

TNF α differentially regulates the expression of pro-inflammatory genes in human airway smooth muscle cells by activation of IFN 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 TNF α induces CD38 expression as well as its role in regulating gene expression in human ASM cells. Using flow cytometry analyses, TNF α enhanced CD38 expression in a manner that was time (0-24hr), concentration (0.1-40 ng/ml) and protein synthesis (cycloheximide blockade) dependent. A selective agonistic antibody against TNFR1 also augmented CD38 expression while anti-TNFR2 antagonistic antibody did not prevent the TNF α response. Inhibition of the JAK/STAT pathways using a soluble inhibitor ((2-(1,1-Dimethylethyl)-9-fluoro-3,6-dihydro-7H-benz-[h]imidaz[4,5f]isoquinolin-7-one) or with neutralizing antibody against IFN β completely abrogated TNF α -induced CD38 expression at both protein and mRNA levels. Combining TNF α (0.1 and 1 ng/ml) and IFN β (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 TNF α -induced IL-6 secretion, while RANTES secretion was suppressed. 8-bromo-cADPr, however, did not affect TNF α -induced cell surface expression of ICAM-1. Together, our study is the first to demonstrate that IFN β -dependent activation of CD38 pathway is a novel component by which TNF α differentially regulates the expression of inflammatory genes in ASM cells.

Introduction

CD38 is a bifunctional ectoenzyme with ADP-ribosyl cyclase activity that converts the cellular intermediary metabolite βNAD^+ to cADPr, a calcium (Ca^{2+})-mobilizing second messenger. In addition, CD38 mediates the degradation of cADPr to ADPR through its cADPr hydrolase activity (reviewed in (Lee, 2001)). CD38 expression occurs widely in many mammalian cells including hematopoietic cells, such as B and T lymphocytes, macrophages, as well as resident cells such as pancreas, heart, brain, liver and lung cells, vascular and uterine smooth muscle (reviewed in (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 (Partida-Sanchez et al., 2001), as well as 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 $\text{C}\gamma$, phosphatidyl inositol 3-kinase and other tyrosine phosphorylated proteins (Shubinsky and Schlesinger, 1997). Together, these studies show 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 (GPCR) in monocytes (Partida-Sanchez et al., 2004), in arterial SM cells (Ge et al., 2003) and in airway SM cells (Prakash et al., 1998; White et al., 2003). We also found that induction of CD38 expression by $\text{TNF}\alpha$ or $\text{IL-1}\beta$ correlated with increases in Ca^{2+} signals to different GPCR agonists (bradykinin, carbachol), an effect that was abrogated by the cADPr antagonist, 8-Br-cADPr (Deshpande et al., 2003). Similar findings were also recently reported in myometrium cells where oxytocin-induced Ca^{2+} responses were enhanced by $\text{TNF}\alpha$, an effect also prevented by 8-Br-cADPr (Barata et al., 2004). Collectively, these data 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 regulate the bronchomotor tone, may represent a key mechanism underlying the development of bronchial hyper-responsiveness to GPCRs, a defining feature of asthma.

In this study, we present the first evidence that the induction of CD38 expression by $\text{TNF}\alpha$ occurs via transcriptional mechanisms involving the synergistic cooperation of endogenous $\text{IFN}\beta$. More importantly, activation of CD38/cADPr pathway by $\text{TNF}\alpha$ differentially regulates the expression of inflammatory genes. Our findings shows that, in addition to its recently shown role in promoting airway hyper-responsiveness, 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). Briefly, adherent cells were washed with PBS, detached by trypsinization (2 min, 37°C) and then washed with Ham's-F12 (10% fetal calf serum) media, centrifuged, and transferred to microfuge tubes (1.5 ml). Following incubation with the mouse anti-human CD38 antibody (2 µg/ml, Santa Cruz Biotechnology, Santa Cruz, CA) and FITC-conjugated goat anti-mouse antibody (Jackson immunoResearch, West Grove, PA), the cells were centrifuged and resuspended in cold PBS in microfuge tubes. Samples were then analyzed using an EPICS XL flow cytometer (Coulter, Hialeah, FL). ICAM-1 expression was assessed using FITC-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).

Sodium Dodecyl Sulfate–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, Santa Cruz, CA).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis. Total RNA was extracted from human ASM cells using RNeasy mini kit (Qiagen) according to the manufacturer's instructions. RT-PCR reactions were performed using human CD38

primers for semi-quantitative analysis as previously described (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 semi-quantitative 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 (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 ELISA. Confluent ASM cells were growth-arrested by incubating the monolayers in Ham's F12 with 0.1% bovine serum albumin for 24 h and stimulated with TNF α (10ng/ml) for 24 h. The concentration of IL-6 and RANTES in the culture medium was determined by ELISA as described previously (Tliba et al., 2003). To investigate the effect of CD38/cADPr pathway on TNF α induced IL-6 and RANTES expression, the 8-Br-cADPr, a membrane permeant antagonist of cADPr (100 μ M) was added 15 min before the addition of TNF α .

