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Inactivation of the Adenosine A_{2A} Receptor Protects Apolipoprotein E–Deficient Mice From Atherosclerosis

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Background—Atherosclerosis is a chronic inflammatory disease of the arterial vessel wall. The A_{2A} receptor ($A_{2A}R$) plays a central role in many antiinflammatory effects of adenosine. However, the role of $A_{2A}R$ in atherosclerosis is not clear.

Methods and Results—The knockout of $A_{2A}R$ in apolipoprotein E–deficient ($Apoe^{-/-}/A_{2A}R^{-/-}$) mice led to an increase in body weight and levels of blood cholesterol and proinflammatory cytokines, as well as the inflammation status of atherosclerotic lesions. Unexpectedly, $Apoe^{-/-}/A_{2A}R^{-/-}$ mice developed smaller lesions, as did chimeric $Apoe^{-/-}$ mice lacking $A_{2A}R$ in bone marrow–derived cells (BMDCs). The lesions of those mice exhibited a low density of foam cells and the homing ability of $A_{2A}R$-deficient monocytes did not change. Increased foam cell apoptosis was detected in atherosclerotic lesions of $Apoe^{-/-}/A_{2A}R^{-/-}$ mice. In the absence of $A_{2A}R$, macrophages incubated with oxidized LDL or in vivo–formed foam cells also exhibited increased apoptosis. $A_{2A}R$ deficiency in foam cells resulted in an increase in p38 mitogen–activated protein kinase (MAPK) activity. Inhibition of p38 phosphorylation abrogated the increased apoptosis of $A_{2A}R$-deficient foam cells.

Conclusion—Inactivation of $A_{2A}R$, especially in BMDCs, inhibits the formation of atherosclerotic leisons, suggesting that $A_{2A}R$ inactivation may be useful for the treatment of atherosclerosis. **(*Arterioscler Thromb Vasc Biol*. 2009;29:1046-1052.)**

Key Word: atherosclerosis ■ adenosine receptor ■ macrophages ■ apoptosis

Atherosclerosis is a chronic inflammatory disease of the arterial vessel wall that involves endothelial cells, vascular smooth muscle cells, mononuclear cells, platelets, growth factors, and inflammatory cytokines.[1–3] Conditions that increase inflammation also exacerbate atherosclerosis in vivo, and most drugs that improve the clinical outcome of atherosclerosis also inhibit inflammation.[2] Therefore, inflammation is considered a therapeutic target in atherosclerosis.[2]

Adenosine is an endogenous regulator of inflammation and tissue injury, and most of its antiinflammatory effects are elicited via $A_{2A}R$.[4] $A_{2A}R$ exists on many inflammatory cells, including neutrophils, monocytes, lymphocytes, macrophages, and platelets,[4,5] and loss of $A_{2A}R$ increases inflammatory responses and tissue damage in vivo.[6,7] In contrast, occupancy of $A_{2A}R$ reduces inflammation and protects tissues from injury.[5] Therefore, $A_{2A}R$ is considered as an inflammatory modulator and promising pharmacological target for the treatment of inflammatory disorders.

$A_{2A}R$ plays a complex role in inflammation and tissue injury. In the context of neurological disease, blocking $A_{2A}R$ appears to be beneficial.[8] Several $A_{2A}R$ antagonists are being developed to treat neurological disorders, and some of these are even being assessed in clinical trials.[9] Notably, many patients with neurodegenerative disease also suffer from vascular disease associated with atherosclerosis. Thus, it is relevant to study whether blocking $A_{2A}R$ also affects atherosclerosis. To date, however, there have been no reports on the effects of blocking or knocking out $A_{2A}R$ on atherosclerosis. Therefore, we evaluated whether $A_{2A}R$ deficiency affects atherosclerosis using mice deficient for both $A_{2A}R$ and Apoe ($Apoe^{-/-}/A_{2A}R^{-/-}$).

Materials and Methods

Mice

$A_{2A}R^{-/-}$ mice in C57BL/6J background[10] were bred with $apoE^{-/-}$ (C57BL/6J background) mice to generate $Apoe^{-/-}/A_{2A}R^{-/-}$ mice and their littermate controls. Chimeric mice with or without $A_{2A}R$ in their bone marrow–derived cells (BMDCs) were produced by bone marrow transplantation, as described.[11] Mice were fed a Western diet for 3 months or 6 months and then euthanized for collection of aortas. All animal experiments and care were approved by the University of Minnesota Animal Care and Use Committee, in accordance with AAALAC guidelines.

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Blood Lipid and Leukocyte Analysis

Blood lipid was determined via an automated enzymatic technique (Boehringer Mannheim GmbH). Blood leukocytes were quantified using an automated blood cell counter (Hemavet 850FS, CDC Technologies).

Measurement of Plasma Cytokines

Cytokine levels were determined by multiplex assay on the Luminex platform using BioPlex software (Bio-Rad) and mouse-specific bead sets according to the manufacturer's instructions (R&D Systems).

Preparation of Mouse Aortas and Quantification of Atherosclerosis

Aortas of atherosclerotic mice were collected and processed for oil red O staining using either en face preparation of whole aortas or cross-sections of aortic sinuses.[12]

Histological Analysis of Atherosclerotic Lesions

Oil red O staining was performed on frozen sections (5 μm thick) of atherosclerotic aortic sinuses. Using specific antibodies, immunostaining to detect expression of macrophage F4/80 (Accurate) and phospho-NF-κB p65 (Cell Signaling) was performed. Slides were examined under a light microscope (Carl Zeiss), and images were digitized into a Macintosh computer. Samples from 10 mice were analyzed per group. Quantification was done by dividing the area of positive staining by the total measured lesion area in digitized images.

TUNEL Assays

Thioglycollate-elicited peritoneal macrophages were recovered from wild-type (wt) or $A_{2A}R^{-/-}$mice, plated in 8-well culture slides (BD) at 0.5×10^6 cells per well, and cultured in DMEM/10% fetal bovine serum for 16 hours. The cells were then incubated with 100 μg/mL of human oxidized LDL (ox-LDL) (Biomedical Technologies) with or without the p38 inhibitor SB203580 at 20 μmol/L (Calbiochem) for 20 hours. Cells were fixed in 4% paraformaldehyde and apoptosis determined using the Dead End Fluorometric TUNEL System (Promega) following the manufacturer's instructions. The same TUNEL staining was also conducted on frozen sections of mouse aortic sinuses to examine the apoptotic cells in atherosclerotic lesions.

Real-Time PCR

Total RNA from atherosclerotic arteries was extracted using Trizol reagent (Invitrogen), and cDNA was synthesized using a first-strand cDNA synthesis kit (Fermentas). PCR was performed with a LightCycler 2.0 thermal cycler (Roche) using SYBR Green as a double-stranded DNA–specific dye. The relative amount of each gene in each sample was estimated by the $\Delta\Delta C_T$ method. Supplemental Table IV (available online at http://atvb.ahajournals.org) lists the sequences of primers for cytokines.

