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Adenosine A_{2A} receptor deficiency in leukocytes increases arterial neointima formation in apolipoprotein E-deficient mice

Huan Wang[1], **Weiyu Zhang**[1], **Rong Tang**[1], **Chuhong Zhu**[1], **Christoph Bucher**[1], **Bruce Blazar**[1], **Jianguo Geng**[1], **Chunxiang Zhang**[2], **Joel Linden**[3], **Chaodong Wu**[4], and **Yuqing Huo**[1]

[1] Department of Medicine, University of Minnesota, Minneapolis, MN

[2] Department of Anesthesiology, New Jersey Medical School, University of Medicine & Dentistry of New Jersey, Newark, NJ

[3] Department of Medicine, University of Virginia Health System, Charlottesville, VA

[4] Department of Nutrition and Food Science, Texas A&M University, College Station, TX

Abstract

Background—The A_{2A} receptor ($A_{2A}R$) plays a complex role in inflammation and tissue injury. In this study, we used the mice deficient in both $A_{2A}R$ and apolipoprotein E ($A_{2A}R^{-/-}$/apo$E^{-/-}$) 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 apo$E^{-/-}$ mice, $A_{2A}R$ deficiency increased the size of arterial neointima in injured carotid arteries by 83%. Arterial neointima formation was also enhanced in bone marrow transplanted chimeric mice that lacked $A_{2A}R$ in their bone marrow-derived cells. Epifluorescence intravital microscopy showed that neutrophil rolling and adherence to the injured arterial area was enhanced by 80% and 110% in $A_{2A}R^{-/-}$/apo$E^{-/-}$ 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 (MAPK), PSGL-1 clustering, and the affinity of b_2 integrins. Inhibition of p38 phosphorylation abrogated the increased PSGL-1 clustering and b_2 integrin affinity, thereby reversing the increased homing ability of $A_{2A}R$-deficient leukocytes.

Conclusion—The deficiency of $A_{2A}R$ enhances the homing ability of leukocytes and increases the formation of arterial neointima after injury. $A_{2A}R$ antagonists are being tested for the treatment of neurodegenerative diseases and other chronic diseases. Our results suggest that an evaluation of the effect of $A_{2A}R$ antagonists on arterial restenosis following arterial angioplasty should be conducted.

Address correspondence to: Yuqing Huo, MD, PhD, Cardiovascular Division & Vascular Biology Center, Department of Medicine, University of Minnesota School of Medicine, 420 Delaware St SE, MMC508, Minneapolis, MN 55455, Phone: 612-626-7055 Fax: 612-626-4411, Yuqing@umn.edu or Chaodong Wu, MD, PhD, Department of Nutrition and Food Science, Texas A&M University, College Station, TX, cdwu@tamu.edu.

Disclosures
None.

Introduction

Adenosine receptor A_2 ($A_{2A}R$) is one of the four 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], while 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], as well as neurodegenerative diseases [7]. Accordingly, many $A_{2A}R$ antagonists are being developed to treat neurological disorders including Parkinson's 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 human, VSMCs predominate neointimal hyperplasia. However, it has been demonstrated that in human neointimal hyperplasia, the number of leukocytes in 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 field of research [11]. The inflammatory response, including the platelet and leukocyte accumulation on the injured arterial areas, as well as the smooth muscle cell migration, is requisite for arterial neointima formation [12–14]. Immediately after arterial injury, platelets interact with the injured area via many factors including glycoprotein Ib and glycoprotein IIb/IIIa [15, 16]. Upon adherence, platelets become activated and orchestrate the leukocyte recruitment and endothelial regeneration on the injured site [17–19]. Studies from our and other groups have demonstrated that the formation of arterial neointima is significantly suppressed following the inhibition of platelet accumulation, leukocyte adhesion, and the improvement of endothelial regeneration on the injured area [20–22].

Many senior patients with neurodegenerative diseases also suffer from 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 the arterial repair following injury. To our best 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$^{-/-}$).

Materials and 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, 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 prior to the wire injury of arteries and maintained on the same diet after wire injury until euthanization. Arterial injury was induced by a guide wire [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 AAALAC guidelines. Detailed methods are available inthe supplemental material.

Results

$A_{2A}R$ deficiency leads to the formation of 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 (Fig. 1a). Additionally, 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 (Fig. 1b and 1c). $A_{2A}R^{-/-}/apoE^{-/-}$ 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 (Supplementary table I and II); however, these differences were not statistically significant.

