LNK/SH2B3 Loss of Function Promotes Atherosclerosis and Thrombosis

Wei Wang, Yang Tang, Ying Wang, Liana Tascau, Joanna Balcerek, Wei Tong, Ross L. Levine, Carrie Welch, Alan R. Tall,* Nan Wang*

Rationale: Human genome-wide association studies have revealed novel genetic loci that are associated with coronary heart disease. One such locus resides in LNK/SH2B3, which in mice is expressed in hematopoietic cells and suppresses thrombopoietin signaling via its receptor myeloproliferative leukemia virus oncogene. However, the mechanisms underlying the association of LNK single-nucleotide polymorphisms with coronary heart disease are poorly understood.

Objective: To understand the functional effects of LNK single-nucleotide polymorphisms and explore the mechanisms whereby LNK loss of function impacts atherosclerosis and thrombosis.

Methods and Results: Using human cord blood, we show that the common TT risk genotype (R262W) of LNK is associated with expansion of hematopoietic stem cells and enhanced megakaryopoiesis, demonstrating reduced LNK function and increased myeloproliferative leukemia virus oncogene signaling. In mice, hematopoietic Lnk deficiency leads to accelerated arterial thrombosis and atherosclerosis, but only in the setting of hypercholesterolemia. Hypercholesterolemia acts synergistically with LNK deficiency to increase interleukin 3/granulocyte-macrophage colony-stimulating factor receptor signaling in bone marrow myeloid progenitors, whereas in platelets cholesterol loading combines with Lnk deficiency to increase activation. Platelet Lnk deficiency increases myeloproliferative leukemia virus oncogene signaling and AKT activation, whereas cholesterol loading decreases SHIP-1 phosphorylation, acting convergently to increase AKT and platelet activation. Together with increased myelopoiesis, platelet activation promotes prothrombotic and proatherogenic platelet/leukocyte aggregate formation.

Conclusions: LNK (R262W) is a loss-of-function variant that promotes thrombopoietin/myeloproliferative leukemia virus oncogene signaling and platelet and leukocyte production. In mice, LNK deficiency is associated with both increased platelet production and activation. Hypercholesterolemia acts in platelets and hematopoietic progenitors to exacerbate thrombosis and atherosclerosis associated with LNK deficiency. (Circ Res. 2016;119:e91-e103. DOI: 10.1161/CIRCRESAHA.116.308955.)

Key Words: atherosclerosis ■ cholesterol ■ hematopoiesis ■ hypercholesterolemia ■ thrombosis

In human epidemiological studies, increased monocyte, neutrophil, and platelet counts predict risk of myocardial infarction and thrombotic stroke.1–3 Augmented production of platelets and myeloid cells, formation of platelet–leukocyte aggregates, and increased generation of platelet and leukocyte inflammatory mediators4 are potential underlying mechanisms that contribute to accelerated atherothrombotic disease. Platelet–leukocyte interactions are thought to trigger a series of events that contribute to the inflammatory reaction of the vessel wall and promotion of atherogenesis and thrombosis.4

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LNK (also called SH2B3) is a member of the SH2B family of adaptor proteins primarily expressed in hematopoietic and endothelial cells.5 In hematopoietic cells, LNK functions as a negative regulator of cytokine signaling and cell proliferation.5,6 Rare loss-of-function mutations in LNK give rise to myeloproliferative neoplasms characterized by platelet and leukocyte overproduction.7–9 Targeted deletion of LNK in mice causes expansion of...
hematopoietic stem cells (HSC), increased myelopoiesis, megakaryopoiesis, thrombocytosis, and leukocytosis,\(^5,10,11\) suggesting lack of negative feedback regulation of thrombopoietin (TPO) and myeloproliferative leukemia virus oncogene (MPL) signaling.\(^5,13\) In human genome-wide association study, the LNK single-nucleotide polymorphism (SNP), rs3184504, causing a missense mutation at position 262 (p.R262W, c.784T>C), is associated with increased risk of coronary heart disease (CHD)\(^12\); the same SNP is also associated with increased platelet counts and leukocytosis,\(^13\) which are risk factors for CHD.\(^11,12,18\) Furthermore, increased hematopoietic stem cells and megakaryocytes in bone marrow (BM) and increases in blood platelets and leukocytes,\(^5,10,11\) suggesting lack of negative feedback regulation of thrombopoietin (TPO) and myeloproliferative leukemia virus oncogene (MPL) signaling,\(^5,13\) have been described in a recent study,\(^21\) which quantified the allelic ratio of TT versus CC individuals, whereas \(^{LNK/SH2B3}\) mRNA levels were reproducibly higher in association with TT (Online Figure II). TPO/MPL signaling increases LNK transcription in human hematopoietic cells.\(^20\) Because we detected increased TPO/MPL signaling in hematopoietic cells of the TT genotype (Figure 1D and 1E), the increase in LNK mRNA may be explained by LNK loss of function and disruption of a LNK/MPL negative feedback loop by the LNK R262W variant.

We also performed studies to assess alternative possibilities that LNK mRNA levels were increased because of distinct regulation of the T allele–specific expression by cis-regulatory or post-transcriptional regulatory effects. This was based on a novel approach described in a recent study,\(^21\) which quantified the allelic ratio of TT versus CC individuals, whereas \(^{LNK/SH2B3}\) mRNA levels were reproducibly higher in association with TT (Online Figure II). TPO/MPL signaling increases LNK transcription in human hematopoietic cells.\(^20\) Because we hypothesized that the increased LNK mRNA levels associated with T allele is because of increased transcription as a result of feedback regulation to compensate for the functional deficiency of LNK, we expect this regulatory impact to be equal for both T- and C-specific LNK alleles in heterozygous samples. Thus, a 1:1 allele-specific expression ratio is expected from these samples despite an increase in total LNK mRNA level. A ratio different from 1:1 would suggest allele-specific cis-regulatory effects or allele-specific post-transcriptional regulation and refute our hypothesis. We first showed, using samples with fixed ratios of TT or CC cDNAs, that the measured allele-specific expression of the 2 alleles closely correlated with the expected ratio (Online Figure IIIa), validating this assay. Then, we assessed the relative T- or C-allele–specific expression of the heterozygous samples, and the ratio of the allele-specific expression was determined. The average of this ratio was indeed 1:1 (Online Figure IIIb), consistent with our hypothesis. Together, these findings show for the first time that LNK acts as a brake on TPO/MPL signaling in humans and suggest that R262W causes a functional defect of LNK that leads to increased TPO/MPL signaling.

**Methods**

Full Methods are provided in the Online Data Supplement.

**Results**

**The TT Risk Allele of LNK Increases Human HSC and Megakaryopoiesis**

To assess the potential impact of rs3184504 on hematopoietic functions, we characterized the HSC compartment in human cord blood samples based on LNK genotype. This revealed a significant increase in the HSC-containing fraction (CD90\(^+\)CD45RA\(^-\))\(^14\) in subjects carrying the TT risk genotype (Figure 1A through 1C). Using phospho flow cytometry, we showed increased phosphorylation of STAT5 and ERK1/2, but not AKT, in response to TPO in TT HSCs (Figure 1D and 1E; Online Figure I), suggesting that HSCs were increased as a result of increased TPO/MPL signaling. Moreover, the number of megakaryocyte colonies derived from the cord blood CD34\(^+\) cells and the overall megakaryocyte counts per colony were increased in association with the TT genotype (Figure 1F through 1H), indicating increased megakaryopoiesis. Because LNK functions as a negative regulator of cytokine signaling in hematopoietic cells, including TPO/MPL signaling, these findings suggest that the T allele is associated with reduced LNK function, which could result from altered protein structure/activity or decreased expression of LNK in hematopoietic stem and progenitor cells (HSPCs) and megakaryocyte progenitors (MkPs). R262 is not conserved in mice, and overexpression studies with LNK R262W in cell lines have not been informative.\(^2\) Thus, an expression quantitative trait loci at rs3184504 or another SNP in linkage disequilibrium (LD) with rs3184504 could be responsible for reduced LNK function. We assessed LNK mRNA levels in cord blood CD34\(^+\) cells. Paradoxically, LNK mRNA levels were increased in subjects with the TT genotype (Figure 1I), which is inconsistent with an expression quantitative trait loci causing reduced LNK activity via a reduction in LNK mRNA. ATXN2, PTEN11, and TRAFD1 are neighboring genes of LNK/SH2B3 on chromosome 12, and there is evidence of LD of rs3184504 with SNPs in these genes.\(^19\) Thus, we measured ATXN2, PTEN11, and TRAFD1 mRNA levels in a new set of cord blood CD34\(^+\) cells but found no significant difference between TT versus CC individuals, whereas LNK/SH2B3 mRNA levels were reproducibly higher in association with TT (Online Figure II). TPO/MPL signaling increases LNK transcription in human hematopoietic cells.\(^20\) Because we detected increased TPO/MPL signaling in hematopoietic cells of the TT genotype (Figure 1D and 1E), the increase in LNK mRNA may be explained by LNK loss of function and disruption of a LNK/MPL negative feedback loop by the LNK R262W variant.

