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Tumor Necrosis Factor-α Differentially Regulates the Expression of Proinflammatory Genes in Human Airway Smooth Muscle Cells by Activation of Interferon-β–Dependent CD38 Pathway

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ABSTRACT

Recent evidence suggests that CD38, an ectoenzyme that converts NAD^+ to cyclic ADP-ribose (cADPr), may play a role in cytokine-induced airway smooth muscle (ASM) cell hyper-responsiveness, a key feature associated with chronic asthma. In the present study, we investigated the major signaling pathways by which tumor necrosis factor-α (TNFα) induces CD38 expression and its role in regulating gene expression in human ASM cells. Using flow cytometry analyses, TNFα enhanced CD38 expression in a manner that was time- (0–24 h), concentration- (0.1–40 ng/ml), and protein synthesis- (cycloheximide blockade) dependent. A selective agonistic antibody against tumor necrosis factor receptor (TNFR) 1 also augmented CD38 expression, whereas anti-TNFR2 antagonistic antibody did not prevent the TNFα response. Inhibition of the Janus activated kinase/signal transducer and activator of transcription pathways using the soluble inhibitor 2-(1,1-dimethylethyl)-9-fluoro-3,6-dihydro-7H-benz-[*h*]imidaz[4,5-*f*]isoquinolin-7-one (DBI) or with neutralizing antibody against interferon β (IFNβ) completely abrogated TNFα-induced CD38 expression at both protein and mRNA levels. Combining TNFα (0.1 and 1 ng/ml) and IFNβ (100 IU/ml) at concentrations alone that had little effect on CD38 expression induced a robust synergistic induction of CD38 mRNA and protein levels. 8-Bromo-cADPr, a cADPr antagonist, significantly augmented TNFα-induced interleukin-6 secretion, whereas regulated on activation normal T cell expressed and secreted secretion was suppressed. 8-Bromo-cADPr, however, did not affect TNFα-induced cell surface expression of intercellular adhesion molecule-1. Our study is the first to demonstrate that IFNβ-dependent activation of CD38 pathway is a novel component by which TNFα differentially regulates the expression of inflammatory genes in ASM cells.

CD38 is a bifunctional ectoenzyme with ADP-ribosyl cyclase activity that converts the cellular intermediary metabolite βNAD^+ to cADPr, a Ca^{2+}-mobilizing second messenger. In addition, CD38 mediates the degradation of cADPr to ADP-ribose through its cADPr hydrolase activity (Lee, 2001). CD38 expression occurs widely in many mammalian cells, including hematopoietic cells, such as B and T lymphocytes and macrophages, and resident cells such as pancreas, heart, brain, liver, and lung cells and vascular and uterine smooth muscle (Deaglio et al., 2001). The cellular function of CD38 remains unclear. Early evidence shows that CD38 plays a critical role in insulin release from pancreatic β cells (Takasawa et al., 1993). Additional studies using different experimental approaches such as monoclonal agonistic antibodies, cADPr antagonist 8-Bromo-cADPr (8-Br-cADPr), or CD38-deficient cells demonstrate a role for CD38 in both B and T cell proliferation (Funaro et al., 1997), cytokine production from B and T cells (Deaglio et al., 2003), neutrophil migration

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ABBREVIATIONS: cADPr, cyclic ADP-ribose; ASM, airway smooth muscle; 8-Br-cADPr, 8 bromo-cADP-ribose; GPCR, G protein-coupled receptor; ICAM, intercellular adhesion molecule; IFN, interferon; JAK, Janus activated kinase; RT-PCR, reverse transcription-polymerase chain reaction; RANTES, regulated upon activation normal T cell expressed and secreted; STAT, signal transducer and activator of transcription; TNFR, tumor necrosis factor receptor; DBI, 2-(1,1-dimethylethyl)-9-fluoro-3,6-dihydro-7H-benz-[*h*]imidaz[4,5-*f*]isoquinolin-7-one; TNFα, tumor necrosis factor α; IL, interleukin; PCR, polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Ab, antibody.

(Partida-Sanchez et al., 2001), and neurotransmission and cardiac contraction (Higashida et al., 2001). Whether all CD38 cellular effects are mediated via cADPr remains controversial; new evidence shows that CD38 signaling in response to activating antibodies occurs independently of its enzymatic activity (Lund et al., 1999). The nature of these signaling pathways is not known, but CD38 ligation can activate multiple molecules such as phospholipase Cγ, phosphatidyl inositol 3-kinase, and other tyrosine phosphorylated proteins (Shubinsky and Schlesinger, 1997). Together, these studies show that the mechanisms that regulate CD38/cADPr expression and function remain unknown.

We and others recently showed that the CD38/cADPr pathway represents a novel component in the regulation of Ca^{2+} homeostasis in response to activation of G protein-coupled receptors (GPCRs) in monocytes (Partida-Sanchez et al., 2004), in arterial smooth muscle cells (Ge et al., 2003), and in airway smooth muscle cells (Prakash et al., 1998; White et al., 2003). We also found that the induction of CD38 expression by TNFα or IL-1β correlated with increases in Ca^{2+} signals to different GPCR agonists (bradykinin and carbachol), an effect that was abrogated by the cADPr antagonist 8-Br-cADPr (Deshpande et al., 2003). Similar findings were also reported recently in myometrium cells in which oxytocin-induced Ca^{2+} responses were enhanced by TNFα, an effect also prevented by 8-Br-cADPr (Barata et al., 2004). These data collectively suggest that the modulation CD38/cADPr pathways by inflammatory cytokines may represent one mechanism in the regulation of cell responsiveness to GPCR agonists. We therefore propose that changes in CD38 expression and/or function in ASM, the main effector tissue that regulates the bronchomotor tone, may represent a key mechanism underlying the development of bronchial hyperresponsiveness to GPCRs, a defining feature of asthma.

