first set of experiments, mice were continuously 
infused with a supraphysiological dose of Ang II (angio-
tensin II) for 28 days. Ang II infusion leads to activation 
of the renin angiotensin-aldosterone system and induces 
cardiac-remodeling characterized by cardiac fibrosis, 
hypertrophy, and dysfunction. Analysis of these end 
points after infusion with Ang II revealed greater car-
diac remodeling in the mice that harbored HSPC with 
the Ppm1d mutation compared with the control non-
targeted sgRNA group (Figure 2). Echocardiographic 
analysis revealed a time-dependent decrease in fractional 
shortening and an increase in left ventricle end-
diastolic and end-systolic diameters in mice that received 
the Ppm1d-mutant cells at the 4-week timepoint after 
Ang II administration (Figure 2A). Heart weights were 
significantly greater in the Ppm1d-mutant group in 
the absence of differences in blood pressure between 
experimental groups as measured by the tail-cuff pleth-
ysmography (Figure 2B and 2C). Autopsy analyses did

Figure 2. Ppm1d (protein phosphatase Mg2+/Mn2+ 
dependent 1D) mutagenesis in hematopoietic stem and progenitor 
cells (HSPC) leads to greater cardiac pathology in response to Ang II (angiotensin II) infusion.
A, Sequential echocardiographic analysis of mice transplanted with HSPC transduced with lentiviral vectors encoding control (Ctrl) or 
Ppm1d-targeting (n=8 per genotype) at 0 (base) 2 and 4 wk (W) post-Ang II infusion. Statistical significance was evaluated by a 2-way repeated ANOVA 
with Sidak multiple comparison tests. B, Heart weight (HW) adjusted by tibia length (TL) following 4 wk Ang II infusion for experimental groups 
(n=6 for Sham and n=7 for Ang II). Statistical significance was evaluated by 2-way ANOVA with Sidak multiple comparison tests. C, Blood 
pressure as measured by tail-cuff plethysmography following 4 wk Ang II infusion for the experimental groups (n=6 for Sham and n=7 for Ang II). Statistical significance was evaluated by 2-way ANOVA with Sidak multiple comparison tests. 
D, Serum BNP levels following 28 days of Ang II infusion (n=6 for Sham and n=7 for Ang II). Statistical significance was evaluated by 2-way ANOVA with Sidak multiple comparison tests. E, Representative images and analysis of Picrosirius red/Fast Green staining of the heart sections from hearts of control and Ppm1d-mutated mice 
at the end of the study (scale bar=1 mm). Representative images were selected to represent the mean value of each condition. F, Representative 
images of wheat germ agglutinin (WGA) staining of the heart sections from hearts of control and Ppm1d-mutated mice at the end of the study 
(scale bar=50 μm). Representative images were selected to represent the mean value of each condition. G, Quantitation of fibrosis in E (n=6 for 
Sham and n=7 for Ang II). Statistical significance was evaluated by 2-way ANOVA with Sidak multiple comparison tests. H, Quantitative analysis 
of myocyte cross-sectional analysis (n=6 for Sham and n=7 for Ang II). Statistical significance was evaluated by 2-way ANOVA with Sidak 
multiple comparison tests.
not reveal aortic dissections/aneurysms in either experimental group. Plasma BNP, a heart failure marker, was significantly increased in the \textit{Ppm1d}-mutant group (Figure 2D). Picrosirius red/Fast Green staining of the heart showed greater levels of fibrosis in the \textit{Ppm1d}-mutant group (Figure 2E and 2G), and an increase cardiomyocyte cross-sectional area size that is consistent with greater cardiac hypertrophy (Figure 2F and 2H).

Three independent sets of experiments were performed to further test the hypothesis that exon 6 mutations in \textit{Ppm1d} promote Ang II-induced cardiac remodeling. First, the time course was extended to 2 months of Ang II infusion by the implantation of a second osmotic pump (Figure IV in the Data Supplement). Compared with the 1-month timepoint, the later time point revealed greater cardiac remodeling by the continuous infusion of Ang II and a greater separation of the data between test and control conditions. In a second set of experiments, \textit{Ppm1d} gene editing was directed by the lentivirus-mediated delivery of a second, independent sgRNA that was also predicted to have at least 4 mismatches with any other coding region in the genome (Figure V in the Data Supplement). Compared with the initial sgRNA targeting the \textit{Ppm1d} gene, there was a nearly identical impact on Ang II-induced cardiac remodeling, indicating that the enhanced cardiac pathology is not due to off-target effects of the sgRNA. Finally, we compared the \textit{Ppm1d}-targeting sgRNA with a control sgRNA that targets an intron in the \textit{Actb} gene (Figure VI in the Data Supplement). The \textit{Actb}-targeting sgRNA species did not detectably augment the hypertrophic response and, compared with this sgRNA control, the \textit{Ppm1d}-targeting condition promoted robust cardiac remodeling in response to Ang II stimulation. Collectively, these data indicate that mice with the gain-of-function, exon 6 \textit{Ppm1d} mutations in hematopoietic cells have normal cardiac function at baseline, but display greater cardiac remodeling and dysfunction following Ang II infusion.

