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Objective—Core2 1 to 6-N-glucosaminyltransferase-I (C2GlcNAcT-I) plays an important role in optimizing the binding functions of several selectin ligands, including P-selectin glycoprotein ligand. We used apolipoprotein E (ApoE)-deficient atherosclerotic mice to investigate the role of C2GlcNAcT-I in platelet and leukocyte interactions with injured arterial walls, in endothelial regeneration at injured sites, and in the formation of arterial neointima.

Methods and Results—Arterial neointima induced by wire injury was smaller in C2GlcNAcT-I–deficient apoE$^{-/-}$ mice than in control apoE$^{-/-}$ mice (a 79% reduction in size). Compared to controls, apoE$^{-/-}$ mice deficient in C2GlcNAcT-I also demonstrated less leukocyte adhesion on activated platelets in microflow chambers (a 75% reduction), and accumulation of leukocytes at injured areas of mouse carotid arteries was eliminated. Additionally, endothelial regeneration in injured lumenal areas was substantially faster in C2GlcNAcT-I–deficient apoE$^{-/-}$ mice than in control apoE$^{-/-}$ mice. Endothelial regeneration was associated with reduced accumulation of platelet factor 4 (PF4) at injured sites. PF4 deficiency accelerated endothelial regeneration and protected mice from neointima formation after arterial injury.

Conclusions—C2GlcNAcT-I deficiency suppresses injury-induced arterial neointima formation, and this effect is attributable to decreased leukocyte recruitment to injured vascular walls and increased endothelial regeneration. Both C2GlcNAcT-I and PF4 are promising targets for the treatment of arterial restenosis. **(*Arterioscler Thromb Vasc Biol.* 2009;29:1053-1059.)**

Key Words: leukocytes ■ endothelial recovery ■ neointima formation

Percutaneous transluminal coronary intervention is a mainstay in the treatment of patients with coronary artery disease. In a great number of patients, however, this intervention results in arterial injury that causes restenosis of the vessel. Arterial restenosis even occurs in the drug-eluting stent area. Restenosis is characterized by a decrease in arterial luminal diameter of 50% or more that results from pathological intimal hyperplasia.[1] Wire-induced neointima formation in the mouse carotid artery is a widely used model for mimicking the pathology of arterial neointima in patients with arterial restenosis.[2]

The accumulation of platelets and leukocytes on injured arterial areas is requisite for neointima formation. Immediately after arterial injury, platelets interact with the injured area via many factors, including glycoprotein Ib and glycoprotein IIb/IIIa.[3,4] On adherence, platelets become activated and express P-selectin, which along with integrins and other platelet-derived factors orchestrates the recruitment of leukocytes to the injured site.[5–7] P-selectin glycoprotein ligand 1 (PSGL-1) is expressed on adherent leukocytes and serves as a platform to recruit more activated platelets.[8] Interactions of platelet P-selectin with PSGL-1 or other P-selectin ligands presented by cells at the injured area contribute to further platelet accumulation.[8] In mice, deletion or blockade of P-selectin or PSGL-1 inhibits this platelet accumulation and leukocyte adhesion, thereby suppressing the formation of arterial neointima.[9–11] The roles of P-selectin and PSGL-1 in neointima formation have also been validated in other models of vascular injury.[12,13]

PSGL-1 contains sialylated and fucosylated oligosaccharides (O-glycans).[8,14] This O-glycan structure is crucial for the optimal binding of PSGL-1 to selectins.[15,16] Core2 1 to 6-N-glucosaminyltransferase-I (C2GlcNAcT-I), an intracellular enzyme in leukocytes, is responsible for the O-glycosylation of PSGL-1.[17] C2GlcNAcT-I is important for the recruitment of Ly-6C^{hi} mouse inflammatory monocytes to

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From the Department of Medicine (H.W., W.Z., R.T., R.P.H., C.Z., Y.H.), University of Minnesota, Minneapolis; the Department of Hematology (M.A.K.), Children's Hospital of Philadelphia, Pa; the Department of Anesthesiology (C.Z.), New Jersey Medical School, University of Medicine and Dentistry of New Jersey, Newark; the Department of Cellular and Molecular Medicine and the Howard Hughes Medical Institute (J.D.M.), University of California San Diego, La Jolla; and Glycobiology/Carbohydrate Chemistry Program (M.F.), Burnham Institute for Medical Research, La Jolla, Calif.

Correspondence to Yuqing Huo, MD, PhD, Cardiovascular Division and Vascular Biology Center, Department of Medicine, University of Minnesota, 420 Delaware St SE, MMC508, Minneapolis, MN 55455. E-mail yuqing@umn.edu, or Chuhong Zhu, MD, PhD, Department of Anatomy, Third Medical University, Gao Tan Yan Street, Shaping Ba District, Chongqing, China, 400038. E-mail zhuxx319@umn.edu.

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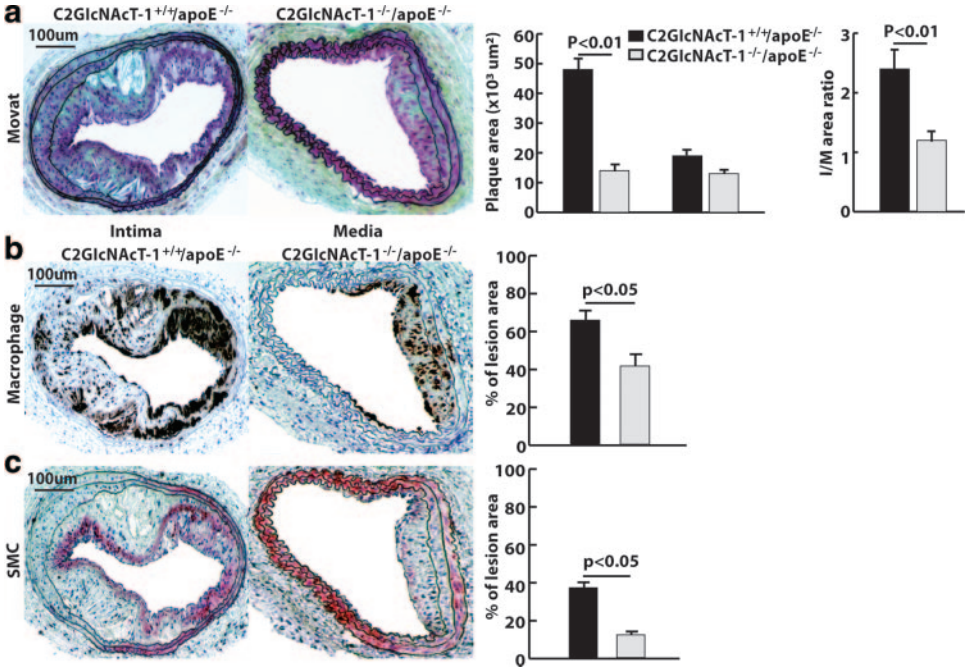

Figure 1. C2GlcNAcT-I deficiency suppresses injury-induced arterial neointima formation. a, Movat staining and size quantification of neointima (I), media (M), and ratio of intima to media. b, Anti–Mac-2 staining of infiltrated macrophages. c, Anti–α-actin staining of smooth muscle cells (SMCs). Data from 12 sections of 12 mice.

arterial vessel walls and the formation of atherosclerotic lesions.[17] However, the role of C2GlcNAcT-I in the regulation of platelet accumulation, leukocyte recruitment, and neointima formation in injured arteries in vivo has yet to be clarified.

