Major Histocompatibility Class II Transactivator Expression in Smooth Muscle Cells from A2b Adenosine Receptor Knock-out Mice

CROSS-TALK BETWEEN THE ADENOSINE AND INTERFERON-γ SIGNALING*$$\text{1}

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Atherosclerosis characterized by sustained inflammation and aberrant extracellular matrix alterations. Our previous investigations have defined major histocompatibility class II transactivator (CIITA) as a key factor in mediating these two processes in smooth muscle cells. Here, we demonstrate that CIITA and major histocompatibility class II expression are elevated in interferon-γ (IFN-γ)-treated smooth muscle cells from A2b adenosine receptor (A2bAR-/-) knock-out mice, as compared with wild type cells. An A2-type adenosine receptor agonist suppresses these effects of IFN-γ in wild type cells, which can be blocked by an A2bAR-specific antagonist. We further identify that increased cellular cAMP levels are responsible for the down-regulation of CIITA expression and, hence, reduced IFN-γ response as evidenced by the following data: 1) direct activation of adenylyl cyclase activity is both necessary and sufficient to suppress the IFN-γ response; 2) inhibition of phosphodiesterase activity attenuates IFN-γ induced transcription events; and 3) direct treatment with cAMP analogs abrogates CIITA activation and IFN-γ response. Therefore, our data establish possible cross-talk between the adenosine signaling through cAMP and IFN-γ during regulation of CIITA expression.

The pathogenesis of cardiovascular diseases, which can be divided into several stages including hemostasis, inflammation, cellular proliferation, and remodeling, involves complex interplay between different tissues and cells (1). Atherogenesis, for example, features the infiltration of smooth muscle cells (SMCs)3 that proliferate, migrate, and synthesize extracellular matrix (ECM). Meanwhile, inflammatory cells are recruited to the vascular cell wall to release cytokines (e.g. IFN-γ) and trigger chronic inflammation. Therefore, targeting the two key characteristics of vascular disease-ECM remodeling and inflammatory response of SMCs can not only provide clues for unveiling the molecular mechanisms underlying atherogenesis but also shed light into potential therapeutic solutions to cardiovascular diseases.

Major histocompatibility complex class II (MHC II) transactivator (CIITA) is a protein capable of regulating both ECM reshuffling and inflammatory responses. CIITA was first identified to be mutated from patients with a rare hereditary disease called bare lymphocyte syndrome. Bare lymphocyte syndrome patients are diagnosed with severe immune deficiency caused by complete loss of MHC II expression, hence establishing CIITA as an essential factor in the regulation of immune response. More recently, CIITA has been suggested to be involved in the transcriptional regulation of a wide range of other genes, including type I collagen (2, 3), the most abundant component of ECM, indicating that CIITA may also play an important role in regulating ECM remodeling.

Adenosine receptors (AR) represent a family of G-protein-coupled receptors that also bear the dual functionality of remodeling ECM and modulating inflammation. The role of adenosine receptors in cell or tissue function has been primarily delineated by studies with agonists and antagonists. A1 and A3AR primarily inhibit adenylyl cyclase (AC), whereas the A2-type AR stimulates AC (4, 5). A2 adenosine receptors can be further categorized into high affinity A2aAR and low affinity A2bAR (6). Adenosine receptors have been reported to alter the expression of ECM proteins including collagen both in cell culture (7, 8) and animal models (9, 10). Meanwhile, the importance of adenosine receptors in modulating the immune response has been highlighted in studies using AR agonists/antagonists as well as in a number of animal models in which ARs are shown to cross-talk with several inflammatory signaling pathways (11–14).

Previously, our laboratory has demonstrated that CIITA is inducible by IFN-γ in both human aortic SMCs and mouse aortic SMCs and that CIITA is responsible for mediating MHC II up-regulation and collagen repression in response to IFN-γ (15, 16). Our earlier investigations have also revealed that the A2b adenosine receptor (A2bAR) is expressed in the vascular scaffold of atherosclerotic plaques. Here, we demonstrate that A2bAR mediates IFN-γ-induced synthetic and secretory functions of SMCs, possibly through cross-talk with CIITA.

