TNF-α dysregulation in asthma: Relationship to ongoing corticosteroid therapy

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S Waserman, J Dolovich, M Conway, JS Marshall. TNF-α dysregulation in asthma: Relationship to ongoing corticosteroid therapy. Can Respir J 2000;7(3):229-237.

BACKGROUND: Tumour necrosis factor-alpha (TNF-α) is a major proinflammatory cytokine that is thought to be important in the pathogenesis of asthma. However, alterations in systemic regulation of this cytokine in asthma have not been examined in the context of corticosteroid therapy.
OBJECTIVES: To examine the ability of peripheral blood mononuclear cells (PBMC) from three different groups of patients with asthma requiring varying amounts of inhaled corticosteroids (ICS) for clinical control, and to examine cells from age- and sex-matched nonasthmatic patients to produce TNF-α.
DESIGN: All patients with asthma had a positive methacholine challenge test. 'High dose' ICS patients with asthma required ICS greater than or equal to 800 µg/day. 'Medium dose' patients with asthma were on less than or equal to 500 µg/day of ICS, whereas 'no ICS' patients with asthma had received no ICS for at least three months. Each patient with asthma was examined in parallel with an age- and sex-matched, nonasthmatic, nonatopic control subject. Cells were cultured (with or without the addition of potential stimulators phytohemagglutinin, lipopolysaccharide, formyl-methionine-leucine-phenylalanine or antihuman CD3), and TNF-α production was assessed by ELISA.
MAIN RESULTS: PBMC from both high dose ICS (n=8) and no ICS (n=11) patients with asthma produced more than twice the amount of TNF-α than cells from matched nonasthmatic control patients ($P<0.01$) when cultured alone or in the presence of each stimulus ($P<0.05$). In contrast, there was no significant difference in TNF-α production between medium dose ICS patients with asthma and control patients. A group of asymptomatic atopic patients (n=6) did not have an increased level of TNF-α production.
CONCLUSIONS: Increases in TNF-α production within the PBMC compartment can be observed in both patients with asthma receiving high dose ICS and in a group of patients with mild asthma receiving no ICS therapy, but not in patients with asthma receiving a medium dose of ICS or atopic patients.

Key Words: *Asthma; Inflammation; Peripheral blood mononuclear cells; T cells; Tumour necrosis factor-alpha*

Pour le résumé, voir page suivante

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Dérèglement de la production de TNF-α et asthme : lien avec la corticothérapie continue

CONTEXTE : On croit que le facteur nécrosant des tumeurs alpha (TNF-α), une cytokine proinflammatoire importante, joue un rôle considérable dans la pathogenèse de l'asthme. Toutefois, les troubles de la régulation générale de cette cytokine dans l'asthme n'ont pas encore fait l'objet d'analyse dans le contexte de la corticothérapie.

OBJECTIF : Examiner la capacité de production de TNF-α par les cellules mononucléaires dans le sang périphérique (PBMC) dans trois groupes de patients asthmatiques devant faire usage de quantités différentes de corticostéroïdes en inhalation (CI) pour la maîtrise clinique de la maladie et examiner des cellules de patients non asthmatiques, appariés selon l'âge et le sexe.

PLAN D'ÉTUDE : Le test de provocation à la méthacholine s'est avéré positif chez tous les patients asthmatiques. On entend par forte dose de CI des quantités égales ou supérieures à 800 µg/jour et par dose moyenne des quantités égales ou inférieures à 500 µg/jour; quant au non-usage de CI, il se définit comme l'absence d'utilisation du médicament depuis trois mois. Chaque asthmatique a été comparé à un témoin non asthmatique, non atopique, apparié selon l'âge et le sexe. Il y a eu culture de cellules (avec ou sans l'ajout de stimulants potentiels : phytohémagglutinine, lipopolysaccharide, formylméthionine-leucine-phénylalanine ou CD3 antihumain), et la production de TNF-α a été évaluée par le test ELISA.

RÉSULTATS : La production de TNF-α par les PBMC chez les patients asthmatiques prenant de fortes doses de CI (n=8) et n'en prenant pas (n=11) a été plus du double de celle des cellules provenant de témoins non asthmatiques (P<0,01), cultivées seules ou avec l'un ou l'autre des stimulants (P<0,05). Par contraste, on n'a pas enregistré de différences sensibles de production de TNF-α entre les patients prenant des doses moyennes de CI et les témoins. Enfin, on n'a pas observé de production accrue de TNF-α dans un groupe de patients atopiques ne présentant pas de symptômes (n=6).

CONCLUSION : On a observé une production accrue de TNF-α dans le compartiment des PBMC tant chez les patients asthmatiques prenant de fortes doses de CI que chez ceux qui n'en faisaient pas usage, mais pas chez les patients asthmatiques prenant des doses moyennes de CI ou chez les patients atopiques.

It has now been clearly established that asthma is accompanied by airway inflammation (1). A marked inflammatory infiltrate with a preponderance of eosinophils and lymphocytes is a prominent feature of the bronchial mucosa in patients with asthma (2-4). Other airway inflammatory cells, including mast cells, alveolar macrophages, neutrophils and epithelial cells, have also been postulated to participate in the inflammatory response. This chronic inflammatory state is considered to be related intimately to the pathogenesis of nonspecific bronchial hyperresponsiveness (BHR), a key feature of asthma (5). The factors responsible for the influx of inflammatory cells are not completely understood; however, a range of cytokines are recognized as important factors orchestrating the allergic inflammatory response. One such cytokine is tumour necrosis factor-alpha (TNF-α).