$A_{2A}R$ deficiency in bone marrow–derived cells increases injury-induced arterial neointima formation

We next determined whether, and to what extent, the presence of $A_{2A}R$ in bone marrow–derived cells (BMDCs) influences the formation of 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 described above. At the time of 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 chimeric mice lacking $A_{2A}R$ in their BMDCs were 60% larger than the lesions in chimeric control mice (Fig. 2 and supplementary Fig. Ia). The arterial neointima was stained heavily for the marker of macrophages but not that of vascular smooth muscle cells (supplementary Figs. Ib and Ic). Additionally, we performed wire injury in chimeric mice that were $A_{2A}R^{-/-}/apoE^{-/-}$ mice receiving 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, which were $A_{2A}R^{+/+}/apoE^{-/-}$ mice receiving 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 due to the deficiency of $A_{2A}R$ in BMDCs

$A_{2A}R$ deficiency increases leukocyte interactions with the injured arteries

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 (Fig. 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 (Fig. 3b and supplementary Fig. 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 (Fig. 3c and supplementary Fig. IIb); the presence of monocytes (macrophages) was rare (Fig. 3d and supplementary Fig. 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 (Fig. 3e, 3f and supplementary Fig. IId, IIe).

$A_{2A}R$ 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 *in vivo* trauma model of 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, and 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 numberof neutrophils passing through the same vessel. Compared to wt mice, $A_{2A}R$-deficient mice exhibited a 2-fold increase in the neutrophil rolling flux fraction (Fig. 4a). Additionally, 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 to 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 (Fig. 4b).

To compare the binding affinity of wt and $A_{2A}R$-deficient neutrophils, we employed an *ex vivo* micro-flow 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 following 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 (Fig. 4c). In addition, $A_{2A}R$ -deficient neutrophils rolled much more slowly than wt neutrophils did (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, LFA-1, CD11b, and CXCR2 were identical between both types of neutrophils (supplementary Fig. 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 (above), the levels of P-selectin binding to wt and $A_{2A}R$-deficient neutrophils were identical (supplementary Fig. IIIb). Thus, under static conditions, PSGL-1 has the same level of P-selectin binding in $A_{2A}R$-deficient and wt neutrophils.

Using confocal microscopy, we investigated the distribution of PSGL-1 following 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 that of wt neutrophils. This result was observed on neutrophils isolated from blood and on those adhering to injured arteries (Fig. 5a and 5b). Also, we explored several intracellular signaling mechanisms – i.e., the activity of phosphoinositide 3-kinase (PI3K)/Akt and mitogen-activated protein kinases (MAPKs) – that might be responsible for the increased homing ability of $A_{2A}R$-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 (Fig. 5c). This phenomenon was also observed in injured arteries (Fig. 5d). In contrast, the levels of phosphorylated Akt and other MAPKs were not significantly different between wt and $A_{2A}R$-deficient neutrophils (supplementary Fig. IV).

Consistent with the PSGL-1 distribution under flow conditions, $A_{2A}R$-deficient neutrophils exhibited greater PSGL-1 binding than wt neutrophils did. This result was demonstrated by an *ex vivo* micro-flow 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 mm,^{2} whereas the whole blood from wt mice presented 800 ± 70 rolling neutrophils per mm^2 (Fig. 5e). In a similar setup, 5-minute perfusion of mouse whole blood through the flow chamber coated with P-selectin and ICAM-1 led to a 2-fold higher number of $A_{2A}R$-deficient neutrophils adhering to the surface than the number of adhered wt neutrophils (Fig. 5f), indicating 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 (1mg/kg/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 ICAM-1 to the levels in wt mice (Figs. 5e and 5f).

Discussion

Our results demonstrate that leukocyte $A_{2A}R$ is critical for the protection of atherosclerotic mice from the 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 TNF-a and superoxide anions [28]. Several studies 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 (fMLP) [29–31]. 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 (VLA-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 fMLP, TNF-a 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. Rather, $A_{2A}R$ deficiency altered the cell membrane distribution of PSGL-1and the affinity of b_2 integrins, thereafter 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 PI3K/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, thereafter 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-in 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* micro-flow 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 [39, 40, 25]. Additionally, monocyte recruitment and activation could also play a role in the increased neointima formation following 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, NF-kB 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 due to the fact that many resolution aspects of inflammation take actions to reduce the size of lesions during a long-term chronic 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, 42, 43, 44]. We have previously shown that $A_{2A}R$-deficiency increases the apoptosis of macrophages [5]. In addition, CD8 T cells target the neovascularization of atherosclerotic arteries to reduce atherosclerosis [45]. Dr. Sitkovsky's group has reported that $A_{2A}R$-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 (personal communication). Therefore, the reduction of atherosclerosis in $A_{2A}R^{-/-}$/apoE$^{-/-}$ mice may also be due to the 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 at 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 $A_{2A}Rs$ [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 (Supplementary Figs. Va to Vc). Thus, different from its pharmacological effect as reported in other studies [50, 49], endothelial $A_{2A}R$ does not play a physiological role in determining the inflammatory status of endothelial cells in the arterial neointima model.

