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Effect of endogenous nitric oxide on tumour necrosis factor-α-induced leukosequestration and IL-8 release in guinea-pigs airways *in vivo*

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1 Tumour necrosis factor-α (TNF-α) is implicated in the pathogenesis of many pulmonary and airway diseases. TNF-α stimulation may release interleukin-8 (IL-8) in airways mediated via an increase in intracellular oxidant stress. In the present study, we have assessed leukosequestration and IL-8 release in the airways in response to intratracheal administration of human recombinant TNF-α, and examined the modulatory role of endogenous NO by pretreatment with a NO synthase inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME).

2 TNF-α (10^2-10^4 u) was administered intratracheally in male guinea-pigs which were anaesthetized with urethane and were ventilated artificially. TNF-α induced a time- and dose-related increase in neutrophil numbers and a concomitant increase in human IL-8 equivalent level retrieved from bronchoalveolar lavage (BAL) with the peak effect at 10^3 u at 6 h of TNF-α injection (late phase). Intratracheal administration of recombinant human (rh)IL-8 (0.025, 0.25, 2.5 ng) producing a similar range of human IL-8 equivalent levels in BAL as measured in our results induced neutrophil recovery in BAL fluid to a similar extent. Administration of anti-IL-8 antibody prevented the late phase of neutrophil recruitment induced by TNF-α or rhIL-8.

3 Pretreatment with L-NAME significantly enhanced the TNF-α (10^3 u)-induced neutrophil recruitment and human IL-8 equivalents production at 6 h, but not at 1 h of TNF-α administration (early phase). L-Arginine reversed the responses to L-NAME. Pretreatment with 0.2% DMSO (i.v.) significantly inhibited TNF-α-induced neutrophil recruitment and human IL-8 equivalents release both in the early and late phase of the responses. Pretreatment with DMSO also inhibited the enhancement effect of L-NAME on the late phase of TNF-α-induced responses. DMSO failed to modify exogenous rhIL-8-induced neutrophil recruitment. Neither L-NAME nor DMSO alone induced any significant change in neutrophil numbers or human IL-8 equivalent level in BAL fluid.

4 Neutrophil depletion by cyclophosphamide pretreatment failed to modify TNF-α-induced human IL-8 equivalent release.

5 The expression of β2-integrin, CD11b/CD18 on neutrophils was increased only in the late but not early phase of TNF-α stimulation. L-NAME failed to modify these responses.

6 In conclusion, we demonstrated that NO may be an important endogenous inhibitor of TNF-α-induced leukocyte chemotaxis via inhibition of IL-8 production. Thus, the production of NO in airway inflammatory diseases may play a negative feedback role in self-limiting the magnitude of inflammatory responses.

Keywords: Nitric oxide; interleukin-8; neutrophil; adhesion molecule; respiratory system; tumour necrosis factor-α

Introduction

Neutrophil accumulation in the airways is incriminated in tissue damage, airway hypersecretion and airway obstruction associated with several inflammatory airway diseases such as idiopathic pulmonary fibrosis, emphysema, bronchiectasis and cystic fibrosis (Sibille & Reynolds, 1990). To reach lung interstitium, neutrophils within the pulmonary vasculature must adhere to and subsequently migrate through endothelium. Similarly, to reach the alveolar space, neutrophils must migrate through pulmonary epithelium.

Tumour necrosis factor-α (TNF-α) is a 17.5 kDa cytokine that is implicated in the pathogenesis of many pulmonary and airway diseases. TNF-α has been shown to be chemotactic for neutrophils at nanomolar concentrations (Horgan *et al*., 1993). TNF-α is mainly produced by alveolar macrophages in the lung, and its expression is enhanced by bacterial or viral infection (Nelson *et al*., 1989; Becker *et al*., 1991) or allergen challenge (Donnelly *et al*., 1993). TNF-α generated by alveolar macrophages may stimulate airway epithelial cells in a paracrine fashion, resulting in the production of soluble chemotactic factors, including interleukin-8 (IL-8) (Kwon *et al*., 1994). Exogenous stimuli like endotoxin (Martich *et al*., 1991), IL-1, TNF-α and TNF-β (Larsen *et al*., 1989) induce IL-8 production in a wide variety of cells including monocytes, T-lymphocytes, neutrophils, vascular endothelial cells (Strieter *et al*., 1989), dermal fibroblasts, keratinocytes, and hepatocytes (Thornton *et al*., 1990). IL-8, a member of the chemokine polypeptides, exhibits chemotactic activities against neutrophils and lymphocytes (Yoshimura *et al*., 1987; Larsen *et al*., 1989). *In vivo*, IL-8 may be a particularly important chemotactic cytokine by virtue of its resistance to protease and consequently long duration of action (Baggiolini *et al*., 1989). Thus, the production of IL-8 may amplify airway inflammation in response to TNF-α stimulation in bacterial or viral infection or asthma.

Endogenous nitric oxide (NO) is synthesized by vascular endothelial cells, epithelial cells and pro-inflammatory cells including neutrophils and monocytes/macrophages from the terminal guanidino nitrogen atom of the amino acid L-arginine (Moncada & Higgs, 1991). This biosynthetic process is inhibited by analogues of L-arginine such as N^G-nitro-L-arginine

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methyl ester (L-NAME). Alteration of endothelial NO production is a cause of subsequent leukocyte adhesion. Inhibition of NO synthesis results in increased leukocyte adherence in postcapillary venules (Kubes *et al*., 1991). Thus, NO is an important endogenous modulator of leukocyte recruitment, although the precise molecular mechanism(s) is not elucidated. One hypothesis is that NO is capable of avidly inactivating superoxide (Del Maestro *et al*., 1982; Rubanyi & Vanhoutte, 1986), a proadhesive molecule that is ambiently produced within all cells (Cross, 1987). Previous work has clearly demonstrated that oxidant stress stimulated IL-8 production in a variety of cell lines, including A549 epithelial cells (DeForge *et al*., 1993). Oxidant tone was also demonstrated to stimulate IL-8 production in epithelial cells infected with respiratory syncytial virus (Mastronarde *et al*., 1995). Thus it is also possible that endogenous NO may interfere with the activity of oxidant to modulate the release of IL-8 and thereby modulate neutrophil recruitment. In the present study, we explored this hypothesis by intratracheal administration of recombinant human TNF-α into guinea-pigs to induce leukosequestration and IL-8 release, and examined the modulatory role of endogenous NO by pretreatment with a NO synthase inhibitor, N^{ω}-nitro-L-arginine methyl ester (L-NAME).

Methods

Animal preparation

Male Dunkin-Hartley guinea-pigs (350–500 g body weight) were obtained in pathogen-free containers and were maintained in closed-circulation cubicles. Intratracheal injections were performed in animals under local anaesthesia with 2% lignocaine. After an incision was made in the soft tissue overlying the trachea, 0.5 ml total volume of TNF-α or sterile 0.9% saline was injected via a 25-gauge needle into the visualized trachea. Thirty minutes or 5 h and 30 min after intratracheal injection, the animals were anaesthetized with urethane (8 mg kg^{-1} body weight) intraperitoneally. The trachea and left external jugular artery were cannulated. The animals were ventilated with a small animal constant volume respiratory pump (Harvard Apparatus, Ltd., Edenbridge, U.K.) operating at 60 strokes min^{-1} of 1 ml of laboratory air per 100 g body weight. The overflow pressure was measured with a differential pressure transducer (Farnell Electronic Components Ltd., Leeds) after a stable state was reached. Drugs were injected via the jugular veins. Blood pressure was monitored continuously via an indwelling cannula filled with heparin-saline (10 u ml^{-1}) in the left carotid artery.