**LNK Loss of Function Interacts With Hypercholesterolemia to Promote Myelopoiesis**

Although R262 is not conserved in mice, Lnk\(^{-/-}\) mice show increased hematopoietic stem cells and megakaryocytes in bone marrow (BM) and increases in blood platelets and leukocytes,\(^5,10,11\) similar to the effects of the T allele in humans. Thus, Lnk\(^{-/-}\) mice

### Nonstandard Abbreviations and Acronyms

<table>
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<tr>
<th>Abbreviation</th>
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<tr>
<td>BM</td>
<td>bone marrow</td>
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<tr>
<td>CBs</td>
<td>common-beta subunit</td>
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<td>CHD</td>
<td>coronary heart disease</td>
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<tr>
<td>HSC</td>
<td>hematopoietic stem cell</td>
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<td>HSPC</td>
<td>hematopoietic stem and progenitor cell</td>
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<td>Lnk−/−</td>
<td>lymphocyte specific adapter protein</td>
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<tr>
<td>MkP</td>
<td>megakaryocyte progenitor</td>
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<tr>
<td>MPL</td>
<td>myeloproliferative leukemia virus oncogene</td>
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<tr>
<td>SHIP-1</td>
<td>SH2 domain-containing inositol phosphatase 1</td>
</tr>
<tr>
<td>TPO</td>
<td>thrombopoietin</td>
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<tr>
<td>WT</td>
<td>wild type</td>
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<td>WTD</td>
<td>Western type diet</td>
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Nonstandard Abbreviations and Acronyms
seem to be a suitable model to assess the effects of reduced LNK function on cardiovascular disease. Humans have much higher levels of low-density lipoprotein cholesterol than mice, and some studies have suggested that increased low-density lipoprotein cholesterol increases platelet reactivity. We therefore assessed the impact of LNK deficiency on hematopoietic functions, thrombosis, and atherosclerosis in both normo- and hypercholesterolemic backgrounds. We transplanted BM from wild-type (WT) or $\text{Llk}^{-/-}$ mice into irradiated WT or $\text{Ldlr}^{-/-}$ mice, followed by feeding either a chow diet (normocholesterolemic WT recipients) or Western type diet (WTD; hypercholesterolemic $\text{Ldlr}^{-/-}$ recipients) for 12 weeks.

Similar to chow-fed $\text{Llk}^{-/-}$ mice, $\text{Llk}^{-/-}$ BM recipients displayed expansion of hematopoietic stem and progenitor cells (HSPCs, defined as $\text{Lin}^{-}\text{Sca-1}^+\text{c-Kit}^+$ LSK cells), myeloid and MkPs in BM (Figure 2A), and monocytosis and neutrophilia on both diets (Figure 2B; Online Figure IV). Interestingly, hypercholesterolemic $\text{Llk}^{-/-}$ BM recipients also showed markedly more pronounced neutrophilia and monocytosis compared with WT or chow-fed $\text{Llk}^{-/-}$ recipients (Figure 2B), reflecting an increase in the BM LSK and GMP (granulocyte-macrophage progenitor) populations (Figure 2A).

This suggested an interaction of LNK deficiency with hypercholesterolemia to expand BM myeloid progenitors. There is evidence that LNK negatively regulates interleukin 3 signaling. Moreover, cholesterol accumulation in HSPCs has been associated with expansion of HSPCs and increased signaling via the interleukin 3/granulocyte-macrophage colony-stimulating factor receptor because of increased levels of the common-beta subunit (CBS) of this receptor on the cell surface. CBS deletion reversed HSPC expansion and the associated leukocytosis. Cell surface CBS levels were decreased in HSPCs in chow-fed $\text{Llk}^{-/-}$ BM recipients (Figure 2C), consistent with increased interleukin 3/granulocyte-macrophage colony-stimulating factor signaling and feedback downregulation of the CBS as a result of disrupted negative feedback regulation by LNK. This decrease in CBS was reversed in HSPCs, and the CBS levels were further increased in $\text{Llk}^{-/-}$ GMPs in the setting of hypercholesterolemia (Figure 2C). Consistent with increased CBS levels, hematopoietic cell signaling in response to interleukin 3 or granulocyte-macrophage colony-stimulating factor treatment, using phosphorylated STAT5 or ErK1/2 as the readout, was increased by the combination of LNK deficiency and hypercholesterolemia (Figure 2D). These findings explain at least in part the marked increase in neutrophilia and monocytosis in hypercholesterolemic mice with hematopoietic LNK deficiency.

Figure 1. Association of the LNK TT risk single-nucleotide polymorphism (SNP) with hematopoietic stem cell (HSC) expansion and increased megakaryopoiesis. Flow cytometry overview of HSCs (Lin$^{-}\text{CD34}^+\text{CD38}^{-}\text{CD90}^+\text{CD45RA}^-$) (A) and HSC percentage in CD34$^+$ (B) or Lin$^{-}\text{CD34}^+\text{CD38}^{-}$ (C) cord blood cell fractions. p-STAT5 (D) and p-ERK1/2 (E) levels in CD34$^+$ cells. Megakaryocyte (Mk) colony number (F) and Mk counts per colony (H). Normalized $\text{Llk}$ mRNA levels in CD34$^+$ cells (I). J, Allele-specific LNK expression of human cord blood CD34$^+$ cells. AU indicates arbitrary unit; FSC, forward-scattered light; MFI, median fluorescence intensity; and SSC, side-scattered light.
Loss of Function Interacts With Hypercholesterolemia to Increase Platelet Activation

Platelet/leukocyte aggregates are thought to have an important role in promoting both atherosclerosis and thrombosis in states of leukocyte and platelet overproduction as exemplified in myeloproliferative neoplasms.27 Platelet/leukocyte aggregates were markedly increased by the combination of hypercholesterolemia and hematopoietic LNK deficiency, involving both increased platelet/neutrophil and increased platelet/monocyte aggregates (Figure 2E). In contrast to leukocytosis, thrombocytosis in Lnk−/− BM recipients was similar in both normo- and hypercholesterolemic backgrounds (Figure 2B), paralleling similar levels of the BM MKP population (Figure 2A). Formation of platelet/leukocyte aggregates is promoted by platelet activation.28 Thus, we further assessed parameters of platelet function. In the resting state, Lnk−/− platelets from either normo- or hypercholesterolemic mice did not show significant alteration of surface presentation of P-selectin or active integrin β3/αIIb, markers of platelet activation, relative to the WT BM recipients (Figure 3A and 3B). However, Lnk−/− platelets from hypercholesterolemic mice showed markedly increased surface exposure of these molecules when activated by PAR4 (protease-activated receptor 4) agonist AYPGKF, whereas Lnk−/− platelets from normocholesterolemic mice showed similar activation (Figure 3A and 3B), indicating an interaction of LNK deficiency with hypercholesterolemia to promote platelet activation. To further assess the impact of hypercholesterolemia on WT or Lnk−/− platelets, we performed a time course study. Because the mice on the WTD developed progressive hypercholesterolemia (Online Figure V), there was a marked increase in cell surface P-selectin and active integrin β3/αIIb.
Figure 3. Hypercholesterolemia markedly increases platelet reactivity in Lnk−/− bone marrow (BM) recipient. Surface P-selectin (A) and active integrin αIIbβ3 (JON/A; B) levels on washed platelets with or without PAR4 (protease-activated receptor 4) agonist (AYPGKF, 100 μmol/L) stimulation (n=5). Surface P-selectin (C) and active integrin αIIbβ3 (JON/A; D) levels on platelets in whole blood with or without AYPGKF (100 μmol/L) stimulation (n=5). Washed platelet aggregation on AYPGKF (100 μmol/L; E) or ADP (20 μmol/L; F) stimulation (n=4–5). Thrombopoietin (TPO) receptor (Mpl) levels on platelet surface (G), platelet TPO internalization (H), and plasma TPO levels (I) in wild-type (WT) or Lnk−/− BM recipient mice (n=3–7). For (A–F), black bars represent chow feeding, and green bars represent Western type diet (WTD) feeding for 12 wk. *, **, and *** denote P<0.05, P<0.01, and P<0.001 for WTD-fed Lnk−/− vs WT or chow-fed Lnk−/− vs WT. ^, ^^, and ^^^ denote P<0.05, P<0.01, and <0.001 for chow-fed Lnk−/− vs WTD-fed Lnk−/− or chow-fed WT vs WTD-fed WT. C-Mpl indicates myeloproliferative leukemia virus oncogene.
α_IIbβ_3 in response to PAR4 activation in \textit{Lnk}^{-/-} platelets but not in WT platelets (Figure 3C and 3D). Similarly, aggregation of \textit{Lnk}^{-/-} platelets in response to PAR4 agonist or ADP was markedly increased only when mice became hypercholesterolemic (Figure 3E and 3F; Online Figure VI). Together, these findings indicate a profound interaction of the \textit{Lnk}^{-/-} genotype with hypercholesterolemia to increase platelet activation.

