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LNK/SH2B3 Loss of Function Promotes Atherosclerosis and Thrombosis

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Abstract

Rationale—Human genome wide association studies (GWAS) have revealed novel genetic loci that are associated with coronary heart disease (CHD). One such locus resides in *LNK/SH2B3* which in mice is expressed in hematopoietic cells and suppresses thrombopoietin (TPO) signaling via its receptor MPL. However, the mechanisms underlying the association of *LNK* snps with CHD are poorly understood.

Objective—To understand the functional effects of *LNK* snps 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 MPL 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 IL-3/GM-CSF receptor signaling in bone marrow myeloid progenitors, while in platelets cholesterol loading combines with Lnk deficiency to increase activation. Platelet LNK deficiency increases MPL signaling and AKT activation, while cholesterol loading decreases SHIP-1 phosphorylation, acting convergently to increase AKT and platelet activation. Together

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DISCLOSURES

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with increased myelopoiesis, platelet activation promotes pro-thrombotic and pro-atherogenic platelet/leukocyte aggregate formation.

Conclusions—LNK (R262W) is a loss of function variant that promotes TPO/MPL signaling, 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.

Keywords

LNK/SH2B3; Hypercholesterolemia; atherosclerosis; thrombosis; platelet; cholesterol

Subject Terms

Lipids and Cholesterol; Stem Cells; Platelets; Atherosclerosis; Thrombosis

INTRODUCTION

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 mediators[4] are potential underlying mechanisms that contribute to accelerated athero-thrombotic 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]

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 TPO and MPL signaling[5,6]. In human GWAS, the *LNK* SNP, rs3184504 causing a missense mutation at position 262 (p.R262W, c.784T>C), is associated with an increased risk of CHD[12]; the same SNP is also associated with increased platelet counts and leukocytosis[13], which are risk factors for CHD[11, 28, 29], suggesting that the risk allele of *LNK* might worsen CHD via its effects on hematopoiesis. However, rs3184504 is associated with pleiotropic effects including autoimmunity, Type 1 diabetes and hypertension[14–17]. Moreover, it is not known if this SNP directly affects LNK expression or hematopoietic functions, and the relationship of *LNK* germline genomic variation to atherosclerosis and thrombosis has not been directly investigated. In this study, we aim to assess the impact of rs3184504 on hematopoiesis and use $Lnk^{-/-}$ mice as a model to assess the effects of LNK loss of function on atherothrombosis.

METHODS

Full Methods are provided in the Online Data Supplement.

RESULTS

The TT risk allele of LNK increases human HSC and megakaryopoiesis

In order 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 ($CD90^+CD45RA^-$)[18] in subjects carrying the TT risk genotype (Fig. 1a–c). Using phospho-flow cytometry, we showed increased phosphorylation of STAT5 and ERK1/2, but not AKT, in response to TPO in TT HSCs (Fig. 1d,e, Online Fig. 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 (Fig. 1f–h), indicating increased megakaryopoiesis. Since 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 HSPCs and megakaryocyte progenitors (MkPs). R262 is not conserved in mice, and overexpression studies with LNK R262W in cell lines have not been informative[7]. Thus, an expression quantitative trait loci (eQTL) at rs3184504 or another SNP in linkage disequilibrium 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 (Fig. 1i), which is inconsistent with an eQTL causing reduced LNK activity via a reduction in *LNK* mRNA. *ATXN2, PTPN11* and *TRAFD1* are neighboring genes of *LNK/SH2B3* on chromosome 12 and there is evidence of linkage disequilibrium (LD) of rs3184504 with SNPs in these genes[19]. Thus, we measured *ATXN2, PTPN11 and TRAFD1* mRNA levels in a new set of cord blood $CD34^+$ cells but found no significant difference between TT vs CC individuals, while *LNK/SH2B3* mRNA levels were reproducibly higher in association with TT(Online Fig. II). TPO/MPL signaling increases *LNK* transcription in human hematopoietic cells[20]. Since we detected increased TPO/MPL signaling in hematopoietic cells of the TT genotype (Fig. 1d, e), 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 carried out studies to assess alternative possibilities that LNK mRNA levels were increased due to 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 allele-specific (in our case, T or C specific) expression of *LNK*. Since we hypothesized that the increased *LNK* mRNA levels associated with T allele is due to 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 two alleles closely correlated with the expected ratio (Online Fig. 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 Fig. 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.

LNK loss of function interacts with hypercholesterolemia to promote myelopoiesis

While 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 appear to be a suitable model to assess the effects of reduced LNK function on cardiovascular disease. Humans have much higher levels of LDL cholesterol than mice and some studies have suggested that increased LDL cholesterol increases platelet reactivity[22]. We therefore assessed the impact of LNK deficiency on hematopoietic functions, thrombosis and atherosclerosis in both normo- and hyper-cholesterolemic backgrounds. We transplanted BM from WT or *Lnk*$^{-/-}$ mice into irradiated WT or *Ldlr*$^{-/-}$ mice, followed by feeding either a chow diet (normocholesterolemic WT recipients) or WTD (hypercholesterolemic *Ldlr*$^{-/-}$ recipients) for 12 weeks.