In this study, we present the first evidence that the induction of CD38 expression by TNFα occurs via transcriptional mechanisms involving the synergistic cooperation of endogenous IFNβ. More importantly, activation of the CD38/cADPr pathway by TNFα differentially regulates the expression of inflammatory genes. Our findings shows that, in addition to its recently shown role in promoting airway hyper-responsiveness, the CD38/cADPr pathway potentially modulates airway inflammation via the transcriptional regulation of inflammatory genes in ASM cells.

Materials and Methods

Cell Culture. Human tracheal tissue culture was obtained from lung transplant donors in accordance with procedures approved by the University of Pennsylvania Committee on Studies Involving Human Beings. The culture of human ASM cells was performed as described elsewhere (Amrani et al., 2001).

Flow Cytometry Analysis. Flow cytometry was performed as described previously (Amrani et al., 1999). In brief, adherent cells were washed with phosphate-buffered saline, detached by trypsinization (2 min at 37°C), and then washed with Ham's F-12 medium (10% fetal calf serum), centrifuged, and transferred to microcentrifuge tubes (1.5 ml). After incubation with the mouse anti-human CD38 antibody (2 μg/ml; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and fluorescein isothiocyanate-conjugated goat anti-mouse antibody (Jackson ImmunoResearch Laboratories Inc., West Grove, PA), the cells were centrifuged and resuspended in ice-cold phosphate-buffered saline in microcentrifuge tubes. Samples were then analyzed using an EPICS XL flow cytometer (Beckman Coulter, Inc., Hialeah, FL). ICAM-1 expression was assessed using fluorescein isothiocyanate-conjugated mouse anti-human ICAM-1 Ab (10 μg/ml; R&D Systems, Minneapolis, MN). CD38 and ICAM-1 expression was expressed as the fold increases in mean fluorescence intensity over basal (untreated cells).

SDS-Polyacrylamide Gel Electrophoresis and Western Blot Analysis. Immunoblot analysis for phospho-STAT1 was performed as described previously (Tliba et al., 2003): To ensure equal loading, the membranes were stripped and reprobed with anti-STAT1 (Santa Cruz Biotechnology).

Reverse Transcription-Polymerase Chain Reaction Analysis. Total RNA was extracted from human ASM cells using RNeasy Mini Kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. RT-PCR reactions were performed using human CD38 primers for semiquantitative analysis as described previously (Deshpande et al., 2003). Each of 35 cycles of the PCR was programmed to carry out denaturation at 94°C for 30 s, primers annealing at 55°C for 45 s, extension at 72°C for 45 s, and a final extension at 72°C for 10 min. The semiquantitative PCR approach of CD38 mRNA was performed in parallel by investigating human GAPDH mRNA levels with the following primers: 5′-ATGGATGATGATATCGCCGC-3′ (sense) and 5′-TTAATGTCACGCACGATTTC-3′ (antisense). The intensity of density area was analyzed using a Gel-Pro Analyzer (MediaCybernetics, Silver Spring, MD). The final PCR product was expressed as the ratio of CD38 to GAPDH used for scanning analysis.

Measurement of IL-6 and RANTES Secretion by ASM Cells by Enzyme-Linked Immunosorbent Assay. Confluent ASM cells were growth-arrested by incubating the monolayers in Ham's F-12 medium with 0.1% bovine serum albumin for 24 h and stimulated with TNFα (10 ng/ml) for 24 h. The concentration of IL-6 and RANTES in the culture medium was determined by enzyme-linked immunosorbent assay as described previously (Tliba et al., 2003). To investigate the effect of CD38/cADPr pathway on TNFα-induced IL-6 and RANTES expression, the 8-Br-cADPr, a membrane-permeant antagonist of cADPr (100 μM), was added 15 min before the addition of TNFα.

Measurement of cADPr Levels. cADPr levels were determined by an enzymatic cycling method developed by Graeff and Lee (2002). ASM cultures were treated in the absence or presence of TNFα for 24 h. The media were then removed by aspiration, and 5 ml of ice-cold 40% (v/v) acetonitrile was added (Grob et al., 2003). The cells were scraped from the dish and frozen. After thawing, the cell extracts were sonicated for 20 s on ice and centrifuged at 2000*g* for 30 min to remove precipitated protein. The supernatant was evaporated to dryness using a Speed-Vac concentrator (Thermo Savant, Holbrook, NY). The cADPr assay depends on the conversion of cADPr to NAD using *Aplysia californica* ADP-ribosyl cyclase, thus necessitating the removal of endogenous NAD before the assay (Graeff and Lee, 2002). To accomplish this, we used reverse-phase high-performance liquid chromatography to separate cellular NAD from cADPr. The chromatography step used an LC18T column (4.6 mm × 15 cm; Supelco, Bellefonte, PA) at a flow rate of 1 ml/min with 10 mM KH_2PO_4, pH 6.0. A gradient from 0 to 10% methanol in 10 mM KH_2PO_4, pH 6.0, from 6 to 15 min was used to separate cADPr from NAD. cADPr elutes between 3 and 4 min, whereas NAD elutes at approximately 14 min. The dried samples were reconstituted in 500 μl of 10 mM KH_2PO_4, pH 6.0, and filtered through 0.22-μm cellulose acetate centrifugal filters (Spin-x; Corning Glassworks, Corning, NY) before injection. Fractions containing cADPr (1-ml fractions collected from 2 to 6 min) were dried on a Speed-Vac concentrator. These fractions were reconstituted in 200 μl of 100 mM sodium phosphate, pH 8.0. Aliquots (40 μl) of the reconstituted fractions were used in the cADPr determination.