We bred C2GlcNAcT-I–deficient mice with apolipoprotein E–deficient atherosclerotic mice to generate double knockout mice (C2GlcNAcT-I$^{-/-}$/apoE$^{-/-}$) and their controls. Using these mice, we investigated the effect of loss of C2GlcNAcT-I on leukocyte and platelet accumulation, endothelial regeneration at injured areas of arteries, and the formation of arterial neointima. Preliminary data from these mice revealed an important role for platelet–leukocyte interactions in endothelial regeneration. To further investigate the molecular mechanisms involved in this process, we used platelet factor 4 (PF4)-deficient mice (PF4$^{-/-}$) to study the role of PF4 in endothelial regeneration and neointima formation after arterial injury.

Methods

C2GlcNAcT-I$^{-/-}$[18] and PF4$^{-/-}$[19] mice were first crossed with C57BL/6J mice for more than 10 times, then bred with apoE$^{-/-}$ mice to generate double-knockout mice and their littermate controls. Carotid arteries of these mice were injured using a guide wire according to a protocol approved by the University of Minnesota Institutional Animal Care and Use Committee. Detailed methods are available in the supplemental material (available online at http://atvb.ahajournals.org).

Results

Formation of Injury-Induced Arterial Neointima in C2GlcNAcT-I–Deficient Mice

To determine the role of C2GlcNAcT-I in the formation of arterial neointima, carotid arteries of C2GlcNAcT-I$^{-/-}$/apoE$^{-/-}$ mice and littermate C2GlcNAcT-I$^{+/+}$/apoE$^{-/-}$ mice were injured with a guide wire. Four weeks later, the carotid arteries were excised and processed for analysis. Injured carotid arteries from C2GlcNAcT-I$^{-/-}$/apoE$^{-/-}$ mice exhibited neointima 4 to 6 times smaller than the neointima of C2GlcNAcT-I$^{+/+}$/apoE$^{-/-}$ mice (Figure 1a). Additionally, the number of macrophages (Figure 1b) and smooth muscle cells (Figure 1c) in the neointima of injured arteries from C2GlcNAcT-I$^{-/-}$/apoE$^{-/-}$ mice was reduced by 45% and 75%, respectively, compared with those from C2GlcNAcT-I$^{+/+}$/apoE$^{-/-}$ mice. No difference was found in collagen content in neointima of both types of mice (supplemental Figure I). Notably, only one-third of arteries from these mice showed neointima and media growth, which was very minor.

Interactions of C2GlcNAcT-I–Deficient Leukocytes With Activated Platelets and Injured Arteries

Using an ex vivo microflow perfusion chamber with a shear stress of 1 dyn/cm^2, we measured the tethering, rolling, and adherence of wild-type (wt) leukocytes to a surface coated with activated platelets. The number of wt leukocytes that rolled or adhered increased over time. After 5 minutes of perfusion, the number of rolling and adhering wt leukocytes was 800±70 per mm^2 and 700±20 per mm^2, respectively. By contrast, the number of rolling and adhering C2GlcNAcT-I–deficient leukocytes after 5 minutes of perfusion was only 200±20 per mm^2 and 180±12 per mm^2, respectively (Figure 2a). C2GlcNAcT-I–deficient leukocytes rolled with higher velocities than wt leukocytes did (data not shown).

We used in vivo mouse models of carotid artery and femoral artery injury and intravital epifluoresence microscopy to examine the interactions of leukocytes and platelets with injured vessel walls. Using a 10× objective, we imaged injured mouse carotid arteries and observed that leukocytes (labeled with rhodamine 6G) rolled and adhered to the injured area in C2GlcNAcT-I$^{+/+}$/apoE$^{-/-}$ mice. However, in C2GlcNAcT-I$^{-/-}$/apoE$^{-/-}$ mice these interactions were almost completely eliminated (Figure 2b). Using a 40× objective, we imaged rhodamine 6G-labeled platelets and leuko-

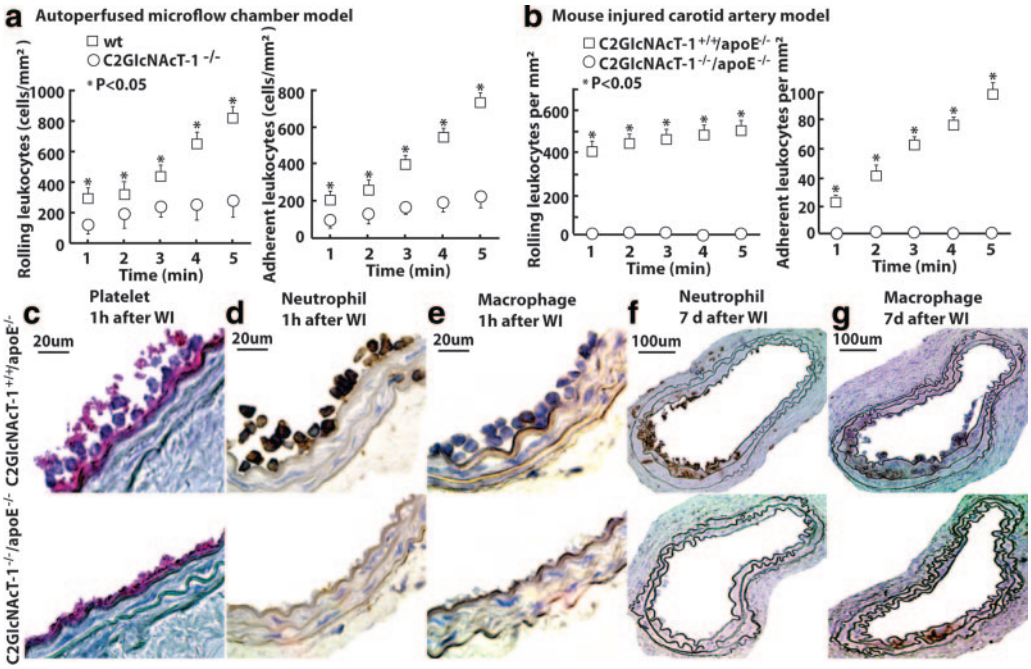

Figure 2. C2GlcNAcT-I deficiency suppresses leukocyte rolling and adhesion. a, Leukocyte rolling and adhesion on activated platelets through microflow chambers. b, Leukocyte rolling and adhesion in injured carotid arteries. c to g, Carotid arteries after wire injury (WI) stained for platelets, neutrophils, and macrophages (n=5).

cytes in injured mouse femoral arteries. In C2GlcNAcT-I$^{+/+}$/apoE$^{-/-}$ mice, platelets appeared as small weakly fluorescent spots and leukocytes appeared as bright/large spots (supplemental Figure II, left). By contrast, spots for leukocytes were almost completely absent and spots for platelet were dramatically reduced in the injured femoral arterial walls of C2GlcNAcT-I$^{-/-}$/apoE$^{-/-}$ mice (supplemental Figure II, right).