Similar to chow-fed *Lnk*$^{-/-}$ mice[5, 10, 11], *Lnk*$^{-/-}$ BM recipients displayed expansion of hematopoietic stem and progenitor cells (HSPCs, defined as *Lin*$^{-}$, *Sca-1*$^{+}$, *c-Kit*$^{+}$ LSK cells), myeloid and megakaryocyte progenitors in BM (Fig. 2a) and monocytosis and neutrophilia on both diets (Fig.2b, Online Fig.IV). Interestingly, hypercholesterolemic *Lnk*$^{-/-}$ BM recipients also showed markedly more pronounced neutrophilia and monocytosis compared to WT or chow-fed *Lnk*$^{-/-}$ recipients (Fig. 2b), reflecting an increase in the BM LSK and GMP populations (Fig. 2a).

This suggested an interaction of LNK deficiency with hypercholesterolemia to expand BM myeloid progenitors. There is evidence that LNK negatively regulates IL-3 signaling [10]. Moreover, cholesterol accumulation in HSPCs has been associated with expansion of HSPCs and increased signaling via the IL-3/GM-CSF receptor due to increased levels of the common-beta subunit (CBS) of this receptor on the cell surface[23, 24]. CBS deletion reversed HSPC expansion and the associated leukocytosis[25]. Cell surface CBS levels were decreased in HSPCs in chow-fed *Lnk*$^{-/-}$ BM recipients (Fig. 2c), consistent with increased IL-3/GM-CSF signaling and feedback down-regulation of the CBS[26] 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 *Lnk*$^{-/-}$ GMPs in the setting of hypercholesterolemia (Fig. 2c). Consistent with increased CBS levels, hematopoietic cell signaling in response to IL-3 or GM-CSF treatment, using phosphorylated STAT5 or ErK1/2 as the readout, was increased by the combination of LNK deficiency and hypercholesterolemia (Fig. 2d). These findings explain at least in part the marked increase in neutrophilia and monocytosis in hypercholesterolemic mice with hematopoietic LNK deficiency.

LNK 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 (Fig. 2e). In contrast to leukocytosis, thrombocytosis in *Lnk*$^{-/-}$ BM recipients was similar in both normo- and hypercholesterolemic backgrounds (Fig. 2b), paralleling similar levels of the BM MkP population (Fig. 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 $\alpha_{IIb}\beta_3$(JON/A), markers of platelet activation, relative to the WT BM recipients (Fig. 3a, b). However, *Lnk*$^{-/-}$ platelets from hypercholesterolemic mice showed markedly increased surface exposure of these molecules when activated by PAR4 agonist AYPGKF, while *Lnk*$^{-/-}$ platelets from normocholesterolemic mice showed similar activation (Fig. 3a, b), 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. As the mice on the WTD developed progressive hypercholesterolemia (Online Fig. V), there was a marked increase in cell surface P-selectin and active integrin $\alpha_{IIb}\beta_3$ in response to PAR4 activation in *Lnk*$^{-/-}$ platelets but not in WT platelets (Fig. 3c, d). Similarly, aggregation of *Lnk*$^{-/-}$ platelets in response to PAR4 agonist or ADP was markedly increased only when mice became hypercholesterolemic (Fig. 3e, f, Online Fig. VI). Together, these findings indicate a profound interaction of the *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 *Lnk*$^{-/-}$ platelets (Fig. 3g), reflecting a defect in internalization of MPL (Fig. 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 (Fig. 3h), likely reflecting the decreased internalization and turnover of MPL and TPO in platelets. Hepatic, splenic or bone marrow TPO mRNA levels showed no difference between wild type and *Lnk*$^{-/-}$ mice, with or without hypercholesterolemia (Online Fig. VII). This novel observation suggests that in addition to the known cell intrinsic effects of LNK deficiency in HSCs[5], 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, while 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 Lnk$^{-/-}$ platelet activation

