Enhanced Megakaryopoiesis and Platelet Activity in Hypercholesterolemic, B6-Ldlr\textsuperscript{−/−}, Cdkn2a-Deficient Mice

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Background—Genome-wide association studies for coronary artery disease/myocardial infarction revealed a 58 kb risk locus on 9p21.3. Refined genetic analyses revealed unique haplotype blocks conferring susceptibility to atherosclerosis per se versus risk for acute complications in the presence of underlying coronary artery disease. The cell proliferation inhibitor locus, CDKN2A, maps just upstream of the myocardial infarction risk block, is at least partly regulated by the noncoding RNA, ANRIL, overlapping the risk block, and has been associated with platelet counts in humans. Thus, we tested the hypothesis that CDKN2A deficiency predisposes to increased platelet production, leading to increased platelet activation in the setting of hypercholesterolemia.

Methods and Results—Platelet production and activation were measured in B6-Ldlr\textsuperscript{−/−}Cdkn2a\textsuperscript{+/−} mice and a congenic strain carrying the region of homology with the human 9p21.3/CDKN2A locus. The strains exhibit decreased expression of CDKN2A (both p16\textsuperscript{INK4a} and p19\textsuperscript{ARF}) but not CDKN2B (p15\textsuperscript{INK4b}). Compared with B6-Ldlr\textsuperscript{−/−} controls, both Cdkn2a-deficient strains exhibited increased platelet counts and bone marrow megakaryopoiesis. The platelet overproduction phenotype was reversed by treatment with cyclin-dependent kinase 4/6 inhibitor, PD0332991/palbociclib, that mimics the endogenous effect of p16\textsuperscript{INK4a}. Western diet feeding resulted in increased platelet activation, increased thrombin/antithrombin complex, and decreased bleeding times in Cdkn2a-deficient mice compared with controls.

Conclusions—Together, the data suggest that one or more Cdkn2a transcripts modulate platelet production and activity in the setting of hypercholesterolemia, amenable to pharmaceutical intervention. Enhanced platelet production and activation may predispose to arterial thrombosis, suggesting an explanation, at least in part, for the association of 9p21.3 and myocardial infarction.

Key Words: arterial thrombosis ■ atherosclerosis ■ genetics ■ mouse ■ platelet
the region of homology with the human CDKN2A/9p21.3 locus and exhibiting decreased Cdkn2a (+p16\textsubscript{INK4a} and +p19\textsubscript{ARF}) but not Cdkn2b (+p15\textsubscript{INK4b}) transcript levels compared with the B6-Ldlr\textsuperscript{−/−} control strain.\textsuperscript{18} The phenotype was recapitulated in irradiated mice receiving bone marrow (BM) from genetically targeted Cdkn2a\textsuperscript{−/−} mice, and the increase in atherosclerosis was associated with altered proliferation of monocyte/macrophage subpopulations.\textsuperscript{18} A similar transplantation experiment with BM derived from transcript-specific, homozygous p16\textsubscript{INK4a}/p19\textsubscript{ARF} mice, which exhibit compensatory upregulation of the nearby cell proliferation inhibitor locus, Cdkn2b, did not show an effect on atherosclerosis in the B6-Ldlr\textsuperscript{−/−} background.\textsuperscript{19} Further, homozygous Cdkn2a\textsuperscript{−/−} or p19\textsubscript{ARF}/−/− alleles in the APO*E3 Leiden transgenic background, also leading to compensatory upregulation of Cdk inhibitor genes, resulted in no effect or decreased atherosclerosis.\textsuperscript{20} These data, along with earlier reports of mRNA compensation in homozygous knockouts in wild-type genetic backgrounds,\textsuperscript{21,22} make it difficult to interpret effects of Cdkn2a transcripts on atherogenesis.