Materials and Reagents. Tissue culture reagents and primers used for PCR were obtained from Invitrogen (Carlsbad, CA). Human rTNFα was provided by Roche Diagnostics (Indianapolis, IN). rIFNγ, rIFNβ, rIFNα, and the different antibodies antagonistic anti-

TNFR1, agonistic anti-TNFR1, neutralizing anti-IFNβ (sheep polyclonal Ab), and isotype-matched goat or mouse IgG were all purchased from R&D Systems. Cycloheximide was purchased from Sigma Chemical (St. Louis, MO). The JAK inhibitor 2-(1,1-dimethylethyl)-9-fluoro-3,6-dihydro-7H-benz-[*h*]imidaz[4,5-*f*]isoquinolin-7-one (DBI) was provided by Calbiochem (San Diego, CA). The anti-TNFR2 antagonistic Ab was obtained from Cell Sciences Inc. (Norwood, MA). 8-Br-cADPr was prepared as described previously (Walseth et al., 1997). The sheep serum was purchased from Jackson ImmunoResearch Laboratories.

Data Analysis. Data points from individual assays represent the mean values of triplicate measurements. Significant differences among groups were assessed with analysis of variance (Bonferroni-Dunn test) or by *t* test analysis, with values of $P < 0.05$ sufficient to reject the null hypothesis for all analyses. Each set of experiments was performed with a minimum of three different human ASM cell lines.

Results

TNFα Stimulates CD38 Expression and cADPr Production in Human ASM Cells. In ASM cells stimulated with 10 ng/ml TNFα for 0 to 24 h, CD38 expression was increased in a time-dependent manner, with a significant increase of 1.8 ± 0.1- and 4.1 ± 0.5-fold at 12 and 24 h, respectively ($P < 0.01$) (Fig. 1A). To determine whether the TNFα effect on CD38 expression was caused by protein synthesis, ASM cells were pretreated with the protein synthesis inhibitor cycloheximide (10 μM for 1 h) as described by Amrani et al. (2000b). As shown in Fig. 1A, cycloheximide completely inhibited TNFα-induced CD38 induction at 24 h ($P < 0.001$). These results suggest that TNFα-induced CD38 expression required de novo protein synthesis. In addition, CD38 expression by TNFα (0.1–40 ng/ml for 24 h) was concentration-dependent, with a net fold increase of 4.3 ± 0.2 and 8.2 ± 0.4 at 10 and 40 ng/ml, respectively ($P < 0.01$) (Fig. 1B). We also found that the increase in CD38 protein expression was associated with increased levels of cADPr, with a net 1600 fmol/mg ($n = 2$, data not shown). For all subsequent experiments, modulation of CD38 expression was examined in ASM cells stimulated with 10 ng/ml of TNFα for 24 h.

CD38 Induction by TNFα Involves the Activation of TNFR1 but not TNFR2. Using a set of agonistic and antagonistic antibodies, we showed that TNFR1 plays an important role in mediating many cellular effects induced by TNFα in ASM cells (Amrani et al., 2001). Incubation of ASM cells with the agonistic antibody to TNFR1 for 24 h stimulates the expression of CD38 with levels similar to those induced by TNFα with a 4.8 ± 0.61- and 5.3 ± 0.45-fold increase over basal, respectively (Fig. 2A). At the same concentration, the isotype-matched antibody had no effect on CD38 levels in basal and TNFα-treated cells. As shown in Fig. 2B, neutralizing anti-TNFR2 antibody (20 μg/ml for 1 h) as used in Amrani et al. (2001) had little effect on CD38 induction by TNFα. These data collectively show that TNFα stimulates CD38 expression in ASM cells mainly by activating TNFR1.

CD38 Induction by TNFα Requires the Autocrine Activation of the JAK/STAT Pathways. Evidence from our laboratory showed that TNFα can activate the JAK/STAT signaling molecules via the autocrine action of endogenous IFNβ (Tliba et al., 2003). Because type I IFNs can stimulate CD38 expression in other cell types (Bauvois et al., 1999), we next examined whether endogenous IFNβ modulated TNFα-induced CD38 expression and cADPr production. As shown in Fig. 3, neutralizing anti-IFNβ antibody significantly suppressed TNFα-induced CD38 expression by more than 85% at both protein (Fig. 3A) and mRNA (Fig. 3B) levels. In contrast, the sheep serum, the antibody diluent, did not have any effect on TNFα-induced CD38 expression. Neutralizing anti-IFNβ antibody also completely abolished TNFα-induced cADPr production at 24 h (95% inhibition, data not shown).

The involvement of JAK/STAT pathways was confirmed by using the recently described inhibitor of IFN receptor-associated kinases JAK1 and Tyk2, DBI (Thompson et al., 2002). We found that DBI at 25 nM was effective in inhibiting IFNγ-coupled signaling pathways, as shown by the dose-dependent inhibition of IFNγ-induced STAT1 phosphorylation (Fig. 4A). At this particular concentration, we also found that DBI abrogated TNFα-induced increases in CD38 protein (Fig. 4B) and mRNA (Fig. 4C). Taken together, these results suggest that the JAK/STAT pathways play an essential role

