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Priming of Signal Transducer and Activator of Transcription Proteins for Cytokine-Triggered Polyubiquitylation and Degradation by the A_{2A} Adenosine Receptor

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ABSTRACT

Here we demonstrate that overexpression of the human A_{2A} adenosine receptor (A_{2A}AR) in vascular endothelial cells confers an ability of interferon-α and a soluble IL-6 receptor/IL-6 (sIL-6Rα/IL-6) trans-signaling complex to trigger the down-regulation of signal transducer and activator of transcription (STAT) proteins. It is noteworthy that STAT down-regulation could be reversed by coincubation with A_{2A}AR-selective inverse agonist 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-*a*][1,3,5]triazin-5-ylamino]ethyl)phenol (ZM241385) but not adenosine deaminase, suggesting that constitutive activation of the receptor was responsible for the effect. Moreover, STAT down-regulation was selectively abolished by proteasome inhibitor *N*-benzoyloxycarbonyl (*Z*)-Leu-Leu-leucinal (MG132), whereas lysosome inhibitor chloroquine was without effect. Down-regulation required Janus kinase (JAK) activity and a Tyr705→Phe-mutated STAT3 was resistant to the phenomenon, suggesting that JAK-mediated phosphorylation of this residue is required. Consistent with this hypothesis, treatment of A_{2A}AR-overexpressing cells with sIL-6Rα/IL-6 triggered the accumulation of polyubiquitylated wild-type but not Tyr705→Phe-mutated STAT3. Support for a functional role of this process was provided by the observation that A_{2A}AR overexpression attenuated the JAK/STAT-dependent up-regulation of vascular endothelial growth factor receptor-2 by sIL-6Rα/IL-6. Consistent with a role for endogenous A_{2A}ARs in regulating STAT protein levels, prolonged exposure of endogenous A_{2A}ARs in endothelial cells with ZM241385 in vitro triggered the up-regulation of STAT3, whereas deletion of the A_{2A}AR in vivo potentiated STAT1 expression and phosphorylation. Together, these experiments support a model whereby the A_{2A}AR can prime JAK-phosphorylated STATs for polyubiquitylation and proteasomal degradation and represents a new mechanism by which an anti-inflammatory seven-transmembrane receptor can negatively regulate JAK/STAT signaling.

Vascular endothelial cells (ECs) comprise a nonthrombotic anticoagulatory surface that resists the onset of inflammation. The shift to a predominantly proinflammatory or "dysfunctional" state in response to injury or infection is triggered by a variety of stimuli, including pathogen-derived molecules, bioactive lipids, and cytokines (Gimbrone, 1995; von der Thüsen et al., 2003). Endothelial dysfunction is also strongly linked to the development of obesity and type II diabetes and underlies the increased susceptibility to cardiovascular disease displayed by individuals with these conditions (Ritchie et al., 2004; Fantuzzi and Mazzone, 2007). The development of the proinflammatory phenotype is now be-

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ABBREVIATIONS: EC, endothelial cell; HUVEC, human umbilical vein endothelial cell; IL, interleukin; JAK, Janus kinase; STAT, signal transducer and activator of transcription; IFN, interferon; AR, adenosine receptor; 7TM, seven transmembrane; AV, adenovirus; MG132, *N*-benzoyloxycarbonyl (*Z*)-Leu-Leu-leucinal; CGS21680, 2-(4-(2-carboxyethyl)phenylethylamino)-5′-*N*-ethylcarboxamidoadenosine; ZM241385, 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-*a*][1,3,5]triazin-5-ylamino]ethyl)phenol; VEGFR2, vascular endothelial growth factor receptor 2; sIL-6Rα, soluble interleukin-6 receptor-α; SLIM, signal transducer and activator of transcription-interacting protein with a LIM domain; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ADA, adenosine deaminase; GFP, green fluorescent protein; TBST, Tris-buffered saline/Tween 20; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; KO, knockout; WT, wild type; NF-κB, nuclear factor κB; C/EBP, CCAAT/enhancer binding protein; LPS, lipopolysaccharide; ERK1,2, extracellular signal-regulated kinases 1 and 2.

lieved to be driven largely by the concerted action of so-called "adipocytokines" released from adipose tissue. Many studies have found that levels of several of these adipocytokines are continuously elevated in obese and diabetic subjects (Ritchie et al., 2004; Tilg and Moschen, 2006; Fantuzzi and Mazzone, 2007) and that those which have largely proinflammatory/atherogenic effects, such as IL-6 and leptin, can accumulate within atherosclerotic plaques and at sites of vascular injury (Schieffer et al., 2000; Schäfer et al., 2004).

IL-6 exerts its effects on target cells by binding to either cell membrane-localized or soluble receptor α-chains. The receptor-cytokine complex then activates a dimeric transmembrane signal transducing component termed "gp130." This allows constitutively bound tyrosine kinases of the Janus kinase (JAK) family to transphosphorylate and activate each other before phosphorylating multiple tyrosine residues on gp130 to enable docking of specific members of the "signal transducer and activator of transcription" (STAT) family and the protein tyrosine phosphatase SHP-2 via their SH2 domains (Heinrich et al., 2003). Recruitment of STAT1 and STAT3 causes their phosphorylation on Tyr701 and Tyr705, respectively, by gp130-associated JAKs, resulting in their homo-/heterodimerization and translocation to the nucleus, in which they can initiate cytokine-inducible target gene transcription (Levy and Darnell, 2002).

It is becoming increasingly apparent that proinflammatory signaling pathways are also subject to regulation by noncytokine stimuli, thus providing a means by which otherwise distinct signaling modules can negatively control cytokine responsiveness. The seven transmembrane (7TM) A_{2A} adenosine receptor (A_{2A}AR) has emerged as an important suppressor of vascular inflammation in vivo (McPherson et al., 2001; Sitkovsky et al., 2004), largely due to receptor expression in neutrophils, monocytes, macrophages, and other inflammatory cell types. For example, A_{2A}AR-selective agonists can inhibit activation of the neutrophil respiratory burst (Sullivan et al., 2001) and elastase release (Anderson et al., 2000) in response to chemotactic peptide *N*-formylmethionyl-leucylphenylalanine. A_{2A}AR activation can also mediate some of the suppressive effects of adenosine on proinflammatory aspects of macrophage function, such as IL-12 production (Haskó et al., 2000) and also enhance the CCAAT/enhancer binding protein (C/EBP)-dependent induction of anti-inflammatory cytokine IL-10 (Csóka et al., 2007). Functional A_{2A}ARs expressed in vascular ECs also have important anti-inflammatory roles, including inhibition of adhesion molecule expression and monocyte adhesion (Sands et al., 2004; Zernecke et al., 2006). One aspect of the A_{2A}AR's effects is an ability to inhibit pro-inflammatory NF-κB activation by multiple cell type-specific mechanisms (Majumdar and Aggarwal, 2003; Sands et al., 2004). However, given its potent anti-inflammatory effects in vivo, it is likely that the receptor inhibits additional proinflammatory signaling mechanisms to limit inflammation and associated tissue damage.

In this study, we examined the effect of A_{2A}AR overexpression on activation of the JAK/STAT pathway. We demonstrate that the A_{2A}AR suppresses STAT phosphorylation in response to multiple cytokines by priming JAK-phosphorylated STATs for ubiquitylation and proteasomal degradation. This reveals a previously unappreciated mechanism by which it may be possible to suppress proinflammatory signaling in the vascular endothelium.