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 presenting 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 has been 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 chronic caffeine intake, is beneficial for the prevention of platelet aggregation [52, 53]. Different from above studies, we did not find increased platelet activation mediated by thrombin in $A_{2A}R$-deficient platelets (Supplementary Fig. 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 (Supplementary Fig. 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 following arterial angioplasty should be considered.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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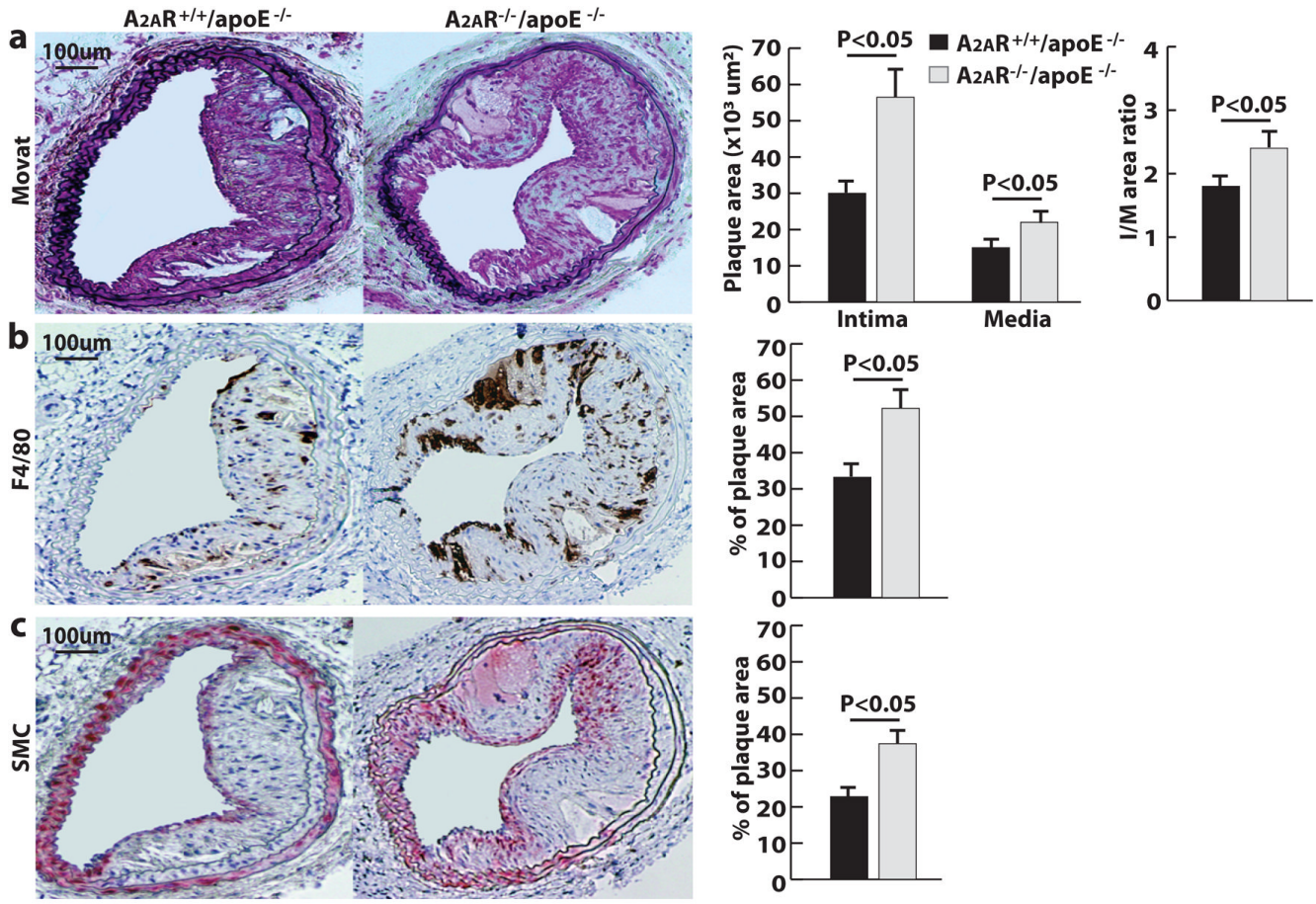

Figure 1. A_{2A}Rdeficiency increases injury-induced arterial neointima formation

a, Movat staining and size quantification of neointima (I), media (M) and ratio of intima to media (I/M). **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.

