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## Essential role of IFN $\beta$ and CD38 in TNF $\alpha$ -induced airway smooth muscle hyper-responsiveness

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### Abstract

We recently identified autocrine interferon (IFN) $\beta$  as a novel mechanism mediating tumor necrosis factor (TNF) $\alpha$ -induced expression of inflammatory genes in airway smooth muscle (ASM) cells, including CD38, known to regulate calcium signaling. Here, we investigated the putative involvement of IFN $\beta$  in regulating TNF $\alpha$ -induced airway hyper-responsiveness (AHR), a defining feature of asthma. Using our pharmacodynamic model to assess *ex vivo* AHR isolated murine tracheal rings, we found that TNF $\alpha$ -induced enhanced contractile responses to carbachol and bradykinin was abrogated by neutralizing anti-IFN $\beta$  antibody or in tracheal rings deficient in CD38. In cultured human ASM cells, where CD38 has been involved in TNF $\alpha$ -induced enhanced calcium signals to carbachol and bradykinin, we found that neutralizing anti-IFN $\beta$  prevented TNF $\alpha$  enhancing action only on carbachol responses but not to that induced by bradykinin. In a well-characterized model of allergic asthma (mice sensitized and challenged with *Aspergillus fumigatus* (Af)), we found heightened expression of both IFN $\beta$  and CD38 in the airways. Furthermore, allergen-associated AHR to methacholine, assessed by lung resistance and dynamic compliance, was completely suppressed in CD38-deficient mice, despite the preservation of airway inflammation. These data provide the first evidence that ASM-derived IFN $\beta$  and CD38 may play a significant role in the development of TNF $\alpha$ -associated AHR.

### Keywords

Allergic asthma; Cytokine; Airway smooth muscle; Inflammation; Calcium signaling; Hypercontractility

### Introduction

Considerable *in vitro* and *in vivo* evidence suggests that tumor necrosis factor (TNF) $\alpha$  plays a key role in the development of non-specific bronchial hyper-responsiveness (AHR), a central feature of asthma (Boushey et al., 1980). In both normal and asthmatic subjects, Thomas and colleagues demonstrated that oral administration of TNF $\alpha$  increased airway responsiveness to methacholine (MCh) (Thomas, 2001; Thomas et al., 1995). Similarly, oral or intra-peritoneal

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administration of TNF $\alpha$  to different animal species induces AHR to different G-protein-coupled receptor (GPCR) agonists (Kips et al., 1992; Wheeler et al., 1992). Using the soluble protein inhibitor Ro-45-2981, Renzetti et al. (1996) provided the evidence for a direct role of TNF $\alpha$  in allergen-associated airway inflammation and AHR in sensitized guinea-pigs and Brown Norway rats. Additional studies performed in receptor knockout mice further confirmed the involvement of both TNF $\alpha$  receptors, TNFR1 and TNFR2, in the development of AHR triggered by allergen challenge in sensitized mice (Kanehiro et al., 2002) or following ozone exposure (Shore et al., 2001). Further, others showed a therapeutic benefit of anti-TNF $\alpha$  antibodies (Etanercept) in patients with severe asthma (Berry et al., 2006; Howarth et al., 2005). The precise mechanisms by which TNF $\alpha$  promotes AHR remain unknown, although studies from our laboratory and others showed that modulation of calcium metabolism in airway smooth muscle (ASM), the main effector tissue regulating bronchoconstriction, may represent a molecular mechanism linking TNF $\alpha$  to AHR (Amrani, 2006).

Using isolated airway preparations from different species, TNF $\alpha$  has been shown to enhance ASM responsiveness to agonists by either augmenting ASM reactivity (upward shift of the dose–response curve) and/or ASM sensitivity (leftward shift of the curve), suggesting the involvement of multiple molecular mechanisms in TNF $\alpha$  effects on ASM (Adner et al., 2002; Amrani and Panettieri, 2002; Anticevich et al., 1995; Sakai et al., 2004a; Sukkar et al., 2001). Additionally, we showed that in cultured human ASM cells TNF $\alpha$  potentiated, also in a non-specific manner, calcium signals in response to different GPCR agonists (Amrani, 2006). Parallel studies further supported the potential role of TNF $\alpha$ –ASM interaction in AHR by showing that TNF $\alpha$  acts by inducing Rho-dependent calcium sensitization (Hunter et al., 2003; Hunter and Nixon, 2006; MacEwan, 2002; McFarlane et al., 2001; Parris et al., 1999; Sakai et al., 2004b). Together, these investigators demonstrate that TNF $\alpha$  promotes AHR by modulating ASM contractility, an effect that could result from an altered GPCR-associated calcium signaling.

In our recent studies, we demonstrated that autocrine interferon (IFN) $\beta$  is a novel signaling molecule that mediates some TNF $\alpha$  effects in ASM cells, including expression of inflammatory proteins such as IL-6 and RANTES (Tliba et al., 2003a, b, 2004). The observation that autocrine IFN $\beta$  also partially participates in mediating TNF $\alpha$ -induced expression of CD38 (Tliba et al., 2004), a critical ectoenzyme that was shown to regulate ASM reactivity to contractile agonists in mice (Deshpande et al., 2004, 2003, 2005) prompted us to investigate the potential role of IFN $\beta$ –CD38 pathways in TNF $\alpha$  induction of *in vitro* and *ex vivo* AHR.

