

ORIGINAL PAPER

Changes in immune regulation in response to examination stress in atopic and healthy individuals

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Clinical and Experimental Allergy

Summary

Background Stress can aggravate the allergic inflammation, but determinants of disturbed immune regulation are largely unknown.

Objective To determine systemic immunological, local inflammatory and functional airway responses to stress in healthy and atopic individuals.

Methods Forty-one undergraduate students, 22 with allergy of whom 16 had asthma, and 19 healthy controls, were studied in a low-stress period and in association with a large exam. Subjects completed questionnaires on stress and health behaviours, underwent lung function tests, bronchial methacholine challenge, measurements of exhaled nitric oxide and urine cortisol. Blood cells were phenotyped, and cytokines from mononuclear blood cells were analysed.

Results Perceived stress and anxiety increased in both groups during the exam period while cortisol increased only in the atopy group. Cytokine production decreased broadly in response to stress in both groups, which was paralleled by an increase in the proportion of regulatory T cells (CD4⁺CD45RO⁺CD25^{bright}). Interestingly, atopic individuals, but not controls, reacted with a decreased T-helper type 1/T-helper type 2 (Th1/Th2) ratio and a decrease in natural killer (NK) cell numbers in response to stress. In control subjects only, exhaled nitric oxide decreased and forced expiratory volume in one second increased during stress.

Conclusion Atopic and non-atopic subjects shared some immune changes in response to stress, such as a dramatic decline in cytokines and an increase in the number of regulatory T cells in peripheral blood. However, other stress-induced immune changes were unique to atopic individuals, such as a skewed Th1/Th2 ratio and reduced NK cell numbers, indicating that some pathogenic mechanisms in atopics may be more strongly affected by stress than others.

Keywords allergy, cytokines, inflammation, regulatory T cells, NK cells

Submitted 20 October 2005; revised 5 February 2006; accepted 6 April 2006

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Introduction

Atopic disease is characterized by a dominance of T-helper type 2 (Th2) cytokines (e.g., IL-4 and IL-5) over T-helper type 1 (Th1) cytokines (e.g., IFN- γ and IL-2). The dominance of Th2 cytokines leads to enhanced Ig class switching to IgE, recruitment of eosinophils to the airways and to down-regulation of cell-mediated immunity, features characteristic of asthmatic and allergic disease [1]. Several mechanisms have been accounted for in the explanation for the shift in Th1/Th2 balance in atopy,

such as genetic predisposition [2], disturbed natural killer (NK) cell function [3], levels of glucocorticoids [4, 5] and deficient function of regulatory T cells [6, 7]. The complexity of diseases such as asthma implies the involvement of several of these factors in parallel. In addition, asthma and allergy have strong psychosocial components. Of particular interest is the possible influence of stress on manifestations of atopic diseases [8–11]. Importantly, stress may induce a drive towards Th2 in the balance between Th1 and Th2 cytokines [4, 5, 12, 13]. In response to stress, health behaviours that can be associated with

immune physiology, such as sleep, can also change [14, 15]. For example, sleep difficulties have been associated with lower Th1/Th2 ratios [16, 17]. Other health behaviours, such as exercise, smoking, caffeine intake or alcohol, may also influence leucocyte count, cytokine balance or markers of inflammation, such as fraction of exhaled nitric oxide (NO) [18–20].

In order to understand how complex diseases, such as asthma and allergy, are influenced by stress and anxiety, interdisciplinary approaches are warranted. Here, we use psychological, physiological and immunological techniques to investigate the relationship between stress, airway obstruction and immune parameters in medical students with or without atopy in response to a stressful examination. We hypothesized that stress associated with a large academic examination would be accompanied by a decrease in the Th1/Th2 balance and that this effect would be larger in atopic subjects. In addition, we aimed to assess the effects of stress on variables associated either with regulation of the allergic inflammation, e.g. NK cells and regulatory T cells, or with outcomes associated with allergic aggravation, such as exhaled NO and lung function, that have been little or not at all explored in earlier stress-allergy studies. Exhaled NO was included as the levels of exhaled NO are greater in subjects with atopic asthma and decrease after steroid therapy, suggesting that NO is a valuable marker of airway inflammation [21]. In addition, because changed health behaviours may have influenced changes in immune parameters in earlier studies, we wanted to include behavioural measures of relevance for allergy-related immune measures. Our data demonstrated that academic stress was associated with an aggravated Th2 dominance in atopic individuals but not in controls. This was accompanied by a significant decrease in NK cells in atopics but not control subjects. Furthermore, we noted, for the first time, an increase in absolute numbers and percentages of regulatory T cells in response to stress. Our interdisciplinary study suggests novel angles from which to study the complex aetiology of atopic disease.

Materials and methods

Subjects

Forty-one undergraduate medical students at Karolinska Institutet, 14 females and eight males (21–31 years, median age 25 years) with verified allergy (see Table 1), and 10 females and nine males (21–27 years, median age 24 years) included as control subjects, were studied. All students but one were Caucasians. The non-Caucasian subject was Asian. The distribution of gender between groups did not differ significantly ($\chi^2 = 1.19$; NS). The weight status, calculated as body mass index, of the included subjects showed that all but five were within the normal weight limits (18.5–24.9). Of the five subjects