Western Blotting

Mouse peritoneal macrophages were lysed and transferred to a polyvinylidene fluoride membrane. Antibodies against p38, phospho-p38, caspase-3, and GAPDH (Cell Signaling) were applied. The blots were incubated with alkaline phosphatase–conjugated secondary antibodies, developed with a chemifluorescence reagent, and scanned by Storm 860 (GE Healthcare).

Electrophoretic Mobility Shift Assay

Nuclear protein was extracted using NucBuster Protein Extraction kit (Novagen). A biotin end-labeled double-stranded oligonucleotide (5′-biotin-GGAGAGTGGGGACTACCCCCTCTGCT-3′) and a nonlabeled oligonucleotide containing the NF-κB consensus sequence were incubated with the extracted nuclear protein. The samples were subjected to SDS-PAGE and transferred to a nylon membrane. The biotin-labeled DNA was detected with the LightShift Chemiluminescent Electrophoretic Mobility Shift Assay kit (Pierce).

Statistical Analysis

Statistical analysis was performed with Instat software (GraphPad). Data are presented as mean±SEM. Data were analyzed with either a 1-way ANOVA followed by a Bonferroni correction posthoc test or a Student t test to evaluate 2-tailed levels of significance. The null hypothesis was rejected at $P<0.05$.

Results

Atherosclerosis in $Apoe^{-/-}/A_{2A}R^{-/-}$ Mice

To determine the role of $A_{2A}R$ in the development of atherosclerotic lesions in vivo, $Apoe^{-/-}/A_{2A}R^{-/-}$ mice and their littermate $Apoe^{-/-}$ mice were fed a chow diet or Western diet for 3 months. These mice exhibited no differences in blood pressure, number of circulating leukocytes, differential counts, or blood glucose (supplemental Tables I through III). The level of blood alanine aminotransferase (ALT) in $Apoe^{-/-}/A_{2A}R^{-/-}$ mice was 4× higher than that in $Apoe^{-/-}$ mice on Western diet (supplemental Table IV). The weight of $Apoe^{-/-}/A_{2A}R^{-/-}$ mice fed a Western diet was 23% higher for males and 12% higher for females compared with sex-matched $Apoe^{-/-}$ mice fed the same diet. Total blood cholesterol was 45% higher in male $Apoe^{-/-}/A_{2A}R^{-/-}$ mice and 25% higher in females compared with $Apoe^{-/-}$ mice on both chow and Western diets; this increase was solely attributable to increased LDL cholesterol (Table; supplemental Table III). Interestingly, lipid profiles were similar in $A_{2A}R^{-/-}$ and wt mice on Western diet. In $A_{2A}R^{-/-}$ and wt mice on both chow and Western diet, blood IL-6 levels were not detectable. In contrast, blood IL-6 levels were detectable and much higher in $Apoe^{-/-}/A_{2A}R^{-/-}$ than in $Apoe^{-/-}$ mice (25±8.2 versus 20±5.8 pg/mL, as shown in supplemental Table V). Despite the higher body weight, blood cholesterol, and proinflammatory cytokine levels, atherosclerotic lesion size in the aortas of $Apoe^{-/-}/A_{2A}R^{-/-}$ mice was decreased by 26% in females and 20% in males compared to $Apoe^{-/-}$ mice (Figure 1b). In addition, the aortic sinuses displayed much smaller lesions in $Apoe^{-/-}/A_{2A}R^{-/-}$ mice than in $Apoe^{-/-}$ mice (Figure 1c).

To examine whether $A_{2A}R$ deficiency could also protect mice from advanced atherosclerosis, $Apoe^{-/-}/A_{2A}R^{-/-}$ mice and their littermate $Apoe^{-/-}$ mice were placed on a Western diet for 6 months. In accordance with the changes in mice fed a Western diet for three months, $Apoe^{-/-}/A_{2A}R^{-/-}$ mice gained more body weight and had a much higher level of blood total cholesterol than $Apoe^{-/-}$ mice (Table). Aortic atherosclerotic lesions in female $Apoe^{-/-}/A_{2A}R^{-/-}$ mice were 51% smaller compared with those in female $Apoe^{-/-}$ mice, and the lesions in male $Apoe^{-/-}/A_{2A}R^{-/-}$ mice were 55% smaller compared with controls (Figure 1a). These results confirmed the data obtained from mice fed a Western diet for 3 months and demonstrated even greater protection against atherosclerosis in $Apoe^{-/-}/A_{2A}R^{-/-}$ mice during a longer period of atherosclerotic challenge.

The cellular components of atherosclerotic lesions in cross-sections of the aortic sinus were also compared. Macrophages and foam cells were mainly located in the cap and shoulders of lesions in $Apoe^{-/-}$ and $Apoe^{-/-}/A_{2A}R^{-/-}$ mice, but the total number of macrophages and foam cells in lesions of $Apoe^{-/-}/A_{2A}R^{-/-}$ mice was greatly diminished (Figure 1d).

Table. Characteristics of Apoe$^{-/-}$/$A_{2A}R^{-/-}$ Mice

Genotype	Gender	n	Body Weight (g)	Total Cholesterol (mg/dL)	LDL (mg/dL)
3 months WD					
Apoe$^{-/-}$/$A_{2A}R^{+/+}$	Male	15	34.5±6.2	1262.5±197.3	1067.4±147.8
Apoe$^{-/-}$/$A_{2A}R^{-/-}$		15	40.7±8.0*	1828.1±203.7*	1426.6±194.0*
			*P=0.012	*P=0.023	*P=0.024
Apoe$^{-/-}$/$A_{2A}R^{+/+}$	Female	15	25.6±3.2	898.8±76.7	657.1±82.7
Apoe$^{-/-}$/$A_{2A}R^{-/-}$		15	28.7±6.2*	1115.8±278.1*	853.9±151.2*
			*P=0.021	*P=0.028	*P=0.021
6 months WD					
Apoe$^{-/-}$/$A_{2A}R^{+/+}$	Male	15	31.2±6.1	1156.2±146.7	957.8±85.6
Apoe$^{-/-}$/$A_{2A}R^{-/-}$		15	38.1±10.8*	1482.4±201.6*	1243.3±176.4*
			*P=0.034	*P=0.012	*P=0.017
Apoe$^{-/-}$/$A_{2A}R^{+/+}$	Female	15	25.6±6.0	670.2±76.4	512.7±65.5
Apoe$^{-/-}$/$A_{2A}R^{-/-}$		15	28.6±4.9*	859.2±92.7*	674.6±81.4*
			*P=0.034	*P=0.023	*P=0.025

This was further supported by the lower levels of mRNA encoding the monocyte marker CD68 in lesions of Apoe$^{-/-}$/$A_{2A}R^{-/-}$ mice than those of Apoe$^{-/-}$ mice (Figure 1e).