## Materials and methods

### Animals

In all, 8–12-week-old female C57BL/6 wild-type (WT) or CD38-deficient (CD38<sup>-/-</sup>) mice (Jackson Laboratories, Bar Harbor, ME), were housed under pathogen-free conditions. This study was approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania. Sensitization and challenge to *Aspergillus fumigatus* (*Af*) was described previously (Haczku et al., 2000, 2001, 2002). Briefly, 27 days after intra-peritoneal sensitization with *Af*, sensitized and control mice were anesthetized by isoflurane inhalation, and 25  $\mu$ l of *Af* extract or vehicle was applied to the left nares, respectively. All mice were sacrificed 24 h after their intranasal treatment, unless specified otherwise, when the peaks of eosinophil infiltration and airway responses were assumed to occur. Naïve mice that received intranasal glycerol treatment alone showed no difference compared to non-sensitized, normal C57BL/6 mice in any of the study's parameters investigated, including lung histology, BAL cellular content, immunoglobulin, cytokine profile and airway responses to acetylcholine (data not shown).

### ***In vivo* measurement of airway responsiveness to acetylcholine (ACh)**

Airway function measurements were carried out as previously described (Haczku et al., 2000, 2001, 2002). Lung function tests were assessed 24 h after the A<sub>f</sub> or saline challenge. Bronchial reactivity to aerosolized MCh was measured using the FlexiVent<sup>®</sup> system (Sireq, Montreal, Canada). Lung mechanics were studied in tracheostomized mice under anesthesia by intra-peritoneal injection of ketamine and xylazine. Mice were ventilated with a tidal volume of 8 ml/kg at a rate of 450 breaths/min and a positive end-expiratory pressure of 2 cm H<sub>2</sub>O by a computerized FlexiVent System. After mechanical ventilation for 2 min, a sinusoidal 1-Hz oscillation was applied to the tracheal tube. The single-compartment model was fitted to these data by multiple linear regression to calculate dynamic resistance and compliance of the airway.

### **BAL analysis for differential cell count, cytokine content**

Lungs were lavaged using 2 ml of sterile saline. The amount of liquid retained after BAL was an average of 1.75 ml. Cytokine levels were determined from cell free supernatant of the BAL by ELISA using antibodies and recombinant cytokines from PharMingen (San Diego, CA). Total and differential cell counts and ELISA analysis were performed as described previously (Haczku et al., 2000, 2001, 2002).

### **Immunohistochemistry**

After BAL, lungs obtained from naïve and sensitized and challenged mice were inflated with 10% formalin in PBS, excised and immunohistochemistry was performed as described previously (Haczku et al., 2006). FITC-conjugated anti-smooth muscle  $\alpha$ -actin was purchased from Sigma Aldrich (St. Louis, MO) and isotype control antibodies were purchased from BD Pharmingen (San Diego, CA). Anti-murine IFN $\beta$  antibody (R&D Systems, MN) was detected using an anti-rabbit Alexa 594 secondary antibody (Molecular Probes, Eugene, OR). After washing, the glass coverslips were mounted onto glass slides, examined under epifluorescence microscopy (Nikon, Tokyo, Japan), and photographed.

### **Measurement of isometric force generation**

Isometric force generation by murine-cultured tracheal rings was performed as described previously (Chen et al., 2003; Kim et al., 2005; Tliba et al., 2003a, b). Briefly, mouse tracheae obtained from 7- to 12-week-old female Balb/c mice were sectioned into 3–4 mm long rings. The tracheal rings were then cultured overnight in Ham's F-12 medium supplemented with 100 mM HEPES, 1.0 M NaOH, 10% fetal bovine serum (Hy-Cone, Logan, UT), 0.2 M glutamine, 1.0 M CaCl<sub>2</sub>, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin. The organ cultures were treated with 50 ng/ml TNF $\alpha$  or diluent alone for 24 h and then washed with Krebs solution containing (in mM) 118 NaCl, 4.7 KCl, 1.2 KH<sub>2</sub>PO<sub>4</sub>, 11.1 dextrose, 1.2 MgSO<sub>4</sub>, 2.8 CaCl<sub>2</sub> and 25 NaHCO<sub>3</sub>. In some experiments, tracheal rings were pre-incubated with 1000 U/ml neutralizing anti-mouse IFN $\beta$  antibody before treatment with TNF $\alpha$  or diluent. Contractile response curves to carbachol (Ch) were obtained by using a range of concentrations from 10<sup>-9</sup> to 10<sup>-5</sup> M, respectively. Tracheae were mounted with a stainless-steel pin, supported by a Plexiglas rod into the base of a double-jacketed, glass organ bath, maintained at 37 °C and bubbled with 95% O<sub>2</sub>, 5% CO<sub>2</sub>, pH of 7.4. Changes in tension of the rings were measured using an FT03 isometric transducer (Astro-Med, Inc., West Warwick, RI) and synchronously recorded with an MP 100WS system (BIOPAC Systems, Inc., Santa Barbara, CA). All initial tensions of tracheal rings were set at approximately 0.5 g. After a 20-min equilibration period, cumulative concentration–isometric force curves were then generated.

### **Human airway smooth muscle cell culture**

Human tracheae were obtained from lung transplant donors in accordance with procedures approved by the University of Pennsylvania Committee on Studies Involving Human Beings

at the University of Pennsylvania. ASM culture was performed as described previously (Panettieri et al., 1989).

### Measurement of intracellular calcium concentration

Fura-2/AM, a cell-permeant, ratiometric calcium indicator dye was used to determine the intracellular calcium concentration in HASM cells. Calcium measurements were determined as described previously (Amrani et al., 1995). Briefly, human ASM cells grown on 100 mm dishes were washed with HBSS containing 10 mM *N*-2-hydroxyethylpiperazine-*N'*-ethane sulfonic acid, 11 mM glucose, 2.5 mM CaCl<sub>2</sub>, and 1.2 mM MgCl<sub>2</sub> (pH 7.4) and incubated with 5 μM fura-2/AM for 30 min at 37 °C and 5% CO<sub>2</sub>. Fura-2-loaded cells were trypsinized, and fluorescence was estimated in cell suspensions by excitation at 340 and 380 nm wavelength. Fluorescence emissions were collected separately for each wavelength at 510 nm using a spectrofluorimeter (Photon Technology International). The ratio of fluorescence intensities at 340 and 380 nm wavelength was determined and converted to the calcium concentrations using the standard equation. The net calcium responses to contractile agonists were calculated by subtracting the basal from that of the peak intracellular calcium concentration. All experiments were performed at 37 °C.