We next considered that there might be a cell-intrinsic effect of cholesterol enrichment of LNK-deficient platelets. We found increased cell surface MPL levels in \textit{Lnk}^{-/-} platelets (Figure 3G), reflecting a defect in internalization of MPL (Figure 3I), suggesting that LNK negatively regulates MPL signaling in part by promoting MPL internalization. Plasma TPO levels were increased in mice with hematopoietic LNK deficiency (Figure 3H), likely reflecting the decreased internalization and turnover of MPL and TPO in platelets. Hepatic, splenic, or BM TPO mRNA levels showed no difference between wild-type and \textit{Lnk}^{-/-} and TPO in platelets. Hepatic, splenic, or BM TPO mRNA levels showed no difference between wild-type and \textit{Lnk}^{-/-} mice, with or without hypercholesterolemia (Online Figure VII). This novel observation suggests that in addition to the known cell-intrinsic effects of LNK deficiency in HSCs, increased plasma TPO levels may contribute to HSC expansion and megakaryopoiesis in hematopoietic LNK deficiency. However, the increase in plasma TPO levels was similar in normocholesterolemic and hypercholesterolemic mice, consistent with similar numbers of MKPs and platelets in these mice. Thus, although an increase in plasma TPO levels likely promoted platelet production and an increase in platelet MPL might lead to increased MPL signaling and priming of platelets, this did not explain the effects of hypercholesterolemia on platelet activation.

**Cholesterol Modulates \textit{Lnk}^{-/-} Platelet Activation**

We next undertook experiments to determine whether cholesterol enrichment or depletion acted directly in platelets to alter their activation. Although the WTD increased total cholesterol mass in platelets from both WT and \textit{Lnk}^{-/-} mice, there was no difference in total and free cholesterol content or in plasma membrane free cholesterol content as assessed by filipin staining and flow cytometry between platelets of the 2 genotypes (Online Figure VIII through ViliiC). To model the effect of hypercholesterolemia-induced cholesterol accumulation in platelets of either genotype, we cholesterol loaded platelets from chow-fed WT mice that had been transfected with WT or \textit{Lnk}^{-/-} BM, using cholesterol-rich liposomes.\(^{50}\) Cholesterol loading increased both WT and \textit{Lnk}^{-/-} platelet P-selectin exposure after PAR4 activation, but the increase was more pronounced and occurred at a lower threshold in \textit{Lnk}^{-/-} platelets (Figure 4A). Increased cell surface levels of P-selectin in \textit{Lnk}^{-/-} platelets indicated increased platelet degranulation. Protein kinase C (PKC)-mediated signaling is a major pathway downstream of PAR4 activation, regulating platelet degranulation.\(^{30}\) An increase in PKC activation after PAR4 activation was observed in \textit{Lnk}^{-/-} platelets only after cholesterol loading, providing an explanation for the synergistic effect of platelet LNK deficiency and cholesterol loading on P-selectin exposure (Figure 4B). Consistently, PKC activation in response to PAR4 agonist AYPEGKF\(^{50}\) was much more pronounced in \textit{Lnk}^{-/-} platelets relative to WT platelets from WTD-fed \textit{Ldlr}^{-/-} recipients, but the difference was less in platelets from chow-fed WT recipients (Online Figure IX). To assess the effects of cholesterol removal, we treated platelets with cholesterol-poor reconstituted high-density lipoprotein (HDL), which has been shown to promote cholesterol efflux and decrease platelet activation in diabetic humans.\(^{41}\) HDL treatment decreased P-selectin exposure in response to PAR4 activation in both \textit{Lnk}^{-/-} and WT platelets and abolished their differential response (Figure 4C). The heightened \textit{Lnk}^{-/-} platelet aggregation induced by PAR4 activation was reversed by HDL treatment (Figure 4D). We also used cyclodextrin, which nonspecifically promotes cellular cholesterol efflux.\(^{52}\) Cyclodextrin, like HDL, reversed elevation of P-selectin exposure and platelet aggregation in LNK deficiency (Figure 4E and 4F). Consistent with the reversal of \textit{Lnk}^{-/-} platelet hyper-reactivity by HDL, HDL also significantly reduced serine phosphorylation of PKC protein substrates in response to PAR4 activation in WT and \textit{Lnk}^{-/-} platelets (Figure 4G). Together, these findings indicate that, in hypercholesterolemic mice with LNK deficiency, increased platelet cholesterol content heightens the response to PAR4 activator, leading to increased PKC signaling, P-selectin exposure, and integrin α_IIbβ_3 activation.

**LNK Loss of Function and Cholesterol Enrichment Increase Platelet AKT Activation**

STAT5, ERK1/2, and AKT are the major downstream signaling pathways in TPO/MPL signaling.\(^{53}\) Consistent with increased TPO/MPL signaling, basal p-STAT5, p-ERK1/2, and p-AKT levels were increased in \textit{Lnk}^{-/-} platelets from the chow- or WTD-fed mice (Figure 5A). Interestingly, hypercholesterolemia further increased p-AKT but not p-STAT5 or p-ERK1/2 levels (Figure 5A; Online FigureXA), suggesting convergent effects of LNK deficiency and hypercholesterolemia on AKT activation. AKT has a critical role in regulating platelet degranulation and aggregation.\(^{54}\) PAR4 activation increases AKT phosphorylation and activation, and AKT also is an important node in the TPO/MPL signaling pathway.\(^{33,35}\) Thus, we next focused on assessing the role of AKT in the heightened activation of \textit{Lnk}^{-/-} platelets from hyperlipidemic mice.

AKT phosphorylation induced by PAR4 activation was increased in both WT and \textit{Lnk}^{-/-} platelets from hypercholesterolemic mice compared with normocholesterolemic mice, with a more pronounced effect in the \textit{Lnk}^{-/-} platelets (Figure 5B; Online Figure XA). Cholesterol-loading increased AKT phosphorylation in response to PAR4 activation in both WT and \textit{Lnk}^{-/-} platelets (Figure 5C), with a more pronounced effect in \textit{Lnk}^{-/-} platelets (Figure 5C). Conversely, removal of cholesterol from platelets by HDL reversed the increased AKT phosphorylation associated with hypercholesterolemia (Figure 5F). To assess the functional impact of increased AKT phosphorylation and activation on platelets, WT or \textit{Lnk}^{-/-} platelets from the chow-fed WT or WTD-fed \textit{Ldlr}^{-/-} recipient were pretreated with ruxolitinib, a JAK2 inhibitor,\(^{16}\) or an AKT inhibitor, MK2206, followed by PAR4 activation. JAK2 acts upstream of AKT in TPO/MPL signaling.\(^{13}\) The increased exposure of surface P-selectin in \textit{Lnk}^{-/-} platelets from the hypercholesterolemic recipient was reversed by inhibition of JAK2 or AKT (Figure 5D), indicating a critical role of AKT in the heightened platelet activation.

PI3K mediates AKT activation by generating PIP(3,4,5)P3, whereas SHIP-1 reduces AKT activation by converting PIP(3,4,5)P3 to PIP(3,4)P2.\(^{37}\) SHIP-1 phosphorylation was increased in \textit{Lnk}^{-/-} versus WT platelets from normocholesterolemic mice (Figure 5E; Online Figure XB), consistent with increased MPL signaling and activation of a negative feedback effects on AKT.
phosphorylation; however SHIP-1 phosphorylation was markedly decreased in both WT and Lnk−/− platelets from hypercholesterolemic mice, relative to the normocholesterolemic mice. Ex vivo cholesterol loading of platelets from normocholesterolemic WT or Lnk−/− BM recipient mice also reduced SHIP-1 phosphorylation (Online Figure XI). Together, these results suggest that cholesterol enrichment increases AKT phosphorylation and activation by decreasing SHIP-1 phosphatase activity.