# The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S3.
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3 The abbreviations used are: SMC, smooth muscle cell; CIITA, class II transactivator; IFN, interferon; MHC II, major histocompatibility complex type II; AR, adenosine receptor; AC, adenylyl cyclase; ECM, extracellular matrix; WT, wild type; DMEM, Dulbecco’s modified Eagle’s medium; IBMX, 3-isobutyl-1-methylxanthine; GFP, green fluorescent protein; NECA, 5’-N-ethylcarboxamidoadenosine; PDE, phosphodiesterase; CREB, cAMP-responsive element-binding protein.
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ture and protects against inflammation, as judged by the augmentation of pro-inflammatory cytokines and consequently enhanced inflammatory responses in a A2bAR knock-out/reporter gene knock-in (A2bAR−/−) mouse model (17). Therefore, there is a possible link between CIITA and A2bAR in regulating ECM remodeling and inflammation. Here we report that CIITA expression is elevated in vascular SMCs from A2bAR−/− mice, which augments MHC II transcription activation and collagen transcription repression in response to IFN-γ.

EXPERIMENTAL PROCEDURES

Cell Isolation—To avoid a potential ambiguity in data interpretation caused by analysis of mice on a mixed background, the A2bAR−/− mice used here were on a C57BL/6 background strain. This was achieved following eight generations of backcross-breeding of the original knock-out mice (17), accompanied by periodical PCR-based gene marker analysis MAX-BAX (Charles River Laboratories) to guide in selecting mice with the highest percentage of C57BL/6/6 background. This combined process of breeding, genotyping for A2bAR, and MAX-BAX analysis yielded A2bAR−/− mice on a 99.8% C57BL/6 background. Following procedures conventionally practiced in the literature, these animals were interbred, and their progeny were used for analyses. SMCs were prepared from aortas of WT (C57BL/6) and A2bAR−/− mice essentially as described previously (18, 19). Briefly, aortas of 19-day-old mice were removed from the aortic arch to the femoral bifurcation and stripped of adventitia. The aortas were minced and subjected to digestion with 0.5 μg/ml bacterial collagenase (Sigma type I) and 0.125 μg/ml porcine pancreatic elastase (Sigma type III) in Dulbecco’s modified Eagle’s medium (DMEM) at 37 °C. Resulting SMCs were collected by centrifugation at 400 g for 10 min at room temperature and grown in DMEM (Invitrogen) supplemented with 0.4% fetal bovine serum for 16 h prior to IFN-γ or different chemicals. The cells were pretreated in DMEM (Invitrogen). One day prior to the experiments, the cells were plated at density of 6 × 10^5 cells/well in 6-well dishes or 3 × 10^6 cells/plate in p100 plates and incubated at 37 °C with 5% CO_2 for 16–24 h. In several studies the cells were treated with IFN-γ or different chemicals. The cells were pretreated in DMEM with 0.4% fetal bovine serum for 16 h prior to IFN-γ (100units/ml), NECA (10 μM), MRS-1754 (10 μM), forskolin (1–10 μM), SQ22536 (10 μM), 3-isobutyl-1-methylxanthine (IBMX; 50 μM), or 8-bromo-cAMP (0.5–1 mM) treatment.

Plasmids and Transfection and Luciferase Assay—The col1a2-luciferase construct (pGL3-COL-LUC) (3) contains sequences from −357 to +55 bp of mouse col1a2 promoter fused to the luciferase reporter gene. The HLA-DRα promoter fused to the luciferase reporter gene was a gift from Dr. Jenny Ting (20). Transfections were performed with the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s protocol. A GFP reporter construct was co-transfected as a control for transfection efficiency. The cells were harvested 24 h post-transfection, and luciferase assays were performed with a luciferase reporter assay system (Promega). The data are normalized to GFP fluorescence and total protein. The experiments were routinely performed in triplicate wells.

RNA Isolation and Real Time PCR—The cells were harvested, and RNA was extracted using an RNeasy RNA isolation kit (Qiagen) according to the manufacturer’s protocol. Reverse transcriptase reactions were performed using a SuperScript first strand synthesis system (Invitrogen) according to the manufacturer’s protocol. Real time PCRs were performed on ABI Prism 7700 sequence detection PCR machine (Applied Biosystems, Foster City, CA) according to the manufacturer’s protocol. The oligonucleotide primers and TaqMan probes were as previously described (16).