### RT-PCR analysis

Total RNA was extracted from tracheal rings cultured with or without TNF $\alpha$  (50 ng/ml) for 24 h and from lung homogenates of naïve and *Af*-sensitized mice using Trizol reagent (Invitrogen) according to the manufacturer's instructions. Reverse transcriptase PCR analysis of IFN $\beta$  was then performed as reported previously (Tliba et al., 2003a, b, 2004). The primer sequences used for amplification are: IFN $\beta$  forward primer 5'-CAC GAC AGC TCT TTC CAT GA-3'; and reverse 5'-AGC CAG TGC TCG ATG AAT CT-3', mu-CD 38 forward primer 5'-GCA ACA TCA CAA GAG AAG ACT ACG C-3' and reverse 5'-ACA CAC TGA AGA AAC CTG GCA GGC C-3', mu- $\beta$ -actin forward 5'-CCC CAT TGA ACA TGG CAT TG-3' and reverse primer 5'-ACG ACC AGA GGC ATA CAG G-3'. Additionally, another set of mu-IFN $\beta$  primers was used in some experiments with murine lung. The sequences of this set are forward primer 5'-CCA CCA CAG CCC TCT CCA TCA ACT AT-3' and the reverse primer 5'-CAA GTG GAG AGC AGT TGA GGA CAT C-3'. PCR products were separated on 1% agarose gels and visualized by staining with ethidium bromide.

### Data analysis

Tension was calculated as milligram tensions per milligram tracheal smooth muscle weight (mg/mg) and expressed as percentage (%) of 10<sup>-5</sup> M Ch-evoked force of the cultured tracheal rings in the absence of TNF $\alpha$  for contraction studies. The concentrations of agonists required to produce half-maximal contraction were determined, and the EC<sub>50</sub> of bradykinin (BK) were then converted to log values (EC<sub>50</sub>). Concentration of agonists required to produce a half-maximal contraction (pD<sub>2</sub>) was determined with -log values of the EC<sub>50</sub>. All values were expressed as means $\pm$ SEM. Comparisons among groups with or without TNF $\alpha$  were performed by a one-way ANOVA. Student's unpaired *t*-test was used to compare the effect of drug treatment. A *p*-value <0.05 was considered significant. For lung function analyses and calcium experiments, statistical analysis was performed with Prism4 software (GraphPad Inc., San Diego, CA). Student *t*-test was used for pair wise comparisons and two-way-ANOVA for analysis of time courses. Data are expressed as means $\pm$ SEM; *p*<0.05 was considered statistically significant.

### Reagents

Tissue culture reagents and primers used for PCR were obtained from Invitrogen (Carlsbad, CA). Human recombinant TNF $\alpha$  was provided by Roche Diagnostics (Indianapolis, IN).

Neutralizing anti-IFN $\beta$  (sheep polyclonal Ab) against both murine and human protein was from PBL Biomedical Laboratories (Piscataway, NJ), isotype-matched goat or mouse IgG were purchased from R&D Systems. Ch, BK, and Fura2/AM were purchased from Sigma (St. Louis, MO, USA). *Af* was bought from Bayer Pharmaceuticals (Elkhart, IN). Aluminum hydroxide was from Pierce (Imject Alum; Rockford, IL).

## Results

### IFN $\beta$ and CD38 are critical for TNF $\alpha$ -induced AHR in murine tracheal rings

We first assessed whether endogenous IFN $\beta$  mediated TNF $\alpha$  modulation of ASM responsiveness to contractile agonists using our *ex vivo* model of airway hyper-responsiveness (AHR) (Chen et al., 2003; Kim et al., 2005; Tliba et al., 2003a, b). As shown in Fig. 1A, compared to untreated murine tracheae, levels of IFN $\beta$  mRNA were significantly up-regulated following 24 h stimulation with 50 ng/ml TNF $\alpha$ . Fig. 1B shows that Ch generates force in murine tracheal rings ( $pD_2$  values of  $6.5 \pm 0.18$ ,  $n = 7$ ) in a concentration-dependent manner while BK had a very modest contractile response. In TNF $\alpha$ -treated murine tracheal rings, the contractile responses induced by Ch and BK were significantly increased when compared with responses of rings pre-exposed to diluent alone (Fig. 1B and C). TNF $\alpha$  treatment had little effect on  $pD_2$  values to Ch ( $6.4 \pm 0.28$ ,  $n = 6$ ) but significantly enhanced murine ASM sensitivity to all BK concentrations ( $pD_2 = 6.7 \pm 0.5$ ,  $n = 6$ ). These data suggest that enhanced contraction to agonists observed in TNF $\alpha$ -treated rings is associated (BK) or not associated (Ch) with changes in receptor affinity. Interestingly, TNF $\alpha$ -induced enhancement of both Ch- or BK-evoked contractile responses were prevented by neutralizing anti-IFN $\beta$  antibody (1000 U/ml, Fig. 1B and C) but not the isotype-matched antibody (data not shown). Because CD38 pathways has been associated with cytokine-induced AHR in cultured ASM cells (Deshpande et al., 2003), we next investigated the involvement of CD38 in this *ex vivo* model of AHR. Interestingly, we found that TNF $\alpha$  failed to increase Ch-induced contractile responses in CD38-deficient murine tracheal rings (Fig. 2B) when compared to those derived from WT animals (Fig. 2A). Collectively, these data show that TNF $\alpha$  enhances ASM responsiveness to both Ch and BK in an IFN $\beta$ - and CD38-dependent manner.

