The A2B adenosine receptor protects against inflammation and excessive vascular adhesion

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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-null/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 IkB-α are the underlying mechanisms for an observed upregulation of adhesion molecules in the vasculature of these A2B-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 A1AR 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 A3AR have been described in several reports. The ability of an A3AR selective ligand to potentiate antigen-dependent degranulation of mast cells, as measured by hexosaminidase release, is lost in mice lacking A3 receptors 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 A3AR (12). Attenuation of LPS-induced TNF-α production is decreased in these A3AR-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 A3AR compared with control mice (13). Interestingly, ischemia-reperfusion injury of mice lacking A3ARs 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 A2ARs, 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 hypogalgesia (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 A2A-null mice, which suggested that caffeine-dependent psychostimulation is mediated by the A2AAR (20). Activation of A2A is required for dopaminergic function, since dopamine-mediated cellular response such as
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 A2BAR 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 for expression in macrophages.
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of protein expression. Figure 1E shows that the A2AR and A2BAR agonist 5’-N-ethylnicarbamidoadenosine (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 A2AR and A2BAR were previously shown to be expressed in WT primary VSMCs (35). The A2BAR selective antagonist MRS1754 eliminated the stimulatory effect of NECA only in WT cells, indicating that A2BAR 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 A2BAR-deficient cells. Similar results were obtained using macrophages (Supplemental Figure 1B).

A2BAR-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 A2BAR-KO mice were used as detailed in Methods.

A2BAR expression in different tissues. By replacing exon 1 of the A2BAR gene with a reporter construct containing β-gal, tissue-specific activation of the A2BAR gene promoter was conve-

Figure 2
β-Gal expression in different tissues. (A) β-Gal staining of different organs derived from WT or KO mice and captured at the indicated magnifications (in parenthesis on the left) with an Olympus IX70 microscope combined with a Hamamatsu charge-coupled device camera (C4742-95). (B) Histological examination of tissue sections derived from the organs shown in A. The arrows point to the VSMC layer. Fold magnifications of captured images are indicated above each column (×200 or ×600). (C) Electron microscopic examination of arteries. In order to ascertain β-gal expression in the endothelial layer and further confirm expression in smooth muscle cells, we applied electron microscopic examination. The red arrow indicates endothelial cells, the light blue arrow elastin, and the dark blue arrow the VSMC layer. Original magnification, ×6,400. The black stain is indicative of β-gal staining. As control, WT mice were subjected to similar staining, showing no expression. (D) β-Gal expression was evident in peritoneal macrophages isolated and processed as described in Methods. The isolation procedure yielded a preparation enriched with macrophages (to 35–40% of total cells).

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The expression of IL-10 during the first few hours after endotoxin injection. This suggests that IL-6 upregulation in LPS-treated A2BAR-KO mice compared with WT mice is associated with the expression 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 A2BAR-KO 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.

The A2BAR controls proinflammatory cytokine production. In view of the significant expression of A2AR 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 A2BAR-KO 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

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**Figure 3**
The levels of proinflammatory cytokines are increased in A2BAR-KO mice. (A) Basal levels of the indicated cytokines were measured in the liver of WT or KO mice. Results are presented as the average of 5 experiments ± SD. (B) LPS-induced cytokine levels in the plasma of WT and KO mice. Mice were injected with LPS (5 μg/g body weight) or vehicle (control [Cont]), and at the indicated hours, plasma cytokine levels were determined. Data are presented as average ± SD for 5 experiments performed in duplicate. TNF-α levels (pg/ml) in WT versus KO mice were 5.64 ± 2.3 versus 11.6 ± 2.8 at baseline; 51 ± 19 versus 106 ± 34 at 3 hours after LPS injection; and 6.2 ± 2.6 versus 18 ± 12 at 24 hours after LPS injection. As to IL-6, the plasma levels (pg/ml) in WT and KO mice were 5.3 ± 3.7 and 5.1 ± 3.8, respectively. (C) For IL-10, the values (pg/ml) at baseline were 6.4 ± 1.1 in WT mice and 7.2 ± 1.5 in KO mice. These relatively low levels are not illustrated clearly in the figure because of scale adjustment.

