CD38 expression is insensitive to steroid action in cells treated with TNFα and IFNγ by a mechanism involving the upregulation of glucocorticoid receptor β isoform.

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ABSTRACT

Evidence shows that the CD38 molecule, recently involved in the two main features of asthma, bronchial hyper-responsiveness and airway inflammation, could represent a new potential therapeutic target for asthma. Here, we investigated whether glucocorticoid (GC), the most effective treatment for lung diseases, can affect CD38 expression in human airway smooth muscle (ASM) cells treated with different pro-inflammatory cytokines such as TNFα and IFNs. We found that CD38 expression induced by TNFα alone was completely abrogated by fluticasone (100 nM), dexamethasone (1 μM) or budesonide (100 nM). In contrast, the synergistic induction of CD38 by the combination of TNFα with IFNγ or IFNβ, but not with IL-1β or IL-13, was completely insensitive to the GC inhibitory effects. We also found that TNFα and IFNγ impaired GC responsiveness by inhibiting steroid induced both i) GRα-DNA binding activity, and ii) GC-Responsive-Elements-(GRE)-dependent gene transcription. While levels of GC receptor (GR) alpha isoform remained unchanged, expression of GRβ, the dominant negative GR isoform, was synergistically increased by TNFα and IFNγ with a GRα/GRβ ratio of 1 to 3. More importantly, fluticasone failed to induce GRE-dependent gene transcription and to suppress TNFα-induced CD38 expression in ASM cells transfected with constitutively active GRβ. We conclude that, upon pro-inflammatory cytokine stimulation, CD38 expression becomes insensitive to GC action by a mechanism involving the upregulation of GRβ isoform, thus providing a novel *in vitro* cellular model to dissect GC resistance in primary cells.

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CD38 is an ectoenzyme that converts the cellular intermediary metabolite βNAD^+ to cyclic ADP ribose (cADPr), a Ca^{2+}-mobilizing second messenger. Previous reports using 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, cytokine production from B and T cells (Funaro et al., 1997), neutrophil migration (Deaglio et al., 2003), and neurotransmission and cardiac contraction (Partida-Sanchez et al., 2001). Interestingly, we showed that CD38 pathways also mediate TNFα-enhancing effect on agonist-evoked Ca^{2+} responses (Deshpande et al., 2003), and proposed that abnormal CD38 signaling may represent a novel mechanism involved in the increased airway narrowing observed in asthmatic patients (Amrani et al., 2004; Tliba et al., 2004). We also found that a combination of pro-asthmatic cytokines such as TNFα and IFNs synergistically increased CD38 expression both at protein and mRNA levels (Tliba et al., 2004). Furthermore, in addition to modulating contractile function, we recently reported a putative role of CD38/cADPr pathway in the regulation of different inflammatory genes, such as IL-6 and RANTES, important in the pathogenesis of asthma (Tliba et al., 2004). Collectively, these observations suggest that abnormal CD38 function and/or expression could play a critical role in two main features of asthma, bronchial hyper-responsiveness and airway inflammation. Thus, a better understanding of both the factors and the mechanisms that regulate CD38 activity will provide new therapeutic options for treating airway inflammatory diseases.

Glucocorticoids (GCs) are the treatment of choice for chronic inflammatory diseases such as asthma (Lemanske and Busse, 2003). Most of their anti-inflammatory effects are mediated via glucocorticoid receptor alpha isoform (GRα) which suppresses expression of inflammatory genes through mechanisms known as transactivation or transrepression (Leung and Bloom,

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2003). Transactivation results from a direct binding of activated GRα to DNA sequences called GC-response elements (GRE) present on inducible anti-inflammatory genes. Transrepression is mediated via the direct interaction between activated GRα and different transcription factors (TF), such as NF-κB and AP-1, thus repressing TF-inducible pro-inflammatory genes (Pujols et al., 2004). Interestingly, in addition to GRα, and as a result of alternative splicing mechanisms, another GR isoform, namely GRβ, has been described (Hollenberg et al., 1985). Although the role of GRβ is not well understood, a previous report in immune and transformed cells demonstrated its dominant negative effects on GRα-dependent transcriptional activities (Leung et al., 1997).

Increasing evidence suggests that ASM cells play a central role in the pathogenesis of asthma (Amrani and Panettieri, 2003; Hunter et al., 2003; Parris et al., 1999). In fact, when exposed to inflammatory conditions, ASM can become a source of different chemokines/cytokines that are capable of orchestrating and/or perpetuating airway inflammation (Howarth et al., 2004). Although the GC effects on human ASM cells have been investigated, their modulatory effects on gene expression are quite complex, and the signaling mechanisms underlying their suppressive effects have been poorly characterized. First, the effects of GC seem to be highly gene-specific. For example, reports showed that dexamethasone can effectively inhibit either cytokine-induced IL-6, RANTES (Ammit et al., 2002), eotaxin (Pang and Knox, 2001) or COX-2 expression (Vlahos and Stewart, 1999), while it has no effect on cytokine-induced ICAM-1 expression (Amrani et al., 1999). Second, GC suppressive effect seems to be time-dependent since dexamethasone partially abrogates cytokine-mediated ICAM-1 expression at early time points, but had no effect at later time points (Amrani et al., 1999). Third, unexpectedly, instead of acting as potent inhibitors, GC synergistically enhanced cytokine-

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induced fractalkine expression and secretion (Sukkar et al., 2004). Finally, GC inhibitory action is also stimuli-specific. Indeed, while dexamethasone inhibits significantly IL-1β-induced GM-GSF secretion, it inhibits only partially GM-GSF secretion induced by thrombin. Collectively, these observations demonstrate that ASM is a unique and complex model where GC actions are differentially modulated by cytokines.

In the present report, we demonstrate that CD38 expression induced by cytokine combination becomes refractory to the suppressive action of steroid. We found that the specific combination of TNFα with IFNs, but not with IL-1β or IL-13, reduces ASM cell responsiveness to different classes of GC including fluticasone, dexamethasone and budesonide. This steroid insensitivity appears to be the result of increased levels of GRβ that impair GRα transcriptional activity.

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MATERIALS AND METHODS

ASM Cell Culture and Treatment - 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 (Panettieri et al., 1989).

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. In preliminary experiments, we determined, for each primer pair, the melting temperature and number of amplification cycles necessary to yield the appropriate hybridization signal. The PCR of CD38, GRα, GRβ and β-actin was performed using previously published primers (Orii et al., 2002; Tliba et al., 2004). 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 was performed in parallel by investigating the β-actin mRNA level. The intensity of the area density was analyzed using a Gel-Pro Analyzer (Media Cybernetics, Silver Spring, MD) and the final PCR product was expressed as a ratio of area density of CD38, GRα, or GRβ to β-actin.

