

Awarded Article, Annual Meeting of JSA

# Association of Tumor Necrosis Factor- $\alpha$ and Neutrophilic Inflammation in Severe Asthma

Shinya Kikuchi<sup>1</sup>, Izumi Kikuchi<sup>1</sup>, Koichi Hagiwara<sup>1</sup>, Minoru Kanazawa<sup>1</sup> and Makoto Nagata<sup>1</sup>

## ABSTRACT

**Background:** There is evidence that neutrophils are increased in the airway of severe disease or acute exacerbations of asthma. The mechanisms by which neutrophils are recruited to the airways and contribute to the pathophysiology of asthma remain to be elucidated. Tumor necrosis factor (TNF- $\alpha$ ), which can induce both tissue accumulation and activation of neutrophils and eosinophils, has been shown to be increased in the airways of severe asthma. The objective of this study is to evaluate whether TNF- $\alpha$  is associated with neutrophilic inflammation in asthma.

**Methods:** Following an inhalation of hypertonic saline, induced sputum was obtained from 9 healthy controls, 9 mild persistent asthma patients who were treated with low-dose inhaled corticosteroids; and 7 severe persistent asthma patients who were treated with combinations of drugs including high-dose inhaled corticosteroids, oral prednisolone, bronchodilators, and leukotriene receptor antagonist. After 0.1% dithiothreitol (DTT) homogenization, they were examined for total cell count, cellular differentiation, and the concentrations of TNF- $\alpha$  and myeloperoxidase (MPO).

**Results:** The concentration of TNF- $\alpha$  was not correlated with neutrophils in healthy controls or mild asthma patients. In sputum from severe asthma patients, however, the concentration of TNF- $\alpha$  is significantly correlated with both the percentage of neutrophils and the concentration of MPO. The concentration of TNF- $\alpha$  is not correlated with the percentage of eosinophils in healthy controls, mild asthma patients, or severe asthma patients.

**Conclusions:** TNF- $\alpha$  may be a contributing molecule for both accumulation and activation of neutrophils in the airways of severe asthma.

## KEY WORDS

myeloperoxidase, neutrophilic inflammation, neutrophils, severe asthma, tumor necrosis factor- $\alpha$

## INTRODUCTION

Asthma is a complex syndrome, characterized by airflow limitation, bronchial hyperresponsiveness, and chronic inflammation. In general, inflammation in asthma is characterized by accumulations of eosinophils, lymphocytes, and mast cells in the bronchial tissue.<sup>1,2</sup> It has been recently shown that neutrophils also accumulate in the airways of asthma patients with severe disease<sup>3-5</sup> and during acute exacerbation.<sup>6</sup> The mechanisms by which neutrophils are recruited to the airways and contribute to the pathophysiology of asthma remains to be elucidated. Tu-

mor necrosis factor (TNF- $\alpha$ ), which can induce both tissue accumulation and activation of neutrophils, has been shown to be increased in the airways of severe asthma.<sup>7</sup> TNF- $\alpha$  is capable of enhancing the expression of intercellular adhesion molecule (ICAM) -1 generating CXC chemokines such as IL-8 from endothelial cells<sup>8,9</sup> and thereby augmenting neutrophilic inflammation. Moreover, TNF- $\alpha$  can directly induce activation of neutrophils.<sup>10-12</sup> Therefore, it is reasonable to postulate that TNF- $\alpha$  may be involved in neutrophilic inflammation in the airways of asthma patients. In this study we report that the concentration of TNF- $\alpha$  is associated with both the accumulation

<sup>1</sup>Department of Respiratory Medicine, Saitama Medical School, Saitama, Japan.  
Correspondence: Makoto Nagata, M.D., Department of Respiratory Medicine, Saitama Medical School, 38-Morohongo, Moroyama-cho, Iruma-gun, Saitama 350-0495, Japan.