To further distinguish platelets and leukocytes adherent on injured arteries, we immunostained cross sections of injured arteries using specific markers. At 1 hour after arterial injury in apoE$^{-/-}$ mice, the denuded luminal surface was covered with platelets and leukocytes, and many platelets bound to the surface of adherent leukocytes (Figure 2c, upper panel). Nearly all of the adherent leukocytes were classified as neutrophils (Figure 2d, upper panel); monocytes were rare (Figure 2e, upper panel). In C2GlcNAcT-I$^{-/-}$/apoE$^{-/-}$ mice, a layer of platelets accumulated on the injured arterial wall, but no leukocytes adhered to these platelets (Figure 2c to 2e, lower panels). At 7 days after wire injury, many neutrophils and macrophages in apoE$^{-/-}$ mice, but only a few in C2GlcNAcT-I$^{-/-}$/ apoE$^{-/-}$ mice, adhered to or infiltrated the injured arterial walls (Figure 2f and 2g).

Endothelial Regeneration in the Injured Arteries in C2GlcNAcT-I–Deficient Mice

Reendothelialization is an important deterrent to neointima formation.[20] We used quantitative Evans blue staining to compare the regeneration of endothelial cells on the injured areas of arteries from atherosclerotic mice with and without C2GlcNAcT-I. In the arteries of C2GlcNAcT-I$^{+/+}$/apoE$^{-/-}$ mice, reendothelialization after wire-induced arterial injury was only 18%, 41%, and 81% at 3, 5, and 7 days, respectively; for arteries of C2GlcNAcT-I$^{-/-}$/apoE$^{-/-}$ mice, however, reendothelialization was 30%, 61%, and 95% (Figure 3a). We also obtained micrographs of cross-sections of injured carotid arteries that were immunostained with anti-CD31 or anti–VE-cadherin. At 3, 5, and 7 days after injury, a greater luminal circumferential area stained positively for these endothelial markers in arteries of C2GlcNAcT-I$^{-/-}$/apoE$^{-/-}$ mice than in C2GlcNAcT-I$^{+/+}$/apoE$^{-/-}$ arteries (Figure 3b).

PF4 and Reendothelialization of Injured Arteries

Recombinant platelet factor 4 (PF4) inhibits endothelial cell proliferation,[21] so we immunostained the injured arteries for PF4. Nearly all areas where platelets accumulated in the injured arteries of C2GlcNAcT-I$^{+/+}$/apoE$^{-/-}$ mice stained positively for PF4, and staining on the surface of adherent leukocytes (where platelets bound) was very robust (Figure 4a). By contrast, PF4 staining was much weaker in the injured arteries of C2GlcNAcT-I$^{-/-}$/apoE$^{-/-}$ mice. This was further confirmed with the results of Western blots. These indicated that PF4 accumulation was much greater in the injured carotid arteries of C2GlcNAcT-I$^{+/+}$/apoE$^{-/-}$ mice than in C2GlcNAcT-I$^{-/-}$/apoE$^{-/-}$ mice (Figure 4b).

To directly address the effect of platelet-released PF4 on endothelial cell proliferation, we used an in vitro model of endothelial wound repair. A sterile pipette tip dragged across confluent endothelial cell monolayers created 1-mm cell-free wounds that recovered nearly completely by ≈12 hours (Figure 4c). This process was not affected by the presence of neutrophils (data not shown). The addition of activated PF4$^{+/+}$ platelets to the wounded endothelial cell monolayers significantly suppressed the recovery (Figure 4d, left panel). Furthermore, the suppression of recovery was amplified by

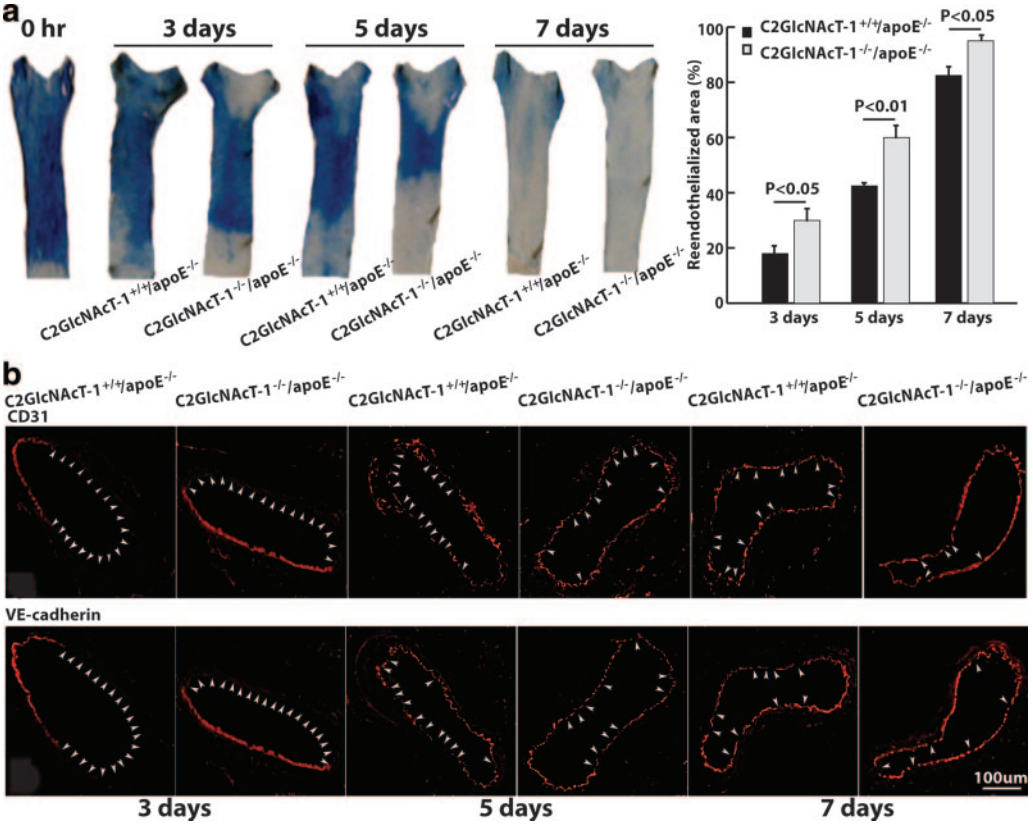

Figure 3. C2GlcNAcT-I deficiency accelerates endothelial regeneration. a, Evans blue staining of injured carotid arteries shows the areas that were not covered with newly generated endothelial cells. b, Representative micrographs of cross-sections of injured carotid arteries immunostained with anti-CD31 or anti–VE-cadherin. Arrowheads show the areas negative for staining.

adding a mixture of isolated neutrophils and activated $PF4^{+/+}$ platelets (Figure 4e, left panel). By contrast, the addition of either activated $PF4^{-/-}$ platelets or a mixture of activated $PF4^{-/-}$ platelets with isolated neutrophils suppressed the recovery of the wounded area with endothelial cells to a much lesser extent than did $PF4^{+/+}$ platelets or a mixture of both $PF4^{+/+}$ platelets and neutrophils, respectively (Figure 4d and 4e, right panels, and Figure 4f for quantitative data).

Analyses of 5-bromodeoxyuridine (BrdU) incorporation into endothelial cells showed that the addition of neutrophils to the wounded monolayers did not significantly affect endothelial cell proliferation. However, the addition of activated $PF4^{+/+}$ platelets inhibited endothelial cell proliferation, and this inhibition was enhanced by the addition of a mixture of activated $PF4^{+/+}$ platelets with isolated neutrophils. Whenever $PF4^{-/-}$ platelets were added, the suppression of endothelial cell proliferation was significantly reduced (Figure 4g). These experiments indicated that PF4 from activated platelets, especially in a context of platelet-neutrophil interactions, inhibited endothelial cell proliferation.