We next undertook experiments to determine if cholesterol enrichment or depletion acted directly in platelets to alter their activation. While the WTD increased total cholesterol mass in platelets from both WT and 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 two genotypes (Online Fig. VIIIa–c). 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 transplanted with WT or *Lnk*$^{-/-}$ BM, using cholesterol-rich liposomes[29]. Cholesterol loading increased both WT and *Lnk*$^{-/-}$ platelet P-selectin exposure after PAR4 activation but the increase was more pronounced and occurred at a lower threshold in *Lnk*$^{-/-}$ platelets (Fig. 4a). Increased cell surface levels of P-selectin in *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 *Lnk*$^{-/-}$ platelets only after cholesterol loading, providing an explanation for the synergistic effect of platelet LNK deficiency and cholesterol loading on P-selectin exposure (Fig 4b). Consistently, PKC activation in response to PAR4 agonist AYPGKF was much more pronounced in *Lnk*$^{-/-}$ platelets relative to WT platelets from WTD-fed *Ldlr*$^{-/-}$ recipients but the difference was less in platelets from chow-fed WT recipients (Online Fig. IX). To assess the effects of cholesterol removal, we treated platelets with cholesterol-poor reconstituted HDL (rHDL), which has been shown to promote cholesterol efflux and decrease platelet activation in diabetic humans[31]. HDL treatment decreased P-selectin exposure in response to PAR4 activation in both *Lnk*$^{-/-}$ and WT platelets and abolished their differential response (Fig. 4c). The heightened *Lnk*$^{-/-}$ platelet aggregation induced by PAR4 activation was reversed by HDL treatment (Fig 4d). We also employed cyclodextrin, which nonspecifically promotes cellular cholesterol efflux[32]. Cyclodextrin, like HDL, reversed elevation of P-selectin exposure and platelet aggregation in LNK deficiency (Fig 4e,f). Consistent with the reversal of *Lnk*$^{-/-}$ platelet hyperreactivity by HDL, HDL also significantly reduced serine phosphorylation of PKC protein substrates in response to PAR4 activation in WT and *Lnk*$^{-/-}$ platelets (Fig 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 $\alpha_{IIb}\beta_3$ activation.

LNK loss of function and cholesterol enrichment increase platelet AKT activation

STATs, ERK1/2 and AKT are the major downstream signaling pathways in TPO/MPL signaling[33]. Consistent with increased TPO/MPL signaling, basal p-STAT5, p-ERK1/2 and p-AKT levels were increased in *Lnk*$^{-/-}$ platelets from the chow- or WTD-fed mice (Fig. 5a). Interestingly, hypercholesterolemia further increased p-AKT but not p-STAT5 or p-ERK1/2 levels (Fig. 5a, Online Fig. Xa), suggesting convergent effects of LNK deficiency and hypercholesterolemia on AKT activation. AKT has a critical role regulating platelet degranulation and aggregation[34]. 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 *Lnk*$^{-/-}$ platelets from hyperlipidemic mice.

AKT phosphorylation induced by PAR4 activation was increased in both WT and *Lnk*$^{-/-}$ platelets from hypercholesterolemic mice compared to normocholestrolemic mice, with a more pronounced effect in the *Lnk*$^{-/-}$ platelets (Fig. 5b and Online Fig. Xa). Cholesterol-loading increased AKT phosphorylation in response to PAR4 activation in both WT and *Lnk*$^{-/-}$ platelets (Fig. 5c), with a more pronounced effect in *Lnk*$^{-/-}$ platelets (Fig. 5c). Conversely, removal of cholesterol from platelets by HDL reversed the increased AKT phosphorylation associated with hypercholesterolemia (Fig. 5f). To assess the functional impact of increased AKT phosphorylation and activation on platelets, WT or *Lnk*$^{-/-}$ platelets from the chow-fed WT or WTD-fed *Ldlr*$^{-/-}$ recipient were pretreated with ruxolitinib, a JAK2 inhibitor[36], or an AKT inhibitor MK2206 followed by PAR4 activation. JAK2 acts upstream of AKT in TPO/MPL signaling[33]. The increased exposure of surface P-selectin in *Lnk*$^{-/-}$ platelets from the hypercholesterolemic recipient was reversed by inhibition of JAK2 or AKT (Fig. 5d), indicating a critical role of AKT in the heightened platelet activation.

PI3K mediates AKT activation by generating PI(3,4,5)P3, while SHIP-1 reduces AKT activation by converting PI(3,4,5)P3 to PI(3,4)P2[37]. SHIP-1 phosphorylation was increased in *Lnk*$^{-/-}$ versus wild type platelets from normocholesterolemic mice (Fig. 5e and Online Fig. Xb), consistent with increased MPL signaling and activation of a negative feedback effects on AKT phosphorylation[38]; 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 Fig. 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[39]. 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 *Lnk*$^{-/-}$ platelets from the hypercholesterolemic mice (Fig. 5f and Online Fig. XII). This idea was further assessed in genetic models expressing a kinase dead mutant of LYN (*Lyn*$^{kd/kd}$)[42]. SHIP-1 phosphorylation was increased in response to PAR4 activation in platelets (not shown) but the increase was markedly reduced in *Lyn*$^{kd/kd}$ platelets (Fig. 5g and Online Fig. 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: *Lyn*$^{kd/kd}$ platelets showed increased P-selectin exposure and integrin $\alpha_{IIb}\beta_3$ activation in response to PAR4 activation (Fig. 5h). Importantly, the priming effect of cholesterol enrichment was abolished in *Lyn*$^{kd/kd}$ platelets (Fig.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.