Materials and Methods

Materials. The generation of plaque-purified adenoviruses (AVs) encoding myc epitope-tagged human A_{2A}AR and GFP have been described previously by us (Sands et al., 2004). AVs encoding Flag epitope-tagged WT and Tyr705→Phe mutated murine STAT3 were generously donated by Brian Foxwell (Kennedy Institute of Rheumatology, London, UK) and Keiko Yamauchi-Takihara (Osaka University Health Care Centre, Osaka, Japan) (Kunisada et al., 1998; Williams et al., 2004). A_{2A}AR knockout (KO) mice and their WT littermates (both on a CD1 background) were generated in a pathogen-free facility using founder heterozygotes. Offspring were genotyped by tail-tipping and polymerase chain reaction amplification of genomic DNA (Ledent et al., 1997). Anti-Flag M5 antibody and M2 antibody-conjugated Sepharose beads were from Sigma-Aldrich (St. Louis, MO). Anti-ubiquitin antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Sources of other materials have been described elsewhere (Sands et al., 2004, 2006).

Cell Culture and AV Infection. HUVECs were propagated at 37°C in a humidified atmosphere containing 5% (v/v) CO_2 in ECM-2 medium supplemented with 2% (w/v) fetal bovine serum, hydrocortisone, ascorbate, and recombinant growth factors as recommended by the supplier (Lonza Nottingham Ltd., Nottingham, UK). Human embryonic kidney 293 cells for AV propagation were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum, L-glutamine, penicillin, and streptomycin. For infection with recombinant AVs, HUVECs were washed in regular growth medium and then incubated overnight with the same medium supplemented with recombinant AV at the multiplicities of infection indicated under *Results*. The next day, the virus-containing medium was aspirated and replaced with normal medium. Cells were used for analysis 24 h later.

Treatment of Mice with Endotoxin. Endotoxic shock in age-matched WT and A_{2A}AR KO mice was induced by intravenous injection of 0.4 mg/kg 0111:B4 *Escherichia coli* lipopolysaccharide (LPS). Mice injected with PBS vehicle were used as injection controls. After 4 h, animals were sacrificed for multiplex analysis of serum cytokine levels and isolation of the aorta for preparation of samples for SDS-polyacrylamide gel electrophoresis (PAGE).

Immunoblotting. Aortae isolated from vehicle or LPS-treated WT and A_{2A}AR KO mice after sacrifice were frozen in liquid nitrogen and pulverized using a pestle and mortar. Pulverized extracts were then lysed directly in SDS-PAGE sample buffer before analysis. Confluent HUVECs in six-well plates were treated as described in the figures before washing in ice-cold PBS and solubilization by scraping into 50 μl/well detergent lysis buffer [50 mM sodium HEPES, pH 7.5, 150 mM sodium chloride, 5 mM EDTA, 10 mM sodium fluoride, 10 mM sodium phosphate, 1% (v/v) Triton X-100, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 0.1 mM phenylmethylsulfonyl fluoride, 10 μg/ml soybean trypsin inhibitor, 10 μg/ml benzamidine and EDTA-free complete protease inhibitor mix]. After brief vortexing, insoluble material was removed by microcentrifugation, and the supernatant was assayed for protein content using a bicinchoninic acid assay. Samples equalized for protein content (typically 10–20 μg/sample) were fractionated by SDS-PAGE. After transfer to nitrocellulose, membranes were blocked for 1 h at room temperature in blocking solution [5% (w/v) skimmed milk in Tris-buffered saline containing 0.1% (v/v) Tween 20 (TBST)]. Membranes were then incubated either overnight at 4°C or for 1 h at room temperature with primary antibody diluted to a final concentration of 1 μg/ml in 5% (w/v) IgG-free bovine serum albumin (BSA) in TBST. After three washes in blocking solution, membranes were incubated for 1 h at room temperature with appropriate horseradish peroxidase-conjugated secondary antibody at a 1:1000 dilution in BSA/TBST. After further washes with TBST and Tris-buffered sa-

line, immunoreactive proteins were visualized by enhanced chemiluminescence. Quantification was by densitometric scanning of non-saturating exposed films using Phoretix version 2.0 imaging software (TotalLab Ltd, Garth Heads, Newcastle upon Tyne, UK).

Immunoprecipitation. Confluent HUVECs in six-well dishes were preincubated with 6 μM *N*-benzoyloxycarbonyl (*Z*)-Leu-Leu-leucinal (MG132) before treatment with or without sIL-6Rα/IL-6 as described in the figure legends before termination of the incubation by placing dishes on ice and washing cell monolayers three times with ice-cold PBS. Cells were solubilized by scraping into 0.1 ml of denaturing lysis buffer [50 mM sodium-HEPES, pH 7.5, 100 mM sodium chloride, 1 mM *N*-ethylmaleimide, 2% (w/v) SDS, 0.1 mM phenylmethylsulfonyl fluoride, 10 μg/ml soybean trypsin inhibitor, 10 μg/ml benzamidine, and EDTA-free complete protease inhibitor mix] and heating to 95°C for 5 min followed by probe sonication. After the addition of 0.9 ml of lysis buffer containing Triton X-100 and sodium deoxycholate to give final concentrations of 1% (v/v) and 0.5% (w/v), respectively, insoluble material was removed by centrifugation, and soluble fractions were equalized for protein content and volume before incubation for 1 h at 4°C with rotation with a 25-μl packed volume of protein A-Sepharose beads in the presence of 0.2% (w/v) IgG-free BSA. Anti-STAT3 antibody (2 μg/sample) was then added, and the incubation continued for 1 h further. Immune complexes were isolated by brief centrifugation and washed three times with detergent lysis buffer before elution of precipitated proteins by the addition of electrophoresis sample buffer. Samples were then fractionated by SDS-PAGE and transferred to nitrocellulose for immunoblotting. Recombinant Flag-tagged STAT3 was immunoprecipitated by the addition of 20-μl packed volume of anti-Flag M2-Sepharose beads and incubation with rotation for 1 h at 4°C before analysis by SDS-PAGE and immunoblotting as described above.

Statistical Analysis. Data are presented in the text as means ± S.D. for the number of experiments indicated, whereas representative experiments are shown in the figures. Concentration-response data were fitted to a sigmoid curve using Prism 4 software (GraphPad Software Inc., San Diego, CA). Statistical significance was assessed by either paired Student *t* tests or analysis of variance using Instat 3 software (GraphPad).

Results

Effect of A_{2A}AR Overexpression and Activation on Cytokine Activation of the JAK/STAT Pathway. A recombinant AV was used to drive overexpression of myc epitope-tagged human A_{2A}ARs in HUVECs. Consistent with our previous study (Sands et al., 2004), 9E10 anti-myc immunoblotting revealed that recombinant A_{2A}ARs migrated as a doublet of 42 and 49 kDa (Fig. 1A). Analysis of receptor function in control GFP-expressing cells demonstrated that treatment with the A_{2A}AR-selective agonist 2-(4-(2-carboxyethyl)phenylethylamino)-5′-*N*-ethylcarboxamidoadenosine (CGS21680) (Jarvis et al., 1989) produced a transient increase in ERK1,2 phosphorylation, consistent with the presence of endogenous functional A_{2A}ARs (Sexl et al., 1997; Sands et al., 2004). However, overexpression of recombinant A_{2A}ARs potentiated ERK1,2 phosphorylation at each time point (Fig. 1A), demonstrating that the recombinant receptor was functional with respect to its ability to couple to downstream signaling pathways in HUVECs.