Endothelial Regeneration and Neointima Formation in Injured Arteries in PF4-Deficient Mice

We used $PF4^{-/-}$ mice to determine the role of platelet PF4 in endothelial regeneration and neointima formation after arterial injury in vivo. Using the 10× objective of the epifluorescence intravital microscope, we imaged injured carotid mouse arteries and observed leukocyte interactions with the injured vessels. No difference was observed in the number of rolling and adhering leukocytes in the carotid arteries of $PF4^{-/-}$ mice and wt mice (supplemental Figure IIIa). In the mouse femoral artery injury model, using the 40× objective of the epifluorescence intravital microscope we observed similar platelet accumulations and leukocyte rolling and adhesion on the injured arteries of $PF4^{-/-}$ mice and wt mice (supplemental Figure IV). In an atherosclerotic background, PF4 immunostaining was performed 1 hour after wire injury of arteries from $PF4^{-/-}/apoE^{-/-}$ mice and $PF4^{+/+}/apoE^{-/-}$ mice. Consistently, PF4 staining was positive on platelets accumulated on injured arteries of $PF4^{+/+}/apoE^{-/-}$ mice and negative on those of $PF4^{-/-}/apoE^{-/-}$ mice (supplemental Figure IIIc). Platelet accumulation and neutrophil adhesion were observed on injured arteries from both $PF4^{-/-}/apoE^{-/-}$ and $PF4^{+/+}/apoE^{-/-}$ mice, and there was no significant difference between the numbers of platelets and neutrophils on the injured arteries from these mice (supplemental Figure IIIb and IIId to IIIe).

Staining of wire-injured carotid arteries with Evans blue showed that at 5 days after injury, reendothelialization was 65% in arteries of $PF4^{-/-}/apoE^{-/-}$ mice but only 40% in arteries of $PF4^{+/+}/apoE^{-/-}$ mice (Figure 5a). Relative to $PF4^{+/+}/apoE^{-/-}$ mice, arterial neointima formation after wire injury was significantly reduced by 35% in $PF4^{-/-}/apoE^{-/-}$ mice, and the size of the media in injured arteries was also reduced, although the difference was not statistically

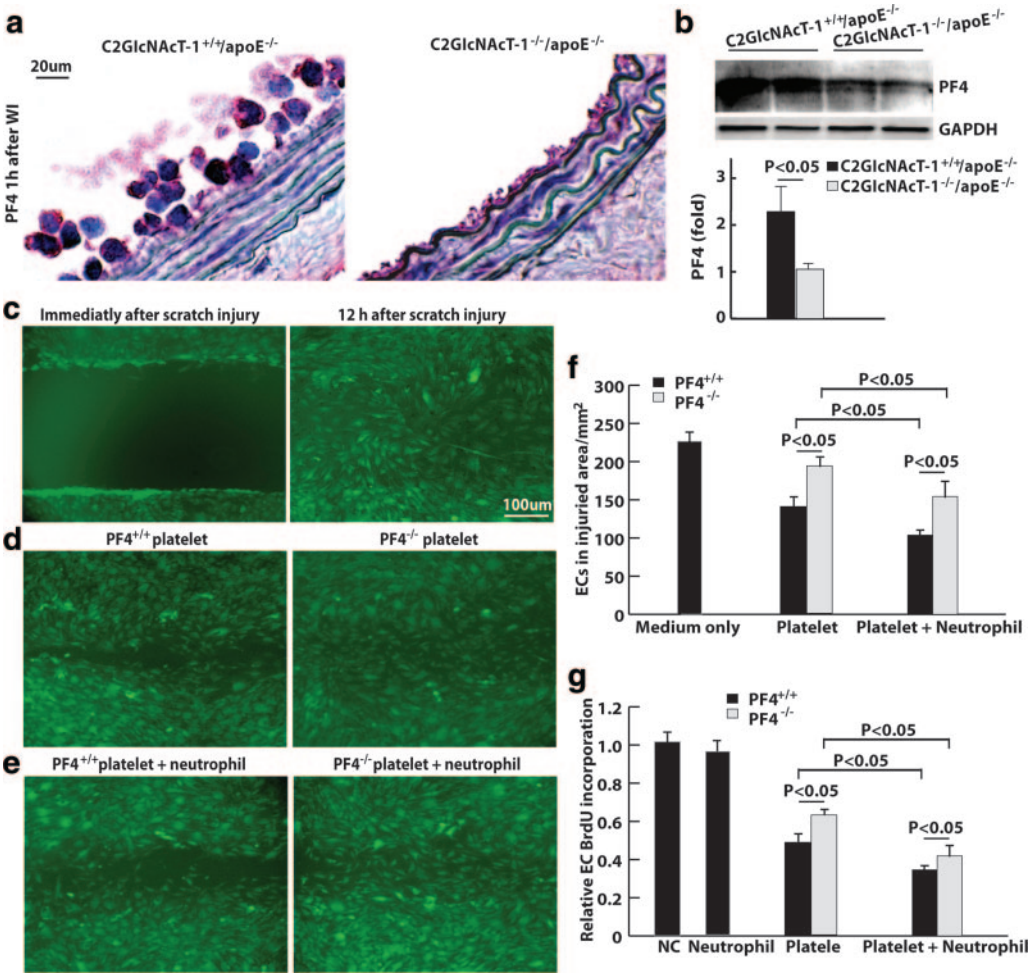

Figure 4. PF4 inhibits endothelial proliferation in the context of platelet-neutrophil interactions. a and b, Anti-PF4 immunostaining (red) and Western blot of injured carotid arteries. c to f, The covering with proliferating endothelial cells (ECs) 12 hours after wounding. g, BrdU incorporation under the above conditions (c to e).

significant (Figure 5b). The number of macrophages and smooth muscle cells in neointima of $PF4^{-/-}/apoE^{-/-}$ mice was reduced by 28% and 25%, respectively, compared to that of $PF4^{+/+}/apoE^{-/-}$ mice (Figure 5c and 5d).

Discussion

This study demonstrates that C2GlcNAcT-I is critical for the formation of injury-induced arterial neointima in atherosclerotic mice. Compared to control mice, C2GlcNAcT-$I^{-/-}$/$apoE^{-/-}$ mice were almost completely incapable of developing significant arterial neointima after wire-induced injury. The inhibition of leukocyte recruitment and rapid endothelialization of the vessel wall are important cellular mechanisms for this protection.

