British Journal of Pharmacology (2009), 156, 201–210

0007-1188/08
www.brjpharmacol.org

RESEARCH PAPER

Etanercept prevents airway hyperresponsiveness by protecting neuronal M_2 muscarinic receptors in antigen-challenged guinea pigs

Zhenying Nie[1], David B. Jacoby[1,2] and Allison D. Fryer[1,2]

[1]*Division of Pulmonary and Critical Care Medicine, Oregon Health and Science University, Portland, OR, USA, and*
[2]*Department of Physiology and Pharmacology, Oregon Health and Science University, Portland, OR, USA*

Background and purpose: Increased tumour necrosis factor-α (TNF-α) is associated with airway hyperreactivity in antigen-challenged animals. In human asthmatics, TNF-α is increased and blocking it prevents airway hyperreactivity in some asthmatic patients. However, the mechanisms by which TNF-α mediates hyperreactivity are unknown. Airway hyperreactivity can be caused by dysfunction of neuronal M_2 muscarinic receptors that normally limit acetylcholine release from parasympathetic nerves. Here we test whether blocking TNF-α receptors with etanercept prevents M_2 receptor dysfunction and airway hyperreactivity in antigen-challenged guinea pigs.
Experimental approach: Ovalbumin-sensitized guinea pigs were challenged by inhalation of antigen. Some animals received etanercept (3 mg kg^{-1} i.p.) 3 h before challenge. 24 h after challenge, airway hyperreactivity and M_2 receptor function were tested. Inflammatory cells in bronchoalveolar lavage, blood and lung were counted. TNF-α and its receptors were detected by real-time RT-PCR and immunocytochemistry in parasympathetic nerves from humans and guinea pigs and in human neuroblastoma cells.
Key results: Antigen-challenged animals were hyperreactive to vagal stimulation and neuronal M_2 receptors were dysfunctional. Both M_2 receptor dysfunction and airway hyperreactivity were prevented by etanercept. Etanercept reduced eosinophils around airway nerves, and in blood, bronchoalveolar lavage and airway smooth muscle. Also, TNF-α decreased M_2 receptor mRNA in human and guinea pig parasympathetic neurons.
Conclusions and implications: Tumour necrosis factor-α may contribute to M_2 receptor dysfunction and airway hyperreactivity directly by decreasing receptor expression and indirectly by promoting recruitment of eosinophils, containing major basic protein, an M_2 antagonist. This suggests that etanercept may be beneficial in treatment of allergic asthma.
British Journal of Pharmacology (2009) **156**, 201–210; doi:10.1111/j.1476-5381.2008.00045.x

Keywords: TNF-α; eosinophil; asthma; airway hyperreactivity

Abbreviations: BAL, bronchoalveolar lavage; CCR3, CC-motif chemokine receptor 3; hTNF-α, human tumour necrosis factor-α; ICAM-1, intercellular adhesion molecule 1; IFN-γ, interferon-gamma; MBP, major basic protein; mTNF-α, mouse tumour necrosis factor-α; RT-PCR, reverse transcriptase PCR; TNF-α, tumour necrosis factor-α; TNFRSF1A, TNF receptor I; TNFRSF1B, TNF receptor II

Introduction

Parasympathetic nerves provide the dominant autonomic control of airway smooth muscle by releasing acetylcholine (ACh) onto muscarinic receptors, causing contraction and bronchoconstriction. Neuronal M_2 muscarinic receptors (M_2 receptors; nomenclature follows Alexander *et al.*, 2008) limit release of ACh from parasympathetic nerves in lungs (Fryer and Maclagan, 1984). Inhibitory M_2 muscarinic receptors do not function normally in airways of some asthmatics (Ayala and Ahmed, 1989; Minette *et al.*, 1989) or in airways of antigen-challenged animals (Fryer and Wills-Karp, 1991; Verbout *et al.*, 2007). Loss of M_2 receptor function mediates hyperreactivity in antigen-challenged animals and may be an important cause of airway hyperreactivity in asthmatics.

The mechanisms of hyperreactivity in antigen-challenged guinea pigs are very well understood. Sensitization increases eosinophils in the lungs and, in particular, increases eosinophils around nerves (Adamko *et al.*, 1999). However, these eosinophils are quiescent as sensitization alone does not result in airway hyperreactivity (Adamko *et al.*, 1999; Proskocil *et al.*, 2008). Antigen challenge causes resident eosinophils to degranulate and release major basic protein (MBP), an

Correspondence: Allison D. Fryer, Division of Pulmonary and Critical Care Medicine, and Department of Physiology and Pharmacology, Oregon Health and Science University, Portland, OR 97239, USA. E-mail: fryera@ohsu.edu
Received 20 March 2008; revised 11 June 2008; accepted 25 September 2008

endogenous antagonist for M_2 receptors (Jacoby *et al.*, 1993). M_2 receptor blockade increases ACh release, increasing bronchoconstriction (Baker *et al.*, 1992). Airway hyperreactivity and neuronal M_2 receptor dysfunction can be prevented by depletion of eosinophils with an antibody to interleukin-5 (IL-5; Elbon *et al.*, 1995), by inhibition of eosinophil recruitment into the airways with an antibody to very late activation antigen-4 (Fryer *et al.*, 1997), by inhibition of eosinophil migration to nerves with a CC-motif chemokine receptor 3 (CCR3) antagonist (Fryer *et al.*, 2006), and by blockade of MBP with an antibody (Evans *et al.*, 1997) or with heparin (Fryer and Jacoby, 1992). In asthmatics (Azzawi *et al.*, 1992; Ohashi *et al.*, 1992; Costello *et al.*, 1997), eosinophils are also recruited to airways where they are found lined up along airway nerves (Costello *et al.*, 1997), suggesting that recruitment of eosinophils to nerves and loss of M_2 receptor function may contribute to airway hyperreactivity in asthma.

Tumour necrosis factor-α (TNF-α) has garnered attention as a potential player in airway hyperreactivity in asthma (Gosset *et al.*, 1991; Broide *et al.*, 1992; Bradding *et al.*, 1994; Thomas, 2001; Berry *et al.*, 2006). In the lung, TNF-α is released from mast cells (Bradding *et al.*, 1994; Kim *et al.*, 2007; Nakae *et al.*, 2007) and macrophages (Careau *et al.*, 2006). TNF-α expression, TNF receptor I (TNFRSF1A) and TNF-α converting enzyme are all increased in bronchoalveolar lavage (BAL) from patients of asthma compared with normal subjects (Thomas, 2001; Berry *et al.*, 2006; Cazzola and Polosa, 2006). Increased TNF-α activity also has been reported in patients with Churg-Strauss syndrome (Grau *et al.*, 1989; Tsukadaira *et al.*, 1999) which is characterized by severe eosinophilia and most patients have bronchial asthma.

Tumour necrosis factor-α can be blocked by etanercept, a TNF-α receptor IgG_1 fusion protein (Lesslauer *et al.*, 1991; Peppel *et al.*, 1991; Suffredini *et al.*, 1995; Ritchlin *et al.*, 2003). Several papers have reported that blocking TNF-α, either pharmacologically or genetically suppresses airway inflammation in antigen-challenged mice (Choi *et al.*, 2005; Kim *et al.*, 2006; Hutchison *et al.*, 2008). In humans, TNF-α blocking agents decrease eosinophils in the circulation and in the lungs in patients with Churg-Strauss syndrome disease (Arbach *et al.*, 2002). In patients with severe and steroid-resistant asthma, anti-TNF-α therapy decreases airway hyperresponsiveness and reduces exacerbation frequency (Howarth *et al.*, 2005; Berry *et al.*, 2006). However, there is little or no effect of etanercept in mild asthma (Rouhani *et al.*, 2005; Erin *et al.*, 2006). Thus, TNF-α may be important in some but not all types of asthma and indications for anti-TNF-α therapy need to be clarified.

