Effects of TNFα on the human nasal mucosa *in vivo*

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Running head: Effects of TNFα on the nasal mucosa

Abstract

Background: TNFα is a cytokine that may contribute to the pathophysiology of airway inflammation. Inhalation of TNFα produces granulocyte recruitment and airway hyper-responsiveness in man. Anti-TNFα treatment may inhibit allergen-induced plasma exudation in guinea-pig airways. Increased nasal mucosal output of TNFα has been demonstrated in allergic rhinitis, but the effect of TNFα on the human nasal mucosa has not been examined *in vivo*.

Objective: To examine effects of topical TNFα on the human nasal mucosa *in vivo*.

Methods: In a dose-finding study, healthy subjects received intranasal TNFα (0-7.5 µg). Nasal lavages were carried out before as well as 10 min and 24 hrs post challenge and α_2-macroglobulin was measured as an index of plasma exudation. In a second study, involving patients with allergic rhinitis examined out of season, a sham-controlled nasal challenge with TNFα (10 µg) was performed and followed 24 hrs later by an allergen challenge. Lavages were performed before the TNFα challenge, 24 hrs thereafter, and 10 min post allergen challenge. α_2-Macroglobulin, eosinophil cationic protein (ECP), myeloperoxidase (MPO), and IL-8 were analyzed as indices of plasma exudation, eosinophil activity, neutrophil activity, and pro-inflammatory cytokine production, respectively.

Results: In the dose-finding study, TNFα produced significant increases in α_2-macro-globulin 24 hrs post challenge ($p<0.01$). In allergic rhinitis, 10 µg of TNFα also produced this effect ($p<0.01$) as well as increases in ECP and IL-8 ($p<0.01$). MPO was increased 24 hrs post challenge, but this change did not reach statistical significance. TNFα did not produce any acute effects and did not affect the responsiveness to allergen.

Conclusion: The present study demonstrates that topical TNFα produces a human nasal inflammatory response. These data suggest a role of TNFα in nasal conditions characterised by mucosal inflammation.

Key words: Eosinophils, exudation, inflammation, IL-8, neutrophils.

Introduction

TNFα is a pro-inflammatory mediator involved in the pathophysiology of a range of inflammatory conditions. Frequently explored areas are autoimmune conditions such as rheumatoid arthritis and Crohn's disease, which today are successfully treated with TNFα-inhibitors [1].

The role of TNFα in conditions characterized by airway inflammation is currently under investigation focusing on the bronchial airways. Yu *et al*. have shown increased bronchoalveolar lavage (BAL) cell expression of TNFα mRNA following airway allergen challenge in allergic mice [2]. Inhalation of TNFα in rats has been demonstrated to produce BAL neutrophilia and airway hyperresponsiveness [3]. In experimental models of asthma, blockade of TNFα activity has been shown to reduce allergen-induced airway extravasation of plasma and granulocyte recruitment [2, 4-6]. In human bronchial airways, TNFα is produced by a number of cell types, including macrophages [7], eosinophils [8, 9], epithelial cells [10, 11], and mast cells [12, 13]. Inhalation of TNFα produces an inflammatory response characterized by increased sputum numbers of neutrophils and eosinophils as well as by development of airway hyperresponsiveness [14, 15]. Furthermore, recent studies have shown that treatment with etanercept, a soluble TNFα receptor-IgG1 Fc fusion protein, is associated with improvement in asthma symptoms, lung function, and airway hyperresponsiveness in refractory asthma [16, 17].

Potentially reflecting the pathophysiological similarities between airway inflammation in allergic rhinitis and asthma, patients suffering from allergic rhinitis have been shown to feature increased tissue expression of TNFα mRNA as well as increased nasal mucosal output of the TNFα protein [18-20]. However, information on the role of TNFα in human nasal airways is limited. Notably, to the best of our knowledge, there is no information on the consequences to the nasal airway and to allergic rhinitis of increased TNFα activity. One way of exploring this, again in analogy with the bronchial airways [14, 15], would be to examine effects of TNFα topically applied on the nasal mucosa.

