The A2B adenosine receptor protects against inflammation and excessive vascular adhesion

Dan Yang,1 Ying Zhang,1 Hao G. Nguyen,1 Milka Koupnova,1 Anil K. Chauhan,2 Maria Makitalo,1 Matthew R. Jones,1 Cynthia St. Hilaire,1 David C. Seldin,1 Paul Toselli,1 Edward Lamperti,1 Barbara M. Schreiber,1 Haralambos Gavras,3 Denisa D. Wagner,2 and Katya Ravid1,3

1Department of Biochemistry and Whitaker Cardiovascular Institute, Boston University School of Medicine, Boston, Massachusetts, USA.
2CBR Institute for Biomedical Research and Department of Pathology, Harvard Medical School, Boston, Massachusetts, USA.
3Department of Medicine, Boston University School of Medicine, Boston, Massachusetts, USA.

Adenosine has been described as playing a role in the control of inflammation, but it has not been certain which of its receptors mediate this effect. Here, we generated an A2B adenosine receptor–knockout/reporter gene–knock-in (A2BAR-knockout/reporter gene–knock-in) mouse model and showed receptor gene expression in the vasculature and macrophages, the ablation of which causes low-grade inflammation compared with age-, sex-, and strain-matched control mice. Augmentation of proinflammatory cytokines, such as TNF-α, and a consequent downregulation of iκB-α are the underlying mechanisms for an observed upregulation of adhesion molecules in the vasculature of these A2BAR-null mice. Intriguingly, leukocyte adhesion to the vasculature is significantly increased in the A2BAR-knockout mice. Exposure to an endotoxin results in augmented proinflammatory cytokine levels in A2BAR-null mice compared with control mice. Bone marrow transplantations indicated that bone marrow (and to a lesser extent vascular) A2BARs regulate these processes. Hence, we identify the A2BAR as a new critical regulator of inflammation and vascular adhesion primarily via signals from hematopoietic cells to the vasculature, focusing attention on the receptor as a therapeutic target.

Introduction

Based on pharmacologic studies, the G protein–coupled adenosine receptors were initially classified into adenylyl cyclase inhibitory (A1 and A3) and stimulatory (A2) categories (1, 2). Further classification of A2 adenosine receptors (A2ARs) into subtypes 2A and 2B (3, 4) was determined by the presence of high-affinity A2AAR and low-affinity A2BAR binding sites (5). Subsequent molecular studies identified all 4 genes encoding these receptors (reviewed in ref. 6), and their classification has been endorsed by the International Union of Pharmacology Committee on Receptor Nomenclature and Drug Classification (7). The role of adenosine receptors in cellular or tissue function has been deduced from studies with adenosine ligands (reviewed in ref. 8). Mouse models engineered to harbor a deletion of a specific adenosine receptor have proven to be important in elucidating the function of different adenosine receptors (reviewed in ref. 6). Generation of mice deficient in A1ARs has supported previous pharmacological research related to the role of the A1AR in central nervous system and kidney physiology (9). These studies demonstrate that the A1AR is necessary for the tubuloglomerular response to increased flow rate and subsequently impacts renal water and sodium retention. Furthermore, plasma renin activity is increased in A1-deficient mice compared with their WT littermates, suggesting that adenosine regulates renin release via A1ARs. Mice lacking functional A1ARs show signs of increased anxiety and hyperalgesia (10). Hypoxia-associated decrease in neuronal activity is less pronounced in A1AR-null mice, as is recovery of neuronal activity after hypoxia (9). Mice carrying a genetic deletion of the A2AR have been described in several reports. The ability of an A2AR selective ligand to potentiate antigen-dependent degranulation of mast cells, as measured by hexosaminidase release, is lost in mice lacking A2AR compared with normal mice (11). Cutaneous vasopermeability is associated with activation and subsequent degranulation of mast cells, and induction of cutaneous vasopermeability by adenosine or inosine, as measured by extravasation of plasma protein, is lost in mice lacking a functional A2AR (12). Attenuation of LPS-induced TNF-α production is decreased in these A2AR-null mice as compared with control mice (11). Studies of BP response to intravenous adenosine injection show a significantly larger drop in BP in mice lacking the A2AR compared with control mice (13). Interestingly, ischemia-reperfusion injury of mice lacking A2ARs results in a significant reduction in infarct size compared with WT mice. No difference in infarct size is seen after preconditioning (14). As to the A3ARs, they were the first adenosine receptors to be genetically deleted in a murine model, and over the years several studies have continued to elucidate the role of these receptors in various physiological processes. These animals demonstrate several central nervous system disturbances, including increased BP, decreased exploratory activity, increased aggressiveness and hypoalgesia (15), compensatory alteration in spinal cord opioid receptors (16), attenuated psychostimulant responses (17, 18), reduced alcohol sensitivity (19), and reduced alcohol withdrawal-induced seizure (20). Exploratory activity in mice is generally increased by caffeine administration; however, the opposite effect was observed in A3AR-null mice, which suggested that caffeine-dependent psychostimulation is mediated by the A3AR (20). Activation of A3AR is required for dopaminergic function, since dopamine-mediated cellular response such as...

Nonstandard abbreviations used: A2AR, A2B adenosine receptor; M-MLV, Moloney murine leukemia virus; NECA, 5′-N-ethylcarboxamideadenosine.

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DARPP-32 phosphorylation and immediately early gene expression in the striatum were abolished in the A2B-deficient mice (21, 22). Inflammatory stimuli induced higher levels of proinflammatory cytokines and increased tissue damage after injury in mice lacking the A2B compared with WT mice (23, 24). Studies of all the above-described adenosine receptor–null mouse models indicate that extracellular adenosine acting at adenosine receptors results in complex action in various tissues.