TNF-α has many proinflammatory actions in vitro that may potentiate allergic inflammation in vivo and hence play a role in asthma. An increased amount of TNF-α is present in the airways of patients with asthma. Bronchoalveolar lavage (BAL) fluid from patients with asthma contains more cells expressing positive hybridization signals following in situ analysis for TNF-α mRNA than BAL from normal volunteers (6). Similarly, Cembrzynska-Nowak et al (7) found increased TNF-α secretion by cultured BAL leukocytes from patients with asthma, both at baseline and after phytohemagglutinin stimulation. Broide et al (8) measured cytokine levels in BAL fluid of asymptomatic and symptomatic patients with asthma. TNF-α, interleukin (IL)-6 and granulocyte-macrophage colony-stimulating factor were increased in the symptomatic group, compared with controls.

TNF-α has a multiplicity of actions that may lead to BHR and airway inflammation. Animal studies carried out in rats (9) and sheep (10) indicate that administered TNF-α causes BHR. The same has now been shown for normal human subjects who inhaled recombinant TNF-α (11). In this case, BHR was accompanied by a sputum neutrophilia. The mechanism of the BHR is not well understood, but may involve the direct effect of TNF-α on bronchial smooth muscle, similar to a direct effect of TNF-α shown in vascular smooth muscle in vitro (12). Similarly, TNF-α is known to cause the release of various inflammatory mediators, including arachidonic acid metabolites (13,14) and platelet activating factor (15), making it possible that TNF-α may increase BHR via these mediators.

TNF-α can also lead to an influx of inflammatory cells into tissues, an important part of asthma pathogenesis, by upregulating the vascular endothelial expression of adhesion molecules such as endothelium leukocyte adhesion molecule-1, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1. These molecules bind complementary ligands on the surface of inflammatory cells and enhance the recruitment of inflammatory cells from the bloodstream (16,17). The importance of TNF-α-induced adhesion has been illustrated in a primate model of allergic airway inflammation. Exposure of ascaris-sensitized Rhesus monkeys to aerosolized ascaris ova induced BHR to methacholine, eosinophils in the airways and increased ICAM-1 expression on the airway endothelium (18). The eosinophil infiltrate and BHR were prevented by pretreatment with anti-ICAM antibodies. Another important feature of TNF-α includes its ability to activate inflammatory leukocytes including neutrophils (19), eosinophils (20), macrophages (13) and lymphocytes (21). TNF-α can also stimulate mononuclear phagocytes and other cell types to produce certain proinflammatory cytokines, including IL-1 (22), IL-6 (23), TNF-α itself (24) and a number of chemokines (25), particularly RANTES

TABLE 1
Characteristics of patients with asthma studied and compared in parallel with age- and sex-matched control patients

Patient initials	Age (years)	Sex	FEV_1 (% predicted)	Atopy (# positive skin tests)	ICS (μg/day)	Other asthma medication
High dose inhaled corticosteroid patients with asthma						
DL	22	F	75	7	800 budesonide	Salbutamol
LH	52	F	61	3	1600 budesonide	nedocromil sodium Procaterol HCl
JA	48	F	21	1	800 budesonide	Ipratropium bromide terbutaline sulfate
SH	36	F	80	7	1600 budesonide	Salbutamol
ND	59	F	60	0	1200 budesonide	Salbutamol
MK	31	F	75	4	1200 budesonide	terbutaline sulfate
EW	64	F	54	1	1200 budesonide	Salbutamol
GB	20	F	78	5	1600 budesonide	Salbutamol
Mean±SD	42±6		63±7			
Medium dose inhaled corticosteroid patients with asthma						
IU	59	F	61	2	400 budesonide	Salbutamol
JK	35	F	67	10	500 beclamethasone dipropionate	Salbutamol
DS	33	M	80	8	100 beclamethasone dipropionate	Salbutamol Salbutamol
MY	57	F	73	8	400 budesonide	
MV	50	F	75	5	500 beclamethasone dipropionate	Salbutamol pirbuterol INH
MB	73	F	65.3	2	250 beclamethasone dipropionate	terbutaline sulfate Salbutamol
RVL	42	M	82.0	12	300 budesonide	terbutaline sulfate
MH	60	F	63.4	14	500 beclamethasone dipropionate	Salbutamol Salbutamol
LH	37	F	96.2	11	400 budesonide	
MC	18	F	71.6	7	400 budesonide	
KF	54	F	47.3	3	200 beclamethasone dipropionate	
Mean±SD	47±5		71±13			
No inhaled corticosteroid patients with asthma						
MF	25	F	120	3	–	Salbutamol
ED	27	F	104	10	–	terbutaline sulfate
JH	43	F	101	9	–	Salbutamol
MT	20	F	93	7	–	Salbutamol
SR	46	F	85	3	–	Salbutamol
MAT	33	F	81	2	–	Salbutamol
JC	20	F	89	1	–	Salbutamol
CB	21	M	88	1	–	Salbutamol
AM	50	M	102	1	–	Salbutamol
LH	34	F	84	8	–	Salbutamol
Mean±SD	32±4		95±12			

FEV_1 Forced expiration volume in 1 s; HCl hydrochloric acid; ICS Inhaled corticosteroids

(26,27). These effects are not limited to immune effector cells but may also involve structural cells such as smooth muscle cells (26). In most cases, corticosteroids have been shown to antagonize the effects of TNF-α (24-27).