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niently determined in various organs and specific cells within those organs. In all tissues tested, including colon, ovary, testis, liver, spleen, fat, muscle, pancreas, retina, brain, lung, heart, kidney, and arteries, the primary site of expression was the vasculature. In the brain and retina, however, there were also other cell types that expressed β-gal. Future studies will examine the exact identity and origin of cells expressing the receptor in these loci. Because of the significant β-gal staining in different vascular beds, our studies focused on the specific distribution of vascular A2BAR expression (Figure 2A). The level of A2BAR gene promoter–induced expression was different in various arteries tested, with the aortic arch, mesenteric artery, femoral artery, and small venules showing a high of level expression (Figure 2, A and B). Within these vessels, expression was evident in the VSMC layer, and to a lesser extent in endothelial cells, but not in the adventitia (Figure 2C and Supplemental Figure 2). Within the vascular smooth muscle layer, the expression was patchy (not all cells expressed the gene), particularly in the aortic arch (Supplemental Figure 2). The expression along the aorta was quite low and generally not detectable in most areas. A patchy expression in blood vessels was also reported for the A1AR gene promoter (36). It is intriguing to speculate that varying intensities of local signals within the vasculature induced by cytokines, shear stress, and other factors affect the expression of the A2BAR gene. This contention will be examined in future studies. In addition to blood vessels, A2BAR gene promoter–directed expression was highly detected in macrophages (Figure 2D) but barely detectable in polymorphonuclear leukocytes (data not shown). This profile of expression was further supported by RT-PCR of RNA extracted from WT tissues (e.g., those shown in Figure 2A).
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 microvénules of 25–35 μm in diameter. Shear rates for all vénules studied were similar in Aβ2AR-KO mice compared with WT mice (Supplemental Table 2). More leukocytes were observed rolling in Aβ2AR-KO mice compared with control mice (Figure 5A; P < 0.0001). We also found that leukocytes rolled at a slower velocity in Aβ2AR-KO mice. Of the total number of leukocytes analyzed for velocity, 65% rolled at a velocity of less than 20 μm/s in Aβ2AR-KO as compared with 23% in control mice (Figure 5C). In addition, we observed that more leukocytes adhered to the endothelium at baseline conditions in Aβ2AR-KO compared with control samples (Figure 5A; P < 0.001). Similar results were observed in the microvénules. More leukocytes rolled on the endothelium in Aβ2AR-KO compared with control mice (Figure 5B; P < 0.005), and more leukocyte adhesion to the endothelium was observed in the former line (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% (n = 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 and 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...
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–knockin 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 A2AAR as an important contributor to these phenomena. We found that the A2AAR-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 A2AAR-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 at 10 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 crossed 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).

