

Allergen Challenge Alters Intracellular Cytokine Expression

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Abstract

The pathophysiology of asthma is complex and engages cascades of events in the cytokine network. We, therefore, investigated the impact of bronchial allergen challenge in humans on the cytokine profile of circulating lymphocytes. Peripheral blood samples from 10 patients with allergic asthma were collected before and 24 h after allergen provocation. Patients who mounted a late-phase reaction were designated dual responders opposite to single responders. Whole blood cells were stimulated by mitogen and intracellular interleukin (IL)-4 and interferon (IFN)- γ were detected by flow cytometry. The allergen challenge induced a decrease in IL-4⁺CD4⁺ cells in the patients ($P=0.05$), and a significant decrease ($P < 0.05$) in IFN- γ ⁺CD4⁺ cells was noted in single, but not dual, responders. In addition, there was a significant difference ($P < 0.01$) with respect to the changes in the IFN- γ ⁺CD4⁺ cells comparing dual and single responders. No corresponding changes were observed in CD8⁺ cells. The data suggest a possible on-going traffic of IFN- γ and IL-4⁺CD4⁺ lymphocytes into the bronchial mucosa in relation to an allergen challenge and generate the hypothesis that a difference exists between single and dual responders in this respect. Because the CD4⁺IFN- γ -producing cells have the capacity to down-regulate the T-helper type 2 response, a reduced capacity in this aspect might contribute to the pathophysiology in dual responders.

Introduction

Allergic asthma is a disease characterized by a recruitment of lymphocytes and eosinophils into the bronchial mucosa. Studies have indicated that the tissue-dwelling lymphocytes are skewed towards a T-helper type 2 (Th2) population and that the Th2 cytokines interleukin (IL)-4, IL-5 and IL-13 play an essential role in the pathogenesis of the bronchial inflammation that features allergic asthma [1–4]. This theory is supported by the finding that the number of IL-4⁺ and IL-5⁺ cells per square millimetre of bronchial submucosa is significantly higher in patients with asthma and eosinophilic bronchitis than in healthy control subjects [5].

The role of interferon (IFN)- γ in allergic asthma is not fully known. The Th2 hypothesis includes the concept that allergic subjects have a relative IFN- γ deficiency, but this has been challenged by data supporting a more complex view on the role of IFN- γ [6–8]. IFN- γ levels in serum and bronchial alveolar lavage fluid (BAL), as well as IFN- γ ⁺ cells, are increased in asthmatic subjects, and serum-IFN- γ levels correlate with disease activity [4]. Krug and coworkers [9] described an increased percentage of

IFN- γ -producing T cells in BAL from the subjects with asthma, compared with atopic and nonatopic controls. Furthermore, Brown and coworkers reported that the percentage of IFN- γ ⁺ T cells in BAL was significantly increased in children with atopic asthma compared with both atopic nonasthmatic subjects and normal controls. On the basis of these observations, they suggested that IFN- γ may play an important role in the pathogenesis of childhood asthma and that asthma is not only a Th2 driven response [10].

Several studies have addressed the question whether there are differences in circulating T-cell populations between asthmatics and healthy subjects, with regard to cytokine profile. Most previous reports on this issue are cross-sectional designed. On the basis of the diversity in results between stable asthma and at exacerbation, the question has emerged whether an active traffic between the circulation and the lung may explain these inconsistencies [11].

To gain more insight into the potential role of the balance between IFN- γ and IL-4⁺ T lymphocytes in allergic asthma, we sought to assess the cytokine profile of

circulating T lymphocytes in allergic asthmatics before and after a bronchial allergen challenge. We hypothesized that an allergen challenge induces an active recruitment of lymphocytes into the bronchial mucosa, which is mirrored by a decrease in the corresponding circulating population.