outside the normal range, four subjects were underweight (17.9, 18.3, 18.3, 18.4) and one subject was overweight (26.3). None of the included subjects was classified as obese. Body mass index did not differ significantly between controls (22.6 (20.6–23.4)) and atopics (21.0 (19.6–23.0)). All students were subjected to screening for total serum IgE with Phadiatop (Pharmacia Diagnostics, Uppsala, Sweden), and when positive quantitative analysis of specific serum IgE was performed by RAST. Atopic subjects (all with rhinitis, 16 of them also with asthma (21–31 years, median age 26 years)) [22, 23] had a history of allergic symptoms and a positive RAST. Selection criteria for asthma included a well-documented history of asthma and bronchial hyper-reactivity with previous use of asthma medication. Students with rhinitis had a history of rhinoconjunctivitis with previous use of antihistamine or local steroids. Control subjects had no history of allergy and a negative Phadiatop. Subjects were not regular nicotine users and the individuals (three controls and five atopics, of which three were asthmatics) who declared that they smoked occasionally had a consumption of a few cigarettes each month at the most. Only 2 of the atopic subjects were using corticoids on a regular basis, and another 6 during the pollen season, before inclusion in the study. No subjects included in the study used any systemic or local corticoids during the study. All such medication was withheld at least 4 weeks before the start of the study. All 41 subjects completed the protocol. Three cases, one control and one with allergy at the low-stress phase, and one with allergy at the stress phase, were excluded due to respiratory infection. The local ethical committee approved the study, and all subjects gave their written informed consent.

Study design

Screening evaluations included medical history, physical examination, Phadiatop, RAST and baseline spirometry. The subjects were then evaluated on two separate occasions: a low-stress evaluation and a stress phase evaluation. The low-stress evaluation was conducted in mid-semester during a period of studies where no oral or written exams were undertaken: neither 2 weeks before nor 2 weeks following the evaluation. The stress phase evaluation was conducted at a day between a major oral and a major written exam that was held during a week of major final exams. Participants were studied at the same time of the day on both occasions to avoid chronobiological variation. The study was conducted out of the pollen season and none of the allergic subjects was exposed to animals in their home environment.

Participants completed a diary each morning and evening for 7 days preceding each evaluation, concerning sleep, exercise, intake of coffee and alcohol and peak expiratory flow (PEF).

Table 1. Descriptive data of atopic subjects

Age (years)	Gender	History of allergy symptoms	S-IgE (kU/L)	Atopy (RAST) class (scale: 1–6)	VC (% pred)	FEV ₁ (% pred)	FEV ₁ /FVC	PEF (% pred)
24	M	R/C	120	Dust mite: 1, timothy: 4	100	105	0.87	84
24	F	R/C	180	Timothy: 4, birch: 1	105	105	0.87	104
24	M	R/C	23	Cat: 3, horse: 1, dog: 2	114	108	0.76	99
28	F	R/C	53	Cat: 1, horse: 2, dog: 2, birch: 3, timothy: 2, mugwort: 3	104	95	0.80	93
21	F	R/C	120	Timothy: 2	88	98	0.98	84
25	F	R/C	62	Cat: 1, birch: 1, timothy: 1	107	111	0.93	127
31	F	A, R/C	400	Timothy: 4, birch: 5, mugwort: 3	117	122	0.91	89
31	F	A, R/C	110	Cat: 3, horse: 2, dog: 2, birch: 2, mugwort: 3	130	109	0.75	84
27	M	A, R/C	130	Cat: 1, timothy: 2, birch: 5	93	90	0.79	104
25	M	A, R/C	140	Cat: 3, horse: 2, dog: 2, timothy: 3, birch: 4	NA	94	0.65	85
21	F	A, R/C	64	Horse: 3, birch: 3	112	118	0.92	116
28	F	A, R/C	71	Cat: 2, dog: 2, horse: 2, birch: 4, timothy: 3, dust mite: 3	97	94	0.84	81
25	F	A, R/C	330	Cat: 4, dog: 3, horse: 3, birch: 4, timothy: 4, mugwort: 2, cladosp.: 2	111	114	0.94	95
24	M	A, R/C	170	Cat: 3, dog: 2, birch: 3, mugwort: 1	111	127	0.91	120
22	F	A, R/C	180	Cat: 3, horse: 1, dog: 2, mugwort: 3	120	103	0.75	88
24	M	A, R/C	87	Dust mite: 3, cat: 1, horse: 1, timothy: 3, birch: 2	100	83	0.74	75
27	F	A, R/C	65	Cat: 2, horse: 1, dog: 2, timothy: 4, birch: 2	106	87	0.72	111
29	M	A, R/C	58	Dust mite: 4, cat: 2, horse: 3, dog: 2	146	157	0.85	141
28	F	A, R/C	880	Dust mite: 2, cat: 3, horse: 3, dog: 3, timothy: 5, birch: 4, mugwort: 2, cladosp.: 3	136	124	0.80	120
30	M	A, R/C	86	Cat: 2, horse: 3, dog: 2, birch: 3	128	127	0.78	106
21	F	A, R/C	440	Dust mite: 2, cat: 2, horse: 3, Dog: 2, timothy: 2, birch: 6, mugwort: 2	86	85	0.86	75
21	F	A, R/C	97	Dust mite: 2, cat: 3, horse: 2, dog: 2, timothy: 3, birch: 5, mugwort: 3	111	108	0.85	100
Median					111	107	0.85	99
25–75th percentile					100–119	94.5–120	0.77–0.91	84–114

M, male; F, female; R/C, rhinoconjunctivitis; A, asthma; S-IgE, serum immunoglobulin E; Cladosp, cladosporium herbarum; VC, vital capacity; pred, predicted; NA, not available; FEV₁, forced expiratory volume in 1 s; FVC, forced vital capacity; PEF, peak expiratory volume.

At both evaluations, current levels of stress and anxiety were measured with a visual analogue scale (VAS) [24] and with the state version of the State-Trait Anxiety Inventory (STAI-S) [25]. Blood was also sampled for blood cell and cytokine analyses (for details, see below). NO in oral single-breath exhalations was determined using an NIOX[®] (Aerocrine AB, Solna, Sweden) using standardized procedures [26]. Forced expiratory volume in 1 s (FEV₁), PEF, vital capacity (VC), forced vital capacity (FVC) and methacholine bronchial challenges were performed using standardized procedures (see below for details). Urine was collected from 10 PM the evening before until the first morning urine on both assessment days, and cortisol concentrations were determined by standard laboratory methods (Auto Delfia Cortisol Kit, Wallac OY, PerkinElmer, Wellesley, MA, USA, with a detection range between 5 and 1600 nmol/L).