We have described previously how potentiating A_{2A}AR overexpression over endogenous levels is sufficient to suppress NF-κB activation, even in the absence of agonist (Sands et al., 2004). Thus, we initially tested the effect of A_{2A}AR overexpression on cytokine activation of the JAK/STAT pathway. In control cells, treatment with an sIL-6Rα/IL-6 trans-signaling complex produced a transient increase in the phosphorylation of STAT3 on Tyr705, which plateaued at 15 and 30 min. In comparison, overexpression of the A_{2A}AR significantly reduced STAT phosphorylation at each time point (Fig. 1B). It is noteworthy that whereas total levels of STAT3 were unaltered by sIL-6Rα/IL-6 treatment of GFP-expressing cells, a marked decrease in the amount of total STAT3 in A_{2A}AR-expressing HUVECs that reached statistical signifi-

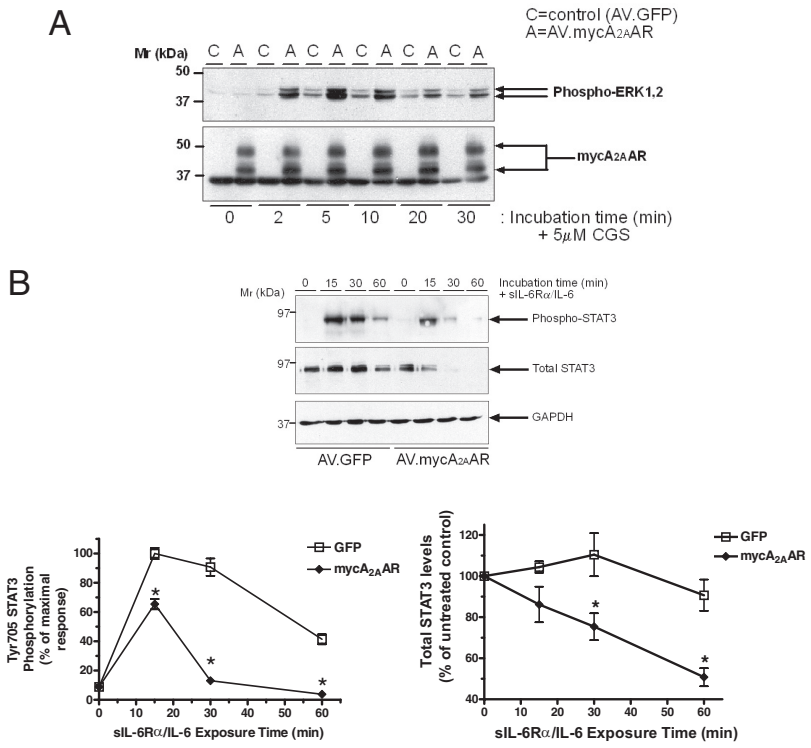

Fig. 1. Effect of A_{2A}AR overexpression on STAT3 phosphorylation and expression in response to sIL-6Rα/IL-6. A, HUVECs were infected with the indicated AVs at a multiplicity of infection of 25 as described under *Materials and Methods* before stimulation with 5 μM CGS21680 (CGS) for the indicated times and preparation of soluble cell extracts for SDS-PAGE and immunoblotting with anti-Thr202/Tyr204 phospho-ERK1,2 and anti-myc 9E10 antibodies to identify recombinant A_{2A}AR. B, HUVECs were infected with recombinant AVs as in A before treatment with or without 25 ng/ml sIL-6Rα/5 ng/ml IL-6 (sIL-6Rα/IL-6) for the indicated times. Soluble cell extracts equalized for protein content were then fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of total and Tyr705 phospho-STAT3 levels from three experiments is presented (*, $p < 0.05$ versus the response observed in AV.GFP-infected cells).

cance 30 min after cytokine stimulation (Fig. 1B). A similar inhibitory effect of the A_{2A}AR on total STAT1 expression and its cytokine-stimulated phosphorylation on Tyr701 was also observed (data not shown). It is noteworthy that cell viability assays revealed that the decrease in STAT levels did not simply reflect reduced HUVEC viability after A_{2A}AR overexpression and cytokine treatment (data not shown).

The effect of on STAT down-regulation was then characterized in more detail. First, we examined the pharmacology of the response using the A_{2A}AR-selective agonist CGS21680 and selective inverse agonist 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-*a*][1,3,5]triazin-5-ylamino]ethyl)phenol (ZM241385) (Poucher et al., 1995; Klinger et al., 2002) on STAT protein levels. Coincubation of sIL-6Rα/IL-6 with either drug did not produce any significant changes in STAT3 expression in control GFP-expressing cells. In contrast, sIL-6Rα/IL-6 treatment of A_{2A}AR-overexpressing cells promoted reductions in both Tyr705-phosphorylated and total STAT3, which were significantly reversed by coincubation with inverse agonist ZM241385. Coincubation with the agonist CGS21680 did not further potentiate the effect of A_{2A}AR overexpression, suggesting that the receptor displays a level of constitutive activity that is sufficient to observe maximal cytokine-stimulated down-regulation of STATs (Fig. 2A). Again, the effect was not restricted to STAT3 because an identical pattern was also observed for STAT1 (data not shown). Additional experiments revealed that ZM241385-mediated reversal of the effect occurred in a concentration-dependent manner (EC_{50} = 4.9 ± 3.0 nM; Fig. 2B), consistent with the reported low nanomolar affinity of the compound at the human A_{2A}AR (Gao et al., 2000). In contrast, pretreatment with 3 U/ml adenosine deaminase (ADA) failed to alter the effect of A_{2A}AR overexpression (58 ± 15% decrease in total STAT3 in the absence of ADA versus 47 ± 16% in the presence of ADA, $p > 0.05$, $n = 3$; Fig. 2C), confirming that the ability of receptor overexpression to prime STATs for down-regulation was due largely to the degree of constitutive activity of the receptor rather than accumulation of the physiological agonist adenosine during the incubation.

To determine whether this effect was restricted to sIL-6Rα/IL-6, we also tested the effect of A_{2A}AR overexpression on responses to interferon α (IFNα), which also activates STAT1 and STAT3 (Brierley and Fish, 2002). Similar to sIL-6Rα/IL-6, extended time courses revealed that both STAT1 and STAT3 protein expression were reduced in parallel to almost undetectable levels after 3 h in response to either cytokine in A_{2A}AR-expressing cells (Fig. 3). In contrast, cytokine exposure of GFP-expressing HUVECs under the same conditions produced no significant changes (Fig. 3). Thus, a requirement for A_{2A}AR overexpression to trigger down-regulation of STATs is shared by multiple cytokines.

Role of JAK-Mediated Phosphorylation in Targeting STATs for Down-Regulation. The observed cytokine dependence raised the possibility that JAK-mediated STAT phosphorylation was a critical step responsible for initiating down-regulation. Two approaches were used to test this hypothesis in more detail. First, we assessed the effect of pharmacological inhibition of JAK activity on STAT down-regulation in A_{2A}AR-overexpressing cells. This demonstrated that a concentration of JAK inhibitor sufficient to abolish sIL-6Rα/IL-6-mediated tyrosine phosphorylation of both JAK1 and JAK2, as well as the subsequent Tyr705 phosphorylation of STAT3, completely blocked STAT3 down-regulation (Fig. 4A and B). Second, HUVECs were coinfected with AVs encoding the A_{2A}AR and either Flag epitope-tagged WT or Tyr705→Phe-mutated STAT3, because mutation of Tyr705 renders STAT3 resistant to phosphorylation by JAKs (Kaptein et al., 1996). Under conditions in which WT STAT3 underwent down-regulation in response to sIL-6Rα/IL-6 similar to the effect observed for endogenous STAT3, levels of Tyr705→Phe STAT3 were not altered (Fig. 4C). Thus, JAK-mediated tyrosine phosphorylation of STATs seems to be essential for promoting their cytokine-mediated down-regulation in A_{2A}AR-overexpressing cells.

Effect of Proteasome and Lysosome Inhibition on STAT Down-Regulation. Regulated degradation is a frequently used mechanism by which cellular levels of transcription factors, such as p53, are controlled (Watson and Irwin, 2006). To determine whether STAT degradation was the mechanism underlying the effect of A_{2A}AR overexpression, we used the proteasome inhibitor MG132. Preincubation with MG132 was found to be sufficient to abolish the effect of the A_{2A}AR on priming STAT3 for down-regulation in response to sIL-6Rα/IL-6 (Fig. 5A). Moreover, MG132 abolished the ability of A_{2A}AR overexpression to inhibit sIL-6Rα/IL-6-mediated STAT3 phosphorylation, suggesting that STAT degradation is the mechanism responsible for this effect (Fig. 5B).