Src family kinases including LNY kinase mediate SHIP-1 tyrosine phosphorylation and activation. We showed...
previously that cellular cholesterol accumulation in MkPs inhibited LYN kinase activation and increased AKT phosphorylation and activation in response to TPO.40 Thus, we assessed the potential role of LYN kinase in SHIP-1 and AKT signaling in platelets. Tolimidone, a LYN kinase activator,41 markedly reduced AKT phosphorylation in response to PAR4 activation in \( \text{Lynkd}^{\text{kd}} \) platelets from the hypercholesterolemic mice (Figure 7F; Online Figure XII). This idea was further assessed in genetic models expressing a kinase-dead mutant of LYN (\( \text{Lynkd}^{\text{kd}} \)).42 SHIP-1 phosphorylation was increased in response to PAR4 activation in platelets (not shown), but the increase was markedly reduced in \( \text{Lynkd}^{\text{kd}} \) platelets (Figure 5G; Online Figure XIII). Together, these results indicate a rate limiting role of LYN kinase in mediating SHIP-1 phosphorylation in platelets. The functional impact on platelet activation was also assessed: \( \text{Lynkd}^{\text{kd}} \) platelets showed increased P-selectin exposure and integrin \( \alpha_{\text{IIb}} \beta_{3} \) activation in response to PAR4 activation (Figure 5H). Importantly, the priming effect of cholesterol enrichment was abolished in \( \text{Lynkd}^{\text{kd}} \) platelets (Figure 5I), suggesting that cholesterol enrichment acts to reduce LYN kinase activity, which decreases SHIP-1 phosphorylation and increases AKT activation in response to PAR4 agonists.

Figure 5. LNK loss of function and cholesterol enrichment increase platelet AKT activation. A, Thrombopoietin (TPO)/myeloproliferative leukemia virus oncogene (Mpl) signaling in resting platelets of wild-type (WT) and Lnk\(^{-/-}\) bone marrow (BM) recipient mice without TPO treatment. B, AKT activity in platelets from WT and Lnk\(^{-/-}\) BM recipient mice with and without platelet agonist AYPGKF stimulation. C, Effect of ex vivo cholesterol loading on AKT activity from platelets of chow-fed mice. D, Effect of Jak2 and AKT inhibitors on platelets of WT and Lnk\(^{-/-}\) BM recipient mice (n=4). E, p-SHIP1 level in platelets of WT and Lnk\(^{-/-}\) BM recipient mice with and without AYPGKF stimulation. F, Change of AKT activity after high-density lipoprotein (HDL) and LYN tyrosine kinase activator tolirimidone treatment in Lnk\(^{-/-}\) platelets from Western type diet (WTD)–fed mice. G, p-SHIP1 levels in WT and \( \text{Lynkd}^{\text{kd}} \) platelets on AYPGKF stimulation. H, Platelet activity of \( \text{Lynkd}^{\text{kd}} \) mice on AYPGKF stimulation. I, Cholesterol-loading effect on platelet activity of \( \text{Lynkd}^{\text{kd}} \) mice. MFI indicates median fluorescence intensity.
Together, these studies suggest that LNK deficiency increases AKT activation by enhancing TPO/MPL signaling, whereas cholesterol enrichment increases AKT activation by reducing LYN kinase and SHIP-1 activation. Thus, LNK deficiency and cholesterol enrichment act independently but converge on AKT to promote platelet activation.

**LNK Loss of Function Promotes Thrombosis and Atherosclerosis**

Increased platelet reactivity in association with thrombocytosis in the hypercholesterolemic *Lnk−/−* BM recipients would be expected to accelerate thrombosis. A previous study suggested that LNK functions to stabilize thrombus formation and LNK deficiency retards arterial thrombosis; notably, however, this study was conducted in normocholesterolemic mice.43 Mice do not develop spontaneously ruptured atherosclerotic plaques giving rise to atherothrombosis. Thus, to evaluate the in vivo impact of LNK deficiency on arterial thrombosis in our hypercholesterolemic model, we used FeCl₃-induced carotid artery thrombosis.44 Carotid artery occlusion was markedly accelerated in the WTD-fed *Lnk−/−* BM recipients relative to controls (Figure 6A). Consistently, tail vein bleeding time was significantly shortened compared with controls (Figure 6B). The accelerated thrombosis in WTD-fed *Lnk−/−* BM recipients could be the consequence of thrombocytosis or platelet hyperreactivity or both. Next, we assessed carotid artery thrombosis in chow or WTD-fed WT or *Lnk−/−* BM recipients. On the chow diet, LNK deficiency did not significantly alter thrombosis, although the chow-fed *Lnk−/−* BM recipients showed marked thrombocytosis (Figure 6C). In contrast, the induced thrombosis was significantly accelerated in the WTD-fed *Lnk−/−* recipients (Figure 6C). These results suggest that the hyper-reactivity of cholesterol-loaded *Lnk−/−* platelets likely has a major role in the accelerated arterial thrombosis in vivo.

Activated platelets, monocytes, neutrophilia, and platelet–leukocyte aggregates all promote atherogenesis.44–47 To assess atherogenesis, a similar BM transplantation protocol to that described above was used; however, normocholesterolemic controls were not included because they do not develop atherosclerosis. Plasma non-HDL and HDL lipoprotein cholesterol levels were similar in the WTD-fed *Ldlr−/−* mice receiving WT or *Lnk−/−* BM (not shown). Hematopoietic LNK deficiency in combination with angiotensin II injection increases blood pressure.48 However, systolic and diastolic blood pressure in WTD-fed *Lnk−/−* and WT BM recipients showed no difference (Online Figure XIV), likely because angiotensin II injection was required to show increased blood pressure in LNK deficiency.48 We also measured blood insulin and glucose levels from the fasting mice that showed no difference between chow- or WT-fed WT and *Lnk−/−* mice (Online Figure XV). Atherosclerotic lesion size was significantly increased in aortic roots of the *Ldlr−/−* mice receiving *Lnk−/−* BM (Figure 7A), after feeding the WTD for 10 weeks. A separate study showed larger lesions and a trend to larger size in the *Lnk−/−* BM recipient after feeding WTD for 12 weeks (P=0.06; Figure 7A). The latter was confirmed as aortic arch oil-red O stained en face lesion area was significantly increased in *Ldlr−/−* mice receiving *Lnk−/−* BM relative to WT BM after feeding WTD for 16 weeks (P<0.01). We also assessed necrotic core area, which is thought to be an index of plaque stability. Necrotic core area was markedly increased both in the 10- and 12-week samples (Figure 7B and 7C). These results indicate that hematopoietic LNK deficiency accelerates atherogenesis.

The presence of neutrophilia, monocytosis, and increased platelet–monocyte or platelet–neutrophil aggregates suggested increased neutrophil and monocyte recruitment into the lesion. Moreover, plasma levels of MCP-1, a potent chemokine for monocytes,49 were increased in WTD-fed *Lnk−/−* relative to WT BM recipients (Online Figure XVI). We assessed Ly6C⁺ monocyte recruitment into atherosclerotic lesions in *Lnk−/−* versus WT BM recipients.48 Ly6C⁺ monocyte recruitment was significantly increased in hypercholesterolemic *Lnk−/−* BM recipients compared with controls (Figure 7D). Consistently, lesional macrophages were markedly increased in *Lnk−/−* BM recipients (Figure 7E). Lesional neutrophils were also increased in *Lnk−/−* BM recipients (Figure 7F). Although circulating T-cell counts were markedly increased in *Lnk−/−* BM recipients (Online Figure IV), the number of lesional T cells was not altered (Online Figure XVII).

**Discussion**

Human genome-wide association studies have revealed many genetic loci associated with CHD. Although many of these loci act by increasing low-density lipoprotein cholesterol or triglyceride level, the majority act through unknown mechanisms.12,51 By analyzing human cord blood samples, we found that a common genetic variant in LNK that has been linked

![Figure 6. LNK deficiency accelerates thrombosis. FeCl₃-induced carotid artery thrombotic occlusion (A) or tail vein bleeding (B) in wild-type (WT)→*Ldlr−/−* vs *Lnk−/−*→*Ldlr−/−* recipients fed Western type diet (WTD) for 10 wk. C, FeCl₃-induced carotid artery thrombotic occlusion in chow-fed (black symbols) or WTD-fed (green symbols) recipient mice (n=7–8).](http://circres.ahajournals.org/)

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to CHD in human genome-wide association study is associated with HSC expansion, increased TPO/MPL signaling, and increased megakaryopoiesis, suggesting that the T allele is a loss-of-function variant that promotes platelet production and myelopoiesis. This likely explains the association of LNK with leukocyte and platelet counts. Our studies in mice provide the first direct evidence that Lnk loss of function promotes atherogenesis and arterial thrombosis and thus suggest that LNK acts at least in part through a similar mechanism in humans. Accelerated atherosclerosis, thrombosis, and hematopoietic abnormalities associated with Lnk deficiency were markedly exacerbated by hyperlipidemia, reflecting increased activation of Lnk−/− platelets by cholesterol loading and enhanced myelopoiesis. Increased MPL signaling in LNK-deficient platelets and reduced SHIP-1 activity because of hypercholesterolemia combined to increase AKT activation and enhance the response of platelets to activation signals.

