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Platelet IKKβ Deficiency Increases Mouse Arterial Neointima Formation via Delayed Glycoprotein Ibα Shedding

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Abstract

Objective—On the luminal surface of injured arteries, platelet activation and leukocyte-platelet interactions are critical for the initiation and progression of arterial restenosis. The transcription factor nuclear factor kappa B (NF-κB) is a critical molecule in platelet activation. Here, we investigated the role of the platelet NF-κB pathway in forming arterial neointima after arterial injury.

Methods and Results—We performed carotid artery wire injuries in LDL receptor-deficient ($LDLR^{-/-}$) mice with a platelet-specific deletion of IκB kinase beta (IKKβ) ($IKK\beta^{fl/fl}/PF4^{cre/}$

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LDLR$^{-/-}$) and in control mice (IKKβ$^{fl/fl}$/LDLR$^{-/-}$). The size of the arterial neointima was 61% larger in the IKKβ$^{fl/fl}$/PF4^{cre}/LDLR$^{-/-}$ mice compared to the littermate control IKKβ$^{fl/fl}$/LDLR$^{-/-}$ mice. Compared to the control mice, the IKKβ$^{fl/fl}$/PF4^{cre}/LDLR$^{-/-}$ mice exhibited more leukocyte adhesion at the injured area. The extent of GPIbα shedding after platelet activation was compromised in the IKKβ-deficient platelets. This effect was associated with a low level of the active form of A Disintegrin And Metalloproteinase 17 (ADAM17), the key enzyme involved in mediating GPIbα shedding in activated IKKβ-deficient platelets.

Conclusions—Platelet IKKβ deficiency increases the formation of injury-induced arterial neointima formation. Thus, NF-κB-related inhibitors should be carefully evaluated for use in patients after an arterial intervention.

Keywords

restenosis; arterial injury; platelets; leukocytes; NF-κB

Introduction

Neointimal hyperplasia following percutaneous interventions, such as balloon angioplasty or stenting, is the principal cause of arterial restenosis.[1] Platelet deposition and subsequent leukocyte-platelet interactions on the injured luminal surface are critical in the repair process after arterial damage.[2, 3, 4, 5] Although platelets are anucleate cells, they contain nearly all of the NF-κB family members.[6] NF-κB plays a complex role in platelet activation. NF-κB inhibitors impair platelet aggregation mediated by ADP, collagen and thrombin and reduce ATP release, thromboxane B2 formation and P-selectin expression stimulated by thrombin.[7] However, arachidonic acid-induced platelet activation is not affected by NF-κB inhibitors.[7] The common pathway that regulates the activation of NF-κB is based on the degradation of IκB from the NF-κB complex, a process initiated by IκB phosphorylation. The latter is catalyzed by a multisubunit protein kinase called IκB kinase (IKK).[8] IKKβ is more active than other IKK subunits in catalyzing IκB phosphorylation.[9] The loss of IKKβ dramatically inhibits NF-κB activation, resulting in embryonic lethality in mice.[10]

We were interested in determining whether the inhibition of platelet NF-κB activation could suppress neointima formation after arterial injury. Using floxed IKKβ (IKKβ$^{fl/fl}$) mice, we generated mice with IKKβ deficiency specifically in platelets by breeding PF4-Cre (PF4^{cre}) mice with these floxed mice and breeding the resulting mice with LDLR$^{-/-}$ mice. The role of platelet IKKβ in arterial neointima formation was evaluated by comparing the size of the neointima and leukocyte-platelet interactions in the injured arteries. Furthermore, the underlying mechanisms contributing to the change in the leukocyte-platelet interactions were explored.

Methods

The PF4-cre mice [11] and the floxed-IKKβ mice [12], provided by the groups of Drs. Radek C. Skoda and Michael Karin, were bred. A mouse 384 single nucleotide polymorphism panel (markers spread across the genome at approximately 7-Mbp intervals; Charles River Laboratories International, Inc., Wilmington, MA) was used to characterize the genetic background of the breeders. Polymorphic markers demonstrated that the IKKβ$^{fl/fl}$PF4^{cre} breeders were 98.17% C57BL/6. The LDLR$^{-/-}$ mice (002207) from the Jackson Lab were bred with IKKβ$^{fl/fl}$PF4^{cre} to generate IKKβ$^{fl/fl}$PF4^{cre}LDLR$^{-/-}$ mice and their littermate IKKβ$^{fl/fl}$LDLR$^{-/-}$ mice. The breeding scheme is detailed in Supplemental Figure I.

The mice were subjected to a guide wire injury in the carotid artery, and the size of the arterial neointima was examined using a method previously described.[3, 13, 14] Leukocyte

interactions with the injured arteries were studied by immunostaining the arteries with specific leukocyte markers or directly observing the injured arteries *in vivo* by intravital epifluorescence microscopy.[3] All of the animal experiments and care were approved by the Georgia Health Sciences University Animal Care and Use Committee, in accordance with AAALAC guidelines. The data are presented as the mean ± SEM and were analyzed by either one-way ANOVA followed by a Bonferroni correction post-hoc test or Student's *t*-test to evaluate two-tailed levels of significance. The null hypothesis was rejected at $P < 0.05$.

