

RESEARCH PAPER

TNF- α -induces airway hyperresponsiveness to cholinergic stimulation in guinea pig airways

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BACKGROUND AND PURPOSE

TNF- α is an inflammatory cytokine implicated in the pathogenesis of asthma and it causes airway inflammation, bronchoconstriction and airway hyperresponsiveness to a number of spasmogens following inhalation.

EXPERIMENTAL APPROACH

We compared contractions of guinea pig isolated trachea incubated with saline or TNF- α for 1, 2 or 4 days to electrical field stimulation (EFS), 5-HT or methacholine. In addition, we compared bronchoconstriction in anaesthetized guinea pigs 6 h after intratracheal instillation of saline or TNF- α to vagal nerve stimulation, i.v. 5-HT or methacholine. Differential counts were performed on the bronchoalveolar lavage fluid (BALF).

KEY RESULTS

Maximum contractions to methacholine, 5-HT and EFS were not different between freshly prepared and saline-incubated tissues. Exposure to TNF- α concentration-dependently potentiated contractions to 5-HT and EFS, but not methacholine. All contractions were atropine-sensitive, but not hexamethonium-sensitive. 5-HT-evoked contractions were inhibited by ketanserin or epithelial denudation. Only EFS-evoked contractions were tetrodotoxin-sensitive. Vagal stimulation, i.v. 5-HT or MCh caused a significant atropine-sensitive, frequency- and dose-dependent bronchoconstriction and decreased blood pressure similarly in both saline and TNF- α pre-treated animals. TNF- α potentiated the bronchoconstriction to vagal stimulation and 5-HT, but not MCh. The BALF from saline-treated animals contained predominantly macrophages, whereas that from TNF- α -treated animals contained neutrophils.

CONCLUSIONS AND IMPLICATIONS

TNF- α caused airway hyperresponsiveness to nerve stimulation *in vivo* and increased contractility *in vitro*. However, responsiveness to MCh was unchanged, suggesting a pre-synaptic action of TNF- α on parasympathetic nerves. TNF- α -induced airway hyperresponsiveness to 5-HT suggested an increased 5-HT_{2A} receptor-mediated acetylcholine release from epithelial cells.

Abbreviations

ACh, acetylcholine; BALF, bronchoalveolar lavage fluid; DMEM, Dulbecco's modified Eagle's medium; EFS, electrical field stimulation; MCh, methacholine; pA₂, antagonist potency; TTX, tetrodotoxin

Introduction

The complex aetiology of asthma consists of contraction of airway smooth muscle, infiltration and injury of the airway

walls by inflammatory cells, oedema of airway mucosa and muscle, increased production of mucus, desquamation of the airway epithelium and stiffening of the airways (Boushey *et al.*, 1980; Gleich, 1990; Aikawa *et al.*, 1992; Spina and Page,

2009; Halwani *et al.*, 2010). These cardinal features of asthma are manifested as an increase in airway responsiveness to a variety of stimuli, which are normally innocuous to the healthy population (Bousquet *et al.*, 2000).

The cascade of events leading to the increase in airway responsiveness and obstruction to airflow are considered to be through a direct and indirect reactivity of the airway muscle to a large number of mediators released from activated inflammatory cell types, including mast cells (Gordon and Galli, 1990; Broide *et al.*, 1991; Bradding *et al.*, 1994; Kim *et al.*, 2007; Nakae *et al.*, 2007), platelets (Pitchford and Page, 2006), macrophages (Gosset *et al.*, 1991; Cembrzynska-Nowak *et al.*, 1993), neutrophils and eosinophils (Bousquet *et al.*, 1990; Gleich, 1990; Lukacs *et al.*, 1995) and from non-inflammatory cell types such as airway smooth muscle, epithelial cells and nerves (Boushey *et al.*, 1980).

The role of inflammatory lipid mediators, for example arachidonic acid metabolites and platelet-activating factor, biogenic amines, such as histamine and 5-HT, and peptides such as bradykinin and substance P, has been extensively explored in terms of their ability to induce airway hyperresponsiveness and bronchospasm. This is particularly during the acute IgE-dependent early inflammatory response in allergic asthmatics upon exposure to an antigen. However, our understanding of the mediators and the mechanism by which they trigger or contribute to the late inflammatory response that occurs 4 to 12 h after antigen exposure and subsequent airway hyperresponsiveness, which leads to the chronic exacerbation of airway function, is less well understood.

A large body of evidence has emerged to implicate a number of pro-inflammatory cytokines in having a wide spectrum of roles spanning the initiation, orchestration and propagation of the inflammation, heightened airway responsiveness and structural remodelling expressed in asthma (Tarantini *et al.*, 2007). In particular, the pleiotropic cytokine TNF- α has been directly linked with the pathogenesis and worsening of asthma symptoms because of its copious production by many activated inflammatory and non-inflammatory cells and the ubiquitous expression of its two receptors, p55TNF and p75TNF (Thomas, 2001; Cazzola and Polosa, 2006; Brightling *et al.*, 2008). This agonist-receptor interaction causes activation of a multitude of signal transduction pathways and genes in response to an inflammatory insult, infection or the innate host defence immune reaction in the lung (Sluss *et al.*, 1994; Winston *et al.*, 1995; Waterman *et al.*, 1996; Amrani *et al.*, 2000).

In the lungs of subjects with asthma, compared with those from normal subjects, increased levels of the mRNA of the p55TNF receptor, the receptor protein (Yoshida *et al.*, 1996) and the TNF-converting enzyme – which cleaves the membrane bound TNF- α form to the equiactive soluble form – have been measured in bronchoalveolar lavage (BAL) fluid (Broide *et al.*, 1992) and sputa (Taki *et al.*, 1991). Administration of inhaled recombinant TNF- α to normal human subjects (Thomas, 2001; Thomas *et al.*, 1995) and rats (Kips *et al.*, 1992), or challenge of rats and mice with antigen, bacterial endotoxin (Ohkawara *et al.*, 1992) or respiratory viruses (Tera-jima *et al.*, 1997), releases large amounts of endogenous TNF- α , which induces neutrophilic and eosinophilic cell infiltration and a potentiation of their cytotoxic effect on structural cells, mucus metaplasia, airway fibrosis, airway

obstruction and heightened airway smooth muscle reactivity to spasmogens (Busse *et al.*, 2005; Choi *et al.*, 2005; Berry *et al.*, 2006).

A suppression of the airway hyperresponsiveness and airway inflammation in allergen-challenged mice after either targeted deletion of the genes encoding the TNF- α protein, the TNF receptors (Rudmann *et al.*, 2000) or the antagonism of the p55TNF receptor using two monoclonal antibodies, etanercept and infliximab (Cazzola and Polosa, 2006; Kim *et al.*, 2006; Deveci *et al.*, 2008; Hutchison *et al.*, 2008) has provided the most convincing evidence for a primary role of TNF- α in the pathogenesis of asthma. Importantly, these observations in experimental animals have been confirmed in recent clinical trials by an improvement in the lung function in humans with severe asthma following treatment with etanercept (Howarth *et al.*, 2005; Berry *et al.*, 2006).

Nevertheless, our understanding of the mechanisms whereby TNF- α can induce airway hyperresponsiveness is not complete. Indirect evidence such as the increased release of other pro-inflammatory cytokines such as IL-1 β and bronchoconstrictor mediators such as acetylcholine (ACh) released because of an impairment of the neuronal M₂ muscarinic autoreceptors (Nie *et al.*, 2009), or a direct potentiation of the bronchospasm to muscarinic cholinergic agonists by increasing intracellular Ca²⁺ levels and inositol turn-over (Amrani *et al.*, 1995, 1996; Parris *et al.*, 1999), greater release of or impairing β_2 -adrenoceptor-mediated relaxation of airway smooth muscle, or release (Wills-Karp *et al.*, 1993) have all been suggested as possible contributing mechanisms (Alexander *et al.*, 2011).

An exaggerated agonist-induced contraction and a parallel decrease in agonist-induced relaxation of the smooth muscle in response to TNF- α could underlie the airway hyperresponsiveness. However, it is plausible that the increased responsiveness of airway smooth muscle in subjects with asthma could also be through an augmented release of endogenous spasmogens or decrease in endogenous airway smooth muscle relaxant mediators rather than their effect on the smooth muscle. To this end, we attempted to investigate the former possibility using two experimental approaches. First, by examining the action of TNF- α alone to modulate the contractions of the guinea pig isolated trachea to electrical field stimulation (EFS), 5-HT or the synthetic muscarinic receptor agonist methacholine (MCh). Second, to extend these *in vitro* observations to investigate the effect of intratracheal instillation of TNF- α on the lung function and airway responsiveness to spasmogens of the anaesthetized guinea pig *in vivo*.

A preliminary account of some of the present data has been communicated to the American Thoracic Society (Makwana *et al.*, 2009; 2010).