Blood analysis

Complete blood count with leucocyte differentials was performed at Karolinska University Laboratory, Solna,

Sweden. Whole blood cells were further characterized using direct-labelled monoclonal mouse antibodies (mAb) as previously described [27]. Anti-CD3, -CD4 and -CD8 mAb (Dakopatts, Glostrup, Denmark) were used to detect T cell subsets. Anti-CD3, -CD56/16 and -CD45 mAb (BD Biosciences, Franklin Lakes, NJ, USA) were used to delineate NK cells. For recognition of regulatory T cells, defined as CD4⁺CD45RO⁺CD25^{bright} cells, anti-CD25, -CD45RO (BD Biosciences) and -CD4 (Dakopatts) were used. Anti-human leucocyte antigen-DR (HLA-DR, BD Biosciences) and anti-CD4 (Dakopatts) mAb were used to identify activated CD4⁺ T cells. Cells were analysed by flow cytometry using a FACS-Calibur flow cytometer (BD, San Jose, CA, USA).

Preparation and stimulation of peripheral blood mononuclear cells

Peripheral blood were obtained from venous puncture and collected in a heparinized vacutainer via routine procedures. Whole blood was diluted 1 : 1 in PBS and peripheral

blood mononuclear cells (PBMC) were separated by gradient centrifugation using Ficoll-Paque PLUS (Pharmacia Biosciences AB, Uppsala, Sweden) at $900 \times g$ for 20 min at room temperature. 1×10^6 PBMC in 500 μ L RPMI 1640, supplemented with 100 U/mL penicillin, 100 μ g/mL streptomycin (penicillin–streptomycin), 2 mM L-glutamine (all Sigma-Aldrich, St Louis, MO, USA) and 10% FBS (Invitrogen, Rockville, MD, USA), were cultured in 24-well flat bottom plates (BD Falcon™, Franklin Lakes, NJ, USA). Cells were cultured for 24 h at 37 °C with 5% CO₂ in the presence of 5 μ g/mL phytohaemagglutinin (PHA) and 10 ng/mL phorbol myristate acetate (PMA, both Sigma-Aldrich) as previously described [11, 28]. Cell culture supernatants were then collected, centrifuged at $400 \times g$ for 10 min at 4 °C and stored in aliquots at –70 °C until analysis.

Enzyme-linked immunosorbent assay

Protein levels of IL-2, IFN- γ , IL-4 and IL-5 in supernatants from peripheral blood mononuclear cells were determined by ELISA (OptEIA™ Human sets, Pharmingen, BD Biosciences, San Jose, CA, USA).

Lung function tests

FEV₁, PEF rate, VC and FVC were determined using the MasterScope direct reading spirometer (Erich Jaeger GmbH, Hoechberg, Germany), software version 2.53.2. Percentage predicted FEV₁ was calculated as measured FEV₁/predicted FEV₁, and percentage predicted PEF was calculated as measured PEF/predicted PEF.

At the screening visit, PEF meters (MiniWright Standard Peak Flow Meter, Clement Clarke Int. Ltd., Essex, UK) were administered to determine PEF rate variability in the diary from 1 week before, and up until, both evaluations. All subjects were trained on how to use the flow meter correctly and instructed to perform three peak flows in the morning and in the evening, before taking asthma medication, and to record the highest value of the three measurements each morning and evening in the diary. Two different PEF indices were calculated from the diaries according to the recommendations in Global Initiative for Asthma (GINA) [22]; diurnal PEF variability (calculated from the daily amplitude of PEF (morning–evening) expressed as a % of the mean daily PEF value averaged over 7 days) and minimum morning PEF, % of recent best (calculated as the lowest daily morning PEF, expressed as % of recent best during the 7-day period).

Bronchial provocation and calculation of log PD₂₀

A methacholine bronchial challenge test was performed using a dosimeter-controlled jet nebulizer Spira Electro 2 (Respiratory Care Center, Hämeenlinna, Finland). After recording the baseline FEV₁, the subject first inhaled saline followed by a methacholine solution. With the nose

clipped, methacholine was inhaled for a specified number of breaths, in concentrations from 14.2 to a maximum cumulative dose of 7256 μ g, with dose increments around every third minute. FEV₁ was measured 2.5 min after each dose of methacholine and the test stopped when FEV₁ had decreased by at least 20% from its post-saline value. The logarithmic methacholine doses were plotted against the percentage of the post-saline FEV₁. PD₂₀ values were calculated from the cumulative dose–response curves by linear interpolation and expressed as log values. After the onset of a positive response, the patient was offered a β_2 -agonist inhalation.

Statistical analysis

Values are presented as medians and 25–75th percentiles in parentheses. Wilcoxon's signed-rank test, or a paired *t*-test for normally distributed data, were used for within-subject comparisons. Mann–Whitney *U*-test, or an unpaired *t*-test for normally distributed data, was used for comparisons between subjects. Levels of cytokines were logarithmically transformed to normalize distributions. A *P* value of less than 0.05 was considered significant. Gender distribution between the atopy and the control group was tested by a χ^2 test. Statistical analyses were performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA, USA).