Bronchoalveolar lavage (BAL)

BAL was performed with a total volume of 50 ml of prewarmed (37°C) sterile 0.9% saline via the tracheal cannula. The fluid was recovered manually, through gentle aspiration with a disposable pyrogen-free syringe. The lavage fluid was immediately centrifuged (10 min, 4°C, 2000 r.p.m.). The supernatant of BAL was stored at −80°C until analysis of the IL-8 content. The cell pellet was washed with phosphate buffered saline (PBS) twice and resuspended for flow cytometric analysis. The absolute cell number was enumerated with a haematocytometer counting chamber. Cytocentrifuged preparations (Shandon, Runcorn, UK) were stained with a modified Wright-Giemsa stain and a differential cell count, based on morphologic criteria, was made on 200 cells. The neutrophil numbers recovery in BAL were calculated by multiplying the ratio of neutrophils.

Flow cytometric assay

An indirect immunofluorescent assay was used to examine the expression of CD11b/CD18 on neutrophils retrieved from BAL. The cell pellet was resuspended with PBS and incubated with mouse monoclonal antibodies against murine Mac-1 molecules on neutrophil surfaces at 4°C for 30 min. The cells were washed, fluorescein-conjugated $F(ab)_2$ fragments of rabbit anti-mouse IgG was added, and then the cells were incubated further at 4°C for 30 min. The cells were washed and the cell surface fluorescence was measured with a FACScan. Analysis was performed with a FACScan flow cytometer and LYSYS II software (Becton Dickson, Mountain View, CA, U.S.A.). All fluorescence was measured by logarithm amplification. Ten thousand events were collected for each sample. Cells were gated on the basis of forward scatter and side scatter into lymphocytes, macrophages and granulocytes. The ability of the gates to segregate cells was checked with CD14 and CD3 antibodies to determine the purity of the macrophage and lymphocyte gates, respectively. Less than 5% cells in the monocyte gate expressed CD3, and less than 0.4% cells in the lymphocyte gate expressed CD14. Controls were used to give a measure of non-specific binding with PE-conjugated $F(ab)_2$ fragments of rabbit anti-mouse IgG2 against which results were expressed as a mean fluorescence intensity in arbitrary units transformed to a linear scale from the $\log_{10}$ channel number of mean fluorescence, for a particular cell marker. Unlabelled cells and isotype controls were run in parallel. FACScan cytometer was operated at the same settings. Relative fluorescence intensity of neutrophils is presented as the mean fluorescent channel. Average intensity of fluorescence emitted by at least 5000 cells was measured.

IL-8 assay

Supernatant of BAL was sampled at various time points and freeze-dried at −76°C under −3.0 ATM for 24 h. The samples were then resuspended in one tenth of original volume of PBS and stored at −80°C for later measurement of IL-8 concentration. The concentration of IL-8 was measured by use of a specific ELISA kit (R & D System, Minneapolis, U.S.A.) which employed the quantitative immunometric sandwich enzyme immunoassay technique. A microtitre plate coated with a murine monoclonal antibody to human IL-8 was provided in the kit. Samples containing standard amounts of rhIL-8 as well as study samples were added to individual wells where any IL-8 present would bind to the immobilized antibody. After 4 washes, which removed any unbound protein, a polyclonal antibody specific for IL-8 and conjugated to horseradish peroxidase was added to the wells. After another four washes, a substrate solution containing hydrogen peroxide and tetramethylbenzidine was added and a colour developed in proportion to the amount of IL-8 present. The reaction was stopped by adding 2 N sulphuric acid. The degree of colour generated was determined by measuring the optical density at 450 nm in a spectrophotometric microtitre plate reader. The standard curve was linearized by means of log/log scale and subjected to regression analysis. The IL-8 concentration of unknown samples was determined by use of the regression equation generated by the standard curve run concurrently with the samples. The limit of detection of IL-8 was 3 pg ml^{-1}. Since anti-human monoclonal antibody to IL-8 was employed in this study, the results are presented as pg of human IL-8 equivalent per ml of BAL fluid.

To determine the specificity of the IL-8 ELISA kits, three peptides with high homology to IL-8, rhGRO-α (growth-related gene product-α), human NAP-2 (neutrophil-activating peptide 2) and rhTNF-β were prepared at concentrations of 5 pg, 100 pg, 5 ng, 100 ng and 1 μg ml^{-1} in PBS to test for cross-reactivity. However, there was no detectable cross-reactivity with our IL-8 antibody in the ELISA kits for any concentration of NAP-2 or rhTNF-β. GRO-α was not detectable at concentrations below 100 ng ml^{-1}. However, at 1 μg ml^{-1} GRO-α, 5.2×10^{-4}% cross-reactivity with the IL-8 antibody was observed. GRO-α activity was also determined in all the samples by a specific ELISA kit against human GRO-α. The limit of detection of human equivalent GRO-α was 5 pg ml^{-1}.

Experimental protocol

To investigate the role of endogenous NO in regulation of TNF-α-induced leukosequestration and IL-8 release, animals were pretreated with L-NAME (10 mg kg^{-1}) or saline intravenously 4–6 min before intratracheal injection of TNF-α, when the vasopressor effect of L-NAME had reached a plateau (Kuo *et al*., 1992). In one group of animals, L-arginine (50 mg kg^{-1}) was administered intravenously, to reverse the response to L-NAME, at the peak of the vasopressor response to L-NAME (~2 min after administration) followed by intratracheal injection when the vasopressor response reverted to the plateau level (~2 min) (Kuo *et al*., 1992).

To investigate the role of intracellular oxidant stress in TNF-α induced IL-8 release, the hydroxyl radical scavenger dimethyl sulphoxide (DMSO) 0.2% (v/v) or its vehicle control, 0.9% saline, was administered at 1 ml kg^{-1}, intravenously, 15 min before TNF-α or 0.9% saline intratracheal injection either in the presence or absence of L-NAME pretreatment. DMSO was chosen here because hydroxyl radicals are the most potent oxidant radicals mediating IL-8 production and DMSO has been shown to inhibit LPS or TNF-α induced IL-8 production (DeForge *et al*., 1992; 1993). In some animals, DMSO was given before intratracheal administration of 0.5 ml of exogenous rhIL-8 (0.25 ng) to determine whether DMSO may directly inhibit the action of IL-8.

To determine the causal relationship between IL-8 production and neutrophil recruitment, anti-human IL-8 neutralizing antibody (0.5 mg kg^{-1}) was given intravenously 5 min before in some animals to inhibit TNF-α-induced leukosequestration. In addition, rhIL-8 (0.025, 0.25 and 2.5 ng) in a volume of 0.5 ml was administered intratracheally to examine whether the human IL-8 equivalent levels measured in BAL fluid may evoke neutrophil recruitment.