Real time PCR - Real time PCR analysis was performed on a Cepheid Smart Cycler (Cepheid, Sunnyvale, CA) by using SYBR-Green kit according to the manufacturer's instructions (Roche Diagnostics, Indianapolis, IN). PCR was performed using the same GRα, GRβ and β-actin primers in a total volume of 25 µl in the presence of SYBR Green PCR Master Mix. For GRα and GRβ PCR amplification, the program consists of 60 s for the initial denaturation period at 95°C, and 45 cycles of 95°C for 1 s, 60°C for 5 s, and 72°C for 15 s. For β-actin, the program consists of 60 s for the initial denaturation period at 95°C, 40 cycles of 95°C for 1 s, 55°C for

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5 s, and 72°C for 18 s. The results were calculated using a quantitation approach termed the comparative cycle threshold (C_t) method as described elsewhere (Ponchel et al., 2003).

GR-DNA binding activity - Nuclear extraction was performed as described previously (Tliba et al., 2003). Ten μg of nuclear extracts were tested for GR-DNA binding activity by using TransAM™ GR kit according to the manufacturer's instructions (Active Motif, Carlsbad, CA). The results (optical densities measured at 450 nm) were expressed as a percentage of increase over untreated cells.

SDS-Polyacrylamide Gel Electrophoresis and Western Blot Analysis - Immunoblot analyses for GRα and GRβ were performed as described previously (Tliba et al., 2003) using specific anti-GRα and anti-GRβ antibodies (Affinity BioReagent, Golden, CO, USA). To ensure equal loading, the membranes were stripped and reprobed with anti-actin antibody (Santa Cruz Biotech. Inc.). The area densities for each GR isoform and the total actin were calculated using a Gel-Pro Analyzer (Media Cybernetics, Silver Spring, MD) and the data were expressed as a ratio of area density of GRα or GRβ to actin.

Transfection of ASM cells - Since most standard transfection methods yield poor transfection efficiencies for ASM cells (<10%), here we have optimized a high-efficiency transfection technique. This technique is an extension of electroporation, using Nucleofector kit for primary smooth muscle (Amaxa Biosystems, Germany), where plasmid DNA is transfected directly into the cell nucleus. Transfection was performed according to the manufacturer's instructions and the program used was D-33. Sixteen to 18 hours after transfection, the media were changed with serum-free media for the next 24 hr. Transfection efficiency of GFP-tagged plasmids was monitored by flow cytometry as described below. The use of this method using GFP (green

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fluorescence protein)-pmax control vector (Amaxa Biosystems, Germany) enabled us to reach a transfection efficiency of 70%.

Recombinant plasmids - To assess the dominant negative activity of GRβ, ASM cells were transfected with constitutively active (CA) GRβ GFP-tagged (Oakley et al., 1996) or with pCMV-GFP empty vector (Clontech, Palo Alto, CA). To monitor fluticasone transactivating activity, cells were co-transfected with GRE (glucocorticoids responsive element)-SEAP (secreted alkaline phosphatase) reporter vector and with pSV-β-galactosidase vector used to normalize transfection efficiencies (Promega, Madison, WI).

SEAP and β-galactosidase Assays - The activities of SEAP and β-galactosidase in the supernatants were evaluated using Great EscAPe SEAP detection kit (Clontech, Palo Alto, CA) and β-galactosidase detection kit (Promega, Madison, WI) respectively according to their manufacturer's instructions (An et al., 2005; Tliba et al., 2003).

Fluorescence-activated cell sorter (FACS) analysis - Flow cytometry was performed as described previously (Tliba et al., 2002). Antibody used for CD38 expression was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Phycoerythrin (PE)-conjugated secondary antibody was bought from Jackson ImmunoResearch Laboratories (West Grove, PA). GRβ-GFP transfection efficiency was monitored by a shift to green fluorescence (FL-1), and CD38 expression level was monitored by a shift to red fluorescence (FL-2). FACS analysis was performed with EPICS XL flow cytometer (Beckman Coulter, Inc., Hialeah, FL) using the Cellquest software.

Materials and Reagents - Tissue culture reagents and primers were obtained from Invitrogen (Carlsbad, CA). Human recombinant (r) TNFα, rIL-1β and rIFNγ were provided by Roche Diagnostics (Indianapolis, IN). Human rIFNβ and rIL-13 were purchased from R&D Systems

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(Minneapolis, MN). Fluticasone propionate (FP) was generously supplied by GlaxoSmithKline, Inc. (King of Prussia, PA). Dexamethasone and budesonide were obtained from Sigma (St. Louis, MO).

Statistical 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.

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RESULTS

Fluticasone inhibits in a dose-dependent manner TNFα-induced CD38 expression – We first investigated the effect of GC on TNFα-induced CD38 expression, a gene potentially involved in modulating airway inflammation via the transcriptional regulation of different inflammatory mediators (Tliba et al., 2004). For this purpose, human ASM cell cultures were pretreated for 2 hr with increasing concentrations of fluticasone (1-100 nM) prior to TNFα treatment, and CD38 expression was measured by RT-PCR. As shown in Fig. 1A, fluticasone at 100 nM completely inhibited TNFα-induced CD38 mRNA expression ($P < 0.05$). In addition, flow cytometry analyses revealed that fluticasone also inhibited, in a concentration-dependent manner, TNFα-induced CD38 expression ($P < 0.05$) with a complete inhibition achieved at 100 nM (CD38 level decreased from 5.2 ± 0.26 to 1.42 ± 0.13 fold increase over basal in cells treated with TNFα in the absence or presence of fluticasone, respectively) (Fig. 1B and 1C). In parallel experiment, using MTT assay (Amrani et al., 1996), we found that fluticasone did not affect ASM cell viability (data not shown). Collectively these data suggest that GC suppresses TNFα-induced CD38 expression by involving transcriptional mechanisms.

TNFα combined to IFNs (β or γ), but not to IL-1β or IL-13, dramatically reduces CD38 sensitivity to fluticasone – Because TNFα cooperates with other cytokines to regulate the expression of different inflammatory genes in ASM cells (Tliba et al., 2003), we next investigated whether cytokine combination would induce a greater increase in CD38 expression and whether this response would be sensitive to steroid. We found that IFNγ (500 UI/ml) (Fig. 2A), IFNβ (500 UI/ml) (Fig. 2B), IL-1β (10 ng/ml) (Fig. 3A), or IL-13 (50 ng/ml) (Fig. 3B) induced a weak but significant increase in CD38 expression with a 2 ± 0.1, 2.2 ± 0.05, 2.25 ±

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0.12 and 1.7 ± 0.09 fold increase over basal, respectively. In addition, while IL-1β and IL-13 showed no additive effect when combined with TNFα (Figs. 3A and 3B), IFNγ and IFNβ enhanced significantly TNFα-induced CD38 expression by 13.8 ± 0.15 and 16.2 ± 0.28 fold increase over basal, respectively (Figs. 2A and 2B). Interestingly, we found that CD38 induction in cells treated with the combination of TNFα with either IFNγ (Fig. 2A) or IFNβ (Fig. 2B) was completely insensitive to effective concentration of fluticasone. However, fluticasone was still effective in preventing CD38 induction in cells treated with the combination of TNFα with either IL-1β (Fig. 3A) or IL-13 (Fig. 3B). Similar results were obtained with other steroids, including dexamethasone (1 μM) or budesonide (100 nM), known to be potent inhibitors of different inflammatory genes in ASM cells (Ammit et al., 2002; Roth et al., 2002) (Fig. 4). Collectively, these data suggest TNFα and IFNs is an effective combination to i) synergistically induce CD38 expression, and ii) to dramatically reduce the effect of different synthetic GCs.