In order to better target anti-TNF-α therapy, the mechanisms by which TNF-α contribute to hyperreactivity need to be determined. We used our well-characterized model of allergic hyperreactivity to test whether TNF-α mediates hyperreactivity through inflammation and M_2 muscarinic receptor function.

Methods

Preparation of animals

All animal procedures and experimental protocols complied with the NIH Guidelines and those of the animal care and use committees of Oregon Health and Science University. Specific pathogen-free Dunkin-Hartley guinea pigs (100–150 g; Elm Hill, Chelmsford MA, USA) were kept in high-efficiency particulate-filtered air. Some guinea pigs were sensitized to ovalbumin (10 mg kg^{-1} i.p. on days 1, 3 and 5). Three weeks after the last ovalbumin injection, they were challenged with 2.5% aerosolized ovalbumin for 5 min or until signs of respiratory distress. Twenty-four hours after antigen challenge guinea pigs were anesthetized with urethane (1.9 g kg^{-1}, i.p.), chemically sympathectomized with guanethedine (5 mg kg^{-1}, i.v.), paralysed with succinylcholine (10 μg kg^{-1} min^{-1}, i.v.) and ventilated via a tracheal cannula (tidal volume 2.5 mL, 100 breaths min^{-1}) as previously described (Evans *et al.*, 1997; Fryer *et al.*, 2006). Pulmonary inflation pressure (Ppi in mmH_2O) was measured and heart rate and blood pressure, from a carotid cannula, were monitored to ensure adequate levels of anesthesia.

Some ovalbumin sensitized guinea pigs were pretreated with etanercept (Immunex) 1.5 h or 3 h before antigen challenge, while some controls (neither sensitized nor challenged) were treated with etanercept 24 h before physiological measurements. Etanercept was chosen over other TNF-α blocking drugs like infliximab, because it has relatively lower cost-effectiveness ratio (Bravo Vergel *et al.*, 2007; Wailoo *et al.*, 2008). The dose of 3 mg kg^{-1} i.p. is similar to doses already used in animal (Kivilcim *et al.*, 2007) and clinical studies (Takei *et al.*, 2001). Pyrilamine (0.5 mg kg^{-1}, Sigma-Aldrich) was injected i.p. 1 h before antigen challenge. Controls treated with IgG were not included in these studies as we have previously shown *in vivo* that IgG does not inhibit airway hyperreactivity in antigen-challenged guinea pigs (Fryer *et al.*, 1997).

Assessment of neuronal M_2 receptor function in guinea pig lungs in vivo

Both vagi were cut. Electrical stimulation of the distal ends (1–15 Hz, 10 V, 0.2 ms pulse duration, for 5 s at 40 s intervals) produced frequency-dependent bronchoconstriction measured as an increase in Ppi that were abolished by atropine, confirming they were mediated by release of ACh onto muscarinic receptors.

Neuronal M_2 receptor function was measured as the ability of gallamine (0.01–10.0 mg kg^{-1} i.v.) to block endogenous activation of M_2 receptors and potentiate vagally induced bronchoconstriction. Both vagus nerves were stimulated (15 Hz, 0.2 ms, for 3 s; voltage was adjusted in order to produce reproducible bronchoconstrictions in the range of 10–20 mmH_2O; mean 14.2 ± 0.7 mmH_2O). The voltages used were not significantly different among groups (control, 4.4 ± 1.69 V; control-etanercept, 3.3 ± 0.6 V; antigen-challenged, 3.0 ± 0.7 V; antigen-challenged-etanercept, 4.6 ± 1.0 V). ACh (1–10 μg kg^{-1} i.v.) was administered to vagotomized animals to test sensitivity of airway smooth muscle to ACh. Lungs were lavaged via the tracheal cannula.

Histochemical analyses of guinea pig lung

Nerves were detected immunohistochemically in paraffin embedded lung by using a mouse monoclonal antibody to

protein gene product 9.5 (PGP 9.5) and visualized with biotinylated goat anti-mouse IgG with streptavidin-linked horseradish peroxide and DAB-Ni (all from Vector), as previously described (Fryer and Jacoby, 1992). Eosinophils were labelled with a 1% solution of chromotrope 2R as previously described (Fryer and Jacoby, 1992). Airway area measured with Metamorph® and eosinophils within airway walls and under the epithelial basement membrane, as well as nerve associated eosinophils (within 10 μm of an airway nerve) were counted in three cartilaginous airways per animal, five animals per group, by investigators unaware of the treatments.

Preparation of human and guinea pig parasympathetic neurons and human neuroblastoma cells

Human tissue was obtained from anonymous organ donors via a tissue bank (Pacific Northwest Transplant Bank) and the age or sex of donors is not known. All families provided informed consent for the use of these tissues. Airway parasympathetic neurons were isolated from human and guinea pig tracheas as previously described (Fryer *et al*., 2006; Nie *et al*., 2007) and grown on Matrigel in serum-free medium for 1 week. SK-N-SH, human neuroblastoma cells, were obtained from ATCC and grown in medium containing 10% FCS. Guinea pig neurons were incubated with 2 ng mL^{-1} mouse TNF-α (315-01A Peprotech) and 1000 U mL^{-1} mouse IFN-γ (315-05 Peprotech) for 4 h. Human neurons were incubated in 2 ng mL^{-1} human TNF-α (T0157 Sigma) for 4 h; while SK-N-SH cells were incubated with 1 ng mL^{-1} human TNF-α for 1–24 h or with 0.03–30 ng mL^{-1} human TNF-α for 4 h. For experiments on mRNA stability, 2 ug mL^{-1} of actinomycin D (A1419 Sigma) was added to neuroblastoma cells in the presence or absence of 2 ng mL^{-1} human TNF-α for 1–24 h.

RT-PCR for M_2 receptors

RNA was isolated using RNeasy Mini Kit (74106; QIAGEN) and reverse transcribed using SuperScript III (18080-051; Invitrogen Corp) with random hexamer primers. Quantitative PCR was carried out in triplicate at 60°C over 45 cycles using Quantitect SYBR Green PCR kit (204143; QIAGEN). PCR products were quantified using the Mx3000P real-time PCR system (Stratagene). Oligonucleotide PCR primer pairs were designed from published sequences as follows: human M_2 muscarinic receptor: sense, TTA AAG TCA ACC GCC ACC TC, antisense, CAA AGG TCA CAC ACC ACA GG; guinea pig M_2 muscarinic receptor: sense, TTT TCC AAT GCT GCT GTC AC, antisense, GCC ATG TTG TTG TTG TTT GG. 18S rRNA: sense, GTAA CCCGTTGAACCCCATT, antisense, CCATCCAATCGGTAG TAGCG. Threshold cycle number was measured, and relative expression of M_2 muscarinic receptor mRNA was normalized to 18S rRNA.

Neuronal TNF-α and M_2 receptors

Guinea pig parasympathetic neurons and human neuroblastoma cells were fixed with 4% paraformaldehyde, blocked with 10% normal goat serum and incubated with 1:1000 dilution of rabbit anti-TNFRSF1A (TNFRSF1A from Abcam) or 1:200 dilution of anti-TNF receptor II (TNFRSF1B from Abcam) or 1:500 dilution of rabbit anti-human M_2 muscarinic receptor (R&D Bio) antibody at 4°C for 24 h. Antibodies were visualized with Alexa Fluor 555 or Alexa Fluor 488 labelled goat anti-rabbit IgG (Molecular Probes).

Data analysis

Responses *in vivo* to nerve stimulation, ACh and gallamine were analysed using repeated-measures analysis of variance. Physiological baselines, lavage cell counts and histological analyses were analysed by two-way ANOVA with Fisher and Bonferroni-Dunn correction using Statview 4.5 (Abacus Concepts); P values < 0.05 were considered significant.

Results

Baseline responses

There was no statistically significant difference in any baseline parameter for Ppi, heart rate and blood pressure among groups (Table 1).