### Infiltrating Monocytes and Macrophages Have an Essential Role in Cardiac Remodeling Response to Ang II Infusion

Since the \textit{Ppm1d} mutation was introduced into the HSPC fraction that repopulates the bone marrow, multiple leukocyte progeny derived from these HSPC will express the edited gene. Furthermore, because relatively little is known about the roles of immune cells in nonischemic heart failure, we examined the content of immune cells in the heart over time following the initiation of Ang II infusion to develop a better understanding of the leukocyte populations that could be contributing to the development of cardiac dysfunction in this t-CH model. Hearts were analyzed by flow cytometry at 0, 3, 7, and 14 days following the initiation of Ang II to characterize the dynamics of the cardiac immune cell content and infiltration over the course of cardiac hypertrophy in WT mice (Figures VII and VIII in the Data Supplement). We observed a significant increase in the absolute numbers of cardiac monocytes (defined as CD45\(^{+}\)CD64\(^{+}\)Ly6C\(^{+}\)), macrophages (defined as CD45\(^{+}\)CD64\(^{+}\)Ly6C\(^{−}\)), and neutrophil (defined as CD45\(^{+}\)CD64\(^{−}\)Ly6G\(^{+}\)) at 3 days. When monocytes and macrophages were further gated for expression of the CCR2 (C-C motif chemokine receptor 2), \textsuperscript{31} it was revealed that the CCR2+ monocyte and macrophage populations displayed the greatest elevation in the heart in response to Ang II stimulation (Figure 3A and 3B). In contrast to these changes in myeloid cells, little or no changes were observed in the absolute numbers of B or T cells within the heart in response to Ang II stimulation (Figure VIII in the Data Supplement).

To explore the functional role of monocytes and macrophages in this system, the cardiac stress response to Ang II was evaluated in mice deficient in CCR2 (\textit{Ccr2-KO}), that is required for monocyte mobilization from bone marrow. Echocardiographic analysis revealed a time-dependent decrease in fractional shortening and an increase in left ventricle end-systolic diameter in the control mice, but these changes were abrogated in the \textit{Ccr2-KO} group (Figure 3C). Compared with WT mice, heart weight was significantly lower in the \textit{Ccr2-KO} group after 28 days of Ang II infusion (Figure 3D). Analysis of transcript expression in the isolated hearts revealed that transcript expression of the proinflammatory cytokines \textit{Il6} and \textit{Il1b} and the heart failure markers \textit{Anf} and \textit{Bnp} were significantly lower in \textit{Ccr2-KO} mice (Figure 3E). Consistent with this reduction in inflammation, Picrosirius red/Fast Green staining of the heart revealed less extensive fibrosis in the \textit{Ccr2-KO} mice (Figure 3F).

While \textit{Ccr2-KO} mice are protected from the effects of Ang II infusion, it is unknown whether this effect is mediated by the loss of CCR2 in bone marrow cells or endothelial cells, fibroblasts, myocytes, etc that also express this gene.\textsuperscript{32} Thus, to analyze this in greater detail, lethally irradiated \textit{Ccr2-KO} mice were transplanted with either WT or \textit{Ppm1d} mutant HSPC and then treated with the continuous Ang II infusion. Following transplantation, \textit{Ccr2-KO} mice acquired susceptibility to Ang II–induced cardiac remodeling, and transplantation with the \textit{Ppm1d}-mutant HSPC conferred a worse cardiac remodeling phenotype compared with the mice with WT HSPC (Figure IX in the Data Supplement). Collectively, these data document the importance of infiltrating, bone marrow-derived monocytes/macrophages in this cardiac remodeling response, and they further corroborate cardiac remodeling phenotype that is conferred by the \textit{Ppm1d} mutant HSPC. Thus, the role of \textit{Ppm1d}...
mutations in the macrophage population was assessed further.

Ppm1d-Mutant Macrophages Exhibit DDR Pathway Impairment and an Augmented Proinflammatory Profile

PPM1D expression was assessed in myeloid cells isolated from mice transplanted with Ppm1d-mutant HSPC. Western blot analysis revealed robust expression of the stabilized (ie, truncated) form of PPM1D in bone marrow derived macrophages (Figure 4A, Figure V in the Data Supplement). Subsequent studies assessed the role of C-terminal Ppm1d mutations in controlling the DDR pathway and cytokine expression in both in murine J774.1 monocyte/macrophage cell line and bone marrow-derived macrophages. PPM1D is a serine-threonine protein phosphatase that dephosphorylates several substrates, including ATM (ataxia telangiectasia mutated) serine/threonine kinase, CHK1 (checkpoint kinase 1), and γH2AX (γH2A histone family member X) and negatively regulates the DDR pathway.33 Thus to examine the effect of the Ppm1d truncation mutation on the DDR pathway, we generated a murine J774.1 cell line that expresses the exon 6-mutated forms of Ppm1d using the CRISPR-Cas9 approach described above. The nature of the CRISPR/Cas9-edited mutations in exon 6 of Ppm1d is shown in Figure X in the Data Supplement. These mutations led to a marked increase in the PPM1D protein expression, but they had little or no impact on the phosphorylation of ATM, CHK1, or γH2AX under nonstimulated conditions (Figure 4B). However, the phosphorylation of these proteins was suppressed by the exon 6 mutations in Ppm1d in cells stimulated with LPS/IFN-γ (interferon-γ). Whereas it has been reported that the complete germline ablation of Ppm1d can enhance inflammation through p65-NFκB activation,34 the gain-of-function Ppm1d mutations examined here had little or no effect on the phosphorylation levels of p65 NFκB or p38 MAPK under these same conditions (Figure XI in the Data Supplement).
To examine the functional consequences of Ppm1d gene editing on the DDR pathway function, J774.1 cells were exposed to LPS to induce DNA-damage and inflammatory cytokine production through the activation of TLR4 (toll-like receptor 4) signaling. The comet assay, which detects cellular DNA-damage, revealed greater DNA fragmentation in the LPS-stimulated Ppm1d-mutant cells compared with LPS-treated control cells (Figure 4C and 4D). These observations were corroborated using LPS-stimulated bone marrow-derived macrophages isolated from mice that were transplanted with WT or Ppm1d-mutated cells (Figure XII in the Data Supplement).