TNFα and IFNγ together alter steroid-induced transactivation activities – The inability of GC to suppress CD38 induced by TNFα and IFNγ (Figs. 2 and 4) may involve an alteration of GC transactivation activities. As shown in Fig. 5A, fluticasone significantly enhanced GR-DNA binding activity by 51 ± 5.2% compared to untreated cells, an effect that was completely abrogated when an excess of wild-type competitor oligonucleotides, but not with mutated control oligonucleotides, was added. Interestingly, as seen with wild-type oligonucleotides, TNFα and IFNγ completely prevented fluticasone-induced GR-DNA binding activity. Further, in ASM cells transfected with a reporter construct containing SEAP gene driven by GRE motifs, we found that fluticasone significantly enhanced SEAP activity by 25 ± 3.1% compared to untreated cells, a response that was dramatically reduced by 70% when TNFα and IFNγ were added (Fig. 5B).

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These data suggest that TNFα and IFNγ together impair GC cellular response by reducing both GR-DNA interaction and GR transactivating activity.

TNFα and IFNγ enhance selectively the expression of GRβ isoform – Evidence suggests that changing the GRα/GRβ cellular ratio in different inflammatory conditions, where GRβ predominates, may promote GC resistance (Pujols et al., 2004). Therefore, we next investigated whether cytokine combination could alter GRα/GRβ ratio in human ASM cells. Using RT-PCR analyses, we found that the steady state of GRα mRNA levels, which is constitutively expressed in untreated cells, was not affected by TNFα or IFNγ either alone or in combination (Fig. 6A). Similar results were obtained by real time PCR (data not shown). Even though TNFα or IFNγ alone have only a slight stimulatory effect on GRβ levels, their combination significantly increased by 470 % the steady state of GRβ mRNA levels (Fig. 7A), a result that was also confirmed by real time PCR (Fig. 7B). Using immunoblot analyses, we found that while the constitutive expression of GRα protein was not affected by TNFα or IFNs (β or γ) either alone or in combination (Fig. 6B), GRβ protein expression was significantly increased by TNFα and IFNs combination (Fig. 7C, 7D). Semi-quantitation analyses of GR isoform expression showed that GRα/GRβ ratio was 8 to 1 in untreated cells, 1 to 1 in TNFα or IFNβ or IFNγ-treated cells, and 1 to 2 and 1 to 3 in TNFα/IFNβ and TNFα/IFNγ-treated cells, respectively. Collectively, these data demonstrate that in cells exposed to cytokine combination, GRβ becomes the predominant GR isoform.

GRβ over-expression prevents GC-mediated transactivation and reduces the sensitivity of CD38 to fluticasone – To further support the association between the reduced action of GC (Figs. 2, 4 and 5) and the enhancement of GRβ expression (Fig. 7) in cells treated with cytokine

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combination, we next examined the effect of GRβ over-expression on GC activities. To determine whether GRβ interferes with GC-induced transactivation activity, ASM cells were co-transfected with 2 μg of CA-GRβ or pCMV empty vector, and 2 μg of SEAP-reporter construct containing GRE motifs. As shown in Fig. 8A, fluticasone-induced GRE-dependent reporter activity was completely abrogated in GRβ– but not in pCMV-transfected cells. To test whether GRβ interferes with GC actions on CD38 expression, ASM cells were transfected with 4 μg of CA-GRβ GFP-tagged then treated with TNFα for 24 hr in the presence or absence of fluticasone, and CD38 expression in GRβ-transfected cells was assessed by two-color flow cytometry. As shown in Fig. 8B, TNFα significantly enhanced CD38 expression in GRβ-transfected cells (35.1 $\pm$ 2.2% fold increase over basal), a response that was completely insensitive to fluticasone action (filled bars). However, fluticasone was still effective in inhibiting TNFα-induced CD38 expression in cells transfected with control vector (pCMV-GFP) (open bars). Collectively, these data suggest that GRβ upregulation is associated with a significant reduction of GC activities in ASM cells.

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DISCUSSION

We and others showed that TNFα and exogenous IFNs synergistically regulate expression of a variety of pro-inflammatory genes including cytokines, chemokines and growth factors in ASM cells (Chung, 2000; Tliba et al., 2004). Our latest studies also showed that CD38, an ectoenzyme recently involved in asthma (Deshpande et al., 2003; Tliba et al., 2004), was also responsive to the synergistic action of TNFα and IFNs in ASM cells (Tliba et al., 2004). In the present study, we report that the induction of CD38 induced by both TNFα and IFNs, but not by cytokine alone, is refractory to the anti-inflammatory action of steroids. To our knowledge, this is the first report showing that expression of CD38 can become refractory to the suppressive action of synthetic GC in clinically relevant cells.

Our data show the TNFα and IFNs render ASM cells insensitive to GC as well as reduce GC-induced transactivation activities. This is an important finding as numerous studies suggest a strong correlation between GC insensitivity and a variety of inflammatory diseases, such as asthma, nasal polyps and inflammatory bowel disease (Adcock et al., 1995; Farrell and Kelleher, 2003; Honda et al., 2000). These observations prompted investigators to examine whether inflammatory mediators could be responsible for the altered GC cellular responses. Impairment of GC activity can be reproduced *in vitro* in immune cells exposed to IL-2 and IL-4 that reduced the inhibitory effect of methylprednisolone on mitogen-induced T cell proliferation (Leung and Bloom, 2003). Other studies in human peripheral blood mononuclear cells (PBMC) showed that the combination of IL-1β, IL-6 and IFNγ also decreased the suppressive action of dexamethasone on mitogen-induced proliferation (Almawi et al., 1991). In T cells, monocytes and neutrophils, GC action is as well impaired when exposed to a single cytokine such as IL-2, IL-7, IL-13, IL-15, or IL-8 (Goleva et al., 2002; Pipitone et al., 2001; Spahn et al., 1996;

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Strickland et al., 2001). While all *in vitro* studies describing GC insensitivity have been performed in either immune cells or transformed cells, very little is known about the modulation of GC signaling in structural cells that are relevant for lung diseases. Interestingly, we now show that the specific combination of TNFα with IFNs reduces ASM cell responsiveness to different classes of GC including fluticasone, dexamethasone and budesonide (Figs 2 and 3). This TNFα- and IFNγ-dependent GC insensitivity was not observed when TNFα was combined with other cytokines, such as IL-1β or IL-13 although these latter cytokines significantly reduced GC cellular response in other cell types (Pariante et al., 1999; Spahn et al., 1996; Webster et al., 2001). Our findings could explain, at least in part, the lack of dexamethasone to suppress fractalkine secretion in ASM cells treated similarly with both TNFα and IFNγ (Sukkar et al., 2004). These data suggest that TNFα and IFNs is a new cytokine combination inducing GC insensitivity in human ASM cells, thus providing a novel in vitro model to dissect GC insensitivity in structural cells. The mechanisms underlying TNFα/IFNs suppressive effects on GC signaling and function are unknown but our study demonstrates the potential role of GRβ.