### Differential role of autocrine IFN $\beta$ in TNF $\alpha$ -induced airway hyper-responsiveness in human ASM cells

We previously made the unique finding that autocrine IFN $\beta$  is essential in mediating TNF $\alpha$ -induced expression of inflammatory genes in human ASM cells (Tliba et al., 2003a, b, 2004). In the present study, we found that autocrine IFN $\beta$  also participates in TNF $\alpha$ -induced AHR in isolated murine tracheal rings. The next logical question was to assess whether autocrine IFN $\beta$  also mediates TNF $\alpha$ -induced *in vitro* AHR by using our cellular model of cytokine-induced enhancement of calcium signaling in human ASM cells (Amrani and Panettieri, 2002). In agreement with our previous findings (reviewed in Amrani, 2006), we found that TNF $\alpha$  (24 h, 10 ng/ml) significantly enhanced calcium responses to Ch from  $248.5 \pm 18.47$  nM ( $n = 10$ ) to  $532.8 \pm 93.26$  nM ( $n = 9$ ;  $p < 0.001$ , Fig. 3). Interestingly, pretreatment of ASM cells with neutralizing anti-IFN $\beta$  completely abrogated TNF $\alpha$ -induced potentiation of calcium responses to Ch (Fig. 3) but did not prevent TNF $\alpha$  enhancing effects on BK responses (data not shown). Anti-IFN $\beta$  antibody alone had no effect on calcium transients in response to Ch (Fig. 3A) or BK (data not shown). These data strongly suggest that autocrine IFN $\beta$  also plays a role in mediating cytokine-induced *in vitro* ASM AHR.

### *Af* sensitization and challenge is associated with increased expression of IFN $\beta$ in the airway tissue

We next used our well-established murine model of allergic AHR (*Af*-exposed mice, (Haczku, 2006)) to assess whether both IFN $\beta$  and CD38 were increased following allergen challenge.

Twenty-four hours after *Af* challenge of sensitized mice, there was a significant AHR to MCh as assessed by lung resistance and dynamic compliance (Fig. 4A). In addition, these mice had increased levels of pro-inflammatory cytokines including IL-4, IL-5 as well as TNF $\alpha$  and had an influx of eosinophils and neutrophils in the airways (Fig. 4B and C). Immunohistochemical studies in lung tissues of mice sensitized and challenged with *Af* showed increased expression of IFN $\beta$  in the ASM and in the epithelium (Fig. 4D) as well as higher levels of IFN $\beta$  and CD38 mRNA in lungs by RT-PCR (Fig. 4E). Allergen-induced CD38 expression started at 6 h while IFN $\beta$  levels were increased only at 12 h post-challenge. Both cytokines were significantly increased at 48 h post-allergen challenge. These data suggest that (i) allergen challenge is a potent inducer of both CD38 and IFN $\beta$  in the lungs and (ii) that ASM and epithelium represent novel sources of IFN $\beta$  in the airways.

### CD38 is essential for *Af* challenge-induced airway hyper-responsiveness

To assess the role of CD38 in allergen (*Af*)-induced AHR, we sensitized and challenged WT and CD38<sup>-/-</sup> mice as described in the “Materials and methods” section. *Af*-induced airway inflammation of these mice was confirmed by increased total BAL cell counts and higher number of eosinophils and neutrophils in both the groups (Fig. 6). WT mice demonstrated a significant AHR to MCh 24 h after *Af* challenge in terms of both lung resistance and dynamic compliance (Fig. 5A and B) while these lung responses to MCh were abrogated in similarly challenged CD38<sup>-/-</sup> mice. Surprisingly, allergen-induced influx of inflammatory cells (macrophages, neutrophils, eosinophils and lymphocytes) in the airways was not affected in CD38-deficient mice (Fig. 6A and B). These data suggest that CD38 pathway is important in mediating allergen-induced AHR but not in the development of airway inflammation.

### Discussion

In previous reports, we made the interesting finding that TNF $\alpha$ -treated ASM cells secrete active IFN $\beta$ , which in turn modulates both cell mitogenesis as well as expression of different inflammatory genes (Tliba et al., 2003a, b, 2004). In this study, we show for the first time that IFN $\beta$ , which is increased in the airways following allergen exposure, is essential for promoting ASM hyper-responsiveness induced by TNF $\alpha$ . We also provide the first preclinical evidence of a role of CD38, an IFN $\beta$ -inducible protein, in the development of AHR in a murine model of allergic asthma.

Accumulating evidence from both clinical (Berry et al., 2006; Howarth et al., 2005) and animal studies (Choi et al., 2005; Kim et al., 2006) strongly support a critical role of TNF $\alpha$  in the pathogenesis of asthma, including its clinical feature, AHR. A direct action of TNF $\alpha$  on ASM function as a plausible mechanism for the enhanced airway responsiveness is evidenced by previous reports showing (i) enhanced *ex vivo* contractility to different contractile agonists (Adner et al., 2002; Sakai et al., 2004a, b; Chen et al., 2003) or (ii) impaired responses to  $\beta$ -adrenoceptor agonists (Chen et al., 2003; Bachar et al., 2005; Wills-Karp et al., 1993) following TNF $\alpha$  treatment. The mechanisms by which TNF $\alpha$  promotes these “pro-asthmatic” responses are still unknown. In previous reports (Tliba et al., 2003a, b, 2004), we made the unique finding that ASM-derived IFN $\beta$  differentially regulates TNF $\alpha$ -induced expression of inflammatory genes in cultured ASM cells including RANTES, IL-6 as well as CD38, an ectoenzyme recently involved in AHR in asthma. Interestingly, we found that TNF $\alpha$  also increases IFN $\beta$  expression in isolated murine tracheal rings, an *ex vivo* model often used to study the cellular and molecular mechanisms associated with AHR. The use of neutralizing antibodies allowed us to demonstrate for the first time that IFN $\beta$ , in an autocrine manner, plays a key role in mediating TNF $\alpha$ -induced *ex vivo* ASM hyper-responsiveness to two different contractile agonists Ch (Fig. 1B) and BK (Fig. 1C). Different mechanisms in the ASM have been proposed to explain the action of TNF $\alpha$  in the development of *ex vivo* AHR. Recent evidence shows that induction