Results

Platelet IKKβ deficiency increases neointimal formation in LDLR$^{-/-}$ mice

IKK$\beta^{fl/fl}$/PF4^{cre}/LDLR$^{-/-}$ mice and their littermate control IKK$\beta^{fl/fl}$/LDLR$^{-/-}$ mice were fed a Western diet for two weeks, followed by carotid artery wire injury. Four weeks later, the arteries were excised and processed for analysis. The two groups of mice were identical in body weight, blood cholesterol levels, and number of peripheral leukocytes (Supplemental Tables I and II). The number of platelets in the IKK$\beta^{fl/fl}$/PF4^{cre}/LDLR$^{-/-}$ mice exhibited a decreasing trend, but this trend did not reach statistical significance when compared to that in IKK$\beta^{fl/fl}$/LDLR$^{-/-}$ mice (Supplemental Table I). Surprisingly, the size of the neointima lesions was 61% larger in the IKK$\beta^{fl/fl}$/PF4^{cre}/LDLR$^{-/-}$ mice compared to the IKK$\beta^{fl/fl}$/LDLR$^{-/-}$ mice (Figure 1A). The size of the media was also increased in the IKK$\beta^{fl/fl}$/PF4^{cre}/LDLR$^{-/-}$ mice compared to the IKK$\beta^{fl/fl}$/LDLR$^{-/-}$ mice (Figure 1A). Notably, the ratio of the intima to the media (I/M) was markedly elevated in the IKK$\beta^{fl/fl}$/PF4^{cre}/LDLR$^{-/-}$ mice compared to the IKK$\beta^{fl/fl}$/LDLR$^{-/-}$ mice (Supplemental Figure II). Macrophages in the injured carotid arteries were stained with F4/80 and found to be significantly increased in the IKK$\beta^{fl/fl}$/PF4^{cre}/LDLR$^{-/-}$ mice compared to the control mice (Figure 1B).

To exclude the effect of IKKβ deficiency in other PF4-expressing cells on the formation of arterial neointima,[15] we used bone marrow transplantation to generate LDLR$^{-/-}$ chimeric mice that lacked IKKβ only in their platelets and the control mice that retained IKKβ in their platelets. The carotid arteries of both groups were injured with a guide wire. Four weeks after the injury, we observed that the neointimal lesions were 64% larger in the LDLR$^{-/-}$ mice that received bone marrow from the IKK$\beta^{fl/fl}$/PF4^{cre}/LDLR$^{-/-}$ mice compared to the control mice. The arterial neointimas from the LDLR$^{-/-}$ mice that received bone marrow from IKK$\beta^{fl/fl}$/PF4^{cre}/LDLR$^{-/-}$ mice also stained more strongly with a macrophage marker (Supplemental Figure III A and B).

Platelet IKKβ deficiency increases leukocyte adhesion to injured carotid arteries and platelet-leukocyte aggregates

To evaluate the leukocyte interactions with the injured arteries, arterial cross-sections were immunostained with specific markers for platelets, neutrophils, and monocytes. One hour after the carotid arterial injury, platelets and leukocytes covered the denuded luminal surface (Figure 2A and 2B), and almost no monocytes were observed on the denuded luminal surface (data not shown). The number of platelets covering the injured area was not significantly different between the IKK$\beta^{fl/fl}$/PF4^{cre}/LDLR$^{-/-}$ and IKK$\beta^{fl/fl}$/LDLR$^{-/-}$ mice (Figure 2A). However, considerably more neutrophils were adhered to the injured area of the carotid arteries in the IKK$\beta^{fl/fl}$/PF4^{cre}/LDLR$^{-/-}$ mice compared to the IKK$\beta^{fl/fl}$/LDLR$^{-/-}$ mice (Figure 2B). Seven days after the wire injury, more infiltrating macrophages were observed in the injured carotid arteries of the IKK$\beta^{fl/fl}$/PF4^{cre}/LDLR$^{-/-}$ mice than in the IKK$\beta^{fl/fl}$/LDLR$^{-/-}$ mice (Figure 2C).

Next, we examined the interactions of the leukocytes with the injured mouse carotid arteries *in vivo* by using intravital epifluorescence microscopy. The circulating leukocytes, which

were labeled with rhodamine 6G, rolled on and adhered to the injured vessel wall after the wire injury. The number of leukocytes rolling on the arterial wall did not differ between the IKKβ$^{fl/fl}$/PF4^{cre}/LDLR$^{-/-}$ and IKKβ$^{fl/fl}$/LDLR$^{-/-}$ mice (Figure 2D). During the early stage after the arterial injury, the number of leukocytes adhering to the arterial wall was not markedly different between the 2 groups. Twenty minutes later, more leukocytes had adhered to the arterial walls of the IKKβ$^{fl/fl}$/PF4^{cre}/LDLR$^{-/-}$ mice compared to the IKKβ$^{fl/fl}$/LDLR$^{-/-}$ mice (Figure 2D).