Little is known about the cell-specific expression of the A2BAR in vivo or about the functional significance of the A2BAR, the latter because there is a lack of specific agonists for this receptor. Despite this limitation, the A2BAR has been implicated in several important biological events, including mediating vasodilation (25, 26), inhibiting growth of rat aortic smooth muscle cells (27, 28), and increasing the production of cytokines, such as IL-6, by vascular cells (29, 30). In vitro studies described the A2BAR as mediating augmentation of cytokine production by vascular cells (29, 30), and adenosine has been described as a regulator of inflammatory response (31). This was attributed to adenosine receptor–mediated control of cytokine production by macrophages (32, 33) and confirmed during analysis of A2BAR-null mice (24, 34). Based on these in vivo studies, among adenosine receptors, the A2BAR has been regarded as a main protector against inflammation. However, the direct influence of A2BAR on cytokine levels, inflammation, and/or vascular adhesion has not yet been examined in vivo, nor has the relative contribution of bone marrow–derived adenosine receptors or other A2BARs to these processes.

The current study describes the generation of a mouse model with targeted deletion of the A2BAR gene. Exon 1 of the A2BAR was replaced by a reporter gene, which allowed examination of endogenous A2BAR-dependent expression in various tissues and cell types in vivo. Results show that there is abundant reporter expression in the vasculature and in macrophages. This new animal model emphasizes a role for the A2BAR in attenuating inflammation, at baseline or in response to endotoxin treatment, by regulating proinflammatory cytokine production and adhesion properties of the vasculature. These effects are primarily mediated via signals from hematopoietic cells to the vasculature, as deduced from bone marrow transplantation experiments. Contrary to the speculated function of A2BAR in vasodilation, the A2BAR-null mice have normal BP at baseline or in response to adenosine infusion.

Results

Generation of A2BAR-deficient mice. A targeting construct was generated to inactivate the A2BAR gene by replacing exon 1 of the A2BAR with a reporter construct containing the β-gal gene (Figure 1A). Successful genomic integration of the mutant allele was determined by Southern blot analysis and PCR as shown in Figure 1, B and C. Furthermore, to confirm A2BAR gene ablation, A2BAR transcripts were quantified in various tissues using RT-PCR (Figure 1D and Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI27933DS1). Because an antibody specific to mouse A2BAR is currently unavailable, the presence or absence of receptor activity was used as an indicator.
of protein expression. Figure 1E shows that the A<sub>2A</sub>AR and A<sub>2B</sub>AR agonist 5'-N-ethylcarboxamidoadenosine (NECA) only mildly increased cAMP levels in primary VSMCs derived from KO mice compared with the significant increase in cAMP production in WT VSMCs. Both A<sub>2A</sub>AR and A<sub>2B</sub>AR were previously shown to be expressed in WT primary VSMCs (35). The A<sub>2B</sub>AR selective antagonist MRS1754 eliminated the stimulatory effect of NECA only in WT cells, indicating that A<sub>2B</sub>AR activity is high in these cells and absent in the KO cells. Administration of forskolin (a direct activator of adenylyl cyclase) demonstrated that adenylyl cyclase activity augmented cAMP levels in both WT and A<sub>2B</sub>AR-deficient cells. Similar results were obtained using macrophages (Supplemental Figure 1B).

A<sub>2B</sub>AR-KO mice bred normally and exhibited normal platelet, red blood, and white cell counts (Supplemental Table 1). For all subsequent studies, age- and sex-matched WT and A<sub>2B</sub>AR-KO mice were used as detailed in Methods.

A<sub>2B</sub>AR expression in different tissues. By replacing exon 1 of the A<sub>2B</sub>AR gene with a reporter construct containing β-gal, tissue-specific activation of the A<sub>2B</sub>AR gene promoter was conve-
The A2BAR controls proinflammatory cytokine production. In view of the significant expression of A2BAR in macrophages, the effect of its deletion on the level of proinflammatory cytokines was examined. As shown in Figure 3A, basal levels of liver-derived TNF-α and IL-6 were approximately 2-fold higher in KO compared with WT mice. Similar results were obtained with primary macrophages derived from these mice (Supplemental Figure 3), suggesting low-grade inflammation. To examine the response of these mice to challenge, they were stimulated with LPS. The level of serum TNF-α was 5-fold higher in the KO mice compared with WT controls at 1 hour after LPS injection (Figure 3B). This observation is in accordance with the reported inhibitory effect of adenosine analogs on the release of TNF-α from macrophages via activation of A2ARs (37). This cytokine is rapidly (within an hour) and transiently elevated after exposure to endotoxin, while the increase in the other proinflammatory cytokine, IL-6, is sustained hours after LPS injection (34). The level of IL-6 was also greatly increased in A2BARKO mice treated with LPS compared with WT mice treated with this endotoxin, and this difference was manifested at 12 to 20 hours after injection. This suggests that IL-6 upregulation in LPS-treated A2AR-KO mice depends upon early signaling and changes induced during the first few hours after endotoxin injection. The extent of induction of the antiinflammatory cytokine IL-10 was reduced in the KO mice compared with WT (Figure 3C). No change was observed in the level of IL-4 (data not shown). At 24–48 hours after LPS exposure, all plasma cytokine levels returned to basal levels in both control and KO mice.