The fact that the inhibitory effect of cytokine combination on GC actions could be mimicked in cells transfected with CA-GRβ (Fig. 8) strongly suggests that GRβ represents one molecular mechanism responsible for the impaired GC activity. We found that TNFα and IFNs combination significantly increased GRβ isoform at both mRNA and protein levels but had a weak effect on GRα expression. In PBMC, where the levels of GRβ were significantly increased by IL-7 or IL-18, no changes in GRα levels were observed (Orii et al., 2002). In neutrophils, however, IL-8 enhanced GRβ expression while the expression of GRα isoform was decreased (Strickland et al., 2001). Moreover, (Torrego et al., 2004) showed that IL-2 and IL-4 combination had no effect on GRβ expression but enhanced significantly GRα expression

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contrasting with the selective induction of GRβ by the same cytokine combination observed in Leung and colleagues study (Leung et al., 1997). Together, these observations suggest that GR isoforms are differentially regulated in a manner that is complex, stimuli- and cell-specific. The important question that remains still unanswered is the nature of the specific mechanisms that regulate the selective induction of GRβ. Importantly, since GRβ expression is a result of alternative splicing (Hollenberg et al., 1985), it is plausible that the selective GRβ induction by TNFα and IFNs may be due to the possible activation of specific alternative splicing factors. Indeed, (Xu et al., 2003) showed that increased levels of serine-arginine rich protein (SR) SRp30 is required for the alternative splicing of GR pre-mRNA to GRβ isoform in neutrophils. In our study, cytokine combination failed to increase SRp30 content in ASM cells (Tliba and Amrani, unpublished observation). Further investigations are needed to determine the alternative splicing factors that are involved in the differential induction of GR isoforms in ASM cells.

To date, the inhibitory role of GRβ on GRα activity remains controversial. (Oakley et al., 1999) found that the transient transfection of increasing amounts of GRβ-expressing vectors into immune and transformed cells inhibits GC actions by blocking the trans-activating ability of GRα. In contrast, (de Lange et al., 1999; Hecht et al., 1997) were unable to confirm the inhibitory role for GRβ when overexpressed into COS-1 cells. These controversial results may be explained by the use of different experimental conditions such as the transfection methods, the promoter used to overexpress GRβ, and the relative amounts of transfected plasmids, which could lead to insufficient GRβ expression. Indeed, a high expression level of GRβ isoform is necessary in order to significantly inhibit GC-mediated gene expression in COS-1 cells (Oakley et al., 1999). This hypothesis is supported in our study by the fact that in cells exposed to cytokine alone (TNFα or IFNγ), where GRβ expression was slightly enhanced reaching a level

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comparable to that of GRα (Figs. 6 and 7), fluticasone was still able to suppress cytokine-induced CD38 expression (Fig. 1). In contrast, we showed that in cells resistant to fluticasone action, i.e., treated with both TNF/IFNs (Fig. 2), GRβ level was significantly predominant over that of GRα (ratio GRα:GRβ was ~ 1:3). The dominant negative properties of GRβ were difficult to demonstrate in primary cells since most standard transfection methods yield poor transfection efficiencies (<10%). To overcome the low transfection efficiencies, we have optimized a high-efficiency transfection technique. This technique is an extension of electroporation, using Nucleofector kit for primary smooth muscle (Amaxa Biosystems, Germany), where plasmid DNA is transfected directly into the cell nucleus. Using this method, we showed that the GC ability to block TNFα-induced CD38 expression as well as to induce GRE-dependent gene transcription was completely impaired in ASM cells transfected with CA-GRβ (Fig 8). To our knowledge, this is the first study in primary cells showing that pro-asthmatic cytokines alter steroid function via the induction of GRβ.

In summary, we found that TNFα and IFNs combination renders CD38 expression refractory to the suppressive action of steroid, by a mechanism involving the GRβ-dependent alteration of GRα signaling and function. Interestingly, the importance of IFNs in promoting GC insensitivity in airway resident cells could explain, at least in part, the increased GC requirements in severe asthma patients experiencing viral infections that produce high levels of IFNs in the airways (Johnston, 1999; Yamada et al., 2000). Our study opens a new area of investigation to determine the molecular mechanisms by which cytokine-induced GRβ expression impairs GC signaling in structural cells.

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FOOTNOTES

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FIGURE LEGENDS

Figure 1. Dose-dependent effect of fluticasone (FP) on TNFα-induced CD38 expression in ASM cells. Cells were pretreated with indicated concentrations of FP for 2 hr and then stimulated with TNFα (10 ng/ml) for 24 hr. (A) Representative Agarose gel showing the CD38 PCR products stained with ethidium bromide. Cells stimulated as indicated were lysed, total mRNA was isolated, and RT-PCR was performed as described in the *Materials and Methods* using specific primers for CD38 and β-actin genes. (B) CD38 protein expression was assessed by flow cytometry as described in the *Materials and Methods* and the results are expressed as the fold increases in mean fluorescence intensity over basal (untreated cells) ± 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, not significant compared with cells treated with TNFα alone. (C) Representative flow cytometry histograms of GC effect on CD38 expression.

Figure 2. Effect of TNFα and IFNs combination on CD38 sensitivity to FP. Human ASM cells were treated with TNFα (10 ng/ml) and IFNγ (500 IU/ml) (A) or TNFα (10 ng/ml) and IFNβ (500 IU/ml) (B) for 24 hr in the presence or absence of FP (100 nM) added 2 hr before. CD38 expression was assessed by flow cytometry as described in the *Materials and Methods*. The results are expressed as the fold increases in mean fluorescence intensity over basal (untreated cells) ± S.E.M. of three separate experiments. *, $P < 0.05$ compared with untreated cells; **, $P < 0.01$ compared with untreated cells; $^{\#}$ and $^{\delta}$, $P < 0.05$ compared with cells treated with TNFα or IFNs alone, respectively; NS, not significant compared with cells treated with TNFα and IFNs combination.

Figure 3. Effect of TNFα and IL-1β or TNFα and IL-13 combination on CD38 sensitivity to FP. Human ASM cells were treated with TNFα (10 ng/ml) and IL-1β (10 ng/ml) (A), or TNFα

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(10 ng/ml) and IL-13 (50 ng/ml) (B) for 24 hr in the presence or absence of FP (100 nM) added 2 hr before. CD38 expression was assessed by flow cytometry as described in the *Materials and Methods*. The results are expressed as the fold increases in mean fluorescence intensity over basal (untreated cells) ± S.E.M. of three separate experiments. *, $P < 0.05$ compared with untreated cells; $^{\#}$ and $^{\delta}$, $P < 0.05$ compared with cells treated with cytokine alone or in combination, respectively.