There are many potential sources of TNF-α, including monocyte and/or macrophages (28-30), mast cells (31), eosinophils (32) and epithelial cells (33), but the major disease-related cell source in asthma is unknown. Gosset et al (34) found that the alveolar macrophages from people with asthma and allergies will produce larger amounts of TNF-α after immunoglobulin (Ig) E-dependent stimulation (IgE cross-linking) compared with controls. TNF-α production is also enhanced in alveolar macrophages examined in people with asthma who develop a late asthmatic response (LAR) after allergen challenge (35). Williams et al (36) showed that there is increased expression of low-affinity IgE receptors on both peripheral blood mononuclear cell (PBMC) phagocytes and alveolar macrophages of patients with asthma compared with controls, which may lead to IgE-dependent production of IL-β and TNF-α. Bradding et al (31) examined immunohistochemically bronchial mucosal biopsies and found increased expression of TNF-α in people with asthma, especially in cells staining positively for the mast cell marker tryptase. Finally, another potential source of TNF-α is the T lymphocyte. Bronchial biopsies from atopic people with asthma contain activated T lymphocytes bearing the IL-2 receptor (37).

The present study examines the regulation of systemic production of TNF-α in asthma. While local TNF-α expression may reflect better the ongoing inflammatory status of

the patient with asthma, examination of the regulation of PBMC cytokine production could provide clues to the nature of the immune dysregulation responsible for the chronic local disease process. TNF-α production was evaluated from PBMC collected from people with asthma of varying inhaled corticosteroid (ICS) requirement, as well as atopic nonasthmatic and control nonatopic, nonasthmatic patients. PBMC were cultured for 24 h and supernatants examined for TNF-α content at baseline by ELISA and after the addition of a variety of stimulators. The cell source was determined by further purifying the PBMC into monocyte and lymphocyte compartments by magnetic-activated cell sorting (MACS).

PATIENTS AND METHODS

Patient groups: Four groups of patients were studied and compared in parallel with age- and sex-matched control patients (Table 1). These included three groups of people with asthma and a fourth group of atopic, nonasthmatic patients. All people with asthma fulfilled American Thoracic Society (37) criteria for asthma. One group (high dose ICS people with asthma) had a forced expiration volume in 1 s (FEV_1) less than or equal to 80% predicted, and was defined as requiring at least 800 μg/day of ICS for disease control. A second group (medium dose ICS patients) was on less than or equal to 500 μg/day ICS. A third group of people with asthma did not use ICS for at least three months before the study. All patients were 18 years of age or older, and were permitted to be on $beta_2$ agonists, sodium cromoglycate, nedocromil, theophylline and antihistamines (Table 1). Atopic nonasthmatic patients had three or more positive skin tests (wheal of 3 mm or greater) to a range of common aeroallergens, and no history of asthma or other allergic disease. All were asymptomatic at the time of the study. Control patients were nonatopic, nonasthmatic and on no medication, and had no history of underlying allergic disease. Exclusion criteria included prednisone use in the previous two months, other ongoing allergic disease, respiratory infection in the previous four weeks, smoking, pregnancy, depression and other steroid-requiring illnesses. The use of patients in these studies was approved by the institutional ethics review board, and subjects gave their informed consent. Allergy skin tests were performed using the modified prick technique with 19 common allergens.

Cell separation and culture: Peripheral blood was drawn from each of the study patients at the same time as from an age- and sex-matched control subject. Blood was separated into PBMC and granulocyte preparations using Dextran (Sigma, St Louis, Missouri) sedimentation followed by Percoll (Sigma) density separation. Allergy skin tests were performed using the modified prick technique with 19 common allergens. Six per cent Dextran in 0.9% sodium chloride was added to 20 mL of peripheral blood in a 5:1 ratio. This mixture was allowed to stand at room temperature for 40 mins and the overlying red cell-depleted layer removed. This layer was diluted 1:1 with normal saline (pH 7.4) and layered over 65% Percoll. This was centrifuged at 1500 *g* at room temperature for 25 mins; the resulting mononuclear cell-containing interface was removed, washed twice by centrifugation and resuspended in RPMI media (Life Technologies, Mississauga, Ontario) with 10% fetal calf serum (FCS). The more dense granulocyte layer was also recovered in some experiments.

Preparation of purified monocytes and lymphocytes: Purified monocyte and lymphocyte populations were obtained from five high dose ICS people with asthma (defined previously) and five age- and sex-matched nonatopic control patients using a combination of MACS and cell adherence. Briefly, PBMC populations were prepared as described above and incubated at a concentration of 1×10^7 cells/mL with anti-human CD14 magnetic micro beads (Miltenyi Biotec, Bergisch Gladbach, Germany) (20 μL/mL) for 40 mins at 4°C in supplemented RPMI media as described in the Patients and Methods section. The cells were then washed by centrifugation and applied to an AS type separation column (Miltenyi Biotec) inserted in the magnetic field of a MACS apparatus. The column was washed extensively with media to remove CD14-negative cells (lymphocyte population) before recovery of the CD14-positive cells (monocyte population) by washing the column with media outside the magnetic field. The lymphocyte population was further depleted of any contaminating monocytes by incubation at 37°C in supplemented RPMI at a concentration of 1×10^6 cells/ mL for 1 h in 24 well tissue culture plates, and the nonadherent population was recovered. Cell purity was assessed on Giemsa stained cytocentrifuge preparations. The isolated cell populations were placed in parallel in 24 h culture as described for unseparated PBMC populations, either unstimulated or in the presence of CD3 antibody (OKT3) (as described below), and the TNF-α content of the cell free supernatants were determined by ELISA.