Email: favre4mn@saitama-med.ac.jp  
Received 1 February 2005. Accepted for publication 16 June 2005.  
©2005 Japanese Society of Allergology

**Table 1** Patient characteristics I

	Healthy (n = 9)	Mild asthma patients (n = 9)	Severe asthma patients (n = 7)
Gender (M/F)	(6/3)	(4/5)	(5/2)
Age (years)	31.9 ± 2.0*	42.3 ± 5.8	54.4 ± 4.2
Sputum volume (mL)	3.4 ± 0.6	4.4 ± 1.0	5.4 ± 1.8
Total sputum cells (10 <sup>5</sup> /mL)	22.3 ± 6.1	20.9 ± 4.7	19.2 ± 8.8
Squamous epithelium (%)	20.1 ± 6.3	27.1 ± 8.5	29.8 ± 10.1
Sputum eosinophils (%)	0.2 ± 0.1	7.2 ± 4.4	3.1 ± 1.2**

\*p &lt; 0.05 vs others \*\*p &lt; 0.01 vs healthy

**Table 2** Patient characteristics II

	Mild asthma patients (n = 9)	Severe asthma patients (n = 7)
%FEV <sub>1.0</sub>	79.5 ± 2.5	63.1 ± 11.9*
Disease duration (yrs)	15.4 ± 3.2	17.4 ± 5.5
IgE (IU/ml)	3114.5 ± 1772.5	839.9 ± 430.0
Positive case of IgE Ab:		
<i>D. farinae</i>	6 (66.7%)	5 (71.4%)
<i>C. albicans</i>	7 (77.8%)	4 (57.1%)
<i>J. cedar</i>	9 (100.0%)	6 (85.7%)

\*p &lt; 0.05 vs mild asthma patients

and the activation of neutrophils in sputum from patients with severe persistent asthma.

## METHODS

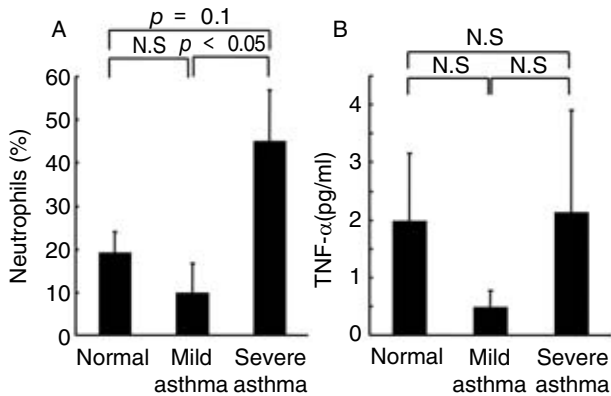
### PATIENTS

The study population consisted of 16 asthma patients recruited from the Department of Respiratory Medicine, Saitama Medical School Hospital, and 9 healthy controls. Subjects who had an episode of cold syndrome during the last four weeks prior to the onset of this study were excluded. In addition, subjects who showed positive c-reactive protein at the time of sampling were excluded. Consequently, the number of healthy controls was 9, and the numbers of patients who were enrolled in this study were as follows: 9 mild persistent asthma patients; and 7 severe persistent asthma patients (Table 1). Asthma was diagnosed based on a history of recurrent wheezing, dyspnea, chest tightness, and either reversible air-flow limitation (forced expiratory volume in one second (FEV<sub>1</sub>): <70% predicted or previous best, which increased by >15% after the inhalation of 200 µg salbutamol) or methacholine-induced airway hyperresponsiveness. Asthma patients were assigned into the following two groups: a group of 9 patients with mild persistent (step 2) asthma treated with 200 µg fluticasone dipropionate dry powder inhaler (FP-DPI) alone; and another group of 7 patients with severe persistent (step 4) asthma treated with a combination of 800 µg FP-DPI, a long-acting beta agonist inhaled

salmeterol, theophylline, leukotriene receptor antagonist (LTRA), and 5 to 10 mg oral prednisolone.