Figure 4. Effect of TNFα and IFNγ on CD38 sensitivity to dexamethasone and budesonide. Human ASM cells were treated with TNFα (10 ng/ml) and IFNγ (500 IU/ml) for 24 hr in the presence or absence of dexamethasone (DEX) (1 μM) or budesonide (BUD) (100 nM) added 2 hr before. CD38 expression was assessed by flow cytometry as described in the *Materials and Methods*. The results are expressed as the fold increases in mean fluorescence intensity over basal (untreated cells) ± S.E.M. of three separate experiments. *, $P < 0.05$ compared with untreated cells; **, $P < 0.01$ compared with untreated cells; $^{\#}$, $P < 0.05$ compared with cells treated with TNFα alone; NS, not significant compared with cells treated with TNFα and IFNγ combination.

Figure 5. TNFα and IFNγ combination impairs GC activities. A. ASM cells were treated with fluticasone (100 nM) for 24 hr in the presence or absence of wild-type oligonucleotide, or mutated oligonucleotide, or TNFα and IFNγ combination. Nuclear extracts were then tested for GR-DNA binding activity using the TransAM^{TM} GR kit as described in *Materials and Methods*. *$*P < 0.05$* compared with untreated cells. $^{\#}$, *$P < 0.05$* compared with cells treated with fluticasone alone. NS, not significant compared to cells treated with fluticasone alone. B. Cells were transfected with 4 μg of SEAP reporter construct containing GRE motifs before fluticasone and cytokines were added. Cells were then lysed, and the SEAP activity was performed as

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described in *Materials and Methods*. *, $P < 0.05$ compared with untreated cells; #, $P < 0.05$ compared with cells treated with fluticasone alone.

Figure 6. Effect of TNFα and IFNγ combination on GRα expression. ASM cells were stimulated with TNFα (10 ng/ml) and IFNγ (500 IU/ml) either alone or in combination for 24 hr. A. Total mRNA (2 μg) was subjected to RT-PCR with β-actin and GRα primers. PCR products were separated on a 1% agarose gel and stained with ethidium bromide. B. In separate experiments, total cell lysates were prepared and assayed for GRα by immunoblot analysis using rabbit anti-human GRα antibody.

Figure 7. Effect of TNFα and IFNγ combination on GRβ expression. ASM cells were stimulated with TNFα (10 ng/ml) and IFNγ (500 IU/ml) either alone or in combination for 24 hr. A. Total mRNA (2 μg) was subjected to RT-PCR with β-actin and GRβ primers. PCR products were separated on a 1% agarose gel and stained with ethidium bromide. B. Real-time PCR analysis of GRβ gene. GRβ mRNA levels were normalized to the corresponding levels of β-actin mRNA using the comparative cycle threshold (C_t) method as described in the *Materials and Methods*. The results are expressed as a percentage of untreated control values. *, $P < 0.05$ compared with untreated cells; **, $P < 0.01$ compared with untreated cells. C. Total cell lysates were prepared and assayed for GRβ by immunoblot analysis using rabbit anti-human GRβ antibody. D. Scanning densitometry of three representative immunoblots with each condition normalized over the area density of the corresponding actin content. The results are expressed as fold increase over basal. *, $P < 0.05$ compared with untreated cells; **, $P < 0.01$ compared with untreated cells.

Figure 8. Effect of GRβ over-expression on FP activities. A. Cells were co-transfected with a 2 μg of pCMV empty vector or CA-GRβ construct and 2 μg of SEAP reporter plasmid

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containing GRE motifs before FP (100 nM) was added for 24 hr. SEAP activity was measured as described in the *Materials and Methods*. The results are expressed as a percentage of control values from untreated cells. Data are representative of three separate experiments. *, $P < 0.05$ compared with untreated cells; $^{\#}$, $P < 0.05$ compared with cells treated with FP alone; NS, not significant compared with cells treated with fluticasone alone. B. Flow cytometry analysis of CD38 expression in GRβ-transfected cells. Cells were transfected with 4 μg of pCMV-empty vector or CA-GRβ construct before FP (100 nM) and TNFα (10 ng/ml) were added for 24 hr. Cells were then harvested by trypsinization, fixed and two-color flow cytometry was performed to assess CD38 expression on GRβ positive cells as described in the *Materials and Methods*. The percentage of double positive cells (i.e., pCMV-GFP/CD38 (open bars) or GRβ-GFP/CD38 (filled bars) was then calculated and presented in bar graph. *, $P < 0.05$ compared with untreated cells; $^{\#}$, $P < 0.05$ compared with cells treated with TNFα alone; NS, not significant compared to cells treated with TNFα alone.