In the present study, we have thus examined effects of topical TNFα on the human nasal mucosa. First, increasing doses were given to healthy subjects, and nasal symptoms and PIF (peak inspiratory flow) were monitored. Also, nasal lavages were carried out and levels of α_2-macroglobulin were monitored as an index of plasma exudation. Secondly, an exudation-producing dose of TNFα was given to patients with seasonal allergic rhinitis (out of season). Nasal lavages were carried out 24 hrs post challenge and lavage fluid levels of α_2-macroglobulin, eosinophil cationic protein (ECP), myeloperoxidase (MPO), and interleukin-8 (IL-8) were measured as indices of inflammation. In addition, a nasal allergen challenge was carried out immediately after the 24 hrs lavage, and this measure was also followed by a lavage and again by analysis of α_2-macroglobulin, ECP, MPO, and IL-8. The rationale for employing an allergen challenge was that patients with allergic rhinitis often feature a variable responsiveness to allergen depending of the underlying inflammatory activity. Accordingly, we hypothesized that an inflammatory effect of TNFα could change the responsiveness to allergen.

Material and methods

Study design

Subjects were challenged intranasally with TNFα. Healthy individuals were recruited to a dose-finding study and, subsequently, patients with allergic rhinitis were recruited to a principal study. Written informed consent was obtained and the study was approved by the regional ethics committee.

Dose-finding study

Ten healthy volunteers (9 males, 1 female) aged between 18 and 27 participated in the study. Prior to the first challenge all subjects were subjected to an interview, a general health examination, a nasal examination, and a skin prick test. Inclusion criteria were: A negative skin prick test, a normal nasal examination, and use of contraceptives (for females). Exclusion criteria were: A history of upper respiratory tract infection within a period of seven days before the start of the study, allergic and non-allergic rhinitis, other nasal disease (structural abnormalities, rhinosinusitis, nasal polyposis), use of nasal decongestants within a period of seven days before the start of the study, other pharmacological treatments (except occasional analgesics) within a period of one month prior to the study.

Intranasal challenges with isotonic saline and escalating doses of TNFα were carried out. The doses of TNFα were 0.3, 1.5, and 7.5 µg, and between the challenges washout periods of seven days were instituted. Nasal lavages were carried out 5 min before, 10 min after, and 24 hrs after every challenge. Also, two 30 sec lavages were carried out immediately prior to the TNFα challenge (these lavages were employed to produce a low baseline and were not collected). Nasal lavage levels of α_2-macroglobulin were measured as an index of plasma exudation. Prior to each nasal lavage the subjects scored nasal symptoms and monitored nasal PIF (peak inspiratory flow) using a specific flow meter (Clement-Clarke, Harlow, UK). Sneezes, secretion, and blockage were each scored on a scale from 0-3. The scores were added to a total nasal symptom score (range 0-9).

Principal study

Sixteen patients with allergic rhinitis (11 males, 5 females) aged between 24 and 35 participated in the study. All subjects were subjected to an interview, a general health examination, a nasal examination, and a skin prick test. Inclusion and exclusion criteria were the same as described above except that these subjects presented a history of at least two years of seasonal allergic rhinitis and a positive skin prick test to relevant aeroallergens.

Nasal spray-challenges with 10 µg of TNFα and isotonic saline were carried out in a crossover, randomized, and sham-controlled design. A washout period of two weeks was instituted between the challenges. Twenty-four hours after each challenge, the patients were subjected to a nasal allergen challenge (1,000 SQ-U of either birch or grass pollen allergen). Nasal saline lavages were carried out 5 min before each TNFα challenge, 24 hrs thereafter (just before the allergen challenge), and 10 min after each allergen challenge. Also, two 30 sec lavages were carried out immediately prior to the TNFα challenge (these lavages were not collected). Nasal lavage levels of α_2-macroglobulin, IL-8, ECP, and MPO, and were measured as indices of plasma exudation, pro-inflammatory cytokine production, eosinophil activity, and neutrophil activity, respectively. Prior to the challenge, 24 hrs after sham/TNFα challenge, and 10 min following the allergen challenge sneezes, secretion, and blockage were scored as described above.

TNFα challenge and nasal lavages

Recombinant human TNFα (210-TA/CF, R&D Systems, Abingdon, UK) was mixed with isotonic saline to concentrations where 100 µl of each solution contained the preferred amounts of TNFα. The TNFα-solution was prepared less then one hour before the challenge and it was administered using a spray-device delivering 100 µl per actuation. At all times the challenges and lavages were administered to the right hand side nasal cavity. The lavages were carried out using a pool-device containing 15 ml of isotonic saline [21]. The lavage fluids were kept in the right nasal cavity for 5 min. The recovered lavage fluid was centrifuged and the supernatant was homogenized, prepared in aliquots, and frozen (-30°C) for later analysis.