To extend these findings, DNA damage was assessed in J774.1 cells in the presence or absence of the genotoxic agent doxorubicin under the following conditions: (1) untreated cells (nonmodified), (2) lentivirus/Cas9/sgRNA that does not target a DNA locus (nontargeted), (3) lentivirus/Cas9/sgRNA treatment that targets an intron of the Actb gene (noncoding; Figure VI in the Data Supplement), (4) lentivirus/Cas9/sgRNA treatment that targets exon 6 of PPM1D gene (Ppm1d), and (5) treatments with a lentivirus vector that overexpresses the truncated form of PPM1D (Ppm1d O/E) and lacks the CRISPR system components (Figure XIII).
in the Data Supplement). In the absence of stimulation, there was little or no DNA damage, suggesting that the DDR is not altered at baseline (Figure XIV in the Data Supplement). After stimulation with doxorubicin, there was detectable DNA damage in all experimental groups, but the DNA damage in Ppm1d gene-edited condition was significantly greater than that of the control groups. Consistent with findings in the mouse model, there was no difference among the different control groups regardless of whether they were not treated, transduced with a nontargeting sgRNA or transduced with a sgRNA that targets the intron of the Actb gene (Figure XIV in the Data Supplement). Collectively, these assays revealed no evidence of DDR activation by the CRISPR system per se. However, the exon 6 mutant form of Ppm1d, produced either by CRISPR gene editing or by the direct expression of this mutant form, led to suppression of the DDR and substantially greater DNA damage when cells were stimulated with a genotoxic agent.

The immune-regulatory properties of the gain-of-function, exon 6 Ppm1d-mutations have not been described previously. Thus, LPS-stimulated, mutant and control J774.1 cells were evaluated for cytokine and chemokine expression, and reactive oxygen species (ROS) production by the dichlorofluorescein assay because DNA damage can activate ROS production. After stimulation with LPS, ROS production was increased in both WT and Ppm1d-mutant cells, but the increase in ROS was markedly greater in Ppm1d-mutant condition (Figure 4E). Treatment with 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl (TEMPOL) suppressed LPS-induced ROS production in both control and Ppm1d-mutant cells, and there was no detectable difference between mutant and control cells after treatment. The Ppm1d-mutant cells also displayed higher levels of Il1b, Il6, and Cxcl2 transcripts following stimulation with LPS (Figure 4F and 4G, Figure XV in the Data Supplement). Higher levels of Il1b were also observed in J774.1 cells that overexpress the truncated form of PPM1D in the absence of the CRISPR system reagents (Figure XVI in the Data Supplement). To further investigate this inflammatory response, cells were incubated with the PPM1D inhibitor GSK2830371 or the antioxidant compound TEMPOL. As expected, the GSK2830371 compound had little or no effect on the expression of Il1b in WT cells, but it markedly inhibited Il1b expression in the Ppm1d-mutant cells (Figure 4F). TEMPOL treatment also suppressed LPS-induced Il1b expression in WT and Ppm1d-mutant cells and eliminated the difference in Il1b expression between the mutant and control conditions (Figure 4G).

To corroborate these findings, cytokine expression levels were analyzed in bone marrow-derived macrophages isolated from mice transplanted with Ppm1d-mutant or WT cells (Figure 4H through 4K). LPS-stimulated macrophages from the Ppm1d-mutant group showed elevated expression of both Il1b and Il18 transcripts (Figure 4H and 4J), and an ELISA analysis revealed a significant increase in the secretion of IL-1β and IL-18 following LPS/IFN-γ/ATP treatment in the Ppm1d-mutant macrophages (Figure 4I and 4K). TEMPOL treatment attenuated the elevated secretion of IL-1β and IL-18 in both control and the Ppm1d-mutant bone marrow-derived macrophages, and there was no detectable difference between these 2 groups in the TEMPOL-treated cells (Figure 4I and 4K). Collectively, these results suggest that activating mutations in the C-terminal exon of Ppm1d lead to the dephosphorylation of PPM1D substrates and inactivation of the DDR pathway. In turn, these events will result in the accumulation of DNA-damage, ROS production, and a proinflammatory phenotype.