Cell culture: Cells were adjusted to a concentration of 0.5 million cells/mL in RPMI media plus 10% FCS and 1% PenStrep for both PBMC and granulocyte preparations. Cells were cultured overnight at 37°C in a carbon dioxide incubator in media alone or with the addition of a variety of stimulators, including phytohemagglutinin (PHA) (Gibco-RBL, Mississauga, Ontario) 1 μg/mL, *Escherichia coli* strain lipopolysaccharide (LPS) (Sigma) 4 μg/mL, IL-1β (Immunex Corp, San Francisco, California) 5 ng/mL, anti-CD3 antibody (OKT3) (Zymed, San Francisco, California) 0.1 μg/mL and formyl-methionine-leucine-phenylalanine (Sigma) 10^{-6} M. After 24 h, the cell cultures were centrifuged for 10 mins at 150 *g* at room temperature. Supernatants were removed and assayed for TNF-α content. Pellets were resuspended in 100 μL of medium, and cytospins were prepared from both the granulocyte and PBMC compartments on APTEX coated slides (3-amino propyltriethoxysilane, Sigma). Slides were air dried and stained with Giemsa. Cell types were assessed by light microscopy. All reagents were confirmed to be endotoxin free (less than 0.06 EU/mL with the ETOXATE kit assay system, Sigma) before use in the culture system.

TNF-α ELISA: Ninety-six well microtitre ELISA plates (Maxi-Sorp, Nunclon, Rochester, New York) were coated for

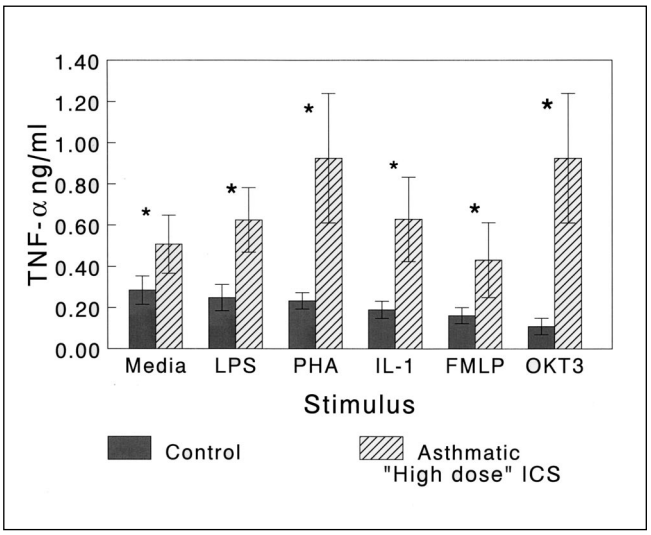

Figure 1) *Tumour necrosis factor-alpha (TNF-α) production by peripheral blood mononuclear cells (PBMC) from high dose inhaled corticosteroid (ICS) patients with asthma receiving greater than 800 μg/day of ICS therapy, and age- and sex-matched control patients. Cells were incubated for 24 h in supplemented RPMI media alone or with the addition of potential activating agents as described in the Patients and Methods section. Mean values are given ± SEM for eight patients and matched controls. Under all incubation conditions PBMC from patients with asthma demonstrated greater TNF-α production than controls (*P<0.05). Both phytohemagglutinin (PHA) and CD3 antibody (OKT3) induced a significant (P<0.05) increase in TNF-α expression compared with media-incubated cells from the same subjects in the patients with asthma group only. FMLP Formyl-methionine-leucine-phenylalanine; IL-1 Interleukin-1; LPS* Escherischia coli *strain lipopolysaccharide*

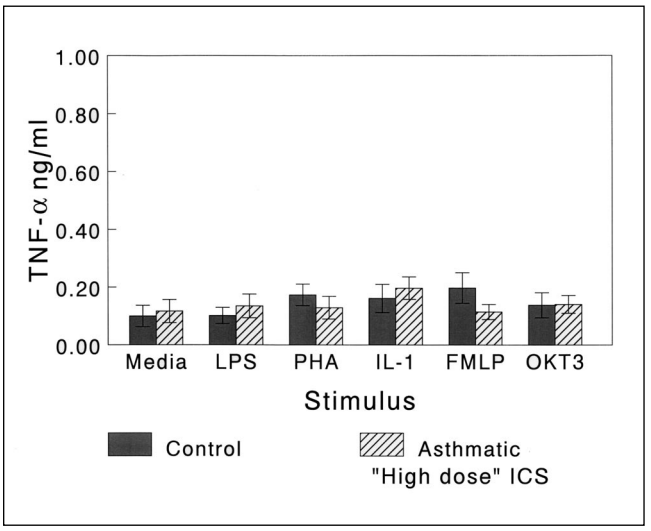

Figure 2) *Tumour necrosis factor-alpha (TNF-α) production by granulocyte preparations from high dose inhaled corticosteroid (ICS) patients with asthma, and age- and sex-matched control patients. Granulocytes were cultured for 24 h in supplemented RPMI media alone or with the addition of potential activating agents as described in the Patients and Methods section. Mean ± SEM are shown for eight subjects and controls. None of the activating agents used induced significant TNF-α expression in either group. There were no significant differences in TNF-α production between patients with asthma and control subjects. FMLP Formyl-methionine-leucine-phenylalanine; IL-1 Interleukin-1; LPS* Escherischia coli *strain lipopolysaccharide; OKT3 CD3 antibody; PHA Phytohemagglutinin*