of CD38, an ADP-ribosyl cyclase that regulates  $\text{Ca}^{2+}$  homeostasis (Malavasi et al., 1992; Mehta et al., 1996; Musso et al., 2001), is playing a key role in mediating TNF $\alpha$ -induced ASM hyper-responsiveness (Deshpande et al., 2004; Kang et al., 2006). The use of CD38-deficient mice allowed us to provide the first experimental evidence showing the implication of CD38 in TNF $\alpha$ -mediating ASM hyper-responsiveness. Our data show that in CD38-deficient murine tracheal rings, not only did TNF $\alpha$  fail to enhance Ch-evoked contractile responsiveness but also that Ch responses were dramatically suppressed in TNF $\alpha$ -treated rings (Fig. 2). It is possible that CD38 signaling pathways are somewhat serving as potent regulators of TNF $\alpha$  signaling. In this regard, we have shown that interfering with CD38 pathways differentially regulates cellular responses induced by TNF $\alpha$  in ASM cells (Tliba et al., 2003a, b). It is therefore conceivable that in isolated airway preparations, a similar phenomenon could occur with TNF $\alpha$  having a dual action on muscle reactivity, i.e. hyper-responsiveness or hypo-responsiveness, depending on the presence or absence of CD38 pathways.

Induction of CD38 by TNF $\alpha$  in ASM cells required the autocrine action of IFN $\beta$  (Tliba et al., 2004), we therefore investigated whether IFN $\beta$  was playing any role in our cellular model of hyper-responsiveness, i.e. cytokine-induced alterations in calcium handling in ASM cells (Amrani, 2006). Synthesis of *de novo* protein was required to mediate TNF $\alpha$  effects (Amrani et al., 1997), although the proteins involved have not been identified. A recent study demonstrated that induction of transient receptor potential C3 mediates TNF $\alpha$ -induced increases in agonist-evoked calcium responses (White et al., 2006). We now found that autocrine IFN $\beta$  is a novel mediator involved in the potentiating effect of TNF $\alpha$  on calcium signals induced by Ch. These *in vitro* data contrast with the *ex vivo* data found in murine tracheal rings where neutralizing IFN $\beta$  prevented TNF $\alpha$ -induced *ex vivo* AHR to both Ch and BK (Amrani, unpublished observation). This discrepancy could be explained by two possibilities: (i) the species differences of our *in vitro* (human) and *in vivo* (mice) models or (ii) the cellular complexity of the *ex vivo* model where TNF $\alpha$  could be acting on different cell types (epithelium and nerves) to promote AHR. Assuming that ASM is the main target of TNF $\alpha$  in the isolated murine airways, then these *in vitro* observations argue against the fact that changes in calcium metabolism is the only mechanism involved in TNF $\alpha$ -induced AHR (here to BK). In agreement with this hypothesis are the recent studies showing that AHR to BK induced by TNF $\alpha$  strongly correlates with changes in receptor levels in the ASM (Zhang et al., 2004). Future studies will delineate the IFN $\beta$ -associated calcium-dependent (transient signals) and -independent (receptor changes) pathways involved in TNF $\alpha$ -induced AHR.

Other novel findings described in this study were made in a well-established mouse model of allergic AHR (i.e., *Af*-sensitized and challenged mice, Fig. 4A) (Haczku et al., 2000,2001, 2006). We found a time-dependent (seen at 6 h) and sustained (up to 48 h) increase in IFN $\beta$  as well as CD38 expression in the lungs (including in the ASM) of hyper-responsive mice following allergen challenge (Fig. 4E). The pathogenic role of CD38 in the allergic response was demonstrated using knockout mice where allergen challenge-associated AHR to MCh seen in WT animals was completely abrogated in CD38<sup>-/-</sup> mice; surprisingly, the inflammatory component of the allergic responses (influx of inflammatory cells) was not affected (Figs. 5 and 6). This observation supports the recent finding by Guedes et al. (2006) describing a central role of CD38 in AHR induced by intranasal administration of exogenous IL-13. The authors also found that IL-13-induced AHR could be abrogated in CD38 knockout mice despite no effect on the airway inflammatory response. This suggests that inflammation independent but CD38-dependent pathways, likely involving changes in ASM function, are playing an important role in the development of allergic AHR. The role of IFN $\beta$  in the allergic responses has not been investigated in the present report but our *ex vivo* studies strongly suggest a potential role of IFN $\beta$  in AHR. Additional studies are clearly needed to investigate this novel hypothesis.

Together, we found that ASM-derived IFN $\beta$  acts as an important factor mediating TNF $\alpha$ -induced ASM hyper-responsiveness to contractile agonists, possibly through its ability to regulate CD38 pathways. These *ex vivo* data combined to the novel observation that CD38 is critical for mediating allergen-induced AHR, strongly suggest that IFN $\beta$ –CD38 axis is a novel area of investigation for studying the cellular/molecular mechanisms underlying AHR in asthma.

