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Objective—To use the mice deficient in both adenosine receptor A_{2A} ($A_{2A}R^{-/-}$) and apolipoprotein E ($apoE^{-/-}$) to investigate the role of $A_{2A}R$ in mediating the interactions of leukocytes with injured arterial walls and the formation of arterial neointima induced by a guide wire.

Methods and Results—In $apoE^{-/-}$ mice, $A_{2A}R$ deficiency increased the size of the arterial neointima in injured carotid arteries by 83%. Arterial neointima formation was also enhanced in chimeric mice that underwent bone marrow transplantation (these mice lacked $A_{2A}R$ in their bone marrow–derived cells). Epifluorescence intravital microscopy showed that neutrophil rolling and adherence to the injured arterial area were enhanced by 80% and 110% in $A_{2A}R^{-/-}/apoE^{-/-}$ mice, respectively. This phenomenon occurred even though the protein levels of homing molecules on $A_{2A}R$-deficient neutrophils were unchanged from those of wild-type neutrophils. $A_{2A}R$-deficient neutrophils exhibited an increase in the phosphorylation of p38 mitogen-activated protein kinase, P-selectin glycoprotein ligand-1 (PSGL-1) clustering, and the affinity of b_2 integrins. The inhibition of p38 phosphorylation abrogated the increased PSGL-1 clustering and β_2 integrin affinity, thus reversing the increased homing ability of $A_{2A}R$-deficient leukocytes.

Conclusion—$A_{2A}R$ plays a complex role in inflammation and tissue injury. The deficiency of $A_{2A}R$ enhances the homing ability of leukocytes and increases the formation of the arterial neointima after injury. $A_{2A}R$ antagonists are being tested for the treatment of neurodegenerative and other chronic diseases. An evaluation of the effect of $A_{2A}R$ antagonists on arterial restenosis after arterial angioplasty should be conducted. **(*Arterioscler Thromb Vasc Biol*. 2010;30:915-922.)**

Key Words: adhesion molecules ■ atherosclerosis ■ carotid arteries ■ leukocytes ■ restenosis

Adenosine receptor A_2 ($A_{2A}R$) is 1 of the 4 G-protein–coupled receptors for adenosine. It is present on many inflammatory cells, including neutrophils, monocytes, platelets, and all vascular cells.[1,2] $A_{2A}R$ plays different roles in inflammation and tissue injury under different conditions. In many acute inflammatory or injury models of peripheral organs, $A_{2A}R$ acts as an anti-inflammatory molecule. For example, loss of $A_{2A}R$ increases inflammatory responses and causes tissue damage in the liver, lung, and spleen in vivo,[3,4] whereas the activation of $A_{2A}R$ with agonists reduces inflammation and protects tissues from injury.[2] In contrast to the anti-inflammatory effect of $A_{2A}R$ in the acute injury or inflammatory models, the absence or blocking of $A_{2A}R$ appears to offer mice protection from chronic diseases, such as atherosclerosis and liver cirrhosis,[5,6] and neurodegenerative diseases.[7] Accordingly, many $A_{2A}R$ antagonists are being developed to treat neurological disorders, including Parkinson disease, and some of them are being evaluated in clinical trials.[8]

Arterial restenosis is a serious complication of angioplasty, including percutaneous transluminal coronary intervention.[9] In humans, vascular smooth muscle cells (VSMCs) predominate neointimal hyperplasia. However, it has been demonstrated that in human neointimal hyperplasia, the number of leukocytes in the neointima correlates with the severity of restenosis,[10] indicating the causal role of infiltrated leukocytes in the formation of arterial restenosis. To study the effect of infiltrated leukocytes on arterial neointima in patients with arterial restenosis, a model of wire-induced neointima formation in the mouse carotid artery has been described and widely used in the research field.[11] The inflammatory response, including platelet and leukocyte accumulation on the injured arterial areas, and smooth muscle cell migration are requisite for arterial neointima forma-

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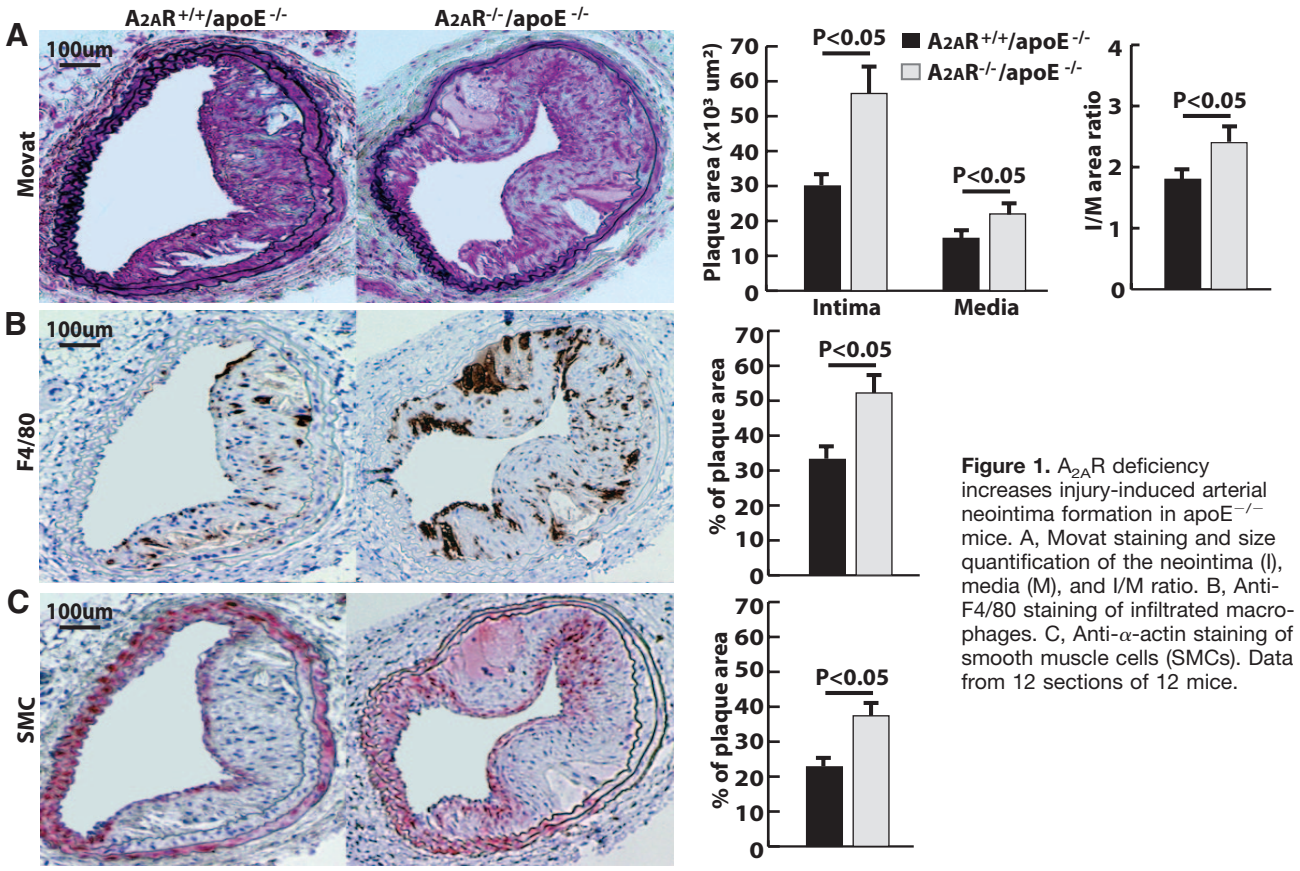

Figure 1. $A_{2A}R$ deficiency increases injury-induced arterial neointima formation in $apoE^{-/-}$ mice. A, Movat staining and size quantification of the neointima (I), media (M), and I/M ratio. B, Anti-F4/80 staining of infiltrated macrophages. C, Anti-α-actin staining of smooth muscle cells (SMCs). Data from 12 sections of 12 mice.

tion.[12–14] Immediately after arterial injury, platelets interact with the injured area via many factors, including glycoprotein Ib and glycoprotein IIb/IIIa.[15,16] On adherence, platelets become activated and orchestrate leukocyte recruitment and endothelial regeneration on the injured site.[17–19] Studies[20–22] have demonstrated that the formation of arterial neointima is significantly suppressed after the inhibition of platelet accumulation, leukocyte adhesion, and the improvement of endothelial regeneration on the injured area.

Many elderly patients with neurodegenerative diseases also have atherosclerotic coronary diseases. Therefore, the patients, who might take $A_{2A}R$ antagonists for the treatment of their neurological disease, could possibly need percutaneous transluminal coronary intervention for their coronary artery disease. Given this clinical scenario, it is relevant to study whether the blocking or inactivation of $A_{2A}R$ affects arterial repair after injury. To our knowledge, no reports have been published on the effects of the blocking or inactivation of $A_{2A}R$ on the formation of arterial neointima. In this study, we evaluated whether $A_{2A}R$ deficiency affects the injury-induced arterial neointima by using the mice deficient in both $A_{2A}R$ and apolipoprotein E ($A_{2A}R^{-/-}/apoE^{-/-}$).