A2AR deficiency enhances the expression of adhesion molecules as well as leukocyte adhesion and rolling. It has been shown that an increased level of circulating TNF-α is associated with the expression of...
adhesion molecules in the vasculature (38, 39). Because basal levels of TNF-α were augmented in Aβ2AR-deficient mice, we examined adhesion molecule expression in KO versus WT mice. Interestingly, E-selectin, P-selectin, and ICAM-1 levels were all upregulated in Aβ2AR-deficient mice (Figure 4A). Previous studies demonstrated that the mechanism of adhesion molecule induction by TNF-α involves NF-κB activation, which depends on downregulation of the NF-κB inhibitor IκB-α (reviewed in refs. 40–42). Selectin upregulation in KO mice is accompanied by reduced IκB compared with WT mice (Figure 4B).

Induced expression of adhesion molecules is the underlying mechanism for leukocyte adhesion to blood vessels (43–45). For this reason, we sought to determine potential differences in leukocyte rolling and adhesion between WT and Aβ2AR-KO mice. These parameters were studied by intravital microscopy in 2 different types of mesenteric venules: large venules of 220–235 μm and microvessels of 25–35 μm in diameter. shear rates for all venules studied were similar in Aβ2AR-KO mice. Despite attempts to dissect an endothelial layer from a mouse artery, the preparation was accompanied with VSMCs. vWF is only expressed in endothelial cells and, hence, serves as an appropriate marker for this cell type. Samples were subjected to Western blotting with the indicated antibodies. (Figure 5A; P < 0.001). We also found that leukocytes rolled at a slower velocity in Aβ2AR-KO mice compared with WT mice (Figure 5B; P < 0.05). The leukocytes adhered to the endothelium for more than 25 seconds (Figure 5D).

Blood cell–derived signaling mediated by the Aβ2AR induces cytokine release and adhesion molecule expression. Cytokines are produced by bone marrow–generated immune cells as well as by other cell types, including VSMCs (29). Bone marrow transplantation experiments were conducted to examine the contribution of these cells to inflammatory cytokine production and to the induction of adhesion molecules in Aβ2AR-KO mice. Genotyping of sex chromosome–linked genes using PCR analysis was used to confirm cross-sex transplantation of bone marrow–derived cells (from male donor to female recipient) as described in Methods and shown in Figure 6A. Transplantation efficiency was determined by the percentage of β-gal–positive bone marrow macrophages in WT mice transplanted with Aβ2AR-KO mouse bone marrow cells (Figure 6B), and it was determined to be 97.5% ± 6.9% (α = 4). Red and white blood cell counts and platelet counts in all transplanted animals varied by up to 15% (data not shown). LPS-induced elevation of the proinflammatory cytokines TNF-α and IL-6 in KO→KO (i.e., bone marrow from Aβ2AR-null mice transplanted into irradiated Aβ2AR-null mice) was about 3- to 5-fold greater than LPS-induced levels in WT→WT (compare white bars in Figure 6C). In nontransplanted mice (Figure 3B), LPS induction of these cytokines was also 3- to 5-fold greater in KO mice compared with WT. As also shown in Figure 6C, after LPS treatment, TNF-α levels were greatly elevated in KO→KO and KO→WT mice compared with those in WT→WT mice or WT→KO. The level of IL-6 followed a trend similar to that exhibited by TNF-α, except that the induction in KO→KO was greater than in KO→WT (Figure 6C). These results indicate that elevated TNF-α levels and a significant portion of IL-6 originate from bone marrow–derived cells. In addition to macrophages, another known source of proinflammatory cytokines is VSMCs (29). We demonstrated that primary cultures of VSMCs and macrophages isolated from Aβ2AR-KO mice produced increased levels of TNF-α (in the case of macrophages) and IL-6 (in the case of VSMCs and macrophages) under basal conditions when exposed to LPS (in both cell types), compared with cultures derived from WT mice (Supplemental Figure 3). These results indicate that, at least under culture conditions (with mitogenic/serum stimuli), VSMCs produce greater levels of proinflammatory cytokines in the absence of Aβ2AR expression compared with control. The effect of Aβ2AR ablation on cytokine production was more prominent, however, in macrophages.

To examine whether selectin induction in the vasculature depends on signals from Aβ2AR-deleted bone marrow–derived cells, ICAM-1, P-selectin, and E-selectin were measured in the vasculature of transplanted mice. As shown in Supplemental Figure 4, KO mice implanted with WT mouse bone marrow cells showed decreased expression of adhesion proteins. This suggested that signals from bone marrow cells stimulate the expression of these proteins. The fold induction, however, was smaller than that in KO mice compared with WT mice (Figure 4B), suggesting that vascular Aβ2ARs somewhat contribute to the observed upregulation.

Deletion of Aβ2AR does not impact BP. Since the Aβ2AR is highly expressed in the vasculature and because some studies implied

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**Figure 4**

E-selectin, P-selectin, and ICAM-1 levels are augmented and IκB-α levels are reduced in the Aβ2AR-KO mouse. (A) Western blot analysis of protein extracts isolated from mesenteric arteries derived from WT and KO mice. Despite attempts to dissect an endothelial layer from a mouse artery, the preparation was accompanied with VSMCs. vWF is only expressed in endothelial cells and, hence, serves as an appropriate marker for this cell type. Samples were subjected to Western blotting with the indicated antibodies. (B) Quantification of the Western blot analyses of WT and Aβ2AR-KO samples shown in A. Probing with anti-actin was used to generally reflect protein loading. Protein levels on the Western blots were assessed and normalized to the level of vWF, which represents the fraction of endothelial cells within the collected protein. Quantification was performed using NIH ImageJ software (version 1.62; http://rsb.info.nih.gov/ij/). The data shown represents the average ± SD of 3 experiments.
its potential role in vascular tone (25, 26), we determined BP in the A2BAR-KO mice under basal conditions and in response to intravenous administration of adenosine at different concentrations. A2BAR deficiency did not impact BP under basal conditions or after adenosine-induced vasodilation (Supplemental Figure 5 and Supplemental Table 3).