Materials and methods

Subject characterization and sampling procedure. Ten patients (nine male and one female) with mild asthma and allergy to birch ($n=8$) or timothy pollen ($n=2$) were randomly included in the study (Table 1). Mean age of the patient group was 35 (22–43) years with mean asthma duration 21 (5–29) years. All except one patient were nonsmokers (six life-time nonsmokers and three ex-smokers). The subjects underwent a bronchial allergen challenge, and peripheral blood samples were collected in heparinized tubs before and 24 h after allergen provocation. Heparinized blood from 10 healthy blood donors served as control. The study was performed outside the pollen season. The patients used no per os or inhaled steroids during the study period, and no patient had an on-going infection. All patients inhaled β_2 -agonists as needed.

Bronchial allergen challenge. The allergen challenge was done with a dosimeter jet nebulizer (Spira Elektro 2, Respiratory Care Center, Hameenlinna, Finland). Standardized and freeze-dried birch or timothy allergen extracts (Aquagen, ALK, Copenhagen, Denmark) were diluted and used at a maximum of four concentrations: 1000, 4000, 16,000 and 64,000 standardized quality (SQ) allergen units per millilitre. The nebulizer was set to nebulize for 0.5 s giving an output of 7.1 μ l/breath. At each concentration, first two and then four breaths could be taken, and if needed followed by eight and 16 breaths at the highest concentration providing doses from 14 to 7040 SQ allergen units. Specific airway resistance (S_{Raw}) and thoracic gas volume were measured 15 min after each dose of allergen. The challenge proceeded until a 100% increase in S_{Raw} was reached (Table 1). Median provocative dose of allergen required to cause 100% increase of S_{Raw} (PD_{S_{Raw}100%}) was calculated by linear interpolation on a logarithmic scale. Forced expiratory volume in 1 s (FEV₁) and S_{Raw} were recorded immediately before and 15 min after each single dose of allergen was inhaled.

Spirometry. The measurements of FEV₁ were made hourly using a portable computerized spirometer (Diary Card spirometer, Micromedical Ltd. Chatham, Kent, UK).

The maximal fall in FEV₁ from allergen challenge to 3–10 h after allergen challenge, as well as the average fall in FEV₁ during this period, was used to measure the asthmatic reaction during the late phase. Results for FEV₁ are provided in litres.

Definition of single and dual responders. Single responders had only an early reaction 15 min after the allergen

challenge with a 100% increase in S_{Raw}. Late-phase reaction was defined as at least 15% decline in FEV₁ 3–10 h after the last dose of allergen challenge. Dual responders had a late-phase reaction in addition to the initial 100% increase in S_{Raw}.

Blood cells' count. Blood samples were collected in 5 ml EDTA tubs, before and 24 h after allergen provocation and analysed in ADVIATM 120 Hematology System, Bayer-HealthCare LLC, Tarrytown, NY, USA.

Stimulation of peripheral blood lymphocytes. Five hundred microlitres of whole blood was incubated at 37 °C (5% CO₂) for 4 h with 20 μ l 1 μ g/ml phorbol 12-myristate-13-acetate (Sigma Chemical, St. Louis, MO, USA) and 2 μ l 500 μ g/ml ionomycin (Sigma Chemical) in presence of 2 μ l 5 mg/ml Brefeldin, BFA (Sigma Chemical) and 0.5 ml Hepes-RPMI. Unstimulated cells, which served as controls, remained in BFA and medium only for 4 h. BFA was used to interrupt the intracellular Golgi-mediated transport and to allow the cytokines to be accumulated inside the cells.

Surface immunostaining. In vitro activation of CD3⁺ cells was assessed by CD69 upregulation, and 90% CD69⁺CD3⁺ was set as the lowest acceptable level. To identify the CD4 and CD8⁺ lymphocytes by flow cytometry, we surface stained the cells with specific antibodies. IgG staining was simultaneously used to exclude unspecific binding. To each 50 μ l of the stimulated blood sample, 5 μ l FITC-conjugated anti-CD4, anti-CD8 or anti-IgG (Becton Dickinson Immunocytometry Systems, CA, USA) was added, and the samples were incubated for 15 min at room temperature (RT) in the dark.