Methods

Animals

All animal care and experiments complied with the Animals (Scientific Procedures) Act 1986 and were approved by the King's College London ethics committee. Male adult Dunkin-Hartley guinea pigs (250–350 g) were used from stock originating at B&K Laboratories (Hull, UK). The animals were

housed in rooms with a controlled temperature ($22 \pm 1^\circ\text{C}$), humidity ($55 \pm 10\%$) and 12 h light–dark cycle. Food and water were available *ad libitum*.

In vitro organ bath studies

Guinea pigs were killed by cervical dislocation followed by exsanguination. Whole tracheae were rapidly removed and immersed into sterile Dulbecco's modified Eagle's medium (DMEM) supplemented with $4500\text{ mg}\cdot\text{L}^{-1}$ D-glucose, $110\text{ mg}\cdot\text{L}^{-1}$ sodium pyruvate, $584\text{ mg}\cdot\text{L}^{-1}$ L-glutamine supplemented with $100\text{ U}\cdot\text{mL}^{-1}$ penicillin and $100\text{ }\mu\text{g}\cdot\text{mL}^{-1}$ streptomycin. Individual tracheae were thereafter dissected free from adherent connective tissue and fat under sterile conditions and divided into four segments, each with three cartilage rings. The tracheal rings were used directly after dissection (fresh) or placed individually in DMEM for culture. Under culture conditions, the tracheal rings were incubated at 37°C in humidified 5% CO_2 in air for 1, 2 and 4 days with either saline or TNF- α at 1, 10, $100\text{ ng}\cdot\text{mL}^{-1}$ in $300\text{ }\mu\text{L}$ DMEM in 96-well plates. Segments were moved into a new well containing fresh media and TNF- α where appropriate every day.

Tracheal smooth muscle contractions were measured in 4 mL organ baths containing Krebs–Henseleit solution (composition in mM; NaCl 118.3, KCl 4.7, MgSO_4 1.2, KH_2PO_4 1.2, NaHCO_3 25, D-glucose 11.1, CaCl_2 2.5) supplemented with the COX inhibitor indomethacin ($10\text{ }\mu\text{M}$). The Krebs–Henseleit solution was gassed with a stream of 95% O_2 and 5% CO_2 and maintained at 37°C . The tracheal rings were placed over two L-shaped metal prongs. One prong was connected to an FT-03 (Grass Instruments, Quincy, MA, USA) force transducer for continuous recording of isometric tension in units of milli Newtons (mN) using Chart recording software (version 5, AD Instruments, Oxford, UK) connected to a PowerLab acquisition system (AD Instruments). The other prong was fixed to a displacement rod connected to a micrometer for enabling adjustment of the distance between the parallel prongs. After 1 h equilibration under a 10 mN tension, the contractile capacity of each tracheal ring was assessed by exposure to MCh ($10\text{ }\mu\text{M}$). Following washout and a 30 min resting period, contractions induced by EFS (1 min trains every 2 min at 1–30 Hz frequency, 0.5 ms pulse width and a voltage 10% greater than the voltage required to elicit maximal contractions) were obtained with each segment. This was followed by constructing cumulative concentration–response curves to 5-HT and MCh. Pharmacological characterization of the 5-HT receptor responses was performed in the absence and presence of a 20 min incubation with atropine ($1\text{ }\mu\text{M}$), hexamethonium ($100\text{ }\mu\text{M}$), ketanserin (100 nM) or denuding the epithelium by gently rubbing the tracheal lumen with forceps.

In vivo lung function studies

Guinea pigs were anaesthetized transiently with an intramuscular injection of ketamine ($40\text{ mg}\cdot\text{kg}^{-1}$) and xylazine ($8\text{ mg}\cdot\text{kg}^{-1}$). A flexible silicone catheter (outer diameter 2 mm, inner diameter 1 mm) was introduced into the trachea, and a blunt Hamilton syringe needle was passed into the trachea through the catheter to enable instillation of $50\text{ }\mu\text{L}$ saline or TNF- α ($2\text{ }\mu\text{g}$; Whelan, 1996) dissolved in $50\text{ }\mu\text{L}$ saline into the airways. After administration, both the catheter and syringe

were withdrawn, and the animals were allowed to recover. Six hours after instillation, the animals were injected with urethane ($1.5\text{ g}\cdot\text{kg}^{-1}$ i.p.) to induce surgical anaesthesia. A tracheotomy was performed to enable cannulation of the trachea, which was attached to a pneumotachograph connected to a Validyne differential pressure transducer ($\pm 2\text{ cm H}_2\text{O}$). Changes in airflow were measured using a Lung Function Recording system (version 9.2; Mumed Systems, London, UK) and displayed in real time on a personal computer (Stone Computers, London, UK). The flow signal was integrated to give a measure of tidal volume. The intercostal muscles were paralysed using suxamethonium ($2\text{ mg}\cdot\text{kg}^{-1}$ s.c.), and the animal was artificially ventilated with $8\text{ mL}\cdot\text{kg}^{-1}$ of room air using a ventilator pump (Harvard Apparatus, Edenbridge, UK) throughout the experiment.

A cannula was inserted into the thoracic cavity between the third and fifth rib and connected to another Validyne pressure differential transducer ($\pm 20\text{ cm H}_2\text{O}$). The positive side of the transducer was connected to the side of the pneumotachograph proximal to the animal to obtain a measure of the transpulmonary pressure (difference between mouth and thoracic pressure). The lung function parameter total airway resistance (R_t ; $\text{cm H}_2\text{O s}\cdot\text{L}^{-1}$) was derived from each measure of flow, tidal volume and transpulmonary pressure by the method of integration. The jugular vein and carotid artery were cannulated for i.v. administration of drug and measurement of mean arterial blood pressure respectively. In some experiments, bilateral vagotomy was also performed to enable the vagi to be threaded through a pair of platinum electrodes for vagal electrical stimulation using a Grass S88 stimulator (Grass Instruments).

Following stable baseline recording of lung air flow resistance, heart rate and mean arterial blood pressure, the vagi were stimulated for 5 s trains every minute at 1–20 Hz frequency, 0.5 ms pulse width and 40 V . This was followed by construction of dose–response curves to 5-HT and MCh. In other experiments, pharmacological characterization of the vagal and 5-HT responses was performed 15 min after a i.v. injection of atropine ($10\text{ }\mu\text{g}\cdot\text{kg}^{-1}$) or ketanserin ($10\text{ }\mu\text{g}\cdot\text{kg}^{-1}$). Before terminating the experiment by killing the animal with an overdose of sodium pentobarbital ($65\text{ mg}\cdot\text{kg}^{-1}$ i.v.), the tracheal cannula was disconnected from the pneumotachograph to enable a lavage of the lungs using 5 mL of 0.9% saline.

Cell count in the lavage fluid

A $100\text{ }\mu\text{L}$ sample of the lavage fluid was added to an equal volume of 50% Turk's stain solution for counting the total number of leucocytes present in the lavage fluid using an improved ($0.2 \times 0.2 \times 0.1\text{ mm}$) Neubauer haemocytometer under light microscopy. The number of cells counted was multiplied by 10^4 to give the concentration of cells $\cdot\text{mL}^{-1}$ present in the lavage sample. To identify the different types of cells present in the lavage fluid as macrophages, neutrophils and eosinophils, a differential cell count was performed after using the REASTAIN Quick-Diff stains on cytopspins of the lavage fluid.

Data analysis

Individual agonist concentration–response curves obtained from the organ bath studies in the absence and presence of a

competing ligand were fitted by non-linear regression to the four-parameter Hill equation (Equation 1), using GraphPad PRISM 5.0 for Windows (GraphPad Software, San Diego, CA, USA):

$$E = \text{Basal} + \frac{E_{\max} - \text{Basal}}{1 + 10^{(\text{Log EC}_{50} - \text{Log}[A])n_H}} \quad (1)$$

where E denotes response, $\text{Log}[A]$ the logarithm of the concentration of an agonist A , n_H the midpoint slope of the curve, Log EC_{50} the logarithm of the midpoint location parameter along the concentration axis and E_{\max} and basal the upper and lower asymptotes respectively. The concentration–response data were plotted as the mean \pm SEM. The concentration ratio for the rightward shift of the agonist concentration–response curve in the presence of a competing ligand was determined as the ratio of concentrations corresponding to the 50% equieffective agonist response level of the curves. The antagonist potency (pA_2) was calculated from the Gaddum–Schild equation (Schild, 1949). The pA_2 represents the negative logarithm of the concentration of the antagonist, which produces a shift of the agonist concentration–response curve to the right by two linear units to give a concentration ratio of two.

Data obtained from *in vivo* studies were calculated in percentage terms as changes from the baseline value determined immediately before administration of an electrical stimulus or spasmogen and reported as the mean \pm SEM of n values, where n represents the number of animals used per experiment.