Cyclin-dependent kinase (CDK) inhibitors have been shown to play an important role in hematopoietic stem cell and progenitor cell self-renewal.\textsuperscript{23–25} The proteins encoded by CDKN2A are functionally unrelated but potent tumor suppressors: p16\textsubscript{INK4a}, an inhibitor of CDK4/6, and p19/ARF, a regulator of p53 stability. Both proteins are mediators of cellular senescence, and competitive BM transplantation experiments showed improved repopulating ability of hematopoietic stem cells derived from p16\textsubscript{INK4a}/−/− mice compared with controls in an age-dependent manner.\textsuperscript{26} Moreover, hematologic toxicity of ionizing radiation in mice was ameliorated through pharmacological inhibition of CDK4/6, mimicking the role of endogenous p16\textsubscript{INK4a}.\textsuperscript{27} Somatic loss of CDKN2A—by deletion, mutation, or epigenetic silencing—is the most frequent genetic alteration detected in human tumors. However, association of platelet counts with a common nucleotide variant of CDKN2A in the general population suggests that CDKN2A may play a role in platelet production in healthy individuals. Using 2 unique mouse models of Cdkn2a deficiency,\textsuperscript{18} we tested the hypothesis that deficiency of one or more transcripts encoded by the Cdkn2a locus predisposes to increased platelet production, leading to increased platelet activation. To more closely mimic the condition in which atherothrombosis occurs in humans, the experiments were performed under the condition of hypercholesterolemia.

Methods

Mice

All experiments were performed in the B6-Ldlr\textsuperscript{−/−} background (www.jaxmice.org stock No 002207). B6-Ldlr\textsuperscript{−/−}/Cdkn2a\textsuperscript{+/−} and B6-Ldlr\textsuperscript{−/−}.MOLFchr4subD mice were described previously.\textsuperscript{15} Heterozygous Cdkn2a knockout mice were used to more closely mimic natural gene expression variation in humans and to avoid the complication of spontaneous tumorigenesis that occurs in homozygous knockouts. B6-Ldlr\textsuperscript{−/−}.MOLFchr4subD is a congenic strain originally developed in an atherosclerosis susceptibility mapping study.\textsuperscript{18} These mice carry a region of mouse chromosome 4 derived from MOLF/Ei on the B6-Ldlr\textsuperscript{−/−} background. The region confers increased susceptibility to atherosclerosis and contains the region of homology with the human 9p21.3 CAD/MI GWAS locus and the CDKN2A/CDKN2B genes. B6-Ldlr\textsuperscript{−/−}/Cdkn2a\textsuperscript{+/−} and B6-Ldlr\textsuperscript{−/−}.MOLFchr4subD mice are deficient in both p16\textsubscript{INK4a} and p19\textsubscript{ARF}, but not related Cdk inhibitor transcripts p15\textsubscript{INK4b} or p14\textsubscript{ARF}. The congenic strain exhibits a more pronounced deficiency of Cdkn2a transcripts without the complication of tumorigenesis.\textsuperscript{18} Mice were fed (1) regular chow, (2) chow+50 mg/kg PD0332991/palbociclib (CDK4/6 inhibitor, Pfizer), or (3) Western-type diet (WTD; Harlan Teklad No TD88137) as indicated. Because there was no effect of sex on the platelet production phenotypes (Figure 1 in the Data Supplement), similar numbers of males and females were used for all experiments, except for the tail vein bleeding assay under the condition of WTD diet feeding, in which there were not enough males available. All procedures were in accordance with Institutional guidelines.

Complete Blood Count

Whole blood collected from the retro-orbital plexus, with 10% volume of acid–citrate–dextrose or EDTA as anticoagulant, was analyzed using a FORCYTE Veterinary Hematology Analyzer (Oxford Science, Inc).

Megakaryocyte Progenitor-CFU Assay

Primary BM cells were plated in collagen-based medium (5000 cells per assay) containing thrombopoietin (50 ng/mL) and interleukin-3 (10 ng/mL) and incubated for 12 days according to the manufacturer’s protocol (Megacult-C, Stemcell Technologies). The collagen gels were then dehydrated, fixed, and stained for acetylcholinesterase activity to identify megakaryocyte progenitor (MkP) cells. Nuclei were counterstained with Harris’ hematoxylin. MkP-colony-forming units (CFUs) were defined by groupings of at least 3 MkP cells. Large MkP-CFUs were defined by groupings of at least 10 MkP cells.

Immunohistochemistry Staining

Bone samples were fixed in 10% formalin for 24 hours and decalciﬁed with EDTA for 48 hours before sectioning. Paraffin sections were incubated with von Willebrand factor antibody (Dako A0082, 1:400 dilution) and visualized using the avidin–biotin complex staining method.