To further define the binding affinity of the IKKβ-deficient and control platelets to leukocytes, an *ex vivo* micro-flow chamber was used.[3, 14] The chamber was coated with thrombin-activated IKKβ-deficient and control platelets, which were isolated from IKKβ$^{fl/fl}$/PF4^{cre} or IKKβ$^{fl/fl}$ mice. Then, whole blood from the wild-type mice was allowed to flow through the chamber. Interestingly, the number of rolling wild-type leukocytes on the chamber coated with activated IKKβ-deficient platelets was low, but these levels did not reach significance when compared to the chamber coated with control platelets. However, the number of adhering leukocytes was much greater in the chamber coated with IKKβ-deficient platelets compared to the chamber coated with control platelets (Figure 2E).

To evaluate the role of platelet IKKβ deficiency in mediating platelet-leukocyte interactions in the circulation, we examined the mouse blood leukocyte population by flow cytometry. Both platelet-neutrophil and platelet-monocyte aggregates were increased 4 to 5 times in the IKKβ$^{fl/fl}$/PF4^{cre}/LDLR$^{-/-}$ mice compared to the IKKβ$^{fl/fl}$/LDLR$^{-/-}$ mice (Supplemental Figure IV). Within those aggregates, the level of GPIbα expression was remarkably higher in IKKβ$^{fl/fl}$/PF4^{cre}/LDLR$^{-/-}$ mice (Supplemental Figure IV). Leukocyte Mac-1 (αMβ2, CD11b/CD18) and platelet GPIbα are critically involved in the formation of leukocyte-platelet aggregates.[16-18] This result suggests that platelet IKKβ deficiency enhances platelet-leukocyte aggregation due to the elevated level of GPIbα on IKKβ-deficient platelets.

Platelet IKKβ deficiency decreases platelet activation, secretion and aggregation

To evaluate the role of IKKβ deficiency in platelet function, platelets from IKKβ$^{fl/fl}$/PF4^{cre} mice and their IKKβ$^{fl/fl}$ littermates were stimulated with thrombin. Flow cytometry revealed that there was no significant difference in P-selectin expression between the resting IKKβ-deficient platelets and the control platelets (Figure 3A). However, P-selectin expression induced with 0.1 U/ml thrombin was diminished in the IKKβ-deficient platelets compared to the control platelets (Figure 3A). In addition, Lumi-Aggregometer-based experiments demonstrated that IKKβ-deficient platelets exhibited reduced ATP release and aggregation compared to control platelets when stimulated with thrombin (Figure 3B). Electron microscopy also indicated that the release of α-granules and platelet aggregation were attenuated in the IKKβ-deficient platelets compared to the control platelets after stimulation with thrombin for 10 minutes (Figure 3C).

IKKβ deficiency attenuates GPIbα shedding in platelets

Platelet GPIbα plays an important role in the interaction between platelets and leukocytes.[16-18] We examined the level of GPIbα on IKKβ-deficient platelets. Platelets were isolated from IKKβ$^{fl/fl}$/PF4^{cre} mice and their littermate IKKβ$^{fl/fl}$ mice. Flow cytometry (Figure 4A) and western blot analyses (Figure 4B) demonstrated that there was no significant difference in GPIbα expression between the resting IKKβ-deficient platelets and the control platelets. Stimulation with thrombin caused glycoprotein shedding from the surface of the platelets. Indeed, thrombin treatment led to rapid GPIbα shedding from the IKKβ$^{fl/fl}$ platelets in a time-dependent manner. However, IKKβ deficiency significantly attenuated the extent of GPIbα shedding (Figure 4A and 4B). Flow cytometry demonstrated slow shedding of GPV in IKKβ-deficient platelets compared to control platelets

(Supplemental Figure VA). Interestingly, stimulation with thrombin did not change the surface levels of GPVI (Supplemental Figure VB), GPIX (Supplemental Figure VC) or αIIbβ3 (Supplemental Figure VD) on the platelets. ADP (5 μM) and collagen IV (20 μg/ml) also caused GPIbα shedding, but no difference was observed between the IKKβ-deficient and control platelets (data not shown). No difference was observed in the protein levels of GPV, GPVI, GPIX or αIIbβ3 on the membrane of the platelets following stimulation with ADP or collagen (data not shown). These results demonstrate that platelet IKKβ plays a critical role in GPIbα and GPV shedding following thrombin stimulation.