Where appropriate, shifts of a concentration–response curve by the presence of the competing ligand were compared by a one-way ANOVA followed by a Dunnett's *post hoc* test for multiple comparisons, or a Student's unpaired *t*-test for comparisons of individual means. Values of $P < 0.05$ were taken to be statistically significant.

Materials

5-HT, ACh, atropine, DMEM, hexamethonium, ketamine, ketanserine, MCh, penicillin, sodium pentobarbital, streptomycin, suxamethonium, urethane and xylazine were all obtained from Sigma-Aldrich, Poole, UK. Tetrodotoxin (TTX) was purchased from Tocris Biosciences UK. Recombinant human TNF- α was purchased from both R&D Systems UK and First Link UK. All other chemicals were purchased from Fisher Scientific UK.

Results

In vitro contraction studies

Contractile effect of TNF- α , EFS, 5-HT and MCh in fresh tracheal segments. Contractions of freshly isolated tracheal rings induced by TNF- α , EFS, 5-HT or MCh were assessed before evaluating the responses of the cultured tissue. Administration of TNF- α (100 ng·mL⁻¹) to the organ baths containing freshly isolated tracheal rings did not elicit a contraction nor a significant change in resting basal tension over a 4 h period following application of the cytokine (data not shown; $n = 6$).

EFS, application of 5-HT (100 μ M) or MCh (10 μ M) to tracheal rings treated for 4 h with TNF- α (100 ng·mL⁻¹) elicited contractions indistinguishable in height to those of fresh tissues not exposed to the cytokine (data not shown; $n = 6$ in each case).

Whilst EFS of the fresh tracheal segments elicited frequency-dependent contractions, both 5-HT and MCh elicited concentration-dependent contractions. Table 1 summarizes the maximal contractions in response to each form of stimulation.

The contractions to each frequency of EFS were transient and predominantly monophasic over the range tested (Figure 1A). Occasionally, a transient relaxation was observed following a contraction at the higher, that is 15–30 Hz frequencies of stimulation. During the construction of the cumulative concentration–response curves to 5-HT, the contractions to this agonist were biphasic with an initial transient peak followed by a contraction that reached a stable plateau. Regardless of the biphasic nature of the contraction, the maximum height of either contraction was measured for plotting the concentration–response curve. Contractions to MCh were monophasic and formed a stable plateau. Table 1 shows that the maximal contraction induced by EFS or 5-HT were comparable, but almost fourfold smaller in amplitude compared with those evoked by MCh in these fresh tracheal segments.

Contractile effect of EFS, 5-HT or MCh following culture of tracheal segments in the absence and presence of TNF- α . Fully defined frequency- and concentration–response curves were obtained to EFS (Figure 2) and the agonists 5-HT (Figure 3) and MCh (Figure 4), respectively, for all segments cultured for 1, 2 and 4 days in the absence and presence of TNF- α (1, 10 and 100 ng·mL⁻¹). In the absence of TNF- α treatment, the maximal contraction and the sensitivity of the tracheal rings to each type of stimulation were not significantly different from that of fresh segments. However, treatment of the segments for 1, 2 and 4 days with 1, 10 and 100 ng·mL⁻¹ TNF- α resulted in a significant time- and concentration-dependent increase in the maximum contraction and sensitivity to EFS and 5-HT (Figures 2 and 3). Figure 1 illustrates the larger contractions of tracheal rings incubated for 4 days with TNF- α (100 ng·mL⁻¹), compared with those of tracheal rings incubated for 4 days with saline.

On day 1 (Figure 3A), only treatment with the highest concentration of TNF- α induced a significantly stronger contraction to 5-HT of $38.1 \pm 4.3\%$ ($n = 6$) compared with the saline-incubated control tracheal segments, whereas at day 2 (Figure 3B) and 4 (Figure 3C), all three concentrations of TNF- α potentiated the contractions to the amine. By day 4, the contractions to 5-HT were potentiated markedly by all three concentrations of TNF- α (45.2 ± 3.8 , 64.1 ± 2.9 and $75.3 \pm 2.3\%$ increase by 1, 10 and 100 ng·mL⁻¹ TNF- α , respectively, $n = 6$). In contrast to the situation with 5-HT, all three concentrations of TNF- α augmented the contractions to EFS from day 1 (Figure 2). The magnitude of the enhancement to each concentration of TNF- α increased progressively with the duration of incubation. By day 4, the contractions to EFS were potentiated significantly by 23.2 ± 4.7 , 45.8 ± 2.9 and $58.3 \pm 3.9\%$ by 1, 10 and 100 ng·mL⁻¹ TNF- α , respectively

Table 1Contractile responses to EFS, 5-HT and MCh in guinea pig tracheal rings, freshly prepared or following 1, 2 and 4 days in organ culture with or without TNF- α (1–100 ng·mL⁻¹)

| Treatment | TNF- α ng·mL ⁻¹ | EFS E_{\max} (mN) at 30 Hz | % increase in EFS E_{\max} from saline control | EFS Frequency provoking 50% of E_{\max} | 5-HT E_{\max} (mN) | % increase in 5-HT E_{\max} from saline control | 5-HT pEC ₅₀ (M) | MCh E_{\max} (mN) | % increase in MCh E_{\max} from saline control | MCh pEC ₅₀ (M) |
|-----------|--------------------------------------|------------------------------------|--------------------------------------------------------|-------------------------------------------------|-------------------------|---------------------------------------------------------|-------------------------------|------------------------|--------------------------------------------------------|------------------------------|
| Fresh | 0 | 10.60 ± 2.46 | – | 1.3 ± 0.2 | 9.65 ± 2.37 | – | 6.11 ± 0.07 | 38.43 ± 4.45 | – | 6.11 ± 0.07 |
| Day 1 | 0 | 10.53 ± 3.64 | – | 1.3 ± 0.4 | 9.45 ± 3.36 | – | 6.53 ± 0.09 | 37.80 ± 2.42 | – | 6.53 ± 0.09 |
| | 1 | 13.95 ± 2.29* | 25.21 ± 3.42 [#] | 1.1 ± 0.2 | 12.05 ± 3.62 | 20.34 ± 3.76 | 6.37 ± 0.11 | 38.45 ± 2.56 | 1.02 ± 2.56 | 6.39 ± 0.09 |
| | 10 | 18.05 ± 4.58* | 43.36 ± 3.71 [#] | 2.3 ± 0.4 | 13.06 ± 5.55 | 26.75 ± 2.57 | 6.03 ± 0.09 | 39.13 ± 2.65 | 2.32 ± 1.24 | 6.31 ± 0.09 |
| | 100 | 22.93 ± 2.26* | 51.34 ± 2.76 [#] | 3.9 ± 0.3 | 15.55 ± 2.66* | 38.13 ± 4.32 [#] | 6.58 ± 0.25 | 35.74 ± 2.75 | 0.14 ± 2.56 | 6.59 ± 0.12 |
| Day 2 | 0 | 13.29 ± 2.43 | – | 2.3 ± 0.5 | 10.87 ± 2.37 | – | 6.57 ± 0.08 | 37.91 ± 2.18 | – | 6.25 ± 0.07 |
| | 1 | 16.75 ± 5.46* | 25.65 ± 4.21 [#] | 2.3 ± 0.4 | 15.30 ± 3.64* | 21.55 ± 3.05 [#] | 6.55 ± 0.10 | 40.41 ± 3.67 | 2.14 ± 1.61 | 6.41 ± 0.09 |
| | 10 | 21.52 ± 2.52* | 39.31 ± 2.61 [#] | 3.2 ± 0.4 | 25.30 ± 4.32* | 56.42 ± 3.63 [#] | 6.23 ± 0.11 | 37.66 ± 3.69 | 0.62 ± 1.20 | 6.32 ± 0.10 |
| | 100 | 29.40 ± 4.07* | 51.78 ± 3.34 [#] | 4.8 ± 0.5 | 27.36 ± 4.03* | 60.13 ± 5.00 [#] | 6.53 ± 0.09 | 37.60 ± 4.55 | 0.53 ± 0.82 | 6.41 ± 0.10 |
| Day 4 | 0 | 17.45 ± 4.86* | – | 2.1 ± 0.1 | 8.67 ± 2.37 | – | 6.69 ± 0.10 | 38.20 ± 4.85 | – | 6.45 ± 0.16 |
| | 1 | 23.71 ± 5.71* | 23.22 ± 4.72 [#] | 2.7 ± 0.3 | 16.24 ± 3.51* | 45.21 ± 3.79 [#] | 6.85 ± 0.09 | 36.83 ± 2.44 | 0.94 ± 0.31 | 6.36 ± 0.10 |
| | 10 | 31.19 ± 4.88* | 45.78 ± 2.87 [#] | 3.6 ± 0.3 | 23.74 ± 4.82* | 64.11 ± 2.85 [#] | 6.47 ± 0.18 | 37.83 ± 3.59 | 1.32 ± 1.13 | 6.36 ± 0.10 |
| | 100 | 36.54 ± 4.82* | 58.34 ± 3.87 [#] | 4.3 ± 0.3 | 33.82 ± 4.12* | 75.34 ± 2.33 [#] | 6.56 ± 0.14 | 38.43 ± 2.72 | 1.06 ± 1.47 | 6.53 ± 0.08 |

E_{\max} values were derived by non-linear regression analysis of the contractions of the isolated tracheal segments in response to EFS (1 min trains every 2 min at 1–30 Hz frequency, 0.5 ms pulse width and a voltage 10% greater than the voltage required to elicit maximal contraction), 5-HT or MCh. – represents values not determined. TNF- α (0 ng·mL⁻¹) represents the saline-treated control. Values shown are means ± SEM, $n = 6$. * $P < 0.05$, significant difference (paired t -test) between the E_{\max} of the TNF- α incubated tissue and contractions of the corresponding freshly dissected tissue in response to EFS (30 Hz) or 5-HT (10⁻⁴ M). [#] $P < 0.05$, significant difference (paired t -test) between the percentage increase in contraction of the TNF- α incubated tissue and contractions of the corresponding freshly dissected tissue in response to EFS (30 Hz) or 5-HT (10⁻⁴ M).