Discussion

Cell culture studies indicated that A2BAR is expressed in immune cells, endothelial cells, and aortic vascular smooth muscle (reviewed in ref. 6). In addition to confirming the results of these previous reports, the A2BAR-KO/reporter gene–knock-in mouse model showed that not all VSMCs express this gene. Our results demonstrated that the A2BAR gene is differentially activated/suppressed in smooth muscle cells within different blood vessels. It is conceivable that varying intensities of local signals within vasculatures induced by cytokines, shear stress, and other factors lead to differential expression of the A2BAR gene in these loci. The underlying mechanism of this phenomenon awaits further investigation.

In comparison to other adenosine receptor subtypes, it was speculated that the A2AAR is the most significant mediator of cytokine production (46). In a study using primary peritoneal macrophages (47), the potency of the A2AAR selective agonist CGS 21680 in decreasing cytokine production indicated an effect on the A2AAR. Consistent with these findings, A2AAR-KO mice were reported to have increased levels of proinflammatory cytokines, albeit only upon challenge with LPS (24). The current study focuses attention on the A2BAR as an important contributor to these phenomena. We found that the A2BAR-KO mice displayed a mild but significant increase in proinflammatory cytokines under baseline conditions. Following a challenge with LPS, there was a large augmentation in production of these cytokines in the KO mice compared with WT controls. Intriguingly, at baseline, the A2BAR-KO mice showed upregulated levels of the adhesion molecules E-selectin, P-selectin, and ICAM-1 in the vasculature. Polymorphonuclear leukocytes are recruited into inflamed tissue via such adhesion molecules. Leukocyte rolling is supported by the endothelial selectins E-selectin and P-selectin (CD62E and CD62P), which are upregulated on the plasma membrane in response to cytokines (48, 49). In large venules as well as microvessels, more rolling of leukocytes and increased adhesion to the vessel were observed in the A2BAR-KO mice compared with control mice, pointing to an important role for this receptor in vascular homeostasis. Direct staining of aortic (along the aorta and in the arch) or mesenteric sections with antibodies against macrophage and leukocyte markers (using procedures described in Methods) showed no significant difference in KO versus WT tissues (data not shown). Although the KO mice exhibited inflammation, we did not necessarily expect accumulation of these cells within the tissue. Unless a chemoattractant is provided, such cells do not spontaneously transmigrate. As inflammation and increased leukocyte adhesion are known to aggravate atherosclerosis, it will be interesting to examine in the future the progression of this pathology in A2BAR-KO mice crossbred with apolipoprotein E–null mice, as the latter are known to provide a genetic background susceptible to atherosclerosis. Of interest, studies using A2AAR-KO mice showed no evidence of inflammation at baseline; however, inflammatory stimuli induced higher levels of proinflammatory cytokines in these KO mice than in WT mice, and the extent of induced liver injury was greater in the KO mice (23, 24).
Augmented levels of cytokines and adhesion molecules could be due to altered signaling in A2BAR-defleted vascular cells and/or due to activation by bone marrow cell–derived signals. Bone marrow transplantation studies indicated that bone marrow–derived A2BAR signals induce cytokine elevation and upregulation of vascular adhesion molecules and suggest a role for the vascular A2BAR as well. In accordance with these findings, primary cultures of aortic VSMCs derived from A2BAR-KO mice produced increased levels of IL-6 under basal conditions and of IL-6 and TNF-α in response to LPS, compared with cultures derived from matched control mice. Of note, bronchial smooth muscle cell A2BARs stimulate IL-6 production (29). In our study, elimination of the A2BAR increased IL-6 production. The difference could be due to application of selective ligands in the former study, versus analysis of KO mice, and/or related to the source of the smooth muscle cells used.

Previous pharmacological studies have suggested a role for A2BAR in determining BP (25, 26). Studies with the A2BAR-KO mouse model, however, ruled out a role for this receptor in modulating BP, under basal conditions and in response to adenosine. Furthermore, A2BAR ablation did not affect the expression or activity of the A2BAR (Supplemental Figure 1) or of other adenosine receptor subtypes (A1, A2a, and A3), which was determined by quantitative RT-PCR (data not shown). A2A-deficient mice on a CD1 genetic background have increased BP, heart rate, and platelet aggregation (15). A2A-deficient mice on either a mixed 129-Steel × C57BL/6 (50) or congenic C57BL/6 (51) genetic background, however, did not display BP differences.

In summary, the study demonstrates the cell-specific anatomic location of endogenous A2BAR gene expression and identifies the A2BAR as a modulator of inflammatory cytokines, expression of adhesion molecules and of leukocyte adhesion, and rolling on blood vessels. Mechanisms related to altered expression of adhesion proteins in the KO mice involve IκB downregulation, primarily due to A2BAR signaling from bone marrow cells to the vasculature. The generation of A2BAR-deficient mice will also allow future examination of other A2BAR-dependent functions in different tissues. For example, A2BAR is expressed in mast cells and may play an important role in the host response to inducers of asthma (reviewed in ref. 52). Similarly, the significance of A2BAR expression in the brain and retina await future investigation.