Cell membrane fixation. Fifty microlitres of Medium A (Fix and Perm Cell permeabilization kit, Caltag Laboratories Inc., Burlingame, CA, USA) was added to each sample tube and incubated 15 min at RT in the dark. The samples were then washed with 2 ml PBS and centrifuged at 188 $\times g$ for 5 min.

Cell membrane permeabilization and immunofluorescence staining of intracellular IFN- γ and IL-4. Monoclonal antibodies designed for intracellular staining and flow cytometry were used for detection of intracellular human IL-4 and IFN- γ . Fifty microlitres of medium B (Fix and Perm Cell permeabilization kit, Caltag Laboratories Inc.) and 10 μ l fluorescent-conjugated intracellular antibodies to IFN- γ and to IL-4, respectively (Becton Dickinson Immunocytometry Systems) were added to each tube and allowed to incubate for 15 min at RT in the dark as described by Källström and coworkers [12]. Samples were washed with 2 ml PBS and centrifuged at 188 $\times g$ for 5 min. The resulting pellet was resuspended with 500 μ l PBS and finally analysed in an EPICS XL (Coulter Inc., Hialeah, FL, USA).

Flow cytometric analysis. The final preparations of samples/lymphocytes were analysed in an EPICS XL (Coulter Inc.) [12]. Discrimination gates were set around the respective cell population, and a minimum of 3000

Table 1 Anthropometric and clinical data

Patient Number	Sex	Age (years)	Length (cm)	Weight (kg)	Smoking	Asthma duration (years)	Allergen inhaled	FEV ₁	FEV ₁ (% pred*)	Skin-prick test (mm†)	Allergen PD _{SRA} 100% SQ units‡	Late-phase reactions§
1	Female	27	175	68	N	10	T	3.34	92	7.5	1117	No
2	Male	38	177	78	E	23	T	4.14	103	15.5	847	No
3	Male	34	190	98	N	22	B	3.98	85	10.5	194	Yes
4	Male	43	186	90	E	27	B	3.57	84	6.5	972	No
5	Male	37	185	93	N	21	B	3.61	82	7	84	Yes
6	Male	33	181	100	N	28	B	3.17	73	7.5	222	No
7	Male	42	183	84	E	23	B	4.82	116	8.5	90	No
8	Male	45	190	103	Y	5	B	4.57	104	7.5	175	Yes
9	Male	31	178	83	N	29	B	4.3	101	12	17	No
10	Male	22	184	80	N	17	B	4.09	86	8.5	33	Yes

N, never smoker; E, ex-smoker; Y, smoker; T, timothy; B, birch.

*% pred: percentage of predicted value.

†Skin prick test >3 mm = positive reaction.

‡Provocative dose of allergen causing a 100% increase in specific airway resistance, SQ = standard quality.

§Late-phase reaction defined as ≥15% decline in forced expiratory volume in 1 s (FEV₁) 3–10 h after the allergen challenge.

lymphocytes were accumulated during analysis. The cells were identified both as CD3, CD69 CD4, CD8⁺ and positive for the respective cytokine. The results are expressed as percentage of accumulated lymphocytes.

The analyses were assessed in samples before and 24 h after allergen provocation.

Statistical methods. Results are given as median and range values or Δ -values, which represent the percentage change between two observations within the same individual. The Wilcoxon test was used to analyse the differences between paired measurements before and after the allergen provocation, and the Mann–Whitney *U*-test was used to analyse the differences of Δ -values between single and dual responders.

Ethics approval. The study was conducted in accordance with national and institutional guidelines for human studies. Approval was obtained from the Ethics Committee of the Karolinska University Hospital, Sweden, prior to commencement of the study.