Measurement of cADPr levels. cADPr levels were determined by an enzymatic cycling method developed by Graeff et al. (Graeff and Lee, 2002). ASM cultures were treated in the absence or presence of TNF α for 24 hours. The media was then removed by aspiration and 5ml 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 seconds on ice and centrifuged at 2000xg for 30 minutes to remove

precipitated protein. The supernatant was evaporated to dryness using a Savant Speed-Vac concentrator. The cADPr assay depends on the conversion of cADPr to nicotinamide adenine dinucleotide (NAD) using *Aplysia* ADP-ribosyl cyclase, thus necessitating removal of endogenous NAD before the assay [Graeff, 2002 #2; Graeff, 2003 #3]. To accomplish this, we used reverse phase HPLC to separate cellular NAD from cADPr. The chromatography step utilized a LC18T column (4.6mm x 15cm, Supelco) at a flow rate of 1 ml/min with 10mM KH₂PO₄, pH 6.0. A gradient from 0-10% methanol in 10mM KH₂PO₄, pH 6.0 from 6 to 15 minutes was utilized to separate cADPr from NAD. cADPr elutes between 3 and 4 minutes while NAD elutes at approximately 14 minutes. The dried samples were reconstituted in 500µl of 10mM KH₂PO₄, pH 6.0 and filtered through 0.22µm cellulose acetate centrifugal filters (Spin-x, Corning, Inc) before injection. Fractions containing cADPr (1 ml fractions collected from 2 to 6 minutes) were dried on a Savant Speed-Vac concentrator. These fractions were reconstituted in 200µl of 100mM sodium phosphate, pH 8.0. Forty µl aliquots of the reconstituted fractions were used in the cADPr determination.

Materials and Reagents. Tissue culture reagents and primers used for PCR were obtained from Life Technologies (Grand Island, NY). Human rTNF α was provided by Boehringer Mannheim (Minneapolis, MN). rIFN γ , rIFN β , rIFN α , and the different antibodies: antagonistic anti-TNFR1, agonistic anti-TNFR1, neutralizing anti-IFN β (sheep polyclonal Ab), isotype-matched goat or mouse IgG were all purchased from R&D Systems (Minneapolis, MN). Cycloheximide was purchased from Sigma (St. Louis, MO). The JAK inhibitor (DBI) (2-(1,1-Dimethylethyl)-9-fluoro-3,6-dihydro-7H-benz[h]imidaz[4,5f]isoquinolin-7-one) 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 previously described (Walseth et al., 1997). The Sheep serum was purchased from Jackson ImmunoResearch (West Grove, PA).

Data analysis. Data points from individual assays represent the mean values of triplicate measurements. Significant differences among groups were assessed with ANOVA (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

TNF α stimulates CD38 expression and cADPr production in human ASM

Cells. In ASM cells stimulated with 10 ng/ml of TNF α for 0-24 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 hr and 24 hr, respectively ($P < 0.01$, Fig. 1A). To determine whether the TNF α effect on CD38 expression was due to protein synthesis, ASM cells were pretreated with the protein synthesis inhibitor cycloheximide (10 μ M for 1 h, as described in (Amrani et al., 2000b)). As shown in Figure 1A, cycloheximide completely inhibited TNF α -induced CD38 induction at 24 hr ($P < 0.001$). These results suggest that TNF α -induced CD38 expression required *de novo* protein synthesis. In addition, CD38 expression by TNF α (0.1-40 ng/ml, 24 hr) 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 TNF α for 24 h.

CD38 induction by TNF α 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 TNF α 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 TNF α 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 TNF α -treated cells. As shown in Figure 2B, neutralizing anti-TNFR2 antibody (20 μ g/ml, 1 hr as used in (Amrani et al., 2001)) had little effect on CD38 induction by TNF α . Collectively, these data show that TNF α stimulates CD38 expression in ASM cells mainly by activating TNFR1.

CD38 induction by TNF α requires the autocrine activation of the JAK/STAT pathways. Evidence from our laboratory showed that TNF α can activate the JAK/STAT signaling molecules via the autocrine action of endogenous IFN β (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 IFN β modulated TNF α -induced CD38 expression and cADPr production. As shown in Figure 3, neutralizing anti-IFN β antibody significantly suppressed TNF α -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 TNF α -induced CD38 expression. Neutralizing anti-IFN β antibody also completely abolished TNF α -induced cADPr production at 24 hr (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, *2-(1,1-Dimethylethyl)-9-fluoro-3,6-dihydro-7H-benz-[h]imidaz[4,5f]isoquinolin-7-one* (DBI) (Thompson et al., 2002). We found that DBI at 25 nM was effective in inhibiting IFN γ -coupled signaling pathways as shown by the dose-dependent inhibition of IFN γ -induced STAT1 phosphorylation (Fig. 4A). At this particular concentration, we also found that DBI abrogated TNF α -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 in the transcriptional activation of CD38 gene induced by TNF α .