Figure 5

Analysis of basal leukocyte rolling and adhesion in mesenteric venules. Analysis of leukocyte rolling and adherence in venules of 220–235 μm in diameter (A) and 25–35 μm in diameter (B). Data shown are mean ± SE for 8 KO and 9 WT mice for venules of 220–235 μm in diameter and 5 KO and 4 WT mice for the 25- to 35-μm-diameter microvenules. (C) Cumulative histogram of rolling velocities (venules of 220–235 μm in diameter) allows direct comparison of distribution among groups. The number of leukocytes analyzed for KO mice was 660 and for WT, 573. P < 0.001. (D) Photographs, taken 25 seconds apart, of the microvenules of WT and KO mice are shown. Arrows indicate adherent leukocytes.
Augmented levels of cytokines and adhesion molecules could be due to altered signaling in A2BAR-deleted 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 A2AR (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. In each case, bone marrow cells were derived from a genetic background (denoted on the left as WT or KO) and transplanted (denoted by an arrow) into a recipient (denoted on the right) (e.g., WT→WT). Similar results were obtained when mice were injected with 1 μg/g body weight LPS (data not shown).
tion signal termination 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 A2bAR 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=10099) under permissive conditions. The human A2aAR 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 A2bAR gene generated by KpnI/XhoI 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 XhoI-EcoRI linker, yielding pPNT-A2bAR-intron. A 6-kb fragment of the A2bAR gene promoter was subcloned into the SphI end of the gene encoding prokaryotic β-gal in the pBS-lacZ plasmid. The 9.4-kb NotI/XhoI A2bAR fragment was then subcloned into pPNT-A2bAR-intron at the NotI/XhoI sites, 5′ of the Neo cassette. Recombinant A2pAR-KO-LacZ ES cells (mouse 129/SvJ derived) were obtained and cultured according to conventional methods (e.g., ref. 15). The positive recombinant ES colonies were first screened with Exend Long PCR (Roche Diagnostics), using the primer pair sense 5′-TGCGCTTCCTTGGCAGTCTT-3′ and antisense 5′-GCTATCAGCTACATCTTTGTC-3′ and antisense 5′-AGCAGGAGTTGCAAGGTTAGGTATT-3′, yielding a 4-kb ampiclon. The positively identified A2pAR-KO-β-gal ES cell clone 23 was then injected into C57BL/6-derived blastocysts, the heterozygous A2bAR fragment was then subcloned into pPNT-A2bAR-intron at the NotI/XhoI sites, 5′ of the Neo cassette. Recombinant A2bAR-KO-LacZ ES cells (mouse 129/SvJ derived) were obtained and cultured according to conventional methods (e.g., ref. 15). The positive recombinant ES colonies were first screened with Exend Long PCR (Roche Diagnostics), using the primer pair sense 5′-TGCGCTTCCTTGGCAGTCTT-3′ and antisense 5′-GCTATCAGCTACATCTTTGTC-3′ and antisense 5′-AGCAGGAGTTGCAAGGTTAGGTATT-3′, yielding a 4-kb ampiclon. The positively identified A2pAR-KO-β-gal ES cell clone 23 was then injected into C57BL/6-derived blastocysts, which 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 upstream primer 5′-CTCACACAGACTCCTTCTT-3′ and downstream primer 5′-TCGCGACAGCAGTTTGT-3′ were used to produce a 485-bp fragment (both primers are located in the intron 1 area). Mutant upstream primer 5′-TGGCGAAGTCTCTTCATCCG-3′ and downstream primer 5′-GGCCCATCTTCCCTAGACAC-3′ were used to generate a 900-bp fragment (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 A2pAR gene mutant mice. In this study, the mice used were 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 A2pAR mutant heterozygous mice generated the same background strain as WT or A2pAR 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 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 A2bAR 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, 1x 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 A2bAR 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 the presence or absence of reverse transcriptase (1 μg RNA, M-MLV reverse transcriptase, 1x first-strand buffer, 0.5 mM dNTP, 5 mM DTT, 0.5 μM RNase inhibitor, 5 μM random primers, all purchased from Invitrogen). The primer pairs were designed to produce a 746-bp fragment within exon 1 (PCR primers: sense, 5′-ATCGGACTGAGCAGCCAAAG-3′; antisense, 5′-GGAGCCCAACACACAGGCAAC-3′). To optimally detect A2bAR 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′-TCACACTCTCCAGGAGG-3′ and antisense, 5′-GCTTACACACCCTTCTT-3′). To amplify the second exon in A2bAR cDNA by RT-PCR, the following primers were employed: sense, 5′-CAAGTGGTGTAGTATGTGG-3′; antisense-5′-TTTCCGGATCATATTCA-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 MacVector sequence alignment program (version 6.5.3; Accelrys) was used to ensure that the homology to A2aAR, A1AR, or A2AR was no greater than 75% (50% in most areas), with little homology at primer ends. Aberrant fragments potentially generated from other adenosine receptors will be of a different size than amplified A2bAR exon 2 (see Supplementary Figure 1A). Additionally, primers were analyzed using UCSC Genome Bioinformatics In-Silico PCR tool (http://genome.ucsc.edu/cgi-bin/hgPerCommand=start) to ensure that there was no amplification of other gene sequences. Total RNA was isolated from mesenteric artery or kidney by immediate homogenization in TRIZol reagent (Invitrogen) or using Versagene RNA Tissue Kit according to the manufacturer’s protocol (Gentre). RNA was treated with DNase I (Promega) for 30 minutes at 37°C prior to reverse transcription consisting of 1 μg RNA, M-MLV reverse transcriptase, 1x first-strand buffer, 0.5 mM dNTP, 5 mM DTT, 0.5 μM RNase inhibitor, 5 μM random primers (all purchased from Invitrogen). A mock reaction lacking reverse transcriptase served as a control for DNA contamination. RT-PCR of exon 2 was performed with the above-mentioned primers with a program of 1 cycle at 95°C for 2 minutes, 30 cycles at 95°C for 55 seconds, 60°C for 55 seconds, 72°C for 55 seconds, and 1 cycle at 72°C for 5 minutes.