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## Abbreviation

<b>Af</b>	Aspergillus fumigatus
<b>AHR</b>	airway hyper-responsiveness
<b>ASM</b>	airway smooth muscle
<b>BK</b>	bradykinin
<b>(Ca<sup>2+</sup>)<sub>I</sub></b>	intracellular calcium
<b>Ch</b>	carbachol
<b>IFN</b>	interferon
<b>MCh</b>	methacholine
<b>TNF</b>	tumor necrosis factor

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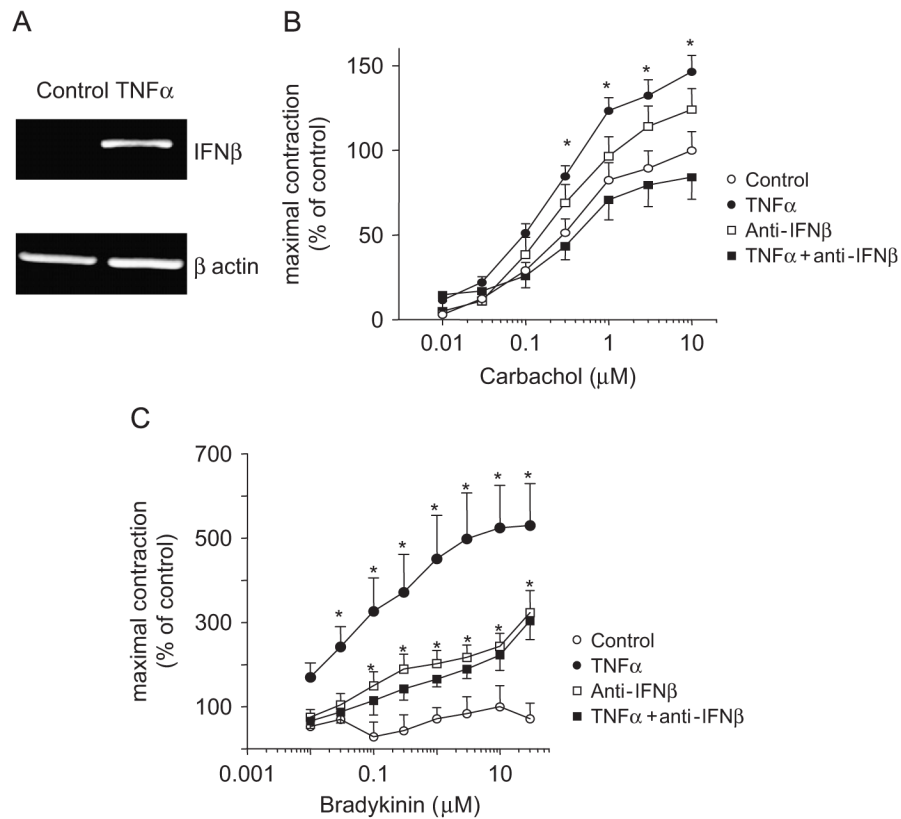
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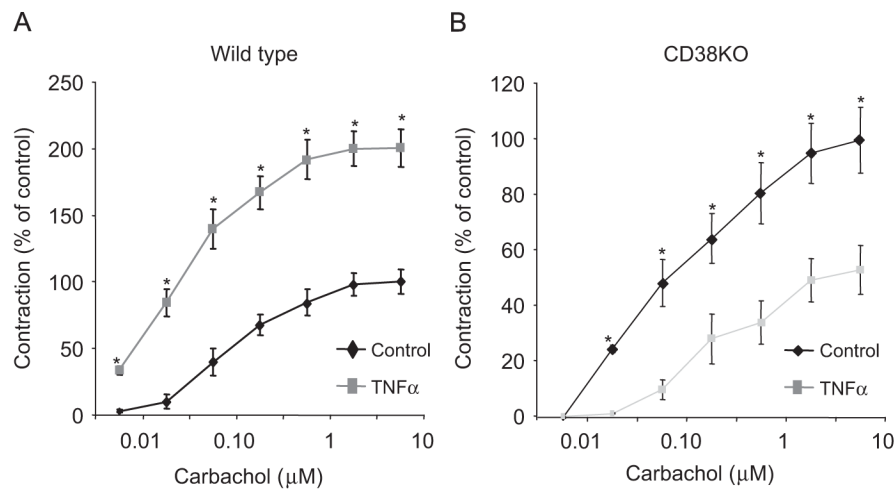
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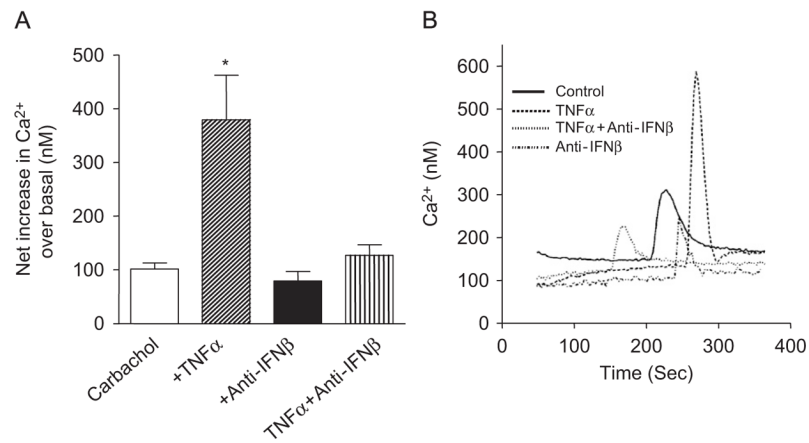
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**Fig. 1.**