18 to 20 h at 4°C with a 1 μg/mL solution of mouse monoclonal antihuman TNF-α antibody (a gift from Genentech, San Francisco, California), 100 μL/well, in 0.2 M borate buffer pH 9.6. The plates were washed three times in phosphate buffered saline (PBS), pH 7.4. Nonspecific binding was blocked with 100 μL/well of PBS plus 1% bovine serum albumin (BSA) for 2h at 37°C. Plates were again washed in PBS. Cell culture supernatants were diluted 1:5 and then 50 μL/well plated and incubated overnight at 4°C. This was carried out in duplicate. After washing, 50 μL/well of biotinylated antihuman TNF-α, 0.5 μg/mL dilution was added. This was incubated for 4 h at 37°C, the plates washed and biotin label detected with 100 μL of alkaline phosphatase labelled streptavidin (Zymed). This was incubated for 1 h and then the colour developed with P-nitro phenol 0.1 M diethanolamine pH 9.6. Optical densities were read on an ELISA plate reader (Dynamex Technologies, Burlington, Ontario) at 405 nm. All dilutions were made in PBS plus 1% weight/volume BSA. This assay had a sensitivity of 1 pg/mL and did not detect other human cytokines including IFN-γ, IL-2, IL-4 and lymphotoxin.

Pilot studies using a combination of this ELISA methodology and culture system have shown that optimal differences between patients are detected after 24 h in culture and that the system has a good level of reproducibility. An inter-assay variation of less than 10% was observed when six people with asthma taking high dose ICS were tested on two occasions, separated by an interval of four months (data not shown).

Statistical analysis: In view of the data distribution, comparisons among matched patients and controls and evaluation of responses to stimulators within groups were performed using the paired nonparametric Wilcoxon signed rank test.

RESULTS

Patient characterization: Subject characteristics of all the patient groups are shown in Table 1. All patients had normal complete blood count and differential (not shown).

Cytokine production in patient groups

High dose ICS patients with asthma: TNF-α production by PBMC from high dose ICS people with asthma is shown in Figure 1. PBMC from high dose ICS people with asthma produced significantly more TNF-α than cells from matched, nonasthmatic control patients when cultured alone or in the presence of each of the stimuli (Wilcoxon signed rank test P<0.01, n=8, see Figure 1). OKT3 and PHA were the most effective inducers of TNF-α production. Peak values approached approximately 0.9 ng TNF-α/mL. The numbers of monocytes and lymphocytes observed in cell preparations from people with asthma and control subjects were not significantly different (data not shown). In six of eight control patients and all patients with asthma, OKT3 or PHA activation induced an increase in TNF-α production.

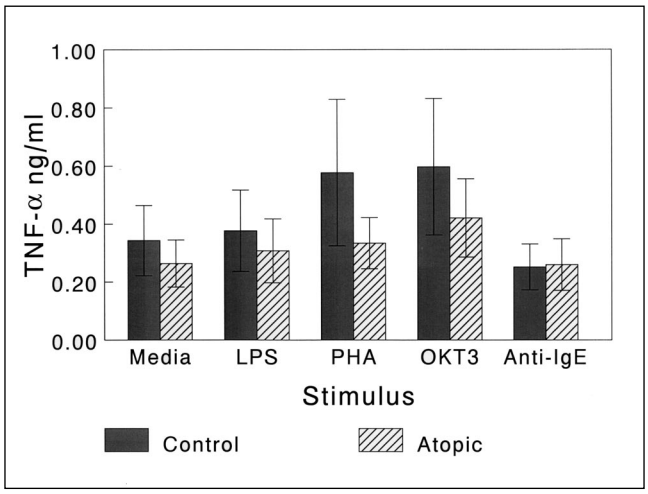

Figure 3) *Tumour necrosis factor-alpha (TNF-α) production by peripheral blood mononuclear cells (PBMC) from atopic nonasthmatic subjects. PBMC were cultured for 24 h as described in the Patients and Methods section with supplemented RPMI media alone or with the addition of activating agents. Mean values ± SEM for eight subjects per group are shown. None of the activating agents used induced significant expression of TNF-α over that produced in cultures with media alone. There were no significant differences in TNF-α production observed between PBMC from atopics and control patients. IgE Immunoglobulin E; LPS* Escherischia coli *strain lipopolysaccharide; OKT3 CD3 antibody; PHA Phytohemagglutinin*

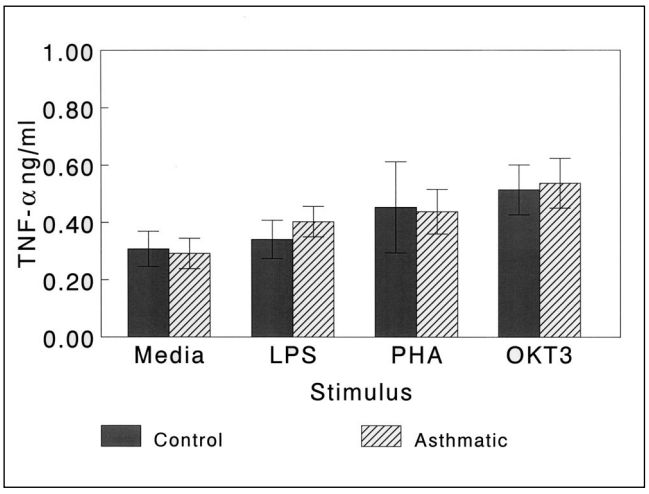

Figure 5) *Tumour necrosis factor-alpha (TNF-α) production by peripheral blood mononuclear cells (PBMC) from medium dose inhaled corticosteroid (ICS) patients with asthma receiving less than or equal to 500 μg/day ICS therapy and controls. PBMC were cultured for 24 h as described in the Patients and Methods section with supplemented RPMI media alone or with the addition of activating agents. Mean values ± SEM are shown for 11 subjects per group. There were no significant differences between patients with asthma and control subjects under any of the culture conditions. Both control and medium dose ICS subjects demonstrated significant (P<0.05) increases in TNF-α production in response to CD3 antibody (OKT3) stimulation (Wilcoxon signed rank test). LPS* Escherischia coli *strain lipopolysaccharide; PHA Phytohemagglutinin*