The compromised binding of selectin ligands in C2GlcNAcT-$I^{-/-}$ mice contributes to the protective effect of C2GlcNAcT-I deficiency on neointima formation. C2GlcNAcT-I deletion reduces neutrophil binding to P-, E-, and L-selectin both in vitro and in vivo.[18,22,23] Recently, we also demonstrated decreased binding of monocytes to these selectins.[17] Other C2GlcNAcT-I–modified molecules such as CD34, CD43, and CD44 are speculated to be responsible for the leukocyte homing phenotype of C2GlcNAcT-$I^{-/-}$ mice.[24] However, in CD34- or CD43-deficient mice, there is no change in the recruitment of

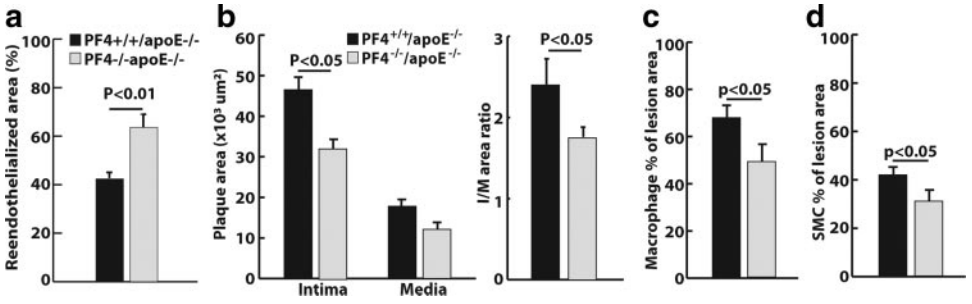

Figure 5. PF4 deficiency accelerates endothelial cell regeneration and suppresses neointima formation. a, Quantification of Evans blue staining of carotid arteries 5 days after injury. b, Quantification of Movat staining 4 weeks after injury. c, Quantification of anti–Mac-2 staining of infiltrated macrophages. d, Quantification of anti–α-actin staining of SMCs.

neutrophils and monocytes to inflammatory sites.[25,26] Also, the binding of CD44 with its major ligand, hyaluronic acid, is not affected by C2GlcNAcT-I deletion.[17] Therefore, C2GlcNAcT-I must affect leukocyte homing through its modification of ligands for P-, E-, and L-selectins.

Our current work illustrates the dynamics of leukocyte interactions with injured arterial areas. Within the first few days of wire-induced injury, the injured arterial area is usually predominated by neutrophils and subsequently by a mixture of neutrophils and monocytes. By approximately 1 week after injury, the injured area is predominated by monocytes (data not shown), consistent with previous studies.[27,28] It has been well established that monocyte infiltration plays an important role in the formation of neointima. Ly-6C^{hi} monocytes—important contributors to the formation of spontaneous lesions and arterial neointima—express high levels of PSGL-1 and bind selectins with high affinity.[10] Without PSGL-1, Ly-6C^{hi} monocytes cannot be effectively recruited to injured vessel walls, leading to the formation of a much smaller neointima.[10] In C2GlcNAcT-I$^{-/-}$ mice, there is a significant defect in the binding function of selectin receptors, including PSGL-1.[17] Consistent with these results, we observed that far fewer monocytes infiltrated the injured vessel wall of C2GlcNAcT-I$^{-/-}$/apoE$^{-/-}$ mice relative to apoE$^{-/-}$ mice. This may be one of the underlying mechanisms for reduced arterial neointima in C2GlcNAcT-I$^{-/-}$/apoE$^{-/-}$ mice.

Neutrophil adhesion to injured arterial areas has been long observed, but the role of neutrophils in neointima formation is uncertain.[27,29] Some studies have suggested that neutrophils directly contribute to neointima formation.[29,30] Interestingly, we found that compared to neutrophil-covered injured arterial areas of apoE$^{-/-}$ mice, elimination of neutrophils at the injured area in C2GlcNAcT-I$^{-/-}$/apoE$^{-/-}$ mice promoted endothelial regeneration at the injured area. In addition, injured C2GlcNAcT-I$^{-/-}$/apoE$^{-/-}$ arteries attracted fewer platelets and had reduced accumulation of PF4. In vitro experiments indicated that the observed inhibition of endothelial regeneration could be attributed to the increased presence of platelets and platelet-released PF4.

Endothelial recovery after percutaneous transluminal coronary intervention is crucial in the prevention of arterial restenosis.[31] Slow endothelial regeneration leads to an increase in platelet accumulation, leukocyte adherence, and released inflammatory factors from leukocytes and platelets. These pathologies initiate and aggravate the inflammatory response in injured arterial wall.[31] Recombinant PF4 is a well-established potent antiangiogenic factor.[21,32] Platelets release PF4 in large quantities,[33] but they also release many other factors, including certain angiogenic factors. Consequently, the role of platelet-released PF4 within the context of the total platelet content has been uncertain with regard to endothelial cell proliferation.[21] Our in vitro and in vivo studies are the first to demonstrate that platelet-released PF4 has a significant inhibitory effect on endothelial proliferation, especially in the context of neutrophil–platelet interactions. There are several possible explanations for this. First, as shown in Figure 4a, neutrophils adhering to the injured area provide a platform for more platelets to bind. Without neutrophils, very few platelets and less PF4 accumulate at the injured site. Second, PSGL-1 on neutrophils may interact with P-selectin on platelets to mediate outside-in signaling so as to activate platelets to release more PF4 from their α-granules. In P-selectin–deficient mice, platelets that accumulate on the injured arterial area are less compact,[34] indicating that platelets are not fully activated in the absence of P-PSGL-1–mediated platelet–leukocyte interactions. Third, neutrophils may release enzymes to cleave PF4 to optimize its antiangiogenic function or release other factors that synergize with PF4 in its antiangiogenic effect.[35] A variety of PF4-derived peptides inhibit endothelial cell proliferation much more potently than full-length PF4.[36]

Neutrophil adhesion at injured areas of PF4$^{-/-}$/apoE$^{-/-}$ arteries was not decreased compared with PF4$^{+/+}$/apoE$^{-/-}$ arteries. This is consistent with the early observation showing that PF4 is devoid of chemotactic activity for neutrophils.[37] In PF4$^{-/-}$/apoE$^{-/-}$ arteries, there was an increase of endothelial regeneration at injured areas. This may be the predominant underlying mechanism for decreased neointima formation in PF4$^{-/-}$/apoE$^{-/-}$ mice, but other mechanisms may also be involved. PF4 released from platelets may bind to newly regenerated endothelium and promote monocyte recruitment. Platelet PF4 may bind to RANTES, and these chemokines cooperate to recruitment monocytes to injured vessel wall.[38,39] Indeed, less accumulation of RANTES was found in the injured area of PF4$^{-/-}$/apoE$^{-/-}$ mice than PF4$^{+/+}$/apoE$^{-/-}$ mice (supplemental Figure V). PF4 also can activate endothelial cells,[40] and activation of newly regenerated endothelial cells on the injured area may be partially responsible for neointima formation in injured arteries of apoE$^{-/-}$ mice. In PF4$^{-/-}$/apoE$^{-/-}$ mice, the level of inflammation of regenerated endothelial cells may be low as a result of PF4 absence. Additionally, protection from arterial injury in PF4$^{-/-}$/apoE$^{-/-}$ mice may also be attributed to the high level of HDL. Consistent with the results in a previous report,[41] PF4$^{-/-}$/apoE$^{-/-}$ mice in this study also had a higher level of HDL than PF4$^{-/-}$/apoE$^{-/-}$ mice (data not shown).

Collectively, our data demonstrate that C2GlcNAcT-I is a promising therapeutic target to curb the formation of arterial neointima. Inhibition of C2GlcNAcT-I compromises selectin receptor function, resulting in suppression of leukocyte and platelet accumulation on the vessel wall. In addition, our results reveal an adverse effect of platelet-released PF4 on endothelial regeneration, suggesting that anti-PF4 treatment might have the beneficial effect of inhibiting arterial neointima formation.

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Disclosures

None.