Flow Cytometry–Based Hematopoietic Stem Cell Profiles

BM cells from mouse femurs and tibias were stained with a cocktail of antibodies as previously described.\textsuperscript{18} Briefly, BM cells were incubated with antibodies to lineage-committed cells (CD45R, CD19, CD11b, CD3e, TER-119, CD2, CD8, CD4, and Ly-6CG, all fluorescein isothiocyanate conjugated; eBioscience), Sca1-Pacific blue (Biologend), and c-Kit-APC cy7 (eBioscience) to identify Lin−Sca1+c-Kit+(LSK) cells and hematopoietic stem and progenitor cells (Lin−Sca1−c-Kit+).

Further, another cocktail of antibodies to lineage-committed cells (same as mentioned above) plus Sca-1 fluorescein isothiocyanate, CD16/CD32 (FcγRII/II/III)-Pacific blue, CD34-PerCP Cy5.5 (BD Biosciences), CD71-phycocerythrin (PE), and CD41-PE cy7 to identify progenitor cell populations, including common myeloid progenitor (Lin−Sca1−c-Kit+CD34+/−FcγRII/II/III+), granulocyte–macrophage progenitor (Lin−Sca1−c-Kit+CD34+/−FcγRII/II/III+), megakaryocyte–erythroid progenitor (Lin−Sca1−c-Kit+CD34+/−FcγRII/II/III+), erythroid progenitor (Lin−Sca1−c-Kit+CD34+/−FcγRII/II/III+CD71−CD41+), and MkP (Lin−Sca1−c-Kit+CD34+/−FcγRII/II/III+CD71−CD41+). For spleen cells, similar staining and gating strategies were used in separating spleen LSK and hematopoietic stem and progenitor cells. Spleen MkP was deﬁned as (Lin−Sca1−c-Kit+CD41+). Samples were run on an LSR II instrument using FACSIdiva software (BD Biosciences). Cell sorting was performed on a BD Influx using BD Software 1.2.0.142 (BD Biosciences) and analyzed with FlowJo software. All antibodies were used at 1:50 dilution.

Flow Cytometry of Platelet–Leukocyte Aggregates

Acid–citrate–dextrose-whole blood collected from the retro-orbital plexus was incubated with an excess of ice-cold lysin buffer plus 1

1.2.0.142 (BD Biosciences) and analyzed with FlowJo software. All antibodies were used at 1:50 dilution.
Reticulated Platelet Detection
Although both young and mature platelets are anuclear, young reticulated platelets have residual RNA content that can be used for detection, providing an indication of relative rates of megakaryopoiesis in the BM. Acid–citrate–dextrose- or EDTA whole blood collected from the retro-orbital plexus was incubated with thiazole orange fluorescent dye (nucleic acid-binding, final concentration 1 μg/mL) and counterstained with a CD41-PE antibody (to identify platelets). Samples were incubated at room temperature for 20 minutes, fixed with 1% formaldehyde in PBS, and analyzed within 45 minutes of blood collection. Data were acquired using logarithmic amplification of light scatter and fluorescence signals. PE-positive cells were gated in a thiazole orange fluorescence versus side scatter dot plot, reducing interferences caused by platelet size and platelet aggregation.30

Platelet Activity Assays
Platelet activation was assessed by platelet surface expression of P-selectin, a marker of platelet degranulation, and surface JON/A, which selectively binds to the high affinity conformation of mouse integrin αIIbβ3. Briefly, acid–citrate–dextrose whole blood was diluted in HEPES-buffered, modified Tyrode’s buffer and analyzed as a pan platelet marker. The reactions were stopped by the addition of 400 μL HEPES-buffered, modified Tyrode’s buffer and analyzed within 30 minutes.

Plasma Thrombin/Antithrombin Complex ELISA
A dual antibody mouse thrombin/antithrombin (T-AT) complex ELISA kit (Innovative Research, IRAPKT175) was used. Plasma samples were diluted 1:50, plated into T-AT antibody-coated plates, and incubated at room temperature for 2 hours. After 6 plate washes, 50 μL of biotinylated mouse antithrombin antibody was added for an incubation period of 2 hours. After washing, streptavidin–peroxidase conjugate secondary antibody and chromogen substrate were added to the wells. Optical density was measured at 450 nm with 570 nm correction using a SpectraMax M2 plate reader. Final concentration was calculated from a standard curve, generated in parallel, on the same plate.