Figure 1

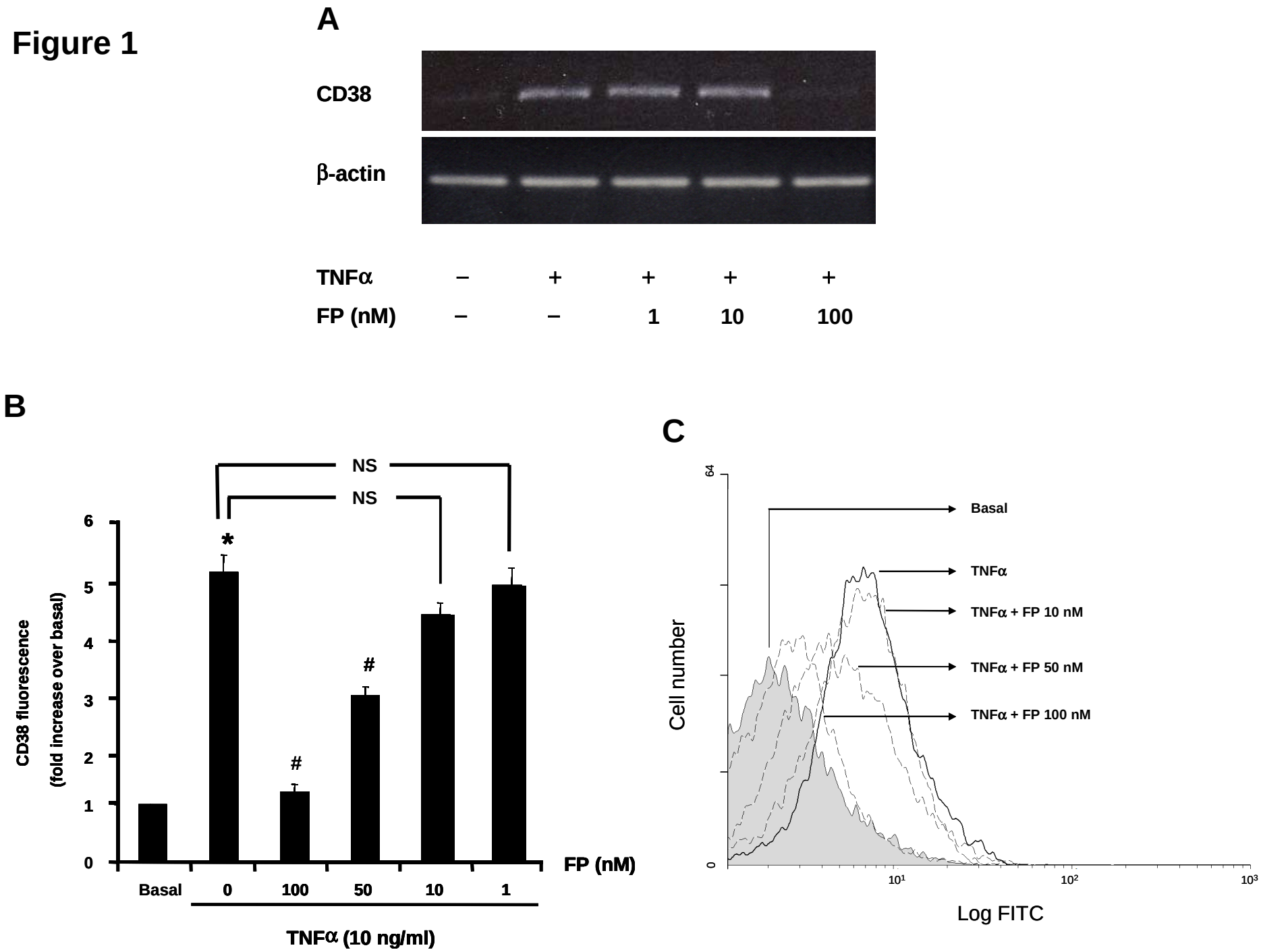

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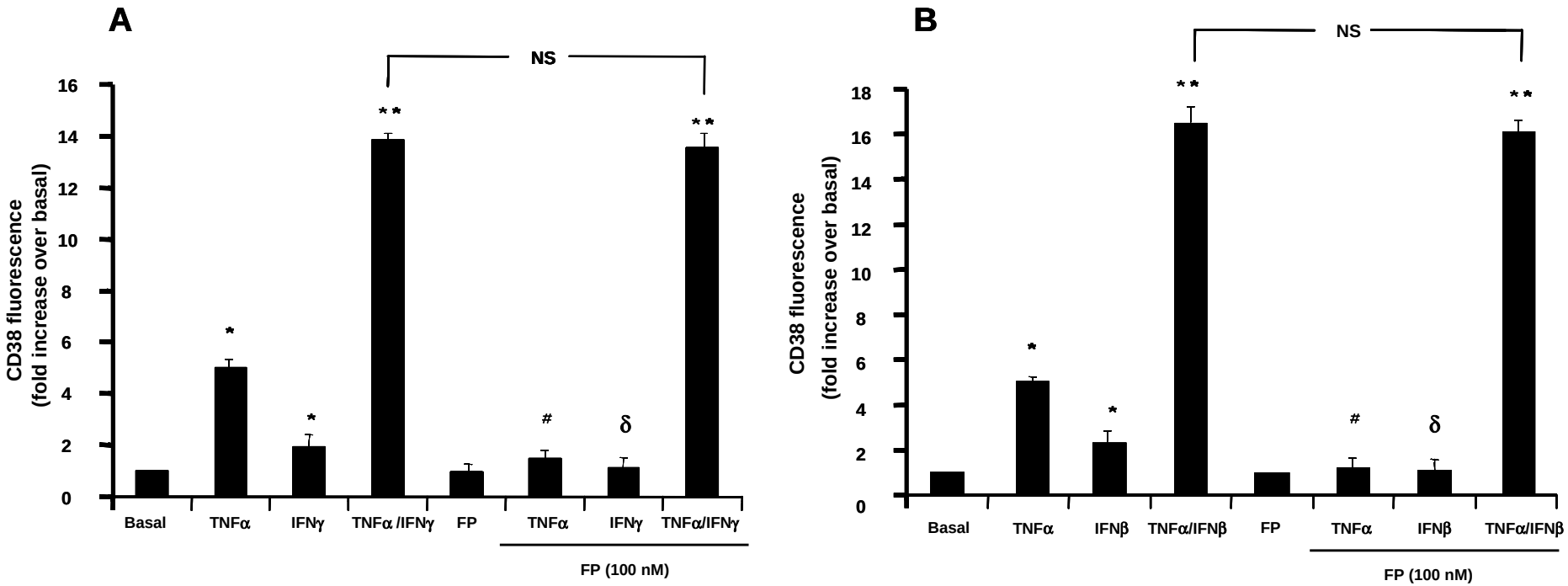
Figure 2



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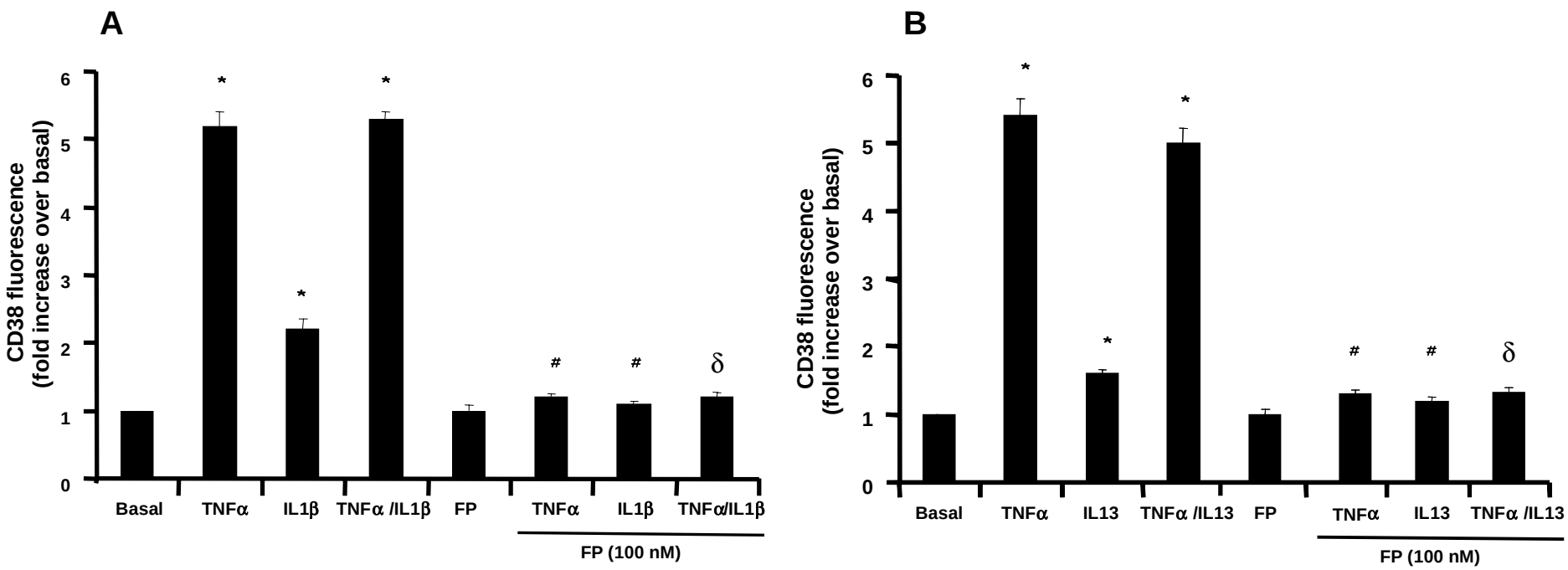