Effects of etanercept on airway hyperreactivity

Electrical stimulation of both vagus nerves produced frequency-dependent bronchoconstriction (measured as a rapid and reversible increase in Ppi) (Fig. 1). Vagally induced bronchoconstriction was significantly increased in antigen-challenged animals, compared with control, which indicated airway hyperreactivity. This potentiation was prevented by treatment with etanercept 3 h before challenge but not with the treatment 1.5 h before challenge (data not shown). Etanercept did not affect airway reactivity to vagal stimulation in control guinea pigs.

Effects of etanercept on responsiveness of airway smooth muscle to ACh

ACh, given i.v., caused dose-dependent bronchoconstriction in vagotomized animals by stimulating M_3 muscarinic recep-

Table 1 Baseline parameters of the experimental groups of guinea pigs

Treatment group	*Ppi mmH_2O*	*Heart rate beats min^{-1}*	*Systolic BP mmHg*	*Diastolic BP mmHg*
Control	115.0 ± 7.4	283.0 ± 17.9	46.5 ± 1.8	26.3 ± 1.6
Control + etanercept	123.3 ± 9.4	271.0 ± 14.2	46.4 ± 2.5	26.4 ± 3.0
Challenge	131.0 ± 5.9	272.8 ± 11.5	45.4 ± 1.7	24.0 ± 1.8
Challenge + etanercept	143.3 ± 15.7	272.1 ± 13.2	41.6 ± 2.1	23.6 ± 1.7

There were no statistically significant differences in any baseline parameter among groups. Etanercept also did not change any baseline measurements in control or in challenged animals. Values shown are means ± SEM from eight to nine animals per group.
BP, blood pressure; Ppi, pulmonary inflation pressure.

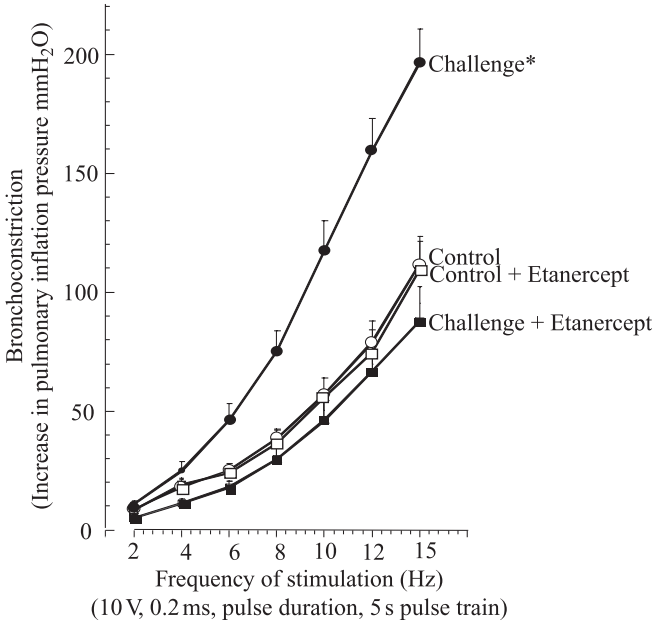

Figure 1 Etanercept prevented airway hyperreactivity in antigen-challenged guinea pigs. Electrical stimulation of both vagus nerves produced frequency-dependent bronchoconstriction measured as an increase in pulmonary inflation pressure. Vagally induced bronchoconstriction was potentiated in antigen-challenged guinea pigs ($n = 8$) compared with controls ($n = 9$). Etanercept (3 mg kg^{-1} i.p.; prior to antigen challenge) prevented potentiation of vagally induced bronchoconstriction in challenged animals ($n = 8$) but did not alter vagally induced bronchoconstriction in control animals ($n = 9$). Data shown are mean ± standard error of the mean. *The entire frequency response is significantly different from controls, using ANOVA.

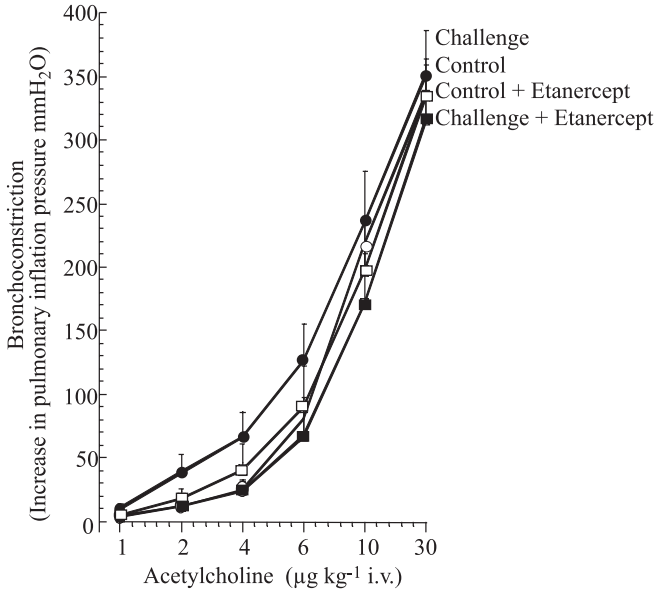

Figure 2 Etanercept (3 mg kg^{-1} i.p.; prior to antigen challenge) did not change M_3 muscarinic receptor function on airway smooth muscle in antigen-challenged guinea pigs. Intravenous ACh induced bronchoconstriction, measured as an increase in pulmonary inflation pressure, was not changed by antigen challenge ($n = 8$) or by etanercept ($n = 8$) as compared with control ($n = 8$). ACh, acetylcholine.

tors on airway smooth muscle (Fig. 2). Neither antigen challenge nor etanercept changed M_3 muscarinic receptor function because there were no significant differences in the ACh dose response curves among control, antigen-challenged and etanercept-treated control or etanercept-treated-challenged animals.

Effects of etanercept on neuronal M_2 muscarinic receptor function

Gallamine, a M_2 muscarinic receptor antagonist, potentiated vagally induced bronchoconstriction in a dose-dependent manner in control guinea pigs (Fig. 3) demonstrating normal M_2 muscarinic receptor function. In antigen-challenged guinea pigs, the ability of gallamine to potentiate vagally induced bronchoconstriction was significantly reduced compared with controls. This indicates that, in antigen-challenged animals in the absence of gallamine, neuronal M_2 muscarinic receptors were less able to inhibit ACh release. Etanercept partially protected M_2 receptor function in antigen-challenged guinea pigs. Etanercept treatment of control animals did not affect M_2 muscarinic receptor function.

Effect of etanercept on M_2 muscarinic receptors in heart

Bradycardia was induced by either electrical stimulation of both vagus nerves or by intravenous injection of ACh. Both procedures decreased heart rate via stimulation of cardiac M_2

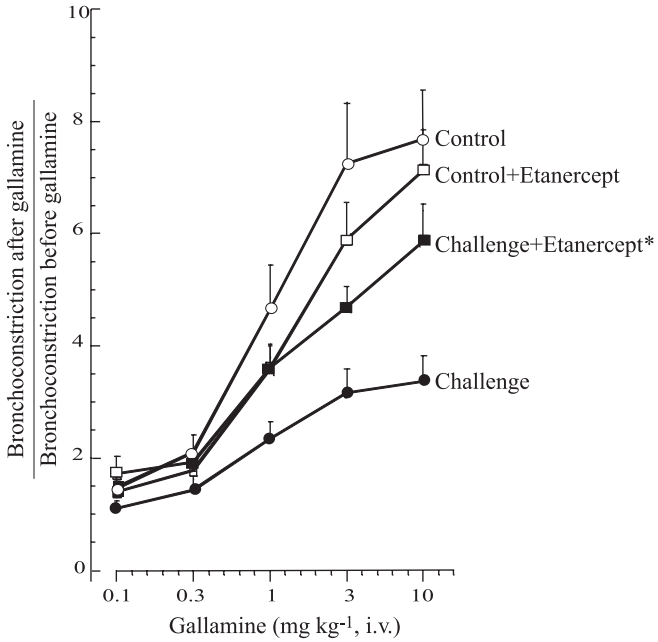

Figure 3 Etanercept (3 mg kg^{-1} i.p.; prior to antigen challenge) partially protected neuronal M_2 muscarinic receptor function in airways of antigen-challenged guinea pigs. In control animals ($n = 6$), gallamine potentiated vagally induced bronchoconstriction by inhibiting M_2 muscarinic receptor function. The ability of gallamine to potentiate vagally induced bronchoconstriction was significantly reduced in antigen-challenged guinea pigs ($n = 8$) indicating that M_2 muscarinic receptors were dysfunctional. Etanercept pretreatment partially restored the ability of gallamine to potentiate vagally induced bronchoconstriction in antigen-challenged animals ($n = 8$) but had no effect in controls ($n = 5$). Data shown are means ± standard error of the mean. *The dose response curve is significantly different from antigen-challenged animals, using ANOVA.

muscarinic receptors (Fig. 4). Bradycardia was not changed by antigen challenge or by etanercept treatment.