The Inflammasome Has an Essential Role in the Ppm1d-Mediated Cardiac Dysfunction

Additional experiments focused on the upregulation of Il1b in the Ppm1d-mutant condition because this cytokine has previously been implicated clonal hematopoiesis and Ang II-mediated cardiac pathologies. Analysis of transcript expression in hearts revealed that the expression of Il1b was significantly higher in mice that had been transplanted with Ppm1d-mutant bone marrow cells (Figure 5A). To assess the major sources of IL-1β and NLRP3 (NLR family pyrin domain containing 3) expression in the heart, fibroblasts (CD45 −Ter119−CD31−PDGFRβ+), endothelial cells (CD45 −CD11b−CD31+CD18+), and myeloid cells (CD45−CD31−CD11b+) were sorted from WT mice at 3 days after treatment with Ang II. The quantitative polymerase chain reaction analysis showed that all cell types express Il1b and Nlpc3; however, their expression is vastly greater in the myeloid cells compared with the other cell types (Figure XVII in the Data Supplement). Immunofluorescence staining of heart tissue also revealed greater numbers of IL-1β-positive macrophages compared with control hearts from mice that had been treated with Ang II (Figure 5B and 5C). Very few dead macrophages were identified in the myocardium as assessed by TUNEL staining, and there was no difference between mice transplanted with either WT or Ppm1d-mutant bone marrow (Figure XVIII in the Data Supplement). To extend these findings, cytokine expression levels were analyzed in macrophages sorted from mice transplanted with Ppm1d-mutant or WT cells after 3 days of Ang II infusion. Indicative of a noncell autonomous effect of clonal hematopoiesis that has been noted by others, both RFP+ (Ppm1d-mutant) and RFP− (WT) macrophages expressed elevated levels of Il1b transcript in the mice transplanted with Ppm1d-mutant cells (Figures 5D and 5E). Further analyses revealed that the heart tissue from Ang II–treated mice transplanted with either WT or Ppm1d-mutant HSPCs did not differ from the control groups.
in the absolute numbers of monocytes, macrophages or neutrophils within the heart between the 2 experimental groups (Figure XIX in the Data Supplement). In addition, the degree of chimerism of TagRFP-positive cells were similar to what was observed in the peripheral blood for each immune cell subset. Collectively, these data suggest that the \textit{Ppm1d} mutated HSPC give rise to proinflammatory cardiac myeloid cells, but these conditions lead to little or no effect on the infiltration of immune cells to the heart.

\textbf{IL-1β} is synthesized as a precursor protein (pro-IL-1β) and requires cleavage by the NLRP3 inflammasome for activity. To assess the functional significance of NLRP3 inflammasome in the augmented cardiac pathology observed in mice transplanted with \textit{Ppm1d} mutant cells, the inflammasome inhibitor MCC950 was administered at the time of Ang II infusion. Co-infusion of MCC950 for 28 days resulted in protection against the enhanced Ang II–induced cardiac remodeling in the mice that had received \textit{Ppm1d}–mutant HSPC (Figure 5F through 5I). The effect of MCC950 treatment was quantitatively greater in the \textit{Ppm1d}–mutant group, and this treatment eliminated the statistical differences in the echocardiographic parameters of left ventricle end-systolic diameters and fractional shortening between mice transplanted with control cells and \textit{Ppm1d}–mutant cells (Figure 5F). Consistent with these data, MCC950 had similar protective effects on the parameters of heart function.
weight to tibia length ratio (Figure 5G), the extent of fibrosis (Figure 5H), and the increase in myocyte CSA at the 28-day time point (Figure 5I). For all of these parameters, treatment with MCC950 inhibited the pathological remodeling in the Ppm1d-mutant group and eliminated the differences in these parameters between mice transplanted with control and mutant cells. Overall, these data suggest that the greater cardiac remodeling and dysfunction observed in mice receiving Ppm1d-mutant HSPC is likely due to an augmentation of NLRP3 inflammasome-mediated cytokine production under conditions of Ang II infusion.

**DISCUSSION**

While the prevalence of clonal hematopoiesis increases with age, genotoxic agents, such as radiation and chemotherapy, have been associated with t-CH in younger individuals who are cancer survivors. Approximately 30% of cancer survivors have at least one detectable clonal event in their white blood cells that display mutations in a set of drivers that include DDR genes. The DDR gene PPM1D, with activating mutations in exon 6, is the most highly enriched t-CH driver gene. Recent studies show that HSPC harboring the gain-of-function PPM1D-mutant clones have the ability to avoid cell death induced by DNA-damage. Thus, these mutations can confer a competitive survival advantage to HSPC under conditions of genotoxic stress and result in the clonal expansion of their progeny leukocytes in blood.

It is widely appreciated that radiation or chemotherapy can contribute to the development of nonischemic heart disease in cancer survivors. While epidemiological data support an association between t-CH and CVD in cancer survivors, causal and mechanistic relationships have never been evaluated. Thus, we developed a mouse model to examine whether somatic hematopoietic mutations in exon 6 of Ppm1d can increase the heart's susceptibility to stress. This model specifically focused on the impact of t-CH on cardiac dysfunction that can occur in cancer survivors long after their therapy has ended, rather than on the shorter term adverse cardiac effects of cancer therapy in patients with preexisting clonal hematopoiesis. It was observed that mice transplanted with Ppm1d-mutant cells exhibit greater pathological cardiac remodeling following Ang II infusion. The results of this experimental study are supported by a recent study that found an association between PPM1D-mediated clonal hematopoiesis and worse outcome in chronic ischemic heart failure. Collectively, these findings raise the possibility that PPM1D-mediated t-CH may be a factor that contributes to the development of late-onset heart failure in cancer survivors.