Results

Health behaviours

Measures of health behaviours did not reveal any general differences between atopic and control subjects, with the exception of exercise frequency, which was higher in the control group compared with the atopy group (*P*=0.03, not shown). In addition, at the examination period, students of both groups decreased their amount of weekly exercise (*P*=0.02) and alcohol intake (*P*=0.001) compared with the low-stress period. A slight decrease was also observed in subjective sleep quality (*P*=0.01) during the examination period and a non-significant trend towards increased sleep time (*P*=0.07). Other measures of health behaviour, such as caffeine consumption, and whether participants felt that they had slept enough and felt refreshed after sleep, did not change in response to stress in any of the groups.

Perceived stress and urine cortisol in response to examination

To evaluate stress and anxiety in response to the examination period, students were evaluated using a VAS and the STAI-S [24, 25]. Both controls and atopic subjects displayed significantly higher VAS (Fig. 1a) and STAI-S (ctrl low stress: 36.5 (30.0–41.0) vs. ctrl stress: 41.0 (30.0–48.0),

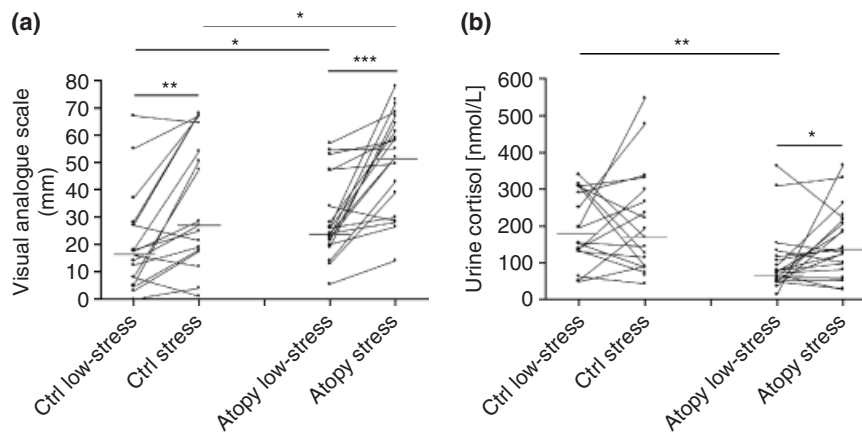


Fig. 1. Effect of examination stress on (a) perceived stress, as determined by visual analogue scale (VAS) and (b) urine cortisol. Medians are indicated in the graphs. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

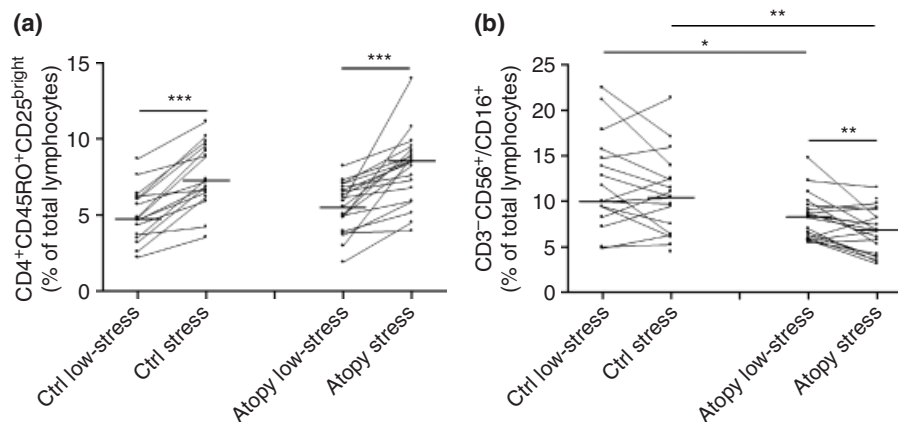


Fig. 2. Effect of examination stress on (a) CD4⁺CD45RO⁺CD25^{bright} (regulatory T cells) and (b) CD3⁻CD56⁺CD16⁺ (NK cells) in peripheral blood. Medians are indicated in the graphs. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

$P < 0.05$; atopy low stress: 37.0 (31.5–41.5) vs. atopy stress: 42.0 (39.0–53.0), $P < 0.01$) scores during the examination period, indicating increased levels of perceived stress and anxiety in both groups at this time compared with the calm study period. VAS scores were significantly higher in atopics compared with controls, indicating higher stress levels in the former group (Fig. 1a). Interestingly, a numerically larger increase in perceived stress in the atopy group was paralleled by a significant increase in urine cortisol in response to stress that was not observed in the control group (Fig. 1b). An intriguing observation was that the atopic students displayed significantly lower urine cortisol levels compared with controls, both during low- and high-stress periods (Fig. 1b).

Effects of examination stress on blood leucocytes and subsets of white blood cells

As expected, atopic subjects showed a tendency towards higher numbers of eosinophils in venous blood in com-

parison with controls (0.20 (0.05–0.30) 10^9 L^{-1} vs. 0.10 (0.00–0.20) 10^9 L^{-1} , $P = 0.07$). No changes in total numbers of monocytes, lymphocytes, neutrophils or eosinophils were seen in response to stress in either group (data not shown). The number of basophils in venous blood was below the detection limit.