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Figure I

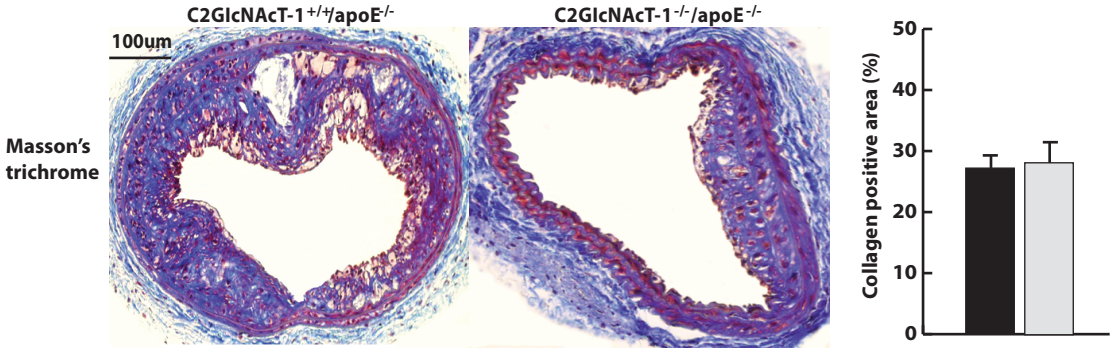


Figure II

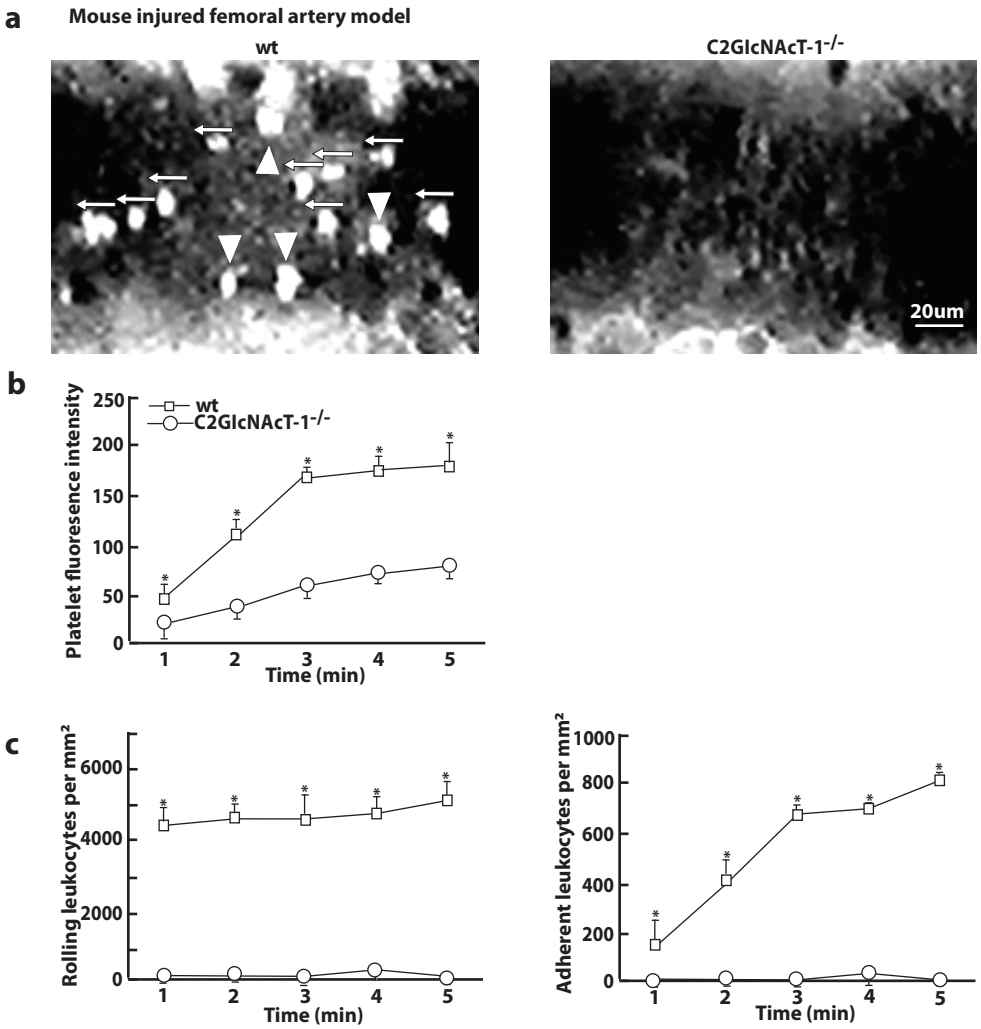


Figure III

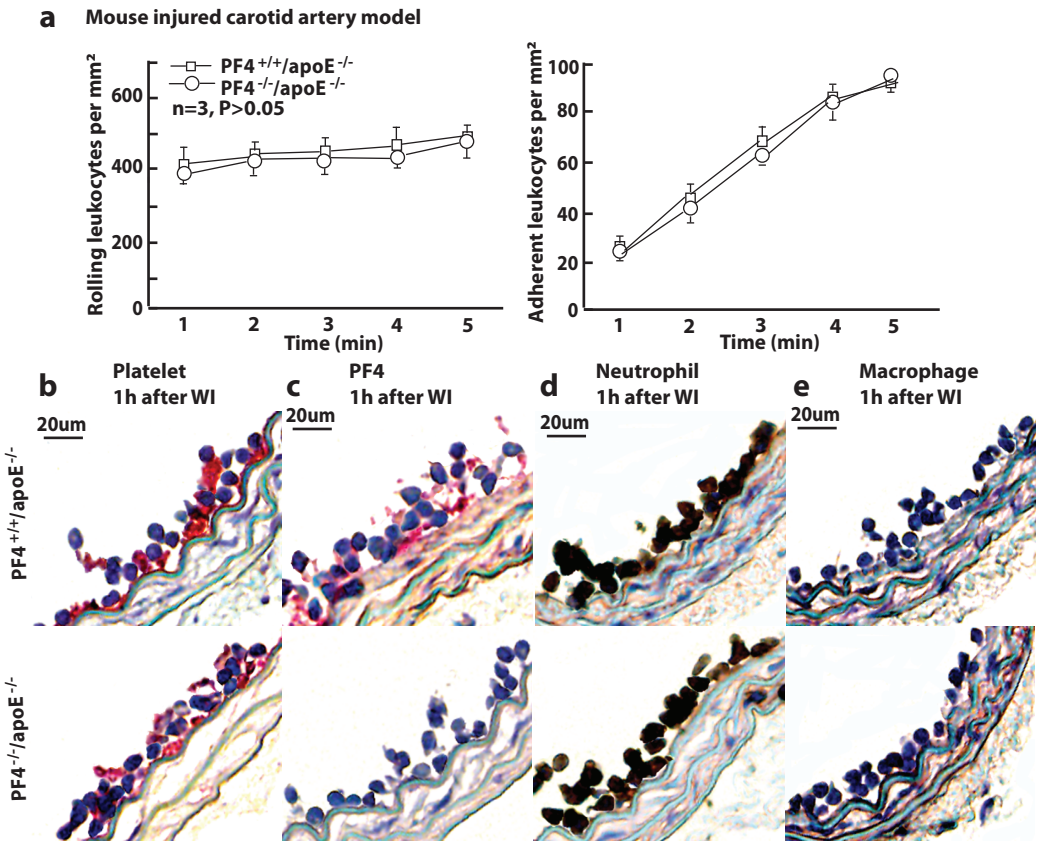


Figure IV

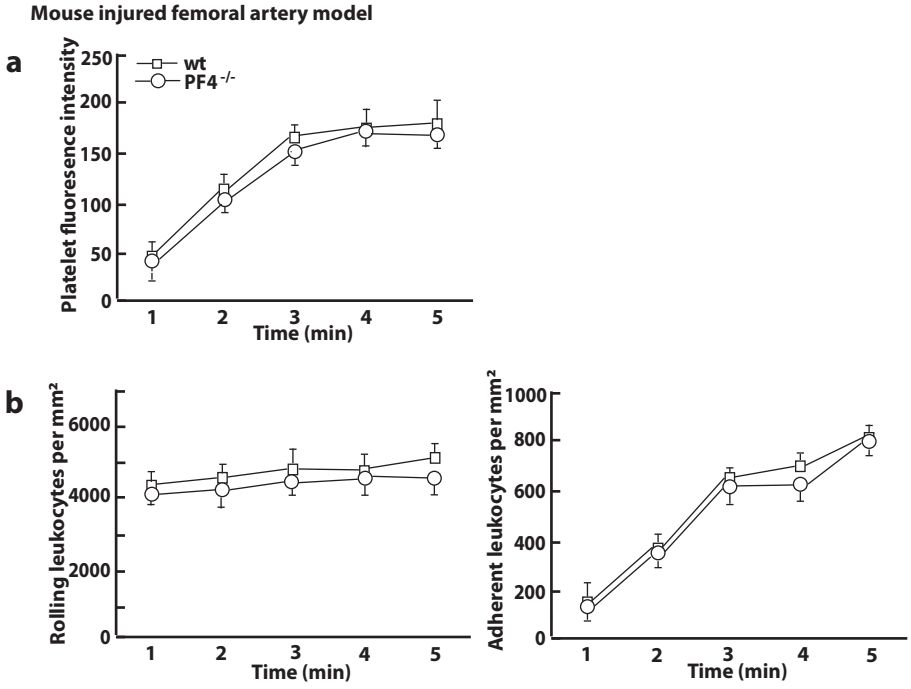
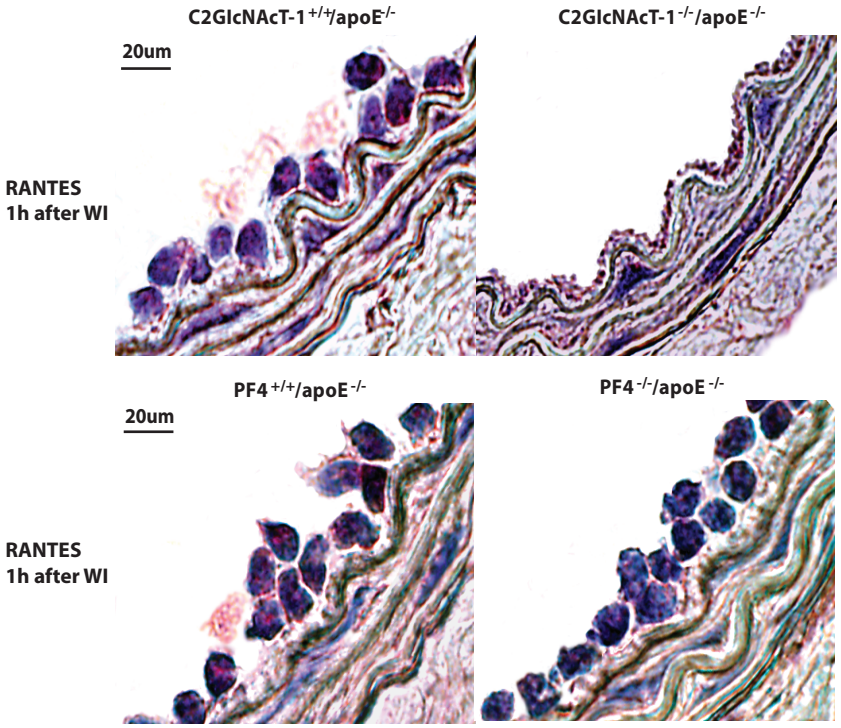


Figure V



Supplemental Materials

Materials and Methods

Mice and mouse carotid artery wire injury model

C2GlcNAcT-I–/– [1] and PF4–/– [2] mice, which have been backcrossed with C57BL/6J mice for more than 10 times, were bred with apoE–/– mice from The Jackson Laboratory (Bar Harbor, ME) to generate C2GlcNAcT-I–/–/apoE–/– mice and C2GlcNAcT-I+/+/apoE–/– littermates, and PF4–/–/apoE–/– mice and PF4+/+/apoE–/– littermates for this study. Eight-week-old male mice were fed a Western diet containing 21% fat, 0.15% cholesterol, and 19.5% casein without sodium cholate for 2 weeks prior to wire injury of arteries. Mice were maintained on the same diet until euthanization.

The arterial wire injury was performed as described.[3] Briefly, mice were anesthetized using an intraperitoneal injection of ketamine (80 mg/kg body weight) and xylazine (5 mg/kg) (Phoenix Scientific, Inc., St. Joseph, MO). After midline neck incision, the left external carotid artery was tied off distally and a 0.014-inch flexible angioplasty guide wire was advanced by 1 cm along the common carotid artery via transverse arteriotomy. Complete and uniform endothelial denudation was achieved by five passes with a rotating motion. At different time points after injury, mice were anesthetized and perfused *in situ* using 4% paraformaldehyde at 100 mm Hg for tissue fixation. Injured arteries were excised and embedded in paraffin. All animal experiments and care were approved by the University of Minnesota Animal Care and Use Committee, in accordance with AAALAC guidelines.