To evaluate whether neutrophils may be a major cellular source for the production of human IL-8 equivalents, one group of animals were pretreated with cyclophosphamide (50 mg kg^{-1}, i.p.) 5 days and one day before experiment to deplete about 90% of leukocytes in circulation and examine the release of human IL-8 equivalents level in BAL fluid in response to TNF-α (10^3 u) stimulation.

Reagents

Highly purified TNF-α from *Escherichia coli* with a specific activity of 3.15×10^7 u mg^{-1} was purchased from Cellular Products (Buffalo, NY, U.S.A.). The preparation contained <15.8 pg of endotoxin per 10^6 u TNF-α activity by standard stimulus assay. TNF-α (10^2–10^4 u) was injected intratracheally. L-NAME (N^{ω}-nitro-L-arginine methyl ester), L-arginine, cyclophosphamide, DMSO, PBS and urethane were purchased from Sigma (St. Louis, MO, U.S.A.); IL-8 and GRO-α ELISA kits, rhGRO-α, anti-IL-8 antibody and rhIL-8 were obtained from R&D Systems Inc. (Minneapolis, U.S.A.). Monoclonal antibodies reacting with human and murine CD11b/CD18 surface molecules (Mac-1 protein) were obtained from Cedarlane Ltd. (Ontario, Canada). As irrelevant isotype-matched antibodies, rabbit IgG2 (for CD11b/CD18) were obtained from Boehringer Mannheim (Mannheim, Germany).

Statistics

Standard formulae were used for the analysis. Data did not approximate a Gaussian distribution, whereby the mean value did not approximate the median value. Nonparametric statistical analyses were therefore employed, and the probability of differences between groups was initially assessed by Kruskal-Wallace analysis. The number of animals in a group was too small to allow for a strict median test between groups, and subsequent analysis was performed by the Mann-Whitney U test (two-tailed) to assess the significance of differences between groups. To minimize the possibility of obtaining chance significance as a result of multiple comparisons, preplanned comparisons between specific groups were made and the significant values confirmed with Newman-Keuls analysis. Spearman rank correlation (*r*s) was used to assess the relationship between IL-8 level and neutrophil numbers. Data are presented as means ± s.e.. The null hypothesis was rejected at $P<0.05$.

Results

TNF-α-induced leukosequestration

Intratracheal injection of TNF-α induced an increase in the numbers of monocyte/macrophages, lymphocytes, eosinophils and most prominently neutrophils in the airways with the maximal responses at 10^3 u (Table 1). At 1 hour of administration (the early phase), TNF-α (10^3 u) significantly increased the neutrophil numbers in the airways ($21.3 \pm 1.0 \times 10^5$ cells, $n=5$, $P<0.01$) compared with saline control ($2.6 \pm 1.0 \times 10^5$ cells, $n=5$). At 6 h of administration (the late phase), there was a further increase in neutrophils recruited in the airways. TNF-α (10^3 u) increased the neutrophil counts retrieved in BAL to $113.2 \pm 15.6 \times 10^5$ cells ($n=11$) which was significantly higher than those in the saline control group ($4.0 \pm 1.0 \times 10^5$ cells, $n=11$, $P<0.01$) and in the early phase of TNF-α stimulation ($P<0.01$) (Figure 1). Intravenous administration of a neutralizing antibody to human IL-8 (500 ug kg^{-1}, i.v. $n=8$) 5 min before significantly inhibited TNF-α (10^3 u)-induced late phase monocytes/macrophages, lymphocyte, eosinophil (Table 1) and neutrophil recruitment in the airways ($7.5 \pm 2.5 \times 10^5$ cells, $n=8$, $P<0.01$) (Figure 1). Administration of anti-IL-8 antibody alone did not significantly influence the neutrophil recovery in the airways ($2.9 \pm 1.4 \times 10^5$ cells, $n=4$) when compared with saline control.

TNF-α-induced human IL-8 equivalents release

TNF-α also caused a dose-related increase in the level of human IL-8 equivalent in BAL (Figure 2). TNF-α only induced a significant increase in human IL-8 equivalent level in the late phase, but not the early phase (Figure 2). The increase in human IL-8 equivalent level was found at the peak of 10^3 u (33.7 ± 7.1 pg ml^{-1}, $n=11$) which was significantly greater than that in the saline control group (4.2 ± 0.3 pg ml^{-1}, *n*-11). However, the human IL-8 equivalent level in animals with TNF-α 10^4 u was significantly lower than that in animals with TNF-α 10^3 u (Figure 2). There was no detectable human IL-8 equivalent level in the BAL fluid in animals pretreated with anti-human IL-8 antibody (500 ug kg^{-1}, i.v., $n=8$) 5 min before TNF-α (10^3 u) stimulation.

Effect of L-NAME on TNF-α-induced leukocyte recruitment and human IL-8 equivalents release

L-NAME 10 mg kg^{-1} did not affect the early phase of TNF-α (10^3 u) induced neutrophil ($21.9 \pm 2.8 \times 10^5$ cells, $n=6$) or recovery of other leukocytes, but significantly increased neutrophil recovery ($215.4 \pm 39.5 \times 10^5$ cells, $n=8$, $P<0.02$) in the late phase of stimulation when compared with the corresponding TNF-α treated groups (Figure 3). L-NAME alone did not significantly change neutrophil numbers recovered in BAL fluid either in the early ($3.2 \pm 1.8 \times 10^5$ cells, $n=5$) or late phase ($3.3 \pm 1.2 \times 10^5$ cells, $n=5$) of saline instillation (Figure 3). L-Arginine (50 mg kg^{-1}, $n=6$) significantly reversed the L-NAME-enhancement effect on neutrophil recruitment ($117.5 \pm 22.3 \times 10^5$ cells, $P<0.03$) (Figure 3).

Pretreatment with L-NAME also enhanced the TNF-α (10^3 u)-induced production of human IL-8 equivalents in the late phase response (120.6 ± 24.7 pg ml^{-1}, $n=8$, $P<0.03$) when compared with TNF-α treatment alone (Figure 4). There was no significant enhancement effect of L-NAME on the early phase response (4.0 ± 2.0 pg ml^{-1}, $n=6$) to TNF-α (10^3 u). L-Arginine (50 mg kg^{-1}, $n=6$) significantly reversed the L-

