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 $P < 0.05$.

Reference List

(1) Chen JF, Huang Z, Ma J, Zhu J, Moratalla R, Standaert D, Moskowitz MA, Fink JS, Schwarzschild MA. A(2A) adenosine receptor deficiency attenuates brain injury induced by transient focal ischemia in mice. *J Neurosci* 1999;19:9192-9200.

(2) Lappas CM, Day YJ, Marshall MA, Engelhard VH, Linden J. Adenosine A2A receptor activation reduces hepatic ischemia reperfusion injury by inhibiting CD1d-dependent NKT cell activation. *J Exp Med* 2006;203:2639-2648.

(3) An G, Wang H, Tang R, Yago T, McDaniel JM, McGee S, Huo Y, Xia L. P-selectin glycoprotein ligand-1 is highly expressed on Ly-6Chi monocytes and a major determinant for Ly-6Chi monocyte recruitment to sites of atherosclerosis in mice. *Circulation* 2008;117:3227-3237.

(4) Wang H, Zhang W, Tang R, Hebbel RP, Kowalska MA, Zhang C, Marth JD, Fukuda M, Zhu C, Huo Y. Core2 1-6-N-Glucosaminyltransferase-I Deficiency Protects Injured Arteries From Neointima Formation in ApoE-Deficient Mice. *Arterioscler Thromb Vasc Biol* 2009;29:1053-1059.

(5) Wang H, Tang R, Zhang W, Amirikian K, Geng Z, Geng J, Hebbel RP, Xia L, Marth JD, Fukuda M, Katoh S, Huo Y. Core2 1-6-N-Glucosaminyltransferase-I Is Crucial for the Formation of Atherosclerotic Lesions in Apolipoprotein E-Deficient Mice. *Arterioscler Thromb Vasc Biol* 2009;29:180-187.

(6) Huo Y, Schober A, Forlow SB, Smith DF, Hyman MC, Jung S, Littman DR, Weber C, Ley K. Circulating activated platelets exacerbate atherosclerosis in mice deficient in apolipoprotein E. *Nat Med* 2003;9:61-67.

(7) Smith ML, Sperandio M, Galkina EV, Ley K. Autoperfused mouse flow chamber reveals synergistic neutrophil accumulation through P-selectin and E-selectin. *J Leukoc Biol* 2004;76:985-993.

(8) Hafezi-Moghadam A, Thomas KL, Prorock AJ, Huo Y, Ley K. L-selectin shedding regulates leukocyte recruitment. *J Exp Med* 2001;193:863-872.

(9) Huo Y, Zhao L, Hyman MC, Shashkin P, Harry BL, Burcin T, Forlow SB, Stark MA, Smith DF, Clarke S, Srinivasan S, Hedrick CC, Pratico D, Witztum JL, Nadler JL, Funk CD, Ley K. Critical role of macrophage 12/15-lipoxygenase for atherosclerosis in apolipoprotein E-deficient mice. *Circulation* 2004;110:2024-2031.

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.