Protein Isolation and Western Blots—Whole cell lysates were obtained by resuspending cell pellets in radioimmune precipitation assay buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100) with freshly added protease inhibitor (Roche Applied Science) and phenylmethylsulfonyl fluoride (100 μg/ml radioimmune precipitation assay buffer). The proteins were quantified with the BCA reagent (Pierce) according to the manufacturer’s protocol, and separated by 10% polyacrylamide gel electrophoresis with prestained markers (Bio-Rad) for estimating molecular weight and efficiency of transfer to blots. The proteins were transferred to nitrocellulose membranes (Bio-Rad) in a Mini-Trans-Blot Cell (Bio-Rad). The membranes were blocked with 5% milk powder in Tris buffered saline (TBST) (0.05% Tween 20, 150 mM NaCl, 100 mM Tris-HCl, pH 7.4) buffer at 4 °C for several hours and incubated with monoclonal anti-CIITA (7-1H, 1:200, Santa Cruz), polyclonal anti-collagen type I (1:5000, Rockland), or monoclonal anti-β-actin (1:5000, Sigma) antibody overnight. After three washes with TBST, the membranes were incubated with appropriate secondary antibodies, either anti-mouse IgG, or anti-rabbit IgG (Amersham Pharmacia) conjugated to horseradish peroxidase, for another hour at room temperature. Then protein blots were visualized using the Supersignal ECL reagent (Pierce) on a Kodak image station (PerkinElmer Life Sciences).

RESULTS

A2bAR−/− Cells Have Elevated CIITA Expression—In a previous study, the mice were generated with deleted A2bAR gene (A2bAR−/−) in which a reporter gene encoding prokaryotic β-galactosidase was introduced immediately under the control of the endogenous A2bAR gene promoter. In the current study, primary cultures of aortic SMCs derived from age-, gender-, and strain-matched (C57BL/6) WT and A2bAR−/− mice were isolated as described previously (21) to determine whether the A2bAR influences the expression of CIITA.

CIITA, the master regulator of MHC II transcription (22), is dramatically induced in SMCs by IFN-γ (15, 16). IFN-γ-induced expression of CIITA protein was compared in WT and A2bAR−/− cells. There was significantly more CIITA protein...
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CIITA has isoforms produced by alternate gene promoters and first exons. Cell type-specific modulation of CIITA expression is controlled largely by promoters I, III, and IV (23, 24). Overexpression of all three isotypes in SMCs activates MHC II transcription and represses collagen transcription (15). However, mouse SMCs express isoforms I and IV only after IFN-γ treatment (16). The primers were designed previously (16) to examine all the CIITA isoforms using a common sequence (Fig. 1B, common). IFN-γ induced twice as much CIITA message in the A2bAR<sup>−/−</sup> cells as in the WT cells (Fig. 1A). The data are expressed as relative RNA levels compared with control levels, normalized to 18 S RNA and presented as the averages ± S.D. A2bAR<sup>−/−</sup> cells expressed significantly more common and type IV CIITA messages compared with the WT (p < 0.01, **).

CIITA expression was most likely due to increased CIITA. Taken together, these data demonstrate that CIITA expression was elevated in the absence of A2bAR.

A2bAR<sup>−/−</sup> Cells Have Elevated MHC II Expression and Enhanced Collagen Repression in Response to IFN-γ—Next, experiments were conducted to determine whether increased CIITA protein induced MHC II expression. SMCs treated with IFN-γ were examined for expression of MHC II, using primers to H2-Eβ, a murine homolog of the human HLA-DR<sub>e</sub> molecule, by quantitative PCR. As expected, there was little expression of MHC II at a basal level that was dramatically stimulated by IFN-γ in WT and A2bAR<sup>−/−</sup> cells. There was a 1.6-fold increase in MHC II expression in the A2bAR<sup>−/−</sup> cells compared with the WT in response to IFN-γ (Fig. 2A), similar to the increase in CIITA (Fig. 1B). Because MHC II plays a central role in regulation of the immune response, an increase in these molecules may be involved in the augmented inflammation observed in the A2bAR<sup>−/−</sup> mice.