Effects of etanercept on inflammatory cells in BAL fluid and peripheral blood

In the BAL, antigen challenge significantly increased the total number of cells, compared with those in BAL from lungs of control guinea pigs (Fig. 5A). This increase was made up predominantly of macrophages and eosinophils. In blood, antigen challenge significantly increased eosinophils and monocytes but not lymphocytes and neutrophils (Fig. 5B). Etanercept also significantly reduced the number of eosinophils in BAL and cause a sharp trend towards decrease in number of macrophages ($P = 0.052$). Etanercept reduced the number of circulating eosinophils in the blood of antigen-challenged animals; it had no effect on the number of eosinophils in control animals.

Effects of etanercept on eosinophil recruitment to airways and airway nerves

In lungs from control guinea pigs, there was a small resident population of eosinophils within the airway smooth muscle (Fig. 6A,D) but not associated with nerves (Fig. 6E,H). After antigen challenge, eosinophils were significantly increased in the airway smooth muscle (Fig. 6B,D) and were associated with airway nerves (Fig. 6F,H). Pretreatment with etanercept, prior to antigen challenge, significantly decreased the number of eosinophils in airway smooth muscle (Fig. 6C,D) and the number of eosinophils associated with airway nerves (Fig. 6G,H).

Expression of TNF-α receptors in primary cultures of airway parasympathetic nerves and human neuroblastoma cells

An antibody for the TNF-α receptor I (TNFRSF1A) labelled SK-N-SH human neuroblastoma cells (Fig. 7A) and guinea pig parasympathetic neurons in primary culture (Fig. 7B), indicating that these neurons expressed TNFRSF1A. In contrast, there was no detectable expression of the TNF-α receptor II (TNFRSF1B) on either parasympathetic neurons (Fig. 7C) or neuroblastoma cells (Fig. 7D).

TNF-α decreases M_2 muscarinic receptor expression in primary cultures of airway parasympathetic neurons from human and guinea pig lungs and human neuroblastoma cells

Both SK-N-SH cells and parasympathetic neurons isolated from human and guinea pig tracheas expressed M_2 muscarinic

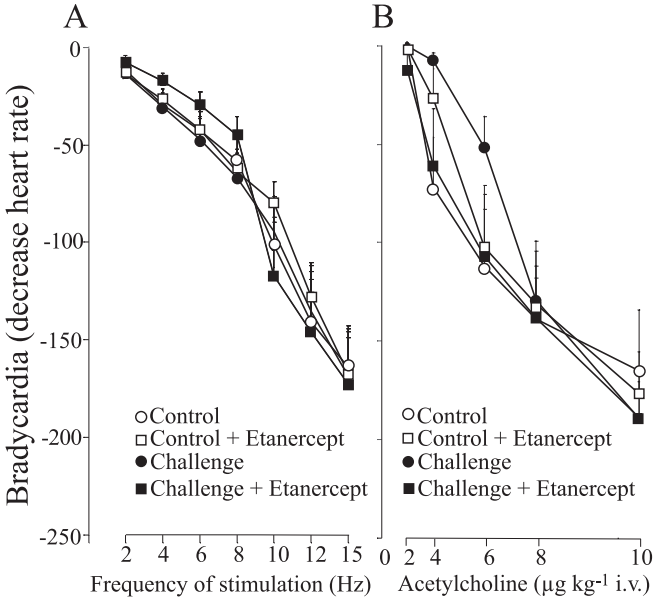

Figure 4 Etanercept (3 mg kg^{-1} i.p.; prior to antigen challenge) did not change M_2 muscarinic receptor function in the heart. Bradycardia induced either by electrical stimulation of both vagus nerves (A) or intravenous ACh (B) was not changed by antigen challenge ($n = 8$), or by etanercept in controls ($n = 9$) or in antigen-challenged animals ($n = 8$). ACh, acetylcholine.

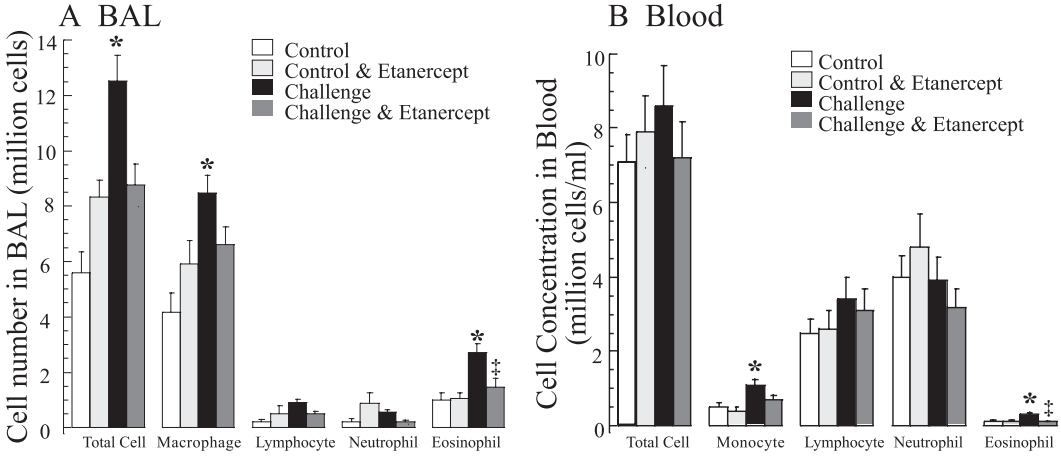

Figure 5 Etanercept (3 mg kg^{-1} i.p.; prior to antigen challenge) decreased eosinophils in bronchoalveolar lavage (BAL; A) and peripheral blood (B) of antigen-challenged guinea pigs. Antigen challenge significantly increased macrophages and eosinophils in BAL, and increased monocytes and eosinophils in peripheral blood. In antigen-challenged animals, etanercept not only prevented the increase in eosinophils in both BAL and blood but also attenuated the increase in macrophages in the BAL and monocytes in the blood. Data shown are means ± standard error of the mean. *Significantly different from controls. ‡Significantly different from challenged. $n = 8$.