Table 1 The total differential cell counts retrieved in BAL fluid

	TC ($\times 10^7$)	*Mac* ($\times 10^5$)	*Lym* ($\times 10^5$)	*Eos* ($\times 10^5$)	*Neu* ($\times 10^5$)
1 hour after injection					
Saline	1.1±0.2	96.8±6.9	9.0±1.8	1.5±0.6	2.6±1.0
TNF 10^2 u	1.4±0.6	119.2±11.1	11.3±4.5	3.9±1.5	5.3±1.4
TNF 10^3 u	1.8±0.5*	135.4±13.8*	15.3±8.1*	7.8±3.8*	21.3±1.0*
TNF 10^4 u	2.6±1.0*	217.8±21.4*	23.1±10.6	7.5±5.2	20.8±8.0*
LN+TNF-α	2.0±0.1	158.0±15.8	11.6±4.2	8.6±4.4	21.9±2.8
D+TNF-α	1.3±0.2#	107.8±10.5	10.9±4.6	3.9±2.0	7.5±1.4#
6 hours after injection					
Saline	2.2±0.2	194.0±7.3	16.9±3.3	4.4±1.8	4.0±1.0
TNF 10^2 u	2.8±0.6*	231.6±11.2*	18.8±4.2	5.0±1.4	26.0±5.9**
TNF 10^3 u	3.7±0.4*	226.8±28.1*	21.5±6.3*	13.3±7.0*	113.2±15.6**
TNF 10^4 u	3.4±0.5*	221.7±31.3*	18.4±11.6	4.6±3.7	97.6±20.4**
LN+TNF-α	5.1±0.5#	261.1±41.3	29.6±6.1	3.6±2.0#	215.4±39.5#
LN+TNF-α+L	3.0±0.4†	156.0±22.5†	16.2±6.0	9.0±4.2	117.5±22.3†
D	2.1±0.3	180.8±25.8	20.4±2.7	5.0±1.0	4.4±1.3
D+TNF-α	2.0±0.2#	164.0±19.2#	19.0±3.2	10.2±3.2	8.0±2.0#
D+TNF-α+LN	2.3±0.4†	186.3±18.9†	23.7±4.4	8.7±1.8†	10.8±5.8†
TNF-α+MAb	1.2±0.1#	101.3±12.6#	5.8±1.8#	5.5±1.1#	7.5±2.5#
rh IL-8 (ng)					
0.025	2.4±0.9	178.8±11.3	22.3±9.8	14.9±3.6*	26.0±17.3*
0.25	3.9±0.4*	254.3±23.0*	17.9±2.7	32.0±14.8*	87.2±33.0**
2.5	4.3±0.3*	196.6±43.0*	28.8±7.1*	27.5±4.7*	195.0±21.5**
0.25+D	3.1±0.7	227.5±16.7	14.9±3.1	14.9±1.9	54.0±12.1
0.25+MAb	1.8±0.5	154.1±17.6	17.5±6.5	5.8±2.0	2.6±1.7
Cycl	1.5±0.2	136.5±8.1*	11.4±3.5	0.9±0.6*	2.4±0.9*
Cycl+TNF-α	1.4±0.2#	116.9±13.9#	9.1±4.3	2.0±0.8#	12.8±4.3#

Data are means±s.e. $*P<0.05$, $**P<0.01$ compared with saline controls; $\#P<0.05$ compared with the corresponding TNF-α treatment alone group; $\dagger P<0.05$ compared with LN+TNF-α group. Abbreviations: D: 0.2% DMSO; LN: L-NAME; L: L-arginine; TNF10^{2-4} u: TNF-α at 10^2, 10^3, 10^4 u; TNF-α: TNF-α at 10^3 u; Cycl; cyclophosphamide pretreatment; MAb: monoclonal antibody to IL-8; TC: total cell count; Mac: monocyte/macrophage; Lym: lymphocyte; Eos: eosinophil; Neu: neutrophil.

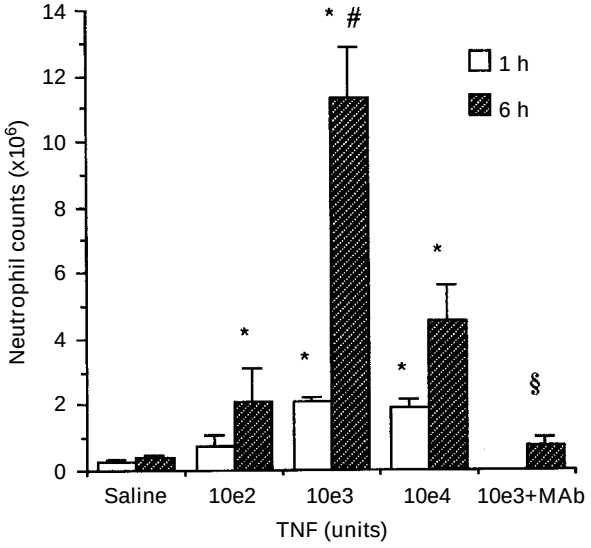

Figure 1 Dose-related neutrophil recovery in BAL fluid retrieved 1 h and 6 h after intratracheal administration of TNF-α (TNF) at 10^2 (10e2, $n=4$ for both times), 10^3 (10e3, $n=5$ and 11, respectively) and 10^4 (10e4, $n=5$ for both times) u or saline ($n=5$ and 11, respectively) in the absence and presence of anti-IL-8 monoclonal antibody (MAb, 500 μg kg^{-1}, $n=8$). Data are means±s.e. $*P<0.01$ compared with the corresponding time-matched saline control; $\#P<0.05$ and $\S P<0.01$ compared with animals treated with TNF-α 10^4 and 10^3 u, respectively.

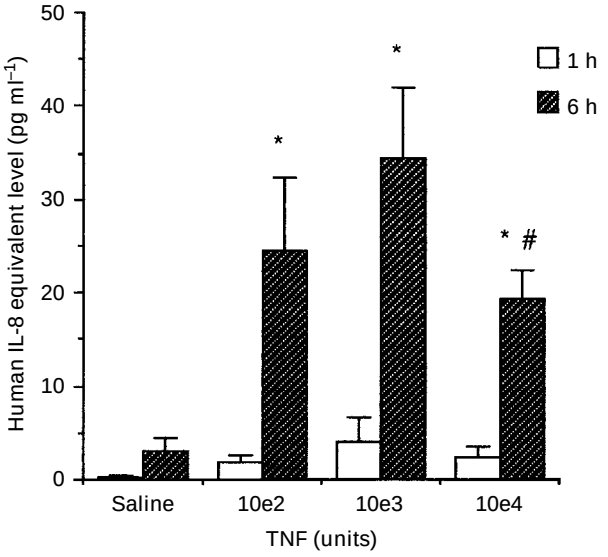

Figure 2 Dose-related human IL-8 equivalent level in BAL fluid retrieved 1 h and 6 h after intratracheal administration of TNF-α (TNF) at 10^2 (10e2, $n=4$ for both times), 10^3 (10e3, $n=5$ and 11, respectively) and 10^4 (10e4, $n=5$ for both times) u or saline ($n=5$ and 11, respectively). Data are means±s.e. $*P<0.01$ compared with time-matched saline control; $\#P<0.05$ compared with animals treated with TNF-α 10^3 u.

NAME-induced effect on human IL-8 equivalent production (47.9±33.4 pg ml^{-1}) (Figure 4). The late phase human IL-8 equivalent level was highly related to the numbers of neutrophil retrieved in BAL (Figure 5). There was no detectable human equivalent GRO-α activity in BAL fluid retrieved from any group of animals.