Results

Airway response and leucocyte counts

In addition to the initial airway response to the allergen challenge, measured as 100% increase in Straw, four of 10 patients mounted a late-phase reaction and were designated dual responders. The given allergen dose is presented in Table 1. There was a significant increase in the numbers of eosinophils 24 h after the allergen provocation [$0.25(0.11 - 0.52) \times 10^9/l$ versus $0.36(0.17 - 0.68) \times 10^9/l$, $P = 0.007$], which was not observed for lymphocytes, neutrophils, monocytes or basophiles.

Lymphocyte cytokine profiles in patients and healthy subjects

The initial percentage of IFN- γ^+ CD4⁺ and IFN- γ^+ CD8⁺ lymphocytes had a tendency to be lower and the initial percentage of IL-4⁺CD4⁺ and IL-4⁺CD8⁺ lymphocytes had a tendency to be higher in the patient group than in the healthy control group (Table 2).

IFN- γ^+ lymphocytes in relation to allergen challenge

No significant change in IFN- γ^+ CD4⁺ lymphocytes before and 24 h after the allergen challenge was noted in

the patient group (Fig. 1A). However, when the patient group was divided into single ($n = 6$) and dual responders ($n = 4$), the single responders showed a significant ($P = 0.046$) decrease, as opposed to the dual responders who showed a noticeable but not significant increase (Fig. 1A) in IFN- γ^+ CD4⁺ lymphocytes. The difference between single and dual responders concerning Δ -values was significant for IFN- γ^+ CD4⁺ lymphocytes [$-1.96(-4.10 - 0.60)\%$ versus $2.98(2.24 - 9.20)\%$, $P < 0.01$] but not for IFN- γ^+ CD8⁺ lymphocytes (Table 3). No significant change in IFN- γ^+ CD8⁺ lymphocytes before and 24 h after the allergen challenge was noted neither in the patient group nor in the subgroups (Fig. 1B).

IL-4⁺ lymphocytes in relation to allergen challenge

A decrease in IL-4⁺CD4⁺ lymphocytes was observed 24 h after the allergen challenge in the patient group (Fig. 1C) ($P = 0.050$). There was no significant difference between single and dual responders with respect to Δ -values for IL-4⁺ lymphocytes [$-0.55(-5.65 - 0.65)\%$ versus $-0.34(-0.70 - 0.20)\%$, $P = \text{not significant}$] (Table 3). However, no significant changes were observed in IL-4⁺CD8⁺ lymphocytes (Fig. 1D) in relation to allergen challenge.

Discussion

The main finding in the present study is that a bronchial allergen challenge induces a substantial switch in peripheral CD4⁺ lymphocyte cytokine profile. A decrease in circulating IL-4⁺CD4⁺ lymphocytes characterized the patients, accompanied by a significant decrease in circulating IFN- γ^+ CD4⁺ lymphocytes featured single responders as opposed to in-dual responders.

A lower level of IFN- γ^+ lymphocytes and a higher level of IL-4⁺ lymphocytes in the patient group than in the healthy control group are in line with findings observed in other atopic disorders and suggest that a defective IFN- γ production is a generalized feature of atopy. These results are in agreement with previous published data by Lee *et al.* as well as with the Th2 paradigm in atopy and asthma [13–15] and support the accuracy of the used techniques.

Table 2 Cytokine expression

	Healthy		Patients before provocation		Patients 24 h after provocation	
	Median	Range	Median	Range	Median	Range
IFN- γ^+ CD4 ⁺	15.3	9.7–25.7	13.4	6.8–33.9	13.7	3.1–36.4
IFN- γ^+ CD8 ⁺	20.1	12.8–29.7	9.5	2.7–37.8	5.3	0.9–29.5
IL-4 ⁺ CD4 ⁺	0.9	0.0–2.5	1.4	0.0–5.6	0.5	0.0–2.5
IL-4 ⁺ CD8 ⁺	0.0	0.0–1.2	0.2	0.0–1.7	0.1	0.0–1.6

IFN, interferon; IL, interleukin. Median and range percent values of the cytokine-producing T cells in the patient group ($n = 10$) before and 24 h after the allergen provocation and in the healthy control group ($n = 10$).