Together, these studies suggest that LNK deficiency increases AKT activation by enhancing TPO/MPL signaling, while 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 athero-thrombosis. Thus, to evaluate the in vivo impact of LNK deficiency on arterial thrombosis in our hypercholesterolemic model, we used $FeCl_3$-induced carotid artery thrombosis[40]. Carotid artery occlusion was markedly accelerated in the WTD-fed *Lnk*$^{-/-}$ BM recipients relative to controls (Fig. 6a). Consistently, tail vein bleeding time was significantly shortened compared to controls (Fig. 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 (Fig. 6c). In contrast, the induced thrombosis was significantly accelerated in the WTD-fed *Lnk*$^{-/-}$ recipients (Fig. 6c). These results suggest that the hyperreactivity of cholesterol-loaded *Lnk* platelets likely has a major role in the accelerated arterial thrombosis in vivo.

Activated platelets, monocytosis, neutrophilia, and platelet-leukocyte aggregates all promote atherogenesis[44–47]. To assess atherogenesis, a similar BM transplantation protocol to that described above was employed, however, normocholesterolemic controls were not included as 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 Fig. 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 which showed no difference between chow- or WT-fed WT and *Lnk*$^{-/-}$ mice (Online Fig. XV). Atherosclerotic lesion size was significantly increased in aortic roots of the *Ldlr*$^{-/-}$ mice receiving *Lnk*$^{-/-}$ BM (Fig. 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=.06). (Fig. 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 (Fig. 7b,c). 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 Fig. XVI). We assessed Ly6C^{hi} monocyte recruitment into atherosclerotic lesions in *Lnk*$^{-/-}$ vs WT BM recipients[50]. Ly6C^{hi} monocyte recruitment was significantly increased in hypercholesterolemic *Lnk*$^{-/-}$ BM recipients compared to controls (Fig. 7d). Consistently, lesional macrophages were markedly increased in *Lnk*$^{-/-}$ BM recipients (Fig. 7e). Lesional neutrophils were also increased in *Lnk*$^{-/-}$ BM recipients (Fig. 7f). While circulating T cell counts were markedly increased in *Lnk*$^{-/-}$ BM recipients (Online Fig. IV), the number of lesional T cells was not altered (Online Fig. XVII).

DISCUSSION

Human GWAS have revealed many genetic loci associated with CHD. While a number of these loci act by increasing LDL 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 to CHD in human GWAS 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[13, 19]. 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 as well as enhanced myelopoiesis. Increased MPL signaling in LNK deficient platelets and reduced SHIP-1 activity due to hypercholesterolemia combined to increase AKT activation and enhance the response of platelets to activation signals.

While rs3184504 is associated with an increased risk of CAD, whether R262W LNK is a causal variant has been contentious. This snp appears to have arisen 1000 to 1500 years ago in European populations as part of a large linkage disequilibrium 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[19]. Snps in multiple genes within this LD block are associated with CHD[19]. 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*[52]. However, in another study[53], 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 eQTL. Moreover, we showed that the other genes in this region that are expressed in hematopoietic tissues i.e. *ATXN2, PTPN11, TRAFD1*[19] did not show altered expression in cord blood CD34^{+} cells, whereas *LNK* mRNA was increased. Moreover, since 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 eQTL 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. Since TPO/MPL signaling increases LNK gene expression[20], our findings suggest that the increase in *LNK* mRNA is secondary to reduced LNK function due to an amino acid change. While 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 appear 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. While our study does not differentiate amongst 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 (Fig. 3a) as well as the increase in circulating leukocytes (Fig. 2b) may explain the increase in platelet/leukocyte aggregate formation and the increased entry of monocytes and neutrophils into plaques (Figs 2e, 7d, 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] While 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 pro-coagulant state[61, 62], likely reflecting multiple underlying mechanisms[62–64]. Earlier studies show that in vitro cholesterol-loading increases human platelet activation[29] while 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-to-total cholesterol ratio, reflecting impaired delivery of cholesterol to the liver[66]. Platelet cholesterol overload but not intrinsic SR-BI deficiency in platelets is responsible for the heightened platelet activation in $Scarb1^{-/-}$ mice[64]. The heightened $Lnk^{-/-}$ platelet activation due to 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 due to 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

(Fig 7g), which is well known to enhance the response to agonists[34]. 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[40], 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 (Fig 5f–i).

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 LDL cholesterol levels. A recent study showed that individuals with a high genetic risk score for CAD (which included rs3184504 in *LNK*) were threefold more likely to benefit from statin therapy compared to those with a low genetic risk score[67]. Novel strategies to decrease leukocyte overproduction[1] and platelet activation[68] such as JAK/STAT or AKT inhibitors[69], or LYN Kinase activators, could also reduce the risk of athero-thrombotic disease in individuals with the LNK TT variant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Nonstandard Abbreviations and Acronyms

LNK	lymphocyte specific adapter protein
CHD	coronary heart disease
GWAS	genome wide association study
MPL	myeloproliferative leukemia virus oncogene
TPO	thrombopoietin
SHIP-1	SH2 domain-containing inositol phosphatase 1
HSC	hematopoietic stem cell
HSPC	hematopoietic stem and progenitor cell
MkP	megakaryocyte progenitor
BM	bone marrow

WTD Western type diet

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Novelty and Significance

What Is Known?