CD38 induction by TNF α requires the synergistic cooperation of endogenous IFN β . Although autocrine IFN β (as shown above) mediated TNF α -induced CD38 expression, the mechanisms by which endogenous IFN β regulates CD38 expression are not clear. In ASM cells treated with exogenous IFN β at concentrations believed to be released by TNF α -treated ASM cells (100-IU/ml in (Tliba et al., 2003)), there was no effect on CD38 expression while 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, IFN α 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 TNF α (0.1 and 1 ng/ml as shown in Fig. 1) and IFN β (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 IFN β with 0.1 and 1 ng/ml TNF α , respectively (Fig. 5A). RT-PCR analyses of cytokine-treated ASM cells revealed that the synergistic action of TNF α and IFN β combination was also observed that the mRNA level (Fig. 5B). Together, these results suggest that the induction of CD38 gene by both TNF α and IFN β is likely to involve cooperative mechanisms that synergistically increase CD38 gene transcription.

cADPr antagonist differentially modulates TNF α -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 the absence of TNF α (Deshpande et al., 2003; White et al., 2003). Here, we

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found that 8-Br-cADPr differentially regulates TNF α -induced IL-6 and RANTES secretion while cell surface induction of ICAM-1 expression was not affected. As shown in Figure 6A, 8-Br-cADPr significantly enhanced IL-6 secretion induced by TNF α . IL-6 levels were increased from 3823 ± 120 to 5231 ± 180 pg/ml in cells treated with TNF α alone or with 8-Br-cADPr, respectively. In addition, 8-Br-cADPr considerably suppressed by 50% TNF α -induced RANTES secretion. RANTES levels were decreased from 11900 ± 523 pg/ml to 5843 ± 120 pg/ml in cells treated with TNF α alone or with 8-Br-cADPr, respectively (Figure 6B). Interestingly, 8-Br-cADPr had no effect on ICAM-1 induction by TNF α treated cells (Figure 6C). These data suggest that activation CD38/cADPr pathway differentially regulates TNF α -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 TNF α occurs via the autocrine action of endogenous IFN β and differentially regulates the expression of different inflammatory genes.

Growing evidence shows that binding of TNF α 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 Ca²⁺ homeostasis in ASM, the main effector tissue that regulates bronchomotor tone (Amrani et al., 2000a; Hunter et al., 2003; Parris et al., 1999). 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., 2001; Amrani et al., 2000b). In the present study, we found that TNF α binding to TNFR1 stimulated the expression of CD38 in human ASM cells, in a manner that was concentration (inducible at 10ng/ml), time (detectable at 12 hr) and protein synthesis (blockade by cycloheximide) dependent. Although a similar finding has been also described in human myometrium cells where induction of CD38 protein by TNF α was associated with an increased cADPr cyclase activity (Barata et al., 2004), the nature of TNF α receptor type involved in CD38 expression was not investigated. This is an important question since ASM cells express both TNFR1 and TNFR2 that mediate some TNF α -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 TNF α -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 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) and present study, completely blocked the induction of CD38 expression induced by TNF α , suggesting the contribution of the JAK/STAT pathway. In a recent report, we showed that TNF α , via the autocrine action of IFN β , activates different members of the JAK/STAT pathways including the kinases JAK1 and Tyk2, as well as 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 TNF α effect on CD38 occurred at the transcriptional level via the secretion of endogenous IFN β . Even though CD38 gene induction by TNF α has also been observed in other excitable cell types such as human myometrium (Barata et al., 2004) as well as mesangial cells (Yusufi et al., 2001), our study is the first demonstration of a role of the autocrine action of IFN β in TNF α -induced CD38/cADPr both 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 (Bauvois et al., 1999; Galibert et al., 1996) but not in others such as hairy leukaemia cells (Hassan et al., 1991). We also found that exogenous IFN γ (Deshpande et al., 2003), or IFN β alone (present study) failed

to effectively increase CD38 levels; however, IFN β strongly enhanced CD38 steady state mRNA and protein levels when combined with ineffective concentrations of TNF α (0.1 and 1 ng/ml, Figure 5). Interestingly, the magnitude of CD38 expression induced by the combination of subthreshold concentrations of TNF α and 100 IU/ml exogenous IFN β was greater than that induced by the effective concentration of TNF α alone (Fig. 3). This apparent discrepancy in CD38 induction may be due to the delayed effects of endogenous IFN β on TNF α -induced CD38 expression, which is only secreted and functional after 3 hr (Tliba et al., 2003). Alternatively, exogenous and endogenous IFN β may also manifest different cellular effects due to access to different cellular compartments. A previous article showed that endogenous IFN β induced intracellular signaling events without being secreted and consequently may act differently from exogenous IFN β (Rousseau et al., 1995). Our observation in Figure 5 underscores the cooperative action between TNF α and IFN β receptor-coupled signaling pathways to achieve maximal CD38 gene expression. Interestingly, CD38 promoter contains binding sites for multiple transcription factors including IRF-1 (Ferrero and Malavasi, 1997), known to be activated by TNF α or type I IFNs (Tliba et al., 2003). It is plausible that CD38 induction by TNF α and IFN β may occur by increasing promoter activation through a synergistic action of IRF-1 with other transcription factors including NF- κ B or STAT1 as described previously (Hiroi and Ohmori, 2003; Saura et al., 1999). The transcriptional cooperation observed between subeffective concentrations of TNF α and IFN β 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 IFN β -induced STAT1 phosphorylation on two key residues, tyrosine 701 and Serine 727,