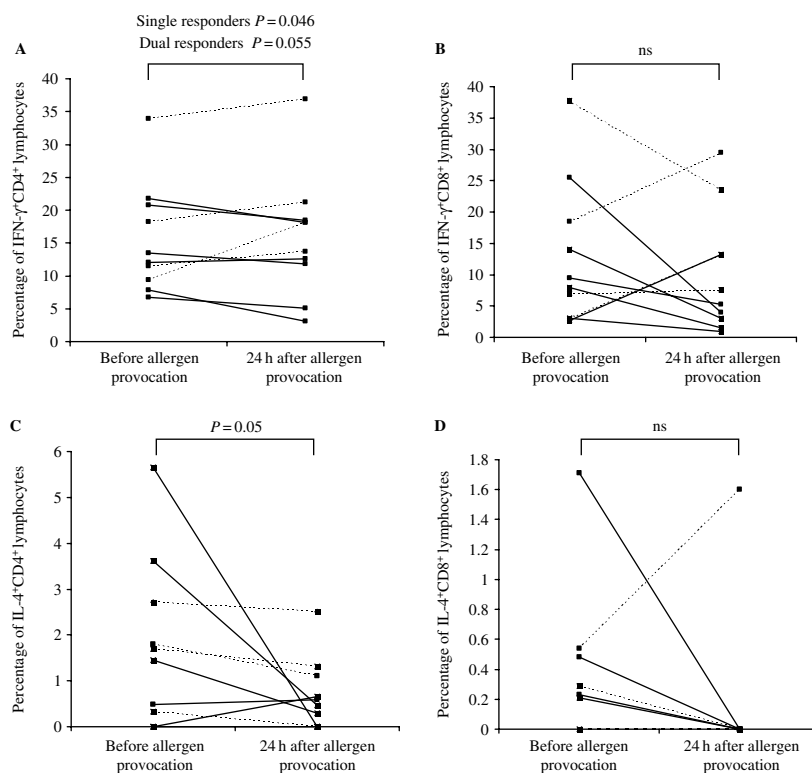


Figure 1 Intracellular cytokine expression before and after allergen provocation in allergic asthma patients. Filled line represents single responder and dotted line dual responder. ns = not significant. (A) percentage of IFN- γ ⁺CD4⁺ cells before and 24 h after allergen provocation. (B) percentage of IFN- γ ⁺CD8⁺ before and 24 h after allergen provocation. (C) percentage of IL-4⁺CD4⁺ before and 24 h after allergen provocation, patient number 6 with 0% before and after provocation. (D) percentage of IL-4⁺CD8⁺ before and 24 h after allergen provocation (dotted line at 0% represents four patients).

The majority of data on T-lymphocyte cytokine profiles in the literature are based on cross-sectional studies, and less data are available on the kinetic nature of the circulating T-lymphocyte population. The rationale for a kinetic approach is based on the proposed importance of an existing active traffic of T lymphocytes into the bronchial mucosa in order to orchestrate the immune response [16]. One potential explanation for the lower IFN- γ ⁺-lymphocyte count in allergic asthmatics is an on-going recruitment to the bronchial mucosa. In support for this hypothesis is our finding that peripheral IFN- γ ⁺CD4⁺ lymphocytes decreased in single responders upon an allergen challenge. A similar pattern was observed in IL-4⁺CD4⁺ lymphocytes, which indicates a concomitant recruitment of IL-4⁺CD4⁺ lymphocytes into bronchial mucosa as a

response to a bronchial allergen challenge. This hypothesis is in line with the current concept of the pathophysiology of allergic asthma [11, 17]. A potential role for IFN- γ - and CD4⁺-producing lymphocytes in allergic asthma is also given by experimental data. For example, using blocking IFN- γ antibodies in a murine OVA-induced allergic asthma model, Matsumoto and coworkers [18] demonstrated that the regulation of asthmatic response is IFN- γ dependent. In addition, Mishima and coworkers [19] demonstrated that CD4⁺ lymphocytes harvested from sensitized rats could transfer airway hyperresponsiveness to allergen to naive rats recipients.