Mechanistically, macrophages with exon 6 Ppm1d mutations exhibited higher expression of IL-1β and IL-18. As there were no detectable changes in the absolute numbers of cardiac monocytes and macrophages between the experimental groups, it would appear that the exon 6 Ppm1d mutation promotes a proinflammatory macrophage phenotype rather than influencing the cardiac infiltration of myeloid cells. Consistent with these observations, editing the Ppm1d gene at exon 6 in bone marrow-derived macrophages or the monocyte/macrophage J774.1 cell line increased the transcription and secretion of the IL-1β and IL-18 in response to stimulation. To test for the involvement of the NLRP3 inflammasome in cardiac remodeling in the Ppm1d-mutant condition, mice were administered the NLRP3 inflammasome inhibitor MCC950 that prevents the processing of IL-1β and IL-18 to their active forms. This treatment effectively reversed the cardiac phenotype caused by the transplantation of the Ppm1d-mutated HSPC, suggesting that inflammasome activation and elevated cytokine production was critical for the enhanced pathological cardiac phenotype.

The immune-regulatory properties of the activating, exon 6 Ppm1d-mutations have not been described previously. With regard to clonal hematopoiesis, our prior studies have shown that mutations in the Tet2 driver gene activate the inflammasome and elevate IL-1β through a HDAC (histone deacetylase)-dependent mechanism. In contrast, it appears that activating mutations in Ppm1d promote IL-1β (and IL-18) expression via a mechanism that involves modulation of the DDR response. Double strand DNA (dsDNA) breaks occur naturally as a result of endogenous metabolic and DNA replicative processes, and this damage is accelerated by exposure to genotoxic agents. In response to this stress, cells mount a canonical DDR to repair the DNA breaks and protect the integrity of the genome via the activation of TP53. In turn, TP53 induces the expression of PPM1D, comprising a negative feedback loop that inactivates the DDR through the inactivating dephosphorylation of TP53, CHK1 and 2, ATM, and γH2AX. The t-CH mutations in exon 6 of PPM1D are activating and will suppress the DDR. As shown here, exon 6 mutations of Ppm1d lead to a greater degree of DNA damage and greater inflammation in stimulated macrophages. Treatment with the ROS scavenger TEMPOL inhibited the induction of Il1b and Il18 and diminished their protein products in Ppm1d-mutant cells, suggesting that ROS contributes to cytokine production under these conditions. Consistent with the notion that inflammation is triggered by DNA damage, it is widely recognized cytoplasmic dsDNA triggers the formation and activation of the AIM2 (absent in melanoma 2) innate immune sensor that mediates the assembly and activation of the inflammasome. Cytoplasmic dsDNA is also sensed by the cGAS (cyclic GMP-AMP synthase)-Stimulator of Interferon Genes pathway which further promotes activation of the NLRP3 and AIM2 inflammasomes in macrophages. Supporting these observations, macrophage inflammasome activity

**Yura et al Clonal Hematopoiesis Promotes Heart Failure**

Circulation Research. 2021;129:684–698. DOI: 10.1161/CIRCRESAHA.121.319314
is promoted by the diminished activity of other DDR components including the DNA-damage repair kinase ATM and the Nbs1 (Nijmegen breakage syndrome 1) proteins that are necessary for the detection of DNA damage. While we cannot rule out any of these parallel mechanisms, our experimental findings are consistent with the hypothesis that Ppm1d stabilization promotes ROS production through the suppression of ATM and other components of the DDR pathway (Figure 4B), thereby leading to activation of the inflammasome in an ROS-dependent manner (Figure 4I and 4K). Overall, the mechanistic findings reported here are consistent with prior studies that have linked the suppression of the DDR pathway to ROS generation and ROS generation to elevated inflammation.

It is reported that germline Ppm1d-knockout (loss-of-function) mice display augmented inflammatory phenotypes that result from abnormalities in B cell, T cell, and neutrophil differentiation. In contrast to these findings, this study and another demonstrate that mice expressing exon 6-specific Ppm1d mutations in the hematopoietic system do not display detectable abnormalities in blood cell differentiation. It has also been reported that HeLa cells that lack functional PPM1D display increased inflammatory cytokine transcript expression via increased p65 NF-κB phosphorylation. However, we did not observe a detectable effect of the exon 6-specific Ppm1d mutations on p65 NF-κB in this system, perhaps because this regulation is dependent on the specific cell type. Thus, the proinflammatory properties of the exon 6-mutated, Ppm1d gain-of-function mice represents a previously unrecognized phenotype that provides insights about the mechanisms by which PPM1D-mediated t-CH mutations contribute to CVD.