Blocking the GPIbα binding site on Mac-1 eliminates increased interactions between IKKβ-deficient platelets and leukocytes

To further determine whether the increased leukocyte adhesion to the injured arteries was due to the reduced GPIbα shedding in IKKβ$^{fl/fl}$/PF4^{cre}/LDLR$^{-/-}$ mice, we utilized an anti-M2 antibody that selectively blocks GPIbα binding site on Mac-1 in mouse injury models.[18] The carotid arteries collected from mice at one hour after the carotid arterial injury were examined by histology. The number of platelets covering the injured area was not significantly different between the IKKβ$^{fl/fl}$/PF4^{cre}/LDLR$^{-/-}$ and the IKKβ$^{fl/fl}$/LDLR$^{-/-}$ mice treated with the control IgG or the anti-M2 antibody (Figure 5A). However, treatment with the anti-M2 antibody significantly reduced neutrophil adherence to the injured area of the carotid arteries in both the IKKβ$^{fl/fl}$/PF4^{cre}/LDLR$^{-/-}$ mice and the IKKβ$^{fl/fl}$/LDLR$^{-/-}$ mice compared to mice treated with control IgG (Figure 5B). More importantly, the increased leukocyte adhesion observed in the control IgG-treated IKKβ$^{fl/fl}$/PF4^{cre}/LDLR$^{-/-}$ mice compared to the control IgG-treated IKKβ$^{fl/fl}$/LDLR$^{-/-}$ mice was abolished when these mice were treated with the anti-M2 antibody (Figure 5B). The same results were observed when we directly examined leukocyte interactions with the injured arteries by intravital epifluorescence microscopy (Figure 5C). In addition, in an *ex vivo* micro-flow chamber, the rolling of leukocytes on the activated IKKβ-deficient platelets or the control platelets did not differ significantly. In line with the *in vivo* data for leukocyte adhesion, the adhesion of the IgG-treated leukocytes to the activated IKKβ-deficient platelets in this *ex vivo* model was much greater than that observed in the activated control platelets. However, treatment with the anti-M2 antibody eliminated the increased leukocyte adhesion on the activated IKKβ-deficient platelets (Figure 5D).

IKKβ deficiency suppresses ADAM17 maturation in platelets

The sheddase ADAM17 is critically involved in platelet surface receptor shedding.[19, 20] To test the involvement of ADAM17 in our system, we stimulated IKKβ-deficient and control platelets with thrombin and detected immature (or pro-) and mature (or active) forms of ADAM17 using western blot analysis. The pro-form of ADAM17 was present in the resting and activated platelets (Figure 6A). The active form of ADAM17 was detectable in the resting control platelets and transiently increased by 2.7-fold during platelet activation. This finding is consistent with earlier studies.[20, 21] IKKβ-deficient platelets had a similar level of active ADAM17 in the resting platelets but a much lower level of active ADAM17 during platelet activation compared to the control platelets (Figure 6A). The level of phosphorylated p38 MAPK has been shown to play a critical role in ADAM-17 activation.[22-24] Thus, we tested the effect of IKKβ deficiency on p38 MAPK phosphorylation. Thrombin remarkably increased p38 MAPK phosphorylation in the IKKβ$^{fl/fl}$ platelets, but no significant increase in p38 MAPK phosphorylation was observed in the IKKβ-deficient platelets after 30 min of thrombin stimulation (Figure 6B). These data indicate that IKKβ is a key molecule in ADAM17 maturation via the regulation of p38 MAPK phosphorylation.

Discussion

The initiation and progression of the neointima formation that underlies restenosis involves the adhesive interactions of platelets with leukocytes and leukocytes with the denuded vessel wall.[4, 5] NF-κB is expressed in platelets and plays a critical role in platelet activation.[6, 7] Here, we showed, *in vivo,* that platelet IKKβ deficiency increases neointima formation after arterial injury due to enhanced leukocyte-platelet interactions. Furthermore, we found that IKKβ deficiency inhibits ADAM17 maturation, resulting in delayed GPIbα shedding in platelets.