### SPUTUM INDUCTION AND PROCESSING

Sputum was induced with an aerosol of hypertonic saline according to a method described in previous articles.<sup>13,14</sup> Informed consent was obtained prior to collection of each sputum sampling. Inhaled salbutamol was administered with a metered dose inhaler at 15 minutes before sputum induction. Subsequently, the patient inhaled 3.5% saline nebulized by an ultrasonic nebulizer at room temperature. The total time for sputum induction was 15 minutes. Mouth washing before each cough was encouraged to minimize salivary contamination. The initial sample from the first cough was discarded. Induced sputum was collected into a 50-mL polypropylene tube, kept at 4°C, and processed within 2 hours after collection. To process induced sputum, 1 ml of Hank's balanced salt solution (HBSS) containing 0.1% dithiothreitol (DTT) (purchased from Sigma, St. Louis, MO, USA) was added to the 1 ml sputum. The mixture was vortexed and repeatedly aspirated at ambient temperature until the sputum was homogenized. Samples were further diluted with HBSS to 5 mL, and centrifuged at 400 xg for 10 minutes. Cell pellets were resuspended, and slides were prepared using Cytospin and stained with May-Giemsa for differential cell counts. At least 500 inflammatory cells were counted for each sample. An adequate sample was defined as having less than 50%



**Fig. 1** The percentages of neutrophils (A) and concentrations of TNF- $\alpha$  (B) in sputum from normal volunteers ( $n = 9$ ), mild asthma patients ( $n = 9$ ), and severe asthma patients ( $n = 7$ ). Data are expressed as means  $\pm$  SEM.

of squamous epithelial cells on Cytospin.

### CONCENTRATIONS OF TNF- $\alpha$ AND MPO IN SPUTUM

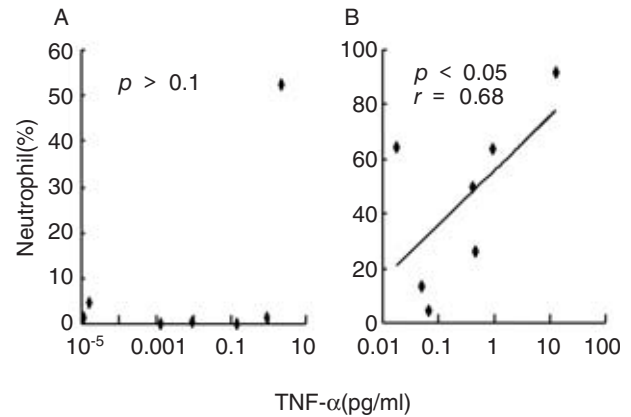
The concentrations of TNF- $\alpha$  and MPO in the supernatants of sputum were determined by ELISA kits (TNF- $\alpha$ ; R&D systems, Minneapolis, MN, USA, MPO; CALBIOCHEM, San Diego, CA, USA).

### STATISTICS

In order to determine statistical significance, the Kruskal-Wallis test was conducted. A value of  $p < 0.05$  was considered to indicate a statistically significant difference. Data are shown as means  $\pm$  SEM. Similarly, Spearman's correlation test was conducted in order to determine statistical correlation.

### RESULTS

Age was significantly lower in healthy controls than in both asthma groups and the percentage of eosinophils in induced sputum was significantly higher in severe asthma patients than in healthy controls (Table 1). There was no significant difference among these three groups with respect to gender distribution, induced sputum volume, total number of sputum inflammatory cells, and squamous epithelium (Table 1). There was no significant difference between the two asthma groups with respect to disease duration, total IgE, positive rate of specific IgE to house-dust mite, candida albicans, or Japanese cedar. However, %FEV<sub>1.0</sub> was significantly lower in severe asthma patients (Table 2). The percentage of neutrophils in induced sputum was significantly higher in severe asthma patients than in mild asthma patients ( $p < 0.05$ ; Fig. 1A). However, there was no significant difference between healthy controls and mild asthma patients (Fig. 1A). There was no significant difference between the three groups with respect to the concen-



**Fig. 2** Relationship between sputum TNF- $\alpha$  and neutrophils in mild asthma patients (A), and severe asthma patients (B). (A):  $p > 0.1$ , (B):  $p < 0.05$ ,  $r = 0.68$ .