The current study also provides evidence supporting the critical role that myeloid cells play in nonischemic heart failure. Previous work has documented the expansion of the cardiac macrophage pool during the early phases of Ang II infarction, but the functional role of monocyte derived-macrophages in this model is controversial. Here, it is shown that the cardiac remodeling response to Ang II stimulation is highly dependent on the participation of bone marrow-derived CCR2+ monocytes and macrophages. Among various leukocyte populations, CCR2+ cells displayed the greatest elevation in the heart in response to Ang II stimulation, and in contrast to a prior report, mice deficient in this chemokine receptor were found to be markedly protected from Ang II-induced cardiac remodeling. Furthermore, when the Ccr2-KO mice were transplanted with Ccr2-positive HSPC, the pathological actions Ang II on the heart were restored and the cardiac pathology was enhanced by the exon 6 mutations in Ppm1d. Collectively, these data indicate that bone marrow-derived myeloid cells are major contributors to the development of nonischemic heart failure, and that inflammasome overactivation in this cellular compartment is a critical feature of the exaggerated pathological responses under the conditions of PPM1D-mediated t-CH.

While our prior work has shown that different mutant driver genes can confer distinct phenotypes to their progeny leukocytes within the clone, a growing body of experimental and epidemiological evidence suggests that activation of IL-1β or IL-6 expression represents a common feature shared by many clonal hematopoiesis driver genes. The experimental findings reported here for exon 6 mutations in Ppm1d further strengthen the connection between overactivation of cytokine signaling and clonal hematopoiesis-mediated cardiovascular pathology. In this regard, there is growing evidence that proinflammatory cytokines affect HSPC behavior and alter their bone marrow niche. It has been reported that Tet2-deficiency also confers a selective advantage to HSPC in response to chronic proinflammatory signals. Thus, it is tempting to speculate that many clonal hematopoiesis driver genes will share this feature of promoting inflammation, particularly via IL-6 and IL-1β, while shielding the mutant HSPC from the suppressive effects of chronic cytokine overactivation. In other words, the upregulation IL-6 and IL-1β may not only be a common feature of the pathological mechanism conferred by multiple clonal hematopoiesis driver genes, but it may also represent a component of a common feedforward mechanism by which multiple clonal hematopoiesis driver genes promote HSPC clonal expansion.

We acknowledge that this study has several limitations. First, while these data support the concept that macrophages have a major role in the Ppm1d-mediated phenotype, we cannot exclude the possibility that other immune cell populations also contribute to cardiac dysfunction in this model. However, it should be noted that myeloid, not lymphoid, cell infiltration of the myocardium was shown to predominate in the Ang II model of cardiac stress. Furthermore, as shown here, macrophages are the main source of the IL-1β cytokine. Second, since our experimental system involves myeloablation before BMT to allow for donor cell engraftment, we cannot exclude the potential confounding effect of irradiation on the cardiac resident immune cell populations in this model. Third, our system, employing CRISPR-Cas9 system, generates homozygous mutation with a relatively high VAF, and this could exaggerate the some of the phenotype observed over the short time course of this study. We note, however, that the murine models of clonal hematopoiesis/CVD are conducted over a short time span compared with the clinical condition. As with many murine models, a higher dose of stimulus is used to compensate for the abbreviated time course. Fourth, recent reports have documented that the CRISPR-Cas9 system can trigger the DDR in some cell types. However, previous studies have employed the CRISPR/Cas9 system to analyze the DDR pathway, and it has been reported that...
HSPC are relatively tolerant of CRISPR-induced dsDNA breaks, exhibiting rapid and transient DDR activation. In the current study, gene editing was performed ex vivo before BMT, and a month before the initiation of Ang II infusion. Furthermore, no cellular or cardiac phenotype could be detected when the CRISPR system was used to create dsDNA breaks using a sgRNA that targets a noncoding region of the gene that encodes for α-actin, and no DDR activation could be observed under baseline conditions in Cas9-expressing cells that were treated with the Ppm1d-targeting sgRNAs. Finally, aspects of these findings were corroborated by expressing the truncated PPM1D protein with an expression system that does not involve CRISPR. Collectively, there were no detectable effects from the CRISPR-Cas9 system in this clonal hematopoiesis model, consistent with reports that CRISPR-induced dsDNA breaks have little if any effect on HSPC function.

CONCLUSIONS

By employing CRISPR-Cas9 technology to introduce mutations in exon 6 of the Ppm1d gene in HSPC, we established a mouse model of t-CH that recapitulates many of the features observed in individuals with PPM1D-mediated clonal hematopoiesis. Mice transplanted with bone marrow containing Ppm1d-mutant cells were more susceptible to stress-induced cardiac remodeling and dysfunction. Ppm1d-mutant macrophages displayed DDR pathway suppression and greater cytokine production in response to stress, and NLRP3 inflammasome inhibition reversed the mouse phenotype that was conferred by the transplantation of the Ppm1d-mutant cells. These data suggest that t-CH, caused by the gain-of-function mutations in PPM1D, can contribute to the late cardiac toxicity observed in cancer survivors. If validated by further studies, these data suggest that observations of t-CH could be predictive of long-term cardiac dysfunction in cancer survivors, and that anti-inflammatory therapies could be useful in treating individuals with this condition.