Atherosclerosis in Chimeric Mice Lacking $A_{2A}R$ and ApoE in Bone Marrow–Derived Cells

To determine the influence of leukocyte $A_{2A}R$ in the formation of atherosclerotic lesions, we studied atherosclerosis in Apoe$^{-/-}$ chimeric mice fed a Western diet for 3 months. Apoe$^{-/-}$ mice lacking $A_{2A}R$ in their BMDCs did not differ from Apoe$^{-/-}$ mice in body weight or blood cholesterol level (data not shown). In the aortic sinuses, a 30% reduction was observed in the average size of lesions in chimeric mice lacking $A_{2A}R$ in their BMDCs compared to that in controls (Figure 1f), suggesting that protection against atherosclerosis in Apoe$^{-/-}$/$A_{2A}R^{-/-}$ mice was mainly attributable to $A_{2A}R$ deficiency in BMDCs.

The presence of macrophages in atherosclerotic lesions of chimeric mice was also assessed; Apoe$^{-/-}$ mice lacking $A_{2A}R$ in their BMDCs demonstrated significantly fewer macrophages in lesions compared with Apoe$^{-/-}$ mice (Figure 1g).

Inflammatory Status of Atherosclerotic Lesions in Apoe$^{-/-}$/$A_{2A}R^{-/-}$ Mice

Atherosclerosis is a chronic inflammatory disease, and disease progression is usually accompanied by increased inflammation.[2] $A_{2A}R^{-/-}$ mice and $A_{2A}R$-deficient macrophages exhibited increased inflammatory phenotype after inflammatory stimulation.[6,7] Because Apoe$^{-/-}$/$A_{2A}R^{-/-}$ mice developed small atherosclerotic lesions, we speculated that $A_{2A}R$-deficient macrophages might react to modified LDL differently from their response to other inflammatory stimuli. To test this possibility, we examined the inflammatory response of $A_{2A}R$-deficient macrophages to ox-LDL in an in vivo peritonitis model. On the third day of thioglycollate-induced peritonitis, mice were injected intraperitoneally with ox-LDL. Peritoneal macrophages were collected 30 minutes after the ox-LDL injections. As shown by an electrophoretic mobility shift assay, both wt and $A_{2A}R$-deficient macrophages displayed significant levels of nuclear P65/P50 binding to the NF-κB consensus sequence, indicating activation of the NF-κB pathway in thioglycollate-elicited macrophages. Compared with wt macrophages, $A_{2A}R$-deficient macrophages showed increased NF-κB activation before and after ox-LDL treatment (Figure 2a).

To determine the level of NF-κB activation in foam cells present in atherosclerotic lesions of Apoe$^{-/-}$/$A_{2A}R^{-/-}$ mice, sections of atherosclerotic lesions were immunostained with phosphor-p65-specific antibody. Phosphorylation of p65 is an indicator of NF-κB activation. Among the macrophages/foam cells present in lesions, many more cells demonstrated positive staining for phospho-p65 in lesions of Apoe$^{-/-}$/$A_{2A}R^{-/-}$ mice than in those of Apoe$^{-/-}$ mice (Figure 2b). In addition to NF-κB signaling, we also determined the mRNA levels of proinflammatory cytokines in lesions by real-time RT-PCR. The levels of IL-1b and IL-6 mRNA were much higher in atherosclerotic lesions of Apoe$^{-/-}$/$A_{2A}R^{-/-}$ mice than in those of Apoe$^{-/-}$ mice (Figure 2c). These results indicate that, in an atherosclerotic environment, $A_{2A}R$-deficient macrophages exhibited an inflammatory phenotype. Notably, the mRNA level of IL-10, an antiinflammatory cytokine, was also increased in lesions of Apoe$^{-/-}$/$A_{2A}R^{-/-}$ mice.

Apoptotic Foam Cells in Atherosclerotic Lesions of Apoe$^{-/-}$/$A_{2A}R^{-/-}$ Mice

Apoptosis of macrophages or foam cells during the early stages of atherosclerosis decreases atherosclerosis.[13–15] To investigate whether this was the mechanism responsible for suppressed atherosclerosis in Apoe$^{-/-}$/$A_{2A}R^{-/-}$ mice, we first performed TUNEL-staining to detect apoptotic cells on cross-sections of atherosclerotic lesions. In lesion areas containing F4/80-positive macrophages, many more cells were positive for TUNEL-staining in lesions of Apoe$^{-/-}$/$A_{2A}R^{-/-}$ mice than in those of Apoe$^{-/-}$ mice (Figure 3a).

Macrophages in the peritoneal cavities of atherosclerotic mice with thioglycollate-induced peritonitis differentiate into foam cells.[16] Using this in vivo foam cell formation model, wt and $A_{2A}R$-deficient foam cells were generated and assayed by

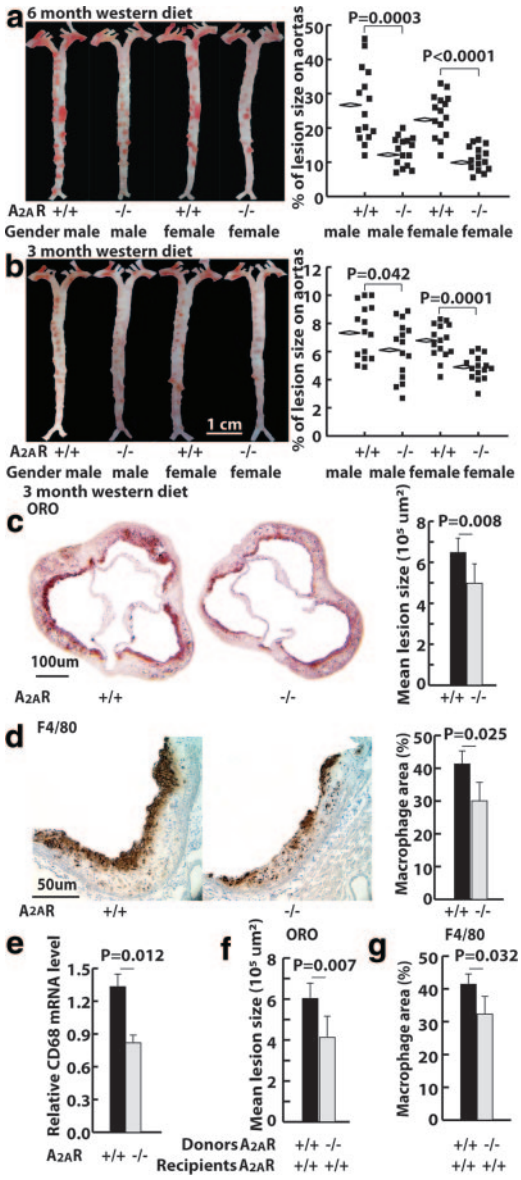

Figure 1. $A_{2A}R$ deficiency decreases atherosclerotic lesion formation in Apoe$^{-/-}$ mice. a and b, Oil red O (ORO) staining of aortas. c and d, ORO and anti-F4/80 staining of aortic sinuses. e, mRNA level of CD68 of aortic sinuses. f and g, Quantitative data of aortic sinuses from chimeric mice.