The total fraction of CD3⁺ T cells was higher in atopic individuals compared with controls (Table 2). When CD4⁺ and CD8⁺ T cell subsets were analysed, the ratio between them (CD4⁺/CD8⁺) was shown to be significantly increased in atopics and with a tendency to be increased in controls in response to stress, resulting from a slight increase in the proportion of CD4⁺ cells and a concomitant slight reduction in the proportion of CD8⁺ cells (Table 2). The increase in CD4⁺ T cells could further be explained by an increase in CD4⁺CD45RO⁺CD25^{bright} cells, the proportion of which increased markedly during stress in both controls and atopics (Fig. 2a). CD4⁺CD45RO⁺CD25^{bright} cells have been described as a specific subset of T cells with regulatory properties [29]. However, CD25 is also an

Table 2. Effects of examination stress on markers of lymphocytes in peripheral blood

	Control		Atopics	
	Low stress	Stress	Low stress	Stress
CD3 ⁺	71.9 (66.1–75.7)	73.2 (66.9–76.8)	76.2 (71.7–79.8) [#]	75.2 (72.0–79.2) [†]
CD4 ⁺	42.5 (40.9–46.8)	45.0 (41.0–49.0) [‡]	44.4 (40.3–47.6)	45.0 (41.0–50.3) [*]
CD8 ⁺	20.8 (18.7–24.3)	20.4 (19.0–23.7) [‡]	24.0 (19.5–28.8)	22.6 (21.1–29.3) [†]
CD4 ⁺ /CD8 ⁺	2.08 (1.67–2.23)	2.13 (1.91–2.49) [‡]	1.91 (1.49–2.34)	2.08 (1.44–2.35) [*]
CD4 ⁺ HLA-DR ⁺	6.34 (4.87–7.40)	5.54 (3.52–7.22) [*]	6.50 (5.46–7.46)	6.94 (5.70–7.60) [†]

With the exception of the CD4⁺/CD8⁺ ratio, the figures denote the percentage of lymphocytes, as defined by flow cytometry through their light scatter properties, expressing the marker(s). Values are presented as medians and 25–75th percentiles in parentheses.

[#]*P* < 0.05 compared with control low-stress value,

^{*}*P* < 0.05 compared with low-stress value within the group,

[†]*P* < 0.1 compared with control stress value,

[‡]*P* < 0.1 compared with low-stress value within the group.

Table 3. Effects of examination stress on PHA/PMA-stimulated cytokine generation from PBMC from peripheral blood

	Controls		Atopics	
	Low stress	Stress	Low stress	Stress
IFN- γ	2270 (48.7–5473)	1472 (105–3542)	979 (220–3714)	550 (179–1936) [*]
IL-2	6584 (966–15 694)	4499 (583–13 255)	2655 (1460–6558)	1300 (328–3364) [†]
IL-4	13.6 (9.29–20.7)	13.0 (7.44–18.9) [*]	15.9 (10.7–20.7)	11.8 (7.74–23.8)
IL-5	97.7 (12.1–276)	105 (11.4–213)	184.0 (90.1–285)	96.9 (41.0–263) [*]

With the exception of cytokine ratios, the figures denote the level of cytokines in pg $\times$ mL⁻¹. To ease comparison, values are presented as medians and 25–75th percentiles in parentheses rather than log values.

^{*}*P* < 0.05 compared with low-stress value within the group,

[†]*P* < 0.01 compared with low-stress values within the group.

PHA, photohaemagglutinin; PMA, Phorbol myristate acetate; PBMC, peripheral blood mononuclear cells; IFN, interferon; IL, interleukin.

activation marker for primed conventional T cells. To exclude that the increase of CD25^{bright} cells reflected a general immune activation in response to stress, we asked whether T cells expressing other activation markers would be increased. However, in contrast to CD4⁺CD45RO⁺CD25^{bright} T cells, the proportion of CD4⁺HLA-DR⁺ cells did not increase during stress in any of the groups (Table 2). In control subjects, those cells even decreased. Thus, the increase in CD4⁺CD45RO⁺CD25^{bright} T cells most likely results from an expansion of regulatory T cells in response to stress.

NK cells have been suggested to play a role in atopy and asthma, not the least due to their capacity to secrete immunomodulatory cytokines such as IFN- γ [30]. The proportion of NK cells (CD3⁺CD56⁺CD16⁺) was significantly lower in atopics compared with controls at the low-stress phase (Fig. 2b). Interestingly, the proportion of NK cells decreased in atopics, but not in controls, during the stress phase (Fig. 2b), suggesting a disturbed control of NK cell homeostasis in atopic individuals.

Changes in percentages of cells were in general paralleled by similar changes in absolute cell numbers (data not shown). However, CD3⁺ T cells were an exception as the numbers of CD3⁺ T cells were similar in atopics and in

controls. The higher percentage of T cells in the group of atopic individuals as measured by flow cytometry therefore likely results from concomitant changes in other cell types within the lymphocyte gate, such as NK cells (Table 2).

Effects of examination stress on cytokine production from peripheral blood mononuclear cells

In the majority of samples, spontaneous secretion of cytokines from unstimulated PBMC was below the detection limit. However, when cytokine levels were measured after PHA/PMA stimulation, significant differences were noted (Table 3). For example, the levels of IFN- γ , IL-2 and IL-5 were significantly reduced in atopics during stress, reductions that were similarly observed but not statistically verified in the control group. In contrast, IL-4 was significantly reduced in response to stress in controls only (Table 3).

Because of a proposed dominant role of Th2 cytokines in the pathogenesis of atopy and allergic asthma [1], we examined the ratio of Th1 and Th2 cytokines in the two groups in response to stress. In the low-stress period, the IL-2/IL-5 ratio was significantly lower in atopics compared