REFERENCES

1. Yura Y, Sano S, Walsh K. Clonal hematopoiesis: a new step linking inflammation to heart failure. *JACC Basic to Transl Sci*. 2020;5:196–207. doi: 10.1016/j.jacbts.2019.08.006
2. Evans MA, Sano S, Walsh K. Cardiovascular disease, aging, and clonal hematopoiesis. *Ann Rev Pathol*. 2020;15:419–438. doi: 10.1146/annurev-pathmedic-012419-032544
3. Shlush LI. Age-related clonal hematopoiesis. *Blood*. 2018;131:496–504. doi: 10.1182/blood-2017-07-746453
4. Steensma DP, Bejar R, Jaiswal S, Lindsley RC, Sekeres MA, Hasserjian RP, Ebert BL. Clonal hematopoiesis of indeterminate potential and its distinction from myelodysplastic syndromes. *Blood*. 2015;126:9–16. doi: 10.1182/blood-2015-03-631747
5. Genovese G, Kähler AK, Handsaker RE, Lindberg J, Rose SA, Bakhour SF, Chambert K, Mick E, Neale BM, Fromer M, et al. Clonal hematopoiesis and blood-cancer risk inferred from blood DNA sequence. *Nat Engl J Med*. 2014;371:2477–2487. doi: 10.1056/NEJMoa1409405
6. Jaiswal S, Fontanillas P, Flannick J, Manning A, Graumann PV, Mar BG, Lindsley RC, Mermel CH, Burtt N, Chavez A, et al. Age-related clonal hematopoiesis associated with adverse outcomes. *Nat Engl J Med*. 2014;371:2488–2498. doi: 10.1056/NEJMoa1408617
7. Xie M, Lu C, Wang J, McLellan MD, Johnson KJ, Wendl MC, McMichael JF, Schmidt HK, Yellapantula V, Miller CA, et al. Age-related mutations associated with clonal hematopoietic expansion and malignancies. *Nat Med*. 2014;20:1472–1478. doi: 10.1038/nm.3733
8. Zink F, Stacey SN, Norddahl GL, Frigge ML, Magnusson OT, Jonsdottir I, Thorgeirsson TE, Sigurdsson A, Guðjónsson SA, Gudmundsson J, et al. Clonal hematopoiesis, with and without candidate driver mutations, is common in the elderly. *Blood*. 2017;130:742–752. doi: 10.1182/blood-2017-02-769869
9. Young AL, Challen GA, Birmann BM, Druley TE. Clonal haematopoiesis harbouring AML-associated mutations is ubiquitous in healthy adults. *Nat Commun*. 2016;7:12484. doi: 10.1038/ncomms12484
10. Lah FR, Genovese G, Handsaker RE, Finucane HK, Reshef YA, Palamara PF, Birmann BM, Tkalcevski ME, Bakhour SF, McCarron SA, et al. Insights into clonal hematopoiesis from 8,342 mosaic chromosomal alterations. *Nature*. 2018;559:350–355. doi: 10.1038/s41586-018-0321-x
11. Bick AG, Pirruccello JR, Griffin GK, Gupta N, Gabriel S, Saleheen D, Libby P, Kathiresan S, Natarajan P. Genetic interleukin 6 signaling deficiency attenuates cardiovascular risk in clonal hematopoiesis. *Circulation*. 2020;141:124–131. doi: 10.1161/CIRCULATIONAHA.119.044362
12. Dorshheimer L, Assmus B, Rasper T, Ortmann CA, Ecke A, Abou-El-Arakat K, Schmidt T, Brune B, Wagner S, Serv H, et al. Association of mutations contributing to clonal hematopoiesis with prognosis in chronic ischemic heart failure. *JAMA Cardiol*. 2019;4:25–33. doi: 10.1001/jamacardio.2018.3965
13. Gibson CJ, Lindsley RC, Thekmedyan V, Mar BG, Shi J, Jaiswal S, Bosworth A, Francisco L, He J, Bansal A, et al. Clonal hematopoiesis associated with adverse outcomes after autologous stem-cell transplantation for lymphoma. *J Clin Oncol*. 2017;35:1598–1605. doi: 10.1200/JCO.2016.71.6712
15. Fuster JJ, MacLauchlan S, Zuriaga MA, Polackal MN, Ostriker AC, Bajpai G, Bredemeyer A, Li W, Zaitsev K, Koenig AL, Lokshina I, Mohan J, Benigni A, Cassis P, Remuzzi G. Angiotensin II revisited: new roles in inflammation, immunology and aging. *EMBO Mol Med* 2012;4:247–257. doi: 10.1002/embo.201100086

16. Sano S, Oshima K, Wang Y, Katanasaka Y, Sano M, Walsh K. CRISPR-mediated gene editing to assess the roles of Tdt and Dnm1l2a in clonal hematopoiesis and cardiovascular disease. *Circ Res* 2018;123:335–341. doi: 10.1161/CIRCRESAHA.120.031719

17. Sano S, Oshima K, Wang Y, MacLauchlan S, Katanasaka Y, Sano M, Zuriaga MA, Yoshiyama M, Goukassian D, Cooper MA et al. Tet2-mediated clonal hematopoiesis accelerates heart failure through a mechanism involving the IL17f/NLRP3 inflammation. *J Am Coll Cardiol* 2018;71:875–886. doi: 10.1016/j.jacc.2017.12.037

18. Sano S, Wang Y, Yura Y, et al. JAK2 (V617F)-mediated clonal hematopoiesis accelerates pathological remodeling in murine heart failure. *JACC Basic to Transl Sci* 2019;4:684–697.