Methods

Generation of A2BAR-KO/β-gal–knock-in mice

The mouse A2BAR gene (GenBank accession number AL596110) was cloned from a mouse ES-129/SvJ bacterial artificial chromosome library, and the A2BAR gene structure is shown in Figure 1A. The targeting vector, A2BAR-KO-β-gal was constructed as depicted in Figure 1A. A strategy was employed to delete exon 1, including the initiation codon, and to introduce instead a reporter gene with a stop codon and poly(A) at the 3′ end to terminate transcription. Exon 1 in the A2BAR gene encodes 3 transmembrane domains of A2BAR (53), and deleting it will remove the initiation methionine. The next in-frame methionine is not present until the fifth transmembrane domain (past the ligand-binding domain) (53). Hence, even if transcription is unexpectedly initiated past the transcrip-

Figure 6
Signals derived from bone marrow cells enhance cytokine production and expression of adhesion molecules in A2BAR KO mice. (A) PCR analysis of DNA from the transplanted mice, including males (M) to females (F), and expected PCR products, as detailed in Methods. (B) Under these conditions, the average efficiency of transplantation was assessed as 97.5% ± 6.9% (SDs of 4 measurements) by monitoring the percentage of β-gal–positive KO peritoneal macrophages in transplanted WT mice (performed as in Figure 2D). This mode of assessment was possible since the total number of macrophages was similar in the different transplanted mice. (C) LPS-induced cytokine levels in the plasma. Mice were injected with vehicle (control) or LPS (5 μg/g body weight), and plasma cytokine levels of TNF-α and of IL-6 were determined after 1 hour and 16 hours, respectively, following the elevation peaks noted in Figure 3B. Results are presented as average ± SD of 4 experiments.
tion signal terminal of the reporter gene, the truncated protein formed is not likely to fold or to be functional, as it will not bind a ligand. To confirm this contention, mRNA expression and functional studies were pursued as detailed below. Also of note, there are no annotated $A_{2B}$AR pseudogenes in the mouse genome listed at the NCBI Nucleotide database (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Nucleotide) or potential unknown pseudogenes, based on using the Basic Local Alignment Search Tool (BLAST, http://www.ncbi.nlm.nih.gov/genome/seq/BlastGen/BlastGen.cgi?taxid=10090) under permissive conditions. The human $A_{2B}$AR pseudogene (GenBank accession number NG_000843) is typical of a retrotransposed pseudogene, meaning that it does not contain introns or the promoter (mRNA was reverse transcribed and then inserted in the genome). This type of pseudogene would not be targeted by our construct, because the flanking homologous regions are those of the promoter and the intron. To generate the targeting vector, a 3.6-kb fragment from intron 1 of the $A_{2B}$AR gene generated by KpnI/Xhol endonuclease restriction digestion was first subcloned into a pPNT vector at KpnI/ EcoRI sites between the neomycin (Neo) gene cassette and thymidine kinase (TK) cassette using a Xhol-EcoRI linker, yielding pPNT-$A_{2B}$AR-intron. A 6-kb fragment of the $A_{2B}$AR gene promoter was subcloned into the $EcoRI$ site of the end gene of the second exon prokaryotic $\beta$-gal in the pBS-lacZ plasmid. The 9.4-kb NotI/Xhol $A_{2B}$AR-$\beta$-gal fragment was then subcloned into pPNT-$A_{2B}$AR-intron at the NotI/Xhol sites, 5′ of the Neo cassette. Recombinant $A_{2B}$AR-KO-LacZ ES cells (mouse 129/SvJ derived) were obtained from The Jackson Laboratory (Bar Harbor, ME) and cells, including smooth muscle cells and macrophages, were subsequently implanted back into FVB foster mothers. Positive chimeric mouse lines were identified by coat color, and their offspring were screened by PCR and Southern blot analysis of tail DNA. PCR WT primers were designed to produce a 746-bp fragment (both primers are located in the intron 1 area). Mutant upstream primer 5′-TCTCACACAGAGCTCCATCTT-3′ and downstream primer 5′-TCTGCGGACAGCTTGTGAT-3′ were used to produce a 488-bp band (both primers are located in the intron 1 area). Mutant upstream primer 5′-CAGCCCTGGTCTCAACCAC-3′ and downstream primer 5′-GGACCTCTCCTCTCAAGACAC-3′ were used to generate a 900-bp band (both primers are located in the Neo cassette). The heterozygous mice were backcrossed to C57BL/6J strain mice for 4 generations to generate congenic C57BL/6J strain $A_{2B}$AR gene mutant mice. In this study, the mice were used of 80% C57BL/6J background strain, confirmed by the PCR-based gene marker analysis MAX-BAX (Charles River Laboratories). Crossbreeding of the 80% C57BL/6J strain $A_{2B}$AR mutant heterozygous mice generated the same background strain as WT or $A_{2B}$AR gene–KO homozygous mice. In the experiments shown, control and knockout mice were strain-, sex-, and age-matched (8–12 weeks old unless otherwise indicated). All procedures were performed according to the Guidelines for Care and Use of Laboratory Animals published by the NIH. Throughout this study, all animals received humane care that was in agreement with the guidelines of and approved by the Institutional Animal Care and Use Committee of the Boston University School of Medicine. All analyses were repeated (n) to obtain averages ± SDs and subjected to 2-tailed Student’s t tests, as indicated in each figure. P values less than 0.05 were considered statistically significant.