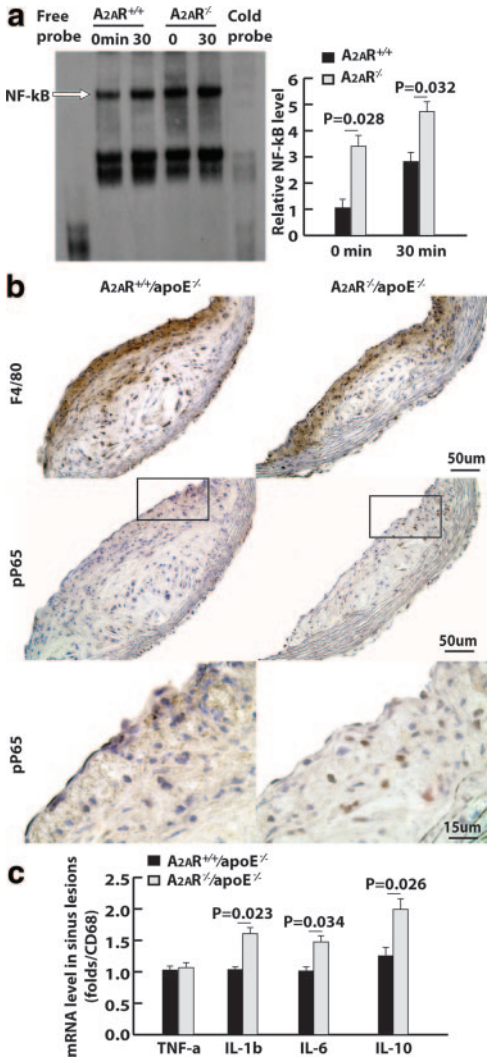

Figure 2. $A_{2A}R$ deficiency elevates inflammatory status of atherosclerotic lesions in Apoe$^{-/-}$ mice. a, Electrophoretic mobility shift assay to assess NF-κB activation induced by ox-LDL in a thioglycollate-induced peritonitis model. b, Immunostaining of phospho-p65 (pP65) and F4/80 of aortic sinuses. c, mRNA expression in atherosclerotic lesions.

flow cytometry. Among the F4/80-positive foam cells, the percentage of annexin V–positive but PI-negative cells was 12% for foam cells from Apoe$^{-/-}$/$A_{2A}R^{-/-}$ mice and 5% for foam cells from Apoe$^{-/-}$ mice (Figure 3b). Similar results were obtained by TUNEL-staining (Figure 3c). Caspase-3 is a critical executioner of apoptosis, and the cleaved p17 fragment represents its active form. The p17 fragment of caspase-3 was detected in foam cells by Western blot. The level of p17 was much higher in $A_{2A}R$-deficient foam cells than wt cells (Figure 3d).

Activation of p38 MAPK in $A_{2A}R$-Deficient Macrophages

Activation of $A_{2A}R$ increases intracellular cAMP,[4] which, in turn, inhibits activation of the intracellular signaling molecule p38 MAPK via the cAMP response element–binding protein–induced dynein light chain.[17] In an in vitro assay using isolated peritoneal macrophages, p38 MAPK activation in response to ox-LDL stimulation was much more robust in $A_{2A}R$-deficient than in wt macrophages (Figure 4a). Furthermore, the level of ox-LDL-induced active caspase-3 was much higher in $A_{2A}R$-deficient macrophages than wt macrophages (Figure 4b). To determine whether p38 activation was a possible mechanism for the increased apoptosis of $A_{2A}R$-deficient macrophages, $A_{2A}R$-deficient macrophages were first pretreated with the p38 inhibitor SB203580, followed by incubation with ox-LDL for induction of apoptosis. Incubation with ox-LDL elicited apoptosis in 20% of $A_{2A}R$-deficient macrophages and 9% of wt macrophages. SB203580 pretreatment decreased ox-LDL–mediated apoptosis in both cases, but this decrease was more pronounced for $A_{2A}R$-deficient macrophages than wt cells. The percentage of apoptotic $A_{2A}R$-deficient macrophages was reduced almost to

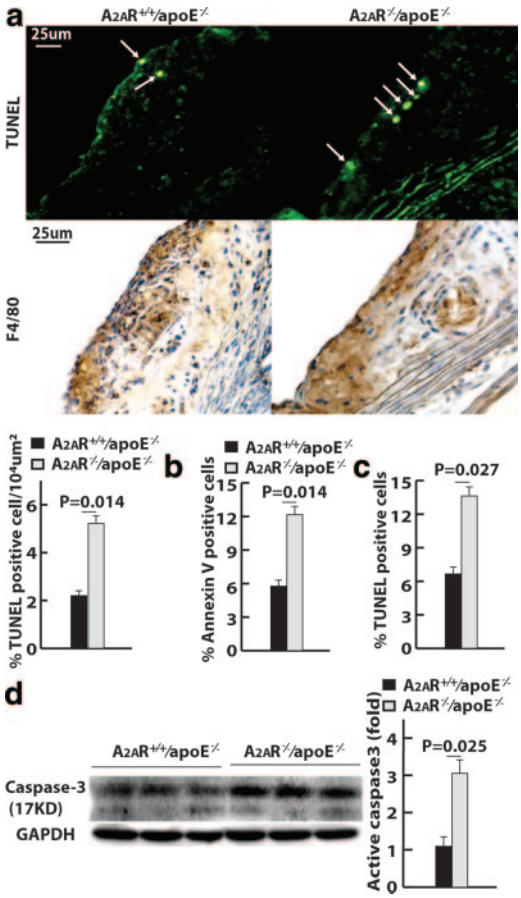

Figure 3. $A_{2A}R$ deficiency increases apoptosis of foam cells. a, Apoptosis and anti-F4/80 staining of atherosclerotic lesions. b and c, Percentages of apoptotic foam cells isolated from the peritoneal cavities on day 3 after thioglycollate injection. d, Western blot showing the level of active caspase-3 fragment in peritoneal foam cells.

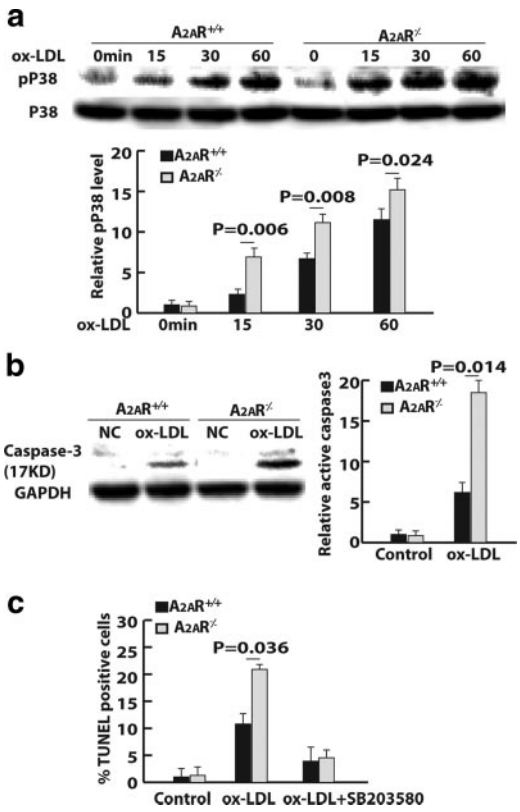

Figure 4. $A_{2A}R$ deficiency increases p38 activation in macrophages. a and b, Western blot showing levels of phosphorylated p38 (pP38) and active caspase-3 in peritoneal macrophages after ox-LDL stimulation (20 hours for b). c, Quantitative data of staining showing the effect of p38 activation on ox-LDL–mediated macrophage apoptosis.

the level measured for wt macrophages, indicating that increased p38 activation is the underlying mechanism for apoptosis of $A_{2A}R$-deficient macrophages (Figure 4c).