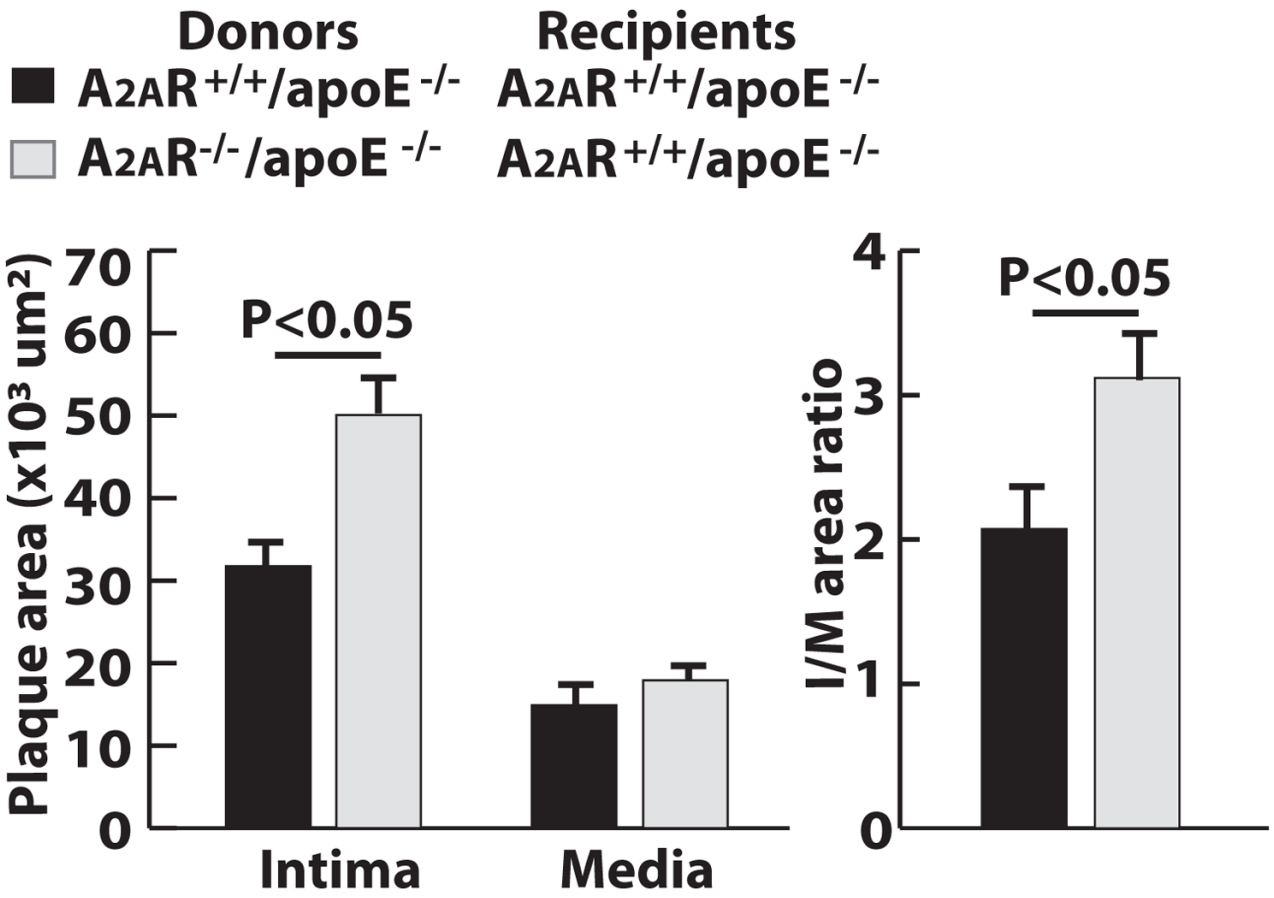

Figure 2. $A_{2A}R$ deficiency in bone marrow derived cells increases injury-induced arterial neointima formation

Size quantification of neointima (I), media (M) and ratio of intima to media (I/M). Data from 12 sections of 12 mice.