Methods

$A_{2A}R^{-/-}$ mice in a C57BL/6J background[23] were bred with $apoE^{-/-}$ (C57BL/6J background) mice to generate $A_{2A}R^{-/-}/apoE^{-/-}$ mice and their littermate controls. Chimeric mice, with or without $A_{2A}R$ in their bone marrow–derived cells (BMDCs), were produced by bone marrow transplantation.[24] The 8-week-old mice were fed a Western diet containing 21% fat, 0.15% cholesterol, and 19.5% casein without sodium cholate for 2 weeks before arterial injury, which was induced by a guide wire; the same diet was maintained after wire injury and until euthanization.[21] Leukocyte interactions with the injured arteries were examined in vivo by intravital epifluorescence microscopy or ex vivo on a coated surface by a technique described previously.[25] All animal experiments and care were approved by the University of Minnesota Animal Care and Use Committee in accordance with Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) guidelines. Detailed methods are available in the Supplemental Material (available online at http://atvb.ahajournals.org).

Results

$A_{2A}R$ Deficiency Leads to the Formation of a Large Neointima in the Injured Arteries of $ApoE^{-/-}$ Mice

To determine the role of $A_{2A}R$ in the formation of arterial neointima, the carotid arteries of $A_{2A}R^{-/-}/apoE^{-/-}$ mice and the littermate $A_{2A}R^{+/+}/apoE^{-/-}$ mice were injured with a guide wire. Four weeks after the injury, their arteries were excised and processed for analysis. The neointima lesions were 83% larger in $A_{2A}R^{-/-}/apoE^{-/-}$ mice than those in $A_{2A}R^{+/+}/apoE^{-/-}$ mice (Figure 1A). In addition, the numbers of macrophages and α-actin–positive smooth muscle cells in the neointima of $A_{2A}R^{-/-}/apoE^{-/-}$ mice were 57% and 68% greater than the numbers in $A_{2A}R^{+/+}/apoE^{-/-}$ mice, respectively (Figure 1B and C). $A_{2A}R^{-/-}/apoE^{-/-}$ mice had slightly higher body weights and blood cholesterol levels than those of $A_{2A}R^{+/+}/apoE^{-/-}$ mice after 6 weeks on a Western diet (Supplemental Table I and Table II); however, these differences were not statistically significant.

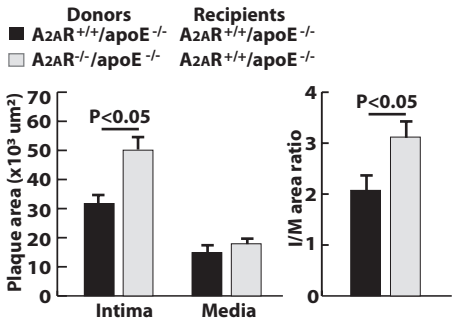

Figure 2. $A_{2A}R$ deficiency in bone marrow–derived cells increases injury-induced arterial neointima formation. Size quantification of neointima (I), media (M), and I/M ratio. Data from 12 sections of 12 mice.

$A_{2A}R$ Deficiency in BMDCs Increases Injury-Induced Arterial Neointima Formation

Next, we determined whether, and to what extent, the presence of $A_{2A}R$ in BMDCs influences the formation of the arterial neointima. We used bone marrow transplantation to generate the apoE$^{-/-}$ chimeric mice that lacked $A_{2A}R$ in their BMDCs and the apoE$^{-/-}$ chimeric control mice that retained $A_{2A}R$ in their BMDCs. Their carotid arteries were injured as previously described. At euthanization, both groups of mice were identical in blood cholesterol level, peripheral leukocyte count, and body weight (data not shown). However, the neointima lesions in the chimeric mice lacking $A_{2A}R$ in their BMDCs were 60% larger than the lesions in chimeric control mice (Figure 2 and Supplemental Figure IA). The arterial neointima was stained heavily for the marker of macrophages but not vascular smooth muscle cells (Supplemental Figure IB and C). In addition, we performed wire injury in chimeric $A_{2A}R^{-/-}$/apoE$^{-/-}$ mice that received bone marrow from $A_{2A}R^{+/+}$/apoE$^{-/-}$ mice. The size of their arterial neointima was similar to that in the $A_{2A}R^{+/+}$/apoE$^{-/-}$ control mice (ie, mice that received bone marrow from $A_{2A}R^{+/+}$/apoE$^{-/-}$ mice) (data not shown). This result suggests that the enhanced neointima formation observed in $A_{2A}R^{-/-}$/apoE$^{-/-}$ mice was mainly the result of the deficiency of $A_{2A}R$ in BMDCs.

$A_{2A}R$ Deficiency Increases Leukocyte Interactions With the Injured Arteries

By using epifluorescence intravital microscopy, we examined the interactions of leukocytes with injured mouse carotid arteries in vivo. Immediately after the arterial injury, circulating leukocytes (fluorescently labeled with rhodamine 6G) were able to roll onto, and adhere to, the injured arterial vessel wall. Within 30 minutes after arterial injury, the number of leukocytes rolling on and adhering to the arterial wall were 1.8- and 1.7-fold greater, respectively, in $A_{2A}R^{-/-}$/apoE$^{-/-}$ mice than in $A_{2A}R^{+/+}$/apoE −/− mice (Figure 3A).

To determine which types of cells were adhering to the injured arteries, we immunostained arterial cross sections with markers specific for platelets, neutrophils, and monocytes. At 1 hour after the arterial injury, platelets and leukocytes covered the denuded luminal surface (Figure 3B and Supplemental Figure IIA). Many more leukocytes bound to the injured area of carotid arteries in $A_{2A}R^{-/-}$/apoE$^{-/-}$ mice than in $A_{2A}R^{+/+}$/apoE$^{-/-}$ mice. Nearly all of the adherent leukocytes were neutrophils (Figure 3C and Supplemental Figure IIB); the presence of monocytes (macrophages) was rare (Figure 3D and Supplemental Figure IIC). At 7 days after wire injury, the adhesion and infiltration of both neutrophils and macrophages were observed in the injured carotid arteries, and more cells were observed in the injured vessel walls of $A_{2A}R^{-/-}$/apoE$^{-/-}$ mice than in $A_{2A}R^{+/+}$/apoE$^{-/-}$ mice (Figure 3E and F and Supplemental Figure IID and E).

$A_{2A}R$ Deficiency Increases the Interactions of Leukocytes With the P-Selectin–Expressing Surface

The homing ability of wild-type (WT) and $A_{2A}R$-deficient neutrophils was further studied by intravital microscopy in an

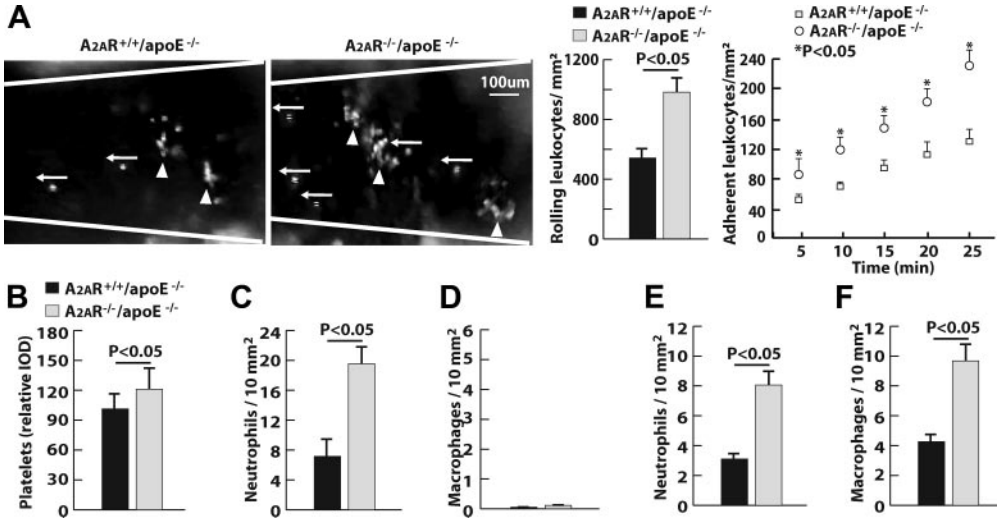

Figure 3. $A_{2A}R$ deficiency increases leukocyte interactions with injured arteries. A, Images and quantification of leukocyte rolling (←) and adhesion (arrowhead) in injured carotid arteries. B to F, Quantification of carotid arteries stained for platelets (B), neutrophils (C), and macrophages (D) 1 hour after wire injury (WI); and neutrophils (E) and macrophages (F) 7 days after WI (n=5).