were not affected by subeffective concentrations of TNF α (Amrani, unpublished observation), 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 TNF α and IFN β found in other cells such as the inhibition of cell proliferation in human tumor-derived cell lines and murine macrophages (Hamilton et al., 1996; Onozaki et al., 1988), and in the enhancement of the anti-viral activity in human fibroblasts (Reis et al., 1989). Whether the failure of TNF α to promote CD38 expression in monocytes (Musso et al., 2001) or in endothelial cells (Favaloro, 1993) is due to the lack to induce IFN β 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 TNF α -treated cells when compared to unstimulated cells (Deshpande et al., 2003). We now confirm that the increased CD38 expression by TNF α 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 Ca²⁺ signals (Deshpande et al., 2004; Deshpande et al., 2003). We now show that ASM cells treated with TNF α 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 since no effect was observed in TNF α -induced ICAM-1 expression. Interestingly, CD38 ligation using monoclonal antibodies can regulate the expression of different inflammatory mediators including IL-2, IL-10, IL-6 in a variety of

lymphoid cells such as T cells, B cells, NK cells (reviewed in (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 TNF α . The fact that autocrine IFN β (Tliba et al., 2003) and 8-Br-cADPr elicited similar modulatory effects on TNF α -inducible inflammatory genes (increased IL-6, reduced RANTES) suggests that the transcriptional cooperation induced by endogenous IFN β and TNF α occurs, at least in part, via the activation of CD38/cADPr pathways.

Our data shows for the first time that activation of CD38/cADPr pathways by TNF α involves the autocrine action of IFN β 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 TNF α as well as the CD38-associated signaling pathways that regulate inflammatory gene expression.

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Footnotes:

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Figure legends

Fig. 1. TNF α induces CD38 expression. ASM cells were incubated with 10 ng/ml of TNF α for the indicated time (*left panel: A*) or for 24 h at the indicated concentrations (*right panel: B*). ASM cells were also treated with cycloheximide (CHX, 10 μ g/ml) added 1 h prior TNF α for 24 hr. CD38 expression was assessed by flow cytometry as described in *Materials and Methods*. The results are expressed as mean of fold increase over basal \pm SEM of three separate experiments. * P < 0.05 and ** P < 0.05 compared with untreated cells. # P < 0.05 compared with cells treated with TNF α alone.

Fig. 2: TNFR1 mediates TNF α -induced CD38 expression. A, human ASM cells were incubated for 24 h with either TNF α (10 ng/ml), agonistic antibody to TNFR1 (5 μ g/ml) or the isotype-matched antibody. B, human ASM cells were incubated for 24 h with TNF α (10 ng/ml) in the presence or the absence of neutralizing antibody to TNFR2 or the isotype-matched antibody (10 μ g/ml, 1 h prior cytokine). CD38 expression was then assessed by flow cytometry as described in *Materials and Methods*. The results are expressed as mean of fold increase over basal \pm SEM of three separate experiments. * P < 0.05 compared with untreated cells.

Figure 3. TNF α , via the autocrine action of secreted IFN β , induces CD38 gene expression. Cells were stimulated for 24 h with TNF α (10 ng/ml) in the presence or absence of neutralizing anti-IFN β (5 μ g/ml) or sheep serum (control) added 15 min before. A, CD38 protein expression was analyzed by flow cytometry as described in *Materials and Methods*. The results are expressed as mean of fold increase over basal \pm SEM of three separate experiments. * P < 0.05 compared with untreated cells. # P < 0.05 compared with cells treated with TNF α alone. B, Cells were lysed, total mRNA was

isolated, and RT-PCR was performed as described in *Materials and Methods* using specific primers for CD38. The lower panel is the scanning densitometric of the representative gel (upper panel) with each value normalized over the mean density of the corresponding GAPDH PCR products.

Figure 4. TNF α induced CD38 expression via the JAK/STAT pathways. A, human ASM cells were treated IFN γ (100UI/ml) for 15 min in the presence or absence of the indicated concentration of JAK inhibitor (called 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 in *Materials and Methods*. Results are representative of three separate blots. B-C, Human ASM cells were stimulated with 10 ng/ml of TNF α in the presence or absence of the JAK inhibitor I (DBI) (25 nM) or DMSO 0.1% added 30 min before. B, CD38 protein expression was analyzed by flow cytometry as described in *Materials and Methods*. The results are expressed as mean of fold increase over basal \pm SEM of three separate experiments. * P < 0.05 compared with untreated cells. # P < 0.05 compared with cells treated with TNF α 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 in *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.

Figure 5. Synergistic activation of CD38 gene by TNF α and IFN β . Cells were stimulated for 24 h with the indicated concentration of TNF α and IFN β (100 U/ml) alone or in combination. A. CD38 protein expression was analyzed by flow cytometry as described

in *Materials and Methods*. The results were expressed as mean of fold increase over basal \pm SEM 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 in *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.