Analyses

α_2-Macroglobulin was measured using a radioimmunoassay sensitive to 7.8 ng/ml. Rabbit anti-human α_2-macroglobulin (Dakopatts, Copenhagen, Denmark) was used as anti-serum and human serum (Behringwerke, Marburg, Germany) as standard. Human α_2-macroglobulin (Capell-Organon, Turnhout, Belgium) was iodinated using a lacto-peroxidase method. Tracer and standard/sample was mixed with antiserum before adding goat anti-rabbit anti-serum (AstraZeneca, Lund, Sweden). The bound fraction was measured using a gamma counter. The intra- and inter- assay coefficients of variation were between 3.8-6.0% and 3.1-7.2%, respectively. IL-8 was measured using a commercially available enzyme-linked immunosorbent assay (R&D Systems, Abingdon, UK). ECP and MPO were also measured using commercially available assays (Pharmacia, Uppsala, Sweden). The limits of detection for IL-8, ECP, and MPO were 31.2 pg/ml, 2.0 ng/ml, and 1.6 ng/ml, respectively.

Statistics

In healthy subjects, differences in levels of α_2-macroglobulin between observation at different TNFα doses were analysed by the Friedman test and, if statistical significance was achieved, by the Wilcoxon signed rank test. In patients with allergic rhinitis, differences in symptoms and lavage fluid levels of α_2-macroglobulin, IL-8, ECP, and MPO were analysed using the Wilcoxon signed rank test. A p-value of <0.05 was considered significant. Data are presented as mean±SEM.

Results

In the dose-finding study, intranasal challenge with TNFα produced a significant increase in lavage fluid levels of α_2-macroglobulin as recorded 24 hrs post challenge ($p<0.01$). In contrast, no such effect was observed 10 min post challenge (Fig. 1). No significant changes in nasal symptoms or nasal PIF were observed at any time point or at any challenge dose (data not shown).

In patients with allergic rhinitis, challenge with 10 µg of TNFα produced significant increases in lavage levels of α_2-macroglobulin 24 hrs post challenge (*c.f.* sham challenge) (Fig 2a). In addition, significant increases were observed for IL-8 and ECP at this time point (Figs. 2b and c). Nasal lavage fluid levels of MPO were also increased 24 hrs post challenge, but this change failed to reach statistical significance ($p=0.09$) (Fig. 2d). TNFα produced low-grade nasal symptoms 24 hrs post challenge (*c.f.* sham challenge) (Table I).

In sham challenged subjects, levels of α_2-macroglobulin were significantly increased 10 min post allergen challenge, compared with pre allergen challenge (24 hrs) levels ($p<0.01$). Also, the allergen challenge produced significant nasal symptoms (Table I): score 5 out of 9 ($p<0.001$).

The symptom score recorded post allergen challenge was not affected by TNFα administration 24 hrs earlier (Table I). Similarly, with the exception of MPO, the lavage fluid indices recorded post allergen challenge were unaffected by prior TNFα administration (*c.f.* sham challenge) (Fig 2a-d). Post allergen challenge MPO levels increased significantly following TNFα administration ($p<0.05$) (*c.f.* sham challenge) (Fig 2d).

In the principal study, at the 24 hour observation (i.e., the point in time when the effect of TNFα was compared to the sham challenge) the overall percentage of nasal lavages with levels of IL-8, ECP, and MPO below the limit of detection was 0, 19, and 0%, respectively.

Discussion

The present study, involving healthy subjects as well as patients with allergic rhinitis, has shown that TNFα produces plasma exudation when applied topically onto the nasal mucosa. This response is associated with pro-inflammatory cytokine production (IL-8) and increased granulocyte activity. The observations are of interest in the context of viewing TNFα as a mediator of potential importance to nasal airway defence as well as to the pathophysiology of nasal conditions characterised by inflammation.

The selection of the first dose of the present challenge-series was based on a study by Thomas *et al*. in which humans inhaled 60 ng of TNFα to the lower airways [14]. At this dose-level, none of eight subjects were reported to experience symptoms suggestive of systemic effects, whereas the authors observed a leftward shift in the dose-response curve to methacholine and a significant rise in the levels of polymorphonuclear cells in sputum. For safety reasons we examined healthy subjects first: As we increased the dose of TNFα from 0.3 to 7.5 μg all subjects remained symptomless and no adverse events occurred. For our principal study, involving patients with allergic rhinitis, 10 μg of TNFα was chosen as challenge dose to assure a positive response. This dose produced only low-grade nasal symptoms (score about 1 on a scale from 0 to 9) and no adverse effect. Accordingly, we conclude that TNFα in the present dose-range is safe as an experimental, nasal challenge agent.