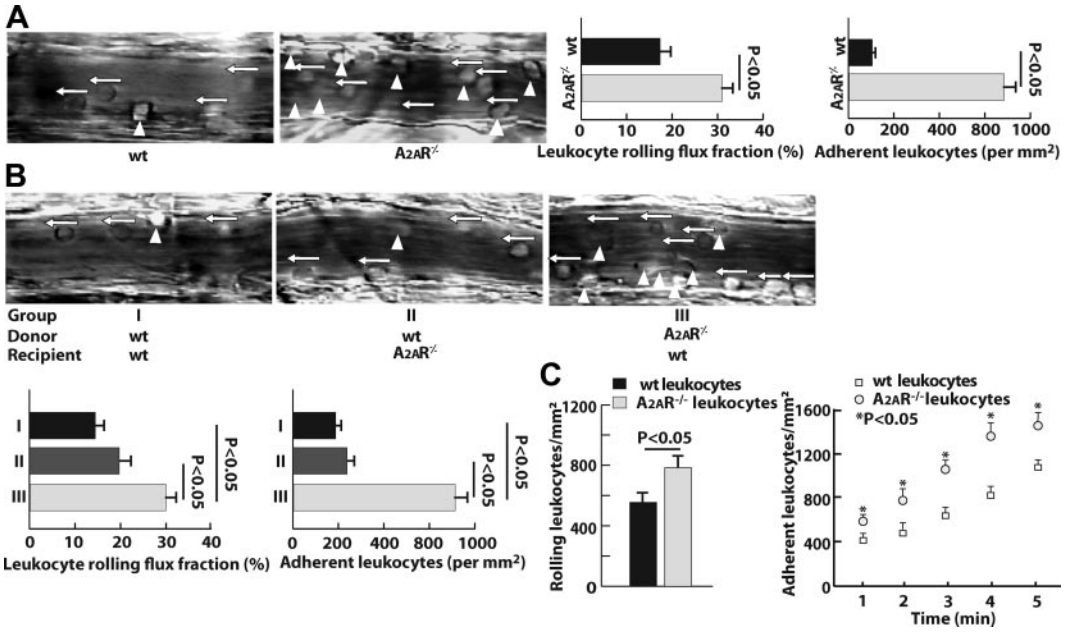

Figure 4. $A_{2A}R$ deficiency increases neutrophil homing ability. A and B, Images and quantification of neutrophil rolling (←) and adhesion (arrowhead) on the endothelium of postcapillary venules of mouse cremaster muscle (n=6). C, Neutrophil rolling and adhesion on activated platelets through microflow chambers (n=6).

in vivo trauma model of the mouse cremaster muscle. Mild trauma caused by the exteriorization of the cremaster muscle is an inflammatory stimulus. This stimulus induces the presentation of P-selectin and fibrinogen on the endothelium of postcapillary venules; it mediates both P-selectin–dependent neutrophil rolling and b2 integrin-dependent neutrophil adhesion.[26] To evaluate the role of $A_{2A}R$ in leukocyte-endothelial interactions, we measured the neutrophil rolling flux fraction, defined as the number of rolling neutrophils divided by the total number of neutrophils passing through the same vessel. Compared with WT mice, $A_{2A}R$-deficient mice exhibited a 2-fold increase in the neutrophil rolling flux fraction (Figure 4A). In addition, many more neutrophils adhered to the endothelium of postcapillary venules in $A_{2A}R^{-/-}$ mice than in WT mice.

To further determine which cellular $A_{2A}R$ is responsible for these increased neutrophil-endothelial interactions in $A_{2A}R^{-/-}$ mice, we performed an intravital microscopy study in chimeric mice with (control) or without $A_{2A}R$ in their BMDCs. Compared with control mice, chimeric mice with $A_{2A}R$-deficient BMDCs had significantly increased neutrophil rolling and adhesion, and chimeric mice with $A_{2A}R$-deficient vessel walls had a similar level of neutrophil rolling and adhesion (Figure 4B).

To compare the binding affinity of WT and $A_{2A}R$-deficient neutrophils, we used an ex vivo microflow chamber[27,25] whose surface was coated with thrombin-activated WT platelets. Whole blood from $A_{2A}R^{+/+}$ or $A_{2A}R^{-/-}$ mice was then perfused through the chamber. Immediately after the perfusion, the numbers of rolling and adherent neutrophils were 60% and 50% higher, respectively, in the blood of $A_{2A}R^{-/-}$ mice than that of $A_{2A}R^{+/+}$ mice. After 5 minutes of perfusion, these numbers further increased to 80% and 90%, respectively (Figure 4C). In addition, $A_{2A}R$-deficient neutrophils rolled much more slowly than WT neutrophils (velocity data not shown).

Mechanisms for the Increased Homing Ability of $A_{2A}R$-Deficient Neutrophils

To determine the influence of $A_{2A}R$ deficiency in neutrophil homing ability, we first measured the expression of homing molecules on WT and $A_{2A}R$-deficient neutrophils by flow cytometry. The levels of PSGL-1, L-selectin, lymphocyte function–associated antigen 1, CD11b, and CXC-chemokine receptor-2 (CXCR2) were identical between both types of neutrophils (Supplemental Figure IIIA).

PSGL-1 is the key molecule responsible for P-selectin–mediated neutrophil rolling on the injured arteries or postcapillary venules.[26] To explore the mechanisms responsible for the increased rolling of $A_{2A}R$-deficient neutrophils, we first used a flow cytometry assay to examine the binding of neutrophil PSGL-1 to P-selectin. In accordance with previously measured levels of PSGL-1 expression, the levels of P-selectin binding to WT and $A_{2A}R$-deficient neutrophils were identical (Supplemental Figure IIIB). Thus, under static conditions, PSGL-1 has the same level of P-selectin binding in $A_{2A}R$-deficient and WT neutrophils.

By using confocal microscopy, we investigated the distribution of PSGL-1 after the immunofluorescent labeling of neutrophils with PSGL-1 antibody. PSGL-1 was much more clustered on the surface of $A_{2A}R$-deficient neutrophils than WT neutrophils. This result was observed on neutrophils isolated from blood and on those adhering to injured arteries (Figure 5A and B). Also, we explored several intracellular signaling mechanisms (ie, the activity of phosphoinositide 3-kinase/Akt and mitogen-activated protein kinase [MAPK]) that might be responsible for the increased homing ability of

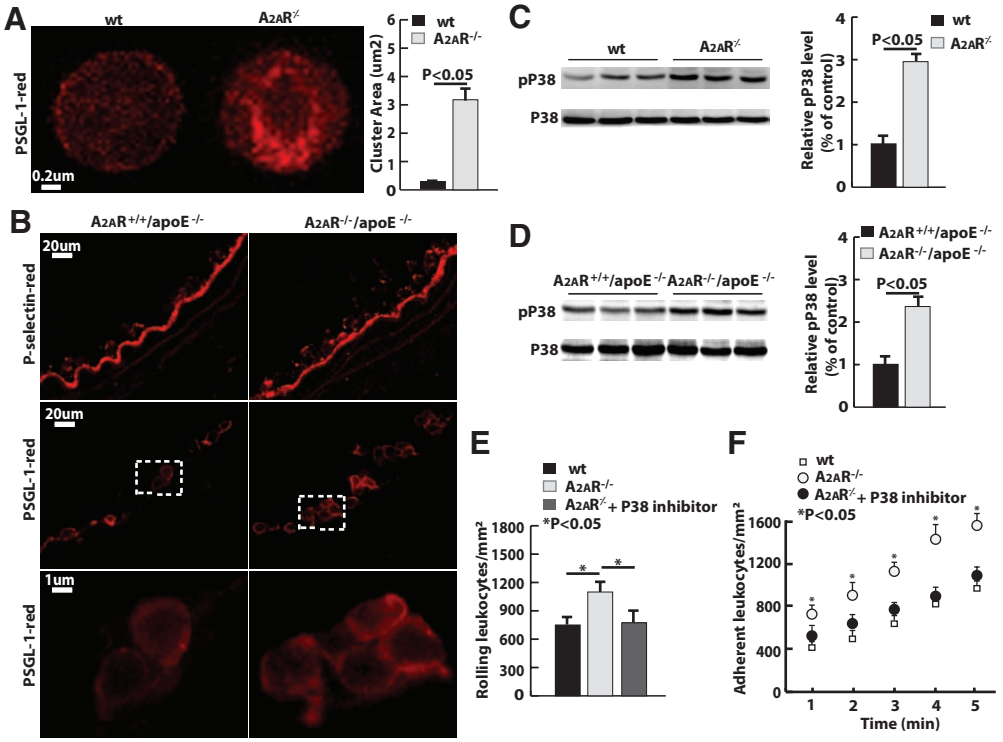

Figure 5. $A_{2A}R$ deficiency increases neutrophil p38 activity and PSGL-1 clustering. A, PSGL-1 distribution on neutrophil membranes. B, P-selectin and PSGL-1 on injured arteries. C and D, Western blot of P38 and phosphorylated p38 (pP38) of spleen neutrophils (C) and carotid arteries (D) 1 hour after injury (n=3). E and F, Leukocyte rolling and adhesion through P-selectin coated (E) and P-selectin and ICAM-1 coated (F) microflow chambers (n=3).