Wire injury-induced neointima formation in the mouse carotid artery is a widely used model to mimic the pathology of arterial neointima formation in patients with arterial restenosis.[25] An excessive leukocyte-platelet interaction and leukocyte infiltration after artery injury has been shown to exaggerate the repair mechanisms and augment neointima formation.[26, 27] After endothelial denudation by mechanical injury, platelets immediately adhere to and accumulate on the injured luminal surface of arteries.[2, 3] P-selectin and many glycoproteins on activated platelets mediate leukocyte rolling and localization and further support leukocyte recruitment.[28, 29] The binding of platelet P-selectin to its leukocyte ligand PSGL-1 initiates leukocyte recruitment to the activated platelets.[30] The interaction of P-selectin and PSGL-1 rapidly activates the leukocyte integrin Mac-1.[31-33] The binding of leukocyte Mac-1 to platelet GPIbα subsequently strengthens the firm adhesion and transmigration of leukocytes to sites of platelet deposition.[16-18] The loss of Mac-1/GPIbα binding leads to reduced leukocyte accumulation after arterial injury and further results in the inhibition of neointima thickening.[18] In this study, increased neointima formation in the $IKK\beta^{fl/fl}/PF4^{cre}/LDLR^{-/-}$ mice was accompanied by an increase in leukocyte adhesion to the injured area. This increased neointima was eliminated by neutrophil depletion (Supplemental Figure VI), indicating that this phenotypic change in neointima formation is due to increased leukocyte adhesion. Furthermore, this increased leukocyte adhesion was abrogated by a blocking antibody that specifically inhibits the binding of leukocyte Mac-1 with platelet GPIbα.[18] These results demonstrate that high levels of GPIbα on activated platelets contribute to the increased neointima formation in $IKK\beta^{fl/fl}/PF4^{cre}/LDLR^{-/-}$ mice.

The dynamic change in GPIbα on activated platelets occurs mainly through ADAM17-mediated GPIbα shedding. ADAM17 is a sheddase on the cell surface that cleaves a variety of substrates, such as heparin-binding epidermal growth factor, transforming growth factor α, tumor necrosis factor receptor, epidermal growth factor receptor, vascular cell adhesion molecule-1 and L-selectin.[34] Following cellular activation, the proform (134 kDa) of ADAM17 is proteolyzed to yield the mature (active) form (98 kDa). The latter active form cleaves its substrates.[35] ADAM-17 activation in leukocytes occurs through p38 and/or the ERK MAPK pathway.[36, 37] ADAM17 is a major sheddase involved in platelet GPIbα shedding.[19] ADAM17 inactivation led to about a 90% reduction in GPIbα shedding in platelets. Brill et al. reported that oxidative stress activates ADAM-17 in platelets in a p38-dependent fashion.[23] Through a similar mechanism, IKKβ deficiency inhibited ADAM-17 maturation, resulting in delayed shedding of platelet GPIbα. This result is consistent with the observation that, in oral squamous cell carcinoma cells, NF-κB inhibition suppresses ADAM-17 maturation.[38]

Platelet IKKβ deficiency did not cause a significant change in platelet adhesion and accumulation on the injured arteries. In this study, we found that IKKβ deficiency delayed GPIbα and GPV shedding. GPIbα and GPV are able to bind vWF and collagen, respectively.[39, 40] High levels of GPIbα and GPV may enhance platelet adhesion to the subendothelial area of the injured arteries.[39, 40] However, many other molecules that are important for platelet adhesion to the subendothelial area, including GPVI, GPIX and

αIIbβ3, did not differ between the IKKβ deficient and the control platelets (Supplemental Figure V).[40-42] In addition, studies from other groups using NF-κB inhibitors have shown that broad NF-κB inhibition causes a defect in platelet adhesion and spreading.[6, 7] All of these factors may explain why decreased shedding of GPIbα and GPV does not result in an increase in platelet adhesion on the injured arteries.

NF-κB is a double-edged sword for activated platelet-mediated pathologies. NF-κB is a key regulator of inflammation, immunity, apoptosis and cell proliferation in all types of nucleated cells.[43] NF-κB affects the progression of inflammatory diseases such as myocardial ischemia, bronchial asthma, arthritis, and cancer.[44] The suppression of NF-κB activation has been shown to inhibit the expression of adhesion molecules and the release of chemokines and cytokines by various inflammatory cells, eventually suppressing the progression of inflammation.[45, 46, 47] However, NF-κB activation during the late stage of inflammation is associated with the resolution of inflammation and anti-inflammatory gene expression.[48] A few reports have indicated that blockade of the NF-κB pathway accelerates the pathology of inflammatory diseases. For example, Kanter et al. revealed that the inhibition of NF-κB activation in macrophages increased atherosclerosis in $LDLR^{-/-}$ mice.[49] Zaph et al. reported that deficient NF-κB activation in the intestinal epithelium is associated with increased inflammation *in vivo*.[50] Consistent with its effect on inflammation, NF-κB activation initiates platelet activation. This process is evident by the reduced level of P-selectin and the suppressed release of granules and ATP in IKKβ-deficient platelets. However, NF-κB activation appears to be necessary for activated platelets to shed their glycoproteins, and the inactivation of NF-κB led to sustained activation of platelets, as demonstrated by much higher levels of GPIbα and GPV on the surface of the activated IKKβ-deficient platelets compared to the wild-type platelets.