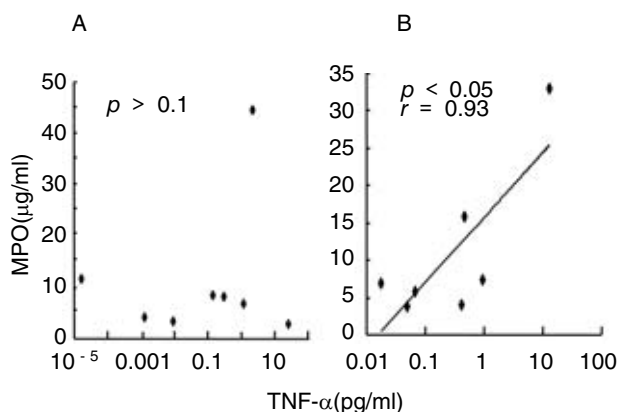
tration of sputum TNF- $\alpha$  ( $p > 0.1$ , respectively; Fig. 1 B).

We next evaluated whether there was an association between the concentration of TNF- $\alpha$  and percentages of neutrophils or eosinophils in sputum. There was no significant correlation between sputum TNF- $\alpha$  and neutrophils in the mild asthma group ( $p > 0.1$ , Fig. 2A) or healthy control subjects ( $p > 0.1$ , data not shown). On the other hand, in severe persistent asthma patients, there was a significant positive correlation between TNF- $\alpha$  and percentage of neutrophils in sputum ( $p < 0.05$ ,  $r = 0.68$ ; Fig. 2B). There was no significant correlation between TNF- $\alpha$  and percentages of eosinophils in healthy controls, mild asthma patients, or severe asthma patients ( $p > 0.1$ , respectively, data not shown).

Finally we evaluated whether there was an association between concentrations of TNF- $\alpha$  and MPO in sputum. In mild asthma patients, there was no significant correlation between sputum TNF- $\alpha$  and MPO ( $p > 0.1$ , Fig. 3A). On the other hand, there was a significant positive correlation between the concentration of TNF- $\alpha$  and MPO in sputum in severe persistent asthma patients ( $p < 0.05$ ,  $r = 0.93$ ; Fig. 3B). Such a positive correlation between TNF- $\alpha$  and MPO was also observed in healthy control subjects ( $p < 0.01$ ,  $r = 0.50$ ; data not shown).

### DISCUSSION

We observed that severe asthma patients showed significantly higher sputum neutrophilia than mild asthma populations. It is noteworthy that these patients were treated with a combination of oral and high dose inhaled corticosteroids. There is evidence that corticosteroids are not capable of suppressing the tissue accumulation or functions of neutrophils,<sup>15,16</sup> suggesting that neutrophils recruited to the airways in severe asthma may still play a role. For ex-



**Fig 3** Relationship between sputum TNF- $\alpha$  and MPO in mild asthma patients (A), and severe asthma patients (B). (A):  $p > 0.1$ , (B):  $p < 0.05$ ,  $r = 0.93$ .

ample, neutrophils are capable of producing chemoattractants for eosinophils such as leukotriene B4 and releasing proteases including matrix metalloprotease (MMP) and elastase which can cause tissue injury<sup>15</sup> and hence may contribute to the deterioration of severe asthma. On the other hand, there was only a trend, but no significant difference, in the percentages of sputum neutrophils between severe asthma and healthy subjects. Although the reason for this remains to be clarified, the lack of difference between these two groups is attributable, at least in part, to the small size of this study. In asthma, association between age and neutrophilic inflammation seems controversial.<sup>17,18</sup> Although the mean age of controls was younger than that of the two asthma groups in our study, it is unclear that this fact adversely affects the interpretation of this study because these subjects are healthy individuals without manifestation of asthma. Furthermore, there was no difference in age between the two asthma groups in our study.