$A_{2A}R$-deficient neutrophils. In a Western blot assay of neutrophils isolated from mouse spleen, the level of phosphorylated p38 MAPK in $A_{2A}R$-deficient neutrophils was 2.5 times higher than that in WT neutrophils (Figure 5C). This phenomenon was also observed in injured arteries (Figure 5 D). In contrast, the levels of phosphorylated Akt and other MAPKs were not significantly different between WT and $A_{2A}R$-deficient neutrophils (Supplemental Figure IV).

Consistent with the PSGL-1 distribution under flow conditions, $A_{2A}R$-deficient neutrophils exhibited greater PSGL-1 binding than WT neutrophils. This result was demonstrated by an ex vivo microflow chamber experiment,[27,25] in which mouse whole blood was perfused through a P-selectin–coated surface. After 5 minutes of perfusion, the whole blood from $A_{2A}R^{-/-}$ mice presented an average of 1200±80 rolling neutrophils per millimeter,[2] whereas the whole blood from WT mice presented 800±70 rolling neutrophils per square millimeter (Figure 5E). In a similar setup, 5-minute perfusion of mouse whole blood through the flow chamber coated with P-selectin and intercellular adhesion molecule 1 led to 2-fold more $A_{2A}R$-deficient versus WT neutrophils adhering to the surface (Figure 5F); this finding indicated an increase in the affinity of b_2 integrins on $A_{2A}R$-deficient neutrophils.

To determine whether the enhanced p38 MAPK activity is responsible for the increased homing ability of $A_{2A}R$-deficient neutrophils, we treated $A_{2A}R^{-/-}$ mice with the p38 inhibitor SB203580, 1 mg/kg per day, intraperitoneally for 3 days before the perfusion. The treatment of SB203580 reduced the number of rolling cells on both the P-selectin–coated surface and the surface coated with P-selectin and intercellular adhesion molecule 1 to the levels in WT mice (Figure 5E and F).

Discussion

Our results demonstrate that leukocyte $A_{2A}R$ is critical for the protection of atherosclerotic mice from injury-induced arterial neointima formation. $A_{2A}R$ deficiency in neutrophils increases p38 activation, resulting in an increase in the clustering of PSGL-1 and the affinity of b_2 integrins. Ultimately, $A_{2A}R$ deficiency enhances neutrophil recruitment to the injured arteries and augments the formation of the injury-induced arterial neointima.

Adenosine is known to inhibit a variety of neutrophil functions, including the production of tumor necrosis factor α and superoxide anions.[28] Several studies[29–31] have demonstrated that adenosine or adenosine receptor agonists inhibit the neutrophil adhesion to endothelial cells and fibrinogen by suppressing the upregulation of neutrophil b_2 integrins, as stimulated by N-formyl-methionyl-leucyl-phenylalanine. When neutrophils are treated with an inhibitor of adenosine kinase (a major intracellular adenosine removal enzyme), adenosine levels increase, and the adhesion of activated neutrophils to cultured endothelial cells decreases through the alteration of L-selectin and the neutrophil cytoskeleton.[32] Recently, adenosine or $A_{2A}R$ agonists have been shown to induce heterologous desensitization of chemokine receptors and suppress the expression of very late antigen 4 on stimulated human neutrophils.[33,34] Notably, these previous studies revealed the pharmacological effects of adenosine or

adenosine receptors on leukocytes. Also, the neutrophils used in the studies were activated in vitro by N-formyl-methionyl-leucyl-phenylalanine, tumor necrosis factor α, or other stimuli. The present study shows a physiological role of $A_{2A}R$ in the regulation of neutrophil homing ability. Under physiological conditions, $A_{2A}R$ deficiency did not affect the expression of adhesion molecules on neutrophils. Instead, $A_{2A}R$ deficiency altered the cell membrane distribution of PSGL-1 and the affinity of b_2 integrins, therefore inhibiting neutrophil recruitment to the injured arteries in vivo.

We explored several possible intracellular signaling mechanisms for the increased homing ability of $A_{2A}R$-deficient neutrophils, including the activity of phosphoinositide 3-kinase/Akt and MAPKs. We found that $A_{2A}R$ regulates the neutrophil homing ability through MAPK p38. Our finding that $A_{2A}R$-deficient neutrophils exhibited increased p38 MAPK activation could result from a change in the signaling associated with intracellular cAMP.[1] $A_{2A}R$ occupancy elevates the intracellular cAMP level, therefore inhibiting the activation of p38 via the cAMP response element–binding protein–induced dynein light chain.[35,36] Leukocyte p38 is usually activated by outside-inside signals, and the activated p38 is able to signal many pathways related to different cellular functions.[35,36] In the early phase of arterial injury, the binding of neutrophil PSGL-1 with platelet P-selectin predominates neutrophilic interactions with the vessel wall. Therefore, we focused on the role of p38 activation on the regulation of PSGL-1 binding function. $A_{2A}R$-deficient neutrophils exhibit increased PSGL-1 clustering. Consequently, these cells have elevated levels of P-selectin binding. In our ex vivo microflow chamber assay, the inhibition of p38 activation significantly reversed the increased neutrophil rolling and adhesion, demonstrating that p38 is a key modulator in regulating $A_{2A}R$-mediated leukocyte homing. The enhanced PSGL-1 clustering can be regulated directly by p38 or indirectly through p38 altered cytoskeletal protein organization.[37,38]

Increased neutrophil recruitment to the injured arteries contributes to neointima formation through many mechanisms.[25,39,40] In addition, monocyte recruitment and activation could also play a role in the increased neointima formation after the arterial injury in $A_{2A}R^{-/-}$/apoE$^{-/-}$ mice. Similar to neutrophils, $A_{2A}R$-deficient monocytes express adhesion molecules at the same levels seen in WT cells.[5] However, $A_{2A}R$-deficient monocytes showed increased adhesion on atherosclerotic endothelium in an ex vivo mouse carotid artery perfusion model (data not shown). Furthermore, nuclear factor κB was highly activated in $A_{2A}R$-deficient macrophages in atherosclerotic lesions.[5] In a short-term arterial neointima formation model, these factors might contribute to the aggravation of arterial neointima formation. However, in a long-term spontaneous atherosclerosis model, $A_{2A}R$ deficiency results in a decrease in atherosclerosis.[5] This discrepancy may be the result of the fact that many resolution aspects of inflammation take actions to reduce the size of lesions during a long-term process of spontaneous lesion formation. These resolution aspects include macrophage apoptosis, macrophage emigration from lesions, and the reduction of the vasa vasorum density of atherosclerotic arteries.[41–44] $A_{2A}R$ deficiency increases the apoptosis of macrophages, as previously shown.[5] In addition, CD8 T cells target the neovascularization of atherosclerotic arteries to reduce atherosclerosis.[45] $A_{2A}R$-deficient T cells are able to suppress or even regress the tumor growth,[46] which may be achieved through the elimination of neovascularization by $A_{2A}R$-deficient CD8 T cells (Michail Sitkovsky, personal oral communication, 2009). Therefore, the reduction of atherosclerosis in $A_{2A}R^{-/-}$/apoE$^{-/-}$ mice may also be the result of suppression of neovascularization by $A_{2A}R$-deficient CD8 T cells.

Increased neointima formation in $A_{2A}R^{-/-}$ mice is not attributed to the $A_{2A}R$ deficiency in endothelial cells. In a mouse carotid artery wire injury model, the regeneration of endothelial cells on the injured area occurred a few days after wire injury. These regenerated endothelial cells were inflamed and expressing a variety of adhesion molecules, which is the key for leukocyte recruitment to the injured arteries and the formation of arterial neointima.[47] In a similar mouse carotid artery injury model, endogenous extracellular adenosine, generated through CD73, protected the regenerated endothelial cells from inflammatory activation.[48] Also, in a mouse carotid artery ligation model, an $A_{2A}R$ agonist inhibited adhesion molecule expression and consequent neointima formation in injured arteries.[49] Thus, it has been speculated that adenosine reduces endothelial activation through $A_{2A}R$s.[1] In this study, we found that $A_{2A}R$ deficiency in endothelial cells does not affect the inflammatory response of endothelial cells that are regenerated on the injured area. This finding is supported by our immunostaining and Western blot results from an in vivo carotid artery injury model and our flow cytometry results of an in vitro cell culture system to determine the role of $A_{2A}R$ deficiency in endothelial inflammatory responses (Supplemental Figure VA-C). Thus, endothelial $A_{2A}R$ does not play a physiological role in determining the inflammatory status of endothelial cells in the arterial neointima model, which is different from its pharmacological effect, as reported in other studies.[49,50]

Augmented arterial neointima formation in $A_{2A}R^{-/-}$ mice may not be associated with the $A_{2A}R$ deficiency in platelets. Immediately after wire injury, platelets accumulated on the injured area. These platelets were activated and presented P-selectin and other integrins to serve as a platform for leukocyte recruitment to the injured arterial vessel wall.[17] In a dog coronary hypoperfusion model, it was demonstrated that platelet $A_{2A}R$ is critically involved in the effect of adenosine on the inhibition of platelet-leukocyte interactions and platelet aggregation.[51] In humans, upregulated $A_{2A}R$ on platelets, as a result of long-term caffeine intake, is beneficial for the prevention of platelet aggregation.[52,53] Different from the previously described studies, we did not find increased platelet activation mediated by thrombin in $A_{2A}R$-deficient platelets (Supplemental Figure VIA). In response to thrombin stimulation, $A_{2A}R$-deficient mouse platelets presented P-selectin and exhibited platelet-leukocyte interactions at the same levels as seen in WT mouse platelets (Supplemental Figure VIB). It is likely that the role of $A_{2A}R$ in platelet activation is strain dependent. Otherwise, adenosine and adenosine receptors are not critically involved in platelet

activation either in response to thrombin or in the milieu of arterial injury.