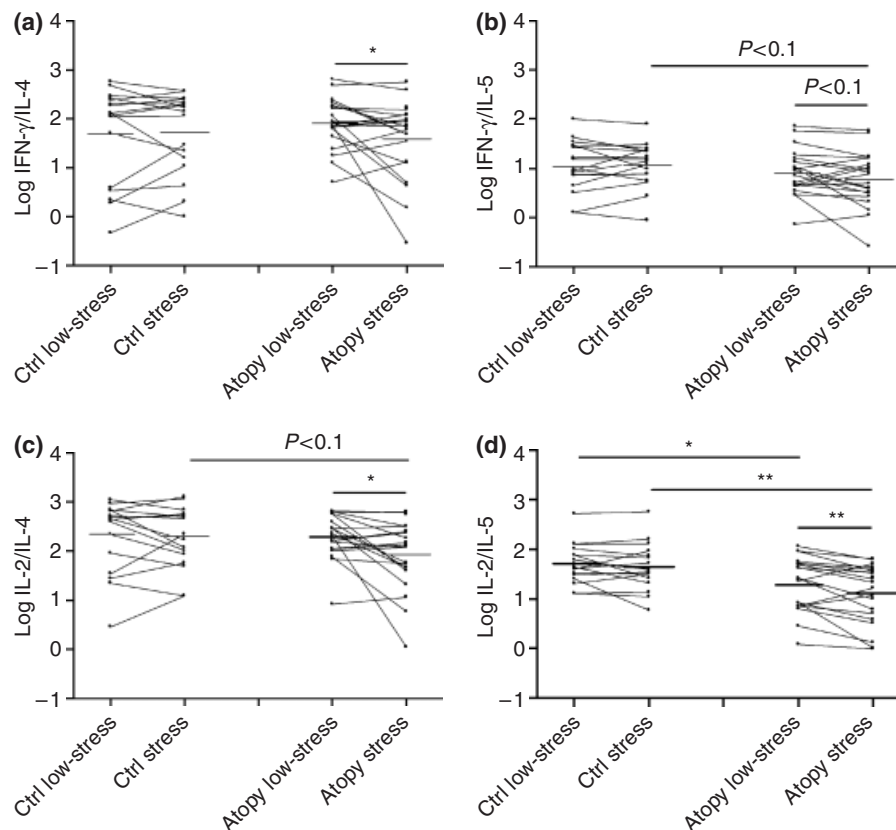


Fig. 3. Effect of examination stress on cytokine production from peripheral blood mononuclear cells. (a) IFN- γ /IL-4, (b) IFN- γ /IL-5, (c) IL-2/IL-4 and (d) IL-2/IL-5. Means are indicated in the graphs. * $P < 0.05$; ** $P < 0.01$.

with controls (Fig. 3). Interestingly, in response to stress, the IFN- γ /IL-4, IL-2/IL-4 and IL-2/IL-5 ratios were also significantly reduced in atopics in comparison with controls (Fig. 3). Together, these data indicate that the regulation of cytokine secretion in atopic subjects is skewed towards a Th2 dominance and that stress may exaggerate this response.

Effects of examination stress on exhaled nitric oxide and lung function

As a local marker of airway inflammation, levels of exhaled NO were assessed. In these analyses, and those for lung function and hyper-reactivity, comparisons were made between atopic subjects with asthma and control subjects. Atopic individuals with asthma exhaled significantly more NO than control subjects (Table 4). The control subjects responded with a small but significant reduction in exhaled NO in response to stress, a feature not evident in the asthmatic subjects (Table 4).

No statistical differences in FEV₁, PEF or FEV₁/FVC were observed between the groups (Table 4). However, atopic students with asthma displayed a non-significant trend towards lower FEV₁/FVC as compared with controls in the low-stress phase ($P = 0.08$), a difference that was statistically significant when assessed at the screening occasion ($P = 0.03$, data not shown). The FEV₁ value

increased slightly but significantly in the group of controls, but not in the group of atopics with asthma, during stress. Consequently, the FEV₁ values of the control group differed significantly from the group of asthmatics during the stress phase (Table 4). No significant differences in PEF variability (amplitude % mean) or in minimum morning PEF (% recent best) were evident between controls and atopics with asthma, nor within these two groups between the two investigated time periods, as calculated from the registrations in the diaries (Table 4).

Effect of examination stress on non-specific bronchial hyper-reactivity

Atopic individuals with asthma responded with a significantly lower log PD₂₀ of methacholine as compared with control subjects, as determined both during the low-stress and stress phase. However, the log PD₂₀ of methacholine was not changed in any of the groups as a function of stress (Table 4).

Analyses based on levels and changes in perceived stress and cortisol

To test whether the immunological differences were related to the elevated levels of baseline stress in atopics,

Table 4. Effects of examination stress on levels of exhaled nitric oxide and lung function parameters

	Controls		Atopics with Asthma	
	Low stress	Stress	Low stress	Stress
FENO (ppb)	15.4 (12.0–20.2)	13.2 (11.1–14.8)*	22.9 (10.6–29.1) [†]	21.5 (11.2–29.4)
FEV ₁ (% pred)	115 (103–118)	118 (105–123)*	112 (94.0–120)	113 (95.0–117)
FEV ₁ /FVC	0.86 (0.83–0.90)	0.86 (0.85–0.90)	0.82 (0.73–0.88) [‡]	0.84 (0.76–0.90)
PEF (% pred)	108 (94.5–126)	109.0 (94.0–120)	101 (85.0–123)	104 (84.0–121)
PEF variability (amplitude % mean)	3.75 (2.15–6.60)	3.00 (1.60–4.95)	4.25 (2.80–6.05)	2.90 (1.95–7.30)
Minimum morning PEF (% recent best)	89.5 (86.0–91.1)	90.3 (86.0–92.1)	90.3 (87.2–94.8)	93.5 (87.2–95.2)
Log PD ₂₀ (ug)	3.73 (3.07–3.86)	3.86 (3.32–3.86)	2.28 (2.16–3.09) [§]	2.52 (2.17–3.16) [¶]

Values are presented as medians and 25–75th percentiles in parentheses.

FENO, fractional exhaled nitric oxide; FEV₁, forced expiratory volume in 1 s; pred, predicted; FVC, forced vital capacity; PEF, peak expiratory flow; PD₂₀, provocative dose causing 20% decrease in FEV₁.

* $P < 0.05$ compared with low-stress values within the group.

[†] $P < 0.05$ compared with control low-stress value.

[‡] $P < 0.1$ compared with control low-stress value.