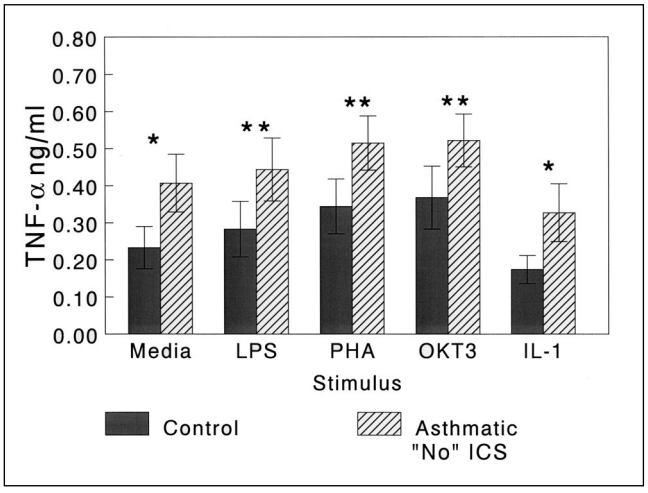

Figure 4) *Tumour necrosis factor-alpha (TNF-α) production by peripheral blood mononuclear cells (PBMC) from patients with asthma receiving no inhaled corticosteroid (ICS) therapy. PBMC were cultured for 24 h as described in the Patients and Methods section with supplemented RPMI media alone or with the addition of activating agents. Mean values ± SEM are shown for 10 patients per group. The production of TNF-α was significantly enhanced in the patients with asthma group compared with the matched control group following incubation with media alone (*P<0.05) or following activation with each of the agents (**P<0.02). CD3 antibody (OKT3) or phytohaemagglutinin (PHA) activation induced a significant (P<0.05) increase in TNF-α production compared with media in subjects with asthma (increased in nine of 10 subjects, Wilcoxon signed rank analysis). Eight of 10 control subjects showed increased TNF-α production in response to OKT3 or PHA stimulation. IL-1 Interleukin-1; LPS* Escherischia coli *strain lipopolysaccharide*

Granulocyte preparations from high dose ICS people with asthma and controls were examined (Figure 2). Only very low levels of TNF-α expression were observed in the granulocyte preparations. The granulocyte preparations produced a similar amount of TNF-α under each culture condition. There was no significant increase in the amount of TNF-α produced in response to any of the stimuli (n=8) (mean ± SEM). No differences were observed in granulocyte TNF-α production between people with asthma and control subjects.

Atopic nonasthmatics: To determine if the increased TNF-α production by PBMC observed in high dose ICS people with asthma was a function of atopic status, atopic nonasthmatic subjects (n=8) were examined (Figure 3). PBMC from atopic and control patients produced a similar amount of TNF-α as measured by ELISA either in unstimulated cultures or following activation of the PBMC populations with a range of agents having selectivity for different cellular targets.

No ICS patients with asthma: Because the high dose ICS patients with asthma were all receiving ICS therapy, which may potentially alter PBMC TNF-α responses, a group of patients with mild asthma not receiving ICS were also identified for study (Table 1). PBMC from these patients with asthma (no ICS) also produced significantly more TNF-α than their matched controls under baseline conditions and in response to each stimulus (Figure 4). (Wilcoxon signed rank test: P<0.05, n=10, see Figure 4). PHA and OKT3 were the most potent inducers of TNF-α. Peak levels of TNF-α were

approximately 0.9 ng/mL. The numbers of lymphocytes and monocytes observed in cultured cell preparations were not significantly different between the patients with mild asthma and control groups (data not shown).

Medium dose ICS patients with asthma: TNF-α production in medium dose ICS patients with asthma who were on 500 µg ICS/day or less (n=11) was similar to control individuals (Figure 5). However, a significant increase in TNF-α production was observed in both groups after stimulation with OKT3 and PDBU plus A23187.

TNF-α production by MACS-purified monocytes and lymphocytes: The mean purity obtained for monocytes was 90% and for lymphocytes 89% after MACS purification. Purified preparations of both monocytes and lymphocytes from high dose ICS patients with asthma (n=8) were noted to produce some TNF-α, either in media alone or following OKT3 stimulation. However, in contrast to unfractionated PBMC, there was no difference observed between high dose ICS patients with asthma and controls in each of the purified cell types (Figure 6). Peak values of separated cells approached 0.3 ng TNF-α/mL. To exclude the possibility that cell separation procedures had lead to inhibited TNF-α responses, the purified cell subsets were recombined in separate experiments and their TNF-α production compared with that of the starting (mixed) population. TNF-α production by recombined cell fractions from no ICS patients ranged between 90% and 94% of that of the starting cell populations examined in parallel (mean 0.67 ng/mL unfractionated, 0.63 ng/mL recombined fractions). Isolated PBMC from control subjects showed very similar levels of TNF-α production in either their unfractionated or fractionated cell populations.