Taken together, the deficiency of $A_{2A}R$ enhances leukocyte recruitment to the injured arteries and aggravates the formation of injury-induced arterial neointima. $A_{2A}R$ antagonists are being tested in clinical trials for the treatment of neurodegenerative diseases and other chronic conditions. The results from this study indicate that the evaluation on the effect of $A_{2A}R$ antagonists on arterial restenosis after arterial angioplasty should be considered.

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Disclosures

None.

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Supplemental Materials and Methods

Mice and mouse carotid artery wire injury model

$A_{2A}R^{-/-}$ mice were produced as described [1]. Congenic $A_{2A}R^{-/-}$ mice in a C57BL/6J background were produced by monitoring 96 microsatellites for five generations of marker-assisted breeding [2]. Wild-type C57BL/6J (wt) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). $A_{2A}R^{-/-}$ mice were bred with $apoE^{-/-}$ mice (C57BL/6J congenic, Jackson Laboratory) to generate $A_{2A}R^{-/-}$ /$apoE^{-/-}$ mice. 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 the wire injury of arteries. Mice were maintained on the same diet until euthanization.

The arterial wire injury was performed as described [3,4]. Briefly, mice were anesthetized by 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 5 passes with a rotating motion. At different time points after injury, mice were euthanized and perfused *in situ* by 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.

Bone marrow transplantation

Bone marrow was harvested from donor mice and transplanted into recipient mice, as previously described [5]. Briefly, recipient mice were lethally irradiated in 2 doses of 600 rads each, approximately 4 hours apart. Donor mice were sacrificed by a lethal injection of sodium pentobartital (Nembutal, Abbott Laboratories, North Chicago, IL). Bone marrow cells from both femurs and tibias were

harvested under sterile conditions. Approximately 50 million nucleated cells were obtained from donor mice. Bones are flushed with RPMI (Life Technologies, Grand Island, NY) (without phenol red) containing 10% fetal calf serum (Atlanta Biologicals, Norcross, GA). Suspended bone marrow cells were washed, and erythrocytes were lysed in 0.15 M NH_4Cl lysing solution. Approximately 2-4 million unfractionated bone marrow cells in 200 μl of media were delivered intravenously through the tail vein of each recipient mouse. Recipient mice were housed in a barrier facility (individually ventilated cages, HEPA-filtered air) under pathogen-free conditions before and after bone marrow transplantation. After bone marrow transplantation, mice were maintained on autoclaved water with antibiotics (5 mM sulfamethoxazole and 0.86 mM trimethoprim) (Sigma, St. Louis, MO) and fed autoclaved food.

Quantitative immunohistochemistry and immunostaining

Serial sections were stained with Movat pentachrome (Sigma, St. Louis, MO)[3,4],. For quantitative comparisons, 10 sections were analyzed from each animal, each section within a standardized distance 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), endothelial cells (VE-cadherin, clone 11D4.1; BD Biosciences, San Diego, CA), and VCAM-1 (Santa Cruz Biotechnology). 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 rhodamine (TRITC)-conjugated

secondary antibody (Jackson Immunoresearch, West Grove, PA).

In vivo examination of leukocyte interactions with the injured arterial wall

An *in vivo* carotid artery wire injury model was used as described [4]. In brief, mice were first anesthetized and intravenously injected with rhodamine 6G. After 10 min, the left carotid arteries were injured with a guide wire to induce vascular injury. With an intravital epifluorescence microscopy system, the fluorescently labeled leukocytes interacting with the injured carotid arteries were observed with 10X objective. Leukocyte interactions with the injured vessel were recorded and analyzed. The interactions of leukocytes with injured arteries include tethering, rolling, and adhesion. Adherent leukocytes are defined as leukocytes that adhere to the vessel wall for at least 30 seconds. Rolling leukocytes are defined as those with continuous movement on the vessel wall for at least 0.5 second. This criteria was used in our previous study [4].

Leukocyte interactions with activated platelets, P-selectin, or ICAM-1 under flow conditions

Mouse platelets were isolated by gel-filtration.[6] The *ex vivo* micro-flow chamber was prepared as previously described [4, 7]. Briefly, platelets were loaded into a rectangular glass capillary tube at a concentration of 2 x 10^9/ml. Platelets adhering to the surface of capillary tube were activated with thrombin (0.1 U/ml; Sigma) and washed with PBS. The micro-flow chamber was then installed on the stage of an epifluorescence intravital microscope. The mouse was anesthetized and injected via the tail vein with 1 mg/ml rhodamine 6G/PBS (50 µl/30 g mouse weight). Mouse blood from the carotid artery was perfused through the micro-flow chamber. Leukocyte interactions with activated platelets were observed and recorded on videotape. For some experiments, the surface of the rectangular glass capillary tube was coated with P-selectin (10 µg/ml) and/or ICAM-1 (10 µg/ml) (R&D Systems Inc., Minneapolis, MN).

Leukocyte-endothelial interactions in postcapillary venules of cremaster muscle

The surgical preparation of the cremaster muscle for intravital microscopy was conducted as described [8]. Briefly, after opening the scrotum, the cremaster muscle was exteriorized and spread over a cover glass. The epididymis and testis were gently pinned to the side, giving full microscopic access to the microcirculation of cremaster muscle. Experiments were recorded *via* a CCD camera system (model VE-1000; Dage-MTI, Inc., Michigan City, IN) on a Panasonic S-VHS recorder. The cremaster muscle was superfused with thermocontrolled (36°C) bicarbonate-buffered saline. The postcapillary venules under observation ranged from 20 to 50 μm in diameter.

Flow cytometric analysis

All antibodies were obtained from BD Biosciences (San Diego, CA) unless otherwise specified. To determine the expression of adhesion molecules on mouse neutrophils, blood leukocytes were incubated with a monoclonal antibody against Ly-6G. Ly-6G positive cells were identified as neutrophils by flow cytometry. Monoclonal antibodies against L-selectin, PSGL-1, LFA-1, CD11b, and CXCR2 were used to analyze the expression of these molecules on neutrophils. To examine the expression of P-selectin on mouse platelets, platelets were isolated as described above and activated with thrombin (0.1 U/ml; Sigma). For the expression of VCAM-1 on mouse aortic endothelial cells, cells were collected and cultured as described, then stimulated with TNF-α (10 ng/ml; Sigma) [9]. After fixation with 1% PFA, these samples were incubated with monoclonal antibody against P-selectin or VCAM-1 to analyze the expression of P-selectin on platelets or the expression of VCAM-1 on aortic endothelial cells. Data were analyzed by CellQuest (Tampa, FL) software. In all cases, appropriate isotype controls were used.

Western blotting

Mouse neutrophils were isolated from the spleen using mouse neutrophil-specific anti-Ly-6G and

magnetic columns (MACS; Miltenyi Biotec, Auburn, CA). Cells were lysed in modified RIPA lysis buffer. Samples were separated by SDS-PAGE and transferred to a polyvinylidene fluoride membrane. Membranes were then blocked with 5% non-fat dry milk in Tris-buffered saline with 0.1% Tween 20 and subsequently incubated with primary antibodies diluted in blocking buffer. The antibodies, including anti-p38, anti-phospho-p38, anti-extracellular signal-regulated kinase (ERK1/2), anti-phospho-ERK1/2, anti-JNK (JNK), anti-phospho-JNK (p-JNK), anti-Akt, and anti-phospho-Akt (Cell Signaling, Danvers, MA) were applied. The blots were incubated with alkaline phosphatase–conjugated secondary antibodies, developed with a chemifluorescence reagent, and scanned by Storm 860 (GE Healthcare).