- Human GWAS have shown that a common SNP in *LNK/SH2B3* (R262W) is associated with leukocytosis, thrombocytosis and an increased risk of coronary heart disease and stroke.
- LNK inhibits 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 (MPL) in hematopoietic stem cells (HSCs)

What New Information Does This Article Contribute?

- Studies using human cord blood HSCs show that the risk SNP reduces LNK function, leading to increased signaling of thrombopoietin via MPL and enhanced megakaryopoiesis.
- Mice with hematopoietic LNK deficiency on an $Ldlr^{-/-}$ background display HSC 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 SHIP-1 activation, leading to increased AKT and platelet priming

LNK is a scaffolding protein that reduces signaling via various cytokine receptors in hematopoietic cells, limiting proliferative responses. Our studies in human cord blood stem cells show that a common SNP in *LNK/SH2B3* results in reduced LNK function and thus increased signaling of thrompopoietin via its receptor (MPL) and enhanced production of leukocytes and platelets. These functions could explain the association of the risk SNP with leukocytosis, thrombocytosis and atherothrombosis. In mice with hematopoietic LNK deficiency in the background of LDL receptor deficiency, hypercholesterolemia combines with LNK deficiency to augment HSC, myeloid and megakaryocyte progenitor expansion, leading to thrombocytosis, leukocytosis, platelet-leukocyte aggregates, accelerated atherosclerosis and thrombosis. LNK deficient platelets show enhanced activation in response to agonists, reflecting convergent activating effects of increased MPL signaling and membrane cholesterol loading on AKT.

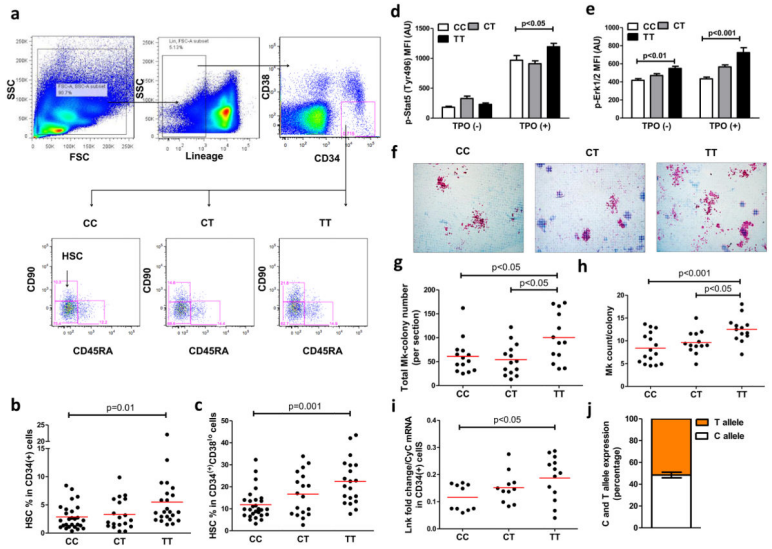

Figure 1. Association of the *LNK* TT risk SNP with HSC expansion and increased megakaryopoiesis

Flow cytometry overview of HSCs ($Lin^{-}CD34^{+}CD38^{lo}CD90^{+}CD45RA^{-}$) **(a)** and HSC percentage in $CD34^{+}$ (**b**) or $Lin^{-}CD34^{+}CD38^{lo}$ **(c)** cord blood cell fractions. p-STAT5 **(d)** and p-ERK1/2 **(e)** levels in $CD34^{+}$ cells. Megakaryocyte (Mk) colony number **(f,g)** and Mk counts per colony **(h)**. Normalized *Lnk* mRNA levels in $CD34^{+}$ cells **(i)**. **j)** Allele-specific *LNK* expression of human cord blood $CD34^{+}$ cells.