Based on preliminary observations on acute plasma exudation producing effects of TNFα in guinea-pig airways (Greiff *et al*., unpubl.), suggesting that TNFα may act as a direct microvascular permeability increasing mediator, we selected nasal lavage fluid levels of the plasma protein α_2-macroglobulin as index in our dose-finding study [22]. We could demonstrate that TNFα induced plasma exudation in the human nasal airway. However, whereas TNFα produced this effect 24 hrs post challenge, no plasma exudation was observed acutely (10 min post challenge). Our observation suggests species differences between humans and rodents. Such differences have previously been shown and may be attributable to a differential ability of sensory nerves to evoke and mediate exudative, neurogenic inflammation [22]. We can not exclude that even higher

doses of TNFα would have produced acute plasma exudation in our challenge model. However, the present observation suggests that TNFα-induced plasma exudation 24 hrs post challenge does not reflect a direct microvascular action of the mediator, but rather a secondary response to increased cellular inflammation (below). Our finding suggests the possibility that TNFα can be a pro-inflammatory mediator in the human nasal airway.

In the present study, the nasal output of ECP and MPO was increased 24 hrs post TNFα challenge. Whereas this effects was statistically significant for ECP, it reached borderline significance for MPO (p=0.09). In addition to ECP and MPO, the output of the pro-inflammatory chemokine IL-8 was also increased. These findings are in keeping with observations in man on increased numbers of granulocytes in sputum following TNFα inhalation [14]. It is also in agreement with reports showing that treatment with a soluble TNFα receptor, reducing the levels of free TNFα, attenuates peribronchial eosinophil and neutrophil infiltration in allergic mice [4]. Furthermore, our data support *in vitro* findings by Yamagishi *et al*. on reduced epithelial cell production of IL-8 following TNFα-antibody treatment [23]. Taken together, available data suggest that TNFα is involved in mounting airway granulocyte responses, in part through increased IL-8 activity [24], and that this mechanism is valid also for the human nasal airway. Additional studies are warranted to further explore this effect of TNFα and tentatively such approaches should comprise lavage as well as tissue observations.

The present allergen challenge performed 24 hrs post sham challenge produced symptoms of allergic rhinitis as well as significant plasma exudation. These responses, and the employed lavage fluid indices with the exception of MPO, were not significantly affected by TNFα administration (*c.f.* sham challenge). Our observations suggest the possibility that TNFα may not be critically involved in the pathophysiology of allergen responsiveness in allergic rhinitis. Somewhat in contrast, Thomas *et al*. [14, 15] demonstrated increased airway responsiveness to methacholine 24 hours after inhalation of TNFα in normal subjects as well as in mild asthmatics. Furthermore, Renzetti *et al*. reported that pre-treatment of allergic guinea-pigs with an anti-TNFα-agent reduced the airway responsiveness to substance P [6]. Differences between

challenge agents (allergen vs. methacholine/substance P), between nasal and bronchial airways, and between species may explain the above discrepancies. Also, we can not exclude that a greater dose of TNFα would have produced a different result in the present study. The strength of our observation lies in the fact that it involves humans and a clean, acute type-1 allergic reaction, as opposed to a more complex inflammatory condition that characterises asthma. Further studies, tentatively comprising anti-TNFα measures, are warranted to explore whether or not TNFα has a role in allergic rhinitis.

Our observation that levels of MPO recorded post allergen challenge was significantly increased by TNFα may suggest a development of "hyperresponsiveness", in this case a situation where the nasal mucosa is prone to mount a neutrophil response. However, the allergen challenge may not *per se* have produced the increase in neutrophil activity. Alternatively, it may be speculated that TNFα *per se* produced a limited neutrophil degranulation, as suggested by the present study, and that the luminal entry of MPO was facilitated by the allergen-induced plasma exudation response. Such a mechanism has previously been demonstrated for other inflammatory mediators [22]. Further studies involving additional neutrophil markers and detailed analyses of nasal biopsies are needed to clarify this issue.