[§] $P < 0.01$ compared with control low-stress value.

^{||} $P < 0.05$ compared with control stress value.

[¶] $P < 0.01$ compared with control stress value.

median splits were performed on the whole group of students to analyse whether grouping based on levels (high or low) of perceived stress (VAS) at baseline would yield similar results in the main immunological findings as grouping based on atopic status. This exploratory approach was applied on selected main findings of the study, including regulatory T cells, NK cells, Th1/Th cytokine ratios and FEV₁.

Both the group with low (14 (6.8–18.5) mm) and high (28 (26–53) mm) VAS at baseline displayed increases in the proportion of CD4⁺CD45RO⁺CD25^{bright} cells between the low-stress and the stress phase ($P < 0.001$, not shown), concordant with a broad and general response to stress in this cell type. Interestingly, the group characterized by high VAS at baseline had higher numbers of CD4⁺CD45RO⁺CD25^{bright} cells than the group with low VAS ($P < 0.05$), possibly consistent with a proposition that numbers of regulatory T cells are related to stress level.

Furthermore, the group with low VAS decreased significantly in the proportion of CD3⁺CD56⁺CD16⁺ between assessments ($P < 0.05$). Thus, the finding that atopic but not healthy students showed a decrease in CD3⁺CD56⁺CD16⁺ in response to stress does not seem to be related to baseline stress levels, because the atopic group had higher rather than lower baseline stress levels (Fig. 1). In addition, our finding that atopics have lower number of NK cells in the low-stress period is probably not related to initial stress levels, because the group with low and high VAS at baseline did not differ with respect to the proportion of NK cells.

The subjects with high VAS at baseline, but not those with low, decreased significantly in the IL-2/IL-5 ratio in

response to stress ($P < 0.05$). This finding could indicate that the change observed in IL-2/IL-5 in atopics could be due to the higher initial VAS levels in this group rather than their atopic status. However, this effect was not observed in any other measure of Th1/Th cytokine balance, which makes it unlikely that the general Th1/2 decrease observed in atopic students (IL-2/IL-5, IL-2/IL-4, IFN- γ /IL-4) is due to baseline differences in perceived stress. In addition, our finding that atopics have a lower IL-2/IL-5 ratio in the low-stress period is probably not related to initial stress levels, because the group with low and high VAS at baseline did not differ from each other in these median split analyses. Altogether, our analyses provide compelling evidence suggesting the importance of atopic status for the immunological differences observed in the exam period.

When grouping students based on levels of perceived stress (VAS) at baseline, no significant difference in FEV₁ was noted in either low or high VAS between the low-stress and the stress period. Analyses based on similar median splits on baseline cortisol levels were not applied, as the high cortisol group only included five atopics. However, median splits on the basis of small or large changes in VAS or cortisol between the low-stress and the stress phase were possible to make. In these analyses, no significant differences in any investigated parameter were found between groups of subjects displaying small or large changes in VAS or cortisol (data not shown).

Discussion

An increased Th2 cytokine dominance has previously been demonstrated in atopic individuals and has been

suggested to be part of the pathogenesis of atopic diseases [1]. Our results corroborate this notion and also confirm that stress may aggravate this phenotype in atopic individuals. In line with previous data [31], our results also demonstrate an overall reduction in cytokine production in cells from stressed individuals. This decline involved both Th1 (IFN- γ , IL-2) and Th2 (IL-4, IL-5) cytokines. Interestingly, while non-atopic students exhibited a similar reduction of Th1 and Th2 cytokines, atopic students showed a more pronounced decrease in Th1 cytokines, resulting in larger Th2 dominance with high stress. A possible reason for a skew towards Th2 could be an increased sensitivity of Th1 cells to glucocorticoids [4, 5, 12]. The Th2 skewing in this study could therefore result from the larger cortisol increase in the former group. On the other hand, this proposition was not supported by analyses of subjects with small or large increases in urine cortisol levels, as changes in cortisol did not relate significantly to changes in immune parameters. As many authors have pointed out, however, cortisol stress dynamics are complexly regulated, including both steroid levels and adaptations in target cell sensitivity to determine the physiological net effect [32]. The lower cortisol values observed by us in atopic students have also been reported by previous investigators [33]. It has been suggested that cortisol dynamics are generally altered in atopic subjects [34], possibly also reflected by the observation that individuals with asthma may be more susceptible to stress-related immune dysregulation [9]. An interesting possibility is therefore that disease-specific patterns of steroid regulation and stress sensitivity contribute to the increased Th2 response in atopic individuals.

An additional explanation to an altered Th1/Th2 balance in atopic individuals in response to stress could be the dramatic decline in the proportion of NK cells in this group. NK cells are major IFN- γ producers *in vivo* [30] and it is possible that the reduction in NK cells numbers in atopic individuals in response to stress may contribute to the reduction in levels of IFN- γ and changed cytokine balance. Glucocorticoids may reduce the capacity of NK cells to respond to IL-12 [12], and changes in the levels of corticoids, as presently observed in atopic individuals in response to stress, may further diminish the IFN- γ -producing capacity of NK cells. However, it should be noted that the role of NK cells in atopy may be more complex. In a murine model of asthma, depletion of NK cells prevented the accumulation of eosinophils and T cells in the airways and diminished Th2 cytokines [35, 36]. In this respect, it is important to realize that NK cells are not only IFN- γ producers but may also produce Th2 cytokines, such as IL-5 and IL-13 [37]. Interestingly, in a model where NK cells promoted disease, they also produce high levels of the Th2 cytokine IL-5 [36]. It is therefore possible that NK cells may play several roles in atopic disease, perhaps depending on what triggers them, where they are trig-

gered and when they are called into action. An increase in NK cells in asthmatic airways in comparison with healthy controls has been reported [38], findings that warrant a more detailed investigation of how NK cells contribute to atopic disease and the role of stress in this process.