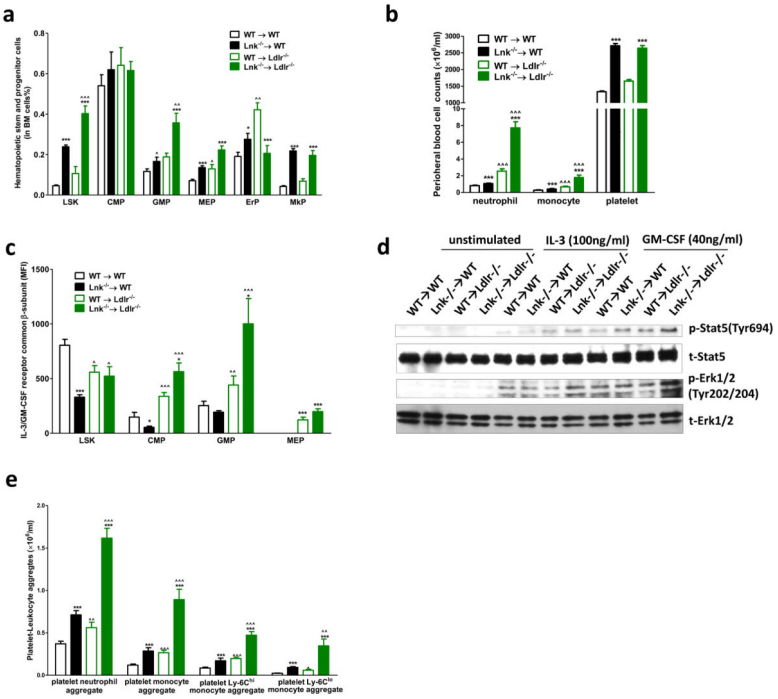

Figure 2. Hypercholesterolemia markedly increases myelopoiesis in *Lnk*$^{-/-}$ BM recipient

Hematopoietic stem and progenitor cells in BM **(a)**, peripheral neutrophil, monocyte and platelet counts **(b),** cell surface CBS levels **(c)** and platelet neutrophil/monocyte aggregates **(e).** n=14–15. **(d)** p-Erk and p-Stat levels in BM cells with and without stimulation of IL-3 (100ng/ml) and GM-SCF (40ng/ml).

Black bars represent chow feeding and green bars represent WTD feeding for 12 weeks. *, **, *** denote p<0.05, <0.01 and <0.001 for WTD-fed *Lnk* $^{-/-}$ vs WT or chow-fed *Lnk* $^{-/-}$ vs WT. ^, ^^, ^^^ denote p<0.05, <0.01 and <0.001 for chow-fed *Lnk* $^{-/-}$ vs WTD-fed *Lnk* $^{-/-}$ or chow-fed WT vs WTD-fed WT.

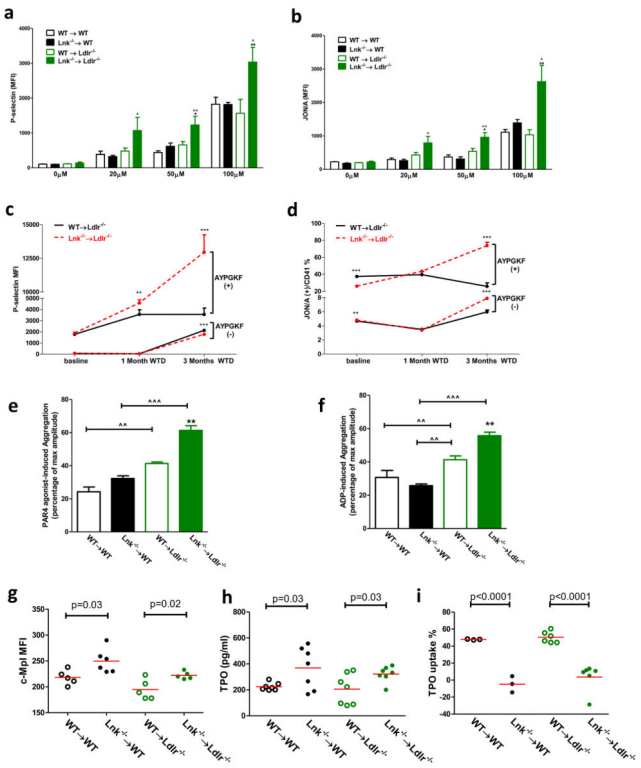

Figure 3. Hypercholesterolemia markedly increases platelet reactivity in *Lnk*$^{-/-}$ BM recipient

Surface P-selectin **(a)** and active integrin $\alpha_{IIb}\beta_3$ (JON/A) **(b)** levels on washed platelets with or without PAR4 agonist (AYPGKF, 100 μM) stimulation (n=5). Surface P-selectin **(c)** and active integrin $\alpha_{IIb}\beta_3$ (JON/A) **(d)** levels on platelets in whole blood with or without AYPGKF (100 μM) stimulation (n=5). Washed platelet aggregation upon AYPGKF (100μM) **(e)** or ADP (20 μM) **(f)** stimulation (n=4–5). TPO receptor (Mpl) levels on platelet surface **(g)**, platelet TPO internalization **(h)** and plasma TPO levels **(i)** in WT or *Lnk*$^{-/-}$ BM recipient mice. (n=3–7)

For 3a–3f, black bars represent chow feeding and green bars represent WTD feeding for 12 weeks. *, **, *** denote p<0.05, <0.01 and <0.001 for WTD-fed *Lnk* $^{-/-}$ vs WT or chow-fed *Lnk* $^{-/-}$ vs WT. ^, ^^, ^^^ denote p<0.05, <0.01 and <0.001 for chow-fed *Lnk* $^{-/-}$ vs WTD-fed *Lnk* $^{-/-}$ or chow-fed WT vs WTD-fed WT.