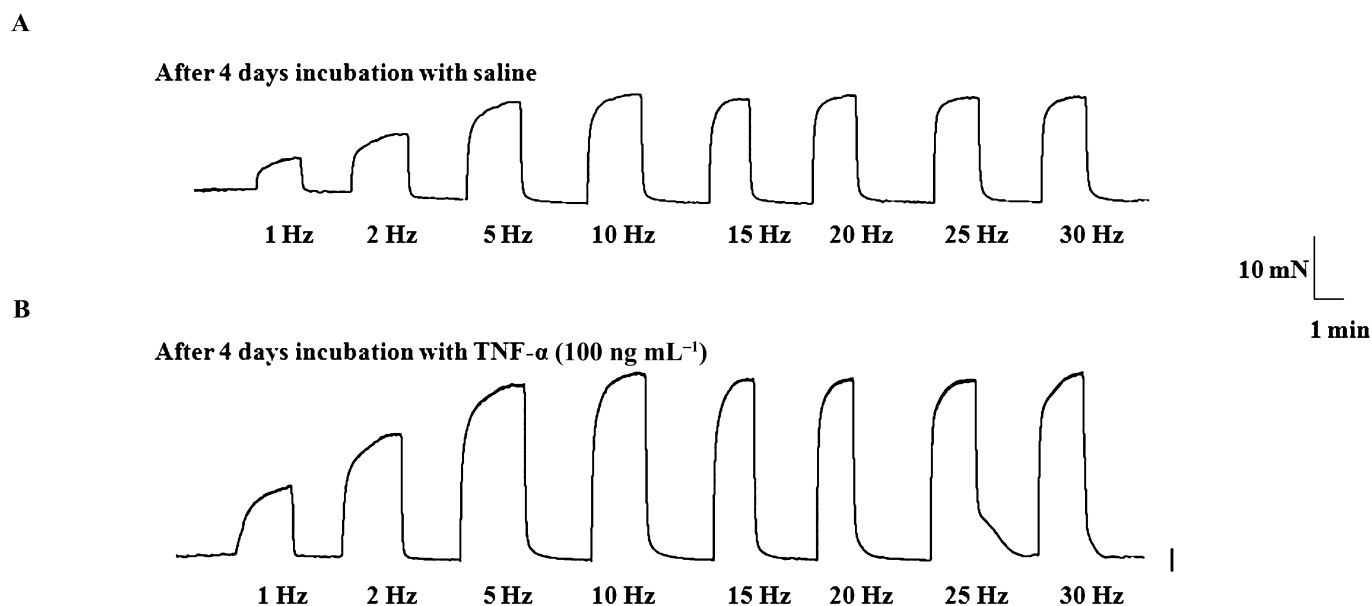


Figure 1

Isometric contractions of the guinea pig isolated trachea in response to EFS after incubation for 4 days with (A) saline and (B) TNF- α (100 ng·mL⁻¹). EFS parameters: 1 min trains every 2 min at 1–30 Hz frequency, 0.5 ms pulse width and a voltage 10% greater than the voltage required to evoke maximal contractions.

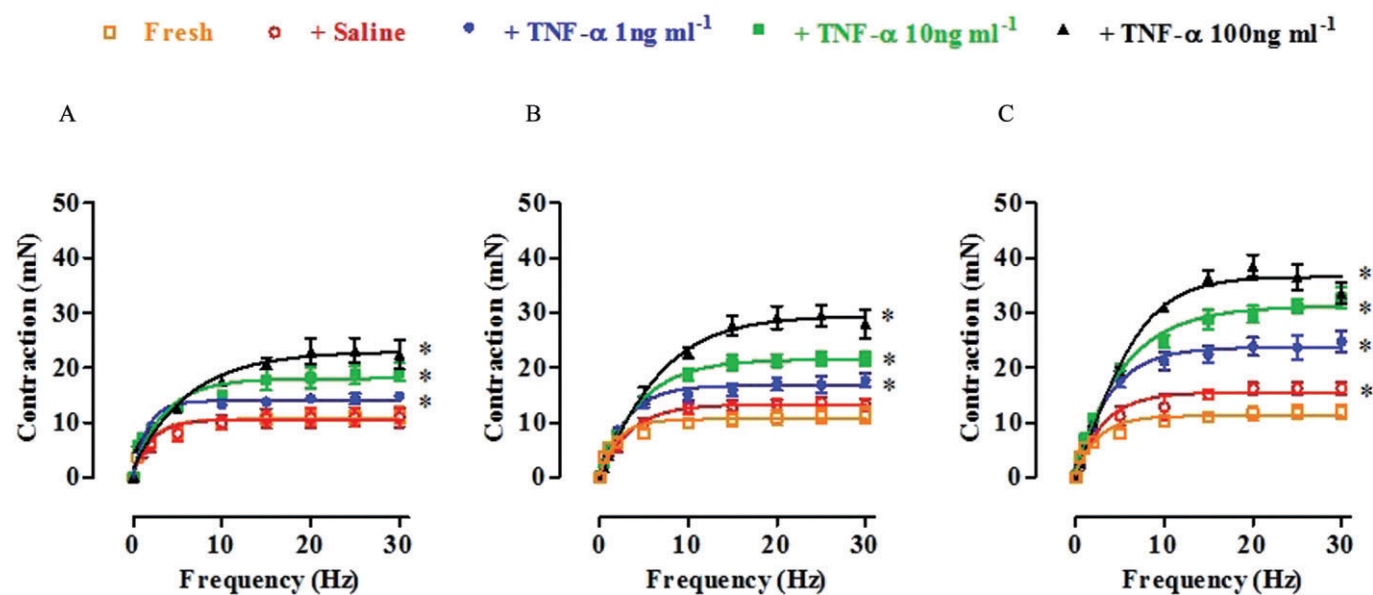


Figure 2

Frequency–response curves of the guinea pig isolated trachea in response to electrical field stimulation (EFS). Response of freshly dissected tracheal rings and rings incubated for (A) 1 day, (B) 2 days and (C) 4 days with saline or TNF- α at 1, 10 or 100 ng·mL⁻¹. Each point represents the mean \pm SEM ($n = 6$). * $P < 0.05$, significant difference between contractions of the TNF- α -incubated tissue and contractions of the freshly dissected tissue, in response to EFS (30 Hz).

($n = 6$, Figure 2C), compared with the saline-incubated control segments. The effect of TNF- α at a concentration higher than 100 ng·mL⁻¹ was not examined to establish the E_{\max} concentration of TNF- α .

In contrast to EFS and 5-HT, the concentration–response curves to MCh were unaltered by the concentration and time of incubation with TNF- α and were identical to the TNF- α untreated control segments tracheal (Figure 4).