NF-κB has been shown to be involved in the transcriptional regulation of more than 150 genes, a significant proportion of which exhibit proinflammatory properties.[51] Therefore, approaches to specifically inhibit the NF-κB pathway are under active development as possible therapeutic interventions. In this study, we demonstrated that platelet IKKβ deficiency enhances leukocyte-platelet interactions, resulting in aggregated neointima formation after arterial injury. The underlying mechanism is that NF-κB inactivation delays ADAM17-mediated-GPIbα shedding, thus strengthening the interaction of leukocytes with platelets. Because the inhibition of the NF-κB pathway is likely to be a promising therapeutic strategy and because tissue-specific therapies are not currently available, the application of NF-κB inhibitors for diseases that have pathologies in which activated platelets are extensive participants, such as arterial injury, sepsis and thrombosis, should be carefully evaluated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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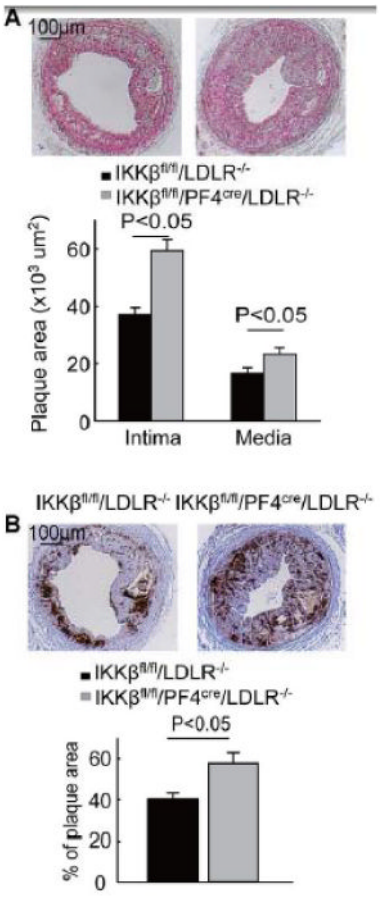

Figure 1.

IKKβ deficiency in platelets augments injury-induced arterial neointima formation in LDLR$^{-/-}$ mice. A, Movat pentachrome staining of the arterial neointima 4 weeks after injury; the size of the neointima and media were measured (n = 12 for each group). B, Immunostaining (with anti-F4/80) of the infiltrating macrophages in the arterial neointima. Twelve cross sections of each mouse carotid artery were analyzed. The percent positive area was calculated by dividing the positive area by the measured lesion area. Twelve injured carotid arteries were included for each group.

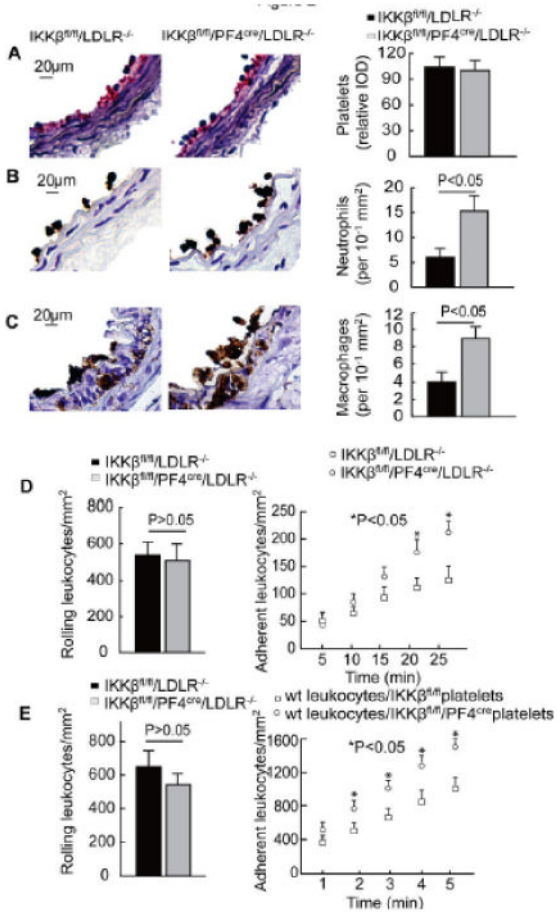

Figure 2.

IKKβ deficiency in platelets increases leukocyte interactions with the injured arteries. A to C, The carotid arteries collected at 1 hour after wire injury were stained for platelets (A) and neutrophils (B), and the carotid arteries collected 7 days after the wire injury were stained for macrophages (C) (n = 5). D, Leukocyte rolling and adhesion in the injured carotid arteries within the first 25 min after injury (n = 5). E, Leukocyte rolling and adhesion on the activated $IKK\beta^{fl/fl}$ or $IKK\beta^{fl/fl}/PF4^{cre}$ platelets in the microflow chambers (n = 5).