To examine a role for lysosomal degradation, we tested the effect of preincubation with chloroquine, an inhibitor of lysosomal acidification (van Weert et al., 1995). Preliminary experiments determined that a concentration of 100 μM was effective in blocking vascular endothelial growth factor-induced down-regulation of ubiquitylated vascular endothelial growth factor receptor VEGFR2 (data not shown), a process which has been shown to be mediated via lysosomal degradation (Ewan et al., 2006). However, chloroquine pretreatment failed to block STAT3 down-regulation in A_{2A}AR-overexpressing cells (Fig. 5C). Therefore, STAT down-regulation was sensitive to inhibition of the proteasome but resistant to inhibition of lysosome function, suggesting that the A_{2A}AR specifically targets STATs for proteasomal degradation.

Cytokine-Stimulated Polyubiquitylation of STATs. Proteins targeted for degradation are typically tagged on one or more lysine residues with Lys48-conjugated polyubiquitin chains, which are recognized as a degradation signal by the 26S proteasome (Liu et al., 2005; Nalepa et al., 2006). To assess directly whether STATs were ubiquitylated in A_{2A}AR-overexpressing HUVECs, cells were treated with sIL-6Rα/IL-6 and MG132, which allows for the accumulation of ubiquitylated proteins by inhibiting proteasome activity. Endogenous STAT3 was immunoprecipitated after denaturing cell lysis to remove any noncovalently associated STAT-binding proteins and inactivate deubiquitinase enzymes. Immunoblotting of STAT3 immunoprecipitates with anti-ubiquitin antibody revealed that sIL-6Rα/IL-6 treatment resulted in the accumulation of a smear of high molecular weight ubiquitylated species only in A_{2A}AR-overexpressing cells (Fig. 6A). Thus, expression of the A_{2A}AR is required to observe cytokine-stimulated ubiquitylation of STAT3 in response to sIL-6Rα/IL-6.

Because down-regulation of STAT3 requires its JAK-mediated phosphorylation on Tyr705 (Fig. 4), we then examined the relationship between STAT3 phosphorylation and ubiquitylation in A_{2A}AR-overexpressing cells. To

achieve this, Flag-tagged WT and Tyr705→Phe-mutated STAT3 were coexpressed in HUVECs with the A_{2A}AR and immunoprecipitated with anti-Flag antibody after denaturing cell lysis after treatment with sIL-6Rα/IL-6 and MG132. This demonstrated that under conditions in which recombinant WT STAT3 is polyubiquitylated similarly to the endogenous protein in response to sIL-6Rα/IL-6 (Fig. 6A), no ubiquitylation of the Tyr705→Phe mutant could be detected despite the presence of both WT and Tyr705→Phe STAT3 proteins in immunoprecipitates (Fig. 6B). Together with the data presented in Fig. 3, these observations are consistent with a model in which A_{2A}AR overexpression specifically primes JAK-phosphorylated STATs for polyubiquitylation, and this triggers their subsequent degradation by the proteasome.

Effect of A_{2A}AR Expression on Regulation of VEGFR2 Expression. To be considered functionally significant, the ability of cytokines to promote the accumulation of STAT target gene products should be compromised in A_{2A}AR-overexpressing cells. In the course of our studies, we identified VEGFR2 as a protein in ECs whose levels are posi-

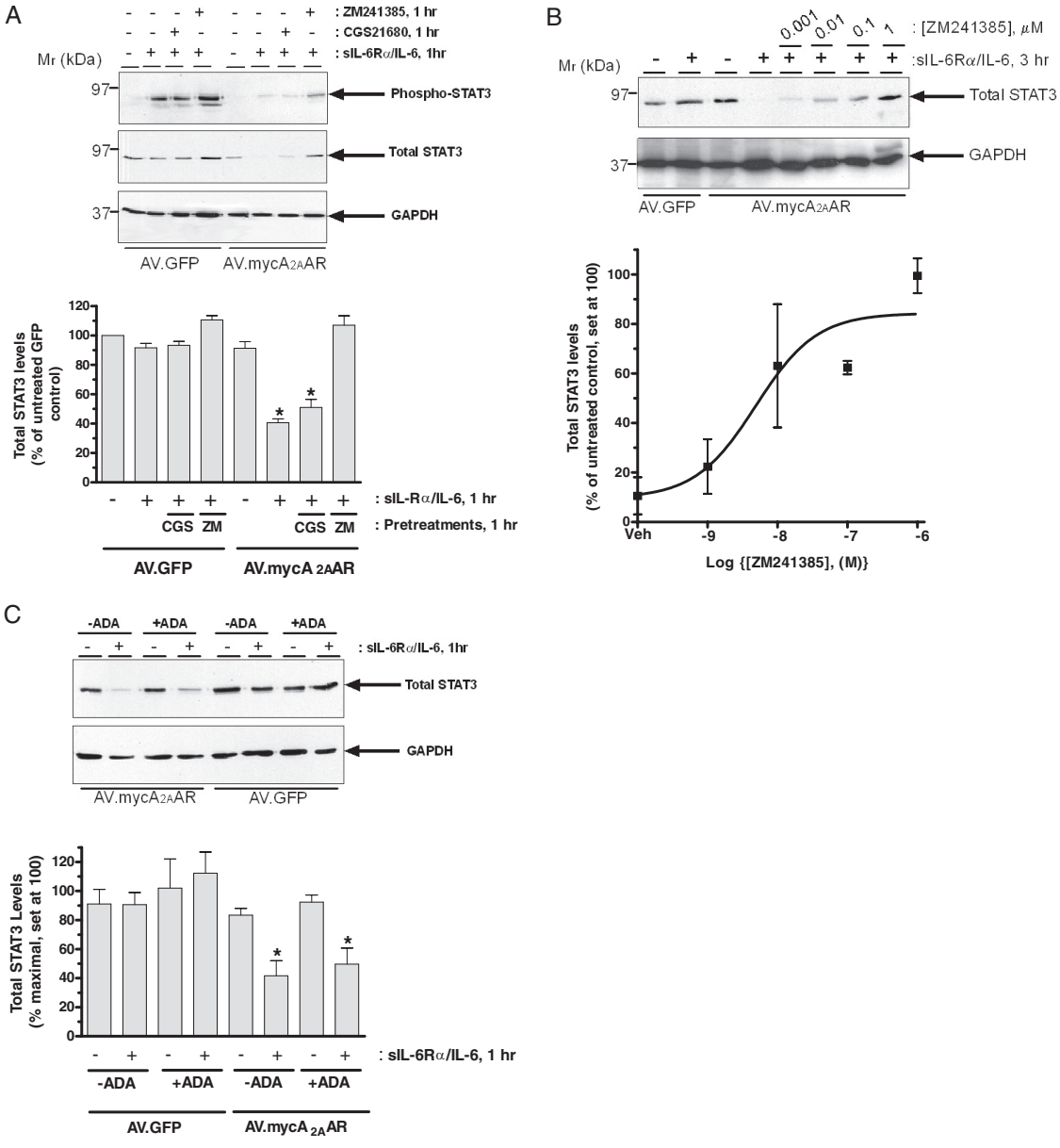

Fig. 2. Pharmacology of the A_{2A}AR effect on STAT3 phosphorylation and expression. A, HUVECs were infected with recombinant AVs before treatment with or without sIL-6Rα/IL-6 in the presence of A_{2A}AR-selective agonist CGS21680 (5 μM) or selective inverse agonist ZM241385 (1 μM) for 1 h as indicated. Soluble cell extracts equalized for protein content were then fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of total STAT3 levels in A_{2A}AR-expressing cells from three such experiments is presented (*, $p < 0.05$ versus levels in AV.mycA_{2A}AR-infected vehicle-treated cells). B, HUVECs were infected with recombinant AVs as in A before treatment with or without sIL-6Rα/IL-6 for 3 h in the presence of increasing concentrations of ZM241385 as indicated. Soluble cell extracts equalized for protein content were then fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of the recovery of total STAT3 levels in A_{2A}AR-expressing cells from three such experiments is presented. C, HUVECs were infected with recombinant AVs as in A before pretreatment with or without 3 U/ml ADA for 3 h followed by treatment with or without sIL-6Rα/IL-6 for 1 h. Soluble cell extracts equalized for protein content were then fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of total STAT3 levels in A_{2A}AR-expressing cells from three such experiments is presented (*, $p < 0.05$ versus levels in vehicle-treated cells).