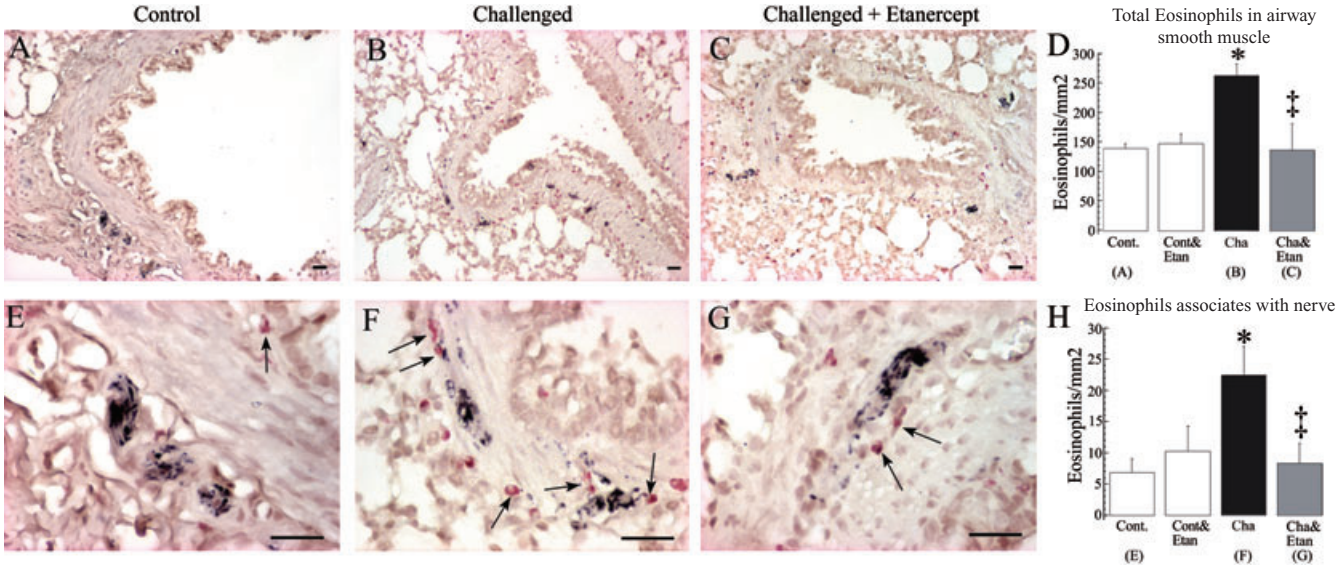

Figure 6 Etanercept decreased eosinophil recruitment to airway smooth muscle and airway nerves in antigen-challenged guinea pigs. Representative cross sections are shown of guinea pig bronchi from control (A and E), antigen-challenged (B and F), and etanercept (3 mg kg^{-1} i.p.) pretreated antigen-challenged guinea pigs (C and G). Nerves were stained blue-gray with monoclonal antibody to PGP 9.5. Eosinophils were stained red with chromotrope 2R. There were few eosinophils in lungs (D) or around nerves (H) in control guinea pig lungs. Following antigen challenge, the number of eosinophils increased within smooth muscle (D) and in close proximity to nerves (H). In antigen-challenged animals, etanercept not only inhibited eosinophil influx into the airway walls (D), but also attenuated recruitment of eosinophils to the airway nerves (H). Data are expressed as the mean number of eosinophils per mm^2 ± standard error of the mean; $n = 5$. *Significantly different from controls. ‡Significantly different from antigen challenged. Arrows point to eosinophils in high powered image. Magnification bars: 50 μm. PGP, protein gene product.

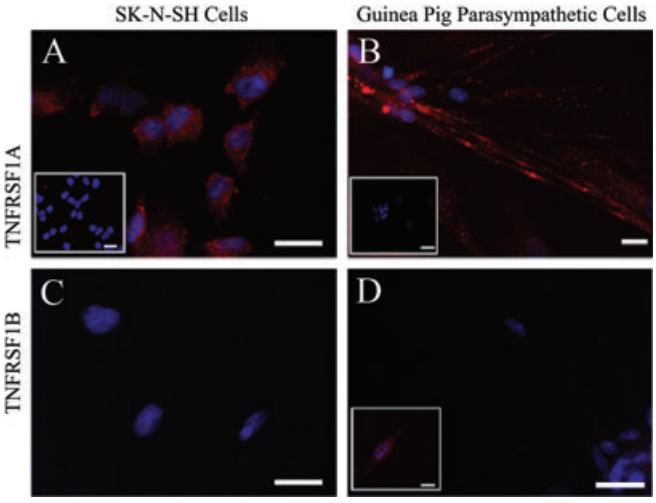

Figure 7 Tumour necrosis factor-α (TNF-α) receptors were present on human neuroblastoma cells (A) and guinea pig airway parasympathetic neurons (B). Neurons were stained with Alexa Fluor 555 labelled antibody against TNFRSF1A (TNF-α receptor I, red). There was no detectable TNFRSF1B (TNF-α receptor II) expression on human neuroblastoma cells (C) or primary cultures of guinea pig airway parasympathetic neurons (D); although this antibody could detect TNFRSF1B (TNF-α receptor II) in non-neuronal cells (insert D). Nuclei of all cells were stained blue with DAPI (4,6-diamidino-2 phenylindole). Only DAPI-stained nuclei were visible in the absence of primary antibody (insert A and B). Magnification bars: 50 μm.

receptors as shown by real time RT-PCR (Fig. 8A). Exposure to a combination of mouse tumour necrosis factor-α (mTNF-α) and mIFN-γ markedly downregulated mRNA for M_2 muscarinic receptors in cultures of guinea pig parasympathetic neurons (Fig. 8A). A similar down-regulation of M_2 muscarinic receptor mRNA was seen in human parasympathetic neurons exposed to human tumour necrosis factor-α (hTNF-α) (Fig. 8A). Decreased M_2 receptor expression in the presence of hTNF-α (2 ng mL^{-1}) was confirmed by immunocytochemistry in SK-N-SH cells (Fig. 8B). The ability of hTNF-α to inhibit M_2 muscarinic receptor expression in SK-N-SH cells was time-dependent (Fig. 8C). hTNF-α started to decrease M_2 muscarinic receptor mRNA 2 h after treatment and the effect plateaued after 16 h. The ability of TNF-α to decrease M_2 muscarinic receptor mRNA was also concentration-dependent (Fig. 8D), over a range of 0.03–1 ng mL^{-1}, with an IC_{50} value of 0.1 ng mL^{-1}.

TNF-α destabilizes M_2 muscarinic receptor mRNA

Incubation of SK-N-SH cells with actinomycin D, which blocks transcription, had no effect on the levels of mRNA for M_2 receptors over 8 h (Fig. 8E). However, in the presence of hTNF-α combined with actinomycin D, M_2 muscarinic receptor mRNA rapidly decayed over the same time period. These data strongly suggest that TNF-α decreases M_2 muscarinic receptor mRNA stability.

Discussion and conclusions

In these studies, we show that etanercept inhibits airway hyperreactivity by decreasing eosinophil recruitment to the airways. Our previous studies showed that airway eosinophils induce M_2 muscarinic receptor dysfunction by releasing MBP,