Tail Vein Bleeding Assay
Mice were anesthetized with isoflurane, and a 5 mm segment of the distal tail tip was transected with a sharp scalpel. Bleeding was monitored by gently dabbing the tail tip on Whatman paper until the cessation of bleeding, as described.31

Statistics
Three-group analyses were done by 1-factor analysis of variance with Bonferroni correction (single measurements), repeated measures analysis of variance (multiple measurements), or Kruskal–Wallis with Dunn’s multiple comparison (small sample size or skewed data) as indicated. Two-group analyses were done by Student’s t test (2-tailed) or Mann–Whitney (small sample size) as indicated. Analyses were performed using Prism 6 (GraphPad Software, Inc). The threshold for significance was P<0.05. Bar graphs indicate mean±SD.

Results
Increased Platelet Production in Chow-Fed Cdkn2a-Deficient Strains in the B6-Ldlr<sup>−/−</sup> Background
Complete blood count analysis revealed 30% to 40% increased circulating platelet counts in B6-Ldlr<sup>−/−</sup>Cdkn2a<sup>−/−</sup> and B6-Ldlr<sup>−/−</sup>. MOLFchr4subD mice compared with B6-Ldlr<sup>−/−</sup> controls (Figure 1A), and the increases were independent of sex (Figure IIA in the Data Supplement). Total white blood cell, neutrophil, and lymphocyte counts were not altered between the strains (Figure IIA in the Data Supplement). Monocyte counts were modestly elevated in the gene-targeted Cdkn2a<sup>−/−</sup> mice compared with controls, and both Cdkn2a<sup>−/−</sup> strains showed increases in the inflammatory subset characterized by high cell surface expression of Ly6C antigen (Figure IIB in the Data Supplement), consistent with our previous observations in these strains.30 Along with the increases in total platelet counts, percentages of immature reticulated platelets were increased in blood samples from the 2 Cdkn2a<sup>−/−</sup> strains (Figure 1A, right-hand graph), suggesting increased megakaryopoiesis in the BM. The increases in reticulated platelets were observed in both males and females of the gene-targeted Cdkn2a<sup>−/−</sup> strain but more heavily influenced by males in the subcongenic strain, possibly because of small sample size (Figure IIA in the Data Supplement).

To test for increased megakaryopoiesis, we looked at hematopoietic stem cell and progenitor profiles by flow cytometry and determined MkP colony-forming potential of the 3 strains. Analysis of LSK (Lin<sup>−</sup>Sca1<sup>−</sup>c-Kit<sup>+</sup>) and progenitor populations revealed no differences in the percentages of LSK cells in total BM cells between the strains but increased percentages of granulocyte–macrophage progenitors and trends toward increased megakaryocyte–erythroid progenitors and significantly increased MkPs but not erythroid progenitors in the Cdkn2a<sup>−/−</sup>-deficient strains compared with controls (Figure 1B). The differences in percentages of granulocyte–macrophage progenitor and MkP cells were independent of sex in both strains (Figure 1B in the Data Supplement). In the spleen, percentages of LSK and MkP cells were significantly increased in the gene-targeted Cdkn2a<sup>−/−</sup> strain compared with controls, with trends toward increased percentages in the subcongenic strain (Figure 1B, right-hand graph). As a measure of MkP production, we measured MkP-CFU<sub>S</sub> in BM-derived cells after 12 days of culture in the presence of thrombopoietin and interleukin-3 growth factors. There was a trend toward increased total colony numbers, and colony size, in BM derived from B6-Ldlr<sup>−/−</sup>Cdkn2a<sup>−/−</sup> mice and significantly increased colony numbers and size in BM derived from B6-Ldlr<sup>−/−</sup>.MOLFchr4subD mice compared with controls (Figure 1C). Finally, immunohistochemical analysis of decalcified bone showed a trend toward increased numbers of von Willebrand factor–positive megakaryocytes in B6-Ldlr<sup>−/−</sup>.MOLFchr4subD mice compared with B6-Ldlr<sup>−/−</sup> controls (Figure IIIA in the Data Supplement). Consistent with these results, we observed decreased Cdkn2a<sub>2</sub>, transcript levels in MkP cells derived from B6-Ldlr<sup>−/−</sup>Cdkn2a<sup>−/−</sup> and B6-Ldlr<sup>−/−</sup>.MOLFchr4subD mice compared with B6-Ldlr<sup>−/−</sup> controls (Figure IIIB in the Data Supplement). These data indicate a role for one or both Cdkn2a transcripts in regulating megakaryopoiesis and platelet production.