tively controlled by STAT3. Thus, incubation of HUVECs with sIL-6Rα/IL-6 increased VEGFR2 expression by 6.8 ± 1.1-fold over untreated controls ($p < 0.05$, $n = 4$), an event that was markedly inhibited by preincubation with a maximally effective concentration of JAK inhibitor (Fig. 7A). In addition, the sIL-6Rα/IL-6-mediated induction of VEGFR2 could be mimicked by transient overexpression of WT STAT3 and abolished by the expression of dominant-negative Tyr705→Phe STAT3 (Kaptein et al., 1996) (Fig. 7B). It is noteworthy that overexpression of the A_{2A}AR also increased VEGFR2 expression versus AV.GFP control cells, although this phenomenon seems to be STAT-inde-

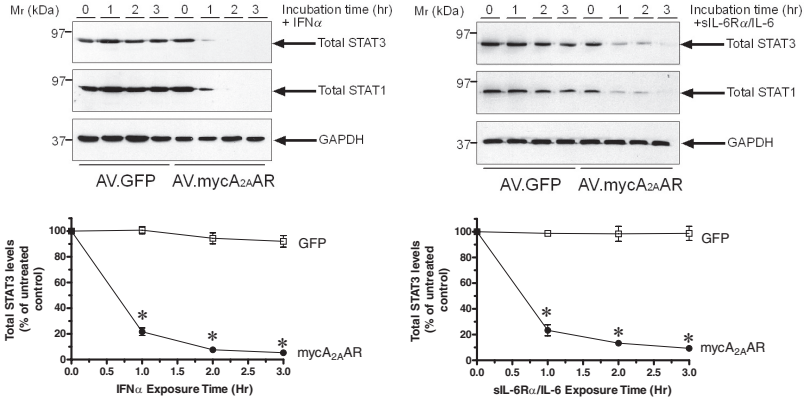

Fig. 3. The effect of A_{2A}AR overexpression on IFNα regulation of STAT1 and STAT3. HUVECs were infected with recombinant AVs before treatment with or without 500 U/ml IFNα or sIL-6Rα/IL-6 for the indicated times. Soluble cell extracts equalized for protein content were then fractionated by SDS-PAGE for immunoblotting with anti-STAT1, STAT3, and GAPDH antibodies. Quantitative analysis of total STAT3 levels from three experiments with each cytokine is presented (*, $p < 0.05$ versus the levels in AV.GFP-infected cells at the given time point).

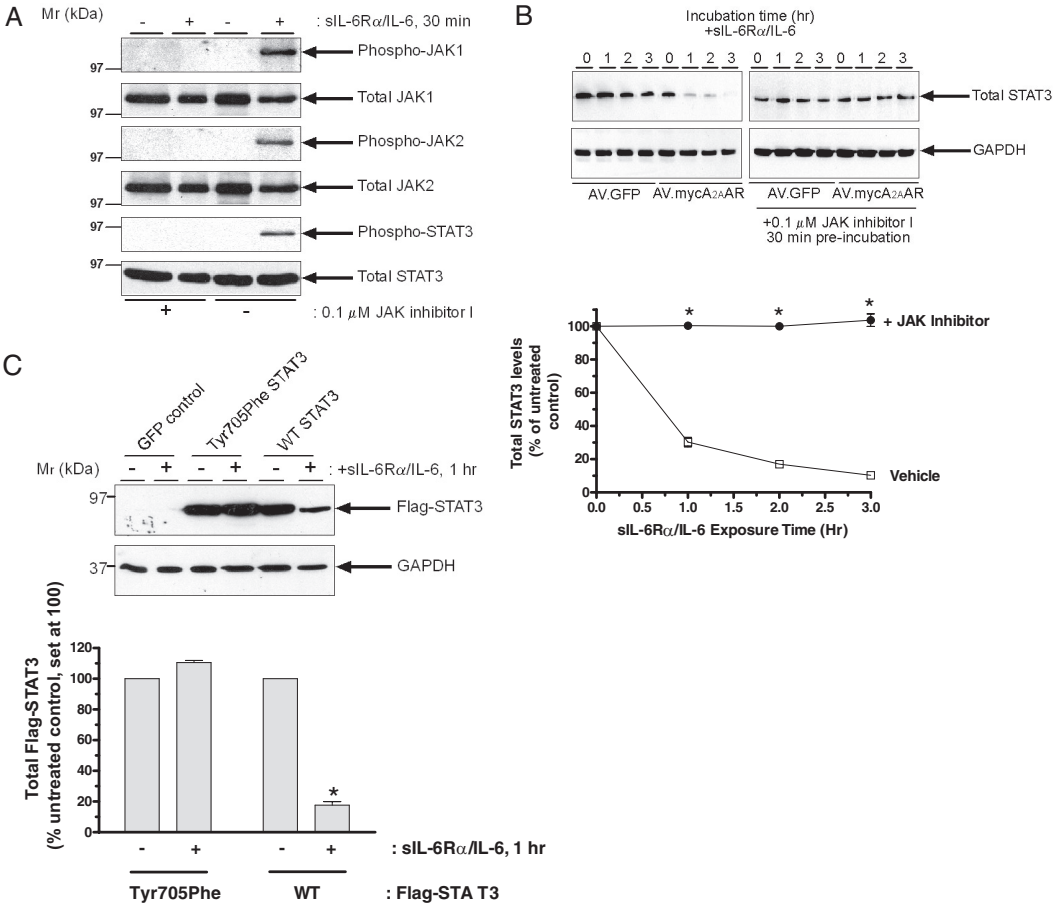

Fig. 4. A role for JAK phosphorylation in triggering cytokine-mediated STAT down-regulation in A_{2A}AR-overexpressing cells. A, HUVECs were preincubated with or without 0.1 μM JAK inhibitor I for 30 min before treatment with or without sIL-6Rα/IL-6 for 30 min as indicated. Soluble cell extracts equalized for protein content were then fractionated by SDS-PAGE for immunoblotting with total and anti-Tyr1022/1023 phospho-JAK1, Tyr1007/1008 phospho-JAK2, and Tyr705 phospho-STAT3 antibodies as indicated. B, HUVECs were infected with the indicated AVs before pretreatment with or without 0.1 μM JAK inhibitor I for 30 min and treatment with sIL-6Rα/IL-6 for up to 3 h as indicated. Soluble cell extracts equalized for protein content were then fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of total STAT3 levels in A_{2A}AR-expressing cells from three experiments is presented (*, $p < 0.05$ versus STAT levels in vehicle-pretreated cells at the given time point). C, HUVECs were coinfected with AV.myc-A_{2A}AR and either AV.Flag-WT STAT3, AV.Flag-Tyr705→Phe STAT3, or AV.GFP (control) before treatment with or without sIL-6Rα/IL-6 for 1 h as indicated. Soluble cell extracts equalized for protein content were then fractionated by SDS-PAGE for immunoblotting with M5 anti-Flag and GAPDH antibodies. Quantitative analysis of total Flag-STAT3 levels from three experiments is presented (*, $p < 0.05$ versus levels in vehicle-treated cells).

pendent because receptor expression alone produces no detectable changes in STAT phosphorylation (Fig. 1). However, subsequent incubation of A_{2A}AR-overexpressing cells with sIL-6Rα/IL-6 for 4 h triggered a 91 ± 6% down-regulation of VEGFR2 compared with levels in untreated controls ($p < 0.05$, $n = 4$; Fig. 7C).