It is of particular interest that the concentration of sputum TNF- $\alpha$  is positively correlated with the airway accumulation of neutrophils in severe persistent asthmatics. Furthermore, we found that concentration of TNF- $\alpha$  is correlated with MPO in severe asthma patients. TNF- $\alpha$  is capable of enhancing the expression of intercellular adhesion molecule (ICAM) -1 on and generation of chemokines from endothelial cells<sup>8,9</sup> and thereby can augment neutrophilic inflammation. Moreover, TNF- $\alpha$  can directly induce adhesion and activation of neutrophils.<sup>10-12</sup> In the airways of severe asthma patients, contributing mechanisms of TNF- $\alpha$  may be augmented in the presence of other inflammatory molecules. Therefore, it is theoretically plausible that TNF- $\alpha$  contributes to both accumulation and activation of neutrophils in the airways of severe asthma patients, which persists even under full corticosteroid therapy. In contrast to its impact on neutro-

philic inflammation, the concentration of TNF- $\alpha$  was not associated with eosinophilic inflammation in this study. Unlike neutrophils, eosinophilic inflammation in asthma may be largely dependent on the ordinary mediators involved in allergic inflammation including Th2 cytokines, CC-chemokines and cysteinyl leukotrienes, and therefore the contributing role of TNF- $\alpha$  may be limited.

The fact that concentrations of TNF- $\alpha$  and MPO are weakly but positively correlated in healthy subjects was not what we expected. This may suggest that TNF- $\alpha$  may be a fundamental mechanism which may be associated with activation of neutrophils in the airways of healthy subjects. Interestingly, although the reason is unclear, such a correlation between TNF- $\alpha$  and MPO was not observed in the mild asthma group. In the mild asthma group, the concentration of TNF- $\alpha$  was low, although it was not significant (Fig. 1B). The cellular sources for providing TNF- $\alpha$  to the airways can be a variety of cells including eosinophils,<sup>19</sup> epithelial cells,<sup>20</sup> airway macrophages,<sup>21</sup> mast cells,<sup>22</sup> and neutrophils by themselves.<sup>15</sup> These mild asthma cases were well controlled with low-dose inhalational corticosteroids, and therefore it is possible that generation of inflammatory cytokines including TNF- $\alpha$  may be effectively suppressed. On the other hand, in severe asthma, inflammatory cells including increased neutrophils, which are insensitive to corticosteroids, may be, at least in part, contributing sources of TNF- $\alpha$  in the airways.

Taken together, the present study suggests that TNF- $\alpha$  is an involved molecule for neutrophilic inflammation seen with severe asthma. Although the clinical significance of neutrophilic inflammation in severe asthma remains to be elucidated, this study may provide important novel information about the pathophysiology of severe asthma and a possible therapeutic target, namely TNF- $\alpha$ , for controlling the particular phenotype of the disease.

## ACKNOWLEDGEMENTS

The authors gratefully acknowledge the technical assistance of Ms. Akemi Yokote.

## REFERENCES

1. Busse WW, Lemanske RF Jr. Asthma. *N. Engl. J. Med.* 2001;**344**:350-362.
2. Bradley BL, Azzawi M, Jacobson M *et al*. Eosinophils, T-lymphocytes, mast cells, neutrophils, and macrophages in bronchial biopsy specimens from atopic subjects with asthma: comparison with biopsy specimens from atopic subjects without asthma and normal control subjects and relationship to bronchial hyperresponsiveness. *J. Allergy Clin. Immunol.* 1991;**88**:661-674.
3. Gibson PG, Simpson JL, Saltos N. Heterogeneity of airway inflammation in patient asthma: evidence of neutrophilic inflammation and increased sputum interleukin-8. *Chest* 2001;**119**:1329-1336.