µM prostaglandin E1 (PGE<sub>1</sub>) to prevent platelet activation and in vitro formation of platelet–leukocyte aggregates as described.29 The blood cells were lysed briefly (1 minute), spun at 4 °C, and stained with a cocktail of antibodies at 1:100 dilution: CD45-Pacific blue (eBioscience), CD11b-PE Cy7 (eBioscience), and CD41-fluorescein isothiocyanate (eBioscience). Platelet Ly-6C<sup>−</sup> monocyte aggregates, platelet Ly-6C<sup>−</sup> monocyte aggregates, and platelet neutrophil aggregates were defined as CD45<sup>+</sup>Gr-1<sup>hi</sup>CD115<sup>+</sup>CD41<sup>+</sup>, CD45<sup>+</sup>Gr-1<sup>−</sup>PerCP Cy5.5 (Ly-6-C/G; BD Biosciences), CD11b-PE Cy7 (eBioscience), and CD41-fluorescein isothiocyanate (eBioscience). Platelet Ly-6C<sup>−</sup> monocyte aggregates, platelet Ly-6C<sup>−</sup> monocyte aggregates, and platelet neutrophil aggregates were defined as CD45<sup>+</sup>Gr-1<sup>+</sup>CD115<sup>−</sup>CD41+, CD45<sup>+</sup>Gr-1<sup>−</sup>CD115<sup>−</sup>CD41+, and CD45<sup>Gr-1</sup>CD115<sup>−</sup>CD41+. 

Statistics
Three-group analyses were done by 1-factor analysis of variance with Bonferroni correction (single measurements), repeated measures analysis of variance (multiple measurements), or Kruskal–Wallis with Dunn’s multiple comparison (small sample size or skewed data) as indicated. Two-group analyses were done by Student’s t test (2-tailed) or Mann–Whitney (small sample size) as indicated. Analyses were performed using Prism 6 (GraphPad Software, Inc). The threshold for significance was P<0.05. Bar graphs indicate mean±SD.
Inhibition of CDK4/6 Reverses the Platelet Overproduction Phenotype of Cdkn2a-Deficient Strains in the B6-Ldlr−/− Background

The Cdkn2a-deficient strains used in this study are partially deficient in both transcripts encoded by the locus: p16 INK4a and p19/ARF. The p16INK4a protein blocks cell proliferation by interfering with the interaction of CDK4 and cyclin D, resulting in hypophosphorylation of the retinoblastoma oncogene, Rb, and subsequent G1 arrest. To test the specific role of p16INK4a in megakaryopoiesis and platelet production, we treated mice with the specific CDK4/6 inhibitor, PD0332991/palbociclib, that mimicks the effect of endogenous p16 INK4a.

The PD0332991 inhibitor was incorporated into regular chow to provide an effective dose of 50 mg/kg. 27 Mice were bled...
at baseline and after 7 and 16 days of treatment. As previously demonstrated with chow feeding, both Cdkn2a-deficient strains had increased circulating platelet counts compared with B6-Ldlr<sup>−/−</sup> mice at baseline. At 7 days of treatment, platelet counts were decreased in all 3 strains, with only B6-Ldlr<sup>−/−</sup>-MOLFChr4subD mice exhibiting increased counts compared with controls (Figure 2A). At 16 days of treatment, there were no further decreases in platelet counts, and the differences between the strains were eliminated (Figure 2A). Flow cytometry analysis of BM cell profiles at 21 days of treatment revealed no differences between the strains in the percentages of LSK or progenitor subpopulations in total BM cells, including megakaryocyte–erythroid progenitor and MkP cells (Figure 2B). The MkP-CFU assay showed that the number and size of MkP colonies derived from both Cdkn2a-deficient strain BM were reduced to at least that of the B6-Ldlr<sup>−/−</sup> controls after PD0332991 treatment (Figure 2C). Finally, we measured an increase in vivo marker of thrombin generation, T-AT complex, as a potential functional consequence of augmented platelet production. Baseline levels of T-AT complex were elevated in B6-Ldlr<sup>−/−</sup>Cdkn2a<sup>−/−</sup> mice, but not B6-Ldlr<sup>−/−</sup>-MOLFChr4subD mice compared with B6-Ldlr<sup>−/−</sup> controls (Figure 2D). Treatment with PD0332991 reduced T-AT complex levels in B6-Ldlr<sup>−/−</sup>Cdkn2a<sup>−/−</sup> mice to that of the controls (Figure 2D). These data are consistent with a transcript-specific role of p16<sub>INK4a</sub> in megakaryopoiesis, platelet production, and thrombin generation in these mouse models.