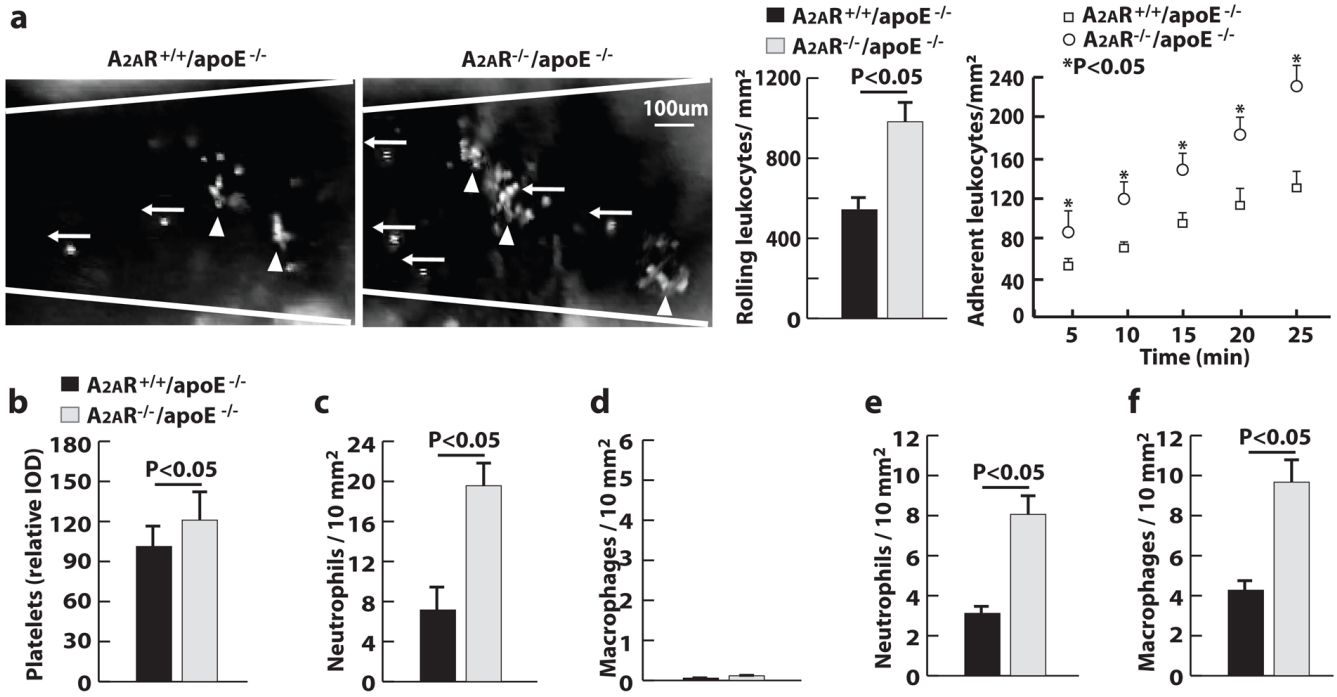

Figure 3. $A_{2A}R$ deficiency increases leukocyte interactions with injured arteries

a, Images and quantification of leukocyte rolling (←) and adhesion (▲) in injured carotid arteries. **b** to **f**, Quantification of carotid arteries stained for (b) platelets 1 hour after wire injury (WI), (c) neutrophils 1 hour after WI, (d) macrophages 1 hour after WI, (e) neutrophils 7 days after WI and (f) macrophages 7 days after WI (n=5).