Discussion

Previous studies have shown that $A_{2A}R$ deficiency exacerbates inflammatory reactions and induces severe tissue injury,[6,7] and the present work demonstrates that $Apoe^{-/-}/A_{2A}R^{-/-}$ mice had increased body weight, considerable hypercholesterolemia, and increased proinflammatory cytokines in the blood. These data would predict a severe atherosclerotic phenotype in $Apoe^{-/-}/A_{2A}R^{-/-}$ mice. Thus, the observed suppression of atherosclerosis in $Apoe^{-/-}/A_{2A}R^{-/-}$ mice was highly unexpected. The initial data showing decreased atherosclerosis in mice fed a Western diet for 3 months were surprising, and led us to subsequently assess mice fed a Western diet for 6 months. $A_{2A}R$ deficiency led to even greater protection against atherosclerosis when mice were provided a Western diet for a longer period. Results from these 2 animal studies unambiguously support a protective role for $A_{2A}R$ inactivation in atherosclerosis.

The protective role of $A_{2A}R$ deficiency or blockade has mostly been observed in neurological disease models.[8] Loss or blockade of $A_{2A}R$ decreases ischemic brain injury and neurotoxicity in models of Parkinson disease and Huntington disease.[10,18–20] Blocking $A_{2A}R$-mediated glutamate release from the ischemic and nonischemic cortex and striatum has been proposed as the mechanism for these beneficial effects.[21] A recent study found that either global or BMDC-specific $A_{2A}R$ deficiency in mice attenuated infarct volumes in an ischemic brain injury model.[22] This protection was associated with a decline in the ischemia-induced expression of several proinflammatory cytokines. Using the same real-time RT-PCR assay, we found that the expression of cytokines in atherosclerotic lesions of $Apoe^{-/-}/A_{2A}R^{-/-}$ mice was higher than that of $Apoe^{-/-}$ mice, indicating that the mechanism for protection against atherosclerosis attributable to $A_{2A}R$ deficiency differs from that involved in neuroprotection.

$A_{2A}R$ deficiency has adverse effects in most animal models of peripheral organ diseases. $A_{2A}R^{-/-}$ mice exhibit extensive liver damage attributable to prolonged and enhanced expression of proinflammatory cytokines (such as TNF-α, IL-6, and IL-12) in concanavalin A– or endotoxin-induced septic shock and ischemic liver injury models.[6] Additionally, in a renal ischemia reperfusion injury model, plasma creatinine and cytokines are significantly increased in $A_{2A}R^{-/-}$ compared to wt mice.[23] In an adenosine deaminase–deficient model of pulmonary inflammation, $A_{2A}R$ deficiency causes enhanced

pulmonary leukocyte infiltration and mucin production in the bronchial airways, as well as elevated levels of MCP-1 and CXCL1.[24] $A_{2A}R$-mediated protection may be achieved via suppression of the generation of reactive oxygen species and proinflammatory cytokines in inflammatory cells.[4] In line with the above studies, we found that proinflammatory cytokines were increased in the circulating blood and atherosclerotic lesions of $Apoe^{-/-}/A_{2A}R^{-/-}$ mice.

Macrophage phenotype is modulated through adenosine $A_{2A}R$ activation. $A_{2A}R$ agonists synergize toll like receptors to switch macrophages from an M1 (inflammatory) phenotype to an M2 (angiogenic) phenotype.[25] Thus, because of the lack of $A_{2A}R$, macrophages in lesions maintain themselves in the M1 phenotype. Indeed, NF-κB activation was enhanced in lesion foam cells of $A_{2A}R^{-/-}/apoE^{-/-}$ mice. In an in vitro assay, $A_{2A}R$-deficient macrophages also exhibited increased NF-κB activation in response to ox-LDL, though ox-LDL may stimulate different receptors compared to minimally modified LDL and the effects of these ligands might discriminate important differences between wt and $A_{2A}R$-deficient macrophages.[26] Nevertheless, results from both in vivo and in vitro setups confirm the inflammatory phenotype of $A_{2A}R$-deficient macrophages and foam cells under atherosclerotic conditions. Contrary to the general concept that suppression of macrophage inflammatory reactions reduces atherosclerosis, inhibition of NF-κB activity by deletion of IKK2 decreases macrophage inflammatory phenotype, but enlarges atherosclerotic lesions.[27] Therefore, enhanced macrophage inflammatory phenotype may not directly lead to an increase of atherosclerotic lesion size.

The size of atherosclerotic lesions is directly related to the number of foam cells within the lesions, which is balanced by monocyte recruitment, macrophage apoptosis, and macrophage emigration from lesions.[2] No significant difference was found in monocyte homing ability between wt and $A_{2A}R$-deficient monocytes (supplemental Figure I). However, the number of macrophages in atherosclerotic lesions of $Apoe^{-/-}/A_{2A}R^{-/-}$ mice was less than that of $Apoe^{-/-}$ mice. This led us to examine whether $A_{2A}R$ deficiency induces macrophage apoptosis in atherosclerotic lesions.

Macrophage or foam cell apoptosis occurs during all stages of atherosclerosis and plays a different role in atherosclerosis depending on the stage at which it occurs.[28,13] During late stages of atherosclerosis, apoptosis contributes to the formation of necrotic cores and to lesion vulnerability.[29] However, during the early stages of atherosclerosis, apoptosis decreases the number of foam cells and the size of atherosclerotic lesions.[14,15] In lesions of $Apoe^{-/-}/A_{2A}R^{-/-}$ mice, most apoptoic cells were localized in the subendothelial space, indicating early apoptosis of foam cells. In response to oxLDL treatment, $A_{2A}R$-deficient macrophages exhibited increased p38 MAPK activation. This may result from a change in signaling associated with intracellular cAMP.[4] Elevation of cAMP following $A_{2A}R$ occupancy inhibits activation of p38 via the cAMP response element–binding protein–induced dynein light chain, and p38 activation has been linked to apoptosis.[17,30] A recent study showed that p38 mediates caspase-3 activation and apoptosis in macrophages stimulated with ATP and H_2O_2. $A_{2A}R$-deficient macrophages challenged with modified LDL may use similar pathways because the p38 inhibitor can inhibit caspase-3 activation and apoptosis.[31] We have attempted to elucidate molecular mechanisms underlying the apoptosis of $A_{2A}R$-deficient macrophages, but we have yet to find a difference in the levels of Bcl-2, Bax, and Bcl-XL between wt and $A_{2A}R$-deficient macrophages.