Although rs3184504 is associated with an increased risk of cardiovascular disease, whether R262W LNK is a causal variant has been contentious. This SNP seems to have arisen 1000 to 1500 years ago in European populations as part of a large LD block that may have endowed an enhanced immune response and protection from infections such as the plague at the expense of increased susceptibility to autoimmune and cardiovascular diseases. SNPs in multiple genes within this LD block are associated with CHD. This leaves open the question of whether rs3184504 is in LD with other SNP(s), which alter expression of several genes in the region. A recent study suggested that phenotypic associations of rs3184504 could be mediated through altered expression of the neighboring gene ATXN2. However,
in another study,51 the authors analyzed 1000 Genomes CEU and ENCODE databases and showed that no other SNPs in LD with rs3184504 [T] would cause nonsynonymous amino acid changes or were associated with an expression quantitative trait loci. Moreover, we showed that the other genes in this region that are expressed in hematopoietic tissues, that is, ATXN2, PTPN11, TRAFD119 did not show altered expression in cord blood CD34+ cells, whereas LNK mRNA was increased. Moreover, because LNK acts to inhibit hematopoietic functions,6 including in the cord blood samples we analyzed, the directionality of change was opposite to that expected for an expression quantitative trait loci effect. This was explained by our functional studies, which showed increased TPO/MPL signaling and megakaryopoiesis in association with rs3184504 [T], indicating reduced LNK inhibition of hematopoiesis. Because TPO/MPL signaling increases LNK gene expression,20 our findings suggest that the increase in LNK mRNA is secondary to reduced LNK function because of an amino acid change. Although our study shows several similarities between LNK(R262W) in humans and LNK deficiency in mice, including increased TPO signaling, HSC expansion, increased myelopoiesis, megakaryopoiesis and accelerated atherosclerosis, and arterial thrombosis, the effects on hematopoiesis seem to be more pronounced in Lnk−/− mice, suggesting that human LNK(R262W) may represent a partial loss of function.

The increase in atherosclerosis and arterial thrombosis in hypercholesterolemic mice with hematopoietic LNK deficiency is likely explained in part by the increase in leukocytes, enhanced platelet activation, and increased platelet/leukocyte aggregates. Although our study does not differentiate among these different factors, there is considerable evidence to implicate each of these mechanisms in accelerated atherosclerosis and thrombosis.46,50,54,55 Infusion of activated platelets into Apoe−/− mice resulted in increased atherosclerosis, reflecting formation of platelet/leukocyte aggregates, which bind to arterial endothelium over atherosclerotic plaques where they release inflammatory chemokines and cytokines and thereby promote the entry of monocytes and neutrophils into the plaque.46,56 The formation of platelet/leukocyte aggregates is mediated by the interaction of platelet P-selectin with P-selectin ligand on leukocytes.57 Thus, the marked increase in platelet P-selectin exposure (Figure 3A) and the increase in circulating leukocytes (Figure 2B) may explain the increase in platelet/leukocyte aggregate formation and the increased entry of monocytes and neutrophils into plaques (Figures 2E, 7D, and 7F). Even though increased activation of platelets ex vivo required exposure to agonists, platelet–leukocyte aggregates were significantly increased in the basal state, likely contributing to accelerated atherogenesis. The marked increase in induced arterial thrombosis likely reflects the enhanced activation of platelets via thrombin generation secondary to tissue factor exposure in the injured vessel.58–60 Although hypertension, erythrocytosis, and hyperglycemia were not present in our hypercholesterolemic Lnk−/− mice, rs3184504 is associated with these risk factors in humans, which likely also contribute to atherothrombosis.

Hyperlipidemia as exemplified by familial hypercholesterolemia is associated with increased platelet activation and an underlying procoagulant state,61,62 likely reflecting multiple underlying mechanisms.62–64 Earlier studies show that in vitro cholesterol-loading increases human platelet activation,29 whereas HDL infusion reduces platelet activation in diabetic subjects, likely by promoting cholesterol efflux from platelets.31 There is also markedly increased platelet activation and thrombosis in Scarb1−/− mice.64,65 Scarb1−/− mice have an unusually high plasma unesterified:total cholesterol ratio, reflecting impaired delivery of cholesterol to the liver.66 Platelet cholesterol overload but not intrinsic SR-B1 (scavenger receptor class B type I) deficiency in platelets is responsible for the heightened platelet activation in Scarb1−/− mice.64 The heightened Lnk−/− platelet activation because of HC is distinct from the Scarb1−/− model in that LNK deficiency does not affect platelet cholesterol content, and moreover, the heightened activation requires intrinsic hematopoietic Lnk deficiency.

Our studies suggest that increased platelet MPL levels and signaling because of LNK deficiency lead to increased AKT activation which, when combined with effects of platelet cholesterol loading to reduce SHIP-1 activation, further increases AKT activation and platelet priming (Figure 7G), which is well known to enhance the response to agonists.39 As a result, there is increased PKC activation in response to agonists such as thrombin, leading to increased platelet degranulation and P-selectin exposure. Moreover, as suggested in an earlier study, LYN kinase may act as a membrane cholesterol sensor in platelets, and our studies suggest that inhibition of LYN kinase in cholesterol-loaded platelets may contribute to reduced SHIP-1 phosphorylation and increased AKT activation (Figure 5F through 5I).

On a therapeutic level, our study suggests that individuals with common genetic variants such as the LNK TT genotype that lead to overproduction of platelets and leukocytes could benefit from early identification and aggressive treatment of low-density lipoprotein cholesterol levels. A recent study showed that individuals with a high genetic risk score for cardiovascular disease (which included rs3184504 in LNK) were 3-fold more likely to benefit from statin therapy compared with those with a low genetic risk score.67 Novel strategies to decrease leukocyte overproduction1 and platelet activation58 such as JAK/STAT or AKT inhibitors,69 or LYN kinase activators, could also reduce the risk of atherothrombotic disease in individuals with the LNK TT variant.

Acknowledgments

We wish to thank Dr Margaret L. Hibbs for kindly providing LynR262W mice for this study.

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Disclosures

Dr Tall is a consultant for Amgen and CSL Behring. The other authors report no conflicts.

References

Novelty and Significance

**What Is Known?**

- Human genome-wide association study have shown that a common single-nucleotide polymorphism in *LNK/SH2B3* (R262W) is associated with leukocytosis, thrombocytosis, and an increased risk of coronary heart disease and stroke.
- LNK inhibition signaling via various cytokine receptors especially in hematopoietic cells suppressing cell proliferation.
- In mice, hematopoietic LNK deficiency leads to increased production of leukocytes and platelets, reflecting increased signaling of thrombopoietin via its receptor (*myeloproliferative leukemia virus oncogene [MPL]*) in hematopoietic stem cells.

**What New Information Does This Article Contribute?**

- Studies using human cord blood hematopoietic stem cells show that the risk single-nucleotide polymorphism reduces LNK function, leading to increased signaling of thrombopoietin via MPL and enhanced megakaryopoiesis.
- Mice with hematopoietic LNK deficiency on an *Ldlr−/−* background display hematopoietic stem cell and myeloid progenitor expansion, increased production of leukocytes, platelets and platelet–leukocyte aggregates, and accelerated arterial thrombosis and atherosclerosis.
- In the setting of hypercholesterolemia, Lnk−/− platelets display increased MPL signaling, which is amplified by the effects of cholesterol loading and reduced SHP-1 activation, leading to increased AKT and platelet priming.

**LNK Deficiency Promotes Atherosclerosis and Thrombosis**

Wang et al


LNK/SH2B3 Loss of Function Promotes Atherosclerosis and Thrombosis
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Supplemental Materials.

Mice, diet and bone marrow transplantation

WT (C57BL/6J) and Ldlr\(^{-/-}\) (B6.129S7-Ldlrtm1Her) mice were purchased from Jackson Laboratory. Bone marrow from WT (C57BL/6J) and Lnk\(^{-/-}\) mice\(^1\) were transplanted into irradiated 7-8 week old female WT (C57BL/6J) or Ldlr\(^{-/-}\) (B6.129S7-Ldlrtm1Her) mice. For atherosclerosis study, female Ldlr\(^{-/-}\) recipient mice were fed a western type diet (TD88137, Harlan Teklad) for the indicated period of time. Lyn\(^{kd/kd}\) mice were generously provided by Dr. Margaret L. Hibbs from Alfred Medical Research and Education Precinct at Monash University.