Effect of DMSO on the TNF-α-induced leukocyte recruitment and human IL-8 equivalents release

Pretreatment with 0.2% DMSO alone did not significantly affect total cell or neutrophil numbers recovered in BAL fluid either in the early phase ($3.2\pm1.3\times10^5$ cells, $n=5$) or the late

phase responses ($4.4 \pm 1.3 \times 10^5$ cells, $n=6$) of animals with intratracheal saline injection. Pretreatment with DMSO significantly inhibited TNF-α (10^3 u)-induced increase in neutrophil recruitment in both early and late phase responses ($7.5 \pm 1.4 \times 10^5$, $n=5$, $P<0.01$ and $8.0 \pm 2.0 \times 10^5$ cells, $n=8$, $P<0.01$, respectively) when compared with the corresponding TNF-α alone treated groups (Figure 6). DMSO also significantly inhibited the L-NAME pretreatment-enhanced increase in neutrophil recruitment ($10.8 \pm 5.8 \times 10^5$ cells, $n=7$, $P<0.01$) in the late phase of TNF-α (10^3 u) stimulation when compared with the corresponding animals without DMSO pretreatment (Figure 6).

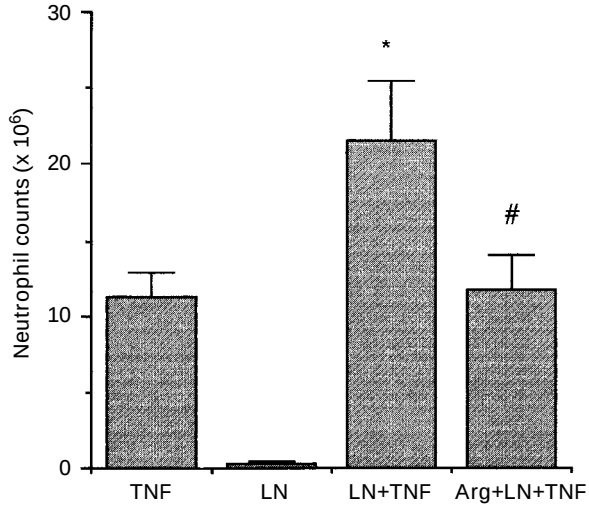

Figure 3 The effect of L-NAME (10 mg kg^{-1}, i.v.) on neutrophil recovery in BAL fluid 6 h after intratracheal administration of saline (LN, $n=5$) or TNF-α at 10^3 u (LN+TNF, $n=8$). L-Arginine (50 mg kg^{-1}, i.v.) reversed the effect of L-NAME on the response to TNF-α administration (Arg+LN+TNF, $n=6$). Data are means ±s.e. *$P<0.02$ compared with TNF-α treatment alone controls (TNF, $n=11$). #$P<0.03$ compared with L-NAME+TNF-α treatment group.

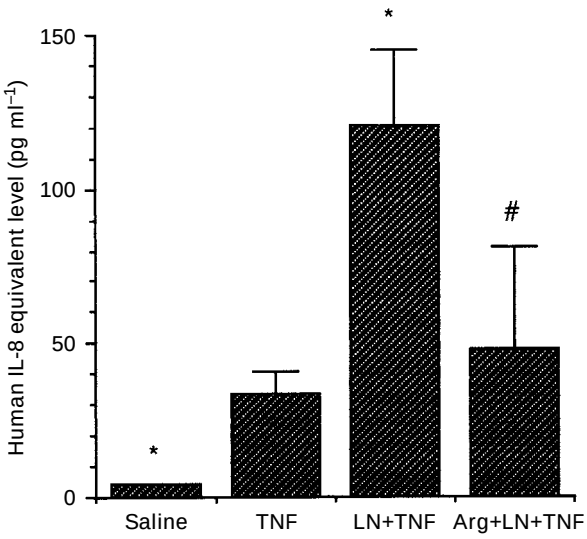

Figure 4 The effect of L-NAME (10 mg kg^{-1}, i.v.) on human IL-8 equivalents recovery in BAL fluid 6 h after intratracheal administration of saline (LN, $n=5$) or TNF-α at 10^3 u (LN+TNF, $n=8$). L-Arginine (50 mg kg^{-1}, i.v.) reversed the effect of L-NAME on the response to TNF-α administration (Arg+LN+TNF, $n=6$). Data are means±s.e. *$P<0.03$ compared with TNF-α treatment alone groups (TNF, $n=11$). #$P<0.05$ compared with L-NAME+TNF-α treatment group.

Administration of DMSO alone did not induce any detectable human IL-8 equivalent level retrieved from BAL fluid either in the early or late phase in saline control groups. DMSO abolished the late phase of TNF-α (10^3 u)-induced human IL-8 equivalents production (2.3 ± 1.3 pg ml^{-1}, $n=8$, $P<0.01$) when compared with TNF-α (10^3 u) alone treated group (Figure 7). The enhanced human IL-8 equivalents production by L-NAME in the late phase of TNF-α-stimulation was also significantly inhibited by DMSO pretreatment (44.5 ± 13.9 pg ml^{-1}, $n=7$, $P<0.01$) when compared with corresponding animals without DMSO pretreatment (Figure 7).

Effect of rhIL-8 on leukocyte recruitment

Intratracheal administration of rhIL-8 (0.025, 0.25 and 2.5 ng) resulted in a recovery of human IL-8 equivalent level (6.8 ± 0.4 pg ml^{-1}, $n=4$; 9.8 ± 1.5 pg ml^{-1}, $n=5$ and 87.1 ± 28.5 pg ml^{-1}, $n=5$, respectively), in BAL fluid, which were in similar concentrations as found in the BAL fluid retrieved 6 h after TNF-α stimulation, and induced increases in

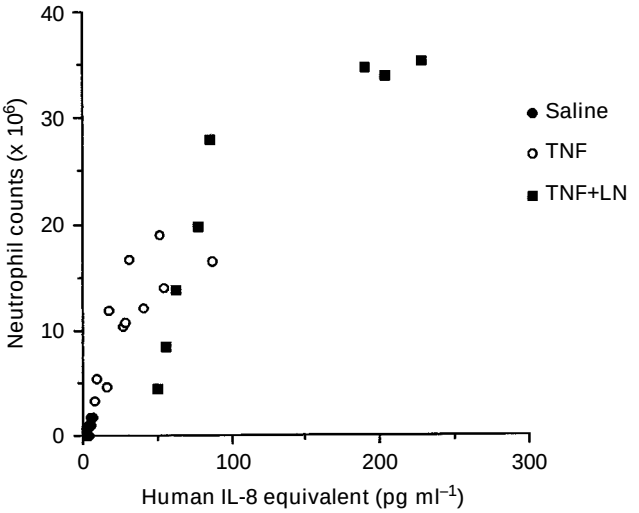

Figure 5 The relationship between human IL-8 equivalent level and neutrophil numbers retrieved from BAL 6 h after saline ($n=11$) or TNF-α (10^3 u) administration alone ($n=11$, TNF) or after L-NAME pretreatment ($n=8$, TNF+LN); $r=0.80$, $n=30$, $P<0.01$.