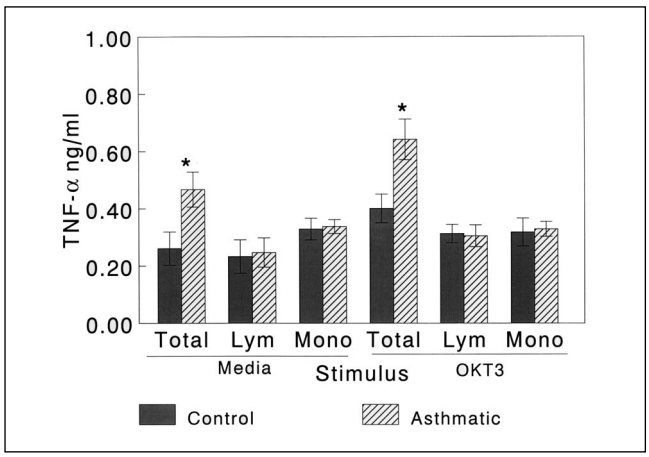

Figure 6) *Tumour necrosis factor-alpha (TNF-α) production over 24 h in culture by fractionated cells from high dose inhaled corticosteroid patients with asthma and matched control subjects. Unfractionated cells (total) were cultured in supplemented RPMI media alone or with the further addition of CD3 antibody (OKT3) in parallel with purified lymphocyte (Lym) and monocyte (Mono) preparations. Mean values of five subjects and controls are given ± SEM. The total numbers of cells cultured per group are equivalent. Only the unfractionated (total) cell populations exhibited a significant difference in TNF-α expression between patients with asthma and control subjects (*P<0.05). OKT3 activation also induced an increase in TNF-α production by unfractionated (total) cells in all subjects but not separated cell preparations. The mean purity of lymphocyte preparations was 90%; mean purity of monocyte preparations was 89%. IgE immunoglobulin E; LPS* Escherischia coli *strain Lipopolysaccharide; PHA Phytohemagglutinin*

DISCUSSION

Cytokines are important in orchestrating the influx of inflammatory cells that characterize the airways of patients with asthma and in the activities of these cells within the tissues. This inflammatory infiltrate is composed mainly of eosinophils and lymphocytes. TNF-α is a potent cytokine, with widespread proinflammatory activities that may potentiate allergic inflammation in vivo. TNF-α has been shown to increase airway hyperresponsiveness when given as an aerosol to humans.

Kips et al (9,39) have speculated on the role of TNF-α in the pathogenesis of asthma. Inhalation of allergen induces mast cell degranulation and release of bronchoconstrictors such as histamine, prostaglandin D2 and leukotriene C4, causing an immediate asthmatic response. TNF-α is also released from antigen-stimulated mast cells and other potential cell sources, such as monocytes or lymphocytes. It increases BHR by increasing the release of inflammatory mediators such as arachidonic acid metabolites or by direct effect on smooth muscle cells. Also, TNF-α increases the expression of endothelial adhesion molecules over the next 4 to 6 h, enhancing the influx of inflammatory cells into the airway mucosa. Furthermore, TNF-α can activate various inflammatory cells including eosinophils and lymphocytes, which can lead to the release of mediators that cause bronchoconstriction. Thus a single mediator, TNF-α, could induce an increase in BHR and the influx of inflammatory cells, such as that seen in the LAR.

The present study has shown that PBMC cultures of certain people with asthma, both high dose ICS-treated patients, or those with mild disease not requiring steroid therapy, will produce significantly more TNF-α than age- and sex-matched nonasthmatic controls. This will occur both when cells are cultured without activating agents and in the presence of a variety of cell stimulators. Interestingly, stimulators that are T cell-specific, such as OKT3, or are relatively more stimulatory for T cells, such as PHA, induced the greatest production of TNF-α in culture supernatants. Monocytes and macrophages are thought to be one of the primary producers of TNF-α; however, in our system, LPS, which activates monocytes effectively in other systems, was not as potent an inducer of TNF-α, suggesting that lymphocytes may be more important contributors or inducers of the TNF-α response in PBMC preparations from this group of patients. Thus, enhanced TNF-α production can occur systemically in the PBMC in asthma, regardless of disease severity, and is not a result of steroid therapy.

Because most of the people with asthma were also atopic, atopic nonasthmatics were studied in comparison with controls to see if the observed elevation of TNF-α production

was a function of atopy. Results indicate that there is no significant difference in the TNF-α production by PBMC of this group compared with nonatopic, nonasthmatic matched control patients. The increased TNF-α observed in the people with asthma is therefore not a function of atopy and could be a 'disease-specific' finding. An examination of a clinically well controlled group of medium dose ICS people with asthma, however, showed that not all groups of people with asthma demonstrated substantial TNF-α dysregulation. In this case, neither unstimulated cultures nor cultures stimulated with a range of agents showed any difference in TNF-α production from matched control subjects examined in parallel. We postulate that this could be a reflection of the relative severity of their disease at the time of sampling, compared with the high dose group; although the ranges of FEV_1 values are similar in the two groups, the lesser requirement for steroidal and nonsteroidal medications to control clinical symptoms in the medium dose group is a sign of relative disease quiescence. The ICS treatment itself may be related to the difference in behaviour between medium and no dose ICS groups. Indeed, it is interesting to speculate that a group matching the clinical parameters for the no ICS patients with asthma but subjected to a course of ICS treatment might yield cells that do not exhibit the upregulated TNF-α production.