VSMCs were isolated from aortas of 6-week-old WT or A2bAR-KO mice by enzymatic dispersion as previously described (54). Eighteen hours after seeding, VSMC culturing media was removed and replenished with 100 μl/well of fresh VSMC culturing media containing 5 μM MRS1754 [8-(4-[[4-cyanophenyl]carbamoylmethyl]oxy)phenyl]-1,3-di(n-propyl)Xanthine; Sigma-Aldrich, catalog no. M-6316]. After a 10-minute pretreatment, cells were stimulated by adding 5 μM MRS1754, 0.5 mM dNTP, 5 mM DTT, 0.5 μM RNase inhibitor, 5 μM random primers (all purchased from Invitrogen). A mock reaction lacking reverse transcriptase served as a control for DNA contamination. RT-PCR of exon 2 was performed with the above-mentioned primers with a program of 1 cycle at 95°C for 2 minutes, 30 cycles at 95°C for 55 seconds, 60°C for 55 seconds, 72°C for 55 seconds, and 1 cycle at 72°C for 5 minutes.

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cAMP levels were measured using Direct Cyclic AMP Kit (Assay Designs, catalog no. 900-066).

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

RNA preparation and PCR conditions were essentially as listed above for the dA2R. For the dA1R, the primers used were: sense, 5′-GGCT-TAGTCCCCTGAGATCAG-3′; antisense, 5′-CCCTTGTCTTAGAG-3′; expected fragment of 436 bp. For the dA3R, the primers were: sense, 5′-TGAAGGCAGGAAGCATCA-3′; antisense, 5′-CCGAG-GTCTTTTGTGAGTCT-3′; expected fragment of 117 bp. For the dA3AR, the primers were: sense, 5′-TAAATGAGAGGCGAGATGAAG-3′; antisense, 5′-GATTTAGGTAGTGTTCAGCA-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 fixative (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 K4Fe(CN)6, 5 mM K3Fe(CN)6·3H2O (Sigma-Aldrich), catalogue nos. P-8131 and P-9287, respectively), 2 mM MgCl2 in PBS) containing a final concentration of 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside (Biovision, catalogue no. 380-5200) or 5-bromo-4-chloro-3-indolyl-β-D-maltoside, as indicated (Blue-gal, Sigma-Aldrich, catalogue 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. Samples 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-galactopyranoside (X-gal); American Bioanalytical, catalogue no. AB2400-1000) or halogenated indolyl-β-D-galactoside, as indicated (Blue-gal; Sigma-Aldrich, catalogue no. B2904) was used in 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-mesh Athene Thin Bar copper grids.

β-gal assay in macrophages, polymorphonuclear cells, and bone marrow cells. Peritoneal macrophages were collected from 6-week-old WT or A2AR-KO mice (4 mice per group). To this end, mice were anesthetized by inhalation of isoflurane and then injected with PBS (5 ml/mouse) in the peritoneal cavities. After 5 minutes of massaging, injected PBS was collected from the peritoneal cavities and spun down at 800 g, for 10 minutes, at 4°C. Red cells were lysed with 8 ml of prewarmed lysis buffer (17 mM Tris, 140 mM NH4Cl in H2O, pH 7.2) at 37°C, for exactly 10 minutes. Cell pellets were washed and centrifuged in 10 ml PBS twice. Cells were resuspended in 1 ml/well serum-free RPMI (RPMI-1640 medium supple-mented with 4 mM L-glutamine, 50 μM 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% CO2, 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, catalogue 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 K4Fe(CN)6, 3H2O, 4 mM K3Fe(CN)6, 1 mM MgCl2, 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 A2AR-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 MgCl2), washed twice with PBS, and then incubated in 1 ml X-gal staining solution for 16–20 hours at 37°C. Cells were mounted onto slides, and blue precipitates (indicative of β-gal activities) were visualized via light microscopy.

**Cytokine measurements in plasma and in liver**

eBiosience ELISA Ready-SET-Go! Kits testing for mouse TNF-α (catalog no. 88-7324-22), IL-6 (catalog no. 88-7064-22), IL-10 (catalog no. 88-7104-22), IL-2 (catalog no. 88-7024-22), IL-4 (catalog no. 88-7044-22), and IFN-γ (catalog no. 88-7314-22) were used to measure cytokine levels 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-β-actin antibody (1:200 dilution; catalog no. sc-14011), goat polyclonal anti-iWF 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-LKB-α 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-2055).

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

Tissues were collected after fixation as described for β-gal staining. They were embedded with 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
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 in 0.9% saline solution, sealed with heat, and attached to the animal’s nape. 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 Axiosvert 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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**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 0.9% saline solution, sealed with heat, and attached to the animal’s nape. 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).

**Bone marrow transplantation experiments**

Generation of chimeric mice by bone marrow transplantation. Ten-week-old 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 used for cytokine measurements.