Transient transfections were performed to examine whether changes of message levels were due to transcriptional alterations resulting from the increased CIITA expression. A luciferase construct driven by the well described human MHC II promoter was transfected into both WT and A2bAR<sup>−/−</sup> SMCs. IFN-γ stimulated the promoter activity significantly higher (5.5-fold) in A2bAR<sup>−/−</sup> cells than in WT cells (3.5-fold; Fig. 2B). RXF5 (regulatory factor for X-box 5), a DNA-binding transcription factor critical for recruitment of CIITA and for MHC II activation (25) during IFN-γ response, was expressed in the same manner in WT and A2bAR<sup>−/−</sup> cells either with or without IFN-γ (data not shown), indicating that elevation of MHC II expression was most likely due to increased CIITA.

We next focused on whether increased CIITA might repress its second target gene, collagen. Our earlier results, using both overexpression (2, 15, 16, 26, 27) and silencing (2) of CIITA, demonstrated that CIITA is crucial for IFN-γ-induced repression of collagen type I in fibroblasts and SMCs. In this study, IFN-γ repressed type I collagen mRNA levels more in the A2bAR<sup>−/−</sup> cells than in WT cells (Fig. 2C). However, the increased repression of collagen mRNA was small, it was consist—Next, we examined whether increased CIITA might repress its second target gene, collagen. Our earlier results, using both overexpression (2, 15, 16, 26, 27) and silencing (2) of CIITA, demonstrated that CIITA is crucial for IFN-γ-induced repression of collagen type I in fibroblasts and SMCs. In this study, IFN-γ repressed type I collagen mRNA levels more in the A2bAR<sup>−/−</sup> cells than in WT cells (Fig. 2C). Although the increased repression of collagen mRNA was small, it was consistent with the increase in MHC II expression. This suggests that adenosine receptor signaling represses CIITA isoform IV promoter in mouse. Taken together, these data demonstrate that CIITA expression was elevated in the absence of A2bAR.

A2bAR<sup>−/−</sup> cells have elevated CIITA expression. WT and A2bAR<sup>−/−</sup> (KO) vascular SMC cells were treated with or without IFN-γ (50 units/ml) for 24 h as described under "Experimental Procedures." A, whole cell lysates were separated by SDS-polyacrylamide gel electrophoresis, and Western blots were performed to measure CIITA. β-Actin was used as protein loading control. B, RNA was harvested, reverse-transcribed into cDNA, and amplified using primers described previously (16) to detect all isoforms (common) or individual CIITA isoforms as indicated. All of the samples were IFN-γ treated. Each experiment was repeated three times in triplicate wells. The data are expressed as relative RNA levels compared with control levels, normalized to 18 S RNA and presented as the averages ± S.D. A2bAR<sup>−/−</sup> cells expressed significantly more common and type IV CIITA messages compared with the WT (p < 0.01, **).

![Figure 1](image_url)

**FIGURE 1.** A2bAR<sup>−/−</sup> cells have elevated CIITA expression. WT and A2bAR<sup>−/−</sup> (KO) vascular SMC cells were treated with or without IFN-γ (50 units/ml) for 24 h as described under "Experimental Procedures." A, whole cell lysates were separated by SDS-polyacrylamide gel electrophoresis, and Western blots were performed to measure CIITA. β-Actin was used as protein loading control. B, RNA was harvested, reverse-transcribed into cDNA, and amplified using primers described previously (16) to detect all isoforms (common) or individual CIITA isoforms as indicated. All of the samples were IFN-γ treated. Each experiment was repeated three times in triplicate wells. The data are expressed as relative RNA levels compared with control levels, normalized to 18 S RNA and presented as the averages ± S.D. A2bAR<sup>−/−</sup> cells expressed significantly more common and type IV CIITA messages compared with the WT (p < 0.01, **).

![Figure 2](image_url)