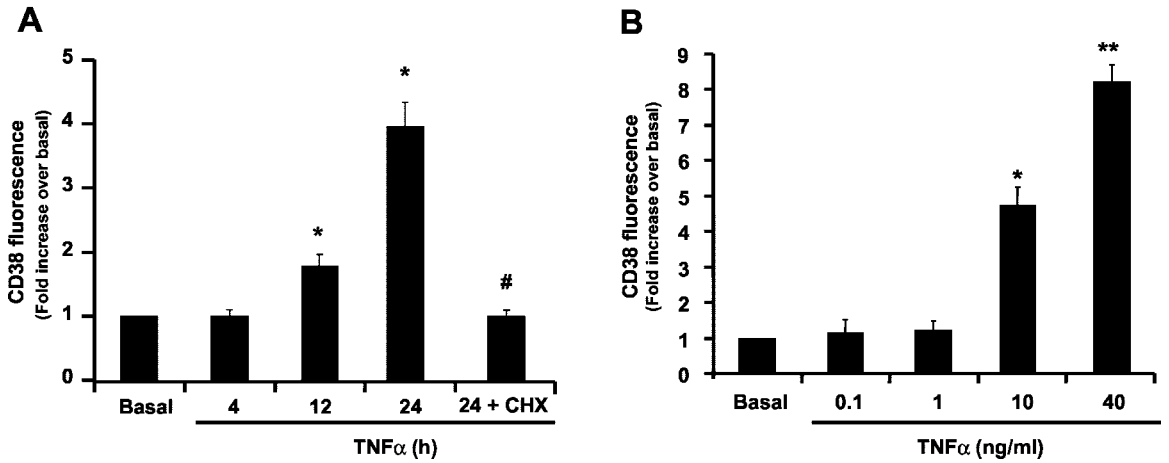

Fig. 1. TNFα induces CD38 expression. ASM cells were incubated with 10 ng/ml TNFα for the indicated time (A) or for 24 h at the indicated concentrations (B). ASM cells were also treated with cycloheximide (CHX, 10 μg/ml) added 1 h before TNFα for 24 h. CD38 expression was assessed by flow cytometry as described under *Materials and Methods*. The results are expressed as the mean of fold increase over basal ± S.E.M. of three separate experiments. ⋆, $P < 0.05$ and ⋆⋆, $P < 0.05$ compared with untreated cells. #, $P < 0.05$ compared with cells treated with TNFα alone.

in the transcriptional activation of CD38 gene induced by TNFα.

CD38 Induction by TNFα Requires the Synergistic Cooperation of Endogenous IFNβ. Although autocrine IFNβ (as shown above) mediated TNFα-induced CD38 expression, the mechanisms by which endogenous IFNβ regulates CD38 expression are not clear. In ASM cells treated with exogenous IFNβ at concentrations believed to be released by TNFα-treated ASM cells [100 IU/ml (Tliba et al., 2003)], there was no effect on CD38 expression, whereas only a modest stimulatory effect was observed at higher concentrations such as 1000 UI/ml (1.48 ± 0.3-fold increase, $n = 3$, data not illustrated). In addition, another type I IFN, IFNα, had no effects on CD38 expression at either 100, 500, or 1000 UI/ml (data not shown). In contrast, there was a significant increase in CD38 levels in ASM cells treated with a combination of ineffective concentrations of TNFα (0.1 and 1 ng/ml as shown in Fig. 1) and IFNβ (100 IU/ml, see above). CD38 expression was increased by 6.1 ± 0.3- and 7.2 ± 0.2-fold when combining 100 IU/ml IFNβ with 0.1 and 1 ng/ml TNFα, respectively (Fig. 5A). RT-PCR analyses of cytokine-treated ASM cells revealed that the synergistic action of the TNFα and IFNβ combination was also observed that the mRNA level (Fig. 5B). Together, these results suggest that the induction of CD38 gene by both TNFα and IFNβ is likely to involve cooperative mechanisms that synergistically increase CD38 gene transcription.

cADPr Antagonist Differentially Modulates TNFα-Induced Gene Expression. In previous reports, the use of 8-Br-cADPr, a cell-permeant cADPr antagonist (Walseth et al., 1997), allowed us to demonstrate the physiological role of cADPr in ASM cells in the presence or absence of TNFα (Deshpande et al., 2003; White et al., 2003). Here, we found that 8-Br-cADPr differentially regulates TNFα-induced IL-6 and RANTES secretion, whereas cell-surface induction of ICAM-1 expression was not affected. As shown in Fig. 6A,

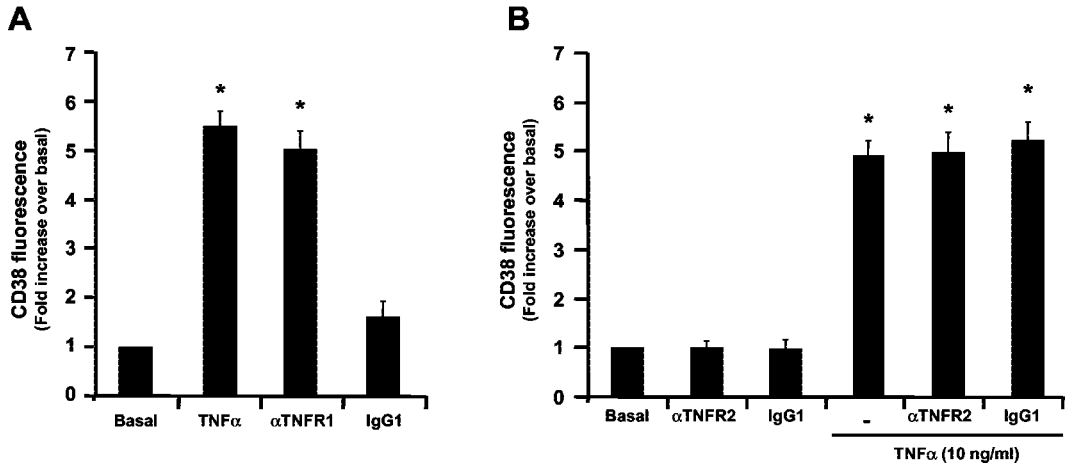

Fig. 2. TNFR1 mediates TNFα-induced CD38 expression. A, human ASM cells were incubated for 24 h with either TNFα (10 ng/ml), agonistic antibody to TNFR1 (5 μg/ml), or the isotype-matched antibody. B, human ASM cells were incubated for 24 h with TNFα (10 ng/ml) in the presence or the absence of neutralizing antibody to TNFR2 or the isotype-matched antibody (10 μg/ml, 1 h before cytokine). CD38 expression was then assessed by flow cytometry as described under *Materials and Methods*. The results are expressed as the mean of fold increase over basal ± S.E.M. of three separate experiments. ⋆, $P < 0.05$ compared with untreated cells.