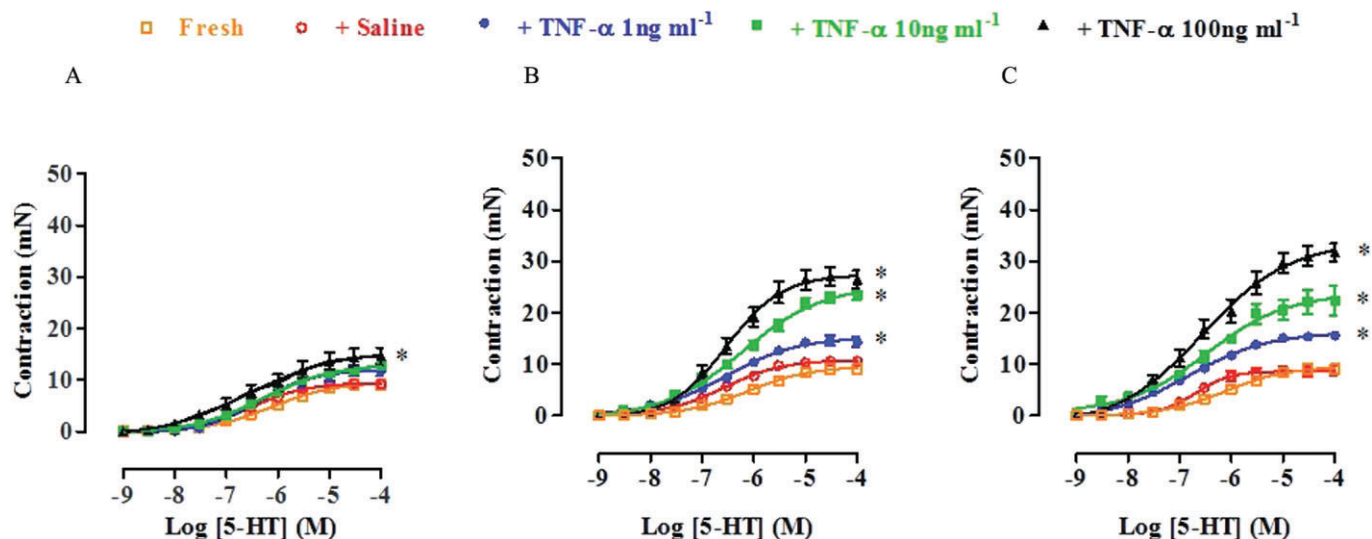


Figure 3

Concentration-response curves of the guinea pig isolated trachea in response to 5-HT. Response of freshly dissected tracheal rings and rings incubated for (A) 1 day, (B) 2 days and (C) 4 days with saline or TNF- α at 1, 10 or 100 ng·mL⁻¹. Each point represents the mean \pm SEM ($n = 6$). * $P < 0.05$, significant difference between contractions of the TNF- α -incubated tissue and contractions of the freshly dissected tissue, in response to 5-HT (10⁻⁴ M).

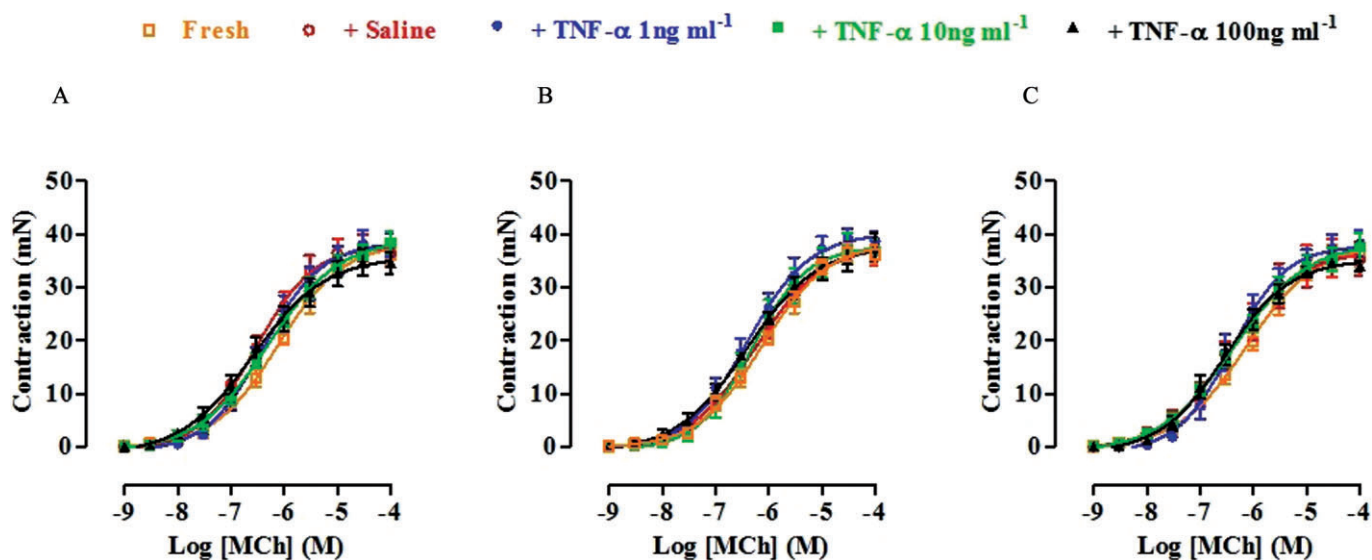


Figure 4

Concentration-response curves of the guinea pig isolated trachea in response to MCh. Response of freshly dissected tracheal rings and rings incubated for (A) 1 day, (B) 2 days and (C) 4 days with saline (O) or TNF- α at 1, 10 and 100 ng·mL⁻¹. Each point represents the mean \pm SEM ($n = 6$).

Pharmacological characterization of the evoked contractions. EFS and 5-HT can stimulate release of acetylcholine from the parasympathetic nerves and tracheal epithelium in mice, respectively (Eum *et al.*, 1999; Moffatt *et al.*, 2004). Therefore, experiments were performed to characterize whether these findings extended to the guinea pig trachea and whether the TNF- α -induced enhancement of contractions to EFS and 5-HT was in response to an increased release of ACh. In

addition, experiments were performed in order to define the receptor or receptors responsible for the contractile action of EFS, 5-HT and MCh.

In tracheal segments incubated for 4 days both in the absence and presence of TNF- α (100 ng·mL⁻¹), a 15 min pre-treatment of the segments with atropine (1 μ M) caused a ~1000-fold parallel rightward shift of the location of the EFS frequency- and 5-HT and MCh concentration-response curve

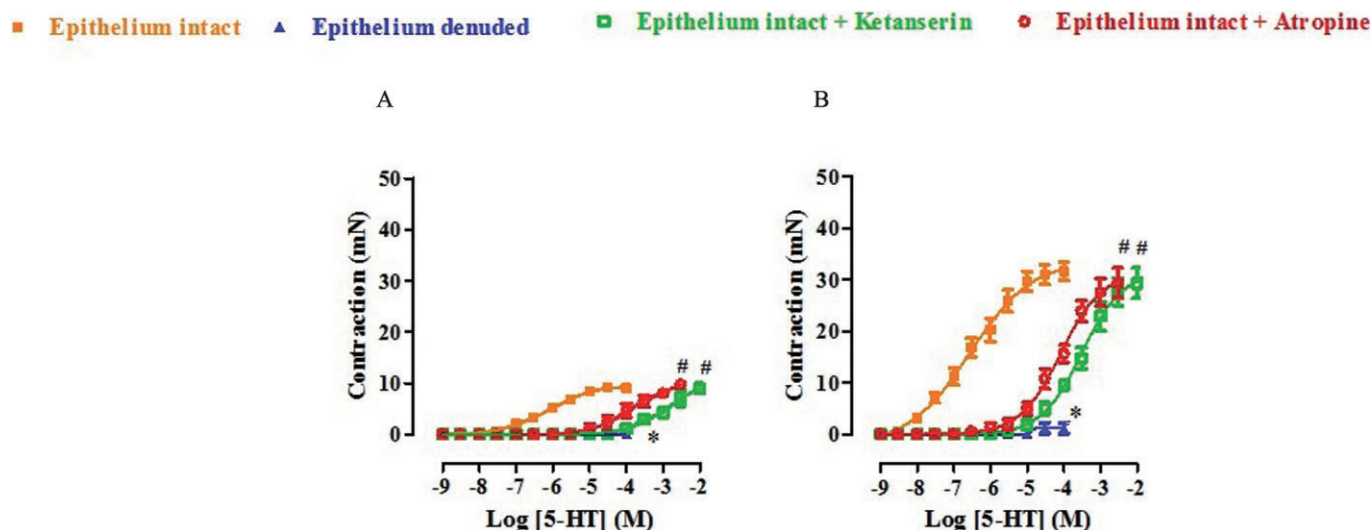


Figure 5

Concentration–response curves of the guinea pig isolated trachea in response to 5-HT. Response of (A) freshly dissected tracheal rings and (B) rings pre-incubated with TNF- α 100 ng·mL⁻¹ for 4 days before and after removal of the epithelium, pre-treatment with atropine 10⁻⁶ M or ketanserin 10⁻⁷ M. Each point represents the mean \pm SEM ($n = 6$). * $P < 0.05$, significant difference between contractions of the epithelium-intact and epithelium-denuded trachea TNF- α -incubated tissue and the contractions of freshly dissected tissue, in response to 5-HT (10⁻⁴ M). # $P < 0.05$, significant difference between the concentration–response curves to 5-HT in the absence and presence of atropine 10⁻⁶ M or ketanserin 10⁻⁷ M.

(data not shown; $n = 6$ in each case) without a change in the maximal contraction. The pA₂ values of atropine for the antagonism of 5-HT and MCh contractions were 9.12 ± 0.45 and 8.36 ± 0.33 , respectively, in the 4 day TNF- α -incubated segments, whereas the pA₂ values of atropine against 5-HT and MCh in the 4 day control segments were 8.54 ± 0.61 and 8.76 ± 0.30 respectively (data not shown; $n = 6$ in each case). The contractions of all segments to each of the three forms of stimulation were unaffected by a pre-treatment with hexamethonium (100 μ M; data not shown; $n = 6$).