Regulatory T cells are able to control a variety of immune responses and their depletion leads to severe autoimmunity in both humans and mice [39]. Recent studies also imply a role for CD25⁺ regulatory T cells in atopic disease [40, 41]. Our study demonstrates, for the first time, that T cells bearing markers associated with regulatory phenotypes (CD4⁺CD45RO⁺CD25⁺) were strongly up-regulated by stress. As regulatory T cells are able to exert dramatic control of inflammatory states in many situations [39, 42], this finding provides a possible explanation for the large decline in several cytokines observed in both groups in response to stress. This observation is also interesting in relation to a recent finding showing an increase in CD4⁺CD25⁺ regulatory T cells after glucocorticoid exposure in mice [43] as well as in asthmatics [44]. In our study, both atopic and non-atopic subjects showed clear increases in the numbers of regulatory T cells at the time of the exam, indicating that it may represent a general physiological response to stress. The proposition that stress affects the number of regulatory T cells is cautiously supported by our finding that subjects with a high perceived stress level (VAS) had significantly higher numbers of CD4⁺CD45RO⁺CD25^{bright} cells than those with a low perceived stress level.

It is interesting to note that several studies imply a dysregulated function of CD4⁺CD25⁺ regulatory T cells in the pathogenesis of atopic disease. For example, CD4⁺CD25⁺ T cells from atopic donors were less efficient in their ability to inhibit allergen-stimulated T cell proliferation and production of IL-5 as compared with cells from non-atopic donors [6]. Similarly, CD4⁺CD25⁺ regulatory T cells from birch-pollen-allergic patients were defective in down-regulating pollen-induced IL-13 and IL-5 production, while their capacity to suppress IFN- γ production was retained [7]. Importantly, this phenomenon was not observed outside of the pollen season and not in control subjects [7]. Further studies are required to test the interesting possibility that healthy and atopic subjects share certain control features of regulatory T cells in a stress response (increase in the numbers of regulatory T cells), but that other features (control of Th2 cytokine production) may differ between groups.

On the behavioural level, marked increases in indices of subjective stress level were observed in both atopic and non-atopic subjects at the time of examination, while relatively few changes were observed in health behaviours. One interesting change was a decrease in subjective sleep quality, which may reflect the subjects' higher levels of experienced stress and anxiety. Because some recent studies indicate that disturbed sleep may be

related to Th2 dominance [16, 17], it cannot be excluded that the deterioration in subjective sleep quality is one factor behind the cytokine changes in the present study. Physical exercise decreased in the stress phase, which would not be consistent with a cytokine change in this direction [19]. It is interesting to note that the changes in neither immune parameters nor lung function were significantly different between subjects displaying small or large changes in perceived stress or cortisol levels. This finding is supported by the meta-analysis of stress and immune function by Segerstrom and Miller [9], where subjective reports of stress generally did not associate with immune changes.

As expected, levels of exhaled NO were generally higher in subjects with asthma. However, we did not observe an increase in local inflammation in the airways measured as levels of exhaled NO during the stress phase. Interestingly, control subjects displayed a decrease in NO in the stress phase. The cellular origins of the changed NO levels in controls are not known, because exhaled NO may stem from multiple sources, such as macrophages or epithelial cells [21]. To our knowledge, no earlier studies have studied the relation between stress and exhaled NO. The absence of significant pulmonary changes in atopic subjects with asthma in the present study may relate to our selection of patients with mild and well-managed asthma out of the pollen season. It should be noted, however, that the empirical support for stress-induced airway obstruction is weak [45]. In fact, we noted a slight increase in FEV₁ in control subjects in the stress phase, leading to a significant difference between the controls and asthmatics in the stress phase only. Increased FEV₁ has earlier been reported in response to acute stress in asthmatic subjects [46]. With activation of an allergic response, it is possible that stress-related differences in immune parameters, in airway inflammation and in lung function would increase. In the present study, it is reasonable that study group differences are underestimated, focusing on relatively healthy undergraduates without allergic activation. In the study by Liu et al. [13], the increase in sputum eosinophils after allergen challenge was amplified during stress, and the percentage of eosinophils correlated negatively with percentage change in FEV₁ in the stress phase.

Atopic diseases are increasing in the western world. At the same time, modern lifestyles are intimately coupled with increased levels of stress. Findings like ours, suggesting several links between stress and disease mechanisms of atopy, may therefore be of importance for public health. The results from the present study suggest that functional differences in immune regulation, such as the control over the allergic inflammation by NK cells and regulatory T cells, may explain why subjects with or without atopy can respond in different ways to stress in terms of cytokine production.

Acknowledgements

The authors wish to thank Dr P. Gyllfors and H. Blomqvist, M. Dahl, G. de Forest, C. Larsson and B. Dahlberg for assistance with screening of patients, lung function tests and laboratory assistance, and students at Karolinska Institutet for participating in the study. We thank Dr P. Hoglund for fruitful discussions.

The present work was funded by the Ekhaga Foundation, Swedish Asthma and Allergy Association, King Oscar II Jubilee Foundation, the Swedish Research Council, Swedish Heart Lung Foundation, Swedish Society of Medicine, Magnus Bergvall's Foundation, Tore Nilson's Foundation, Lars Hierta Foundation, Torsten and Ragnar Söderberg's Foundations, Åke Wiberg's Foundation and Karolinska Institutet. C. O. H. was funded by the Swedish Research Council, C. K. by the Swedish Heart Lung Foundation and the Swedish Asthma and Allergy Association, J. A. and S. J. by the Centre for Allergy Research at Karolinska Institutet and Y. S. by the Swedish Asthma and Allergy Association.

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