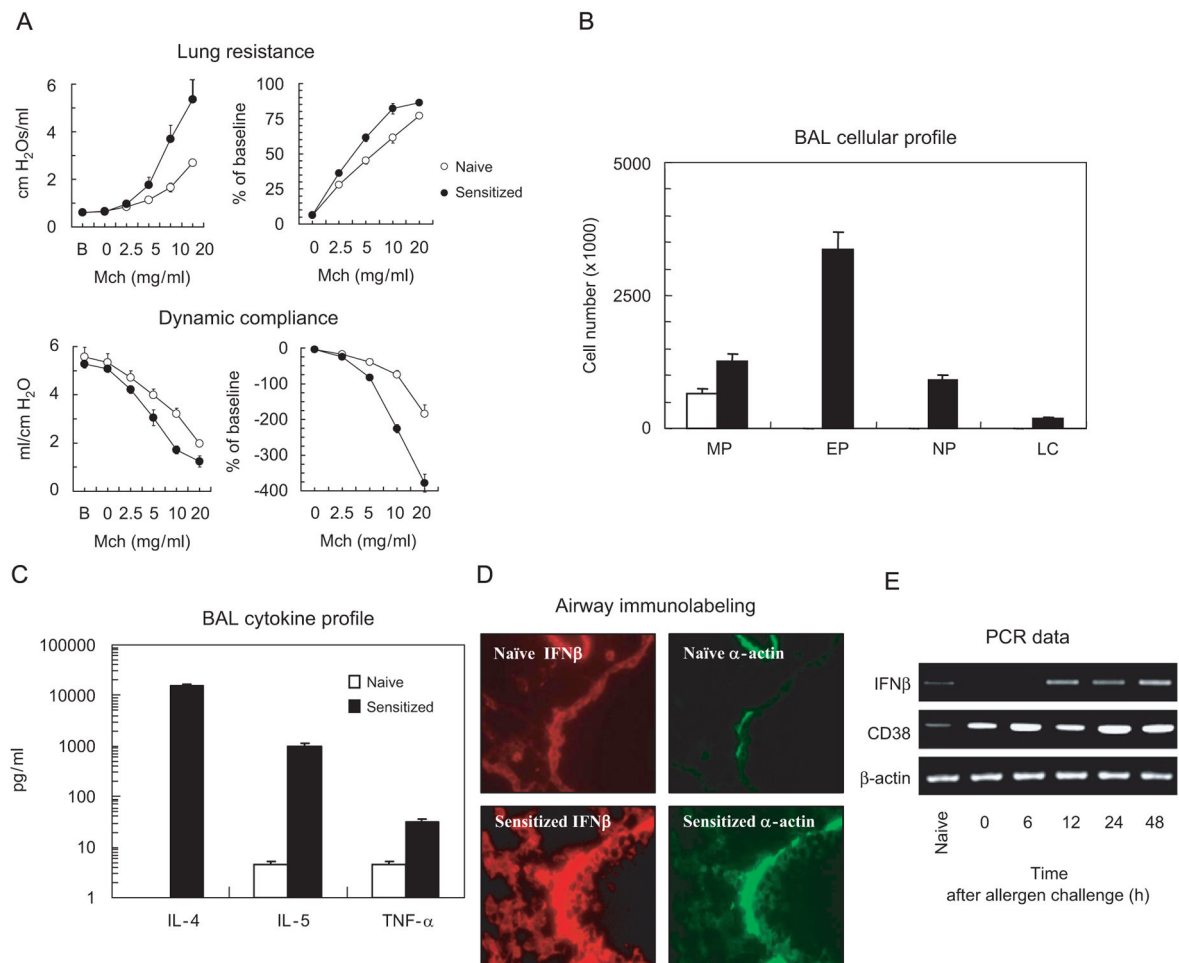
Role of IFN $\beta$  in mediating TNF $\alpha$ -induced augmentation of agonist-induced contraction in cultured tracheal rings. (A) Tracheae were dissected from Balb/c mice and stimulated with TNF $\alpha$  for 24 h as described in Methods. Total mRNA was reverse transcribed and amplified with IFN $\beta$  and  $\beta$ -actin-specific primers. PCR products were separated on a 1% agarose gel. Data are representative of mRNA obtained from three different experiments. Cumulative concentration–response curves to carbachol (B) or to bradykinin (C) were completed in cultured control ( $n = 4–5$ ) or in the presence of TNF $\alpha$  (50 ng/ml) ( $n = 7$ ). In some experiments ( $n = 7–8$ ), tracheal rings were treated with anti-IFN $\beta$  (1000 U/ml) alone or for 30 min prior to incubation with TNF $\alpha$ . Isometric measurements of tracheal reactivity were calculated as changes in milligram tensions per milligram weight (mg/mg) and expressed as percentage of  $10^{-5}$  mol/l carbachol-induced tensions in control rings. All tension measurements from groups were expressed as means  $\pm$  SEM, \* $p < 0.05$  compared with rings treated with diluent alone. Contractile studies in response to BK were done in the presence of  $10^{-5}$  M D-thiorphan.