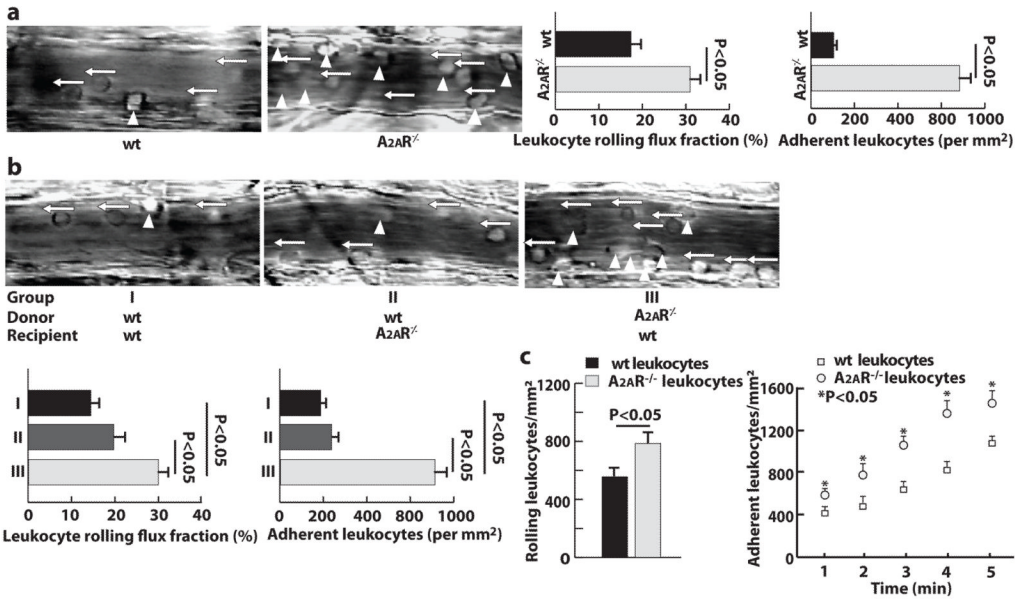

Figure 4. $A_{2A}R$ deficiency increases neutrophil homing ability

a and b, Images and quantification of neutrophil rolling (←) and adhesion (▲) on the endothelium of postcapillary venules of mouse cremaster muscle (n=6). **c and d,** Neutrophil rolling and adhesion on activated platelets through micro-flow chambers (n=6).

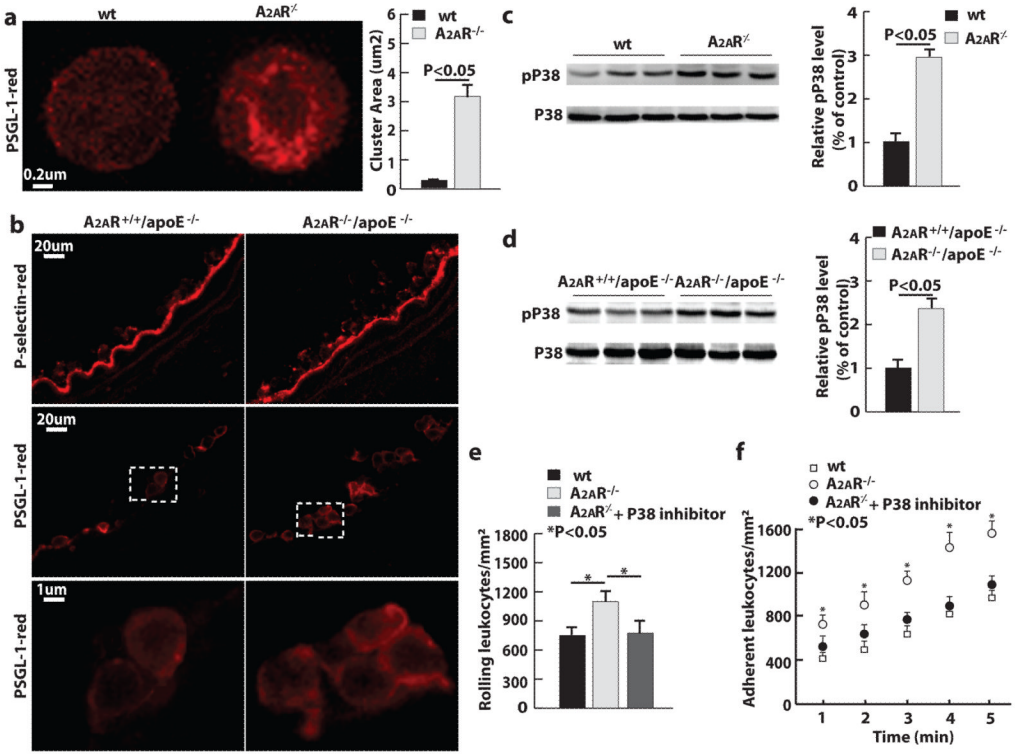

Figure 5. $A_{2A}R$ deficiency increases neutrophil p38 activity, and PSGL-1 clustering

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 (c) spleen neutrophils and (d) carotid arteries 1 hour after injury (n = 3). **e** and **f**, Leukocyte rolling and adhesion through (e) P-selectin coated and (f) P-selectin and ICAM-1 coated micro-flow chambers (n=3).