Activation of $A_{2A}R$ using agonists dramatically inhibits inflammation and protects against tissue injury. $A_{2A}R$ activation protects against ischemia in the myocardium, kidney, liver, spinal cord, and brain.[5] Additionally, administration of $A_{2A}R$ agonists improves survival in mouse models of endotoxemia and sepsis,[32] and attenuates inflammation and injury in lipopolysaccharide-induced lung injury,[33] diabetic nephropathy,[34] and inflammatory bowel disease.[35] Recent studies have shown that $A_{2A}R$ agonists inhibit foam cell formation and vascular remodeling after injury.[36,37] It is very likely that $A_{2A}R$ agonists inhibit the formation of atherosclerotic lesions. The antiatherosclerotic effects of $A_{2A}R$ deficiency do not rule out the potential efficacy of $A_{2A}R$ agonists in the treatment of atherosclerosis.

In summary, our data provide evidence that $A_{2A}R$ inactivation protects against atherosclerosis. $A_{2A}R$ deficiency increases p38 activation in macrophages and foam cells, and this modulation in signaling induces activation of caspase-3. The latter drives foam cells toward apoptosis, thus reducing the size of atherosclerotic lesions. This study suggests that $A_{2A}R$ inactivation represents a new direction for antiatherosclerotic therapies.

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Disclosures

None.

References

1. Ross R. Atherosclerosis–an inflammatory disease. *N Engl J Med*. 1999; 340:115–126.
2. Libby P. Inflammation in atherosclerosis. *Nature*. 2002;420:868–874.
3. Glass CK, Witztum JL. Atherosclerosis. the road ahead. *Cell*. 2001;104: 503–516.
4. Hasko G, Cronstein BN. Adenosine: an endogenous regulator of innate immunity. *Trends Immunol*. 2004;25:33–39.
5. Lappas CM, Sullivan GW, Linden J. Adenosine A2A agonists in development for the treatment of inflammation. *Expert Opin Investig Drugs*. 2005;14:797–806.
6. Ohta A, Sitkovsky M. Role of G-protein-coupled adenosine receptors in downregulation of inflammation and protection from tissue damage. *Nature*. 2001;414:916–920.
7. Lukashev D, Ohta A, Apasov S, Chen JF, Sitkovsky M. Cutting edge: Physiologic attenuation of proinflammatory transcription by the Gs protein-coupled A2A adenosine receptor in vivo. *J Immunol*. 2004;173: 21–24.
8. Chen JF, Sonsalla PK, Pedata F, Melani A, Domenici MR, Popoli P, Geiger J, Lopes LV, de MA. Adenosine A2A receptors and brain injury: broad spectrum of neuroprotection, multifaceted actions and "fine tuning" modulation. *Prog Neurobiol.* 2007;83:310–331.