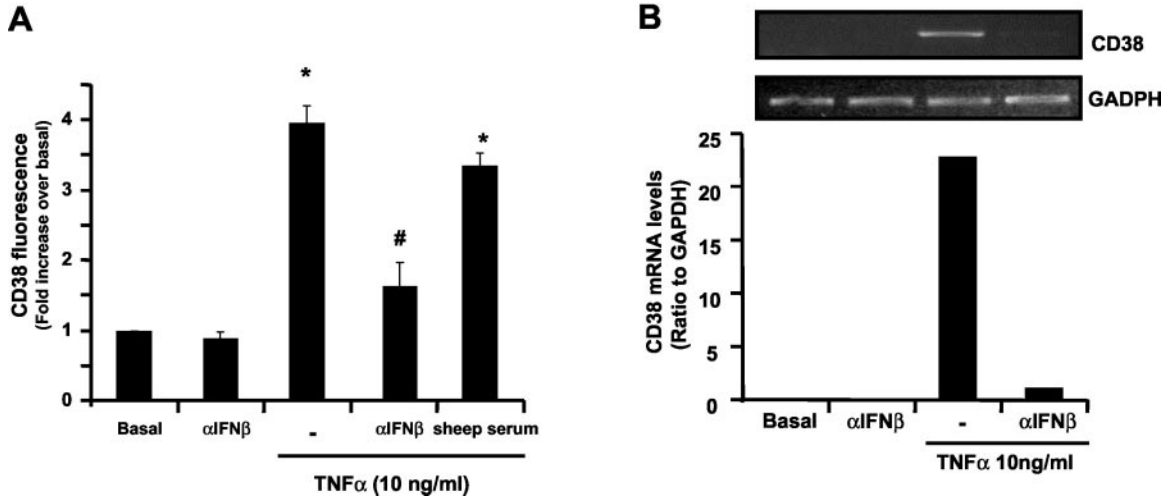

Fig. 3. TNFα, via the autocrine action of secreted IFNβ, induces CD38 gene expression. Cells were stimulated for 24 h with TNFα (10 ng/ml) in the presence or absence of neutralizing anti-IFNβ (5 μg/ml) or sheep serum (control) added 15 min before. A, CD38 protein expression was analyzed by flow cytometry as described under *Materials and Methods*. The results are expressed as the mean of fold increase over basal ± S.E.M. of three separate experiments. ⋆, $P < 0.05$ compared with untreated cells; #, $P < 0.05$ compared with cells treated with TNFα alone. B, cells were lysed, total mRNA was isolated, and RT-PCR was performed as described under *Materials and Methods* using specific primers for CD38. Bottom, scanning densitometric of the representative gel (top) with each value normalized over the mean density of the corresponding GAPDH PCR products.

8-Br-cADPr significantly enhanced IL-6 secretion induced by TNFα. IL-6 levels were increased from 3823 ± 120 to 5231 ± 180 pg/ml in cells treated with TNFα alone or with 8-Br-cADPr, respectively. In addition, 8-Br-cADPr considerably suppressed by 50% TNFα-induced RANTES secretion. RANTES levels were decreased from 11,900 ± 523 to 5843 ± 120 pg/ml in cells treated with TNFα alone or with 8-Br-cADPr, respectively (Fig. 6B). It is interesting that 8-Br-cADPr had no effect on ICAM-1 induction by TNFα-treated cells (Fig. 6C). These data suggest that activation of CD38/cADPr pathway differentially regulates TNFα-induced expression of inflammatory genes in ASM cells.

Discussion

Recent reports from our laboratories suggest that CD38 expression and activation by inflammatory cytokines in ASM may represent a key molecular mechanism for the development of bronchial hyper-responsiveness, a defining feature of asthma (Amrani et al., 2000a). The present work provides the unique demonstration that activation of CD38/cADPr pathways by TNFα occurs via the autocrine action of endogenous IFNβ and differentially regulates the expression of different inflammatory genes.

Growing evidence shows that the binding of TNFα to TNFR1 in ASM activates multiple signaling pathways and genes that may be critical to the pathogenesis of asthma (Amrani et al., 2000a). TNFR1 engagement promotes bronchial hyper-responsiveness by altering Ca^{2+} homeostasis in ASM, the main effector tissue that regulates bronchomotor tone (Parris et al., 1999; Amrani et al., 2000a; Hunter et al., 2003). Activation of TNFR1 in ASM may also regulate airway inflammation, another major characteristic of asthmatics, by promoting the secretion and/or expression of different inflammatory molecules, including cytokines and chemokines (Amrani et al., 200b, 2001). In the present study, we found that TNFα binding to TNFR1 stimulated the expression of CD38 in human ASM cells in a manner that was dependent on concentration (inducible at 10 ng/ml), time (detectable at 12 h), and protein synthesis (blockade by cycloheximide). Although a similar finding has been also described in human myometrium cells in which the induction of CD38 protein by TNFα was associated with an increased cADPr cyclase activity (Barata et al., 2004), the nature of TNFα receptor type