PSGL-1 clustering on neutrophils

To observe the clustering of PSGL-1 on neutrophils, 100 ul of mouse blood was collected by using heparin anticoagulant and fixed in 1% paraformaldehyde. Red blood cells were lysed and labeled with PE conjugated PSGL-1 antibody (BD Biosciences, San Diego, CA). Nuclear material was stained with DAPI (Invitrogen, Eugene, OR) for neutrophil identification. For MAPK p38 inhibition, mice were intraperitoneally pretreated with SB203580 (100mg/kg dissolved in 5% dimethyl sulfoxide, Sigma) for 3 days. Images were taken by inverted confocal microscopy (Olympus, Melville, NY). The micrographs are representative of 30–50 cells (n = 3). All fluorescent images were analyzed and processed with Image Pro Plus v4.5 software (Media Cybernetics, Silver Spring, MD). For the quantitation of PSGL-1 clustering, a cluster was defined as a localized region of the membrane, in which the pixel intensity is at least 3-fold greater than the background fluorescent intensity. Pixel intensity values range from 0 to 255. Based on these values and the image of the cell, a threshold intensity value was chosen to represent the average background intensity over the surface. This value typically ranged from 80 to 120. Clusters reaching the off-scale values were assigned a maximum intensity value of 255 by default. After thresholding on the background fluorescence, the number of

fluorescent clusters (frequency) and the surface area per cluster were obtained. The cluster area was calculated by timing the number of clusters with the surface area per cluster. The images of 30 neutrophils were analyzed for each group.

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's t test to evaluate two-tailed levels of significance. The null hypothesis was rejected at $P < 0.05$.

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Figure legends for supplementary figures

Figure I. $A_{2A}R$ deficiency in bone marrow derived cells results in the formation of large neointima after the arterial injury

a, Movat pentachrome staining of arterial neointima in bone marrow transplanted chimeric mice. Images show the cross-sections of carotid arteries from the irradiated mice that received the bone marrow from $A_{2A}R^{+/+}$/apoE$^{-/-}$ mice (left) or $A_{2A}R^{-/-}$/apoE$^{-/-}$ mice (right). The size of neointima (I) and media (M), and the ratio of intima to media (I/M) were quantified (n = 12 for both groups). **b** and **c** Immunostaining (with anti-F4/80 and α-actin antibody) of the infiltrated macrophages and vascular

smooth muscle cells in arterial neointima. The areas stained positive for macrophages were calculated by analyzing 12 cross-sections of each mouse carotid artery. Twelve injured carotid arteries were included for each group.

Figure II. $A_{2A}R$ deficiency increases leukocyte interactions with injured arteries. a to **e**, Images of carotid arteries from $A_{2A}R^{+/+}$/apoE$^{-/-}$ and $A_{2A}R^{-/-}$/apoE$^{-/-}$ mice stained for (a) platelets 1 hour after wire injury (WI), (b) neutrophils 1 hour after WI, (c) macrophages 1 hour after WI, (d) neutrophils 7 days after WI and (e) macrophages 7 days after WI (n=5).

Figure III. $A_{2A}R$ deficiency does not affect levels of PSGL-1, L-selectin, LFA-1, CD11b, CXCR2 and P-selectin binding on neutrophils

a, Representative flow cytometry results showing the levels of PSGL-1, L-selectin, LFA-1, CD11b, and CXCR2 on wt and $A_{2A}R$-deficient neutrophils. **b,** Representative flow cytometry results showing the levels of P-selectin binding to wt and $A_{2A}R$-deficient neutrophils. Results are representative of three independent experiments described in (a) and (b).

Figure IV. Signaling alternation in the injured arteries of $A_{2A}R$ deficient mice

a to **c,** Western blot showing levels of Akt, Erk, JNK, and their phosphorylated forms in arteries from $A_{2A}R^{+/+}$/apoE$^{-/-}$ and $A_{2A}R^{-/-}$/apoE$^{-/-}$ mice at 1 hour after wire injury. n = 3.

Figure V. $A_{2A}R$ deficiency does not affect the expression of VCAM-1 on endothelial cells

a, Representative immunofluorescence staining of VE-cadherin and VCAM-1 on carotid arteries from $A_{2A}R^{+/+}$/apoE$^{-/-}$ and $A_{2A}R^{-/-}$/apoE$^{-/-}$ mice at 7 days after wire injury. **b**, Western blot analysis showing the levels of VCAM-1 expressed in carotid arteries from $A_{2A}R^{+/+}$/apoE$^{-/-}$ and $A_{2A}R^{-/-}$/apoE$^{-/-}$ mice at

7 days after wire injury. **c,** Representative flow cytometry results showing VCAM-1 expression on cultured wt or $A_{2A}R$-deficient mouse aortic endothelial cells. Endothelial cells were treated with TNF-α (10 ng/ml) for 6 hours.

Figure VI. $A_{2A}R$ deficiency does not affect thrombin-mediated platelet activation

a, Representative flow cytometry results showing the levels of P-selectin on wt and $A_{2A}R$-deficient platelets. The platelets were treated with thrombin (0.1 U/ml) for 10 minutes. Results are representative of 3 experiments. **b,** Leukocyte rolling and adhesion to a surface coated with the thrombin-activated wt or $A_{2A}R^{-/-}$ platelets at 1.0 ± 0.1 dyn/cm^2. Data were collected within 5 min after whole blood from wt or $A_{2A}R^{-/-}$ mice was perfused through *ex vivo* micro-flow chambers.