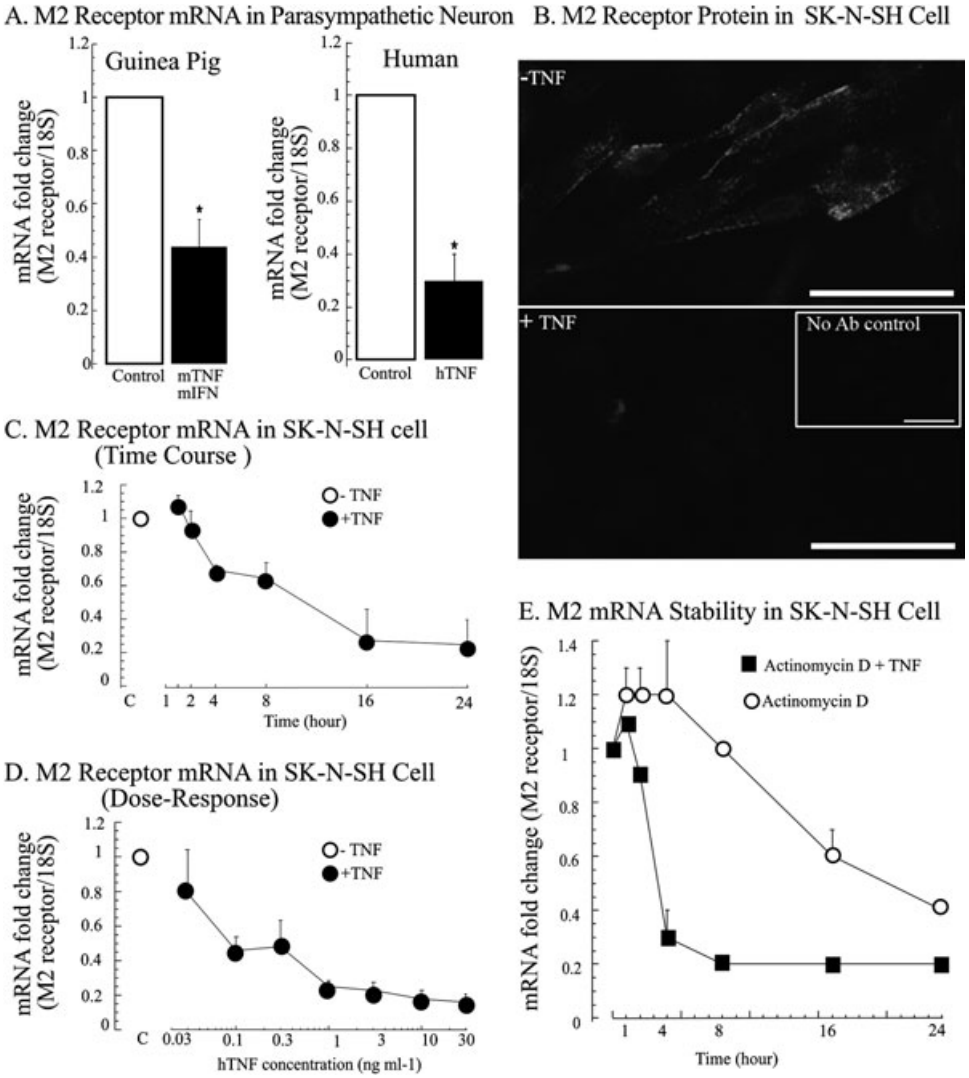

Figure 8 Tumour necrosis factor-α (TNF-α) reduced M_2 muscarinic receptor expression in human and guinea pig parasympathetic neurons. In (A), mTNF-α (2 ng mL^{-1}) combined with mIFN-γ (1000 U mL^{-1}) reduced M_2 muscarinic receptor mRNA in guinea pig parasympathetic nerves and hTNF-α (2 ng mL^{-1}) reduced M_2 muscarinic receptor mRNA in human parasympathetic neurons. In (B), M_2 muscarinic receptor expression in SK-N-SH cells (detected with fluorescence and anti-human M_2 muscarinic receptor antibody) was reduced after 24 h incubation with hTNF-α (2 ng mL^{-1}). Cells receiving no primary antibody are shown in the inset. Magnification bars: 50 μm. In (C), hTNF-α induced inhibition of M_2 muscarinic receptor expression was time and concentration (in D) dependent in SK-N-SH cells. In (E), destabilization of M_2 muscarinic receptor mRNA by TNF-α is shown. In the absence of TNF-α, actinomycin D caused a gradual decrease in M_2 muscarinic receptor mRNA over 24 h that was markedly accelerated in the presence of TNF-α. Data shown are the means ± standard error of the mean; $n = 3$. *Significantly different from control by using paired two-tailed Student *t*-tests $P < 0.01$.

which binds to M_2 muscarinic receptors, blocking their function (Fryer and Jacoby, 1992; Jacoby *et al.*, 1993; Elbon *et al.*, 1995; Costello *et al.*, 1997; Evans *et al.*, 1997; Verbout *et al.*, 2007). Intercellular adhesion molecule 1 (ICAM-1) (Sawatzky *et al.*, 2002) and eotaxin (Fryer *et al.*, 2006) are key factors in eosinophil chemoattraction to the nerves. Blockade of either ICAM or eotaxin receptors specifically inhibits recruitment of eosinophils to airway nerves. TNF-α not only upregulates eotaxin (Ghaffar *et al.*, 1999) and ICAM-1 (Nie *et al.*, 2007), but also increases IL-5 (Zhang *et al.*, 1997). Thus, TNF-α may indirectly increase eosinophil proliferation (IL-5), migration (eotaxin) and adhesion (ICAM) to parasympathetic nerves. Treatment with etanercept, prior to challenge, significantly decreased eosinophils in blood (Fig. 5) and in airway smooth muscle (Fig. 6), as well as decreasing the number of eosinophils associated with airway nerves (Fig. 6). Thus, preventing eosinophil migration to the lung or adhesion to parasympathetic nerves may be a mechanism by which etanercept protects M_2 muscarinic receptor function (Fig. 3) and prevents airway hyperreactivity (Fig. 1).

In addition, both airway parasympathetic neurons and human neuroblastoma cells express TNF-α receptor I (Fig. 7). We showed that TNF-α reduced M_2 muscarinic receptor mRNA in primary cultures of human and guinea pig parasympathetic neurons and in human neuroblastoma cells. In human airway parasympathetic neurons, this was due to

destabilization of M_2 muscarinic receptor mRNA (Fig. 8). This may be another mechanism by which TNF-α induces airway hyperactivity.

The effects of TNF-α are cell-specific. In a fibroblast cell line, TNF-α did not decrease M_2 muscarinic receptor mRNA unless it was combined with IL-1β (Haddad *et al.*, 1996). Furthermore, in these fibroblasts cells, TNF-α had no effect on M_2 muscarinic receptor mRNA stability, probably reducing expression at the transcription level (Haddad *et al.*, 1996). In contrast, we found that in parasympathetic nerves from lung, TNF-α alone decreased expression of the mRNA for M_2 receptors, probably by decreasing message stability (Fig. 8). Furthermore, M_2 muscarinic receptors also undergo tissue-specific regulation. M_2 receptors on cardiac muscle, which mediate bradycardia, were not affected by antigen challenge (Fig. 4A) while the prejunctional M_2 muscarinic receptors on nerves in the lung were dysfunctional after challenge (Fig. 3). If the effect of TNF-α is specific to neurons, then it strengthens the rationale for using TNF-α antagonists in conditions with neurogenic inflammation.

M_2 muscarinic receptor dysfunction plays a key role in allergic asthma. Dysfunctional M_2 muscarinic receptors have been reported in asthmatic patients (Ayala and Ahmed, 1989; Minette *et al.*, 1989; Chanez *et al.*, 2007) and in antigen-challenged guinea pigs (Fryer and Maclagan, 1984; Fryer and Wills-Karp, 1991; Elbon *et al.*, 1995; Evans *et al.*, 1997). In addition, treatments that protect M_2 muscarinic receptor function inhibit airway hyperreactivity (Elbon *et al.*, 1995; Evans *et al.*, 1997; Fryer *et al.*, 1997). Hyperreactivity in challenged animals is not due to changed M_3 muscarinic receptor function on airway smooth muscle because the sensitivity of airway smooth muscle to exogenous ACh is not changed (Fryer and Maclagan, 1984; Fryer and Wills-Karp, 1991; Elbon *et al.*, 1995; Evans *et al.*, 1997).

In some (Howarth *et al.*, 2005; Berry *et al.*, 2006), but not all (Rouhani *et al.*, 2005; Erin *et al.*, 2006) clinical trials of etanercept treatment in asthma, lung function improved. In the studies showing no effect, etanercept was given to patients with mild to moderate asthma (Rouhani *et al.*, 2005; Erin *et al.*, 2006), while studies showing a beneficial effect of etanercept were in patients with more severe corticosteroid-dependent asthma (Howarth *et al.*, 2005; Berry *et al.*, 2006; Cazzola *et al.*, 2006). Patients with severe asthma have higher levels of TNF-α, compared with those in patients with mild asthma (Howarth *et al.*, 2005; Cazzola *et al.*, 2006). This may explain why anti-TNF-α therapy successfully reduced asthma attacks in severely asthmatic patients (Howarth *et al.*, 2005; Berry *et al.*, 2006; Cazzola *et al.*, 2006) but not in studies of those with mild asthma (Rouhani *et al.*, 2005; Erin *et al.*, 2006). As severe asthma is often poorly controlled by inhaled corticosteroids (Chanez *et al.*, 2007), there is an important clinical need for additional treatments for these severe asthma patients. It is severe asthma patients who have the highest morbidity and mortality (Chanez *et al.*, 2007), and it is in the severe, steroid-resistant patients that etanercept was most effective (Howarth *et al.*, 2005; Berry *et al.*, 2006; Cazzola *et al.*, 2006).