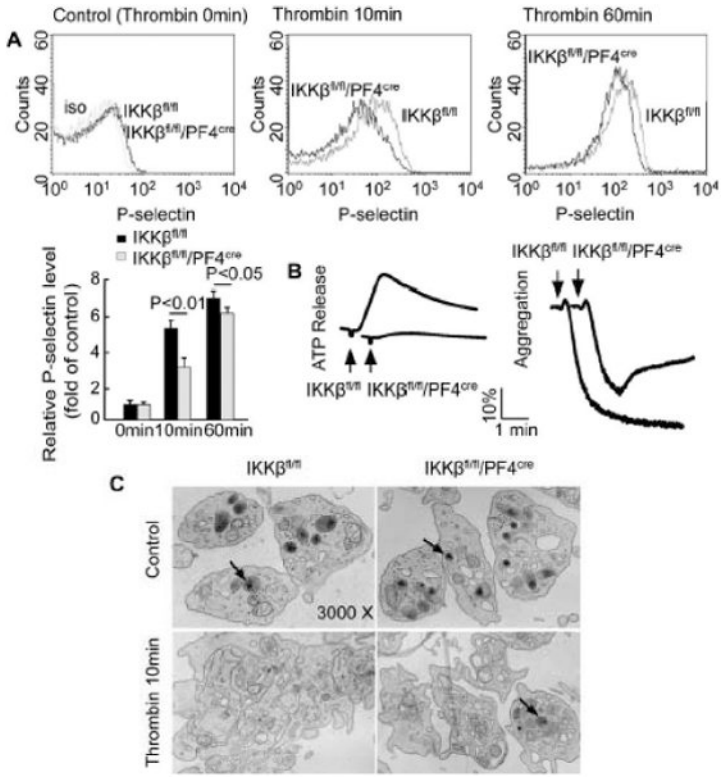

Figure 3.

IKKβ deficiency in platelets decreases platelet activation, secretion and aggregation. The washed platelets from the IKKβ$^{fl/fl}$/PF4^{cre} and the IKKβ$^{fl/fl}$ mice were stimulated with or without thrombin. A, Platelet P-selectin expression was analyzed by flow cytometry after stimulation with 0.1 U/ml thrombin for 0, 10, and 60 min at 22°C, and the relative expression of P-selectin (mean fluorescence intensity) was compared (n = 5). B, ATP release and platelet aggregation were examined in a Lumi-Aggregometer after the platelets were incubated with 0.025 U/ml thrombin. The data represent 5 independent experiments. C, Platelet aggregation and α-granule release (arrowhead) were evaluated by electron microscopy after incubation with 0.1 U/ml thrombin for 10 min at 22°C. The data represent 5 independent experiments.

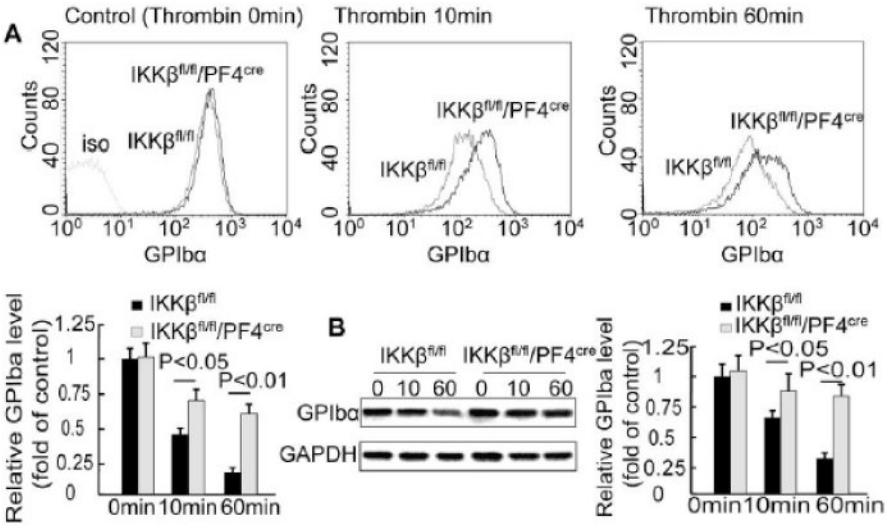

Figure 4.

IKKβ deficiency inhibits thrombin-induced GPIbα shedding in platelets. Platelets isolated from IKKβ$^{fl/fl}$/PF4^{cre} and IKKβ$^{fl/fl}$ mice were stimulated with 0.1 U/ml thrombin. A, Representative flow cytometric histograms and the relative expression of GPIbα at different times after stimulation (n = 5). B, An immunoblot of platelet GPIbα at different times after stimulation (n = 5).