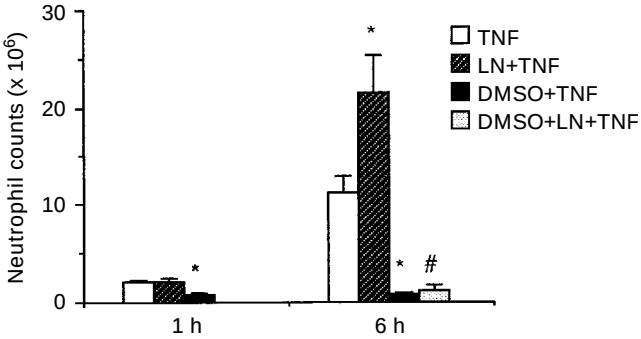

Figure 6 The effect of dimethyl sulphoxide (DMSO, 0.2%, 1 ml kg^{-1}, i.v.) pretreatment on neutrophil recovery in BAL fluid at 1 h or 6 h after intratracheal administration of TNF-α (TNF, 10^3 u, $n=5$ and 8, respectively). Dimethyl sulphoxide inhibited the enhancement effect of L-NAME (LN, 10 mg kg^{-1}, i.v.) on TNF-α (10^3 u)-induced neutrophil recruitment 6 h after administration ($n=7$). Data are means±s.e. *$P<0.01$ compared with corresponding time-matched TNF-α treatment alone group ($n=5$ and 11, respectively). #$P<0.01$ compared with L-NAME+TNF-α group ($n=8$).

neutrophil numbers in BAL fluid 6 h ($26.0 \pm 17.3 \times 10^5$, $87.2 \pm 33.0 \times 10^5$ and $195.0 \pm 21.5 \times 10^5$ cells, respectively), but not 2 h after instillation (Figure 8). Administration of rhIL-8 also induced a significant increase in the numbers of monocyte/macrophages, lymphocytes and eosinophils (Table 1). Pretreatment with intravenous anti-human IL-8 antibody abolished the effect of rhIL-8 (0.25 ng) on neutrophil ($2.6 \pm 1.7 \times 10^5$ cells, $n=4$) (Figure 8) and eosinophil recruitment (Table 1). The human IL-8 equivalent level in the BAL fluid was not detectable after anti-IL-8 antibody pretreatment. Pretreatment with DMSO failed to modify significantly the recruitment of neutrophils into airways response to rhIL-8 (0.25 ng) ($54.0 \pm 12.1 \times 10^5$ cells, $n=4$) (Figure 8).

Effect of cyclophosphamide pretreatment on TNF-α-induced leukosequestration and human IL-8 equivalents release

Pretreatment with cyclophosphamide depleted about 90% of leukocytes in circulation but did not significantly affect the total cell counts retrieved in BAL fluid (Table 1) either in saline control or after TNF-α (10^3 u) stimulation. Cyclophosphamide pretreatment significantly reduced the late phase neutrophil recruited in the BAL fluid by TNF-α (10^3 u) ($12.8 \pm 4.3 \times 10^5$ cells, $n=10$), but did not significantly influence TNF-α (10^3 u)-induced human IL-8 equivalents production (65.6 ± 27.5 pg ml^{-1}).

Effect on surface molecule expression

The CD11b/CD18 expression (expressed as mean fluorescence intensity) on neutrophils were significantly increased in the late phase of TNF-α (10^3 u) stimulation (407.7 ± 36.4, $n=8$, $P<0.03$) compared with saline control (311.2 ± 28.9, $n=8$) (Figure 9). There is no significant change in CD11b/CD18 expression on neutrophils recovered in the early phase of TNF-α (10^3 u) treatment (228.2 ± 31.5, $n=5$) compared to saline control (227.3 ± 19.8, $n=5$) (Figure 9). L-NAME did not significantly influence the magnitude of CD11b/CD18 expression in response to TNF-α either in the early (234.5 ± 21.1, $n=5$) or late phase responses (409.8 ± 41.5, $n=8$) (Figure 9).

Effect on airway and blood pressure

Intratracheal injection of TNF-α caused no significant change in the mean airway pressure either in the early or late phase of responses in the presence or absence of L-NAME (10 mg kg^{-1}) or DMSO pretreatment (Table 2). TNF-α ($10^2 - 10^4$ u) did not significantly affect the mean blood pressure in the early phase responses, while TNF-α ($10^3 - 10^4$ u) significantly ($P<0.05$) elevated the mean blood pressure to 40.2 ± 2.8 mmHg ($n=8$) and 42.6 ± 3.1 mmHg ($n=5$), respectively, in the late phase of responses, when compared with the saline control (33.1 ± 3.0 mmHg) (Table 2). Intravenous injection of L-NAME significantly elevated the mean blood pressure to 53.7 ± 3.5 mmHg ($n=9$) which reached a plateau around 4–5 min and remained elevated for at least 6 h (Table 2). However, L-NAME did not change the mean blood pressure after TNF-α stimulation (Table 2). L-Arginine significantly reversed the responses to L-NAME (Table 2). Pretreatment with DMSO did not affect the vasopressor responses to L-NAME or TNF-α (Table 2).

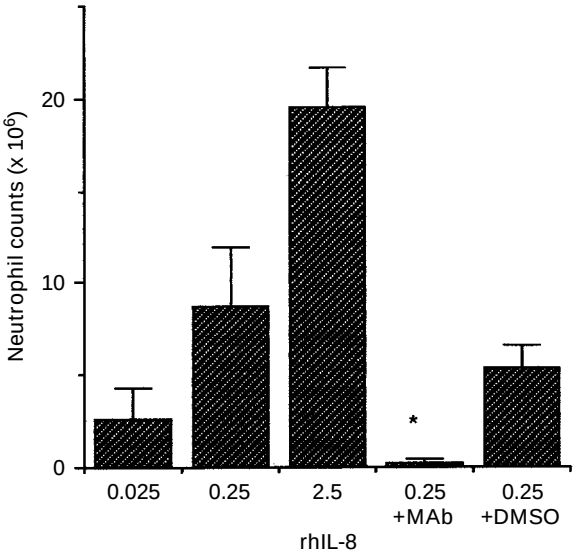

Figure 8 Effect of intratracheal administration of rhIL-8 (0.025, 0.25 and 2.5 ng, $n=4-5$) on neutrophil recruitment in the airways in the absence and presence of DMSO (0.2%, 1 ml kg^{-1}, i.v., $n=4$) or anti-IL-8 monoclonal antibody (MAb, 500 μg kg^{-1}, i.v., $n=4$) pretreatment. *$P<0.05$ compared with rhIL-8 treatment alone group.

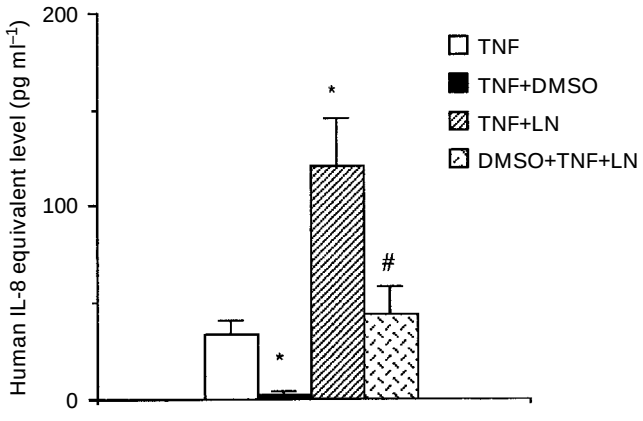

Figure 7 The effect of dimethyl sulphoxide (DMSO, 0.2%, 1 ml kg^{-1}, i.v.) pretreatment on human IL-8 equivalent release in BAL fluid 6 h after intratracheal administration of TNF-α (TNF, 10^3 u, $n=8$). Dimethyl sulphoxide inhibited the enhancement effect of L-NAME (LN, 10 mg kg^{-1}, i.v.) on TNF-α (10^3 u)-induced human IL-8 equivalent release 6 h after administration ($n=7$). Data are means ± s.e. *$P<0.01$ compared with TNF-α treatment alone group ($n=11$); #$P<0.01$ compared with TNF-α + L-NAME treatment group ($n=8$).