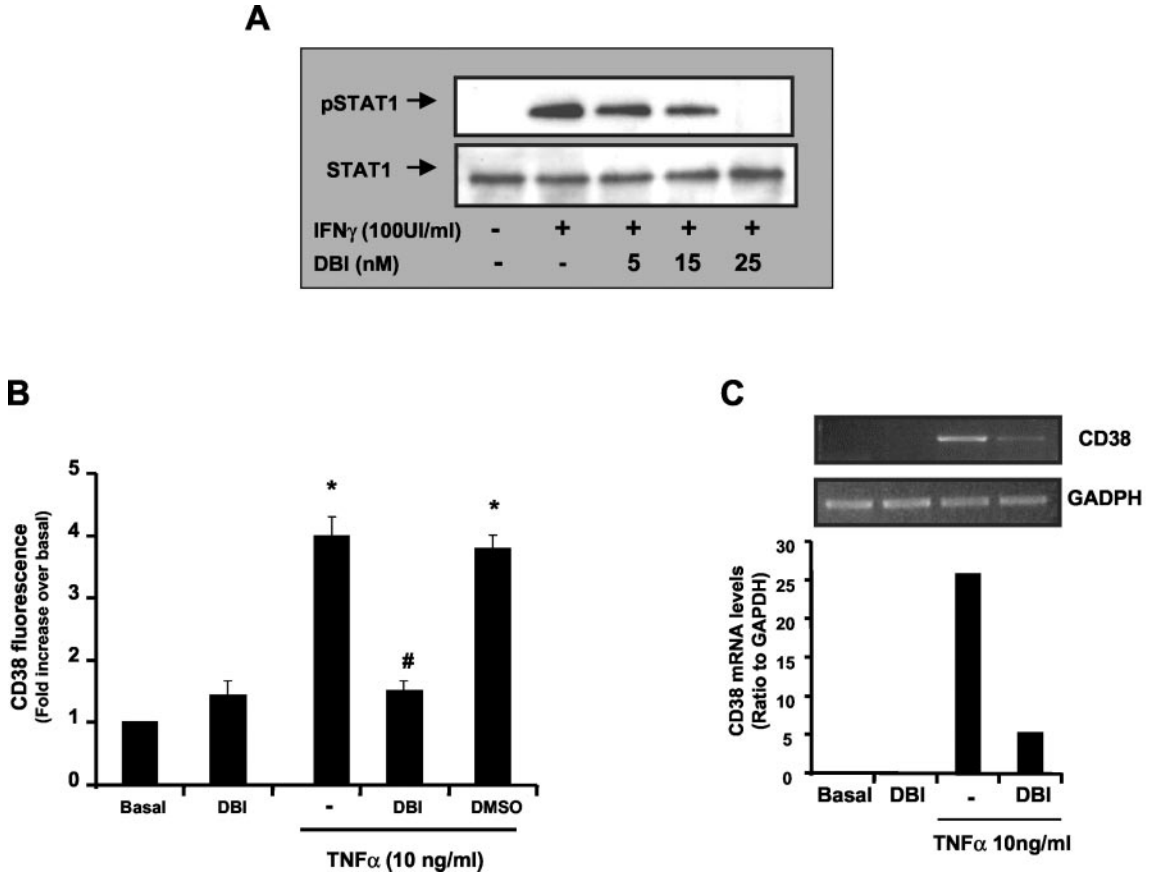

Fig. 4. TNFα induced CD38 expression via the JAK/STAT pathways. A, human ASM cells were treated IFNγ (100 IU/ml) for 15 min in the presence or absence of the indicated concentration of JAK inhibitor (DBI) added 30 min before. Cells were then lysed, and nuclear extracts were prepared and assayed for the phosphorylated and nonphosphorylated forms of STAT1 by immunoblot analysis as described under *Materials and Methods*. Results are representative of three separate blots. B and C, human ASM cells were stimulated with 10 ng/ml TNFα in the presence or absence of the JAK inhibitor I (DBI) (25 nM) or dimethyl sulfoxide 0.1% added 30 min before. B, CD38 protein expression was analyzed by flow cytometry as described under *Materials and Methods*. The results are expressed as the mean of fold increase over basal ± S.E.M. of three separate experiments. ⋆, $P < 0.05$ compared with untreated cells; #, $P < 0.05$ compared with cells treated with TNFα alone. C, representative agarose gel showing the CD38 PCR products stained with ethidium bromide. Cells were lysed, total mRNA was isolated, and RT-PCR was performed as described under *Materials and Methods* using specific primers for CD38. Below is the scanning densitometric of the representative gel with each value normalized over the mean density of the corresponding GAPDH PCR products.