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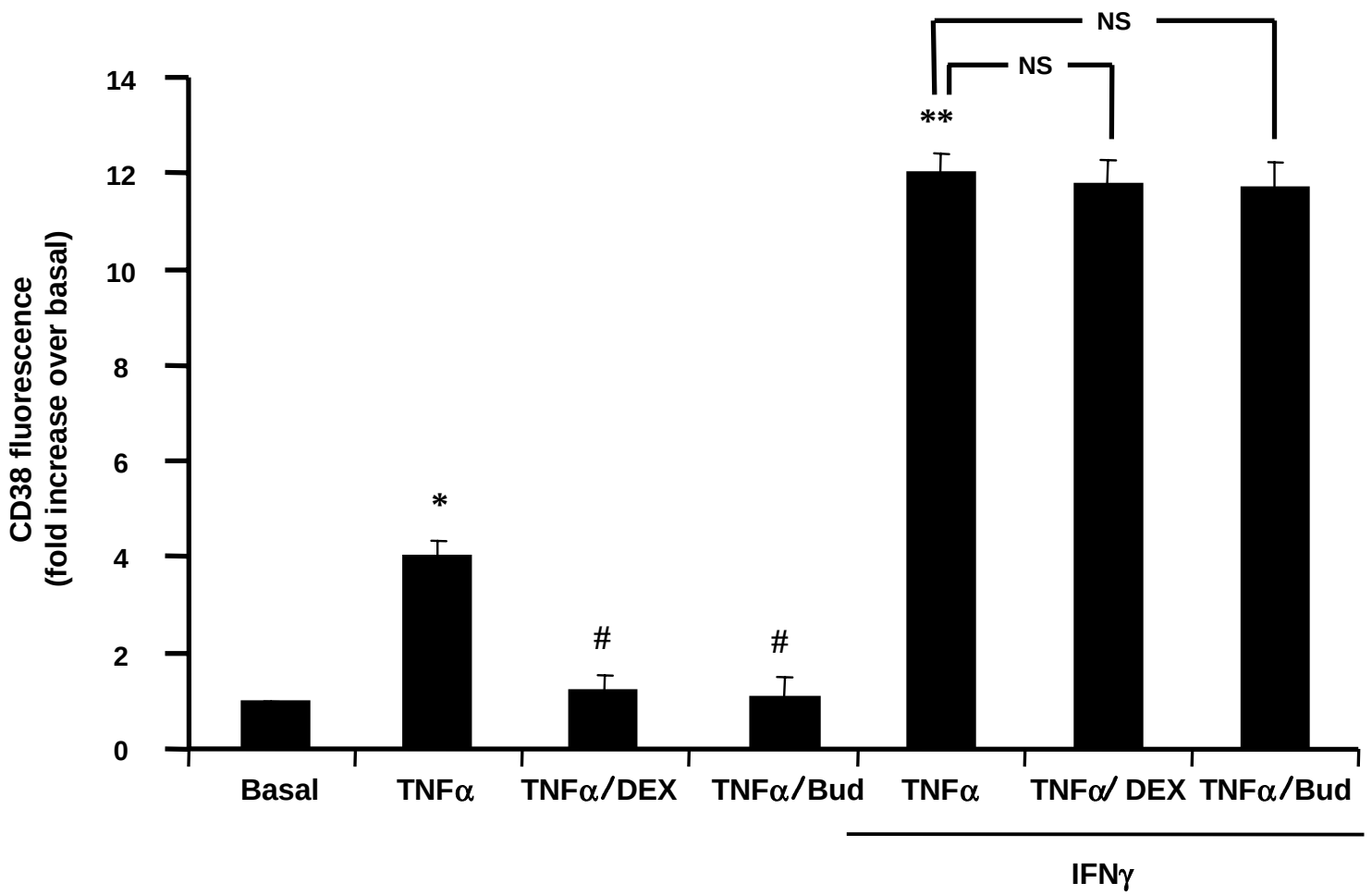

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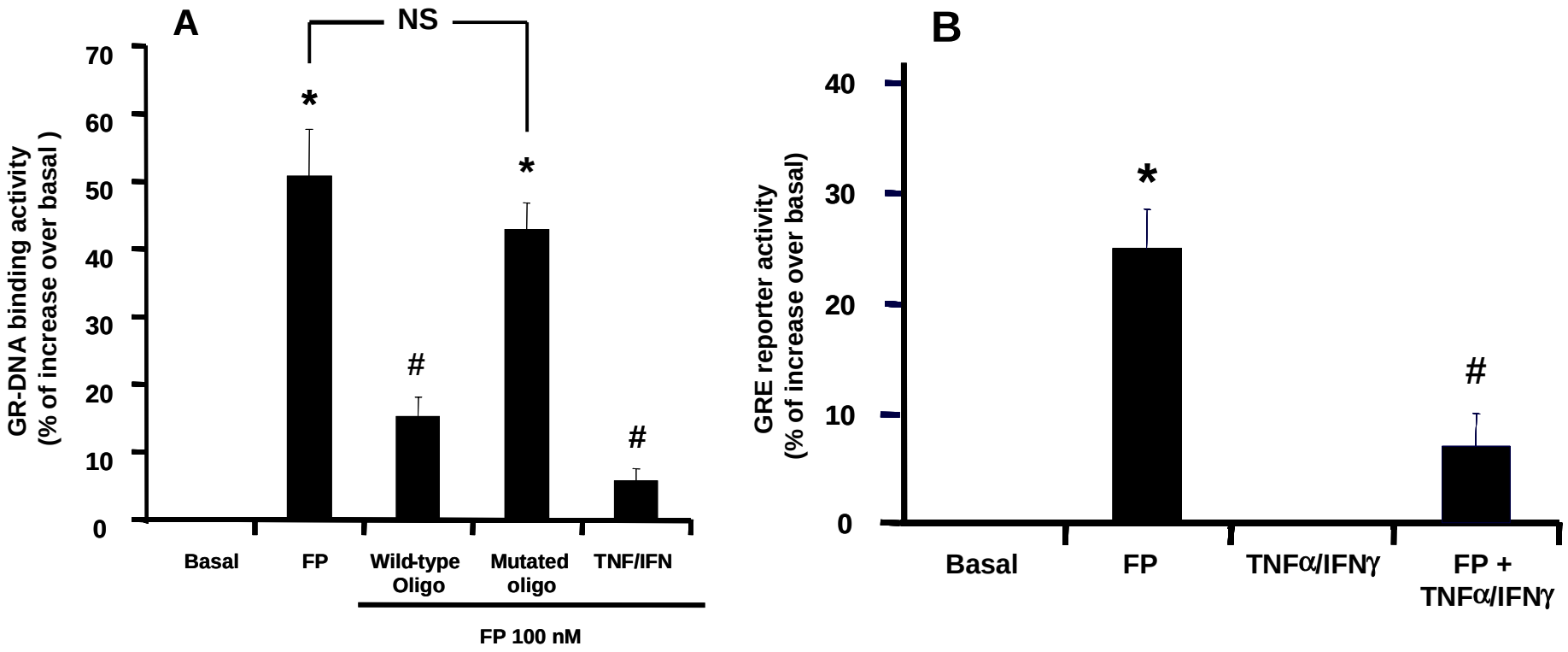