Histology analysis and immunofluorescent staining of atherosclerotic lesion

Tissues and ascending aorta were serially paraffin sectioned and section were stained with Hematoxylin and eosin (H&E staining) for morphometric lesion analysis as previously described. The aortic lesion size of each mouse was calculated as the mean of the lesion areas in five aortic sections. Identification of macrophages, neutrophils, lymphocytes in atherosclerotic lesions were performed using anti-mouse CD107b (Mac-3) (BD), anti-mouse Ly-6G (BioRad) and anti-mouse CD3 (BioRad) respectively. Primary antibodies were incubated with tissue sections at 4\(^\circ\)C overnight. After three times of washing with PBS, sections were incubated with Alexa-594 or Alexa-488 (Life Technologies) at room temperature for 30 minutes. Sections were counterstained with DAPI to identify nuclei before mounting. Images were taken using Nikon microscope and quantification of the images was performed using Image J.

Monocyte infiltration experiment

To track newly recruited monocytes in atherosclerotic lesions in studying the monocyte infiltration, the Ly-6C\(^{hi}\) subset of monocytes was labeled with fluorescent beads as described previously\(^2\). Briefly, mice were injected intravenously with 250ul clodronate-containing liposome (http://www.clodronateliposomes.com/clodronate-liposomes/clodronate-liposomes) to deplete monocytes 96 hours before harvesting the heart. After 48 hours, mice were injected with Fluoresbrite Plain YG microspheres (polysciences, FITC channel) with a total volume 25oul (1:4 dilution in PBS). After another 48 hours, peripheral blood samples were collected and analyzed by FACS to quantify the efficiency of beads labeling of Ly-6C\(^{hi}\) monocyte. Mice were then euthanized and heart and aortic tissues were process as described previously. The newly recruited beads labeled monocytes in atherosclerotic lesions were visualized by fluorescence microscope and quantified using Image J.
Thrombosis assays: Mouse carotid artery FeCl₃-injury model and tail vein bleeding

For FeCl₃-injury thrombosis assay, mice were anesthetized with isoflurane and the carotid artery was exposed. A filter paper soaked in 5% FeCl₃ was applied to the artery for 3 minutes and then blood flow was monitored with an ultrasound flow probe (Transonics). The time to total occlusion was defined as from the time point when FeCl₃ filter paper was applied to a complete occlusion of the artery with zero blood flow. Under the circumstance when there was no complete occlusion of the vessel or the vessel re-opened after a partial thrombi formation in the lumen, the whole procedure was stopped at 40 minutes and the occlusion time was recorded as 40 minute. After the procedure, all mice were etherized immediately. Tail bleeding assays were performed on Ldlr⁻/⁻ mice transplanted with either WT or Lnk⁻/⁻ bone marrow cells who were fed on western type diet for 10 weeks. Mice were anesthetized with isofluorane with a 5mm segment of the distal tip of the tail was cut off. Tail bleeding times were defined as the time required for the bleeding to stop.

Complete blood count

Complete blood counts were performed using whole blood collected from retro-orbital bleeding. 10% volume of acid-citrate-dextrose (ACD) was used as the anticoagulant and 25ul whole blood was analyzed using a FORCYTE Veterinary Hematology Analyzer (Oxford Science, Inc.)

Flow cytometry of platelet-leukocyte aggregates

100ul of whole blood was collected using retro-orbital bleeding and anti-coagulated with 10% volume of ACD. Red blood cells (RBCs) were lysed briefly at 4°C for less than 1 minute, and washed with staining buffer. Cells were then stained with CD45-Pacific blue (eBioscience), CD115-APC (eBioscience), Gr-1-PerCP cy5.5 (Ly6-C/G; BD Biosciences), CD11b-PE Cy7 (eBioscience) and CD41-FITC (eBioscience) at 1:500 dilution for 30 min on ice. The cells were washed with staining buffer, resuspended in FACS buffer and run on an LSRII flow cytometer (BD Biosciences) to detect platelet Ly-6Chi monocyte aggregates defined as CD45⁺Gr-1⁺CD115⁺CD41⁺, platelet Ly-6Clo monocyte aggregates defined as CD45⁺Gr-1⁺CD115⁻CD41⁺ and platelet neutrophil aggregates defined as CD45⁺Gr-1⁻CD115⁻CD41⁺. Leukocyte activation was further analyzed as reflected by mean fluorescent intensity (MFI) of CD11b.
Flow cytometry of hematopoietic stem cell profile in mouse bone marrow and spleen, and human cord blood

Bone marrow and spleen cells were stained and analyzed as previously described\textsuperscript{5}. Briefly, bone marrow cells from mouse femurs and tibias were stained with a cocktail of antibodies to lineage-committed cells (CD45R, CD19, CD11b, CD3e, TER-119, CD2, CD8, CD4 and Ly-6C/G, all FITC conjugated; Bioscience), Sca 1-Pacific blue and c-Kit-APC cy7 to identify LSK (Lin\textsuperscript{−}Sca1\textsuperscript{+}c-Kit\textsuperscript{+}) cells and HPC (Lin\textsuperscript{−}Sca1\textsuperscript{−}c-Kit\textsuperscript{+}) cells. Further, another set of antibodies including a cocktail of antibodies to lineage-committed cells (same as mentioned above), Sca-1-FITC, CD16/CD32 (FcyRII/III)-Pacific blue, CD34-PerCP Cy5.5, CD71-PE and CD41-PE cy7 to identify progenitor cell populations including CMP (Lin\textsuperscript{−}Sca1\textsuperscript{−}c-Kit\textsuperscript{+}CD34\textsuperscript{int}FcyRII/III\textsuperscript{int}), GMP (Lin\textsuperscript{−}Sca1\textsuperscript{−}c-Kit\textsuperscript{+}CD34\textsuperscript{int}FcyRII/III\textsuperscript{hi}) and MEP (Lin\textsuperscript{−}Sca1\textsuperscript{−}c-Kit\textsuperscript{+}CD34\textsuperscript{int}FcyRII/III\textsuperscript{low}) cell populations. In MEP population, ErP was defined as (Lin\textsuperscript{−}Sca1\textsuperscript{−}c-Kit\textsuperscript{+}CD34\textsuperscript{int}FcyRII/III\textsuperscript{int}CD71\textsuperscript{−}CD41\textsuperscript{−}) and MkP as (Lin\textsuperscript{−}Sca1\textsuperscript{−}c-Kit\textsuperscript{+}CD34\textsuperscript{int}FcyRII/III\textsuperscript{int}CD71\textsuperscript{−}CD41\textsuperscript{+})\textsuperscript{5,6}. In mouse spleen, similar staining and gating strategy were used in separating spleen LSK and HSPC. Spleen MkP was defined as (Lin\textsuperscript{−}Sca1\textsuperscript{−}c-Kit\textsuperscript{+}CD41\textsuperscript{−}). All antibodies were used at 1:50 dilution.

In human cord blood, mononuclear cells were isolated using Ficoll-Paque PREMIUM (density 1.078, GE healthcare life science). Mononuclear cells were frozen down with 90% FBS and 10% DMSO in -80°C for further experiments. For flow cytometry study, frozen mononuclear cells were thawed and stained with human Hematopoietic Lineage FITC Cocktail (ebioscience), anti-human CD34-APC, CD38-PE cy7, CD90-Alexa 700 and CD45RA-Pacific Blue. Hematopoietic stem cells (HSCs) were defined as Lin\textsuperscript{−}CD34\textsuperscript{+}CD38\textsuperscript{lo}CD90\textsuperscript{−}CD45RA\textsuperscript{−} cells\textsuperscript{7}.