Effect of Endogenous A_{2A}ARs on STAT Protein Levels. To assess any potential relevance of the mechanisms we observed for overexpressed A_{2A}ARs on endogenous A_{2A}AR function, we used two distinct approaches. First, we assessed the effects of prolonged incubation with A_{2A}AR-selective inverse agonist ZM241385 on STAT3 protein levels, because sustained (24 h or longer) treatment with inverse agonists has been shown to unmask effects of low level constitutive activation of endogenous 7TM receptors (Berg et al., 1999). In contrast to the lack of any effect of coincubation with ZM241385 observed in control HUVECs (Fig. 2A), these experiments demonstrated that prolonged preincubation of HUVECs with ZM241385 for 24 h was able to produce a significant increase in STAT3 protein levels, which was associated with an increase in sIL-6Rα/IL-6-stimulated STAT3 phosphorylation on Tyr705 (Fig. 8A). Similar results were observed if the incubation time was further extended to 48 h (data not shown). Second, we used mice in which both copies of the A_{2A}AR gene had been deleted (Ledent et al., 1997). Consistent with other studies using this model (Ohta and Sitkovsky, 2001), the absence of A_{2A}ARs potentiated the proinflammatory effect of LPS administration, as determined by the significantly elevated serum levels of multiple proinflammatory cytokines, such as IL-1, IL-6, tumor necrosis factor-α, and granulocyte cell-stimulating factor, detectable in LPS-treated A_{2A}AR KO animals versus WT controls (data not shown). Analysis of STAT expression and phosphorylation in aortic extracts from treated animals revealed that LPS administration significantly increased the levels of Tyr701-phosphorylated STAT1 and Tyr705-phosphorylated STAT3 in both WT and A_{2A}AR KO animals versus vehicle controls. However, total STAT1 expression and its phosphorylation on Tyr701 were both significantly enhanced in A_{2A}AR KO animals versus WT. It is interesting that this effect was restricted to STAT1, because total and Tyr705-phosphorylated STAT3 levels were not significantly different between WT and A_{2A}AR KO animals (Fig. 8B), potentially reflecting a dominance of STAT1-mobilizing stimuli at this time point.

Discussion

The A_{2A}AR has been identified as a protective anti-inflammatory 7TM receptor protein not only from pharmacological studies (McPherson et al., 2001; Lappas et al., 2005) but also from several studies characterizing changes in the inflammatory response in mice in which both copies of the A_{2A}AR gene have been deleted (Ohta and Sitkovsky, 2001; Haskó and Pacher, 2008). Gene dosage studies have provided evidence to show that, at least in T-lymphocytes, there is no A_{2A}AR reserve (Armstrong et al., 2001). As a consequence, pathophysiological conditions that alter A_{2A}AR expression, such as EC exposure to Th1 cytokines (Nguyen et al., 2003) or hypoxia (Kobayashi and Millhorn, 1999), are likely to alter cellular responsiveness to inflammatory stimuli. Multiple mechanisms have been proposed to account for its potent anti-inflammatory properties across different cell types; these include inhibition of degranulation and superoxide release from neutrophils, suppression of IL-12 and tumor necrosis factor-α release, and increased IL-10 production from monocytes and macrophages and the ability of the receptor to suppress activation of proinflammatory p38 and NF-κB signaling path-

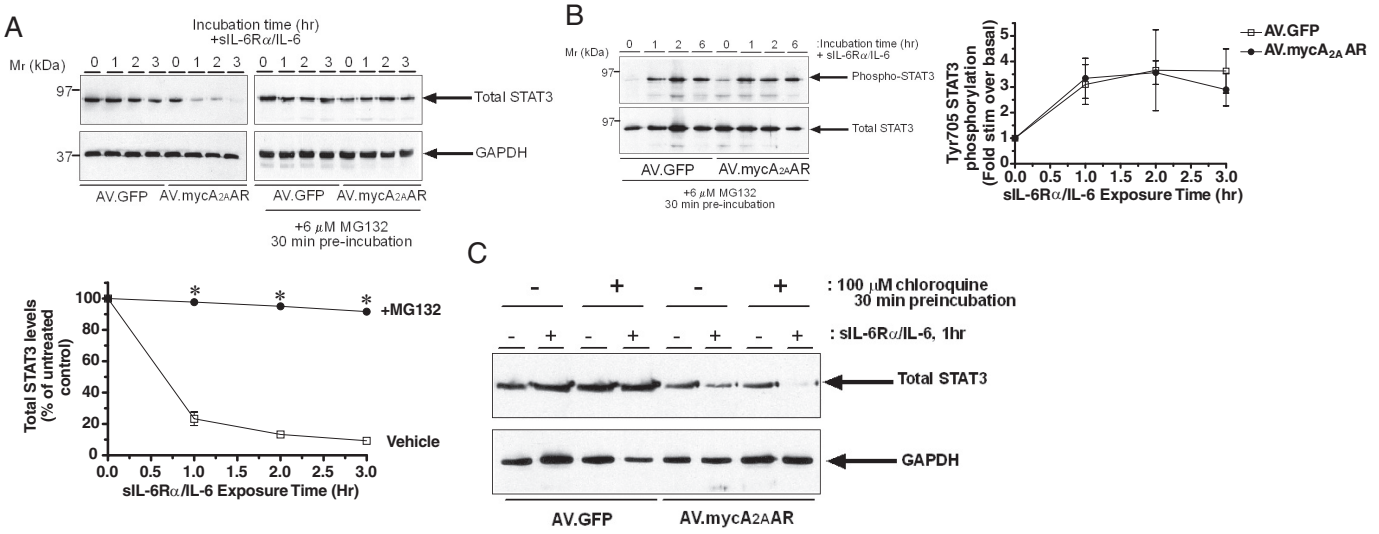

Fig. 5. Effect of proteasome and lysosome inhibitors on sIL-6Rα/IL-6-induced STAT3 phosphorylation and down-regulation in A_{2A}AR-overexpressing HUVECs. C, HUVECs were infected with the indicated AVs before pretreatment with or without 6 μM MG132 for 30 min and treatment with sIL-6Rα/IL-6 for up to 3 h as indicated. Soluble cell extracts equalized for protein content were then fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of total STAT3 levels in A_{2A}AR-expressing cells from three experiments is presented (*, $p < 0.05$ versus STAT3 levels in vehicle-pretreated cells at the given time point). B, HUVECs were infected with the indicated AVs before pretreatment with MG132 and sIL-6Rα/IL-6 as in A. Soluble cell extracts equalized for protein content were then fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of Tyr705 phospho-STAT3 levels from three experiments is presented. C, HUVECs were infected with the indicated AVs before pretreatment with or without 100 μM chloroquine for 30 min and treatment with sIL-6Rα/IL-6 for 1 h as indicated. Soluble cell extracts equalized for protein content were then fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. This is one of three experiments that produced similar results.

ways (Haskó et al., 2007). From the results of this study, we have extended these observations by demonstrating that the A_{2A}AR overexpression can prime cytokine-activated STATs for polyubiquitylation and subsequent degradation by the proteasome, a mechanism that may explain the elevated levels of STAT1 protein observed upon A_{2A}AR depletion in vivo and the elevated levels of STAT3 seen upon sustained incubation of endothelial cells in vitro with A_{2A}AR-selective inverse agonist ZM241385.

Although the process described here represents a new mechanism by which 7TM receptors can negatively regulate the JAK/STAT pathway, several studies have already shown that cytokines using the JAK/STAT pathway can be regulated by distinct A_2AR subtypes. For example, Xu et al. (2008) have observed that the A_{2B}AR is an important repressor of IFNγ-mediated induction of major histocompatibility complex II transactivator in aortic smooth muscle cells. The sensitivity of the SOCS-3 gene to induction via a cAMP-stimulated pathway involving "exchange protein directly activated by cyclic AMP 1" and C/EBP transcription factors also represents a level of cross-talk between A_2AR-activated signaling pathways and the inhibition of ERK1,2 and JAK/STAT signaling from defined suppressor of cytokine signaling-3-regulated cytokine receptors (Sands et al., 2006; Yarwood et al., 2008). Such a mechanism may also explain the ability of A_{2A}AR activation to induce IL-10 in a C/EBP-dependent manner (Csóka et al., 2007).