19. Wang Y, Sano S, Yura Y, Ke Z, Sano M, Oshima K, Ogawa H, Horitani K, Min KD, Miura-Yura E, et al. Tet2-mediated clonal hematopoiesis in non-mitotically active cancer cells accelerates age-associated cardiac dysfunction. *JCI Insight* 2020;5:e135204. doi: 10.1172/jci.insight.135204

20. Coombs CC, Zehir A, Devlin SM, Kelly D, Patel M, Berthon A, Syed A, Yabe M, et al. Cancer therapy shapes the fitness landscape of clonal hematopoiesis. *Nat Genet* 2020;52:1219–1226. doi: 10.1038/s41438-020-00710-0

21. Hsu JI, Dayaram T, Töyö A, De Braekeleer E, Hsiao HM, Weinheimer C, et al. Tissue resident CCR2- and CCR2+ cardiac macrophages differentially orchestrate monocyte recruitment and fate specification following myocardial injury. *Circ Res* 2019;124:263–278.

22. Bolton KL, Ptashkin RN, Gao T, Braunstein L, Devlin SM, Kelly D, Patel M, Hsu JI, Dayaram T, Töyö A, De Braekeleer E, Jeong M, Wang F, Zhang L, Zhao Y. Lipopolysaccharide-mediated reactive oxygen species generation through the H2AX-Nox1/Rac1 pathway. *J Neurosci* 2019;39:6378–6394. doi: 10.1523/JNEUROSCI.0779-19.2019

23. Pereira-Lopes S, Tur J, Calatayud-Subias JA, Lloberas J, Stracker TH, Celada A. NBS1 is required for macrophage homeostasis and functional activity in mice. *Blood* 2015;126:2502–2510. doi: 10.1182/blood-2015-04-63737

24. Shioh Y, Ziv Y, The ATM protein kinase: regulating the cellular response to genotoxic stress, and more. *Rev Mol Cell Biol* 2013;14:197–210.

25. Ray P, Hu Y, Wen MH. Lipopolysaccharide-mediated reactive oxygen species and signal transduction in the regulation of interleukin-1 gene expression. *J Biol Chem* 2002;277:22131–22139. doi: 10.1074/jbc.M111883200

26. Wang P, Su H, Zhang L, Chen H, Hu X, Yang F, Lv J, Zhang L, Zhao Y. Phosphatase wild-type p53-induced phosphatase 1 controls the development of TH9 cells and allergic airway inflammation. *J Allergy Clin Immunol* 2018;141:2186–2186. doi: 10.1016/j.jaci.2017.06.026

27. Patel B, Bansal SS, Ismail MA, et al. CCR2(-) monocyte-derived infiltrating macrophages are required for adverse cardiac remodeling during pressure overload. *JACC Basic to Transl Sci* 2018;3:230–244. doi: 10.1016/j.jacbts.2017.12.006

28. Wang Y, Sano S, Oshima K, Sano M, Watanabe Y, Katanasaka Y, Yura Y, Jung C, Arzani A, Swirski FK, et al. Wnt5a-mediated neutrophil recruitment has an obligatory role in pressure overload-induced cardiac dysfunction. *Circulation* 2019;140:479–499. doi: 10.1161/CIRCULATIONAHA.118.038890

29. Epelman S, Lavine KJ, Beaudin AE, Sojka DK, Carrero JA, Calderon B, Brija I, Epelman S, Lavine KJ, Beaudin AE, Sojka DK, Carrero JA, Calderon B, Brija I. The can-
angiotensin II-induced cardiac fibrosis. Am J Physiol Heart Circ Physiol. 2011;301:H538–H547. doi: 10.1152/ajpheart.01114.2010
52. Bick AG, Weinstock JS, Nandakumar SK, Fulco CP, Bao EL, Zekavat SM, Szeto MD, Liao X, Leventhal MJ, Nasser J, et al; NHLBI Trans-Omics for Precision Medicine Consortium. Inherited causes of clonal hematopoiesis in 97,691 whole genomes. Nature. 2020;586:763–768. doi: 10.1038/s41586-020-2619-2
53. Pietras EM. Inflammation: a key regulator of hematopoietic stem cell fate in health and disease. Blood. 2017;130:1693–1698. doi: 10.1182/blood-2017-06-780882
54. Cai Z, Kotzin JJ, Ramdas B, Chen S, Nelamuthula S, Palam LR, Pandey R, Mali RS, Liu Y, Kelley MR, et al. Inhibition of inflammatory signaling in Tet2 mutant preleukemic cells mitigates stress-induced abnormalities and clonal hematopoiesis. Cell Stem Cell. 2018;23:833–849.e5. doi: 10.1016/j.stem.2018.10.013
55. Haapaniemi E, Botla S, Persson J, Schmierer B, Taipale J. CRISPR-Cas9 genome editing induces a p53-mediated DNA damage response. Nat Med. 2018;24:927–930. doi: 10.1038/s41591-018-0049-z
56. Schiroli G, Conti A, Ferrari S, Della Volpe L, Jacob A, Albano L, Beretta S, Calabria A, Vavassori V, Gasparini P, et al. Precise gene editing preserves hematopoietic stem cell function following transient p53-mediated DNA damage response. Cell Stem Cell. 2019;24:551–565.e8. doi: 10.1016/j.stem.2019.02.019