Quantitative immunohistochemistry and immunofluorescence

Serial sections (5 μm) were stained with Movat pentachrome (Sigma, St. Louis, MO). For quantitative comparisons, 10 sections were analyzed from each animal, each section within a

standardized distance (1200 μm) from the bifurcation to the common carotid artery. The areas of the lumen, internal elastic lamina, and external elastic lamina were determined by planimetry using NIH Image software. Plaque, medial, overall vessel area, and intima/media ratio were calculated.

To determine the cellular components of the injured vessel wall, arterial cross sections were stained with monoclonal antibodies to identify platelets (MWReg30; Santa Cruz Biotechnology, Santa Cruz, CA), macrophages (Mac-2, clone M3/38; Accurate Chemical, Westbury, NY), neutrophils (anti-mouse neutrophil, clone 7/4; Accurate Chemical), smooth muscle cells (alkaline phosphatase–conjugated α-actin, clone 1A4; Sigma), and endothelial cells (CD31, clone M-20; and VE-cadherin polyclonal antibody, Santa Cruz Biotechnology). A rabbit anti–mouse PF4 (Bethyl Laboratories, Montgomery, TX) and a rabbit anti-mouse RANTES (Perpotech Inc. Rocky Hill, NJ) were used to determine the presence of these chemokines in the injured area of mouse carotid arteries. Specific antibody staining was visualized by using an avidin/biotin peroxidase-linked detection system (Vector Laboratories, Burlingame, CA), Fast Red Substrate (Dako, Carpinteria, CA), or a secondary rhodamine (TRITC)-conjugated antibody (Jackson Immunoresearch, West Grove, PA). Re-endothelialization of the luminal surface was expressed as the percentage of CD31-positive or VE-cadherin-positive luminal lining over the total luminal circumference.