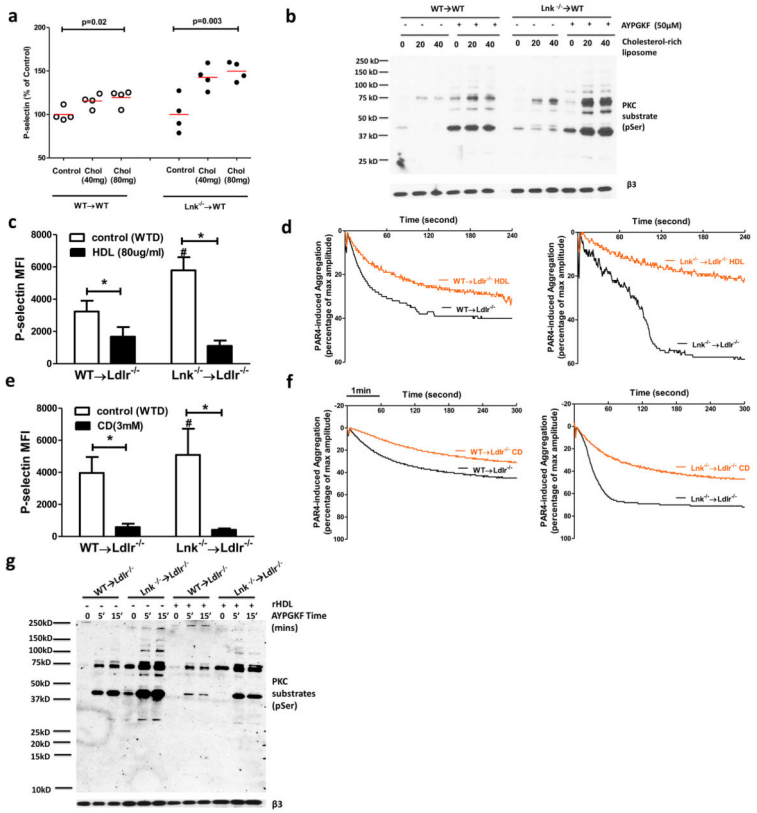

Figure 4. Cholesterol loading increases while cholesterol unloading decreases platelet and PKC activity

(a) Platelets from the chow-fed recipients were loaded with or without cholesterol and then stimulated with AYPGKF (100μM). Surface P-selectin levels were shown (n=4). **(b)** Platelets from the chow-fed recipients were loaded with or without cholesterol and then stimulated with or without AYPGKF (50μM). PKC activity was estimated (n=3). AYPGKF-induced surface P-selectin exposure **(c)** or aggregation **(d)** of platelets from the WTD-fed recipients, with or without rHDL (50 μg/ml) treatment. **(e, f)** similar as in **(c)** or **(d)** except that cyclodextrin (CD) (3mM) replaced HDL for the treatment (n=4). **(g)** PKC activity in platelets from WTD-fed recipients was estimated, with and without AYPGKF (100μM) and HDL (50μg/ml) treatment (n=3).

For 3c and 3e, * $p<0.05$ between indicated groups; # $p<0.05$ between control (WTD) and CD or HDL treatment groups.