**FIGURE 2.** A2bAR<sup>−/−</sup> cells have elevated MHC II expression and enhanced collagen repression in response to IFN-γ. WT and A2bAR<sup>−/−</sup> (KO) vascular SMC cells were treated with or without IFN-γ (50 units/ml) for 24 h as described under "Experimental Procedures." A and C, RNA was harvested, reverse-transcribed into cDNA, and amplified using primers to detect MHC II (A) and type I collagen (C) messages. Each experiment was repeated at least twice in triplicate wells. The data are expressed as relative RNA levels compared with control levels, normalized to 18 S RNA, and presented as averages ± S.D. A2bAR<sup>−/−</sup> cells had significantly higher MHC II and lower type I collagen mRNA in response to IFN-γ than the WT (p < 0.01, **). B and D, WT and A2bAR<sup>−/−</sup> vascular SMC cells were transfected with either an MHC II promoter construct (~300 DRA) (B) or two type I collagen promoter constructs (D) as indicated. A cytomegalovirus-GFP construct was co-transfected to normalize for transfection efficiency. One day following transfection, the cells were treated with or without IFN-γ (50 units/ml) for additional 24 h before harvesting. The average luciferase activities were normalized by both protein concentration and GFP fluorescence and presented as the averages ± S.D. This representative experiment was repeated twice in triplicate wells. Activation of MHC II promoter and repression of collagen promoters were significantly higher in A2bAR<sup>−/−</sup> cells than the WT (p < 0.01, **).
CIITA and Collagen Expression in SMCs from A2bAR^{−/−} cells alter CIITA expression either by itself or in conjunction with MRS-1754 (data not shown). On the other hand, there was more CIITA protein being induced in the A2bAR^{−/−} cells (lane 7), but neither NECA nor MRS-1754 resulted in any significant changes in CIITA expression (lanes 8–10). This suggests that the NECA effect was specifically through the A2bAR. The same pattern was also observed with CIITA mRNA levels (Fig. 3B). NECA and its antagonist had no effect on A2bAR^{−/−} cells as expected if the animals are truly A2bAR^{−/−} for this receptor.

Next, the effect of adenosine receptor agonist/antagonist on MHC II expression was examined. NECA down-regulated MHC II expression by more than 60% in the WT cells (Fig. 3B, left panel), likely through repressing CIITA levels. The specific A2bAR antagonist MRS-1754 restored the up-regulation of MHC II expression, overcoming the NECA effect, confirming that NECA is signaling through the A2bAR, not A2aAR. The A2bAR^{−/−} SMCs expressed more IFN-γ-induced mRNA, and NECA did not alter the MHC II mRNA levels in the A2bAR^{−/−} SMCs (Fig. 3B) similar to the changes in CIITA (Fig. 3, A and B).

To examine whether or not NECA could directly alter MHC II transcription during IFN-γ response, transient transfections were performed with a luciferase construct driven by the MHC II promoter. IFN-γ activated the promoter activity by more than 2-fold, which was inhibited after the addition of NECA (Fig. 3D). MRS-1754 by itself did not interfere with the IFN-γ stimulation of MHC II transcription, but it did block the effects of NECA activation of MHC II expression. This suggests that the A2bAR signaling might directly control MHC II transcription. Again, NECA and its antagonist had no effect on promoter activity in the A2bAR^{−/−} SMCs but had an effect on the wild type SMCs. This suggests that A2bAR activation dampens CIITA expression and, thereby, lowers MHC II activation.

Adenylyl Cyclase Activity Is Both Necessary and Sufficient to Counteract the IFN-γ Response—When adenosine (or NECA) binds and activates A2-type adenosine receptors, the level of the second messenger cAMP is greatly elevated in vascular SMCs as we reported earlier (8, 28). Here, several approaches were taken to evaluate the role of cAMP on CIITA expression during the IFN-γ response.

First, murine SMCs were treated with or without an activator of AC, forskolin. Indeed, in WT and A2bAR^{−/−} SMCs, forskolin significantly down-regulated the IFN-γ-induced expression of CIITA protein (Fig. 4A) and message (Fig. 4B). Moreover, diminished expression of CIITA in the presence of forskolin was accompanied by reduction of MHC II expression (Fig. 4C) as well as decreased MHC II transcription (Fig. 4D). This indicates that the activation of AC is sufficient to counter CIITA induction by IFN-γ and therefore abrogate MHC II transcriptional activation even in the A2bAR null cells. Activation of AC mimicked activation of A2bAR in regards to effects on MHC II, suggesting that A2bAR may signal through AC activation.