Kuo and coworkers [17] have reported an increase in IL-4 and IFN- γ ⁺CD4⁺ lymphocytes in peripheral blood in patients with acute asthma attacks compared with at

Table 3 Δ -Values (%) of cytokine expression before and 24 h after the allergen challenge

Patient number	Δ -value IFN- γ ⁺ CD4 ⁺	Δ -value IFN- γ ⁺ CD8 ⁺	Δ -value IL-4 ⁺ CD4 ⁺	Δ -value IL-4 ⁺ CD8 ⁺
1	-1.62	-11.01	0.65	0.00
2	-1.66	-4.20	-1.15	-0.21
Dual responder 3	2.24	0.60	-0.33	-0.29
4	-4.10	-6.53	-5.65	-0.23
Dual responder 5	3.00	-14.30	-0.70	0.00
6	0.60	-2.11	0.00	0.00
7	-3.70	-21.50	0.09	-0.48
Dual responder 8	2.95	10.97	-0.20	1.06
9	-2.30	10.50	-3.15	-1.71
Dual responder 10	9.20	10.17	-0.37	0.00

IFN, interferon; IL, interleukin.

stable condition. As the study was carried out in patients with asthma exacerbations, multiple confounding factors such as infections had to be taken into consideration. Moreover, it is also well known that approximately half of the asthmatics are dual responders, and the authors' aim was not to identify single and dual responders. It is of notice that IFN- γ ⁺CD4⁺ lymphocytes for our total patient group did not change significantly.

Studies in BAL fluid have shown a higher number of IFN- γ ⁺ lymphocytes in asthmatics than in healthy controls [9, 10] and that a decrease in these cells occurs after allergen challenge [20–22]. These data might contrast our hypothesis that IFN- γ ⁺ cells are recruited upon an allergen challenge. However, these studies did not distinguish between dual and single responders, and it is also not established whether the conditions in BAL fluid actually mirrors the inflammatory state in bronchial mucosa [23]. To study cytokine profiles of circulating T lymphocytes in response to allergen challenge became an established method [24]. We used intracellular cytokine method documented in several studies [12, 25]. To measure intracellular cytokines, we stimulated the cells by mitogens *in vitro*. This does not affect the number of T cells but identifies cells that have the capacity to produce a specific cytokine, thereby reflecting the alterations caused by the specific allergen exposure *in vivo*. Different kinetic pattern in T-lymphocyte cytokine profile in single and dual responders has recently been reported by Matsumoto and coworkers. They reported that IL-10⁺CD4⁺ lymphocytes increased after an allergen challenge in single, but decreased in dual, responders [24]. This finding goes in line with our IFN- γ data, because an existing antagonistic balance between IFN- γ and IL-10 has been suggested [26]. Together, these studies indicate a complex kinetic pattern in the cytokine profile of circulating T lymphocytes in relation to the clinical manifestation. However, the size of our study group is rather small, and randomized studies including well-characterized patient groups with respect to clinical manifestations are warranted.

In summary, an allergen challenge provokes a switch in peripheral T-lymphocyte cytokine profile, indicating a possible on-going traffic of IFN- γ and IL-4⁺CD4⁺ lymphocytes into the bronchial mucosa in relation to the challenge. Moreover, the data generate the hypothesis that a difference exists between single and dual responders in this respect. The recruitment of CD4⁺IFN- γ -producing cells featured single responders, and a reduced capacity in this aspect might contribute to the pathophysiology in dual responders.

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