Figure I

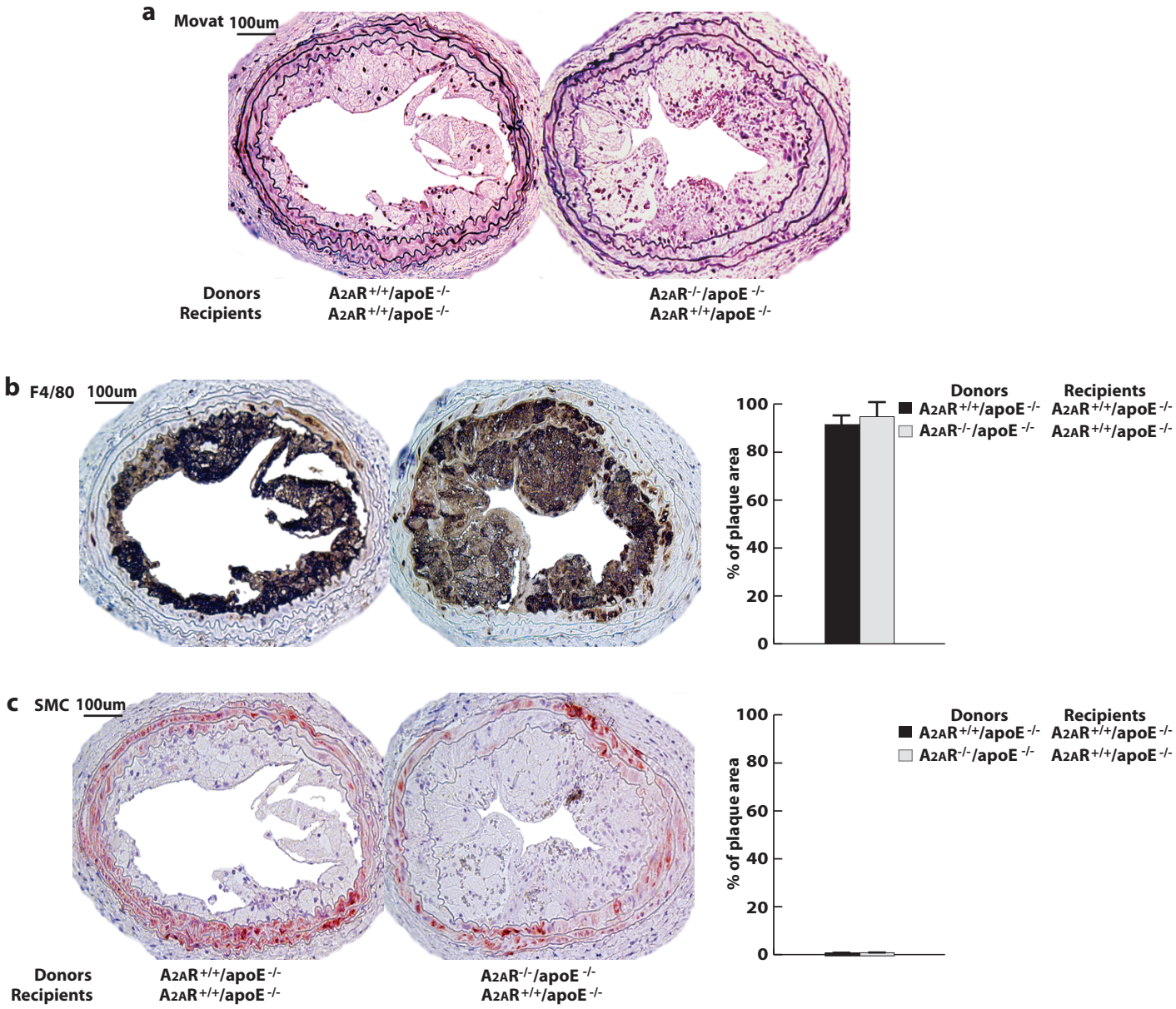


Figure II

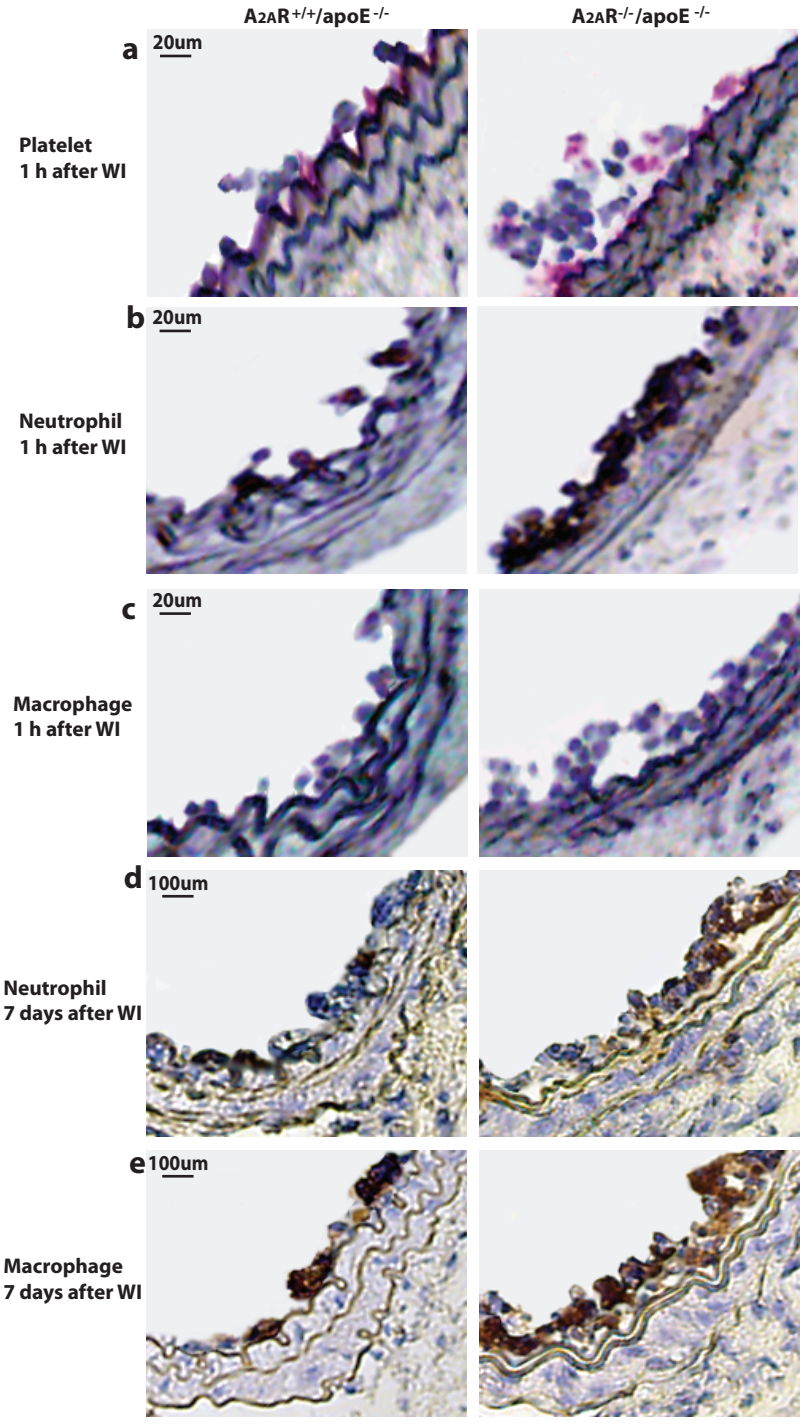


Figure III

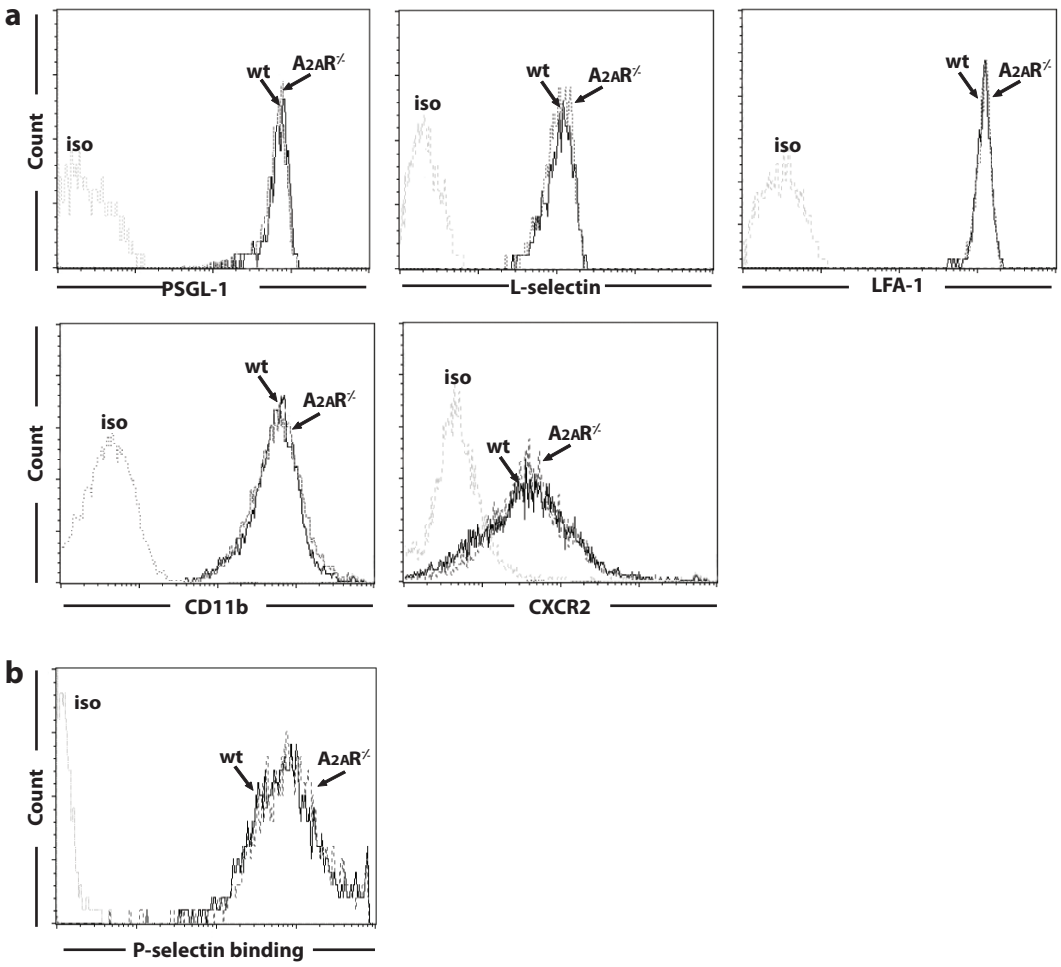


Figure IV

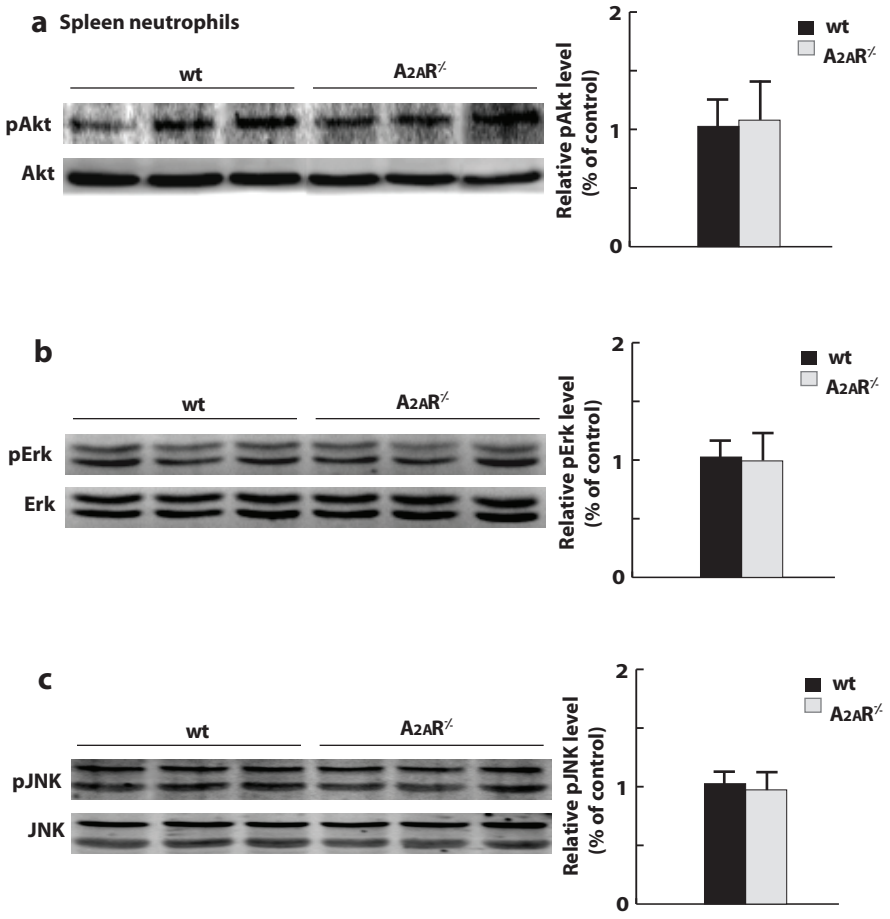
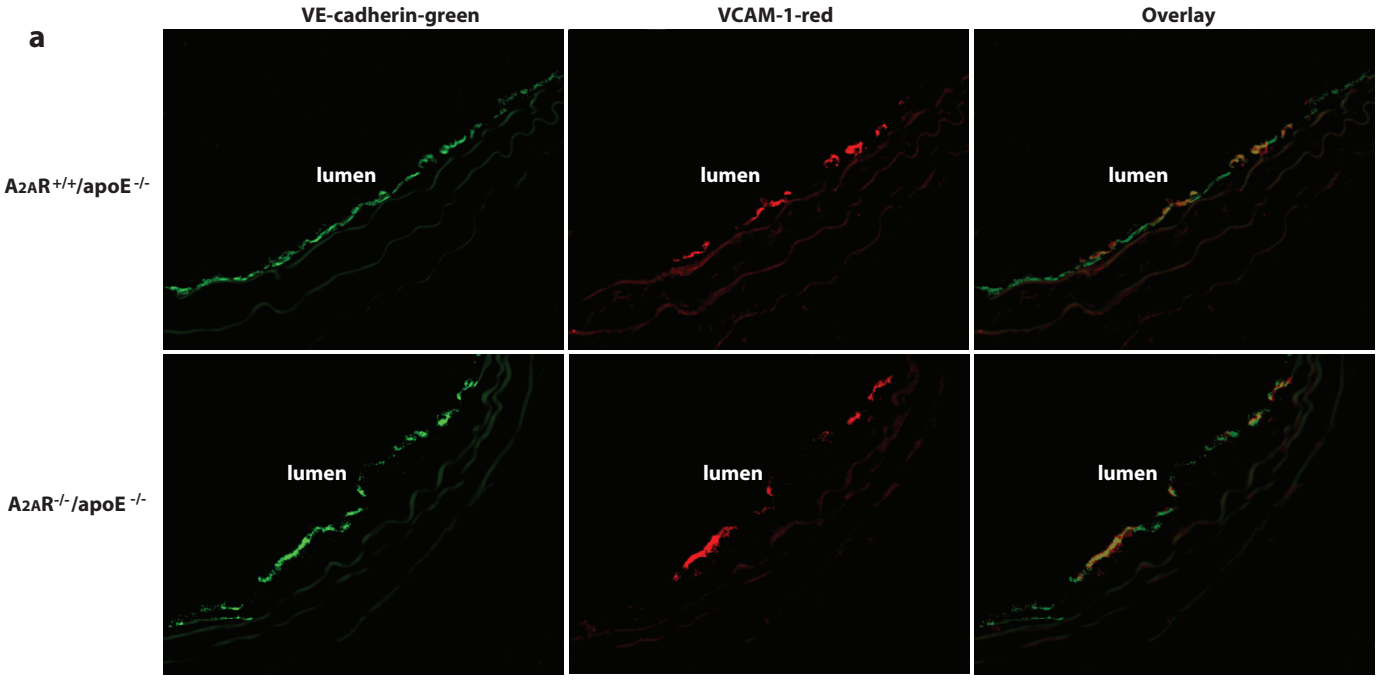
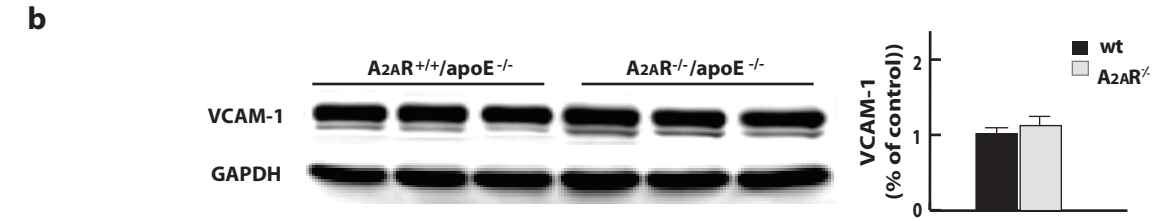


Figure V





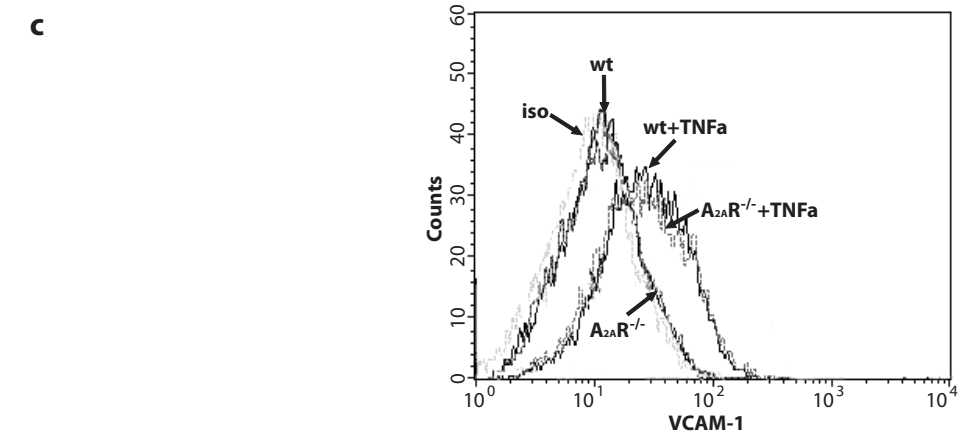
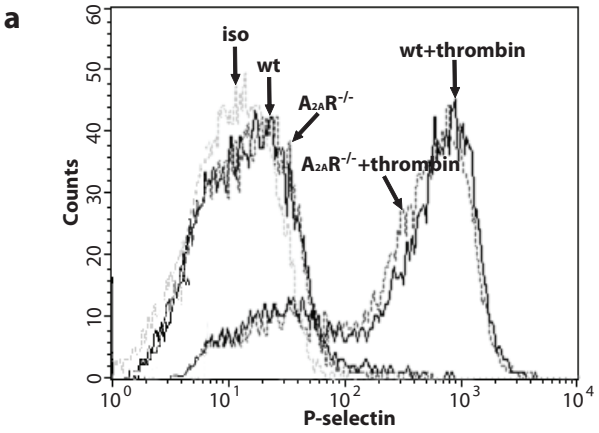


Figure VI



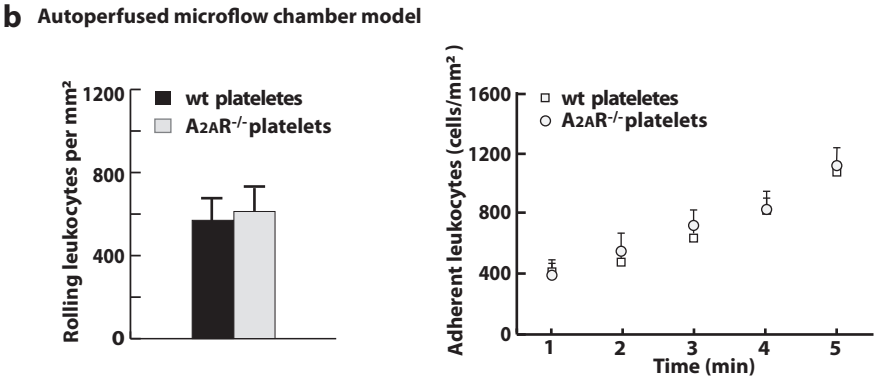


Table I, II

Table I

Genotype	BW (g)	Bp (mmHg)	Cholesterol (mg/dl)	Triglyceride (mg/dL)	LDL (mg/dl)	VLDL (mg/dL)	HDL (mg/dL)
$Apoe^{-/-}/A_{2A}R^{+/+}$	32.8±6.4	112.1±15.3	1321.5±167.2	126.0±24.6	965.6±137.3	26.7±4.7	327.5±43.4
$Apoe^{-/-}/A_{2A}R^{-/-}$	35.2±8.1	108.8±13.2	1486.6±246.3	137.9±32.4	1043.8±186.7	28.3±4.2	331.3±56.7

Table II

Genotype	WBC (k/uL)	NE (k/uL)	LY (k/uL)	MO (k/uL)	NE%	LY%	MO%	PTL (k/uL)
$Apoe^{-/-}/A_{2A}R^{+/+}$	5.3±2.6	1.6±1.2	2.6±1.4	0.4±0.2	35.5±11.2	56.4±8.3	5.6±4.2	1004.5±335.8
$Apoe^{-/-}/A_{2A}R^{-/-}$	6.1±3.8	2.4±2.0	3.4±1.7	0.5±0.3	36.6±14.7	54.2±13.9	6.1±4.6	946.7±236.1