A better understanding of the mechanisms of TNF-α induced airway hyperreactivity are crucial in order to evaluate anti TNF-α therapy and develop new strategies for prevention and treatment of asthma. Additionally, inflammation of nerves is also characteristic of other chronic inflammatory diseases, including atopic dermatitis (Kakurai *et al.*, 2006), pancreatitis (Keith *et al.*, 1985) and inflammatory bowel disease (Hogan *et al.*, 2001). ACh release from nerves in these other organs is also under the control of M_2 receptors (Love, 2000; Bernardini *et al.*, 2002; Coulson *et al.*, 2004). Therefore, agents that target TNF-α or its receptors may be beneficial in restoring normal neural function in these other chronic inflammatory diseases. The present findings may have important implications regarding the role of TNF-α in severe steroid-resistant asthma and may be applicable to other diseases with neurogenic inflammation.

Acknowledgements

We thank the Pacific Northwest Transplant Bank, Portland, OR, for human organ donor tracheas. We thank Jesse Lorton for technical assistance.

Conflict of interest

None.

References

Adamko DJ, Yost BL, Gleich GJ, Fryer AD, Jacoby DB (1999). Ovalbumin sensitization changes the inflammatory response to subsequent parainfluenza infection. Eosinophils mediate airway hyperresponsiveness, m(2) muscarinic receptor dysfunction, and antiviral effects. *J Exp Med* **190**: 1465–1478.

Alexander SPH, Mathie A, Peters JA (2008). Guide to Receptors and Channels (GRAC), 3rd edn. *Br J Pharmacol* **153** (Suppl. 2): S1–S209.

Arbach O, Gross WL, Gause A (2002). Treatment of refractory Churg-Strauss-Syndrome (CSS) by TNF-alpha blockade. *Immunobiology* **206**: 496–501.

Ayala LE, Ahmed T (1989). Is there loss of protective muscarinic receptor mechanism in asthma? *Chest* **96**: 1285–1291.

Azzawi M, Johnston PW, Majumdar S, Kay AB, Jeffery PK (1992). T lymphocytes and activated eosinophils in airway mucosa in fatal asthma and cystic fibrosis. *Am Rev Respir Dis* **145**: 1477–1482.

Baker DG, Don HF, Brown JK (1992). Direct measurement of acetylcholine release in guinea pig trachea. *Am J Physiol* **263**: L142–L147.

Bernardini N, Roza C, Sauer SK, Gomeza J, Wess J, Reeh PW (2002). Muscarinic M2 receptors on peripheral nerve endings: a molecular target of antinociception. *J Neurosci* **22**: RC229.

Berry MA, Hargadon B, Shelley M, Parker D, Shaw DE, Green RH *et al.* (2006). Evidence of a role of tumor necrosis factor alpha in refractory asthma. *N Engl J Med* **354**: 697–708.

Bradding P, Roberts JA, Britten KM, Montefort S, Djukanovic R, Mueller R *et al.* (1994). Interleukin-4, -5, and -6 and tumor necrosis factor-alpha in normal and asthmatic airways: evidence for the human mast cell as a source of these cytokines. *Am J Respir Cell Mol Biol* **10**: 471–480.

Bravo Vergel Y, Hawkins NS, Claxton K, Asseburg C, Palmer S, Woolacott N *et al.* (2007). The cost-effectiveness of etanercept and infliximab for the treatment of patients with psoriatic arthritis. *Rheumatology (Oxford)* **46**: 1729–1735.

Broide DH, Lotz M, Cuomo AJ, Coburn DA, Federman EC, Wasserman SI (1992). Cytokines in symptomatic asthma airways. *J Allergy Clin Immunol* **89**: 958–967.

Careau E, Proulx LI, Pouliot P, Spahr A, Turmel V, Bissonnette EY (2006). Antigen sensitization modulates alveolar macrophage functions in an asthma model. *Am J Physiol Lung Cell Mol Physiol* **290**: L871–L879.

Cazzola M, Polosa R, Erzurum SC, Berry MA, Hargadon B, Shelley M *et al.* (2006). Anti-TNF-alpha and Th2 cytokine directed therapies for the treatment of asthma. *Curr Opin Allergy Clin Immunol* **6**: 43–50.

Chanez P, Wenzel SE, Anderson GP, Anto JM, Bel EH, Boulet LP *et al.* (2007). Severe asthma in adults: what are the important questions? *J Allergy Clin Immunol* **119**: 1337–1348.

Choi IW, Sun K, Kim YS, Ko HM, Im SY, Kim JH *et al.* (2005). TNF-alpha induces the late-phase airway hyperresponsiveness and airway inflammation through cytosolic phospholipase A(2) activation. *J Allergy Clin Immunol* **116**: 537–543.

Costello RW, Schofield BH, Kephart GM, Gleich GJ, Jacoby DB, Fryer AD (1997). Localization of eosinophils to airway nerves and effect on neuronal M2 muscarinic receptor function. *Am J Physiol* **273**: L93–L103.

Coulson FR, Jacoby DB, Fryer AD (2004). Insulin regulates neuronal M2 muscarinic receptor function in the ileum of diabetic rats. *J Pharmacol Exp Ther* **308**: 760–766.

Elbon CL, Jacoby DB, Fryer AD (1995). Pretreatment with an antibody to interleukin-5 prevents loss of pulmonary M2 muscarinic receptor function in antigen-challenged guinea pigs. *Am J Respir Cell Mol Biol* **12**: 320–328.

Erin EM, Leaker BR, Nicholson GC, Tan AJ, Green LM, Neighbour H *et al.* (2006). The effects of a monoclonal antibody directed against tumor necrosis factor-alpha in asthma. *Am J Respir Crit Care Med* **174**: 753–762.

Evans CM, Fryer AD, Jacoby DB, Gleich GJ, Costello RW (1997). Pretreatment with antibody to eosinophil major basic protein prevents hyperresponsiveness by protecting neuronal M2 muscarinic receptors in antigen-challenged guinea pigs. *J Clin Invest* **100**: 2254–2262.

Fryer AD, Jacoby DB (1992). Function of pulmonary M2 muscarinic receptors in antigen-challenged guinea pigs is restored by heparin and poly-L-glutamate. *J Clin Invest* **90**: 2292–2298.

Fryer AD, Maclagan J (1984). Muscarinic inhibitory receptors in pulmonary parasympathetic nerves in the guinea-pig. *Br J Pharmacol* **83**: 973–978.

Fryer AD, Wills-Karp M (1991). Dysfunction of M2-muscarinic receptors in pulmonary parasympathetic nerves after antigen challenge. *J Appl Physiol* **71**: 2255–2261.

Fryer AD, Costello RW, Yost BL, Lobb RR, Tedder TF, Steeber DA *et al.* (1997). Antibody to VLA-4, but not to L-selectin, protects neuronal M2 muscarinic receptors in antigen-challenged guinea pig airways. *J Clin Invest* **99**: 2036–2044.

Fryer AD, Stein LH, Nie Z, Curtis DE, Evans CM, Hodgson ST *et al.* (2006). Neuronal eotaxin and the effects of CCR3 antagonist on airway hyperreactivity and M2 receptor dysfunction. *J Clin Invest* **116**: 228–236.