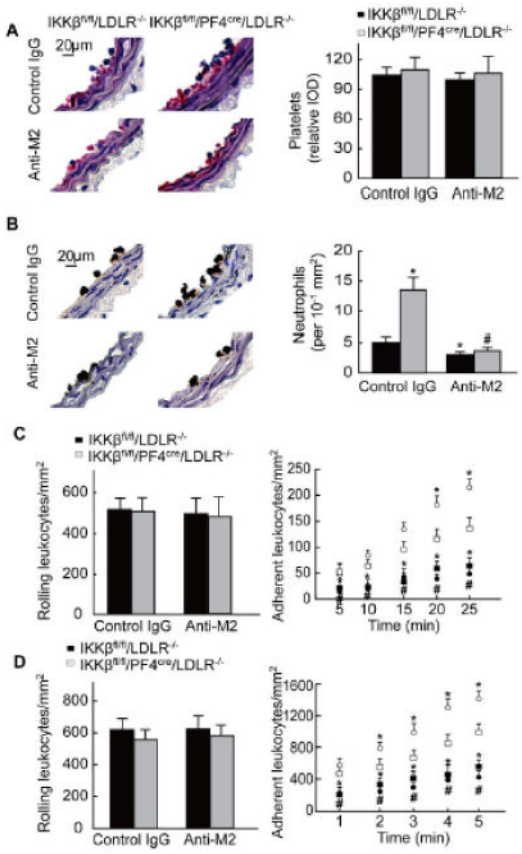

Figure 5.

Blocking the GPIbα binding site on Mac-1 prevents the increase in leukocyte interactions with injured arteries in IKKβ$^{fl/fl}$/PF4^{cre}/LDLR$^{-/-}$ mice. A to B, Immunostaining of platelets and neutrophils on carotid arteries collected 1 hour after wire injury (n = 5). *P < 0.05 vs. IKKβ$^{fl/fl}$/LDLR$^{-/-}$ mice treated with control IgG; $^{\#}P$ < 0.05 vs. IKKβ$^{fl/fl}$/PF4^{cre}/LDLR$^{-/-}$ mice treated with control IgG. C, Leukocyte rolling and adhesion in the carotid arteries within the first 25 min after injury (n = 5). White squares: IKKβ$^{fl/fl}$/LDLR$^{-/-}$ mice treated with control IgG; white circles: IKKβ$^{fl/fl}$/PF4^{cre}/LDLR$^{-/-}$ mice treated with control IgG; black squares: IKKβ$^{fl/fl}$/LDLR$^{-/-}$ mice treated with anti-M2 antibody; black circles: IKKβ$^{fl/fl}$/PF4^{cre}/LDLR$^{-/-}$ mice treated with anti-M2 antibody. *P < 0.05 vs. IKKβ$^{fl/fl}$/LDLR$^{-/-}$ mice treated with control IgG; $^{\#}P$ < 0.05 vs. IKKβ$^{fl/fl}$/PF4^{cre}/LDLR$^{-/-}$ mice treated with control IgG. D, Leukocyte rolling and adhesion on the activated IKKβ-deficient and control platelets in the micro-flow chambers within the first 5 minutes (n = 5). White squares: wild type mice were treated with control IgG, and their blood was passed through a micro-flow chamber coated with control platelets; white circles: wild type mice were treated with control IgG, and their blood was passed through a micro-flow chamber coated with IKKβ-deficient platelets; black squares: wild type mice were treated with anti-M2 antibody, and their blood was passed through a micro-flow chamber coated with control platelets; black circles: wild type mice were treated with anti-M2 antibody, and their blood was passed through a micro-flow chamber coated with IKKβ-deficient platelets. *P<0.05 vs. wild type mice were treated with the control IgG and blood was passed through a micro-flow chamber coated with the control platelets; $^{\#}P$<0.05 vs. wild type mice treated with control IgG and blood was passed through a micro-flow chamber coated with IKKβ-deficient platelets.

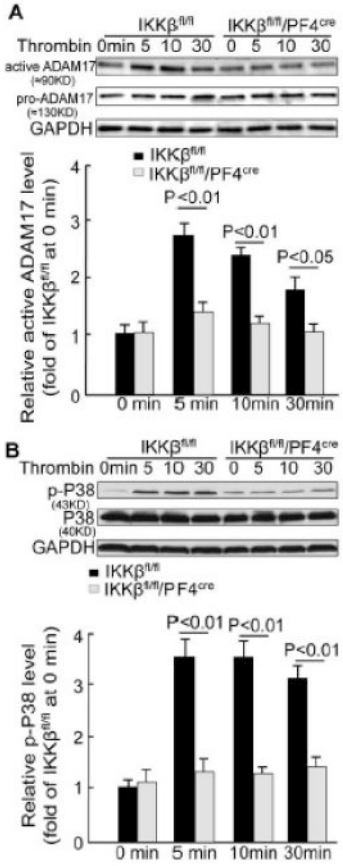

Figure 6.

IKKβ deficiency suppresses ADAM17 maturation in platelets. Platelets from IKKβ$^{fl/fl}$/PF4^{cre} and IKKβ$^{fl/fl}$ mice were stimulated with 0.1 U/ml thrombin. A, Western blot analysis showing the levels of the immature (or pro) and mature (or active) ADAM17 in the platelets (n = 5). B, Western blot analysis showing the level of phosphorylated p38 MAPK in the platelets (n = 5).