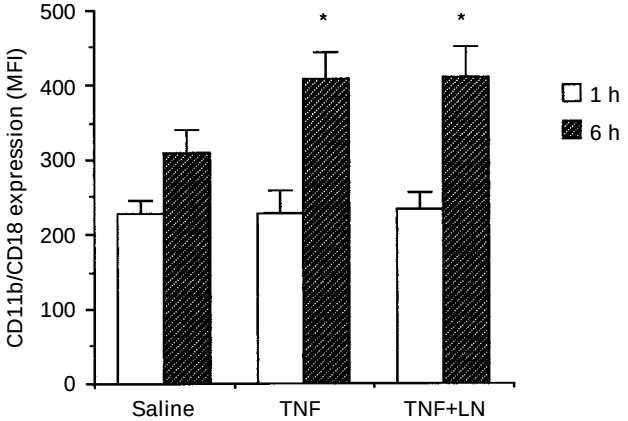

Figure 9 The expression of adhesion molecule CD11b/CD18 on neutrophils retrieved from BAL fluid one and 6 h after intratracheal administration of saline ($n=5$ and 8, respectively) or TNF-α (TNF, 10^3 u, $n=5$ and 9, respectively) in the absence and presence of L-NAME pretreatment (LN, 10 mg kg^{-1}, i.v., $n=5$ and 8, respectively). Data are mean fluorescence intensity (MFI) ± s.e. *$P<0.03$ compared with time-matched saline control.

Table 2 Effect of TNF-α on mean blood and airway pressure in the presence and absence of 0.2% DMSO or L-NAME pretreatment

		Mean blood pressure	*Mean airway pressure*
1 hour after injection	(n)	(mmHg)	(cmH_2O)
Saline	(5)	35.1±0.3	11.7±1.1
TNF-α 10^2 u	(4)	38.9±0.4	11.1±1.0
TNF-α 10^3 u	(5)	34.5±1.5	13.5±1.9
TNF-α 10^4 u	(5)	33.5±1.4	13.5±2.1
L-NAME + TNF-α 10^3 u	(5)	53.7±3.5#	13.0±3.2
L-NAME	(5)	52.0±4.8**	12.1±2.3
L-NAME + TNF-α 10^3 u + Larg	(5)	42.5±2.2	12.5±1.4
DMSO + TNF-α 10^3 u	(5)	47.6±2.4	10.6±0.3
DMSO	(5)	52.0±6.4	10.0±0.4
6 hour after injection	(n)		
Saline	(11)	32.7±1.8	10.4±0.4
TNF-α 10^2 u	(4)	33.8±5.4	11.6±1.5
TNF-α 10^3 u	(11)	41.6±2.1*	11.2±1.0
TNF-α 10^4 u	(5)	42.6±3.1*	10.3±0.9
L-NAME + TNF-α 10^3 u	(8)	52.6±3.5#	10.6±1.1
L-NAME	(5)	53.0±9.2**	10.3±0.4
L-NAME + TNF-α 10^3 u + Larg	(5)	41.3±4.8	12.6±0.6
DMSO + TNF-α 10^3 u	(8)	42.3±6.8	10.6±0.7
DMSO	(6)	39.7±3.5	8.9±0.2
DMSO + TNF-α + L-NAME	(7)	63.9±4.5#	10.6±0.6

Data are means±s.e.*$P<0.05$, **$P<0.01$ compared with saline controls; #$P<0.05$ compared with the corresponding TNF-α treatment alone group. Abbreviations: DMSO: 0.2% DMSO; Larg: L-arginine.

Discussion

Endogenous NO has been demonstrated to regulate vasodilator tone, platelet aggregability, neurotransmission, host defence cytotoxic mechanisms (Moncada & Higgs, 1991) as well as vascular permeability (Kuo *et al*., 1992). Our present study provides evidence showing a role for endogenous NO in modulation of TNF-α-induced leukosequestration and human IL-8 equivalents release in guinea-pigs *in vivo*. We demonstrated that intratracheal injection of TNF-α induced a time- and dose-related increase in neutrophil accumulation and human IL-8 equivalent level in BAL fluid. Inhibitory of endogenous NO production by L-NAME enhanced neutrophil recruitment and the release of human IL-8 equivalent in the late but not the early phase of TNF-α stimulation. This effect of L-NAME was reversed by L-arginine, suggesting this response was mediated via the generation of endogenous NO. The concomitant increase in human IL-8 equivalent level and neutrophil numbers in BAL fluid in the late, but not the early response to TNF-α suggests the late phase of leukosequestration might be related to human IL-8 equivalents release. This is further supported by the high correlation between the neutrophil numbers and human IL-8 equivalent level. In addition, pretreatment with anti-IL-8 monoclonal antibody significantly inhibited the late phase of neutrophil recruitment induced by TNF-α, indicating the causal relationship between human IL-8 equivalents and the late phase of neutrophil recruitment. This inhibitory effect also discounted the possibility that TNF-α is itself a neutrophil chemoattractant. These findings indicate that endogenous NO plays an important physiological role in preventing neutrophil recruitment, by modulating human IL-8 equivalent production in the guinea-pig airways in response to intratracheal administration of TNF-α.

Guinea-pig IL-8 has 70% amino acid sequence similarity to human IL-8 (Yoshimura & Johnson, 1993) and acts as a potent chemoattractant for neutrophils. In this study, we did not use specific ELISA kits for the measurement of guinea-pig IL-8 levels, therefore, the human IL-8 equivalent level we measured in this study may not completely represent the actual level of guinea-pig IL-8. Nevertheless, intratracheal administration of rhIL-8 which produced a similar range of IL-8 concentrations as measured in BAL in our results, induced neutrophil recovery in BAL fluid to a similar magnitude. In addition, the late phase of leukosequestration induced by either TNF-α- or rhIL-8 was prevented by pretreating animals with a neutralizing antibody against human IL-8. These results indicated that the human IL-8 equivalents released in the BAL fluid was responsible for the TNF-α-induced leukosequestration and functionally similar to human IL-8, as a potent neutrophil chemoattractant. Although the human IL-8 equivalents may not represent all the biological activity of guinea-pig IL-8, our study suggests guinea-pigs are an ideal animal model to study the modulatory effect of endogenous NO on human IL-8 activity, compared to rodents such as the mouse and rat in which the IL-8 gene is absent (Yoshimura & Johnson, 1993). It is not certain whether antibodies that recognize human IL-8 equivalents may cross-react with other neutrophil chemoattractants such as GRO-α or NAP-2, although their biological activities are not yet documented in guinea-pigs. There was no detectable human GRO-α equivalent in the BAL fluid in our study. The IL-8 ELISA kits used in this study were shown not to be cross-reactive with NAP-2. Only trivial cross-reactivity with GRO-α could be detected at concentrations up to 1 $\mu g\ ml^{-1}$. Thus, GRO-α or other neutrophil chemoattractants may not play an important role in the IL-8-related responses in this study.