It was important to try to isolate the cellular source(s) of the observed increased TNF-α production in people with asthma. To clarify this further, MACS analysis was performed on high dose ICS patients with asthma to prepare enriched populations of lymphocytes and monocytes, and the individual populations were set up in identical culture conditions as the PBMC. Initial culture results have suggested an important role for lymphocytes, because stimulation by the T cell-specific stimulator OKT3 induced the greatest increase in TNF-α production. However, this may be a direct or indirect effect of lymphocyte activation because these cells can produce a number of factors known to induce TNF-α production by monocytes, such as interferon-gamma. TNF-α production by granulocytes of patients with asthma in culture was very low and not significantly different from that of controls. Examination of purified preparations of monocytes and lymphocytes revealed that both cell types contributed to TNF-α production; however, the previously observed difference between people with asthma and controls was no longer seen when cell types were cultured separately. This suggested that some degree of cell interaction may be important for TNF-α production. While it is possible that the cell separation procedures interfered with the ability of isolated subsets to produce TNF-α, the role for interaction between the cell types is supported by the fact that recombination of the subsets resulted in restoration of TNF-α production to levels almost identical to those from the unseparated cell population (92% to 94%). It should also be noted that in control subjects, both isolated cell populations and mixed populations produced almost identical levels of TNF-α.

CONCLUSIONS

The present study demonstrates that substantial change in TNF-α regulation within both mild and severe patients with asthma can be detected in all of the peripheral blood, but no such dysregulation is observed in well controlled, medium dose ICS patients with asthma. The precise mechanisms for these alterations in TNF-α regulation remain to be determined but probably involve the participation of more than one cell type.

ACKNOWLEDGEMENTS: We acknowledge the generous support of ASTRA-Pharma (Canada), the Medical Research Council of Canada and Inspiraplex-Respiratory Health Network of Centres of Excellence. Thanks also to Jolanta Bryk, Ula Kadela-Stolarz and Anna Zganiac for their expert technical assistance and to Paulette Malcolm for her secretarial expertise.

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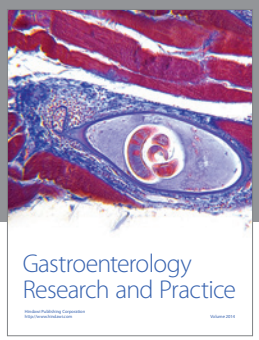

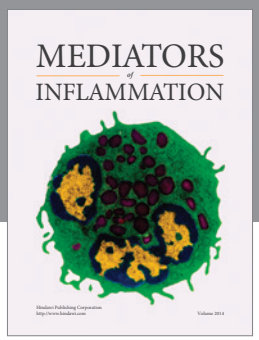

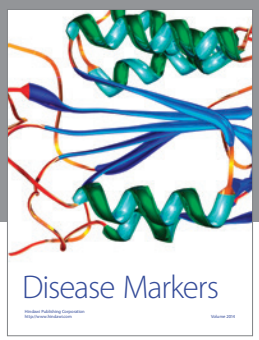

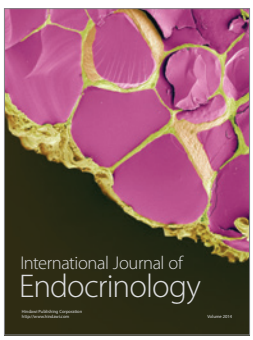

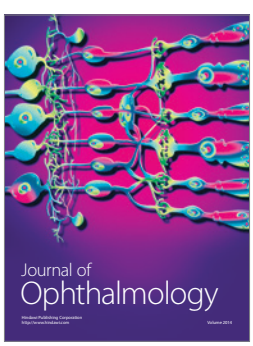

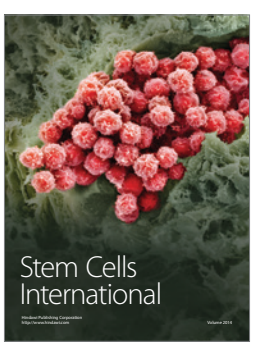

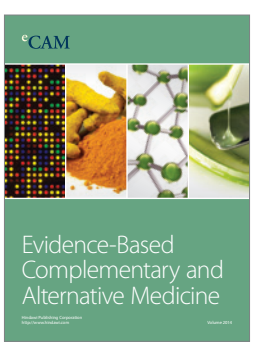

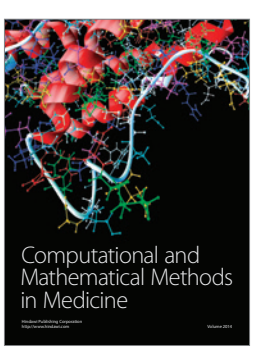

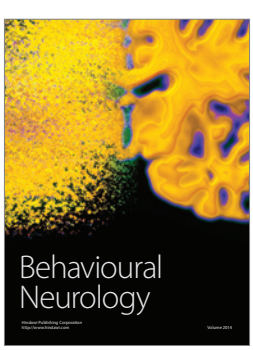

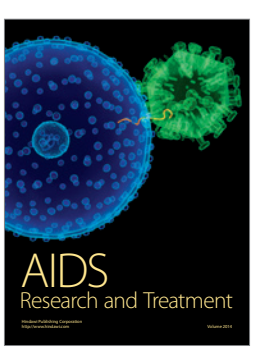

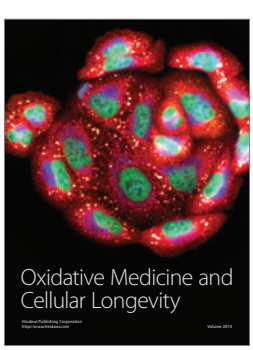

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