To further determine whether A2bAR signals through AC, murine SMCs were treated with an AC antagonist SQ22536 in the presence or absence of NECA. This antagonist blocked the NECA effect and brought CIITA protein to the control level in WT and A2bAR^{−/−} cells (Fig. 5A, compare lane 5 with lane 2 and lane 10 with lane 7). SQ22536 by itself did not alter CIITA protein levels significantly in WT or A2bAR^{−/−} cells (Fig. 5A,
compare lane 4 with lane 2 and lane 9 with lane 7), probably because A2bAR signaling was not active without agonist in these cells. CIITA mRNA levels exhibited similar patterns under these treatments (Fig. 5B). SQ22536 also blocked the NECA down-regulation of MHC II mRNA (Fig. 5C) as well as MHC II transcription (Fig. 5D) during the IFN-γ response.

The IFN-γ-induced collagen repression was lowered by forskolin treatment in WT and A2bAR−/− cells (supplemental Fig. S1, A and B). In addition, NECA alleviated the IFN-γ-induced repression of collagen, whereas SQ22536 restored the IFN-γ-induced effect in the WT but not in the A2bAR−/− cells (supplemental Fig. S1, C and D). IFN-γ consistently down-regulated type I collagen levels more efficiently in A2bAR−/− cells than in the WT cells.

Combined together, these data strongly implicate AC activity as part of the A2bAR signaling in SMCs. AC is both necessary and sufficient to down-regulate IFN-γ induced MHC II activation and collagen repression by restoring CIITA expression.

The Phosphodiesterase Inhibitor IBMX Decreases CIITA Activation by IFN-γ—The cellular level of cAMP in response to the adenosine receptor signaling is controlled not only by its synthesizing enzymes, the adenylyl cyclases, but also by its degrading enzymes, the phosphodiesterases (PDEs). Therefore, the role of PDE in the expression of CIITA during the IFN-γ response was examined. As shown in Fig. 6A, a universal PDE
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Inhibition of MHC II transcription (Fig. 6), whereas vascular SMCs play important roles in hypertension, restenosis, and atherosclerosis (10). The pathogenesis of atherosclerosis is highlighted by chronic inflammation, when cytokines (e.g. IFN-γ) are secreted by infiltrating immune cells to activate MHC II expression and to alter synthesis and degradation of extracellular matrix proteins (e.g. collagen). SMCs are involved in both of these processes, which are mediated by the transcription modulator CIITA as we reported previously (15). This process may contribute to the instability of atherosclerotic plaques. On the other hand, our earlier findings indicate that the adenosine receptor A2b is expressed in the vasculature, and there is augmented inflammation in A2bAR−/− mouse, suggesting that A2bAR may play a key role in atherogenesis (17). Here we propose that acute inflammation in A2bAR−/− mouse can be explained by an elevated response to IFN-γ as evidenced by increased expression of CIITA protein by IFN-γ slightly (Fig. 6A, compare lane 4 with lane 2 and lane 9 with lane 7). Moreover, in presence of NECA, IBMX treatment reduced the IFN-γ mediated CIITA protein expression (Fig. 6A, compare lane 5 with lane 2 and lane 10 to lane 7). CIITA mRNA levels also decreased in the presence of IBMX (Fig. 6B).

In accordance with previous results, the reduction of CIITA protein levels by the addition of IBMX also led to the down-regulation of MHC II message levels (Fig. 6C) as well as the inhibition of MHC II transcription (Fig. 6D). These data suggest that increased cAMP levels are important in decreasing IFN-γ-induced expression of CIITA, which up-regulates MHC II.

Cyclic AMP (8-Bromo-cAMP) Attenuates the Expression of CIITA—In control mouse SMCs treated with NECA or forskolin, cAMP is elevated by about 5- and 15-fold, respectively (17). In SMCs derived from A2bAR null mice, NECA elevates cAMP by 1.2 (because of marginal activity of the A2aAR), whereas the effect of forskolin on cAMP is similar to control cells (17). Our results strongly indicate that an increase in cellular cAMP levels (either through activating A2bAR by NECA, activating adenylyl cyclase by forskolin, or inhibiting phosphodiesterase by IBMX) leads to down-regulation of IFN-γ-induced expression of CIITA. Therefore, the effect of cAMP on CIITA expression was examined by direct addition of the cAMP analog 8-bromo-cAMP to the WT and A2bAR−/− SMCs in conjunction with IFN-γ. IFN-γ induced more CIITA protein in the A2bAR−/− cells than in the WT, which was attenuated in the presence of 1 μM 8-bromo-cAMP (data not shown). Both CIITA and MHC II message levels were reduced by the addition of cAMP (Fig. 7A and B). HLA-DRα promoter-driven luciferase activity was decreased in the presence of cAMP in WT and A2bAR−/− cells (Fig. 7C), suggesting that the regulation was probably at least in part at the transcriptional level. Furthermore, cAMP treatment ameliorated the down-regulation of type I collagen synthesis by IFN-γ (data not shown).