Administration of TNF-α not only induced neutrophil accumulation but also monocytes/macrophages, lymphocytes and eosinophils in both the early and the late phase of responses. The human IL-8 equivalents seemed to be implicated in the late phase response, since pretreatment with a neutralizing antibody against human IL-8 almost completely blocked these chemotactic activities. IL-8 has been demonstrated to exhibit chemotactic activities against lymphocytes (Larsen *et al*., 1989) and eosinophils (Collins *et al*., 1993). Our results also showed that intratracheal administration of rhIL-8 into guinea-pigs recruited monocytes/macrophages, lymphocytes and eosinophils into airways. However, the L-NAME-enhanced human IL-8 equivalents release did not induce further eosinophils, lymphocytes or monocytes/macrophages accumulation. Probably, at higher concentrations of IL-8 primarily neutrophils are attracted (Matsushima & Oppenheim, 1989). It is also possible that IL-8 may operate via an indirect mechanism for the chemotactic activities against inflammatory cells other than neutrophils (Collins *et al*., 1993).

Neutrophils may also produce IL-8 in response to TNF-α or LPS stimulation (Fujishima *et al*., 1993). However, the capacity of a single neutrophil to produce IL-8 is about one seventieth of that of a single monocyte/macrophage (Fujishima *et al*., 1993). Thus, the contribution of recruited neutrophils to the IL-8 release in BAL fluid may be trivial, considering the capacity to produce IL-8 alveolar macrophages, the majority cell population in BAL, and other cellular sources, such as airway epithelial cells (Kwon *et al*., 1994) and endothelial cells (Villarete & Remick, 1995). In addition, depletion of neutrophils by cyclophosphamide pretreatment failed to decrease the effect of TNF-α on IL-8 release in BAL fluid, suggesting neutrophils are not the major cellular source of IL-8.

The production of IL-8 is highly related to intracellular oxidative stress (DeForge *et al*., 1992; 1993). The hydroxyl radical scavengers, DMSO or dimethyl thiourea, selectively inhibit IL-8 production in response to LPS, TNF-α or IL-1β, aggregated immune complexes or phytohaemagglutinin (DeForge *et al*., 1992). In the present study, DMSO was shown to suppress TNF-α-induced human IL-8 equivalents release and IL-8 related leukocyte accumulation in the airways, but failed

to modulate the rhIL-8-induced neutrophil recruitment in the airways, suggesting that the inhibitory effect of DMSO is not directly on neutrophils, and reactive oxygen metabolites, especially hydroxyl radicals, play an important role in the regulation of TNF-α -induced IL-8 production. The increased oxidative flux by TNF-α was demonstrated to be apparent as early as 30 min (Niu *et al*., 1994). However, IL-8 is produced *de novo* in the presence of an inflammatory stimulus, therefore, the oxidative flux has to wait for a latent period for enhanced IL-8 production As shown in the present study, a significant level of human IL-8 equivalent release was only demonstrated in the late phase, but not the early phase of TNF-α stimulation. DMSO also almost completely inhibited the early phase of TNF-α-induced neutrophil recruitment, indicating ROI generation may also mediate this response, probably by producing pre-existing neutrophil chemoattractant factors, such as C5a and leukotriene B_4 (Shingu & Nobunaga, 1984; Ivey *et al*., 1995) which may also be responsible for the TNF-α-induced early phase of eosinophils accumulation (Collins *et al*., 1993).

NO reacts with superoxide anion to produce peroxynitrite which can undergo homolytic cleavage to produce the hydroxyl radical (Stamler *et al*., 1992). However, NO is capable of inhibiting superoxide production mediated via a direct inhibition of a membrane component of NADPH oxidase (Clancy *et al*., 1992). Inhibition of NO synthesis by L-NAME was shown to increase intracellular oxidative flux (Niu *et al*., 1994). Therefore, different capacities of various cell types to replenish reduced glutathione could be an important individual determinant of the response to NO. Our results showed that pretreatment with DMSO significantly reduced the enhancement effect of L-NAME on TNF-α-induced human IL-8 equivalents release as well as neutrophil recruitment, suggesting that intracellular oxidative stress, especially hydroxyl radicals mediate the modulatory effect of NO on IL-8 generation. Our results are in agreement with previous data showing an inhibitory effect of NO on cytokine production (Marcinkiewicz & Chain, 1993; Eigler *et al*., 1995). Conversely, NO was recently shown to stimulate IL-8 production from endothelial cells (Villarete & Remick, 1995). The reason for this discrepancy is unknown. It is likely that the intracellular response to NO may be different in different cell types and experimental procedures.

The β2 integrins especially CD11b/CD18 molecules mediate neutrophil-endothelial cell adherence and TNF-α-induced neutrophil transmigration across epithelial cells (Nelson *et al*., 1986; Arnaout, 1990; Simms & D'Amico, 1991; Laurent *et al*., 1994). Our results showed that CD11b/CD18 expression on neutrophils was upregulated in the late, but not the early phase of TNF-α-induced leukosequestration, suggesting the late phase of TNF-α-induced leukosequestration might be CD11b/CD18-dependent. Recently, NO was found to limit neutrophil adherence to endothelium by downregulation of CD11b/CD18 expression or by interfering with the ability of constitutively expressed CD11/CD18 to form an adhesive bond with the endothelial cell surface (Kubes *et al*., 1991). Although our results showed that inhibition of NO synthesis did not modify the CD11b/CD18 expression on neutrophils, we cannot exclude the possibility that endogenous NO might change the adhesiveness, but not the density of CD11b/CD18 of neutrophils to modulate their emigration. CD11b/CD18 was also shown to mediate IL-8 release from neutrophils (Au *et al*., 1994). A qualitative rather than quantitative change in CD11b/CD18 expression by inhibition of NO synthesis may also explain, in part, the enhanced IL-8 release in response to TNF-α.

Inhibition of NO production causes vasoconstriction and a reduction in the shear forces that tend to push neutrophils along venular endothelium. Reductions in shear rate were shown to elicit a reversible adherence of neutrophils in mesenteric venules (Perry & Granger, 1991). However, the shear rate-dependent neutrophil adherence accounts for only a small fraction of the adhesion response associated with inhibition of NO production (Kubes *et al*., 1991). Furthermore, L-NAME in the presence or absence of TNF-α induced vasoconstrictor responses to a similar magnitude, but L-NAME alone failed to affect neutrophil recruitment into airspace, suggesting vasoconstrictor responses did not significantly affect neutrophil adherence or emigration.

In conclusion, the results of this study indicate that NO may be an important endogenous inhibitor of TNF-α-induced leukocyte chemotaxis via inhibition of IL-8 production. Thus, the production of NO in airway inflammatory diseases may play a negative feedback role in self-limiting the magnitude of inflammatory responses.

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