Pre-treatment of the tracheal segments incubated for 4 days in the absence and presence of TNF- α (100 ng·mL⁻¹) with TTX (1 μ M) only abolished the contractions to EFS. Pre-treatment with ketanserin (100 nM) caused a parallel rightward displacement of the 5-HT concentration–response curve without a reduction in the maximal response with pA₂ values of 10.13 ± 0.32 and 9.42 ± 0.65 respectively (Figure 5; $n = 6$ in each case). Ketanserin (100 nM) did not alter the contractions to EFS and MCh. Therefore, the pA₂ values of atropine against 5-HT and MCh, and of ketanserin against 5-HT in the absence and presence of 4 days of incubation with TNF- α (100 ng·mL⁻¹) were not significantly different.

Removal of the epithelium from the tracheal segments before incubation for 4 days in the absence and presence of TNF- α (100 ng·mL⁻¹) prevented the tissues responding to 5-HT. However, these denuded tissues responded to EFS with a slightly, non-significant, reduced maximum contraction compared with that achieved in the epithelial-intact TNF- α -incubated tracheal segments (data not shown; $n = 6$ in each case). The concentration–response curves for MCh in the TNF- α -incubated epithelial-denuded tracheal segments did not differ from the epithelial-intact TNF- α -incubated segments.

In vivo lung function studies

Baseline responses. *In vivo* studies were performed on guinea pigs exposed only to a single 2 μ g dose of TNF- α per animal, instilled intratracheally, because of ethical considerations towards the use of a large number of animals and the high cost of the cytokine for defining a dose–response curve.

Guinea pigs treated with either saline or TNF- α did not show any signs of breathing difficulties or differences in overt behaviour upon recovery from the ketamine- and xylazine-induced transient anaesthesia, used during the intratracheal instillation. Under urethane-induced surgical anaesthesia, initially, the baseline total lung resistance to airflow of the TNF- α -treated guinea pigs was significantly higher by $7.1 \pm 2.2\%$ ($n = 6$) than that of the saline-treated guinea pigs (Table 2). Inflation of the lungs by occluding the air outlet of the ventilator caused the elevated baseline to fall to the baseline total lung resistance of the saline-treated animals. There was no statistically significant difference in the resting mean arterial blood pressure and heart rate between the two treatment groups (Table 2).

Effect of TNF- α on bronchoconstriction induced by vagal EFS, 5-HT or MCh. EFS of the distal ends of vagus nerves, 5-HT and MCh (given i.v.) all provoked frequency- and dose-dependent, rapid and reversible increases in total lung resistance in all animals respectively (Figure 6). These increases in total lung resistance from baseline were taken as a measure of bronchoconstriction. The bronchoconstriction to vagal EFS (Figures 6A and 7) and 5-HT (Figure 6B), but not MCh (Figure 6C), was significantly augmented in the TNF- α -treated animals, compared with the saline-treated control animals. For instance, the frequency of vagal EFS eliciting an increase in total lung resistance by 200% above baseline in

Table 2

Baseline parameters of the experimental groups of guinea pigs

| Treatment group | Total Resistance (water L ⁻¹ .s ⁻¹) | Heart rate (beats.min ⁻¹) | Mean arterial blood pressure (mmHg) |
|-----------------|---------------------------------------------------------------|------------------------------------------|----------------------------------------|
| Control | 144 ± 7.1 | 290 ± 16.1 | 46.7 ± 3.1 |
| +TNF-α | 154 ± 6.7* | 287 ± 13.5 | 43.6 ± 5.3 |

Values are represented as mean ± SEM, *n* = 6. **P* < 0.05, significant effect (paired *t*-test) of TNF-α treatment.

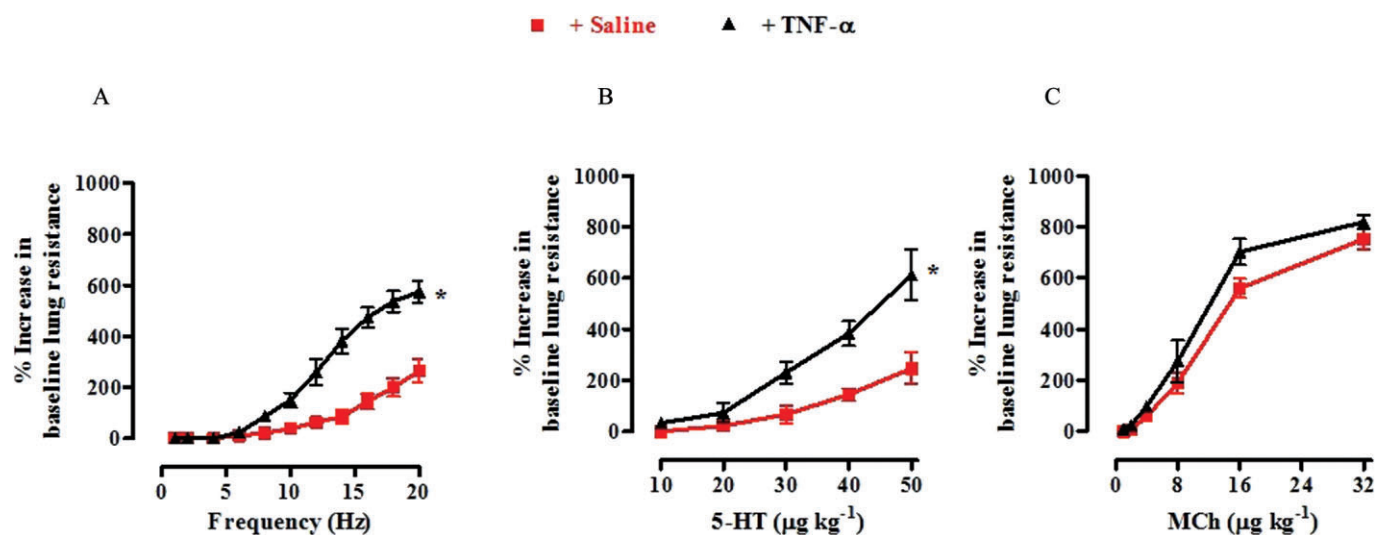


Figure 6

Increases in airway resistance above baseline of anaesthetized guinea pigs to (A) vagal stimulation, (B) 5-HT or (C) MCh 6 h after intratracheal instillation of 50 μL of either saline or 2 μg of human TNF-α. Each point represents the mean ± SEM (*n* = 6). **P* < 0.05, significant difference between the increase in airway lung resistance in response to vagal stimulation or i.v./ 5-HT after tracheal instillation of saline or TNF-α.

the saline- and TNF-α-treated animals was 18 ± 2 and 11 ± 3 Hz, respectively (*n* = 6, *P* < 0.05 unpaired *t*-test), whereas the dose of 5-HT provoking a similar increase in total lung resistance above baseline in the respective animal groups was 44 ± 3 versus 28 ± 2 μg.kg⁻¹ (*n* = 6, *P* < 0.05 unpaired *t*-test).

Pharmacological characterization of the TNF-α augmented bronchoconstriction induced by vagal EFS, 5-HT MCh. A role of endogenous ACh in mediating the bronchoconstriction to vagal EFS and 5-HT and its enhancement by TNF-α was investigated in animals following pre-treatment (15 min) with either atropine (10 μg.kg⁻¹ i.v.) or ketanserin (10 μg.kg⁻¹ i.v.). While atropine abolished the bronchoconstriction to vagal EFS, 5-HT and MCh in both saline- and TNF-α-treated animals, ketanserin only abolished the bronchoconstriction in response to 5-HT (data not shown; *n* = 5–6).

Effect of TNF-α on heart rate and mean arterial blood pressure in response to vagal EFS, 5-HT or MCh. All three forms of stimulation caused rapid and transient decreases in heart rate and mean arterial blood pressure at all frequencies of vagal EFS and doses of agonists used in both saline- and TNF-α-

challenged animals (Figure 8). The maximum falls in heart rate and blood pressure to the highest frequency of vagal EFS and the highest dose of 5-HT were seven- and threefold of the fall caused by MCh (data not shown; *n* = 6), and they did not differ between the TNF-α- and saline-treated guinea pigs.

Cell count in the lavage fluid. The total number of leukocytes per mL in the BAL fluid recovered from guinea pigs treated with TNF-α was significantly (31.9 ± 4.4%) greater than in BAL fluid obtained from saline-treated animals (Figure 9A). A differential cell count showed that the increase in total number of leukocytes was mostly due to a marked increase of neutrophils in the BAL fluid from the TNF-α-treated guinea pigs (Figure 9B). Eosinophils were largely absent from the BAL fluid obtained from either saline- or TNF-α-treated animals.