Figure 6. 8-Br-cADPr differentially regulates TNF α -induced expression of IL-6, RANTES and ICAM-1. Cells were stimulated for 24 h with TNF α (10 ng/ml) in the presence or absence of 8-Br-cADPr (100 μ M, added 15 min). Secretion of IL-6 (A) and RANTES (B) or expression of ICAM-1 (C) were analyzed as described in *Materials and Methods*. Values shown are mean \pm SEM of three separate experiments. $*P < 0.05$ compared with untreated cells. $^{\#}P < 0.05$ compared with cells treated with TNF α alone. NS, non-significant when compared with cells treated with TNF α alone.

Figure 1

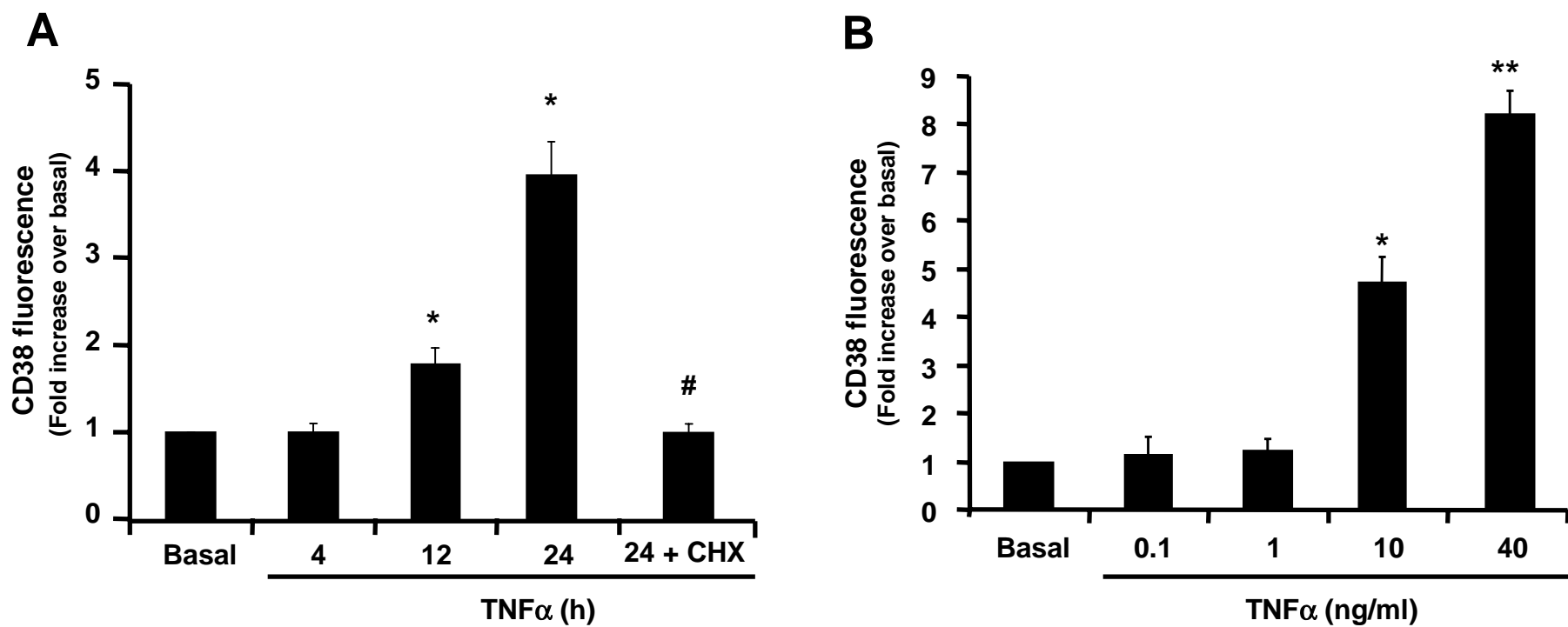


Figure 2

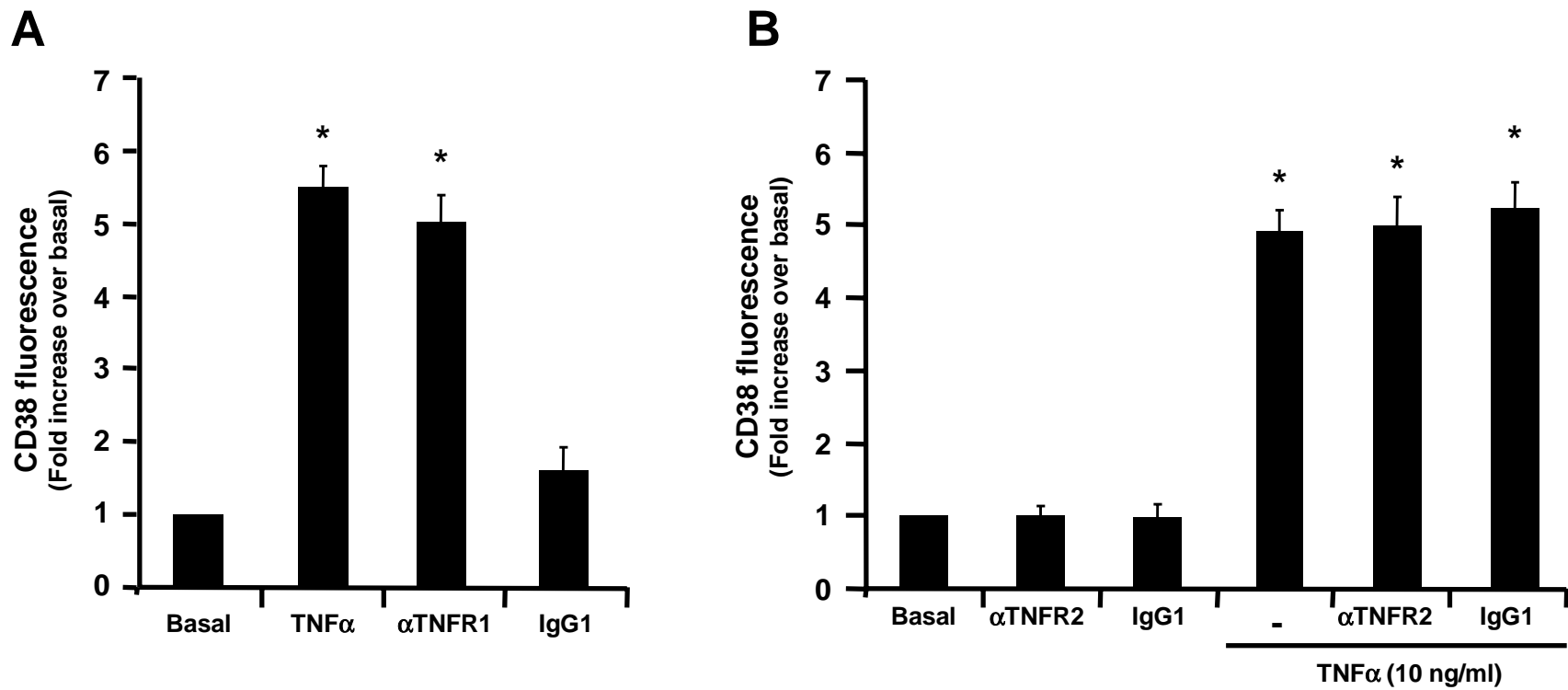


Figure 3

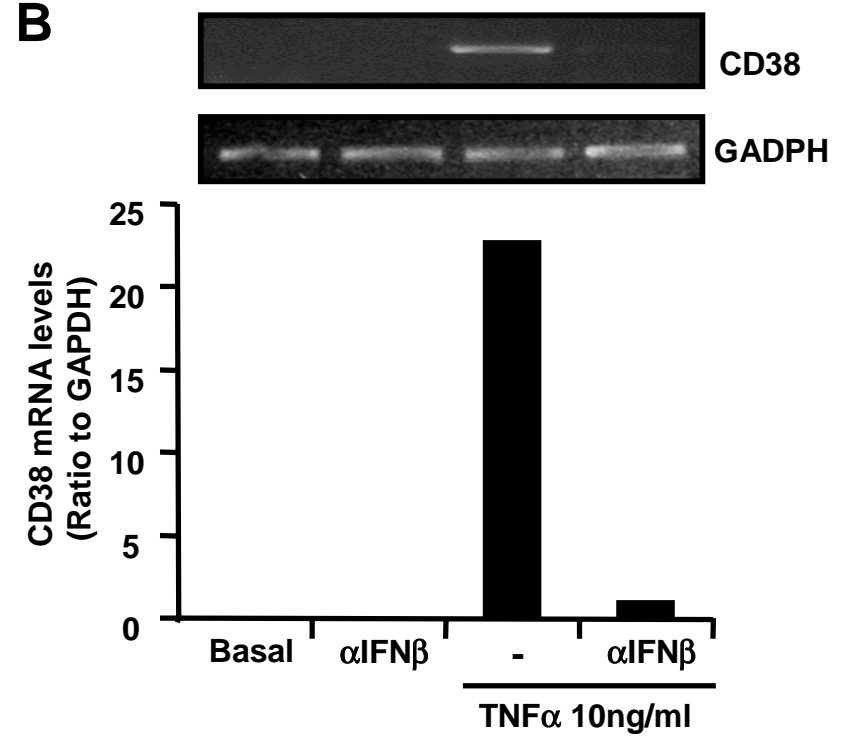
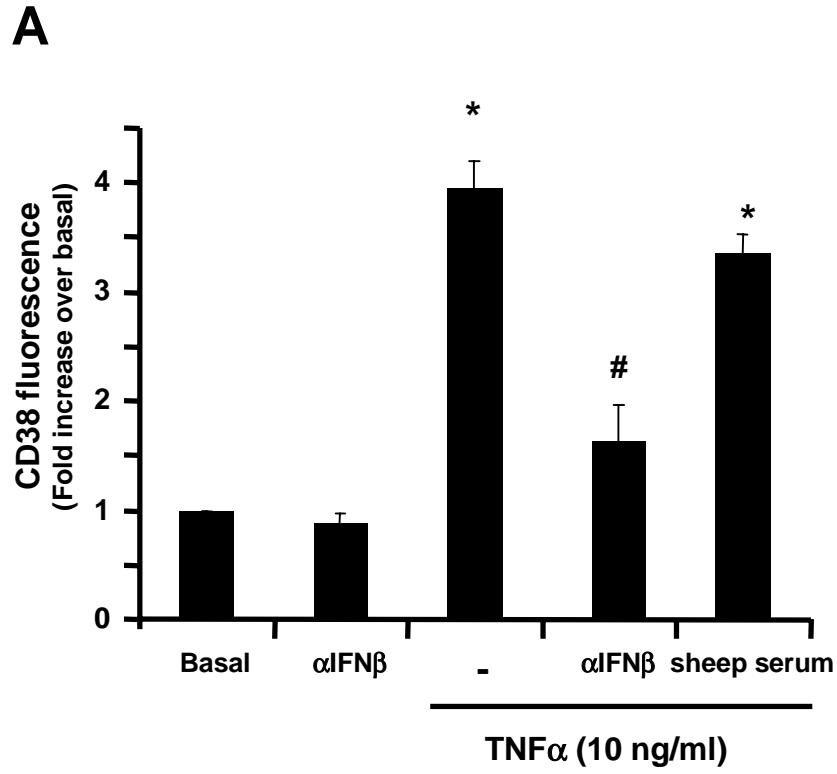
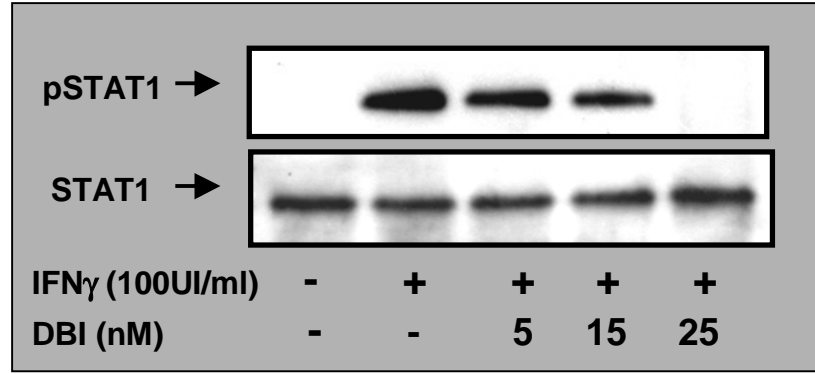
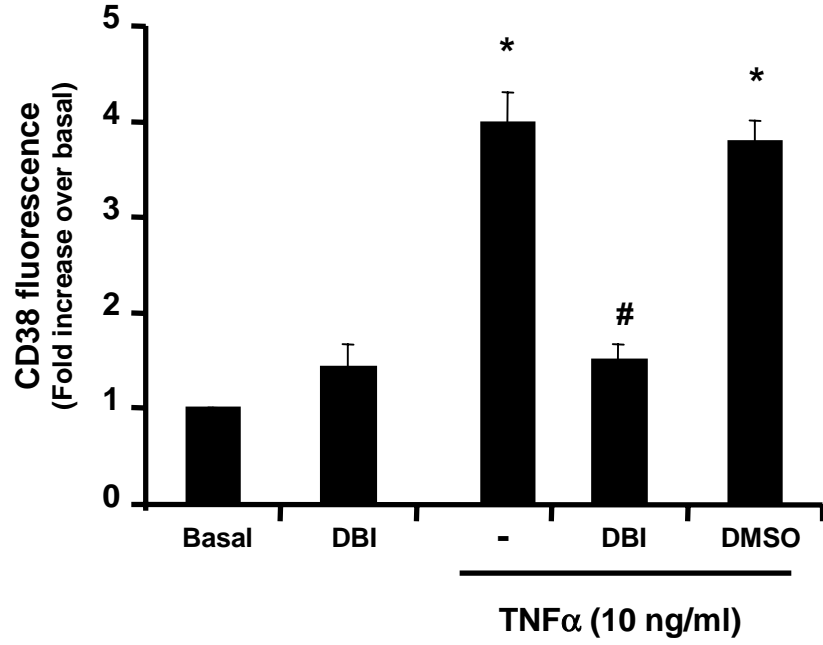