Phosphorylation flow cytometry of hematopoietic stem cell in human cord blood

CD34+ cells from human cord blood were isolated using magnetic beads (CD34 MicroBead Kit, human, Miltenyi Biotec) according to the protocol from manufacture. Cells were then treated with human recombinant TPO (20ng/ml) for 10 minutes at 37°C followed by wash with cold phosphate buffered saline (PBS) and permeabilized with a proprietary buffer (BD™ Phosflow Perm Buffer III; 558050, BD) on ice for 30 minutes. After two washes with PBS/FBS buffer (554657, BD), cells were incubated with antibodies to anti-human Stat5 (pY694)-PE (562007,BD), ERK1/2 (pT202/pY204)-PE (561991, BD) or AKT (Thr308) (9088S, Cell signaling), and analyzed by flow cytometry using a BD LSR-II flow cytometer\textsuperscript{8}. 
Cord blood genotyping, CD34+ cell enrichment from cord blood mononuclear cells and megakaryocyte colony forming unit (MK-CFU) assay

Genotyping of single nucleotide polymorphism (SNP) of Lnk rs3184504 of cord blood was performed used TaqMan® Assays (C_2981072_10, 4351379, Thermo Fisher). DNA was isolated from whole blood using DNA isolation column. Cord blood mononuclear cells isolated using Ficoll-Paque Premium was enriched with CD34+ cells using magnetic beads (CD34 MicroBead Kit, human, Miltenyi Biotec). MK-CFU was performed using a MegaCult™-C collagen-based assay (Stem cell Technologies). Briefly, a total of 5000 cells were seeded into a semi-collagen gel culture medium and cultured in two chamber slides for 12 days with cytokines including TPO (50ng/ml), IL-3 (10ng/ml) and IL-6 (10ng/ml). Cells were fixed with 1:3 methanol:acetone solution at room temperature for 20 minutes. Slides were stained using immunohistochemistry staining protocol by blocking the slides with human serum, incubating with anti-human GPIIb/IIIa primary antibody (1:100 dilution) and then a biotin-conjugated goat anti-mouse IgG secondary antibody (1:300 dilution), and finally colorized using avidin alkaline phosphatase conjugate. Last, the slides were counterstained with Evans Blue. CFU-Mk colonies usually ranged in size from three to several hundred megakaryocytes per colony. Small size colony referred to a colony with 3 to 20 cells per colony, while a medium or a large colony was defined as a colony with 21 to 49 cells per colony, or ≥ 50 cells per colony. Large Mk colonies arise from more primitive Mk progenitors whereas the smaller Mk colonies are produced from more mature Mk progenitors.

Immunoblot

Whole cell protein extracts were obtained by lysing platelets in Laemmli sample buffer (Bio-Rad) and heating to 100°C for 5 minutes. Samples were electrophoresed in 8% to 16% gradient Tris-glycine gels (Invitrogen) and transferred to nitrocellulose membranes, which were then blocked for 1 hour with blocking buffer (927-40000, Odyssey). Membranes were incubated with the appropriate primary antibody in Odyssey blocking buffer with 0.1% Tween 20 (16 hours, 4°C), washed with Tris-buffered saline-Tween 20 (TBST), and incubated with the anti-rabbit or anti-mouse HRP secondary antibody at room temperature for 30 minutes. Primary antibodies used included anti-phospho-p44/42 MAPK (p-Erk1/2) (Thr202/Tyr204), anti-p44/42 MAPK (Erk1/2), anti-phospho-AKT (Thy 308), anti-AKT (pan), anti-phospho-Stat5, anti-Stat5, anti PKC substrates (pSer), anti-phospho-SHIP1 (Tyr 1020), and anti-integrin β3 (all from Cell Signaling). Protein bends were detected using Supersignal West Pico-enhanced chemiluminescent solution (Pierce).
Platelet isolation and platelet aggregation function study using aggregometer

Platelet-rich plasma was prepared and platelets were pelleted with 1μM PGE1 to inhibit platelet activation. Platelet were resuspended in HEPES-buffered modified Tyrodes buffer (HBMT) at a concentration of 3.0 X 10⁵ /μl. After incubation in 37°C water bath for 30 minutes, platelet aggregation was initiated with AYPGKF (final concentration 100 μM) or ADP (final concentration 20 μM) and monitored in a PAP-8E aggregometer (BioData Corporation) at 37°C with stirring. The light transmission rate was recorded by the software.

Cholesterol loading on platelets using cholesterol-rich liposome

Cholesterol-rich liposome was prepared using a modified protocol9-11. Briefly, 20mg, 40mg and 80mg cholesterol (Sigma) were mixed with 1,2-dimyristoyl-sn-glycero-3-phosphocholine(DMPC)(Avanti Polar Lipids) and dissolved in chloroform to make cholesterol-rich liposome. These cholesterol-rich liposomes were dried onto glass vessel walls under a stream of nitrogen gas and re-suspended in 10ml HBMT buffer which then were subjected to 70W for 45 minutes with a probe sonication at room temperature. The preparations were then centrifugated at 10,000 g for 20 minutes and supernatant was collected and store in aliquot at 4°C. The concentration of cholesterol-rich liposome was 2mg/ml, 4mg/ml and 8mg/ml respectively. Platelets were isolated from mice using the protocol mentioned above and re-suspended in 30μl HBMT and loaded with 30μl 2mg/ml, 4mg/ml and 8mg/ml cholesterol-rich liposome for 2 hours at 37°C before AYPGKF (Ala-Tyr-Pro-Gly-Lys-Phe-NH2 peptide, PAR4 agonist, Sigma) stimulation at 100μM for 10 minutes.

Filipin staining of cell surface free cholesterol levels on platelets using both flow cytometry and confocal microscope

Platelets were isolated and washed with HBMT before stained with filipin for 2 hours. Samples then were run on LSR-II using DAPI channel. Imaging of filipin stained platelets was performed on an AxioObserver Z.1 microscope (Zeiss, Thornwood, NY) using a 100x/1.3 Plan-Neofluar objective lens and an Orca ER cooled CCD camera (Hamamatsu Photonics, Bridgewater, NJ). Filipin was excited with a 365-nm LED (Zeiss Colibri) and emission was collected through a 455-nm beamsplitter and a 480/40 nm emission filter (Zeiss filter set 47 with excitation filter removed). Hardware was controlled by ZEN software (Zeiss).

Cholesterol content of platelets
Platelets were isolated and a small aliquot was saved to determine protein content using Pierce™ BCA Protein Assay Kit (23225, Thermo Scientific). Platelet total lipids were extracted using methanol/chloroform (v:v 1:2). The lipids in chloroform were then dried down under nitrogen gas and resuspended in 50 mM Tris, pH 7.5 and 0.1% Triton X-100. Total cholesterol content was measured using Cholesterol E kit (435-35801, Wako) and normalized to platelet protein content.

Reverse-transcriptase quantitative polymerase chain reaction (RT-qPCR) of LNK, ATXN2, TRAFD1 and PTPN11 mRNA levels in cord blood CD34+ cells

CD34+ cells from human cord blood were isolated using magnetic beads as mentioned above. Cells were harvested and RNAs were isolated using RNasey Mini kit (74104, Qiagen) according to the manufacturer’s protocol. RNA with an A260/280 of >1.8 was used for cDNA synthesis using Maxima First Strand cDNA Synthesis Kit (K1671, Thermo Scientific) and qPCR was performed in a 7500 Real-Time PCR system (Applied Biosystem) using SYBR green chemistry. Primers were purchased from Integrated DNA Technologies. Forward primer for LNK was 5’-CCAGGAGAAGCACCTTGGT-3’ and reverse primer for LNK was 5’-CCGACAGACATCTTTGAGG-3’. Forward primer for ATXN2 was 5’-AGTTGTGGCTCCAAATGTGA-3’ and reverse primer for ATXN2 was 5’-CATGTGGCCGCATCAAGTACCA-3’. Forward primer for TRAFD was 5’-GACCAGGAAACTCGACTGTGA-3’ and reverse primer for TRAFD was 5’-AGTCTCCATGTCAGATTTGGGAA-3’. Forward primer for PTPN11 was 5’-CCGCTCATGACTATACGCTAAG-3’ and reverse primer for PTPN11 was 5’-AGACCGTTCTCTCCGTATCC -3’. Forward primer for CYC was 5’-GCCATCCAACCACCTAGTCT-3’ and reverse primer for CYC was 5’-ATGTGCAGGGTGGTGACT-3’.

TPO mRNA levels in mouse liver, kidney and bone marrow

For RNA isolation from bone marrow cells, bone marrow cells were flushed out of tibia and wash twice with Hank’s Balanced Salt Solution (HBSS) and lysed in RLT buffer from the kit of RNeasy Mini kit from Qiagen (74104, Qiagen). and RNAs were isolated according to the instruction from the manufacturer. For liver and kidney RNA isolation, 50mg tissues were homogenized in 1 ml of TRIZOL reagent using a power homogenizer. 0.2 ml of chloroform was added to the Trizol and undergone centrifugation at 15,000 xg for 10 minutes to separate phases. The top 200 ul from the top layer was collected and be9ig added to 700 ul of Qiagen
RLT buffer in a new tube. RNA isolation from RLT cell lysates from bone marrow, liver and kidney were isolated using RNeasy Mini kit. cDNA synthesis was performed using Maxima First Strand cDNA Synthesis Kit (K1671, Thermo Scientific) and qPCR was performed in a 7500 Real-Time PCR system (Applied Biosystem) using Taqman mouse TPO gene expression assay (Mm00456355_m1, 4331182, Thermo Fisher). GAPDH was used as internal control (Mm00186825_cn, 4400291, Thermo Fisher).