Discussion

Pro-inflammatory cytokines such as TNF-α play a central role in the induction and propagation of the asthma pathology by

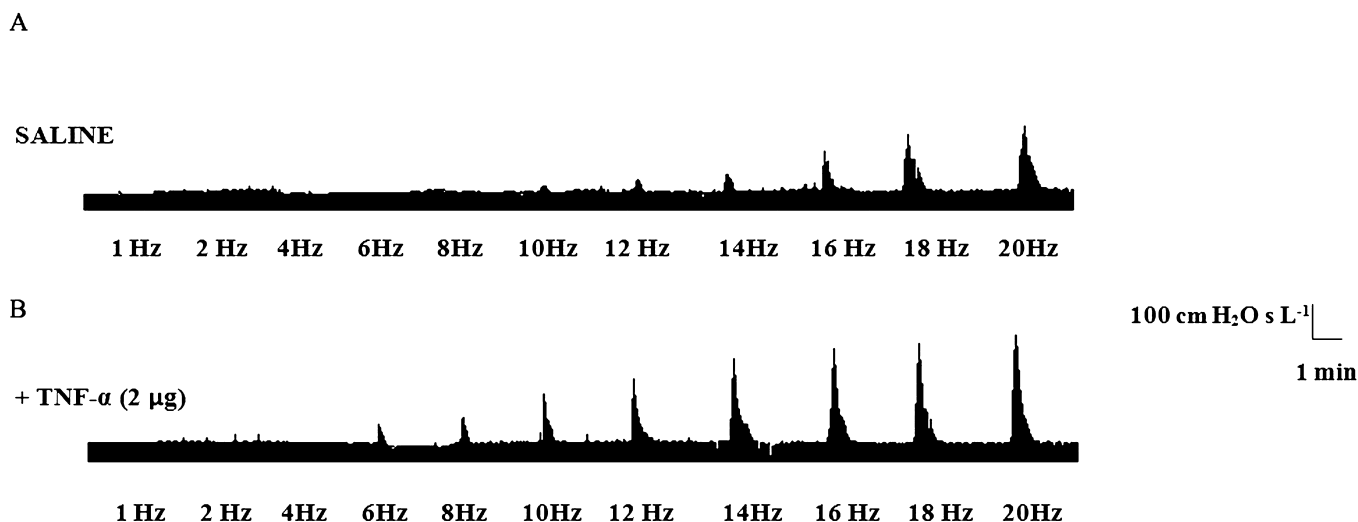


Figure 7

Increases in airway lung resistance, as a measurement of bronchoconstriction, in anaesthetized guinea pigs in response to EFS of the vagi 6 h after tracheal instillation of (A) saline and (B) TNF- α (2 μ g). EFS parameters: 5 s trains every minute at 1–20 Hz frequency, 0.5 ms pulse width and 40 V.

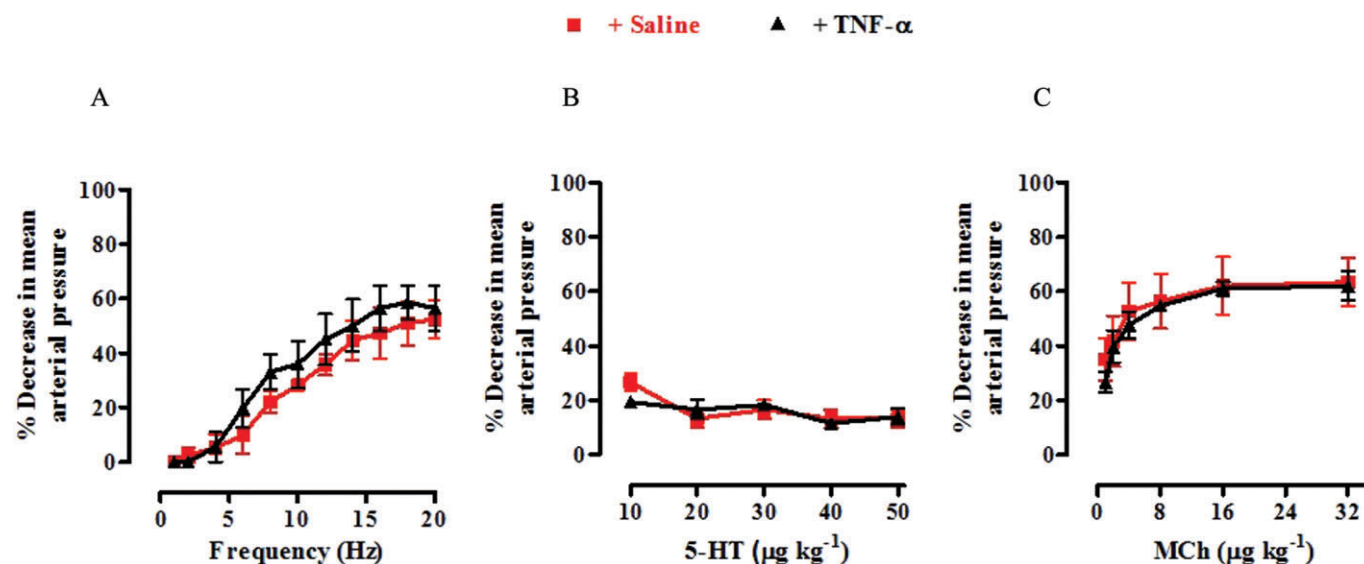


Figure 8

Decreases in mean arterial blood pressure of anaesthetized guinea pigs to (A) vagal stimulation, (B) 5-HT and (C) MCh 6 h after intratracheal instillation of 50 μ L of either saline or 2 μ g of human TNF- α . Each point represents the mean \pm SEM ($n = 6$).

causing hyperresponsiveness, inflammation and remodelling of the airways. Our aim was to use a comparable *in vitro* and *in vivo* protocol to understand the mechanisms of TNF- α -induced airway hyperresponsiveness.

These data obtained from the organ bath studies showed that guinea pig isolated tracheal segments cultured for up to 4 days in the absence of TNF- α maintained their contractile phenotype by responding reproducibly to EFS, 5-HT and MCh, as effectively as fresh tissues. All forms of stimulation caused contractions of the tracheal segments through activa-

tion of nerves, epithelium and airway smooth muscle, respectively (see below), which suggested that each of these cell types, together with their receptors and intracellular signalling pathways remained intact and stable during the 4 day culture procedure. Control tracheal segments incubated for 8 days did not contract uniformly in response to EFS, 5-HT or MCh, and therefore, incubation for this longer period was not continued. Cultured isolated airway segments have previously been used to study the effects of inflammatory mediators. With the exception of the mouse trachea (Adner *et al.*,

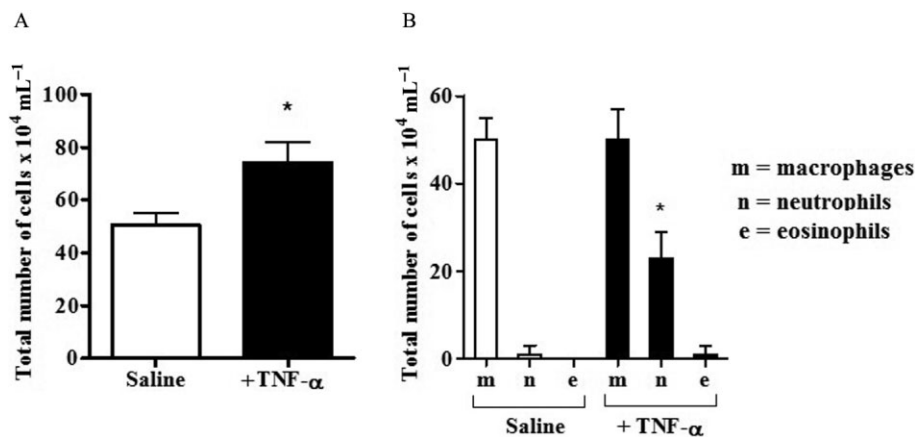


Figure 9

Total (A) and differential (B) count of the leukocytes in lavage fluid (BAL) from guinea pig lung, 6 h after intratracheal instillation of 50 µL of either saline or 2 µg of human TNF-α. In (B), differential counts of macrophages, neutrophils and eosinophils are shown. *P < 0.05, significantly different from the corresponding values in saline-treated animals.

2002), culture of tracheal segments with TNF-α from larger species such as man (Amrani *et al.*, 1995; 1997; Sukkar *et al.*, 2001), cow (Nakatani *et al.*, 2000), rabbit (Hakonarson *et al.*, 1996), guinea pig (Pennings *et al.*, 1998; Renzetti *et al.*, 1996; White *et al.*, 1997; Parris *et al.*, 1999), rat (Renzetti *et al.*, 1996) and sheep (Reynolds *et al.*, 2000) have not typically exceeded 1 day. The mouse trachea can survive in culture for up to 4 days without a loss in reactivity to 5-HT and carbachol, and without changes in global gene expression, possibly because of the ease of diffusion of nutrients to the cells through its thin (~1 mm) tracheal wall.