Similar to the effect we observed on suppression of NF-κB in two separate cell systems (Sands et al., 2004), overexpression of the A_{2A}AR was sufficient to prime STATs for degradation in the absence of any exogenous agonist. It is possible that over the course of the experiments, endogenous adenosine released by HUVECs in vitro reaches extracellular levels sufficient to cause A_{2A}AR activation. Endothelial cells are an abundant source of adenosine in vivo because of expres-

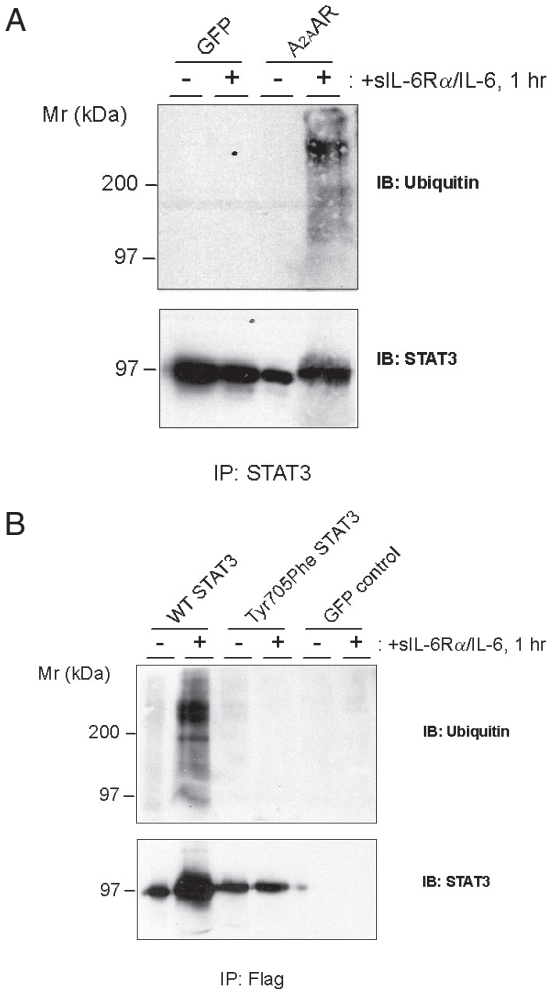

Fig. 6. Effect of A_{2A}AR overexpression on STAT ubiquitylation in response to cytokine stimulation. A, HUVECs were infected with the indicated AVs before pretreatment with 6 μM MG132 for 30 min and incubation with or without sIL-6Rα/IL-6 for 1 h as indicated. Samples were then denatured by heating in SDS-containing buffer before dilution into excess nonionic detergent for preparation of clarified extracts, equalization of protein content, and immunoprecipitation of STAT3. Immunoprecipitates were fractionated by SDS-PAGE for immunoblotting with anti-ubiquitin and STAT3 antibodies. B, HUVECs were coinfected with AV.myc-A_{2A}AR and either AV.GFP (control), AV.Flag-WT STAT3, or AV.Flag-Tyr705→Phe STAT3 before treatment with MG132 and incubation with or without sIL-6Rα/IL-6 for 1 h as indicated. Clarified extracts were then prepared for immunoprecipitation of Flag-STAT3 proteins using M2 antibody-coupled Sepharose beads. Samples were then fractionated by SDS-PAGE before immunoblotting with anti-ubiquitin and STAT3 antibodies.

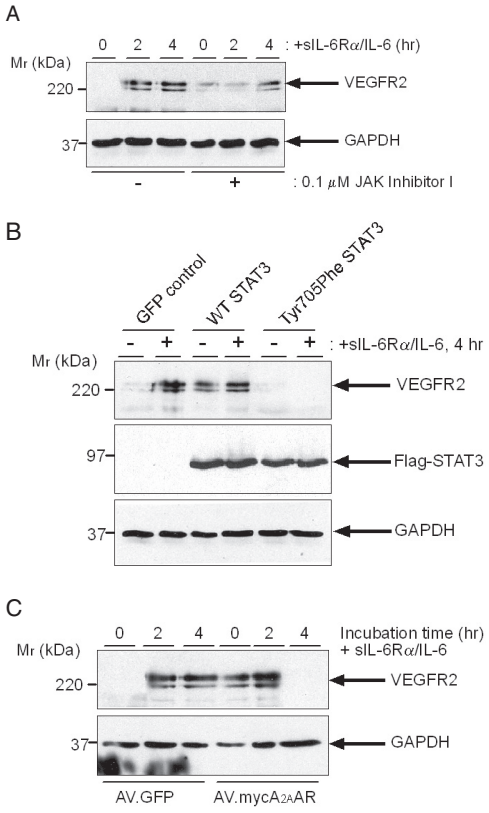

Fig. 7. Effect of A_{2A}AR overexpression on STAT3-regulated induction of VEGFR2 by sIL-6Rα/IL-6. A, HUVECs were preincubated with or without 0.1 μM JAK inhibitor I for 30 min before treatment with or without sIL-6Rα/IL-6 for the indicated times. Soluble cell extracts equalized for protein content were then fractionated by SDS-PAGE for immunoblotting with anti-VEGFR2 and GAPDH antibodies. B, HUVECs were infected with AV.GFP (control), AV.Flag-WT STAT3, or AV.Flag-Tyr705→Phe STAT3 before treatment with or without sIL-6Rα/IL-6 for 4 h. Soluble cell extracts equalized for protein content were then fractionated by SDS-PAGE for immunoblotting with anti-VEGFR2, anti-Flag M5, and GAPDH antibodies. C, HUVECs were infected with the indicated AVs before treatment with sIL-6Rα/IL-6 for 2 or 4 h as indicated. Soluble cell extracts equalized for protein content were then fractionated by SDS-PAGE for immunoblotting with anti-VEGFR2 and GAPDH antibodies.

sion of CD39, an ectoapyrase that catalyzes ATP hydrolysis, and CD73, an ecto-5′-nucleotidase that converts the resulting AMP to adenosine (Zernecke et al., 2006). The accumulation of adenosine that ensues in large blood vessels is believed to play an important protective role in vivo by limiting endothelial cell activation and subsequent monocyte attachment (Zernecke et al., 2006). However, it is also possible that the receptor may be sufficiently active in the absence of agonist to trigger its associated signaling pathways. This phenomenon has been described not only upon overexpression of many 7TM receptors but also for endogenously expressed receptors and virally encoded 7TM receptors such as Kaposi's sarcoma-associated herpes virus ORF74 (Vischer et al., 2006). Reversal of the effect of receptor overexpression on STAT down-regulation in a concentration-dependent manner by inverse agonist ZM241385 (Fig. 2, A and B), coupled with the lack of any significant effect of coincubation with ADA (Fig. 2C), would suggest that the receptor's constitutive activity is mainly responsible. It is noteworthy that the reported effect of A_{2B}AR deletion in enhancing IFNγ-mediated induction of complex II transactivator in vitro (Xu et al., 2008) would tend to suggest that constitutive signaling is not restricted to the A_{2A}AR subtype.