**Allele-specific qPCR of C and T alleles of LNK expression levels**

CD34+ HSCs from cord blood were isolated using magnetic beads (CD34 MicroBead Kit, human, Miltenyi Biotec). RNA isolation and cDNA preparation were performed as mentioned above. The SNP genotyping TaqMan assays for allele-specific LNK probes and were provided by the Thermo Fisher (assay ID: C___2981072_10). Assay validation was performed by mixing a certain ratio of CC and TT homozygous samples and a standard curve was draw as reported in recently publication12.

**TPO uptake and enzyme-linked immunosorbent assay (ELISA) of TPO levels**

Wash platelets were obtained as stated previously and resuspended in HBMT buffer. Platelets were then incubated with 2000 pg/ml recombinant TPO at °C for 1 hour. After incubation, platelets were palleted and the supernatant was used without further dilution in the Quantikine TPO ELISA. TPO uptake was calculated as follows: uptake% = (2000-ELISA read-out)*100/2000. TPO levels in plasma and HBMT buffer were measured using Mouse/Rat CCL2/JE/MCP-1 Quantikine ELISA Kit (DCP00, R&D) and Mouse IFN-gamma Quantikine ELISA Kit (MIF00, R&D) according to the manufacture’s protocols.

**Statistics**

T-test was used in comparing the difference between two groups in the mouse studies. One-way ANOVA was used in comparing the difference among more than three groups in the mouse studies and cord blood sample analysis. The difference between two specific groups from one-way ANOVA was further analyzed using post-hoc Bonferroni test. For the analysis of genotype and diet/cholesterol loading interaction, two-way ANOVA was used. Two-tail analysis was performed in all statistical analysis except the gene expression levels in cord blood in supplementary figure 2 where one-tail t test was performed. p value less than 0.05 was considered as a significant difference.
References:


Supplemental Figure Legend

Supplementary Fig. I: p-AKT levels in CD34+ cells from cord blood.

Supplementary Fig. II: *LNK, ATXN2, TRAFD1* and *PTPN11* mRNA levels in cord blood CD34+ cells. (a) *LNK*, (b) *ATXN2*, (c) *TRAFD1* and (d) *PTPN11*. (p values of one-tail t-test were reported).

Supplementary Fig. III: Allele specific PCR to quantify the mRNA levels of Lnk with either C or T alleles. Allele-specific *LNK* expression analysis of allele-biased samples with indicated allele ratios to verify the assay.

Supplementary Fig. IV: Lnk−/− BM recipient mice had leukocytosis and lymphocytosis. The BM recipient mice were fed with chow (black bars) or WTD (green bars) for 12 weeks. *** p<0.01 and <0.001 between donor genotype (WT→WT vs. Lnk−/−→WT, or WT→Ldlr−/− vs. Lnk−/−→Ldlr−/−). ^^^ <0.001 between recipient genotype (diet) (WT→WT vs. WT→Ldlr−/−, or Lnk−/−→WT vs. Lnk−/−→Ldlr−/−).

Supplementary Fig. V: Time course of plasma cholesterol levels in mice fed with WTD.

Supplementary Fig. VI: Washed platelet aggregation.
The BM recipient mice were fed with chow (black line) or WTD (green line) for 12 weeks. Shown are representative of washed platelet aggregation. (a) PAR4 agonist (AYPGKF 100μM) induced aggregation and (b) ADP induced aggregation.

Supplementary Fig. VII: TPO mRNA levels in liver, kidney and bone marrow from mice on WTD or chow diet.

Supplementary Fig. VIII: Cholesterol content of platelets from mice on WTD or chow diet. (a) flow cytometry of Fillipin stained platelets for free cholesterol estimation. (b) total cholesterol levels in platelets. (c) confocal microscope of Fillipin stained platelets. Abbreviations: n.s. not significant.

Supplementary Fig. IX: PKC activity of platelets in response to PAR4 agonist (AYPGKF) stimulation. Platelets from chow-fed (WT→WT and Lnk−/−→WT) or WTD-fed (WT-Ldlr−/− and Lnk−/−→Ldlr−/−) recipients were stimulated with AYPGKF (100 μM) for 10 min and PKC activity was estimated by Western analysis.
Supplementary Fig. X: western blot quantification of signaling in platelets. (a) p-Akt in resting and AYPGKF activated platelets and (b) p-SHIP-1 in AYPGKF activated platelets of chow-fed (WT→WT and Lnk-/→WT) or WTD-fed (WT-Ldlr-/ and Lnk-/→ Ldlr-/ ) recipients.

Supplementary Fig. XI: effect of ex vivo cholesterol loading on p-SHIP1 from platelets of chow fed mice.

Supplementary Fig. XII: Quantification of tolimidone effect on p-Akt in WT and Lnk⁻⁻ platelets from chow diet fed mice.

Supplementary Fig. XIII: Quantification of p-SHIP1 in platelets from both WT and Lyn⁻⁻⁻⁻⁻⁻ mice after AYPGKF stimulation.

Supplementary Fig. XIV: Diastolic (a) and systolic (b) blood pressure in WT and Lnk⁻⁻⁻⁻⁻⁻ BM recipient mice on WTD diet for 10 weeks.

Supplementary Fig. XV: Glucose (a) and insulin (b) levels of WT and Lnk⁻⁻⁻⁻⁻⁻ BM recipient mice.

Supplementary Fig. XVI : Plasma MCP-1 levels of WT and Lnk⁻⁻⁻⁻⁻⁻ BM recipient mice on WTD for 10 weeks.

Supplementary Fig. XVII: CD3⁺ T-cells of atherosclerotic lesion of WT and Lnk⁻⁻⁻⁻⁻⁻ BM recipient mice. (a) quantification and (b) representative images.
Supplementary Fig. I

The figure shows the measurement of p-Akt (Tyr308) MFI (AU) under different conditions.

- **TPO (-)**
  - CC
  - CT
  - TT

- **TPO (+)**
  - CC
  - CT
  - TT

The y-axis represents the p-Akt (Tyr308) MFI in arbitrary units (AU), with the x-axis indicating the presence or absence of TPO treatment.
Supplementary Fig. III

![Graphs showing allele expression percentages for different allele combinations](Image)
Supplementary Fig. IV

Peripheral blood cell counts (×10^6/ml)
Supplementary Fig. V

![Graph showing plasma total cholesterol levels over time for WT→Ldlr−/− and Lnk−/−→Ldlr−/− genotypes. The x-axis represents time (week) ranging from 0 to 15, and the y-axis represents plasma total cholesterol (mg/dL) ranging from 0 to 1500. The graph shows a significant increase in cholesterol levels over time for both genotypes.]
Supplementary Fig. VI

(a) PAR4 agonist-induced Aggregation (percentage of max amplitude)

(b) ADP-induced Aggregation (percentage of max amplitude)
Supplementary Fig. VII

Liver
Kidney
Bone Marrow

0.0000
0.0000
0.0000
0.0001
0.005
0.010
0.015
0.020
0.025

WT

o

WT

o

Lnk^-/^-WT

o

WT-Ldlr^-/^

o

Lnk^-/-Ldlr^-/-

TPO/GAPDH mRNA (AU)

WT->WT

Lnk^-/-->WT

WT->Ldlr^-/^

Lnk^-/-->Ldlr^-/-

Liver
Kidney
Bone Marrow

TPO/GAPDH mRNA (AU)
Supplementary Fig. IX
Supplementary Fig. X

(a) 

Resting

AYPGKF 100µM stimulated

p=0.02
p=0.008

p<0.05
p=0.02

p<0.001

(b) 

AYPGKF 100µM

p=0.03
p=0.02

p=0.07

p=0.07
Supplementary Fig. XII

- + - + + +
0 0 30 0 0 30 60
HDL Tolimidone AYPGKF 100μM

WT→WT Lnk→WT

p<0.05

p<0.05

p<0.05

p<0.05

p-Akt/t-Akt (AU)
Supplementary Fig. XIII

![Graph showing p-SHIP/β3 (AU) for Lyn+/- and Lynkd/-. The p-value is 0.003.](image)
Supplementary Fig. XIV

(a) Diastolic blood pressure (mmHg)

(b) Systolic blood pressure (mmHg)
Supplementary Fig. XV
Supplementary Fig. XVII

(a) 

![Bar chart showing CD3(+) T cell/section comparison between WT→Ldlr$^{-/-}$ and Lnk$^{-/-}$→Ldlr$^{-/-}$](image)

(b) 

![Immunofluorescence images comparing WT→Ldlr$^{-/-}$ and Lnk$^{-/-}$→Ldlr$^{-/-}$] (CD3 (T-cell) and DAPI (nuclei))