Ghaffar O, Hamid Q, Renzi PM, Allakhverdi Z, Molet S, Hogg JC *et al.* (1999). Constitutive and cytokine-stimulated expression of eotaxin by human airway smooth muscle cells. *Am J Respir Crit Care Med* **159**: 1933–1942.

Gosset P, Tsicopoulos A, Wallaert B, Vannimenus C, Joseph M, Tonnel AB *et al.* (1991). Increased secretion of tumor necrosis factor alpha and interleukin-6 by alveolar macrophages consecutive to the development of the late asthmatic reaction. *J Allergy Clin Immunol* **88**: 561–571.

Grau GE, Roux-Lombard P, Gysler C, Lambert C, Lambert PH, Dayer JM *et al.* (1989). Serum cytokine changes in systemic vasculitis. *Immunology* **68**: 196–198.

Haddad EB, Rousell J, Lindsay MA, Barnes PJ (1996). Synergy between tumor necrosis factor alpha and interleukin 1beta in inducing transcriptional down-regulation of muscarinic M2 receptor gene expression. Involvement of protein kinase A and ceramide pathways. *J Biol Chem* **271**: 32586–32592.

Hogan SP, Mishra A, Brandt EB, Royalty MP, Pope SM, Zimmermann N *et al.* (2001). A pathological function for eotaxin and eosinophils in eosinophilic gastrointestinal inflammation. *Nat Immunol* **2**: 353–360.

Howarth PH, Babu KS, Arshad HS, Lau L, Buckley M, McConnell W *et al.* (2005). Tumour necrosis factor (TNFalpha) as a novel therapeutic target in symptomatic corticosteroid dependent asthma. *Thorax* **60**: 1012–1018.

Hutchison S, Choo-Kang BS, Bundick RV, Leishman AJ, Brewer JM, McInnes IB *et al.* (2008). Tumour necrosis factor-alpha blockade suppresses murine allergic airways inflammation. *Clin Exp Immunol* **151**: 114–122.

Jacoby DB, Gleich GJ, Fryer AD (1993). Human eosinophil major basic protein is an endogenous allosteric antagonist at the inhibitory muscarinic M2 receptor. *J Clin Invest* **91**: 1314–1318.

Kakurai M, Monteforte R, Suto H, Tsai M, Nakae S, Galli SJ (2006). Mast cell-derived tumor necrosis factor can promote nerve fiber elongation in the skin during contact hypersensitivity in mice. *Am J Pathol* **169**: 1713–1721.

Keith RG, Keshavjee SH, Kerenyi NR (1985). Neuropathology of chronic pancreatitis in humans. *Can J Surg* **28**: 207–211.

Kim J, McKinley L, Natarajan S, Bolgos GL, Siddiqui J, Copeland S *et al.* (2006). Anti-tumor necrosis factor-alpha antibody treatment reduces pulmonary inflammation and methacholine hyperresponsiveness in a murine asthma model induced by house dust. *Clin Exp Allergy* **36**: 122–132.

Kim YS, Ko HM, Kang NI, Song CH, Zhang X, Chung WC *et al.* (2007). Mast cells play a key role in the development of late airway hyperresponsiveness through TNF-alpha in a murine model of asthma. *Eur J Immunol* **37**: 1107–1115.

Kivilcim M, Peyman GA, Kazi AA, Dellacroce J, Ghobrial RN, Monzano R (2007). Intravitreal toxicity of high-dose etanercept. *J Ocul Pharmacol Ther* **23**: 57–62.

Lesslauer W, Tabuchi H, Gentz R, Brockhaus M, Schlaeger EJ, Grau G *et al.* (1991). Recombinant soluble tumor necrosis factor receptor proteins protect mice from lipopolysaccharide-induced lethality. *Eur J Immunol* **21**: 2883–2886.

Love JA (2000). Electrical properties and synaptic potentials of rabbit pancreatic neurons. *Auton Neurosci* **84**: 68–77.

Minette PA, Lammers JW, Dixon CM, McCusker MT, Barnes PJ (1989). A muscarinic agonist inhibits reflex bronchoconstriction in normal but not in asthmatic subjects. *J Appl Physiol* **67**: 2461–2465.

Nakae S, Ho LH, Yu M, Monteforte R, Iikura M, Suto H *et al.* (2007). Mast cell-derived TNF contributes to airway hyperreactivity, inflammation, and TH2 cytokine production in an asthma model in mice. *J Allergy Clin Immunol* **120**: 48–55.

Nie Z, Nelson CS, Jacoby DB, Fryer AD (2007). Expression and regulation of intercellular adhesion molecule-1 on airway parasympathetic nerves. *J Allergy Clin Immunol* **119** (6): 1415–1422.

Ohashi Y, Motojima S, Fukuda T, Makino S (1992). Airway hyperresponsiveness, increased intracellular spaces of bronchial epithelium, and increased infiltration of eosinophils and lymphocytes in bronchial mucosa in asthma. *Am Rev Respir Dis* **145**: 1469–1476.

Peppel K, Crawford D, Beutler B (1991). A tumor necrosis factor (TNF) receptor-IgG heavy chain chimeric protein as a bivalent antagonist of TNF activity. *J Exp Med* **174**: 1483–1489.

Proskocil BJ, Bruun DA, Lorton JK, Blensly KC, Jacoby DB, Lein PJ *et al.* (2008). Antigen sensitization influences organophosphorus pesticide-induced airway hyperreactivity. *Environ Health Perspect* **116**: 381–388.

Ritchlin CT, Haas-Smith SA, Li P, Hicks DG, Schwarz EM (2003). Mechanisms of TNF-alpha- and RANKL-mediated osteoclastogen-

esis and bone resorption in psoriatic arthritis. *J Clin Invest* **111**: 821–831.

Rouhani FN, Meitin CA, Kaler M, Miskinis-Hilligoss D, Stylianou M, Levine SJ (2005). Effect of tumor necrosis factor antagonism on allergen-mediated asthmatic airway inflammation. *Respir Med* **99**: 1175–1182.

Sawatzky DA, Kingham PJ, Court E, Kumaravel B, Fryer AD, Jacoby DB *et al*. (2002). Eosinophil adhesion to cholinergic nerves via ICAM-1 and VCAM-1 and associated eosinophil degranulation. *Am J Physiol Lung Cell Mol Physiol* **282**: L1279–L1288.

Suffredini AF, Reda D, Banks SM, Tropea M, Agosti JM, Miller R (1995). Effects of recombinant dimeric TNF receptor on human inflammatory responses following intravenous endotoxin administration. *J Immunol* **155**: 5038–5045.

Takei S, Groh D, Bernstein B, Shaham B, Gallagher K, Reiff A (2001). Safety and efficacy of high dose etanercept in treatment of juvenile rheumatoid arthritis. *J Rheumatol* **28**: 1677–1680.

Thomas PS (2001). Tumour necrosis factor-alpha: the role of this multifunctional cytokine in asthma. *Immunol Cell Biol* **79**: 132–140.

Tsukadaira A, Okubo Y, Kitano K, Horie S, Momose T, Takashi S *et al*. (1999). Eosinophil active cytokines and surface analysis of eosinophils in Churg-Strauss syndrome. *Allergy Asthma Proc* **20**: 39–44.

Verbout NG, Lorton JK, Jacoby DB, Fryer AD (2007). Atropine pretreatment enhances airway hyperreactivity in antigen-challenged guinea pigs through an eosinophil-dependent mechanism. *Am J Physiol Lung Cell Mol Physiol* **292**: L1126–L1135.

Wailoo AJ, Bansback N, Brennan A, Michaud K, Nixon RM, Wolfe F (2008). Biologic drugs for rheumatoid arthritis in the Medicare program: a cost-effectiveness analysis. *Arthritis Rheum* **58**: 939–946.

Zhang K, Gharaee-Kermani M, McGarry B, Remick D, Phan SH (1997). TNF-alpha-mediated lung cytokine networking and eosinophil recruitment in pulmonary fibrosis. *J Immunol* **158**: 954–959.