9. Schwarzschild MA, Agnati L, Fuxe K, Chen JF, Morelli M. Targeting adenosine A2A receptors in Parkinson's disease. *Trends Neurosci*. 2006; 29:647–654.
10. Chen JF, Huang Z, Ma J, Zhu J, Moratalla R, Standaert D, Moskowitz MA, Fink JS, Schwarzschild MA. A(2A) adenosine receptor deficiency attenuates brain injury induced by transient focal ischemia in mice. *J Neurosci*. 1999;19:9192–9200.
11. Huo Y, Zhao L, Hyman MC, Shashkin P, Harry BL, Burcin T, Forlow SB, Stark MA, Smith DF, Clarke S, Srinivasan S, Hedrick CC, Pratico D, Witztum JL, Nadler JL, Funk CD, Ley K. Critical role of macrophage 12/15-lipoxygenase for atherosclerosis in apolipoprotein E-deficient mice. *Circulation*. 2004;110:2024–2031.
12. Wang H, Tang R, Zhang W, Amirikian K, Geng Z, Geng J, Hebbel RP, Xia L, Marth JD, Fukuda M, Katoh S, Huo Y. Core2 1-6-N-glucosaminyltransferase-I is crucial for the formation of atherosclerotic lesions in apolipoprotein E-deficient mice. *Arterioscler Thromb Vasc Biol*. 2009;29:180–187.
13. Tabas I. Consequences and therapeutic implications of macrophage apoptosis in atherosclerosis: the importance of lesion stage and phagocytic efficiency. *Arterioscler Thromb Vasc Biol*. 2005;25:2255–2264.
14. Arai S, Shelton JM, Chen M, Bradley MN, Castrillo A, Bookout AL, Mak PA, Edwards PA, Mangelsdorf DJ, Tontonoz P, Miyazaki T. A role for the apoptosis inhibitory factor AIM/Spalpha/Api6 in atherosclerosis development. *Cell Metab*. 2005;1:201–213.
15. Wang Z, Liu B, Wang P, Dong X, Fernandez-Hernando C, Li Z, Hla T, Li Z, Claffey K, Smith JD, Wu D. Phospholipase C beta3 deficiency leads to macrophage hypersensitivity to apoptotic induction and reduction of atherosclerosis in mice. *J Clin Invest*. 2008;118:195–204.
16. Li AC, Brown KK, Silvestre MJ, Willson TM, Palinski W, Glass CK. Peroxisome proliferator-activated receptor gamma ligands inhibit development of atherosclerosis in LDL receptor-deficient mice. *J Clin Invest*. 2000;106:523–531.
17. Zhang J, Bui TN, Xiang J, Lin A. Cyclic AMP inhibits p38 activation via CREB-induced dynein light chain. *Mol Cell Biol*. 2006;26:1223–1234.
18. Phillis JW. The effects of selective A1 and A2a adenosine receptor antagonists on cerebral ischemic injury in the gerbil. *Brain Res*. 1995; 705:79–84.
19. Chen JF, Xu K, Petzer JP, Staal R, Xu YH, Beilstein M, Sonsalla PK, Castagnoli K, Castagnoli N, Jr., Schwarzschild MA. Neuroprotection by caffeine and A(2A) adenosine receptor inactivation in a model of Parkinson's disease. *J Neurosci.* 2001;21:RC143.
20. Fink JS, Kalda A, Ryu H, Stack EC, Schwarzschild MA, Chen JF, Ferrante RJ. Genetic and pharmacological inactivation of the adenosine A2A receptor attenuates 3-nitropropionic acid-induced striatal damage. *J Neurochem*. 2004;88:538–544.
21. Marcoli M, Raiteri L, Bonfanti A, Monopoli A, Ongini E, Raiteri M, Maura G. Sensitivity to selective adenosine A1 and A2A receptor antagonists of the release of glutamate induced by ischemia in rat cerebrocortical slices. *Neuropharmacology*. 2003;45:201–210.
22. Yu L, Huang Z, Mariani J, Wang Y, Moskowitz M, Chen JF. Selective inactivation or reconstitution of adenosine A2A receptors in bone marrow cells reveals their significant contribution to the development of ischemic brain injury. *Nat Med*. 2004;10:1081–1087.
23. Day YJ, Huang L, McDuffie MJ, Rosin DL, Ye H, Chen JF, Schwarzschild MA, Fink JS, Linden J, Okusa MD. Renal protection from ischemia mediated by A2A adenosine receptors on bone marrow-derived cells. *J Clin Invest*. 2003;112:883–891.
24. Mohsenin A, Mi T, Xia Y, Kellems RE, Chen JF, Blackburn MR. Genetic removal of the A2A adenosine receptor enhances pulmonary inflammation, mucin production, and angiogenesis in adenosine deaminase-deficient mice. *Am J Physiol Lung Cell Mol Physiol*. 2007;293: L753–L761.
25. Pinhal-Enfield G, Ramanathan M, Hasko G, Vogel SN, Salzman AL, Boons GJ, Leibovich SJ. An angiogenic switch in macrophages involving synergy between Toll-like receptors 2, 4, 7, and 9 and adenosine A(2A) receptors. *Am J Pathol*. 2003;163:711–721.
26. Miller YI, Chang MK, Binder CJ, Shaw PX, Witztum JL. Oxidized low density lipoprotein and innate immune receptors. *Curr Opin Lipidol*. 2003;14:437–445.
27. Kanters E, Pasparakis M, Gijbels MJ, Vergouwe MN, Partouns-Hendriks I, Fijneman RJ, Clausen BE, Forster I, Kockx MM, Rajewsky K, Kraal G, Hofker MH, de Winther MP. Inhibition of NF-kappaB activation in macrophages increases atherosclerosis in LDL receptor-deficient mice. *J Clin Invest*. 2003;112:1176–1185.
28. Kockx MM. Apoptosis in the atherosclerotic plaque: quantitative and qualitative aspects. *Arterioscler Thromb Vasc Biol*. 1998;18:1519–1522.
29. Schrijvers DM, De Meyer GR, Kockx MM, Herman AG, Martinet W. Phagocytosis of apoptotic cells by macrophages is impaired in atherosclerosis. *Arterioscler Thromb Vasc Biol*. 2005;25:1256–1261.
30. Zarubin T, Han J. Activation and signaling of the p38 MAP kinase pathway. *Cell Res*. 2005;15:11–18.
31. Noguchi T, Ishii K, Fukutomi H, Naguro I, Matsuzawa A, Takeda K, Ichijo H. Requirement of reactive oxygen species-dependent activation of ASK1-p38 MAPK pathway for extracellular ATP-induced apoptosis in macrophage. *J Biol Chem*. 2008;283:7657–7665.
32. Sullivan GW, Fang G, Linden J, Scheld WM. A2A adenosine receptor activation improves survival in mouse models of endotoxemia and sepsis. *J Infect Dis*. 2004;189:1897–1904.
33. Reutershan J, Cagnina RE, Chang D, Linden J, Ley K. Therapeutic anti-inflammatory effects of myeloid cell adenosine receptor A2a stimulation in lipopolysaccharide-induced lung injury. *J Immunol*. 2007;179: 1254–1263.
34. Awad AS, Huang L, Ye H, Duong ET, Bolton WK, Linden J, Okusa MD. Adenosine A2A receptor activation attenuates inflammation and injury in diabetic nephropathy. *Am J Physiol Renal Physiol*. 2006;290:F828–F837.
35. Odashima M, Bamias G, Rivera-Nieves J, Linden J, Nast CC, Moskaluk CA, Marini M, Sugawara K, Kozaiwa K, Otaka M, Watanabe S, Cominelli F. Activation of A2A adenosine receptor attenuates intestinal inflammation in animal models of inflammatory bowel disease. *Gastroenterology*. 2005;129:26–33.
36. Reiss AB, Rahman MM, Chan ES, Montesinos MC, Awadallah NW, Cronstein BN. Adenosine A2A receptor occupancy stimulates expression of proteins involved in reverse cholesterol transport and inhibits foam cell formation in macrophages. *J Leukoc Biol*. 2004;76:727–734.
37. McPherson JA, Barringhaus KG, Bishop GG, Sanders JM, Rieger JM, Hesselbacher SE, Gimple LW, Powers ER, Macdonald T, Sullivan G, Linden J, Sarembock IJ. Adenosine A(2A) receptor stimulation reduces inflammation and neointimal growth in a murine carotid ligation model. *Arterioscler Thromb Vasc Biol,*. 2001;21:791–796.