Pharmacological characterization of the contractions of tracheal segments with and without TNF-α treatment showed that the EFS-evoked contractions were indirect. Moreover, they were mediated by activation of the muscarinic receptors located on the smooth muscle by ACh released from the innervating post-ganglionic neurones. We inferred this because the contractions were inhibited by blockade of the tracheal smooth muscle M₃ muscarinic receptors with atropine (pA₂ ~9.0) and nerve conduction with the voltage-gated Na⁺ channel blocker TTX, but not by blockade of the autonomic ganglia with the nicotinic receptor antagonist hexamethonium. The EFS-evoked contractions were refractory to the 5-HT receptor antagonist ketanserin and to epithelial denudation, suggesting that EFS did not release 5-HT or an epithelium-derived spasmogen.

As with fresh tissues, 5-HT-evoked contractions of tracheal segments exposed to TNF-α were also indirect. However, these contractions appeared to be mediated by activation of the muscarinic receptors by ACh released from the tracheal epithelium in response to activation of the 5-HT_{2A} receptor by 5-HT. This interpretation was based on the attenuation of the contractions by atropine (pA₂ ~9.0), ketanserin (pA₂ ~10.0) and following removal of the epithelial layer, but not by blockade of nerve or ganglionic transmission. The pA₂ values of atropine and ketanserin were similar to that obtained at the M₃ muscarinic receptors and 5-HT_{2A} receptors in the mouse (Moffatt *et al.*, 2004) and guinea pig trachea (Macquin-Mavier *et al.*, 1991). Furthermore, these pA₂

values were not significantly different between fresh, 4 day TNF-α-treated and -untreated tracheal segments.

The contractions of both the TNF-α-treated and -untreated tracheal segments in response to MCh were likely to be mediated by a direct stimulation of the smooth muscle through the activation of the M₃ muscarinic receptors and not due to secondary release of a nerve- or an epithelium-derived spasmogen. This was deduced from the antagonism with atropine (pA₂ ~9.0), but not hexamethonium, ketanserin, TTX or following denudation of epithelium.

Culture of guinea pig isolated tracheal segments with TNF-α for 1, 2 and 4 days induced a marked and selective increase in the contractility in response to both EFS and 5-HT, but not to MCh. The augmented contractility to the two former forms of stimulation was dependent on both the concentration and duration of TNF-α incubation. The contractions in response to EFS were greater than those induced by 5-HT before and after treatment with TNF-α, suggesting that more ACh release occurred from the epithelium than from nerves. These findings could imply a greater reserve pool of ACh vesicles stored in the epithelium whose exocytosis machinery is primed for release by TNF-α, or that TNF-α causes an up-regulation in the number of 5-HT_{2A} receptors and their functional coupling in the epithelial cells. *In vivo*, it has been demonstrated that rats exposed to aerosolized TNF-α also have an increased responsiveness to 5-HT (Kips *et al.*, 1992). Parasympathetic nerves releasing ACh also express presynaptic inhibitory M₂ muscarinic receptors to limit the release of ACh itself from the nerves. TNF-α has been shown to contribute to M₂ muscarinic receptor dysfunction by decreasing the expression of the mRNA of the receptor and this may underlie the increased release of ACh and the consequently greater contractions to EFS (Nie *et al.*, 2009).

The potency and maximal contractions elicited by MCh were similar in control and TNF-α-treated segments cultured for the same time period, indicating that TNF-α did not alter the functional capacity of the contractile proteins in the airway smooth muscle. This finding is in agreement with earlier studies using isolated guinea pig and rabbit trachea

cultured for up to 18 h (Wills-Karp *et al.*, 1993; Hakonarson *et al.*, 1996) and mouse trachea cultured for up to 8 days (Adner *et al.*, 2002), where no differences in potency were observed to carbachol. Perhaps, the unchanged potency and maximal contraction in response to MCh in the guinea pig tracheal smooth muscle after TNF- α treatment is attributed to a lack of effect on the number of spare muscarinic receptors, or MCh being a full agonist, which therefore induces a contraction close to the capacity of the tracheal segments respectively.

In this study, we also extended the findings obtained from the contractions of the isolated trachea to the bronchoconstrictor response of the anaesthetized guinea pig. Intratracheal instillation of TNF- α in the guinea pigs did not cause noticeable breathing difficulties upon recovery from the instillation. This finding complemented the lack of a direct contractile action of TNF- α on isolated tracheas and suggested that the cytokine did not, acutely, release sufficient amounts of endogenous spasmogens, such as ACh, to cause airway smooth muscle contraction. The slightly higher baseline total lung resistance measured in the TNF- α -treated animal could be attributed to an occlusion of the airways by increased bronchial secretions in response to TNF- α because inflation of the lungs reduced the baseline total lung resistance to the level of the saline-treated animals.

TNF- α instillation significantly potentiated the bronchoconstrictor response to both vagal EFS and 5-HT, but not to MCh, and consolidated the results from the organ bath studies. The bronchoconstrictor responses to both EFS and 5-HT were mediated indirectly by ACh acting on the airway smooth muscle, like MCh, because of their antagonism by atropine. Furthermore, the contractions to 5-HT but not EFS or MCh were antagonized by ketanserin at a dose selective for the 5-HT_{2A} receptor. This indicated that 5-HT activated 5-HT_{2A} receptors which then released ACh from the epithelium to cause bronchospasm. Importantly, this experiment also confirmed *in vitro* findings that EFS did not release 5-HT to trigger the release of ACh from the epithelium, which thereafter caused the bronchoconstriction.

Previous studies (Pennings *et al.*, 1998; Wills-Karp *et al.*, 1993; Parris *et al.*, 1999) have reported that short-term incubation (not exceeding 24 h) of guinea pig isolated trachea with TNF- α can lead to the development of a hypercontractile phenotype of the airway smooth muscle in response to bronchoconstrictors that act primarily on the smooth muscle, such as carbachol and high K⁺. The pathways by which TNF- α causes a potentiation of the contractility of smooth muscle have not been clearly established, but in addition to a possible alteration in the affinity, expression and effector pathways of a receptor for a bronchoconstrictor, or an impaired response to bronchorelaxant agonists, an alteration in calcium influx and calcium sensitivity of the airway smooth muscle has been postulated.

With respect to the well-established central role of Ca²⁺ ions in regulating airway smooth muscle contractility, there is evidence to suggest that TNF- α can directly impair airway smooth muscle contractility by altering Ca²⁺ regulatory mechanisms in the muscle cells through a sensitization of the small monomeric G-protein, RhoA. This protein can enhance muscle contractility to a spasmogen by increasing levels of myosin light chain phosphorylation through a Rho-activated

kinase-dependent suppression of myosin light chain phosphatase (Amrani and Panettieri, 2002; Amrani, 2006). However, the data from the present study suggest that RhoA-dependent mechanisms may not have a role in the development of airway hyperresponsiveness in response to TNF- α in the our experimental models because contractions of the isolated trachea and bronchoconstrictor response of the guinea pig to MCh were not augmented by treatment with TNF- α .

It was noted that the duration for onset of airway hyperresponsiveness in response to TNF- α under *in vivo* conditions was considerably shorter compared with time taken for development of hyperresponsiveness *in vitro*. The precise mechanism to account for the difference is not clear. However, it is well known that afferent neural reflex pathways and local axon reflexes play an important role in the modulation of airway function *in vivo* through a feedback regulation of airway smooth muscle tone. Moreover, sensitization of the airway reflexes by inflammatory mediators is known to contribute to the development of airway hyperresponsiveness, bronchoconstriction, mucus and fluid secretion, airway inflammation and cough. Therefore, it is conceivable that the exposure of the guinea pig airways to TNF- α *in vivo* could have resulted in a sensitization of airway neural reflexes, which thereby accelerated the induction of airway hyperresponsiveness. The absence of afferent airway reflexes operating in the isolated trachea could be responsible for the delayed onset of hyperresponsiveness *in vitro*.

Bradycardia and hypotension was induced by vagal EFS, 5-HT and MCh but were not influenced by TNF- α treatment, suggesting a local pulmonary site of action of TNF- α . Airway inflammation to TNF- α was characterized by neutrophil migration into the lungs. The absence of significant eosinophilia suggested that severe inflammation was not present despite the presence of airway hyperresponsiveness. However, whilst neutrophils have been suggested to contribute to airway hyperresponsiveness, the ability of TNF- α to induce hyperresponsiveness *in vitro* suggests they are unlikely to play a major role.

Taken together, the data obtained showed that exposure to TNF- α regulated the phenotype of the airways by selectively potentiating the neuronal and epithelium-derived release of ACh, without altering its contractile action on the muscle. Furthermore, the present findings have important implications regarding the mechanism by which TNF- α modulates cholinergic airway hyperresponsiveness.

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Conflict of interest

N Gozzard is an employee of UCB Celltech.

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