### Measurement of $A_{2B}$AR Expression and Activity in Different Tissues and Cells, Including Smooth Muscle Cells and Macrophages

Total RNA from different mouse tissues was prepared with TRIZol (according to the manufacturer’s protocol; Invitrogen). RT-PCR was used (1 μg RNA, Moloney murine leukemia virus [M-MLV] reverse transcriptase, 1× first-strand buffer, 0.5 mM dNTP, 5 mM DTT, 0.5 μM RNase inhibitor, 5 μM random primers, all purchased from Invitrogen) to measure $A_{2B}$AR mRNA expression. RNA was treated with RQ1 RNase-Free DNase (according to the manufacturer’s instructions; Promega, catalog no. M6101) and this was followed by RT-PCR performed in a 96-well plate according to the manufacturer’s instruction. The primer pairs were designed to produce a 746-bp fragment within exon 1 (PCR primers: sense, 5′-CTGCGGACAGCTTGTGAT-3′; antisense, 5′-GGACCTCTCCTCTCAAGACAC-3′). To optimally detect $A_{2B}$AR mRNA, the number of PCR cycles in our system was set at 30. GAPDH mRNA expression, used as control, was set at 20 cycles (GAPDH primers generate 554-bp fragments and were: sense, 5′-TCCACATCCCTCCAGGAGG-3′ and antisense, 5′-GCTCTCACACCTTCTTCTGTG-3′). To amplify the second exon in $A_{2B}$AR cDNA by RT-PCR, the following primers were employed: sense, 5′-CAAGTGGGTGAATGTTGGAAGG-3′; antisense, 5′-TTCCCAGGATCTACTAC-3′ (to generate a 448-bp fragment). The primers were selected using Primer3 primer design tool (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3 www.cgi). The MacVctor sequence alignment program (version 6.5.3; Accelrys) was used to ensure that there was no amplification of other gene sequences. Total RNA was isolated from mesenteric artery or other gene sequences. Total RNA was isolated from mesenteric artery or other gene sequences. Total RNA was isolated from mesenteric artery or other gene sequences. Total RNA was isolated from mesenteric artery or other gene sequences. Total RNA was isolated from mesenteric artery or other gene sequences.
cAMP levels were measured using Direct Cyclic AMP Kit (Assay Designs, catalog no. 900-066).

**Primer used to amplify all adenosine receptor mRNAs by PCR**

RNA preparation and PCR conditions were essentially as listed above for the A<sub>2A</sub> AR. For the A<sub>1</sub> AR, the primers were used: sense, 5′-GCT-TAGTCCTCAGATACAG-3′; antisense, 5′-CCCTTGTCTTAGAG-GTCCA-3′; expected fragment of 436 bp. For the A<sub>3</sub> AR, the primers were: sense, 5′-TGAAGGCGAAGGGCATCA-3′; antisense, 5′-CGCAG-GTCTTGTGAGGATTCT-3′; expected fragment of 117 bp. For the A<sub>2B</sub> AR, the primers were: sense, 5′-TAATGGGAAGGCAGGGATAAG-3′; antisense, 5′-GATGATTGATGTTGTCAGCCA-3′; expected fragment of 284 bp. The PCR conditions included: 1 cycle at 95°C for 2 minutes, 95°C for 50 seconds, 60°C for 50 seconds, 72°C for 50 seconds, for 28 or 30 cycles, and 1 cycle of 5 minutes at 72°C.

**Analysis of β-gal expression in tissue sections and in cells**

β-gal assay in tissue sections at the light-microscopic level. Mice were anesthetized with isoflurane and perfused through the left heart ventricle with 20 ml PBS (pH 7.4) at a rate of 4 ml/min. Perfusion with fixed (30 ml freshly made 2% paraformaldehyde in PBS [pH 7.4]) continued for 15 minutes at 2 ml/min, followed by perfusion with PBS for 10 minutes. Various tissues were dissected from the perfused mouse and stored in ice-cold PBS, prior to staining for β-gal activity. Individual organs were cut into 1- to 2-mm-thick slices and stained with X-gal staining solution (5 mM K<sub>3</sub>Fe(CN)<sub>6</sub>, 5 mM K<sub>4</sub>Fe(CN)<sub>6</sub>·3H<sub>2</sub>O (Sigma-Aldrich, catalogue nos. P-8131 and P-9287, respectively), 2 mM MgCl<sub>2</sub> in PBS) containing a final concentration of 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside (X-gal; American Bioanalytical, catalog no. AB2400-1000) or halogenated indolyl-β-d-galactoside, as indicated (Blue-gal; Sigma-Aldrich, catalog no. B2904). Samples were incubated at 37°C for 6–12 hours on a rocking platform. After staining, samples were rinsed with PBS and stored in 4% paraformaldehyde at 4°C. Sections were embedded in paraffin and cut at a thickness of 5 μm. Sections were stained with H&E.

Analysis of β-gal expression at the ultrastructural level. Mouse tissue was stained for β-gal activity as described above, except that Blue-gal (halogenated indolyl-β-d-galactoside from Invitrogen; catalog no. 15519-010) was used as a substrate instead of X-gal. Tissues were processed for electron microscopy using a modification of a previously published protocol (36). Briefly, 25% glutaraldehyde (Polysciences Inc.) was diluted to 4.3% with 0.03 M sodium barbital–sodium acetate buffer (pH 7.4) in 0.07 M potassium chloride. The samples were kept in this glutaraldehyde solution overnight at 4°C. The samples were rinsed 3 times with sodium barbital–sodium acetate buffer containing potassium chloride for 15 minutes each. This was followed by dehydration in a graded series of ethanol starting with 50% ethanol, embedded in a 1:1 mixture of Araldite 502 (Ted Pella Inc.) and dodecenyl succinic anhydride at 60°C. After polymerization of the Araldite mixture, sections were cut on an LKB Ultratome V. Ribbons of sections showing gray, silver, or slightly gold interference colors were picked up on uncoated 200-μm thick poly-l-lysine slide and transferred overnight at 4°C to Immobilon-P PVDF membranes (Millipore), and Western blotting was performed as described in ref. 58. The following antibodies (purchased from Santa Cruz Biotechnology Inc.) were used: rabbit polyclonal anti-E-selectin antibody (1:200 dilution; catalog no. sc-14011), goat polyclonal anti-vWF antibody (1:200 dilution; catalog no. sc-8068), rabbit polyclonal anti-ICAM-1 antibody (1:200 dilution; catalog no. sc-1511), mouse monoclonal anti–VCAM-1 antibody (1:1,000 dilution; catalog no. sc-13506), goat polyclonal anti–P-selectin antibody (1:200 dilution; catalog no. sc-6943), rabbit polyclonal anti–L-selectin antibody (1:1,000 dilution; catalog no. sc-371), mouse monoclonal anti-actin antibody (1:1,000 dilution; catalog no. sc-1616). Secondary antibodies included: goat anti-rabbit IgG-HRP (catalog no. sc-2004), donkey anti-goat IgG-HRP (catalog no. sc-2056), and goat anti-mouse IgG-HRP (catalog no. sc-205).