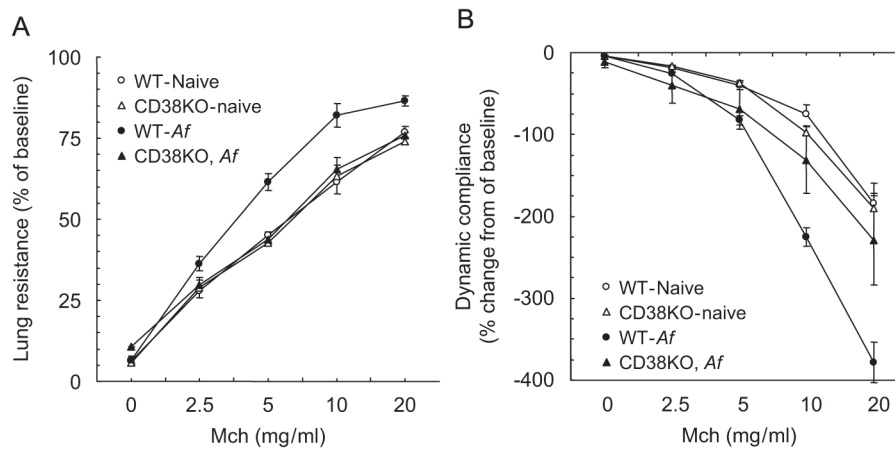
**Fig. 2.** TNF $\alpha$ -induced enhancement of contractile agonist-evoked contractile force is abrogated in CD38 knockout mice. Cumulative concentration–response curves to carbachol in WT (A) and CD38 $^{-/-}$  mice (B) in the presence or absence of TNF $\alpha$  (100 ng/ml) treatment for 24 h. All tension measurements from groups are expressed as means $\pm$ SEM,  $n = 6-7$ , \* $p < 0.05$  compared with untreated rings.

**Fig. 3.**

TNF $\alpha$  potentiation of carbachol-mediated calcium transients is abrogated by anti-IFN $\beta$ . Human ASM were loaded with Fura-2/AM as described in Materials and methods. (A) TNF $\alpha$  activates carbachol-mediated intracellular Ca<sup>2+</sup> increases in cultured HASM which can be abrogated by pre-treating the cells with anti-IFN $\beta$ . Values are means $\pm$ SEM of three independent experiments ( $n = 9-10$  for control and TNF $\alpha$  and three for TNF $\alpha$ +anti-IFN $\beta$ ). \* $p < 0.001$  compared with control. (B) Each tracing represents a cytosolic calcium transient from a single sample.

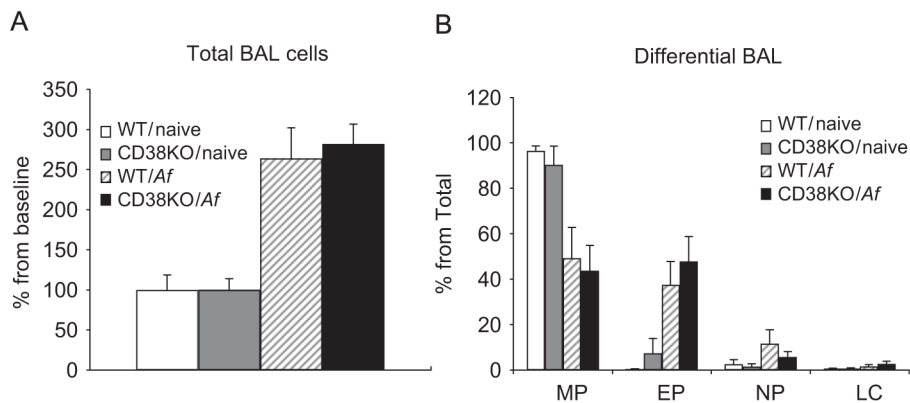


**Fig. 4.** *Af*-induced allergic airway inflammation and hyper-responsiveness is associated with release of TNF $\alpha$  and an increased expression of IFN $\beta$  and CD38 in the airways and lung. Sensitized mice were challenged with *Af* as described and studied 24 h later. (A) Lung function measurements were performed using the FlexiVent system. (B) BAL cell profile was determined by performing total and differential cell counts on cytospin preparations. (C) Cytokine profile was determined by ELISA (means $\pm$ SEM of  $n = 10$ – $12$ ). (D) Immunohistochemistry was performed using a direct FITC-conjugated anti-smooth muscle  $\alpha$ -actin monoclonal antibody or anti-IFN $\beta$  primary antibody followed by secondary Alexa 594 antibody. Data are representative of immunostaining performed in three different experiments. (E) Total mRNA was extracted at various time points after sensitization and challenge with *Af* and the level of IFN $\beta$  and CD38 mRNA was determined using RT-PCR as described in Methods. Data are representative of three different experiments.



**Fig. 5.** *Af*-induced AHR is abrogated in CD38<sup>-/-</sup> mice. Sensitized mice were challenged with *Af* as described and studied 24 h later using the FlexiVent system. Lung resistance (A) and dynamic compliance (B) was measured after inhalation of saline (0) or different concentrations of methacholine (MCh). While allergen-challenged wild-type animals showed significantly increased lung resistance to increasing doses of MCh compared to naive animals ( $*p < 0.0001$ ), allergen challenge of CD38<sup>-/-</sup> mice did not significantly increase their lung resistance. In fact lung resistance in *Af*-challenged CD38<sup>-/-</sup> mice was significantly lower ( $p = 0.0135$ , two-way ANOVA) when compared to *Af*-challenged WT animals. Similarly, dynamic compliance ( $C_{dyn}$ ) did not decrease significantly in *Af*-challenged CD38<sup>-/-</sup> mice compared to naive controls. However,  $C_{dyn}$  in *Af*-challenged wild-type mice was significantly reduced ( $p < 0.0001$ , two-way ANOVA) compared to naive animals. Values are expressed as % change from baseline. Means  $\pm$  SEM of  $n = 5-6$ .





**Fig. 6.** *Af*-induced inflammatory cell influx in the airways is not affected in CD38<sup>-/-</sup> mice. The total number of BAL cells (A) was derived from the differential counts in Giemsa-stained cytopsin preparations and the total cell counts (B) in each BAL sample as described in Materials and methods. Data are expressed as means $\pm$ SEM of  $n = 8-12$  mice for each condition.