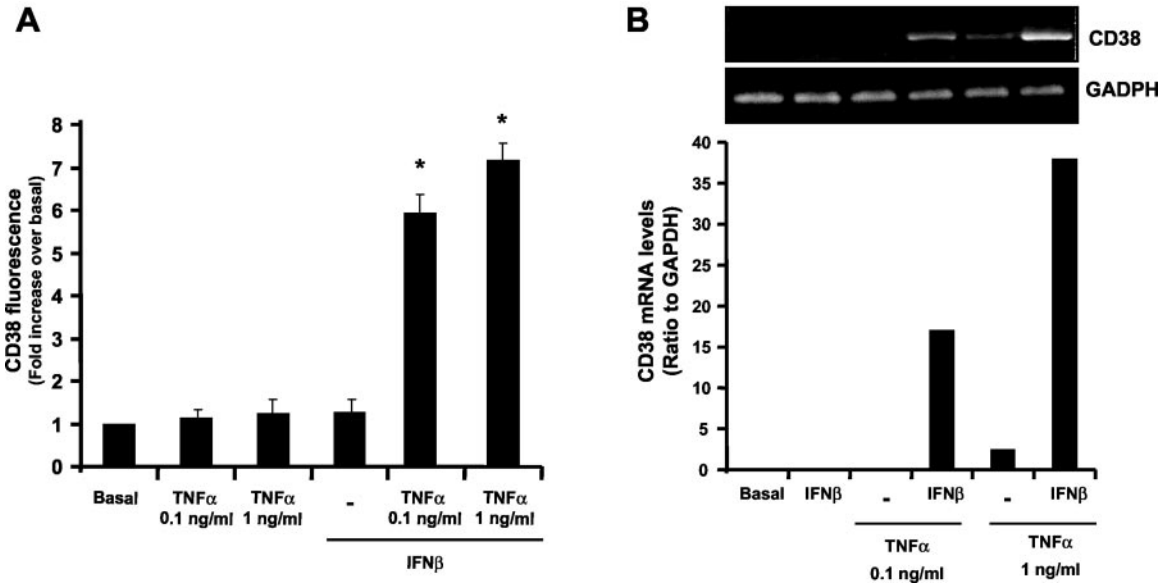

Fig. 5. Synergistic activation of CD38 gene by TNFα and IFNβ. Cells were stimulated for 24 h with the indicated concentration of TNFα and IFNβ (100 U/ml) alone or in combination. A, CD38 protein expression was analyzed by flow cytometry as described under *Materials and Methods*. The results were expressed as the mean of fold increase over basal ± S.E.M. of three separate experiments. ⋆, $P < 0.05$ compared with untreated cells. B, representative agarose gel showing the CD38 PCR products stained with ethidium bromide. Cells were lysed, total mRNA was isolated, and RT-PCR was performed as described under *Materials and Methods* using specific primers for CD38. Below is the scanning densitometric of the representative gel with each value normalized over the mean density of the corresponding GAPDH PCR products.

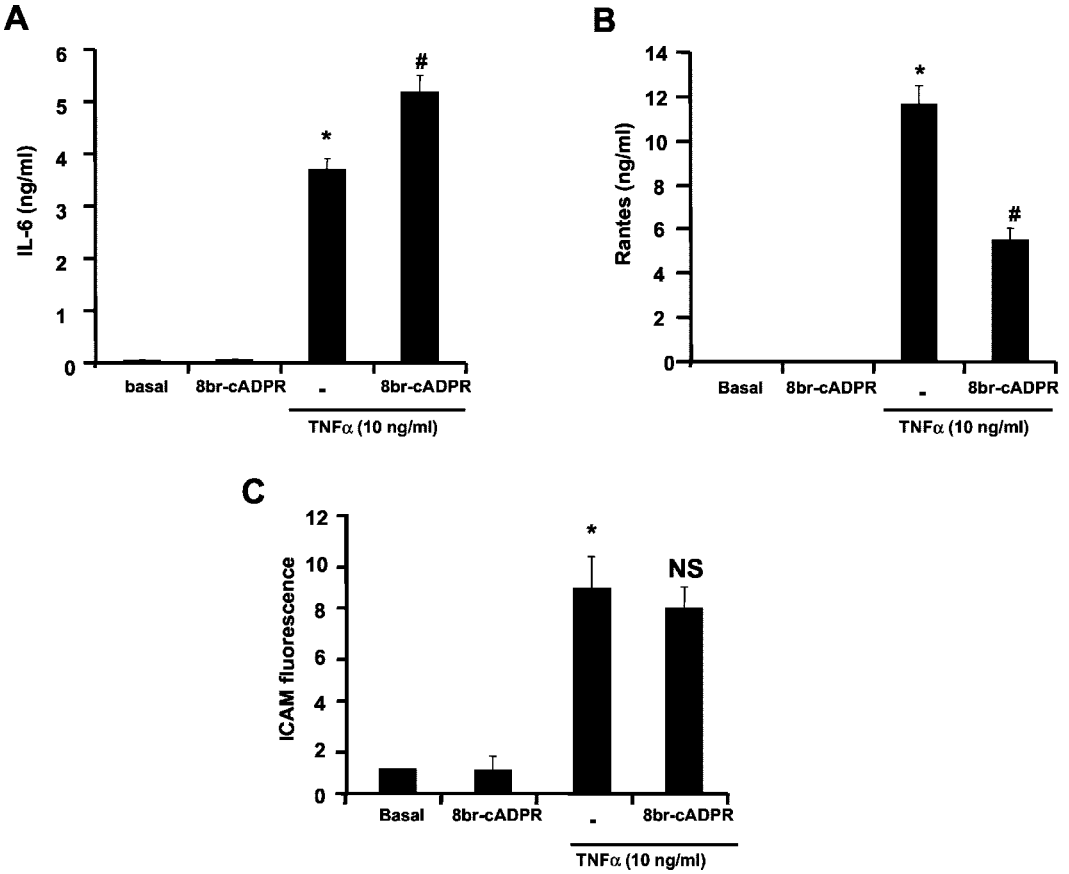

Fig. 6. 8-Br-cADPr differentially regulates TNFα-induced expression of IL-6, RANTES, and ICAM-1. Cells were stimulated for 24 h with TNFα (10 ng/ml) in the presence or absence of 8-Br-cADPr (100 μM, added for 15 min). Secretion of IL-6 (A) and RANTES (B) or expression of ICAM-1 (C) were analyzed as described under *Materials and Methods*. Values shown are mean ± S.E.M. of three separate experiments. ⋆, $P < 0.05$ compared with untreated cells; #, $P < 0.05$ compared with cells treated with TNFα alone. NS, nonsignificant compared with cells treated with TNFα alone.

involved in CD38 expression was not investigated. This is an important question, because ASM cells express both TNFR1 and TNFR2 that mediate some TNFα-induced cellular responses, such as RANTES expression (Amrani et al., 2001). Other reports performed in Hela and 293 cells also showed the contribution of both TNFR2 and TNFR1 in TNFα-induced cellular function, including apoptotic responses (Fotin-Mleczek et al., 2002) or antiviral activities (Chan et al., 2003). Our present findings suggest that activation of the CD38 gene solely involves TNFR1-associated signaling molecules, although the nature of these pathways remains unknown.