**Immunohistochemistry of macrophages and leukocytes in aortic and mesenteric tissue sections**

Tissues were collected after i.p. injection as described for β-gal staining. They were embedded in paraffin and cut at a thickness of 5 μm. After deparaffinization, rehydration and high-temperature antigen retrieval were performed as follows. Sections were placed in 10 mM citric acid, pH 6, and 10 mM 2-mercaptoethanol, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.1 mM nonessential amino acids and seeded on a 24-well plate. After 3 hours of incubation at 37°C and 5% CO<sub>2</sub>, the media with nonadherent cells (non-macrophages, polymorphonuclear cells) were removed. Attached macrophages were rinsed with PBS twice and fixed with 0.5% glutaraldehyde (Sigma-Aldrich, catalog no. G-7651) in PBS for 10 minutes at room temperature. Macrophages were then rinsed 2 times with PBS and stained in X-gal solution (4 mM K<sub>3</sub>Fe(CN)<sub>6</sub>·3H<sub>2</sub>O, 4 mM K<sub>4</sub>Fe(CN)<sub>6</sub>, 1 mM MgCl<sub>2</sub>, 1 mg/ml X-gal in PBS) for 7 hours at 37°C. Bone marrow cells were isolated from the femurs of 6-week-old WT or A<sub>B</sub>AR-KO mice as described previously (55). Cell pellets were resuspended in fixative buffer for 20 minutes (0.5% glutaraldehyde, 0.02% Nonidet P-40 [NP-40] in 1× PBS without MgCl<sub>2</sub>), washed twice with PBS, and then incubated in 1 ml X-gal staining solution for 16–20 hours at 37°C. Cells were mounted on slides, and blue precipitates (indicative of β-gal activities) were visualized via light microscopy.

**Cytokine measurements in plasma and in liver**

Cytokine measurements in plasma and in liver according to the manufacturer's protocol. Liver protein was extracted as described in "Western blot analysis" below.

**Blood cell count**

Blood was harvested and blood cells were counted as we have described elsewhere (56, 57).

**Western blot analysis**

Tissues were homogenized and lysed on ice with radioimmunoprecipitation assay buffer (RIPA) buffer (1× PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 mg/ml PMSF, and aprotinin [2 μg/ml] freshly supplemented with 1× protease inhibitor cocktail (Roche Diagnostics)). Lysates were vigorously vortexed and incubated on ice for 30 minutes, then frozen in liquid nitrogen and thawed at 37°C. Finally, lysates were cleared by centrifugation at 800 g for 10 minutes at 4°C. Fifty micrograms/sample of total protein was electrophoresed on 6% or 10% SDS-PAGE gels and transferred overnight at 4°C to Immobilon-P PVDF membranes (Millipore), and Western blotting was performed as described in ref. 58. The following antibodies (purchased from Santa Cruz Biotechnology Inc.) were used: rabbit polyclonal anti–E-selectin antibody (1:200 dilution; catalog no. sc-14011), goat polyclonal anti–vWF antibody (1:200 dilution; catalog no. sc-8068), rabbit polyclonal anti–ICAM-1 antibody (1:200 dilution; catalog no. sc-1511), mouse monoclonal anti–VCAM-1 antibody (1:1,000 dilution; catalog no. sc-13506), goat polyclonal anti–P-selectin antibody (1:200 dilution; catalog no. sc-6943), rabbit polyclonal anti–αvβ<sub>3</sub> antibody (1:1,000 dilution; catalog no. sc-371), mouse monoclonal anti-actin antibody (1:1,000 dilution; catalog no. sc-1616). Secondary antibodies included: goat anti-rabbit IgG-HRP (catalog no. sc-2004), donkey anti-goat IgG-HRP (catalog no. sc-2056), and goat anti-mouse IgG-HRP (catalog no. sc-205).
heated by 700-W microwave for 2 minutes 3 times, with 2-minute intervals between each time, followed by cooling down for 20 minutes. Paraffin sections were blocked with 10% normal goat serum (Vector Laboratories, catalog no. S-1000) for 1 hour at 37°C and then incubated overnight at 4°C with rat anti-mouse CD43 monoclonal antibody diluted 1:25 (BD Biosciences, catalog no. 552366) to detect leukocytes or with rat anti-mouse F4/80 monoclonal antibody (Serotec, catalog no. MCA497R) to detect macrophages. The staining was revealed using goat anti-rat biotinylated secondary antibody (Vector Laboratories, catalog no. BA-9400) at a dilution of 1:200 with an incubation time of 1 hour at 37°C. After 1x PBS wash, sections were incubated with ABC reagent (Vector Laboratories, catalog no. PK-6100) for 30 minutes at 37°C, and Vector DAB substrate (Vector Laboratories, catalog no. SK-4100) was used to develop the brown positive signal by incubating the sections for 5 minutes. Sections were counterstained with Shandon Gill Tg [UBC-GFP] 30Scha/J, stock no. 004353) were transplanted into WT or also subjected to Western blot analysis, and blood cell count was determined, all as described above.