Leukocyte interactions with activated platelets under flow conditions

Mouse platelets were isolated by using a gel-filtration method[4] and then loaded into a rectangular glass capillary tube at a concentration of 2×10^9/ml. After 1 h, the tube was gently perfused with PBS to wash away platelets not adhering on the tube internal wall, followed with a loading of PBS containing thrombin (0.1 U/ml; Sigma) at 37°C for 10 min. Extra thrombin was neutralized

with an equimolar dose of hirudin (Sigma). The tube was washed with PBS before placed in the setup of the flow chamber.

A rectangular glass coated with activated platelets was connected with silastic tubing (ID 0.58 mm, OD 0.965 mm; Dow Corning Corp., Midland, MI) at each end and then connected to a mouse carotid artery and a jugular vein with a silastic PE 10 tube to form an auto-perfused micro-flow chamber.[5] Each mouse was anesthetized and placed on a 37°C heating pad. A calculated volume of 1 mg/ml rhodamine 6G/PBS (50 µl/30 g mouse weight) was perfused into the mouse via the jugular catheter. Ten minutes after the perfusion of rhodamine 6G, the mouse was used for the micro-flow chamber study. When blood from the carotid artery was perfused through the micro-flow chamber, microscopic observation was made on an intravital epifluorescence microscope (Axioskop, Carl Zeiss, Inc., NY) with a saline immersion objective (SW 20/0.5). Recordings were made through a CCD camera (model VE-1000CD, Dage-MTI, Michigan City, IN) on a Panasonic S-VHS recorder. For each experiment, leukocyte interactions with activated platelets were recorded for 5 min.

Leukocyte interactions with the injured arterial wall in vivo

A carotid artery wire injury model and a mouse femoral artery ligation injury model were used. In brief, mice were first anesthetized and intravenously injected with rhodamine 6G. After 10 min, either mouse left carotid arteries were injured with a guide wire or the femoral arteries were ligated vigorously for 5 min to induce vascular injury. With an intravital epifluorescence microscopy system, 10X and 40X objectives were used to view the injured carotid and femoral arteries. The leukocyte and platelet interactions with the injured vessel were recorded and analyzed as described.[7]

Evans Blue staining of injured arteries

Endothelial regeneration on the injured arterial area was evaluated by staining of the denuded areas with Evans blue dye (Sigma). Mice were anesthetized then injected intravenously with 300 µl of saline containing 2% Evans blue. Ten minutes later, mice were euthanized, followed by perfusion of 4% paraformaldehyde/PBS for 5 min. The injured artery was opened longitudinally and placed *en face* on Parafilm. The Evans blue-stained luminal area indicated the area not covered with endothelial cells. The endothelial regenerated area was calculated as the percentage of the non-blue area over the total injured luminal surface of the artery.

Endothelial wound injury repair assay

Mouse aortic endothelial cells were isolated and cultured as described.[8] Cells in Dulbecco's Modified Eagles Medium containing 10% fetal bovine serum were grown to confluency in 12-well culture plates and labeled with Calcein AM (Molecular Probes, Invitrogen, Carlsbad, CA). Endothelial wounds were induced by dragging a sterile pipette tip across the cell monolayers to create a 1-mm cell-free path.

Mouse platelets were isolated and activated as described above. Mouse neutrophils were isolated using mouse neutrophil-specific anti-Ly-6G and magnetic columns (MACS; Miltenyi Biotec, Auburn, CA). Activated platelets (10^8/well of a 12-well culture plate) or a mixture of activated platelets with neutrophils (10^8 activated platelets plus 10^6 neutrophils per well of a 12-well culture plate) were added for 12 h after injury. The growth of Calcein AM–labeled endothelial cells was observed and imaged through an Olympus BH-2 system with a Dage-MTI DC-330 color camera (Dage-MTI). In this way, endothelial cells, but not added platelets and neutrophils, were observed and analyzed microscopically.

Endothelial cell proliferation assay

Mouse aortic endothelial cells were seeded in 96-well plates. When ~80% confluent, the cells

were incubated with 5-bromodeoxyuridine (BrdU, 10 μM), and either mouse neutrophils (2×10^5/well), platelets (2×10^7/well), or both were added for 12 h at 37°C in a 5% CO_2 atmosphere. Cells were fixed, denatured, and incubated with a biotinylated antibody against BrdU (Zymed BrdU staining kit, Zymed Laboratories, South San Francisco, CA) followed by addition of the streptavidin-fluorescein isothiocyanate substrate. Absorbance was measured at 530 nm.

Blood lipid and leukocyte analyses

Plasma triglycerides, LDL, HDL, and total cholesterol were determined using an automated enzymatic technique (Boehringer Mannheim GmbH, Mannheim, Germany). The number of total and differential leukocytes was measured from an aliquot of 20 μl of blood using an automated blood cell counter (Hemavet 850FS, CDC Technologies, Oxford, CT).

Statistical analyses

Statistical analyses were performed with Instat software (GraphPad Software). Data are presented as the mean ± SE. Data were compared with either one-way ANOVA followed by the Bonferroni correction post-hoc test or Student t test to evaluate two-tailed levels of significance. The null hypothesis was rejected at $P < 0.05$.

Legends of supplementary figures

Figure I. Collagen content in injury-induced arterial neointima

Cross-sections of arterial neointima stained with Masson's trichrome and quantification of the collagen positive area (blue). The averaged percentages of collagen area in the neointima were obtained by analyzing 12 cross sections from 12 injured carotid arteries from mice.

Figure II. C2GlcNAcT-I deficiency suppresses leukocyte and platelet accumulation in injured femoral arteries

a, Platelet accumulation, leukocyte rolling and adhesion in injured mouse femoral arteries within the first 5 min after injury. Images were obtained from videotape recordings of the epifluorescence intravital microscopy study, illustrating rolling (←) and adhering (▲) leukocytes in the injured arteries of wt (left) and C2GlcNAcT-I–/– mice (right). The data points in **b** and **c** represent the means of three separate experiments. *$P < 0.05$, C2GlcNAcT-I–/– vs. wt mice.

Figure III. PF4 deficiency does not affect leukocyte-platelet interaction in the injured area of carotid arteries

a, The number of rolling leukocytes and adherent leukocytes in injured mouse carotid arteries at 5 min after injury. Data were obtained from videotape recordings of the epifluoresence intravital microscope with 10X objective. The data points represent the means of three separate experiments. **b** to **e**, Injured carotid arteries of PF4+/+/apoE–/– and PF4–/–/apoE–/– mice were collected at 1 h after wire injury (WI) and immunostained with antibodies specific for platelets (b), PF4 (c), neutrophils (d), and macrophages (e). Carotid arteries of five mice were analyzed

for each group.

Figure IV. PF4 deficiency does not affect leukocyte and platelet accumulation in injured femoral arteries

Platelet accumulation (a), leukocyte rolling and adhesion (b) in injured mouse femoral arteries within the first 5 min after injury. The data represent the means of three separate experiments. *P < 0.05, PF4–/– vs. wt mice.

Figure V. RANTES deposition in injured arteries of C2GlcNAcT-I+/+/apoE–/–, C2GlcNAcT-I–/–/apoE–/–, PF4+/+/apoE–/–, and PF4–/–/apoE–/– mice

Anti-RANTES immunostaining with an antibody specific for RANTES (red) of cross-sections of injured carotid arteries of C2GlcNAcT-I+/+/apoE–/–, C2GlcNAcT-I–/–/apoE–/–, PF4+/+/apoE–/–, and PF4–/–/apoE–/– mice. Carotid arteries were collected at 1 hour after wire injury.

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