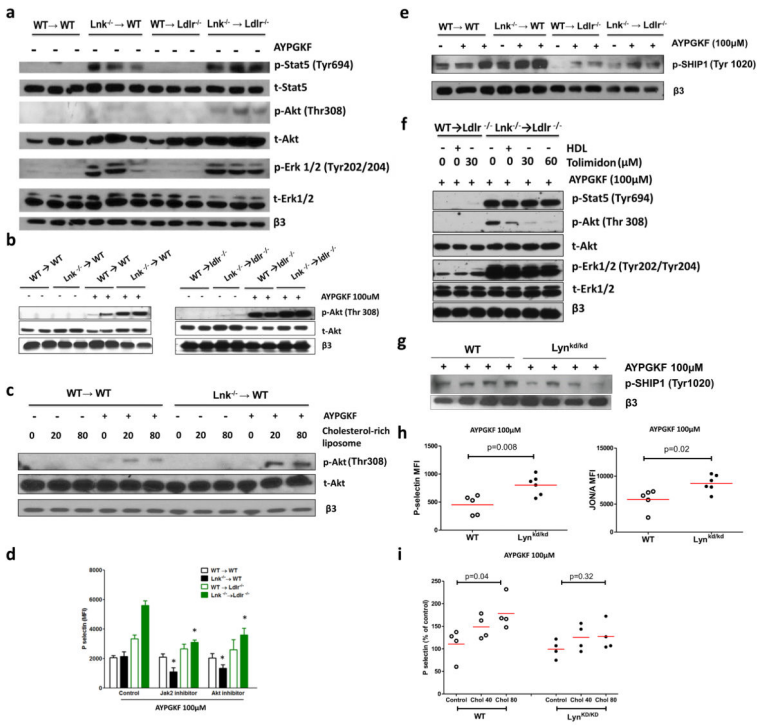

Figure 5. LNK loss of function and cholesterol enrichment increase platelet AKT activation. (a) TPO/Mpl signaling in resting platelets of WT and Lnk $^{-/-}$ BM recipient mice without TPO treatment. **(b)** AKT activity in platelets from WT and *Lnk* $^{-/-}$ BM recipient mice with and without platelet agonist AYPGKF stimulation. **(c)** effect of ex 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 HDL and Lyn tyrosine kinase activator Tolimidone treatment in Lnk$^{-/-}$ platelets from WTD fed mice. **(g)** p-SHIP1 levels in WT and *Lyn*$^{kd/kd}$ platelets upon AYPGKF stimulation. **(h)** platelet activity of *Lyn*$^{kd/kd}$ mice upon AYPGKF stimulation. **(i)** cholesterol loading effect on platelet activity of *Lyn*$^{kd/kd}$ mice.

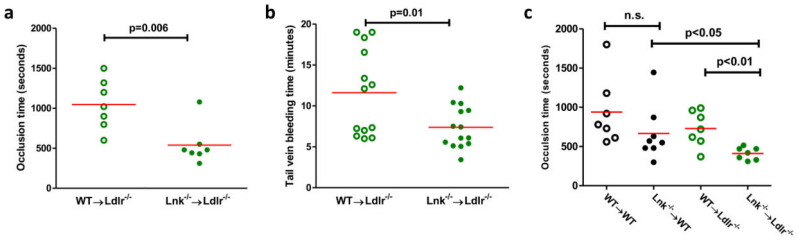

Figure 6. LNK deficiency accelerates thrombosis

$FeCl_3$-induced carotid artery thrombotic occlusion **(a)** or tail vein bleeding **(b)** in WT→*Ldlr*$^{-/-}$ vs. *Lnk* $^{-/-}$→ *Ldlr*$^{-/-}$ recipients fed WTD for 10 weeks. **(c)** $FeCl_3$-induced carotid artery thrombotic occlusion in chow-fed (black symbols) or WTD-fed (green symbols) recipient mice (n=7–8).

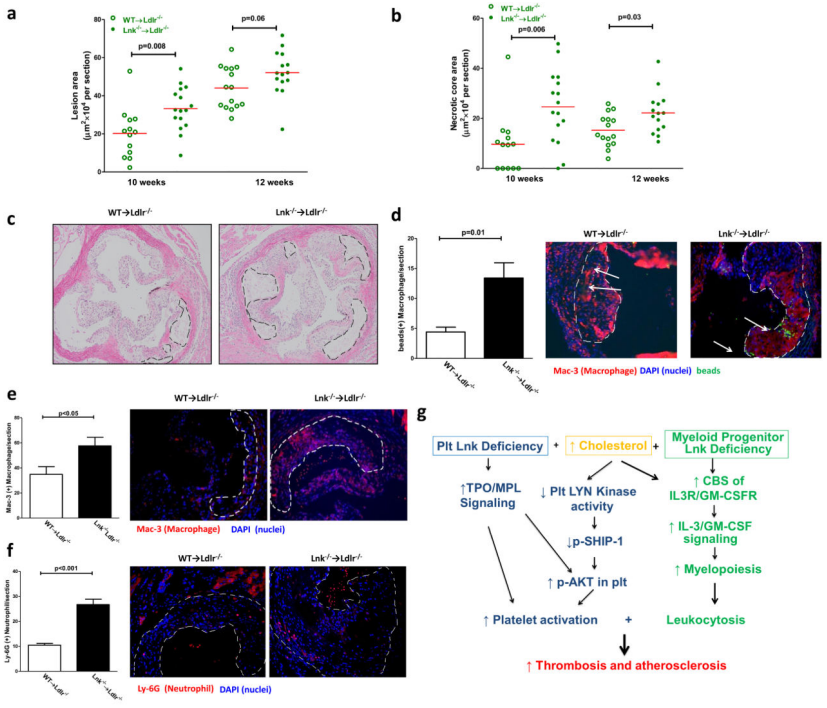

Figure 7.

(a) Atherosclerotic lesion area and **(b)** necrotic core area at aortic roots in *Ldlr*$^{-/-}$ recipients fed with WTD for 10 and 12 weeks respectively (n=14–16). **(c)** Representative images of H&E staining of aortic root atherosclerosis lesions of WT and *Lnk* $^{-/-}$ BM recipient mice. **(d)** Accumulation of fluorescence bead labeled monocytes in atherosclerotic lesions. Atherosclerotic lesional macrophage **(e)** and neutrophil **(f)** staining from mice on WTD for 10 weeks. N=14–16. **(g)** Schematic summary. Hypercholesterolemia enriches platelet membrane, including lipid rafts, with cholesterol and inhibits LYN Kinase activity. Together with increased TPO/MPL signaling due to LNK deficiency, hypercholesterolemia and LNK deficiency act convergently to activate AKT and platelets in a 2 hit model.