A study involving patients with allergic rhinitis examined out of season has shown that treatment with a clinical dose of an antihistamine (fexofenadine) may not affect elevated levels of TNFα in nasal secretions observed following nasal allergen-challenge [20]. Similarly, Benson *et al*. have demonstrated that a topical corticosteroid (budesonide) does not reduce the levels of TNFα in nasal fluids obtained from children with seasonal allergic rhinitis [25]. In the context of viewing anti-TNFα drugs as treatment candidates for airway conditions characterised by allergic airway inflammation, it is of interest to note that TNFα production may be little affected by such key anti-allergy pharmaceuticals. Accordingly, drugs that reduce TNFα activities may add to current airway therapeutic approaches. A role for anti-TNFα in the treatment of inflammatory airway conditions is suggested by the present observations and by other airway observations on pro-inflammatory effects of TNFα as well as by recent reports of

beneficial effects of anti-TNFα treatment in asthma [14-17]. A role in allergic inflammation may not be excluded by the present observation that TNFα (*c.f.* sham challenge) did not affect the response to an allergen challenge since the dose-response relationship, as well as the time-response relationship, has not been fully explored. Whether or not TNFα inhibiting drugs have a role in the treatment of nasal conditions characterised by inflammation, including allergic rhinitis, remains to be clarified.

We conclude that TNFα produces pro-inflammatory effects in human nasal airways, but that TNFα may not affect the responsiveness to allergen.

Acknowledgement

We thank Mrs. L. Glantz-Larsson and Mrs. C. Cervin-Hoberg for technical assistance. The study was supported in parts by grants from the Swedish Research Council, Skåne County Council, and Lund University.

Tables

Table I. Nasal symptom scores (mean±SEM, range 0-9) recorded prior to any challenge, 24 hrs after sham/TNFα challenge, and following allergen challenge (performed 24 hrs after the sham/TNFα challenge). TNFα produced low-grade but significant symptoms (*c.f.* sham challenge). Allergen produced marked symptoms, which were unaffected by prior TNFα administration. (** denotes $p<0.01$).

	Sham	**TNFα**
Pre challenge	0.4±0.2	0.6±0.2
24 hrs post challenge	0.4±0.2	1.1±0.3**
Post allergen challenge	5.0±0.6	5.9±0.3

Legends

Fig. 1. Effects of intranasal TNFα and sham challenge on nasal lavage fluid levels of α_2-macroglobulin in healthy subjects. The challenges were separated by at least seven days. Nasal lavages were carried out before each challenge (pre-challenge) as well as 10 min and 24 hrs thereafter. TNFα produced significant increases in lavage fluid levels of α_2-macroglobulin 24 hrs post challenge, whereas no such effect was observed at 10 min post challenge. (** denotes $p<0.01$)

Fig. 2. Effects of nasal TNFα and sham challenge on lavage fluid levels of α_2-macroglobulin (a), IL-8 (b), ECP (c), and MPO (d) in patients with allergic rhinitis examined outside the pollen season. The lavages were carried out before each challenge (pre-challenge) and 24 hrs thereafter (24 hrs). In addition, lavages were carried out 10 min after an allergen challenge that in turn was carried out 24 hrs after the sham/TNFα challenge (24 hrs + allergen). TNFα significantly increased the lavage fluid levels of IL-8, ECP, and α_2-macroglobulin. An increase in MPO was also observed following TNFα, but this change only reached borderline significance ($p=0.09$). The responsiveness to allergen was significantly increased following TNFα challenge for MPO (d), but not for the other employed lavage fluid indices. (* denotes $p<0.05$, ** denotes $p<0.01$).

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Fig. 1

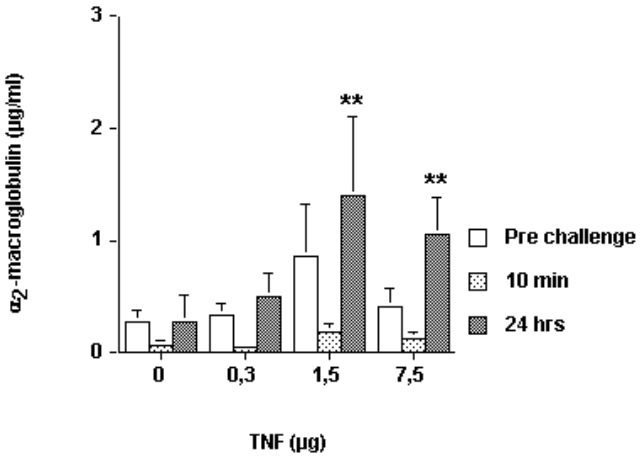

3
2
1
0
**
**
α2-macroglobulin (µg/ml)
Pre challenge
10 min
24 hrs
0
0,3
1,5
7,5
TNF (µg)