Figure I

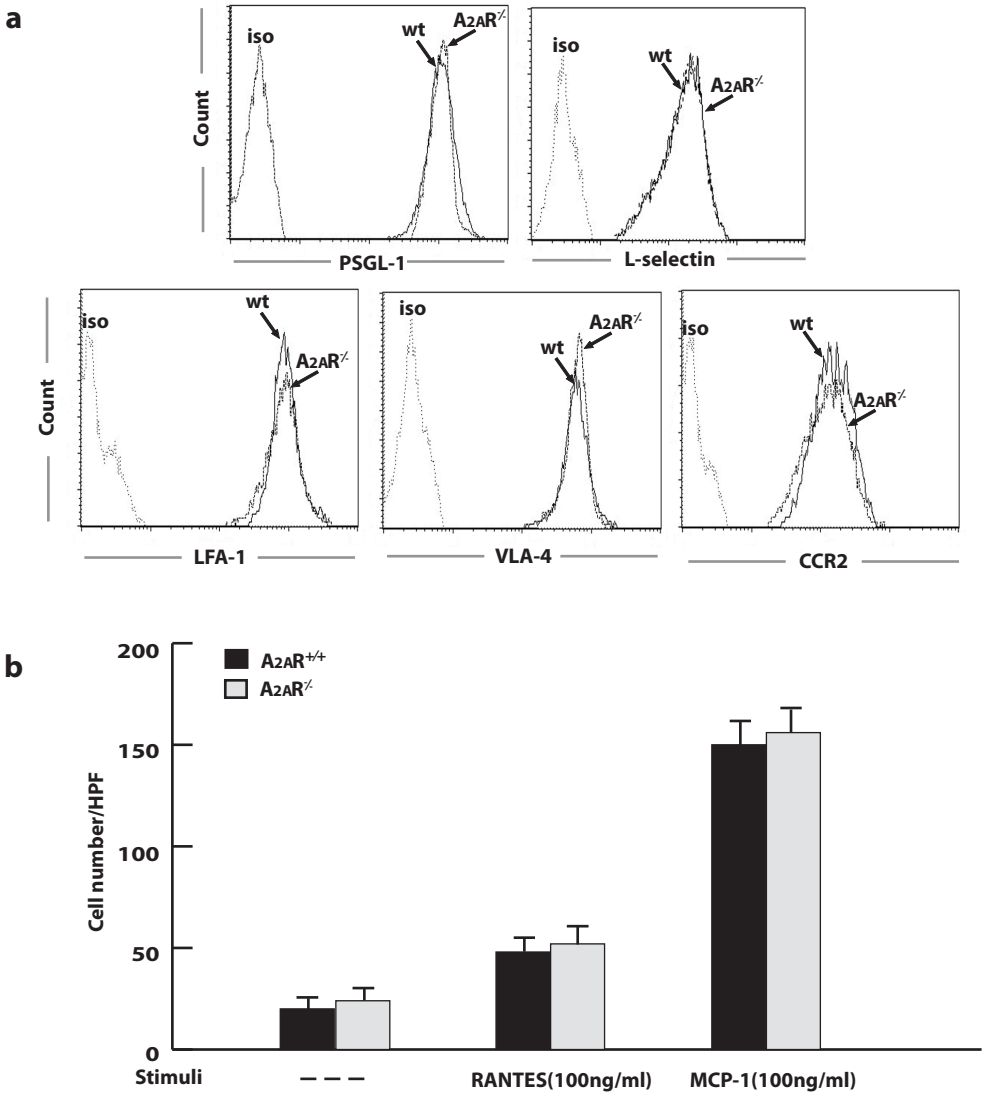

Table I, II

Table I

Genotype	Gender	n	WBC (k/uL)	NE (k/uL)	LY (k/uL)	MO (k/uL)	NE%	LY%	MO%	PTL (k/uL)
3 months WD										
Apoe$^{-/-}$/$A_{2A}R^{+/+}$	M	15	5.0±2.9	1.7±1.0	2.9±1.8	0.3±0.2	36.7±10.8	55.4±8.5	5.8±4.8	1011.5±343.5
Apoe$^{-/-}$/$A_{2A}R^{-/-}$		15	6.7±4.2	2.6±2.3	3.5±1.9	0.5±0.4	36.4±13.5	52.2±14.5	6.2±4.2	921.6±245.8
Apoe$^{-/-}$/$A_{2A}R^{+/+}$	F	15	6.1±3.3	1.8±1.0	3.3±2.1	0.4±0.3	33.3±8.5	53.5±10.2	7.9±4.6	647.1±255.1
Apoe$^{-/-}$/$A_{2A}R^{-/-}$		15	6.4±5.1	2.4±1.8	3.0±2.4	0.4±0.3	37.3±7.9	50.6±8.4	6.1±2.3	666.3±298.6
6 months WD										
Apoe$^{-/-}$/$A_{2A}R^{+/+}$	M	15	2.8±1.4	1.1±0.5	1.5±0.9	0.2±0.1	40.8±11.3	50.4±10.1	4.8±3.6	1084.8±259.3
Apoe$^{-/-}$/$A_{2A}R^{-/-}$		15	3.4±1.7	1.6±1.0	1.5±0.7	0.3±0.3	45.0±11.9	46.2±10.3	8.4±6.6	969.3±301.0
Apoe$^{-/-}$/$A_{2A}R^{+/+}$	F	15	2.1±1.5	1.0±0.7	1.1±0.9	0.1±0.1	46.8±15.0	46.2±14	5.1±3.0	796.5±327.6
Apoe$^{-/-}$/$A_{2A}R^{-/-}$		15	2.6±1.0	1.2±0.6	1.2±0.5	0.1±0.1	46.8±11.7	46.1±10.7	5.9±4.9	796.2±273.3

Table II

Genotype	Gender	n	Bp (mmHg)	Triglyceride (mg/dL)	VLDL (mg/dL)	HDL (mg/dL)	Glucose (mg/dL)
3 months WD							
Apoe$^{-/-}$/$A_{2A}R^{+/+}$	Male	15	114.2±17.4	148.2±23.1	29.9±4.3	367.1±47.6	226.5+/-56.7
Apoe$^{-/-}$/$A_{2A}R^{-/-}$		15	109.4±12.6	143.9±22.9	28.7±4.6	370.1±53.3	225.0+/-58.8
Apoe$^{-/-}$/$A_{2A}R^{+/+}$	Female	15	104.4±15.7	107.0±34.6	19.1±6.7	215.1±24.1	107.0+/-35.6
Apoe$^{-/-}$/$A_{2A}R^{-/-}$		15	101.6±11.2	107.6±16.6	21.6±3.4	235.5±38.9	107.6+/-37.8

Table III

Genotype	Gender	n	Cholesterol (mg/dL)	Triglyceride (mg/dL)	LDL (mg/dL)	VLDL (mg/dL)	HDL (mg/dL)
3 months CD							
$Apoe^{-/-}/A_{2A}R^{+/+}$	Male	6	390.8±46.4	124.3±32.5	246.5±26.2	30.5±6.3	234.1±34.6
$Apoe^{-/-}/A_{2A}R^{-/-}$		6	567.5±66.6*	138.4±35.9	374.6±34.0*	32.7±5.1	201.5±43.8
$Apoe^{-/-}/A_{2A}R^{+/+}$	Female	6	353.4±42.3	117.7±42.6	196.8±21.6	28.1±7.5	217.1±36.2
$Apoe^{-/-}/A_{2A}R^{-/-}$		6	456.3±41.1*	123.7±34.1	306.2±31.2*	23.3±5.8	198.4±39.2
3 months WD							
$A_{2A}R^{+/+}$	Male	6	221.8±36.5	119.2±36.8	39.2±6.8	33.6±5.2	254.4±64.6
$A_{2A}R^{-/-}$		6	234.5±29.7	117.3±36.5	37.3±6.5	34.6±6.8	222.5±48.3
$A_{2A}R^{+/+}$	Female	6	179.8±25.1	104.5±38.1	34.5±8.1	31.4±5.7	239.5±43.1
$A_{2A}R^{-/-}$		6	186.4±35.4	106.7±35.3	36.7±5.3	29.3±6.2	224.5±39.4

*P<0.05 vs $Apoe^{-/-}/A_{2A}R^{+/+}$

Table IV

Genotype	n	ALT (U/L)
3 months WD		
$Apoe^{-/-}/A_{2A}R^{+/+}$	6	34.8±4.1
$Apoe^{-/-}/A_{2A}R^{-/-}$	6	158.5±36.2*
3 months CD		
$Apoe^{-/-}/A_{2A}R^{+/+}$	6	39.6±6.8
$Apoe^{-/-}/A_{2A}R^{-/-}$	6	41.3±8.2
3 months WD		
$A_{2A}R^{+/+}$	6	50.1±10.8
$A_{2A}R^{-/-}$	6	60.2±12.2
3 months CD		
$A_{2A}R^{+/+}$	6	36.4±6.8
$A_{2A}R^{-/-}$	6	37.1±8.2

*P<0.05 vs $Apoe^{-/-}/A_{2A}R^{+/+}$

Table V

Table V

Genotype	n	IL-1b	IL-2	IL-4	IL-6	IL-10	IL-12p70	IFN-g	JE	TNF-a
$Apoe^{-/-}/A_{2A}R^{+/+}$	30	NS	NS	125±18.6	20±5.8	9.4±1.5	NS	NS	NS	NS
$Apoe^{-/-}/A_{2A}R^{+/+}$	30	NS	NS	128±20.3	25±8.2*	9.8±2.1	NS	NS	NS	NS
Sensitivity		28.0	8.0	40.0	12.0	8.0	40.0	80.0	200	12.0

*P=0.036 The units of all these proteins are pg/mL.