4. Wenzel SE, Schwartz LB, Langmack EL *et al.* Evidence that severe asthma can be divided pathologically into two inflammatory subtypes with distinct physiologic and clinical characteristics. *Am. J. Respir. Crit. Care Med.* 1999; **160**:1001-1008.
5. Wenzel SE, Szefler SJ, Leung DY, Sloan I, Rex MD, Martin RJ. Bronchoscopic evaluation of severe asthma: persistent inflammation associated with high dose glucocorticoids. *Am. J. Respir. Crit. Care Med.* 1997; **156**(3 pt 1): 737-743.
6. Fahy JV, Kim KW, Liu J, Boushey HA. Prominent neutrophilic inflammation in sputum from subjects with asthma exacerbation. *J. Allergy Clin. Immunol.* 1995; **95**:843-852.
7. Tillie-Leblond I, Pugin J, Marquette CH *et al.* Balance between proinflammatory cytokines and their inhibitors in bronchial lavage from patients with status asthmatics. *Am. J. Respir. Crit. Care Med.* 1999; **159**:487-494.
8. Pober JS, Gimbrone MA Jr, Lapierre LA *et al.* Overlapping patterns of activation of human endothelial cells by interleukin 1, tumor necrosis factor, and immune interferon. *J. Immunol.* 1986; **137**:1893-1896.
9. Smart SJ, Casale TB. TNF-alpha-induced transendothelial neutrophil migration is IL-8 dependent. *Am. J. Physiol.* 1994; **266**:238-245.
10. Ferrante A. Activation of neutrophils by interleukins-1 and -2 and tumor necrosis factors. *Immunology Series* 1992; **57**:417-436.
11. Henson PM, Wenzel SE. Neutrophils and their mediators in asthma. In: Busse WW, Holgate ST (eds). *Asthma & Rhinitis*. Oxford, Blackwell Science, 2000;503-530.
12. Meyer JD, Yurt RW, Duhanev R *et al.* Tumor necrosis factor-enhanced leukotriene B4 generation and chemotaxis in human neutrophils. *Arch. Surg.* 1988; **123**:1454-1458.
13. Jatakanon A, Uasuf C, Maziak W, Lim S, Chung KF, Barnes PJ. Neutrophilic inflammation in severe persistent asthma. *Am. J. Respir. Crit. Care Med.* 1999; **160**:1532-1539.
14. Keatings VM, Evans DJ, O'Connor BJ, Barnes PJ. Cellular profiles in asthmatic airways: a comparison of induced sputum, bronchial washings, and bronchoalveolar lavage fluid. *Thorax* 1997; **52**:372-374.
15. Cox G. Glucocorticoid treatment inhibits apoptosis in human neutrophils. Separation of survival and activation outcomes. *J. Immunol.* 1995; **154**:4719-4725.
16. Meagher LC, Cousin JM, Seckl JR, Haslett C. Opposing effects of glucocorticoids on the rate of apoptosis in neutrophils and eosinophilic granulocytes. *J. Immunol.* 1996; **156**:4422-4428.
17. Vignola AM, Bonanno A, Profita M *et al.* Effect of age and asthma duration upon elastase and alpha1-antitrypsin levels in adult asthmatics. *Eur. Respir. J.* 2003; **22**:795-801.
18. Woodruff PG, Khashayar R, Lazarus SC *et al.* Relationship between airway inflammation, hyperresponsiveness, and obstruction in asthma. *J. Allergy Clin. Immunol.* 2001; **108**:753-758.
19. Costa JJ, Matossian K, Resnick MB *et al.* Human eosinophils can express the cytokines tumor necrosis factor alpha and macrophage inflammatory protein-1 alpha. *J. Clin. Invest.* 1993; **91**:2673-2684.
20. Khair OA, Devalia JL, Abdelaziz MM, Sapsford RJ, Tarraf H, Davies RJ. Effect of Haemophilus influenzae endotoxin on the synthesis of IL-6, IL-8, TNF alpha and expression of ICAM-1 in cultured human bronchial epithelial cells. *Eur. Respir. J.* 1994; **7**:2109-2116.
21. Gosset P, Tsicopoulos A, Wallaert B *et al.* 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.* 1991; **88**:561-571.
22. Coward WR, Okayama Y, Sagara H, Wilson SJ, Holgate ST, Church MK. NF-kappa B and TNF-alpha: a positive autocrine loop in human lung mast cells? *J. Immunol.* 2002; **169**:5287-5293.