The recently described JAK inhibitor DBI (Thompson et al., 2002) in the present study completely blocked the induction of CD38 expression induced by TNFα, suggesting the contribution of the JAK/STAT pathway. In a recent report, we showed that TNFα, via the autocrine action of IFNβ, activates different members of the JAK/STAT pathways, including the kinases JAK1 and Tyk2 and the transcription factors STAT1 and STAT2 (Tliba et al., 2003). The observations that neutralizing antibodies completely blocked increased CD38 expression (at both protein and mRNA levels) and activity (cADPr production) suggest that the TNFα effect on CD38 occurred at the transcriptional level via the secretion of endogenous IFNβ. Even though CD38 gene induction by TNFα has also been observed in other excitable cell types such as human myometrium (Barata et al., 2004) and mesangial cells (Yusufi et al., 2001), our study is the first demonstration of a role of the autocrine action of IFNβ in TNFα-induced CD38/cADPr expression and activation. The effect of exogenous type I IFNs on CD38 induction remains controversial; CD38 is induced by Type I IFNs in some cell types, such as leukemic B cells and resting B lymphocytes (Galibert et al., 1996; Bauvois et al., 1999), but not in others, such as hairy leukemia cells (Hassan et al., 1991). We also found that exogenous IFNγ (Deshpande et al., 2003), or IFNβ alone (present study) failed to effectively increase CD38 levels; however, IFNβ strongly enhanced CD38 steady-state mRNA and protein levels when combined with ineffective concentrations of TNFα (0.1 and 1 ng/ml) (Fig. 5). It was interesting that the magnitude of CD38 expression induced by the combination of subthreshold concentrations of TNFα and 100 IU/ml exogenous IFNβ was greater than that induced by the effective concentration of TNFα alone (Fig. 3). This apparent discrepancy in CD38 induction may be caused by the delayed effects of endogenous IFNβ on TNFα-induced CD38 expression, which is only secreted and functional after 3 h (Tliba et al., 2003). On the other hand, exogenous and endogenous IFNβ may also manifest different cellular effects caused by access to different cellular compartments. A previous article showed that endogenous IFNβ induced intracellular signaling events without being secreted and consequently may act differently from exogenous IFNβ (Rousseau et al., 1995). Our observation in Fig. 5 underscores the cooperative action between TNFα and IFNβ receptor-coupled signaling pathways to achieve maximal CD38 gene expression. It is interesting that CD38 promoter contains binding sites for multiple transcription factors, including interferon regulatory factor-1 (Ferrero and Malavasi, 1997), known to be activated by TNFα or type I IFNs (Tliba et al., 2003). It is plausible that CD38 induction by TNFα and IFNβ may occur by increasing promoter activation through a synergistic action of interferon regulatory factor-1 with other transcription factors, including nuclear factor κB or STAT1, as described previously (Saura et al., 1999; Hiroi and Ohmori, 2003). The transcriptional cooperation observed between subeffective concentrations of TNFα and IFNβ may also involve an increased STAT phosphorylation either on tyrosine residues that augment STAT activity or on serine residues required for maximal transcription of the target gene. We found that 100 IU/ml IFNβ-induced STAT1 phosphorylation on two key residues, tyrosine 701 and serine 727, was not affected by subeffective concentrations of TNFα (Y. Amrani, unpublished observations), suggesting that additional mechanisms may explain the synergistic induction of CD38 gene by both cytokines. Such transcriptional cooperation may explain the functional synergism induced by the combination of TNFα and IFNβ found in other cells, such as the inhibition of cell proliferation in human tumor-derived cell lines and murine macrophages (Onozaki et al., 1988; Hamilton et al., 1996) and in the enhancement of the antiviral activity in human fibroblasts (Reis et al., 1989). Whether the failure of TNFα to promote CD38 expression in monocytes (Musso et al., 2001) or in endothelial cells (Favaloro, 1993) is caused by the inability to induce IFNβ in these cells is an interesting hypothesis that remains to be explored.

Our laboratories showed that CD38 enzymatic activity was increased by 3.7-fold in TNFα-treated cells compared with unstimulated cells (Deshpande et al., 2003). We now confirm that the increased CD38 expression by TNFα is associated with a 1.37-fold increase in the production of cADPr. The functional consequence of cADPr accumulation on ASM function has not been completely investigated, but our earlier studies showed that cADPr-dependent pathways are playing a critical role in mediating cytokine effects on agonist-evoked Ca^{2+} signals (Deshpande et al., 2003, 2004). We now show that ASM cells treated with TNFα in the presence of 8-Br-cADPr, a membrane-permeant antagonist of cADPr (Walseth et al., 1997; White et al., 2003), released more IL-6 and produced less RANTES. Moreover, the effect of 8-Br-cADPr seems to be gene-specific, because no effect was observed in TNFα-induced ICAM-1 expression. It is interesting that CD38 ligation using monoclonal antibodies can regulate the expression of different inflammatory mediators including IL-2, IL-10, and IL-6 in a variety of lymphoid cells such as T cells, B cells, and NK cells (Deaglio et al., 2001). Our study, however, is the first to implicate CD38/cADPr pathways in the differential induction of inflammatory cytokines in response to a physiological stimulus, TNFα. The fact that autocrine IFNβ (Tliba et al., 2003) and 8-Br-cADPr elicited similar modulatory effects on TNFα-inducible inflammatory genes (increased IL-6, reduced RANTES) suggests that the transcriptional cooperation induced by endogenous IFNβ and TNFα occurs, at least in part, via the activation of CD38/cADPr pathways.

Our data show for the first time that activation of CD38/cADPr pathways by TNFα involves the autocrine action of IFNβ and differentially regulates the expression of inflammatory genes in human ASM cells. Further studies are needed to delineate the molecular mechanisms underlying CD38 expression by TNFα as well as the CD38-associated signaling pathways that regulate inflammatory gene expression.

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