**Cdkn2a Deficiency Promotes Platelet Activation in B6-Ldlr<sup>−/−</sup> Strains Fed WTD**

Platelet overproduction has been shown to lead to platelet activation in Abcg4- and Abcc6-deficient mouse models. Thus, we next studied markers of platelet activation in the Cdkn2a-deficient strains. In vivo ligation of the platelet surface receptor, PAR-4, by thrombin results in increased surface expression of cell adhesion molecules that facilitate platelet-leukocyte interactions, platelet-endothelial interactions, and intercellular signaling. Ex vivo treatment of whole blood with synthetic peptide AYPGKF, a PAR-4 agonist, resulted in increased mean fluorescence intensity of surface P-selectin in chow-fed B6-Ldlr<sup>−/−</sup>-MOLFChr4subD (1.4-fold), but not B6-Ldlr<sup>−/−</sup>-MOLFChr4subD mice compared with B6-Ldlr<sup>−/−</sup> controls (Figure 3A). We also observed increased levels of sP-selectin in blood from B6-Ldlr<sup>−/−</sup>-MOLFChr4subD mice (Figure 3B). In addition, both Cdkn2a-deficient strains exhibited increased bleeding times compared with controls in a tail vein bleeding assay (Figure 3C). Overall, partial Cdkn2a deficiency in the B6-Ldlr<sup>−/−</sup> background resulted in modest effects on platelet activation with chow feeding.

To more closely mimic the conditions under which atherothrombosis occurs in humans, we next studied platelet activation in WTD-fed mice. The 8-week time point represents a period of active atherogenesis but precedes age-dependent decline in hematopoietic stem cell function. As demonstrated with chow feeding, B6-Ldlr<sup>−/−</sup>Cdkn2a<sup>−/−</sup> and B6-Ldlr<sup>−/−</sup>-MOLFChr4subD mice had increased circulating platelet counts and increased percentages of young, reticulated platelets, compared with B6-Ldlr<sup>−/−</sup> mice (Figure IVA and IVB in the Data Supplement). However, there was no evidence of monocytosis or neutrophilia (Figure V in the Data Supplement), phenotypes observed in other models with WTD feeding. We measured surface expression of both P-selectin and active integrin α<sub>Iβ<sub>3</sub></sub> (JON/A). In the resting state, no differences between the strains were observed for levels of platelet P-selectin or JON/A (Figure VIA and VIB in the Data Supplement). After stimulation with PAR-4 agonist AYPGKF, whole blood from both Cdkn2a-deficient strains exhibited markedly increased surface levels of P-selectin (Figure 4A) and JON/A (Figure 4B). For P-selectin, the increases were 8.5- and 39-fold for B6-Ldlr<sup>−/−</sup>Cdkn2a<sup>−/−</sup> and B6-Ldlr<sup>−/−</sup>-MOLFChr4subD relative to the controls, respectively. For JON/A, the increases were 4- and 9-fold for B6-Ldlr<sup>−/−</sup>Cdkn2a<sup>−/−</sup> and B6-Ldlr<sup>−/−</sup>-MOLFChr4subD relative to the controls, respectively. To test for a direct role of Cdkn2a/p16<sub>INK4a</sub> deficiency on platelet activation, we measured PAR-4-stimulated P-selectin and JON/A expression after ex vivo treatment of whole blood with PD0332991. Little to no differences were observed between treated and untreated samples, and no differential strain effects were observed (Figure VII in the Data Supplement). Together, the data are consistent with a primary effect of Cdkn2a deficiency on platelet overproduction, leading to increased platelet activation.