Overall, these data implicate adenosine receptor signaling through cAMP in down-regulation of IFN-γ-induced CIITA expression. In our model (Fig. 8), multiple agonists and antagonists used in the investigation point to a central role for a cAMP that dampens IFN-γ-induced CIITA expression. Activation of A2bAR leads to activation of AC and increased cAMP levels that block the IFN-γ-induced expression of CIITA. This transcription co-activator is essential for regulation of MHC II, a central protein in antigen presentation and innate immunity. In addition, CIITA may play a role in remodeling extracellular matrix by decreasing collagen expression during inflammation. Therefore, the role of A2bAR in SMCs may be to reduce inflammation within atherosclerosis when IFN-γ is present.

DISCUSSION

SMCs from different tissues participate in a wide variety of pathophysiological events. For instance, SMCs lining the airway are implicated in asthma (29) and pulmonary fibrosis (30), whereas vascular SMCs play important roles in hypertension, restenosis, and atherosclerosis (10). The pathogenesis of atherosclerosis is highlighted by chronic inflammation, when cytokines (e.g. IFN-γ) are secreted by infiltrating immune cells to activate MHC II expression and to alter synthesis and degradation of extracellular matrix proteins (e.g. collagen). SMCs are involved in both of these processes, which are mediated by the transcription modulator CIITA as we reported previously (15). This process may contribute to the instability of atherosclerotic plaques. On the other hand, our earlier findings indicate that the adenosine receptor A2b is expressed in the vasculature, and there is augmented inflammation in A2bAR−/− mouse, suggesting that A2bAR may play a key role in atherogenesis (17). Here we propose that acute inflammation in A2bAR−/− mouse can be explained by an elevated response to IFN-γ as evidenced.
by increased CIITA expression that activates MHC II transcription and reduces type I collagen transcription in A2bAR−/− SMCs compared with WT SMCs (Fig. 2 and data not shown). Moreover, we attribute the up-regulation of IFN-γ response to an increased CIITA expression (Fig. 1), a key mediator of transcriptional control of both MHC II and type I collagen genes by IFN-γ. CIITA is specifically responsible for the IFN-γ effect because RFX5 expression, which is also up-regulated by IFN-γ (15, 31) is not affected in A2bAR−/− SMCs (data not shown). The specificity of A2bAR in opposing the IFN-γ response is further illustrated by the observation that the A2 agonist NECA attenuates IFN-γ mediated MHC II activation and type I collagen repression, which is blocked by the A2bAR-specific antagonist MRS-1754 in WT but not A2bAR−/− SMCs, whereas an A2aAR-specific agonist CGS-21680 fails to affect the IFN-γ response (Fig. 3 and data not shown). Therefore, the adenosine signaling through A2bAR may antagonize the IFN-γ response in up-regulating CIITA expression to modulate the inflammatory response and extracellular matrix reshufling.

Adenosine receptors are G-protein-coupled receptors capable of launching a wide range of transcriptional events depending on their associated downstream messengers. In the case of adenosine receptor A2b, the low affinity A2 receptor investigated in this manuscript, there is primarily an interaction with Gi that stimulates AC leading to cAMP accumulation, which acts as a second messenger (8, 17, 32). The cross-talk between AC and IFN-γ-induced transcriptional events remains controversial and seems to depend on specific cell types and specific genes. For example, it has been demonstrated that whereas IFN-γ induced expression of intercellular adhesion molecule (ICAM-1) is decreased by AC agonist in human glioma cells (33), it is not altered in human vascular SMCs (34). The data presented here clearly demonstrate that stimulation of AC activity by forskolin in SMCs suppresses IFN-γ induced MHC II expression (Fig. 4), whereas inhibition of AC activity by SQ22536 overcomes the NECA effect and restores the IFN-γ response (Fig. 5), suggesting that the AC activity is both necessary and sufficient to suppress the IFN-γ response. In some cells, A2bAR can also interact with Gi that stimulates the phospholipase C/protein kinase C pathway (35). Although we cannot rule out the possibility that this alternative pathway is involved in the suppression of the IFN-γ response by A2bAR in our cells, most findings in the literature speak against this scenario. For example, there is evidence suggesting that activation of protein kinase C results in CIITA up-regulation and MHC II overproduction in macrophages, HeLa cells and central nervous system-infiltrated encephalitogenic T cells (36–38). Protein kinase C has even been shown to stimulate MHC II synthesis by directly phosphorylating CIITA to enhance its transcriptional activity (39).