Fig. 2a

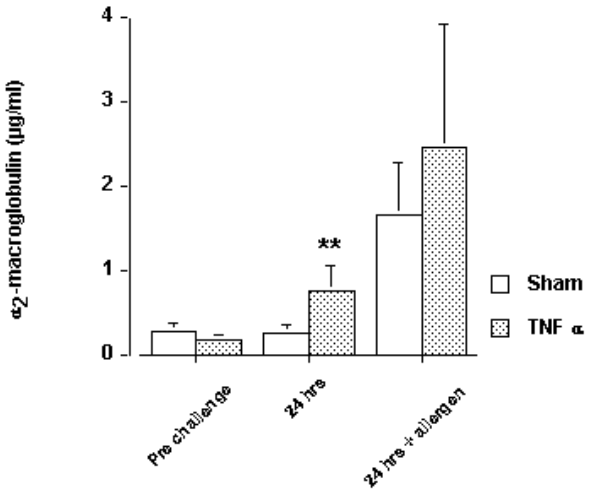

α2-macroglobulin (μg/ml)
4
3
2
1
0
**
Sham
TNF α
Pre challenge
24 hrs
24 hrs + allergen

Fig. 2b

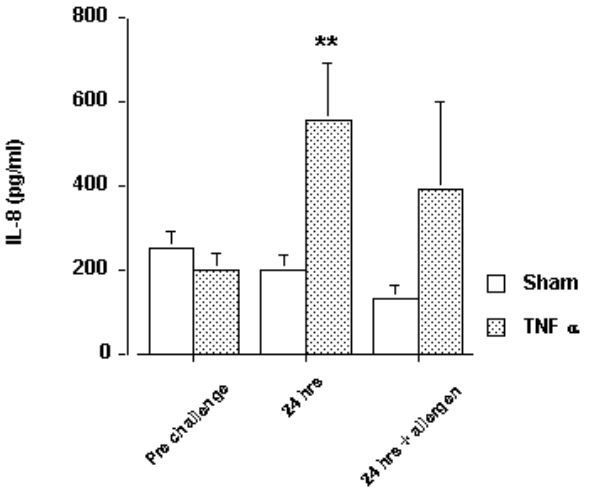

IL-8 (pg/ml)
800
600
400
200
0
**
Sham
TNF α
Pre challenge
24 hrs
24 hrs + allergen

Fig. 2c

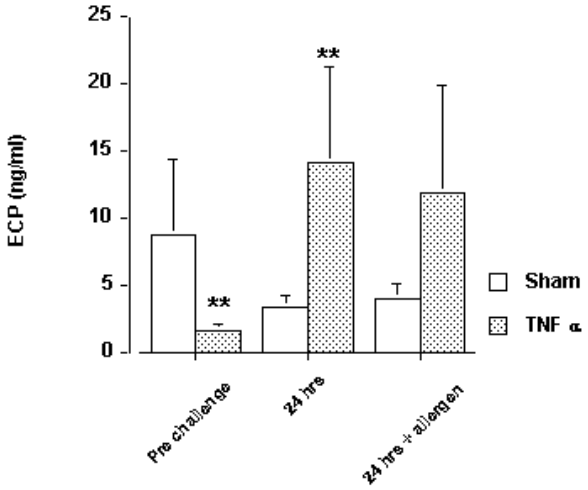
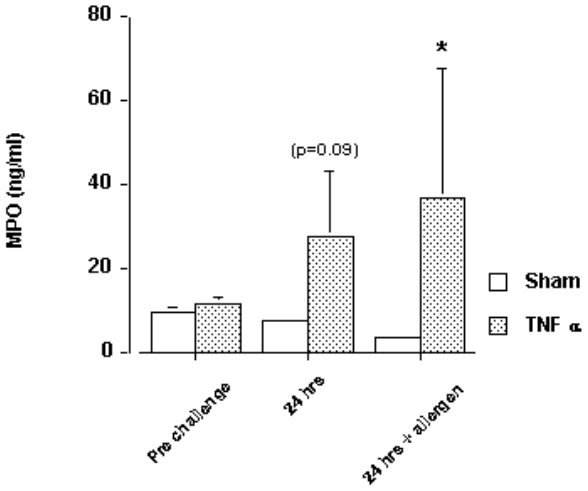

ECP (ng/ml)
25
20
15
10
5
0
**
**
Sham
TNF α
Pre challenge
24 hrs
24 hrs + allergen

Fig. 2d


MPO (ng/ml)
80
60
40
20
0
(p=0.09)
*
Sham
TNF α
Pre challenge
24 hrs
24 hrs + allergen