**LPS-induced acute inflammation**

WT or AβAR-KO mice were given a single i.p. injection of LPS (E. coli serotype 0127:B8, Sigma-Aldrich, catalog no. L-4516) at 5 μg/g of body weight or of saline in a total volume of up to 100 μl (control). Mice were sacrificed at 1 hour, 3 hours, 16 hours, and 24 hours after LPS or saline administration and subjected to blood and tissue collection. Plasma and liver protein were collected at 0 hours, 1 hour, and 16 hours after LPS injection. Plasma was collected via the retro-orbital sinus and prepared for blood DNA isolation (Gentra VERSAGENE DNA Blood Kits, catalog no. VGD-0050B1). Determination of repopulation efficiency of bone marrow–derived cells in chimeric mice. Genotyping of sex chromosome–linked genes (Jaridld and Jaridlc) was used to confirm cross-sex transplantation of bone marrow–derived cells (from male donor to female recipient) as reported before. Jaridld and Jaridlc PCR upstream primer 5′-CCGGCTGCCAAATTCTTTGG-3′ and downstream primer 5′-TGAAGCTTGGCTTTGAG-3′ were used to produce a 300-bp fragment in female mice and both 300-bp and 330-bp fragments in male mice. Six weeks after transplantation, bone marrow cells were isolated from the femurs of all 4 groups of mice (WT → WT, KO → WT, KO → KO, WT → KO) and used for β-gal staining according to the method described above. For each group, β-gal–positive (blue) cells were counted under microscopy in randomly chosen fields (with native AβAR-KO and WT mice as controls). To further test the efficiency of transplantation under our protocol, bone marrow cells from GFP mice (Jackson Laboratory, strain name C57BL/6-Tg [UBC-GFP] 30Sc/a/J, stock no. 004353) were transplanted into WT or AβAR-KO mice, which we used as transplant recipients. Six weeks after transplantation, bone marrow cells were isolated from recipients and mounted on slides. GFP-positive cells were counted under a fluorescence microscope with GFP transgenic mice and WT mice as controls (data not shown).

Sample collection from transplant-recipient chimeric mice and related assays. Six weeks after transplantation, chimeric mice were injected i.p. with 1 μg/g or 5 μg/g body weight of LPS. Blood and liver samples were collected at 0 hours, 1 hour, and 16 hours after LPS injection. Plasma was used for cytokine measurements as we described above. Proteins were also subjected to Western blot analysis, and blood cell count was determined, all as described above.

**Tail-cuff and direct BP measurements, adenosine infusion-catheterization, and determination of heart rates**

Tail-cuff systolic BP was obtained at baseline using a computerized tail-cuff system (BP 2000, Visitech Systems) as described in ref. 59. Arterial and venous catheterization was performed under anesthesia induced by sodium pentobarbital (50 mg/kg, i.p.) (60). A modified polyethylene catheter (PE-50) was introduced into the right iliac artery for direct BP recording, and silastic tubing was placed into the right iliac vein for adenosine infusion. Both catheters were tunneled subcutaneously and exteriorized at the back of the animal’s neck. Subsequently, they were filled with heparin in 9.0% saline solution, sealed with heat, and attached to the animal’s pate. After the surgery, the animals were returned to their cages and allowed an overnight recovery period. On the following day, the arterial catheter was connected to a BP transducer attached to a recorder (model 220S; Gould) for direct BP monitoring. Control BP was recorded for no less than 30 minutes, when the BP became stable. The venous catheter was connected to a Harvard infusion pump (Harvard Apparatus), and adenosine was administered at a dose of 200–600 μg/kg/min for 60 minutes. Heart rates were measured as described previously (13).

**Analysis of leukocyte rolling by intravital microscopy**

Mice were anesthetized with 2.5% tribromoethanol (0.15 ml/10 g), and a midline incision was made through the abdominal wall to expose the mesentery and mesenteric venules of 200- to 300-μm diameters. Exposed mesentery was kept moist by periodic superfusion using PBS (without Ca2+ or Mg2+) warmed to 37°C. The mesentery was transilluminated with a 12-V, 100-W, DC stabilized source. The shear rate was calculated using an optical Doppler velocity meter as described previously (61). Venules were visualized using a Zeiss Axiovert 135 inverted microscope (objective ×32) connected to an SVHS video recorder (AG-6730; Panasonic) using a silicon-intensified tube camera (C2400; Hamamatsu). Leukocyte interaction with the endothelium vessel wall was recorded for 10 minutes each in 2–3 unbranched venules per mouse. Recorded images were analyzed as follows: the number of leukocytes passing a given plane perpendicular to the vessel axis during 1 minute was counted; leukocyte rolling/minute/venule for each mouse was determined by taking the average of four 1-minute counts during the entire 10-minute recording. The rolling velocity was determined as the number of leukocytes that traversed over a 250-μm-long and 200- to 300-μm-wide segment. A leukocyte was considered to be adherent if it remained stationary for more than 20 seconds.

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Address correspondence to: Katya Ravid, Department of Biochemistry, K225, Boston University School of Medicine, Boston, Massachusetts 02118, USA. Phone: (617) 638-5053; Fax: (617) 638-5054; E-mail: ravid@biochem.bumc.bu.edu.