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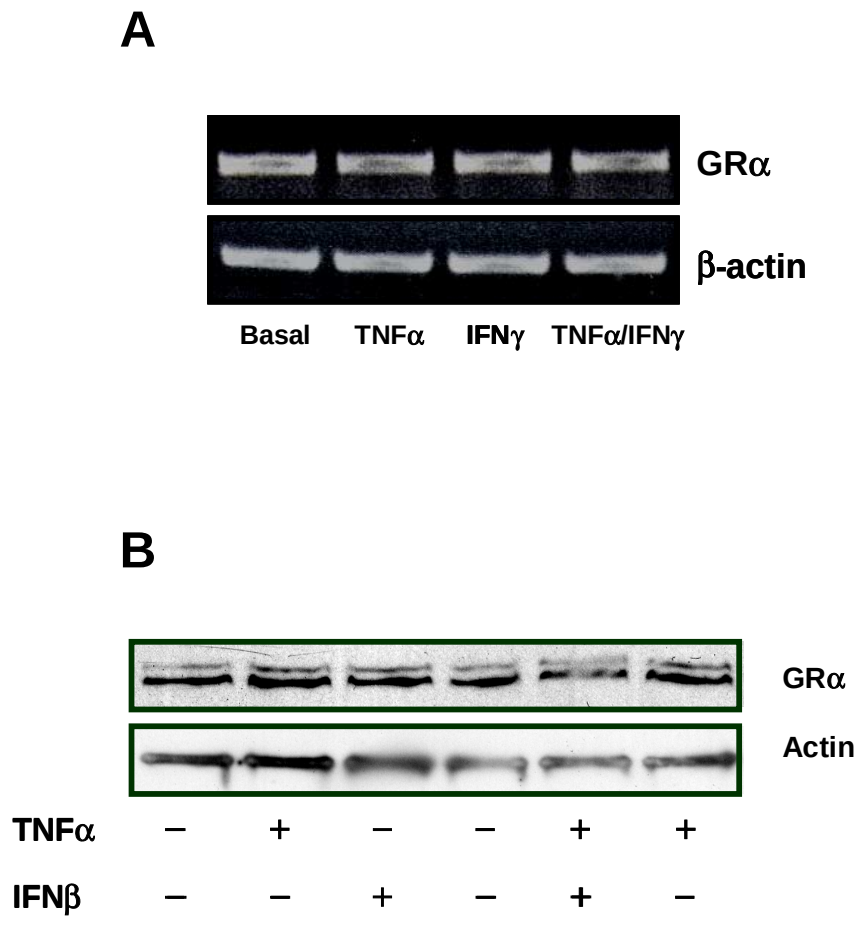

Figure 7

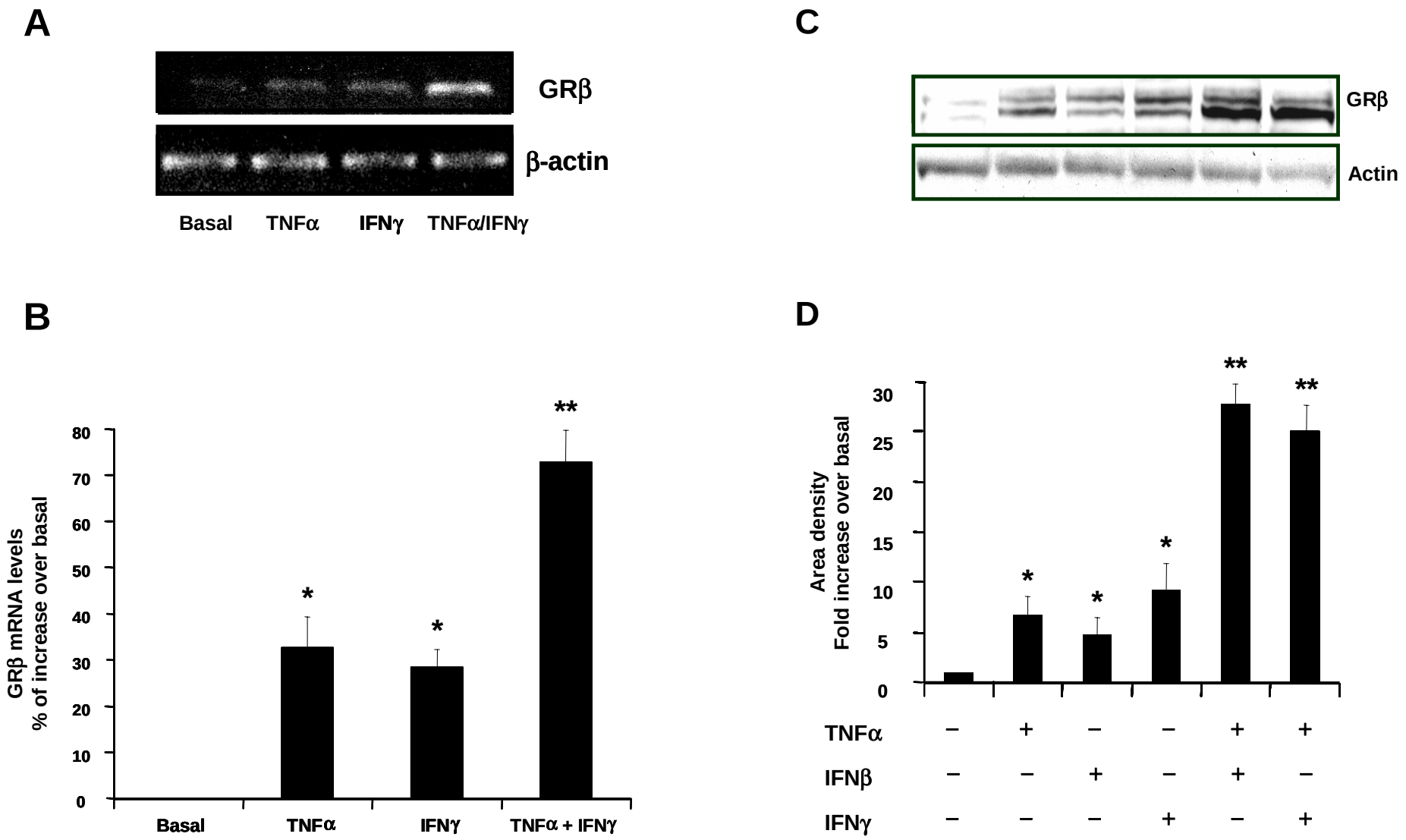

Figure 8