In addition, we observed increases in platelet:leukocyte aggregates with Cdkn2a deficiency, especially in the B6-Ldlr<sup>−/−</sup>-MOLFChr4subD strain. The percentages of platelet:neutrophil and platelet:monocyte aggregates in total white blood cells were increased in B6-Ldlr<sup>−/−</sup>-MOLFChr4subD mice, with a trend toward increased platelet:neutrophil aggregates in B6-Ldlr<sup>−/−</sup>Cdkn2a<sup>−/−</sup> mice, compared with controls (Figure 5A and 5B). The increase in total platelet:monocyte aggregates in the B6-Ldlr<sup>−/−</sup>-MOLF Chr4subD strain was because of increases in both platelet:Ly<sub>6C<sub>−</sub></sub> monocyte and platelet:Ly<sub>6C<sup>w</sup></sub> monocyte aggregates (Figure 5C and 5D).

Under the condition of WTD feeding, the in vivo marker of thrombin generation, T-AT complex, was significantly elevated in the circulation of both Cdkn2a-deficient strains by 2-fold compared with B6-Ldlr<sup>−/−</sup> controls (Figure 6A). In addition, both Cdkn2a-deficient strains exhibited significantly decreased tail vein bleeding times (Figure 6B), indicating an effect on hemostasis. Together, the data indicate that partial Cdkn2a deficiency promotes platelet overproduction, leading to platelet activation in the setting of hypercholesterolemia.

**Discussion**

In the present study, we have identified an important role for Cdkn2a in platelet production, leading to platelet activation in hypercholesterolemic B6-Ldlr<sup>−/−</sup> mice. Gene-targeted heterozygous deficiency of Cdkn2a and naturally occurring inbred strain variation observed in B6-Ldlr<sup>−/−</sup>-MOLFChr4subD mice, both resulting in decreased expression of both p16<sub>INK4a</sub> and p19<sub>ARF</sub> but not p15<sub>INK4b</sub> transcripts, led to increased platelet counts in the circulation and augmented megakaryopoiesis in the BM. These phenotypes were reversed by pharmacological treatment with the CDK4/6 inhibitor, PD0332991, consistent with a transcript-specific effect of p16<sub>INK4a</sub> on platelet production that warrants further testing. Although some strain effects on platelet activation were observed with chow diet feeding, Cdkn2a deficiency in
the setting of WTD feeding resulted in more robust effects on platelet activation and downstream functional effects, such as platelet:leukocyte aggregate formation, T-AT complex generation, and decreased bleeding times. Together, the data indicate that Cdkn2a deficiency promotes platelet overproduction, leading to platelet activation.
In humans and mice, complete deficiency of CDKN2A/Cdkn2a, including both p16INK4a and p19ARF, leads to spontaneous development of a wide spectrum of tumors because of increased mitogenic activity of hematopoietic stem and progenitor cells. Preclinical studies with the specific CDK4/6 inhibitor, PD0332991, in mice resulted in tumor regression because of decreased proliferative capacity of early myeloid progenitors without effects on apoptosis. Thus, it is possible that hypercholesterolemia may exaggerate the effect of Cdkn2a deficiency on platelet production. Moreover, the effects of Cdkn2a deficiency on platelet overproduction were associated with increased platelet activation, which was enhanced with WTD feeding. Platelet production and activation have been implicated in inflammatory pathways contributing to both thrombosis and atherosclerosis development. Thus, it is possible that platelet overproduction may have contributed to the augmented atherogenesis observed in our previous BM transplantation study; however, we did not measure platelet production in that model. It will be interesting to determine whether Cdkn2a deficiency has a greater effect on arterial thrombosis.

Hypercholesterolemia has been shown to increase platelet activation by multiple mechanisms, including increased uptake of oxidized lipoproteins via platelet surface scavenger receptors. We observed increased markers of platelet atherogenesis associated with increased ratios of inflammatory Ly6C\(^{hi}\)/Ly6C\(^{lo}\) monocytes in the circulation. Moreover, the B6-Ldl\(^{-}\)/Cdkn2a\(^{-}\) recipients exhibited increased cell proliferation of circulating Ly6C\(^{hi}\) monocytes and tissue macrophages compared with controls transplanted with B6-Ldl\(^{-}\) BM. In the current study using whole-body Cdkn2a deficiency models, we have observed an additional effect of partial Cdkn2a deficiency in the B6-Ldl\(^{-}\) background—a pronounced effect on circulating platelet counts. Although an effect on platelet counts is not surprising, the amplitude of the effect in the models described herein suggests that hypercholesterolemia may exaggerate the effect of Cdkn2a deficiency on platelet production.
activation by multiple measures in WTD-fed Cdkn2a-deficient strains compared with chow-fed mice. Although chow-fed B6-Ldlr−/−.MOLFch4subD mice exhibited elevated markers of platelet activation (Figure 3), both strains fed WTD exhibited significantly elevated platelet surface P-selectin and JON/A, after PAR-4 stimulation (Figure 4). Further, WTD feeding resulted in increased levels of circulating platelet:leukocyte aggregates in B6-Ldlr−/−.MOLFch4subD (Figure 5) and elevated T-AT levels in both strains (Figure 6A), indicative of a prothrombotic phenotype. Consistent with these findings, we previously reported a platelet overproduction phenotype with BM-specific Abcg4 deficiency that was also associated with WTD feeding.28 These data strongly suggest an augmented effect of hypercholesterolemia on Cdkn2a-dependent platelet overproduction, leading to platelet activation.