The A2bAR/cAMP pathway is further delineated by the investigation of PDE involvement. A universal PDE inhibitor IBMX inhibits IFN-γ-mediated MHC II activation and type I collagen repression both by itself and in conjunction with NECA (Fig. 6), indicating that A2bAR acts through elevating the cAMP concentration inside the cells. Finally, cAMP alone is sufficient to antagonize IFN-γ-induced transcriptional events (Fig. 7). There has been documentation in the past that cAMP directly counteracts IFN-γ-mediated transcriptional events. For instance, it suppresses IFN-γ-induced FcγRIII expression in peripheral eosinophils, although the in vivo pathway responsible is unclear (40). Our results strongly suggest that this may happen through the A2bAR pathway. The downstream signaling may result in activation of protein kinase A, which can phosphorylate CIITA directly to inactivate CIITA without altering its expression (41), or influence MHC II transcription through phosphorylation of CREB (42). Alternatively, IFN-γ stimulates an inducible cAMP early repressor that could form heterodimers with CREB (43), sequestering CREB from the MHC II promoter.

CIITA is constitutively expressed in a few immune cell lines such as B cells, macrophages, and dendritic cells, but it can be induced by IFN-γ in other cell types such as fibroblasts (2, 22) and SMCs (15, 16). Transcription of CIITA can be initiated from one or more of its four promoters, giving rise to four different isoforms. Previously we have shown that isoform I and, to a larger extent, isoform IV are the two major IFN-γ inducible CIITA isoforms in murine vascular SMCs (16), whereas in human aortic SMCs isoform III and isoform IV isoforms are activated by IFN-γ (15). CIITA isoform IV message, but not isoform I, is clearly up-regulated in A2bAR−/− SMCs (Fig. 1B). Our recent publication demonstrates that isoform IV CIITA interacts with peroxisome proliferator-activated receptor γ, which also regulates its expression, in mediating MHC II activation and type I collagen repression (26). This suggests that there may be a broader, as of yet unknown functional role associated with isoform IV CIITA. However, the mechanism underlying the suppression of CIITA expression by the A2bAR signaling remains to be elucidated. It is likely that A2bAR functions through inhibiting Stat-1 activity (44), which is essential for CIITA transcription, by promoting cellular cAMP levels.

The immune protective role of A2aAR has been intensively studied. In several animal models, A2aAR exerts potent anti-inflammatory effect in protecting against airway allergy, inflammation related skin ulcer, hepatic reperfusion injury, and atherosclerotic lesions (45–48). It is not until recently, however, that the role of A2bAR has been assessed in immune protection. Our data presented in this manuscript demonstrate for the first time that the adenosine signaling pathway, specifically through the A2bAR, suppresses IFN-γ-induced MHC II transcription activation and type I collagen transcription repression in mouse vascular SMCs by down-regulating CIITA expression in vascular smooth cells, hence providing a possible link between the adenosine signaling and IFN-γ signaling. Changes in collagen levels are a hallmark of vascular lesion during atherosclerosis and restenosis. Here, we describe relatively small changes in collagen expression in response to A2bAR activation in a primary SMC culture system. However, often, several small changes over a life span lead to larger effects on accumulation of collagen in vivo. In this situation, we hypothesize that if inflammation occurs with secretion of active IFN-γ, then nearby cells, in this case SMCs, produce less collagen and more MHC II to become nonprofessional antigen producing cells. This process may be active throughout periods of atherosclerosis or restenosis development, with small, but cumulative changes in collagen level over time. Because of the potential importance of the
above described signaling pathways in atherogenesis, our findings provide insights into uncovering novel therapeutic solutions for this disease. Hence, future studies will explore changes in CIITA, MHC, and collagen during vascular injury in A2bAR-deficient mice.

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