Figure 4

A



B



C

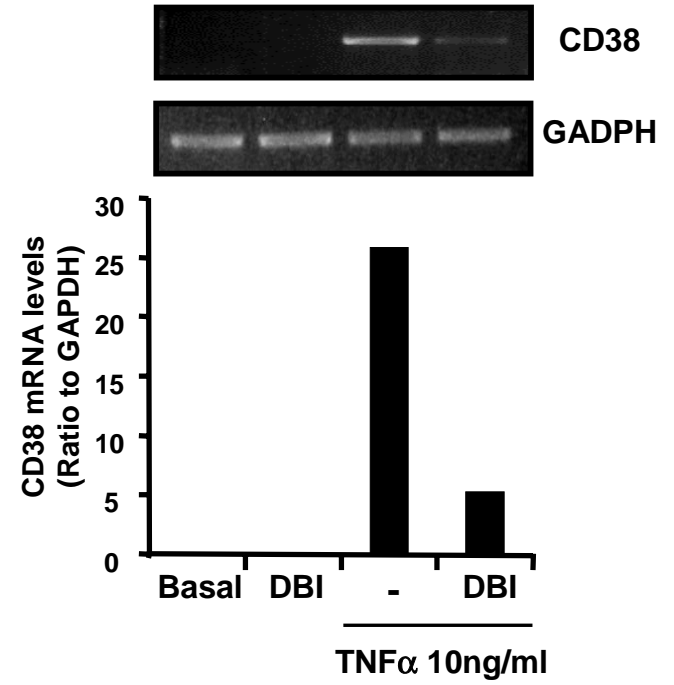


Figure 5

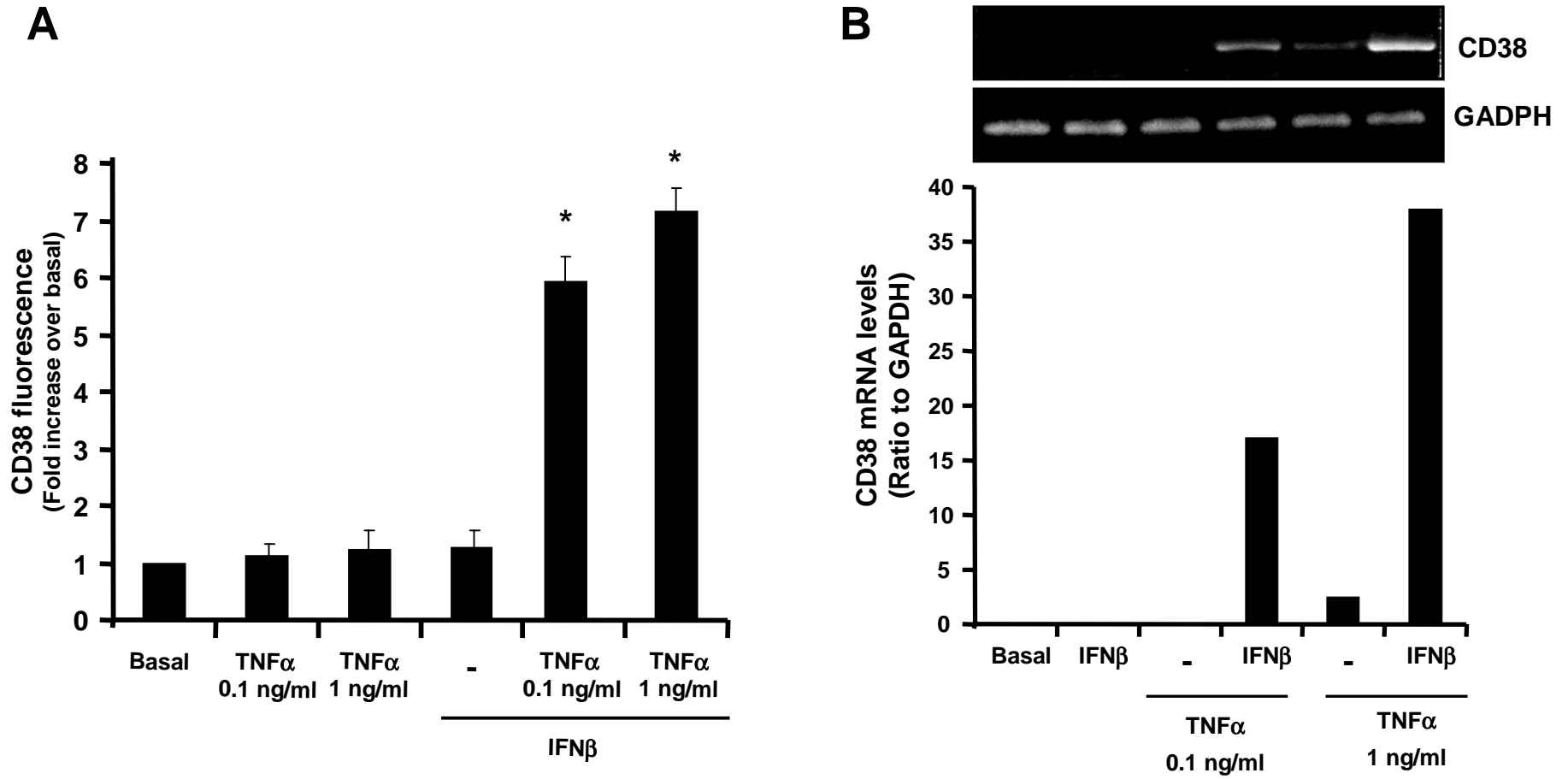


Figure 6