Although both Cdkn2a-deficient strains studied exhibited enhanced megakaryopoiesis, platelet overproduction, and increased platelet activation, the phenotype of the congenic B6-Ldlr+/−.MOLFch4subD strain tended to be more robust than that of the gene-targeted B6-Ldlr−/−.Cdkn2a−/− strain. The strain-specific differences in the platelet overproduction phenotype are likely because of differences in underlying genetic variation at the Cdkn2a locus. We previously reported 3- to 8-fold versus 2.5- to 4.5-fold decreases in expression levels of p16INK4a in B6-Ldlr−/−.MOLFch4subD versus B6-Ldlr+/−.Cdkn2a−/− mice compared with B6-Ldlr−/− controls, dependent on the tissue/condition queried.18 Thus, the more dramatic phenotype of B6-Ldlr−/−.MOLFch4subD mice is likely due, at least in part, to differences in the level of gene expression.

Overexpression of p16INK4a resulted in increased venous thrombosis in a murine vascular injury model,43 in contrast to our hypothesis that Cdkn2a deficiency promotes a prothrombotic phenotype. However, there are substantial pathobiologic differences between venous and arterial thrombosis.43,44 Venous thrombosis occurs with stasis and hypercoagulability, and fibrin and red blood cells are the main constituents of venous thrombi. On the other hand, endothelial damage, platelets, lipids, and inflammation play a much larger role in arterial thrombosis. The disease risk genes identified for arterial thrombosis are often distinct from those for venous thrombosis.45 For example, inherited thrombophilia factors that are strongly associated with high risk for venous thrombosis are not associated, or less strongly, with arterial thrombosis.45 In a mouse model of fibronectin haploinsufficiency, delayed thrombus formation was observed in arterioles but not venules.46 Thus, it is highly plausible that Cdkn2a/p16INK4a may have different effects in arterial versus venous thrombosis.

Platelet activation is strongly associated with MI risk in humans. Moreover, therapeutic interventions that inhibit platelet activation, such as aspirin and clopidigrel, decrease risk of atherothrombotic events.47 Association of the 9p21.3 adverse outcomes/MI block with platelet reactivity suggests an underlying mechanism for risk of disease.11,12 Although most treatment regimens focus on antiplatelet drugs, there is increasing evidence suggesting that therapies directed at abnormal hematopoiesis, including excessive platelet production, may be beneficial in preventing atherothrombosis/MI.39 The increased platelet production, platelet activation,

Figure 5. Mixed platelet (PLT):leukocyte aggregates in the circulation of Western-type diet-fed mice. A, Platelet:neutrophil aggregates. B, Platelet:monocyte aggregates. C, Platelet:Ly-6C+ monocyte aggregates. D, Platelet:Ly-6C+ monocyte aggregates. One-way analysis of variance (ANOVA; A–D).
Figure 6. Increased thrombin/antithrombin (T-AT) complex and decreased tail vein bleeding times in Western-type diet–fed Cdkn2a-deficient strains. A, T-AT complex measured in sodium citrate plasma by commercially available ELISA. B, Time to cessation of tail vein bleeding after tail clip injury. One-way ANOVA (A and B).

and increased T-AT complex phenotype of hypercholesterolemic Cdkn2a-deficient mice suggests a prothrombotic phenotype. Although mouse models of atherosclerosis are highly resistant to thrombosis, vascular injury models have been used to verify gene-specific effects on thrombosis that are often relevant to human thrombosis. Together, these data may provide a causal explanation, at least in part, for the association of 9p21.3 and MI.

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Disclosures

None.

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