Despite its obvious importance in controlling the expression and function of various transcription factors such as p53 (Watson and Irwin, 2006) and NF-κB (Chen, 2005), relatively few reports have examined STAT degradation. Indeed, although the ability of V proteins encoded by paramyxoviruses to function as STAT E3 ubiquitin ligases is a well established mechanism by which they subvert the interferon response (Horvath, 2004), native cellular mechanisms controlling STAT degradation are rather less well defined. The first description of STAT degradation centered around the observation that proteasome inhibition produced a more robust accumulation of tyrosine-phosphorylated STAT1 in HeLa cells after exposure to IFNγ, suggesting that proteasomal degradation is an important mechanism by which STAT1 function is turned off in these cells (Kim and Maniatis, 1996). Thereafter, it was shown that IL-3 exposure results in a time-dependent proteasomal degradation of STAT5A but not STAT1, -2, or -3 in 32D myeloid cells (Wang et al., 2000), although nuclear translocation rather than tyrosine phosphorylation per se seems to be required for degradation to occur (Chen et al., 2006). Another study has shown that the degradation of STAT3 in H4IIE hepatoma cells could be triggered by hyperosmotic stress and occurred indepen-

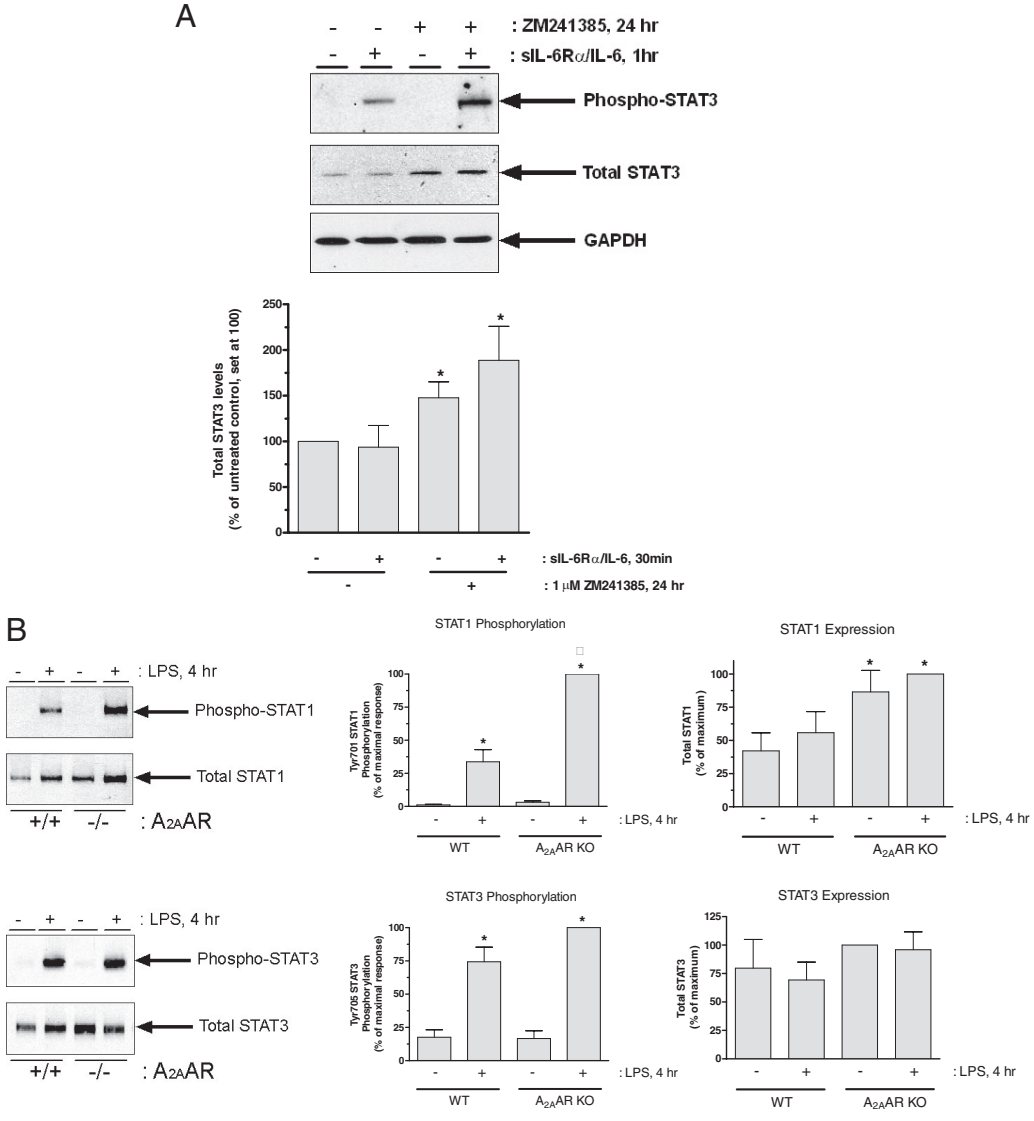

Fig. 8. Effect of endogenous A_{2A}ARs on STAT phosphorylation and expression. A, HUVECs were pretreated with either vehicle or A_{2A}AR-selective inverse agonist ZM241385 (1 μM) for 24 h as indicated before further incubation with or without sIL-6Rα/IL-6 for 1 h as indicated. Soluble cell extracts equalized for protein content were then fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of total STAT3 levels from three experiments is presented (*, $p < 0.05$ versus STAT3 levels in cells treated with vehicle alone). B, A_{2A}AR(+/+) (WT) and homozygous A_{2A}AR(−/−) KO mice were injected with either PBS vehicle or 0.4 mg/kg LPS for 4 h before isolation of aortae for solubilization in sample buffer, SDS-PAGE, and immunoblot analysis of total and tyrosine-phosphorylated STATs 1 and 3 as indicated. Quantitative analysis of eight animals per group is shown (STAT1 and -3 phosphorylation graphs; *, $p < 0.001$ versus vehicle-treated animals, ψ, $p < 0.001$ versus LPS-treated WT animals; STAT expression graph; *, $p < 0.05$ versus WT animals).

dently of phosphorylation on Tyr705 (Lornejad-Schäfer et al., 2005). Taken as a whole, none of these observations is consistent with a single unifying mechanism. Thus, although the recent identification of the protein "SLIM" (*S*TAT-interacting protein with *LIM* domain) as an E3 ubiquitin ligase able to trigger the polyubiquitylation of STAT1 and STAT4 is an important advance (Tanaka et al., 2005; Gao et al., 2007), it is unlikely to account for all of the STAT degradation phenomena reported in the literature. Related to this issue, we found that inhibition of proteasome function was sufficient to block the inhibitory effect of the A_{2A}AR on STAT3 phosphorylation, demonstrating that priming of STATs for degradation is the only mechanism responsible for the reduced cytokine-stimulated STAT phosphorylation observed in A_{2A}AR-overexpressing cells. This contrasts with the observation that exogenous expression of SLIM in HEK293 cells was able to inhibit cytokine-stimulated tyrosine phosphorylation of STAT4 as well as promoting its polyubiquitylation and degradation by the proteasome (Tanaka et al., 2005). This might suggest the involvement of another E3 ubiquitin ligase in HUVECs, and consistent with this hypothesis we have been unable to detect SLIM message or protein in HUVECs after cytokine stimulation or A_{2A}AR-overexpressing cells (M. M. A. Sahfi, W. A. Sands, T. M. Palmer, unpublished observations). In addition, although tyrosine phosphorylation is clearly the critical step in targeting STATs for degradation in A_{2A}AR-overexpressing cells, it is unclear as to whether it functions simply as a classic phosphodegron, or whether the nuclear translocation that occurs as a result of phosphorylation is also important for localizing the phosphorylated STAT dimer with the relevant E3 ubiquitin ligase.

More generally, the identification in this study of a new mechanism by which cytokine signaling can be turned off by targeting tyrosine-phosphorylated STATs for degradation has significant implications for diseases associated with altered regulation of the JAK/STAT pathway. It also reinforces the argument that potentiation of A_{2A}AR function might prove to be a useful strategy with which to down-regulate proinflammatory responses by virtue of its capacity to inhibit multiple proinflammatory processes used by distinct stimuli. However, it also raises the issue that any beneficial effects of A_{2A}AR-selective inverse agonists in neurodegenerative conditions such as Huntington's and Parkinson's diseases (Jenner et al., 2009) might also have undesirable proinflammatory side effects.

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